

## Research Article

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# A new species of *Echinostoma* (Trematoda: Echinostomatidae) from the 'revolutum' group found in Brazil: refuting the occurrence of *Echinostoma miyagawai* (= *E. robustum*) in the Americas

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**Abstract**

Although *Echinostoma robustum* (currently a synonym of *E. miyagawai*) was reported in the Americas based on molecular data, morphological support on adult parasites is still required. Herein, a new species of *Echinostoma* is described based on worms found in a chicken from Brazil. Molecular phylogenetic analyses based on 28S (1063 bp), ITS (947 bp) and Nad-1 (442 bp) datasets reveal the inclusion of the new species within *Echinostoma* 'revolutum' species complex. Moreover, it was verified the conspecificity between cercariae previously identified as *E. robustum* in Brazil [identical ITS and only 0.3% of divergence (1 nucleotide) in Nad-1]. Species discovery analyses show that these two isolates form an independent lineage (species) among *Echinostoma* spp. Compared to *E. miyagawai*, the new species presents relatively high divergence in Nad-1 (7.88–9.09%). Morphologically, the specimens are distinguished from all nominal species from the 'revolutum' species complex by the more posterior position of the testes (length of post-testicular field as a proportion of body length about 20%). They further differ from *E. miyagawai* and South American *Echinostoma* spp. by the higher proportion of forebody to the body length. Therefore, combined molecular and morphological evidence supports the proposal of the species named here as *Echinostoma pseudorobustum* sp. nov.

**Introduction**

The trematodes of the genus *Echinostoma* Rudolphi, 1809 (Echinostomatidae Looss, 1899) are intestinal parasites of birds and mammals with a worldwide distribution. Species bearing 37 spines in the head collar and sharing morphological and biological similarities are considered as belonging to the 'revolutum' species complex, referring to the type species of the genus, *Echinostoma revolutum* (Frölich, 1802) (Kanev, 1994; Kostadinova *et al.*, 2003; Fried and Graczyk, 2004; Georgieva *et al.*, 2013, 2017; Faltýnková *et al.*, 2015). This speciose group of echinostomes has a controversial taxonomy mostly due to morphological similarities between species, the existence of cryptic diversity and the superficial or inaccurate description of some species (Kostadinova and Gibson, 2000; Esteban and Muñoz-Antoli, 2009; Georgieva *et al.*, 2014; Toledo *et al.*, 2014; Chai *et al.*, 2020).

In order to better understand the interrelationships and actual diversity within the 'revolutum' group, more robust analyses based on integrative taxonomy approaches are needed for most species. This approach has shown compelling evidence to support the taxonomic reappraisal of several species that had their affiliation clarified, mainly from Europe and Asia (Georgieva *et al.*, 2013, 2014, 2017; Faltýnková *et al.*, 2015; Chai *et al.*, 2021). Among the species that had its taxonomy recently illuminated with new morphological and molecular data is *Echinostoma miyagawai* Ishii, 1932. This species, originally described from Asia, had its occurrence in Oceania and Europe confirmed, as well as its conspecificity with *Echinostoma friedi* Toledo *et al.*, 2000, and *E. revolutum* sensu Morgan and Blair (1995, 1998a, b) – both synonyms of *E. miyagawai* (Kostadinova *et al.*, 2003; Georgieva *et al.*, 2014, 2017; Faltýnková *et al.*, 2015). Recently, some isolates from Bangladesh identified as *Echinostoma robustum* Yamaguti, 1935 were shown to be molecularly indistinguishable from *E. miyagawai* (Mohanta *et al.*, 2019; Heneberg, 2020; Chai *et al.*, 2021). Based on these findings, the synonymy between *E. robustum* and *E. miyagawai* was recently proposed (Chai *et al.*, 2021).

More complexity for this scenario comes with the report of *E. robustum* in the New World based on larval and adult parasites from the USA and Brazil (Detwiler *et al.*, 2010). The Brazilian isolate was identified after the molecular study of cercariae found in *Biomphalaria glabrata* (Say, 1818), for which adult forms are unknown. Although different authors have accepted the occurrence of *E. robustum* in the Americas mostly relying on the findings of Detwiler *et al.* (2010), there is molecular evidence that these parasites belong to as-of-yet undescribed species of the 'revolutum' species complex or known species for which molecular data

are not available (Georgieva *et al.*, 2014; Heneberg, 2020; Chai *et al.*, 2021). In the present study, 37-collar-spined echinostomes found in a free-range chicken from Brazil were subject to morphological and molecular studies. Results obtained revealed that the isolate previously identified in the country as *E. robustum* is a new species herein described.

## Materials and methods

### Morphological study

The gastrointestinal tracts of 46 free-range chickens slaughtered for consumption were donated by owners from the municipality of Viçosa (20°45'14"S, 42°52'55"W), Minas Gerais, Brazil, in June 2018. Each segment of the gastrointestinal tract was individually opened with scissors in a longitudinal section and washed with tap water over a 100 µm mesh sieve. The helminths recovered were separated in Petri dishes containing physiological saline (0.9% NaCl). Specimens of trematodes were compressed between glass slides and fixed in 10% formalin. Subsequently, they were stained with alum acetocarmine, dehydrated in ethanol series, diaphanized in beechwood creosote and mounted on permanent slides with Canada balsam.

Morphological and morphometric studies of permanent preparations were performed under an Olympus BH2 optical microscope (Olympus, Tokyo, Japan). Measurements were obtained with the aid of a micrometre eyepiece and are expressed in micrometres, unless otherwise indicated. The abbreviations used in the morphological description are as follows: BL, body length; BW, maximum body width; BW/BL (%), body width as a proportion of the body length = shape of the body; FORE, forebody length; FORE (%), forebody as a proportion of body length; CL, head collar length; CW, head collar width; CW/BW (%), head collar as a proportion of body width; ASL, angular spines length; ASW, angular spines width; LSL, lateral spines length; LSW, lateral spines width; DSL, dorsal spines length; DSW, dorsal spines width; OSL, oral sucker length; OSW, oral sucker width; VSL, ventral sucker length; VSW, ventral sucker width; OSW/VSW, oral sucker to ventral sucker width ratio; ESL, esophagus length; ESL/BL (%), esophagus length as a proportion of body length; PL, prepharynx length; PHL, pharynx length; PHW, pharynx width; ATL, anterior testis length; ATW, anterior testis width; PTL, posterior testis length; PTW, posterior testis width; TEND, length of post-testicular region; TEND (%), length of post-testicular field as a proportion of body length; OVL, ovary length; OVW, ovary width; OVAR, distance between ovary and posterior margin of ventral sucker; OVAR(%), distance between ovary and posterior margin of ventral sucker as a proportion of the body length.

