# First molecular phylogeny of the freshwater planarian genus Girardia (Platyelminthes, Tricladida) unveils hidden taxonomic diversity and initiates resolution of its historical biogeography

| | |
|---|---|
| Journal: | *Zoological Journal of the Linnean Society* |
| Manuscript ID | ZOJ-03-2022-4908.R1 |
| Manuscript Type: | Original Article |
| Keywords: | biogeography < Geography, North America < Geography, Central America < Geography, South America < Geography, Bayesian analysis < Phylogenetics, phylogenetics < Phylogenetics, new species < Taxonomy |
| Abstract: | The genus Girardia (Platyhelminthes: Tricladida) comprises several species of which some have spread from their original areas of distribution in the Americas to other parts of the globe. Due to great anatomical similarity between species, morphology-based phylogenetic analyses struggled to resolve the affinities between species and species-groups. This problem is exacerbated by the fact that populations of Girardia may show only asexual reproduction by fissiparity and, thus, do not exhibit a copulatory apparatus, which hampers taxonomic identification of phylogenetic characters. In the present work this problem has been resolved by constructing a molecular phylogeny of the genus. Although our samples do not include representatives of all known species, they cover a large part of the original distributional range of the genus Girardia. Our phylogenetic results suggest the presence of two main clades, which are genetically and karyologically highly differentiated. North and South American populations of G. tigrina actually constitute two sibling species that are not even closely related. The South American form is here described as a new species. The phylogenetic tree brings to light that Girardia arose on the South American portion of Gondwanaland, from which it, subsequently, dispersed to the Nearctic Region, probably more than once. |
| | |

## Abstract

The genus *Girardia* (Platyhelminthes: Tricladida) comprises several species of which some have spread from their original areas of distribution in the Americas to other parts of the globe. Due to great anatomical similarity between species, morphology-based phylogenetic analyses struggled to resolve the affinities between species and species-groups. This problem is exacerbated by the fact that populations of *Girardia* may show only asexual reproduction by fissiparity and, thus, do not exhibit a copulatory apparatus, which hampers taxonomic identification of phylogenetic characters. In the present work this problem has been resolved by constructing a molecular phylogeny of the genus. Although our samples do not include representatives of all known species, they cover a large part of the original distributional range of the genus *Girardia*. Our phylogenetic results suggest the presence of two main clades, which are genetically and karyologically highly differentiated. North and South American populations of *G. tigrina* actually constitute two sibling species that are not even closely related. The South American form is here described as a new species. The phylogenetic tree brings to light that *Girardia* arose on the South American portion of Gondwanaland, from which it, subsequently, dispersed to the Nearctic Region, probably more than once.

**Keywords**: *Girardia*, evolutionary relationships, historical biogeography, hypogean diversity, introduced species, taxonomy, Tricladida, new species

## INTRODUCTION

The genus *Girardia* comprises about 59 valid species, the natural distribution of which covers the Americas, from Southern Argentina and Chile to Southern Canada, albeit that in North America it is not the most species rich genus of freshwater planarian (Sluys et al. 2005). Furthermore, species of *Girardia* have been introduced into many other regions of the world (Stocchino et al. 2019 and references therein). For Australia, occurrence of introduced *G. tigrina* was established (Sluys et al. 1995 and references therein), apart from three presumed autochthonous species of *Girardia* (cf. Grant et al. 2006; Sluys & Kawakatsu 2001). However, recent molecular work (Grant 2017) revealed that the latter three species (*G. sphincter* Kawakatsu & Sluys, 2001; *G. graffi* (Weiss, 1909); *G. informis* Sluys & Grant, 2006) do not belong to the genus *Girardia*.

Since the most recent, more comprehensive account on species of *Girardia* from the South American continent and the Caribbean Region by Sluys et al. (2005), 13 new species have been described (Chen et al. 2015; Souza et al. 2015; Souza et al. 2016; Hellmann et al. 2018, 2020; Lenguas Francavilla et al. 2021; Morais et al. 2021). Phylogenetic analyses of the genus *Girardia* are limited to the study of Sluys (2001), while historical biogeographic studies focusing on the genus are basically absent.

Due to great anatomical similarities between species, morphology-based phylogenetic analyses struggled to resolve the affinities between species and species-groups (cf. Sluys 2001). This problem is exacerbated by the fact that populations of *Girardia* may show only asexual reproduction by fissiparity and, thus, do not exhibit a copulatory apparatus, which hampers taxonomic identification, as well as the detection of phylogenetic characters. Currently, the use of molecular markers allows workers to overcome some of the limitations of morphological characters in the delimitation of species and in the reconstruction of the evolutionary history of the triclads. The nuclear gene *Elongation Factor 1 alpha* (*EF1a*) has been used in several phylogenetic and phylogeographic studies of triclads (see Álvarez-Presas & Riutort 2014), while the mitochondrial gene *Cytochrome C Oxidase I* (*COI*) has been used for taxonomic studies as well as for species delimitation in the genus *Dugesia* (Sluys et al. 2013; Solà et al. 2013; Dols-Serrate et al. 2020; Leria et al. 2020). In all of these studies, an integrated approach, combining molecular and morphological data, proved to be highly successful in furthering our knowledge on the systematics of the various groups.

Here, we present the first molecular phylogeny of the genus *Girardia*, which resulted in several new insights into its taxonomic diversity, particularly in Mexican and South American territories, and into the biogeographic history of the genus.

## MATERIALS AND METHODS

### Taxon sampling

For molecular analyses, samples of *Girardia* were obtained from Asia, Australia, Hawaii, the Americas, and Europe, with greater representation of the two last-mentioned geographical areas. Most of the samples were collected by the authors, while the rest was made available by various colleagues. Individuals were fixed in absolute ethanol. Specimens were identified to species level when both external and internal morphology could be examined (Table S1). When no anatomical information was available, individuals were simply classified as *Girardia* sp. In addition, all available *Girardia* sequences of *Cytochome Oxidase I* (*COI*) and *Elongation factor 1 alpha* (*EF1α*) were downloaded from GenBank. During our analyses some of the latter were excluded because of one or more of the following reasons: (a) low quality or short length of the sequences, (b) uncertain classification of the specimen, (c) avoidance of multiple sequences from a single locality (Table S2).

Figure 1 shows the distribution map of our samples, while an interactive map that allows a better resolution of the information on each locality is available at https://www.ub.edu/planarian-maps/. When available, we used the coordinates of the original samples, otherwise, approximated geographical coordinates were obtained from Google Earth (https://www.google.com/earth/index.html; last visited 12 March 2019) by entering the sampling localities (Table S1). We placed the data points on an open-source map (https://www.openstreetmap.org/) by using a custom script (not available in this publication) of JavaScript (ECMAScript 2015).

### DNA extraction, gene amplification and sequencing

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

A portion of the mitochondrial *COI* and of the nuclear *EF1α* regions were amplified by Polymerase Chain Reaction (PCR), using 100 ng of template DNA and specific primers (Table 1) in 25µl of final reaction volume with $MgCl_2$ (2.5mM), dNTPs (30µM), primers (0.4µM) and 0.75U of Go Taq® DNA polymerase enzyme (Promega Madison, Wisconsin, USA) with its buffer (1x). The amplification program consisted of 2 minutes (m) for initial denaturation at 95°C and 35 cycles of: 50 seconds (sec.) at 94°C, 45 sec. at annealing temperature (Table 1) and 50 sec. at 72°C; with a final extension step of 4 min. at 72°C.

PCR products were run in agarose gels (1%) to check whether the correct band had been amplified. PCR products were purified by ultrafiltration in a Merck Millipore MultiScreen System (Darmstadt, Germany). For those samples that showed a faint PCR band on the electrophoresis, remaining 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) with the same primers as used in the amplification. In order to obtain the final contigs, chromatograms were analysed with Genious v.10 (Kearse et al. 2012).

## Sequence alignment and datasets

Sequences of *COI* and *EF1α*, were aligned independently with ClustalW on the BioEdit Sequence Alignment Editor (Hall 1999). Each gene was translated into amino acids with the corresponding genetic code to check for the absence of stop codons and to produce the alignment, and, thereafter, converted again to nucleotides. Two alignments for each gene were generated, one including only *Girardia* sequences and the other comprising sequences of the closely related outgroup genera *Schmidtea* and *Dugesia* (cf. Álvarez-Presas & Riutort, 2014). We obtained the following four datasets for single gene alignments: (a) *COI* no outgroup (Dataset1), (b) *COI* with outgroup (Dataset2), (c) *EF1α* no outgroup (Dataset3), (d) *EF1α* with outgroup (Dataset4) (Table 2). The number of individuals sequenced for *COI* and *EF1α* differ for the following reasons: (1) *COI* sequences were obtained first from nearly all samples, whereafter the phylogenetic tree was used to select samples for *EF1α* sequencing by including individuals from different clades, as well as different localities; (2) for some samples, *COI* amplification was impossible and, thus, only *EF1α* was obtained; (3) some species from GenBank have sequences for only one of the two markers (Appendix 1).

A concatenated dataset of both genes without outgroup (Dataset5) (Table 2) was obtained in Mesquite v3.04 (Maddison & Maddison 2015), including all individuals for which sequences of both genes were available, as well as a few samples lacking one of the sequences. However, the latter sequences had to be included because they concerned the only available representatives for particular clades. Missing data were coded by Ns.

## Phylogenetic Inference

The best sequence evolution model and partition scheme for each gene alignment was estimated independently with PartitionFinder v2.1.1 (Lanfear et al. 2012), thereby considering the score for the Bayesian Information Criterion (BIC). As a preliminary step we hypothesized three partitions, corresponding with the first, second and third codon position for each gene. The results of the PartionFinder program validated this codon partition scheme, both for *COI* and *EF1α*. For each partition the best model was General Time Reversible + Gamma Distribution + Invariable Sites (GTR + Γ + I). This codon partition scheme was then implemented in phylogenetic inference analyses, with the estimations of the parameters for each partition being independent.

Because nucleotide substitution saturation may decrease phylogenetic information contained in the sequences, a saturation test (Xia et al. 2003; Xia and Lemey 2009) was run, using DAMBE (Xia 2017). Third codon positions were analysed alone, while first and second positions were analysed together, including only fully resolved sites. Since the test can only analyse 32 Operational Taxonomic Units (OTUs) at a time, 10,000 replicates of subsets of 4, 8, 16, and 32 OTUs were performed. The proportion of invariant sites was calculated and included in the saturation analysis. The alignments for each gene, including outgroups, showed no saturation for any codon position, as determined by the saturation test (in all four tests Iss was significantly lower than Iss.c, thus indicating no saturation).

Bayesian Inference (BI) and Maximum Likelihood (ML) methods were applied on the five data sets (Table 2) to infer the best tree. BI trees and posterior probabilities (PP) were inferred using MrBayes v3.2.2 (Ronquist et al. 2012). The chains were parameterized to 10 million generations, sampling every 1000 generations, and a 25% burn-in (default setting) was applied. Convergence of parameter values and topologies was examined by checking that the average standard deviation of split frequencies was below 0.01. Estimated sample size values (ESS) of each run were inspected in Tracer v1.5 (Rambaut et al. 2018) to check that the values were over 200. Maximum Likelihood (ML) trees were obtained with IQTREE software (Minh et al. 2020) using 1 million of replicates of ultrafast bootstrap approximation (Hoang et al. 2018).

## Sequences and alignments

A total of 124 *Girardia* sequences of *COI* (109 obtained in this study, 15 downloaded from GenBank) and 81 of *EF1α* (of which 80 new) were used in the final analyses, representing localities from all over the range of the genus (Appendix 1, Fig. 1). Several representatives of the genera *Dugesia* and *Schmidtea* were used as outgroups (Appendix 1). Sequences were analysed in individual gene alignments or combined into five datasets (Table 2).

