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Description of two new species of allocreadiid trematodes (Digenea: Allocreadiidae) in middle American freshwater fishes using an integrative taxonomy approach

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Abstract

Integrative taxonomy uses several sources of information to establish more robust species delimitation criteria. In this study, we followed that approach to describe two new species of allocreadiid trematodes from freshwater fish distributed in locations across Middle America. The new species were first recognized by using sequences of the domains D1–D3 of the ribosomal 28S rRNA gene and then, morphological data (light and scanning electron microscopy (SEM)), host association, and geographical distribution were considered as additional sources of information to complement the species delimitation and description. *Auriculostoma tica* n. sp. was found in the intestine of *Gymnotus maculosus* Albert and Miller in Costa Rica, while *Wallinia anindoi* n. sp. was found in the intestine of *Astyanax aeneus* (Günther) in Oaxaca and Chiapas, Mexico, as well as in a location of Guatemala. A phylogenetic analysis combining newly generated sequences of the two new species along with those available for other allocreadiids in GenBank, revealed that isolates of each species are reciprocally monophyletic, and also their interrelationships: *Au. tica* n. sp. is a sister taxon of *Auriculostoma totonacapanensis* Razo-Mendivil et al., 2014, and *W. anindoi* n. sp. is a sister taxon of *Wallinia brasiliensis* Dias, et al., 2018. Genetic distances for the 28S rRNA gene were estimated among the American species of allocreadiids and further supported the validity of the new species.

Keywords Middle-America · 28S rDNA · Molecular phylogeny · *Auriculostoma* · *Wallinia*

Introduction

The Allocreadiidae represents an important component of the helminth parasite fauna of freshwater fishes (Caira and Bogéa 2005; Choudhury et al. 2007). Our knowledge about species diversity and interrelationships of allocreadiid trematodes in Middle-American freshwater fishes has increased over the last 15 years. Several descriptions have been published for species occurring primarily in small-bodied characids (tetras), but also in cyprinodontiformes of the families Poeciliidae Garman (guppies) and Profundulidae Hoedeman and Bronner (killifishes) (Choudhury et al. 2002, 2006; Scholz et al. 2004; Razo-Mendivil et al. 2014a, b; Bautista-Hernández et al. 2015; Pérez-Ponce de León et al. 2015, 2016; Hernández-Mena et al. 2016). The first species descriptions of allocreadiids in that geographical area were solely based on morphological characters (see Choudhury et al. 2002; Scholz et al. 2004); however, most new species have been described following an integrative taxonomy approach (Dayrat 2005) where DNA sequence data was used in combination with morphological analyses of phenotypic differences, mostly through scanning electron microscopy observations (SEM), host association and geographic distribution, to assess more robust species limits (e.g., Pérez-Ponce de León et al. 2015; Hernández-Mena et al. 2016). Three additional Middle-American species of allocreadiids have been described following that approach within the genera *Auriculostoma* Scholz

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et al., 2004, and *Wallinia* Pearse, 1920, i.e., *Auriculostoma totonacapanensis* Razo-Mendivil et al., 2014, as a parasite of *Astyanax mexicanus* (De Filippi) from Veracruz, Mexico; *Auriculostoma lobata* Hernández-Mena et al., 2016, from *Brycon guatemalensis* Regan, in Chiapas, Mexico (Razo-Mendivil et al. 2014a; Hernández-Mena et al. 2016); and one species of *Wallinia*, *Wallinia mexicana* Pérez-Ponce de León et al., 2015 in *As. mexicanus* from central Mexico (Pérez-Ponce de León et al. 2015). In a continuation of our efforts to uncover and describe the allocreadiid diversity in neotropical freshwater fishes, a new phylogenetic analysis of the Allocreadiidae was performed using sequences of the 28S ribosomal rRNA gene of newly sampled specimens, from our field collections of 2015 and 2016 in several localities of southern Mexico, Guatemala, and Costa Rica, resulting in the discovery of two new species, one belonging to *Wallinia*, from the intestine of *As. aeneus* Günther and one to *Auriculostoma*, from the intestine of *Gymnotus maculosus* Albert & Miller. Specimens processed for morphology and SEM showed distinctive characters when compared with congeneric species allocated into these two genera and therefore, in this study, we describe the two new species; the species delimitation criteria are then based on information from 28S rDNA sequences, morphological data, geographical distribution, and host association.

Material and methods

Sample collections

Specimens of allocreadiids were collected from their freshwater fish between 2015 and 2016 in two locations in Costa Rica (*Gy. maculosus* $n = 3$), three in Mexico (*As. aeneus*, $n = 15$), and one in Guatemala (*As. aeneus*, $n = 11$) (Table 1). Fish were collected with seine nets and electrofishing, kept alive, and necropsied 4 h after capture. The gastrointestinal tract was removed from each host and placed in Petri dishes with 6.5% saline solution and examined under a stereomicroscope. Some specimens were fixed in hot (nearly boiling) tap water and kept in vials with 4% formalin for subsequent morphological study. Other specimens were placed directly in vials with 100% ethanol for molecular analyses.

Molecular work

Specimens were individually digested overnight at 56 °C in a solution containing 10 mM Tris-HCl (pH 7.6), 20 mM NaCl, 100 mM Na2 EDTA (pH 8.0), 1% Sarkosyl, and 0.1 mg/ml proteinase K. DNA was isolated from the supernatant using the DNAzol (Molecular Research Center, Cincinnati, OH) following the manufacturer's instructions. The domains D1–D3 of the 28S rRNA gene were amplified using the forward primer 391 (5′-AGC GGA GGA AAA GAA ACT AA-3′; Nadler and Hudspeth 1998) and the reverse primer 536 (5′-CAG CTA TCC TGA GGG AAA C-3′; García-Varela and Nadler 2005). Polymerase chain reaction (PCR) amplifications were carried out with 2 μl of DNA, 1 μl of each PCR primer (10 μM), 2.5 μl of 10× buffer, 1.5 μl of 25 mM of $MgCl_2$, 0.5 μl of dNTPs (10 mM), and 1 U of Taq DNA polymerase. The following amplification profile was used: 95 °C for 5 min (denaturation); 35 cycles of 94 °C for 1 min, 50 °C for 1 min, and 72 °C for 1 min; and a final extension of 72 °C for 10 min. PCR products for 28S rDNA were sequenced with PCR primers plus internal primers 503 (5′-CCT TGG TCC GTG TTT CAA GAC G-3′; Stock et al. 2001) and 504 (5′-CGT CTT GAA ACA CGG ACT AAG G-3′; García-Varela and Nadler 2005). PCR products were sequenced using an ABI 3730xl Genetic Analyzer (Applied Biosystems). The consensus sequences were obtained from the individual sequences amplified from each primer using the software Geneious Pro 4.8.4® (Biomatters Ltd. Auckland, New Zealand). Sequences were submitted to GenBank (Table 1).

