University of Sassari

Dissertation for the Degree of Doctor of Philosophy in Environmental Biology

presented at Sassari University in 2013

XXVI cycle

MOLECULAR MARKERS AS A TOOL FOR PHYLOGENETIC STUDIES IN PROSERIATA (PLATYHELMINTHES: NEOOPHORA)

PH.D. CANDIDATE: ***Dr. Fabio Scarpa***

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Abstract

The order Proseriata is one of the most abundant groups of the meiofauna, particularly common in high-energy habitats. Studying Proseriata is difficult, as they are very minute animals with few morphological features that can be used for taxonomic analysis. The lack of diagnostic characters hinders the study of Proseriata by means of traditional methods, and molecular analyses are in most cases needed to reconstruct the phylogeny of Proseriata and to provide reliable taxonomic assessment. Here, the results of five study cases were reported: i) the new recently discovered species, *Pseudomonocelis paupercula* was studied by means of an integrative taxonomic approach that include morphology, karyology, molecular phylogeny, reproductive biology and parentage analyses; ii) the *Parotoplana tubifera* species group was also investigated by an integrative approach that includes morphology, karyology and molecular phylogeny, and four new *Parotoplana* species were described; iii) phylogenetic relationships within the genus *Monocelis*, and within the family Monocelididae were investigated, and two new Mediterranean species belonging to the genus *Monocelis* was retrieved; iv) the first molecular clock for Proseriata was calibrated, that can be an appealing integration to the integrative taxonomic approach, and can provide phylogenetic reconstruction more accurate in future analyses; v) universal primers were designed to obtain a wider molecular sampling in Proseriata, so far very scarce.

Key words: Proseriata; Molecular phylogeny; Molecular clock; Ribosomal DNA; ISSR.

Riassunto

L'ordine Proseriata costituisce uno dei più abbondanti gruppi della meiofauna, i cui rappresentanti sono particolarmente comuni in habitat ad alta energia. Gli studi sui Proseriata sono particolarmente problematici, in quanto l'ordine è rappresentato da organismi di ridotte dimensioni con pochi caratteri diagnostici utili ai fini tassomici. Per questo motivo i Proseriata non possono essere studiati solamente con metodi tradizionali e negli ultimi anni ci si è avvalsi di un approccio tassonomico integrato, comprendente analisi molecolari, sia per le ricostruzioni filogenetiche, sia per l'inquadramento tassonomico. Questo elaborato è costituito da cinque linee di ricerca: i) una nuova specie di recente scoperta, *Pseudomonocelis paupercula*, è stata descritta e analizzata mediante approccio tassonomico integrato; ii) quattro nuove specie appartenenti al gruppo di specie *Parotoplana tubifera* sono state descritte e sono state studiate le relazioni filogenetiche all'interno del gruppo e della famiglia Otoplanidae; iii) sono state chiarite le relazioni filogenetiche all'interno del genere *Monocelis* e della famiglia Monocelididae; questo studio ha portato alla scoperta di due nuove specie del genere *Monocelis*; iv) è stata eseguita la prima calibrazione dell'orologio molecolare nei Proseriati, che consente di implementare l'approccio tassonomico integrato; v) sono stati disegnati primer universali per i Proseriati, al fine di ampliare l'attuale campionamento molecolare sinora esiguo.

Parole chiave: Proseriata; Filogenesi molecolare; Orologio molecolare; DNA ribosomiale; ISSR.

Introduction

The term Biodiversity refers to biological diversity, given by the variety of ecosystems, species, population within species, and genetic diversity of organisms (Frankham et al., 2006). Species richness, which concern the number of species in a given area, has been so far the most widely used metric to depict biodiversity, and much has been written about how many species may exist on land and in the sea (May, 2002; Hamilton et al., 2010). Nevertheless, using species richness to investigate biodiversity is not an easy goal, as obtaining a complete picture on the actual extent of variety through which life expresses itself presents many difficulties. Indeed, the number of species, not yet described, that potentially exist, amount from about 300,000 to over 10,000,000 (Grassle and Maciolek, 1992; van der Land, 1994).

Studying marine biodiversity is even more problematic (see Mathews, 2006), due to the vastness of marine environment, the higher dispersal capacity, and the more and more number of cryptic species, i.e. morphologically identical species, distinguishable only on molecular bases. Accordingly, marine biodiversity is likely to be underestimated (see Appeltans et al., 2012), and at present there is a strong belief that current estimates of marine biodiversity are based upon a non-representative dataset, which needs a re-evaluation (Sala, 2002). The number of described marine species is deemed under-representative (Bouchet et al., 2002): nowadays the number of described species vary from 150,000 to 274,000 (Winston, 1992; van der Land, 1994; Reaka-Kudla, 1996; Gibbons et al., 1999; Gordon, 2001; Bouchet, 2006; Appeltans et al., 2012).

The problem of species underestimation in marine environment is particularly felt for meiofauna, i.e. organisms that can pass through a 500 µm mesh (see Lambshead, 1993;

Godfray and Lawton, 2001; Blaxter, 2003). Four main constraints hinder the appraisal of the actual number of meiofaunal species: 1) the small size of the specimens; 2) the morphological homogeneity (Fontaneto et al., 2009); 3) the non-informative species descriptions (Godfray, 2002); and 4) the shortage of expert taxonomists (Curini-Galletti et al., 2012).

The order Proseriata

One of the most abundant meiofaunal groups is represented by the taxon Proseriata (Platyhelminthes: Rhabditophora) (Remane, 1933; Reise, 1988), which is a cosmopolitan, species-rich and diverse taxon of free-living Rhabditophora. Most Proseriata are minute interstitial organisms with an elongated body, occurring in all marine habitats, from supra- to sub-littoral environments, and in all kinds of sediments (Curini-Galletti and Martens, 1990). They are particularly common in high-energy habitats, with medium to coarse sediments (Reise, 1988), where they may be the dominant metazoan species (Martens and Schockaert, 1986). Proseriata may be very common in brackish-water biotopes (Curini-Galletti and Martens, 1990), but few representatives occur in freshwater (Cannon, 1986). Proseriata are carnivores and/or scavengers (Martens and Schockaert, 1986), and their impact on meiofaunal communities may be considerable (Littlewood et al., 2000). The order Proseriata has been divided into two suborders on morphological basis: 1) the Lithophora, to which belong taxa with a statocyst in front of the brain, and without pigment in the mantle cells of rhabdomeric receptors; 2) the Unguiphora, composed by taxa without statocyst and with pigment in the mantle cells of rhabdomeric receptors (Sopott-Ehlers, 1985; and see Curini-Galletti et al., 2010 for details about this scenario).

Studying Proseriata is notoriously difficult, as they are very minute animals with relatively few morphological features that can be used for taxonomic analysis, and thus species delimitation. A crucial issue related to the scarcity of morphological characters is indeed represented by the pervasive occurrence of cryptic species, also known as sibling species. Moreover, within the order are also present species complexes (i.e. group of closely related species, where the exact demarcation between species is often unclear or cryptic owing to their recent and usually, incomplete reproductive isolation) and species groups (i.e. an informal taxonomic rank into which an assemblage of closely related species within a genus are grouped because of their morphological similarities, and their identity as a biological unit with a single monophyletic origin).

Therefore, the lack of diagnostic characters hinders the study of Proseriata by means of traditional methods, and molecular analyses are in most cases needed to reconstruct the phylogeny of Proseriata and to provide reliable taxonomic assessment. For the latter point, it is important to underline that, for the species delimitations in "taxonomically difficult" taxa, such as Proseriata, it is advisable to use the so-called integrative taxonomic approach (Schlick-Steiner et al., 2010), in which traditional analyses (morphology, karyology, reproductive biology) are flanked by molecular survey.

Molecular phylogeny

Molecular phylogeny exploits the information embedded within target DNA sequences to assess the evolutionary relationships among groups of organisms. Since from its first use, molecular phylogeny based on DNA sequences, provided a significant contribution to phylogeny and taxonomy at different hierarchical levels (see e.g., Carranza et al., 1997; Ruiz-Trillo et al., 2002; Baguña et al., 2008), which leads, in the last decades, to

many changes in phylogenetic reorganization of several taxa. For example, one of the most important contributions of molecular phylogeny to taxonomy attains the Bilateria, which are no longer classified according to the presence or absence of the coelom, whose importance has been downgraded. At present, Bilateria have been subdivided in Ecdysozoa (Aguinaldo et al. in 1997), Lophotrochozoa (Halanych et al., 1995) and Deuterostomia (Halanych et al., 1995). Phylogenetic trees based upon the nuclear ribosomal DNA 18S genes suggest the condition of Ecdysozoa, Lophotrochozoa and Deuterostomia as monophyletic groups. Within Lophotrochozoa, a group that underwent a deep phylogenetic reorganization is the Phylum Platyhelminthes, which includes a large number of free-living and parasitic species, from small to medium size, heterogeneous regarding their habitat (marine, freshwater, terrestrial). Indeed, the use of molecular techniques evidenced Platyhelminthes are actually a polyphyletic group (Ruiz-Trillo et al., 2004). In particular, the taxa Acoela and Nemertodermatida have been thus removed from Platyhelminthes, and they have been acknowledged as basal Phyla of Bilateria (Ruiz-Trillo et al., 1999, 2002; Jondelius et al., 2002).

Molecular tools have been successfully applied to reconstruct phylogeny within Proseriata (see e.g., Kuznedelov and Timoshkin, 1995; Katayama et al., 1996; Litvaitis et al., 1996; Jondelius, 1998; Litvaitis and Rhode, 1999; Littlewood et al., 1999; Littlewood et al., 2000; Noren and Jondelius, 2002; Lockyer et al., 2003; Willems et al., 2006; Casu and Curini-Galletti, 2004; 2006; Sanna et al., 2009; Casu et al., 2009; Curini-Galletti et al., 2010; Casu et al., 2011; Chapter 1, 3, 4). At present, the molecular markers mainly used are the complete nuclear small subunit rRNA (18S) gene and the partial nuclear large subunit rRNA (28S) fragment (spanning variable domains D1-D6). These two markers also showed their potential in discriminating new species (see Curini

et al., 2010; Chapter 1, 3, 4, and references therein). To date, due to the poor knowledge of their genome, and the absence in literature of specific pairs of primers (but see, Sanna et al., 2009), 18S and 28S are the only molecular markers that give satisfactory results in terms of reliable amplification, and sequences length, throughout the Proseriata, and for which a large database is available.

Integrative taxonomic approach

Taxonomy is central to explore and understand biodiversity. Traditionally, taxonomy is based on formal morphological description of the species. However, traditional morphology-based taxonomy, used as approach for delimiting species, shows some limitations (Dayrat, 2005), in particular when organisms lack of clearly distinguishable taxonomic characters (i.e. morphological traits used as evidence for species discrimination) (Mayr, 1969). Since the advent of molecular tools and the discover of PCR (Polymerase Chain Reaction) (Mullis and Faloona, 1987), new ways were opened to overcome the limit given by the species delimitations performed using only morphological information (Moritz and Hillis, 1996), and recently some scientist invoked the possibility to use only DNA sequences to obtain "simple" and reliable species discriminations. One of the most-known method proposed to delimit species by molecules is the so-called DNA barcoding (Hebert et al., 2003; Tautz et al., 2003; Blaxter, 2004; Gaston and O'Neill, 2004), which is based on the sequencing of a almost 650 base pairs long mitochondrial region coding for the sub-unit I of the cytochrome c oxidase (COI) gene, the so-called Folmer region (Folmer et al., 1994). DNA barcoding relies on the assumption that each species has its own 'diagnostic' sequence in that gene (i.e. a unique set of base pair mutations), that can serve as the core of a global bio-

identification system for animals (Hebert et al., 2003). However, it is worth to note that the Folmer region failed as “barcoding molecule” in several instance, e.g.: 1) in the mollusc group of Cypraeidae (Meyer and Paulay, 2005) COI was unable to detect cryptic speciation; 2) in two different lake Baikal freshwater sponge genera (*Lubomirskia* and *Baikalospongia*) COI bear identical sequences (Schröder et al., 2003); 3) in the *Astrosclera willeriana* COI did not aid the identification of a species complex (Erpenbeck et al., 2006); 4) in the Anthozoa (Cnidaria), where Shearer et al., (2002) evidenced a very slow rate of mitochondrial DNA sequence evolution. Additional hindrances to the extensive employ of the Folmer region are that: 1) in some cases, the high variability of the sequences adjacent to the priming region makes the “universal” primers unusable (Sanna et al., 2009); and 2) COI), usually performed to make inferences on the genetic relationships of different species, is not suitable to separate species younger than 3 to 4 millions of years (Bouzid et al., 2008). Remarkably, in the case of meiofaunal organisms, the at least limited dispersal capabilities may result in marked divergence among allopatric populations, and the degree of intraspecific vs. interspecific differentiation should be tested on extensive taxonomic and geographic samplings.

In this context, the “integrative taxonomic approach”, which considers species boundaries from multiple, complementary perspectives, has been recently advocated as the best way to overcome potential caveats of any species delimitation methods (Padial et al., 2010). This approach allows to merge several biological information, in order to obtain an as detailed as possible reconstruction of the species biology (Wilson, 2003, 2004; Dayrat, 2005; Vogler, 2007; Knapp, 2008). A general view has emerged that the best way to perform taxonomical analysis is to construct a more "integrative taxonomy",

that would accommodate new concepts and methods (Dayrat, 2005; Will et al., 2005; Valdecasas et al., 2005; Schlick-Steiner et al., 2010), and a considerable number of researches have already echoed the new term "integrative taxonomy" (e.g. Fonseca et al., 2008; Gibbs, 2009; Glaw et al., 2010; Lumley and Sperling, 2010). To date, integrating different perspectives is a standard procedure for many groups, from mammals, where the vast majority of the species are already known (see e.g., Wada et al., 2003), to groups where morphological features are limited, such as bacteria (see e.g., Oren, 2004).

The integrative taxonomic approach has been widely applied also for Proseriata (see Litvaitis et al., 1996; Littlewood et al., 2000; Curini-Galletti et al., 2010; Casu et al., 2004, 2006, 2009; Chapter 1) taking into consideration morphological description, karyology, reproductive biology (breeding experiments and anatomical reconstructions) and molecular tools.

Background and objectives

When my PhD course started, one of the most recent and complete research on in-group and out-group phylogenetic relationships within the order Proseriata was that performed by Curini-Galletti et al. (2010). That research was carried out by means of an integrative approach that included both molecular (18S and 28S regions) and morphological surveys. The phylogenetic survey of the above-mentioned study has provided several results: the sister taxa relationship between the two suborder Unguiphora and Lithophora is confirmed; Lithophora is a monophyletic taxon; the family Coelogynoporidae are also monophyletic; the families Calviriidae and Coeloginoporidae show a sister-taxa relationship, as well as the Calviriidae +

Coeloginoporidae clade and the clade composed by the remaining Lithophora (Monocelididae, Archimonocelididae and Otoplanidae). However, there are still open issue that need to be investigated by future studies.

In this context, my PhD project was aimed to increase the knowledge about the order Proseriata by using molecular technique. This generic goal was achieved by means of more targeted and specific aims, such as:

- To clarify the phylogenetic relationships of several taxa belonging to the order Proseriata;
- To solve doubts concerning species complexes;
- To provide a taxonomic assessment of not yet described species;
- To expand molecular sampling available.

In my PhD thesis I have reported five study cases, structured in seven chapters, corresponding to seven manuscripts:

1) The first study case deals with a newly discovered species, *Pseudomonocelis paupercula* Curini-Galletti, Casu & Lai, 2011. This new species lives in brackish-water habitats of the Mediterranean Sea. It is a very rare species; indeed *P. paupercula* was found only in four localities, Porto Pozzo (Sardinia, Italy), Maliakós, (Greece), Alexandria (Egypt), and Akko (Israel), although several sampling campaigns over some years in the Mediterranean Sea were carried out. This species is strictly confined to sectors characterized by the presence of fresh-water outlets and mixed, silty sediment. This condition make these populations potentially vulnerable, and, for this reason, exposed to the risk of (local) extinction. *Pseudomonocelis paupercula* is morphologically characterized by the presence of the copulatory organ provided with a stylet, combined with lack of vagina and the presence of a muscular organ close to the

female pore. An integrative approach that include morphology, karyology, molecular phylogeny (performed by means of the 18S and 28S D1-D6 fragment), reproductive biology (breeding experiments and anatomical reconstructions) and parentage analyses (performed by means of ISSR - Inter Simple Sequence Repeat - markers) of offspring was used. The first paper (**Chapter 1**) reports the species description and new insights about phylogenetic relationship within the genus *Pseudomonocelis*; the second paper (**Chapter 2)** underlines the presence and the relevance of self-fertilization in *P. paupaercula*.

2) The second study case refers the *Parotoplana tubifera* species group (**Chapter 3**). This group is composed by four new species of Otoplanidae belonging to the genus *Parotoplana*, (*Parotoplana tubifera* Curini-Galletti & Delogu, 2013; *Parotoplana ambrosolii* Curini-Galletti & Delogu, 2013; *Parotoplana impastatoi* Curini-Galletti & Delogu, 2013; *Parotoplana livatinoi* Curini-Galletti & Delogu, 2013) with distribution restricted to the central Mediterranean. This species group is morphologically characterized by the presence of a tubular copulatory stylet (hence their name) and by a bursa lacking an open connection to the atrium. The four species differ mainly in details of the morphology of sclerotized structures of the copulatory organ. The paper, based on the results obtained by an integrative approach that includes morphology, karyology and molecular phylogeny (performed by means of the 18S and 28S D1-D6 fragment), presents the description of these four new species and partly clarify their generic attribution.

3) The third study case was aimed to provide further insight on the phylogenetic relationship within the genus *Monocelis*, and within the family Monocelididae (**Chapter 4**). Furthermore, a revision of the taxonomic status of the *M. lineata* species complex

(the existence of at least four Atlantic and Mediterranean cryptic species was suggested by Casu and Curini-Galletti, 2004, based on allozymes evidence) was performed. In particular, my research evidenced the occurrence of two new Mediterranean species of the complex, whose populations are geographically very close.

4) The fourth study case was focused to calibrate the molecular clock for Proseriata. Indeed molecular clock data can provide phylogenetic reconstruction easier and more accurate (Lemey and Posada, 2009), and they can be an appealing integration to the integrative taxonomic approach.

This study was possible as, during several intense sampling campaigns performed on both sides of the Isthmus of Panama, we were able to find, at two different times, two pairs of geminate species (i.e., morphologically similar sister species that live in allopatric conditions and occur on opposite sides of a geographic barrier), that represent the only way to calibrate molecular clock when fossil records are absent. Furthermore, during further sampling campaigns, we retrieved three pairs of sister taxa (belonging to families Otoplanidae, Monocelididae, and Archimonocelididae), which was used to test the reliability of our calibration. Two papers were produced based on this research. The first paper (**Chapter 5**) reports the results of the preliminary and explorative study, where the mutation rate per million years for two nuclear ribosomal genes (18S and 28S) were estimated using the first-found pair of geminate species (*Minona gemella* species pair). In the second paper (**Chapter 6**), results obtained adding a second geminate species pair (*Parotoplana* sp. nov. species pair) was reported. Our calibration of the molecular clock was here tested on the Atlantic and Pacific species belonging to the genus *Kata* (Otoplanidae), belonging to the genus *Duplominona* (Monocelididae), and to the genus *Archimonocelis* (Archimonocelididae).

5) The fifth study case was aimed to obtain a wider molecular sampling for Proseriata. For molecular studies in Proseriata the 18S and 28S rDNA fragments are very popular, and they have been extensively used. Nowadays, their sequences represent the only large database available for Proseriata. Although these two genes have proved to be highly informative as molecular markers for several study cases both in Proseriata (see Littlewood et al., 2000; Willems et al., 2006; Curini-Galletti et al., 2010; Chapter 1, 3, 4) and in Platyhelminthes in general (see Carranza et al., 1997; Littlewood et al., 1999; Littlewood and Olson, 2001; Lockyer et al., 2003; Baguñà and Riutort, 2004; Willems et al., 2006; Larsson and Jondelius, 2008), the need of a wider molecular sampling was needed (see Curini-Galletti et al., 2010and Chapters 1, 3, 5, 6) as most universal primers for amplifying other nuclear and mitochondrial genes/fragment for invertebrate, such as those for the cytochrome c oxidase (COI) (Folmer et al., 1994), do not often provide satisfactory results, and specific primers are not available, except for few species (see Sanna et al., 2009; Casu et al., 2011). In this context, the goal of this research was to find further markers, designing a set of primers that allow a cross-use within the whole order (**Chapter 7**).

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Chapter 1

A reappraisal of the monophyly of the genus *Pseudomonocelis* Meixner, 1943 (Platyhelminthes: Proseriata), with the description of a new species from the Mediterranean

Marco Casu, Piero Cossu, Daria Sanna, Tiziana Lai, Fabio Scarpa, Marco Curini Galletti

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A reappraisal of the monophyly of the genus *Pseudomonocelis* Meixner, 1943 (Platyhelminthes: Proseriata), with the description of a new species from the Mediterranean

MARCO CASU, PIERO COSSU, DARIA SANNA, TIZIANA LAI,
FABIO SCARPA & MARCO CURINI-GALLETTI[1]

Dipartimento di Zoologia e Genetica Evoluzionistica, Università di Sassari, Via F. Muroni 25, 07100 Sassari, Italy. Centro di Eccellenza dell'Università di Sassari

[1]Corresponding author. E-mail: curini@uniss.it

Abstract

Pseudomonocelis paupercula **nov. sp.** is described from brackish-water habitats of the Mediterranean. It is distinguished from other members of the genus by the copulatory organ provided with a stylet, combined with lack of vagina and presence of a muscular organ close to the female pore. Its phylogenetic relationships have been investigated sequencing complete 18S rRNA gene and partial 28S rRNA gene, spanning variable domains D1-D6. Both BI and ML suggest a sister-taxon relationships of *P. paupercula* **nov. sp.** with the east African *P.* cf *cavernicola.* However, statistical support is low. Conversely, MP indicates *P. paupercula* **nov. sp.** as sister-taxon to all the remaining *Pseudomonocelis* and *Minona ilenae*. Overall, results of the combined analysis do not support the monophyly of the genus *Pseudomonocelis*. The need for wider molecular and taxonomic sampling is stressed.

Key words: *Pseudomonocelis paupercula*, microturbellarian, karyotype, taxonomy, biodiversity, molecular phylogeny, 18S, lrsDNA

Introduction

The Monocelididae Hofsten, 1907 (Platyhelminthes: Proseriata) include marine interstitial flatworms, with a comparatively simple morphology. Among the few morphological characters on which their systematics is based, particular weight has been traditionally attributed to the position of the ovaries in relation to the pharynx (Martens, 1983). In most Monocelididae the ovaries are placed just in front of the pharynx, while a few species display postpharyngeal ovaries. However, the paucity of characters available for taxonomy and the chances of parallel evolution raise suspicions that groupings of taxa exclusively based on relative position of the ovaries may be homoplastic (Martens, 1983; Curini-Galletti *et al.*, 2010). Among the genera with posterior ovaries, the genus *Pseudomonocelis* Meixner, 1943 appears indeed particularly heterogeneous. It includes species showing all stages from the presence of a fully functional accessory organ provided with a stylet (*Pseudomonocelis hoplites* Curini-Galletti, 1997) to a reduced state which has lost both the stylet and the glandular function (*P. cavernicola* Schockaert & Martens, 1987), to the total loss of the organ (Curini-Galletti, 1997; Curini-Galletti & Casu, 2005; Curini-Galletti *et al.*, 2011; Schockaert & Martens, 1987). So far, the monophyly of the genus has never been tested using gene sequence data. *Pseudomonocelis hoplites* has not been sequenced yet. *Pseudomonocelis* cf *cavernicola*, in a molecular study of the *Pseudomonocelis agilis* complex, clustered with the rest of the *Pseudomonocelis* species (Casu *et al.*, 2009). However, branch support was very low, and the molecular sample too limited to allow any sound conclusions.

Recently, during sampling campaigns performed in the Mediterranean under the sponsorship of the project BIOIMPA ('Biodiversity of Inconspicuous Organisms in Marine Protected Areas'), a new species of *Pseudomonocelis* was found which, similarly to *P.* cf *cavernicola*, presents an accessory organ without stylet. Here we present

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the description of this species, as well as new insights on the phylogenetic relationships of the species currently attributed to the genus *Pseudomonocelis*.

Material and methods

Sampling and karyological analysis. The specimens were found in samples collected manually from the following localities: Porto Pozzo, Sardinia, Italy (PP); Maliakós, Greece (MA); Alexandria, Egypt (AL) and Akko, Israel (AK) (see species description for further information on the stations). In all instances, the sediment consisted of silty sand. Extraction of the animals was through the $MgCl_2$ decantation technique (Martens, 1984), modified according to the quantity of silt in the sediment. After about 10 min immersion in a $MgCl_2$ solution isotonic to sea water, the sediment was stirred, and the suspension filtered through comparatively coarse sieves of decreasing mesh size (300 µm to 140 µm). The sieves were rinsed with sea-water in Petri dishes till silt was washed away. They were then placed to rest, until the animals crawled across the sieves in the Petri dish, and were retrieved with the aid of a stereomicroscope.

Whole mounts were done with polyvinyl-lactophenol. For microscopical analysis material was fixed in Bouin's fluid, embedded in 60º C Paraplast and cut into serial sagittal sections at 4 µm, stained with Hansen's haematoxylin and eosin-orange and mounted in Eukitt. For molecular analysis, specimens were fixed in 95% ethanol and stored at 4° C until DNA extraction.

Karyotype was determined from acetic-orcein-stained spermatogonial mitoses as described by Curini-Galletti *et al.* (1985). Relative lengths (r.l. = length of chromosome × 100/total length of haploid genome), centrometric indices (c.i. = length of short arm × 100/length of entire chromosome) and haploid genome length (g.l.) were obtained from measurements of camera lucida drawings of metaphase plates. The fundamental number (F.N.) is derived according to Matthey (1949) and chromosome nomenclature follows Levan *et al.* (1964).

Comparisons among karyotypes were based on plates obtained from 10 specimens per population. From each specimen, one plate, showing average contraction, was measured. Unifactorial ANOVA was performed on seven karyological variables. 'Population' factor was treated as fixed. Interpopulational differences were deemed as significant with $P \leq 0.01$. Cochran's *C*-test was used to test assumption of homogeneity of variances.

The holotype is deposited in the collections of the Swedish Museum of Natural History (Stockholm, Sweden) (SMNH). Paratypes and additional material are deposited in the collection of the Zoological Museum of the University of Sassari (Italy) (CZM).

Molecular analysis. Genomic DNA was extracted using the QIAGEN DNeasy Tissue kit (QIAGEN Inc.) according to the supplier's instructions. After extraction, the DNA was stored as a solution at 4 °C.

Six individuals belonging to each population were sequenced at complete nuclear small subunit rRNA (18S) gene, and partial nuclear large subunit rRNA (28S) fragment, spanning variable domains D1-D6 (see Table 1 for GenBank Accession numbers). Amplifications for both regions were carried out using the following external primers (see Littlewood *et al.*, 2000): for 18S: A (forward) AMC TGG TGG ATC CTG CCA G, and B* (reverse) TGA TCC ATC TGC AGG TTC ACC T; for 28S: LSU5 (forward) TAG GTC GAC CCG CTG AAY TTA AGC A, and LSUD6-3* (reverse) GGA ACC CTT CTC CAC TTC AGT C.

The polymerase chain reaction (PCR), carried out in a total volume of 25 µl, contained 5 ng/µl of total genomic DNA on average, 2.5 U of Taq DNA Polymerase (Euroclone), 1× reaction buffer, 1.25 mM of $MgCl_2$, 0.4 µM of primers, and 200 µM of dNTPs. PCR amplification was carried out in a MJ PTC-100 Thermal Cycler (MJ research) programmed as follows: 1 cycle of 3 min at 94° C, 45 cycles of 40 s at 94° C, 45 s at 54° C (18S / 28S D1-D6 primers' annealing temperature), and 1 min and 40 s at 72° C. At the end, a post-treatment for 5 min at 72° C and a final cooling at 4° C were carried out. Both positive and negative controls were used to test the effectiveness of the PCR reagents.

Electrophoresis was carried out on 2% agarose gels, prepared using 0.5× Tris-Borate-EDTA buffer, at 4 V/cm for 20 min and stained with ethidium bromide (10 mg/ml). PCR products, purified by ExoSAP-IT (USB Corporation, under license from GE Healthcare) following the manufacturer's instructions, were sequenced for both forward and reverse 18S and 28S D1-D6 strands, using an external sequencing core service (Macrogen Inc., Korea).

We also sequenced for both 18S and 28S D1-D6 one individual belonging to each of the monocelidid species listed in Table 1. Taxonomic information and full geographical data for these species are given in Casu *et al.*, 2009, 2011; Curini-Galletti & Casu, 2005; Curini-Galletti *et al.,* 2011; Lai *et al.*, 2008.

Sequences of the outgroup *Archimonocelis staresoi* Martens & Curini-Galletti, 1993 (Proseriata: Archimonocelididae) were those of Littlewood *et al.* (2000). No further species belonging to Monocelidinae were added to the dataset, since published sequences of both fragments were not available.

TABLE 1. List of species sampled and sequences used for this study. Accession numbers refer to GenBank codes; accession numbers of new sequences are in bold.

Species	Locality	SSU	LSU D1-D6
Pseudomonocelis paupercula **nov. sp.**	see text	**JN224901-904**	**JN224915-918**
Pseudomonocelis ophiocephala (Schmidt, 1861)	Porto Torres (Sardinia, Italy)	**JN224895**	**JN224907**
Pseudomonocelis agilis (Schultze, 1851)	Helsingør (Denmark)	**JN224897**	**JN224912**
Pseudomonocelis cf *cavernicola* Schockaert & Martens, 1987	Dongwe (Zanzibar)	**JN224900**	**JN224914**
Pseudomonocelis cetinae Meixner, 1943	Omiš (Croatia)	**JN224899**	**JN224913**
Pseudomonocelis occidentalis Curini-Galletti, Casu & Lai, 2011	Porto Pozzo (Sardinia, Italy)	**JN224894**	**JN224909**
Pseudomonocelis orientalis Curini-Galletti, Casu & Lai, 2011	Maliakós (Greece)	**JN224896**	**JN224908**
Minona ileanae Curini-Galletti, 1997	Great Bitter Lake (Egypt)	**JN224905**	**JN224910**
Monocelis lineata (Müller, 1773)	Ferrol (Galicia, Spain)	**JN224906**	**JN224911**
Archimonocelis staresoi Martens & Curini-Galletti, 1993	Porto Cesareo (Lecce, Italy)	AJ270152	AJ270166

The 18S and 28S D1-D6 sequences were aligned using Clustal W (Thompson *et al.*, 1994), implemented in BioEdit 7.0.5.2 software (Hall, 1999).

The best probabilistic model of sequence evolution was determined for 18S and 28S D1-D6 after evaluation by JModeltest 0.1 (Posada, 2008) with a maximum likelihood optimised search, using the Akaike Information Criterion (AIC). The model GTR+G for both 18S and 28S D1-D6 has been calculated as the best fitting.

Phylogenetic relationships among individuals and species were investigated using the Bayesian Inference (BI), the Maximum Likelihood (ML) and the Maximum Parsimony (MP), using the combined dataset of 18S and 28S D1-D6.

The software MrBayes 3.1.2 (Ronquist & Huelsenbeck, 2003) was employed for the BI, specifying a partitioned model and settings N_{ST} = 6, rates = gamma, ngammacat = 4. Two independent runs each consisting of four Metropolis-coupled MCMC chains (one cold and three heated chains) were run simultaneously for 5,000,000 generations, sampling trees every 1,000 generations. We allowed each partition to have his own set of parameters and a potentially different overall evolutionary rate. The first 25% of sampled trees were discarded. Convergence of chains was checked following the procedures described by Huelsenbeck & Ronquist (2001) and by Gelman *et al.* (1995). Nodes with a percentage of posterior probability lower than 95% are considered not highly supported.

ML was obtained using the genetic algorithm implemented in GARLI 2.0 (Zwickl, 2006). In order to find the best tree the configuration file for partitioned models was set up to perform 10 replicate searches (searchreps = 10). Model parameters: ratematrix = (0 1 2 3 4 5), ratehetmodel = gamma, numratecats = 4, corresponding to the evolution model suggested by JModeltest (GTR+G) for both 18S and 28S D1-D6 were used. In order to allow independent estimates of the parameters for each gene the option link was set to 0. In addition, since we have two models, Modweight was set to 0.0015. Finally, node support was assessed by 1000 bootstraps (bootstrapreps = 1000).

For MP (software PAUP*, Swofford, 2003), heuristic analyses with tree-bisection-reconnection (TBR) branch-swapping were used with 100 iterations each beginning with random starting trees. MP bootstrap support was assessed with 1000 bootstrap iterations (full TBR heuristic searches). For ML and MP, nodes with a percentage of bootstrap lower than 50% are considered not highly supported.

Fabio Scarpa

Molecular markers as a tool for phylogenetic studies in Proseriata (Platyhelminthes: Neoophora)

Tesi di Dottorato in Scienze della Natura e delle sue Risorse - Indirizzo: Biologia Ambientale - XXVI ciclo

Università degli studi di Sassari

Results

Taxonomy

Family Monocelididae Hofsten, 1907

Genus *Pseudomonocelis* Meixner, 1943

***Pseudomonocelis paupercula* Curini-Galletti, Casu & Lai nov. sp.**
(Figs 1, 2)

Material. Holotype: Italy, Sardinia, Porto Pozzo (41°11'20.22"N; 9°17'11.03"E), lower intertidal in brackish water; very silty mixed sand (May 2004); sagittally-sectioned (SMNH Type-8082). Paratypes: three specimens sagittally-sectioned (CZM 408 - 410); two whole mounts (in one slide) (CZM 411), same data as holotype. Additional material from the type locality: 10 specimens for karyology.

Greece, Gulf of Maliakòs, near Stylida (38°54'26.05"N; 22°37'22.25"E), lower intertidal in mixed, silty sand, near a fresh-water outlet (March 2004). 11 specimens, sagittally sectioned (CZM 412 - 422); 10 specimens for karyology.

Israel, Akko, Western Wall (32°55'29.94"N; 35°4'5.55"E), mixed sediment in rocky pools (October 1997). 10 specimens, sagittally sectioned (CZM 423 - 432); two specimens used for karyology.

Egypt, Alexandria, Montazah Palace (31°17'16.68" N; 30°0'35.16"E), mixed sediment at the mouth of a fresh-water outlet (October 2003). 15 specimens, sagittally sectioned) (CZM 433 - 447); 10 specimens for karyology.

Etymology. From lat. *pauper* poor. Named for the small and poorly visible copulatory organ, the unusually small chromosomes for the genus, and its overall, non-descript appearance.

Note. The authorship of the species is limited to the three authors who most effectively contributed to the species description.

Diagnosis. Unpigmented *Pseudomonocelis* with copulatory organ provided with a stylet 15-29 µm long. With swollen female duct, and an unarmed accessory organ close to the female pore.

Description. Fixed mature specimens up to 2.8 mm long (holotype: 2.5 mm; paratype: 2.2 mm), elongate and cylindrical (Fig. 1A). Living animals appear opaque due to gut content and internal organs, especially vitellaria. Without pigmented eyespots. Epithelium with insunk nuclei, ciliated. Cilia about 2.5 µm long ventrally, 2 µm dorsally. Ventral side entirely ciliated, apart from the area surrounding the female pore. Dorsally, the caudalmost area is unciliated. Frontal end with oily droplets. Ovoid rhabdoids 11-15 µm in length. 10-12 rhabdoid glands, particularly large and conspicuous, and easily noticeable in living specimens (Figs 1A, 2E), are present caudally. These glands lie deeply imbedded in the parenchyma (Figs 1D, 2D). Adhesive glands arranged radially at the posterior tip of the body. Animals can quickly modify the shape of the tail, from narrowly elongate to spatulate, and adhere strongly to the substrate.

Statocyst about 25 µm in diameter; containing one statolith about 15 µm long. The ovoid brain, 55 µm long and 50 µm wide in the holotype, abuts the statocyst. Body musculature formed by a very thin outer circular layer, and a thicker inner longitudinal layer. With a few transverse muscular fibres. Pharynx in mid-body, comparatively small, 150-200 µm long in mature specimens. Internally, it is ciliated in its distal half, with cilia 3-3.5 µm long; externally, it is uniformly ciliated (cilia 1–1.5 µm long), apart from the distal tip, where pharyngeal glands discharge. With a well developed oesophagus, about half the length of the pharynx. Musculature of pharynx inverted with respects to body musculature. The inner circular component is particularly strong distally, while it is not present below the oesophagus. Pharyngeal glands well developed, extending anteriorly in the body, and easily seen in living, squeezed specimens.

Male genital organs. With very few (5–10), ventral testes. Copulatory organ of the simplex-type (see Litvaitis *et al.*, 1996), placed close to the ovaries. In some large, otherwise mature animals the copulatory organ was totally lacking (CZM 412, 422, 436, 439) or presumably non functional (CZM 435, 440). The copulatory organ is formed by a seminal vesicle lined by a muscular sheath and a penis papilla provided with a stylet (Fig. 1C). Shape and size of the bulb strongly depend on contraction during fixation and amount of sperm content. It is 87 µm high, 81 µm wide in the holotype (Fig. 2C); much smaller and regularly spherical (32 µm across) in the paratype, where sperm was not present. The bulb of specimens from Akko ranged from 40 µm high and 35 µm wide to 105 µm high and

93 µm wide. In specimens from Maliakos, the bulb ranged 28–40 µm high, and 34–53 µm wide (Figs 2A, B). In specimens from Alexandria it ranged 24–43 µm and 20–64 µm respectively. The bulb is lined by an outer layer of circular musculature, and an inner layer of longitudinal musculature. The thickness of the muscular wall depends on its state of contraction. It is about 2 µm thick in the holotype, and ranges from up to 7.5 µm in specimens with a small, contracted bulb to less than 1 µm in diameter in the largest bulbs of specimens from Akko. The seminal vesicle is lined by a thin epithelium, which becomes high and glandular distally, where it is pierced by the outlet of prostatic glands, whose nuclei lie outside the bulb. The prostatic glands are few, and poorly developed. The penis papilla is small and conical, and is formed mostly by circular musculature. It is provided with a copulatory stylet. The stylet is pen nib-shaped, with a broader basis and a distal, gutter shaped pointed tip. The stylet is 26 µm long in the holotype (Fig. 2C), and ranged 15–29 µm in the sample. The stylet is wrapped by the penis papilla, and its basis may protrude into the bulb. It has been observed in all the sectioned specimens from the type locality, which were large, mature animals. In the other populations, it has been detected in most specimens; it was absent in few specimens only, with the smallest copulatory bulbs. The stylet is thus presumably formed at full male maturity. Nonetheless, due to its small size and poor sclerification, the stylet can be detected in mature living specimens with great difficulty. On the contrary, it is easily noticeable in sectioned specimens, as it is intensely eosinophilous. The male antrum is small and unciliated, and opens to the outside through the male pore.

