■ ORIGINAL ARTICLE

Echinostoma mekongi n. sp. (Digenea: Echinostomatidae) from Riparian People along the Mekong River in Cambodia

Jaeeun Cho[1], Bong-Kwang Jung[1], Taehee Chang[1], Woon-Mok Sohn[2] ⓘ, Muth Sinuon[3], Jong-Yil Chai[1,4,*] ⓘ

[1]*Institute of Parasitic Diseases, Korea Association of Health Promotion, Seoul 07649, Korea;* [2]*Department of Parasitology and Tropical Medicine, and Institute of Health Sciences, Gyeongsang National University College of Medicine, Jinju 52727, Korea;* [3]*National Center for Parasitology, Entomology and Malaria Control, Phnom Penh, Cambodia;* [4]*Department of Tropical Medicine and Parasitology, Seoul National University College of Medicine, Seoul 03080, Korea*

Abstract: *Echinostoma mekongi* n. sp. (Digenea: Echinostomatidae) is described based on adult flukes collected from humans residing along the Mekong River in Cambodia. Total 256 flukes were collected from the diarrheic stool of 6 echinostome egg positive villagers in Kratie and Takeo Province after praziquantel treatment and purging. Adults of the new species were 9.0-13.1 (av. 11.3) mm in length and 1.3-2.5 (1.9) mm in maximum width and characterized by having a head collar armed with 37 collar spines (dorsal spines arranged in 2 alternative rows), including 5 end group spines. The eggs in feces and worm uterus were 98-132 (117) μm long and 62-90 (75) μm wide. These morphological features closely resembled those of *Echinostoma revolutum*, *E. miyagawai*, and several other 37-collar-spined *Echinostoma* species. However, sequencing of the nuclear ITS (ITS1-5.8S rRNA-ITS2) and 2 mitochondrial genes, *cox*1 and *nad*1, revealed unique features distinct from *E. revolutum* and also from other 37-collar-spined *Echinostoma* group available in GenBank (*E. bolschewense*, *E. caproni*, *E. cinetorchis*, *E. deserticum*, *E. miyagawai*, *E. nasincovae*, *E. novaezealandense*, *E. paraensei*, *E. paraulum*, *E. robustum*, *E. trivolvis*, and *Echinostoma* sp. IG). Thus, we assigned our flukes as a new species, *E. mekongi*. The new species revealed marked variation in the morphology of testes (globular or lobulated), and smaller head collar, collar spines, oral and ventral suckers, and cirrus sac compared to *E. revolutum* and *E. miyagawai*. Epidemiological studies regarding the geographical distribution and its life history, including the source of human infections, remain to be performed.

Key words: *Echinostoma mekongi*, 37-collar-spined echinostome, Kratie Province, Takeo Province, Mekong River, Cambodia

INTRODUCTION

Echinostoma spp. (family Echinostomatidae) of the 37-collar-spined group, or '*Echinostoma revolutum* group', are taxonomically diverse consisting of at least 15 (excluding *E. mekongi* of this study) valid and 10 validity-retained species worldwide [1]. The 15 valid species include *E. revolutum* (Froelich, 1802) Dietz, 1909, *E. bolschewense* (Kotova, 1939) Nasincova, 1991, *E. caproni* Richard, 1964, *E. cinetorchis* Ando & Ozaki, 1924, *E. deserticum* Kechemir et al., 2002, *E. lindoense* Sandground & Bonne, 1940, *E. luisreyi* Maldonado et al., 2003, *E. miyagawai* Ishii, 1932, *E. nasincovae* Faltýnková et al., 2015, *E. novaezealandense* Georgieva et al., 2017, *E. paraensei* Lie &

Basch, 1967, *E. paraulum* Dietz, 1909, *E. robustum* Yamaguti, 1935, *E. trivolvis* (Cort, 1914) Kanev, 1985, and *Echinostoma* sp. IG of Georgieva et al., 2013 [1]. All of these species, except *E. lindoense* and *E. luisreyi*, have unique molecular data deposited in GenBank. The 10 validity-retained species include *E. acuticauda* Nicoll, 1914, *E. barbosai* Lie & Basch, 1966, *E. chloephagae* Sutton & Lunaschi, 1980, *E. echinatum* (Zeder, 1803) de Blainville, 1828, *E. jurini* (Skvortsov, 1924) Kanev, 1985, *E. nudicaudatum* Nasir, 1960, *E. parvocirrus* Nassi & Dupouy, 1988, *E. pinnicaudatum* Nasir, 1961, *E. ralli* Yamaguti, 1934, and *E. rodriguesi* Hsu et al., 1968. Seven species among them, including *E. revolutum*, *E. cinetorchis*, *E. echinatum* (needs confirmation), *E. lindoense*, *E. miyagawai* (experimental infection), *E. paraulum*, and possibly *E. paraensei* (from the coprolite of a human mummy), were regarded as human-infecting zoonotic echinostomes [1-3].

