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Phylogeny and phylogeography of the family Hyalidae (Crustacea: Amphipoda) along the northeast Atlantic coasts

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“...Nothing at first can appear more difficult to believe than that the more complex organs and instincts should have been perfected, not by means superior to, though analogous with, human reason, but by the accumulation of innumerable slight variations, each good for the individual possessor…” (Darwin 1859)

1) Index

2) Abstract

The family Hyalidae comprises more than one hundred species, distributed worldwide. They are common and abundant in the littoral and shallow sublittoral habitats and they play an important role in the coastal food chain. Most studies about this family were dealing with taxonomy and ecology, while very little is known about phylogenetic relationship among genera and species.

In the present study we aim to achieve the first approach of the phylogenetic patterns of this family in NE Atlantic Ocean and Mediterranean Sea, and to perform the first insight into the phylogeography *Apohyale prevostii* along both the North Atlantic coasts. In order to do that, eight species belonging to the genera *Apohyale*, *Hyale*, *Serejohyale* and *Protohyale* were investigated using the mitochondrial COI-5P barcode region. Specimens were collected along European and Moroccan Atlantic rocky shores, including Iceland, the British Isles, Macaronesia and in the Mediterranean Sea. Sequences of *A. prevostii,* from the NW Atlantic Ocean, available in BOLD and GenBank, were retrieved.

As expected, phylogenetic analyses showed highly-divergent clades, clearly discriminating among different species clusters, confirming their morphology-based identifications. Although, within *A. perieri*, *A. media, A. stebbingi, P. (Protohyale) schmidtii* and *S. spinidactylus*, high genetic diversity was found, revealing putative cryptic species. The clade of *A. prevostii* and *A. stebbingi* appears well supported and divided from the other two congeneric species, and *P. (Protohyale) schmidtii* shows a basal divergence.

The north-western Atlantic coasts were recently colonized by *A. prevostii* after the last glacial maximum from the European populations showing also a common haplotype in every population analysed.

The use of the COI-5P as DNA barcode provided a good tool to underline the necessity of a revision of this emblematic family, as well as to discern taxonomically the possible new species flagged with this molecular device.

3) Introduction

a) Hyalidae Bulycheva, 1957

The family Hyalidae Bulycheva, 1957 is part of the superfamily Talitroidea, which, according to Serejo (2004), also includes three other families – Chiltoniidae J.L. Barnard, 1972, Dogielinotidae Gurjanova, 1953 and Talitridae Rafinesque, 1815. Bousfield & Hendrycks (2002) revised the hyalids, based on the North Pacific fauna and split the large *Hyale* Rathke, 1837 (**Fig. 1**) genus into five additional new genera and created 13 new species. After a further revision (Horton *et al.* 2015; Serejo 2004) the family Hyalidae was subdivided into two subfamilies (Hyacheliinae Bousfield & Hendrycks, 2002 and Hyalinae Bulycheva, 1957) with 11 genera and more than 110 species worldwide.

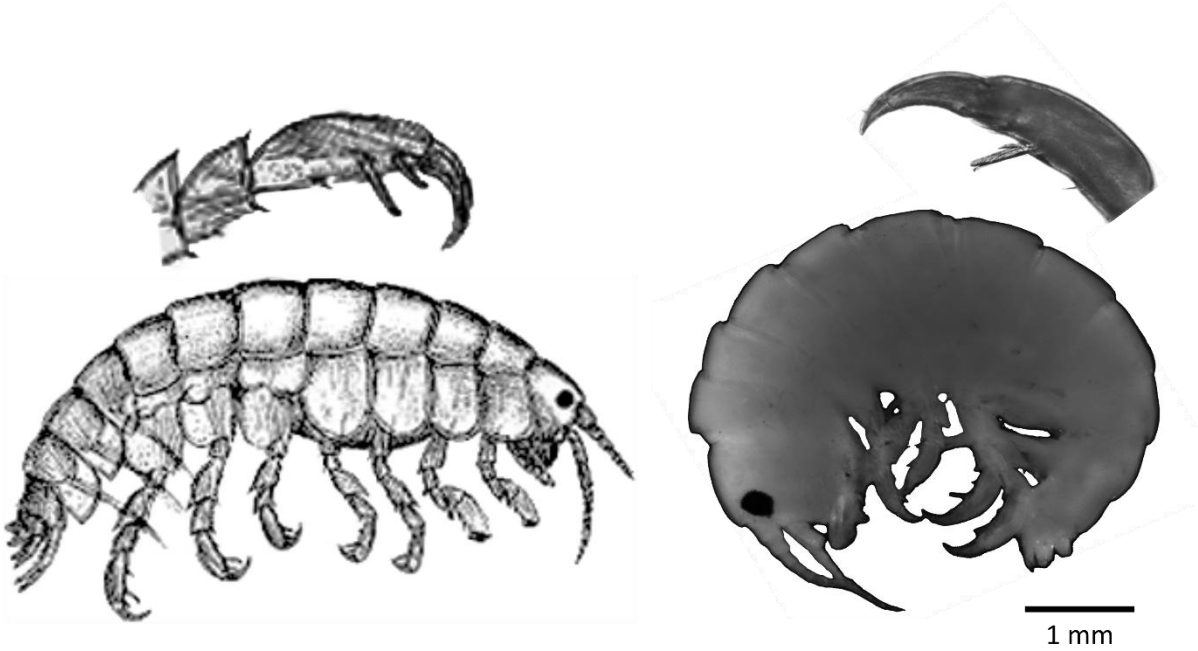


Fig.1. *Hyale pontica* type species of the genus *Hyale.* On the left, draw of a female of *H. lubbochiana* (synonymous of *H. pontica*) from Sars 1895 plate 11/2 with the distinctive strong spine on the propodous of the pereopod. On the right, photo of a female *H. pontica* and the same character.

The family Hyalidae is predominantly and commonly found among algae of the intertidal and shallow subtidal areas of tropical and subtropical zones (Serejo & Sittrop 2009), although a few species are reported at higher latitudes (McBane & Croker 1984). Like majority of the peracaridean, hyalids have a direct development, lacking the larval phase, which is one of the most relevant way of dispersal in marine

ecosystems. Several species of hyalids are related to the complexity of the fronds of the algae that they inhabit, preferring more filamentous algae (*Pterosiphonia, Gymnogongrus*) in the juvenile, choosing less ramificate until foliaceus algae in the adults (*Sargassum, Gelidium, Ulva*) (Dubiaski-Silva & Masunari 1998; McBane & Croker 1983; Moore 1976). They are mainly detritivores in marine and estuarine habitats and serve as food for many fishes and birds and, like many other amphipods, they play an important role in the food chain (Serejo 2004).

Presently, a complete and corrected checklist of this family, at least for the North Atlantic coasts, is missing. For instance, in the World Register of Marine Species database (WoRMS; Horton *et al.* 2015), several species of the genus *Hyale* Rathke, 1837, which now are assigned to different genera, are still included as accepted species with more than one name (eg. *H. stebbingi* and *Apohyale stebbingi, H. schmidti* and *Protohyale* (*Protohyale*) *schmidtii*).

Taking into account only the new nomenclature, 25 species of hyalids are reported in the North Atlantic Ocean and Mediterranean Sea, of which only 16 are reported from the Northeast Atlantic coasts and Mediterranean Sea, all of them of the sub-family Hyalinae (De Broyer *et al.* 2007; LeCroy 2007; Ruffo 2006).

b) Phylogeny

Understand the distribution of biodiversity around the World is one of the main goals of the biologists. A deeper knowledge of this vast field could give more prospects for its conservation at every level of diversity: genetic, specific and ecosystemic one. Species represent different amounts of evolutionary history, which is reflected in morphological and genetic diversity accumulated in different amounts of time and diverse conditions. The relationships among species are investigated through phylogenetic works.

The methodology of phylogenetic work rests on two approaches at present: numerical taxonomy (phenetics) and phylogenetic systematics (cladistics). Phenetics classifies species by using as many anatomical characteristics as possible and arranges them by similarity, regardless of any evolutionary relationships (Heywood & McNeill 1964). The idea behind cladistic is that members of a group share a common evolutionary history, and are more related to members of the same group than to other organisms (Hennig 1965). These groups are recognized by sharing unique features, which were not present in distant ancestors. These shared derived characteristics are called synapomorphies; conversely, characters that do not unite a clade because they are primitive are called plesiomorphies (Hennig 1965).

Species taxonomy has been fundamental in estimating biodiversity levels and designing conservation strategies. Although the amount of phylogenetically informative characters provided from morphology is limited, if organisms with reduced or conserved body plan, like amphipods, are taken into account, the number of informative characters would decrease (Hou *et al.* 2007). Morphology is also frequently subject to parallel and convergent evolution (Losos 2011), increasing the possibilities of misjudgement in the classification of the characters. For these reasons, the systematists are taking into account molecular information, in order to address the questions that morphology is not able to answer alone.

Furthermore, in conservation, the maximization of Phylogenetic Diversity should be a priority (Crozier 1997; Faith 1992; Witting & Loeschcke 1995). In order to avoid the extinction of a species in an old, monotypic or species-poor clade, which would therefore result in a greater loss of biodiversity than that incurred when a young species with many close relatives disappears, (Mace *et al.* 2003; May 1990),

conservation strategies relying on the “originality of a species” (Pavoine *et al.* 2005) have been developed (Isaac *et al.* 2007). In order to fulfil these goals, the most accurate possible phylogeny is required.

i) Phylogeny of Hyalidae

Despite being such important inhabitants of the rocky shore benthos, the cladistics phylogeny of hyalids has never been specifically studied. However, two independent studies proposed phylogenies that include hyalid species. Serejo (2004) presented a cladistics taxonomical revision of the talitroidean amphipods with a focus on this family. Recently, Hiwatari (Hiwatari *et al.* 2011) studied the 28S rRNA gene of some talitroidean amphipods including 6 species of hyalids and proposed a different phylogeny (**Fig. 2**).

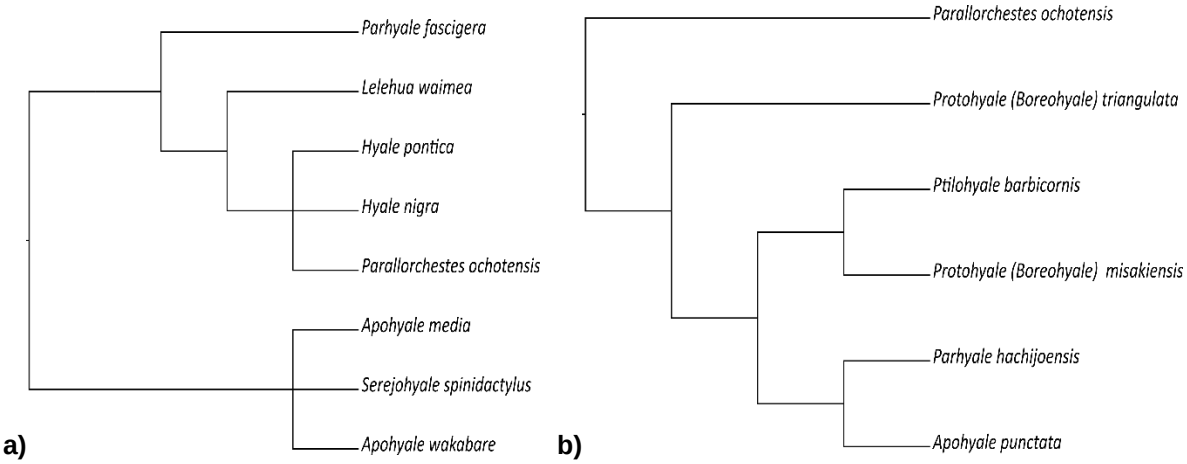


Fig. 2, Schematic cladograms of the family Hyalidae of Serejo (a) and Hiwatari (b). The trees were manually written in newick format and edited on Figtree v 1.4.2 (http://tree.bio.ed.ac.uk/software/figtree/).

Hiwatari underlined that the trait of the maxilla palp uni-articulate in *Parallorchestes ochotensis,* described from Serejo as apomorphic, should be considered as a plesiomorphic trait (Hiwatari *et al.* 2011; Serejo 2004). In fact, in the different trees the position of this species changes drastically from a shallow position in the same clade with *Hyale pontica* and *H. nigra*, in the analysis of Serejo, to a basal position in Hiwatari as the most primitive in hyalid (**Fig. 2 a-b**). It is also to highlight the absence

of the genera *Protohyale* and *Ptilohyale* in the analysis of Serejo, as for the genus *Lelehua* for Hiwatari.

Serejo (2004) herself underlined the "*necessity of more details and wide-ranging study, to have more confident results about the phylogenetic relationship in this family*".

c) The DNA barcode

In the present study we used the DNA barcode technique to investigate the phylogeny of the family Hyalidae. DNA barcodes are recognised, standardised molecular tags for species identification. They are based on the evidence that a single short region of the genome can provide information for species discrimination (Hebert *et al.* 2003a). The DNA barcode region established for most groups is the mitochondrial gene cytochrome c oxidase subunit I (COI-5P). The suitability of the COI gene to deliver species-diagnostic barcode in different vertebrate and invertebrate taxa is well documented (Costa *et al.* 2007; Ward *et al.* 2005). Moreover, DNA barcoding may lead to species discovery by flagging cryptic species, although more data than COI sequences are necessary for describing a new species (Radulovici *et al.* 2009).

d) *Apohyale prevostii* (Milne Edwars, 1830)

Fig.3. From the top clockwise: male of *Apohyale prevostii*; urosome; dactylus of the pereopod 5, with short and slender seta (1/5 or less length of dactylus) in red.

