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Abstract

The planarian suborder Cavernicola Sluys, 1990 was originally created to house five species of triclad flatworms with special morphological features and a surprisingly discontinuous and broad geographic distribution. These five species could not be accommodated with any degree of certainty in any of the three taxonomic groups existing at that moment, viz., Paludicola Hallez, 1892, Terricola Hallez, 1892, and Maricola Hallez, 1892. The scarce representation of the group and the peculiarities of the morphological features of the species, including several described more recently, have complicated new tests of the monophyly of the Cavernicola, the assessment of its taxonomic status, as well as the resolution of its internal relationships. Here we present the first molecular study including all genera currently known for the group, excepting one. We analysed newly generated 18S and 28S rDNA data for these species, together with a broad representation of other triclad flatworms. The resulting phylogenetic trees supported the monophyly of the Cavernicola, as well as its sister-group relationship to the Maricola. The sister-group relationship to the Maricola and affinities within the Cavernicola falsify the morphology-based phylogeny of the latter that was proposed previously. The relatively high diversity of some cavernicolan genera suggests that the presumed rarity of the group actually may in part be due to a collecting artefact. Ancestral state reconstruction analyses suggest that the ancestral habitat of the group concerned epigean freshwater conditions. Our results point to an evolutionary scenario in which the Cavernicola (a) originated in a freshwater habitat (b) as the sister clade of the marine triclads, and (c) subsequently radiated and colonized both epigean and hypogean environments. Competition with other planarians, notably members of the Continenticola, or changes in epigean habitat conditions are two possible explanations -still to be tested- for the loss of most epigean diversity of the Cavernicola, which is currently reflected in their highly disjunct distributions.

Keywords	Gondwana, Pangea, molecular phylogeny, taxonomy, troglobitic flatworms
Taxonomy	Zoology, Evolutionary Biology

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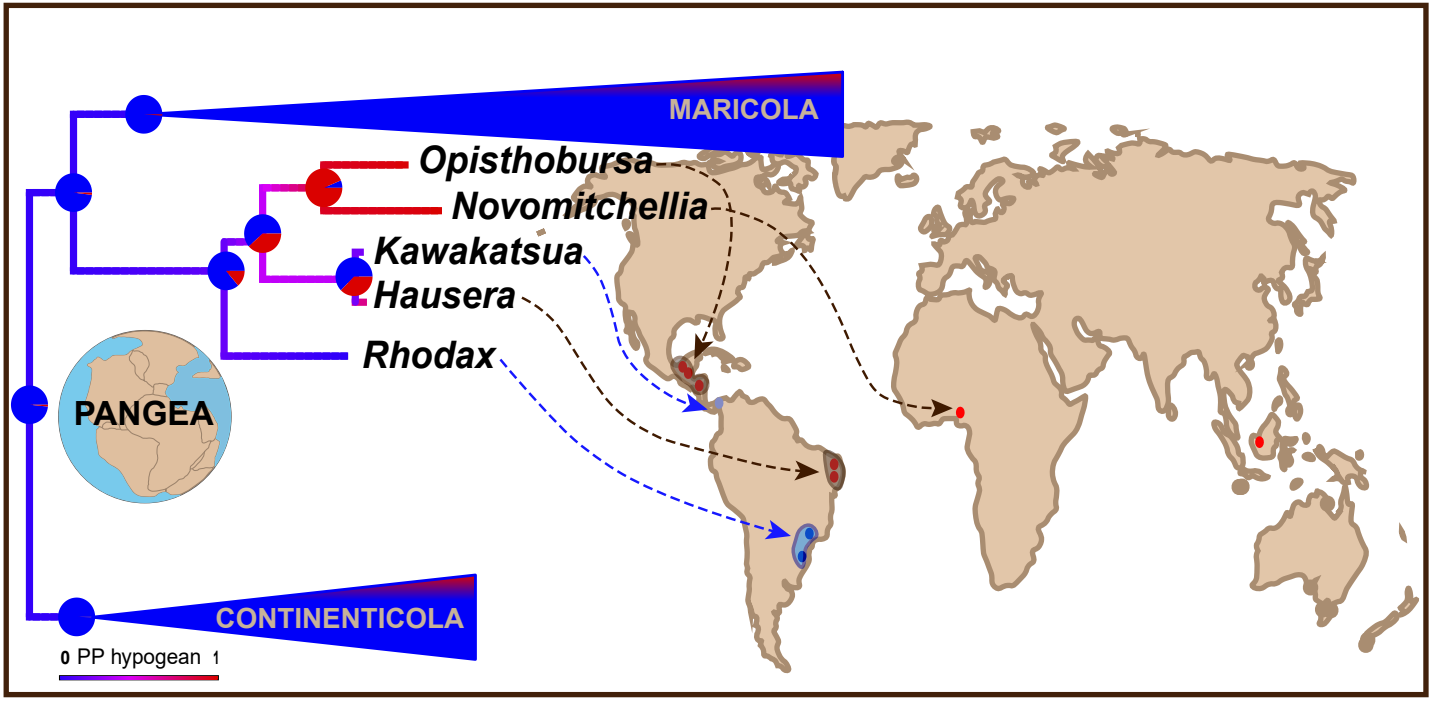
Highligths

- First molecular phylogeny of a rare (11 species) planarian suborder, Cavernicola
- confirms monophyly, Maricola sistership and their status as a distinct suborder
- freshwater and epigean in origin, colonized both epigean and hypogean habitats
- disjunct distribution may be due to Gondwana origin, and loss of most epigean diversity
- genetic diversity and new findings foreshadow more species to be discovered


MARICOLA
Opisthobursa
Novomitchellia
Kawakatsua
Hausera
Rhodax
PANGEA
CONTINENTICOLA
0 PP hypogean 1

hylogeny and biogeography of the Cavernicola (Platyhelminthes: Tricladida): relicts of an epigean group sheltering in caves?

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Abstract

The planarian suborder Cavernicola Sluys, 1990 was originally created to house five species of triclad flatworms with special morphological features and a surprisingly discontinuous and broad geographic distribution. These five species could not be accommodated with any degree of certainty in any of the three taxonomic groups existing at that moment, viz., Paludicola Hallez, 1892, Terricola Hallez, 1892, and Maricola Hallez, 1892. The scarce representation of the group and the peculiarities of the morphological features of the species, including several described more recently, have complicated new tests of the monophyly of the Cavernicola, the assessment of its taxonomic status, as well as the resolution of its internal relationships. Here we present the first molecular study including all genera currently known for the group, excepting one. We analysed newly generated 18S and 28S rDNA data for these species, together with a broad representation of other triclad flatworms. The resulting phylogenetic trees supported the monophyly of the Cavernicola, as well as its sister-group relationship to the Maricola. The sister-group relationship to the Maricola and affinities within the Cavernicola falsify the morphology-based phylogeny of the latter that was proposed previously. The relatively high diversity of some cavernicolan genera suggests that the presumed rarity of the group actually may in part be due to a collecting artefact. Ancestral state reconstruction analyses suggest that the ancestral habitat of the group concerned epigean freshwater conditions. Our results point to an evolutionary scenario in which the Cavernicola (a) originated in a freshwater habitat (b) as the sister clade of the marine triclads, and (c) subsequently radiated and colonized both epigean and hypogean environments. Competition with other planarians, notably members of the Continenticola, or changes in epigean habitat conditions are two possible explanations -still to be tested- for the loss of most epigean diversity of the Cavernicola, which is currently reflected in their highly disjunct distributions.

1. INTRODUCTION

Between 1946 and 1983 five species of planarian flatworms (Platyhelminthes, Tricladida) had been described that consistently defied the taxonomic schemes developed by planarian systematists. Four out of these five species (*Opisthobursa mexicana* Benazzi, 1972; *O. josephinae* Benazzi 1975; *Balliania thetisae* Gourbault, 1978; *Novomitchellia sarawakana* (Kawakatsu & Chapman, 1983) usually had been assigned to the marine triclads of the Suborder Maricola Hallez, 1892. The fifth species, *Rhodax evelinae* Marcus, 1946, was considered to belong to the freshwater triclads or Paludicola Hallez, 1892. It should be noted that the Suborder Paludicola is no longer valid; its representatives, together with terrestrial planarians –the now obsolete Suborder Terricola Hallez, 1892- are currently classified in the Suborder Continenticola Carranza et al., 1998 (Sluys et al. 2009; Riutort et al. 2012). In all cases, however, some doubt was expressed about the taxonomic assignments of these five species (Ball 1974, Sluys 1990). At long last, Sluys (1990) resolved the taxonomic confusion surrounding these five species by showing, on the basis of morphological characters, that they formed a monophyletic group that represented a new and different clade in the phylogenetic tree of the triclad flatworms. At that time three major clades, at the level of suborder or infraorder, were recognized within the Tricladida Lang, 1884, viz., Paludicola, Maricola, and Terricola. For his new, fourth branch on the tree of the planarian flatworms Sluys (1990) erected a new taxon for which he coined the name Cavernicola Sluys, 1990, presently being ranked as a Suborder (Sluys et al. 2009). Although most of its constituent species had a hypogean habitat and exhibited adaptations to life in caves (unpigmented body, lack of eyes), Sluys (1990) stressed the notion that the name of the new taxon had no ecological connotation.

With respect to the phylogenetic position of the new suborder within the Tricladida, Sluys (1990) suggested a possible close relationship between the Cavernicola and the

Paludicola, based on the fact that the cavernicolan *Opisthobursa josephinae* exhibits one of the three presumed autapomorphies of the Paludicola, viz., sperm transfer by means of a spermatophore. However, he considered that character distribution as too weak to formally propose presence of a spermatophore as a synapomorphy for the Cavernicola and the Paludicola. Relationships within the Cavernicola were analysed also by Sluys (1990). The fact that the species possess a mixture of primitive features (Marcus, 1946, Sluys 1990) greatly complicated resolution of their phylogenetic affinities.

After this, it took a long time before the number of species for the Cavernicola started to increase slowly. Two new species and one new genus were described in recent years, viz., *Hausera hauseri* Leal-Zanchet & Souza, 2014 from Brazil, and *Novomitchellia bursaelongata* Harrath, Sluys & Riutort, 2016 from Africa; both species live in a hypogean habitat (Leal-Zanchet et al., 2014; Harrath et al., 2016). In addition, Laumer and Giribet (2014) reported 18S and 28S rRNA sequences for a new, undescribed species of Cavernicola. It was only recently that this new species acquired its proper taxonomic designation when it was described as the new genus and species *Kawakatsua pumila* Sluys, 2019 (Sluys and Laumer, 2019). It is noteworthy that this species was found in a basically terrestrial habitat. Addition of these new species to the Cavernicola made even more evident a conspicuous feature of this small group of species, i.e., their highly disjunct distributions (Fig. 1).

The present study is the first to include molecular data for all cavernicolan taxa, excepting *Balliania* Gourbault, 1978. In our analyses we have incorporated also representatives of 15 genera of triclads belonging to the other two suborders, thus allowing us to test for the first time the previously hypothesized monophyly of the Cavernicola, to analyse its relationships within the Tricladida, as well as the affinities between its constituent taxa.

2. MATERIALS AND METHODS

97 **2.1 Taxon sampling and identification**

98 We obtained samples from six either new or already known localities from South and
99 North America (Southern Mexico), and combined our data with sequences obtained from
100 GenBank, thus including all genera of the Cavernicola presently known, excepting *Balliania*
101 (Table 1, Fig. 1). New specimens of *Opisthobursa mexicana* and *Hausera hauseri* were
102 sampled at the original type localities of these two species, viz., Las Grutas de Coconá,
103 Tabasco, Mexico and Crotes cave, Rio Grande do Norte, Brazil, respectively. In the case of
104 *Rhodax*, the type-locality of *Rhodax evelinae* (the only described species for the genus) no
105 longer exists as it was dramatically transformed due to urbanization, hence representatives of
106 this genus in our study come from other localities. In first instance, we assigned these new
107 representatives to the genus *Rhodax* on the basis of their external features, combined with
108 anatomical and histological features. All new specimens present the following characteristics
109 of the genus *Rhodax*: rounded anterior tip with an adhesive organ; eyes present; pigmented
110 body; short, cylindrical pharynx (Appendix A). Specimens of *Rhodax* spp. 1 and 2, sampled
111 in surface waters located in Tavares and Pinheirinhos, respectively, in southern Brazil, did not
112 have reproductive organs. Specimens of *Rhodax* sp. 2 showed asexual reproduction in the
113 laboratory, similarly to what was described by Marcus (1946) in the original description of
114 the species. Specimens of *Rhodax* sp. 3 from surface water in Tramandaí, southern Brazil,
115 presented a reproductive system, which is characterized by the presence of testes tubes, a
116 large common spermiducal vesicle, and a connection between the copulatory apparatus and
117 the intestine (Leal-Zanchet et al., unpublished results). With respect to their female copulatory
118 organs, these animals show some differences with *R. evelinae*, such as a longer female genital
119 duct, which may be due to intraspecific variation. *Hausera* sp., which was sampled in a cave
120 from northeastern Brazil, is a typical troglobitic animal with an unpigmented body and
121 absence of eyes (Appendix A), similar to *H. hauseri*. *Hausera* sp. also matches other

diagnostic features of the genus, such as sperm ducts separately penetrating the penis bulb, the female genital duct communicating with the intestine, ovovitelline ducts without caudal dichotomy, uniting to form a common ovovitelline duct. *Hausera* sp. differs from *H. hauseri* in the shape of the penis papilla and bulbar cavity, the course of the sperm ducts when approaching the penis bulb, and the shape and length of the female genital duct (Hellmann et al., unpublished results).

