## Universidade do Minho

Escola de Ciências

# Marcos André Machado Lima Teixeira 

Phyllodocida (Annelida, Polychaeta) of the North East Atlantic as a model for the investigation of cryptic species

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Phyllodocida (Annelida, Polychaeta) of the North East Atlantic as a model for the investigation of cryptic species

Ph.D. Thesis in Biology
Specialization in Integral Management of the Sea

Work supervised by
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## STATEMENT OF INTEGRITY

I hereby declare having conducted this academic work with integrity. I confirm that I have not used plagiarism or any form of undue use of information or falsification of results along the process leading to its elaboration.

I further declare that I have fully acknowledged the Code of Ethical Conduct of the University of Minho.


#### Abstract

Molecular techniques have been effective in signalling potential hidden diversity in species displaying similar morphology and presumed widespread distribution. In this study, members of Phyllodocida collected along European coasts were used as a model taxon to investigate this topic, by employing a combination of multi-locus molecular data (mtCOI-5P, 16SrRNA, ITS regions and 28SrRNA), together with morphological and morphometric examination. This work identified a large number of undescribed cryptic lineages within 6 morphospecies, namely: Eumida sanguinea (22 Molecular Operational Taxonomic Units - MOTUs); Eulalia clavigera (9 MOTUs); Hediste diversicolor (5 MOTUs); Platynereis dumerilii(10 MOTUs); Perinereis cultrifera (14 MOTUs) and Trypanosyllis zebra (10 MOTUs). In total 70 lineages were uncovered, of which 43 are unique to this work. Five of these morphospecies have a dedicated chapters where an integrative approach allowed the description of 13 new species to science and the clarification of ambiguities regarding previously descriptions. The Macaronesian islands and especially, the western part of the Mediterranean Sea, are hotspots of cryptic diversity, with a total of 10 and 30 unique lineages for each region, respectively. Mediterranean MOTUs appear to be genetically closer to the ones from Macaronesia islands, instead of the NE Atlantic lineages. A total of 2171 new sequences ( 1012 COI, 307 16S, 320 ITS and 532 28S) were added to the reference libraries (GenBank and BOLD systems) and will be publicly available upon publication in peer-reviewed journals. Upon minute morphological examination of the specimens, it become apparent that several lineages with obvious morphological differences have been overlooked in the literature, being commonly misidentified to the morphologically closer described species. Morphological stasis was challenged, since it appears that the older the ancestral split resulting from the different geological event periods, the higher is the probability of finding slight phenotypic disparities in cryptic lineages, previously thought to be morphological identical. Evidence for this can be seen in the deep divergence between major phylogenetic clades within some of the analysed species complex, and the perfect match of each clade to the specific morphological variation (e.g. complexes within Perinereis, Platynereis and Eulalia). In spite of these contributes, the analyses indicated that only $11 \%$ of the existing Phyllodocida species have DNA barcodes publicly available. Naming molecular lineages which lacked enough specimens with structural integrity, further sampling in subtidal regions and additional bio-informatic tools to explore the cryptic phenomena from an evolutionary and phylogeographic point of view is desirable in future works.


Keywords: Cryptic species; Integrative taxonomy; Phylogeography; Polychaetes; Systematics

## Resumo

Técnicas moleculares têm vindo a ser eficazes na sinalização de diversidade oculta em espécies com uma ampla distribuição geográfica. Nesta tese, membros dos Phyllodocida coletados ao longo das costas europeias foram utilizados como um táxon modelo para investigar espécies crípticas, usando uma combinação de dados moleculares multi-locus (mtCOI-5P, rRNA16S, regiões ITS e rRNA28S), morfológicos e morfométricos. Este estudo identificou um grande número de linhagens cripticas não descritas em 6 morfo-espécies distintas: Eumida sanguinea (22 Unidades moleculares taxonómicas operacionais - MOTUs); Eulalia clavigera (9 MOTUs); Hediste diversicolor(5 MOTUs); Platynereis dumerilii (10 MOTUs); Perinereis cultrifera (14 MOTUs) e Trypanosyllis zebra (10 MOTUs). No total, foram descobertas 70 linhagens, das quais 43 aparentam ser exclusivas deste trabalho. Cinco dessas morfoespécies têm nesta tese um capítulo dedicado, onde uma abordagem integrativa permitiu a descrição de 13 novas espécies para a ciência e a remoção de ambiguidades em relação a descrições anteriores. As ilhas da Macaronésia e a parte ocidental do Mar Mediterrâneo, são hotspots de especiação críptica, tendo-se encontrado um total de 10 e 30 linhagens únicas para cada região, respetivamente. MOTUs mediterrâneos aparentam ser geneticamente mais próximos das ilhas da Macaronésia, com as linhagens do Nordeste Atlântico aparentando ser mais distantes. Um total de 2171 novas sequencias ( 1012 COI, 307 16S, 320 ITS e 532 28S) foram adicionadas às bibliotecas de referência (GenBank e BOLD) e estarão disponíveis publicamente após publicação. Ao examinar mais detalhadamente o grau real de semelhança morfológica entre algumas destas supostas linhagens crípticas, fica claro que um numero considerável possui diferenças morfológicas que foram negligenciadas e erroneamente identificadas. A estase morfológica foi desafiada, uma vez que parece que quanto mais antiga a divisão ancestral resultante dos diferentes períodos geológicos, maior é a probabilidade de encontrar pequenas disparidades fenotípicas em linhagens que inicialmente aparentavam ser morfologicamente idênticas. A evidência disso pode ser vista na divergência profunda entre os principais clados filogenéticos em alguns dos complexos aqui analisados e a combinação perfeita de cada clado com uma variação morfológica especifica (por exemplo, nos complexos Perinereis, Platynereis e Eulalia). Além do mais, verificou-se neste estudo que apenas $11 \%$ das espécies existentes na ordem dos Phyllodocida têm códigos de barra de ADN disponíveis ao público. Linhagens moleculares por nomear, mais amostragens em regiões subtidais e ferramentas bioinformáticas adicionais são necessárias para continuar a explorar este fenômeno críptico do ponto de vista evolutivo e filogeográfico.

Palavras-chave: Espécies crípticas; Filogeografia; Poliquetas; Sistemática; Taxonomia integrativa

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## Abbreviations

| ft | Nucleotide diversity |
| :---: | :---: |
| ${ }^{\circ} \mathrm{C}$ | Celsius |
| $\mu \mathrm{l}$ | Microlitre |
| $\mu \mathrm{m}$ | Micrometre |
| a.a. | Amino acid |
| ABGD | Automatic Barcode Gap Discovery |
| ANOSIM | One-way analysis of similarity |
| BI | Bayesian Inference |
| BIN | Barcode Index Number |
| BOLD | Barcode of Life Data System |
| bp | DNA Base pair |
| bPTP | Poisson Tree Processes |
| COI | Cytochrome c oxidase subunit I |
| CPR | Checklist Progress Report |
| DNA | Deoxyribonucleic Acid |
| et al. | Et alia |
| F | Forward reads |
| GTR | General Time Reverse model |
| GYMC | Generalized Mixed Yule Coalescent |
| h | Number of haplotypes |
| Hd | Haplotype diversity |
| HKY | Hasegawa-Kishino-Yano |
| HMDS | Hexamethyldisilazane solution |
| ITS | Internal transcribed spacer |
| K2P | Kimura-2-parameters |
| JTT | Jones-Taylor-Thornton |
| MAC | Macaronesia islands |
| MDS | Multidimensional scaling |
| MED | Mediterranean Sea |
| ML | Maximum likelihood |


| MOTUs | Molecular Operational Taxonomic Units |
| :--- | :--- |
| mtCOI-5P | $5^{\prime}$ ' end of the mitochondrial cytochrome c oxidase subunit I gene corresponding to the |
|  | barcode region |
| $n$ | Number of specimens or sequences |
| NE | North East |
| NEA | North East Atlantic |
| NJ | Neighbour-joining |
| PCA | Principal Component Analysis |
| PCR | Polymerase Chain Reaction |
| PRIMER-E | Plymouth Routines in Multivariate Ecological Research |
| R | Reverse reads |
| rDNA 28S | Large subunit (LSU) 28S Ribosomal Ribonucleic Acid |
| RESL | Refined Single Linkage |
| rRNA 16S | Small subunit 16S Ribosomal Ribonucleic Acid |
| S | Segregating sites |
| SEM | Scanning electron microscopy |
| SIMPER | Similarity Percentages analysis |
| s.s. | Sensu stricto |
| sp. nov. | Species nova |
| TCS | Templeton, Crandall and Sing method |
| WoRMS | World Register of Marine Species |
| WPD | World Polychaeta Database (a subset of WoRMS) |
| WRiMS | World Register of Introduced Marine Species (a subset of WoRMS) |

## Chapter 1

General introduction

### 1.1 Integrative taxonomy

Carl Linnaeus introduced the practice of using the binomial nomenclature system to name species. The description and classification of most eukaryotic species is typically rooted in the anatomical body plan and unique morphological features observed in the specimens. The naming of new species follows a strict protocol according to the International Code of Nomenclature by which species have unique binomial scientific names (a generic name plus a specific name) and are linked to type specimens (from type localities) preserved in museum collections (Polaszek, 2005; Zhang, 2008). Species are the central unit for taxonomy and a fundamental reference of biological information, with the majority of the questions regarding evolutive biology, ecology, biogeography and conservation biology being highly dependent of species inventories and its associated metadata (Savage, 1995; Dayrat, 2005). Morphological approaches have been underpinning the description and naming of thousands of species every year (Polaszek, 2005; Zhang, 2008), but in the last decade it became evident that the existent biodiversity is much greater than the available operational infrastructure and human resources dedicated to its study (Costa and Antunes, 2012).

By themselves, morphology-based approaches have some limitations which hinders the sustainable progress of cataloguing all biological diversity, a task of enormous complexity and massive scale. They are time consuming and lack appropriate funding (Radulovici et al., 2010). The difficulties, often linked to unreliable species diagnosis, include a shortage of taxonomic experts, the use of incomplete identification keys and the collection of degraded or damaged specimens caused by sampling techniques (Knowlton, 1993; Hebert et al., 2003). Moreover, taxonomic ambiguities and uncertainties are frequently generated by the presence of complex life stages, sexually dimorphic species or those with large phenotypic plasticity and cryptic or hidden complexes (Jarman et al., 2000; Bickford et al., 2007; Ekrem et al., 2007; Nygren, 2014). In particular, the marine benthic invertebrate fauna possess great morphological complexity and it is estimated that most of its diversity is still yet to be discovered (Radulovici et al., 2010; Mora et al., 2011; Pamungkas et al., 2019). Besides invertebrates, most of the species-level diversity and a significant proportion of biomass is concentrated in taxonomically poorly known taxa like bacteria, insects and fungi, that are often referred to as "dark taxa" (Hausmann et al., 2020), or as "dark taxon impediment" (Meier et al., 2021). Because of the resulting taxonomic impediment and the current progress in classifying life (Bouchet 2006) the predicted timeframe for an inventory of marine biodiversity alone is more than 1000 years. Considering also the rates of biodiversity loss, it is evident that many species will go extinct before we even know they existed (Mora et al., 2011).

These inherent limitations to morphology-based identifications signal the need of an alternative or complementary approaches for species recognition, discovery and delimitation. The rapid development of molecular methodologies provided researchers the opportunity to clarify many ambiguities in the traditional taxonomy (Hebert et al., 2004; Jörger et al., 2012). Small fragments from the mitochondrial (mtDNA) and nuclear genes encoding ribosomal RNA have been the most commonly used for inferring species phylogenies and define species boundaries. They are easily accessible, have primers of broad taxonomic scope available and enable the discrimination of closely related species, making these loci very important to investigate systematics questions (Wakeley, 2004). These markers, which have substitutions that are considered selectively neutral or of little or no functional consequence to the organism (Kimura, 1983), have a degree of polymorphisms proportional to the underlying rate of mutation (Drake et al., 1998). This has the potential to provide resolution across multiple time scales, with different genes displaying different evolutionary rates (Hillis 1987). Nuclear markers, however, usually have a lower substitution rate and consequently a slower evolution compared to the mitochondrial ones, making them more suitable to resolve deeper phylogenetic nodes preferentially (Moriyama and Powell, 1997). In particular, the mtCOI is being used as a universal molecular marker (DNA barcoding) for the identification of animal life (Hebert et al., 2003a). Since the mitochondria are present in large copy numbers in each cell, thus becoming easy to amplify when DNA is degraded or from small amounts of tissue, mtDNA markers are usually preferred to nuclear DNA. Additionally, indels are absent or infrequent and the existence of standard protocols including a large array of available primers, makes the mtDNA with a higher probability of being amplified in a wide range of species (Hebert et al., 2003a; Ratnasingham \& Hebert, 2013). Lastly, it possesses a higher evolutionary rate and due to maternal inheritance, there is generally no recombination (Galtier et al., 2009).

For the past decades, an agreement was established where species are separately evolving lineages of populations or metapopulations (Wiley, 1978), with disagreements remaining only about where separate lineages should be recognized as distinct species along the divergence continuum (Hey, 2006; Mallet, 2008). This allow taxonomy to be integrated with new data and methodologies of population biology, phylogenetics, and other evolutionary disciplines (Sites and Marshall, 2004; Tan et al., 2010). A detailed investigation combining morphological characters, identification keys, phylogenetic analyses with multiple molecular markers, and ecological data is currently accepted as the long-term goal in the taxonomic study of most organisms, which can produce high quality species hypotheses (Will et al., 2005; Burns et al., 2008; Pante, Schoelinck \& Puillandre, 2015; Janzen et al., 2017).

### 1.1.1 Minimalist revision approach

Some authors claim integrative taxonomy is not enough to tackle the taxonomic impediment, at least in particular groups of hyper diverse and understudied taxa (Meierotto et al., 2019; Sharkey et al., 2021b). To address this issue, Meierotto et al. (2019) endorse a minimalist approach based on DNA barcodes to be employed in the description and naming of tens of thousands of insect species of Ichneumonoidea and many other undescribed species-rich taxa, often found with molecular data. Each description consists of a short diagnosis based solely on COI barcode nucleotide differences, a lateral image of the specimen, and the holotype specimen information required by the International Code of Zoological Nomenclature. Similarly, Sharkey et al. (2021a), proposed the "minimalist revision" methodology to serve as a first pass approach that can later be completed with additional information, to describe 403 new species in 11 subfamilies of Costa Rican braconid parasitoid wasps, based on barcode clusters ("BINs") computed by BOLD Systems (Ratnasingham and Hebert, 2013). These authors argue that there are too many species and too little time, as well the many difficulties patent in biodiversity assessment using current approaches, giving several examples, one of which, regarding the marine faunal inventories usually failing to identify one third of specimens to the species level when using morphological methods (Meierotto et al., 2019; Sharkey et al., 2021a). Criticisms of these protocols were soon echoed (Zamani et al., 2021; Meier et al., 2021), citing issues such as: ignoring previously described species; the proved unreliability and instability of BINs as a single molecular operational taxonomic unit (MOTU) delineation method for certain taxa and cryptic lineages; lack of multi locus approach to test congruence between nuclear and mitochondrial markers to better reinforce the species units identified; and possible COI disadvantages like introgression, lineage sorting and maternal inheritance of mtDNA which can obfuscate the species-level signal (Will and Rubinoff, 2004; DeSalle et al., 2005). Furthermore, the COI protein is under strong stabilizing selection in closely related taxa and is characterized by synonymous substitutions, with modifications concentrated mostly in the 3rd positions for an evolutionary change at the DNA level (Roe \& Sperling, 2007; Kwong et al., 2012; Pentinsaari et al., 2016). Meier et al. (2021) suggested that nucleotide fixation in the 3rd position is likely caused by genetic drift with COI distances between BINs and closely related species mostly measuring time of divergence, which is only correlated but may not be identical to the probability of speciation. All of this could cause a large number of possible superficial species descriptions which can only be resolved by consulting type specimens ("superficial description impediment"), thus creating a new impediment by trying to solve an existing taxonomic impediment (Meier et al., 2021).

Sharkey et al. (2021a) argues that if a taxonomist wishes to integrate additional information or diagnoses to any of the reviewed taxa in their study, they will have a great starting point and resources by using the existing first pass barcode-based descriptions. This is possible because the method is described as "an iterative approach to solve the taxonomic impediment of megadiverse and undertaxonomically resourced groups that standard technical and biopolitical approaches have not been able to tackle". The authors insist that effective morphological keys may be written and old museum specimens may regain their value to go along with their barcodes, when a large number of specimens, from a wide geographic range, are barcoded (e.g., Janzen et al., 2017). Furthermore, accordingly to Sharkey et al. (2021b) morphological descriptions, old type specimens scattered in museums (often impossible to sequence due to tissue degradation) and keys are not useful when the COI barcode is the only reliable source for identification. An example of slight morphological variations in two insect species was given by the former mentioned study, questioning how one can decide if such features reflect intraspecific variation or are evidence of distinct species, especially when there are only a few available specimens, if no molecular data is used to signal the existence of two independent species.

Undoubtedly, hyper diverse taxa can be problematic and a middle ground between the minimalist revision and critics could be established in the near future to solve this issue. For example, COI-only protocols could be viable but instead of just using BINs, multiple MOTU delineation methods could be applied. To minimize the overestimation of species, a multi locus approach could be used only when uncertain BINs with low COI divergence (e.g. when compared to previously established species from the same family) and/or no consensus MOTUs are found. But, as pointed out by Meier et al. (2021), the disadvantages created by the superficial description impediment may outweigh the advantages offered by the minimalist revision in the quest to solve the dark taxon impediment. Further $21^{\text {st }}$ century solutions that could help tackling the taxonomic impediment are also already available, such as large throughput imaging and sequencing (Hebert et al., 2018; Ärje et al., 2020; Wührl et al., 2021; Srivathsan et al., 2021), from the fields of machine learning and integrative species delimitation (Solis-Lemus et al., 2015; Favret and Sieracki, 2016) and frameworks to pair eDNA metabarcoding and morphological approaches (Pereira et al. 2021). Moreover, data can now be analysed with increasingly sophisticated algorithms that will provide taxonomists with a solid foundation for species descriptions that can be based on multiple sources of data (Puillandre et al., 2012; Hartop et al., 2021). Meier et al. (2021) suggest that such data will be particularly suitable for generating automatic species descriptions that are resilient and future ready.

### 1.2 Polychaeta (Annelida) and the order Phyllodocida

The phylum Annelida, corresponding to the segmented worms, is currently composed of two valid classes (Polychaeta and Clitellata) according to the World Register of Marine Species (WoRMS, http://www.marinespecies.org/index.php, Read and Fauchald, 2020). WoRMS follows the classification of Rouse and Pleijel (2001), but recent phylogenomic insights about the Polychaeta (marine worms) provided a new picture of annelid phylogeny. Besides a basal grade comprising taxa such as e.g. the polychaete families Chaetopteridae, Oweniidae, and Magelonidae, the vast majority of polychaetes form a major clade "Pleistoannelida" (Struck, 2011), with Errantia and Sedentaria as the highest ranked sister groups. This clade is defined as the last common ancestor of the polychaete subclasses Errantia and Sedentaria, with the latter containing the former polychaete subclass Echiura and the former class Clitellata (leeches and earthworms), which unlike polychaetous annelids, are characterized by the presence of a clitellum - the 'collar' that forms a reproductive cocoon during part of their life cycles. Several groups formerly placed outside the phylum Annelida, e.g. Sipuncula, grouped with the Annelida as well. Because of this, WoRMS partially integrated the new data in the website by making some adjustments to the classification of Rouse and Pleijel (2001). The subclass Errantia is now substituting the former Aciculata and the subclass Sedentaria include the former Canalipalpata and Scolecida. Furthermore, a temporary superclass (Annelida incertae sedis) was erected to accommodate taxa corresponding to the several groups formerly placed outside Annelida phylum, albeit not including the Sipuncula. Additionally, the interstitial annelid families (including former "archiannelids"), together with some other taxa of obscure or Polychaeta-basal affinities are now awaiting assignment under the temporary subclass "Polychaeta incertae sedis". However, WoRMS still assigns the Clitellata as a distinct class from the polychaetes and suggest that Pleistoannelida may be superfluous if Polychaeta is retained.

Polychaetes, have two pre-segmental regions, the prostomium and the peristomium, with sensory and/or feeding appendages, a segmented trunk (metastomium), that may differentiate into the thoracic and abdominal regions, and a post-segmental pygidium. Typically, each segment has a pair of parapodia associated with chaetae, or only a few chaetae arising directly from the tegument. However, this class of marine invertebrates have high morphological variability (Glasby et al., 2000) and in some cases can present unusually high COI congeneric genetic divergences ( $>25 \%$ ) almost as high as within family distances (Lobo et al., 2016), reflecting the diversity of lifestyles found among these animals. Polychaetes also exhibit a wide variety of reproductive strategies which can be sexual or asexual. They are typically dioecious, but many species are also hermaphrodites. Many of the distinguishing features among
polychaetes are absent or reduced in smaller and interstitial animals, which makes their affinities with the respective family obscure (Glasby et al., 2000).

The first polychaetes were formally named by Linnaeus (1758) and since then thousands of these organisms have been described. According to Rouse and Pleijel (2001), the number of accepted species reaches 9,000, however, a few thousand more were meanwhile named, while others are currently considered invalid. A more recent review (Pamungkas et al., 2019) of the discovery progress of polychaete worms (Annelida) based in the WoRMS, found that 11,456 valid species of recent polychaetes (1417 genera, 85 families) have been named by 835 first authors since 1758 . Over this period, three discovery phases of the fauna were identified: the initial phase (from 1758 to mid-nineteenth century) where nearly 500 species were described by few taxonomists; the second phase (from the 1850's to mid-twentieth century) where almost 5,000 species were largely described by some very productive taxonomists; and the third phase (from the 1950's to modern times) in which about 6,000 species were described by the most taxonomists ever. Pamungkas et al. (2019) also noted that the six polychaete families with the most species were Syllidae (993 species), Polynoidae (876 species), Nereididae (687 species), Spionidae (612 species), Terebellidae ( 607 species) and Serpulidae ( 576 species). Yet still many more remain undiscovered or waiting to be described, as this group represent an important component in the diversity of marine animals. This fact is exemplified in studies on the variety of polychaetes in small areas. For example, Grassle and Grassle (1974) found 1441 polychaete specimens on a single piece of coral that weighed only a few pounds. These polychaetes were separated into 103 groups of nominal species which represented two thirds of all macrofauna collected. A few years later, studies on the diversity of deep-sea polychaetes have shown a similar pattern, namely in terms of dominance of individuals and taxa (e.g. Grassle and Maciolek, 1992; Patterson et al., 1998). In particular, the number of undescribed polychaetes found in some studies, e.g. 64\% by Grassle and Maciolek (1992). Accordingly to Pamungkas et al. (2019), their modelling predict that 5,200 more annelid species will be discovered between 2019 and the year 2100, with the total number of polychaete species of the world by the end of this century anticipated to be about 16,700 species.

Polychaetes play an important role as trophic links of food chains functioning as predominant prey for many species with ecological and conservational relevance (e.g. fish, birds and mammals), and are as well responsible for prominent ecosystem functions such as nutrient cycling and ecosystem engineering through bioturbation and bioirrigation activities due to the holes created by their locomotion in the sediment (Kristensen et al., 1985; Volkenborn et al., 2007). Many species can also be used as
bioindicators of environmental quality and have a considerable economic weight in the fishing industry serving as fish bait (Glasby et al., 2000; Scaps, 2002).

The order Phyllodocida is the largest and one of the most phylogenetically diverse among polychaetes (Nygren, 2014; Ravara et al., 2017). It is considered monophyletic with strong molecular support (Struck et al., 2011; Weigert and Bleidorn, 2016), and presents unique morphological features such as the ventral position of sensory palps, the presence of anterior enlarged cirri, the loss of dorsolateral folds, the presence of an axial muscular proboscis and the presence of compound chaetae with a single ligament (Rouse and Fauchald, 1997). Currently, more than 6,600 species-level taxa are part of the Phyllodocida, of which, around 4,627 are considered valid in WoRMS, belonging to 27 families and 566 valid genera. These organisms can be found from marine benthic environments, to brackish waters, freshwater and even terrestrial areas, being more prevalent in the former. In the latter case, the environments are usually moist, often completely flooded, so the terrestrial nature of these taxa is more apparent than real (Glasby et al., 2000). Several families are also holoplanktonic (Jumars et al., 2015). Benthic phyllodocids can live in different habitats, from intertidal and subtidal depths to the deep sea (Rouse and Pleijel, 2001), including extreme environments such as hydrothermal vents (McCowin and Rouse, 2018; Wu et al., 2019). Usually present in the surface of the seabed, either attached to objects on the bottom or free-moving, burrowing in muddy and sandy bottoms, under rocks, mixed sediments, or hiding in crevices in hard surfaces (Rouse and Pleijel, 2001). Their dietary habits can be diverse, but most members are predators feeding on other invertebrates, including other polychaetes. Some species are also herbivorous, carrion-feeders and filter feeders, instead (Jumars et al., 2015).

### 1.3 Cryptic species

The definition of cryptic species is usually accepted as two or more morphological very similar species but genetically distinct (Nygren, 2014). However, this definition has some level ambiguity, as there is no way to measure the degree of the morphological similarity that characterizes a cryptic complex. The term "pseudo-cryptic" can be used to distinguish between new undescribed similar species that can still present micro morphological variations such as coloration (Nygren and Pleijel, 2011; Lindsay and Valdés, 2016), morphometry (Ragionieri et al., 2009; Martin et al., 2017) or even small deviations in particular morphological characters (Barroso et al., 2010; Cerca et al., 2020), from true cryptic species which are apparent identical between each other (Álvarez-Campos et al., 2017; Langeneck et al., 2020). Still, discussions about the reference levels of 'crypticitism' are common and often do not find a common ground. For example, several peracaridean species were initially assumed to be complexes of species,
but some degree of morphologic variation was later found. However, this variation is not enough to consider them as different species accordingly to some authors (e.g., Krapp-Schickel and Vader 1998; Bruce and Holdich, 2002; Vader and Krapp-Schickel, 2012). For this thesis purpose, all the new molecular lineages which are phylogenetically close to each other and apparently belong to a described morphospecies, will be considered a cryptic complex.

The taxonomic challenge posed by cryptic species and by using morphological characters alone has been recognized for some time, but integrative taxonomy with the emergence of relatively inexpensive and rapid DNA sequencing to complement traditional morphological identifications has provided biologists with a proficient tool for detecting and discriminating morphologically similar species (Bickford et al., 2007). Even in well-known animal groups like mammals, as much as $60 \%$ of the newly described species since 1993 derived from cryptic complexes (Ceballos and Ehrlich, 2009). In some cases, with the help of molecular data, the morphological, ecological and behavioural differences, that were once overlooked, could be elucidated after further examination of divergent taxa (Hebert et al., 2004). Understanding all biodiversity is fundamental for ecological research and a key factor in maintaining a healthy environment, interpreting biogeographical patterns and predicting or detecting climate change induced events (Nygren, 2014). The occurrence of cryptic complexes appears to be particularly frequent in polychaetes, one of the most prominent bioindicator groups among the marine benthic invertebrates (Brasier et al. 2016). However, many of these are not formally described following their discovery (Egge and Simons, 2006; Lobo et al., 2016; Delić et al., 2017), staying as unnamed species that rarely are taken into consideration in biological research and conservation programs (Bickford et al., 2007; Pante et al., 2015).

Multiple studies using molecular data already showed cases of cryptic diversity among the Phyllodocida that were thought to be cosmopolitan species like, e.g. Notophyllum foliosum (Sars, 1835) (Nygren et al., 2010), Eurythoe complanata (Pallas, 1766) (Barroso et al., 2010) or the Eumida sanguinea (Örsted, 1843) complex, the latter comprising up to ten putative species (Nygren and Pleijel, 2011). A screening of shallow water and deep-sea species from the NE Atlantic, using cytochrome oxidase I sequences (COI / DNA barcodes), revealed potential hidden diversity in multiple species, as for example Phyllodoce madeirensis Langerhans, 1880, which displayed three deeply divergent lineages (Ravara et al., 2017) or Trypanosyllis zebra (Grube, 1860) which was distributed among four lineages, diverging in average 27\% (Lobo et al., 2016). Notably, the ragworm Hediste diversicolor displayed an exceedingly complex pattern of COI diversity, with outstanding levels of intraspecific molecular diversity compared to other polychaetes and marine invertebrates (Virgilio et al., 2009; Lobo et al., 2016; Tosuji et al., 2019), with at least 64 unique haplotypes found around Europe (Virgilio et al., 2009). Specimens collected in
different estuaries of Portugal could be assigned to seven different MOTUs, with high intraspecific differences (> $6 \%, K 2 P$ ), sometimes even within the same estuary and collection site (Lobo et al., 2016).

Morphological stasis has been pointed to as a possible justification for morphological similarities within cryptic complexes, wherein some members retain a high degree of morphological similarity over extended periods (Costa and Carvalho, 2010; Cerca et al., 2020b). Although it has been investigated by combining comprehensive data on genomic and phenotypic traits to statistically test for significant differences in rates of phenotypic disparity between cryptic and non-cryptic species (Struck et al., 2018), stasis remains a controversial issue in evolutionary biology (Crossman et al., 2016; Fraïsse et al., 2016; Fišer et al., 2018). Morphological characters and their variation are important to identify and discriminate specimens and species; therefore, their absence is often interpreted as a potential failure to capture and study biodiversity (Futuyma, 2010). Finding new cryptic lineages and combining molecular tools with occasional small morphological trait changes in lineages displaying stasis is essential to help comprehend this evolutionary phenomenon.

### 1.4. Study area

Estuaries and coastal areas harbour a high diversity of benthic invertebrates, where polychaetes are one of the most dominant taxa (Sousa et al., 2006; Sousa et al., 2008). The Northeast Atlantic is home to many Phyllodocida species (Rouse and Pleijel, 2001; Pleijel, 1993), often with evidence of cryptic complexes (Lobo et al., 2016). Possible occurrence of cryptic diversity within the same apparent morphospecies spread across other European regions is highly likely, but still understudied (Bianchi et al, 2012; Vieira et al., 2021). It has been suggested that future cryptic species research should focus on appropriately designed case-studies using combined approaches. Moreover, large-scale bulk sample analyses using high-throughput sequencing and improvement of the DNA barcode reference libraries, may also contribute to answer the pending biodiversity questions (Nygren, 2014). A fair number of polychaetes with presumed cosmopolitan distributions have been revealed as complexes of cryptic and pseudo cryptic species, often displaying geographically restricted distributions, where the range of the cryptic lineages is typically smaller than the parent morphospecies (Hutchings \& Kupriyanova, 2018; Cerca et al., 2018). The speciation of these lineages is usually linked either to local environmental adaptation or, in most cases, to the evolutionary geodynamic events of each region.

Unravelling this biodiversity will prove vital not only for the general ecological research but also to the construction and improvement of the DNA barcode reference library for polychaetes in Europe, that together with high-throughput sequencing technologies can be applied in large scale biomonitoring
programmes under the Water Framework Directive (2000/60/EC) and Marine Strategy Framework Directive (2008/56/EC).

### 1.4.1 North East Atlantic

Originated during the break-up of Pangea in the Jurassic Period, the North Atlantic Ocean englobes the area in the Northern Hemisphere, between the continents of Africa and Europe with the American continent (Seton et al., 2012). This region suffered several climatic oscillations during its history, leading to a rapid cooling in the late Eocene changing the seascape from subtropical to temperate and cold. Such environmental changes impacted local fauna and led to biological diversification (Golikov and Tzvetkova 1972). Later, additional taxa invaded the North Atlantic across the Arctic basin, in a period of climatic warming allowing a successful trans-Arctic dispersal due to the opening of the Bering Strait (Vermeij, 1991). Further changes in the marine biodiversity were influenced by the relatively recent Quaternary glaciations (2.8 MY, Maggs et al., 2008), during the glacial and interglacial phases (Wares and Cunningham, 2001; Bianchi et al., 2012). Such periods were characterized by the presence of isolated ice-free areas that may have allowed pockets of diversity to persist (Stewart and Lister, 2001; Rowe et al., 2004; Provan and Bennett, 2008). These glacial refugia are areas where taxa evolved and survived in unfavourable periods, with organisms of the same kind being either extinguished in surrounding areas or retracted to more favourable locations in the south (Andersen and Borns, 1994). The Last Glacial Maximum (LGM, 20000 years ago) was the last period in which Europe was covered by massive ice sheets, and when large parts of the continental shelf were uncovered due to low sea levels resulting from the glacial formations (Maggs et al., 2008).

### 1.4.2 Mediterranean Sea

The Mediterranean Sea is a semi-enclosed intercontinental sea encircled by the Atlantic Ocean on the west, Asia on the east, and separating Europe from Africa. It is the deepest (average 1460 m ) and largest (2969.000 km²) enclosed sea on Earth (Mannino et al., 2017). The western end of this Sea connects with the Atlantic Ocean by the narrow and shallow channel of the Strait of Gibraltar, which is roughly 13 km wide at its narrowest point. This strait is fundamental for the circulation and productivity (the rate of generation of organic matter) of the Mediterranean, an extremely oligotrophic sea, largely due to a poor nutrient supply (Dugdale and Wilkerson, 1988). The northeast extremity is connected with the strait of the Bosporus through the Marmara Sea (Boxer et al., 2019). On the other end, in the South
eastern part of the sea, the Suez Canal provides an artificial navigable connection to the Red Sea (Mannino et al., 2017).

The Mediterranean is currently affected by different pressures, mainly driven by human activities such as climate change and bio invasions, with hot dry summers and low input from rivers making it a concentration basin (Pérèz and Picard, 1964; Mannino et al., 2017). Estimations of the number of marine species living in the region were already attempted (Bianchi and Morri, 2000; Boudouresque, 2004; Coll et al., 2010), showing a variation of 8500 to 17,000 macroscopic species, with one quarter of the whole Mediterranean biota being endemic to the region (Tortonese, 1985; Fredj et al., 1992; Giaccone, 1999). However, such numbers may still be heavily underestimated since even in conspicuous, best known and popular taxa such as fish, more than 100 Mediterranean species were added to the collection in just 30 years (1980 to 2010, Coll et al., 2010). It is expected that the increment in numbers for possible unreported species to be even larger, either for smaller and little-known taxa or undersampled habitats such as submarine caves and deep waters (Bianchi et al., 2012).

Although the Mediterranean Sea is only $0.82 \%$ in surface area and $0.32 \%$ in volume of the world ocean, the region harbours somewhere between 4 and $18 \%$ of the world's marine species, with large differences according to the phylum under consideration (Bianchi and Morri, 2000). Apart from the high rate of introduction of exotic species in this area (Zenetos et al., 2008; Galil, 2009), paleogeographic events and ecological features of the different basins within the Sea, may explain the high biodiversity found in such a small portion of the planet (Boudouresque, 2004; Lejeusne et al., 2010; Coll et al., 2010). The role of the alternating glacial and interglacial stages has been often suggested as a possible reason for the "biodiversity pump" in the Mediterranean (Bianchi et al., 2011). Sub-tropical and boreal species introduced from the Atlantic to the Mediterranean may have also experienced reduced gene flow between populations. Complete isolation of Mediterranean populations is known to have occurred during the Messinian event, when the Gibraltar strait closed due to low sea levels, around 5.96 to 5.33 million years ago (Bianchi et al., 2011; Hupało et al., 2019).

### 1.4.3 The Macaronesian Biogeographic Region

As defined by the European Environment Agency, the Portuguese archipelagos of Madeira, Savage isles and Azores, and the Spanish archipelago of the Canary Islands comprises the Macaronesia biogeographic region. These volcanic islands are present in the Northeast Atlantic Ocean, off the coast of the Iberian Peninsula and North Africa (Fernández-Palacios and Dias, 2001; Fernández-Palacios, 2010). The Macaronesia also include the Cape Verde archipelago, which is not part of the European Union, and
was not considered in this thesis. Recent studies also suggest that Cape Verde differs significantly from the other Macaronesian archipelagos and appears to be a sub province within the West African Transition province (Freitas et al., 2019).

The Macaronesia islands were formed at different geological times, with the oldest island, Selvagem Grande, arising 27 Million years ago (MYa) (Geldmacher et al., 2001) and the newest, Pico island in the Azores, only 0.27 MYa (Carine and Schaefer, 2010). They are hundreds of kilometres apart at distances from the continental shores varying from 96 to 1500 km , possess a range of unique geological and climatic conditions and their biota is shaped through dispersal from other sources after island formation (Cowie and Holland, 2006). This makes Macaronesia and other volcanic islands natural laboratories for evolutionary diversification as well for natural extinction processes (Valente et al., 2014). Additionally, the Paleo Macaronesia are a group of seamounts which were former islands when sea levels were lower; e.g. Seine and Dacia, which emerged around 22 and 47 MYa from the Madeira and Canarian volcanic provinces hotspots, respectively (Fernández-Palacios et al., 2010). Available plate tectonic scenarios (Smith et al., 1994; Scotese, 2004) suggest that when these seamounts, located closer to the Iberian Peninsula, were still islands, they may have been affected by the east-to-west warm circumequatorial marine current that flowed through the Tethys Sea. This could have facilitated the colonization of these former islands from Iberia and North Africa. Furthermore, these islands could have served as stepping stones for colonization of the present day configuration of Macaronesia (Ávila, 2000; Juan et al., 2000; Fernandez-Palacios et al., 2015).

### 1.5. Aims and structure of the thesis

The main goal of this thesis was to investigate overlooked cryptic species within the order Phyllodocida (Annelida, Polychaeta) in subtidal or intertidal rocky shores from Portugal, Mediterranean Sea and along NE Atlantic coastal areas. Two of the most dominant families in these geographic areas and habitat: Nereididae and Phyllodocidae, were the focus of the research, in which was employed an integrative taxonomic approach. By thoroughly examining the incidence of cryptic species among families of this "cryptic-rich" order, this thesis aim to use it as a model to gain insights into this still poorly understood evolutionary phenomenon. More specifically, the objectives of the present thesis were:

- Analyse all the public COI generated data and access the worldwide DNA barcode coverage for the Phyllodocida species present in the BOLD platform
- To clarify taxonomic ambiguities and detect potential hidden or cryptic diversity
- Formally describe additional cryptic lineages discovered either in unknown or well-established species complexes using a combination of a multi locus approach, complemented with either biogeographic data, morphometric data, drawings or microscopic pictures.
- Improve our knowledge of the biodiversity and true geographic distribution of the different cryptic lineages of European polychaetes species
- To contribute to the understanding of the role of Macaronesia islands and the Mediterranean Sea in the diversification and evolution of annelids

This thesis is divided in 9 chapters, seven of which (Chapters 2 to 8) consist of the studies performed in the scope of this thesis and organized in individual sections (Abstract, Keywords, Introduction, Material and Methods, Results, Discussion and Conclusions/Final Remarks). Chapters 2 to 7 each correspond to one or more published articles in an indexed peer-reviewed international scientific journal or manuscripts in preparation to be submitted, which are listed further bellow. Chapter 2 was part of a larger review study on the diversity of Phyllodocida in collaboration with other authors, where each author was responsible for a specific topic within the Special Issue, with Chapter 2 contributing with the molecular gap-analysis section of the paper. Additionally, molecular data for the Trypanosyllis zebra complex was generated under the scope of this thesis but is only available at the appendix material (Phylogenetic trees: Figs. A1 (COI), A2 (16S) and A3 (28S-D2); Specimen data: Table A1) and was neither used for further analysis nor has a dedicated chapter.

Chapter 1 corresponds to the general introduction. Chapter 2 summarizes the current status of the publicly available DNA barcodes within the order Phyllodocida, based on data deposited in the BOLD platform. Chapter 3 updates the taxonomy and distribution of the well-known Eumida sanguinea (Phyllodocidae) species complex with twelve additional lineages, in which six of them are described as new species. Chapter 4 reports five undescribed lineages belonging to the Eulalia viridis/clavigera (Phyllodocidae) pseudo-cryptic complex, with description of three new species. Chapter 5 unveils ten new lineages within the Platynereis dumerilii (Nereididae) apparent morphotype, with a formal description to five of them. Five unknown molecular operational taxonomic units sharing some similarities to $P$. dumerilii juvenile forms were uncovered as well, which are unique either to the Mediterranean Sea or the Canary Islands. In chapter 6, a phylogeographic analysis of the common ragworm Hediste diversicolor (Nereididae) is performed, together with the description of two additional species from the five lineages composing this cryptic complex. Chapter 7, reveals Perinereis cultrifera (Nereididae) as a complex of thirteen divergent evolutionary lineages, with the Macaronesia islands and the Mediterranean Sea
emerging as hotspots of cryptic diversity. Chapter 8 uses the revealed species complexes in this thesis, to further explore the phylogeny and perform a comparative analysis on the divergence patterns between populations in continental Europe and Macaronesia. Lastly, Chapter 9 consists in the global appraisal of the thesis, with the concluding remarks and future perspectives.

This thesis is not to be regarded as a publication in the sense of the International Code of Zoological Nomenclature (ICZN, 1999; 2012), and scientific names mentioned in it should not be cited in any form.

Seven articles have been produced on the course of this PhD thesis, which have been published or will be submitted for publication in due course:

## Chapter 2

Martin D., Aguado M.T., Fernández Álamo M.-A., Britayev T.A., Böggemann M., Capa M., Faulwetter S., Fukuda M.V., Helm C., Petti M.A.V., Ravara A., Teixeira M.A.L. (2021). On the Diversity of Phyllodocida (Annelida: Errantia), with a Focus on Glyceridae, Goniadidae, Nephtyidae, Polynoidae, Sphaerodoridae, Syllidae, and the Holoplanktonic Families. Diversity , 13, 131. https://doi.org/10.3390/d13030131.

## Chapter 3

Teixeira M.A.L., Vieira P.E., Pleijel F., Sampieri B.R., Ravara A., Costa F.O., Nygren A. (2020). Molecular and morphometric analyses identify new lineages within a large Eumida (Annelida) species complex. Zoologica Scripta. 49, 222-235. https://doi.org/10.1111/zsc.12397.

Teixeira M.A.L., Vieira P.E., Ravara A., Costa F.O., Nygren A. (2020). From 13 to 22 in a second stroke: revisiting the European Eumida sanguinea (Phyllodocidae: Annelida) species complex. Zoological Journal of the Linnean Society. 196, 169-197.
https://doi.org/10.1093/zoolinnean/zlab100.

## Chapter 4

Teixeira M.A.L., Vieira P.E., Pleijel F., Langeneck J., Sampieri B.R., Hernandez J.C., Ravara A., Costa F.O., Nygren A. Revealing the diversity of the green Eulalia (Annelida, Phyllodocidae)
species complex along the European coast, with description of three new species. Submitted to Organisms Diversity \& Evolution.

## Chapter 5

Teixeira M.A.L., Bakken T., Vieira P.E., Langeneck J., Sampieri B.R., Kasapidis P., Ravara A., Nygren A., Costa F.O. The curious and intricate case of the European Hediste diversicolor (Annelida, Nereididae) species complex, with description of two new species. Accepted in Systematics and Biodiversity.

## Chapter 6

Teixeira M.A.L., Langeneck J., Vieira P.E., Hernandez J.C., Sampieri B.R., Kasapidis P., Mucciolo S., Bakken T., Ravara A., Nygren A., Costa F.O. Reappraisal of the hyperdiverse Platynereis dumerilii (Annelida: Nereididae) species complex in the North Atlantic, with the description of two new species. Accepted in Invertebrate Systematics.

## Chapter 7

Teixeira M.A.L., Langeneck J., Vieira P.E., Hernandez J.C., Sampieri B.R., Kasapidis P., Bakken T., Ravara A., Nygren A., Costa F.O. Large cryptic hotspot in the Mediterranean: the striking case of the Perinereis cultrifera (Annelida: Nereididae) species complex. To be submitted.

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Chapter 2

# On the Diversity of Phyllodocida (Annelida: Errantia), gap-analysis and annotated reference library 


#### Abstract

The large amount of molecular data being generated in the past decade can be very useful for several different analysis ranging from metabarcoding, to complementing species identification or even in large scale biomonitoring projects. DNA reference libraries, such as the Barcode of Life Data System (BOLD) or GenBank, are thus essential for these tasks, however, due to incompleteness, its general application can be limited. One of the main goals of this chapter was to assemble a comprehensive reference library of DNA barcodes for the worldwide Phyllodocida species, to assess gaps in species coverage and examine data ambiguities. First, a checklist for the Phyllodocida based on WoRMS was compiled, comprising 27 families, 566 genera, 4680 species, and 161 subspecies. Then, a total of 6,361 public DNA sequences of the cytochrome coxidase subunit I barcode region (COI-5P) were compiled in a BOLD dataset corresponding to 620 Phyllodocida species. Taxonomic discordances were evaluated and cases of deep intraspecific divergence flagged. The main findings revealed that the number of DNA barcodes assigned to different taxa levels among Phyllodocida was highly variable, with $59.5 \%$ having species names, $11.9 \%$ having only genus names, $8.8 \%$ having family or subfamily names, $1.4 \%$ having just the order assigned, and $18.4 \%$ barcodes with user generated tag codes added to the species. Additionally, 1,215 Barcode Index Numbers (BINs) were assigned to the dataset, of which 34\% showed no apparent taxonomic conflict (i.e., concordant), $6.7 \%$ had taxonomic conflicts (i.e., discordant), $44.1 \%$ of the records were singletons (single barcode), and $15.2 \%$ of the BINs include possible cryptic complexes. Furthermore, by using only the records identified with species names and comparing it to the Phyllodocida checklist, only $10.26 \%$ of the species (480) and $0.62 \%$ of the subspecies (1) from the checklist have barcodes. Of this, the family Syllidae held the highest number of sequenced species (138) representing $12.35 \%$ of its species in the checklist. Nereididae displayed the lowest level of completion (11.2\%), while Glyceridae had the highest percentage (28\%). Polynoidae, Nereididae, Phyllodocidae and Syllidae also appear to be the most afflicted families with multiple BINs, some of which displaying cryptic evidence.

The library here analysed still has considerable gaps, numerous poorly represented species, and potential misidentifications or other errors in barcode generation. The proportion of species flagged for possible cryptic diversity was also considerable, which certainly merits further analyses.


Keywords: Annelida; Phyllodocida; DNA barcoding; reference library; cytochrome c oxidase subunit I

### 2.1 Introduction

Phyllodocida are among the most phylogenetically diverse groups of organisms and the largest order among the annelids (Nygren et al., 2014; Ravara et al., 2017). The key roles this group play in marine ecosystems makes it a demanding component for morphology-based biomonitoring (Borja et al., 2000). Moreover, molecular tools are also being increasingly integrated in regular and large-scale biomonitoring initiatives thanks, for instance, to high-throughput sequencing technologies (Leese et al. 2018; Pennisi 2019). However, to achieve their full potential, the creation and constant improvement of DNA barcode libraries is an essential task to support species identification. Together with the emergence of DNA metabarcoding and eDNA-based approaches for ecological and biological research (Deiner et al. 2018), the need to update molecular libraries becomes crucial (Weigand et al., 2019) not only for already known species, but also for the remarkable hidden diversity that is being continuously revealed with the support of molecular data (Nygren and Pleijel, 2011; Delić et al., 2017; Fišer et al., 2018). Taking this into account, this chapter aims at analysing all public Barcode of Life Data System (BOLD, Ratnasingham and Hebert, 2007) data to assess the worldwide DNA barcode coverage for the species of the order Phyllodocida. This will allow to evaluate taxonomic uncertainties, as well as to analyse species phylogenetic diversity, to improve DNA metabarcoding studies at the taxonomic assignment step (Weigand et al., 2019) and to highlight the existing knowledge gaps and the main still pending taxonomic revisions.

### 2.2 Material and methods

### 2.2.1 Species lists

All taxa lists were compiled and kindly provided by Sarah Faulwetter (University of Patras, Greece): A list of species and subspecies for Phyllodocida were downloaded from the World Polychaeta Database (WPD; Read and Fauchald, 2020) on 06-09-2020, using the Worrms library (Chamberlain, 2019) in R 3.6.1 (R Foundation, Vienna, Austria) (R Core Team, 2013). Subsequently, taxa with an unclear taxonomic status (nomen nudum, interim unpublished, temporary name, uncertain, taxon inquirendum) were excluded. Alternative representations of names were treated as objective synonyms (all data and scripts available via figshare, DOI: https://doi.org/ 10.6084/m9.figshare.13678570, posted on 2 March 2020). A list of non-indigenous species and their regions of introduction was compiled from the World Register of Introduced Marine Species (WriMS) (Ahyong et al., 2020) and additional literature sources (Çinar, 2013; Faulwetter et al., 2017; Keppel et al., 2015, 2019; López et al., 2017; Langeneck et al., 2020a).

Non-indigenous species are defined as "species introduced outside of their natural range (past or present) and outside of their natural dispersal potential" (Olenin et al., 2010).

### 2.2.2 Data mining and BOLD Dataset creation

The list of selected taxa of Phyllodocida mentioned above was upload to BOLD (Ratnasingham and Hebert, 2007) (CL-MTVPP, DOI: https://doi.org/10.6084/m9.figshare.13678570, posted on 02-$03-2020$ ), comprising 27 families, 566 genera, 4680 species, and 161 subspecies. The list of species considered non-indigenous (CL-MTAPP, DOI: https://doi.org/10.6084/m9.figshare. 13 678570, posted on 2 March 2020), containing 13 families, 44 genera, 62 species, and one subspecies was uploaded to BOLD as well. BOLD platform was used to search for all the publicly available COI-5P sequences belonging to Phyllodocida, including from GenBank, to create the dataset DS-MTAPP (DOI: https://doi.org/ 10.5883/DS-MTAPP, posted on 02-03-2020) for the analysis. A species was considered successfully barcoded if at least one COI-5P sequence (>300 bp) was available. COI sequences without information on species name and with less than 300 base pairs, lacking BINs and flagged for contamination, stop codons or indels were disregarded. The initial dataset contained 11,799 sequences corresponding to 1,418 species. However, only 7,831 barcodes (from 830 species) were publicly available. Using the methods described above a final dataset was obtained (also used for statistical analyses) that included 6,361 DNA barcodes from 620 species ( 3,509 exclusive to BOLD and 2,852 mined from GenBank making). Since most GenBank records lack metadata (e.g., GPS coordinates, depth), GenBank-only records were excluded from the species list to generate a new dataset with 3,509 records that was also uploaded to BOLD (DS-MTBPP, DOI: https://doi.org/10.5883/DS-MTBPP, posted on 02-03-2020) to analyse bathymetric patterns in barcode availability.

### 2.2.3 Data processing and analyses

A global gap-analysis was conducted by comparing the available barcoded species of Phyllodocida by 04-04-2020 and its congruence with the total number of valid species (Weigand et al., 2019; Leite et al., 2020; Duarte et al., 2020). The species list CL-MTVPP was compared with all publicly available COI5 P sequence records using the BOLD checklist tool to obtain the percentage of barcoded species. Only the records identified at the species level were included and those with tag codes added by BOLD users were discarded. Tag codes are often used either to distinguish lineages within cryptic complexes or between different populations in certain BOLD projects to make use of the several available analytical
tools in the platform. As such, these records are considered as different species by the Checklist Progress Report (CPR) tool in BOLD. Thus, they will not match with the corresponding species found by the CPR tool (e.g., 'Nereis pelagica CMC01' will be considered a different species from 'Nereis pelagica').

All species in the dataset had a Barcode Index Number (BIN, Ratnasingham and Hebert, 2013). These BINs were annotated with one of four possible taxonomic congruency grades: Discordant (i.e. more than one nominal species assigned to the same BIN, which often include conflicts with sequences of species labelled with tag codes), Complex (i.e., one nominal species assigned to more than one BIN), Concordant (i.e., one species assigned to a single BIN) and Singletons (nominal species with just one available sequence). A careful inspection was performed in the Discordant BINs by checking their placement in Neighbouring-joining (NJ) phenograms, looking for valid species names, synonyms or contaminations, and by inspecting BINs' content on BOLD database. BINs were considered as "Complex" when the same species had more than two sequences for at least two different BINs and were close to each other in the phylogenetic tree. Also, if the same species have two BINs with more than two sequences and a third BIN with one sequence, the third BIN was considered as part of the complex as well, instead of a singleton. The BIN system clusters COI sequence data into Molecular Operational Taxonomic Units (MOTUs) independent of prior taxonomic assignment. As such, it allows us to confirm barcode sequence clusters vs. species designations concordance. This validation was performed by comparing the taxonomy on input records against all others in the same BINs, including those submitted and managed by other users (Ratnasingham and Hebert, 2007). The worldwide barcode map based on georeferenced data was built with the dggridR package in R. The accumulation Curve tool within BOLD was used to visualize the total number of sequences, species and BINs over time, for the whole order and for each family of Phyllodocida. Further data analyses were represented by histogram and pie charts created with Microsoft Excel.

### 2.3 Results

### 2.3.1 Barcode availability

A total of 620 species of Phyllodocida have 6,361 sequences published in BOLD, while the total number of BINs is 1,215 (Fig. 2.1.A). The discordance between sequences and BINs is caused by the assignment of some sequences to higher taxonomic ranks (genus or family), but also to wrong taxonomic assignment.


Fig. 2.1. (A) Accumulation curve using all records from the dataset DS-MTAPP. The number of species and number of BINs by number of published/public sequences submitted to BOLD over time from 2008-2019. (B) The number of available sequences per family; records lacking family assignations (unknown) correspond to sequences only identified at the order level. (C) The number of species, BINs, and the total number of sequences for the most represented families.

In terms of number of sequences per family, the Polynoidae took the largest share (24\%), followed by the Nereididae (20\%), Phyllodocidae and Syllidae ( $11 \%$ each), Hesionidae (10\%), Nephtyidae and Glyceridae (4\% each), and only $2 \%$ are identified at order level (Fig. 2.1.B, C). All remaining families (excluding Nautiliniellidae -presently within the Chrysopetalidae- and Pisionidae that are currently not accepted in WoRMS) represent 14\% of the total (Fig. 2.1.B). However, the Syllidae held the highest number of sequenced species and Polynoidae, Nereididae, Phyllodocidae and Syllidae also appeared as the most afflicted with multiple BINs (Fig. 2.1C).

### 2.3.2 Barcode progress report

The number of DNA barcodes assigned to different taxa levels among Phyllodocida was highly variable (Fig. 2.2.A), with 3,787 ( $59.5 \%$ ) having species names, 754 ( $11.9 \%$ ) having only genus names, 559 (8.8\%) having family or subfamily names, and 94 (1.4\%) having just the order assigned. In turn,

1,169 ( $18.4 \%$ ) barcodes had tag codes added to the species name. However, only the records of sequences associated with species names could be compared against the worldwide Phyllodocida species-level list (CL-MTAPP), which results in only $10.26 \%$ of the species (480) and $0.62 \%$ of the subspecies (1) from the species list having barcodes by 04-04-2020 (Fig. 2.2.B). Using the same approach, $32.63 \%$ (185) of the genera and $78.57 \%$ (22) of the families were represented with DNA barcodes (Fig. 2.3.B, C). Overall, from the 6,361 sequences, it was only possible to analyse 4,917 barcodes, which imply that there are at least 1,400 sequences misidentified and/or with invalid, misspelled or synonymized names. As mentioned above, Polynoidae and Nereididae had by far the highest number of representative sequences. However, at the same time they are also by far the families showing the lowest level of completion (Fig. 2.3.A, $10.5 \%$ and $11.2 \%$, respectively). Conversely, the Glyceridae and the Nephtyidae doubled these numbers ( $28 \%$ and $26 \%$, respectively). When analysing the information at the species level, the Syllidae was the richest family, with $26.7 \%$ (138) of the sequenced species, while the Glyceridae was the poorest ( $4.8 \%, 25$ species). These data are still more informative and the lack of knowledge may be better assessed if taking into account the extremely disparate number of valid taxa of these families: 1117 for Syllidae, 926 for Polynoidae, 736 for Nereididae, 89 for Glyceridae and 154 for Nephtyidae.


Fig. 2.2. (A) The number of DNA barcodes with species names, barcodes identified only at the genus, family and subfamily, order and barcodes with tag codes added to the species name. (B) The number of barcoded records with species name present in the list of Phyllodocida (CL-MTAPP).


Fig. 2.3. (A) The percentage of barcoded species and species still missing molecular data for the most represented families based on the list of Phyllodocida (CL-MTAPP). (B) The percentage of barcoded genera. (C) The percentage of families with DNA barcodes. Records identified only at the order were discarded

### 2.3.3 Barcode distribution and BIN discordance report

As for the biogeographic distribution, although the total number of sequenced species in the DSMTAPP dataset having georeferenced coordinates is certainly still very low (only 4,145 records), barcoding in Phyllodocida showed similar biogeographic trends (Fig. 2.4) as those reported for the taxa and a similar bias. Most records came from North America $(2,382)$, followed by Southeast Asia (688) and Europe (484), there is also a considerable amount that have unspecific locations (358). As for the number of BINs (Fig. 2.5), from a total of 1,215 , most of them (220 species, 34\%) showed no apparent taxonomic conflict (i.e., concordant), while there were taxonomic conflicts (i.e., discordant) for 108 species (6.7\%). Moreover, although 44.1\% of the records (i.e., 500) were singletons (i.e., having just a single barcode), a significative number of them were identified only at the genus/family level or had tag codes. Thus, this analysis proved that there were only 257 species identified at the species level and having a single
available sequence, while 35 "species" ( $15.2 \%$ of the BINs) were possible cryptic complexes (See Table S2.1 for the species with multiple BINs considered "Complex").


Fig. 2.4. Worldwide barcode distribution for the Phyllodocida using the dataset DS-MTBPP


Fig. 2.5. Number of barcode index numbers (BINs) according to congruency grades. Concordant: The number of BINs with no apparent taxonomic conflict; Discordant: taxonomic conflict within BINs; Singletons: BINs with just one single barcode record; Complex: one species assigned to more than one BIN.

### 2.3.4 Bathymetric barcode patterns and exotic species

From the DS-MTBPP dataset (3,509 barcodes from 277 species), only 1,666 sequences were identified at the species level (and had no tag codes) allowing to analyse the respective bathymetric trends. Accordingly, barcoding appeared to be mostly available for shallow areas (Fig. 2.6.A), while deepsea species showed a significantly low number of sequences bellow 100 m depth. Some non-native species of Phyllodocida listed in CL-MTAPP have been upload to BOLD indicating that they are considered invasive in certain areas (Table 2.1). However, the total number of barcoded alien Phyllodocida is relatively low (24, ca. $40 \%$ ), and only four of them have been sequenced in the location reported as being "invaded" (Fig. 2.6.B, Table 2.1).



Fig. 2.6. (A) Number of species with barcode and number of BINs. Values on the top of each bar refer to the total number of sequences. (B) Number of barcoded species belonging to reported alien species found in literature

Table 2.1. Non-native species. PO: possible origin; AOI: area of introduction; BNN: number of sequences (seq) barcoded non-native areas; BOA: number of sequences (seq) barcoded in other areas.

| Family | Species | PO | AOI | BNN | BOA | Source |
| :---: | :---: | :---: | :---: | :---: | :---: | :---: |
| Nereididae | Alitta succinea | ?NW Atlantic | Australian |  | USA Atlantic (8 seq) | Ahyong et al. 2020 |
|  |  |  | Exclusive |  |  |  |
|  |  |  | Economic Zone |  |  |  |
| Nereididae | Alitta succinea |  | Argentina |  |  | Cinar, 2013 |
| Nereididae | Alitta succinea |  | Caribbean Sea |  |  | Ahyong et al. 2020 |
| Nereididae | Alitta succinea | ?NW Atlantic | Hawaii |  |  | Cinar, 2013 |
| Nereididae | Alitta succinea | ?NW Atlantic | Japan |  |  | Cinar, 2013 |
| Nereididae | Alitta succinea | ?NW Atlantic | South Africa |  |  | Cinar, 2013 |
| Nereididae | Alitta succinea | ?NW Atlantic | USA Pacific |  |  | Cinar, 2013 |
| Nereididae | Alitta virens | ? | Baltic Sea |  | Russia Arctic (3 seq) | Cinar, 2013 |
| Nereididae | Alitta virens | ? | North Sea |  | USA Atlantic (50 seq) | Cinar, 2013 |
| Syllidae | Amblyosyllis speciosa | Japan | USA Pacific | $x$ (3seq) |  | Cinar, 2013 |
| Chrysopetalidae | Bhawania goodei |  | Mediterranean Sea |  | USA (1 seq) | Ahyong et al. 2020 |
| Syllidae | Branchiosyllis exilis |  | Aegean Sea |  | Western Australia (1seq) | Ahyong et al. 2020 |
| Syllidae | Branchiosyllis exilis | Indo- <br> Pacific/Red Sea | USA Pacific |  |  | Cinar, 2013 |
| Phyllodocidae | Eumida sanguinea | ?NE Atlantic | Hawaii |  | NE Atlantic (29seq) | Cinar, 2013 |


| Family | Species | PO | AOI | BNN | BOA | Source |
| :---: | :---: | :---: | :---: | :---: | :---: | :---: |
| Syllidae | Eusyllis kupfferi |  | Cypriote part of the Mediterranean Sea - Eastern Basin |  | Western Australia (1seq) | Ahyong et al. 2020 |
| Glyceridae | Glycera capitata | E Atlantic | Black Sea |  | Arctic Russia (9 seq) and Canada (10 seq); India (1seq.) | Cinar, 2013 |
| Nephtyidae | Inermonephtys inermis |  | Red Sea |  | China (3seq) | Cinar, 2013 |
| Nereididae | Leonnates decipiens | Indo-Pacific | Mediterranean |  | India (1seq) | Cinar, 2013 |
| Nereididae | Namalycastis abiuma | Indo-Pacific | Hawaii |  | China (2seq); India (5seq) | Cinar, 2013 |
| Nereididae | Neanthes acuminata | W Atlantic | USA Pacific | $x$ ( 54 seq ) | Portugal (5seq); Hawai (1seq), USA Atlantic (5 seq.); Pacific Mexico (6 seq.) | Cinar, 2013 |
| Paralacydoniidae | Paralacydonia paradoxa | Mediterranean | Red Sea |  | China (18 seq) | Cinar, 2013 |
| Polynoidae | Paralepidonotus ampulliferus | Indo-Pacific | New Zealand |  | No GPS data (3seq) | Cinar, 2013 |
| Nereididae | Perinereis aibuhitensis | ?Korea | Japan |  | No GPS data (7 seq); China (1seq) | Cinar, 2013 |
| Nereididae | Perinereis aibuhitensis | Indo-Pacific | Portugal |  |  | Cinar, 2013 |
| Nereididae | Perinereis nuntia | Indian | Mediterranean |  | Indonesia (1seq.) | Cinar, 2013 |

(Table 2.1. Continuation)

| Family | Species | PO | AOI | BNN | BOA | Source |
| :---: | :---: | :---: | :---: | :---: | :---: | :---: |
| Sigalionidae | Pisione guanche |  | Turkish part of the Mediterranean <br> Sea - Eastern Basin | $\begin{aligned} & \text { x (1seq - } \\ & \text { Spain) } \end{aligned}$ |  | Ahyong et al. 2020 |
| Hesionidae | Podarkeopsis capensis |  | Turkish part of the Aegean Sea | $\begin{aligned} & \text { x (1seq - } \\ & \text { Spain) } \end{aligned}$ |  | Ahyong et al. 2020 |
| Nereididae | Pseudonereis anomala |  | Mediterranean Sea |  | Australia (21 seq) | Ahyong et al. 2020 |
| Pilargidae | Sigambra parva |  | Turkish part of the Aegean Sea |  | India (2seq) | Ahyong et al. 2020 |
| Polynoidae | Subadyte pellucida | Mediterranean | Red Sea |  | Cádiz - Spain (2seq) | Cinar, 2013 |
| Syllidae | Syllis bella |  | Lebanese part of the Mediterranean <br> Sea - Eastern Basin |  | Philipines (1seq) | Ahyong et al. 2020 |
| Syllidae | Syllis gracilis | ?Mediterranean | Argentina |  | Peru (5seq); Australia (2seq); <br> Pacific USA (12); Phillipines (8seq); <br> Italy (2seq); Spain (4seq) | Cinar, 2013 |
| Syllidae | Syllis nipponica | Japan | USA Pacific |  | Japan (1seq) | Cinar, 2013 |

(Table 2.1. Continuation)

### 2.4 Discussion

Even though the number of sequences and barcoded species have grown almost exponentially since 2008, these results highlight the apparent difficulty of having molecular data with correct identifications among Phyllodocida, with less than $60 \%$ of the records being usable to species-level in statistical analysis. Additionally, less than $11 \%$ of the compiled worldwide Phyllodocida list had barcodes. This might be partly justified by other factors, such as possible contaminations, misidentifications, outdated taxonomic identifications and synonyms. For example, two families in the BOLD dataset are now invalid (Read and Fauchald, 2020): Nautiliniellidae and Pisionidae, with the accepted names being Calamyzinae Hartmann-Schröder, 1971 (subfamily for Chrysopetalidae Ehlers, 1864) and Sigalionidae Kinberg, 1856, respectively. Also, the species Glycera tridactyla Schmarda, 1861, identified as "Glycera convoluta", a subjective synonym; or the species Sphaerodoridium minutum (Webster \& Benedict, 1887), being identified as "Sphaerodoropsis minuta", a superseded subsequent combination. Indeed, less than $80 \%$ of the species were found barcoded in the list (i.e., 481 of 620 ), while there where 81 discordant BINs and 535 singletons (Fig. 2.5). The latter are subject to high uncertainty and low confidence due to the lack of comparable sequences and sources from multiple studies. Even if all species from the analysed dataset could be found in the list, it still is a far cry compared to the current 4,627 valid species of Phyllodocida (Read and Fauchald, 2020). This could be due to the marine biodiversity assessment challenge caused by the large-scale geographical sampling effort required, which can affect community richness outcomes (Bergsten et al., 2012). However, the number of studies dedicated to this annelid group and, consequently, that of the associated barcoding projects must also be taken into account (Weigand et al., 2019). For example, in the case of fishes, the amount of dedicated projects is significantly higher and, thus, the barcode library closer to completion (Costa et al., 2012; Oliveira et al., 2016; Cariani et al., 2017), which is not the case for macroinvertebrate barcoding projects and the current state of its molecular libraries (Duarte et al., 2020).

Another incipient aspect revealed by this analysis were annelids collected in the deep sea (Fig. 2.6.A) which show a significant low number of sequences bellow 100 meters depth. Not only it is more costly to sample in such locations, but also it is often exceptionally hard to identify and sequence deepsea specimens because of tissue degradation due to the combined effect of different environmental pressures and sampling techniques (Ravara et al. 2017). Indeed, most deep-sea records of sequenced Phyllodocida, correctly identified at the species level, came from a few papers, e.g., Ravara et al. (2017) and Carr et al. (2011), which certainly indicates that further efforts must be addressed in barcoding deepsea members of the group. In addition, from the few species having specimens collected from significant
different depth levels (more than 100 m apart), three showed again possible evidence of cryptic complexes with lineages specific to each depth layer: Phyllodoce madeirensis Langerhans, 1880 (BINs: BOLD:AAZ1549, BOLD:AAZO051 and BOLD:AAZ0052 at 246, 392 and 660 m depth, respectively); Glycera kerguelensis McIntosh, 1885 (BINs: BOLD:AAA8690 and BOLD:AAA8688 at 5000 and 2000 m depth, respectively) and Eunereis longissima (Johnston, 1840) (BINs: BOLD:AAY3565 and BOLD:AAZ1159 at 300 and 700 m depth, respectively). There is a still unknown number of possible cryptic species complexes, which were inferred, in part, from BINs and records having "tag codes" usually attributed by BOLD users to differentiate between cryptic lineages. For instance, "Nereis pelagicaCMC01" and "Nereis pelagica CMCO3", which display high COI intraspecific divergence appearing on different BINs. Over the last decade, cryptic species have been increasingly reported, thereby emerging as a substantial fraction of biodiversity and as a much more widespread and frequent phenomenon than previously thought, especially in marine invertebrates (Brasier et al., 2016; Sá-Pinto et al., 2008; Vieira et al. 2019; Desiderato et al., 2019). Dedicated studies about this topic can highly increase the representativeness of sequences belonging to these groups in genetic databases. Thirty-five species were considered possible cryptic species complexes, corresponding in total to 185 BINs. Some notorious examples are Platynereis bicanaliculata (Baird, 1863) (six BINs), Treptopale homalos Watson, 2010 (seven BINs) and Pseudonereis anomala Gravier, 1899 (seven BINs). Syllis gracilis Grube, 1840 (six BINs) was already a target study for cryptic diversity (Langeneck et al., 2020b), with the authors refraining from naming the new species due to the existence of multiple lineages in the same type locality with no apparent morphological differences and the inability to access the holotype for sequencing. An extreme case with a unique genetic fragmentation by presenting intraspecific divergence higher than usual compared to other annelids ( $>3 \%$ ) but still not enough to be considered different species for most cases (<8\%) (Carr et al., 2011; Lobo et al., 2016), is that of Hediste diversicolor (0.F. Müller, 1776). It was already documented by Audzijonyte et al. (2008) and Virgilio et al. (2009), and in the present study, 140 sequences were found allocated in 37 BINS. Hediste diversicolor, together with Hediste atoka Sato \& Nakashima, 2003 ( 10 BINs in the present analysis), seem to be outliers where the number of MOTUs clearly and far surpasses the number of possible species within the complex (Tosuji et al. 2019). Overall, Polynoidae and Nereididae showed the highest number of representative sequences while having lower levels of completion ( $10.5 \%$ and $11.2 \%$, respectively; Fig. 2.3.A), which might be underestimated due to possible hidden diversity. Integrative taxonomy is thus essential to solve this kind of situations and to allow naming the involved undescribed species. Otherwise, most molecular data providing enough
support for species hypothesis (Fujita et al. 2012) will continue to be unused, and large biodiversity sections would remain unnoticed (Fontaneto et al. 2015).

The problem of cryptic species is, to some extent, intrinsically linked to the detection of exotic species. In some cases, supposedly non-indigenous or introduced species belonging to cryptic complexes. These complexes require detailed morphological studies, often combined with molecular data, to resolve the delimitation of the involved species, often leading to new species descriptions. Obviously, Phyllodocida is not an exception (Álvarez-Campos et al., 2017; Aguado et al., 2018; Gastaldi, 2019; Lindgren et al., 2019). An advantage of metabarcoding studies is the ability to easily detect invasive species in certain locations or even to report species in previously undocumented locations. However, a relatively low number (24, ca. 40\%) of Phyllodocida have been uploaded to BOLD with indications that they are considered invasive in certain areas (Table 2.1), while only two (i.e., one syllid and one nereidid) have been sequenced in the location reported as being "invaded" (Fig. 2.7, Table 2.1). In some cases, the populations from the invaded area or nearby have different sequences in each of these areas, which also differ from that in type locality. This certainly raises the question whether these species are actually nonnative or just overlooked cryptic complexes, which certainly merits further analyses.

### 2.5 Final remarks

To assess this and other complex taxonomic and biogeographic problems, recent tools, like the R-based application Barcode, Audit and Grade System (BAGS), may potentially be a valuable addition to forthcoming DNA metabarcoding studies, as it may long-term contribute to globally improve the quality and reliability of the public reference libraries. BAGS can quickly screen reference libraries to gauge data congruence and to facilitate the triage of ambiguous records for posterior review, allowing researchers to obtain the most useful and reliable data by highlighting and segregating records according to their congruency) (Fontes et al., 2021). These analyses show the key importance of keeping libraries adequately curated, together with the need of adding metadata (e.g., GPS coordinates, depth) to public databases. This is especially critical as the library here analysed still has considerable gaps, numerous poorly represented species, and potential misidentifications or other errors in barcode generation. Certainly, this opens the door to future works that will allow to obtain a more precise picture of the biodiversity within Phyllodocida and, by extension, through the whole tree of life.

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Chapter 3
From eleven to twenty-two in a second stroke: revisiting the European Eumida sanguinea (Annelida, Phyllodocidae) species complex


#### Abstract

Eumida sanguinea is a recognized polychaete species complex which, in previous studies, has been reported to have additional undescribed diversity. Eleven additional lineages were detected by analysing DNA sequences (mitochondrial: COI, 16 S rRNA and nuclear loci: ITS region and 28 S rRNA) of $E$. sanguinea morphotype populations from a broader sampling effort in European marine waters. Customary morphological features failed to provide consistent differences or unique characters that could be used to distinguish these Eumida species. However, by complementing DNA data with morphometrics, geographic range, colour, and pigmentation patterns, six new species were revealed. One of these undescribed species derived from the previously signalled Eumida lineages S21, which is now named as E. schanderisp. nov.. Five other species based on newly discovered lineages, namely E. mackieisp. nov., E. fenwicki sp. nov.., E. fauchaldi sp. nov., E. pleijeli sp. nov., and E. langenecki sp. nov. From the six new lineages remaining, three are represented by less than two exceptionally well-preserved specimens, which prevented further comprehensive analysis. The last three lineages were only distinct with mitochondrial markers. Integrative taxonomy is essential to elucidate evolutionary phenomena and eventually allow informed use of species complexes, exhibiting stasis in biomonitoring or other ecological studies.


Keywords: Phyllodocidae, Eumida, Polychaeta, Europe, molecular data, morphometrics, cryptic species

### 3.1 Introduction

The species Eumida sanguinea (Örsted, 1843) was originally described from the Danish coast (WoRMS Editorial Board, 2021) and has been commonly reported in the Atlantic northern hemisphere (Eibye-Jacobsen, 1991), including the northern Iberian Peninsula (Leite et al., 2019), as well as in Madagascar, Mozambique (Day et al., 1967), and New Zealand (Glasby et al., 2009). It is usually found in sandy-muddy substrates or gravel and among algae in shallow subtidal habitats, ranging from a few to hundreds of meters in depth (Eibye-Jacobsen, 1991), including estuaries and coastal lagoons (Walker \& Rees, 1980). As a phyllodocid, it is believed to be a carnivore (Jumars et al., 2015), but no published study has yet described its specific feeding habits. Although its planktotrophic larvae enable a large-scale dispersal (Pleijel, 1993; Rouse, 2006) its cosmopolitan status has recently been challenged. In European seas, 10 different lineages have already been reported to belong to the Eumida sanguinea species complex (Essc). By combining multi-locus molecular data with the white pigmentation pattern observed in live animals, Nygren and Pleijel (2011) defined nine of those lineages as nominal species: Eumida sanguinea s.s.; Eumida notata (Langerhans, 1880); Eumida alkyone Nygren \& Pleijel, 2011; E. asterope Nygren \& Pleijel, 2011; E. elektra Nygren \& Pleijel, 2011; E. kelaino Nygren \& Pleijel, 2011; E. maia Nygren \& Pleijel, 2011; E. merope Nygren \& Pleijel, 2011; and E. taygete Nygren \& Pleijel, 2011. The remaining putative species [Eumida F22 and Eumida S21 from Nygren and Pleijel (2011)] could not be described because only one specimen of each was available, which is not ideal, especially when the description is heavily based on molecular data (Churchill et al., 2014; Delić et al., 2017) or morphometric analyses (Ravara, et al., 2010; Martin et al., 2017). All these putative species appeared to be sympatric with at least one other species of the complex, except for E. notata that is exclusive to Madeira Island (Portugal) and possesses a unique white pigmentation pattern among the Essc.

The new EsSC illustrated in Nygren and Pleijel (2011) were described based on systematic molecular analyses, an approach applicable when there are no evident morphological differences (cryptic species). Apart from the white pigmentation pattern in live worms, the morphology of antennae, anterior cirri, and parapodia provided no consistent differences to be used to distinguish species. Moreover, all chaetae are composite within the entire genus (Pleijel, 1993). Reproductive features and gametogenesis may be a useful alternative in discriminating closely related species, as seen in Sampieri et al. (2020), in which two cryptic Laeonereis (family Nereididae) lineages were distinguished using both COI and histological data. However, specimens have to be directly preserved in a special preservation solution (e.g., $10 \%$ glutaraldehyde) instead of ethanol, which, in turn, may affect DNA amplification success.

In this study, twelve new Essc lineages were uncovered in the European NE Atlantic and Mediterranean Sea, with six of them being erected to accommodate the previously undescribed Eumida S21 (Nygren \& Pleijel, 2011) and five of them unravelled for the first time. The lineages were defined based on four different loci and supplemented by data on morphometrics, geographic range, colour, and pigmentation patterns. Furthermore, new sequences were provided for the previously described species, both from populations already located such as E. maia from Great Britain (Plymouth), E. taygete from France (Banyuls), and E. alkyone from Norway (Bergen and Drøbak), as well as unreported locations like E. kelaino from Great Britain (Plymouth), France (Roscoff), and Norway (Sandefjord and Bergen); E. merope from Great Britain (Plymouth) and France (Roscoff); E. elektra from France (Roscoff); E. sanguinea s.s. from Great Britain (Plymouth); and lastly E. taygete from Great Britain (Plymouth) and western Italy (Ischia). The close molecular similarity between some of the new lineages was discussed from an evolutionary perspective, and the ESSC case was used to investigate links between morphological stasis and cryptic diversity.

### 3.2 Material and methods

### 3.2.1 Taxon sampling, image capture, and molecular data retrieval

Two hundred and twenty-one Eumida specimens were collected from Portugal (Madeira - PTM), Norway (Agdenes - NOA; Bergen - NOB; Drøbak - NOD; and Sandefjord - NOS), Sweden (Bohuslän SWB), France (Roscoff - FRR and Banyuls - FRB), Great Britain (Plymouth - GBP and Cornwall - GBC), and Italy (Ischia - ITI; Taranto - ITT; Antignano - ITA; Naples - ITN; and Orbetello - ITO) and fixed in 96\% ethyl alcohol for molecular analysis. Photographs of live and preserved specimens were taken with a Canon EOS1100D camera. The specimens from Norway are deposited at the University Museum of Bergen (ZMBN), and the remaining ones at the Biological Research Collection (Marine Invertebrates) of the Department of Biology of the University of Aveiro (COBI at DBUA), Portugal. The two specimens of $E$. taygete, MTANE128-19 and MTANE129-19, had all their tissue used for DNA extraction purposes, and no voucher is available.

Sequences of mitochondrial cytochrome oxidase subunit I (mtCOI-5P) were obtained from all the new available 221 specimens, and mitochondrial 16 rRNA, nuclear ITS-regions (i.e., ITS1, 5.8 r rRNA, and ITS2), and 28 S rRNA for a representative number of specimens per location. For comparison purposes, a compilation of 88 published sequences from the COI and ITS-regions corresponding to the EsSc, and the respective outgroups were mined from the GenBank, originally from the study of Nygren
and Pleijel (2011). Moreover, 35 novel 16 S and 73 original 28 S rRNA sequences were retrieved during this work from specimens used in the previous study. Molecular data from Eumida bahusiensis Bergström, 1914; Eumida ocke/manni Eibye-Jacobsen, 1987; and Sige fusigera Malmgren, 1865 were used as outgroups for all alignments to comprise the final dataset. The full dataset and associated metadata, including GenBank accession numbers, can be accessed at the Barcode of Life Data Systems (BOLD), under the project "Five new species - Eumida sanguinea complex (DS-MTANE2)" and in the following link: DOI: https://dx.doi.org/10.5883/DS-MTANE2. Supplemental Table S3.1 details the sampling locations, public BIN accession numbers and voucher data for the original data. Supplemental Table S3.2 details the voucher and GenBank accession numbers for sequences used for comparison purposes from other studies.

DNA extraction was performed using either the E.Z.N.A. Mollusc DNA Kit (Omega Bio-tek) according to manufacturer instructions, or the QuickExtract™ DNA Extraction Solution (Lucigen) using 50 $\mu l$ of the reagent per tube. The tubes from QuickExtract are then transferred to a heat block at $65^{\circ} \mathrm{C}$ for 30 minutes and then additional two minutes at $95^{\circ} \mathrm{C}$. Depending of the specimen size, only a small amount of tissue or the whole animal was used. PCR reactions based on E.Z.N.A. extractions were performed in a $25 \mu \mathrm{l}$ volume containing $2.5 \mu \mathrm{l}$ of 10X PCR Buffer, $2.5 \mu \mathrm{l}$ of $25 \mathrm{mM} \mathrm{MgCl} 2,1 \mu \mathrm{l}$ of 10 mM dNTPs, $0.2 \mu$ of Taq polymerase (ThermoScientific) and $0.55-1.25 \mu$ of each primer ( 10 mM ). DNA template varied between $1 \mu$ and $4 \mu$ l. Ultrapure water was added until the final volume. PCR reactions based on QuickExtract were performed using a premade PCR mix from VWR containing 10 ul per tube of Red Taq DNA polymerase Master Kit, $0.5 \mu \mathrm{l}$ of each primer and $1 \mu \mathrm{l}$ of DNA template in a total $12 \mu \mathrm{l}$ volume reaction. Table 3.1 displays the PCR conditions and primers used.

Amplification success was screened in a $1.5 \%$ agarose gel, using either 1 or $3 \mu$ of PCR product depending of the chosen PCR protocol, with the larger volume used for E.Z.N.A. reactions. When COI or 16 primers failed to amplify the respective DNA fragment, alternative primers were used instead (see Table 3.1). Successful PCR products were then purified using the Exonuclease I and Shrimp Alkaline Phosphatase (ExoSAP, New England BioLabs) protocol, according to manufacturer instructions. Cleanedup amplicons were sent to external sequencing service suppliers (Macrogen Spain, or Eurofins Europe), for bidirectional sequencing.

Table 3.1. Primers and PCR conditions used in this thesis.


### 3.2.2 Phylogenetic analysis and genetic distances

A methodology similar to that of Nygren and Pleijel (2011) was applied for the phylogenetic analysis of the different loci by maximum likelihood (ML) and Bayesian inference (BI). In brief, mitochondrial markers (COI and 16S) were concatenated and aligned in MEGA 10.0.5 software (Kumar et al., 2018) with Clustal W (Thompson et al., 1994). Nuclear markers (ITS regions and 28S) were also concatenated and aligned with MAFFT online (https://mafft.cbrc.jp/alignment/server/; Katoh and Standley, 2013). Table 3.1 included all marker sequence lengths. Highly variable regions, extensive gaps, and poorly aligned positions, which were extensively present only in the concatenated nuclear alignment, were eliminated using Gblocks 0.91 b (http://molevol.cmima.csic.es/castresana/Gblocks_server.html; Castresana, 2000). The options for a less stringent selection and to not allow many contiguous nonconserved positions were selected, making it more suitable for phylogenetic analysis.

MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003) was used to conduct the Bayesian analysis. Best-fit models were selected using the Akaike Information Criterion in the JModeltest software (Guindon and Gascuel, 2003; Darriba et al., 2012). For COI, it was applied the Hasegawa-Kishino-Yano with gamma-distributed rates across sites (HKY +G) for the third position and the General Time-Reversible (GTR) model with equal rates across sites (GTR) for the first two positions. The latter was also applied to the 16 S analysis. Regarding the concatenated ITS region with 28S, the GTR model with gammadistributed rates across sites ( $G T R+G)$ was applied. The number of generations was set to 10000000 , and the sampling frequency to 500 . Twenty-five per cent of the samples were discarded as burn-in (burninfrac $=0.25$ ). The resulting tree files were successfully checked for convergence in Tracer 1.6 software (Rambaut et al., 2018) and then analysed in Figtree 1.4.3 (http://tree.bio.ed.ac.uk/software/figtree/). The final version of the trees for each alignment was edited with the software Inkscape 0.92 .3 (https://www.inkscape.org). Maximum likelihood phylogenies were performed in MEGA 10.0.5 with 1000 bootstrap runs, using the GTR model with equal rates across sites for both concatenated datasets. Only the BI tree was displayed in the results and, in the case of a similar topology, with the addition of the ML support values. The alignments (FASTA and NEXUS formats) for each marker and the concatenated ones are all publicly available online at Figshare (DOI: https://dx.doi.org/10.6084/m9.figshare.12114528).

The mean genetic distances (Kimura-2-parameters, K2P) within and between MOTUs were calculated in MEGA 10.0.5, using the same GBlock alignment from above for the nuclear loci.

### 3.2.3 MOTU clustering

To depict MOTUs, three delineation methods were applied to both the concatenated mitochondrial and nuclear alignments except for COI, to which the Barcode Index Number (BIN) was also applied, implemented in BOLD (Ratnasingham and Hebert, 2013), that is exclusive to this locus. The Automatic Barcode Gap Discovery (ABGD, Puillandre et al., 2012) approach was implemented on a web interface (https://bioinfo.mnhn.fr/abi/public/abgd/abgdweb.html) with default settings and K2P distance matrix. Both Generalized Mixed Yule Coalescent (GYMC) single threshold model (Fujisawa and Barraclough, 2013) and Poisson Tree Processes (bPTP, Zhang et al., 2013) were applied on a web interface (https://species.h-its.org/). BEAST 2.4.6 (Bouckaert et al., 2014) was used to generate a Bayesian ultrametric tree for the GYMC, with the appropriate best model (based on AIC criteria; GTR equal rates) and four independent runs for 50000000 MCMC generations, sampled every 5000 generations. Tracer 1.6 software was used to estimate convergence in effective sampling sizes (ESSs > 200) for all parameters. A consensus tree was obtained using TreeAnnotator 2.4.6 (Bouckaert et al., 2014) and loaded into the Figtree software. The ML phylogenies obtained in the "phylogenetic analysis" section contributed to the bPTP results. Consensus MOTUs were defined based on the majority rule and, in case of a draw, an intermediate MOTU was chosen.

### 3.2.4 Genetic diversity and structure

To evaluate the relationship between haplotypes and their geographical distribution, haplotype networks were built through the PopART software (Leigh and Bryant, 2015) using the Templeton, Crandall and Sing method (TCS, Clement et al., 2002). No GBlocks were applied in this analysis to avoid underestimating the number of nuclear haplotypes. Indices of genetic diversity, namely number of haplotypes (h), haplotype diversity (HD), polymorphic sites (S), nucleotide diversity ( $\pi$ ), and Fu \& Li D and Tajima D statistical tests, were estimated based on COI for each MOTU, using the DNASP 5.10 software (Librado and Rozas, 2009).

### 3.2.5 Morphometry

Three objectives were proposed for the morphometric analysis. The first objective (1) explore if genetically very similar species belonging to E. notata, E. merope, and the new British lineage E. aff. merope can be separated using this methodology; (2) to complement molecular results with morphometric data to help describe the new species $E$. schanderi sp. nov., $E$. fenwickisp. nov., and $E$.
fauchaldisp. nov.. Samples from E. elektra were used for comparison purposes against the new species. This species was chosen for being usually located in the middle of the phylogenetic tree and within the average Essc genetic distances. These first two objectives make use of scatter plots between pairs of morphological characters mentioned bellow. Lastly, the remaining objective (3) is to compare the new species E. mackiei, against E. notata, E. maia and E. sanguinea s.s. using Principal Component Analysis (PCA). The remaining new lineages were represented by very small and/or less than three specimens and thus were not used for this analysis. At least nine preserved specimens under ideal conditions (i.e., with the morphological characters proposed herein and, if possible, of similar sizes) were chosen per lineage for the scatter plot analysis, and a minimum of twenty-five specimens of each lineage were used for the PCA methodology. Additional preserved specimens of E. sanguinea s.s., E. notata and E. maia were kindly loaned by the Swedish Museum of Natural History (SMNH, Table S3.1) for the morphometric analysis. To reach the minimum of twenty-five specimens of each species required to perform an adequate PCA comparison (Martin et al., 2017), additional specimens from the above species were collected (see Table S3.1 for sampling locations). All the different morphological characters were measured directly from the specimen, without dissecting specific structures.

The following characters were selected and measured (Fig. 3.1.A, B): number of segments (NS); the lengths (in mm ) of worm (WL), chaetigerous lobes (CLL), terminal antennae (AL), palps (PL), median antenna (MAL), cirri on segment 1 and dorsal cirri on segment 2 (CS1L, DCS2L), dorsal and ventral cirri on median segments ( $\mathrm{DCL}, \mathrm{VCL}$ ), and head ( HL ); the widths (in mm ) of worm with parapodia (WWP) and without parapodia (WW), head (HW), and dorsal and ventral cirri of median segments (DCW, VCW); and distance between eyes (DE), as well as height (mm) of chaetigerous lobes (CLH). Although the first two segments are fused, this study refer to them as segments 1 and 2 , with the latter having a pair of cirri (dorsal and ventral). WW and WWP were measured from the worm's widest part, usually from either segment 27 or 40, depending on the worm's size. The distance between eyes was measured from the centre of the eyespots to avoid possible different individual responses to fixation as is the case of hesionids (Martin et al., 2017). All measurements were done with a LEICA MC170 HD stereo microscope, with an incorporated measurement software. Supplementary Table S3.2 shows detailed morphometric values for each specimen. To minimize bias based on size variability, measurements taken for inter-lineage analysis were converted to ratios of taxonomically relevant character proportions, i.e.: AL/CS1L, AL/DCS2L, AL/HL, AL/HW, AL/PL, AL/MAL, PL/MAL, HL/MAL, HW/MAL, CS1L/DCS2L, DCS2L/HL, CS1L/HL, PL/CS1L, PL/DCS2L, DE/HL, DE/WW, HL/HW, WW/WWP, WW/NS, WL/NS, WW/WL, HL/MAL, DCL/VCL, DCL/DCW, VCL/VCW, DCL/CLL, VCL/CLL, and CLL/CLH.


