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Treasure island: DNA data reveals unknown diversity in Cuban freshwater planarians (Platyhelminthes: Tricladida)

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ABSTRACT. Freshwater planarians constitute an important component in aquatic ecosystems as predators. They are, nonetheless, delicate animals used as indicators of water quality. This group has been little studied in The Antilles, where only seven species of *Girardia* Ball, 1974 have been reported. Those records date from the last two centuries and were identified based on morphology, leaving several specimens unidentified. Furthermore, the anatomical similarities among species and the lack of the copulatory apparatus in fissiparous populations make it necessary to use molecular data to perform accurate species delimitations and phylogenetic studies. The Cuban archipelago is the reservoir of the highest species diversity in the Caribbean. However, only one species of freshwater triclad has been described, *Girardia cubana* (Codreanu & Balcesco, 1973), which is endemic to Cuba. Recent samplings in the western part of the island molecularly identified *Girardia sinensis* Chen & Wang, 2015. At present, we are performing broad samplings all around Cuba. As a first result, we here present a phylogeny-based identification of freshwater planarians, collected in four localities of eastern Cuba, inferred using nuclear and mitochondrial markers. The presence of *G. sinensis* in the eastern part of the island is reported and two other lineages of the genus are identified, at least one could be a new species. Moreover, we found a lineage belonging to Cavernicola, of which there are no previous records in The Antilles. These findings support that the planarian richness of Cuba has been underestimated and new species could be described, providing relevant biogeographic information about the group in the Caribbean.

KEY WORDS. Antilles, Caribbean, Cavernicola, Dugesiidae, *Girardia*, *Rhodax*.

INTRODUCTION

Flatworms constitute an important component in freshwater ecosystems as predators and are used as biological indicators of water quality (Schockaert et al. 2008). However, little attention has been paid to turbellarians (free-living Platyhelminthes) in biodiversity studies related to freshwater habitats, although these animals usually present high species richness and abundance (Schockaert et al. 2008, Reyes et al. 2022). Approximately one-third of the known

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freshwater turbellarian species are triclads (Schockaert et al. 2008). These triclads have received more attention than the other turbellarian groups due to their larger size (1–5 cm or more), and their "popularity" as models for regeneration studies (Sánchez 2000, Reddien and Sánchez 2004, Rink 2018).

The few studies related to freshwater planarians diversity and distribution conducted in America, recorded 102 species: 66 from the Nearctic and 36 from the Neotropics (Kenk 1989, Schockaert et al. 2008, Benítez-Álvarez et al. 2020, 2023b). Therefore, the known taxonomic composition of freshwater planarians is uneven in the American continent, being lower in the Neotropics (Sluys et al. 2005). In Central and South America, the greatest species richness belongs to *Girardia* Ball, 1974, and a few species are part of other five genera. Representatives of *Girardia* extend their distribution to North America, but have a smaller number of species compared to the other 11 genera distributed in the region (Schockaert et al. 2008).

The Caribbean is a global biodiversity hotspot, characterized by a high species richness in marine, freshwater, and terrestrial habitats (Gould et al. 2020). Nonetheless, only seven species of *Girardia* have been reported so far: *Girardia aurita* (Kennel, 1888), *G. festae* (Borelli, 1889), *G. antillana* (Kenk, 1941), *G. arimana* (Hyman, 1957), *G. cubana* (Codreanu & Balcesco, 1973), *G. bursalacertosa* Sluys, 2005, and *G. sinensis* Chen & Wang, 2015 (for distribution details see Sluys 1992: fig. 8, except for *G. sinensis* and *G. bursalacertosa* the latter only present on Jamaica) (Sluys 1992, Sluys et al. 2005, Benítez-Álvarez et al. 2023b). Six of those species were collected during the 19th and 20th centuries as part of expeditions or punctual studies and were identified based on morphology, leaving several other specimens unidentified (see Sluys 1992: fig. 8) (e.g., Kenk 1941, Codreanu and Balcesco 1973, Sluys 1992). Recently, the first molecular phylogenetic study on the genus has been carried out; however, apart from *G. sinensis*, no other Antillean species were included (Benítez-Álvarez et al. 2023b).

The Cuban archipelago is a reservoir of the highest species diversity in the Caribbean (Herrera 2007). Among this diversity, only one species of freshwater triclad has been described, *G. cubana*, for which no type locality or type material were declared. The original description was made from material sampled in four localities of the central-eastern region of Cuba (Fig. 1). Reconstructions of the copulatory apparatus were made only from the specimens sampled in the center of the island (Codreanu and Balcesco 1973). Afterward, Sluys (1992) analyzed specimens sampled in the western region (Fig. 1, Gran Caverna de Santo Thomas), and found differences in the structure of the penis papilla and the bursal canal with respect to the material described by Codreanu and Balcesco (1973) but no taxonomic action was taken.

Since the description of *G. cubana*, all populations of freshwater planarians catalogued on the island have been identified either as *G. cubana* or Dugesiidae, with no detailed analysis of their morphology. Even values of the Biological Monitoring Working Party-Cuba (BMWP-Cu) have been standardized to evaluate the water quality based on this identification (e.g., Naranjo et al. 2005, Olivares-Calzado et al. 2012, Naranjo-López and López del Castillo 2013). The validation of triclad species requires a detailed study of their internal morphology, especially the reproductive systems (Sluys and Riutort 2018). However, the great morphological similarity between species and the lack of a copulatory apparatus in many fissiparous populations make it necessary to use molecular data to perform accurate species identification and phylogenetic studies (Benítez-Álvarez et al. 2023b). In fact, recent sampling in Matanzas, on the west of the island, allowed the molecular identification of *G. sinensis* (Fig. 1, El Huequito) (Benítez-Álvarez et al. 2023b).

