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Research paper

# Integrative taxonomy of *Goezia spinulosa* (Nematoda: Raphidascarididae) from arapaimas in the northwestern Brazil

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ABSTRACT

*Arapaima gigas*, a fish with a high market value, has been farmed in different localities within Brazil. Among its parasites, adults of *Goezia spinulosa* are reported to cause ulcers in the stomach and to result in the death of farmed fingerlings. Due to the veterinary importance of this nematode in cultured arapaimas, an integrative taxonomic study is proposed, combining morphological, ultrastructural and genetic profiles of this parasite. The fish were obtained from semi-intensive fish farming in Acre State, Brazil. The fish measured 7–42 cm in total length and the intensity of infection was 1–60 parasites per fish. The site of infection was mainly the stomach. Morphological and ultrastructural analyses of *G. spinulosa* showed the importance of its spiny body in firmly attaching the worm to the gastric mucosa, resulting in lesions, ulcers and deep gastric perforations of the stomach wall. New sequences for partial 18S rDNA, ITS1, 5.8S and ITS2 rDNA, partial 28S rDNA, *cox1* mtDNA and for *cox2* mtDNA are presented. Phylogenetic reconstructions based on the partial 18S and 28S rDNA shows species of *Goezia* occur in a clade well separated from other genera in both analyses. Both the partial 18S and 28S rDNA genes represented good genetic markers for distinguishing genera of the Raphidascarididae, with exception of *Hysterothylacium*. This integrated taxonomic study produced a robust profile for *G. spinulosa* that will aid the diagnosis of both adults and larval stages from arapaimas and possible intermediate hosts.

## 1. Introduction

The nematode *Goezia spinulosa* (Diesing, 1839) has been reported parasitizing *Arapaima gigas* (Schinz, 1822) in different geographical localities of Brazil (Moravec, 1998; Thatcher, 2006; Santos and Gibson, 2007; Araujo et al., 2009; Santos and Moravec, 2009; Eiras et al., 2010; Marinho et al., 2013; Andrade-Porto et al., 2015 and Silva et al., 2016). Adults of *G. spinulosa* are reported to cause ulcers in the stomach of their fish hosts and to cause the death of farmed fingerlings (Santos and Moravec, 2009).

Recently, we studied the helminth community structure of *A. gigas* from semi-intensive and intensive culture systems in the State of Acre, Brazil (Silva et al., 2016). These results indicated that fish from the semi-intensive system had nine parasite species with significantly higher levels of infection than those from the intensive system, where only a single monogenean species was found. In the semi-intensive system, the prevalence of *G. spinulosa* was 29.7% (Silva et al., 2016).

Due to the veterinary importance of *G. spinulosa* in farmed arapaimas, an integrative taxonomic study, including morphological, ultrastructural and genetic data, was undertaken in order to better characterize this nematode parasite in arapaimas and thus contribute to a better specific diagnosis of adult and larval stages.

## 2. Materials and methods

### 2.1. Ethics statement

This study was authorized by the Brazilian Institute of Environment and Renewable Natural Resources (IBAMA, license no. 39106/2013) in accordance with the guidelines of the Brazilian College for Animal

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Experiments (COBEA).

### 2.2. Study areas and collection of parasites

The fish were obtained from Fazenda Boa Esperança, a semi-intensive fish farm, in the municipality of Bujari (9°45′24.5″S 68°04′25.0″W), Acre State, associated with the southwestern reaches of the Amazon. The organs of 64 arapaimas were examined in saline medium under a stereomicroscope. Any parasites recovered were fixed in 70% ethanol. Some specimens were cleared and examined as temporary mounts using glycerine. Drawings were made with the aid of a drawing tube and redrawn using Adobe Illustrator CS6. Measurements are presented in micrometres as the range followed by the mean in parentheses, unless otherwise stated. Specimens were deposited at the Helminthological Collection of Instituto Oswaldo Cruz, Brazil, no. 38.520.

### 2.3. Scanning electron microscopy (SEM)

The specimens were fixed in 4% formaldehyde solution, washed in 0.1 M sodium cacodylate buffer and post-fixed overnight in a solution 1% osmium tetroxide and potassium ferrocyanide 0.8% in the dark. Specimens were then dehydrated in an alcohol series (30–100%), critical point dried with $CO_2$, coated with gold and observed in a JEOL JSM 6390 LV SEM microscope.

