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Two new species of *Phyllodistomum* Braun, 1899 (Digenea: Gorgoderidae), from freshwater fishes (Cyprinodontiformes: Goodeidae: Goodeinae) in central Mexico: An integrative taxonomy approach using morphology, ultrastructure and molecular phylogenetics

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Abstract

An integrative taxonomy approach is used to characterise the diversity of gorgoderid trematodes that parasitize freshwater fishes of the subfamily Goodeinae in central Mexico. Records of *Phyllodistomum* sp. and *Dendrorchis* sp. from the urinary bladder of goodeines have been previously published, although the identification at species level was not achieved. A few specimens were collected and fixed to conduct a scanning electron microscopy study, and to obtain sequences of a mitochondrial (COI) and nuclear (28S rRNA) gene, to be analysed in the context of the molecular phylogeny of gorgoderid trematodes. Based on the new findings, two new species of *Phyllodistomum* Braun, 1899 are described. *Phyllodistomum cribbi* **n. sp.** was found in *Zoogoneticus quitzeoensis* (Bean), *Allotoca zacapuensis* Meyer, Radda & Domínguez-Domínguez, *Hubbsina turneri* de Buen and *Z. purhepechus* Domínguez-Domínguez, Pérez-Rodríguez & Doadrio from Zacapu Lake, and La Luz Spring, in Michoacan, central Mexico. *Phyllodistomum wallacei* **n. sp.** parasitized *Xenotaenia resolanae* Turner, *Ilyodon furcidens* (Jordan & Gilbert), and *Allodontichthys tamazulae* Turner from the Cuzalapa, Ayuquila and Tamazula Rivers in Jalisco, western Mexico. These species are compared with several freshwater *Phyllodistomum* species from different areas of the world, especially a group of eight species that comprise a monophyletic clade in recent phylogenetic hypotheses of the Gorgoderidae Looss, 1899. The two new species are distinguished from other close relatives by the combination of morphological traits such as the body shape, sucker ratio, shape of the gonads, and extension of intestinal ceca. The new species are distinct in some ultrastructural characters of the body surface when compared with those species where scanning electron micrographs (SEM) and/or microphotographs are available. Data of two molecular markers (28S rRNA and COI genes) demonstrate that the two new species are distinct from each other and from those species of *Phyllodistomum* Braun, 1899 for which sequences are available.

Key words: 28S, COI, Cuzalapa River, Gorgoderidae, new species, *Phyllodistomum cribbi*, *Phyllodistomum wallacei*, scanning electron microscopy, species tree, Trematoda, *Xenotaenia resolanae*, Zacapu Lake, *Zoogoneticus quitzeoensis*

Introduction

Phyllodistomum Braun, 1899 is probably the most diverse genus within the Digenea, with more than 120 species, and with a worldwide distribution, containing parasites of amphibians, and both marine and freshwater fishes (Campbell 2008; Ho *et al*. 2014; Pérez-Ponce de León *et al*. 2015; Nakao 2015). In Mexico, nine nominal species of *Phyllodistomum* have been recorded thus far, four of them in marine or brackish water fishes, and five in freshwater fishes, including two undescribed cryptic species discovered in ictalurid catfishes (Mendoza-Garfias & Pérez-Ponce de León 2005; Rosas-Valdez *et al*. 2011; Razo-Mendivil *et al*. 2013; Pérez-Ponce de León *et al.* 2015). Even though the inventory of the helminth parasite fauna of Goodeinae, an endemic subfamily of freshwater fish cyprinodontiforms occurring in central Mexico (comprising 41 species) is thought to be complete (see

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Martínez-Aquino *et al.* 2014b), several published accounts reported the presence of specimens of gorgoderid trematodes inhabiting the urinary bladder of their goodeine hosts identified either as *Phyllodistomum* sp. (in Zacapu Lake and La Luz Spring, in the Lerma River Basin [Martínez-Aquino *et al*. 2011, 2012]) or as *Dendrorchis* sp. (in the Ayuquila, Tamazula and Cuzalapa River drainages, on the west coast of Mexico [Salgado-Maldonado *et al*. 2004; Martínez-Aquino *et al*. 2009, 2014b]). However, taxonomic identification at species level was not achieved, mainly because morphological traits were not useful to either allocate them into one of the recognized congeneric species or to establish a morphological distinction to describe them as new species. In the absence of a well-defined host-specificity pattern in a species-rich genus such as *Phyllodistomum*, morphology may not be enough to establish robust species delimitation criteria. Other sources of information are required to complete the task.

The recent finding of a few additional specimens in our sample that were preserved for SEM and molecular studies allowed us to study the ultrastructure of the body surface and to obtain sequences of two molecular markers. This integrative taxonomy study revealed that these few additional specimens represented two undescribed species of *Phyllodistomum*. We describe these two new species herein, and we provide microphotographs of the body surface through SEM. We also applied state-of-the-art phylogenetic tools on sequences of the COI and 28S rRNA genes to obtain robust species delimitation criteria and to establish the systematic position of both new species within the phylogeny of the Gorgoderidae Looss, 1899.

Material and methods

Specimens of *Phyllodistomum* used in this study were collected from the urinary bladder of *Xenotaenia resolanae* Turner in the Cuzalapa River and Tamazula River, in Jalisco State, Mexico (19°30'32.1"N, 104°17'45.6"W, 19°43'22.7"N, 103°12'08.5"W, respectively) in August 2008 and July 2010 (Martínez-Aquino *et al.* 2014b) (Fig. 1). Four specimens were sequenced and one specimen was used for SEM. Originally, these specimens had been reported as *Dendrorchis* sp. by Martínez-Aquino *et al*. (2009). Another set of specimens were collected from *Zoogoneticus quitzeoensis* (Bean) in Zacapu Lake, Michoacan, Mexico (19°49'35"N, 101°47'10"W). Five specimens were sequenced and one more was used for SEM. The specimens had been identified as *Phyllodistomum* sp. (Martínez-Aquino *et al*. 2012). Additional specimens deposited at the Colección Nacional de Helmintos (CNHE), Mexico City, were also observed since they were identified either as *Phyllodistomum* sp. or *Dendrorchis* sp.: *Dendrorchis* sp., ex *Ilydon furcidens* (Jordan & Gilbert), Ayuqila River (CNHE 4786) (Salgado-Maldonado *et al*. 2004); *Phyllodistomum* sp., ex *Zoogoneticus purhepechus* Domínguez-Domínguez, Pérez-Rodríguez & Doadrio, La Luz Spring (CNHE 7791) (Martínez-Aquino *et al*. 2011). Specimens had been originally fixed in either 4% hot formalin for SEM study or in 100% ethanol for molecular study. The description of the new species is thus based on specimens already deposited at the CNHE. Specimens were measured and drawings were made with the aid of a drawing tube attached to an Olympus BX51 microscope, and the measurements are presented in micrometers with the ranges followed by the means in parentheses. For the scanning electron microscopy study, specimens were dehydrated through a graded series of ethyl alcohol and then critical point dried with carbon dioxide. The specimens were mounted on metal stubs with silver paste, then coated with gold and examined in a Hitachi Stereoscan Model SU1510. Variable pressure SEM at 10 kV.

The protocols of the molecular biology laboratory, including DNA extraction, primers used in PCR, PCR reactions (amplification and sequencing), product purification, and sequencing, were realized following Razo-Mendivil *et al*. (2013). Sequences obtained in this study were edited using the platform Geneious Pro v5.1.7. (Drummond *et al.* 2010). All sequences, together with published representative outgroup sequences of Allocreadiidae Looss, 1902, Callodistomidae Odhner, 1910, Dicrocoeliidae Looss, 1899 and Encyclometridae Mehra, 1931 used previously by Cutmore *et al*. (2013), plus those included in this study, were aligned using an interface available in MAFFT v7 (Katoh & Standley 2013) within Geneiuos Pro, with a final edition by eye in the same platform (accession numbers in Appendices 1–2). The software JModelTest 2.1.3 (Darriba *et al.* 2012) was used to select evolution models through the Bayesian Information Criterion (BIC) (Schwarz 1978) for each data set separately (COI and 28S). The COI data set was partitioned into first-, second- and third-codon positions with the appropriate nucleotide substitution model implemented for each codon position (TrN+I for the first [Tamura & Nei 1993]; TPM3uf+I for the second [Kimura 1981]; and HKY+I for the third codon position [Hasegawa *et al.* 1985]).

