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Molecular evidence for resurrection of *Plesiochorus elongatus* (Digenea: Gorgoderidae): an urinary bladder parasite of sea turtles

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Abstract

Trematodes of the genus *Plesiochorus* were recovered from the urinary bladder of a stranded female adult loggerhead sea turtle, *Caretta caretta*, on a beach in Rio de Janeiro State, Brazil. Morphological analysis of the specimens revealed characteristics resembling the sub-species *Plesiochorus cymbiformis elongatus* rather than the recently synonymised *Plesiochorus cymbiformis*. Molecular phylogenetic analysis of the ITS2 region also showed that *P. c. elongatus* was distinct from *P. cymbiformis* and related taxa. Further analysis of the ITS2 revealed substantial differentiation between *P. cymbiformis* from the USA and Brazil and the newly sequenced *P. c. elongatus* from Brazil, while a previously unspecified *Plesiochorus* sp. from the USA closely related to the novel Brazilian *P. c. elongatus* was reconciled as a USA isolate of *P. c. elongatus*. Based on both the morphological and molecular data it is suggested

that *P. c. elongatus* should be referred to as *Plesiochorus elongatus* and be considered as the second species in the genus.

Keywords: Digenea; Gorgoderidae; *Plesiochorus*, *Plesiochorus cymbiformis*; *Plesiochorus elongatus*

Introduction

The Gorgoderidae Looss, 1899 are a distinctive group of trematodes which have radiated among a wide range of aquatic vertebrate hosts including fish, amphibians and reptiles [1,2]. Those infecting chondricthyan fish are found in the body cavity and those found largely associated with urinary-bladder infections parasitize actinopterygians and tetrapods [1]. Morphologically the Gorgoderidae have a wide range of characters, including a non-spinous tegument, a highly restricted vitellarium but extensive uterus, and simple male terminal genitalia [1,2].

Recent molecular studies have illustrated the phylogenetic stability of the Gorgoderidae and confirmed the family to be formed of three distinct well supported subfamilies, namely the Degeneriinae Cutmore et al., 2013, the Anaporrhutinae Looss, 1901a and the Gorgoderinae Looss, 1899. However, all recent phylogenetic studies have highlighted the paucity of available data needed to disentangle the interrelationships between closely related species, especially those species that had historically been erected based solely on morphological characters before the advent of molecular systematics and phylogenetic analyses [2,3,4,5]. Cutmore et al. [2] highlighted this point showing that, based on phylogenetic reconstruction using ribosomal gene markers, the genera *Gorgodera* and *Xystretrum* were in fact nested within the diverse genus *Phyllodistomum*, illustrating that the application of molecular techniques is crucial to delineate genera.

The subfamily Anaporrhutinae, which is composed of seven genera including the turtle parasites within the genus *Plesiochorus*, has received little attention relative to the Gorgoderinae. *Plesiochorus* Looss 1901 was proposed by Looss [6] [type species *Plesiochorus cymbiformis* (Rudolphi, 1819) Looss, 1901, syn. *Distoma cymbiforme* Rudolphi 1819]. The parasite is cosmopolitan and is found infecting the urinary bladders of sea turtles worldwide [see 7]. Currently, *P. cymbiformis* is regarded as the only species within the genus. Pigulevski [8] proposed the subspecies *Plesiochorus cymbiformis elongatus* for some worms reported by Looss [9]. He appears to have misunderstood the host and collection data provided in Looss' paper and claimed that the worms were from a freshwater turtle from New Guinea Island. In fact, Looss' specimens were from loggerhead sea turtle (*Caretta caretta*) in the Mediterranean Sea. Pigulevski [8] apparently regarded characteristics such as longer body and internal organs as being sufficient to merit naming a new subspecies, but this was never confirmed by molecular analyses. Studies on freshwater gorgoderids [10,11] illustrated the use of ribosomal markers, particularly the internal transcribed spacer region (ITS), for disentangling true cryptic species which were impossible to differentiate on morphology alone. We have applied the same approach to the genus *Plesiochorus*. In this current study we report here molecular analysis of *Plesiochorus* species infecting loggerhead sea turtle on the east coast of Brazil, which most closely resemble *P. c. elongatus* morphologically. However, levels of molecular divergence between *Plesiochorus* isolates reveal two distinct species rather than subspecies, therefor the authors suggest that the Brazilian specimens of *P. c. elongatus* should now be referred to as *Plesiochorus elongatus*.