Photographs were taken with a Leica ICC50 HD digital camera coupled to the microscope Leica DM500 and analysed in the Leica Application software suite (LAZ EZ), version 2.0 (Leica Microsystems, Wetzlar, Germany). Drawings were made using a camera lucida. The type material is deposited in the Helminthological Collection of the Instituto Oswaldo Cruz (CHIOC) and Collection of Trematodes of the Federal University of Minas Gerais (UFMG-TRE).

### Molecular study

An ethanol-fixed specimen was used in the molecular study. DNA was extracted using the Wizard® Genomic DNA Purification kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. The concentration of the extracted DNA was estimated using a microvolume spectrophotometer NanoDrop® ND-1000 (Thermo Fisher Scientific, Wilmington, DE, USA).

Partial regions of the genes 28S, ITS and Nad-1 were amplified using the primer pairs Dig-12/1500R, BD1/BD2 and NDJ11/NDJ2a, respectively (Luton *et al.*, 1992; Morgan and Blair, 1995; Kostadinova *et al.*, 2003; Tkach *et al.*, 2003). PCR reactions were performed in a final volume of 25 µL, which included 12.5 µL of GoTaq® G2 Hot Start Polymerase DNA Polymerase (Promega), 1.25 pmol of each primer, about 50 ng of template DNA and sterile ddH<sub>2</sub>O. PCR products were electrophoresed on a 1.5% agarose gel, and the band of the expected size was purified with polyethylene glycol (PEG) [20% PEG 8000 (Promega) in 2.5 M NaCl solution]. Purified DNA was sequenced in both directions by capillary electrophoresis in an ABI3730 sequencer using the Big Dye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA).

Sequence chromatograms obtained were aligned and edited using the ChromasPro v.2.0.1 (Technelysium Pty Ltd, Australia) and contigs were used for phylogenetic analyses. They were compared with the sequences of species of *Echinostoma* available in GenBank obtained with Basic Local Alignment Search Tool – BLAST\* (Altschul *et al.*, 1990) searches (<https://blast.ncbi.nlm.nih.gov>). The sequences were assembled into three alignments with selected echinostomes using the MUSCLE implemented in MEGA X (Kumar *et al.*, 2018). Both nuclear and mitochondrial datasets were used according to the main goals of the molecular analyses, as follows: 28S data were used to confirm that our isolate is nested within *Echinostoma* in the Echinostomatidae, and the ITS and Nad-1 were used to assess its phylogenetic position within the genus. The alignments were trimmed to match the shortest sequence prior to the phylogenetic analyses. The evolutionary models used for the analyses were determined by Bayesian information criterion in MEGA X, corresponding to GTR + G + I for 28S, K2 + G for ITS and HKY + G + I for Nad-1. Outgroups for each dataset were chosen based on previous molecular phylogenetic studies including *Echinostoma* representatives and related taxa (Tkach *et al.*, 2016). Maximum Likelihood (ML) and Bayesian Inference (BI) were the methods used to generate phylogenetic hypotheses. ML analyses were performed in MEGA X and the nodal support was measured using the bootstrap test with 1000 replicates. BI analyses were performed with MrBayes v.3.2.6 (Ronquist *et al.*, 2012) using Markov chain Monte Carlo (MCMC) for 1 000 000 generations and sampling every 1000 generations. The 'burn-in' was established for the first 25% of the trees sampled, and nodal support was estimated with posterior probability.

For testing the taxonomic hypothesis of *Echinostoma pseudorobustum* sp. nov. as revealed by morphological evidence (see below), besides phylogenetic position and genetic distances, we used three species discovery methods (not requiring prior species assignments), namely the distance-based Assemble Species by Automatic Partitioning (ASAP) (Puillandre *et al.*, 2021), the tree-based Poisson Tree Process (PTP) and multi-rate PTP (mPTP) models (Zhang *et al.*, 2013; Kapli *et al.*, 2017). These analyses were performed using the Nad-1 dataset as this gene is considered suitable for barcoding species of *Echinostoma* (Georgieva *et al.*, 2014). The latter (mPTP) was used especially because it has been shown to be more accurate for species delimitation in datasets with uneven sampling as in our Nad-1 data (Blair and Bryson, 2017). ASAP analyses were run online (<https://bioinfo.mnhn.fr/abi/public/asap/>) using the final Nad-1 alignment as input and default parameters for the three-distance metrics available, i.e. Jukes-Cantor (JC69), Kimura (K80) and simple distance (*p*-distance); only the partition showing the lowest ASAP-score for each metric was considered. PTP analyses were performed in the bPTP and mPTP web servers (<https://species.h-its.org/ptp/>; <https://mptp.h-its.org/#/tree>), using the BI tree as input and default parameters, except the MCMC for the bPTP analysis

that was run for 500 000 generations; the convergence of chains was checked in the likelihood trace plot. Sequences obtained were deposited in GenBank.

## Results

From the total of 46 chickens evaluated during the helminthological study, only one bird (2.2%) was found harbouring three morphologically similar specimens of echinostomes; one individual was used in the molecular analyses and the other two (paragenophores voucher specimens *sensu* Pleijel *et al.*, 2008) were used in the morphological description. The morphology and molecular data gathered revealed a hitherto unknown species of 37-collar-spined *Echinostoma* from the 'revolutum' group, which is described here as follows.

Family Echinostomatidae Looss, 1899

Genus *Echinostoma* Rudolphi, 1809

***Echinostoma pseudorobustum* sp. nov.**

[syn. *Echinostoma robustum* ex *Biomphalaria glabrata* of Detwiler *et al.*, 2010]

Type host: *Gallus gallus domesticus*

Type-locality: Viçosa, State of Minas Gerais, Brazil

Type-material: Holotype – CHIOC 39709

Paratype – UFMG-TRE 121

Site in host: caeca

Prevalence: 2.2% (1/46); infected chicken was found harbouring three adult echinostomes.

