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Phylogenetic position of *Magnivitellinum* Kloss, 1966 and *Perezitrema* Baruš & Moravec, 1967 (Trematoda: Plagiorchioidea: Macroderoididae) inferred from partial 28S rDNA sequences, with the establishment of Alloglossidiidae n. fam.

David Iván Hernández-Mena · Berenit Mendoza-Garfias ·
Claudia Patricia Ornelas-García · Gerardo Pérez-Ponce de León

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Abstract The systematic position of two genera of Macroderoididae McMullen, 1937, *Perezitrema* Baruš & Moravec, 1967 and *Magnivitellinum* Kloss, 1966 is reviewed based on a phylogenetic analysis of the interrelationships of 15 species of the family allocated into six genera, along with 44 species of plagiorchioid trematodes, using partial sequences of the 28S rRNA gene. Sequences were analysed through parsimony, maximum likelihood and Bayesian inference. The obtained topologies show *Perezitrema* as the sister taxon of three species of *Macroderoides* Pearse, 1924; the latter genus appears to be paraphyletic since another three species are not included in this group. Instead, *Magnivitellinum* was placed as the sister taxon of *Alloglossidium* Simer, 1929. These relationships are well supported by high bootstrap and posterior probability values. The resulting trees demonstrate that the family Macroderoididae, as currently conceived in taxonomic treatments, is not monophyletic. *Magnivitellinum simplex* Kloss, 1966 and *Alloglossidium* spp. were nested as sister taxa of members of the family Leptophallidae Dayal, 1938, whereas *Perezitrema bychowskii* Baruš & Moravec, 1967 and species of *Macroderoides* and *Paramacroderoides* Venard, 1941 were grouped with *Auridistomum chelydrae* (Stafford, 1900), a monotypic member of Auridistomidae Stunkard, 1924. Based on our results, a new family, Alloglossidiidae n. fam. was established to accommodate the genera *Magnivitellinum* and *Alloglossidium*.

Introduction

The family Macroderoididae McMullen, 1937 was erected to include intestinal parasites of freshwater fish, although the group now includes forms that mature in marine fish, and some even in crustaceans and leeches (Font & Lotz, 2008a). The assignment of genera into this family of plagiorchioid trematodes, and as a consequence its species composition, is still not settled and has been controversial due to the inadequate choice of morphological characters (e.g. excretory vesicle shape), and insufficient information regarding their life-cycles (Font & Lotz, 2008a). The current classification of the Macroderoididae includes trematodes that mostly infect freshwater fish and are

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D. I. Hernández-Mena · B. Mendoza-Garfias ·
G. Pérez-Ponce de León (✉)
Laboratorio de Helmintología, Instituto de Biología,
Universidad Nacional Autónoma de México, Mexico, DF,
Mexico
e-mail: ppdleon@ib.unam.mx

D. I. Hernández-Mena
Posgrado en Ciencias Biológicas, Universidad Nacional
Autónoma de México, Mexico, DF, Mexico

C. P. Ornelas-García
Laboratorio de Ictiología, Instituto de Biología,
Universidad Nacional Autónoma de México, Mexico, DF,
Mexico

diagnosed by having a spined tegument, elongate, blind caeca, usually extending to near posterior extremity, I-shaped excretory vesicle, and exceptionally forming cyclocoel. However, as discussed by Kostadinova & Pérez-del-Olmo (2014) the search of apparent non-homoloplasious morphological characters at the higher taxonomic levels of trematodes has been controversial, and requires the addition of molecular data to obtain a more accurate classification scheme. This seems to be the case for the family Macroderoididae.

Font & Lotz (2008a) discussed in detail the complex taxonomic history of the family and recognised only nine valid genera: *Alloglossidium* Simer, 1929; *Cirkennedya* Gibson & Bray, 1979; *Gauhatiana* Gupta, 1955; *Macroderoides* Pearse, 1924; *Rauschiella* Babero, 1951; *Magnivitellinum* Kloss, 1966; *Paramacroderoides* Venard, 1941; *Perezitrema* Barus & Moravec, 1967; and *Malawitrema* Bray & Hendrix, 2007. Likewise, Font & Lotz (2008a) also recognised that the assignment of genera such as *Gauhatiana* and *Rauschiella* to Macroderoididae, both of which possess Y-shaped vesicles, should be considered provisional. Further, Pojmańska et al. (2008) considered some genera of trematodes that have been included at different times and by different authors in the family as *incertae sedis*, i.e. *Wallinia* Pearse, 1920; *Glossidium* Looss, 1899, *Vietosoma* Van Cleave & Mueller, 1932; and *Pseudomagnivitellinum* Dronen & Underwood, 1980. Razo-Mendivil et al. (2006) obtained molecular evidence that the genus *Rauschiella* does not belong to the Macroderoididae. Pérez-Ponce de León et al. (2007a) used 28S rDNA sequence data to show that *Wallinia* belongs to the Allocreadiidae Looss, 1902. The other three genera, i.e. *Glossidium*, *Vietosoma* and *Pseudomagnivitellinum* exhibit morphological characters of the family Macroderoididae, and are parasites of siluriform freshwater fishes (Khalil, 1972; Hoffman, 1999; Dronen & Underwood, 1980).