## RESULTS

### Phylogenetic analyses

Phylogenetic trees were obtained from the five datasets using both BI (Figs 2, 3, S1, S2A-B) and ML (Figs S3A-D) methods. Both methods resulted in very similar topologies, with differences affecting nodes with low support. ML trees showed in general less fully supported nodes than BI. The lower supports presumably are a consequence of bootstrap resampling strategy (Efron, Halloran, & Holmes 1996; Felsenstein 1985) being affected by a high proportion of missing data in some sequences (principally those downloaded from GeneBank and/or missing one gene); the resampling process will result in fewer information for the nodes including those sequences. Nonetheless, studies based on empirical and simulated data support the benefit of including missing data over the total elimination of genes or taxa (Fulton & Strobeck, 2006; Jiang, Chen, Wang, Li, & Wiens 2014; Wiens 2003, 2006) and the outperformance of the BI method when analysing alignments with high missing data content (Wiens & Morrill 2011 and references therein). Furthermore, posterior probabilities have increased sensitivity to phylogenetic signal than bootstrap values. Therefore, posterior probabilities allow us to achieve high confidence in a correct result in analyses based on fewer characters (Alfaro et al, 2004). For these reasons, in the following we will refer only to BI posterior probabilities.

All phylogenetic trees delimited the same major groups and singletons (in the trees denoted with letters A to R), while the composition of clades does not change between datasets. We denoted with letters also five singletons (C, D, H, I, J), which may represent real clades from which, however, we only have one representative. The lettered clades are fully supported (>0.99 PP) in the concatenated dataset, with the only exception of clade K (0.67 PP). In the following, we will first describe the composition of the various clades and, where possible, the species assignments, followed by an account on the phylogenetic relationships between the clades.

*Species assignment of the terminals and clades*

Sequences of individuals of *G. schubarti* (Marcus, 1946) from GenBank and from two localities in southern Brazil constitute a monophyletic group, together with some unidentified specimens from two other localities in southern Brazil (clade B; Figs. 2, 3A, 3B). This clade was highly differentiated from the rest of the OTUs in the tree, suggesting that it could be identified as *G. schubarti*. However, given its high diversity, clade B may actually correspond to a complex of species closely related to *G. schubarti*.

OTUs of *G. multidiverticulata* de Souza et al., 2015 (clade F), *G. biapertura* Sluys, 1997 (D), *G. anderlani* (Kawakatsu & Hauser, 1983) (J) and *G.* aff. *arenicola* Hellmann & Leal-Zanchet, 2018 (I) all group into their own clades, thus representing distinctly separated lineages (Fig. 3A, B). The branch of *G. sanchezi* (Hyman, 1959), represented by two individuals from the type locality in Chile, constitute clade K. Although this is the only clade with rather low support (0.67 PP), its two representatives together with *Girardia tomasi* (clade L) constitute a maximum supported clade that is well-differentiated from all other OTUs, including other unidentified samples from Chile (clade G). OTUs of *G. tomasi* Lenguas Francavilla et al., 2021 and *G. somuncura* Lenguas Francavilla et al., 2021 from Argentina group in their respective clades L and M. Among the new specimens included in our analyses, there are five individuals from Brazil that had been identified as *G. tigrina*. Four of these individuals group into clade N, together with three non-identified individuals from Brazil, all from Rio Grande do Sul, thus suggesting that all of these OTUs belong to this species (Fig. 3A, B).

Clade P was assigned to *G. tigrina* because it included a sequence (downloaded from GenBank) from an individual of this species belonging to a sexual population from southern France (Vila-Farré et al, 2004). We were unable to include into our analyses any unequivocally identified *G. tigrina* individual from North America, nonetheless clade P comprises OTUs from Michigan, USA (679sp_USADL) and Nova Scotia, Canada (659sp_CanNS) (Fig. 3B).

There are two other species whose specimens were taxonomically identified and included in our study, viz., *G. sinensis* Chen & Wang, 2015 from China and *G. dorotocephala* (Woodworth, 1897) from North America. The first-mentioned species was represented by a sequence available from GenBank and the second by specimens purchased from Carolina Biological Supply Company that were collected from the USA, albeit that exact provenance of this sample was not known. The sequences of these two species group into two separate clades, viz., clade Q (*G. sinensis*; GB_Gsi_China) and clade R (*G. dorotocephala*; 466.2do_USA) (Fig. 3B). However, apart from *G. dorotocephala,* clade R houses also non-identified OTUs from USA, Mexico, Canada, Europe, Japan, Hawaii, and Brazil. Surprisingly, clade Q not only comprises *G. sinensis* from China, but also non-identified OTUs from Australia, China, Cuba, and Europe.

Four clades (A, E, G, and O), and two singletons (C, H) cannot be assigned to any known species of *Girardia*. Clade A comprises OTUs from distant localities,, viz., Los Tuxtlas region in Mexico (1062, 1063, 1056, 1059), and two localities from the South of USA, (Texas (InoueA_CS103) and New Mexico (InoueB_ES201)). Terminal C is an unclassified OTU from Los Tuxtlas, Mexico (1070), while clade E is formed by unclassified samples from two very distant (2018 km) Brazilian caves (401, 1178). Clade G comprises unclassified samples from Huinay Research Station, Chile (295, 298, 299), while H is an unclassified sample (402) from Santa Catarina, Brazil. Clade O houses unclassified individuals (1181, 1179) from Xochimilco Mexico (Fig. 3B).

*Phylogenetic relationships between clades*

The phylogenetic trees based on the individual genes and including the outgroup taxa reveal the presence of two main, well-differentiated lineages within the genus *Girardia* (Figs 2, S1). One of these lineages, with OTUs from Mexico, USA, and Brazil, includes two sister clades (A+B), each of which is highly supported (1 PP), and that are well-separated from each other by long branches. The other main lineage comprises all remaining *Girardia* samples, with OTUs from North, Central and South America, which group in the clades C-R (these clades are collapsed in Figs 2 and S1). Three of the lineages in this second main group (clades P, Q, R: *G. tigrina, G. sinensis*, and *G. dorotocephala,* respectively), concern OTUs that have been introduced into other parts of the world, outside of the native range of *Girardia*.

In view of these results, we replaced in further phylogenetic analyses the initial outgroup taxa (species of *Dugesia* and *Schmidtea*) by the A+B clade, in order to avoid rooting with outgroup taxa that might be too distantly related to the ingroup. In this way, we attempted to avoid long-branch attraction (Felsenstein 1978) and systematic error due to highly divergent outgroup taxa (Graham et al. 2002).

Phylogenetic trees resulting from analyses of both concatenated and individual-gene datasets, rooted with clade A+B, are shown in Figure 3 and Figures S2 and S3A-B. Individual-gene analyses (Figs. S2 and S3A-B) recovered fewer internal nodes that are fully supported than the analysis of the concatenated dataset (Fig. 3), probably due to synergetic information in the molecular markers. In the following we describe the relationships and supports found in the concatenated tree (Fig. 3).

With respect to the ingroup C-R, an unclassified OTU from Los Tuxtlas (C) is sister to a major branch comprising all remaining clades, with good support (0.96 PP).

One branch of this major clade, comprising the groups D-J, does not receive high support (0.79 PP), and concerns several South American lineages with unresolved affinities, such as: *G. biapertura* (D); OTUs from two Brazilian caves (E), and from Santa Catarina (H); *G. anderlani* (J) and the troglobitic *G.* aff. *arenicola* (I); troglobitic *G. multidiverticulata* (F), and OTUs from Chile (G).

The second major branch on the tree, comprising clades K-R, is highly supported (1.0 PP) (Fig. 3A, B). It contains a clade formed by the two sister species *G. sanchezi* (K) and *G. tomasi* (L), as well as clades of the following six well-differentiated taxa: *G. somuncura* (M), *G. tigrina* from Brazil (N), unclassified OTUs from Xochimilco, Mexico (O), *G. tigrina* from North America (P), *G. sinensis* (Q), and *G. dorotocephala* (R). All nodes within the K R group receive high to maximum support values, ranging between 0.91 and 1.0. Hence, the topology of this portion of the tree (Fig. 3) shows well-supported relationships, in contrast to clade D – J.

## DISCUSSION

### *Girardia*: genetical and chromosomal divergences

Our phylogenetic tree indicated the existence of two major lineages of *Girardia*, one constituted by the sister taxa *G. schubarti* sensu lato (clade B) and the taxonomically unidentified clade A, and the other comprising all other *Girardia* OTUs and taxa (C R) (Fig. 3). In a morphological phylogenetic analysis, *G. schubarti* grouped with other species of *Girardia* and formed a clade together with *G. arizonensis* (Kenk, 1975) and *G. azteca* (Benazzi & Giannini, 1971) (Sluys 2001). Previous molecular studies have shown similar topologies to the one presented here but analysed fewer *Girardia* species

(Lázaro et al., 2011; Álvarez-Presas & Riutort, 2014; Inoue et al. 2020). Evidently, as our study includes more OTUs than these previous studies, a more complex pattern of genealogical affinities is to be expected. In addition to the clear sister-group relationship between clades A+B and C R, the great genetic distance between these two clades is noteworthy (Fig. 2), since the length of the branches is comparable with the distance between the two sister genera *Schmidtea* and *Dugesia*, which presumably diverged about 135.9 million years ago (Mya) (Solà et al. 2022).

*Girardia schubarti* is also differentiated from other *Girardia* species by its number of chromosomes, having a basic haploid complement of n=4 (Kawakatsu et al. 1984; Jorge et al. 2000; Knakievicz et al. 2007), albeit that similar chromosome portraits are found in *G. arizonensis* and *G. jenkinsae* (Benazzi & Gourbault 1977; Benazzi 1982), species not included in the present study (unless they are represented by some of our unidentified specimens from Mexico or the USA). On the other hand, *G. tigrina*, *G. dorotocephala, G. sanchezi, G. anceps*, *G. tahitiensis*, and *G. festae* exhibit haploid complements of n=8 (Gourbault 1977; Puccinelli & Deri 1991), while *G. anderlani*, *G. biapertura* and *G. cubana* (Codreanu & Balcesco, 1973) have n=9 (Benazzi 1982; Jorge et al. 2000; Benya et al. 2007; Knakievicz et al. 2007). For *G. nonatoi,* Marcus (1946) counted in oocytes 10 chromosomes during meiosis in the haploid phase, so that the full complement presumably consists of 20 chromosomes. Unfortunately, the chromosome portraits of other species of *Girardia* are unknown. Despite this paucity of information on chromosome number in the genus *Girardia*, a pattern emerges when the complements are plotted on the phylogenetic tree: clade A+B includes *G. schubarti* with n=4, D J includes two species (*G. anderlani*, *G. biapertura*) with n=9 , and clade K R clade includes three species (*G. tigrina*, *G. dorotocephala, G. sanchezi*) with n =8. If the chromosomal numbers found in the few species within each of these major clades (A+B, D-J, and K-R) are presumed to be common for all species within each of these groups, it may be hypothesized that the origin of the main clades of *Girardia* was associated with events of genomic duplications and/or chromosomal rearrangements.

Differences in chromosome number between closely related species of triclads are relatively common and have been related to speciation events in the genera *Schmidtea* and *Dugesia* (Leria et al. 2018, 2020). However, with the present information available for *Girardia*, it cannot be excluded that chromosomal changes were not the drivers of the speciation process but accumulated only after speciation had taken place. Therefore, it is only through future, more comprehensive and integrative studies that it may be determined whether the great genetical and chromosomal divergences of the A+B clade, as compared to its congeners, warrant taxonomic recognition in the form of a separate genus, or merely represent highly evolved autapomorphic features for a particular branch within the genus *Girardia*.

## Genetic differentiation within clades of *Girardia*

Although we only have scattered samples from all over the Americas (Appendix 1, Fig. 1), our molecular-based phylogenetic results revealed a high genetic diversity and structure within *Girardia*, particularly in Mexico and Brazil.

Mexico showed the highest molecular diversity, despite the rather low number of samples. Eleven individuals were analysed, two of unknown origin and the rest coming from five localities (Appendix 1), which exhibited clear structure and genetic differentiation and comprised four different clades (A, C, O, and R, Fig. 3). From these four clades, particularly clade A is noteworthy because of its high internal diversity, albeit that it does not include any taxonomically identified species. However, genetic structure within clade A suggests that it contains more than one species. This is in accordance with the suggestion made by Inoue et al. (2020), who delimited two putative species on the basis of short fragments of *COI*, coded InoueA and InoueB in the present work (Fig. 3). Clades C and O (sister to D-R clade and P+Q+R clade, respectively) comprise only animals from Mexico, while some Mexican individuals occur also in clade R (*G. dorotocephala*). Evidently, at this moment it remains undecided whether the observed genetic diversity concerns new species of *Girardia* or merely reflects the presence of already known species of which molecular data is still lacking.

