

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

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1 **A 18S and 28S-based clock calibration for marine Proseriata**  
2 **(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.

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



## 52 **1. Introduction**

53 Interstitial meiofauna is among the most diverse and species-rich components  
54 of marine biodiversity (Kennedy and Jacoby, 1999). Knowledge of many aspects  
55 of the biology of these minute organisms is however limited, even in  
56 comparatively well-studied areas (Curini-Galletti et al., 2012), and patterns of  
57 spatial distribution are particularly poorly understood. Early studies pointed to  
58 the existence of large, amphi-Atlantic or even cosmopolitan, distributions in  
59 meiofaunal taxa (Sterrer, 1973; Westheide and Schmidt, 2003). Such large-  
60 scale ranges in species lacking any obvious means of dispersal is at the basis  
61 of the so-called 'meiofauna paradox' (Giere, 2009). Ancient vicariance events,  
62 followed by evolutionary stasis, were hypothesized to be responsible of the  
63 observed patterns (Sterrer, 1973); this, however, would imply a surprisingly old  
64 phylogenetic age for these species (see Sepkoski, 1998). Although recent  
65 molecular surveys showed that, in many instances, these vast distributions are  
66 the result of the lack of resolution of cryptic species complexes (i.a., Casu et al.,  
67 2009; Fontaneto et al., 2009; Jörger and Schrödl, 2013; Todaro et al., 1996), at  
68 least some cases of large-scale distribution of meiofaunal taxa have been  
69 supported by molecular data (see e.g., Dericke et al., 2008; Jörger et al., 2012;  
70 Meyer-Wachsmuth et al., 2014; Tulchinsky et al., 2012), leaving open the  
71 choice between great antiquity of lineages, or unsuspected capabilities for  
72 dispersal.

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

78 evolutionary divergence is based on the molecular clock hypothesis (MCH)  
79 (Zuckerlandl and Pauling, 1965), which assumes a relatively constant rate of  
80 molecular evolution over time and across taxa (see Kimura, 1968, and  
81 references therein). However, recent empirical studies have demonstrated the  
82 existence of a significant variation in the rate of molecular evolution  
83 (Bromham and Penny, 2003; Thomas et al., 2006), and the use of a more  
84 sophisticated approach, such as a relaxed clock model, has been  
85 recommended (see Lepage et al., 2007 and references therein). In order to  
86 assign concrete dating, a molecular clock needs to be calibrated against  
87 independent evidence (Benton and Donoghue, 2007). The most common  
88 calibration of the molecular clock is achieved by using fossil records (e.g.,  
89 Blanton et al., 2013; Mulcahy et al., 2012; Ronquist et al., 2012a) or, when  
90 possible, by means of both fossil records and paleogeographic events (e.g.,  
91 Heads, 2005a).

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

104 to fossil records as calibration points for a molecular clock, and can be used in  
105 turn to estimate divergence times between related species (e.g., Lessios, 1998;  
106 Knowlton and Weigt, 1998; Marko and Moran, 2009). For this purpose, one of  
107 the most used geographical barriers is the Isthmus of Panama (see, e.g., Heads,  
108 2005b). Geological literature suggests that complete isolation between the  
109 Caribbean Sea and the Pacific Ocean occurred about 3.1-3.5 million years (Myr)  
110 ago, due to the final emergence of the Isthmus (Allmon, 2001; Collins et al.,  
111 1996; Jackson and Budd, 1996; Knowlton and Weig, 1998).

112 This approach is however not without criticisms (see Heads, 2005b). A major  
113 objection is that identification of a species pair as geminate species may be  
114 biased by the taxonomic sampling available (Lessios, 2008). Furthermore,  
115 establishing the time of separation between geminate species may be fraught  
116 with problems, as the emergence of the isthmian landmass was a prolonged  
117 geological process, and not all geminate pairs were simultaneously separated  
118 by the emerging Isthmus (Knowlton, 1993; Knowlton and Weigt, 1998; Marko,  
119 2002). Populations of intertidal meiofauna may be ideal candidates for the  
120 calibration of the molecular clock, as they were more likely to maintain  
121 continuity of habitat and gene flow across the emerging isthmus, until the  
122 separation of eastern Pacific and Caribbean was completed. However, they  
123 have never been studied in this context.

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

130 the reduced mobility of adults, their potential for dispersal is limited (Curini-  
131 Galletti et al., 2012). Indeed, setting the molecular clock for taxa belonging to  
132 the order of Proseriata, would be of particular interest, as support for  
133 phylogeographic studies (Casu et al., 2011) or setting up the evolutionary time-  
134 frame in cases of allopatric distributions (Delogu and Curini-Galletti, 2009; Casu  
135 et al., 2014; Curini-Galletti et al., 2011). Furthermore, the existence of many  
136 supra-specific taxa with anti-tropical distributions (Laumer et al., 2014) could  
137 be ideally interpreted with information on the timing of their divergence.  
138 Notwithstanding the current, different ecological conditions at the two sides of  
139 the isthmus (Lessios, 2008), morphologically similar congeneric species were  
140 found, which could be tested as potential geminate species. We sequenced  
141 further morphologically similar congeners, allopatrically distributed along the  
142 Pacific shores of Panama and in western Atlantic (South Brazil). These latter,  
143 trans-American species were used as potential test cases, as their age of  
144 divergence should pre-date that of trans-isthmian geminates.  
145 We calibrated the molecular clock on two ribosomal genes, the complete  
146 nuclear small subunit rDNA (18S) gene and the partial nuclear large subunit  
147 rDNA (28S) gene fragment (spanning variable domains D1-D6), since their  
148 sequences constitute the only large database available for Proseriata.

