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ORIGINAL PAPER

Integrative taxonomy identifies a new species of *Phyllodistomum* (Digenea: Gorgoderidae) from the twospot livebearer, *Heterandria bimaculata* (Teleostei: Poeciliidae), in Central Veracruz, Mexico

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Abstract *Phyllodistomum inecoli* n. sp. is described from the twospot livebearer, *Heterandria bimaculata* (Teleostei: Poeciliidae), collected in the Río La Antigua basin, Veracruz, Mexico. The new species is described and characterised by using a combination of morphology, scanning electron microscopy, and sequences of nuclear and mitochondrial genes. Diagnostic characters of the new species of *Phyllodistomum* include a genital pore opening at the level of the caecal bifurcation; oval vitellarium, situated just posterior to the ventral sucker and not extended laterally and anterior extracaecal uterine loops variable in extension (reaching the anterior, median or posterior margin of the ventral sucker). *P. inecoli* n. sp. most closely resembles *P. brevicecum*, a species described as a parasite of the central mudminnow, *Umbra limi*, in other parts of North America; however, the genital pore in *P. brevicecum* is situated between the caecal bifurcation and the ventral sucker, the ovary is larger, the vitellarium is lobed and extended laterally and the anterior portion of the uterus extends to the posterior margin of the ventral sucker. Comparison of about 1,500–2,200 nucleotides of *cox*1 and 28S rDNA and ITS1 strongly supports the status of *P. inecoli* as a new species. Bayesian inference analysis of combined datasets of 28S rDNA and *cox*1 sequences showed that *P. inecoli* n. sp. and the other species found in freshwater fishes of Mexico, including the species complex of *P. lacustri*, are not sister species. Phylogenetic analysis based on 28S rDNA sequences of several gorgoderid taxa revealed the close relationship of *P. inecoli* n. sp. with several species of *Phyllodistomum*, *Gorgodera* and *Gorgoderina* with cystocercous cercariae developing in sphaeriid bivalves. Dot-plot analysis of ITS1 sequences of *P. inecoli* n. sp. revealed the presence of eight repetitive elements with different length, which together represent almost half the length of ITS1.

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Introduction

Phyllodistomum Braun, 1899 (Digenea: Gorgoderidae) includes parasites that typically inhabit the urinary bladder of amphibians, and both marine and freshwater fishes (Campbell 2008; Rosas-Valdez et al. 2011). The genus has a worldwide distribution and is one of the most speciose groups within the Digenea, with around 120 species having been described (Cribb et al. 2002; Helt et al. 2003). In Mexico, five nominal species of *Phyllodistomum* have been recorded thus far, four of them in marine or brackish water fishes: *Phyllodistomum carangis* Manter, 1947, *P. marinae* Bravo-Hollis and Manter 1957, *P. mirandai* Lamothe, 1969, and *P. centropomi* Mendoza-Garfias and Pérez-Ponce de León 2005 (Bravo and Manter 1957; Lamothe-Argumedo et al. 1997; Mendoza-Garfias and Pérez-Ponce de León 2005), and *P. lacustri* (Loewen, 1929) Lewis, 1935, which is the only species found in freshwater fishes (i.e., ictalurids and cichlids)

(Vidal-Martínez et al. 2001; Salgado-Maldonado 2006; Pérez-Ponce de León et al. 2007; Rosas-Valdez and Pérez-Ponce de León 2008). Recently, Rosas-Valdez et al. (2011) analysed nuclear and mitochondrial sequences of specimens of *P. lacustri* across its geographical range, from Mexico, through USA, to Canada, and recognized the presence of three cryptic species as parasites of the catfishes *Ictalurus dugesii* (Bean) and *Ictalurus punctatus* (Rafinesque) from Río Lerma, *Ictalurus pricei* (Rutter) from Río Nazas, and *I. punctatus* from Río Pantepec, respectively.

The combination of morphological and molecular data has become a common practice in the description and characterisation of new species of digeneans (e.g., Curran et al. 2013; Tkach et al. 2013). Sequences of nuclear (28S rDNA and ITSs) and mitochondrial (*cox*1) genes have been widely used to discriminate and characterise closely related species of digeneans (Parker et al. 2010; Razo-Mendivil and Pérez-Ponce de León 2011). The analysis of *cox*1 and ITS1 sequences has allowed the discovery of cryptic species of digeneans parasitising freshwater fish in Mexico (Razo-Mendivil et al. 2010; Rosas-Valdez et al. 2011).

Continuing with our efforts to survey the helminth parasite fauna of Mexican freshwater fishes, we collected several specimens of an apparently undescribed species of *Phyllodistomum* from the urinary bladder of the poeciliid *Heterandria bimaculata* (Heckel) from one creek in Agua Bendita, in the locality of Xico, which belongs to the Río La Antigua basin, Veracruz, Mexico. In this paper, we describe the new taxon, employing both light and scanning electron microscopic (SEM) observations, and present its molecular characterisation based on the sequences of the 28S rDNA and *cox*1 genes and ITS1 region.

Material and methods

Collection of host and parasites, and light and scanning electron microscopy

In June 2012, 55 specimens of the twospot livebearer or Guatopote manchado, *H. bimaculata*, were caught by electrofishing in a creek at Agua Bendita, Xico, Veracruz, Mexico (19°24′44.33″N, 97°00′51.53″W). Hosts were transported alive to the laboratory and examined for helminths immediately after sacrifice through pithing. Digeneans were removed from the urinary bladder of their host and placed in saline solution (0.65 %). Several worms were fixed by immersion in hot (steaming) 4 % formalin without flattening, and stored in 4 % formalin; others were washed twice with clean saline solution, and immediately preserved in 100 % ethanol. Specimens were stained with Gomori's trichrome or Mayer's paracarmine, dehydrated in graded ethanol series, cleared in methyl salicylate and mounted as permanent slides using Canada balsam. Several specimens were permanently mounted between coverslips and held in Cobb slides. Drawings were made with the aid of a drawing tube attached to the microscope. Measurements are presented in micrometers (μm) with the range followed by the mean (in parentheses). Specimens were deposited in the Colección Nacional de Helmintos (CNHE), Instituto de Biología, Universidad Nacional Autónoma de México (UNAM); and some paratypes were also deposited at the United States National Parasite Collection (USNPC) Beltsville, MD. Three individuals fixed with hot 4 % formalin were used for SEM studies. Worms were post fixed in 1 % OsO_4 for 1 h, 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, coated with gold and examined in a Hitachi Stereoscan Model S-2469 N at 15 kV.

For morphological comparisons, the following specimens deposited at the CNHE were examined: *Phyllodistomum lacustri* ex *Ictalurus dugesii* and *Gobiomorus dormitor* Lacepède from Lago de Chapala, Jalisco (CNHE 1278) and Tecolutla, Veracruz (CNHE 1523), respectively; *Phyllodistomum carangis* ex *Citula dorsalis* (Gill) from María Cleofas, Nayarit (CNHE 1112) and Mazatlán, Sinaloa (CNHE 1798), and ex *Trachinotus rhodopus* Gill from Chamela, Jalisco (CNHE 3109); *Phyllodistomum centropomi* ex *Centropomus parallelus* (Poey) from Río Papaloapan, Veracruz (CNHE 4505) and *Phyllodistomum mirandai* ex *Sphoeroides annulatus* (Jenyns) from Salina Cruz, Oaxaca (CNHE 924). We also reviewed several of the published descriptions of *Phyllodistomum* species known to occur in North America, including *P. americanum* Osborn, 1903, *P. brevicecum* Steen 1938, *P. bufonis* Frandsen, 1957, *P. carangis*, *P. carolini* Holl, 1929, *P. caudatum* Steelman, 1938, *P. centropomi*, *P. etheostomae* Fischthal, 1942, *P. fausti* Pearse, 1924, *P. funduli* Helt et al. 2003, *P. lacustri*, *P. lohrenzi* Crowell, 1949, *P. lysteri* Miller, 1940, *P. marinae*, *P. mirandai*, *P. nocomis* Fischthal, 1942, *P. notropidus* Fischthal, 1942, *P. pearsei* Holl, 1929, *P. scrippsi* Brooks and Mayes, 1975, *P. semotili*, Fischthal, 1942, *P. singulare* Lynch 1936, *P. staffordi* Pearse, 1924, *P. superbum* Stafford, 1904, *P. trinectes* Corkum, 1961 and *P. undulans* Steen, 1938.