This high genetic Mexican diversity is not geographically structured. Within clade A, the long branch separating samples from the Biological Station (1056, 1059) and individuals from Laguna Escondida (1062, 1063) suggests that two genetically highly differentiated species are present at these two localities, although the collection sites are only 2 Km apart. Clade C is only formed by individual 1070 from a second collection site at the Biological Station. All of this points to a possible co-occurrence of two highly differentiated species in the same river within the Biological Station. At the Xochimilco locality we found three specimens, two constituting the sister clade (O) of the group including clades P, Q and R, while the third specimen (1180) belongs to clade R (*G. dorotocephala*). This mix of genetically distant species at sites that are geographically in close proximity to each other, suggests a complex history for the diversification and evolution of *Girardia* in the Americas or, alternatively could be the results of human introductions.

Another interesting fact that surfaced in our analyses was the relatively high diversity of cave-dwelling species in Brazil, with *G. multidiverticulata* being the first troglobitic continenticolan to be reported from South America. Although its distinctive characters differ from other species of *Girardia*, it shares with *G. anderlani* the presence of a large and branched bulbar cavity (Souza et al. 2015). Unfortunately, our molecular trees did not show sufficient resolution to support a sister-group relationship between *G. multidiverticulata* and *G. anderlani.* In point of fact, the trees suggested a closer relationship between epigean *G. anderlani* and specimen 261, which probably represents troglobitic *G.* aff. *arenicola*, both showing dorsal testes and a branched bulbar cavity (Hellmann et al. 2018).

Among our Brazilian samples there are two other specimens that originated from hypogean habitats, viz., OTUs 401 and 1178, together constituting clade E (Fig. 3). These two individuals are genetically quite distinct, while their sampling localities are far apart. This suggests that clade E comprises two new cave-dwelling species of *Girardia*. This recently discovered flourishing of hypogean *Girardia* species in Brazil (see Morais et al. 2021) may be an indication that the genus is highly successful in adapting to life in caves and that future studies of those habitats in other regions in the Americas may unveil further diversity.

To date, nine species of *Girardia* have been recorded from Mexico, USA, and Canada, with *G. tigrina* and *G. dorotocephala* being the most widely distributed ones (Sluys et al. 2005). The present study adds *G. sinensis*, described from a locality in China (Chen et al. 2015), since we identified it molecularly from Cuba (Figs 1, 3A). Moreover, the close phylogenetic relationship that this species shares with *G. dorotocephala* and *G. tigrina*, both of North American origin, also clearly point to that region as the original area of distribution of *G. sinensis*. In a recent molecular study on freshwater planarians from New Mexico and Texas, Inoue et al. (2020) identified two putative new species (InoueC and InoueD in our trees) that were closely related to *G. tigrina* and *G. dorotocephala*. However, in our analyses, both of their sequences grouped among representatives of *G. dorotocephala* (Fig. 3). Several other sequences from individuals collected outside of the Americas fall into the three clades P, Q, and R, thus corroborating the North American origin of the introduced populations.

## Nominal *Girardia tigrina*

A very interesting result of our molecular analysis concerns the positions in the phylogenetic tree of the North and South American *G. tigrina* samples, showing that the Brazilian clade (N) is not even sister to the North American one (clade P) (Fig. 3). This corroborates the conclusion of Sluys et al. (2005) that the North and South American forms are different species. According to Sluys et al. (2005), the only anatomical difference between them resides in the coat of muscles around the bursal canal. In North American *G. tigrina* this coat of muscles consists of a thin, subepithelial layer of circular muscle, followed by an equally thin layer of longitudinal muscle fibres. In contrast, the South American form possesses a bursal canal musculature that consists of a well-developed coat of intermingled circular and longitudinal muscle fibres. In other characters the two forms are very similar, but our results clearly show that they are genetically well-differentiated and are not even sister species. Therefore, the South American form is here described and named as the new species *Girardia clandistina* Sluys & Benítez-Álvarez, sp. nov. (for differential diagnosis, see Appendix 2). This taxonomic action is not unimportant, since *G. tigrina* is the type species of the genus *Girardia* (Kenk 1974) and, therefore, it is necessary to know the precise boundaries of the taxon and the extension of the species name.

## Historical biogeography of *Girardia*

Considering the origin of the samples analysed (Fig. 3A), it is possible to comment the biogeographical history of the genus *Girardia*, at least for the taxa included in our study. However, it is important to take into account a number of issues that complicate geographic interpretation of the tree. For example, *G. sinensis*, although described from China, appears to be of North American origin. Moreover, *G. sinensis, G. tigrina*, and *G. dorotocephala* have been introduced from North America into other parts of the world, and, therefore, any country outside of the North American subcontinent should be disregarded when considering the natural distribution of the genus. However, South America is an exception to this rule, in that the present study shows that presumed *G. tigrina* from this subcontinent actually concerns the new, sibling species *G. clandistina* Sluys & Benítez-Álvarez, sp. nov

Our phylogenetic tree suggests that *Girardia* evolved on the South American subcontinent and from there colonized North America. Previous studies argued that the family Dugesiidae, including *Girardia*, had already diversified on Gondwanaland (Ball 1975) or even at pre-Pangaean times and, thus, must have diversified also already on Pangaea (Sluys et al. 1998). According to our results, *Girardia* diversified on the South American portion of Gondwanaland, and, subsequently, ancestors of the A, C, and O-R clades migrated to North America, possibly in three different waves, and diversified there. The relatively short inter-branches among the clades on the North American subcontinent (clades O, P, Q, R; Fig. 3B) imply that diversification of *Girardia* in North America is rather recent, and hence that the northward migration for this group probably did take place only after complete closure of the Isthmus of Panama at about 2.8 Mya (O'Dea et al. 2016).

Interestingly, presence of the basal lineages A in Mexico and the USA, and clade C in Mexico, and of the crown group O-R in Mexico, USA, and Canada, suggests that the North American subcontinent was populated by at least three independent waves of dispersal from the Neotropics. Although in these cases the available data do not allow us to infer whether this northward migration was relatively recent or took place in more remote epochs, again, it would have been possible only through freshwater tracks in the intermittent connections during emergence of the Isthmus of Panama or once it was fully established (McGirr et al. 2021). Unfortunately, lack of OTUs from the northern parts of South America prevents further elucidation of the precise routes taken by neotropical *Girardia*'s during their dispersal into the Nearctic.

**Data availability**

All sequences have been deposited in GenBank.

**Conflicts of interest**

All authors declare no conflict of interest to disclose.

**Funding statement**

This work was supported by the Brazilian Research Council - CNPq, Brazil [grants 306853/2015-9 and 313691/2018-5] and Ministerio de Economía y Competitividad, Spain [projects CGL2015-63527-1P and PGC2018-093924-B-100]. Fieldwork in Mexico was funded by the Unión Iberoamericana de Universidades (UIU), Spain [Project BIO02/2017].

**References**

Alfaro, M. E., Zoller, S., & Lutzoni, F. (2003). Bayes or Bootstrap? A simulation study comparing the performance of Bayesian Markov Chain Monte Carlo sampling and bootstrapping in assessing phylogenetic confidence. Molecular Biology and Evolution, 20(2), 255–266. https://doi.org/10.1093/molbev/msg028

Álvarez-Presas, M., & Riutort, M. (2014). Planarian (Platyhelminthes, Tricladida) diversity and molecular markers: A new view of an old group. *Diversity*, *6*, 323–338. https://doi.org/10.3390/d6020323

Ball, I. R. (1975). Nature and formulation of biogeographical hypotheses. *Systematic Zoology*, *24*(4), 407–430.

Benazzi, M. (1982). Speciation events evidenced in Turbellaria. In C. Barigozzi (Ed.), *Mechanisms of Speciation* (pp. 307–344). New York: Alan R. Liss.

Benazzi, M., & Gourbault, N. (1977). *Dugesia jenkinsae* n. sp., a fresh-water triclad (Turbellaria) from Texas. *Transactions of the American Microscopical Society, 96*, 540–543

Benya, E. G. F., Leal-Zanchet, A. M., Santos, W. H., Hauser, J., & Erdtmann, B. (2007). Chromosome polymorphism and complements in populations of *Girardia* species (Platyhelminthes, Tricladida, Paludicola) from southern Brazil. *Brazilian journal of Biology*, *67*(4 Suppl), 951–5. https://doi.org/10.1590/S1519-69842007000500021

Chen, X., Chen, Y., Wu, C., & Wang, A. (2015). A new species of the genus *Girardia* (Tricladida: Dugesiidae) from China. *Zoological Systematics*, *40*(2), 166–178. https://doi.org/10.11865/zs.20150202

Dols-Serrate, D., Leria, L., Aguilar, J. P., Stocchino, G. A., & Riutort, M. (2020). *Dugesia hepta* and *Dugesia benazzii* (Platyhelminthes: Tricladida): two sympatric species with occasional sex? *Organisms Diversity and Evolution, 20*(3), 369–386. https://doi.org/10.1007/s13127-020-00438-z

Efron, B., Halloran, E., & Holmes, S. (1996). Bootstrap confidence levels for phylogenetic trees. *Proceedings of the National Academy of Sciences of the United States of America, 93*(23), 13429–13434. https://doi.org/10.1073/pnas.93.23.13429

Felsenstein, J. (1978). Cases in which parsimony or compatibility methods will be positively misleading. *Systematic Zoology*, *53*(1), 401–410. https://doi.org/10.1080/10635150490265012

Felsenstein, J. (1985). Confidence limits on phylogenies: An approach using the bootstrap. *Evolution, 39*(4), 783. https://doi.org/10.2307/2408678

Fulton, T. L., & Strobeck, C. (2006). Molecular phylogeny of the Arctoidea (Carnivora): Effect of missing data on supertree and supermatrix analyses of multiple gene data sets. *Molecular Phylogenetics and Evolution, 41*(1), 165–181. https://doi.org/10.1016/j.ympev.2006.05.025

Gourbault, N. (1977). Etude descriptive et cytotaxonomique d'une planaire polynesienne *Dugesia tahitiensis* n. sp. (Turbellarie, Triclade). *Annales de Limnologie*, *13*(2), 211–220.

Graham, S. W., Olmstead, R. G., & Barrett, S. C. H. (2002). Rooting phylogenetic trees with distant outgroups: A case study from the commelinoid monocots. *Molecular Biology and Evolution*, *19*(10), 1769–1781. https://doi.org/10.1093/OXFORDJOURNALS.MOLBEV.A003999

Grant, L. J., Sluys, R., & Blair, D. (2006). Biodiversity of Australian freshwater planarians (Platyhelminthes: Tricladida: Paludicola): New species and localities, and a review of paludicolan distribution in Australia. *Systematics and Biodiversity*, *4*(4), 435–471. https://doi.org/10.1017/S1477200006002064

Grant, L. J. (2017). Biodiversity, Biogeography and Phylogeny of Australian Freshwater Triclads (Platyhelminthes: Tricladida: Continenticola). PhD thesis, James Cook University. https://researchonline.jcu.edu.au/51512/1/51512-grant-2016-thesis.pdf

Hall, T. A. (1999). BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucl. Acids. Symp. Ser.*, *41*, 95–98.

