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ORIGINAL PAPER

A new species of *Wallinia* Pearse, 1920 (Digenea: Allocreadiidae) collected from *Astyanax fasciatus* (Cuvier, 1819) and *A. lacustris* Lucena and Soares, 2016 (Characiformes: Characidae) in Brazil based on morphology and DNA sequences

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Abstract

Wallinia brasiliensis n. sp. is described from the intestine of two species of tetras—*Astyanax fasciatus* (Cuvier, 1819) and *Astyanax lacustris* Lucena and Soares, 2016—collected from the Batalha River in São Paulo State, Brazil. The new species can be clearly distinguished from the other three congeneric species by its vitelline follicles extending from the genital pore to the end of the caeca, eggs lacking operculum, a larger egg size with a consequently lower number relative to the other three species, and the ovary located opposite the anterior testis. The validity of the new species was confirmed through a phylogenetic analysis of the 28S rRNA gene which showed that the new species is the sister taxon to *Wallinia mexicana* Pérez-Ponce de León, Razo-Mendivil, Mendoza-Garfía, Rubio-Godoy and Choudhury, 2015, a species infecting *Astyanax mexicanus* (De Filippi, 1853) in Mexico.

Keywords *Astyanax fasciatus* · *Astyanax lacustris* · *Wallinia* · South America · 28S rDNA

Introduction

The order Characiformes includes 210 genera and 1674 species, and its members inhabit exclusively freshwater habitats (Nelson 2006). The Characidae is the fourth most diversified family (Eschmeyer and Fricke 2009), and its distribution range extends from the Patagonia region of Argentina to the US-Mexico border (Mirande 2010). The two species of characids studied herein have different distributional ranges; according to FishBase (http://www.fishbase.org), *Astyanax fasciatus* is distributed from Argentina to Mexico, though Ornelas-García et al. (2008) argues that the species is not found in Mexico; meanwhile, *A. lacustris* is exclusively distributed across South America, particularly in eastern Brazil. Both species are preferentially omnivorous (Bennemann et al. 2005). At the time of this study, the species *Astyanax altiparanae* Garutti and Britski, 2000 was considered valid, but Lucena and Soares (2016) redescribed two nominal species in Brazil: *Astyanax lacustris* and *A. abramis* (Jenyns, 1842), through comparisons of orbit diameter, the number of anal fin rays and other meristic and morphometric results and recognized the species *Astyanax jacuhiensis* (Cope, 1894); *A. asuncionensis* Géry, 1972; and *A. altiparanae* as new junior synonyms of *A. lacustris*. Therefore, in this study, specimens of *A. altiparanae* have been considered *A. lacustris*.

The trematode fauna of the two characid species in Brazil includes species from five genera *Zonocotyloides* Padilha, 1978; *Genarchella* Travassos, Artigas & Pereira, 1928; *Prosorhynchus* Odhner, 1905; *Prosthenhystera* Travassos, 1922; and *Halipegus* Looss, 1899 and also

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metacercariae from five genera: *Herpetodiplostomum* Dubois, 1936; *Clinostomum* Leidy, 1856; *Tylodelphys* Diesing, 1850; *Ascocotyle* Looss, 1899; and *Antorchis* Linton, 1911 (Thatcher 2006; Kohn et al. 2007; Camargo et al. 2016). There are also records of unidentified parasites belonging to the family Bucephalidae Poche, 1907 (Lizama et al. 2008). However, no members of the family Allocreadiidae (Looss, 1902) Stossich, 1903 have been previously reported.

The genus *Wallinia* Pearse, 1920 is characterized by having morphological traits such as an elongated body, oral sucker without papillae, developed pharynx, and testes in diagonal or tandem position (Choudhury et al. 2002). The species included in this genus were included in several families before they were considered within the family Allocreadiidae (Pérez-Ponce de León et al. 2007).

