Parasitology Research
https://doi.org/10.1007/s00436-023-07863-x

RESEARCH

Phylogenetic position of the South American freshwater *Rhipidocotyle santaensis* (Digenea:Bucephalidae) based on partial 28S rDNA

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Received: 18 October 2022 / Accepted: 1 May 2023

Abstract

The family Bucephalidae is comprised of nine subfamilies, the most important being Bucephalinae with eight genera. Among these, the genus *Rhipidocotyle* has been found in marine and freshwater environments all over the world. Previous studies of *Rhipidocotyle santanaensis* have dealt with its morphology or host's ecology. Here, we provide a phylogenetic analysis based on two 28S rDNA sequences from *R. santanaensis* parasitizing the freshwater fish *Acestrorhynchus pantaneiro* from the Ibera Lagoon (Corrientes Province, Argentina). The 28S rDNA tree showed that it clustered together with *Rhipidocotyle* species from Middle and North America, suggesting a common history. Bucephalinae appears to have undergone four evolutionary processes: first, the diversification within the same host family; second, more than one successful infection of the same host family in different geographic regions; third, "jumping" between host families; and, finally, successful invasion of the freshwater environment (occurring in at least four different events in the subfamily). We hypothesize that *R. santanaensis* entered the freshwater environment by a "jumping" event from some unknown marine host family when a seawater ingression took place in South America during the Late Quaternary. This is the first sequenced Bucephalinae species from South America. Further sequencing will help shed light on the evolutionary relationships between South American members of this group from marine and, especially, freshwater environments.

Keywords *Rhipidocotyle santanaensis* · Argentina · Freshwater · 28S rDNA · *Acestrorhynchus pantaneiro*

Introduction

The family Bucephalidae is comprised of nine subfamilies, with Bucephalinae being the largest. According to WoRMS (2022), this subfamily includes the following genera: *Aenigmatrema* Corner, Cribb & Cutmore, 2020 with five species; *Alcicornis* MacCallum, 1917 with 13 species; *Bucephalus* von Baer, 1827 with 53 species; *Parabucephalopsis* Tang & Tang, 1963 with one species; *Prosorhynchoides* Dollfus, 1929 with 62 species; *Pseudobucephalopsis* Long & Lee, 1964 with two species; *Pseudorhipidocotyle* Wang & Pan in Long & Lee, 1964 with two species; and *Rhipidocotyle* Diesing, 1858 with 59 species.

The relationships within the subfamily Bucephalinae are uncertain, as they were based mainly on general morphological traits. In particular, the morphology of the rhynchus—an apical holdfast organ disassociated from the digestive system (Overstreet and Curran 2002) which was previously used to distinguish genera—was found to be an unreliable diagnostic character (Corner et al. 2020). Molecular studies have provided evidence supporting the rhynchus evolved independently at least twice in the highly speciose genera *Bucephalus*, *Rhipidocotyle*, and *Prosorhynchoides* (Nolan et al. 2015; Hammond et al. 2020).

The genus *Rhipidocotyle* inhabits marine and freshwater fish from all over the world. In South America, *Rhipidocotyle adbaculum* Manter, 1940, *Rhipidocotyle angusticolle*

Section Editor: Shokoofeh Shamsi

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Published online: 24 May 2023

Chandler, 1941, *Rhipidocotyle fluminensis* Vicente & Santos, 1973, *Rhipidocotyle pentagonum* (Ozaki, 1924) Eckmann, 1932, and *Rhipidocotyle quadriculatum* Kohn, 1961 were reported from marine environments, whereas *Rhipidocotyle gibsoni* Kohn & Fernandes, 1994; *Rhipidocotyle jeffersoni* (Kohn, 1970) Overstreet & Curran, 2002; and *Rhipidocotyle santanaensis* Lunaschi, 2004 were reported from freshwater environments (Kohn et al. 2007).

Rhipidocotyle santanaensis was described parasitizing the following freshwater fishes from South America: *Acestrorhynchus pantaneiro* Menezes from Corrientes Province, Argentina; *Ac. pantaneiro* and *Astyanax lacustris* Lütken from southern Brazil (Pedro et al. 2016); and *Acestrorhynchus falcirostris* Cuvier from Amazonas State, Brazil (Fernandes et al. 2017). These studies focused on the morphology of this parasite or the ecology of its hosts, and no phylogenetic analysis has been performed so far.

The previous phylogenetic analysis of Bucephalinae took into account both marine and freshwater species, but did not include sequences from South America (Corner et al. 2020, Atopkin et al. 2022). The aims of this study were to provide the first sequences of a member from the Bucephalinae in South America, specifically Argentina, and to elucidate the phylogenetic position of *R. santanaensis* using partial sequences of 28S rDNA.

Material and methods

One specimen of *Ac. pantaneiro* was collected from Ibera Lagoon, Colonia Carlos Pellegrini (28° 32′ 25″ S, 57° 10′ 48″ W), Corrientes Province, Argentina (Fig. 1), in spring of 2014. The fish host was caught by a fishing rod and transported alive to the field laboratory, where it was euthanized with an overdose of Eugenol® (Dickinson, Argentina) and necropsied. Digeneans were found in the pyloric caecae; specimens used for morphological studies were killed in hot water and fixed in 10% formalin. These specimens were stained with hydrochloric carmine, dehydrated in a graded ethanol series according to laboratory protocols (Pritchard and Kruse 1982), cleared in Eugenol, and mounted in Canada balsam. Measurements are expressed in micrometers (µm). Voucher specimens were deposited at the Helminthological Collection of the Museo de La Plata, Buenos Aires, Argentina (MLP).