Hellmann, L., Ferreira, R. L., Rabelo, L., & Leal-Zanchet, A. M. (2020). Enhancing the still scattered knowledge on the taxonomic diversity of freshwater triclads (Platyhelminthes: Dugesiidae) in caves from two Brazilian Biomes. *Studies on Neotropical Fauna and Environment*, *00*(00), 1–16. https://doi.org/10.1080/01650521.2020.1829901

Hellmann, L., Leal-Zanchet, A. M., & Lopes Ferreira, R. (2018). Two new sympatric troglobitic freshwater flatworms (platyhelminthes: Dugesiidae) from a hotspot of subterranean biodiversity in the neotropics. *Zootaxa*, *4438*(3), 561–574. https://doi.org/10.11646/zootaxa.4438.3.8

Hoang, D. T., Chernomor, O., Haeseler, A. von, Minh, B. Q., & Vinh, L. S. (2018). UFBoot2: Improving the ultrafast bootstrap approximation. *Molecular Biologyand Evolution, 35*, 518–522. https://doi.org/10.1093/molbev/msx281

Inoue, K., Pohl, A. L., Sei, M., Lang, B. K., & Berg, D. J. (2020). Use of species delimitation approaches to assess biodiversity in freshwater planaria (Platyhelminthes, Tricladida) from desert springs. *Aquatic Conservation: Marine and Freshwater Ecosystems*, aqc.3273. https://doi.org/10.1002/aqc.3273

Jiang, W., Chen, S.-Y., Wang, H., Li, D.-Z., & Wiens, J. J. (2014). Should genes with missing data be excluded from phylogenetic analyses? *Molecular Phylogenetics and Evolution, 80*, 308–318. https://doi.org/10.1016/j.ympev.2014.08.006

Jorge, M. A. P., Alvarez, L. E., & Almeida, E. J. C. (2000). Occurrència de mixoploidia em exemplares de Girardia schubarti (Platyhelminthes, Tricladida, Paludicola). *Genetics & Molecular Biology*, *20*(3), Suppl.: 5-6.

Kawakatsu, M., Oki, I., Tamura, S., Yamayoshi, T., Hauser, J., & Friedrich, S. M. G. (1984). Morphological, karyological and taxonomic studies of freshwater planarians from South Brazil. VI. *Dugesia schubarti* (Marcus, 1946) from the vicinity of São Leopoldo, Estado de Rio Grande do Sul (Turbellaria, Tricladida, Paludicola). *Bulletin of Fuji Women's College*, *No. 22,* (ser.II), 45–62.

Kearse, M., Moir, R., Wilson, A., Stones-Havas, S., Cheung, M., Sturrock, S., et al. (2012). Geneious Basic: An integrated and extendable desktop software platform for the organization and analysis of sequence data. *Bioinformatics*, *28*(12), 1647–1649. https://doi.org/10.1093/bioinformatics/bts199

Kenk, R. (1974). Index of the genera and species of the freshwater triclads (Turbellaria) of the world. *Smithsonian Contributions to Zoology*, *183*, 1–90. https://doi.org/10.5479/si.00810282.183

Knakievicz, T., Lau, A. H., Prá, D., & Erdtmann, B. (2007). Biogeography and karyotypes of freshwater planarians (Platyhelminthes, Tricladida, Paludicola) in southern Brazil. *Zoological Science*, *24*(2), 123–129. https://doi.org/10.2108/zsj.24.123

Lanfear, R., Calcott, B., Ho, S. Y. W., & Guindon, S. (2012). PartitionFinder: Combined selection of partitioning schemes and substitution models for phylogenetic analyses. *Molecular Biology and Evolution*, *29*(6), 1695–1701. https://doi.org/10.1093/molbev/mss020

Lázaro, E. M., Harrath, A. H., Stocchino, G. A., Pala, M., Baguñà, J., & Riutort, M. (2011). *Schmidtea mediterranea* phylogeography: an old species surviving on a few

Mediterranean islands? *BMC Evolutionary Biology*, *11*(1), 274. https://doi.org/10.1186/1471-2148-11-274

Lenguas Francavilla, M., Negrete, L., Martínez-Aquino, A., Damborenea, C., & Brusa, F. (2021). Two new freshwater planarian species (Platyhelminthes: Tricladida: Dugesiidae) partially sympatric in Argentinean Patagonia. *Canadian Journal of Zoology*, *99*, 269–278. https://doi.org/10.1139/cjz-2020-0169

Leria, L., Sluys, R., & Riutort, M. (2018). Diversification and biogeographic history of the Western Palearctic freshwater flatworm genus *Schmidtea* (Tricladida: Dugesiidae), with a redescription of *Schmidtea nova*. *Journal of Zoological Systematics and Evolutionary Research*, *56*(3), 335–351. https://doi.org/10.1111/jzs.12214

Leria, L., Vila-Farré, M., Álvarez-Presas, M., Sánchez-Gracia, A., Rozas, J., Sluys, R., & Riutort, M. (2020). Cryptic species delineation in freshwater planarians of the genus *Dugesia* (Platyhelminthes, Tricladida): Extreme intraindividual genetic diversity, morphological stasis, and karyological variability. *Molecular Phylogenetics and Evolution*, *143*, 106496. https://doi.org/10.1016/j.ympev.2019.05.010

Maddison, W. P., & Maddison, D. R. (2015). Mesquite: A modular system for evolutionary analysis. Version 3.04. *http://www.mesquiteproject.org*.

Marcus, E. (1946). Sôbre Turbellaria límnicos brasileiros. *Boletim da Faculdade de Filosofia, Ciências e Letras, Zoologia. Universidade de São Paulo*, *11*, 5–254.

McGirr, R., Seton, M., & Williams, S. (2021). Kinematic and geodynamic evolution of the Isthmus of Panama region: Implications for Central American Seaway closure. *GSA Bulletin*, *133*(3–4), 867–884. https://doi.org/10.1130/B35595.1

Minh, B. Q., Schmidt, H. A., Chernomor, O., Schrempf, D., Woodhams, M. D., Haeseler, A. von, & Lanfear, R. (2020). IQ-TREE 2: New models and efficient methods for phylogenetic inference in the genomic era. *Molecular Biology and Evolution, 37*, 1530–1534. https://doi.org/10.1093/molbev/msaa015

Morais, A. L., Bichuette, M. E., Chagas-Júnior, A., & Leal-Zanchet, A. (2021). Under threat: Two new troglobitic species of *Girardia* (Platyhelminthes: Tricladida) from sandstone and limestone caves in Brazil. *Zoologischer Anzeiger*, *293*, 292–302. https://doi.org/10.1016/J.JCZ.2021.06.015

O'Dea, A., Lessios, H. A., Coates, A. G., Eytan, R. I., Restrepo-Moreno, S. A., Cione, A. L., et al. (2016). Formation of the Isthmus of Panama. *Science Advances*, *2*(8). https://doi.org/10.1126/sciadv.1600883

Puccinelli, I., & Deri, P. (1991). Comparative karyological analysis of some American planarians belonging to the genus *Dugesia* (subgenus *Girardia*) (Platyhelminthes, Tricladida). *Caryologia*, *44*(3–4), 225–232. https://doi.org/10.1080/00087114.1991.10797189

Rambaut, A., Drummond, A. J., Xie, D., Baele, G., & Suchard, M. A. (2018). Posterior summarization in Bayesian phylogenetics using Tracer 1.7. *Systematic Biology*, *67*(5), 901–904. https://doi.org/10.1093/SYSBIO/SYY032

Ronquist, F., Teslenko, M., van der Mark, P., Ayres, D. L., Darling, A., Höhna, S., et al. (2012). Mrbayes 3.2: Efficient Bayesian phylogenetic inference and model choice across a large model space. *Systematic Biology*, *61*(3), 539–542. https://doi.org/10.1093/sysbio/sys029

Sluys, R. (2001). Towards a phylogenetic classification and characterization of dugesiid genera (Platyhelminthes, Tricladida, Dugesiidae): a morphological perspective. In

D. T. J. Littlewood & R. D. Bray (Eds.), *Interrelationships of the Platyhelminthes. Syst. Ass. Spec. Vol. Ser. 60* (pp. 57–73). London, England: Taylor & Francis.

Sluys, R., Joffe, B., & Cannon, L. R. G. (1995). An alien flatworm in Australian waters. *Memoirs of the Queensland Museum*, *38*(2), 642.

Sluys, R., & Kawakatsu, M. (2001). Contribution to an inventory of the freshwater planarians of Australia and New Zealand (Platyhelminthes, Tricladida, Dugesiidae), with distribution maps of the species examined. *Beaufortia/Bulletin Zoölogisch Museum Universiteit van Amsterdam*, *51*(10), 163–198.

Sluys, R., Kawakatsu, M., & Ponce de León, R. (2005). Morphological stasis in an old and widespread group of species: Contribution to the taxonomy and biogeography of the genus *Girardia* (Platyhelminthes, Tricladida, Paludicola). *Studies on Neotropical Fauna and Environment*, *40*, 155–180.

Sluys, R., Kawakatsu, M., & Winsor, L. (1998). The genus *Dugesia* in Australia, with its phylogenetic analysis and historical biogeography (Platyhelminthes, Tricladida, Dugesiidae). *Zoologica Scripta*, *27*(4), 273–289. https://doi.org/10.1111/j.1463-6409.1998.tb00461.x

Sluys, R., Solà, E., Gritzalis, K., Vila-farré, M., Mateos, E., & Riutort, M. (2013). Integrative delineation of species of Mediterranean freshwater planarians (Platyhelminthes: Tricladida: Dugesiidae). *Zoological Journal of the Linnean Society, 169*, 523–547. https://doi.org/10.1111/zoj.12077

Solà, E., Sluys, R., Gritzalis, K., & Riutort, M. (2013). Fluvial basin history in the northeastern Mediterranean region underlies dispersal and speciation patterns in the genus *Dugesia* (Platyhelminthes, Tricladida, Dugesiidae). *Molecular Phylogenetics and Evolution*, 66(3), 877–888. https://doi.org/10.1016/j.ympev.2012.11.010

Solà, E., Leria L, Stocchino GA, Bagherzadeh R, Balke M, Daniels SR, Harrath AH, Khang TF, Krailas D, Kumar B, Li M-H, Maghsoudlou A, Matsumoto M, Naser N, Oben B, Segev O, Thielicke M, Tong X, Zivanovic G, Manconi R, Baguñà J, & Riutort M (2022). Three dispersal routes out of Africa: A puzzling biogeographical history in freshwater planarians. *Journal of Biogeography,* online https://doi.org/10.1111/jbi.14371.

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*(1), 107–121.

Souza, S. T., Morais, A. L. N., Cordeiro, L. M., & Leal-Zanchet, A. M. (2015). The first troglobitic species of freshwater flatworm of the suborder Continenticola (Platyhelminthes) from South America. *ZooKeys*, *16*(470), 1–16. https://doi.org/10.3897/zookeys.470.8728

Stocchino, G. A., Sluys, R., Harrath, A. H., Mansour, L., & Manconi, R. (2019). The invasive alien freshwater flatworm *Girardia tigrina* (Girard, 1850) (Platyhelminthes, Tricladida) in Western Europe: new insights into its morphology, karyology and reproductive biology. *Contributions to Zoology*, 88, 1–21. https://doi.org/10.1163/18759866-20191406

Vila-Farré, M., Sancho, M., Cardona, A., Fernández, J., & Romero, R. (2004). Nueva poblacion sexuada de *Girardia tigrina* (Girard, 1850) Kenk, 1974 (Platyhelminthes, Turbellaria, Tricladida, Dugesidae), en el sur de Francia. *Boletín de La Real Sociedad Española de Historia Natural Seccion Biologica*, 99(1–4), 49–53.

