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GENETIC DISTANCE AND PHYLOGENETIC RELATIONSHIPS OF SOME *ECHINOSTOMA* SPECIES (*E. MALAYANUM, E. REVOLUTUM, E. MIYAGAWAI*) AND *HYPODERAEUM CONOIDEUM* (FAMILY ECHINOSTOMATIDAE) INFERRED FROM PARTIAL 28S rDNA SEQUENCE ANALYSIS

Le Thanh Hoa[1,2,✉], Pham Thi Khanh Linh[1,3], Nguyen Thi Khue[1,2], Do Thi Roan[1,2], Le Thi Kim Xuyen[1,2], Doan Thi Thanh Huong[1,2]

[1] *Institute of Biotechnology, Vietnam Academy of Science and Technology , 18 Hoang Quoc Viet Road, Cau Giay District, Hanoi, Vietnam*
[2] *Graduate University of Science and Technology, Vietnam Academy of Science and Technology, 18 Hoang Quoc Viet Road, Cau Giay District, Hanoi, Vietnam*
[3] *University of Science and Technology of Hanoi, Vietnam Academy of Science and Technology, 18 Hoang Quoc Viet Road, Cau Giay District, Hanoi, Vietnam*

✉To whom correspondence should be addressed. E-mail: imibtvn@gmail.com

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SUMMARY

Echinostomiasis is a neglected disease caused by the intestinal flukes (family Echinostomatidae, suborder Echinostomata) and is common in communities in Asian countries, such as India, Indonesia, the Philippines, China, Malaysia, Singapore, Korea, Japan, Thailand, Laos, Cambodia and Vietnam. The genetic markers from the nuclear ribosomal transcription units are commonly used in genetic studies and phylogenetic analyses. A portion of the 28S rDNA sequence (domains D1–D3, of 1062–1067 bp for the final use) was obtained from the zoonotic *Echinostoma malayanum* (strain E/Amala-EMI3-TH), *E. revolutum* (strain Erevo-MSD15-TH), *E. miyagawai* (Emiya-RED11-TH), and *Hypoderaeum conoideum* (Hcono-RED42-TH) species; and used to perform an alignment for genetic distance estimation and phylogenetic analysis. The alignment was performed using 62 strains of 42 species from 19 genera of the family Echinostomatidae, including *Echinoparyphium*, *Echinostoma*, *Artyfechinostomum*, *Patagifer*, *Neoacanthoparyphium*, *Hypoderaeum*, *Echinoparyphium*, *Drepanocephalus*, *Euparyphium*, *Chaunocephalus*, *Neopetasiger*, *Ribeiroia*, *Cathaemasia*, *Rhopalias*, *Isthmiophora*, *Petasiger*, *Moliniella*, *Pegosomum*, and *Schistosoma* (family Schistosomatidae). The genetic distance estimation among 16 strains/10 species has shown a low intra-specific divergence level between strains within the same species, such as *E. miyagawai* (0–0.10%), *E. revolutum* (0.10–0.50%), and *H. conoideum* (0–0.10%), while between strains within the same genus it was higher (normally over 1.0%) and among strains/species between genera it was the highest (3.06–4.12%). The 28S rDNA sequence analysis and phylogenetic relationship well supported the *Echinostoma/ Artyfechinostomum malayanum* intergeneric taxonomy and the topology indicated clear, well-supported positions of member species in different genera in the family Echinostomatidae of the suborder Echinostomata. More *sensu lato* samples of the genera, are required for sequencing, particularly those of zoonotic species in the five genera: *Artyfechinostomum*, *Echinostoma*, *Hypoderaeum*, *Echinoparyphium*, and *Isthmiophora*. The resultant mitochondrial and nuclear data obtained from these species will be a good source to use to clearly assess the taxonomic and generic relationships.

Keywords: 28S rDNA sequence, *Artyfechinostomum*, *Echinostoma*, Echinostomatidae, Echinostomata, genetic distance, *Hypoderaeum*, phylogenetic analysis, ribosomal transcription unit