To date, three species of the genus *Wallinia* Pearse, 1920 have been described, all parasitizing the intestine of freshwater fishes: *Wallinia valenciae* Pearse, 1920 from *Gephyrocharax valencia* (Eigenmann, 1920) in Venezuela (Pearse 1920); *Wallinia chavarriae* Choudhury, Daverdin & Brooks, 2002 from *Astyanax aeneus* (Günther, 1860) and from *Bryconamericus scleroparius* (Regan, 1908) in Costa Rica (Choudhury et al. 2002); and *Wallinia mexicana* from *Astyanax mexicanus* in Mexico (Pérez-Ponce de León et al. 2015).

In this study, we describe a new species of *Wallinia* from the intestines of *A. lacustris* and *A. fasciatus*. We used an integrative taxonomy approach involving morphological data in combination with DNA sequences from the D1-D3 domains of the 28S rDNA gene. By this, we were able to validate the taxonomic status of the two new species and to establish their phylogenetic relationships with other members of the genus. This represents the first record of the genus *Wallinia* in Brazil.

Materials and methods

Between June 2013 and July 2014, 31 *A. fasciatus* specimens and 75 *A. lacustris* specimens were collected from the Batalha River (22° 24′ 46″ S and 49° 08′ 05″ W) in the city of Piratininga, São Paulo State, Brazil. Adult digeneans were removed from the intestine and rinsed in 0.65% saline. Next, some specimens were fixed in absolute ethanol for DNA extraction, and others were fixed using hot (steaming) 4% formalin for morphological identification. Some specimens were stained with Gomori's trichrome and mounted in Canada balsam. Measurements are given in micrometers (μm) unless otherwise stated and are expressed as the range followed by the mean in parenthesis and were obtained using the Motic Images Plus™ software, version 2.0. Drawings were made with the aid of a drawing tube mounted on a Hund Wetzlar H-600 phase contrast microscope. Specimens were deposited at the National Institute of Amazon Research (INPA), located in the city of Manaus, Amazonas State, Brazil.

To ensure the identity of each specimen, the medial portion of each digenean was cut and the anterior portion was kept in lamina for visualization of the morphological characters and use as a molecular voucher (hologenophore; see Astrin et al. 2013). Meanwhile, the posterior half was used for molecular characterization. Genomic DNA was extracted from a portion of a single specimen using a DNeasy® Blood & Tissue Kit (Qiagen) according to the manufacturer's protocol, with final volume of 30 μl. DNA fragments were amplified using a partial 28S rDNA gene containing D1/D3 divergent domains with 28digl2 primers (5-AAG CAT ATC ACT AAG CGG-3′) and 1500 R (5′-GCTATCCTGAGGGAAACTTCG-3′) (Curran et al. 2011). Amplification reactions consisted of 25 μl primary PCR amplifications with 3 μl DNA extract, 0.5 or 1.0 μl of each primer, and Ready-to-Go PCR beads (Pure Taq™ Ready-to-Go™ PCR beads, GE Healthcare). The solution consisted of the stabilizers BSA, dATP, dCTP, dGTP, and dTTP, as well as 2.5 U of puReTaq DNA polymerase and reaction buffer. With the bead reconstituted to a final volume of 25 μl, the makeup and concentration of each dNTP was 200 μM in 10 mM Tris-HCl (pH 9.0 at room temperature), 50 mM KCl, and 1.5 mM MgCl2. PCR products (2 μl) were run on 1% agarose gel using GelRed and loading buffer to confirm amplicon size and yield. PCR products were purified using a QIAquick PCR Purification Kit (Qiagen), and automated sequencing was performed directly on purified PCR products from specimens using a BigDye v.3.1 Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems, Foster City, CA, USA) for cycle sequencing. Sequences were read on an Applied Biosystems ABI 3500 DNA genetic analyzer. Contiguous sequences were assembled and edited using SequencherTM v. 5.2.4 (Gene Codes, Ann Arbor, MI) and submitted to GenBank (accession number MH520995; sequences will be submitted to GenBank after the manuscript is accepted for publication).

