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

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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.

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., Derycke 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

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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., Knowlton and Weigt, 1998; Lessios, 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 Weigt, 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 (Casu et al., 2014; Curini-Galletti et al., 2011; Delogu and Curini-Galletti, 2009). Furthermore, the existence of many supraspecific 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. *M. 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,

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 italic.

Family	Species	Locality[a]	18S	28S D1–D6
Otoplanidae	*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
	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*
	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*
Archimonocelididae	*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*
	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*

Table 1 *(continued)*

Family	Species	Locality[a]	18S	28S D1–D6
Monocelididae	*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***

[a] For newly sequenced taxa only.

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 Coelogynoporidae (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 transisthmian geminate species pairs, by means of the software Mega 6.06 (Tamura et al., 2011) with 1000 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 1000 bootstraps (bootstrapreps = 1000). Consensus tree was computed using TreeAnnotator 1.7.4 (Drummond and Rambaut, 2007) and visualized 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 1000 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/).

Fig. 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.

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 (*M. gemella* + *M.* 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

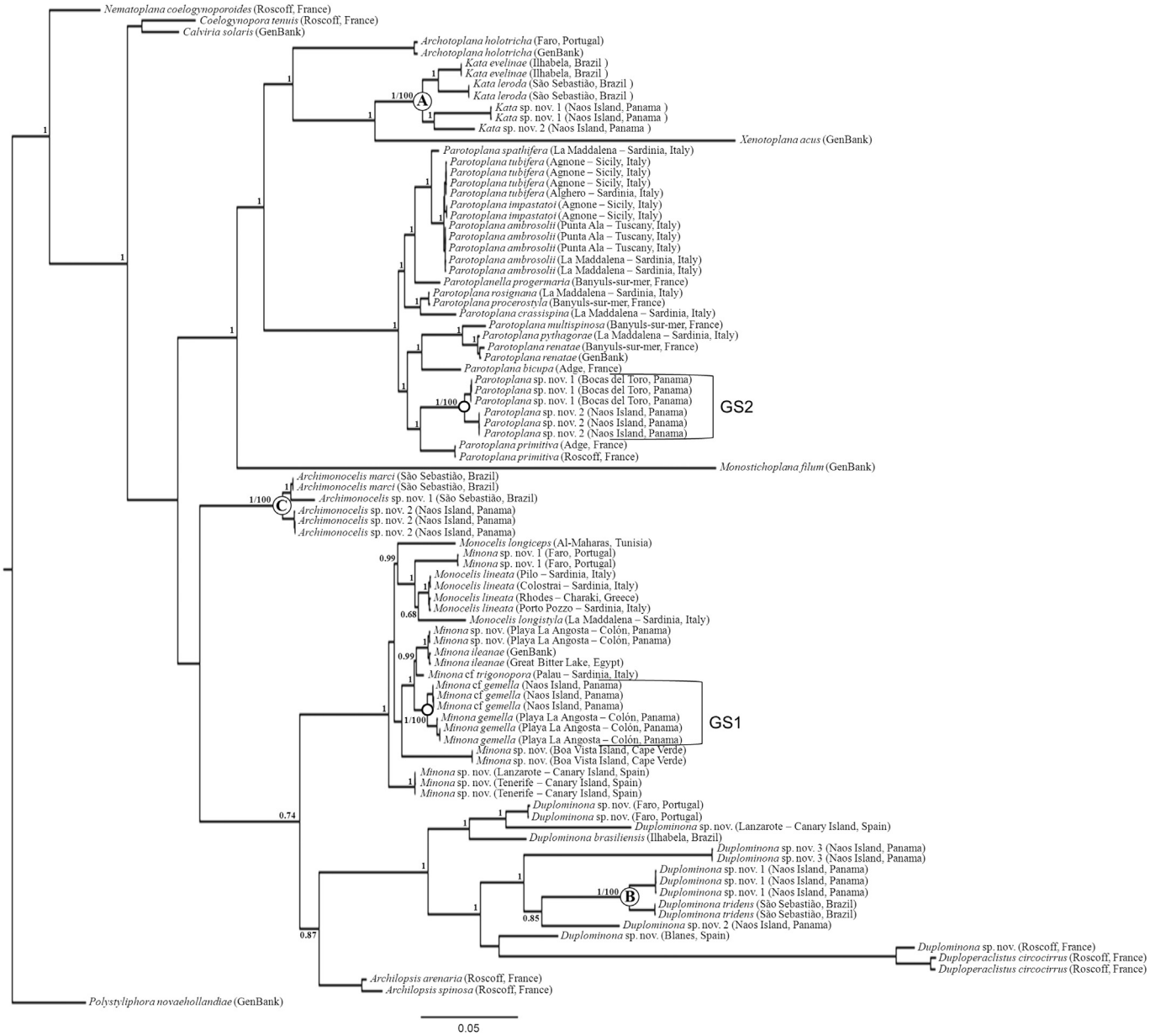

Fig. 2. Phylogenetic tree. Tree obtained by BI showing the interrelationships of the species based on combined 18S + 28S D1–D6. The branch length scale refers to the number of substitutions per site. Nodal supports are indicated for BI as posterior probability (PP). For the three test cases and the two 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.

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 normal distribution with lower, central 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 with the accession number TB2: S16597.

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 *M. gemella* + *Minona* cf *gemella* and *Parotoplana* sp. nov. 1 + *Parotoplana* sp. nov. 2: $K2P = 0.0085 \pm 0.0022$ and $K2P = 0.0115 \pm 0.0027$, for the 18S; and $K2P = 0.0345 \pm 0.0045$ and $K2P = 0.0361 \pm 0.0049$, 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).

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* *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

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 is 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 of the sampling campaign can be assessed only after morphological and molecular analyses in the laboratory (see e.g., Casu et al., 2014). In this context, it is noteworthy that an inadequate taxonomic coverage may lead to the use of false geminate species for the calibration of the molecular clock, and thus to the use of species pairs separated well before the last closure of the isthmus which results in an overestimation of