149

## 150 **2. Materials and methods**

151

### 152 *2.1. The species*

#### 153 *2.1.1. Trans-isthmian species pairs*

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

156 Species found at the opposite ends of the Canal (Table 1; Fig. 1), in intertidal  
157 habitats. *Minona gemella* (type locality: Bermuda) is characterized by the  
158 presence of two accessory prostatoid organs, one anterior and one posterior to  
159 the copulatory organ - a unique feature for species of the genus *Minona* Marcus  
160 1946 (Ax and Sopott-Ehlers, 1985). The Pacific counterpart appears identical in  
161 morphology, as reconstructed from observations on living, semi-squeezed  
162 specimens, and from histological sections, as well as for all measurable  
163 characters of the sclerotized structures. The only appreciable differences have  
164 been detected in their karyotypes: Caribbean specimens from Panama and  
165 Puerto Rico have chromosome II metacentric, while it is more heterobrachial in  
166 Pacific specimens (Curini-Galletti, 1991; unpubl. data).

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

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

173

#### 174 *2.1.2 Trans-American species*

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

176 The four described species of the genus *Kata* are distributed on both American  
177 coasts: *Kata evelinae* Marcus, 1949 and *Kata leroda* Marcus, 1950 from South  
178 Brazil (both of which were here sequenced); *Kata galapagoensis* Ax and Ax,  
179 1974 from Galapagos Island; and *Kata galea* Ax and Sopott-Ehlers, 1987 from  
180 Bermuda. The two new species from the Pacific coast of Panama (*Kata* sp. nov.  
181 1 and *Kata* sp. nov. 2) differ from each other and the other species of the  
182 genus for details of the morphology of the sclerotized pieces of the copulatory  
183 organ (unpubl. data). All species occur intertidally.

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

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

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

192 The American species here sequenced, *Archimonocelis marci* Curini-Galletti,  
193 2014 and *Archimonocelis* sp. nov. 1 from Brazil, and *Archimonocelis* sp. nov. 2,  
194 from Pacific Coast of Panama are morphologically similar, as they share a  
195 simple structure of the copulatory organ, with a stylet surrounded by a girdle of  
196 short, nearly identical spines (Curini-Galletti, 2014; unpubl data). All species  
197 occur in shallow subtidal habitats.

198

## 199 2.2. Sampling, DNA extraction, amplification and sequencing

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

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

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

211 Genomic DNA was extracted using the Macherey-Nagel NucleoSpin Tissue  
212 (Macherey-Nagel GmbH and Co. KG) according to the supplier's instructions.  
213 After extraction, DNA was stored as a solution at 4 °C. Complete 18S and  
214 partial 28S (D1-D6) sequences were analyzed for a total of 92 individuals; 62  
215 were newly obtained specifically for this study, and 30 taken from GenBank (for  
216 details see Table 1). The dataset was built with 40 sequences of individuals  
217 belonging to the family Otoplanidae (15 of which newly sequenced, Table 1),  
218 42 to the family Monocelididae (39 of which newly sequenced, Table 1), 6 to  
219 the family Archimonocelididae (all of which newly sequenced, Table 1), one to  
220 the family Calviridae (from GenBank), one to the family Coeloginoporidae  
221 (newly sequenced), and two to the suborder Unguiphora (one of which newly  
222 sequenced). Amplifications for 18S and 28S D1-D6 regions were carried out  
223 using the following primers: 18S: A (forward) GCG AAT GGC TCA TTA AAT CAG,  
224 and B (reverse) CTT GTT ACG ACT TTT ACT TCC (Littlewood and Olson, 2001);  
225 28S: LSU5 (forward) TAG GTC GAC CCG CTG AAY TTA AGC A, and LSUD6-3  
226 (reverse) GGA ACC CTT CTC CAC TTC AGT C (Littlewood et al., 2000).

227 PCRs were carried out in a total volume of 25  $\mu$ l containing about 25 ng (5 ng/ $\mu$ l)  
228 of total genomic DNA on average, 1.0 U of Taq DNA Polymerase (EuroTaq by  
229 Euroclone), 1 $\times$  reaction buffer, 3.5 mM of MgCl<sub>2</sub>, 0.32  $\mu$ M of each primer, and  
230 200  $\mu$ M of each dNTP. PCR amplifications were performed in a MJ PTC 200  
231 Thermal Cycler (Biorad) programmed as follows: 1 cycle of 2 min at 94° C, 35  
232 cycles of 1 min at 94° C, 1 min at 54° C (18S / 28S primers' annealing  
233 temperature), and 1 min and 30 s at 72° C. At the end, a post-treatment for 5  
234 min at 72° C and a final cooling at 4° C were carried out. Both positive and  
235 negative controls were used to test the effectiveness of the PCR reagents, and  
236 the absence of possible contaminations. Electrophoreses were carried out on  
237 2% agarose gels, prepared using 1 $\times$  SBA buffer (sodium boric acid, pH 8.2) and  
238 stained with a 1  $\mu$ l/20 ml ethidium bromide solution. PCR products were  
239 purified by ExoSAP-IT (USB Corporation) and sequenced for both forward and  
240 reverse 18S and 28S strands, using an external sequencing core service  
241 (Macrogen Inc., Europe). The sequencing runs were repeated twice in order to  
242 verify the reliability of results.