Representative DNA sequences: 28S OK586835, ITS OK586836, Nad-1: OK564515

ZooBank registration: urn:lsid:zoobank.org:act:273DDBE0-EFF8-4EEA-ACD9-E5915495C382

Etymology: The new species is named based on the previous assignment of its larval stages to *Echinostoma robustum* by Detwiler *et al.* (2010). The species name derives from the prefix *pseudo-* (false) added to the epithet *robustum*.

## Description

[Based on two specimens (Figs 1 and 2); measurements are presented in Table 1]. Elongated body, flattened dorsoventrally, narrow, with maximum width at the level of ventral sucker (Figs 1 and 2). Body length/body width ratio 6–7:1. Forebody 19.3% (holotype) and 18.5% (paratype) of body length. Tegument with small spines from head collar to median region of body. Head collar well-developed, reniform, 45.8% and 53.2% of body width, with 37 spines, arranged as follows: 5 angular spines on each edge of head collar, 6 lateral spines on each side, and 15 dorsal spines in a double row. Oral sucker muscular, prepharynx present, small, pharynx ovoid, muscular, oesophagus occupying 9.8 and 10% of total body length, bifurcating in intestinal caeca next to ventral sucker. Blind caeca lateral, almost reaching the posterior end, overlapping vitellaria. Ventral sucker muscular, oval, in the equatorial region of the body. Average ratio between the diameter of oral sucker and ventral sucker 1:2.5. Two testes, rounded or subglobular, tandem, similar in size, in the final quarter of body, at 2 and 2.28 mm from the posterior end, corresponding to 18.4 and 19.8% of total body length. Cirrus-sac oval, between the level of intestinal bifurcation and the anterior margin of ventral sucker, containing seminal vesicle, pars prostatica, ejaculatory duct and cirrus. Genital pore under intestinal bifurcation. Ovary oval, in the third quarter of the body. Mehlis' gland contiguous with ovary and anterior testis. Vitellaria in two lateral fields, from pre-equatorial region to posterior end of the body, overlapping caeca; fields enlarged towards post-testicular region but non-confluent. Uterus intercecal, with many coils, between the posterior margin of ventral sucker and ovary, occupying

38.8% and 44.4% of total body length. Egg oval, yellow, operculated, immature.

## Remarks

The new species can be readily distinguished from the remaining *Echinostoma* of the 'revolutum' group by having the posterior testis located at the shortest distance to the posterior end, representing <20% of the body length (TEND 18.4 and 19.8%). *Echinostoma pseudorobustum* sp. nov. further differs from its congeners described in South America, namely *Echinostoma barbosae* Lie and Basch, 1966; *Echinostoma chloephaegae* Sutton and Lunaschi, 1980; *Echinostoma erraticum* Lutz, 1924; *Echinostoma luisreyi* Maldonado *et al.*, 2003; *Echinostoma mendax* Dietz, 1910; *Echinostoma microrchis* Lutz, 1924; *Echinostoma neglectum* Lutz, 1924; *Echinostoma nephrocystis* Lutz, 1924, and *Echinostoma rodriguesi* Hsu *et al.*, 1968, by its larger body size (BL 10.8 and 11.5 mm vs <10.8 mm) and longer forebody (FORE representing at least 18.5% of the body length, instead of <18% in the above-mentioned species) (see Table 2). The other species with a similar body size is *Echinostoma paraensei* Lie and Basch, 1967, which can also be easily differentiated by its smaller forebody (FORE 18.5 and 19.3% vs 11.3%), oesophagus length as a proportion of body length (ESL/BW 9.8 and 10% vs 4.3%), head collar as a proportion of body width (CW/BW 45.8 and 53.2% vs 36%) and uterine field (OVAR 38.8 and 44.4% vs 31.3%) but more importantly by the size of dorsal collar spines. Regarding *Echinostoma lindoense* Sandground and Bonne, 1940, a species described in Asia and reported in Brazil by Lie (1968), the new species is differentiated in having a smaller body size (BL 10.8 and 11.5 mm vs 12.4–17.2 mm). This species is very similar to *E. miyagawai*, but considered distinct from it due to the limits for the body length and width, head collar and ventral sucker width and size of cirrus-sac, ovary and testes (Kostadinova *et al.*, 2000).

Even though molecularly characterized cercariae of the new species was identified as *E. robustum* by Detwiler *et al.* (2010) (see below), currently accepted as *E. miyagawai*, adult morphology clearly shows the distinctiveness of *E. pseudorobustum* sp. nov. Comparing with the original description of *E. miyagawai* by Ishii (1932), our specimens are much smaller (BL 10.8 and 11.5 mm vs 21–26 mm), more robust (BW/BL 14.3 and 16.1% vs 11.7%), have rounded testes instead of lobulated ones, longer oesophagus (ESL/BL 9.8 and 10% vs 5.4%) and uterine field (OVAR 38.8 and 44.4% vs 30.5%). In relation to the most recent reports of *E. miyagawai* from Eurasia, *E. pseudorobustum* sp. nov. also shows differences in the morphology of testes (smooth vs irregularly lobed), (Kostadinova *et al.*, 2000; Toledo *et al.*, 2000; Faltýnková *et al.*, 2015; Mohanta *et al.*, 2019; Chai *et al.*, 2021), while comparing to the original description of *E. robustum* of Yamaguti (1935) (Fig. 1B), our specimens were larger and narrower (BW/BL 14.3 and 16.1% vs 23.2%), possess larger forebody (FORE 18.5 and 19.3% vs 16%), head collar width (CW/BW 45.8 and 53.2% vs 43.3%), and uterine field (OVAR 38.8 and 44.4% vs 28%), and smooth testes, unlike the irregularly lobulated ones.

The description of *E. pseudorobustum* sp. nov. led us to propose a slight modification in the diagnosis of *Echinostoma*. It remains as provided by Kostadinova (2005) with the following modification: Testes tandem, elongated-oval, smooth, indented or lobed, contiguous or separated, just post-equatorial; occasionally, anterior testis equatorial (TEND = 18–35%).