Sequences of the 28S rRNA gene of 13 species of allocreadiids were downloaded from GenBank. *Phyllodistomum angulatum* (Linstow, 1907) and *Prosthenhystera obesa* (Diesing, 1850) (AY222206) were used as the outgroup for rooting the tree. Previous phylogenetic analyses have demonstrated that callodistomids (e.g., *P. obesa*) and gorgoderids (e.g., *P. angulatum*) are sister taxa of Allocreadiidae and represent the best choice as outgroups for phylogenetic analyses (see Choudhury et al., 2017; Pérez-Ponce de León et al., 2015). Sequences were aligned using the MUSCLE software (Edgar 2004), implemented in the Geneious Server database, version 7.1.3, using default settings (Kearse et al. 2012) with the extremes of the alignment trimmed. The index of substitution saturation (I_{ss}) was estimated in DAMBE 5 to evaluate the occurrence of substitution

saturation (Xia 2013). The number of base substitutions per site among the sequences was calculated. Standard error estimates were obtained using a bootstrap procedure (2000 replicates). Analyses were performed using the Kimura 2-parameter model. Evolutionary analyses were conducted in MEGA6 (Kimura 1980; Tamura et al. 2013). The best-fit model for nucleotide substitution in the resulting matrix was determined by the Akaike information criterion (AIC) in jModelTest (Posada 2008). Phylogenetic analyses were performed using the Bayesian Inference (BI) implemented in MrBayes (Ronquist and Huelsenbeck 2003), and maximum likelihood (ML) was run in PhyML v3.0 (Guidon et al. 2003). The Bayesian analysis was run with the following nucleotide substitution model settings: lset nst = 6, rates = invgamma, ncat = 4, shape = estimate, inferrates = yes, and basefreq = empirical, which correspond to the GTR+I+G model. For the search on the Markov chain, Monte Carlo (MCMC) chains were run with 10,000,000 generations, saving one tree every 1000 generations. The first 25% generations were discarded as burn-in, and the consensus trees were estimated using the remaining trees. The log-likelihood scores were plotted, and only the final 75% of trees were used to produce the consensus trees by setting the burn-in parameter at 1000 generations. The nodal support for ML was determined by performing 1000 bootstrap replicates; posterior probability values were calculated for BI. The trees were generated and edited in FigTree v1.3.1 (Rambaut 2009).

Results

Allocreadiidae (Looss, 1902) Stossich, 1903
Wallinia Pearse, 1920
Wallinia brasiliensis n. sp.
(Fig. 1a–c)

Description (based on six whole-mounted specimens) Allocreadiidae. Body 1.114–1.165 mm (1.136 mm) long; maximum width 245–299 (257) in ventral sucker region; anterior extremity bluntly rounded; body widening behind oral sucker region, narrowing after testicular field and terminating in narrower tapered end. Remnants of eye spots present at level of pharynx. Oral sucker 114–166 (141) long and 110–166 (134) wide; anteriorly directed oral opening without papillae. Ventral sucker 134–166 (152.9) long and 134–146 (136) wide with rounded opening; ratio of oral sucker length to ventral sucker length 1:0.85–1.15 (1:1). Ratio of oral sucker width to ventral sucker width 1:0.80–0.90 (1:0.9). Prepharynx absent. Pharynx muscular 35–40 (38) long and 41–50 (44) wide. Esophagus narrow, relatively long, sinuous 82–124 (102) long. Gland-cells forming lateral clusters on either side of pharynx reaching anterior third of esophagus in some specimens. Caecal bifurcation short distance anterior to ventral sucker; caeca terminating blindly near posterior end of body 204–278 (254) from posterior end.