In this study, we incorporate 28S rDNA sequences for species of two genera of the Macroderoididae (*Magnivitellinum* and *Perezitrema*) to the database that has been built for different members of the family (see Tkach et al., 1999, 2001, 2008, 2010; Tkach & Mills, 2011; Tkach & Kinsella, 2011; Kasl et al., 2014). We investigate the systematic position of these two genera of macroderoidids through rDNA sequences in the context of the phylogeny of the Plagiorchioidea Lühe, 1901, and we discuss the implications of our findings for the classification scheme of the family that resulted in the establishment of a new family to accommodate some of the genera, and we further discuss the host and historical biogeography of the host-parasite associations.

Materials and methods

Specimen collection

Adult specimens of two species belonging to the genera *Magnivitellinum* and *Perezitrema*, were collected from freshwater fishes in seven localities across Mexico, between October 2013 and March 2015 (Table 1). Worms recovered from their hosts were rinsed in 0.6% saline solution; some individuals were fixed in 100% ethanol for molecular analyses and some individuals were fixed in hot (steaming) 4% formalin for further morphological study. Some specimens were stained with Mayer's paracarmine and mounted in Canada balsam for identification; voucher specimens were deposited in the Colección Nacional de Helmintos (CNHE), Mexico City, with the accession numbers 9974-9976 (*Magnivitellinum simplex* Kloss, 1966) and 9973 (*P. bychowskyi* Baruš & Moravec, 1967).

Light and scanning electron microscopy

Specimens were stained with Gomori's trichrome or Mayer's paracarmine, dehydrated in a graded ethanol series, cleared in methyl salicylate, and mounted as permanent slides in Canada balsam. Four individuals (two of each species) fixed in 4% formalin were used for scanning electron microscopy (SEM) studies. Worms were dehydrated through a graded ethanol series, critical point-dried with carbon dioxide, mounted on metal stubs with carbon adhesive tabs, gold coated and examined at 15 kV in a Hitachi Stereoscan Model SU1510 SEM (Hitachi Ltd., Tokyo, Japan).

DNA extraction, amplification and sequencing

Eight trematodes identified as *Magnivitellinum simplex*, from five localities and ten identified as *Perezitrema bychowskyi* from two localities, were individually digested overnight at 56°C in a solution containing 10 mM Tris-HCl (pH 7.6), 20 mM NaCl,

Table 1 Digenean species used in phylogenetic analyses (in alphabetical order by family and species) with GenBank accesion numbers, hosts and localities of the isolates sequenced

Species	GenBank acc. no.	Host species (Order)	Locality
Allocreadiidae Looss, 1902			
Auriculostoma astyanace Scholz, Aguirre-Macedo & Choudhury, 2004	HQ833707	*Astyanax fasciatus* (Cuvier) (Characiformes)	Río Animas, Costa Rica
Auridistomidae Stunkard, 1924			
Auridistomum chelydrae (Stafford, 1900)	AY116872	*Chelydra serpentina* (L.) (Testudines)	Jackson County, USA
Brachycoeliidae Looss, 1899			
Brachycoelium salamandrae (Frölich, 1789)	AF151935	*Salamandra salamandra* (L.) (Caudata)	Zakarpatska, Ukraine
Parabrachycoelium longicaecum Pérez-Ponce de León, Mendoza-Garfias, Razo & Parra-Olea, 2011	HQ165754	*Chiropterotriton* Taylor, 1944 (Caudata)	Tlaquilpa, Mexico
Cephalogonimidae Looss, 1899			
Cephalogonimus americanus Stafford, 1902	HM137615	*Ambystoma velasci* Dugès (Caudata)	Quechulac, Mexico
Cephalogonimus retusus (Dujardin, 1845)	AY222276	*Pelophylax ridibundus* (Pallas) (Caudata)	Kokaljane, Bulgaria
Choanocotylidae Jue Sue & Platt, 1998			
Choanocotyle nematoides Jue Sue & Platt, 1998	AY116862	*Emydura macquarii* Gray (Testudines)	MacCleay River, Australia
Glypthelminthidae Cheng, 1959			
Glypthelmins brownorumae Razo-Mendivil, León-Règagnon & Pérez-Ponce de León, 2004	AY875674	*Rana brownorum* (Sanders) (Anura)	Villahermosa, Mexico.
Haematoloechidae Freitas & Lent, 1939			
Haematoloechus abbreviatus (Bychowsky, 1932)	AF184251	*Bombina variegata* (L.) (Anura)	Zakarpatska region, Ukraine
Leptophallidae Dayal, 1938			
Leptophallus nigrovenosus (Bellingham, 1844)	AF151914	*Natrix natrix* (L.) (Squamata)	Kiev region, Ukraine
Macrodera longicollis (Abildgaard, 1788)	AF151913	*Natrix natrix* (L.) (Squamata)	Kiev region, Ukraine
Metaleptophallus gracillimus (Lühe, 1909)	AF151912	*Natrix natrix* (L.) (Squamata)	Kiev region, Ukraine
Paralepoderma cloacicola (Lühe, 1909)	AF151910	*Natrix natrix* (L.) (Squamata)	Kiev region, Ukraine
Macroderoididae McMullen, 1937			
Alloglossidium corti (Lamont, 1921)	JF440783	*Ameiurus melas* Rafinesque (Siluriformes)	Pine Lake, USA