The Vienna RNA Website (Lorenz *et al*. 2011) was used to detect ambiguously aligned hypervariable regions in the 28S data set according to a secondary structure model that were excluded from the analyses (see Gillespie *et al*. 2006; Ceccarelli & Zaldivar-Riveron 2013). Phylogenetic reconstruction was performed using Bayesian Inference (BI) with MrBayes 3.2.3 (Ronquist *et al.* 2012), applying the same parameters and those used previously by Martínez-Aquino *et al.* (2013). A combined dataset (COI+28S) was used to perform a multispecies coalescent analysis—species tree—in *BEAST v. 1.8.1 (Heled & Drummond 2010). All phylogenetic analyses were run through the CIPRES Science Gateway V. 3.3 [Miller *et al.* 2010].

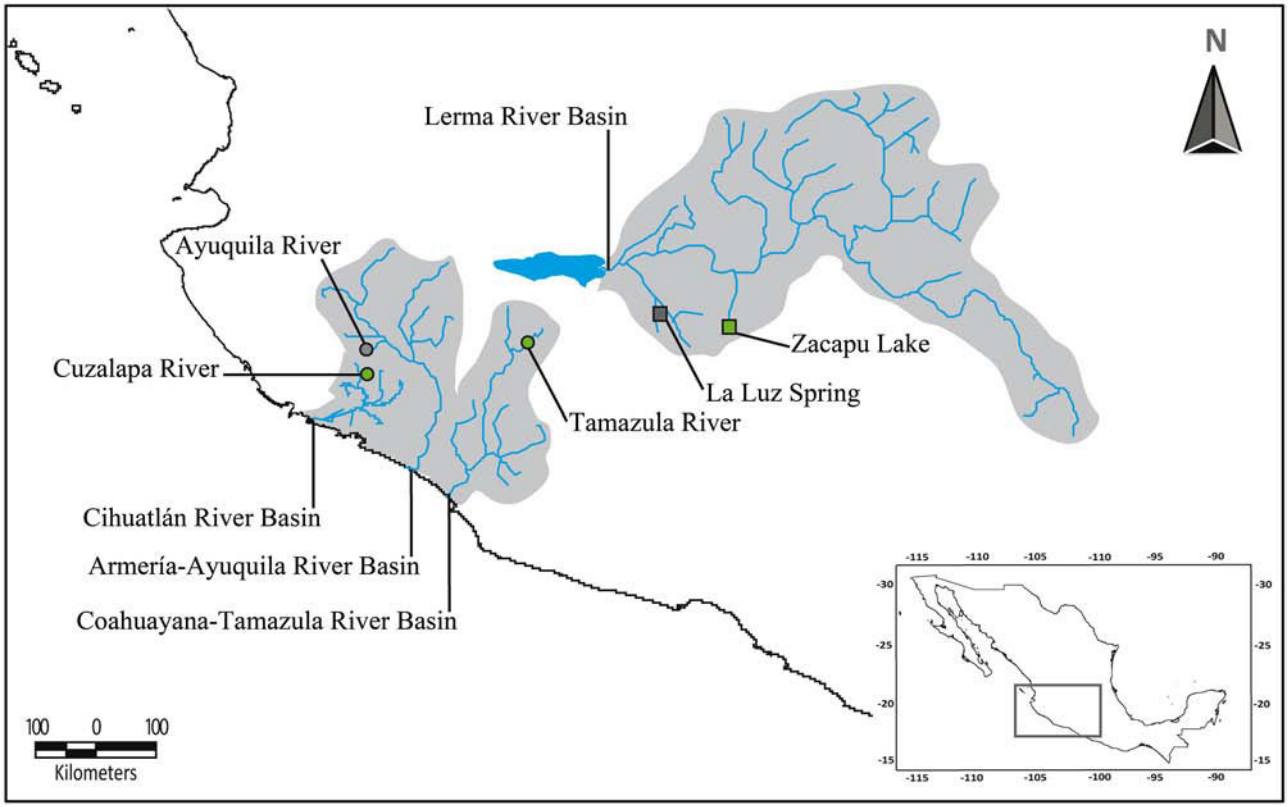


FIGURE 1. Hydrological systems and collection sites for *Phyllodistomum cribbi* **n. sp.** (square) and *P. wallacei* **n. sp.** (circles) in central Mexico. Full green circles and square correspond to localities where specimens were collected for molecular phylogenetic analyses; full grey circle and square indicate specimens identified either as *Dendrorchis* sp. or *Phyllodistomum* sp. from previous studies. These records were not included in the phylogenetic analyses in this study.

Results

Family Gorgoderidae Looss, 1899

Genus *Phyllodistomum* Braun, 1899

Phyllodistomum cribbi n. sp.

(Figs. 2–3, 6–9)

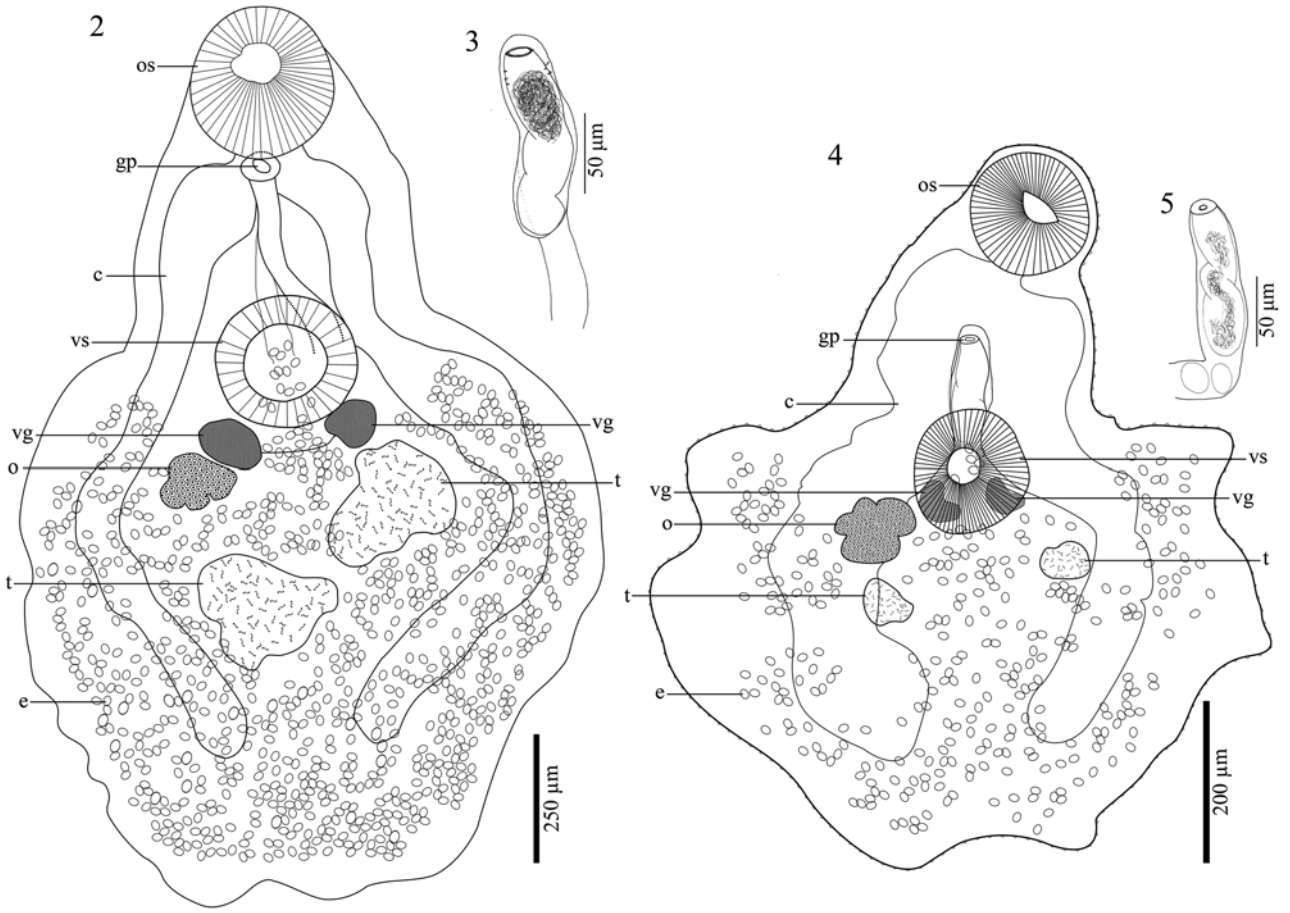
Synonym: *Phyllodistomum* sp. of Martínez-Aquino *et al*. (2012).