Materials and Methods

Parasite material

In March 2014, the carcass of a stranded female adult loggerhead sea turtle (94.2 cm curved carapace length) was found on "Praia do Sul" beach – São Francisco de Itabapoana City (21°29´04.80´´S and 41°03´36.36´´W) the State of Rio de Janeiro, Brazil. At post mortem examination the loggerhead sea turtle showed linear marks around the flippers and neck indicating interaction with fishing net. During the necropsy eight specimens of the *Plesiochorus* sp. were collected from the urinary bladder and fixed in 70% alcohol for morphological and molecular identification. Examples of the helminths (n=3) collected were deposited in the Helminthological Collection of the Biosciences Institute (CHIBB number 7454) São Paulo State University (UNESP), Botucatu, São Paulo.

Morphological analysis

For morphometric analysis the specimens were stained with carmine and cleared with eugenol. Data were determined with the aid of an image analysis program (ImageJ, National Institutes of Health) and drawings were made using a drawing tube. The identification keys of genera by Campbell [1], the original descriptions by Looss [6] and Pigulevski [8] and papers by Caballero y Caballero [12], Blair and Limpus [7] and Santoro and Morales [13] were used for the morphological and morphometric comparison.

DNA extraction, PCR and Sequencing

Total genomic DNA was extracted from three, of the eight, Brazilian *Plesiochorus* i following the manufacturer's specifications for the Qiagen DNeasy Blood and Tissue Kit. Due to the limited amount of comparable molecular data available for species within the genus *Plesiochorus*, only the second internal transcribed spacer region (ITS2) of the nuclear rDNA was sequenced as there were three available comparable sequences of *Plesiochorus* species available on GenBank, two representing *P. cymbiformis* from the USA (Acc: KC494054) and Brazil (Acc: KF578463) and the other representing an unspecified *Plesiochorus* species also

from the USA (KF013154). Unlike other studies on the identification of Gorgoderidae, which have favored the use of 28S rDNA to resolve interspecies relationships, there is currently only a single 28S rDNA sequence available for *Plesiochorus* which was not identified to species and cannot therefore be used as species reference sequence. Furthermore, the ITS region has been used extensively for platyhelminth molecular systematics due to high rates of evolutionary change, providing variation that can be used to infer the existence of cryptic species [2,14,15,16]. The complete ITS region was amplified using primers and cycling conditions described by Wang et al. [17] and PCR reactions were performed using 12.5 µl of DreamTaq^{TM} PCR master mix (2X DreamTaq buffer, 0.4 mM of each dNTP, 4mM $MgCl_2$) and 1-2 ng/µl DNA. The final reactions were made up to 25 µl with PCR-grade water and reactions took place on a Veriti 96 well thermal cycler (Applied Biosystems^{TM}). Using only 5 µl of the PCR product, the resultant amplicons were visualized on a 1% agarose gel stained with gel red (Bioline). The remaining 20 µl PCR products were sequenced at the Natural History Museum, London, using fluorescent dye terminator sequencing kits (Applied Biosystems^{TM}), then run on an Applied Biosystems 3730KL automated sequencer.

Alignments and phylogenetic analysis

Forward and reverse ITS sequences were visualised and assembled into contigs using Bioedit [18] and then subjected to BLASTn searches for initial identification performed against the GenBank sequence database housed at NCBI (http://www.ncbi.nlm.nih.gov/). This showed the novel ITS sequences generated in this study (Acc: MK577499-MK577501) to be most closely related to *Plesiochorus* ITS2 sequences represented by KC494054, KF013154 and KF578463 as described above. Phylogenetic analysis was performed on the obtained sequences using alignments with published ITS sequences from other Gorgoderidae. Species representing the three subfamilies were used to provide substantial diversity across the family to validate the true identification of the Brazilian *Plesiochorus sequences*. The analysis