Table 1 continued

Species	GenBank acc. no.	Host species (Order)	Locality
Alloglossidium fonti Tkach & Mills, 2011	JF440763	*Ameiurus melas* Rafinesque (Siluriformes)	Mud Lake, USA
Alloglossidium geminum (Mueller, 1930)	JF440771	*Ameiurus melas* Rafinesque Siluriformes)	Red River, USA
Alloglossidium kenti Simer, 1929	JF440806	*Ictalurus punctatus* Rafinesque (Siluriformes)	Pascagoula River, USA
Alloglossidium floridense Kasl, Fayton, Font & Criscione, 2014	KC812276	*Noturus leptacanthus* Jordan (Siluriformes)	Santa Fe River, USA
Macroderoides flavus Van Cleave & Mueller, 1932	HQ680851	*Esox niger* Lesueur (Esociformes)	Connecticut, USA
Macroderoides minutus Tkach & Kinsella, 2011	HQ680850	*Lepisosteus platyrhincus* DeKay (Lepisosteiformes)	Orange Lake, USA
Macroderoides spiniferus Pearse, 1924	EU850400	*Lepisosteus platyrhincus* DeKay (Lepisosteiformes)	Nueces River, USA
Macroderoides texanus Tkach, Strand & Froese, 2008	EU850398	*Atractosteus spatula* Lacépède (Lepisosteiformes)	Nueces River, USA
Macroderoides trilobatus Tyler, 1978	EU850406	*Lepisosteus platyrhincus* Dekay (Lepisosteiformes)	Cross Creek, USA
Macroderoides typicus (Winfield, 1929)	HQ680846	*Lepisosteus platyrhincus* DeKay (Lepisosteiformes)	Orange Lake, USA
Magnivitellinum simplex **Kloss, 1966**	**KU535683** **KU535684**	*Astyanax aeneus* (Günther) (Characiformes)	Metzabok Lake, Chiapas, Mexico
Magnivitellinum simplex **Kloss, 1966**	**KU535677** **KU535678**	*Astyanax. mexicanus* De Filippi (Characiformes)	Micos, Qeretaro Mexico
Magnivitellinum simplex **Kloss, 1966**	**KU535679**	*Astyanax. mexicanus* De Filippi (Characiformes)	Media Luna, San Luis Potosí, Mexico
Magnivitellinum simplex **Kloss, 1966**	**KU535682**	*Astyanax aeneus* (Günther) (Characiformes)	Pichucalco River, Tabasco Mexico
Magnivitellinum simplex **Kloss, 1966**	**KU535680** **KU535681**	*Astyanax aeneus* (Günther) (Characiformes)	Raudal River, Veracruz, Mexico
Paramacroderoides kinsellai Tkach, Pulis & Overstreet, 2010	HM137661	*Lepisosteus oculatus* Winchell (Lepisosteiformes)	Oxbow Lake, USA

Table 1 continued

Species	GenBank acc. no.	Host species (Order)	Locality
Perezitrema bychowskyi (Caballero & Caballero, 1975)	**KU535685** **KU535688-KU535691**	*Atractosteus tropicus* Gill (Lepisosteiformes)	Emiliano Zapata, Pantanos de Centla, Campeche, Mexico
Perezitrema bychowskyi (Caballero & Caballero, 1975)	**KU535686-KU535687** **KU535692-KU535694**	*Atractosteus tropicus* Gill (Lepisosteiformes)	San Pedro, Pantanos de Centla, Tabasco, Mexico
Rauschiella poncedeleoni Razo-Mendivil & León-Règagnon, 2001	AY875678	*Leptodactylus melanonotus* (Hallowell) (Anura)	San Pedro las Playas, Guerrero, Mexico
Rauschiella tineri (Babero, 1951)	AY875677	*Leptodactylus melanonotus* (Hallowell) (Anura)	San Pedro las Playas, Guerrero, Mexico
Mesocoeliidae Dollfus, 1929			
Mesocoelium sp.	AF433677	–	–
Omphalometridae Looss, 1899			
Neoglyphe sobolevi (Shaldybin, 1953)	AF300329	*Sorex araneus* L. (Soricomorpha)	Vilkovo, Ukraine
Omphalometra flexuosa Rudolphi, 1809	AF300333	*Talpa europaea* L. (Soricomorpha)	Kosewo GoÂrne, Poland
Rubenstrema exasperatum (Rudolphi, 1819)	AF300331	*Sorex araneus* L. (Soricomorpha)	Vilkovo, Ukraine
Plagiorchiidae Luhe, 1901			
Aptorchis aequalis Nicoll, 1914	EF014729	*Emydura krefftii* (Gray) (Testudines)	Ross River, Australia
Choledocystus hepatica (Lutz, 1928)	AY875679	*Rhinella marina* (L.) (Anura)	San Pedro las Playas, México
Haplometra cylindracea (Zeder, 1800)	AF151933	*Rana arvalis* Nilsson (Anura)	Carpathian Mountains, Ukraine
Lecithopyge rastellus Perkins, 1928	AF151932	*Bombina variegata* (L.) (Anura)	Carpathian Mountains, Ukraine
Plagiorchis elegans (Rudolphi, 1802)	AF151911	–	–
Skrjabinoeces similis (Looss, 1899)	AY222279	*Pelophylax ridibundus* (Pallas) (Anura)	Kokaljane, Bulgaria
Reniferidae Pratt, 1902			
Dasymetra nicolli Holl & Allison, 1935	AF433672	*Nerodia rhombifer* Hallowell (Squamata)	USA
Lechriorchis tygarti Talbot, 1933	JF820603	*Lithobates sylvaticus* Leconte (Anura)	North Dakota, USA