Type-Host. *Zoogoneticus quitzeoensis* (Bean); Goodeidae: Goodeinae; picotee goodeid.

Type-Locality. Zacapu Lake, Michoacan, Mexico (19°49’35”N, 101°47’10” W).

Other locality. La Luz Spring, Michoacan, Mexico (19º56’10.4”N, 102º17’57.84” W).

Other hosts. Goodeidae; Goodeinae; *Allotoca zacapuensis* Meyer, Radda & Domínguez-Domínguez; and highland splitfin *Hubbsina turneri* de Buen (Zacapu); Goodeidae; Goodeinae; La Luz splitfin *Zoogoneticus purhepechus* Domínguez-Domínguez, Pérez-Rodríguez & Doadrio (La Luz Spring).



FIGURES 2–5. Line drawings of the holotype of *P. cribbi* **n. sp.** and *P. wallacei* **n. sp.**, and detail of the male reproductive system of both species **2.** *Phyllodistomum cribbi* **n. sp.** from *Zoogoneticus quitzeoensis*, ventral view. **3.** Detail of the cirrus sac of *P. cribbi* **n. sp. 4.** *Phyllodistomum wallacei* **n. sp.** from *Ilyodon furcidens*, ventral view. **5.** Detail of the cirrus sac of *P. wallacei* **n. sp.** Symbols: os = oral sucker, gp = genital pore, c = cecum, vs = ventral sucker, vg = vitelline gland, o = ovary, t = testis, e = eggs .

Site of infection. Urinary bladder.

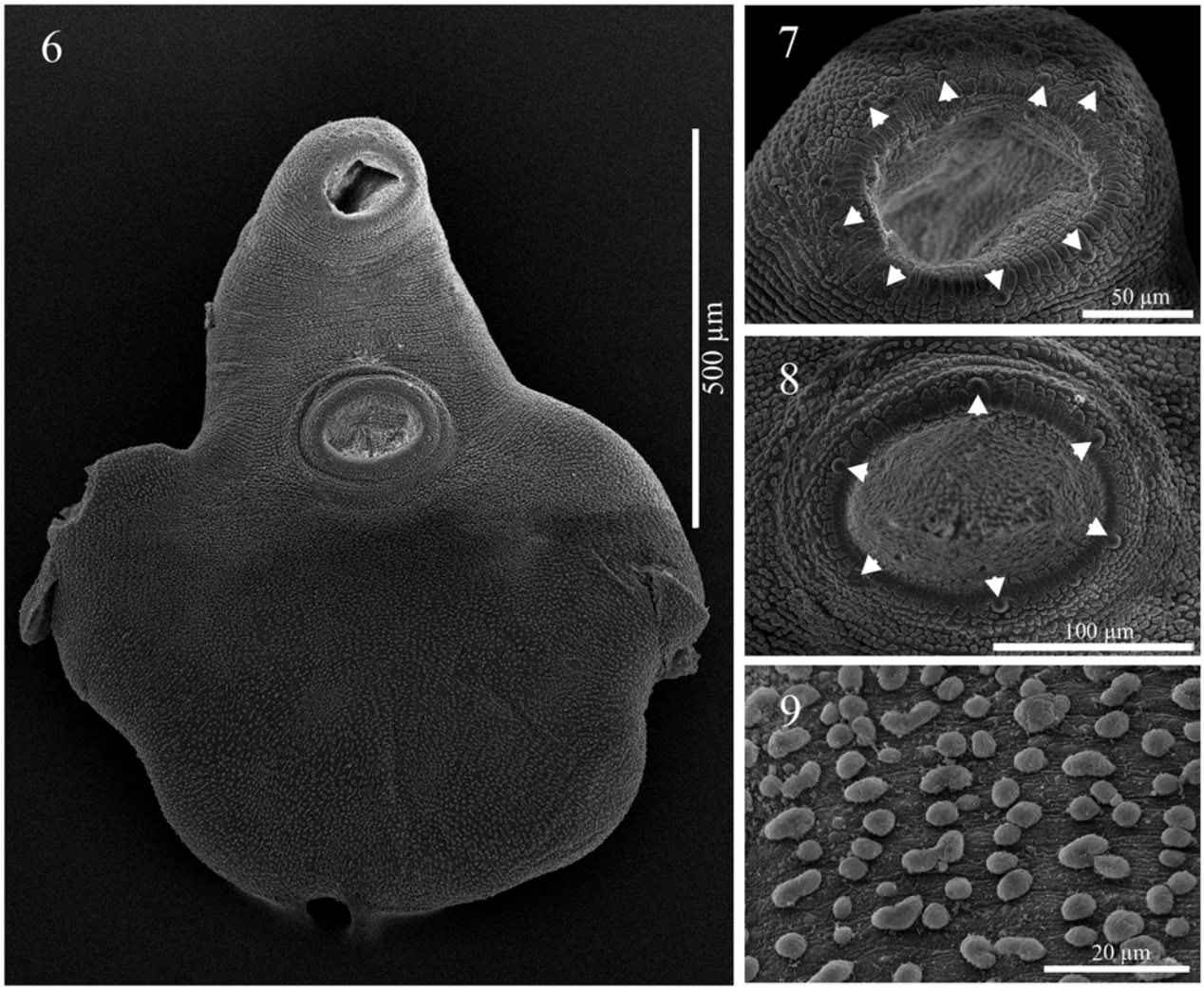
Specimens deposition. CNHE, Holotype **7815**, paratypes: 9798, 7813, 7814, 7791.

Genbank accession numbers. COI (KT376727- KT376731, ZACA Zqu1-5), 28S (KT376718 - KT376722, ZACA Zqu1-5

Etymology. The new species is named after Dr. Tom Cribb, University of Queensland, Australia, for his great contribution to our understanding of fish trematode diversity, and particularly his contribution to the species descriptions of *Phyllodistomum* species.

Description. Measurements are of 10 gravid specimens. Body spatuled, with lateral margins smooth, 926–1,841 (1,465) long, 477–1,224 (900) wide; maximum width at level of hindbody; body length/width 1.2–1.6 (1.5). Forebody relatively short, 423–665 (552) long, 323–590 (469) wide, representing 33–46% (39%) of total body length. Hindbody discoid, 237–1,201 (825) long, 477–1,224 (954) wide. Tegument with numerous tiny papillae. Oral sucker opening subterminally, rounded, 174–292 (239) long, 147–289 (239) wide, bearing eight well-developed papillae. Ventral sucker slightly wider than long, 145–252 (201) long, 149–277 (221) wide, bearing six well-developed papillae, one anterior, 4 lateral and 1 posterior. Oral sucker/ventral sucker length and width ratio 1:0.83–0.86 (1:0.84), 1:0.96–1.01 (1:0.92), respectively. Prepharynx and pharynx absent. Oesophagus very short (not visible in holotype), straight, 34–67 (55) long. Intestinal bifurcation 199–373 (296) from anterior end. Caeca wide, running laterally into the hindbody, ending blindly half the distance between testes and posterior end of body; right caecum 52–341 (220), left caecum 77–350 (221) from posterior end. Testes 2, entire to slightly lobed, oblique. Anterior testis 121–271(192) long, 96–267 (186) wide; posterior testis 80–300 (177) long, 104–283 (186) wide. Seminal vesicle saccular, relatively short. Pars prostatica not observed. Genital pore median, at level of caecal bifurcation, 262–439 (321) from anterior end. Ovary lobed (3–5 lobes), amphitypic, dextral in 6 specimens,

sinistral in 2, lateral to anterior testis, 63–179 (110) long, 121–159 (139) wide. Two compact vitelline glands, ovoid to almost round, entire, immediately posterior to ventral sucker; right vitelline gland 48–93 (69) long, 62–146 (114) wide, left vitelline gland 54–100 (79) long, 60–143 (103) wide. Laurer´s canal not observed. Uterus extensively coiled, occupying all hindbody, inter- and extracaecal. Eggs oval to slightly rounded, 18–37 (28) long, 13–26 (20) wide. Excretory vesicle not observed. Excretory pore subterminal.



FIGURES 6–9. Scanning electron microscopy of a specimen of *Phyllodistomum cribbi* **n. sp. 6.** Adult, ventral view. **7.** Oral sucker, showing 4 pairs of papillae. **8.** Ventral sucker, showing 3 pairs of papillae. **9.** Ventral surface of hindbody exhibiting papillae on the tegument.

***Phyllodistomum wallacei* n. sp.**
(Figs. 4–5, 10–13)

Synonym: *Dendrorchis* sp. of Martínez-Aquino *et al*. (2009, 2014b).