### 2.4. Genetic analysis

The genomic DNA was extracted using a QIAamp DNA Mini Kit (QIAGEN) according to the manufacturer's recommendations. The partial region spanning the 18S rDNA was amplified by PCR using set of primers SSU18A (5′- AAAGATTAAGCCATGCATG −3′) and SSU26R (5′- CATTCTTGGCAAATGCTTTC −3′) with the PCR following Floyd et al. (2002). The rDNA region spanning the ITS1, 5.8S and ITS2 was amplified by PCR using the primers NC5 (5′- GTAGGTGAACCTGCGG-AAGGATCATT-3′) (Zhu et al., 1998) and BD2 (5′-TATGCTTAARTTCA-GCGGGT-3′) (Luton et al., 1992) under the following conditions: 94 °C for 3 min, followed by 40 cycles of 95 °C, 30 s, 55 °C, 30 s, 72 °C, 60 s and 72 °C for 7 min. The partial region spanning 28S rRNA was amplified by PCR using the primers C1 (5′-ACCCGCTGAATTTAAGCA-T-3′) and D2 (5′-TGGTCCGTGTTTCAAGAC-3′) (Hassouna et al., 1984; after Chisholm et al., 2001) under the following conditions: 95 °C for 5 min, 40 cycles at 95 °C, 60 s, 56.2 °C, 45 s, 72 °C, 60 s and 72 °C for 5 min. The mtDNA citochrome oxidase subunit 1 (*cox1*) region was amplified by PCR using a set primer “cocktail” as described in Prosser et al. (2015). The mtDNA citochrome oxidase subunit 2 (*cox2*) region was amplified by PCR using 210 (5′- CACCAACTCTTAAAATTATC −3′) and 211 (5′- TTTTCTAGTTATATAGATTGRTTTYAT −3′) (Nadler and Hudspeth, 2000). PCR assays were carried out in a total volume of 15 μl containing 7.5 μl of 2 × GoTaq® Colorless Master Mix (Promega), 0.5 μl $Mg^{2+}$ (50 mM concentration), 1.5 μl of each primers with final concentration at 0.5 μM, 2.0 μl of cDNA sample and ultrapure water to complete.

PCR products were visualized after electrophoresis on a 1.5% agarose gel stained with SYBR green (Invitrogen). Amplified PCR products were purified with ExoSAP-IT (Affymetrix) following the manufacturer instructions. DNA cycle sequencing reactions were performed using BigDye v.3.1 chemistry (Applied Biosystems). The sequencing was performed in an ABI Prism 3100 sequence analyser using the same primer set. Sequences of both strands were generated, edited and aligned using the CLUSTAL W algorithm of the MEGA 6.0 package (Thompson et al., 1994; Tamura et al., 2011). The comparison for similarities with sequences from GenBank was performed using BLAST 2.0 (Altschul et al., 1990).

**Table 1**
List of the species of Raphidascarididae and outgroup used in the phylogenetic reconstructions using sequences of the partial 28S and 18S rDNA genes.

| Species | GenBank access numbers | |
|---|---|---|
| | 28S | 18S |
| *Goezia spinulosa** | KY198734 | KY198732 |
| *Goezia spinulosa* | – | JF803924 |
| *Goezia pelagia* | U94758 | U94372 |
| *Hysterothylacium fortalezae* | U94760 | U94374 |
| *Hysterothylacium pelagicum* | U94761 | U94375 |
| *Raphidascaroides moraveci* | KP726279 | KP726278 |
| *Raphidascaroides brasiliensis* | KP726275 | KP726274 |
| *Raphidascaris lanfrediae* | KX859075 | KX859076 |
| *Hysterothylacium reliquens* | U94762 | U94376 |
| *Hysterothylacium reliquens* | KX815300 | – |
| *Heterocheilus tunicatus* | U94759 | U94373 |

### 2.5. Phylogenetic analysis

The best-fit substitution model of partial 18S and 28S dataset was the GTR + I + G model of nucleotide substitution selected under the Akaike information criterion (AIC) using MrModelTest 2 with the aid of PAUP4.0a147 (Nylander, 2004). The dataset was aligned using the E-INS-i algorithm of the program MAFFT (Katoh et al., 2002). Tree reconstructions were carried out using Bayesian Inference (BI) via the software MrBayes 3.2.6, where the Markov chain Monte Carlo (MCMC) was set to $4 \times 10^6$ generations, with sampling frequency every $4 \times 10^3$ generations and discarding the initial 1/4 of sampled trees ($1 \times 10^6$) as burn-in (Pereira and Luque, 2016). The remaining trees were used to generate a consensus tree and to calculate the Bayesian posterior probabilities (Bpp) of all branches using a majority-rule consensus approach. Maximum-likelihood (ML) analysis was constructed with PhyML 3.1 and nodal support was estimated by performing 1000 bootstrap replicates (Swofford, 2002). Trees were rooted by *Heterocheilus tunicatus*, based upon previous phylogenies of the Ascaridoidea using similar genes (Nadler and Hudspeth 1998, 2000). Tree topologies were visualized in FigTree 1.4.2 (2014). Taxa, for which sequences from GenBank were used for phylogenetic analysis, are listed in Table 1.

## 3. Results

### 3.1. Fish and intensity of infection

The fish measured 7–42 cm in total length and weighed 2–392 g. The site of infection was mainly the stomach, less often the intestine. The prevalence was 29.69%, and the intensity of infection with *Goezia spinulosa* was 1–60 parasites per fish.