The species differentiation of 37-collar-spined echinostomes has been done mostly based on the morphology, biology, life-cycle, and host characteristics of each species [4]. However,

identification of these species only by morphology is difficult and confusing, and molecular analyses are a highly useful alternative method [5,6]. When sequences of the nuclear internal transcribed spacer region (ITS1-5.8S rRNA-ITS2) or mitochondrial genes, including cytochrome *c* oxidase 1 (*cox*1) and NADH dehydrogenase subunit 1 (*nad*1), were analyzed, remarkable genetic variation has been noted in *E. revolutum* and other 37-collar-spined *Echinostoma* spp. from Europe [7-9], North America [10,11], Oceania [6,12], Southeast Asia [5,13-15], and various continents and localities [16]. There were 2 genetic lineages of *E. revolutum*; Eurasian and American by *nad*1 and Southeast Asian and American by *cox*1 analyses [5]. *E. miyagawai* also revealed 2 genetic lineages, Australian and Eurasian by *nad*1 analysis [5]. Other 37-collar-spined group deposited in GenBank, which are genetically distinct, include *E. bolschewense*, *E. caproni*, *E. cinetorchis*, *E. deserticum*, *E. nasincovae*, *E. novaezealandense*, *E. paraensei*, *E. paraulum*, *E. robustum*, *E. trivolvis*, and *Echinostoma* IG of Georgieva et al., 2013 [1,5-12].

We collected adult flukes of 37-collar-spined *Echinostoma* species from 6 riparian people in 2 localities (Kratie and Takeo Province) of Cambodia along the Mekong River, after treatment with praziquantel and purging. The flukes were morphologically difficult to differentiate from *E. revolutum* and several other members of 37-collar-spined *Echinostoma* group. Therefore, the nuclear ITS gene and 2 mitochondrial genes (*cox*1 and *nad*1) were sequenced, and the flukes were found to be genetically distinct from the 13 ever-known 37-collar-spined *Echinostoma* species available in GenBank, including *E. revolutum* and *E. miyagawai*. The other species of 37-collar-spined *Echinostoma* group unavailable in GenBank were morphologically differed from our flukes. Therefore, our flukes were assigned as a new species, i.e., *E. mekongi* n. sp.

MATERIALS AND METHODS

Patients and worm recovery

A total of 256 adult flukes of 37-collar-spined echinostomes, *E. mekongi* n. sp., were collected from 6 riparian people (1-157 worms by individual) living along the Mekong River (4 people from Kratie Province and 2 from Takeo Province), Cambodia in May 2011 (Table 1). Some of them complained of abdominal discomfort, indigestion, and other mild gastrointestinal troubles; the others had little clinical symptoms. The procedures of worm recovery were as described previously [17].

Table 1. Worm recovery of *Echinostoma mekongi* n. sp. from 6 riparian people in 2 provinces of Cambodia

Province	Village name	Patient (age, sex)	No. of echinostome eggs/gram of feces[a]	No. of *E. mekongi* adult specimens collected[b]
Kratie	Talous	25 F	2,976	157
	Rokakandal	37 F	264	53
	Talous	17 F	1,896	33
	Talous	15 F	0	1
Takeo	Ang Svay Chek	11 F	1,152	6
	Ang Svay Chek	10 F	1,032	6
Total				256

[a]No. of eggs were counted on Kato-Katz fecal smears and multiplied by 24 [40].
[b]Adult specimens were collected from the diarrheic stool of each patient after treatment with praziquantel (40 mg/kg) and purging with MgSO₄ (30-40 g in water).

Some flukes were fixed in 10% neutral formalin for morphological studies, and the others were fixed and preserved in 70-80% ethanol for molecular analyses. The formalin-fixed samples were washed with water and stained with Semichon's acetocarmine, dehydrated with a graded series of ethanol, cleared in xylene, and mounted in Permount. The ethanol-fixed samples were used for molecular analyses. Informed consent was obtained from each enrolled person or guardians. The procedures of worm collection from the people were permitted under the agreement between the National Center for Parasitology, Entomology, and Malaria Control, Phnom Penh, Cambodia and the Korea Association of Health Promotion, Seoul, the Republic of Korea (2009-2011).