Molecular protocols

Genomic DNA of ten gravid worms representing the new taxon and between 1–5 specimens belonging to three nominal species of *Phyllodistomum* (Table 1) were extracted using the DNeasy® Blood & Tissue Kit (Qiagen, Valencia, CA, USA) following the manufacturer's protocol. Around 350 base pairs (bp) of the cytochrome *c* oxidase subunit 1 (*cox*1), between ~550 and 1,200 bp of the internal transcribed spacer 1 (ITS1) + partial 5.8S gene, and a fragment of the 28S rDNA gene of

Table 1 Species of *Phyllodistomum* analysed molecularly with information of their host, locality and GenBank accession numbers for each molecular dataset

Species	Host	Locality	GenBank accession number		
			*cox*1	ITS1	28S
Phyllodistomum inecoli n. sp.	*Heterandria bimaculata*	Agua Bendita, Xico, Veracruz, Mexico	KC760169– KC760178	KC760184–KC760189	KC760199– KC760203
P. brevicecum	*Umbra limi*	Brokenhead, Manitoba, Canada	KC760179–KC760183	KC760190– KC760195	KC760204– KC760208
P. staffordi	*Ameirus melas*	La Salle River, Winnipeg, Manitoba, Canada		KC760196– KC760197	
P. lacustri	*Noturus flavus*	Assiniboine River, Manitoba, Canada		KC760198	

approximately 1,250 bp (including the domains D1–D3), were amplified by polymerase chain reaction (PCR) using the primer pairs JB3 (5′-TTTTTTGGGCATCCTGAGGTTTAT-3′) and JB4.5 (5′-TAAAGAAAGAACATAATGAAAATG-3′) (Bowles et al. 1995), Glyp1 (5′-GCTGAGAAGACGACCA AACTTGAT-3′) and Re5.8S (5′-AATGTGCGTTCAA GATGTCGAT-3′) (Razo-Mendivil et al. 2010), and 28digl2 (5′-AAGCATATCACTAAGCGG-3′) and 1500R (5′-GCTATCCTGAGGGAAACTTCG-3′) (Curran et al. 2011), respectively. All PCR reactions were performed in a final volume of 12.5 μl, including 1.25 μl 10× PCR buffer containing 15 mM $MgCl_2$, 0.25 μl 10 mM dNTPs mixture (200 μM each), 0.1 μl of each primer (10 pmol), 1 μl template DNA, 0.0625 μl of Taq DNA polymerase (0.312 units), and 9.8 μl of sterile distilled water. PCR were run in a thermocycler (MJ mini; Bio-Rad) under the following conditions: initial denaturation at 94 °C for 3 min, followed by 35 cycles of 94 °C for 45 s, annealing at 45 °C (*cox*1) or 55 °C (28S rDNA gene and ITS1) and extension at 72 °C for 90 s; the reactions were incubated at 72 °C for 10 min to complete extension and then brought to 8 °C. Amplicons were visualized on ethidium bromide stained 1 % agarose gels and then purified by filtration using Sephadex™ G50 (Sigma-Aldrich, St. Louis, MO, USA). Sequencing reactions were carried out using Big Dye Terminator chemistry, incorporating the same primers as those used in PCR, and cleaned by filtration with Sephadex™G50. The sequenced products were read on an ABI PRISM 3100 automated DNA sequencer (Applied Biosystems, Foster City, CA, USA). Electropherograms were visually inspected using FinchTV (Geospiza Inc., Seattle, WA, USA), and overlapping fragments of forward and reverse sequences were assembled using the computer program BioEdit v. 7.0.9 (Hall 1999).

Sequence alignment and phylogenetic analysis

Five distinct alignments were constructed using MUSCLE v. 3.5 (Edgar 2004), implemented in the software SEAVIEW v. 4.2 (Galtier et al. 1996). First, sequences of *cox*1 and 28S rDNA genes and ITS1 region obtained in this study were aligned independently. Then, a concatenated alignment of 28S rDNA and *cox*1 was constructed with sequences from the new species, *P. brevicecum* and several sequences of *Phyllodistomum* spp. used by Rosas-Valdez et al. (2011) to prospect for cryptic species within the *P. lacustri* species complex, and those available in GenBank. Sequences of ITS1 were not included in this alignment due to the inequality of sequence length among species (Table 1), and this dataset was analysed separately. Finally, partial 28S rDNA sequences of five specimens of the new species and of five individuals of *P. brevicecum* were aligned along with several sequences of gorgoderid taxa available in GenBank, and generated by Cutmore et al. (2013) to test phylogenetic relationships within Gorgoderidae. Sequences generated in this study were

deposited in GenBank with the accession numbers KC760169–KC760183 (*cox* 1), KC760184–KC760198 (ITS1) and KC760199–KC760208 (28S rDNA).

Tree searches were conducted under Bayesian inference (BI) analysis. Sequences of *Prosthenhystera obesa* (HQ325029, HQ325057) and *Gorgoderina* sp. (HQ325007, HQ325032) were used as outgroups, and for rooting the trees obtained from the combined analysis of 28S rDNA and *cox* 1 dataset. Sequences of *Brachylecithum lobatum* (Railliet, 1900) (AY222260), *Dicrocoelium* sp. (AY222261), *Encyclometra colubrimurorum* (Rudolphi, 1819) (AF184254), *Lyperosomum collurionis* (Skrjabin and Issatchikov, 1927) (AY222259), *Paracreptotrematina limi* Amin and Myer, 1982 (HQ833706) and *Prosthenhystera obesa* (Diesing, 1850) (AY222206) were used as outgroups in the analysis of 28S rDNA sequences (including other gorgoderid taxa). Prior to BI, Bayesian Information Criterion (BIC) (Schwarz 1978), implemented in jModelTest 2 (Guindon and Gascuel 2003; Darriba et al. 2012), was used as model selection strategy to determine the best-fit models of nucleotide substitution and parameter settings for *cox* 1 and 28S rDNA datasets and for 28S rDNA dataset (including other gorgoderid taxa). The selected likelihood models were HKY + G, TIM3 + G, and TVM + I + G for *cox* 1, 28S rDNA and 28S rDNA (including gorgoderid taxa) datasets, respectively. BI analysis was performed using MrBayes v. 3.2.1 (Ronquist et al. 2012). For the concatenated dataset (*cox* 1+28S rDNA), a partitioned analysis permitting separate parameter estimates for each molecular partition was set to be independent, using the unlink statefreq (all), revmat = (all), shape = (all), pinvar = (all) command in MrBayes. Independently of the dataset, BI analyses were run over 2.0×10^6 generations, which led to convergence in analysis as estimated by split frequencies (<0.01). Parameter settings used were: nst=2, rates = gamma and nst=6, rates = gamma for *cox* 1 and for both 28S rDNA dataset, respectively. Appropriate burn-in values were determined after log-likelihood values of trees were plotted against the generation time, then 4,000 trees sampled prior to Ln stationarity were discarded. A 50 % majority-rule consensus tree representing the posterior probability (pp) distribution of clades was produced from the remaining trees. Finally, uncorrected pairwise sequence divergences (**p** distances) were calculated for each dataset obtained herein using PAUP* 4.0b10 (Swofford 2003).