### 3.2. *Goezia spinulosa* (Diesing, 1839)

#### 3.2.1. Male (measurements of 12 specimens)

Length of body 7.8–16.6 (10.9) mm; maximum width 425–720 (595). Length (height) of lips 35–70 (50); width of body at level of lips 140–220 (169) (Figs. 1 A and B, and 2 A and B ). Oesophagus 500–900 (727) long (4–9% of body length) (Fig. 1A and B). Nerve-ring, excretory pore and deirids 170–350 (248), 200–245 (220) and 260–580 (402), respectively, from anterior extremity (Figs. 1A and B). Ventriculus 60–150 (96) × 50–175 (103); ventricular appendix 2.0–2.9 (2.4) mm long (Figs. 1A and B). Intestinal caecum 100–350 (199) long (Fig. 1A and B). Length ratio of caecum and ventricular appendix 1:10–24, that of caecum and oesophageal length 1:2–7. Spicules alate, equal, 350–630 (493) long (3.0–6.0% of body length) (Figs. 1 C and 2 C and D). Genital papillae sessile, 21 pairs: pre-cloacal pairs 19–21 divided into 2 series; smallest with 7–8 lateral pairs closer to cloaca and anterior

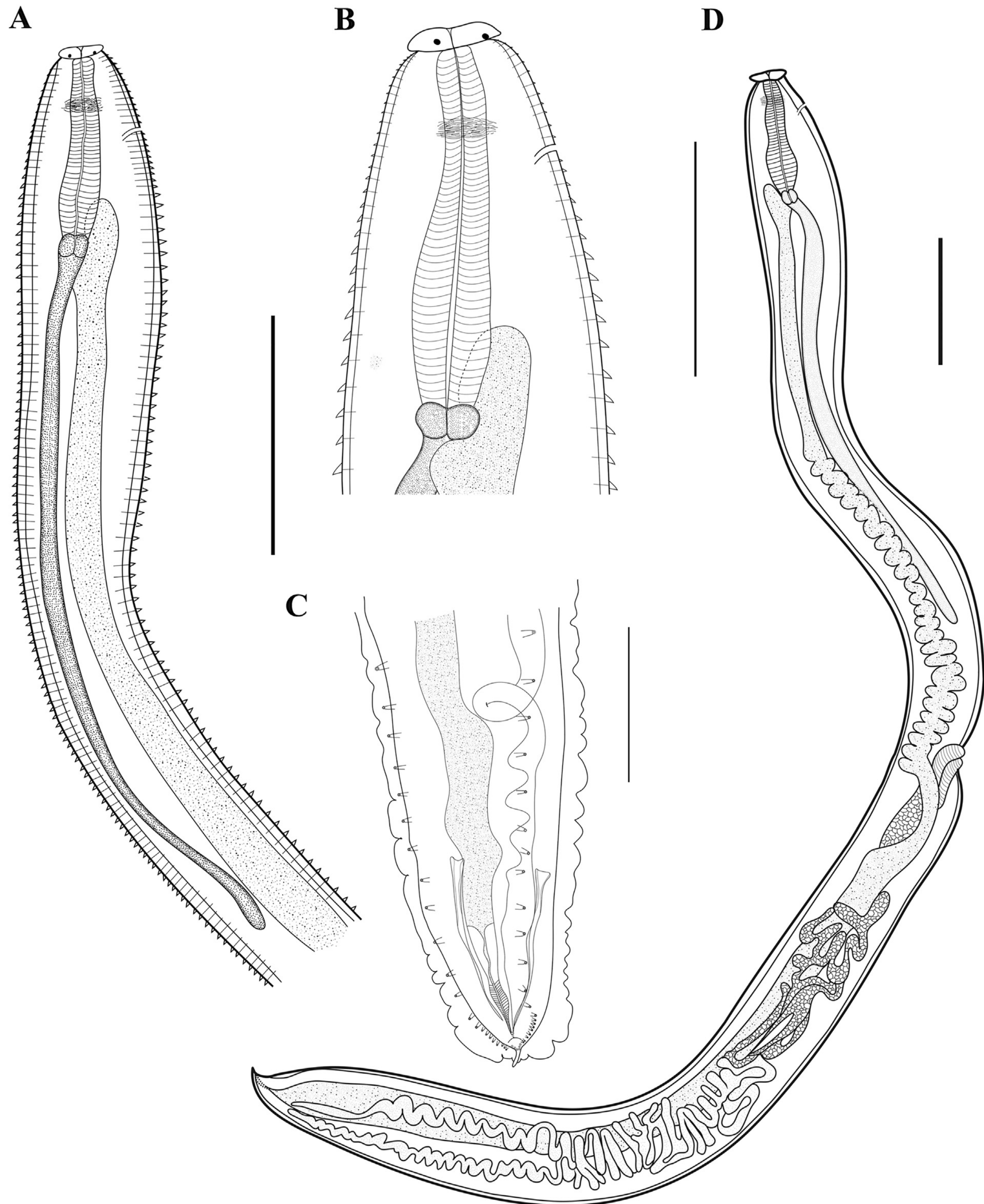


**Fig. 1.** *Goezia spinulosa* (Diesing, 1839). A − anterior end of body, showing intestinal caecum, lateral view; B − detail of anterior end of body, showing nerve-ring and excretory pore, lateral view; C − posterior end of male, ventral view; D − female, whole worm. Scale bars: A and D = 1000 μm; B and C = 500 μm.

series with 12–13 larger pairs; single unpaired plus single pair of adanal papillae; and 4 pairs of postanal papillae (Figs. 1 C and 2 C and D). Tail conical, 65–125 (93) long (Figs. 1 C and 2 C and D).