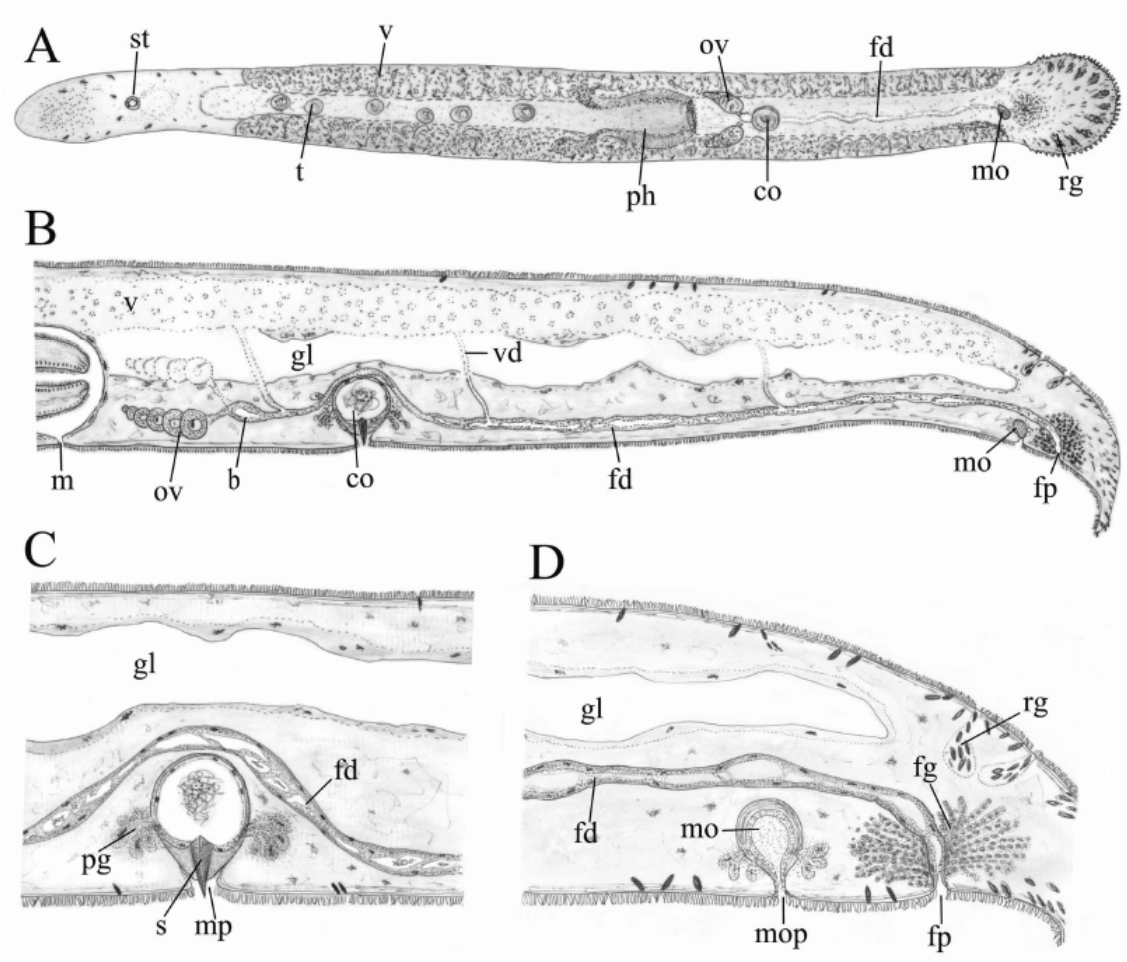

FIGURE 1. *Pseudomonocelis paupercula* **nov. sp.** A: general appearance; B–D: sagittal reconstructions of the post-pharyngeal regions (B), copulatory organ (C); female genital organ and muscular organ (D). Abbreviations: b: bursa; co: copulatory organ; fd: female duct; fg: female glands; fp: female pore; gl: gut lumen; m: mouth; mo: muscular organ; mop: muscular organ pore; mp: male pore; ov: ovaria; pg: prostatic glands; ph: pharynx; rg: rhabdoid glands; s: stylet; st: statocyst; t: testes; v: vitellaria; vd: vitelloduct.

Fabio Scarpa

Molecular markers as a tool for phylogenetic studies in Proseriata (Platyhelminthes: Neoophora)

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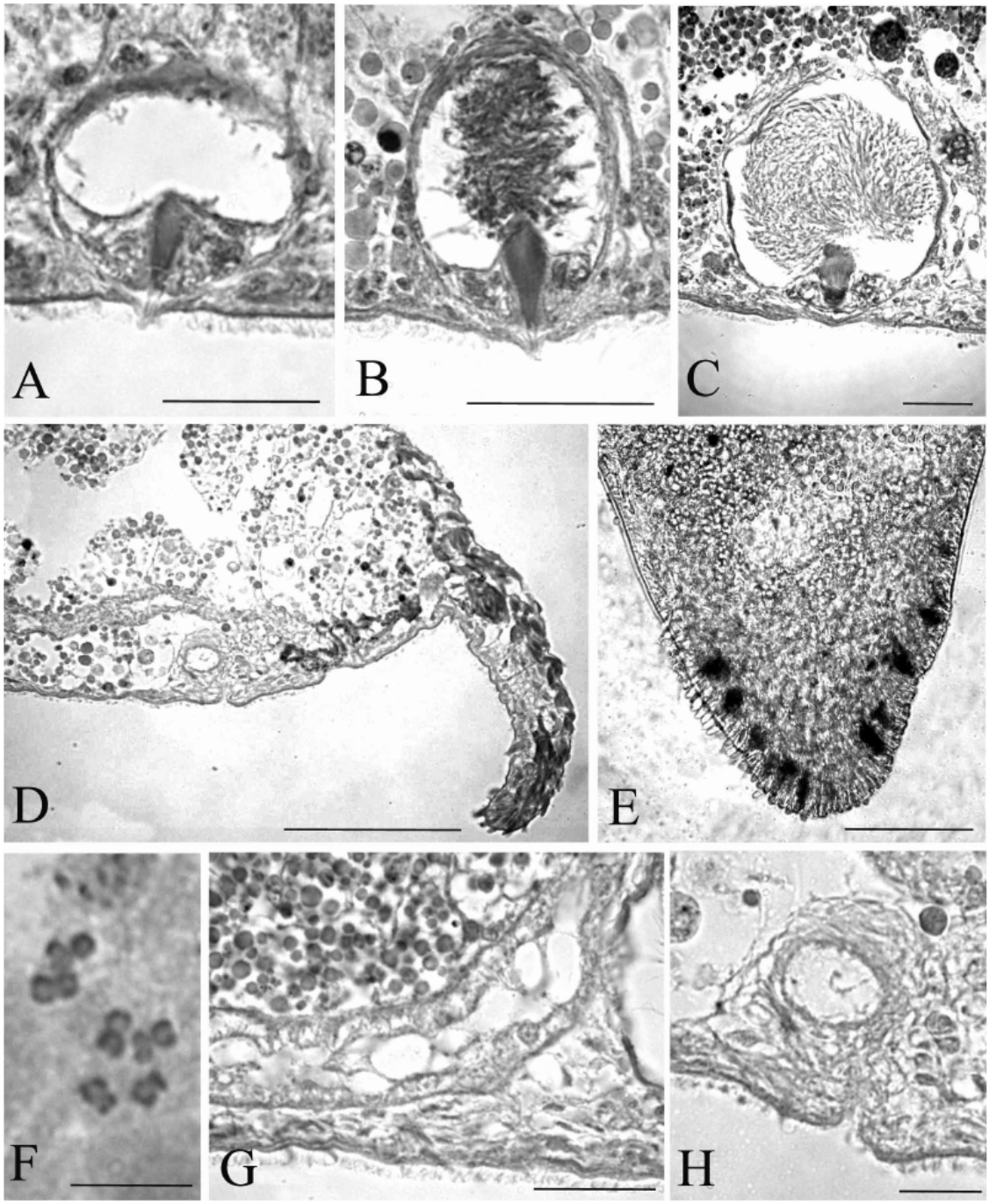

FIGURE 2. *Pseudomonocelis paupercula* **nov. sp.** A–C: sagittal sections of copulatory organs (A: CZM 413; B: CZM 414; C: SMNH Type-8082); D: sagittal section of the caudal area (CZM 408); E: caudal tip of a living specimen, showing the radial arrangement of rhabdoid glands; F: spermatogonial plate; G: detail of the female duct (SMNH Type-8082); H: detail of the muscular organ (CZM 408). Scale bar: A–C: 25 µm; D: 50 µm; E: 100 µm; F: 5 µm; G,H: 10 µm.

Female genital organs. With large, thick vitellaria, extending from behind the brain till the level of the copulatory organ. They are easily visible, due to their development, in living slightly squeezed specimens (Fig. 1A). Ovaria post-pharyngeal. Up to 15 oocytes per ovary are discernible in sections, the largest (up to 33 µm in diame-

ter) being the caudalmost. Shortly behind the ovaria, the two oviducts fuse into the female duct, which runs posteriorly and ventrally over the copulatory organ and opens to the outside through the female pore, just in front of the caudal end of the body (Fig. 1B). At the joining of the two oviducts, a few specimens show a widening, up to about 20 µm across, which may be interpreted as a small bursa (Fig. 1B). The entire female duct system is irregularly swollen along its course and is lined by a high, vacuolar epithelium (Fig. 2G). The development of vacuoles is to such an extent that the entire female duct, in sections, appears as lacking a continuous lumen. This peculiar lining extends to the oviducts, which are surrounded by vacuoles. Duct and vacuoles are often filled with sperm. Vitelloducts consisting of several, independent ducts (Fig. 1B). In some specimens, up to four vitelloducts could be traced, scattered along the length of the female duct, presumably connected to the overlying portion of the vitellaria. The most anterior vitelloducts join the oviducts, and are presumably connected to prepharyngean vitellaria. The female pore is surrounded by large, and numerous female glands (Fig. 1D).

Accessory organ. It is an ovoid, muscular organ, 14-17 µm wide, 16-25 µm high, located just in front of the female pore (Figs 1D, 2H). It is lined with a muscular sheath 2-2.5 µm thick, consisting mostly of longitudinal fibres, which are pierced by ducts of numerous glands, whose cell body lies outside the organ. The organ appears filled with the granular content produced by these glands, and opens to the outside through a short, narrow duct, very close to the female pore. Due to the reduced size of the organ, it is extremely difficult to discern in living specimens. In sections, it is easily detectable in large mature specimens only, and is absent in subadult specimens.

Karyotype. All populations showed karyotypes with haploid number n = 3, and F.N. = 6 (Fig. 2F). Chromosomes small and slightly differing in size: Chromosome 3 is about 75% of the length of Chromosome 1. Chromosomes 1 and 2 are metacentric; Chromosome 3 is submetacentric, with high index value. Karyometrical data of the populations used for statistical analysis (PP, MA, AL) are reported in Table 2. A unifactorial ANOVA based on the relative length and centromeric index of each chromosome and the haploid genome length was not significant, for any variable, at $P \leq 0.01$ (Table 2). Karyometrical data from two specimens from Akko closely corresponded to the other populations: Chrom. I = r.l.: 38.24 ± 3.32; c.i.: 46.33 ± 0.59; Chrom. II = r.l.: 32.45 ± 1.99; c.i.: 45.85 ± 1.45; Chrom. III = r.l.: 28.93 ± 5.74; c.i.: 35.65 ± 0.07;g.l.: 7.2 ± 0.8 µm.

TABLE 2. Karyometrical data (means ± SD) and results of the analysis of variance of three populations of *Pseudomonocelis paupercula* **nov. sp.**

		PP	MA	AL	$F_{(2,27)}$	P	
Chromosome 1	r.l.	38.15 ± 0.48	39.13 ± 0.45	38.61 ± 0.48	2.58	0.0944	n.s.
	c.i.	46.33 ± 0.65	47.18 ± 0.44	46.43 ± 0.27	0.93	0.408	n.s.
Chromosome 2	r.l.	32.96 ± 0.34	33.84 ± 0.22	32.43 ± 0.44	2.5	0.1009	n.s.
	c.i.	45.73 ± 0.53	46.41 ± 0.35	46.32 ± 0.49	0.64	0.5349	n.s.
Chromosome 3	r.l.	28.89 ± 0.69	27.03 ± 0.56	28.96 ± 0.84	3.95	0.031	n.s.
	c.i.	35.79 ± 1.13	35.61 ± 0.70	35.15 ± 0.93	0.12	0.884	n.s.
Haploid genome length (in µm)		7.5 ± 0.3	7.1 ± 0.2	7.2 ± 0.2	0.78	0.4682	n.s.

Molecular phylogenetic analysis

Sequences of 1672, and 1566 *bp* were obtained for 18S and 28S D1-D6 regions, respectively.

No sequence variation at both regions in any of the studied specimens was found. Therefore, in the trees (Figs 3-4), individuals of the same population have been joined into one terminal.

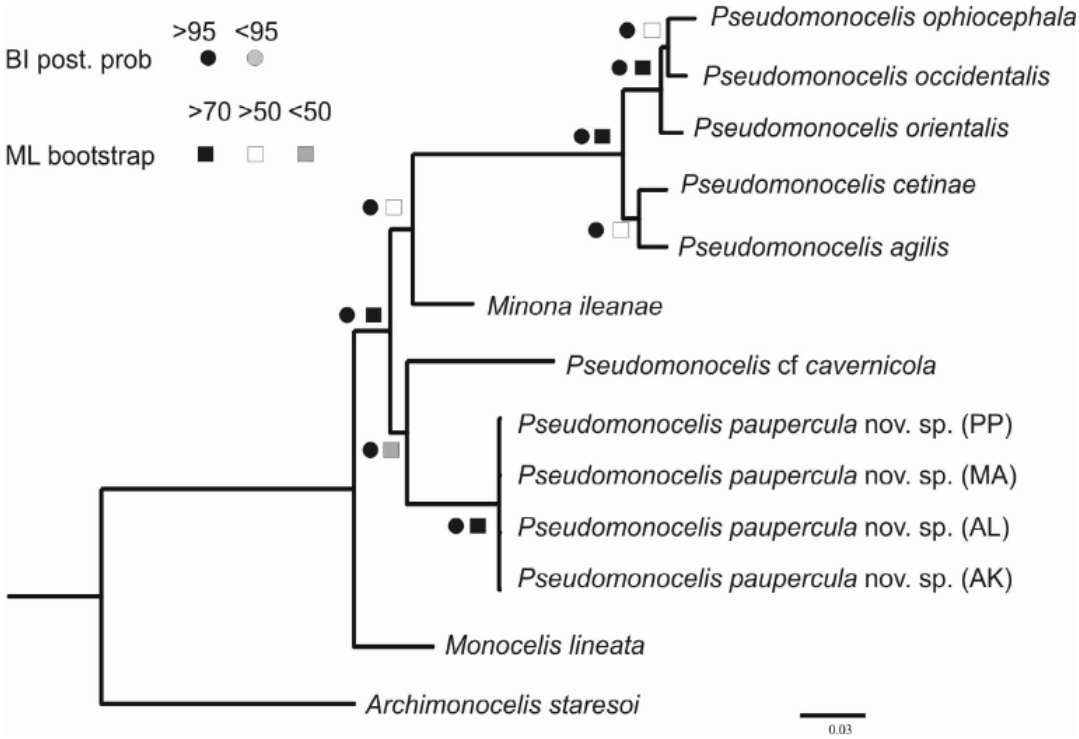

FIGURE 3. Tree obtained by BI showing the interrelationships of the species based on combined 18S+28S D1-D6. The branch length scale refers to the number of substitutions per site. Supports of main nodes are indicated for both BI (posterior probability) and ML (bootstrap) as a percentage.

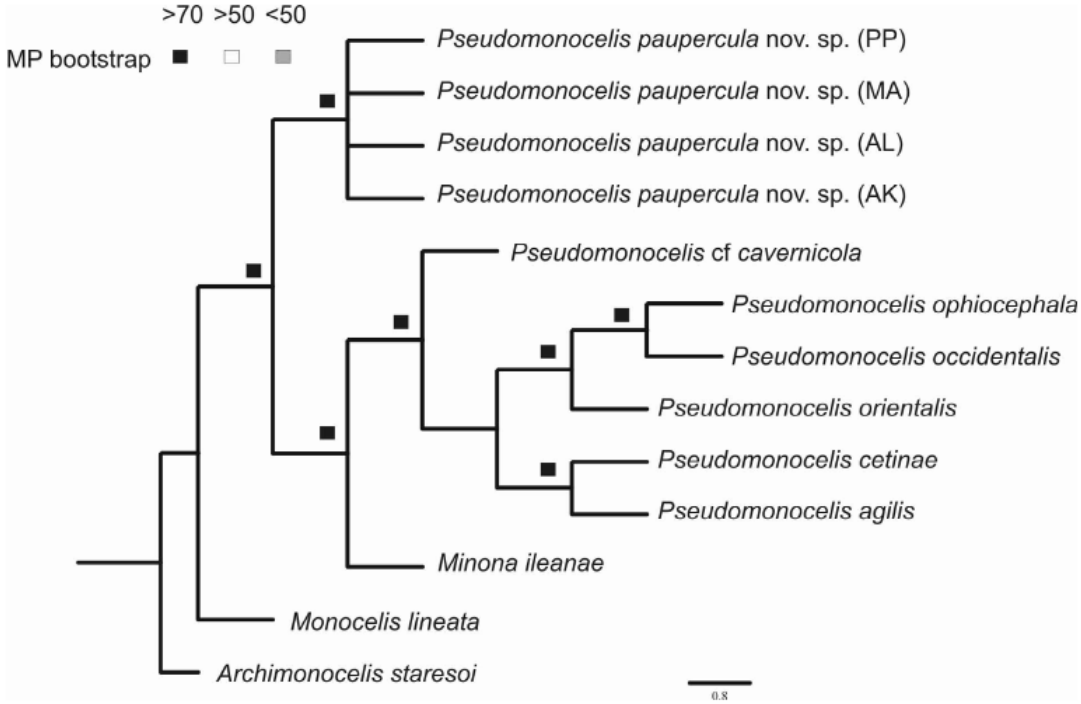

FIGURE 4. Tree obtained by MP showing the interrelationships of the species based on combined 18S+28S D1-D6. The branch length scale refers to the number of substitutions per site. Bootstrap nodal support is indicated as a percentage.

BI and ML were consistent in showing trees with the same topology, thus we reported the Bayesian trees only in Fig. 3. Both analyses do not resolve the genus *Pseudomononocelis* as monophyletic, since *M. ileanae* nests within it. *Pseudomonocelis paupercula* **nov. sp.** and *P.* cf *cavernicola* form a distinct clade (although not highly supported by both posterior probability and bootstrap values).

In the MP tree (Fig. 4), the new species of *Pseudomonocelis* is set as sister taxon of the remaining *Pseudomonocelis* species. Similarly to what is evidenced by BI and ML trees (Fig.3), *M. ileanae* is nested within the genus *Pseudomonocelis*. All nodes of the MP tree are highly supported.

Discussion

Species justification. Populations of *Pseudomonocelis paupercula* **nov. sp.** do not differ noticeably either in morphology and karyology (Table 2) across the range, which encompasses most of the Mediterranean. The new species is clearly distinct from congenerics, as it is the only species in the genus with a copulatory organ provided with a stylet. Its accessory organ is similar in position, size and, at least in part, morphology with that of *P. cavernicola.* However, in the latter species a glandular function has been excluded (Schockaert & Martens, 1987). Furthermore, *P. cavernicola* has a large bursa, provided with a vagina (Schockaert & Martens, 1987). The new species has the smallest haploid genome length (7.1-7.5 µm) known for the genus *Pseudomonocelis.* In the other species, genome length ranges from 9.1 µm (*Pseudomonocelis hoplites*) to 15.5 µm (*Pseudomonocelis caribbea* Curini-Galletti & Casu, 2005) (Curini-Galletti, 1997; Curini-Galletti & Casu, 2005). Sequences from *P. paupercula* **nov. sp.** form a highly supported and well separated cluster, meriting the designation of a new species.

Phylogenetic relationships. Our molecular sample, although limited, included species of the genus *Pseudomonocelis*, with postpharyngeal ovary, and species with prepharyngeal ovary (*Monocelis lineata*, *Minona ileanae,* and *Archimonocelis staresoi*). One of these species, *Minona ileanae,* is provided with an accessory (prostatoid) organ, with a stylet. This feature was given particularly relevant phylogenetic weight, and was used to split the Monocelididae into two subfamilies, based on the presence (Minoninae) or absence (Monocelidinae) of the organ (Karling, 1966). Later, Litvaitis *et al.* (1996), based on molecular, morphological and karyological data, considered the presence of the accessory organ as the plesiomorphic feature for the Monocelididae. Present systematic arrangement of the Monocelididae is mainly based on the morphology of the copulatory bulb, of the simplex-type in the Monocelidinae and of the duplex-type in the Duplomonocelidinae (Litvaitis *et al.*, 1996). However, the presence/absence of the accessory organ is still used to support the validity of otherwise morphologically identical genera, such as *Minona* and *Monocelis* in the Monocelidinae, and *Duplominona* Karling, 1966 and *Archilina* Ax, 1959 in the Duplomonocelidinae, respectively with and without this feature (Litvaitis *et al.*, 1996; Curini-Galletti *et al.*, 2010).

Results of the combined 18S + 28S D1-D6 analysis are conflictual with current, morphology-based definition of genera. In fact, the nesting of *M. ileanae* within *Pseudomonocelis* species shown by BI, ML and MP does not support the monophyly of the genus *Pseudomonocelis* (Figs 3-4). Furthermore, this placement of *M. ileanae* is particularly difficult to justify on morphological grounds. Not only would it, in fact, require repeated losses of the accessory organ or of its stylet, but the most parsimonious explanation of the prepharyngeal position of the ovary in *M. ileanae* would then be that a reversal of the backward migration of the ovary has occurred in this species, further jeopardising present tenets in the taxonomy of the group. Interestingly, BI and ML trees (Fig. 3) suggest a sister-species relationships between *P.*cf *cavernicola* and *P. paupercula* **nov. sp.**. This would give further strength to the hypothesis that the presence of the accessory, muscular organ without stylet close to the female pore is a synapomorphic feature for the two species. However, this solution is poorly supported and the position of *P.* cf *cavernicola* is not consistent between BI and ML trees and the MP tree (Fig. 4). Thus, the limited sampling and the risks of parallelism in the group do not justify further (phylogenetic and biogeographical) considerations.

Conclusions

The present study highlights the problems of genera delimitation in a taxon such as the Monocelidinae, with comparatively very few morphological diagnostic characters, and where combination of character states may arise by parallel evolution. Given the conflicting results shown by the trees constructed on the combined 18S and 28S D1-D6 dataset, the need of a more extensive molecular and taxonomic sampling is apparent. On the other hand, this

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research provides further evidence on the usefulness of rRNA fragments as markers for the molecular taxonomy of the Monocelidinae in which group the universal primers of the Cytochrome c Oxidase subunit 1 (the so-called Folmer region) often fail to produce adequate sequences (see e.g. Sanna *et al.*, 2009).

Finally, it is worth mentioning that *Pseudomonocelis paupercula* **nov. sp.** has been only very rarely encountered during samplings in the Mediterranean and, even in the stations where it has been found, was strictly confined to sectors characterised by the presence of fresh-water outlets and mixed, silty sediment. Given the threats that coastal fresh-water habitats are facing in the Mediterranean, the species appears potentially vulnerable.

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Chapter 2

First evidence of self-fertilization in a marine microturbellarian (Platyhelminthes)

Marco Casu, Piero Cossu, Tiziana Lai, Fabio Scarpa, Daria Sanna, Gian Luca Dedola, Marco Curini Galletti

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Marco Casu *, Piero Cossu, Tiziana Lai, Fabio Scarpa, Daria Sanna, Gian Luca Dedola, Marco Curini-Galletti

Dipartimento di Scienze della Natura e del Territorio, Università di Sassari, Via F. Muroni 25, 07100 Sassari, Italy

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ABSTRACT

The reproductive strategy of the simultaneous hermaphrodite *Pseudomonocelis paupercula* (Platyhelminthes: Proseriata) was investigated using multiple approaches including breeding experiments, anatomical reconstruction, and parentage analysis of offspring. The 18 allozyme loci tested were monomorphic. Conversely, the ISSR markers showed differences among the populations, and allowed us to ascertain whether the offspring were derived from selfing or cross-breeding. The results suggest that selfing is the most common mode of reproduction in this species, with only 8% of the offspring resulting from cross-reproduction. Age at first reproduction of selfers does not differ from that of paired, potentially cross-breeding, specimens. The presence of sperm in the female ducts of individuals that have been isolated since birth suggests the existence of a connection between the male and female reproductive systems that allows self-fertilization. Habitat is suggested to be the key factor shaping the reproductive strategy of the species. *P. paupercula* is found in highly fragmented brackish-water microhabitats, and selfing may allow for colonization of new habitats that can start from single, unfertilized specimens.

1. Introduction

Hermaphroditism occurs in the majority of the animal phyla and is prevalent in major taxa including *Platyhelminthes*, *Gastrotricha*, *Pulmonata*, *Clitellata* and *Ascidiacea*. However, relatively few cases of selfing have been reported in animals (Jarne and Auld, 2006; Lamy et al., 2012). In fact, the negative genetic and evolutionary consequences of selfing (see Jarne and Charlesworth, 1993) are so significant that many hermaphroditic species display differential timing in the maturation of gonads, and simultaneous hermaphrodites may delay the onset of reproduction in the absence of mates (Escobar et al., 2011).

Yet, in marine habitats selfing may confer a significant advantage to poorly vagile or sessile species when the densities of conspecifics are low and mate availability is problematic. In the case of hermaphroditic, free-spawning species, self-fertilization may be determinant for assuring reproductive success in cases of sperm limitation and/or dilution (Brazeau et al., 1998). Furthermore, isolated, self-fertile individuals can rapidly establish populations in new habitat spaces (Ryland and Bishop, 1990), and high rates of selfing combined with phylopatry may allow for local adaptation (Parker, 1991).

A potentially viable alternative could be a mixed-mating system, where individuals shift between selfing and cross-breeding, according to the situation. The rarity of this reproductive strategy in animals has been explained by genetic models, which predict evolution towards pure selfing or pure outcrossing as a result of the disruptive selection against intermediate selfing rates (Lande and Schemske, 1985). However, in ecological models, forces external to the genetic costs and the benefits of selfing may lead to mixed-mating (Goodwillie et al., 2005), and the extent to which selfing occurs may reflect the diversity of the selective forces operating in the particular ecological setting where the species is present (Husband and Schemske, 1996).

The predominantly simultaneous hermaphroditic *Platyhelminthes* are among the taxa where alternative strategies to obligate cross-breeding are present. However, although selfing has been reported in parasitic taxa and in *Tricladida* (Jarne and Auld, 2006; Sluys, 1989), it has not been previously documented in marine microturbellarians, which include minute, predominantly interstitial, free-living marine *Platyhelminthes*. These organisms lack dispersal stages and occurring at low densities in the sediment (Curini-Galletti, 2001), set a plausible ecological scenario for the evolution of selfing.

Preliminary observations of isolated specimens of a recently described species, *Pseudomonocelis paupercula* Curini-Galletti, Casu and Lai, 2011 (Platyhelminthes: Proseriata), laying fertile cocoons suggested that the species may be capable of self-fertilization. However, on the basis of chance observations alone, the possibility of the long-term viability of allosperm in the female reproductive tract could not be ruled out. In this paper, the presence and relevance of selfing in this species was studied using multiple approaches including breeding experiments, anatomical reconstructions and parentage analysis of offspring.

* Corresponding author. Tel.: +39 079 228924; fax: +39 079 228665.
E-mail address: marcasu@uniss.it (M. Casu).

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2. Materials and methods

2.1. Sampling

The specimens of *P. paupercula* used in this study were collected from the following locations:

- Porto Pozzo (PP), Sardinia, Italy (Western Mediterranean) (41°11′ 20.22″N; 9°17′11.03″E);
- Maliakós (MA), Greece (Aegean Sea, Eastern Mediterranean) (38°54′26.05″N; 22°37′22.25″E);
- Alexandria (AL), Egypt (Levantine Sea, Eastern Mediterranean) (31°17′16.68″ N; 30°0′35.16″E);
- Akko (AK), Israel (Levantine Sea, Eastern Mediterranean) (32°55′ 29.94″N; 35°4′5.55″E).

At each station, 2 l of sediment was collected from the water line to approximately 20 cm deep by scraping the upper surface of the sand. The specimens were isolated from the sediment using the $MgCl_2$ technique (see Casu et al., 2011a for details).

Cultures of the specimens were maintained in the laboratory at 18 ± 0.5 °C and 37‰ salinity and fed weekly with crushed *Sphaeroma* sp. (Isopoda).

2.2. Experiments on reproductive biology

The main questions addressed were as follows: i) is selfing present in the species and is it relevant compared to cross-breeding and ii) what is the pathway through which selfing is achieved?

1) Testing selfing and its relevance. To quantify the number of offspring produced by isolated specimens and by pairs, a set of experiments was performed using newly hatched individuals (NHI) from the populations from Akko and Porto Pozzo:
   - Isolated specimens: 20 NHI per population were isolated into 20 ml containers filled with seawater, and maintained under the culture conditions described for the stock populations;
   - Paired specimens: 20 pairs, each pair consisting of two NHI from the same population, maintained as above.

   The specimens who died before reaching maturity were replaced with others maintained in individual containers as backups, to obtain the necessary number of replicates.

   The offspring were produced by specimens kept in pairs and in isolation. The offspring were counted and relocated weekly. To avoid bias due to senescence, only the 10 weeks following the hatching of the first offspring were considered in the calculation of the reproductive output.

   ANOVAs were used to analyze differences in a) the age of first reproduction by singles and pairs of the same population (i.e., the age when the first cocoons were laid – because it was impossible to assign the cocoon to one individual in the paired specimens, the laying of the first cocoon was taken as the onset of reproduction for both individuals) and b) the reproductive output (i.e., the number of offspring produced per individual per week) of individuals and pairs of specimens from the same population – the offspring were equally subdivided between parents for the paired specimens. To test the quantitative relevance of selfing, both the hypotheses, H_1: the average number of offspring produced by the isolated individuals = the average number of offspring produced by the pairs; and H_2: the average number of offspring produced by the isolated individuals × 2 = the average number of offspring produced by the pairs, were tested.

   One-way ANOVA was run for the variable "the age of first reproduction" separately for each population where the culture condition (isolation vs. pairs) was treated as a fixed factor. The variable "reproductive output" was analyzed by means of a one-way ANOVA, with population (PP and AK) and culture condition as random and fixed factor respectively. The Cochran's C-test was used to test the assumption of the homogeneity of variance, and the log-normal transformation was used to remove heteroscedasticity. The differences were deemed to be significant when $P \leq 0.05$. The Student–Newman–Keuls (SNK) test was used to compare the means following significant effects found in the ANOVA (Underwood, 1997).

2) Morphological study. Twenty NHI from Porto Pozzo were kept in individual containers until the birth of the first offspring. The specimens were then fixed in Bouin's fluid, embedded in 60 °C Paraplast and cut into 4-μm serial sagittal sections and stained with Hansen's haematoxylin and eosin-orange, and then mounted in Eukitt. The voucher slides were deposited in the collection of the Zoological Museum of the University of Sassari (Italy) (CZM).

2.3. Molecular analysis

Molecular analyses were performed to test the i) within and among population variability and ii) to ascertain whether the offspring produced by the specimens kept in pairs were derived from selfing or cross-breeding.

The genetic structure of *P. paupercula* was evaluated using both allozyme electrophoresis and ISSR (Inter-Simple Sequence Repeat) techniques (Zietckiewicz et al., 1994). Forty wild-caught individuals (ten per each population) were analyzed using both molecular markers.

To assess the origin of the offspring (i.e., whether they were derived from cross-breeding or from selfing, and in the latter case, from which parent) we performed a series of experiments pairing specimens from populations, which, based on results of the genetic population studies, showed adequate differences in the ISSR banding patterns. Thus, five pairs of NHI consisting of the combination AL × MA or AK × PP were formed and maintained in the culture conditions described above. Additionally, as a control of the uniformity of the ISSR banding pattern in offspring obtained by selfing, two NHI specimens per population (AL, MA, AK, and PP) were isolated and reared individually in the same culture conditions. The offspring produced by the pairs and isolated individuals were removed and fixed in 95% ethanol for molecular analysis. The parents were then fixed. All of the specimens were stored at 4 °C until the DNA was extracted. The ISSRs were used for the parentage analysis.

2.3.1. Allozyme electrophoresis

A total of 22 enzyme systems were assayed in Tris EDTA maleate electrode buffer [Tris (0.1 M), EDTA (0.01 M), $MgCl_2$ (0.001 M), corrected to pH 7.8 with maleic acid] (Table 1). Each specimen was placed in a 25 μl microwell containing 5 μl of 0.05 M Tris pH 8.0 grinding buffer, where it was manually homogenized. The supernatant fractions were applied to cellulose acetate membranes for electrophoresis (25–30 min at 350 V). The samples were maintained at less than 5 °C at all stages. The enzyme staining was performed according to the procedures described by Pasteur et al. (1987), with slight modifications. Enzymes with low activity and resolution were discarded, resulting in a focus on 12 enzymes (Table 1). Before estimating within and among-population genetic variability (see Casu and Curini-Galletti, 2006 for the routine analyses performed on allozymes), the arbitrary value of 100 was assigned to the most common allele.

2.3.2. ISSRs

The genomic DNA was extracted using the QIAGEN DNeasy Tissue kit (QIAGEN Inc.) according to the supplier's instructions. After extraction, the DNA was stored in solution at 4 °C. When amplification was poor, we used the GenomiPhi DNA Amplification Kit (GE Healthcare), a whole genome amplification kit that can perform unlimited DNA tests from small samples. A set of 18 primers provided by Proligo Primers and Probes (Proligo France SAS) was used as a preliminary screening to achieve wide genome coverage (Table 2). We chose to use only primers anchored at the 3′ end, to obtain greater reaction specificity and to minimize problems due to homoplasy and/or homology of bands.

Table 1
Enzyme systems screened in *Pseudomonocelis paupercula* and total number of loci and alleles detected. EC: enzyme commission number; EA: enzymatic activity NL: number of loci detected; NA: number of alleles detected.

Code	Enzyme	EC	EA	NL	NA
ADH	Alcohol dehydrogenase	1.1.1.1	–	nr	nr-
AK	Adenylate kinase	2.7.4.3	–	nr	nr-
ALD	Aldolase	4.1.2.13	–	nr	nr-
ALKP	Alkaline phosphatase	3.1.3.1	–	nr	nr-
AO	Aldehyde oxidase	1.2.3.1	–	nr	nr-
APK	Arginine kinase	2.7.3.3	+	1	1
FH	Fumarate hydratase	4.2.1.2	–	nr	nr-
FK	Fructose kinase	2.7.1.3	–	nr	nr-
GAPDH	Glyceraldheyde-3-P dehydrogenase	1.2.1.12	+	3	1,1,1
GLD	Glutamate dehydrogenase	1.4.1.2	–	nr	nr-
αGPD	Glycerol-3-phosphate dehydrogenase	1.1.1.8	+	2	1,1
GPI	Glucose-6-phosphate isomerase	5.3.1.9	+	1	1
G6PDH	Glucose-6-P dehydrogenase	1.1.1.49	+	2	1,1
HK	Hexokinase	2.7.1.1	–	nr	nr-
IDH	Isocitrate dehydrogenase	1.1.1.42	+	1	1
LDH	L-lactate dehydrogenase	1.1.1.27	+	2	1,1
MDH	Malate dehydrogenase	1.1.1.37	+	2	1,1
ME	Malic enzyme (NADP+)	1.1.1.40	+	1	1
MPI	Mannose-6-phosphate isomerase	5.3.1.8	–	nr	nr-
PEP	Leu-Gly peptidase	3.4.11.26	+	1	1
PGDH	Phosphogluconate dehydrogenase	1.1.1.44	+	1	1
PGM	Phosphoglucomutase	5.4.2.2	+	1	1

+: excellent or good; –: scarce or absent; nr: not resolved.

The PCR reaction mixture and the amplification conditions were performed as described in Casu et al. (2008). For all primers, negative controls and replicates were included in the amplifications to check for the occurrence of PCR artifacts and to verify the repeatability of the results. The PCR products were analyzed with a 100 base pair (bp) ladder by electrophoresis (4 V/cm for 3 h) using a 2% agarose gel in 1× Tris-acetate-EDTA buffer, and subsequently stained by soaking the gel in a 1 μl/10 ml ethidium bromide solution for the UV visualization.

The method proposed by Zhivotovsky (1999) was chosen to estimate the allelic frequencies from the datasets of dominant markers. We assumed a moderate ($F_{IS}=0.10$) Hardy–Weinberg disequilibrium because our research on other Proseriates (Casu and Curini-Galletti, 2004, 2006; Casu et al., 2009a) showed a low to medium deficit of heterozygotes. Under this hypothesis, the number of polymorphic bands (percentage of polymorphisms at the 99% cut-off level), and heterozygosity (H) were calculated by using AFLP-SURV 1.0 (Vekemans, 2002). Unique bands (Luque et al., 2002) for each population and within the Western and Eastern Mediterranean (Aegean and Levantine seas) were also computed.

Table 2
ISSR primer names and primer sequences, range of molecular weight in base pairs (bp) and number of (polymorphic) bands per primer. BR: banding resolution; NB: number of bands; NPB: number of polymorphic bands (P_{99} cut-off criterion).

Primer	Sequence (5′-3′)	BR	Size range of bands	NB	NPB
IT1	$(CA)_8GT$	–	nr	nr	nr
IT2	$(CA)_8AC$	–	nr	nr	nr
IT3	$(CA)_8AG$	–	nr	nr	nr
PT1	$(GT)_8C$	–	nr	nr	nr
SAS1	$(GTG)_4GC$	+	1000-430	14	7
SASS2	$(CTC)_4GC$	–	nr	nr	nr
SAS3	$(GAG)_4GC$	–	nr	nr	nr
SAS4	$(CAC)_5GC$	–	nr	nr	nr
SAS5	$(GTG)_5TC$	–	nr	nr	nr
SAS6	$(AT)_8G$	–	nr	nr	nr
SAS7	$(GC)_8C$	–	nr	nr	nr
UBC809	$(AG)_8G$	+	600	1	0
UBC811	$(GA)_8C$	+	1250-450	12	4
UBC827	$(AC)_8G$	+	1500-450	15	11
UBC868	$(GAA)_6$	–	nr	nr	nr
814	$(CT)_8TG$	–	nr	nr	nr
844A	$(CT)_8AC$	–	nr	nr	nr
844B	$(CT)_8GC$	–	nr	nr	nr

+: excellent or good; –: scarce or absent; nr: not resolved.