In the Hyalidae family, only *Apohyale prevostii* (**Fig. 3**) has been studied for the North Atlantic Ocean, it is also one of the few species of the hyalids already studied worldwide (Dubiaski-Silva & Masunari 1998; Hiwatari & Kajihara 1984; Tsoi & Chu 2005). *A. prevostii* is reported to be an amphi-Atlantic species (occurring in North Northwest and Northeast Atlantic coasts) (Bousfield 1973). It is known to inhabit cold water and to be frequent in intertidal and estuarine waters (McBane & Croker 1984). *A. prevostii* has a lifespan of approximately one year with two generations per year

(McBane & Croker 1984). The relationship between this hyalid and the fronds of algae present in the intertidal area is well studied from both Atlantic coasts, showing a segregation between adults and juveniles in the Scottish coasts (as described for the other species of hyalids), while along the Canadian coasts the releasing of juveniles coincided with the appearance of ephemeral algae (*Ulva intestinalis*, *Porphyra umbilicalis*) (den Hartog 1963; McBane & Croker 1983, 1984; Moore 1976). Regardless the wide distribution and the deep knowledge of the biology of this species, the phylogeography and the history of *A. prevostii* has never been inspected.

e) Area of the study

The Northeast Atlantic Ocean has a wide range of climatic conditions (from subtropical to subarctic), experienced complex geological and climatological changes during its history (e.g. the Pleistocene glaciation) and has a highly diverse biota. These conditions provide an interesting case study to understand the patterns of genetic diversity and their drivers (Xavier *et al.* 2010).

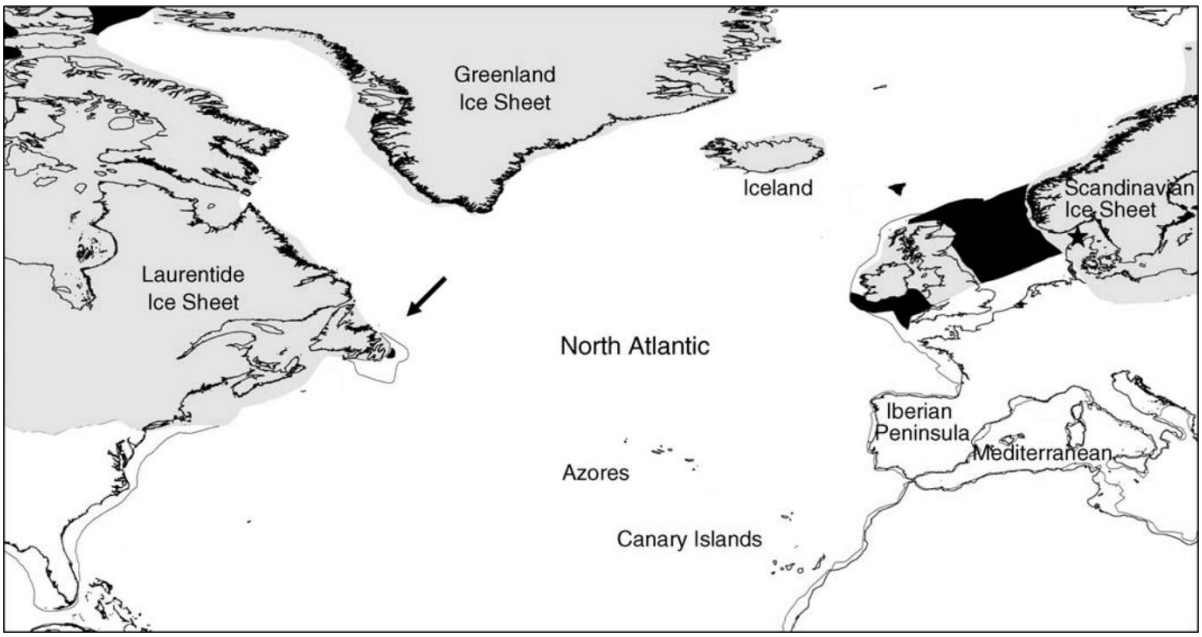


Fig. 4. Northern North Atlantic Ocean at the Last Glacial Maximum. Ice sheets are shown in gray, and solid fill indicates the areas of uncertain conditions (the tip of Newfoundland, arrowed; the Faroes; southwest of the British Isles; and most of the North Sea, with a deep trench indicated by a star) in contrasting maximum and minimum CLIMAP models (Bowen *et al.* 2002; Clark & Mix 2002; Dyke *et al.* 2002; Pflaumann *et al.* 2003). The paleocoastline (a finer line) and ice sheets were drawn in ArcView 9.4 (ESRI, Redlands, California, USA) from shapefiles at http://lgb.unige.ch/;ray/lgmveg/index.html, modified following Pflaumann et al. (2003). (from (Maggs *et al.* 2008))

It is important to underline that the Last Glacial Maximum (LGM) was around 20 kya and, after that, the global sea level arose by approximately 130 m, which means that the old intertidal area was totally submerged and only the animals that were able to move and recolonize the new part survived driving to a lower species and genetic diversity (Lambeck *et al.* 2002) (**Fig. 4)**. During glacial periods, marine species from temperate regions were forced to retract their ranges into warmer glacial refugial areas, ‘‘areas where some plants or animals survived an unfavorable period ... when plants or animals of the same kind were extinguished in surrounding areas’’ (Andersen and Borns 1994), whereas during inter-glacial eras, organisms were able to recolonize warming northern areas (Hewitt 1996). The southern locations of the Northeast Atlantic, such as Macaronesian archipelagos, the Iberian Peninsula, and the Atlantic coasts of North Africa, were likely to have been glacial refugia for many temperate species (Almada *et al.* 2001; Domingues *et al.* 2005).

Historical events such as glaciation are expected to leave genetic signatures on marine populations (Cunningham & Collins 1998; Kelly & Palumbi 2010) especially in species with low dispersal capacity such as the hyalids.

f) Aims of the study

The aims of this study are threefold: i) to explore the phylogenetic relationships in the family Hyalidae; ii) to investigate the genetic variability of the COI-5P gene within all the species along a vast area that includes the North Atlantic Ocean and the Mediterranean Sea; iii) to provide a first insight into the phylogeography of *Apohyale prevostii* along the Northwest and Northeast Atlantic coasts.

4) Material and Methods

a) Specimens collection

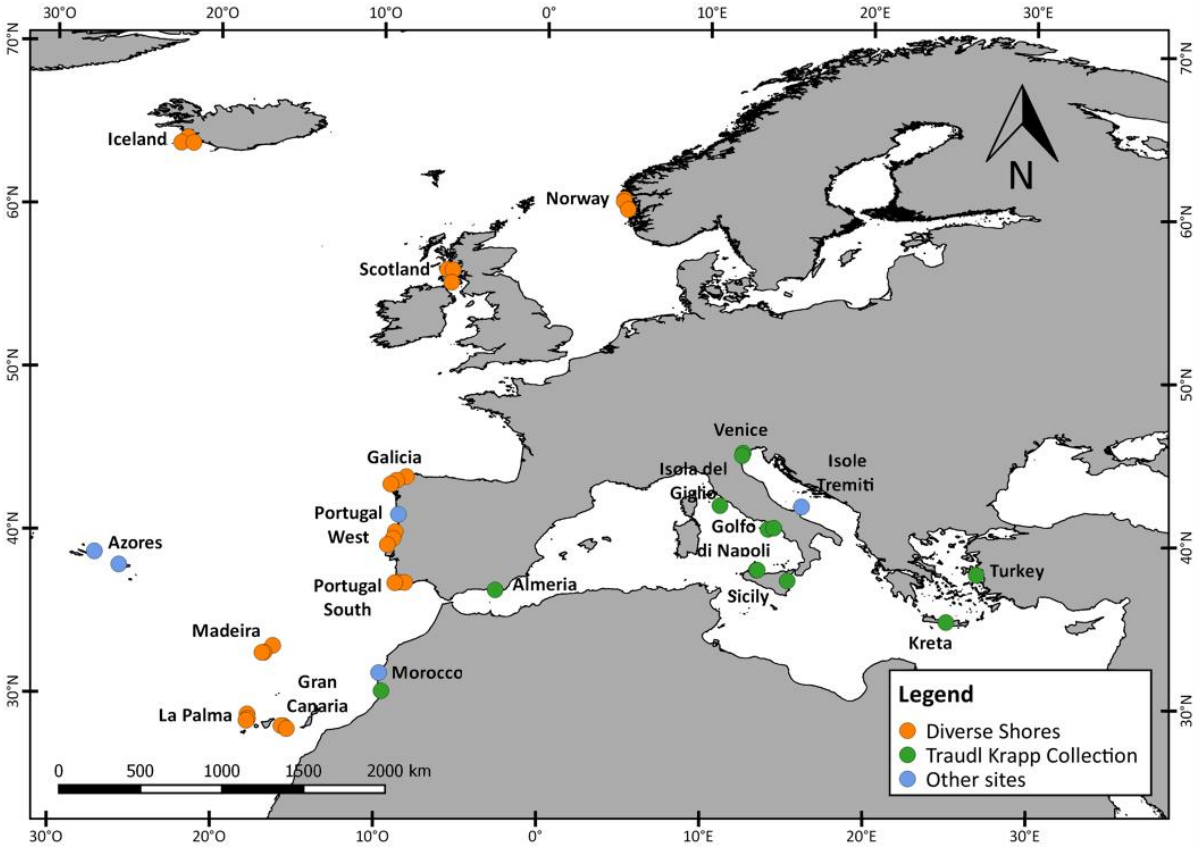


Fig.5. Sampling sites of the Diverse Shores project and of the Traudl Krapp-Schickel's private collection, and the other collecting sites.

During 2011, samples from the intertidal zone of Northeast Atlantic rocky shores (from Iceland to Norway and Scotland to Macaronesia archipelagos of Madeira and Canaries; **Fig.5**) were taken within the Diverse Shores project (Fundação para a Ciência e Tecnologia, PTDC/BIA-BIC/114526/2009). Each sample was preserved in 96% alcohol and later sorted in the laboratory. Between 2011 and 2015, additional samples were collected and preserved in 96% alcohol from mainland Portugal, Azores, Morocco and Italy (**Fig.5**). In order to have more information about the possible variability between Atlantic Ocean and Mediterranean Sea, specimens of hyalids were also retrieved from the private collection of Traudl Krapp-Schickel (**Tab.1**) (**Fig.5**) (**Annex 1**).

Tab.1. Species and number of individuals used for the DNA extraction from the private collection of Traudl Krapp-Schickel, with indication of site of collection.

Species	Site (number of specimens)	Total
Apohyale perieri	Agadir (3), Golfo di Napoli (4)	**7**
Apohyale stebbingi	Golfo di Napoli (1), Ischia (1), Palermo-Sferracavallo (3)	**5**
Hyale camptonix	Aguadulce (4), Almeria (2), Capo Molini (2), Golfo di Napoli (1), Kreta (2), Malamocco (2), Palermo-Sferracavallo (2)	**15**
Parhyale eburnea	Ischia (3)	**3**
Parhyale sp	Chioggia (1)	**1**
Protohyale (Protohyale) schmidtii	Almeria (1), Aquamarina (2), Bodrum (2), Capo Molini (2), Isola del Giglio (4), Golfo di Napoli (3), Kreta (2), West-Kreta (2), Palermo-Addaura (2), Roquetas (2), Sicily (2), Urla (2)	**26**

All specimens were morphologically identified to species level or to the lowest possible taxonomic rank, using an Olympus ZX16 stereomicroscope, and the descriptions were taken from DELTA database (Dallwitz 2010) based on the Hyalidae World genera species. A dichotomic key has been produced to perform a fast identification for the hyalid found in the Northeast Atlantic coasts (**Annex 2**). A picture of each species for both sexes was taken with an IDS UI-2280SE camera. The validity of species names were checked in the online WoRMS database (Horton *et al.* 2015).

b) DNA extraction and amplification

From each sample a piece of isolated trunk muscle tissue, few pereopods or central part of body (between 2° and 3° pereosomite if the specimen was particularly small), was isolated. DNA extraction was carried out using the E.Z.N.A Mollusc DNA Kit (Omega Biotek), following the manufacturer's instructions. The barcode region of the mtDNA gene cytochrome oxidase I (COI-5P) was amplified in a MyCycler™ Thermal Cycler (Bio-Rad) thermal cycler using a pre-made PCR master mix and five alternative primer pairs (**Tab. 2**), depending on amplification success. PCR thermal cycling conditions for each primer pair are also presented in **Tab. 2**. Each reaction

contained 2.5 µl 10× PCR buffer, 3 µl of 25 mM $MgCl_2$, 1 µl of 10 mM dNTP mixture, 0.2 µl of 5 U/ µl of DNA Taq polymerase plus 10 µM of each primer (1.25 µl for LoboF1/LoboR1; 0.5 µl for LCO1490/HCO2198; 0.55 µl for ArR5), 2-4 µl of DNA template and completed with sterile milli Q-grade water to make up a total volume of 25 µl.