Recently, Diez et al. (2023), on an analysis of the diversity of free-living flatworms, indicated that The Antilles, particularly Cuba, is an understudied region with a high undiscovered diversity of turbellarians. Overall, previous findings suggest that the species richness for Cuba is underestimated and new species of planarians could be described, which would provide relevant biogeographic information about the group in The Caribbean. The aim of this study is to summarize the molecular species identification, after preliminary samplings performed in four localities of the eastern region of Cuba.

MATERIAL AND METHODS

Specimens sampling and preservation

Samples were taken in four rivers in eastern Cuba from October, 2021 to August, 2022 (Fig. 2, Table 1, Supplementary Table S1). These rivers are associated with two of the most important mountain systems in the archipelago, Sierra Maestra and Nipe-Sagua-Baracoa, characterized by a high biodiversity associated with them. Two of the sampled localities are located in urbanized areas (Cauto and San Juan Rivers) (Fig. 2D, E).

Planarians were extracted from vegetation and organic material by the oxygen depletion method (Schockaert 1996). For this, samples were placed in 2 L beakers, bringing them to

Figure 1. Distribution of the previous records of planarian species in Cuba. Circles represent localities of *Girardia cubana*, the localities in the Central-Eastern region correspond to the original description. Diamond represents *G. sinensis*.

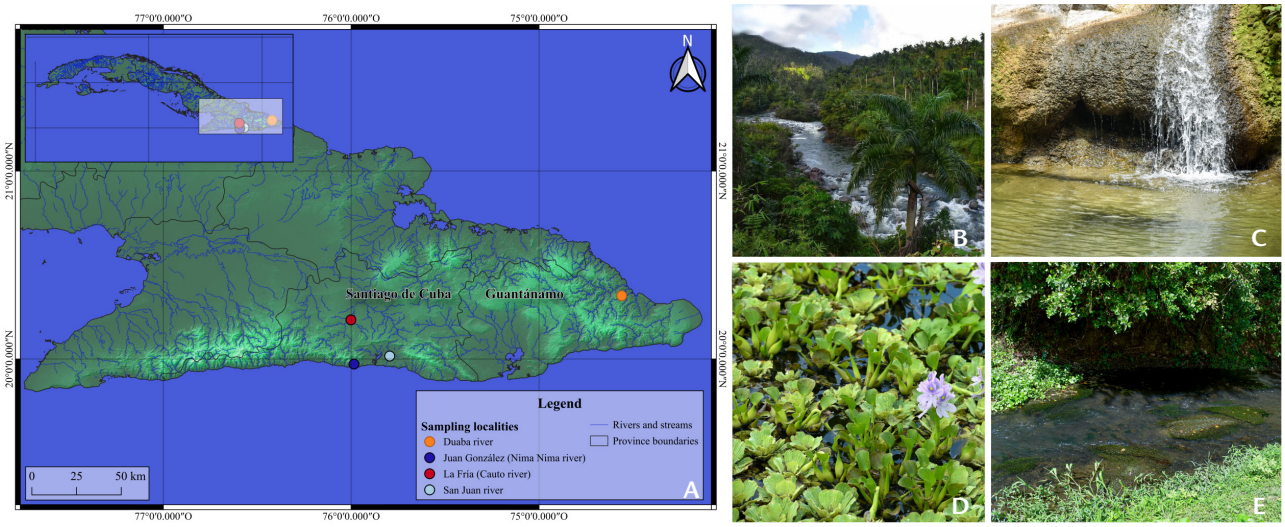

Figure 2. Sampling localities of freshwater planarians in eastern Cuba: (A) map with the distribution of the sampling localities; (B, C) rivers on not urbanized areas where B: Duaba River and C: Juan González, Nima Nima River; (D, E) rivers in urbanized areas where D: *Eichhornia crassipes* and *Pistia stratiotes* in La Fría, Cauto River and E: San Juan River note the presence of *Elodea* sp. in the water.

Table 1. New sequences obtained in this study, with indication of sample codes, sampling localities, taxonomic assignment before and after analyses, codes used in the text and figures, and GenBank accession numbers for COI, EF1α and 18S rDNA sequences. See Supplementary Table S1 for more details on localities and collectors.