The underlying genetic structure and the occurrence of cross-breeding was inferred by an individual-based approach using the Bayesian model-based clustering algorithm implemented by the STRUCTURE 2.2.3 software (Falush et al., 2003; Pritchard et al., 2000). To assess the number of genetic clusters that best fits the data, we ran an algorithm for K ranging from 1 to 4. For each value of K, ten independent runs were performed applying the admixture model with correlated allelic frequencies (Falush et al., 2003), each one consisting of 100,000 iterations after a 100,000 burn-in period (see Pritchard and Wen, 2004 for the method). Following Pritchard and Wen (2004), we used the highest posterior probability of data averaged over the ten runs to choose the model that best fit the available data.

The method implemented in STRUCTURE was also adapted to identify individuals derived from cross-breeding. The individuals are probabilistically assigned to populations or, in the case of admixed ancestry, jointly to parental populations. In a genetic admixture analysis with the a priori assumption that $K=2$, it is assumed that there are two populations contributing to the gene pool of the sample. The admixed ancestry is modeled by assuming that an individual (i) has inherited some fraction (q) of its genome from ancestors in the population (k) (Pritchard et al., 2000). The individuals derived from cross-breeding can then be inferred when they are intermediate between the two clusters in terms of the Q-value. Following Vähä and Primmer (2006), the distinction between individuals derived from cross-breeding and individuals derived from selfing was assessed by applying a threshold Q-value of 0.2, i.e., any individual with Q-value between 0.2 and 0.8 is classified as a "hybrid". Five independent runs assuming two populations ($K=2$) with 100,000 iterations after a burn-in of 100,000 under the admixture model with correlated allelic frequencies were performed.

We also used a second method, implemented in NEWHYBRIDS 1.1 (Anderson and Thompson, 2002), which is more specifically aimed at detecting hybrids (in our case, specimens derived from cross-breeding) and estimates the probability of individuals belonging to different genotype classes (hybrid, non-hybrid). In the model, each individual's genotype frequency class (i.e., hybrid category) is inferred providing a posterior probability that reflects the level of certainty that an individual belongs to a certain hybrid group. A posterior probability value of 50% was used as a threshold for assigning an individual to a specific class. The posterior probability values for all hybrid classes for an individual were summed and used as one estimate to determine the accuracy/efficiency with which hybrids, regardless of hybrid class, could be distinguished from non-hybrids. Runs were based on 100,000 MCMC sweeps following a burn-in period of 100,000 iterations and assuming Jeffrey's priors to estimate the mixing proportions and allelic frequencies. Longer runs were then tested, but the results did not improve.

3. Results

3.1. Reproductive biology

P. paupercula, as most mesopsammic flatworms, has a direct development. The juveniles hatch singly from the adhesive cocoons approximately 10 days after deposition and immediately start feeding.

3.1.1. Age at first reproduction

The experiments revealed no significant differences in the age of the deposition of the first cocoons between isolated specimens and pairs from Akko and Porto Pozzo (Table 3).

There were, however, significant differences between the two populations either among single specimens ($F=98.55$; $P<0.01$) and pairs ($F=34.23$; $P<0.05$).

Table 3
Comparison of age at first reproduction (in days ± standard deviation) of specimens from different populations (Akko and Porto Pozzo) reared individually and in pairs. n.s.: not significant at the 5% level.

Population	Isolated specimens	Paired specimens	Cochran's C test	MS	F	P
Akko	45.67 ± 3.37	49.25 ± 2.24	0.6391 n.s.	77.0417	0.78	0.3865 n.s.
Porto Pozzo	55.66 ± 2.82	55.79 ± 3.33	0.5811 n.s.	2.6667	0.02	0.8799 n.s.

3.1.2. Quantitative relevance of selfing

During the ten week period of the experiment, the isolated specimens from Akko produced 10.75 ± 2.31 offspring per week per specimen. Conversely, the paired specimens produced 19.65 ± 2.59 offspring per week per pair. In the parallel experiments with specimens from Porto Pozzo, the isolated individuals produced 12.15 ± 1.92 offspring per week, while pairs produced 20.7 ± 1.46 offspring. The reproductive outputs of isolated specimens of the two populations, as well as that of paired specimens, were not significantly different ($F = 0.25$; $P = 0.6168$ and $F = 0.16$; $P = 0.6926$ respectively). Thus, the one-way ANOVA was run on the pooled population data (Table 4). H_1 was rejected (P significant at the 5% level). The SNK test showed that pairs produced significantly more offspring than isolated individuals. Alternatively, H_2 could not be rejected (P not significant at the 5% level), and the SNK test did not reveal any difference in the means between pairs and isolated individuals.

Thus, for both populations, the number of offspring produced by the pairs was significantly higher than the number produced by the isolated individuals, whereas the number of offspring produced by the pairs was not significantly different than twice the number produced by the isolated individuals, suggesting that each specimen, regardless of whether it was isolated or paired, produced a comparable number of offspring.

3.1.3. Anatomy

Of the 20 specimens reared in individual containers, slides of adequate quality were obtained from 15 specimens (CZM 457–471). Two of the specimens died before reaching maturity, and mishandling during the process of inclusion and sectioning accounted for the additional losses.

The morphology of the species has been described in detail by Casu et al. (2011a). Of the 15 specimens that were sectioned, 11 specimens showed the presence of sperm in the female ducts. In particular, in most specimens, an accumulation of sperm was observed in the oviducts in close proximity to the eggs (Fig. 1A). The four specimens where no sperm was clearly detected in the female ducts were also the low-quality mounts, and this may account for the lack of observable sperm. In all of the specimens, sperm was observed in the seminal vesicle, which is included within the copulatory bulb (Fig. 1B). No direct connection between the seminal ducts and the female reproductive tract could be traced.

Table 4
Comparison of reproductive output (number of offspring produced per week ± standard deviation) of specimens from different populations (Akko and Porto Pozzo) reared individually and in pairs. Hypotheses tested: H_1 = significance of the difference in the number of offspring produced by the isolated individuals vs. the number of offspring produced by the pairs; H_2 = significance of the difference of the double of the number of offspring produced by the isolated individuals vs. the number of offspring produced by the pairs. n.s.: not significant at the 5% level.

Populations		Cochran's C test	MS	F	P
Akko + Porto Pozzo	H_1	0.3476 n.s.	14.7061 n.s.	17.13	0.0001
	H_2	0.4009 n.s.	0.1222 n.s.	0.24	0.6249 n.s.

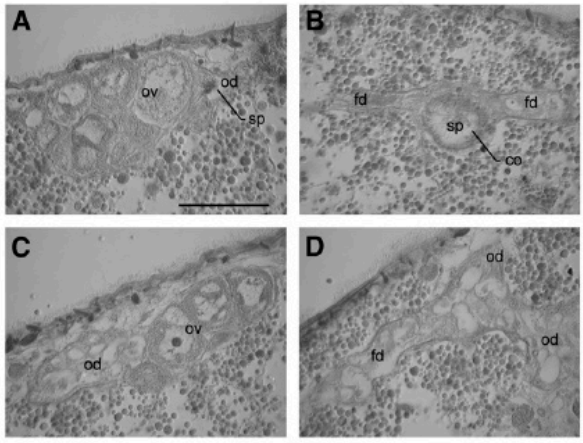

Fig. 1. *Pseudomonocelis paupercula*. Sagittal sections of specimens isolated since birth, showing: A: the presence of sperm in the oviducts; B: the female duct wrapping the copulatory organ; C, D: the extent of the vacuolarity of the oviducts and female duct. A, B, from specimen CZM-457; C, D, from specimen CZM-458. Scale bar = 50 μm. Abbreviations: co = copulatory organ; fd = female duct; od = oviduct; ov = ovary.

3.2. Molecular analysis

3.2.1. Allozyme electrophoresis

The 12 enzymes assayed showed 18 loci. Each was monomorphic across all individuals of all populations (Table 1). Therefore, lacking any allozyme variability, no statistical analyses could be performed.

3.2.2. ISSR

3.2.2.1. Population genetics. Four of the 18 ISSR primers that were scored (SAS1, UBC809, UBC811, UBC827) yielded banding patterns that were clear and could be scored with confidence (Table 2). The four primers amplified 42 fragments, which ranged from 430 (SAS1) to 1500 (UBC827) bp (Electronic Appendix Table 1A, B). The number of bands obtained from each ISSR primer ranged from one (UBC809) to 15 (UBC827) (Electronic Appendix Table 1A, B). The number of polymorphic bands (NPB) and heterozygosity (H) were similar for the four samples (PP, MA, AL, AK) analyzed and were between NPB = 83.3–85.7%, and $H = 0.176$–0.220 (Table 5). On a total of forty individuals, we found three unique bands (UBC811: 900; UBC827: 600, 520) in the Western Mediterranean sample (PP), whereas no unique bands were found in any of the Eastern Mediterranean samples (MA, AL, AK) (Table 5; Electronic Appendix Table 1A, B). However, the three Eastern Mediterranean samples shared 5 bands not present in PP (SAS1: 600, 480; UBC811: 700, 670; UBC827: 620). Two additional bands (UBC827: 1100, 700) were uniquely shared by the Levantine Sea populations (AL, AK) (Electronic Appendix Table 1A, B). Note that different ISSR band profiles occur within each population (Electronic Appendix Table 1B).

Results of STRUCTURE analysis (Fig. 2) showed the occurrence of three genetic clusters. Individuals from Porto Pozzo (Western Mediterranean) and from Maliakós (Aegean Sea) were grouped into distinct clusters. The third cluster grouped together individuals from the Levantine Sea (Alexandria and Akko) (Fig. 2).

3.2.2.2. Parentage analysis. Of the ten pairs (five each of Al × MA and AK × PP), results were obtained from all AL × MA pairs (which produced a total of 32 offspring) (Electronic Appendix Table 2A) and from one out of five AK × PP pair (which produced a total of five offspring) (Electronic Appendix Table 2B). The loss of data from the AK × PP cross-breeding experiment was due to a failure in the DNA extraction and/or amplification of the parents and/or offspring. The genotypes of the offspring were

Table 5
Summary of ISSR-PCR products per sampling station. %PB: percentage of polymorphic bands; *H*: expected heterozygosity; SE: standard error; NUB: number of unique bands; PP: Porto Pozzo; MA: Maliakós; AL: Alexandria; AK: Akko; W-Med.: Western Mediterranean; E-Med: Eastern Mediterranean.

	%PB	*H* (± SE)	NUB
PP (W-Mediterranean)	83.3	0.209 ± 0.024	3
MA (Aegean Sea)	85.7	0.176 ± 0.019	0
AL (Levantine Sea)	85.7	0.216 ± 0.024	0
AK (Levantine Sea)	85.7	0.220 ± 0.025	0
AL, AK (Levantine Sea)	–	–	2
MA, AL, AK (E-Mediterranean)	–	–	7

also determined for eight individuals (two from each population) from the selfing experiments (Electronic Appendix Table 2A, B).

Two independent datasets were analyzed: the AL × MA group which consists of 67 individuals, 25 of which were derived from the selfing experiment and 42 were derived from the pairings (10 parents and 32 offspring) (Electronic Appendix Table 2A) and the AK × PP group which consists of 29 individuals, 22 of which were derived from the selfing experiment and seven of which were derived from the pairings (two parents and five offspring) (Electronic Appendix Table 2B). For each of the two datasets, the five independent runs of the STRUCTURE algorithm gave highly consistent results bearing exactly the same solution. Each individual was clearly distinguished according to their population of origin (Figs. 3A, 4A). Three out of the five AL × MA crosses showed offspring derived from cross-breeding, one offspring out of nine, five, and seven, in the 2nd, 3rd, and 4th crosses, respectively (2 OFa 6, 3 OFa 2, and 4 OFa 4, in Electronic Appendix Table 2A). In contrast, all the offspring from the cross between AK and PP appeared to be derived from selfing and showed either an AK-like genotype or a PP-like genotype (Fig. 4A). These results were confirmed by NEWHYBRIDS (Figs. 3B, 4B), which determined that the same individuals (2 OFa 6, 3 OFa 2, and 4 OFa 4, in Electronic Appendix Table 2A) as found in the STRUCTURE analysis were hybrids ($P > 0.95$). It is noteworthy that both the STRUCTURE and NEWHYBRIDS analyses assigned each parent used for the crossing experiments to the population of origin (Figs. 3, 4). The proportion of specimens derived from cross-breeding of the overall offspring number was low (3 out of 37 individuals, 8.1% of the offspring) (Fig. 3A).

4. Discussion

Platyhelminthes, with very few exceptions, are simultaneous hermaphrodites with complex reproductive organs. In general, the female

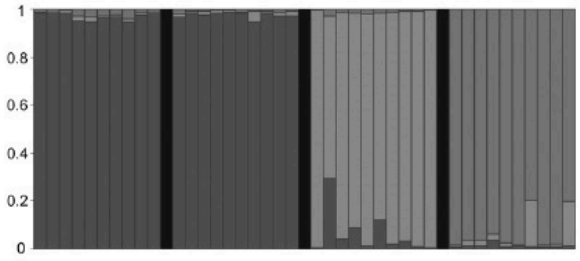

Fig. 2. Bayesian analysis (STRUCTURE) showing the genetic structuring of the four populations analyzed. The bar plot displays the assignment proportion of each individual to the inferred genetic clusters. Each segmented vertical bar corresponds to an individual. The thicker black lines separate each sampling location (from left to right): Akko, Alexandria, Maliakós, Porto Pozzo.

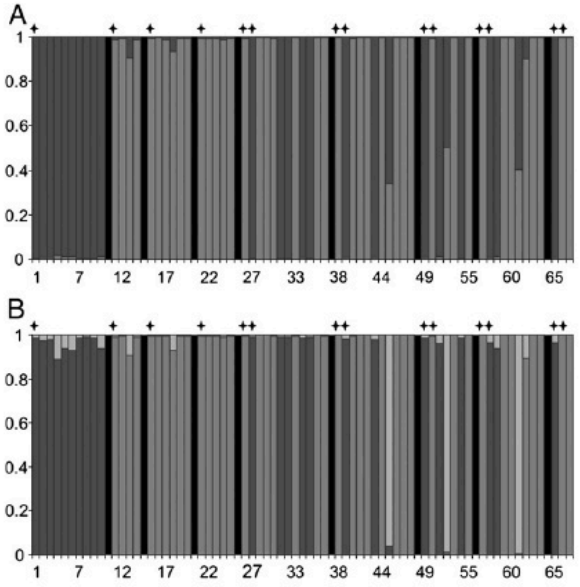

Fig. 3. The cross-breeding results for crosses between Alexandria and Maliakós. Vertical bars corresponding to parents are marked by a "star". A) STRUCTURE analysis: individuals derived from cross-breeding are expected to show admixed ancestry in the two parental clusters, denoted by a coefficient membership (Q-value) between 0.2 and 0.8. According to this threshold, the individuals 45, 52, and 61 could be classified as hybrids. B) NEWHYBRIDS analysis: the bar plot displays the probability of individuals having a pure or hybrid genotype. Here, three classes of genotypes are taken into account: the pure Alexandria genotype; the pure Maliakós genotype; the hybrid genotype. Individuals 45, 52, and 61 have a probability of > 95% to be hybrids.

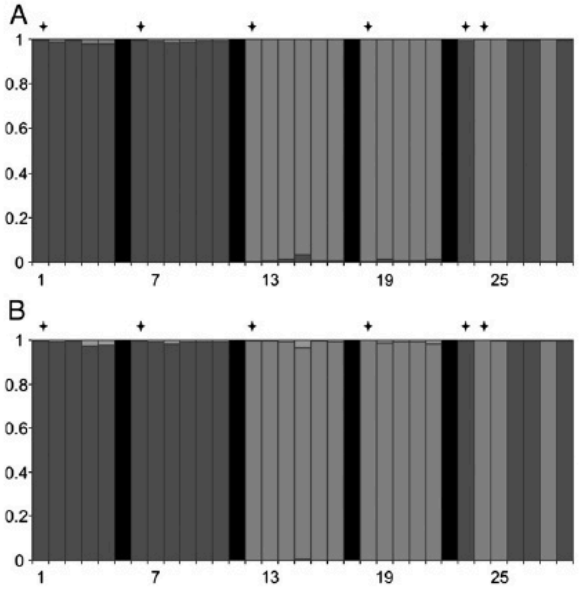

Fig. 4. Cross-breeding results for crosses between Akko and Porto Pozzo. Vertical bars corresponding to parents are marked by a "star". A) STRUCTURE analysis: individuals derived from cross-breeding are expected to show admixed ancestry in the two parental clusters, denoted by a coefficient membership (Q-value) between 0.2 and 0.8. According to this threshold, no individual could be classified as hybrid. B) NEWHYBRIDS analysis: the bar plot displays the probability of individuals having a pure or hybrid genotype. Here, two classes of genotypes are taken into account: the pure Akko genotype; the pure Porto Pozzo genotype. No individual has a probability of > 95% to be hybrid.

and male reproductive systems, although in close proximity, are separated (see, i.a., Hyman, 1951), and most free-living species are obligate cross-breeders (Gremigni, 1992). Alternative reproductive strategies, spanning from asexual reproduction to gynogenesis and parthenogenesis, have been reported in fresh-water representatives of the phylum (Benazzi and Benazzi Lentati, 1976; Benazzi Lentati, 1966; D'Souza and Michiels, 2006). Self-fertilization appears to be exceedingly rare and confined to the fresh-water Tricladida *Cura pinguis*, *Balliana tethysae* and *Polycelis nigra* (Benazzi, 1991; Gourbalt, 1978). The few reports of selfing in marine triclads (e.g. Gremigni and Nigro, 1983) rely on chance observations, and have been questioned by Sluys (1989).

The present study demonstrated that mixed mating occurs in *P. paupercula* and that, in this species, cross-breeding appears to be a rare occurrence. In fact, all isolated specimens that attained maturity were able to reproduce, and the percentage of selfing was higher than 90%, even when a mate was available. Furthermore, no significant difference in the reproductive output of the specimens kept singly or in pairs was detected, meaning that outcrossing did not significantly enhance, at least quantitatively, the reproductive performance of the species. Nevertheless, we cannot a priori rule out that these results reflect some confounding factors, such as mechanisms preventing cross-breeding between, but not within, populations. Indeed, it is possible that higher rates of cross-breeding within populations are present, but, because of the lack of resolution due to low differences evidenced in ISSR banding pattern of individuals from the same sampling site, we are not able to detect it. Thus, in such context, we are reduced to speculate that selfing appears to be favored with respect to cross-breeding in *P. paupercula*.

The fact that selfing is a stable and preferential component of the reproductive strategy of this species is also shown by the presence of two features frequently encountered in selfers:

1) Absence of delayed selfing. Theory predicts that in preferentially outcrossing animals, isolated individuals should delay their age at first reproduction to wait for future partners (Escobar et al., 2011). In *P. paupercula*, no differential timing of the deposition of the first cocoons was detected between isolated and paired specimens (Table 3);
2) Absence of allozyme genetic variability. A unique trait of the species is the fixation of all of the allozymic loci investigated. Indeed, all Proseriate species tested thus far showed high values of observed heterozygosity (H_o). For example, in the congeneric *P. ophiocephala* and *P. agilis* species, complexes, H_o varied from 0.006 to 0.061 and from 0.020 to 0.106, respectively (Casu and Curini-Galletti, 2004; Casu et al., 2009a). While, in the *Monocelis lineata* species complex, H_o varied from 0.015 to 0.113 (Casu and Curini-Galletti, 2004). Convergent selection and/or selective sweep (see Gompert et al., 2006; Johansson et al., 2008; Simonato et al., 2007) may interact to configure the unique genetic structure of the species. Nonetheless, ISSR results revealed a level of genetic variability similar to (Casu and Curini-Galletti, 2006; Lai et al., 2008) or higher than (Casu et al., 2009a; Casu et al., 2011b) those obtained for other Proseriate species using dominant markers. Hence the observed lack of allozyme genetic variability may be due to phenomena of genetic drift. In any case, the lack of allozymic variability suggests that no inbreeding depression occurs in the species, possibly as a result of the progressive purge of recessive deleterious alleles (Day et al., 2003, and references therein).

The presence of sperm in the female reproductive tract of isolated specimens suggests that autogamy (i.e., the singamy of gametes originating from different meioses in a single individual) (Mogie, 1986) is the modality of selfing in the species. The anatomical reconstructions failed to show a direct connection between the male and female reproductive systems. However, *P. paupercula* is very unusual among Proseriates due to the presence of a high, vacuolar epithelium lining the entire female duct system, including the oviducts (Fig. 1B-D). The vacuoles may be so developed that the entire female duct, in sections, appears swollen and lacking a continuous lumen (see Casu et al., 2011a). Furthermore, *P. paupercula* is unique in the way the female duct wraps around the copulatory organ, whose musculature appears thin, with muscular fibers unusually spaced in comparison with congenerics (Fig. 1B). In other species of *Pseudomonocelis* a somewhat vacuolar, irregular lining of the female duct is only present in the area abutting the bursa (Schockaert and Martens, 1987, Fig. 7, p. 112; Curini-Galletti et al., 2011, Fig. 1, p. 51). Because no direct connection between the female duct and the bursa is present in these species, the vacuolar area was assumed to allow the passage of the sperm from the partner stored in the bursa to the female duct, and hence to the oviducts (Schockaert and Martens, 1987). The extreme development of the vacuolar lining of the female ducts in *P. paupercula* may similarly allow the passage of the spermatozoa from the closely lying sperm ducts, as well as from the copulatory organ.

Other than in flatworms, selfing has been reported in sessile or highly sedentary, free-spawning marine species, where sperm dilution in the water column may be a limiting factor for reproduction (Brazeau et al., 1998). Brooding corals may self-fertilize at high frequencies (34–49%), and this also occurs in the sea anemone *Epiactis prolifera* (3.8–100%) (Edmands and Potts, 1997). Selfing has also been reported in Bryozoa, where it appears to be an "emergency option" when no mate is available (Hunter and Hughes, 1993). However, most of the studies have been conducted on brooding species, which allow for an easy genetic assay of the offspring (Carlon, 1999), and the incidence of the phenomenon in the sea may be underestimated.

Given the rarity of selfing in free-living flatworms and in marine species as a whole, one may question why it has arisen in *P. paupercula*. Darwin (1876) proposed an ecological explanation for the evolution of selfing, which still holds true (Jarne and Auld, 2006). Under the "reproductive-assurance hypothesis", selfing evolves to provide a guarantee of reproduction: i.e., outcrossing is favored, but selfing can evolve if ecological conditions vary and mate availability becomes problematic, so that reproduction is always possible. Environmental instability has indeed been shown to promote selfing in plants (Morgan et al., 1997). The environmental constraints may indeed be the key factor shaping the reproductive strategy of *P. paupercula*. The species occurs in extremely confined, silty microhabitats that are subject to wide salinity ranges in scattered locations across the Mediterranean (Casu et al., 2011a). It has been rarely recorded, and the four populations that were sampled in this study are the only known populations. Recent samples from the Porto Pozzo area failed to find the species at the station where it was previously found (pers. obs.), and local extinctions of populations may occur in the species. Similar to all Proseriates, *P. paupercula* has a direct development. This, and the interstitial habitus of the species, reduces the chances for dispersal. Colonization of the confined microhabitats must rely on chance events of the dispersal of juveniles and adults in the water column. Selfing and a lack of inbreeding depression may thus allow for the establishment of viable populations from just one, unfertilized individual. Recent, theoretical studies have demonstrated that poor dispersal and selfing are indeed correlated (Cheptou and Massol, 2009). However, complete selfing, when maintained long-term, will eventually lead to the same evolutionary inability to adapt to changing ecological conditions as was theoretically predicted for parthenogenetic populations (Hughes, 1989). The low but constant rate of outcrossing that was observed in the species may be a strategy to avoid this fate, as postulated for the presence of occasional sex in parthenogenetic populations of freshwater planarians (D'Souza and Michiels, 2006).

This study further demonstrates the usefulness of ISSR markers in detecting genetic polymorphisms (Casu et al., 2009b, 2012; Cossu et al., 2012; Machkour-M'Rabet et al., 2009; Maltagliati et al., 2006; Roux et al., 2007) when results from other codominant markers were inconclusive (allozymes) or not achievable (no specific microsatellites primers are presently available for Proseriates). Remarkably, Mariette et al. (2002) showed that dominant markers may be as efficient as the codominant markers in estimating the genetic diversity.

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Chapter 3

Biodiversity patterns in interstitial marine microturbellaria: a case study within the genus *Parotoplana* (Platyhelminthes: Rhabditophora) with the description of four new species

Marco Casu, Fabio Scarpa, Valentina Delogu, Piero Cossu, Tiziana Lai, Daria Sanna, Marco Curini Galletti

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Biodiversity patterns in interstitial marine microturbellaria: a case study within the genus *Parotoplana* (Platyhelminthes: Rhabditophora) with the description of four new species

MARCO CASU*, FABIO SCARPA, VALENTINA DELOGU, PIERO COSSU, TIZIANA LAI, DARIA SANNA, MARCO CURINI-GALLETTI

Dipartimento di Scienze della Natura e del Territorio – Sezione di Zoologia, Archeozoologia e Genetica, Università di Sassari, Via F. Muroni 25, 07100 Sassari, Italy - Centro di Eccellenza dell'Università di Sassari

*Corresponding author: Marco Casu

Dipartimento di Scienze della Natura e del Territorio –

Sezione di Zoologia, Archeozoologia e Genetica,

Università di Sassari,

Via F. Muroni 25,

07100 Sassari, Italy

e-mail: marcasu@uniss.it

phone: +39 079 228924

fax: +39 079 228665

Abstract

Four new species of Otoplanidae (Platyhelminthes: Rhabditophora: Proseriata) with ranges restricted to the central Mediterranean are described. These species are characterised by the presence of a tubular copulatory stylet and by a bursa lacking an open connection to the atrium. Both characters are novel for the family Otoplanidae. The four species differ mainly in details of the morphology of the sclerotized structures of the copulatory organ. Generic attribution of the new species has been problematic. Morphological characters shared with either *Parotoplanina* or *Parotoplanella* were detected. Molecular data (based on *18S rRNA* and *28S D1-D6 rRNA* genes) evidenced that the new species constitute a monophyletic group falling within species of *Parotoplana*, with a sister-taxon relationship with *Parotoplana spathifera*. The genus *Parotoplana*, however, appears to be paraphyletic, as *Parotoplanella progermaria* nests within *Parotoplana* species. The inadequate molecular sampling, combined with the lack of sequences from the type species of both *Parotoplana* and *Parotoplanina*, suggested a cautionary taxonomic approach, and the new species are therefore attributed to the earliest generic available taxon, *Parotoplana*.

Key words: Microturbellarians; taxonomy; biodiversity; molecular analysis; phylogeny.

Introduction

The recent register of the world's marine species singled out the Rhabditophora as a taxon with a higher than average percentage of unknown species (Appeltans et al. 2012). This lack of knowledge is particularly acute for free-living marine interstitial groups (collectively known as microturbellarians), whose inventory is far from complete. Recent metagenetic evidence has revealed that microturbellarians may be particularly abundant in meiofaunal communities, although they are often neglected due to the extraction techniques used in traditional ecological research (Fonseca et al. 2010). Given that most microturbellarians occupy top predator roles in benthic ecosystems (Martens and Schockaert 1986), their prominence demonstrates that conventional diversity assessments may provide a distorted perspective of the relationships within the benthos.

The discrepancy between the diversity and the ecological relevance of this group and its state of knowledge is obvious. The study of these minute interstitial organisms is, admittedly, problematic, as external morphology provides no clues to taxonomy and both living and sectioned specimens must be obtained for accurate observations (Cannon and Faubel 1988).

The Proseriata (Rhabditophora: Neoophora) are among the largest taxa of marine microturbellarians; however, the over 400 species known so far are thought to comprise only a fraction of their worldwide diversity (Curini-Galletti 2001; Appeltans et al. 2012). Furthermore, information on the actual geographic ranges of the Proseriata, and of microturbellarians in general, is particularly limited. This lack of information on distribution further complicates estimates of the global species richness of the taxon.

Over the course of several sampling periods in the Mediterranean, we discovered specimens of Otoplanidae (Proseriata), belonging to four different species, that shared a hitherto unknown construction of the male sclerotised system, and differed in subtle, yet constant, morphological details. The generic attribution of these new species was particularly problematic, either on morphological and molecular grounds. These species are presented and discussed here.

Materials and methods

Sampling and morphological study

Samples were collected manually by scooping up the superficial layer of sediment.

Extraction of the animals from the sediment was accomplished using MgCl2 decantation (Martens 1984). Each specimen was first studied alive by slight squeezing under the cover slip, and then retrieved and treated for further analyses

For microscopical study, specimens were fixed in Bouin's fluid, embedded in 60 ºC Paraplast, serial sagittal sections were obtained at 4 µm intervals, stained with Hansen's haematoxylin and eosin-orange and mounted in Eukitt (see Beccari and Mazzi 1966 for details).

The contribution to the description of the species by the various authors differs, and the authorship of the new species is limited to the authors who contributed to morphological description.

Type material is stored in the collections of the Swedish Museum of Natural History (Stockholm, Sweden) (SMNH). Additional voucher material is stored in the collection of the Zoological Museum of the University of Sassari (Italy) (CZM).

Measurements of sclerotised structures of the male copulatory organ were taken on squeezed, karyological slides (see below). We measured copulatory stylet, two companion spines, and two lateral spines (see species descriptions). In species diagnoses, the ratio among sclerotised pieces is given as: a) length of the copulatory stylet = 1; b) length of companion spines/length of copulatory stylet; c) length of lateral spines/length of copulatory stylet.

For statistical analysis, the following parameters were measured (see species descriptions for explanation of the morphological terminology used):

1) length of copulatory stylet; 2) length of basal lobe / total length of copulatory stylet; 3) width-to-length ratio (W/L) of copulatory stylet; 4) length of companion spines; 5) length ratio between companion spines and copulatory stylet; 6) length of distal tip / total length of companion spines; 7) W/L of companion spines; 8) length of lateral spines; 9) length ratio between lateral spines and copulatory stylet; 10) length ratio between lateral spines and companion spines; 11) length of distal tip / total length of lateral spines; 12) W/L of lateral spines.

Multivariate patterns of morphological variability were explored by a non-metric Multi-Dimensional Scaling (nMDS) (Fig. 1), carried out on the matrix of squared individual Euclidean distances. We performed an Analysis Of SIMilarities (ANOSIM) on the matrix of squared Euclidean distances to test for differences at the level of species (1,000 permutations). Furthermore, groups of individuals were also investigated by K-means clustering with 1,000 iterations, considering 2 up to 6 groups (the maximum number of populations). The most likely number of groups was detected applying the criterion of Calinski (Calinski and Harabasz 1974). Multivariate analyses were

performed with Vegan 2.0-5 (Oksanen et al. 2013), a package that works within the R Statistical environment 2.15.0 (R Development Core Team 2012).

Abbreviations used in figures

a: atrium; b: bursa; br: brain; cgp: common genital pore; co: copulatory organ; cs: companion spine; fd: female duct; fp: female pore; gl: gut lumen; ls: lateral spine; m: musculature; mp: male pore; ov: ovaria; ph: pharynx; s: stylet; st: statocyst; sv: seminal vesicle; t: testis; ta: thickened area of the basal lobes of the stylet; tp: 'tubular portion' of the ejaculatory duct; vi: vitellaria; vt: vacuolar tissue.

Karyology

The karyotype was determined from acetic orcein-stained spermatogonial mitoses, as described by Curini-Galletti et al. (1989). Relative lengths (r.l. = length of chromosome x 100/total length of haploid genome) and centromeric indices (c.i. = length of short arm x 100/length of entire chromosome) were obtained from measurements of the camera lucida drawings of metaphase plates. Karyotype formula was represented as follows: absolute length of haploid genome in μm; fundamental number; relative length and centromeric index of each chromosome; chromosome nomenclature within parentheses (m = metacentric; sm = submetacentric; st = subtelocentric; t = acrocentric). The fundamental number (FN) (i.e. the number of chromosome arms in the karyotype) was derived according to Matthey (1949) and the chromosome nomenclature used was that of Levan et al. (1964). After karyological study, slides were made permanent by lactophenol.

Molecular analysis

Whenever possible, some of the specimens identified alive by light squeezing under a cover slip were fixed in absolute ethanol (95° ethanol) for molecular study. The reduced size of these minute organisms did not allow for the deposition of voucher material. Genomic DNA was extracted using the Macherey-Nagel NucleoSpin Tissue (MACHEREY-NAGEL GmbH & Co. KG; Neumann Neander Str. 6-8 D-52355 Düren, Germany) according to the supplier's instructions. After extraction, DNA was stored as a solution at 4 °C.

Two nuclear genes were analysed: the *18S rRNA* gene (complete sequence; *18S*) and the *28S rRNA* gene (partial sequence spanning the variable domains *D1-D6*; *28S*). These sequences were analysed in 23 individuals. Moreover, the corresponding sequences for seven individuals were taken from GenBank (Table 1). The dataset was built using 11 sequences from the unknown Otoplanidae specimens found. To test the monophyly of this group we added sequences of nine *Parotoplana* species (sequenced by the authors, Table 1). Four species belonging to the family Otoplanidae (one sequenced by the authors and three taken from GenBank, Table 1) were added to infer phylogenetic relationships within the family. As outgroups, three species belonging to the family Monocelididae (two sequenced by authors and one taken from GenBank, Table 1), one to Archimonocelididae (from GenBank), one to Calviridae (from GenBank), and one member of the suborder Unguiphora (from GenBank) were also added (Table 1). Amplifications for *18S* and *28S D1-D6* regions were carried out using the following primers: *18S*: A (forward) GCG AAT GGC TCA TTA AAT CAG, and B (reverse) CTT GTT ACG ACT TTT ACT TCC (Littlewood and Olson 2001); *28S D1-D6*: LSU5

(forward) TAG GTC GAC CCG CTG AAY TTA AGC A, and LSUD6-3 (reverse) GGA ACC CTT CTC CAC TTC AGT C (Littlewood et al. 2000).

PCR was carried out in a total volume of 25 µl containing 5 ng/µl of total genomic DNA on average, 1.0 U of Taq DNA Polymerase (Euroclone), 1× reaction buffer, 3.5 mM of $MgCl_2$, 0.32 µM of each primer, and 200 µM of each dNTP. PCR amplification was performed in a MJ PTC 200 Thermal Cycler (Biorad) programmed as follows: 1 cycle of 2 min at 94° C, 35 cycles of 1 min at 94° C, 1 min at 54° C (*18S / 28S D1-D6* primers' annealing temperature), and 1 min and 30 s at 72° C. At the end, a post-treatment for 5 min at 72° C and a final cooling at 4° C were carried out. Both positive and negative controls were used to test the effectiveness of the PCR reagents, and the absence of possible contaminations.

Electrophoresis was carried out on 2% agarose gels, prepared using 1× SBA buffer (sodium boric acid, pH 8.2) and stained with a 1 µl/20 ml ethidium bromide solution. PCR products were purified by ExoSAP-IT (USB Corporation) and sequenced for both forward and reverse *18S* and *28S D1-D6* strands, using an external sequencing core service (Macrogen Europe).

The *18S* and *28S D1-D6* sequences were aligned separately using the algorithm Q-INS-I implemented in Mafft 6.903 (Katoh and Toh, 2008), which is appropriate for non-coding RNA as it considers RNA secondary structure. After the alignment, sequences of 1698 and 1629 *bp* were obtained for the *18S* and *28S D1-D6* regions, respectively (see Table 1 for the GenBank accession numbers). The best probabilistic model of sequence evolution was determined after evaluation by JModeltest 0.1 (Posada 2008), with a maximum likelihood optimised search, using the Akaike Information Criterion (AIC). The model GTR+G (Tavaré 1986) for both *18S* and *28S D1-D6* has been estimated as

the best fitting to our sequences. Phylogenetical relationships among individuals and species were investigated using both Maximum Likelihood (ML) and Bayesian Inference (BI) on the combined *18S* and *28S D1-D6* sequences. We set as outgroup for the analyses the species *Polystyliphora novaehollandiae* Curini-Galletti, 1998 (Proseriata: Unguiphora). ML was performed using the genetic algorithm implemented in Garli 2.0 (Zwickl 2006). In order to find the best tree, the configuration file for partitioned models was set up to perform 10 replicate searches (searchreps = 10). Model parameters: ratematrix = (0 1 2 3 4 5), statefrequencies = estimated, ratehetmodel = gamma, numratecats = 4, corresponding to the evolution model calculated by JModeltest, were used. In order to allow independent estimates of the parameters for each gene, the option link was set to 0. The parameter modweight was set to 0.0015, as we have two models. Finally, node support was assessed by 1,000 bootstraps (bootstrapreps = 1000). Consensus tree was computed using TreeAnnotator 1.7.4 (part of the Beast package) (Drummond and Rambaut 2007) and visualised by FigTree 1.4.0 (http://tree.bio.ed.ac.uk/software/figtree/).

BI was carried out using the software MrBayes 3.2.1 (Ronquist et al. 2012), specifying a partitioned model and setting as model parameters: NST = 6, rates = gamma, ngammacat = 4. Two independent runs each consisting of four Metropolis-coupled Markov Chain-Monte Carlo chains (one cold and three heated chains) were run simultaneously for 5,000,000 generations, sampling trees every 1,000 generations. We allowed each partition to have its own set of parameters and a potentially different overall evolutionary rate. The first 25% of sampled trees were discarded. Convergence of chains was checked first scrolling down the Average Standard Deviation of Split Frequencies (ASDSF), that approaches 0, if the chains' convergence has been reached

(Ronquist et al. 2012), and then checking the Potential Scale Reduction Factor (PSRF), that compares the variance among runs with the variance within runs (Gelman and Rubin 1992). Initially, variance among runs will be higher than the variance within runs. If chains converge the variances will became more similar and the PSRF will approach 1. Nodes with a percentage of posterior probability lower than 95% are considered not highly supported. Phylogenetic consensus tree was visualised using FigTree 1.4.0.

Because BI and ML generated trees with identical topology, we reported only the Bayesian tree (see Fig. 2).

Results and Discussion

Species justification

The four species described here (see Appendix), *Parotoplana tubifera* sp. nov., *P. impastatoi* sp. nov., *P. ambrosolii* sp. nov, and *P. livatinoi* sp. nov. (henceforth called the *P. tubifera* species group), present unique characters for the family Otoplanidae:

- a tube-like stylet, with convex, pointed basal lobes;
- a funnel shaped-apex of lateral spines, through which (prostatic?) glands discharge;
- a bursa embedded in the dorsal wall of the atrium, without a direct connection to the atrium.

These synapomorphies indicate the existence of a distinct, closely related group of species (see below for a discussion on the generic placement), and the monophyly of at least three species of this group (*Parotoplana tubifera* sp. nov., *P. impastatoi* sp. nov., and *P. ambrosolii* sp. nov., for which molecular data are available) is also supported on molecular grounds (Fig. 2).