## Molecular study

Molecular sequences of 28S (1063 bp), ITS (960 bp) and Nad-1 (442 bp) genes were successfully obtained for a specimen

**Table 1.** Morphometric comparisons between *Echinostoma pseudorobustum* sp. nov. and other morphologically similar species of *Echinostoma*

Species	<i>Echinostoma pseudorobustum</i> sp. nov.		<i>Echinostoma miyagawai</i>				<i>Echinostoma lindoense</i>		<i>Echinostoma paraensei</i>		
	References	Present study	Ishii (1932)	Yamaguti (1935)	Kostadinova et al. (2000)	Faltýnková et al. (2015)	Chai et al. (2021)	Sandground and Bonne (1940)	Lie (1968)	Lie and Basch (1967)	Maldonado Jr et al. (2001)
BL (mm)	10.85 <sup>H</sup>	11.49 <sup>P</sup>	21.0–26.0	7.8–9.8	9.2–11	8.88–13.16	6.19–8.54	13–15	12.42–17.2	7.49–16.03	15.49–16.32
BW (mm)	1.75	1.64	2.0–3.5	1.33–2.18	1.2–1.5	1.15–2.02	1.01–1.42	2.5–3.0	1.4–2.23	0.79–1.95	2.4–2.41
BW(%)	16.1	14.3	11.7*	22.4*	12.9*	11.3–15.4	14.2–20.9	16.8*	–	12.2*	13.8*
FORE (mm)	2.09	2.13	–	1.31*	1.25–1.53	1.53–2.01	0.91–1.27	2.06*	–	1.62*	2.0*
FORE (%)	19.3	18.5	10.7*	16.8*	13–15	13.6–17.2	14–17.2	13.3*	–	11.3*	9.6*
CL	255	309	–	437.5*	393–505	380–597	395–599	543*	–	375*	429*
CW	801	855	890–1100	670–850	598–682	577–842	528–725	780	720–948	396–746	545–657
CW/BW (%)	45.8	53.2	41.2*	39.3*	46–57	37.7–52.2	43.1–58.4	41.7*	–	36*	25*
ASL	39–77	44–76	84–108#	69–87	52–96	93–134	71–83	84–95#	65–92	36–99	37–57
ASW	23–41	24–31	26–28#	20–30	17–29	28–36	19–25	22–28#	–	–	–
LSL	36–107	53–82	–	87–105	67–87	66–124	75–91	–	–	–	52–74
LSW	27–36	23–32	–	21–30	20–29	15–34	18–24	–	–	–	–
DSL	30–80	51–84	–	87–123	58–75	78–109	69–79	–	–	22–33	44–79
DSW	14–32	19–26	–	20–36	19–26	16–34	18–24	–	–	–	–
OSL	200	255	200–240	312.5*	262–337	230–421	227–307	435*	295–392	375*	255–338
OSW	309	346	–	310–400	262–355	241–421	233–412	230–510	280–363	216–408	274–384
VSL	746	765	–	810–1.08	655–748	686–1090	594–830	1.196*	–	625*	824–870
VSW	837	801	714–1090	800–960	655–785	621–971	544–910	600–1380	720–1027	456–1.008	917–930
OSW/VSW	1: 2.7	1: 2.3	1: 2*	1: 3*	1: 2.2–2.8	1: 2.4	1: 2.6*	1:2.7*	–	1:2*	1:4.1
ESL	1090	1130	1260–1290	770	542–655	708–854	569–790	543*	780–1185	236–664	388–405
ESL/BL (%)	10	9.8	7*	8.8*	13	–	10.2*	3.5*	–	4.3*	2.1*
PL	123	87	–	–	0–37	40–88	4–41	–	30–68	72–120	129–139
PHL	182	182	–	250–330	262–337	219–304	230–292	326*	250–316	180–348	198–287
PHW	182	182	221–346	220–280	187–355	183–263	197–335	180–400	200–268	180–288	161–231
ATL	710	528	1020–1310	500–620	430–692	511–818	403–653	1.304*	730–1058	348–732	752–844
ATW	583	492	850–1100	410–660	337–505	453–889	588–982	1.304*	490–730	312–612	717–763
PTL	765	564	986–1190	437.5*	449–655	438–1030	477–674	1.522*	750–1058	408–852	917–940
PTW	710	473	884–1020	562.5*	337–524	467–819	593–787	1.304*	474–680	312–516	741–750

TEND (mm)	2	2.28	-	2*	2.82–3.33	2.65–3.75	2.24–2.79	1.92*	-	5.12*	6.0*
TEND (%)	18.4	19.8	29.5*	25.6*	30–32	25.9–32.2	31.2–36.7	29.4*	-	35.6*	29*
OVL	364	273	544–595	210–310	243–355	219–392	198–342	435*	-	375*	476–490
OYW	492	328	612–731	400–500	337–449	336–558	290–620	300–500	316–505	250–408	682–712
OVAR (mm)	4.82	4.46	-	2.12*	2.78*	4.41–5.93	3.54–4.31	3.91*	-	4.5*	7.7*
OVAR (%)	44.4	38.8	32*	27.2*	27.4*	47.7–49.7	50.3–57.7	25.2*	-	31.3*	37.2*
Eggs	92–106	92–135	104–112	111–129	94–96	99–113	79–105	92–124	104–116	104–122	104–112
	57–71	42–64	64–72	60–69	60	51–70	56–70	65–76	64–74	74–86	74–85