Tab.2. Primers, number of successfully amplified specimens and cycling conditions.

Reference (number of specimens)	**Primer**	**Primer Direction (5' – 3')**	**PCR thermal cycling conditions**	**bp**
Folmer *et al.* 1994 (52)	LCO1490 HCO2198	(F) GGTCAACAAATCATAAAGATATTGG (R) TAAACTTCAGGGTGACCAAAAAATCA	1) 94°C (1 min); 2) 5 cycles: 94°C (30 s), 45°C (1 min 30 s), 72°C (1 min); 3) 35 cycles: 94°C (30 s), 51°C (1 min 30 s), 72°C (1 min); 4) 72°C (5 min).	658
Gibson *et al.* 2014 (44)	LoboF1 ArR5	(F) KBTCHACAAAYCAYAARGAYATHGG (R) GTRATIGCICCIGCIARIACIGG	1) 94°C (2 min); 2) 35 cycles: 94°C (30 s), 46°C (1 min), 72°C (1 min); 3) 72°C (5 min).	550
Lobo *et al.* 2013 (18)	LoboF1 LoboR1	(F) KBTCHACAAAYCAYAARGAYATHGG (R) TAAACYTCWGGRTGWCCRAARAAYCA	1) 94°C (1 min); 2) 5 cycles: 94°C (30 s), 45°C (1 min 30 s), 72°C (1 min); 3) 45 cycles: 94°C (30 s), 54°C (1 min 30 s), 72°C (1 min); 4) 72°C (5 min).	658

The PCR products were purified from primers and free nucleotides with the High PCR purification Kit Roche according to manufacturer instructions and then sequenced bidirectionally using the BigDye Terminator 3 kit, and run on an ABI 3730XL DNA analyser (all from Applied Biosystems™) by STAB Vida Lda (Portugal).

c) Data analysis

Each trace file was edited individually and manually. The resultant sequences were aligned using Clustal W implemented in MEGA v. 6.0 (Tamura et al. 2013) and inspected for eventual anomalies. Sequences of different length were obtained: 658bp amplified with primers LCO1490/HCO2198 and LoboF1/LoboR1; 550bp with LoboF1/ArR5. In order to avoid the problem of increasing artificially the differences, the smallest common fragment of 550bp was used for diversity and phylogenetic analyses. The alignment was translated into amino-acids to assess the presence of stop-coding sites.

i) Estimate of genetic diversity

To confirm the identity of the specimens and to check possible contaminations a BLAST search was performed with MEGA v. 6.0 (Tamura et al. 2013). Sequences of the same species, including those with known synonymous species names, were retrieved and included in the alignment.

A preliminary analysis revealed a very large divergence within the species *Apohyale perieri*, *A. media*, *A. stebbingi*, *Protohyale (Protohyale) schmidtii* and *Serejohyale spinidactylus*, suggesting the presence of putative species (Costa *et al.* 2009; Hebert *et al.* 2003a). To accommodate these observations with the genetic distance we defined separate molecular operational taxonomic units (MOTU; Floyd *et al.* 2002). This approach allowed the assignment of putative species to clusters that emerged from the molecular divergence data, and hence enabled testing species groupings. In this case, we attributed separate MOTUs to reciprocally monophyletic groups of specimens with more than 3% divergence (Hebert *et al.* 2003b; Costa *et al.* 2009; **Annex 2**).

Uncorrected *p*-distances were calculated in MEGA version v. 6.0 (Tamura et al. 2013) and used to estimate genetic distance between pairs of taxa within the same species, between different MOTUs, and between different species.

The minimum threshold between intra- and inter-specific distance was detected through the software R (www.r-project.org) with the libraries APE (Paradis *et al.* 2004) and SPIDER (function 'localMinima'; Brown *et al.* 2012). Measures of genetic

diversity, haplotype diversity (Hd), and nucleotide diversity (π) were estimated for each species, using DnaSP (Librado & Rozas 2009).

All the sequences, the metadata and the photographs of the specimens genetically examined were uploaded to BOLD and are publicly available in the project titled "Hyalidae Diverse Shores" (DSHYA).

ii) Phylogenetic analyses

Phylogenetic analyses of the COI were conducted with the maximum parsimony (MP), maximum likelihood (ML) and the Bayesian inference (BI) methods (Cabezas *et al.* 2013). One sequence per variant haplotype was extracted with the function 'haplotype', of the library PEGAS (Paradis 2010) using R, and was used for the analyses.

The MP analysis was performed with MEGA version 6.0 (Tamura et al. 2013), using 1 x 10^3 bootstraps analyses to estimate branch support. The function Best fitting ML model of MEGA version 6.0 (Tamura et al. 2013) was used to search for the most appropriate model of evolution for our dataset. The TN93 +I +G model was found to be the best-fit model for the data. The ML tree was reconstructed using the software package PhyML (Guindon *et al.* 2010). Branch support was inferred by 1 x 10^3 bootstraps. Bayesian phylogenetic analyses were performed with the software MrBayes on XSEDE (3.2.6) (Ronquist *et al.* 2012) (https://www.phylo.org/portal2/createTask!selectTool.action?selectedTool=MRBAYES_XSEDE) through CIPRES Science Gateway (Miller et al. 2010). Two independent runs were conducted with 2 x 10^8 generations each. Parameters were sampled every 1 x 10^3 generations. In the end a Majority rule consensus tree was reconstructed with a burn-in of 10%, discarding the first 2 x 10^6 generations for each run. Each tree was constructed using as outgroup *Microdeutopus chelifer* (Bate, 1962). A outgroup significantly distant from this family, infraorder Corophiida Leach, 1814 (sensu Lowry & Myers, 2013), was chosen to root the trees because, in preliminary analyses, the position of closer talitroidean amphipods was uncertain.

In order to investigate possible differences, nucleotides were translated to amino-acid sequences and used to build a neighbour-joining (NJ) tree in MEGA version 6.0

(Tamura et al. 2013) based on the Jones–Taylor–Thornton (JTT) matrix (Jones *et al.* 1992) and determining branch support with 1 x 10^3 bootstrap replicates (Costa et al., 2009).

iii) Phylogeography of *Apohyale prevostii* (Milne Edwards, 1830)

Due to the large amount of data retrieved from BOLD (Ratnasingham & Hebert, 2007) and GenBank (Benson *et al.* 2013) (**Annex 2**), the phylogeography of *Apohyale prevostii* was investigated by building a haplotype network with 53 sequences of 649bp using R (www.r-project.org) with the libraries APE (Paradis *et al.* 2004) and PEGAS (Paradis 2010). The haplotype distribution map was done with QGIS (Quantum GIS Development Team 2012).

The tables were done with Microsoft Excel 2013©. The maps were originated with QGIS (Quantum GIS Development Team 2012). The trees were edited with Figtree v 1.4.2 (http://tree.bio.ed.ac.uk/software/figtree/). All the pictures were edited using the open source vector graphics programme Inkscape (http://www.inkscape.org/) and Adobe© Photoshop© Cs4.

5) Results

The COI gene was amplified for a total of 114 individuals (**Annex 2**), belonging to seven species of four genera: *Apohyale perieri* (Lucas, 1849), *A. media* (Dana, 1853), *A. prevostii* (Milne Edwars, 1830), *A. stebbingi* (Chevreux, 1888), *Hyale pontica* Rathke, 1847, *Protohyale (Protohyale) schmidtii* (Heller, 1866), *Protohyale* sp. Bousfield & Hendricks, 2002, *Serejohyale spinidactylus* (Chevreux, 1926) (**Tab.3**).

Tab.3. Number of sequences for each source and total number of sequences for each species.

Species	n° sequences	Source	Total sequences
Apohyale prevostii	13	Diverse Shores	**53**
	40	BOLD + Genbank	
Apohyale stebbingi	24	Diverse Shores	**24**
Apohyale perieri	15	Diverse Shores	**18**
	3	Other	
Apohyale media	10	Diverse Shores	**10**
Hyale pontica	6	Diverse Shores	**7**
	1	Other	
Serejohyale spinidactylus	17	Diverse Shores	**19**
	2	Other	
Protohyale schmidtii	19	Diverse Shores	**20**
	1	Other	
Protohyale sp	3	Other	**3**

Unfortunately, the amplification of the COI fragment of the specimens from the collection of Traudl Krapp was not possible, due to the age of the samples and the consequently DNA degradation.

The only species from the Atlantic Ocean already included, with the COI sequence, in BOLD and GenBank was *A. prevostii* (**Tab. 3**). A total of 154 sequences (outgroup excluded) were aligned and used for the analyses.

a) Estimate of genetic diversity

From the overall analysis of the polymorphism 265 variable sites were found, of which 255 were parsimony informative, excluding outgroup species. A total of 79 haplotypes were observed, of which 10 singletons. For the mtDNA COI gene, overall, both haplotype (Hd = 0.939) and nucleotide (π = 0.177) diversities were high.

Tab.4. Number of sequences (N), number of haplotypes (H), haplotype diversity (Hd) and nucleotide diversity (**π**) for the Hyalidae species included in the present study. Yellow and red indicate the lowest and highest values, respectively.

Species	N	H	Hd	Π
A. perieri	18	8	0.699	0.03798
A. media	10	4	0.711	0.02105
A. prevostii	53	12	0.551	0.00135
A. stebbingi	24	18	0.971	0.11935
H. pontica	7	4	0.810	0.00277
P. schmidtii	20	14	0.932	0.06400
Protohyale sp.	3	3	1.000	0.00364
S. spinidactylus	19	16	0.982	0.11493

Haplotype diversity ranged from 0.511 in *Apohyale prevostii*, despite the large geographic distribution of the samples (Northwest and Northeast Atlantic coasts), to 1.000 in *Protohyale sp.* (**Table 4**). Nucleotide diversity ranged from 0.00135 in *A. prevostii* to 0.11935 in *A. stebbingi*.

The analysis of pairwise COI nucleotide divergences for all Hyalidae species in our dataset showed a very high divergence among individuals, both between species and within species (**Annex 2**). While the overall average distance was 17.8%, the within-species divergence averaged was 4.6% (range of 0–19.5) (**Tab. 5a**), while between-species average divergence was close to 21% (range of 16–26.7) (**Tab. 5b**). The

maximum within-species distance corresponded to distances between two MOTUs of *A. stebbingi* (19.5%) (**Annex 4**).

Tab.5a. Average, minimum and maximum distances within species. In red the highest value.

Species	Min	Average	Max
A. perieri	0.000	0.038±0.004	0.116
A. media	0.000	0.021±0.003	0.096
A. prevostii	0.000	0.001±0.000	0.007
A. stebbingi	0.000	0.119±0.008	0.195
H. pontica	0.000	0.003±0.001	0.005
P. schmidtii	0.000	0.064±0.006	0.129
Protohyale sp.	0.002	0.004±0.002	0.005
S. spinidactylus	0.000	0.115±0.009	0.176

Tab.5b. Average, minimum and maximum distances between species. In yellow and red the lowest and highest values respectively.

Species	Min	Average	Max
A. perieri	0.226	0.223±0.016	0.256
A. media	0.196	0.223±0.016	0.264
A. prevostii	0.160	0.197±0.016	0.242
A. stebbingi	0.167	0.218±0.015	0.267
H. pontica	0.176	0.203±0.016	0.231
P. schmidtii	0.182	0.213±0.015	0.264
Protohyale sp.	0.160	0.199±0.016	0.249
S. spinidactylus	0.189	0.224±0.015	0.267

The average distance between MOTU 17 and 18 was the lowest (3.5%) and closest to the threshold of 3% chosen according to literature to separate different species

(**Annex 4**). However in only 3 out of 8 species analysed (*A. prevostii*, *Hyale pontica*, *Protohyale sp*.) the within-species distance was lower than 3% (**Tab.5a**).