Figures ID	Locality	Taxonomic Identification before analysis	Taxonomic Identification after analysis	COI	EF1α	18S
MR1423-1 *Girardia* sp. (Duaba River)	Cuba, Baracoa, Duaba River	*Girardia*-like morph	*Girardia* sp. 1	PQ537315	PQ533389	–
MR1423-2 *Girardia* sp. (Duaba River)	Cuba, Baracoa, Duaba River	*Girardia*-like morph	*Girardia* sp. 1	PQ537316	–	–
MR1425-1 *Girardia* sp. (Nima Nima River)	Cuba, Guamá, Nima Nima River	*Girardia*-like morph	*Girardia* sp. 2	PQ537317	–	–
MR1425-2 *Girardia* sp. (Nima Nima River)	Cuba, Guamá, Nima Nima River	*Girardia*-like morph	*Girardia* sp. 2	PQ537318	PQ533389	–
MR1424-1 *G. sinensis* (San Juan River)	Cuba, Santiago de Cuba, San Juan River	*Girardia*-like morph	*G. sinensis*	PQ537319	–	–
MR1424-2 *G. sinensis* (San Juan River)	Cuba, Santiago de Cuba, San Juan River	*Girardia*-like morph	*G. sinensis*	PQ537320	–	–
MR1426-1 Cavernicola Cuba	Cuba, Santiago de Cuba, Cauto River	Unknown morph	*Rhodax* sp.	–	–	PQ530958

the bottom and the volume was completed with water. They were allowed to rest and checked every 30 to 60 minutes for 72 hours. When climbing, the worms were captured with the help of soft tweezers, brushes and pipettes. In absence of aquatic vegetation, animals were directly collected under submerged rocks using soft brushes. From all sampled localities, worms were separated into morphotypes (Fig. 3) and from each of them some specimens were fixed in 100% ethanol for molecular analysis.

Only one planarian morphotype was identified at each sampled locality. The morphotypes can be classified into two groups: A *Girardia*-like morph characterized by having auricles (Fig. 3A–C) and an Unknown morph where auricles are not present (Fig. 3D). However, at least two different *Girardia*-like morphs were identified according to the shape of the auricles and the color pattern. One morph has pointed auricles and solid dorsal brownish or blackish color (Fig. 3A, B) and the other has triangle auricles and a dotted color pattern (Fig. 3C).

DNA extraction, gene amplification and sequencing

Total DNA was extracted from single individuals using Wizard® Genomic DNA Purification Kit (Promega) and DNAzol® Reagent (Thermo Fisher Scientific, USA), according to the manufacturer's instructions. The extraction products were quantified using a NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific, USA).

Three genes were amplified by polymerase chain reaction (PCR) using approximately 25 ng of template DNA and specific primers (see Table 2) in 25 µL final reaction volume with $MgCl_2$ (2.5 mmol/L), dNTPs (30 µmol/L), primers (0.4 µmol/L) and 0.75 U of Go Taq DNA polymerase enzyme (Promega Madison, Wisconsin, USA) with its buffer (1×). The amplification protocol for COI (BarS-PlatRGi) consisted of 2' for initial denaturation at 95 °C and 35 cycles of: 50" at 94 °C, 45" at annealing temperature (AT) (Table 2) and 50" at 72 °C; with a final extension step of 4' at 72 °C. For EF-1α the 35 cycles were: 45" at 94 °C, 45" at AT (Table 2) and 45" at 72 °C. For 18S rDNA the number of cycles was increased

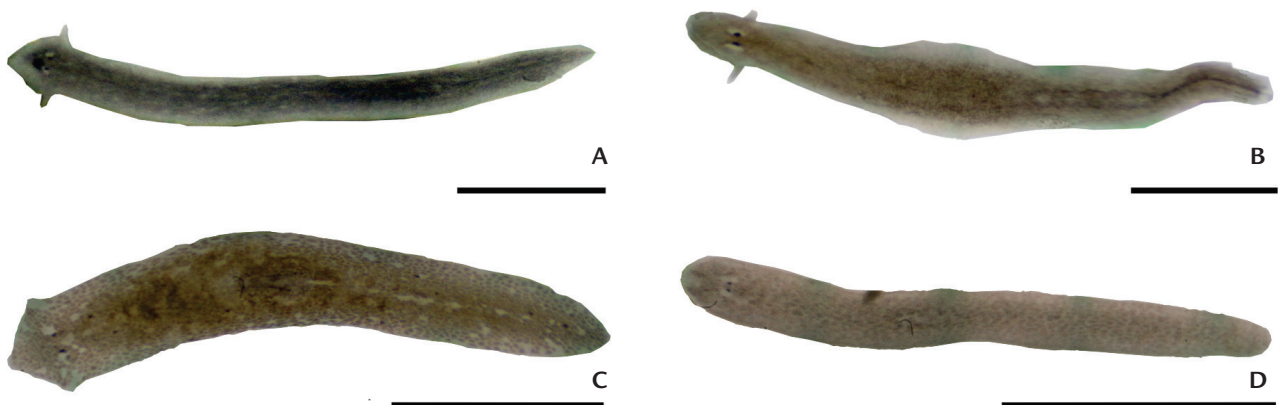

Figure 3. Different morphological types sampled from: (A) Duaba River, (B) Juan González, Nima Nima River, (C) San Juan River, (D) La Fría, Cauto River. (A–C) *Girardia*-like morph: (A, B) present pointed auricles; (C) triangle shaped auricles and a dotted color pattern; (D) the unknown morph without auricles. Scale bars: 2 mm.