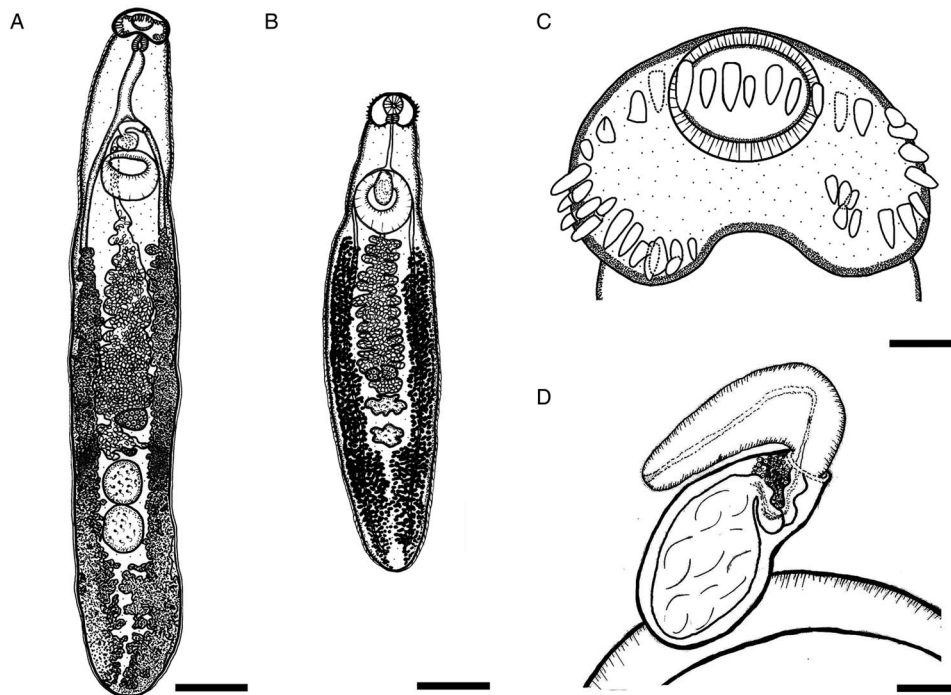
Measurements are in  $\mu\text{m}$ , unless stated otherwise. Abbreviation of the morphological structures are as presented in the text. Asterisk indicates data taken from original drawings. Hash indicates the range for all collar spines. Measurements not provided or missing drawings are indicated by a dash. Superscripted H and P indicate data for Holotype and Paratype, respectively. Data by Yamaguti (1935) correspond to the original description of *Echinostoma robustum*, currently a synonymy of *Echinostoma miyagawai*.

*E. pseudorobustum* sp. nov. obtained in the same host. After alignment and trimming, datasets composed of 1063 bp of 28S (56 sequences from 55 species/species-level lineages); 947 bp of ITS (12 sequences from 11 species/species-level lineages) and 442 bp of Nad-1 (156 sequences from 21 species/species-level lineages) were used in the subsequent analyses. Regarding 28S, >99% of similarity was verified between the Brazilian isolate and all 13 species of *Echinostoma* included in the analysis. Despite being of limited use for tackling the intrageneric relationships, the phylogenetic trees based on this gene clearly show that our isolate is nested within the representatives of *Echinostoma* (Fig. 3). However, the nodal supports for the different clades verified in the 28S tree were most of the time low. On the other hand, phylogenetic trees resulting from ML and BI analyses of both ITS and Nad-1 genes showed the newly sequenced *Echinostoma* clustering in a clade together with a Brazilian isolate identified as *E. robustum* lineage D by Detwiler *et al.* (2010) and also confirmed that the new species belongs to the 'revolutum' group of *Echinostoma* (Figs 4 and 5).

Considering the ITS sequences, *E. pseudorobustum* sp. nov. differed 0.11–2.27% from other 9 species belonging to the genus *Echinostoma* included in the analyses. The newly-generated sequence falls into a clade containing two isolates [one from Brazil (GQ463133 – 0.11% of divergence – 1 nucleotide) and another from the USA (GQ463132 – 0.21% of divergence – 2 nucleotides)] identified as *E. robustum* by Detwiler *et al.* (2010) (Fig. 4).

The analyses of the Nad-1 dataset showed high sequence similarity of *E. pseudorobustum* sp. nov. with *E. robustum* lineage D (GQ463055) (0.23% of divergence – 1 nucleotide), confirming their conspecificity. These Brazilian sequences clustered in a well-supported clade, sister to *E. robustum* lineage C (GQ463054) from the USA (Fig. 5). The latter most likely represent either an undescribed species or a known taxon yet not characterized molecularly as well as *E. robustum* lineage A (GQ463053), also from the USA, which appeared in a distantly related clade, sister to a clade composed by 35 sequences previously assigned to different taxa: *E. friedi* (Marcilla *et al.*, unpublished), *E. revolutum* by Morgan and Blair (1998a, b), *E. miyagawai* by Georgieva *et al.* (2014, 2017), Nagataki *et al.* (2015), Li *et al.* (2019), Fu *et al.* (2019), and *E. robustum* by Mohanta *et al.* (2019). Currently, these isolates are treated under a single species with Eurasian + Oceanian distribution, *E. miyagawai*, given the rough similarity in the Nad-1 sequences (0.23–2.94%), which falls in the intraspecific range of variability (Georgieva *et al.*, 2014; Nagataki *et al.*, 2015; Chai *et al.*, 2021).

The distance- and tree-based methods of species discovery based on Nad-1 yielded similar results (Supplementary Fig. 1), recognizing the conspecificity between the Brazilian isolate (GQ463055) of Detwiler *et al.* (2010) with our newly sequenced specimen and their independent status as *E. pseudorobustum* sp. nov., yet the mPTP analysis also recognized in this new taxon the isolate GQ463054 of the same authors (see below). All distance metrics of the ASAP method recognized 19 species of *Echinostoma*, whereas the bPTP and mPTP methods informed us 20 and 18 taxa, respectively; this difference is due to the disputed affiliation of isolates assigned to *Echinostoma caproni* Richard, 1964 and those potentially belonging to the new species. While the ASAP and mPTP recognized a single entity for all isolates of *E. caproni*, the bPTP admitted two discrete entities. In turn, the bPTP and ASAP support the conspecificity of our isolate only with GQ463055, which further includes the North American isolate GQ463054 from *Ladislavella elodes* (Say, 1821) (= *Lymnaea elodes*) considering the mPTP method. Agreeing with the phylogenetic analyses, all methods indicate that the American isolates assigned to *E. robustum* by Detwiler *et al.*



**Fig. 1.** *Echinostoma pseudorobustum* sp. nov. found in chicken from Brazil: (A) Line drawing of the holotype. (B) *Echinostoma robustum* (currently a synonym of *Echinostoma miyagawai*), a taxon that was previously associated with larval stages of the new species here described (redrawn from Yamaguti, 1935). (C) Line drawing of the collar of spines. (D) Detail of the cirrus-sac. Scale bars: A and B: 1 mm; C and D: 100  $\mu$ m.



**Fig. 2.** *Echinostoma pseudorobustum* sp. nov. found in a chicken from Brazil: (A) Micrograph of the paratype. (B) Micrograph of collar spines showing angular (\*) and lateral (#) spines. (C) Micrograph of dorsal spines (+). Scale bars: A: 1 mm; B and C: 100  $\mu$ m.