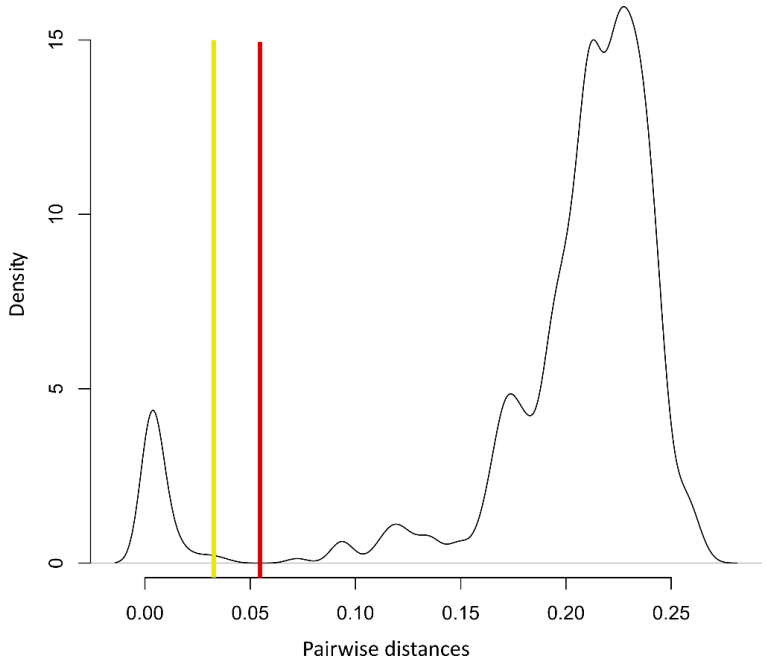


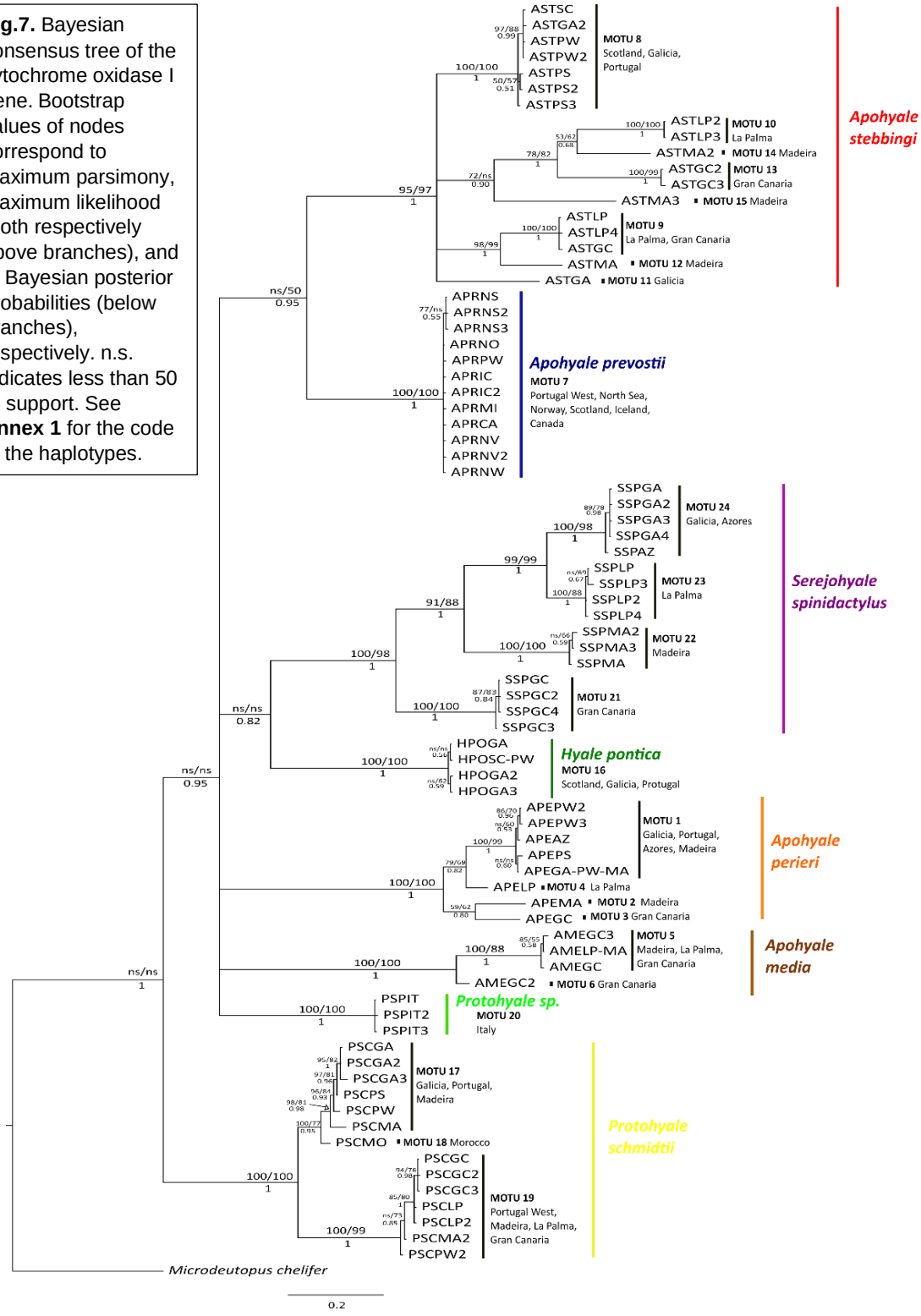
Fig.6. Density plot of the genetic distances. The red line is the minimum transition between intra- and interspecific distances found with the software R using the function 'localminima' of the library SPIDER; the yellow line is the threshold used for the delimitation of the MOTUs.

The minimum distance among species was detected between *A. prevostii* and *Protohyale sp.* (16%). The minimum threshold, as possible transition between intra- and inter-specific distances found with 'localminima' of SPIDER, was 5.4% (**Fig.6**).

a) Phylogenetic analyses

The topology of the 3 nucleotide trees was virtually identical for the shallow and highly supported nodes of the tree, allowing clear species discrimination by the observation of the clustering patterns. All pre-defined MOTUs clustered generally in well supported monophyletic groups, independently of the evolutionary model and tree-building method used (**Fig.7**). Deeper nodes of the trees showed an overall decrease in node support and more differences among topologies.

Fig.7. Bayesian consensus tree of the cytochrome oxidase I gene. Bootstrap values of nodes correspond to maximum parsimony, maximum likelihood (both respectively above branches), and to Bayesian posterior probabilities (below branches), respectively. n.s. indicates less than 50 % support. See **Annex 1** for the code of the haplotypes.



In general the only method that showed high support for the deeper nodes is the BI. For instance, in contrast to the ML and BI trees that grouped *Protohyale (Protohyale) schmidtii* in a different clade from the other species, the MP tree showed a basal node

with *Apohyale media* isolated, but such rearrangement was not associated with high bootstrap support.

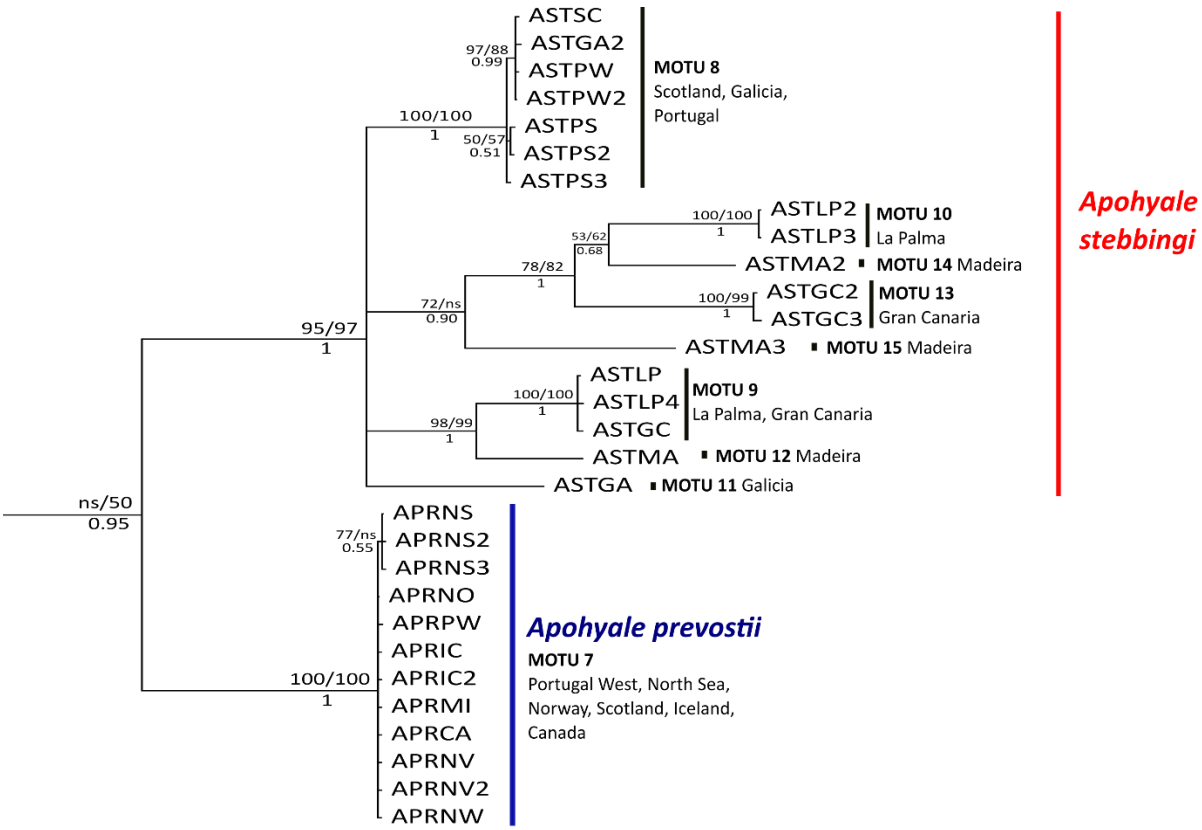


Fig. 8. Sub-tree detail (from **Fig.7**) of section corresponding to *Apohyale stebbingi* and *A. prevostii.*

The most complex clade was the one of *A. stebbingi* with 8 different MOTUs (**Fig. 8**). It was to be partitioned in three main groups of which one included 4 different MOTUs (10-13-14-15) all of them from the Macaronesia. The other two groups were specimens from Scotland (ASTSC), Galicia (ASTGA) and Portugal (ASTPW-ASTPS) (MOTU 8), and Gran Canaria (ASTGC), La Palma (ASTLP) and Madeira (ASTMA) (MOTU 9-12). This pattern reflected the general trend in the species that were present also in the Macaronesia, displaying one or more different MOTUs in the islands, compared to the European continental coasts and the northern islands that were usually clustered together.

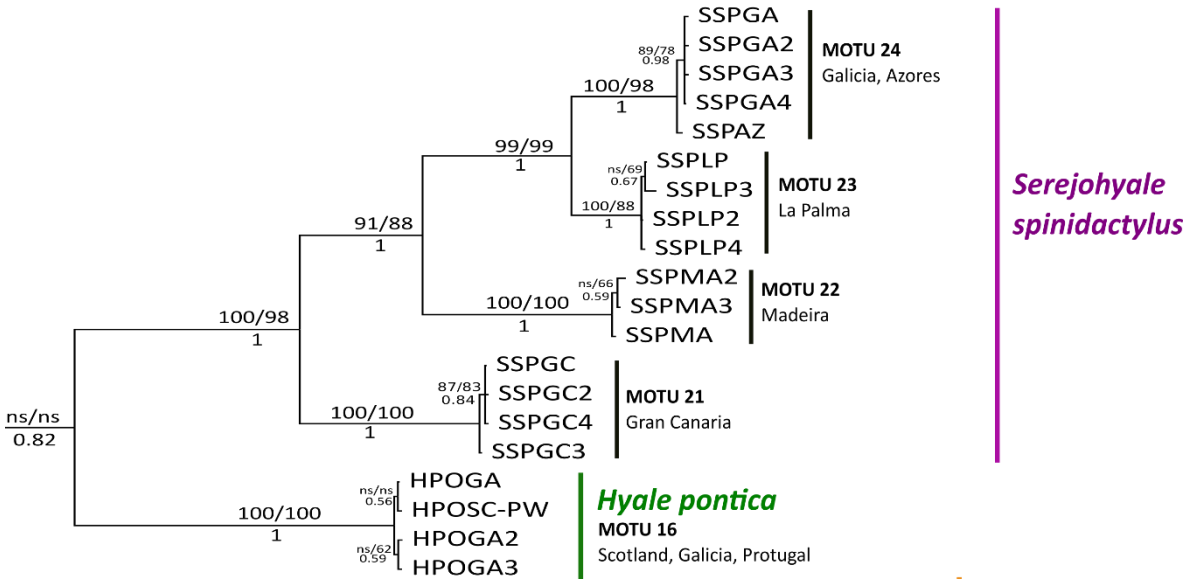


Fig. 9. Sub-tree detail (from **Fig. 7**) of section corresponding to *Serejohyale spinidactylus* and *Hyale pontica.*

Serejohyale spinidactylus displayed four different MOTUs (21-22-23-24), with high support, each belonging to a different island except for MOTU 24, which included Galicia (SSPGA) and Azores (SSPAZ) (**Fig. 9**).