For molecular analysis, live specimens were preserved in cold 96% ethanol and stored until DNA extraction. The total genomic DNA was extracted from two individual specimens using PURO-Genomic DNA (Productos Bio-logicos SA) according to the manufacturer's protocol. The partial fragment of 28S rDNA was amplified with the forward primer LSU-5 (5′–TAG GTC GAC CCG CTG AAY TTA AGC A–3′) (Littlewood et al. 2000) and the reverse primer 1500R

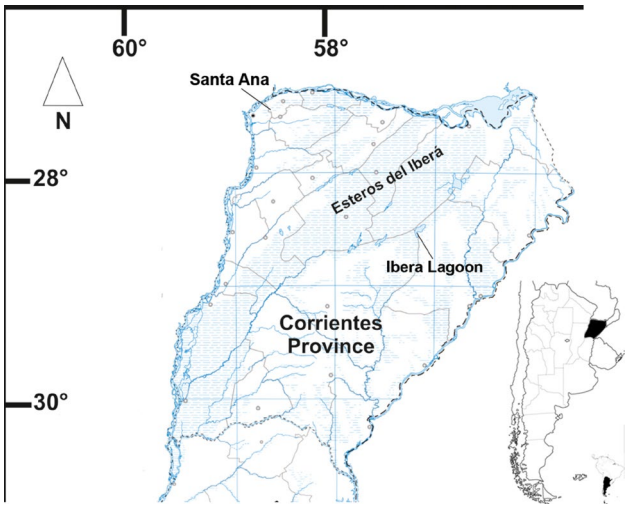

Fig. 1 Map of Argentina with details of the Corrientes Province showing the collection site of the specimen used for the original description of *Rhipidocotyle santanaensis* Lunaschi, 2014 in Santa Ana and the new record from the Iberá Lagoon in this study

(5′–GCT ATC CTG AGG GAA ACT TCG–3′) (Tkach et al. 2003) using polymerase chain reaction (PCR) on an Eppendorf mastercycler thermal cycler.

The PCR was performed with Master Mix (PCR, Productos Bio-logicos S.A) following the protocols described by Tkach et al. (2003). PCR products were sent to Macrogen Inc. (Korea) to be purified and Sanger sequenced. Sequences were assembled using the platform Geneious 5.0.4.

The resulting 28S sequences were used to search for homologous sequences (Table 1) in GenBank with the BLASTn tool. Sequences were aligned using the online version of MAFFT 7 (Katoh et al. 2019). Ambiguously aligned, hypervariable regions in the 28S dataset were removed with Gblocks online version 0.91b (Talavera and Castresana 2007), according to a secondary structure model, with the parameter settings of a less stringent selection (allowing smaller final blocks, gap positions within the final blocks and less strict flanking positions). *Olssonium terneri* and *Pleorchis uku* were used as outgroups on the basis of the tree topology published by Nolan et al. (2015). The best partitioning scheme and substitution model for the DNA partition were selected by the Akaike information criterion (Posada and Buckley, 2004) using MEGA X (Kumar et al., 2018). The appropriate nucleotide substitution model implemented for 28S rDNA matrix was the general time-reversible model with estimates of invariant sites and gamma-distributed among site variation (GTR + G + I) model.

Phylogenetic reconstruction was performed using Bayesian inference (BI) through MrBayes 3.2.3 (Ronquist et al. 2012). Phylogenetic trees were constructed using two parallel analyses of Metropolis-Coupled Markov Chain Monte Carlo (MCMC) for 20 million generations each, to estimate

Table 1 Information on Bucephalinae species used to construct the phylogenetic tree showed in Fig. 1

Species	Host	Environment	Location	28S Sequence	Reference
Aenigmatrema inopinatum Corner, Cribb & Cutmore, 2020	*Sphyraena obtusata* Cuvier	M, B	Moreton Bay, Queensland, (Australia)	MT809144	Corner et al. (2020)
Aenigmatrema grandiovum Corner, Cribb & Cutmore, 2020				MT809145	
Aenigmatrema undecimtentaculatum Corner, Cribb & Cutmore, 2020				MT809141, MT809143	
Bucephalus cynoscion Hopkins, 1956	*Cynoscion arenarius* Ginsburg	M, B	Mississippi River (USA)	KT273396	Nolan et al. (2015)
	Bairdiella chrysoura Lacepède	M, B, F		KT273397	
Bucephalus gorgon (Linton, 1905) Eckmann, 1932	*Seriola dumerili* Risso	M	North-Central Gulf of Mexico	KT273400	
Bucephalus margaritae Ozaki & Ishibashi, 1934	*Caranx crysos* Mitchill	M, B		KT273395	
Bucephalus polymorphus von Baer, 1827	*Dreissena polymorpha* Pallas	M	Lepelskoe lake (Belarus)	AY289238, AY289242-47	Stunžėnas et al. (2004)
	Perca fluviatilis Linnaeus	B, F	Curonian Lagoon (Lithuania)	JQ346717	Petkevičiūtė et al. (2014)
Bucephalus skrjabini Akhmerov, 1963	*Siniperca chuatsi* Basilewsky	F	Amur River (Russia)	OM615426-30	Atopkin et al. (2022)
Bucephalus varicus Manter, 1940	*Caranx caballus* Günther	M, B	Chamela, Jalisco (Mexico)	MK648266	Pérez-Ponce de León and Hernandez Mena (2019)
Olssonium turneri Bray & Gibson, 1980	*Alepocephalus agassizii* Goode & Bean	M	United Kingdom	AY222283	Olson et al. (2003)
Parabucephalopsis parasiluri Wang, 1985	*Silurus biwaensis* Tomoda	F	Shiga, Seta River (Japan)	AB640884	Baba et al. (2012)
Paurorhynchus hiodontis Dickerman, 1954	*Hiodon alosoides* Rafinesque	F	Minnesota (USA)	KT273401	Nolan et al. (2015)
Pleorchis uku Yamaguti, 1970	*Aprion virescens* Valenciennes	M	Lizard Island, GBR (Australia)	DQ248216	Bray et al. (2005)
Prosorhynchoides sp.	*Plecoglossus altivelis altivelis*Temminck & Schlegel	M, B, F	Wakayama (Japan)	LC498576	Shirakashi et al. (2020)
Prosorhynchoides apogonis Bott & Cribb, 2005	*Cheilodipterus macrodon* (Lacepède)	M	Off Heron Island, Queensland, Australia	KT213576	Nolan et al. (2015)
Prosorhynchoides caecorum (Hopkins, 1956) Bott & Cribb, 2005	*Sciaenops ocellatus* Linnaeus	M, B	Mississippi River (USA)	KT273392	Nolan et al. (2015)
	B. chrysoura	M, B, F		KT273393	
Prosorhynchoides cutmorei Hammond, Cribb & Bott, 2018	*Tylosurus gavialoides* Castelnau	M, B	Moreton Bay, Queensland (Australia)	MG953232	Hammond et al. (2018)
Prosorhynchoides galaktionovi Hammond, Cribb, Nolan & Bott, 2020	*Tylosurus crocodilus* Péron & Lesueur	M	Off Lizard Island (Australia)	MN310395-96	Hammond et al. (2020)
Prosorhynchoides gracilescens (Rudolphi, 1819) Stunkard, 1976	*Lophius piscatorius* Linnaeus	M	North Sea, UK	AY222224	Olson et al. (2003)