Wiens, J. J. (2003). Missing data, incomplete taxa, and phylogenetic accuracy. *Systematic Biology, 52*(4), 528–538. https://doi.org/10.1080/10635150390218330

Wiens, J. J. (2006). Missing data and the design of phylogenetic analyses. *Journal of Biomedical Informatics, 39*(1), 34–42. https://doi.org/10.1016/j.jbi.2005.04.001

Wiens, J. J., & Morrill, M. C. (2011). Missing data in phylogenetic analysis: Reconciling results from simulations and empirical data. *Systematic Biology, 60*(5), 719–731. doi.org/10.1093/sysbio/syr025

Xia, X. (2017). DAMBE6: New tools for microbial genomics, phylogenetics, and molecular evolution. *Journal of Heredity*, *108*(4), 431–437. https://doi.org/10.1093/jhered/esx033

Xia, X., & Lemey, P. (2009). Assessing substitution saturation with DAMBE. In P. Lemey, M. Salemi, & A.-M. Vandamme (Eds.), *The phylogenetic handbook: a practical approach to DNA and protein phylogeny* (2nd ed., pp. 615–630). Cambridge: Cambridge University Press. https://doi.org/10.1017/CBO9780511819049.022

Xia, X., Xie, Z., Salemi, M., Chen, L., & Wang, Y. (2003). An index of substitution saturation and its application. *Molecular phylogenetics and evolution*, *26*, 1–7. https://doi.org/10.1016/s1055-7903(02)00326-3

# LEGENDS TO FIGURES, TABLES, SUPPLEMENTARY FIGURES, AND APPENDICES

**Figure 1.** Maps showing the sampling localities for all individuals analysed in the present study, including those corresponding to GenBank sequences. A: The Americas; B: Western Europe; C: Hawaii; D: Asia and Oceania. For a finer resolution, visit the interactive map at: https://www.ub.edu/planarian-maps/

**Figure 2**. Bayesian Inference tree inferred from Dataset2 (*COI* with outgroup). Clades C to R have been collapsed for the sake of clarity. Clade A is constituted by unclassified samples from Mexico and Texas (USA); Clade B includes identified individuals of *Girardia schubarti* from Brazil and other unidentified Brazilian individuals. Outgroup is composed of several representatives of genera *Dugesia* and *Schmidtea* downloaded from GenBank (Appendix 1). Values at nodes correspond to posterior probability. Scale bar: number of substitutions per nucleotide position.

**Figure 3**. Bayesian Inference tree inferred from Dataset5 (concatenated no outgroup). Different clades indicated by letters and colours. (A) Schematic representation of the tree with collapsed clades (triangles), and singletons (rectangles) showing species identifications, when available, and countries of origin of the various terminals. The relationships between groups D, E, F, G, H, I, and J have been collapsed due to lack of resolution; shaded circles at nodes indicate posterior probability support, with values below 0.9 considered to be unsupported. (B) circular tree with all terminals; values at nodes correspond to posterior probability support. Scale bar: number of substitutions per nucleotide position.

**Table 1.** Primers used in this study, sequences, references, and annealing temperature (AT).

**Table 2.** Datasets analysed in this study, with their shorthand description, indication of the phylogenetic trees resulting from the analysis, number of species belonging to either *Dugesia* or *Schmidtea* used as outgroups, number of gene sequences, and the total length of these sequences in nucleotides.

**Appendix 1.** Sequences included in this study, with indication of sample codes, sampling localities (for *Girardia* samples), taxonomic assignment before and after analyses, codes used in the text and figures, and GenBank accession numbers for *COI* and *EF1α* sequences. Sequence codes in bold concern new sequences reported in this study. See Supplementary Table S1 for exact localities, collectors, and criteria used for taxonomic assignment previously to our analysis.

**Appendix 2.** Differential diagnosis of *Girardia clandistina* Sluys & Benítez-Álvarez, sp. nov.

## SUPPORTING INFORMATION

Figure S1. Bayesian Inference tree from Dataset4 (EF1a with outgroup). The sister group of *Girardia schubarti* (clade A) and unclassified samples from Mexico (clade B) has been collapsed. Values at nodes correspond to posterior probability support. Scale bar: number of substitutions per nucleotide position.

Figure S2. Bayesian Inference trees from A: Dataset1 (*COI* without outgroup) and B: Dataset3 (*EF1α* without outgroup). Values at nodes correspond to posterior probability support. Scale bar: number of substitutions per nucleotide position.

Figure S3. Maximum Likelihood trees obtained with IQTREE software. A: ML tree, COI no outgroup (Dataset2). B: ML tree, EF1$\alpha$ with outgroup (Dataset4). C: ML tree, COI without outgroup (Dataset1). D: ML tree, EF1$\alpha$ without outgroup (Dataset3). E: ML tree, concatenated COI and EF1$\alpha$ without outgroup (Dataset5). Values at nodes correspond to ultrafast bootstrap support. Scale bar: number of substitutions per nucleotide position.

Table S1. Samples included in this study, geographical coordinates, collectors, and identification method. The asterisk indicates approximated coordinates based on sampling locality description.

Table S2. *COI* and *EF1$\alpha$* sequences of *Girardia* genus present in GenBank. In bold are indicated the sequences included in the analyses. Reason for exclusion is indicated for certain sequences not included in the present study.

**Table 1.**

| Primer | Sequence (5´-3´) | Reference | AT |
|---|---|---|---|
| **Cytochrome C Oxidase I** | | | |
| BarS (Forward) | GTTATGCCTGTAATGATTG | Alvarez-Presas et al., 2011 | 43ºC |
| BarT (Forward) | ATGACDGCSCATGGTTTAATAATGAT | Alvarez-Presas et al., 2011 | |
| COIR (Reverse) | CCWGTYARMCCHCCWAYAGTAAA | Lazaro et al., 2009 | |
| PlatR-Gi (Reverse) | CATCCTGAGGTTTATATWTTGATT | This study | |
| **Elongation Factor 1α** | | | |
| EF2a (Forward) | GARGCYCARGARATGGGWAAAGGWTC | Barney et al., 2000 | 54ºC |
| EF9a (Reverse) | TCNGCRAAYTTGCARGCAATRTGWGC | Barney et al., 2000 | |
| ef1aF (Forward) | ATACGCTTGGGTTTTGG | This study | 47ºC |
| ef1aR (Reverse) | ATGRATTTGACCTGGGTG | This study | |
| EFGi-2F (Forward) | CCT TCA AAT ACG CTT GGG | This study | 51ºC |
| EFGi-2R (Reverse) | GRATTTGACCTGGRTGATTC | This study | |

**Table 2.**

| **Data sets** | **Description** | **Tree figure** | **Outgroup** | | **Gene sequences** | | **OTUs** | **Length** |
|---|---|---|---|---|---|---|---|---|
| | | | ***Dugesia*** | ***Schmidtea*** | **COI** | **EF1a** | | |
| Dataset 1 | COI no outgroup | Figure S2 A | | | 124 | | 124 | 837 |
| Dataset 2 | COI with outgroup | Figure 2 | 6 | 3 | 133 | | 133 | 840 |
| Dataset 3 | EF1a no outgroup | Figure S2 B | | | | 81 | 81 | 879 |
| Dataset 4 | EF1a with outgroup | Figure S1 | 4 | 1 | | 86 | 86 | 879 |
| Dataset 5 | Concatenated no outgroup | Figure 3 | | | 94 | 78 | 98 | 1716 |

**Appendix 1**.

| **Sample ID** | **Locality** | **Taxonomic Identification before analysis** | **Taxonomic Identification after analysis** | **ID in Figures** | **COI** | **EF1α** |
|---|---|---|---|---|---|---|
| **84** | France, River Lez | *Girardia* sp. | *G. dorotocephala* | 84sp_FrLez | **OM307073** | **OM349486** |
| **86.1** | France, River Laderge | *Girardia* sp. | *G. dorotocephala* | 86.1sp_FrLad | **OM307074** | |
| **86.2** | France, River Laderge | *Girardia* sp. | *G. dorotocephala* | 86.2sp_FrLad | **OM307075** | |
| **87** | France, Lunaç | *Girardia* sp. | *G. tigrina* | 87sp_FrLun | **OM307076** | **OM418671** |
| **109** | Spain, Catalonia, St. Llorenç de la Muga, River Muga | *Girardia* sp. | *G. tigrina* | 109sp_CatMug | **OM307077** | **OM418675** |
| **116** | Spain, Catalonia, Riba-roja d'Ebre, River Ebro | *Girardia* sp. | *G. tigrina* | 116sp_CatEbr | **OM307078** | **OM418673** |
| **125** | Brazil, Rio Grande do Sul, Constantina | *G. schubarti* | *G. schubarti* | 125sc_BrRGScon | **OM307079** | **OM418648** |
| **126** | Brazil, Rio Grande do Sul, Constantina | *G. schubarti* | *G. schubarti* | 126sc_BrRGScon | **OM307080** | **OM418649** |
| **127** | Brazil, Rio Grande do Sul, São Leopoldo | *Girardia* sp. | *G. tigrina* Brazil | 127sp_BrRGSleo | **OM307081** | **OM418688** |

|  |  |  |  |  |  |  |
|---|---|---|---|---|---|---|
| **131.1** | Brazil, Rio Grande do Sul, São Sebastião de Caí | *G. biapertura* | *G. biapertura* | 131.1bi_BrRGSseb | **OM307082** |  |
| **131.2** | Brazil, Rio Grande do Sul, São Sebastião de Caí | *G. biapertura* | *G. biapertura* | 131.2bi_BrRGSseb | **OM307083** |  |
| **132** | Brazil, Rio Grande do Sul, Gramado | *G. tigrina* | *G. tigrina* Brazil | 132ti_BrRGSgra | **OM307084** |  |
| **133** | Brazil, Rio Grande do Sul, São Leopoldo | *G. tigrina* | *G. tigrina* Brazil | 133ti_BrRGSleo | **OM307085** | **OM418690** |
| **134** | Brazil, Rio Grande do Sul, Salvador do Sul | *G. tigrina* | *G. tigrina* Brazil | 134ti_BrRGSsalv | **OM307086** | **OM418689** |
| **135.1** | France, Gorges de l'Ardeche | *Girardia* sp. | *G. tigrina* | 135.1sp_FrArd | **OM307087** | **OM418677** |
| **135.2** | France, Gorges de l'Ardeche | *Girardia* sp. | *G. tigrina* | 135.2sp_FrArd |  | **OM418679** |
| **137** | France, Ispagnac | *Girardia* sp. | *G. tigrina* | 137sp_FrIsp | **OM307088** | **OM418682** |
| **138.1** | Spain, Catalonia, Girona, Orfes, River Fluvià | *Girardia* sp. | *G. sinensis* | 138.1sp_CatFlu | **OM307089** | **OM418669** |
| **138.2** | Spain, Catalonia, Girona, Orfes, River Fluvià | *Girardia* sp. | *G. sinensis* | 138.2sp_CatFlu | **OM307090** | **OM418664** |
| **138.3** | Spain, Catalonia, Girona, Orfes, River Fluvià | *Girardia* sp. | *G. tigrina* | 138.3sp_CatFlu | **OM307091** | **OM418681** |
| **138.4** | Spain, Catalonia, Girona, Orfes, River Fluvià | *Girardia* sp. | *G. tigrina* | 138.4sp_CatFlu | **OM307092** |  |
| **138.5** | Spain, Catalonia, Girona, Orfes, River Fluvià | *Girardia* sp. | *G. sinensis* | 138.5sp_CatFlu | **OM307093** |  |

| | | | | | | |
|---|---|---|---|---|---|---|
| **138.6** | Spain, Catalonia, Girona, Orfes, River Fluvià | *Girardia* sp. | *G. sinensis* | 138.6sp_CatFlu | **OM307094** | |
| **138.7** | Spain, Catalonia, Girona, Orfes, River Fluvià | *Girardia* sp. | *G. sinensis* | 138.7sp_CatFlu | **OM307095** | |
| **138.8** | Spain, Catalonia, Girona, Orfes, River Fluvià | *Girardia* sp. | *G. sinensis* | 138.8sp_CatFlu | **OM307096** | **OM418663** |
| **139.1** | Spain, Menorca, Algendar | *Girardia* sp. | *G. sinensis* | 139.1sp_EsMen | **OM307097** | |
| **139.2** | Spain, Menorca, Algendar | *Girardia* sp. | *G. sinensis* | 139.2sp_EsMen | **OM307098** | **OM418657** |
| **140.1** | Italy, Sardinia, R.Fungarone, Putifigari | *Girardia* sp. | *G. sinensis* | 140.1sp_ItCer | **OM307099** | **OM418655** |
| **140.2** | Italy, Sardinia, R.Fungarone, Putifigari | *Girardia* sp. | *G. sinensis* | 140.2sp_ItCer | **OM307100** | |
| **143** | Italy, Toscana, Torr.Vincio | *Girardia* sp. | *G. tigrina* | 143sp_ItTos | **OM307101** | **OM418683** |
| **247** | Spain, Pontevedra, Gondomar | *Girardia* sp. | *G. sinensis* | 247sp_EsPon | **OM307102** | **OM418653** |
| **248** | Spain, Cuenca, Reíllo | *Girardia* sp. | *G. dorotocephala* | 248sp_EsCue | **OM307103** | **OM349494** |
| **250** | Portugal, Cheleiros | *Girardia* sp. | *G. dorotocephala* | 250sp_PrCh | **OM307104** | **OM349490** |
| **252** | France, Issalès | *Girardia* sp. | *G. tigrina* | 252sp_FrIss | **OM307105** | **OM418674** |
| **253** | Spain, Salamanca, Ciudad Rodrigo | *Girardia* sp. | *G. sinensis* | 253sp_EsSal | **OM307106** | **OM418654** |