In order to determine the phylogenetic position of the Cavernicola within Tricladida, we included in our analyses one representative sequence of each genus of the Cavernicola and also sequences of representative taxa of the suborders Maricola and Continenticola. We also included as outgroup species belonging to groups most closely related to the Tricladida, according to previous studies (Laumer et al., 2015; Norén and Jondelius, 2002; Riutort et al., 2012), viz., Fecampiidae and Prolecitophora (Table 2). For determining the relationships within the suborder Cavernicola we used as ingroup all available sequences assigned to this suborder and as outgroup two maricolan taxa. In order to reconstruct ancestral character states related to habitat (epigean / hypogean) and salinity tolerance (freshwater / marine) some recently published sequences were included of marine triclads that show a tolerance to freshwater (Table 2).

2.2 DNA extraction, gene amplification and sequencing

Genomic DNA was extracted from specimens preserved in absolute ethanol by Wizard® Genomic DNA Purification Kit (Promega), according to the manufacturer's instructions. The extraction product was quantified using a NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific, USA). Genomic DNA was used to amplify a fragment of the nuclear genes 18S rRNA (18S) and 28S rRNA (28S) through a polymerase chain reaction (PCR). For 18S amplification we used the primers 18S1F, 18S4F, 18S5R and

18S9R (Carranza et al., 1996) to amplify two overlapping fragments. For 28S amplification we used the primers 28S1F, 28S2F, 28S4R and 28S6R (Álvarez-Presas et al., 2008). The PCR reactions were performed in a final volume of 25µl, with final concentrations as follows: $MgCl_2$ 2.5mM, dNTPs 30µM, primers 0.4µM each, 0.75U Go Taq® DNA polymerase enzyme (Promega Madison, Wisconsin, USA) with its corresponding buffer (1X), and approximately 100 ng of template DNA. The amplification program for both fragments of 18S consisted of 30 cycles in the following manner: 30 seconds at 94°C, 45 seconds at 50°C (AT: annealing temperature), and 1 minute at 72°C, with 2 minutes for initial denaturation at 95°C and 4 minutes for final extension at 72°C. The program for both fragments of 28S was 35 cycles in the following manner: 45 seconds at 94°C, 45 seconds at 55°C (AT), 45 seconds at 72°C, as well as 1 minute of initial denaturalization at 94°C and 3 minutes of final extension at 72°C. The PCR products were purified by ultrafiltration in the Merck Millipore MultiScreen System (Darmstadt, Germany). Both chains of purified fragments were sequenced by Macrogen Inc., (Macrogen Europe, Amsterdam). The chromatograms were revised and edited with Geneious v. 10 (https://www.geneious.com) to obtain the final contigs.

2.3 Sequence alignment

Sequences of both genes were independently aligned with MAFFT v7 (Katoh and Standley, 2013) using the web server http://mafft.cbr.jp/alignment/server/ (last visited January 15th, 2019) with the G−INS−i algorithm. The following two principal sets of species were allocated for the phylogenetic analyses. The first (~~Sp-~~dataset -I), was designed to test the monophyly of the Cavernicola, as well as its taxonomic position within the Tricladida. This ~~Sp-~~dataset -I included one representative of each cavernicolan genus, as well as one representative per genus for a series of genera belonging to other suborders of the Tricladida and the outgroup taxa (Table 2). From ~~Sp-~~dataset -I three ~~subsequent~~ datasets were generated,

viz., two sets for the alignment of the individual genes 18S and 28S (named as ~~Sp-set~~dataset-I-[18S] and ~~Sp-set~~dataset -I-[28S], respectively), and another for the concatenation of both genes (named ~~Sp-~~dataset -I-[18S+28S]).

The second major species set, ~~Sp-~~dataset -II, comprised all available sequences of cavernicolan representatives, as well as sequences of two maricolan taxa that were used as outgroups (Table 2). This species set was used to infer phylogenetic relationships within the Cavernicola. From ~~Sp-set~~dataset -II three subsequent datasets were generated, two for the individual genes 18S and 28S (called ~~Sp-set~~dataset -II-[18S] and ~~Sp-set~~dataset -II-[28S], respectively), and a third dataset for the concatenation of both genes (called ~~Sp-set~~dataset -II-[18S+28S]).

For the ancestral state reconstructions, another major dataset was created on the basis of an 18S alignment, including as ingroup taxa (a) one representative for each cavernicolan genus, and (b) one representative per genus for a series of genera belonging to other suborders of Tricladida; in the following this dataset is abbreviated as ASR-18S. In this dataset some recently published maricolan taxa were included (Table 2) that live in freshwater or are freshwater-tolerant, in order to have a better representation in this group of the feature "salinity tolerance"; representatives of the Fecampiidae were included as outgroup taxa.

Gblocks (Castresana, 2000) was used to remove regions of ambiguous alignment; the parameters used for each alignment are shown in Supplementary Material Table S1. Each alignment was edited by hand to trim the ends and the code N was assigned to sites with missing data. The concatenated alignments were obtained from the alignments of each gene in Mesquite v. 3.04 (Maddison and Maddison, 2008); in these alignments missing sequences were assigned the code N.

We used Xia's method (Xia and Lemey, 2009), implemented in DAMBE6 software (Xia, 2017), to assess the nucleotide substitution saturation. This test is based on the

196 concept of entropy in information theory and calculates an index of substitution saturation
197 (Iss), which is statistically compared to a critical value (Iss.c) that defines a threshold for
198 significant saturation in the data at which the sequences will begin to fail to recover the true
199 tree (Xia and Lemey, 2009). We analyzed each alignment by including all sites and using the
200 proportion of invariant sites previously calculated by the same program.

201 **2.4 Phylogenetic Inference**

202 In order to infer the best sequence evolution model for our datasets we used the
203 jModeltest 2.1 software (Darriba et al., 2012), taking into account the scores of the Bayesian
204 Information Criterion (BIC). The best model for both genes, calculated independently, was
205 GTR + Γ + I (General Time Reversible + Gamma Distribution + Invariable Sites). A gene
206 partition was defined in all the concatenated datasets analyses, so that the estimation of the
207 parameters for each partition was independent. We used two phylogenetic inference methods,
208 viz., Maximum Likelihood (ML) and Bayesian Inference (BI). Both approaches were used to
209 analyze each gene independently, as well as for analyzing the concatenated datasets. ML
210 analyses were performed with RAxML v8.2.4 software (Stamatakis, 2014) under the
211 GTRGAMMA model, taking into account the author's recommendations. A rapid bootstrap
212 analysis with 2000 pseudoreplicates was conducted to obtain bootstrap support values (bs) for
213 the nodes. We ran Bayesian analyses in MrBayes v3.2.2 software (Ronquist et al., 2012) with
214 5 million generations, sampling frequency every 1000 and 25% burn-in to obtain the
215 consensus tree and posterior probability values (pp). Convergence of the topology and
216 parameter values for the two runs was examined by observing that the average standard
217 deviation of split frequencies was below 0.01. Furthermore, the *.p* file of each run was
218 inspected in Tracer v1.5 software (Rambaut and Drummond, 2007) to ensure that the
219 effective sample size (ESS) values of each parameter were above 200.

2.5 Ancestral states reconstruction

For the Ancestral States Reconstruction analysis (ASR), we obtained a phylogenetic tree with BI using the ~~ASR-18S dataset~~dataset III-[18S]. Because the Maricola clade shows a good number of species with various degrees of salinity tolerance, we included in this ~~ASR-18S dataset~~dataset III-[18S] (Table 2) 15 species covering the freshwater tolerance diversity in this group. In the salinity tolerance state reconstruction analysis the state for terrestrial planarians was coded as freshwater, since the animals generally depend on the humidity of forests soils, which usually will be formed by freshwater. This tree was used to independently estimate the ancestral states for habitat (epigean/hypogean) and salinity tolerance (freshwater/freshwater-marine/marine) by using the Phytools package v.0.6.60 of R (Revell, 2012). The posterior probability for each state on the nodes was determined from stochastic character-state maps by applying the empirical Bayes method (Bollback, 2006). For this, we used an all-rates-different (ARD) model and applied the make.simmap function with Markov Chain Monte Carlo (MCMC), and ran 10000 simulations. In the case of salinity tolerance, which has polymorphic states (freshwater/freshwater-marine/marine), we used the fitpolyMk function integrated with the make.simmap function.

3. RESULTS

3.1. Datasets

The length of the amplified 18S and 28S fragments was approximately 1800 base pairs (bp) and 1500 bp, respectively. For unknown reasons, which may range from problems in the fixation of the specimens, conditions of preservation during transport to intrinsic characteristics of these animals, many of our attempts to obtain good quality DNA for amplification of the genes were unsuccessful. Fortunately, eventually a total of seven new sequences of 18S and of 28S were obtained. After Gblocks processing, the ~~Sp-set~~dataset - I-

[18S] dataset contained 29 sequences with a total length of 1602 sites, while the ~~Sp-set-I-28S~~dataset_I-[28S] data set included 27 sequences with a total length of 1336 sites (Table 2 and Supplementary Material Table S1); these two alignments were concatenated in a ~~Sp-set-I-18S+28S~~dataset_I-[18S+28S] dataset (2939 bp and 29 OTUs) and used to infer phylogenetic relationships within the Tricladida. Another dataset with concatenated alignments (~~Sp-set-II-18S+28S~~dataset_II-[18S+28S], with 3199 positions: 1710 for 18S; 1489 for 28S) with 11 OTUs including only cavernicolan taxa, as well as *Procerodes dohrni* Wilhelmi, 1909 and *Bdelloura candida* (Girard, 1850) as outgroups, was obtained and analyzed to infer the phylogenetic relationships within the Cavernicola. Finally, the ~~ASR-18S-dataset~~dataset_III-[18S] included 28 sequences (1709 bp after Gblocks processing; Supplementary material Table S1) and was used for the ancestral character state reconstruction analysis.

Saturation analysis of the alignments for each gene, including outgroups, showed no saturation for our datasets; the proportion of invariant sites was 0.17 and 0.22 for 18S and 28S, respectively.

3.2 Phylogeny

The trees obtained from the three ~~Sp-set~~datsets -I ~~datasets~~ (~~Sp-set-I-18S~~[18S], ~~Sp-set-I-28S~~[28S], and ~~Sp-set-I-18S+28S~~[18S+28S]) by both inference strategies (ML and Bayesian), all group the cavernicolan taxa into a well-supported monophyletic clade that is sister to Maricola; in turn, Cavernicola + Maricola is sister group of ~~phylogenetically separated from the~~ Continenticola ~~and the Maricola~~ (Fig. 2; Appendix B). However, the topology of the phylogenetic tree inferred from the 28S alone differs in two points from the results obtained with the18S and concatenated datasets. While the ~~Sp-set-I-18S~~dataset_I-[18S] and the ~~Sp-set-I-18S+28S~~dataset_I-[18S+28S] trees (Appendix B.1 Fig. 2) position the Cavernicola as the sister-group of the Maricola with high support (94% bs; 1.00 pp for the 18S tree and 100% bs;

1.00 pp for the concatenated), the ~~Sp-set-I-28S~~dataset I-[28S] tree (Appendix B.2) fails to resolve the relationships between the three triclad suborders, as the Cavernicola groups with Continenticola at a poorly supported node (49% bs; 0.6 pp). Further, in the ~~Sp-set-I-18S~~dataset I-[18S] and the ~~Sp-set-I-18S+28S~~dataset I-[18S+28S] trees the genus *Opisthobursa* groups with *Novomitchellia* with high support, and together with *Hausera* and *Kawakatsua* they form a monophylum that is highly supported for 18S (87% bs; 1.00 pp) and receives a low or reasonably good support, depending on the method, for the concatenated trees (58% bs; 0.96 pp). In the ~~Sp-set-I-28S~~dataset I-[28S] tree there is no information on *Novomitchellia bursaelongata* (no data in GenBank), while in this phylogeny *Opisthobursa* is the sister genus of *Rhodax* with a reasonable good support (73% bs; 0.96 pp).

The analyses of the three ~~Sp-~~datasets-II ~~datasets~~ (~~Sp-set-II-18S~~ [18S], ~~Sp-set-II-28S~~ [28S], and ~~Sp-set-II-18S+28S~~[18S+28S]) resulted in phylogenetic trees in which *Opisthobursa* and *Novomitchellia* (only *Opisthobursa* in the case of ~~Sp-set-II-28S~~dataset II-[28S]) were positioned as the sister-group of a clade formed by *Hausera* and *Kawakatsua,* with good support in the 18S tree (76% bs; 0.97 pp) and in the concatenated tree (76% bs; 0.99 pp), but with low support in the 28S tree (59% bs; 0.65 pp) (Appendix C, Fig.~~ure~~ 3).

