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

note finali coverpage

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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.

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

78 evolutionary divergence is based on the molecular clock hypothesis (MCH)

79 (Zuckerkandl 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 significative 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

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).

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 µl containing about 25 ng (5 ng/µl)

228 of total genomic DNA on average, 1.0 U of Taq DNA Polymerase (EuroTaq by

229 Euroclone), 1× reaction buffer, 3.5 mM of $MgCl_2$, 0.32 µM of each primer, and

230 200 µM 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× SBA buffer (sodium boric acid, pH 8.2) and

238 stained with a 1 µl/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 (Katoh 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* 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

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);

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 ucld.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 the18S, 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

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.

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

473

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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 coast

719 + *Parotoplana* sp. nov. 2 - Pacific coast). Node A: Atlantic *Kata evelinae* and

720 *Kata leroda* + Pacific *Kata* sp. nov. 1 and *Kata* sp. nov. 2. Node B: Atlantic

721 *Duplominona tridens* + Pacific *Duplominona* sp. nov. 1. Node 3: Atlantic

722 *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.

Table 1.

Family	Species	Locality*	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*
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*

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

	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

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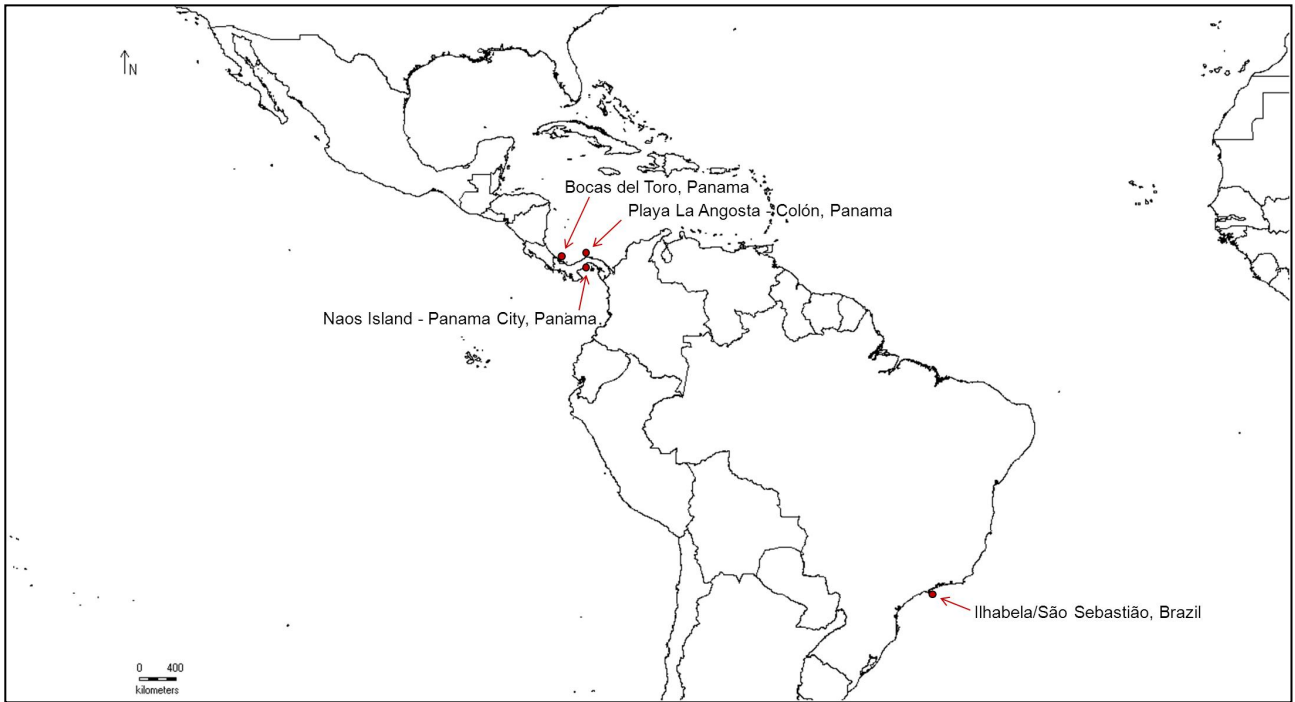
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760 **Figure 1**