Fig. 3.1. Schematic diagrams of the $E$. sanguinea morphotype showing measurements used in the morphometric analysis. (A) anterior end. (B) parapodium. Abbreviations: CLL, the length of the chaetigerous lobes; CLH, the height of the chaetigerous lobes; AL, the length of the antennae; PL, the length of the palps; MAL, the length of the middle antenna; CS1L, cirri on segment 1; DCS2L, dorsal cirri on segment 2; DCL, the length of the dorsal cirri; VCL, the length of the ventral cirri; HL , the length of the head; WWP, the width of the worm with parapodia; WW, the width of the worm without parapodia; HW, the width of the head; DCW, the width of the dorsal cirri; VCW, the width of the ventral cirri; DE, distance between the eyes

Principal Component Analysis (PCA) was based on normalised data. The significance of the interlineage differences was explored by one-way analysis of similarity (ANOSIM) based on Euclidean distance resemblance matrices, while the contribution of each measured character to the distance within and between the four species was assessed by the Similarity Percentages analysis (SIMPER) based on Euclidean distance. Both SIMPER and ANOSIM also used the normalized proportion dataset. PCA and SIMPER analyses were conducted using PRIMER version 6.1.11, copyright by PRIMER-E Ltd. 2008 (Clarke and Warwick, 2001) and scatter plots were build in Microsoft Excel (Office 365 ProPlus).

Although not used in the above analysis due to lack of available specimens in optimal conditions to allow the creation of morphometric clusters, additional measurements were also collected for two

Italian lineages (E. pleijeli sp. nov. and $E$. langenecki sp. nov.). The ratio of common morphological structures used to separate Eumida species might provide additional information to be used as differential diagnoses against the remaining analysed lineages. Emphasis was given to: antennae, palps, cirri on segment 1 , dorsal cirri on segment 2 , dorsal cirri of median segments and ventral cirri of median segments.

### 3.3 Results

### 3.3.1 Phylogenetic reconstruction

The BI phylogenetic trees (Fig. 3.2.A, B) were created from a dataset of 297 COI, 94 16S, 192 ITS, and 28 S sequences belonging to specimens of the Essc and four outgroup species (E. bahusiensis, E. ockelmanni, S. fusigera, and E. aff. ockelmanni. Support values over 0.85 are shown in the BI trees. Since BI and ML trees display a different topology, ML bootstrap values are not shown in the BI tree.

Both mitochondrial and nuclear loci showed evidence of at least eight new Eumida MOTUs compared to the previous study, with mitochondrial markers also revealing a distinct British MOTU sister to E. merope, hereafter referred to as E. aff. merope (MOTU 11, Fig. 3.2.A); a new Mediterranean MOTU sister to E. kelaino, hereafter referred to as E. aff. kelaino (MOTU 17, Fig. 3.2.A); another British MOTU sister to E. fauchaldi sp. nov., hereafter referred to as E. aff. fauchaldi (MOTU 13, Fig. 3.2.A); and lastly an additional unnamed Italian lineage Eumida ORB997 (MOTUs 2 and 23, Fig. 3.2.A, B) close to the new species E. pleijeli sp. nov.. In total 12 new lineages were detected for the Eumida sanguinea complex.

Apart from outgroups, the number of consensus MOTUs range between 18 (Fig. 3.2B) and 22 (Fig. 3.2.A). Most of them are present either in Great Britain, Scandinavia, or southern France. The newly described species, E. fauchaldi sp. nov., is present in the British Isles and northern France (MOTU 12 and 25, Fig. 3.2.A, B); E. pleijeli sp. nov. (MOTUs 3 and 23, Fig. 3.2.A, B) and E. langenecki sp. nov. (MOTUs 5, Fig. 3.2.A, B) are both in Western Italy; E. schanderisp. nov. [previously referred to as Eumida unnamed species S21 from Nygren \& Pleijel (2011)] exclusively in Norway and Sweden (MOTUs 22 and 26, Fig. 3.2.A, B); E. fenwickisp. nov. in both Scandinavia and Great Britain (MOTU 6, Fig. 3.2.A, B), and lastly $E$. mackiei sp. nov. that seems to be unique to Great Britain (MOTU 1, Fig. 3.2.A, B).


Fig. 3.2. Phylogenetic trees reconstructed using Bayesian inference for the Essc, comparing 296 COI and 9416 S concatenated mitochondrial sequences (A) against 192 combined nuclear markers from the ITS-region and 28 S sequences (B), with information regarding the different MOTU delineation methods. BINs were used only for COI. Collapsed clades have less than $3.5 \%$ genetic divergence. Numbers in parenthesis indicate the number of sequences used for each MOTU and in the case of the mitochondrial markers the first correspond to COI and the second to 16S. Eumida ockelmanni, Eumida aff. ockelmanni, Sige fusigera and Eumida bahusiensis used as outgroups (OUTG). Only bootstrap values over 0.85 BI support are shown. Each consensus MOTU (Cons. MOTU) is represented by a unique number, with the coloured ones corresponding to the described species and new lineages found in this chapter.

The closely related species E. notata (MOTU 9, Fig. 3.2.A) and E. merope (MOTU 10, Fig. 3.2.A), including the new British lineage $E$. aff. merope (MOTU 11, Fig. 3.2.A), could be completely sorted using mitochondrial loci, forming highly supported clades in the BI tree. However, only one of the clustering algorithms could split these lineages into three distinct MOTUs by using nuclear markers, with the remaining ones being clustered together in a low supported monophyletic clade instead (MOTU 24, Fig. 3.2.B). A similar pattern is also observed between Eumida pleijeli sp. nov. and Eumida ORB997 (MOTU 23, Fig. 3.2.B), between E. aff. fauchaldi and E. fauchaldi sp. nov. (MOTU 25, Fig. 3.2.B), and between E. aff. kelaino and E. kelaino (MOTU 27, Fig. 3.2.B).

Distinct marker-dependent MOTU sorting cases are also observed for E. schanderi sp. nov., in which MOTU 26 was delimited only with nuclear markers. This sorting is recorded independently for both ITS and 28S, as evidenced in the haplotype networks detailed further below.

### 3.3.2 Genetic distances and Eumida aff. ockelmanni

Assuming E. aff. merope, E. aff. kelaino, and E. aff. fauchaldi as valid species, the global mean genetic distances for the whole complex can be found in Table 3.2, including the distances of the most similar and divergent MOTUs for the nearest and farthest neighbours, respectively. The mean intraspecific distances are $0.59(0-3.8) \%$ for COI and $0.18(0-0.8) \%$ for 16 S , while average congeneric distances are $16.7(5.5-23.4) \%$ and $7.6(0.3-15.1) \%$ respectively. The distances for ITS-region range between $0.44(0-5.8) \%$ and $9.2(0.5-18.1) \%$ for intra- and interspecific divergence, respectively, whereas for 28S, the corresponding distances are $0.06(0-0.8) \%$ and $1.7(0-4.5) \%$, respectively. The two MOTUs found in E. schanderi are responsible for the high intraspecific maximum distances reported for the nuclear loci.

At first, E. aff. ockelmanni was assigned to the Essc based on morphological similarity; however, genetic distances and BI phylogenetic tree topology signalled otherwise. The two available specimens are very small (less than 2 mm in length), which can sometimes lead to misidentifications in Eumida. Upon a more careful morphological analysis, I concluded that this MOTU is closer to the outgroup belonging to E. ockelmanni. This seems to corroborate the molecular data, in which unusually high molecular distances are observed compared to the remaining Essc. This is true especially regarding nuclear markers (maximum distances up to 38.2 and $9.7 \%$ for ITS region and 28 S, respectively), and yet much closer to E. ocke/manni (maximum distances up to 13.8 and $1.8 \%$ for ITS region and 28 S, respectively), which might indicate a species complex still undescribed for this group as well.

Table 3.2. Mean intra and interspecific genetic distances (K2P) among all the Essc for the four analysed markers (COI, 16S, ITS and 28S), with focus on the distances between MOTUs in relation to the three closest and distant neighbours

|  | Marker | MOTUs | Minimum Distance (\%) | Mean Distance (\%) | Maximum distance (\%) |
| :---: | :---: | :---: | :---: | :---: | :---: |
| Within <br> MOTUs | COI | All | 0 | 0.59 | 3.8 |
|  | 16 S |  | 0 | 0.18 | 0.8 |
|  | ITS |  | 0 | 0.44 | $5.8{ }^{*}$ |
|  | 28S |  | 0 | 0.06 | 0.8* |
| Between MOTUs | COI | All | 5.5 | 16.7 | 23.4 |
|  | 16S |  | 0.3 | 7.6 | 15.1 |
|  | ITS |  | 0.5 | 9.2 | 18.1 |
|  | 28 S |  | 0 | 1.7 | 4.5 |
| Most similar MOTUS | COI | 9 vs 11 | 7.6 | 8.2 | 8.7 |
|  |  | 9 vs 10 | 5.5 | 6.7 | 7.4 |
|  | 16S | 10 vs 11 | 9.8 | 10.2 | 10.6 |
|  |  | 10 vs 11 | 0.3 | 0.3 | 0.5 |
|  |  | 9 vs 11 | 0.4 | 0.4 | 0.5 |
|  | ITS | 9 vs 10 | 0.5 | 0.7 | 0.8 |
|  |  | $17 \text { vs } 18$ | 0.5 | 0.5 | 0.9 |
|  |  | $2 \text { vs } 3$ | 1.5 | 1.5 | 1.5 |
|  | 28 S | 12 vs 13 | 1.4 | 1.9 | 3.4 |
|  |  | $10 \text { vs } 11$ | 0 | 0 | 0 |
|  |  | 20 vs 16 | 0.1 | 0.1 | 0.1 |
|  |  | 17 vs 18 | 0.3 | 0.3 | 0.4 |
| Most distant MOTUs | COI | 1 vs 13 | 20.5 | 21.7 | 23.4 |
|  |  | 1 vs 5 | 21.1 | 22 | 22.6 |
|  | 16S | 2 vs 12 | 21.1 | 21.8 | 22.9 |
|  |  | 2 vs 15 | 15.1 | 15.1 | 15.1 |
|  |  | 2 vs 22 | 14.1 | 14.4 | 14.4 |
|  | ITS | 1 vs 15 | 13.7 | 13.8 | 14.1 |
|  |  | 1 vs 14 | 17.4 | 17.6 | 18.1 |
|  |  | 3 vs 14 | 15 | 15.1 | 15.2 |
|  | 28 S | 2 vs 14 | 15.4 | 15.5 | 15.6 |
|  |  | 2 vs 12 | 3.7 | 3.8 | 4.5 |
|  |  | 2 vs 13 | 3.5 | 3.6 | 3.7 |
|  |  | 5 vs 13 | 3.4 | 3.5 | 3.6 |

### 3.3.3 Haplotype networks

All haplotype networks (COI, Fig. 3.3; ITS, 28S, 16S, Fig. 3.4.A-C) show that the six new species, as well as the new unnamed Eumida lineages, are completely sorted from each other and the remaining Essc. This is even observed in 28 S haplotypes (Fig. 3.4.B), which is a slowly evolving gene and may fail to exhibit complete classification when others do, especially when dealing with closely related species. The only exception to this pattern is observed in the Mediterranean and British lineages from E. merope and E. aff. merope, which shared haplotypes both in 28 , and ITS loci. The low number of mutational steps between nuclear haplotypes, such as evidenced in the ITS and 28 S networks (Fig. 3.4.A, B), may be responsible for their lower phylogenetic resolution when it comes to delineating MOTUs $23,24,25$, and 27 (Fig. 3.2.B). All of the MOTUs are sympatric with at least one other MOTU within the Essc, except the MOTUs from Italy and $E$. notata.

Additionally, two distinct ITS and 28 S haplotypes for $E$. schanderi sp . nov. are found, which correspond to different MOTUs in the BI tree (22 and 26, Fig. 3.2.B). Also, two distinct groups of haplotypes for $E$. alkyone could be distinguished based on ITS alone, in which no sharing is observed between Norwegian and Swedish specimens. Three completely sorted COI haplotype groups are also found within E. taygete, splitting the Mediterranean and British populations and adding a unique shared haplotype in samples from both regions, with seven mutations apart from the remaining ones. Some of the species show comparatively little geographic sorting, especially E. sanguinea s.s., which frequently has the same haplotypes present in several different locations.

No MOTU has a central position from which every other derived in any of the networks, and a large amount of circular COI mutation paths are found mainly in E. notata, E sanguinea s.s., E. alkyone, E merope, and E. aff. merope.

Haplotype diversity within the Essc is relatively high for COI (Table 3.3), with E. fenwickisp. nov., E. mackiei sp. nov., and E. maia being the only ones with significant negative Tajima D or Fu and Li's D tests. Therefore, the population might be in expansion after a recent bottleneck or linkage to a swept gene, with the neutral model of nucleotide substitutions being accepted for the remaining MOTUs. Eumida mackiei sp. nov. and E. fauchaldi sp. nov. have the highest haplotype diversity (Hd [COI]: 0.99) and segregating sites ( $\mathrm{S}=37$ and 39 respectively).

Table 3.3. Indices of genetic diversity estimated, based on COI for each MOTU. Number of sequences (n); nucleotide diversity ( tt ), number of haplotypes (h), haplotype diversity ( Hd ) and number of variables sites (S). Values in bold are significative. Region abbreviations as stated in the methods, with the addition of: DENH, Denmark, Helsingør; CROI, Croatia, Istra and GBS, Great Britain, Scilly Islands.

|  | Region | N | h | Hd | S | t | Fu and Li's D | $\underset{\text { D }}{\text { Tajima's }}$ |
| :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: |
| E. schanderi sp. nov. | SWB, NOB | 13 | 6 | 0.72 | 4 | 0.0015 | $\begin{gathered} -1.60955 \\ P>0.10 \end{gathered}$ | $\begin{aligned} & \hline-1.43759 \\ & P>0.10 \end{aligned}$ |
| E. fenwicki sp. nov. | $\begin{aligned} & \text { NOA, GBC, } \\ & \text { GBP, FRR } \end{aligned}$ | 23 | 10 | 0,73 | 11 | 0,0022 | $\begin{aligned} & -1,33065 \\ & P>0.10 \end{aligned}$ | $\begin{aligned} & -1,87001 \\ & P<0.05 \end{aligned}$ |
| E. fauchaldi <br> sp. nov. | GBP | 26 | 24 | 0.99 | 39 | 0.0089 | $\begin{gathered} -2.10801 \\ 0.10>P> \\ 0.05 \end{gathered}$ | $\begin{gathered} -1.77253 \\ 0.10>P> \\ 0.05 \end{gathered}$ |
| $\begin{aligned} & \text { Eumida RO174- } \\ & 180 \end{aligned}$ | FRR | 2 | 2 | 1.0 | 1 | 0,0016 | - | - |
| E. aff. fauchaldi | FRR, GBP | 2 | 2 | 1.0 | 3 | 0,0048 | - | - |
| E. aff. kelaino | FRB | 2 | 1 | 0.0 | - | - | - | - |
| Eumida ANT002 | ITA | 1 | 1 | - | - | - | - | - |
| E. pleijelisp. nov. | ITN | 2 | 1 | 0.0 | - | - | - | - |
| E. langeneckisp. nov. | ITA | 5 | 5 | 1.0 | 11 | 0,0073 | $\begin{aligned} & -0,92693 \\ & P>0.10 \end{aligned}$ | $\begin{aligned} & -0,92693 \\ & P>0.10 \end{aligned}$ |
| Eumida ORB997 | ITO | 1 | 1 | - | - | - | - |  |
| E. aff. merope | FRR, GBP | 13 | 8 | 0,86 | 11 | 0,0033 | $\begin{gathered} -1,94450 \\ 0.10>P> \\ 0.05 \end{gathered}$ | $\begin{gathered} -1,70303 \\ 0.10>P> \\ 0.05 \end{gathered}$ |
| E. merope | FRB, CROI | 10 | 8 | 0.93 | 32 | 0,0060 | $\begin{aligned} & 0,19975 \\ & P>0.10 \end{aligned}$ | $\begin{aligned} & 0,84167 \\ & P>0.10 \end{aligned}$ |
| E. notata | PTM | 11 | 10 | 0,98 | 24 | 0,0092 | $\begin{gathered} -1,27851 \\ P>0.10 \end{gathered}$ | $\begin{gathered} -1,47163 \\ P>0.10 \end{gathered}$ |
| E. mackiei | GBP | 28 | 26 | 0,99 | 37 | 0,0081 | $\begin{gathered} -2,69971 \\ P<0.05 \end{gathered}$ | $\begin{gathered} -1,71525 \\ 0.10>P> \\ 0.05 \end{gathered}$ |
| E. sanguinea | SWB, NOF, <br> NOB, GBP, <br> GBS, DENH | 31 | 14 | 0,82 | 26 | 0,0073 | $\begin{aligned} & -0,06960 \\ & P>0.10 \end{aligned}$ | $\begin{gathered} -1,06336 \\ P>0.10 \end{gathered}$ |
| E. maia | GBP, GBC, FRB | 39 | 24 | 0,91 | 42 | 0,0084 | $\begin{gathered} -2,61094 \\ P<0.05 \end{gathered}$ | $\begin{gathered} -1,71386 \\ 0.10>P> \\ 0.05 \end{gathered}$ |
| E. alkyone | $\begin{aligned} & \text { NOB, NOD, } \\ & \text { SWB } \end{aligned}$ | 8 | 7 | 0,96 | 16 | 0,0082 | $\begin{aligned} & -1,09777 \\ & P>0.10 \\ & \hline \end{aligned}$ | $\begin{aligned} & -0,85599 \\ & P>0.10 \\ & \hline \end{aligned}$ |


|  | Region | N | h | Hd | S | tt | Fu and Li's D | $\underset{\text { D }}{\text { Tajima's }}$ |
| :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: |
| E. elehtra | NOB, FRR | 15 | 4 | 0,70 | 5 | 0,0030 | $\begin{aligned} & 0,48090 \\ & P>0.10 \end{aligned}$ | $\begin{aligned} & 0,76339 \\ & P>0.10 \end{aligned}$ |
| E. taygete | $\begin{gathered} \text { FRB, GBC, } \\ \text { GBP, ITI, } \\ \text { CROI } \end{gathered}$ | 22 | 18 | 0,98 | 35 | 0,0111 | $\begin{aligned} & -0,49631 \\ & P>0.10 \end{aligned}$ | $\begin{gathered} -1,07113 \\ P>0.10 \end{gathered}$ |
| E. kelaino | $\begin{aligned} & \text { SWB, GBP, } \\ & \text { NOS, NOB, } \\ & \text { FRR } \end{aligned}$ | 21 | 10 | 0,81 | 10 | 0,0038 | $\begin{gathered} -1,00506 \\ P>0.10 \end{gathered}$ | $\begin{aligned} & -0,50678 \\ & P>0.10 \end{aligned}$ |
| E. asterope | FRB | 2 | 2 | 1.0 | 1 | 0,0016 | - | - |
| Eumida F22 | FRB | 1 | 1 | - | - | - | - | - |

(Table 3.3. Continuation)

### 3.3.4 Live photographs and pigmentation data

A summary of the different white pigmentation combinations [types A to H , following Nygren and Pleijel (2011)] observed for all the species in the complex is given in Table 3.4. Live photographs of specimens exhibiting white pigmentation patterns and colour, belonging to the newly described species and unnamed Eumida lineages (RO174-180, ANT002, and ORB997), including E. aff. merope, E. aff. kelaino and E. aff. fauchaldi can be found in Fig. 3.5.A-F, Fig. 3.6.A-E , Fig. 3.7.A-C and Fig.3.8.A. Three of the six new species (E. schanderi sp. nov., E. fenwickisp. nov., and E. fauchaldisp. nov.) share type B pigmentation, which corresponds to the absence of white pigmentation. However, E. schanderisp. nov. (Fig. 3.5.A) and E. fauchaldi sp. nov. (Fig. 3.5.D) are polymorphic, with some specimens also exhibiting type $D$ (dorsally on segment 2 only, Fig. 3.5.B) and type F (Fig. 3.5.C) pigmentation, respectively (see Table 3.4). Type F pigmentation was defined by Nygren and Pleijel (2011) as a single longitudinal line of white pigmentation and erroneously assigned to one specimen designated as Eumida unnamed species S21, here named as $E$. schanderi sp. nov. This specimen presents type B pigmentation, i.e., no white pigmentation. Type F pigmentation is here redefined as white transverse dorsal lines present on most segments. Eumida fenwickisp. nov. also possesses type B pigmentation (Fig. 3.5.E), while E. pleijeli sp. nov. (Fig. 3.6.D) has a green colour with type C, characterized by the presence of a longitudinal white line together with white pigmentation dorsally on segment 2. Eumida mackiei sp . nov. presents a characteristic green colour pattern, mostly present in the anterior region, with type A pigmentation (Fig. 3.8.A), present dorsally on segment 2 and with dorsal transverse lines in most segments.


Fig. 3.3. Haplotype networks based on COI for all the Essc and respective outgroups. Each haplotype is represented by a circle whose size represents number of haplotypes according to the displayed scale; colours indicate the geographic location of the haplotype. Numbers correspond to the number of mutational steps between haplotypes. Lines without numbers mean only one mutation between haplotypes.


Fig. 3.4. Haplotype networks based on ITS (A), 28 (B) and $16 S$ (C) for all the EsSc and respective outgroups. Each haplotype is represented by a circle whose size represents number of haplotypes according to the displayed scale; colours indicate the geographic location of the haplotype. Numbers correspond to the number of mutational steps between haplotypes. Lines without numbers means only one mutation between haplotypes.

Two other pigmentation types are newly defined in this study, namely: type $G$ and type $H$. Type G refers to white pigmentation from the prostomium to the middle of the eyes of worms, similar to type E , but with the addition of small dorsal transverse white dots, which seems to be unique to $E$. langenecki sp. nov. (Fig. 3.6.C).

Table 3.4. Patterns of white pigmentation in the Eumida sanguinea species complex with eight unique combinations (types A-H). Species in bold have polymorphic pigmentation types.

| Combination Type | On Prostomium | Dorsally on Segment 2a | Dorsal Transverse Lines ${ }^{\text {b }}$ | Dorsal Longitudinal Line | Dorsal Transverse Dots | Dorsal Eye-like Pattern | Species |
| :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: |
| $A \ni$ | - | X | X | - | - | - | E. alkyone; E. maia; E. merope; E. aff. merope, E. mackiei; |
| $B \bigcirc$ | - | - | - | - | - | - | E. asterope; E. elektra; E. taygete; $\boldsymbol{E}$. fauchaldi sp. nov. ; $E$. schanderi sp. nov.; <br> E. fenwickisp. nov.; Eumida R0174-180; E. aff. fauchaldi |
| $c \bigoplus$ | - | X | - | X | - | - | E. kelaino; E. aff. kelaino; Eumida pleijeli sp. nov. |
| $D \circlearrowleft$ | - | X | - | - | - | - | E. sanguinea; E. merope; E aff, merope, $\boldsymbol{E}$. taygete, Eumida F22; E. schanderi sp. nov.; Eumida ANT002; E. elektra |
| $E \bigcirc$ | X | - | - | - | - | - | E. notata |
| $F \Theta$ | - | - | X | - | - | - | E. fauchaldi sp. nov.; E. aff. fauchaldi |
| $G \cong$ | X | - | - | - | X | - | E. langeneckisp. nov. |
| $H \rightleftharpoons$ | - | X | - | - | - | X | Eumida ORB997 |

[^0]

Fig. 3.5. Live, relaxed Eumida specimens exhibiting different types of white pigmentation patterns and coloration. (A) Eumida schanderi, specimen ZMBN_134559 (size: 3.3 mm ), with green coloration and type B pigmentation. (B) specimen ZMBN_134556 (size: 3.7 mm, holotype) with type D pigmentation and focus on the prostomium. (C) Eumida fauchaldi, specimen DBUA0002400.01 (size: 15 mm , holotype) exhibiting type F pigmentation. (D) specimen DBUA0002400.03 (size: 13 mm ) exhibiting type B pigmentation. (E) Eumida fenwicki, specimen DBUA0002396.01 (size 5 mm , holotype) exhibiting type B pigmentation. (F) Eumida RO174-180, specimen DBUA0002403.01 (size: 10 mm ) exhibiting type $B$ pigmentation. Darker yellow colour results from the stomach content.


Fig. 3.6. Live, relaxed Eumida specimens exhibiting different types of white pigmentation patterns and coloration. (A) Eumida aff. merope, specimen DBUA0002393.01 exhibiting type A pigmentation. (B) specimen DBUA0002395.02 displaying type D pigmentation. (C) Eumida langenecki, specimen DBUA0002408.01, with type $G$ pigmentation. (D) Eumida pleijeli, specimen DBUA0002407.02, displaying its characteristic green coloration mixed with type C pigmentation. (E) Eumida ORB997, specimen DBUA0002410.01, with type H pigmentation. All specimens are similar in size measuring around 12 mm , except for DBUA0002410.01 measuring around 6.3 mm .

Type H pigmentation, spotted in the currently unnamed Eumida ORB997 (Fig. 3.6.E), is defined by the presence of white pigmentation dorsally on segment 2 but with a non-white eye-like pattern dorsally between segments along the whole body of the worm. Eumida ORB997 has a very distinct pigmentation among all members of the Essc, including E. pleijeli sp. nov., even though these two species share the same nuclear MOTU.

Eumida aff. merope is also polymorphic and shares the same type D (Fig. 3.6.B) and A (Fig. 3.6.A) white pattern as the Mediterranean counterpart. Eumida aff. fauchaldi possesses both type B and F (Fig. 3.7.C) pigmentation types, following the same pattern as its sister lineage E. fauchaldi sp. nov.. Eumida. aff. kelaino has type C (Fig. 3.7.A) similar to E. kelaino, while the unnamed Eumida R0174-180 has type B (Fig. 3.5.F) and lastly the unnamed Eumida ANT002 (Fig. 3.7.C) has type D.


Fig. 3.7. Live, relaxed Eumida specimens exhibiting the different types of white pigmentation patterns and coloration. (A) Eumida aff. kelaino., specimen DBUA0002404.01 exhibiting type C pigmentation. (B) Eumida aff. fauchaldi, specimen DBUA0002401.01 displaying type F pigmentation with small transverse lines. (C) Eumida ANT002, specimen DBUA0002405.01 exhibiting type D pigmentation. All specimens are similar in size measuring around 14 mm , except for DBUA0002405.01 measuring around 3 mm .

The new British E. taygete population has an additional pigmentation (type B) compared to the Mediterranean populations (type D). E. elektra population from northern France also has a distinct pigmentation (type D) when compared to the Scandinavian populations (type B). Apart from E. schanderi sp. nov., E. elektra, E. merope, E. aff. merope, E. taygete, E. fauchaldisp. nov., and E. aff. fauchaldi, the remaining lineages of the Essc only have a single pigmentation type so far.

In total, the EsSc is composed of eight variable pigmentation types distributed among 22 distinct COI clades. Based on geographic distribution and pigmentation types, ESSC belonging species can be significantly narrowed down without using molecular data, distinguishing some only based on these criteria (see the Essc key in the taxonomic section).

### 3.3.5 Morphometric measurements

### 3.3.5.1 PCA and SIMPER analysis

PCA analysis of the morphometric proportion data individualize three distinct clusters that correspond to the species E. maia, E. notata and E. mackiei sp. nov., segregating them into three clear groups (Fig. 3.8.A). Morphometric data from specimens of E. sanguinea s.s. is scattered and partially overlapping with the three remaining species, therefore failing to produce a fully segregated group (Fig. 3.8.B). Possible justifications for this result will be discussed later. Because of this, E. sanguinea s.s. was removed from the following data analysis. Seventeen character proportions were used in the PCA discrimination (Fig. 3.8.A, B), with Axes 1 (eigenvalue $=3.93$ ) and 2 (eigenvalue $=3.45$ ) explaining 23.1\% and $20.3 \%$ of the variation, respectively, for Fig. 3.8.A. The ANOSIM test indicated significant differences between the morphometric data of the three species (Global $R=0.552$; significance level at $0.1 \%$ ).

The average morphometric variation within species was $11.41 \%$ for $E$. mackiei sp. nov., $15.12 \%$ for E. maia and $9.67 \%$ for E. notata. The average inter-species distance was $34.81 \%$ (E. mackiei sp. nov. / E. notata), $44.13 \%$ (E. mackiei sp. nov. / E. maia), and $37.27 \%$ (E. notata / E. maia). The most significant proportions for the intra-species similarity were CLL/CLH; WWP/WW; CS1L/HL; DCL/DCW; DE/HL and PL/HL for E. mackie/sp. nov.. WL/WW; CS1L/DCS2L, WWP/WW; DE/HW and CS1L/HL for E. notata. $\mathrm{DCL} / \mathrm{DCH} ; \mathrm{DE} / \mathrm{HL} ; \mathrm{DE} / \mathrm{HW}$; VCL/CLL and $\mathrm{HL} / \mathrm{HW}$ for $E$. maia with a contribution of $>7.50 \%$. Regarding the inter-species dissimilarity (Table 3.5), NS/WW; CLL/CLH; PL/HL and CS1L/HL (E. mackiei sp. nov. / E. notata); AL/PL; DCL/VCL; VCL/VCW and CLL/CLH (E. mackiei sp. nov. / E. maia), and DCS2L/HL; AL/HL; CS1L/DCS2L; AL/PL and VCL/CLL (E. notata / E. maia) were the proportions that mainly explained the differences between the respective species, also with a contribution of $>7.50 \%$.


Fig. 3.8. Principal Component Analysis (PCA) plots based on proportion data. Seventeen character proportions were used. (A) Comparison excluding Eumida sanguinea s.s., with a live photo of the new species E. mackiei (specimen DBUA0002331.12, worm length: 11.4 mm ) with the characteristic green colour pattern with type A pigmentation. (B) Comparison including E. sanguinea s.s..

### 3.3.5.2 Scatter plots

The different morphometric proportions seen in the scatter plots in Fig. 3.9.A-H are the only ones displaying significant visible differences, with the formation of independent clusters among the analysed MOTUs. A variation of either nine or ten specimens per lineage were analysed.

Table 3.5. List of the most contributing proportions to the inter-population dissimilarities based on the SIMPER analyses. Measurement abbreviations: Av.Value, average value; Av.Sq.Dist, average square distance; Sq.Dist/SD, square distance divided by standard deviation; Contrib\%, percentage of contribution; Cum.\%, cumulative percentage of contribution. Negative values are the result of normalizing the data.

|  | Av.Value <br> E.mackiei <br> sp. nov. | Av.Value E. notata | Av.Sq.Dist | Sq.Dist/SD | Contrib\% | Cum. \% |
| :---: | :---: | :---: | :---: | :---: | :---: | :---: |
| NS/WW | -1.04 | 1.02 | 4.73 | 1.63 | 13.60 | 13.60 |
| CLL/CLW | 0.862 | -0.42 | 3.08 | 0.84 | 8.85 | 22.44 |
| PL/HL | 0.737 | -0.566 | 3.05 | 0.85 | 8.76 | 31.20 |
| CS1L/HL | 0.391 | -0.511 | 2.64 | 0.75 | 7.60 | 38.80 |
| WWP/WW | 0.46 | -0.1 | 2.33 | 0.78 | 6.69 | 45.50 |
| VCL/VCW | 0.991 | -0.193 | 2.25 | 0.98 | 6.46 | 51.96 |
| WL/WW | -0.516 | 0.216 | 2.19 | 0.68 | 6.28 | 58.24 |
| HL/HW | -0.349 | 0.593 | 2.15 | 0.93 | 6.17 | 64.41 |
| DE/HL | 0.436 | -0.337 | 1.96 | 0.80 | 5.63 | 70.04 |
| DCS2L/HL | 3.21E-2 | -0.837 | 1.81 | 0.76 | 5.19 | 75.24 |
| DCL/DCW | 0.296 | 0.146 | 1.56 | 0.68 | 4.48 | 79.72 |
|  | E.mackiei sp. nov. | E. maia |  |  |  |  |
| AL/PL | 0.757 | 1.04 | 4.3 | 0.79 | 9.74 | 9.74 |
| DCL/VCL | 0.855 | 0.849 | 4.2 | 0.91 | 9.53 | 19.27 |
| VCL/VCW | 0.991 | -0.799 | 4.09 | 1.16 | 9.26 | 28.53 |
| CLL/CLW | 0.862 | -0.442 | 3.32 | 0.84 | 7.51 | 36.04 |
| DCL/DCW | 0.296 | 0.15 | 2.65 | 0.65 | 6.00 | 42.04 |
| VCL/CLL | 0.247 | -0.664 | 2.56 | 0.80 | 5.80 | 47.84 |
| WWP/WW | 0.46 | -0.36 | 2.45 | 0.74 | 5.56 | 53.40 |
| PL/HL | 0.737 | -0.17 | 2.43 | 0.72 | 5.50 | 58.90 |
| DE/HL | 0.436 | -9.96E-2 | 2.4 | 0.75 | 5.45 | 64.35 |
| CS1L/DCS2L | 0.309 | -0.711 | 2.34 | 0.86 | 5.31 | 69.66 |
| DE/HW | 0.178 | -0.377 | 2.27 | 0.75 | 5.15 | 74.81 |
|  | E. notata | E. maia |  |  |  |  |
| DCS2L/HL | -0.837 | 0.805 | 3.77 | 1.00 | 10.13 | 10.13 |
| AL/HL | 0.793 | 0.894 | 3.7 | 1.15 | 9.92 | 20.05 |
| CS1L/DCS2L | 0.403 | -0.711 | 3.05 | 0.76 | 8.18 | 28.23 |
| AL/PL | -0.279 | 1.04 | 2.97 | 0.64 | 7.96 | 36.19 |
| VCL/CLL | 0.417 | -0.664 | 2.92 | 0.81 | 7.84 | 44.03 |
| HL/HW | 0.593 | -0.244 | 2.5 | 0.77 | 6.71 | 50.74 |
| DE/HW | 0.199 | -0.377 | 2.37 | 0.67 | 6.37 | 57.11 |
| WL/WW | 0.216 | 0.3 | 2.19 | 0.75 | 5.88 | 62.99 |
| CS1L/HL | -0.511 | 0.12 | 1.87 | 0.70 | 5.03 | 68.02 |
| DE/HL | -0.337 | -9.96E-2 | 1.87 | 0.83 | 5.02 | 73.04 |
| DCL/DCW | 0.146 | 0.15 | 1.84 | 0.68 | 4.95 | 77.99 |

The use of morphometric proportions of antenna length (AL) against head length or width (HL; HW), palp length (PL), cirri on segment 1 or dorsal cirri on segment 2 (CS1L; DCS2L), and median antenna length (MAL) seems to be effective in distinguishing E. fauchaldi sp. nov., E. schanderi sp. nov., E. fenwicki sp. nov., and E. elektra from each other (main morphometric findings summarized in Table 3.5). The larger number of segments and worm length is also very distinct in E. fauchaldi sp. nov. (Fig. 3.9.G, H). The short antennal length recorded for one of the $E$. elektra specimens (around 0.158 mm ), which might be due to damages during sampling, could be the reason for the overlap with the remaining analysed species.

Even though there are not enough available specimens to form morphometric clusters for Eumida pleijeli sp. nov. and $E$. langenecki sp. nov., these species can still be described with unique features that distinguish them from the remaining Essc. To do so, a combination of pigmentation type, live colouration, and geographic range (Table 3.6) is needed and complemented with the molecular data seen above (Fig. 3.2.A, B).

As for finding possible morphometric variations between the sister lineages $E$. merope and $E$. aff. merope, the data (Fig. 3.10.A-F) reveal high intraspecific variation within E. aff. merope, whose morphometric measurements are scattered around the other analysed species for most of the proportions, except when comparing antennae (AL) and palp (PL) lengths (Fig. 3.10.B). Some partial overlaps between E. notata and E. merope are also observed. However, E. merope, E. schanderisp. nov., and $E$. fauchaldi sp. nov. seem to have palps longer than antennae. This is contrary to the remaining species analysed in this study, which either have antennae larger than palps or of the same proportion. Besides $\mathrm{AL} / \mathrm{PL}$ ratio, $E$. notata can be differentiated from E. elektra, E. merope, and $E$. aff. merope by comparing worm width (WW) with worm width with parapodia (WWP) (Fig. 3.10.A). Some morphometric clusters may also overlap with E. elektra, probably due to how genetically close this species is against the remaining analysed ones. Moreover, the mean COI distances between this species and the closest neighbours are shared with E. notata, E. aff. merope, and E. alkyone, with K2P values of 13.8, 13.5, and $12.6 \%$, respectively.

A description of the six new species can be found in the taxonomic section below, with their respective Zoobank Isid registration codes. Diagnoses based on pigmentation patterns, geographic distribution, molecular and morphometric data, and type designations are present to fulfil the requirements of the International Code of Zoological Nomenclature.


Fig. 3.9. Scatterplots with the most considerable proportions in distinguishing E. fenwicki, E. schanderi, E. fauchaldi sp. nov. and $E$. elehtra from each other. Morphometric proportions between the length of the antennae - AL and (A), cirri on segment 1 - CS1L; (B), dorsal cirri on segment 2 - DCS2L; (C), head width - HW; (D), head length - HL; (E), palp length - PL; (F), length of the middle antenna - MAL. Measurements between the number of segments - NS against $(G)$, worm width $-W W$ and $(H)$, worm length $-W L$.

Table 3.6. Summary of the most relevant morphometric findings based on scatter plots rating from 1 (smaller proportions) to 4 (larger proportions), number of segments (NS), ratio between the length and width of the dorsal cirri of median segments ( $\mathrm{DCL}>\mathrm{DCW}$ ), ventral cirri of media segments (VCL>VCW) and the length between the dorsal cirri on segment 2 against the cirri on segment 1 (DCS2L>CS1L), worm length (WL), pigmentation type, live coloration and geographical range regarding the new described species and $E$. elektra. Data in bold has the most distinct differences when combined.

|  | E. fenwicki sp. nov. | E. schanderisp. nov. | E. fauchaldi sp. nov. | E. elektra | E. pleijelis sp. nov. | E. langeneckisp. nov. |
| :---: | :---: | :---: | :---: | :---: | :---: | :---: |
| AL/PL | $2(\mathbf{A L ~ > ~ P L ) ~}$ | 1 (AL < PL) | 3 ( $\mathbf{A L}$ < PL) | $4(\mathrm{AL} \mathrm{>} \mathrm{PL)}$ | AL > PL | AL > PL |
| AL/HL | 2 | 1 | 3 (larger head) | 4 | - | - |
| AL/HW | 2 | 1 | 3 (larger head) | 4 | - | - |
| AL/MAL | 2 | 1 | 2 | 3 | - | - |
| AL/STL | 2 | 1 | 3 (larger tentacles) | 4 | - | - |
| AL/LTL | 2 | 1 | 3 (larger tentacles) | 4 | - | - |
| NS (mean) | 56 | 45 | 94 | 61 | 70 | 59 |
| DCL>DCW | 1,5x | 1.9x | 1.5 x | 1.5x | 1.5x | 1.45 x |
| VCL>VCW | 2 x | 2x | 2 x | 2 x | 1.5 x | 2x |
| DCS2L>CS1L | 2 x | 2 x | 2 x | 2 x | 2.7x | 2 x |
| WL (mean, mm) | 5 | 4 | 14 | 7 | 9 | 12 |
| Pigmentation | B | $B$ and D | $B$ and $\mathbf{F}$ | B | C | G |
| Live Coloration | Light Yellow | Greenish | Yellowish-brown | Yellowish | Green Western | Yellow Western |
| Distribution | NE Atlantic | Scandinavia | Great Britain | NE Atlantic | Mediterranean Sea | Mediterranean Sea |



Fig. 3.10. Scatterplots with the most considerable proportions in distinguishing E. notata, E. merope, E. aff. merope and E. elektra from each other. Morphometric measurements between (A), worm width - WW and worm width with parapodia - WWP. (B) antennae length - AL and palp length - PL. (C) head length - HL with antennae length - AL. (D) distance between the eyes - DE and head width - HW. (E) ventral cirri length - VCL with dorsal cirri length - DCL. (F) chaetigorous lobe length - CLL against the ventral cirri length - VCL.

### 3.3.6 Taxonomic section

## Eumida sanguinea species complex (Essc)

Diagnosis (amended from Nygren \& Pleijel, 2011)
Eumida with cordate dorsal cirri, near-symmetrical along the longitudinal axis, 1.25-1.9 times longer than wide. Colour varies between light yellow, yellowish-brown and green, distributed among eight different pigmentation types (Table 4). Small to medium-sized worm, usually between 3 to 30 mm in length and 30 to 110 segments. High intraspecific morphometric variation.

## Remarks

Eumida sanguinea species complex is an informal name for a clade that includes fifteen described species in north-east Atlantic waters (Nygren \& Pleijel 2011) including the new ones described herein, with an addition of four undescribed lineages (Eumida F22, R0174-180, ANT002, and ORB997) and three distinct mitochondrial sister lineages (E. aff. merope, E. aff. kelaino, and E. aff. fauchald). This designation should be applied for identifications based on the morphology of preserved specimens, in which white pigmentation has disappeared and no molecular data is available.

Recorded egg sizes are 85-95 $\mu \mathrm{m}$ for specimens from Danish waters (Eibye-Jacobsen, 1991) and $90 \mu \mathrm{~m}$ for specimens from the English Channel and Sweden (Pleijel, 1993). Egg sizes up to $110 \mu \mathrm{~m}$ were also observed by Nygren \& Pleijel (2011) for some members of the complex. Cazaux (1970) described the development from trochophore to newly settled stages from Bordeaux in France.

Eumida bahusiensis Bergstrom, 1914 is phylogenetically very close to Essc species and can therefore be part of it. The species can be distinguished morphologically by its broader dorsal and ventral cirri distally pointed and by the green colour with white type A pigmentation in live animals (Nygren et al. 2017). However, it can often be confused with Eumida mackiei ap. nov. which has the same background colour and pigmentation, as well as median ventral cirri, approaching the broader form of $E$. bahusiensis. The two species are genetically very distinct with $21 \%$ COI average divergence and are not sister species.

Key to Essc species based on pigmentation types and geographic distribution

This key should only be used for identifications where pigmentation and colour of live specimens were recorded. Table 3.4 displays the pigmentation types.

## 1) Scandinavia

a) Type A pigmentation ..... E. alkyoneb) Type B pigmentation
b1) Palps longer than antennae. E. schanderisp. nov.

$\qquad$b2) Palps shorter than antennaeE. elektra, E. fenwickisp. nov.(The distinction between these two species is only possible with molecular data)c) Type C pigmentation
$\qquad$ E. kelaino
d) Type $D$ pigmentation $\qquad$ E. sanguinea s.s., E. schanderisp. nov. (The distinction between these two species is only possible with molecular data)

## 2) Great Britain + Brittany, France

a) Type A pigmentation
a1) Greenish colour
a1.1) Palps as long as antennae $\qquad$ .E. mackieisp. nov.
a1.2) Palps shorter than antennae E. maia a2) Yellowish-brown colour
a2.1) Palps longer than antennae $\qquad$ E. fauchaldisp. nov.; E. aff. fauchaldi (The distinction between these two species is only possible with molecular data)
a2.2) Palps shorter than antennae
E. aff. merope
b) Type B pigmentation $\qquad$ E. taygete, Eumida R0174-180, E. fenwickisp. nov. (The distinction between these three species is only possible with molecular data)
c) Type C pigmentation $\qquad$ E. kelaino
d) Type $D$ pigmentation .E. sanguineas.s., E. elektra; E. aff. merope (The distinction between these three species is only possible with molecular data)
e) Type F pigmentation $\qquad$ .E. fauchaldisp. nov.; E. aff. fauchaldi
(The distinction between these two species is only possible with molecular data)

## 3) Madeira Island (Portugal)

a) Type E pigmentation
E. notata

## 4) Western Mediterranean

a) Type A pigmentation E. maia
b) Type B pigmentation

$\qquad$
E. asterope
c) Type $C$ pigmentation
$\qquad$c2) Yellowish colour
$\qquad$ E. aff. kelaino
d) Type $D$ pigmentation $\qquad$ E. merope, Eumida F22, E. taygete; Eumida ANT002 (The distinction between these four species is only possible with molecular data)
e) Type G pigmentation $\qquad$ E. langeneckisp. nov.
f) Type H pigmentation $\qquad$ Eumida ORB997

## 5) Eastern Mediterranean

a) Type A pigmentation
E. merope
b) Type $D$ pigmentation
E. taygete

## Eumida fenwickisp. nov.

 urn:Isid:zoobank.org:act: DA689D94-E20B-4126-8DA4-575576EB5C86Material examined
Type material. Great Britain, Cornwall: 1 spm , holotype and hologenophore, DBUA0002396.01, $50^{\circ} 21.5^{\prime} \mathrm{N}-04^{\circ} 08.9^{\prime} \mathrm{W}, 10 \mathrm{~m}$, coarse shell gravel, dredge, $16 / 03 / 2011 ; 1 \mathrm{spm}$, paratype and paragenophore, DBUA0002396.02, $50^{\circ} 21.5^{\prime} \mathrm{N}-04^{\circ} 08.9^{\prime} \mathrm{W}, 10 \mathrm{~m}$, coarse shell gravel, dredge, $16 / 03 / 2011$. The type specimens were collected by David Fenwick.

Other material. Great Britain, Cornwall: 5 spms, DBUA0002397.01-05, $50^{\circ} 06^{\prime} 12.0^{\prime \prime} \mathrm{N}$ 5³2'49.4"W, pontoon scrapings, 14/04/2015, 02/04/2015, 04/05/2015, 07/05/2015 and 28/05/2015 respectively; 1spm, DBUA0002397.06, $50^{\circ} 06^{\prime} 12.0^{\prime \prime} \mathrm{N}-5^{\circ} 32^{\prime} 49.4^{\prime \prime} \mathrm{W}$, pontoon scrapings, 13/07/2016; Norway, Agdenes: 9 spms, ZMBN_134523-134530; DBUA0002398.01, 63³5.721'N $9^{\circ} 33.100^{\prime} \mathrm{E}, 10 \mathrm{~m}$, sand, shell-fragments, dredge, 05/09/2016; 3 spms, ZMBN_134531-134533, $63^{\circ} 35.721^{\prime} \mathrm{N}-9^{\circ} 33.100^{\prime} \mathrm{E}, 2 \mathrm{~m}$, coarse gravel and rocks, dredge, 05/09/2016; France, Roscoff: 2 spms, DBUA0002399.01-02, 48044'55.2"N-354'23.3"W, 45m, gravel, 01/02/2018. British specimens were
collected by David Fenwick, while the Norwegian ones were collected by the students from the ForBio programme. All specimens are preserved in ethanol $96 \%$.

## Diagnosis

Member of Essc with type B pigmentation (Table 3.4), i.e., without white pigmentation (Fig. 3.5.E). Live specimens present light-yellowish colouration. Antennae slightly longer than palps. Proportions between antenna length and cirri length on segment 1, dorsal cirri length on segment 2, or head length and width smaller than those found in $E$. fauchaldi sp. nov. and $E$. elektra, but greater than those of $E$. schanderi sp. nov. Palp length, cirri on segment 1 and dorsal cirri on segment 2, and head length and width are considerable smaller when compared to the same length of $E$. fauchaldi sp. nov. antennae. Dorsal cirri on segment 2 usually twice as long as cirri on segment 1 . Head wider rather than longer. Dorsal cirri of median segments 1.5 times longer rather than wider. Ventral cirri of median segments twice as long rather than wider. Proboscis not observed. Worms small, usually between 3 to 10 mm long, with 45 to 75 segments.

## Molecular data

COI, 16S, ITS, and 28S sequences as in specimens DBUA0002396.01-06; ZMBN_134523 to 134533; DBUA0002398.01; DBUA0002399.01-02 (Table S3.1). Phylogenetic relationship as in Fig. 3.2.A-B, with high support values and low intraspecific ( $<3 \%$ ) genetic divergence for both mitochondrial and nuclear markers (MOTU 6). Mean interspecific COI distances to the nearest and farthest neighbours are $14.8 \%$ (K2P, E. aff. merope) and $21.1 \%$ (K2P, E. schanderisp. nov.), respectively. DOI for the species' Barcode Index Number (BIN): dx.doi.org/10.5883/BOLD:ADG3938.

Etymology
The new species is named after David Fenwick to recognize his kindness in collecting and photographing a large number of Eumida specimens on the behalf of the co-supervisor of this thesis, Arne Nygren.

## Distribution and habitat

Atlantic Ocean - from Norway to the British Isles, 2 to 10 m depth, on coarse gravel and rocks.

Remarks
Morphologically similar to E. sanguinea sensu stricto (Örsted, 1843), except for pigmentation pattern. Pigmentation type B shared with E. asterope Nygren \& Pleijel, 2015; E. elektra Nygren \& Pleijel, 2015; some specimens of $E$. schanderi sp. nov.; the British population of E. taygete Nygren \& Pleijel, 2011; Eumida R0174-18; and some specimens from E. fauchaldisp. nov. and E. aff. fauchaldi. However, those species differ from E. fenwickisp. nov. at the molecular level, with mean interspecific COI distances (K2P, \%) of 19.4, 15.1, 21.1, 17.3, 17.7, 16.6, and 16.5 respectively. Morphometric proportions of the antennal length against either head length or width, palp length, cirri on segment 1 , and dorsal cirri on segment 2 seem to be effective in distinguishing this species from E. fauchaldi, E. schanderi, and E. elektra.

## Eumida schanderisp. nov.

 urn:Isid:zoobank.org:act: 4780C5B7-EC23-44A5-B2CC-88E9FE8C5891
## Material examined

Type material. Norway, Bergen: 1 spm, holotype and hologenophore, ZMBN_134556, $60^{\circ} 14^{\prime} 11.9^{\prime \prime} \mathrm{N}-5^{\circ} 12^{\prime} 02.1^{\prime \prime} \mathrm{E}, 27 \mathrm{~m}$, algae, gravel, triangular dredge, $24 / 07 / 2014$; 11 spms, paratypes and paragenophores, ZMBN_134550 - 134555; ZMBN_134557 - 134561, $60^{\circ} 14^{\prime} 11.9^{\prime \prime} \mathrm{N}$ $5^{\circ} 12^{\prime} 02.1^{\prime \prime} \mathrm{E}, 27 \mathrm{~m}$, algae, gravel, triangular dredge, $24 / 07 / 2014$. The type specimens were collected by the crew aboard R/V Hans Brattström owned by the University of Bergen and operated by the Institute of Marine Research.

Other material. Sweden, Bohuslän: 1spm, SMNH 110614, $58^{\circ} 52^{\prime} 00.0^{\prime \prime N}-11^{\circ} 06^{\prime} 00.0^{\prime \prime} \mathrm{E}, 40 \mathrm{~m}$, gravel, dredge, 12/05/2005. Collected by Fredrik Pleijel.

## Diagnosis

Member of EsSC with type D pigmentation (Table 3.4), i.e., with white pigmentation present dorsally on segment 2 and anterior cirri (Fig. 3.5.B). Type B pigmentation, i.e., without white pigmentation (Fig. 3.5.A) also observed in some paratypes and other analysed material. Live specimens present greenish colouration. Antennae slightly shorter than palps. Proportions between the antenna length and head length or width, median antenna length, cirri on segment 1, and dorsal cirri on segment 2 smaller than those in E. fauchaldi sp. nov., E. fenwickisp. nov., and E. elektra. Palp length, cirri on segment 1,
and dorsal cirri on segment 2 , or head width similar to those of $E$. fenwicki. Head almost twice as wide as long. Dorsal cirri on segment 2 usually twice as long as cirri on segment 1 . Dorsal cirri of median segments very large, almost twice as long rather than wider. Ventral cirri of median segments longer rather than wider, usually twice as long. Proboscis with numerous minute papillae evenly distributed (Fig. 3.5.B). Worms small, usually between 3 to 7 mm long, with 40 to 60 segments.

## Molecular data

COI, 16S, ITS, and 28S sequences as in specimens ZMBN_134550 to 134561 and SMNH 110614 (Table S3.1). Phylogenetic relationship as in Fig. 3.2.A-B, with high support values and low intraspecific (<3\%) genetic divergence for mitochondrial loci (MOTU 22). However, introgression of mtDNA from a non-sampled lineage may be present in nuclear markers with two strongly-supported sister MOTUs with 5.8 and $0.6 \%$ mean genetic divergence for ITS and 28S, respectively (MOTUs 22 and 26). Mean interspecific COI distances to the nearest and farthest neighbours are $15.6 \%$ (K2P, E. aff. merope) and 21.8\% (K2P, E. langenecki sp. nov.), respectively. DOI for the species' Barcode Index Number (BIN): dx.doi.org/10.5883/BOLD:ACQ6378.

## Etymology

The new species is named to honour the memory of Christoffer Schander (1960-2012), a muchappreciated former colleague to the co-supervisor of this thesis, Arne Nygren.

Distribution and habitat
Atlantic Ocean - Norway and Sweden, from 27 to 40 m depth, on gravel with algae.

## Remarks

Morphologically similar to E. sanguinea sensu stricto (Örsted, 1843), including pigmentation pattern. Pigmentation type D shared with E. sanguinea s.s.; E. merope Nygren \& Pleijel, 2015; E. aff. merope; Eumida F22 Nygren \& Pleijel, 2011; Eumida ANT002; and the Mediterranean population of $E$. taygete Nygren \& Pleijel, 2011. Pigmentation type B shared with E. fenwickisp. nov.; E. asterope Nygren \& Pleijel, 2011; E. elektra Nygren \& Pleijel, 2011; E. fauchaldi sp. nov.; and the British population of $E$. taygete Nygren \& Pleijel, 2011. However, those species differ from E. schanderi at the molecular level, with mean interspecific COI distances (K2P, \%) of 15.1, 17.3, 15.6, 18.6, 16.5, 21.1, 16.5, 18.8, and 18.4, respectively. Proboscis has papillae (Fig. 3.5.B), unlike the one reported in E. sanguinea s.s., which
is almost smooth with sparsely distributed minute papillae, arranged in six more-or-less distinct rows (Pleijel, 1993). Morphometric proportions of the antenna-palp ratio and antenna length against head length or width, palp length, cirri on segment 1, and dorsal cirri on segment 2, and median antenna seem to be effective in distinguishing this species from E. fauchaldi sp. nov., E. schanderi sp. nov., and E. elektra.

## Eumida fauchaldisp. nov.

urn:Isid:zoobank.org:act:B974C7EA-E00D-4D8A-B791-5A82BAEAAADE

Material examined
Type material. Great Britain, Plymouth: 1 spm, holotype and hologenophore, DBUA0002400.01, $50^{\circ} 21^{\prime} 30.0^{\prime \prime} \mathrm{N}-4^{\circ} 08^{\prime} 54.0^{\prime \prime} \mathrm{W}, 15 \mathrm{~m}$, coarse shell gravel, dredge, $16 / 03 / 2011 ; 8$ spms, paratypes and paragenophores, DBUA0002400.02-09, $50^{\circ} 21^{\prime} 30.0^{\prime \prime} \mathrm{N}-4^{\circ} 08^{\prime} 54.0^{\prime \prime} \mathrm{W}, 15 \mathrm{~m}$, coarse shell gravel, dredge, $16 / 03 / 2011$. Collected by the crew aboard R/V SEPIA (Marine Biological Association) and Fredrik Pleijel.

Other material. Great Britain, Plymouth: 17 spms, DBUA0002400.10-26, $50^{\circ} 21.59^{\prime \prime} \mathrm{N}$ $4^{\circ} 09.03$ "W, $8-13 m$, coarse shell gravel, dredge, 27/03/2017. Collected by the crew aboard R/V SEPIA (Marine Biological Association) and Fredrik Pleijel.

## Diagnosis

Member of Essc with type F pigmentation (Table 3.4), i.e., with transverse dorsal lines across segments (Fig. 3.5.C). Type B pigmentation, i.e., without white pigmentation (Fig. 3.5.D) also observed in some paratypes and other analysed material. Live specimens present yellowish-brown colouration. Antennae are shorter than palps. Proportions of the antenna length against head length or width, median antenna length, cirri on segment 1 , and dorsal cirri on segment 2 larger than those of $E$. schanderi sp. nov. and $E$. fenwicki sp. nov., but smaller than those of $E$. elektra. Despite a considerable larger worm size, antenna length with similar morphometric measurements as E. fenwicki sp. nov. Head wider than longer. Dorsal cirri on segment 2 usually twice as long as cirri on segment 1 . Dorsal cirri of median segments large, 1.5 times longer rather than wider. Ventral cirri of median segments twice longer than wider. Proboscis with numerous minute papillae evenly distributed (Fig. 3.5.C). Worms small- to mediumsized, usually between 10 to 20 mm long, with 80 to 105 segments.

## Molecular data

COI, 16S, ITS, and 28S sequences as in specimens DBUA0002400.01-26 (Table S3.1). Phylogenetic relationship as in Fig. 3.2A-B, with high support values and low intraspecific (<3\%) genetic divergence for both mitochondrial and nuclear markers (MOTU 13). Mean interspecific COI distances to the nearest and farthest neighbours are 13\% (K2P, E. aff. fauchald) and 21.8\% (K2P, Eumida ORB997), respectively. DOI for the species' Barcode Index Number (BIN): dx.doi.org/10.5883/BOLD:AEA3142.

## Etymology

The new species is named in memory of Kristian Fauchald (1935-2015).

Distribution and habitat
Atlantic Ocean - British Isles, 8-15 m depth, on coarse shell gravel.

## Remarks

Morphologically similar to E. sanguinea sensu stricto (Örsted, 1843), except for pigmentation pattern. Type F pigmentation is unique for this species (including E. aff. fauchald). Type B pigmentation is shared with E. fenwickisp. nov.; E. asterope Nygren \& Pleijel, 2011; E. elektra Nygren \& Pleijel, 2011; E. aff. fauchaldr; Eumida R0174-180; some specimens of E. schanderi sp. nov;; and the British population of $E$. taygete Nygren \& Pleijel, 2011. However, those species differ from E. fauchaldi sp. nov. at the molecular level, with mean interspecific COI distances (K2P, \%) of 16.6, 15.5, 16.6, 13.0, 14.5, 18.4, and 16.0, respectively. Proboscis has papillae (Fig. 5C), unlike the one reported in E. sanguinea s.s., which is almost smooth with sparsely distributed minute papillae, arranged in six more-or-less distinct rows (Pleijel, 1993). The number of segments, worm length, antennae/palps ratio, as well as morphometric proportions of the antenna length against head length or width, palp length, cirri on segment 1 , dorsal cirri on segment 2 , and median antenna, seem to be very effective in distinguishing this species from E. fenwickisp. nov., E. schanderisp. nov., and E. elektra.

## Eumida pleijelisp. nov.

urn:Isid:zoobank.org:act: F2B43974-0771-4B9A-9CC9-42FF33CEB454

## Material examined

Type material. Italy, Naples: 1 spm, holotype and hologenophore, DBUA0002407.02, $40^{\circ} 49^{\prime} 48.0^{\prime \prime} \mathrm{N}-14^{\circ} 14^{\prime} 13.2^{\prime \prime} \mathrm{E}, 6 \mathrm{~m}$, coarse shell gravel, dredge, 05/05/2010; 1 spm , paratype and paragenophore, DBUA0002407.01, $40^{\circ} 49^{\prime} 48.0^{\prime \prime} \mathrm{N}-14^{\circ} 14^{\prime} 13.2^{\prime \prime} \mathrm{E}, 6 \mathrm{~m}$, coarse shell gravel, dredge, 05/05/2010. Collected by Joachim Langeneck.

## Diagnosis

Member of Essc with green colouration mixed with type C pigmentation (Table 4), i.e., white pigmentation dorsally on segment 2 and anterior cirri, and with a longitudinal mid-dorsal line (Fig. 6D). Antennae longer than palps. Dorsal cirri on segment 2 almost three times as long as cirri on segment 1, unlike the smaller ratio (twice as long) in E. fenwicki sp. nov., E. schanderi sp. nov, E. fauchaldi sp. nov. E. langeneckisp. nov. and E. elektra. Dorsal cirri of median segments large, 1.5 times longer rather than wider, with similar ratio as $E$. fenwickisp. nov., E. fauchaldisp. nov. E. langeneckisp. nov. and E. elektra, but smaller than E. schanderi sp. nov. (usually twice as long). Ventral cirri of median segments 1.5 times longer rather than wider, but with smaller ratio (usually twice as long) as E. fenwickisp. nov., E. schanderi sp. nov, E. fauchaldisp. nov. E. langeneckisp. nov. and E. elektra. Proboscis not observed. Worms smallto medium-sized, usually between 10 to 15 mm long, with 55 to 65 segments.

## Molecular data

COI, 16S, ITS, and 28S sequences as in specimens DBUA0002407.01-02 (Table S3.1). Phylogenetic relationship as in Fig. 3.2.A-B, with high support values and low intraspecific (<3\%) genetic divergence for both mitochondrial and nuclear markers (MOTU 3). However, nuclear markers (ITS and 28 S) group this species into the same MOTU (23, Fig. 3.2B) as the unnamed Eumida ORB99, even though high interspecific COI divergence is found (18\%, K2P). Mean interspecific COI distances to the nearest and farthest neighbours are 16.8\% (K2P, E. mackie) and 21.6 (K2P, E. langenecki sp. nov.), respectively. DOI for the species' Barcode Index Number (BIN): dx.doi.org/10.5883/BOLD:AEH2033

## Etymology

The new species is named after Fredrik Pleijel to honour his passion and dedication to the study of the Phyllodocidae.

Distribution and habitat
Western Mediterranean Sea - Italy, 6 m depth, on coarse shell gravel.

Remarks
Morphologically similar to E. sanguinea sensu stricto (Örsted, 1843), except for pigmentation pattern. Type C pigmentation is shared only with E. kelaino and its Mediterranean counterpart E. aff. kelaino. However, E. kelaino and E. aff. kelaino differ greatly from E. pleijelii sp. nov. at the molecular level, with mean interspecific COI distances (K2P, \%) of 19.0 and 19.1, respectively, and share a different colouration. This species can share the same nuclear MOTU (ITS+28S) as the unnamed EumidaORB997, but the latter has a very distinct pigmentation (Type H) among all members of the Essc. Eumida pleijelii sp. nov. can be identified based only on colour, pigmentation, and geographic distribution jointly.

## Eumida langenecki sp. nov.

urn:Isid:zoobank.org:act: 3E870E7A-C1D6-4918-BCE0-737E5B81418A

## Material examined

Type material. Italy, Antignano: 1 spm, holotype and hologenophore, DBUA0002409.02, $43^{\circ} 29^{\prime} 31.2^{\prime \prime} \mathrm{N}-10^{\circ} 19^{\prime} 01.2^{\prime \prime} \mathrm{E}, 6 \mathrm{~m}$, coarse shell gravel, dredge, 22/05/2020; 2 spms, paratypes and paragenophores, DBUA0002409.04-05, $43^{\circ} 29^{\prime} 31.2^{\prime \prime} \mathrm{N}-10^{\circ} 19^{\prime} 01.2^{\prime \prime} \mathrm{E}, 6 \mathrm{~m}$, coarse shell gravel, dredge, 22/05/2020. Collected by Joachim Langeneck.

Other material. Italy, Ischia: 1 spm , DBUA0002408.01, $40^{\circ} 44^{\prime} 42.0^{\prime \prime} \mathrm{N}-13^{\circ} 56^{\prime} 20.4^{\prime \prime} \mathrm{E}, 6 \mathrm{~m}$, coarse shell gravel, dredge, 10/05/2010; Italy, Antignano: 1 spm, DBUA0002409.01, $43^{\circ} 277^{\prime} 57.6^{\prime \prime N}$ $10^{\circ} 20^{\prime} 24.0^{\prime \prime} \mathrm{E}, 4 \mathrm{~m}$, coarse shell gravel, dredge, 05/05/2010; 1 spm, DBUA0002409.03, $43^{\circ} 29^{\prime} 31.2^{\prime \prime} \mathrm{N}-10^{\circ} 19^{\prime} 01.2^{\prime \prime} \mathrm{E}, 6 \mathrm{~m}$, coarse shell gravel, dredge, 22/05/2020. Collected by Joachim Langeneck.

Diagnosis
Member of Essc with type G pigmentation (Table 3.4), i.e., with white pigmentation dorsally on prostomium and white transverse dorsal dots across segments (Fig. 3.6C). Live specimens present yellow colouration. Antennae slightly longer than palps. Dorsal cirri on segment 2 twice as long as cirri on segment 1 , with similar ratio as $E$. fenwicki sp. nov., $E$. schanderi sp. nov, $E$. fauchaldi sp. nov and $E$.
elektra, but smaller than E. pleijeli sp. nov. (almost three times as long). Dorsal cirri of median segments large, 1.45 times longer rather than wider, sharing a similar ratio as E. fenwicki sp. nov., E. pleijeli sp. nov., E. fauchaldi sp. nov and E. elektra, but smaller than E. schanderi sp. nov (twice as long). Ventral cirri of median segments almost twice as long rather than wider, sharing a similar ratio as E. fenwickisp. nov., E. schanderi sp. nov., E. fauchaldi sp. nov and E. elektra, but greater than E. pleijeli sp. nov (1.5 times as long). Proboscis not observed. Worms small, usually between 7 to 11 mm long, with 70 segments.

## Molecular data

COI, 16S, ITS, and 28S sequences as in specimens DBUA0002408.01; DBUA0002409.01-05 (Table S3.1). Phylogenetic relationship as in Fig. 3.2.A-B, with high support values and low intraspecific $(<3 \%)$ genetic divergence for both mitochondrial and nuclear markers (MOTU 5). Mean interspecific COI distances to the nearest and farthest neighbours are 15.4\% (K2P, E. maia) and 22.0 (K2P, E. mackien), respectively. DOI for the species' Barcode Index Number (BIN): dx.doi.org/10.5883/BOLD:AEH2035

Etymology
The new species is named after Joachim Langeneck for his sampling efforts and kindness in providing unique Mediterranean Eumida specimens on the behalf of the author of this thesis.

## Distribution and habitat

Western Mediterranean Sea - Italy, 3-6 m depth, on coarse shell gravel.

## Remarks

Morphologically similar to E. sanguinea sensu stricto (Örsted, 1843), except for pigmentation pattern. Type G pigmentation is unique among the Essc and can solely be used to identify this species. Type G and E pigmentation, are the only pigmentation types with white pigmentation dorsally on the prostomium up to the middle of the eyes. The latter being exclusive to $E$. notata found only in Madeira Island (Portugal).

## Eumida mackieisp. nov.

urn:Isid:zoobank.org:act: 59EB632B-76A5-41FC-91D2-3D4CF50A383D

Material examined
Type material. Great Britain, Plymouth: 1 spm, holotype and hologenophore, DBUA0002331.01, $50^{\circ} 21.59^{\prime} \mathrm{N}-4^{\circ} 9.03^{\prime} \mathrm{W}, 8-13 \mathrm{~m}$, coarse shell gravel, dredge, 27/03/2017; 24 spms, paratypes and paragenophores, DBUA0002331.02-23; DBUA0002331.25; DBUA0002331.26, 50º 21.59' N-40.03' W, 8-13 m, coarse shell gravel, dredge, 27/03/2017. Collected by the crew aboard R/V SEPIA (Marine Biological Association).

Other material. Great Britain, Plymouth: 2 spms, DBUA0002331.24; DBUA0002331.27; DBUA0002418.01, $50^{\circ} 21.59^{\prime} \mathrm{N}-4^{\circ} 9.03^{\prime} \mathrm{W}, 8-13 \mathrm{~m}$, coarse shell gravel, dredge, 27/03/2017. Collected by Fredrik Pleijel.

Diagnosis
Member of EsSc with Type A pigmentation, i.e. white pigmentation dorsally on segment 2 and anterior cirri with transverse lines. Live specimens present a greenish colouration (Fig. 3.8.A). Antennae and palps about the same size. Proportions between the number of segments and worm width, the length and height of the chaetigorous lobe, the length between the palps length against the head length and antennae length, the ventral cirri length against the length of the dorsal cirri, and width of the ventral cirri larger than those of E. maia and E. notata. Head wider than longer. Dorsal cirri on segment 2 twice as long as cirri on segment 1 , larger than those of $E$. notata and $E$. maia. Dorsal cirri of median segments large, 1.6 times longer rather than wider, sharing a similar ratio as $E$. maia but smaller than $E$. notata (1.8 times as long). Ventral cirri of median segments twice as long rather than wider, sharing a similar ratio as $E$. maia and $E$. notata. Proboscis with numerous minute papillae evenly distributed (Fig. 3.8.A). Worms small, usually between 3 to 12 mm long, with 24 to 68 segments.

## Molecular data

COI, 16S, ITS and 28S sequences as in specimens DBUA0002331.01-27 (Table S3.1). Phylogenetic relationship as in Fig. 3.2A-B with high support values and low intraspecific ( $<3 \%$ ) genetic divergence for both mitochondrial and nuclear markers (MOTU 1). Mean interspecific COI distances to the nearest and farthest neighbours are 15.2\% (K2P, Eumida ORB997) and 22.0 (K2P, E. langenecki sp.
nov.), respectively. DOI for the species' Barcode Index Number (BIN): dx.doi.org/10.5883/BOLD:ADY9496.

## Etymology

The new species is named after Dr. Andy Mackie in recognition of his outstanding knowledge on polychaetes.

Remarks.
This species is morphologically identical to E. sanguinea sensu stricto (Örsted, 1843), except for some slight variations in the size of specific morphological characters and the pigmentation of the live specimens that present a distinctive greenish band similar to $E$. bahusiensis, with some additions of white pigmentation. The pigmentation type is shared with E. maia, E. alkyone and E. merope type two. Eumida mackieisp. nov. can be distinguished from E. maia and $E$. notata by the larger distance between the eyes (DE), longer ventral cirri (VCL) and chaetigerous lobe (CLL), and wider head (HW). Also the proportions between the number of segments (NS)/worm width (WW), CLL/chaetigorous lobe height (CLH), palp length $(\mathrm{PL}) /$ head length $(\mathrm{HL})$, antennae length $(\mathrm{AL}) / \mathrm{PL}$, dorsal cirri length ( DCL )/VCL and VCL/ventral cirri width (VCW) are different for the three species and possess the greatest dissimilarity results (SIMPER) in relation to the new species. The $D E$ is significatively larger when compared to $E$. maia and $E$. notata (Fig. S3.3.D). This is especially remarkable when comparing against specimens from E.sanguinea s.s. given the size difference. E. mackiei sp . nov. (average worm length of 8 mm ) still maintains similar measurements for the DE as the larger specimens from E. sanguinea s.str. ( 30 mm in length). The antennae and palps have similar length as opposed to $E$. maia and $E$. notata where the antennae are clearly longer than the palps. Proboscis has papillae (Fig. 3.8.A), unlike the one reported in E. sanguinea s.s., which is almost smooth with sparsely distributed minute papillae, arranged in six more-or-less distinct rows (Pleijel, 1993)

### 3.4 Discussion

### 3.4.1 New species and unnamed lineages

This chapter reflect once again the high level of hidden diversity in polychaetes (Nygren, 2014; Nygren et al, 2018), this time within the Essc, which prior to this study already had eleven lineages recognized, with five of them present in boreal waters, another five present in the Mediterranean and one
unique to Portugal, in the island of Madeira (Nygren and Pleijel, 2011). The results from this chapter builds upon this data and adds additional eleven new Eumida lineages, with six of them described in the taxonomic section. All members of the Essc, including the six new species, displayed COI genetic distances comparable to those found among established species of polychaetes (e.g., Carr et al., 2011; Lobo et al., 2016; Sampieri et al., 2021). However, the results of nuclear markers in E. schanderi sp. nov. are unexpected due to their customary low divergence with COI. Vieira et al. (2019) also found a similar occurrence when comparing COI and 18S rRNA for one of the MOTUs of Dynamene magnitorata Holdich, 1968 (Isopoda), reporting evidence of cryptic lineages between the Iberian Peninsula and Macaronesia islands. This could be a case of heterozygosity at nuclear loci (Sota \& Vogler, 2003); however, overlapping spikes were not found when analysing the trace files for E. schanderi sp. nov. Besides, the distance between these haplotypes was perhaps too large to be attributed to heterozygotic variation. Another more plausible scenario is that hybridization and introgression of mtDNA from a nonsampled lineage could explain the presence of the same COI haplotype in two different sister lineages (Bachtrog et al., 2006).

Although molecular data from this study support species hypothesis for most new lineages, there are a few exceptions in the nuclear MOTUs $23,24,25$, and 27 . Each of them is composed of at least two corresponding distinct mitochondrial lineages. At first, these patterns can either be explained by hybridization or differential substitution rates among loci. It is because some loci would display more consolidated lineage sorting stages than others. However, except for E. merope and E. aff. merope (MOTU 24, Fig. 3.2.B), no nuclear haplotypes are shared (Fig. 3.4.A, B). Therefore, although broader sampling and balanced representation of sequences from different loci are needed, hybridization could be discarded. MOTU 24 also did not share haplotypes between E. notata and either $E$ merope or $E$. aff. merope. However, regardless of the species status, it is evident these lineages have diverged recently. When considering only the COI genetic distances (for which there is extensive data), they appear to be on the lower boundary of customary congeneric distances reported either within consolidated Eumida species or even compared to polychaetes in general (Nygren et al., 2009; Ravara et al., 2017). Indeed, E. aff. merope displays a mean COI genetic distance of 10 and $8 \%$ to E. merope and E. notata, respectively, with limited morphometric differences (Fig. 3.10) as well. Similar values can be found between E. kelaino (MOTU 18) and E. aff. kelaino (MOTU 17) or between E. fauchaldi sp. nov. (MOTU 12) and E. aff. fauchaldi (MOTU 13), with 12 and $13 \%$ mean COI divergence, respectively, and each pair sharing the same nuclear MOTU. It seems that EsSC species with COI divergence below $10 \%$ may have little or no differentiation in 16S, ITS or 28 S (Table 3.2). Some exceptions to this pattern can be found in
E. pleijeli sp. nov. (MOTU 3) and Eumida ORB997 (MOTU 2). In these, a mean COI divergence of 18\% was observed associated with different pigmentation types, but only $1.5 \%$ in the ITS region. Alternative divergence patterns between nuclear and mitochondrial markers have been reported for other cryptic species as well (e.g., Notophylum Örsted, 1843). In this regard, although low mean COI K2P distances were found between shallow and deep water populations (8.5\%), mean distances for ITS1 (4.9\%) were still higher compared to some Eumida species analysed here (Nygren et al., 2010). The animal mitochondrial genome, and in particular COI and 16S genes, have been documented as fast evolving genes, at least compared with more conserved genome regions such as 18 S and 28 S (Borges et al., 2012; Jörger et al., 2012). Because of this, differences in the number and clustering pattern of putative MOTUs between nuclear and mitochondrial genes is anticipated, usually resulting in a higher number recovered in the latter case (e.g., Borda et al., 2013; Desiderato et al, 2019; Vieira et al, 2019). Therefore, a combination of mitochondrial and nuclear loci is advised to better assess species boundaries and unravel cryptic diversity (Grabowski et al., 2017; Jörger and Schrödl, 2013).

### 3.4.2 Phylogeografic insights

Most Essc species have extreme COI haplotype diversity (Table 3.3). This is comparable to Terebellides Sars, 1835 in Nygren et al. (2018), wherein almost all specimens sampled and sequenced had a unique haplotype in some species. Therefore, additional larvae may have been recruited from other populations (Meibner et al., 2014). Other polychaete species, such as Hediste Malmgren, 1867, have also shown more than 80 haplotypes in 100 sequences recorded in species " $A$ " and " $B$ " for both $H$. diversicolor (0.F. Müller, 1776) and H. atoka Sato \& Nakashima, 2003 (Tosuji et al., 2019). Such high haplotype diversity within species could also be related to the Pleistocene glaciation (initiated circa 2.8 MY, Maggs et al. 2008). Isolated northern ice-free areas may have allowed pockets of diversity to persist (Stewart and Lister, 2001; Rowe et al., 2004; Provan and Bennett, 2008). These glacial refuges are areas where some plants or animals survived during this unfavourable period, with organisms of the same kind extinguished nearby or retracted southwards to more favourable locations (Andersen and Borns, 1994). It has been proposed that the Western English Channel is one of the possible locations of coastal glacial refuges (Maggs et al., 2008), thereby close to the Plymouth and Cornwall area, which is home to eight different Essc species, including two of the new species (MOTUs 6 and 12, Fig. 3.2.A, B). Isolation into refugia reduces geographical ranges and population sizes, resulting in high genetic diversity and high dissimilarity between refugee populations (Comes and Kadereit, 1998; Willis et al., 2004).

The high genetic distance between MOTUs within the Essc of the northeast Atlantic suggests that their diversification likely pre-dates the Pleistocene glaciations. This potential survival of divergent lineages in common refugia may explain the high level of sympatry currently observed in this region (Highsmith, 1985; Desiderato et al., 2019).

### 3.4.3 Morphometry-based insights

Previous morphometric studies among polychaetes have been used independently of molecular analyses to successfully resolve the taxonomy of several cryptic complexes or very similar species, often leading to the description of new species. A few extra steps to this type of methodology were first added by Ford and Hutchings (2005) and more recently by Martin et al. (2017) and Meca et al. (2019) with the incorporation of statistical dissimilarities derived from the SIMPER routine of the PRIMER software (Clarke and Warwick, 2001) based on a matrix of morphometric measurements in order to distinguish between morphologically similar species. The results from this study succeeded in separating three of the four lineages through the PCA analysis. Comparing the measurements taken from E. sanguinea s.s. regarding the three main morphological characters that are indicators of polychaete growth (Number of Segments - NS, Worm Width - WW and Worm Length - WL) (Pleijel, 1993; Seaver et al., 2005) this lineage had by far the biggest disparity in specimen size distributed among the 25 individuals analysed, with more than five times the values for WL, three times the values for WW and more than twice the number of segments, highlighting the possibility of the existence of juveniles and thus disrupting the proportion data analysis. The SIMPER data (Table 3.5) illustrate the importance of the body width and length as well as the segment number in the distinction between similar species in morphometric analysis. Previous studies on other polychaete families have shown similar results (e.g. MacCord and Amaral, 2005; Ravara et al., 2010). Indeed, E. maia also displayed some differences in specimen size but to a lesser extent when compared to $E$. sanguinea s.s.. Given the existence of other lineages belonging to the Essc with just one specimen (Fig. 3.2.A, MOTU 4, 6 and 7), this could motivate a future study comparing their morphometric features against specimens of $E$. sanguinea s.s. of similar size, to verify whether this pattern in the current work is still replicated.

Apart from E. sanguinea s.s and focusing on the three remaining species from the PCA analysis, independent clustering patterns were evident (Fig. 3.8.A). Despite their similar morphology, SIMPER was also able to discriminate these lineages with inter-species dissimilarity values between E. mackiei sp. nov., E. maia and E. notata being at least three times higher than intra-species dissimilarities. This SIMPER result was similar to the one found in Oxydromus (Martin et al., 2017), though none of the
analysed Eumida species reached the high value of $21.4 \%$ intra-population divergence. In the case of the Oxydromus, the inter-population dissimilarity reached $46 \%$ against the average one of $39 \%$ from the three Eumida species. In a study of Owenia (Ford and Hutchings, 2005) SIMPER values of populations from different regions averaged $16.23 \%$ and ranged between $5 \%$ to $21 \%$ in individual areas, being almost two times lower compared to my data. Significant ANOSIM results were also higher in the current study $(0.552 \%$ at $0.1 \%)$ compared to Oxydromus ( $0.421 \%$ at $0.1 \%$ ) and Owenia (for differences between locations a Global R of 0.341 at $0.1 \%$ and for differences between regions a Global R of 0.614 at $0.2 \%$ ).

Scatter plots were also very successful in the separation between several cryptic lineages (Fig. 3.9), however failed to separate MOTUs with comparatively low genetic distances between them (<14\% COI, Fig. 3.10). Rice et al. (2008) compared genetic distances and reproductive compatibility in Polydora cornuta Bosc, 1802 populations. They reported that signs of partial larval development can be found between populations with $8 \%$ mean COI distances, but not between those with COI divergence above $15 \%$. Some exceptional cases have also been reported in marine invertebrates, namely in copepods (Handschumacher et al., 2010). This study showed that, despite the genetic COI distances of above 23\% between the Pacific population of Tigriopus californicus (Baker, 1912) and Icelandic Tigriopus brevicornis (Müller O.F., 1776), their crossing can produce mature F1 and F2 hybrids. This, in turn, challenges the restrictive biological species concept.

### 3.4.4 Pigmentation data, the Essc and morphological stasis

Recent studies have suggested that cryptic complexes may remain morphologically identical due to long periods of morphological stasis (Struck et al., 2018). For example, rates of morphological evolution in the Stygocapitella complex are significantly slower than in closely related non-cryptic taxa from Nerillidae Levinsen, 1883 and Orbiniidae Hartman, 1942 (Cerca et al., 2020b). Besides the geographic distribution, colouration, and pigmentation, all the new Eumida species examined in this study fail to display any stable and diagnostic morphological differences, even though slight morphometric variations on the size and shape of the cirri and prostomial appendages can be found at least between 4 lineages. Notably, the outgroup E. bahusiensis, which normally occurs paraphyletically within the Essc (Fig. 3.2), possesses the same white pigmentation pattern (type B) and the distinct greenish band as does E. mackiei sp. nov.. However, the dorsal cirrus has a visible and larger width that can be identified through traditional morphological approaches (Nygren et al., 2017). Such micromorphological variations within cryptic and pseudo-cryptic species are seldom detected. For instance, most Stygocapitel/a lineages lack diagnostic characters and morphological differences that could allow an unambiguous identification to the species
level, including morphometric data (Cerca et al., 2020a). In this study, E. sanguinea s.s. also failed to produce a separated morphometric PCA cluster against three other species from the complex (Fig. 3.8.B). Even though in this particular case it could be attributed to bias towards juveniles among the examined specimens, the likelihood of finding overlapping morphometric variation is still high when dealing with more than fifteen different Eumida species. This PCA result and, in particular, the morphometric data for E. aff. merope (Fig. 3.10) could also be indicative of phenotypic plasticity (e.g., in the proportions of several morphological structures). This phenomenon is widespread across invertebrates since different phenotypes occur associated with particular environmental conditions (Fusco and Minelli, 2010; Forsman, 2015), but still scarcely studied in polychaetes (Nygren and Pleijel, 2011; Syomin et al., 2017). Environmental features could be an explanation for this variation. In the Syllis gracilis Grube, 1840 complex (Langeneck et al., 2020), a univariate analysis of morphological characters showed that marine specimens sampled on intertidal algal communities are differentiated from brackish-water and Sabellariaassociated individuals.

Pronounced phenotype changes without molecular divergence are also patent in the Essc. Aside from the lack of apparent correlation between geographic occurrence and species with colour polymorphism (such as E. merope, E aff. merope, E. schanderisp. nov., E. fauchaldisp. nov., and E. aff. fauchaldd, E. elektra and E. taygete populations had all their sequences grouped in the same respective MOTU, but geographically different populations had distinct pigmentations. The latter had an exceptionally wide geographical range within the Essc, from Great Britain to the western and eastern Mediterranean. Yet, the individuals from the British population possess a distinct pigmentation type (Table 3.4) and several unique haplotypes (Fig. 3.3). Interestingly, reports have often attributed the deep divergence in invertebrates between eastern and western Mediterranean to the Messinian salinity crisis around 6 MY (e.g., Hupało et al., 2019; Rögl, 1999). The same is not observed in E. taygete, suggesting recent colonization of the Mediterranean. In this case, neither the morphotype nor geographic location alone could be indicative of a new species within the Eumida complex unless complemented with molecular data. Such a contrast, for example between Eumida pleijeli sp. nov. and Eumida langenecki sp. nov., where a combination among collection location, live colouration, and white pigmentation type is sufficient to successfully identify these species within the Essc.

### 3.5 Conclusion

The combination of morphometric and genetic data successfully validated the existence of four new undescribed species within the Essc, namely E. mackieisp. nov., E. schanderisp. nov., E. fenwicki
sp. nov., and $E$. fauchaldi sp. nov. Since morphometric scatter plots seem to be informative only for at least five specimens with optimal conditions, such methodology cannot be used for the remaining eight newly detected MOTUs. However, combining colour, white pigmentation types, and geographic distribution was enough to successfully identify two additional new species, $E$. pleijeli sp. nov. and $E$. langenecki sp. nov., rising to fifteen the total number of species described within this complex. These results also suggest that morphometric data alone may not provide enough resolution for the most genetically close Eumida species (i.e., with about less than $14 \%$ COI divergence) and/or cases where nuclear data fail to split into the same number of MOTUs as mtDNA. Moreover, the probability of finding overlapping morphometric variation for any of the analysed proportions is high when dealing with more than fifteen different Eumida species. Although genetically similar, the sister species E. notata and E. merope had at least two different morphometric markers, did not share haplotypes, and also differed in pigmentation type and geographic distribution, strengthening their status as independent species. Ideally, studies examining reproductive compatibility between populations could help clarify the species status of the lineages referred to as E. aff. merope, E aff. kelaino, and E. aff. fauchaldi compared to their respective sister species. The remaining three new undescribed Eumida lineages in this work (RO174-180, ANT002 and ORB997) will join Eumida F22 from Nygren and Pleijel (2011) as putative species within the Essc, with further sampling efforts still needed to clarify their status.

The underlying mechanisms behind morphological stasis are still unknown and remain controversial in evolutionary biology (Fišer et al., 2018). In this sense, combining molecular phylogenetic tools and examination of small morphological changes can help understand stasis in species complexes. This can eventually allow for more formal and widespread recognition of cryptic and pseudo-cryptic biodiversity in biomonitoring and ecological studies.

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Chapter 4

From subtidal to intertidal: Revealing the diversity of the Eulalia clavigera (Annelida, Phyllodocidae) species complex along the European coast


#### Abstract

The green phyllodocids Eulalia clavigera and E. viridis are a known European pseudo-cryptic complex, but questions about its distribution and evidence of additional lineages in previous studies call for an investigation of the real diversity within the complex.

The analysis of DNA sequence data (COI, ITS-regions rRNA and 28 S rRNA) of populations of the apparent E. clavigera morphotype from intertidal and subtidal marine waters along the North East Atlantic, Mediterranean Sea and the Macaronesia islands (Madeira, Savage islands, Azores and Canaries) provided compelling evidence for the existence of six additional divergent evolutionary lineages. Three of the most abundant lineages are described here as new species: Eulalia feliciae sp. nov., intertidal and unique to the west Mediterranean, Eulalia madeirensis sp. nov., a subtidal variant unique to the Madeira island (Portugal), and Eulalia xanthomucosa sp. nov., occurring mostly in subtidal habitats of the British Isles and southern France. The molecular data was complemented with morphometric methodologies and compared against the parent morphospecies (E. clavigera s.s.). Eulalia feliciae sp. nov. and E. madeirensis sp. nov. formed two independent morphometric clusters, while measurements for $E$. xanthomucosa sp. nov. often overlapped with E. clavigera. However, the latter new species presents an unique yellow coloration produced by the worm's mucus and has larger parapodial cirri on median segments in relation to its body size.

Recent biotechnological findings using E. clavigerahighlights the importance of formally describing cryptic complexes, since their chemistry might be unique to each lineage and can have a range of distinct effects and applications.


Keywords: Eulalia clavigera, Phyllodocidae, integrative taxonomy, cryptic species, morphometry

### 4.1 Introduction

Biodiversity comprises three levels of variation: genetic, species and ecosystem. Molecular tools have been enabling the in-depth appraisal of the true diversity present in animals, namely by detecting cryptic or pseudo-cryptic species. The latter constitute a substantial fraction of biodiversity, and appear to be a frequent phenomenon among marine benthic invertebrates (Miglietta et al., 2011; Nygren, 2014), in well-known taxa and studied areas (e.g. Bleidorn et al., 2006; Carr et al., 2011; Grosse et al., 2021; Jolly et al., 2006; Leite et al., 2020). Despite the increasing evidence for extensive occurrence of cryptic species, the lack of formal taxonomic description (Fernandez-Triana, 2022) hinders accurate estimates of their contribution to biodiversity (Delić et al., 2017; Fišer et al., 2018; Hutchings and Kupriyanova, 2018), therefore limiting our understanding of their evolutionary and ecological significance, as well as their recognition in large scale biomonitoring programs using high throughput sequence technologies.

The homogeneously green phyllodocid, Eulalia viridis (Linnaeus, 1767), has been reported from throughout the northern hemisphere (Eibye Jacobsen, 1991, 1993) and is common in intertidal and subtidal coastal areas and marinas, at depths until 50 m (Ushakov, 1972). This species usually lives on rocky reefs in crevices, among algae, mussel beds, Balanus spp. blocks, Dendropoma reefs, Posidonia oceanica meadows and coralligenous formations (Bonser et al., 1996; Viéitez et al., 2004; Çinar, 2005). However, it does not occur in the Sabellaria alveolata (Linnaeus, 1767) reefs from the Mediterranean, where it is replaced by Eulalia ornataSaint-Joseph 1888, another greenish species morphologically highly similar to E. viridis except for the pigmentation pattern (Schimmenti et al., 2016). In a study from 1996, Bonse and colleagues, using isoelectric focusing and morphological data, found a correlation between exclusive isoenzymes and protein patterns, the morphology and size of the midbody dorsal cirri, and the size of the proboscideal papillae, that allowed to discriminate between two distinct groups of Eulalia populations. One morphotype, sampled in the North Sea and Scandinavia coast, with smaller papillae and slender dorsal cirri, corresponded to $E$. viridis, while the other one, occurring in France and England, showing larger papillae and significantly thicker dorsal cirri, was attributed to Eulalia clavigera (Audouin \& Milne Edwards, 1833), hitherto considered synonymous with E. viridis. The reproductive biology of these species in particular, and of phyllodocids in general, is poorly known. These species have a planktonic larval stage and reproduce once a year (Meyer, 1938), but local populations along the coasts of Northern Europe also differ in the time of reproduction with reproductive cycle starting 4 to 6 weeks earlier in Swedish specimens compared to the ones from the English and French coasts (Olive, 1975; Pleijel, 1993). Molecular studies based on the mitochondrial cytochrome c oxidase subunit I (COI) locus (Hardy et al., 2011; Lobo et al., 2016) also allowed the separation of populations identified as Eulalia
viridis from Kandalaksha Bay (Russia) and Portugal, respectively with more than 20\% Kimura's two parameter (K2P) genetic divergence. The highly similar morphology and the large number of genetic markers discriminating between this eastern and the western group implies the existence of a pseudocryptic species complex. Given the high number of species already found within complexes from other phyllodocids such as Notophyllum (Nygren et al., 2010) or Eumida (Nygren and Pleijel, 2011), and even in other polychaete families (Lobo et al., 2016, Sampieri et al., 2021, Martin et al., 2017, 2020), the actual diversity and distribution of the Eulalia viridis/clavigera species group in Europe is questioned. Langeneck et al. (2019) collected a large amount of E. clavigera specimens from Nuevo Gulf, Patagonia (South-western Atlantic Ocean) and using the mitochondrial COI marker detected no genetic structure between the north-eastern and south-western Atlantic, supporting a non-native origin of the Patagonian population. However, a distinct Mediterranean lineage was found when compared against the Patagonia and the NE Atlantic clade.