Phylogenetic analyses

The newly generated sequences of the nuclear 28S rRNA gene were aligned with available sequences of other species of allocreadiids and callodistomids using ClustalW (Thompson et al. 1997), implemented in the website http://www.genome.jp/tools/clustalw/. Based on previously phylogenetic trees of Allocreadiidae (Hernández-Mena et al. 2016; Pérez-Ponce de León et al. 2016), sequences of other species of allocreadiids were included, i.e., *Paracreptotrematoides heterandriae* (Salgado-Maldonado et al., 2012) Pérez-Ponce de León et al., 2016, *Paracreptotrema blancoi* Choudhury et al., 2006, and *Paracreptotrema rosenthali* Bautista-Hernández et al., 2015 (see Table 1). Species of *Prosthenhystera* Travassos, 1922 (Callodistomidae) were used as outgroups for rooting the trees. Phylogenetic analyses were run under maximum likelihood (ML) and Bayesian inference (BI), employing the model of nucleotide evolution GTR+GAMMA-I selected with jModelTest v2 (Darriba et al. 2012). ML inference (20 replicates), model parameters and bootstrap (Bt) support (1000 repetitions) were estimated with RAxML v. 7.0.4 (Stamatakis 2006). BI analysis was carried out using MrBayes v. 3.2 (Ronquist et al. 2012), running two independent Markov Chain Monte Carlo runs of two chains each run (a heating parameter value of 0.5) for 10 million generations and sampling tree topologies every 1000 generations (printfreq = 1000, samplefreq = 1000, diagnfreq = 10,000). Burn-in periods were set to the first 500 generations. A 50% majority-rule consensus tree and nodal support estimated as posterior probability (PP) values were calculated from the remaining trees. The phylogenetic trees obtained

Table 1 Collection data and GenBank accession numbers for allocreadiid species analyzed in this study. New species in bold

Digenean species	GenBank accession numbers	Host species	Locality	Reference
Allocreadiidae Looss, 1902				
Auriculostoma astyanace Scholz et al., 2004	HQ833707	*Astyanax aeneus* (Günther)	Tempisquito River, Guanacaste, Costa Rica	Curran et al. (2011)
	KF631422	*Astyanax faxciatus* (Cuvier)	Sapoa River, Guanacaste, Costa Rica	Razo-Mendivil et al. (2014a)
Auriculostoma lobata Hernández-Mena et al.	KX954170 KX954174	*Brycon guatemalensis* Regan	El Managal Lagoon, Tenosique, México	Hernández-Mena et al. (2016)
***Auriculostoma tica* n. sp.**	**MH997001-02**	***Gymnotus maculosus* Albert & Miller**	**Orosí River, creek at Pitaya, affluent to the Orosí River, Costa Rica**	**This study**
Auriculostoma totonacapenensis Razo-Mendivil et al., 2014	KF631417 KF631418	*Astyanax mexicanus* (De Filippi)	Filipinas, Veracruz, Mexico	Razo-Mendivil et al. (2014a)
Creptotrematina aguirrepequenoi Jiménez-Guzmán, 1973	HQ833708 HQ833709	*Astyanax aeneus* (Günther)	Tempisquito River, Guanacaste, Costa Rica	Curran et al. (2011)
Paracreptotrema blancoi Choudhury et al., 2006	KT833285	*Priapichthys annectens* (Regan)	Orosí River, Costa Rica	Pérez-Ponce de León et al. (2015)
Paracreptotrema heterandriae Salgado-Maldonado et al., 2012	KF697693	*Heterandria bimaculata* (Heckel)	Agua Bendita, Xico, Veracruz, Mexico	Razo-Mendivil et al. (2014b)
Paracreptotrema rosenthali Bautista-Hernández et al., 2015	KT833287	*Xiphophorus malinche* Rauchenberger et al.	Malila River, Hidalgo, Mexico	Pérez-Ponce de León et al. (2015)
Wallinia brasiliensis Dias et al., 2018	MH520995	*Astyanax fasciatus* (Cuvier) *A. lacustris*	State of São Paulo, Brazil	Dias et al. (2018)
Wallinia chavarriae Choudhury et al., 2002	HQ833703	*Astyanax aeneus* (Günther)	Animas River, Guanacaste, Costa Rica	Curran et al. (2011)
***Wallinia anindoi* n. sp.**	**MH997003-12 MH997013 MH997014-15**	***Astyanax aeneus* (Günther)**	**San Juan, Oaxaca, Mexico Río San Juan, Chiapas, Mexico Río las Cabezas, El Progreso, Guatemala**	**This study**
Wallinia mexicana Pérez-Ponce de León et al., 2015	KJ535504	*Astyanax mexicanus* (De Filippi)	Covadonga River, Durango, Mexico	Pérez-Ponce de León et al. (2015)
	KJ535505	*Astyanax mexicanus*	Huichihuayan River, San Luis Potosí, Mexico	
Outgroup taxa Callodistomidae Odhner, 1910				
Prosthenhystera caballeroi Jiménez-Guzmán, 1973	KM871185	*Astyanax aeneus* (Günther)	Tampisquto River, Guanacaste, Costa Rica	Tkach and Curran (2015)
Prosthenhystera obesa (Diesing, 1850) Travassos, 1922	AY222206	*Hoplias* sp. Gill	Rio Itaya, Iquitos, Peru	Olson et al. (2003)
Prosthenhystera oonastica Tkach & Curran, 2015	KM871181	*Pylodictis olivaris* (Rafinesque)	Pearl River, Mississippi, USA	Tkach and Curran (2015)

from both analyses were visualized in FigTree v. 1.4.2. Genetic distances for the 28S data set were calculated as uncorrected *p* distance using MEGA v6 (Tamura et al. 2013).

Morphological study

Specimens were stained with Mayer's paracarmine and Gomori's trichrome, dehydrated in a graded ethanol series, cleared in methyl salicylate, and examined as permanent mounts in Canada balsam. Specimens were examined using an Olympus BX51 microscope (Olympus Corporation, Tokyo, Japan) with differential interference contrast optics. Measurements were taken using the Olympus Quick-Photo Image-Program, and are presented in micrometers (μm) with the range followed by the mean in parentheses. Drawings were made with a drawing tube attached to the microscope. Paragenophores were deposited in the Colección Nacional de Helmintos (CNHE), Instituto de Biología, Universidad Nacional Autónoma de México (UNAM). For morphological comparison, the following type specimens were studied: *Auriculostoma astyanace* ex *As. fasciatus* 1 holotype (CNHE 4923) and 1 paratype (CNHE 4924), from Loonku Creek, Bluefields, Región Autónoma del Atlántico del Sur (RAAS), Nicaragua; *Au. lobata* ex *B. guatemalensis* 1 holotype (CNHE 1081), and 8 paratypes (CNHE 1082), from El Mangal Lagoon, Tenosique, Tabasco, Mexico, plus 6 voucher specimens from Río Usumacinta, Chiapas, Mexico (CNHE 7505); *Au. totonacapanensis* ex *As. mexicanus* 1 holotype and 7 paratypes Mexico (CNHE 8471), from Filipinas and Veracruz and 1 paratype US National Parasite Collection (USNPC 107880); *W. mexicana* ex *As. mexicanus* 1 holotype (CNHE 8453), 15 paratypes from Río Covadonga at Peñon Blanco, Durango, Mexico, (CNHE 6311–6313), and six specimens from Río Huichihuayán, San Luis Potosí, Mexico (CNHE 7411).