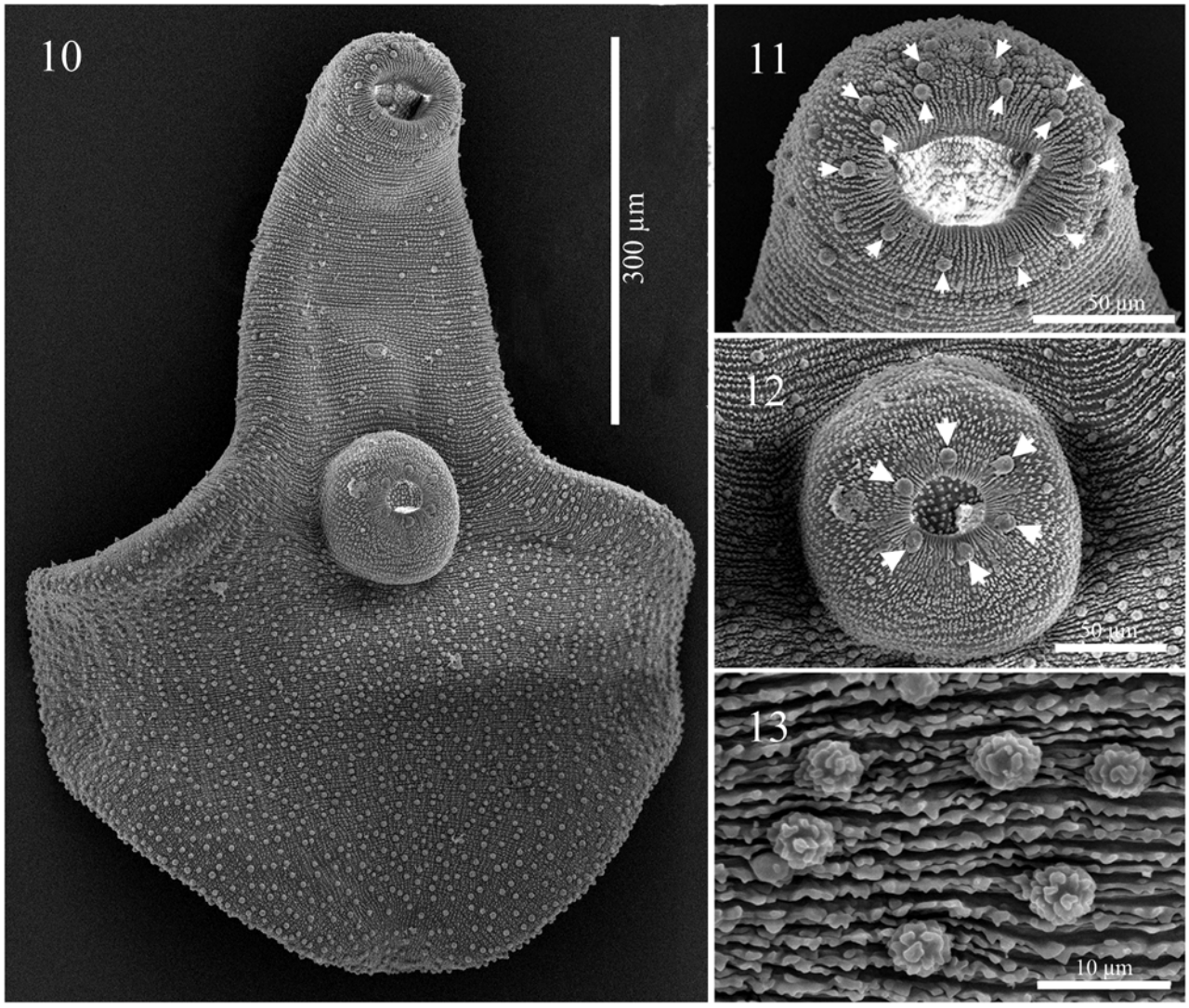
Type-Host. *Xenotaenia resolanae* Turner; Goodeidae: Goodeinae; leopard splitfin.

Type-Locality. Arroyo Durazno, Cuzalapa River, Jalisco, Mexico (19°30’32.1”N, 104°17’45.6”W).

Other localities. Tamazula River, Jalisco, Mexico (19°43’22.7”N, 103°12’08.5”W), Ayuquila River, Jalisco, Mexico (19°37’15”N, 104°12’10”W)

Other hosts. Goodeidae: Goodeinae; Tuxpan splitfin *Allodontichthys tamazulae* Turner (Tamazula); Goodeidae: Goodeinae; Goldbreast goodeid *Ilyodon furcidens* (Jordan & Gilbert) (Ayuquila).

Site of infection. Urinary bladder.



FIGURES 10–13. Scanning electron microscopy of a specimen of *Phyllodistomum wallacei* **n. sp. 10.** Adult, ventral view, with scattered dome-like papillae on hind- and forebody. **11.** Oral sucker, showing 7 pairs of papillae. **12.** Ventral sucker, showing 3 pairs of papillae. **13.** Dome-like papillae with small projections.

Specimens deposition. CNHE, Holotype **9799**, paratypes 6881, 4786, **9800**, **9801**.

Genbank accession numbers. COI (KT376723-KT376726, CUZA Xre1-2, TAMA Ata1-2), 28S (KT376714-KT376717, CUZA Xre1-2, TAMA Ata1-2)

Etymology. The new species is named after Alfred Russell Wallace, co-discoverer of the Theory of Natural Selection and founding father of biogeography.

Description. Measurements are of 7 specimens. Body spatuled to elongate, with lateral margins smooth, 712–1,619 (1,245) long, 445–1,222 (799) wide; maximum width at level of hindbody; body length/width 1.1–1.6 (1.5). Forebody relatively short, 355–650 (515) long, 187–506 (356) wide, representing 29–49% (39%) of total body length, bearing an irregular number of dome-like papillae on the ventral surface. Hindbody discoid to irregular, 554–1,223 (995) long, 488–1,045 (830) wide, bearing large number of dome-like papillae on the ventral surface. Oral sucker opening subterminally, rounded, 142–280 (194) long, 115–322 (198) wide, bearing 14 well-developed papillae, arranged in four pairs along the anterior edge, 1 pair in the middle, and 2 pairs along the posterior edge. Ventral sucker rounded, protuberant, 119–188 (160) long, 96–218 (165) wide, bearing six well-developed papillae, one anterior, 4 lateral and 1 posterior. Oral sucker/ventral sucker length and width ratio 1:0.67–0.84 (1:0.82), 1:0.67–0.84 (1:0.83), respectively. Prepharynx and pharynx absent. Oesophagus narrow, straight, 88–150 (119) long (not visible in the holotype). Intestinal bifurcation 271–349 (320) from anterior end. Caeca wide, running laterally into hindbody, ending blindly half distance between testes and posterior end of body; right caecum 131–

306 (232), left caecum 125–287 (218) from posterior end. Testes 2, lobed, oblique, smaller than ovary. Anterior testis 63–251 (124) long, 71–135 (105) wide; posterior testis 97–196 (134) long, 80–169 (112) wide. Seminal vesicle saccular, short. Pars prostatica not observed. Genital pore median, immediately post-bifurcal, 262–419 (334) from anterior end. Ovary deeply lobed (5 lobes), sinistral, lateral to anterior testis, usually larger than testes, 58–207 (135) long, 121–159 (139) wide. Two compact vitelline glands, bilobed, dorsal or immediately posterior to ventral sucker; right vitelline gland 36–115 (71) long, 38–133 (88) wide, left vitelline gland 35–116 (69) long, 39–130 (88) wide. Laurer´s canal not observed. Uterus extensively coiled, occupying all hindbody, inter- and extracaecal. Eggs oval to slightly rounded, 19–32 (25) long, 13–23 (18) wide. Excretory vesicle not observed. Excretory pore subterminal.