In summary, with respect to the phylogenetic position of the Cavernicola within Tricladida, the ~~Sp-set-I-28S~~dataset I-[28S] tree showed a polytomy for the three suborders. In contrast, analyses of the 18S and the concatenated datasets for Sp-set-I returned a highly supported clade for Cavernicola + Maricola. With respect to relationships within the Cavernicola, analysis of the ~~Sp-set-I-28S~~dataset I-[28S] dataset yielded moderate support for the clade *Rhodax* + *Opisthobursa*, taking into account that *Novomitchelia* is missing from that dataset. The other five trees resulting from analyses of the Sp-set-I and Sp-set-II datasets (Fig.~~ure~~ 2, Fig.~~ure~~ 3, Appendix B and C) show *Rhodax* as the sister-group of a clade including *Opisthobursa* + *Novomitchelia* and *Hausera* + *Kawakatsua,* generally with high support.

These results suggest, in our opinion, that the data at hand strongly support the topology shown in Fig.~~ure~~ 2.

3.3 Ancestral habitat

We inferred the ancestral character states for the habitat types (epigean/hypogean) and for salinity tolerance (marine/freshwater) on the nodes of the phylogenetic tree obtained with BI from the ~~ASR-18S dataset~~dataset III-[18S] (Fig. 4; Appendix D). This dataset was used because it renders the same topology as shown in Fig. 2 and does not contain missing data, so that branch lengths will be more accurate than those in the tree resulting from the concatenated dataset. The hypothesis of an epigean habitat for the ancestor of the clade Maricola + Cavernicola was strongly supported by the ancestral state reconstruction analyses (pp=0.97; node 10, Fig 4A, Appendix D.1). In addition, an epigean habitat of the ancestor of the Cavernicola is supported with a high posterior probability value (pp=0.80, node 11, Fig. 4A, Appendix D.1). Furthermore, a high support value (pp=0.98; node 11, Fig. 4B, Appendix D.2~~7~~) suggests that this ancestor lived in a freshwater habitat, while the common ancestor of Maricola + Cavernicola has a nearly equal probability for being either a freshwater animal or exhibiting a tolerance to changes in salinity (pp=0.59~~5~~ freshwater; 0.40~~1~~ freshwater/marine; node 10, Fig. 4B, Appendix D.2).

4. DISCUSSION

4.1 Monophyly of the Cavernicola and its relationship to other Suborders of the Tricladida

The phylogenetic trees obtained in the present study corroborate the monophyly of the Cavernicola, as proposed by Sluys (1990). Monophyly of the Cavernicola was proposed on the basis of three apomorphic features: (a) penis bulb with gland cells, (b) horizontal orientation of the bursal canal or female genital duct, combined with the dorsal opening of the

317 common oviduct, or diverticulum, and (c) location of the ovaries at some distance posterior to
318 the brain (Sluys 1990; Fig. 6). Two of the new species described since Sluys' (1990)
319 monographic study also possess these three features, reinforcing their value as diagnostic
320 characters for the suborder (Leal-Zanchet et al., 2014; Harrath et al., 2016) . However,
321 *Kawakatsua pumila* does not possess a penis bulb with gland cells, while *Rhodax* does neither
322 exhibit the character “gland cells in the penis bulb” (character 1 in Sluys 1990; see also Fig.
323 6). Thus, currently there are two species of cavernicolans, among the eight species known at
324 present, that lack this presumed apomorphic character state of the Cavernicola, one species
325 (*Rhodax*) being positioned as sister to the rest of genera in the phylogenetic tree and the other
326 species (*Kawakatsua*) positioned at one of its tips. Under present conditions absence of this
327 character state in these two taxa is probably best interpreted as being the result of secondary
328 loss.

329 With respect to the third presumed apomorphic character for the Cavernicola postulated
330 by Sluys (1990), viz., "ovaries situated at some distance posterior to the brain", this character
331 condition is present in *B. thetisae*, *R. evelinae, O. mexicana*, *N. bursaelongata* , and
332 *Kawakatsua pumila* (Sluys, 1990; Harrath et al., 2016; Sluys and Laumer 2019), while it is
333 absent in *O. josephinae* and *H. hauseri* (Sluys, 1990; Leal-Zanchet et al., 2014) and
334 ambiguous for *N. sarawakana* (Sluys, 1990). This character state distribution casts some
335 doubt on the presumed synapomorphy for the Cavernicola, as the condition for the
336 cavernicolan ancestor, in view of the topology of our tree (Fig. 4), probably downgrades to
337 being equivocal.

338 The five currently known cavernicolan genera are housed in a single family,
339 Dimarcusidae Mitchell & Kawakatsu, 1972, which is supported by the fact that these genera
340 together form a monophyletic clade in our analysis (Fig. 2). In addition, its sister-group
341 relationship to the Maricola and the sister-group relationship shared between Dimarcusidae +

342 Maricola and the Continenticola in our phylogenetic trees lends further support to Sluys'
343 (1990) proposal of including all cavernicolan species in a separate suborder. However, in
344 contrast to his hypothesis that the Cavernicola is more closely related to freshwater planarians
345 than to marine triclads, our present phylogeny (Fig. 2) corroborates the conclusions of two
346 previous molecular studies (Laumer and Giribet, 2014; Harrath et al., 2016) that the
347 Cavernicola is most closely related to the Maricola. Our results do not support inclusion of the
348 Dimarcusidae in the suborder Maricola, as suggested by Mitchell and Kawakatsu (1972), but
349 do agree with Sluys' (1990) arguments that the Dimarcusidae does not possess the presumed
350 apomorphous character state of the Maricola, nor the derived features of more restrictive
351 groups of marine triclads.

352 Our results falsify the presumed close relationship between the Cavernicola and the
353 freshwater planarians. This is in agreement with the fact that cavernicolans lack two out of the
354 three apomorphies hypothesized for the Paludicola (see Sluys, 1989), viz., the probursal
355 condition, and body musculature with an extra outer layer of subepidermal longitudinal fibers.
356 Furthermore, although the third apomorphic feature proposed for the Paludicola, presence of a
357 spermatophore, has been described for *O. josephinae* by Benazzi (1975), it has not been
358 observed in any other cavernicolan species (Sluys 1990; AMLZ, pers. obs.). In point of fact,
359 Sluys (1990, p. 26) himself recognized that "… the data set at hand suggests little else than a
360 sistergroup relationship between the Paludicola and the Dimarcusidae, although this presumed
361 affinity remains poorly supported by apomorphous characters".

362 In summary, currently we can recognize three suborders within the Tricladida, viz.,
363 Cavernicola, Maricola, and Continenticola, which show clear differences in their morphology
364 (Sluys 1990) and are genetically highly differentiated.

365 **4.2 Relationships and diversity within the Cavernicola**

366 The phylogenetic relationships within the Cavernicola as revealed by molecular data
367 (Figs 2 and 3) differ from those proposed on the basis of morphological apomorphies (Fig. 5).
368 In the phylogeny presented here, *Rhodax* is sister to all other taxa of the Cavernicola. This
369 implies some changes in our interpretation of the evolution of several morphological features,
370 as will be discussed below.

371 The clade formed by *Opisthobursa* and *Novomitchellia* is highly supported (Fig. 3), as
372 well as its sister-group relationship to the clade comprising *Hausera* and *Kawakatsua*. This
373 casts some doubt on the four synapomorphies proposed for the sister-group relationship
374 between *Rhodax* and *Opisthobursa* as proposed by Sluys (1990): (a) ciliation being confined
375 to the posterior section of the bursal canal or female genital duct, (b) vitellaria being situated
376 medially to the testes, (c) loss of the primary copulatory bursa, and (d) presence of testes
377 tubes, i.e., fused testicular follicles (characters 5-8 in Fig. 5). In fact, *Opisthobursa* presents a
378 copulatory bursa, but Sluys (1990) pointed out that it is a secondary bursa and not a primary
379 one. In view of the present phylogenetic results, loss of the primary copulatory bursa in both
380 *Rhodax* and *Opisthobursa* may be interpreted as having evolved independently.

381 Fused testicular follicles or testis tubes, as present in *R. evelinae* and *O. josephinae*
382 (character 8 in Fig. 5), represent a rare condition among triclad flatworms (see Sluys and
383 Riutort, 2018). In view of the fact that *O. mexicana* has discrete testes follicles (Sluys 1990),
384 presence of testis tubes in *R. evelinae* and *O. josephinae* is presently best interpreted as the
385 result of convergent evolution.

386 Sluys (1990) hypothesized that oviducts running medially to the ventral nerve cords
387 represent a derived character linking the genera *Rhodax*, *Opisthobursa* and *Novomitchellia*,
388 not shared with *Balliania*, which situated the latter genus as sister to the rest of Cavernicola.
389 Unfortunately, molecular data for *Balliania* is not available and, therefore, we cannot put

390 forward a hypothesis on its position within the Cavernicola. However, for the genus *Hausera*
391 the situation is different, in that the oviducts are exactly dorsal to the nerve cords (Leal-
392 Zanchet et al., 2014); the precise character state for the course of the oviducts in relation to
393 the ventral nerve cords is not known for *Kawakatsua pumila* (Sluys and Laumer, 2019).
394 Under the present phylogeny "oviducts running medially to the ventral nerve cords" may still
395 be postulated as a synapomorphy for all cavernicolans included in our analysis (Fig. 3), under
396 the assumption that at least *Hausera* has evolved another character state in which the oviducts
397 run dorsally to the ventral nerve cords.

398 The high differentiation found in the tree (Fig. 3) between some of the representatives
399 of *Rhodax* included in our study may point to the presence of more than one species, but this
400 issue may be resolved only by a thorough study that also includes morphological data, which
401 currently is unavailable. Even more surprising is the low genetic differentiation between the
402 genera *Kawakatsua* and *Hausera*, while these are well differentiated at the morphological
403 level (Sluys and Laumer, 2019) and are geographically distant. This situation clearly shows,
404 on the one hand, that a broader sampling most probably will reveal new species for the
405 Cavernicola, and, on the other hand, that within this suborder levels of genetic diversification
406 may vary among different groups.

407 **4.3 Origin and evolution of the Cavernicola**

408 One of the most conspicuous features of this group is its rarity (only a handful of
409 genera, each with merely one or two species, mostly present at a single locality), together with
410 their disjunct distributions (Fig. 1). The fact that currently known species occur at tropical
411 latitudes and that cavernicolans thus far have not been reported from relatively well sampled
412 areas such as Europe and North America suggests that the Cavernicola fauna has a
413 predominantly intertropical distribution. However, it is important to realize that the current

414 distribution, including its disjunctions, may simply reflect a collector's artefact, due to
415 comparatively low investments in research of subterranean habitats in most regions of the
416 world, excepting Europe and North America.

417 Our phylogenetic trees suggest that the Cavernicola forms an old group. Although no
418 strict time calibration of the entire order Tricladida has been performed, the few molecular
419 timetrees published for *Dugesia* (Solà, 2014; Solà et al., 2013) have situated the
420 diversification of this continenticolan freshwater genus at approximately 150 million years
421 ago (Mya), which implies that the origin of the Continenticola lies even much further back in
422 time. From this perspective, the present phylogenetic tree suggests a great antiquity also for
423 the Cavernicola. This agrees with one of the possibilities for the evolution of the Cavernicola
424 proposed by Sluys (1990), i.e., that the group had differentiated on Gondwana and perhaps
425 already on Pangea. This hypothesis seems plausible given the sister-group relationship
426 between *Opisthobursa* and *Novomitchellia*, genera present in Mexico and Benin, respectively;
427 in turn, this clade is sister to the clade formed by *Hausera* and *Kawakatsua*, from northeastern
428 Brazil and Panama, respectively (Figs 1, 3). This suggests that the common ancestor of these
429 four lineages may have lived on Gondwana before this supercontinent started to breakup
430 around 200 Mya (McLoughlin, 2001; Storey, 1995). As Panama and Mexico were not part of
431 Gondwanaland, this scenario presumes that after breakage some descendants of the American
432 lineage dispersed to North America via the Panamanian isthmus when North and South
433 America were eventually connected with each other.

