An 18S and 28S-based clock calibration for marine Proseriata (Platyhelminthes)

A 18S and 28S-based clock calibration for marine Proseriata (Platyhelminthes)

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*

[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 are a powerful tool for calibrating the molecular clock in marine organisms, and their adoption is mandatory for soft-bodied taxa, which lack fossil records. The first attempt to calibrate the molecular clock in taxa belonging to meiofaunal microturbellaria (Platyhelminthes: Proseriata) based on geminate species is presented here. We used two species pairs from both sides of the Isthmus of Panama: *Minona gemella* (Caribbean) and *Minona* cf *gemella* (Pacific); *Parotoplana* sp. nov. 1 (Caribbean) and *Parotoplana* sp. nov. 2 (Pacific). The mutation rates per million years were estimated for both geminate species pairs on two ribosomal regions, the complete nuclear small subunit rDNA (18S) gene and the partial nuclear large subunit rDNA (28S) gene fragment (spanning variable domains D1-D6). Similar values of mutation rates per million years were found in both species pairs, ranging 0.12-0.16 % for 18S and 0.49-0.52% for 28S. The values obtained were used as calibration points at minimum age, in order to estimate the divergence times within the phylogenetic tree of the whole dataset, and tested on three cases of trans-American (not-geminate) species from Pacific Panama and S-E Brazil, belonging to the genera *Kata*, *Archimonocelis* and *Duplominona*. They consistently showed higher divergence times (ranging 9.4-17.9 Myr) than geminate, trans-isthmian pairs. These results suggest potential usefulness of our molecular clock calibration, for future research on phylogeography and evolution of Proseriata.

Key words: Meiofauna; Isthmus of Panama; mutation rates; relaxed molecular clock; divergence time; calibration point.

51

1. Introduction

Interstitial meiofauna is among the most diverse and species-rich components of marine biodiversity (Kennedy and Jacoby, 1999). Knowledge of many aspects of the biology of these minute organisms is however limited, even in comparatively well-studied areas (Curini-Galletti et al., 2012), and patterns of spatial distribution are particularly poorly understood. Early studies pointed to the existence of large, amphi-Atlantic or even cosmopolitan, distributions in meiofaunal taxa (Sterrer, 1973; Westheide and Schmidt, 2003). Such large-scale ranges in species lacking any obvious means of dispersal is at the basis of the so-called ‘meiofauna paradox’ (Giere, 2009). Ancient vicariance events, followed by evolutionary stasis, were hypothesized to be responsible of the observed patterns (Sterrer, 1973); this, however, would imply a surprisingly old phylogenetic age for these species (see Sepkoski, 1998). Although recent molecular surveys showed that, in many instances, these vast distributions are the result of the lack of resolution of cryptic species complexes (i.a., Casu et al., 2009; Fontaneto et al., 2009; Jörger and Schrödl, 2013; Todaro et al., 1996), at least some cases of large-scale distribution of meiofaunal taxa have been supported by molecular data (see e.g., Dericke et al., 2008; Jörger et al., 2012; Meyer-Wachsmuth et al., 2014; Tulchinsky et al., 2012), leaving open the choice between great antiquity of lineages, or unsuspected capabilities for dispersal.

In order to provide an adequate coverage of information, systematic, biogeographic and phylogenetic studies should ideally be flanked by the estimation of divergence time among clades (see i.a., Heads, 2005a; Ree and Smith, 2008), which may allow inferences on the time-scale of speciation processes. The modern molecular phylogenetic approach applied to date

evolutionary divergence is based on the molecular clock hypothesis (MCH) (Zuckerkandl and Pauling, 1965), which assumes a relatively constant rate of molecular evolution over time and across taxa (see Kimura, 1968, and references therein). However, recent empirical studies have demonstrated the existence of a significative variation in the rate of molecular evolution (Bromham and Penny, 2003; Thomas et al., 2006), and the use of a more sophisticated approach, such as a relaxed clock model, has been recommended (see Lepage et al., 2007 and references therein). In order to assign concrete dating, a molecular clock needs to be calibrated against independent evidence (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; Ronquist et al., 2012a) or, when possible, by means of both fossil records and paleogeographic events (e.g., Heads, 2005a).

In the case of meiofaunal organisms, however, dating of divergence is particularly difficult, as these taxa (and especially the soft-bodied component) do not leave any fossil record that can be used as calibration points (e.g., Blanton et al., 2013; Mulcahy et al., 2012). Therefore, the adoption of geminate species - i.e. morphologically indistinguishable sister species that live in allopatric conditions and occur at the opposite sides of a (datable) geographic barrier (Jordan, 1908) - and the MCH constitute an almost inevitable strategy (Coyne and Orr, 2004; Lessios, 2008). Geminate species represent a widely cited model of allopatric speciation (Coyne and Orr, 2004; Lessios, 2008; Vermeij, 1978), constituting a ‘natural experiment’ that can describe evolutionary divergence and its causes (Lessios, 2008). Indeed, several studies have demonstrated that geminate species may represent a suitable alternative

to fossil records as calibration points for a molecular clock, and can be used in turn to estimate divergence times between related species (e.g., Lessios, 1998; Knowlton and Weigt, 1998; Marko and Moran, 2009). For this purpose, one of the most used geographical barriers is the Isthmus of Panama (see, e.g., Heads, 2005b). Geological literature suggests that complete isolation between the Caribbean Sea and the Pacific Ocean occurred about 3.1-3.5 million years (Myr) ago, due to the final emergence of the Isthmus (Allmon, 2001; Collins et al., 1996; Jackson and Budd, 1996; Knowlton and Weig, 1998).

This approach is however not without criticisms (see Heads, 2005b). A major objection is that identification of a species pair as geminate species may be biased by the taxonomic sampling available (Lessios, 2008). Furthermore, establishing the time of separation between geminate species may be fraught with problems, as the emergence of the isthmian landmass was a prolonged geological process, and not all geminate pairs were simultaneously separated by the emerging Isthmus (Knowlton, 1993; Knowlton and Weigt, 1998; Marko, 2002). Populations of intertidal meiofauna may be ideal candidates for the calibration of the molecular clock, as they were more likely to maintain continuity of habitat and gene flow across the emerging isthmus, until the separation of eastern Pacific and Caribbean was completed. However, they have never been studied in this context.

We aimed to assess the molecular clock on species belonging to different families of meiobenthic, intertidal free-living microturbellarians (Platyhelminthes: Proseriata), using geminate species from the Isthmus of Panama. Representatives of Proseriata may be numerically abundant and characterize entire soft-bottom communities (Reise, 1988; Remane, 1933). As most meiofaunal organisms, Proseriata lack larval stages, and, combined with

the reduced mobility of adults, their potential for dispersal is limited (Curini-Galletti et al., 2012). Indeed, setting the molecular clock for taxa belonging to the order of Proseriata, would be of particular interest, as support for phylogeographic studies (Casu et al., 2011) or setting up the evolutionary time-frame in cases of allopatric distributions (Delogu and Curini-Galletti, 2009; Casu et al., 2014; Curini-Galletti et al., 2011). Furthermore, the existence of many supra-specific taxa with anti-tropical distributions (Laumer et al., 2014) could be ideally interpreted with information on the timing of their divergence. Notwithstanding the current, different ecological conditions at the two sides of the isthmus (Lessios, 2008), morphologically similar congeneric species were found, which could be tested as potential geminate species. We sequenced further morphologically similar congeners, allopatrically distributed along the Pacific shores of Panama and in western Atlantic (South Brazil). These latter, trans-American species were used as potential test cases, as their age of divergence should pre-date that of trans-isthmian geminates.

We calibrated the molecular clock on two ribosomal genes, the complete nuclear small subunit rDNA (18S) gene and the partial nuclear large subunit rDNA (28S) gene fragment (spanning variable domains D1-D6), since their sequences constitute the only large database available for Proseriata.

2. Materials and methods

2.1. The species

2.1.1. Trans-isthmian species pairs

- *Minona gemella* Ax and Sopott-Ehlers, 1985 (Caribbean) / *Minona* cf *gemella* (Pacific) (Proseriata: Monocelididae).

Species found at the opposite ends of the Canal (Table 1; Fig. 1), in intertidal habitats. *Minona gemella* (type locality: Bermuda) is characterized by the presence of two accessory prostatoid organs, one anterior and one posterior to the copulatory organ - a unique feature for species of the genus *Minona* Marcus 1946 (Ax and Sopott-Ehlers, 1985). The Pacific counterpart appears identical in morphology, as reconstructed from observations on living, semi-squeezed specimens, and from histological sections, as well as for all measurable characters of the sclerotized structures. The only appreciable differences have been detected in their karyotypes: Caribbean specimens from Panama and Puerto Rico have chromosome II metacentric, while it is more heterobrachial in Pacific specimens (Curini-Galletti, 1991; unpubl. data).

- Genus *Parotoplana* Meixner, 1938 (Proseriata: Otoplanidae).

Parotoplana sp. nov. 1 (Caribbean coast of Panama) and *Parotoplana* sp. nov. 2 (Pacific coast of Panama) are morphologically very similar, differing for minute details of the sclerotized structures (unpubl. data), only detectable on strongly squeezed, karyological slides, where tissues have been macerated with acetic acid (see Curini-Galletti et al., 1989). Both species occur intertidally.