The four new species can be distinguished by features of their sclerotised pieces. In particular, the species differ in the morphological details of the apices of their companion spines, that are as follows:

i) bilobed, with a comparatively small, obtuse basal lobe and an obtuse, flat distal lobe, placed at an angle of approximately 45° to the axis of the spine, in *P. tubifera* sp. nov.;

ii) bilobed, with a comparatively large, spherical basal lobe and a distal, very thin, recurve, and acutely pointed distal lobe, in *P. livatinoi* sp. nov.;

iii) triangular, placed at an angle of about 90° to the axis of the spine, in *P. ambrosolii* sp. nov.;

iv) elongate, with a slightly hooked distal tip and a basal apophysis, and similar in morphology to the lateral spines, in *P. impastatoi* sp. nov.

Furthermore, *P. ambrosolii* sp. nov. is the only species in which the bases of the companion spines are not fused with the basal lobes of the stylet.

In addition to these distinctive apex morphologies, more subtle differences exist in the morphology and size relationships of the three sclerotised pieces. Indeed, the ANOSIM detected a significant difference between individuals grouped at the species level ($R = 0.935$, $P = 0.001$). However, according to the nMDS plot (Fig. 1), only three species (*P. tubifera* sp. nov., *P. livatinoi* sp. nov., *P. impastatoi* sp. nov.) which occur sympatrically in eastern Sicily (Agnone) are clearly separated from each other. One of them (*P. livatinoi* sp. nov.) is not differentiated from the northern Tyrrhenian *P. ambrosolii* sp. nov. based on the parameters considered in the nMDS analysis.

The two species are however clearly differentiated for the shape of the distal part of their companion spines, as well as for the proximal part of these spines, fused and not fused respectively, with the stylet. Karyotypes seem to differ among species; in

particular, *P. livatinoi* sp. nov. and *P. tubifera* sp. nov. appear to have the lowest number of heterobrachial chromosomes. However, the limited karyometrical data available did not allow further analysis.

Molecular data (only available for three of the four species due to the lack of available material of *P. livatinoi* sp. nov.) showed that these species are grouped into a highly supported monophyletic group, with specimens of *P. impastatoi* sp. nov. and *P. tubifera* sp. nov. placed in distinct clusters, and *P. ambrosolii* sp. nov. being paraphyletic. Therefore, although *18S* and *28S D1-D6* markers have shown to be appropriate for species delimitation in several case studies on Proseriata (e.g. Litvaitis et al. 1996; Littlewood et al. 1999, 2000; Curini-Galletti et al. 2010; Casu et al. 2009, 2011), this study shows their limits for taxonomic purposes (see also Tang et al. 2012).

Generic placement

Determining the phylogenetic position of the *P. tubifera* species group has proven problematic. Part of the problem lies in the present systematic arrangement of the family Otoplanidae, derived by Ax (1956) and most recently revised by Miller and Faubel (2003), which splits the family into five subfamilies. The diagnoses of these subfamilies appear to be riddled with exceptions, and there are cases of overlapping definitions. Although no thorough molecular study of the taxon has previously been performed, the available molecular data (Littlewood et al. 2000; Curini-Galletti et al. 2010) do not support the current system.

The characters of the species here described fall within the definition of the Parotoplaninae Ax, 1956, diagnosed as "Otoplanidae with short collar-like pharynx diagonally or vertically oriented in relation to the ventral side. Pharynx always

internally ciliated, outside often without cilia. Intestine above the pharynx with wide lumen. Accessory genital glands compact at the opening of the germovitelloducts into the genital atrium (exception, *Pseudosyrtis* Ax, 1956)" (from Miller and Faubel 2003).

However, the four species of the *P. tubifera* group have a long, cylindrical pharynx, mostly held horizontally, and the "accessory genital glands" (corresponding to the higher, glandular epithelium surrounding the female pore in our descriptions) are poorly developed, when they are even observed. The length and orientation of the pharynx may not be a reliable phylogenetic marker: species of *Parotoplana* have been described with long pharynges, and due to its mobility, the position of the pharynx is variable even over the course of short observations on living specimens (see above, under *P. tubifera*). The development of the 'accessory genital glands' appears to be variable even within the same species and is possibly linked to maturity state (pers. obs.).

On the other hand, the species here described lack the characters considered most relevant in the diagnoses of the other subfamilies (Miller and Faubel 2003). In particular, they lack the following characteristics:

i) the tube-like narrowing of the gut above the pharynx (as in the Otoplaninae Hallez, 1910);

ii) a bulbous pharynx with inverted muscular layers (i.e., with outer circular and inner longitudinal fibers) (as in the Bulbotoplaninae Ax, 1956);

iii) a copulatory cirrus (as in Cirroplaninae Miller & Faubel, 2003);

iv) an entirely ciliated body (as in the Archotoplaninae Ax, 1956).

Therefore, the species here described are assigned to the Parotoplaninae (whose diagnosis needs to be emended). At present, the Parotoplaninae are comprised of 13 genera (see Miller and Faubel 2003). The new species of the *P. tubifera* group share the

following diagnostic characters with *Parotoplanina geminoductus* Ax, 1956, the type (and single) species of its genus:

i) position of the epithelial nuclei (intraepithelial in unciliated areas; insunk in ciliated areas):

ii) lack of a distinct prostatic vesicle.

The synapomorphy of the latter character may be debatable. *P. geminoductus* has a glandular (prostatic) area confined to the distalmost portion of the ejaculatory duct, and entirely comprised within the copulatory organ. Such condition is to some extent shared by the new species; however, the presence of lateral spines connected to presumably prostatic glands is unique to the *P. tubifera* species group.

A further diagnostic feature for the genus *Parotoplanina* is the morphology of the bursa, placed dorsally to the copulatory organ and connected to the atrium through two distinct bursal canals (see Ax 1956, Figs 191-193, p. 737), quite unlike the new species.

The position of the epithelial nuclei is also shared with the genus *Parotoplanella* Ax, 1956 (type: *P. progermaria* Ax, 1956, and containing two known species). However, this genus has frontal ovaries placed among the testes, vitellaria running anteriorly lateral to the testes, and an internally unciliated pharynx. The species of the genus *Parotoplanella* also have one bursal canal.

Overall, our species appear similar to species of the genus *Parotoplana* Meixner, 1938, (type: *P. capitata* Ax, 1956), which includes 15 species in the Mediterranean alone and is the most species-rich of the family Otoplanidae (Delogu and Curini-Galletti 2009). The genus is heterogeneous in body and pharynx shape, the presence/absence of bursal nozzles and the proximal/distal fusion of the oviducts. However, all *Parotoplana*

species described from sectioned material present one bursal canal and epithelial cells with intraepithelial nuclei (see, i.a. Ax 1956).

The remaining genera of Parotoplaninae differ even further from our species (see Miller and Faubel 2003 for extended diagnoses):

- *Galapagoplana* Ax & Ax, 1974 has an epithelium with insunk nuclei and a bursa resorbiens dorsal to the copulatory organ;

- *Paraplana* Ax, 1956 has an epithelium with insunk nuclei and lacks a bursa.

- *Polyrhabdoplana* Ax & Ax, 1967 has postpharyngeal testes follicles;

- *Pseudosyrtis* Ax, 1956 and *Philosyrtis* Giard, 1904, among other differences, lack a bursa;

- *Triporoplana* Ax, 1956; *Kataplana* Ax, 1956 and *Otoplanidia* Meixner, 1938 all have an additional, secondary bursa that opens to the outside through an independent pore;

- *Praebursoplana* Ax, 1956 has a ciliated bursal canal which opens to the genital atrium anterior to the copulatory organ; the bursa is provided with spermatic ducts connected to the ovaries;

- *Postbursoplana* Ax, 1956 has an opening of the female duct in the anteriormost part of the genital atrium, anterior to the opening of the bursal canal.

Considering the situation presented above, the erection of a new genus for the species described here would not be unjustified. However, we refrained from this course of action, as the available information from molecular data show a far less clear cut scenario as indicated by present genus diagnoses. The three new species sequenced (*Parotoplana tubifera* sp. nov., *P. impastatoi* sp. nov., and *P. ambrosolii* sp. nov.) are in fact the sister taxon of *P. spathifera*; together, these species are the sister taxon of a group composed of the remaining *Parotoplana* species and *Parotoplanella*

progermaria. Both *Parotoplanella progermaria* and the four new species have ciliated epithelial cells with insunk nuclei, but they clustered in different groups.

Furthermore, a careful re-examination of the sectioned material on which the original description of *P. spathifera* was based (vouchers CZM44 - CZM50) shows that this species indeed presents an epithelium with intraepithelial nuclei overall, a very obvious, large bursal canal, and a large prostatic vesicle, well-separated and distinct from the seminal vesicle. These results suggest that even a character such as 'position of the epithelial nuclei' may be acquired/lost more than once, and the phylogenetic weight of such a character may be less relevant than previously assumed in the systematics of Otoplanidae.

The placement of *Parotoplanella progermaria* inside the cluster containing the species of *Parotoplana* also suggests either that the well-known genus *Parotoplana* is paraphyletic (see also Bursey et al. 2012), or that *Paratoplanella* is not a valid genus. The situation is further complicated by the lack of sequences for the type species of both *Parotoplanina* and *Parotoplana*, whose range is limited to the North Sea/ Channel area. Despite several attempts by one of the authors (MCG), specimens of these species have yet to be retrieved.

Distribution and inferences on speciation

Assessments of the global diversity of microturbellarians worldwide are complicated by the few and scattered data presently available (Appeltans et al. 2012). The necessity of facilities for the extraction of these minute organisms from the sediment and of microscopes for observations on living specimens has limited comprehensive species lists to relatively few areas, all of which are close to the home institutions of the

authorities on the group or to Marine Biological Research centres. As a result, information on the actual range of most microturbellarians, and therefore on patterns of geographic-scale species diversity, is limited, and it is difficult to extrapolate the magnitude of species diversity within the group. The existence of restricted geographic ranges for certain species of Proseriata has been already suggested (see Casu and Curini 2006; Casu et al. 2009; Delogu and Curini-Galletti 2009). However, the possibility of bias resulting from undersampling cannot be eliminated from these studies.

The evidence presented in this work strengthens the case for limited geographic ranges: the species involved are comparatively large, striking and unmistakable; quite common and obvious in parts of the central Mediterranean; and not found elsewhere. Indeed, numerous sampling efforts to assess the composition of soft bodied meiofauna along the Mediterranean and European Atlantic coasts, the results of which have only partially been published (see Curini et al. 2012), failed to reveal the species described here, even in contiguous and at least as intensively sampled, areas (Tunisia, Lampedusa Is., East Spain, Gulf of Lyon, Ionian and Adriatic Seas) (MCG, unpubl. results).

Among the Otoplanidae, the only other species-group studied in such detail (*Parotoplana jondelii* group) showed a somewhat comparable distribution pattern: the seven species described have restricted and mostly non-overlapping ranges, suggesting allopatric divergence as the main driving force in their speciation processes (Delogu and Curini-Galletti 2009).

Conclusion

The results of this research offer several points which may be of more general relevance:

- there are indications that the present systematic arrangement of the Otoplanidae is inadequate. A more extensive molecular and taxonomic samplings is necessary and the monophyly of most of its genera should be carefully evaluated. In this regard, the fact that the type species of many genera are either rare or restricted in distribution is particularly regrettable;

- different morphological characters may not have the phylogenetic weight that has been traditionally attributed to them. In our case, the position of epithelial cell nuclei differs even among sister species, suggesting that the transition from one state to the other may have occurred multiple times. It is worth mentioning that differing morphologies of epithelial cells have been used to justify the erection of distinct genera in several taxa of the Platyhelminthes (see, e.g., Marcus 1949). Similarly, the shape and position of the pharynx, generally of paramount importance in the systematics of the Platyhelminthes, as well as its patterns of ciliation, may be subject to species-specific modification;

- the geographical ranges of microturbellarian species, which are in some instances more restricted than is generally considered, may imply that current assessments underestimate the magnitude of species richness in the group (Appeltans et al. 2012);

- the fact that such common, littoral, and striking species were not discovered yet points to the amount of field work which remains towards obtaining adequate knowledge of the composition and diversity of the group.

Appendix

Species description

Family Otoplanidae Hallez, 1892

Genus *Parotoplana* Meixner, 1938

***Parotoplana tubifera* Curini-Galletti & Delogu sp. nov.**

(Figs 3 A, G, I, J; 4 A-C; 5 A-C, E, F)

Holotype: one karyological slide made permanent with lactophenol (SMNH Type-8556).

Type locality: Le Bombarde beach, Alghero (Sardinia, Italy) (Lat. 40°35'1.33"N; Long. 8°15'38.69"E).

Additional material: 2 karyological slides made permanent with lactophenol (CZM 472; 473), 8 sagittally (CZM 474-481) and 2 transversally (CZM 482; 483) sectioned specimens, all from type locality.

Other locality: Agnone (Sicily, Italy) (Lat. 37°18'37.24"N; Long. 15°6'21.47"E); medium volcanic sand, intertidal: 7 karyological slides, containing a total of 14 specimens) slides made permanent with lactophenol (CZM 484-490), 3 specimens sagittally sectioned (CZM 491-493).

Etymology: from latin *tubus* (tube) and *ferre* (to carry), referring to the tubular shape of the copulatory stylet.

Description

A large, flattened otoplanid: living specimens up to 4-6 mm, fixed specimens about 2 mm long; unpigmented. Living specimens appear ribbon-shaped, gradually tapering at both extremities (Figs 3 A; 5 A). The anterior end is markedly set-off from the rest of the body and provided with a ciliated sensory furrow, and long sensory bristles. The caudal end is distinctly fan-shaped, and provided with numerous adhesive papillae.

The epithelial cells, with intraepithelial nuclei, are unciliated, with the exception of a ciliated creeping sole, that runs as a narrow, median band from the anterior end to the level of the genital pore; cilia are 6 to 7 µm in length. The nuclei of this ciliated sole are sunk below the basal lamina. Rhabdoids are rod-shaped, 5 to 10 µm long and about 1.5 µm wide, and arranged in clusters of 3-4 rhabdoids each. These clusters form numerous longitudinal rows dorsally, while they are sparser and more irregularly arranged ventrally. The encapsulated brain is ovoid and measures about 60 to 65 µm in its widest axis. It abuts the circular statocyst which reaches about 25 µm in diameter.

Body musculature consisting of an outer, thin circular layer and an inner longitudinal layer, particularly well developed ventrally.

The rather short, tubular pharynx (of the *plicatus tubiformis* type) is located at the beginning of the second half of the body. In all living specimens it appeared horizontally oriented (Fig. 5 A). However, in a few contracted sectioned specimens, it is ventrally oriented. The epithelium, with insunk nuclei, lining the inner and outer surfaces of the pharynx is ciliated, with cilia up to 3 µm in length, slightly longer internally. The distal tip of the pharynx, where the poorly developed pharyngeal glands discharge, is unciliated. The pharynx musculature is reversed with respects to body musculature, with a thin, outer longitudinal layer and stronger, inner circular muscles. A

ciliated epithelium, overlaying a well developed musculature similar to that of the pharynx, lines the mouth cavity for some distance from the pharynx, and its contraction/extension may allow the shift in pharynx shape and orientation observed in living versus fixed specimens. The rest of the mouth cavity is lined with a thin, unciliated epithelium with intraepithelial nuclei, and lacks a muscular cell lining. The small mouth is ciliated. The gut extends from behind the brain almost to the caudal tip; its lumen does not appear noticeably narrower compared to the pharynx.

Male genital system

The numerous, follicular testes (about 80) are arranged into two regular rows at both sides of the body, in front of the ovaries (Fig. 3 A: t).

The copulatory organ is post-pharyngeal. In squeezed living specimens, a conspicuous sclerotised apparatus (Fig. 5 B) and a long, winding vesicle containing sperm are apparent. Due to its circumvolutions, the actual length and shape of this vesicle are difficult to appreciate in sectioned specimens. The proximal, caudal part of the vesicle is irregularly ovoid in shape, up to 65 µm across, lined with a thin epithelium with intraepithelial nuclei, and contains large amounts of sperm in all specimens sectioned (Fig. 3 J: sv). More distally, the seminal vesicle narrows progressively, until it becomes tubular (up to 100 µm long and up to 10 µm wide), surrounded by circular muscle cells, and does not contain sperm (Fig. 3 J: tp). Although this portion is comparable in position to the prostatic vesicle of other Parotoplaninae (see, i.a., Ax 1956: p.718, Fig. 150), it is not vesicular, and the glandular nature of its epithelial lining could not be ascertained on the material available, nor any glandular cell body was noticed in its proximity. The distalmost portion of the ejaculatory duct enters into the basal part of the

stylet, runs through its whole length and opens into the genital atrium through the distal opening of the stylet. The epithelial lining of this distal portion of the ejaculatory duct is high, and appears glandular, at least where it enters the basal part of the stylet. Within the stylet, the lining appears irregularly swollen and vacuolar, and not clearly glandular in nature.

The stylet, 118.3 ± 2.9 µm long in specimens from Le Bombarde, Sardinia (range: 115-122 µm, three measurements), and 129.1 ± 3.0 µm in specimens from Agnone, Sicily (range: 117-141 µm, 14 measurements), is an intracellular structure (Figs 3 I, J; 5 B: s). The cell-forming nuclei of the stylet are large and flattened. Proximally, the basis of the stylet consists of two long, acutely pointed, triangular lobes, which wrap the 'glandular' portion of the ejaculatory duct, and are thus somehow convex in shape. These lobes fuse distally into a narrow tube, 50-65 µm long, and 13-18 µm in diameter. A suture line runs along the length of the tube, and evidences its origin by fusion of two elements. The tube widens distally to 23-25 µm; its distal opening is truncated, V-shaped from one side, and provided with a narrow, poorly sclerotised tip, opposite in position to V-shaped notch. The stylet is thin and diaphanous – however, it contains internally a losange-shaped, noticeably more thickened area (Fig. 3 I: ta). This strengthened area is broad and triangular in the portion contained within the basal lobes, narrow and pointed at the basis of the tubular part, where it progressively disappears. Thick muscle bundles (Fig. 3 J: m) run from the basal part of the stylet to the wall of the male portion of the atrium (see below), and appear more or less limited to the area above the losange-shaped thickening.

Two companion spines, 122.2 ± 2.2 µm (range: 119-124 µm) and 132.7 ± 8.9 µm (range: 117.5- 150 µm) long in Sardinian and Sicilian specimens respectively, are

placed at both sides of the stylet (Figs 3 I, J: cs). They are intracellular, although the cell nuclei are few and not noticeable at least distally. Their bases, for a length of about 30 µm (range: 21-40 µm in the sample), are fused to the basal lobes of the stylet. Very thick muscle bundles are attached to the outer side of the spines, for at least 2/3 of the their length. They join the similar bundles attached to the basis of the stylet. The distal part of these companion spines is provided with a two-lobed apex. The basal lobe is obtuse and poorly developed. The distal lobe is similarly obtuse, but it is slender, flat, and placed at an angle of about 45° with the axis of the spine. This distal lobe is 10 ± 1.4 µm long in Sardinian specimens (Figs 3 I; 4 C) where it may even be slightly bilobed (Fig. 5 F), while it is somewhat shorter (7.2 ± 2.1 µm) in Sicilian specimens (Figs 3 G; 4 A, B; 5 E).

Two arrow-shaped lateral spines are placed lateral to the stylet-companion spines complex (Figs 3 I; 5 B: ls). They are 110.7 ± 4.0 µm (range: 104.5-113 µm) and 114.9 ± 7.1 µm (range: 103-130 µm) long in Sardinian and Sicilian specimens respectively. Distally, they widen and form a tubular apex, 38.0 ± 1.7 and 30.9 ± 3.6 µm long respectively in the two populations, which tapers into a sickle-shaped tip. The distal opening is provided, on the concave side, of an acutely pointed tip. Bundles of muscles connect the inner area of this distal portion to the wall of the atrium. Numerous, thick glands, with coarse-grained secretion, enter the tubular apex. These glands are very long, and extend well beyond the basis of the spines.

The male sclerotised structures are placed in the frontal part of the genital atrium (Fig. 3 J: a). This male portion is surrounded by muscle cells, to which the muscle bundles departing from stylet and spines are connected. In addition, some of these muscle cells

appear to be connected to the longitudinal, ventral body musculature. Overall the atrium is lined with a flat epithelium with intraepithelial nuclei.

The atrium opens to the outside through the common genital pore (Figs 3 J; 5 C: cgp). In some of the specimens sectioned, the epithelium of this pore is ciliated at the anterior side, with cilia joining the ventral ciliated sole, and unciliated at the opposite, posterior side. In others, it is overall unciliated.

Female genital system

Two ovaries are placed just posterior to the testes (Fig. 3 A: ov). Two rows of vitellaria extend from just behind the ovaries to the genital area (Fig. 3 A: vi). Part of them (about 10 per side) are placed in front of the pharynx, the remaining (about 20 per side) behind it.

The female duct could be traced only close to its outlet in the posterior, female part of the common atrium, where it is surrounded by female glands. It is internally ciliated and lined with a thin infranucleated epithelium (Fig. 3 J: fd; fp). The epithelium of the atrium, close to the female pore, is high and presumably glandular.

A bursa is present in the dorsal part of the atrium (Fig. 3 J: b). It is ovoid, up to 50 µm across, and contains large amounts of sperm, apparently in various stages of degeneration. In none of the specimens sectioned, a connection of this bursa with the atrium was apparent. Instead, in all specimens the bursa was separated from the atrium by a bar of irregular and vacuolar tissue (Fig. 3 J: vt).

Karyotype

Chromosome number: 2n = 18. A part from Chromosomes I and II, the rest of the chromosomes of the set are small, and their morphology could not always be easily appreciated. Only few plates were thus suitable for karyometrical analysis.

Sardinian specimens (based on two plates, from two specimens) = Chromosome I: 18.52 ± 0.82; 42.32 ± 0.49 (m); Chromosome II: 15.12 ± 0.53; 32.69 ± 2.6 (sm); Chromosome III: 11.2 ± 0.7; 39.72 ± 1.76 (m); Chromosome IV: 10.9 ± 0.39; 30.05 ± 0.64 (sm); Chromosome V: 9.6 ± 0.61; 27.84 ± 3.1 (sm); Chromosome VI: 9.16 ± 0.38; 31.02 ± 4.72 (sm); Chromosome VII: 8.35 ± 0.25; 19.20 ± 5.21 (st); Chromosome VIII: 8.32 ± 0.27; 24.05 ± 6.77 (st); Chromosome IX: 8.23 ± 0.54; 29.37 ± 7.83 (sm). FN=16.

Sicilian specimens (based on four plates, from three specimens) = Chromosome I: 19.42 ± 0.97; 39.3 ± 4.26 (m); Chromosome II: 15.14 ± 1.22; 27.86 ± 5.66 (sm); Chromosome III: 11.18 ± 0.66; 28 ± 9.68 (sm); Chromosome IV: 10.43 ± 0.34; 37.98 ± 3.6 (m); Chromosome V: 9.79 ± 0.1; 26.24 ± 2.46 (sm); Chromosome VI: 9.3 ± 0.45; 30.9 ± 5.72 (sm); Chromosome VII: 8.62 ± 0.35; 24.26 ± 6.87 (st); Chromosome VIII: 8.4 ± 0.33; 42.76 ± 3.18 (m); Chromosome IX: 7.67 ± 0.53; 27.99 ± 6.87 (sm). FN=17.

Based on the data available, the two populations appear karyologically very similar. Both have sets with one large metacentric pair (Chromosome I), followed by a submetacentric Chromosome II. The rest of the set consists of smaller, almost evenly sized chromosomes, mostly submetacentric or at the border between submetacentric and subtelocentric, or between submetacentric and metacentric. This fact, together with the very small size of the sample, may explain the discrepancies observed in the karyotype formulas above.

Diagnosis: Parotoplaninae with tubular copulatory stylet with two convex, triangular basal lobes. With two companion spines fused basally to the stylet, and provided with two-lobed apices: basal lobe poorly developed; distal lobe obtuse, flat, slender, placed at an angle of about 45° with the axis of the spine. With two lateral spines, provided with a tubular, recurve, pointed distal tip. Ratio among sclerotised structures: 1: 1.03: 0.9. Bursa without open connection to the atrium. Chromosome number: 2n = 18; FN = 16-17.

***Parotoplana ambrosolii* Curini-Galletti & Delogu sp. nov.**

(Figs 3 B, E; 4, D, E; 5 G, H)

Holotype: one karyological mount made permanent with lactophenol (SMNH Type-8557).

Type locality: Punta Ala (Tuscany, Italy), at the Castle (Lat. 42°48'1.57"N; Long. 10°44'10.52"E); midlittoral in coarse sand/fine gravel (June 2008).

Additional material: 3 karyological mounts, made permanent with lactophenol (CZM 494-496), 1 specimen sagittally sectioned (CZM 497); all from type locality; midlittoral. One karyological mount from the type locality, from coarse, clean sand among rocks, about 2.5 m deep.

Other localities: Tuscany: Punta Ala, Cala Civette (Lat. 42°50'43.63"N; Long. 10°46'31.84"E); midlittoral in coarse sand: 3 karyological mounts, one of which made permanent with lactophenol (June 2008) (CZM 498).

Tuscany: Castiglione della Pescaia, Le Rocchette (Lat. 42°46'24.33"N; Long. 10°47'26.27"E); midlittoral in coarse sand: one karyological mount (June 2008).

Sardinia: La Maddalena, Cala Corsara (Lat. 41°13'50.87"N; Long. 9°20'33.15"E); midlittoral in coarse sand, one karyological mount with 2 specimens (September 2010).

Etymology: the species name honours the memory of Giorgio Ambrosoli (1933-1979), Italian lawyer and bourgeois hero, who paid with his life his dedication to duty.

Description

A large, ribbon-shaped species (Fig. 3 B). Living, extended specimens up to 5-6 mm; cephalic and caudal regions not distinctly set-off from the rest of the body. Arrangement of the epithelial cells nuclei and ciliation as in the previous species. Rhabdoids slender (10 µm long and about 1 µm wide), in clusters of 3-4 rhabdoids each, arranged in few, widely spaced longitudinal rows.

Body musculature consisting of an outer circular layer, overall thicker than in the previous species, and of inner longitudinal muscles, particularly well developed ventrally.

Horizontal pharynx nearly in mid-body (Fig. 3 B: ph). In living specimens, it appeared liable to modify its shape from very long, tubular, to short, bell-shaped or even to almost round. In the sectioned specimen, the pharynx is horizontal, at least 250 µm long and about 50 µm across. The proximal part of the pharynx is strongly muscular. Strong circular muscles extend around the basis of the pharynx, and line the pharyngeal cavity. With a very small, unciliated esophagus. A few pharyngeal glands run through the length of the pharynx, and open at its tip, in a small, unciliated area.

Male genital system

With 20-50 testes arranged into two regular rows at both sides of the body, in front of the ovaries, and at some distance from the head. Copulatory organ at the posterior end of body, similar to the previous species in observations on living, squeezed specimens. Seminal vesicle convoluted; posterior region with a thin, cellular lining; frontal region with a very thin layer of circular musculature. Close to the stylet, the epithelium is higher, and presumably glandular. This 'prostatic area' is very small, about 25 µm long and 13 µm wide, and not vesicular. The sclerotised structures are formed by a central stylet and two pairs of spines.

The tube-shaped stylet is overall very similar in morphology to the previous species, with two sharply pointed, triangular, somehow convex basal lobes, that join to form a tube with a wide distal opening, with a V-shaped sinus and an opposite, elongated, poorly sclerotised tip (Figs 4 D, E). The inner, basal thickening is similar in position and shape to the previous species. The stylet is 90 µm long in the holotype (ranging 80-100 µm in sample from Tuscany (mean 88.78 ± 6.62 N= 9) and 103-106 (two measurements) in the Sardinian specimens.

The two companion spines are 79.1 ± 5.2 µm (range: 72-90 µm) and 90.5 ± 1 µm (range: 89.5-91.5 µm) long in Tuscan and Sardinian specimens respectively. In all specimens studied, their bases are not fused to the proximal part of the stylet, but rather appear to be nested between the basal lobes, without direct connection, and presumably held in place by musculature: hard squeezing results in their displacement (Fig. 4 E). Thick muscle bundles are attached to the outer side of the spines, at a distance of about 20 µm from the apex. These muscles can be traced till they join the musculature surrounding the atrium. The distal part of the companion spines is provided with a

somewhat triangular apex, 5-9 µm long, placed at an angle of about 90° with the axis of the spine (Figs 3 E; 5 G). There is some variation in the sample as to apex morphology: the distalmost margins can be more or less rounded, and, in rare instances, both are slightly protruding, so that the outline of the apex is somewhat bilobed (see Fig. 5 H).

The two lateral spines are 87.9 ± 8.9 µm (range: 70-103.5 µm) and 105 ± 7 µm (range: 98-112 µm) long in Tuscan and Sardinian specimens respectively (Fig. 4 D). They are very similar in morphology to the previous species. They are connected to a glandular complex, and to bundles of muscles which join those originating from the companion spines.

Female genital system

Two ovaries are placed caudal to the testes. Two rows of vitellaria extend from behind the ovaries to the genital area (Fig. 3 B). Large, presumably fully mature specimens had up to 12 vitellaria follicles per side in front of the pharynx, and 20-24 behind it. A few follicles are present lateral to the pharynx as well.

The female pore is located in the posterior, female part of the common atrium, surrounded an area with high and presumably glandular epithelium. A few female glands are present.

The vacuolar bursa is small (about 20 µm across) and devoid of sperm in the sectioned specimens. Similarly to the previous species, the bursa is separated from the atrium by a bar of vacuolar tissue.

Karyotype

Chromosome number: 2n = 18. Length proportions among chromosome pairs of the set very similar to those of the previous species. On the contrary, a higher number of chromosome pairs are heterobrachial, and the Fundamental Number consequently lower.

Karyotype formula (based on five plates, from three specimens from the type locality): Chromosome I: 18.6 ± 2.27; 37.83 ± 4.08 (m); Chromosome II: 15.29 ± 2.64; 21.75 ± 3.1 (st); Chromosome III: 12.12 ± 0.28; 21.73 ± 3.54 (st); Chromosome IV: 10.76 ± 0.38; 36.85 ± 5.59 (sm); Chromosome V: 10.02 ± 0.75; 24.05 ± 3.56 (st); Chromosome VI: 8.99 ± 0.8; 38.40 ± 3.99 (m); Chromosome VII: 8.94 ± 1.09; 21.98 ± 4.16 (st); Chromosome VIII: 8.36 ± 1.42; 38.71 ± 3.88 (m); Chromosome IX: 6.78 ± 0.81; 20.95 ± 6.46 (st). FN=13.

Diagnosis: Parotoplaninae with tubular copulatory stylet with two convex, triangular basal lobes. With two companion spines not fused to the stylet, and provided with triangular apices placed at an angle of about 90° with the axis of the spine. With two lateral spines, provided with a tubular, recurve, pointed distal tip. Ratio among sclerotised structures: 1: 0.9: 1. Bursa without open connection to the atrium. Chromosome number: 2n = 18; FN = 13.

***Parotoplana impastatoi* Curini-Galletti & Delogu sp. nov.**

(Figs 3 C, H; 4 F, G; 5 D, I, J)

Holotype: one whole mount made permanent with lactophenol (SMNH Type-8558).

Type locality: Agnone (Sicily, Italy) (Lat. 37°18'37.24"N; Long. 15°6'21.47"E), in fine-medium sand, at a depth of about 1.5-2 m.

Additional material: five karyological slides (containing 13 specimens) made permanent with lactophenol (CZM 499-503), 2 specimens sagittally sectioned (CZM 504; 505), one specimen horizontally sectioned (CZM 506).

Etymology: the species name honours the memory of Peppino Impastato (1948-1978), a Sicilian political activist and civic hero, who was killed by Mafia for his tireless exposures of crimes and dealings of local Mafiosi.

Description

Living animals appear ribbon-shaped, narrower and more elongate than the previous two species (Fig. 3 C). Cilia of the ventral creeping sole about 6 µm long, reaching 9 µm cephalically. Tail distinctly fan shaped, very rich in adhesive glands and long, stiff cilia. Rhabdoids somehow broader than the previous species (10 µm long and about 2 µm wide), arranged in clusters of 3-4 rhabdoids in few longitudinal rows. Body musculature with a circular, external layer particularly thick and obvious ventrally; longitudinal muscles very strong on both sides.

Pharynx nearly at mid-body, horizontal and elongate (at least 170 µm long in sectioned specimens), ciliated at both sides, with a wide unciliated area at the pharynx' tip, where the well developed pharyngeal glands open to the outside. Outer cilia longer than inner cilia (3-3.5 µm and 2-2.5 µm long respectively). Musculature layers reversed with respects to body musculature; circular component particularly well developed. Strong circular fibres surround the basis of the pharynx. A very small esophagus could be observed.

Male genital organs

With 25 testes per side in front of the ovaries. Copulatory organ provided with a large vesicle clearly divided into two parts. The posterior part is about 100 µm long and 45 µm wide, it is lined with a thin and flattened epithelium, and contains sperm. The narrow and tubular anterior part, about 60 µm long and 12-15 µm wide, curves over the bursa and enters the basis of the stylet. This part is surrounded by a thin layer of circular musculature, and its epithelium is cubic to high. The presence of heavily pigmented 'dots' in this epithelium (particularly inside the stylet) suggests its glandular nature.

The stylet is similar in morphology to the previous species (Figs 4 F, G). It is 97µm long in the holotype (ranging 90-110 µm in the sample, mean 98.2 µm ± 5.1 N= 13), with diverging, pointed basal lobes, and a tubular part 12-15 µm across at its narrower point, widening towards the distal opening (up to 27 µm wide). Similarly with the other species, the basal lobes and the most proximal part of the stylet are strengthened by two much thicker, losange-shaped inner areas.

The two companion spines are 84.5 ± 2.4 µm long, ranging 79-89 µm. They are arrow-shaped, similar in morphology to the lateral spines, with a long, pointed, distally hooked apex, and an apophysis, to which bundles of muscles are attached, placed at about 15-21 µm from the distal tip (Figs 3 H; 4 F, G; 5 I, J). Their bases are fused to the proximal part of the stylet for a length of 15-25 µm.

With two lateral spines (length: 95 – 96 µm in the holotype; range: 87 – 105 µm, mean 98.3 µm ± 4.3, N=14) similar to the two previous species (Fig. 4 G). Their tubular, pointed, sickle-shaped apex is 30 – 35 µm long (30 µm long in the holotype). Also in this case, large bundles of muscles are attached to the apophysis, while very large glands enter the basis of the apex, through which they presumably discharge.

Female genital system

With two ovaries caudal to the testes, and frontal to vitellaria. A few vitellarian follicles (3-5 per side) in front of pharynx; most (up to 15 per side) behind it. Bursa embedded in the dorsal wall of the genital atrium (Fig. 5 D). The bursal area is wide (up to 65 µm x 40 µm), and consists of several, irregular vacuoles, some of which contained sperm at different stages of degeneration. A central larger vacuole is present; it contained sperm apparently not degenerated. Similarly to the previous species, there is no indication of a direct connection between bursa and atrium. They are separated by a bar of very irregular, vacuolar tissue.

The two oviducts fuse behind the pharynx and form a ciliated female duct, that enters the atrium with a pore surrounded by high and glandular epithelium. Atrium lined with irregular epithelium with intraepithelial nuclei. Common genital pore ciliated anteriorly, unciliated posteriorly.

Karyotype:

Chromosome number: 2n = 18. Karyotype similar to the previous species in general morphology. Chromosome I: 20.1 ± 0.98; 42.29 ± 4.07 (m); Chromosome II: 14.79 ± 1.2; 28.06 ± 3.78 (sm); Chromosome III: 11.97 ± 0.57; 43.33 ± 1.75 (m); Chromosome IV: 10.78 ± 0.68; 21.63 ± 5.31 (st); Chromosome V: 9.69 ± 0.32; 38.99 ± 2.93 (m); Chromosome VI: 9.37 ± 0.57; 23.32 ± 2.85 (st); Chromosome VII: 8.26 ± 0.48; 19.09 ± 2.33 (st); Chromosome VIII: 8.15 ± 0.18; 25.52 ± 6.02 (sm); Chromosome IX: 6.84 ± 0.36; 19.18 ± 6.15 (st). FN=14 (based on 4 plates).

Diagnosis: Parotoplaninae with tubular copulatory stylet with two convex, triangular basal lobes. With two companion spines fused basally to the stylet, and provided with pointed, slightly hooked apices 15-21 µm long, with a small apophysis. With two lateral spines, provided with a tubular, recurve, pointed distal tip. Ratio among sclerotised structures: 1: 0.9: 1. Bursa without open connection to the atrium. Chromosome number: 2n = 18; FN = 14.

Parotoplana livatinoi **Curini-Galletti & Delogu sp. nov.**

(Figs 3 D, F; 4 H; 5 K, L)

Holotype: one whole mount made permanent with lactophenol (SMNH Type-8559).

Type locality: Agnone (Sicily, Italy) (Lat. 37°18'37.24"N; Long. 15°6'21.47"E), in medium sand, 1-1.5 m deep.

Additional material: 2 karyological slides from the type locality, made permanent with lactophenol (CZM 507; 508).

Etymology: the species name honours a Sicilian judge and civic hero, Rosario Livatino, born in Canicattì in 1952, murdered by Mafia killers near Agrigento on September 21, 1990, as he travelled unescorted to court.

Description

Living animals (Fig. 3 D) comparatively short and broad, lacking the distinctive parallel-sided, ribbon shape of the previous species. Pharynx tubular, held horizontally.

With about 40-50 testes arranged in two rows, in front of the ovaries. Vitellaria posterior to ovaries, partly in front (about 5 follicles per side) and partly behind (about 15 follicles per side) the pharynx.

The sclerotised apparatus consists of a central stylet and two pairs of spines.

The tube-shaped stylet is similar to that of the previous species (Fig. 4 H). It is 108 µm long in the holotype (mean 109 ± 1 µm, range: 108-110 µm; N= 3), about 11 µm broad at its middle, not distinctly widening at its top, and with two evident losange-shaped strengthenings in its proximal part.

The two companion spines are about 97 µm long in the holotype (mean 89 ± 7, range: 80–97 µm; N= 3). They are fused to the basal lobes of the stylet for a length of about 20 µm. Their apex is distinctly bilobed, about 7 µm long, and provided with one proximal, comparatively large, obtuse lobe, and a narrow distal, recurve, acutely pointed tip (Figs 3; 5 K, L).

The two lateral spines are about 95 µm long in the holotype (mean 88.3 ± 6.2 µm, range: 80-95 µm; N= 3), similar in morphology to the previous species (Fig. 2 H). The sickle-shaped apex is about 30 µm in length in the holotype (range 26-30 µm).

Karyotype

Chromosome number: 2n = 18. Karyotype similar to the previous species in general morphology, with most chromosomes meta- or submetacentric. Chromosome I: 17.89 ± 0.1; 43 ± 1.4 (m); Chromosome II: 15.35 ± 0.14; 32.61 ± 3.88 (sm); Chromosome III: 12.88 ± 1.37; 35.57 ± 1.21 (sm); Chromosome IV: 10.51 ± 0.19; 27.71 ± 5.86 (sm); Chromosome V: 10.14 ± 1.64; 36.87 ± 1.89 (sm); Chromosome VI: 9.57 ± 0.87; 41.57 ± 0.46 (m); Chromosome VII: 8.38 ± 0.31; 30.67 ± 5.93 (sm); Chromosome VIII: 7.85

± 0.59; 32.97 ± 6.54 (sm); Chromosome IX: 7.24 ± 0.85; 19.67 ± 4.55 (st). FN = 17 (based on 3 plates).