243

### 244 *2.3. Estimates of genetic distance and phylogenetic analysis*

245 The 18S and 28S sequences were aligned separately using the algorithm Q-  
246 INS-I, implemented in Mafft 6.903 (Kato and Toh, 2008), which is appropriate  
247 for non-coding RNA as it considers RNA secondary structure. The best  
248 probabilistic model of sequence evolution was determined after evaluation by  
249 jModeltest 2.1.1 (Posada, 2008), with a maximum likelihood optimized search,  
250 using the Akaike Information Criterion (AIC) and the Bayesian Information  
251 Criterion (BIC). Both criterions selected the GTR+I+G (Tavaré, 1986) as the  
252 best fitting model for both 18S and 28S datasets. The pairwise genetic

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

259 Phylogenetic relationships among individuals and species were investigated  
260 using both Maximum Likelihood (ML) and Bayesian Inference (BI) on the  
261 combined 18S and 28S sequences. We set as outgroup for the analyses the  
262 species *Polystyliphora novaehollandiae* Curini-Galletti, 1998. ML was performed  
263 using the genetic algorithm implemented in Garli 2.01 (Zwickl, 2006). In order  
264 to find the best tree, the configuration file for partitioned models was set up to  
265 perform 10 replicate searches (searchreps = 10). Model parameters:  
266 ratematrix = (0 1 2 3 4 5), statefrequencies = estimated, ratehetmodel =  
267 gamma, numratecats = 4, corresponding to the evolution model calculated by  
268 JModeltest, were used. In order to allow independent estimates of the  
269 parameters for each gene, the option link was set to 0. The parameter  
270 modweight was set to 0.0015, as we have two partitions. Finally, node support  
271 was assessed by 1,000 bootstraps (bootstrapreps = 1000). Consensus tree was  
272 computed using TreeAnnotator 1.7.4 (Drummond and Rambaut, 2007) and  
273 visualised by FigTree 1.4.0 (<http://tree.bio.ed.ac.uk/software/figtree/>).

274 BI was carried out using the software MrBayes 3.2.2 (Ronquist et al., 2012b),  
275 specifying a partitioned model and setting as model parameters: NST = 6,  
276 rates = invgamma, ngammacat = 4. We allowed each partition to have its own  
277 set of parameters and a potentially different overall evolutionary rate. Two  
278 independent runs, each consisting of four Metropolis-coupled MCMC chains

279 (one cold and three heated chains), were run simultaneously for 5,000,000  
280 generations, sampling trees every 1,000 generations. The first 25% of the  
281 10,000 sampled trees was discarded as burnin.

282 In order to assess the convergence of chains we checked that the Average  
283 Standard Deviation of Split Frequencies (ASDSF), approached 0 (Ronquist et al.,  
284 2012b), and the Potential Scale Reduction Factor (PSRF) was around 1 (Gelman  
285 and Rubin, 1992). Nodes with a percentage of posterior probability lower than  
286 95% are considered not highly supported. Phylogenetic tree was visualized  
287 using FigTree 1.4.0 (<http://tree.bio.ed.ac.uk/software/figtree/>).

288

## 289 *2.4. Calibration of molecular clock*

### 290 *2.4.1. Estimation of the mutation rates per million years*

291 After phylogenetic analysis showed that the trans-isthmian species (*Minona*  
292 *gemella* + *Minona cf gemella* and *Parotoplana* sp. nov. 1 + *Parotoplana* sp. nov.  
293 2) represent two pairs of sister species, and may thus be considered as  
294 geminate species (see section 3.1 below and Fig. 2), the mutation rates per  
295 million years ( $r$ ) between species from both sides of the isthmus were  
296 estimated for each gene. We used the formula  $r = K (K2P \text{ genetic distance}) / 2T$   
297 (time of divergence multiplied by 2 to account for the age of each lineage) (Li  
298 and Graur, 1991). The obtained mutation rates per million years (two for each  
299 species pair) were used for calibrating the timetree, in order to estimate the  
300 divergence time throughout the whole dataset.

301

### 302 *2.4.2. Estimation of divergence time*

303 The software package Beast 1.7.4 (Drummond and Rambaut, 2007) was used  
304 to estimate the divergence time for all of the clades evidenced by the

305 phylogenetic tree. Site parameters (Substitution Model = GTR; Bases  
306 Frequencies = Estimated; Site Heterogeneity Model = Gamma + Invariant Sites;  
307 Number of Gamma Categories = 4) have been set according to the best-fitting  
308 evolution model selected by jModeltest. For the molecular clock rate variation  
309 model, the lognormal uncorrelated relaxed clock was chosen because it  
310 assumes independent rates on different branches. Moreover, the use of the  
311 lognormal uncorrelated relaxed clock model gives an indication of how clock-  
312 like data is (measured by the *uclid.stdev* parameter). If the *uclid.stdev*  
313 parameter estimate is close to 0, then the data is quite clock-like, while if it has  
314 an estimated value much greater than 1, then data exhibits very substantial  
315 rate heterogeneity among lineages. For the tree prior the Yule prior process to  
316 the speciation model was applied. The priors for model parameters and  
317 statistics have been set for calibrating the timetree assuming the mutation  
318 rates per million years estimated separately for each of the two regions (18S  
319 and 28S) on the *Minona* and *Parotoplana* species pairs. Divergence times were  
320 estimated using a uniform distribution with lower and upper values set  
321 according to the mutation rate per million years of the two species pairs (see  
322 Table 2). Operator parameters have been set following the instructions on the  
323 user manual. In order to obtain the Effective Sample Size (ESS) greater than  
324 200 for all of the statistic parameters, a run of 400,000,000 generations was  
325 performed, sampling a tree every 40,000 generations.