2.1.2 Trans-American species

- Genus *Kata* Marcus, 1950 (Proseriata: Otoplanidae).

The four described species of the genus *Kata* are distributed on both American coasts: *Kata evelinae* Marcus, 1949 and *Kata leroda* Marcus, 1950 from South Brazil (both of which were here sequenced); *Kata galapagoensis* Ax and Ax, 1974 from Galapagos Island; and *Kata galea* Ax and Sopott-Ehlers, 1987 from Bermuda. The two new species from the Pacific coast of Panama (*Kata* sp. nov. 1 and *Kata* sp. nov. 2) differ from each other and the other species of the genus for details of the morphology of the sclerotized pieces of the copulatory organ (unpubl. data). All species occur intertidally.

- Genus *Duplominona* Karling, 1966 (Proseriata: Monocelididae).

The two species tested share a unique feature of the posterior end, deeply split into a ‘trident’ shape. *Duplominona tridens* Marcus, 1954 is a south Brazilian species (Marcus, 1954a). The Pacific counterpart (*Duplominona* sp. nov. 1) is identical in external morphology and general topography of organs, but differs for characters of the sclerotized structures of the copulatory organ (Curini-Galletti, 2014). Both species occur intertidally.

- Genus *Archimonocelis* Meixner, 1938 (Proseriata: Archimonocelididae).

The American species here sequenced, *Archimonocelis marci* Curini-Galletti, 2014 and *Archimonocelis* sp. nov. 1 from Brazil, and *Archimonocelis* sp. nov. 2, from Pacific Coast of Panama are morphologically similar, as they share a simple structure of the copulatory organ, with a stylet surrounded by a girdle of short, nearly identical spines (Curini-Galletti, 2014; unpubl data). All species occur in shallow subtidal habitats.

2.2. Sampling, DNA extraction, amplification and sequencing

Samples were collected manually by scooping up the superficial layer of sediment. All necessary permits for samplings in protected areas were

obtained. No specific permits were required for other sites, which were not privately owned or protected.

Extraction of the animals from the sediment was accomplished using $MgCl_2$ decantation (Martens, 1984). Each specimen was studied alive by slight squeezing under the cover slip. Whenever possible, vouchers were prepared, consisting of whole mounts of posterior body regions of the specimens sequenced, and are maintained in the collections of the Zoological Museum of the University of Sassari (CZM). For information about sampling localities see Table 1 and Fig. 1.

Genomic DNA was extracted using the Macherey-Nagel NucleoSpin Tissue (Macherey-Nagel GmbH and 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 analyzed for a total of 92 individuals; 62 were newly obtained specifically for this study, and 30 taken from GenBank (for details see Table 1). The dataset was built with 40 sequences of individuals belonging to the family Otoplanidae (15 of which newly sequenced, Table 1), 42 to the family Monocelididae (39 of which newly sequenced, Table 1), 6 to the family Archimonocelididae (all of which newly sequenced, Table 1), one to the family Calviridae (from GenBank), one to the family Coeloginoporidae (newly sequenced), and two to the suborder Unguiphora (one of which newly sequenced). 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).

PCRs were carried out in a total volume of 25 µl containing about 25 ng (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 amplifications were 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. Electrophoreses were 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 Inc., Europe). The sequencing runs were repeated twice in order to verify the reliability of results.

2.3. 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 2.1.1 (Posada, 2008), with a maximum likelihood optimized 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 pairwise genetic

distances corrected according to the Kimura two-parameter model (*K2P*) (Kimura, 1980) were estimated between population's representatives to the trans-isthmian geminate species pairs, by means of the software Mega 6.06 (Tamura et al., 2011) with 1,000 bootstrap replications. *K2P* distances were estimated singularly for each gene in order to insert them into the formula proposed by Li and Graur (1991) (see section 2.4.1).

Phylogenetic relationships among individuals and species were investigated using both Maximum Likelihood (ML) and Bayesian Inference (BI) on the combined 18S and 28S sequences. We set as outgroup for the analyses the species *Polystyliphora novaehollandiae* Curini-Galletti, 1998. ML was performed using the genetic algorithm implemented in Garli 2.01 (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 3.2.2 (Ronquist et al., 2012b), specifying a partitioned model and setting as model parameters: NST = 6, rates = invgamma, 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., 2012b), 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 visualized using FigTree 1.4.0 (http://tree.bio.ed.ac.uk/software/figtree/).

2.4. Calibration of molecular clock

2.4.1. Estimation of the mutation rates per million years

After phylogenetic analysis showed that the trans-isthmian species (*Minona gemella* + *Minona* cf *gemella* and *Parotoplana* sp. nov. 1 + *Parotoplana* sp. nov. 2) represent two pairs of sister species, and may thus be considered as 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) (Li 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.

2.4.2. 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 separately for each of the two regions (18S and 28S) on the *Minona* and *Parotoplana* species pairs. Divergence times were estimated using a uniform distribution with lower and upper values set according to the mutation rate per million years of the two species pairs (see Table 2). 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 1.6 (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 to estimate node ages. TreeAnnotator and FigTree were used for drawing and visualizing the timetree, respectively. Alignments and Bayesian tree-files are deposited and available in Treebase

331 (TB2: S16487.

3. Results

3.1. Estimates of genetic distance and phylogenetic analysis

After the alignment, sequences of 1632 *bp* and 1650 *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 provided comparable values between the pairs *Minona gemella* + *Minona* cf *gemella* and *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; additionally, in both trees the nodes of our interest are highly supported. We therefore reported the BI tree obtained by the software MrBayes only (Fig. 2). This phylogenetic tree shows that *M. gemella* (Atlantic coast) and *Minona* cf *gemella* (Pacific coast) (GS1) are in a sister-taxon relationship, as well as *Parotoplana* sp. nov. 1 (Atlantic coast) and *Parotoplana* sp. nov. 2 (Pacific coast) (GS2); the corresponding nodes are highly supported both for posterior probability and bootstrap values (Fig. 2). Therefore, according to Jordan's definition (1908)**,** they can be considered as geminate species, and they will be used 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 Otoplanidae, species belonging to the genus *Kata* were separated into two geographic clusters, one grouping the Atlantic *K. evelinae* + *K. leroda*, and one the Pacific *Kata* sp. nov. 1 + *Kata* sp. nov. 2 (node A in Fig. 2);

- Within Monocelididae, node B (Fig. 2) splits the Atlantic specimens of *Duplominona tridens* from the Pacific specimens of *Duplominona* sp. nov. 1;
- Within Archimonocelidae, node C (Fig. 2) splits the Atlantic *A. marci* + *Archimonocelis* sp. nov. 1 from the Pacific *Archimonocelis* sp. nov. 2. For each of these three cases, nodes are highly supported (Fig. 2).

3.2. Mutation rates per million years and divergence time

The estimated ucld.stdev parameter amounts to 0.842 and 0.677 for the 18S and 28S, respectively, indicating that our dataset is clock-like. The mutation rate per million years between *M. 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: 0.16% for the18S, 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. On these bases, we estimated a divergence time for node A (splitting Atlantic *K. evelinae* and *K. leroda* from the Pacific *Kata* sp. nov. 1 and *Kata* sp. nov. 2) of about 17.9 Myr, ranging 12.9 - 23.8 Myr (Fig. 3); for node B (splitting Atlantic *Duplominona tridens* from the Pacific *Duplominona* sp. nov. 1) of about 13.9 Myr, ranging 8.9 - 20.0 Myr (Fig. 3); and for node C (splitting the Atlantic *A. marci* and *Archimonocelis* sp. nov. 1, from the Pacific *Archimonocelis* sp. nov. 2) of 9.4 Myr, ranging 5.5 - 14.9 Myr (Fig. 3).

4. Discussion

Molecular tools and the MCH have provided new clues on past evolutionary processes and mechanisms driving molecular evolution (Bromham and Penny, 2003). However, several authors have shown perplexity about the wide applicability of the MCH (see e.g., Heads, 2005b; Lessios, 2008; Palumbi, 1997), and the use of the molecular clock to infer divergence time elicits criticisms, mostly concerning the way the clock is calibrated (Peterson et al., 2004). The use of paleogeographic events, which represents the only possible alternative of calibration in absence of fossil records, is a contentious issue (Coyne and Orr, 2004; Lessios, 2008). It should be noticed that estimates on geminate species assume the final closure of a given geographic barrier as a minimum age calibration; hence, time since divergence may have been underestimated if taxa diverged before this date. 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 potentially may be more complex (see Knowlton and Weigt, 1998).