Table 2. Primers used in this study, sequences, references and annealing temperature (AT)

Primer	Direction	Primer sequence (5'–3')	Reference	AT
COI				
BarS	Forward	GTTATGCCTGTAATGATTG	Álvarez-Presas et al. (2011)	44 °C
PlatR-Gi	Reverse	CATCCTGAGGTTTATATWTTGATT	Benítez-Álvarez et al. (2023b)	
EF1-α				
EFGi-2F	Forward	CCT TCA AAT ACG CTT GGG	Benítez-Álvarez et al. (2023b)	51 °C
EFGi-2R	Reverse	GRATTTGACCTGGRTGATTC	Benítez-Álvarez et al. (2023b)	
18S rDNA				
18S_1F	Forward	TACCTGGTTGATCCTGCCAGTA	Carranza et al. (1996)	45–51 °C depending on pairs
18S_5R	Reverse	CTTGGCAAATGCTTTCGC	Carranza et al. (1996)	
18S_5F	Forward	GCGAAAGCATTTGCCAAGAA	Carranza et al. (1996)	
18S_9R	Reverse	GATCCTTCCGCAGGTTCACCTAC	Carranza et al. (1996)	
18S_4F	Forward	CCAGCAGCCGCGCTAATTC	Carranza et al. (1996)	
18S_7R	Reverse	GCATCACAGACCTGTTATTGC	Carranza et al. (1996)	

to 38 and the AT and extension times varied depending on pairs of primers used, 1-7R AT 46 °C and extension 1'30", 4F-9R AT 48 °C and extension 1'30", 1F-5R and 4F-7R AT 50 °C and extension 1', 5F-9R AT 42 °C and extension 1'.

PCR products were run in agarose gels (1%) to check whether there had been amplification of a fragment of the expected size. PCR primers and dNTPs were digested by ExoSAP, a mix of two hydrolytic enzymes (Exonuclease I and Shrimp Alkaline Phosphatase; Thermo Fisher Scientific, USA) in a 3:1 ratio (amplified product: ExoSAP). Both strands of purified fragments were sequenced by Macrogen Inc., (Macrogen Europe, Madrid) or at the Centres Científics i Tecnològics (CCiT, Universitat de Barcelona) with the same primers as used in the amplification. In order to obtain the final contigs, chromatograms were analyzed with Genious v. 10 (Kearse et al. 2012).

Sequence alignment

The newly obtained COI and EF1-α sequences of *Girardia* were aligned with a set of sequences downloaded from GenBank (Supplementary Table S2) to include the maximum diversity of the genus. The sequences were aligned with ClustalW on the BioEdit Sequence Alignment Editor (Hall 1999). The mitochondrial gene was translated into amino acids with the corresponding genetic code (GenBank code 9) while the nuclear genes were translated using the universal code to check for the absence of stop codons and to produce the alignment and, thereafter, converted again to nucleotides. Three alignments were generated to infer the phylogenetic relationships of the Cuban *Girardia*-like morphs: one including COI sequences of *Girardia*, one with EF1-α sequences, and a concatenated of COI and EF1-α sequences. The concatenated alignments were obtained with Mesquite v. 3.04 (Maddison and Maddison 2023). In these alignments, missing bases were substituted by the code N.

To infer the phylogenetic position of the Unknown-morph, an 18S rDNA gene alignment was performed. The 18S sequences for a set of species representing a series of genera belonging to all the suborders of Tricladida and the outgroup taxa were downloaded from GenBank (Supplementary Table S3). Sequences were aligned with MAFFT v7 (Katoh and Standley 2013) using the web server http://maff.cbr.jp/alignment/server/ (last visited October, 2023) using the E-INS-i method and default parametrization. Since it has been noted that automatic filtering of alignments may result in loss of information (Tan et al. 2015), and a previous study using the same dataset, except for the new sequence, found no significant differences between filtering or not for ambiguous regions on topology or support values (Stocchino et al. 2021), we inferred phylogenetic trees from the original alignments without any filtering. Each alignment nonetheless was edited by hand to trim the ends and the code N was assigned to sites with missing data.

Phylogenetic inference

In all phylogenetic analyses, for each coding gene, we defined three partitions, corresponding with the first-, second-, and third-codon. In consequence, in the concatenated dataset (dataset 3) six partitions were considered.

Maximum likelihood (ML) and Bayesian inference (BI) methods were applied to the four datasets to infer the best tree. Maximum likelihood trees were obtained with the IQTREE software (Minh et al. 2020) using 10,000 ultrafast bootstrap alignments and iterations (Hoang et al. 2018) and 10,000 replicates for the SH-aLRT single-branch test. The substitution model was set to auto for the software to esti-

mate the best model for each partition (Table 3). BI trees and posterior probabilities (PP) were inferred using MrBayes on XSEDE (3.2.7a) (Ronquist et al. 2012) in the CIPRES Science Gateway server (https://www.phylo.org/, last visited October, 2023) (Miller et al. 2010). The chains were parameterized to ten million generations, sampling every 1000 generations, and a 25% burn-in (default setting) was applied. Convergence between runs of parameter values and topologies was examined by checking that the average standard deviation of split frequencies was below 0.01. The evolutionary models were set to General Time Reversible+Gamma Distribution+Invariable Sites (GTR+Γ+I) for all datasets and partitions with unlinked parameters estimation.

Table 3. Substitution model estimated by IQTree chosen according to BIC for the three partitions of each coding gene and for 18S rDNA.

Gene	Codons partitions	Substitution model
COI	1st	TPM3u+F+G4
	2nd	TIM2+F+G4
	3rd	TIM2+F+I+G4
EF1-α	1st	TPM2u+F+G4
	2nd	TIM2+F+I+G4
	3rd	JC+I+G4
18S		GTR+F+I+G4

The *Girardia* trees were rooted on the node separating the clade constituted by *Girardia schubarti* (Marcus, 1946) and a group of North American species (A+B clade in Fig. 4) from the rest, following the results of Benítez-Álvarez et al. (2023b).