Dot-plot analysis

In order to identify repetitive elements within the complete ITS1 sequences of *Phyllodistomum* spp. (Table 1) obtained in this study, a dot-plot analysis was performed for each sequence. Dot-plot method is a useful tool to visualise in two-dimensional plots, regions that are similar between two sequences and repeats within a single sequence (see Gibbs and McIntyre 1970). We used the dot-plot option implemented in SEAVIEW v. 4.2 (Galtier et al. 1996) to analyse each ITS1 sequence of *Phyllodistomum* spp. against itself with a window size of 25 and a match/window of 21, which means that a dot will be placed in the graph only if at least 21 of 25 paired residues in the two oligonucleotides (l=25 pb) compared have identical residues. The number of repetitive elements was determined following the formula $N=N_d+1$, where N is the number of repetitive elements and N_d is the number of lateral diagonals on one side of the central diagonal (Dvořák et al. 2002).

Results

Phyllodistomum inecoli n. sp

Description (based on holotype and 28 paratypes): Body spatulate, aspinose, lateral margins smooth, 1,156–2,089 (1,600) long, maximum width 492–1,132 (809) at level of ovarian–testicular region; ratio of body length/width 1.77–2.40 (2.00); anterior end rounded; bluntly widening anterior to ventral sucker region, gradually narrowing behind posterior testis, finishing in an attenuated tapered end (Figs. 1, 2, 3 and 4). Forebody subcylindrical, 501–831 (664) long by 269–564 (413) wide at level of shoulders, representing 36–47 % (41 %) of total body length (TBL). Hindbody 52–63 % (58 %) of TBL. Tegument with numerous papillae, arranged in paired longitudinal rows, each row constituted by seven to eight dome-like papillae, and occurring symmetrically on ventral, lateral and dorsal surfaces of forebody; papillae randomly distributed on hindbody (Fig. 3a). Remains of stylet situated dorsal to anterior margin of oral sucker, flanked by one dorsal and three lateral pairs of dome-like papillae (Fig. 3b). Cephalic glands present, composed of oval and filiform gland cells, slightly dispersed or overlapped. Glands extend from oral sucker to caecal bifurcation running parallel to oesophagus; at level of oral sucker, glands pass dorsally on both sides. Ducts of gland cells pass dorsally on each side of oral sucker and open into dorsal and lateral surfaces of remains of stylet (Fig. 3b). Oral sucker subterminal, slightly oval, 168–300 (235) long by 149–258 (210) wide, bearing five and four symmetrical pairs of dome-like papillae on outer and inner edges of oral sucker, respectively (Fig. 3c). Ventral sucker 147–273 (200) long by 141–270 (191) wide, bears (6, 4, and 6) dome-like papillae decreasing in size from outer to inner margins (Fig. 3d). Oral sucker/ventral sucker length and width ratio 1:0.64–1:1.05 (1:0.85), 1:0.74–1:1.12 (1:0.90), respectively. Prepharynx and pharynx lacking. Oesophagus narrow, moderately sinuous, 103–208 (150) long by 19–47 (29) wide. Caecal bifurcation approximately half distance between posterior margin of oral sucker and anterior edge of ventral sucker, 312–495 (407) from anterior extremity. Symmetrical caeca end blindly, running

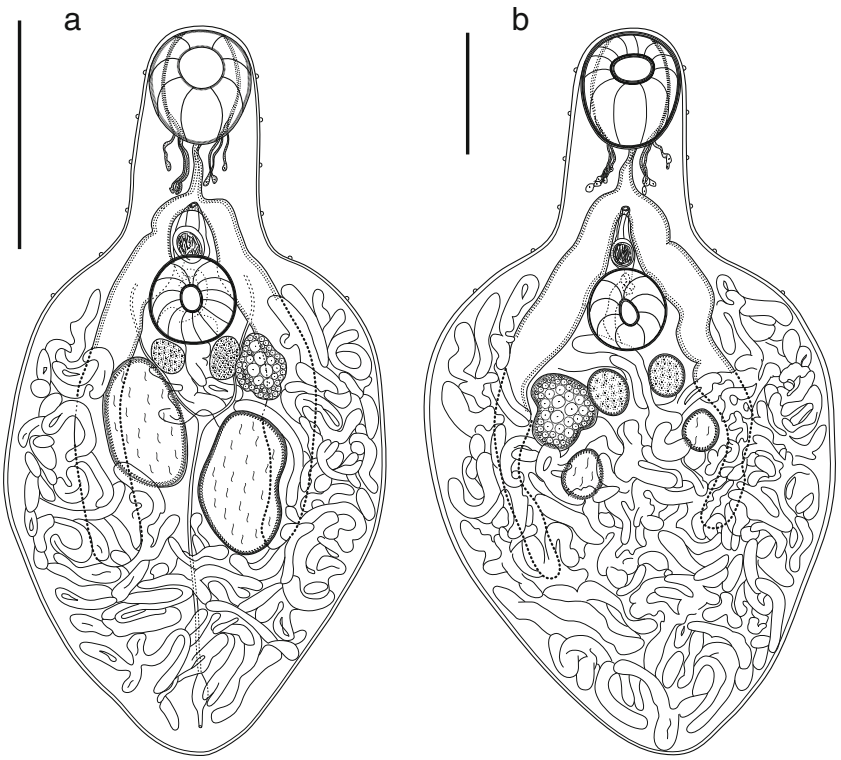

Fig. 1 *Phyllodistomum inecoli* n. sp. from *Heterandria bimaculata*: **a** Holotype (CNHE 8401), ventral view. Scale bar = 500 μm. **b** Paratype (CNHE 8402), dorsal view. Scale bar = 300 μm

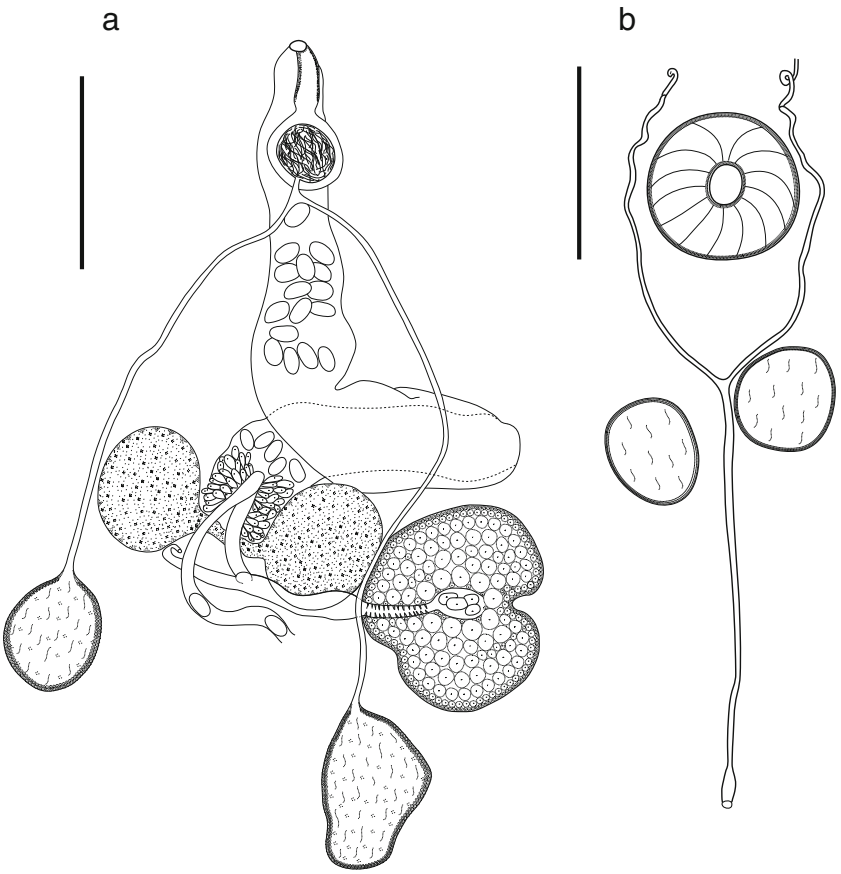

Fig. 2 *Phyllodistomum inecoli* n. sp. from *Heterandria bimaculata*: **a** Detail of male and female reproductive complex, dorsal view. Scale bar = 200 μm. **b** Excretory vesicle, dorsal view. Scale bar = 200 μm