434 Despite the presumed antiquity of the group, one could argue, alternatively, that it
435 exhibits signs of recent dispersal since both Panama and Tahiti are the result of recent (in the
436 last 5 million years) volcanism, with the latter being a true oceanic island with no connection
437 to any continental landmass. Therefore, an alternative hypothesis might be that there must
438 have been a mechanism for dispersal. But if cavernicolans could disperse, relatively recently,

to such habitats, it may similarly have been possible for them to disperse away from Gondwana in ancient times, subsequent to its breakup, or even to Gondwana from elsewhere. One may be tempted to favour such dispersal explanations in view of the situation that an hypothesis about Gondwanan origins of the Cavernicola currently lacks any representation from South Africa, southern South America, Australia, and New Zealand. Generally, much of the evidence for true Gondwanan relicts in other taxa hinges on representation from such areas and even then molecular timetrees may falsify presumed evolution on Gondwana, as was the case with ratite birds (Reilly, 2019 and references therein). ~~However, it may well have been the case that a taxon had a distribution that was restricted already to a certain region of Gondwana, which may have applied also to the Cavernicola.~~

Although calibrated timetrees are as yet not available for the Cavernicola, what is known about the absolute age of triclad flatworms (see above) suggests that the group is ancient. Accepting the antiquity of the group, one may wonder whether the cavernicolans had already evolved their troglobitic adaptations on Pangea or Gondwana. Harrath et al. (2016) put forward two alternative hypotheses for the origin of the Cavernicola. According to their first hypothesis, which was based on the close phylogenetic relationship of the Cavernicola to the Maricola, marine ancestors were forced to invade the underground habitat due to gradual recession of the sea, after which the worms adapted to the hypogean freshwater habitat. A similar scenario was suggested to explain the ecology of *Hausera hauseri* from the karstic Jandaíra formation in northeastern Brazil (Leal-Zanchet et al., 2014). Under their first scenario, Harrath et al. (2016) proposed that epigean *R. evelinae* would have evolved from stygobiont populations and have again acquired the eyes that were lost in its underground ancestors, a possibility that has also been hypothesized for some crustaceans (Humphreys, 2000, and references therein). The second scenario sketched by Harrath et al. (2016) for the evolution of the Cavernicola proposed that an ancestral brackish- and freshwater-tolerant

epigean maricolan species led to a brackish water-tolerant *Rhodax* ancestor and to a lineage that invaded the phreatic habitat, possibly to escape presumed competition with continenticolans. In this scenario presence of pigmentation and eyes in *Rhodax* simply reflects retention of the ancestral character states.

Our ancestral states analyses revealed that the character conditions for the ancestor of the Cavernicola are most probably epigean and freshwater (0.80 and 0.98~~7~~ posterior probability, respectively~~, appendix D~~, Fig. 4, Appendix D), implying diversification of the cavernicolans from worms originally adapted to continental epigean freshwater habitats. In this scenario presence of pigmentation and eyes in *Rhodax* then most probably reflects retention of the ancestral conditions, while for *Kawakatsua pumila* the probability of having retained the ancestral epigean character state is lower (its ancestor with *Hausera* has a 0.51 probability of having been epigean). With respect to salinity tolerance, our analyses show that the ancestor of the Maricola + Cavernicola has a higher probability of being a freshwater animal, or at least being tolerant to freshwater, than that it was a marine species (0.55 and 0.41 vs. 0.03, node 10, Fig. 4A, Appendix D.1~~, Figure 4~~); therefore, the Cavernicola may not have had a marine ancestor. This lends support to the second scenario for the evolution of the Cavernicola sketched by Harrath et al. (2016). On the other hand, our results falsify the scenario suggested by Leal-Zanchet et al. (2014) that *Hausera* evolved directly from marine ancestors that entered the caves and then adapted to the freshwater environment.

We hypothesize here that the scenario for the Cavernicola, with dispersal of freshwater animals and colonization of hypogean habitats, resembles cases currently known for the Continenticola, such as representatives of *Girardia* from South America (Souza et al., 2015, 2016; Hellmann et al., 2018), and many species of the Dendrocoelidae in Europe (Stocchino et al., 2017, and references therein) that occur in caves or in surface waters. However, in contrast to *Girardia,* in which epigean species outnumber hypogean taxa, epigean species of

the Cavernicola presently represent a minority, as compared with hypogean members of the same suborder (for which at least three more undescribed species occur in the Jandaíra formation; AMLZ, unpublished data), or with epigean species of the Continenticola. This scarcity of cavernicolans in general and that of epigean ones in particular, may be the result of a loss of diversity due to climatic changes or competition with other groups. Specifically, the loss of suitable epigean habitats, and/or competition with continenticolan species, the latter group showing a broad radiation in the same biogeographic regions that also house cavernicolans, could underlie the paucity of epigean cavernicolans. Although these explanations may seem highly speculative with the data at hand, the karstic Jandaíra formation in northeastern Brazil, where *Hausera* species inhabit (Appendix E1 and E2), may be an example of such a loss of suitable epigean habitat. In this area, surface karstification forms recharge zones, favouring the storage and flow of subterranean water (Miranda, 2012), constituting the only water source in most of the region, where epigean streams are scarce (Fernandes et al., 2005). Therefore, no epigean species are expected to be able to survive under these conditions. Thus, under this tentative scenario, caves may have become a refuge for the cavernicolans.

In summary, our results lend support to the hypothesis of a freshwater ancestor of the Cavernicola that colonized continental epigean and phreatic habitats and, subsequently, radiated to form a diverse group with a broad distribution~~, probably on Gondwanaland~~. Under this scenario the evolution of the Cavernicola constitutes a classical example of evolutionary diversification, followed by independent adaptations to hypogean habitats, where caves may have become a refuge habitat for the group for reasons still not fully understood.

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7. REFERENCES

Álvarez-Presas, M., Baguñà, J., Riutort, M., 2008. Molecular phylogeny of land and freshwater planarians (Tricladida, Platyhelminthes): From freshwater to land and back. Mol. Phylogenet. Evol. 47, 555–568.

Ball, I.R., 1977. On the phylogenetic classification of aquatic planarians. Acta Zool. Fenn. 154, 21–35.

Ball, I.R., 1974. A contribution to the Phylogeny and Biogeography of the Freshwater Triclads (Platyhelminthes: Turbellaria), in: Riser, N.W., Morse, M.P. (Eds.), Biology of the Turbellaria, McGraw-Hill, NY. New York, pp. 339-401.

536 Benazzi, M., 1975. *Opisthobursa josephinae*, a new troglobitic planarian from Chiapas,
537 Mexico. Atti della Accad. Nazi onale dei Lincei LIX, 533–536.

538 Bollback, J., 2006. SIMMAP: Stochastic character mapping of discrete traits on phylogenies.
539 BMC Bioinformatics 7, 88. https://doi.org/10.1186/1471-2105-7-88

540 Carranza, S., Giribet, G., Ribera, C., Riutort, M., 1996. Evidence that two types of 18S rDNA
541 coexist in the genome of *Dugesia* (Schmidtea) *mediterranea* (Platyhelminthes,
542 Turbellaria, Tricladida). Mol. Biol. Evol. 13, 824–832.
543 https://doi.org/10.1093/oxfordjournals.molbev.a025643

544 Castresana, J., 2000. Selection of conserved blocks from multiple alignments for their use in
545 phylogenetic analysis. Mol Biol Evol 17, 540–552.

546 Darriba, D., Taboada, G.L., Doallo, R., Posada, D., 2012. JModelTest 2: More models, new
547 heuristics and parallel computing. Nat. Methods 9, 772.
548 https://doi.org/10.1038/nmeth.2109

549 Fernandes, M.A.B., Santiago, M.F., Gomes, D.F., Filho, J.M., Frischkorn, H., Lima, J.G.,
550 2005. A origm dos cloretos nas águas subterrâneas na Chapada do apodi-Ceará. Águas
551 subterrâneas 19, 25–34.

552 Harrath, A.H., Mansour, L., Lagnika, M., Sluys, R., Boutin, C., Alwasel, S., Poch, A., Riutort,
553 M., 2016. A molecular analysis of the phylogenetic position of the suborder Cavernicola
554 within the Tricladida (Platyhelminthes), with the description of a new species of
555 stygobiont flatworm from Benin. Zool. J. Linn. Soc. 178, 482-491.
556 https://doi.org/10.1111/zoj.12430

557 Humphreys, W.., 2000. Relict fauna and their derivation, in: Wilkens, H., Culver, D.,
558 Humphreys, W. (Eds.), Subterranean Ecosystems. Elsevier, Amsterdam, pp. 417–432.

559 Katoh, K., Standley, D.M., 2013. MAFFT Multiple Sequence Alignment Software Version 7:
560 Improvements in Performance and Usability. Mol. Biol. Evol. 30, 772–780.
561 https://doi.org/10.1093/molbev/mst010

562 Kumar, S., Stecher, G., Li, M., Knyaz, C., Tamura, K., 2018. MEGA X: Molecular
563 Evolutionary Genetics Analysis across Computing Platforms. Mol. Biol. Evol. 35, 1547–
564 1549. https://doi.org/10.1093/molbev/msy096

565 Laumer, C.E., Giribet, G., 2014. Inclusive taxon sampling suggests a single, stepwise origin
566 of ectolecithality in Platyhelminthes. Biol. J. Linn. Soc. 111, 570–588.
567 https://doi.org/10.1111/bij.12236

568 Laumer, C.E., Hejnol, A., Giribet, G., 2015. Nuclear genomic signals of the
569 "microturbellarian" roots of platyhelminth evolutionary innovation. Elife 4, 1–31.
570 https://doi.org/10.7554/eLife.05503

571 Leal-Zanchet, A.M., De Souza, S.T., Ferreira, R.L., 2014. A new genus and species for the
572 first recorded cave-dwelling Cavernicola (Platyhelminthes) from South America.
573 Zookeys 15, 1–15. https://doi.org/10.3897/zookeys.442.8199

574 Maddison, W.P., Maddison, D.R., 2008. Mesquite: A modular system for evolutionary
575 analysis. Evolution (N. Y). 62, 1103–1118. https://doi.org/http://mesquiteproject.org

576 Marcus, E., 1946. Sôbre Turbellaria límnicos brasileiros. Bol. Fac. Filos. Ciênc. Let., Zool.
577 Univ. São Paulo 11, 5–254.

578 McLoughlin, S., 2001. The breakup history of Gondwana and its impact on pre-Cenozoic
579 floristic provincialism. Aust. J. Bot. 49, 271-300. https://doi.org/10.1071/BT00023

580 Miranda, T.S. De, 2012. Estudo do comportamento geológico-estrutural dos depósitos

581 Cretácicos do sistema aquífero Apodi, Bacia Potiguar, Ne do Brasil. Estud. Geológicos
582 22, 3–19.

583 Mitchell, R.W., Kawakatsu, M., 1972. A new family, genus, and species of cave-adapted
584 planarian from Mexico (Turbellaria, Tricladida, Maricola). Occas. Pap. Museum Texas
585 Tech Univ. 8, 1–16.

586 Norén, M., Jondelius, U., 2002. The phylogenetic position of the Prolecithophora
587 (Rhabditophora, 'Platyhelminthes'). Zool. Scr. 31, 403–414.
588 https://doi.org/10.1046/j.1463-6409.2002.00082.x

589 Rambaut, A., Drummond, A.J., 2007. Tracer v1.4. Disponible en: beast.bio.ed.ac.uk/Tracer.

590 Reilly, J., 2019. The Ascent of Birds – How Modern Science is Revealing their Story. Pelagic
591 Publishing, Exeter.

592 Revell, L.J., 2012. phytools: an R package for phylogenetic comparative biology (and other
593 things). Methods Ecol. Evol. 3, 217–223. https://doi.org/10.1111/j.2041-
594 210X.2011.00169.x

595 Riutort, M., Álvarez-Presas, M., Lazaro, E.M., Solà, E., Paps, J., 2012. Evolutionary history
596 of the Tricladida and the Platyhelminthes: an up-to-date phylogenetic and systematic
597 account. Int. J. Dev. Biol. 56, 5–17. https://doi.org/10.1387/ijdb.113441mr

598 Ronquist, F., Teslenko, M., van der Mark, P., Ayres, D.L., Darling, A., Höhna, S., Larget, B.,
599 Liu, L., Suchard, M. a, Huelsenbeck, J.P., 2012. MrBayes 3.2: efficient Bayesian
600 phylogenetic inference and model choice across a large model space. Syst. Biol. 61,
601 539–42. https://doi.org/10.1093/sysbio/sys029

602 Sluys, R., 1990. A monograph of the Dimarcusidae (Platyhelminthes, Seriata, Tricladida).

603 Zool. Scr. 19, 13–29.

604 Sluys, R., 1989. Phylogenetic relationships of the triclads (Platyhelminthes, Seriata,
605 Tricladida). Bijdr. Dierk. 59, 3–25.

606 Sluys, R., Kawakatsu, M., Riutort, M., Baguñà, J., 2009. A new higher classification of
607 planarian flatworms (Platyhelminthes, Tricladida). J. Nat. Hist. 43, 1763–1777.
608 https://doi.org/10.1080/00222930902741669

609 Sluys, R., Laumer, C., 2019. A new genus and species of a terrestrial cavernicolan planarian
610 from Barro Colorado Island, Panama (Platyhelminthes, Tricladida, Cavernicola).
611 Zootaxa 4586, 187–193.

612 Sluys, R., Riutort, M., 2018. Planarian Diversity and Phylogeny, in: Rink, J.C. (Ed.),
613 Planarian Regeneration: Methods and Protocols. Methods in Molecular Biology, Vol.
614 1774. Springer, New York, pp. 1–56. https://doi.org/10.1007/978-1-4020-6682-5

615 Sluys, R., Vila-Farré, M., Álvarez-Presas, M., Riutort, M., Kawakatsu, M., Tulp, A.S., 2015.
616 The diet and distribution of *Pentacoelum kazukolinda* (Kawakatsu & Mitchell, 1984), a
617 maricolan planarian with a freshwater ecology. Zool. Scr. 44, 72–91.
618 https://doi.org/10.1111/zsc.12084

619 Solà, E., 2014. Systematics and historical biogeography of the genus Dugesia. Unpublished
620 PhD thesis. Universitat de Barcelona.