A single individual of *Auriculostoma* and three specimens of *Wallinia* were used for scanning electron microscopy (SEM) study. The specimens were dehydrated through a graded series of ethyl alcohol, and then critical point dried with carbon dioxide. Specimens were mounted on a metal stub with carbon adhesive tabs, then gold coated, and examined at 15 kV in a Hitachi Stereoscan Model SU1510 SEM (Hitachi Ltd., Tokyo, Japan).

Results

Phylogenetic relationships and genetic divergence

The data set of the 28S rRNA gene consisted of 34 sequences representing 15 species, 4 of which are members of *Auriculostoma* and 4 of *Wallinia*; the remaining species represented 4 species of allocreadiids, and 3 of the callodistomid genus *Prosthenhystera*. The final alignment consisted of 1424 bp. Nucleotide frequencies were $A = 0.218$, $C = 0.212$, $G = 0.314$, and $T = 0.256$. The ML tree had a value of − ln of 3949.29. Both the ML and BI analyses yielded the same phylogenetic relationships. The phylogenetic tree (Fig. 1) showed that sequences used in our study for allocreadiids are a monophyletic group, with high nodal support. Within allocreadiids, a monophyletic group was formed including species of *Creptotrematina* Yamaguti, 1954; *Auriculostoma*, and *Wallinia*, typical inhabitants of small-bodies characids, although with a low bootstrap support value. Both phylogenetic trees showed that two isolates of *Creptotrematina aguirrepequenoi* Jiménez, 1973 are the sister of *Auriculostoma* and *Wallinia*, both recovered as monophyletic assemblages. The new species of *Auriculostoma* (two isolates) was recovered as the sister species of *Au. totonacapanensis*, with *Au. astyanace* Scholz et al., 2004, and *Au. lobata* as their sister species. The 13 isolates of the new species of *Wallinia* were reciprocally monophyletic, with the highest values of nodal support, and were nested as the sister species of *W. brasiliensis* Dias et al., 2018. *W. mexicana* + *W. chavarriae* Choudhury et al., 2002 were their sister group.

Genetic distance of the 28S rDNA sequences among genera was relatively high, varying from 2.66 to 5.51% between *Auriculostoma* and *Wallinia*, 3.76 to 4.49% between *Auriculostoma* and *Creptotrematina*, and 3.67 to 5.33% between *Wallinia* and *Creptotrematina*. Likewise, the interspecific genetic distance between the new species and *Au. totonacapanensis*, its sister species, was 2.11%, whereas between the new species and *Au. lobata* and *Au. astyanace* was 1.47 and 1.38%, respectively. The genetic distance between the sister species pair that included the new species and *W. brasiliensis* was 1.19%; meanwhile, between the new species and the other two congeneric species varied from 3.03 to 4.04%. Intraspecific genetic divergence of the 28S rDNA sequences between the two isolates of the new species of *Auriculostoma* and among the 13 isolates of the new species of *Wallinia* was null.

Family Allocreadiidae Looss, 1902.

Genus *Auriculostoma* Scholz et al., 2004

Auriculostoma tica n. sp. (Figs. 2, 3)

Description (Based on four adult unflattened specimens from Orosí River). Body elongate, 1551–2118 (1778) long, narrow at the pharynx level; maximum width 424–568 (478) in the region between ovary and anterior testis. Tegument smooth. Forebody length 563–654 (600). Oral sucker subterminal, funnel-shaped, well developed; single pair of dorsolateral muscular lobes located on either side, with an inconspicuous base, stretching from ventrolateral to dorsolateral side,