Morphometric examinations

Twenty-six acetocarmine-stained specimens were used for morphological observations, measurements (Table 2), and description of worms. The comparison of our specimens with 37-collar-spined *Echinostoma* spp. was based on morphological characters given by previous authors [4,6,18]. Photomicrographs of the worms were taken with a digital camera (Olympus DP72, Tokyo, Japan) on an Olympus CKX41 microscope (Tokyo, Japan). Measurements were taken from digital images with the aid of CellSens Standard v1.5 image analysis software.

The following morphological characters and measurements were used for species comparison of 37-collar-spined *Echinostoma* group [6,18]. They included the body length, body width at 3 different levels (intestinal bifurcation, posterior border of ventral sucker, and mid-way between ventral sucker and ova-

Table 2. Measurements of *Echinostoma mekongi* n. sp. (adults) in comparison with other 37-collar-spined *Echinostoma* species (unit: μm)

Species	*E. mekongi* n. sp.		*E. revolutum* [18]		*E. miyagawai* [18]		*E. paraulum* [18]		*E. lindoense* [23]
No. of specimens	n=20		n=16		n=13		n=10		Several hundreds
	Mean	Range	Mean	Range	Mean	Range	Mean	Range	Range
Body length (BL)	11,278	8,970-13,123	10,531	9,454-11,846	9,990	9,163-11,014	6,345	5,600-6,862	13,000-15,000
Body width 1 (BW1)	1,031	774-1,293	1,298	1,182-1,454	1,159	1,029-1,272	1,043	923-1,108	2,033[a]
Body width 2 (BW2)	1,280	903-1,615	1,618	1,303-1,815	1,026	898-1,178	1,308	1,138-1,446	2,033[a]
Body width 3 (BW3)	1,933	1,336-2,503	1,815	1,454-2,092	1,305	1,197-1,459	1,514	1,231-1,662	2,500-3,000
Head collar length (CL)	372	336-425	392	351-424	434	393-505	488	432-553	390[a]
Head collar width (CW)	555	468-649	607	552-652	656	598-692	729	644-781	780
Oral sucker length (OSL)	231	199-263	316	261-358	305	262-337	287	265-318	230-510 (diam.)
Oral sucker width (OSW)	244	200-297	282	246-303	293	262-355	335	280-371	230-510 (diam.)
Angle spine length (ASL)	54	35-86	74	58-92	70	52-93	100	73-124	60[a]
Angle spine with (ASW)	17	9-32	17	11-24	22	17-29	21	15-28	18[a]
Lateral spine length (LSL)	55	40-85	79	64-91	76	67-87	101	85-120	62[a]
Lateral spine width (LSW)	15	9-38	17	12-21	23	20-26	21	17-25	20[a]
Dorsal spine length (DSL)	45	24-95	85	71-94	68	58-75	99	90-105	60[a]
Dorsal spine width (DSW)	12	6-25	18	13-23	21	19-26	21	19-23	18[a]
Prepharynx length (PL)	119	31-149	41	7-99	10	0-37	45	15-68	78[a]
Pharynx length (PHL)	201	170-230	237	209-258	299	262-337	324	273-379	180-400 (diam)
Pharynx width (PHW)	179	144-217	219	194-258	250	187-355	238	212-280	180-400 (diam.)
Esophagus length (ESL)	586	333-783	408	318-569	585	542-655	408	265-546	519[a]
Cirrus sac length (CSL)	401	262-564	552	410-735	506	468-598	413	318-531	300-500
Cirrus sac width (CSW)	249	190-326	316	243-403	300	281-355	281	205-326	300[a]
Seminal vesicle length (SVL)	308	201-457	336	224-440	-	-	238	152-303	-
Seminal vesicle width (SVW)	147	100-216	146	60-212	-	-	134	114-144	-
Ventral sucker length (VSL)	603	516-694	873	796-1,038	706	655-748	667	569-766	600-1,380 (diam.)
Ventral sucker width (VSW)	573	448-648	893	796-1,061	738	655-785	731	705-766	600-1,380 (diam.)
Ovary length (OVL)	248	187-393	349	288-394	283	243-355	150	114-182	300-500 (diam.)
Ovary width (OVW)	295	252-437	416	291-493	397	337-449	246	212-288	300-500 (diam.)
Mehlis' gland length (MEL)	425	321-811	399	326-455	386	337-468	153	106-190	385[a]
Mehlis' gland width (MEW)	631	504-932	734	582-849	373	318-411	310	258-341	674[a]
Ant. testis length (ATL)	645	470-800	788	629-932	564	430-692	384	341-455	1,011 (diam.)[a]
Ant. testis width (ATW)	572	406-809	597	425-750	409	337-505	377	326-447	1,011 (diam.)[a]
Post. testis length (PTL)	694	489-828	879	627-1,061	562	449-655	406	296-515	1,064[a]
Post. testis width (PTW)	555	287-884	579	395-705	411	337-524	415	288-531	837[a]
Egg length (EL)	117	98-132	114	108-125	95	94-96	113	104-122	111.3 (92-124)
Egg width (EW)	75	62-90	65	57-75	60	59-60	62	53-70	70.6 (65-76)
Forebody length (FORE)	1,937	1,163-3,008	1,369	1,200-1,662	1,408	1,253-1,533	1,218	985-1,600	1,925[a]
ODIV	1,439	1,080-1,883	1,027	923-1,262	1,181	1,103-1,324	1,074	923-1,354	1,887[a]