To overcome such limitations, the use of different genes or loci, and different calibration points has been recommended (Marko and Moran, 2009). In the case of Proseriata, the number of genes/loci we could use are limited, because 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 provide satisfactory results, and specific primers are not available except for a few species (see Casu et al., 2011; Sanna et al., 2009). Furthermore, since a limited number of sequences of Proseriata is at present available in Genbank, the number of calibration points depends on sampling’s success, and the adequacy

406 of the sampling campaign can be assessed only after morphological and
407 molecular analyses in laboratory (see e.g., Casu et al., 2014). In this context, it
408 is noteworthy that an inadequate taxonomic coverage may lead to the use of
409 false geminate species for the calibration of the molecular clock, and thus to
410 the use of species pairs separated well before the last closure of the isthmus
411 which results in an overestimation of the mutation rates per million years
412 (Heads, 2005a; Knowlton and Weigt, 1998). Consequently, the use of an higher
413 rate may cause an underestimation of the divergence time among groups in
414 the timetree.
415 Albeit it might be questionable whether our taxonomic coverage is extensive
416 enough to assess sister species relationships reliably, the Atlantic *M. gemella*
417 and *Parotoplana* sp. nov. 1, and their Pacific counterparts (*Minona* cf *gemella*
418 and *Parotoplana* sp. nov. 2, respectively) are reciprocally monophyletic and
419 morphologically indistinguishable at the routine level of morphological
420 observation, and are thus highly suggestive of geminate lineages. Furthermore,
421 the two pairs show very similar values of mutation rate per million years in
422 both genes. It is noteworthy that these similar values have been found in
423 species pairs belonging to two different families (Monocelididae and
424 Otoplanidae), and may thus prove applicable across the Proseriata. Finally, in
425 the three trans-American species used as test cases (*Kata* spp., *Duplominona*
426 spp. and *Archimonocelis* spp.), the obtained divergence times are greater than
427 the final closure of the Isthmus of Panama - ranging from 9.4 Myr (time of
428 divergence between *Archimonocelis* spp.) and about 17.9 Myr (time of
429 divergence between *Kata* spp.) - and therefore not conflicting with the values
430 obtained with trans-isthmian species.

Our results are consistent to those found for other trans-American species pairs (see e.g., Beu, 2001; Coates and Obando, 1996; Collins, 1996; Jackson et al., 1993; Roopnarine, 2001; Vermeij, 2001). For instance, the calibration on COI and ITS (Internal Transcribed Spacer) sequences revealed a time of divergence of 17.4-27.0 Myr, and 14.5-18.8 Myr, respectively, between trans-American populations of the subgenus *Acar* (Bivalvia) (Marko and Moran, 2009).
In the cases of *Kata* spp, *Duplominona* spp., and *Archimonocelis* spp., dispersal between ocean basins along the southern tip of South America would obfuscate interpretation of our results. However, no member of the species pairs involved was found in previous research in Chile, Uruguay, Terra del Fuego, or sub-Antarctic islands (Marcus, 1954b; Schockaert et al., 2009, 2011). Furthermore, species of the genus *Kata* are only known from tropics; similarly, *Duplominona* and *Archimonocelis* species, with few exceptions, occur in tropical to warm-temperate areas (Martens and Curini-Galletti, 1993; Tyler et al., 2006, 2012). At least in recent times, therefore, the rigid conditions of extreme south of South America acted as barrier to dispersal of these organisms.

4.1.Conclusions

The study of geminate species of Proseriata across the Isthmus of Panama allowed the first calibration of the molecular clock for a meiofaunal taxon. Results of our research open potentials for the use of intertidal meiofauna for MCH. Among the major objections of the MCH, in fact, is that speciation among geminate pairs may predate the final emergence of the isthmus. However, meiofaunal, intertidal/shallow-water taxa may have shown continuity of habitat until final emergence of the barrier, and their divergence may indeed reflect

456 the final stage of the isthmian formation. A similar suggestion was advanced
457 for species from brackish-water and mangrove habitats (see Miura et al., 2010).
458 Although further tests on a larger dataset and on other test-cases are deemed
459 necessary, data obtained (both mutation rates and divergence times) might
460 prove invaluable to provide further insights into the phylogenetic relationships
461 and evolution of Proseriata.
462
463
464 **Acknowledgements**
465 We acknowledge partial funding support of the field work by an award from the
466 Smithsonian Institution's Marine Science Network to JLN. We are grateful to
467 staff at the Smithsonian Tropical Research Institute's Naos Marine Laboratory
468 for space and assistance of many sorts. We also acknowledge for partial
469 funding support ASSEMBLE grant agreement no. 227799 for specimens
470 sampled in Roscoff and in Faro. Finally we are grateful to Prof. Ulf Jondelius and
471 to an anonymous referee for their constructive suggestions who helped to
472 improve the paper.
473

References

Allmon, W.D., 2001. Nutrients, temperature, disturbance, and evolution: a model for the late Cenozoic marine record of the western Atlantic. Paleogeogr. Paleoclimatol. Paleoecol. 166, 9- 26.

Ax, P., Sopott-Ehlers B., 1985. Monocelididae (Plathelminthes, Proseriata) von Bermuda. Microfauna Marina 2, 371-382.

Benton, M.J., Donoghue, P.C.J., 2007. Paleontological evidence to date the tree of life. Mol. Biol. Evol. 24 (1), 26-53.

Beu, A.G., 2001. Gradual Miocene to Pleistocene uplift of the Central American Isthmus: evidence from tropical American tonnoidean gastropods. J. Paleontol. 75, 706-720.

Blanton, R.E., Page, L.M., Hilber S.A., 2013. Timing of clade divergence and discordant estimates of genetic and morphological diversity in the Slender Madtom, *Noturus exilis* (Ictaluridae). Mol. Phylogenet. Evol. 66, 679-693.

Bromham, L., Penny, D., 2003. The modern molecular clock. Nat. Rev. Genet. 4 (3), 216-224.

Casu, M., Lai, T., Sanna, D., Cossu, P., Curini-Galletti, M., 2009. An integrative approach to the taxonomy of the pigmented European *Pseudomonocelis* Meixner, 1943 (Platyhelminthes: Proseriata). Biol. J. Linnean Soc. 98, 907-922.

Casu, M., Sanna, D., Cossu, P., Lai, T., Francalacci, P., Curini-Galletti, M., 2011. Molecular phylogeography of the microturbellarian *Monocelis lineata* (Platyhelminthes: Proseriata) in the North-East Atlantic. Biol. J. Linn. Soc. 103, 117-135.

Casu, M., Scarpa, F., Delogu, V., Cossu, C., Lai, T., Sanna D., Curini-Galletti, M., 2014. Biodiversity patterns in interstitial marine microturbellaria: a case study within the genus *Parotoplana* (Platyhelminthes: Rhabditophora) with the description of four new species. J. Zool. Syst. Evol. Res. 52 (3), 190-202.

Coates, A.G., Obando, J.A., 1996. The geologic evolution of the Central American Isthmus, in: Jackson, J.B.C., Budd, A.F., Coates, A.G. (Eds.), Evolution and environment in tropical America, University of Chicago Press, Chicago, pp. 21-56.

Collins, L.S., Coates, A.G., Berggren, W.A., Aubry, M.P., Zhang, J., 1996. The late Miocene Panama Isthmian strait. Geology 24, 687-690.

Coyne, J.A., Orr, H.A., 2004. Speciation. Sinauer Associates Inc., Sunderland, Ma.

Curini-Galletti, M., 1991. Monocelididae (Platyhelminthes, Proseriata) from Puerto Rico. I. Genera *Minona* and *Monocelis*. Proc. Biol. Soc. Wash. 104 (2), 229-240.

Curini-Galletti, M., 2014. Contribution to the knowledge of the Proseriata (Platyhelminthes: Rhabdithophora) from southeast Brazil. Mar. Biodiv. 44 (3), 287-312.

Curini-Galletti, M., Artois, T., Delogu, V., De Smet, W.H., Fontaneto, D., Jondelius, U., Leasi, F., Martínez, A., Meyer-Wachsmuth, I., Nilsson, K.S., Tongiorgi, P., Worsaae, K., Todaro A.M., 2012. Patterns of diversity in soft-bodied meiofauna: dispersal ability and body size matter. PloS One 7 (3), e33801.

Curini-Galletti, M., Casu, M., Lai, T., 2011. On the *Pseudomonocelis agilis* (Schultze, 1851) complex (Platyhelminthes: Proseriata), with description of two new species. Meiofauna Marina, 19, 49-59.