Table 1 continued

Species	GenBank acc. no.	Host species (Order)	Locality
Ochetosoma kansense (Crow, 1913)	AF433671	*Drymarchon corais* Holbrook (Squamata)	USA
Renifer aniarum (Leidy, 1890)	HQ665459	*Natrix natrix* (L.) (Squamata)	Calabria, Italy
Telorchiidae Looss, 1899			
Opisthioglyphe ranae (Frölich, 1791)	AF151929	*Rana arvalis* Nilsson (Anura)	Ivano-Frankivsk region, Ukraine
Telorchis assula (Dujardin, 1845)	AF151915	*Natrix natrix* (L.) (Squamata)	Kiev region, Ukraine

100 mM Na2 EDTA (pH 8.0), 1% Sarkosyl, and 0.1 mg/ml proteinase K. Genomic DNA was extracted from the supernatant using the DNAzol (Molecular Research Center, Cincinnati, Ohio, USA). Partial fragments (domains D1-D3) of the large subunit of the nuclear ribosomal DNA (28S) were amplified using polymerase chain reaction (PCR), using the forward primer BD3 5′-GAA CAT CGA CAT CTT GAA CG-3′ and the reverse primer 536 5′-CAG CTA TCC TGA GGG AAA C-3′ (García-Varela & Nadler, 2005). PCR cycling parameters consisted of denaturation at 94°C for 1 min, followed by 35 cycles of 94°C for 1 min, annealing at 50°C for 1 min, and extension at 72°C for 1.30 min, followed by a post-amplification incubation at 72°C for 10 min. Sequencing reactions were performed using four internal primers: BD2 5′-TAT GCT TAA ATT CAG CGG GT-3′ (Luton et al., 1992); 502 5′-CAA GTA CCG TGA GGG AAA GTT GC-3′ (García-Varela & Nadler, 2005); 503 5′-CCT TGG TCC GTG TTT CAA GAC G-3′ (Stock et al., 2001); and 504 5′-CGT CTT GAA ACA CGG ACT AAG G-3′ (García-Varela & Nadler, 2005). Reaction products were separated and detected using an ABI 3730 capillary DNA sequencer. Contiguous sequences were assembled using Geneious Pro 4.8.4 (Biomatters Ltd., Auckland, New Zealand). Sequences were deposited in the GenBank database (see accession numbers in Table 1).

Alignment and phylogenetic analyses

Sequences of the 28S rRNA gene of *Magnivitellinum simplex* and *Perezitrema bychowskyi* were aligned with sequences of species of other genera of the family Macroderoididae available in Genbank (*Alloglossidium*, *Macroderoides* and *Paramacroderides* Venard, 1941) for testing their phylogenetic relationships within the Macroderoididae. The monophyly of the Macroderoididae was also tested by including sequence data from other 13 families of the Plagiorchioidea (see Table 1). Sequences were aligned with the software ClustalW (Thompson et al., 1997), implemented in the web http://www.genome.jp/tools/clustalw/. No alignment positions were excluded prior to the analysis due to the fact that the 28S rRNA gene posseses conserved fragments and show a low substitution rate. Tree searches were conducted under Maximum Parsimony (MP), Maximum Likelihood (ML) and Bayesian inference (BI). *Auriculostoma astyanace* Scholz, Aguirre-Macedo & Choudhury, 2004 (Allocreadidae) was used as the outgroup for rooting the trees.

Parsimony analyses were performed using New Technology Search with Ratchet, tree drifting and tree fusing algorithms with 100 repetitions using the TNT software v 1.1 (Goloboff et al., 2008). All characters in the analyses were given the same weight, and were treated as unordered, and non-additive. Gaps were treated as missing data. Branch support was estimated with 1,000 bootstrap replicates with ten random addition sequences and TBR branch swapping. jModeltest version 3.0 (Guindon & Gascuel, 2003; Darriba et al., 2012) was used for inferring the best nucleotide substitution model and parameter settings for the 28S dataset. The ML trees were inferred using RAxML 7.0.4 (Stamatakis, 2006). Analyses were executed with 100 replicates and GTR CAT+I model was used. Each analysis was initiated with a random starting tree, using the rapid hill-climbing algorithm (-x) (Stamatakis et al., 2007). Clade support was assessed through 10,000 bootstrap replicates, and the rapid-hill climbing algorithm was also implemented

(Stamatakis et al., 2008). The software MrBayes v. 3.2.1 (Ronquist et al., 2012) was used to perform the BI analysis. The settings were two simultaneous runs of the Markov chain Monte Carlo (MCMC) for 10 million generations, sampling every 200 generations, a heating parameter value of 0.2 and a 'burn-in' of 10%. A 50% majority-rule consensus tree representing the posterior probability distribution of clades was produced from 20,000 trees. All phylogenetic trees were visualised with the software FigTree version 1.4.2. Sequence divergences were estimated using uncorrected p-distance model with the program PAUP* 4.0b10 (Swofford, 2003).