Table 2. Continued

| Species | E. mekongi n. sp. | | E. revolutum [18] | | E. miyagawai [18] | | E. paraulum [18] | | E. lindoense [23] |
| No. of specimens | n=20 | | n=16 | | n=13 | | n=10 | | Several hundreds |
	Mean	Range	Mean	Range	Mean	Range	Mean	Range	Range
OVAR	5,876	4,674-6,558	4,461	3,969-5,046	4,856	4,507-5,348	3,043	2,739-3,385	6,740[a]
TEND	2,742	1,673-4,014	3,653	3,000-4,308	3,081	2,824-3,329	2,059	1,262-2,431	3,370[a]
OSW/PHW	1.21	1.01-1.39	1.29	1.15-1.54	0.97	0.78-1.11	1.41	1.24-1.58	1.27[a]
BW1/BL (%)	9.2	6.32-12.3	12.4	11.2-14.3	11.6	11.2-12.5	16.5	15.0-19.8	15.6[a]
BW2/BL (%)	11.5	7.29-16.9	15.4	13.8-17.4	10.3	9.7-10.9	20.7	18.9-24.7	15.6[a]
BW3/BL (%)	17.6	12.0-27.7	17.3	15.2-19.5	13.1	12.4-13.6	23.9	21.5-26.9	20.2[a]
FORE/BL (%)	17.5	9.53-31.7	13	10.9-14.8	14.1	13.4-14.9	19.3	15.5-26.3	14.8[a]
CW/BW3 (%)	29.3	20.9-38.0	33.6	28.3-38.3	50.4	46.2-56.9	48.4	42.0-57.3	28.4[a]
OVID/BL (%)	13.0	9.67-19.1	9.8	8.3-11.2	11.8	11.2-12.5	17.0	14.5-23.7	14.5[a]
OVAR/BL (%)	52.4	41.0-61.0	42.5	39.1-46.2	48.6	47.7-49.7	48.1	43.5-57.0	29.6[a]
TEND/BL (%)	24.1	17.4-35.9	34.6	31.4-37.4	30.9	30.1-31.9	32.3	22.0-35.4	25.9[a]

ODIV, distance from anterior extremity to intestinal bifurcation; OVAR, distance from the posterior margin of ventral sucker to ovary; TEND, length of post-testicular region [18].
[a]Estimated from the original drawing of the worm [23].

ry), head collar, angle spine, lateral spine, dorsal spine, oral sucker, pre-pharynx length, pharynx, esophagus length, cirrus-sac, seminal vesicle, ventral sucker, ovary, Mehlis' gland, anterior testis, posterior testis, eggs, forebody length, distance from anterior extremity to intestinal bifurcation, distance between posterior margin of ventral sucker and ovary, length of post-testicular region, oral sucker to pharynx width ratio, body width to body length ratio, forebody length to body length, collar width to maximum body width ratio, distance from anterior extremity to intestinal bifurcation to body length ratio, length of pre-ovarian region to body length ratio, and length of post-testicular region to body length ratio [18].

Molecular analyses (ITS region, *cox*1, and *nad*1)

For molecular analyses, worms preserved in 70-80% ethanol were used. If combined morphological and molecular analyses were preferred, worms mounted on glass slides were photographed and then removed from the slides using a slide heater or xylene for molecular analyses. Genomic DNA was extracted using the Spin-Column Protocol of DNeasy® Blood & Tissue kit (QIAGEN, Hilden, Germany). PCR and nested-PCR were then conducted using specific primers designed to amplify ITS (ITS1-5.8S rRNA-ITS2) [19] and *cox*1 and *nad*1 [16] genes in echinostomes. The primers for ITS gene were BD1 and BD2 [19], and the those for *cox*1 were JB3 and JB13 [16]. The primers for *nad*1 were JB11 and JB12 in the first PCR and EchND1/inF and EchND1/inR for the second PCR of the inner region [16]. The PCR products were sequenced using the BigDye® Terminator v3.1 cycle sequencing kit by ABI 3730XL DNA analyzer (Applied Biosystems, Foster City, California, USA). For evaluation of the genetic identity of the samples, the basic local alignment search tool (BLAST; http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used. Using the Geneious® version 6.1.6 (Biometers Ltd., Auckland, New Zealand), we aligned the obtained sequences with GenBank reference ITS, *cox*1, and *nad*1 sequences of 37-collar-spined *Echinostoma* species. Phylogenetic information was assessed via maximum-likelihood (ML) analyses using the MEGA v6 program applying Tamura-nei model of nucleotide substitution with 1,000 bootstrap replications [20].

