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Characterization of *Echinostoma revolutum* and *Echinostoma robustum* from ducks in Bangladesh based on morphology, nuclear ribosomal ITS2 and mitochondrial *nad1* sequences

Uday Kumar Mohanta[a,b], Takuya Watanabe[a], Anisuzzaman[c], Yuma Ohari[a,b], Tadashi Itagaki[a,b,*]

[a] *Laboratory of Veterinary Parasitology, Faculty of Agriculture, Iwate University, Ueda, 3-18-8, Morioka 020-8550, Japan*
[b] *Department of Pathogenic Veterinary Science, United Graduate School of Veterinary Science, Gifu University, Yanagido 1-1, Gifu 501-1193, Japan*
[c] *Department of Parasitology, Bangladesh Agricultural University, Mymensingh 2202, Bangladesh*

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ABSTRACT

Precise discrimination of *Echinostoma* species within the '*revolutum*' group is quite difficult because of their morphological similarities. The objective of this study was to precisely characterize the echinostomes of ducks from Bangladesh based on both morphological and molecular characteristics. Two *Echinostoma* species were identified: *E. revolutum* and *E. robustum*. In the phylogenetic trees (ITS2 and *nad1*), *E. revolutum* and *E. robustum* belonged to their respective Eurasian clade, which is distinct from the American clade. These results suggest that both species have two distinct and geographically separated lineages, Eurasian and American. Our molecular and morphological data combined with previously published data supports the synonymy of *E. robustum*, *E. miyagawai*, and *E. friedi* previously based on either molecular or morphological evidence. This study thus improves our understanding of species diversity of the '*revolutum*' group, particularly in Asia.

1. Introduction

The genus *Echinostoma* Rudolphi, 1809 (Trematoda: Echinostomatidae) consists of > 120 nominal species [1] and exhibits a cosmopolitan distribution. Members of this genus have a complex life cycle, using aquatic snails as intermediate hosts and vertebrates, typically birds, as the final host. *Echinostoma* is characterized by the number of collar spines, which varies from 27 to 51, around the oral sucker [2]. A group of species within the genus containing 37 collar spines known as the *Echinostoma* '*revolutum*' group has been widely examined [1–7]. Species identification of members within the group is difficult because of their morphological similarity, extensive synonymy, and inadequate descriptions [8]. In addition to the morphology of adult flukes, the morphology and biology of the developmental stages are regarded as crucial factors for identification [5,9]. Geographic separation may result in phenotypic differences and genetic separation of trematodes [10]. Therefore, precise identification and greater taxonomic resolution can be achieved by analyzing DNA sequences [11].

Nuclear rDNA (ITS1, 5.8S, and ITS2) is useful for discriminating closely related genera in eukaryotes, including 7 nominal species in 37-collar-spine and 28-collar-spine echinostomes [7]. Mitochondrial nicotinamide adenine dinucleotide dehydrogenase subunit 1 (*nad1*) was found to be effective for exploring relationships and intraspecific variations within 37-collar-spine echinostomes [6]. Therefore, DNA sequence analysis is crucial for identifying and characterizing echinostomes. Although much advancement towards the taxonomic resolution of 37-collar-spine echinostomes has been made in Europe and America, a few studies have examined these organisms in Asia. There are some reports on the prevalence and pathology of echinostomes from domestic ducks in Bangladesh [12,13]; however, there is no report of the detailed morphology and molecular characteristics of echinostomes. Therefore, we characterized two echinostomes, *E. revolutum* and *E. robustum*, based on their detailed morphological and molecular characteristics.

2. Materials and methods

2.1. Specimen collection, processing and preservation

Echinostomes were recovered from the small and large intestines of domestic ducks (*Anas platyrhynchos domesticus*) purchased from local markets in Mymensingh district in Bangladesh. A total of 31 flukes were collected from 11 ducks (1–7 flukes/duck) in January 2015. The flukes were washed with saline and observed under a light microscope. After preliminary observation, the flukes were fixed under slight pressure

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[*] Corresponding author at: Laboratory of Veterinary Parasitology, Faculty of Agriculture, Iwate University, Ueda, 3-18-8, Morioka 020-8550, Japan.
E-mail address: itagaki@iwate-u.ac.jp (T. Itagaki).