621 Solà, E., Sluys, R., Gritzalis, K., Riutort, M., 2013. Fluvial basin history in the northeastern
622 Mediterranean region underlies dispersal and speciation patterns in the genus *Dugesia*
623 (Platyhelminthes, Tricladida, Dugesiidae). Mol. Phylogenet. Evol. 66, 877–888.

624 Souza, S., Morais, A.L., Bichuette, M.E., Leal-Zanchet, A.M., 2016. Two new species of

freshwater flatworms (Platyhelminthes: Tricladida: Continenticola) from South American caves. Zootaxa 4092, 107–121.

Stamatakis, A., 2014. RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. Bioinformatics 30, 1312–1313. https://doi.org/10.1093/bioinformatics/btu033

Stocchino, G.A., Sluys, R., Kawakatsu, M., Sarbu, S.M., Manconi, R., 2017. A new species of freshwater flatworm (Platyhelminthes , Tricladida , Dendrocoelidae) inhabiting a chemoautotrophic groundwater ecosystem in Romania. Eur. J. Taxon. 342, 1–21.

Storey, B.C., 1995. The role of mantle plumes in continental breakup: case histories from Gondwanaland. Nature 377, 301–308. https://doi.org/10.1038/377301a0

Xia, X., 2017. DAMBE6: New tools for microbial genomics, phylogenetics, and molecular evolution. J. Hered. 108, 431–437. https://doi.org/10.1093/jhered/esx033

Xia, X., Lemey, P., 2009. Assessing substitution saturation with DAMBE., in: Lemey, P., Salemi, M., Vandamme, A.-M. (Eds.), The Phylogenetic Handbook: A Practical Approach to DNA and Protein Phylogeny. Cambridge University Press, Cambridge, pp. 615–630. https://doi.org/10.1017/CBO9780511819049.022

LEGENDS TO THE FIGURES AND TABLES

Figure 1. Distribution of cavernicolan taxa; all known sites are shown. The taxa included in this study are highlighted in boldface.

Figure 2. Bayesian Inference tree inferred from the dataset including 18S and 28S sequences of representatives of the various Suborders of the Tricladida (~~Sp-set-I-18S+28S~~dataset_I-

[18S+28S]). Values at nodes correspond to posterior probability/bootstrap support. *: 1.00 and 100% values for BI and ML, respectively. Scale bar: number of substitutions per nucleotide position.

Figure 3. Relationships within the Cavernicola inferred by Bayesian Inference from the ~~Sp-set-II-18S+28S~~dataset II-[18S+28S] dataset. Numbers at nodes indicate posterior probability/bootstrap supports for BI and ML, respectively. *: 1.00 and 100% values for BI and ML, respectively. Scale bar indicates number of substitutions per site.

Figure 4. Results of the Ancestral States Reconstruction analysis based on the Bayesian Inference tree obtained from the ~~ASR-18S dataset~~dataset III-[18S]. Pie charts at nodes represent the posterior probabilities of ASR analysis for A: epigean (green) and hypogean (yellow) habitat and B: freshwater (red), freshwater-marine (purple) and marine (blue) salinity tolerance. Terrestrial species were scored for the freshwater condition, as they are only able to survive in a humid habitat. For exact posterior probabilities obtained at each node, see Appendix D.

Figure 5. Morphological phylogeny of Cavernicola based on Sluys (1990). Black rectangles represent morphological apomorphies.

Table 1. Geographic coordinates of the type localities, and habitat as well as stygobiont conditions of cavernicolan taxa.

Table 2. Species names, higher taxa, and GenBank accession numbers for sequences used in the analyses. Species selected to represent each of the cavernicolan genera highlighted in boldface.

LEGENDS TO THE APPENDICES

Appendix A. Dorsal view of live specimens of *Rhodax* sp. 3 (A1) from surface water in Tramandaí, southern Brazil, and *Hausera* sp. (A2) from Furna Feia cave, northeastern Brazil. Anterior end to the left.

Appendix B. Bayesian Inference tree of Tricladida suborders based on the ~~Sp-set-I-18S~~dataset I-[18S] (B1) and ~~Sp-set-I-28S~~dataset I-[28S] (B2) datasets. Numbers at nodes indicate posterior probability/bootstrap supports for BI and ML, respectively. *: 1.00/100% values for BI/ML. Scale bar indicates number of substitutions per site.

Appendix C. Bayesian Inference tree of Suborder Cavernicola inferred from the ~~Sp-set-II-18S~~dataset II-[18S] (C1) and ~~Sp-set-II-28S~~dataset II-[28S] (C2) data sets. Numbers at nodes indicate posterior probability/bootstrap supports for BI and ML, respectively. *: 1.00/100% values for BI/ML. Scale bar indicates number of substitutions per site.

Appendix D. Posterior probability values at each node in Figure 4 for the conditions habitat (epigean/hypogean) (D1) and salinity tolerance (freshwater/freshwater-marine/marine) (D2), obtained in the Ancestral States Reconstruction analysis using the make.simmap fuction from the R package Phytools.

Appendix E. Habitats of cavernicolan taxa included in this study. E1: Crotes cave, northeastern Brazil (type locality of *Hausera hauseri*); E2: Furna Feia cave, northeastern Brazil (*Hausera* sp.); E3: Parakou, Benin (type locality of *Novomitchellia bursaelongata*); E4: Grutas de Coconá cave, México (type locality of *Opisthobursa mexicana*); E5: Mostardas, southern Brazil (*Rhodax* sp.1); E6: Tavares, southern Brazil (*Rhodax* sp.1); E7: Santo Antonio de Patrulha, southern Brazil (*Rhodax* sp.2); E8: Tramandaí, southern Brazil (*Rhodax* sp.3).

693

694 **Table 1.** Geographic coordinates of the type localities, and habitat as well as stygobiont conditions of cavernicolan taxa.

Taxon	Locality	Geographic coordinates	Habitat	Stygobiont features
Rhodax evelinae Marcus, 1946	Sao Paulo city, São Paulo, Brazil	-	Lotic and lentic superficial waters	No
Opisthobursa mexicana Benazzi, 1972	Las Grutas de Coconá, Teapa, Tabasco, Mexico	17.616667, -92.966667	Cave	Yes
Opisthobursa josephinae Benazzi, 1975	Pozza Casa Bell, San Cristobal de las Casas, Estado de Chiapas, Mexico	16.716667, -92.666667	Cave	Yes
Balliania thetisae Gourbault, 1978	Maraa, Paea, Tahiti[a]	-	Phreatic layer	Yes
Opisthobursa sp. Kawakatsu & Mitchell, 1983	Grutas de Languín, Alta Verapaz, Guatemala	15.573611, -89.980556	Cave	Yes
Kawakatsua pumila Sluys, 2019	Barro Colorado, Panama	9.15265, -79.85172	Large pile of humid leaf mulch between two buttress roots of an old broadleaf tree	Yes
Hausera hauseri Leal-Zanchet & Souza, 2014	Crotes cave, Felipe Guerra, Rio Grande do Norte, Brazil	-5.592344, -37.686183	Cave	Yes
Novomitchellia sarawakana (Kawakatsu & Chapman, 1983)	Water Polo Cave, Gunung Mulu National Park, Sarawak, Malaysia	4.000000, 114.852778	Cave	Yes
Novomitchellia bursaelongata Harrath, Sluys & Riutort, 2016	Parakou, Benin Republic	9.272972, 2.581861	Waterhole (7.8 m depth and 6.6 m height)	Yes
Hausera sp. 1	Furna Feia cave, Baraúna, Rio Grande do Norte, Brazil	-5.036877, -37.560177	Cave	Yes
Rhodax sp. 1_1	Tavares, Rio Grande do Sul, Brazil	-31.280277, -51.060555	Coastal wetland	No
Rhodax sp. 1_2	Tavares, Rio Grande do Sul, Brazil	-31.317500, -51.122777	Coastal wetland	No
Rhodax sp. 2	Pinheirinhos, Santo Antônio da Patrulha, Rio Grande do Sul, Brazil	-29.71205, -50.638233	Rice field	No
Rhodax sp. 3	Tramandaí, Rio Grande do Sul, Brazil	-30.087777, -50.170833	Coastal wetland	No

695 [a] Temporary water course close to the Insectarium of the *Institut de Recherches médicales* «Louis Malardé»

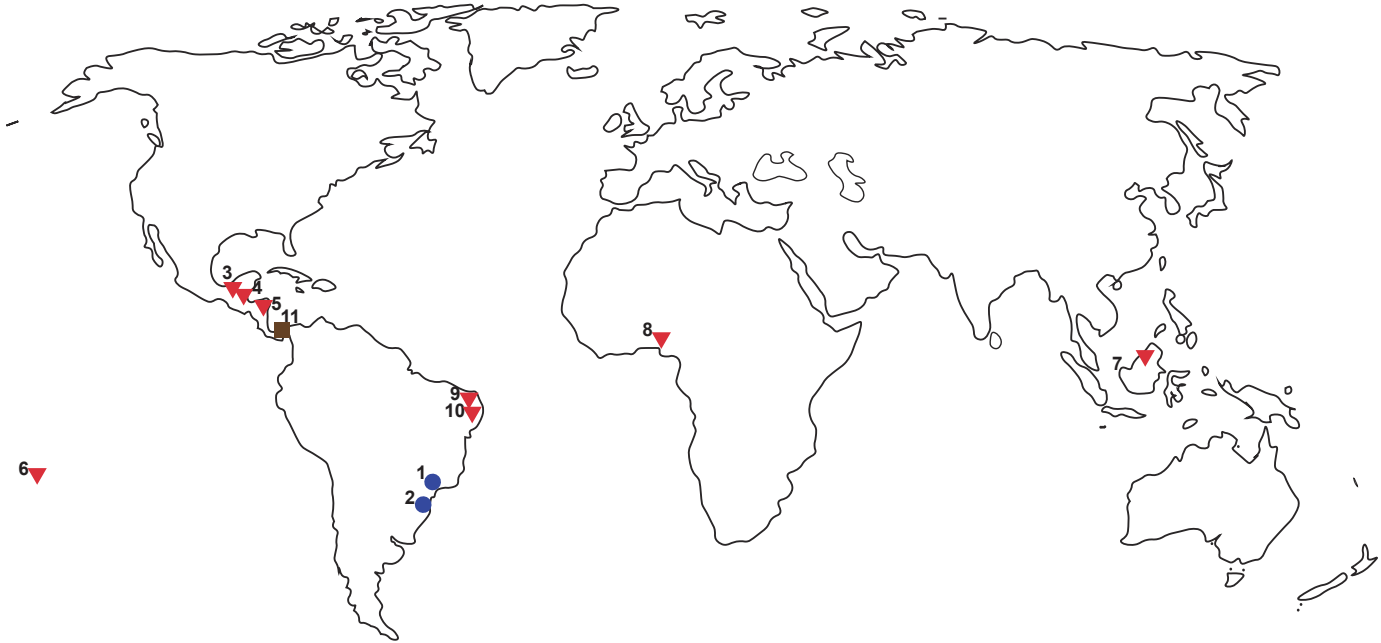
696 **Table 2.** Species names, taxonomic classification, and GenBank accession numbers for the Plathyheminthes sequences used in the analyses.
697 Species selected to represent each of the cavernicolan genera are highlighted in boldface.