RESULTS

Datasets

For the *Girardia*-like morph the final datasets consisted of: Dataset 1, 100 COI sequences with a length of 837 bp (6 obtained in this study); Dataset 2, 80 EF1-α sequences with a length of 879 bp (4 obtained in this study) including representatives from all over the range of the genus in order to assign the new specimens from Cuba accurately (Table 1, Supplementary Table S2); Dataset 3, the concatenation of dataset 1 and 2, with a total of 104 sequences and length 1715 bp, includes a 29% of missing data, due to the lack one or the other gene for some individuals or of shorter sequences.

For the Unknown-morph the 18S rDNA dataset contained 15 sequences (1 obtained in this study, Table 1, Supplementary Table S3) and a length of 1545 bp.

Phylogeny of *Girardia*, and *Girardia*-like morph specimens' assignment

The Maximum Likelihood (ML) and Bayesian (BI) phylogenetic trees obtained from datasets 1 and 2 are shown in the supplementary data (Supplementary Figs S1–S4), and the trees obtained from dataset 3 (concatenated COI and EF1-α) are shown in Fig. 4 and Supplementary Fig. S5.

The trees inferred by both Maximum Likelihood and Bayesian inference methods from the same dataset have a similar topology, showing discrepancies only on nodes with low support. Following the groupings established in Benítez-Álvarez et al. (2023b), we have defined three main groups on the trees, although they are not always monophyletic: An unresolved South American clade (clade G-L), a North American + South American clade (clade M–T) which includes a clade of only North American species (clade Q-T). However, the inclusion of the new sequences from Cuba has resulted in some changes in the composition of the South American clade, since *Girardia* sp. from a Brazilian cave (clade F) is not situated within that clade in some of our analyses (Fig. 4, Supplementary Figs S2, S4, S5).

The specimens from San Juan River (Santiago de Cuba) were assigned to *G. sinensis*, in the North American clade (clade Q-T). The specimens sampled on Duaba River (Baracoa) and Nima Nima River (Guamá) always form a highly supported monophyletic group (pp = 1; SH-aLRT = 99; UF-boot = 99.5 in the concatenated tree). Moreover, according to the length of the branches separating them, they might be two different species. These two sibling species were not identical or closely related to any of the species of *Girardia* with sequences available in GenBank. Except when analyzing dataset 1 (COI alone), their position in the trees is close to the base as one of the first offshoots of the ingroup, before the South American clade emerges. In most trees they are nested in a low supported clade (clade C–E) with *Girardia* sp. from Mexico (singleton E) leaving their relationship unresolved (Fig. 4, Supplementary Figs S2, S4, S5).

Unknown-morph specimen

The phylogenetic tree based on the 18S rDNA sequences to place the Unknown-morph specimens is shown in Fig. 5 and Supplementary Fig. S6. This analysis recovered monophyletic Continenticola, Cavernicola and Maricola, showing a closer relationship between the latter two clades. Within this tree, the Unknown-morph specimen from Cauto River clusters together with a specimen of *Rhodax* coming from Brazil in a highly supported clade (pp = 1; SH-aLRT = 99.9; UF-boot = 100). Nonetheless, our new sequence and *Rhodax*