included species from the genera *Nagmia*, *Staphylorchis* and *Anaporrhutum* as representatives of the Anaporrhutinae and species within the genera *Phyllodistomum*, *Gorgodera* and *Xystretrum* to represent the Gorgoderinae. As in Cutmore et al. [2] *Bunodera luciopercae* (Acc: FJ874917) and *Paracreptorematina limi* (Acc: HQ833706) were used as outgroups. DNA sequence alignments were performed using the MUSCLE sequence alignment tool (http://www.ebi.ac.uk) and the ends of the alignment were edited by eye using Bioedit [18] to ensure that the relative lengths of the sequences within the alignment were equal. Final curation of the alignment was performed using the Gblocks server (http://molevol.cmima.csic.es/castresana/Gblocks_server.html) [19] with default parameters for block selection to remove any alignment gaps and ambiguities that could have caused erroneous phylogenetic inferences during analysis. Phylogenetic analysis was performed on a final alignment of approximately 290bp containing the most phylogenetically informative characters and both maximum likelihood (ML) and maximum parsimony (MP) phylogenetic analysis were implemented in MEGA6 [20]. The Kimura 2-parameter model with gamma distribution of evolutionary rates (K2+G) was identified as the most suitable nucleotide substitution model by MEGA6 [20] based upon the lowest Bayesian information criterion scores relative to the other models tested, and was used to perform the ML phylogenetic reconstruction. The Subtree-Pruning-Regrafting (SPR) algorithm with a search level of 5 was used to perform the MP phylogenetic analysis. Subsequent trees in the MP analysis were obtained by random addition of sequences (10 replicates) from which a consensus tree was drafted. In both ML and MP reconstructions all positions containing gaps and missing data were eliminated from the analysis and nodal support values were estimated using 1000 bootstrap replicates.

A second alignment was constructed only containing ITS2 sequences from *Plesiochorus* in order to identify any species-specific nucleotide substitutions and to measure

divergence between sequences which could arise between distinct species. For clear species comparisons the alignment was edited based on the smallest available *Plesiochorus* ITS2 sequence producing a final alignment of 261bp. Using MEGA6 [20] the total number of substitutions was calculated between each sequence and uncorrected *p*-distance was also calculated as a measure of divergence between sequences also.

Results

Morphological description and morphometric analysis of the Brazilian *Plesiochorus* reveals the distinct species *Plesiochorus elongatus*

Description (Fig. 1, Table 1): Parasites with rounded posterior and anterior, without tegumental spines and constricted in ventral sucker region; anterior region elongated and more narrow than posterior region, posterior region of body completely filled by uterine loops containing numerous eggs; oral sucker subterminal; muscular pharynx present, rounded in shape; oesophagusshort and sinuous; caeca thin following body margin to end near posterior end of body where they are obscured by uterine loops; testes, two, large, opposite, irregular in shape and deeply lobed, in hind body posterior to level of ovary, occupy much to body width; ventral sucker large and rounded, located about one-third body length from anterior end; genital pore between caecal bifurcation and ventral sucker; vitelline follicles located after constriction of body, ventral to cecacum, with quite distinct lobes resembling "petals of a flower"; ovary at level of vitelline follicles, oval in shape, submedian, left; Mehlis' gland oval, between ovary and ventral sucker; uterus replete with eggs and filling posterior of body [a characteristic that distinguishes the Brazilian *Plesiochorus* as *P. elongatus* rather than *P. cymbiformis*(see Pigulevski, 1953], near the testicular region largely median, passing between testes then dorsal in body until genital pore.

Molecular phylogenetic analysis of the ITS2 fragments

Both the ML and MP analysis produced congruent phylogenies with high nodal support (bootstrap >50) with the MP analysis appearing to have the highest level of support with 12/14 nodes within the Gorgoderidae in group supported with bootstraps of 100 (Fig. 2). Although the overall tree topology was the same in the ML reconstruction only 11/14 nodes within the in-group were supported with bootstraps >50 of which only six had bootstrap values higher than 90 but none at 100. However, two distinct clades representing the subfamilies Anaporrhutinae and Gorgorderinae emerged with same interspecies relationships reported in previous ITS2 phylogenetic analysis by Cutmore et al. [2]. All three of the Brazilian *Plesiochorus* sequences fell within the Anaporrhutinae clade clustering most closely with other species of *Plesiochorus*. The shark parasites *Nagmia* sp. (KF013168), *Staphylorchis cymatodes* (HM486321) and *Anaporrhutum* sp. (KF013159) formed a monophyletic clade distinct from the *Plesiochorus* as reported previously [2]. In both ML and MP analysis sequences of *Plesiochorus* formed two clear sub-clades illustrating that *P. cymbiformis* and Brazilian *Plesiochorus* were in fact distinct sister taxa infecting the loggerhead sea turtle from the USA and Brazil. Sub-clade A contained the two *P. cymbiformis* ITS2 sequences from the USA (Acc: KC494954) and Brazil (Acc: KF578463) and sub-clade B appeared as a polytomy containing all the Brazilian *Plesiochorus* sequence generated in this study including the previously unspecified *Plesiochorus* species (KF013154) from the USA (Fig. 2).