Diagnosis: Parotoplaninae with tubular copulatory stylet with two convex, triangular basal lobes. With two companion spines fused basally to the stylet, and provided with two-lobed apices: basal lobe large, obtuse; distal lobe narrow, recurve and pointed. With two lateral spines, provided with a tubular, recurve, pointed distal tip. Ratio among sclerotised structures: 1: 0.8: 0.8. Chromosome number: 2n = 18; FN = 17.

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Table 1. List of specimens investigated in the present study (GenBank accession numbers in bold), as well as sequences retrieved from GenBank. * For newly sequenced taxa only.

Family	Species	Reference number	Locality*	*18S*	*28S D1-D6*
Otoplanidae	*Parotoplana tubifera* Curini-Galletti & Delogu sp. nov.	1	Le Bombarde beach (Sardinia, Italy)	**KC971058**	**KC971081**
	Parotoplana tubifera Curini-Galletti & Delogu sp. nov.	2	Agnone (Sicily, Italy)	**KC971046**	**KC971069**
	Parotoplana tubifera Curini-Galletti & Delogu sp. nov.	3	Agnone (Sicily, Italy)	**KC971047**	**KC971070**
	Parotoplana tubifera Curini-Galletti & Delogu sp. nov.	4	Agnone (Sicily, Italy)	**KC971049**	**KC971072**
	Parotoplana ambrosolii Curini-Galletti & Delogu sp. nov.	5	Punta Ala (Tuscany, Italy)	**KC971043**	**KC971066**
	Parotoplana ambrosolii Curini-Galletti & Delogu sp. nov.	6	Punta Ala (Tuscany, Italy)	**KC971044**	**KC971067**
	Parotoplana ambrosolii Curini-Galletti & Delogu sp. nov.	7	Punta Ala (Tuscany, Italy)	**KC971045**	**KC971068**
	Parotoplana ambrosolii Curini-Galletti & Delogu sp. nov.	8	La Maddalena (Sardinia, Italy)	**KC971056**	**KC971079**
	Parotoplana ambrosolii Curini-Galletti & Delogu sp. nov.	9	La Maddalena (Sardinia, Italy)	**KC971057**	**KC971080**
	Parotoplana impastatoi Curini-Galletti & Delogu sp. nov.	10	Agnone (Sicily, Italy)	**KC971048**	**KC971071**
	Parotoplana impastatoi Curini-Galletti & Delogu sp. nov.	11	Agnone (Sicily, Italy)	**KC971050**	**KC971073**
	Parotoplana spathifera Delogu & Curini-Galletti, 2007	12	La Maddalena (Sardinia, Italy)	**KC971053**	**KC971076**
	Parotoplana pythagorae Delogu & Curini-Galletti, 2007	13	La Maddalena (Sardinia, Italy)	**KC971052**	**KC971075**
	Parotoplana renatae Ax, 1956	14	Banyuls-sur-mer (Languedoc-Roussillon, France)	**KC971062**	**KC971085**
	Parotoplana multispinosa Ax, 1956	15	Banyuls-sur-mer (Languedoc-Roussillon, France)	**KC971061**	**KC971084**
	Parotoplana bicupa Sopott-Ehlers, 1976	16	Agde (Languedoc-Roussillon, France)	**KC971063**	**KC971086**
	Parotoplana primitiva Ax, 1956	17	Agde (Languedoc-Roussillon, France)	**KC971060**	**KC971083**
	Parotoplanella progermaria Ax, 1956	18	Banyuls-sur-mer (Languedoc-Roussillon, France)	**KC971059**	**KC971082**
	Parotoplana crassispina Delogu & Curini-Galletti, 2009	19	La Maddalena (Sardinia, Italy)	**KC971051**	**KC971074**
	Parotoplana rosignana Lanfranchi & Melai, 2008	20	La Maddalena (Sardinia, Italy)	**KC971054**	**KC971077**
	Parotoplana procerostyla Ax,1956	21	Banyuls-sur-mer (Languedoc-Roussillon, France)	**KC971055**	**KC971078**
	Archotoplana holotricha Ax, 1956	22	GenBank	AJ243676	AJ270165
	Monostichoplana filum (Meixner,1938)	23	GenBank	AJ270158	AJ270173
	Xenotoplana acus Ax, Weidemann & Ehlers, 1978	24	GenBank	AJ270155	AJ270181
Monocelididae	*Monocelis longiceps* (Duges, 1830)	25	Al-Maharas (Tunisia)	**KC971064**	**KC971087**
	Minona ileanae Curini-Galletti, 1997	26	GenBank	JN224905	JN224910
	Monocelis longistyla Martens & Curini-Galletti, 1987	27	La Maddalena (Sardinia, Italy)	**KC971065**	**KC971088**
Archimonocelididae	*Archimonocelis staresoi* Martens & Curini-Galletti, 1993	28	GenBank	AJ270152	AJ270166
Calviriidae	*Calviria solaris* Martens & Curini-Galletti, 1993	29	GenBank	AJ270153	AJ270168
Unguiphora	*Polystyliphora novaehollandiae* Curini-Galletti, 1998	30	GenBank	AJ270161	AJ270177

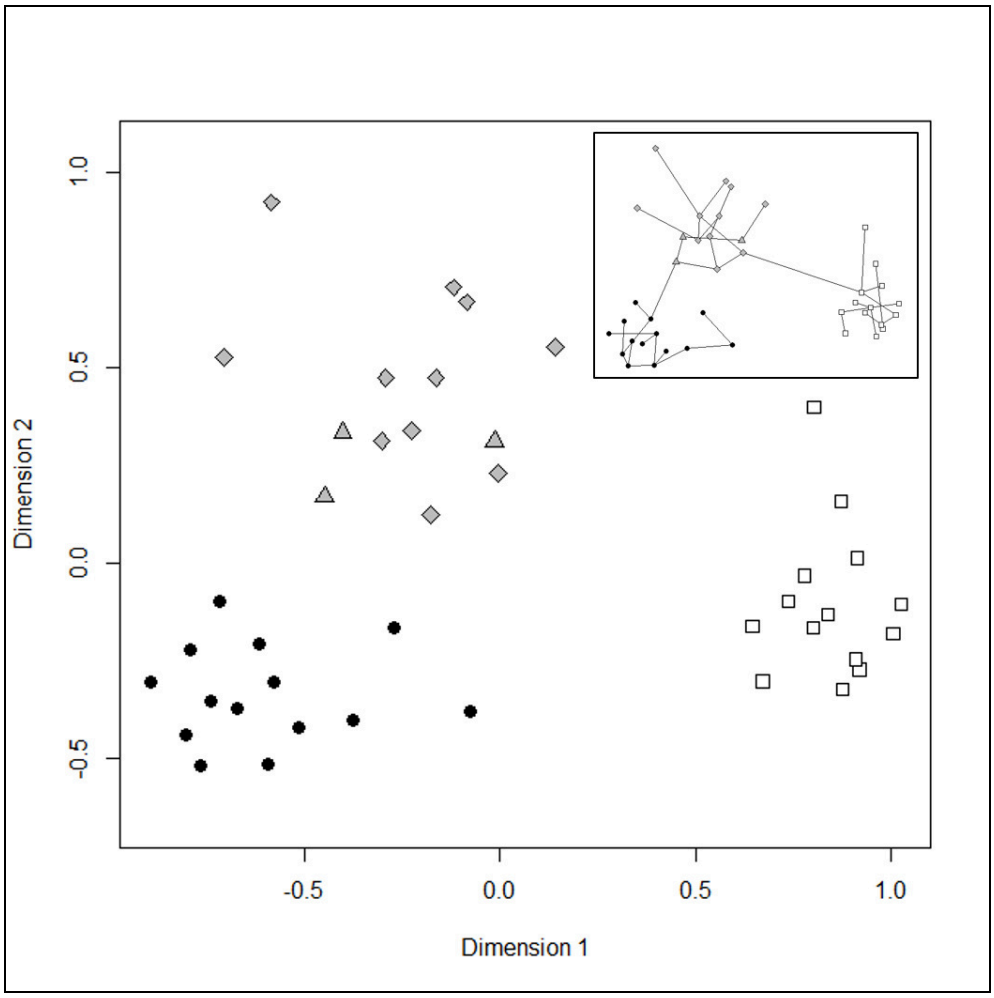

Figure 1. Multivariate analysis of morphological traits: non-metric Multi-Dimensional Scaling (nMDS) ordination based upon squared individual Euclidean distances (stress = 0.074). Specimens belonging to the four putative species are represented by different symbols: *Parotoplana tubifera* sp. nov. (black circles); *P. ambrosolii* sp. nov. (grey diamonds); *P. livatinoi* sp. nov. (grey triangles) and *P. impastatoi* sp. nov. (white squares). Colors correspond to the more likely grouping of individuals (three groups) of the k-means clustering superimposed on the ordination. The inset in the upright corner of the figure depicts a minimum spanning network superimposed on the same ordination of the main figure.

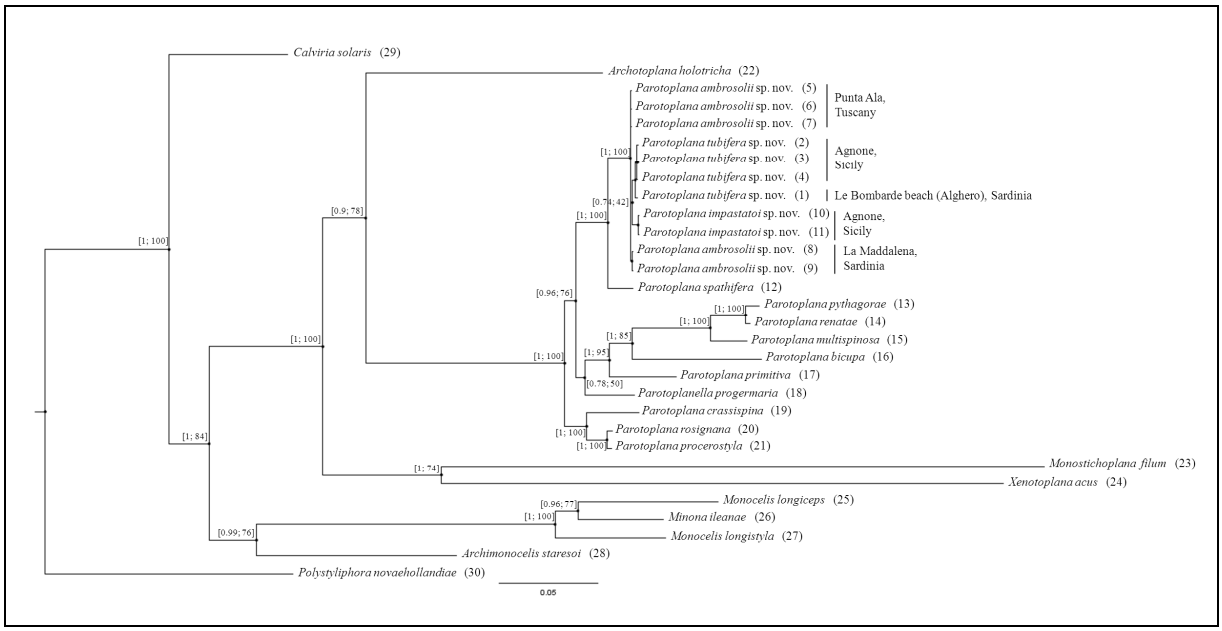

Figure 2. Tree obtained by BI based on combined *18S* / *28S D1-D6*, showing the interrelationships of the species. The branch length scale refers to the number of substitutions per site. Nodal support is indicated for BI (posterior probability, pp) and ML (bootstrap values, bv) with the numbers in square brackets [pp; bv]. Numbers on the right of each specimens refers to the species reference numbers in Table 1.

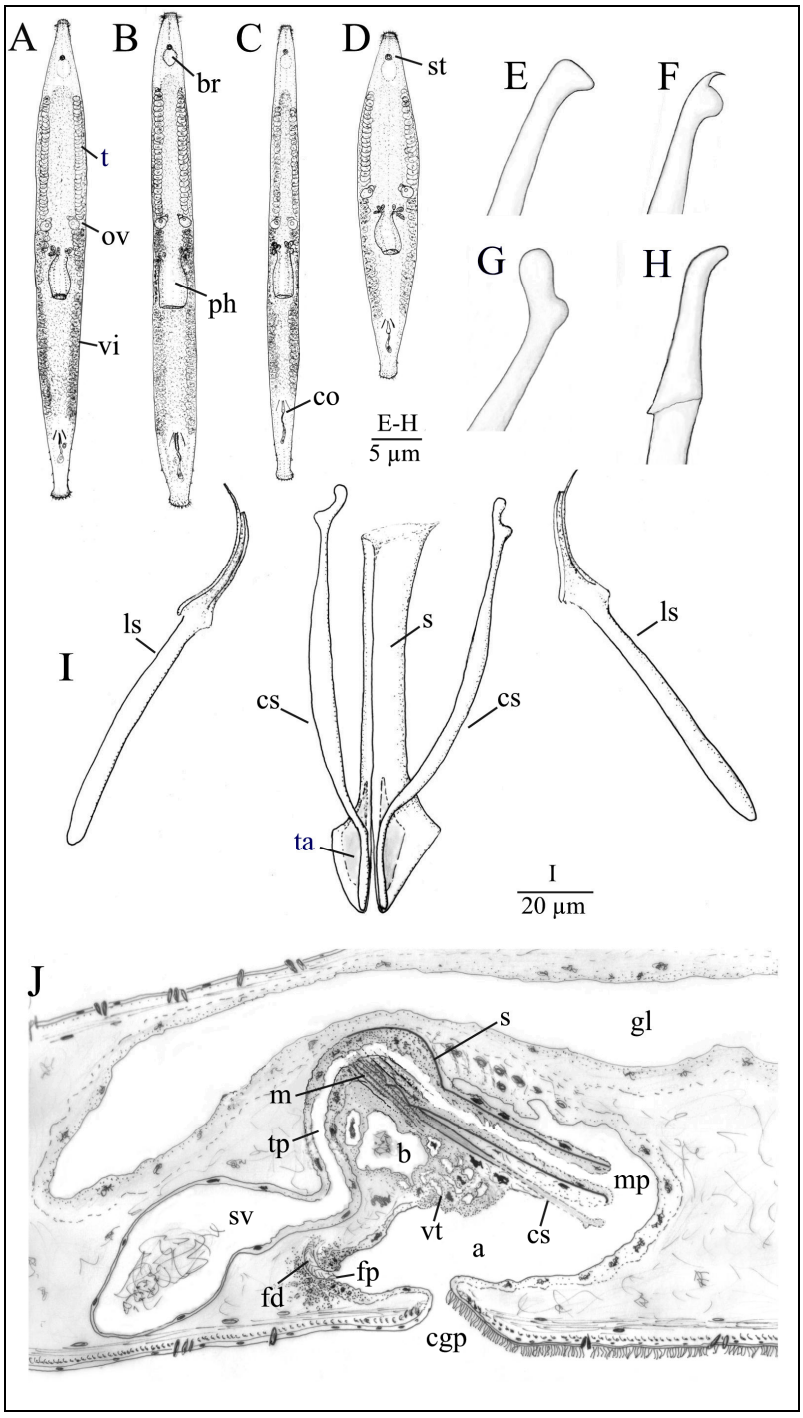

Figure 3. A-D: habitus of living specimens of *Parotoplana tubifera* sp. nov. (A); *P. ambrosolii* sp. nov. (B); *P. impastatoi* sp. nov. (C); *P. livatinoi* sp. nov. (D). E-H: apices of the companion spines of *P. ambrosolii* (E); *P. livatinoi* (F); *P. tubifera* (G); *P. impastatoi* (H). I, J: *P. tubifera*: sclerotised structures of the copulatory organ (I); reconstruction of the genital organs from sagittal sections (J).

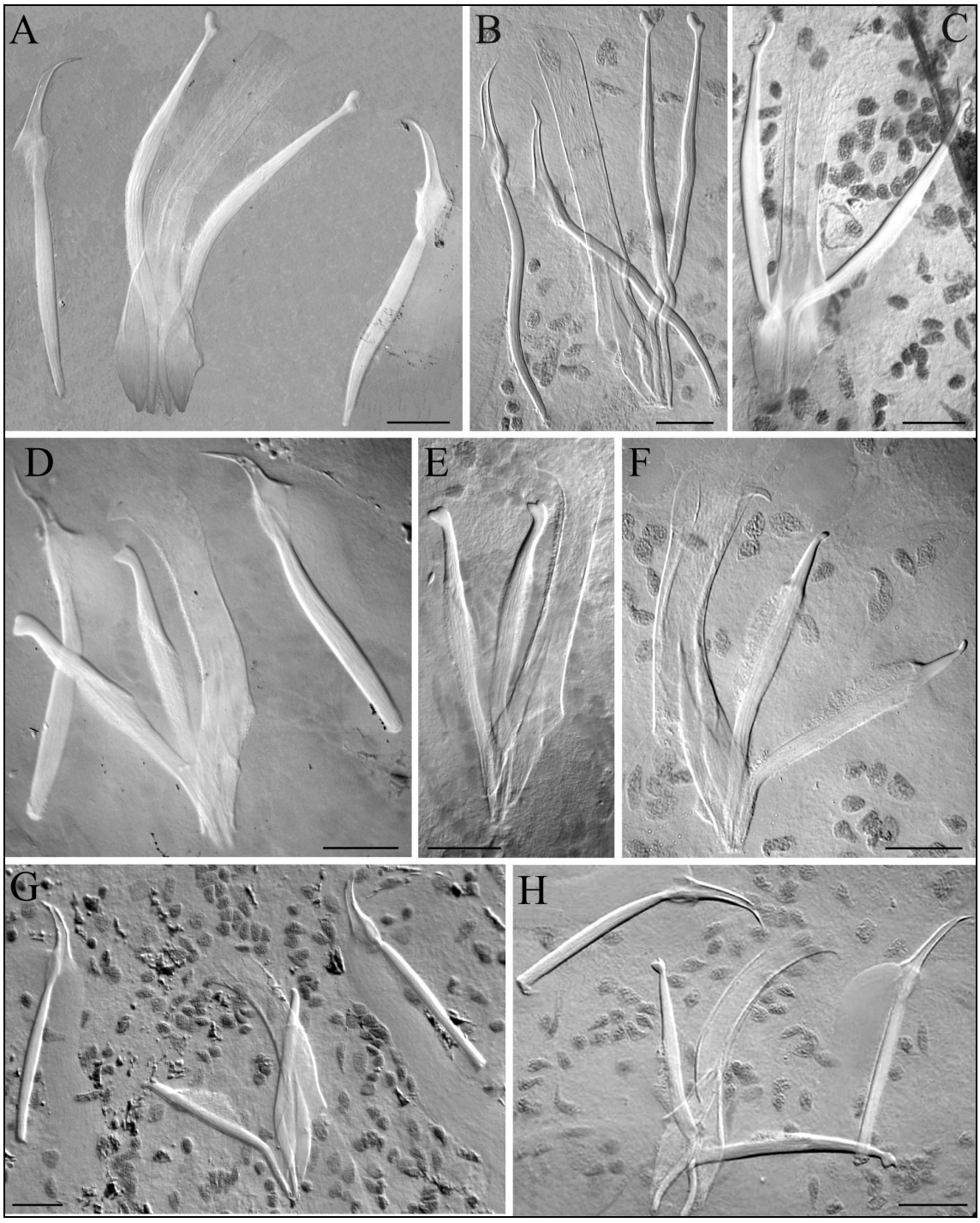

Figure 4. Sclerotised structures of *Parotoplana tubifera* sp. nov. (A: CZM 489; B: CZM 485; C: Holotype); *P. ambrosolii* sp. nov. (D: Holotype; E: CZM 498); *P. impastatoi* sp. nov. (F: CZM 499; G: Holotype); *P. livatinoi* sp. nov. (H: Holotype). Scale bar = 20 µm.

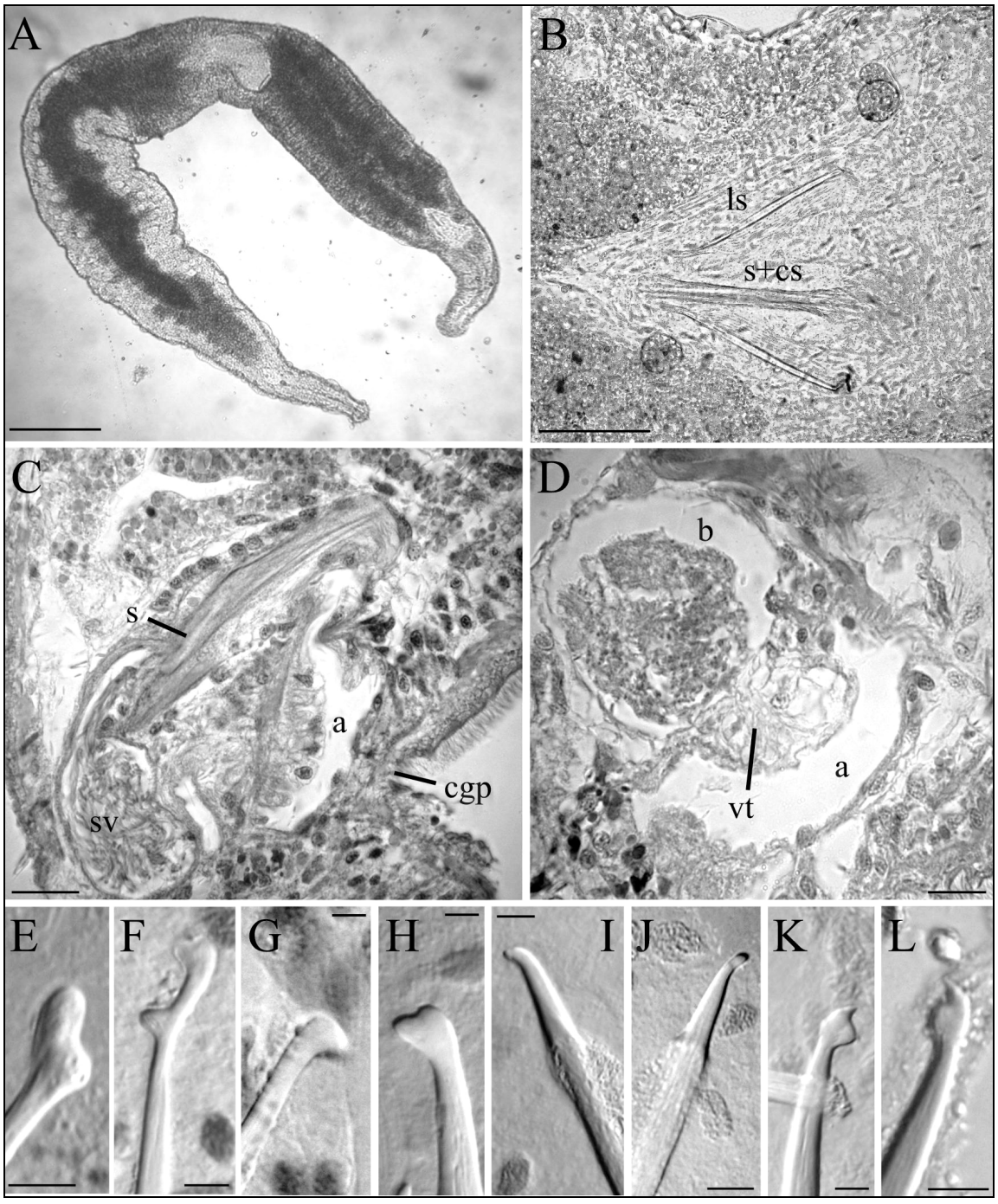

Figure 5. A-C: *Parotoplana tubifera* sp. nov. (A: habitus; B: sclerotised structures from a living specimen; C: sagittal section of the copulatory organ). D: sagittal section of bursa of *P. impastatoi* sp. nov. E-N: apices of companion spines of *P. tubifera* (E, F); *P. ambrosolii* sp. nov. (G, H); *P. impastatoi* (I, J); *P. livatinoi* sp. nov. (K, L). Scale bar = A: 400 µm; B: 50 µm; C, D: 20 µm; E-L: 5 µm.

Chapter 4

Molecular approach to the phylogeny of the genus *Monocelis* O.F. Müller, 1774 (Platyhelminthes: Proseriata) with two new species from the Mediterranean Sea

Fabio Scarpa, Tiziana Lai, Daria Sanna, Piero Cossu, Marco Curini Galletti, Marco Casu

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Molecular approach to the phylogeny of the genus *Monocelis* O.F. Müller, 1774 (Platyhelminthes: Proseriata) with two new species from the Mediterranean Sea

FABIO SCARPA, TIZIANA LAI, DARIA SANNA, PIERO COSSU, MARCO CURINI-GALLETTI, MARCO CASU*

Dipartimento di Scienze della Natura e del Territorio – Sezione di Zoologia, Archeozoologia e Genetica, Università di Sassari, Via F. Muroni 25, 07100 Sassari, Italy - Centro di Eccellenza dell'Università di Sassari

*Corresponding author: Marco Casu

Dipartimento di Scienze della Natura e del Territorio –

Sezione di Zoologia, Archeozoologia e Genetica

Università di Sassari

Via F. Muroni 25

07100 Sassari, Italy

e-mail: marcasu@uniss.it

phone: +39 079 228924

fax: +39 079 228665

Abstract

Monocelis lineata is a species complex spread in midlittoral habitats of the Mediterranean and the Atlantic. Previous studies evidenced the occurrence of at least four taxonomic unit (*M. lineata sensu stricto*), and pointed out on the possible occurrence of two further species of the complex, with a very narrow distribution (Cala Rossa and Porto Puddu, North Sardinia, Mediterranean). This study was addressed to unravel the actual extent the species complex of *M. lineata* in the Mediterranean Sea, to gain new insight on the taxonomic status of the two populations from North Sardinia. Moreover, phylogenetic relationships within the genus *Monocelis*, and within the family Monocelididae, were investigated. We used as molecular markers the complete nuclear small subunit rRNA (18S) gene and the partial nuclear large subunit rRNA (28S) fragment (spanning variable domains D1-D6). Results showed that the individuals from Cala Rossa and those from Porto Puddu grouped in two different clusters. Particularly, Porto Puddu comes out as sister taxon of the Atlantic species *M. fusca*, whereas Cala Rossa clustered externally to the remaining Atlantic and Mediterranean *M. lineata sensu stricto*. Furthermore, within Mediterranean samples of *M. lineata sensu stricto*, the occurrence of at least three species was confirmed. Therefore, our study showed the occurrence of two new species of the genus *Monocelis*, with limited geographic distribution. Furthermore, monophyly of the *M. lineata* species complex was not supported, as well as relationships within Atlantic and Mediterranean populations of *M. lineata sensu stricto* were not resolved. The need for wider molecular and taxonomic sampling is stressed.

Key words: Proseriata; *Monocelis*; 18S; 28S D1-D6; species complex; molecular phylogeny.

1. Introduction

The taxon Proseriata represents a cosmopolitan, species-rich and diverse order of free-living Neoophora (Platyhelminthes). Most Proseriata are minute interstitial organisms with an elongate body, occurring in all marine habitats, from supra- to sublittoral environments, and in all kinds of sediments (Curini-Galletti & Martens, 1990). They are particularly common in high-energy habitats, with medium to coarse sediments (Reise, 1988), where they may be the dominant metazoan species (Martens & Schockaert, 1986). Proseriata are carnivores and/or scavengers (Martens & Schockaert, 1986), and their impact on meiofaunal organisms may be considerable (Littlewood *et al*., 2000). Within the order Proseriata, the family Monocelididae Hofsten, 1907 is the largest and most diverse Curini-Galletti *et al*., 2010). This family represents a wide and heterogeneous taxon (Tyler *et al*., 2006), whose phylogenetic relationship has been investigated in several studies, but even to date they are not completely solved. Among the members of Monocelididae, the most well known and cited species is *Monocelis lineata* OF Müller, 1774. It is one of the most common and characteristic flatworms of its range, which includes both coasts of the North Atlantic, the Mediterranean and the Black Sea. The species occurs in brackish to marine habitats, on any kind of substrates (Ax, 1956). Due to its abundance and widespread occurrence, it is often taken as the typical Proseriata in zoological textbooks, and is among the very few 'microturbellarians' mentioned in identification guides to the European marine fauna (e.g. Riedl, 1983). It is a morphologically 'simple' species lacking sclerotised structures, which usually aid species identification.

A molecular research carried out by means of allozymes electrophoresis (Casu & Curini-Galletti, 2004) suggested that the nominal species *Monocelis lineata* is a species

complex (i.e., a group of closely related species, where the exact demarcation between species is often unclear or cryptic owing to their recent and as yet, usually, incomplete reproductive isolation (Allaby, 1999)) composed by at least four taxonomic units, candidate species (*sensu* Alonso *et al*., 2012), one species (defined as species "F", in Casu & Curini-Galletti, 2004) from the Atlantic Ocean (for details about Atlantic populations' phylogeography of *M. lineata* see Casu *et al*., 2011a), and three from the Mediterranean Sea: two brackish population with eye pigment (defined as species "A" and "B", in Casu & Curini-Galletti, 2004); and one marine population without the eye pigment (defined as species "C", in Casu & Curini-Galletti, 2004). Casu & Curini-Galletti (2004) highlighted an unexpected result for two geographically close Mediterranean populations of the *M. lineata* complex from North Sardinia (Cala Rossa and Porto Puddu). Indeed, individuals belonging to the Cala Rossa (marine, found on algae) and Porto Puddu (found in a small brackish area), both with pigmented eye-spots, did not cluster within the Mediterranean populations clade, but they were placed as an outgroup, external even to the Atlantic population. Genetic distance found in Casu & Curini-Galletti (2004) between these two North Sardinian populations, and among them and the other populations, suggested to consider them as two further species of the complex (defined as species "D" (Cala Rossa), and "E" (Porto Puddu)). Interestingly, from a morphologic perspective specimens from Cala Rossa and Porto Puddu showed a few shared features, and several morphological differences from the species complex referable to *Monocelis lineata sensu stricto* (species "A", "B", "C", and "F", in Casu & Curini-Galletti, 2004) (Curini-Galletti pers. obs., in prep.). However, lacking further molecular data, the question about the occurrence of a *M. lineata* species complex

remains to date unsolved, and the phylogenetic position of the Mediterranean specimens from Cala Rossa and Porto Puddu is still puzzling.

Our research was therefore aimed to provide further insight on the phylogenetic relationship within the genus *Monocelis*, and within the family Monocelididae, focusing on the two populations from Cala Rossa and Porto Puddu, that may contribute to estimate the actual extent the species complex of *M. lineata* in the Mediterranean Sea.

For these purposes we chose to use two nuclear ribosomal markers, the complete nuclear small subunit rRNA (18S) gene and the partial nuclear large subunit rRNA (28S) fragment (spanning variable domains D1-D6). These molecular markers are widely applied to molecular systematic in Proseriata (see Litvaitis *et al*., 1996; Littlewood *et al*., 1999; 2000; Curini *et al*., 2010), and in several cases they showed their potential in discriminate groups of populations referable to distinct species (see Casu *et al*., 2011b and references therein). To gain a wider landscape of the genetic and specific relationship of the genus *Monocelis* , we designed a dataset with all of the available species. This dataset also allow us to use already identified *Monocelis* species as reference for a better understanding about the possible taxonomic rank of the species here studied.

2. Materials and methods

2.1. Sampling, DNA extraction, amplification and sequencing

Samples were collected manually by scooping up the superficial layer of sediment.

Extraction of the animals from the sediment was accomplished using MgCl2 decantation (Martens, 1984). Each specimen was first studied alive by slight squeezing under the cover slip. For information about sampling localities see Table 1.

Genomic DNA was extracted using the Macherey-Nagel NucleoSpin Tissue (MACHEREY-NAGEL GmbH & Co. KG) according to the supplier's instructions. After extraction, DNA was stored as a solution at 4 °C.

Complete 18S and partial 28S (spanning variable domains D1-D6) were analysed for a total of 56 specimens, 46 of which newly sequenced specifically for this study by us, and 10 taken from GenBank (for details see Table 1). The dataset was built using 10 individuals belonging to the new species here under study (five for each population). In addition, to verify the monophyly of the taxon, 36 individuals belonging to the genus *Monocelis* (all sequenced by us for this study, Table 1) were added. To investigate phylogenetic relationship within the family nine species of Monocelididae (from GenBank, Table 1) were added. One member to the family Archimonocelididae (from GenBank) was added as outgroup (Table 1).

Amplifications for 18S and 28S D1-D6 regions were carried out using the following primers: 18S: A (forward) GCG AAT GGC TCA TTA AAT CAG, and B (reverse) CTT GTT ACG ACT TTT ACT TCC (Littlewood & Olson 2001); 28S D1-D6: LSU5 (forward) TAG GTC GAC CCG CTG AAY TTA AGC A, and LSUD6-3 (reverse) GGA ACC CTT CTC CAC TTC AGT C (Littlewood *et al*., 2000).

PCR was carried out in a total volume of 25 µl containing 5 ng/µl of total genomic DNA on average, 1.0 U of Taq DNA Polymerase (Euroclone), 1× reaction buffer, 3.5 mM of $MgCl_2$, 0.32 µM of each primer, and 200 µM of each dNTP. PCR amplification was performed in a MJ PTC 200 Thermal Cycler (Biorad) programmed as follows: 1 cycle of 2 min at 94° C, 35 cycles of 1 min at 94° C, 1 min at 54° C (18S / 28S D1-D6 primers' annealing temperature), and 1 min and 30 s at 72° C. At the end, a post-treatment for 5 min at 72° C and a final cooling at 4° C were carried out. Both positive

and negative controls were used to test the effectiveness of the PCR reagents, and the absence of possible contaminations.

Electrophoresis was carried out on 2% agarose gels, prepared using 1× SBA buffer (sodium boric acid, pH 8.2) and stained with a 1 µl/20 ml ethidium bromide solution. PCR products were purified by ExoSAP-IT (USB Corporation) and sequenced for both forward and reverse 18S and 28S D1-D6 strands, using an external sequencing core service (Macrogen Europe).

2.2. Phylogenetic analysis and genetic distances

The 18S and 28S D1-D6 sequences were aligned separately using the algorithm Q-INS-I implemented in Mafft 6.903 (Katoh & Toh, 2008), which is appropriate for non-coding RNA as it considers RNA secondary structure. The best probabilistic model of sequence evolution was determined after evaluation by JModeltest 0.1 (Posada, 2008), with a maximum likelihood optimised search, using the Akaike Information Criterion (AIC) and the Bayesian Information Criterion (BIC). The model GTR+G (Tavaré, 1986) both for 18S and 28S D1-D6 has been calculated as the best fitting model to our sequences by either criterions.

The genetic pairwise distance corrected according to the Kimura two-parameter model (*K2P*) (Kimura, 1980) was estimated by means of the software Mega 5.2 (Tamura *et al*., 2011) with 1,000 bootstrap replications.

Phylogenetic relationships among individuals and species were investigated using both Maximum Likelihood (ML) and Bayesian Inference (BI) on the combined 18S and 28S D1-D6 sequences. We set as outgroup for the analyses the species *Archimonocelis staresoi* Martens & Curini-Galletti 1993. ML was performed using the genetic

algorithm implemented in Garli 2.0 (Zwickl, 2006). In order to find the best tree, the configuration file for partitioned models was set up to perform 10 replicate searches (searchreps = 10). Model parameters: ratematrix = (0 1 2 3 4 5), statefrequencies = estimated, ratehetmodel = gamma, numratecats = 4, corresponding to the evolution model calculated by JModeltest, were used. In order to allow independent estimates of the parameters for each gene, the option link was set to 0. The parameter modweight was set to 0.0015, as we have two partitions. Finally, node support was assessed by 1,000 bootstraps (bootstrapreps = 1000). Consensus tree was computed using TreeAnnotator 1.7.4 (part of the BEAST package) (Drummond & Rambaut, 2007) and visualised by FigTree 1.4.0 (http://tree.bio.ed.ac.uk/software/figtree/).

BI was carried out using the software MrBayes 3.2.1 (Ronquist *et al*., 2012), specifying a partitioned model and setting as model parameters: NST = 6, rates = gamma, ngammacat = 4. Two independent runs each consisting of four Metropolis-coupled MCMC chains (one cold and three heated chains) were run simultaneously for 5,000,000 generations, sampling trees every 1,000 generations. We allowed each partition to have its own set of parameters and a potentially different overall evolutionary rate. The first 25% of sampled trees were discarded. Convergence of chains was checked first scrolling down the Average Standard Deviation of Split Frequencies (ASDSF), that approaches 0 if the chains convergence has been reached (Ronquist *et al*., 2012), and then checking the Potential Scale Reduction Factor (PSRF), that compares the variance among runs with the variance within runs (Gelman & Rubin, 1992). Initially, variance among runs will be higher than the variance within runs. If chains converge the variances will became more similar and the PSRF will approach 1.

Nodes with a percentage of posterior probability lower than 95% are considered not highly supported. Phylogenetic tree was visualised using FigTree.

3. Results and Discussion

After the alignment, sequences 1656 and 1596 *bp* long were obtained for the 18S and 28S D1-D6 regions, respectively (see Table 1 for the GenBank accession numbers). Because BI and ML generated trees with consistent topology, we reported only the Bayesian tree (Fig. 1).

The most striking results obtained in this study is the clear separation in two well defined cluster of individuals which correspond to Cala Rossa, *Monocelis* sp. "D", following the nomenclature given in Casu & Curini-Galletti (2004) (cluster D in Fig. 1), and of Porto Puddu, *Monocelis* sp. "E" (cluster E in Fig. 1). In particular, phylogenetic tree shows that individuals of *Monocelis* sp. "E" clustered in a clade that presents a sister-taxon relationship with *Monocelis fusca* (Fig. 1). Similarly, individuals of *Monocelis* sp. "D", clustered in a clade reciprocally monophyletic with the cluster in which the remaining Atlantic and Mediterranean *Monocelis lineata sensu stricto* specimens group. This clade shows a sister-taxa relationship with the above-described cluster which comprises *Monocelis fusca* plus *Monocelis* sp. "E".

Within the cluster which comprises all individuals referable to *Monocelis lineata sensu stricto*, the Mediterranean samples constitute a monophyletic group (Fig. 1). This group is split into two reciprocally monophyletic clades, one composite by marine samples (species "C", in Casu & Curini-Galletti, 2004), and one by brackish samples, further separated in two cluster, representative of the two brackish taxonomic units well separated also by allozyme electrophoresis analyses (species "A" and "B", in Casu &

Curini-Galletti, 2004). Conversely, individuals belonging to the Atlantic populations do not form a unique distinct clade, but they appear as a paraphyletic group (Fig. 1). It is noteworthy that two Atlantic individuals from Ferrol (Galicia) clustered externally to the cluster of Mediterranean population, as their sister group. This is suggestive of the fact that some genetic exchange occurs (or has occurred) between Mediterranean populations and the Atlantic populations geographically more related (Fig. 1).