In this study a multi-locus approach and morphometric data is used to investigate the possible occurrence of additional diagnosable species within the Eulalia viridis/clavigera complex, comparing the E. clavigera species reported in Europe, from the United Kingdom to Portugal, the Macaronesia islands (Azores, Madeira and Canaries) and the Western and Eastern Mediterranean Sea.

### 4.2 Material and methods

### 4.2.1 Taxon sampling and molecular data retrieval

A total of 134 Eulalia specimens presumably belonging to the Eulalia clavigera/viridis complex and 1 Phyllodoce species distributed along the European coasts and Macaronesia Islands were sampled (Fig. 4.1). Worms were collected at low tide in rocky beaches among the algae and mussels, marinas or subtidal areas up to 34 meters in depth. The specimens were fixed in $96 \%$ ethanol. Samples were harvested in continental Portugal (Canto Marinho, Leixoes, Aveiro, Nazaré) as well as in Santa Maria and Madeira islands, Spain (Coruna, Tenerife, Gran Canaria and La Palma), France (Roscoff, Morgat, Banyuls and Corsica), Great Britain (Plymouth and Cornwall), Norway (Espevaer, Grimstad, Bergen, Trondheim and Finmark), Sweden (Koster), Italy (Livorno, Ischia island and Taranto) and Croatia (IStria). Sample sites and abbreviations can be found in Table 4.1.

A partial segment of the 5' end of the mitochondrial cytochrome oxidase subunit I (mtCOI-5P) was sequenced from 119 specimens, and a representative number per location for the ITS-regions (i.e. ITS1, 5.8 S rRNA, and ITS2) and 28 S rRNA.


Fig. 4.1. Map with the sampling sites used for this study. Abbreviations as seen in Table 4.1.

Four mitochondrial sequences (COI) belonging to Eulalia cf. clavigera sampled in the Mediterranean Sea (Capraia island and port of Stintino, Italy) from Langeneck et al. (2019) were mined from GenBank for comparison purposes (MG253799 - MG253802). Molecular data of Eulalia aurea Gravier, 1896 and Phyllodoce sp. Lamarck, 1818 were used as outgroups for all the analysed loci to comprise the final dataset. DNA was extracted, amplified, sequenced, and assembled as described in the Chapter 3 of this thesis. Regarding PCR conditions, primers and sequence lengths for the different markers see Chapter 3, Table 3.1. Supplemental Table S4.1 details the sampling locations, public BIN accession numbers and voucher data for the original material. As only a few parapodia or a small portion of the posterior end were used for the extraction, the majority of the specimens included in this study have been deposited in the Research Collection of Marine Invertebrates of the Department of Biology of the University of Aveiro (COBI at DBUA) and are available for further morphological and molecular study.

Two specimens from Corsica were deposited in Muséum national d'Histoire naturelle (MNHN) and the French Mediterranean specimen BI-2014/15-077 was donated to SCRIPPS Oceanography. Additionally, the following specimens are stored in Arne's Nygren private collection and were assigned only with the Process ID from the BOLD systems (http://v4.boldsystems.org/): MTE040-20, MTE042-20, MTE05220, MTE053-20, MTE054-20, MTE055-20, MTE057-20, MTE079-20, MTE080-20, MTE081-20 and MTE088-20.

Table 4.1. Number of specimens acquired for this study, the respective sampling area and code abbreviation for the different sampling locations.

| Code | Region | Location | n |
| :--- | :--- | :--- | ---: |
| PTA | NE European Coast | Portugal, Aveiro | 6 |
| PTL | NE European Coast | Portugal, Marina of Leixões | 1 |
| PTC | NE European Coast | Portugal, Canto Marinho | 10 |
| PTN | NE European Coast | Portugal, Nazaré | 3 |
| SPC | NE European Coast | Spain, Coruña | 5 |
| FRM | NE European Coast | France, Morgat | 2 |
| FRR | NE European Coast | France, Roscoff | 8 |
| GBP | NE European Coast | Great Britain, Plymouth | 12 |
| GBC | NE European Coast | Great Britain, Cornwall | 7 |
| SK | Scandinavia, Skagerrak | Sweden, Koster | 3 |
| NOE | Scandinavia, Skagerrak | Norway, Grimstad | 3 |
| NOG | Scandinavia, North Sea | Norway, Espevaer | 3 |
| NOB | Scandinavia, North Sea | Norway, Bergen | 1 |
| NOT | Scandinavia, Norway Sea | Norway, Trondheim | 4 |
| NOF | Scandinavia, Barents Sea | Norway, Finmark | 1 |
| FRBA | West Mediterranean Sea | France, Banyuls | 2 |
| FRC | West Mediterranean Sea | France, Corsica | 14 |
| ITL | West Mediterranean Sea | Italy, Livorno | 2 |
| ITI | West Mediterranean Sea | Italy, Ischia island | 3 |
| ITT | East Mediterranean Sea | Italy, Taranto | 2 |
| CI | East Mediterranean Sea | Croatia, Istria | 1 |
| AM | Macaronesia archipelagos | Azores, Santa Maria | 1 |
| MF | Macaronesia archipelagos | Madeira, Funchal | 2 |
| MP | Macaronesia archipelagos | Madeira, Porto Moniz | 8 |
| TE | Macaronesia archipelagos | Canary islands, Tenerife | 4 |
| GC | Macaronesia archipelagos | Canary islands, Gran Canaria | 11 |
| LP | Macaronesia archipelagos | Canary islands, La Palma | 10 |
| SI | Macaronesia archipelagos | Savage islands | 1 |

The full dataset and its metadata can be accessed at BOLD Systems under the project "Eulalia Species Complex (DS-MTE)", except for the four COI sequences from Langeneck et al. (2019), which cannot be found in BOLD. The dataset will be publicly available upon this chapter's acceptance for publication in a peer reviewed journal.

### 4.2.2 Phylogenetic analysis and genetic distances

Maximum likelihood (ML) and Bayesian inference (BI) were used to perform the phylogenetic analyses of the different loci. The nuclear markers (ITS-regions and 28S) and the mitochondrial COI locus were concatenated with MEGA 10.0.05 (Kumar et al., 2018) and aligned with MAFFT online (https://mafft.cbrc.jp/alignment/server/, Katoh and Standley, 2013). Highly variable regions, extensive gaps and poorly aligned positions, which were mainly present in the ITS-regions, were eliminated using Gblocks 0.91b (http://molevol.cmima.csic.es/castresana/Gblocks_server.html; Castresana, 2000), allowing all the options for a less stringent selection and not allowing many contiguous non-conserved positions. MrBayes 3.1.2 (Ronquist and Huelsenbeck 2003) was used to conduct the Bayesian analysis. Best-fit models were selected using the Akaike Information Criterion in the JModeltest software (Darriba et al., 2012; Guindon and Gascuel, 2003). For COI the Hasegawa-Kishino-Yano gamma distributed rates across sites ( $H K Y+G$ ) was applied for the first two positions and the HKY model with equal rates across sites for the third position. Regarding the concatenated ITS with 28S, the General Time Reversible model with equal rates across sites (GTR) was applied. Number of generations was set to 10000 000, and sample frequency to 500. Twenty-five percent of the samples were discarded as burn-in (burninfrac = 0.25 ). The resulting tree files were checked for convergence in the effective sampling sizes (ESSs >200) with Tracer 1.6 software (Rambaut et al., 2018) and then analysed in Figtree 1.4.3 (http://tree.bio.ed.ac.uk/software/figtree/). The final version of the concatenated tree was edited with the software Inkscape 0.92 .3 (https://www.inkscape.org). Maximum Likelihood phylogenies were performed in MEGA 10.0.05 with 1000 bootstrap runs with the GTR model with equal rates across sites for the concatenated dataset. Only the BI tree was displayed in the results and if a similar topology is found, with the addition of the ML support values.

The mean genetic distances (K2P) within and between molecular operational taxonomic units (MOTUs) were calculated in MEGA 10.0.05 using the same GBlock alignment from above for the nuclear loci.

### 4.2.3 MOTU clustering

To depict Molecular Operational Taxonomic Units (MOTUs), three delineation methods to the concatenated alignment were applied based on ABDG (Puillandre et al., 2012), bPTP (Zhang et al., 2013) and GYMC (Fujisawa and Barraclough, 2013) as detailed in Chapter 3 of this thesis. BEAST 2.4.6 (Bouckaert et al., 2014) was used to generate the Bayesian ultrametric tree for the GMYC based on AIC criteria, with GTR model and equal rates across sites. The Barcode Index Number (BIN) was applied as well for the COI, which makes use of the Refined Single Linkage (RESL) algorithm implemented in BOLD (Ratnasingham and Hebert, 2013), exclusive to this locus. A final consensus MOTU was chosen using the majority rule (i.e. most common number of MOTUs).

### 4.2.4 Genetic diversity and structure

Haplotype networks were made through the PopART software (Leigh and Bryant, 2015) using the method of Templeton, Crandall and Sing (TCS, Clement et al., 2002) to evaluate the relationship between the haplotypes and their geographical distribution. No GBlocks were applied in this analysis to avoid underestimating the number of nuclear haplotypes. Indices of genetic diversity, namely number of haplotypes (h), haplotype diversity (hd), polymorphic sites (S), nucleotide diversity ( $\pi$ ), Fu \& Li D and Tajima D statistical tests, were estimated based on COI for each MOTU using DNASP 5.10 (Librado and Rozas 2009).

### 4.2.5 Morphometric analysis

Specimens from four Eulalia lineages were used for morphometric analysis and compared against each other to complement the molecular data. The remaining lineages had less than three available specimens with a very small size (therefore unsuitable for morphometric studies) and were not named or used in this analysis. A minimum of 5 specimens with optimal conditions (i.e. specimens with the presence of the proposed morphological characters for this study and whenever possible, similar in size) per population were chosen.

The following characters were selected and measured (Fig. 4.2.A, B): the number of segments (NS); the length (mm) of the worm (WL), chaetigerous lobes (CLL), terminal antennae (AL), palps (PL), middle antenna (MAL), dorsal and ventral tentacular cirrus from the second segment (DTL, VTL, respectively), dorsal and ventral cirri ( $\mathrm{DCL}, \mathrm{VCL}$ ) and head ( HL ); the width ( mm ) of the worm with parapodia (WWP) and without parapodia (WW), head (HW) and dorsal and ventral cirri (DCW, VCW); and
the distance between the eyes (DE) as well the height (mm) of the chaetigerous lobes (CLH). WW, WWP and the different parapodia structures were measured from the worm's widest part. The distance between the eyes was measured from the center of the eyespots to avoid possible different individual responses to fixation as is the case of hesionids in Martin et al. (2017).


Fig. 4.2. Schematic of the Eulalia clavigera morphotype showing the measurements used in the morphometric analysis (A, B). (A) Anterior end. (B) Parapodia. Abbreviations: CLL, the length of the chaetigerous lobes; CLH, the height of the chaetigerous lobes; $A L$, the length of the antennae; $P L$, the length of the palps; MAL, the length of the middle antenna; DTL, dorsal tentacular cirri on segment 2; VTL, ventral tentacular cirri on segment 2; DCL, the length of the dorsal cirri; VCL, the length of the ventral cirri; HL, the length of the head; WWP, the width of the worm with parapodia; WW, the width of the worm without parapodia; HW, the width of the head; DCW, the width of the dorsal cirri; VCW, the width of the ventral cirri; DE , distance between the eyes.

To minimize bias based on size variability, measurements taken to analyse the inter-lineage differences were converted to ratios and used to create scatter plots between relevant morphological characters found in Phyllodocids similar to previous studies (Chapter 3 of this thesis). All remaining analyses were conducted using Microsoft Excel (Office 365 ProPlus). Measurements were done with a LEICA MC170 HD stereo microscope, with an incorporated measurement software.

### 4.3 Results

### 4.3.1 Genetic distances

The Global intra- and interspecific mean distances for the nine MOTUs and two outgroups for each marker are provided in Table 4.2. Apart from the outgroups and Eulalia IT2-1 (MOTU 8, Fig. 4.3.A), the mean intraspecific distance for COI is $0.93(0.0-3.3) \%$, while the average congeneric distance is $17.9(7.1-25.5) \%$. For the ITS-region it ranges between $1.4(0.0-3.9) \%$ and $17.2(4.4-32.6) \%$ for intra- and interspecific divergence, respectively, while for 28 S the corresponding distances are 0.04 (0 $0.4) \%$ and 2.7 ( $0-5.9$ )\%, respectively. The populations between the continental Europe and the Macaronesia islands from E. clavigera (MOTU 4, Fig. 4.3.A) only have COI maximum distances up to $3.3 \%$ and no significative divergence $(<1 \%)$ in the nuclear markers. Eulalia IT2-1 has a particularly high interspecific distance in the nuclear markers, reaching values higher than 60\% for the ITS region and $12 \%$ for the 28 S locus, similar to the ones found in the Phyllodoce sp. (Outgroup). This lineage belongs to a very small specimen which at first, seemed to fit the E. viridis morphotype based on the small size, pointed midbody dorsal cirri and bright red eyes, however molecular data is very divergent, showing evidence of an entirely new Eulalia group yet to be described.

### 4.3.2 Phylogenetic reconstruction

Without any variation in the different delineation methods, nine MOTUs are retrieved from the concatenated Bayesian phylogenetic tree (Fig. 4.3.A), belonging to monophyletic clades with low divergence $(<3 \%$ ). Apart from the previously described E. clavigera (MOTU 4) and E. viridis (MOTU 7), molecular evidence for six new Eulalia species can be found with the addition of MOTU GB1 from Langeneck et al. (2019). Major clade A englobes four MOTUs which are genetically close to E. clavigera with high bootstrap support. This is composed of MOTU 1, unique to the western Mediterranean, MOTU 2, unique to the subtidal habitats from the island of Madeira (Portugal) the unnamed Eulalia KRO53 (MOTU 3) occurring in the Eastern Mediterranean Sea and lastly, the Mediterranean Eulalia cf. clavigera (MOTU GB1).

MOTUs 5 and 6 are within major clade $B$, are sister to each other and genetically closer to $E$. viridis and the outgroup $E$. aurea instead, the latter revealing itself to be an actual ingroup for this complex. MOTU 5 is present both in the British Isles and Western Mediterranean, while the subtidal samples from MOTU 6, together with MOTUs 3 and 8 have few and very small specimens in relatively poor conditions or exhausted in the DNA analysis, and thus were not named or used in the morphometric analysis.

Table 4.2. Mean intra (in BOLD) and inter-species genetic distances (K2P) for the 3 analysed markers (COI, ITS, 28S), for the 9 Eulalia lineages and 2 outgroups.

|  |  | Loci | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 |
| :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: |
| E. clavigera | 1 | COI | $1.4 \pm 0.3$ |  |  |  |  |  |  |  |  |  |  |
|  |  | ITS | $1.7 \pm 0.2$ |  |  |  |  |  |  |  |  |  |  |
|  |  | 28 S | $0.0 \pm 0.0$ |  |  |  |  |  |  |  |  |  |  |
| Eulalia KRO53 | 2 | COI | $7.5 \pm 1.1$ | NA |  |  |  |  |  |  |  |  |  |
|  |  | ITS | $4.8 \pm 0.5$ | NA |  |  |  |  |  |  |  |  |  |
|  |  | 28 S | $0.0 \pm 0.0$ | NA |  |  |  |  |  |  |  |  |  |
| E. madeirensis sp. nov. | 3 | COI | $11.4 \pm 1.3$ | $11.8 \pm 1.2$ | $0.8 \pm 0.2$ |  |  |  |  |  |  |  |  |
|  |  | ITS | $4.6 \pm 0.5$ | $6.9 \pm 0.7$ | $0.1 \pm 0.1$ |  |  |  |  |  |  |  |  |
|  |  | $28 \mathrm{~S}$ | $0.2 \pm 0.1$ | $0.1 \pm 0.1$ | $0.0 \pm 0.0$ |  |  |  |  |  |  |  |  |
| E. feliciae sp. nov. | 4 | $\mathrm{COI}$ | $13.9 \pm 1.5$ | $14.4 \pm 1.6$ | $14.2 \pm 1.6$ | $1.0 \pm 0.2$ |  |  |  |  |  |  |  |
|  |  | ITS | $5.4 \pm 0.6$ | $7.6 \pm 0.7$ | $7.0 \pm 0.7$ | $2.1 \pm 0.4$ |  |  |  |  |  |  |  |
|  |  | $28 \mathrm{~S}$ | $0.1 \pm 0.1$ | $0.1 \pm 0.1$ | $0.2 \pm 0.1$ | $0.2 \pm 0.1$ |  |  |  |  |  |  |  |
| E. viridis | 5 | $\mathrm{COI}$ | $23.3 \pm 1.9$ | $23.3 \pm 1.9$ | $22.0 \pm 1.7$ | $21.2 \pm 1.8$ | $0.8 \pm 0.2$ |  |  |  |  |  |  |
|  |  | ITS | $29.3 \pm 1.8$ | $30.5 \pm 1.8$ | $30.8 \pm 1.8$ | $28.7 \pm 1.7$ | $2.7 \pm 0.4$ |  |  |  |  |  |  |
|  |  | 28 S | $5.6 \pm 0.9$ | $5.6 \pm 0.9$ | $5.7 \pm 0.9$ | $5.7 \pm 0.9$ | $0.0 \pm 0.0$ |  |  |  |  |  |  |
| xanthomucosa sp. nov. | 6 | COI | $20.1 \pm 1.7$ | $20.1 \pm 1.8$ | $19.9 \pm 1.6$ | $20.4 \pm 1.9$ | $20.3 \pm 1.9$ | $1.3 \pm 0.3$ |  |  |  |  |  |
|  |  | ITS | $17.3 \pm 1.3$ | $18.6 \pm 1.4$ | $18.3 \pm 1.4$ | $16.4 \pm 1.3$ | $27.8 \pm 1.8$ | $0.3 \pm 0.1$ |  |  |  |  |  |
|  |  | 28 S | $2.7 \pm 0.6$ | $2.7 \pm 0.6$ | $2.9 \pm 0.6$ | $2.8 \pm 0.6$ | $5.1 \pm 0.9$ | $0.0 \pm 0.0$ |  |  |  |  |  |
| Eulalia IS-BA | 7 | COI | $18.3 \pm 1.6$ | $19.7 \pm 1.7$ | $19.9 \pm 1.7$ | $19.6 \pm 1.8$ | $23.7 \pm 2.1$ | $12.1 \pm 1.5$ | $0.3 \pm 0.2$ |  |  |  |  |
|  |  | ITS | $17.7 \pm 1.3$ | $19.3 \pm 1.4$ | $18.9 \pm 1.4$ | $16.2 \pm 1.2$ | $27.5 \pm 1.3$ | $7.6 \pm 0.9$ | $0.8 \pm 0.2$ |  |  |  |  |
|  |  | 28 S | $2.7 \pm 0.6$ | $2.7 \pm 0.6$ | $2.9 \pm 0.6$ | $2.8 \pm 0.6$ | $5.7 \pm 0.9$ | $0.5 \pm 0.3$ | $0.0 \pm 0.0$ |  |  |  |  |
|  |  | COI | $17.7 \pm 1.6$ | $18.4 \pm 1.6$ | $19.7 \pm 1.8$ | $17.9 \pm 1.5$ | $21.4 \pm 1.9$ | $15.4 \pm 1.6$ | $17.5 \pm 1.5$ | $1.4 \pm 0.3$ |  |  |  |
| E. aurea (OUTG) | 8 | ITS | $11.4 \pm 1.0$ | $11.8 \pm 1.0$ | $13.0 \pm 1.1$ | $11.2 \pm 0.9$ | $29.1 \pm 1.9$ | $17.3 \pm 1.7$ | $19.4 \pm 1.4$ | $1.7 \pm 0.3$ |  |  |  |
|  |  | $28 \mathrm{~S}$ | $0.6 \pm 0.2$ | $0.5 \pm 0.2$ | $0.7 \pm 0.3$ | $0.6 \pm 0.2$ | $5.6 \pm 0.9$ | $3.3 \pm 0.6$ | $3.0 \pm 0.6$ | $0.3 \pm 0.2$ |  |  |  |
|  |  | $\mathrm{COI}$ | $24.1 \pm 2.0$ | $23.9 \pm 2.1$ | $23.4 \pm 1.9$ | $24.2 \pm 1.9$ | $23.7 \pm 2.0$ | $21.8 \pm 1.9$ | $24.0 \pm 1.9$ | $22.3 \pm 1.9$ |  |  |  |
| Eulalia IT2-1 | 9 | ITS | $63.9 \pm 3.4$ | $65.1 \pm 3.5$ | $62.5 \pm 3.3$ | $61.8 \pm 3.4$ | $59.0 \pm 3.3$ | $55.8 \pm 3.3$ | $55.5 \pm 3.3$ | $62.4 \pm 3.5$ | NA |  |  |
|  |  | $28 \mathrm{~S}$ | $12.5 \pm 1.4$ | $12.5 \pm 1.4$ | $12.6 \pm 1.4$ | $12.5 \pm 1.4$ | $14.1 \pm 1.5$ | $11.5 \pm 1.3$ | $11.5 \pm 1.3$ | $12.1 \pm 1.3$ | NA |  |  |
| Eulalia cf. clavigera. (GB1) | 10 | COI | $15.9 \pm 1.5$ | $16.3 \pm 1.3$ | $16.9 \pm 1.6$ | $17.0 \pm 1.6$ | $21.6 \pm 1.8$ | $19.8 \pm 1.7$ | $17.9 \pm 1.7$ | $16.8 \pm 1.6$ | $21.5 \pm 2.0$ | $0.5 \pm 0.2$ |  |
| Phyllodoce sp. (OUTG) | 11 | COI | $19.3 \pm 1.6$ | $21.3 \pm 1.8$ | $21.6 \pm 1.8$ | $22.6 \pm 1.8$ | $23.2 \pm 1.8$ | $18.1 \pm 1.6$ | $16.7 \pm 1.6$ | $22.2 \pm 1.8$ | $24.6 \pm 1.9$ | $20.9 \pm 1.9$ | NA |
|  |  | ITS | $48.6 \pm 2.5$ | $50.2 \pm 2.6$ | $47.9 \pm 2.5$ | $48.3 \pm 2.5$ | $48.6 \pm 2.9$ | $42.2 \pm 2.4$ | $43.5 \pm 2.4$ | $50.2 \pm 2.6$ | $54.9 \pm 3.0$ | NA | NA |
|  |  | 28 S | $8.1 \pm 1.1$ | $8.1 \pm 1.1$ | $8.2 \pm 1.1$ | $8.2 \pm 1.1$ | $9.0 \pm 1.2$ | $8.4 \pm 1.2$ | $8.4 \pm 1.1$ | $8.7 \pm 1.1$ | $12.8 \pm 1.4$ | NA | NA |



Fig. 4.3. (A) Phylogenetic tree reconstructed using Bayesian inference based on concatenated COI, ITS and $28 S$ sequences, with information regarding the different MOTU delineation methods. BINs were used only for COI. MOTU GB1 only have COI sequences and was not present in BOLD systems preventing BIN analysis. Only the bootstrap values over 0.85 BI and 85 ML support are shown. Each different consensus MOTU is represented by the respective number, with the different colours corresponding to the respective geographic distribution. Live photo belong to the specimen DBUA0002474.02.v01, measuring around 45 mm in length and exhibiting greenish colour. (B) Haplotype network based on COI for all the analysed MOTUs and outgroups (OUTG). Each haplotype is represented by a circle and number of haplotypes are according to the displayed scale. Colours indicate the geographic location of the haplotype. Numbers correspond to the number of mutational steps between haplotypes. Lines without numbers means only one mutation between haplotypes.

### 4.3.3 Haplotype networks

Only the 28S network (Fig. 4.4.B) fail to discriminate all the identified MOTUs from the concatenated dataset and is characterized by a star-shape phylogeny, with most of the unique haplotypes closely related to the common central haplotype which is composed by MOTUs 1, 2 and 4. However, MOTU 1 also has a distinct haplotype, with a similar number of mutations apart as the outgroup $E$. aurea, from the common one. The ancestral central haplotype might suggest the possibility of vicariance-driven speciation through a single colonization event and subsequent diversification.


Fig. 4.4. Haplotypes networks based on ITS (A) and 28 (B) for all MOTUs and outgroups, except MOTU GB1. Each haplotype is represented by a circle and number of haplotypes are according to the displayed scale. Colours indicate
the geographic location of the haplotype. Numbers correspond to the number of mutational steps between haplotypes. Lines without numbers means only one mutation between haplotypes.

The COI (Fig. 4.3.B) and ITS (Fig. 4.4.A) networks reveal geographically structured populations within MOTU 4, between continental Europe and the Macaronesia archipelagos (Azores, Canary and Savage islands). This correspond to the two distinct clades found in the Bl tree, but have not enough divergence to be divided into two separate MOTUs. Other biogeographical signals, where certain haplotypes or parts of the haplotype network can be correlated with a specific biogeographic region, can be found in the Madeira island (MOTU 2), Scandinavia (MOTU 7), eastern Mediterranean (MOTU 3) and south of France (MOTU 1).

Table 4.3. Indices of genetic diversity estimated for each Eulalia species and outgroups (OUTG), based on COI. Number of sequences ( n ); nucleotide diversity ( tt ), number of haplotypes ( h ), haplotype diversity ( Hd ) and number of variables sites (S). Region abbreviations as stated in Table 4.1.

|  | Region | N | h | Hd | S | tt | Fu and Li's D* | Tajima's D |
| :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: |
| E. clavigera | PTA; PTN; PTC; SPC; FRR; FRM; GBP; ITL; AM; SI; LP;TE; GC | 63 | 34 | 0.970 | 46 | 0.01326 | $\begin{gathered} -1,64660 \\ P>0.10 \end{gathered}$ | $\begin{gathered} -0,48425 \\ P>0.10 \end{gathered}$ |
| Eulalia KRO53 | Cl | 1 | 1 | - | - | - | - | - |
| E. madeirensis sp. nov. | MP; MF | 12 | 11 | 0.985 | 22 | 0.00838 | $\begin{gathered} -1.58386 \\ P>0.10 \end{gathered}$ | $\begin{gathered} -1.22113 \\ P>0.10 \end{gathered}$ |
| E. feliciae sp. nov. | FRBA | 10 | 8 | 0.956 | 18 | 0.00957 | $\begin{aligned} & 0.01523 \\ & P>0.10 \end{aligned}$ | $\begin{gathered} -0.24985 \\ P>0.10 \end{gathered}$ |
| E. viridis | SK. NOG; NOE; NOB; NOT; NOF | 14 | 8 | 0.890 | 23 | 0.00802 | $\begin{gathered} -1.45830 \\ P>0.10 \end{gathered}$ | $\begin{gathered} -1.27765 \\ P>0.10 \end{gathered}$ |
| E. xanthomucosa sp. nov. | FRBA; GBC;FRC | 11 | 9 | 0.964 | 22 | 0.01139 | $\begin{gathered} -0.13958 \\ P>0.10 \end{gathered}$ | $\begin{gathered} -0.17886 \\ P>0.10 \end{gathered}$ |
| Eulalia IS-BA | FRBA; ITI | 3 | 3 | 1 | 3 | 0.00318 | - | - |
| E. aurea | GBP | 3 | 3 | 1 | 13 | 0.01378 | - | - |
| Eulalia IT2-1 | ITT | 1 | 1 | - | - | - | - | - |


|  | Region | N | h | Hd | $\mathbf{S}$ | tt | Fu and Li's <br> $\mathbf{D}^{*}$ | Tajima's D |
| :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: |
| Eulalia sp. <br> (GB1) <br> Phyllodoce $s p$. | ITC; ITS | 4 | 4 | 1 | 5 | 0.00486 | -0.20080 <br> $P>0.10$ | -0.21249 |

(Table 4.3. Continuation)

The COI haplotype diversity is relatively high ( $\mathrm{Hd}>0.89$ to 0.985 , Table 4.3 ) and in some cases it can be extreme, with almost all specimens having an unique haplotype as seen in MOTU 1 (8 haplotypes in 10 specimens) and MOTU 2 ( 11 haplotypes in 12 specimens). None of the MOTUs have a significant Tajima $D$ and Fu and Li's $D$ tests, with the neutral model of nucleotide substitutions being accepted for all the lineages.

### 4.3.4 Morphometric measurements

The most noticeable morphometric proportions can be seen in the scatter plots in Figs. 4.5.A-F, displaying considerable visible differences, with the formation of independent clusters among the analysed species. The exception is represented by specimens of MOTU 5 (Fig. 4.6.B) and MOTU 4 which often overlap the same morphometric cluster. However, specimens from the latter species are considerably larger (both in number of segments and the worm's length and width) than the ones from MOTU 5. Despite the worm's size difference, MOTU 5 is characterized by the presence of large morphological structures such as the dorsal and ventral cirri, head, dorsal cirri on segment 2 and the antennae. No considerable differences are found in E. clavigera between the populations from continental Europe and the Canary islands. Nevertheless, partial morphometric clusters between the number of segments compared to the worm width and the ratio between the middle antenna with the head length are the only morphometric proportions able to partially differentiate between these two populations (Fig. 4.5.G, H)

The use of morphometric proportions between the head length against either the head width, the length of the antennae or dorsal cirri on segment; and between the length of the ventral cirri against either the length of the chaetigorous lobe, dorsal cirri and width of the ventral cirri seems to be effective in distinguishing MOTU 2 (Fig. 4.6.C), MOTU 1 (Fig. 4.6.A) and E. clavigera from each other. MOTU 2 has smaller proportions when compared to the remaining analysed species, with MOTU 1 appearing in the
middle clusters (main morphometric findings, coloration, depth and geographic distribution summarized in Table 4.4).


Fig. 4.5. Scatter plots with the most considerable proportions in distinguishing E. clavigera (populations from mainland Europe and Canary islands), E. feliciae sp. nov., E. madeirensis sp. nov. and E. xanthomucosa sp. nov (A-H). (A) Morphometric proportions between the length of the ventral cirri (VCL) and the length of the dorsal cirri (DCL). (B) between the length of the dorsal cirri (DCL) and the width of the dorsal cirri (DCW). (C) between the length of the
chaetigorous lobe (CLL) and the length of the ventral cirri (VCL). (D) between the length of the head (HL) and the width of the head $(\mathrm{HW})$. (E) between the length of the head (HL) and dorsal tentacular cirri on segment 2 (DTL). (F) between the length of the head $(\mathrm{HL})$ and the length of the antennae $(\mathrm{AL})$. $(\mathrm{G})$ between the length of the head $(\mathrm{HL})$ and length of the median antenna (MAL). (H) between the width of the worm of median segments (WW) and the number of segments (NS).

### 4.3.5 Taxonomic section

Eulalia clavigera (Audouin \& Milne Edwards, 1833)
(Fig. 4.1; Fig. 4.3.A)
Phyllodoce clavigera Audouin and Milne-Edwards 1833: 226-228, PL. XVI, fig. 9-13
Eulalia clavigera: Bonse et al. 1996: 40-45, Fig. 14 (redescr., syn.); Alós 2004: 193-196, Fig. 69 (SEM photographs)
? Eulalia viridis: Morgado and Amaral 1984: 51 (non Linnaeus, 1767)

Material examined

Portugal, Aveiro: 6 spms, DBUA0002468.01.v01-v06, $40^{\circ} 33^{\prime} 32.4^{\prime \prime} \mathrm{N}-8^{\circ} 46^{\prime} 19.2^{\prime \prime} \mathrm{W}$, low tide, among rocks with algae and mussels, collected by Marcos AL Teixeira and Ascensão Ravara, 27/07/2018. Portugal, Canto Marinho: 7 spms, DBUA0002469.02.v01-v07, $41^{\circ} 44^{\prime} 13.2^{\prime \prime} \mathrm{N}$ $8^{\circ} 52^{\prime} 33.6^{\prime \prime}$ W, low tide, among rocks with algae and mussels, collected by Marcos AL Teixeira, 20/05/2019. Portugal, Areosa: 3 spms, DBUA0002469.01.v01-v03, $41^{\circ} 42^{\prime} 36.0^{\prime \prime} \mathrm{N}-8^{\circ} 52^{\prime} 12.0^{\prime \prime} \mathrm{W}$, low tide, among rocks with algae and mussels, collected by Marcos AL Teixeira, 20/03/2018. Portugal, Leixões: 1 spm, DBUA0002470.01.v01, $41^{\circ} 10^{\prime} 58.8^{\prime \prime} \mathrm{N}-8^{\circ} 42^{\prime} 18.0^{\prime \prime} \mathrm{W}$, marina in pontoon scrappings, collected by Sofia Duarte, 23/06/2020. Portugal, Nazaré: 3 spms, DBUA0002493.01.v01-v03, $39^{\circ} 36^{\prime} 13.0^{\prime \prime} \mathrm{N}-9^{\circ} 04^{\prime} 44.0^{\prime \prime} \mathrm{W}$, among rocks, collected by Ascensão Ravara, 26/07/2021. Portugal, Santa Maria (Azores): 2 spm, DBUA0002477.01.v01-v02, $36^{\circ} 57^{\prime} 03.6^{\prime \prime} \mathrm{N}-25^{\circ} 01^{\prime} 04.8^{\prime \prime} \mathrm{W}$, low tide, among rocks with algae and mussels, collected by Ana Costa, 07/05/2019. Portugal, Savage islands: 1 spm, MB29-000385, $30^{\circ} 08^{\prime} 23.9^{\prime \prime} \mathrm{N}-15^{\circ} 51^{\prime} 57.6^{\prime \prime} \mathrm{W}$, low tide, among rocks with algae, kindly provided by the National Museum of Science and Natural History (Portugal), collected in 22/06/2010. Spain, Ferrol lagoon: 5 spms, DBUA0002473.01.v01-v05, $43^{\circ} 30^{\prime} 07.2^{\prime \prime} \mathrm{N}-8^{\circ} 09^{\prime} 32.4^{\prime \prime} \mathrm{W}$, low tide, among rocks with algae and mussels, collected by Julio Parapar, 03/02/2015. Spain, Tenerife (Canary islands): 11 spms, DBUA0002476.01.v01-v11, $28^{\circ} 34^{\prime} 15.6^{\prime \prime} \mathrm{N}-16^{\circ} 20^{\prime} 02.4^{\prime \prime} \mathrm{W}$, low tide, among rocks with algae, collected by Marcos AL Teixeira and Pedro E Vieira, 05/04/2019. Spain, La Palma (Canary islands): 10
spms, DBUA0002476.02.v01-v10, $28^{\circ} 48^{\prime} 18.0^{\prime \prime} \mathrm{N}-17^{\circ} 45^{\prime} 43.2^{\prime \prime} \mathrm{W}$, low tide, among rocks with algae, collected by Marcos AL Teixeira and Pedro E Vieira, 09/04/2019. Spain, Gran Canaria (Canary islands): 5 spms, DBUA0002476.03.v01-v05, $27^{\circ} 59^{\prime} 06.0^{\prime \prime} \mathrm{N}-15^{\circ} 22^{\prime} 33.6^{\prime \prime} \mathrm{W}$, low tide, among rocks with algae, collected by Marcos AL Teixeira and Pedro E Vieira, 06/04/2019. France, Roscoff: 8 spm, DBUA0002471.01.v01-v08, $48^{\circ} 43^{\prime} 33.6^{\prime \prime} \mathrm{N}-3^{\circ} 58^{\prime} 40.8^{\prime \prime} \mathrm{W}$, low tide, among rocks with algae and mussels, collected by Arne Nygren, 20/03/2018. France, Morgat: 2 spms, DBUA0002472.01.v01-v02, low tide, among rocks with algae, $48^{\circ} 13^{\prime} 20.3^{\prime \prime} \mathrm{N}-4^{\circ} 29^{\prime} 42.5^{\prime \prime} \mathrm{W}$, collected by Nicolas Lavesque, 16/06/2018. Great Britain, Plymouth: 12 spms, DBUA0002474.01.v01-v05, DBUA0002474.02.v01v05, DBUA0002474.03.v01-v02, $50^{\circ} 21^{\prime} 25.2^{\prime \prime} \mathrm{N}-4^{\circ} 07^{\prime} 40.8^{\prime \prime} \mathrm{W}$, low tide, among rocks with algae and mussels, collected by Arne Nygren and Fredrik Pleijel, 18/03/2006. Italy, Livorno: 3 spms, DBUA0002475.01.v01-v03, $43^{\circ} 32^{\prime} 24.0^{\prime \prime} \mathrm{N}-10^{\circ} 18^{\prime} 00.0^{\prime \prime} \mathrm{E}$, marina in pontoon scrapings, collected by Joachim Langeneck, 20/09/2019.

Diagnosis (updated from Pleijel, 1993)
Body anteriorly stout and posteriorly tapered. Complete specimens with up to 275 segments and 68 mm total length, up to 2 mm maximum width if parapodia included (smallest specimens: 30 mm long, 1.6 mm wide, 155 chaetigers). Living specimens are deep green (Fig. 4.3.A), once preserved the pigment fades off into a greenish hue and can turn into brownish once aged. Prostomium rounded triangular, wider than long. Eyes medium-sized, rounded and occasionally partly covered by segment I. Distance between the eyes about the same length of the head. Median antenna $f$ similar size as the terminal ones situated well in front of the eyes. Palps about the same size as antennae. Proboscis widest distally, densely covered with rounded to conical papillae. Terminal ring with varying number of papillae. Tentacular cirri shorter than the body width. Tentacular cirri of segment 1 reaching about segment 3 and half the size of the largest tentacular cirri found in segment 2. Dorsal tentacular cirri of segment 2 and tentacular cirri from segment 3 reaching about segment 7 . Ventral tentacular cirri from segment 2 reaching about segment $3-4$, often thick and slightly flattened. Dorsal cirri of median segments asymmetrically lanceolate, about twice longer than wider. Ventral cirri rounded slightly longer than wider and smaller than the chaetigorous lobes. Chaetae usually present from segment 3, occasionally one or two chaetae arising from anterior side of ventral cirrophores of segment 2.

COI, ITS and 28 S sequences as in specimens DBUA0002468.01.v01-v05, DBUA0002469.01.v01-v03, DBUA0002469.02.v01-v05, DBUA0002470.01.v01, DBUA0002471.01. v01-v05, DBUA0002472.01.v01-v02, DBUA0002473.01.v01-v05, DBUA0002474.01.v01-v05, DBUA0002474.02.v01-v05, DBUA0002474.03.v01-v02, DBUA0002475.01.v01-v03, DBUA000 2476.01.v01-v05, DBUA0002476.02.v01-v07, DBUA0002476.03.v01-v05, DBUA0002477.01.v01-v02 and MB29-000385 (Table S4.1). Phylogenetic relationship as in Fig. 4.3.A, where E. clavigera is clearly distinct from the remaining species of the complex, grouping in MOTU 4. Interspecific COI mean distances to the closest and distant neighbour are $7.5 \%$ (K2P, Eulalia KRO53) and $23.3 \%$ (K2P, E. viridis) respectively. DOI for the species' Barcode Index Number (BIN): upon paper publication.

Distribution and habitat
From the NE Atlantic Ocean (United Kingdom, France, Iberian Peninsula) to the western Mediterranean (western Italy). Present as well in the archipelagos of the Canaries, Azores and Savage islands. Type locality: Brittany, France. It was also recorded as an introduced species in the South-western Atlantic Ocean (Langeneck et al., 2019).

Usually present in intertidal rocky areas surrounded by algae, mussels and associated with Sabellaria reefs. Also present in marinas among the algae attached to the pontoons.

Reproduction
The reproductive biology of this species is poorly known and available data most likely represent different lineages, corresponding to E. viridis from Scandinavian samples and E. clavigera from the English and French coasts. This species have a planktonic larval stage and reproduce once a year (Meyer, 1938), but local populations along the coasts of Northern Europe also differ in the time of reproduction with reproductive cycle starting 4 to 6 weeks earlier in Swedish specimens compared to the ones from the English and French coasts (Olive 1975; Pleijel, 1993)

Remarks

Bonse et al. (1996) redescribed E. viridis and reinstated E. clavigera (Audouin \& Milne-Edwards, 1834) which have been previously synonymised by McIntosh (1908). These two species have slight differences in prostomial, parapodial and pharynx papillation features that allow their distinction.

According to Bonse et al. (1996), the length-to-width ratio of dorsal cirri is the most useful character to distinguish between $E$. viridis and $E$. clavigera. Smaller papillae and slender dorsal cirri, corresponded to E. viridis, while E. clavigera have larger papillae and significantly thicker dorsal cirri. Eulalia viridis is also unique to the Scandinavia and Northern Sea and seems to be a northern boreal and sub-arctic species both in intertidal or subtidal waters. Eulalia clavigera is a temperate species mostly found in intertidal rocky beaches, ranging from Great Britain to the western Mediterranean Sea, being present as well in the Azores, Savage islands and widespread in the Canary islands.

Audouin \& Milne Edwards (1833) erected the species Phyllodoce gervilleifrom Granville (France), stating that it is identical to $P$. clavigera, with the exception of the missing median antenna and smaller tentacular cirri. McIntosh (1908) synonymised both species with E. viridis, considering that the absence of antennae in $P$. gervillei may have been accidental. However, given the type locality of $P$. gervillei, that species is most probably a synonym of $E$. clavigera.

Specimens from the type locality of E. clavigera (Brittany, France) were collected for this study and grouped in MOTU 4 (Fig. 4.3.A). The number of segments compared to the worm width and the ratio between the middle antenna with the head length were the only morphometric proportions able to better separate the continental European populations from the ones found in the Canary islands (Fig. 4.5.G,H). This lack of variation is also reflected in the molecular data where these two populations, although present in two distinct clades, only diverge up to $3.3 \%$ (COI) between each other, grouping in the same MOTU. Eulalia clavigera usually possess larger proportions in most of the diagnostic characters when compared against the other three species from the complex described here, especially the ratio between the length of the dorsal and ventral cirri, between the length of the chaetigorous lobe and ventral cirri, the length to width ratio in the ventral cirri, as well as the ratio between the length of the head against either the length of the dorsal cirri on segment 2, antennae or the width of the head. The exception to this can sometimes be found against specimens from E. xanthomucosa sp. nov. (described below), which can often share the same cluster measurements, however, analysed specimens from E. clavigera were considerable larger in size (number of segments; worm's length and width).

Table 4.4. Summary of the most relevant morphometric findings rating from 1 (smaller proportions) to 4 (larger proportions), number of segments (NS), worm length (WL), worm width (WW), live and preserved coloration, depth and geographical range between the new described species and E. clavigera. Abbreviations for the morphometric proportions as stated in the methods.

|  | E. clavigera | E. madeirensis sp. nov. | E. feliciae sp. nov. | E. xanthomucosa sp. nov. |
| :---: | :---: | :---: | :---: | :---: |
| VCL/DCL | 3 | 1 | 2 | 3 |
| VCL/VCW | 3 | 1 | 2 | 3 |
| VCL/CLL | 4 | 1 | 2 | 3 |
| HL/HW | 4 (larger width only) | 1 | 2 | 3 |
| HL/DTL | 3 | 1 | 2 | 3 |
| HL/AL | 3 | 1 | 2 | 3 |
| AL/PL | 2 (AL=PL) | 1 ( $\mathrm{AL}<\mathrm{PL}$ ) | $2(\mathrm{AL}<\mathrm{PL})$ | 2 (AL=PL) |
| NS (mean) | 164 / continent 221 / islands | 70 | 113 | 103 |
| WL (mean, mm) | 39.1 / continent 52.5 / islands | 6.6 | 12.2 | 16 |
| WW (mean, mm) | 1.3 | 0.209 | 0.381 | 0.551 |
| Color: Live specimens | Green | Yellowish/light green | Emerald green | Yellow |
| Color: Preserved specimens | Green; Greenish brown; Brown | Greenish brown; Brown | Green; Greenish brown | Brown |
| Depth (meters) | Usually intertidal | Subtidal (5-25) | Usually Intertidal | Intertidal, but mostly subtidal (134) |
| Distribution | NE Atlantic; Macaronesia islands; Western Mediterranean | Island of Madeira (Portugal) | Mediterranean France | Great Britain; Mediterranean France |



Fig. 4.6. Live, relaxed Eulalia specimens exhibiting the different types of coloration corresponding to the new described species and information regarding the specimen size (WL: worm length). (A) Eulalia feliciae sp. nov., specimen DBUA0002478.01.v07, dorsal view, exhibiting greenish colour. (B) Eulalia xanthomucosa sp. nov., specimen from the Natural History Museum, live photo by David Fenwicki (left) and specimen BI-2014/15-077 (right), dorsal view, exhibiting yellow colouration. (C) Eulalia madeirensis sp. nov., specimen DBUA0002479.01.v03, dorsal view, exhibiting a faint yellowish/light green colour.

# Eulalia madeirensis sp. nov. 

(Fig. 4.6.C)
urn:Isid:zoobank.org:act: upon paper publication

Material examined
Type material. Portugal, Madeira (Funchal): 1 spm, holotype and hologenophore, DBUA0002479.01.v02, $32^{\circ} 38^{\prime} 09.6^{\prime \prime} \mathrm{N}-16^{\circ} 55^{\prime} 51.6^{\prime \prime} \mathrm{W}$, subtidal, 11 m depth, collected by Arne Nygren, 21/09/2009; 4 spms, paratypes and paragenophores, DBUA0002479.01.v01, DBUA0002479.01.v03, DBUA0002479.01.v04-v06, $32^{\circ} 38^{\prime} 09.6^{\prime \prime} \mathrm{N}-16^{\circ} 55^{\prime} 51.6^{\prime \prime} \mathrm{W}$, subtidal, 11 m depth, collected by Arne Nygren, 21/09/2009.

Other material. Portugal, Madeira (Funchal): 1 spm, MTE052-20, 32º38'09.6"N $16^{\circ} 55^{\prime} 51.6^{\prime \prime}$ W, subtidal, 11 meters depth, collected by Arne Nygren, 21/09/2009. Portugal, Madeira (Porto Moniz), 4 spms, DBUA0002479.02.v01, MTE053-20, MTE055-20 and MTE057-20, $32^{\circ} 51^{\prime} 38.6^{\prime \prime} \mathrm{N} 17^{\circ} 09^{\prime} 06.3^{\prime \prime} \mathrm{W}$, subtidal, 11 meters depth, collected by Arne Nygren, 30/09/2009.

## Diagnosis

Small worms both in width, length and number of segments; complete specimens with up to 115 segments and 10 mm total length and 0.4 mm maximum width if parapodia included (smallest specimen: 4 mm long, 0.3 mm wide, 52 chaetigers). Holotype lacking posterior end, 10 mm in length, 0.4 mm in width and 115 chaetigers. Living specimens are yellowish to light green (Fig. 4.6.C), once preserved the pigment fades off into greenish brown. Prostomium rounded triangular, wider than long. Eyes mediumsized, rounded and occasionally partly covered by segment I. Distance between the eyes about the same length of the head. Median antenna of similar size as the terminal ones, situated well in front of the eyes. Palps slightly larger than the antennae. Proboscis widest distally, densely covered with rounded to conical papillae. Tentacular cirri of segment 1 reaching segment $3-4$. Dorsal tentacular cirri of segment 2 usually 1.7 times the size of the ventral tentacular from the same segment. Ventral tentacular cirri from segment 2 often thick and slightly flattened, reaching segment 4-5. Dorsal tentacular cirri of segment 2 and 3 reaching about segment 8 . Dorsal cirri of median segments asymmetrically lanceolate, about twice longer than wider. Ventral cirri of median segments rounded slightly longer than wider and half the length of the chaetigorous lobes, especially in the posterior half of the worm. Chaetae usually present from segment 3 , occasionally one or two chaetae arising from anterior side of ventral cirrophores of segment 2.

Molecular data
COI, ITS and 28 S sequences as in specimens DBUA0002479.01.v01-v06, DBUA0002479.02.v01, MTE052-20 - MTE055-20 and MTE057-20 (Table S4.1). Phylogenetic relationship as in Fig. 4.3.A, where E. madeirensis sp. nov. is clearly distinct from the remaining species of the complex, grouping in MOTU 2. Interspecific COI mean distances to the closest and distant neighbour are $11.4 \%$ (K2P, E. clavigera) and 23.3\% (K2P, E. viridis) respectively. DOI for the species' Barcode Index Number (BIN): upon paper publication.

Etymology
The new species is named after the main Madeira island, the unique remote location where this species can be found so far.

Distribution and habitat
Atlantic ocean: Exclusive to the Madeira island (Portugal), in subtidal environments up to 11 meters depth.

Remarks
Member of the Eulalia clavigera species complex, subtidal variant and mostly morphological similar to $E$. clavigera. Besides the molecular data and its geographical distribution unique to the Madeira island (Portugal), E. madeirensis sp. nov. can be distinguished from E. clavigera and the remaining species of the complex mainly by the yellowish light green coloration of live specimens and smaller worm size (Table 4.4). It also shows smaller morphometric proportions in most of the diagnostic characters when compared against the other three species from the complex, especially the ratio between the length of the dorsal and ventral cirri, between the length of the chaetigerous lobe and ventral cirri, the length to width ratio in the ventral cirri, as well the ratio between the length of the head against either the length of the dorsal cirri on segment 2, antennae or the width of the head.

# Eulalia feliciae sp. nov. 

(Fig. 4.6.A)
urn:Isid:zoobank.org:act: upon paper publication

## Material examined

Type material. France, Banyuls: 1 spm, holotype and hologenophore, DBUA0002478.01.v05, $42^{\circ} 28^{\prime} 48.0^{\prime \prime} \mathrm{N}-3^{\circ} 08^{\prime} 06.0^{\prime \prime} \mathrm{E}$, near shore at $0.5-1 \mathrm{~m}$ depth, rocky beach, collected by Arne Nygren and Fredrik Pleijel, 22/04/2001; 5 spms, paratype and paragenophores, DBUA0002478.01.v01-v04 and DBUA0002478.01.v06, $42^{\circ} 28^{\prime} 48.0^{\prime \prime} \mathrm{N}-3^{\circ} 08^{\prime} 06.0^{\prime \prime} \mathrm{E}$, near shore at $0.5-1 \mathrm{~m}$ depth, rocky beach, collected by Arne Nygren and Fredrik Pleijel, 22/04/2001.

Other material. France, Banyuls: 2 spms, DBUA0002478.01.v07 and MTEO40-20, $42^{\circ} 28^{\prime} 48.0^{\prime \prime} \mathrm{N}-3^{\circ} 08^{\prime} 06.0^{\prime \prime} \mathrm{E}$, subtidal at 10 m depth, among algae, rocks and mussels, collected by Arne Nygren and Fredrik Pleijel, 02/04/2009; 2 spms, DBUA0002478.01.v08 and MTE042-20, $42^{\circ} 28^{\prime} 48.0^{\prime \prime} \mathrm{N}-3^{\circ} 08^{\prime} 06.0^{\prime \prime} \mathrm{E}$, subtidal at 10 m depth, among rocks with hydroids, collected by Arne Nygren and Fredrik Pleijel, 05/04/2009.

## Diagnosis

Small worm both in width, length and number of segments; complete specimens with up to 135 segments and 14 mm total length and 0.6 mm maximum width if parapodia included (smallest: 9 mm long, 0.5 mm wide, 93 chaetigers). Holotype lacking posterior end, 14 mm in length, 0.6 mm in width and 135 chaetigers. Living specimens are deep emerald green (Fig. 4.6.A) once preserved the pigment fades off into a greenish hue and can retain this colour once aged. Prostomium rounded triangular, wider than long. Eyes medium-sized, rounded and occasionally partly covered by segment I. Distance between the eyes shorter than the length of the head. Median antenna of similar size as the terminal ones, situated well in front of the eyes. Palps larger than the antennae. Proboscis widest distally, densely covered with rounded to conical papillae. Cirri of segment 1 reaching segment $3-4$. Dorsal tentacular cirri of segment 2 usually 1.8 times the size of the ventral tentacular cirri from the same segment. Ventral tentacular cirri from segment 2 often thick and slightly flattened, reaching segment 4. Dorsal tentacular cirri of segment 2 and 3 reaching about segment 6-7. Dorsal cirri of median segments asymmetrically lanceolate, about 2.4 times longer than wider. Ventral cirri of median segments twice as long as wide. Ventral cirri slightly shorter than the chaetigerous lobes. Chaetae usually present from segment 3 , occasionally one or two chaetae arising from anterior side of ventral cirrophores of segment 2.

## Molecular data

COI, ITS and 28S sequences as in specimens DBUAO002478.01.v01-v08, MTE040-20 and MTE042-20 (Table S4.1). Phylogenetic relationship as in Fig. 4.3.A, where E. feliciae sp. nov. is clearly distinct from the remaining seven species of the complex, grouping in MOTU 1. Interspecific COI mean distances to the closest and distant neighbour are 13.9\% (K2P, E. clavigera) and 22\% (K2P, E. viridis) respectively. DOI for the species' Barcode Index Number (BIN): upon paper publication.

## Etymology

The new species is named after Felicia Ulltin, a former master student under the supervision of Arne Nygren, the co-supervisor of this thesis, whose enthusiasm and love for polychaetes is unmatched and an inspiration for future marine researchers.

Distribution
Mediterranean Sea: South of France. Usually present in intertidal or subtidal rocky areas among algae, hydroids and mussels.

Remarks
Member of the Eulalia clavigera species complex and morphological similar to E. clavigera. Besides the molecular data and its geographical distribution unique to the western Mediterranean Sea, E. feliciae sp. nov. can be distinguished from E. clavigera and the remaining species from the complex mostly by the deep emerald green coloration of the live specimens and the small to medium sized morphometric proportions. It shows larger morphometric proportions in most of the diagnostic characters when compared to $E$. madeirensis sp. nov. but smaller against $E$. clavigera and $E$. xanthomucosasp. nov. (described below). The most significative proportions are the ratio between the length of the dorsal and ventral cirri, between the length of the chaetigorous lobe and ventral cirri, the length to width ratio in the ventral cirri, as well the ratio between the length of the head against either the length of the dorsal cirri on segment 2, antennae or the width of the head.

## Eulalia xanthomucosa sp. nov.

(Fig. 4.6.B).
urn:Isid:zoobank.org:act: upon paper publication

Material examined
Type material. United Kingdom, Cornwall (Newlyn Marina): 1 spm, holotype and hologenophore, DBUA0002480.01.v07, $50^{\circ} 06^{\prime} 10.8^{\prime \prime} \mathrm{N}-5^{\circ} 32^{\prime} 49.2^{\prime \prime} \mathrm{W}$, subtidal at 25 m depth, among coralligenous samples, collected by David Fenwicki, 02/06/2016; 3 spms, paratypes and paragenophores, DBUA0002480.01.v01-v03, $50^{\circ} 06^{\prime} 10.8^{\prime \prime} \mathrm{N}-5^{\circ} 32^{\prime} 49.2^{\prime \prime} \mathrm{W}$, lowershore in a rock crevice, collected by David Fenwicki, 02/07/2016; 3 spms, paratypes and paragenophores, DBUA0002480.01.v04-v06, $50^{\circ} 06^{\prime} 10.8^{\prime \prime} \mathrm{N}-5^{\circ} 32^{\prime} 49.2^{\prime \prime} \mathrm{W}$, subtidal at 25 m depth, in rock crevices at Laminaria zones and among coralligenous, collected by David Fenwicki, 22/08/2017.

Other material. France, Banyuls: $1 \mathrm{spm}, \mathrm{BI}-2014 / 15-077,42^{\circ} 28^{\prime} 48.0^{\prime \prime} \mathrm{N}-3^{\circ} 08^{\prime} 06.0^{\prime \prime} \mathrm{E}$, subtidal at 25 m depth, among algae and boulders, collected by Fredrik Pleijel, 07/04/2009; 1 spm , DBUA0002481.01.v01, $42^{\circ} 50^{\prime} 37.0^{\prime \prime} \mathrm{N}-3^{\circ} 14^{\prime} 12.0^{\prime \prime} \mathrm{E}$, subtidal at 25 m depth, among coralligenous, collected by Felicia Ultin, 15/09/2020. France, Corsica island, 2 spms, MNHN-IA-2021-654 and MNHN-IA-2021-655, $41^{\circ} 26,8^{\prime} \mathrm{N}-008^{\circ} 54^{\prime} \mathrm{E}$, subtidal at 34 m depth, collected by the CORSICABENTHOS expeditions, 23/10/2020.

## Diagnosis

Complete specimens with up to 230 segments and 104 mm total length and 2.378 mm maximum width if parapodia included (smallest specimen: 12 mm long, 0.397 mm wide, 89 chaetigers). Holotype lacking the posterior end, 26 mm in length, 1.2 mm in width and 128 chaetigers. Living specimens present a yellow coloration provided by the worm's mucus (Fig. 4.6.B), once preserved the pigment fades off into a brownish colour. Prostomium rounded triangular, wider than long. Eyes small to medium-sized, rounded and occasionally partly covered by segment I. Distance between the eyes shorter than the length of the head. Median antenna of similar size as the terminal ones, situated well in front of the eyes. Palps about the same size as antennae. Proboscis not examined. Cirri of segment 1 reaching segment 4-5. Dorsal tentacular cirri of segment 2 usually 1.8 times the size of the ventral tentacular cirri from the same segment. Ventral tentacular cirri from segment 2 often thick and slightly flattened, reaching segment 5-6. Dorsal tentacular cirri of segment 2 and 3 reaching about segment 8-9. Dorsal cirri of median segments asymmetrically lanceolate, about 2.3 times longer than wider. Ventral cirri of median segments 1.5 times longer than wide. Ventral cirri slightly shorter than the chaetigerous lobes. Chaetae usually present from segment 3 , occasionally one or two chaetae arising from anterior side of ventral cirrophores of segment 2.

## Molecular data

COI, ITS and 28 S sequences as in specimens DBUA0002480.01.v01-v07, DBUA0002481.01.v01, BI-2014/15-077, MNHN-IA-2021-654 and MNHN-IA-2021-655 (Table S4.1). Phylogenetic relationship as in Fig. 4.3.A, where E. xanthomucosa sp. nov. is clearly distinct from the remaining Eulalia species, grouping in MOTU 5. Interspecific COI mean distances to the closest and distant neighbour are 12.1\% (K2P, Eulalia IS-BA) and 20.4\% (K2P, E. feliciae sp. nov.) respectively. DOI for the species' Barcode Index Number (BIN): upon paper publication.

## Etymology

The new species is named based on its unique bright yellow ("xantho" from ancient Greek) colouration produced by the worm's mucus.

Distribution and habitat
Atlantic Ocean: United Kingdom, Cornwall; Mediterranean Sea: France, Banyuls. Occasional lower intertidal but typically shallow sublittoral in rock crevices at Laminaria zones, among coralligenous material in marinas.

Remarks
This species was registered at the Natural History Museum as Eulalia sp. "Emits Yellow Mucus A" (tvk NHMSYS0021180023, https://www.aphotomarine.com/worm_eulalia_species_28-09-11.html). The species can easily be distinguished from E. clavigera using the live coloration (yellow instead of green), but may be confused with $E$. aurea due to similar yellowish coloration. However, the unusually large dorsal cirri of median segments in relation to the worm size is very distinct compared to both $E$. clavigera and E. aurea. Based on my observations, E. clavigera and E. xanthomucosa sp. nov. can generally be found together in marinas, but so far only confirmed at Newlyn Marina (Cornwall, United Kingdom). Usually, E. clavigera occurs higher on the shore than E. xanthomucosa sp. nov..

Eulalia xanthomucosa sp . nov. presents larger morphometric proportions in most of the diagnostic characters when compared against $E$. feliciae sp. nov., and $E$. madeirensis sp. nov., especially the ratio between the length of the dorsal and ventral cirri; between the length of the chaetigorous lobe and ventral cirri; the length to width ratio in the ventral cirri; as well the ratio between the length of the head against either the length of the dorsal cirri on segment 2, antennae or the width of the head. The exception to this can sometimes be found against specimens from E. clavigera, which can often share
the same cluster measurements, however, the analysed specimens from E. clavigera were considerably larger in size (number of segments; worm's length and width). Similar ratio between the antennae and palps is also shared with E. clavigera. Some specimens from E. xanthomucosa sp. nov. can reach similar worm sizes compared to E. clavigera, as seen in the specimen DBUA0002481.01.v01, up to 230 segments, 104 mm total length and 2.378 mm maximum width if parapodia included).

### 4.4 Discussion

With the use of molecular tools, it was possible to unravel hidden diversity in the Eulalia genus. Compelling evidence for six additional European MOTUs within the E. clavigera and E. viridis pseudocryptic complex was found. Based on the combination of different approaches (molecular, morphometric, coloration and geographical distribution data), three of these lineages, are here described as new species. Mean COI distances (17.9\%) between lineages are within fit the range usually reported in other annelids (Nygren et al., 2018; Ravara et al., 2017; Sampieri et al., 2021), including other Phyllodocids (Nygren and Pleijel, 2011) and the MOTU delineation was congruent among all the delineation methods employed. There is a clear geographic structure for most of the retrieved European MOTUs. In this study, E. viridis (MOTU 7) is unique to the Scandinavia and Northern Sea and seems to be a northern boreal and subarctic species, both in intertidal or subtidal waters, in agreement with previous works (Bonse et al., 1996; Kato et al., 2001). Eulalia clavigera s.s. (MOTU 4) is a temperate species mostly found in intertidal rocky shores, ranging from Great Britain to the western Mediterranean Sea, being present as well in the Azores, Savage islands and widespread in the Canary islands. Its presence was also confirmed in Argentina (Langeneck et al., 2019). Based in the sampling campaigns for this thesis and personal observations, this species seems to be one of the most dominant taxa present in the rocky beaches from the island of Tenerife and can even be found in very large quantities close to artificial pools in tourist zones, despite the heavy human presence in these areas. It should be noted that Langeneck et al. (2019) reported the occurrence of individuals morphologically similar to E. clavigera collected in Brazil, although it was not possible to obtain molecular data. It is also possible that specimens identified as $E$. viridis from southern Brazil (Morgado and Amaral, 1983) might actually belong to E. clavigera instead.

Langeneck et al. (2019) suggested the possibility of $E$. clavigera being a relict species in the Mediterranean Sea, while the majority of the Mediterranean shallow-water green Eulalia probably belong to one or more different species. The new species, E. feliciae sp. nov. (MOTU 1) seems to co-exist in sympatry with E. clavigera (MOTU 4) and E. xanthomucosa sp. nov. (MOTU 5) in the western Mediterranean Sea. Together with a specimen of E. clavigera reported in Langeneck et al. (2019), these

3 MOTUs were collected in Banyuls-sur-Mer and can be found in the intertidal zone. However, so far, $E$. xanthomucosa sp. nov. seems to be more abundant in subtidal regions (mainly from recreational marinas), it is also present in Great Britain and possesses an characteristic coloration (yellowish instead of the characteristic green) similar to the ingroup E. aurea. Live coloration is one of the most important features in the taxonomy of this genus, as most of the different Eulalia species are almost impossible to distinguish based solely on morphologic features of the discoloured preserved specimens (Schimmenti et al. 2016). Eulalia xanthomucosa sp. nov. was indeed the most divergent MOTU found in the complex and, besides coloration, displayed some other visible phenotypic features comparable to the E. clavigera morphotype. In particular, parapodia showed a larger size of the dorsal and ventral cirri compared to the worm size. These morphological differences appear to parallel the molecular divergence data, e.g. the interspecific nuclear genetic distances tripled when compared to the distances found between MOTUs within the major "clavigera" clade (clade A, Fig. 4.3.A). This clade, with the exception of the population from Madeira, also shared 28 S haplotypes, but this seems to be a common occurrence in other closely related marine species (Borges et al., 2012; Vieira et al., 2019). Ribosomal nuclear loci (due to the lower evolutionary rates) are not suitable to species-level discrimination in invertebrates (e.g. Jörger et al., 2012) being more efficient in reconstructing deeper phylogenies instead (e.g. Weitschek et al., 2014).

The unnamed lineage from Croatia (MOTU 3) is genetically close to E. clavigera (COI, 7.5\%; ITS, $4.8 \%$; no 28 variation), which suggests that the speciation might be recent and unlikely to be driven by the Messinian salinity crisis (from 6 to 5.33 MY , Hupało et al. 2019). This important event is usually referred to explain the emergence of geographic barriers preventing gene flow not only between the NE Atlantic and the Mediterranean, but also between the Western and Eastern part of this Sea. However, selection associated with the environmental features of the different habitats, which promoted local adaptation (Peijnenburg et al. 2004), might also explain this apparently recent speciation. The small-size morphotype and the type locality of MOTU 3 is close to Eulalia virens Ehlers, 1868, currently considered a junior synonym of $E$. viridis described for the Adriatic sea, mainly characterized by the low number of segments (54) and small size (length, 7 mm ; width, 0.5 mm ). Further sampling and examination of Eulalia specimens from this locality might elucidate if both designations belong to the same morphotype.

At least four different Eulalia MOTUs seem to be exclusive to the Mediterranean Sea (Fig. 4.3), a known biodiversity hotspot (Bianchi and Morri, 2000), including for cryptic species (Calvo et al., 2009; Langeneck et al., 2020; Taboada et al., 2017) and exotic species (Galil, 2009; Zenetos et al., 2008). The role of the alternating glacial and interglacial stages has been often suggested as one of the reasons reason for the high number of species in this Sea. Under the conditions of a characteristic interglacial
period, the Mediterranean region had a warm and arid climate and a deficient water balance, where the input of Atlantic surface water into the Mediterranean through the Strait of Gibraltar plays an important role. This may allow the possible introduction and maintenance of (sub)tropical littoral biota in this period (Bianchi et al., 2012), with boreal species from the NE Atlantic introduced to Mediterranean refugia areas during glacial periods (Gómez and Lunt, 2007; Maggs et al., 2008; Schmitt et al., 2021). The survival of part of this fauna despite the water temperature fluctuation and different environmental and depth conditions over time, sustains the hypothesis of the Mediterranean "biodiversity pump", a possible outcome of the climatic events of the Quaternary (Bianchi and Morri, 2000).

In spite of the recent indication of high incidence of marine invertebrate endemisms in the Macaronesia archipelagos (Desiderato et al., 2019; Vieira et al., 2019) no additional intertidal MOTUs were recorded in the Azores and Canary islands. These volcanic islands never had contact with the mainland continent, were formed at different times, are hundreds of kilometres apart, possess a range of unique geological and climatic conditions, and their biota is the result of dispersal from distant geographical sources and in situ evolution and diversification (Fernández-Palacios et al. 2011). However, no appreciable differentiation was observed when compared to the continental populations, apart from two partial morphometric markers and completely sorted COI and ITS haplotypes (Figs. 4.3.B; 4.4.A; 4.5.G,H). Only intertidal samples were collected in these islands, contrasting to the new lineage found in the subtidal populations from Madeira (MOTU 2, E. madeirensis sp. nov.). Evidence of cryptic species among lineages inhabiting at different depths has been found, as for example, for the species Phyllodoce madeirensis Langerhans, 1880 where three different MOTUs were reported, each corresponding to different sampling depths (Martin et al., 2021). Additional sampling efforts in the subtidal habitats of the Canary or the Azores archipelagos may reveal new Eulalia species yet to be discovered. Intertidal Eulalia populations from the South Eastern Atlantic (Patagonia, Argentina) also failed to display any molecular or morphological divergence from the European E. clavigera (Langeneck et al., 2019). This may suggest a recent colonization by anthropogenic activities for both the Canary islands and the South American populations. Indeed, as reported by J. M. Orensanz in a personal communication to the authors from the previously mentioned study, neither E. clavigera or $E$. viridis were recorded during the intensive surveys done in the 70's, unlike the abundant populations observed recently in Puerto Madryn, Argentina. Furthermore, according to the Biodiversity Data Bank of the Canary islands (BDBC, https://www.biodiversidadcanarias.es/biota/?lang=en), the first records of the E. clavigerain the Spanish archipelago date at least from 1976 (Sosa et al., 1976; Núñez et al., 2005). However, unlike the Patagonia populations, the specimens from the Macaronesia islands do not share COI or ITS haplotypes
with mainland Europe (Figs. 4.3.B, 4.4.A, respectively), suggesting instead, an older non-anthropogenic driven colonization compared to the South East populations. Schwindt et al. (2014) hypothesized a recent unintentional introduction of $E$. clavigera due to shipping activities, either with ballast waters or in fouling communities. Other studies also show evidence of many small benthic marine fishes, chordate species or small-sized invertebrates and plankton, introduced as eggs, larvae or juveniles, being first recorded from regions with major commercial ports and international shipping as the most probable vector (Cuesta et al., 2016; Lockett and Gomon, 2001; Wonham et al., 2000).

Additional unsampled European MOTUs of Eulalia might still be uncovered. For example, Audouin and Milne Edwards (1833) erected the species Phyllodoce gervilleifrom Granville (France), stating that it is identical to $P$. clavigera, with the exception of the missing median antenna and smaller tentacular cirri. McIntosh (1908) synonymised both species with E. viridis, considering that the absence of antennae in $P$. gervillei may have been accidental. However, given the type locality of $P$. gervillei, that species is most probably a synonym of E. clavigera. Furthermore, the species Eulalia (Eumida) microceros Claparède, 1868, also a current synonym of $E$. viridis, is described for the Gulf of Naples and is characterized by its large size (Length, 5 cm ; width, 3 mm ; number of segments, 300). This far surpasses any of the analysed green Eulalia specimens from continental Europe in this study (Table 4.5, Table S4.2), suggesting that this is either a larger specimen belonging to E. clavigera based on type locality and figures from the original description (PL. XVI, fig.4), or another large species with a similar morphotype, different from what was analysed in this study.

### 4.5 Conclusion

In this study six additional MOTUs within Eulalia were found, which appear to be rarer and mainly restricted to a particular region. Nevertheless, available data on E. clavigera s.s. continues to indicate that this species is quite widespread in Europe. It is very abundant in temperate areas from the western Mediterranean to the NE Atlantic, including the Savage islands, Azores and Canary islands. Despite the close genetic proximity between the NE Atlantic and the Macaronesia populations, the lack of shared haplotypes between these regions suggests that recent anthropogenic introduction through shipping may not be the reason for this divergence, unlike the southern American population (Langeneck et al., 2019), and instead, an older colonization of these islands could be possible. Its successful establishment in these temperate and sub-tropical areas and recent observations of large populations in both regions, might change trophic interactions within the native fauna. Given that $E$. clavigera is a predator feeding mostly
on mussels and barnacles (Rodrigo et al., 2015), with scavenger habits also observed (Morton, 2011), the demography and effect of this species on local fauna deserve close monitoring

Recently, a hidden biotechnological potential was uncovered in marine invertebrates, which might offer a wide array of natural products, showing properties compatible with anaesthetics, fluorescent probes, and even antibiotics and pesticides (Rodrigo and Costa, 2019). By analysing the phylogeny of toxin mixtures, Rodrigo et al. (2021a) show that annelids are uniquely positioned in the evolution of animal venoms. In particular, using the toxin-containing mucus present in the green Eulalia, which based on collection site (mainland Portugal) corresponds to E. clavigera s.s. in this chapter, revealed possible applications in anti-cancer therapeutics (Rodrigo et al., 2021b) and fluorescent probes for biotechnological applications using a protein mixture from the mucus (Rodrigo, 2020). This once again highlights the importance of formally describing cryptic complexes, since biochemical features might be unique to each lineage and can have a range of distinct effects and applications.

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Chapter 5

## The curious and intricate case of the European Hediste diversicolor (Annelida, Nereididae) species complex, with description of two new species


#### Abstract

Past molecular studies using mtDNA sequences and alloenzymes signalled the existence of at least two cryptic species within the Hediste diversicolor morphotype, in European coasts. However, to this day, no new species descriptions have been made. In this study, it was identified five completely sorted lineages using a multi locus approach, including the mitochondrial DNA cytochrome oxidase I gene (COI-5P) and the nuclear markers ITS2 rRNA and 28S rRNA. The molecular data was complemented with morphometric measurements examined through multivariate statistical analysis and the incorporation of statistical dissimilarities. Apart from the Baltic Sea, where three of the lineages occur in sympatry, Hediste diversicolor comprise four deeply divergent allopatric lineages in the remaining Europe. They group populations from the NE Atlantic and part of the Western Mediterranean Sea; from the Tyrrhenian Sea; from the Adriatic and lonian Sea; and, lastly, from the Caspian, Black and the northern Aegean Seas. The lineage from the Ionian Sea revealed low genetic distances compared to the one from the Adriatic Sea and lacked enough specimens for the morphometric analysis, preventing further conclusions about its independent status. Three independent morphometric clusters were identified based on worm size, number of segments and the length of several prostomial appendages. Two sympatric lineages present in the Baltic Sea, showed evidence of possible hybridization and lacked significant PCA morphometric variation between them. The two remaining lineages were formally described as new species, namely Hediste pontii sp. nov. (Adriatic Sea) and Hediste astae sp. nov. (northern Aegean, Caspian and Black Seas). These new species can now be formally recognized and used in biomonitoring or other relevant ecological studies. Finally, a neotype is defined for $H$. diversicolor, whose usage is restricted to the NE Atlantic lineage.


Keywords: Hediste, Nereididae, morphometry, molecular data, cryptic species

### 5.1 Introduction

The common ragworm Hediste diversicolor (0. F. Müller, 1776) (Nereididae) is a widespread omnivorous species which occurs in estuarine environments and brackish waters among mud, sand, gravel, and turf of the Atlantic coasts of Europe, and in the Mediterranean, Black and Caspian Seas (Scaps, 2002). It is suspected that this species was also introduced to North America prior to 1880, hence earlier than the first biological surveys of the intertidal sediment in the NW Atlantic (Einfeldt et al., 2014), where the most probable vector was the dry ballast, i.e. stored sediment or soil from the intertidal onto ships to adjust buoyancy (Galil et al., 2011). This seems to be corroborated by the occurrence of this species in estuarine sediment associated with historic shipping ports and the brooding of larvae lacking a pelagic phase (Scaps, 2002; Faulwetter et al., 2014). This species lacks a true planktonic larval phase in early development and its life cycle is completed within the low-salinity regions of estuaries, without epitokous metamorphosis and reproductive swarming in adults (Smith, 1950). The larvae burrow immediately after emergence, resulting in limited dispersal that is expected to promote genetic isolation among populations separated by stretches of unsuitable habitat at different spatial ranges (BartelsHardege and Zeeck, 1990; Scaps, 2002). Hediste diversicolor is an efficient bioturbator that builds U- or Y-shaped burrows at densities documented to exceed 3500 individuals $\mathrm{m}^{2}$, and has an important role in the biogeochemical and ecological processes of estuarine environments, as well as representing an important prey for many invertebrate and vertebrate species (Cuny et al., 2007; Bowser et al., 2013). This species is also one of the few nereidids of economic importance, used as bait in recreational fishing and as food in aquaculture (Scaps, 2002; Younsi et al., 2010). It is commonly used in ecotoxicological studies, bioaccumulation assays (Virgilio et al., 2005; Burlinson and Lawrence, 2007; Durou et al., 2007) and displays a wide tolerance to temperature changes (Wolff, 1973), hypoxia (Kristensen, 1983) and salinity variation, thriving in habitats ranging from freshwater to twice the normal salinity found in seawater (Wolff, 1973; Neuhoff, 1979). However, it is susceptible to anthropogenic stress, experiencing reduced fecundity and fitness when exposed to elevated levels of toxic trace metals (Scaps, 2002; Durou et al., 2005; Moreira et al., 2006). Yet, still possesses higher tolerance to heavy metals compared to other nereidid species (Hateley et al., 1992), making it a resilient bio-indicator in many marine and brackish water habitats.

Past studies have suggested inter-population morphological, biochemical and physiological differences within this species in individuals from different areas and different environmental conditions, which may be related to the limited dispersal capacity of the species (Scaps, 2002). For example, differences in the number of paragnaths were reported by Maltagliati et al. (2006) but no geographical
pattern of morphological variation was detected by multidimensional scaling. This suggests that the variation found among populations may reflect local differences in diet or dominant mode of feeding, and thus be the consequence of phenotypic plasticity (Fusco and Minelli, 2010; Forsman, 2015). Genetic data also hinted at the existence of at least two cryptic species. Using both mitochondrial and nuclear markers, Audzijonyte et al. (2008) divided Hediste diversicolor into species A and B, both sympatric in the Baltic Sea. Later, Virgilio et al. (2009) found haplotypes of species B in the western Mediterranean, Adriatic Sea, as well as in the Black and Caspian Seas, with three deeply divergent mtDNA lineages with a nearly disjunct geographical distribution and suggested Species B was introduced from these areas to the Baltic in two or more colonisation events. Species A was also reported in north America from the Bay of Fundy and Maine, with the Maine population having unique haplotypes and most likely originated from unsampled European populations (Einfeldt et al., 2014). More recently, Vasileiadou et al. (2016) analysed populations in the Greek Amvrakikos Gulf and found unique COI haplotypes which are distinct from the ones reported in the previous studies.

The aim of this study was to employ a multi-locus approach together with morphometric analysis to complement the existing evidence of separate species within the European Hediste diversicolor populations. Naming of newly-found cryptic species is fundamental for their subsequent routine recognition and to achieve realistic estimates of biodiversity (Delić et al., 2017; Fišer et al., 2018). Failure to do so prevent their use in large scale biomonitoring programs, even those employing DNA-based approaches, and limits our understanding of their evolutionary and ecological significance, generating biased interpretations in ecotoxicological, bioaccumulation and in other relevant ecological studies (Volkenborn et al., 2007; Hutchings and Kupriyanova, 2018).

### 5.2 Materials and methods

### 5.2.1 Taxon sampling and molecular data retrieval

A total of 269 Hediste specimens distributed along the European coasts (Table 5.1) were gathered by digging out 10-20 cm thick of sediment and washing it through a 1 mm sieve in low tide or near the shore at $0.5-1 \mathrm{~m}$ depth. From Portugal, samples were collected in the estuaries of Sado, Lima and Minho, as well in the Aveiro lagoon. From Spain, specimens were collected in Vigo (Lagares estuary) and Coruña (Ferrol Lagoon). Specimens were also collected in north of France (Brest), south Norway (Grimstad and Sandefjord), middle Norway (Trondheim), Sweden (Tjärnö-Saltö canal), and Italy, from both the western Mediterranean (Navicelli Canal, Pisa) and eastern Mediterranean Sea (Venezia Lagoon).

Table 5.1. Number of specimens acquired for this study, the respective sampling area, code abbreviation for the sampling location and the institution responsible for storing the samples.

| Code | Region | Location | n | Coordinates |  | Storing Institution |
| :---: | :---: | :---: | :---: | :---: | :---: | :---: |
|  |  |  |  | Latitude | Longitude |  |
| SA | NE European Coast | Portugal, Sado estuary | 18 | 38*29'52.8"N | 850'16.8'W | DBUA |
|  |  |  | 9 | $38^{\circ} 29^{\prime} 24.0^{\prime \prime} \mathrm{N}$ | $8^{\circ} 48^{\prime} 54.0^{\prime \prime} \mathrm{W}$ |  |
| AV | NE European Coast | Portugal, Aveiro Lagoon | 25 | $40^{\circ} 38^{\prime} 02.4{ }^{\prime \prime} \mathrm{N}$ | $8^{\circ} 40 \cdot 30.0^{\prime \prime} \mathrm{W}$ |  |
| LI | NE European Coast | Portugal, Lima estuary | 23 | $41^{\circ} 42^{\prime} 03.6{ }^{\prime \prime} \mathrm{N}$ | $8^{\circ} 44^{\prime} 56.4{ }^{\prime \prime} \mathrm{W}$ |  |
| MI | NE European Coast | Portugal, Minho estuary | 25 | $41^{\circ} 52^{\prime} 55.2^{\prime \prime} \mathrm{N}$ | $8^{\circ} 49^{\prime} 44.4{ }^{\prime \prime W}$ |  |
| LA | NE European Coast | Spain, Lagares estuary | 5 | $42^{\circ} 12^{\prime} 07.2^{\prime \prime} \mathrm{N}$ | $8^{\circ} 466^{\prime} 40.8^{\prime \prime} \mathrm{W}$ |  |
| FE | NE European Coast | Spain, Ferrol Lagoon | 10 | $43^{\circ} 29^{\prime} 34.8^{\prime \prime} \mathrm{N}$ | $8^{\circ} 14^{\prime} 56.4^{\prime \prime} \mathrm{W}$ |  |
| BR | NE European Coast (Celtic Sea) | France, Brest | 10 | $48^{\circ} 24^{\prime} 21.6^{\prime \prime} \mathrm{N}$ | $4^{\circ} 22^{\prime} 01.2^{\prime \prime} \mathrm{W}$ |  |
| TD | North European Sea | Norway, Trondheim | 2 | $63^{\circ} 26^{\prime} 09.6^{\prime \prime} \mathrm{N}$ | 10²9'56.4"E |  |
| GM | Skagerrak | Norway, Grimstad | 5 | $58^{\circ} 17^{\prime} 52.8{ }^{\prime \prime} \mathrm{N}$ | $8^{\circ} 32^{\prime 2} 2.44^{\prime \prime} \mathrm{E}$ | NTNU |
| SF | Skagerrak | Norway, Sandefjord | 1 | $59^{\circ} 07 \cdot 37.2^{\prime \prime} \mathrm{N}$ | $10^{\circ} 14^{\prime} 24.0^{\prime \prime} \mathrm{E}$ |  |
| TJ | Kattegat Sea | Sweden, Tjärnö-Saltö canal | 52 | 5852'26.4"N | $11^{\circ} 08^{\prime} 42.0^{\prime \prime} \mathrm{E}$ |  |
| NA | Tyrrhenian Sea (Mediterranean) | Italy, Navicelli Canal | 10 | $43^{\circ} 40^{\prime} 19.2^{\prime \prime} \mathrm{N}$ | 10²2'15.6"E | DBUA |
| VE | Adriatic Sea (Mediterranean) | Italy, Venezia Lagoon | 28 | $45^{\circ} 20^{\prime} 13.2^{\prime \prime} \mathrm{N}$ | $12^{\circ} 16^{\prime} 30.0^{\prime \prime} \mathrm{E}$ |  |
| AM | Ionian Sea (Mediterranean) | Greece, Amvrakikos Lagoon | 5 | $39^{\circ} 02^{\prime} 45.6^{\prime \prime} \mathrm{N}$ | 20* $46{ }^{\prime} 15.6^{\prime \prime} \mathrm{E}$ | DNA only |
| NAS | Northern Aegean Sea (Mediterranean) | Greece, Evros Lagoon | 30 | $40^{\circ} 44^{\prime} 38.4{ }^{\prime \prime} \mathrm{N}$ | 26*02'13.2"E | DBUA |
|  |  | Greece, Ptelea Lagoon | 8 | $40^{\circ} 56^{\prime} 13.2{ }^{\prime \prime} \mathrm{N}$ | 25*14'49.2"E |  |
|  |  | Greece, Aliky Lagoon | 6 | $40^{\circ} 57^{\prime} 00.0{ }^{\prime \prime} \mathrm{N}$ | $25^{\circ} 12^{\prime} 50.4$ " E |  |
|  |  | Greece, Nestos Lagoon | 4 | $40^{\circ} 54^{\prime} 36.0^{\prime \prime} \mathrm{N}$ | 24*52'22.8"E |  |
|  |  | Greece, Axios Lagoon | 4 | $40^{\circ} 30^{\prime} 28.8{ }^{\prime \prime} \mathrm{N}$ | $22^{\circ} 43^{\prime} 40.8^{\prime \prime} \mathrm{E}$ |  |

Lastly, additional specimens were obtained from the Ionian Sea in the Amvrakikos lagoon (Greece), as well in eastern Greece from lagoons in the Thracian Sea, or most commonly known as northern Aegean Sea (Evros, Nestos, Alyki, Axios and Ptelea lagoons). Twenty-five specimens from Evros Lagoon (DBUA0002466.28-52) were preserved in formaldehyde and the remaining ones were all preserved in 96\% ethanol.

Two hundred and eleven Hediste specimens were sequenced for the mitochondrial cytochrome oxidase subunit I (mtCOI-5P). A representative number of specimens per location for the ITS2 region and 28 S rRNA were used (with 93 nuclear sequences in total). Molecular data from 12 specimens of Alitta virens (M. Sars, 1835) were used as outgroup for all the analysed loci (12 COI and 3 ITS2/28S sequences). "Species A" and "Species B" were defined after Audzijonyte et al. (2008), and representative sequences from the Baltic Sea corresponding to each of the obtained MOTUs were used for comparison purposes. Additionally, GenBank sequences from the "Species B" (Virgilio et al., 2009) from the western Mediterranean, Adriatic Sea and Black and Caspian Seas, together with sequences of the "Species A" from Great Britain, Germany and Netherlands were added to the alignment. Lastly, sequences from Vasileiadou et al. (2016) corresponding to the new Mediterranean haplotypes were added as well to comprise the final dataset. DNA was extracted, amplified, sequenced, and assembled as described in the Chapter 3 of this thesis. Regarding PCR conditions, primers and sequence lengths for the different markers see Chapter 3, Table 3.1. Supplemental Table S5.1 details the sampling locations, public BIN accession numbers and voucher data for the original material. Supplemental Table S5.2 details the GenBank accession numbers for sequences used for comparison purposes from other studies.

The dataset used for molecular analysis and its metadata can be accessed at the BOLD Systems under the project "Hediste species complex (DS-MTHD)", which will be public available upon this chapter's acceptance for publication in a peer reviewed journal. The biological material is deposited at the Research Collection of Marine Invertebrates of the Department of Biology of the University of Aveiro (COBI at DBUA), Portugal. Specimens from Norway deposited at the Norwegian University of Science and Technology, NTNU University Museum (Bakken et al., 2021). Specimens which were exhausted in the DNA analysis were assigned only with the Process ID from the BOLD systems (http://v4.boldsystems.org/), corresponding to the ones from the Amvrakikos lagoon (MTHD178-20, MTHD180-20, MTHD183-20, MTHD184-20 and MTHD187-20), Ferrol Iagoon (MTHD015-20) and TjärnöSaltö canal (MTHD145-20).

### 5.2.2 Phylogenetic analysis

The phylogenetic analyses were performed through maximum likelihood (ML) and Bayesian inference (BI). Mitochondrial COI sequences and the nuclear markers (ITS2 and 28S) were concatenated with MEGA 10.0.05 (Kumar et al., 2018) and aligned with MAFFT online (https://mafft.cbrc.jp/alignment/server/; Katoh and Standley, 2013). Highly variable regions, extensive gaps and poorly aligned positions in the concatenated alignment were eliminated using Gblocks 0.91b (http://molevol.cmima.csic.es/castresana/Gblocks_server.html; Castresana, 2000), allowing all the options for a less stringent selection and not allowing many contiguous non-conserved positions, so that it becomes more suitable for phylogenetic analysis. MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003) was used to conduct the Bayesian analysis. Best-fit models were selected using the Akaike Information Criterion in the JModeltest software (Guindon and Gascuel, 2003; Darriba et al., 2012). For COI the Kimura-2-parameter model with gamma distributed rates across sites (K2P+G) was applied for the first two positions and Hasegawa-Kishino-Yano (HKY) with equal rates across sites for the third position. The latter was also applied to the ITS2 and 28S loci. Number of generations was set to 10000 000, and sample frequency to 500 . Twenty-five percent of the samples were discarded as burn-in (burninfrac = $0.25)$. The resulting tree file was checked for convergence in the effective sampling sizes (ESSs $>200$ ) with Tracer 1.6 software (Rambaut et al., 2018) and then analysed in Figtree 1.4.3 (http://tree.bio.ed.ac.uk/software/figtree/). The final version of the concatenated tree was edited with the software Inkscape 0.92 .3 (https://www.inkscape.org). Maximum Likelihood phylogenies were performed in MEGA 10.0.05 with 1000 bootstrap runs with the General Time Reversible (GTR) model with equal rates across sites for the concatenated dataset. The BI tree was displayed in the results with the addition of the ML support values if a similar topology is found.

### 5.2.3 MOTU clustering

To depict Molecular Operational Taxonomic Units (MOTUs), three delineation methods to the concatenated alignment were applied based on ABDG (Puillandre et al., 2012), bPTP (Zhang et al., 2013) and GYMC (Fujisawa and Barraclough, 2013) as detailed in Chapter 3 of this thesis. BEAST 2.4.6 (Bouckaert et al., 2014) was used to generate the Bayesian ultrametric tree for the GMYC based on AIC criteria, with HKY model and equal rates across sites. The Barcode Index Number (BIN) was applied as well for the COI, which makes use of the Refined Single Linkage (RESL) algorithm implemented in BOLD
(Ratnasingham and Hebert, 2013), exclusive to this locus. The final consensus on MOTUs was chosen using the majority rule.

### 5.2.4 Genetic diversity and structure

Genetic distances (Kimura-2-parameters, K2P) between all records (for each marker and concatenated data) were calculated using MEGA 10.0.05 and plotted two-dimensionally (Multidimensional scaling - MDS) using R 3.6.0 software and the package "stats" (function cmdscale: distances) (R core Team, 2019; www.r-project.org). The mean genetic distances (Kimura-2-parameters, K2P) within and between MOTUs for each individual genetic marker were calculated in MEGA 10.0.05.