524 Curini-Galletti, M., Puccinelli I., Martens, P.M., 1989. Karyometrical analysis of
525 10 species of the subfamily Monocelidinae (Proseriata, Platyhelminthes)
526 with remarks on the karyologicai evolution of the Monocelididae. Genetica
527 78, 169-178.
528 Delogu, V., Curini-Galletti, M., 2009. The *Parotoplana jondelii* species-group
529 (Platyhelminthes: Proseriata): a microturbellarian radiation in the
530 Mediterranean. Contrib. Zool. 78 (3), 99-112.
531 Derycke, S., Remerie, T., Backeljau, T., Vierstraete, A., Vanfleteren, J., Vincx, M.,
532 Moens, T., 2008. Phylogeography of the *Rhabditis (Pellioditis) marina*
533 species complex: evidence for long-distance dispersal, and for range
534 expansions and restricted gene flow in the northeast Atlantic. Mol. Ecol. 17
535 (14), 3306-3322.
536 Drummond, A.J., Rambaut, A., 2007. BEAST: Bayesian evolutionary analysis by
537 sampling trees. BMC Evol. Biol. 7, 214.
538 Folmer, O., Black, M., Hoeh, W., Lutz, R., Vrijenhoek, R., 1994. DNA primers for
539 amplification of mitochondrial cytochrome c oxidase subunit I from diverse
540 metazoan invertebrates. Mol. Mar. Biol. Biotechnol. 3, 294-299.
541 Fontaneto, D., Kaya, M., Herniou, E.A., Barraclough, T.G., 2009. Extreme levels
542 of hidden diversity in microscopic animals (Rotifera) revealed by DNA
543 taxonomy. Mol. Phylogenet. Evol., 53 (1), 182-189.
544 Gelman, A., Rubin, D.B., 1992. Inference from iterative simulation using
545 multiple sequences. Stat. Sci. 7, 457-472.
546 Giere, O., 2009. Meiobenthology: The microscopic motile fauna of aquatic
547 sediments. Second ed. Springer Verlag, Berlin.
548 Heads, M., 2005a. Dating nodes on molecular phylogenies: a critique of
549 molecular biogeography. Cladistics 21, 62-78.

Heads, M., 2005b. Towards a panbiogeography of the seas. Biol. J. Linnean Soc. 84, 675-723.

Jackson, J.B.C., Jung, P., Coates, A.G., Collins, L.S., 1993. Diversity and extinction of tropical American mollusks and emergence of the Isthmus of Panama. Science 260, 1624-1626.

Jackson, J.B.C., Budd, A.F., 1996. Evolution and environment: introduction and overview, in: Jackson, J.B.C., Budd, A.F., Coates, A.G. (Eds.), Evolution and environment in tropical America. University of Chicago Press, Chicago, IL, pp. 1-20.

Jordan, D.S., 1908. The law of geminate species. Am. Nat. 17, 73-80.

Jörger, K.M., Schrödl, M., 2013. How to describe a cryptic species? Practical challenges of molecular taxonomy. Front. Zool. 10, 59.

Jörger, K.M., Norenburg, J.L., Wilson, N.G., Schrödl, M., 2012. Barcoding against a paradox? Combined molecular species delineations reveal multiple cryptic lineages in elusive meiofaunal sea slugs. Evol. Biol. 12, 245.

Katoh, K., Toh, H., 2008. Recent developments in the MAFFT multiple sequence alignment program. Brief. Bioinform. 9, 286-298.

Kennedy, A.D., Jacoby, C.A., 1999. Biological indicators of marine environmental health: meiofauna – a neglected benthic component? Environ. Monit. Assess. 54, 47-68.

Kimura, M., 1968. Evolutionary rate at the molecular level. Nature 217, 624-626.

Kimura, M., 1980. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J. Mol. Evol. 16, 111-120.

575 Knowlton, N., 1993. Sibling species in the sea. Annu. Rev. Ecol. Systemat. 24,
576 189-216.
577 Knowlton, N., Weigt, L.A., 1998. New dates and new rates for divergence across
578 the Isthmus of Panama. Proc. R. Soc. B-Biol. Sci. 265, 2257-63.
579 Laumer, C.E., Giribet, G., Curini-Galletti, M., 2014. *Prosogynopora riseri* gen. et
580 spec. nov., a phylogenetically problematic lithophoran proseriate
581 (Platyhelminthes: Rhabditophora) with inverted genital pores from the
582 New England coast. Invertebr. Syst. In press
583 Lepage, T., Bryant, D., Philippe, H., Lartillot, N., 2007. A General Comparison of
584 Relaxed Molecular Clock Models. Mol. Biol. Evol. 24, 1-12.
585 Lessios, H.A., 2008. The great American Schism: divergence of marine
586 organisms after the rise of the Central American Isthmus. Annu. Rev. Ecol.
587 Evol. Syst. 39, 63-91.
588 Li, W.S., Graur, D., 1991. Fundamentals of molecular evolution. Sinaeur,
589 Sunderland.
590 Littlewood, D.T.J., Curini-Galletti, M., Herniou, E.A., 2000. The interrelationships
591 of Proseriata (Platyhelminthes: Seriata) tested with molecules and
592 morphology. Mol. Phylogenet. Evol. 16, 449-466.
593 Littlewood, D.T.J., Olson, P.D., 2001. Small subunit rDNA and the
594 Platyhelminthes: Signal, noise, conflict and compromise, in: Littlewood,
595 D.T.J., Bray, R.A. (Eds.), Interrelationships of the Platyhelminthes. Taylor
596 and Francis Inc, New York, pp. 262-278.
597 Marcus, E., 1954a. Turbellaria Brasileiros (11). Papéis Avulsos Dep. Zool. Secr.
598 Agric. Sao Paulo 11, 419-489.
599 Marcus, E., 1954b. Turbellaria. Reports of the Lund University Expedition 1948-
600 49. Kungl. Fysiograf. Saellsk. Handl. NF 64, 1-115.

601 Marko, P.B., 2002. Fossil calibration of molecular clocks and the divergence
602 times of geminate species pairs separated by the Isthmus of Panama. Mol.
603 Biol. Evol. 19, 2005-2021.
604 Marko, P.B., Moran A.L., 2009. Out of sight, out of mind: high cryptic diversity
605 obscures the identities and histories of geminate species in the marine
606 bivalve subgenus *Acar*. J. Biogeogr. 36, 1861-1880.
607 Martens, P.M., 1984. Comparison of three different extraction methods for
608 Turbellaria. Mar. Ecol.-Prog. Ser. 14, 229-234.
609 Martens, P.M., Curini-Galletti, M., 1993. Taxonomy and Phylogeny of the
610 Archimonocelididae Meixner, 1938 (Platyhelminthes Proseriata). Bijdragen
611 Dierkunde 63, 65-102.
612 Meyer-Wachsmuth I., Curini-Galletti, M., Jondelius U., 2014. Hyper-Cryptic
613 Marine Meiofauna: Species Complexes in Nemertodermatida. PloS One 9
614 (9), e107688.
615 Miura, O., Torchin, M.E., Bermingham, E., 2010. Molecular phylogenetics
616 reveals differential divergence of coastal snails separated by the Isthmus
617 of Panama. Mol. Phylogenet. Evol. 56, 40-48.
618 Mulcahy, D.G., Noonan, B.P., Moss, T., Townsend, T.M., Reeder, T.W., Sites Jr,
619 J.W., Wiens, J.J., 2012. Estimating divergence dates and evaluating dating
620 methods using phylogenomic and mitochondrial data in squamate reptiles.
621 Mol. Phylogenet. Evol. 65, 974-991.
622 Palumbi, S.R., 1997. Molecular biogeography of the Pacific, in: Lessios, H.A.,
623 McIntyre, I.G., (Eds.), Proceedings of the 8th International Coral Reef
624 Symposium (Panama) Balboa: Smithsonian Tropical Research Institute 1,
625 91-96 (Also published in Coral Reefs 1997, 16, S47-S52).

626 Peterson, K.J., Lyons, J.B., Nowak, K.S., Takacs, C.M., Wargo, M.J., McPeek M.A.,
627 2004. Estimating metazoan divergence times with a molecular clock. Proc.
628 Natl. Acad. Sci. U. S. A. 101(17), 6536-6541.
629 Posada, D., 2008. jModelTest: Phylogenetic model averaging. Mol. Biol. Evol. 25,
630 1253-1256.
631 Rambaut, A., Drummond, A.J., 2009. Tracer Version 1.5.0.
632 http://beast.bio.ed.ac.uk.
633 Ree, R.H., Smith, S.A., 2008. Maximum Likelihood Inference of Geographic
634 Range Evolution by Dispersal, Local Extinction, and Cladogenesis. Syst.
635 Biol. 57 (1), 4-14.
636 Reise, K., 1988. Plathelminth diversity in littoral sediments of the island of Sylt
637 in the North Sea. Fortschr. Zool. 36, 469-480.
638 Remane, A., 1933. Verteilung und Organisation der bentonischen Mikrofauna
639 der Kieler Bucht. Helgol. Wiss. Meeresunters. (Abt. Kiel) 21, 161-221.
640 Ronquist, F., Klopfstein, S., Vilhelmsen, L., Schulmeister, S., Murray, D. L.,
641 Rasnitsyn, A. P., 2012a. A total-evidence approach to dating with fossils,
642 applied to the early radiation of the Hymenoptera. Syst. Biol. 61, 973-999.
643 Ronquist, F., Teslenko, M., van der Mark, P., Ayres, D.L., Darling, A., Höhna, S.,
644 Larget, B., Liu, L., Suchard, M.A., Huelsenbeck, J.P., 2012b. MrBayes 3.2:
645 efficient Bayesian phylogenetic inference and model choice across a large
646 model space. Syst. Biol. 61, 539-542.
647 Roopnarine, P.D., 2001. A history of diversification, extinction, and invasion in
648 tropical America as derived from species-level phylogenies of chionine
649 genera (Family Veneridae). J. Paleontol. 75, 644-657.
650 Sanna, D., Lai, T., Francalacci, P., Curini-Galletti, M., Casu, M., 2009. Population
651 structure of the *Monocelis lineata* (Proseriata, Monocelididae) species