Molecular phylogenetic analysis

Appendices 1 and 2 show the phylogenetic trees resulting from 28S rRNA and COI data sets analysed separately. Both trees clearly show that the two new species represent independent lineages, and are part of a monophyletic group, with *P. cribbi* **n. sp.** as the sister taxon to a clade containing at least seven *Phyllodistomum* species, including the second new species, *P. wallacei* **n. sp.** which nests as the sister taxon of two recently described species i.e., *P. inecoli* Razo-Mendivil, Pérez-Ponce de León & Rubio-Godoy, 2013 and *P. spinopapillatum* Pérez-Ponce de León, Pinacho-Pinacho, Mendoza-Garfias & García-Varela, 2015 (Razo-Mendivil *et al*. 2013; Pérez-Ponce de León *et al*. 2015). The alignment of the 28S rRNA consists of 85 terminals for members of the Gorgoderidae (following the data provided by Cutmore *et al*. 2013, Razo-Mendivil *et al.* 2013 and Pérez-Ponce de León *et al.* 2015), plus another nine xiphidiatan trematodes, and three species of *Prosthenhystera* Travassos, 1922 that were used as outgroups (Appendix 1). The COI alignment consisted of a smaller number of terminals, since only a few species of gorgoderids have been sequenced for that molecular marker (i.e., Rosas-Valdez *et al.* 2011; Razo-Mendivil *et al.* 2013; Urabe *et al*. 2015). *Prosthenhystera* spp. were also used as an outgroup (Appendix 2). Irrespective of the number of taxa included in analyses, Bayesian inference of both molecular markers yielded the same interspecific relationships for the members of the Gorgoderidae herein considered. To corroborate the phylogenetic position and status as independent taxonomic operational units (OUT´s), a species tree analysis was conducted for the combined data matrix of both markers, and to establish more accurately the validity of the lineages that corresponded with separate species (Fig. 14). The species tree corroborates that *P. wallacei* **n. sp.** is the sister species of *P. inecoli* and *P. spinopapillatum*, while *P. cribbi* **n. sp.** is included in a clade along with *P. brevicecum* Steen, 1938 and *Gorgoderina* sp. which is a parasite of the urinary bladder of amphibians.

Discussion

Both new species conform to the diagnosis of the genus *Phyllodistomum* given by Campbell (2008); i.e., spatuled hindbody, blind caeca, and uterus strongly coiled extending throughout the hindbody but not into the forebody. Even though both species possess an amphitypic ovary, and were found parasitizing freshwater fishes within the Goodeinae, a subfamily of the Cyprinodontiformes endemic to river basins of Central and Northwestern Mexico (Domínguez-Domínguez *et al*. 2010), they can be readily distinguished from each other. *Phyllodistomum wallacei* **n. sp.** possesses dome-like papillae on the ventral surface of the fore- and hindbody, vitelline glands that are bilobed and testes that are smaller than the ovary. In contrast, *P. cribbi* **n. sp.** lacks dome-like papillae, possesses vitelline glands that are entire, and its ovary is smaller than the testes. Further, *P. cribbi* **n. sp.** is only found in La Luz Spring and Zacapu Lake, in the Lerma River basin, and it infects goodeines belonging to the tribes Chapalichthyni and Girardinichthyni, whereas *P. wallacei* **n. sp.** is found in the Ayuquila, Tamazula, and Cuzalapa River drainages in western Mexico, and it only parasitizes members of the tribe Ilyodontini (Domínguez-Domínguez *et al.* 2010). These two new species of *Phyllodistomum* follow a similar distribution pattern as that exhibited by the allocreadiid *Margotrema* Lamothe-Argumedo, 1970, whose only two species described thus far are found, one in the Cuzalapa River in western Mexico, and the other in the Lerma River Basin (Martínez-Aquino *et al.* 2014a), although both *Phyllodistomum* species are not closely related; actually, *P. cribbi* **n. sp.** is apparently more closely related to species from other continents than to *P. wallacei* **n. sp.** We have not an explanation about

the relationship of *P. cribbi* **n. sp.** with other group of species from various areas, but most likely it should be the result of incomplete sampling, since more sequences for species of *Phyllodistomum* are required. The latest description of a new species of *Phyllodistomum*, *P. kanae*, as a parasite of the Ezo salamander *Hynobius retardatus* Dunn in Japan (Nakao 2015) illustrates the problematic classification scheme for members of this speciose group of trematodes as revelaled by DNA studies. In the 28S rRNA phylogenetic tree of Nakao (2015) *P. kanae* is nested as the sister species of *P. magnifucum*, a species of freshwater fishes in Australia and New Zealand (Cribb 1987); likewise, in the COI tree, *P. kanae* is the sister taxa of *Gorgoderina* sp., a morphologically very distinct genera (with a long body and large ventral sucker), whose species are common parasites of amphibians (see Mata-López and León-Règanon, 2006).

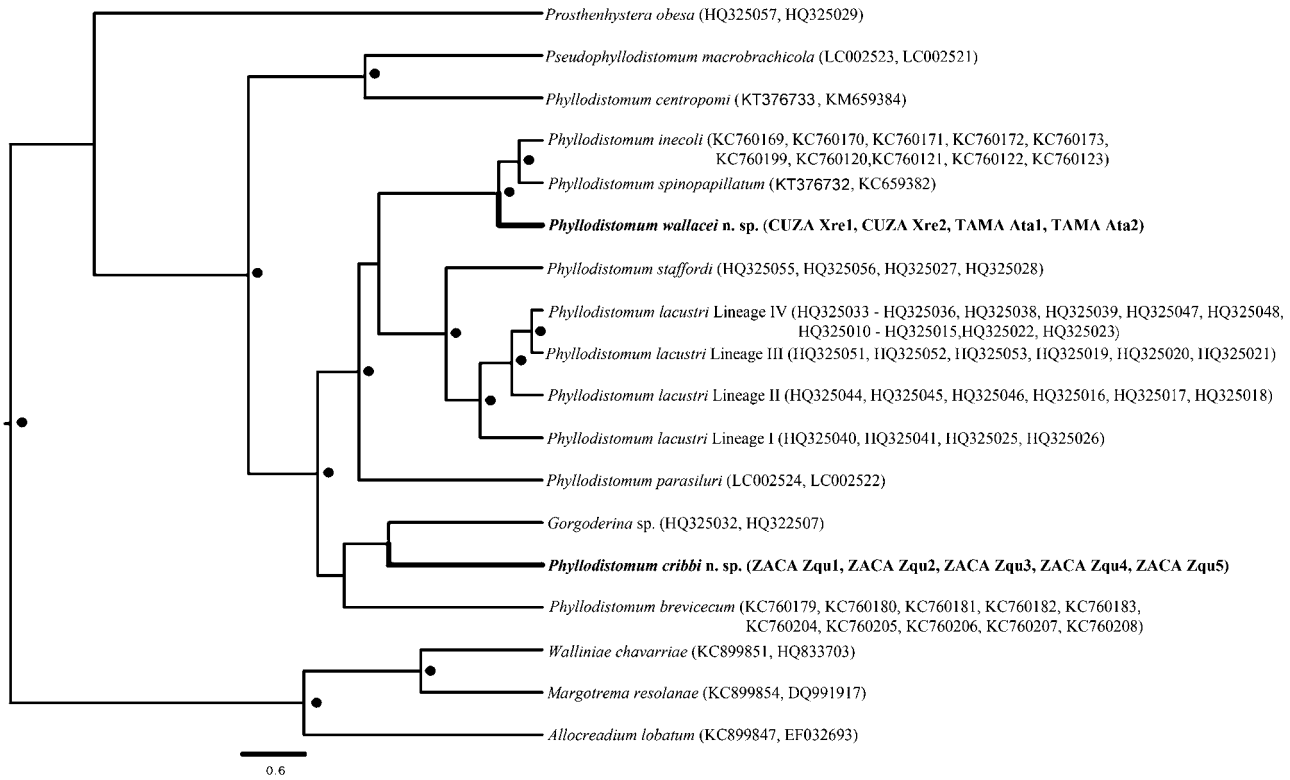


FIGURE 14. Coalescent-based phylogenetic tree obtained from the species tree analysis of the combined data set (COI+28S). The scale bar represents the number of nucleotide substitutions per site. Filled circles above/below branches represent Bayesian posterior probability ≥ 0.95. GenBank accession numbers of the new species are given in the taxonomic remarks section.