RESULTS

Description of worms
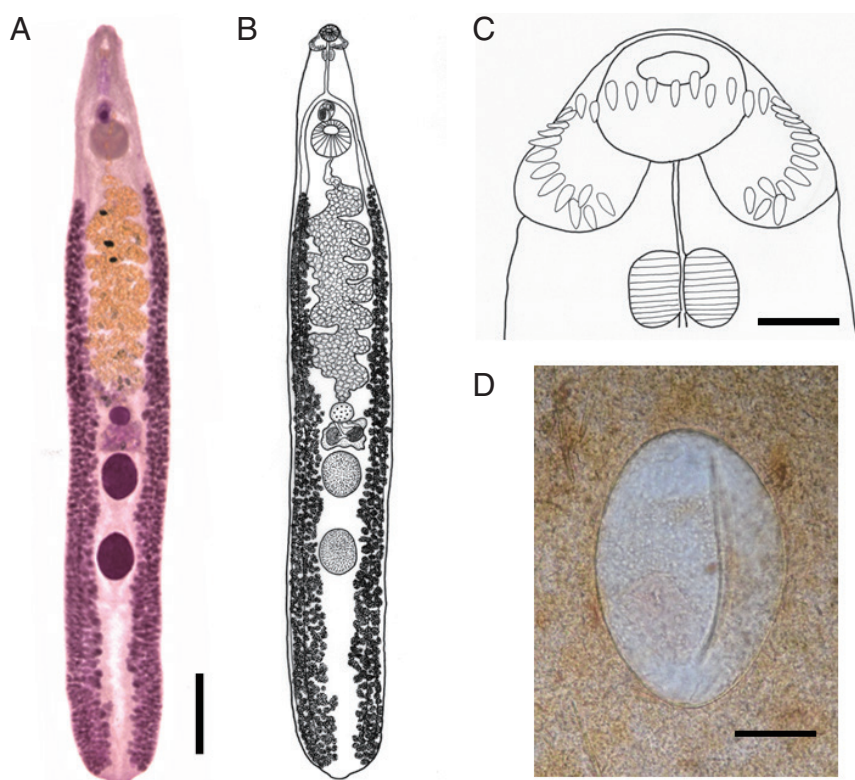*Echinostoma mekongi* n. sp. (Table 2; Fig. 1A-D)

Fig. 1. *Echinostoma mekongi* n. sp. from Cambodia. (A) An adult worm (type) collected from a riparian person (37-year-old female). Acetocarmine-stained. Ventral view. Scale bar = 1.4 mm. (B) Line drawing of the worm in Fig. 1A. (C) Head collar with 37 collar spines. Scale bar = 140 μm. (D) An egg in Kato-Katz fecal smear of a patient from Kratie Province. Scale bar = 33 μm.

Family Echinostomatidae Looss, 1899

Subfamily Echinostomatinae Looss, 1899

Genus *Echinostoma* Rudolphi, 1809

Adults: Body dorsoventrally flattened, muscular, elongated leaf-like with slightly attenuated both ends, 8.97-13.12 (av. 11.28) mm in length and 1.34-2.50 (1.93) mm in maximum width at mid-uterine or ovarian region (n = 20) (Table 2; Figs. 1A, B, 2). Tegument beset with triangular spines, less dense posteriorly, extending to level of posterior testis. Forebody long representing about 17.5% of whole body length. Anterior end with characteristic features of an echinostome, equipped with an oral sucker and a head collar. Oral sucker small, muscular, spherical, subterminal, about 2/5 of the size of the ventral sucker. Head collar small but prominent, muscular, and reniform armed with collar spines (Fig. 1C). Collar spines 37 in total number, with the formula of 5-6-6-3-6-6-5, including angle (corner) spines 5+5, lateral spines 6+6, dorsal spines 6+3+6; dorsal spines arranged in 2 alternating rows (Fig. 1C). Collar spines relatively small in length and width, moderately pointed (not sharply pointed nor blunt-ended). Prepharynx