652 complex assessed by phylogenetic analysis of the mitochondrial
653 cytochrome c oxidase subunit I (COI) gene. Genet. Mol. Biol. 32, 864-867.
654 Schockaert, E.R., Curini-Galletti, M., De Ridder, W., Volonterio, O., Artois, T.,
655 2009. A new family of lithophoran Proseriata (Platyhelminthes), with the
656 description of seven new species from the Indo-Pacific and South America,
657 and the proposal of three new genera. Zool. J. Linnean Soc. 155, 759-773.
658 Schockaert, E.R., Curini-Galletti, M., De Ridder, W., Artois, T., 2011. On the
659 Calviriidae Martens and Curini-Galletti, 1993 (Platyhelminthes, Proseriata),
660 with the description of three new species. Zootaxa 3034, 32-46.
661 Sepkoski, J.J. Jr, 1998. Rates of speciation in the fossil record. Philos. T. R. Soc.
662 B. 353 (1366), 315-326.
663 Sterrer, W., 1973. Plate tectonics as a mechanism for dispersal and speciation
664 in interstitial sand fauna. Neth. J. Sea Res. 7, 200-222.
665 Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., Kumar, S., 2011.
666 MEGA5: molecular evolutionary genetics analysis using maximum
667 likelihood, evolutionary distance, and maximum parsimony methods. Mol.
668 Biol. Evol. 28 (10), 2731-2739.
669 Tavaré, S., 1986. Some probabilistic and statistical problems in the analysis of
670 DNA sequences, in: Miura, R.M. (Eds.), Some mathematical questions in
671 biology - DNA sequence analysis. American Mathematical Society,
672 Providence, pp. 57-86.
673 Thomas, J.A., Welch, J.J., Woolfit, M., Bromham L., 2006. There is no universal
674 clock for invertebrates, but rate variation does not scale with body size.
675 Proc. Natl. Acad. Sci. U. S. A. 103 (19), 7366-7371.
676 Todaro, M.A., Fleeger, J.W., Hu, Y.P., Hrinchevich, A.W., Foltz, D.W., 1996. Are
677 meiofaunal species cosmopolitan? Morphological and molecular analysis of

Xenotrichula intermedia (Gastrotricha: Chaetonotida). Mar. Biol. 125 (4), 735-742.

Tulchinsky, A., Norenburg, J., Turbeville, J., 2012. Phylogeography of the marine interstitial nemertean *Ototyphlonemertes parmula* (Nemertea, Hoplonemertea) reveals cryptic diversity and high dispersal potential. Mar. Biol., 159 (3), 661-674.

Tyler, S., Schilling, S., Hooge, M., Bush, L.F., (comp.) 2006-2012. Turbellarian taxonomic database. Version 1.7 http://turbellaria.umaine.edu.

Vermeij, G.J., 1978. Biogeography and Adaptation. Harvard University Press, Cambridge, MA.

Vermeij, G.J., 2001. Community assembly in the sea: geologic history of the living shore biota, in: Bertness, M.D., Gaines, S.D., Hay, M.E. (Eds.), Marine Community Ecology. Sinauer Associates, Sunderland, Massachusetts, pp. 39-60.

Westheide, W., Schmidt, H., 2003. Cosmopoltan versus cryptic meiofaunal polychaete species: an approach to molecular taxonomy. Helgol. Mar. Res. 57 (1), 1-16.

Zuckerkandl, E., Pauling, L., 1965. Evolutionary divergence and convergence in proteins, in: Bryson, V., Vogel H.J. (Eds.), Evolving Genes and Proteins. Academic Press, New York, pp. 97-166.

Zwickl, D.J., 2006. Genetic algorithm approaches for the phylogenetic analysis of large biological sequence datasets under the maximum likelihood criterion. Ph.D. dissertation, The University of Texas at Austin. URL: www.bio.utexas.edu/faculty/ antisense/garli/Garli.html.

FIGURE AND TABLE LEGENDS

Figure 1. Sampling localities.

Map of the four trans-American and geminate species sampling localities: Bocas del Toro, Panama; Playa La Angosta - Colón, Panama; Naos Island - Panama City, Panama; Ilhabela/São Sebastião, Brazil.

Figure 2. Phylogenetic tree.

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 BI as posterior probability (PP). For the three test cases and the two geminate species pairs ML bootstrap values are also reported at each node.
GS1: geminate species 1 (*Minona gemella* - Atlantic coast + *Minona* cf *gemella* - Pacific coast). GS2: geminate species 2 (*Parotoplana* sp. nov. 1 - Atlantic coast + *Parotoplana* sp. nov. 2 - Pacific coast). 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. Node 3: Atlantic *Archimonocelis marci* and *Archimonocelis* sp. nov. 1 + Pacific *Archimonocelis* sp. nov. 2.

Figure 3. Timetree.

Tree obtained by the software Beast showed divergence time among taxa. Nodes indicated with A, B and C correspond to the nodes showed in Fig. 2. Values within brackets represent the median values of divergence time of the node. Only for the three test cases (nodes A, B and C) within brackets are

730 showed both median values and the range (underlined) of divergence time of
731 the node.
732
733
734 **Table 1.** List of species sampled and sequences used for this study. Accession
735 numbers refer to GenBank codes; accession numbers of new sequences are in
736 italic.
737
738 **Table 2.**
739 18S and 28S mutation rates for the two geminate species pairs. *K2P*: genetic
740 distance corrected according to the Kimura two-parameters model (Kimura,
741 1980) and standard error; *r*: mutation rates per million years.

742 **Table 1.**

Family	Species	Locality*	18S	28S D1-D6
	Archotoplana holotricha Ax, 1956	GenBank	**AJ243676**	**AJ270165**
	Xenotoplana acus Ax, Weidemann and Ehlers, 1978	GenBank	**AJ270155**	**AJ270181**
	Parotoplana ambrosolii Curini-Galletti and Delogu, 2014	GenBank	**KC971043**	**KC971066**
	Parotoplana ambrosolii Curini-Galletti and Delogu, 2014	GenBank	**KC971044**	**KC971067**
	Parotoplana ambrosolii Curini-Galletti and Delogu, 2014	GenBank	**KC971045**	**KC971068**
	Parotoplana tubifera Curini-Galletti and Delogu, 2014	GenBank	**KC971046**	**KC971069**
	Parotoplana tubifera Curini-Galletti and Delogu, 2014	GenBank	**KC971047**	**KC971070**
	Parotoplana tubifera Curini-Galletti and Delogu, 2014	GenBank	**KC971049**	**KC971072**
	Parotoplana tubifera Curini-Galletti and Delogu, 2014	GenBank	**KC971058**	**KC971081**
Otoplanidae	*Parotoplana impastatoi* Curini-Galletti and Delogu, 2014	GenBank	**KC971048**	**KC971071**
	Parotoplana impastatoi Curini-Galletti and Delogu, 2014	GenBank	**KC971050**	**KC971073**
	Parotoplana ambrosolii Curini-Galletti and Delogu, 2014	GenBank	**KC971056**	**KC971079**
	Parotoplana ambrosolii Curini-Galletti and Delogu, 2014	GenBank	**KC971057**	**KC971080**
	Parotoplana spathifera Delogu and Curini-Galletti, 2007	GenBank	**KC971053**	**KC971076**
	Parotoplana pythagorae Delogu and Curini-Galletti, 2007	GenBank	**KC971052**	**KC971075**
	Parotoplana renatae Ax, 1956	GenBank	**AJ012517**	**AJ270176**
	Parotoplana renatae Ax, 1956	GenBank	**KC971062**	**KC971085**
	Parotoplana multispinosa Ax, 1956	GenBank	**KC971061**	**KC971084**
	Parotoplana primitiva Ax, 1956	GenBank	**KC971060**	**KC971083**

	Parotoplana bicupa Sopott-Ehlers, 1976	GenBank	**KC971063**	**KC971086**
	Parotoplanella progermaria Ax, 1956	GenBank	**KC971059**	**KC971082**
	Parotoplana crassispina Delogu and Curini-Galletti, 2009	GenBank	**KC971051**	**KC971074**
	Parotoplana rosignana Lanfranchi and Melai, 2008	GenBank	**KC971054**	**KC971077**
	Parotoplana procerostyla Ax,1956	GenBank	**KC971055**	**KC971078**
	Monostichoplana filum (Meixner,1938)	GenBank	**AJ270158**	**AJ270173**
	Archotoplana holotricha Ax, 1956	Faro (Portugal)	***KJ682322***	***KJ682384***
	Kata evelinae Marcus, 1949	Ilhabela (Brazil)	***KJ682323***	***KJ682385***
	Kata evelinae Marcus, 1949	Ilhabela, (Brazil)	***KJ682324***	***KJ682386***
	Kata leroda Marcus, 1950	São Sebastião (Brazil)	***KJ682325***	***KJ682387***
	Kata leroda Marcus, 1950	São Sebastião, (Brazil)	***KJ682326***	***KJ682388***
	Kata sp. nov. 1	Naos Island (Panama)	***KJ682327***	***KJ682389***
	Kata sp. nov. 1	Naos Island (Panama)	***KJ682328***	***KJ682390***
	Kata sp. nov. 2	Naos Island (Panama)	***KJ682329***	***KJ682391***
	Parotoplana sp.nov. 1	Bocas del Toro (Panama)	***KJ682330***	***KJ682392***
Otoplanidae	*Parotoplana* sp.nov. 1	Bocas del Toro (Panama)	***KJ682331***	***KJ682393***
	Parotoplana sp.nov. 1	Bocas del Toro (Panama)	***KJ682332***	***KJ682394***
	Parotoplana sp. nov. 2	Naos Island (Panama)	***KJ682333***	***KJ682395***
	Parotoplana sp. nov. 2	Naos Island (Panama)	***KJ682334***	***KJ682396***
	Parotoplana sp. nov. 2	Naos Island (Panama)	***KJ682335***	***KJ682397***
	Parotoplana primitiva Ax, 1956	Roscoff (France)	***KJ682336***	***KJ682398***