Ovary smooth, oval-shaped, immediately posterior to and not overlapping ventral sucker, 95–104 (97.3) long and 46–66 (55) wide and ventrally situated on dextral position; proximal region of ovary containing larger oocytes. Mehlis' gland conspicuous, beside seminal receptacle and ovary and composed of numerous gland cells of various sizes. Seminal receptacle rounded 51–62 (55) long and 53–112 (82) wide with thick muscular wall immediately posterior to ovary. Laurer's canal conspicuous, running laterally and slightly posterolateral and opening outside cecal field at level of anterior testis. Vitellarium follicular, extracecal in two ventrolateral fields extending from level of cecal bifurcation/genital pore to posterior end of body at ends of caeca. Vitelline ducts unite posteriorly to ootype to form vitelline reservoir. Intercecal tubular uterus passing posteriorly through intertesticular space forming convoluted loops extending to posterior end of body. Distal arm of uterus winding anteriorly and traversing pretesticular region medially, forming weakly differentiated terminal ventral metraterm leading to genital pore. Eggs relatively large and ovoid embryonated with no operculum 47–64 (53) long and 31–40 (35) wide ($n = 20$). Genital pore median between cecal bifurcation and anterior border of ventral sucker.

Testes two, oblique and ellipsoidal, intercecal, with smooth margins; anterior testis 97–117 (109) long and 65–88 (76) wide; posterior testis 107–136 (127) long and 74–102 (86) wide, 363–413 (390) from posterior extremity of body; distance between testes 188–382 (299). Cirrus-sac elongated, median and dorsal, overlapping ventral sucker and extending slightly posterior to margin of ventral sucker with maximum width of 34–64 (50), containing muscular sinuous ejaculatory duct lined with cupola-like structures forming middle circles on internal wall of duct and surrounded by scattered gland cells; pars prostatica indistinct, rounded; seminal vesicle folded, tubular, occupying most of cirrus-sac; cirrus not observed. Excretory vesicle I-shaped. Excretory pore terminal.

Taxonomic Summary Type host: *Astyanax fasciatus* (Cuvier, 1819) (Actinopterygii: Characidae)

Other hosts: *Astyanax lacustris* Lucena & Soares, 2016 (Actinopterygii: Characidae)

Location: Batalha River, located on the border between the cities of Bauru and Piratininga (22° 24′ 46″ S; 49° 05′ 05″ W)

Site of infection: Intestine

Number of hosts: *Astyanax fasciatus* (31) and *Astyanax lacustris* (75)

Prevalence: *Astyanax fasciatus* (16.13%) and *Astyanax lacustris* (9%)

Mean intensity: *A. fasciatus* (two worms per infected host) and *A. lacustris* (six worms per infected host)