Phylogenetic analyses

The final 28S rDNA data set consisted of 63 sequences (32 representing 16 species of the family Macroderoididae), and included 1,273 nt positions, of which 450 were parsimony-informative. MP analysis recovered 32 equally parsimonious trees with a C.I. = 0.4286, R.I. = 0.7950 and a length of 1,363 steps. ML yielded a single tree with -ln = 8687.369841. The MP strict consensus tree, the ML tree and the 50% majority-rule consensus tree of BI yielded similar topologies (Fig. 1), and some relationships were supported by relatively high bootstrap and posterior probability support values. The phylogenetic trees showed *Magnivitellinum simplex* nested as the sister taxon of the members of *Alloglossidium*, while *Perezitrema bychowskyi* nested as the sister taxon of three species of *Macroderoides* (*M. texanus* Tkach, Strand & Froese, 2008; *M. minutus* Tkach & Kinsella, 2011; and *M. spiniferus* Pearse, 1924), rendering *Macroderoides* as paraphyletic (Fig. 1). The phylogenetic trees also revealed that Macroderoididae, as currently conceived, is not monophyletic. The family is split in two large sister clades. One clade contains the monotypic *Auridistomum chelydrae* (Auridistomidae), as the sister taxon of a group containing three species of *Macroderoides* (*M. typicus* Winfield, 1929; *M. flavus* Van Cleave & Mueller 1932; and *M. trilobatus* Tyler, 1978) + *Paramacroderoides kinsellai* Tkach, Pulis & Overstreet, 2010 + other three species of *Macroderoides* (mentioned above) + *Perezitrema.* The second large clade contains the Leptophallidae, including *Leptophallus nigrovenosus* (Bellingham, 1844), *Macrodera longicollis* (Abildgaard, 1788), *Metaleptophallus gracillimus* (Lühe, 1909), and *Paralepoderma cloacicola* (Lühe, 1909), as the sister group of a clade formed by *Alloglossidium* (five species) + *Magnivitellinum* (see Fig. 1).

Discussion

The MP, ML an BI analyses of the 28S rDNA dataset conducted in this study consistently show that the family Macroderoididae is not monophyletic, since some of its members, at least the ones for which sequence data are available, are sister taxa to other families of plagiorchioid trematodes such as the Auridistomidae and the Leptophallidae. First, the type-genus of the family, i.e. *Macroderoides*, nests as the siter taxon of *Auridistomum chelydrae*, a parasite of freshwater turtles. In addition to that, our phylogenetic analysis also shows unequivocally that the genus *Macroderoides* is paraphyletic. Tkach et al. (2010) were the first to notice that at least one species of *Macroderoides* was genetically closer to *Paramacroderoides* than to other *Macroderoides* spp. However, a year later, Tkach & Kinsella (2011) used *Paramacroderoides* as an outgroup in a phylogenetic analysis of *Macroderoides* based on partial sequences of the 18S and 28S rRNA genes and the complete ITS region, and these results supported the monophyly of *Macroderoides*, with two distinct clades for the North American species, and *Paramacroderoides* as their sister taxon. Although we only used partial 28S rDNA sequences, the inclusion of more members of the Plagiorchioidea resulted in the placement of *Paramacroderoides* as the sister taxon of *Perezitrema* and three species of *Macroderoides*, *M. spiniferus* and *M. texanus* + *M. minutus* (Fig. 1), providing further support for the paraphyly of *Macroderoides*.

The taxonomic history of *Perezitrema* has been controversial. The genus was originally included in the Acanthostomidae by Baruš & Moravec (1967) and subsequently assigned to the Opisthorchiidae Looss, 1899 (see Yamaguti, 1971), the Plagiorchiidae (see Brooks, 1980), and the Macroderoididae (see Moravec & Salgado-Maldonado, 2002). Our study provides further molecular evidence to demonstrate that *Perezitrema* does belong in the Macroderoididae, and its taxonomic position is now settled.