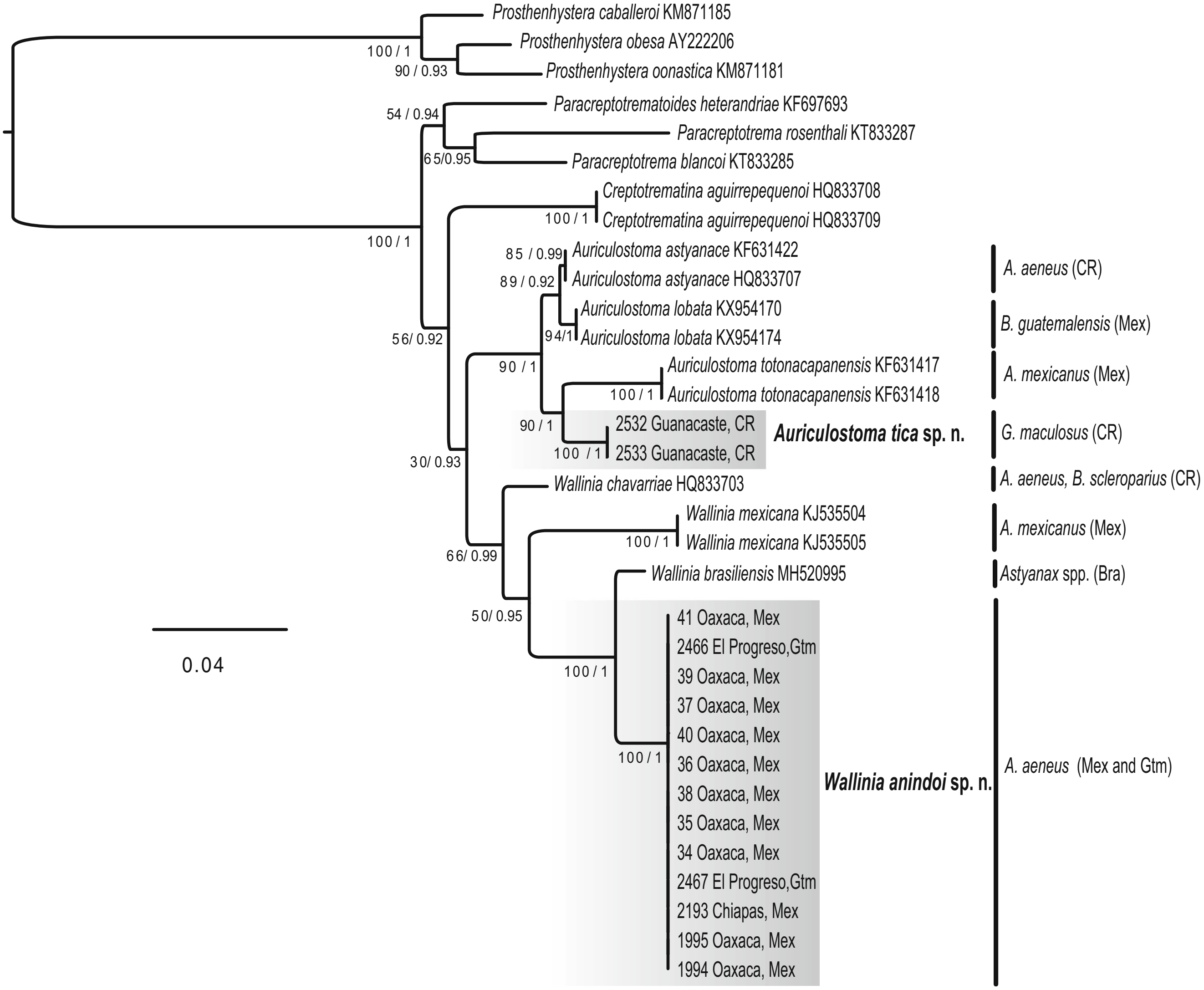

Fig. 1 Phylogenetic relationships among allocreadiid trematodes resulting from maximum likelihood based on the partial sequence of the 28S rDNA gene. Bootstrap support and posterior probability values are shown near the nodes. The two new species described here are highlighted in bold. Host species and geographical distribution of each species on the tree is indicated. Mex = Mexico, CR = Costa Rica, Bra = Brazil, Gtm = Guatemala. The scale bar indicates the number of substitutions per site

forming short rounded "free" ends. Oral sucker 240–270 (260) long, 216–260 (241) wide, excluding lateral lobes, and 285–335 (316) wide including lateral lobes; oral sucker with 21 dome-like papillae arranged in 4 rows: 4 apical, 8 anterior (Fig. 3b, d), 4 on inner surface, and 5 on outer surface. Ventral sucker spherical, muscular, 143–179 (164) long, 161–192 (181) wide, slightly smaller than oral sucker; ratio of oral sucker length to ventral sucker length 1:0.57–0.74 (1:0.68); ratio of oral sucker width to ventral sucker width not considering lateral lobes 1:0.62–0.85 (1:0.75), and ratio of oral sucker width to ventral sucker width considering lateral lobes 1:0.48–0.67 (1:0.57). Mouth subterminal. Prepharynx absent. Pharynx muscular, globular, 74–98 (86) long, 81–115 (100) wide. Esophagus curved. Intestinal bifurcation at anterior margin of ventral sucker. Caeca long and wide, reaching near to posterior extremity. Post-caecal space length = 59–198 (132). Two testes of similar size, oval, entire, oblique, in posterior half of body. Anterior testis submedial, 135–172 (149) long by 83–110 (97) wide. Posterior testis medial, 135–182 (158) long by 109–144 (121) wide. Post-testicular space length: 386–642 (487), representing 25–30% (27%) of body length. Cirrus-sac relatively long, dorsal, median, straight, extending from genital pore to mid-level between ventral sucker and ovary, containing wide muscular ejaculatory duct, pars prostatica, and bi-partite internal seminal vesicle; cirrus not observed. Genital pore median, opening on ventral surface, between pharynx (at mid-level of esophagus), and intestinal bifurcation; distance of genital pore from anterior end of body 363–454 (400). Ovary bean-shaped, entire, posterior to ventral sucker, pre-

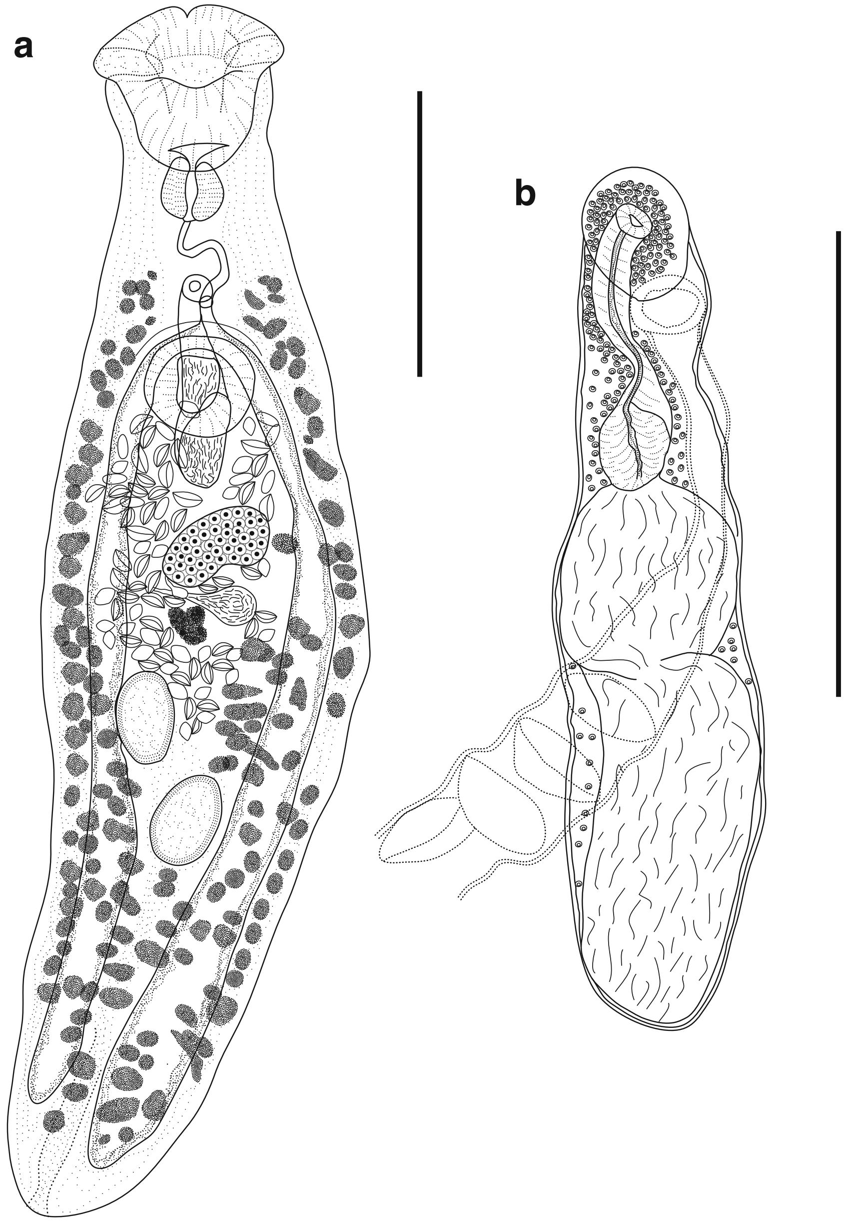

Fig. 2 *Auriculostoma tica* n. sp. **a** Ventral view of holotype. Scale bar = 500 μm. **b** Ventral view of terminal genitalia showing that the ejaculatory duct and the uterus open to the genital atrium (reconstruction made from the observation of several specimens). Scale bar = 200 μm