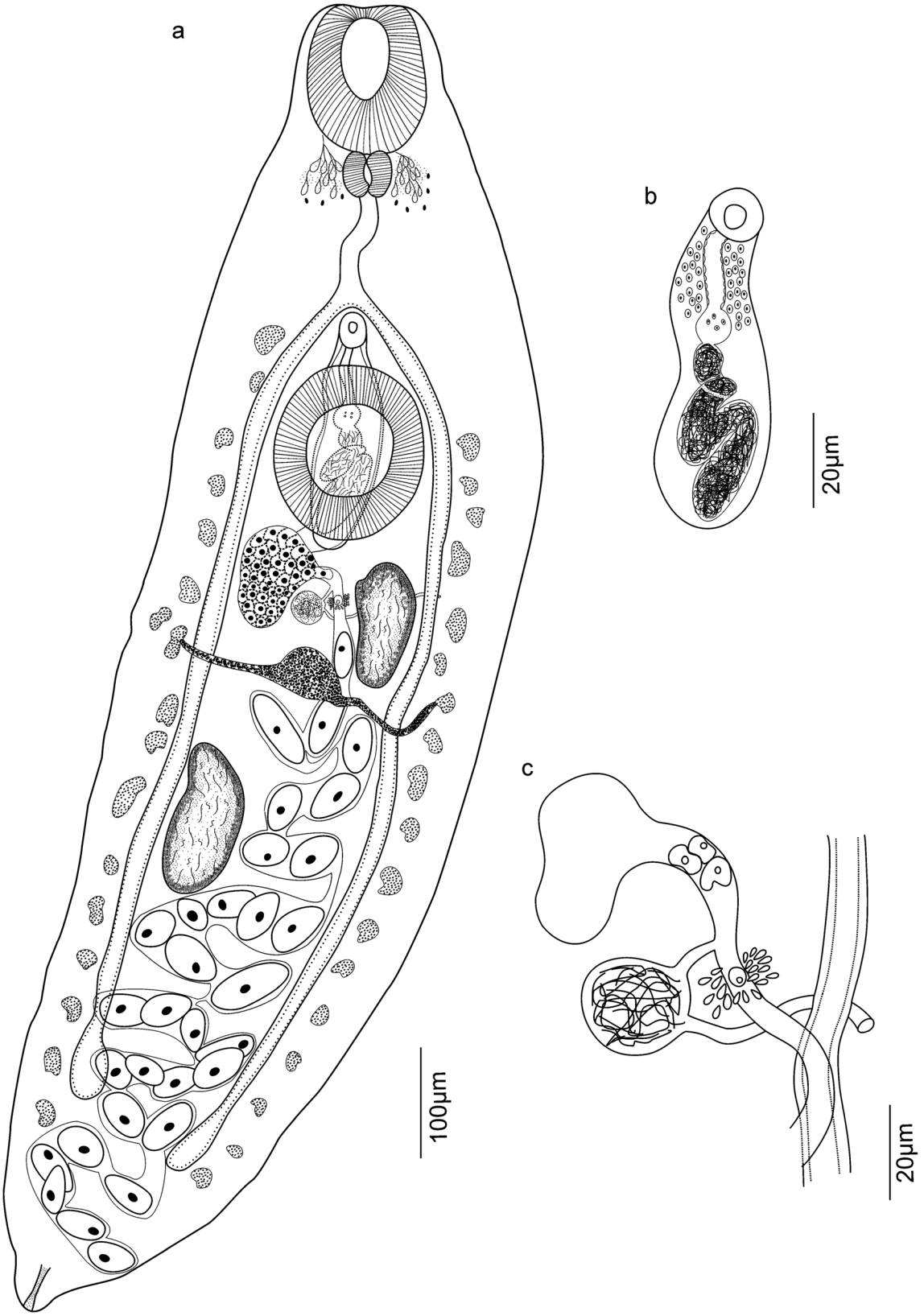

Fig. 1 *Wallinia brasiliensis* n. sp. from *Astyanax lacustris* and *Astyanax fasciatus*. **a** *Astyanax lacustris* holotype: ventral view of whole mount specimen. **b** Ventral view of the cirrus sac of a paratype from *A. fasciatus*. **c** Female reproductive complex from an *A. lacustris* holotype

Specimens deposited: holotype and paratype specimens and molecular voucher deposited in the Zoological Collection at the INPA under holotype number INPA 782 and paratype numbers INPA 783 and molecular voucher INPA 784 (will be deposited after manuscript acceptance).

Etymology: The specific epithet *brasiliensis* refers to the country (Brazil) where the parasite was discovered.

Remarks

Wallinia brasiliensis n. sp. has been allocated to the genus *Wallinia* because it exhibits morphological characteristics typical of this allocreadiid genus, e.g., unspined tegument, median genital pore, well-developed cirrus-sac, and the uterus passes between the testes on its way to occupying an extensive zone of the postesticular area (Pearse 1920; Choudhury et al. 2002).

Wallinia valenciae was the first species described from this genus as a parasite of the characid *Gephyrocharax valenciae* in Lake Valencia, Venezuela (Pearse 1920). The description was based on a single specimen (holotype USNPC 7569), which makes it difficult to compare the description to the other species described as belonging to the genus *Wallinia*. Two additional congeneric species have been described thus far: *W. chavarriae* from Costa Rica and *W. mexicana* from Mexico.