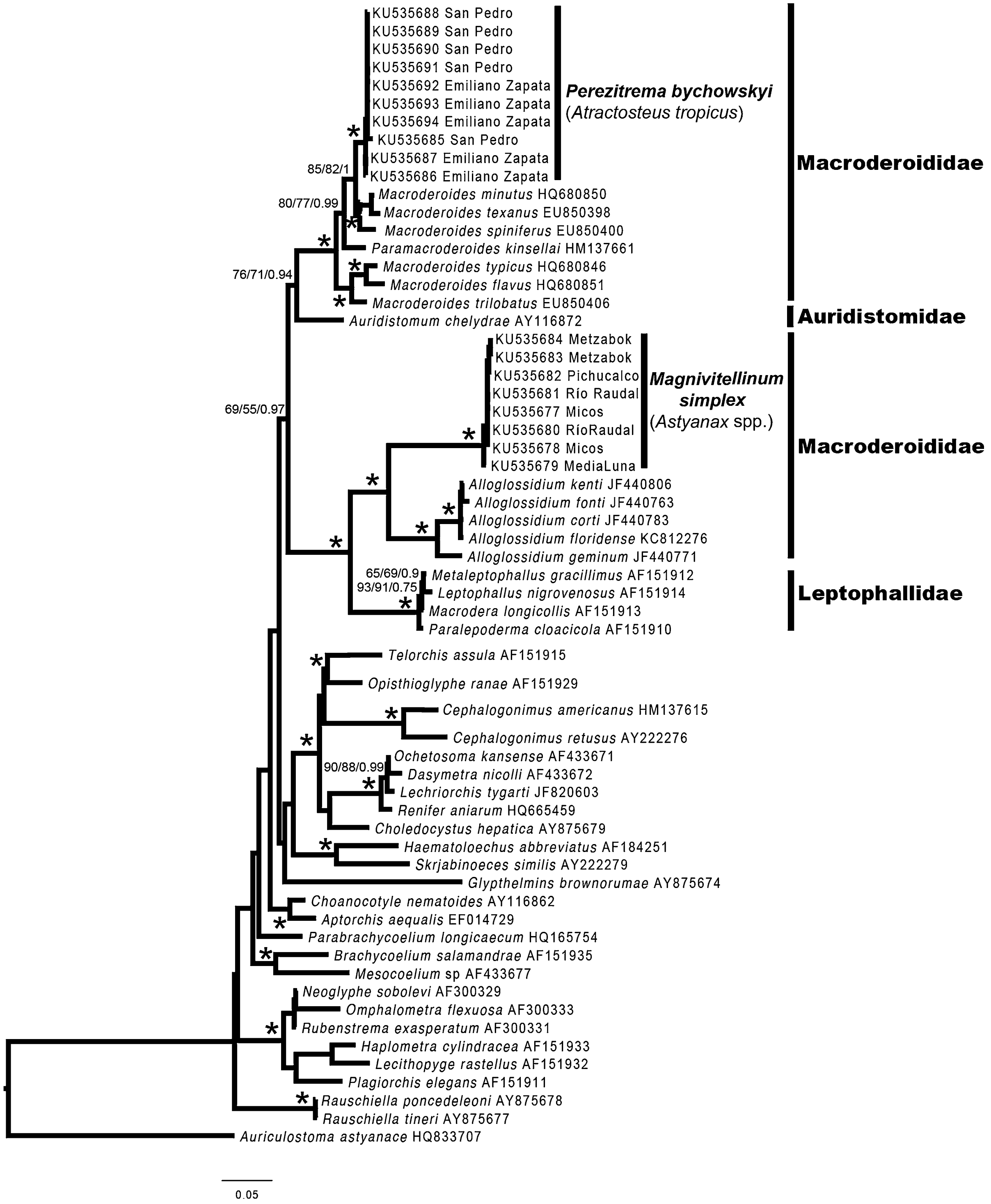

Fig. 1 Phylogenetic tree inferred through maximum likelihood of the 28S rDNA dataset. Numbers near internal nodes show MP/ML bootstrap clade frequencies and posterior probability clade frequencies (*= values above 90% for both ML and MP, and above 0.9 for BI)

Macroderoides is the type-genus of Macroderoididae, and the family is characterised by containing trematodes with the following characters: body fusiform to elongate, typically bearing tegumental spines, ventral sucker relatively small in the anterior end of body, cirrus-sac claviform, containing a bipartite seminal vesicle, ejaculatory duct unarmed, uterine seminal vesicle present, follicular vitellarium forming symmetrical lateral fields of variable extent, and I-shaped excretory vesicle. In their taxonomic revision of the family, Font & Lotz (2008a) modified the diagnosis to include characters exhibited by *Perezitrema*, i.e. a cyclocoel and a funnel-shaped oral sucker, characteristic of the genus (Moravec & Salgado-Maldonado, 2002). The presence of a cyclocoel in *Perezitrema* might be regarded as a morphological autapomorphy of the genus, in the same way *Paramacroderoides* is diagnosed by having a slender and elongate body with an oral sucker armed with two circular rows of large spines. Since both genera are valid and possess unique characters to diagnose them, then a new genus will be needed to accommodate the aforementioned three species of *Macroderoides*; however this requires further molecular phylogenetic analyses and a detailed assessment of their morphology.

A second result of our molecular phylogenetic analysis reveals that *Alloglossidium* and *Magnivitellinum* are sister taxa, and that these two genera are the sister group of members of the family Leptophallidae (Fig. 1). The latter comprise exclusively parasites of reptiles and possess a unique morphological characteristic uniting all four genera, i.e. the presence of an external seminal vesicle (Tkach et al., 1999). The position of this clade within the phylogeny of the plagiorchioid trematodes provides evidence that the current circumscription of the Macroderoididae is not satisfactory, since the family is not monophyletic. This is not the first time that the relationship between *Alloglossidium* and *Macroderoides* has been questioned. Based on a personal communication from V. V. Tkach, Bray & Hendrix (2007) (p. 861) mentioned that these genera do not cluster together and therefore, that the former although a plagiorchioid was not a macroderoidid. The results of our study provide the molecular evidence in support of this observation. Based on the new evidence, a new family has to be established to accommodate these two genera because clearly, just by host association, distribution, and phylogenetic interrelationships, they are not members of the Leptophallidae. Alloglossidiidae n. fam. is therefore proposed with the following diagnosis.

Alloglossidiidae n. fam.

Diagnosis: Body elongate. Tegument bearing spines, dense in the anterior extremity, decreasing in number at mid-level of hindbody. Oral sucker round, subterminal. Ventral sucker round, typically in anterior half of body. Prepharynx short or long. Pharynx well developed. Oesophagus distinct. Intestinal bifurcation about halfway between pharynx and ventral sucker. Caeca elongate, usually terminating between posterior testis and posterior extremity of body. Testes two, tandem or diagonal, typically entire, in hindbody. Cirrus-sac straight or curved, usually extending posteriorly beyond ventral sucker, contains bipartite seminal vesicle and coiled cirrus. Genital pore median or submedian, immediately anterior to ventral sucker. Ovary median or submedian, between ventral sucker and testes, sometimes close to or overlapping ventral sucker, spherical to oval, entire. Uterine seminal receptacle present. Uterus extends to posterior extremity of body, completely filling postcaecal space, with ascending and descending coils passing between testes; transverse uterine loops overlapping caeca or expanding into extracaecal space. Metraterm poorly differentiated. Eggs numerous, oval, operculate. Vitellarium consists of large follicles forming lateral fields; anterior margin of vitelline fields at different levels between ventral sucker and pharynx; posteriorly vitelline follicles usually extend to intertesticular area, confluent or not. Excretory vesicle I-shaped, pore terminal. In the intestine of freshwater fish (Ictaluridae, Characidae) or in freshwater crustaceans and leeches as progenetic metacercariae. Nearctic and Neotropical regions. Type-genus *Alloglossidium* Simer, 1929.