Table 1 (continued)

Species	Host	Environment	Location	28S Sequence	Reference
Prosorhynchoides karvei (Bhalerao, 1937) Srivastava & Chauhan, 1973	*Strongylura strongylura* van Hasselt	M	Halong Bay East	OM615431-34	Atopkin et al. (2022)
Prosorhynchoides kohnae Hammond, Cribb, Nolan & Bott, 2020	*T. crocodilus*	M	Off Lizard Island (Australia)	MN310397	Hammond et al. (2020)
Prosorhynchoides longoviferus Manter, 1940) Curran & Overstreet, 2009	*Sphyraena barracuda* Edwards	M, B	Florida (USA)	KT273387	Nolan et al. (2015)
Prosorhynchoides megacirrus (Riggin & Sparks, 1962) Curran & Overstreet, 2009	*S. ocellatus*	M, B	Mississipi (USA)	KT273391	
Prosorhynchoides moretonensis Hammond, Cribb & Bott, 2018	*T. gavialoides*	M, B	Moreton Bay, Queensland (Australia)	MG953230	Hammond et al. (2018)
Prosorhynchoides ovatus (Linton, 1900) Dollfus, 1929	*Lobotes surinamensis* Bloch	M, B	Mississippi River (USA)	KT273399	Nolan et al. (2015)
Prosorhynchoides ozakii (Nagaty, 1937) Margolis & Arthur, 1979	*S. biwaensis*	F	Kyoto, Uji River (Japan)	AB640885	Baba et al. (2012)
Prosorhynchoides paralichthydis (Corkum, 1961) Curran & Overstreet, 2009	*Paralichthys lethostigma* Jordan & Gilbert	M, B	Mississippi River (USA)	KT273398	Nolan et al. (2015)
Prosorhynchoides scomberomorus (Corkum, 1968) Curran & Overstreet, 2009	*Scomberomorus maculatus* Mitchill	M		KT273388	
	Scomberomorus cavalla Cuvier	M	North-Central Gulf of Mexico	KT273389	
Prosorhynchoides waeschenbachae Hammond, Cribb & Bott, 2018	*T. gavialoides*	M, B	Moreton Bay, Queensland (Australia)	MG953231	Hammond et al. (2018)
Rhipidocotyle sp.	*Kyphosus elegans* Peters	M	Chamela, Jalisco (Mexico)	MK648267	Pérez-Ponce de León and Hernandez Mena (2019)
Rhipidocotyle angusticolle Chandler, 1941	*Euthynnus alletteratus* Rafinesque	M, B	Louisiana (USA)	KT273383	Nolan et al. (2015)
Rhipidocotyle campanula Chandler, 1941	*Anodonta anatina* Linnaeus	F	Saravesi Lake (Finland)	JQ346710	Petkevičiūtė et al. (2014)
			Vilkoksnis Lake (Lithuania)	JQ346711	
			Chesnava River (Russia)	JQ346712	
			Smolka River (Ukraine)	KF184356	
	Unio crassus Philipsson	F	Kiauna River (Lithuania)	JQ346714	
				KF184357	
	A. anatina	F	Kaunas Water Reservoir (Lithuania)	JQ346713	
Rhipidocotyle fennica Gibson, Taskinen & Valtonen, 1992	*A. anatina*	F	Saravesi Lake (Finland)	JQ346715-16	
			Smolka River (Ukraine)	KF184364	
			Vilkoksnis Lake (Lithuania)	KF184361-63	
	Esox lucis Linnaeus	F		KM068119	Stunžėnas et al. (2004)

Table 1 (continued)

Species	Host	Environment	Location	28S Sequence	Reference
Rhipidocotyle galeata (Rudolphi, 1819) Eckmann, 1932	*Eutrigla gurnardus* Linnaeus	M, B	North Sea (United Kingdom)	AY222225	Olson et al. (2003)
Rhipidocotyle husi Atopkin, Shedko, Rozhkovan, Nguyen & Besprozvannykh, 2022	*Huso dauricus* Georgi	M, B, F	Amur River (Russia)	OM615411-25	Atopkin et al. (2022)
Rhipidocotyle lepisostei Hopkins, 1954	*Lepisosteus oculatus* Winchell	B, F	Mississippi River (USA)	KT273390	Nolan et al. (2015)
Rhipidocotyle santanaensis Lunaschi, 2004	*Acestrorhynchus pantaneiro* Menezes	F	Ibera (Argentina)	OQ244080-81	Present study
Rhipidocotyle transversalis Chandler, 1935	*Strongylura marina* Walbaum	M, B, F	Mississippi River (USA)	KT273394	Nolan et al. (2015)
Rhipidocotyle tridecapapillata Curran & Overstreet, 2009	*Micropterus salmoides* Lacepède	F		KT273384	

New sequences in bold. Abbreviation: *B*, brackish; *F*, freshwater; *M*, marine

the posterior probability (PP) distribution. Topologies were sampled every 1000 generations, and the average standard deviation of split frequencies was observed to be less than 0.01 at the end of the run, as suggested by Ronquist et al. (2012). The robustness of the clades was assessed using Bayesian posterior probability (PP), where PP > 0.95 was considered strongly supported. A majority consensus tree with branch lengths was reconstructed for each run after discarding the first 25% trees sampled as "burn-in".