Archimonocelidae	*Archimonocelis marci* Curini-Galletti, 2014	São Sebastião (Brazil)	***KJ682337***	***KJ682399***
	Archimonocelis marci Curini-Galletti, 2014	São Sebastião (Brazil)	***KJ682338***	***KJ682400***
	Archimonocelis sp. nov. 1	São Sebastião (Brazil)	***KJ682339***	***KJ682401***
	Archimonocelis sp. nov. 2	Naos Island (Panama)	***KJ682340***	***KJ682402***
	Archimonocelis sp. nov. 2	Naos Island (Panama)	***KJ682341***	***KJ682403***
	Archimonocelis sp. nov. 2	Naos Island (Panama)	***KJ682342***	***KJ682404***
Monocelididae	*Minona ileanae* Curini-Galletti, 1997	GenBank	**JN224905**	**JN224910**
	Monocelis longiceps (Duges, 1830)	GenBank	**KC971064**	**KC971087**
	Monocelis longistyla Martens and Curini-Galletti, 1987	GenBank	**KC971065**	**KC971088**
	Minona ileanae Curini-Galletti, 1997	Great Bitter Lake (Egypt)	***KJ682343***	***KJ682405***
	Minona sp. nov.	Playa La Angosta, Colón (Panama)	***KJ682344***	***KJ682406***
	Minona sp. nov.	Playa La Angosta, Colón (Panama)	***KJ682345***	***KJ682407***
	Minona cf *trigonopora* Ax, 1956	Palau (Sardinia, Italy)	***KJ682346***	***KJ682408***
	Minona gemella Ax and Sopott-Ehlers, 1985	Playa La Angosta, Colón (Panama)	***KJ682347***	***KJ682409***
	Minona gemella Ax and Sopott-Ehlers, 1985	Playa La Angosta, Colón (Panama)	***KJ682348***	***KJ682410***
	Minona gemella Ax and Sopott-Ehlers, 1985	Playa La Angosta, Colón (Panama)	***KJ682349***	***KJ682411***
	Minona cf *gemella* Ax and Sopott-Ehlers, 1985	Naos Island (Panama)	***KJ682350***	***KJ682412***
	Minona cf *gemella* Ax and Sopott-Ehlers, 1985	Naos Island (Panama)	***KJ682351***	***KJ682413***
	Minona cf *gemella* Ax and Sopott-Ehlers, 1985	Naos Island (Panama)	***KJ682352***	***KJ682414***
	Minona sp. nov.	Boa Vista Island (Cape Verde)	***KJ682353***	***KJ682415***
	Minona sp. nov.	Boa Vista Island (Cape Verde)	***KJ682354***	***KJ682416***

	Monocelis lineata OF Müller, 1774	Porto Pozzo (Sardinia, Italy)	***KJ682355***	***KJ682417***
	Monocelis lineata OF Müller, 1774	Charaki (Rhodes, Greece)	***KJ682356***	***KJ682418***
	Monocelis lineata OF Müller, 1774	Pilo (Sardinia, Italy)	***KJ682357***	***KJ682419***
	Monocelis lineata OF Müller, 1774	Colostrai (Sardinia, Italy)	***KJ682358***	***KJ682420***
	Minona sp. nov. 1	Faro (Portugal)	***KJ682359***	***KJ682421***
	Minona sp. nov. 1	Faro (Portugal)	***KJ682360***	***KJ682422***
	Minona sp. nov.	Lanzarote, Canary Island (Spain)	***KJ682361***	***KJ682423***
	Minona sp. nov.	Tenerife, Canary Island (Spain)	***KJ682362***	***KJ682424***
	Minona sp. nov.	Tenerife, Canary Island (Spain)	***KJ682363***	***KJ682425***
Monocelididae	*Duplominona* sp. nov.	Lanzarote, Canary Island (Spain)	***KJ682364***	***KJ682426***
	Duplominona sp. nov.	Faro (Portugal)	***KJ682365***	***KJ682427***
	Duplominona sp. nov.	Faro (Portugal)	***KJ682366***	***KJ682428***
	Duplominona brasiliensis Curini-Galletti, 2014	Ilhabela (Brazil)	***KJ682367***	***KJ682429***
	Duplominona sp. nov. 1	Naos Island (Panama)	***KJ682368***	***KJ682430***
	Duplominona sp. nov. 1	Naos Island (Panama)	***KJ682369***	***KJ682431***
	Duplominona sp. nov. 1	Naos Island (Panama)	***KJ682370***	***KJ682432***
	Duplominona tridens (Marcus, 1954)	São Sebastião (Brazil)	***KJ682371***	***KJ682433***
	Duplominona tridens (Marcus, 1954)	São Sebastião (Brazil)	***KJ682372***	***KJ682434***
	Duplominona sp. nov. 2	Naos Island (Panama)	***KJ682373***	***KJ682435***
	Duplominona sp. nov. 3	Naos Island (Panama)	***KJ682374***	***KJ682436***
	Duplominona sp. nov. 3	Naos Island (Panama)	***KJ682375***	***KJ682437***

	Duplominona sp. nov.	Roscoff (France)	***KJ682376***	***KJ682438***
	Duploperaclistus circocirrus Martens, 1983	Roscoff (France)	***KJ682377***	***KJ682439***
	Duploperaclistus circocirrus Martens, 1983	Roscoff (France)	***KJ682378***	***KJ682440***
	Duplominona sp. nov.	Blanes (Spain)	***KJ682379***	***KJ682441***
	Archilopsis spinosa (Jensen, 1878)	Roscoff (France)	***KJ682380***	***KJ682442***
	Archilopsis arenaria Martens, Curini-Galletti & Pucinelli, 1989	Roscoff (France)	***KJ682381***	***KJ682443***
Calviriidae	*Calviria solaris* Martens and Curini-Galletti, 1993	GenBank	**AJ270153**	**AJ270168**
Coelogynoporidae	*Coelogynopora tenuis* Meixner, 1938	Roscoff (France)	***KJ682382***	***KJ682444***
Unguiphora	*Polystyliphora novaehollandiae* Curini-Galletti, 1998	GenBank	**AJ270161**	**AJ270177**
	Nematoplana coelogynoporoides Meixner, 1938	Roscoff (France)	***KJ682383***	***KJ682445***

*For newly sequenced taxa only.

746 **Table 2.**

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

747

748

749

750

751

752

753

754

755

756

757

758

759

Figure 1


N
Bocas del Toro, Panama
Playa La Angosta - Colón, Panama
Naos Island - Panama City, Panama
Ilhabela/São Sebastião, Brazil
0 400
kilometers