### 3.2.2. Female (measurements of 9 specimens)

Body spined, 10.0–17.0 (12.1) mm long, 200–810 (562.2) wide (Fig. 1D). Length (height) of lips 35–60 (48.6). Width of body at level of lips 100–195 (169.7) (Fig. 1A). Oesophagus 0.7–1.03 (0.8) mm long (6–8% of body length). Nerve-ring, excretory pore and deirids 150–250 (188.5), 190–270 (227.8) and 350–425 (393.7), respectively, from anterior extremity (Fig. 1D). Ventriculus 65–120 (101.2) × 50–230 (98.1); ventricular appendix 2.2–3.1 (2.4) mm long. Intestinal caecum 150–450 (206.8) long. Ratio of caecum and ventricular appendix 1:12–19, that of caecum and oesophageal length 1:2.0–7.0. Vulval opening 3.3–9.0 (5.5) mm or 34–53% of body length from anterior extremity; vulval lips not elevated (Fig. 1D). Vagina directed posteriorly

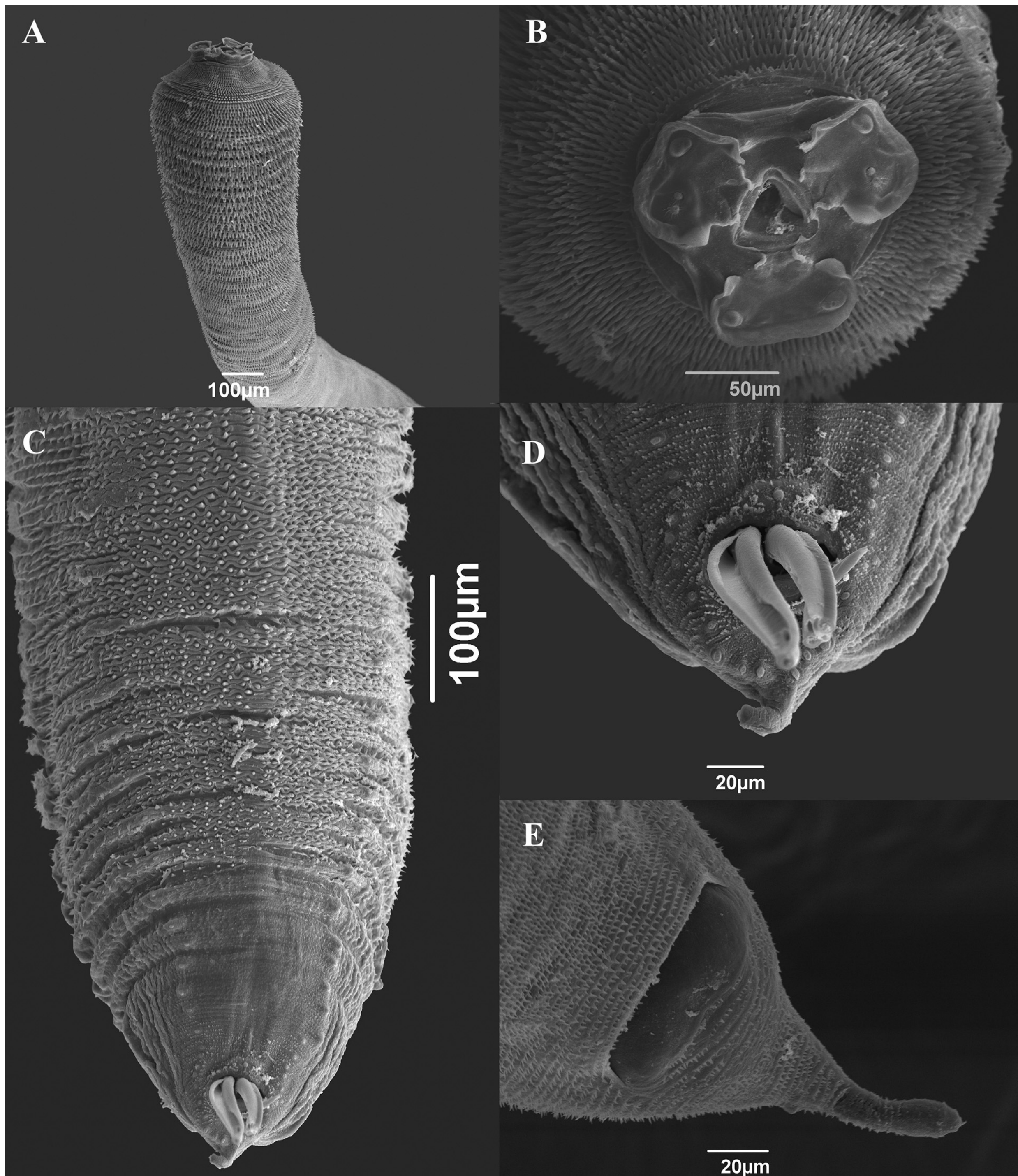


**Fig. 2.** *Goezia spinulosa* (Diesing, 1839), scanning electron micrographs. A − anterior end of body, lateral view; B − cephalic end, apical view; C − posterior end of male, ventral view; D − detail of male tail showing papillae distribution and spicules; E − female tail, ventral view.

from vulva. Eggs almost spherical, 45–50 (48.3) in diameter. Tail conical, 130–210 (167.7) in length (Fig. 2E).

### 3.3. Ultrastructure

Body covered with thick cuticle armed with transverse rows of spines, enlarged in anterior region but with constriction close to lips (Fig. 2A). Lips approximately equal in size, with alate margins; inner part of each lip with 2 distinct lobes oriented towards oral aperture (Fig. 2B). Dorsal lip with 2 double papillae; ventrolateral lips each with single lateral amphid, 1 single papilla and 1 double papilla (Fig. 2B). Oral aperture triangular (Fig. 2B). Size of spines on body of male decrease towards cloacal aperture; spicules alate, forming well delimited gutter for sperm transfer (Fig. 2C and D). Precloacal papillae distributed along ventro-lateral sides of body; single unpaired precloacal papilla visible on anterior margin of cloaca; as single pair of adanal papillae; postanal papillae disposed in two subventral rows (Fig. 2D). Tail of female is spined as far as terminal digitiform process (Fig. 2E).

### 3.4. Genetic data

The new sequences were deposited in GenBank under the accession

numbers: KY198732for the partial 18S rDNA region with 899 bp; KY198733 for the ITS1, 5.8S and ITS2 rDNA with 987 bp; KY198734 for the partial 28S rDNA with 717 bp; KY198735 for the *cox1* mtDNA with 618 bp; and KY198736 for the *cox2* mtDNA with 542 bp.