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Table 1

Comparative morphometric properties of *E. revolutum* recovered in this study with that from different geographic origin.

	E. revolutm in this study (n = 16)	*E. revolutum*		
		Faltynkova et al. [3] (n = 16)	Kanev [9] (n = 100)	McDonald [24]
Body length (mm)	4.45–10.36	9.45–11.84	6.46–30.0	10.0–22.0
Body width (mm)	1.24–1.83	1.45–2.09	0.62–1.63	–
Fore body length (mm)	0.52–1.14	1.2–1.66	–	–
Fore body length/body length (%)	9.73–15.63	10.9–14.8	–	–
Head collar (μm)	302–478 × 332–697	351–424 × 552–652	500–600	520–600
Head collar/body width (%)	25.7–39.2	28.3–38.3	–	–
Collar spines	37	37	37	37
Angle spines (μm)	70–114	58–92	–	68–103
Lateral spines (μm)	71–108	64–91	–	–
Dorsal spines (μm)	53–102	71–94	–	–
Oral sucker (μm)	184–350 × 169–355	261–358 × 246–303	180–296 × 100–180	250–300
Ventral sucker (μm)	310–1149 × 309–1088	796–1038 × 796–1061	460–620	–
Constriction at the level of Ventral Sucker	None	None	–	–
Pharynx (μm)	131–261 × 146–225	209–258 × 194–258	130–240 × 100–180	–
Oesophagus length (μm)	162–511	318–569	–	Shorter than the ventral sucker
Testes	Oval, irregular margin	Oval, smooth margin	Smooth, sometimes irregular margin	Branched in different degrees
Anterior (μm)	215–869 × 374–681	629–932 × 425–750	260–480 × 150–540	–
Posterior (μm)	278–933 × 312–667	627–1061 × 395–705	314–614 × 172–515	–
Ovary (μm)	121–321 × 293–653	288–394 × 291–493	130–460	–
Vitelline glands	Not connected behind the posterior testis	–	–	Not connected behind the posterior testis
Egg (μm)	91–108 × 55–67	108–125 × 71–94	–	99-132

–: no data.

between two slides and preserved in 70% ethanol for morphological and molecular analysis.

2.2. Morphological studies

The body length, maximum body width, and forebody length (measured here as the distance from the anterior extremity of the fluke to the upper edge of the ventral sucker) were measured under an optical microscope before staining and tissue separation. The specimens were measured from total mounts.

2.3. DNA analyses

Total DNA was extracted from the excised tissue of the individual fluke with a High Pure PCR Template Preparation Kit (Roche, Mannheim, Germany) according to the manufacturer's instructions and stored at −20 °C. To amplify the ITS2 region including partial 5.8S and 28S rRNA genes, the primers ITS2-F and ITS2-R [14] were used and the reaction was conducted under common cycling conditions with an annealing temperature of 65 °C. The *nad1* sequences were amplified by using the primer set JB11 [6] and NDJ2a [1] under common reaction conditions at an annealing temperature of 48 °C. The PCRs were performed using the GenAmp PCR system 2700 (Applied Biosystems, Foster City, CA, USA). PCR products were purified by gel extraction using a NucleoSpin Gel and PCR clean-up kit (Macherey−Nagel, Düren, Germany). Amplicons of the ITS2 region and *nad1* were sequenced directly in both directions with the same primers used for PCR amplification and a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) on an ABI3500 Genetic Analyzer (Applied Biosystems). The resulting sequences were initially assembled using ATGC ver. 6.0.3 (Genetyx Co., Tokyo, Japan), and the haplotypes were distinguished by GENETYX ver. 10 (Genetyx Co.).