(2010) belong to at least two species other than *E. miyagawai*, including *E. pseudorobustum* sp. nov. and at least one undescribed or non-sequenced species. All methods confirmed that only isolates of Eurasian distribution can be assigned to *E. revolutum* (*sensu stricto*), setting them apart from the North American *E. revolutum* (*sensu lato*) (Georgieva et al., 2013), and that the large clade composed of 35 sequences represents a single species,

*E. miyagawai*, with a wide geographic distribution (Chai et al., 2021).

### Discussion

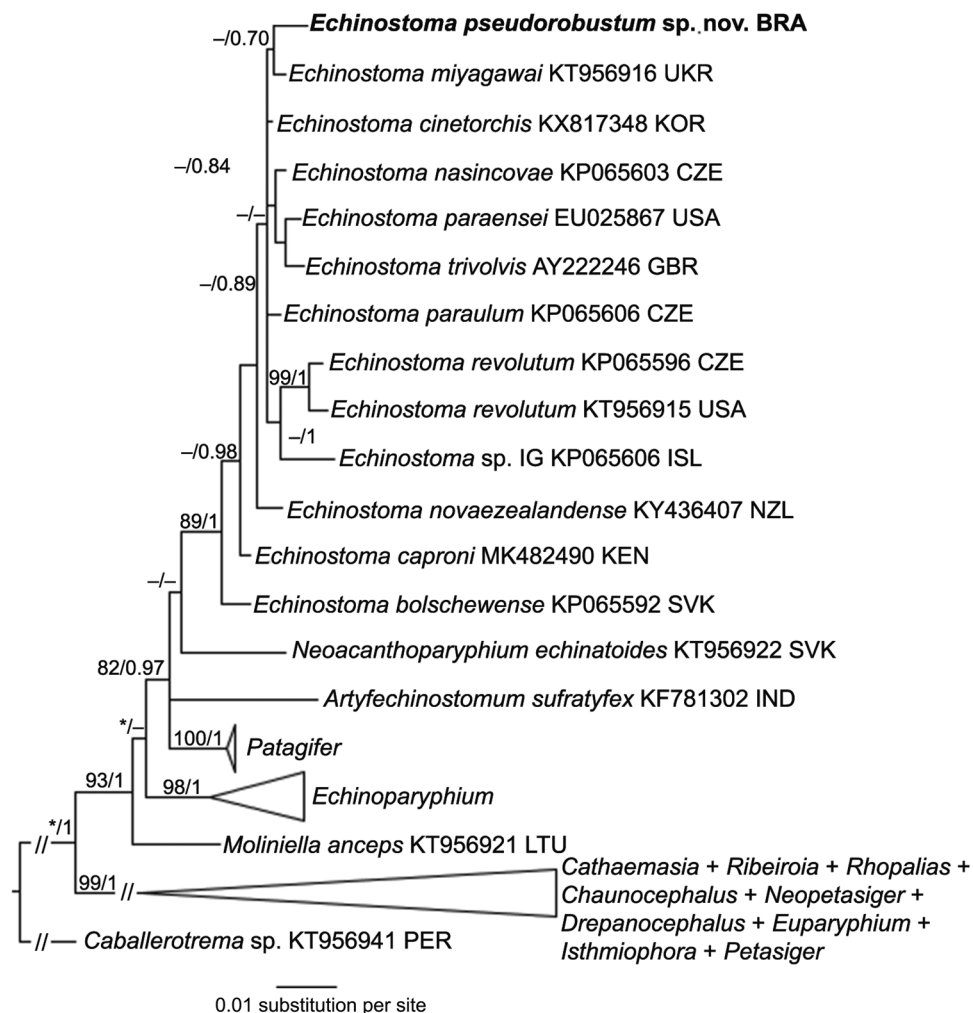
The actual diversity, host-associations, phylogenetic relationships and distribution of the 'revolutum' species complex within *Echinostoma* have been obscured partially because, in most contributions, authors have relied only on a single line of evidence (e.g. morphology or DNA) to support their species hypotheses, which ultimately rendered a dubious taxonomic status for several species (Georgieva et al., 2014, 2017; Faltýnková et al., 2015; Chai et al., 2020). In the present study, thorough morphological evaluation along with phylogenetic reconstructions based on three loci (nuclear 28S and ITS, and mitochondrial Nad-1 markers; the latter also used in the species discovery analyses) made it possible to describe *E. pseudorobustum* sp. nov. from a chicken in Brazil and to confirm its conspecificity with cercariae previously found in *B. glabrata* from the same country identified as *E. robustum* (Detwiler et al., 2010). The results obtained reinforce, as already advocated by others (Georgieva et al., 2014, 2017; Nagataki et al., 2015; Chai et al., 2021), that isolates identified as *E. robustum* from the Americas, in fact, belongs to yet undescribed or molecularly non-characterized species, one of them here described as new. The morphological data for *E. pseudorobustum* sp. nov. clearly separate this species from *E. robustum* *sensu* Yamaguti (1935), currently considered a synonym of *E. miyagawai* (Chai et al., 2021). Moreover, all but one molecular analysis indicates that the other two American isolates identified as *E. robustum* represent two distinct species that need further scrutiny for a better characterization. These two North American isolates were identified after morphological study of experimentally obtained adults (Detwiler et al., 2010), but a detailed morphological description or deposition of vouchers was not provided, hampering the advance in this taxonomic issue.

In the last years, new species belonging to the 'revolutum' species complex, some of them with overlapping morphological

**Table 2.** Comparative data between body length, forebody (FORE%) and post-testicular (TEND%) distance proportional to the body between *Echinostoma pseudorobustum* sp. nov. and *Echinostoma* spp. belonging to the 'revolutum' group reported in South America