The new species proposed here possesses morphological traits that set it apart from the three other described species. First, *W. brasiliensis* n. sp. is the smallest species in the genus: its body length ranges from 1.114 to 1.165 mm (1.136 mm), while the lengths of *W. chavarriae*, *W. mexicana*, and *W. valenciae* range from 2.250 to 3.350 mm (2.739 mm), from 2.520 to 3.420 mm (2.880 mm), and 1.340 mm (contracted specimen), respectively. Egg size in the new species is larger than in the other species, and *W. brasiliensis* n. sp. exhibits a relatively lower number of eggs which are not operculate. Further, the maximum body width is at the testes level in *W. chavarriae* and *W. mexicana*, whereas the new species' maximum width is at the level of the ventral sucker, as in the case of *W. valenciae*. *Wallinia brasiliensis* n. sp. also differs in the posterior extension of the vitelline follicles: the follicles extend from the genital pore to the ends of the caeca, while in the other three species, the vitelline follicles are more restricted. In *W. valenciae*, the follicles extend to the end of the ovary, while in *W. chavarriae*, they extend to the level of the posterior testis, and in *W. mexicana*, they extend to the halfway point between the posterior margin of posterior testis and the end of caeca; in the new species, as in *W. valenciae*, the vitellarium is extracecal, while in the other two species, the follicles are extracecal and intracecal; finally, the anterior testis of *W. brasiliensis* n. sp. is located at the same level as the ovary and on opposite sides, while in the other three species, the ovary is always clearly oblique relative to the anterior testis.

Molecular characterization

Only one specimen was sequenced successful for DNA. We are aware that unreplicated sequences can be problematic

Table 1 Genetic divergence (Kimura-2-parameter, expressed in percent) estimated using partial 28S rDNA among Allocreadiidae species retrieved from GenBank and *Wallinia brasiliensis* n. sp. described in this study. The species *Phyllodistomum angulatum* (KJ729531) and *Prosthenhystera obesa* (AY222206) were used as an outgroup

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1- *Phyllodistomum angulatum*																		
2- *Prothenhystera obesa*	21																	
3- *Allocreadium isoporum*	21	13																
4- *Allocreadium lobatum*	20	11	04															
5- *Bunodera* sp.	22	11	08	07														
6- *Bunodera lucioperca*	22	12	08	07	01													
7- *Creptotrematina aguirrepequenoi*	20	13	09	08	08	08												
8- *Paracreptotrematoides heteriandriae*	21	13	09	08	07	07	04											
9- *Megalogonia ictaluri*	22	12	07	07	06	06	04	03										
10- *Crepidostomum illinoiense*	20	08	07	06	06	06	05	04	03									
11- *Crepidostomum cornutum*	20	12	07	07	05	05	05	03	03	02								
12- *Wallinia mexicana*	22	14	10	09	08	08	05	04	05	06	05							
13- *Wallinia mexicana*	22	14	10	09	08	08	05	04	05	06	05	00						
14- *Wallinia brasiliensis* n. sp.	22	14	10	09	09	09	05	05	05	06	05	04	04					
15- *Wallinia chavarriae*	21	13	09	08	07	07	04	04	04	04	04	03	03	03				
16- *Auriculostoma totonacapanensis*	21	14	10	08	08	08	05	05	05	06	05	05	05	05	04			
17- *Auriculostoma astyanace*	21	12	09	07	07	07	04	04	03	04	04	04	04	04	03	02		
18- *Auriculostoma astyanace*	21	12	09	07	07	07	04	04	03	04	04	04	04	04	03	02	00	

and that having more than one consensus sequenced is ideal for taxonomic decision. In this case, specimens were collected and fixed for morphology and only one specimen was fixed for DNA work. The final 28S ribosomal gene alignment consisted of 18 sequences representing 13 allocreadiid species from the Americas. The final alignment was 1082 bp long. The percentage of K2P pairwise identity between the *Wallinia* species and the putative new species was 4% when it was compared to *W. mexicana*, and 3% relative when it was compared to *W. chavarriae* (Table 1). The genetic divergence between *W. brasiliensis* and other allocreadiid genera ranged from 3 to 10% (Table 1). The I_{ss} indicated no saturation in either transitions or transversions. Critical index of substitution saturation ($I_{ss.c}$) values were greater than the I_{ss} values. The ML and Bayesian phylogenetic inference methods recovered identical tree topologies with well-supported values in basal and terminal nodes (Fig. 2), with the exception of the clade that includes *Wallinia* and *Auriculostoma* species (Fig. 2). *Wallinia brasiliensis* n. sp. nested as the sister species of *W. mexicana*, and *W. chavarriae* is the sister species to the other two species.