2.4. Phylogenetic analysis

Phylogenetic analyses inferred from the sequences of ITS2 (427 bp) and *nad1* (448 bp) were conducted using Bayesian inference (BI) analyses using MrBayes5D [15], a modified version of MrBayes v.3.1.2 [16] and the maximum likelihood (ML) method with MEGA 6 [17]. In the Bayesian analyses, the best fitting nucleotide substitution model was determined based on the Kakusan4 [18]. The Hasegawa-Kishino-Yano model with discrete Gamma distribution (HKY + G) was selected for constructing the Bayesian tree inferred from the ITS2 and *nad1* sequences. Bayesian trees were estimated over 1,000,000 generations via 4 simultaneous Metropolis-coupled Markov Chain Monte Carlo algorithm (nchains = 4) with a sampling frequency of 100 generations. Tracer v.1.6 was used to assess stationarity and that the sample sizes for each parameter were adequate (effective sample size > 200). The first 10,001 trees were discarded as burn-in. The remaining trees were used to construct the 50% majority rule consensus tree and to estimate the nodal support as posterior probability values [19]. The mean divergence values (*p*-value) for the *nad1* sequences were calculated using MEGA 6 [17]. In the ML analyses, the best nucleotide substitution model was determined based on the Bayesian Information Criterion. The Kimura 2 parameter model was selected for constructing the ML tree inferred from the ITS2 sequences, whereas HKY + G with 5 rate categories was selected for the *nad1* sequences. All the positions containing gaps or missing data were eliminated. In both the trees, bootstrap analyses were conducted using 1000 replicates. The mean divergence values (*p*-value) for the *nad1* sequences were calculated using MEGA 6 [17].

3. Results

3.1. Species identification based on morphology and morphometry

All flukes were 4.45–10.80 mm long and 1.24–2.12 mm wide and had a well-developed head collar bearing 37 spines (5 angle spines and 6 lateral spines on each side, and 15 dorsal spines). The esophagus was shorter than the diameter of the ventral sucker. These morphological properties matched those of the members of 37-collar-spined echinostomes (*E. revolutum* group), including *E. paraulum*, *E. revolutum*, *E.*

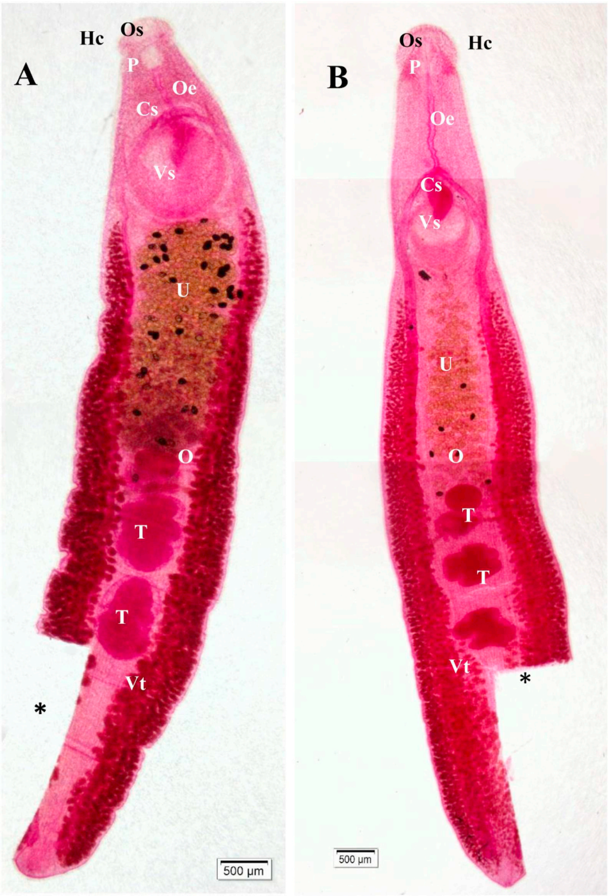

Fig. 1. Representative *Echinostoma* flukes from the intestine of duck. A: Whole *E. revolutum* fluke. B: Whole *E. robustum* fluke. *the part separated for DNA extraction. Hc: head collar; Oe: esophagus; Cs: cirrus sac; Vs: ventral sucker; U: uterus; O: ovary; T: testis; Vt: vitelline glands. Os: oral sucker; P: pharynx.