INTRODUCTION

Tens of species from at least five genera in the family Echinostomatidae Looss, 1899 (Trematoda: Platyhelminthes) frequently cause human infections worldwide (Toledo, Esteban, 2016; Chai, 2019). Included among these zoonotic genera are *Echinostoma* Rudolphi, 1809; *Hypoderaeum* Dietz, 1909; *Echinoparyphium* Dietz, 1909; *Isthmiophora* Lühe, 1909; and the recently identified genus *Artyfechinostomum* (Prasad *et al*., 2019; Chai, 2019). Human infections by Echinostomatidae species are common in communities in Asian countries, such as India, Indonesia, the Philippines, China, Malaysia, Singapore, Korea, Japan, Thailand, Laos, Cambodia, and Vietnam (Chai, 2009; Saijuntha *et al*., 2011; Sohn *et al*., 2017; Toledo, Esteban, 2016; Chai, Jung, 2020). The family Echinostomatidae belongs to the suborder Echinostomata and exhibits a substantial taxonomic diversity, among which tens of species belong to the genus *Echinostoma*, which is the most complex genus (Chai, 2019). Echinostomes have been differentiated by morphological characteristics, particularly the presence of “collar-spines” around the oral sucker, among which the most important "*revolutum*" group (*E. revolutum*) has 37-collar-spines, other *Echinostoma* species have varying numbers, such as 25–29 (*E. hortense*), 31 (*E. anseries*), 43 (*Echinostoma/Artyfechinostomum malayanum*), and 43–45 (*E. aegyptiacum*) while *Hypoderaeum conoideum* has 41–45 collar-spines (Kostadinova, 2005; Georgieva *et al*., 2014; Saijuntha *et al*., 2011; Sohn *et al*., 2017; Chai, 2019; Le *et al*., 2020). *Echinostoma malayanum* (Leiper, 1911) was the first described as infecting people in Malaysia in 1911 (Mukherjee, Ghosh, 1968), causing considerable taxonomic controversy, originally being synonymised with *A. surfrartyfex* (Lane, 1915; Prasad *et al*., 2019), and now, in fact, being considered as *Artyfechinostomum malayanum* (Chai, 2019; Pham *et al*., 2022). Traditionally, spine numbers and morphological characteristics have been used for species differentiation of echinosomes (Georgieva *et al*., 2014; Faltýnková *et al*., 2015; Chai, 2019). However, it is not true since these can vary between individuals of the same species, and morphological characters and spines can also be lost during sample preparation, leading to species-misidentification.

The major challenge in taxonomy of echinosomes and Echinostomatidae systematics is a gap needing to be filled in the understanding of evolutionary and phylogenetic relationships of species within the family Echinostomatidae of the suborder Echinostomata (Tantrawatpan *et al*., 2013; Georgieva *et al*., 2013; 2014; Faltýnková *et al*., 2015; Chai, 2009; 2019). DNA sequences are commonly used for molecular diagnosis, providing a basis for the development of accurate diagnostic tools and systematic/phylogenetic studies. The use of molecular markers has solved the specific classification and phylogenetic relationships of particular species of *Echinostoma* and genera within the family Echinostomatidae, and between families of the suborder Echinostomata (Kostadinova, 2005; Georgieva *et al*., 2014; Tkach *et al*., 2016; Chai, 2019; Chai, Jung, 2020).

The DNA markers from the nuclear ribosomal transcription unit (rTU) (including 18S, ITS1, ITS2, and 28S) have been shown to be crucial in resolving taxonomic issues for parasitic worms (Tkach *et al*., 2016; Le *et al*., 2020). The 18S and 28S rDNA sequences as well as the intergenic regions (ITS-1, ITS-2) were used as reliable molecular markers in the analysis of phylogenetic and molecular evolutionary relationships between species and taxonomic classification (Weider et al., 2005; Blair, 2006; Tkach *et al*., 2016; Pérez-Ponce de León *et al*., 2019). A detailed ribosomal phylogenetic analysis of taxonomically confused echinostomes, particularly those related to *Echinostoma*/*Artyfechinostomum* and *Hypoderaeum* and their generic congeners, will facilitate clarification of inter-relationships among species of the family Echinostomatidae (and Echinostomata suborder).

Thus, the aim of this paper is to present the use of 28S rDNA sequences to assess the genetic distance and an in-depth phylogenetic approach to resolve the interrelationship between *E. malayanum* (synonym: *Artyfechinostomum malayanum*), *E. miyagawai, E. revolutum, H. conoideum* and their trematode congeners in the genera *Echinostoma*, *Artyfechinostomum*, and *Hypoderaeum* in the Echinostomatidae of the suborder Echinostomata.

MATERIALS AND METHODS

Parasite samples, DNA extraction, and species identification

Adult *E. revolutum, E. miyagawai* and *H. conoideum* flukes were obtained from the intestines of naturally infected domestic ducks from abattoirs; adult *Echinostoma malayanum* (syn. *Artyfechinostomum malayanum*) flukes were recovered from the intestines of experimental hamsters fed on cysts containing metacercariae collected from the freshwater snail *Indoplanorbis exustus* in Khon Kaen province, Thailand. The samples were obtained from Assoc. Prof. Dr. Weerachai Saijuntha (Walai Rukhavej Botanical Research Institute, Biodiversity and Conservation Research Unit, Mahasarakham University, Mahasarakham 44150, Thailand). The flukes were thoroughly washed in physiological saline, morphologically examined, and verified by molecular analysis.

This study involved four strains of four Echinostomatidae species, *E. malayanum* (E/Amala-EMI3-TH), *E. revolutum* (Erevo-MSD15-TH), *E. miyagawai* (Emiya-RED11-TH), and *H. conoideum* (Hcono-RED42-TH) (Table 1). Due to its synonymy with the generic name of *Artyfechinostomum* (as it is *Artyfechinostomum malayanum*) (Chai, 2019; Pham *et al*., 2022), the abbreviation of the *E. malayanum* strain, is changed to E/Amala-EMI3-TH for use.