Likewise, the species *P. wallacei* **n. sp.** closely resembles *P. inecoli* and *P. spinopapillatum* sharing the same body shape and possessing a ventral surface with numerous dome-like papillae as evidenced through SEM. The papillae of the new species and *P. inecoli* are morphologically very similar, however they differ in the number of papillae on the hindbody (a larger number), and by having a different number and arrangement of entire dome-like papillae on the oral and ventral sucker. The new species possesses 14 instead of 18 papillae on the oral sucker, and only six instead of 16 papillae on the ventral sucker. Additionally, *P. wallacei* **n. sp.** is a parasite of goodeids to which it exhibits some level of host-specificity, meanwhile *P. inecoli* is a parasite of poeciliids (Razo-Mendivil *et al*. 2013; Pérez-Ponce de León *et al*. 2015). Further, the new species bears roughly the same number of dome-like papillae on the ventral surface of the hindbody as in *P. spinopapillatum*, but the shape of these papillae is very different; they have a multilobed structure, but not spinulated as in *P. spinopapillatum*. Both species further differ in the number of entire dome-like papillae on the oral sucker (14 instead of 8) and ventral sucker (6 instead of 18), and in the fact that *P. spinopapillatum* is exclusively found in killifishes of the genus *Profundulus* Hubbs and not in goodeids. Interestingly, goodeids and profundulids are sister taxa (Domínguez-Domínguez *et al*. 2010). This points to a possible host-specificity pattern that resulted from a common evolutionary history. The aforementioned three species, along with *P. funduli* Helt, Janovy & Ubelaker, 2003, a species described from the plains topminnow, *Fundulus sciadicus* Cope, in Nebraska, U.S.A. (Helt *et al*. 2003) for which no DNA sequences are available thus

far, all parasitise members of the Order Cyprinodontiformes. Even though the distributional range of the families to which these hosts belong (Goodeidae Jordan, Profundulidae Hoedeman & Bronner, Fundulidae Jordan & Gilbert, Poeciliidae Garman) mostly do not overlap (Miller *et al*. 2005), they harbour their own but related species of *Phyllodistomum*, indicating a historical association with their hosts.

Phyllodistomum cribbi **n. sp.** is morphologically very similar to *P. brevicecum*, but it can be readily distinguished by the lack of cephalic glands, a uterus occupying the entire hindbody and caeca extending half the distance between the posterior testis and the posterior end of the body. In addition, apparently *P. brevicecum* lacks dome-like papillae on the ventral surface, although no SEM studies have been conducted on this species when reported in North American freshwater fishes (see Fischthal 1947; 1950; Peckham & Dineen 1957; Hoffman 1999). Additionally, *P. brevicecum* occurs as a parasite of the urinary bladder of the central mudminnow, *Umbra limi* (Kirtland), a member of the family Esocidae (Cuvier), in Indiana, U.S.A. (Steen 1938), whereas the new species known only in goodeines in La Luz Spring and Zacapu Lake, Michoacan, central Mexico.

The results we present in this paper corroborate the contention that the use of sequence data is essential to properly describe parasite diversity within the context of an integrative taxonomic approach (Pérez-Ponce de León & Choudhury 2010). Apparently, the species richness within the genus *Phyllodistomum* is underestimated and the continuous use of DNA will demonstrate the need to keep describing new species (e.g. Ho *et al*. 2014; Pérez-Ponce de León *et al* 2015; Nakao 2015). Cribb (1987) discussed the difficulties to proper identification among *Phyllodistomum* species because of the great intraspecific morphological variation in many species and numerous inadequate morphological descriptions. The advancements in the use of molecular tools in the study of digenean taxonomy over the last decade demonstrate that we need to take a closer look at methods used to describe parasite diversity. Following the contention by Cribb (1987), the case of *Phyllodistomum* will present a challenge because it represents one of the most speciose genera within the Digenea, and it seems that in the following years, we will witness the description of new congeneric species and even the recognition of cryptic species in what once was thought to be a single species (see Rosas-Valdez *et al*. 2011 for the case of *P. lacustri* [Loewen, 1929]). The major challenge is to accomplish the proper description of the new species making use of the available morphological characters and overcome the difficulties expressed by Cribb (1987).

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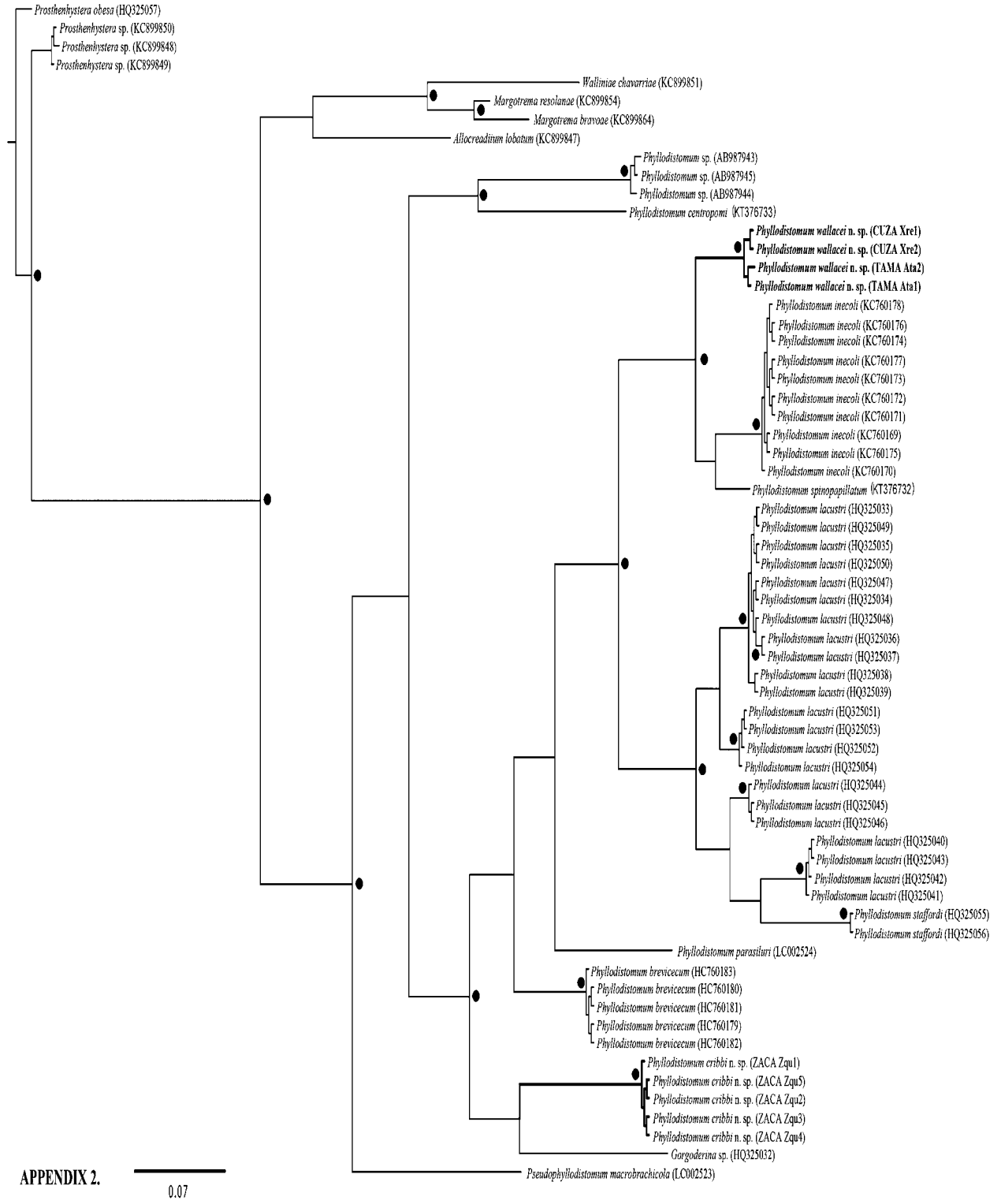
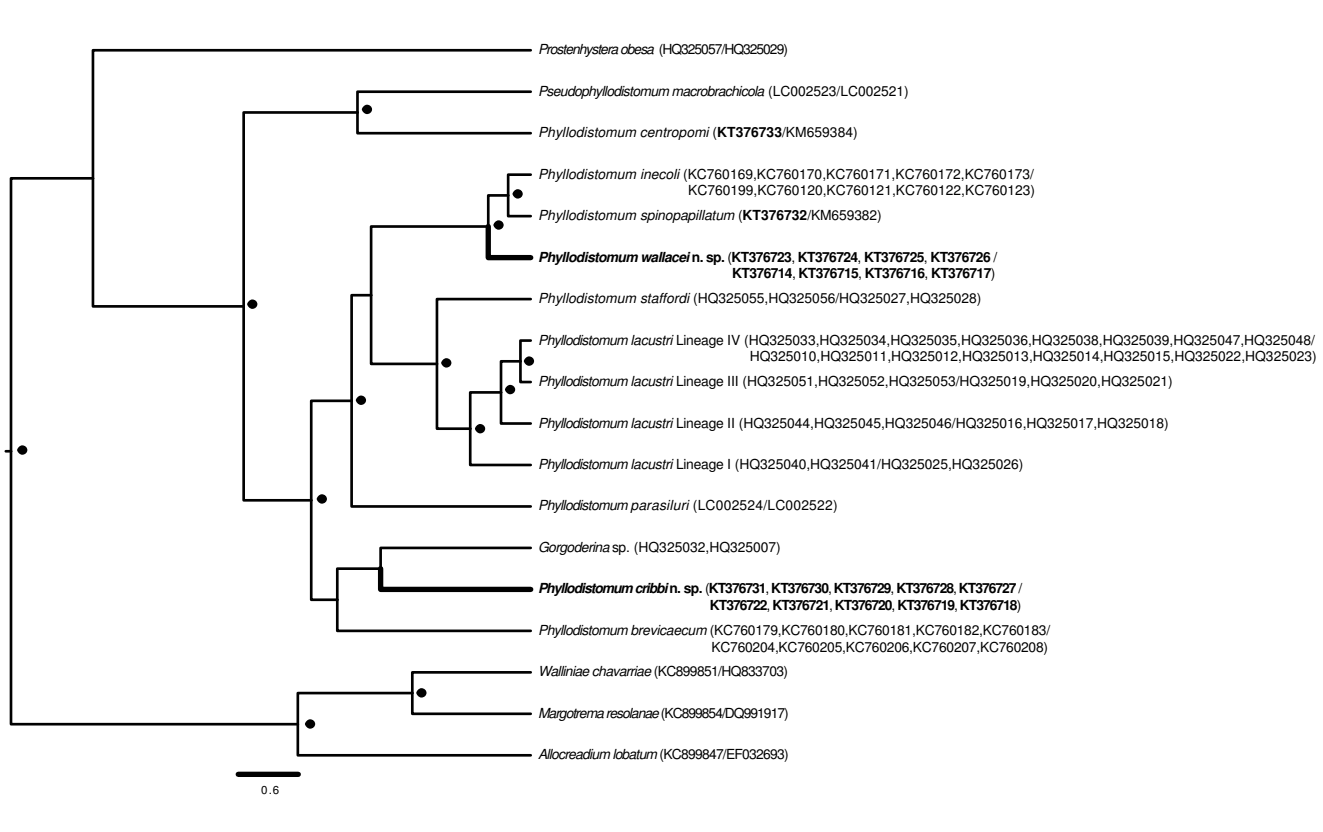
APPENDIX 1.