Comparisons of ITS2 sequences between *Plesiochorus* species

When all six sequences representing species only from the genus *Plesiochorus* were analyzed, 14 polymorphic sites were identified at positions 67 – 69, 73, 135, 168, 174, 209, 221, 232,

234, 246, 249 and 254 of which all were considered to be parsimoniously informative except for mutations at 174 -. Nucleotide diversity within the genus was relatively high (π = 0.0281± 0.00855) within an average of 7.333 nucleotide differences. Variation within each sub-clade was extremely low with sub-clade A showing 1 nucleotide difference between the USA and Brazilian *P. cymbiformis* (nucleotide diversity of π = 0.00383± 0.00192). Low levels of variation were also seen between sequences within sub-clade B (nucleotide diversity of π = 0.00192± 0.00102). All three of the novel Brazilian *Plesiochorus* had identical ITS haplotypes but differed from the USA unspecified *Plesiochorus* species by a single nucleotide at position 246. The uncorrected *p*-distance also showed substantial differentiation between the USA and Brazilian *P. cymbiformis* and the novel *P. elongatus* sequences from Brazil with a 5-5.4% divergence (Table 2). Conversely, the unspecified *Plesiochorus* species from the USA (KF013154) only showed a 0.4 % divergence relative to the novel Brazilian *P. elongatus* sequences (Table 2).

Discussion

Plesiochorus cymbiformis elongatus was described by Pigulevsky [8] who analysed different morphological features of *P. cymbiformis* presented by Looss [9]. Unfortunately, the Pigulevsky` description includes apparent errors and lacks discussion. Pigulevsky [8] (see page 567) cites *P. c. elongatus* as collected from the bladder of *Carettochelys insculpta* (syn. *Thalassochelys corticata*), (i.e. a freshwater turtle found only in Australia and New Guinea), from New Guinea. An observation of the manuscript of Looss [9] [see p. 469, as quoted by Pigulevsky [8] the author describes the occurrence of *P. cymbiformis* in loggerhead sea turtle and green turtle (*Chelonia mydas*), implicating them as the apparent hosts of the parasites around the coast of Egypt. There is strong evidence that Pigulevisky [8] made a mistake in transcribing the data originally presented by Looss [9]. This information had previously been observed by Blair and Limpus [7] in a review of the genus.

Although only subtle differences in morphoetric data were found between *P. cymbiformis* and the Brazilian *Plesiochorus* (Table 1), the esophagus width and the ovary length were greater in the latter. However, when the morphometric data of only Brazilian *Plesiochorus* was compared with the specimens reported by Pigulevsky [8], all measurements were in the same range as those in Pigulevsky [8] except the Brazilian isolates from this study were slightly shorter in body length, which the authors interpret as typical of intra population variation. Despite the morphological similarities between *P. cymbiformis* and the Brazilian *Plesiochorus* the uterine loops in the latter are closer to the edge of the parasite than in specimens of *P. cymbiformis*, and it appears to have a more elongated body shape. These two characteristics were used to identify the Brazilian *Plesiochorus* originally as *P. c. elongatus* in the present study and are practically identical to the observations made by Pigulevsky [8]. However, it is important to note that there is no reference to the number of parasites reported by Pigulevsky [8], who only provided body length and width morphometrics records, as well as oral and ventral sucker diameter and eggs dimensions. Thus, the current study provides new detailed morphometric data which supports the creation of P. *elongatus* as a separate species, which is also supported by molecular phylogenetic analyses as described below. However, we agree that there is great body shape and morphological variation in the genus as observed by various authors [see 7, 9,12,13,21 and present study)] and that further study is needed between these two species.

The phylogenetic analysis of ITS2 resolved the *Plesiochorus* species as a monophyletic clade distinct from other anaporrhutine species which infect elasmobranchs as shown by Cutmore et al. [2]. The *Plesiochorus* clade contains two well supported sub-clades separating *P. cymbiformis* from the Brazilian *Plesiochorus* again supporting the occurrence of *P. elongatus* as a distinct species owing to the occurrence of two distinct evolutionary lineages within the genus. Initially, it was suspected that these two sub-clades represented potential geographical