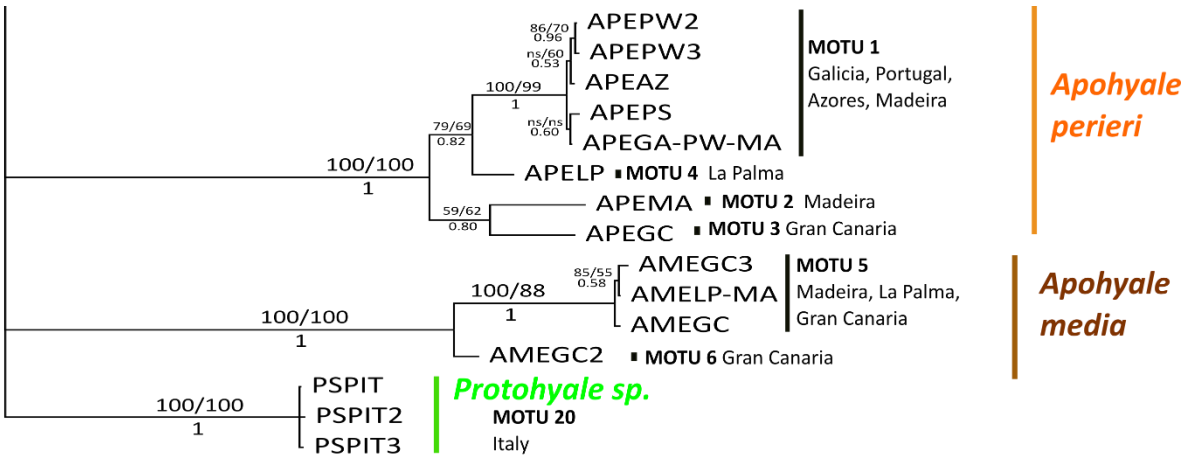


Fig. 10. Sub-tree detail (from **Fig. 7**) of section corresponding to *Apohyale perieri, A. media* and *Protohyale sp.*

A. perieri showed a subdivision in two main groups, one with Madeira (APEMA, MOTU 2) and Gran Canaria (APEGC, MOTU 3), and one with La Palma (APELP,

MOTU 4) and MOTU 1. MOTU 1 grouped together haplotypes from geographically distant sites: Portugal (APEPW, APEPS), Azores (APEAZ), Galicia and Madeira that shared a haplotype also with the Portugal West (APEGA-PW-MA) (**Fig.10**). Moreover, four specimens (A0.1-2-3-15 see **Annex 2**) were preliminarily identified as *A. crassipes*, but also, the pereopods 3-7 dactylus, with long and slender seta (1/3 or more length of dactylus), described in Delta database as typical of *A. crassipes*, was present in all the individuals of *A. perieri.* Also, *A. crassipes* was described by Ruffo (1982) as endemic of the Mediterranean Sea, although it was reported in the literature along Atlantic coasts (Horton *et al.* 2015). There was the possibility that in the Atlantic this species had been misidentified and confused for *A. perieri.* In addition, all the specimens of *A. crassipes* shared the same two haplotypes of *A. perieri* (APEPS and APEGA-PW-MA). For these reasons, in this work, only the species name *A. perieri* is used until further analyses, in order to understand if the two morpho-species cannot be detected with COI barcode, or if they are actually the same species.

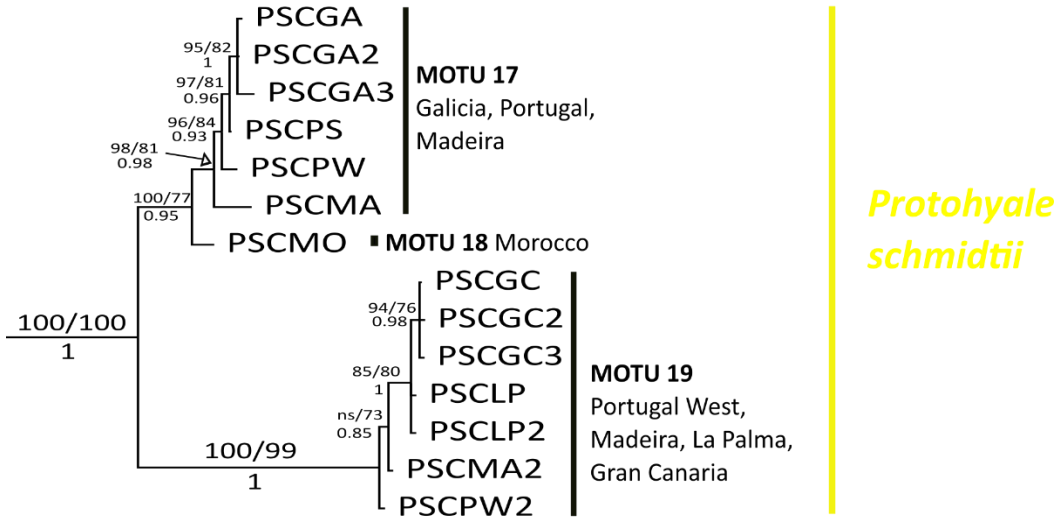


Fig. 11. Sub-tree detail (from Fig. 7) of section corresponding to *Protohyale (Protohyale) schmidtii.*

P. (*Protohyale*) *schmidtii* displayed two big clusters, one mainly from the continental coasts with haplotypes from Morocco (PSCMO, MOTU 18), Galicia (PSCGA), Portugal (PSCPW-PSCPS) and Madeira (PSCMA), all of the same MOTU 17; the

second group, as basal node, showed the divergence between a haplotype from the Portugal West (PSCPW2) and the other haplotypes entirely from the Macaronesia, subdivided into sub-clusters of each island (**Fig.11**).

A. media showed only one haplotype that diverged significantly from the main cluster (**Fig. 10**). Besides, two specimens were included in this group that morphologically were identified as *S. spinidactylus*, but genetically were totally equal to *A. media.*

The other species (*A. prevostii, Hyale pontica* and *Protohyale* sp.), displayed only one lineage (**Fig. 8-9-10**), according with the MOTUs delineated, including haplotypes from all sites.

Exception for the node between *A. prevostii* and *A. stebbingi*, which was displayed again with 68% of bootstrap support, the amino-acid NJ tree showed the same clusters of species with high bootstrap support, and no statistical significance (lower than 50%) for deeper nodes (**Fig.12**) as for the nucleotide trees.

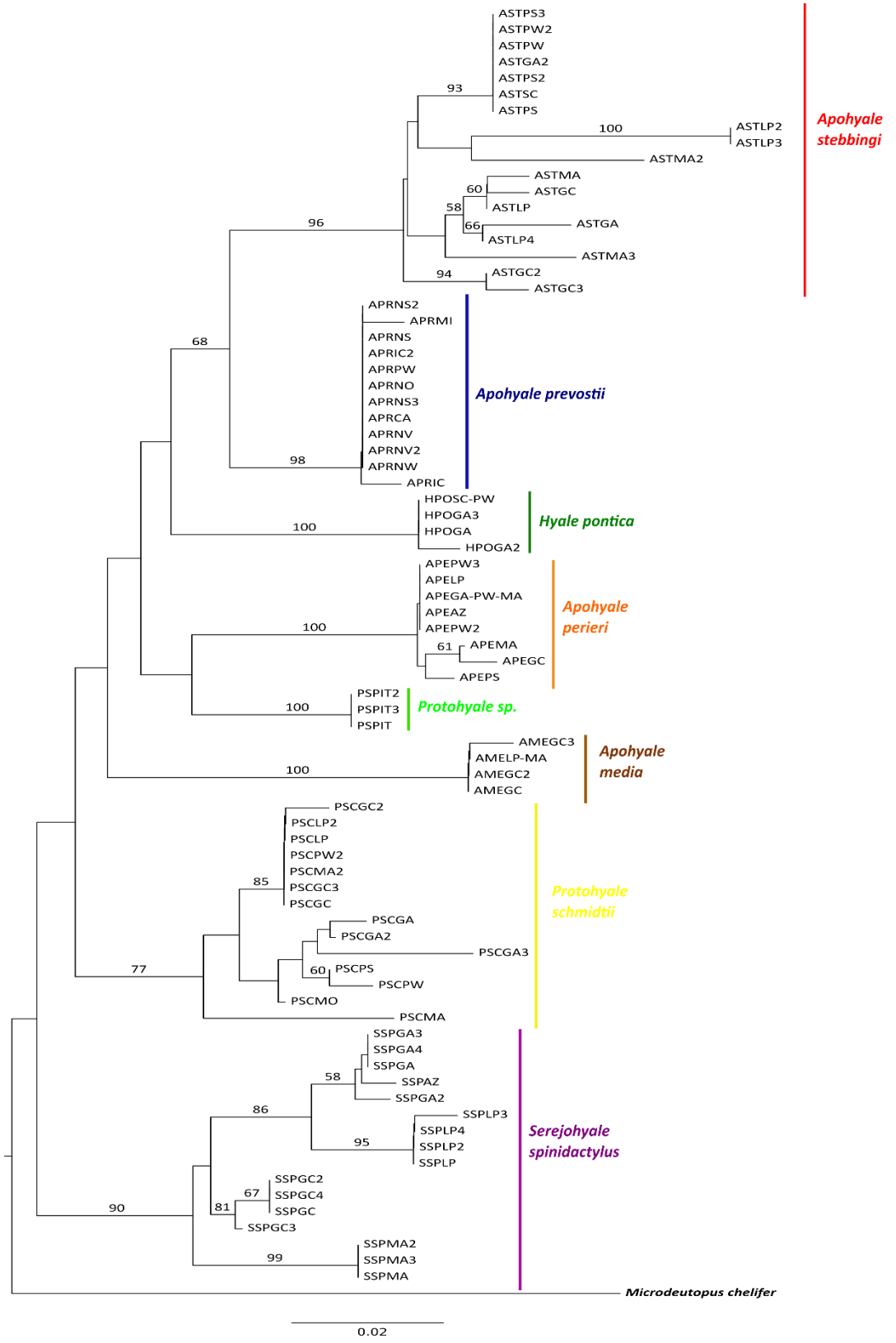


Fig.12. Neighbour-joining tree based on amino-acid sequences of the cytochrome oxidase I gene.

The cluster of *A. stebbingi* showed some differences in the arrangement of the sub-clusters (**Fig. 9**). For instance, the complex group of four MOTUs, found in the nucleotide trees (**Fig. 4**), was not statistically significant (< 50%). In contrast, the haplotype from Galicia (ASTGA) resulted clustered within the MOTUs 9 and 12. The cluster of *A. perieri* showed a change, removing the distance between the MOTUs 4 and 1.

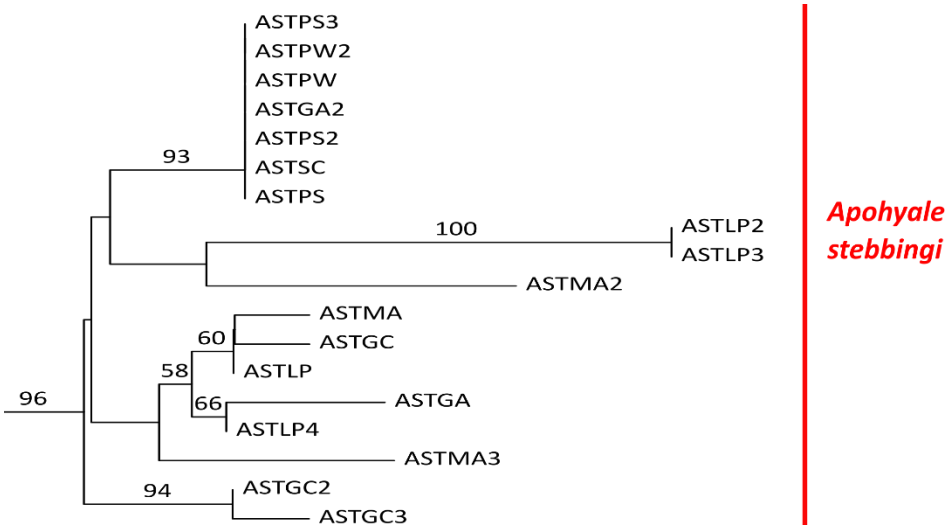


Fig.13. Sub-tree (from Fig.12) of section corresponding to *Apohyale stebbingi*

In general, the topology of the nucleotide trees was preserved as the majority of distances is proportionally conserved. All the different rearrangements were not supported by high bootstrap values.

b) Phylogeography of *Apohyale prevostii* (Milne Edwars, 1830)

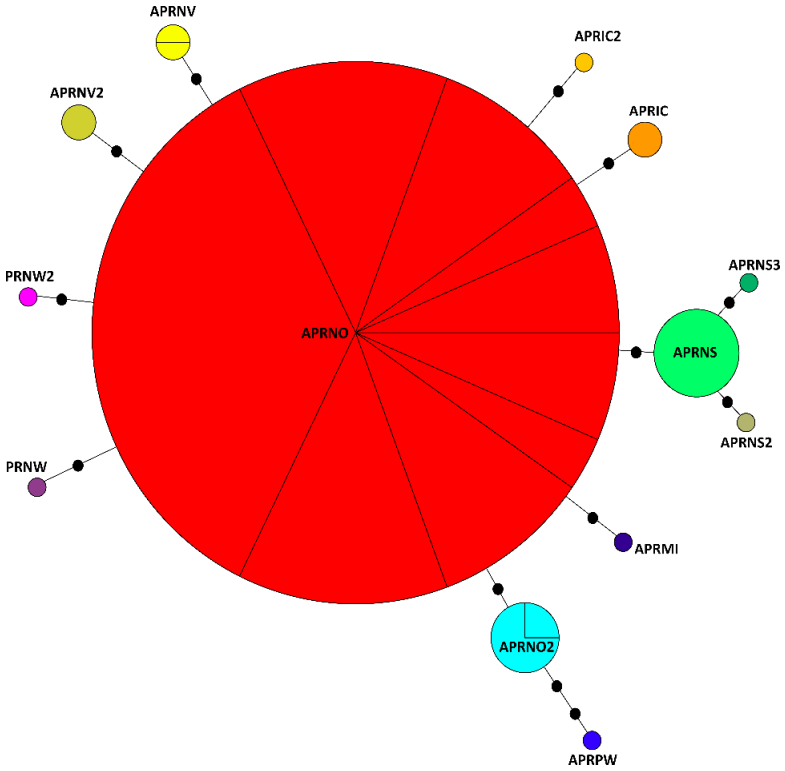


Fig. 14a. Haplotype network of *Apohyale prevostii.* Each black spot represents a mutation.