From a phylogenetic perspective, our results do not support the monophyly of the genus *Monocelis*; caused by the placement of *M. longiceps* that is not inserted within the *Monocelis* clade, but is positioned as outgroup of the the clade composed by all specimens belonging to the genus *Pseudomonocelis* and *Minona ileanae*.

Since *Monocelis* sp. "E" and "D" are nested within the group of species belonging to the genus *Monocelis* our results indicate their correct affiliation to the genus, as suggested on morphologic bases (Curini-Galletti pers. obs.). In this context, the presence in the dataset of other well-known and clearly morphologically and karyologically different species belonging to the genus (*M. fusca*, *M. longistyla* and *M. longiceps*) becomes crucial. Indeed, their position in the phylogenetic tree can be used as reference for species discrimination, and their pairwise genetic distances corrected according to the *K2P* model were used as landmark. Interestingly, values obtained between *Monocelis* sp. "E", "D" and other well-known species are consistent to those obtained among *M. fusca*, *M. longistyla* and *M. longiceps* (see Table 2 and Table 3), they seem to show level of genetic diversity ascribable to specific differentiation. Moreover, the condition of candidate species (*sensu* Alonso *et al*., 2012), is underlined by the fact that *Monocelis* sp. "E" and *Monocelis* sp. "D" are clearly distinct (with high node support) from *M. fusca*, *M. longistyla* and *M. longiceps*. In future researches, it would be desirable to

perform combined analysis using either morphology, karyology and reproductive biology, in order to obtain an integrative taxonomic approach that can allow us to unravel a picture as clear as possible of their taxonomical position.

Because molecular analysis does not show that *Monocelis* sp. "E" and *Monocelis* sp. "D" are phylogenetically closely related, we suggest that their shared morphologic characters (Curini-Galletti pers. obs.) may not have a phylogenetic relevance, but are either homoplastic or, rather, plesiomorphic features. It is hoped that the study, both molecular and morphological, of the new species of *Monocelis* which have been recently discovered (Curini-Galletti, unpubl. data) will help to disentangle this matter.

It is worth mentioning that sampling campaigns over several years aimed to find new samples of *Monocelis* sp. "E" and *Monocelis* sp. "D" in the Mediterranean did not produce further individuals which can be attribute to one of these to taxonomic units. This suggests that the two new candidate species have a very limited geographic distribution, which may potentially represents a threat to their conservation. This condition for two potentially new species, still undescribed, pointed out the importance of performing meiofauna surveys, in order to avoid that species become extinct even before their find and formally description. In a poorly known taxon as Proseriata, local extinctions may be not rare events. For example, during data collection for a research aimed to investigate the occurrence of a sibling species complex of *Pseudomonocelis ophiocephala sensu lato*, a local extinction of at least one population (Calvi, Corsica) belonging to one of the four sibling species detected, *Pseudomonocelis caputserpentis* Casu & Curini-Galletti, 2006, occurred (Casu & Curini-Galletti, 2006). This means that, if local extinction should happen for a species which occur only in one locality (such as

the case of *Monocelis* sp. “E” and *Monocelis* sp. “D”) then the local extinction may result in an irrecoverable loss of a species.

Finally, although results obtained in this research provided an important answers to our questions (namely the occurrence of two candidate species of the genus *Monocelis*), they evidenced at the same time the presence of still open issues: first, since the genus *Monocelis* seems to be not monophyletic, phylogenetic relationship within the genus should be further investigated; second, while analysis suggested that, consistently to previous allozyme results (Casu & Curini-Galletti, 2004), a *Monocelis lineata* complex in the Mediterranean exists, nonetheless the questions remains not fully solved, as well as the relationship between Atlantic and Mediterranean populations. In both cases further analyses with a wider both taxonomic and molecular sampling are needed. In particular more individuals belonging to marine and brackish-water Mediterranean populations, and hopefully further Atlantic samples geographically very close to the Mediterranean, especially from the areas close to the Strait of Gibraltar, should be added. Regarding the molecular sampling, further molecular markers should be searched for and tested, in order to unravel a finer resolution within the *M. lineata* species complex. Indeed, the lack of resolution of species complexes in meiofaunal organisms may contribute to the underestimation of the rate of marine extinctions, so far exclusively known for macrofaunal organisms (Roberts & Hawkins, 1999).

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Table 1. List of species sampled and sequences used for this study. Accession numbers refer to GenBank codes; accession numbers of new sequences are not yet available. *For newly sequenced taxa only. [#] Population also used in Casu & Curini-Galletti (2004). [a] The suffixes "O" and "X" indicate the presence and the absence of the eye pigment, respectively.

Family	Species	Abbreviation[a]	Locality*	18S	28S D1-D6
Monocelididae	*Monocelis* sp. "E"	MSPE O [#]	Porto Puddu (Sardinia, Italy)	XXXX	XXXX
	Monocelis sp. "D"	MSP D O [#]	Cala Rossa (Sardinia, Italy)	XXXX	XXXX
	Monocelis lineata OF Müller, 1774	MPP X [#]	Porto Pozzo (Sardinia, Italy)	XXXX	XXXX
	Monocelis lineata OF Müller, 1774	MCH X [#]	Charaki (Rhodes, Greece)	XXXX	XXXX
	Monocelis lineata OF Müller, 1774	MPL O [#]	Pilo (Sardinia, Italy)	XXXX	XXXX
	Monocelis lineata OF Müller, 1774	MCS O [#]	Casaraccio (Sardinia, Italy)	XXXX	XXXX
	Monocelis lineata OF Müller, 1774	MCO O [#]	Colostrai (Sardinia, Italy)	XXXX	XXXX
	Monocelis lineata OF Müller, 1774	MAL O	Alexandria (Egypt)	XXXX	XXXX
	Monocelis lineata OF Müller, 1774	MKQ O	Kilmore Quay (Ireland)	XXXX	XXXX
	Monocelis lineata OF Müller, 1774	MGA O	Ferrol (Galicia, Spain)	XXXX	XXXX
	Monocelis lineata OF Müller, 1774	MKF O [#]	Keflavik (Iceland)	XXXX	XXXX
	Monocelis lineata OF Müller, 1774	MTJ O	Tjärnö (Sweden)	XXXX	XXXX
	Monocelis lineata OF Müller, 1774	MTJ X	Tjärnö (Sweden)	XXXX	XXXX
	Monocelis lineata OF Müller, 1774	MAR O	Ardrossan (Scotland)	XXXX	XXXX
	Monocelis lineata OF Müller, 1774	MAR X	Ardrossan (Scotland)	XXXX	XXXX
	Monocelis lineata OF Müller, 1774	MRO O	Roscoff (France)	XXXX	XXXX
	Monocelis lineata OF Müller, 1774	MRO X	Roscoff (France)	XXXX	XXXX
	Monocelis lineata OF Müller, 1774	MHE O	Helsingor (Denmark)	XXXX	XXXX
	Monocelis longiceps (Duges, 1830)	MLG	Al-Maharas (Tunisia)	KC971064	KC971087
	Monocelis longistyla Martens & Curini-Galletti, 1987	MLY	La Maddalena (Sardinia, Italy)	XXXX	XXXX
	Minona ileanae Curini-Galletti, 1997	MIL	GenBank	JN224905	JN224910
	Monocelis fusca Örsted, 1843	MFU	Ardrossan, Scotland	XXXX	XXXX
	Archiloa rivilaris de Beauchamp, 1910	ARI	GenBank	U70077	U40049
	Pseudomonocelis ophiocephala (Schmidt, 1861),	POP	GenBank	JN224895	JN224907
	Pseudomonocelis occidentalis Curini-Galletti, Casu & Lai, 2011	POC	GenBank	JN224894	JN224909
	Pseudomonocelis orientalis Curini-Galletti, Casu & Lai, 2011	POR	GenBank	JN224896	JN224908
	Pseudomonocelis agilis (Schultze, 1851)	PAG	GenBank	JN224897	JN224912
	Pseudomonocelis cetinae Meixner, 1943	PCE	GenBank	JN224899	JN224913

Family	Species	Abbreviation[a]	Locality*	18S	28S D1-D6
Monocelididae	*Pseudomonocelis* cf *cavernicola* Schockaert & Martens, 1987	PCFC	GenBank	JN224900	JN224914
	Pseudomonocelis paupercula Curini-Galletti, Casu & Lai, 2011	PPA	GenBank	JN224901	JN224915
Archimonocelididae	*Archimonocelis staresoi* Martens & Curini-Galletti, 1993	AST	GenBank	AJ270152	AJ270166

Table 2. Pairwise genetic distance, corrected according the Kimura two parameters model, between groups on the 18S fragment. Values above the diagonal represent the standard error. Values above the diagonal represent the standard error. For the abbreviation codes see Table 1.

	MFU	MLY	MLG	MPP X	MCH X	MPL O	MCO O	MAL O	MGA O	MKQ O	MTJ X	MKF O	MHE O	MSPE O	MSPD O	MCS O	MTJ O	MAR X	MAR O	MRO O	MRO X	ARI	POP	POR	POC	PAG	PCE	PCFC	PPA	AST	MIL
MFU	-	0.0066	0.0075	0.0029	0.0029	0.0031	0.0029	0.0029	0.0030	0.0030	0.0030	0.0030	0.0030	0.0033	0.0037	0.0029	0.0030	0.0032	0.0030	0.0031	0.0030	0.0148	0.0147	0.0145	0.0146	0.0131	0.0150	0.0092	0.0089	0.0246	0.0064
MLY	0.0404	-	0.0076	0.0054	0.0054	0.0056	0.0054	0.0054	0.0053	0.0053	0.0053	0.0053	0.0053	0.0055	0.0064	0.0054	0.0053	0.0055	0.0053	0.0054	0.0053	0.0148	0.0151	0.0150	0.0148	0.0141	0.0139	0.0091	0.0086	0.0273	0.0068
MLG	0.0482	0.0560	-	0.0069	0.0069	0.0069	0.0069	0.0069	0.0070	0.0070	0.0070	0.0070	0.0070	0.0069	0.0070	0.0069	0.0070	0.0072	0.0070	0.0071	0.0070	0.0169	0.0138	0.0136	0.0137	0.0128	0.0130	0.0097	0.0090	0.0287	0.0077
MPP X	0.0120	0.0311	0.0411	-	0.0000	0.0009	0.0000	0.0000	0.0009	0.0009	0.0009	0.0009	0.0009	0.0017	0.0023	0.0000	0.0009	0.0012	0.0009	0.0012	0.0009	0.0143	0.0134	0.0136	0.0133	0.0121	0.0134	0.0077	0.0077	0.0238	0.0055
MCH X	0.0120	0.0311	0.0411	0.0000	-	0.0009	0.0000	0.0000	0.0009	0.0009	0.0009	0.0009	0.0009	0.0017	0.0023	0.0000	0.0009	0.0012	0.0009	0.0012	0.0009	0.0143	0.0134	0.0136	0.0133	0.0121	0.0134	0.0077	0.0077	0.0238	0.0055
MPL O	0.0134	0.0318	0.0411	0.0013	0.0013	-	0.0009	0.0009	0.0013	0.0013	0.0013	0.0013	0.0013	0.0019	0.0024	0.0009	0.0013	0.0014	0.0013	0.0014	0.0013	0.0143	0.0136	0.0139	0.0135	0.0120	0.0132	0.0079	0.0078	0.0238	0.0056
MCO O	0.0120	0.0311	0.0411	0.0000	0.0000	0.0013	-	0.0000	0.0009	0.0009	0.0009	0.0009	0.0009	0.0017	0.0023	0.0000	0.0009	0.0012	0.0009	0.0012	0.0009	0.0143	0.0134	0.0136	0.0133	0.0121	0.0134	0.0077	0.0077	0.0238	0.0055
MAL O	0.0120	0.0311	0.0411	0.0000	0.0000	0.0013	0.0000	-	0.0009	0.0009	0.0009	0.0009	0.0009	0.0017	0.0023	0.0000	0.0009	0.0012	0.0009	0.0012	0.0009	0.0143	0.0134	0.0136	0.0133	0.0121	0.0134	0.0077	0.0077	0.0238	0.0055
MGA O	0.0120	0.0294	0.0412	0.0013	0.0013	0.0027	0.0013	0.0013	-	0.0000	0.0000	0.0000	0.0000	0.0013	0.0022	0.0009	0.0000	0.0006	0.0000	0.0006	0.0000	0.0142	0.0131	0.0133	0.0130	0.0119	0.0132	0.0076	0.0076	0.0237	0.0054
MKQ O	0.0120	0.0294	0.0412	0.0013	0.0013	0.0027	0.0013	0.0013	0.0000	-	0.0000	0.0000	0.0000	0.0013	0.0022	0.0009	0.0000	0.0006	0.0000	0.0006	0.0000	0.0142	0.0131	0.0133	0.0130	0.0119	0.0132	0.0076	0.0076	0.0237	0.0054
MTJ X	0.0120	0.0294	0.0412	0.0013	0.0013	0.0027	0.0013	0.0013	0.0000	0.0000	-	0.0000	0.0000	0.0013	0.0022	0.0009	0.0000	0.0006	0.0000	0.0006	0.0000	0.0142	0.0131	0.0133	0.0130	0.0119	0.0132	0.0076	0.0076	0.0237	0.0054
MKF O	0.0120	0.0294	0.0412	0.0013	0.0013	0.0027	0.0013	0.0013	0.0000	0.0000	0.0000	-	0.0000	0.0013	0.0022	0.0009	0.0000	0.0006	0.0000	0.0006	0.0000	0.0142	0.0131	0.0133	0.0130	0.0119	0.0132	0.0076	0.0076	0.0237	0.0054
MHE O	0.0120	0.0294	0.0412	0.0013	0.0013	0.0027	0.0013	0.0013	0.0000	0.0000	0.0000	0.0000	-	0.0013	0.0022	0.0009	0.0000	0.0006	0.0000	0.0006	0.0000	0.0142	0.0131	0.0133	0.0130	0.0119	0.0132	0.0076	0.0076	0.0237	0.0054
MSPE O	0.0136	0.0294	0.0413	0.0041	0.0041	0.0054	0.0041	0.0041	0.0027	0.0027	0.0027	0.0027	0.0027	-	0.0028	0.0017	0.0013	0.0015	0.0013	0.0014	0.0013	0.0142	0.0132	0.0134	0.0131	0.0124	0.0132	0.0078	0.0075	0.0246	0.0052
MSPD O	0.0190	0.0392	0.0459	0.0090	0.0090	0.0104	0.0090	0.0090	0.0090	0.0090	0.0090	0.0090	0.0090	0.0120	-	0.0023	0.0022	0.0024	0.0022	0.0023	0.0022	0.0159	0.0146	0.0145	0.0146	0.0130	0.0149	0.0088	0.0088	0.0269	0.0061
MCS O	0.0120	0.0311	0.0411	0.0000	0.0000	0.0013	0.0000	0.0000	0.0013	0.0013	0.0013	0.0013	0.0013	0.0041	0.0090	-	0.0009	0.0012	0.0009	0.0012	0.0009	0.0143	0.0134	0.0136	0.0133	0.0121	0.0134	0.0077	0.0077	0.0238	0.0055
MTJ O	0.0120	0.0294	0.0412	0.0013	0.0013	0.0027	0.0013	0.0013	0.0000	0.0000	0.0000	0.0000	0.0000	0.0027	0.0090	0.0013	-	0.0006	0.0000	0.0006	0.0000	0.0142	0.0131	0.0133	0.0130	0.0119	0.0132	0.0076	0.0076	0.0237	0.0054
MAR X	0.0128	0.0302	0.0422	0.0020	0.0020	0.0034	0.0020	0.0020	0.0007	0.0007	0.0007	0.0007	0.0007	0.0034	0.0098	0.0020	0.0007	-	0.0006	0.0008	0.0006	0.0143	0.0133	0.0135	0.0132	0.0120	0.0133	0.0077	0.0077	0.0240	0.0054
MAR O	0.0120	0.0294	0.0412	0.0013	0.0013	0.0027	0.0013	0.0013	0.0000	0.0000	0.0000	0.0000	0.0000	0.0027	0.0090	0.0013	0.0000	0.0007	-	0.0006	0.0000	0.0142	0.0131	0.0133	0.0130	0.0119	0.0132	0.0076	0.0076	0.0237	0.0054
MRO O	0.0128	0.0302	0.0421	0.0020	0.0020	0.0034	0.0020	0.0020	0.0007	0.0007	0.0007	0.0007	0.0007	0.0034	0.0097	0.0020	0.0007	0.0013	0.0007	-	0.0006	0.0144	0.0133	0.0135	0.0133	0.0120	0.0134	0.0077	0.0077	0.0238	0.0054
MRO X	0.0120	0.0294	0.0412	0.0013	0.0013	0.0027	0.0013	0.0013	0.0000	0.0000	0.0000	0.0000	0.0000	0.0027	0.0090	0.0013	0.0000	0.0007	0.0000	0.0007	-	0.0142	0.0131	0.0133	0.0130	0.0119	0.0132	0.0076	0.0076	0.0237	0.0054
ARI	0.1121	0.1172	0.1389	0.1069	0.1069	0.1079	0.1069	0.1069	0.1057	0.1057	0.1057	0.1057	0.1057	0.1059	0.1222	0.1069	0.1057	0.1072	0.1057	0.1069	0.1057	-	0.0256	0.0247	0.0244	0.0248	0.0250	0.0168	0.0145	0.0171	0.0135
POP	0.1198	0.1191	0.1198	0.1074	0.1074	0.1100	0.1074	0.1074	0.1047	0.1047	0.1047	0.1047	0.1047	0.1048	0.1191	0.1074	0.1047	0.1061	0.1047	0.1060	0.1047	0.2087	-	0.0030	0.0021	0.0062	0.0050	0.0169	0.0145	0.0379	0.0127
POR	0.1210	0.1206	0.1152	0.1117	0.1117	0.1143	0.1117	0.1117	0.1089	0.1089	0.1089	0.1089	0.1089	0.1090	0.1206	0.1117	0.1089	0.1103	0.1089	0.1102	0.1089	0.2017	0.0112	-	0.0026	0.0063	0.0051	0.0163	0.0137	0.0365	0.0125
POC	0.1169	0.1162	0.1169	0.1047	0.1047	0.1073	0.1047	0.1047	0.1020	0.1020	0.1020	0.1020	0.1020	0.1021	0.1162	0.1047	0.1020	0.1034	0.1020	0.1033	0.1020	0.1999	0.0061	0.0090	-	0.0063	0.0050	0.0161	0.0130	0.0352	0.0124
PAG	0.1053	0.1119	0.1067	0.0954	0.0954	0.0954	0.0954	0.0954	0.0928	0.0928	0.0928	0.0928	0.0928	0.0955	0.1060	0.0954	0.0928	0.0941	0.0928	0.0941	0.0928	0.2073	0.0369	0.0377	0.0360	-	0.0055	0.0149	0.0147	0.0358	0.0128
PCE	0.1147	0.1058	0.1065	0.1026	0.1026	0.1012	0.1026	0.1026	0.1000	0.1000	0.1000	0.1000	0.1000	0.1001	0.1169	0.1026	0.1000	0.1013	0.1000	0.1012	0.1000	0.1995	0.0270	0.0287	0.0262	0.0305	-	0.0159	0.0138	0.0399	0.0130
PCFC	0.0635	0.0667	0.0721	0.0494	0.0494	0.0513	0.0494	0.0494	0.0493	0.0493	0.0493	0.0493	0.0493	0.0515	0.0607	0.0494	0.0493	0.0504	0.0493	0.0503	0.0493	0.1353	0.1278	0.1246	0.1248	0.1146	0.1212	-	0.0080	0.0271	0.0070
PPA	0.0583	0.0565	0.0666	0.0487	0.0487	0.0495	0.0487	0.0487	0.0467	0.0467	0.0467	0.0467	0.0467	0.0468	0.0603	0.0487	0.0467	0.0477	0.0467	0.0476	0.0467	0.1212	0.1083	0.1072	0.1031	0.1101	0.1020	0.0554	-	0.0264	0.0078
AST	0.1952	0.2111	0.2271	0.1852	0.1852	0.1865	0.1852	0.1852	0.1814	0.1814	0.1814	0.1814	0.1814	0.1862	0.2071	0.1852	0.1814	0.1835	0.1814	0.1818	0.1814	0.1397	0.2951	0.2936	0.2821	0.2983	0.3122	0.2121	0.2065	-	0.0232
MIL	0.0372	0.0425	0.0475	0.0283	0.0283	0.0300	0.0283	0.0283	0.0266	0.0266	0.0266	0.0266	0.0266	0.0267	0.0361	0.0283	0.0266	0.0275	0.0266	0.0275	0.0266	0.1076	0.0987	0.0987	0.0961	0.0963	0.0995	0.0445	0.0506	0.1789	-

Table 3. Pairwise genetic distance, corrected according the Kimura two parameters model, between groups on the 28S fragment. Values above the diagonal represent the standard error. Values above the diagonal represent the standard error. For the abbreviation codes see Table 1.

	MFU	MLY	MLG	MPP X	MCH X	MPL O	MCO O	MAL O	MGA O	MKQ O	MTJ X	MKF O	MHE O	MSPE O	MSPD O	MCS O	MTJ O	MAR X	MAR O	MRO O	MRO X	ARI	POP	POR	POC	PAG	PCE	PCFC	PPA	AST	MIL
MFU	-	0.0112	0.0122	0.0052	0.0052	0.0058	0.0058	0.0058	0.0049	0.0049	0.0046	0.0048	0.0047	0.0054	0.0049	0.0058	0.0048	0.0046	0.0050	0.0046	0.0046	0.0251	0.0265	0.0267	0.0237	0.0234	0.0261	0.0134	0.0127	0.0291	0.0122
MLY	0.0624	-	0.0158	0.0096	0.0095	0.0097	0.0097	0.0097	0.0091	0.0091	0.0090	0.0089	0.0087	0.0094	0.0094	0.0097	0.0089	0.0089	0.0091	0.0089	0.0089	0.0282	0.0297	0.0308	0.0273	0.0274	0.0293	0.0171	0.0148	0.0297	0.0174
MLG	0.0711	0.0968	-	0.0108	0.0107	0.0111	0.0111	0.0111	0.0110	0.0110	0.0113	0.0111	0.0112	0.0115	0.0113	0.0111	0.0111	0.0112	0.0111	0.0112	0.0112	0.0248	0.0254	0.0249	0.0234	0.0217	0.0243	0.0145	0.0118	0.0308	0.0132
MPP X	0.0198	0.0523	0.0616	-	0.0004	0.0027	0.0027	0.0027	0.0018	0.0018	0.0023	0.0026	0.0027	0.0041	0.0037	0.0027	0.0026	0.0023	0.0029	0.0023	0.0023	0.0243	0.0272	0.0267	0.0227	0.0232	0.0261	0.0141	0.0114	0.0293	0.0129
MCH X	0.0190	0.0514	0.0606	0.0006	-	0.0026	0.0026	0.0026	0.0018	0.0018	0.0023	0.0025	0.0026	0.0041	0.0037	0.0026	0.0025	0.0022	0.0028	0.0022	0.0022	0.0241	0.0269	0.0264	0.0224	0.0230	0.0259	0.0140	0.0112	0.0291	0.0128
MPL O	0.0221	0.0531	0.0628	0.0059	0.0052	-	0.0000	0.0000	0.0032	0.0032	0.0035	0.0037	0.0037	0.0049	0.0038	0.0000	0.0037	0.0035	0.0039	0.0035	0.0035	0.0245	0.0255	0.0262	0.0231	0.0234	0.0264	0.0142	0.0118	0.0298	0.0138
MCO O	0.0221	0.0531	0.0628	0.0059	0.0052	0.0000	-	0.0000	0.0032	0.0032	0.0035	0.0037	0.0037	0.0049	0.0038	0.0000	0.0037	0.0035	0.0039	0.0035	0.0035	0.0245	0.0255	0.0262	0.0231	0.0234	0.0264	0.0142	0.0118	0.0298	0.0138
MAL O	0.0221	0.0531	0.0628	0.0059	0.0052	0.0000	0.0000	-	0.0032	0.0032	0.0035	0.0037	0.0037	0.0049	0.0038	0.0000	0.0037	0.0035	0.0039	0.0035	0.0035	0.0245	0.0255	0.0262	0.0231	0.0234	0.0264	0.0142	0.0118	0.0298	0.0138
MGA O	0.0176	0.0478	0.0646	0.0032	0.0026	0.0079	0.0079	0.0079	-	0.0000	0.0014	0.0017	0.0019	0.0038	0.0036	0.0032	0.0017	0.0012	0.0021	0.0012	0.0012	0.0241	0.0264	0.0260	0.0220	0.0227	0.0255	0.0139	0.0112	0.0288	0.0130
MKQ O	0.0176	0.0478	0.0646	0.0032	0.0026	0.0079	0.0079	0.0079	0.0000	-	0.0014	0.0017	0.0019	0.0038	0.0036	0.0032	0.0017	0.0012	0.0021	0.0012	0.0012	0.0241	0.0264	0.0260	0.0220	0.0227	0.0255	0.0139	0.0112	0.0288	0.0130
MTJ X	0.0169	0.0470	0.0675	0.0052	0.0045	0.0099	0.0099	0.0099	0.0019	0.0019	-	0.0014	0.0015	0.0036	0.0033	0.0035	0.0014	0.0006	0.0019	0.0006	0.0006	0.0246	0.0265	0.0269	0.0227	0.0234	0.0263	0.0136	0.0116	0.0292	0.0130
MKF O	0.0176	0.0461	0.0644	0.0059	0.0052	0.0106	0.0106	0.0106	0.0026	0.0026	0.0019	-	0.0018	0.0038	0.0036	0.0037	0.0000	0.0012	0.0013	0.0012	0.0012	0.0249	0.0258	0.0262	0.0222	0.0228	0.0255	0.0137	0.0113	0.0282	0.0131
MHE O	0.0169	0.0453	0.0677	0.0065	0.0059	0.0113	0.0113	0.0113	0.0032	0.0032	0.0026	0.0032	-	0.0040	0.0035	0.0037	0.0018	0.0014	0.0018	0.0014	0.0014	0.0248	0.0266	0.0270	0.0228	0.0235	0.0264	0.0138	0.0116	0.0292	0.0130
MSPE O	0.0205	0.0530	0.0720	0.0126	0.0119	0.0176	0.0176	0.0176	0.0106	0.0106	0.0099	0.0106	0.0112	-	0.0044	0.0049	0.0038	0.0035	0.0041	0.0035	0.0035	0.0241	0.0272	0.0274	0.0233	0.0236	0.0266	0.0137	0.0111	0.0273	0.0131
MSPD O	0.0191	0.0479	0.0664	0.0113	0.0106	0.0106	0.0106	0.0106	0.0106	0.0106	0.0099	0.0106	0.0113	0.0147	-	0.0038	0.0036	0.0033	0.0038	0.0033	0.0033	0.0244	0.0272	0.0274	0.0242	0.0243	0.0272	0.0143	0.0120	0.0289	0.0131
MCS O	0.0221	0.0531	0.0628	0.0059	0.0052	0.0000	0.0000	0.0000	0.0079	0.0079	0.0099	0.0106	0.0113	0.0176	0.0106	-	0.0037	0.0035	0.0039	0.0035	0.0035	0.0245	0.0255	0.0262	0.0231	0.0234	0.0264	0.0142	0.0118	0.0298	0.0138
MTJ O	0.0176	0.0461	0.0644	0.0059	0.0052	0.0106	0.0106	0.0106	0.0026	0.0026	0.0019	0.0000	0.0032	0.0106	0.0106	0.0106	-	0.0012	0.0013	0.0012	0.0012	0.0249	0.0258	0.0262	0.0222	0.0228	0.0255	0.0137	0.0113	0.0282	0.0131
MAR X	0.0162	0.0461	0.0664	0.0045	0.0039	0.0092	0.0092	0.0092	0.0013	0.0013	0.0006	0.0013	0.0019	0.0092	0.0092	0.0092	0.0013	-	0.0018	0.0000	0.0000	0.0245	0.0262	0.0266	0.0225	0.0232	0.0260	0.0136	0.0115	0.0289	0.0129
MAR O	0.0191	0.0479	0.0664	0.0072	0.0065	0.0120	0.0120	0.0120	0.0039	0.0039	0.0032	0.0013	0.0032	0.0119	0.0120	0.0120	0.0013	0.0026	-	0.0018	0.0018	0.0254	0.0262	0.0267	0.0226	0.0233	0.0259	0.0140	0.0115	0.0282	0.0132
MRO O	0.0162	0.0461	0.0664	0.0045	0.0039	0.0092	0.0092	0.0092	0.0013	0.0013	0.0006	0.0013	0.0019	0.0092	0.0092	0.0092	0.0013	0.0000	0.0026	-	0.0000	0.0245	0.0262	0.0266	0.0225	0.0232	0.0260	0.0136	0.0115	0.0289	0.0129
MRO X	0.0162	0.0461	0.0664	0.0045	0.0039	0.0092	0.0092	0.0092	0.0013	0.0013	0.0006	0.0013	0.0019	0.0092	0.0092	0.0092	0.0013	0.0000	0.0026	0.0000	-	0.0245	0.0262	0.0266	0.0225	0.0232	0.0260	0.0136	0.0115	0.0289	0.0129
ARI	0.1636	0.1871	0.1626	0.1627	0.1612	0.1616	0.1616	0.1616	0.1612	0.1612	0.1655	0.1671	0.1660	0.1621	0.1616	0.1616	0.1671	0.1640	0.1704	0.1640	0.1640	-	0.0346	0.0328	0.0321	0.0286	0.0306	0.0205	0.0202	0.0234	0.0218
POP	0.1816	0.1985	0.1725	0.1839	0.1822	0.1714	0.1714	0.1714	0.1787	0.1787	0.1799	0.1747	0.1805	0.1881	0.1839	0.1714	0.1747	0.1781	0.1781	0.1781	0.1781	0.2503	-	0.0047	0.0066	0.0093	0.0097	0.0204	0.0229	0.0375	0.0215
POR	0.1816	0.2075	0.1691	0.1827	0.1810	0.1747	0.1747	0.1747	0.1776	0.1776	0.1822	0.1771	0.1828	0.1904	0.1839	0.1747	0.1771	0.1805	0.1805	0.1805	0.1805	0.2389	0.0148	-	0.0064	0.0091	0.0089	0.0205	0.0223	0.0370	0.0233
POC	0.1667	0.1896	0.1621	0.1592	0.1577	0.1604	0.1604	0.1604	0.1547	0.1547	0.1589	0.1544	0.1593	0.1664	0.1692	0.1604	0.1544	0.1574	0.1574	0.1574	0.1574	0.2314	0.0280	0.0281	-	0.0087	0.0092	0.0189	0.0202	0.0328	0.0192
PAG	0.1589	0.1841	0.1475	0.1600	0.1585	0.1558	0.1558	0.1558	0.1554	0.1554	0.1596	0.1550	0.1600	0.1640	0.1644	0.1558	0.1550	0.1581	0.1581	0.1581	0.1581	0.2090	0.0510	0.0496	0.0458	-	0.0033	0.0160	0.0171	0.0333	0.0179
PCE	0.1753	0.1945	0.1631	0.1763	0.1747	0.1719	0.1719	0.1719	0.1714	0.1714	0.1759	0.1709	0.1764	0.1805	0.1810	0.1719	0.1709	0.1742	0.1742	0.1742	0.1742	0.2199	0.0545	0.0494	0.0492	0.0079	-	0.0178	0.0189	0.0328	0.0201
PCFC	0.0767	0.1086	0.0825	0.0837	0.0826	0.0829	0.0829	0.0829	0.0826	0.0826	0.0817	0.0829	0.0820	0.0826	0.0874	0.0829	0.0829	0.0806	0.0851	0.0806	0.0806	0.1452	0.1381	0.1381	0.1301	0.1054	0.1152	-	0.0099	0.0266	0.0131
PPA	0.0748	0.0922	0.0653	0.0672	0.0662	0.0685	0.0685	0.0685	0.0662	0.0662	0.0691	0.0661	0.0693	0.0659	0.0722	0.0685	0.0661	0.0681	0.0681	0.0681	0.0681	0.1333	0.1550	0.1519	0.1382	0.1095	0.1201	0.0499	-	0.0226	0.0148
AST	0.1801	0.1924	0.1874	0.1846	0.1829	0.1874	0.1874	0.1874	0.1793	0.1793	0.1840	0.1785	0.1829	0.1743	0.1837	0.1874	0.1785	0.1821	0.1785	0.1821	0.1821	0.1535	0.2705	0.2614	0.2405	0.2348	0.2307	0.1719	0.1488	-	0.0297
MIL	0.0678	0.1080	0.0727	0.0707	0.0697	0.0761	0.0761	0.0761	0.0717	0.0717	0.0726	0.0736	0.0727	0.0756	0.0719	0.0761	0.0736	0.0716	0.0757	0.0716	0.0716	0.1577	0.1397	0.1522	0.1295	0.1174	0.1285	0.0763	0.0877	0.2014	-

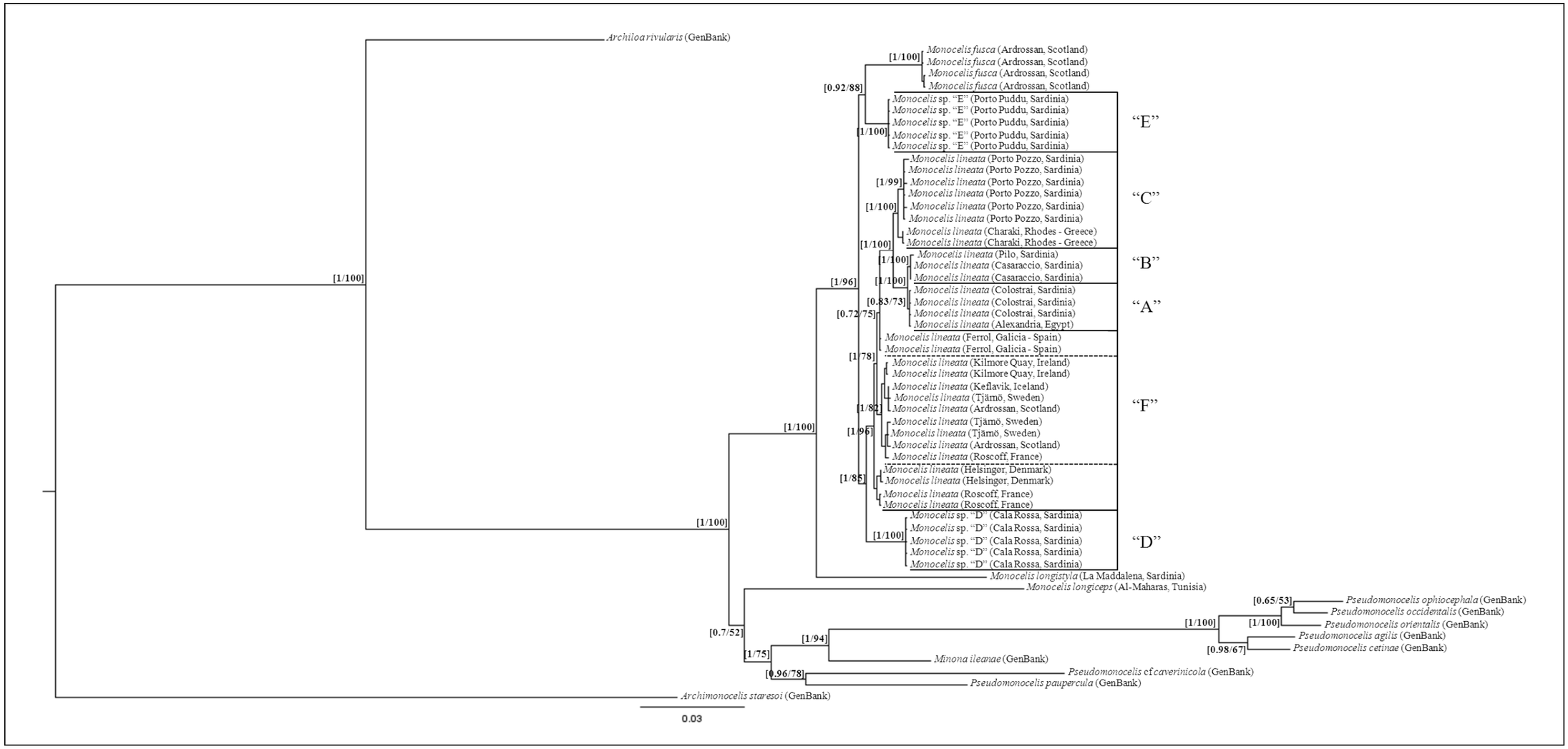

Figure 1. Tree obtained by BI showing the interrelationships of the species based on combined 18S+28S D1-D6. The branch length scale refers to the number of substitutions per site. Nodal supports are indicated for both BI (posterior probability) and ML (bootstrap). Letters on the right refer to the nomenclature given in Casu and Curini-Galletti (2004).

Chapter 5

Preliminary study for calibrating molecular clock in marine Platyhelminthes using geminate species

Fabio Scarpa, Piero Cossu, Daria Sanna, Tiziana Lai, Gian Luca Dedola, Valentina Delogu, Marco Curini Galletti, Marco Casu

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F. Scarpa, P. Cossu, D. Sanna, T. Lai, G.L. Dedola, V. Delogu,
M. Curini-Galletti, M. Casu

Dip. di Scienze della Natura e del Territorio, Sez. di Zoologia, Archeozoologia e Genetica,
Università di Sassari, Via F. Muroni, 25 - 07100 Sassari, Italy.
fscarpa@uniss.it

PRELIMINARY STUDY FOR CALIBRATING MOLECULAR CLOCK IN MARINE PLATYHELMINTHES USING GEMINATE SPECIES

STUDIO PRELIMINARE SULLA CALIBRAZIONE DELL'OROLOGIO MOLECOLARE NEI PLATELMINTI MARINI MEDIANTE L'USO DI GEMINATE SPECIES

Abstract *- Geminate species are a powerful tool, allowing calibration of the molecular clock in marine organisms. Species pairs at both sides of the Isthmus of Panama have been widely used for this purpose. In this work we focused on geminate species of Panamian Proseriata in order to provide a first estimate of the mutation rate per million years for the group.*

Key-words: *Proseriata, geminate species, molecular clock, molecular phylogeny, mutation rate.*

Introduction - The modern molecular phylogenetic approach for dating and solving evolutionary divergence between species is based on the molecular clock hypothesis (MCH) (Zuckerkandl and Pauling, 1965), which hypotheses a relatively constant rate of molecular evolution. Unfortunately, opportunities for the calibration of the molecular clock are relatively rare, especially in the absence of fossil record. For this purpose, in marine organisms, the so-called geminate species (i.e., pairs of morphologically similar sister species that occur on opposite sides of a geographic barrier) represent a powerful tool. Indeed, several studies have demonstrated that geminate species pairs may allow a calibration point for molecular clock, and may be used in turn to estimate the divergence times of related species. One of the most studied cases of geminate species is given by taxa that live on the two sides of the Isthmus of Panama, because their divergence, due to the closure of the Isthmus, is relatively recent and well-dated. Geological evidences in fact, date the final emergence of the Isthmus at about 3.1-3.5 million years ago (Knowlton and Weigt, 1998). In this study, we aimed to individuate geminate species pairs belonging to the order Proseriata (Meixner, 1938) at both sides of the Isthmus of Panama in order to provide a first estimate of the mutation rates per million years, which should be used in the future to calculate the divergence time among other taxa of Proseriata and marine Platyhelminthes.