Total genomic DNA was extracted from an individual worm, using the DNA extraction kit (QIAGEN, Hilden, Germany) as instructed, eluted in 100 μL, and stored at −20°C until use (Saijuntha *et al*., 2011; Tantrawatpan *et al*., 2013). The DNA concentration was estimated using a Thermo Scientific Nanodrop 1000 UV/VIS spectrophotometer and diluted to a working 50 ng/μL and 2 μL was used as a template in a PCR of 50 μL volume.

Primers, amplification and sequence annotation

Primers, including forward LSU1F (5’ CTTAAGCATATCACTAAGCGGAGG 3’) and reverse LSU3R (5′ GCTATCCTGAGGGAAACTTCG 3′) were designed based on the alignment of 28S rDNA sequences available from GenBank and previous publications. These primers were used for amplification of a 28S rDNA region (i.e., the D1–D3 domain) to obtain a PCR amplicon of about 1.3 kb in length, and they were also used as the flanking primers for direct sequencing from both ends. PCR reactions of 50 μL were prepared using 25 μL of DreamTaq PCR Master Mix (2X) (Thermo Fisher Scientific Inc., MA, USA) and 2 μL of DNA template (50 ng/μL), 2 μL of each primer (10 pmol/μL), 2 μL DMSO (dimethyl sulphoxide) and 17 μL of water, performed in an MJ PTC-100 thermal cycler. Initiation was at 94°C for 5 min, followed by 35 cycles consisting of denaturation for 30 s at 94°C, annealing at 52°C for 30 s, extension at 72°C for 6 min, and a final extension at 72°C for 10 min. The PCR products (10 μL of each) were examined on a 1% agarose gel, stained with ethidium bromide and visualized under UV light (Wealtec, Sparks, NV, USA). The amplicons were sent to the sequencing services (Nam Khoa company, Ho Chi Minh City) for direct sequencing.

The partial 28S rDNA sequence for each of the four Echinostomatidae species was obtained after editing chromatograms (using Chromas 2.6.6; http://technelysium.com.au/wp/chromas/) and was 1120–1230 bp for analysis, respectively, and deposited in the GenBank database (NCBI).

Sequence analysis

A total of 62 partial 28S rDNA sequences, approximately 1.1–1.3 kb in length, from 62 strains of 43 trematode species of the Echinostomatidae, including *E. revolutum*, *E. miyagawai*, *E. malayanum*, *H. conoideum*, was used in this study. Included in the alignment are trematodes from the genera *Echinoparyphium*, *Echinostoma*, *Euparyphium*, *Hypoderaeum*, *Artyfechinostomum*, *Chaunocephalus*, *Cathaemasia*, *Drepanocephalus*, Echinostomatidae sp., *Isthmiophora*, *Moliniella*, *Neoacanthoparyphium*, *Neopetasiger*, *Pegosomum*, *Patagifer*, *Neopetasiger*, and *Rhopalias* of Echinostomatidae. The sequences were aligned using GENEDOC 2.7 (available at: http://iubio.bio.indiana.edu/soft/molbio/ibmpc/genedoc-readme.html). A 28S rDNA sequence of *Schistosoma haematobium* (family Schistosomatidae) was used as an outgroup (listed in Table 1). The final alignment block containing sequences of 1062–1067 bp was used for estimation of genetic distance and assessment of phylogenetic relationships.

Table 1. List and information on strains and species that provide partial nuclear ribosomal 28S rDNA sequences for phylogenetic analysis and tree construction for the assessment of the taxonomic relationships of the family Echinostomatidae (Trematoda: Platyhelminthes).