Discussion

The genus *Wallinia* has been included in the family Allocreadidae, despite its controversial taxonomic history, which placed it either as a member of the family Allocreadiidae or the family Macroderoididae McMullen, 1937. Pérez-Ponce de León et al. (2007) showed that the genus *Wallinia* represents bona fide allocreadiids and not macroderoidids. The current geographic distribution of most members of the genus *Wallinia* is largely restricted to Middle America, though *W. valenciae* was described as a parasite of *G. valenciae* in Lake Valencia, Venezuela (Pearse 1920). The other two described species are *W. chavarriae*, distributed in the Area de Conservación de Guanacaste, Costa Rica (in the characids *B. sceloparius* and *A. aeneus*), and more recently, it was found in *Geophyrocharax atricaudatus* in Panama; *W. mexicana* was recorded as a parasite of *A. mexicanus* in two localities across central Mexico (Pérez-Ponce de León et al. 2015; Choudhury et al. 2017). *Wallinia brasiliensis* n. sp. is the fourth species described for the genus *Wallinia* and, similar to the other species, it was found in the intestine of small-

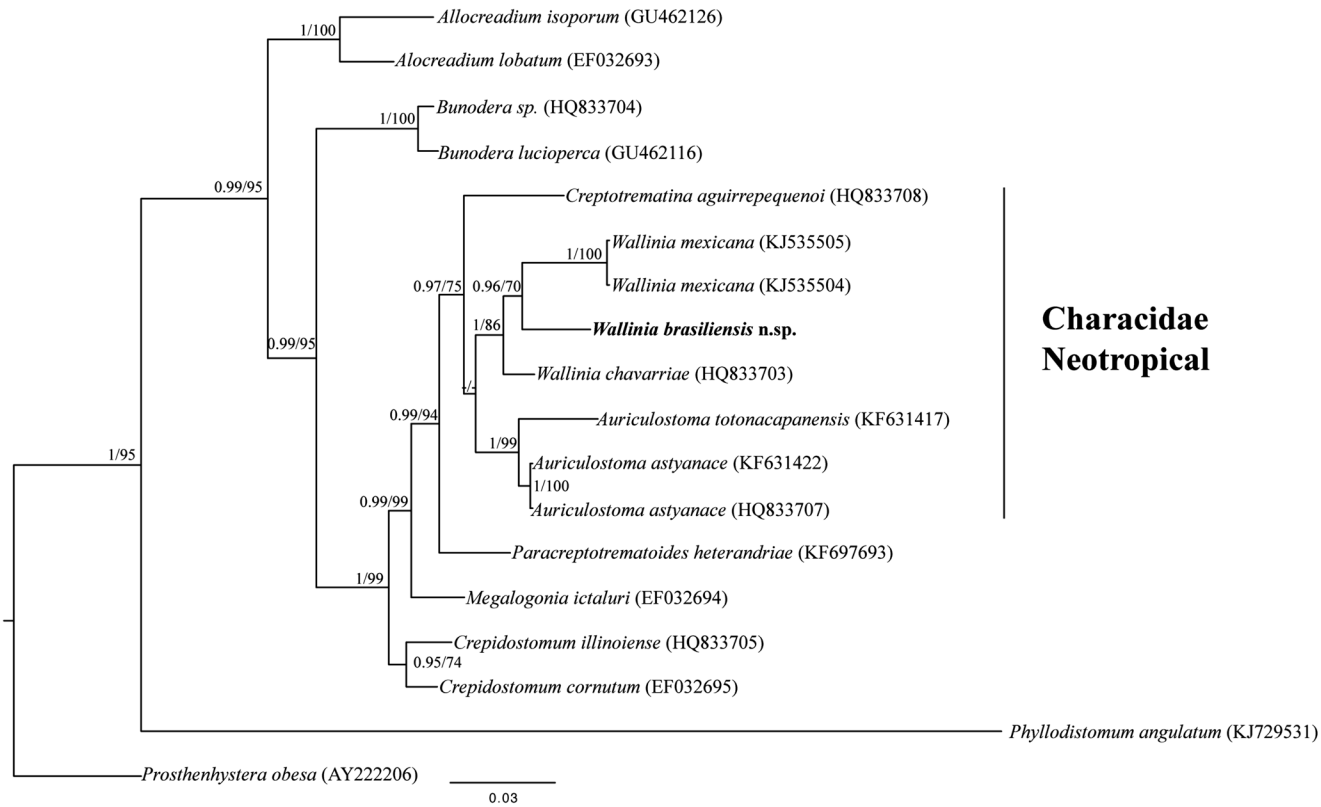

Fig. 2 Bayesian topology based on partial 28S ribosomal DNA sequences of some members of the Allocreadiidae. GenBank accession numbers are next to species names. Support values are above the nodes as follows: posterior probabilities for the Bayesian analyses and bootstrap for maximum likelihood analyses. The best-fit model used for both analyses was GTR+I+G. Dashes represent branches not supported by analyses (posterior probabilities < 0.90 and bootstrap scores < 70). Branch length scale bar indicates number of substitutions per site. The species *Phyllodistomum angulatum* (KJ729531) and *Prosthenhystera obesa* (AY222206) were used as an outgroup

bodied characids (*A. lacustris* and *A. fasciatus*). Both species of characids are found only in South America; the former is endemic to the Paraná River basin, and the latter has a wider distribution range across South America (Galvis et al. 1997; Reis et al. 2003).