Species	Body length (mm)	FORE (%)	TEND (%)	Source
<i>Echinostoma pseudorobustum</i> sp. nov.	10.85–11.49	18.4–19.8	18.4–19.2	This study
<i>Echinostoma barbosa</i>	3.65–7.83	11.6*	36.8*	Lie and Basch (1966)
<i>Echinostoma chloephagae</i>	5.13–6.91	14.7*	30.1*	Sutton and Lunaschi (1980)
<i>Echinostoma erraticum</i>	7–8	17.9*	28.4*	Lutz (1924)
<i>Echinostoma lindoense</i>	12.42–17.2	-	-	Lie (1968)
<i>Echinostoma luisreyi</i>	5.25–6.5	12.3*	38.6*	Maldonado Jr. et al. (2003)
<i>Echinostoma mendax</i>	6–9.5	12.2*	33.3*	Dietz (1910)
<i>Echinostoma microrchis</i>	5	14.7*	33*	Lutz (1924)
<i>Echinostoma neglectum</i>	np	14*	30.1*	Lutz (1924)
<i>Echinostoma nephrocystis</i>	2.5–4.75	18*	27*	Lutz (1924)
<i>Echinostoma paraensei</i>	7.49–16.03	11.3*	35.6*	Lie and Basch (1967)
<i>Echinostoma rodriguessi</i>	3.87–6.8	13.3*	36.4*	Hsu et al. (1968)

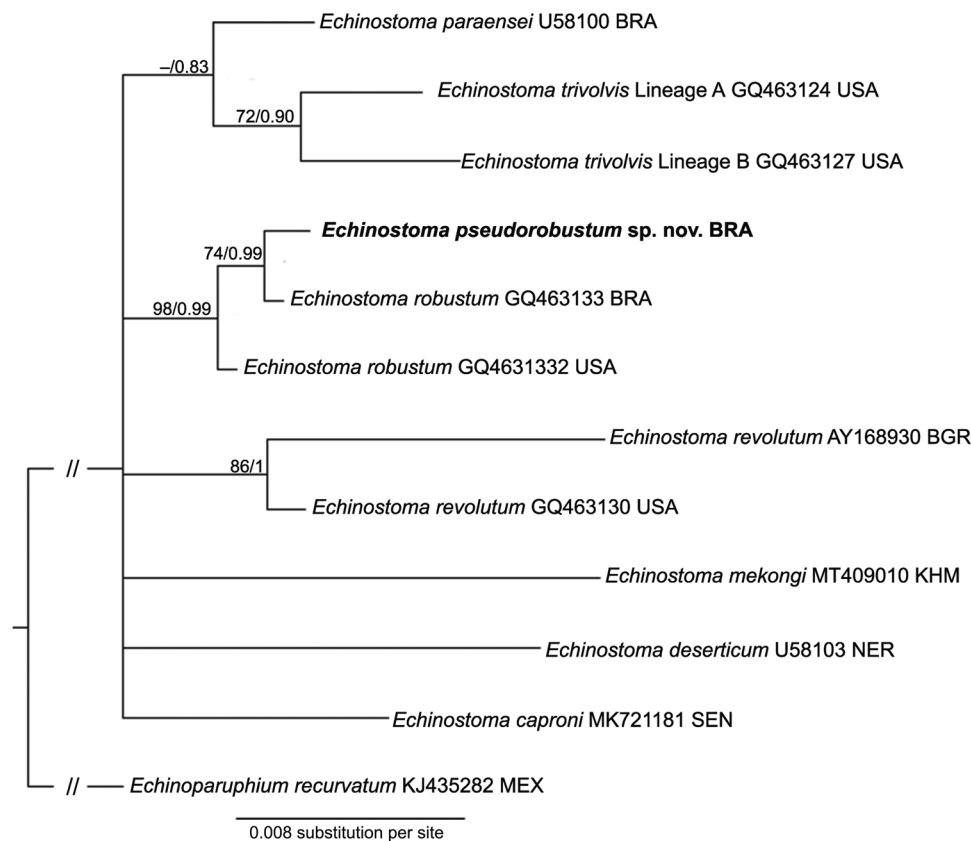
Asterisk indicates data taken from original drawings. Measurement not provided or missing drawings are indicated by a dash.



**Fig. 3.** Phylogenetic relationship between *Echinostoma pseudorobustum* sp. nov. (in bold) and other 37-collar-spined *Echinostoma* species inferred from sequences of 28S rDNA (1063 bp) based on Maximum Likelihood (ML) and Bayesian Inference (BI) analyses. Nodal support is indicated as ML/BI; values <0.70 (BI) and <0.70 (ML) are indicated by a dash. BRA, Brazil; UKR, Ukraine; KOR, South Korea; CZE, Czech Republic; USA, United States; GBR, United Kingdom; ISL, Iceland; NZL, New Zealand; KEN, Kenya; SVK, Slovakia; IND, India; LTU, Lithuania; PER, Peru.

characteristics, have been described after molecular studies, revealing a greater diversity than previously thought for this group (Georgieva et al., 2013, 2014, 2017; Faltýnková et al.,

2015; Chai et al., 2021). In South America, 13 species of *Echinostoma* have already been described with 37 head collar spines, but molecular studies are extremely scarce. In fact, *E.*



**Fig. 4.** Phylogenetic relationship between *Echinostoma pseudorobustum* sp. nov. (in bold) and other 37-collar-spined *Echinostoma* species inferred from sequences of internal transcribed spacer (947 bp) based on Maximum Likelihood (ML) and Bayesian Inference (BI) analyses. Nodal support is indicated as ML/BI; values <0.70 (BI) and <70 (ML) are indicated by a dash. BRA, Brazil; USA, United States; BGR, Bulgaria; KHM, Cambodia; NER, Niger; SEN, Senegal; MEX, Mexico.

*pseudorobustum* sp. nov. is the second species of the genus for which molecular data were generated (the other one is *E. paraensei*). This fact greatly hinders the advance in taxonomic aspects and life cycle, especially considering morphologically similar species. Despite these difficulties, the new species described here can be separated from other South American 37-collar-spined echinostomes by the longest forebody (highest FORE%) and the shortest post-testicular distance (testes in the last quarter of the body, i.e. lowest TEND%).