Haplotype networks were made for the original sequences through the PopART software (Leigh and Bryant, 2015) using the using the method of Templeton, Crandall and Sing (TCS, Clement et al., 2002) to evaluate the relationship between the haplotypes and their geographical distribution. No GBlocks were applied in this analysis to avoid underestimating the number of nuclear haplotypes. Indices of genetic diversity, namely number of haplotypes ( h ), haplotype diversity (Hd), polymorphic sites (S), nucleotide diversity ( $\pi$ ), Fu \& Li D and Tajima D statistical tests, were estimated based on COI for each MOTU using DNASP 5.10 (Librado and Rozas, 2009).

### 5.2.5 Morphometric and morphological analysis

Specimens from the different Hediste MOTUs (NE Atlantic and Norway; Adriatic Sea; northern Aegean Sea; Sweden and Western Mediterranean) were used for morphometric analysis and compared against each other to complement the molecular data. A total of 25 specimens with optimal conditions (i.e. specimens with the presence of the proposed morphological characters for this study and whenever possible, similar in size) per MOTU were chosen.

The following characters were selected and measured (Fig. 5.1.A.B): the number of segments (NS); the length (mm) of the entire worm (WL), parapodium up to the median ligule (CLL), antennae (AL), palps (PL), antero-dorsal cirri and postero-dorsal cirri (DSTL, DLTL, respectively), dorsal and ventral cirri of median segments (DCL, VCL), dorsal and ventral ligule of median segments (DLL, VLL) and head (HL); the width (mm) of the worm with parapodia (WWP) and without parapodia (WW), head (HW), dorsal and ventral ligule (DLW, VLW); and the distance between the anterior eyes (DAE), distance between the posterior eyes (DPE), distance between the anterior and posterior eyes (DAPE) as well the height (mm) of the parapodium (CLH). WW, WWP and the different parapodia structures were measured from the
worm's widest part, usually from segment 20 to 45 depending on the worm size. The distance between the eyes was measured from the center of the eyespots to avoid possible different individual responses to fixation as in the case of hesionids in Martin et al. (2017). To minimize bias based on size variability, measurements taken to analyse the inter-population differences were converted to ratios and submitted to two types of analysis: 1) taxonomically relevant character proportions through a PCA analysis (i.e., AL/PL, DLTL/DSTL, AL/DLTL, AL/DSTL, PL/DLTL, PL/DSTL, AL/HL, PL/HL, AL/HW, PL/HW, HL/HW, DAE/DPE, DAPE/HL, DAE/HW, DPE/HW, WW/WWP, WL/WW, NS/WW, NS/WL, DCL/VCL, DLL/VLL, DLL/DLW, VLL/VLW, DCL/DLL, CLL/CLH, CLL/VCL, CLL/DCL) and 2) raw data used to create scatter plots between morphological characters with particularly high SIMPER dissimilarity in case point 1) failed to produce independent clusters.


Fig. 5.1. Schematic of the Hediste diversicolor morphotype showing the measurements used in the morphometric analysis. (A) Anterior end. (B) Parapodia. Abbreviations: the length of the parapodium up to the median ligule (CLL), antennae (AL), palps (PL), antero-dorsal cirri and postero-dorsal cirri (DSTL, DLTL, respectively), dorsal and ventral cirri of median segments (DCL, VCL), dorsal and ventral ligule of median segments (DLL, VLL) and head (HL); the width of the worm with parapodia (WWP) and without parapodia (WW), head (HW), dorsal and ventral ligule (DLW, VLW); and the
distance between the anterior eyes (DAE), distance between the posterior eyes (DPE), distance between the anterior and posterior eyes (DAPE) as well the height of the parapodium (CLH).

Principal Component Analysis (PCA) was based on normalised data. The significance of the interpopulation differences was explored by one-way analysis of similarity (ANOSIM) based on Euclidean distance resemblance matrices. The contribution of each measured character to the distance within and between the four species was assessed by the Similarity Percentages analysis (SIMPER) based on Euclidean distance. Both SIMPER and ANOSIM also used the normalized proportion dataset and were conducted using PRIMER version 6.1.11, copyright by PRIMER-E Ltd. 2008 (Clarke and Warwick, 2001). All measurements were done with a LEICA MC170 HD stereo microscope, with an incorporated measurement software.

Representative specimens from each Hediste lineage were used for scanning electron microscopy (SEM). These specimens were transferred to 100\% ethanol, dehydrated for 2 hours with hexamethyldisilazane (HMDS, $\geq 99 \%$ ) and left to dry overnight. No coating was applied. Images were obtained using a TM3030Plus tabletop microscope (Hitachi). Morphological observations were carried out with an Olympus stereo microscope equipped with a camera lucida for line drawings.

Stereo microscope images were taken with a Canon EOS1100D camera. Compound microscope images of parapodia and chaetae were obtained with a Zeiss Axioplan 2 imaging light microscope (Carl Zeiss, Oberkochen, Germany), equipped with a DP70 Olympus camera (Olympus Corp., Tokyo, Japan), after mounting the parapodia on a slide preparation using Aquamount (Gurr) liquid. The software Inkscape 0.92.3 (https://www.inkscape.org) was used to create the final images for the drawings of the parapodia.

Parapodial and chaetal terminology in the taxonomic section follows Bakken and Wilson (2005) with the modifications made by Villalobos-Guerrero and Bakken (2018). Pharynx paragnath terminology follows Bakken et al. (2009). Chaetigers after segment 15 are considered part of the worm's mid-body, with the first 15 segments considered the anterior region of the body.

### 5.3 Results

### 5.3.1 Phylogenetic reconstruction

The concatenated BI Tree (Fig. 5.2.A, B.) show evidence of at least five different species belonging to the Hediste diversicolor complex. There is a MOTU consensus, corresponding to each of the monophyletic clades with low divergence, belonging to MOTU 1, MOTU 3, MOTU 4 and MOTU 5. However,
none of the species delineation methods or the morphometric data provided by the PCA and SIMPER analysis, reached a consensus to define the previously defined "Species A" as a single entity. This massive clade varied between 1 to 35 MOTUs and has genetic distances above $3 \%$, reaching almost $7 \%$ in two of the four sub-clades present in the BI tree. The bPTP method grouped all the Hediste populations into a single entity and the PCA grouped MOTUs 1 and 2 together. To achieve consensus as MOTU 2 corresponding to Species A , the most conservative result within the major clade was chosen (SIMPER).


Fig. 5.2. MrBayes tree from concatenated analysis of three markers and MOTU distribution. (A), Phylogenetic tree reconstructed for the Hediste diversicolor complex using Bayesian inference based on concatenated COI, ITS2 and 28S sequences, with information regarding the different MOTU delineation methods. BINs were used only for COI. Only the bootstrap values over 0.85 BI support are shown. Each different consensus MOTU is represented by the respective number, with the different colours corresponding to the respective geographic distribution. The outgroup (OUTG) belong to the species Alitta virens. (B), Geographic distribution in Europe for the five retrieved MOTUs based on the original sequences (non-bold abbreviations) and data from the previous studies (bold abbreviations). Region abbreviations as stated in Table 5.1, with the addition of: GER, Germany; GB, Great Britain; NL, Netherlands; BAS, Baltic Sea; MOR, Morocco; MAR, Marseille (France), OR, Oristano (Italy); LEC, Leece (Italy); CRO, Croatia; BS, Black Sea; CS, Caspian Sea.

The phylogeographic structure of the European Hediste diversicolor comprises at least four divergent lineages (Fig. 5.2.B). MOTU 1 occurs in the western part of the Mediterranean (Tyrrhenian Sea), north-east Skagerrak, Kattegat Sea and in the Baltic Sea. MOTU 2 can be found in all of the NE Atlantic and Scandinavia, ranging from Portugal and Morocco to Norway (excluding the north-eastern part of the Skagerrak), French part of the Western Mediterranean (based on a cytb sequence from Breton et al. (2003)), and also in the Baltic Sea in sympatry with two other lineages (MOTUs 1 and 5). The cytb sequence grouped in the same clade as the COI sequences from Virgilio et al. (2009), which in turn corresponds to the clade identified as MOTU 2 in this study. MOTU 3 is exclusive to the Adriatic Sea, biogeographically part of the eastern Mediterranean, while MOTU 4 is present only in Greece in the Ionian Sea. Lastly, MOTU 5 is located in eastern Greece (northern Aegean Sea) and corresponds to the same MOTU found in the Caspian and Black Sea from previous studies.

### 5.3.2 Haplotype networks

The COI (Fig. 5.3.A) and ITS2 (Fig. 5.3.B) haplotypes completely sorted all MOTUs, and no haplotype have a central position in the networks. MOTU sorting is also supported by the genetic distances between records, with five clear clusters visible (Supplemental Fig. S5.1; except for 28S). However, haplotype sharing between MOTUs are found in the 28S network (Fig. 5.3.C), not only between populations from Norway and Brest (MOTU 2) with Sweden (MOTU 1), but also between the Adriatic Sea (MOTU 3) and the Ionian Sea (MOTU 4). Interestingly, even though MOTUs 3 and 4 are separated by 26 mutations in the COI network, compared to the 7 mutations found between the two geographically structured populations within MOTU 1 (western Mediterranean and Scandinavia), the latter presents a completely different topology and higher number of mutations in the nuclear haplotypes for the different populations. In contrast, nuclear haplotypes from the Adriatic and Ionian Sea show evidence of belonging to the same lineage. COI haplotype diversity is relatively high in MOTUs 2 and 5 ( $\mathrm{Hd}>0.94$ to 0.98 , respectively; Table 5.2). However, lower values can be found in MOTU 1 (Hd: 0.54) and MOTU 3 (Hd: 0.76 ), with the latter being the only one with significant Tajima D and Fu and Li's D tests. The negative values indicate either a population expansion after a recent bottleneck, or linkage to a swept gene, while the neutral model of nucleotide substitutions accepted for the remaining MOTUs.

The high haplotype numbers in MOTU 2 are mostly present within the populations from Norway and in the estuaries of Minho, Lima, and Lagares (Table 5.3). Together with the high number of mutations between haplotypes from these populations, this can explain the unusual number of potential lineages
identified by some of the species delineation methods and the formation of two sub-clades with high intraspecific divergence (>3\%) in the BI tree.


Fig. 5.3. Haplotypes networks for $\mathrm{COI}(5)$, ITS2 (6) and $28 \mathrm{~S}(7)$ for all the five MOTUs based on the original Hediste data and Alitta virens as outgroup. Each haplotype is represented by a circle and number of haplotypes are according to the displayed scale. Colours indicate the geographic location of the haplotype. Numbers correspond to the number of mutational steps between haplotypes. Lines without numbers means only one mutation between haplotypes.

The Norwegian ITS2 haplotypes from MOTU 2 seem to form an independent group, unlike the scattered topology seen in the COI network, while haplotypes from Brest are still scattered among the different Iberian estuaries. This contrasts with the populations from Aveiro, Sado and Ferrol which instead present a similar network structure to the remaining MOTUs.

Table 5.2. Indices of genetic diversity estimated for each MOTU, based on COI and from the original data. Number of sequences ( n ); nucleotide diversity ( tt ), number of haplotypes ( h ), haplotype diversity ( Hd ) and number of variable sites (S). Region abbreviations as stated in Table 5.1. Values in bold are significative.

|  | Region | N | h | Hd | S | tt | Fu and Li's D* | Tajima's D |
| :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: |
| MOTU 1 | NV, TJ, SF | 41 | 7 | 0.5 | 15 | 0,00475 | -1,48049 | -0,34310 |
|  |  |  |  | 4 |  |  | P $>0.10$ | P $>0.10$ |
| MOTU 2 | SA, AV, FE, | 117 | 7 | 0,9 | 190 | 0,03497 | -2,21398 | -1,51333 |
|  | BR, GM, TD, |  | 7 | 8 |  |  | $0.10>P>0.05$ | P $>0.10$ |
|  | LI, MI, LA |  |  |  |  |  |  |  |
| MOTU 3 | VE | 21 | 9 | 0.7 | 20 | 0,00337 | -3,30826 | -2,25699 |
|  |  |  |  | 6 |  |  | P < 0.02 | P < 0.01 |
| MOTU 4 | AM | 5 | 1 | 0 | 0 | 0 | - | - |
| MOTU 5 | NAS | 27 | 1 | 0.9 | 32 | 0,01155 | 0,12419 | -0,31253 |
|  |  |  | 4 | 4 |  |  | P $>0.10$ | P $>0.10$ |

Table 5.3. Haplotype, number of BINs and genetic distances (COI) comparisons within the different populations from the five retrieved MOTUs. Values in BOLD are unusual, with high haplotype diversity, as well with more than $3.5 \%$ COI distances. Coloured values with the same colour share the same BINs.

|  | Populations <br> (COI) | $\mathbf{n}$ | $\mathbf{h}$ | Hd | BINs <br> (shared) | Mean <br> Distance <br> (\%) | Maximum <br> distance (\%) |
| :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: |
| MOTU 1 | Tjärnö | 30 | 4 | 0,251 |  | 0.1 | 0.9 |
|  | Navicelli | 10 | 3 | 0,378 | 1 | 0.1 | 0.3 |
|  | Minho | 20 | 19 | 0,995 | $\mathbf{6 ( 7 )}$ | $\mathbf{4 . 1}$ | $\mathbf{6 . 9}$ |
|  | Lima | 23 | 19 | 0,984 | $\mathbf{8}(9)$ | $\mathbf{4 . 5}$ | $\mathbf{6 . 8}$ |
|  | Lagares | 5 | 5 | 1 | $\mathbf{3 ( 2 )}$ | $\mathbf{3 . 2}$ | $\mathbf{4 . 4}$ |
| MOTU 2 | Grimstad | 5 | 5 | 1 | $\mathbf{5}$ | $\mathbf{4 . 6}$ | $\mathbf{6 . 4}$ |
|  | Trondheim | 2 | 2 | 1 | $\mathbf{2}$ | $\mathbf{4 . 4}$ | $\mathbf{4 . 4}$ |
|  | Aveiro | 20 | 10 | 0,905 | 1 | 0.5 | 1.1 |
|  | Sado | 22 | 11 | 0,818 | 1 | 0.5 | 1.4 |
|  | Brest | 10 | 4 | 0,644 | $1(1)$ | 1.3 | 3.6 |
| MOTU 3 | Ferrol | 10 | 3 | 0,511 | 1 | 0.3 | 0.9 |
| MOTU 4 | Adriatic Sea | 21 | 9 | 0,757 | 1 | 0.3 | 1.7 |
| MOTU 5 | Amvrakikos | 5 | 1 | 0 | 1 | 0.0 | 0.0 |

### 5.3.3 Genetic distances

Global intra- and interspecific distances for the five different MOTUs and each marker are provided in Table 5.4. For COI, the mean intraspecific distance is $1.13(0.0-7.5) \%$, while the average congeneric distance is $6.9(4.1-10.1) \%$. For the ITS2-region it ranges between $2.6(0.0-10) \%$ and $4.6(0.3-$ 11.6)\% for intra- and interspecific divergence, respectively, while for 28 S the corresponding distances are $0.5(0-1.7) \%$ and $0.7(0-2.4) \%$, respectively.

Table 5.4. Mean intra (in bold) and inter-MOTU genetic distances (K2P) for the three analysed markers (COI, ITS2, 28S), for the five retrieved Hediste MOTUs, based on the original data.

|  | Loci | 1 | 2 | 3 | 4 | 5 |
| :---: | :---: | :---: | :---: | :---: | :---: | :---: |
| MOTU 1 <br> (Species B1) | COI | $0.5 \pm 0.4$ |  |  |  |  |
|  | ITS2 | $4.8 \pm 0.4$ |  |  |  |  |
|  | 285 | $0.6 \pm 0.2$ |  |  |  |  |
| MOTU 2 <br> (Species A) | COI | $7.8 \pm 0.9$ | $3.6 \pm 0.2$ |  |  |  |
|  | ITS2 | $6.5 \pm 0.6$ | $4.3 \pm 0.5$ |  |  |  |
|  | 28 S | $0.8 \pm 0.2$ | $0.8 \pm 0.2$ |  |  |  |
| MOTU 3 <br> (Species B2) | COI | $5.7 \pm 0,9$ | $7.5 \pm 0.9$ | $0.3 \pm 0.1$ |  |  |
|  | ITS2 | $4.6 \pm 0.5$ | $5.4 \pm 0.6$ | $1.8 \pm 0.3$ |  |  |
|  | 28 S | $0.9 \pm 0.2$ | $0.7 \pm 0.2$ | $0.2 \pm 0.1$ |  |  |
| MOTU 4 <br> (Species B3) | COI | $6.1 \pm 0.9$ | $7.8 \pm 0.9$ | $4.4 \pm 0.8$ | $0.0 \pm 0.0$ |  |
|  | ITS2 | $4.3 \pm 0.6$ | $4.9 \pm 0.6$ | $1.4 \pm 0.3$ | $0.1 \pm 0.1$ |  |
|  | 28 S | $0.9 \pm 0.2$ | $0.7 \pm 0.2$ | $0.1 \pm 0.0$ | $0.0 \pm 0.0$ |  |
| MOTU 5 <br> (Species B4) | COI | $7.0 \pm 1.0$ | $8.2 \pm 0.9$ | $7.3 \pm 1.0$ | $7.7 \pm 1.0$ | $1.2 \pm 0.2$ |
|  | ITS2 | $5.9 \pm 0.7$ | $6.7 \pm 0.8$ | $3.5 \pm 0.5$ | $2.8 \pm 0.5$ | $1.7 \pm 0.3$ |
|  | 28 S | $0.8 \pm 0.2$ | $0.8 \pm 0.2$ | $0.7 \pm 0.2$ | $0.6 \pm 0.2$ | $0.4 \pm 0.1$ |

MOTUs 3 and 4 have low genetic divergence between them, with just 4.4\% COI and 1.4\% ITS2 genetic distances (K2P), which is lower than the intraspecific divergence found within MOTU 2. The latter shows unusual high genetic distances within populations of the same estuary as seen in Table 5.3. These high genetic distances are present in the estuaries of Lima, Minho, Lagares and in the Norwegian specimens, where the number of BINs and haplotypes are unusually high as well. The Lima estuary in particular not only has maximum COI distances reaching almost $7 \%$, but also has 17 BINs , with 8 of them being unique to the estuary and the remaining 9 being shared with populations from Minho and Lagares (Table 5.3). In contrast, Hediste populations from the estuaries of Sado, Aveiro and Ferrol, also from

MOTU 2, have less than $1.5 \%$ intraspecific COI divergence, with the Brest population having a mixed genetic variation corresponding to the two different BINs.

No genetic structure (i.e. genetic populations sorted geographically) seems to be patent within MOTU2 (Supplemental Fig. S5.2).

### 5.3.4 Morphology

No consistent morphological differences between specimens from the different MOTUs was found. The paragnath patterns present in the worm's pharynx (Fig. 5.4.A, B) are consistent with the descriptions of $H$. diversicolor (Müller, 1776) with some variation in the numbers found between the MOTUs. The low number of paragnaths in MOTUs 1, 3 and 5 (detailed in the taxonomic section), especially in areas III and IV, can sometimes reach half the number as the ones found in MOTU 2 and may be a diagnostic feature, but phenotypic variation is high among the specimens. The main types of chaetae found in all the lineages from the complex can be seen in Fig. 5.4.C-D, being characterized by the presence of neuropodial heterogomph spinigers (Figs. 5.4.C; 5.5.A) and neuropodial heterogomph falcigers (Figs. 5.4.D; 5.5.C) in the ventral fascicle. Neuropodial homogomph spinigers and neuropodial heterogomph falcigers can also be found in the dorsal fascicle. Additionally, large homogomph spinigers are the only type of chaetae present in the notopodium (Fig. 5.5.B). In the posterior part of the body, the neuropodial heterogomph falcigers from the dorsal fascicle are replaced by a large fused falciger, apparently after chaetiger 40 depending on specimen size (Fig. 5.5.D).

Parapodia morphotypes from anterior parapodia (Fig. 5.4.E) and after chaetiger 20 (Fig. 5.4.F), both with short, thick ligules. No major differences were found in the parapodia structures between the four analysed MOTUs (Figs. 5.6.A-L), with the complex having dorsal ligules longer and wider than ventral ligules and dorsal cirri longer than ventral cirri, both reaching around half the size of the respective ligules. However, the proportions between the parapodial structures seem to differ slightly for the different MOTUs and were further analysed using morphometric measurements.


Fig. 5.4. Representative SEM images for the Hediste diversicolor morphotype. (A) Paragnath patterns in the worm's pharynx, dorsal view. (B) Paragnath patterns in the worm's pharynx, ventral view. (C) Mid-body neuropodial heterogomph spiniger. (D) Mid-body neuropodial heterogomph falciger. (E) Morphotype of the parapodia after chaetiger 20, anterior view. (F) Morphotype of the anterior parapodia (< chaetiger 15), posterior view.


Fig. 5.5. Microscopic scans of all the different chaetae types found in the Hediste diversicolor complex. (A) Neurochaeta: heterogomph spiniger, chaetiger 30. (B) Notochaeta: homogomph spiniger, chaetiger ten. (C) Neurochaeta: heterogomph falciger, chaetiger 30. (D) Neurochaeta: fused falciger, chaetiger 45.

### 5.3.5 Morphometry

The morphometric proportion data in the PCA analysis individualized three distinct clusters corresponding to the combined data between MOTU 1/MOTU 2 against MOTU 3 and MOTU 5, segregating them into three clear groups (Fig. 5.7.A). Photos from preserved specimens belonging to MOTU 3 and MOTU 5 can be seen in Fig. 5.7.B and Fig. 5.7.C, respectively. Morphometric measurements from the specimens belonging to MOTUs 1 and 2 are scattered and overlapping, failing to produce two separated groups. Specimens from both the western Mediterranean and Sweden were used in MOTU 1, while Norwegian and Portuguese samples (mainly from Minho and Sado) were used for the measurements in MOTU 2.


B


E



C

MOTU 1 Hediste sp. B1
$\qquad$


G


L


Fig. 5.6. Drawings of the main morphological features found in the parapodia from different parts of the worm's body. Scale bars $=500 \mu \mathrm{~m}$. MOTU 1 (Hediste sp. B1, specimen DBUA0002463.01.v24): (A) Parapod 10, posterior view. (B) Parapod 30, posterior view. (C) Parapod 49, posterior view; MOTU 2 (Hediste diversicolor s.s., specimen NTNU-VM 82084): (D) Parapod 10, posterior view. (E) Parapod 28, posterior view. (F) Parapod 60, posterior view; MOTU 3 (Hediste pontii sp. nov., specimen DBUA0002465.01.v01): (G) Parapod 10, posterior view. (H) Parapod 31, posterior view. (I) Parapod 61, posterior view; MOTU 5 (Hediste astae sp. nov., specimen DBUA0002466.02.v05): (J) Parapod 10, posterior view. (K) Parapod 30, posterior view. (L) Parapod 59, posterior view.

No significant differences were found between and within these populations. Twenty-seven character proportions were used in the PCA discrimination, with Axes 1 (eigenvalue $=9.18$ ) and 2 (eigenvalue $=4.55$ ) explaining $34.0 \%$ and $16.9 \%$ of the variation, respectively. The ANOSIM test indicates
significant differences between the morphometric data of the four analysed MOTUs (Global $R=0.756$; significance level at $0.1 \%$ ).


Fig. 5.7. Principal Component Analysis (PCA) plots based on proportion data. (A) Plot between MOTUs $1,2,3$ and 5 . Twenty-seven character proportions were used. (B) Scale bar $=2 \mathrm{~mm}$. Photo of a preserved specimen (DBUA0002465.01.v03) from MOTU 3. (C) Scale bar $=500 \mu \mathrm{~m}$. Photo of a preserved specimen (DBUA0002466.01.v09) from MOTU 5.

The average morphometric variation within species provided by the SIMPER results is $17.51 \%$ for MOTU 2, $11.90 \%$ for both MOTU 1 and MOTU 3 and $16.37 \%$ for MOTU 5 . The average inter-species distance range between 37.64\% (MOTU 1/MOTU 2) and 91.47\% (MOTU 3/MOTU 5), with greater distances when involving MOTU 5 (Table 5.5). The three most significant proportions for the inter-species dissimilarity are summarized in Table 5.5, all with more than $4.50 \%$ of contribution. The length and width of the head (HL, HW), head appendages (AL, PL, DLTL, DSTL) and distance between the posterior eyes (DPE) are the features that, when combined, most contributed to the divergence between all the analysed
species. Worm width and length (WW and WL, respectively) and number of segments (NS) are also highlighted when comparing MOTU 2 against MOTU 3 , and MOTU 3 with MOTU 5.

Traditional morphometric approaches based on scatter plots by using relevant combinations of the most significant characters revealed by the SIMPER analysis (the length of the antennae against either the length of the head, the antero dorsal cirri and postero dorsal cirri), have enough divergence to display two partial clusters for each of the analysed lineages as seen in Fig. 5.8A-C. These are the only combinations with distinct clusters between MOTUs 1 and 2, which explain the PCA result and low interspecies morphometric distance reported above.

### 5.3.6 Taxonomic section

> Hediste diversicolor (O.F. Müller, 1776) s.s.
> Neanthes diversicolor (Müller, 1776)
> Nereis (Hediste) diversicolor 0.F. Müller, 1776
> Nereis (Nereis) diversicolor Müller, 1776
> Nereis brevimanus Johnston, 1840
> Nereis depressa Frey \& Leuckart, 1847
> Nereis diversicolor Müller, 1776
> Nereis sarsii Rathke, 1843
> Nereis versicolor [misspelling for diversicolor]
> Nereis viridis Johnston, 1840

Material examined
Type material. Norway, Grimstad: 1 spm, neotype and hologenophore, NTNU-VM82082, $58^{\circ} 17^{\prime} 52.8^{\prime \prime} \mathrm{N}-8^{\circ} 32^{\prime} 20.4^{\prime \prime} \mathrm{E}$, low tide, muddy sand and gravel, collected by Eivind Oug, 14/04/2019.

Other material. Portugal, Sado estuary: 9 spms, DBUA0002458.01.v01, DBUA0002458.02.v01-v03, DBUA0002458.03.v01-v05, $38^{\circ} 29^{\prime} 24.0^{\prime \prime} \mathrm{N}-8^{\circ} 48^{\prime} 54.0^{\prime \prime} \mathrm{W}$, low tide, muddy sand and gravel, collected by Marcos AL Teixeira, Pedro E Vieira, Bruno R Sampieri, Jorge Lobo and Claudia Hollatz, 31/07/2018; 18 spms, DBUA0002457.01.v01-v18, $38^{\circ} 29^{\prime} 52.8^{\prime \prime} \mathrm{N}-8^{\circ} 50^{\prime} 16.8^{\prime \prime W}$, low tide, muddy sand and gravel, collected by Marcos AL Teixeira, Pedro E Vieira, Bruno R Sampieri, Jorge Lobo and Claudia Hollatz, 28/02/2018. Portugal, Lima estuary: 23 spms, DBUA0002459.01.v01-v23, $41^{\circ} 42^{\prime} 03.6^{\prime \prime} \mathrm{N}-8^{\circ} 44^{\prime} 56.4^{\prime \prime} \mathrm{W}$, low tide, muddy sand and gravel, collected by Marcos AL Teixeira, Pedro E Vieira and Bruno R Sampieri, 20/03/2018. Portugal, Aveiro lagoon: 25 spms, DBUA0002460.01.v01-
v25, $40^{\circ} 38^{\prime} 02.4^{\prime \prime} \mathrm{N}-8^{\circ} 40^{\prime} 30.0^{\prime \prime} \mathrm{W}$, low tide, muddy sand and gravel, collected by Marcos AL Teixeira, Pedro E Vieira, Bruno R Sampieri and Ascensão Ravara, 28/02/2018. Portugal, Minho estuary, 25 spms, DBUA0002461.01.v01-v25, $41^{\circ} 52^{\prime} 55.2^{\prime \prime} \mathrm{N}-8^{\circ} 49^{\prime} 44.4^{\prime \prime} \mathrm{W}$, low tide, muddy sand and gravel, collected by Marcos AL Teixeira, 28/02/2018. Spain, Lagares: 5 spms, DBUA0002455.01.v01-v05, $42^{\circ} 12^{\prime} 07.2^{\prime \prime} \mathrm{N}-8^{\circ} 46^{\prime} 40.8^{\prime \prime} \mathrm{W}$, low tide, muddy sand and gravel, collected by Marcos AL Teixeira, 23/10/2017. Spain, Ferrol, 10 spms, DBUA0002456.01.v01-v09 and MTHD015-20, 43²9'34.8"N $8^{\circ} 14^{\prime} 56.4^{\prime \prime W}$, low tide, muddy sand and gravel, collected by Julio Parapar, 26/06/2018. France, Brest: 10 spms, DBUA0002462.01.v01-v10, $48^{\circ} 24^{\prime} 21.6^{\prime \prime} \mathrm{N}-4^{\circ} 22^{\prime} 01.2^{\prime \prime} \mathrm{W}$, low tide, muddy sand and gravel, collected by Juan Pardo, 14/09/2019. Norway, Grimstad: 4 spms, NTNU-VM 82080-82081 and NTNUVM 82083-82084, $58^{\circ} 17^{\prime} 52.8^{\prime \prime N}-8^{\circ} 32^{\prime} 20.4^{\prime \prime E}$, low tide, muddy sand and gravel, collected by Eivind Oug, 14/04/2019. Norway, Trondheim: 2 spms, NTNU-VM 76340 and NTNU-VM 76341, 63² $26^{\prime} 09.6^{\prime \prime} \mathrm{N}$ $-10^{\circ} 29^{\prime} 56.4^{\prime \prime} \mathrm{E}$, low tide, muddy sand and gravel, 04/09/2018.

Diagnosis
Body with a prominent dorsal blood vessel. Medium-sized worm of normal tapering shape. Analysed specimens vary between $20-39 \mathrm{~mm}$ in length, with 50 to 83 segments. Neotype lacking the posterior end, 27 mm in length for 58 chaetigers, 1.503 mm in width. Colour variable between greenish, yellowish-brown, and orange. Head wider than long. Pharynx with conical paragnaths (Figs. 5.4.A, B). Area I with 2-6 paragnaths forming a longitudinal line or a shapeless group. Area II with 9-23 paragnaths forming a diagonal thick line. Area III with a shapeless group of 24-52 paragnaths. Area IV with 19-38 paragnaths forming a " C " shape group. Area V absent. Area VI with 3-8 conical paragnaths. Area VII-VIII with two rows of paragnaths, the posterior row with twice $(20-26)$ as many paragnaths as the anterior one (10-13). Most tentacular cirri as long as body width or shorter. The postero-dorsal cirri can sometimes surpass the body's width and usually doubles the length of the shorter tentacular cirri; 2.6 times as long as the antero-dorsal cirri. Distance between the anterior eyes larger than that between the posterior ones. Antennae markedly shorter than palps, around half the palp's length. Parapodia with short, thick ligules (Figs. 5.4.C, D; 5.6.D-F). Based on measurements posterior to chaetiger 20, dorsal cirri shorter than the dorsal ligule, but not less than half the ligule's length. Dorsal ligule slightly longer than the ventral ligule. Dorsal and ventral ligules twice longer than wide. Ventral cirri shorter than the ventral ligule.

Table 5.5. List of the three most contributing proportions to the inter-population dissimilarities based on the SIMPER analyses.

|  | Proportions | Contribution (\%) | Ratio | Average Intervariation (\%) | ANOSIM |
| :---: | :---: | :---: | :---: | :---: | :---: |
| MOTUs 2 vs 1 | AL/DLTL <br> AL/DSTL <br> AL/HL | $\begin{aligned} & 5.43 \\ & 5.37 \\ & 4.54 \\ & \hline \end{aligned}$ | MOTU 1 > AL; similar DLTL, DSTL and HL | 37.64 | 0.376 at 0.1\% |
| MOTUs 2 vs 3 | DLL/VLL <br> WL/WW <br> CLL/CLH | $\begin{aligned} & \hline 7.16 \\ & 7.10 \\ & 7.00 \\ & \hline \end{aligned}$ | MOTU 3 > proportions | 58.08 | 0.752 at 0.1\% |
| MOTUs 2 vs 5 | WW/WWP <br> DPE/HW <br> PL/HW | $\begin{aligned} & \hline 7.28 \\ & 6.54 \\ & 6.04 \\ & \hline \end{aligned}$ | MOTU 5 < proportions | 69.86 | 0.858 at 0.1\% |
| MOTUs 1 vs 3 | $\begin{gathered} \mathrm{AL} / \mathrm{HL} \\ \mathrm{AL} / \mathrm{PL} \\ \mathrm{DAPE} / \mathrm{HL} \end{gathered}$ | $\begin{aligned} & 8.66 \\ & 8.52 \\ & 7.65 \end{aligned}$ | MOTU $3>\mathrm{HL}, \mathrm{PL}$ but similar AL; > DAPE/HL | 44.84 | 0.744 at 0.1\% |
| MOTUs 1 vs 5 | DLTL/DSTL AL/DSTL DPE/HW | $\begin{aligned} & 7.94 \\ & 7.83 \\ & 6.28 \\ & \hline \end{aligned}$ | MOTU 5 < proportions | 71.12 | 0.946 at 0.1\% |
| MOTUs 3 vs 5 | $\begin{gathered} \hline \text { NS/WL } \\ \text { PL/DSTL } \\ \text { NS/WW } \\ \hline \end{gathered}$ | $\begin{aligned} & \hline 7.46 \\ & 6.92 \\ & 6.15 \\ & \hline \end{aligned}$ | MOTU 3 > proportions | 91.47 | 0.961 at 0.1\% |

Large homogomph spiniger chaetae in notopodia (Fig. 5.5.B) and in dorsal fascicle of neuropodia, with variable sizes. Heterogomph spinigers with variable sizes and long blade falcigers in neuropodia, ventral fascicle (Fig. 5.5A, C, respectively). One large fused falciger in each neuropodium, most commonly found in the posterior chaetigers (Fig. 5.5.D), replacing heterogomph falcigers in the dorsal fascicle.

## Molecular data

COI, ITS2 and 28S sequences as in specimens DBUA0002458.01.v01, DBUA0002458.02.v01v03, DBUA0002457.01.v01-v18, DBUA0002459.01.v01-v23, DBUA0002460.01.v01-v20, DBUA0002461.01.v01-v20, DBUA0002455.01.v01-v05, DBUA0002456.01.v01-v09 and MTHD01520, NTNU-VM 82080-82084 and NTNU-VM 76340-76341 (Table S5.1). Genetic distances are given in Table 5.4. Phylogenetic relationship as in Fig. 5.2.A, belonging to MOTU 2 and characterized by the high intraspecific divergence within some populations that can reach up to $7.5 \% \mathrm{COI} \mathrm{K} 2 \mathrm{P}$. These distances can be achieved even in specimens from the same estuary (e.g. Minho and Lima estuaries). Interspecific COI mean distances to the closest and distant neighbour are $7.5 \%$ (K2P, MOTU 3) and 8.2\% (K2P, MOTU 5) respectively. High number of BINs (35, Fig. 5.2A, Table 5.3) and COI haplotypes (Fig. 5.3.A, Table 5.2) also characterize this MOTU. DOI for the neotype specimens' Barcode Index Number (BIN): upon paper publication.

## Distribution and habitat

Northeast Atlantic Ocean, from Norwegian Sea to Morocco; Baltic Sea. Also reported in North America (Einfeldt et al., 2014). In the Baltic Sea and Skagerrak, it occurs in sympatry with Hediste sp. B1 and $H$. astae sp. nov. (described below) (Fig. 5.2.B). Mostly found in intertidal areas, making burrows in black muddy sand, often under brackish conditions. Commonly used as bait by anglers.

## Reproduction

Available data on reproduction have been accumulated over time and most likely represent different lineages. Reproduction, including spawns and broods of embryos at ten week trochophore / demersal nectochaeta stage, occurs at favorable levels of 5-27 salinity. Egg sizes between 200-250 $\mu \mathrm{m}$ were reported for the North Atlantic coastal populations (Müller 1776; Dales, 1950; Smith, 1964; Christensen 1980; Bartels-Hardege and Zeeck, 1990; Scaps, 2002).

## Remarks

The taxonomic history of Hediste diversicolor is intricate and has been difficult to unravel. Müller (1776) provided a short and vague diagnosis of the species, based on previous records referring to material from Denmark (likely, Copenhagen) and western Norway (Ström, 1762), with no illustrations. Notwithstanding this being considered as the formal original description of the species (Oug et al., 2014), more detailed descriptions and illustrations were given in those previous records (Salazar-Vallejo et al., 2021). Müller kept a large collection of specimens (Anker, 1950), but no original material is presently known to exist (Oug et al., 2014). Knowing now that Hediste diversicolor is a species complex with multiple genetically evolved entities, it is necessary to select a neotype to provide nomenclatural stability and a physical specimen preserved for later reference. In accordance with the results from this study, it is reasonable to select a specimen from MOTU 2 (H. diversicolor s.s.), collected in Norway, as neotype.

The specimens of $H$. diversicolor s.s. examined herein present a higher SIMPER intramorphometric variation between the analysed proportions when compared to the rest of the complex, similarly to the molecular results regarding the intraspecific COI divergence. Hediste diversicolor s.s. have similar proportions to Hediste sp. B1, and the morphometric distinction between the two species can only be partially achieved if comparing the antenna length (shorter measurements) against either the similar length of the postero-dorsal cirri, antero-dorsal cirri and head (Fig. 5.8.A-C, Table 5.5). These two lineages seem to have an higher number of neuropodial heterogomph falciger in the ventral fascicle against the number of heterogomph spinigers, when compared to the remaining lineages where the inverse is observed. The latter observation is more evident in Hediste sp. B1 and may be a diagnostic feature. Evidence of hybridization between H. diversicolors.s. and Hedistesp. B1 is seen in the molecular nuclear data and alloenzymes (Audzijonyte et al., 2008), which might not support reproductive isolation in the scope of the more restrictive biological species concept. The other two lineages studied herein, corresponding to $H$. astae sp. nov. and $H$. pontii sp. nov. described below, present smaller and larger morphometric proportions, respectively, when comparing to H. diversicolor s.s. The most significant distinguishing proportions are the length of the dorsal / ventral ligules, the length / width of the worm, the length / height of the parapodia, the width of the worm with / without parapodia, and both the distance of the posterior eyes and the length of the palps / width of the head (Table 5.5). Hediste diversicolor s.s. is further distinguished from the other three species by the higher number of paragnaths (sometimes twice the amount), especially in the areas III and IV, which may be a diagnostic feature. However, phenotypic variation is high among the specimens.

For a major review of the biology, ecology and potential use of Hediste diversicolor see Scaps (2002). The complete mitochondrial genome from an adult H. diversicolor specimen, collected by Andreas Hagemann in Trondheim Fjor (Leangbukta, Norway at $63^{\circ} 26^{\prime} 20.9^{\prime \prime} \mathrm{N}-10^{\circ} 28^{\prime} 28.6^{\prime \prime} \mathrm{E}$ ), was sequenced by Gomes-dos-Santos et al. (2021). The specimen is deposited at the Interdisciplinary Center of Marine and Environmental Research - CIIMAR (Prof. Filipe Castro, filipe.castro@ciimar.up.pt) under the voucher number 4HDIV3 and GenBank accession number MW377219.


Fig. 5.8. Scatter plots with the most significative proportions in distinguishing MOTU 2 (Hediste diversicolor s.s.) from MOTU 1 (Hediste sp. B1). (A) Morphometric proportions between the length of the antennae (AL) and the length of the
postero-dorsal cirri (DLTL). (B) Morphometric proportions between the length of the antennae (AL) and the length of head (HL). (C) Morphometric proportions between the length of the antennae (AL) and the length of the antero-dorsal cirri (DSTL).

## Hediste pontii sp. nov.

(Fig. 5.7.B)
urn:Isid:zoobank.org:act: upon paper publication

## Material examined

Type material. Italy, Venezia lagoon: 1 spm, Holotype and hologenophore, DBUA0002465.01.v03, $45^{\circ} 20^{\prime} 13.2^{\prime \prime} \mathrm{N}-12^{\circ} 16^{\prime} 30.0^{\prime \prime} \mathrm{E}$, near shore at $0.5-1 \mathrm{~m}$ depths, muddy sand and gravel, collected by Massimo Ponti, 10/07/2018; 20 spms, Paratypes and paragenophores, DBUA0002465.01.v01-v02 and DBUA0002465.01.v04-v21, $45^{\circ} 20^{\prime} 13.2^{\prime \prime} \mathrm{N}-12^{\circ} 16^{\prime} 30.0^{\prime \prime} \mathrm{E}$, near shore at $0.5-1 \mathrm{~m}$ depths, muddy sand and gravel, collected by Massimo Ponti, 10/07/2018.

Other material. Italy, Venezia lagoon: 7 spms, DBUA0002465.01.v22-v28, $45^{\circ} 20^{\prime} 13.2^{\prime \prime} \mathrm{N}$ $12^{\circ} 16^{\prime} 30.0^{\prime \prime} \mathrm{E}$, near shore at $0.5-1 \mathrm{~m}$ depths, muddy sand and gravel, collected by Massimo Ponti, 10/07/2018.

## Diagnosis

Medium-sized worm of normal tapering shape. Analysed specimens vary between $20-39 \mathrm{~mm}$ in length, 1.9 to 2.9 mm in width and 60 to 96 segments. Dorsal blood vessel in worm's body may not be visible. Holotype lacking posterior end, 62 mm in length for 74 chaetigers, 2.157 mm in width. Colour mainly yellowish-brown. Head wider than long. Pharynx with conical paragnaths (Fig. 5.4.A, B). Area I with 1-3 paragnaths that can form either a longitudinal line or a shapeless group. Area II with 10-17 paragnaths forming a diagonal tick line. Area III with a shapeless group of 22-25 paragnaths. Areas IV form together a " C " shape group of 17-20 paragnaths. Area V absent. Area VI with $3-8$ paragnaths in each group. Area VII-VIII with two rows of paragnaths, the posterior row with twice (20-24) as many as the anterior row (10-12). Most tentacular cirri as long as body width or shorter. Postero-dorsal cirri 2.6 times as long as the antero-dorsal cirri. Distance between the anterior eyes larger than between the posterior ones. Antennae markedly shorter (usually 2.4 times) than palps. Parapods (Figs. 5.6.G-I) with short, thick ligules. Based on measurements posterior to chaetiger 20, dorsal cirri shorter than the respective ligule, but not less than half the ligule's length. Large homogomph spiniger chaetae in
notopodia (Fig. 5.5.B) and in dorsal fascicle of neuropodia, with variable sizes. Heterogomph spinigers with variable sizes and long blade falcigers in neuropodia, ventral fascicle (Fig. 5.5A, C, respectively). One large fused falciger in each neuropodium, most commonly found in the posterior chaetigers (Fig. 5.5.D), replacing heterogomph falcigers in the dorsal fascicle.

## Molecular data

COI, ITS2 and 28S sequences as in specimens DBUA0002465.01.v01-v21 (Table S5.1). COI haplotype information and genetic distances as in Tables 5.2 and 5.4 , respectively. Phylogenetic relationships as in Fig. 5.2.A, belonging to MOTU 3, with high support values and low intraspecific (<3\%) genetic divergence for both the mitochondrial and nuclear markers. Interspecific COI mean distances to the closest and distant neighbour are 4.4\% (K2P, MOTU 4) and 7.5\% (K2P, MOTU 2) respectively. DOI for the species' Barcode Index Number (BIN): upon paper publication

## Etymology

The new species is named after Massimo Ponti to recognize his great kindness in collecting a large number of Hediste specimens from the Adriatic Sea on the behalf of the author of this thesis.

Distribution and habitat
Mediterranean, restricted to the Adriatic Sea (Fig. 5.2.B). Intertidal, making burrows in black muddy sand, usually under high salinity waters. Commonly used as bait by anglers

Remarks
Hediste pontii sp. nov. is a member of the European Hediste diversicolor species complex, thus morphologically highly similar to $H$. diversicolors.s. and the remaining species of the complex. However, some variations in the size of specific morphological characters can be found. Specimens from this species usually present a higher number of segments, wider and longer body, and overall larger morphometric proportions compared to the remaining species of the complex. General PCA and SIMPER data shows considerable morphometric differences, with the most significative proportions being the length of the dorsal / ventral ligules of median segments, the length / height of the parapodia of median segments, the worm's length / width, and the length of the palps / antero-dorsal cirri. Proportions for the length of the antennae and head are larger for $H$. pontiisp. nov. than for $H$. diversicolors.s. and $H$. astae sp. nov. (described below). However, the morphometric proportions used to distinguish H. pontiisp. nov.
from $H$. diversicolor s.s. and $H$. astae sp. nov., usually have the same values as for Hediste sp. B1 (MOTU 1) and mostly cannot be used to separate the latter from $H$. pontii sp. nov. Nevertheless, despite a considerably longer head size and palps, antennae length have similar morphometric measurements as Hediste sp. B1. Furthermore, H. pontii sp. nov. have larger proportions between the length of the head when compared to the distance between the posterior and anterior eyes of Hediste sp. B1 (Table 5.5).

A lower number of paragnaths (sometimes down to half), especially in areas III and IV, further distinguishes $H$. pontii sp. nov. from $H$. diversicolor s.s., although there is a high phenotypic variation within the latter species preventing this feature to be $100 \%$ accurate.

Very low intraspecific COI variation and clear MOTU delineation also separates this species from the remaining species described from the complex. It is possible that Hediste populations from Greece in the Amvrakikos lagoon (lonian Sea, Hediste sp. B3) might belong to this species based on nuclear haplotypes, however more than $4 \%$ divergency is present in the COI loci. There is the possibility that unsampled haplotypes occur in the area between Venice and Amvrakikos Lagoon, that hosts several potentially suitable habitats for this species. Thus, eastern Ionian Sea and Northern Adriatic Sea haplotypes might well be two extremes of a continuum of unsampled populations. No morphometric or reproduction data is yet available to confirm the status between Adriatic and Ionian populations.

## Hediste astae sp. nov.

(Fig. 5.7.C).
urn:Isid:zoobank.org:act: upon paper publication

Material examined
Type material. Greece, Nestos lagoon: 1 spm, Holotype and hologenophore, DBUA0002466.03.v04, $40^{\circ} 54^{\prime} 36.0^{\prime \prime} \mathrm{N}-24^{\circ} 52^{\prime} 22.8^{\prime \prime} \mathrm{E}$, near shore at $0.5-1 \mathrm{~m}$ depths, muddy sand and gravel, collected by Sarah Faulwetter, 28/05/2018; 7 spms, Paratypes and paragenophores, DBUA0002466.03.v01-v03 and DBUA0002466.03.v05-v08, $40^{\circ} 54^{\prime} 36.0^{\prime \prime} \mathrm{N}-24^{\circ} 52^{\prime} 22.8^{\prime \prime E}$, near shore at 0.5-1 m depths, muddy sand and gravel, collected by Sarah Faulwetter, 28/05/2018.

Other material. Greece, Evros lagoon: 5 spms, DBUA0002466.01.v01-v05, $40^{\circ} 44^{\prime} 38.4^{\prime \prime N}$ $26^{\circ} 02^{\prime} 13.2^{\prime \prime}$ E, near shore at $0.5-1 \mathrm{~m}$ depths, muddy sand and gravel, $25 / 05 / 2018$. Greece, Ptelea lagoon: 4 spms, DBUA0002466.04.v01-v04, $40^{\circ} 56^{\prime} 13.2^{\prime \prime} \mathrm{N}-25^{\circ} 14^{\prime} 49.2^{\prime \prime} \mathrm{E}$, near shore at $0.5-1 \mathrm{~m}$ depths, muddy sand and gravel, collected by Sarah Faulwetter, 26/05/2018. Greece, Alyki lagoon: 6
spms, DBUA0002466.02.v01-v06, $40^{\circ} 57^{\prime} 00.0^{\prime \prime} \mathrm{N}-25^{\circ} 12^{\prime} 50.4^{\prime \prime} \mathrm{E}$, near shore at $0.5-1 \mathrm{~m}$ depths, muddy sand and gravel, collected by Sarah Faulwetter, 26/05/2018. Greece, Axios lagoon: 4 spms, DBUA0002466.05.v01-v04, $40^{\circ} 30^{\prime} 28.8^{\prime \prime} \mathrm{N}-22^{\circ} 43^{\prime} 40.8^{\prime \prime} \mathrm{E}$, near shore at $0.5-1 \mathrm{~m}$ depths, muddy sand and gravel, collected by Sarah Faulwetter, 21/05/2018; 25 spms, DBUA0002466.01.v06-v30, $40^{\circ} 44^{\prime} 38.4^{\prime \prime} \mathrm{N}-26^{\circ} 02^{\prime} 13.2^{\prime \prime} \mathrm{E}$, near shore at $0.5-1 \mathrm{~m}$ depths, muddy sand and gravel, collected by Sarah Faulwetter, 25/05/2018.

## Diagnosis

Small to medium sized worm of normal tapering shape. Analysed specimens vary between 7.6 to 32 mm in length, 0.542 to 1.334 in width and 40 to 82 chaetigers. Dorsal blood vessel in worm's body may not be visible. Holotype lacking the posterior end, 20 mm in length for 42 chaetigers, 1.155 mm in width. Colour variable between yellowish, or orange. Head wider than long. Pharynx with conical paragnaths (Fig. 5.4.A, B). Area I with 1-2 paragnaths forming a longitudinal line or a shapeless group. Area II with 5-15 paragnaths forming a diagonal thick line. Area III with a shapeless group of 19-29 paragnaths. Area IV with $13-22$ paragnaths forming a " C " shape group. Area V absent. Area VI with 3-8 conical paragnaths. Area VII-VIII with two rows of 11-15 paragnaths each. Most tentacular cirri as long as body width or shorter. Postero-dorsal cirri 1.9 times as long as the antero-dorsal cirri. Distance between the anterior eyes larger than between the posterior pair. Antennae markedly shorter than palps, usually half the palp's length. Parapods (Fig. 5.6.J-L) with short, thick ligules. Based on measurements posterior to chaetiger 20, dorsal cirri shorter than the respective ligule, but not less than half the ligule's length. Dorsal ligule slightly longer than the ventral ligule and 1.8 times longer than wider. Ventral ligule 1.7 times longer than wider. Ventral cirri shorter than the ventral ligule. Large homogomph spiniger chaetae in notopodia (Fig. 5.5.B) and in dorsal fascicle of neuropodia, with variable sizes. Heterogomph spinigers with variable sizes and long blade falcigers in neuropodia, ventral fascicle (Fig. 5.5A, C, respectively). One large fused falciger in each neuropodium, most commonly found in the posterior chaetigers (Fig. 5.5.D), replacing heterogomph falcigers in the dorsal fascicle.

## Molecular data

COI, ITS2 and 28 S sequences as in specimens DBUA0002466.01.v01-v05, DBUA0002466.02.v01-v06, DBUA0002466.03.v01-v08, DBUA0002466.04.v01-v04, DBUA0002466. 05.v01-v04 (Table S5.1). COI Haplotype information and genetic distances as in Tables 5.2 and 5.4, respectively. Phylogenetic relationship as in Fig. 5.2.A, belonging to MOTU 5, with high support values
and low intraspecific ( $\mathrm{COI}<3.5 \%$, usually in the higher end of the spectrum) genetic divergence for both the mitochondrial and nuclear markers. Interspecific COI mean distances to the closest and distant neighbour are 7.0\% (K2P, MOTU 1) and 8.2\% (K2P, H. diversicolor s.s.), respectively. DOI for the species' Barcode Index Number (BIN): upon paper publication.

## Etymology

The new species is named after Asta Audzijonyte to recognize her earlier contribution in the detection and separation between "species A" and "B" from the European Hediste diversicolor complex.

## Distribution and habitat

Mediterranean, restricted to the Aegean Sea (Greece). Also present in the Black and Caspian Seas. In the Baltic Sea it occurs in sympatry with Hediste diversicolor s.s. and Hediste sp. B1 (Fig. 5.2.B). Intertidal, making burrows in black muddy sand, usually under high salinity waters. Commonly used as bait by anglers

## Remarks

Hediste astae sp. nov. is a member of the European Hediste diversicolor species complex, morphological highly similar to H. diversicolor s.s., Hediste sp. B1 and H. pontii sp. nov. regarding paragnath patterns, chaetae types, parapodial structure and head features. Specimens from this species usually present a low number of segments and have a smaller body, when comparing to the other species of the complex. Though, some specimens, not used in the morphometric analysis, were very large reaching 84 mm in length, 4.369 mm in width and 90 number of segments (e.g., specimen DBUA0002466.02.v05). General PCA and SIMPER data shows considerable morphometric differences, compared to the other species of the complex, and usually smaller proportions. The most significative distinguishing proportions are the width of the worm with / without parapodia, both the distance between the posterior eyes and length of the palps with the width of the head, both the length of the antennae and palps with the length of the antero-dorsal cirri, and the length of the postero-dorsal cirri / antero-dorsal cirri (Table 5.5). A lower number of paragnaths (sometimes down to half), especially in areas III and IV, further distinguishes $H$. astae sp. nov. from $H$. diversicolor s.s., and may be a diagnostic feature, although phenotypic variation is high among the specimens. Low intraspecific COI variation (although it may reach values slightly higher than 3\%) and clear MOTU delineation also separates this species from the remaining described ones from the complex.

### 5.4 Discussion

As observed by Virgilio et al. (2009), and confirmed in this study, the phylogeographic structure of the European Hediste diversicolor comprises at least three deeply divergent allopatric lineages. Excluding the Baltic Sea, where sympatry seems to occur between three different MOTUs (1, 2 and 5), these allopatric lineages include populations from the NE Atlantic and part of the western Mediterranean Sea (MOTU 2, H. diversicolor s.s.); from the Tyrrhenian Sea (MOTU 1, Hediste sp. B1); and lastly from the Caspian and Black Seas with the addition of populations from the northern Aegean Sea (MOTU 5, Hediste astae sp. nov.). In this study, integrative taxonomy supports the addition of a fourth divergent lineage in the Adriatic Sea (MOTU 3, H. pontii sp. nov.) as well. Hediste pontii sp. nov. displayed an independent morphometric cluster in the PCA (Fig. 5.7.A) with a mean inter-cluster variation of $64.80 \%$ (SIMPER), which is far higher than those observed in similar polychaete studies (Ford and Hutchings, 2005; Martin et al., 2017). Molecular evidence for a possible fifth lineage unique to the Ionian Sea (MOTU 4, Hediste sp. B3) was also observed, but additional specimens are needed to complement this information with morphological data. The molecular distances between H. pontii sp. nov. and Hediste sp. B3 are relatively low (4.4\% mean COI divergence, Table 5.4). However, instances of low or even nonexistent COI divergence can also be found in other Hediste species, e.g. between H. diadroma Sato \& Nakashima, 2003 and "form B" of H. atoka Sato \& Nakashima, 2003, both endemic to south of Japan. These sympatric taxa cannot be discriminated using only the COI gene (Tosuji et al., 2019), and the morphology is almost indistinguishable in sexually immature worms (atokes). Yet, their differentiation is still possible but only through the presence of an unique epitokous metamorphosis in H. diadroma and egg sizes (Sato and Nakashima, 2003).

The occurrence of different European lineages can be possibly explained by vicariance events, either caused by the emergence of land barriers, by isolation within glacial refugia or by changes in oceanic currents. These events are known to have triggered allopatric divergence, genetic isolation and speciation in several marine organisms in the region (Wares and Cunningham, 2001; Patarnello et al., 2007; Xavier and Van Soest, 2012). Additionally, divergent selection related to environmental features can lead to genetic differentiation among lineages, promoting local adaptation (Peijnenburg et al., 2004). For example, evidence of different salinity preferences was found between "Species A" and "Species B" of $H$. diversicolor, that could affect their success in competition for habitat in the Baltic regions, despite being both euryhaline (Audzijonyte et al., 2008). The lack of pelagic phase can also facilitate a rapid increase of genetic differentiation between populations (Breton et al., 2003; Virgilio and Abbiati, 2006).

### 5.4.1 Sympatry and possible hybridization in the Baltic Sea

The three sympatric lineages found in the Baltic Sea (Hediste sp. B1, H. diversicolor s.s. and H. astae sp. nov.) constitute an exception compared to the phylogeographic patterns observed in other European regions. Populations of $H$. diversicolors.s. (Species A) and the remaining sympatric lineages (Hediste sp. B1 and H. astae sp. nov. (Species B4) seem to split in the Skagerrak area, but alloenzyme data indicating sympatry between "Species A" and "Species B", or just the presence of Species B, were found in the Danish Ringkøbing fjord (Röhner et al., 1997) and as well in the Weser Estuary (German North Sea coast, Fong and Garthwaite, 1994). Additional sampling in these areas could clarify if sympatry is indeed restricted only to the Skagerrak, Baltic and Kattegat Seas, or if it extends across the North Sea.

Cases of mismatch between alloenzymes and mitochondrial DNA in the Baltic Sea were interpreted by Audzijonyte et al. (2008) as indications of occasional hybridization some generations ago, that has led to mitochondrial introgression among Baltic lineages. This could justify the unusual intraspecific divergence patent in the ITS2 sequences of Hediste sp. B1, especially between the Mediterranean and Swedish populations. The ITS2 data also shows that Mediterranean haplotypes of Hediste sp. B1 displayed a high number of mutation steps, being clearly separated from the Swedish samples, whereas the Swedish haplotypes appear closer to H. diversicolor s.s. instead (Fig. 5.3.B). Furthermore, the presence of phylogenetically related haplotypes in the 28 S locus between lineages from Norway and north of France (H. diversicolor s.s.) and the Swedish population (Hediste sp. B1), suggests that some level of gene flow may have occurred relatively recently. However, the occurrence of shared 28 S haplotypes between different but closely related lineages (sorted by mitochondrial data) is not uncommon (e.g. Vieira et al., 2019). This nuclear locus is known for its reliability in the reconstruction of deep phylogenies (e.g. Weitschek, et al., 2014), but can often fail to discriminate between species in many groups of animals (e.g. Jörger et al., 2012).

Virgilio et al. (2009), hypothesized that Species A (H. diversicolor s.s.), colonized the Baltic from the North European Coasts after the Last Glacial Maximum. Given that the other sympatric lineages are missing from the NE Atlantic, they were probably introduced in the Baltic by human vectors through waterways from other European Seas (Black, Caspian or/and western Mediterranean Sea). An example of this can be seen in the fish Neogobius melanostomus (Pallas, 1814), or the Marenzelleria Mesnil, 1896 polychaete species (Leppäkoski and Olenin, 2000; Sapota, 2004). This was also corroborated by both the current and Audzijonyte et al. (2008) data, where the lack of genetic variability in the Baltic samples (Species B) and especially the low COI haplotype diversity in the Swedish population (MOTU 1, Table 5.3) suggests a recent bottleneck where the population would have been originated by a small
number of colonisers, and did not have time for replenishing the variation through new mutations. Similar low diversity patterns were recorded for the European littoral prawn Palaemon elegans Rathke, 1836, where human vector-derived introductions into the Baltic from the Black Sea were also suggested (Reuschel et al., 2010). Much of the present biological diversity of the Baltic is reported to be of foreign origin, composed of species intentionally or unintentionally moved by humans over intrinsic geographic barriers (Leppäkoski and Olenin, 2000).

As might have been expected, alloenzymes used in Audzijonyte et al. (2008) were not able to distinguish between Hediste sp. B1 and H. astae sp. nov., which corresponds to the species there referred to as "Species B". The phylogenetic clades recovered in their study (BII and BIII corresponding to H . astae sp. nov. and BIV corresponding to Hediste sp. B1) did not indicate a subdivision into another pair of reproductively isolated biological lineages, since in the transition zone heterozygotes at the GOT-2 locus (alloenzyme) were commonly found. Indeed, the molecular data in the present study might not support complete reproductive isolation for the Swedish population (Hediste sp. B1), which also possesses low morphometric differentiation from H. diversicolor s.s. (Figs. 5.7.A; 5.8.A-C). Additional ecological data is needed to reach more definitive conclusions, thereby this lineage remains unnamed in this study. On the other hand, H. astae sp. nov. (northern Aegean Sea) was both genetically and morphometrically very distinct, and no evidence of current hybridization was found in this chapter.

### 5.4.2 New Mediterranean species

The Mediterranean Basin is a known biodiversity hotspot, in which taxa evolved and survived the Pleistocene cold phases, initiated circa 2.8 Ma (Hewitt, 1999; Myers et al., 2000; Schmitt, 2007; Maggs et al., 2008) and even reaching back to the Neogene, initiated circa 20.45 Ma (Husemann et al., 2014). The presence of several closely related Hediste species in this region (Hediste sp. B1, H. pontiisp. nov., Hediste sp. B3 and $H$. astae sp. nov., Figs. 5.2.A, B) could be associated with the alternating glacial and interglacial stages. Assuming that the cytb sequences from Hediste samples collected in Marseille (Breton et al., 2003) were not a result of anthropogenic transport, the Gibraltar strait does not seem to be a contemporary barrier preventing gene flow between populations of $H$. diversicolor s.s. from the western Mediterranean and the northeast Atlantic. However, a geographic split separating Hediste sp. B1 from $H$. diversicolor s.s. appears to exist between the coast of Tuscany/Sardinia (Italy) and the Mediterranean coast of France. Additional samples from the Alboran Sea, Balearic Sea and South of France could be useful to check the occurrence of these lineages at a finer spatial scale.

The ancestral split of eastern Mediterranean lineages (H. pontii sp. nov., Hediste sp. B3 and $H$. astae sp. nov.) may be explained by the refugia in the Balkan Peninsula and Anatolia. There is a possibility that these refugia are not a single homogeneous unit but further sub-structured into a number of geographically small subunits, in which distinct lineages could have evolved geographically separated (Gómez and Lunt, 2007; Schmitt et al., 2021). Furthermore, the low genetic diversity detected in the populations from the Adriatic Sea and the significant negative values found in the Tajima test for H. pontii sp. nov. (MOTU 3, Table 5.2) could be interpreted as an indication of a recent extinction and recolonization in this region. The particular topography and partially enclosed circulation of the Adriatic Sea (Artegiani et al., 1993) may have promoted the genetic isolation of these Adriatic populations. Similarly, the unique haplotypes observed in Hediste sp. B3 could be related to the isolation of the Amvrakikos Gulf and the periodic hypoxic conditions during its formation history (Vasileiadou et al., 2016). Moreover, there is the possibility that unsampled haplotypes occur in the area between Venice and Amvrakikos Lagoon, that hosts several potentially suitable habitats for this species. By comparing the pattern of Hediste to other brackish-water taxa, it could fit the one-direction stepping stone model, as observed in Aphanius fasciatus (Valenciennes, 1821); but with greater divergences due to shorter generations times and the very limited dispersal capability of any form of the life cycle (Langeneck et al., 2021). Thus, eastern Ionian Sea and northern Adriatic Sea haplotypes might well be two extremes of a continuum of unsampled populations.

The emergence of $H$. astae sp. nov., could be attributed to the different paleoclimatic history of the Mediterranean and Black Seas. These two regions have specific environmental conditions (e.g. salinity, sea surface temperature) which may have promoted the selection-driven divergence between the Mediterranean lineages (Peijnenburg et al., 2004, 2006). The colonization history of H. diversicolor in the Caspian Sea is probably recent since it is suspected that the species was introduced from the Black Sea in 1939-1941 (Grigorovich et al., 2003). The samples from the northern Aegean Sea group in the same MOTU (Fig. 5.2.A) and have very low divergence compared to some sequences from the Caspian and Black Seas (Fig. S5.1). It is possible that either Hediste astae sp. nov. has been transferred from the Baltic to the Black and Caspian Sea, or the opposite. I suspect it is primarily a Black Sea species that was secondarily introduced in the Baltic, because of i) the parallel introduction in the Caspian Sea and ii) the fact that this lineage appears closer to the eastern Mediterranean ones (H. pontii and Hediste sp. B3).

### 5.4.3 Intraspecific variation in H . diversicolor s.s.

Hediste diversicolor s.s. comprises a fair number of specimens (117) and sites sampled (9), extending from Portugal to Norway, where a clear genetic or geographic structure is hard to perceive (Figs. 5.2.A, B; S5.3). Although within-clade COI genetic distances (up to $7.5 \%$ ) are not as high as typical values found between congeneric polychaete species, they are much higher than what is usually observed within species, or even within species clades (<3\%, Glasby, 2005; Paiva et al., 2019). High intraspecific COI variability was also observed in $H$. astae sp. nov., but to a far lesser extent (up to $3.5 \%$ ). Mitochondrial genes have faster rates of nucleotide substitution compared to nuclear markers (Hebert et al., 2003a) and it is expected to find higher genetic distances in COI when compared to ITS2 or 28S loci. However, COI distances between Hediste lineages were also within the lower boundaries (max. divergence up to $10.1 \%$ ) when compared to other polychaete studies (> 15\%, Carr et al., 2011; Lobo et al., 2016; Ravara et al., 2017; Sampieri et al., 2021), implying either a recent divergence or a case of an outlier species complex among polychaetes in what concerns patterns of COI variation. Interestingly, ITS2 intra and interspecific distances were very similar to COI, and even had higher intra-specific values than this mitochondrial marker, including when separating the populations from Sweden and the western Mediterranean in MOTU 1 as well (Table 5.4). These findings deviate considerably from the typical pattern of low within-clade variation in DNA barcodes (COI) that has been reported for multiple animal taxa (Hebert et al., 2003b; Costa and Carvalho, 2010). The fact that representative specimens of H. diversicolor s.s. from Portugal and Norway were ascribed respectively to as much as 26 and 7 different BINs, illustrates the uniqueness of this case. Seventy seven haplotypes were recorded in 117 specimens, and, within the relatively small Lima estuary only, as much as 17 BINs have been attributed, 8 exclusive to this site (Table 5.3). The morphometric variation was also the highest among all the analysed lineages ( $17.51 \%$, Table 5.5). Indeed, the unusually high level of variability in COI , and the absence of distinct "barcoding gaps" (Hebert et al., 2003b) within this highly variable lineage, contrasts with typical patterns of aggregation of COI barcodes in well-sorted clusters, which are commonly found, not only in polychaetes, but in other marine invertebrates as well (Sá-Pinto et al., 2008; Varela and Haye, 2012; Delić et al., 2017; Nygren et al., 2018; Desiderato et al., 2019).

A range of possibilities could be proposed at this point to explain these observations, from mutation rates, through drift and selection. For example, Audzijonyte et al. (2008), reported high levels of mtDNA genetic diversity in some Baltic samples within Species $A$, and suggested that long-term isolation and subsequent mixing could have generated that pattern. However, the non-structured genealogy observed within the $H$. diversicolor s.s. clade is not suggestive of such history. Incipient
speciation, may also be an explanation. Svante Martinsson and Christer Erséus have discussed this phenomenon in cryptic Clitellata (Annelida) species, where a more restrictive approach to the species delimitation methods was taken. In particular, the species Fridericia magna Friend, 1899, failed to segregate using nuclear markers, despite having a large mitochondrial genetic variation (up to seven deep divergent lineages were retrieved). Hence the authors concluded it does not constitute a complex of cryptic species (Martinsson et al., 2020), and suggested that each case should be seen as unique instead (Dupuis et al., 2012; Martinsson and Erséus, 2021).

The genetic structure of $H$. diversicolor s.s. within estuaries could also be caused by a combination of stochastic biological and microevolutionary processes (i.e. short larval dispersal, sweepstake recruitment and genetic drift). Other alternative processes could be related to genetic adaptation of populations to environmental stressors. Toxicological studies showed that H. diversicolor can develop local ecotypes tolerant to high concentrations of heavy metals (Bryan and Hummerstone, 1971, 1973). The hypothesis of a genetic control of tolerance was supported by laboratory experiments demonstrating that tolerance to copper and zinc had a heritable component (Grant et al., 1989). Patterns of differentiation in alloenzymes, which could be related to the contamination levels, were found as well by Virgilio et al. (2003).

Currently available data for H. diversicolor s.s. is insufficient to attempt to provide any supported explanation for the patterns observed in this lineage. However, the exceptionality of this case merits detailed examination in future studies, which, due to its peculiarity, would require further and extensive sampling along the NE Atlantic to characterize as comprehensively as possible the genetic variability and the ecological features of this lineage.

### 5.5 Conclusion

Formal description of cryptic species is particularly challenging since it depends largely on molecular data for which there is no established consensus on universal boundaries to delimit species (Westheide and Hass-Cordes, 2001; Moritz and Cicero, 2004; Lefébure et al., 2006; Martinsson and Erséus, 2021). According to the phylogenetic species concept (Mishler \& Theriot 2000), and the mtDNA phylogroups definition (Avise and Walker, 1999), reciprocal monophyly among mitochondrial clusters could be used as a criterion to consider all the five MOTUs of $H$. diversicolor as new species. However, according to the more restrictive biological species concept (Mayr, 1942), the molecular evidence obtained does not clearly support full reproductive isolation between two of the three sympatric lineages in the Baltic Sea (Hediste sp. B1 and H. diversicolor s.s.). Therefore, Hediste sp. B1 was not named in
this study, requiring further reproductive and ecological data to clarify its taxonomic status. Similarly, low genetic distances between $H$. pontii sp. nov. and Hediste sp. B3, as well as lack of sufficient samples to test morphometric differentiation in the latter, also prevented reaching clear conclusions about that MOTU. Hence, the status of Hediste sp. B3 will remain uncertain until further samples can be examined, ideally through quantitative morphometric analysis, and additional data on reproductive and ecological features is available.

Describing and naming these species and similar cryptic complexes is essential, as understanding biodiversity is fundamental to ecological research and key to maintaining a healthy environment, understanding biogeographic patterns, or assess and predict climate change-induced impacts. Furthermore, considering the widespread use of Hediste diversicolor as a model organism or live bait, failing to recognize its true diversity may lead to undesired consequences. Different lineages can have different scope of environmental tolerance, making it difficult to compare between independent studies, and failure to appreciate the various genetically or reproductively isolated lineages will probably affect the sustainability of their harvest.

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Chapter 6
The small polychaete Platynereis dumerilif (Annelida, Nereididae) revealed as a large species complex of ten MOTUs in European marine habitats


#### Abstract

Morphologically similar species are often overlooked, but molecular techniques have been effective in signalling potential hidden diversity, thereby boosting the documentation of unique evolutionary lineages and ecological diversity. Platynereis dumerilijand Platynereis massiliensis are part of a recognized species complex, where only differences in the reproductive biology have been reported so far. Recent studies integrating cytochrome c oxidase subunit I (COI) sequence data with reproductive features and life-history observations, found evidence of additional undescribed diversity for these species in the Mediterranean Sea. Analyses of DNA sequence data (COI, 16S rRNA and D2 region of the 28 S rRNA) of populations of the apparent $P$. dumerilii morphotype, obtained from a broader sampling area along European marine waters and the Macaronesia islands (Madeira, Azores and Canaries), provided compelling evidence for the existence of at least 10 divergent evolutionary lineages. Complementing the genetic data, morphological observations of the better represented lineages revealed two major groups with distinctive paragnath patterns. Other morphological characters, such as differences in the size of the tentacular cirri, number of segments, shape of the parapodia, serration type in the spiniger chaetae and pigmentation patterns, compared between topotypic material and from other locations, were also useful in the diagnosis of two new Platynereis species: P. macaronesiensis sp. nov. widespread in the Macaronesia islands and P. jourdei sp. nov., restricted to the western Mediterranean. The new combination P. agilis is proposed for the Nereis agilis, previously unaccepted, for one of the lineages present both in the NE Atlantic and western Mediterranean. Platynereis dumerilii is also redescribed based on topotypic material. However, the uncertainty in the identity of $P$. massiliensis due to the original incomplete description, and the absence of type and topotypic material prevents its unequivocal assignment to the lineage assumed in this and in other previous studies. The remaining five lineages are represented by only a few small specimens with morphological features poorly preserved, thus were not described in this study. Lastly, two small nereidid species that share the same habitat and can often be misidentified as $P$. dumerilii juveniles, one unique to the Macaronesia islands and the other present both in the Mediterranean and Macaronesia, may be new species or new pseudo cryptic lineages belonging to an existing group. Additional sampling effort and further morphological examination are needed to clarify the status of these lineages.


Keywords: Platynereis, Nereididae, integrative taxonomy, cryptic species

### 6.1 Introduction

An increasing number of studies have been challenging the broadly-distributed or cosmopolitan status of multiple marine benthic invertebrates (e.g. Nygren et al., 2018; Hupało et al., 2019; Sampieri et al., 2021), unveiling instead the occurrence of complexes of cryptic or pseudo cryptic species with more restricted geographic distributions (Struck et al., 2018; Hutchings and Kupriyanova, 2018; Cerca et al., 2020). Morphologically similar species are often overlooked, but molecular techniques have been extremely effective in signalling potential hidden species. Their detection, associated to detailed morphological descriptions, has the ability to boost the documentation of unique evolutionary lineages and associated diversity of ecological attributes (Nygren, 2014; Langeneck et al., 2020; Martin et al., 2020).

Species with no clear and stable morphological differences but with two or more molecular lineages involved., i.e. cryptic species, can sometimes be distinguished by their life history traits. Evidence of this apparent morphological stasis can be exemplified by the annelids Platynereis dumerilii (Audouin \& Milne-Edwards, 1833) and P. massiliensis (Moquin-Tandon, 1869). Based on previous descriptions, these sibling species can mainly be distinguished by reproductive and developmental traits, but also including biology and physiology (Hauenschild, 1951; Schneider et al., 1992; Valvassori et al., 2015). Platynereis dumerilii is gonochoric and semelparous (with a single reproductive event in life), with males and females being attracted to each other by pheromones (Zeeck et al., 1988; Zeeck et al., 1998), transforming into a pelagic epitokous form called heteronereis (Zantke et al., 2014). The larval stage has a planktotrophic development (Zeeck et al., 1988; Fischer and Dorresteijn, 2004). Whereas, P. massiliensis shows no epitokous transformation and is a protandrous hermaphrodite, characterized by egg brooding and lecithotrophic larval stages with a semi-direct development (Schneider et al., 1992).

Platynereis dumerilii is a meso-herbivore species (Ricevuto et al., 2015) first described from the French Atlantic coast (type locality: La Rochelle by Audouin and Milne-Edwards (1833)). It is also reported throughout the Mediterranean inhabiting shallow hard bottoms covered by seaweeds (Giangrande, 1988; Gambi et al., 2000), where it is often misidentified and sympatrically-distributed with P. massiliensis (type locality: Marseille, France by Moquin-Tandon (1869)). Outside the Mediterranean, P. dumerillii has also been reported from other parts of the world such as the Gulf of Mexico (Hartman, 1951), Cuba (Ibarzábal, 2006), North Africa to the Irish Sea and the Isefjord in Denmark (Hartmann-Schroeder, 1996), the Black Sea (Popa et al., 2014) and South Africa (Day, 1967). It is considered a bioindicator of organic pollution (Bellan, 1980), a model species for basic biology and Evo-Devo studies (Fischer and Dorresteijn, 2004; Helm et al., 2015; Özpolat et al., 2021) and can also be used as a model to address various aspects of
acclimatization and adaptation to ocean acidification (Wäge et al., 2017), as it is one of the dominant species present in volcanic $\mathrm{CO}_{2}$ vents (Ricevuto et al., 2015). Although reported in several Mediterranean locations, e.g., Naples (Hauenschild, 1951), Banyuls (Schneider et al., 1992), in Villefranche-sur-Mer as a host of gregarines Lecudina platynereidis (Theodorides, 1969) and in Mediterranean Spain (Coll et al., 2010), P. massiliensis is often overlooked and not included in Mediterranean polychaete check-lists and revisions (e.g. Arvanitidis, 2000; Mikac, 2015; Faulwetter et al., 2017). Based on reproductive biology studies, Valvassori et al. (2015) also found evidence of the occurrence of $P$. massiliensis in the $\mathrm{CO}_{2}$ vents system of the Italian island of Ischia.

Evidence of additional lineages belonging to the $P$. dumerilii were found by Wäge et al. (2017) after integrating cytochrome c oxidase subunit I (COI) sequence data with reproductive biology and lifehistory observations on some selected populations thriving in the vent areas from the Italian islands of Ischia and Vulcano. This analysis highlighted the presence of four distinct Platynereis lineages, two of them primarily present in $\mathrm{CO}_{2}$ vents, and presumably all brooders, and the other two clades dominating the non-acidified sites, appearing to be epitokous free spawners. Based on this genetic data and the fact that there is no evidence of accidental human translocation of $P$. dumerilii to other regions (Read, 2007), it is highly probable that at least some of the 28 previously synonymised species (Read and Fauchald, 2022) with P. dumerilii are actually valid distinct species. These synonyms belong to at least 13 different type localities, ranging from the Atlantic to the Pacific Ocean, nine of which reported for European coasts and might correspond to morphotype variants within the $P$. dumerilii cryptic complex, that were inadequately synonymised. Recently, a South African taxon formerly thought to be P. dumerilii was ascribed to a new species (P. entshonae Kara, Santos, Macdonald \& Simon, 2020) mainly based on molecular data, with principal component analysis scores revealing no separation based on morphological characters (Kara et al., 2020). However, a shorter postero-dorsal tentacular cirri (up to chaetigers 6-8) and an unique bidentate notopodial homogomph falciger, distinguish this species from the original $P$. dumerilii.

To investigate the possible existence of additional hidden Platynereis species within the $P$. dumerili, and attempting to resolve the current existing European complex in this group, a multi-locus approach was used, as well as morphological data, to examine populations from Scandinavia, NE Atlantic, Macaronesia islands (Azores, Madeira and Canaries) and the Western and Eastern Mediterranean Sea.

### 6.2 Material and methods

### 6.2.1 Taxon sampling

Nereidid specimens were collected in several localities along the Atlantic and Mediterranean coasts of Europe, including the Macaronesia islands, and at Mazagan (Morocco). The Atlantic localities include: Norway (Stavanger, Bergen and Trondheim), Sweden (Tjärnö), Great Britain (Plymouth), France (Morlaix Bay, La Rochelle, Arcachon Bay), Portugal (northern beach of Canto Marinho, Azorean islands of Santa Maria, São Miguel and Terceira, Madeira and Porto Santo islands), and Spain (Canary islands of Tenerife, Gran Canaria, El Hierro, La Palma, Lanzarote and Fuerteventura). The Mediterranean localities include: France (Banyuls), Spain (Calpe), Italy [Tuscany area (Calafuria, Antignano, Ardenza, Vada, Livorno and the islands of Montecristo, Pianosa and Elba), Trieste (Adriatic Sea) and Taranto (Ionian Sea)], and Greece (Mazoma and in Crete Island (Paralia Skinaria)). Detailed number of Platynereis and Nereis specimens per locality and respective coordinates can be found in Table 6.1. The specimens were picked among algae in rocky beaches, at low tide or by scuba diving down to 10 meters depth, and fixed in $96 \%$ ethanol. Additionally, specimens from the Arrabida Natural Park (Lisbon, Portugal) were provided by the National Museum of Science and Natural History (MUHNAC, Portugal).

### 6.2.2 Molecular procedures and data mining

DNA sequences of the $5^{\prime}$ end of the mitochondrial cytochrome oxidase subunit I (mtCOI-5P, approximately 658 bp ) were obtained for all the 193 Platynereis specimens. Sequences of 16 S rDNA (approximately 368 bp ) and D2 region of 28 S rDNA (approximately 420 bp ) were also obtained for a representative number of specimens per location. For comparison purposes, molecular data from Pseudonereis sp. (Treadwell, 1923) specimens, collected at Crete island, and 33 small Nereis specimens from the Mediterranean and Macaronesia islands were used as outgroup for all the analysed loci, as well as COI sequences from Perinereis marionii (Audouin \& Milne Edwards, 1833) specimens collected in NW Portugal (Canto Marinho) and Great Britain (Plymouth). Additionally, COI sequences from the four Platynereis lineages obtained by Wäge et al. (2017) and Platynereis sequences from Kara et al. (2019) and (Calosi et al. 2013) were mined from GenBank. Furthermore, COI sequences belonging to the outgroups Neanthes fucata (Savigny, 1822), Nereis zonata (Malmgren, 1867), Nereis pelagica Linnaeus, 1758, Nereis heterocirrata Treadwell, 1931 and Ceratonereis tantaculata Kinberg, 1865 were mined from GenBank and completed the final dataset used for the phylogenetic analysis.

DNA was extracted, amplified, sequenced, and assembled as described in the Chapter 3 of this thesis. Regarding PCR conditions, and primers for the different markers see Chapter 3, Table 3.1. Supplemental Table S6.1 details the sampling locations, public BIN accession numbers and voucher data for the original material. Supplemental Table S6.2 details the GenBank accession numbers for sequences used for comparison purposes from other studies. As only a few parapodia or a small portion of the posterior end were used for the DNA extraction, DNA voucher specimens are deposited at the Research Collection of Marine Invertebrates of the Department of Biology of the University of Aveiro (COBI at DBUA), Portugal, and available for further morphological or molecular study. Specimens which were exhausted in the DNA analysis were assigned only with the Process ID from the BOLD systems (http://v4.boldsystems.org/), corresponding to the ones from northern Greece (MTPD194-20-MTPD20120) and the specimens MTPD191-20 (France, Morlaix) and MTPD144-20 (Spain, Gran Canaria). The specimens from Norway are deposited at NTNU University Museum (Bakken et al. 2021). The full dataset (excluding the sequences from Calosi et al., 2013), which cannot be found in BOLD) and its metadata can be accessed at BOLD Systems under the project "Platynereis Species Complex (DS-MTPD)", which will be publicly available upon this chapter's acceptance for publication in a peer reviewed journal.

### 6.2.3 Phylogenetic analysis

The phylogenetic analyses of the different loci was performed through maximum likelihood (ML) and Bayesian inference (BI). Sequences from the mtDNA COI-5P, rRNA 16S and the D2 region of the rRNA 28 S were aligned and concatenated in MEGA 10.0.5 software (Kumar et al., 2018) with Clustal W (Thompson et al. 1994). MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003) was used to conduct the Bayesian analysis. Best-fit models were selected using the Akaike Information Criterion in the jModeltest software (Guindon and Gascuel, 2003; Darriba et al., 2012). For COI, the Hasegawa-Kishino-Yano gamma distributed rates across sites ( $\mathrm{HKY}+\mathrm{G}$ ) was applied for the first two positions and the General Time Reversible model with gamma distributed rates across sites $(G T R+G)$ for the third position. The latter model was also applied to the remaining loci (16S and 28S-D2). Number of generations was set to 10000 000 , and sample frequency to 500 . Twenty-five percent of the samples were discarded as burn-in (burninfrac $=0.25$ ). The resulting tree files were checked for convergence in the effective sampling sizes (ESSs >200) with Tracer 1.7 software (Rambaut et al., 2018) and then analysed in Figtree 1.4.3 (http://tree.bio.ed.ac.uk/software/figtree/).

Table 6.1. Number of Platynereis and Nereis specimens acquired for this study ( n ), the respective sampling area, code abbreviation for the sampling location and the institution responsible for storing the samples.

| Code | Region | Location | n | Coordinates |  | Storing Institution |
| :---: | :---: | :---: | :---: | :---: | :---: | :---: |
|  |  |  |  | Latitude | Longitude |  |
| PTA | Northeast Atlantic | Portugal, Arrabida Natural Park | 16 | $38^{\circ} 26.22^{\prime} \mathrm{N}$ | $9^{\circ} 3.78{ }^{\text {' W }}$ | MUHNAC |
| PTC | Northeast Atlantic | Portugal, Canto Marinho | 15 | $41^{\circ} 44.22^{\prime} \mathrm{N}$ | $8^{\circ} 52.56{ }^{\prime} \mathrm{W}$ |  |
| FRA | Northeast Atlantic | France, Arcachon Bay | 1 | $44^{\circ} 39.72^{\prime} \mathrm{N}$ | $1^{\circ} 9.18^{\prime} \mathrm{W}$ |  |
| FRR | Northeast Atlantic | France, La Rochelle | 17 | $46^{\circ} 8.79^{\prime} \mathrm{N}$ | $1^{\circ} 12.6{ }^{\text {W W }}$ | DBUA |
| FRM | Northeast Atlantic | France, Morlaix Bay | 2 | $48^{\circ} 43.8^{\prime} \mathrm{N}$ | $3^{\circ} 59.16^{\prime} \mathrm{W}$ | DBUA |
| GBP | Northeast Atlantic | Great Britain, Plymouth | 1 | $50^{\circ} 21.59^{\prime} \mathrm{N}$ | $4^{\circ} 9.03{ }^{\text {W }}$ |  |
| SWT | Kattegat Sea, North European Coast | Sweden, Tjärnö-Saltö canal | 11 | $58^{\circ} 52 \cdot 26.4$ " N | $11^{\circ} 08^{\prime} 42.0^{\prime \prime} \mathrm{E}$ |  |
| NOT | Norway Sea | Norway, Trondheim | 1 | $63^{\circ} 26^{\prime} 09.6^{\prime \prime} \mathrm{N}$ | $10^{\circ} 29^{\prime} 56.4$ " E |  |
| NOB | North European Sea | Norway, Bergen | 2 | $60^{\circ} 23.76{ }^{\prime} \mathrm{N}$ | $5^{\circ} 19.5{ }^{\text { }}$ E | NTNU |
| SPC | Balearic Sea, Western Mediterranean | Spain, Calpe | 19 | $38^{\circ} 38.3966^{\prime} \mathrm{N}$ | $0^{\circ} 3.5{ }^{\prime} \mathrm{E}$ |  |
| FRB | Western Mediterranean | France, Banyuls-sur-Mer | 3 | $42^{\circ} 28.8983{ }^{\prime} \mathrm{N}$ | $3^{\circ} 8.005^{\prime} \mathrm{E}$ | DBUA |
| ITT | Tyrrhenian Sea, Western Mediterranean | Italy, Tuscany Area | 48 | $43^{\circ} 32.765^{\prime} \mathrm{N}$ | $10^{\circ} 18.1433^{\prime} \mathrm{E}$ |  |
| ITR | Adriatic Sea, Eastern Mediterranean | Italy, Trieste | 1 | $45^{\circ} 38.86{ }^{\prime} \mathrm{N}$ | $13^{\circ} 45.54833^{\prime} \mathrm{E}$ |  |
| ITTA | Ionian Sea, Eastern Mediterranean | Italy, Taranto | 1 | $40^{\circ} 27.9833^{\prime} \mathrm{N}$ | $17^{\circ} 14.3333^{\prime} \mathrm{E}$ | DBUA |
| GRA | Ionian Sea, Eastern Mediterranean | Greece, Amvrakikos Lagoon | 8 | $39^{\circ} 02^{\prime} 45.6^{\prime \prime N}$ | $20^{\circ} 46^{\prime} 15.6^{\prime \prime} \mathrm{E}$ | DNA only |
| GRC | Sea of Crete, Eastern Mediterranean | Greece, Crete, Skinaria Beach | 14 | $35^{\circ} 9.96{ }^{\prime} \mathrm{N}$ | $24^{\circ} 25.2833^{\prime} \mathrm{E}$ | DBUA |
| TER | Macaronesia islands, Northeast Atlantic | Portugal, Azores, Terceira | 3 | $38^{\circ} 41^{\prime} \mathrm{N}$ | $27^{\circ} 3.4517^{\prime} \mathrm{W}$ |  |
| SMA | Macaronesia islands, Northeast Atlantic | Portugal, Azores, Santa Maria | 3 | $36^{\circ} 56.995{ }^{\prime} \mathrm{N}$ | $25^{\circ} 5.7^{\prime} \mathrm{W}$ |  |
| SMI | Macaronesia islands, Northeast Atlantic | Portugal, Azores, Santa Miguel | 3 | $37^{\circ} 54{ }^{\prime} \mathrm{N}$ | $25^{\circ} 49.08^{\prime} \mathrm{W}$ |  |
| MA | Macaronesia islands, Northeast Atlantic | Portugal, Madeira, Funchal | 4 | $32^{\circ} 38.7667{ }^{\prime} \mathrm{N}$ | $16^{\circ} 49.45{ }^{\prime} \mathrm{W}$ |  |
| PS | Macaronesia islands, Northeast Atlantic | Portugal, Madeira, Porto Santo | 1 | $33^{\circ} 4.38^{\prime} \mathrm{N}$ | $16^{\circ} 17.76^{\prime} \mathrm{W}$ |  |
| LP | Macaronesia islands, Northeast Atlantic | Spain, Canaries, La Palma | 9 | $28^{\circ} 48.3296{ }^{\prime} \mathrm{N}$ | $17^{\circ} 45.6932^{\prime} \mathrm{W}$ |  |
| EH | Macaronesia islands, Northeast Atlantic | Spain, Canaries, El Hierro | 2 | $27^{\circ} 47.085{ }^{\prime} \mathrm{N}$ | $18^{\circ} 0.695{ }^{\text {W }}$ | DBUA |
| TE | Macaronesia islands, Northeast Atlantic | Spain, Canaries, Tenerife | 3 3 | $\begin{aligned} & 28^{\circ} 25.1142^{\prime} \mathrm{N} \\ & 28^{\circ} 34.2854^{\prime} \mathrm{N} \end{aligned}$ | $\begin{aligned} & 16^{\circ} 32.9752^{\prime} \mathrm{W} \\ & 16^{\circ} 20.0175^{\prime} \mathrm{W} \end{aligned}$ |  |
| GC | Macaronesia islands, Northeast Atlantic | Spain, Canaries, Gran Canaria | 13 | $27^{\circ} 59.108^{\prime N}$ | $15^{\circ} 22.5493{ }^{\prime} \mathrm{W}$ |  |
| FV | Macaronesia islands, Northeast Atlantic | Spain, Canaries, Fuerteventura | 8 | $28^{\circ} 3.995^{\prime} \mathrm{N}$ | $14^{\circ} 30.415^{\prime} \mathrm{W}$ |  |
| LA | Macaronesia islands, Northeast Atlantic | Spain, Canaries, Lanzarote | 6 | $29^{\circ} 13.0883{ }^{\prime} \mathrm{N}$ | $13^{\circ} 26.5067{ }^{\prime} \mathrm{W}$ |  |
| MOR | Northwest African coast | Morocco, Mazagan | 3 | $3315.8417^{\prime} \mathrm{N}$ | 830.64331 W |  |

The final version of the tree was edited with the software Inkscape 0.92.3 (https://www.inkscape.org). Maximum Likelihood phylogenies were performed in MEGA 10.0.5 with 1000 bootstrap runs with the GTR model with gamma distributed rates across sites (GTR +G) for the concatenated dataset. A maximum likelihood amino acid radiation tree was also performed in MEGA 10.0.5, using the Jones-Taylor-Thornton model with equal rates across sites (JTT) for all the COI Platynereis lineages to visualize amino acid differences between lineages. The BI tree was displayed in the results with the addition of the ML support values if a similar topology is found.