miyagawai, and *E. robustum* [20]. According to their detailed morphological and morphometric properties, two groups of flukes were discriminated in this study. One of these two groups, 16 of 31 flukes had a short forebody (0.52–1.14 mm) and the distance between the anterior extremity of the fluke and cranial border of the ventral sucker was 9.73–15.63% of the body length (Table 1). The flukes did not have constriction at the level of the ventral sucker. Testes were elongated with smooth to somewhat rough margins, tandem in position, and slightly separated from each other. The anterior testis was shorter and wider than the posterior testis (Fig. 1A). The cirrus sac was oval and located transversely between the level of intestinal bifurcation and anterior border of ventral sucker. The ovary was oval, median, and transversely located between the posterior end of uterus and cranial margin of the anterior testis. Based on these morphological characteristics, the specimens in this group were identified as *E. revolutum.*

The other group consisting of 15 flukes had a comparatively long forebody (1.16–2.11 mm), comprising 16.4–20.9% of the fluke length (Table 2), and a distinctive constriction at the level of the ventral sucker (Fig. 1B). The cirrus sac was elongate-oval, extending from the level of intestinal bifurcation to the middle of the ventral sucker. The testes were median, tandem in position, and markedly separated. These features are common in *E. robustum*, *E. miyagawai,* and *E. friedi*. The testes in our specimens were deeply lobed (2–3 lobes) and transversely extended. The esophagus was 9.2–12.6% of the fluke length. Based on these morphological properties along with the morphometric values (Table 2), the flukes were identified as *E. robustum*.

3.2. Sequence and phylogenetic analyses

The nucleotide sequences (589 bp) inferred from ITS2 region of the 16 flukes, which were identified morphologically as *E. revolutum*, were completely identical. The ITS2 sequence was 430 bp and named as Erev-ITS2 (accession number: LC224085). Erev-ITS2 was identical to the sequences (KM980472, KM520150 and KP342419) from Eurasian *E. revolutum*. The sequences (588 bp) of the ITS2 region of the 15 flukes identified morphologically as *E. robustum* were also identical and the ITS2 was 429 bp and named as Erob-ITS2 (accession number: LC224084). Erob-ITS2 was identical to the reference sequences of *E. friedi* (KJ848450 and AJ564383) and *E. revolutum* (U58102) from Eurasia, whereas, the sequence had only 2–3 nucleotide substitutions (5–6 if gaps are included) with those (GQ463132 and GQ463133) of *E. robustum* reported from America [21]. The alignments of Erev-ITS2 and Erob-ITS2 differed in 9 nucleotide positions (10, if gap is included), and interspecific variation between the two species was 2.33%. In the Bayesian tree (Fig. 2), Erev-ITS2 constituted a monophylogenic group together with *E. revolutum* references except one reference (U58102) and was included in the Eurasian cluster. Erob-ITS2 was included in the cluster (Eurasian *E. robustum*) formed by *E. friedi* (AJ564383 and KJ848450) and *E. revolutum* (U58102) and was distinct from the clade formed by the American isolates (GQ463132 and GQ463133) of *E. robustum*. The tree topology was almost the same as that constructed by ML method (data not shown).