The BLAST result for the partial 18S rDNA region indicated a 100% identity with 95% query cover and a maximum score of 1580 for *G. spinulosa* (JF803924) and a 99% identity with 100% query cover and a maximum score of 1650 for *Goezia pelagia* (U94372). The ITS1, 5.8S and ITS2 rDNA sequences indicated a 92% identity with 57% query cover and a maximum score of 795 for *Hysterothylacium* sp. (KU705468). For the partial 28S rDNA sequences, the BLAST indicated a 97% identity with 100% query cover and a maximum score of 1195 for *G. pelagica* (U94758). For the *cox1* mtDNA sequences, it indicated a 89% identity with 98% query cover and a maximum score of 686 for *Toxascaris leonina* (KC902750). For the *cox2* mtDNA sequences it indicated a 87% identity with 99% query cover and a maximum score of 636 for *G. pelagia* (AF179912).

### 3.5. Phylogenetic analysis

Our sequences of partial 18S and 28S rDNA were aligned with other similar sequences of members of the Raphidascarididae available from GenBank. The topology of the phylogenetic trees generated using BI and ML were similar. In general view, BI appears to have the major values with better statistical support than ML (Figs. 3 and 4 ).

The phylogenetic reconstruction based on the partial sequence spanning the 18S rDNA shows that our sequence of *G. spinulosa* is grouped with *G. spinulosa* (JF803924) and *G. pelagia* (U94372), with a statistical support of BI = 0.99 and ML = 89%. Each genus studied appears to cluster in the same clade, with the exception of *Hysterothylacium* that clustered in different clades (Fig. 3). On the partial 28S rDNA tree, our sequence of *G. spinulosa* is grouped with *G. pelagia* (U94358) (BI = 1 and ML = 100%). However, *Hysterothylacium* again appears in a different clade (Fig. 4). The species of *Goezia* appear in a clade well separated from the other genera in both phylogenetic reconstructions.

### 3.6. Pathogenicity

Multiple lesions were observed in the stomach of the arapaimas due to the presence of *G. spinulosa.* These parasites were strongly attached to the gastric mucosa and the spined body caused ulcers and deep gastric perforations that penetrate the stomach wall (Fig. 5A and D).

## 4. Discussion

*Arapaima gigas*, a fish with a high market value, has been farmed in different localities in Brazil. Recently, we reported the first occurrence of *Goezia spinulosa* in Acre State (Silva et al., 2016). Previously, this species has been reported from *A. gigas* in the states of Amazonas (Travassos et al., 1928 and Araújo et al., 2009), Pará (Santos and Moravec, 2009) and Mato Grosso (Santos et al., 2008 and Menezes et al., 2011).