variants of the same species as ITS2 has been shown to vary between populations of trematode species, with haplotypes found to be associated with specific geographical locations [22, 23,24,25]. However, the occurrence of genotypes from both the USA and Brazil in both sub-clade A and B supports strong separation of the two non-location specific lineages which appear to occur sympatrically. There was low variation within each sub-clade with KC49054 and KF578463 (sub-clade A) being identical, except for a single substitutional change at position at 174bp. Similarly, in sub-clade B, KF013154 was distinct from the novel sequences by a single base change at position 246. However, nucleotide divergence and the *p*-distance analysis between the sub-clades showed up to 5% divergence. Such divergence between ribosomal sequences falls within the range of those recorded between cryptic species of Gorgoderidae [10,26] and for other marine trematodes [25]. Therefore, based on the molecular evidence the authors conclude again that the Brazilian *Plesiochorus* is distinct from *P. cymbiformis* and not a sub species, and thus should be considered as the separate species *Plesiochorus elongatus*.

The current study illustrates the challenge of identifying closely related parasites that have poor morphological distinguishing features and the need to use molecular tools for accurate species identification and to provide insights into the evolution and radiation of such parasites. Both *P. elongatus* and *P. cymbiformis* do share hosts (Table 3) and are sympatric with genotypes of both species being found in loggerhead sea turtles from the USA and Brazil. Loggerhead sea turtles are found across the world and known to migrate great distances. There is genetic evidence of movement of loggerhead sea turtles from South East Asia, to South America and then up into the waters of North America [45]. The distribution of parasites is intimately linked to the movement of hosts [22, 23, 24]: given the global connectivity of loggerhead sea turtle populations, the occurrence of such sympatry in populations of *P. cymbiformis* and *P. elongatus* in the USA and Brazil is not surprising.

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Figure legends

Fig. 1 *Plesiochorus elongatus* Pigulevski 1953 (Digenea: Gorgoderidae) found in *Caretta caretta* Linnaeus 1758 (Testudines, Cheloniidae) from Brazil. (scale bar = 500 µm)

Fig. 2 The phylogenetic relationships between *Plesiochorus cymbiformis* and *Plesiochorus elongatus* in the context of other gorgoderid species. Both maximum likelihood (A) and maximum parsimony (B) analysis of the ITS2 fragment show the over all same topology separating *P. cymbiformis* and *P. c. elongatus* into separate sub-clades and distinguishing them from each other. Bootstrap values are used to illustrate nodal support but values <50 are not shown

Tables

Table 1. Morphometric data of the genus *Plesiochorus* (Digenea: Gorgoderidae) from marine turtles (Testudines: Chelonidae). Data are presented as range (mean only or Mean ± SD) and measurements are in millimeters

Table 2. Genetic distances between ITS2 sequences of *Plesiochorus* species from the USA and Brazil represented as uncorrected *p*-distances in the bottom left and numbers of

nucleotide substitutions between sequences in the top right. The shaded regions illustrate the major differences between *Plesiochorus cymbiformis* and *Plesiochorus elongatus*

Table 3. *Plesiochorus* species reported in sea turtles.

Table 1. Morphometric data of the genus *Plesiochorus* (Digenea: Gorgoderidae) from marine turtles (Testudines: Chelonidae). Data are presented as range (mean only or Mean ± SD) and measurements are in millimeters.

	Plesiochorus cymbiformis			*Plesiochorus elo*	
	Caballero (1954)	Blair & Limpus (1982)	Santoro & Morales (2007)	Pigulevsky, 1953	Pres
	C. mydas	*C. caretta*	*L. olivacea*	*C. caretta*	*C*
	Panamá	Australia	Costa Rica	New Guine	
	UB	UB	UB	UB	
es	3		31 (10 mens.)	?	8
	2.5–3.2	2.97–7.21 (5.47)	12.8–15.7(14±1.0)	12	6.8–7
	1.21–1.27	1.31–3.7 (2.2)	4.4–5.4 (5.1±0.2)	2.4	2.5–3
	0.13–0.24	0.41–0.86 (0.62)	0.84–1.07 (0.96 ± 0.86)	0.77*	0.66–0.8
	0.28–0.29	0.42–0.85 (0.64)	1.14–1.32 (1.24 ± 0.05)	-	0.74–0.8
	0.038–0.057	-	0.31–0.56 (0.44 ± 0.10)	-	0.21–0.
	0.038–0.046	-	-	-	0.08–0.1
	0.068–0.076	0.15–0.3 (0.21)	0.25–0.37 (0.32 ± 0.04)	-	0.23–0.2
	0.099–0.10	0.18–0.37 (0.25)	0.36–0.37 (0.37 ± 0.005)	-	0.26–0.
	0.076–0.114			-	0.09–0.1
gth	0.37–0.39	0.5–1.26 (0.95)	1.51–1.89 (1.72 ± 0.12)	1.3*	1.1–1
th	0.40–0.41	0.62–1.37 (0.95)	1.57–1.89 (1.73 ± 0.11)	-	1.0–1.
	0.27–0.28	0.48–1.52 (0.9)	2.39–3.46 (2.83 ± 0.37)	-	1.3–1.
	0.19–0.2	0.43–1.15 (0.7)	1.57–2.14 (1.93 ± 0.19)	-	1.0–1.
	0.26–0.33	0.48–1.52 (0.9)	2.4–3.4 (2.9 ± 0.31)	-	1.2–1.
	0.11–0.17	0.43–1.15 (0.7)	1.63–2.2 (1.95 ± 0.17)	-	1.0–1.
gth	0.2–0.28	–	–	-	