equatorial, in middle line of body, 90–112 (103) long by 75–129 (105) wide. Seminal receptacle sac-shaped, post-ovarian, 61 long by 95 wide. Mehlis' gland posterior to ovary and seminal receptacle. Laurer's canal not observed. Vitelline follicles small, numerous, intra- and extracaecal along body, extending from genital pore to posterior end of body, confluent at post-testicular area. Vitelline reservoir ventral to Mehlis' gland. Uterus pre-testicular, between posterior border of anterior testis and genital pore. Metraterm relatively short. Eggs 41–70 (54) long by 23–39 (29) wide. Excretory vesicle I-shaped, reaching to anterior margin of anterior testis. Excretory pore terminal.

Type material Holotype (CNHE 10872); paratypes: three specimens from Orosí River (CNHE 10873).

Type host *Gymnotus maculosus* Albert & Miller (Gymnotidae).

Type locality Orosí River, Costa Rica (11° 02′ 50″ N, 85° 22′ 48″ W).

Other Localities Creek at Pitaya, affluent to the Orosí River, Costa Rica (11° 03′ 05″ N, 85° 24’ 30″ W).

Site in host Intestine.

Etymology This specific epithet refers to the Costa Rican people, who refer to themselves as "ticos," in recognition of their friendship while we conducted field work in particular areas of the country.

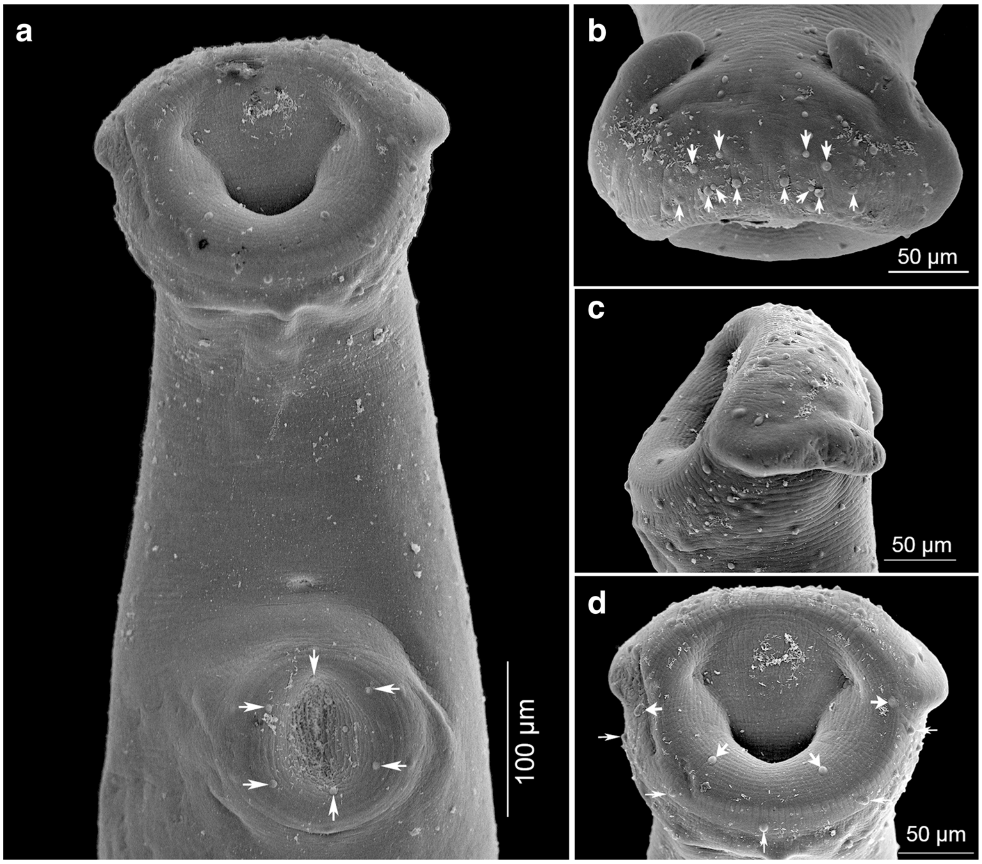

Fig. 3 Scanning electron micrographs of *Auriculostoma tica* n. sp. **a** Anterior end with oral sucker bearing muscular lobe on either side, genital atrium and ventral sucker showing six dome-like papillae (white arrows). **b** Pair of muscular lobes with a short posterior "free" end and four apical dome-like papillae. **c** Lateral view of muscular lobe. **d** Distribution of dome-like papillae over oral sucker, eight anterior, four on the inner surface, and five on the outer surface (white arrows)

Remarks

Auriculostoma tica n. sp. conforms to the diagnosis of *Auriculostoma* in having a single pair of muscular lobes on either side of the oral sucker (Scholz et al. 2004; Razo-Mendivil et al. 2014a). Overall, the new species is morphologically more similar to *Auriculostoma diagonale* Curran et al., 2011; *Auriculostoma platense* (Szidat 1954) Scholz et al., 2004; and *Au. totonacapanensis* by possessing entire testes in oblique position; in some specimens of *Au. totonacapanensis*, testes seem to be in tandem, but in most of them, testes are oblique (see Razo-Mendivil et al. 2014a). In both *Au. tica* n. sp. and *Au. platense*, the anterior extension of the vitelline follicles reaches the mid-level of esophagus, whereas in *Au. diagonale*, follicles reach pharynx level, and in *Au. totonacapanensis*, follicles reach the intestinal bifurcation. Additionally, the new species differs from these species by the posterior extension of the cirrus-sac and the position of the genital pore: in *Au. tica* n. sp., the cirrus-sac extends to mid-level of the region between the ventral sucker and ovary, and the genital pore reaches the mid-level of esophagus; in *Au. platense*, the cirrus-sac does not extend posteriorly beyond the ventral sucker and the genital pore is located between the anterior margin of the ventral sucker and the intestinal bifurcation; in *Au. diagonale*, the cirrus-sac extends to the anterior margin of the ovary and the genital pore is located between the anterior margin of the ventral sucker and intestinal bifurcation; finally, in *Au. totonacapanensis*, the cirrus-sac extends to the mid-level or to the posterior margin of the ovary but does not surpass the ovarian region, and the genital pore is located at the level of the intestinal bifurcation.