The *nad1* sequences (479 bp) of *E. revolutum* had 14 variable sites yielding 9 haplotypes (Erev-nad1-G1–G9) (accession numbers: LC224097–LC224105). Fourteen nucleotide substitutions were observed in the *nad1* sequences (479 bp) of *E. robustum*, constituting 11 haplotypes (Erob-nad1-G1–G11) (accession numbers: LC224086–LC224096). The intraspecific variation in *E. revolutum* and *E. robustum* was 0.1–0.5% and 0.1–0.6%, respectively. The interspecific variation between *E. revolutum* and *E. robustum* was 6.7–7.7%. In the Bayesian tree (Fig. 3), Erev-nad1-G1 − G9 constituted a monophylogenic group along with the *E. revolutum* references except for one reference (AF025832) and was included in the Eurasian *E. revolutum* cluster. Erob-nad1-G1 − G11 were included in the Eurasian cluster comprising *E. miyagawai* (KP065625, KP065640, KP455622, KP455623, and KP455624), *E. friedi* (AJ564379), and *E. revolutum* (AF025832), and clearly separated from the clade formed by the American isolates (GQ463054 and GQ463055) of *E. robustum*. The tree topology was almost the same as that constructed by ML method (data not shown).

4. Discussion

Precise identification of *Echinostoma* species in the 37-collar-spined ‘*revolutum*’ group is difficult because of their morphological similarity and contradictory data from different hosts and geographic regions. In this study, adult echinostomes from ducks in Bangladesh were subjected for detailed morphological examination, and therefore, were identified *E. revolutum* and *E. robustum*, on the basis of the morphological characteristics and descriptions used previously for species delineation within the 37-collar-spined echinostomes [3,4,8,9]. The morphological characteristics and various morphometric measurements of *E. revolutum* in this study were consistent with those reported previously [3,8]. The position and lobulation of the testes and other morphological properties of *E. robustum* were consistent with the description of the species reported previously [20,22]. The testes of *E. miyagawai* have an irregular surface, are not lobed, and are extended

Table 2
Comparative morphometric properties of *E. robustum* recovered in this study with other closely related echinostomes.

	E. robustum in this study (n = 15)	*E. robustum*		*E. miyagawai*		*E. friedi*
		Yamaguti [25]	McDonald [24]	Faltynkova [3] (n = 13)	McDonald [24]	Toledo [27] (n = 70)
Body length (mm)	5.64–10.8	7.8–9.8	6–9.8	9.16–11.01	9.3–13	7.45–13.31
Body width (mm)	1.28–2.12	1.33–2.18	–	1.19–1.46	–	1.2–2.21
Fore body length (mm)	1.16–2.11	–	–	1.25–1.53	–	1.06–1.78
Fore body length/body length (%)	16.4–20.9	–	–	13.4–14.9	–	12–20
Head collar (μm)	360–707 × 568–889	670–850 (width)	–	393–505 × 598–692	680–960 (width)	241–494 × 540–793
Head collar/body width (%)	36.6–47.8	–	–	46.2–56.9	–	34–44
Collar spines	37	37	37	37	37	37
Angle spines (μm)	87–108	69–87	–	52–93	83–132	–
Lateral spines (μm)	80–107	87–105	–	67–87	–	–
Dorsal spines (μm)	63–85	87–123	–	58–75	–	–
Oral sucker (μm)	225–395 × 270–428	310–400	340–484	262–337 × 262–355	–	222–356 × 253–368
Ventral sucker (μm)	629–964 × 654–876	810–1080 × 800–960	–	655–748 × 655–785	–	620–919 × 643–862
Constriction at the level of Ventral Sucker	Present	Present	–	Present	–	Present
Prepharynx length (μm)	17–62	–	< 250	0–37	–	0–86
Pharynx (μm)	212–344 × 154–235	250–330 × 220–280	130–240 × 100–180	262–337 × 187–355	–	172–333 × 172–345
Oesophagus length (μm)	373–1292	780–1780	–	542–655	–	310–689
Oesophagu/body length (%)	9.2–12.6	9	–	–	–	5
Testes	Deeply lobed	Deeply lobed	Lobed, horizontally extended	Round, irregular margin	3 lobed	3 lobed
Anterior (μm)	264–502 × 467–790	500–620 × 410–660	–	430–692 × 337–505	840–1000	460–885 × 436–988
Posterior (μm)	260–621 × 488–862	–	–	449–655 × 337–524		552–1046 × 414–1034
Ovary (μm)	151–392 × 122–659	210–310 × 400–500	130–460	243–355 × 337–449	–	184–402 × 230–597
Vitelline glands	Not connected	–	Not connected		Connected behind the posterior testis	Almost connected behind the posterior testis
Egg (μm)	86–99 × 51–56	111–129	81–103	94–96 × 59–60	88–107	–

–: no data.