| | | | | | | |
|---|---|---|---|---|---|---|
| **260.1** | Brazil, Rio Grande do Sul, São Jose do Norte | *Girardia* sp. | *G. tigrina Brazil* | 260.1sp_BrRGSsj | **OM307107** | **OM418691** |
| **260.2** | Brazil, Rio Grande do Sul, São Jose do Norte | *Girardia* sp. | *G. tigrina Brazil* | 260.2sp_BrRGSsj | **OM307108** | |
| **261** | Brazil, São Paulo, Iporanga (cave) | *Girardia* aff. *arenicola* | *Girardia* aff. *arenicola* | 261ar_BrSPip | **OM264750** | **OM418632** |
| **262** | Brazil, Mato Grosso do Sul, Bodoquena (cave) | *G. multidiverticulata* | *G. multidiverticulata* | 262mu_BrMGSbod | **OM307109** | **OM418642** |
| **263** | Brazil, Rio Grande do Sul, São Jose do Norte | *Girardia sp.* | *G. tigrina* Brazil | 263sp_BrRGSsj | **OM307110** | **OM418692** |
| **269** | Brazil, Rio Grande do Sul, Constantina | *Girardia sp.* | *G. dorotocephala* | 269sp_BrRGScon | **OM307111** | **OM349488** |
| **270.1** | USA, Baltimore | *Girardia sp.* | *G. dorotocephala* | 270.1sp_USAbalt | **OM307112** | **OM349498** |
| **270.2** | USA, Baltimore | *Girardia sp.* | *G. dorotocephala* | 270.2sp_USAbalt | **OM307113** | **OM349499** |
| **295** | Chile, Los Lagos, Huinay Research Station | *Girardia* sp. | *Girardia* sp. | 295sp_ChiLHrs | **OM307114** | **OM418636** |
| **296** | Chile, Los Lagos, Huinay Research Station | *Girardia* sp. | *Girardia* sp. | 296sp_ChiLHrs | **OM307115** | |
| **297** | Chile, Los Lagos, Huinay Research Station | *Girardia* sp. | *Girardia* sp. | 297sp_ChiLHrs | **OM307116** | |
| **298.1** | Chile, Pumalin Park | *Girardia* sp. | *Girardia* sp. | 298.1sp_ChiPP | **OM307117** | |

| | | | | | | |
|---|---|---|---|---|---|---|
| **298.2** | Chile, Pumalin Park | *Girardia* sp. | *Girardia* sp. | 298.2sp_ChiPP | **OM307118** | **OM418638** |
| **299** | Chile, Los Lagos, Peninsula Huequi | *Girardia* sp. | *Girardia* sp. | 299sp_ChiLPh | **OM307119** | **OM418637** |
| **300** | Chile, Los Lagos, Port Montt | *Girardia* sp. | *Girardia* sp. | 300sp_ChiLPm | **OM307120** | |
| **308.1** | France, Montpellier | *Girardia* sp. | *G. sinensis* | 308.1sp_FrMon | **OM307121** | **OM418656** |
| **308.2** | France, Montpellier | *Girardia* sp. | *G. sinensis* | 308.2sp_FrMon | **OM307122** | |
| **325** | Brazil, Mato Grosso do Sul, Bonito (cave) | *G. multidiverticulata* | *G. multidiverticulata* | 325mu_BrMGSbon | **OM307123** | **OM418641** |
| **327** | USA, Michigan, Ann Arbor | *Girardia* sp. | *G. dorotocephala* | 327sp_USAmich | **OM307124** | **OM349489** |
| **336** | Brazil, Rio Grande do Sul, Severiano de Almeida | *G. anderlani* | *G. anderlani* | 336an_BrRGScon | **OM232748** | |
| **337** | Brazil, Mato Grosso do Sul, Bodoquena (cave) | *G. multidiverticulata* | *G. multidiverticulata* | 337mu_BrMGSbod | **OM307125** | **OM418643** |
| **338** | Brazil, Mato Grosso do Sul, Bodoquena (cave) | *G. multidiverticulata* | *G. multidiverticulata* | 338mu_BrMGSbod | **OM307126** | |
| **373** | Spain, Catalonia, Riera de Mura | *Girardia* sp. | *G. tigrina* | 373sp_CatMur | **OM307127** | **OM418680** |
| **377** | Spain, Catalonia, Girona, Fluvià, Vilert | *Girardia* sp. | *G. sinensis* | 377sp_CatFlvil | **OM307128** | **OM418658** |

| | | | | | | |
|---|---|---|---|---|---|---|
| **383** | Hawaii, Upper Manoa | *Girardia* sp. | *G. dorotocephala* | 383sp_HawUM | **OM307129** | **OM349501** |
| **384** | Hawaii, Middle Manoa | *Girardia* sp. | *G. dorotocephala* | 384sp_HawMM | **OM307130** | **OM349500** |
| **399** | Brazil, Rio Grande do Sul, Maquine | *Girardia* sp. | *G. schubarti* | 399sp_BrRGSmaq | **OM307131** | **OM418646** |
| **401** | Brazil, Mato Grosso, Chapada Guimaraes (cave) | *Girardia* sp. | *Girardia* sp. | 401sp_BrMatcha | **OM307132** | |
| **402** | Brazil, Santa Catarina, Chapeco | *Girardia sp.* | *Girardia* sp. | 402sp_BrSCchap | **OM307133** | **OM418635** |
| **403** | Brazil, Rio Grande do Sul, São Leopoldo | *G. tigrina* | *G. tigrina* Brazil | 403ti_BrRGSleo | **OM307134** | **OM418686** |
| **466.1** | USA, Carolina Enterprise† | *G. dorotocephla*<br>*Dugesia dorotocephala*‡ | *G. dorotocephala* | 466.1do_USA | **OM307135** | |
| **466.2** | USA, Carolina Enterprise† | *G. dorotocephla*<br>*Dugesia dorotocephala*‡ | *G. dorotocephala* | 466.2do_USA | **OM307136** | **OM349491** |
| **467.1** | USA, Carolina Enterprise† | *Girardia* sp<br>Brown planaria‡ | *G. dorotocephala* | 467.1brown_USA | **OM307137** | |
| **467.2** | USA, Carolina Enterprise† | *Girardia* sp<br>Brown planaria‡ | *G. dorotocephala* | 467.2brown_USA | **OM307138** | **OM349502** |
| **468.1** | USA, Carolina Enterprise† | *Girardia* sp<br>Black planaria‡ | *G. dorotocephala* | 468.1black_USA | **OM307139** | |
| **468.2** | USA, Carolina Enterprise† | *Girardia* sp<br>Black planaria‡ | *G. dorotocephala* | 468.2black_USA | **OM307140** | **OM349492** |

| | | | | | | |
|---|---|---|---|---|---|---|
| **469.1** | Spain, Catalonia, Montjüic | *Girardia* sp. | *G. tigrina* | 469.1sp_CatMj | **OM307141** | **OM418670** |
| **469.2** | Spain, Catalonia, Montjüic | *Girardia* sp. | *G. tigrina* | 469.2sp_CatMj | **OM307142** | **OM418684** |
| **534** | Brazil, Rio Grande do Sul, São Francisco de Paula | *G. schubarti* | *G. schubarti* | 534sc_BrRGSpau | | **OM418647** |
| **535** | Brazil, Rio Grande do Sul, São Leopoldo | *G. tigrina* | *G. tigrina* Brazil | 535ti_BrRGSleo | **OM307143** | **OM418687** |
| **542** | Germany, Pillnitz | *Girardia* sp. | *G. tigrina* | 542sp_GerPill | **OM307144** | **OM418678** |
| **543** | USA, Virginia | *Girardia* sp. | *G. dorotocephala* | 543sp_USAvirg | **OM307145** | **OM349495** |
| **544** | Australia, Tasmania, Derwent River | *Girardia* sp. | *G. sinensis* | 544sp_Austderw | **OM307146** | **OM418667** |
| **545** | USA, Virginia, Ashburn, Janelia Farm Research Campus | *Girardia* sp. | *G. dorotocephala* | 545sp_USAvirgJFRC | **OM307147** | **OM349496** |
| **546** | Francia, Lez | *Girardia* sp. | *G. dorotocephala* | 546sp_FrLez | **OM307148** | |
| **547** | Spain, Catalonia, Barcelona, Vallvidrera | *Girardia* sp. | *G. tigrina* | 547sp_CatBarcVald | **OM307149** | |
| **548** | Mexico | *Girardia* sp. | *G. dorotocephala* | 548sp_Mex | **OM307150** | |
| **550** | Mexico | *Girardia* sp. | *G. dorotocephala* | 550sp_Mex | **OM307151** | **OM349497** |
| **551** | France, River Herault | *Girardia* sp. | *G. sinensis* | 551sp_FrHer | **OM307152** | |

| | | | | | | |
|---|---|---|---|---|---|---|
| **552** | Germany, Zschorna | *Girardia* sp. | *G. sinensis* | 552sp_GerZsch | **OM307153** | **OM418666** |
| **553** | Germany, Pillnitz | *Girardia* sp. | *G. tigrina* | 553sp_GerPill | **OM307154** | |
| **554** | Netherlands, Leiden | *Girardia* sp. | *Girardia sinensis* | 554sp_NethLeid | **OM307155** | **OM418665** |
| **556** | Spain, Catalonia, Arenys d' Empordà, Fluvià River | *Girardia* sp. | *G. tigrina* | 556sp_CatFlEmp | **OM307156** | **OM418685** |
| **558** | Spain, Catalonia, Arenys d' Empordà, Fluvià River | *Girardia* sp. | *G. sinensis* | 558sp_CatFlEmp | **OM307157** | |
| **559.1** | Spain, Catalonia, Arenys d' Empordà, Fluvià River | *Girardia* sp. | *G. sinensis* | 559.1sp_CatFlEmp | **OM307158** | **OM418668** |
| **559.2** | Spain, Catalonia, Arenys d' Empordà, Fluvià River | *Girardia* sp. | *G. sinensis* | 559.2sp_CatFlEmp | **OM307159** | |
| **659** | Canada, Nova Scotia, Ainslie Lake | *Girardia* sp. | *G. tigrina* | 659sp_CanNS | **OM307160** | **OM418676** |
| **660** | Australia, Queensland, UQ Lakes | *Girardia* sp. | *G. sinensis* | 660sp_AustQUQ | **OM307161** | **OM418659** |
| **661** | Australia, Queensland | *Girardia* sp. | *G. sinensis* | 661sp_AustQCC | **OM307162** | **OM418660** |
| **679** | USA, Michigan, Douglas Lake | *Girardia* sp. | *G. tigrina* | 679sp_USADL | **OM307163** | **OM418672** |
| **683.1** | Japan, Hoshikuki-cho, Mizu-no-sato Park, Miyako River | *Girardia* sp. | *G. dorotocephala* | 683.1sp_Jap | **OM307164** | |
| **684.1** | Japan, Hoshikuki-cho, Mizu-no-sato Park, Miyako River | *Girardia* sp. | *G. dorotocephala* | 684.1sp_Jap | **OM307165** | |