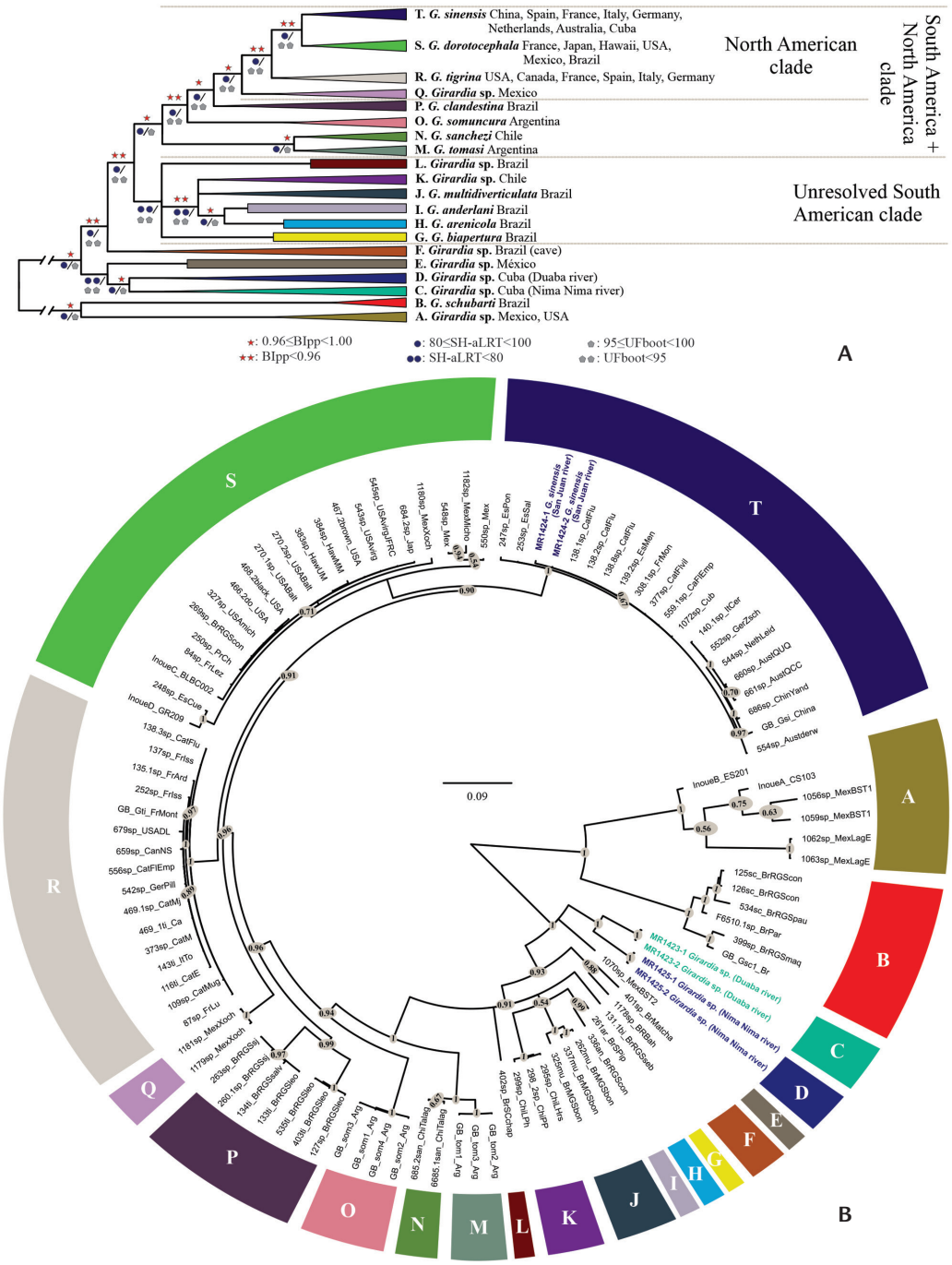

Figure 4. Phylogenetic relationships within *Girardia* based on dataset 3 (COI+EF) including Cuban specimens. Groups indicated by letters and colours highlight the clades delimited in Benítez-Álvarez et al. (2023b). (A) Consensus of Bayesian and Maximum Likelihood analyses with collapsed clades (triangles) and singletons (rectangles) showing species identifications, when available, and countries of origin of the various terminals. The nodes with a support below 0.5 or 50 have been collapsed. Symbols above branches: posterior probability values. Symbols below branches: ultrafast bootstrap and SH-aLRT support. (B) Bayesian circular tree with all terminals; values at nodes correspond to posterior probability support. The colours in the terminals indicate Cuban specimens. Scale bar represents substitutions per nucleotide.

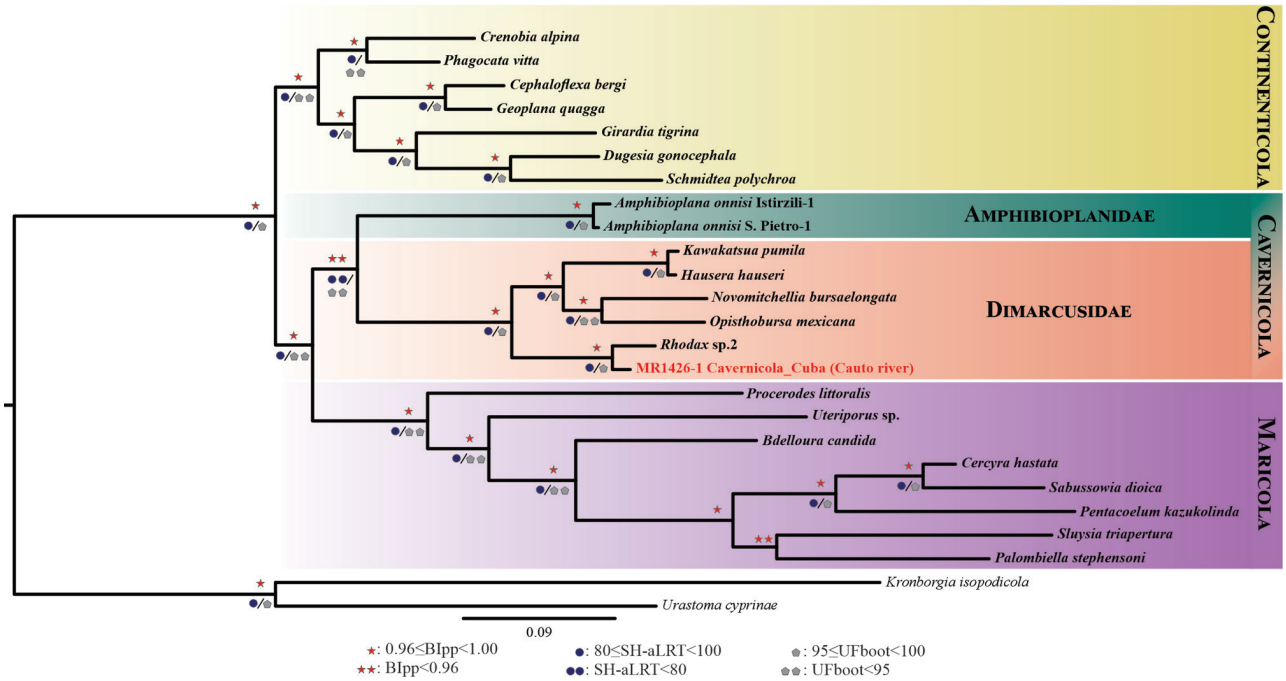

Figure 5. Bayesian tree showing the phylogenetic relationships within Tricladida based on 18S rDNA sequences and the situation of the Unknown-morph found in Cuba (in red). Symbols above branches: posterior probability values. Symbols below branches: ultrafast bootstrap and SH-aLRT support. Scale bar represents substitutions per nucleotide.

sp. 2 are separated by branches of relatively long length, which may indicate that the Cuban specimen belongs to a different species.