No	Species	Abbrevi-ation	Sequence designation	Country of isolation	GenBank accession No
1	*Echinoparyphium aconiatum*	Eacon	Eacon-(Lstag)-CZ	Czech	KT956912
2	*Echinoparyphium cinctum*	Ecinc	Ecinc-UA(sub)	n/a	AF184260
3	*Echinoparyphium ellisi*	Eelli	Eelli-EEAP2-NZ	New Zealand	KY436410
4	*Echinoparyphium mordvilkowi*	Emord	Emord-L563-LT	Lithuania	KJ542642
5	*Echinoparyphium poulini*	Epoul	Epou-EPCA2-NZ	New Zealand	KY436409
6	*Echinoparyphium recurvatum*	Erecu	Erecu-(Rovat)-UK	United Kingdom	KT956913
7	*Echinoparyphium rubrum*	Erubr	Erubr-2(Pcolc)-US	United States	JF820595
8	*Echinostoma bolschewense*	Ebols	Ebols-EBG13-SK	Slovakia	KP065591
9	*Echinostoma bolschewense*	Ebols	Ebols-EBG14-SK	Slovakia	KP065592
10	*Echinostoma cinetorchis*	Ecine	Ecine-1- SK(sub)	South Korea	KX817344
11	*Echinostoma malayanum*	Emala	**Emala-EMI3-TH**	Thailand	This study
12	*Echinostoma miyagawai*	Emiya	Emiya-EMT2-CZ	Czech	KP065593
13	*Echinostoma miyagawai*	Emiya	Emiya-HLJ-CN	China	MH748722
14	*Echinostoma miyagawai*	Emiya	**Emiya-RED11-TH**	Thailand	This study
15	*Echinostoma novaezealandense*	Enova	Enova-ENCA-NZ	New Zealand	KY436407
16	*Echinostoma paraensei*	Eparae	Eparae-(hamster)-US	United States	EU025867
17	*Echinostoma paraulum*	Eparau	Eparau-EPM1-DE	Germany	KP065604
18	*Echinostoma paraulum*	Eparau	Eparau-EPT1-CZ	Czech	KP065605
19	*Echinostoma revolutum*	Erevo	Erevo-ERBA1-CZ	Czech	KP065594
20	*Echinostoma revolutum*	Erevo	Erevo-ERT1-CZ	Czech	KP065596
21	*Echinostoma revolutum*	Erevo	Erevo-ERVD1-CZ	Czech	KP065595
22	*Echinostoma revolutum*	Erevo	**Erevo-MSD15-TH**	Thailand	This study
23	*Echinostoma revolutum*	Erevo	Erevo-VVT2015-US	United States	KT956915
24	*Euparyphium capitaneum*	Ecapi	Ecapi-3(Aanhi)-US	United States	KP009618
25	*Euparyphium capitaneum*	Ecapi	Ecapi-5(Aanhi)-US	United States	KP009620
26	*Euparyphium melis*	Emel	Emeli- UA(sub)	n/a	AF151941
27	*Euparyphium cf. murinum*	Emuri	Emuri cf.-VVT2015-UG	Uganda	KT956917
28	*Hypoderaeum conoideum*	Hcono	Hcon-AF261-FI	Finland	MZ409814
29	*Hypoderaeum conoideum*	Hcono	Hcon-AK44-CZ	Czech	KP065607
30	*Hypoderaeum conoideum*	Hcono	Hcono-Kherson-UA	Ukraine	KT956918

31	*Hypoderaeum conoideum*	Hcono	Hcono-NA-US	United States	KT956919
32	*Hypoderaeum conoideum*	Hcono	**Hcono-RED42-TH**	Thailand	This study
33	*Artyfechinostomum sufrartyfex*	Asufr	Asufr-Shillong-IN	India	KF781303
34	*Chaunocephalus ferox*	Cfero	Cfero-(Cnig)-UA	Ukraine	KT447522
35	*Cathaemasia hians*	Chian	Chian-(Ppla)-CZ	Czech	KT956947
36	*Drepanocephalus auritus*	Dauri	Dauri-MJGDA-US	United States	KP053259
37	*Drepanocephalus mexicanus*	Dmexi	Dmexi-DNA1519-MX	Mexico	MF351544
38	*Drepanocephalus spathans*	Dspat	Dspat-HCC-US	United States	JN993270
39	Echinostomatidae sp.	Ech-sp	Ech-spCMA2010a-US	United States	GU270100
40	*Isthmiophora hortensis*	Ihort	Ihort-Waka-JP	Japan	AB189982
41	*Isthmiophora melis*	Imeli	Imeli-(Aagra)-PL	Poland	KT359583
42	*Moliniella anceps*	Mance	Mance-Jodk-LT	Lithuania	KT956921
43	*Neoacanthoparyphium echinatoides*	Nechi	Nechi-Gabci-SK	Slovakia	KT956922
44	*Neopetasiger islandicus*	Nisla	Nisla-(Aocci)-US	United States	KT956924
45	*Neopetasiger islandicus*	Nisla	Nisla-MGC6-CA	Canada	KT831344
46	*Pegosomum asperum*	Paspe	Paspe-Biber-DE	Germany	KY945919
47	*Pegosomum saginatum*	Psagi	Psagi-Bibe-DE	Germany	KY945918
48	*Patagifer bilobus*	Pbilo	Pbilo-Kherson-UA	Ukraine	KT956945
49	*Patagifer vioscai*	Pvios	Pvios-(Ealbu)-US	United States	KT956946
50	*Petasiger exaeretus*	Pexae	Pexae-Kherson-UA	Ukraine	KT956923
51	*Petasiger exaeretus*	Pexae	Pexae-KM4-HU	Hungary	KY284009
52	*Petasiger exaeretus*	Pexae	Pexae-PA3-HU	Hungary	KY284001
53	*Petasiger phalacrocoracis*	Ppha	Ppha-KM1-HU	Hungary	KY284006
54	*Petasiger phalacrocoracis*	Ppha	Ppha-KM3-HU	Hungary	KY284008
55	*Petasiger phalacrocoracis*	Ppha	Ppha-PA2-HU	Hungary	KY284000
56	*Petasiger radiatus*	Pradi	Pradi-(Pcarb)-UA	Ukraine	KT956927
57	*Petasiger radiatus*	Pradi	Pradi-KM5-HU	Hungary	KY284010
58	*Neopetasiger islandicus*	Pisla	Pisla-AK231-IS	Iceland	JQ425592
59	*Rhopalias macracanthus*	Rmacr	Rmacr-1-8-MX	Mexico	MK648280
60	*Ribeiroia ondatrae*	Ronda	Ronda-JAM17N33-US	United States	MK321661
61	*Ribeiroia ondatrae*	Ronda	Ronda-(Peryt)-US	United States	KT956956
62	*Schistosoma haematobium*	Shaem	Shaem-N10-ML*	Mali	AY157607