dth	0.038–0.057	–	–	-	
	0.11–0.14	0.23–0.37 (0.29)	0.44–0.63 (0.53 ± 0.07)	-	0.5–0.8
	0.084–0.087	0.21–0.37 (0.29)	0.37–0.69 (0.57 ± 0.09)	-	0.41–0.8
h	0.065–0.095	-	0.18–0.31 (0.28 ± 0.04)	-	0.24–0.4
n	0.095–0.152	-	0.18–0.37 (0.28 ± 0.05)	-	0.24–0.5
cles length	–	0.19–0.42 (0.32)	0.56–0.94 (0.79 ± 0.16)	-	0.45–0.
cles width	–	0.19–0.5 (0.28)	0.31–0.63 (0.50 ± 0.11)	-	0.3–0.7
es length	–	0.19–0.42 (0.32)	0.56–0.88 (0.73 ± 0.11)	-	0.35–0.8
es width	–	0.19–0.5 (0.28)	0.50–0.56 (0.57 ± 0.05)	-	0.48–0.7
	0.042–0.046	0.03–0.047 (0.038)	0.032–0.039 (0.034± 0.003)	0.038 – 0.04	0.076–0
	0.027–0.030	0.022–0.041 (0.031)	32	0.052 – 0.034	0.052–0.0
erior end	0.614–0.797	-	-	-	1.7–2.

Legend: *= diameter

Table 2. Genetic distances between ITS2 sequences of *Plesiochorus* species from the USA and Brazil represented as uncorrected *p*-distance in the bottom left and numbers of nucleotide substitutions between sequences in the top right. The shaded regions are

	KC494054 *Plesiochorus cymbiformis* USA	KF578463 *Plesiochorus cymbiformis* Brazil	KF013154 *Plesiochorus* sp. SCC-2013	*Plesiochorus cymbiformis elongatus* 1 Brazil	*Plesiochorus cymbiformis elongatus* 2 Brazil	*Plesiochorus cymbiformis elongatus* 3 Brazil
KC494054 *Plesiochorus cymbiformis* USA		1	12	13	13	13
KF578463 *Plesiochorus cymbiformis* Brazil	0.004		13	14	14	14
KF013154 *Plesiochorus sp*. SCC-2013	0.046	0.05		1	1	1
Plesiochorus cymbiformis elongatus 1 Brazil	0.05	0.054	0.004		0	0
Plesiochorus cymbiformis elongatus 2 Brazil	0.05	0.054	0.004	0.00		0
Plesiochorus cymbiformis elongatus 3 Brazil	0.05	0.054	0.004	0.00	0.00	

illustrate the major differences between *Plesiochorus cymbiformis* and *Plesiochorus c. elongatus*

Table 3. *Plesiochorus* species reported in sea turtles.

	Host	Locality	Reference
Plesiochorus cymbiformis	*Chelonia mydas*	Brazil	[27]
		Egypt	[6, 9, 28, 29]
		India	[21]
		Italy	[30]
		Panama	[12]
		USA	[31]
	Eretmochelys imbricata	India	[21]
		Puerto Rico	[32]
		USA	[31]
	Caretta caretta	Australia	[7]
		Brazil	[33]
		Egypt	[6, 9, 28, 29, 34]
		Spain	[35]
		Greece	[36]
		Italy	[37, 38, 39, 40]
		USA	[31, 41, 42]
	Lepidochelys olivacea	Costa Rica	[13]
		Japan	[43]
		Mexico	[44]
Plesiochorus elongatus	*Caretta caretta**	Brazil	Present report

	Egypt*	[8]