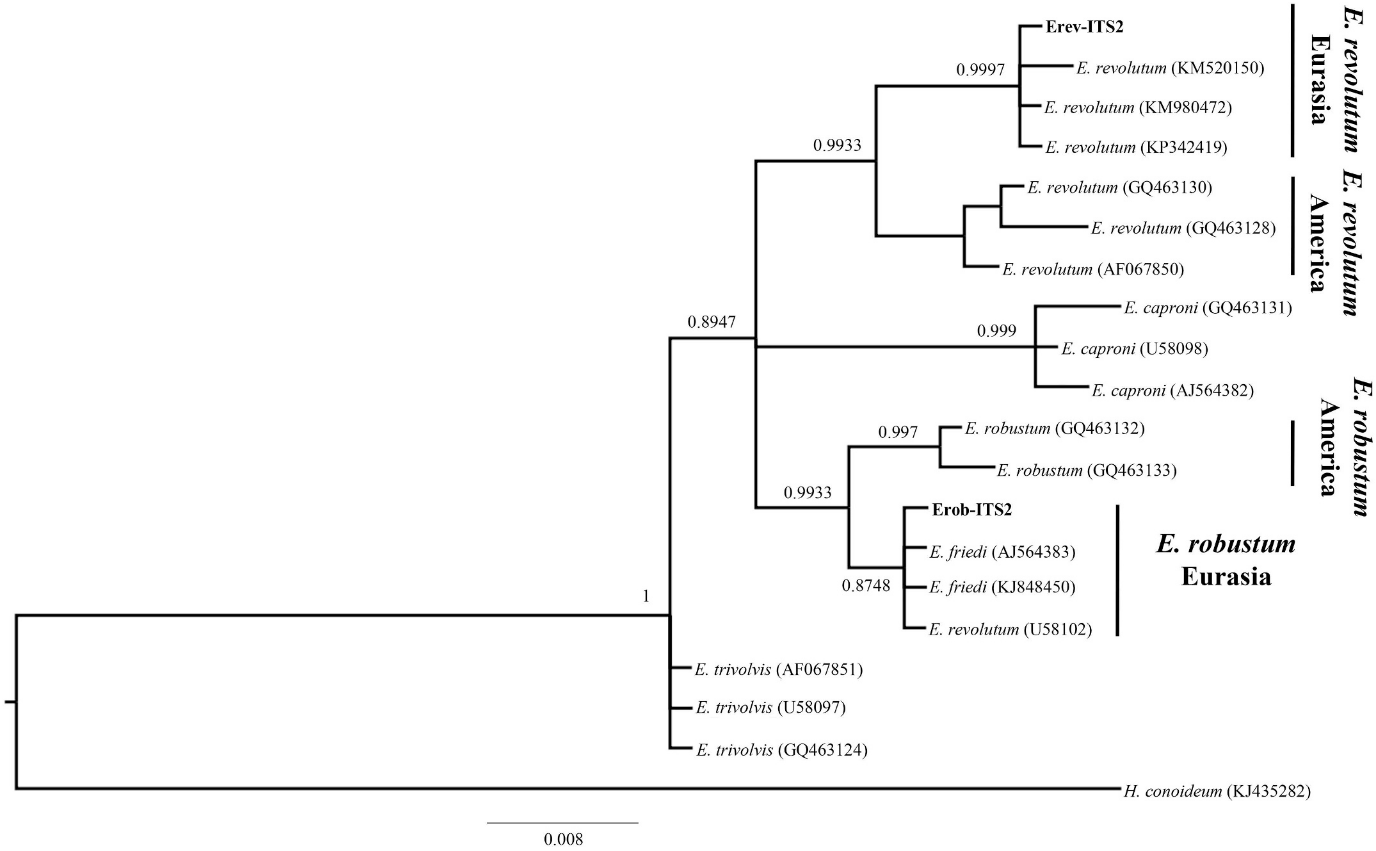

Fig. 2. Phylogenetic relationships of echinostomes based on the ITS2 sequences (427 bp) inferred from Bayesian (support values). Erev-ITS2 and Erob-ITS2 show genotypes of *E. revolutum* and *E. robustum,* respectively generated in the present study. Support values > 0.80 are shown near the node.