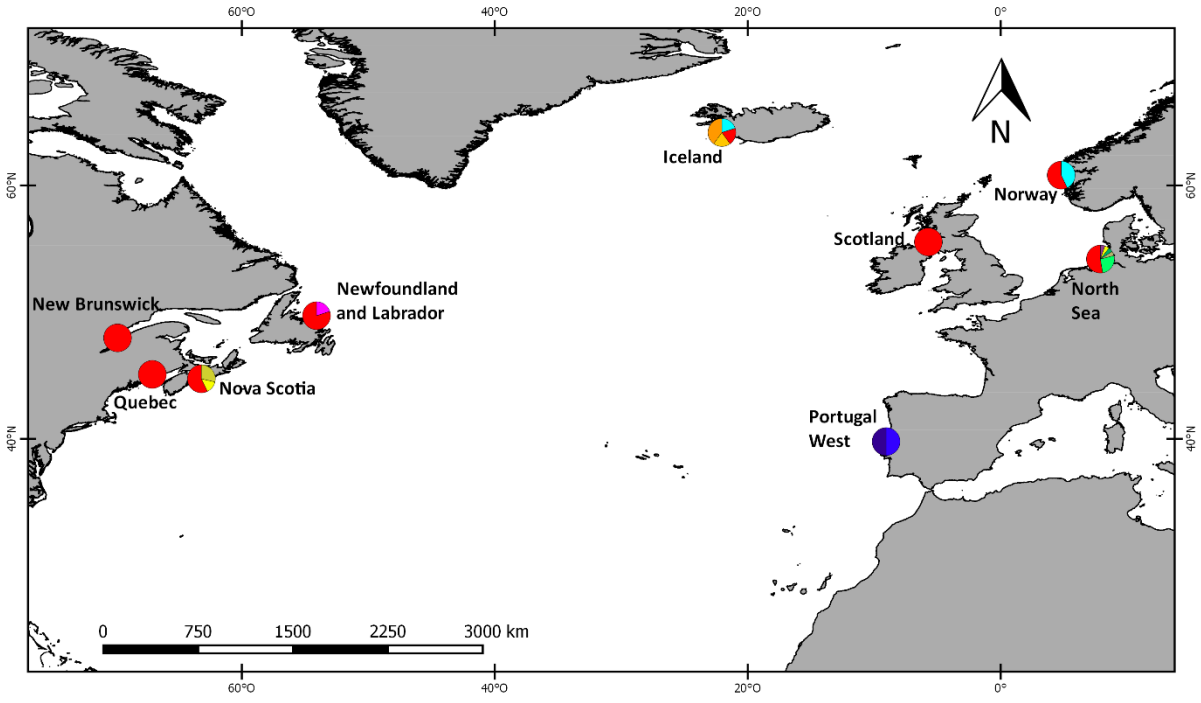


Fig.14b. Distribution map of the different haplotypes.

The haplotype network of *Apohyale prevostii* showed that one of 13 haplotypes was shared among all the coasts and the 53 sequences considered, exception done for Portugal West (**Fig.14a-b**). Only three haplotypes had more than one mutation difference from the most common haplotype: one in Portugal (APRPW/APRNO = 3 mutations) and two in the North Sea (APRPW/APRNO = 3 mutations) (APRNS2-3/APRNO = 2 mutations) (**Fig.14a**).

6) Discussion

A first evidence in our study was the low ratio of success (46.53%, 114/245 individuals) in the amplification and sequentiation of the COI, underlying the difficulty of working with old samples of this family at molecular level. The main problem was the age of the samples. It is known that after 10 years in 90% alcohol, retrieving a full sequence of 650 bp is difficult, due to DNA degradation (Hajibabaei & McKenna 2012). Most of the samples from the Traudl Krapp-Schickel were almost five years old, and the oldest specimen was collected in 1973. For this reason, different combinations of primers were adopted, amplifying smaller fragments of the Barcode region, ranging from 658 to 550 bp, increasing the success from 28.57% (70/245) to the actual ratio. While it is known that fragments of 100 bp are sufficiently informative to discriminate among species (Meusnier *et al.* 2008), to perform phylogenetic analyses we chose a longer sequence in order to have more informative sites. Building bigger fragments by the combination of smaller ones, will be the only possibility to handle these old samples, and will be the approach for an advanced study.

A second evidence is that this study includes only 6 (plus *Protohyale* sp.) of the 16 hyalids present in the Atlantic Ocean and Mediterranean Sea, belonging to four of the six genera. Moreover two sub-genera of *Protohyale* Bousfield & Hendricks, 2002, *P. (Boreohyale)* Bousfield & Hendricks, 2002 and *P. (Diplohyale)* Bousfield & Hendricks, 2002, are missing (Ruffo 2006; LeCroy 2007; Horton *et al.* 2015).

It is also important to underline that this study is the first record of *A. media* (Dana, 1853) from the Northeast Atlantic. This species is cosmopolitan in tropical and subtropical waters (Serejo 1999). It occurs along the coasts the South Atlantic Ocean, particularly East coasts (Serejo 1999; De Broyer *et al.* 2007), and in the Gulf of Mexico (Nelson 1995; LeCroy 2007). It is also documented along the coasts of Pacific and Indian Oceans (Serejo 1999; Martín & Díaz 2003), but many records of this species in the literature, especially Pacific records, may actually refer to other species (LeCroy 2007).

a) Phylogeography of *Apohyale prevostii* (Milne Edwars, 1830)

The occurrence of a common haplotype in every site across most of the North Atlantic is a clear suggestion of the recent spreading of this species. Additionally, the presence of haplotypes only one mutation away from the shared one suggests that they are all recent haplotypes (Maggs *et al.* 2008). On the opposite, in Europe there are three haplotypes with more than one base distance (APRPW, APRNS2-3) which are private of the respectively sites.

Slow mutation rates of the organellar markers mean that most polymorphisms involving more than one base difference certainly pre-date the LGM, about 20 kya (Anderson *et al.* 2006). Moreover both the sites (Portugal and North Sea) are known to be refugia during the LGM. Additionally, Iceland looks particularly rich in private haplotypes, 2 out of 4, suggesting that it may also have acted as a refugium, or that it has been recolonized from the close Foroe Islands, as suggested for *Idotea balthica* (Pallas, 1772) and *Carcinus maenas* (Linnaues, 1758) (Wares 2001a; Maggs *et al.* 2008). This hypothesis is supported by the fact that both Iceland and the Faroe were below single ice sheets, and in Faroe probably with a small ice cap. The presence of a constant layer of ice (CLIMAP Project Members 1981) could have maintained the temperature of surface water constant, avoiding physiologically stressful changes. The absence of the haplotype APRNO in Portugal could be due to the presence of only two sequences from this coast in our database. Perhaps that, with more specimens, this southern site would give important information regarding its role as a glacial refugium.

Like other peracarideans, *A. prevostii* could have colonized the coasts of the West Atlantic Ocean after the end of LGM (Wares 2001b). Obviously, a study on the population dynamic of this species is essential to better understand the history of this species.

b) Estimate of genetic diversity

Remarkably, the species present in the Macaronesia region displayed a higher nucleotide diversity than *Apohyale prevostii*, of which the sequences were from both the East and the West Atlantic coasts.

The minimum threshold between intra- and inter-specific distances, found with SPIDER, was higher than the 3% suggested by previous works (Hebert *et al.* 2003b; Costa *et al.* 2009). Re-drawing the MOTUs with the threshold of 5.4 found with "localminima", only MOTU 18, including the singleton of *Protohyale* (*Protohyale*) *schmidtii* from Morocco, joined the one from mainland (MOTU 17). Strict phylogenetic species criteria have been criticized for over-splitting taxa to avoid paraphyletic species (Harrison 1998; Avise 2004; Witt *et al.* 2006). The other MOTUs remained unchanged, strongly suggesting the possibility of 23 cryptic species. The 5.4% threshold distance found could be due to the large geographic scale of our study. Notwithstanding, this reasoning seems to be contradicted by the reduced distances between the northern haplotypes from Scotland and the ones from Portugal and Galicia in *Apohyale stebbingi* and *Hyale pontica*, or between Canada and Europe in *A. prevostii*. Although, the lower diversification in the northern regions could be accounted for the recent recolonization after the LMG (Maggs *et al.* 2008).

Even if in amphipods cryptic speciation has been widely reported (Witt *et al.* 2006; Costa *et al.* 2009; Radulovici *et al.* 2009), the high within-species genetic distance exceeds the expectations for the taxa existing in the Macaronesia. For instance, the situation of *Serejohyale spinidactylus* is emblematic, reporting four well-supported MOTUs with a considerably high average divergence of 15.2% among them (9.4-17.1%). Is possible that this family used the Macaronesia region as refugium during the glaciations and recolonized the mainland afterwards. This hypothesis is strengthened by the reduced haplotype distances in the MOTUs of mainland in both nucleotide and amino-acid trees. Moreover, the presence of the haplotypes from Azores, in *S. spinidactylus* and *A. perieri,* within the same MOTUs of the haplotypes from mainland, suggests a colonization from these islands to the mainland. This hypothesis is in line with the common opinion that postulates the Azores as glacial refugium (Chevolot *et al.* 2006; Xavier *et al.* 2010). The only species that displayed a

different pattern in the amino-acid tree was *P. (Protohyale) schmidtii*, which had an inverted arrangement suggesting a more recent colonization of the Macaronesia.

The importance of islands for species evolution is worldwide accepted (Selmi & Boulinier 2001; Villacorta *et al.* 2008; Losos & Ricklefs 2009). It is well documented that vicariance events, caused either by the emergence of land barriers or by the isolation within glacial refuges, have prompted allopatric divergence and speciation in many marine organisms (Quesada *et al.* 1995; Wares & Cunningham 2001; Patarnello *et al.* 2007; Xavier 2011). Nonetheless, the colonization of oceanic islands is strictly dependent of the species dispersal capability. In amphipods, which lack a larval phase, dispersal mechanisms are limited to rafting objects and anthropic mediated transport (Thiel & Gutow 2005; Cowie & Holland 2006; Wildish & Pavesi 2012; Cabezas *et al.* 2013). The biology of the hyalids as inhabitants of algae with a high rafting dispersal potential, such as species of the genus *Sargassum* (Dubiaski-Silva & Masunari 1998), increase the possibility of these events (Deysher & Norton 1981; Poore 2005).

High mtDNA difference with few shared haplotypes (only one in *A. perieri* and one in *A. media*) indicates a low genetic exchange and suggests the isolation of the different populations (MOTU). As for the possibility of well-described allopatric speciation in remote islands, in the clade of the morpho-species *A. stebbingi*, there are more MOTUs belonging to same site. For instance, the divergence between MOTU 14 and MOTU 15 (16.9%), both from Madeira, shows the appearance of possible sympatric cryptic species.

Sympatric speciation is a phenomenon that is not completely understood. For Mayr (1947), in sympatric speciation, populations first become reproductively isolated and then diverge (Mayr 1947). This is usually related to a shift in ecological preference of the divergent species, as for the soil predilection of the palms of Lord Howe Island (Savolainen *et al.* 2006), or the plant host for phytophagous insects (Berlocher & Feder 2002). The possibility of a shift in the ecological habits of some hyalids, such as the preference for different algae during their life cycle, is a strong possibility.

However, allopatric speciation in a vicariance scenario is also a possibility, if it would have occurred concurrently with the evolution of the Macaronesia. In fact, it is acknowledged that the present-day Macaronesia is only the residual of a bigger

complex that now is submerged for a major part (Fernández-Palacios *et al.* 2011). The emerged seamounts during the Pleistocene, now eroded and submerged, could have worked as stepping stones for the colonization of recent islands from Europe and Africa (Carine *et al.* 2004). Similarly, they could have allowed the dispersion between the different archipelagos of the Macaronesia, especially to the Azores from the Josephine or Great Meteor archipelagos (Van Den Broeck *et al.* 2008; Fernández-Palacios *et al.* 2011). Yet, because of the low success in the amplification of the COI, our data are still insufficient to test this hypothesis.

c) Phylogeny

Some significant topics emerged comparing the tree generated from our analysis and the two trees present in literature (Serejo 2004; Hiwatari *et al.* 2011). As expected, being both at molecular level, the phylogeny based on the 28S proposed by Hiwatari shows some congruencies with ours.