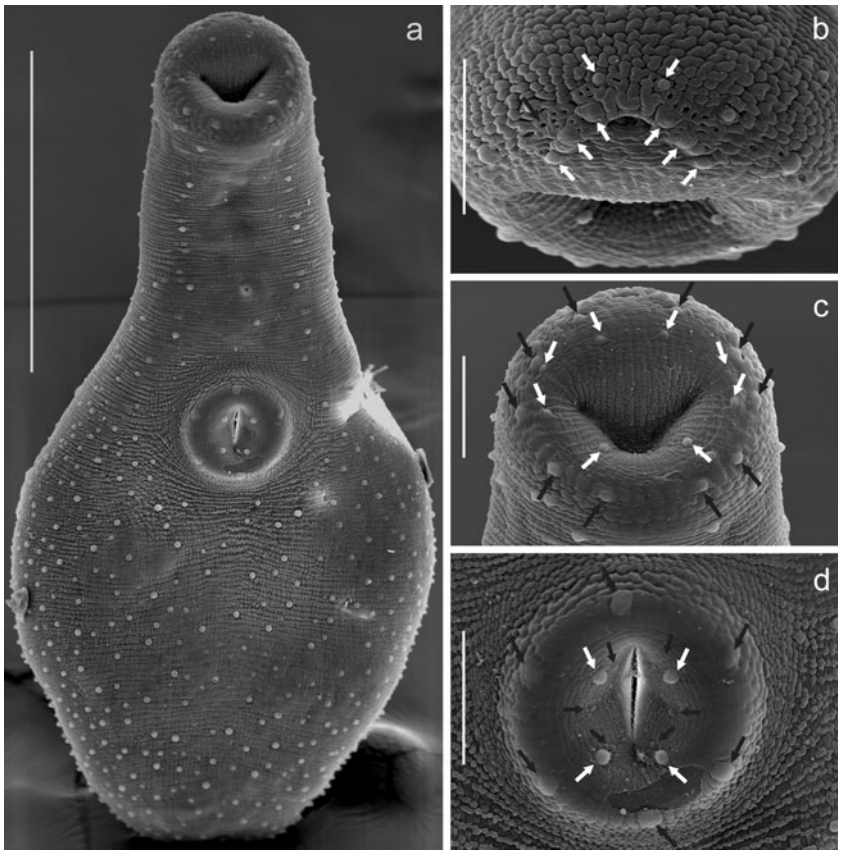

Fig. 3 Scanning electron microscopy of a specimen of *Phyllodistomum inecoli* n. sp.: **a** gravid adult, ventral view; **b** remains of the stylet flanked by three lateral pairs and one dorsal pair of dome-like papillae (*white arrows*) and opening of ducts of cephalic glands (*black arrows*); **c** oral sucker, showing five pairs of external papillae (*black arrow*) and four pairs of internal papillae (*white arrows*); **d** ventral sucker showing three pairs of external papillae (*black arrow*), two pairs of medial dome-like papillae (*white arrow*) and three pairs of internal dome-like papillae (*small black arrow*)

lateral to ventral sucker, reaching just into post-testicular area; right caecum ends at 254–730 (488) and left caecum at 255–807 (501) from posterior extremity.

Testes two, entire to lobed, elongate along longitudinal axis, oblique, equatorial, slightly overlapping caeca. Anterior testis usually at same level as ovary, 107–391(233) long by 66–214 (133) wide; posterior testis, postovarian, 86–399 (232) long by 66–216 (141) wide. *Vasa efferentia* arising from anterior margin of each testis, passing forward lateral to vitellarium, then post-lateral to ventral sucker, joining to form short vas deferens, which opens into seminal vesicle. Seminal vesicle saccular, situated immediately anterior to ventral sucker, opening via genital pore, 51–127 (73) long by 36–109 (62) wide. Pars prostatica not observed, ejaculatory duct straight. Genital pore median, at level of caecal bifurcation, 57–180 (132) from anterior margin of ventral sucker. Ovary amphitypic, on left and rigth sides of 16 and 13 specimens, respectively; ovoid, moderately lobed (2–4 lobes) to almost entire, equatorial, lateral to vitellarium, slightly overlapping caeca; smaller than testes, 59–201 (132) long by 64–174 (112) wide. Vitellarium paired, entire to rarely bilobed, ovoid, immediately posterior to ventral sucker, usually in contact with ventral sucker, ovary and anterior testis; dextral vitellarium 60–128 (92) long and 49–103 (76) wide, sinistral vitellarium 61–160 (96) long and 48–108 (72) wide. Oviduct arises from ovary dorsally, runs transversely toward vitellarium and gives rise to Laurer's canal in distal third. Laurer's canal extends to postero-lateral margin of vitellarium opposite ovary, turns backward and opens dorsally. After the formation of Laurer's canal, oviduct turns anteriorly, receives common vitelline duct and opens into ootype surrounded by Mehlis' gland. Ootype ascends between vitellarium, turns dorsally, then descends and immediately forms uterus. Uterus extensively coiled, occupying entire hindbody. Descending uterus runs ventral to caeca and between testes and extends to lateral and posterior margins of body. Anterior extent of extracaecal uterine coils variable; coils frequently extend to level of anterior margin of ventral sucker, rarely reach middle or posterior levels of ventral sucker. Ascending uterus passes ventral to oviduct and ootype, running forward dorsal to ventral sucker, then narrows to form metraterm which passes ventral to seminal vesicle and opens into genital pore. Eggs 23–28 (26) long, 14–18 (16) wide. Excretory vesicle narrow, tubular, dorsal to uterus, extends to anterior testis forming two slender collecting ducts that run anteriorly around ventral sucker, reaching level of