Legend: * see discussion

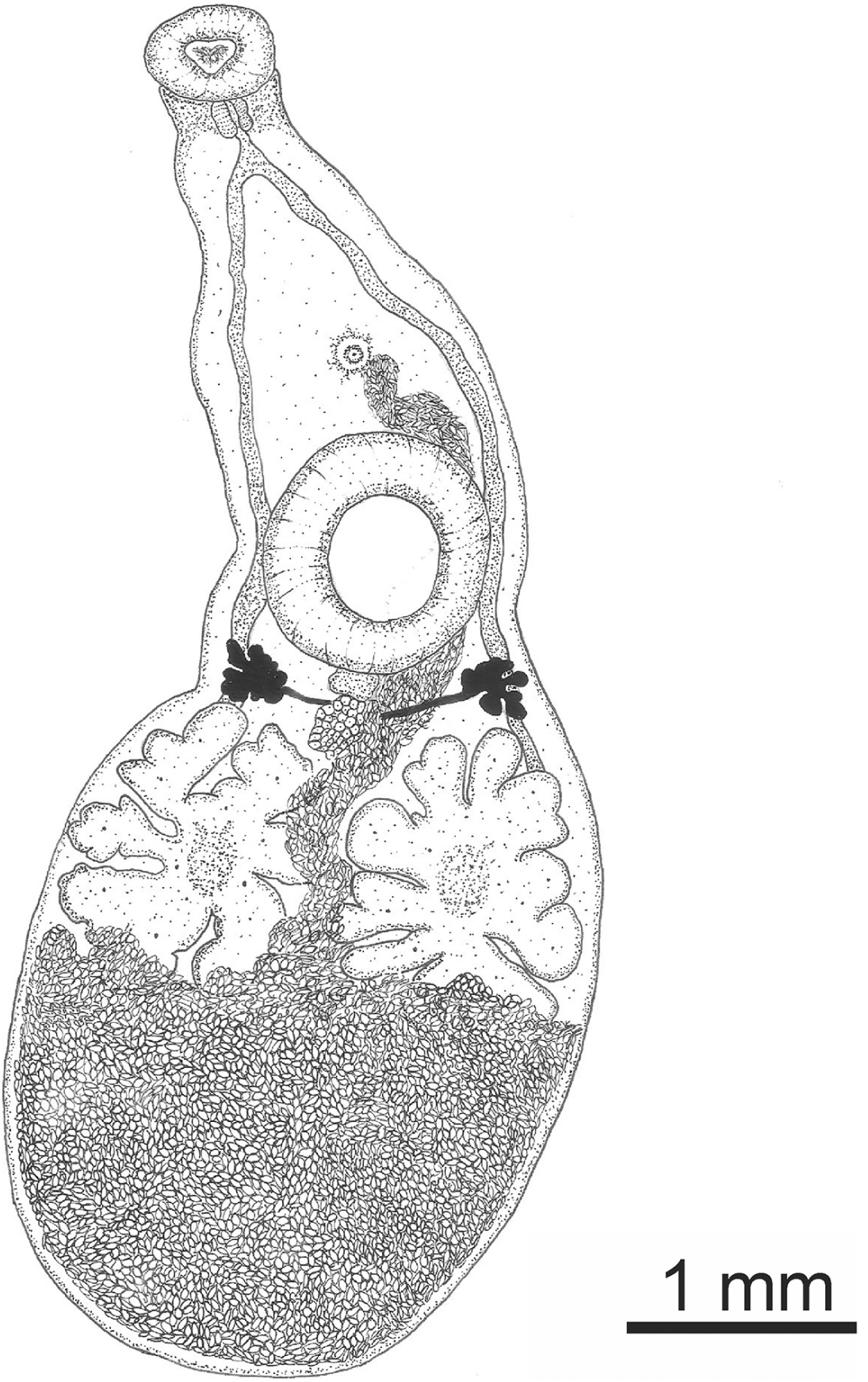

Figure 1

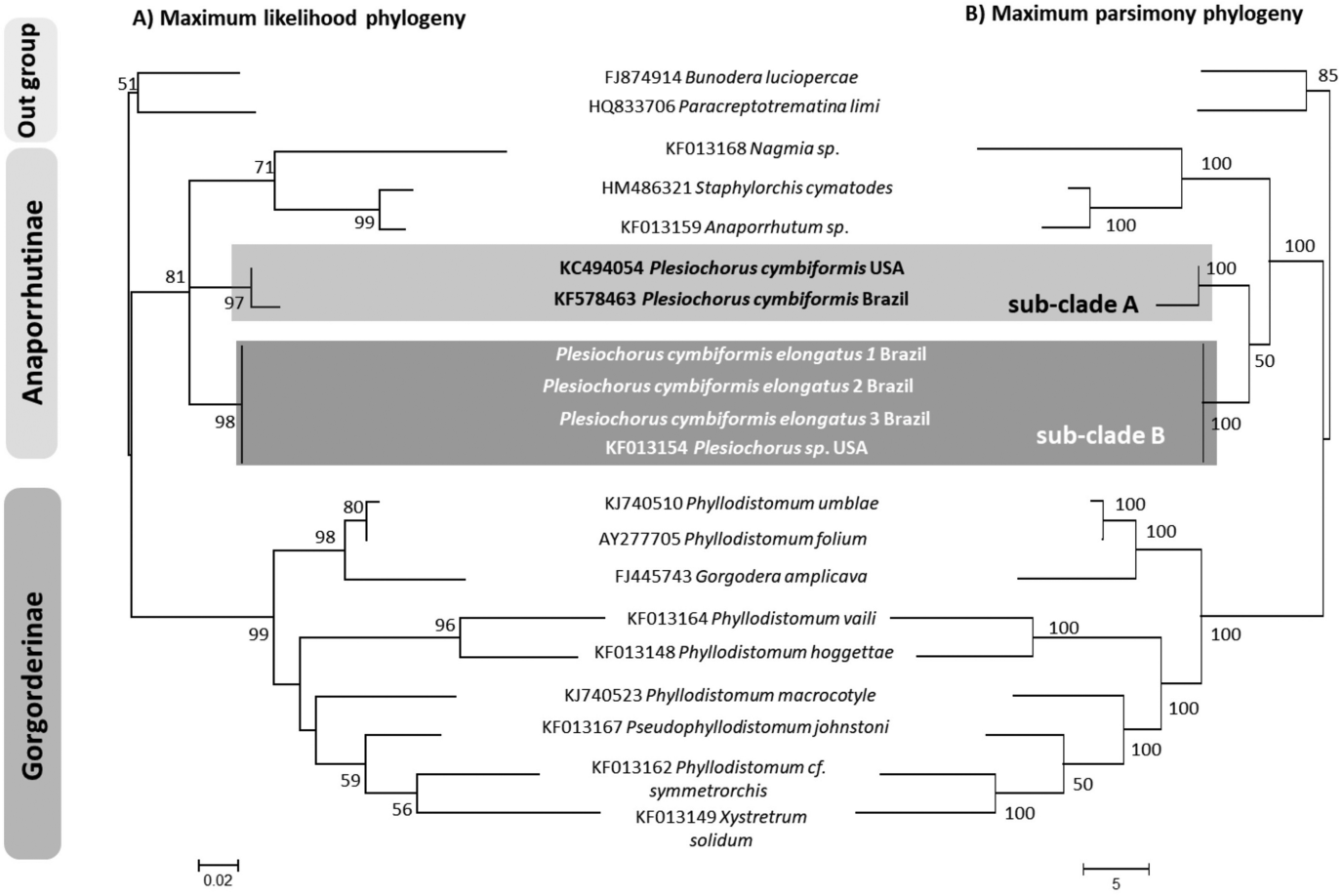
A) Maximum likelihood phylogeny
B) Maximum parsimony phylogeny
Out group
Anaporrhutinae
Gorgorderinae
FJ874914 Bunodera luciopercae
HQ833706 Paracreptotrematina limi
KF013168 Nagmia sp.
HM486321 Staphylorchis cymatodes
KF013159 Anaporrhutum sp.
KC494054 Plesiochorus cymbiformis USA
KF578463 Plesiochorus cymbiformis Brazil
sub-clade A
Plesiochorus cymbiformis elongatus 1 Brazil
Plesiochorus cymbiformis elongatus 2 Brazil
Plesiochorus cymbiformis elongatus 3 Brazil
KF013154 Plesiochorus sp. USA
sub-clade B
KJ740510 Phyllodistomum umblae
AY277705 Phyllodistomum folium
FJ445743 Gorgodera amplicava
KF013164 Phyllodistomum vaili
KF013148 Phyllodistomum hoggettae
KJ740523 Phyllodistomum macrocotyle
KF013167 Pseudophyllodistomum johnstoni
KF013162 Phyllodistomum cf. symmetrorchis
KF013149 Xystretrum solidum
0.02
5

Figure 2