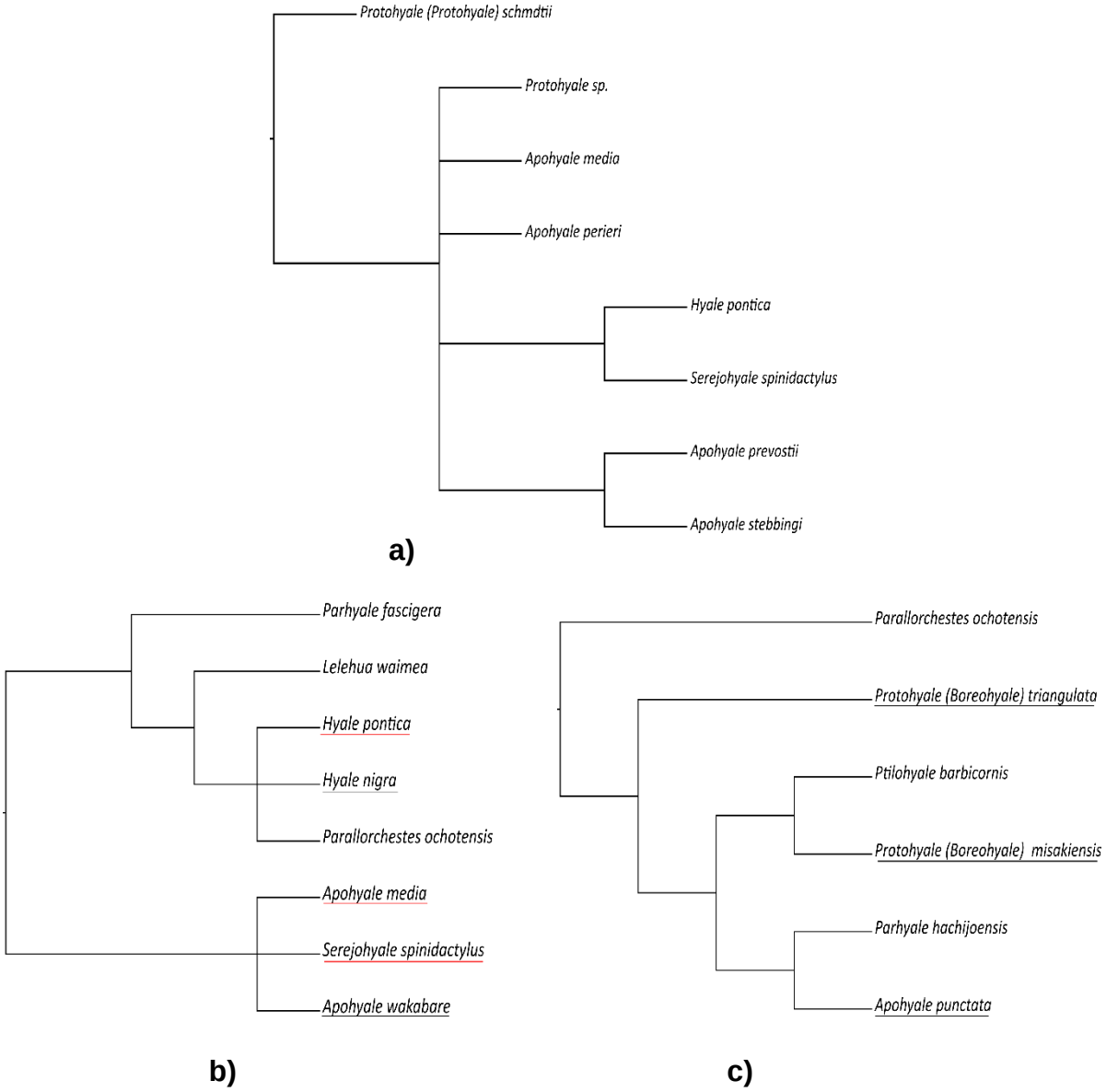


Fig.15. Schematic cladograms of the family Hyalidae: **a)** phylogeny proposed by this work; **b)** phylogeny proposed by Serejo (2004); **c)** phylogeny proposed by Hiwatari (2011). Underlined the taxa used also in this study: in red the species, in black the genera. The trees were manually written in newick format and edited on Figtree v 1.4.2 (http://tree.bio.ed.ac.uk/software/figtree/).

For instance, the basal node between *Protohyale* (*Protohyale*) *schmidtii* and the other species, including *Protohyale* sp., displayed in our study, finds a partial congruence with the tree of Hiwatari, where *P.* (*Boreohyale*) *misakiensis* is in a lineage closer to the genus *Apohyale* than *P.* (*Boreohyale*) *triangulata*, which diverges in a basal node. In contrast, our results do not appear to agree with the phylogeny proposed by Serejo. For instance, *Serejohyale spinidactylus* and *Hyale pontica*, which in our study constitute a possible cluster, are represented divergent in the systematic analysis of Serejo, which organizes together the genera *Serejohyale* and *Apohyale*. Also, one of the characters used to group the family Hyalidae is the uniarticulate maxilla 1 palp, but in *A. prevostii* and *A. stebbingi* the maxilla 1 palp is biarticulate, invalidating it as synapomorphic trait.

The appearance of this apomorphic character, could confirms the monophily of the clade of these two species. Moreover, the lineage formed by *A. prevostii* and *A. stebbingi* is displayed in all the trees with different levels of support. On the other hand, *A. perieri* and *A. media* do not seem to be related either with this clade or to each other. Briefly, the genus *Apohyale* seems to be paraphyletic, and could possibly include more than one genus.

Our analyses showed a large amount of polytomies and unsolved nodes that require more species to be solved. Actually, the largest limitation of this study is the insufficiency of species, only eight out of sixteen for the Northeast Atlantic coasts, but regarding more than 110 in the Hyalidae. As well as the use of only one gene that undoubtedly have restriction in phylogenetic analysis (Maddison 1997). Also a deeper analysis of the morphological characters may could help to solve the phylogeny of this family, as for *A. prevostii* and *A. stebbingi.*

7) Conclusions

This work is the first in our knowledge that investigates the phylogenetic relationship of the Hyalidae family. Even if the COI might not be the best gene for phylogenetic analyses and the hypotheses generated need more loci for confirmation (Collins & Cruickshank 2012), this insight into the phylogeny of this family, shows occurrence of cryptic species not morphologically detectable, highlighting the amount of work needed to better understand the phylogenetic relationships within the group. Moreover, the Macaronesia region had a fundamental effect on the evolution and differentiation of this family, underlining the importance in the conservation of these islands and the genetic diversity that they preserve.

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9) Annex

Annex 1. Table with coordinates of each site.

Samples	Coast	Site	Latitude	Longitude
Diverse Shores	Iceland	Reykjavik	64°09'46.12"N	22°00'32.75"W
		Grindavik	63°49'33.80"N	22°24'41.14"W
		Strandarkirkja	63°49'22.46"N	21°39'35.04"W
	Norway	Baloy	60°48'16.40"N	4°48'20.66"E
		Hellesoy	60°39'45.53"N	4°47'13.79"E
		Viksoy	60°10'28.22"N	5°02'30.38"E
	Scotland	Carsaig	56°19'8.67"N	5°57'54.32"W
		Easdale	56°17'17.42"N	5°38'5.34"W
		Bellochantuy	55°31'31.72"N	5°42'40.46"W
	Galicia	Pedreira	43°33'22.21"N	8°16'29.79"W
		Barizo	43°19'19.61"N	8°52'22.02"W
		Muxía	43°05'34.19"N	9°13'24.35"W
	Portugal West	Buarcos	40°10'33.51"N	8°54'2.06"W
		S. Pedro Moel	39°45'28.85"N	9°01'59.39"W
		Peniche	39°22'20.76"N	9°22'39.18"W
	Portugal South	Dona Ana	37°05'13.09"N	8°40'03.78"W
		Arrifes	37°04'36.32"N	8°16'34.03"W
		Ingrina	37°02'42.93"N	8°52'40.97"W
	Madeira	Porto dos Frades	33°04'21.27"N	16°17'44.40"W
		Reis Magos	32°38'46.00"N	16°49'27.00"W
		Ponta da Cruz	32°37'59.24"N	16°56'37.11"W
	La Palma	La Fajana	28°50'32.19"N	17°47'39.57"W
		La Salemera	28°34'40.75"N	17°45'38.00"W
		El Faro	28°27'27.16"N	17°51'01.22"W
	Gran Canaria	Bañaderos	28°08'58.77"N	15°32'24.65"W
		Caleta	28°09'47.47"N	15°41'57.37"W
		Playa Melenara	27°59'20.01"N	15°22'13.74"W
Traudl Krapp collection	Venice	Malamocco	45°22'11"N	12°20'15"E
	Sicily	Palermo-Sferracavallo	38°11'56"N	13°16'35"E
		Palermo-Addaura	38°11'27"N	13°20'58"E
		Capo Mulini	37°34'22"N	15°10'16"E
	Almeria	Roquetas	36°43'06"N	2°43'26"W

		Aguadulce	36°48'46"N	2°33'58"W
	Golfo di Napoli	Spiaggia degli inglesi	40°42'52"N	13°56'34"E
		Golfo di Napoli	40°47'49"N	14°17'53"E
		Ischia	40°43'10"N	13°53'35"E
	Turkey	Urla	38°07'18"N	26°45'09"E
		Bodrum	37° 00'19"N	27°23'34"E
	Kreta	Kreta	35°11'30"N	24°56'39"E
		West Kreta	35°24'16"N	23°33'2"E
	Venice	Chioggia	45°12'01"N	12°16'52"E
	Morocco	Agadir	30°24'17"N	9°36'05"W
	Isola del Giglio	Isola del Giglio	38°34'57"N	6°07'14"E
Other sites	Morocco	Akhfenir	28°05'51.09"N	2°03'02.52"W
	Isole Tremiti	Isole Tremiti	42°07'32.29"N	15°31'01.56"E
	Azores	Ponta da Ferreirinha	37°51'39.78"N	25°51'17.27"W
		Mosteiros	37°54'00.55"N	5°49'04.35"W
	Portugal West	Agudela	41°14'26.61"N	8°43'39.17"W

Annex 2. Table with the sample.id for BOLD accession, respective haplotype code, coast and site of the collection and MOTU of membership.

Species	Sample.id	Haplotype	Coast	Site or Source	MOTUs
Microdeutopus chelifer	OUT.MC	*Microdeutopus _chelifer*			
Apohyale perieri	A0.1	APEPS	Portugal South	Arrifes	MOTU 1
	A0.15	APEGA-PW-MA	Galicia	Pedreira	
	A0.2	APEGA-PW-MA	Galicia	Pedreira	
	A0.3	APEGA-PW-MA	Madeira	Ponta da Cruz	
	A1.1	APEGA-PW-MA	Galicia	Barizo	
	A1.10	APEGA-PW-MA	Galicia	Muxia	
	A1.12	APEAZ	Azores	Ponta da Ferreirinha	
	A1.13	APEGA-PW-MA	Galicia	Pedreira	
	A1.14	APEPW2	Portugal West	S. Pedro Moel	
	A1.2	APEGA-PW-MA	Galicia	Barizo	
	A1.3	APEGA-PW-MA	Galicia	Barizo	
	A1.4	APEPW3	Portugal West	Buarcos	
	A1.8	APEGA-PW-MA	Portugal West	Figueira da Foz	
	A1.9	APEGA-PW-MA	Portugal West	Agudela	
	A1.11	APEMA	Madeira	Ponta da Cruz	MOTU 2
	A1.5	APEGC	Gran Canaria	Caleta	MOTU 3

	A1.6	APELP	La Palma	El Faro	MOTU 4
	A1.7	APELP	La Palma	La Fajana	
Apohyale media	A2.1	AMEGC	Gran Canaria	Banhaderos	MOTU 5
	A2.2	AMEGC	Gran Canaria	Banhaderos	
	A2.3	AMEGC	Gran Canaria	Banhaderos	
	S0.17	AMEGC3	Gran Canaria	Banhaderos	
	A2.5	AMELP-MA	La Palma	La Salemera	
	A2.6	AMELP-MA	La Palma	La Salemera	
	A2.7	AMELP-MA	La Palma	La Salemera	
	A2.8	AMELP-MA	La Palma	La Salemera	
	S0.16	AMELP-MA	Madeira	Ponta da Cruz	
	A2.4	AMEGC2	Gran Canaria	Banhaderos	MOTU 6
Apohyale prevostii	A3.1	APRNO	Norway	Baloy	MOTU 7
	A3.10	APRNO	Norway	Viksoy	
	A3.13	APRPW	Portugal West	S. Pedro Moel	
	A3.11	APRIC	Iceland	Reykjavik	
	A3.14	APRIC	Iceland	Reykjavik	
	A3.6	APRNO	Iceland	Reykjavik	
	A3.7	APRNO	Iceland	Grindavik	
	A3.8	APRIC2	Iceland	Strandarkirkja	
	A3.2	APRNO	Norway	Baloy	
	A3.3	APRNO	Scotland	Bellochantuy	
	A3.4	APRNO	Scotland	Easdale	
	A3.5	APRNO	Norway	Hellesoy	
	A3.9	APRNO	Norway	Viksoy	
	BBAY004-01	APRNO	Newfoundland and Labrador	BOLD	
	BBAY004-02	APRNO	Newfoundland and Labrador	BOLD	
	BBAY010-02	APRNO	Newfoundland and Labrador	BOLD	
	MT02075	APRNS	North Sea	BOLD	
	MT02076	APRNS	North Sea	BOLD	
	MT02077	APRNO	North Sea	BOLD	
	MT02078	APRNO	North Sea	BOLD	
	MT03293	APRNS	North Sea	BOLD	
	MT00029	APRNO	North Sea	BOLD	
	MT00030	APRNS2	North Sea	BOLD	
	MT00031	APRNO	North Sea	BOLD	
	MT00032	APRNS	North Sea	BOLD	
	MT00033	APRNS3	North Sea	BOLD	
	MT00034	APRNS	North Sea	BOLD	
	MT00035	APRNO	North Sea	BOLD	
	SFCM18-001	APRMI	Portugal West	BOLD	
	AF520435	APRCA	Canada	GenBank	