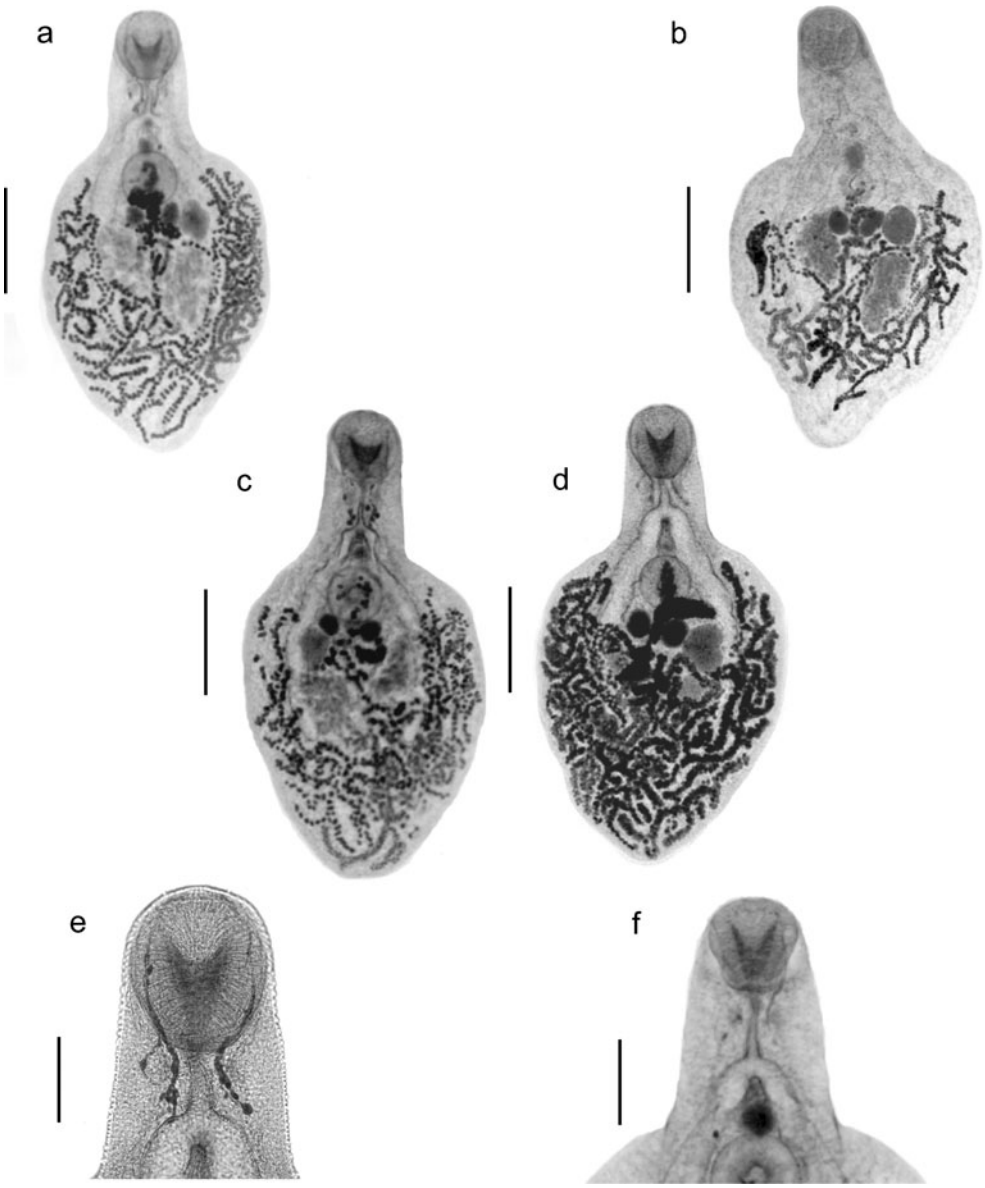

Fig. 4 Microphotographs of four specimens of *P. inecoli* n. sp. showing some variable morphological characters (e.g., dextral or sinistral ovary, anterior extension of uterine loops): **a** Holotype, ventral view. **b**–**d** Paratypes, ventral view. Scale bars = 300 μm. **e** Forebody of holotype showing cephalic glands stained with Gomori's trichrome. **f** Forebody of one paratype stained with Mayer's paracarmine. Scale bars = 100 μm

seminal vesicle. Distal extremity of excretory vesicle slightly expanded just anterior to opening by subterminal excretory pore.

Type-host: *Heterandria bimaculata* (Heckel) (Teleostei: Poeciliidae).

Type-locality: Creek at Agua Bendita, Xico, Veracruz, Mexico (19°24′44.33″N, 97°00′51.53″W), upper basin of the Río La Antigua.

Site of infection: Urinary bladder.

Type specimens: Holotype (CNHE 8401); paratypes: 25 specimens (CNHE 8402); vouchers: eight specimens (CNHE 8403), collected from *H. bimaculata*, Agua Bendita, Xico, Veracruz, Mexico; two paratypes and two vouchers at the U.S. National Parasite Collection (USNPC 107208 and 107209).

Prevalence, *mean intensity and abundance*: Creek at Agua Bendita: 53 of 55 infected fish (96 % prevalence); 310 digeneans collected; intensity range, 1–19 individuals per parasitised host; mean intensity: 5.9±3.98 per infected host; mean abundance: 5.6±4.04 per analysed host.

Etymology: The specific epithet refers to the Instituto de Ecología, A.C. (commonly known as INECOL), a research centre dedicated to the study of Mexico's natural resources, conservation and biodiversity.

Previous records: *Phyllodistomum* sp. from *H. bimaculata* from Agua Bendita, Veracruz, Mexico, reported by Salgado-Maldonado (2006), may be conspecific with *P. inecoli*, but voucher specimens were not studied because material referred to by Salgado-Maldonado (2006) was not deposited in any scientific collection.

Remarks

Cribb (1987) discussed the difficulties to accomplish the proper identification of *Phyllodistomum* species, because of great intraspecific morphological variation in many species and their inadequate descriptions. In the present study, among the 39 specimens used to describe the new species, of which 29 were studied morphologically and ten from which DNA sequences were obtained, some degree of intraspecific morphological variation was detected in the following characters: size of the oral and ventral suckers, ovary position and the anterior extension of the uterus (Fig. 4a–d). Overall, the oral sucker is larger than the ventral sucker, but the ventral sucker of six specimens was slightly larger and/or wider than the oral sucker. Considering the position of the ovary in the hindbody, sinistral position was observed in 22 specimens, whereas dextral in 17 specimens.

The anterior extension of the uterus was also a variable character since 22 out of the 39 individuals showed extracaecal uterine loops extending to the level of the anterior margin of the ventral sucker, seven to the mid-level, and in ten the uterus hardly reached the posterior margin of the ventral sucker. We found no correlation among variation patterns of a specific character (i.e., individuals with dextral ovary and uterus extending to the anterior margin of the ventral sucker, while specimens with the sinistral ovary show the same pattern of extension of the uterus). Based on the premise that the inadequate characterisation of morphological variability in a particular species might result in the erroneous description of two or more species, we decided to complement the morphological data with molecular information obtained from the sequencing of three molecular markers (see below) from five to ten individuals. This strategy allowed us to confirm that the presence of different morphs in the population of the new species is just a case of intraspecific morphological variability, and enabled us to discount the potential existence of two or more species of *Phyllodistomum* in the poeciliid *H. bimaculata* from Agua Bendita, Veracruz, Mexico.

In our specimens, the observation of the cephalic glands depended upon the stain technique used, either Gomori's trichrome (Fig. 4e) or Mayer's paracarmine (Fig. 4f). In all 16 specimens stained with Gomori's trichrome, cephalic glands and ducts were observed. In contrast, in all worms stained with Mayer's paracarmine, cephalic glands and ducts were indistinguishable. It is thus probable that the alleged absence of glands in other species of *Phyllodistomum* is in fact caused by the application of different staining techniques, and that these structures are ubiquitous. The exact number of cephalic glands was not determined because they are often overlapped. However, SEM observations allowed us to determine the presence of at least 24 pores, representing the openings of the gland ducts; these pores are situated laterally and dorsally to the remains of the stylet (Fig. 3b). We were unable to determine if the number of pores is correlated with the number of cephalic glands and if they are functional in adult worms. In this context, we highlight the importance of using different staining techniques to demonstrate the presence or absence of cephalic glands in specimens belonging to species of *Phyllodistomum* previously described, or in the description of new species.

Genetic variation and phylogenetic analyses

Alignment sequences of the *cox* 1 gene included the sequences of ten isolates of the new species of *Phyllodistomum*, five isolates of *P. brevicecum* and all sequences deposited in GenBank by Rosas-Valdez et al. (2011) for *P. staffordi* and for cryptic species of *P. lacustri*. The length of alignment was 309 bp (without outgroups). No intra-specific variation was exhibited among ten isolates of *P. inecoli* n. sp. and among five individuals of *P. brevicecum*. Comparison between nominal species showed a great amount of nucleotide differences, ranging from 13.0 % to 16.2 %. The new species differed from *P. staffordi* by 13.7 %, and from *P. brevicecum* by 14.2 %. With respect to cryptic species of *P. lacustri*, the divergence ranged from 13.0 % to 16.2 % (Table S1).