Note: Species: full name; abbreviation: five letters with the first capital letter from the genus and next four are the first letters from the species name. Sequence designation: the strain name is in between the abbreviation of the species and the country names (two letters) in which the bolded names indicate the sequences obtained in this study. *Outgroup sequence (from *Schistosoma haematobium* (Schistosomatidae)).

Genetic distance estimation

A pairwise distance analysis was also performed and estimated as a measure of genetic distance (p-distance) between 16 strains of 10 species of three genera (*Echinostoma*, *Artyfechinostomum*, and *Hypoderaeum*) in the family Echinostomatidae. The 16 sequence alignment was imported into MEGA X. The analysis was set for distance estimation using the "Maximum Composite Likelihood" model/method with 1000 bootstrap replications (Kumar *et al*., 2018).

Phylogenetic reconstruction

To examine the phylogenetic position of the Echinostomatidae species relative to their congeners and other trematodes, a phylogenetic tree was reconstructed from the alignment of 62 partial 28S rDNA sequences (listed in Table 1), including the outgroup sequence, using the maximum likelihood (ML) analysis in the MEGA X program. The substitution model with the best score according to the Bayesian information criterion was the (GTR+G+I) model, with residue frequencies estimated from the data (GTR), rate variation along the length of the alignment (+G), and allowing for a proportion of invariant sites (+I) (Kumar *et al*., 2018).

RESULTS

Pairwise genetic distances among *Echinostoma, Artyfechinostomum,* and *Hypoderaeum* species

The partial 28S rDNA sequences (1062–1067 bp) of 16 strains of nine species of the genera *Echinostoma*, *Artyfechinostomum* and *Hypoderaeum* including E/Amala-EMI3-TH of *Echinostoma/Artyfechinostomum malayanum* (Pham *et al*., 2022), Emiya-RED11-TH of *Echinostoma miyagawai*, Erevo-MSD15-TH of *Echinostoma revolutum*, and Hcono-RED42-TH of *Hypoderaeum conoideum*, respectively were used to estimate the genetic distance using the alignment of the sequences.

The p-distance calculations for the partial 28S rDNA nucleotide sequences showed the lowest level of divergence among strains within each species at 0–0.1% for three strains of *E. miyagawai* (Emiya-RED11-TH, Emiya-HLJ-CN, and Emiya-EMT2-CZ), 0–0.50% for three strains of *E. revolutum* (Erevo-MSD15-TH, Erevo-ERBA1-CZ, and Erevo-VVT2015-US), and 0–0.1% for three strains of *H. conoideum* (Hcono-RED42-TH, Hcono-AK44-CZ, and Hcono-NA-US). This divergence indicates an intra-species variation level or intra-specific genetic divergence among strains within a species (Table 2). Table 2 also showed the pairwise genetic distance estimated among strains between three genera, as indicated by the block highlights. In the first column, it was between *Echinostoma*/*Artyfechinostomum malayanum* (E/Amala-EMI3-TH) and *Echinostoma* (3.07–4.02%), and between this species and *Hypoderaeum* species (4.02–4.12%). In the last row, it was between *H. conoideum* (Hcono-NA-US) and *Artyfechinostomum* (3.69–4.12%); and between *Artyfechinostomum* and *Echinostoma* (3.06–3.80%) species. This divergence indicates an inter-generic variation or inter-generic genetic divergence among the genera (Table 2).

Overall, a relatively high divergence was seen between *E. revolutum* (Erevo-MSD15-TH and Erevo-VVT2015-US) at 3.80%, while the highest divergence was between *A. malayanum* and *H. conoideum* (Hcono-AK44-CZ and Hcono-NA-US), at 4.12%. Between E/Amala-EMI3-TH of *Echinostoma/ Artyfechinostomum malayanum* and Asufr-Shillong-IN (KF781303) of *Artyfechinostomum sufrartyfex,* the genetic distance was shown at 1.0%, which was too low in respect of an interspecific (between species) variation level. It was debated that the extremely close interrelationship between *E. malayanum* (or A. *malayanum*) and *A. sufartyfex* makes it possible to consider as an intraspecific variation level. Or indeed, as to which taxonomic validity is for this fluke, *Echinostoma malayanum*, or *Artyfechinostomum sufartyfex,* or *Artyfechinostomum malayanum* or all should be unified into one. The generic name for this species has been recently suggested to be retaken by *Artyfechinostomum malayanum* based on the complete mitochondrial genome analysis (Pham *et al*., 2022).