longitudinally [3,20]. These three species can be discriminated based on the ratio of the esophagus to the fluke length, which is 5% in *E. friedi* [22], 11–13% in *E. miyagawai* [23] and 9.8% in *E. robustum* [24]. In this study, the esophagus was 9.2–12.6% of the fluke length. However, the morphometric values often overlapped among species. Although the present *E. revolutum* specimens had a comparatively smaller body and forebody length than those described previously [3,8], the other morphological features were consistent with the reports. Intraspecific differences in the morphometric values of *E. revolutum* may be because of their divergent geographic origins (Europe, America, and Bangladesh) and final host (pigeons and ducks) as well as the source of adult flukes, which were recovered from natural or experimental infection [24]. In contrast, the morphological and morphometric characteristics of the present *E. robustum* specimens were consistent with those reported previously [20,22].

In this study, we analyzed adult echinostomes based on their nuclear ITS2 and mitochondrial *nad1* sequences. The present *E. revolutum* and *E. robustum* specimens showed no intraspecific variation in ITS2. This result is supported by previous findings, in which no intraspecific variation was found in any of the 7 species of echinostomes [7]. The sequence of Erob-ITS2 was identical to those of *E. friedi* (AJ564383 and KJ848450) and *E. revolutum* (U58102). Furthermore, the haplotypes (Erob-nad1-G1–G11) of *E. robustum* belonged to the same clade as *E. friedi* (AJ564379), *E. revolutum* (AF025832), and *E. miyagawai* (KP065625, KP065640, KP455622, KP455623, and KP455624) in the *nad1* Bayesian tree. These three *Echinostoma* species are members of the 'revolutum' group and share many morphological characteristics, making species identification difficult [5]. In fact, *E. revolutum* (U58102) was later re-described as *E. robustum* based on the detailed morphology of the voucher specimen [1].

In *nad1*, the sequences of *E. robustum* differed by 0–0.8% from *E. miyagawai* (KP455622–455624, KP065625, KP065640), 0–0.3% from *E. friedi* (AJ564379), and 0.3–0.8% from *E. revolutum* (AF025832). These values suggest that these *Echinostoma* isolates, which were identified as different species, are conspecific. Georgiva et al. [25] indicated that *E. robustum* (AF025832) and *E. friedi* (AJ564379) were conspecific because their genetic divergence was 0.8%. Furthermore, Faltnková et al. [3] reported that there are no justifying characters to differentiate *E. friedi* from *E. miyagawai*, and therefore these two species are considered as synonymous. Our molecular and morphological data combined with previously published data supports the synonymy of *E. robustum*, *E. miyagawai*, and *E. friedi* previously based on either molecular or morphological evidence. Two geographically distinct lineages of *E. revolutum*, Eurasian and American, have been recognized through molecular phylogenetic analyses [26]. This study also supported the occurrence of the lineages because *E. revolutum* from Bangladesh belonged to the Eurasian clade in both the ITS2 and *nad1* trees. Additionally, we found that *E. robustum* can be divided into the Eurasian and American lineages. However, further morphological and molecular studies are needed to verify this.