Figure 2

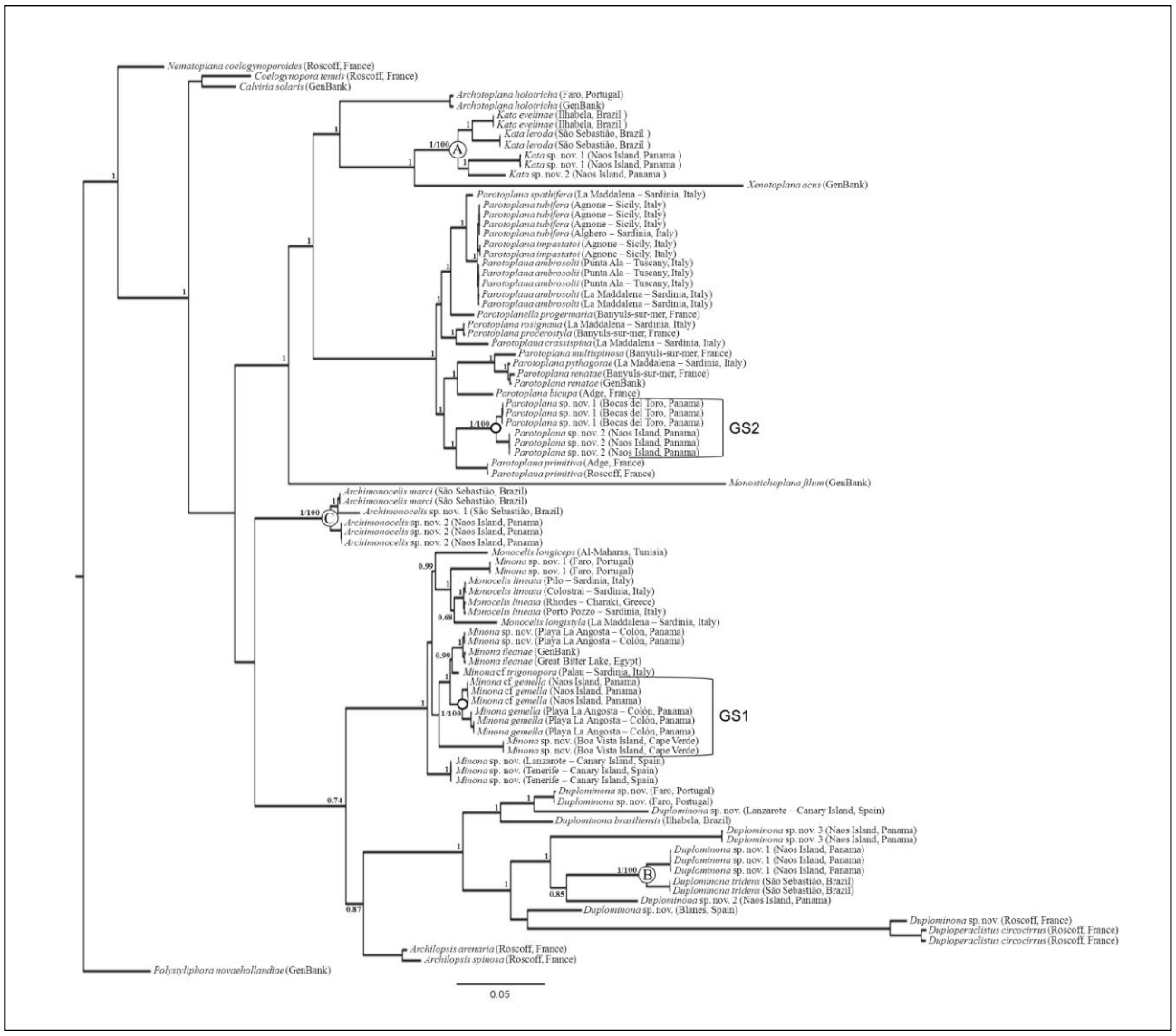
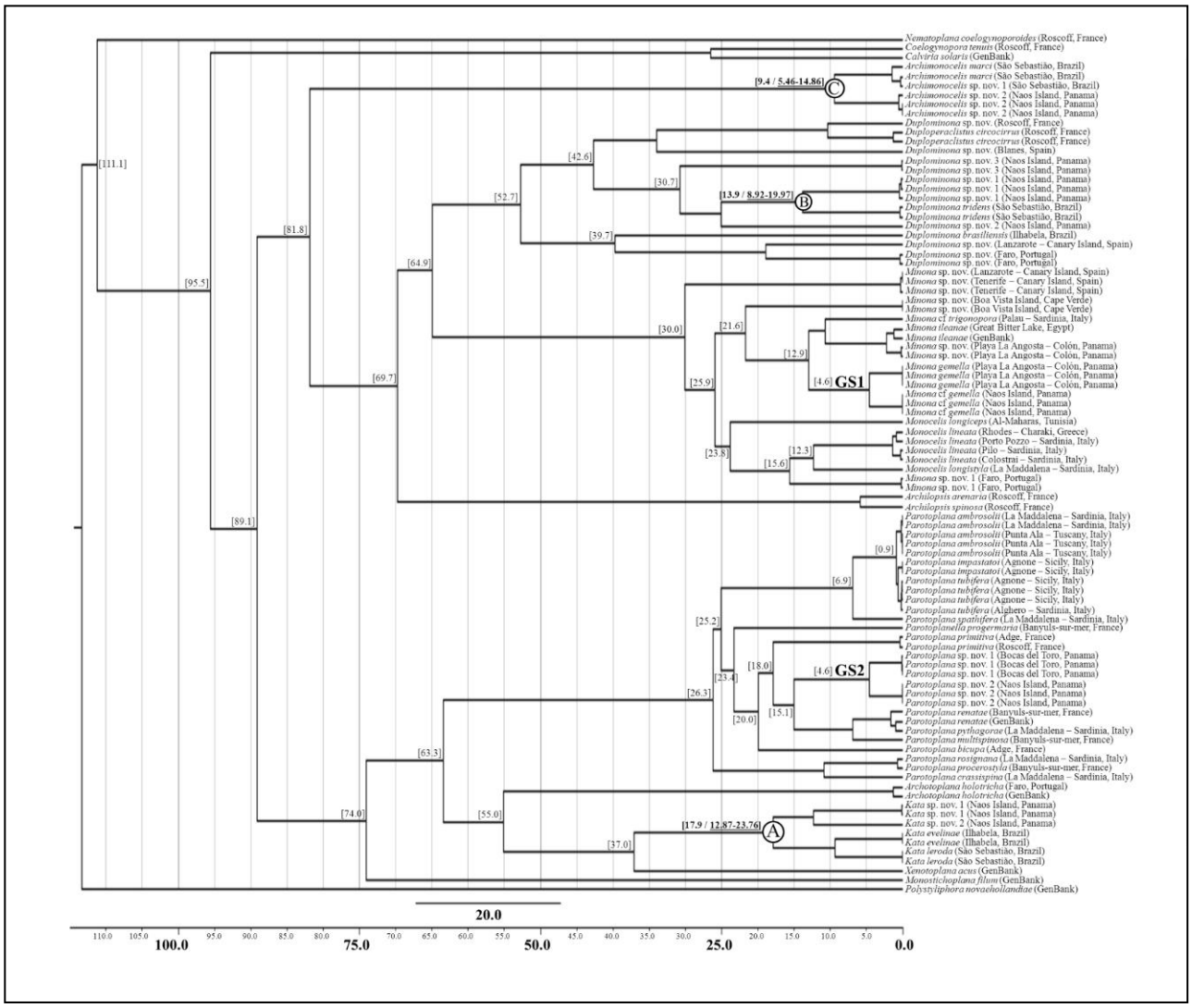

Nematoplana coelogynoporoides (Roscoff, France)
Coelogynopora tenuis (Roscoff, France)
Calviria solaris (GenBank)
Archotoplana holotricha (Faro, Portugal)
Archotoplana holotricha (GenBank)
Kata evelinae (Ilhabela, Brazil)
Kata evelinae (Ilhabela, Brazil)
Kata leroda (São Sebastião, Brazil)
Kata leroda (São Sebastião, Brazil)
Kata sp. nov. 1 (Naos Island, Panama)
Kata sp. nov. 1 (Naos Island, Panama)
Kata sp. nov. 2 (Naos Island, Panama)
Xenotoplana acus (GenBank)
Parotoplana spathifera (La Maddalena – Sardinia, Italy)
Parotoplana tubifera (Agnone – Sicily, Italy)
Parotoplana tubifera (Agnone – Sicily, Italy)
Parotoplana tubifera (Agnone – Sicily, Italy)
Parotoplana tubifera (Alghero – Sardinia, Italy)
Parotoplana impastatoi (Agnone – Sicily, Italy)
Parotoplana impastatoi (Agnone – Sicily, Italy)
Parotoplana ambrosoli (Punta Ala – Tuscany, Italy)
Parotoplana ambrosoli (Punta Ala – Tuscany, Italy)
Parotoplana ambrosoli (Punta Ala – Tuscany, Italy)
Parotoplana ambrosoli (La Maddalena – Sardinia, Italy)
Parotoplana ambrosoli (La Maddalena – Sardinia, Italy)
Parotoplanella progermaria (Banyuls-sur-mer, France)
Parotoplana rosignana (La Maddalena – Sardinia, Italy)
Parotoplana procerastyla (Banyuls-sur-mer, France)
Parotoplana crassispina (La Maddalena – Sardinia, Italy)
Parotoplana multispinosa (Banyuls-sur-mer, France)
Parotoplana pythagorae (La Maddalena – Sardinia, Italy)
Parotoplana renatae (Banyuls-sur-mer, France)
Parotoplana renatae (GenBank)
Parotoplana bicupa (Adge, France)
Parotoplana sp. nov. 1 (Bocas del Toro, Panama)
Parotoplana sp. nov. 1 (Bocas del Toro, Panama)
Parotoplana sp. nov. 1 (Bocas del Toro, Panama)
Parotoplana sp. nov. 2 (Naos Island, Panama)
Parotoplana sp. nov. 2 (Naos Island, Panama)
Parotoplana sp. nov. 2 (Naos Island, Panama)
GS2
Parotoplana primitiva (Adge, France)
Parotoplana primitiva (Roscoff, France)
Monostichoplana filum (GenBank)
Archimonocelis marci (São Sebastião, Brazil)
Archimonocelis marci (São Sebastião, Brazil)
Archimonocelis sp. nov. 1 (São Sebastião, Brazil)
Archimonocelis sp. nov. 2 (Naos Island, Panama)
Archimonocelis sp. nov. 2 (Naos Island, Panama)
Archimonocelis sp. nov. 2 (Naos Island, Panama)
Monocelis longicops (Al-Maharas, Tunisia)
Minona sp. nov. 1 (Faro, Portugal)
Minona sp. nov. 1 (Faro, Portugal)
Monocelis lineata (Pilo – Sardinia, Italy)
Monocelis lineata (Colostrai – Sardinia, Italy)
Monocelis lineata (Rhodes – Charaki, Greece)
Monocelis lineata (Porto Pozzo – Sardinia, Italy)
Monocelis longistyla (La Maddalena – Sardinia, Italy)
Minona sp. nov. (Playa La Angosta – Colón, Panama)
Minona sp. nov. (Playa La Angosta – Colón, Panama)
Minona tleanae (GenBank)
Minona tleanae (Great Bitter Lake, Egypt)
Minona cf. trigonopora (Palau – Sardinia, Italy)
Minona cf. gemella (Naos Island, Panama)
Minona cf. gemella (Naos Island, Panama)
Minona cf. gemella (Naos Island, Panama)
Minona gemella (Playa La Angosta – Colón, Panama)
Minona gemella (Playa La Angosta – Colón, Panama)
Minona gemella (Playa La Angosta – Colón, Panama)
Minona sp. nov. (Boa Vista Island, Cape Verde)
Minona sp. nov. (Boa Vista Island, Cape Verde)
GS1
Minona sp. nov. (Lanzarote – Canary Island, Spain)
Minona sp. nov. (Tenerife – Canary Island, Spain)
Minona sp. nov. (Tenerife – Canary Island, Spain)
Duplominona sp. nov. (Faro, Portugal)
Duplominona sp. nov. (Faro, Portugal)
Duplominona sp. nov. (Lanzarote – Canary Island, Spain)
Duplominona brasiliensis (Ilhabela, Brazil)
Duplominona sp. nov. 3 (Naos Island, Panama)
Duplominona sp. nov. 3 (Naos Island, Panama)
Duplominona sp. nov. 1 (Naos Island, Panama)
Duplominona sp. nov. 1 (Naos Island, Panama)
Duplominona sp. nov. 1 (Naos Island, Panama)
Duplominona tridens (São Sebastião, Brazil)
Duplominona tridens (São Sebastião, Brazil)
Duplominona sp. nov. 2 (Naos Island, Panama)
Duplominona sp. nov. (Blanes, Spain)
Duplominona sp. nov. (Roscoff, France)
Duploperaclistus circocirrus (Roscoff, France)
Duploperaclistus circocirrus (Roscoff, France)
Archilopsis arenaria (Roscoff, France)
Archilopsis spinosa (Roscoff, France)
Polystylphora novaehollandiae (GenBank)
0.05