Phylogenetic interrelationships and taxonomic position of *Echinostoma, Artyfechinostomum,* and *Hypoderaeum* species

To examine the phylogenetic interrelationships and taxonomic position of some *Echinostoma, Artyfechinostomum*, and *Hypoderaeum* species in the family Echinostomatidae within the suborder Echinostomata, an ML tree was constructed from a phylogenetic analysis of 62 partial 28S rDNA sequences for 43 trematode species belonging to 18 genera of the family Echinostomatidae and an outgroup species, *Schistosoma haematobium* (Schistosomatidae) (Table 1; Fig. 1).

In the tree presented in Fig. 1, 62 sequences were placed in 21 groups/branches that were distinguished from each other. Besides the outgroup sequence (*S. haematobium*), the majority of groups/branches were clearly performed from the sequences of strains/species of 18 genera they belong to, including *Echinoparyphium*, *Echinostoma*, *Artyfechinostomum*, *Patagifer*, *Neoacanthoparyphium*, *Hypoderaeum*, *Echinoparyphium*, *Drepanocephalus*, *Euparyphium*, *Chaunocephalus*, *Neopetasiger*, *Ribeiroia*, *Cathaemasia*, *Rhopalias*, *Isthmiophora*, *Petasiger*, *Moliniella*, and *Pegosomum*.

The topology indicated well in the phylogenetic tree (Fig. 1), that the genus *Echinostoma* with *E. miyagawai* (strain Emiya-RED11-TH) and *E. revolutum* (strain Erevo-MSD15-TH) in this study was placed as a "sister" group to *Artyfechinostomum*, formed by E/Amala-EMI3-TH of *Echinostoma/ Artyfechinostomum malayanum* and Asufr-Shillong-IN of *Artyfechinostomum sufrartyfex*. The *E. malayanum* species was resolved as a sister taxon to *A. sufrartyfex* and was confirmed as a member of the genus *Artyfechinostomum*. The group of *Hypoderaeum conoideum*, including the strain Hcono-RED42-TH of this study, was rendered as paraphyletic (Fig. 1).

Table 2. Pairwise genetic distances (%) among 16 strains/10 species and between the genera of *Echinostoma*, *Artyfechinostomum*, and *Hypoderaeum* estimated based on the analysis of the partial 28S rDNA sequences.

		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
1	**E/Amala-EMI3-TH**															
2	Asufr-Shillong-IN (KF781303)	1.00														
3	**Emiya-RED11-TH**	3.28	2.85													
4	Emiya-HLJ-CN (MH748722)	3.39	2.96	**0.10**												
5	Emiya-EMT2-CZ (KP065593)	3.28	2.85	**0.00**	**0.10**											
6	**Erevo-MSD15-TH**	4.02	3.38	0.90	1.00	0.90										
7	Erevo-ERBA1-CZ (KP065594)	3.92	3.27	0.80	0.90	0.80	**0.10**									
8	Erevo-VVT2015-US (KT956915)	3.92	3.38	0.90	1.00	0.90	**0.50**	**0.40**								
9	Ebols-EBG13-SK (KP065591)	3.48	3.06	0.80	0.90	0.80	1.71	1.61	1.71							
10	Ecine (KX817344)	3.07	2.64	0.20	0.30	0.20	0.90	0.80	0.90	1.00						
11	Enova-ENCA-NZ (KY436407)	3.28	2.85	0.60	0.70	0.60	1.20	1.10	1.21	1.00	0.40					
12	Eparae-(hamster) -US (EU025867)	3.28	2.96	0.50	0.60	0.50	1.20	1.10	1.00	1.10	0.50	0.70				
13	Eparau-EPM1-DE (KP065604)	3.28	2.85	0.20	0.10	0.20	0.90	0.80	0.90	1.00	0.20	0.60	0.50			
14	**Hcono-RED42-TH**	4.02	3.58	2.96	3.06	2.96	3.69	3.59	3.69	3.16	2.96	3.17	3.06	2.96		
15	Hcono-AK44-CZ (KP065607)	4.12	3.69	3.06	3.17	3.06	3.80	3.69	3.80	3.27	3.06	3.27	3.17	3.06	**0.10**	
16	Hcono-NA-US (KT956919)	4.12	3.69	3.06	3.17	3.06	3.80	3.69	3.80	3.27	3.06	3.27	3.17	3.06	**0.10**	**0.00**

Note: Information for strain/ species is given in Table 1. The sequences for the strains/species of this study are bolded (Nos 11, 14, 22, 32). The different highlighted blocks in the first column (E/Amala-EMI3-TH) and the last row (Hcono-NA-US) indicate the pairwise genetic divergence between these species and members of each genus. The intra-specific genetic distance of strains within each species (*E. miyagawai*, *E. revolutum*, *H. conoideum*) is squared.