We recognized that the type and only known definitive host of *E. pseudorobustum* sp. nov., the chicken, a non-native domestic bird, probably is not the main final host for this echinostome species. In fact, the low prevalence of infection and worm burden, ultimately, suggests we are dealing with an accidental host. The process involving spillover of wild hosted echinostomes to chicken was previously reported in the Americas by Mello (1933), even though the identification as *E. revolutum* requires molecular confirmation. Moreover, the experimental susceptibility of *G. g. domesticus* to some South American species of *Echinostoma* (e.g. *E. barbosa*, *E. erraticum*, *E. lindoense* and *E. rodriguez*) was shown by different authors (Lie and Basch, 1966; Hsu et al., 1968; Lie, 1968; Komma, 1972). Information on the exact environment in which the infected chicken was established is not available. We speculate that it became infected in the rural area of the city, in which the birds are reared in free-ranging system and with access to an aquatic environment containing molluscs or amphibians harbouring echinostome metacercariae. Probably, a wild bird is the natural definitive host of *E. pseudorobustum* sp. nov. Given the natural host of the new species here described is likely distinct from the type-host here described, the existence of some level of host-induced phenotypic plasticity cannot be ruled out. However, clear morphological differences that set the studied specimens

apart from the other *Echinostoma* spp. of the 'revolutum' group (see remarks) hardly could be associated with some host-induced variability. Moreover, a high level of morphological variation has not been reported to other species of *Echinostoma* known from experimental hosts (including chicken) compared to natural hosts (e.g. *E. trivolvis*, *E. caproni* and *E. miyagawai*) (Beaver, 1937; Jeyarasasingam et al., 1972; Fried and Huffman, 1996; Kostadinova et al., 2000). It is also worth mentioning that, yet the validity of the new species is supported by morphological and molecular data, further morphological studies based on a larger number of specimens may evidence a wider breadth of intraspecific variability in the taxon but still not overlapping key diagnostic features with other species.

The incorrect assignment to some sequences of *Echinostoma* ('revolutum' group) that are available in the GenBank database probably stems from an inaccurate morphological identification of the sequenced isolates. It is also likely true that a finer morphological study combined with molecular findings may reveal that those so-called cryptic species, e.g. the three independent lineages under the same name in the GenBank, *E. trivolvis*, are in fact discrete entities, even morphologically, that each deserves their own name. Therefore, integrated approaches are extremely important to unravel the actual species diversity in *Echinostoma* and make it more reliable information available in the genetic repositories. In the present study, the levels of morphological variation, combined with phylogenetic support, confirm that the clades in which the new species here described as *E. pseudorobustum* sp. nov. nested and the one that grouped the Australasian lineage of *E. miyagawai* represent genetically distinct species.

In general, molecular data have not supported the occurrence of the same species of *Echinostoma* in the New and Old World. For instance, a widely known issue is related to the distribution





enough phylogenetic signal for the distinction between these closely related lineages. However, considering the substantial body of evidence for the utility of the Nad-1 in unravelling the interrelations of *Echinostoma* spp. (Georgieva et al., 2013, 2014, 2017; Chai et al., 2021, present study), which in many cases corroborates the morphological data, further studies based on a wider taxon coverage for Cox-1 data, obviously including isolates of *E. miyagawai* from Europe, should be performed before accepting such assumption. Ideally, gathering complete mitogenomes may indicate more precisely which genes are more reliable and informative (individually or in concatenated analyses); currently, complete mitogenome is available only to four species, i.e. *E. caproni*, *E. miyagawai*, *E. paraensei* and *E. revolutum*, and mostly used to assess higher-level phylogenetic relationships (Fu et al., 2019; Le et al., 2020).

The species delimitation methods handle one of the most problematic issues in describing the biological diversity, which is identifying taxa at the species level (Carstens et al., 2013). Even though these approaches cannot be considered the panacea to the species problem, a bulk of methods have been recently employed to strengthen the hypotheses delineated from more traditional pieces of evidence (e.g. morphology, ecology and behaviour), and often unravelling cryptic diversity (Carstens et al., 2013). Two categories of species delimitation methods can be recognized: the species discovery, such as the GMYC, PTP and ASAP, which do not require prior assignments for the putative species, and the species validation ones, which demand the discrimination of the lineages *a priori* (e.g. \*BEAST, BPP, spedeTEM) (Carstens et al., 2013; da Cruz and Weksler, 2018). Both strategies have been recently used for parasitic helminths such as trematodes (Pérez-Ponce de León et al., 2016), acanthocephalans (Pinacho-Pinacho et al., 2018), nematodes (Ailán-Choke et al., 2020) and cestodes (Alves et al., 2021), and their usefulness is acknowledged in these works. In the present study, the species discovery methods were useful to confirm the conspecificity between our newly sequenced specimen of *E. pseudorobustum* sp. nov. with the Brazilian isolate previously found in the first intermediate host (GQ463055), and to refute the occurrence of *E. miyagawai* in the Americas, as previously suggested by other authors (Georgieva et al., 2013, 2014; Chai et al., 2021). Even though Izrailskaia et al. (2021) suggested that isolates identified as *E. revolutum* from North America and Eurasia represent a single species, the results obtained reported them as independent lineages similarly as found by other workers (Georgieva et al., 2014, 2017; Chai et al., 2021). The differences observed in the number of species recognized by the mPTP (18), ASAP (19) and bPTP (20) are expected as each method has its own limitations, e.g. overlumping or oversplitting (Dellicour and Flot, 2018; Puillandre et al., 2021), therefore incongruencies among them are common (Carstens et al., 2013). Considering the amount of genetic divergence between the two Brazilian isolates, including the newly generated one, and the poorly known North American isolate GQ463054, we do not consider the latter isolated in our concept of *E. pseudorobustum* as suggested by the mPTP analysis, yet this decision needs verification based on adult specimens for allowing morphological comparisons, and higher taxon sampling (accounting for the intraspecific variability) to be more useful for certain species delimitation methods.

Integrative taxonomy approach in the study of species of *Echinostoma* from South America is in its infancy. New broader approach studies are necessary to advance the knowledge of life history and host-parasite relationship involving this complex group of trematodes. Besides the description of a new species, data presented here suggest that *E. miyagawai* (= *E. robustum*) must be considered a species with distribution restricted to Eurasia and Oceania, not occurring in New World.

**Supplementary material.** The supplementary material for this article can be found at <https://doi.org/10.1017/S0031182021001864>.

**Author contribution.** M.C.V., P.V.A. and H.A.P. conceived and designed the study. M.C.V. conducted fieldwork. M.C.V., D.L.H. and P.V.A. performed molecular and phylogenetic analyses. M.C.V., D.L.H., P.V.A. and H.A.P. wrote the article. H.A.P. supervising, reviewing and editing the article.

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**Conflict of interest.** None.

**Ethical standards.** The use of parasites from chickens was in accordance with the local animal research ethics committees of Universidade Federal de Viçosa (protocol CEUA/UFV 69/2017).

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