326 We used Tracer 1.6 (Rambaut and Drummond, 2009) for viewing the resulting  
327 log file, in order to ensure convergence of parameter values, to verify whether  
328 ESS values exceeded 200, and to estimate node ages. TreeAnnotator and  
329 FigTree were used for drawing and visualizing the timetree, respectively.

330 Alignments and Bayesian tree-files are deposited and available in Treebase

331 (TB2: S16487.

### 332 **3. Results**

#### 333 *3.1. Estimates of genetic distance and phylogenetic analysis*

334 After the alignment, sequences of 1632 bp and 1650 bp were obtained for the  
335 18S and 28S regions, respectively (see Table 1 for the GenBank accession  
336 numbers). For each region, the genetic pairwise distance corrected according  
337 to the K2P model provided comparable values between the pairs *Minona*  
338 *gemella* + *Minona cf gemella* and *Parotoplana sp. nov. 1* + *Parotoplana sp. nov.*  
339 *2*:  $K2P = 0.0085 \pm 0.0022$  and  $K2P = 0.0115 \pm 0.0027$ , for the 18S; and  $K2P =$   
340  $0.0345 \pm 0.0045$  and  $K2P = 0.0361 \pm 0.0049$ , for the 28S D1-D6, respectively  
341 (Table 2).

342 ML and BI generated consistent trees with negligible differences in topology;  
343 additionally, in both trees the nodes of our interest are highly supported. We  
344 therefore reported the BI tree obtained by the software MrBayes only (Fig. 2).  
345 This phylogenetic tree shows that *M. gemella* (Atlantic coast) and *Minona cf*  
346 *gemella* (Pacific coast) (GS1) are in a sister-taxon relationship, as well as  
347 *Parotoplana sp. nov. 1* (Atlantic coast) and *Parotoplana sp. nov. 2* (Pacific coast)  
348 (GS2); the corresponding nodes are highly supported both for posterior  
349 probability and bootstrap values (Fig. 2). Therefore, according to Jordan's  
350 definition (1908), they can be considered as geminate species, and they will be  
351 used for estimating the mutation rate per million years.

352 Furthermore, the tree confirmed the sister-taxa relationship between Atlantic  
353 and Pacific clusters of species belonging to the families Otoplanidae (node A),  
354 Monocelididae (node B) and Archimonocelididae (node C) (Fig. 2). In particular:  
355 - Within Otoplanidae, species belonging to the genus *Kata* were separated into  
356 two geographic clusters, one grouping the Atlantic *K. evelinae* + *K. leroda*, and  
357 one the Pacific *Kata sp. nov. 1* + *Kata sp. nov. 2* (node A in Fig. 2);

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

### 363 364 3.2. Mutation rates per million years and divergence time

365 The estimated ucl.d.stdev parameter amounts to 0.842 and 0.677 for the 18S  
366 and 28S, respectively, indicating that our dataset is clock-like. The mutation  
367 rate per million years between *M. gemella* and *Minona* cf *gemella* amounts to  
368 0.12% for the 18S, and 0.49% for the 28S (Table 2). Slight higher values were  
369 obtained between *Parotoplana* sp. nov. 1 and *Parotoplana* sp. nov. 2: 0.16% for  
370 the 18S, and 0.52% for the 28S (Table 2). Analysis performed by means of the  
371 software Beast produced a tree whose topology is consistent to those obtained  
372 by both Garli and MrBayes. On these bases, we estimated a divergence time  
373 for node A (splitting Atlantic *K. evelinae* and *K. leroda* from the Pacific *Kata* sp.  
374 nov. 1 and *Kata* sp. nov. 2) of about 17.9 Myr, ranging 12.9 - 23.8 Myr (Fig. 3);  
375 for node B (splitting Atlantic *Duplominona tridens* from the Pacific *Duplominona*  
376 sp. nov. 1) of about 13.9 Myr, ranging 8.9 - 20.0 Myr (Fig. 3); and for node C  
377 (splitting the Atlantic *A. marci* and *Archimonocelis* sp. nov. 1, from the Pacific  
378 *Archimonocelis* sp. nov. 2) of 9.4 Myr, ranging 5.5 - 14.9 Myr (Fig. 3).

379

#### 380 **4. Discussion**

381 Molecular tools and the MCH have provided new clues on past evolutionary  
382 processes and mechanisms driving molecular evolution (Bromham and Penny,  
383 2003). However, several authors have shown perplexity about the wide  
384 applicability of the MCH (see e.g., Heads, 2005b; Lessios, 2008; Palumbi, 1997),  
385 and the use of the molecular clock to infer divergence time elicits criticisms,  
386 mostly concerning the way the clock is calibrated (Peterson et al., 2004). The  
387 use of paleogeographic events, which represents the only possible alternative  
388 of calibration in absence of fossil records, is a contentious issue (Coyne and Orr,  
389 2004; Lessios, 2008). It should be noticed that estimates on geminate species  
390 assume the final closure of a given geographic barrier as a minimum age  
391 calibration; hence, time since divergence may have been underestimated if  
392 taxa diverged before this date. In addition, considering merely geminate  
393 species as species pairs originated after the rise of a geographic barrier could  
394 be an oversimplification of their evolutionary path, since the evolutionary  
395 history of many nominal geminate species potentially may be more complex  
396 (see Knowlton and Weigt, 1998).