Materials and methods - The sampling campaign on both sides of the Isthmus of Panama (founded by the sponsorship with the Smithsonian Institution, U.S.A.) produced two populations morphologically referable to *Minona gemella* Ax and Sopott-Ehlers, 1985 (type locality: Bermuda). Notably, populations of Proseriata found in allopatry often have shown to be a complex of (cryptic) species on molecular basis (e.g. see Casu and Curini-Galletti, 2006). For this reason, the first step of our analysis was testing whether these two populations represent different species. Phylogenetic analysis were performed using Bayesian Inferences [software MrBayes 3.2.1 (Huelsenbeck and Ronquist, 2001)] on the sequences of complete 18S gene and partial 28S gene for a total of 46 species belonging to the family of Otoplanidae Hallez, 1892, Monocelididae Hofsten, 1907, and Archimonocelididae Meixner, 1938. Indeed, these molecular markers have shown to be appropriate for species delimitation in Proseriata (see Casu *et al.*, 2011 and references therein). To

calculate the mutation rate per million years the following formula was used: $r = K2P$ (Kimura 2-Parameters) * $(2T)^{-1}$ (time of divergence, multiplied by 2 to account for the age of each lineage) (Li and Graur, 1991).

Results and conclusions - Phylogenetic results suggest that the two transisthmian populations referable to *M. gemella* are different, morphologically undistinguishable species. Tree shows that they are also sister species, thus falling into the definition of geminate species. On this basis, the mutation rates per million years estimated between the *Minona* species pair amount to 0.1% for 18S and 0.36% for 28S. Molecular clock calibration for geminate species is a contentious issue (see Marko and Moran, 2009), and requires a carefully understanding and interpretation. To our knowledge, this is the first work aimed to the detection of a calibration point, not only for Proseriata but also for marine Platyhelminthes in general. The correct use of these mutation rates may thus offer a remarkable contribution for a better understanding of the evolutionary divergence within the taxon. Indeed, the next step of our research will focus on the application of these mutation rates throughout the whole dataset of Proseriata by means of the software Beast 1.7.4 (Drummond and Rambaut, 2007), which provides a phylogenetic tree in which for each node is shown the time of the most recent common ancestor (TMRCA) among the taxa included in that clade. Further analyses should be performed on a more substantial sample of species pairs separated by the emergence of the Isthmus of Panama (presently under study), and species separated by a different geographic barrier (with a well-known timing of the geological event), in order to corroborate our results, and test their applicability across marine Platyhelminthes.

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Chapter 6

Nuclear rDNA clock calibration for Proseriata flatworm

Fabio Scarpa, Piero Cossu, Daria Sanna, Tiziana Lai, Jon L. Norenburg, Marco Curini Galletti, Marco Casu

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Nuclear rDNA clock calibration for Proseriata flatworm

FABIO SCARPA[1], PIERO COSSU[1], DARIA SANNA[1], TIZIANA LAI[1], JON L. NORENBURG[2], MARCO CURINI-GALLETTI[1], MARCO CASU[1]*

[1]*Dipartimento di Scienze della Natura e del Territorio – Sezione di Zoologia, Archeozoologia e Genetica, Università di Sassari, Via F. Muroni 25, 07100 Sassari, Italy - Centro di Eccellenza dell'Università di Sassari*

[2]*Department of Invertebrate Zoology, National Museum of Natural History, Smithsonian Institution, Washington, DC 20560, USA*

*Corresponding author: Marco Casu

Dipartimento di Scienze della Natura e del Territorio –

Sezione di Zoologia, Archeozoologia e Genetica

Università di Sassari

Via F. Muroni 25

07100 Sassari, Italy

e-mail: marcasu@uniss.it

phone: +39 079 228924

fax: +39 079 228665

Abstract

Geminate species represent a powerful tool for calibrating the molecular clock in marine organisms. Their use may be even more important for soft-bodied organisms, such as the free-living flatworm Proseriata, which miss fossil records because of the lack of hard-parts. Here we present the first attempt to calibrate the molecular clock for the order Proseriata, based on two ribosomal regions, the complete nuclear small subunit rRNA (18S) gene and the partial nuclear large subunit rRNA (28S) fragment (spanning variable domains D1-D6). For this purpose we used two geminate species pairs from both sides of the Isthmus of Panama, the most frequently used geographical barrier for this kind of researches. On the Atlantic coast of the isthmus we found the species *Minona gemella* which is morphologically undistinguishable from its counterpart *Minona* cf *gemella*, found on the Pacific coast. As well, morphologically similar individuals belonging to the genus *Parotoplana* Meixner, 1938 (belonging to the *Parotoplana turgida* group), *Parotoplana* sp. nov. 1 (Atlantic coast) and *Parotoplana* sp. nov. 2 (Pacific coast) were found on opposite coasts of the isthmus. The mutation rates per million years were estimated for both geminate species pairs on the two ribosomal genes. The obtained values were subsequently used as calibration points at the minimum age, in order to calibrate the timetree and estimating the divergence time within the phylogenetic tree of the whole dataset. Similar values of mutation rate per million years were found in both species pairs, either for 18S and the 28S regions. Relying upon the divergence time of three test cases (populations from the Atlantic and Pacific Ocean), the obtained mutation rates exhibits the possibility of a cross-use at least within and among the families Otoplanidae, Monocelididae and Archimonocelididae.

This research represent the first molecular clock calibration proposed not only for Proseriata but also for marine Platyhelminthes in general.

These data point out the potential usefulness of the molecular clock calibration, that should be applied in future researches aimed to provide further insights into the phylogenetic relationships of Proseriata.

Key words: Proseriata; molecular clock; divergence time; calibration point; 18S; 28S D1-D6.

Introduction

The modern molecular phylogenetic approach applied to date evolutionary divergence between species, has been based for a long time on the molecular clock hypothesis (MCH) (Zuckerkandl and Pauling, 1965), which assumed a relatively constant rate of molecular evolution over time and across taxa (see Kimura, 1968, and references therein; but see also Thomas et al., 2006). This theory is based on the assumptions that the genetic distance between the same nucleotide regions belonging to two isolated, different species, increases linearly with the divergence time of the two given species (Lemey and Posada, 2009). The molecular clock is used not only to estimate time of divergence and evolutionary rates, but it is also a tool for a formal description of the substitution process, which is fundamental for a better understanding of the evolutionary pathways concerning a given taxon (Lemey and Posada, 2009).

In order to assign concrete datations, molecular clock needs to be calibrated against independent evidence about datations (Benton and Donoghue, 2007). The most common calibration of the molecular clock is achieved by using fossil records (e.g., Blanton et al., 2013; Mulcahy et al., 2012) or, when allowed, by means of both fossil records and paleogeographic events (e.g., Heads, 2005a). Unfortunately, fossil records are not available for many taxa; in particular, the so-called soft-bodied organisms, lacking hard parts, show to be extremely rare within the fossil records, which represents a hindrance to the reconstruction of their evolutionary history. This problem is largely felt in the marine environment, in which several taxa of soft-bodied invertebrates are present. Among them, an overwhelming and important component of marine biodiversity is represented by meiofauna (Kennedy and Jacoby, 1999), defined as animals that can pass

through a 500-µm mesh, whose small dimensions further decrease the possibility to leave fossil records.

To overcome these problems Jordan (1980) suggested the use of the so-called geminate species, i.e. morphologically similar sister species that live in allopatric conditions and occur on opposite sides of a geographic barrier, to calibrate the molecular clock. The geminate species represent a largely cited model of allopatric speciation (Lessios, 2008; Vermeij, 1978; Coyne and Orr, 2004), constituting a "natural experiment" that can well describe the evolutionary divergence and its causes (Lessios, 2008). Indeed, several studies have demonstrated that geminate species may represent a valid alternative to the fossil records as calibration points for molecular clock, and then can be used in turn to estimate divergence times between related species (e.g., Marko and Moran, 2009; Knowlton and Weigt, 1998; Lessios, 1998). For this purpose, one of the most used geographical barriers is the Isthmus of Panama, whose last closure is relatively recent and well dated (Allmon, 2001; Jackson and Budd, 1996; and references therein). Geological literature suggests that the isolation between the Caribbean Sea and the Pacific Ocean occurred at the last time of about 3.1-3.5 million years ago (Collins, 1996), due to the final emergence of the Isthmus of Panama (Knowlton and Weig, 1998).

In this context, the aim of this study was to set up the first calibration of the molecular clock for the order Proseriata (Platyhelminthes) (Meixner, 1938), using the last closure of the Isthmus of Panama to estimate the time of divergence at the minimum age, i.e. assuming that the taxa used to calibrate the molecular clock were separated from at least 3.1-3.5 million years. Proseriata is a meiofaunal soft-bodied taxon of free-living

flatworm, whose representatives may be numerically abundant and characterizing entire soft-bottom communities (Reise, 1988; Remane, 1933). As for most meiofaunal organisms, Proseriata lacks larval stages. This, together with the reduced mobility of adults, provides limited potential for an active dispersal. Passive recruitment in the water column is the main way of dispersal, and thus for genes exchange between geographically separated populations (see Palmer, 1988). Although geminate species belonging to Proseriata may represent an interesting study case to calibrate molecular clock throughout a geographic barrier, researches with this aim have never been carried out so far. The mainly reason is given by the fact that studies on Proseriata are notoriously difficult because they are very minute animals, with relatively very few morphological features which aid the taxonomic analysis, as demonstrated by the pervasive occurrence of sibling species (e.g., Casu and Curini-Galletti, 2006, 2004). Accordingly, Proseriata cannot be reliably studied by means of only traditional methods, and molecular analyses are very often needed (see, e.g., Casu et al., 2013, 2011a, 2009, 2006, 2004; Curini-Galletti et al., 2010; Littlewood et al., 2000; Litvaitis et al., 1996).

For this study several sampling campaigns were carried out in the Central and South America, on both sides of the Isthmus of Panama, which provided two pairs of geminate species. On the Atlantic coast of the isthmus we found the species *Minona gemella* Ax & Sopott-Ehlers, 1985 (type locality: Bermuda), whereas on the Pacific coast we found a *Minona* cf *gemella*, morphologically undistinguishable from *M. gemella*. As well, morphologically similar individuals belonging to the genus *Parotoplana* Meixner, 1938 (belonging to the *Parotoplana turgida* group) hereafter called *Parotoplana* sp. nov. 1

(Atlantic coast) and *Parotoplana* sp. nov. 2 (Pacific coast) were found on both coasts of the Isthmus of Panama. The finding of these two pairs of geminate species allow us to estimate the mutation rate per million years between individuals belonging to the two trans-isthmian populations, using the last closure of the Isthmus of Panama as time of divergence. The obtained mutation rates were subsequently used as calibration points at the minimum age, in order to calibrate the timetree and estimating the divergence time within the phylogenetic tree of the whole dataset. The concurrent finding of trans-isthmian species pairs belonging to the families of Otoplanidae, Monocelididae, and Archimonocelididae during the above-mentioned sampling campaigns further allow us to verify the consistency between our first calibration of the molecular clock and the geographical event of the last closure of the Isthmus of Panama, and the possible cross-use of these rates among the species belonging to the order Proseriata. We calibrated the molecular clock on two ribosomal regions, the complete nuclear small subunit rRNA (18S) gene and the partial nuclear large subunit rRNA (28S) fragment (spanning variable domains D1-D6).

2. Materials and methods

2.1. Sampling, DNA extraction, amplification and sequencing

Samples were collected manually by scooping up the superficial layer of sediment.

Extraction of the animals from the sediment was accomplished using MgCl2 decantation (Martens, 1984). Each specimen was studied alive by slight squeezing under the cover slip. For information about sampling localities see Table 1 and Fig.1.

Genomic DNA was extracted using the Macherey-Nagel NucleoSpin Tissue (Macherey-Nagel GmbH & Co. KG) according to the supplier's instructions. After extraction, DNA was stored as a solution at 4 °C. Complete 18S and partial 28S D1-D6 sequences were analysed for a total of 100 individuals, 69 were newly obtained specifically for this study and 31 taken from GenBank (for details see Table 1). The dataset was built with 40 sequences of individuals belonging to the family Otoplanidae (25 of which from GenBank, Table 1), 43 to the family Monocelididae (3 from GenBank, Table 1), 13 to the family Archimonocelididae (1 from GenBank, Table 1), one to the family Calviridae (from GenBank), one to the family Coeloginoporidae (from GenBank), and two to the suborder Unguiphora (from GenBank). Amplifications for 18S and 28S D1-D6 regions were carried out using the following primers: 18S: A (forward) GCG AAT GGC TCA TTA AAT CAG, and B (reverse) CTT GTT ACG ACT TTT ACT TCC (Littlewood and Olson, 2001); 28S: LSU5 (forward) TAG GTC GAC CCG CTG AAY TTA AGC A, and LSUD6-3 (reverse) GGA ACC CTT CTC CAC TTC AGT C (Littlewood et al., 2000).

PCR was carried out in a total volume of 25 µl containing 5 ng/µl of total genomic DNA on average, 1.0 U of Taq DNA Polymerase (EuroTaq by Euroclone), 1× reaction buffer, 3.5 mM of $MgCl_2$, 0.32 µM of each primer, and 200 µM of each dNTP. PCR amplification was performed in a MJ PTC 200 Thermal Cycler (Biorad) programmed as follows: 1 cycle of 2 min at 94° C, 35 cycles of 1 min at 94° C, 1 min at 54° C (18S / 28S primers' annealing temperature), and 1 min and 30 s at 72° C. At the end, a post-treatment for 5 min at 72° C and a final cooling at 4° C were carried out. Both positive and negative controls were used to test the effectiveness of the PCR reagents, and the

absence of possible contaminations. Electrophoresis was carried out on 2% agarose gels, prepared using 1× SBA buffer (sodium boric acid, pH 8.2) and stained with a 1 µl/20 ml ethidium bromide solution. PCR products were purified by ExoSAP-IT (USB Corporation) and sequenced for both forward and reverse 18S and 28S strands, using an external sequencing core service (Macrogen Europe).

2.2. Estimates of genetic distance and phylogenetic analysis

The 18S and 28S sequences were aligned separately using the algorithm Q-INS-I, implemented in Mafft 6.903 (Katoh and Toh, 2008), which is appropriate for non-coding RNA as it considers RNA secondary structure. The best probabilistic model of sequence evolution was determined after evaluation by JModeltest 0.1 (Posada, 2008), with a maximum likelihood optimised search, using the Akaike Information Criterion (AIC) and the Bayesian Information Criterion (BIC). Both criterions selected the GTR+I+G (Tavaré, 1986) as the best fitting model for both 18S and 28S datasets. The genetic distance corrected according to the Kimura two-parameter model (*K2P*) (Kimura, 1980) was estimated by means of the software Mega 5.2 (Tamura et al., 2011) with 1,000 bootstrap replications. *K2P* distances were estimated in order to insert it into the formula proposed by Liu and Graur (1991) (see section 2.3.1).

Phylogenetic relationships among individuals and species were investigated using both Maximum Likelihood (ML) and Bayesian Inference (BI) on the combined 18S and 28Ssequences. We set as outgroup for the analyses the species *Polystyliphora novaehollandiae* Curini-Galletti, 1998. ML was performed using the genetic algorithm implemented in Garli 2.0 (Zwickl, 2006). In order to find the best tree, the configuration

file for partitioned models was set up to perform 10 replicate searches (searchreps = 10). Model parameters: ratematrix = (0 1 2 3 4 5), statefrequencies = estimated, ratehetmodel = gamma, numratecats = 4, corresponding to the evolution model calculated by JModeltest, were used. In order to allow independent estimates of the parameters for each gene, the option link was set to 0. The parameter modweight was set to 0.0015, as we have two partitions. Finally, node support was assessed by 1,000 bootstraps (bootstrapreps = 1000). Consensus tree was computed using TreeAnnotator 1.7.4 (Drummond and Rambaut, 2007) and visualised by FigTree 1.4.0 (http://tree.bio.ed.ac.uk/software/figtree/).

BI was carried out using the software MrBayes 3.2.1 (Ronquist et al., 2012), specifying a partitioned model and setting as model parameters: NST = 6, rates = gamma, ngammacat = 4. We allowed each partition to have its own set of parameters and a potentially different overall evolutionary rate. Two independent runs, each consisting of four Metropolis-coupled MCMC chains (one cold and three heated chains), were run simultaneously for 5,000,000 generations, sampling trees every 1,000 generations. The first 25% of the 10,000 sampled trees was discarded as burnin.

In order to assess the convergence of chains we checked that the Average Standard Deviation of Split Frequencies (ASDSF), approached 0 (Ronquist et al., 2012), and the Potential Scale Reduction Factor (PSRF) was around 1 (Gelman and Rubin, 1992). Nodes with a percentage of posterior probability lower than 95% are considered not highly supported. Phylogenetic tree was visualised using FigTree 1.4.0 (http://tree.bio.ed.ac.uk/software/figtree/).

2.3. Calibration of molecular clock

2.3.1. Estimation of the mutation rates per million years

After phylogenetic analysis showed that the two trans-isthmian populations couple (*Minona gemella* + *Minona* cf *gemella* and *Parotoplana* sp. nov. 1 + *Parotoplana* sp. nov. 2) represent two pairs of sister species (and thus geminate species) (see section 3.1 below and Fig. 2), the mutation rates per million years (*r*) between species from both sides of the isthmus were estimated for each gene. We used the formula $r = K$ (*K2P* genetic distance) / $2T$ (time of divergence multiplied by 2 to account for the age of each lineage) (Liu and Graur, 1991). The obtained mutation rates per million years (two for each species pair) were used for calibrating the timetree, in order to estimate the divergence time throughout the whole dataset.

2.3.2. Estimation of divergence time

The software package Beast 1.7.4 (Drummond and Rambaut, 2007) was used to estimate the divergence time for all of the clades evidenced by the phylogenetic tree. Site parameters (Substitution Model = GTR; Bases Frequencies = Estimated; Site Heterogeneity Model = Gamma + Invariant Sites; Number of Gamma Categories = 4) have been set according to the best-fitting evolution model selected by JModeltest. For the molecular clock rate variation model, the lognormal uncorrelated relaxed clock was chosen because it assumes independent rates on different branches. Moreover, the use of the lognormal uncorrelated relaxed clock model gives an indication of how clock-like data is (measured by the ucld.stdev parameter). If the ucld.stdev parameter estimate is close to 0, then the data is quite clock-like, while if it has an estimated value much

greater than 1, then data exhibits very substantial rate heterogeneity among lineages. For the tree prior the Yule prior process to the speciation model was applied. The priors for model parameters and statistics have been set for calibrating the timetree assuming the mutation rates per million years estimated singularly for the two regions (18S and 28S) on the *Minona* and *Parotoplana* species pairs. For all four partitions we have set both the initial value (see Table 2), and the values corresponding to lower and upper values of mutation rate per million years for each gene, using a uniform distribution. Operator parameters have been set following the instructions on the user manual. In order to obtain the Effective Sample Size (ESS) greater than 200 for all of the statistic parameters, a run of 400,000,000 generations was performed, sampling a tree every 40,000 generations.

We used Tracer 1.5 (Rambaut and Drummond, 2009) for viewing the resulting log file, in order to ensure convergence of parameter values, to verify whether ESS values exceeded 200 , and the estimate of node age. TreeAnnotator and FigTree were used for drawing and visualizing the time tree respectively.

3. Results

3.1. Estimates of genetic distance and phylogenetic analysis

After the alignment, sequences of 1657 *bp* and 1703 *bp* were obtained for the 18S and 28S regions, respectively (see Table 1 for the GenBank accession numbers). For each region, the genetic pairwise distance corrected according to the *K2P* model has provided comparable values between the couple composed by *Minona gemella* + *Minona* cf *gemella* and that composed by *Parotoplana* sp. nov. 1 + *Parotoplana* sp. nov. 2: *K2P* =

0.0085 ±0.0022 and *K2P* = 0.0115 ±0.0027, for the 18S; and *K2P* = 0.0345 ±0.0045 and *K2P* = 0.0361 ±0.0049, for the 28S D1-D6, respectively (Table 2).

ML and BI generated consistent trees with negligible differences in topology, thus we reported only the BI tree obtained by the software MrBayes (Fig. 2). The phylogenetic tree shows that both the couple composed by *Minona gemella* + *Minona* cf *gemella*, and the couple *Parotoplana* sp. nov. 1 (Atlantic coast) + *Parotoplana* sp. nov. 2 (Pacific coast) present a sister-taxa relationship (in both cases highly supported both for posterior probability and bootstrap values) (Fig. 2); therefore, according to the Jordan's definition, they are also geminate species. For this reason we could use both species pairs for estimating the mutation rate per million years.

Furthermore, the tree confirmed the sister-taxa relationship between Atlantic and Pacific clusters of species belonging to the families Otoplanidae (node A), Monocelididae (node B) and Archimonocelididae (node C) (Fig. 2). In particular:

- Within the Otoplanidae clade, the species belonging to the genus *Kata* Marcus, 1949 were separated into two geographic clusters, one grouping the Atlantic *Kata evelinae* Marcus, 1949 + *Kata leroda* Marcus 1950, and one the Pacific *Kata* sp. nov. 1 + *Kata* sp. nov. 2 (node A in Fig. 2);

- Within the Monocelididae clade, the node splits the Atlantic specimens of *Duplominona tridens* from the Pacific specimens of *Duplominona* sp. nov. 1 and *Duplominona* sp. nov. 2 (*Duplominona tridens* group) (node B in Fig. 2);

- Within the Archimonocelidae clade, the node splits the Pacific *Archimonocelis* sp. nov. from the cluster which groups all the Atlantic *Archimonocelis* specimens (node C in Fig. 2).

For each of these three cases nodes are highly supported (Fig. 2).

3.2.Mutation rates per million years and divergence time

The mutation rate per million year, estimated between *Minona gemella* and *Minona* cf *gemella*, amounts to 0.12% for the 18S, and 0.49% for the 28S (Table 2). Slight higher values were obtained between *Parotoplana* sp. nov. 1 and *Parotoplana* sp. nov. 2, that amount to 0.16% for the 18S, and 0.52% for the 28S (Table 2). Analysis performed by means of the software Beast produced a tree whose topology is consistent to those obtained by both Garli and MrBayes. Regarding the clade datations, we estimate for the node A (that splits the Atlantic *Kata evelinae* and *Kata leroda* from the Pacific *Kata* sp. nov. 1 and *Kata* sp. nov. 2) a time of divergence of about 15.2 Myr (Million years), with a range between 11.5 and 19.3 Myr (Fig. 3); the node B (that splits Atlantic *Duplominona tridens* from the Pacific *Duplominona* sp. nov. 1 and *Duplominona* sp. nov. 2) is dated at about 15.5 Myr, with a range between 10.1 and 20.9 Myr (Fig. 4); the node C (that splits the Pacific *Archimonocelis* sp. nov. from its Atlantic counterparts) is dated about 11.0 Myr, with a range between 8.4 and 13.8 Myr (Fig. 5).

The ucld.stdev parameter estimated amount to 0.807 and 0.787 for the 18S; and 0.535 and 0.540 for the 28Sin the *Minona* and *Parotoplana* species pairs, respectively. Since all of these values are smaller than 1, this result suggests that our dataset does not exhibit a substantial rate heterogeneity among lineages, but it seems to be clock-like.

4. Discussion

To date, molecular tools and the MCH provided new clues on the mechanisms driving molecular evolution and the understanding of past evolutionary processes (Bromham and Penny, 2004). However, since its earliest applications, several authors have shown perplexity about the wide applicability of the MCH and the occurrence of universal molecular clock (see e.g., Lessios, 2008; Heads, 2005b; Palumbi, 1997; but see also Thomas et al., 2006). In particular, the use of the molecular clock to infer divergence time conceals several difficulties related on how the molecular clock is calibrated (Peterson et al., 2004).

In this context, the use of geminate species, that represent the only alternative way in absence of fossil records, is a further contentious issue (Lessios, 2008). Indeed, the main problem is given by the fact that we cannot known how many time elapsed between the separation of taxa due to the rise of a geographic barrier and the moment in which we use them to estimate the datation, a problem, however, which is also present when fossil records are used (Lessios, 2008). In addition, considering merely geminate species as species pairs originated after the rise of a geographic barrier could be an oversimplification of their evolutionary path, since the evolutionary history of many nominal geminate species may be potentially more complex (see Knowlton and Weigt, 1998).

To overcome such limitations, the best way to calibrate a molecular clock as accurately as possible is to use i) different genes or loci, and ii) different calibration points (Marko and Moran, 2009). In the case of Proseriata, the above-mentioned issues are not negligible: i) the number of genes/loci we could use are very limited, because most of

"universal" primers for invertebrates, such as those for the cytochrome c oxidase (COI) Folmer's region (Folmer et al., 1994) do not always provide satisfactory results, and specific primers are not available except for a few species (see Casu et al., 2011b; Sanna et al., 2009); ii) the number of calibration points for the molecular clock depends on the sampling's success and this is a crucial problem for Proseriata, because in most cases (it) is possible to verify the adequacy of the sampling campaign only after molecular analyses (see e.g., Casu et al., 2013). The main error which may be caused by an inadequate sampling is the use of not-true geminate species. Indeed, the use of more distantly related species for calibrating molecular clock may cause an overestimation of the divergence time of the molecular clock.

To avoid and/or minimize these shortcomings, in the present study we used coupled 18S and 28S D1-D6 regions, because their sequences constitute the only large database available for Proseriata. Furthermore, we carried out several sampling campaigns from both Atlantic and Pacific coast of the Isthmus of Panama, and from Brazil over several years (from 2011 to 2013), after which we were able to compose an overall dataset of a hundred individuals, 35 of which representative of a wide geographical area around the Isthmus of Panama and South America. Moreover, in order to obtain a dataset as representative as possible of a worldwide geographic area, we also included representatives of the order Proseriata from the Mediterranean, and from other Atlantic and Pacific localities. The achieved dataset proved to be very functional for our scopes. Indeed, phylogenetic results evidenced, not only the occurrence of two pairs of geminate species, used as calibration point in the divergence tree, but also the usefulness of our molecular clock calibration for Atlantic and Pacific representatives of

three different Proseriata families (Otoplanidae, Monocelididae and Archimonocelididae). Two main results point out the potential usefulness of our molecular clock calibration for Proseriata:

- A similar values of mutation rate per million years in both species pairs, either for 18S and the 28S regions was found. This is a remarkable data in the perspective of a cross-use of these mutation rates within the Proseriata, because the two geminate species pairs, *Minona gemella* + *Minona* cf *gemella* and *Parotoplana* sp. nov. 1 + *Parotoplana* sp. nov. 2, belong to different families, Monocelididae and Otoplanidae respectively;
- The estimates of the divergence time at the three nodes that we used as test cases (nodes A, B, and C in Fig. 2, Figs. 3, 4, 5) are consistent with the datation of the last closure of the Isthmus of Panama. Indeed, since our calibration was performed at the minimum age, we assumed that the taxa considered (Atlantic and Pacific species belonging to the genus *Kata*, Otoplanidae; Atlantic and Pacific species belonging to the genus *Duplominona*, Monocelididae; Atlantic and Pacific species belonging to the genus *Archimonocelis*) live in allopatric condition from no more than 3.1-3.5 Myr; for these three nodes, finding a lower estimate of the divergence time would have meant an incorrect datation. Moreover, divergence time estimates were similar across the different families used as test-cases; this is a further clue of the goodness of our estimated mutation rates per million years.

Although other studies used the Isthmus of Panama as geographic barrier to estimate divergence time on invertebrates (see e.g., Marko and Moran, 2009; Marko, 2002), our study represents the first attempt to calibrate the molecular clock for marine Platyhelminthes in general. Unfortunately, this means also the lack of data with which

we can perform a comparison, and the impossibility, to date, to unravel if a universal molecular clock for marine Platyhelminthes exists.

In conclusion the mutation rates estimated in this study exhibits the possibility of a cross-use at least within and among the families Otoplanidae, Monocelididae and Archimonocelididae. Thus, mutation rates we obtained should be used in the future to test their applicability in other families of Proseriata, if possible including more pairs of geminate species also from other areas with geographic barrier. Finally, the data obtained could be used for researches aimed to provide further insights into the phylogenetic relationships of Proseriata, as the molecular clock may improve phylogenetic reconstructions (Lemey and Posada, 2009).

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Table 1. List of species sampled and sequences used for this study. Accession numbers refer to GenBank codes; accession numbers of new sequences are not yet available. * Only for taxa sequenced by us.

Family	Species	Locality*	18S	28S D1-D6
	Archotoplana holotricha Ax, 1956	GenBank	AJ243676	AJ270165
	Archotoplana holotricha Ax, 1956	Faro (Portugal)	XXXX	XXXX
	Xenotoplana acus Ax, Weidemann & Ehlers, 1978	GenBank	AJ270155	AJ270181
	Kata evelinae Marcus, 1949	Ilhabela (Brazil)	XXXX	XXXX
	Kata evelinae Marcus, 1949	Ilhabela, (Brazil)	XXXX	XXXX
	Kata leroda Marcus, 1950	São Sebastião (Brazil)	XXXX	XXXX
	Kata leroda Marcus, 1950	São Sebastião, (Brazil)	XXXX	XXXX
	Kata sp. nov. 1	Naos Island (Panama)	XXXX	XXXX
	Kata sp. nov. 1	Naos Island (Panama)	XXXX	XXXX
	Kata sp. nov. 2	Naos Island (Panama)	XXXX	XXXX
	Parotoplana ambrosolii Curini-Galletti & Delogu sp. nov.	Punta Ala (Tuscany, Italy)	KC971043	KC971066
	Parotoplana ambrosolii Curini-Galletti & Delogu sp. nov.	Punta Ala (Tuscany, Italy)	KC971044	KC971067
	Parotoplana ambrosolii Curini-Galletti & Delogu sp. nov.	Punta Ala (Tuscany, Italy)	KC971045	KC971068
	Parotoplana tubifera Curini-Galletti & Delogu sp. nov.	Agnone (Sicily, Italy)	KC971046	KC971069
	Parotoplana tubifera Curini-Galletti & Delogu sp. nov.	Agnone (Sicily, Italy)	KC971047	KC971070
	Parotoplana tubifera Curini-Galletti & Delogu sp. nov.	Agnone (Sicily, Italy)	KC971049	KC971072
	Parotoplana tubifera Curini-Galletti & Delogu sp. nov.	Le Bombarde beach - Alghero (Sardinia, Italy)	KC971058	KC971081
	Parotoplana impastatoi Curini-Galletti & Delogu sp. nov.	Agnone (Sicily, Italy)	KC971048	KC971071
	Parotoplana impastatoi Curini-Galletti & Delogu sp. nov.	Agnone (Sicily, Italy)	KC971050	KC971073
	Parotoplana ambrosolii Curini-Galletti & Delogu sp. nov.	La Maddalena (Sardinia, Italy)	KC971056	KC971079
	Parotoplana ambrosolii Curini-Galletti & Delogu sp. nov.	La Maddalena (Sardinia, Italy)	KC971057	KC971080
	Parotoplana spathifera Delogu & Curini-Galletti, 2007	La Maddalena (Sardinia, Italy)	KC971053	KC971076
	Parotoplana pythagorae Delogu & Curini-Galletti, 2007	La Maddalena (Sardinia, Italy)	KC971052	KC971075
	Parotoplana renatae Ax, 1956	GenBank	AJ012517	AJ270176
	Parotoplana renatae Ax, 1956	Banyuls-sur-mer (Languedoc-Roussillon, France)	KC971062	KC971085
	Parotoplana multispinosa Ax, 1956	Banyuls-sur-mer (Languedoc-Roussillon, France)	KC971061	KC971084
	Parotoplana sp. nov. 1	Bocas del Toro (Panama)	XXXX	XXXX
	Parotoplana sp. nov. 1	Bocas del Toro (Panama)	XXXX	XXXX
	Parotoplana sp. nov. 1	Bocas del Toro (Panama)	XXXX	XXXX
	Parotoplana sp. nov. 2	Naos Island (Panama)	XXXX	XXXX
Otoplanidae				

Family	Species	Locality*	18S	28S D1-D6
Otoplanidae	*Parotoplana* sp. nov. 2	Naos Island (Panama)	XXXX	XXXX
	Parotoplana sp. nov. 2	Naos Island (Panama)	XXXX	XXXX
	Parotoplana primitiva Ax, 1956	Agde (Languedoc-Roussillon, France)	KC971060	KC971083
	Parotoplana primitiva Ax, 1956	Roscoff (France)	XXXX	XXXX
	Parotoplana bicupa Sopott-Ehlers, 1976	Agde (Languedoc-Roussillon, France)	KC971063	KC971086
	Parotoplanella progermaria Ax, 1956	Banyuls-sur-mer (Languedoc-Roussillon, France)	KC971059	KC971082
	Parotoplana crassispina Delogu & Curini-Galletti, 2009	La Maddalena (Sardinia, Italy)	KC971051	KC971074
	Parotoplana rosignana Lanfranchi & Melai, 2008	La Maddalena (Sardinia, Italy)	KC971054	KC971077
	Parotoplana procerostyla Ax,1956	Banyuls-sur-mer (Languedoc-Roussillon, France)	KC971055	KC971078
	Monostichoplana filum (Meixner,1938)	GenBank	AJ270158	AJ270173
Archimonocelididae	*Archimonocelis staresoi* Martens & Curini-Galletti, 1993	GenBank	AJ270152	AJ270166
	Archimonocelis marci Curini-Galletti, 2013	São Sebastião (Brazil)	XXXX	XXXX
	Archimonocelis sp. nov.	São Sebastião (Brazil)	XXXX	XXXX
	Archimonocelis sp. nov.	São Sebastião (Brazil)	XXXX	XXXX
	Archimonocelis sp. nov.	Tenerife, Canary Island (Spain)	XXXX	XXXX
	Archimonocelis sp. nov.	Faro (Portugal)	XXXX	XXXX
	Archimonocelis crucifera Martens & Curini-Galletti, 1993	Lampedusa Island (Italy)	XXXX	XXXX
	Archimonocelis sp. nov.	Banyuls-sur-mer (Languedoc-Roussillon, France)	XXXX	XXXX
	Archimonocelis carmelitana Martens & Curini-Galletti, 1993	Banyuls-sur-mer (Languedoc-Roussillon, France)	XXXX	XXXX
	Archimonocelis carmelitana Martens & Curini-Galletti, 1993	Banyuls-sur-mer (Languedoc-Roussillon, France)	XXXX	XXXX
	Archimonocelis carmelitana Martens & Curini-Galletti, 1993	Banyuls-sur-mer (Languedoc-Roussillon, France)	XXXX	XXXX
	Archimonocelis sp. nov.	Naos Island (Panama)	XXXX	XXXX
	Archimonocelis sp. nov.	Naos Island (Panama)	XXXX	XXXX
Monocelididae	*Minona ileanae* Curini-Galletti, 1997	GenBank	JN224905	JN224910
	Minona ileanae Curini-Galletti, 1997	Great Bitter Lake (Egypt)	XXXX	XXXX
	Minona sp. nov.	Playa La Angosta, Colón (Panama)	XXXX	XXXX
	Minona sp. nov.	Playa La Angosta, Colón (Panama)	XXXX	XXXX
	Minona cf *trigonopora* Ax, 1956	Palau (Sardinia, Italy)	XXXX	XXXX
	Minona gemella Ax & Sopott-Ehlers, 1985	Playa La Angosta, Colón (Panama)	XXXX	XXXX
	Minona gemella Ax & Sopott-Ehlers, 1985	Playa La Angosta, Colón (Panama)	XXXX	XXXX
	Minona gemella Ax & Sopott-Ehlers, 1985	Playa La Angosta, Colón (Panama)	XXXX	XXXX

Family	Species	Locality*	18S	28S D1-D6
Monocelididae	*Minona* cf *gemella* Ax & Sopott-Ehlers, 1985	Naos Island (Panama)	XXXX	XXXX
	Minona cf *gemella* Ax & Sopott-Ehlers, 1985	Naos Island (Panama)	XXXX	XXXX
	Minona cf *gemella* Ax & Sopott-Ehlers, 1985	Naos Island (Panama)	XXXX	XXXX
	Minona sp. nov.	Boa Vista Island (Cape Verde)	XXXX	XXXX
	Minona sp. nov.	Boa Vista Island (Cape Verde)	XXXX	XXXX
	Monocelis longiceps (Duges, 1830)	Al-Maharas (Tunisia)	KC971064	KC971087
	Monocelis longistyla Martens & Curini-Galletti, 1987	La Maddalena (Sardinia, Italy)	KC971065	KC971088
	Monocelis lineata OF Müller, 1774	Porto Pozzo (Sardinia, Italy)	XXXX	XXXX
	Monocelis lineata OF Müller, 1774	Charaki (Rhodes, Greece)	XXXX	XXXX
	Monocelis lineata OF Müller, 1774	Pilo (Sardinia, Italy)	XXXX	XXXX
	Monocelis lineata OF Müller, 1774	Colostrai (Sardinia, Italy)	XXXX	XXXX
	Minona sp. nov. 1	Faro (Portugal)	XXXX	XXXX
	Minona sp. nov. 1	Faro (Portugal)	XXXX	XXXX
	Minona sp. nov.	Lanzarote, Canary Island (Spain)	XXXX	XXXX
	Minona sp. nov.	Tenerife, Canary Island (Spain)	XXXX	XXXX
	Minona sp. nov.	Tenerife, Canary Island (Spain)	XXXX	XXXX
	Duplomonona sp.nov.	Lanzarote, Canary Island (Spain)	XXXX	XXXX
	Minona sp. nov. 2	Faro (Portugal)	XXXX	XXXX
	Minona sp. nov. 2	Faro (Portugal)	XXXX	XXXX
	Duplominona brasiliensis Curini-Galletti, 2013	Ilhabela (Brazil)	XXXX	XXXX
	Duplominona sp. nov. 1	Naos Island (Panama)	XXXX	XXXX
	Duplominona sp. nov. 1	Naos Island (Panama)	XXXX	XXXX
	Duplominona sp. nov. 2	Naos Island (Panama)	XXXX	XXXX
	Duplominona tridens (Marcus, 1954)	São Sebastião (Brazil)	XXXX	XXXX
	Duplominona tridens (Marcus, 1954)	São Sebastião (Brazil)	XXXX	XXXX
	Duplominona sp. nov. 3	Naos Island (Panama)	XXXX	XXXX
	Duplominona sp. nov. 4	Naos Island (Panama)	XXXX	XXXX
	Duplominona sp. nov. 4	Naos Island (Panama)	XXXX	XXXX
	Duplominona sp. nov.	Roscoff (France)	XXXX	XXXX
	Duploperaclistus circocirrus Martens, 1983	Roscoff (France)	XXXX	XXXX
	Duploperaclistus circocirrus Martens, 1983	Roscoff (France)	XXXX	XXXX

Family	Species	Locality*	18S	28S D1-D6
Monocelididae	*Duplominona* sp. nov.	Blanes (Spain)	XXXX	XXXX
	Archilopsis spinosa (Jensen, 1878)	Roscoff (France)	XXXX	XXXX
	Archilina duplaculeata (Ax & Armonies, 1990)	Roscoff (France)	XXXX	XXXX
	Monocelidinae sp. nov.	Faro (Portugal)	XXXX	XXXX
Calviriidae	*Calviria solaris* Martens & Curini-Galletti, 1993	GenBank	AJ270153	AJ270168
Coelogynoporidae	*Coelogynopora tenuis* Meixner, 1938	Roscoff (France)	XXXX	XXXX
Unguiphora	*Polystyliphora novaehollandiae* Curini-Galletti, 1998	GenBank	AJ270161	AJ270177
	Nematoplana coelogynoporoides Meixner, 1938	Roscoff (France)	XXXX	XXXX

Table 2. 18S and 28S mutation rates for the two geminate species pairs. K2P: genetic distance corrected according to the Kimura two-parameters model (Kimura, 1980) and standard error; r: mutation rates per million years.