*Goezia spinulosa* was redescribed by Santos and Moravec (2009), based on material from the delta of the Amazon, as having 21 pairs of sessile papillae, with 16 precloacal pairs of different sizes, an unpaired

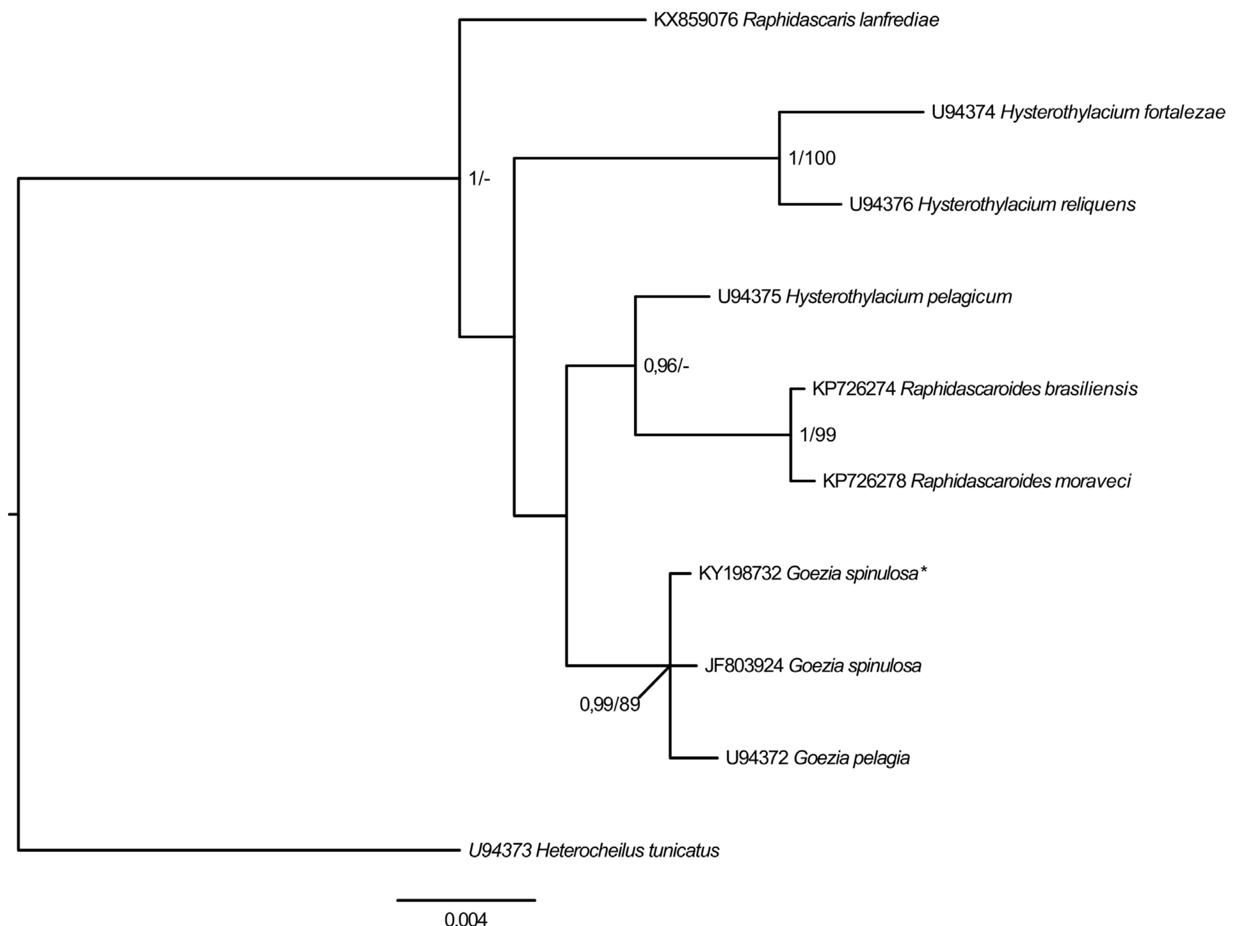


**Fig. 3.** Phylogenetic reconstruction generated from Bayesian Inference analysis of sequences of the 18S of members of the Raphidascarididae. First number of branch support gives the Bayesian posterior probability ($4 \times 10^6$ generations, sampling frequency = $4 \times 10^3$, burn-in = $1 \times 10^6$); second indicates the maximum likelihood (ML) bootstrap values (1000 replications). Asterisk specifies species from the present study.