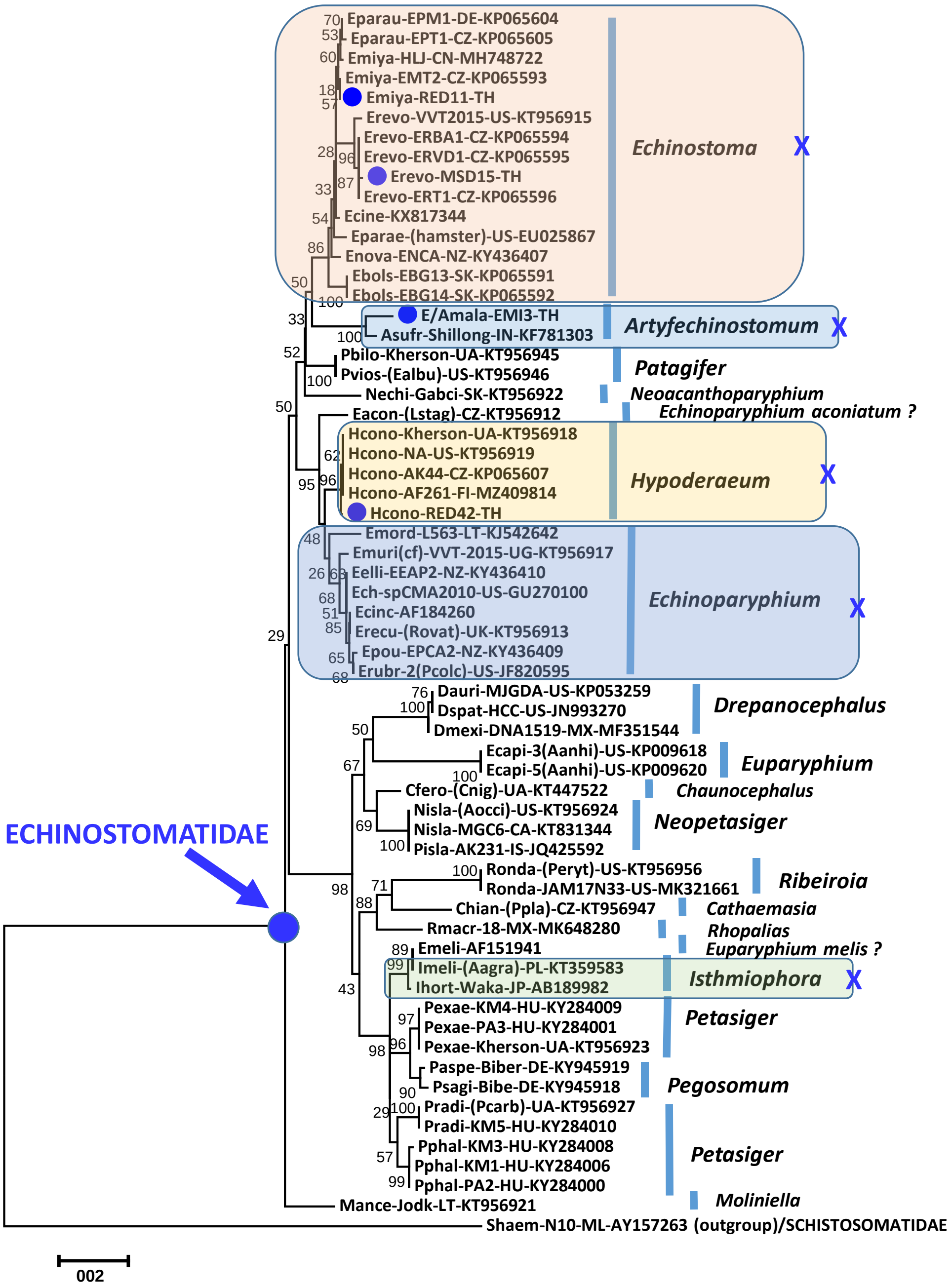

Figure 1. A maximum likelihood phylogenetic tree showing the interrelationships and taxonomic position of *Echinostoma, Artyfechinostomum*, and *Hypoderaeum* in this study (solid circle symbol) and other members of the family Echinostomatidae based on analysis of the partial 28S rDNA sequence data from 62 strains/species. This tree was reconstructed by the MEGA X program (Kumar *et al*., 2018) from 18 genera of 43 trematode species of the family Echinostomatidae and an outgroup species (*Schistosoma haematobium* of Schistosomatidae). Five genera marked by X (*Echinostoma, Artyfechinostomum, Hypoderaeum, Echinoparyphium*, and *Isthmiophora*) that contain species capable of infecting humans, are framed and highlighted. Nodal support values evaluated using 1000 bootstrap resamplings are shown on each branch. An abbreviation name is given for each species/strain, followed by the strain abbreviation (if available) and the country name (in two capital letters) of their origin (where available) (according to (http://www.nationsonline.org/oneworld/country_code_list.htm). For example, CZ: Czech; UA: Ukraine; US: United States. GenBank accession numbers are given at the end of each sequence. The scale bar represents the number of substitutions per site.