397 To overcome such limitations, the use of different genes or loci, and different  
398 calibration points has been recommended (Marko and Moran, 2009). In the  
399 case of Proseriata, the number of genes/loci we could use are limited, because  
400 most of 'universal' primers for invertebrates, such as those for the cytochrome  
401 c oxidase subunit I (COI) Folmer's region (Folmer et al., 1994) do not provide  
402 satisfactory results, and specific primers are not available except for a few  
403 species (see Casu et al., 2011; Sanna et al., 2009). Furthermore, since a limited  
404 number of sequences of Proseriata is at present available in Genbank, the  
405 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 (Hedges, 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.

431 Our results are consistent to those found for other trans-American species pairs  
432 (see e.g., Beu, 2001; Coates and Obando, 1996; Collins, 1996; Jackson et al.,  
433 1993; Roopnarine, 2001; Vermeij, 2001). For instance, the calibration on COI  
434 and ITS (Internal Transcribed Spacer) sequences revealed a time of divergence  
435 of 17.4-27.0 Myr, and 14.5-18.8 Myr, respectively, between trans-American  
436 populations of the subgenus *Acar* (*Bivalvia*) (Marko and Moran, 2009).

437 In the cases of *Kata* spp, *Duplominona* spp., and *Archimonocelis* spp., dispersal  
438 between ocean basins along the southern tip of South America would obfuscate  
439 interpretation of our results. However, no member of the species pairs involved  
440 was found in previous research in Chile, Uruguay, Terra del Fuego, or sub-  
441 Antarctic islands (Marcus, 1954b; Schockaert et al., 2009, 2011). Furthermore,  
442 species of the genus *Kata* are only known from tropics; similarly, *Duplominona*  
443 and *Archimonocelis* species, with few exceptions, occur in tropical to warm-  
444 temperate areas (Martens and Curini-Galletti, 1993; Tyler et al., 2006, 2012).  
445 At least in recent times, therefore, the rigid conditions of extreme south of  
446 South America acted as barrier to dispersal of these organisms.

447

#### 448 *4.1. Conclusions*

449 The study of geminate species of Proseriata across the Isthmus of Panama  
450 allowed the first calibration of the molecular clock for a meiofaunal taxon.  
451 Results of our research open potentials for the use of intertidal meiofauna for  
452 MCH. Among the major objections of the MCH, in fact, is that speciation among  
453 geminate pairs may predate the final emergence of the isthmus. However,  
454 meiofaunal, intertidal/shallow-water taxa may have shown continuity of habitat  
455 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

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473

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702

703

## 704 FIGURE AND TABLE LEGENDS

705

706 **Figure 1. Sampling localities.**

707 Map of the four trans-American and geminate species sampling localities:

708 Bocas del Toro, Panama; Playa La Angosta - Colón, Panama; Naos Island -

709 Panama City, Panama; Ilhabela/São Sebastião, Brazil.

710

711 **Figure 2. Phylogenetic tree.**

712 Tree obtained by BI showing the interrelationships of the species based on

713 combined 18S+28S D1-D6. The branch length scale refers to the number of

714 substitutions per site. Nodal supports are indicated for BI as posterior

715 probability (PP). For the three test cases and the two geminate species pairs ML

716 bootstrap values are also reported at each node.

717 GS1: geminate species 1 (*Minona gemella* - Atlantic coast + *Minona cf gemella*718 - Pacific coast). GS2: geminate species 2 (*Parotoplana* sp. nov. 1 - Atlantic coast719 + *Parotoplana* sp. nov. 2 - Pacific coast). Node A: Atlantic *Kata evelinae* and720 *Kata leroda* + Pacific *Kata* sp. nov. 1 and *Kata* sp. nov. 2. Node B: Atlantic721 *Duplominona tridens* + Pacific *Duplominona* sp. nov. 1. Node 3: Atlantic722 *Archimonocelis marci* and *Archimonocelis* sp. nov. 1 + Pacific *Archimonocelis*

723 sp. nov. 2.