Additionally, uncorrected *p* distance was calculated using MEGA X. Newly generated sequences were submitted to GenBank.

Results

Morphological analysis of *Rhipidocotyle santanaensis*

Description of new specimens: Based on 10 specimens (average followed by a range). Body fusiform, 1299 (1106–1427) × 335 (255–378), entirely covered with spines. Rhynchus sucker-like, with two lateral papilliform projections, without dorsal hood, small, 144 (120–174) × 133 (101–159), representing 11% (9–15%) of body length, without deep cavity, aperture ventral, subterminal. Pharynx, oesophagus, and intestinal caecum not observed. Pre-oral region 483 (424–510), post-oral region 662 (474–779). Testes and ovary located in middle third of body. Testes two, entire, irregularly oval, arranged in tandem but sometimes oblique, dextral, at anterior half of post-oral region. Anterior testis equatorial, 167 (158–181) × 145 (110–178), posterior testis 139 (122–160) × 131 (124–143). Cirrus sac long, occupies 31% (30–33%) of body length, sinistral, uniform in width, with proximal extremity at level of first testis, within posterior half of body; complex cirrus sac-genital lobe 422 (392–453) × 84 (71–94), occupies 31% (30–33%) of body length. Seminal vesicle oval, 110 (97–120) × 77 (67–104), pars prostatica straight, ejaculatory duct short. Genital atrium large, associated with gland cells, enclosing genital lobe. Genital lobe with strong muscular ring at proximal end. Genital pore subterminal. Ovary opposite to anterior testis, 122 (106–136) × 109 (91–138). Vitelline follicles large, forming arch anterior to caecum or in two parallel lateral rows. Uterine loops occupying area between vitelline arch and posterior end of body. Eggs numerous, 19 (18–20) × 12 (12–13).

Taxonomy summary

Type host: *Acestrorhynchus pantaneiro* Menezes (Acestrorhynchidae).
Site of infection: pyloric ceca.
Locality: Colonia Carlos Pellegrini, Corrientes Province, Argentina (28° 32′ 25″ S, 57° 10′ 48″ W)
Type voucher: MLP- He 7981

Remarks

Except for their larger size, the morphology of our specimens matched the original description of *R. santanaensis* collected from the same host (*Ac. pantaneiro*) and province (Corrientes Province, Argentina) (Lunaschi 2004). However, the original description by Lunaschi (2004) may not have

demonstrated the true morphological variation of the species based on the low number of specimens measured (n = 3). The egg-filled uterus and the stained parenchyma in our specimens hindered the visualization of the pharynx, esophagus, and caecum. The distribution of the vitelline follicles described by Lunaschi (2004) was forming an arch anterior to caecum. In our material, the vitellarium was arranged as either an arch anterior to caecum or in two parallel lateral rows (Supp. material 1)

Egg size and extension of the uterine loops were consistent between our specimens and the original materials. These features are the most important diagnostic features for distinguishing between the South American freshwater species. *Rhipidocotyle santanaensis* and *R. gibbsoni* exhibit a similar egg sizes, while *R. jeffersoni* possesses longer eggs (16–20 vs 30–42, respectively). In turn, *R. santanaensis* differs from *R. gibbsoni* in the distribution of the uterine loops, which reach the base of the vitelline follicles in the former and the rhynchus in the latter.

Molecular analysis

Two 28S rDNA sequences of *R. santaensis* with lengths of 1236 and 1305 bp were generated. The phylogenetic tree (Fig. 2) was constructed with a matrix of 84 taxa based on 1022 bp as result of the exclusion of the ambiguous sites.