The length of the 28S rDNA sequences of five isolates of the new species was 1,305 bp long, and 1,250 bp for five specimens of *P. brevicecum*. However, the alignment length among *Phyllodistomum* species was of 854 bp (without outgroups) because sequences of four species of *Phyllodistomum* available in Genbank were shorter than our sequences. Isolates of *P. inecoli* n. sp. and *P. brevicecum* did not show any intraspecific variation; however, when comparing our sequences of *P. brevicecum* with those deposited in GenBank, a 0.12 % variation was detected. Sequence variation among nominal species ranged from 3.2 % to 4.4 %. Sequence variation between the new species and the remaining species of *Phyllodistomum* was 3.3 %, 3.3–3.5 %, and 4.4 % with respect to *P. staffordi*, and the complex of cryptic species of *P. lacustri* and *P. brevicecum*, respectively (Table S2).

Sequence lengths of complete ITS1 showed high disparity among species: in *P. inecoli* the ITS1 was 1,108 bp long, while it was 448 bp for *P. lacustri*, 449 bp for *P. staffordi*, and 455 bp for *P. brevicecum*. Due to the inequality of sequence length among species and the ambiguity in some regions of the alignment, it was trimmed at the 5′ end and only 377 bp were analysed. Null intra-specific variation was observed for isolates of *P. inecoli* n. sp. and *P. brevicecum*. High genetic variation was detected among species ranging from 8.3 % to 14.7 %. Nucleotide difference of the new species from congeners was 8.3 % (*P. lacustri*), 9.6 % (*P. staffordi*) and 14.7 % (*P. brevicecum*) (Table S3).

The concatenated alignment using both *cox* 1 and 28S rDNA sequences consisted of 30 samples (including *P. obesa*

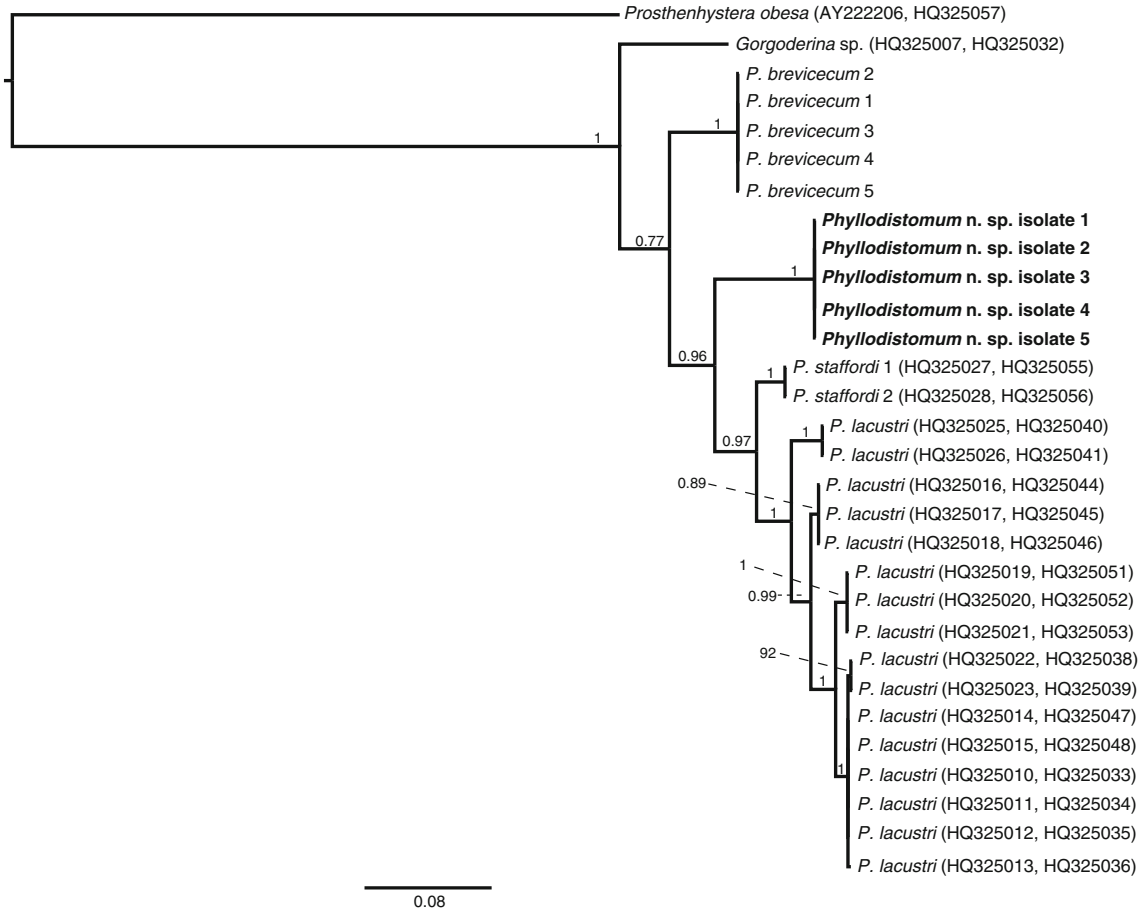

Fig. 5 Bayesian 50 % majority-rule consensus phylogram inferred of simultaneous analysis of 1,166 nucleotide positions (309 from *cox*1 and 857 from 28S rDNA), from four nominal species of *Phyllodistomum*, three cryptic species of *P. lacustri* and two outgroups. Phylogram rooted with *Prosthenhystera obesa*. Bayesian posterior probabilities are given above the internode

and *Gorgoderina* sp. as outgroups), and included 1,166 nucleotide positions, with 142 parsimony-informative characters. The BI tree (Fig. 5) showed the five isolates of *P. inecoli* forming a highly supported group (1 of posterior probabilities [pp]). The new species appeared as sister group to the clade constituted by *P. staffordi* Pearse, 1924 and the *P. lacustri* species complex, which includes three cryptic species (Rosas-Valdez et al. 2011). Phylogenetic relationships among these species were well supported, with 0.96 and 0.97 values of pp. Finally, five isolates of *P. brevicecum* constituted a highly supported clade (1 pp), which occupied the most basal position among species of *Phyllodistomum*.

The second alignment of 28S rDNA was composed by 70 sequences, and included five individuals of *P. inecoli* n. sp, five specimens of *P. brevicecum*, 54 sequences of different gorgoderid taxa and six sequences of species of Allocreadiidae, Callodistomidae, Dicrocoeliidae and Encyclometridae, which were used as outgroups. 28S rDNA alignment consisted of 839 total characters, with 421 parsimony-informative characters. Because several sequences of *P. lacustri* available in GenBank (e.g., HQ325023 and HQ325025) were shorter than 28S rDNA sequences obtained in this study and those of Cutmore et al. (2013) the alignment was trimmed and its total length was 839 nucleotide positions, with 421 parsimony-informative characters. The BI tree (Fig. 6) recovered a similar topology as those obtained by Cutmore et al. (2013) in their phylogenetic analysis of Gorgoderidae. A well-supported clade (1 pp) included *P. staffordi* (GenBank accession numbers, EF032692, HQ325027 and HQ325028), *P. lacustri* (HQ325016–HQ325017 and HQ325023–HQ325026), *P. inecoli* n. sp. (this study), *P. folium* (Olfers, 1817) (AY277707), *Phyllodistomum* sp. (AY277706), *P. brevicecum* (this study and HQ325008 and 325009), *P. magnificum* Cribb 1987 (KF013186 and KF013189), *Gorgodera cygnoides* (Zeder, 1800) (AF151938 and AY222264) and *Gorgoderina* sp. (HQ325007).