698

Species name	Taxon	18S	28S
Protomonotresidae sp.[a]	Rhabditophora: Prolecithophora: Protomonostresidae	KC869820	KC869873
Acanthiella sp.[a]	Rhabditophora: Prolecithophora: Protomonotresidae	KC869786	KC869839
Reisingeria hexaoculata[a]	Rhabditophora: Prolecithophora: Pseudostomidae	AF065426	AY157157
Plagiostomum stellatum[a]	Rhabditophora: Prolecithophora: Plagiostomidae	KC869819	KC869872
Plagiostomum whitmani[a]	Rhabditophora: Prolecithophora: Plagiostomidae	KC869818	KC869871
Plicastoma cuticulata[a]	Rhabditophora: Prolecithophora: Plagiostomidae	AF065422	AY157158
Notentera ivanovia[a]	Rhabditophora: Rhabdocoela: Dalyellioida: Fecampiidae	AJ287546.1	AY157167.1
Kronborgia isopodicola[a]	Rhabditophora: Rhabdocoela: Dalyellioida: Fecampiidae	AJ012513.1	AF022862.1
Urastoma cyprinae[a]	Rhabditophora: Mediofusata: Urastomidae	AF065428.2	AY157165.1
Bdelloura candida	Tricladida: Maricola: Bdellouroidea: Bdellouridae	Z99947.1	AJ270167.1
Nerpa fistulata[b]	Tricladida: Maricola: Bdellouroidea: Bdellouridae	MH916614.1	-
Palombiella stephensoni	Tricladida: Maricola: Bdellouroidea: Bdellouridae	DQ666008.2	DQ665988.1
Pentacoelum kasukolinda	Tricladida: Maricola: Bdellouroidea: Bdellouridae	JN009784.1	JN009787.1
Pentacoelum sinensis[b]	Tricladida: Maricola: Bdellouroidea: Bdellouridae	MK140782.1	-
Sluysia triapertura	Tricladida: Maricola: Bdellouroidea: Uteriporidae	MF383119.1	MF383122.1
Paucumara falcata[b]	Tricladida: Maricola; Bdellouroidea; Uteriporidae	MH916612.1	-
Miroplana shenzhensis[b]	Tricladida: Maricola; Bdellouroidea; Uteriporidae	MK140778.1	-
Ectoplana limuli[b]	Tricladida: Maricola; Bdellouroidea; Uteriporidae	D85088.1	-
Obrimoposthia wandeli[b]	Tricladida: Maricola; Bdellouroidea; Uteriporidae	MH108586.1	-
Uteriporus sp	Tricladida: Maricola: Bdellouroidea; Uteriporidae	AF013148.1	-
Cercyra hastata	Tricladida: Maricola: Cercyroidea: Cercyridae	KM200902.1	DQ665962.1
Sabussowia dioica	Tricladida: Maricola: Cercyroidea: Cercyridae	JN009785.1	JN009788.1
Oregoniplana geniculata[b]	Tricladida: Maricola: Cercyroidea: Cercyridae	MH916614.1	-

Procerodes littoralis	Tricladida: Maricola: Procerodoidea: Procerodidae	Z99950.1	DQ665985.1
***Hausera hauseri* ***	**Tricladida: Cavernicola: Dimarcusidae**	MN719501	**MN719494**
Hausera sp. 1*	Tricladida: Cavernicola: Dimarcusidae	MN719502	MN719495
Kawakatsua pumila	Tricladida: Cavernicola: Dimarcusidae	KC869823.1	KC869876.1
Novomitchellia bursaelongata	Tricladida: Cavernicola: Dimarcusidae	KU096054.2	-
*Opisthobursa mexicana**	Tricladida: Cavernicola: Dimarcusidae	MN719503	MN719496
Rhodax sp. 1_1*	Tricladida: Cavernicola: Dimarcusidae	MN719504	MN719497
Rhodax sp. 1_2*	Tricladida: Cavernicola: Dimarcusidae	MN719505	MN719498
Rhodax* sp. 2	**Tricladida: Cavernicola: Dimarcusidae**	MN719506	MN719499
Rhodax sp. 3*	Tricladida: Cavernicola: Dimarcusidae	MN719507	MN719500
Dugesia gonocephala	Tricladida: Continenticola: Geoplanoidea: Dugesiidae	DQ666002.1	DQ665965.1
Girardia sp.	Tricladida: Continenticola: Geoplanoidea: Dugesiidae	AF013156.1	DQ665977.1
Schmidtea polychroa	Tricladida: Continenticola: Geoplanoidea: Dugesiidae	AF013154.1	DQ665993.1
Geoplana quagga	Tricladida: Continenticola: Geoplanoidea: Geoplanidae	KC608497.1	KC608380.1
Cephaloflexa bergi	Tricladida: Continenticola: Geoplanoidea: Geoplanidae	KJ599712.1	KC608355.1
Phagocata vitta	Tricladida: Continenticola: Planarioidea: Planariidae	DQ665998.1	DQ665989.1
Crenobia alpina	Tricladida: Continenticola: Planarioidea: Planariidae	M58345.1	DQ665960.1

*: sequences obtained in this study, Gen Bank accession number pending
[a]: outgroup sequences
[b]: Maricola species included only in the Ancestral Reconstruction States analysis



1 *Rhodax evelinae* Marcus, 1946
2 ***Rhodax*** **spp.**

3 ***Opisthobursa mexicana*** **Benazzi, 1972**
4 *Opisthobursa josephinae* Benazzi, 1975
5 *Opisthobursa* sp.

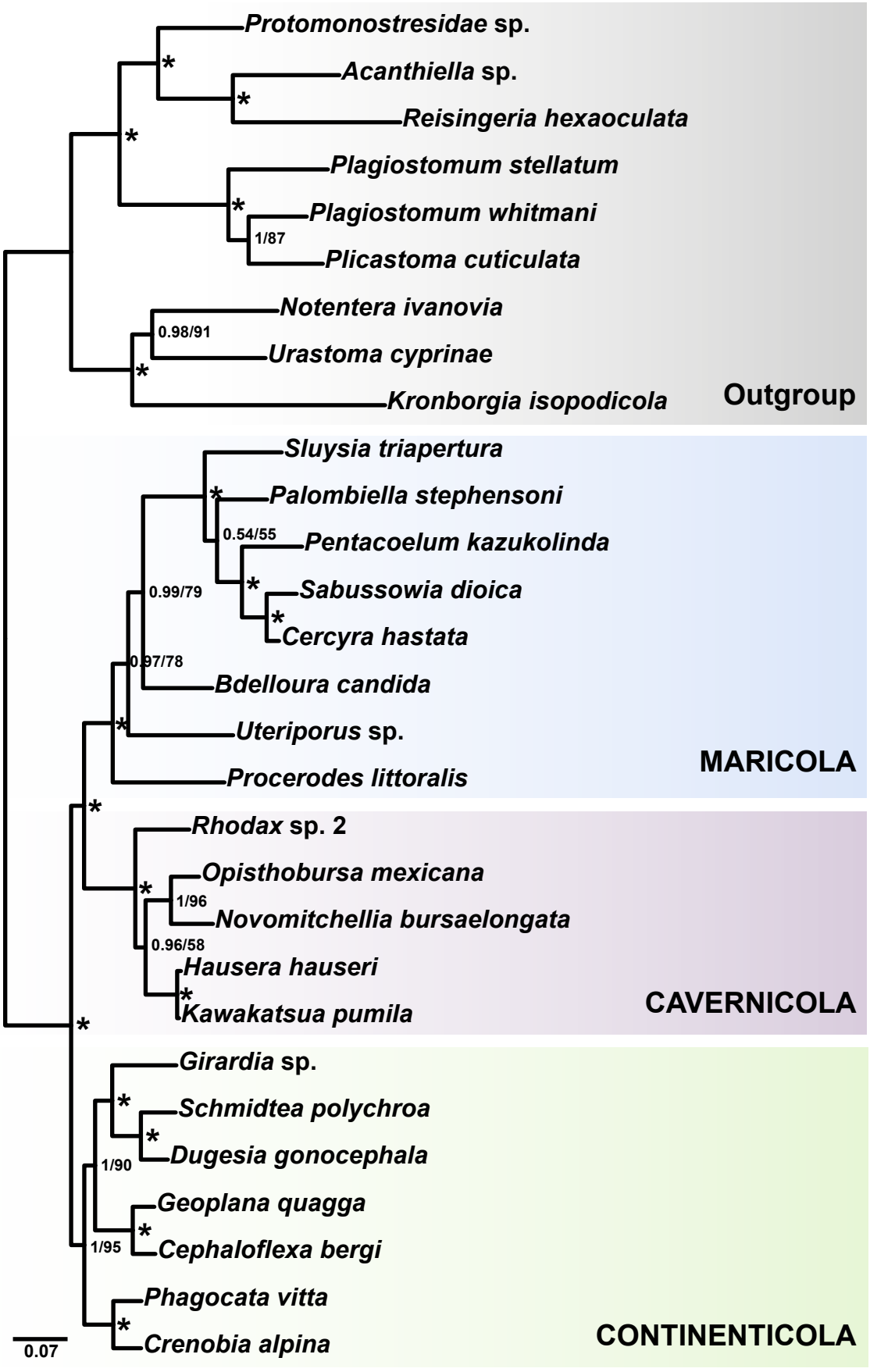
6 *Balliania thetisae* Goubault, 1978

7 *Novomitchellia sarawakana* (Kawakatsu & Chapman, 1983)
8 ***Novomitchellia bursaelongata*** **Harrath, Sluys & Riutort, 2016**