However, there are some exceptions. The positions of the *Echinoparyphium aconiatum* (Eacon-(Lstag)-CZ) and *Euparyphium melis* (Emeli-UA(sub)) species and the *Petasiger* and *Pegosomum* genera were the exceptions. According to the topology, *Echinoparyphium aconiatum* was removed from the cluster of *Echinoparyphium* species (being placed as paraphyletic from *Echinoparyphium* and the *Hypoderaeum* group was bracketed in), and *Euparyphium melis* was removed from the *Euparyphium* cluster (was placed in the *Isthmiophora* group). Another interesting placement is that the genus *Pegosomum* was bracketed between two clades of the genus *Petasiger* clusters (Table 1; Fig. 1). The mispositions may be involved in the taxonomic misidentification of these species and the wrong deposition in GenBank.

DISCUSSION

The Echinostomatidae are one of the largest families in the class Trematoda that exhibits an extremely high level of diversity. The morphological characters that have been used for species identification and taxonomic analysis are not sufficient to clarify the closely related taxa (Georgieva *et al*., 2013; 2014; Faltýnková *et al*., 2015). This has been a particular issue for the genus *Echinostoma* since there are multiple synonyms and newly described species, leading to frequent revision of Echinostomatidae systematics (Tantrawatpan *et al*., 2013; Chai, 2009; 2019). The spine collar examination can often be a tenuous characteristic for species differentiation, and the exact criteria for species taxonomy and family systematics have not been determined (Georgieva *et al*., 2014; Tkach *et al*., 2016; Chai 2019; Le *et al*., 2020).

In this study, four 28S rDNA sequences from *Echinostoma/Artyfechinostomum malayanum* (E/Amala-EMI3-TH), *Echinostoma miyagawai* (Emiya-RED11-TH), *Echinostoma revolutum* (Erevo-MSD15-TH), and *Hypoderaeum conoideum* (Hcono-RED42-TH), respectively, were included in the alignment for estimation of genetic distance and phylogenetic analysis of the Echinostomatidae species. The genetic distance between strains within the same species (intra-specific divergence level) of *E. miyagawai*, *E. revolutum* and *H. conoideum* was very low (0–0.50%), while between strains within the same genus was higher (normally over 1.0%), while among strains/species between genera was the highest (3.06–4.12%).

The phylogenetic analyses have resolved well-supported monophyletic clusters for the majority of genera, especially for the genera *Echinostoma*, *Artyfechinostomum*, and *Hypoderaeum*. The validity of the generic *Artyfechinostomum* for *Echinostoma malayanum* has been solved to retake the generic name of *Artyfechinostomum malayanum*, as previously suggested (Mehlhorn, 2015; Sohn *et al*., 2017; Chai, 2019), and recently confirmed by mitochondrial sequence analysis (Pham *et al*., 2022). The 28S rDNA sequence analysis indicates that the family Echinostomatidae presents broad systematic and taxonomic challenges and validates the combination of using morphological and molecular, both mitochondrial and ribosomal genomic datasets to ensure the taxonomic and generic relationships of member species in the family Echinostomatidae of the suborder Echinostomata (Olson *et al*., 2003; Tkach *et al*., 2016; Chai, 2019; Pérez-Ponce de León *et al*., 2019; Le *et al*., 2020; Pham *et al*., 2022). To clarify the genetic and taxonomic situations, more *sensu lato* samples of the genera are required for sequencing, particularly those of zoonotic species in the five genera: *Artyfechinostomum*, *Echinostoma*, *Hypoderaeum*, *Echinoparyphium*, and *Isthmiophora*. The resultant mitochondrial and nuclear data obtained from these species will be a good source to use to clearly assess the taxonomic and generic relationships.

CONCLUSION

In conclusion, the present study determined the genetic distance and phylogenetic interrelationships of

Echinostoma/Artyfechinostomum malayanum (E/Amala-EMI3-TH), *Echinostoma miyagawai* (Emiya-RED11-TH), *Echinostoma revolutum* (Erevo-MSD15-TH), and *Hypoderaeum conoideum* species with other echinosome species from 21 genera in the family Echinostomatidae. The genetic distance estimation has shown a low intraspecific rate between strains within the same species, *E. miyagawai*, *E. revolutum* and *H. conoideum*. The 28S rDNA sequence analysis and phylogenetic relationship well supported the *Echinostoma/Artyfechinostomum malayanum* intergeneric taxonomy and the clear, well-supported positions of member species in the family Echinostomatidae in the suborder Echinostomata.

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