Repetitive elements

Dot-plot analysis of ITS1 sequences for the species *P. brevicecum*, *P. lacustri* and *P. staffordi* did not show repetitive elements. In contrast, the ITS1 sequences of *P. inecoli* contained two regions with four repetitive elements each

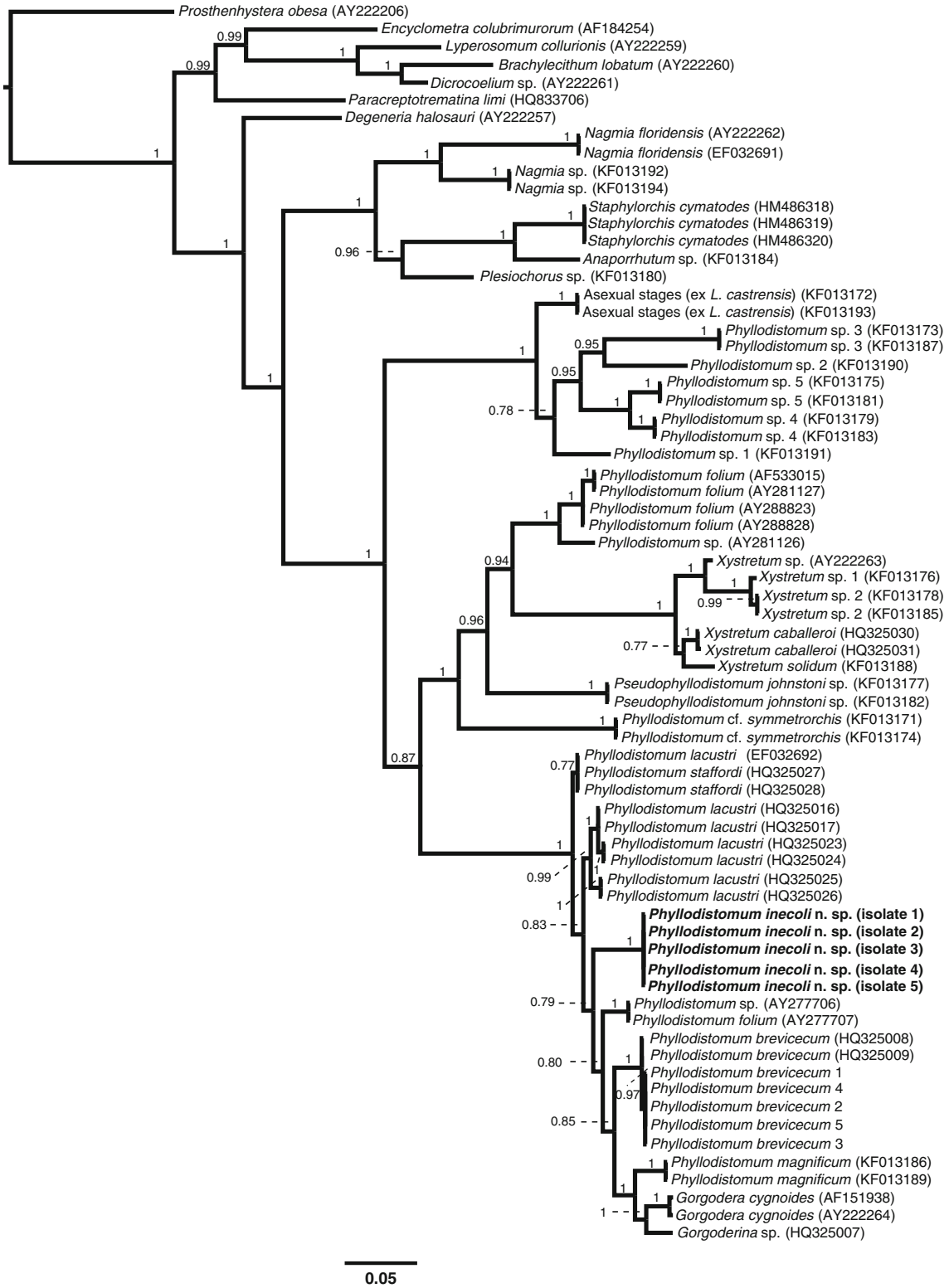

Fig. 6 Fifty percent majority-rule consensus phylogram of relationships of *Phyllodistomum inecoli* n. sp. and other gorgoderid taxa based on Bayesian analysis of 839 bp of the 28S rDNA gene. Phylogram rooted with *Prosthenhystera obesa*. Bayesian posterior probabilities are given above the internode

(Fig. S1). The first region had a length of 542 bp and began with the first nucleotide of ITS1 at the 5′ end, and continued to 542 bp at the 3′ end. This region contained four tandemly repeated copies, three of them constituted by 150 bp (1–150, designated as repetitive element 1, 151–300, designated as repetitive element 1a, 301–450, designated as repetitive

element 1b) and one shorter with a length of 92 bp (451–542, designated as repetitive element 1c). A high degree of sequence similarity was observed between the repetitive elements (1 vs. 1a: 84 %; 1 vs. 1b: 86 %; 1 vs. 1c: 96.7 %). The second region was composed by four non-tandemly repetitive elements constituted by 70 and 106 bp. The fragments from 546 at the 5′ end to 616 at the 3′ end (designated as repetitive element 2), and from 617 at the 5′ end to 972 at the 3′ end (designated as repetitive element 3) have their single-copy counterparts from 769 at the 5′ end to 839 at the 3′ end (designated as repetitive element 2a) and 867 at the 5′ end to 972 at the 3′ end (designated as repetitive element 3a), respectively. Sequence identity between repetitive elements was 80 % and 93.3 %, respectively.

Discussion

The new species most closely resembles *P. brevicecum* a species described from the urinary bladder of the central mudminnow, *Umbra limi* Kirtland, in Indiana, USA (Steen 1938). Both species possess cephalic glands, short caeca, amphitypic ovary, and uterus occupying the entire hindbody; however, *P. inecoli* n. sp. differs from *P. brevicecum* by having a smaller ovary: 59–201 (132) μm long by 67–174 (112) μm wide in the new species, 144–238 (185) μm long by 128–161 (148) μm wide in *P. brevicecum*; and by the oval vitellarium situated just posterior to the ventral sucker, whereas in *P. brevicecum* the vitellarium is lobed and extends laterally. Additionally, in *P. brevicecum* the genital pore is situated between the ventral sucker and the caecal bifurcation, and the uterine coils do not extend anteriorly beyond the posterior margin of the ventral sucker, whereas in the new species the genital pore opens to the level of the caecal bifurcation and uterine coils reach the anterior, median or posterior margins of the ventral sucker.

Besides *P. brevicecum*, two species, *P. singulare*, described from the urinary bladder of the California giant salamander *Dicamptodon ensatus* (Eschscholtz) in Oregon, USA (Lynch 1936), and *P. magnificum* Cribb 1987, from the urinary bladder of several Australian and New Zealand freshwater fishes (Cribb 1987), also possess prominent cephalic glands. Nevertheless, *P. singulare* and *P. magnificum* possess a larger body 3270–3950 (3600) μm and 1926–4542 (2935), respectively; and their intestinal caeca extend posteriorly to the testes. In addition, *P. singulare* has a noticeably crinkled hindbody, a deeply lobed vitellarium and is a parasite of amphibians of the order Caudata.

Of the five species of *Phyllodistomum* recorded in Mexico, *P. lacustri*, a species originally described from the Channel catfish *Ictalurus punctatus* in Minnesota, USA, is the only species parasitizing freshwater fishes in Mexico, and differs from *P. inecoli* n. sp. by having a crenulate margin of the hindbody, a larger body size, a ventral sucker larger than the oral sucker, caeca extending to the posterior end of the body, and a genital pore situated between the ventral sucker and the caecal bifurcation. Even though the three cryptic species of *P. lacustri* remain undescribed (Rosas-Valdez et al. 2011), none of them are morphologically similar to the new species we describe herein, and these three species are also characterised by possessing a crenulate margin of the hindbody.