The new species can be distinguished from the other congeners by a combination of several morphological characters and measurements; *Au. tica* n. sp. differs from the remaining species, except from *Auriculostoma stenopteri* (Mañé-Garzón and Gascón 1973) Aguirre-Macedo & Scholz, 2004, because the genital pore is at the mid-level of the esophagus; in *Auriculostoma foliaceum* Curran et al., 2011, *Au. lobata*, and *Auriculostoma macrorchis* (Szidat 1954) Scholz et al., 2004, the genital pore is located between the anterior margin of ventral sucker and intestinal bifurcation. *Au. tica* n. sp. further differs from these four species by having oblique testes with entire margins rather than testes in tandem, with irregular or lobulated margins, by the anterior extension of the vitelline follicles, and by the OS/VS width ratio; in *Au. tica* n. sp., the vitelline follicles reach the mid-level of esophagus, the genital pore is also located in that position, and the OS/VS width ratio is 1:0.48–0.67; whereas in *Au. foliaceum*, follicles are absent in the forebody, and the OS/VS ratio is 1:0.5; in *Au. lobata*, follicles reach the intestinal bifurcation and the OS/VS ratio is

1:1.21–1-52; and in *Au. macrorchis* and *Au. stenopteri*, follicles extend up to the level of the oral sucker with OS/VS ratios of 1:1–1.3 and 1:1.3, respectively. Additionally, the new species differs from *Au. foliaceum* by having a terminal excretory pore; in *Au. foliaceum*, the excretory pore is subterminal.

Even though the new species and *Au. astyanace* are distributed in the same geographic area in Costa Rica, they are morphologically different. *Au. tica* n. sp. possesses oblique testes while in *Au. astyanace*, testes are in a tandem configuration. Furthermore, the cirrus-sac in the new species is shorter than in *Au. astyanace*; in terms of host association, the new species is a parasite of gymnotids while *Au. astyanace* is a common parasite of characids. Differences in morphology and host association may result in the fact that they are not each other's sister species, even occurring in sympatry (Fig. 1). Finally, the ultrastructure of the body surface of *Au. tica* n. sp., *Au. lobata*, and *Au. totonacapanensis* provide additional information for species delimitation; unfortunately, the ultrastructure of *Au. astyanace* has not been described yet. The SEM photographs (Fig. 3b, d) show that specimens of the new species possess 21 dome-like papillae on the oral sucker surface arranged in four rows; the new species possesses two more apical papillae than *Au. lobata* and *Au. totonacapanensis*. The three species possess a single pair of muscular lobes located on either side of the oral sucker. However, they can be readily distinguished because the base of the dorsolateral oral lobes is less developed in the new species (see Fig. 3).

Genus *Wallinia* Pearse, 1920

Wallinia anindoi n. sp. (Figs. 4, 5)

Description (Based on 10 adult unflattened specimens from San Juan del Río, Oaxaca, Mexico, and 5 adult specimens from Río las Cabezas at Saranate, El Progreso, Guatemala). Body elongate, 2045–3346 (2740) long, maximum width 585–807 (699) at posterior third of body; anterior end bluntly rounded. Body widening gradually behind oral sucker region, terminating in narrower bluntly rounded end. Tegument smooth. Remnants of eyespots present at level of pharynx. Forebody 415–659 (533) long; forebody length: total body length ratio 0.16–0.21 (0.20); forebody without tegumental papillae on lateral sides. Oral sucker spherical, subterminal, well-developed, 241–332 (276) long, 246–331 (281) wide. Oral sucker with a variable number of papillae (Fig. 5c, d). Ventral sucker subspherical, smaller than oral sucker, muscular, 203–339 (265) long, 229–338 (277) wide. Ratio of oral sucker length to ventral sucker length 1:0.81–1.13 (1:0.95); ratio of oral sucker width to ventral sucker width 1:0.83–1.17 (1:0.99). Mouth subterminal. Prepharynx absent. Pharynx muscular, well-developed 93–143 (120.5) long, 110–163 (144) wide. Esophagus curved and narrow 51–113 (78) long. Cluster of gland cells situated laterally on either side of pharyngeal and esophageal region. Intestinal bifurcation at anterior margin of ventral sucker, 366–518 (450) from anterior end of body; caeca terminating between testes and posterior end of body, 548–797 (668) from posterior end. Testes two, oblique, rounded, entire, submedial. Anterior testis 190–384 (264) long by 128–262 (207) wide. Posterior testis contiguous with anterior testis, 192–337 (276) long by 140–272 (228) wide, 692–1453 (1029) from posterior end of body. Intertesticular space 99–368 (213). Cirrus-sac elongate, median, dorsal, and extending beyond posterior margin of ventral sucker; cirrus sac containing distal, tubular and muscular ejaculatory duct surrounded by scattered gland cells; ovoid pars prostatica obscured by surrounding gland cells; unipartite folded seminal vesicle continuous occupying most of cirrus sac with narrower distal tubular portion; cirrus not observed. Genital pore median at intestinal bifurcation. Ovary 183–274 (211) long by 126–215 (168) wide, rounded, margins unlobed, not overlapping ventral sucker, pre-testicular, pre-equatorial, sinistral (or median in few specimens), with distinct distal region containing larger oocytes. Seminal receptacle ovoid 118–153 (129) long by 84.7–144.9 (115) wide, immediately posterior and not overlapping ovary. Mehlis' gland composed of scattered gland cells obscuring ootype, median, between ovary, anterior testis and seminal receptacle. Laurer's canal traversing dorsal side of body laterally and opening to dorsal surface just past outer edge of caecum at level of ovary, seminal receptacle, or anterior testis; opening dextrally. Vitelline follicles in two ventrolateral fields extending usually from anterior margin of ventral sucker to halfway between posterior margin of posterior testis and end of caeca; follicles overlapping caeca ventrally; vitelline ducts uniting immediately posterior to ootype to form vitelline reservoir. Uterus extending between intertesticular space and alongside testes, forming convoluted loops, mostly intercaecal, filling post-testicular space and extending to posterior end of body; distal arm of uterus winding anteriorly, traversing pre-testicular region medially, forming ventral metraterm leading to genital pore. Eggs numerous, ovoid, operculated, embryonated, 47–64 (51) long, 24–39 (29) wide. Excretory bladder narrow, I-shaped; excretory pore slightly dorso-terminal.

Type material Holotype (CNHE 10874); paratypes: 11 specimens from San Juan del Río, Oaxaca, Mexico (CNHE 10875) and 5 specimens from Río Las Cabezas, Saranate, Guatemala (CNHE 10876).

Type host *Astyanax aeneus* (Günther).