	HUNTSAMP0070	APRNO	New Brunswick	BOLD	
	L90AR7-03	APRNO	Quebec	BOLD	
	L164AR1-01	APRNV	Nova Scotia	BOLD	
	L164AR1-03	APRNO	Nova Scotia	BOLD	
	L159AR1-04	APRNO	New Brunswick	BOLD	
	L159AR1-05	APRNO	New Brunswick	BOLD	
	L164AR1-06	APRNV	Nova Scotia	BOLD	
	L167AR2-01	APRNO	Nova Scotia	BOLD	
	L167AR2-02	APRNV2	Nova Scotia	BOLD	
	L155AR1-11	APRNO	New Brunswick	BOLD	
	L246AR1-08	APRNO	Newfoundland and Labrador	BOLD	
	L242AR1-05	APRNW	Newfoundland and Labrador	BOLD	
	L250AR1-07	APRNO	Newfoundland and Labrador	BOLD	
	L240AR1-06	APRNO	Newfoundland and Labrador	BOLD	
	L240AR1-07	APRNO	Newfoundland and Labrador	BOLD	
	NORKA-06	APRNO	Norway	BOLD	
	NORKA-07	APRNO	Norway	BOLD	
	NORKA-08	APRNO	Norway	BOLD	
	L202AR1-01	APRNO	Nova Scotia	BOLD	
	L202AR1-02	APRNV2	Nova Scotia	BOLD	
	L202AR1-03	APRNO	Nova Scotia	BOLD	
	L177AR1-01	APRNO	Canada	BOLD	
	L177AR1-02	APRNO	Canada	BOLD	
Apohyale stebbingi	A4.1	ASTPS	Portugal South	Arrifes	MOTU 8
	A4.8	ASTSC	Scotland	Carsaig	
	A4.9	ASTSC	Scotland	Carsaig	
	A4.10	ASTSC	Scotland	Carsaig	
	A4.14	ASTPS2	Portugal South	Dona Ana	
	A4.16	ASTGA2	Galicia	Pedreira	
	A4.18	ASTGA2	Galicia	Pedreira	
	A4.19	ASTPW	Portugal West	Peniche	
	A4.2	ASTPS3	Portugal South	Ingrina	
	A4.22	ASTPW2	Portugal West	S. Pedro Moel	
	A4.23	ASTPW2	Portugal West	S. Pedro Moel	
	A4.25	ASTPW2	Portugal West	Peniche	
	A4.20	ASTLP4	La Palma	La Salemera	MOTU 9
	A4.21	ASTLP4	La Palma	La Salemera	
	A4.3	ASTGC	Gran Canaria	Caleta	
	A4.11	ASTLP	La Palma	El Faro	
	A4.12	ASTLP2	La Palma	El Faro	MOTU 10

	A4.13	ASTLP3	La Palma	El Faro	
	A4.15	ASTGA	Galicia	Muxia	MOTU 11
	A4.20	ASTMA	Madeira	Reis Magos	MOTU 12
	A4.7	ASTGC3	Gran Canaria	Playa Melenara	MOTU 13
	A4.4	ASTGC2	Gran Canaria	Caleta	
	A4.5	ASTMA2	Madeira	Ponta da Cruz	MOTU 14
	A4.6	ASTMA3	Madeira	Ponta da Cruz	MOTU 15
Hyale pontica	H0.1	HPOGA	Galicia	Barizo	MOTU 16
	H0.2	HPOGA2	Galicia	Barizo	
	H0.3	HPOSC-PW	Scotland	Easdale	
	H0.4	HPOSC-PW	Portugal West	Agudela	
	H0.5	HPOGA	Galicia	Muxia	
	H0.6	HPOGA3	Galicia	Muxia	
	H0.7	HPOGA	Galicia	Muxia	
Protohyale (Protohyale) schmidtii	P0.10	PSCPS	Portugal South	Arrifes	MOTU 17
	P0.19	PSCPS	Portugal South	Arrifes	
	P0.12	PSCGA	Galicia	Pedreira	
	P0.13	PSCMA	Madeira	Porto dos Frades	
	P0.18	PSCGA2	Galicia	Barizo	
	P0.14	PSCGA2	Galicia	Pedreira	
	P0.15	PSCGA2	Galicia	Barizo	
	P0.2	PSCPS	Portugal South	Arrifes	
	P0.6	PSCGA2	Galicia	Barizo	
	P0.16	PSCPW	Portugal West	Buarcos	
	P0.8	PSCGA2	Galicia	Muxia	
	P0.9	PSCGA3	Galicia	Muxia	
	P0.1	PSCMO	Morocco	Akhfenir	MOTU 18
	P0.3	PSCGC	Gran Canaria	Banhaderos	MOTU 19
	P0.4	PSCGC2	Gran Canaria	Banhaderos	
	P0.5	PSCGC3	Gran Canaria	Banhaderos	
	P0.17	PSCMA2	Madeira	Ponta da Cruz	
	P0.6	PSCPW2	Portugal West	Peniche	
	P0.20	PSCLP	La Palma	La Salemera	
	P0.7	PSCLP2	La Palma	El Faro	
Protohyale sp.	P1.1	PSPIT	Italy	Isole Tremiti	MOTU 20
	P1.2	PSPIT2	Italy	Isole Tremiti	
	P1.3	PSPIT3	Italy	Isole Tremiti	
Serejohyale spinidactylus	S0.1	SSPGC	Gran Canaria	Banhaderos	MOTU 21
	S0.15	SSPGC2	Gran Canaria	Banhaderos	
	S0.12	SSPGC3	Gran Canaria	Playa Melenara	
	S0.13	SSPGC4	Gran Canaria	Playa Melenara	
	S0.14	SSPGC4	Gran Canaria	Banhaderos	
	S0.6	SSPMA	Madeira	Ponta da Cruz	MOTU 22

	S0.8	SSPMA2	Madeira	Reis Magos	
	S0.9	SSPMA3	Madeira	Reis Magos	
	S0.10	SSPLP	La Palma	La Salemera	MOTU 23
	S0.11	SSPLP2	La Palma	La Salemera	
	S0.18	SSPLP3	La Palma	La Fajana	
	S0.19	SSPLP4	La Palma	La Fajana	
	S0.2	SSPGA	Galicia	Barizo	MOTU 24
	S0.3	SSPGA2	Galicia	Barizo	
	S0.4	SSPAZ	Azores	Mosteiros	
	S0.20	SSPGA3	Galicia	Muxia	
	S0.21	SSPGA4	Galicia	Muxia	
	S0.5	SSPGA5	Galicia	Muxia	
	S0.7	SSPAZ	Azores	Ponta da Ferreirinha	

Annex 3. Dichotomic key to the Hyalidae species found in the project Diverse Shores, plus the genus *Parhyale*.

1A: Uropod 3 uniramous.	2
1B: Uropod 3 biramous, inner ramus minute, scale-like.	Genus *Parhyale*
2A: Pereopods 5-7 propodus with large robust striated seta/e.	3
2B: Pereopods 5-7 propodus without large robust striated seta/e.	4
3A: Uropod 1 peduncule with enlarged distolateral robust seta.	*Apohyale media*
3B: Uropod 1 peduncule without enlarged distal robust setae.	*Hyale pontica*
4A: Pereopods 3-7 dactylus of medium length (¼ to ½ length of the propodus).	*Protohyale (Protohyale) schmidtii*
4B: Pereopods 3-7 dactylus short (¼ length of the propodus).	5
5A: Pereopods 5-7 propodus with tuft of setae at mid length of posterior margin.	6
5B: Pereopods 5-7 propodus without setae on posterior margin.	7
6A: Pereopods 3-7 dactylus with long and very thick seta (1/3 or more length of dactylus).	*Serejohyale spinidactylus*
6B: Pereopods 3-7 dactylus with short and slender seta (1/5 or less length od dactylus)	*A. stebbingi*

7A: Pereopods 3-7 dactylus with long and slender seta (1/3 or more length of dactylus). *A. perieri*

7B: Pereopods 3-7 dactylus with short and slender seta (1/5 or less length of dactylus) *A. prevostii*

Annex 4. Table with average pairwise p-distances between MOTUs (below the line), between species (above the line).

Species		*Apohyale perieri*				*Apohyale media*		*Apohyale prevostii*	*Apohyale stebbingi*								*Hyale pontica*	*Protohyale schmidtii*			*Protohyale sp*	*Serejohyale spinidactylus*			
	MOTUs	MOTU1	MOTU2	MOTU3	MOTU4	MOTU5	MOTU6	MOTU7	MOTU8	MOTU9	MOTU10	MOTU11	MOTU12	MOTU13	MOTU14	MOTU15	MOTU16	MOTU18	MOTU17	MOTU19	MOTU20	MOTU21	MOTU23	MOTU24	MOTU22
Apohyale perieri	MOTU1					0.245		0.223	0.230								0.213	0.226			0.199	0.224			
	MOTU2	0.113																							
	MOTU3	0.112	0.087																						
	MOTU4	0.072	0.098	0.093																					
Apohyale media	MOTU5	0.247	0.247	0.249	0.240			0.209	0.230								0.216	0.219			0.204	0.241			
	MOTU6	0.226	0.218	0.224	0.209	0.093																			
Apohyale prevostii	MOTU7	0.222	0.224	0.239	0.227	0.208	0.203		0.184								0.182	0.199			0.166	0.214			
Apohyale stebbingi	MOTU8	0.225	0.217	0.213	0.210	0.226	0.212	0.173									0.207	0.230			0.210	0.233			
	MOTU9	0.244	0.230	0.232	0.231	0.239	0.225	0.179	0.136																
	MOTU10	0.255	0.246	0.243	0.240	0.238	0.236	0.214	0.178	0.187															
	MOTU11	0.221	0.216	0.220	0.209	0.229	0.213	0.196	0.126	0.156	0.184														
	MOTU12	0.246	0.220	0.235	0.229	0.233	0.224	0.195	0.149	0.106	0.181	0.145													
	MOTU13	0.237	0.216	0.233	0.220	0.239	0.221	0.202	0.171	0.189	0.154	0.184	0.180												
	MOTU14	0.228	0.227	0.231	0.213	0.238	0.227	0.212	0.172	0.181	0.134	0.178	0.191	0.140											
	MOTU15	0.228	0.225	0.231	0.211	0.233	0.224	0.203	0.191	0.184	0.175	0.169	0.191	0.170	0.169										
Hyale pontica	MOTU16	0.215	0.206	0.220	0.207	0.218	0.210	0.182	0.211	0.208	0.206	0.210	0.202	0.196	0.191	0.200		0.192			0.201	0.211			
Protohyale schmidtii	MOTU18	0.223	0.218	0.224	0.222	0.221	0.205	0.198	0.226	0.237	0.223	0.231	0.229	0.201	0.229	0.233	0.190				0.197	0.230			
	MOTU17	0.226	0.216	0.226	0.227	0.219	0.213	0.203	0.225	0.237	0.234	0.229	0.233	0.211	0.233	0.240	0.188	0.035							
	MOTU19	0.231	0.224	0.235	0.225	0.217	0.217	0.192	0.233	0.241	0.242	0.235	0.236	0.230	0.232	0.225	0.199	0.117	0.121						
Protohyale sp	MOTU20	0.198	0.205	0.201	0.194	0.205	0.198	0.167	0.197	0.210	0.247	0.213	0.222	0.217	0.231	0.228	0.202	0.194	0.198	0.199		0.217			
Serejohyale spinidactylus	MOTU21	0.203	0.215	0.227	0.217	0.237	0.235	0.210	0.212	0.226	0.219	0.222	0.215	0.212	0.215	0.229	0.193	0.212	0.222	0.235	0.214				
	MOTU23	0.243	0.231	0.213	0.240	0.245	0.238	0.213	0.234	0.241	0.244	0.236	0.245	0.220	0.225	0.244	0.227	0.223	0.226	0.243	0.227	0.165			
	MOTU24	0.220	0.229	0.227	0.223	0.235	0.227	0.210	0.241	0.240	0.254	0.228	0.237	0.224	0.231	0.249	0.211	0.215	0.215	0.259	0.211	0.161	0.094		
	MOTU22	0.238	0.244	0.234	0.231	0.260	0.253	0.230	0.236	0.244	0.260	0.267	0.256	0.244	0.233	0.248	0.219	0.230	0.234	0.240	0.224	0.170	0.148	0.171	

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