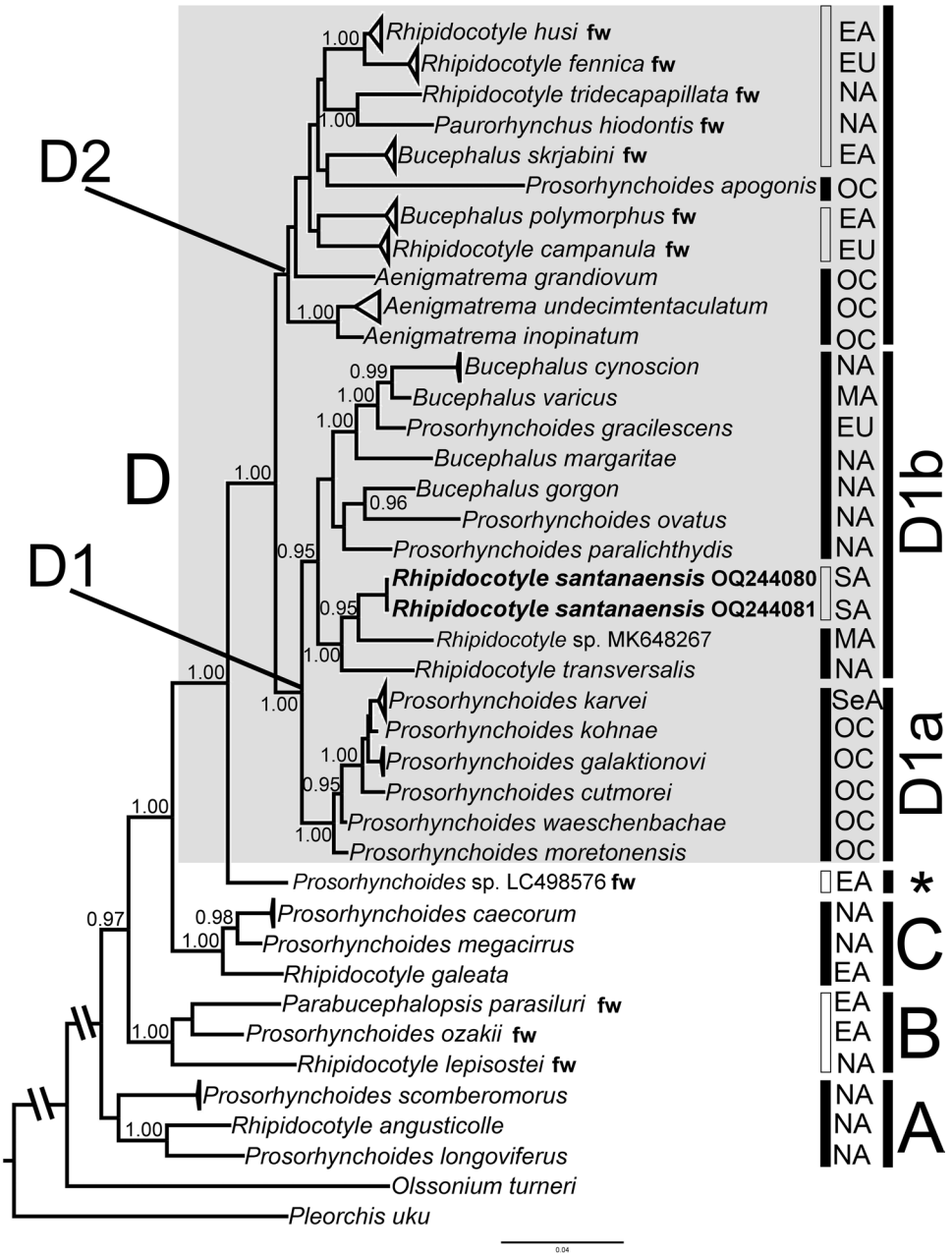

Fig. 2 Phylogram resulting from Bayesian inference (20,000,000 generations) of partial 28S rDNA gene sequences showing the relationships of *Rhipidocotyle santanaensis* Lunaschi, 2014 compared to other Bucephalinae species. Branch support values indicate posterior probabilities. Abbreviations: *clade without name; EA, East Asia; EU, Europe; fw, freshwater; MA, Middle America; NA, North America; OC, Oceania; SA, South America; SeA, South East Asia

The clade A was weakly supported (0.81 PP) and contained *P. scomberomorus* followed by a clade with *R. angusticolle* and *P. longoviferus* from marine waters of North America.

The clade B was highly supported and included *Parabucephalopsis parasiluri*, *P. ozakii* and *R. lepisostei* from freshwater fishes from East Asia and North America.

The clade C was strongly supported and was composed of a clade with *P. caecorum* and *P. megacirrus*, followed by *R. galeata*, all of which are parasites of marine fishes from North America and Europe.

The clade D, with strong PP support, comprised highly diversified taxa distributed worldwide from hosts of different families inhabiting different environments. *Prosorhynchoides* sp. from freshwater fishes of family Plecoglossidae from East Asia emerged at its base. The clade D was divided into subclades D1 with high support and D2 with weak support (0.72 PP).

The subclade D1 further separated into clusters D1a and D1b. The D1a cluster contained *Prosorhynchoides* spp. from marine waters of South-Eastern Asia and Oceania, and all the internal clades were highly supported.

The cluster D1b was mainly composed of marine species from North America, Europe, and Middle America and South America. It was partitioned into two clusters. The first cluster was weakly supported (0.77 pp) and included *B. cynoscion*, *B. varicus*, *P. gracilescens*, *B. margaritae*, *B. gorgon*, *P. ovatus*, and *P. paralichthydis* parasitizing marine fishes from Middle America, North America, and Europe. The second cluster was highly supported and included *R. santanaensis* from freshwater hosts in South America as sister to *Rhipidocotyle* sp. and *Rhipidocotyle transversalis* Chandler, 1935 from marine hosts in Middle and North America, respectively.

The subclade D2 was weakly supported (0.72 pp) and was further divided into two clusters. One of them was weakly supported and composed of species from freshwaters of East Asia, Europe, North America, and Oceania. The pairs *R. husi* - *R. fennica* and *R. tridecapapillata* - *P. hiodontis* had strong support. The other cluster was formed by *Aenigmatrema* spp. from marine fishes in Oceania.

Table 2 shows the genetic divergence between *R. santanaensis* and other Bucephalinae species based on uncorrected p-distances for 28S. *Rhipidocotyle santaensis* has little genetic distance (p-distance of 3%) from most species of clade D1, except for *P. gracilienscens*, *B. margaritae*, *B. varicus* and *P. ovatus* (4%), and *B. cynoscion* (5%).

Discussion

The freshwater species *R. santaensis* belongs to subfamily Bucephalinae; this subfamily is poorly represented in GenBank with sequences of only 6% of species available. We have provided the first sequence of a Bucephalinae species from South America.

The morphology of the rhynchus has been largely used to assign species to Bucephalinae genera. However, Corner et al. (2020) suggested that it is unsuitable as a diagnostic character and underlined the necessity of sequencing more taxa and performing a systematic re-evaluation of morphology, ecology, and life cycles to resolve this problem.

Nolan et al. (2015) proposed that Bucephalinae members could be taxonomically grouped based on host distributions in freshwater or marine environments. Corner et al. (2020) argued that the transition between freshwater and marine hosts probably occurred at least twice or more. Despite the small number of freshwater Bucephalinae sequenced, our 28S phylogeny suggests the occurrence of at least four successful freshwater invasions represented by the following clades: clade B from East Asia and North America; *Prosorhynchoides* sp. (LC498576) from East Asia in hosts of the Plecoglossidae family; clade D2 with the clades comprising species from East Asia, Europe, and North America; and finally *R. santanaensis* in the clade D1b. It is noteworthy that *R. santanaensis* clusters together with other *Rhipidocotyle* spp. from Middle and North America, suggesting a common evolutionary history.