Type locality San Juan del Río, Oaxaca, Mexico (16° 53′ 57″ N, 96° 09′ 57″ W).

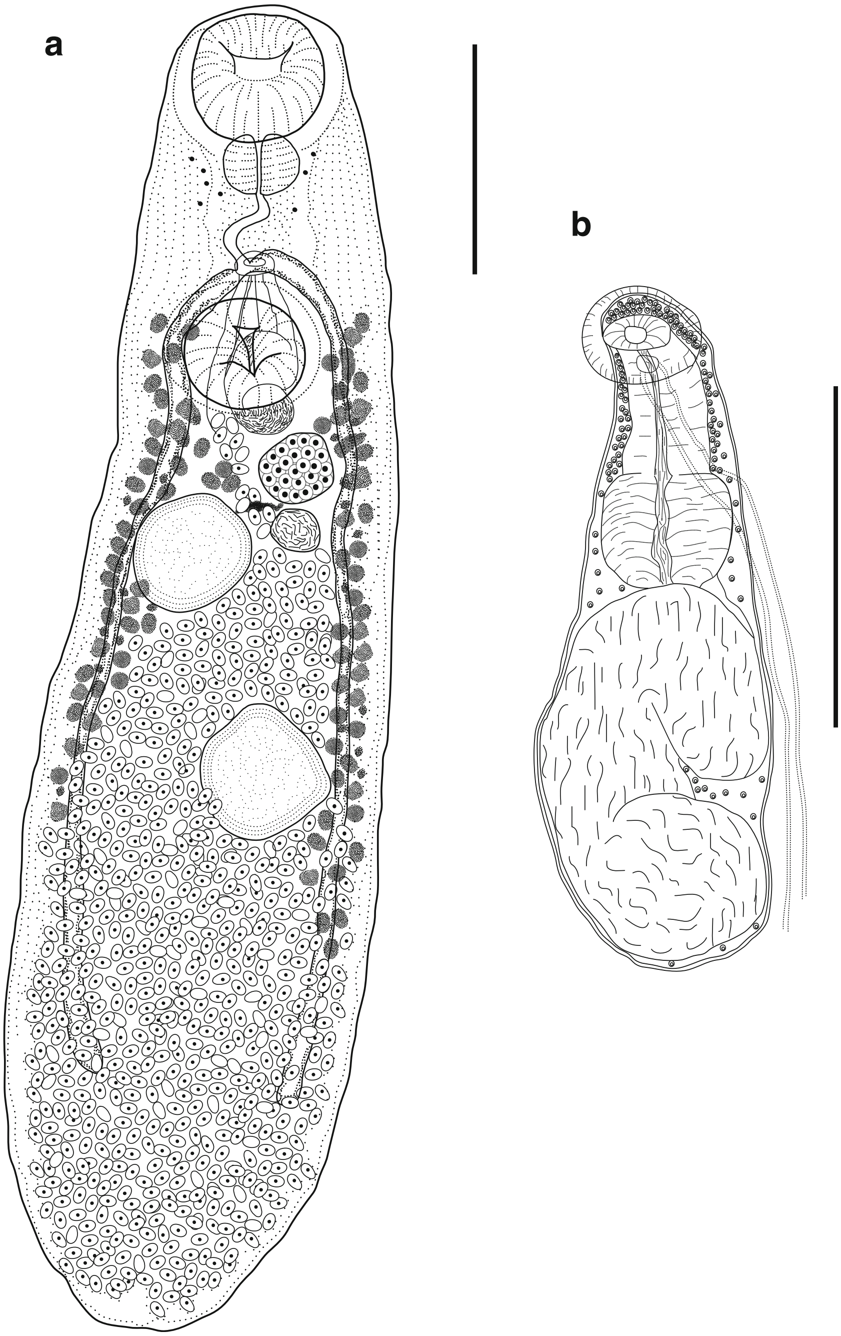

Fig. 4 *Wallinia anindoi* n. sp. **a** Ventral view of holotype. Scale bar = 500 μm. **b** Ventral view of terminal genitalia showing that the ejaculatory duct and the uterus opening to the genital atrium (reconstruction made from the observation of several specimens). Scale bar = 200 μm

Other localities Río San Juan, Chiapas, Mexico (16° 21′ 00″ N, 93° 30′ 54″ W); Río las Cabezas at Sanarate, El Progreso, Guatemala (14° 44′ 23″ N, 90° 04′ 52″ W).

Site in host Intestine.

Etymology The species is named after Dr. Anindo Choudhury, our friend and colleague, for his wide contribution to the study of freshwater fish parasites, and particularly his contributions to the taxonomy of Allocreadiidae.

Remarks

Wallinia anindoi n. sp. is placed in *Wallinia* because it shows the diagnostic characters that distinguish this genus from other allocreadids, i.e., an unspined body, well-developed cirrus-sac, and medial genital pore (Choudhury et al. 2002). The new species is morphologically most similar to *W. mexicana*, a parasite of the Mexican tetra, *As. mexicanus* (De Filippi) in Mexico, although they are not each other's closest relatives (Fig. 1); however, they differ in the ovary shape and in the anterior extension of the