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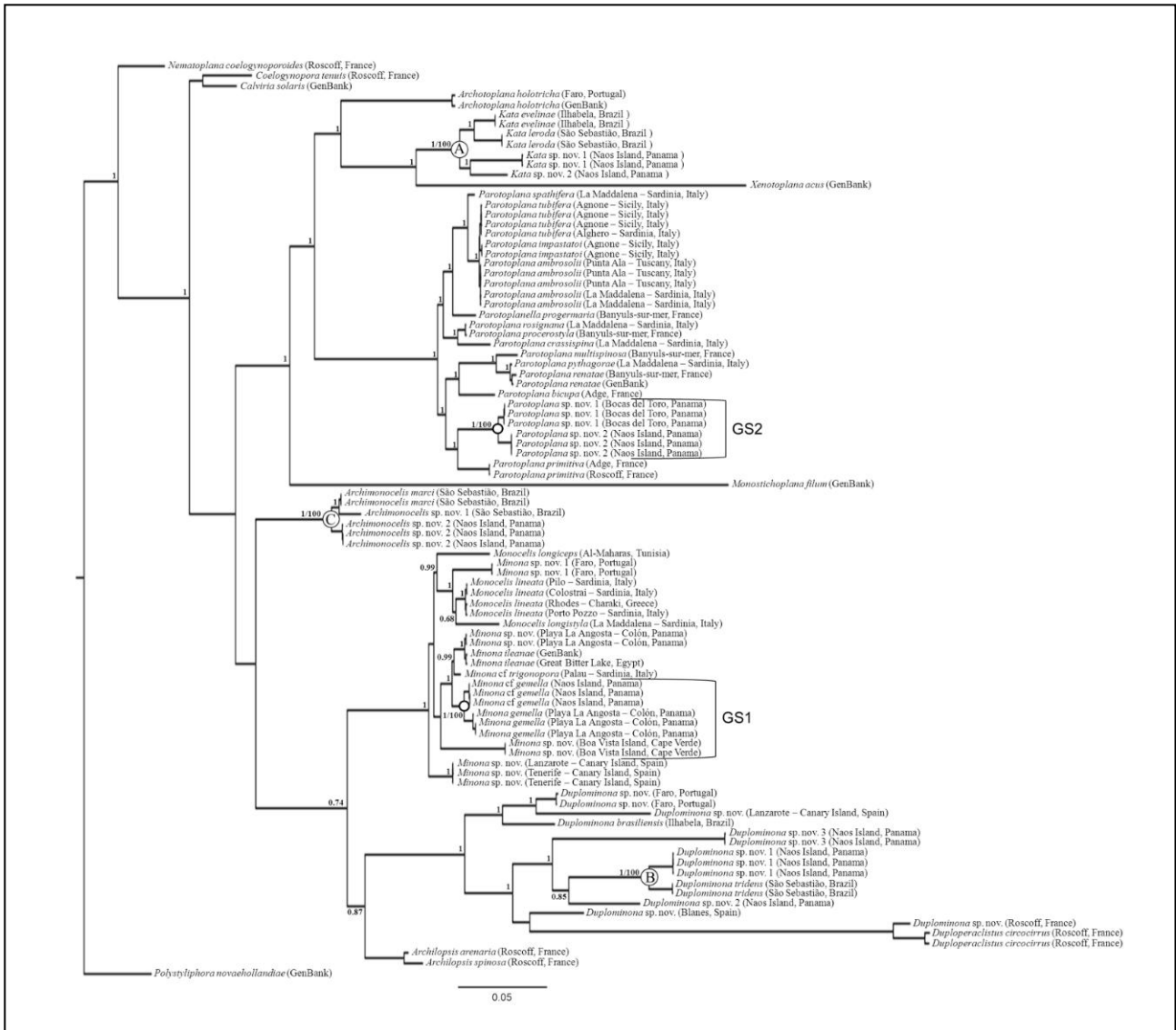
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777 **Figure 2**

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788 **Figure 3**

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