Figure 3


Nematoplana coelogynoporoides (Roscoff, France)
Coelogynopora tenuis (Roscoff, France)
Calviria solaris (GenBank)
Archimonocelis marci (São Sebastião, Brazil)
Archimonocelis marci (São Sebastião, Brazil)
Archimonocelis sp. nov. 1 (São Sebastião, Brazil)
Archimonocelis sp. nov. 2 (Naos Island, Panama)
Archimonocelis sp. nov. 2 (Naos Island, Panama)
Duplominona sp. nov. (Roscoff, France)
Duploperaclistus citrocirrus (Roscoff, France)
Duploperaclistus citrocirrus (Roscoff, France)
Duplominona sp. nov. (Blanes, Spain)
Duplominona sp. nov. 3 (Naos Island, Panama)
Duplominona sp. nov. 3 (Naos Island, Panama)
Duplominona sp. nov. 1 (Naos Island, Panama)
Duplominona sp. nov. 1 (Naos Island, Panama)
Duplominona sp. nov. 1 (Naos Island, Panama)
Duplominona tridens (São Sebastião, Brazil)
Duplominona tridens (São Sebastião, Brazil)
Duplominona sp. nov. 2 (Naos Island, Panama)
Duplominona brasiliensis (Ilhabela, Brazil)
Duplominona sp. nov. (Lanzarote – Canary Island, Spain)
Duplominona sp. nov. (Faro, Portugal)
Duplominona sp. nov. (Faro, Portugal)
Minona sp. nov. (Lanzarote – Canary Island, Spain)
Minona sp. nov. (Tenerife – Canary Island, Spain)
Minona sp. nov. (Tenerife – Canary Island, Spain)
Minona sp. nov. (Boa Vista Island, Cape Verde)
Minona sp. nov. (Boa Vista Island, Cape Verde)
Minona cf. trigonopora (Palau – Sardinia, Italy)
Minona fleanae (Great Bitter Lake, Egypt)
Minona fleanae (GenBank)
Minona sp. nov. (Playa La Angosta – Colón, Panama)
Minona sp. nov. (Playa La Angosta – Colón, Panama)
Minona gemella (Playa La Angosta – Colón, Panama)
Minona gemella (Playa La Angosta – Colón, Panama)
Minona gemella (Playa La Angosta – Colón, Panama)
Minona cf. gemella (Naos Island, Panama)
Minona cf. gemella (Naos Island, Panama)
Minona cf. gemella (Naos Island, Panama)
Monocelis longiceps (Al-Maharas, Tunisia)
Monocelis lineata (Rhodes – Charaki, Greece)
Monocelis lineata (Porto Pozzo – Sardinia, Italy)
Monocelis lineata (Pilo – Sardinia, Italy)
Monocelis lineata (Colostrai – Sardinia, Italy)
Monocelis longisyla (La Maddalena – Sardinia, Italy)
Minona sp. nov. 1 (Faro, Portugal)
Minona sp. nov. 1 (Faro, Portugal)
Archilopsis arenaria (Roscoff, France)
Archilopsis spinosa (Roscoff, France)
Parotoplana ambrosolii (La Maddalena – Sardinia, Italy)
Parotoplana ambrosolii (La Maddalena – Sardinia, Italy)
Parotoplana ambrosolii (Punta Ala – Tuscany, Italy)
Parotoplana ambrosolii (Punta Ala – Tuscany, Italy)
Parotoplana ambrosolii (Punta Ala – Tuscany, Italy)
Parotoplana impastatoi (Agnone – Sicily, Italy)
Parotoplana impastatoi (Agnone – Sicily, Italy)
Parotoplana tubifera (Agnone – Sicily, Italy)
Parotoplana tubifera (Agnone – Sicily, Italy)
Parotoplana tubifera (Agnone – Sicily, Italy)
Parotoplana tubifera (Alghero – Sardinia, Italy)
Parotoplana spathifera (La Maddalena – Sardinia, Italy)
Parotoplanella progermaria (Banyuls-sur-mer, France)
Parotoplana primitiva (Adge, France)
Parotoplana primitiva (Roscoff, France)
Parotoplana sp. nov. 1 (Bocas del Toro, Panama)
Parotoplana sp. nov. 1 (Bocas del Toro, Panama)
Parotoplana sp. nov. 1 (Bocas del Toro, Panama)
Parotoplana sp. nov. 2 (Naos Island, Panama)
Parotoplana sp. nov. 2 (Naos Island, Panama)
Parotoplana sp. nov. 2 (Naos Island, Panama)
Parotoplana renatae (Banyuls-sur-mer, France)
Parotoplana renatae (GenBank)
Parotoplana pythagorae (La Maddalena – Sardinia, Italy)
Parotoplana multispinosa (Banyuls-sur-mer, France)
Parotoplana bicupa (Adge, France)
Parotoplana rosignana (La Maddalena – Sardinia, Italy)
Parotoplana procerostyla (Banyuls-sur-mer, France)
Parotoplana crassispina (La Maddalena – Sardinia, Italy)
Archotoplana holotricha (Faro, Portugal)
Archotoplana holotricha (GenBank)
Kata sp. nov. 1 (Naos Island, Panama)
Kata sp. nov. 1 (Naos Island, Panama)
Kata sp. nov. 2 (Naos Island, Panama)
Kata eveltnae (Ilhabela, Brazil)
Kata eveltnae (Ilhabela, Brazil)
Kata ieroda (São Sebastião, Brazil)
Kata ieroda (São Sebastião, Brazil)
Xenotoplana aeus (GenBank)
Monostichoplana filum (GenBank)
Polystyliphora novaehollandiae (GenBank)
[111.1]
[95.5]
[81.8]
[89.1]
[74.0]
[63.3]
[55.0]
[37.0]
[52.7]
[42.6]
[30.7]
[39.7]
[64.9]
[69.7]
[30.0]
[25.9]
[23.8]
[21.6]
[12.9]
[4.6] GS1
[12.3]
[15.6]
[9.4 / 5.46-14.86] C
[13.9 / 8.92-19.97] B
[17.9 / 12.87-23.76] A
[0.9]
[6.9]
[25.2]
[23.4]
[26.3]
[18.0]
[4.6] GS2
[15.1]
[20.0]
20.0
110.0 105.0 100.0 95.0 90.0 85.0 80.0 75.0 70.0 65.0 60.0 55.0 50.0 45.0 40.0 35.0 30.0 25.0 20.0 15.0 10.0 5.0 0.0