9 ***Hausera*** **sp.**
10 ***Hausera hauseri*** **Leal-Zanchet & Souza, 2014**

11 ***Kawakatsua pumila*** **Sluys, 2019**

Habitat:
▼: freshwater hypogean
●: freshwater epigean
■: terrestrial epigean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Acanthiella sp.
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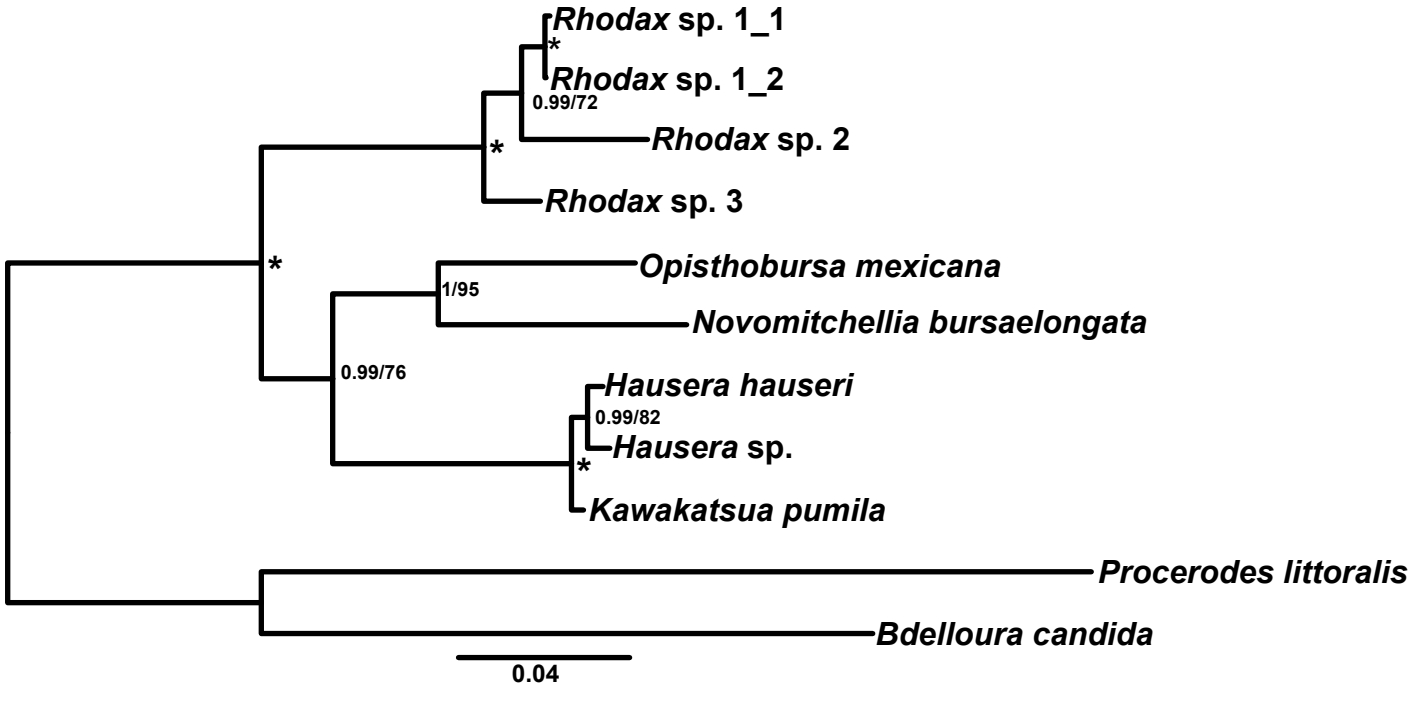
Plagiostomum stellatum
Plagiostomum whitmani
1/87
Plicastoma cuticulata
Notentera ivanovia
0.98/91
Urastoma cyprinae
Kronborgia isopodicola
Outgroup
Sluysia triapertura
Palombiella stephensoni
0.54/55
Pentacoelum kazukolinda
0.99/79
Sabussowia dioica
Cercyra hastata
0.97/78
Bdelloura candida
Uteriporus sp.
Procerodes littoralis
MARICOLA
Rhodax sp. 2
Opisthobursa mexicana
1/96
Novomitchellia bursaelongata
0.96/58
Hausera hauseri
Kawakatsua pumila
CAVERNICOLA
Girardia sp.
Schmidtea polychroa
Dugesia gonocephala
1/90
Geoplana quagga
1/95
Cephaloflexa bergi
Phagocata vitta
Crenobia alpina
CONTINENTICOLA
0.07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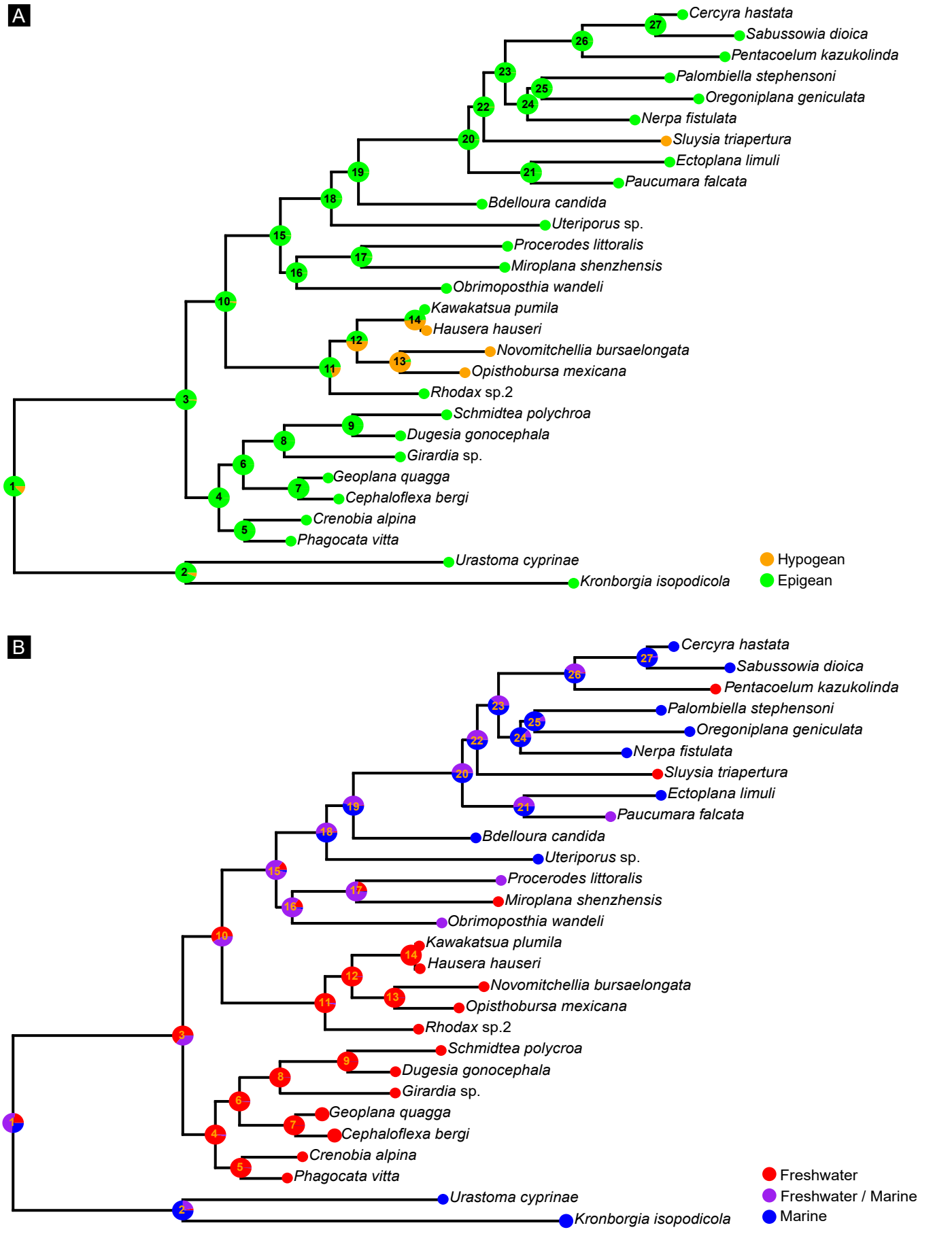
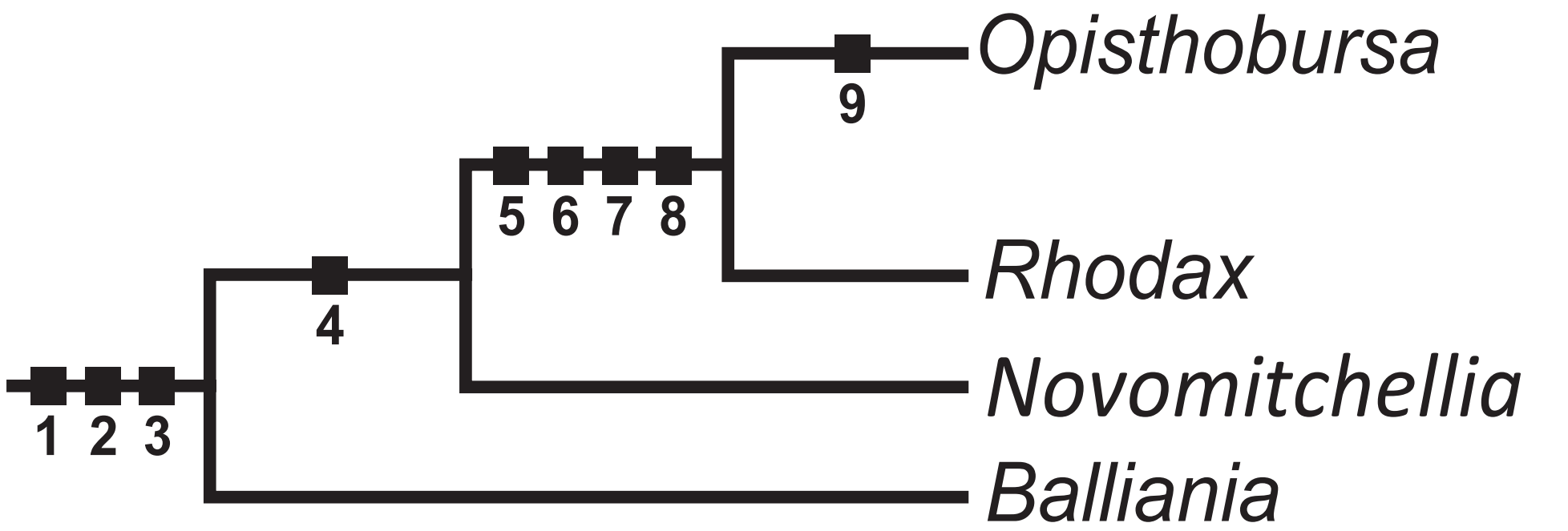
Rhodax sp. 1_2
0.99/72
Rhodax sp. 2
Rhodax sp. 3
Opisthobursa mexicana
1/95
Novomitchellia bursaelongata
0.99/76
Hausera hauseri
0.99/82
Hausera sp.
Kawakatsua pumila
Procerodes littoralis
Bdelloura candida
0.04


A
Cercyra hastata
Sabussowia dioica
Pentacoelum kazukolinda
Palombiella stephensoni
Oregoniplana geniculata
Nerpa fistulata
Sluysia triapertura
Ectoplana limuli
Paucumara falcata
Bdelloura candida
Uteriporus sp.
Procerodes littoralis
Miroplana shenzhensis
Obrimoposthia wandeli
Kawakatsua pumila
Hausera hauseri
Novomitchellia bursaelongata
Opisthobursa mexicana
Rhodax sp.2
Schmidtea polychroa
Dugesia gonocephala
Girardia sp.
Geoplana quagga
Cephaloflexa bergi
Crenobia alpina
Phagocata vitta
Urastoma cyprinae
Kronborgia isopodicola
Hypogean
Epigean
B
Cercyra hastata
Sabussowia dioica
Pentacoelum kazukolinda
Palombiella stephensoni
Oregoniplana geniculata
Nerpa fistulata
Sluysia triapertura
Ectoplana limuli
Paucumara falcata
Bdelloura candida
Uteriporus sp.
Procerodes littoralis
Miroplana shenzhensis
Obrimoposthia wandeli
Kawakatsua plumila
Hausera hauseri
Novomitchellia bursaelongata
Opisthobursa mexicana
Rhodax sp.2
Schmidtea polycroa
Dugesia gonocephala
Girardia sp.
Geoplana quagga
Cephaloflexa bergi
Crenobia alpina
Phagocata vitta
Urastoma cyprinae
Kronborgia isopodicola
Freshwater
Freshwater / Marine
Marine

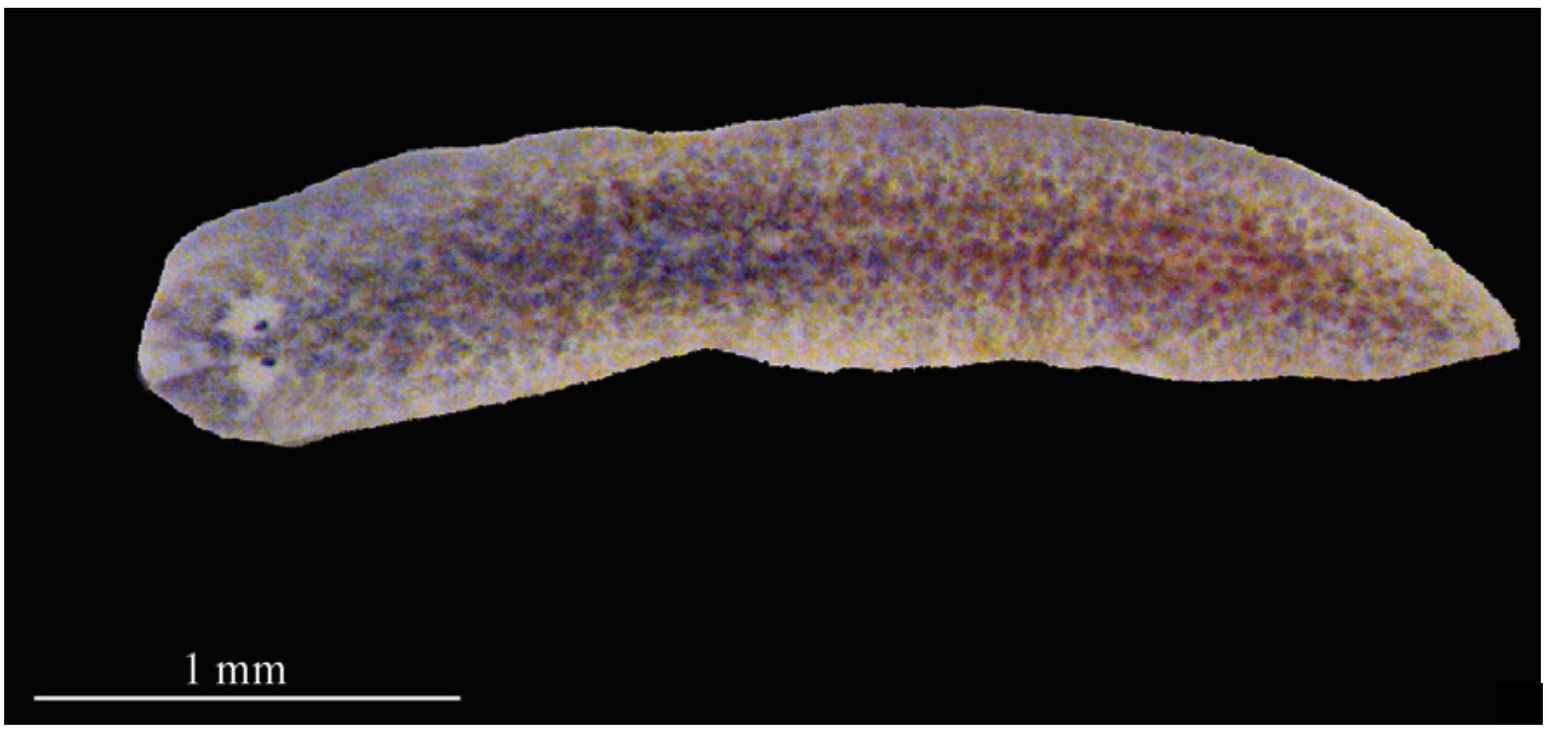


1: penis bulb with gland cells
2: T-junction bursal canal/female genital duct and common oviduct/diverticulum
3: ovaries at some distance posterior to the brain
4: oviducts medially to ventral nerve cords
5: posterior part bursal canal/female genital duct ciliated
6: vitellaria medially to the testes
7: absence of primary copulatory bursa
8: testes tubes
9: two gonopores