Fig. 2. *Echinostoma mekongi* n. sp. adult flukes (n = 157) collected from a riparian person (37-year-old female) along the Mekong River in Kratie Province, Cambodia.

relatively long; pharynx muscular, elongated oval. Esophagus long; intestinal bifurcation from anterior extremity at about 13% of total body length; ceca blind, narrow, overlapped by vitelline follicles, ending before the posterior termination of vitelline follicles. Cirrus sac transversely oval, with muscular walls, located between intestinal bifurcation and anterior mar-

gin of ventral sucker, containing seminal vesicle (with elongate-oval anterior portion and narrow saccular posterior portion), well-developed pars prostatica, coiled ejaculatory duct, muscular cirrus with smooth unarmed surface. Genital atrium and genital pore median, just posterior to intestinal bifurcation, receiving female (metraterm) and male reproductive (ejaculatory) duct. Uterus intercecal, long, slender, with numerous transverse coils between ventral sucker and ovary, containing a large number of eggs. Metraterm short, weakly muscular, connected to genital pore. Ovary spherical, median or slightly submedian, almost equatorial, between uterus and Mehlis' gland. Mehlis' gland transversely oval, median, connected with ovary and uterine tubule. Uterine seminal receptacle present, ventral to Mehlis' gland; Laurer's canal absent. Vitelline follicles extensive, extending laterally forming 2 lateral groups, from the level slightly posteriorly to ventral sucker to near posterior extremity; the 2 groups of vitellaria not merge until their posterior extremities. Two testes tandem, globular, entire in more than 2/3 of the specimens or 5-8 lobulated in about 1/3 of the specimens, located in posterior field of body, more or less separated from each other. Eggs numerous, yellowish, immature containing a germ cell, operculate with a small, inconspicuous operculum, and a small abopercular thickening or wrinkling at the abopercular end, oval, 98-132 (117) μm long and 62-90 (75) μm wide (n = 20) (Fig. 1D). Excretory vesicle Y-shaped, bifurcates just posterior to posterior testis; excretory pore terminal.

Taxonomic summary

Type host: *Homo sapiens* (natural infection)

Site of infection: Small intestine

Type locality: Kratie and Takeo Province, Cambodia

Deposition of specimens: The type specimen is deposited in the Parasite Museum, Institute of Parasitic Diseases, Korea Association of Health Promotion, Seoul, Republic of Korea (no. 2020-0011-01, holotype, and no. 2020-0011-02~24, subtypes). Voucher specimens are deposited in Meguro Parasitological Museum, Tokyo, Japan (MPM Coll. No. 21675).

Etymology: The specific name refers to the name of the river (the Mekong River); the infected humans were residents living nearby the river.

Molecular analyses

A phylogenetic tree based on ITS region (992 bp after trimming) was constructed using the ML method. The tree com-prised of sequences of 7 species of 37-collar-spined *Echinostoma* group, including our samples (*E. mekongi* n. sp.). The sequences of our samples (10 isolates; GenBank accession nos. MT409010-409019) clustered together (100% homologous) with high bootstrap values and constructed a new lineage distinct from any other 37-collar-spined *Echinostoma* species deposited in GenBank (Fig. 3). Although *E. mekongi* n. sp. appeared to be a sister group to *E. revolutum*, a morphologically highly similar species, the sequence comparison showed only 97.6% similarity between the 2 species (Table 3). In addition, the sequences of *E. paraensei* and *E. robustum* showed 96.4% and 97.9% similarities with the new species, respectively (Table 3).

A phylogenetic tree based on *cox*1 (184 bp after trimming) sequences was constructed using the ML analyses. The tree comprised of 7 species of 37-collar-spined *Echinostoma* group, including Southeast Asian and American lineages of *E. revolutum* and *E. mekongi* n. sp. The sequences of our samples (10 isolates; GenBank accession nos. MT449681-449690) clustered together (99.6%) with high bootstrap values and constructed a new lineage distinct from any other 37-collar-spined *Echinostoma* species deposited in GenBank (Fig. 4). Although our samples appeared to be a sister group to *E. caproni*, the sequence showed only 92.6-92.7% similarity between the 2 species (Table 3). In addition, the sequences of *E. trivolvis*, *E. paraensei*, *E. miyagawai*, *E. robustum*, and *E. revolutum* having similar morphology with our samples showed only 89.3-92.7% homology with our samples (Table 3).