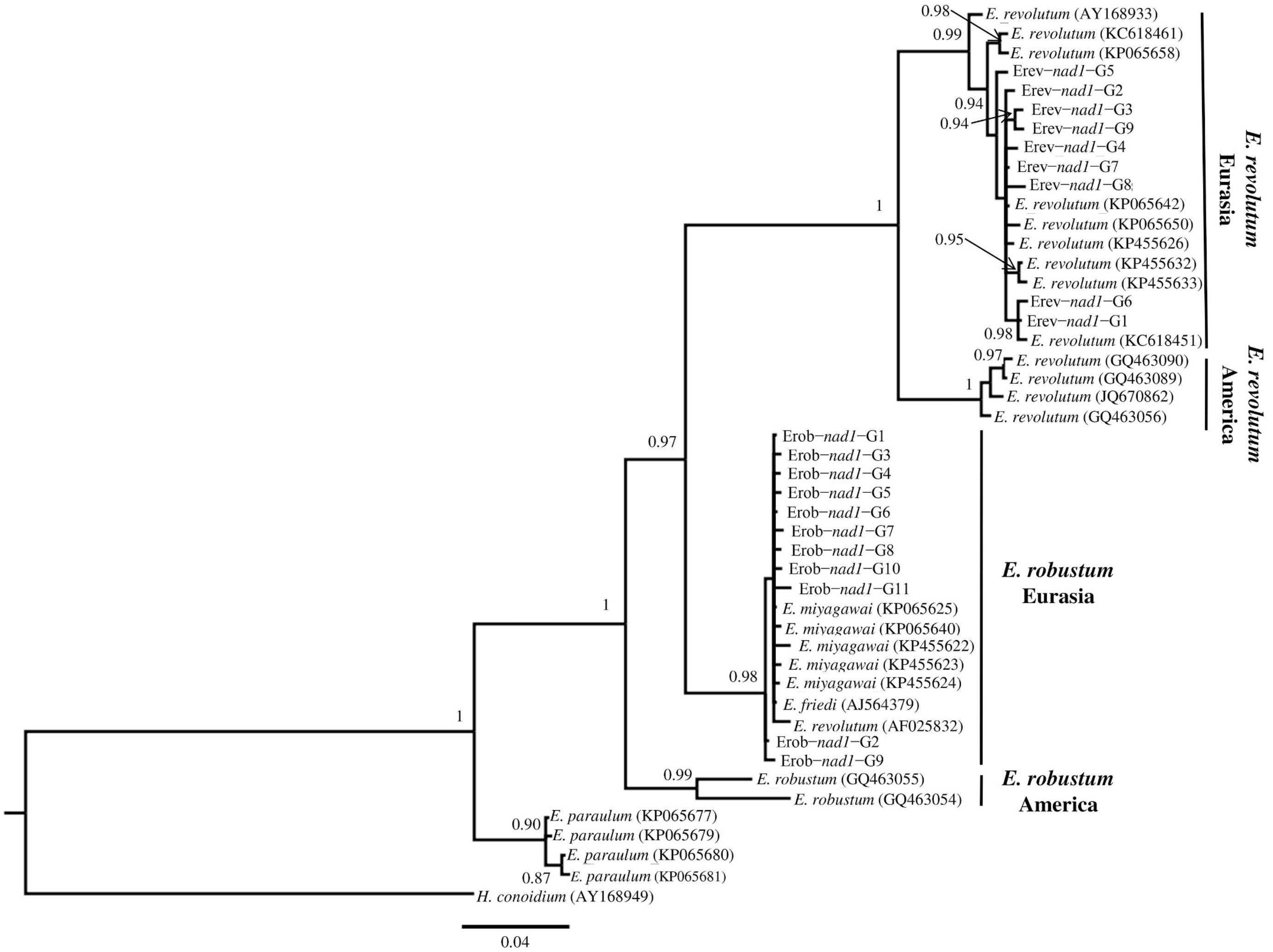

Fig. 3. Phylogenetic relationships of echinostomes based on the *nad1* sequences (448 bp) inferred from Bayesian (support values). Erev-*nad1*-G1 to G9 and Erob-*nad1*-G1 to G11 show genotypes of *E. revolutum* and *E. robustum*, respectively generated in the present study. Support values > 0.80 are shown near the node.

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