The subfamily Bucephalinae is likely to have undergone four evolutionary processes: first, diversification within the same host family; second, more than one successful infection of the same host family in different geographic regions; third, "jumping" between host families, and fourth, successful invasion of the freshwater environment (occurring in at least four different events in the subfamily). The first case was demonstrated with a well-supported clade containing *Prosorhynchoides* species from belonid fishes from Australia (Hammond et al. 2020). Our 28S phylogeny suggests a similar process for the North American species *B. cynoscion* (from clade D1b) and *P. caecorum* and *P. megacirrus* (from clade C) infecting Scienidae hosts. In the second case, belonid hosts were infected at least twice, i.e., by *Prosorhynchoides* in Oceania and South East Asia and by *R. transversalis* in North America (Hammond et al. 2020). In addition, Corner et al. (2020) proposed that sphyraenids were adopted as definitive hosts in at least two different occasions, i.e., by *Aenigmatrema* spp. in Oceania and by *P. longoviferus* in North America. According to our phylogeny, the third case involving "jumping" of parasites between host families may have occurred in the majority of the clades and in marine or freshwater environments worldwide. For example, in clade A, *P. scomberomorus* and *R. angusticolle* are parasites of the family Scombridae, while *P. longoviferus* "jumped" to hosts of the family Sphyraenidae. The transition from marine to freshwater environments may represent a fourth evolutionary process in the history of Bucephalinae. An example of this can be observed in our phylogeny (Fig. 2) by the freshwater

Table 2 Genetic divergence among Bucephalinae species estimated through uncorrected p-distances (in percentage) for the 28S rDNA gene. Variable sites including gaps are based on pairwise comparisons of 1022 sites

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36
1	*R. waeschenbachae*																																				
2	*P. moretonensis*	1																																			
3	*P. galaktionovi*	1	1																																		
4	*P. kohnae*	1	1	0																																	
5	*P. karvei*	1	1	0	0																																
6	*P. cutmorei*	1	1	1	1	1																															
7	*R. santanaensis*	3	3	3	3	3	3																														
8	*B. gorgon*	3	3	3	3	3	3	3																													
9	*P. paralichthydis*	3	3	3	3	3	3	3	3																												
10	*R. transversalis*	4	4	4	4	4	4	3	4	4																											
11	*R.* MK648267	4	4	4	4	4	4	3	5	4	5																										
12	*P. graciliescens*	3	3	3	3	4	4	4	4	4	5	5																									
13	*B. cynoscion*	4	4	5	5	5	5	5	5	5	5	6	3																								
14	*B. margaritae*	4	4	5	5	5	5	4	4	4	5	5	3	4																							
15	*B. varicus*	3	3	3	4	4	4	4	4	4	5	5	2	3	3																						
16	*P.* LC498576	4	4	4	5	5	5	4	5	6	6	6	4	5	6	5																					
17	*B. polymorphus*	4	4	4	4	4	4	4	4	5	5	6	4	5	6	4	4																				
18	*R. fennica*	5	5	5	5	5	5	5	6	5	6	6	6	7	7	6	6	4																			
19	*R. husi*	3	3	3	3	3	4	4	5	4	4	5	5	5	5	5	4	3	1																		
20	*R. campanula*	4	4	5	5	5	5	5	5	5	5	6	5	6	6	5	5	3	4	3																	
21	*R. tridecapapillata*	5	5	5	5	5	5	5	6	6	5	6	6	6	7	6	6	4	5	4	5																
22	*A. grandiovum*	4	4	4	4	4	4	5	5	5	5	6	4	6	6	4	4	4	5	4	5	5															
23	*A. undecimtentaculatum*	4	3	4	4	4	4	4	5	5	5	6	5	6	6	5	5	4	5	4	5	5	4														
24	*A. inopinatum*	4	4	4	4	4	4	5	5	5	5	6	5	6	6	5	5	4	5	4	4	5	4	1													
25	*B. skrjabini*	4	4	4	4	4	4	4	5	5	5	6	5	5	5	5	5	4	4	3	4	5	4	5	4												
26	*P. ovatus*	4	4	5	5	5	5	4	4	4	5	6	5	5	5	5	6	6	7	5	6	7	6	6	6	6											
27	*P. megacirrus*	6	6	6	6	6	6	5	6	6	7	7	6	7	7	6	5	6	7	6	6	7	5	7	6	6	7										
28	*P. caecorum*	6	6	6	6	6	6	6	7	7	7	7	6	7	8	6	5	6	7	6	6	7	6	6	6	6	6	2									
29	*R. galeata*	6	6	6	6	6	6	6	6	6	7	7	6	7	7	6	5	7	7	6	6	7	6	7	7	7	7	2	3								
30	*P. scomberomorus*	8	8	8	8	8	8	8	8	8	8	9	8	9	8	8	7	8	9	8	8	9	8	7	7	8	9	6	6	6							
31	*R. angusticolle*	7	8	8	8	8	8	7	8	8	8	9	8	9	9	8	7	8	9	8	8	9	8	8	8	8	9	6	6	7	5						
32	*P. longoviferus*	8	8	9	9	9	8	8	8	8	9	9	9	9	9	9	7	9	9	8	8	9	9	8	8	9	9	7	7	7	5	4					
33	*Para. parasiluri*	7	8	8	8	8	8	7	9	8	9	9	8	9	9	9	7	8	8	7	7	9	8	8	8	8	9	7	7	7	6	7	7				
34	*P. ozakii*	7	7	7	7	7	7	7	8	7	8	8	7	8	8	8	6	7	8	6	7	8	7	7	7	7	8	6	6	7	6	6	7	4			
35	*R. lepisostei*	8	8	8	9	8	9	8	9	8	9	10	8	9	9	9	7	9	9	8	8	9	8	8	9	9	9	7	7	7	7	8	8	5	5		
36	*P. apogonis*	7	7	8	8	8	8	8	8	8	8	8	8	9	9	8	8	7	8	7	8	7	7	6	7	7	8	9	9	10	10	11	10	10	10	11	
37	*P. hiodontis*	5	4	5	5	5	5	4	6	5	5	6	5	6	6	5	6	5	5	4	5	4	5	5	4	4	6	6	7	7	8	9	9	8	8	9	7

species present in clade B and base of clade D (*Prosorhynchoides* sp.), D1b (*R. santanaensis*), and D2.