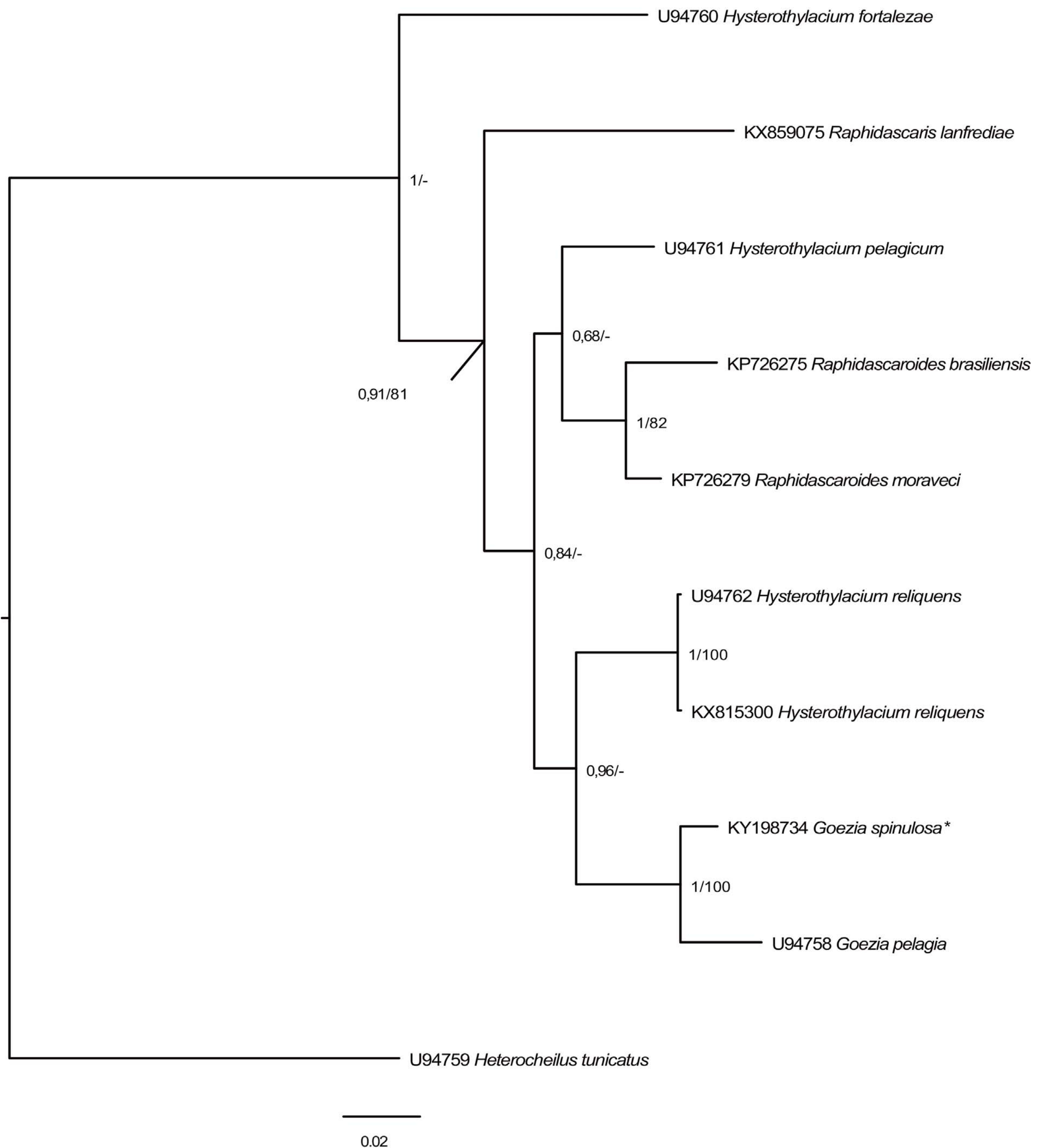


**Fig. 4.** Phylogenetic reconstruction generated from Bayesian Inference analysis of sequences of the 28S rDNA of members of the Raphidascarididae. First number of branch support gives the Bayesian posterior probability ($4 \times 10^6$ generations, sampling frequency = $4 \times 10^3$, burn-in = $1 \times 10^6$); second indicates the maximum likelihood (ML) bootstrap values (1000 replications). Asterisk specifies species from the present study.

precloacal papilla, one pair of adanals and four pairs of postcloacals. The ultrastructure of *G. spinulosa* from the Acre State, in the northwestern Brazilian region, showed that the number and disposition of male papillae vary but are in accordance to those reported by Costa et al. (1995). *G. brasiliensis* Moravec et al., 1994; *G. brevicaeca* Moravec et al., 1994 and *G. leporini* Martins and Yoshitoshi, 2003, reported as parasitizing different fish species in Brazil, were differentiated by having 14, 24 and 24–29 pairs of papillae, respectively (Moravec et al., 1994; Martins and Yoshitoshi, 2003 and Santos and Moravec, 2009). However, if we consider that a specific variation in the number of papillae in the male may occur, the genetic profile of congeneric species would help to better differentiate these species.

In the present study, the body length of females of *G. spinulosa* was smaller in comparison to those reported by Santos and Moravec (2009) from arapaimas in fish farms at Mexiana Island, possibly indicating different stages of maturation. The differences observed can also be a result of intraspecific variability or of fish host age, as in our study only fingerlings were analyzed.