### 6.2.4 MOTU clustering

To depict Molecular Operational Taxonomic Units (MOTUs), three delineation methods to the concatenated alignment were applied based on ABDG (Puillandre et al., 2012), bPTP (Zhang et al., 2013) and GYMC (Fujisawa and Barraclough, 2013) as detailed in Chapter 3 of this thesis. BEAST 2.4.6 (Bouckaert et al., 2014) was used to generate the Bayesian ultrametric tree for the GMYC based on AIC criteria, with GTR model and equal rates across sites. The Barcode Index Number (BIN) was applied as well for the COI, which makes use of the Refined Single Linkage (RESL) algorithm implemented in BOLD (Ratnasingham and Hebert, 2013), exclusive to this locus. A final consensus MOTU was chosen using the majority rule (i.e. most common number of MOTUs across different delimitation methods and in case of draw, MOTUs were separated if more than $3.5 \%$ COI genetic divergence was present).

### 6.2.5 Genetic distances, diversity and structure

The mean genetic distances (Kimura-2-parameters, K2P) within and between MOTUs were calculated in MEGA 10.0.5. Haplotype networks were made for the original sequences through the PopART software (Leigh and Bryant, 2015) using the method of Templeton, Crandall and Sing (TCS, Clement et al., 2002) to evaluate the relationship between the haplotypes and their geographical distribution. Indices of genetic diversity, namely number of haplotypes (h), haplotype diversity (hd), polymorphic sites (S), nucleotide diversity (п), Fu \& Li D and Tajima D statistical tests, were estimated based on COI for each MOTU using DNASP 5.10 (Librado and Rozas 2009).

### 6.2.6 Morphological analysis

Morphological observations were carried out with an Olympus stereo microscope equipped with a camera lucida for line drawings. Stereo microscope images were taken with a Canon EOS1100D camera. Compound microscope images of parapodia and chaetae were obtained with a Zeiss Axioplan 2 imaging light microscope (Carl Zeiss, Oberkochen, Germany), equipped with a DP70 Olympus camera (Olympus Corp., Tokyo, Japan), after mounting the parapodia on a slide preparation using Aquamount (Gurr) liquid. The software Inkscape 0.92.3 (https://www.inkscape.org) was used to create the final images for the drawings of the parapodia, pharynx and anterior part of the worm's body.

Parapodial and chaetal terminology in the taxonomic section follows (Bakken and Wilson, 2005) with the modifications made by (Villalobos-Guerrero and Bakken, 2018). Pharynx paragnath terminology follows (Bakken et al., 2009).

### 6.3 Results

### 6.3.1 Phylogenetic reconstruction

The BI tree (Fig.6.1.A) is split into two major clades. The first clade (Clade A, including MOTUs 1-10) generally complies with the description of the $P$. dumerilij pseudo cryptic complex, while the second clade includes $P$. entshonae, a sibling species of $P$. dumerillii distinguished mainly at the molecular level (Kara et al., 2020), a group of Nereis sp. that share the same habitat and some morphological similarities with juveniles of Platynereis species (Clade B, including MOTUs 11-15), and all the outgroup species included in the analysis. Clade $A$ is further divided into three sub-clades (A1: MOTU 1; A2: MOTUs 2-6, A3: MOTUs 7-10) based on close genetic distances, topology, information regarding the reproductive biology and paragnath variations.

A total of 15 unique consensus MOTUs were obtained, four of which are singletons with only one sequence available (MOTUs $8,13,14$ and 15). The remaining MOTUs correspond to monophyletic clades with low divergence ( $\mathrm{COI}<3 \%, \mathrm{~K} 2 \mathrm{P}$ ) and are collapsed in Figure 6.1.A. Apart from the outgroups, additional MOTUs from other studies are also represented in the tree (GB1-4). From these, GB2 and GB3 (included in Clade A3) present low support values ( $<0.85$ ) and lack well-defined bifurcated clades, and might belong to MOTU 9. However, morphological analysis would need to be done to confirm this. MOTU GB1 seems to be a new Platynereis lineage from South Africa and MOTU GB4 is the recently described species P. entshonae. In general, the Macaronesia (particularly the Canary Islands) and the whole

Mediterranean Sea appear to be a cryptic hotspot, with several localities with more than two sympatric MOTU's (see map on Fig. 6.1.B).


Fig. 6.1. (A) Phylogenetic tree reconstructed using Bayesian inference based on concatenated COI, 16 S and 28S-D2 sequences, with information regarding the different MOTU delineation methods. BINs were used only for COI. Outgroups ("OUTG" and "GB"), with the exception of Pseudonereis sp., only have COI sequences. Collapsed clades have less than $3.5 \%$ genetic divergence. Only the bootstrap values over 0.85 Bl and 85 ML support are shown. Each different consensus MOTU is represented by the respective number, with the different colours corresponding to the respective geographic distribution. (B) Geographic distribution in Europe for the 15 retrieved MOTUs. (C) Maximum likelihood amino acid (a.a.) radiation tree based on COI sequences belonging to MOTUs 1-10 (clade A). Abbreviations for the geographical regions as seen in Table 6.1.

Focusing only on Clade A (P. dumerilij complex), three MOTUs are unique to the Macaronesia (MOTUs 5, 7 and 8) of which one occurs exclusively in Porto Santo Island (MOTU 8) and two sympatric ones are present in the Gran Canaria and Lanzarote islands alone (MOTUs 5 and 7). Additionally, three lineages are present exclusively in the Mediterranean (MOTU 1 and MOTU 6 in the western part and MOTU 3 in the Eastern part of the Sea) of which MOTU 1 was only found at Banyuls (France) and MOTU 3 present only in the Ionian Sea (Northern Greece) so far. Three sympatric MOTUs were identified in the southeast of Spain (MOTUs 4, 6 and 10) and in the Northern Tyrrhenian Sea (MOTUs 4, 6 and 9). Four different MOTU's were found in the NE Atlantic, three of them shared with the Mediterranean (MOTUs 4, 9 and 10) and one exclusive to this part of the European coastline (MOTU 2). The specimens from the type locality of $P$. dumerilii species (La Rochelle, France) grouped all within MOTU 4. This particular lineage is the most widespread and easy to find among all the mainland samples, being present both in NE Atlantic and the whole Mediterranean Sea, while MOTU 7 was the most widespread and abundant one among the Macaronesia islands.

A radiation amino acid tree based on COI sequences from the 10 retrieved Platynereis' MOTUs was also able to separate the three main sub-clades (A1, A2 and A3) found in the BI tree, with MOTUs 1, 2,5 and 7 not sharing the same amino acids with any of the remaining lineages (Fig. 6.1.C).

### 6.3.2 Genetic distances

The global mean genetic distances (K2P) for the clades $A$ and $B$ can be found in Table 6.2. Regarding only the Platynereis complex (clade A), the mean intra-MOTU distance was $0.2(0-3.5) \%$ for COI and $0.3(0-1.4) \%$ for 16S, while the average inter-MOTU distances were $19.4(4.4-26.6) \%$ and 6.2 (1.5-9.9)\% respectively. For the 28S-D2 region, it ranged between $0.2(0-1.4) \%$ and 1.1 ( 0.1 3.9)\% for intra- and inter-MOTU divergence, respectively. Detailed mean genetic distances for the three genetic markers between each MOTU can be found in Table S3. When comparing between major clade A and B, the maximum interspecific genetic distances are significantly higher in all loci, especially for 16 S and 28SD2. In this scenario, maximum divergences of $32.6 \%$ COI, $35.7 \% 16 S$ and $36.9 \%$ 28SD2 were recorded, as opposed to the $26.9 \%, 9.9 \%$ and $3.9 \%$ found only within clade A, based on the same respective loci.

Table 6.2. Mean intra and inter-MOTU genetic distances (K2P) for the three analysed markers (COI, 16S, 28S-D2), either only for the 10 MOTUs corresponding to Clade A (Fig. 6.1A.), or using the additional 5 MOTUs from Clade B (Fig. 6.1.A).

|  | Marker | Minimum Distance <br> $\mathbf{( \% )}$ | Mean Distance <br> $(\%)$ | Maximum distance <br> (\%) |
| :--- | :--- | :--- | :--- | :--- |
| Within All | COI | 0 | 1.4 | 5.3 |
| MOTUs | 16S | 0 | 0.4 | 2.0 |
|  | 28S-D2 | 0 | 0.4 | 3.1 |
| Between All | COI | 4.4 | 22.6 | 32.6 |
| MOTUs | 16S | 0.9 | 19.1 | 35.7 |
| Within | COI | 0 | 16.6 | 36.9 |
| MOTUs | 16S | 0 | 0.2 | 3.5 |
| 1-10 | 28S-D2 | 0 | 0.3 | 1.4 |
| Between | COI | 4.4 | 0.2 | 1.4 |
| MOTUs | 16S | 1.5 | 19.4 | 26.6 |
| $1-10$ | 28S-D2 | 0.1 | 6.2 | 9.9 |

### 6.3.3 Haplotype networks and diversity

All COI (Fig. 6.2.A) and 16S (Fig. 6.2.C) haplotypes were completely sorted among MOTUs, i.e. no haplotypes were shared among more than one MOTU. However, some MOTUs (4, 5 and 6; 12 and 13; 14 and 15) shared the same haplotype in the 28S-D2 loci (Fig. 6.2.B). The 28S-D2 network provided two major groups segregating clade B as seen in the BI, with more than 90 mutations separating it from clade A. The COI network also revealed geographically structured populations within MOTU 9 and 10, corresponding to the 5 distinct BINs shown in the BI (Fig. 6.1.A), except the populations from North of France and south of Great Britain that did not split into separate BINs in MOTU 10. By contrast not all populations from different Atlantic islands were completely sorted in MOTU 7, with the presence of shared haplotypes between all islands, except Gran Canaria and La Palma. Further geographic sorting in the COI network can also be identified within MOTU 4 regarding populations from the western and eastern Mediterranean Sea.

For the most sampled MOTUs (4, 6, 7, 9, 10), COI haplotype diversity is relatively high (Hd > 0.89 to 0.99 , Table 6.3), except for MOTU 6 ( $\mathrm{Hd}: 0.65$ ). The latter, together with MOTU 4 , are the only cases with a significant Tajima D and Fu and Li's D tests, where the negative values indicate possible population expansion after a recent bottleneck or the occurrence of selective sweeps, with the neutral model of nucleotide substitutions accepted for the remaining MOTUs.


Fig. 6.2. Haplotypes networks based on $\mathrm{COI}(\mathrm{A}), 28 \mathrm{~S}-\mathrm{D} 2(\mathrm{~B})$ and $16 \mathrm{~S}(\mathrm{C})$ for all the 15 MOTUs based on the original Platynereis and Nereis data, and Pseudonereis sp. as outgroup. Each haplotype is represented by a circle and number of haplotypes are according to the displayed scale. Colours indicate the geographic location of the haplotype. Numbers correspond to the number of mutational steps between haplotypes. Lines without numbers means only one mutation between haplotypes. Country abbreviations: PT, Portugal; SP, Spain; FR, France; GB, Great Britain; NO, Norway; SW, Sweden; IT, Italy; GR, Greece; MOR, Morocco.

### 6.3.4 Platynereis dumerilii pseudo-cryptic complex (clade A): Morphological findings

A compilation of European species currently considered as synonyms of $P$. dumerilii either close to places from the type locality or same regions of the Platynereis specimens sampled in this study, with their main distinctive morphological traits based on the original descriptions is given in Table 6.4. Platynereis nadiae Abbiati \& Castelli, 1992 was included in this table, despite being currently accepted by WoRMS, given the similarity of this species' description with juveniles from Platynereis dumerili. A similar summary was made for the ten different Platynereis MOTUs analysed in this study (Table 6.5). Two new species are described in the taxonomic section, below, corresponding to the MOTUs 6 (Figs. 6.3.A-E; 6.4.A-E) and MOTU 7 (Figs. 6.5.A-D; 6.6.A-D). Additionally, the previous synonymized name

Nereis agilis Keferstein, 1862 is reinstated as Platynereis agilis comb. nov. for MOTU 10 and redescribed (Figs. 6.7.A-D; 6.8.A-D). Amended diagnosis of P. dumerilli (MOTU 4) and P. c.f. massiliensis (MOTU 9) are also provided, using the specimens studied herein (Figs. 6.9.A-D; 6.10.A-E and Figs. 6.11.A-F; 6.12.AE , respectively).

Table 6.3. Indices of genetic diversity estimated for each MOTU, based on COI. Number of sequences (n); nucleotide diversity (tt), number of haplotypes (h), haplotype diversity (Hd) and number of variables sites (S). Region abbreviations as seen in Table 6.1

|  | Region | N | h | Hd | S | t | Fu and Li's D* | $\begin{gathered} \text { Tajima's } \\ \text { D } \end{gathered}$ |
| :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: |
| MOTU 1 | FRB | 2 | 2 | 1.00 | 1 | 0.00152 | - | - |
| MOTU 2 | SWT, NOB, PTA | 4 | 4 | 1.00 | 19 | 0.0165 | $\begin{gathered} 0.46, \\ P>0.10 \end{gathered}$ | $\begin{gathered} 0.46, \\ P>0.10 \end{gathered}$ |
| MOTU 3 | GRA | 6 | 3 | 0,60 | 3 | 0.00152 | $\begin{gathered} -1.26013, \\ P>0.10 \end{gathered}$ | $\begin{gathered} -1.23311 \\ P>0.10 \end{gathered}$ |
| MOTU 4 | PTC, SPC, GC, LA, FRR, FRA, NOT, SWT, ITT, ITTR, ITTA, GRA, GRC | 62 | 32 | 0.89 | 66 | 0.00826 | $\begin{gathered} 2.99788, \\ P<0.05 \end{gathered}$ | $\begin{gathered} \text { 2.11036, } \\ P<0.05 \end{gathered}$ |
| MOTU 5 | LP, GC | 2 | 2 | 1.00 | 16 | 0.02432 | - | - |
| MOTU 6 | FRB, SPC, ITT | 30 | 13 | 0.65 | 27 | 0.00364 | $\begin{gathered} \text { 3.91387, } \\ P<0.02 \end{gathered}$ | $\begin{gathered} 2.37595 \\ P<0.01 \end{gathered}$ |
| MOTU 7 | TER, SMA, LP, EH, TE, GC, FV, LA, MA, MOR | 45 | 32 | 0.97 | 53 | 0.01949 | $\begin{gathered} -1.43196, \\ P>0.10 \end{gathered}$ | $\begin{aligned} & -0.05629 \\ & P>0.10 \end{aligned}$ |
| MOTU 8 | PS | 1 | 1 | - | - | - | - | - |
| MOTU 9 | PTC, MOR, ITT | 18 | 13 | 0.95 | 39 | 0,00154 | $\begin{gathered} -0,77408 \\ P>0.10 \end{gathered}$ | $\begin{gathered} -0,91319 \\ P>0.10 \end{gathered}$ |
| MOTU 10 | GBP, PTA, SPC, FRM | 23 | 13 | 0.81 | 26 | 0,00673 | $\begin{gathered} -1,26506 \\ P>0.10 \end{gathered}$ | $\begin{gathered} -0,91717 \\ P>0.10 \end{gathered}$ |
| MOTU 11 | LP, EH, GC, SPC, GRC | 19 | 16 | 0.98 | 59 | 0.02870 | $\begin{aligned} & 0.06698, \\ & P>0.10 \end{aligned}$ | $\begin{aligned} & 0.26444, \\ & P>0.10 \end{aligned}$ |
| MOTU 12 | LP, GC, FV, SMI | 7 | 7 | 1.00 | 39 | 0,02345 | $\begin{gathered} -0.63865, \\ P>0.10 \end{gathered}$ | $\begin{aligned} & -0.44925, \\ & P>0.10 \end{aligned}$ |
| MOTU 13 | LP | 1 | 1 | - | - | - | - | - |
| MOTU 14 | GRC | 1 | 1 | - | - | - | - | - |
| MOTU 15 | GRC | 1 | 1 | - | - | - | - | - |

The remaining MOTUs are represented by a smaller number of specimens in suboptimal conditions and thus are not fully described here. However, they seem to share the same morphological features from the respective phylogenetically nearest neighbours (see Fig. 6.1.A), except for a few different characteristics shown by MOTUs 2 and 5 . In MOTU 2 the morphology of parapodia and tentacular cirri is closer to MOTU 10 instead of the remaining MOTUs from clade A2, while in MOTU 5 the tentacular cirri are similar to MOTU 9 (Table 6.5). Specimens from MOTU 3 were very small with the entire worm being used for DNA extraction, thus only a very preliminary morphological analysis was done. MOTU 1 seems to be morphologically similar to MOTU 4 (Table 6.4) and seems to share a similar pigmentation as the Livorno population from MOTU 9.

All the analysed MOTUs from clade A seem to share the typical dorsal and ventral parapodial cirri variation described in the topotypic material ( $P$. dumerilii), with the dorsal cirrus being at least twice the length of the dorsal ligule, whereas the ventral cirrus is short and may reach half the size of the ventral ligule for the mid- body region. Differences in the size of the tentacular cirri, paragnath patterns, number of segments and serration type in the spiniger chaetae contributed for the main differences between lineages.

Pigmentation does not seem to be always a useful character since it can sometimes be absent in very small specimens or completely lost upon fixation in ethanol. However, generally speaking, it is possible to identify a designated MOTU based on the pigmentation patterns as seen in the respective figures, except between MOTUs 4 and 6 where some specimens might share similar pigmentation density and pattern. Another apparently relevant morphological character is the number of teeth in the jaws of adult specimens, considering the stability of the reported numbers, either 8 or 11 (Table 6.4). Due to the difficulty of dissecting small organisms such as Platynereis specimens, the pharynx and jaws of the studied specimens could only be examined in a few worms. Nevertheless, generally, MOTUs from clade A3 seem to have a higher number of teeth, between 7-8 against the 5-6 from clade A2 (Table 6.5).

### 6.3.5 Nereis spp. (clade B): Morphological findings

Five additional MOTUs, belonging to small sized nereidid specimens, were retrieved and may be confused with small juvenile specimens of other Platynereis species, if the pharynx is not possible to be dissected and observed, which often happens in very small specimens. Apart from the genetic evidence (Fig. 6.1A, Table 6.1) and considering morphological features alone (particularly, the chaetae types, the tentacular cirri and pharynx paragnaths), it is clear that MOTUs 11, 12 and 13 belong to a different genus, most probably Nereis Linnaeus, 1758.

Compared to descriptions and figures in Fauvel (1923) and Fauna Iberica (Núñez, 2004), MOTU 11 (Fig. 6.14), present in the Mediterranean and Macaronesia islands, is morphologically close to Nereis zonata Malmgren, 1867, with similar proportions between the antennae in relation to the palps and very short tentacular cirri. However, some differences in the paragnath patterns are found (see Núñez, 2004). The latter species may also display high degree of variation in the paragnath arrangements, some of which may be similar to the ones described for Neanthes fucata (Núñez, 2004; Gravina et al., 2016). This was also observed in the collected specimens from MOTU 11. However, parapodia from the posterior part do not have the characteristic leaf-like dorsal ligules found in $N$. fucata or in some other species belonging to Neanthes. Furthermore, the presence of homogomph falcigers (Fig. 6.14G), which are lacking in Neanthes, resemble Nereis species instead.

Based on photos deposited in BOLD (Zhou et al., 2010), MOTUs 12-13, unique to the Macaronesia showed some resemblance with specimens identified as Nereis heterocirrata Treadwell, 1931, grouping very closely in the phylogenetic tree as well (Fig. 6.1.A). The most noticeable feature of the latter species, however, relates to the anteroventral cirri, which is much more swollen than the remaining tentacular cirri (Treadwell 1931), but this feature is not observed in the specimens from this study (Fig. 6.13). This species is only reported in Eastern Asia and no reports in the Atlantic were found so far (Read \& Fauchald 2021, https://www.marinespecies.org/polychaeta/aphia.php? $\mathrm{p}=$ taxdetails\&id=329658).

The outgroups from GenBank identified as $N$. fucata grouped with samples from this study identified as Perinereis marionii (Audouin \& Milne Edwards, 1833). The latter species possess a characteristic paragnath pattern in the oral ring with a dorso-ventral continuous band composed of multiple small paragnaths and an irregular line of larger paragnaths in the anterior margin, especially in area V with a large conical paragnath with triangular shape and areas VI with a small transverse bar. Parapodia is also characterized with the presence of a very long dorsal ligule in the posterior parapodia (Núñez, 2004; see photo of the specimen from P. marionii collected in this study in the supplemental material Fig. S6.1). This result strongly suggest misidentifications in the genetic databases for this group.

Table 6.4. List of currently unaccepted European synonyms of $P$. dumerilii based on WoRMS database, with their main distinctive morphological traits based on the original descriptions. Platynereis nadiae was included, given the similarity of this species' description with juveniles from Platynereis dumerili.

| Species (original comb.) | Type locality | Live colour | Length (mm) / NS | Number of jaw teeth | Paragnaths (dorsal view) | Pairs of tentacular cirri | Parapodia (mid-body) | Reproduction | Chaetae |
| :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: |
| Nereis <br> dumerilij <br>  <br> Milne-Edwards, <br> 1833 | La <br> Rochelle, <br> North <br> France | Yellowish with brown spots at the basis of parapodia | 80 / 80 | $11 ?$ | Present in the maxillary ring. Present in double rows in the oral ring | 1 long ( $1 / 5$ of the body length) and 3 short | Dorsal cirri twice the length of dorsal ligule; Ventral cirri much shorter than ventral ligule. | Gonochoristic + <br> heteronereis | Homogomph falcigers |
| Nereis <br> zostericola <br> Örsted, 1843 | Hellebæk, Denmark | Yellowish with many brownish spots | $50 / 70$ | ? | ? | 1 long, reaching chaetiger 9, and 3 short | Notopodia with 2 ligules, $1 / 2$ the length of neuropodia. Neuropodia with 3 ligules. | ? | Homogomph spinigers and heterogomph falcigers |
| Nereis taurica Grube, 1850 | Crimea, Black Sea | White, with seethrough blood vessels | $\begin{gathered} 38 / 73- \\ 74 \end{gathered}$ | 4-5 | ? | 1 long, reaching chaetiger 9 or 10 , and 3 short | Notopodia longer than neuropodia. Ligules shaped as an irregular triangle | ? | ? |
| Nereis agilis <br> Keferstein, $1862$ | St. Vaast, <br> North <br> France | Pale with scattered brown and red spots | $\begin{gathered} 10-15 / \\ 40-42 \end{gathered}$ | 8 | ? | 1 long and 3 short | Four ligules are noticeable, although the third is very short | Simultaneous hermaphrodite with different gonads in different parts of the body | ? |


| Species <br> (original comb.) | Type locality | Live colour | Length (mm) / NS | Number of jaw teeth | Paragnaths (dorsal view) | Pairs of tentacular cirri | Parapodia (mid-body) | Reproduction | Chaetae |
| :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: |
| Nereis megodon <br> Quatrefages, 1866 | Bretagne, <br> North of France | ? | $\begin{gathered} 15-20 / \\ 60-80 \end{gathered}$ | 11 | ? | Not clear, but most likely 1 long and 3 short | Dorsal cirri $1 / 3$ longer than dorsal ligule; Ventral cirri shorter than ventral ligule. | Gonochoristic + no heteronereis? | Two chaetaebearing humps |
| Nereis peritonealis Claparède, 1868 | Gulf of <br> Naples, Italy | More or less colourless, with pigmented violet cells in the peritoneum | 45 / 65 | 8-9 <br> (curved <br> jaws) | Absent in the maxillary ring. No further data. | 1 long and 3 short | Dorsal ligule slightly inflated. Dorsal cirri longer than do dorsal ligule | Gonochoristic? <br> Heteronereis? | ? |
| Nereis <br> massiliensis <br> Moquin-Tandon, $1869$ | Marseille, <br> South <br> France | Greenish-brown, mottled with wine-purple | $\begin{gathered} 40-50 / \\ 60-70 \end{gathered}$ | 12 | Absent | 2 long (dorsal ones; 2 short (ventral ones) | Parapodia similar to those of Nereis bilineata | Simultaneous hermaphrodite with commingled sexual products. | ? |
| Platynereis <br> nadiae Abbiati <br> \& Castelli, 1992 | Capraia <br> Island, <br> Tyrrhenian <br> Sea | Pale with large, scattered purple spots (A. Castelli pers. comm.) | 5-10 / 43 | 7-8 | Absent in the maxillary ring. Single rows in the oral ring. | 4 short, irregularly annulate | With variable morphology along the boby. Dorsal cirri long and tapered in posterior segments, with indistinct annulation (similar to tentacular cirri). | ? | Heterogomph and homogomph falcigers and spinigers |

(Table 6.4. Continuation)

Table 6.5. Summary of the main morphological observations for the 10 different Platynereis MOTUs analysed in this study. Species in bold correspond to the ones described in the taxonomic section.

| Species | Distribution | Pigmentation | Length (mm) / NS | Number of jaw teeth | Paragnaths (distinct characteristics only) | Posterodorsal tentacular cirri | Anterior parapodia dorsal ligule | Spiniger chaetae | Reproduction |
| :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: |
| MOTU 1 Platynereis sp. | Mediterranean <br> Sea (Banyuls, France) | Similar to MOTU $4$ | $6 / 40$ | ? | ? | Very long (similar to MOTU 4) | Similar to <br> MOTU 4 | ? | Hermaphrodite, egg brooding, lecithotrophic larval stages |
| MOTU 2 - <br> Platynereis sp. | NE Atlantic (Scandinavia, Portugal) | Absent | $\begin{gathered} 12-15 / \\ 35-45 \end{gathered}$ | ? | ? | Reaching chaetiger 15 | Similar to <br> MOTU 10 | ? | Unknown. |
| MOTU 3 - <br> Platynereis sp. | Ionian Sea (Greece) | $?$ | Very <br> small | ? | ? | ? | ? | ? | Unknown. |
| MOTU 4 - <br> P. dumerilifis.s. | European <br> Atlantic and Mediterranean coast Type locality: La Rochelle, France. | Small dots covering most of the anterior region (Fig. 6.9.A). | $\begin{gathered} 1.5-20 / \\ 30-80 \end{gathered}$ | 5-6 | Area II - double rows; VI - double rows; VII-VIII continuous band of double rows (Fig. 6.9.A-B). | Reaching chaetiger 912 (Fig. 6.9.A). | Rounded, much shorter than in midbody parapodia (Fig. 6.9.C-D). | Lightly serrated (Fig. 6.10.B) | Gonochoric, heteronereis, larval stage with planktotrophic development |
| MOTU 5 - <br> Platynereis sp. | Atlantic (Canary Islands) | Similar to MOTU 4 | $\begin{gathered} 10-20 / \\ 45 \\ \hline \end{gathered}$ | ? | ? | Long (similar to MOTU 9) | Similar to MOTU 4 | ? | Unknown. |
| MOTU 6 - $P$. jourdei sp. nov. | Mediterranean Sea (E Spain, Italy) <br> Type locality: <br> Calpe, Spain | Similar to MOTU 4 but with less amount of dot density (Fig. 6.3.A; Fig. 6.4.A-B). | $\begin{gathered} 1.5-16 / \\ 30-63 \end{gathered}$ | 5 | Similar to MOTU $4$ | Similar to MOTU 4 | Triangular, slightly shorter than in midbody parapodia (Fig. 6.3.C-D). | Lightly serrated (Fig. 6.4.C) | Gonochoric, heteronereis, larval stage with planktotrophic development |


| Species | Distribution | Pigmentation | Length (mm) / NS | Number of jaw teeth | Paragnaths (distinct characteristics only) | Postero- <br> dorsal <br> tentacular <br> cirri | Anterior parapodia dorsal ligule | Spiniger chaetae | Reproduction |
| :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: |
| MOTU 7 - $\boldsymbol{P}$. macaronesiensi $\boldsymbol{s} \mathbf{s p}$. nov. | $N$ Atlantic (Macaronesia Islands, Morocco) Type locality: Tenerife, Spain | Usually ring-like in segment 1; semi ring-like pattern may appear in the anterior region (Fig. 6.5.A; Fig. 6.6.A). | $\begin{aligned} & 5-15 / \\ & 40-49 \end{aligned}$ | 7-8 | Area II - absent; VI - group of 3 rows; <br> VII-VIII continuous band of single rows (Fig. 6.5.A-B). | Reaching chaetiger 6-8 (Fig. 6.5.A). | Similar to midbody parapodia (Fig. 6.5.C-D) | Lightly serrated (Fig. 6.6.B) | Unknown. |
| MOTU 8 Platynereis sp . | NE Atlantic (Porto Santo island, Madeira) | Similar to MOTU 7 | $\begin{gathered} 11-15 / \\ 40 \end{gathered}$ | ? | ? | Similar to MOTU 7 | Similar to MOTU 7 | ? | Unknown. |
| MOTU 9 - P. cf. massiliensis | S. European Atlantic and Mediterranean coast, Morocco Type locality: Marseille, France | Ring-like in most of the anterior segments or as scattered dots throughout the body (Fig. 6.11.A, E-F; Fig. 6.12.A). | $\begin{aligned} & 3.5-26 \\ & / 35-45 \end{aligned}$ | 8-9 | Similar to MOTU 7 | Reaching chaetiger 5-8 (Fig. 6.11.A). | Triangular, slightly shorter than in midbody parapodia (Fig. 6.11.C-D) | Coarsely serrated (Fig. 6.12B) | Hermaphrodite, egg brooding, lecithotrophic larval stages |
| MOTU 10 P. agilis | European <br> Atlantic and W <br> Mediterranean <br> Spanish coast <br> Type locality: <br> St. Vaast, <br> France | Absent (Fig. <br> 6.7.A; Fig. <br> 6.8.A) | $\begin{aligned} & 5-20 / \\ & 45-50 \end{aligned}$ | 7-8 | Similar to MOTU 7 | Reaching <br> chaetiger 10- <br> 15 (Fig. <br> 6.7.A). | Triangular, slightly shorter than in midbody (Fig. 6.7.C-D) | Coarsely serrated (Fig. 6.8B) | Unknown. |

(Table 6.5. Continuation)

### 6.3.6 Taxonomic section

Genus Platynereis Kinberg, 1865
Iphinereis Malmgren, 1865
Leontis Malmgren, 1867
Nectonereis Verrill, 1873
Nereis (Platynereis) (Kinberg, 1865)
Pisenoe Kinberg, 1865
Uncinereis Chamberlin, 1919

Type species. Platynereis magalhaensis Kinberg, 1865

Diagnosis (emended from Bakken and Wilson 2005)
Prostomium cordiform with entire anterior margin, two pairs of eyes in trapezoid arrangement, one pair of antennae, one pair of palps, four pairs of tentacular cirri with distinct cirrophores. One apodous anterior segment, usually larger in length than chaetiger 1. Pharynx maxillary and oral rings with rod-like paragnaths arranged in tight rows: Areas I and V - absent; II - absent or present in small groups; III, IV and VI - present; VII and VIII - present, arranged in isolated patches or in one or more irregular lines forming a continuous band. Jaws with dentate cutting edge. Parapodia with dorsal ligule, prechaetal notopodial lobe may be present, median ligule, and ventral ligule on anterior chaetigers. Neuropodial postchaetal lobe present or absent. Dorsal cirrus simple, lacking basal cirrophore. Ventral cirri single. Notoaciculae absent from segments 1 and 2. Notochaetae: homogomph spinigers, homogomph falcigers may be present. Neurochaeta, dorsal fascicle: homogomph spinigers, heterogomph falcigers; ventral fascicle: heterogomph spinigers, heterogomph falcigers.

## Remarks

Platynereis was originally described and has been accepted as lacking paragnaths in areas I, II and V of the pharynx (Kinberg 1865, Bakken and Wilson, 2005). However, these structures were found to be present in pharynx-area II of specimens belonging to $P$. dumerilii s.s. and to a new species described herein. The diagnosis of the genus is therefore emended accordingly.

## Platynereis jourdei sp. nov.

(Figs. 6.3; 6.4)
urn:Isid:zoobank.org:act: upon paper acceptance

Material examined
Type material. Spain, Calpe, 1 spm, holotype and hologenophore, DBUA0002431.01.v02, $38^{\circ} 38^{\prime} 23.8^{\prime \prime} \mathrm{N}-0^{\circ} 03^{\prime} 30.0^{\prime \prime} \mathrm{E}$, low tide, among algae, 05/08/2019. 8 spms, paratypes and paragenophores, DBUA0002431.01.v01, DBUA0002431.01.v03-v09, $38^{\circ} 38^{\prime} 23.8^{\prime \prime} \mathrm{N}-0^{\circ} 03^{\prime} 30.0^{\prime \prime} \mathrm{E}$, low tide, among algae, collected by Pedro E Vieira, 05/08/2019.

Other material. Italy, Pianosa island: 5 spms, DBUA0002432.04.v01-v06, $42^{\circ} 34^{\prime} 59.8^{\prime \prime} \mathrm{N}-$ $10^{\circ} 05^{\prime} 56.0^{\prime \prime}$ E, low tide, among algae, collected by Joachim Langeneck, 22/09/2020; Italy, Calafuria: 1 spm, DBUA0002432.01.v01, $43^{\circ} 27^{\prime} 57.6^{\prime \prime} \mathrm{N}-10^{\circ} 20^{\prime} 24.0^{\prime \prime} \mathrm{E}$, low tide, among algae, collected by Joachim Langeneck, 11/01/2019. Italy, Antignano: 1 spm, DBUA0002432.02.v02, 43²29'32.0"N $10^{\circ} 19^{\prime} 01.2^{\prime \prime} \mathrm{E}, 6 \mathrm{~m}$ in depth, among Posidonia oceanica rhizomes, collected by Joachim Langeneck, 20/09/2019; 4 spms, DBUA0002432.02.v03-v06, $43^{\circ} 29^{\prime} 32.0^{\prime \prime} \mathrm{N}$ - $10^{\circ} 19^{\prime} 01.2^{\prime \prime} \mathrm{E}, 3 \mathrm{~m}$ depth, among algae, collected by Joachim Langeneck, 10/09/2019; 3 spms, DBUA0002432.02.v07-v09, $43^{\circ} 29^{\prime} 32.0^{\prime \prime} \mathrm{N}-10^{\circ} 19^{\prime} 01.2^{\prime \prime} \mathrm{E}, 3 \mathrm{~m}$ depth, among algae, collected by Joachim Langeneck, 27/06/2019; Italy, Montecristo island: 6 spms, DBUA0002432.03.v01-v06, $42^{\circ} 20^{\prime} 05.9^{\prime \prime} \mathrm{N}-10^{\circ} 17^{\prime} 22.3^{\prime \prime} \mathrm{E}$, low tide, among algae, collected by Joachim Langeneck, 05/09/2020. France, Banyuls: 1 spm, DBUA0002433.01.v01, $42^{\circ} 28^{\prime} 53.9^{\prime \prime} \mathrm{N}-3^{\circ} 08^{\prime} 00.3^{\prime \prime} \mathrm{E}$, low tide, among red algae, collected by Felicia Ultin, 20/09/2020.

## Diagnosis

Small-sized worms (1.5-28 mm long, 30-71 segments), tapering posteriorly. Holotype complete, 26 mm long for 71 segments, very low pigmentation density. Preserved specimens yellowish-brown, with fainted scattered pigmentation dots covering most of the anterior region varying in density (when visible) and the prostomium area adjacent to the eyes (Fig. 6.3.A, Fig. 6.4.A). The apodous anterior segment lacks a well-defined ring-like dot pattern, but this pattern may appear after the first few segments, varying in terms of pigment density (Fig. 6.3.E, Fig. 6.4.B). Prostomium cordiform, with two pairs of eyes in trapezoid arrangement. Antennae and palps similar in length. Palps consisting of a palpophore and ovalshaped palpostyles. Four pairs of tentacular cirri usually longer than the body width, with the longer postero-dorsal cirri reaching up to chaetiger 9-12 (Fig. 6.3.A).


Fig. 6.3. Drawing of the main morphological features in the anterior region, pigmentation and parapodia in $P$. jourdei sp. nov. (MOTU 6). (A) dorsal view of the anterior region with dot-like pigmentation; prostomium and pharynx. (B) ventral view of the pharynx. (C) 10th parapod, posterior view. (D) 30th parapod, posterior view. (E) Pigmentation absent in the apodous anterior segment and well-defined ring-like dot pattern present after the first few segments.

Pharynx maxillary and oral rings with rod-like paragnaths arranged in tight rows (Fig. 6.3.A-B): Area I and V - absent, II - forming double parallel rows, III - forming a group of short rows, IV - forming several long rows in pyramidal arrangement, VI - forming double parallel rows, VII-VIII - arranged in double parallel short rows forming a continuous band. Jaws are finely toothed until a short distance from the tip, usually with five teeth. Anterior parapodia with rounded to triangular ligules (Fig. 6.3.C) slightly shorter than in mid-body parapodia, notopodial ligules equal in length from mid-body chaetigers (Fig. 6.3.D). Neuroacicular ligule short digitiform, longer than a round ventral ligule in anterior chaetigers, triangular and equal in length as a digitiform ventral ligule from mid-body chaetigers (Fig. 6.3.C-D). Dorsal cirri three times the length of parapodial dorsal ligule. Ventral cirri slightly shorter than ventral ligule (Fig. 6.3.C-D).

Notochaetae: homogomph spinigers, serrated almost to the end of blade (Fig. 6.4.C). Neurochaetae, dorsal fascicle: homogomph serrated spinigers, heterogomph falcigers, incurved with a distinct terminal tendon, serrated $1 / 3$ length of blade (Fig. 6.4.E); ventral fascicles: heterogomph serrated spinigers, heterogomph falcigers incurved with a terminal tendon, serrated $2 / 3$ length of blade (Fig. 6.4.D).

## Molecular data

COI, 16S and 28SD2 sequences as in specimens DBUA0002431.01.v01-v09, DBUA0002432.01.v01, DBUA0002432.02.v02-v09, DBUA0002432.03.v01-v06, DBUA0002432. 04.v01-v05 and DBUA0002433.01.v01 (Table S6.1). Phylogenetic relationship within the Platynereis dumerilij pseudo cryptic complex as in Fig. 6.1.A, belonging to MOTU 6, with high support values and low intraspecific (<3\%) genetic divergence for both the mitochondrial and nuclear markers. Interspecific COI mean distances to the closest and distant neighbour are 18.5\% (K2P, P. dumerilii s.s.) and 25.2\% (K2P, MOTU 1) respectively. DOI for the species' holotype Barcode Index Number (BIN): upon paper publication.

## Etymology

The species is named after Jérôme Jourde for his sampling efforts and kindness in providing Platynereis dumerilii specimens from the type locality on the behalf of the author of this thesis.

Distribution and habitat
Western Mediterranean Sea, in subtidal or low tide among algae and Posidonia oceanica rhizomes. Also present in $\mathrm{CO}_{2}$ vents (Wäge et al., 2017).

Reproduction
It is a gonochoric species, with a single reproductive event in life (semelparous) transforming into a pelagic epitokous form (heteronereis) and a larval stage with planktotrophic development (Wäge et al., 2017).


Fig. 6.4. Dorsal view of the anterior region and chaetae types in P. jourdei sp. nov. (MOTU 6). (A) Pigmentation as seen in a preserved specimen (DBUA0002432.03.v01), with high dot density scattered around the anterior region. (B) Pigmentation as seen in a preserved specimen (DBUA0002432.02.v03), with a ring-like dot pattern in the anterior segments. (C) Notochaetae, chaetiger 30: homogomph spinigers lightly serrated. (D) Notochaetae, ventral fascicles: heterogomph falcigers, chaetiger 30. (E) Neurochaetae, dorsal fascicle: heterogomph falcigers (1), homogomph spinigers (2), chaetiger 30.

## Remarks

Platynereis jourdei sp . nov. is morphologically very similar and genetically close to $P$. dumerilii s.s, with both species grouping close together within clade A2 of the phylogenetic tree (Fig. 6.1.A). The two species are barely distinguishable by body pigmentation of live specimens and slightly different parapodia morphology. Platynereis jourdeisp. nov. usually presents lower pigmentation density and some specimens may have ring-like pigment dot pattern in the anterior region (excluding the first few segments)
and parapodia from mid-body segments has shorter triangular ligules. Despite the morphological and phylogenetic proximity of the two species, the molecular interspecific difference between them $(18.5 \%$, COI K2P) justifies the erection of the new species.

Platynereis jourdeisp. nov. and P. dumerilii s.s. are often sympatric in the western Mediterranean Sea, thus requiring some caution in their identification.

## Platynereis macaronesiensis sp. nov.

(Figs. 6.5; 6.6)
urn:Isid:zoobank.org:act: upon paper publication

Material examined
Type material. Spain - Canary Islands, Tenerife: 1 spm, holotype and hologenophore DBUA0002429.01.v03, $28^{\circ} 25^{\prime} 53.3^{\prime \prime} \mathrm{N}-16^{\circ} 32^{\prime} 57.2^{\prime \prime} \mathrm{W}$, low tide, among red algae, collected by Marcos AL Teixeira, 10/04/2019; 2 spms, paratypes and paragenophores DBUA0002429.01.v01-v02, $28^{\circ} 25^{\prime} 53.3^{\prime \prime} \mathrm{N}-16^{\circ} 32^{\prime} 57.2^{\prime \prime} \mathrm{W}$, low tide, rocky beach among red algae, 10/04/2019.

Other material. Spain - Canary Islands, Tenerife: 3 spms, DBUA0002429.02.v01-v03, $28^{\circ} 34^{\prime} 17.1^{\prime \prime} \mathrm{N}-16^{\circ} 20^{\prime} 01.1^{\prime \prime} \mathrm{W}$, low tide, rocky beaches among algae, collected by Marcos AL Teixeira, 05/04/2019; Spain - Canary islands, Lanzarote: 5 spms, DBUA0002429.03.v01-V05, low tide, rocky beaches among algae, $29^{\circ} 13^{\prime} 05.3^{\prime \prime} \mathrm{N}-13^{\circ} 26^{\prime} 30.4^{\prime \prime} \mathrm{W}$, collected by Marcos AL Teixeira, 04/04/2019. Spain - Canary islands, Gran Canaria: 11 spms, DBUA0002429.04.v01-v11, low tide, rocky beaches among algae, $27^{\circ} 59^{\prime} 06.5^{\prime \prime} \mathrm{N}-15^{\circ} 22^{\prime} 33.0^{\prime \prime} \mathrm{W}$, collected by Marcos AL Teixeira, 06/04/2019. Spain Canary islands, La Palma: 5 spms, DBUA0002429.05.v01-v05, low tide, rocky beaches among algae, $28^{\circ} 48^{\prime} 19.8^{\prime \prime} \mathrm{N}-17^{\circ} 45^{\prime} 41.6^{\prime \prime} \mathrm{W}$, collected by Marcos AL Teixeira, 09/04/2019. Spain - Canary islands, Fuerteventura: 5 spms, DBUA0002429.06.v01-v05, low tide, rocky beaches among algae, $28^{\circ} 03^{\prime} 59.7^{\prime \prime} \mathrm{N}-14^{\circ} 30^{\prime} 24.9^{\prime \prime} \mathrm{W}$, collected by Marcos AL Teixeira, 02/04/2019. Spain - Canary islands, El Hierro: 1 spm, DBUA0002429.07.v01, low tide, rocky beaches among algae, $27^{\circ} 47^{\prime} 05.1^{\prime \prime} \mathrm{N}-$ $18^{\circ} 00^{\prime} 41.7^{\prime \prime} \mathrm{W}$, collected by Pedro E Vieira, 2014; Morocco, Mazagan: 2 spms, DBUA0002430.01.v01v02, low tide, rocky beaches among algae, $33^{\circ} 15^{\prime} 50.5^{\prime \prime} \mathrm{N}-8^{\circ} 30^{\prime} 38.6^{\prime \prime} \mathrm{W}$, collected by Pedro E Vieira, 2014. Portugal, Madeira: 4 spms, DBUA0002428.03.v01-v04, $32^{\circ} 38^{\prime} 46.0^{\prime \prime} \mathrm{N}-16^{\circ} 49^{\prime} 27.0^{\prime \prime} \mathrm{W}$, low tide, rocky beaches among algae, collected by Pedro E Vieira, 2011. Portugal - Azores, Terceira island: 3 spms, DBUA0002428.02.v01-v03, $38^{\circ} 40^{\prime} 60.0^{\prime \prime} \mathrm{N}-27^{\circ} 03^{\prime} 27.1^{\prime \prime} \mathrm{W}$, low tide, rocky beaches among
algae, collected by Pedro E Vieira, 2015. Portugal - Azores, Santa Maria island: 3 spms, DBUA0002428.01.v01-v03, $36^{\circ} 56^{\prime} 59.7^{\prime \prime} \mathrm{N}-25^{\circ} 05^{\prime} 42.0^{\prime \prime} \mathrm{W}$, low tide, rocky beaches among algae, collected by Pedro E Vieira, 2014.

## Diagnosis

Small-sized worms ( $5-18 \mathrm{~mm}$ long, 40-49 segments), tapering posteriorly. Holotype lacking posterior end of the worm, 15 mm long for 44 segments. Preserved specimens yellowish-red or yellowishbrown, with a well-defined ring-like pigmentation pattern on the apodous segment and semi ring-like pattern in other anterior segments (Fig. 6.5.A, Fig. 6.6.A). Pigmentation may not be visible in some preserved specimens and may also be present in a prostomium area adjacent to the eyes. Prostomium cordiform, with two pairs of eyes in trapezoid arrangement. Antennae and palps similar in length. Palps consisting of a palpophore and ovalshaped palpostyles. Four pairs of tentacular cirri usually as long as body width, with the longer postero-dorsal cirri reaching up to chaetiger 6-8 (Fig. 6.5.A). Pharynx maxillary and oral rings with rod-like paragnaths arranged in tight rows (Fig. 6.5.A-B): Areas I, II and V - absent. III - forming a group of short rows, IV - forming several long rows in pyramidal arrangement. VI - forming a group of three transverse rows, VII-VIII - arranged in single rows forming a continuous band. Jaws are finely toothed until a short distance from the tip, usually with 7-8 teeth. Notopodial ligule digitiform in anterior parapodia, median ligule rounded, similar in length (Fig. 6.5.C). Mid-body parapodia similar in length, dorsal ligule slightly triangular, median ligule digitiform (Fig. 6.5.D). Neuroacicular ligule triangular, longer than ventral digitiform ligule in anterior chaetiger, shorter than ventral ligule from mid-body chaetigers. Dorsal cirrus more than twice the length of the dorsal ligule and ventral cirrus about the same length or slightly shorter than ventral ligule (Fig. 6.5.C-D). Notochaetae: homogomph spinigers, serrated $2 / 3$ length of blade (Fig. 6.6.B). Neurochaeta, dorsal fascicle: homogomph spinigers, heterogomph falcigers short blades incurved with a small terminal tendon, serrated $2 / 3$ length of blade (Fig. 6.6.C); ventral fascicle: heterogomph spinigers, heterogomph falcigers short blades incurved with a distinct terminal tendon, serrated $1 / 3$ length of blade (Fig. 6.6.D). Spiniger chaetae lightly serrated (Fig. 6.6.B).


Fig. 6.5. Drawing for the main morphological features in the anterior region, pigmentation and parapodia in $P$. macaronesiensis sp . nov. (MOTU 7). (A) dorsal view of the anterior region with a well-defined ring-like dot pigmentation in the apodous anterior segment; prostomium and pharynx. (B) ventral view of the pharynx. (C) 10th parapod, posterior view. (D) 30th parapod, posterior view.

## Molecular data

COI, 16S and 28SD2 sequences as in specimens DBUA0002428.01.v01-v03, DBUA0002428.02.v01-v03, DBUA0002428.03.v01-v04, DBUA0002429.01.v01-v03, DBUA0002429. 02.v01-v03, DBUA0002429.03.v01-V05, DBUA0002429.04.v01-v11, DBUA0002429.05.v01-v05, DBUA0002429.06.v01-v05, DBUA0002429.07.v01, DBUA0002430.01.v01-v02 (Table S6.1). Phylogenetic relationship within the Platynereis dumerilii pseudo cryptic complex as in Fig. 6.1.A, belonging to MOTU 7 , with high support values and low intraspecific ( $<3 \%$ ) genetic divergence for both
the mitochondrial and nuclear markers. Interspecific COI mean distances to the closest and distant neighbour are 13.5\% (K2P, MOTU 8) and 24\% (K2P, P. jourdeisp. nov.) respectively. DOI for the species' holotype Barcode Index Number (BIN): upon paper publication.

## Etymology

The species is named after the regional area (Macaronesia) it is restricted within.


Fig. 6.6. Dorsal view of the anterior region and chaetae types in P. macaronesiensis sp. nov. (MOTU 7). (A) Pigmentation as seen in a preserved specimen (DBUAO002429.04.v03), with presence of well-defined ring-like dot pattern. (B) Notochaetae: homogomph spinigers lightly serrated, chaetiger 10. (C) Neurochaeta, ventral fascicle: heterogomph falcigers (1), chaetiger 10. (D) Neurochaeta, ventral fascicle: heterogomph falcigers, chaetiger 30.

Distribution and habitat
Macaronesia islands (Madeira, Azores and Canary Islands); it occurs in the western coast of Morocco as well, in intertidal rocky beaches among green and red algae. It seems it is not present in the island of Porto Santo (Madeira), being instead replaced by MOTU 8 (Fig. 6.1.A-B), although a greater sampling effort in Porto Santo is needed to confirm this.

Remarks
Platynereis macaronesiensis sp. nov. can be easily distinguished from P. dumerilii s.s. by the lower number of chaetigers (almost half the number of chaetigers for worms of similar size), the shorter tentacular cirri (reaching chaetiger 8, instead of chaetiger 12), the higher number of jaw teeth (with the presence of two or three more teeth) and the distinct paragnath arrangement and pigmentation pattern (see Table 4). Regarding the latter two characters, P. macaronesiensis sp. nov. is closer to P. massiliensis in having a ring-like pigmentation pattern and a similar paragnath arrangement. However, these two species differ in the blades of the spinigerous chaetae, which in P. massiliensis are coarsely serrated, while in $P$. macaronesiensis sp . nov. the blades are narrower and the spinulation is lighter. Genetic distances (mean $15.5 \%$ COI K2P) and distinct geographic distribution also distinguished these two species. Additionally, some pigmentation details in the anterior segments are distinct from P. massiliensis, with the presence of semi ring-like dot patterns.

Unlike most other species from the complex, that are widely distributed along the Atlantic and Mediterranean coast of Europe, P. macaronesiensis sp. nov. is unique to the Macaronesia islands and western coast of Morocco. No reproductive studies were done for this species, but given the genetic proximity to the nearest neighbour (MOTU 9 - P. cf. massiliensis), it is probable it shares the same hermaphrodite features, egg brooding and lecithotrophic larval stages.

Platynereis agilis (Keferstein, 1862) comb. nov.
(Figs. 6.7; 6.8)
Nereis agilis Keferstein, 1862

Material examined
Spain, Calpe: 5 spms, DBUA0002421.01.v01-v05, $38^{\circ} 38^{\prime} 23.8^{\prime \prime} \mathrm{N}, 0^{\circ} 03^{\prime} 30.0^{\prime \prime E}$, low tide, among algae, collected by Pedro E Vieira, 05/08/2019. Portugal, Arrabida Natural Park (Lisbon): 15 spms, MB29-000369 - MB29-000375, MB29-000377 - MB29-000383, 38²6'13.1"N - 9º 03'47.3"W, 9 m in depth, among algae, kindly provided by the National Museum of Science and Natural History (Portugal), 22/09/2014. France, Morlaix Bay: 2 spms, DBUA0002422.01.v01, MTPD191-20, $48^{\circ} 43^{\prime} 48.0^{\prime \prime} \mathrm{N}, 3^{\circ} 59^{\prime} 09.6^{\prime \prime}$ W, low tide, among algae, collected by Celine Houbin, 17/09/2020. Great Britain, Plymouth: 1 spm, DBUA0002423.01.v01, $50^{\circ} 21^{\prime} 35.4^{\prime \prime} \mathrm{N}, 4^{\circ} 09^{\prime} 01.8^{\prime \prime} \mathrm{W}$, low tide, among algae, collected by Felicia Ultin, 27/03/2017.


Fig. 6.7. Drawing of the main morphological features in the anterior region, pigmentation and parapodia in $P$. agilis comb. nov. (MOTU 10). (A) dorsal view of the anterior region with absence of pigmentation; prostomium and pharynx. (B) ventral view of the pharynx. (C) 10th parapod, posterior view. (D) 28th parapod, posterior view.

Diagnosis (based on the original description of Nereis agilis Keferstein, 1862, emended)
Small-sized worms (5-20 mm long, 45-50 segments), tapering posteriorly. Preserved specimens yellowish, with no pigmentation (Fig. 6.7.A, Fig. 6.8.A). Prostomium cordiform, with two pairs of eyes in trapezoid arrangement. Antennae and palps similar in length. Palps consisting of a palpophore and ovalshaped palpostyles. Four pairs of tentacular cirri at least as long as body width, with the longer postero-dorsal cirri reaching up to chaetiger 10-15 (Fig. 6.7.A). Pharynx maxillary and oral rings with rodlike paragnaths arranged in tight rows (Fig. 6.7.A-B): Areas I, II and V - absent. III - forming a group of short rows, IV - forming several long rows in pyramidal arrangement. VI - forming a group of three transverse rows, VII-VIII - arranged in single rows forming a continuous band. Jaws are finely toothed until a short distance from the tip, usually with 7-8 teeth. Dorsal notopodial ligule in anterior parapodia
digitiform to triangular as long as median triangular ligule (Fig. 6.7.C), from mid-body parapodia dorsal ligule triangular similar in length as median ligules (Fig. 6.7.D). Neuroacicular ligule large triangular longer than ventral ligule in anterior parapodia, triangular and shorter than ventral ligule from mid-body parapodia. Dorsal cirrus three times longer than dorsal ligule (Fig. 6.7.C-D). Ventral cirrus about the same size or slightly shorter than ventral ligule (Fig. 6.7.C-D).

Notochaetae: homogomph spinigers with coarsely serrated $3 / 4$ length of blade (Fig. 6.8.B). Neurochaeta, dorsal fascicle: homogomph spinigers serrated, heterogomph falcigers with distinct tendon, serrated $1 / 3$ of the blade (Fig. 6.8.D); ventral fascicle: heterogomph spinigers, heterogomph falcigers with tendon, serrated $1 / 2$ length of the blade (Fig. 6.8.C).

## Molecular data

COI, 16S and 28SD2 sequences as in specimens MB29-000369 - MB29-000375, MB29-000377 - MB29-000383, DBUA0002421.01.v01-v05, DBUA0002422.01.v01, MTPD191-20 and DBUA0002423.01.v01 (Table S6.1). Phylogenetic relationship within the Platynereis dumerilij pseudo cryptic complex as in Fig. 6.1.A, belonging to MOTU 10, with high support values and low intraspecific $(<3 \%)$ genetic divergence for both the mitochondrial and nuclear markers. Interspecific COI mean distances to the closest and distant neighbour are 5\% (K2P, P. cf. massiliensis) and 24.2\% (K2P, MOTU 5) respectively. DOI for the species' Barcode Index Number (BIN): upon paper publication.

## Distribution and habitat

NE Atlantic to the Western Mediterranean Sea, from Great Britain to Mediterranean Spain. Found in rocky beaches among algae in intertidal or subtidal habitats.

Reproduction
The claim by Keferstein (1862) of hermaphroditism has not been confirmed by recent studies, but given the genetic proximity for this species to the nearest neighbour (MOTU 9, P. cf. massiliensis), it is possible it shares the same hermaphrodite features, egg brooding and lecithotrophic larval stages (Wäge et al. 2017).


Fig. 6.8. Dorsal view of the anterior region and chaetae types in P. agilis comb. nov. (MOTU 10). (A) Absence of pigmentation as seen in a preserved specimen (MB29-000373). (B) Notochaetae: homogomph spinigers with coarsely serrated blades, chaetiger 10. (C) Neurochaeta, ventral fascicle: heterogomph falcigers, chaetiger 10. (D) Neurochaeta, ventral fascicle: heterogomph falcigers, chaetiger 28.

Remarks
Platynereis agilis, originally described as Nereis agilis (Keferstein 1862) from St. Vaast (North France) and until now considered as a junior synonym of $P$. dumerili, is clearly part of the $P$. dumerilii species complex, given its similar morphology and the genetic proximity to the other species of the complex (Fig. 6.1, Table 6.5). However, visible differences can easily be found against $P$. dumerilii s.s. with almost half the segments in worms of similar size, distinct paragnath arrangement, no pigmentation (although this is not always a reliable character due to fixation in ethanol), anterior parapodia with longer triangular-like ligules and spinigerous chaetae with coarsely serrated blades. All these differences, along with the genetic distances (mean 21.8\% COI K2P), justify the removal from synonymy and reestablishment of the species. Platynereis agilis shares a similar paragnath arrangement and the coarsely serrated chaetae with the species $P$. cf. massiliensis, but greatly differs from the latter due to lack of pigmentation and regarding the longer size of the postero-dorsal cirri, reaching up to chaetiger 15, instead of chaetiger 8, even in specimens with equal or smaller size. Despite the low genetic COI distance (mean

5\% K2P) compared to P. cf. massiliensis, the distinct morphological differences justify the resurrection of this species.

Platynereis dumerilii (Audouin \& Milne-Edwards, 1833) s.s.
(Figs. 6.9; 6.10)
Eunereis africana Treadwell, 1943
Heteronereis fucicola Örsted, 1843
Heteronereis maculata Bobretzky, 1868
Heteronereis malmgreni Claparède, 1868
Iphinereis fucicola (Örsted, 1843)
Leontis dumerilii (Audouin \& Milne Edwards, 1833)
Leptonereis maculata Treadwell, 1928
Mastigonereis quadridentata Schmarda, 1861
Mastigonereis striata Schmarda, 1861
Nereilepas variabilis Örsted, 1843
Nereis (Platynereis) dumerilii Audouin \& Milne Edwards, 1833
Nereis (Platynereis) dumerilii striata (Schmarda, 1861)
Nereis (Platynereis) striata (Schmarda, 1861)
Nereis agilis Keferstein, 1862
Nereis alacris Verrill, 1879
Nereis antillensis McIntosh, 1885
Nereis dumerilii Audouin \& Milne Edwards, 1833
Nereis glasiovi Hansen, 1882
Nereis gracilis Hansen, 1882
Nereis megodon Quatrefages, 1866
Nereis peritonealis Claparède, 1868
Nereis taurica Grube, 1850
Nereis zostericola Örsted, 1843
Platynereis dumerili [auctt. misspelling]
Platynereis jucunda Kinberg, 1865
Platynereis striata (Schmarda, 1861)

Material examined
Sweden, Tjärnö: 10 spms, DBUA0002435.01.v01-v10, $58^{\circ} 52^{\prime} 27.6^{\prime \prime} \mathrm{N}-11^{\circ} 08^{\prime} 43.4^{\prime \prime E}, 3-5$ meters, among algae, collected by Felicia Ultin and Marcos AL Teixeira, 20/12/2018. Norway, Trondheim: 1 spm, NTNU-VM-76216, $63^{\circ} 26^{\prime} 24.0^{\prime \prime} \mathrm{N}-10^{\circ} 30^{\prime} 14.4$ "E , 2 meters depth, among algae, collected by Torkild Bakken, 04/09/2018. France, La Rochelle: 17 spms, DBUA0002438.01.v01-v17, $46^{\circ} 08^{\prime} 47.4^{\prime \prime} \mathrm{N}-1^{\circ} 12^{\prime} 36.0^{\prime \prime} \mathrm{W}$, low tide, among red algae, collected by Jérôme Jourde, 18/09/2020. France, Arcachon Bay: 1 spm, DBUA0002439.01.v01, $44^{\circ} 39^{\prime} 44.2^{\prime \prime N}-1^{\circ} 09^{\prime} 10.0^{\prime \prime} \mathrm{W}$, low tide, among algae, collected by Nicolas Lavesque, 18/09/2020. Portugal, Canto Marinho: 1 spm, DBUA0002436.01.v01, $41^{\circ} 44^{\prime} 13.2^{\prime \prime} \mathrm{N}-8^{\circ} 52^{\prime} 33.6^{\prime \prime} \mathrm{W}$, low tide, among algae, collected by Marcos AL Teixeira, 20/05/2019. Spain, Calpe: 2 spms, DBUA0002434.01.v01-v02, $38^{\circ} 38^{\prime} 23.8^{\prime \prime} \mathrm{N}$ $0^{\circ} 03^{\prime} 30.0^{\prime \prime} \mathrm{E}$, low tide, among algae, collected by Pedro E Vieira, 05/08/2019. Italy, Antignano: 2 spms, DBUA0002437.01.v01-v02, $43^{\circ} 29^{\prime} 32.0^{\prime \prime} \mathrm{N}-10^{\circ} 19^{\prime} 01.2^{\prime \prime} \mathrm{E}, 3 \mathrm{~m}$, among algae, collected by Joachim Langeneck, 10/09/2019; 3 spms, DBUA0002437.01.v04-v06, $43^{\circ} 29^{\prime} 32.0^{\prime \prime} \mathrm{N}-10^{\circ} 19^{\prime} 01.2^{\prime \prime} \mathrm{E}, 6 \mathrm{~m}$, among Posidonia oceanica rhizomes, collected by Joachim Langeneck, 20/09/2019; 1 spm, DBUA0002437.01.v03, $43^{\circ} 29^{\prime} 32.0^{\prime \prime} \mathrm{N}-10^{\circ} 19^{\prime} 01.2^{\prime \prime} \mathrm{E}, 3 \mathrm{~m}$, among algae, collected by Joachim Langeneck, 27/06/2019. Italy, Ardenza: 5 spms, DBUA0002437.02.v01-v05, $43^{\circ} 30^{\prime} 43.3^{\prime \prime} \mathrm{N}$ $10^{\circ} 18^{\prime} 52.3^{\prime \prime} \mathrm{E}, 2 \mathrm{~m}$, gravel with Posidonia oceanica debris, collected by Joachim Langeneck, 18/09/2019. Italy, Vada: 4 spms, DBUA0002437.03.v01-v04, $43^{\circ} 18^{\prime} 39.8^{\prime \prime} \mathrm{N}-10^{\circ} 25^{\prime} 54.6^{\prime \prime E}, 10 \mathrm{~m}$, among algae, collected by Joachim Langeneck, 26/10/2019. Italy, Elba Island: 3 spms, DBUA0002437.04.v01-v03, $42^{\circ} 48^{\prime} 41.1^{\prime \prime} \mathrm{N}-10^{\circ} 19^{\prime} 23.7$ "E, 3 m , among algae, collected by Joachim Langeneck, 15/01/2020. Italy, Montecristo Island: 7 spms, DBUA0002437.05.v01-v07, $42^{\circ} 20^{\prime} 05.9^{\prime \prime} \mathrm{N}$ - $10^{\circ} 17^{\prime} 22.3^{\prime \prime E}$, low tide, among algae, collected by Joachim Langeneck, 05/09/2020. Italy, Taranto: 1 spm, DBUA0002437.06.v01, $40^{\circ} 27^{\prime} 59.0^{\prime \prime} \mathrm{N}-17^{\circ} 14^{\prime} 20.0^{\prime \prime} \mathrm{E}, 12 \mathrm{~m}$ depth, on mud with shell fragments, collected by Joachim Langeneck, 20/03/2019. Italy, Trieste: 1 spm, DBUA0002437.07.v02, $45^{\circ} 38^{\prime} 51.6^{\prime \prime} \mathrm{N}-13^{\circ} 45^{\prime} 32.9^{\prime \prime} \mathrm{E}$, low tide, among algae, collected by Joachim Langeneck, 19/02/2020. Greece, Mazoma: 2 spm, MTPD200-20, MTPD201-20, $39^{\circ} 03^{\prime} 21.3^{\prime \prime} \mathrm{N}-20^{\circ} 50^{\prime} 00.5^{\prime \prime} \mathrm{E}$, low tide, among algae, collected by Katerina Vasileidou, 01/01/2017. Greece, Crete: 1 spm, DBUA0002440.01.v01, $35^{\circ} 09^{\prime} 57.6^{\prime \prime} \mathrm{N}-24^{\circ} 25^{\prime} 17.0^{\prime \prime} \mathrm{E}$, 5-10 meters, among algae, collected by Giorgos Chatzigeorgiou, 14/03/2020.

## Diagnosis

Small-sized worms (1.5-26 mm long, 30-80 segments), tapering posteriorly. Preserved specimens yellowish-brown, with small pigmentation dots covering most of the anterior region and the prostomium area adjacent to the eyes (Fig. 6.9.A, Fig. 6.10.A). The apodous anterior segment is similar in size to the first chaetigers and lacks a well-defined ring-like dot pattern. Prostomium cordiform, with two pairs of eyes in trapezoid arrangement. Antennae and palps similar in length (Fig. 6.9.A). Palps consisting of a palpophore and ovalshaped palpostyle. Four pairs of tentacular cirri usually longer than body's width, with the longer postero-dorsal cirri reaching chaetiger 9-12 (Fig. 6.9.A), rarely to chaetiger 15. Pharynx maxillary and oral rings (Fig. 6.9.A-B) with rod-like paragnaths arranged in tight rows: Area I and V - absent, II - forming double parallel rows, III - forming a group of short rows, IV - forming several long rows in pyramidal arrangement, VI - forming double parallel rows, VII-VIII - arranged in double parallel short rows forming a continuous band. Jaws are finely toothed until a short distance from the tip, usually with 5 or 6 teeth. Anterior parapodia (Fig. 9C) with rounded to triangular ligules much shorter than in mid-body parapodia (Fig. 6.9.D). Dorsal notopodial ligule triangular from mid-body chaetigers, median notopodial ligule digitiform, equal in length as dorsal ligule (Fig. 6.9.D). Neuroacicular ligule short rounded in anterior chaetigers, triangular and slightly shorter than ventral ligule from mid-body chaetigers. Dorsal cirri three times longer than the parapodial dorsal ligule. Ventral cirri much shorter than ventral ligule (Fig. 6.9.C-D). Notochaetae: homogomph spinigers, serrations present in about $3 / 4$ length of blades; homogomph falcigers, short blades incurved with a terminal tendon, serrated $1 / 2$ length of blade (Fig. 6.10.E). Neurochaeta, dorsal fascicle: homogomph spinigers, serrations present in about $3 / 4$ length of blades (Fig. 6.10.B), heterogomph falcigers short blades incurved with a terminal tendon, serrated $2 / 3$ length of blade (Fig. 6.10.C); ventral fascicle: heterogomph spinigers, serrations present in about $3 / 4$ length of blades (Fig. 6.10.D).

## Molecular data

COI, 16S and 28SD2 sequences as in specimens DBUA0002434.01.v01-v02, DBUA0002435.01.v01-v10, NTNU-VM-76216, DBUA0002436.01.v01, DBUA0002437.01.v01-v06, DBUA0002437.02.v01-v05, DBUA0002437.03.v01-v04, DBUA0002437.04.v01-v03, DBUA0002437. 05.v01-07, DBUA0002437.06.v01, DBUA0002437.07.v02, DBUA0002438.01.v01-v17, DBUA00024 39.01.v01, DBUA0002440.01.v01, MTPD200-20 and MTPD201-20 (Table S6.1). Phylogenetic relationship within the Platynereis dumerilii pseudo cryptic complex as in Fig. 6.1.A, belonging to MOTU 4 , with high support values and low intraspecific $(<3 \%)$ genetic divergence for both the mitochondrial and
nuclear markers. Interspecific COI mean distances to the closest and distant neighbour are 8.2\% (K2P, MOTU 5) and 21.8\% (K2P, MOTU 10) respectively.

Distribution and habitat
NE Atlantic, from Scandinavia to Mediterranean Sea, among green or red algae and gravel with Posidonia oceanica rhizomes, in subtidal or intertidal areas.


Fig. 6.9. Drawing of the main morphological features in the anterior region, pigmentation and parapodia in $P$. dumerilii s.s. (MOTU 4). All terminology used is based on the references mentioned in the Methods. (A) dorsal view of the anterior region with dot-like pigmentation; prostomium and pharynx. (B) ventral view of the pharynx. (C) 10th parapod, posterior view. (D) 30th parapod, posterior view.

Reproduction
It is a gonochoric species, with a single reproductive event in life (semelparous) transforming into a pelagic epitokous form (heteronereis) and a larval stage with planktotrophic development (Wäge et al., 2017).

## Remarks

The holotype of $P$. dumerilii could not be found, thus preventing an effective morphological or molecular comparison. The National Museum of Natural History (France), which is home for major polychaete collections described by French authors, lack the type specimens for this species. However, all the specimens collected in the type locality, presenting morphological characteristics that fit the overall original description by Audouin \& Milne-Edwards (1833), grouped in a single MOTU (Fig. 6.1.A, MOTU 4). Minor differences concern the pigmentation pattern and pharynx jaws compared to the original description. The holotype was reported as being yellowish with some brown spots at the basis of parapodia, although it is not clear whether it refers to live or preserved organisms. Instead, the preserved specimens studied herein were yellowish with brown pigmentation covering most of the anterior region. The pharynx and jaws are incompletely described by Audouin \& Milne-Edwards (1833), but from the original illustrations (PI. XIII, fig. 12), jaws seem to have 11 teeth far surpassing the 5-6 observed in the topotypes examined herein. Furthermore, the original description presents some morphological discrepancies compared to all other Platynereis species. The posterior parapodia are described as having an overgrown neuropodial postchaetal lobe and a clear separation between the notopodium and neuropodium, suggesting that the specimens studied by those authors could be developing into a heteronereis stage. The presence of paragnaths in Area II of the pharynx also distinguishes $P$. dumerilii from the remaining Platynereis species described up to date. Given the apparent loss of the holotype, and to provide taxonomic stability, a neotype was selected from among the specimens collected in the type locality. For a Platynereis review in the use of the species as a model system for genetics, regeneration, reproduction biology, development, evolution, chronobiology, neurobiology, ecology, ecotoxicology, and most recently also for connectomics and single-cell genomics, see Özpolat et al., (2021).


Fig. 6.10. Dorsal view of the anterior region and chaetae types in P. dumerilii s.s. (MOTU 4). (A) pigmentation as seen in a preserved specimen (DBUA0002438.01.v14), with high dot density scattered around the anterior region. (B) Neurochaeta, dorsal fascicle: homogomph spinigers with lightly serrated blades (1), chaetiger 30. (C) Neurochaeta, ventral fascicle: heterogomph falcigers, chaetiger 30. (D) Neurochaeta, ventral fascicle: heterogomph spinigers (1), chaetiger 30. (E) Notochaetae: homogomph falciger, chaetiger 57.

## Platynereis cf. massiliensis

(Figs. 6.11; 6.12)

Material examined
Portugal, Canto Marinho: 14 spms, DBUA0002424.01.v01-v03, DBUA0002425.01.v01-v11, $41^{\circ} 44^{\prime} 13.2^{\prime \prime} \mathrm{N}-8^{\circ} 52^{\prime} 33.6^{\prime \prime} \mathrm{W}$, low tide, among algae, collected by Marcos AL Teixeira, 20/05/2019. Morocco, Mazagan: 1 spm, DBUA0002426.01.v01, low tide, rocky beaches among algae, $33^{\circ} 15^{\prime} 50.5^{\prime \prime} \mathrm{N}, 8^{\circ} 30 ' 38.6^{\prime \prime} \mathrm{W}$, collected by Pedro E Vieira, 2014. Italy, Livorno: 3 spms,

DBUA0002427.01.v01-v03, $43^{\circ} 32^{\prime} 45.6^{\prime \prime} \mathrm{N}-10^{\circ} 18^{\prime} 07.2^{\prime \prime} \mathrm{E}$, marina, pontoon scrapings among algae, 23/10/2019.

## Diagnosis

Small-sized worms ( $3.5-26 \mathrm{~mm}$ long, $35-45$ segments), tapering posteriorly. Preserved specimens yellowish-brown, with a ring-like pigmentation pattern in most of the anterior segments (Fig. 6.11.A, Fig. 6.12.A), or a high amount of dots scattered throughout the body, varying in dot size and density except on the apodous segment (Fig. 6.11.E, F). Pigmentation may also be present in prostomium, adjacent to the eyes. Prostomium cordiform, with two pairs of eyes in trapezoid arrangement. Antennae and palps similar in length. Palps consisting of a palpophore and ovalshaped palpostyles. Four pairs of tentacular cirri usually as long as body width, with the longer postero-dorsal cirri reaching up to chaetiger 6-8 (Fig. 6.11.A). Pharynx maxillary and oral rings with rod-like paragnaths arranged in tight rows (Fig. 6.11.A-B): Areas I, II and V - absent. III - forming a group of short rows, IV forming several long rows in pyramidal arrangement. VI - forming a group of three transverse rows, VIIVIII - arranged in single rows forming a continuous band. Jaws are finely toothed until a short distance from the tip, usually with $8-9$ teeth. Anterior dorsal parapodial ligules (Fig. 6.11.C) digitiform slightly longer than median triangular ligule, neuropodial acicular ligule triangular, as long as a rounded ventral ligule. In mid-body chaetigers dorsal notopodial ligule slightly longer than median digitiform ligule, neuropodial acicular ligule round shorter than lanceolate ventral ligule (Fig. 6.11.D). Dorsal cirrus more than twice the length of the dorsal ligule and ventral cirrus about the same size or slightly shorter than ventral ligule (Fig. 6.11.C-D). Notochaetae: homogomph spinigers with coarsely serrated blades (Fig. 6.12.B). Neurochaeta, dorsal fascicle: homogomph spinigers (Fig. 6.12.C), heterogomph falcigers with short blades incurved with a distinct terminal tendon, serrated $1 / 2$ length of blade (Fig. 6.12.D); ventral fascicle: heterogomph spinigers (Fig. 6.12.C), heterogomph falcigers short blades incurved with a distinct terminal tendon, serrated $1 ⁄ 2$ length of blade (Fig. 6.12.E).

## Molecular data

COI, 16S and 28SD2 sequences as in specimens DBUA0002424.01.v01-v03, DBUA0002425.01.v01-v11, DBUA0002426.01.v01 and DBUA0002427.01.v01-v03 (Table S6.1). Phylogenetic relationship within the Platynereis dumerilii pseudo cryptic complex as in Fig. 6.1.A, belonging to MOTU 9 , with high support values and low intraspecific ( $<3 \%$ ) genetic divergence for both the mitochondrial and nuclear markers. Interspecific COI mean distances to the closest and distant
neighbour are 5\% (K2P, MOTU 10) and 24\% (K2P, MOTU 5) respectively. DOI for the species' Barcode Index Number (BIN): upon paper publication.


Fig. 6.11. Drawing of the main morphological features in the anterior region, pigmentation and parapodia in P. c.f. massiliensis (MOTU 9). (A) dorsal view of the anterior region with ring-like dot pigmentation pattern; prostomium and pharynx. (B) ventral view of the pharynx. (C) 10th parapod, posterior view. (D) 30th parapod, posterior view. (E) Pigmentation absent in the apodous anterior segment and large circular-like dot patterns scattered after the first few segments, typically found in populations from Porto di Livorno (Italy). (F) Pigmentation absent in the apodous anterior segment and scattered dot patterns after the first few segments.

Distribution and habitat
NE Atlantic to the Western Mediterranean Sea, from Portugal and Morocco to western Italy. Found in rocky beaches among algae in intertidal or subtidal habitats, including $\mathrm{CO}_{2}$ vents (Wäge et al., 2017).


Fig. 6.12. Dorsal view of the anterior region and chaetae types in P. cf. massiliensis. (A) pigmentation as seen in a preserved specimen (DBUA0002425.01.v03), with ring-like dot pigmentation pattern present in the apodous anterior segment and in the remaining anterior segments. (B) Notochaetae: homogomph spinigers with coarsely serrated blades, chaetiger 30. (C) Neurochaeta ventral fascicle: heterogomph spinigers (1); dorsal fascicle: homogomph spinigers (2), chaetiger 10. (D) Neurochaeta, dorsal fascicle: heterogomph falcigers, chaetiger 30. (E) Neurochaeta, ventral fascicle: heterogomph falcigers, chaetiger 30.

## Reproduction

Reproduction without epitokous transformation; it is a protandrous hermaphrodite, characterized by egg brooding and lecithotrophic larval stages with a semi-direct development (Schneider et al. 1992, Wäge et al. 2017). In the original description by Moquin-Tandon (1869), it was described as simultaneous hermaphrodite, instead.

Remarks
The original description by Moquin-Tandon is very poor (type locality: Marseille, France), and the identification of specimens as $P$. massiliensis is mostly tentative. Wäge et al. (2017) genetically pinpointed two lineages sharing the same reproductive features as $P$. massiliensis (egg brooders), mainly present in acidic waters. Despite the lack of the type material, the congruence of their developmental observations with other studies (Hauenschild, 1951, Schneider et al., 1992; Helm et al., 2015) suggests that their Platynereis population from Ischia represents P. massiliensis (MOTU 9 in this chapter, Fig. 6.1). The Vulcano population (egg brooder), grouped in MOTU 1 (Fig. 6.1), which also have sequences from Banyuls. However, this MOTU 1 is closer to the original type locality reported for P. massiliensis (Marseille, France). Further sampling and reproductive studies in the topotypic material is needed to confirm if this lineage actually corresponds to specimens found in Marseille.

In the current study, the MOTU attributed to Platynereis massiliensis differs from P. dumerilii s.s. mainly in having much shorter dorsal tentacular cirri, different paragnath arrangement with absence of paragnaths on area II, coarsely serrated chaetae, and different pigmentation in some of its specimens. Additionally, high molecular distances (mean 21.6\% COI K2P) and different reproductive strategies and life history distinguishes this species from P. dumerilii s.s. (Wäge et al., 2017).

This species possesses diverse pigmentation patterns, one of which is very distinct and apparently unique to the population from Porto di Livorno (Italy). This pigmentation pattern has a high amount of dots scattered throughout the body and is characterized by the larger dot size (almost circular-like) when compared to the NE Atlantic populations. An independent COI clade with $3.3 \%$ K2P mean distances distinguishes the Livorno variant against the NE Atlantic populations, however without enough divergence to be separated by any of the applied MOTU delineation methods (Fig. 6.1).

### 6.4 Discussion

Some species within the family Nereididae have morphological features with very small variations which can often lead to misidentifications (Bakken and Wilson, 2005). This is especially true when comparing small specimens belonging to different species where one is significantly more abundant than the other. The difficulty found in identifying the rare and lesser-known species, might be attributed to the presence of juveniles, damage or loss of features, e.g. the size of the tentacular cirri due to the sampling techniques, and the pharynx might not be everted as well, which can be difficult and sometimes impossible to dissect in small specimens. All of this may lead to wrong taxonomic conclusions. This was the case in my samples between the clades A and B, where molecular data and a more careful
morphological analysis found considerable differences between the two. However, it is still possible to find P. dumerilii assigned to MOTU 11 (GenBank: KC591811.1) in the genetic databases, and the earlier first-pass assessment of some specimens from clade B led to incorrect identifications as well (Teixeira et al., 2021). Maximum genetic distances between these two major clades were very high (see Table 6.2), especially in the 28SD2 locus where values rose to $36.9 \%$, as opposed to the $3.9 \%$ found between MOTUs in clade A. Other annelid studies about cryptic complexes also reported similarly low 28S distances among neighbouring MOTUs (e.g. Sampieri et al., 2021). This nuclear locus is known for its poor utility in specieslevel discrimination in many groups of animals (Jörger et al., 2012), but it is very efficient for reconstructing deeper phylogenies (Weitschek et al., 2014). Based on this preliminary data it is clear that either entirely new unreported species, or new pseudo cryptic lineages belonging to an existing group, were found in clade B , but a larger sampling effort and further morphological examination is needed to confirm this.

Regarding the major clade A, the combined molecular data from three different loci provided compelling evidence for the existence of at least 10 deeply divergent and completely sorted lineages within the $P$. dumerilii complex in Europe. These deep genetic distances are a strong indication of longterm isolation, thereby the lineages involved can qualify for recognition as separate species (Bickford et al., 2007; Churchill et al., 2014; Delić et al., 2017). Complementing the molecular data, some morphological variations within the most abundant MOTUs (4, 6, 7, 9, 10) were found as well (Table 6.5). The genetic COI distances recorded in this clade (mean 19.8\%, K2P) fit within the range reported for congeneric distances in comprehensive studies of COI variation targeting polychaetes. For example, mean COI distances (K2P) of 16.5\%, 24.0\% and 22\% were found in the regional polychaete fauna of the Arctic (Carr et al., 2011), north-eastern Atlantic (Lobo et al., 2016) or between cryptic populations of Eurythoe complanata (Pallas, 1766) from eastern Pacific (Panama) and Atlantic samples (Barroso et al., 2010), respectively. The only exception to this are MOTUs 5 and 10 where the COI distances to the nearest neighbours (MOTUs 4 and 9 ) were much lower, namely $8.6 \%$ and $6.4 \%$ respectively, which is still a fair genetic distance and much higher than the usual intraspecific variation found in Nereidids (Glasby, 2005; Paiva et al., 2019).


Fig. 6.13. Drawing for the main morphological features in the anterior region, pigmentation and parapodia in Nereis sp. (MOTUs 12 and 13). (A) dorsal view of the anterior region with absence of pigmentation; prostomium and pharynx. (B) ventral view of the pharynx. (C) 11th parapod, posterior view. (D) 24th parapod, posterior view. (E) Neurochaeta, chaetiger 1: heterogomph falciger. (F) Neurochaeta, chaetiger 24: heterogomph spiniger (3), heterogomph falciger (2), homogomph spiniger (4); Notochaetae homogomph falciger (1). (G) Notochaetae chaetiger 11, homogomph spiniger (1); Neurochaeta parapod 11, heterogomph falciger (2).

### 6.4.1 Untangling the Platynereis complex

The original description for P. massiliensis by Moquin-Tandon (1869) is incomplete and does not include any reliable morphological character or figures (Moquin-Tandon, 1869). Instead, the main reproductive features were highlighted and further studies have been treating this species as morphological similar to P. dumerilii (Hauenschild, 1951; Schneider et al. 1992; Valvassori et al., 2015). Based on Wäge et al. (2017), it was possible to genetically pinpoint two lineages sharing the same reproductive features as $P$. massiliensis (egg brooders), mainly present in acidic waters and two other lineages matching $P$. dumerilii (heteronereis stage), mostly living in non-acidic waters. These latter two $P$. dumerilii lineages grouped in clade A2, more specifically in MOTUs 4 and 6, with the first one occurring in the type locality. MOTU 4 (P. dumerilii s.s.) and MOTU 6 ( $P$. jourdeisp. nov.) have a distinct paragnath pattern from the ones found in clade A3 (MOTUs 7, 9 and 10), where sequences of $P$. massiliensis from

Wäge et al. (2017) grouped with. As stated by the previous mentioned study, despite the lack of type material, the congruence of their developmental observations with other studies (Hauenschild, 1951, Schneider et al., 1992; Helm et al., 2015) suggests that their Platynereis population from Ischia represents $P$. massiliensis, and group together with sequences specifically from MOTU 9. The Vulcano population, also a brooder, grouped in MOTU 1 (clade A1) together with two original sequences from Banyuls, but it was not possible to observe the pharynx and confirm if similar paragnath patterns to MOTU 9 could be identified as well.

Given that MOTU 7 (P. macaronesiensis sp. nov.) and MOTU 8 are endemic to the Macaronesia islands, MOTU GB1 has been reported from South Africa, and MOTUs GB2 and GB3 probably belonging either to MOTU 9 or MOTU 10, among the analysed material only MOTU 10, present in the Western Mediterranean, could also qualify as possible source for the originally described $P$. massiliensis. MOTU 10 is genetically close to MOTU 9 (max distances of $6.4 \%$ COI K2P) and it's very likely that they share the same reproductive traits; however, it shows some visible morphological differences when compared to the latter. These differences seem to fit the description of Nereis agilis Keferstein, 1862, described for the NE Atlantic (type locality: St. Vaast, France) and hitherto considered as an unaccepted subjective synonym for $P$. dumerilii. In the original description, the analysed specimens seem to be simultaneous hermaphrodites without heteronereis stage, tentacular cirri and dorsal cirri are longer than the ones usually reported for P. dumerilii and on parapodia four ligules are noticeable, although the third [starting from the dorsal side] is very short, but no mention to the pharynx is done (Keferstein, 1862).

Another unaccepted subjective synonym described for the Gulf of Naples (Italy), Nereis peritonealis Claparède, 1868, describes a similar paragnath pattern as the one presented here for the clade A3 (massiliensistype). However, even though there is no detailed data on the reproductive mode, the reported small size of mature eggs (Claparede, 1868) would suggest that this is not a species with direct development, i.e. not a brooder, but it might have a planktonic larvae stage (Sato and Masuda, 1997). This goes in line with MOTUs 4 and 6 instead, even though paragnath patterns do not match (dumerilïtype).

An interesting note regarding the description of Nereis agilis is that ovaries and testes are separated in two different sectors of the body (Keferstein, 1862), while in P. massiliensis they should occur in the same segments (Moquin-Tandon, 1869). From the biological point of view, the latter arrangement is very surprising, as it would imply a high risk of selffertilisation; nonetheless, such discrepancies might depend on different interpretations of the same structures by different scholars, and what is interpreted as a developing gonad might be a glandular structure in other sources. This calls for
a new observation on the reproductive features and a description based on topotypic material to compare against this thesis interpretation of $P$. massiliensis and confirm if the lineage identified in this and in other previous studies match the topotypic samples.


Fig. 6.14. Drawing for the main morphological features in the anterior region, pigmentation and parapodia in Nereis aff. zonata (MOTU 11). (A) dorsal view of the anterior region with absence of pigmentation; prostomium and pharynx. (B) ventral view of the pharynx. (C) 10th parapod, posterior view. (D) 31th parapod, posterior view. (E) Notochaetae, chaetiger 10: heterogomph spiniger (1), homogomph spiniger (2). (F) Neurochaeta, chaetiger 30: heterogomph falciger. (G) Notochaetae, chaetiger 30: homogomph falciger.

Other species with currently unaccepted names in European type localities are also available (Table 6.4) but they are incomplete and an unequivocal attribution to any of the MOTUs found in clade A seems impossible. Three additional European synonyms historically synonymized with P. dumerili, i.e., Heteronereis fucicola Örsted, 1843, Nereilepas variabilis Örsted, 1843, and Heteronereis malmgreni

Claparède, 1868, were not included in this table because all refer to epitoke forms, that at the time were believed to be different species from the atoke forms. It is not possible to reconstruct their morphological correspondence to the atoke specimens studied in this work, but we can exclude that they are synonymous with P. massiliensislike brooders. Taxa from Denmark described by Örsted represent different stages of the epitoke modification or different sexes, and based on distribution of MOTUs, might correspond either to P. dumerilii s.s. (MOTU 4) or to MOTU2. Heteronereis malmgreni was instead described for the Gulf of Naples and it is probably a description of the epitoke form of Nereis peritonealis. Heteronereis maculata Bobretzky, 1868 described for the Black Sea is also synonymized, however further details from this species were not included in table 6.4 due the difficulty in translating the original Russian description, but together with $N$. taurica, might be related to the unnamed MOTU3 based on type locality proximity.

### 6.4.2 Reproduction strategies in Platynereis

The suggested reproduction modes based on genetic proximity done in this study, being fixed at the basis of the two major retrieved clades by Wäge et al. (2017), might not be correct. Instances of reproductive plasticity were reported in other Nereididae species, e.g. the suppression of epitoky as a probable answer to environmental pressures within the same lineage (Prevedelli and Cassai, 2001; Daas et al., 2011). However, as no genetic data complemented these studies, this could also be a clue to unreported cryptic species as well. Several references, pointed out in Daas et al. (2011), that stress the presence of atokous and epitokous "races" or "forms" in Perinereis cultrifera (Grube, 1840) (e.g. Marcel, 1962; Zghal and Ben Amor, 1989; Scaps et al., 1992; Rouhi et al., 2008), might actually be linked to the evidence of cryptic species within this taxon, which was reported in other studies. For example, upon further examination, Perinereis populations from North of France and Algeria have distinct alloenzymes, number of paragnaths and number of teeth per half jaw (Scaps et al. 2000). Nevertheless, this would still question if sister lineages or other phylogenetically close species might have, or not, completely different reproduction modes. Without actual studies on reproductive biology complemented with genetic data, we should not discard the possibility of different reproductive features within the Platynereis complex or even possible reproductive plasticity within the same MOTU.