Based on our results, we hypothesize that the ancestor of *R. santanaensis* entered the freshwater environment as a consequence of a "jumping" event from some unknown marine host family to the freshwater family Acestrorhynchidae. Evidence supporting this hypothesis is given by *R. transversalis*, which is located at the base of the clade that shares a common ancestor with *R. santanaensis* and was found on *Strongylura marina* Walbaum, a fish belonging to Belonidae. Moreover, Iberá wetland originated as a floodplain of the Paraná River and later became disconnected about 10,000 years ago (Úbeda et al. 2013). It is possible that a belonid ancestor could have come from the Parana River basin, which indeed is currently known to harbor belonid species such as *Potamorrhaphis eigenmanni* Miranda Ribeiro and *Pseudotylosurus angusticeps* Guenther (Mirande and Koerber 2020). An alternative hypothesis is that the distribution range of the ancestral host family of *R. santanaensis* no longer overlaps that of the Acestrorhynchidae family. In this case, it may not identifiable. Regardless of the exact ancestral host family, we hypothesize that Bucephalinae "jumped" to a new freshwater host species when a seawater ingression took place in South America during the Late Quaternary (Pereyra et al. 2004).

Our study highlights the importance of sequencing South American Bucephalinae species from marine and, especially, freshwater environments such as *R. gibsoni* (in *Acestrorhynchus lacustris* (Lütken)), *R. jeffersoni* (in *Cynopotamus humeralis* (Valenciennes), *Salminus brasili*ensis Cuvier and *Salminus hilarii* (Valenciennes), and specimens of *R. santanaensis* infecting *Ac. falcirostris* and *As. lacustris*. Additional molecular data will continue to shed light on the evolutionary relationships among taxa of this intriguing group of digeneans.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00436-023-07863-x.

Acknowledgements We are grateful to the authorities of Municipio Colonia Carlos Pellegrini, Corrientes Province, Argentina, for granting us the permits to carry out fieldwork, to the park rangers of the Iberá Provincial Reserve, to the people of Colonia C. Pellegrini for their kind assistance, and to CEPAVE for providing the facilities for this work. Finally, special thanks are due to Silvia Pietrokovsky and PhD Tyler J. Achatz for the revising our use of the English language in the manuscript.

Author contribution Martin M. Montes: Specimen collection, morphological and molecular study of parasites, phylogenetic analysis, principal writer of the manuscript.

Clara Vercellini: Secondary writer of the manuscript, text formatting.

Nicolas Ostoich: Morphological study of parasites.

Marina Ibañez Shimabukuro: Molecular study of parasites.

Gastón Cavallo: Parasite imaging and figure preparation.

German Reig Cardarella: Manuscript editing.

Sergio Martorelli: Obtaining funding, manuscript preparation supervision.

Funding This research was partially supported by the Agencia Nacional de Promoción Científica y Técnica (PICT 2020 SERIE A-01531), Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET, PIP 1713), and Universidad Nacional de La Plata (PPID N902 and N039).

Data availability Slides are deposited in the MLP under accessions MLP- He 7981. DNA sequences are deposited in GenBank under accessions OQ244080-81.

Declarations

Ethics approval The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national and institutional guides on the care and use of animals.

Consent to participate All the authors give their consent to participate in this work.

Consent for publication All the authors give their consent to the publication of this work.

Conflict of interest The authors declare no competing interests.

References

Atopkin D, Shedko M, Rozhkovan K, Nguyen H, Besprozvannykh V (2022) *Rhipidocotyle husi* n. sp. and three known species of Bucephalidae Poche, 1907 from the East Asian Region: morphological and molecular data. Parasitology 149:774–785. https://doi.org/10.1017/S0031182022000208

Baba T, Nakamura D, Hosoi M, Urabe M (2012) Molecular identification of larval Bucephalids, *Prosorhynchoides ozakii* and *Parabucephalopsis parasiluri*, infecting the golden mussel *Limnoperna fortunei*, by PCR-RFLP. J Parasitol 98:669–673. https://doi.org/10.1645/GE-2837.1

Bray RA, Webster BL, Bartoli P, Littlewood DTJ (2005) A molecular phylogenetic study of the Acanthocolpidae (Digenea). Acta Parasitol 50:281–291

Corner RD, Cribb TH, Cutmore SC (2020) A new genus of Bucephalidae Poche, 1907 (Trematoda: Digenea) for three new species infecting the yellowtail pike, *Sphyraena obtusata* Cuvier (Sphyraenidae), from Moreton Bay, Queensland, Australia. Syst Parasitol 97:455–476. https://doi.org/10.1007/s11230-020-09931-7

Fernandes BMM, Justo MCN, Anjos CSD, Malta JCO, Dumbo JC (2017) Digenea parasites of *Acestrorhynchus falcirostris* (Osteichthyes, Acestrorhynchidae) in the state of Amazonas, Brazil. Rev Bras Parasitol Vet 26:439–445. https://doi.org/10.1590/S1984-29612017059

Hammond MD, Cribb TH, Bott NJ (2018) Three new species of *Prosorhynchoides* (Digenea: Bucephalidae) from *Tylosurus gavialoides* (Belonidae) in Moreton Bay, Queensland, Australia. Parasitol Int 67:454–464. https://doi.org/10.1016/j.parint.2018.04.004