724

725 **Figure 3. Timetree.**

726 Tree obtained by the software Beast showed divergence time among taxa.

727 Nodes indicated with A, B and C correspond to the nodes showed in Fig. 2.

728 Values within brackets represent the median values of divergence time of the

729 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.**

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

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

<b>Archimonocelididae</b>	<i>Archimonocelis marci</i> Curini-Galletti, 2014	São Sebastião (Brazil)	<u><b>KJ68233</b></u> <u><b>7</b></u>	<u><b>KJ682399</b></u>
	<i>Archimonocelis marci</i> Curini-Galletti, 2014	São Sebastião (Brazil)	<u><b>KJ68233</b></u> <u><b>8</b></u>	<u><b>KJ682400</b></u>
	<i>Archimonocelis</i> sp. nov. 1	São Sebastião (Brazil)	<u><b>KJ68233</b></u> <u><b>9</b></u>	<u><b>KJ682401</b></u>
	<i>Archimonocelis</i> sp. nov. 2	Naos Island (Panama)	<u><b>KJ68234</b></u> <u><b>0</b></u>	<u><b>KJ682402</b></u>
	<i>Archimonocelis</i> sp. nov. 2	Naos Island (Panama)	<u><b>KJ68234</b></u> <u><b>1</b></u>	<u><b>KJ682403</b></u>
	<i>Archimonocelis</i> sp. nov. 2	Naos Island (Panama)	<u><b>KJ68234</b></u> <u><b>2</b></u>	<u><b>KJ682404</b></u>
<b>Monocelididae</b>	<i>Minona ileanae</i> Curini-Galletti, 1997	GenBank	<u><b>JN22490</b></u> <u><b>5</b></u>	<u><b>JN224910</b></u>
	<i>Monocelis longiceps</i> (Duges, 1830)	GenBank	<u><b>KC9710</b></u> <u><b>64</b></u>	<u><b>KC971087</b></u>
	<i>Monocelis longistyla</i> Martens and Curini-Galletti, 1987	GenBank	<u><b>KC9710</b></u> <u><b>65</b></u>	<u><b>KC971088</b></u>
	<i>Minona ileanae</i> Curini-Galletti, 1997	Great Bitter Lake (Egypt)	<u><b>KJ68234</b></u> <u><b>3</b></u>	<u><b>KJ682405</b></u>
	<i>Minona</i> sp. nov.	Playa La Angosta, Colón (Panama)	<u><b>KJ68234</b></u> <u><b>4</b></u>	<u><b>KJ682406</b></u>
	<i>Minona</i> sp. nov.	Playa La Angosta, Colón (Panama)	<u><b>KJ68234</b></u> <u><b>5</b></u>	<u><b>KJ682407</b></u>
	<i>Minona</i> cf <i>trigonopora</i> Ax, 1956	Palau (Sardinia, Italy)	<u><b>KJ68234</b></u> <u><b>6</b></u>	<u><b>KJ682408</b></u>
	<i>Minona gemella</i> Ax and Sopott-Ehlers, 1985	Playa La Angosta, Colón (Panama)	<u><b>KJ68234</b></u> <u><b>7</b></u>	<u><b>KJ682409</b></u>
	<i>Minona gemella</i> Ax and Sopott-Ehlers, 1985	Playa La Angosta, Colón (Panama)	<u><b>KJ68234</b></u> <u><b>8</b></u>	<u><b>KJ682410</b></u>
	<i>Minona gemella</i> Ax and Sopott-Ehlers, 1985	Playa La Angosta, Colón (Panama)	<u><b>KJ68234</b></u> <u><b>9</b></u>	<u><b>KJ682411</b></u>
	<i>Minona</i> cf <i>gemella</i> Ax and Sopott-Ehlers, 1985	Naos Island (Panama)	<u><b>KJ68235</b></u> <u><b>0</b></u>	<u><b>KJ682412</b></u>
	<i>Minona</i> cf <i>gemella</i> Ax and Sopott-Ehlers, 1985	Naos Island (Panama)	<u><b>KJ68235</b></u> <u><b>1</b></u>	<u><b>KJ682413</b></u>
	<i>Minona</i> cf <i>gemella</i> Ax and Sopott-Ehlers, 1985	Naos Island (Panama)	<u><b>KJ68235</b></u> <u><b>2</b></u>	<u><b>KJ682414</b></u>
	<i>Minona</i> sp. nov.	Boa Vista Island (Cape Verde)	<u><b>KJ68235</b></u> <u><b>3</b></u>	<u><b>KJ682415</b></u>
	<i>Minona</i> sp. nov.	Boa Vista Island (Cape Verde)	<u><b>KJ68235</b></u> <u><b>4</b></u>	<u><b>KJ682416</b></u>

	<i>Monocelis lineata</i> OF Müller, 1774	Porto Pozzo (Sardinia, Italy)	<u><b>KJ68235</b></u> <u><b>5</b></u>	<u><b>KJ682417</b></u>
	<i>Monocelis lineata</i> OF Müller, 1774	Charaki (Rhodes, Greece)	<u><b>KJ68235</b></u> <u><b>6</b></u>	<u><b>KJ682418</b></u>
	<i>Monocelis lineata</i> OF Müller, 1774	Pilo (Sardinia, Italy)	<u><b>KJ68235</b></u> <u><b>7</b></u>	<u><b>KJ682419</b></u>
	<i>Monocelis lineata</i> OF Müller, 1774	Colostrai (Sardinia, Italy)	<u><b>KJ68235</b></u> <u><b>8</b></u>	<u><b>KJ682420</b></u>
	<i>Minona</i> sp. nov. 1	Faro (Portugal)	<u><b>KJ68235</b></u> <u><b>9</b></u>	<u><b>KJ682421</b></u>
	<i>Minona</i> sp. nov. 1	Faro (Portugal)	<u><b>KJ68236</b></u> <u><b>0</b></u>	<u><b>KJ682422</b></u>
	<i>Minona</i> sp. nov.	Lanzarote, Canary Island (Spain)	<u><b>KJ68236</b></u> <u><b>1</b></u>	<u><b>KJ682423</b></u>
	<i>Minona</i> sp. nov.	Tenerife, Canary Island (Spain)	<u><b>KJ68236</b></u> <u><b>2</b></u>	<u><b>KJ682424</b></u>
	<i>Minona</i> sp. nov.	Tenerife, Canary Island (Spain)	<u><b>KJ68236</b></u> <u><b>3</b></u>	<u><b>KJ682425</b></u>
<b>Monocelididae</b>	<i>Duplominona</i> sp. nov.	Lanzarote, Canary Island (Spain)	<u><b>KJ68236</b></u> <u><b>4</b></u>	<u><b>KJ682426</b></u>
	<i>Duplominona</i> sp. nov.	Faro (Portugal)	<u><b>KJ68236</b></u> <u><b>5</b></u>	<u><b>KJ682427</b></u>
	<i>Duplominona</i> sp. nov.	Faro (Portugal)	<u><b>KJ68236</b></u> <u><b>6</b></u>	<u><b>KJ682428</b></u>
	<i>Duplominona brasiliensis</i> Curini-Galletti, 2014	Ilhabela (Brazil)	<u><b>KJ68236</b></u> <u><b>7</b></u>	<u><b>KJ682429</b></u>
	<i>Duplominona</i> sp. nov. 1	Naos Island (Panama)	<u><b>KJ68236</b></u> <u><b>8</b></u>	<u><b>KJ682430</b></u>
	<i>Duplominona</i> sp. nov. 1	Naos Island (Panama)	<u><b>KJ68236</b></u> <u><b>9</b></u>	<u><b>KJ682431</b></u>
	<i>Duplominona</i> sp. nov. 1	Naos Island (Panama)	<u><b>KJ68237</b></u> <u><b>0</b></u>	<u><b>KJ682432</b></u>
	<i>Duplominona tridens</i> (Marcus, 1954)	São Sebastião (Brazil)	<u><b>KJ68237</b></u> <u><b>1</b></u>	<u><b>KJ682433</b></u>
	<i>Duplominona tridens</i> (Marcus, 1954)	São Sebastião (Brazil)	<u><b>KJ68237</b></u> <u><b>2</b></u>	<u><b>KJ682434</b></u>
	<i>Duplominona</i> sp. nov. 2	Naos Island (Panama)	<u><b>KJ68237</b></u> <u><b>3</b></u>	<u><b>KJ682435</b></u>
	<i>Duplominona</i> sp. nov. 3	Naos Island (Panama)	<u><b>KJ68237</b></u> <u><b>4</b></u>	<u><b>KJ682436</b></u>
	<i>Duplominona</i> sp. nov. 3	Naos Island (Panama)	<u><b>KJ68237</b></u> <u><b>5</b></u>	<u><b>KJ682437</b></u>