It is speculated that the low dispersal rate in many marine brooding species with a direct or semidirect development without planktonic larval stage can promote genetic divergence and help to explain the genetic isolation of populations, while the free-swimming larvae easily migrate, resulting in higher chances of gene flow among populations (Palumbi and Baker, 1994; Teske et al., 2011). Evidence that
stressful conditions (e.g. hydrothermal vents, port environments or brackish-water habitats) are better tolerated in the survival of Platynereis populations with a brooding strategy was noted in several studies (Lucey et al., 2015; Gambi et al., 2016; Wäge et al., 2017). Being volcanic in origin, the Macaronesia islands harbour in its vicinities a large amount of $\mathrm{CO}_{2}$ vents characterized by the low pH waters (Viveiros et al., 2020; González-Delgado et al., 2021), which might favour the proliferation of brooder worms instead of free spawners. Sampling in the $\mathrm{CO}_{2}$ vents or in subtidal habitats in general could also provide additional Platynereis lineages yet to be explored, which could be unique to each island, given how important the Macaronesia archipelagos seem to be in the cryptic diversity of marine invertebrates (Desiderato et al., 2019; Vieira et al., 2019).

### 6.5 Final remarks

Among the 10 different Platynereis lineages from Europe uncovered with molecular data, seven of them had particular geographical distributions, either confined to the western (MOTUs 1 and 6) or eastern (MOTU 3) Mediterranean Sea, NE Atlantic (MOTU 2), Macaronesia islands (MOTU 7) and sometimes exclusive to a single island (MOTU 8) or limited to a few islands within a single archipelago (MOTU 5), indicating also a high level of endemism. MOTUs 4, 9 and 10 were sympatric with at least two other lineages from the group, with MOTUs 9 and 10 revealing geographically structured populations through their COI haplotypes. No considerable genetic structure was found in each sampled island within MOTU 7 despite the existence of two other lineages in this region of the Atlantic. These findings call for a better recognition of the role of both the Macaronesian archipelagos and the Mediterranean Sea as promoters of extensive diversification of marine invertebrates and emphasize the importance of the conservation of the biodiversity of the intertidal rocky shore of these regions. Despite the two new species erected in this study ( $P$. macaronesiensis sp. nov. and $P$. jourdei sp. nov.) and further clarification regarding the status of $P$. agilis, $P$. dumerilii and $P$. massiliensis, five other lineages still remain unnamed and in need of further sampling effort and morphological examination. This is particularly the case with MOTU 2, an apparently rarer lineage from the NE Atlantic, that seems to be easy to find in Norway based on sampling campaigns under the Norwegian projects (BIN: BOLD:AAC5474, BOLD Systems). Three other unaccepted synonyms are reported for this area as well (e.g. Denmark), but descriptions are very incomplete or referring only to epitoke forms. Topotypic specimens of $P$. massiliensis and further studies on its reproductive biology are also needed to pinpoint if this species actually corresponds to the lineage assumed in this, and in the previous studies (Wage et al., 2017; Calosi et al., 2013; Kara et al., 2020).

Failure to recognise this hidden biodiversity may compromise the accuracy and the interpretation of biomonitoring and ecological data for Platynereis and its use as a model species (Özpolat et al., 2021). Integrative taxonomy is thus essential to solve these uncertainties and to allow naming the involved undescribed species. Otherwise, most molecular data providing enough support for species hypothesis will continue to be unused, and large fractions of biodiversity will persist unnoticed.

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Chapter 7

# Large cryptic hotspot in the <br> Mediterranean: the striking case of the Perinereis cultrifera (Annelida: Nereididae) species complex 


#### Abstract

Molecular data have been suggesting the existence of a cryptic species complex within the Perinereis cultrifera taxon, which has not been confirmed yet. In this study, it is performed a morphological and DNA analysis (COI, 16S rRNA and 28S rRNA) of Perinereis specimens from different European localities, extending from the NE Atlantic to the Mediterranean Sea, including the Macaronesia islands (Azores and Canary islands). Two major phylogenetic clades with at least 13 divergent and completely sorted lineages were uncovered, eight of which occurred solely in the Mediterranean Sea. An additional lineage belonging to $P$. oliveirae, coexisting with the NE Atlantic lineage of the complex, was also retrieved as ingroup. Careful morphological inspection, combined with the deep divergence between the two major molecular clades and the perfect match of each clade to the specific paragnath and chaetal types, highlighted the existence of two very different groups of European Perinereis species: clade A, which shows features matching historical descriptions of $P$. cultrifera, and clade B corresponding to a poorly known and overlooked morphotype described as $P$. rullieri. Although paragnaths show a similar pattern in the two clades, their sizes are considerable smaller in $P$. rullieri and the chaetae are characterized by the coarse serration at the basis of the spiniger blades, as opposed to the lightly serrated blades from P. cultrifera. Furthermore, clade $A$ is composed of nine lineages, five of which are present in the western Mediterranean, three are unique to the Macaronesia islands, and a single one is distributed along the NE Atlantic coast. Clade B totals four different lineages, three inhabiting the Mediterranean, two of which from brackish-water environments, and one unique to the islands of Tenerife and Lanzarote. Morphometric data and paragnath counts also complemented the analysis with the former being able to distinguish between some of the lineages using several proportions between the length of the dorsal cirri and dorsal ligule, postero-dorsal cirri and head, and lastly, the number of segments and body length and width of the worm.

Formal description for most of the new lineages are in preparation and will be submitted in due time.


Keywords: Perinereis, Nereididae, paragnaths, morphometry, molecular data, cryptic species

### 7.1 Introduction

Over the last decades, molecular studies of broadly distributed benthic invertebrates have been revealing shared patterns of variation, namely the occurrence of populations with unusually high levels of divergence within the same morphospecies (Miglietta et al., 2011; Nygren, 2014). Further investigations of these deeply divergent lineages often lead to the recognition of diagnostic morphological features that were previously overlooked (Langeneck et al., 2020; Martin et al., 2020). Annelids in particular, seem to be a very diverse and cryptic group where morphologically similar species previously considered as a single cosmopolitan entity have been reported in a number of studies (e,g. Nygren and Pleijel, 2011; Cerca et al., 2020; Sampieri et al., 2021). A notable example is shared in a study by Brasier et al. (2016), which uncovered cryptic diversity in as much as $50 \%$ of 15 targeted polychaete morphospecies, in the Southern Ocean using mitochondrial DNA sequences.

Polychaetes are widely studied in an attempt to understand which features of their life-strategies have permitted colonization and survival in unpredictable habitats (Grassle and Grassle, 1974; Levin, 1984). Colonization of brackish waters has required strategies that involve many phases of the biological cycle, in particular, regarding reproduction and dispersion. Many organisms living in brackish water environments have a marine origin, which is the case of nereidids, a particular family of worms very abundant in these types of habitats (Mettam, 1980). Perinereis cultrifera (Grube, 1840) (type locality: Naples, Italy) usually occurs among rocky shores with algae and/or mussels attached to the rocks, or burrowed in sediment under stones and cobbles in the intertidal or upper subtidal areas. However, in spite of being mostly a marine species, it can also be present in brackish environments, buried in mud and found together with specimens of Marphysa sanguinea (Montagu, 1813) (Prevedelli and Simonini, 2003). It is known to occur along the Western coasts of Europe and the Mediterranean (Fauvel, 1923), and it has been also recorded in the Western Atlantic Ocean, Red Sea, Arabian Sea, Indian Ocean, Yellow Sea, and Pacific Ocean (Hutchings et al., 1991; Wehe and Fiege, 2002; Park and Kim, 2017). Evidence for the existence of cryptic diversity within this species was already reported by Scaps et al. (2000) who identified a clear divergence between populations from the North of France and Algeria. The authors used alloenzymes complemented with morphological features, such as the mean number of paragnaths and the number of teeth per half jaw to distinguish between populations. Later, Maltagliati et al., (2001), were also able to differentiate two $P$. cultrifera populations from the Elba Island (Western Italy), using similar techniques, corresponding to a brackish-water habitat and an adjacent marine site. More recently, Park and Kim (2017) described a new species belonging to the P. cultrifera complex by comparing Asian specimens from Korea, China, Taiwan and Japan to Portuguese samples using the mitochondrial COI
gene. The new species Perinereis euiini Park \& Kim, 2017 is characterized by the absence of lateral groups of paragnaths on area III (Fig. 4.A, B), in contrast to the presence of those in P. cultrifera as well as notopodial dorsal ligules greatly expanded in posterior parapodia.

Perinereis cultrifera, like many other nereidids, is a semelparous species and has been reported to be either atokous (Marcel, 1962) or undergo epitokous metamorphosis prior to reproduction (Cassai and Prevedelli, 1998; Scaps et al., 2000). Semelparous species usually die after reproduction and generally use a large proportion of their resources in the production of gametes (Eckelbarger, 1994). In Europe, differences in the reproductive period and mode were found between the Northeast Atlantic and Mediterranean populations. Reproduction in the latter has been reported as both epitokous (Scaps et al., 1992; Cassai and Prevedelli, 1998) and atokous (Marcel, 1962), with sexually mature individuals spotted as early as March (Ansaloni et al., 1986) in the Venice Lagoon, and in May near the Golf of Tunis (Zghal and Ben Amor, 1989). In the English Channel, only epitokous reproduction is reported (Cazaux, 1965) and spawning occurs from May to June and sometimes July (Scaps et al., 2000).

These differences in the reproduction and the recent description of new species within the $P$. cultrifera complex, raises the suspicion about the existence of multiple lineages awaiting discovery within this taxon. To this end, this chapter used a multi-locus approach, combined with morphological and geographic data, to check for the suspected existence of hidden species in several European populations of P. cultrifera from the NE Atlantic, the Macaronesia islands (Azores and Canaries) and the Mediterranean Sea.

### 7.2 Material and methods

### 7.2.1 Specimen sampling

A total of 166 Perinereis specimens were hand-collected, apparently belonging to the $P$. cultrifera morphospecies, in rocky shores among algae or mussels, or in the sediment under cobbles during low tide, along the European coasts and Macaronesia islands. Sampling in brackish-water environments was also performed in the Mediterranean. The specimens were preserved in $96 \%$ ethanol.

In Portugal, samples were collected in the northern beaches of Canto Marinho and Areosa, as well as in the São Miguel island from the Azores. In Spain, specimens were collected in Basque country, and in the Canary islands of Tenerife, Gran Canaria, Lanzarote, Fuerteventura and El Hierro. Specimens were also collected in France, at the West (Arcachon Bay and Marennes-Oleron), and Northwest (Roscoff and Morlaix) coasts, and in the south of Norway (Stavanger). The Mediterranean was a main target for
this work, especially in the western part of the basin, where specimens were collected in the Tyrrhenian Sea off Corsica Island (France) and along the coast of Tuscany (Italy: Montecristo and Pianosa Islands, Calafuria and in the brackish-water Portoferraio Saltern of Elba Island). Lastly, additional specimens were obtained from the Eastern Mediterranean region from the Adriatic Sea (Trieste and Ravenna, Italy) and from the island of Crete (Greece).

Additional 19 Nereidid specimens belonging to Neanthes nubila (Savigny, 1822) from Praia Norte (Portugal), Perinereis marionii (Audouin \& Milne Edwards, 1833) from Plymouth (Great Britain) and Perinereis aibuhitensis (Grube, 1878) from South Korea were collected for comparison purposes and used as outgroups in the DNA analysis detailed below. The biological material is deposited at the Biological Research Collection of the Department of Biology of the University of Aveiro (COBI at DBUA), Portugal; specimens from Norway deposited at the Norwegian University of Science and Technology, NTNU University Museum (Bakken et al., 2021) and specimens from Corsica will be deposited in the Muséum national d'Histoire naturelle (MNHN).

### 7.2.2 Molecular data and storing institution

DNA sequences of the $5^{\prime}$ end of the mitochondrial cytochrome oxidase subunit I (mtCOI-5P, approximately 658 bp ) were obtained for 165 Perinereis specimens and respective outgroups (19 specimens). A representative number of specimens per location were also sequenced for the mitochondrial 16 S rRNA and D2 region of nuclear 28S rRNA, resulting in a total number of 93 sequences for 16 S and 77 sequences for $28 \mathrm{~S}-\mathrm{D} 2$, except for $P$. aibuhitensis which lack $28 \mathrm{~S}-\mathrm{D} 2$ sequences. Lastly, COI sequence data (KY249122 - KY249124; Park and Kim, 2017) from P. euiini, an Asian pseudo-cryptic lineage described for the P. cultrifera complex, collected at Gyeongsangnam-do (South Korea) completed the final dataset.

DNA was extracted, amplified, sequenced, and assembled as described in the Chapter 3 of this thesis. Regarding PCR conditions, primers and sequence lengths for the different markers see Chapter 3, Table 3.1. Supplemental Table S7.1 details the sampling locations, public BIN accession numbers and voucher data for the original material.

The dataset used for molecular analysis and its metadata can be accessed at the BOLD Systems under the project "Perinereis species complex (DS-MTPC)" and will be publicly available upon this chapter's acceptance for publication in a peer reviewed journal. Specimens that were exhausted in the DNA analysis were assigned only with the Process ID from the BOLD systems
(http://v4.boldsystems.org/), corresponding to the ones from the Azores (MTPC062-20-MTPC064-20), Marennes-Oleron (MTPC139-20) and Fuerteventura (MTPC048-20).

### 7.2.3 Phylogenetic analysis

Sequences from the mtDNA COI-5P, rRNA 16 S and the D2 region of the rRNA 28 S were concatenated in MEGA 11.0.10 software (Tamura et al., 2021) and aligned with MAFFT online (https://mafft.cbrc.jp/alignment/server/, Katoh and Standley, 2013). The phylogenetic analyses of the concatenated loci were performed through maximum likelihood (ML) and Bayesian inference (BI) for the concatenated dataset. Best-fit models were selected using the Akaike Information Criterion in the jModeltest software (Guindon and Gascuel, 2003; Darriba et al., 2012). Maximum Likelihood phylogenies were performed in MEGA 11.0.10 with 1000 bootstrap runs with the GTR model with equal rates across sites (GTR). MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003) was used to conduct the Bayesian analysis. For COI the Hasegawa-Kishino-Yano gamma distributed rates across sites (HKY +G) was applied for the first two positions and the General Time Reversible model with equal rates across sites (GTR) for the third position. The latter model was also applied to the remaining loci (16S and 28S-D2). Number of generations was set to 10000000 , and sample frequency to 500 . Twenty-five percent of the samples were discarded as burn-in (burninfrac $=0.25$ ). The resulting tree files were checked for convergence in the effective sampling sizes (ESSs >200) with Tracer 1.7 software (Rambaut et al., 2018) and then analysed in Figtree 1.4.3 (http://tree.bio.ed.ac.uk/software/figtree/). The final version of the tree was edited with the software Inkscape 0.92 .3 (https://www.inkscape.org). The BI tree was displayed in the results with the addition of the ML support values if a similar topology is found.

### 7.2.4 MOTU clustering

Four delineation methods to the concatenated alignment were applied to obtain Molecular Operational Taxonomic Units (MOTUs). Two are distance-based methodologies, such as the Barcode Index Number (BIN), which makes use of the Refined Single Linkage (RESL) algorithm implemented in BOLD (Ratnasingham and Hebert, 2013), exclusive only to the COI locus; and the new Assemble Species by Automatic Partitioning (ASAP, Puillandre et al., 2021), implemented in a web interface (https://bioinfo.mnhn.fr/abi/public/asap/asapweb.html) with default settings using the K2P distance matrix. Additionally, by making use of a dedicated web interface (https://species.h-its.org/), two other delineation methods were applied, but based on tree topologies: The Generalized Mixed Yule Coalescent
(GMYC) single threshold model (Fujisawa and Barraclough, 2013) and the Poisson Tree Processes (bPTP; Zhang et al., 2013). BEAST 2.4.6 (Bouckaert et al., 2014) was used to generate the Bayesian ultrametric tree for the GMYC, with the GTR model with equal rates across sites (based on AIC criteria) and four independent runs for 50000000 MCMC generations, sampled every 5000 generations. Tracer 1.6 software was used to estimate convergence ESSs > 200 for all parameters. The consensus tree was obtained using TreeAnnotator 2.4.6 (Bouckaert et al., 2014) and loaded into the Figtree software. ML phylogenies obtained above in the "phylogenetic analysis" section were employed for the bPTP results. A final consensus on MOTUs was chosen using the majority rule and in case of draw, the most conservative MOTU was selected.

### 7.2.5 Genetic diversity and structure

Haplotype networks were made through the PopART software (Leigh and Bryant, 2015) using the method of Templeton, Crandall and Sing (TCS, Clement et al., 2002) based on the original data to evaluate the relationship between the haplotypes and their geographical distribution. COI-based indices of genetic diversity were estimated for each MOTU, namely number of haplotypes (h), haplotype diversity (Hd), polymorphic sites (S), nucleotide diversity ( $\pi$ ), Fu \& Li D and Tajima D statistical tests using DNASP 5.10 (Librado and Rozas, 2009). The mean genetic distances (Kimura-2-parameters, K2P) within and between MOTUs were calculated in MEGA 11.0.10.

### 7.2.6 Morphometric and morphological analysis

Representative specimens from different Perinereis MOTUs were used for morphometric analysis and compared against each other to complement the molecular data. Lineages with less than three specimens available, very small specimens or with compromised structural integrity (therefore unsuitable for morphometric studies) were not used in this analysis. A minimum of 5 specimens with optimal conditions (i.e. specimens with the morphological characters proposed for this study and whenever possible, similar in size) per MOTU were chosen.

The following characters were selected and measured (Fig. 7.1.A, B): the number of segments $(\mathrm{NS})$; the length ( mm ) of the entire worm (WL), parapodium up to the median ligule (CLL), antennae (AL), palps (PL), antero-dorsal cirri and postero-dorsal cirri (DSTL, DLTL, respectively), dorsal and ventral cirri of median segments ( $\mathrm{DCL}, \mathrm{VCL}$ ), dorsal and ventral ligule of median segments ( $\mathrm{DLL}, \mathrm{VLL}$ ) and head (HL); the width (mm) of the worm with parapodia (WWP) and without parapodia (WW), head (HW), dorsal and
ventral ligule (DLW, VLW); and the distance between the anterior eyes (DAE), distance between the posterior eyes (DPE), distance between the anterior and posterior eyes (DAPE) as well the height (mm) of the parapodium (CLH). WW, WWP and the different parapodia structures were measured from the worm's widest part, usually from segment 30 to 60 depending on the worm size. The distance between the eyes was measured from the centre of the eyespots to avoid possible different individual responses to fixation as in the case of hesionids in Martin et al. (2017).


Fig. 7.1. Schematic of the Perinereis cultrifera morphotype showing the measurements used in the morphometric analysis. (A) Anterior end. (B) Parapodia. (C) Paragnath areas. Abbreviations: the length of the parapodium up to the median ligule (CLL), antennae (AL), palps (PL), antero-dorsal cirri and postero-dorsal cirri (DSTL, DLTL, respectively), dorsal and ventral cirri of median segments (DCL, VCL), dorsal and ventral ligule of median segments (DLL, VLL) and head (HL); the width of the worm with parapodia (WWP) and without parapodia (WW), head (HW), dorsal and ventral ligule (DLW, VLW); and the distance between the anterior eyes (DAE), distance between the posterior eyes (DPE), distance between the anterior and posterior eyes (DAPE) as well the height of the parapodium (CLH).

To minimize bias based on size variability, measurements taken to analyse the inter-lineage differences were based on ratios and used to create scatter plots between relevant morphological characters (i.e., AL/PL, DLTL/DSTL, AL/DLTL, AL/DSTL, PL/DLTL, PL/DSTL, AL/HL, PL/HL, AL/HW, PL/HW, HL/HW, DAE/DPE, DAPE/HL, DAE/HW, DPE/HW, WW/WWP, WL/WW, NS/WW, NS/WL, DCL/VCL, DLL/VLL, DLL/DLW, VLL/VLW, DCL/DLL, CLL/CLH, CLL/VCL, CLL/DCL). The measurements were done with a LEICA MC170 HD stereo microscope, with an incorporated measurement software. All remaining analyses were conducted using Microsoft Excel (Office 365 ProPlus).

Morphological observations were carried out with an Olympus SZX9 stereo microscope equipped with a camera lucida for line drawings. Stereo microscope images were taken with a Canon EOS1100D camera. Compound microscope images of parapodia and chaetae were obtained with a Zeiss Axioplan 2 imaging light microscope (Carl Zeiss, Oberkochen, Germany), equipped with a DP70 Olympus camera (Olympus Corp., Tokyo, Japan), after mounting the parapodia on a slide preparation using Aquamount (Gurr) liquid. The software Inkscape 0.92.3 (https://www.inkscape.org) was used to create the final images for the drawings of the parapodia. The morphotype for P. cultrifera bears small denticles on its pharynx called paragnaths (Fig. 7.1.A, C), which are often used to distinguish species within nereidids (Maltagliati et al., 2001; Scaps et al., 2000). Paragnath counts were performed from several specimens belonging to different lineages to see if any distinct patterns could be spotted.

Parapodial and chaetal terminology in the taxonomic section follows Bakken and Wilson (2005) with the modifications made by Villalobos-Guerrero and Bakken (2018). Pharynx paragnath terminology follows Bakken et al. (2009).

### 7.3 Results

### 7.3.1 Phylogenetic reconstruction

The number of consensus MOTUs obtained supported by the concatenated Bayesian tree (Fig. 7.2.A) was 13 . These lineages group in monophyletic clades with low intra-clade divergence ( $<3.6 \%$ ) and with high bootstrap values ( $>0.90$ ). Major clade A includes nine MOTUs whose morphology closely conforms to the original description of Perinereis cultrifera and that share similar large-sized paragnath.


Fig. 7.2. MrBayes tree from concatenated analysis of three markers and MOTU distribution. (A) Phylogenetic tree reconstructed for the Perinereis cultrifera complex using Bayesian inference based on concatenated COI, 16S and 28S
sequences, with information regarding the different MOTU delineation methods. BINs were used only for COI. Only the bootstrap values over 0.85 BI support are shown. Each different consensus MOTU is represented by the respective number, with the different colours corresponding to the respective geographic distribution. The outgroup (OUTG) belong to the species Perinereis aibuhitensis, Perinereis marionii and Neanthes nubila. MOTU GB1 correspond to the new Asian lineage from the Perinereis cultrifera complex. (B) Geographic distribution in Europe for the 14 retrieved MOTUs.

Major clade B is characterized by the presence of four MOTUs, morphologically similar to $P$. cultrifera but with small-sized paragnaths, with two of them only found in brackish-water environments (MOTUS 11 and 13). Clade B also includes the previously described species Perinereis oliveirae as an ingroup, which share morphological similarities with $P$. cultrifera.

Both clades include samples collected in the NE Atlantic, Mediterranean Sea, and the Macaronesia islands, with two major cryptic diversity hotspots found in the latter two regions (Fig. 7.2.B). Eight lineages are present in the Mediterranean, mostly focused at the western part of the sea, with five of them being sympatric, all belonging to clade A. Sympatry is observed between MOTUs 4, 6, 7, 8 and 9 in the Northern Tyrrhenian Sea. The Macaronesia islands harbour four distinct MOTUs, three of them present in the Canary Islands. The island of Gran Canaria in particular hosts two sympatric lineages, also belonging to clade A. The described Asian lineage corresponding to the P. cultrifera morphotype, groups in a distinct major clade (MOTU GB1, P. euiiin).

### 7.3.2 Genetic distances

The Global intra- and interspecific mean genetic distances of the 14 MOTUs are provided for each marker in Table 7.1. The mean intra-MOTU distance for COI is $0.4(0.0-3.6) \%$, while the average interMOTU distance is $20.9(7.5-30.4) \%$. For the 16S it ranges between $0.25(0.0-1.2) \%$ and $9.5(1.25-$ 16.3)\% for intra and inter-MOTU divergence, respectively, while for 28 S the corresponding distances are $0.9(0-6.5) \%$ and $6.5(0-18.8) \%$, respectively. The unusually high intra-MOTU values found in 28S-D2 from MOTU 4 did not originate from specimens from different populations and might be instead related to possible heterozygosity. In contrast, MOTUs 1 and 2, which have lineages from distant archipelagos, show almost no divergence in 28S, even though populations in MOTU 1 between El Hierro and Gran Canaria were responsible for the maximum intra-MOTU distances in COI and 16S markers. The most distant MOTUs were always between major clade A and B from the BI tree (Fig. 7.2), especially against
the ingroup $P$. oliveirae (MOTU 14), while the most similar MOTUs were recorded between MOTUs 11 vs 13; MOTUs 1 vs 2; MOTUs 3 vs 4 and MOTUs 12 vs 13 across all analysed markers.

Table 7.1. Mean intra and interspecific genetic distances (K2P) among the Perinereis complex for the three analysed markers (COI, 16S and 28S-D2), with focus on the distances between MOTUs in relation to the three closest and distant neighbours.

|  | Marker | MOTUs | Minimum Distance (\%) | $\begin{gathered} \text { Mean } \\ \text { Distance (\%) } \end{gathered}$ | Maximum distance (\%) |
| :---: | :---: | :---: | :---: | :---: | :---: |
| Within <br> MOTUs | COI | 1-14 | 0 | 0.4 | 3.6 (M1) |
|  | 16S |  | 0 | 0.25 | 1.2 (M1) |
|  | 28SD2 |  | 0 | 0.9 | 6.5 (M4) |
| Between MOTUs | COI | 1-14 | 7.5 | 20.9 | 30.4 |
|  | 16 S |  | 1.25 | 9.5 | 16.3 |
|  | 28SD2 |  | 0 | 6.5 | 18.8 |
| Most similar MOTUS | COO | 1 vs 2 | 7.5 | 8.6 | 9.7 |
|  |  | 12 vs 13 | 8.1 | 8.4 | 9.0 |
|  |  | 3 vs 4 | 9.3 | 10.2 | 11.2 |
|  | 16S | 1 vs 2 | 1.2 | 2.2 | 2.7 |
|  |  | 3 vs 4 | 1.7 | 2.2 | 2.7 |
|  |  | 12 vs 13 | 2.5 | 3.0 | 4.3 |
|  | 28SD2 | 1 vs 2 | 0 | 0.4 | 0.9 |
|  |  | 13 vs 11 | 0.2 | 0.3 | 0.4 |
|  |  | 10 vs 13 | 0.2 | 0.3 | 0.9 |
| Most distant MOTUs | COI | 7 vs 14 | 29.2 | 30.1 | 30.4 |
|  |  | 8 vs 14 | 27.8 | 28.7 | 29.5 |
|  |  | 3 vs 14 | 27.2 | 28.2 | 29.4 |
|  | 16 S | 12 vs 5 | 15.1 | 15.5 | 16.3 |
|  |  | 12 vs 1 | 14.2 | 15.3 | 16.3 |
|  |  | 5 vs 13 | 15.1 | 15.4 | 15.7 |
|  | 28SD2 | 4 vs 14 | 10.9 | 15.0 | 18.8 |
|  |  | 7 vs 14 | 13.6 | 14.4 | 15.7 |
|  |  | 8 vs 14 | 12.1 | 13.3 | 14.9 |

### 7.3.3 Haplotype networks

The COI network (Fig. 7.3) shows high number of mutations between all the different MOTUs and reveals geographically structured populations within four MOTUs: 1) MOTU 3, where haplotypes are shared between populations from Norway and the Atlantic part of France, but different when compared
to populations from Atlantic France and Portugal; 2) MOTU 12 has distinct haplotypes between populations from the Adriatic Sea (Ravenna, Italy) and the island of Crete (Greece); 3) MOTU 1, with the separation between the islands of Gran Canaria and EI Hierro; 4) MOTU 4, where haplotypes are sorted between the Western (Tuscany area) and Eastern (Trieste) Mediterranean. Haplotype sorting in MOTUs 1 and 4 reflects the two distinct sub-clades found in each of them, as patent in the BI tree, which however were not consistently diagnosed as separate MOTUs among the 4 delimitation methods (Fig. 7.1).


Fig. 7.3. COI haplotype network for all the 14 MOTUs based on the original Perinereis data and respective outgroups. Each haplotype is represented by a circle and number of haplotypes are according to the displayed scale. Colours indicate the geographic location of the haplotype. Numbers correspond to the number of mutational steps between haplotypes. Lines without numbers means only one mutation between haplotypes.

Similarly to COI, the 16 S network (Fig. 7.4.A) completely sorted all MOTUs, and no haplotype has a central position in the networks. The 28S-D2 network (Fig. 7.4.B) reveals closely related haplotypes with low number of mutations between MOTUs 6,8 and 9 ; and between MOTUs 11 and 13. Haplotype sharing can be found between MOTU 1 and 2. However, no 28 S haplotypes were shared between the populations from El Hierro and Gran Canaria within MOTU 1. The high number of mutations between

28 S haplotypes from MOTU 4 are responsible for the high \% intra-specific distances found in this marker. Excluding this MOTU 4, the mean intraspecific distance would decrease to $0.54 \%$, and would become similar to the mean values found for 16 S and CO , albeit with considerable lower maximum distances ( $\approx 1.8 \%$ ).

The COI haplotype diversity is relatively low (mean $\mathrm{Hd}<0.60$, Table 7.2), sometimes very low as seen in MOTU $11(\mathrm{Hd}=0)$. However, an exception to this low diversity pattern is found in MOTUs 3, 4, 6 and 10 which can surpass the 0.85 Hd value. Except for MOTU 1, none of the remaining MOTUs have a significant Tajima $D$ and Fu and Li's $D$ tests, with the neutral model of nucleotide substitutions being accepted for all lineages, but MOTU 1.


Fig. 7.4. Haplotypes networks for $16 S$ (A) and 28S-D2 (B) for all the 13 MOTUs based on the original Perinereis data and respective outgroups. The outgroup P. aibuhitensis is not present in the 28 S network. Each haplotype is represented by a circle and number of haplotypes are according to the displayed scale. Colours indicate the geographic location of the haplotype. Numbers correspond to the number of mutational steps between haplotypes. Lines without numbers means only one mutation between haplotypes.

Table 7.2. Indices of genetic diversity estimated for each MOTU, based on COI and from the original data. Number of sequences ( n ); nucleotide diversity ( tt ), number of haplotypes ( h ), haplotype diversity ( Hd ) and number of variable sites (S). Region abbreviations as stated in Fig. 7.2.B. Values in bold are significative.

|  | Region | N | h | Hd | S | tt | $\begin{gathered} \text { Fu and Li's } \\ \mathbf{D}^{\star} \end{gathered}$ | $\begin{gathered} \text { Tajima's } \\ \text { D } \end{gathered}$ |
| :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: |
| MOTU 1 | GC; EH | 13 | 3 | 0.295 | 23 | 0.00538 | $\begin{gathered} -2.92928 \\ P<0.02 \end{gathered}$ | $\begin{aligned} & -2.25711 \\ & P<0.001 \end{aligned}$ |
| MOTU 2 | SMA | 3 | 3 | 1 | 8 | 0.00811 | - | - |
| MOTU 3 | PTC; PTA; <br> FRO; FRA; <br> FRR; FRM; NOS | 16 | 9 | 0.925 | 13 | 0.00426 | $\begin{gathered} -0.99787 \\ P>0.10 \end{gathered}$ | $\begin{gathered} -1.10030 \\ P>0.10 \end{gathered}$ |
| MOTU 4 | FRC; ITT; ITTR | 11 | 7 | 0.873 | 21 | 0.01375 | $\begin{aligned} & 0.42805 \\ & P>0.10 \end{aligned}$ | $\begin{aligned} & 0.57249 \\ & P>0.10 \end{aligned}$ |
| MOTU 5 | GC; FV | 8 | 4 | 0.758 | 8 | 0.00413 | $\begin{aligned} & -0.19689 \\ & P>0.10 \end{aligned}$ | $\begin{aligned} & -0.58166 \\ & P>0.10 \end{aligned}$ |
| MOTU 6 | FRC; ITT | 7 | 5 | 0.857 | 10 | 0.00557 | $\begin{gathered} -1.05316 \\ P>0.10 \end{gathered}$ | $\begin{aligned} & -0.85853 \\ & P>0.10 \end{aligned}$ |
| MOTU 7 | ITT | 2 | 1 | 0 | 0 | 0 | . | . |
| MOTU 8 | ITT; FRC | 12 | 5 | 0.576 | 10 | 0.00338 | $\begin{aligned} & -1.34389 \\ & P>0.10 \end{aligned}$ | $\begin{gathered} -1.53514 \\ P>0.10 \end{gathered}$ |
| MOTU 9 | FRC | 8 | 2 | 0.250 | 1 | 0.00041 | $\begin{aligned} & -1.12639 \\ & P>0.10 \end{aligned}$ | $\begin{gathered} -1.05482 \\ P>0.10 \end{gathered}$ |
| MOTU 10 | TE; LA | 20 | 9 | 0.863 | 14 | 0.00486 | $\begin{gathered} -1.02857 \\ P>0.10 \end{gathered}$ | $\begin{gathered} -0.69707 \\ P>0.10 \end{gathered}$ |
| MOTU 11 | ITT (brackish waters) | 6 | 1 | 0 | 0 | 0 | - | - |
| MOTU 12 | ITRA; GRC | 30 | 11 | 0.789 | 24 | 0.00896 | $\begin{gathered} -0.73145 \\ P>0.10 \end{gathered}$ | $\begin{gathered} -0.09323 \\ P>0.10 \end{gathered}$ |
| MOTU 13 | ITT (brackish waters) | 8 | 3 | 0.607 | 5 | 0.00364 | $\begin{aligned} & 0.74709 \\ & P>0.10 \end{aligned}$ | $\begin{aligned} & 1.09226 \\ & P>0.10 \end{aligned}$ |
| MOTU 14 | $\begin{gathered} \text { PTC; PTA; } \\ \text { SPB } \end{gathered}$ | 21 | 7 | 0.629 | 15 | 0.00256 | $\begin{gathered} -2.64799 \\ P<0.05 \end{gathered}$ | $\begin{gathered} -2.17192 \\ P<0.01 \end{gathered}$ |

### 7.3.4 Morphological findings

The paragnath patterns present in the worm's pharynx are consistent with the descriptions of $P$. cultrifera (Fig. 7.1, Grube, 1840). However, a considerable difference in paragnath size is evident when comparing between the lineages from clade A (MOTUs 1, 3-9, Figs. 7.5-7.7) with larger paragnaths, against clade B (MOTUs 10-14, Figs. 7.8-7.9) with smaller paragnaths. In the case of MOTU 2, morphological analysis was impossible and therefore only genetic data are available for this work.


Fig. 7.5. Exposed pharynx and respective paragnath patterns in several lineages from the Perinereis cultrifera complex, belonging to the clade A from Fig. 7.2. (A) MOTU 1, specimen DBUA0002498.01.v03, dorsal view. (B) MOTU 1, ventral view. (C) MOTU 3, specimen DBUA0002510.01.v01, dorsal view. (D) MOTU 1, ventral view. (E) MOTU 4, specimen DBUA0002507.01.v01, dorsal view. (F) MOTU 4, ventral view.

MOTU 14 ( $P$. oliveirae) is characterized by longer bar-shaped paragnaths in areas VI (Fig. 7.10.C) compared to the P. cultrifera morphotype, which often break due to fixation in ethanol giving the impression of the existence of 4 bars instead of 2 (Fig. 7.10.A). Additionally, MOTU 14 also has higher paragnath numbers in area IV, often doubling the amount found in the remaining MOTUs. Furthermore, this lineage is also distinguished by the very noticeable shorter tentacular cirri (Fig. 7.10.C) and shorter length in the parapodia ligules and cirri (Fig. 7.11.M-H).


Fig. 7.6. Exposed pharynx and respective paragnath patterns in several lineages from the Perinereis cultrifera complex, belonging to the clade A from Fig. 7.2. (A) MOTU 5, specimen DBUA0002497.01.v01, dorsal view. (B) MOTU 5, ventral view. (C) MOTU 6, specimen DBUA0002504.01.v03, dorsal view. (D) MOTU 6, ventral view. (E) MOTU 7, specimen DBUA0002509.01.v02, dorsal view. (F) MOTU 7, ventral view.

Regarding the remaining MOTUs, although there is some phenotypic variation in paragnath numbers (Table 7.3), considerable differences appear to be present in MOTUs 11 and 12 with a higher amount of paragnaths in area IV (around 18 to 25) and a lower amount in areas VII and VIII in MOTU 11 compared to the other lineages. The latter also have a distinctive small paragnath bar in areas VI .


Fig. 7.7. Exposed pharynx and respective paragnath patterns in several lineages from the Perinereis cultrifera complex, belonging to the clade A from Fig. 7.2. (A) MOTU 8, specimen MNHN8-2, dorsal view. (B) MOTU 8, specimen MNHN8-2, ventral view. (C) MOTU 9, specimen MNHN7-3, dorsal view. (D) MOTU 9, specimen MNHN7-3, ventral view.

Additional paragnath characteristics can be pointed out regarding MOTUs 1 and 5 (Canary islands) with lighter, smooth paragnaths in the maxillary ring (areas I-IV, Fig. 7.1), having the appearance of button-like shape (Fig. 7.5.A, B; Fig. 7.6.A, B), instead of spines as observed in the remaining MOTUs. Number of teeth per half jaw remain more or less stable in the complex (Table 7.3), except in MOTU 4 with only 4 teeth against the 5-6 found in the other MOTUs. The presence of dorsal papillae-like protuberances (Fig. 7.10.E) seem to be unique to the sister MOTUs 3 (NE Atlantic, Fig. 7.5.C; Fig. 7.10.E) and 4 (Western and Eastern Mediterranean Sea, Fig. 7.5.E).

Schemes for the parapodia morphotypes from the first 10 anterior setigers, median segments and last 10 posterior setigers are detailed in Figures 7.11-7.13 for all the MOTUs, except MOTU 2. Apart from MOTU 14, no major differences were found in the parapodia structures of median segments between the analysed MOTUs, with the complex having dorsal ligules longer and wider than ventral ligules and dorsal cirri longer than ventral cirri.


Fig. 7.8. Exposed pharynx and respective paragnath patterns in several lineages from the Perinereis rullieri complex, belonging to the clade B from Fig. 7.2. (A) MOTU 10, specimen DBUA0002496.02.v05, dorsal view. (B) MOTU 10, ventral view. (C) MOTU 11, specimen DBUA0002515.01.v02, dorsal view. (D) MOTU 11, ventral view.


Fig. 7.9. Exposed pharynx and respective paragnath patterns in several lineages from the Perinereis rullieri complex, belonging to the clade B from Fig. 7.2. (A) MOTU 12, specimen DBUA0002501.01.v06, dorsal view. (B) MOTU 12, ventral view. (C) MOTU 13, specimen DBUA0002516.01.v03, dorsal view. (D) MOTU 13, ventral view.

Additionally, dorsal cirri are usually about the same size; however, MOTUs 11 and 13 are characterized by the presence of dorsal ligules larger than dorsal cirri (Fig. 7.11.D-F and Fig. 7.11.J-L, respectively). MOTUs 10, 11 and 13 also show a considerable variation with the presence of an overgrown dorsal ligule in the posterior parapodia (Fig. 7.11.C). The shape of the ligules (not including the neuracicular ligule) for all the MOTUs is either oval or triangular.

Table 7.3. Paragnath counts for eight MOTUs ( $1,3-9$ ) within the $P$. cultrifera complex and for four MOTUs ( $10-13$ ) within the $P$. rullieri complex, including the ingroup corresponding to $P$. oliveirae (MOTU 14). Dorsal and ventral areas as exemplified in Fig. 7.1.C. Information regarding the number of teeth per jaw is also available for all the analysed MOTUs except MOTUs 2, 7 and 11. Data in bold show considerable deviations from the standard values and observations.

|  | Dorsal |  |  |  |  |  | Ventral | No <br>  Area I |
| :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: |
| Area II | Area V | Area VI | Area III | Area IV | Area VII <br> + VIII | Jaws |  |  |
| MOTU 1 | $\mathbf{2 - 5}$ | $8-11$ | 3 | 2 | $5-7$ | $15-19$ | 33 | $5-6$ |
| MOTU 2 | $?$ | $?$ | $?$ | $?$ | $?$ | $?$ | $?$ | $?$ |
| MOTU 3 | $1-2$ | $9-13$ | $2-3$ | 2 | $5-11$ | $10-18$ | $35-39$ | $5-6$ |
| MOTU 4 | 2 | 11 | 3 | 2 | 7 | 15 | 34 | $\mathbf{4}$ |
| MOTU 5 | $1-3$ | $7-11$ | 3 | 2 | $6-8$ | $12-16$ | $35-38$ | $5-6$ |
| MOTU 6 | $1-2$ | $8-10$ | 3 | 2 | $6-7$ | $12-15$ | $33-34$ | $5-6$ |
| MOTU 7 | $?$ | $?$ | $\mathbf{4}$ | 2 | $?$ | $?$ | 34 | $?$ |
| MOTU 8 | 2 | $6-11$ | $1-\mathbf{4}$ | 2 | $8-10$ | $12-19$ | $30-33$ | $5-6$ |
| MOTU 9 | $1-2$ | $11-14$ | $2-3$ | 2 | $9-11$ | $15-19$ | $32-33$ | $5-6$ |
| MOTU 10 | $1-2$ | $\mathbf{5 - 7}$ | $1-3$ | 2 | $6-10$ | $10-14$ | $34-39$ | $5-6$ |
| MOTU 11 | 1 | 9 | 3 | 2 (short) | $\mathbf{1 4 - 1 6}$ | $\mathbf{2 3}$ | $\mathbf{2 2 - 2 8}$ | $?$ |
| MOTU 12 | 1 | $6-11$ | 3 | 2 | $7-9$ | $\mathbf{1 8 - 2 5}$ | $29-33$ | $5-6$ |
| MOTU 13 | 1 | 8 | 3 | 2 | 10 | 19 | 36 | $5-6$ |
| MOTU 14 | $2-4$ | $9-14$ | $\mathbf{1}$ | 2 (long) | $\mathbf{2 1 - 2 8}$ | $\mathbf{2 7 - 4 0}$ | $39-42$ | $5-6$ |

All the 14 analysed MOTUs shared the same type of chaetae, which can be seen in Fig. 7.14. These are characterized by the presence of neuropodial and notopodial homogomph spinigers (Fig. 7.14.A, C, respectively), neuropodial heterogomph spiniger (Fig. 7.14.E) and neuropodial heterogomph falciger chaetae (Fig. 7.14.B), including a short version of the latter type (Fig. 7.14.D), which is usually found in the first 10 anterior chaetiger, but may appear in mid-segments as well. However, excluding the ingroup (MOTU 14), lineages from clade B (MOTUs 10-13) are characterized by the presence of
compound spiniger chaetae with blades coarsely serrated at its basis (Fig. 7.14.F), as opposed to blades lightly serrated along its edge from clade A (MOTUs 1, 3-9; Fig. 7.14.A, C, E).

The major morphologic highlights are summarized in Table 7.4, including some of the most relevant morphometric markers described below.

### 7.3.5 Morphometric measurements

Scatter plots with the most considerable morphometric proportions can be seen in Figs. 7.15 and 7.16. The original version (left) includes all the analysed MOTUs which often overlap with each other, and the cleaner version (right) displays only the MOTUs with the formation of partial or independent clusters between each other.

MOTUs 12 and 13 overlap with other MOTUs in most of the analysed proportions, showing high morphometric plasticity. In particular, the variation within MOTU 12 mainly results from differences in the measurements between the Greek and Adriatic populations, which are, however, not mirrored by the molecular divergence. Generally speaking, lineages with larger paragnaths (clade A, MOTUs1-9) are often separated from the ones with smaller paragnaths (clade B, MOTUs 10-14). Lineages isolated by large geographic distances and/or totally different regions (e.g. Islands vs Mediterranean vs NE Atlantic) also got independent morphometric clusters from each other for the most part. However, MOTUs 3 (NE Atlantic) and 10 (Canaries) usually partially overlap with each other, despite the high molecular divergence, unique geographic locations and very distinct paragnath sizes. Overlapped clusters are mostly seen between lineages within the same major clade.

Highlighted in Table 7.4, the proportions between the length of the dorsal cirri (DCL) with the dorsal ligule (DLL, Fig. 16E, F), the length of the postero-dorsal cirri (DLTL) with either the length of the head (HL, Fig. 16A, B) or antennae (AL, Fig. 7.16.C, D), the worm size (Fig. 7.15.A-D) based on the number of segments (NS), worm width (WW) and worm length (WL), and lastly the ratio between the length of the antennae (AL) and palps (PL, Fig. 7.15.E, F) seem to be the most effective markers in distinguishing between most of the analysed MOTUs. In particular, the proportion DCL/DLL is exceptionally good in distinguishing MOTUs 11, 13 (both from brackish water) and 14 by having ligules from median segments considerable larger than the dorsal cirri (>1.5 times), compared to the reversed ratio in MOTU 14 and apparent equal ratios found in the remaining MOTUs (Table 7.4). Furthermore, the latter proportion is also good in the separation of the MOTUs 9, 8, 1 and 3 varying in ascending measurement values, despite similar ratios (Fig. 7.16.F).


Fig. 7.10. Exposed pharynx and respective paragnath patterns for the ingroup Perinereis oliveirae within clade $B$ from Fig. 7.2: (A) MOTU 14, specimen DBUA0002495.01.v01, dorsal view, with partially broken paragnath bars; (B) MOTU 14, ventral view; (C) MOTU 14, specimen MTCM39, dorsal view, with well-preserved paragnath bars; (D) MOTU 14, ventral view. Focus on the papillae in the worm's dorsal body: (E) as seen in MOTU 3, specimen DBUA0002512.01.v04.

Table 7.4. Major highlights in morphologic and morphometric features, habitat type, distribution and sympatry status from the different MOTUs within P. cultrifera and P. rullieri, including the ingroup P. oliveirae. Ligule shape and ratio between the dorsal ligule and cirri are based on median segments. Ligule shape do not include neuracicular ligules. Abbreviations: DLTL, length of the postero-dorsal cirri; HL, head's length; DCL, length of the dorsal cirri; DLL, length of the dorsal ligule; Post. DL, posterior dorsal ligule; Par. size, Paragnath size. Data in bold show considerable differences.

|  | Dorsal papillae | Par. size | DLTL / HL | DCL / DLL | Ligule shape | Post. DL | Spiniger chaetae | Number Segments | Habitat | Sympatry | Distribution |
| :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: |
| MOTU 1 | Absent | Large <br> (maxillary ring smooth) | 2 x | 1.1 x | oval | Regular | Lightly serrated | $\begin{aligned} & 77 \\ & (75-80) \end{aligned}$ | Intertidal | 5 | Gran Canaria; El Hierro |
| MOTU 2 | ? | ? | ? | ? | ? | ? | ? | ? | Intertidal | - | Azores |
| MOTU 3 | Present | Large | 2.8 x | 1.1 x | triangular | Slightly expanded | Lightly serrated | $\begin{aligned} & 72 \\ & (45-100) \end{aligned}$ | Intertidal | 14 | NE Atlantic |
| MOTU 4 | Present | Large | 2 x | 1 x | triangular | Regular | Lightly serrated | 55 | Intertidal | 6; 7; 8; 12 | Western and Eastern Mediterranean |
| MOTU 5 | Absent | Large <br> (area VI <br> broad; <br> (maxillary ring smooth) | 2.2 x | 0.9 x | oval | Regular | Lightly serrated | 60 | Intertidal | 1 | Gran Canaria; Fuerteventura |
| MOTU 6 | Absent | Large | 2.8 x | 0.9 x | oval | Regular | Lightly serrated | 57 | Intertidal | 4; 7; 8 | Western Mediterranean |
| MOTU 7 | Absent | Large | 1.4 x | 0.9 x | triangular | Regular | Lightly serrated | 51 | Intertidal | 4; 6; 8 | Tuscany area (Italy) |
| MOTU 8 | Absent | Large | 3.1 x | 1.2 x | oval | Slightly expanded | Lightly serrated | $\begin{aligned} & 63 \\ & (60-67) \end{aligned}$ | Intertidal | 4; 6; 7 | Western Mediterranean |


|  | Dorsal papillae | Par. size | DLTL / HL | DCL/ DLL | Ligule shape | Post. DL | Spiniger chaetae | Number <br> Segments | Habitat | Sympatry | Distribution |
| :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: |
| MOTU 8 | Absent | Large | 3.1 x | 1.2 x | oval | Slightly expanded | Lightly serrated | $\begin{aligned} & \hline 63 \\ & (60-67) \end{aligned}$ | Intertidal | 4; 6; 7 | Western <br> Mediterranean |
| MOTU 9 | Absent | Large | 3.9 x | 1.2 x | triangular | Regular | Lightly serrated | $\begin{aligned} & 67 \\ & (55-77) \end{aligned}$ | Intertidal | 4; 6; 8 | Corsica island (France) |
| MOTU 10 | Absent | Small | 3.2 x | 1.1 x | triangular | Greatly expanded | Coarsely serrated | $\begin{aligned} & 110 \\ & (92-115) \end{aligned}$ | Intertidal | - | Tenerife; Lanzarote |
| MOTU 11 | Absent | Small (area VI very short) | 2.5 x | 1.65 x | triangular | Expanded | Coarsely serrated | 88 | Brackish waters | 13 | Elba island (Italy) |
| MOTU 12 | Absent | Small | 2.5 x | $1.15 \times$ | triangular | Slightly expanded | Coarsely serrated | $\begin{aligned} & 68 \\ & (55-88) \end{aligned}$ | Intertidal | 4 (Adriatic) | Eastern <br> Mediterranean |
| MOTU 13 | Absent | Small | $3 \times$ | 1.7 x | triangular | Greatly expanded | Coarsely serrated | $\begin{aligned} & 93 \\ & (89-105) \end{aligned}$ | Brackish waters | 11 | Elba island (Italy) |
| MOTU 14 (ingroup) | Absent | Small <br> (area VI very long) | 0.8 x | $\begin{aligned} & 0.80 \\ & x \end{aligned}$ | triangular | Regular | Lightly serrated | $\begin{aligned} & 112 \\ & (100-126) \end{aligned}$ | Intertidal | 3 | Northern Iberia |

(Table 7.4. Continuation)

A


Parapod 11

B


C


G


H


Parapod 30

I



Fig. 7.11. Drawings of the main morphological features found in the parapodia from different parts of the worm's body based on lineages from the "rullien""-type group and the ingroup $P$. oliveirae. MOTU 10, specimen

DBUA0002496.01.v01: (A) Parapod 11, posterior view. (B). Parapod 50, posterior view. (C) Parapod 100, posterior view; MOTU 11, specimen DBUA0002515.01.v01: (D) Parapod 10, anterior view. (E) Parapod 45, posterior view. (F) Parapod 75, posterior view; MOTU 12, specimen DBUA0002501.01.v03: (G) Parapod 10, posterior view. (H) Parapod 30, posterior view. (I) Parapod 60, posterior view; MOTU 13, specimen DBUA0002516.01.v01: (J) Parapod 10, posterior view. (K) Parapod 46, posterior view. (L) Parapod 80, posterior view; MOTU 14, P. oliveirae, specimen DBUA0002494.02.v02: (M) Parapod 9, posterior view. (N) Parapod 39, posterior view. (H) Parapod 60, posterior view.

The proportion DLTL/HL and DLTL/AL also show high variation between MOTUs. In particular the postero-dorsal cirri (DLTL) are about three times longer than the length of the head (HL) in MOTUs 3, 8, 9 (almost four times larger), in MOTUs 10 and 11 when compared to the remaining "cultrifera" and "rullierl" MOTUs (about two times) and in particular, MOTU 7, only about 1.4 times higher (Table 7.4). Perinereis oliveirae (MOTU 14) is the most distinct one by completely reversing the ratio and having the head slightly longer than the postero-dorsal cirri.

### 7.4 Discussion

The DNA sequence analysis of several specimens from different European regions confirmed the existence of a cryptic species complex under the name Perinereis cultrifera, already suspected by many authors (Scaps et al., 2000; Maltagliati et al., 2001; Park and Kim, 2017), thereby questioning its cosmopolitan status. A recent meiofauna review (Cerca et al., 2018) and recent polychaete studies (e.g. Tilic et al., 2019; Martin et al., 2020) also demonstrate that cryptic and pseudo-cryptic species often have geographically restricted distributions, with the range of cryptic species smaller than the parent morphospecies. However, previous Perinereis cultrifera studies in Europe only highlighted the possibility of two to three lineages based in populations from different habitat types in the same region (e.g. marine vs brackish, Maltagliati et al. 2001) or between different regions (e.g. NE Atlantic vs Mediterranean, Scaps et al. 2000). In this work, the combined molecular data from three different loci provided evidence for the existence of at least 13 deeply divergent and completely sorted lineages in Europe, eight of which occur in the Mediterranean Sea alone. An additional lineage belonging to $P$. oliveirae (MOTU 14, Fig. 10.A-D) is often mistaken with P. cultrifera (e.g. Lobo et al., 2016) given the similar morphological characters used to identify both species and their occurrence in the same habitat. Specimens collected in north of Portugal mostly belong to $P$. oliveirae, while $P$. cultrifera seem to be a rarer species in the region (around a $1 / 10$ ratio). This pattern is inverted further north in French coasts, where no single specimen from $P$. oliveirae
was collected. Additionally, the deep divergence between clade $A$ and $B$ and the perfect match of each clade to the specific paragnath and chaetal types, highlighted the existence of two clearly different groups within Perinereis. Clade A, which corresponds to the traditional descriptions of P. cultrifera (Grube, 1840; Fauvel, 1923; Hutchings et al., 1991; Núñez, 2004) and clade B, which corresponds to a poorly known and overlooked morphotype described as Perinereis rullieri Pilato, 1974.

### 7.4.1 Clade B, Perinereis rullieri species complex

The description of $P$. rullieri given by Pilato (1974) is very complete from the morphological point of view, but it is not easily accessible since the text is written in Italian and it has not been deposited in any digital repository, as far as I know. Moreover, Pilato (1974) did not establish a holotype, nor did he attribute a type locality, stating instead that the material examined was collected along the Eastern Sicilian Coast (i.e. Ionian Sea), along a stretch going from Acitrezza to Augusta. Upon further review of the original description, the main differences pointed by the author between $P$. cultrifera and $P$. rullieri correspond exactly to the main features spotted in this chapter regarding clade A and B (Fig. 7.2, Table 7.4), respectively. As observed by Pilato, even though paragnath patterns are very similar to $P$. cultrifera, their size is considerable smaller in $P$. rullieri and the chaetae are characterised by the coarse serration at the basis of spiniger blades, opposed to the lightly serrated blades from $P$. cultrifera. These features were confirmed as well based on two vials stored at the University of Pisa (Italy), corresponding to P. rullieri. According to Alberto Castelli (personal communication), these specimens were sampled off Catania and identified by Pilato himself, and are part of the material (preserved in formalin) mentioned in the original description. These specimens can therefore be considered as lectotypes of $P$. rullieri..

While the mentioned morphological features do not allow to unequivocally identify any of the MOTUs in clade $B$ as $P$. rullieri, further clues come from geographical and ecological data. The stretch going from Acitrezza to Augusta corresponds to approximately 30 km of coastline, and is characterized by the prevalence of fully marine environments and by the estuaries of two minor rivers. Conversely, brackish-water coastal ponds are completely absent from this area. Within the "rullierilike" clade B (Fig. 7.2.A), MOTUs 11 and 13 are from brackish-water samples, whereas MOTU 10 is unique to two islands in the Canary archipelago, and was not recorded in the Mediterranean. The reasonable inference would be that MOTU 12, which occurs in marine sites from the Eastern Mediterranean, corresponds to the original description of $P$. rullieri. The shape of the parapodia (MOTU 12, Fig. 7.11.G-I) also seems to match the original description.


Parapod 10

E

H

K


Parapod 30
C

Parapod 60
F

I

L

Parapod 54

Fig. 7.12. Drawings of the main morphological features found in the parapodia from different parts of the worm's body based on lineages from the "cultrifera"-type group. MOTU 1, specimen DBUA0002498.01.v03: (A) Parapod 10, posterior view. (B). Parapod 30, posterior view. (C) Parapod 60, posterior view; MOTU 3, specimen DBUA0002512.01.v02: (D) Parapod 10, posterior view. (E) Parapod 40, anterior view. (F) Parapod 70, posterior view; MOTU 4, specimen DBUA0002506.01.v01: (G) Parapod 9, posterior view. (H) Parapod 30, posterior view. (I) Parapod

60, posterior view; MOTU 5, specimen DBUA0002497.01.v02: (J) Parapod 12, posterior view. (K) Parapod 30, posterior view. (L) Parapod 54, posterior view

Pilato (1974) did not report any information regarding reproductive features of $P$. rullieri; however, several studies have analysed populations of this species from brackish-water environments (Cassai and Prevedelli, 1998; Prevedelli and Cassai, 2001; Prevedelli and Simonini, 2003). These populations might correspond to the same brackish-water lineages unveiled in this study (MOTUs 11 and 13, Fig. 7.2), which share similar morphological characters as the one reported in the original description.

In reproductive studies performed in samples from the Venice Lagoon (eastern Italy), even though belonging to the same genus and living in the same brackish environment, $P$. rullieri and P. cultrifera displayed different reproductive modalities. Perinereis rullieri reproduces in the atokous phase with large egg sizes, whereas P. cultrifera has conserved epitoky in its life-cycle similarly to the marine populations, with small egg sizes and greater number of oocytes (Cassai and Prevedelli, 1998; Prevedelli and Cassai, 2001). Confusion in the identification between these two species is probably the reason as to why different reproductive modes were previously reported for $P$. cultrifera, especially when comparing between the Atlantic and Mediterranean populations. Evidence that stressful conditions (e.g. hydrothermal vents, port environments or brackish-water habitats) are better tolerated in the survival of Nereidid species with a atokous reproductive strategy was noted in several studies (e.g. Lucey et al., 2015; Wäge et al., 2017). Moreover, P. rullieri encapsulates its eggs in a jelly matrix anchored to the substrate. Previous studies suggest this jelly matrix may be an adaptation to stressful environments, commonly used by estuarine species which tend to avoid free spawning (Smith, 1958; Cognetti-Varriale, 1971; Strathman, 1982). As noted by Prevedelli and Cassai (2001), other functions are thought to be linked to the jelly matrix as well, such as promoting successful fertilization (Sato and Osanai, 1996), protecting the embryos during early development (Vance, 1973; Schroeder and Hermans 1975), providing food supply for the developing embryos (Bookhout and Horn, 1949; Sato et al., 1982) and limiting dispersal of the young to keep them in a suitable habitat (Chapman, 1965; Gibbs, 1968; Nishihira et al., 1984). Given the morphological similarities of the brackish-water lineages to the original description of $P$. rullieri, it is not surprising seeing former studies using the reproductive differences between the latter species and $P$. cultrifera as a paradigmatic example of adaptation to brackish-water environments (Prevedelli and Cassai, 2001; Prevedelli and Simonini, 2003). However, MOTU 12 is a Mediterranean exclusive marine species and the most probable candidate to match the original description of $P$. rullieri by Pilato.
A


Parapod 6

B


Parapod 17


Parapod 25

C


F
Parapod 45
I



Parapod 4

H


Parapod 25


Parapod 9


Parapod 30

L

Parapod 50

Fig. 7.13. Drawings of the main morphological features found in the parapodia from different parts of the worm's body based on lineages from the "cultrifera"-type group. MOTU 6, specimen DBUA0002504.01.v02: (A) Parapod 6, posterior view. (B). Parapod 17, posterior view. (C) Parapod 19, posterior view; MOTU 7, specimen DBUA0002509.01.v01: (D) Parapod 5, anterior view. (E) Parapod 25, posterior view. (F) Parapod 45, posterior view; MOTU 8, specimen DBUA0002502.01.v01: (G) Parapod 4, posterior view. (H) Parapod 25, anterior view. (I) Parapod

48, posterior view; MOTU 9, specimen MNHN4-1: (J) Parapod 9, posterior view. (K) Parapod 30, posterior view. (L) Parapod 50, posterior view.


Fig. 7.14. Microscopic scans of all the different chaetae types found in the Perinereis complex. (A), Neurochaeta: homogomph spiniger with lightly serrated blades, chaetiger 12. (B) Neurochaeta: heterogomph falciger, chaetiger 50. (C) Notochaeta: homogomph spiniger with lightly serrated blades, chaetiger 19. (D) Neurochaeta: heterogomph short falciger, chaetiger 10. (E) Neurochaeta, chaetiger 5: heterogomph spiniger with lightly serrated blades (1); heterogomph short falciger (2). (F) Neurochaeta, chaetiger 50: homogomph spiniger with coarsely serrated blades (1); heterogomph falciger (2).

When comparing the paragnath numbers to the brackish-water lineage from Maltagliati et al. (2001), some phenotypic variation can be seen against MOTUs 11 and 13 . The minimum number of paragnaths reported in Area III are way higher in this chapter (10 vs 3), with the remaining Areas having values within similar range.

### 7.4.2 Clade A, Perinereis cultrifera species complex

Hutchings et al. (2001) examined what were probably syntypes (Naples, Italy) on the original material of $P$. cultrifera. That publication presents parapodia drawings very similar to MOTU 8 from tis current study. Additionally, parapodia drawings from the $P$. cultrifera population described by Pilato (1974) also resemble the original morphotype, with the pharynx having as well the characteristic large paragnaths as seen in all specimens from clade A (Figs. 7.5-7.7). Analysis of paragnath counts show some variability between MOTUs, especially between clades $A$ and $B$, with the latter having a large amount in Areas III and IV for some lineages, but no considerable variation within clade A. When comparing to the P. cultrifera population from Maltagliati et al. (2001), the obtained values are within or very close to their reported range, except in Area I, with considerably lower values (max: 5 paragnaths) compared to their study (max: 11 paragnaths). Scaps et al. (2000), also reported similar values to this chapter, except in their Algerian Mediterranean population with an impressive number of paragnaths in Areas VII/VIII, reaching values as high as 72 against 42 from this study. There is a high probability of the Algerian population being an additional new MOTU, and further COI data is desirable to confirm this hypothesis.

Currently, according to WoRMS (Read and Fauchald, 2022), out of the seven unaccepted synonymies attributed to P. cultrifera, five are described from the NE Atlantic, with only two being Mediterranean species: Perinereis hedenborgiKinberg, 1865 (type locality: Alexandria, Egypt), with a very brief description, and Spio ventilabrum Delle Chiaje, 1827 (type locality: somewhere in the Mediterranean sea), tagged as a questionably synonym of species listed, which might belong to a different group entirely. Due to the extremely scanty descriptions, it is not possibly to reconstruct their morphological correspondence to the MOTUs here examined. However, when switching to the single NE Atlantic lineage from clade $A$ (MOTU 3), some clues can be found when reviewing the descriptions for the available synonyms. For instance, none of the previous descriptions mention the unique papillae-like protuberances in the dorsal body plan found both in MOTUs 3 and 4 (Figs. 7.5.E, 7.10.E) and can be excluded as possible variants that were inadequately synonymised.


Fig. 7.15. Scatter plots with the most considerable morphometric proportions in distinguishing nine MOTUs (left) which often overlap with each other, and the cleaner version (right) displaying only the MOTUs with the formation of partial or independent clusters between each other. (A) The worm's length (WL) and width (WW). (B) The worm's length (WL) and width (WW), excluding MOTUs 10, 12 and 14. (C) Comparison between the number of segments (NS) and worm's width (WW). (D) Comparison between the number of segments (NS) and worm's width (WW), excluding MOTUs 3, 10 and 12 ( E ) Morphometric proportions between the length of the palps (PL) and the length of the antennae (AL). (F) Morphometric proportions between the length of the palps (PL) and the length of the antennae (AL), excluding MOTUs $8,10,12,13$ and 14 .

Apart from the pseudo-cryptic lineages of $P$. rullieri, which have considerable morphological differences from clade A, lineages found in this clade still correspond to five MOTUs uniquely present in the Mediterranean, all sympatric with each other in the western part of the sea. The Mediterranean is already recognized as a biodiversity hotspot (Bianchi and Morri, 2000), including the presence of cryptic species (Calvo et al., 2009; Taboada et al., 2017; Langeneck et al., 2020) and exotic species (Zenetos
et al., 2008; Galil, 2009). The role of the alternating glacial and interglacial stages has been often suggested as a possible reason for the "biodiversity pump" in the Mediterranean, which is a possible outcome of the climatic events of the Quaternary (Bianchi and Morri, 2000; Bianchi et al., 2011). In particular, the Italian Peninsula is considered an independent sub-centre of differentiation and glacial persistence, also confirmed by molecular analyses (Taberlet et al., 1998; Hewitt, 1999). Other areas such as Maghreb, Iberia, the Balkan Peninsula and Anatolia are also considered biogeographic substructures of the Mediterranean refugia (Dugdale and Wilkerson, 1988; Gómez and Lunt, 2007), in which distinct species could have evolved through vicariance (Gómez and Lunt, 2007; Schmitt et al., 2021). This could explain the presence of multiple lineages, both in the western Mediterranean and between the western and the eastern regions of the sea.


Fig. 7.16. Scatter plots with the most considerable morphometric proportions in distinguishing nine MOTUs (left) which often overlap with each other, and the cleaner version (right) displaying only the MOTUs with the formation of
partial or independent clusters between each other. (A) Morphometric proportions between the length of the posterodorsal cirri ( DLTL ) and the length of the head ( HL ). (B) Morphometric proportions between the length of the posterodorsal cirri (DLTL) and the length of the head (HL), excluding MOTUs 5, 12 and 13. (C) Morphometric proportions between the length of the postero-dorsal cirri (DLTL) and the length of the antennae (AL). (D) Morphometric proportions between the length of the postero-dorsal cirri (DLTL) and the length of the antennae (AL), excluding MOTUs $1,8,10$ and 12. (E) Morphometric proportions between the length of the dorsal ligule (DLL) and the length of the dorsal cirri (DCL). (F) Morphometric proportions between the length of the dorsal ligule (DLL) and the length of the dorsal cirri (DCL), excluding MOTUs 10 and 12.

Besides the private MOTUs of the Mediterranean basin, three lineages from clade A also appear to be unique to the Macaronesia islands with two sympatric MOTUs present in the island of Gran Canaria. These islands are spread along the Northeast Atlantic Ocean and have never been connected with mainland, spanning a wide range of climatic conditions with dynamic geological (islands' emergence and volcanic activity) and sea-level changes (e.g., Pleistocene glaciations) over relatively long periods. Because of this, their biota is a result of dispersal from distant geographical sources and in situ evolution and diversification (Fernández-Palacios et al., 2011) and provide a unique case-study to investigate evolution and phylogeography. In this study, Macaronesian populations are indeed distinct from mainland ones, which indicate some level of differentiation and highlight the importance of these islands in the isolation of Perinereis species. This was also reported for other polychaete studies, e.g. Eumida (Nygren and Pleijel, 2011) and even in other groups of invertebrates such as amphipods (Desiderato et al., 2019), isopods (Vieira et al., 2019) and gastropods (Sá-Pinto et al., 2008). Interestingly, MOTUs from P. rullieri and $P$. cultrifera do not share the same island of the same archipelago (e.g. Canaries) with the former being widespread both in Tenerife and Lanzarote and the latter mainly present in Gran Canaria. Because long term absence of dispersal among geographically close populations of marine invertebrates is very unlikely, this pattern seems to fit the "founder takes all" density-dependent process (Waters et al., 2013). Under this hypothesis, the first founders rapidly colonize a new habitat, but subsequent migrants are incapable of successfully colonizing the pre-empted space, hence leading to the genetic segregation of geographically close populations. The same pattern was also observed in Dynamene edwardsi (Lucas, 1849) (Isopoda), where specimens from Tenerife (Canaries) and Madeira grouped in a MOTU deeply divergent from the one of Porto Santo (part of the Madeira archipelago). Furthermore, MOTUs specific to each of the islands of La Palma, El Hierro, Gran Canaria, Selvagens and S. Miguel (Azores) were found within populations of this morphotype as well (Vieira et al., 2019). This Isopod and several species
complexes from the Hyalidae (Crustacea, Amphipoda) family, are examples of benthic invertebrates from the Macaronesia displaying an exceptional amount of cryptic lineages (Desiderato et al., 2019). The latter was especially true in the Canary Islands, where two to four deeply divergent lineages were found, endemic to specific islands (Tenerife, Gran Canaria, El Hierro and La Palma). Instances with the same island also harbouring two different sympatric MOTUs is the case for Apohyale media (Dana, 1853) and A. stebbingi Chevreux, 1888. All these lineages displayed genetic distances comparable to those found among established species of peracarids (Crustacea) and, accordingly to Vieira et al. (2019), "seem to exhibit a long, rich and deep phylogeographic history in Macaronesia, where founder effects in conjunction with the geodynamics of the islands and subsequent lack of gene flow among populations, creates patterns based on geographical proximity of targeted populations". These studies collectively suggest a much larger role of oceanic islands in the diversification of marine invertebrates than previously anticipated.

### 7.5 Final remarks

Integrative taxonomy has proved to be fundamental for the study of Perinereis species in European waters. Besides the often-overlooked morphological differences between clades A (P. cultrifera complex) and B ( $P$. rulliericomplex), molecular data was also essential in uncovering hidden multiple and deeply divergent lineages within these two major clades.

Similarly to Perinereis, the recent study with Syllis gracilis Grube, 1840 complex (Langeneck et al., 2020) was unable to exactly pinpoint the lineage corresponding to Grube's (1840) original description, because of the impossibility to sequence the holotype and the common co-occurrence of different lineages in the same type-locality within the Mediterranean. Because of this, the authors suggest the possibility that Grube's material itself might have included more than one lineage since there is an apparent inability to distinguish these same lineages based on morphological features alone (ÁlvarezCampos et al., 2017), highlighting in addition the presence of phenotypic plasticity that further conceals informative morphological differences. The possibility of MOTU 8 corresponding to the original description of $P$. cultrifera is highly based on available parapodia drawings, geographic distribution and paragnath patterns. However, to further advance this study, specimens from the type locality are needed to confirm which lineages are present in the region and consequent formal taxonomic description of the new lineages from the complex. Regarding $P$. rullieri, MOTU 12 seems to match all the criteria to be considered the original described species. Given the very distinct geographic distribution and habitats from the remaining
three MOTUs of the complex (clade B), further formal taxonomic description should not pose any constrain.

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## Chapter 8

## Evolutionary insights based on cryptic marine invertebrate complexes


#### Abstract

The lack of an established consensus on universal boundaries to delimit cryptic species and the uncertainty as to whether morphological stasis is a taxonomic artefact, an authentic biological phenomenon or a mix of these two conditions, may create difficulties for formal species descriptions. In an attempt to better understand the cryptic species phenomenon, the molecular results from the previous chapters of this thesis are here assembled and patterns emerging from these combined data further explored. To this end and using polychaetes (2042 sequences, COI, 16s, ITS and 28S) from the main analysed geographical regions (NE Atlantic, Mediterranean and Macaronesia), a comparison based on distances and ratios between the different genetic markers, amino acid phylogenies and CO variation patterns was performed. This analysis showed that among the five polychaete complexes (Eumida, Eulalia, Hediste, Platynereis and Perinereis) here investigated, the higher is the COI divergence between lineages or between major clades of closely related lineages, the greater is the probability of finding overlooked morphological differences. This is best exemplified by the amino acid phylogenetic tree from the pseudo-cryptic Perinereis cultrifera complex, where four very distinct groups are patent: Group I, composed of MOTUs 3 and 4 which are the only lineages showing unique papillae-like protuberances in the dorsal body; Group II with MOTUs 1, 2, 5-9 corresponding to the traditional descriptions of $P$. cultrifera; Group III, with the presence of MOTU 14 which is an ingroup with several distinctive morphological characters, and MOTU 11, which is characterized by the very unique small size of the bar shaped paragnaths, when compared to the remaining "P. rullierilike" clade appearing in group IV (MOTUs 10, 12 and 13). Based on COI distances, the Macaronesia MOTUs from three distinct polychaete complexes seem to be closer to the ones found in the Mediterranean, instead of the NE Atlantic. Moreover, distance ratios between the different genetic markers vary considerably between complexes, with nuclear markers apparently being the best suited loci to screen for potential lineages that may not belong to a particular complex of closely related species, despite low COI genetic distances and apparent morphologic similarity. The lack of a universal pattern of molecular divergence is probably related with particular ecological and life history features associated with local habitats as well as with previous geological events. This strongly suggests that each species complex should be seen as unique and that a "minimalist revision", the method applied to insects in Sharkey et al. (2021), in cryptic annelids should not be encouraged, unless complemented with additional data.


Keywords: Europe; Annelida; macroinvertebrates; cryptic species; mtCOI-5P, rRNA 16S, ITS-region, rRNA 28 S

### 8.1 Introduction

The existence of morphological highly similar and true cryptic species is proving to be a significant part of the real biodiversity in the planet, especially in marine invertebrates (Sá-Pinto et al., 2008; Vieira et al., 2019; Hupało et al., 2019), including polychaetes (Nygren, 2014; Langeneck et al., 2020; Martin et al., 2020). The evolutionary reason behind morphological stasis remains mostly unexplored and discussions on whether these cryptic lineages originated from bias of a morphologically oriented classification of biodiversity or resulted from underlying biological phenomena are still ongoing (Korshunova et al., 2017). Since there is no established consensus on universal criteria to describe cryptic species (Westheide and Hass-Cordes, 2001; Moritz and Cicero, 2004; Martinsson and Erséus, 2021), even when using molecular data, often are the cases where the clear separation of lineages is hard to perceive. Instances of low interspecific COI distances, which might not be enough to distinguish molecular operational taxonomic units and, instead, may reflect regional variation (Meier et al., 2021); mismatching species boundaries between mitochondrial and nuclear markers (Zamani et al., 2021; Meier et al., 2021); or even cases of heterozygosis and introgression (Sota and Vogler, 2003; DeSalle et al., 2005), may prove to be a barrier to formal species description.

Despite molecular data not being customarily considered self-sufficient for species descriptions, it is still fundamental in detecting hidden biodiversity in several regions of the globe (Nygren et al., 2018; Tosuji et al., 2019; Sampieri et al., 2021). Barcode data (COI-5P) is widely used by the scientific community and increasingly available in the public domain (Grant et al. 2021), either through GenBank or the Barcode of Life Database (BOLD, Ratnasingham and Hebert, 2007). Because COI is a standardized region, data can be assessed and compared within and among the many specimens and species that have barcode sequences from several different geographic regions. In particular, the Macaronesia islands and Mediterranean Sea are a cryptic hotspot for several invertebrate species (e.g. amphipods and isopods, Desiderato et al., 2019; Vieira et al., 2019) and provide a unique case-study to investigate evolution and phylogeography (Bianchi and Morri, 2000; Valente et al., 2014; Fernandez-Palacios et al., 2015).

In an attempt to better understand the cryptic species phenomenon, the molecular results from the previous chapters of this thesis are here assembled and patterns emerging from these combined data further explored. Distances and ratios between the different genetic markers are compared and COI distance patterns between lineages from several distinct geographic basins are analysed. A comparative analysis focused on the mitochondrial COI gene between polychaetes with several lineages unique to the

Macaronesian islands was also performed. Finally, links between morphological stasis and cryptic diversity is discussed and investigated.

### 8.2 Material and methods

### 8.2.1 DNA sequences

Molecular data from five Annelid species complexes from the previous chapters corresponding to Eumida sanguinea (Chapter 3), Eulalia clavigera/viridis (Chapter 4), Hediste diversicolor (Chapter 5), Platynereis dumerilii/massiliensis (Chapter 6) and Perinereis cultrifera/rullieri (Chapter 7), were used in this analysis (Fig. 8.1). For the sake of simplicity, from this point on, these species complexes will be referred only by their respective genus. The number of sequences for each available marker and number of MOTUs per geographic region are detailed in Table 8.1. MOTU numbers for each complex correspond to the same that were recognized from the previous chapters. MOTU 14 (ingroup) from the Perinereis complex was included in this analysis, given the very close genetic proximity to the $P$. rullieri clade. ITS1 sequences from the Hediste complex were not available due to the difficulty in amplifying this nuclear region. However, from the few sequences I was able to amplify, the genetic interspecific distances were equivalent to the ITS2 region (data not shown). Similarly, the single D2 region from the 28 SRNA locus, used in the Platynereis and Perinereis complexes, contains by far most of the inter-MOTU variation in all the analysed complexes and thus can be compared with the entire 28 S region obtained for the Eumida, Eulalia and Hediste complexes.

### 8.2.2 Distances and correlations

Mean genetic distances were calculated between MOTUs within each complex (Table 8.1) using K2P distances MEGA 11.0.10 software (Tamura et al., 2021). This data was organized in Microsoft Excel and used to 1) create correlations of the distances between each pair of markers for all the five Annelid species complexes; 2) examine the distance ratios between the distinct molecular marker pairs and interMOTU distances in each annelid species complex for each available loci; 3) build histograms based on mean COI genetic distances to inspect the variations of the barcoding gap, i.e. when maximum intraspecific values do not overlap with minimum interspecific distances (Hebert et al., 2003) for all the groups in Table 8.1; and lastly 4) compare the mean COI genetic distances between the different geographic regions (NE Atlantic vs. Mediterranean vs. Macaronesia) in three widespread Annelid complexes (Platynereis, Perinereis and Eulalia).


Fig. 8.1. Number of lineages detected, main habitat where they are commonly found and sampling regions for the six species complexes investigated in this thesis. *Trypanosyllis zebra has no dedicated chapter (see appendix material) and was not used in this chapter's analyses. Numbers in parenthesis correspond to the total number of MOTUs currently known for each species complex, while regular numbers correspond to the lineages revealed exclusively by this thesis to the best of my knowledge. The ingroup, Perinereis oliveirae, was included in the $P$. cultrifera species complex. Abbreviations: NEA, North East Atlantic; MED, Mediterranean Sea; MAC, Macaronesia islands; PNA, Pacific North America.

### 8.2.3 Amino acid analysis

A maximum likelihood amino acid radiation tree was also built in MEGA 11.0.10, using the Jones-Taylor-Thornton model (JTT) with equal rates across sites for all the annelid species complexes, in order to visualize main amino acid-based lineages emerging from the COI-P locus for each complex and compare them with nucleotide-derived MOTUs.

Table 8.1. Number of sequences per marker and number of MOTUs per geographic region for each of the species complexes analysed in this chapter. Numbers in parenthesis are not unique to a single geographic region. Abbreviations: NEA, North East Atlantic; MED, Mediterranean Sea; MAC, Macaronesia islands.

| Species complexes | No of sequences |  |  |  | № of MOTUs |  |  |  |
| :--- | :--- | :---: | :---: | :---: | :---: | :---: | :---: | :---: |
|  | COI | $\mathbf{1 6 S}$ | ITS <br> region | $\mathbf{2 8 S}$ | NEA | MED | MAC | Total |
|  | Eumida | 278 | 79 | 184 | 184 | 12 | $8(2)$ | 1 |
|  |  |  |  |  |  |  |  |  |
|  | Eulalia | 119 | - | 43 | 43 | 3 | $5(1)$ | $1(1)$ |
|  | Platynereis | 193 | 100 | - | 100 | 4 | $3(3)$ | 3 |
|  | Perinereis | 165 | 86 | - | 71 | 2 | 8 | 4 |
| Total | Hediste | 211 | - | 93 | 93 | 3 | $2(3)$ | - |

### 8.3 Results

### 8.3.1 Genetic COI distances

The global COI mean distances for the five analysed polychaete species complexes (Platynereis, Perinereis, Hediste, Eumida and Eulalia) (Fig. 8.2.F) range from 4-30\%, with the values between 14-20\% being the most common ones. Some instances of very low interspecific distances, between 2 and $10 \%$, are also present between cryptic lineages. In all of the cases, the genetic mean intraspecific values never surpassed $3 \%$, although maximum distances up to $6 \%$ can be found in one lineage from the Hediste complex (Chapter 5).

When displaying the mean genetic distances for each polychaete complex (Fig. 8.2.A-E), specific patterns for each group emerged. In Hediste (Fig. 8.2.E), the mean interspecific distances are the lowest among the five complexes (reaching only up to a maximum of $9 \%$ mean COI distances) and is the only one characterized for the lack of a clear barcoding gap, with a continuum set of distance values of at least 5-6\% between specimens from the same MOTU (MOTU 2, Chapter 5). In Platynereis, there is a prevalence
of inter-MOTU distances from a minimum of $5 \%$ up to $25 \%$ (Fig. 8.2.A). In Perinereis, there is a formation of two main groups with mean values between 14-18\% and 24-28\% (Fig. 8.2.C). Overall, the phyllodocids show a similar pattern, varying only in the most common percentages of divergence, with Eumida (Fig. 8.2.D) having lower values ranging between 14-19\%, compared to the 19-24\% found in Eulalia (Fig. 8.2.B).


Fig. 8.2. Comparisons between intra and interspecific COI genetic distances (K2P) for each specific polychaete species complex. (A) Based on 10 MOTUs from the Platynereis complex. (B) Based on nine MOTUs from the Eulalia complex. (C) Based on 14 MOTUs from the Perinereis complex (D) Based on twenty-two MOTUs from the Eumida complex. (E) Based on the five MOTUs from the Hediste complex. (F) Comparisons between intra and interspecific COI genetic distances (K2P), based on all the MOTUs from the five polychaete species complexes analysed in this thesis.

### 8.3.2 Correlations between different markers

Of the five complexes studied, Hediste is the one showing the lowest values and the lowest variation of inter-MOTU genetic distance (for COI, 28 S and ITS; maximum: 8.2, 0.9 and $6.7 \%$ respectively, Fig. 8.3.D-F), while Eulalia displayed the highest genetic values and genetic variation for any of the genetic marker, notably in the ITS region (between 4.8 and $30.9 \%$ ) (Fig. 8.3.A-C). COI is the genetic marker with the highest genetic distances for all complexes (except the ITS region in Eulalia) and 28 S is the lowest, which is the only genetic marker with inter-MOTU genetic distance cases of zero (Figs. 8.3-8.6).


Fig. 8.3. Mean genetic distance (K2P) correlations between MOTUs using three distinct markers for each polychaete species complex. Eulalia: (A) Comparisons between COI and ITS. (B) Comparisons between COI and 28S. (C) Comparisons between ITS and 28S. Hediste: (D) Comparisons between COI and ITS2. (E) Comparisons between COI and 28S. (F) Comparisons between ITS2 and 28 S.


Fig. 8.4. Genetic distance (K2P) correlations between four distinct markers for the Eumida sanguinea species complex. (A) Comparisons between COI and 16S. (B) Comparisons between 28 S and ITS. (C) Comparisons between COI and ITS. (D) Comparisons between ITS and 16S. (E) Comparisons between COI and 28S. (F) Comparisons between 28S and 16S.

Linear correlation between pairs of genetic markers (i.e. inter-MOTU genetic distance between the same MOTUs for two different markers) showed, in general, a positive correlation between genetic markers (i.e. increase/decrease of genetic distances between MOTUs is patent in all markers; Figs. 8.38.5). Eulalia complex is the one with the highest correlation between markers ( $\mathrm{R}^{2}>0.8 ; \mathrm{COI} / \mathrm{ITS}, \mathrm{COI} / 28 \mathrm{~S}$, ITS/28S, Fig. 8.3.A-C). The pairs with 28 S seem to be the ones with less correlation ( $R^{2}$ values usually bellow 0.45 ), except for the pair 28 S/ITS in Eulalia which is an outlier and the most positive correlated $\left(\mathrm{R}^{2}=0.98\right)$ among all pairs tested. Additionally, COI seem to correlate the most with 16 S (Platynereis, Fig. 8.5.A; Perinereis, Fig. 8.5.D; Eumida, Fig. 8.4.A), followed by COI/ITS (Eulalia, Fig. 8.3.A; Hediste, Fig. 8.3.D; Eumida, Fig. 8.4.C), while the pairs $16 \mathrm{~S} / 28 \mathrm{~S}$ and $\mathrm{COI} / 28 \mathrm{~S}$ displayed the lowest correlation ( $\mathrm{R}^{2}<0.5$; Perinereis, Fig. 8.5.F; Platynereis, Fig. 8.5.B-C; Eumida, Fig. 8.4.E-F).


Fig. 8.5. Genetic distance (K2P) correlations between three distinct markers for each polychaete species complex. Platynereis. (A) Comparisons between COI and 16 S . (B) Comparisons between COI and 28 S . (C) Comparisons between 16 S and 28S. Perinereis. (D) Comparisons between COI and 16S. (E) Comparisons between COI and 28S. (F) Comparisons between 16S and 28S.

Boxplots with COI inter-MOTU genetic distance were on average 2.3 times higher than ITS and 2.8 higher than 16S, while for 28 S it was 16 times higher (Fig. 8.6.B). In some cases, such in Eulalia and Platynereis, the average $\mathrm{COI} / 28 \mathrm{~S}$ ratio was close to 50 and 30 times higher for the mitochondrial marker, respectively. ITS is on average 7 times higher than 28 S, while 16 S is around 6 times higher than 28S. Although only compared within Eumida, 16S genetic distances were on average very similar with ITS (Fig. 8.6.A, B). Perinereis and Hediste displayed similar genetic values among the different markers, while Eulalia and Platynereis displayed the highest variation (Fig. 8.6.B).


Fig. 8.6. Genetic distance (K2P) boxplots between different markers for each polychaete species complex. (A) InterMOTU distances for each loci per species complex. (B) Distance ratios between a combination of two distinct markers.

### 8.3.3 Amino-acid phylogenies

The phyllodocids and the nereidids display very distinct results (Fig. 8.7 and Fig. 8.8, respectively). In all the three nereidid complexes (Platynereis, Perinereis and Hediste) it was possible to recognize a considerable number of MOTUs using amino acids.


Fig. 8.7. Amino acid phylogenies within cryptic Nereidids for: (A) Platynereis dumerilii species complex; (B) Hediste diversicolor species complex; (C) Perinereis cultrifera species complex. Each colour and number correspond to a specific MOTU. Colour areas without any branch (black or white lines) show no amino acid differences, including between different MOTUs.

However, in Perinereis (Fig. 8.7.C), despite still having five well distinguished MOTUs, it is the nereidid complex with the most synonym substitutions with 9 lineages failing to properly segregate in distinct amino acid-based clusters (MOTUs 10 and 12; MOTUs 11 and 14; MOTUs 1, 5, 6, 8, 9). These
three distinct clusters grouped lineages from the Canary islands (Tenerife and Lanzarote, MOTU 10) with the ones from eastern Mediterranean sea (MOTU 12); lineages from Gran Canaria (MOTUs 1 and 5) with the western Mediterranean (MOTUs 6, 8 and 9); and lastly, an ingroup that seem to be unique to the Northern Iberian Peninsula with considerable morphological differences compared to the rest of the complex (MOTU 14), grouping together with MOTU 11, which appear only in brackish waters from the Italian Tuscany area.

In Hediste (Fig. 8.7.B), the western and eastern Mediterranean lineages grouped together (MOTUs 1, 3 and 4), and in Platynereis (Fig. 8.7.A) only MOTUs 2 (unique to NW Greece), 4 (cosmopolitan in mainland Europe) and 6 (unique to the western Mediterranean), have the same nucleotide substitutions for most of the specimens. Additionally, MOTU 8 (unique to Porto Santo) also shares the same amino acid sequences with some specimens from MOTUs 10 and 9 (both present in the NE Atlantic and Western Mediterranean).

The amino acid data of the phyllodocid complexes (Eumida and Eulalia) mostly failed to reconstruct any of their respective nucleotide-based MOTUs, with few exceptions, as for example, seen in MOTUs 1,18 and 19 from a total of 22 Eumida lineages (Fig. 8.8.A). MOTU 8 (Eulalia, Fig. 8.8.B) show a very significant difference with amino-acid distances far surpassing what was obsenved for the remaining lineages, which all shared the same amino acids for most of the specimens.

### 8.3.4 Macaronesia-focused complexes

Using only the polychaete lineages which were not only present, but also intensively sampled in the Macaronesia islands (Perinereis, Platynereis and Eulalia), a comparison was performed between each other based on mean intra and inter-MOTU genetic distances. Additionally, a comparison was also made with COI distances to the nearest neighbour between the lineages from the NE Atlantic against the ones from the Mediterranean and the Macaronesia islands. Both the Macaronesia and Mediterranean showed the lowest genetic distances within the same region and between these two regions (Fig. 8.9.A and Fig.8.9.B, respectively).

The COI distance comparisons between MOTUs from the NE Atlantic against the ones from other regions always generated the highest values, except when analysing nearest neighbour distances against the Mediterranean (Fig.8.10.B).


Fig. 8.8. Amino acid phylogenies within cryptic Phyllodocids for: (A) Eumida sanguinea species complex; (B) Eulalia clavigera species complex. Each colour and number correspond to a specific MOTU. Colour areas without any branch (black or white lines) show no amino acid differences, including between different MOTUs.


Fig. 8.9. Mean genetic distances based on COI comparing MOTUs present in the North East Atlantic (NEA) against lineages either from the Macaronesia islands (MAC) or in the Mediterranean Sea (MED). Only the three polychaete species complexes heavily widespread in the Macaronesia islands (Eulalia, Platynereis and Perinereis) were used. (A) Intra and interspecific distances, as well as the distances to the nearest neighbour (NN dist) in MOTUs only present in a specific region. (B) Comparison between MOTUs from different regions. MOTUs present in the same region were excluded.