DISCUSSION

Diversity of *Girardia* in Cuba

Our results show that at least three different species of *Girardia* inhabit the eastern region of the island of Cuba. In the San Juan River was identified the presence of *G. sinensis* (Fig. 3C), a species for which there is a record from the western region of the archipelago (Fig. 1), also based on a molecular study (Benítez-Álvarez et al. 2023b). This is the first record of the species in the eastern region of Cuba. *Girardia sinensis* was originally described from China (Chen et al. 2015), but later it was found to be an invasive species and it was concluded that it originated from North America and had been recently introduced worldwide by human activities (Benítez-Álvarez et al. 2023a, 2023b). Up to date, Cuba is the only known record of *G. sinensis* in The Americas (Benítez-Álvarez et al. 2023b) and Cuba is situated on the south border of the North American plate (Iturralde-Vinent and MacPhee 1999, Iturralde-Vinent and Macphee 2023). Taking into account the high dispersal potential of this species (Benítez-Álvarez et al. 2023a) and the complex geology of the American continent, at the moment it is not possible to establish whether the species is autochthonous to the island or may have arrived transported by human activities or animals.

The specimens coming from the Duaba (Fig. 3A) and Nima Nima rivers (Fig. 3B), respectively, constitute sister groups; however, the long branches separating them in the trees are indicative of a high genetic differentiation, which suggest they are two different species. These two species were sampled in localities near those of eastern Cuba where Codreanu and Balcesco (1973) described *G. cubana* and, therefore, one of the two molecularly identified species in the present work may correspond to *G. cubana* (Figs 1, 2). A morphological analysis is needed to corroborate whether any of the populations of both localities show the characteristics defined for *G. cubana*. However, the lack of type material or designation of a type locality in the original description of the species, together with the fact that the morphology of the copulatory apparatus of *G. cubana* from most of the localities was not analyzed (Codreanu and Balcesco 1973), renders the classification of new specimens

as *G. cubana* difficult. We suggest designating Topes de Collantes on Central Cuba as type locality for *G. cubana*, since the only anatomical description of the reproductive systems belongs to specimens from that locality (Codreanu and Balcesco 1973).

Cuban *Girardia* evolutionary relationships

The phylogenetic trees of *Girardia* obtained from the concatenated dataset show similar major phylogenetic relationships as those estimated by Benítez-Álvarez et al. (2023b). Nonetheless, the South American clade was already defined as an unsupported and unresolved group in Benítez-Álvarez et al. (2023b). Thus, more data and a focused study are necessary to resolve its relationships. As for the two newly sequenced Cuban putative species, they show an ancient relationship to the rest of the *Girardia* ingroup. The two Cuban lineages and a Mexican species constitute the first lineages to diverge before the South American clade diversifies, although the order of divergences among these three groups is not supported and they form a trichotomy (Fig. 4).

Sluys (1992) suggested that species of *Girardia* from The Antilles are related to those of South America by comparing the morphology of their copulatory apparatus. However, excluding *G. sinensis*, in the present molecular analyses the Cuban species do not group within the South American or the North American + South American clades but, otherwise, are rather related to a species from Mexico (Fig. 4). The Cuban biota had three major sources of colonization (North, Central, and South America) related to the geological history of the island (Hedges 2001). Our results suggest that the colonization of the Cuban archipelago by representatives of *Girardia* could originate from Central America, probably in a quite ancient event considering the basal branches leading to the two Cuban lineages in our trees. On the other hand, a previous biogeographic hypothesis suggested a single-species island endemism for the freshwater planarians on The Antilles (Ball 1983). However, our results show a different scenario for the Cuban archipelago indicating that each island may harbor more than one species, suggesting an ancient colonization and posterior speciation occurring on the islands. As proposed by Sluys (1992), the previously observed pattern is the result of sampling biases rather than the real biogeographic history of the group in the region. To have a better understanding of the distribution pattern of planarians in The Antilles, sampling unexplored areas not only in Cuba but also in the other Antillean islands and the continental regions surrounding them is necessary.

Cavernicola in Cuba

Our first and preliminary samplings in Cuba have rendered another interesting and unexpected result, the presence of a species of Cavernicola. This is the first record of this suborder in The Antilles. The specimens found in Cauto River (Fig. 3D) are closely related to a specimen of *Rhodax* coming from Brazil, which suggests that they belong to this genus (Fig. 5). The species of *Rhodax*, until now, are only known from southern Brazil. Therefore, our findings extend its distribution to The Antilles, suggesting that the known distribution of the genus (and of Cavernicola) is related to sampling biases and more samplings are needed on unexplored areas to infer biogeographic patterns of the group as it was proposed by Benítez-Álvarez et al. (2020).