Taxonomic remarks

Molecular phylogenetic analyses have provided a framework to discuss the interrelationships among members of different trematode groups, and have challenged the traditional classification schemes based on morphological grounds. For instance, a recent molecular phylogeny of the Echinostomatoidea Looss, 1899 (Tkach et al., 2016) resulted in a new phylogeny-based classification of the group, where a number of systematic and nomenclatural changes were required. Our study follows that direction, although it was conducted at a lower scale across the hierarchy of the trematode classification, and a new family is described based on the molecular phylogenetic analysis, and the evaluation of some morphological traits.

The new family can be readily distinguished from members included traditionally within the Macroderoididae by the combination of the following characters: tegumental spines are very dense at the anterior extremity and the number of spines decreases at the mid-level of hindbody; in macroderoidids the entire tegument is covered with spines. Herein we provide evidence of the external surface of *M. simplex* through SEM for the first time, showing that no spines are found on the tegument in the posterior third of the body (Fig. 2). This character seems to be controversial since Smythe & Font (2001) used three character states, i.e. body fully spined, spines extending from anterior extremity of worms to mid-body and spines restricted to anterior extremity. However, the most recent descriptions of congeneric species (see Tkach & Mills, 2011; Tkach et al., 2013; Kasl et al., 2014) clearly show that spines become sparser toward posterior extremity of body and descriptions actually indicate that spine number and size decrease towards the posterior extremity of the body. While describing *A. demshini* Tkach, Greiman & Steffes, 2013 the authors pointed out that spines were not observed posterior to the level of pharynx under light microscope, although under SEM they are seen to reach the level of ventral sucker (Tkach et al., 2013). Based on the aforementioned arguments we suggest that other species of *Alloglossidium* require SEM studies to verify the spine distribution, and be used as a reliable character to separate species. Also, in the new family, the vitelline follicles extend from the level between the pharynx and the anterior end of the ventral sucker, and the intertesticular area, not surpassing the posterior testis; in macroderoidids, the vitellarium extends from the posterior margin of ventral sucker to the posterior extremity of the body, surpassing the level of posterior testis. The post-testicular space is wide in all members of Alloglossidiidae n. fam., and no members of the family possess a funnel-shaped oral sucker, or a cyclocoel, as some members of the Macroderoididae.

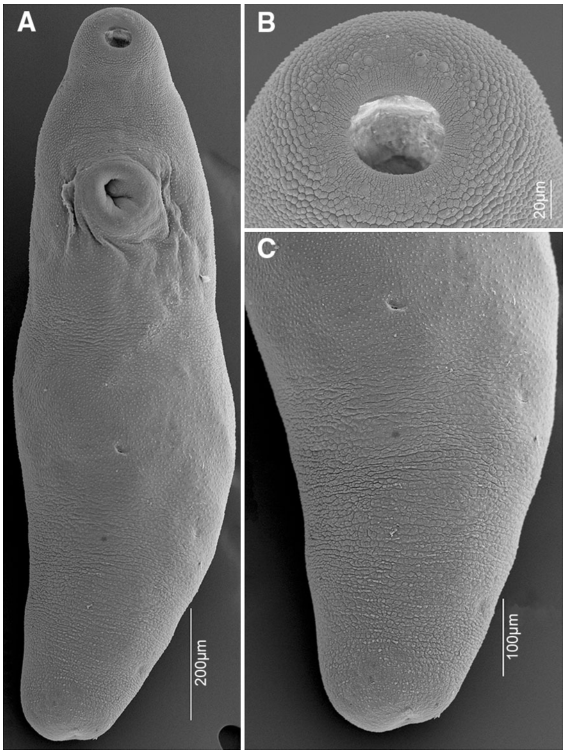

Fig. 2 Scanning electron images of *Magnivitellinum simplex*. A, Total view; B, Oral sucker region; C, Posterior half of the body showing the lack of spines on the body surface in the posterior third of the body

The inclusion of other genera traditionally allocated in the Macroderoididae into the new family, such as *Gauhatiana*, *Cirkennedya*, and *Malawitrema* (see Font & Lotz, 2008a), as well as those considered *incertae sedis* by Pojmańska et al. (2008), i.e. *Vietosoma* and *Pseudomagnivitellinum*, require further verification. No sequence data are available for these genera yet, rendering their inclusion into one of these families problematic. All of the species in these genera possess a spined tegument and in general show some morphological resemblance with members of both, the Macroderoididae or the Alloglossidiidae n. fam., except for species of *Vietosoma* Van Cleave & Mueller, 1932 and *Malawitrema*, which possess symmetrical testes and a different extent of the vitellarium. *Vietosoma parvum* Van Cleave & Mueller, 1932 possesses follicles distributed from the level of oral sucker to the posterior extremity of the body, whereas *Malawitrema staufferi* Bray & Hendrix, 2007 possesses restricted vitellarium, between the testes and ovary region. However, considering the aspect of host association, we believe that they could be clustered within the new family erected herein: both species are parasites of siluriform fishes, with ictalurid catfishes in North America (the channel catfish *Ictalurus punctatus* Rafinesque) as the type-host of *V. parvum*, and the African siluriforms *Clarias gariepinus* (Burchell) and *Bagrus meridionalis* Günther, as hosts of *M. staufer*i. Two additional species are parasites of siluriforms, *Gauthiana batrachii* Gupta, 1955 in the catfish *Clarias batrachus* (Linnaeus) in India and Philippines (Gupta, 1955; Arthur & Lumanlan-Mayo, 1997), and *Pseudomagnivitellinum ictalurum* in the black bullhead *Ictalurus melas* Rafinesque in North America (Dronen & Underwood, 1980). The validity of *Pseudomagnivitellinum* has been recently questioned (Pojmanska, 2008), while *Gauthiana* is accepted as a member of the Macroderoididae by Font & Lotz (2008a). Based on their morphological traits *G. batrachii* and *P. ictalurum* seem to be, in our opinion, valid species; however their taxonomic validity and possible inclusion into the family is pending new sequence data for a molecular phylogenetic analysis. If valid, we also believe that, by host associations and by sharing some morphological traits, both genera will be also members of Alloglossidiidae n. fam.