APPENDIX 1. Phylogenetic tree obtained through Bayesian inference for the 28S rRNA dataset. Filled circles above/below branches represent Bayesian posterior probability ≥ 0.95.

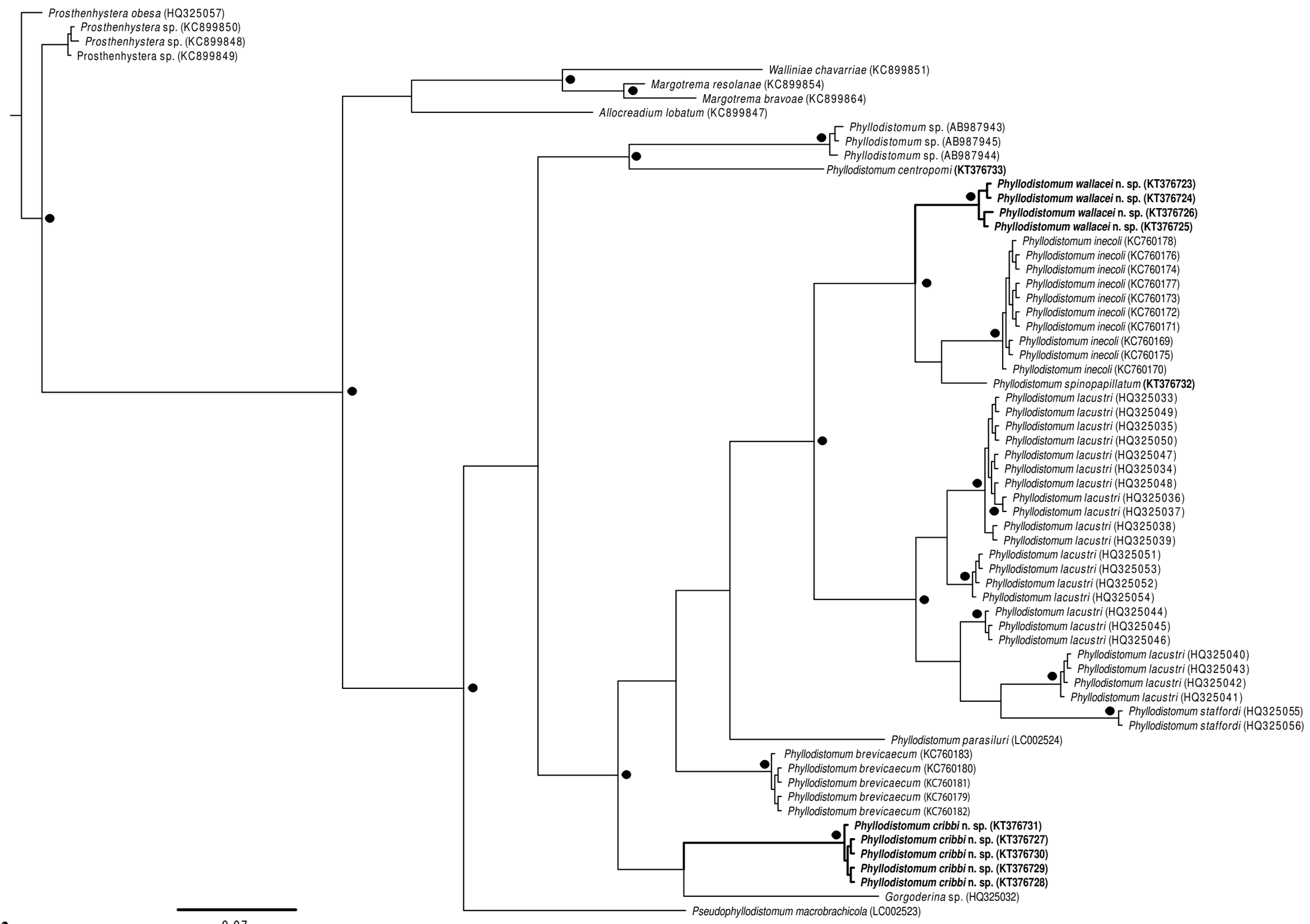


APPENDIX 2. Phylogenetic tree obtained through Bayesian inference for the COI dataset. Filled circles above/below branches represent Bayesian posterior probability ≥ 0.95.


Prostenhystera obesa (HQ325057/HQ325029)
Pseudophyllodistomum macrobrachicola (LC002523/LC002521)
Phyllodistomum centropomi (**KT376733**/KM659384)
Phyllodistomum inecoli (KC760169,KC760170,KC760171,KC760172,KC760173/ KC760199,KC760120,KC760121,KC760122,KC760123)
Phyllodistomum spinopapillatum (**KT376732**/KM659382)
***Phyllodistomum wallacei* n. sp.** (**KT376723**, **KT376724**, **KT376725**, **KT376726** / **KT376714**, **KT376715**, **KT376716**, **KT376717**)
Phyllodistomum staffordi (HQ325055,HQ325056/HQ325027,HQ325028)
Phyllodistomum lacustri Lineage IV (HQ325033,HQ325034,HQ325035,HQ325036,HQ325038,HQ325039,HQ325047,HQ325048/ HQ325010,HQ325011,HQ325012,HQ325013,HQ325014,HQ325015,HQ325022,HQ325023)
Phyllodistomum lacustri Lineage III (HQ325051,HQ325052,HQ325053/HQ325019,HQ325020,HQ325021)
Phyllodistomum lacustri Lineage II (HQ325044,HQ325045,HQ325046/HQ325016,HQ325017,HQ325018)
Phyllodistomum lacustri Lineage I (HQ325040,HQ325041/HQ325025,HQ325026)
Phyllodistomum parasiluri (LC002524/LC002522)
Gorgoderina sp. (HQ325032,HQ325007)
***Phyllodistomum cribbi* n. sp.** (**KT376731**, **KT376730**, **KT376729**, **KT376728**, **KT376727** / **KT376722**, **KT376721**, **KT376720**, **KT376719**, **KT376718**)
Phyllodistomum brevicaecum (KC760179,KC760180,KC760181,KC760182,KC760183/ KC760204,KC760205,KC760206,KC760207,KC760208)
Walliniae chavarriae (KC899851/HQ833703)
Margotrema resolanae (KC899854/DQ991917)
Allocreadium lobatum (KC899847/EF032693)
0.6