A phylogenetic tree based on *nad*1 (472 bp after trimming) sequences constructed using the ML method is shown in Fig. 5. The tree composed of sequences of 14 different species of 37-collar-spined *Echinostoma* group available in GenBank, including *E. mekongi* n. sp., Eurasian and Australian lineages of *E. miyagawai*, and Eurasian and American lineages of *E. revolutum*. The sequences of our samples (12 isolates; GenBank accession nos. MT431426-431437) clustered together (99.4-99.8%) with high bootstrap values constructing a new genetic lineage distinct from any other 37-collar-spined *Echinostoma* species (Fig. 5). In this *nad*1 phylogenetic tree, the lineage of our samples appeared as a sister group to *E. deserticum*; however, the sequence similarity between the 2 was only 87.1-87.2% (Table 3). In addition, the sequences of *E. revolutum* and *E. miyagawai* having similar morphologies with our samples showed only 85.7-86.6% and 86.0-87.7% sequence homologies with our samples, respectively (Table 3).
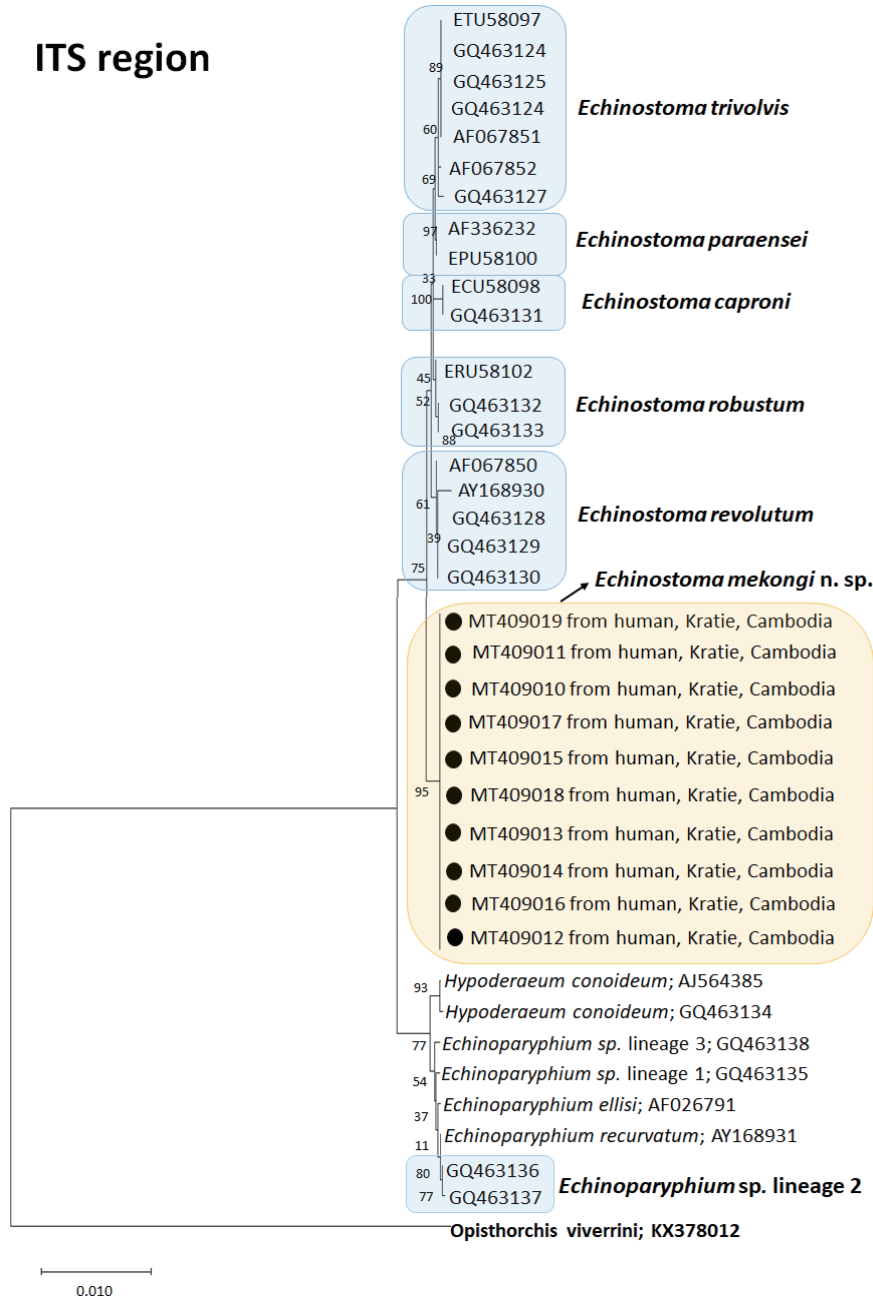
ITS region

Fig. 3. A phylogenetic tree of *Echinostoma mekongi* n. sp. and other 37-collar-spined '*E. revolutum* group' constructed based on 992 bp of nuclear ribosomal ITS (ITS1-5.8S rRNA-ITS2) gene sequences.