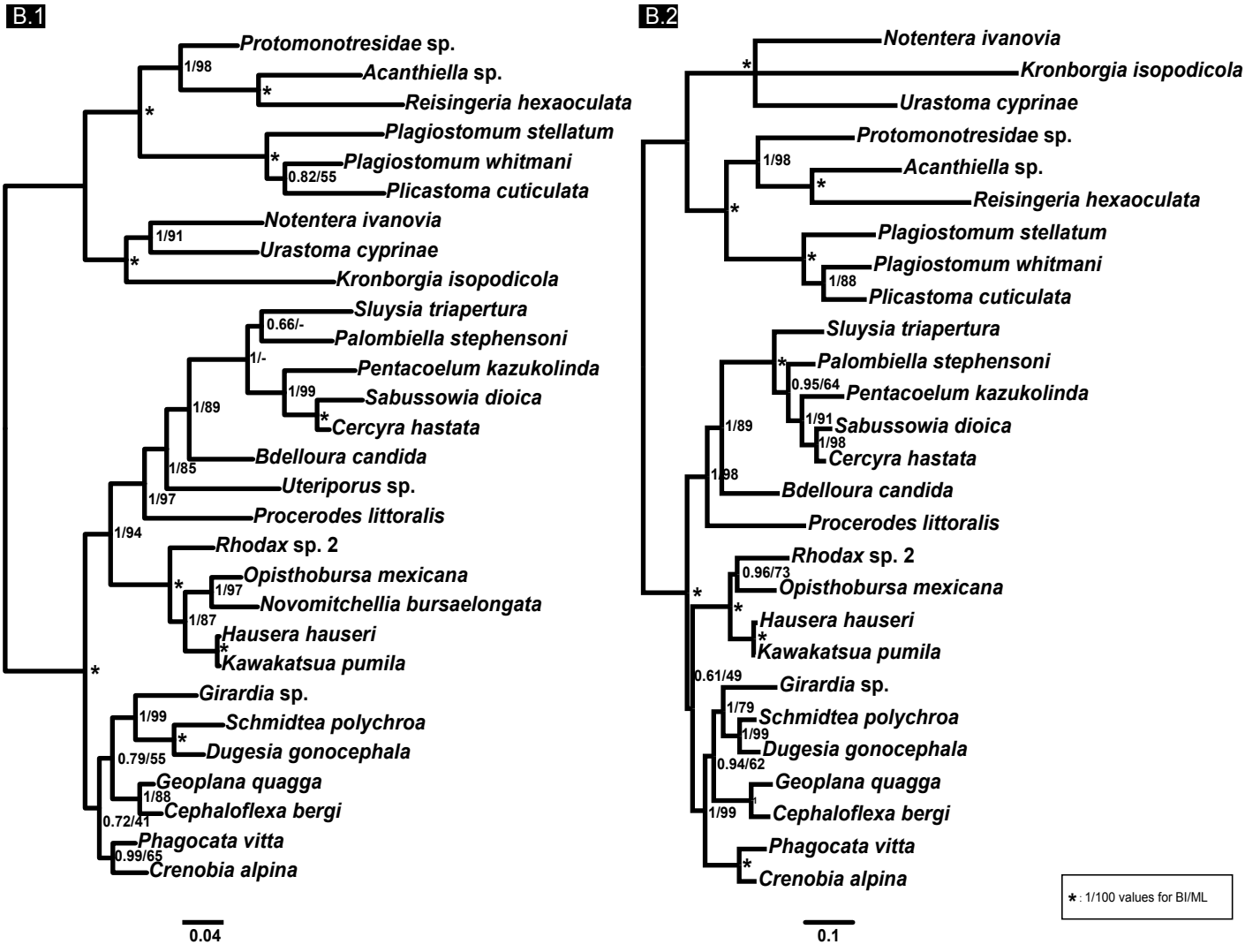
Author contibution statement

MR and AML-Z conceived the project. MR, AML-Z and AO-F obtained the funding suport. AO-F, RLF, DMB, JB contributed to the samplings. LB-A did all the wet laboratory and data analyses. MR and LB-A wrote the first draft of the manuscript. All the authors participated in the discussions of the results and on the writing of the manuscript. All authors read and approved the final version of the manuscript.

A.1



A.2


B.1
Protomonotresidae sp.
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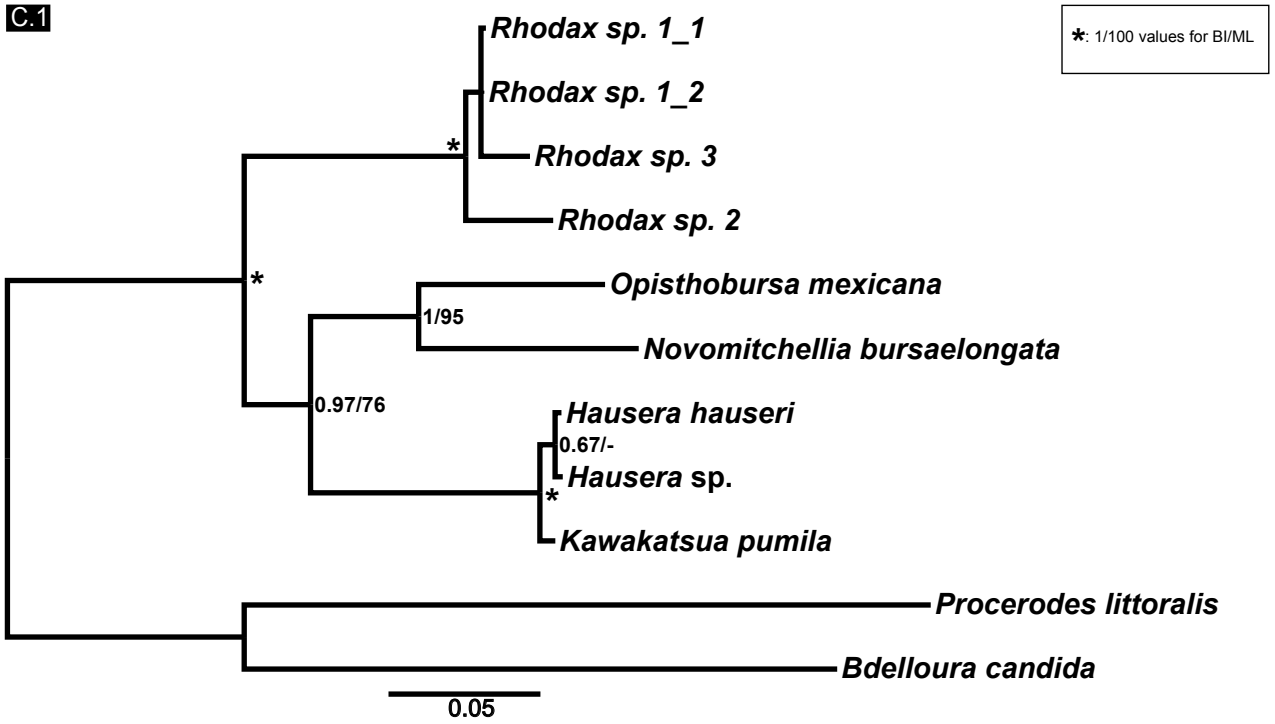
Plagiostomum stellatum
Plagiostomum whitmani
Plicastoma cuticulata
Notentera ivanovia
Urastoma cyprinae
Kronborgia isopodicola
Sluysia triapertura
Palombiella stephensoni
Pentacoelum kazukolinda
Sabussowia dioica
Cercyra hastata
Bdelloura candida
Uteriporus sp.
Procerodes littoralis
Rhodax sp. 2
Opisthobursa mexicana
Novomitchellia bursaelongata
Hausera hauseri
Kawakatsua pumila
Girardia sp.
Schmidtea polychroa
Dugesia gonocephala
Geoplana quagga
Cephaloflexa bergi
Phagocata vitta
Crenobia alpina
1/98
0.82/55
1/91
0.66/-
1/-
1/99
1/89
1/85
1/97
1/94
1/97
1/87
1/99
0.79/55
1/88
0.72/41
0.99/65
0.04
B.2
Notentera ivanovia
Kronborgia isopodicola
Urastoma cyprinae
Protomonotresidae sp.
Acanthiella sp.
Reisingeria hexaoculata
Plagiostomum stellatum
Plagiostomum whitmani
Plicastoma cuticulata
Sluysia triapertura
Palombiella stephensoni
Pentacoelum kazukolinda
Sabussowia dioica
Cercyra hastata
Bdelloura candida
Procerodes littoralis
Rhodax sp. 2
Opisthobursa mexicana
Hausera hauseri
Kawakatsua pumila
Girardia sp.
Schmidtea polychroa
Dugesia gonocephala
Geoplana quagga
Cephaloflexa bergi
Phagocata vitta
Crenobia alpina
1/98
0.95/64
1/91
1/98
1/89
1/98
0.96/73
0.61/49
1/79
1/99
0.94/62
1/99
1/88
0.1
* : 1/100 values for BI/ML

C.1


*: 1/100 values for BI/ML
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Rhodax sp. 1_2
Rhodax sp. 3
Rhodax sp. 2
Opisthobursa mexicana
1/95
Novomitchellia bursaelongata
0.97/76
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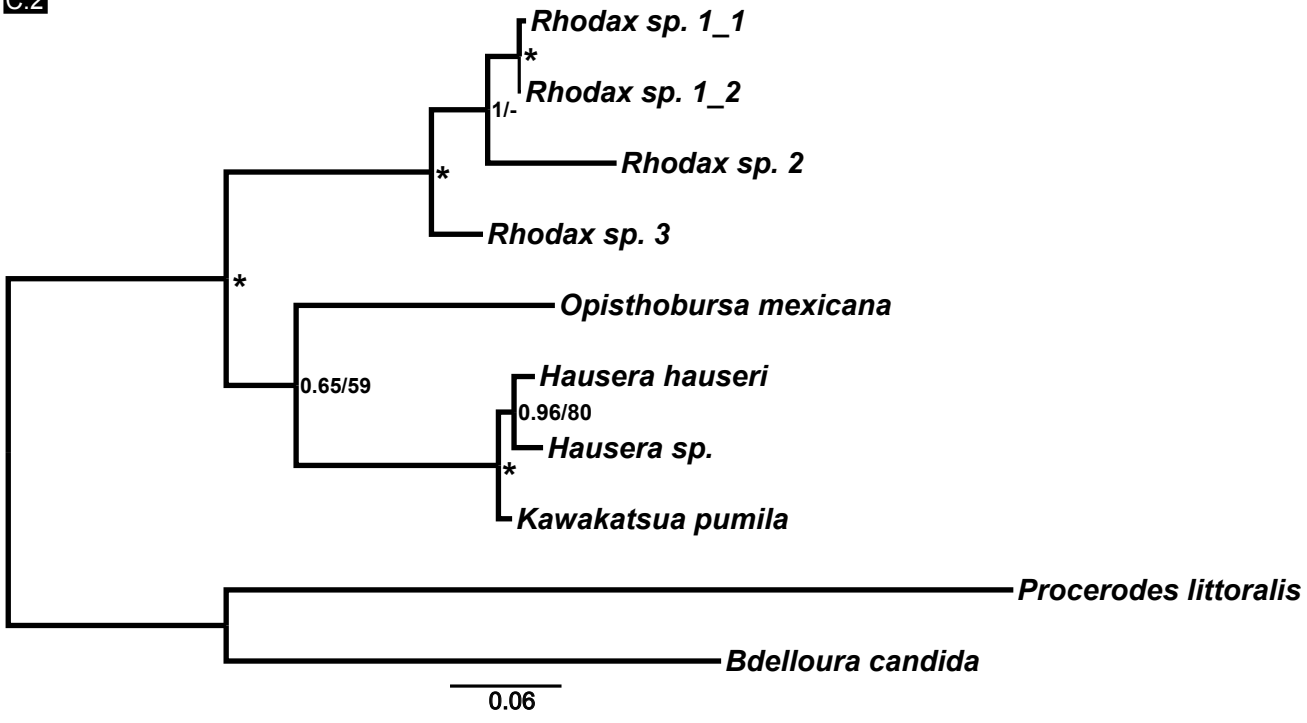
0.67/-
Hausera sp.
Kawakatsua pumila
Procerodes littoralis
Bdelloura candida
0.05

C.2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1/-
Rhodax sp. 2
Rhodax sp. 3
Opisthobursa mexicana
0.65/59
Hausera hauseri
0.96/80
Hausera sp.
Kawakatsua pumila
Procerodes littoralis
Bdelloura candida
0.06

Appendix D. Posterior probabilities of each condition (D.1: habitat and D.2: salinity) obtained in the ancestral reconstruction states analysis.

D.1

Node	Probability	
	Epigean	Hypogean
1	0,88	0,13
2	0,93	0,07
3	0,98	0,02
4	1,00	0,00
5	1,00	0,00
6	1,00	0,00
7	1,00	0,00
8	1,00	0,00
9	1,00	0,00
10	0,97	0,03
11	0,80	0,20
12	0,50	0,50
13	0,05	0,95
14	0,51	0,49
15	0,99	0,01
16	0,99	0,01
17	0,99	0,01
18	0,99	0,01
19	0,99	0,01
20	0,99	0,01
21	1,00	0,00
22	0,99	0,01
23	1,00	0,00
24	1,00	0,00
25	1,00	0,00
26	1,00	0,00
27	1,00	0,00

D.2

Node	Probability		
	Freshwater	Freshwater / Marine	Marine
1	0.22	0.52	0.26
2	0.03	0.22	0.75
3	0.64	0.35	0.01
4	0.97	0.03	0.00
5	0.99	0.01	0.00
6	0.99	0.01	0.00
7	1.00	0.00	0.00
8	1.00	0.00	0.00
9	1.00	0.00	0.00
10	0.59	0.40	0.01
11	0.98	0.03	0.00
12	0.99	0.01	0.00
13	1.00	0.00	0.00
14	1.00	0.00	0.00
15	0.15	0.81	0.04
16	0.15	0.82	0.03
17	0.22	0.76	0.01
18	0.02	0.50	0.48
19	0.01	0.45	0.54
20	0.01	0.49	0.51
21	0.00	0.52	0.48
22	0.01	0.48	0.51
23	0.00	0.38	0.62
24	0.00	0.13	0.87
25	0.00	0.09	0.91
26	0.02	0.40	0.58
27	0.00	0.03	0.97


E.1


E.2


E.3


E.4


E.5


E.6


E.7


E.8