Host associations and biogeographical considerations

The two genera analysed in this study exhibit a different pattern of historical biogeography and the fact that the families to which they belong, the Macroderoididae (containing *Perezitrema*) and the Alloglossidiidae n. fam. (containing *Magnivitellinum*) include species parasitic in freshwater fish, with the sister taxa for each of them parasitising reptiles, poses an interesting host association and biogeographical scenario, where the two families seem to follow independent colonisation events *via* host-switching from ancestors occurring in other aquatic ectotherm vertebrates, such as amphibians and reptiles, to freshwater fish, considering also that the sister taxa of both families such as telorchids, cephalogonimids, ochetosomatids, etc., are all parasites of amphibians or reptiles (see Fig. 1). In the first case, *Auridistomum chelydrae* is found in freshwater turtles in North America (Yamaguti, 1971; Font & Lotz, 2008b), whereas six of the eight species of *Macroderoides* (as currently conceived) are mostly parasites of gars (*Atractosteus* Rafinesque and *Lepisosteus* Gill), bowfin (*Amia calva* Linnaeus) and pickerel (*Esox* Linnaeus) in the USA. Also the three North American species of the genus *Paramacroderoides* are found in gars (Tkach et al., 2010). Interestingly, the two valid species of *Perezitrema* are common parasites of gars, *P. bychowskyi* in the tropical gar (*A. tropicus* Gill) along the Gulf of Mexico and Atlantic slopes of Mexico and Nicaragua (Moravec & Salgado-Maldonado, 2002), and *P. viguerasi* in the Cuban gar *Atractosteous tristoechus* in Cuba (Baruš & Moravec, 1967).

In the second case, the Leptophallidae, formed by species that infect the digestive tract and lungs of snakes in Europe, Asia and North Africa (Tkach, 2008) is the sister group of the new family whose members are mainly parasitic in freshwater fish. In addition, the two genera currently included in the new family exhibit a particular host association and distribution pattern; *Alloglossidium* is found in ictalurid catfishes (a Nearctic freshwater fish group), or in crustaceans and leeches in North America (see Kasl et al., 2014). The five species for which sequences are available in the GenBank database are non-precocious and are parasites of ictalurids across Canada, USA and Mexico (see Gibson, 1996; Pérez-Ponce de León et al, 2007b; Kasl et al., 2014), exhibiting a remarkable host lineage specificity (see Choudhury et al., 2016). *Magnivitellinum*, the sister taxon of *Alloglossidium* has a Neotropical affinity since its species are found in characids and siluriform catfishes; this genus currently contains two species, *M. corvitellinum* Lacerda, Takemoto & Pavanelli, 2009 from a siluriform catfish in Brazil (Lacerda et al., 2009), and *M. simplex*, occurring across a wide geographic range from Argentina to northern Mexico, in close association with characids (Kohn et al., 2007; Pérez-Ponce de León et al., 2007b). Unfortunately, the origin and interrelationships among the genera allocated into the new family are more difficult to explain. The geographical disjunction between the Nearctic and Neotropical biogeographical regions, and the fact that these three fish groups are not closely related, challenges a plausible explanation of the phylogenetic affinities herein uncovered.

Notwithstanding, other trematodes exhibit a similar pattern of host association and geographical distribution. Tkach & Curran (2015) conducted a molecular phylogenetic analysis for the species of *Prosthenhystera* Travassos, 1922 and discovered that *P. caballeroi* Jiménez, 1973, a parasite of *Astyanax* spp. in Middle America, is the sister species of *P. obesa* (Diesing, 1850), a parasite of characids (and apparently in other Neotropical fish groups) in Brazil (see Kohn et al., 2007), and *P. oonastica* Tkach & Curran, 2015, a parasite of ictalurids in the USA. Although the relationships between sister trematode taxa among Nearctic ictalurid and Neotropical siluriforms and characids cannot be explained, the results of our study set the grounds to keep collecting more data to unravel this complex host-parasite association, and to incorporate other lines of evidence to explain this host and biogeographical relationships. Choudhury et al. (2016) discussed that along with exploratory and opportunistic surveys aimed at describing new taxa, we should consider more strategic hypothesis-driven sampling which can yield data for uncovering phylogenetic relationships of widely distributed taxa such as *Magnivitellinum simplex*, as well as patterns and processes of diversification across and within drainages and biogeographical regions.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All applicable institutional, national and international guidelines for the care and use of animals were followed. Hosts were collected under the Cartilla Nacional de Colector Científico de Flora y Fauna Silvestre FAUT-0057 issued to GPPL by the Secretaria del Medio Ambiente y Recursos Naturales.

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