### 8.4 Discussion

The merged analysis of several annelid cryptic complexes in this thesis revealed extraordinary levels of hidden diversity. Molecular data is essential for uncovering apparent cryptic species with hidden multiple deeply divergent lineages. However, after detailed morphological inspection, it became clear that several lineages with obvious morphological differences have been overlooked and misidentified as the
morphologically closest described species. By looking at the genetic distances of the analysed complexes, some patterns can easily be identified and seem to be correlated to the degree of morphological variability. Lineages within a cryptic complex are a group of closely related species and are expected to share lower genetic divergence compared to non-cryptic species or species from different genera (Nygren, 2014). True cryptic lineages depend largely on molecular data for which there is no established consensus on universal boundaries to delimit species (Lefébure et al., 2006; Martinsson and Erséus, 2021). For example, compared to other complexes here investigated, the entire Hediste complex is characterized by low COI divergences between sequence clusters (Fig. 8.2.E,F), where a lack of barcoding gap and any stable diagnostic morphological character between the lineages makes it particularly challenging for a formal description. In contrast, the largest molecular distances are mostly found between pseudo-cryptic lineages showing some degree of morphological divergence, e.g. between clades corresponding to $P$. dumerilii vs $P$. massiliensis (Chapter 6) or E. clavigera vs E. xanthomucosa sp. nov. (Chapter 4). This is even more noticeable in the case of $P$. cultrifera (Chapter 7), with the formation of bimodal distribution of divergences frequencies as seen in Figs. 8.2.C; 8.5.D-F, that correspond to the distances between the "cultrifera" and "rullierl" clades, with maximum COI distances mostly related to the ingroup P. oliveirae. Moreover, when switching to nuclear markers, it is expected to generally find low interspecific divergence compared to COI, as seen in my data, as well as for other species complexes (e.g. Nygren et al., 2010; Nygren and Pleijel, 2011). This data also led to a considerable correlation between COI, 16 S and ITS for all the analysed complexes. For example, in Eumida (EsSc), for which there is a very large pool of samples and four distinct loci, it appears that ITS and 16S divergences are generally about half those of COI , and 28 S less than one tenth those of COI. If divergence rates are relatively consistent within the group, we could anticipate that any Essc species diverging less than about 10\% in COI have a good chance of having little or no differentiation in 16S, ITS or 28S, hence preventing separation in distinct MOTUs (methods highly based on distances), although they may still be distinguished through haplotype networks or molecular diagnostic characters (Délic et al, 2017). These ratios vary considerably between complexes: e.g. in Platynereis and Perinereis, where COI divergence is almost three times higher than 16S (Figs. 8.5.A,D; 8.6.A), or in both Eulalia and Hediste showing an approximate 1:1 ratio between COI and ITS (Fig. 8.6.A), but with very distinct mean distance values between these complexes (Fig. 8.3.A,D). However, the 28 S locus show a large variation and low $\mathrm{R}^{2}$ values when paired to other loci, including in Eumida.

Despite the existence of high COI interspecific distances between the NE Atlantic (NEA) and Mediterranean (MED), some lineages between these regions show comparatively low values in relation to
their respective nearest neighbour. These low mean interspecific genetic distances, close to $15 \%$ (Fig. 8.9.A), suggest a direct common ancestor. Additionally, the Macaronesia (MAC) MOTUs seem to be closer to the ones found in the Mediterranean, instead of the NEA (Fig. 8.9.B). The common perception for Macaronesia's marine invertebrate fauna is that many species are shared with mainland coasts of NW Africa and Iberia, hence a basal faunistic continuity is assumed (Xavier et al., 2016; Cabezas et al., 2013). However, it seems that Mediterranean cryptic lineages might be more closely related to these oceanic islands than previously expected, with other studies suggesting as well an extensive and profound genetic differentiation between peracarid populations from Atlantic Iberian Peninsula and MAC (Vieira et al., 2021). Furthermore, MOTUs unique either to the Mediterranean and Macaronesia, display mean COI distances values lower than $12 \%$ when compared to the nearest neighbour, with the average interspecific divergence reaching very close to 20\% (Fig. 8.9.A).

### 8.4.1 Mitochondrial, amino acids and nuclear markers

The nuclear markers seem to be the best suited loci to screen for potential lineages that may not belong to a particular complex of closely related species. This is clearly patent in the Eulalia complex, where the 28 S and especially, the ITS-region, got much closer to, or even exceeded COI mean values (Fig. 8.6.A) in large part due to a particular single lineage (MOTU 8). This MOTU 8 has a large interspecific distance in the nuclear markers, reaching maximum values higher than $60 \%$ for the ITS region and $12 \%$ for the 28 S locus, similar to the ones found in outgroups (Chapter 4). As previously stated in Chapter 4, this lineage belongs to a very small specimen which at first, seemed to fit the $E$. viridis morphotype based on the small size and pointed midbody dorsal cirri and bright red eyes, however molecular data is very divergent. Even when analysing the COI amino-acids (a.a.), the amount of non-synonym substitutions was very high and clearly deviated from the pattern found in the remaining lineages from the complex (MOTU 8, Fig. 8.8.B) or even among other phyllodocids (Eumida, Fig. 8.8.A). Since nuclear markers and COI a.a. are more efficient in reconstructing deeper phylogenies (e.g. Weitschek et al., 2014), this data show evidence of an entirely new Eulalia group yet to be described, similar to the Eumida ockelmannicomplex pointed out in Chapter 3, despite low COI genetic distances and apparent morphologic similarity between these lineages. Amino acids also appear to be very useful in detecting multiple lineages within the analysed species complex in the nereidids from this study (Fig. 8.7.A-C), although it does not seem to be useful for the studied phyllodocids (Fig. 8.8.A-B). The latter result could be related to a more recent divergence time from the common ancestor compared to the nereidids here examined. When comparing a.a results with morphological data, an obvious and interesting correlation can be seen, especially in $P$.
cultrifera, with the formation of four very distinct groups (Fig. 8.7.C): Group I, composed of MOTUs 3 and 4 which are the only lineages showing unique papillae-like protuberances in the dorsal body; Group II with MOTUs 1, 2, 5-9 corresponding to the traditional descriptions of P. cultrifera (Grube, 1840; Hutchings et al., 1991); Group III, with the presence of MOTU 14 which is an ingroup with several distinctive morphological characters and MOTU 11, which is characterized by the very unique small size of the bar shaped paragnaths, when compared to the remaining "rullierilike" clade appearing in group IV (MOTUs 10, 12 and 13 , Pilatos, 1974).

### 8.4.2 Morphological stasis

The lack of an universal pattern of molecular divergence is probably related with particular ecological and life history features associated with local habitats (Peijnenburg et al., 2004) as well as with previous geological events (e.g. alternating glacial and interglacial stages, volcanic islands as ideal natural laboratories for evolutionary diversification, or the Messiniah salinity crisis) (Bianchi and Morri, 2000; Valente et al., 2014; Hupało et al., 2019). This strongly suggests that each species complex should be seen as unique (Dupuis et al., 2012; Martinsson and Erséus, 2021) and that a "minimalist revision", the method applied to insects in Sharkey et al. (2021), in cryptic annelids should not be encouraged, unless complemented with additional data besides the COI barcode sequence and an image.

It seems that the deeper the divergence between major phylogenetic clades, the higher is the probability of finding slight morphological variations in cryptic lineages previously thought to be morphological identical, which often display a perfect match between molecular and morphological data. The four distinct Perinereis groups mentioned above correspond to four morphotypes with slightly morphological variations present in 13 unique lineages. This low degree of morphological variation is similar to the Eulalia complex, where at least three stable morphotypes can be found ("clavigeralike" phylogenetic clade, "viridis" clade and "xanthomucosa" clade, Chapter 4), based mainly on the size of papillae present in the pharynx and worm's coloration. Furthermore, the Platynereis complex have at least two very stable and distinct morphotypes, where changes in paragnath patterns suggests the need for an amendment to the entire genus (see taxonomic section in Chapter 6) and questions if it can still be considered a cryptic complex to begin with, despite its lineages being genetically very close. In contrast, Hediste (Chapter 5) show no stable morphological variation and is apparently a true cryptic species, while in Eumida (Chapter 3), differences are mostly found in eight pigmentation types mixed with specific colorations, with the most divergent lineage - E. mackiei sp. nov. (MOTU 1) - also showing parapodia variations. Slight morphological differences, e.g. in chaetae, have also been observed in cryptic lineages
from other polychaete groups such as Eurythoe (Barroso et al., 2010) and Stygocapitella (Cerca et al., 2020). The first study found one distinguishable morphotype in three distinct COI clades, while the latter reports four different morphotypes with subtle differences, combined with molecular data supporting 10 reproductively isolated clades. This, however, seem to go in line with the conceptual framework that recognizes cryptic species based on their low levels of phenotypic disparity relative to their degree of genetic differentiation and divergence times as compared with non-cryptic species (Struck et al., 2018). This way of delimiting and perceiving cryptic species as an evolutionary phenomenon, resulting from the deceleration of morphological evolution, provides a means to differentiate these complexes from taxonomic artefacts such as erroneous descriptions, or from poorly sampled and preserved data (Zachos et al., 2013) and can make the debates about levels of 'crypticity' to be more nuanced (Struck et al. 2018). The reason as to why morphological stasis prevails is still an incognita, but punctuated equilibrium could support the occurrence of long periods of stasis which are disrupted by rapid change during speciation implying that adaptive and selective processes are insignificant during substantial parts of the evolutionary histories of species (See Pagel et al. 2006 and Bokma, 2008 for more details). The study of morphological similarity can also benefit from predictions, models, and evidence from paleontological stasis (Cerca et al., 2020), which positions that stasis may result from constraints, selective pressures on physiology and/or behaviour, stabilizing selection and niche conservatism (Hansen and Houle, 2004; Estes and Arnold, 2007; Futuyma, 2010).

### 8.5 Final remarks

Species complexes have a complicated history and the commonly used genetic markers have limitations in the study of possible hybridization and cryptic introgression (Currat et al., 2008). This may prevent further evolutionary insights into the origin of the cryptic phenomena and certain unused genes may be passing across species boundaries, and how these interact with the recipient genome (Twyford and Ennos, 2012). Moreover, the occasional lack of closure about the species status between populations within a particular MOTU or between two MOTUs (e.g. Hediste: MOTU 2, MOTUs 3 and 4, respectively, Chapter 5) demand further methodologies to be explored. As pointed out by Cerca et al. (2021), species are expected to remain morphologically identical in scenarios where bottlenecks reduce genetic variation and consequent constrain of the evolution of morphology, or scenarios where genetic variation is shared, such as hybridization and incomplete lineage sorting. To further explore these evolutionary hypotheses, the use of a whole-genome amplification method (WGA) coupled with double-digestion restriction-site associated DNA sequencing (ddRAD) to reconstruct the evolutionary history species complexes was tested
and applied (Cerca et al. 2021). This can lead to additional options for those working with population genomics and phylogenomics even in microscopic eukaryotes as well, at an affordable cost. Future research in complicated MOTUs or in closely related lineages could reach similar results as Cerca et al. (2021), where the three closely related species in the Stygocapitella (Annelida) complex shared genetic variance probably from incomplete lineage sorting and ancient admixture. The authors further speculate that the degree of shared variance might underlie morphological similarity in this Atlantic species complex, resulting in the retention of symplesiomorphic morphological states (Futuyma, 2010).

Additional studies making use of concatenated alignments of commonly used genetic markers from several species' complexes, applied to ancestral state reconstructions mapping the different degree of morphological variation between lineages could also be used (Cerca et al. 2020). This can further explore the phylogeny and graphically represent a history of character evolution on the phylogenetic tree (e.g. Mesquite software by Maddison and Maddison, 2019). Finding new cryptic lineages and combining molecular tools with, when available, small changes in morphological traits in lineages displaying stasis is essential to help comprehend this evolutionary phenomenon.

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## Chapter 9

Concluding remarks

## Overview: Context and originality

Thanks to high-throughput sequencing technologies, molecular tools are being increasingly integrated in regular and large-scale biomonitoring initiatives (Leese et al., 2018; Pennisi, 2019). But these methodologies largely depend upon the continuing improvement and eventual completion of DNA reference libraries (Grant et al., 2021; Radulovici et al., 2021, Rimet et al., 2021). This thesis contributed not only to considerable progress on the knowledge of unknown European cryptic lineages from several Phyllodocida taxa, but also reinforced the prominent role of the Macaronesian archipelagos and the Mediterranean Sea in promoting genetic diversification, evolution and speciation of annelids, and possibly other marine invertebrates.

Integrative taxonomy is essential to elucidate evolutionary phenomena and eventually to allow informed use of species complexes exhibiting stasis in a variety of different studies. Despite their morphological similarity, cryptic complexes are in fact different species and may not share the same biological characteristics. This implies the possibility of these lineages having a different reproductive biology and life history characteristics; distinct feeding biology; and unique temperature, salinity, habitat, depth or other ecological preferences (Nygren, 2014). All of this can create biased data, since different lineages used as the same species, might produce distinct results in studies such e.g.: bioaccumulation, evolutionary development biology, ecotoxicology, biomonitoring, ocean acidification or even in biotechnological applications as seen with the case of the green phyllodocid, Eulalia clavigera (Rodrigo et al., 2021). Furthermore, apparent cosmopolitan species are not a priority for conservation, contrary to endemic species which are limited to a certain area, often with unique habitat conditions (Hutchings and Kupriyanova, 2018). This scenario is very similar to what happens with some lineages within a cryptic complex, like the ones found exclusively in the Madeira island (Portugal) and nowhere else in the world. With such a limited distribution, small population sizes and possibly adaptation to much more stringent environmental conditions, endemic species may be more susceptible to climate change, pollution or other environmental disturbances. This can lead to higher probability for species extinction and biodiversity loss (Pimm et al., 2014), thus the need for extra care regarding cryptic species in future conservation practices, which can also be applied to other more economically relevant groups, like fish species.

An improved and detailed knowledge of marine invertebrate diversity, including polychaetes, is also fundamental for the successful implementation of the Marine Strategy Framework Directive (2008/56/EC), and of other marine conservation and sustainability regulations. Therefore, there is a need for molecular diagnoses and attribution of Linnean names to newly discovered cryptic species (Delić et al., 2017), with available vouchers (Pleijel et al., 2008), that can later be complemented with additional
ecological and morphological information. Otherwise, if most of the molecular data that can provide support for species hypothesis (Fujita et al., 2012) continues to be unused, large sections of biodiversity will remain unnoticed by the scientific community (Fontaneto et al., 2015).

## Overview: Main findings

The main contribution of this thesis was the detection of a large number of undescribed cryptic lineages within 6 species complexes (Eumida sanguinea; Eulalia clavigera; Hedisite diversicolor, Platynereis dumerilir, Perinereis cultrifera and Trypanosyllis zebra) distributed along European waters. In total, 70 completed sorted lineages (i.e. MOTUs) were uncovered, of which 43 are unique to this work to the best of my knowledge. Five of these taxa had a dedicated chapter where an integrative approach based on multi-locus phylogenetic analysis and morphological data allowed the formal description of 13 new species to science. Additionally, this work also contributed to the clarification of ambiguities regarding previous descriptions that were inadequately synonymised, as well as overlooked and poorly known descriptions misidentified to other popular taxa deemed cosmopolitan. The Macaronesian islands and the western part of the Mediterranean Sea in particular, were once again confirmed as cryptic hotspots, with a total of 10 and 30 unique MOTUs for each region, respectively. Lastly, a fair amount of DNA barcodes were supplied to the global Polychaeta reference libraries, that will be publicly available for the scientific community in BOLD (and GenBank), together with the relevant metadata (Rimet et al., 2021) and voucher specimens deposited in biological collections: total of 1012 COI, 307 16S, 320 ITS and 53228 novel sequences. Molecular data from species used in outgroups, namely Eumida ockelmanni, Sige fusigera, Eumida bahusiensis, Eulalia aurea, Pseudonereis sp., Perinereis marionii, Perinereis aibuhitensis, Neanthes nubila and Alita virens will be also made available, including some undetermined Nereis species that might be new to science. Furthermore, detailed molecular and morphological data on the phylogenetic and ecologically diverse species complex here studied, provide an important contribution to the continuing investigation of the origin, evolution and phylogeography of cryptic species and its relationships with morphological stasis.

The variation of molecular divergence patterns within and between each complex is probably related with particular ecological and life history features associated with local habitats, as well as with previous geological events. As the most probable examples: 1) the alternating glacial and interglacial stages as one of the possible causes for the "biodiversity pump" in the Mediterranean (see Bianchi and Morri, 2000); 2) The Messinian salinity crisis, a time period usually referred to explain the emergence of geographic barriers preventing gene flow not only between the NE Atlantic with the Mediterranean, but
also between the Western and Eastern part of this Sea (e.g. Mediterranean Gammarus amphipods, Hupalo et al. 2019); 3) additionally, the importance of the remote Atlantic volcanic islands in the speciation by acting as ideal natural laboratories for evolutionary diversification not only of terrestrial organisms but apparently also for marine invertebrates (see Vieira et al., 2022). It appears that the older the ancestral split resulting from the different geological event periods, the higher is the probability of finding slight phenotypic disparities in cryptic lineages, previously thought to be morphological identical. Evidence for this can be seen in the deep divergence between major phylogenetic clades within some of the analysed species complex, and the perfect match of each clade to the specific morphological variation (e.g. the studied complexes within Perinereis, Platynereis and Eulalia). In contrast, there appears to be a higher chance for species complexes or lineages with lower molecular divergences to be true cryptic species displaying morphological stasis (e.g. Hediste diversicolor species complex). Furthermore, a noteworthy observation was that Mediterranean cryptic lineages seem to be more closely related phylogenetically to the Macaronesian islands' lineages than to the NE Atlantic ones, suggesting a phylogeographic affinity somewhat unanticipated (Xavier et al., 2016; Cabezas et al., 2013).

A second significant contribution was the analysis of all the publicly available COI DNA barcode data and current state of the worldwide coverage of Phyllodocida species present in the BOLD platform. This barcode reference library is still in its incipient state, not only because it just includes around $11 \%$ of the total described Phyllodocida species, with DNA barcodes assigned at the species level, but also with $44 \%$ of the records only having a single sequence. Deep sea species were also poorly represented. From the 3509 barcodes corresponding to 277 species with information about sampling depths, only 65 sequences ( 30 species) were from specimens sampled at depths below 100 meters. The analysis also revealed evidence of an impressive amount of hidden biodiversity with 185 BINs corresponding to 35 morphospecies being part of possible undescribed cryptic complexes. Another issue found in the analysis of the reference library was the addition of tag codes to the species names by BOLD users, preventing an accurate diversity assessment. Tags are usually added to differentiate cryptic lineages or populations in order to make use of the several bioinformatic tools available in the platform. Almost $20 \%$ of the records had tag codes and the creation of these distinct groups need to be independent of the species name of the records, to avoid user-made ambiguities. The improvement and curation of these databases such as BOLD systems (e.g. Radulovici, 2021), allows the better evaluation of taxonomic uncertainties and to analyse species phylogenetic diversity. Such analysis can also improve DNA metabarcoding studies at the taxonomic assignment step and highlight the gaps that still need to be filled out and possible need of dedicated taxonomic revisions.

In the molecular analysis of the Trypanosyllis zebra (Grube, 1860) complex (Appendix section), the phylogenetic data for each genetic marker (COI, 16 S and 28 S ) provided evidence for the existence of at least ten deeply divergent evolutionary lineages, nine of which are present in Europe. All lineages grouped in monophyletic clades with low intra-clade divergence, most of which were present in the Mediterranean Sea. The lineage from mainland Portugal, which is also shared with data from the Atlantic part of the United States of America (Leray and Knowlton, 2015) seem to deviate considerably from the remaining complex and might instead, be related to Trypanosyl/is krohniïClaparède, 1864, a morphotype very similar to T. zebra (Álvarez-Campos et al., 2017).

## Future perspectives

This study emphasizes the different kinds of methods and criteria that can be used for the identification of cryptic species. Although, the morphological and phylogenetic methodologies offer limitations (see Hey, 2006 for details), the complementary aspects of both approaches tend to convey more accurate species delineations. This thesis extended considerably the knowledge of biodiversity of several European species complexes from what was generally thought to be well-known cosmopolitan taxa. Despite the description of a considerable number of new species, one of the biggest gaps still left by this thesis, is the high number of unnamed lineages / MOTUs, thus unavailable to be formally recognized in a variety of different studies. This was due to the low number of available specimens per lineage, small sized organisms which were used in their entirety for molecular work and/or the lack of morphological structural integrity preventing a proper analysis of the specimens. However, these newly lineages provide a great starting point by using the obtained first pass barcode data and knowledge of the specific areas where they can be sampled from. As a follow up to this work, additional sampling effort and further morphological examination are needed to clarify the status of these lineages and if possible, to name them. In particular, sampling specimens from the type locality (Naples, Italy) for P. cultrifera s.s (original description) is advised to name all the remaining 11 unnamed lineages from the complex. Additionally, the currently available data for H. diversicolor s.s. is insufficient to attempt to provide any supported explanation for the unusual intraspecific patterns observed in this lineage. The exceptionality of this case merits detailed examination in future studies, which, due to its peculiarity, would require further and extensive sampling along the NE Atlantic and in the western Mediterranean areas close to France and Spain to characterize as comprehensively as possible the genetic variability and the ecological features of this lineage. Furthermore, the subtidal rare complex belonging to the Trypanosyl/is zebra
morphotype still have not enough DNA sequences or specimens per lineage for a proper attempt to describe its diversity and, at the current state, only a very simple phylogenetic analysis can be done.

The relation of the different cryptic lineages unique to the NE Atlantic, Mediterranean and Macaronesia can also be further explored with additional bioinformatic tools or even making use of wholegenome amplification methods (WGA) coupled with double-digestion restriction-site associated DNA sequencing (ddRAD) to reconstruct the evolutionary history of the species complexes (Cerca et al., 2021). Moreover, the addition of other major marine invertebrate groups displaying morphological stasis such as gastropods, amphipods, isopods or other crustacea, for comparison purposes in the search of particular phylogeographic patterns and unique molecular signals, could help better understand the cryptic phenomenon.

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## Appendices

## Molecular data for the Trypanosyllis zebra (Grube, 1860) complex



Fig. A1. Maximum likelihood tree with the General Time Reversal model with equal rates across sites (GTR) based on mtCOI-5P for 45 original sequences belonging apparently to the Trypanosyllis zebra morphotype and information regarding specimen's geographical distribution. Two additional sequences were mined from GenBank (MOTU 10), one from Portugal (Lobo et al., 2016) and another from the

Atlantic area of the United States of America (Leray and Knowlton, 2015). MOTU delineation method is based on BINs (Ratnasingham and Hebert, 2013; See Chapter 3 for more details). Bootstrap values based on 1000 bootstrap runs. The outgroup with six original sequences belong to the species Trypanosyllis aeolis Langerhans, 1879.


Fig. A2. Maximum likelihood tree based on 16 S rRNA for 41 original sequences belonging apparently to the Trypanosyllis zebra morphotype and information regarding specimen's geographical distribution.

MOTU delineation method is based on ASAP (Puillandre et al., 2021; See Chapter 7 for more details). Bootstrap values based on 1000 bootstrap runs. The outgroup with six original sequences belong to the species Trypanosyllis aeolis Langerhans, 1879.


Fig. A3. Maximum likelihood tree based on 28S-D2 rRNA for 41 original sequences belonging apparently to the Trypanosyllis zebra morphotype and information regarding specimen's geographical distribution.

MOTU delineation method is based on ASAP (Puillandre et al., 2021; See Chapter 7 for more details). MOTU A englobes MOTUs 1 and 2 found in COI and 16S. MOTU B englobes MOTUs 4 and 5 found in COI and 16S. Bootstrap values based on 1000 bootstrap runs. The outgroup with four original sequences belong to the species Trypanosyllis aeolis Langerhans, 1879.

Table A1. Sample IDs, MOTU designation and location for the analysed specimens from the Trypanosyl/is zebra complex and respective outgroup. All sequence data was uploaded in BOLD under the project "MTTZ - Trypanosyllis Species Complex" and will be publicly available after submission to a peer review journal.

| Species | Vial Code | BOLD Code | BIN | Location |
| :---: | :---: | :---: | :---: | :---: |
| Trypanosyllis zebra MOTU 1 | MTAN8 | MTTZO23-20 | B0LD:AAW8651 | Spain near Cadíz, El Cabo de Trafalgar |
| Trypanosyllis zebra MOTU 1 | MTAN9 | MTTZO24-20 | BOLD:AEE1672 | Spain near Cadiz, Sancti Petri <br> - Chiclana |
| Trypanosyllis zebra MOTU 1 | MTAN11 | MTTZ029-20 | B0LD:AAW8651 | France Banyuls, Outside Pier |
| Trypanosyllis zebra MOTU 1 | MTAN17 | MTTZ030-20 | B0LD:AAW8651 | France, off Banyuls |
| Trypanosyllis zebra MOTU 1 | MTAN28 | MTTZ025-20 | BOLD:AEE1672 | Spain near Cadiz, El Cabo de Trafalgar |
| Trypanosyllis zebra MOTU 1 | MTAN30 | MTTZ026-20 | BOLD:AEE1672 | Spain near Cadiz, Sancti Petri <br> - Chiclana |
| Trypanosyllis zebra MOTU 1 | MTAN31 | MTTZ027-20 | BOLD:AEE1672 | Spain near Cadiz, Sancti Petri <br> - Chiclana |
| Trypanosyllis zebra MOTU 1 | MTAN32 | MTTZO28-20 | BOLD:AEE1672 | Spain near Cadiz, Sancti Petri <br> - Chiclana |
| Trypanosyllis zebra MOTU 1 | MTAN38 | MTTZ031-20 | B0LD:AAW8651 | France, off Banyuls |
| Trypanosyllis zebraMOTU 2 | MTAN21 | MTTZ019-20 | B0LD:AEE5373 | Madeira, Funchal |
| Trypanosyllis zebraMOTU 2 | MTAN22 | MTTZO20-20 | B0LD:AEE5373 | Madeira, Funchal |
| Trypanosyllis zebraMOTU 2 | MTAN23 | MTTZ021-20 | B0LD:AEE5373 | NW Madeira, E Porto Moniz |
| Trypanosyllis zebraMOTU 2 | MTAN24 | MTTZO22-20 | B0LD:AEE5373 | NW Madeira, E Porto Moniz |
| Trypanosyllis zebraMOTU 3 | MTAN35 | MTTZ006-20 | BOLD:AEE1716 | Croatia, Istra - off Rovinj, Sveti Ivan |
| Trypanosyllis zebraMOTU 3 | MTAN36 | MTTZ007-20 | BOLD:AEE1716 | Croatia, Istra - off Rovinj, Sveti Ivan |
| Trypanosyllis zebraMOTU 3 | MTAN12 | MTTZ008-20 | BOLD:AEE1716 | Croatia, Istra - off Rovinj, Sveti Ivan |


| Species | Vial Code | BOLD Code | BIN | Location |
| :---: | :---: | :---: | :---: | :---: |
| Trypanosyllis zebra MOTU 4 | MTAN19 | MTTZO39-20 | B0LD:ADJ6033 | France, off Banyuls |
| Trypanosyllis zebra MOTU 4 | MTAN15 | MTTZ038-20 | B0LD:ADJ6033 | France, Banyuls - llê Grosse |
| Trypanosyllis zebra MOTU 5 | MTAN25 | MTTZO12-20 | BOLD:AEE5372 | Great Britain, Plymouth |
| Trypanosyllis zebra MOTU 5 | MTAN7 | MTTZO13-20 | BOLD:AEE5372 | Great Britain, Plymouth |
| Trypanosyllis zebra MOTU 5 | MTR015 | MTTZ014-20 | BOLD:AEE5372 | France, Roscoff |
| Trypanosyllis zebra MOTU 5 | MTRO16 | MTTZ015-20 | BOLD:AEE5372 | France, Roscoff |
| Trypanosyllis zebra MOTU 5 | MTRO45 | MTTZ016-20 | BOLD:AEE5372 | France, Roscoff |
| Trypanosyllis zebra MOTU 5 | MTRO46 | MTTZ017-20 | B0LD:AEE5372 | France, Roscoff |
| Trypanosyllis zebra MOTU 5 | MTR086 | MTTZO18-20 | BOLD:AEE5372 | France, Roscoff |
| Trypanosyllis zebra MOTU 5 | MTR087 | MTTZ043-20 | BOLD:AEE1619 | France, Roscoff |
| Trypanosyllis zebra MOTU 6 | MTAN29 | MTTZ010-20 | BOLD:AEE1672 | Spain near Cadiz, Sancti Petri - Chiclana |
| Trypanosyllis zebra MOTU 6 | MTAN33 | MTTZ011-20 | BOLD:AEE1672 | Spain near Cadiz, Sancti Petri <br> - Chiclana |
| Trypanosyllis zebra - <br> MOTU 6 | MTAN10 | MTTZ009-20 | BOLD:AEE1672 | Spain near Cadiz, Sancti Petri <br> - Chiclana |
| Trypanosyllis zebra MOTU 7 | MTAN13 | MTTZ001-20 | B0LD:AEE5374 | Croatia, Istra - off Rovinj, Sveti Ivan |
| Trypanosyllis zebra MOTU 7 | MTAN14 | MTTZ002-20 | B0LD:AEE5374 | Unknown |
| Trypanosyllis zebra MOTU 7 | MTAN37 | MTTZ003-20 | B0LD:AEE5374 | Croatia, Istra - off Rovinj, Sveti Ivan |
| Trypanosyllis zebra MOTU 7 | MTAN16 | MTTZ004-20 | BOLD:AEE5374 | France - Banyuls |
| Trypanosyllis zebra MOTU 7 | MTAN18 | MTTZ005-20 | BOLD:AEE5374 | France - Banyuls |
| Trypanosyllis zebraMOTU 8 | MTGC5-1 | MTTZ046-20 | B0LD:ACB6890 | Greece, Crete |
| Trypanosyllis zebraMOTU 8 | MTGC5-2 | MTTZ047-20 | B0LD:ACB6890 | Greece, Crete |
| Trypanosyllis zebraMOTU 8 | MTGC5-3 | MTTZ048-20 | B0LD:ACB6890 | Greece, Crete |
| Trypanosyllis zebra MOTU 8 | MTGC5-4 | MTTZ049-20 | B0LD:ACB6890 | Greece, Crete |
| Trypanosyllis zebra MOTU 9 | MTAN39 | MTTZ034-20 | BOLD:ADJ7140 | USA, San Diego |
| Trypanosyllis zebraMOTU 9 | MTAN40 | MTTZ035-20 | B0LD:ADJ7140 | USA, San Diego |
| Trypanosyllis zebraMOTU 9 | MTAN42 | MTTZ036-20 | B0LD:ADJ7140 | USA, San Diego |

(Table A1. Continuation)

| Species | Vial Code | BOLD Code | BIN | Location |
| :---: | :---: | :---: | :---: | :---: |
| Trypanosyllis zebraMOTU 9 | MTAN43 | MTTZ037-20 | BOLD:ADJ7140 | USA, San Diego |
| Trypanosyllis zebraMOTU 9 | MTAN26 | MTTZ033-20 | BOLD:ADJ7140 | USA, San Diego |
| Trypanosyllis aeolis Outgroup | MTAN1 | MTTZ051-20 | BOLD:AEH0164 | Spain, near Cadiz |
| Trypanosyllis aeolis Outgroup | MTAN2 | MTTZO52-20 | BOLD:AEH0165 | Spain, near Cadiz |
| Trypanosyllis aeolis Outgroup | MTAN3 | MTTZ053-20 | BOLD:AEH0164 | France Banyuls |
| Trypanosyllis aeolis Outgroup | MTAN4 | MTTZ054-20 | BOLD:AEH0165 | France Banyuls |
| Trypanosyllis aeolis Outgroup | MTAN5 | MTTZ055-20 | BOLD:AEH0165 | France Banyuls |
| Trypanosyllis aeolis Outgroup | MTAN6 | MTTZ056-20 | BOLD:AEH0165 | Croatia, Istra |
| Trypanosyllis sp. MOTU 10 | GENBANK | KR916953 | BOLD:ACH7277 | Sado estuary, Portugal (Lobo et al. 2016) |
| Trypanosyllis sp.MOTU 10 | GENBANK | KP254915 | BOLD:ACH7277 | Virginia, USA (Leray and Knowlto, 2015) |

(Table A1. Continuation)

## Annexes

## Annexes of chapter 2

Table S2.1. Species with multiple BINs considered as "Complex". Species in bold are currently not accepted in WoRMS.

| Species | No BINs |
| :--- | :--- |
| Austrolaenilla antarctica Bergström, 1916 | 5 |
| Bathykurila guaymasensis Pettibone, 1989 | 2 |
| Eteone longa (Fabricius, 1780) | 7 |
| Eurysyllis tuberculata Ehlers, 1864 | 3 |
| Glycinde gurjanovae Uschakov \& Wu, 1962 | 2 |
| Glycera dibranchiata Ehlers, 1868 | 2 |
| Glycera americana Leidy, 1855 | 4 |
| Gyptis robertscrippsi Rouse, Carvajal \& Pleijel, 2018 | 3 |
| Harmothoe imbricata (Linnaeus, 1767) | 9 |
| Harmothoe rarispina (M. Sars, 1861) | 3 |
| Hediste atoka Sato \& Nakashima, 2003 | 10 |
| Hediste diversicolor (0.F. Müller, 1776) | 37 |
| Laeonereis culveri (Webster, 1879) | 8 |
| Lepidonotus squamatus (Linnaeus, 1758) | 4 |
| Neogyptis jeffruoccoi Rouse, Carvajal \& Pleijel, 2018 | 2 |
| Neanthes acuminata (Ehlers, 1868) | 6 |
| Nereis vexillosa Grube, 1851 | 3 |
| Nereis pelagica Linnaeus, 1758 | 3 |
| Nereis denhamensis Augener, 1913 | 4 |
| Nepthys caeca (Fabricius, 1780) | 4 |
| Nephtys punctata Hartman, 1938 | 2 |
| Namalycastis abiuma (Grube, 1872) | 2 |
| Oxydromus obscurus (Verrill, 1873) | 2 |
| Pelagomacellicephala iliffeiPettibone, 1985 | 6 |
| Phyllodoce medipapillata Moore, 1909 | 2 |
| Phyllodoce groenlandica Örsted, 1842 | 5 |
| Platynereis bicanaliculata (Baird, 1863) | 6 |
| Pseudonereis anomala Gravier, 1899 | 7 |
| Platynereis dumerilii (Audouin \& Milne Edwards, 1833) | 2 |
| Sigambra bassi (Hartman, 1945) | 3 |
| Syllis gracilis Grube, 1840 | 8 |
| Syllis elongata Day, 1949 | 2 |
| Syllis alternata Moore, 1908 | 4 |
| Treptopale homalos Watson, 2010 | 6 |
| Treptopale paromolos Watson, 2010 | 7 |

## Annexes of chapter 3

Table S3.1. Public BIN accession numbers, museum voucher codes and location for each original specimen in chapter 3.

| Species | Vial code | Voucher Code | BIN | Location |
| :---: | :---: | :---: | :---: | :---: |
| Eumida mackieisp. nov. - MOTU 1 | PLY2017_010 | DBUA0002331.01 | BOLD:ADY9496 | Great Britain, Plymouth |
| Eumida mackieisp. nov. - MOTU 1 | PLY2017_011 | DBUA0002331.02 | BOLD:ADY9496 | Great Britain, Plymouth |
| Eumida mackieisp. nov. - MOTU 1 | PLY2017_012 | DBUA0002331.03 | BOLD:ADY9496 | Great Britain, Plymouth |
| Eumida mackieisp. nov. - MOTU 1 | PLY2017_016 | DBUA0002331.04 | BOLD:ADY9496 | Great Britain, Plymouth |
| Eumida mackieisp. nov. - MOTU 1 | PLY2017_017 | DBUA0002331.05 | BOLD:ADY9496 | Great Britain, Plymouth |
| Eumida mackieisp. nov. - MOTU 1 | PLY2017_019 | DBUA0002331.06 | BOLD:ADY9496 | Great Britain, Plymouth |
| Eumida mackieisp. nov. - MOTU 1 | PLY2017_028 | DBUA0002331.07 | BOLD:ADY9496 | Great Britain, Plymouth |
| Eumida mackiei sp. nov. - MOTU 1 | PLY2017_029 | DBUA0002331.08 | BOLD:ADY9496 | Great Britain, Plymouth |
| Eumida mackieisp. nov. - MOTU 1 | PLY2017_030 | DBUA0002331.09 | BOLD:ADY9496 | Great Britain, Plymouth |
| Eumida mackieisp. nov. - MOTU 1 | PLY2017_067 | DBUA0002331.10 | BOLD:ADY9496 | Great Britain, Plymouth |
| Eumida mackieisp. nov. - MOTU 1 | PLY2017_068 | DBUA0002331.11 | BOLD:ADY9496 | Great Britain, Plymouth |
| Eumida mackieisp. nov. - MOTU 1 | PLY2017_096 | DBUA0002331.12 | BOLD:ADY9496 | Great Britain, Plymouth |
| Eumida mackieisp. nov. - MOTU 1 | PLY2017_120 | DBUA0002331.13 | BOLD:ADY9496 | Great Britain, Plymouth |
| Eumida mackieisp. nov. - MOTU 1 | PLY2017_121 | DBUA0002331.14 | BOLD:ADY9496 | Great Britain, Plymouth |
| Eumida mackiei sp. nov. - MOTU 1 | PLY2017_122 | DBUA0002331.15 | BOLD:ADY9496 | Great Britain, Plymouth |
| Eumida mackieisp. nov. - MOTU 1 | PLY2017_123 | DBUA0002331.16 | BOLD:ADY9496 | Great Britain, Plymouth |
| Eumida mackieisp. nov. - MOTU 1 | PLY2017_124 | DBUA0002331.17 | BOLD:ADY9496 | Great Britain, Plymouth |
| Eumida mackieisp. nov. - MOTU 1 | PLY2017_125 | DBUA0002331.18 | BOLD:ADY9496 | Great Britain, Plymouth |
| Eumida mackieisp. nov. - MOTU 1 | PLY2017_126 | DBUA0002331.19 | BOLD:ADY9496 | Great Britain, Plymouth |
| Eumida mackieisp. nov. - MOTU 1 | PLY2017_128 | DBUA0002331.20 | BOLD:ADY9496 | Great Britain, Plymouth |
| Eumida mackieisp. nov. - MOTU 1 | PLY2017_099 | DBUA0002331.21 | BOLD:ADY9496 | Great Britain, Plymouth |
| Eumida mackieisp. nov. - MOTU 1 | PLY2017_100 | DBUA0002331.22 | BOLD:ADY9496 | Great Britain, Plymouth |


| Species | Vial code | Voucher Code | BIN | Location |
| :---: | :---: | :---: | :---: | :---: |
| Eumida mackieisp. nov. - MOTU 1 | PLY2017_102 | DBUA0002331.24 | BOLD:ADY9496 | Great Britain, Plymouth |
| Eumida mackiei sp. nov. - MOTU 1 | PLY2017_103 | DBUA0002331.25 | BOLD:ADY9496 | Great Britain, Plymouth |
| Eumida mackieisp. nov. - MOTU 1 | PLY2017_104 | DBUA0002331.26 | BOLD:ADY9496 | Great Britain, Plymouth |
| Eumida mackieisp. nov. - MOTU 1 | PLY2017_105 | DBUA0002331.27 | BOLD:ADY9496 | Great Britain, Plymouth |
| Eumida mackieisp. nov. - MOTU 1 | PLY2017_106 | DBUA0002333.01 | BOLD:ADY9496 | Great Britain, Plymouth |
| Eumida mackieisp. nov. - MOTU 1 | PLY2017_098 | DBUA0002418.01 | BOLD:ADY9496 | Great Britain, Plymouth |
| Eumida aff. merope MOTU 11 | G142 | DBUA0002393.01 | B0LD:AEB6473 | Great Britain, Cornwall |
| Eumida aff. merope MOTU 11 | G143 | DBUA0002394.01 | B0LD:AEB6473 | Great Britain, Cornwall |
| Eumida aff. merope MOTU 11 | G144 | DBUA0002394.02 | BOLD:AEB6473 | Great Britain, Cornwall |
| Eumida aff. merope MOTU 11 | G145 | DBUA0002394.03 | B0LD:AEB6473 | Great Britain, Cornwall |
| Eumida aff. merope MOTU 11 | G146 | DBUA0002394.04 | BOLD:AEB6473 | Great Britain, Cornwall |
| Eumida aff. merope MOTU 11 | G147 | DBUA0002394.05 | B0LD:AEB6473 | Great Britain, Cornwall |
| Eumida aff. merope - <br> MOTU 11 | RO2018_144 | DBUA0002395.01 | B0LD:AEB6473 | France, Roscoff |
| Eumida aff. merope MOTU 11 | RO2018_187 | DBUA0002395.02 | B0LD:AEB6473 | France, Roscoff |
| Eumida aff. merope MOTU 11 | RO2018_189 | DBUA0002395.03 | BOLD:AEB6473 | France, Roscoff |
| Eumida aff. merope MOTU 11 | RO2018_199 | DBUA0002395.04 | BOLD:AEB6473 | France, Roscoff |
| Eumida aff. merope MOTU 11 | RO2018_224 | DBUA0002395.05 | BOLD:AEB6473 | France, Roscoff |
| Eumida aff. merope MOTU 11 | RO2018_235 | DBUA0002395.06 | B0LD:AEB6473 | France, Roscoff |
| Eumida aff. merope MOTU 11 | RO2018_236 | DBUA0002395.07 | B0LD:AEB6473 | France, Roscoff |
| Eumida schanderisp. nov. - MOTU 22 | N112 | ZMBN_134550 | B0LD:ACQ6378 | Norway, Bergen |
| Eumida schanderisp. nov. - MOTU 22 | N113 | ZMBN_134551 | B0LD:ACQ6378 | Norway, Bergen |
| Eumida schanderisp. nov. - MOTU 22 | N114 | ZMBN_134552 | B0LD:ACQ6378 | Norway, Bergen |
| Eumida schanderisp. nov. - MOTU 22 | N115 | ZMBN_134553 | B0LD:ACQ6378 | Norway, Bergen |
| Eumida schanderisp. nov. - MOTU 22 | N116 | ZMBN_134554 | B0LD:ACQ6378 | Norway, Bergen |
| Eumida schanderisp. nov. - MOTU 22 | N117 | ZMBN_134555 | B0LD:ACQ6378 | Norway, Bergen |

(Table S3.1. Continuation)

| Species | Voucher Code | BIN | Location |
| :--- | :--- | :--- | :--- |
| Eumida schanderisp. <br> nov. - MOTU 22 | N119 | ZMBN_134557 | BOLD:ACQ6378 | Norway, Bergen

(Table S3.1. Continuation)

| Species | Vial code | Voucher Code | BIN | Location |
| :--- | :--- | :--- | :--- | :--- |
| Eumida fenwicki sp. <br> nov. - MOTU 6 <br> Eumida fenwicki sp. <br> nov. - MOTU 6 | PLY2017_22 | RO2018_201 | DBUA0002332.01 | BOLD:ADG39388 | | Great Britain, |
| :--- |
| Plymouth |,

(Table S3.1. Continuation)

| Species | Vial code | Voucher Code | BIN | Location |
| :---: | :---: | :---: | :---: | :---: |
| Eumida fauchaldi sp. nov. - MOTU 12 | PLY06DNA57_(3)_7 | DBUA0002400.24 | BOLD:AEA3142 | Great Britain, Plymouth |
| Eumida fauchaldi sp. nov. - MOTU 12 | PLY06DNA57_(4)_7 | DBUA0002400.25 | BOLD:AEA3142 | Great Britain, Plymouth |
| Eumida fauchaldi sp. nov. - MOTU 12 | PLY06DNA57_(5)_7 | DBUA0002400.26 | BOLD:AEA3142 | Great Britain, Plymouth |
| Eumida R0174-180 <br> - MOTU 16 | RO2018_174 | DBUA0002403.01 | BOLD:AEH2031 | France, Roscoff |
| Eumida R0174-180 MOTU 16 | RO2018_180 | DBUA0002403.02 | BOLD:AEH2031 | France, Roscoff |
| Eumida aff. fauchaldi <br> - MOTU 13 | RO2018_119 | DBUA0002402.01 | BOLD:AEH2030 | France, Roscoff |
| Eumida aff. fauchaldi <br> - MOTU 13 | PLY2017_060 | DBUA0002401.01 | BOLD:AEH2030 | Great Britain, Plymouth |
| Eumida aff. kelaino <br> - MOTU 17 | BA2020_05 | DBUA0002404.01 | BOLD:AEH2036 | France, Banyuls |
| Eumida aff. kelaino MOTU 17 | BA2020_08 | DBUA0002404.02 | BOLD:AEH2036 | France, Banyuls |
| Eumida ANT002 <br> - MOTU 14 | JLIA2-2 | DBUA0002405.01 | BOLD:AEH2034 | Western Italy, Antignano |
| Eumida pleijeli sp. nov. <br> - MOTU 3 | IT10DNA007 | DBUA0002407.01 | BOLD:AEH2033 | Italy, Naples |
| Eumida pleijeli sp. nov. <br> - MOTU 3 | IT10DNA009 | DBUA0002407.02 | BOLD:AEH2033 | Italy, Naples |
| Eumida langeneckisp. nov. - MOTU 5 | IT10DNA032 | DBUA0002408.01 | BOLD:AEH2035 | Italy, Ischia |
| Eumida langeneckisp. nov. - MOTU 5 | WS14DNA995 | DBUA0002409.01 | BOLD:AEH2035 | Italy, Antignano |
| Eumida langeneckisp. nov. - MOTU 5 | JLIA2-1 | DBUA0002409.02 | BOLD:AEH2035 | Italy, Antignano |
| Eumida langeneckisp. nov. - MOTU 5 | JLIA2-3 | DBUA0002409.03 | BOLD:AEH2035 | Italy, Antignano |
| Eumida langeneckisp. nov. - MOTU 5 | JLIA2-4 | DBUA0002409.04 | BOLD:AEH2035 | Italy, Antignano |
| Eumida langeneckisp. nov. - MOTU 5 | JLIA2-5 | DBUA0002409.05 | BOLD:AEH2035 | Italy, Antignano |
| Eumida ORB997 <br> - MOTU 2 | WS14DNA997 | DBUA0002410.01 | BOLD:AEH2029 | Italy, Orbetello |
| Eumida maia <br> - MOTU 4 | BA09-049 | DBUA0002339.01 | BOLD:ACQ7431 | France, Banyuls |
| Eumida maia <br> - MOTU 4 | BA09-125 | DBUA0002339.02 | B0LD:ACQ7431 | France, Banyuls |
| Eumida maia <br> - MOTU 4 | BA09-151 | DBUA0002339.03 | BOLD:ACQ7431 | France, Banyuls |
| Eumida maia <br> - MOTU 4 | BA09-152 | DBUA0002339.04 | BOLD:ACQ7431 | France, Banyuls |
| Eumida maia <br> - MOTU 4 | BA09-251 | DBUA0002339.05 | B0LD:ACQ7431 | France, Banyuls |
| Eumida maia <br> - MOTU 4 | BA09-286 | DBUA0002339.06 | B0LD:ACQ7431 | France, Banyuls |

(Table S3.1. Continuation)

| Species | Vial code | Voucher Code | BIN | Location |
| :---: | :---: | :---: | :---: | :---: |
| Eumida maia <br> - MOTU 4 | BA09-291 | DBUA0002339.07 | BOLD:ACQ7431 | France, Banyuls |
| Eumida maia <br> - MOTU 4 | BA09-296 | DBUA0002339.08 | B0LD:ACQ7431 | France, Banyuls |
| Eumida maia <br> - MOTU 4 | BA09-297 | DBUA0002339.09 | BOLD:ACQ7431 | France, Banyuls |
| Eumida maia <br> - MOTU 4 | BA09-298 | DBUA0002339.10 | BOLD:ACQ7431 | France, Banyuls |
| Eumida maia <br> - MOTU 4 | BA09-300 | DBUA0002339.11 | B0LD:ACQ7431 | France, Banyuls |
| Eumida maia <br> - MOTU 4 | BA09-301 | DBUA0002339.12 | BOLD:ACQ7431 | France, Banyuls |
| Eumida maia <br> - MOTU 4 | BA09-304 | DBUA0002339.13 | BOLD:ACQ7431 | France, Banyuls |
| Eumida maia <br> - MOTU 4 | BA09-050 | DBUA0002339.14 | BOLD:ACQ7431 | France, Banyuls |
| Eumida maia <br> - MOTU 4 | BA09-051 | DBUA0002339.15 | BOLD:ACQ7431 | France, Banyuls |
| Eumida maia <br> - MOTU 4 | BA09DNA12 | DBUA0002339.16 | BOLD:ACQ7431 | France, Banyuls |
| Eumida maia <br> - MOTU 4 | BA09DNA33 | DBUA0002339.17 | BOLD:ACQ7431 | France, Banyuls |
| Eumida maia <br> - MOTU 4 | BA09DNA41 | DBUA0002339.18 | B0LD:ACQ7431 | France, Banyuls |
| Eumida maia <br> - MOTU 4 | BA09DNA57 | DBUA0002339.19 | BOLD:ACQ7431 | France, Banyuls |
| Eumida maia <br> - MOTU 4 | BA09DNA65 | DBUA0002339.20 | B0LD:ACQ7431 | France, Banyuls |
| Eumida maia <br> - MOTU 4 | PLY2011DNA094 | DBUA0002338.03 | B0LD:ACQ7431 | Great britain, Plymouth |
| Eumida maia <br> - MOTU 4 | PLY2011 DNA106 | DBUA0002338.01 | BOLD:ACQ7431 | Great britain, Plymouth |
| Eumida maia <br> - MOTU 4 | PLY2011 DNA162 | DBUA0002338.02 | BOLD:ACQ7431 | Great britain, Plymouth |
| Eumida maia <br> - MOTU 4 | PLY2017DNA153 | DBUA0002411.01 | BOLD:ACQ7431 | Great britain, Plymouth |
| Eumida maia <br> - MOTU 4 | G73 | DBUA0002411.02 | BOLD:ACQ7431 | Great Britain, Cornwall |
| Eumida maia <br> - MOTU 4 | G72 | DBUA0002411.03 | BOLD:ACQ7431 | Great Britain, Cornwall |
| Eumida maia <br> - MOTU 4 | G74 | DBUA0002411.04 | BOLD:ACQ7431 | Great Britain, Cornwall |
| Eumida maia <br> - MOTU 4 | G75 | DBUA0002411.05 | BOLD:ACQ7431 | Great Britain, Cornwall |
| Eumida maia <br> - MOTU 4 | G76 | DBUA0002411.06 | BOLD:ACQ7431 | Great Britain, Cornwall |
| Eumida maia <br> - MOTU 4 | G77 | DBUA0002411.07 | BOLD:ACQ7431 | Great Britain, Cornwall |
| Eumida maia <br> - MOTU 4 | G78 | DBUA0002411.08 | BOLD:ACQ7431 | Great Britain, Cornwall |

(Table S3.1. Continuation)

| Species | Vial code | Voucher Code | BIN | Location |
| :---: | :---: | :---: | :---: | :---: |
| Eumida sanguinea <br> - MOTU 19 | N131 | DBUA0002334.01 | BOLD:ACQ1561 | Norway, Finnmark |
| Eumida sanguinea <br> - MOTU 19 | N132 | DBUA0002334.02 | BOLD:ACQ1561 | Norway, Finnmark |
| Eumida sanguinea <br> - MOTU 19 | N133 | DBUA0002334.03 | BOLD:ACQ1561 | Norway, Finnmark |
| Eumida sanguínea <br> - MOTU 19 | N134 | DBUA0002334.04 | BOLD:ACQ1561 | Norway, Finnmark |
| Eumida sanguinea <br> - MOTU 19 | N135 | DBUA0002334.05 | BOLD:ACQ1561 | Norway, Finnmark |
| Eumida sanguinea <br> - MOTU 19 | MAR14DNA093 | DBUA0002334.06 | BOLD:ACQ1561 | Norway, Finnmark |
| Eumida sanguínea <br> - MOTU 19 | MAR14DNA104 | DBUA0002334.07 | BOLD:ACQ1561 | Norway, Finnmark |
| Eumida sanguinea <br> - MOTU 19 | MAR14DNA106 | DBUA0002334.08 | BOLD:ACQ1561 | Norway, Finnmark |
| Eumida sanguinea <br> - MOTU 19 | MAR14DNA109 | DBUA0002334.09 | BOLD:ACQ1561 | Norway, Finnmark |
| Eumida sanguínea <br> - MOTU 19 | MAR14DNA149 | DBUA0002334.10 | BOLD:ACQ1561 | Norway, Finnmark |
| Eumida sanguinea <br> - MOTU 19 | SL037 | DBUA0002334.11 | BOLD:ACQ1561 | Norway, Finnmark |
| Eumida sanguinea <br> - MOTU 19 | POLYSKAG 2014052 | DBUA0002335.02 | BOLD:ACQ1561 | Norway, Bergen |
| Eumida sanguínea <br> - MOTU 19 | N137 | DBUA0002336.01 | BOLD:ACQ1561 | Norway, Bergen |
| Eumida sanguinea <br> - MOTU 19 | N138 | DBUA0002336.02 | BOLD:ACQ1561 | Norway, Bergen |
| Eumida sanguínea <br> - MOTU 19 | WS14DNA697 | DBUA0002337.01 | BOLD:ACQ1561 | Norway, Droebak |
| Eumida sanguínea <br> - MOTU 19 | N136 | DBUA0002335.01 | BOLD:ACQ1561 | Norway, Droebak |
| Eumida sanguinea <br> - MOTU 19 | PLY2017_031 | DBUA0002412.01 | BOLD:ACQ1561 | Great britain, Plymouth |
| Eumida sanguínea <br> - MOTU 19 | PLY6DNA_57_(6)_7 | DBUA0002412.02 | BOLD:ACQ1561 | Great britain, Plymouth |
| Eumida notata <br> - MOTU 9 | MA09_117 | DBUA0002340.16 | B0LD:ACQ6102 | Portugal, Madeira |
| Eumida notata <br> - MOTU 9 | MA09_119 | DBUA0002340.18 | B0LD:ACQ6102 | Portugal, Madeira |
| Eumida notata <br> - MOTU 9 | MA09_120 | DBUA0002340.19 | B0LD:ACQ6102 | Portugal, Madeira |
| Eumida notata <br> - MOTU 9 | MA09_121 | DBUA0002340.20 | B0LD:ACQ6102 | Portugal, Madeira |
| Eumida notata <br> - MOTU 9 | MA09_122 | DBUA0002340.21 | B0LD:ACQ6102 | Portugal, Madeira |
| Eumida kelaino | G124 | DBUA0002413.01 | BOLD:ACQ1138 | Great Britain, Plymouth |
| Eumida kelaino <br> - MOTU 18 | G125 | DBUA0002413.02 | BOLD:ACQ1138 | Great Britain, Plymouth |

(Table S3.1. Continuation)

| Species | Vial code | Voucher Code | BIN | Location |
| :---: | :---: | :---: | :---: | :---: |
| Eumida kelaino <br> - MOTU 18 | G127 | DBUA0002413.04 | BOLD:ACQ1138 | Great Britain, Plymouth |
| Eumida kelaino <br> - MOTU 18 | N128 | ZMBN_134544 | B0LD:ACQ1138 | Norway, Sandefjord |
| Eumida kelaino <br> - MOTU 18 | N129 | ZMBN_134545 | BOLD:ACQ1138 | Norway, Bergen |
| Eumida kelaino <br> - MOTU 18 | N130 | ZMBN_134546 | B0LD:ACQ1138 | Norway, Bergen |
| Eumida kelaino <br> - MOTU 18 | RO2018_142 | DBUA0002414.01 | BOLD:ACQ1138 | France, Roscoff |
| Eumida kelaino <br> - MOTU 18 | RO2018_146 | DBUA0002414.02 | BOLD:ACQ1138 | France, Roscoff |
| Eumida kelaino <br> - MOTU 18 | RO2018_148 | DBUA0002414.03 | BOLD:ACQ1138 | France, Roscoff |
| Eumida kelaino <br> - MOTU 18 | RO2018_188 | DBUA0002414.04 | BOLD:ACQ1138 | France, Roscoff |
| Eumida kelaino <br> - MOTU 18 | RO2018_200 | DBUA0002414.05 | BOLD:ACQ1138 | France, Roscoff |
| Eumida kelaino <br> - MOTU 18 | RO2018_223 | DBUA0002414.06 | BOLD:ACQ1138 | France, Roscoff |
| Eumida elektra <br> - MOTU 8 | R02018_131 | DBUA0002415.01 | BOLD:ACQ7892 | France, Roscoff |
| Eumida elektra <br> - MOTU 8 | RO2018_132 | DBUA0002415.02 | BOLD:ACQ7892 | France, Roscoff |
| Eumida elektra <br> - MOTU 8 | RO2018_139 | DBUA0002415.03 | BOLD:ACQ7892 | France, Roscoff |
| Eumida elektra <br> - MOTU 8 | RO2018_145 | DBUA0002415.04 | BOLD:ACQ7892 | France, Roscoff |
| Eumida elektra <br> - MOTU 8 | RO2018_147 | DBUA0002415.05 | BOLD:ACQ7892 | France, Roscoff |
| Eumida elektra <br> - MOTU 8 | RO2018_185 | DBUA0002415.06 | BOLD:ACQ7892 | France, Roscoff |
| Eumida elektra <br> - MOTU 8 | R02018_186 | DBUA0002415.07 | BOLD:ACQ7892 | France, Roscoff |
| Eumida elektra <br> - MOTU 8 | RO2018_198 | DBUA0002415.08 | B0LD:ACQ7892 | France, Roscoff |
| Eumida elektra <br> - MOTU 8 | RO2018_202 | DBUA0002415.09 | BOLD:ACQ7892 | France, Roscoff |
| Eumida elektra <br> - MOTU 8 | RO2018_222 | DBUA0002415.10 | BOLD:ACQ7892 | France, Roscoff |
| Eumida elektra <br> - MOTU 8 | RO2018_225 | DBUA0002415.11 | BOLD:ACQ7892 | France, Roscoff |
| Eumida taygete <br> - MOTU 21 | IT99 | MTANE128-19 | BOLD:ACQ4605 | Italy, Ischia |
| Eumida taygete <br> - MOTU 21 | IT100 | MTANE129-19 | BOLD:ACQ4605 | Italy, Ischia |
| Eumida taygete <br> - MOTU 21 | G107 | DBUA0002416.01 | BOLD:ACQ4605 | Great Britain, Cornwall |
| Eumida taygete <br> - MOTU 21 | G102 | DBUA0002416.02 | BOLD:ACQ4605 | Great Britain, Cornwall |

(Table S3.1. Continuation)

| Species | Vial code | Voucher Code | BIN | Location |
| :--- | :--- | :--- | :--- | :--- |
| Eumida taygete <br> - MOTU 21 | G104 | DBUA0002416.04 | BOLD:ACQ4605 | Great Britain, <br> Eumida taygete |
| - MOTU 21 | G105 |  | DBUAll |  |
| Eumida taygete |  |  |  |  |
| - MOTU 21 |  |  |  |  |

(Table S3.1. Continuation)

Table S3.2. Geographic location, voucher and GenBank accession numbers for sequences belonging to other studies and used for comparison purposes

| Species | Designation (MOTU) | Museum Code | GenBank COI | GenBank ITS | Location | References |
| :---: | :---: | :---: | :---: | :---: | :---: | :---: |
| Eumida maia | MOTU 4 | SMNH T-7987 | HM358655 | HM358739 | France, Banyuls | Nygren and Pleijel, 2011 |
| Eumida | MOTU 4 | SMNH T-7988 | HM358656 | HM358740 | France, | Nygren and |
| maia <br> Eumida |  |  |  |  | Banyuls | Pleijel, 2011 |
| maia | MOTU 4 | SMNH T-7989 | HM358657 |  | Banyuls | Pleijel, 2011 |
| Eumida <br> maia | MOTU 4 | SMNH T-7990 | HM358658 |  | France, Banyuls | Nygren and Pleijel, 2011 |
| Eumida | MOTU 4 | SMNH T-7998 | HM358659 |  | France, Banyuls | Nygren and Pleijel, 2011 |
| Eumida <br> maia | MOTU 4 | SMNH T-7991 | HM358660 |  | France, Banyuls | Nygren and Pleijel, 2011 |
| Eumida <br> maia | MOTU 4 | SMNH T-7992 | HM358661 |  | France, Banyuls | Nygren and Pleijel, 2011 |
| Eumida <br> maia | MOTU 4 | SMNH T-7999 | HM358662 |  | France, Banyuls | Nygren and Pleijel, 2011 |
| Eumida <br> maia | MOTU 4 | SMNH T-7993 | HM358663 |  | France, Banyuls | Nygren and Pleijel, 2011 |
| Eumida <br> maia | MOTU 4 | SMNH T-7997 | HM358664 |  | France, Banyuls | Nygren and Pleijel, 2011 |
| Eumida <br> maia | MOTU 4 | SMNH T-8000 | HM358665 |  | France, Banyuls | Nygren and Pleijel, 2011 |
| Eumida <br> maia | MOTU 4 | SMNH T-7994 | HM358666 |  | France, Banyuls | Nygren and Pleijel, 2011 |
| Eumida <br> maia | MOTU 4 | SMNH T-7995 | HM358667 |  | France, Banyuls | Nygren and Pleijel, 2011 |
| Eumida <br> maia | MOTU 4 | SMNH T-8016 | HM358668 | HM358752 | France, Banyuls | Nygren and Pleijel, 2011 |
| Eumida <br> maia | MOTU 4 | SMNH T-7996 | HM358669 | HM358753 | France, Banyuls | Nygren and Pleijel, 2011 |
| Eumida <br> sanguinea | MOTU 19 | SMNH_110598 | HM358695 |  | Sweden, Bohuslän | Nygren and Pleijel, 2011 |
| Eumida sanguinea | MOTU 19 | SMNH_110599 | HM358696 |  | Sweden, <br> Bohuslän | Nygren and Pleijel, 2011 |
| Eumida sanguinea | MOTU 19 | SMNH_110600 | HM358699 |  | Sweden, Bohuslän | Nygren and Pleijel, 2011 |
| Eumida sanguinea | MOTU 19 | SMNH_110606 | HM358701 |  | Sweden, Bohuslän | Nygren and Pleijel, 2011 |
| Eumida sanguinea | MOTU 19 | SMNH_110601 | HM358702 |  | Sweden, Bohuslän | Nygren and Pleijel, 2011 |
| Eumida sanguinea | MOTU 19 | SMNH_110595 | HM358704 |  | Sweden, Bohuslän | Nygren and Pleijel, 2011 |
| Eumida sanguinea | MOTU 19 | SMNH_110603 | HM358705 |  | Sweden, Bohuslän | Nygren and Pleijel, 2011 |
| Eumida sanguinea | MOTU 19 | SMNH_110604 | HM358707 |  | Sweden, Bohuslän | Nygren and Pleijel, 2011 |


| Species | Designation (MOTU) | Museum Code | GenBank COI | GenBank ITS | Location | References |
| :---: | :---: | :---: | :---: | :---: | :---: | :---: |
| Eumida sanguinea | MOTU 19 | SMNH_110602 | HM358703 | HM358787 | Denmark, Helsingør | Nygren and Pleijel, 2011 |
| Eumida sanguinea | MOTU 19 | SMNH 110597 | HM358697 |  | Norway, Bergen | Nygren and Pleijel, 2011 |
| Eumida sanguinea | MOTU 19 | SMNH_110596 | HM358700 |  | Norway, Bergen Great | Nygren and Pleijel, 2011 |
| Eumida sanguinea | MOTU 19 | SMNH 110607 | HM358698 |  | Britain, Scilly islands | Nygren and Pleijel, 2011 |
| Eumida notata | MOTU 9 | SMNH_110608 | HM358708 | HM358792 | Portugal, Madeira | Nygren and Pleijel, 2011 |
| Eumida notata | MOTU 9 | SMNH_110609 | HM358709 | HM358793 | Portugal, Madeira | Nygren and Pleijel, 2011 |
| Eumida notata | MOTU 9 | SMNH_110610 | HM358710 | HM358794 | Portugal, Madeira | Nygren and Pleijel, 2011 |
| Eumida notata | MOTU 9 | SMNH_110611 | HM358711 | HM358795 | Portugal, Madeira | Nygren and Pleijel, 2011 |
| Eumida notata | MOTU 9 | SMNH_110612 | HM358712 | HM358796 | Portugal, Madeira | Nygren and Pleijel, 2011 |
| Eumida notata | MOTU 9 | SMNH_110613 | HM358713 | HM358797 | Portugal, Madeira | Nygren and Pleijel, 2011 |
| Eumida kelaino | MOTU 18 | SMNH T-7979 | HM358683 | HM358767 | Sweden, Bohuslän | Nygren and Pleijel, 2011 |
| Eumida kelaino | MOTU 18 | SMNH T-7981 | HM358684 | HM358768 | Sweden, <br> Bohuslän | Nygren and Pleijel, 2011 |
| Eumida kelaino | MOTU 18 | SMNH T-7983 | HM358685 | HM358769 | Sweden, Bohuslän | Nygren and Pleijel, 2011 |
| Eumida kelaino | MOTU 18 | SMNH T-7982 | HM358686 | HM358770 | Sweden, Bohuslän | Nygren and Pleijel, 2011 |
| Eumida kelaino | MOTU 18 | SMNH T-7980 | HM358687 | HM358771 | Sweden, Bohuslän | Nygren and Pleijel, 2011 |
| Eumida kelaino | MOTU 18 | SMNH T-7984 | HM358688 | HM358772 | Sweden, Bohuslän | Nygren and Pleijel, 2011 |
| Eumida kelaino | MOTU 18 | SMNH T-7986 | HM358689 | HM358773 | Sweden, Bohuslän | Nygren and Pleijel, 2011 |
| Eumida kelaino | MOTU 18 | SMNH T-7985 | HM358690 | HM358774 | Sweden, Bohuslän | Nygren and Pleijel, 2011 |
| Eumida elektra | MOTU 8 | SMNH T-7975 | HM358677 | HM358761 | Norway, Bergen | Nygren and Pleijel, 2011 |
| Eumida elektra | MOTU 8 | SMNH T-7976 | HM358678 | HM358762 | Norway, Bergen | Nygren and Pleijel, 2011 |
| Eumida elektra | MOTU 8 | SMNH T-7977 | HM358679 | HM358763 | Norway, Bergen | Nygren and Pleijel, 2011 |
| Eumida elektra | MOTU 8 | SMNH T-7978 | HM358680 | HM358764 | Norway, Bergen | Nygren and <br> Pleijel, 2011 |
| Eumida <br> taygete | MOTU 21 | SMNH T-8011 | HM358670 |  | Croatia, Istra | Nygren and Pleijel, 2011 |
| Eumida taygete | MOTU 21 | SMNH T-8012 | HM358671 |  | France, Banyuls | Nygren and Pleijel, 2011 |

(Table S3.2. Continuation)

| Species | Designation (MOTU) | Museum Code | GenBank COI | GenBank ITS | Location | References |
| :---: | :---: | :---: | :---: | :---: | :---: | :---: |
| Eumida <br> taygete | MOTU 21 | SMNH T-8014 | HM358673 |  | France, Banyuls | Nygren and Pleijel, 2011 |
| Eumida taygete | MOTU 21 | SMNH T-8015 | HM358674 | HM358758 | France, Banyuls | Nygren and Pleijel, 2011 |
| Eumida <br> alkyone | MOTU 20 | SMNH T-7970 | HM358691 | HM358775 | Sweden, Bohuslän | Nygren and Pleijel, 2011 |
| Eumida alkyone | MOTU 20 | SMNH T-7971 | HM358693 | HM358777 | Sweden, Bohuslän | Nygren and Pleijel, 2011 |
| Eumida alkyone | MOTU 20 | SMNH T-7972 | HM358692 | HM358776 | Sweden, Bohuslän | Nygren and Pleijel, 2011 |
| Eumida alkyone | MOTU 20 | SMNH T-7969 | HM358694 | HM358778 | Norway, Bergen | Nygren and Pleijel, 2011 |
| Eumida merope | MOTU 10 | SMNH T-8003 | HM358714 | HM358798 | France, Banyuls | Nygren and Pleijel, 2011 |
| Eumida merope | MOTU 10 | SMNH T-8002 | HM358715 | HM358799 | France, Banyuls | Nygren and Pleijel, 2011 |
| Eumida merope | MOTU 10 | SMNH T-8001 | HM358716 | HM358800 | France, Banyuls | Nygren and <br> Pleijel, 2011 |
| Eumida merope | MOTU 10 | SMNH T-8004 | HM358717 | HM358801 | France, Banyuls | Nygren and Pleijel, 2011 |
| Eumida merope | MOTU 10 | SMNH T-8005 | HM358719 | HM358803 | France, Banyuls | Nygren and Pleijel, 2011 |
| Eumida merope | MOTU 10 | SMNH T-8006 | HM358720 | HM358804 | France, Banyuls | Nygren and Pleijel, 2011 |
| Eumida merope | MOTU 10 | SMNH T-8007 | HM358721 | HM358805 | France, Banyuls | Nygren and Pleijel, 2011 |
| Eumida merope | MOTU 10 | SMNH T-8008 | HM358722 | HM358806 | France, Banyuls | Nygren and Pleijel, 2011 |
| Eumida merope | MOTU 10 | SMNH T-8009 | HM358723 | HM358807 | France, Banyuls | Nygren and Pleijel, 2011 |
| Eumida merope | MOTU 10 | SMNH T-8010 | HM358718 | HM358802 | Croatia, Istra | Nygren and Pleijel, 2011 |
| Eumida asterope | MOTU 15 | SMNH T-7973 | HM358681 | HM358765 | France, Banyuls | Nygren and Pleijel, 2011 |
| Eumida asterope | MOTU 15 | SMNH T-7974 | HM358682 | HM358766 | France, Banyuls | Nygren and Pleijel, 2011 |
| Eumida F22 | MOTU 7 | SMNH 110615 | HM358676 | HM358760 | France, Banyuls | Nygren and Pleijel, 2011 |
| EumidaS21 | MOTU 22 | SMNH_110614 | HM358675 | HM358759 | Sweden, Bohuslän | Nygren and Pleijel, 2011 |
| Eumida bahusiensis | Outgroup | SMNH 110638 | HM358649 | HM358733 | Sweden, Bohuslän | Nygren and Pleijel, 2011 |
| Eumida bahusiensis | Outgroup | SMNH 110639 | HM358650 | HM358734 | Sweden, Bohuslän | Nygren and Pleijel, 2011 |
| Eumida bahusiensis | Outgroup | SMNH 110640 | HM358651 | HM358735 | Sweden, Bohuslän | Nygren and Pleijel, 2011 |
| Eumida bahusiensis | Outgroup | SMNH 110641 | HM358652 | HM358736 | Sweden, Bohuslän | Nygren and Pleijel, 2011 |
| Eumida bahusiensis | Outgroup | SMNH 110642 | HM358653 | HM358737 | Sweden, Bohuslän | Nygren and <br> Pleijel, 2011 |

(Table S3.2. Continuation)

| Species | Designation (MOTU) | Museum Code | GenBank COI | GenBank ITS | Location | References |
| :---: | :---: | :---: | :---: | :---: | :---: | :---: |
| Eumida ockelmanni | Outgroup | SMNH 110631 | HM358642 |  | Sweden, Bohuslän | Nygren and Pleijel, 2011 |
| Eumida ockelmanni | Outgroup | SMNH 110632 | HM358643 | HM358727 | Sweden, Bohuslän | Nygren and Pleijel, 2011 |
| Eumida ockelmanni | Outgroup | SMNH 110634 | HM358645 | HM358729 | Sweden, Bohuslän | Nygren and Pleijel, 2011 |
| Eumida ockelmanni | Outgroup | SMNH 110635 | HM358646 |  | Sweden, Bohuslän | Nygren and Pleijel, 2011 |
| Eumida ockelmanni | Outgroup | SMNH 110633 | HM358644 | HM358728 | Denmark, Helsingør | Nygren and Pleijel, 2011 |
| Eumida ockelmanni | Outgroup | SMNH 110636 | HM358647 |  | Denmark, Helsingør | Nygren and Pleijel, 2011 |
| Eumida ockelmanni | Outgroup | SMNH 110637 | HM358648 |  | Denmark, Helsingør | Nygren and Pleijel, 2011 |
| Sige fusigera | Outgroup | SMNH 110629 | HM358640 | HM358724 | Sweden, Bohuslän | Nygren and Pleijel, 2011 |
| Sige fusigera | Outgroup | SMNH 110630 | HM358641 | HM358725 | Sweden, Bohuslän | Nygren and Pleijel, 2011 |

(Table S3.2. Continuation)

## Annexes of chapter 4

Table S4.1. Public BIN accession numbers, museum voucher codes and location for each original specimen in chapter 4.

| Species | Vial code | Museum Voucher | BIN | Location |
| :--- | :--- | :--- | :--- | :--- |
| E. clavigera- <br> MOTU 4 <br> E. clavigera- <br> MOTU 4 | MTPA05 | DBUA0002468.01.v01 | BOLD:AAY5110 | Portugal, Aveiro |
| E. clavigera- <br> MOTU 4 | MTPA07 | DBUA0002468.01.v02 | BOLD:AAY5110 | Portugal, Aveiro |
| E. clavigera- <br> MOTU 4 <br> E. clavigera- | MTPA08 | MTPA11 | DBUA0002468.01.v04 | BOLD:AAY5110 | Portugal, Aveiro


| Species | Vial code | Museum Voucher | BIN | Location |
| :--- | :--- | :--- | :--- | :--- |
| E. clavigera - <br> MOTU 4 | Ro2018-056 | DBUA0002471.01.v05 | BOLD:AAY5110 | France, Roscoff |
| E. clavigera - | NLFB1-1 | DBUA0002472.01.v01 | BOLD:AAY5110 | France, Morgat |
| MOTU 4 |  |  |  |  |
| E. clavigera - <br> MOTU 4 | NLFB1-2 | DBUA0002472.01.v02 | BOLD:AAY5110 | France, Morgat |
| E. clavigera - | JPEC1 | Deach |  |  |
| MOTU 4 |  |  |  |  |

(Table S4.1 Continuation)

| Species | Vial code | Museum Voucher | BIN | Location |
| :---: | :---: | :---: | :---: | :---: |
| E. clavigeraMOTU 4 | MTCT2.5 | DBUA0002476.01.v05 | BOLD:AAY5110 | Spain, Canary, Tenerife |
| E. clavigera MOTU 4 | MTCP2.1 | DBUA0002476.02.v01 | BOLD:AAY5110 | Spain, Canary, La Palma |
| E. clavigera MOTU 4 | MTCP2.2 | DBUA0002476.02.v02 | BOLD:AAY5110 | Spain, Canary, La Palma |
| E. clavigeraMOTU 4 | MTCP2.3 | DBUA0002476.02.v03 | BOLD:AAY5110 | Spain, Canary, La Palma |
| E. clavigeraMOTU 4 | MTCP2.4 | DBUA0002476.02.v04 | BOLD:AAY5110 | Spain, Canary, La Palma |
| E. clavigeraMOTU 4 | MTCP2.5 | DBUA0002476.02.v05 | BOLD:AAY5110 | Spain, Canary, La Palma |
| E. clavigeraMOTU 4 | MTCP2.6 | DBUA0002476.02.v06 | BOLD:AAY5110 | Spain, Canary, La Palma |
| E. clavigeraMOTU 4 | MTCP2.7 | DBUA0002476.02.v07 | BOLD:AAY5110 | Spain, Canary, La Palma |
| E. clavigera MOTU 4 | MTCC2.1 | DBUA0002476.03.v01 | BOLD:AAY5110 | Spain, Canary, Gran Canaria |
| E. clavigeraMOTU 4 | MTCC2.2 | DBUA0002476.03.v02 | BOLD:AAY5110 | Spain, Canary, Gran Canaria |
| E. clavigeraMOTU 4 | MTCC2.3 | DBUA0002476.03.v03 | BOLD:AAY5110 | Spain, Canary, Gran Canaria |
| E. clavigera MOTU 4 | MTCC2.4 | DBUA0002476.03.v04 | BOLD:AAY5110 | Spain, Canary, Gran Canaria |
| E. clavigeraMOTU 4 | MTCC2.6 | DBUA0002476.03.v05 | BOLD:AAY5110 | Spain, Canary, Gran Canaria |
| E. clavigera MOTU 4 | MTAS1 | DBUA0002477.01.v01 | BOLD:AAY5110 | Portugal, Azores, S.Maria |
| E. clavigeraMOTU 4 | MTAS2 | DBUA0002477.01.v02 | BOLD:AAY5110 | Portugal, Azores, S.Maria |
| E. clavigeraMOTU 4 | JPAM02 | MB29-000385 | BOLD:AAY5110 | Portugal, Savage islands |
| E. feliciae sp . nov. <br> - MOTU 1 | BA01DNA067 159 | DBUA0002478.01.v01 | BOLD:AEC0502 | France, Banyuls |
| E. feliciae sp. nov. <br> - MOTU 1 | BA01DNA067 161 | DBUA0002478.01.v02 | BOLD:AEC0502 | France, Banyuls |
| E. feliciae sp . nov. <br> - MOTU 1 | BA01DNA067 155 | DBUA0002478.01.v03 | BOLD:AEC0502 | France, Banyuls |
| E. feliciae sp . nov. <br> - MOTU 1 | BA01DNA067 156 | DBUA0002478.01.v04 | BOLD:AEC0502 | France, Banyuls |
| E. feliciae sp . nov. <br> - MOTU 1 | BA01DNA067 157 <br> (72) | DBUA0002478.01.v05 | BOLD:AEC0502 | France, Banyuls |
| E. feliciae sp . nov. <br> - MOTU 1 | BA09DNA43 | MTE040-20 | BOLD:AEC0502 | France, Banyuls |
| E. feliciae sp . nov. <br> - MOTU 1 | BA09DNA18 | MTE042-20 | BOLD:AEC0502 | France, Banyuls |
| E. feliciae sp . nov. <br> - MOTU 1 | BA01DNA55 | DBUA0002478.01.v06 | BOLD:AEC0502 | France, Banyuls |
| E. feliciae sp . nov. <br> - MOTU 1 | BA09DNA123 | DBUA0002478.01.v07 | BOLD:AEC0502 | France, Banyuls |

(Table S4.1 Continuation)

| Species | Vial code | Museum Voucher | BIN | Location |
| :---: | :---: | :---: | :---: | :---: |
| E. feliciae sp. nov. <br> - MOTU 1 | BA09DNA195 (43) | DBUA0002478.01.v08 | BOLD:AEC0502 | France, Banyuls |
| E. madeirensis sp . nov. - MOTU 2 | MA09-101 | DBUA0002479.01.v01 | BOLD:AEC0503 | Portugal, Madeira Funchal |
| E. madeirensis sp . nov. - MOTU 2 | MA09-102 | DBUA0002479.01.v02 | BOLD:AEC0503 | Portugal, Madeira Funchal |
| E. madeirensis sp . nov. - MOTU 2 | MA09-008 | DBUA0002479.01.v03 | BOLD:AEC0503 | Portugal, Madeira Funchal |
| E. madeirensis sp. nov. - MOTU 2 | MA09-243 | DBUA0002479.02.v01 | BOLD:AEC0503 | Portugal, Madeira - <br> Porto Moniz |
| E. madeirensis sp . nov. - MOTU 2 | MA09-045 | DBUA0002479.01.v04 | BOLD:AEC0503 | Portugal, Madeira Funchal |
| E. madeirensis sp . nov. - MOTU 2 | MA09-066 | DBUA0002479.01.v05 | BOLD:AEC0503 | Portugal, Madeira Funchal |
| E. madeirensis sp . nov. - MOTU 2 | MA09DNA-052 | MTE052-20 | BOLD:AEC0503 | Portugal, Madeira Funchal |
| E. madeirensis sp . nov. - MOTU 2 | DNA49FP | MTE053-20 | BOLD:AEC0503 | Portugal, Madeira - <br> Porto Moniz |
| E. madeirensis sp . nov. - MOTU 2 | MA09DNA-14 | MTE054-20 | BOLD:AEC0503 | Portugal, Madeira Funchal |
| E. madeirensis sp . nov. - MOTU 2 | MA09DNA-51 | MTE055-20 | BOLD:AEC0503 | Portugal, Madeira - <br> Porto Moniz |
| E. madeirensis sp . nov. - MOTU 2 | MA09-95 | DBUA0002479.01.v06 | BOLD:AEC0503 | Portugal, Madeira Funchal |
| E. madeirensis sp . nov. - MOTU 2 | MA09-50 | MTE057-20 | BOLD:AEC0503 | Portugal, Madeira Porto Moniz |
| E. xanthomucosa sp. nov. - MOTU 5 | 4880.0 i kuvert (spm3) | DBUA0002480.01.v01 | BOLD:AEC0501 | Great Britain, Cornwall |
| E. xanthomucosa sp. nov. - MOTU 5 | BA09DNA72 | BI-2014/15-077 | BOLD:AEC0501 | France, Banyuls |
| E. xanthomucosa sp. nov. - MOTU 5 | 4878.0 i kuvert (spm1) | DBUA0002480.01.v02 | BOLD:AEC0501 | Great Britain, Cornwall |
| E. xanthomucosa sp. nov. - MOTU 5 | 5482.0 i kuvert (spm2) | DBUA0002480.01.v03 | BOLD:AEC0501 | Great Britain, Cornwall |
| E. xanthomucosa sp. nov. - MOTU 5 | ANBC1-1 | DBUA0002480.01.v04 | BOLD:AEC0501 | Great Britain, Cornwall |
| E. xanthomucosa sp. nov. - MOTU 5 | ANBC1-3 | DBUA0002480.01.v05 | BOLD:AEC0501 | Great Britain, Cornwall |
| E. xanthomucosa sp. nov. - MOTU 5 | ANBC1-4 | DBUA0002480.01.v06 | BOLD:AEC0501 | Great Britain, Cornwall |
| E. xanthomucosa sp. nov. - MOTU 5 | ANBC1-5 | DBUA0002480.01.v07 | BOLD:AEC0501 | Great Britain, Cornwall |
| E. xanthomucosa sp. nov. - MOTU 5 | BA20_02 | DBUA0002481.01.v01 | BOLD:AEC0501 | France, Banyuls |
| E. xanthomucosa sp. nov. - MOTU 5 | MNHN1-1 | MNHN-IA-2021-654 | BOLD:AEC0501 | France, Corsica |
| E. xanthomucosa sp. nov. - MOTU 5 | MNHN1-2 | MNHN-IA-2021-655 | BOLD:AEC0501 | France, Corsica |
| Eulalia IS-BA - <br> MOTU 6 | IT2010 DNA233 | MTE079-20 | BOLD:AEC0306 | Italy, Ischia |

(Table S4.1 Continuation)

| Species | Vial code | Museum Voucher | BIN | Location |
| :---: | :---: | :---: | :---: | :---: |
| Eulalia IS-BA MOTU 6 | BA09DNA25 | MTE080-20 | BOLD:AEC0306 | France, Banyuls |
| Eulalia IS-BA MOTU 6 | IT2010 DNA198 | MTE081-20 | BOLD:AEC0306 | Italy, Ischia |
| Eulalia KRO53MOTU 3 | KR08DNA53 | DBUA0002482.01.v01 | BOLD:AEC0305 | Croatia, Istra |
| Eulalia IT2-1 MOTU 8 | JLIT2-1 | DBUA0002483.01.v01 | BOLD:AEA0429 | Eastern Italy, Taranto |
| E. viridis MOTU 7 | POLYSKAGDNA43 | DBUA0002484.01.v01 | BOLD:AAE3409 | Norway, Grimstad |
| E. viridis MOTU 7 | HMDNA011 | DBUA0002484.02.v01 | BOLD:AAE3409 | Norway, Espevaer |
| E. viridis MOTU 7 | MAR14DNA100 | DBUA0002484.03.v01 | BOLD:AAE3409 | Norway, Finnmark |
| E. viridis MOTU 7 | MAR14DNA110 | DBUA0002484.03.v02 | BOLD:AAE3409 | Norway, Finnmark |
| E. viridis MOTU 7 | NO02DNA102 | MTE088-20 | BOLD:AAE3409 | Norway, Trondheim |
| E. viridis MOTU 7 | BE2014 163 | DBUA0002484.04.v01 | BOLD:AAE3409 | Norway, Bergen |
| E. viridis MOTU 7 | BE2014 167-127 | DBUA0002484.05.v01 | BOLD:AAE3409 | Norway, Bergen |
| E. viridis MOTU 7 | POLYSKAG DNA041 | DBUA0002484.01.v02 | BOLD:AAE3409 | Norway, Grimstad |
| E. viridis MOTU 7 | BE2014 234 | DBUA0002484.05.v02 | BOLD:AAE3409 | Norway, Bergen |
| E. viridis MOTU 7 | BE2014 052 | DBUA0002484.05.v03 | BOLD:AAE3409 | Norway, Bergen |
| E. viridis MOTU 7 | POLYSKAGDNA01 | DBUA0002484.06.v01 | BOLD:AAE3409 | Norway, Grimstad |
| E. viridis MOTU 7 | TJ2010 003 | DBUA0002485.01.v01 | BOLD:AAE3409 | Sweden, Koster |
| E. viridis MOTU 7 | TJ2010 004 | DBUA0002485.01.v02 | BOLD:AAE3409 | Sweden, Koster |
| E. viridis MOTU 7 | TR2016_081 | DBUA0002485.01.v03 | BOLD:AAE3409 | Sweden, Koster |
| E. aurea Outgroup | ANBC1-2 | DBUA0002486.01.v01 | BOLD:AED9731 | Great Britain, Cornwall |
| E. aurea Outgroup | PLY2017_14 | DBUA0002487.01.v01 | B0LD:AED9731 | Great Britain, Plymouth |
| E. aurea Outgroup | PLY2017_110 | DBUA0002487.01.v02 | B0LD:AED9731 | Great Britain, Plymouth |
| Phyllodoce sp. - <br> Outgroup | BA2020-11 | DBUA0002488.01.v01 | BOLD:ADZ9517 | France, Banyuls |
| Eulalia clavigera | RO2018-55 | DBUA0002471.01.v06 | Morphometry only | France, Roscoff |
| Eulalia clavigera | R02018-57 | DBUA0002471.01.v07 | Morphometry only | France, Roscoff |

(Table S4.1 Continuation)

| Species | Vial code | Museum Voucher | BIN | Location |
| :---: | :---: | :---: | :---: | :---: |
| Eulalia clavigera | R02018-58 | DBUA0002471.01.v08 | Morphometry only | France, Roscoff |
| Eulalia clavigera | MTCM27 | DBUA0002469.02.v06 | Morphometry only | Portugal, Canto Marinho |
| Eulalia clavigera | MTCM18 | DBUA0002469.02.v07 | Morphometry only | Portugal, Canto Marinho |
| Eulalia clavigera | PLY2011-116 | DBUA0002474.02.v04 | Morphometry only | Great Britain, Plymouth |
| Eulalia clavigera | PLY2011-117 | DBUA0002474.02.v05 | Morphometry only | Great Britain, Plymouth |
| Eulalia clavigera | MTCT2-17 | DBUA0002476.01.v06 | Morphometry only | Spain, Canaries - <br> Tenerife |
| Eulalia clavigera | MTCT2-20 | DBUA0002476.01.v07 | Morphometry only | Spain, Canaries - <br> Tenerife |
| Eulalia clavigera | MTCT2-21 | DBUA0002476.01.v08 | Morphometry only | Spain, Canaries - <br> Tenerife |
| Eulalia clavigera | MTCT2-22 | DBUA0002476.01.v09 | Morphometry only | Spain, Canaries - <br> Tenerife |
| Eulalia clavigera | MTCT2-26 | DBUA0002476.01.v10 | Morphometry only | Spain, Canaries - <br> Tenerife |
| Eulalia clavigera | MTCT2-31 | DBUA0002476.01.v11 | Morphometry only | Spain, Canaries - <br> Tenerife |
| Eulalia clavigera | MTCP2-14 | DBUA0002476.02.v08 | Morphometry only | Spain, Canaries - <br> La Palma |
| Eulalia clavigera | MTCP2-15 | DBUA0002476.02.v09 | Morphometry only | Spain, Canaries - <br> La Palma |
| Eulalia clavigera | MTCP2-29 | DBUA0002476.02.v10 | Morphometry only | Spain, Canaries - <br> La Palma |

(Table S4.1 Continuation)