The length of the branches separating the new sequences from the individuals of *Rhodax* sp. 2 in our phylogenetic analysis may indicate that they belong to a different species (Fig. 5). In a previous study including multiple specimens of *Rhodax* coming from different localities in Brazil (Benítez-Álvarez et al. 2020), the authors already pointed out that the differentiation found in their tree (based on 18S and 28S rDNA) among specimens of *Rhodax* could point to the presence of different species within the genus. New material and more sequences, including both rDNA genes and morphological analyses, are needed to clarify the species assignment of the Cuban species and the diversity of this poorly studied animal group.

The specimens sampled in Cauto River were extracted from aquatic vegetation (roots of *Eichhornia crassipes* and *Pistia stratiotes*) on lentic water in a eutrophicated area of the river near the city of Palma Soriano (Fig. 2D). In the original description of *Rhodax evelinae* (the only described species of the genus up to date), Marcus (1946) sampled the specimens in a habitat with similar conditions to those where the Cuban specimens were found: "in dirty ponds and clear water brooks but both with abundant organic matter near the city of São Paulo". Nonetheless, Marcus (1946) also found animals in clean waters. These descriptions of habitat characteristics suggest that, although specimens of *Rhodax* may originally inhabit clean waters, water bodies with abundance of organic matter in anthropogenic habitats (close to cities) could also be an ideal habitat for them. The tolerance for anthropogenic habitats has been shown to be a relevant characteristic in invasive planarian species, such as some species of *Girardia* (Benítez-Álvarez et al. 2023a), and *Dugesia sicula* Lepori, 1948 (Leria et al. 2022). Considering all this reasoning, it is possible that the species of Cavernicola had been introduced in Cuba through human activities,

e.g., through the international trade in aquatic plants and the activity of aquarists. However, with the present data not showing a total identity with previously sequenced species, we cannot rule out that they are autochthonous from Cuba.

Final remarks

The sampling in only four localities in the eastern part of the Cuban archipelago has shown the presence of an unexpected diversity of Tricladida, with each locality housing a different species of planarians. These preliminary samplings make us foresee a promising future regarding our analysis of the planarian fauna diversity and evolution in Cuba once the whole island has been sampled, which will provide relevant biogeographic information about the group in the Caribbean.

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Competing Interests

The authors have declared that no competing interests exist.

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Supplementary material 1

Figure S1. Maximum likelihood circular tree with all terminals showing the phylogenetic relationships of *Girardia* based on dataset 1 (COI); values at nodes correspond to ultrafast bootstrap and SH-aLRT support.

Authors: A. Catalá, L. Benítez-Álvarez, Y.L. Diez, G. Blasco, M. Riutort.

Data type: Species phylogenetic data.

Supplementary material 2

Figure S2. Maximum likelihood circular tree with all terminals showing the phylogenetic relationships of *Girardia* based on dataset 2 (EF); values at nodes correspond to ultrafast bootstrap and SH-aLRT support.

Authors: A. Catalá, L. Benítez-Álvarez, Y.L. Diez, G. Blasco, M. Riutort.

Data type: Species phylogenetic data.

Supplementary material 3

Figure S3. Bayesian circular tree with all terminals showing the phylogenetic relationships of *Girardia* based on dataset 1 (COI); values at nodes correspond to posterior probability support.

Authors: A. Catalá, L. Benítez-Álvarez, Y.L. Diez, G. Blasco, M. Riutort.

Data type: Species phylogenetic data.

Supplementary material 4

Figure S4. Bayesian circular tree with all terminals showing the phylogenetic relationships of *Girardia* based on dataset 2 (EF); values at nodes correspond to posterior probability support.

Authors: A. Catalá, L. Benítez-Álvarez, Y.L. Diez, G. Blasco, M. Riutort.

Data type: Species phylogenetic data.

Supplementary material 5

Figure S5. Maximum likelihood circular tree with all terminals showing the phylogenetic relationships of *Girardia* based on dataset 3 (COI + EF); values at nodes correspond to ultrafast bootstrap and SH-aLRT support.

Authors: A. Catalá, L. Benítez-Álvarez, Y.L. Diez, G. Blasco, M. Riutort.

Data type: Species phylogenetic data.

Supplementary material 6

Figure S6. Maximum likelihood tree showing the phylogenetic relationships of Tricladida based on 18S rDNA sequences and the situation of the Unknown-morph found

in Cuba (in red), values at nodes correspond to ultrafast bootstrap and SH-aLRT support.
Authors: A. Catalá, L. Benítez-Álvarez, Y.L. Diez, G. Blasco, M. Riutort.
Data type: Species phylogenetic data.

Supplementary material 7
Table S1. Samples analysed in this study, localities and geographical coordinates, date, collectors, and sampling method (ODM: Oxigen Depletion Method).
Authors: A. Catalá, L. Benítez-Álvarez, Y.L. Diez, G. Blasco, M. Riutort.
Data type: Species phylogenetic data.

Supplementary material 8
Table S2. *Girardia* sequences downloaded from GenBank included in this study, with indication of sample codes used in the text and figures, sampling localities, taxonomic assignment, and accession numbers for COI and EF1α sequences.
Authors: A. Catalá, L. Benítez-Álvarez, Y.L. Diez, G. Blasco, M. Riutort.
Data type: Species phylogenetic data.

Supplementary material 9
Table S3. Taxonomic classification and GenBank accession number of the downloaded sequences used in the Tricladida trees.
Authors: A. Catalá, L. Benítez-Álvarez, Y.L. Diez, G. Blasco, M. Riutort.
Data type: Species phylogenetic data.

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