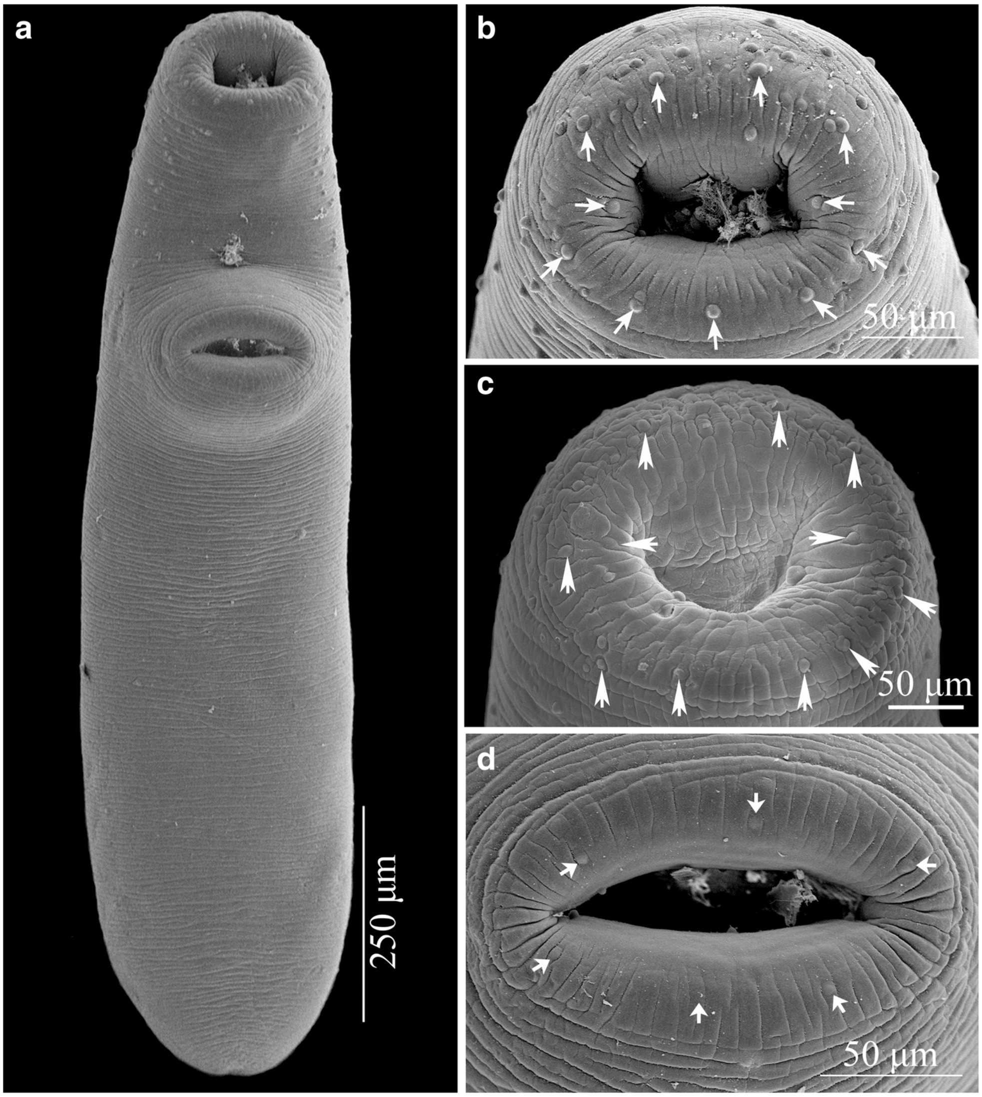

Fig. 5 Scanning electron micrographs of *Wallinia anindoi* n. sp. **a** Whole specimens, ventral view. **b**, **c** Oral sucker showing the arrangement of dome-like papillae (white arrows). **d** Ventral sucker showing six dome-like papillae (white arrows)

vitelline follicles; in *W. anindoi* n. sp., the ovary is oval and the vitelline follicles do not extend anteriorly to the anterior margin of ventral sucker, while in *W. mexicana*, the ovary is triangular and vitelline follicles reach the intestinal bifurcation. Even though these two species are morphologically similar, the genetic divergence for the 28S rRNA gene is very high, reaching values of 4%. The new species can be also distinguished from the other three congeners. *W. chavarriae*, a parasite of the banded tetra, *As. aeneus*, and the creek tetra, *Bryconamericus scleroparius*, (Regan) in Costa Rica, differs from the new species by having a more restricted vitellarium; in the new species, vitelline follicles extend posteriorly to reach half the distance between the posterior testis and the end of the caeca, and they possess a long esophagus and oblique testes; instead, *W. chavarriae* possess vitelline follicles reaching the posterior margin of posterior testis, a short esophagus, and testes in tandem (or slightly oblique in some specimens) and almost contiguous. The closest relative of the new species is *W. brasiliensis*, a species recently described from the intestine of two species of tetras from the Batalha River in São Paulo State, Brazil: *As. fasciatus* (Cuvier) and *Astyanax lacustris* Lucena & Soares, 2016 (Dias et al. 2018). This species pair exhibits the smallest genetic divergence for the 28S rRNA gene with 1.19%. The Brazilian species differs from *W. anindoi* n. sp. by having a shorter body (1136 vs 2749), by the extension of the vitelline follicles almost reaching the posterior end of the body, by the testes shape (elongated rather than rounded), and by having relatively larger eggs. Finally, the type species for which sequence data has not been obtained yet, *Wallinia valenciae* Pearse, 1920, a parasite of *Gephyrocharax valencia* (Eigenmann) in Venezuela (Pearse 1920), differs from the new species by having tubular vitelline follicles rather than rounded and by possessing a smaller oral sucker (relative to the ventral sucker).

Discussion

In this paper, we present the description of two new species of allocreadiids that were first recognized through a phylogenetic analysis using 28S rDNA sequences; intergeneric and interspecific levels of genetic divergence recovered in our study are similar to those obtained for other allocreadiids in previous studies (Razo-Mendivil et al. 2014a; Pérez-Ponce de León et al. 2015, 2016; Hernández-Mena et al. 2016); levels of genetic divergence in combination with the reciprocal monophyly of the isolates of each of the new species corroborated they represented two candidate species. Morphological study of the specimens allowed us to formally describe both as new species; the description of the two species increase the diversity of freshwater fish parasites in Middle America, raising the number of congeneric species to five for the genus *Wallinia*, and to nine for *Auriculostoma* (Hernández-Mena et al. 2016; Dias et al. 2018). The species diversity of allocreadiids in neotropical freshwater fishes has increased in the last years as a result of a more detailed study of the morphological traits, in combination with DNA sequences and molecular phylogenetic analyses. New species and even new genera have been uncovered (see Pérez-Ponce de León et al. 2016). Particularly within the genera *Auriculostoma* and *Wallinia*, six additional species have been described following that approach, four in *Auriculostoma*, i.e., *Au. foliaceum* in *Bryconops caudomaculatus* (Günther); *Au. diagonale* in *Stethaprion erythrops* Cope, both from Peru; *Au. totonacapanensis* in *As. mexicanus* from Veracruz, Mexico, and *Au. lobata* in *Br. guatemalensis* from Chiapas, Mexico (Curran et al. 2011; Razo-Mendivil et al. 2014a; Hernández-Mena et al. 2016); and two species in *Wallinia* also have been described, including *W. mexicana* in *As. mexicanus* from central Mexico, and *W. brasiliensis* from *As. fasciatus* and *As. lacustris* in Brazil (Pérez-Ponce de León et al. 2015; Dias et al. 2018). Species in both genera are mainly parasites of characids across the neotropical biogeographical region. The five species of *Wallinia* and six of the nine species of *Auriculostoma* are exclusively parasites of characids. Two species of the latter genera, *Au. platense* (Szidat 1954) Scholz et al., 2004, and *Au. macrorchis* (Szidat 1954) Scholz et al., 2004 are parasites of siluriforms in Argentina (see Kohn et al. 2007; Ostrowski de Núñez et al. 2017) and the new species we describe herein is a parasite of a gymnotid fish and was found in northwestern Costa Rica. Our results indicate that it would be likely to find a higher diversity of *Auriculostoma* in other Neotropical freshwater fishes and that an integrative taxonomy approach will be needed to identify such species diversity.

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Compliance with ethical standards

All applicable international, national, and/or institutional guidelines for the use and care of animals were followed.

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

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