Geminate species	*K2P*	*r* (%)
18S		
Minona gemella (Atlantic Coast)		
Vs	0.0085 ±0.0022	0.12
Minona cf *gemella* (Pacific Coast)		
Parotoplana sp. nov. 1 (Atlantic Coast)		
Vs	0.0115 ±0.0027	0.16
Parotoplana sp. nov. 2 (Pacific Coast)		
28S		
Minona gemella (Atlantic Coast)		
Vs	0.0345 ±0.0045	0.49
Minona cf *gemella* (Pacific Coast)		
Parotoplana sp. nov. 1 (Atlantic Coast)		
Vs	0.0361 ±0.0049	0.52
Parotoplana sp. nov. 2 (Pacific Coast)		

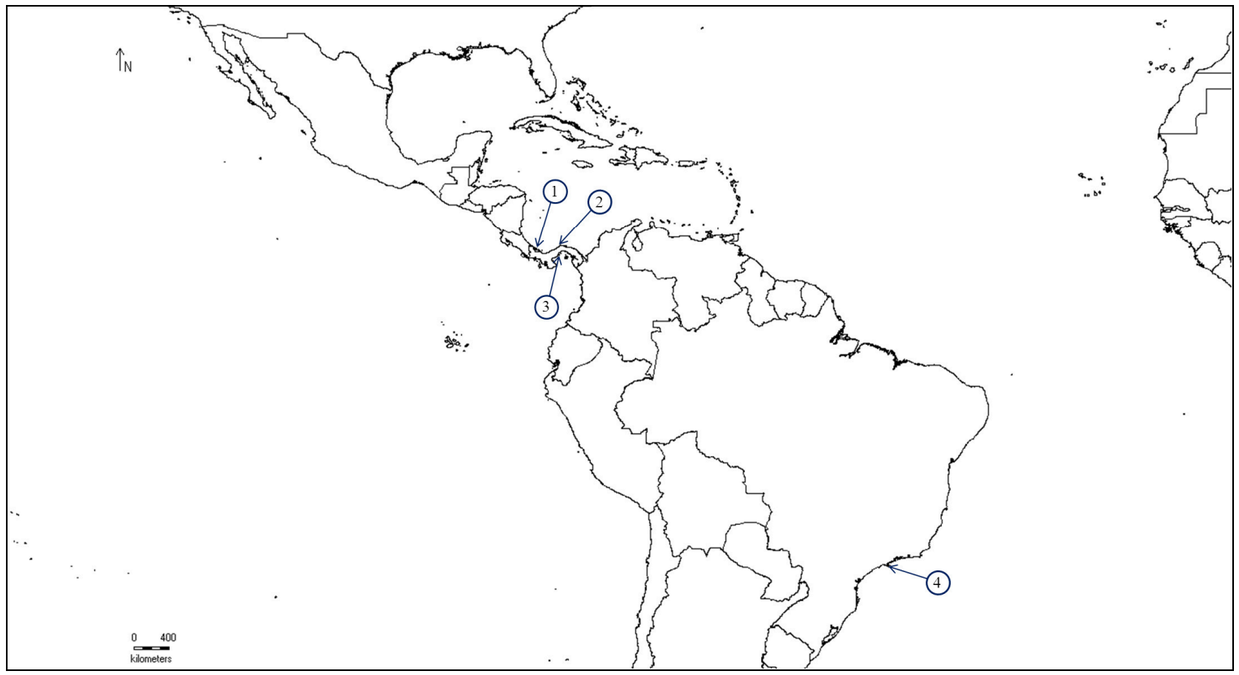

Figure 1. Sampling localities of the specimens belonging to Central and South America. 1: Bocas del Toro, Panama; 2: Playa La Angosta - Colón, Panama; 3: Naos Island - Panama City, Panama; 4: Ilhabela/São Sebastião, Brazil.

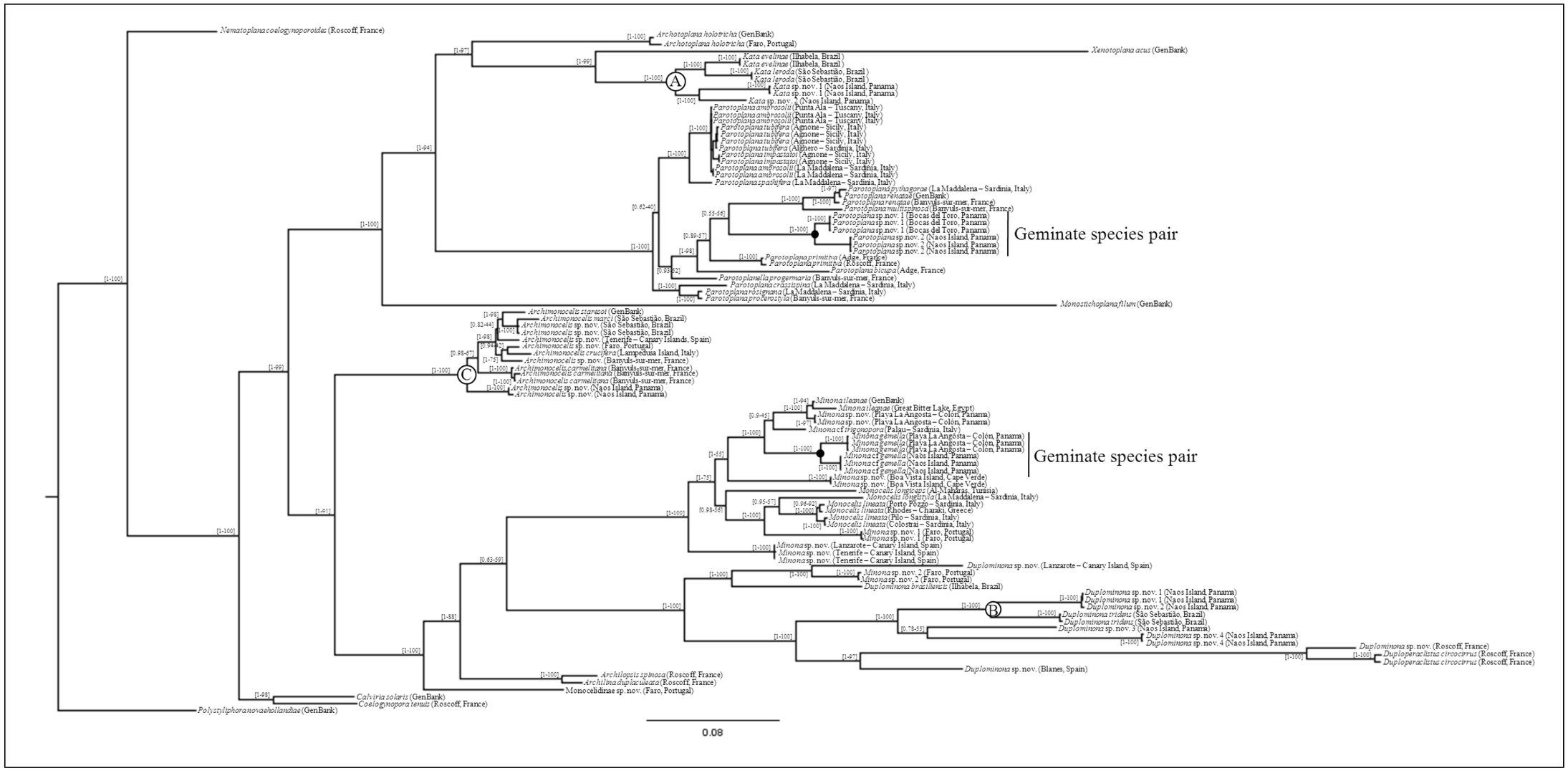

Figure 2. Tree obtained by BI showing the interrelationships of the species based on combined 18S+28S D1-D6. The branch length scale refers to the number of substitutions per site. Nodal supports are indicated for both BI (posterior probability) and ML (bootstrap). Node A: Atlantic *Kata evelinae* and *Kata leroda* + Pacific *Kata* sp. nov. 1 and *Kata* sp. nov. 2. Node B: Atlantic *Duplominona tridens* + Pacific *Duplominona* sp. nov. 1 and *Duplominona* sp. nov. 2. Node 3: Pacific *Archimonocelis* sp. nov. + Atlantic *Archimonocelis* clade.

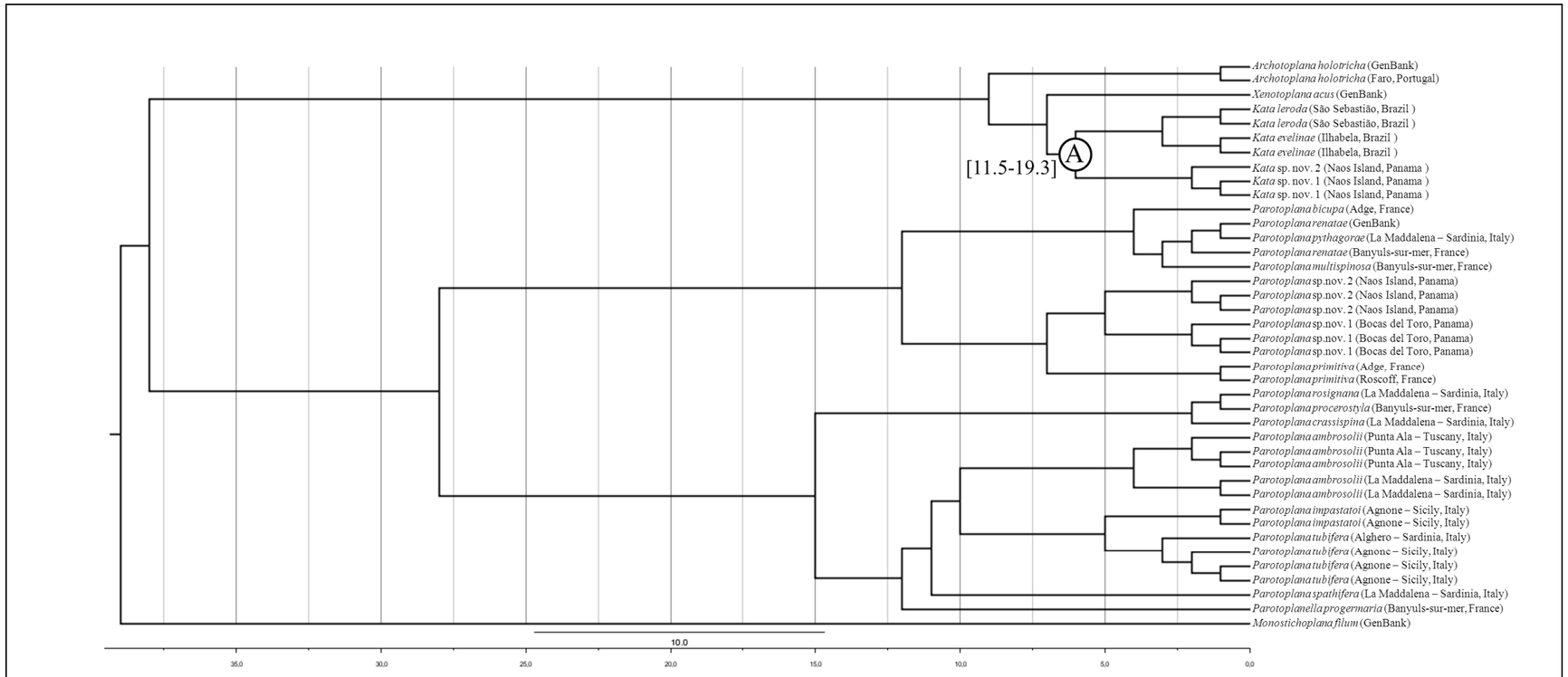

Figure 3. Timetree obtained by the software Beast on the clade in which groups all individuals belonging to the family Otoplanidae. Node indicated with A correspond to the node A showed in Fig. 2. Values within brackets represent the range of divergence time of the node.

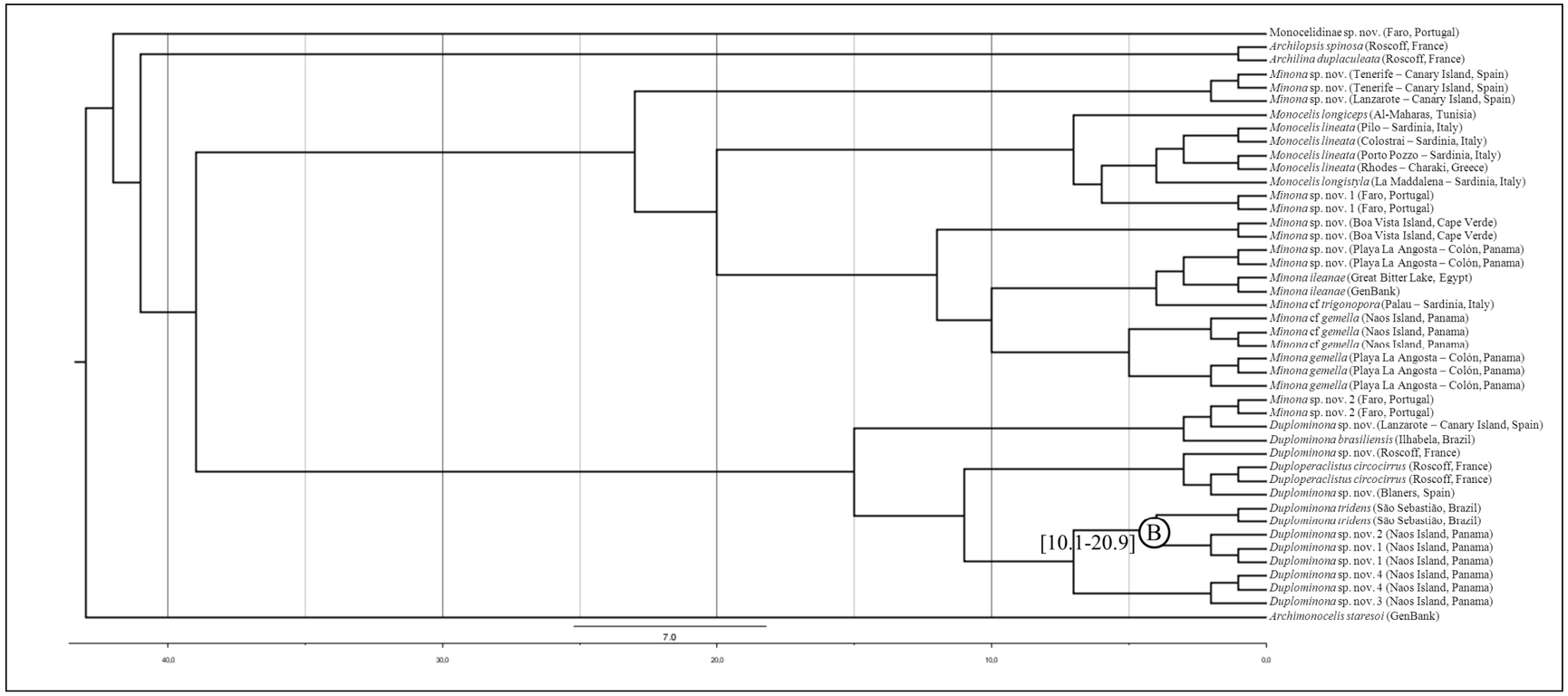

Figure 4. Timetree obtained by the software Beast on the clade in which groups all individuals belonging to the family Monocelididae. Node indicated with B correspond to the node B showed in Fig. 2. Values within brackets represent the range of divergence time of the node.

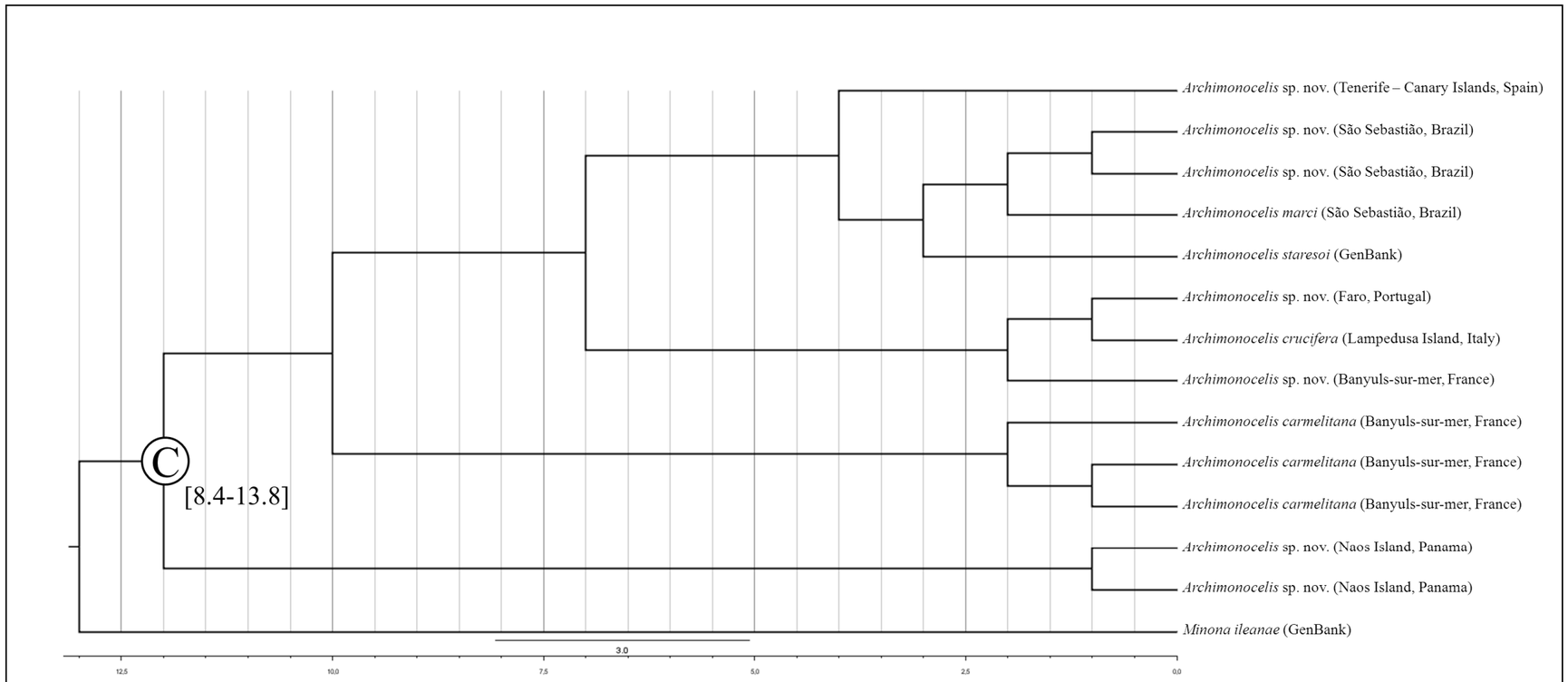

Figure 5. Timetree obtained by the software Beast on the clade in which groups all individuals belonging to the family Archimonocelididae. Node indicated with C correspond to the node C showed in Fig. 2. Values within brackets represent the range of divergence time of the node.

Chapter 7

Novel set of nuclear primers for the ribosomal regions in Proseriata

Marco Casu, Fabio Scarpa, Tiziana Lai, Daria Sanna, Piero Cossu, Marco Curini Galletti

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Novel set of nuclear primers for the ribosomal regions in Proseriata

MARCO CASU, FABIO SCARPA*, TIZIANA LAI, DARIA SANNA, PIERO COSSU, MARCO CURINI-GALLETTI

Dipartimento di Scienze della Natura e del Territorio – Sezione di Zoologia, Archeozoologia e Genetica, Università di Sassari, Via F. Muroni 25, 07100 Sassari, Italy - Centro di Eccellenza dell'Università di Sassari

*Corresponding author: Fabio Scarpa

Dipartimento di Scienze della Natura e del Territorio–

Sezione di Zoologia, Archeozoologia e Genetica,

Università di Sassari,

Via F. Muroni 25,

07100 Sassari, Italy

e-mail: fscarpa@uniss.it

phone: +39 079 228924

fax: +39 079 228665

Abstract

The 18S and 28S rDNA genes have been extensively used as phylogenetic markers in several eukaryotic organisms. These two markers have shown to be useful also for phylogenetic studies in Proseriata (Platyhelminthes: Rhabditophora). Here we provided a set of novel nuclear primers which may allow sequencing the whole ribosomal region in Proseriata, in order to obtain a broad choice of markers to be used.

Key words: Platyhelminthes; Proseriata; nucleolus; rDNA; specific primers, ITS, IGS.

Introduction

To date, all of the eukaryotic species studied showed the presence of several tandem copies of genes encoding the four type of nuclear non-coding (nc) ribosomal RNA (rRNA): 18S, 5.8S, 28S, and 5S (Russel, 2007). With only few exceptions (e.g. species belonging to the genus *Dictyostelium* and some yeasts), in all eukaryotes studied to date, the 5S gene is placed on a different chromosome (Karp, 2008), while the other genes are located on the same chromosome following the order below reported 18S - 5.8S - 28S. They are separated by regions of spacers that include internal and external transcribed and non-transcribed sequences of DNA (Raué et al., 1990). Each cluster composed by rDNA and their spacers, is usually repeated from 100 to 1,000 times, depending on the species (Russel, 2010). The 18S and 5.8S are separated by ITS (Internal-Transcribed Spacer) 1, while 5.8S and 28S are separated by ITS2 sequence. Between the 28S and 18S there is the IGS (Inter-Genic Spacer) region that include ETS (External-Transcribed-Spacer) and NTS (Non-Transcribed-Spacer) (Star et al., 2003), which serves to separate each rDNA cluster from the following one (Palumbi, 1996).

Since the ribosomal genes cluster, contains different domains that evolve at varying rates, they are extensively used as phylogenetic markers in several eukaryotic organisms (Palumbi, 1996). In particular, the 18S and 28S rDNA fragments, which represent the most utilized, contain both variable and conserved regions. They evolve relatively slowly being useful in a great deals of inter-specific phylogenetic analysis. Their nature of orthologous and conserved genes, allows to design universal primers for the amplification of these regions also in a broad range of taxa whose genome information are currently lacking.

The 18S and 28S rDNA genes have been extensively used also among Proseriata (Platyhelminthes: Rhabditophora) (see Littlewood et al., 2000; Willems et al., 2006; Larsson et al., 2008; Curini-Galletti et al., 2010; Casu et al., 2011a) and other Platyhelminthes in general (see Carranza et al., 1997; Littlewood et al., 1999; Littlewood and Olson, 2001; Lockyer et al., 2003; Baguñà and Riutort, 2004; Willems et al., 2006; Larsson and Jondelius, 2008). In particular, due to their potential in depicting genetic variability at inter-specific level, they have shown to be appropriate markers for phylogenetic analysis. In addition, the low level of intra-specific variation of these regions make also them powerful tools for molecular systematics (see Litvaitis et al., 1996; Littlewood et al., 1999; 2000; Curini et al., 2010; Casu et al., 2011a).

Nowadays the 18S and 28S D1-D6 fragment represent the most complete molecular dataset available for Proseriata. However, the high level of conservation of 18S and 28S may represent a limit, thus affecting their effectiveness in detecting more recent evolutionary radiations. Unfortunately, knowledge on the genome of Proseriata are very poor, and most of universal primers for invertebrates, such as those for the cytochrome c oxidase subunit I (COI) Folmer's region (Folmer et al., 1994) do not always provide satisfactory results. Specific primers are available for few species (see Sanna et al., 2009; Casu et al., 2011b), but there is a lack of universal primer for a cross-use within the whole order.

On these bases we aimed to obtain a large set of universal primer for Proseriata to amplify different regions, in order to obtain a broad choice of markers to be used. In this work we used the available sequences of 18S and 28S D1-D6 (see Table 1) for designing a set of universal primers for Proseriata to amplify the neighboring region, the

two internal transcribed spacer (ITS), the 5.8S rDNA gene and the inter-genic spacer (IGS), by means of a primer walking technique.

Methods

Designing primers for ribosomal DNA sequences

In order to design specific primers for the flanking regions of the 18S and 28S genes, two highly conserved regions were identified both within the 18S and 28S sequences in 31 species of Proseriata belonging to four different families (see Table 1). The dataset was built by means of the software BioEdit 7.0.5.2 (Hall, 1999), including sequences present in the GenBank's database and sequences obtained for this study. Both 18S and 28S D1-D6 sequences were aligned separately using the algorithm Q-INS-I implemented in Mafft 6.903 (Katoh and Toh, 2008), which is appropriate for non-coding RNA as it considers RNA secondary structure.

All of the primer here provided were designed using the software Primer Premier 6.12 (PREMIER Biosoft International, Palo Alto, CA).

The first forward primer (318S) was designed within a region which is completely conserved in many animals, from fruit fly to man (Ji et al., 2003), placed almost at the end of the 18S gene (Fig.1). The first primer reverse (528S) was designed within a conserved region (in Proseriata) placed almost at the beginning of the 28S D1-D6 region (Fig.1). The successful of these primers was tested performing cross-priming on at least one species of each genus present in our own dataset, by means of standard PCR, whose mixture, amplification parameters and electrophoresis runs were as described by Casu et al. (2011a). The PCR products did not show any occurrence of

aspecificity. They were purified and sequenced for both forward and reverse strands, using an external sequencing core service (Macrogen Europe®), and the corresponding sequencing runs were repeated twice in order to verify the reliability of results.

After the first obtained fragment that includes ITS1+5.8S+ITS2 was sequenced, further specific primers for amplifying singularly the ITS1 and ITS2 regions were designed.

A reverse primer (ITS rev) that works coupled with the 318S (forward) and a forward primer (ITS for) that works coupled with the 528S (reverse), were designed within a conserved region that, on the basis of alignment with other related species, should correspond to the 5.8S gene (Fig.1).

To design primer for amplifying the IGS region, it was used the same alignment used for the fragment ITS1+5.8S+ITS2. The primers forward (IGS for) and reverse (IGS rev) were designed in a conserved region placed almost 300 base pair before the end of the 28S D1-D6 fragment and almost 200 base pair after the beginning of the 18S fragment respectively (Fig. 1).

Finally, thanks to the obtained fragments, it was possible to design primers to amplify the complete 28S region.

All primers were designed to obtain fragments easy to be overlapped in order to gain a complete sequences of the Proseriata ribosomal region. The effectiveness of each primers designed was tested performing PCR on all available specimens (see Table 2).

Results and Discussion

Proseriata is a diverse and species-rich taxon of flatworm. Due to their poor morphological features and their small size, it is a very difficult taxon to study. For this

reason the use of molecular markers technique to investigate their phylogenetic relationship and their taxonomic assessment is of great relevance. In fact, nowadays most of studies about Proseriata are carried out by means an integrative approach that include morphology, karyology, reproductive biology and genetic analysis using 18S and 28S D1-D6 sequences. To date, sequences of 18S and 28S D1-D6 are very popular molecular markers for Proseriata, accordingly they represent the only large dataset available for this particular taxon. Knowledge on Proseriata are so poor, in particular on their genome, therefore, a widening of the molecular markers available for the analyses can be useful in order to acquire further information on the taxon for different purposes such as phylogenetic relationship, species delimitation, and population genetics.

Here we provided a set of novel nuclear primers by means of which it will be possible sequencing the whole ribosomal region in Proseriata. This region, that allows to obtain more than double molecular characters, can provide a useful genetic information for future analysis on Proseriata. Indeed, the first fragment obtained (that includes ITS1+5.8S+ITS2) is 2800 base pairs long, and the second fragment (IGS region) is about 1500 *bp* long. Thus we can extend our molecular dataset from the current length of about 3200 *bp* (18S+28S D1-D6) to about 7500 *bp*. Moreover, here we proposed a couple of primers specific for the single amplification of the complete 28S region.

Future analyses will be focused to test the potential of the new set of primer in different specific study cases.

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Table 1. List of species used in this study for designing the first couple of primer (318S and 528S) with which amplify the fragment IT1+5.8S+ITS2. Accession numbers refer to GenBank codes; accession numbers of new sequences are not yet available. * Only for taxa sequenced by us.

Family	Species	Locality*	18S	28S D1-D6
Monocelididae	*Monocelis* sp. "E"	Porto Puddu (Sardinia, Italy)	XXXX	XXXX
	Monocelis sp. "D"	Cala Rossa (Sardinia, Italy)	XXXX	XXXX
	Monocelis lineata OF Müller, 1774	Porto Pozzo (Sardinia, Italy)	XXXX	XXXX
	Monocelis lineata OF Müller, 1774	Charaki (Rhodes, Greece)	XXXX	XXXX
	Monocelis lineata OF Müller, 1774	Pilo (Sardinia, Italy)	XXXX	XXXX
	Monocelis lineata OF Müller, 1774	Casaraccio (Sardinia, Italy)	XXXX	XXXX
	Monocelis lineata OF Müller, 1774	Colostrai (Sardinia, Italy)	XXXX	XXXX
	Monocelis lineata OF Müller, 1774	Alexandria (Egypt)	XXXX	XXXX
	Monocelis lineata OF Müller, 1774	Kilmore Quay (Ireland)	XXXX	XXXX
	Monocelis lineata OF Müller, 1774	Ferrol (Galicia, Spain)	XXXX	XXXX
	Monocelis lineata OF Müller, 1774	Keflavik (Iceland)	XXXX	XXXX
	Monocelis lineata OF Müller, 1774	Tjärnö (Sweden)	XXXX	XXXX
	Monocelis lineata OF Müller, 1774	Tjärnö (Sweden)	XXXX	XXXX
	Monocelis lineata OF Müller, 1774	Tjärnö (Sweden)	XXXX	XXXX
	Monocelis lineata OF Müller, 1774	Ardrossan (Scotland)	XXXX	XXXX
	Monocelis lineata OF Müller, 1774	Ardrossan (Scotland)	XXXX	XXXX
	Monocelis lineata OF Müller, 1774	Roscoff (France)	XXXX	XXXX
	Monocelis lineata OF Müller, 1774	Roscoff (France)	XXXX	XXXX
	Monocelis lineata OF Müller, 1774	Helsingør (Denmark)	XXXX	XXXX
	Monocelis lineata OF Müller, 1774	Ismailia, Timsah Lake (Egypt)	XXXX	XXXX
	Monocelis longiceps (Duges, 1830)	Al-Maharas (Tunisia)	KC971064	KC971087
	Monocelis longistyla Martens & Curini-Galletti, 1987	La Maddalena (Sardinia, Italy)	XXXX	XXXX
	Minona ileanae Curini-Galletti, 1997	Great Bitter Lake (Egypt)	JN224905	JN224910
	Monocelis fusca Örsted, 1843	Ardrossan, Scotland	XXXX	XXXX
	Archiloa rivilaris de Beauchamp, 1910	GenBank	U70077	U40049
	Pseudomonocelis ophiocephala (Schmidt, 1861),	Porto Torres (Sardinia, Italy)	JN224895	JN224907
	Pseudomonocelis occidentalis Curini-Galletti, Casu & Lai, 2011	Porto Pozzo (Sardinia, Italy)	JN224894	JN224909
	Pseudomonocelis orientalis Curini-Galletti, Casu & Lai, 2011	Maliakós (Greece)	JN224896	JN224908
	Pseudomonocelis agilis (Schultze, 1851)	Helsingør (Denmark)	JN224897	JN224912
	Pseudomonocelis cetinae Meixner, 1943	Omiš (Croatia)	JN224899	JN224913
	Pseudomonocelis cf *cavernicola* Schockaert & Martens, 1987	Dongwe (Zanzibar)	JN224900	JN224914

Family	Species	Locality*	18S	28S D1-D6
Monocelididae	*Pseudomonocelis paupercula* Curini-Galletti, Casu & Lai, 2011	Porto Pozzo (Sardinia, Italy)	JN224901	JN224915
Archimonocelididae	*Archimonocelis staresoi* Martens & Curini-Galletti, 1993	Porto Cesareo (Lecce, Italy)	AJ270152	AJ270166
Otoplanidae	*Parotoplana tubifera* Curini-Galletti & Delogu sp. nov.	Le Bombarde beach (Sardinia, Italy)	KC971058	KC971081
	Parotoplana tubifera Curini-Galletti & Delogu sp. nov.	Agnone (Sicily, Italy)	KC971046	KC971069
	Parotoplana tubifera Curini-Galletti & Delogu sp. nov.	Agnone (Sicily, Italy)	KC971047	KC971070
	Parotoplana tubifera Curini-Galletti & Delogu sp. nov.	Agnone (Sicily, Italy)	KC971049	KC971072
	Parotoplana ambrosolii Curini-Galletti & Delogu sp. nov.	Punta Ala (Tuscany, Italy)	KC971043	KC971066
	Parotoplana ambrosolii Curini-Galletti & Delogu sp. nov.	Punta Ala (Tuscany, Italy)	KC971044	KC971067
	Parotoplana ambrosolii Curini-Galletti & Delogu sp. nov.	Punta Ala (Tuscany, Italy)	KC971045	KC971068
	Parotoplana ambrosolii Curini-Galletti & Delogu sp. nov.	La Maddalena (Sardinia, Italy)	KC971056	KC971079
	Parotoplana ambrosolii Curini-Galletti & Delogu sp. nov.	La Maddalena (Sardinia, Italy)	KC971057	KC971080
	Parotoplana impastatoi Curini-Galletti & Delogu sp. nov.	Agnone (Sicily, Italy)	KC971048	KC971071
	Parotoplana impastatoi Curini-Galletti & Delogu sp. nov.	Agnone (Sicily, Italy)	KC971050	KC971073
	Parotoplana spathifera Delogu & Curini-Galletti, 2007	La Maddalena (Sardinia, Italy)	KC971053	KC971076
	Parotoplana pythagorae Delogu & Curini-Galletti, 2007	La Maddalena (Sardinia, Italy)	KC971052	KC971075
	Parotoplana renatae Ax, 1956	Banyuls-sur-mer (Languedoc-Roussillon, France)	KC971062	KC971085
	Parotoplana multispinosa Ax, 1956	Banyuls-sur-mer (Languedoc-Roussillon, France)	KC971061	KC971084
	Parotoplana bicupa Sopott-Ehlers, 1976	Agde (Languedoc-Roussillon, France)	KC971063	KC971086
	Parotoplana primitiva Ax, 1956	Agde (Languedoc-Roussillon, France)	KC971060	KC971083
	Parotoplanella progermaria Ax, 1956	Banyuls-sur-mer (Languedoc-Roussillon, France)	KC971059	KC971082
	Parotoplana crassispina Delogu & Curini-Galletti, 2009	La Maddalena (Sardinia, Italy)	KC971051	KC971074
	Parotoplana rosignana Lanfranchi & Melai, 2008	La Maddalena (Sardinia, Italy)	KC971054	KC971077
	Parotoplana procerostyla Ax,1956	Banyuls-sur-mer (Languedoc-Roussillon, France)	KC971055	KC971078
	Archotoplana holotricha Ax, 1956	GenBank	AJ243676	AJ270165
	Monostichoplana filum (Meixner,1938)	GenBank	AJ270158	AJ270173
	Xenotoplana acus Ax, Weidemann & Ehlers, 1978	GenBank	AJ270155	AJ270181
Calviriidae	*Calviria solaris* Martens & Curini-Galletti, 1993	GenBank	AJ270153	AJ270168

Table 2. List of primers designed in this study. #Ta: Annealing temperature. *R = A+G; **Y = C+T

Region	Ta#	Primer name	Sequence 3'-5'
ITS1+5.8S+ITS2	60°C	318S (for)	CCC TGC CCT TTG TAC ACA CCG CCC
		528S (rev)	ATT TAG CCT TR*G ATG GAG TTT ACC
ITS1	54°C	318S (for)	CCC TGC CCT TTG TAC ACA CCG CCC
		ITS (rev)	AGT TGT ATT TCT CTT AGG ATT TC
ITS2	58°C	ITS (for)	TGT GCA AGG TTT AAA GAG AA
		528S (rev)	ATT TAG CCT TR*G ATG GAG TTT ACC
28S	55°C	28S (for)	GGT AAA CGG CGG GAG TAA CTA TGA C
		28S (rev)	CCT TGT TAC GAC TTT TAC TTC CTC T
IGS	54°C	IGS (for)	ATC AAC CAG Y**CC TGA AAA TG
		IGS (rev)	TGC TGC CTT CCT TR*G ATG TG

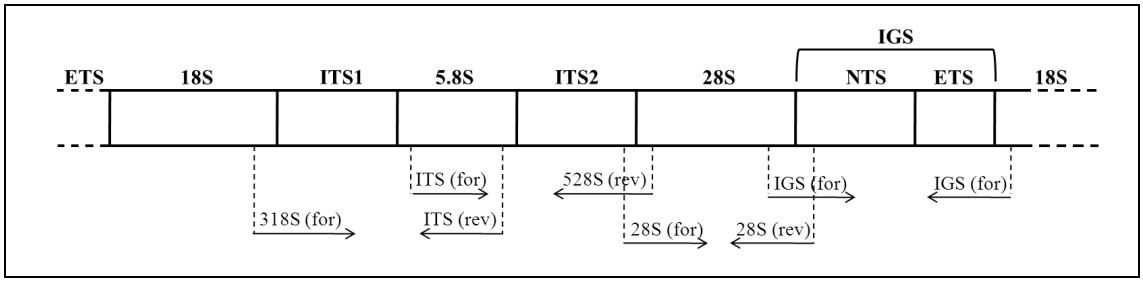

Figure 1. Scheme of nuclear ribosomal DNA regions. Orientation and approximate position of annealing sites for each primers designed in this study are indicated by arrows (not drawn to scale).