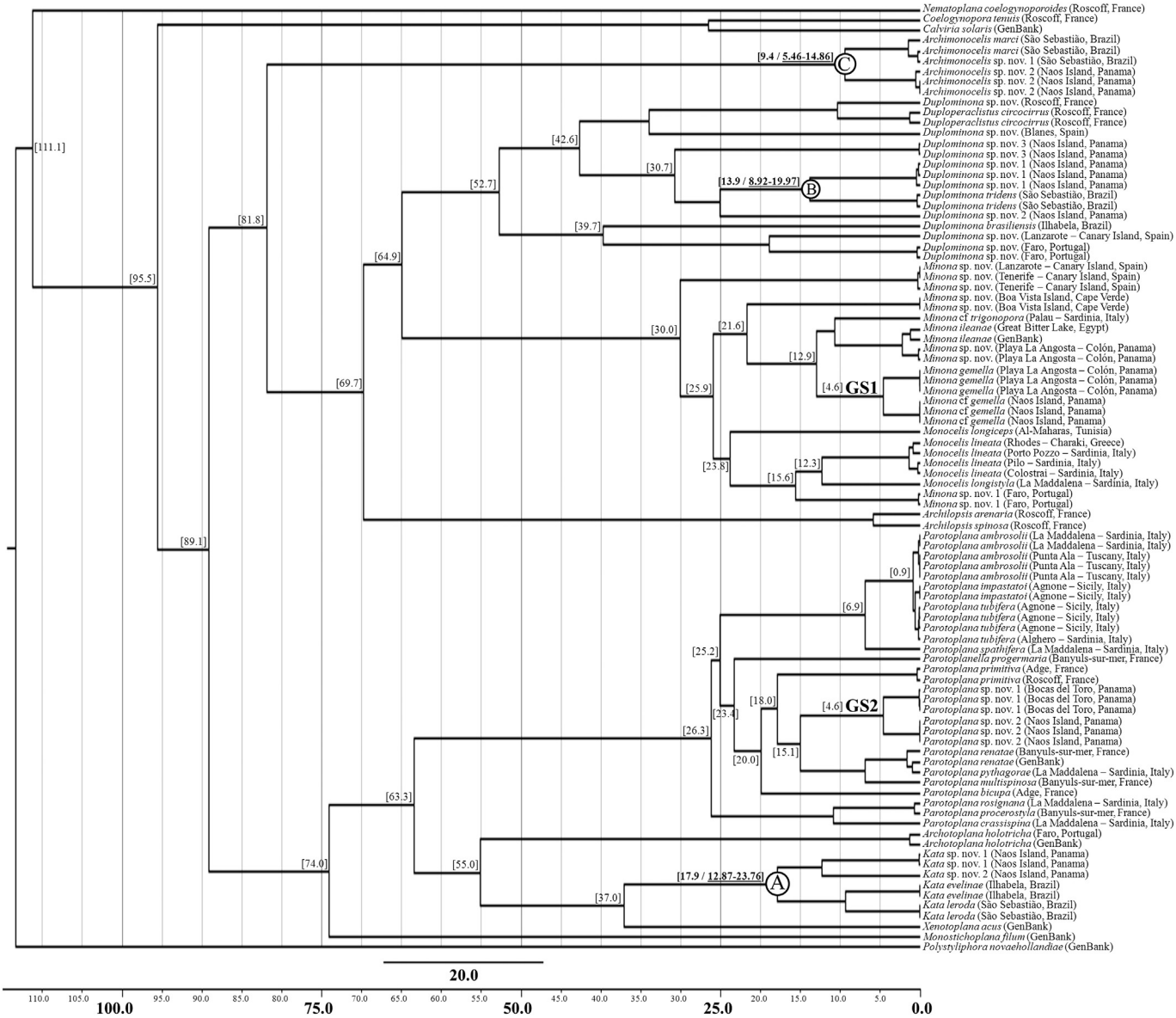

Fig. 3. Timetree. Tree obtained by the software Beast showed divergence time among taxa. Nodes indicated with A, B and C correspond to the nodes showed in Fig. 2. Values within brackets represent the median values of divergence time of the node. Only for the three test cases (nodes A, B and C) within brackets are showed both median values and the range (underlined) of divergence time of the node.

the mutation rates per million years (Heads, 2005a; Knowlton and Weigt, 1998). Consequently, the use of a higher rate may cause an underestimation of the divergence time among groups in the timetree.

Albeit it might be questionable whether our taxonomic coverage is extensive enough to assess sister species relationships reliably, the Atlantic *M. gemella* and *Parotoplana* sp. nov. 1, and their Pacific counterparts (*Minona* cf *gemella* and *Parotoplana* sp. nov. 2, respectively) are reciprocally monophyletic and morphologically indistinguishable at the routine level of morphological observation, and are thus highly suggestive of geminate lineages. Furthermore, the two pairs show very similar values of mutation rate per million years in both genes. It is noteworthy that these similar values have been found in species pairs belonging to two different families (Monocelididae and Otoplanidae), and may thus prove applicable across the Proseriata. Finally, in the three trans-American species used as test cases (*Kata* spp., *Duplominona* spp. and *Archimonocelis* spp.), the obtained divergence times are greater than the final closure of the Isthmus of Panama – ranging from 9.4 Myr (time of divergence between *Archimonocelis* spp.) and about 17.9 Myr (time of divergence between *Kata* spp.) – and therefore not conflicting with the values 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 et al., 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., 2006-2013). 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 the final stage of the isthmian formation. A similar suggestion was advanced for species from brackish-water and mangrove habitats (see Miura et al., 2010).

Although further tests on a larger dataset and on other test-cases are deemed necessary, data obtained (both mutation rates and divergence times) might prove invaluable to provide further insights into the phylogenetic relationships and evolution of Proseriata.

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