| | | | | | | |
|---|---|---|---|---|---|---|
| **684.2** | Japan, Hoshikuki-cho, Mizu-no-sato Park, Miyako River | *Girardia* sp. | *G. dorotocephala* | 684.2sp_Jap | **OM307166** | **OM349493** |
| **685.1** | Chile, Talagante, Mapocho River | *Girardia sanchezi* | *G. sanchezi* | 685.1san_ChiTalag | **OM307167** | **OM418644** |
| **685.2** | Chile, Talagante, Mapocho River | *Girardia sanchezi* | *G. sanchezi* | 685.2san_ChiTalag | **OM307168** | **OM418645** |
| **686** | China, Conghua, Yadongxi River | *Girardia* sp. | *G. sinensis* | 686sp_ChinYand | **OM307169** | **OM418662** |
| **687** | Chile, Los Lagos, Huinay Research Station | *Girardia* sp. | *Girardia* sp. | 687sp_ChiLHrs | **OM307170** | |
| **1056** | Mexico, Biological Station Los Tuxtlas 1 | *Girardia* sp. | *Girardia* sp. | 1056sp_MexBST1 | | **OM418694** |
| **1059** | Mexico, Biological Station Los Tuxtlas 1 | *Girardia* sp. | *Girardia* sp. | 1059sp_MexBST1 | | **OM418639** |
| **1062** | Mexico, Los Tuxtlas, Laguna Escondida | *Girardia* sp. | *Girardia* sp. | 1062sp_MexLagE | **OM307171** | **OM418640** |
| **1063** | Mexico, Los Tuxtlas, Laguna Escondida | *Girardia* sp. | *Girardia* sp. | 1063sp_MexLagE | **OM307172** | |
| **1070** | Mexico, Biological Station Los Tuxtlas 2 | *Girardia* sp. | *Girardia* sp. | 1070sp_MexBST2 | **OM307173** | **OM418633** |
| **1072.2** | Cuba, Matanzas, Martí, El Huequito | *Girardia* sp. | *G. sinensis* | 1072.2sp_Cub | **OM307174** | **OM418661** |
| **1178** | Brazil, Bahía, Chapada Diamantina, Vale do Pati (cave) | *Girardia* sp. | *Girardia* sp. | 1178sp_BrBah | **OM307175** | **OM418634** |
| **1179** | Mexico, Mexico City, Xochimilco, Cuemanco | *Girardia* sp. | *Girardia* sp. | 1179sp_MexXoch | **OM307176** | **OM41869** |

| | | | | | | |
|---|---|---|---|---|---|---|
| **1180** | Mexico, Mexico City, Xochimilco, Cuemanco | *Girardia* sp. | *G. dorotocephala* | 1180sp_MexXoch | **OM307177** | **OM349487** |
| **1181** | Mexico, Mexico City, Xochimilco, Cuemanco | *Girardia* sp. | *Girardia* sp. | 1181sp_MexXoch | **OM307178** | |
| **1182** | Mexico, Michoacán | *Girardia* sp. | *G. dorotocephala* | 1182sp_MexMicho | **OM307179** | |
| **F6510.1** | Brazil, Paraná, Toledo, Cerro da Lola | *Girardia* sp. | *G. schubarti* | F6510.1sp_BrPar | | **OM418651** |
| **F6510.2** | Brazil, Paraná, Toledo, Cerro da Lola | *Girardia* sp. | *G. schubarti* | F6510.2sp_BrPar | | **OM418652** |
| **F6510.3** | Brazil, Paraná, Toledo, Cerro da Lola | *Girardia* sp. | *G. schubarti* | F6510.3sp_BrPar | | **OM418650** |
| InoueA | USA, Texas, Caroline Spring, Independence Creek | *Girardia* sp. | *Girardia* sp. | InoueA_CS103 | MN652340.1 | |
| InoueB | USA, New Mexico, Palomas Creek, Emrick Spring | *Girardia* sp. | *Girardia* sp. | InoueB_ES201 | MN652378.1 | |
| InoueC | USA, Texas, Bitter Lake, Bitter Creek | *Girardia* sp. | *G. dorotocephala* | InoueC_BLBC002 | MN652301.1 | |
| InoueD | USA, New Mexico, West Fork of the Gila River | *Girardia sp.* | *G. dorotocephala* | InoueD_GR209 | MN652373.1 | |
| GB_G.an1 | Brazil | *G. anderlani* | *G. anderlani* | GB_Gan1_Br | DQ666038.1 | |
| GB_G.si | China, Guangdong Province Xinghu Lake in Zhaoqing | *G. sinensis* | *G. sinensis* | GB_Gsi_China | KP091895.1 | |
| GB_G.ti | France, Montpellier | *G. tigrina* | *G. tigrina* | GB_Gti_FrMont | DQ666042.1 | |

| | | | | | | |
|---|---|---|---|---|---|---|
| GB_G.sc1 | Brazil | *G.schubarti* | *G.schubarti* | GB_Gsc1_Br | DQ666041.1 | |
| GB_G.sc2 | Brazil, Rio Grande do Sul, Constantina | *G.schubarti* | *G.schubarti* | GB_Gsc2_BrRGScon | | KJ599691.1 |
| GB_G.som1 | Argentina, Somuncurá Plateau, Head of Valcheta Stream | *G. somuncura* | *G. somuncura* | GB_som1_Arg | MW271865 | |
| GB_G.som2 | Argentina, Somuncurá Plateau, Head of Valcheta Stream | *G. somuncura* | *G. somuncura* | GB_som2_Arg | MW271866 | |
| GB_G.som3 | Argentina, Somuncurá Plateau, Head of Valcheta Stream | *G. somuncura* | *G. somuncura* | GB_som3_Arg | MW271867 | |
| GB_G.som4 | Argentina, Somuncurá Plateau, Head of Valcheta Stream | *G. somuncura* | *G. somuncura* | GB_som4_Arg | MW271869 | |
| GB_G.tom1 | Argentina, Somuncurá Plateau, Head of Valcheta Stream | *G. tomasi* | *G. tomasi* | GB_tom1_Arg | MW271863 | |
| GB_G.tom2 | Argentina, Somuncurá Plateau, Head of Valcheta Stream | *G. tomasi* | *G. tomasi* | GB_tom2_Arg | MW271864 | |
| GB_G.tom2 | Argentina, Somuncurá Plateau, Head of Valcheta Stream | *G. tomasi* | *G. tomasi* | GB_tom2_Arg | MW271868 | |
| GB_S.med1 | Tunisia, Lebna | *Schmidtea mediterranea* | *S. mediterranea* | GB_*S.mediterranea*1 | JF837060.1 | |
| GB_S.med2 | Italy, Sardinia | *S. mediterranea* | *S. mediterranea* | GB_*S.mediterranea*2 | JF837061.1 | |
| GB_S.med3 | Italy, Sicily | *S. mediterranea* | *S. mediterranea* | GB_*S.mediterranea*3 | JF837062.1 | |
| GB_D.sic1 | Spain | *Dugesia sicula* | *Dugesia sicula* | GB_*D.sicula*1 | KC577350.1 | |

| | | | | | | |
|---|---|---|---|---|---|---|
| GB_D.sic2 | Italy | *D. sicula* | *D. sicula* | GB_*D.sicula*2 | KC577351.1 | |
| GB_D.ari | Greece | *D. ariadnae* | *D. ariadnae* | GB_*D.ariadnae* | KF308713.1 | |
| GB_D.arc | Greece | *D. arcadia* | *D. arcadia* | GB_*D.arcadia* | KF308723.1 | |
| GB_D.mal | Greece | *D. malickyi* | *D. malickyi* | GB_*D.malickyi* | KF308777.1 | |
| GB_D.cre | Greece | *D. cretica* | *D. cretica* | GB_*D.cretica* | KF308794.1 | |
| GB_S.me43 | Europe | *S. mediterranea* | *S. mediterranea* | GB_*S. mediterranea*4 | | KJ599709.1 |
| GB_D.sic3 | Europe | *D. sicula* | *D. sicula* | GB_*D. sicula*3 | | KJ599689.1 |
| 1124 | South Africa | *D. afromontana* | *D. afromontana* | *D. afromontana* | | **OM460743** |
| 135 | Japan | *Dugesia* sp. | *Dugesia* sp. | *Dugesia* sp1_Jap | | **OM460745** |
| 1336 | Japan | *Dugesia* sp. | *Dugesia* sp. | *Dugesia* sp2_Jap | | **OM460746** |

---

[†]: Carolina Enterprise Word-Class Support for Science & Math

[‡]: classification according to Carolina Biological Supply Company [https://www.carolina.com/living-organisms/classroom-animals/invertebrates/platyhelminthes/10531.ct?Nr=product.siteId%3A100001]

# Appendix 2

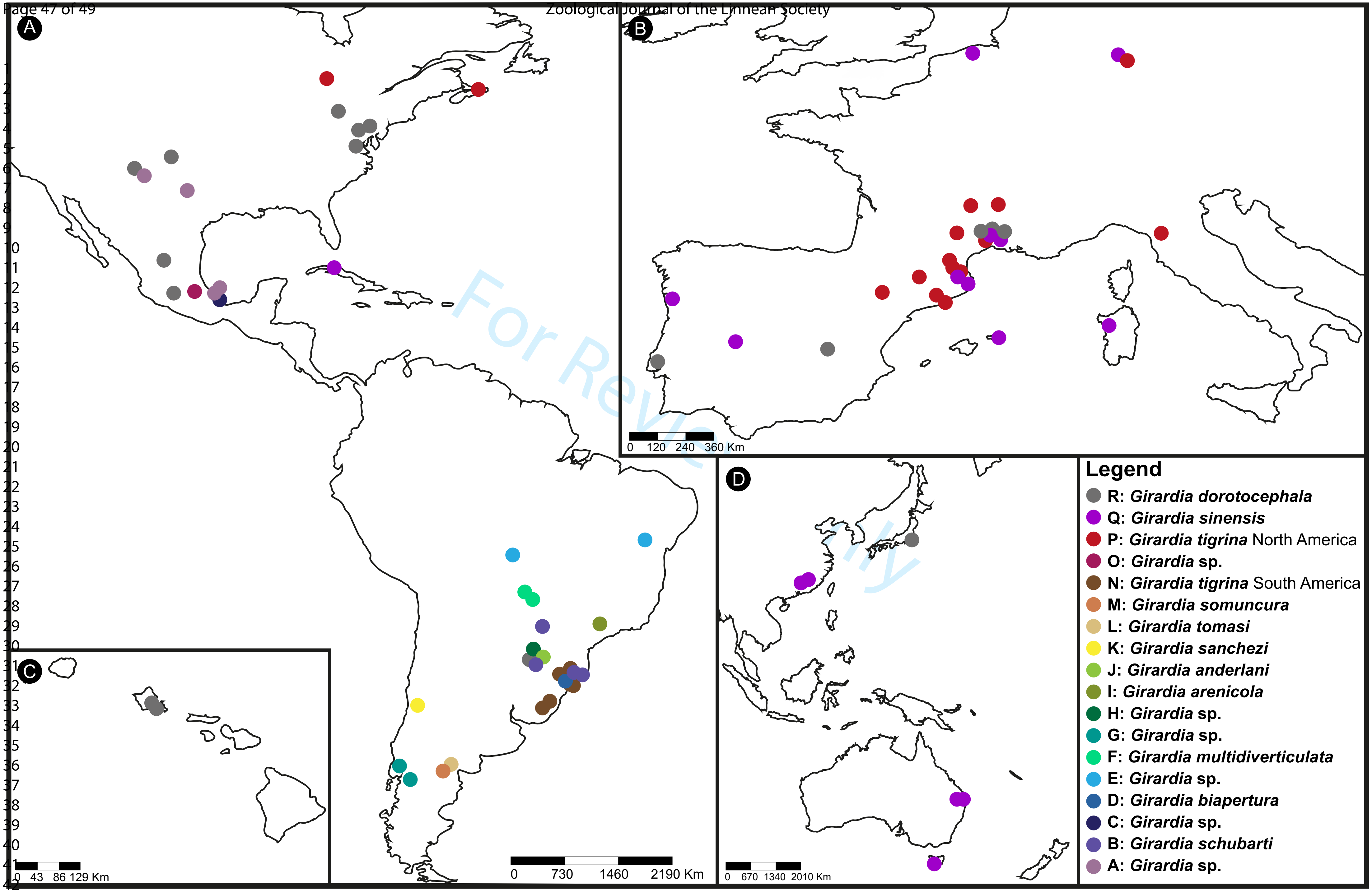
***Girardia clandistina*** **Sluys & Benítez-Álvarez, sp. nov.**