	<i>Duplominona</i> sp. nov.	Roscoff (France)	<u><b>KJ68237</b></u> <u><b>6</b></u>	<u><b>KJ682438</b></u>
	<i>Duploperaclistus circocirrus</i> Martens, 1983	Roscoff (France)	<u><b>KJ68237</b></u> <u><b>7</b></u>	<u><b>KJ682439</b></u>
	<i>Duploperaclistus circocirrus</i> Martens, 1983	Roscoff (France)	<u><b>KJ68237</b></u> <u><b>8</b></u>	<u><b>KJ682440</b></u>
	<i>Duplominona</i> sp. nov.	Blanes (Spain)	<u><b>KJ68237</b></u> <u><b>9</b></u>	<u><b>KJ682441</b></u>
	<i>Archilopsis spinosa</i> (Jensen, 1878)	Roscoff (France)	<u><b>KJ68238</b></u> <u><b>0</b></u>	<u><b>KJ682442</b></u>
	<i>Archilopsis arenaria</i> Martens, Curini-Galletti & Pucinelli, 1989	Roscoff (France)	<u><b>KJ68238</b></u> <u><b>1</b></u>	<u><b>KJ682443</b></u>
<b>Calviriidae</b>	<i>Calviria solaris</i> Martens and Curini-Galletti, 1993	GenBank	<u><b>AJ27015</b></u> <u><b>3</b></u>	<u><b>AJ270168</b></u>
<b>Coelogynoporidae</b>	<i>Coelogynopora tenuis</i> Meixner, 1938	Roscoff (France)	<u><b>KJ68238</b></u> <u><b>2</b></u>	<u><b>KJ682444</b></u>
<b>Unguiphora</b>	<i>Polystyliphora novaehollandiae</i> Curini-Galletti, 1998	GenBank	<u><b>AJ27016</b></u> <u><b>1</b></u>	<u><b>AJ270177</b></u>
	<i>Nematoplana coelogynoporoides</i> Meixner, 1938	Roscoff (France)	<u><b>KJ68238</b></u> <u><b>3</b></u>	<u><b>KJ682445</b></u>

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744 \*For newly sequenced taxa only.