Prosthenhystera oonastica (KM871182)
Prosthenhystera obesa (HQ325029)
Prosthenhystera caballeroi (KM871183)
Allocreadium lobatum (EF032693)
Walliniae chavarriae (HQ833703)
Margotrema bravoae (DQ991917)
Paracreptotrematina limi (HQ833706)
Encyclometra colubrimurorum (AF184254)
Lyperosomum collurionis (AY222259)
Dicrocoelium sp. (AY222261)
Brachylecithum lobatum (AY222260)
Degeneria halosauri (AY222257)
Nagmia floridensis (EF032691)
Nagmia floridensis (AY222262)
Nagmia sp. (KF013194)
Nagmia sp. (KF013192)
Plesiochorus sp. (KF013180)
Anaporrhutum sp. (KF013184)
Staphylorchis cymatodes (HM486318)
Staphylorchis cymatodes (HM486319)
Staphylorchis cymatodes (HM486320)
Asexual stages ex. L. castrensis (KF013193)
Asexual stages ex. L. castrensis (KF013172)
Phyllodistomum sp. 1 (KF013191)
Phyllodistomum sp. 2 (F013190)
Phyllodistomum sp. 3 (KF013187)
Phyllodistomum sp. 3 (KF013173)
Phyllodistomum sp. 4 (KF013179)
Phyllodistomum sp. 4 (KF013183)
Phyllodistomum sp. 5 (KF013175)
Phyllodistomum sp. 5 (KF013181)
Phyllodistomum cf. symmetrorchis (KF013171)
Phyllodistomum cf. symmetrorchis (KF013174)
Phyllodistomum centropomi (KM659384)
Pseudophyllodistomum johnstoni (KF013182)
Pseudophyllodistomum johnstoni (KF013177)
Phyllodistomum sp. (KF013180)
Phyllodistomum folium (AF533015)
Phyllodistomum folium (AY281127)
Phyllodistomum folium (AY288823)
Phyllodistomum folium (AY288828)
Xystretrum solidum (KF013188)
Xystretrum solidum (HQ325031)
Xystretrum solidum (HQ325030)
Xistretum sp. (AY222263)
Xystretrum sp. 2 (KF013178)
Xystretrum sp. 2 (KF013185)
Xystretrum sp. 1 (KF013176)
Phyllodistomum wallacei n. sp. (KT376717)
Phyllodistomum wallacei n. sp. (KT376716)
Phyllodistomum wallacei n. sp. (KT376715)
Phyllodistomum wallacei n. sp. (KT376714)
Phyllodistomum inecoli (KC760199)
Phyllodistomum inecoli (KM659383)
Phyllodistomum inecoli (KC760123)
Phyllodistomum inecoli (KM659389)
Phyllodistomum inecoli (KC760122)
Phyllodistomum inecoli (KM659380)
Phyllodistomum inecoli (KM659386)
Phyllodistomum inecoli (KM659387)
Phyllodistomum inecoli (KC760120)
Phyllodistomum inecoli (KC760121)
Phyllodistomum inecoli (KM659385)
Phyllodistomum spinopapillatum (KM659388)
Phyllodistomum spinopapillatum (KM659379)
Phyllodistomum spinopapillatum (KM659382)
Phyllodistomum spinopapillatum (KM659381)
Phyllodistomum lacustri (HQ325010)
Phyllodistomum lacustri (HQ325013)
Phyllodistomum lacustri (HQ325011)
Phyllodistomum lacustri (HQ325024)
Phyllodistomum lacustri (HQ325015)
Phyllodistomum lacustri (HQ325023)
Phyllodistomum lacustri (HQ325012)
Phyllodistomum lacustri (HQ325020)
Phyllodistomum lacustri (HQ325014)
Phyllodistomum lacustri (HQ325021)
Phyllodistomum lacustri (HQ325019)
Phyllodistomum lacustri (HQ325022)
Phyllodistomum lacustri (HQ325017)
Phyllodistomum lacustri (HQ325016)
Phyllodistomum lacustri (HQ325018)
Phyllodistomum lacustri (HQ325025)
Phyllodistomum lacustri (HQ325026)
Phyllodistomum staffordi (EF032692)
Phyllodistomum staffordi (HQ325027)
Phyllodistomum staffordi (HQ325028)
Phyllodistomum folium (AY277707)
Phyllodistomum folium (AY277706)
Phyllodistomum brevicaecum (HQ325008)
Phyllodistomum brevicaecum (HQ325009)
Phyllodistomum brevicaecum (KC760204)
Phyllodistomum brevicaecum (KC760205)
Phyllodistomum brevicaecum (KC760207)
Phyllodistomum brevicaecum (KC760208)
Phyllodistomum brevicaecum (KC760206)
Gorgodera cygnoides (AF151938)
Gorgodera cygnoides (AY222264)
Gorgoderina sp. (HQ325007)
Phyllodistomum magnificum (KF013186)
Phyllodistomum magnificum (KF013189)
Phyllodistomum cribbi n. sp. (KT376718)
Phyllodistomum cribbi n. sp. (KT376721)
Phyllodistomum cribbi n. sp. (KT376719)
Phyllodistomum cribbi n. sp. (KT376720)
Phyllodistomum cribbi n. sp. (KT376722)
0.05

APPENDIX 1.


Prosthenhystera obesa (HQ325057)
Prosthenhystera sp. (KC899850)
Prosthenhystera sp. (KC899848)
Prosthenhystera sp. (KC899849)
Walliniae chavarriae (KC899851)
Margotrema resolanae (KC899854)
Margotrema bravoae (KC899864)
Allocreadium lobatum (KC899847)
Phyllodistomum sp. (AB987943)
Phyllodistomum sp. (AB987945)
Phyllodistomum sp. (AB987944)
Phyllodistomum centropomi (KT376733)
Phyllodistomum wallacei n. sp. (KT376723)
Phyllodistomum wallacei n. sp. (KT376724)
Phyllodistomum wallacei n. sp. (KT376726)
Phyllodistomum wallacei n. sp. (KT376725)
Phyllodistomum inecoli (KC760178)
Phyllodistomum inecoli (KC760176)
Phyllodistomum inecoli (KC760174)
Phyllodistomum inecoli (KC760177)
Phyllodistomum inecoli (KC760173)
Phyllodistomum inecoli (KC760172)
Phyllodistomum inecoli (KC760171)
Phyllodistomum inecoli (KC760169)
Phyllodistomum inecoli (KC760175)
Phyllodistomum inecoli (KC760170)
Phyllodistomum spinopapillatum (KT376732)
Phyllodistomum lacustri (HQ325033)
Phyllodistomum lacustri (HQ325049)
Phyllodistomum lacustri (HQ325035)
Phyllodistomum lacustri (HQ325050)
Phyllodistomum lacustri (HQ325047)
Phyllodistomum lacustri (HQ325034)
Phyllodistomum lacustri (HQ325048)
Phyllodistomum lacustri (HQ325036)
Phyllodistomum lacustri (HQ325037)
Phyllodistomum lacustri (HQ325038)
Phyllodistomum lacustri (HQ325039)
Phyllodistomum lacustri (HQ325051)
Phyllodistomum lacustri (HQ325053)
Phyllodistomum lacustri (HQ325052)
Phyllodistomum lacustri (HQ325054)
Phyllodistomum lacustri (HQ325044)
Phyllodistomum lacustri (HQ325045)
Phyllodistomum lacustri (HQ325046)
Phyllodistomum lacustri (HQ325040)
Phyllodistomum lacustri (HQ325043)
Phyllodistomum lacustri (HQ325042)
Phyllodistomum lacustri (HQ325041)
Phyllodistomum staffordi (HQ325055)
Phyllodistomum staffordi (HQ325056)
Phyllodistomum parasiluri (LC002524)
Phyllodistomum brevicaecum (KC760183)
Phyllodistomum brevicaecum (KC760180)
Phyllodistomum brevicaecum (KC760181)
Phyllodistomum brevicaecum (KC760179)
Phyllodistomum brevicaecum (KC760182)
Phyllodistomum cribbi n. sp. (KT376731)
Phyllodistomum cribbi n. sp. (KT376727)
Phyllodistomum cribbi n. sp. (KT376730)
Phyllodistomum cribbi n. sp. (KT376729)
Phyllodistomum cribbi n. sp. (KT376728)
Gorgoderina sp. (HQ325032)
Pseudophyllodistomum macrobrachicola (LC002523)
0.07

APPENDIX 2.