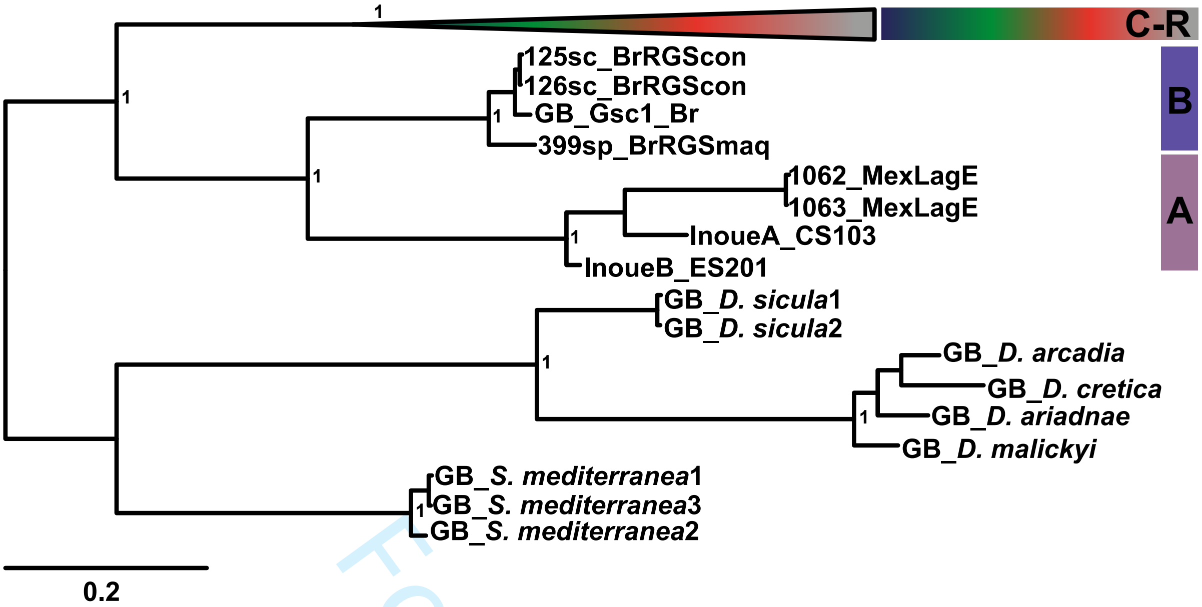
**Holotype**: Naturalis Biodiversity Center, ZMA V.Pl. 976.4, Arroyo Sauce, near Sauce, Departamento Canelones, near Montevideo, Uruguay, 1-3 January 1987, sagittal sections on 6 slides.

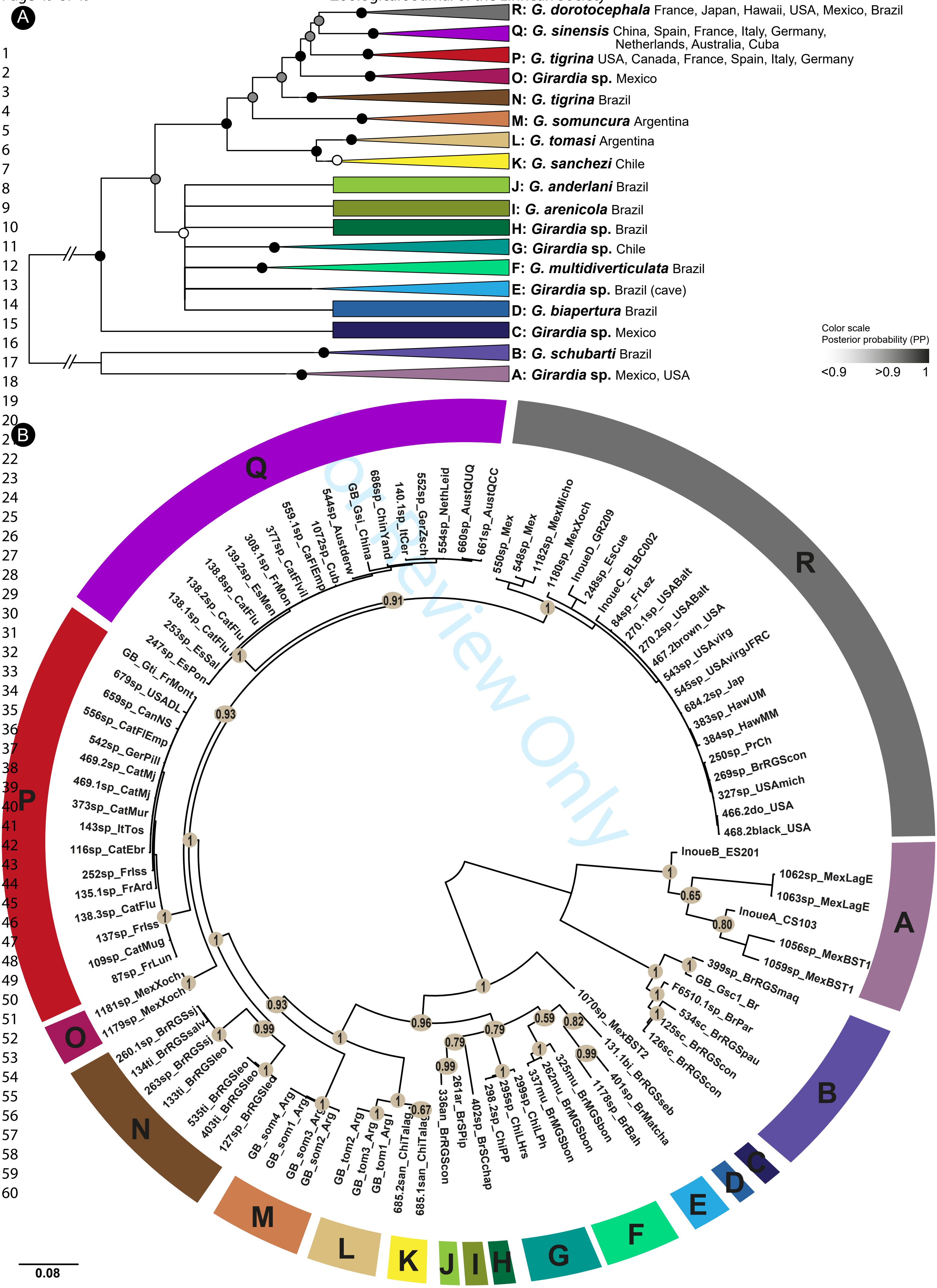
**Etymology**: The specific epithet is based on the Latin adjective *clandistinus*, secret, concealed, and alludes to the fact that it concerns a sibling species.

**Differential diagnosis**:

A species of *Girardia* with low triangular head with bluntly pointed tip and short, broad auricles. Dorsal body colouration variable, being of a reticulated type with darkish spots and also a pair of dark stripes, separated by a pale mid-dorsal streak, or composed of a dark background interspersed with white splotches and with a pale middorsal line, or variations on these two major patterns. Reproductive complex basically as in *G. tigrina*, the only, but consistent, anatomical difference between the two species residing in the coat of muscles around the bursal canal. In North American *G. tigrina* this coat of muscles is simple, consisting of a thin subepithelial layer of circular muscle, followed by an equally thin layer of longitudinal muscle fibres. In contrast, *G. clandistina* possesses a bursal canal musculature that consists of a well-developed coat of intermingled circular and longitudinal muscle fibres.


A
B
C
D
0 120 240 360 Km
0 43 86 129 Km
0 730 1460 2190 Km
0 670 1340 2010 Km
Legend
● R: *Girardia dorotocephala*
● Q: *Girardia sinensis*
● P: *Girardia tigrina* North America
● O: *Girardia* sp.
● N: *Girardia tigrina* South America
● M: *Girardia somuncura*
● L: *Girardia tomasi*
● K: *Girardia sanchezi*
● J: *Girardia anderlani*
● I: *Girardia arenicola*
● H: *Girardia* sp.
● G: *Girardia* sp.
● F: *Girardia multidiverticulata*
● E: *Girardia* sp.
● D: *Girardia biapertura*
● C: *Girardia* sp.
● B: *Girardia schubarti*
● A: *Girardia* sp.


1
C-R
125sc_BrRGScon
126sc_BrRGScon
GB_Gsc1_Br
399sp_BrRGSmaq
B
1
1
1
1062_MexLagE
1063_MexLagE
InoueA_CS103
InoueB_ES201
A
1
GB_D. sicula1
GB_D. sicula2
1
GB_D. arcadia
GB_D. cretica
GB_D. ariadnae
GB_D. malickyi
1
GB_S. mediterranea1
GB_S. mediterranea3
GB_S. mediterranea2
1
0.2


A
R: G. dorotocephala France, Japan, Hawaii, USA, Mexico, Brazil
Q: G. sinensis China, Spain, France, Italy, Germany, Netherlands, Australia, Cuba
P: G. tigrina USA, Canada, France, Spain, Italy, Germany
O: Girardia sp. Mexico
N: G. tigrina Brazil
M: G. somuncura Argentina
L: G. tomasi Argentina
K: G. sanchezi Chile
J: G. anderlani Brazil
I: G. arenicola Brazil
H: Girardia sp. Brazil
G: Girardia sp. Chile
F: G. multidiverticulata Brazil
E: Girardia sp. Brazil (cave)
D: G. biapertura Brazil
C: Girardia sp. Mexico
B: G. schubarti Brazil
A: Girardia sp. Mexico, USA
Color scale
Posterior probability (PP)
<0.9
>0.9
1
B
Q
R
P
O
N
M
L
K
J
I
H
G
F
E
D
C
B
A
0.91
1
0.93
1
1
1
1
0.93
0.99
1
1
1
1
0.67
0.96
0.99
0.79
0.79
1
1
0.59
0.82
0.99
1
1
1
1
1
0.65
0.80
1
0.08
686sp_ChinYand
GB_Gsi_China
544sp_Austderw
1072sp_Cub
559.1sp_CaFlEmp
377sp_CatFlvil
308.1sp_FrMon
139.2sp_EsMen
138.8sp_CatFlu
138.2sp_CatFlu
138.1sp_CatFlu
253sp_EsSal
247sp_EsPon
140.1sp_ItCer
552sp_GerZsch
554sp_NethLeid
660sp_AustQUQ
661sp_AustQCC
550sp_Mex
548sp_Mex
1182sp_MexMicho
1180sp_MexXoch
InoueD_GR209
248sp_EsCue
InoueC_BLBC002
84sp_FrLez
270.1sp_USABalt
270.2sp_USABalt
467.2brown_USA
543sp_USAvirg
545sp_USAvirgJFRC
684.2sp_Jap
383sp_HawUM
384sp_HawMM
250sp_PrCh
269sp_BrRGScon
327sp_USAmich
466.2do_USA
468.2black_USA
InoueB_ES201
1062sp_MexLagE
1063sp_MexLagE
InoueA_CS103
1056sp_MexBST1
1059sp_MexBST1
399sp_BrRGSmaq
GB_Gsc1_Br
F6510.1sp_BrPar
534sc_BrRGSpau
125sc_BrRGScon
126sc_BrRGScon
1070sp_MexBST2
131.1bi_BrRGSseb
401sp_BrMatcha
1178sp_BrBah
325mu_BrMGSbon
262mu_BrMGSbon
337mu_BrMGSbon
299sp_ChiLPh
295sp_ChiLHrs
298.2sp_ChiPP
402sp_BrSCchap
261ar_BrSPip
336an_BrRGScon
685.1san_ChiTalag
685.2san_ChiTalag
GB_tom1_Arg
GB_tom3_Arg
GB_tom2_Arg
GB_som2_Arg
GB_som3_Arg
GB_som1_Arg
GB_som4_Arg
127sp_BrRGSled
403ti_BrRGSleo
535ti_BrRGSleo
133ti_BrRGSleo
263sp_BrRGSsj
134ti_BrRGSsalv
260.1sp_BrRGSsj
1179sp_MexXoch
1181sp_MexXoch
87sp_FrLun
109sp_CatMug
137sp_FrIss
138.3sp_CatFlu
135.1sp_FrArd
252sp_FrIss
116sp_CatEbr
143sp_ItTos
373sp_CatMur
469.1sp_CatMj
469.2sp_CatMj
542sp_GerPill
556sp_CatFlEmp
659sp_CanNS
679sp_USADL
GB_Gti_FrMont