## Annexes of chapter 5

Table S5.1. Public BIN accession numbers, museum voucher codes and location for each original specimen in chapter 5

| Species | Vial code | Museum Voucher | BIN | Location |
| :---: | :---: | :---: | :---: | :---: |
| Hediste diversicolor sp. A | MTES121 | DBUA0002455.01.v01 | BOLD:AEE4299 | Spain, Vigo |
| Hediste diversicolor sp. A | MTES122 | DBUA0002455.01.v02 | BOLD:AEE4833 | Spain, Vigo |
| Hediste diversicolor sp. A | MTES14 | DBUA0002455.01.v03 | BOLD:AEE0972 | Spain, Vigo |
| Hediste diversicolor sp. A | MTESM4 | DBUA0002455.01.v04 | BOLD:AEE3851 | Spain, Vigo |
| Hediste diversicolor sp. A | MTESM5 | DBUA0002455.01.v05 | BOLD:ACE9624 | Spain, Vigo |
| Hediste diversicolor sp. A | JPEC6 | DBUA0002456.01.v01 | BOLD:AAY5198 | Spain, Coruña: <br> Ferrol lagoon |
| Hediste diversicolor sp. A | JPEC7 | DBUA0002456.01.v02 | BOLD:AAY5198 | Spain, Coruña: <br> Ferrol lagoon |
| Hediste diversicolor sp. A | JPEC8 | DBUA0002456.01.v03 | BOLD:AAY5198 | Spain, Coruña: <br> Ferrol lagoon |
| Hediste diversicolor sp. A | JPEC9 | DBUA0002456.01.v04 | BOLD:AAY5198 | Spain, Coruña: <br> Ferrol lagoon |
| Hediste diversicolor sp. A | JPEC10 | DBUA0002456.01.v05 | BOLD:AAY5198 | Spain, Coruña: <br> Ferrol lagoon |
| Hediste diversicolor sp. A | JPEC11 | DBUA0002456.01.v06 | BOLD:AAY5198 | Spain, Coruña: <br> Ferrol lagoon |
| Hediste diversicolor sp. A | JPEC12 | DBUA0002456.01.v07 | BOLD:AAY5198 | Spain, Coruña: <br> Ferrol lagoon |
| Hediste diversicolor sp. A | JPEC13 | DBUA0002456.01.v08 | BOLD:AAY5198 | Spain, Coruña: <br> Ferrol lagoon |
| Hediste diversicolor sp. A | JPEC14 | DBUA0002456.01.v09 | BOLD:AAY5198 | Spain, Coruña: <br> Ferrol lagoon |
| Hediste diversicolor sp. A | JPEC15 | MTHD015-20 | BOLD:AAY5198 | Spain, Coruña: <br> Ferrol lagoon |
| Hediste diversicolor sp. A | MTPSb11 | DBUA0002457.01.v01 | BOLD:AAY5198 | Portugal, Sado estuary |
| Hediste diversicolor sp. A | MTPSb12 | DBUA0002457.01.v02 | BOLD:AAY5198 | Portugal, Sado estuary |
| Hediste diversicolor sp. A | MTPSb13 | DBUA0002457.01.v03 | BOLD:AAY5198 | Portugal, Sado estuary |
| Hediste diversicolor sp. A | MTPSb14 | DBUA0002457.01.v04 | BOLD:AAY5198 | Portugal, Sado estuary |
| Hediste diversicolor sp. A | MTPSb16 | DBUA0002457.01.v05 | BOLD:AAY5198 | Portugal, Sado estuary |
| Hediste diversicolor sp. A | MTPSb17 | DBUA0002457.01.v06 | BOLD:AAY5198 | Portugal, Sado estuary |


| Species | Vial code | Museum Voucher | BIN | Location |
| :---: | :---: | :---: | :---: | :---: |
| Hediste diversicolor sp. A | MTPSb18 | DBUA0002457.01.v07 | B0LD:AAY5198 | Portugal, Sado estuary |
| Hediste diversicolor sp. A | MTPSb20 | DBUA0002457.01.v09 | BOLD:AAY5198 | Portugal, Sado estuary |
| Hediste diversicolor sp. A | MTPSb1 | DBUA0002457.01.v10 | B0LD:AAY5198 | Portugal, Sado estuary |
| Hediste diversicolor sp. A | MTPSb2 | DBUA0002457.01.v11 | B0LD:AAY5198 | Portugal, Sado estuary |
| Hediste diversicolor sp. A | MTPSb3 | DBUA0002457.01.v12 | BOLD:AAY5198 | Portugal, Sado estuary |
| Hediste diversicolor <br> sp. A | MTPSb4 | DBUA0002457.01.v13 | BOLD:AAY5198 | Portugal, Sado estuary |
| Hediste diversicolor sp. A | MTPSb5 | DBUA0002457.01.v14 | BOLD:AAY5198 | Portugal, Sado estuary |
| Hediste diversicolor sp. A | MTPSb6 | DBUA0002457.01.v15 | BOLD:AAY5198 | Portugal, Sado estuary |
| Hediste diversicolor sp. A | MTPSb7 | DBUA0002457.01.v16 | B0LD:AAY5198 | Portugal, Sado estuary |
| Hediste diversicolor <br> sp. A | MTPSb8 | DBUA0002457.01.v17 | B0LD:AAY5198 | Portugal, Sado estuary |
| Hediste diversicolor sp. A | MTPSb9 | DBUA0002457.01.v18 | BOLD:AAY5198 | Portugal, Sado estuary |
| Hediste diversicolor <br> sp. A | MTPSJ3 | DBUA0002458.01.v01 | BOLD:AAY5198 | Portugal, Sado estuary |
| Hediste diversicolor sp. A | MTPSJ4 | DBUA0002458.02.v01 | B0LD:AAY5198 | Portugal, Sado estuary |
| Hediste diversicolor sp. A | MTPSJ5 | DBUA0002458.02.v02 | BOLD:AAY5198 | Portugal, Sado estuary |
| Hediste diversicolor sp. A | MTPSJ7 | DBUA0002458.02.v03 | BOLD:AAY5198 | Portugal, Sado estuary |
| Hediste diversicolor sp. A | MTPLa1 | DBUA0002459.01.v01 | BOLD:AAY5198 | Portugal, Lima estuary |
| Hediste diversicolor sp. A | MTPLb1 | DBUA0002459.01.v02 | BOLD:AC05140 | Portugal, Lima estuary |
| Hediste diversicolor sp. A | MTPLb2 | DBUA0002459.01.v03 | B0LD:AEE0973 | Portugal, Lima estuary |
| Hediste diversicolor sp. A | MTPLd1 | DBUA0002459.01.v04 | B0LD:AEE0531 | Portugal, Lima estuary |
| Hediste diversicolor sp. A | MTPLd2 | DBUA0002459.01.v05 | BOLD:AEE6354 | Portugal, Lima estuary |
| Hediste diversicolor sp. A | MTPLd3 | DBUA0002459.01.v06 | BOLD:AC05139 | Portugal, Lima estuary |
| Hediste diversicolor sp. A | MTPLd4 | DBUA0002459.01.v07 | BOLD:ABZ5903 | Portugal, Lima estuary |
| Hediste diversicolor sp. A | MTPLd5 | DBUA0002459.01.v08 | BOLD:AEE5540 | Portugal, Lima estuary |
| Hediste diversicolor sp. A | MTPLd6 | DBUA0002459.01.v09 | BOLD:ACP3870 | Portugal, Lima estuary |
| Hediste diversicolor sp. A | MTPLd7 | DBUA0002459.01.v010 | BOLD:AEE0973 | Portugal, Lima estuary |

(Table S5.1 Continuation)

| Species | Vial code | Museum Voucher | BIN | Location |
| :--- | :--- | :--- | :--- | :--- |
| Hediste diversicolor <br> sp. A | MTPLd8 | DBUA0002459.01.v11 | BOLD:AEE0530 | Portugal, Lima <br> estuary |
| Hediste diversicolor <br> sp. A | MTPLd10 | DBUA0002459.01.v13 | BOLD:AEE4299 | Portugal, Lima <br> Hediste diversicolor |
| MTPLd11 <br> sp. A | DBUA0002459.01.v14 | BOLD:AEE4300 | Portugal, Lima <br> Hediste diversicolor | MTPLd12 |

(Table S5.1 Continuation)

| Species | Vial code | Museum Voucher | BIN | Location |
| :--- | :--- | :--- | :--- | :--- |
| Hediste diversicolor <br> sp. A | MTPA114 | DBUA0002460.01.v14 | BOLD:AAY5198 | Portugal, Aveiro <br> lagoon |
| Hediste diversicolor <br> sp. A | MTPA50 | DBUA0002460.01.v16 | BOLD:AAY5198 | Portugal, Aveiro <br> Hediste diversicolor |
| MTPA51 | DBUA000n |  |  |  |
| sp. A |  |  |  |  |

(Table S5.1 Continuation)

| Species | Vial code | Museum Voucher | BIN | Location |
| :---: | :---: | :---: | :---: | :---: |
| Hediste diversicolor sp. A | MTEM20 | DBUA0002461.01.v20 | BOLD:AEE8078 | Portugal, Minho estuary |
| Hediste diversicolor sp. A | MTFB2 | DBUA0002462.01.v02 | BOLD:ACE9624 | North France, Brest |
| Hediste diversicolor sp. A | MTFB3 | DBUA0002462.01.v03 | B0LD:AAY5198 | North France, Brest |
| Hediste diversicolor sp. A | MTFB4 | DBUA0002462.01.v04 | BOLD:AAY5198 | North France, Brest |
| Hediste diversicolor sp. A | MTFB5 | DBUA0002462.01.v05 | BOLD:ACE9624 | North France, Brest |
| Hediste diversicolor sp. A | MTFB6 | DBUA0002462.01.v06 | BOLD:AAY5198 | North France, Brest |
| Hediste diversicolor sp. A | MTFB7 | DBUA0002462.01.v07 | BOLD:AAY5198 | North France, Brest |
| Hediste diversicolor sp. A | MTFB8 | DBUA0002462.01.v08 | BOLD:AAY5198 | North France, Brest |
| Hediste diversicolor sp. A | MTFB9 | DBUA0002462.01.v09 | BOLD:AAY5198 | North France, Brest |
| Hediste diversicolor sp. A | MTFB10 | DBUA0002462.01.v10 | BOLD:AAY5198 | North France, Brest |
| Hediste diversicolor sp. A | EONS1 | NTNU-VM82080 | BOLD:AEE1422 | Norway, Grimstad |
| Hediste diversicolor sp. A | EONS2 | NTNU-VM82081 | BOLD:AAB1936 | Norway, Grimstad |
| Hediste diversicolor sp. A | EONS3 | NTNU-VM82082 | BOLD:ACF4936 | Norway, Grimstad |
| Hediste diversicolor sp. A | EONS4 | NTNU-VM82083 | B0LD:AEE0970 | Norway, Grimstad |
| Hediste diversicolor sp. A | EONS5 | NTNU-VM82084 | BOLD:AEE0971 | Norway, Grimstad |
| Hediste diversicolor sp. A | 76340 | NTNU-VM76340 | BOLD:AEH0113 | Norway, Trondheim |
| Hediste diversicolor sp. A | 76341 | NTNU-VM76341 | BOLD:ABZ8120 | Norway, Trondheim |
| Hediste sp. B1 | MTST16 | DBUA0002463.01.v01 | BOLD:AAC7123 | Sweden, Tjärnö- <br> Saltö canal |
| Hediste sp. B1 | MTST17 | DBUA0002463.01.v02 | BOLD:AAC7123 | Sweden, TjärnöSaltö canal |
| Hediste sp. B1 | MTST18 | DBUA0002463.01.v03 | BOLD:AAC7123 | Sweden, TjärnöSaltö canal |
| Hediste sp. B1 | MTST19 | DBUA0002463.01.v04 | BOLD:AAC7123 | Sweden, Tjärnö- <br> Saltö canal |
| Hediste sp. B1 | MTST20 | DBUA0002463.01.v05 | BOLD:AAC7123 | Sweden, TjärnöSaltö canal |
| Hediste sp. B1 | MTST21 | DBUA0002463.01.v06 | BOLD:AAC7123 | Sweden, TjärnöSaltö canal |
| Hediste sp. B1 | MTST22 | DBUA0002463.01.v07 | BOLD:AAC7123 | Sweden, Tjärnö- <br> Saltö canal |
| Hediste sp. B1 | MTST23 | DBUA0002463.01.v08 | BOLD:AAC7123 | Sweden, Tjärnö- <br> Saltö canal |

(Table S5.1 Continuation)

| Species | Vial code | Museum Voucher | BIN | Location |
| :---: | :---: | :---: | :---: | :---: |
| Hediste sp. B1 | MTST24 | DBUA0002463.01.v09 | BOLD:AAC7123 | Sweden, Tjärnö- <br> Saltö canal |
| Hediste sp. B1 | MTST27 | DBUA0002463.01.v11 | BOLD:AAC7123 | Sweden, TjärnöSaltö canal |
| Hediste sp. B1 | MTST28 | DBUA0002463.01.v12 | BOLD:AAC7123 | Sweden, TjärnöSaltö canal |
| Hediste sp. B1 | MTST29 | DBUA0002463.01.v13 | BOLD:AAC7123 | Sweden, TjärnöSaltö canal |
| Hediste sp. B1 | MTSTM16 | DBUA0002463.01.v14 | BOLD:AAC7123 | Sweden, TjärnöSaltö canal |
| Hediste sp. B1 | MTST31 | DBUA0002463.01.v15 | BOLD:AAC7123 | Sweden, TjärnöSaltö canal |
| Hediste sp. B1 | MTST32 | DBUA0002463.01.v16 | BOLD:AAC7123 | Sweden, TjärnöSaltö canal |
| Hediste sp. B1 | MTST33 | DBUA0002463.01.v17 | BOLD:AAC7123 | Sweden, TjärnöSaltö canal |
| Hediste sp. B1 | MTSTM17 | DBUA0002463.01.v18 | BOLD:AAC7123 | Sweden, TjärnöSaltö canal |
| Hediste sp. B1 | MTST35 | DBUA0002463.01.v19 | BOLD:AAC7123 | Sweden, TjärnöSaltö canal |
| Hediste sp. B1 | MTST36 | DBUA0002463.01.v20 | BOLD:AAC7123 | Sweden, TjärnöSaltö canal |
| Hediste sp. B1 | MTSTM1 | DBUA0002463.01.v21 | BOLD:AAC7123 | Sweden, TjärnöSaltö canal |
| Hediste sp. B1 | MTSTM3 | DBUA0002463.01.v22 | BOLD:AAC7123 | Sweden, TjärnöSaltö canal |
| Hediste sp. B1 | MTSTM4 | DBUA0002463.01.v23 | BOLD:AAC7123 | Sweden, TjärnöSaltö canal |
| Hediste sp. B1 | MTSTM6 | DBUA0002463.01.v24 | BOLD:AAC7123 | Sweden, TjärnöSaltö canal |
| Hediste sp. B1 | MTSTM 7 | DBUA0002463.01.v25 | BOLD:AAC7123 | Sweden, TjärnöSaltö canal |
| Hediste sp. B1 | MTSTM10 | DBUA0002463.01.v26 | BOLD:AAC7123 | Sweden, TjärnöSaltö canal |
| Hediste sp. B1 | MTSTM11 | DBUA0002463.01.v27 | BOLD:AAC7123 | Sweden, TjärnöSaltö canal |
| Hediste sp. B1 | MTSTM12 | DBUA0002463.01.v28 | BOLD:AAC7123 | Sweden, TjärnöSaltö canal |
| Hediste sp. B1 | MTSTM13 | DBUA0002463.01.v29 | BOLD:AAC7123 | Sweden, TjärnöSaltö canal |
| Hediste sp. B1 | MTSTM14 | MTHD145-20 | BOLD:AAC7123 | Sweden, TjärnöSaltö canal |
| Hediste sp. B1 | 76499 | NTNU-VM76499 | BOLD:AAC7123 | Norway, Sandefjord |
| Hediste sp. B1 | JLIM1 | DBUA0002464.01.v01 | BOLD:AEE3441 | Western Italy, Navicelli Canal |
| Hediste sp. B1 | JLIM2 | DBUA0002464.01.v02 | BOLD:AEE3441 | Western Italy, Navicelli Canal |
| Hediste sp. B1 | JLIM3 | DBUA0002464.01.v03 | BOLD:AEE3441 | Western Italy, Navicelli Canal |

(Table S5.1 Continuation)

| Species | Vial code | Museum Voucher | BIN | Location |
| :--- | :--- | :--- | :--- | :--- |
| Hediste sp. B1 | JLIM4 | DBUA0002464.01.v04 | BOLD:AEE3441 | Western Italy, <br> Navicelli Canal |
| Hediste sp. B1 | JLIM5 | DBUA0002464.01.v05 | BOLD:AEE3441 | Western Italy, |
| Navicelli Canal |  |  |  |  |
| Hediste sp. B1 | JLIM7 | DBUA0002464.01.v07 | BOLD:AEE3441 | Western Italy, |
| Nediste sp. B1 | JLIM8 | DBUAClli Canal |  |  |

(Table S5.1 Continuation)

| Species | Vial code | Museum Voucher | BIN | Location |
| :---: | :---: | :---: | :---: | :---: |
| Hediste pontii sp. nov. | MPIN55 | DBUA0002465.01.v20 | B0LD:ADW0792 | Italy, Adriatic Sea, Veneza lagoon |
| Hediste pontii sp. nov. | MPIN57 | DBUA0002465.01.v21 | B0LD:ADW0792 | Italy, Adriatic Sea, Veneza lagoon |
| Hediste sp. B3 | NER039 | MTHD178-20 | BOLD:ACQ6664 | Greece, Tsopeli lagoon |
| Hediste sp. B3 | NER053 | MTHD187-20 | BOLD:ACQ6664 | Greece, Tsopeli lagoon |
| Hediste sp. B3 | NER136 | MTHD183-20 | BOLD:ACQ6664 | Greece, Tsopeli lagoon |
| Hediste sp. B3 | NER158 | MTHD184-20 | BOLD:ACQ6664 | Greece, Tsoukalio lagoon |
| Hediste astae sp. nov. | SFGE1-2 | DBUA0002466.01.v01 | BOLD:AAC7124 | Greece, Evros lagoon |
| Hediste astae sp. nov. | SFGE1-3 | DBUA0002466.01.v02 | BOLD:AAC7124 | Greece, Evros lagoon |
| Hediste astae sp. nov. | SFGE1-4 | DBUA0002466.01.v03 | BOLD:AAC7124 | Greece, Evros lagoon |
| Hediste astae sp. nov. | SFGE1-7 | DBUA0002466.01.v04 | BOLD:AAC7124 | Greece, Evros lagoon |
| Hediste astae sp. nov. | SFGE1-8 | DBUA0002466.01.v05 | BOLD:AAC7124 | Greece, Evros lagoon |
| Hediste astae sp. nov. | SFGI1-3 | DBUA0002466.02.v01 | BOLD:AAC7124 | Greece, Alyki lagoon |
| Hediste astae sp. nov. | SFGI1-4 | DBUA0002466.02.v02 | BOLD:AAC7124 | Greece, Alyki lagoon |
| Hediste astae sp. nov. | SFGI1-6 | DBUA0002466.02.v03 | BOLD:AAC7124 | Greece, Alyki lagoon |
| Hediste astae sp. nov. | SFGI1-7 | DBUA0002466.02.v04 | BOLD:AAC7124 | Greece, Alyki lagoon |
| Hediste astae sp. nov. | SFGI1-10 | DBUA0002466.02.v05 | BOLD:AAC7124 | Greece, Alyki lagoon |
| Hediste astae sp. nov. | SFGI1-2 | DBUA0002466.02.v06 | BOLD:AAC7124 | Greece, Alyki lagoon |
| Hediste astae sp. nov. | SFGN1-1 | DBUA0002466.03.v01 | BOLD:AAC7124 | Greece, Nestos lagoon |
| Hediste astae sp. nov. | SFGN1-3 | DBUA0002466.03.v02 | BOLD:AAC7124 | Greece, Nestos lagoon |
| Hediste astae sp. nov. | SFGN1-4 | DBUA0002466.03.v03 | BOLD:AAC7124 | Greece, Nestos lagoon |
| Hediste astae sp. nov. | SFGN1-5 | DBUA0002466.03.v04 | BOLD:AAC7124 | Greece, Nestos lagoon |
| Hediste astae sp. nov. | SFGN1-6 | DBUA0002466.03.v05 | BOLD:AAC7124 | Greece, Nestos lagoon |
| Hediste astae sp. nov. | SFGN1-7 | DBUA0002466.03.v06 | BOLD:AAC7124 | Greece, Nestos lagoon |
| Hediste astae sp. nov. | SFGN1-8 | DBUA0002466.03.v07 | BOLD:AAC7124 | Greece, Nestos lagoon |
| Hediste astae sp. nov. | SFGN1-2 | DBUA0002466.03.v08 | BOLD:AAC7124 | Greece, Nestos lagoon |

(Table S5.1 Continuation)

| Species | Vial code | Museum Voucher | BIN | Location |
| :--- | :--- | :--- | :--- | :--- |
| Hediste astae sp. <br> nov. | SFGP1-2 | DBUA0002466.04.v01 | BOLD:AAC7124 | Greece, Ptelea <br> lagoon |
| Hediste astae sp. <br> nov. | SFGP1-1 | DBUA0002466.04.v02 | BOLD:AAC7124 | Greece, Ptelea |
| lagoon |  |  |  |  |
| Hediste astae sp. | SFGP1-5 | DBUA0002466.04.v04 | BOLD:AAC7124 | Greece, Ptelea <br> nov. |
| Hediste astae sp. | SFGS1-6 | DBUA0002466.05.v01 | BOLD:AAC7124 | Greece, Axios |
| nov. |  |  |  |  |

(Table S5.1 Continuation)

| Species | Vial code | Museum Voucher | BIN | Location |
| :--- | :--- | :--- | :--- | :--- |
| Hediste diversicolor <br> sp. A <br> Hediste diversicolor <br> sp. A | MTPA33 | DBUA0002460.01.v22 | Morphometry only | Portugal, Aveiro <br> lagoon |
| Hediste diversicolor <br> sp. A | MTPA48 | DBUA0002460.01.v23 | Morphometry only | Portugal, Aveiro <br> lagoon |
| Hediste diversicolor <br> sp. A | MTPSJ21 | DBUA0002458.03.v01 | Morphometry only | Portugal, Sado <br> Hediste diversicolor |
| estuary |  |  |  |  |
| sp. A <br> Hediste diversicolor | MTPSJ32 | DBUA0002458.03.v03 | Morphometry only | Portugal, Sado <br> sp. A |
| Hediste diversicolor |  |  |  |  |
| sp. A | MTPSJ34 | DBUA000002458.03.v04 | Morphometry only | Portugal, Sado <br> Hediste diversicolor |
| estuary |  |  |  |  |

(Table S5.1 Continuation)

| Species | Vial code | Museum Voucher | BIN | Location |
| :---: | :---: | :---: | :---: | :---: |
| Hediste astae sp. nov. | 26TW1b_01 | DBUA0002466.01.v06 | Morphometry only | Greece, Evros Lagoon |
| Hediste astae sp. nov. | 26TW1b_02 | DBUA0002466.01.v07 | Morphometry only | Greece, Evros Lagoon |
| Hediste astae sp. nov. | 26TW1b_04 | DBUA0002466.01.v09 | Morphometry only | Greece, Evros Lagoon |
| Hediste astae sp. nov. | 26TW1b_05 | DBUA0002466.01.v10 | Morphometry only | Greece, Evros Lagoon |
| Hediste astae sp. nov. | 26TW1b_06 | DBUA0002466.01.v11 | Morphometry only | Greece, Evros Lagoon |
| Hediste astae sp. nov. | 26TW1b_07 | DBUA0002466.01.v12 | Morphometry only | Greece, Evros Lagoon |
| Hediste astae sp. nov. | 26TW1b_08 | DBUA0002466.01.v13 | Morphometry only | Greece, Evros Lagoon |
| Hediste astae sp. nov. | 26TW1b_09 | DBUA0002466.01.v14 | Morphometry only | Greece, Evros Lagoon |
| Hediste astae sp. nov. | 26TW1b_10 | DBUA0002466.01.v15 | Morphometry only | Greece, Evros Lagoon |
| Hediste astae sp. nov. | 26TW1b_11 | DBUA0002466.01.v16 | Morphometry only | Greece, Evros Lagoon |
| Hediste astae sp. nov. | 26TW1b_12 | DBUA0002466.01.v17 | Morphometry only | Greece, Evros Lagoon |
| Hediste astae sp. nov. | 26TW1b_13 | DBUA0002466.01.v18 | Morphometry only | Greece, Evros Lagoon |
| Hediste astae sp. nov. | 26TW1a_01 | DBUA0002466.01.v19 | Morphometry only | Greece, Evros Lagoon |
| Hediste astae sp. nov. | 26TW1a_02 | DBUA0002466.01.v20 | Morphometry only | Greece, Evros Lagoon |
| Hediste astae sp. nov. | 26TW1a_03 | DBUA0002466.01.v21 | Morphometry only | Greece, Evros Lagoon |
| Hediste astae sp. nov. | 26TW1a_04 | DBUA0002466.01.v22 | Morphometry only | Greece, Evros Lagoon |
| Hediste astae sp. nov. | 26TW1a_05 | DBUA0002466.01.v23 | Morphometry only | Greece, Evros Lagoon |
| Hediste astae sp. nov. | 26TW1a_06 | DBUA0002466.01.v24 | Morphometry only | Greece, Evros Lagoon |
| Hediste astae sp. nov. | 26TW1a_07 | DBUA0002466.01.v25 | Morphometry only | Greece, Evros Lagoon |
| Hediste astae sp. nov. | 26TW1a_08 | DBUA0002466.01.v26 | Morphometry only | Greece, Evros Lagoon |
| Hediste astae sp. nov. | 26TW1a_09 | DBUA0002466.01.v27 | Morphometry only | Greece, Evros Lagoon |
| Hediste astae sp. nov. | 26TW1a_10 | DBUA0002466.01.v28 | Morphometry only | Greece, Evros Lagoon |
| Hediste astae sp. nov. | 26TW1a_11 | DBUA0002466.01.v29 | Morphometry only | Greece, Evros Lagoon |
| Hediste astae sp. nov. | 26TW1a_12 | DBUA0002466.01.v30 | Morphometry only | Greece, Evros Lagoon |

(Table S5.1 Continuation)

Table S5.2. Geographic location and GenBank accession numbers for sequences belonging to other studies and used for comparison purposes

| Species | Designation (MOTU) | GenBank COI | Location | References |
| :---: | :---: | :---: | :---: | :---: |
| H. diversicolor | Species A (MOTU 2) | FJ030956 | Baltic Sea | Audzijonyte et al. (2008) |
| H. diversicolor | Species A (MOTU 2) | FJ030957 | Baltic Sea | Audzijonyte et al. (2008) |
| H. diversicolor | Species B4 (MOTU 5) | FJ030985 | Baltic Sea | Audzijonyte et al. (2008) |
| H. diversicolor | Species B4 (MOTU 5) | FJ030986 | Baltic Sea | Audzijonyte et al. (2008) |
| H. diversicolor | Species B1 (MOTU 1) | FJ030991 | Baltic Sea | Audzijonyte et al. (2008) |
| H. diversicolor | Species B1 (MOTU 1) | FJ030992 | Baltic Sea | Audzijonyte et al. (2008) |
| H. diversicolor | Species A (MOTU 2) | EU300733 | Great Britain, Bath | Virgilio et al. (2009) |
| H. diversicolor | Species A (MOTU 2) | EU300734 | Great Britain, Bath | Virgilio et al. (2009) |
| H. diversicolor | Species A (MOTU 2) | EU300703 | Netherlands, <br> Zeeland | Virgilio et al. (2009) |
| H. diversicolor | Species A (MOTU 2) | EU300704 | Netherlands, <br> Zeeland | Virgilio et al. (2009) |
| H. diversicolor | Species A (MOTU 2) | EU300723 | Germany, Nordfriesland | Virgilio et al. (2009) |
| H. diversicolor | Species A (MOTU 2) | EU300724 | Germany, Nordfriesland | Virgilio et al. (2009) |
| H. diversicolor | Species B1 (MOTU 1) | EU300773 | Tyrrhenian Sea, Italy: Oristano | Virgilio et al. (2009) |
| H. diversicolor | Species B1 (MOTU 1) | EU300774 | Tyrrhenian Sea, Italy: Oristano | Virgilio et al. (2009) |
| H. diversicolor | Species B2 (MOTU 3) | EU300637 | Adriatic Sea, Croatia: Novigrad | Virgilio et al. (2009) |
| H. diversicolor | Species B2 (MOTU 3) | EU300651 | Adriatic Sea, Italy: Leece | Virgilio et al. (2009) |
| H. diversicolor | Species B3 (MOTU 4) | KF737343 | Ionian Sea, Greece: <br> Amvrakikos | Vasileiadou et al. (2016) |
| H. diversicolor | Species B3 (MOTU 4) | KF737345 | Ionian Sea, Greece: <br> Amurakikos | Vasileiadou et al. (2016) |
| H. diversicolor | Species B4 (MOTU 5) | EU300741 | Black Sea, Ukraine | Virgilio et al. (2009) |
| H. diversicolor | Species B4 (MOTU 5) | EU300742 | Black Sea, Ukraine | Virgilio et al. (2009) |
| H. diversicolor | Species B4 (MOTU 5) | EU300754 | Caspian Sea, Russia | Virgilio et al. (2009) |
| H. diversicolor | Species B4 (MOTU 5) | EU300755 | Caspian Sea, Russia | Virgilio et al. (2009) |



Figure S5.1. Multidimensional scaling representation of the genetic distances (Kimura-2-parameters, K2P) between all records for COI, ITS, 28S and concatenated data.


Fig. S5.2. Multidimensional scaling representation of the genetic distances (Kimura-2-parameters, K2P) between all records within MOTU 2 for COI, ITS and concatenated data.

## Annexes of chapter 6

Table S6.1. Public BIN accession numbers, museum voucher codes and location for each original specimen in chapter 6.

| Species | Vial code | Voucher code | BIN | Location |
| :---: | :---: | :---: | :---: | :---: |
| MOTU 10 - |  |  |  | Portugal, Lisbon |
| Platynereis agilis comb. nov. - | JAPM06 | MB29-000377 | BOLD:AEE0899 | (Parque Natural Arrábida) |
| MOTU $10-$ |  |  |  | Portugal, Lisbon |
| Platynereis agilis comb. nov. | JAPM07 | MB29-000378 | BOLD:AEE0899 | (Parque Natural Arrábida) |
| MOTU 10 - |  |  |  | Portugal, Lisbon |
| Platynereis agilis comb. nov. | JAPM08 | MB29-000379 | BOLD:AEE0899 | (Parque Natural Arrábida) |
| MOTU $10-$ |  |  |  | Portugal, Lisbon |
| Platynereis agilis comb. nov. | JAPM10 | MB29-000380 | BOLD:AEE0899 | (Parque Natural Arrábida) |
| MOTU 10 - |  |  |  | Portugal, Lisbon |
| Platynereis agilis comb. nov. | JAPM11 | MB29-000381 | BOLD:AEE0899 | (Parque Natural Arrábida) |
| MOTU $10-$ |  |  |  | Portugal, Lisbon |
| Platynereis agilis comb. nov. | JAPM12 | MB29-000382 | BOLD:AEE0899 | (Parque Natural Arrábida) |
| MOTU 10 - |  |  |  | Portugal, Lisbon |
| Platynereis agilis comb. nov. | JAPM16 | MB29-000383 | BOLD:AEE0899 | (Parque Natural Arrábida) |
| MOTU 10 - |  |  |  | Portugal, Lisbon |
| Platynereis agilis comb. nov. | JAPM27 | MB29-000370 | BOLD:AEE0899 | (Parque Natural Arrábida) |
| MOTU 10 - |  |  |  | Portugal, Lisbon |
| Platynereis agilis comb. nov. | JAPM28 | MB29-000371 | BOLD:AEE0899 | (Parque Natural Arrábida) |
| MOTU 10 - |  |  |  | Portugal, Lisbon |
| Platynereis agilis comb. nov. | JAPM29 | MB29-000372 | BOLD:AEE0899 | (Parque Natural Arrábida) |
| MOTU 10 - |  |  |  | Portugal, Lisbon |
| Platynereis agilis comb. nov. | JAPM30 | MB29-000373 | BOLD:AEE0899 | (Parque Natural Arrábida) |
| MOTU $10-$ |  |  |  | Portugal, Lisbon |
| Platynereis agilis comb. nov. | JAPM31 | MB29-000374 | BOLD:AEE0899 | (Parque Natural Arrábida) |
| MOTU $10-$ |  |  |  | Portugal, Lisbon |
| Platynereis agilis comb. nov. | JAPM34 | MB29-000375 | BOLD:AEE0899 | (Parque Natural Arrábida) |
| MOTU $10-$ |  |  |  | Portugal, Lisbon |
| Platynereis agilis comb. nov. | JAPM05 | MB29-000384 | BOLD:AEE0899 | (Parque Natural Arrábida) |


| Species | Vial code | Voucher code | BIN | Location |
| :--- | :--- | :--- | :--- | :--- |
| MOTU 10 - <br> Platynereis agilis <br> comb. nov. | PVEC30 | DBUA0002421.01.v01 | BOLD:AEE0900 | South Spain, Calpe |
| MOTU 10 - |  |  |  |  |

(Table S6.1 Continuation)

| Species | Vial code | Voucher code | BIN | Location |
| :---: | :---: | :---: | :---: | :---: |
| MOTU 9 - Platynereis cf. massiliensis | PVM4 | DBUA0002426.01.v01 | BOLD:AEE5803 | Morocco, Mazagan |
| MOTU 9 - Platynereis cf. massiliensis | JLIL2 | DBUA0002427.01.v01 | B0LD:ACP6515 | Western Italy, Livorno |
| MOTU 9 - Platynereis cf. massiliensis | JLIL3 | DBUA0002427.01.v02 | B0LD:ACP6515 | Western Italy, Livorno |
| MOTU 9 - Platynereis cf. massiliensis | JLIL4 | DBUA0002427.01.v03 | B0LD:ACP6515 | Western Italy, Livorno |
| MOTU 8 - Platynereis sp. | PVMS1.1 | DBUA0002441.01.v01 | BOLD:AEE2958 | Portugal, Madeira Porto Santo |
| MOTU 7 - Platynereis macaronesiensis sp. nov. | PVAS1.2 | DBUA0002428.01.v01 | BOLD:AEE3434 | Portugal, Azores Santa Maria |
| MOTU 7 - Platynereis macaronesiensis sp. nov. | PVAS1.3 | DBUA0002428.01.v02 | BOLD:AEE3434 | Portugal, Azores Santa Maria |
| MOTU 7 - Platynereis macaronesiensis sp. nov. | PVAS1.5 | DBUA0002428.01.v03 | BOLD:AEE3434 | Portugal, Azores Santa Maria |
| MOTU 7 - Platynereis macaronesiensis sp. nov. | PVAT1.3 | DBUA0002428.02.v01 | BOLD:AEE3434 | Portugal, Azores Terceira |
| MOTU 7 - Platynereis macaronesiensis sp. nov. | PVAT1.6 | DBUA0002428.02.v02 | BOLD:AEE3434 | Portugal, Azores Terceira |
| MOTU 7 - Platynereis macaronesiensis sp. nov. | PVAT1.9 | DBUA0002428.02.v03 | BOLD:AEE3434 | Portugal, Azores Terceira |
| MOTU 7 - Platynereis macaronesiensis sp. nov. | PVMF1.2 | DBUA0002428.03.v01 | BOLD:AEE1367 | Portugal, Madeira Funchal |
| MOTU 7 - Platynereis macaronesiensis sp. nov. | PVMF1.1 | DBUA0002428.03.v02 | BOLD:AEE3434 | Portugal, Madeira Funchal |
| MOTU 7 - Platynereis macaronesiensis sp. nov. | PVMF1.3 | DBUA0002428.03.v03 | BOLD:AEE3434 | Portugal, Madeira Funchal |
| MOTU 7 - Platynereis macaronesiensis sp. nov. | PVMF1.4 | DBUA0002428.03.v04 | BOLD:AEE3434 | Portugal, Madeira Funchal |
| MOTU 7 - Platynereis macaronesiensis sp. nov. | MTCT1.1 | DBUA0002429.01.v01 | BOLD:AEE3434 | Spain, Canary - <br> Tenerife |
| MOTU 7 - Platynereis macaronesiensis sp. nov. | MTCT1.2 | DBUA0002429.01.v02 | BOLD:AEE1366 | Spain, Canary Tenerife |
| MOTU 7 - Platynereis macaronesiensis sp. nov. | MTCT1.4 | DBUA0002429.01.v03 | BOLD:AEE1367 | Spain, Canary Tenerife |

(Table S6.1 Continuation)

| Species | Vial code | Voucher code | BIN | Location |
| :---: | :---: | :---: | :---: | :---: |
| MOTU 7 - Platynereis macaronesiensis sp. nov. | MTCT1.11 | DBUA0002429.02.v01 | BOLD:AEE1367 | Spain, Canary - <br> Tenerife |
| MOTU 7 - Platynereis macaronesiensis sp. nov. | MTCT1.12 | DBUA0002429.02.v02 | BOLD:AEE1367 | Spain, Canary - <br> Tenerife |
| MOTU 7 - Platynereis macaronesiensis sp. nov. | MTCT1.15 | DBUA0002429.02.v03 | BOLD:AEE1366 | Spain, Canary Tenerife |
| MOTU 7 - Platynereis macaronesiensis sp. nov. | MTCL1.1 | DBUA0002429.03.v01 | BOLD:AEE1367 | Spain, Canary - <br> Lanzarote |
| MOTU 7 - Platynereis macaronesiensis sp. nov. | MTCL1. 2 | DBUA0002429.03.v02 | BOLD:AEE1367 | Spain, Canary - <br> Lanzarote |
| MOTU 7 - Platynereis macaronesiensis sp. nov. | MTCL1.3 | DBUA0002429.03.v03 | BOLD:AEE3434 | Spain, Canary - <br> Lanzarote |
| MOTU 7 - Platynereis macaronesiensis sp. nov. | MTCL1.5 | DBUA0002429.03.v04 | BOLD:AEE1367 | Spain, Canary - <br> Lanzarote |
| MOTU 7 - Platynereis macaronesiensis sp. nov. | MTCL1.6 | DBUA0002429.03.v05 | BOLD:AEE1367 | Spain, Canary - <br> Lanzarote |
| MOTU 7 - Platynereis macaronesiensis sp. nov. | MTCC1.1 | DBUA0002429.04.v01 | BOLD:AEE1367 | Spain, Canary - <br> Gran Canaria |
| MOTU 7 - Platynereis macaronesiensis sp. nov. | MTCC1.2 | DBUA0002429.04.v02 | BOLD:AEE1367 | Spain, Canary - <br> Gran Canaria |
| MOTU 7 - Platynereis macaronesiensis sp. nov. | MTCC1.3 | DBUA0002429.04.v03 | BOLD:AEE1367 | Spain, Canary - <br> Gran Canaria |
| MOTU 7 - Platynereis macaronesiensis sp. nov. | MTCC1.4 | DBUA0002429.04.v04 | BOLD:AEE1367 | Spain, Canary - <br> Gran Canaria |
| MOTU 7 - Platynereis macaronesiensis sp. nov. | MTCC1.5 | DBUA0002429.04.v05 | BOLD:AEE1367 | Spain, Canary - <br> Gran Canaria |
| MOTU 7 - Platynereis macaronesiensis sp. nov. | MTCC1.6 | DBUA0002429.04.v06 | BOLD:AEE1367 | Spain, Canary - <br> Gran Canaria |
| MOTU 7 - Platynereis macaronesiensis sp. nov. | MTCC1.12 | DBUA0002429.04.v07 | BOLD:AEE1367 | Spain, Canary - <br> Gran Canaria |
| MOTU 7 - Platynereis macaronesiensis sp. nov. | MTCC1.15 | DBUA0002429.04.v08 | BOLD:AEE1367 | Spain, Canary Gran Canaria |

(Table S6.1 Continuation)

| Species | Vial code | Museum Voucher | BIN | Location |
| :---: | :---: | :---: | :---: | :---: |
| MOTU 7 - Platynereis macaronesiensis sp. nov. | MTCC1.20 | DBUA0002429.04.v09 | BOLD:AEE1367 | Spain, Canary <br> Gran Canaria |
| MOTU 7 - Platynereis macaronesiensis sp. nov. | MTCC1.25 | DBUA0002429.04.v11 | BOLD:AEE1367 | Spain, Canary <br> Gran Canaria |
| MOTU 7 - Platynereis macaronesiensis sp. nov. | MTCP1.1 | DBUA0002429.05.v01 | BOLD:AEE0404 | Spain, Canary - La Palma |
| MOTU 7 - Platynereis macaronesiensis sp. nov. | MTCP1.2 | DBUA0002429.05.v02 | BOLD:AEE0404 | Spain, Canary - La Palma |
| MOTU 7 - Platynereis macaronesiensis sp. nov. | MTCP1.3 | DBUA0002429.05.v03 | BOLD:AEE0404 | $\begin{aligned} & \text { Spain, Canary - La } \\ & \text { Palma } \end{aligned}$ |
| MOTU 7 - Platynereis macaronesiensis sp. nov. | MTCP1.4 | DBUA0002429.05.v04 | BOLD:AEE0404 | Spain, Canary - La Palma |
| MOTU 7 - Platynereis macaronesiensis sp. nov. | MTCP1.5 | DBUA0002429.05.v05 | BOLD:AEE0404 | Spain, Canary - La Palma |
| MOTU 7 - Platynereis macaronesiensis sp. nov. | MTCF1.1 | DBUA0002429.06.v01 | BOLD:AEE3434 | Spain, Canary Fuerteventura |
| MOTU 7 - Platynereis macaronesiensis sp. nov. | MTCF1.2 | DBUA0002429.06.v02 | BOLD:AEE3434 | Spain, Canary Fuerteventura |
| MOTU 7 - Platynereis macaronesiensis sp. nov. | MTCF1.3 | DBUA0002429.06.v03 | BOLD:AEE3434 | Spain, Canary Fuerteventura |
| MOTU 7 - Platynereis macaronesiensis sp. nov. | MTCF1.6 | DBUA0002429.06.v04 | BOLD:AEE1367 | Spain, Canary Fuerteventura |
| MOTU 7 - Platynereis macaronesiensis sp. nov. | MTCF1.8 | DBUA0002429.06.v05 | BOLD:AEE3434 | Spain, Canary Fuerteventura |
| MOTU 7 - Platynereis macaronesiensis sp. nov. | PVCH3 | DBUA0002429.07.v01 | BOLD:AEE1367 | Spain, Canary - El Hierro |
| MOTU 7 - Platynereis macaronesiensis sp. nov. | PVM1 | DBUA0002430.01.v01 | BOLD:AEE3434 | Morocco, Mazagan |
| MOTU 7 - Platynereis macaronesiensis sp. nov. | PVM3 | DBUA0002430.01.v02 | BOLD:AEE3434 | Morocco, Mazagan |
| MOTU 2 - Platynereis sp. | MTSTF7 | DBUA0002442.01.v01 | BOLD:AAC5474 | Sweden, Tjärnö |
| MOTU 2 - Platynereis sp. | JAPM21 | MB29-000376 | BOLD:AAC5474 | Portugal, Lisbon (Parque Natural Arrábida) |

(Table S6.1 Continuation)

| Species | Vial code | Museum Voucher | BIN | Location |
| :---: | :---: | :---: | :---: | :---: |
| MOTU 2 - Platynereis sp. | 76323 | NTNU-VM-76323 | B0LD:AAC5474 | Norway, Bergen |
| MOTU 2 - Platynereis sp. | 75155 | NTNU-VM-75155 | B0LD:AAC5474 | Norway, Stavanger |
| MOTU 3 - Platynereis sp. | N73 | MTPD195-20 | BOLD:AEH0633 | Greece, Mazoma |
| MOTU 3 - Platynereis sp. | N74 | MTPD196-20 | BOLD:AEH0633 | Greece, Mazoma |
| MOTU 3 - Platynereis sp. | N75 | MTPD197-20 | BOLD:AEH0633 | Greece, Mazoma |
| MOTU 3 - Platynereis sp. | N79 | MTPD198-20 | BOLD:AEH0633 | Greece, Mazoma |
| MOTU 3 - Platynereis sp. | N146 | MTPD199-20 | BOLD:AEH0633 | Greece, Mazoma |
| MOTU 6 - Platynereis jourdei sp. nov. | PVEC2 | DBUA0002431.01.v01 | B0LD:ADW1653 | Spain, Calpe |
| MOTU 6 - Platynereis jourdei sp. nov. | PVEC3 - <br> holotype | DBUA0002431.01.v02 | B0LD:ADW1653 | Spain, Calpe |
| MOTU 6 - Platynereis jourdei sp. nov. | PVEC5 | DBUA0002431.01.v03 | B0LD:ADW1653 | Spain, Calpe |
| MOTU 6 - Platynereis jourdei sp. nov. | PVEC6 | DBUA0002431.01.v04 | B0LD:ADW1653 | Spain, Calpe |
| MOTU 6 - Platynereis jourdei sp. nov. | PVEC8 | DBUA0002431.01.v05 | B0LD:ADW1653 | Spain, Calpe |
| MOTU 6 - Platynereis jourdei sp. nov. | PVEC22 | DBUA0002431.01.v06 | B0LD:ADW1653 | Spain, Calpe |
| MOTU 6 - Platynereis jourdei sp. nov. | PVEC24 | DBUA0002431.01.v07 | B0LD:ADW1653 | Spain, Calpe |
| MOTU 6 - Platynereis jourdei sp. nov. | PVEC31 | DBUA0002431.01.v08 | B0LD:ADW1653 | Spain, Calpe |
| MOTU 6 - Platynereis jourdei sp. nov. | PVEC48 | DBUA0002431.01.v09 | B0LD:ADW1653 | Spain, Calpe |
| MOTU 6 - Platynereis jourdei sp. nov. | JLIC1-1 | DBUA0002432.01.v01 | B0LD:ADW1653 | Western Italy, Calafuria |
| MOTU 6 - Platynereis jourdei sp. nov. | JLIAn1 | DBUA0002432.02.v02 | B0LD:ADW1653 | Western Italy, Antignano |
| MOTU 6 - Platynereis jourdei sp. nov. | JLIHB2 | DBUA0002432.02.v03 | B0LD:ADW1653 | Western Italy, Antignano |
| MOTU 6 - Platynereis jourdei sp. nov. | JLIHB4 | DBUA0002432.02.v04 | BOLD:ADW1653 | Western Italy, Antignano |
| MOTU 6 - Platynereis jourdei sp. nov. | JLIHB6 | DBUA0002432.02.v05 | B0LD:ADW1653 | Western Italy, Antignano |
| MOTU 6 - Platynereis jourdei sp. nov. | JLIHB10 | DBUA0002432.02.v06 | BOLD:ADW1653 | Western Italy, Antignano |
| MOTU 6 - Platynereis jourdei sp. nov. | JLIHA2 | DBUA0002432.02.v07 | BOLD:ADW1653 | Western Italy, Antignano |
| MOTU 6 - Platynereis jourdei sp. nov. | JLIHA3 | DBUA0002432.02.v08 | B0LD:ADW1653 | Western Italy, Antignano |
| MOTU 6 - Platynereis jourdei sp. nov. | JLIHA6 | DBUA0002432.02.v09 | BOLD:ADW1653 | Western Italy, Antignano |

(Table S6.1 Continuation)

| Species | Vial code | Museum Voucher | BIN | Location |
| :--- | :--- | :--- | :--- | :--- |
| MOTU 6 - Platynereis <br> jourdei sp. nov. | JLIM1-1 | DBUA0002432.03.v01 | BOLD:ADW1653 | Western Italy, <br> MOTU 6 - Platynereis |
| JLIM1-2 | DBUA0002432.03.v02 | BOLD:ADW1653 Island | Western Italy, <br> jourdei sp. nov. <br> MOTU 6 - Platynereis | JLIM1-5 |

(Table S6.1 Continuation)

| Species | Vial code | Museum Voucher | BIN | Location |
| :---: | :---: | :---: | :---: | :---: |
| MOTU 4 - Platynereis dumerilii | MTCM10 | DBUA0002436.01.v01 | BOLD:AAH9446 | Portugal, Canto Marinho |
| MOTU 4 - Platynereis dumerilii | JJFR1-1 | DBUA0002438.01.v01 | BOLD:AAH9446 | North France, La Rochelle |
| MOTU 4 - Platynereis dumerilii | JJFR1-2 | DBUA0002438.01.v02 | BOLD:AAH9446 | North France, La Rochelle |
| MOTU 4 - Platynereis dumerilii | JJFR1-4 | DBUA0002438.01.v04 | BOLD:AAH9446 | North France, La Rochelle |
| MOTU 4 - Platynereis dumerilii | JJFR1-5 | DBUA0002438.01.v05 | BOLD:AAH9446 | North France, La Rochelle |
| MOTU 4 - Platynereis dumerilii | JJFR1-6 | DBUA0002438.01.v06 | BOLD:AAH9446 | North France, La Rochelle |
| MOTU 4 - Platynereis dumerilii | JJFR1-7 | DBUA0002438.01.v07 | BOLD:AAH9446 | North France, La Rochelle |
| MOTU 4 - Platynereis dumerilii | JJFR1-8 | DBUA0002438.01.v08 | BOLD:AAH9446 | North France, La Rochelle |
| MOTU 4 - Platynereis dumerilii | JJFR1-9 | DBUA0002438.01.v09 | BOLD:AAH9446 | North France, La Rochelle |
| MOTU 4 - Platynereis dumerilii | JJFR1-10 | DBUA0002438.01.v10 | BOLD:AAH9446 | North France, La Rochelle |
| MOTU 4 - Platynereis dumerilii | JJFR1-11 | DBUA0002438.01.v11 | BOLD:AAH9446 | North France, La Rochelle |
| MOTU 4 - Platynereis dumerilii | JJFR1-12 | DBUA0002438.01.v12 | BOLD:AAH9446 | North France, La Rochelle |
| MOTU 4 - Platynereis dumerilii | JJFR1-13 | DBUA0002438.01.v13 | BOLD:AAH9446 | North France, La Rochelle |
| MOTU 4 - Platynereis dumerilii | JJFR1-14 | DBUA0002438.01.v14 | BOLD:AAH9446 | North France, La Rochelle |
| MOTU 4 - Platynereis dumerilii | JJFR1-15 | DBUA0002438.01.v15 | BOLD:AAH9446 | North France, La Rochelle |
| MOTU 4 - Platynereis dumerilii | JJFR1-16 | DBUA0002438.01.v16 | BOLD:AAH9446 | North France, La Rochelle |
| MOTU 4 - Platynereis dumerilii | JJFR1-17 | DBUA0002438.01.v17 | BOLD:AAH9446 | North France, La Rochelle |
| MOTU 4 - Platynereis dumerilii | NLFA1-1 | DBUA0002439.01.v01 | BOLD:AAH9446 | North France, Arcachon Bay |
| MOTU 4 - Platynereis dumerilii | JLIM1-3 | DBUA0002437.05.v01 | BOLD:AAH9446 | Western Italy, Montecristo Island |
| MOTU 4 - Platynereis dumerilii | JLIM1-6 | DBUA0002437.05.v02 | BOLD:AAH9446 | Western Italy, Montecristo Island |
| MOTU 4 - Platynereis dumerilii | JLIM1-7 | DBUA0002437.05.v03 | BOLD:AAH9446 | Western Italy, Montecristo Island |
| MOTU 4 - Platynereis dumerilii | JLIM1-9 | DBUA0002437.05.v04 | BOLD:AAH9446 | Western Italy, Montecristo Island |
| MOTU 4 - Platynereis dumerilii | JLIM1-10 | DBUA0002437.05.v05 | BOLD:AAH9446 | Western Italy, Montecristo Island |
| MOTU 4 - Platynereis dumerilii | JLIM1-11 | DBUA0002437.05.v06 | BOLD:AAH9446 | Western Italy, Montecristo Island |
| MOTU 4 - Platynereis dumerilii | JLIM1-12 | DBUA0002437.05.v07 | BOLD:AAH9446 | Western Italy, Montecristo Island |

(Table S6.1 Continuation)

| Species | Vial code | Museum Voucher | BIN | Location |
| :---: | :---: | :---: | :---: | :---: |
| MOTU 4 - Platynereis dumerilii | JLIHB1 | DBUA0002437.01.v01 | BOLD:AAH9446 | Western Italy, Antignano |
| MOTU 4 - Platynereis dumerilii | JLIHB3 | DBUA0002437.01.v02 | BOLD:AAH9446 | Western Italy, Antignano |
| MOTU 4 - Platynereis dumerilii | JLHIHA10 | DBUA0002437.01.v03 | BOLD:AAH9446 | Western Italy, Antignano |
| MOTU 4 - Platynereis dumerilii | JLIAn3 | DBUA0002437.01.v05 | BOLD:AAH9446 | Western Italy, Antignano |
| MOTU 4 - Platynereis dumerilii | JLIAn4 | DBUA0002437.01.v06 | BOLD:AAH9446 | Western Italy, Antignano |
| MOTU 4 - Platynereis dumerilii | JLIA1 | DBUA0002437.02.v01 | BOLD:AAH9446 | Western Italy, Ardenza |
| MOTU 4 - Platynereis dumerilii | JLIA2 | DBUA0002437.02.v02 | BOLD:AAH9446 | Western Italy, Ardenza |
| MOTU 4 - Platynereis dumerilii | JLIA3 | DBUA0002437.02.v03 | BOLD:AAH9446 | Western Italy, Ardenza |
| MOTU 4 - Platynereis dumerilii | JLIA4 | DBUA0002437.02.v04 | BOLD:AAH9446 | Western Italy, Ardenza |
| MOTU 4 - Platynereis dumerilii | JLIA5 | DBUA0002437.02.v05 | BOLD:AAH9446 | Western Italy, Ardenza |
| MOTU 4 - Platynereis dumerilii | JLIV1 | DBUA0002437.03.v01 | BOLD:AAH9446 | Western Italy, Vada |
| MOTU 4 - Platynereis dumerilii | JLIV2 | DBUA0002437.03.v02 | BOLD:AAH9446 | Western Italy, Vada |
| MOTU 4 - Platynereis dumerilii | JLIV3 | DBUA0002437.03.v03 | BOLD:AAH9446 | Western Italy, Vada |
| MOTU 4 - Platynereis dumerilii | JLIV4 | DBUA0002437.03.v04 | BOLD:AAH9446 | Western Italy, Vada |
| MOTU 4 - Platynereis dumerilii | JLIP1-1 | DBUA0002437.04.v01 | BOLD:AAH9446 | Western Italy, Elba Island - Portoferraio |
| MOTU 4 - Platynereis dumerilii | JLIP1-2 | DBUA0002437.04.v02 | BOLD:AAH9446 | Western Italy, Elba Island - Portoferraio |
| MOTU 4 - Platynereis dumerilii | JLIP1-3 | DBUA0002437.04.v03 | BOLD:AAH9446 | Western Italy, Elba Island - Portoferraio |
| MOTU 4 - Platynereis dumerilii | JLIT1-1 | DBUA0002437.06.v01 | BOLD:AEH1226 | Eastern Italy, Taranto |
| MOTU 4 - Platynereis dumerilii | MTGC1-1 | DBUA0002440.01 | BOLD:AEH1225 | Greece, South Crete |
| MOTU 4 - Platynereis dumerilii | N78 | MTPD200-20 | BOLD:AEH1226 | Greece, Mazoma |
| MOTU 4 - Platynereis dumerilii | N60 | MTPD201-20 | BOLD:AEH1226 | Greece, Mazoma |
| MOTU 4 - Platynereis dumerilii | JLITR1-1 | DBUA0002437.07.v02 | BOLD:AEH1226 | Eastern Italy, Trieste |
| MOTU 5 - Platynereis sp. | MTCC1-7 | DBUA0002443.01.v01 | BOLD:AEE7752 | Spain, Canary - <br> Gran Canaria |
| MOTU 5 - Platynereis sp. | MTCL1-4 | DBUA0002443.02.v01 | BOLD:AEE7753 | Spain, Canary - <br> Lanzarote |
| MOTU 1 - Platynereis sp. | BA2020_41 | DBUA0002444.01.v01 | BOLD:AEH0315 | South France, Banyuls |

(Table S6.1 Continuation)

| Species | Vial code | Museum Voucher | BIN | Location |
| :---: | :---: | :---: | :---: | :---: |
| MOTU 1 - Platynereis sp. | BA2020_43 | DBUA0002444.01.v02 | BOLD:AEH0315 | South France, Banyuls |
| MOTU 11 - Nereis sp. | PVEC18 | DBUA0002446.01.v01 | BOLD:AEE0551 | Spain, Calpe |
| MOTU 11 - Nereis sp. | PVEC21 | DBUA0002446.01.v02 | BOLD:AEE2959 | Spain, Calpe |
| MOTU 11 - Nereis sp. | PVEC28 | DBUA0002446.01.v03 | BOLD:AEE0551 | Spain, Calpe |
| MOTU 11 - Nereis sp. | PVCH2 | DBUA0002445.01.v01 | BOLD:AEE2960 | Spain, Canary - El Hierro |
| MOTU 11 - Nereis sp. | MTGC1-2 | DBUA0002448.01.v01 | BOLD:AEE1368 | Greece, South Crete |
| MOTU 11 - Nereis sp. | MTGC1-4 | DBUA0002448.01.v02 | BOLD:AEE2959 | Greece, South Crete |
| MOTU 11 - Nereis sp. | MTGC1-5 | DBUA0002448.01.v03 | BOLD:AEE1368 | Greece, South Crete |
| MOTU 11 - Nereis sp. | $\begin{aligned} & \text { MTGC1-6 } \\ & \text { (GC3-1) } \end{aligned}$ | DBUA0002448.01.v04 | B0LD:AEE0551 | Greece, South Crete |
| MOTU 11 - Nereis sp. | $\begin{aligned} & \text { MTGC1-7 } \\ & \text { (GC3-2) } \end{aligned}$ | DBUA0002448.01.v05 | BOLD:AEE0551 | Greece, South Crete |
| MOTU 11 - Nereis sp. | $\begin{aligned} & \text { MTGC1-8 } \\ & \text { (GC3-3) } \end{aligned}$ | DBUA0002448.01.v06 | BOLD:AEE1368 | Greece, South Crete |
| MOTU 11 - Nereis sp. | $\begin{aligned} & \text { MTGC1-9 } \\ & \text { (GC3-4) } \end{aligned}$ | DBUA0002448.01.v07 | BOLD:AEE1368 | Greece, South Crete |
| MOTU 11 - Nereis sp. | $\begin{aligned} & \text { MTGC1-11 } \\ & \text { (GC3-6) } \end{aligned}$ | DBUA0002448.01.v08 | BOLD:AEE0551 | Greece, South Crete |
| MOTU 11 - Nereis sp. | $\begin{aligned} & \text { MTGC1-12 } \\ & \text { (GC3-7) } \end{aligned}$ | DBUA0002448.01.v09 | B0LD:AEE0551 | Greece, South Crete |
| MOTU 11 - Nereis sp. | $\begin{aligned} & \text { MTGC1-13 } \\ & \text { (GC3-8) } \end{aligned}$ | DBUA0002448.01.v10 | BOLD:AEE2959 | Greece, South Crete |
| MOTU 11 - Nereis sp. | $\begin{aligned} & \text { MTGC1-15 } \\ & \text { (GC3-10) } \end{aligned}$ | DBUA0002448.01.v11 | BOLD:AEE0551 | Greece, South Crete |
| MOTU 11 - Nereis sp. | MTCP1-38 | DBUA0002447.01.v02 | BOLD:AEE2960 | Spain, Canary - La <br> Palma |
| MOTU 11 - Nereis sp. | MTCP1-42 | DBUA0002447.01.v03 | BOLD:AEE2960 | Spain, Canary - La Palma |
| MOTU 11 - Nereis sp. | MTCP1-43 | DBUA0002447.01.v04 | BOLD:AEE2960 | Spain, Canary - La Palma |
| MOTU 14-Nereis sp. | $\begin{aligned} & \text { MTGC1-10 } \\ & \text { (GC3-5) } \end{aligned}$ | DBUA0002451.01.v01 | BOLD:AEH2506 | Greece, South Crete |
| MOTU 15-Nereis sp. | $\begin{aligned} & \text { MTGC1-14 } \\ & \text { (GC3-9) } \end{aligned}$ | DBUA0002451.01.v02 | BOLD:AEH2507 | Greece, South Crete |
| MOTU 12-Nereis sp. | PVAM1.2 | DBUA0002449.01.v01 | BOLD:AEE2961 | Portugal, Azores São Miguel |
| MOTU 12 - Nereis sp. | PVAM1.3 | DBUA0002449.01.v02 | BOLD:AEE2961 | Portugal, Azores São Miguel |
| MOTU 12 - Nereis sp. | PVAM1.4 | DBUA0002449.01.v03 | BOLD:AEE2961 | Portugal, Azores São Miguel |
| MOTU 12 - Nereis sp. | MTCC1-8 | MTPD144-20 | BOLD:AEE2961 | Spain, Canary Gran Canaria |
| MOTU 12 - Nereis sp. | MTCF1-4 | DBUA0002450.01.v01 | BOLD:AEE2961 | Spain, Canary Fuerteventura |

(Table S6.1 Continuation)

| Species | Vial code | Voucher code | BIN | Location |
| :--- | :--- | :--- | :--- | :--- |
| MOTU 12 - Nereis sp. | MTCF1-7 | DBUA0002450.01.v02 | BOLD:AEE2961 | Spain, Canary - <br> Fuerteventura <br> Spain, Canary - |
| MOTU 12 - Nereis sp. | MTCF1-9 | DBUA0002450.01.v03 | BOLD:AEE2961 | Fuerteventura <br> Spain, Canary - La |
| MOTU 13- Nereis sp. | MTCP1-41 | DBUA0002450.02.v01 | BOLD:AEE3443 | Palma <br> Greece, South |
| Outgroup - <br> Pseudonereis sp. <br> Outgroup - | MTGC1-3 | DBUA0002452.01.v01 | BOLD:AEH1610 | Crete <br> Pseudonereis sp. <br> Outgroup - Perinereis <br> marionii <br> Outgroup - Perinereis <br> marionii |
| MTGC6-1 | MLY2017_69 | DBUUA0002452.01.v02 | BOLD:AEH1610 | Greece, South <br> Crete |

(Table S6.1 Continuation)

Table S6.2. Geographic location and GenBank accession numbers for sequences belonging to other studies and used for comparison purposes

| Species | Designation <br> (MOTU) | GenBank COI | Location | References |
| :--- | :--- | :--- | :--- | :--- |
| Platynereis sp. | MOTU 1 | KT124712 | Italy: Vulcano | Wäge et al. (2017) |
| Platynereis sp. | MOTU 1 | KT124716 | Italy: Vulcano | Wäge et al. (2017) |
| "P. dumerili' | MOTU 11 | KC591811 | Italy | Calosi et al. (2013) |
| P. dumerilii | MOTU 4 | KF737174 | India | Singh et al., |
| P. dumerilii | MOTU 4 | MH114981 | Germany | Tilic et al., |
| P. dumerilii | MOTU 4 | KC591825 | Italy: S. Pietro | Calosi et al. (2013) |
| P. dumerilii | MOTU 4 | KT124685 | Italy: Ischia | Wäge et al. (2017) |
| P. jourdei sp. nov. | MOTU 6 | KC591880 | Italy: Ischia | Calosi et al. (2013) |
| P. jourdei sp. nov. | MOTU 6 | KT124684 | Italy: Ischia | Wäge et al. (2017) |
| Platynereis sp. | MOTU GB4 | MT196851 | South Africa | Kara et al. (2020) |
| Platynereis sp. | MOTU GB4 | MT196856 | South Africa | Kara et al. (2020) |
| Platynereis sp. | MOTU GB4 | MT196857 | South Africa | Kara et al. (2020) |
| Platynereis sp. | MOTU GB2 | KT124674 | South of Spain: | Wäge et al. (2017) |
| Platynereis sp. | MOTU GB3 | KC591870 | Blanes | Italy: Ischia |
| Platynereis sp. | MOTU GB3 | KC591872 | Italy: Ischia | Calosi et al. (2013) |
| Platynereis sp. | MOTU GB3 | KC591875 | Italy: Ischia | Calosi et al. (2013) |
| P. cf. massiliensis | MOTU 9 | KC591833 | Italy: Ischia | Calosi et al. (2013) |
| (Livorno variant) |  |  | Wäge et al. (2017) |  |
| P. cf. massiliensis | MOTU 9 |  | KT124681 | Italy: Ischia |


| Species | Designation (MOTU) | GenBank COI | Location | References |
| :---: | :---: | :---: | :---: | :---: |
| Platynereis entshonae | MOTU GB1 | MT196859 | South Africa | Kara et al. (2020) |
| Platynereis entshonae | MOTU GB1 | MT196867 | South Africa | Kara et al. (2020) |
| Platynereis entshonae | MOTU GB1 | MT196888 | South Africa | Kara et al. (2020) |
| Nereis heterocirrata | Outgroup | MN256591 | South East Asia | Xing and Zhang (2019) |
| Nereis heterocirrata | Outgroup | MN256590 | South East Asia | Xing and Zhang (2019) |
| Nereis heterocirrata | Outgroup | MN256589 | South East Asia | Xing and Zhang (2019) |
| Nereis pelagica | Outgroup | JN852947 | Sweden | Norlinder et al. (2012) |
| Nereis pelagica | Outgroup | KR916895 | Portugal | Lobo et al. (2016) |
| Nereis pelagica | Outgroup | KR916894 | Portugal | Lobo et al. (2016) |
| "Neanthes fucata" | Outgroup | KR916880 | Portugal | Lobo et al. (2016) |
| "Neanthes fucata" | Outgroup | KR916879 | Portugal | Lobo et al. (2016) |
| "Neanthes fucata" | Outgroup | KR916876 | Portugal | Lobo et al. (2016) |
| "Neanthes fucata" | Outgroup | KU714731 | Northern Spain | Miralles et al. (2016) |
| "Neanthes fucata" | Outgroup | KU714730 | Northern Spain | Miralles et al. (2016) |
| Nereis zonata | Outgroup | HQ024404 | Arctic Canada | Carr et al. (2011) |
| Nereis zonata | Outgroup | HQ024401 | Arctic Canada | Carr et al. (2011) |
| Nereis zonata | Outgroup | HQ024403 | Arctic Canada | Carr et al. (2011) |
| Ceratonereis tentaculata | Outgroup | MW277910 | USA: Hawaii | Paulay et al. (2018) |
| Ceratonereis tentaculata | Outgroup | MW277876 | USA: Hawaii | Paulay et al. (2018) |

(Table S6.2 Continuation)


Fig. S6.1. Dorsal view, focused on the worm's head with exposed pharynx. Perinereis marionii, Specimen DBUA0002453.01

## Annexes of chapter 7

Table S7.1. Public BIN accession numbers, museum voucher codes and location for each original specimen in chapter 7 .

| Species | Vial code | Voucher code | BIN | Location |
| :---: | :---: | :---: | :---: | :---: |
| Perinereis oliveirae <br> - MOTU 14 | MTA1 | DBUA0002494.01.v01 | BOLD:AAY5413 | Portugal, Praia Areosa |
| Perinereis oliveirae <br> - MOTU 14 | MTA2 | DBUA0002494.01.v02 | BOLD:AAY5413 | Portugal, Praia Areosa |
| Perinereis oliveirae <br> - MOTU 14 | MTA3 | DBUA0002494.01.v03 | BOLD:AAY5413 | Portugal, Praia Areosa |
| Perinereis oliveirae <br> - MOTU 14 | MTA4 | DBUA0002494.01.v04 | BOLD:AAY5413 | Portugal, Praia Areosa |
| Perinereis oliveirae <br> - MOTU 14 | MTA5 | DBUA0002494.01.v05 | BOLD:AAY5413 | Portugal, Praia Areosa |
| Perinereis oliveirae <br> -MOTU 14 | MTCM40 | DBUA0002494.02.v01 | BOLD:AAY5413 | Portugal, Canto Marinho |
| Perinereis oliveirae <br> - MOTU 14 | MTCM41 | DBUA0002494.02.v02 | BOLD:AAY5413 | Portugal, Canto Marinho |
| Perinereis oliveirae <br> - MOTU 14 | MTCM43 | DBUA0002494.02.v03 | BOLD:AAY5413 | Portugal, Canto Marinho |
| Perinereis oliveirae <br> - MOTU 14 | MTCM44 | DBUA0002494.02.v04 | BOLD:AAY5413 | Portugal, Canto Marinho |
| Perinereis oliveirae <br> - MOTU 14 | MTCM45 | DBUA0002494.02.v05 | BOLD:AAY5413 | Portugal, Canto Marinho |
| Perinereis oliveirae <br> - MOTU 14 | MTCM47 | DBUA0002494.02.v06 | BOLD:AAY5413 | Portugal, Canto Marinho |
| Perinereis oliveirae <br> - MOTU 14 | MTCM48 | DBUA0002494.02.v07 | BOLD:AAY5413 | Portugal, Canto Marinho |
| Perinereis oliveirae <br> - MOTU 14 | MTCM49 | DBUA0002494.02.v08 | BOLD:AAY5413 | Portugal, Canto Marinho |
| Perinereis oliveirae <br> - MOTU 14 | MTCM59 | DBUA0002494.02.v10 | BOLD:AAY5413 | Portugal, Canto Marinho |
| Perinereis oliveirae <br> - MOTU 14 | MTCM60 | DBUA0002494.02.v11 | BOLD:AAY5413 | Portugal, Canto Marinho |
| Perinereis oliveirae <br> - MOTU 14 | MTCM61 | DBUA0002494.02.v12 | BOLD:AAY5413 | Portugal, Canto Marinho |
| Perinereis oliveirae <br> - MOTU 14 | MTCM62 | DBUA0002494.02.v13 | BOLD:AAY5413 | Portugal, Canto Marinho |
| Perinereis oliveirae <br> - MOTU 14 | MTCM63 | DBUA0002494.02.v14 | BOLD:AAY5413 | Portugal, Canto Marinho |
| Perinereis oliveirae <br> - MOTU 14 | MTCM64 | DBUA0002494.02.v15 | BOLD:AAY5413 | Portugal, Canto Marinho |
| Perinereis oliveirae <br> - MOTU 14 | NLSB1-1 | DBUA0002495.01.v01 | BOLD:AAY5413 | North Spain, Basque coast |
| Perinereis sp. - <br> MOTU 10 | MTCT3-1 | DBUA0002496.01.v01 | BOLD:AEE3598 | Canary, Tenerife |
| Perinereis sp. - <br> MOTU 10 | MTCT3-2 | DBUA0002496.01.v02 | BOLD:AEE3598 | Canary, Tenerife |


| Species | Vial code | Voucher code | BIN | Location |
| :---: | :---: | :---: | :---: | :---: |
| Perinereis sp. - <br> MOTU 10 | MTCT3-4 | DBUA0002496.01.v04 | BOLD:AEE3598 | Canary, Tenerife |
| Perinereis sp. - <br> MOTU 10 | MTCT3-5 | DBUA0002496.01.v05 | BOLD:AEE3598 | Canary, Tenerife |
| Perinereis sp. MOTU 10 | MTCT3-6 | DBUA0002496.01.v06 | BOLD:AEE3598 | Canary, Tenerife |
| Perinereis sp. - <br> MOTU 10 | MTCT3-7 | DBUA0002496.01.v07 | BOLD:AEE3598 | Canary, Tenerife |
| Perinereis sp. - <br> MOTU 10 | MTCT3-8 | DBUA0002496.01.v08 | BOLD:AEE3598 | Canary, Tenerife |
| Perinereis sp. MOTU 10 | MTCT3-9 | DBUA0002496.01.v09 | BOLD:AEE3598 | Canary, Tenerife |
| Perinereis sp. - <br> MOTU 10 | МТСТЗ-10 | DBUA0002496.01.v10 | BOLD:AEE3598 | Canary, Tenerife |
| Perinereis sp. - <br> MOTU 10 | MTCL2-1 | DBUA0002496.02.v01 | BOLD:AEE3598 | Canary, Lanzarote |
| Perinereis sp. - <br> MOTU 10 | MTCL2-2 | DBUA0002496.02.v02 | BOLD:AEE3598 | Canary, Lanzarote |
| Perinereis sp. - <br> MOTU 10 | MTCL2-3 | DBUA0002496.02.v03 | BOLD:AEE3598 | Canary, Lanzarote |
| Perinereis sp. - <br> MOTU 10 | MTCL2-4 | DBUA0002496.02.v04 | BOLD:AEE3598 | Canary, Lanzarote |
| Perinereis sp. - <br> MOTU 10 | MTCL2-5 | DBUA0002496.02.v05 | BOLD:AEE3598 | Canary, Lanzarote |
| Perinereis sp. - <br> MOTU 10 | MTCL2-6 | DBUA0002496.02.v06 | BOLD:AEE3598 | Canary, Lanzarote |
| Perinereis sp. - <br> MOTU 10 | MTCL2-7 | DBUA0002496.02.v07 | BOLD:AEE3598 | Canary, Lanzarote |
| Perinereis sp. - <br> MOTU 10 | MTCL2-8 | DBUA0002496.02.v08 | BOLD:AEE3598 | Canary, Lanzarote |
| Perinereis sp. - <br> MOTU 10 | MTCL2-9 | DBUA0002496.02.v09 | BOLD:AEE3598 | Canary, Lanzarote |
| Perinereis sp. - <br> MOTU 10 | MTCL2-10 | DBUA0002496.02.v10 | BOLD:AEE3598 | Canary, Lanzarote |
| Perinereis sp. - <br> MOTU 5 | MTCC3-2 | DBUA0002497.01.v01 | BOLD:AEE3637 | Canary, Gran Canaria |
| Perinereis sp. - <br> MOTU 5 | MTCC3-4 | DBUA0002497.01.v02 | BOLD:AEE3637 | Canary, Gran Canaria |
| Perinereis sp. - <br> MOTU 5 | MTCC3-6 | DBUA0002497.01.v03 | BOLD:AEE3637 | Canary, Gran Canaria |
| Perinereis sp. MOTU 5 | MTCC3-9 | DBUA0002497.01.v04 | BOLD:AEE3637 | Canary, Gran Canaria |
| Perinereis sp. MOTU 5 | MTCC3-10 | DBUA0002497.01.v05 | BOLD:AEE3637 | Canary, Gran Canaria |
| Perinereis sp. MOTU 5 | MTCC3-14 | DBUA0002497.01.v06 | BOLD:AEE3637 | Canary, Gran Canaria |
| Perinereis sp. MOTU 5 | MTCF2-1 | DBUA0002497.02.v01 | BOLD:AEE3637 | Canary, Fuerteventura |
| Perinereis sp. MOTU 5 | MTCF2-5 | MTPC048-20 | BOLD:AEE3637 | Canary, Fuerteventura |

(Table S7.1 Continuation)

| Species | Vial code | Voucher code | BIN | Location |
| :---: | :---: | :---: | :---: | :---: |
| Perinereis sp. MOTU 1 | MTCC3-3 | DBUA0002498.01.v02 | BOLD:AEE3596 | Canary, Gran Canaria |
| Perinereis sp. MOTU 1 | MTCC3-5 | DBUA0002498.01.v03 | BOLD:AEE3596 | Canary, Gran Canaria |
| Perinereis sp. MOTU 1 | MTCC3-7 | DBUA0002498.01.v04 | BOLD:AEE3596 | Canary, Gran Canaria |
| Perinereis sp. MOTU 1 | MTCC3-8 | DBUA0002498.01.v05 | BOLD:AEE3596 | Canary, Gran Canaria |
| Perinereis sp. MOTU 1 | MTCC3-11 | DBUA0002498.01.v06 | BOLD:AEE3596 | Canary, Gran Canaria |
| Perinereis sp. MOTU 1 | MTCC3-12 | DBUA0002498.01.v07 | BOLD:AEE3596 | Canary, Gran Canaria |
| Perinereis sp. MOTU 1 | MTCC3-13 | DBUA0002498.01.v08 | BOLD:AEE3596 | Canary, Gran Canaria |
| Perinereis sp. MOTU 1 | MTCC3-15 | DBUA0002498.01.v09 | BOLD:AEE3596 | Canary, Gran Canaria |
| Perinereis sp. MOTU 1 | MTCC3-16 | DBUA0002498.01.v10 | BOLD:AEE3596 | Canary, Gran Canaria |
| Perinereis sp. MOTU 1 | MTCC3-17 | DBUA0002498.01.v11 | BOLD:AEE3596 | Canary, Gran Canaria |
| Perinereis sp. MOTU 1 | MTCC3-18 | DBUA0002498.01.v12 | BOLD:AEE3596 | Canary, Gran Canaria |
| Perinereis sp. MOTU 1 | MTCH1 | DBUA0002498.02.v01 | BOLD:AEE7675 | Canary, El Hierro |
| Perinereis sp. MOTU 2 | PVAM2-1 | MTPC062-20 | BOLD:AEE3597 | Azores, Santa Maria |
| Perinereis sp. MOTU 2 | PVAM2-2 | MTPC063-20 | BOLD:AEE3597 | Azores, Santa Maria |
| Perinereis sp. MOTU 2 | PVAM2-3 | MTPC064-20 | BOLD:AEE3597 | Azores, Santa Maria |
| Perinereis sp. MOTU 12 | MPIN2-1 | DBUA0002500.01.v01 | BOLD:AEF0096 | Eastern Italy, <br> Adriatic |
| Perinereis sp. MOTU 12 | MPIN2-2 | DBUA0002500.01.v02 | B0LD:AEF0096 | Eastern Italy, Adriatic |
| Perinereis sp. MOTU 12 | MPIN2-3 | DBUA0002500.01.v03 | BOLD:AEF0096 | Eastern Italy, <br> Adriatic |
| Perinereis sp. MOTU 12 | MPIN2-4 | DBUA0002500.01.v04 | BOLD:AEF0096 | Eastern Italy, <br> Adriatic |
| Perinereis sp. MOTU 12 | MPIN2-6 | DBUA0002500.01.v05 | BOLD:AEF0096 | Eastern Italy, Adriatic |
| Perinereis sp. MOTU 12 | MPIN2-7 | DBUA0002500.01.v06 | BOLD:AEF0096 | Eastern Italy, <br> Adriatic |
| Perinereis sp. MOTU 12 | MPIN2-8 | DBUA0002500.01.v07 | BOLD:AEF0096 | Eastern Italy, Adriatic |
| Perinereis sp. MOTU 12 | MPIN2-9 | DBUA0002500.01.v08 | BOLD:AEF0096 | Eastern Italy, Adriatic |
| Perinereis sp. MOTU 12 | MPIN2-10 | DBUA0002500.01.v09 | BOLD:AEF0096 | Eastern Italy, <br> Adriatic |
| Perinereis sp. MOTU 12 | MPIN2-11 | DBUA0002500.01.v10 | BOLD:AEF0096 | Eastern Italy, <br> Adriatic |

(Table S7.1 Continuation)

| Species | Vial code | Voucher code | BIN | Location |
| :---: | :---: | :---: | :---: | :---: |
| Perinereis sp. MOTU 12 | MPIN2-13 | DBUA0002500.01.v11 | BOLD:AEF0096 | Eastern Italy, <br> Adriatic |
| Perinereis sp. MOTU 12 | MPIN2-13 | DBUA0002500.01.v12 | BOLD:AEF0096 | Eastern Italy, <br> Adriatic |
| Perinereis sp. - <br> MOTU 12 | MPIN2-14 | DBUA0002500.01.v13 | BOLD:AEF0096 | Eastern Italy, <br> Adriatic |
| Perinereis sp. - <br> MOTU 12 | MPIN2-15 | DBUA0002500.01.v14 | BOLD:AEF0096 | Eastern Italy, Adriatic |
| Perinereis sp. MOTU 12 | MPIN2-16 | DBUA0002500.01.v15 | BOLD:AEF0096 | Eastern Italy, <br> Adriatic |
| Perinereis sp.- <br> MOTU 12 | MTGC2-2 | DBUA0002501.01.v02 | BOLD:AEF0096 | Greece, Crete |
| Perinereis sp. MOTU 12 | MTGC2-3 | DBUA0002501.01.v03 | BOLD:AEF0096 | Greece, Crete |
| Perinereis sp. - <br> MOTU 12 | MTGC2-4 | DBUA0002501.01.v04 | BOLD:AEF0096 | Greece, Crete |
| Perinereis sp. - <br> MOTU 12 | MTGC2-5 | DBUA0002501.01.v05 | BOLD:AEF0096 | Greece, Crete |
| Perinereis sp. MOTU 12 | MTGC2-6 | DBUA0002501.01.v06 | BOLD:AEF0096 | Greece, Crete |
| Perinereis sp. MOTU 12 | MTGC2-7 | DBUA0002501.01.v07 | BOLD:AEF0096 | Greece, Crete |
| Perinereis sp. MOTU 12 | MTGC2-8 | DBUA0002501.01.v08 | BOLD:AEF0096 | Greece, Crete |
| Perinereis sp.- <br> MOTU 12 | MTGC2-9 | DBUA0002501.01.v09 | BOLD:AEF0096 | Greece, Crete |
| Perinereis sp.- <br> MOTU 12 | MTGC2-10 | DBUA0002501.01.v10 | BOLD:AEF0096 | Greece, Crete |
| Perinereis sp. - <br> MOTU 12 | MTGC2-11 | DBUA0002501.01.v11 | BOLD:AEF0096 | Greece, Crete |
| Perinereis sp. - <br> MOTU 12 | MTGC2-12 | DBUA0002501.01.v12 | BOLD:AEF0096 | Greece, Crete |
| Perinereis sp. - <br> MOTU 12 | MTGC2-13 | DBUA0002501.01.v13 | BOLD:AEF0096 | Greece, Crete |
| Perinereis sp. - <br> MOTU 12 | MTGC2-14 | DBUA0002501.01.v14 | BOLD:AEF0096 | Greece, Crete |
| Perinereis sp. - <br> MOTU 12 | MTGC2-15 | DBUA0002501.01.v15 | BOLD:AEF0096 | Greece, Crete |
| Perinereis sp.MOTU 12 | MTGC2-16 | DBUA0002501.01.v16 | BOLD:AEF0096 | Greece, Crete |
| Perinereis sp. MOTU 8 | JLIC2-1 | DBUA0002502.01.v01 | BOLD:AEE9098 | Western Italy, Calafuria |
| Perinereis sp. MOTU 8 | JLIM2-3 | DBUA0002503.01.v01 | BOLD:AEE9098 | Western Italy, Montecristo island |
| Perinereis sp. MOTU 8 | MNHN6-2 | To be submitted to the MNHN museum | BOLD:AEE9098 | France, Corsica |
| Perinereis sp. MOTU 8 | MNHN6-3 | To be submitted to the MNHN museum | BOLD:AEE9098 | France, Corsica |
| Perinereis sp. MOTU 8 | MNHN6-4 | To be submitted to the MNHN museum | BOLD:AEE9098 | France, Corsica |

(Table S7.1 Continuation)

| Species | Vial code | Voucher code | BIN | Location |
| :---: | :---: | :---: | :---: | :---: |
| Perinereis sp. MOTU 8 | MNHN8-2 | To be submitted to the MNHN museum | BOLD:AEE9098 | France, Corsica |
| Perinereis sp. - <br> MOTU 8 | MNHN8-3 | To be submitted to the MNHN museum | BOLD:AEE9098 | France, Corsica |
| Perinereis sp. - <br> MOTU 8 | MNHN8-4 | To be submitted to the MNHN museum | BOLD:AEE9098 | France, Corsica |
| Perinereis sp. MOTU 8 | MNHN8-6 | To be submitted to the MNHN museum | BOLD:AEE9098 | France, Corsica |
| Perinereis sp. MOTU 8 | MNHN8-7 | To be submitted to the MNHN museum | B0LD:AEE9098 | France, Corsica |
| Perinereis sp. - <br> MOTU 8 | MNHN8-9 | To be submitted to the MNHN museum | BOLD:AEE9098 | France, Corsica |
| Perinereis sp. - <br> MOTU 6 | JLIC2-2 | DBUA0002504.01.v01 | BOLD:AEE9096 | Western Italy, Calafuria |
| Perinereis sp. - <br> MOTU 6 | JLIC2-5 | DBUA0002504.01.v02 | BOLD:AEE9096 | Western Italy, Calafuria |
| Perinereis sp. - <br> MOTU 6 | JLIC2-8 | DBUA0002504.01.v03 | BOLD:AEE9096 | Western Italy, Calafuria |
| Perinereis sp. - <br> MOTU 6 | JLIC2-9 | DBUA0002504.01.v04 | BOLD:AEE9096 | Western Italy, Calafuria |
| Perinereis sp. - <br> MOTU 6 | MNHN5-2 | To be submitted to the MNHN museum | BOLD:AEE9096 | France, Corsica |
| Perinereis sp. - <br> MOTU 6 | MNHN5-3 | To be submitted to the MNHN museum | BOLD:AEE9096 | France, Corsica |
| Perinereis sp. - <br> MOTU 6 | JLIM2-2 | DBUA0002505.01.v01 | BOLD:AEE9096 | Western Italy, Montecristo Island |
| Perinereis sp. - <br> MOTU 4 | JLIC2-3 | DBUA0002506.01.v01 | BOLD:AEE9097 | Western Italy, Calafuria |
| Perinereis sp. - <br> MOTU 4 | JLIC2-4 | DBUA0002506.01.v02 | BOLD:AEE9097 | Western Italy, Calafuria |
| Perinereis sp. - <br> MOTU 4 | JLITR2-1 | DBUA0002507.01.v01 | BOLD:AEE9097 | Eastern Italy, Trieste |
| Perinereis sp. - <br> MOTU 4 | JLITR2-2 | DBUA0002507.01.v02 | BOLD:AEE9097 | Eastern Italy, Trieste |
| Perinereis sp. - <br> MOTU 4 | JLITR2-3 | DBUA0002507.01.v03 | BOLD:AEE9097 | Eastern Italy, Trieste |
| Perinereis sp. - <br> MOTU 4 | JLIPI2-1 | DBUA0002508.01.v01 | BOLD:AEE9097 | Western Italy, Pianosa Island |
| Perinereis sp. - <br> MOTU 4 | JLIM2-1 | DBUA0002508.02.v01 | BOLD:AEE9097 | Western Italy, <br> Montecristo Island |
| Perinereis sp. - <br> MOTU 4 | MNHN8-1 | To be submitted to the MNHN museum | BOLD:AEE9097 | France, Corsica |
| Perinereis sp. - <br> MOTU 4 | MNHN8-5 | To be submitted to the MNHN museum | BOLD:AEE9097 | France, Corsica |
| Perinereis sp. - <br> MOTU 4 | MNHN8-8 | To be submitted to the MNHN museum | BOLD:AEE9097 | France, Corsica |
| Perinereis sp. MOTU 7 | JLIC2-6 | DBUA0002509.01.v01 | BOLD:AEE9095 | Western Italy, Calafuria |
| Perinereis sp. - <br> MOTU 7 | JLIC2-7 | DBUA0002509.01.v02 | BOLD:AEE9095 | Western Italy, Calafuria |

(Table S7.1 Continuation)

| Species | Vial code | Voucher code | BIN | Location |
| :---: | :---: | :---: | :---: | :---: |
| Perinereis sp. MOTU 3 | MTCM42 | DBUA0002510.01.v01 | BOLD:ACH5486 | Portugal, Canto Marinho |
| Perinereis sp. MOTU 3 | MTCM46 | DBUA0002510.01.v02 | BOLD:ACH5486 | Portugal, Canto Marinho |
| Perinereis sp. MOTU 3 | DFPN09 | DBUA0002511.01.v01 | BOLD:ACH5486 | Portugal, Areosa |
| Perinereis sp. MOTU 3 | CHFM2-2 | DBUA0002512.01.v01 | BOLD:ACH5486 | North France, Bay of Morlaix |
| Perinereis sp. MOTU 3 | CHFM2-3 | DBUA0002512.01.v02 | BOLD:ACH5486 | North France, Bay of Morlaix |
| Perinereis sp. MOTU 3 | CHFM2-4 | DBUA0002512.01.v03 | BOLD:ACH5486 | North France, Bay of Morlaix |
| Perinereis sp. MOTU 3 | CHFM2-5 | DBUA0002512.01.v04 | BOLD:ACH5486 | North France, Bay of Morlaix |
| Perinereis sp. MOTU 3 | CHFM2-6 | DBUA0002512.01.v05 | BOLD:ACH5486 | North France, Bay of Morlaix |
| Perinereis sp. MOTU 3 | CHFM2-7 | DBUA0002512.01.v06 | BOLD:ACH5486 | North France, Bay of Morlaix |
| Perinereis sp. MOTU 3 | CHFM2-12 | DBUA0002512.01.v07 | BOLD:ACH5486 | North France, Bay of Morlaix |
| Perinereis sp. MOTU 3 | RO2018-70 | DBUA0002513.01.v01 | BOLD:ACH5486 | North france, Roscoff |
| Perinereis sp. MOTU 3 | RO2018-72 | DBUA0002513.01.v02 | BOLD:ACH5486 | North france, Roscoff |
| Perinereis sp. MOTU 3 | NLFA2-1 | DBUA0002514.01.v01 | BOLD:ACH5486 | North France, Arcachon Bay |
| Perinereis sp. MOTU 3 | NLFA2-2 | DBUA0002514.01.v02 | BOLD:ACH5486 | North France, Arcachon Bay |
| Perinereis sp. MOTU 3 | JJFO1-1 | MTPC139-20 | BOLD:ACH5486 | North France, Marennes-Oleron |
| Perinereis sp. MOTU 3 | B749 | NTNU-VM75758 | BOLD:ACH5486 | Norway, Stavanger |
| Perinereis sp. <br> MOTU 11 | JLIP2-2 | DBUA0002515.01.v01 | BOLD:AEH1977 | Western Italy, Elba Island - <br> Portoferraio |
| Perinereis sp. MOTU 11 | JLIP2-4 | DBUA0002515.01.v02 | BOLD:AEH1977 | Western Italy, Elba Island - <br> Portoferraio |
| Perinereis sp. <br> MOTU 11 | JLIP2-6 | DBUA0002515.01.v03 | BOLD:AEH1977 | Western Italy, Elba Island - <br> Portoferraio |
| Perinereis sp. <br> MOTU 11 | JLIP2-9 | DBUA0002515.01.v04 | BOLD:AEH1977 | Western Italy, Elba Island - <br> Portoferraio |
| Perinereis sp. MOTU 11 | JLIP2-11 | DBUA0002515.01.v05 | BOLD:AEH1977 | Western Italy, Elba Island - <br> Portoferraio |
| Perinereis sp. <br> MOTU 11 | JLIP2-12 | DBUA0002515.01.v06 | BOLD:AEH1977 | Western Italy, Elba Island - <br> Portoferraio |

(Table S7.1 Continuation)

| Vial code | Voucher code | BIN | Location |
| :--- | :--- | :--- | :--- |
| Species |  |  | Western Italy, Elba |
| Perinereis sp. - <br> MOTU 11 | JLIP2-12 | DBUA0002515.01.v06 | BOLD:AEH1977 | | Island - |
| :--- |
| Portoferraio |

(Table S7.1 Continuation)

| Species | Vial code | Voucher code | BIN | Location |
| :--- | :--- | :--- | :--- | :--- |
| Outgroup - <br> Perinereis <br> aibuhitensis <br> Outgroup - <br> Perinereis <br> aibuhitensis <br> Outgroup - | ARK012 | DBUA0002518.01.v03 | BOLD:AAY5413 | South Korea |
| Perinereis <br> aibuhitensis <br> Outgroup - <br> Perinereis <br> aibuhitensis <br> Outgroup - | ARK014 | ARKO15 | DBUA0002518.01.v05 | BOLD:AAY5413 |

(Table S7.1 Continuation)


[^0]:    ${ }^{2}$ Specimens with pigmentation dorsally on segment 2 may also have various amounts of pigmentation on the anterior cirri and in the prostomium.
    ${ }^{-}$Transverse lines in some specimens from Eumida merope, Eumida cf merope and E. aff. fauchaldi are very short, approaching spots.