The profile of *G. spinulosa* from *A. gigas* is now better characterized by the addition of new genetic sequences of partial 28S rDNA, ITS1, 5.8S, ITS2, *cox1* and *cox2* mtDNA. This molecular prospecting as discussed by Nadler and Pérez-Ponce de León (2011) is important not only for future studies and for comparisons with other species, but also for giving support to applied research to improve development and understanding of diagnostics, control and potential eradication of parasitic diseases.

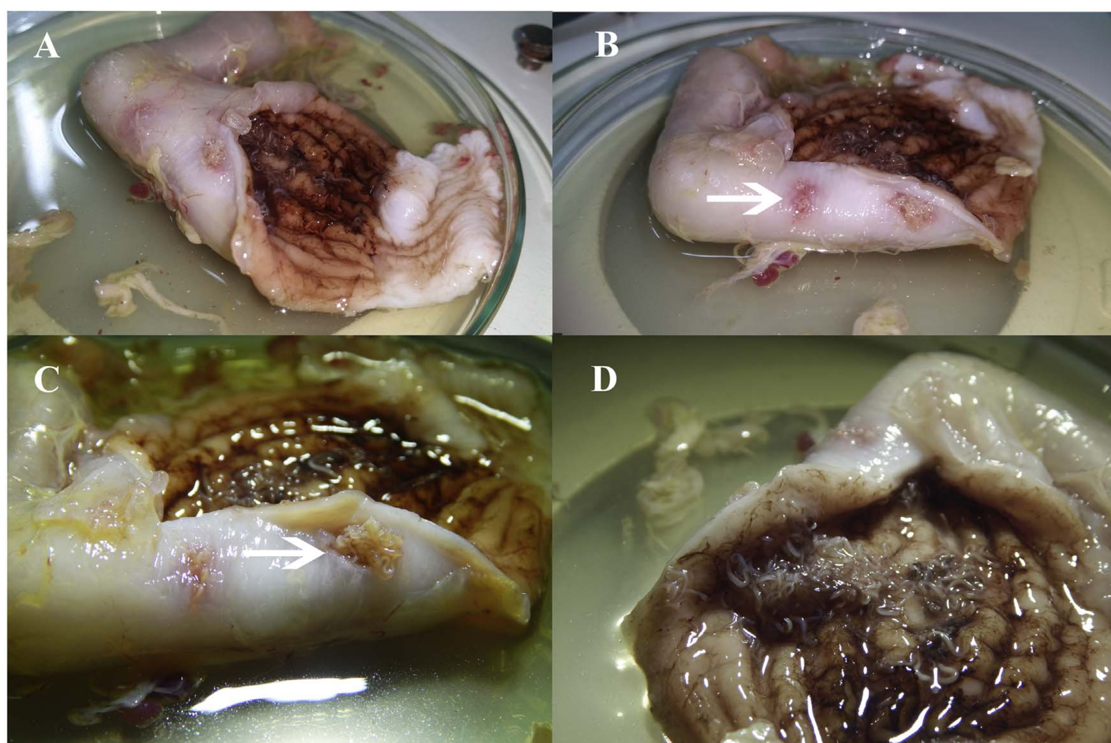


**Fig. 5.** Stomach of *Arappaima gigas.* A − Stomach of juvenile arapaima showing the infection with specimens of *G. spinulosa;* B–C − Perforations in the stomach wall; D − *G. spinulosa* distributed throughout the stomach of *A. gigas.*

The phylogenetic reconstructions based on the 18S and 28S rDNA showed that congeneric species appear in well supported clades. The exceptions were species of *Hysterothylacium* which appeared in different clades, thus confirming their non-monophyly, as previously reported by Nadler and Hudspeth (1998, 2000), Pereira et al. (2015) and Pereira and Luque (2016). In both trees, *Goezia* spp. appeared in well-separated clades without conflicts with other genera. In addition, the 18S and 28S rDNA genes represented good genetic markers for distinguishing genera of the Raphidascarididae, with exception of *Hysterothylacium*.

The parasites negatively influence the health condition of their host (s). Among the most pathogenic parasites of arapaimas is *G. spinulosa*, which causes high mortality rates in cultured fingerlings (Santos and Moravec, 2009). This parasite is able to cause lesions on the stomach mucosa and even perforate the stomach wall, resulting in the death of the host (Santos and Moravec, 2009). The high pathogenicity of *G. spinulosa* was confirmed in a study of tissue changes in adult arapaimas, where a thickening of the gastric mucosa, necrosis of gastric glands and severe inflammatory reaction of the mucosa, submucosa and muscle layer were reported (Menezes et al., 2011). Such damage was also reported to cause digestive problems and secondary infections. The macroscopic observations of the stomach and intestine of arapaimas in Acre State also revealed ulcers and perforations of gastric mucosa, corroborating the previous reports of Santos and Moravec (2009) and Menezes et al. (2011).

The life cycle of *G. spinulosa* has not yet been completely established, but the present integrated taxonomic study involving morphological, ultrastructural and molecular data has resulted in a robust profile for this species, which will aid the diagnosis of both adult and larval stages in arapaimas and potential intermediate hosts.

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