Our findings corroborate the argument that *Wallinia* species are part of the biogeographical core helminth parasite fauna of characids (Pérez-Ponce de León and Choudhury 2005). These authors argued that parasites show characteristic associations with particular lineages of hosts (families, orders, etc.) and that the persistence of core faunas in different areas of the distributional range of these lineages suggests that historical biogeographical processes have shaped host-parasite associations. In this context, species of *Wallinia* have only been found in members of the family Characidae, and more commonly in *Astyanax* spp. across a geographic range that comprise South and Middle America, corresponding to the geographic range of these freshwater fishes. Whether or not species of *Wallinia* will be found in other characids needs to be determined by increasing sampling efforts in the future. Some other species of small-bodied characids have been studied in South America (Kohn et al. 2007), but detailed survey work may result in finding the same species, or probably discovering other undescribed species of the genus.

Considering the fact that previous studies highlight the importance of molecular studies and phylogenetic analyses to correctly assign a species to its genus (Pérez-Ponce de León and Choudhury 2010), future taxonomic studies will need to use DNA sequence data to corroborate morphological differences between species or to uncover the existence of cryptic species complexes (Pérez-Ponce de León and Nadler 2010).

Finally, phylogenetic analysis using 28S rRNA gene corroborates our findings on the evolutionary relationships among allocreadiids that commonly infect characids, such as those reported by Curran et al. (2011) and Razo-Mendivil et al. (2014). Our analysis provides evidence of a close relationship between *Wallinia* and *Auriculostoma* species, although this result is not well-supported by bootstrap and posterior probability values. Sequence data on the type of species (*W. valenciae*) are necessary to establish a more robust phylogenetic framework required to further discuss the evolutionary relationships among species of *Wallinia*. Unfortunately, we were not able to amplify with good quality the DNA of only one specimen for the phylogenetic analyses, because no more specimen was sampled in the locality.

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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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