Hammond MD, Cribb TH, Nolan MJ, Bott NJ (2020) Two new species of *Prosorhynchoides* (Digenea: Bucephalidae) from *Tylosurus crocodilus* (Belonidae) from the Great Barrier Reef and French Polynesia. Parasitol Int 75:102005. https://doi.org/10.1016/j.parint.2019.102005

Katoh K, Rozewicki J, Yamada K (2019) MAFFT online service: multiple sequence alignment, interactive sequence choice and visualization. Brief Bioinform 20:1160–1166. https://doi.org/10.1093/bib/bbx108

Kohn A, Fernandes BM, Cohen SC (2007) South American trematodes parasites of fishes. Express Ltda, Sao Paulo

Kumar S, Stecher G, LiM KC, Tamura K (2018) Mega X: molecular evolutionary genetics analysis across computing platforms. Mol Biol Evol 35:1547–1549. https://doi.org/10.1093/molbev/msy096

Littlewood DTJ, Curini-Galletti M, Herniou EA (2000) The interrelationships of Proseriata (Platyhelminthes: Seriata) tested with molecules and morphology. Mol Phylogenet Evol 16:449–466. https://doi.org/10.1006/mpev.2000.0802

Lunaschi LI (2004) Two new species of bucephalids (Digenea: Bucephalidae) parasitic in freshwater fishes of Argentina. Parasitol Int 53:229–234. https://doi.org/10.1016/j.parint.2004.01.014

Mirande J, Koerber J (2020) Checklist of the freshwater fishes of Argentina. Ichthiol Contr Peces Criollos 72:1–81

Nolan MJ, Curran SS, Miller TL, Cutmore SC, Cantacessi C, Cribb TH (2015) *Dollfustrema durum* n. sp. and *Heterobucephalopsis perardua* n. sp. (Digenea: Bucephalidae) from the giant moray eel, *Gymnothorax javanicus* (Bleeker) (Anguilliformes: Muraenidae), and proposal of the Heterobucephalopsinae n. subfam. Parasitol Int 64:59–570. https://doi.org/10.1016/j.parint.2015.07.003

Olson PD, Cribb TH, Tkach VV, Bray RA, Littlewood DTJ (2003) Phylogeny and classification of the Digenea (Platyhelminthes: Trematoda). Int J Parasitol 33:733–755. https://doi.org/10.1016/S0020-7519(03)00049

Overstreet RM, Curran SS (2002) Superfamily Bucephaloidea Poche, 1907. In: Keys to the Trematoda: Volume 1. CABI Publishing, Wallingford UK, pp 67–110

Pedro NHO, Pelegrini LS, Azevedo RK, Abdallah VD (2016) First record of *Rhipidocotyle santanaensis* (Digenea) parasitizing *Acestrorhynchus lacustris* from Batalha River, Brazil. Braz J Biol 76:878–883. https://doi.org/10.1590/1519-6984.02515

Pereyra FX, Baumann V, Altinier V, Ferrer J, Tchilinguirian P (2004) Soil genesis and landscape evolution in the Parana River Delta. Rev Asoc Geol Arg 59:229–242. http://hdl.handle.net/20.500.12110/paper_00044822_v59_n2_p229_Pereyra. Accessed 9 Jul 2022

Pérez-Ponce de León G, Hernandez Mena DI (2019) Testing the higher-level phylogenetic classification of Digenea (Platyhelminthes, Trematoda) based on nuclear rDNA sequences before entering the age of the 'next-generation' Tree of Life. J Helminthol 93:260–276. https://doi.org/10.1017/S0022149X19000191

Petkevičiūtė R, Stunžėnas V, Staneviciute G (2014) Differentiation of European freshwater bucephalids (Digenea:Bucephalidae) based on karyotypes and DNA sequences. Syst Parasitol 87:199–212. https://doi.org/10.1007/s11230-013-9465-0

Posada D, Buckley TR (2004) Model selection and model averaging in phylogenetics: advantages of Akaike information criterion and Bayesian approaches over likelihood ratio tests. Syst Biol 53:793–808

Pritchard MH, Kruse GOW (1982) The collection and preservation of animal parasites. University of Nebraska Press, Nebraska, p 141

Ronquist F, Teslenkovan M, van der Mark P, Ayres DL, Darling A, Hohna S, Larget B, Liu L, Suchard MA, Huelsenbeck JP (2012) MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across large model space. Syst Biol 61:539–542. https://doi.org/10.1093/sysbio/sys029

Shirakashi S, Waki T, Ogawa K (2020) Bucephalid metacercarial infection in wild larval and juvenile Ayu *Plecoglossus altivelis*. Fish Pathol 54:93–100. https://doi.org/10.3147/jsfp.54.93

Stunžėnas V, Cryan JR, Molloy DP (2004) Comparison of rDNA sequences from colchicine treated and untreated sporocysts of *Phyllodistomum folium* and *Bucephalus polymorphus* (Digenea). Parasitol Int 53:223–228. https://doi.org/10.1016/j.parint.2003.12.003

Talavera G, Castresana J (2007) Improvement of phylogenies after removing divergent and ambiguously aligned blocks from protein sequence alignments. Syst Biol 56:564–577. https://doi.org/10.1080/10635150701472164

Tkach V, Littlewood D, Olson P, Kinsella J, Swiderski Z (2003) Molecular phylogenetic analysis of the Microphalloidea Ward, 1901 (Trematoda: Digenea). Syst Parasitol 56:1–15. https://doi.org/10.1023/A:1025546001611

Úbeda B, Di Giacomo AS, Neiff JJ, Loiselle SA, Guadalupe Poi AS, Gálvez JA, Casco S, Cózar A (2013) Potential Effects of Climate Change on the Water Level, Flora and Macro-fauna of a Large Neotropical Wetland. PLoS ONE 8:e67787. https://doi.org/10.1371/journal.pone.0067787

WoRMS (2022) Bucephalinae Poche, 1907. https://www.marinespecies.org/ aphia.php?p=taxdetails&id =579575 accessed 7 July 2022