SEM of some *Phyllodistomum* species has revealed the presence (e.g., *P. folium*, *P. conostomum*, *P. magnificum*) or absence (e.g., *P. funduli*) of tegumental papillae. Furthermore, papillae differ in number, arrangement and type between species. *P. inecoli* n. sp. possesses several tegumental papillae arranged in paired longitudinal rows, each row constituted by seven to eight dome-like papillae, which occur symmetrically on ventral, lateral and dorsal surfaces of the forebody. Presence of longitudinal rows of papillae occurring on the ventral and lateral surface has also been reported in *P. conostomum* and *P. folium* (Bakke 1985; Bakke and Ždárská 1985). *Phyllodistomum conostomum* and *P. folium* possess nine and ten symmetrical pairs of dome-like papillae over the oral sucker, respectively. The oral sucker of *P. inecoli* also bears nine pairs of dome-like papillae, five pairs on the outer edge and four on the inner edge; this arrangement of papillae is also observed in *P. folium*, but is in contrast with the five and four pairs of papillae exhibited by *P. conostomum*. The two species mentioned above and the new species have three and two pairs of dome-like papillae over the outer and inner edges, respectively; however, in *P. inecoli* n. sp., there are three additional pairs of small papillae.

Approximately 120 species of the genus *Phyllodistomum* have been described thus far as parasites of marine and freshwater fishes, and amphibians around the world (Cribb et al. 2002). In the Americas, at least 42 nominal species of *Phyllodistomum* (Yamaguti 1971; Hoffman 1999; Helt et al. 2003; Kohn et al. 2007; Pérez-Ponce de León et al. 2007) have been described from several marine and freshwater families of fishes. In Mexico, the only species recorded from freshwater fishes is *P. lacustri*, which has been recorded in several species belonging to Cichlidae, Eleotridae and Ictaluridae inhabiting distinct river basins, although it seems to be a member of the core helminth fauna of ictalurids (Pérez-Ponce de León and Choudhury 2002), and the records in non-ictalurid hosts are regarded as doubtful (see Pérez-Ponce de León et al. 2007). *P. inecoli* n. sp. is the first species of the genus described from poeciliid fish. Members of Poeciliidae (28 genera and around 220 species) are endemic to the New World, with the majority of the species occurring in Mexico, Central America and the Antilles (Rosen and Bailey 1963; Lucinda and Reis 2005; Miller et al. 2005; Hrbek et al. 2007). Studies on the helminth fauna of Mexican poeciliid fishes are relatively scarce; to date, 54

species of helminths have been recorded in 24 of the 84 poeciliid species distributed in the Mexican territory (see Salgado-Maldonado 2006; Pérez-Ponce de León and Choudhury 2010; Rubio-Godoy et al. 2010). Since the type host and only host, *H. bimaculata* is distributed in river basins of southeastern Mexico, it is quite possible that the new species we described will be found in other localities, and potentially in other poeciliid species.

Genetic variation, phylogenetic relationships and repetitive elements

Even though *Phyllodistomum* is one of the most diverse genera within the Digenea, few species have been studied from a molecular perspective. As a consequence, few sequences of *cox*1 and 28S rDNA genes and the ITS region are available to molecularly characterise particular species of *Phyllodistomum*. Molecular comparison of specimens of the new species with respect to other three or four congeners (depending on the gene compared) revealed high levels of genetic variation in the three molecular markers analysed. Genetic variation between *P. inecoli* n. sp. and the remaining species ranged from 3.3 % to 4.4 % for 28S rDNA, from 8.3 % to 14.7 % for ITS1, and from 13.0 % to 16.2 % for *cox*1. Similar variation values were also found between the other species pairs (e.g., *P. brevicecum* vs. *P. lacustri*). Morphologically, *P. inecoli* n. sp. and *P. brevicecum* are similar, although they showed high levels of sequence variation: 4.3–4.4 % (28S rDNA), 14.7 % (ITS1), and 14.2 % (*cox*1). The level of divergence we found in the three molecular markers corresponds with that reported in a series of studies that have been conducted on several digeneans (e.g., Parker et al. 2010; Razo-Mendivil and Pérez-Ponce de León 2011; Snyder and Tkach 2011; Pulis et al. 2013). Molecular information clearly supports and reinforces the establishment of the new species we describe herein.

Our phylogenetic analyses of the combined datasets (*cox*1+28S rDNA) showed that *P.inecoli* n. sp. is not closely related to *P. lacustri*, which is the other species recorded in freshwater fishes in Mexico. Although they show a close morphological resemblance, the new species and *P. brevicecum* are not each other's closest relatives. Phylogenetic analysis of 28S rDNA sequences including several species of gorgoderid taxa (including data reported by Cutmore et al. 2013) reinforce the finding that *P. inecoli* n. sp. is not closely related to either *P. lacustri* or *P. brevicecum*. Phylogenetic analyses of Gorgoderidae performed by Cutmore et al. (2013) employing 28S rDNA and ITS2, demonstrated that *Phyllodistomum* is paraphyletic; and these authors recovered one well-supported clade, including species of *Phyllodistomum*, *Gorgodera* and *Gorgoderina* with cystocercous cercariae developing in sphaeriid bivalves. Our analyses showed that *P. inecoli* n. sp. is closely related to species of Gorgoderidae with cystocercous cercariae. Further study about the life cycle of the new species should reveal the type of cercariae it produces, and if the first intermediate host is a sphaeriid bivalve. Likewise, our phylogenetic results reinforce the idea that *Phyllodistomum* does not represent a natural group (Cutmore et al. 2013).

Differences in sequence length and either the presence or the lack of repetitive elements in ITS1 sequences of *Phyllodistomum* spp. was observed in this study. The length of sequences of the complete ITS1 region varied from 448 to 455 bp in the species considered in this study, except for *P. inecoli* n. sp., in which we discovered that the ITS1 region was 1,108 bp long. Extreme length and length variation is associated with long repetitive elements identified in *P. inecoli* n. sp.; this species showed eight repetitive fragments (Fig. S1) of variable length (70–150 bp) which correspond to 572 bp (150+150+92+70+106=572) of a total of 1,108 bp. No repetitive elements were detected in the remaining species of *Phyllodistomum*. Extreme length and length variation of ITS1 has been observed in several groups of metazoans (von der Schulenburg et al. 2001) and the digeneans, as demonstrated here, are not the exception. Size variation and repetitive elements have been documented in species of *Levinseniella* Stiles and Hassall, 1901, *Paragonimus* Braun, 1899, *Schistosoma* Weinland, 1858 and *Trichobilharzia* Skrjabin and Zakharow, 1920 (van Herwerden et al. 1998, 1999; Dvořák et al. 2002; Warberg et al. 2005). Maximum ITS1 size has been observed in the species *Trichobilharzia regenti* Horák, Kolářová and Dvořák, 1998 with 1,325 bp, *Schistosoma japonicum* Katsurada, 1904 with 1,400 bp and *Levinseniella brachysoma* (Creplin, 1837) Stiles and Hasall, 1901 with 1,500 bp. These species exhibited several repeats of variable length ranging from ~39 to ~90 bp (*S. japonicum*), from 48 to 80 bp (*L. brachysoma*) and from 85 to 112 bp (*T. regenti*) (van Herwerden et al. 1998; Dvořák et al. 2002; Warberg et al. 2005). Currently, no biological function has been associated with the extreme larger size of ITS1 and the repeats within it.

At present, establishment of new taxa should not only be morphology-based, but rather use an integrative taxonomy approach (Will et al. 2005; Wheeler 2008). The central purpose of integrative taxonomy is to make use of the combination of a wide range of sources of information (morphology, phylogeny, molecular biology, ecology) to increase the rigour in species delimitation (Schlick-Steiner et al. 2010). Proper analysis of the current diversity of *Phyllodistomum*, recognized as one of the most diverse genera of digeneans, requires the use of such an integrative approach. We will only be able to demonstrate whether the diversity of this group of digeneans has been overestimated or underestimated, when different sources of taxonomic information have been duly considered.

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