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746 **Table 2.**

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

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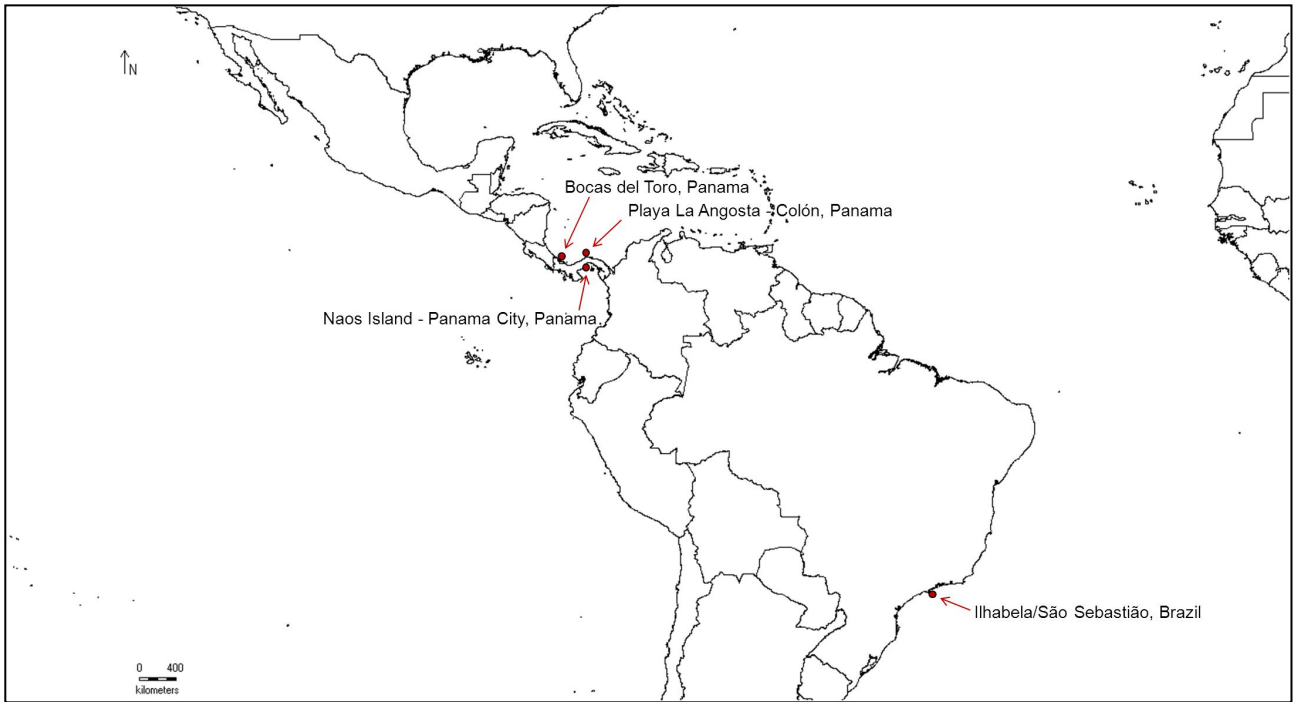
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760 **Figure 1**



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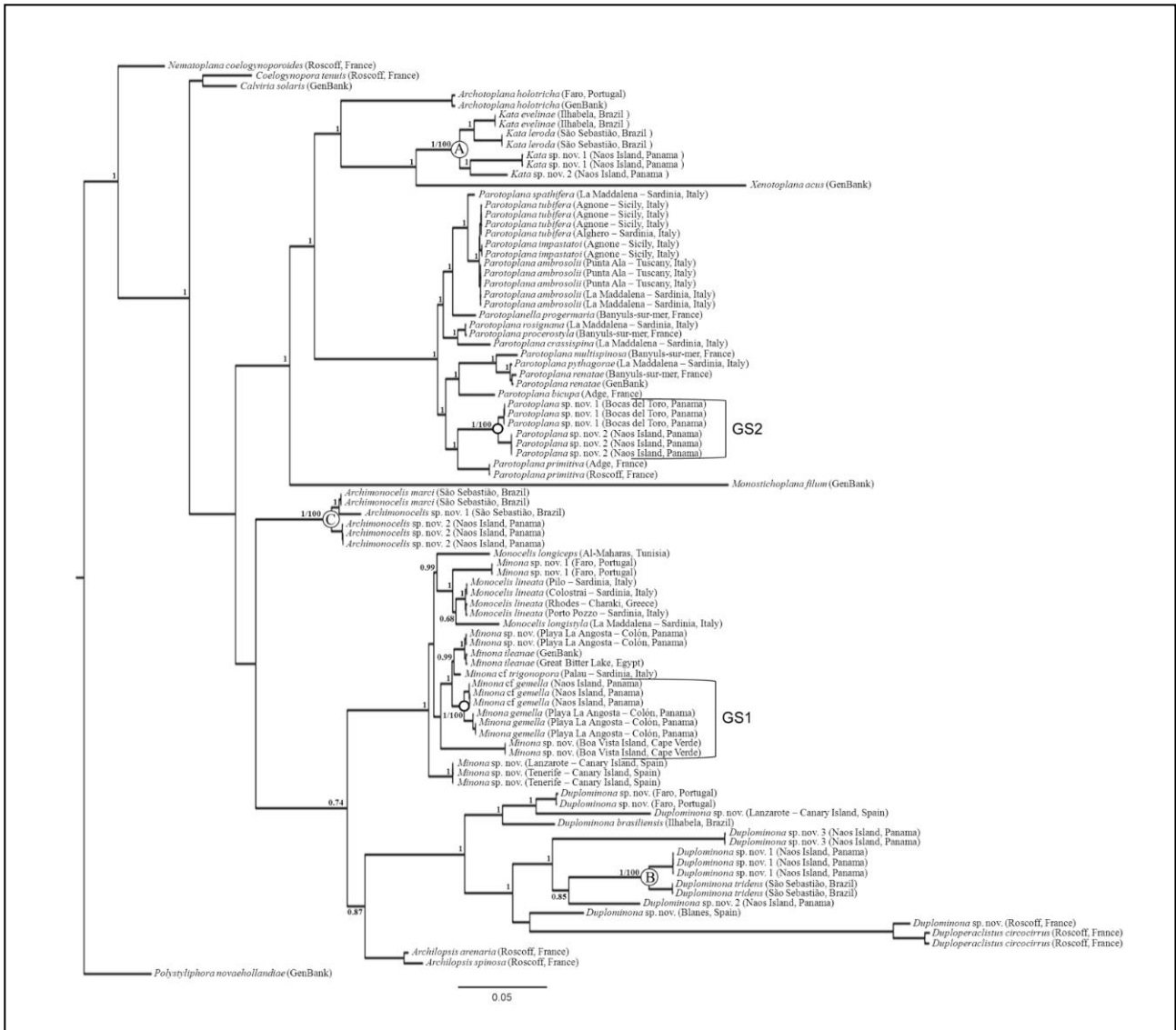
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777 **Figure 2**



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788 **Figure 3**