Remarks

Compared with the 15 valid and 10 validity-retained species of '*E. revolutum* group', the new species morphologically differed from most of them, in particular, those reported from Europe, Africa, North and South America, and Oceania [1]. However, the new species appeared to be comparable with those reported from Asia which included *E. revolutum, E. ci-*

netorchis, E. lindoense, E. miyagawai, E. paraulum, E. ralli, and *E. robustum* [1]. The new species most closely resembled 4 of them, *E. revolutum, E. lindoense, E. miyagawai,* and *E. paraulum* (Table 2). *E. cinetorchis* differed from *E. mekongi* in that *E. cinetorchis* has abnormally located or reduced number of testes [1-3] whereas no such specimen was found in the new species. *E. ralli* differed from *E. mekongi* in that the former has 4 end

Table 3. Sequence comparison of 37-collar-spined *Echinostoma* spp. in GenBank based on ITS region, *nad*1, and *cox*1 genes

ITS region		cox1		nad1	
Between isolates (*E. mekongi* n. sp.)	100		99.6		99.4-99.8
E. revolutum	97.6	*E. revolutum* (Southeast Asian lineage)	90.7-91.2	*E. revolutum* (Eurasian lineage)	85.7-86.0
E. robustum	97.9	*E. revolutum* (American lineage)	89.4-89.9	*E. revolutum* (American lineage)	86.3-86.6
E. caproni	97.0	*E. miyagawai*	90.0	*E. miyagawai* (Eurasian lineage)	86.0-86.3
E. trivolvis	97.6	*E. robustum*	89.4	*E. miyagawai* (Australian lineage)	87.4-87.7
E. paraensei	96.4	*E. caproni*	92.6-92.7	*E. robustum*	87.4-87.7
		E. trivolvis	91.0	*E. paraulum*	88.0-88.2
		E. paraensei	89.3	*E. caproni*	82.2-85.3
				E. trivolvis	83.5-83.8
				E. nasincovae	81.2-81.5
				E. novaezealandense	86.4-86.7
				E. deserticum	87.1-87.2
				E. bolschewense	82.0-82.6
				E. paraensei	85.1-85.4
				E. cinetorchis	87.6-87.7
				Echinostoma sp. IG	79.8-80.4

group spines [21], whereas the new species has 5 end group spines. *E. robustum* could be distinguished from *E. mekongi* in the morphology of testes, globular or slightly lobulated, and vitellaria not merging post-testicularly in the new species and irregularly lobed and horizontally extended testes and 2 lateral vitellaria merging beyond the posterior testis level in *E. robustum* [22].

E. mekongi differed from the 4 closely related species in that it had a smaller head collar (av. 555 μm in collar width) compared to *E. revolutum* (av. 607 μm), *E. miyagawai* (av. 656 μm), *E. paraulum* (av. 729 μm), and *E. lindoense* (780 μm), and smaller collar spines in comparison with the same 4 species (Table 2). The collar spines of *E. mekongi* were not so long and not sharply pointed as those of *E. revolutum* or *E. miyagawai* [1]. The oral and ventral suckers were also smaller (av. 244×231 μm and 603×573 μm, respectively) compared with those of *E. revolutum* (av. 316×282 μm and 893×873 μm, respectively), *E. miyagawai* (av. 305×293 μm and 738×706 μm, respectively), and *E. paraulum* (av. 335×287 μm and 731×667 μm, respectively) [18]. The cirrus sac was also smaller (av. 401×249 μm) than that of *E. revolutum* (av. 552×316 μm), *E. miyagawai* (av. 506×300 μm), and *E. paraulum* (av. 413×281 μm) [18]. The egg size of *E. mekongi* (av. 117×75 μm) was similar to the eggs of *E. revolutum*, *E. paraulum*, and *E. lindoense* but larger than that of *E. miyagawai* (av. 95×60 μm) [18,23] (Table 2). The

forebody length, the distance from the anterior extremity to intestinal bifurcation, and the distance from the posterior margin of ventral sucker to ovary were longer in *E. mekongi* than in *E. revolutum*, *E. miyagawai*, and *E. paraulum* but almost equal to or shorter than that in *E. lindoense* [18,23] (Table 2). The vitelline follicles of *E. mekongi* were distributed laterally from the level of some distance from the posterior margin of the ventral sucker to the posterior extremity, without merging beyond the posterior testis level which resembled *E. revolutum* and *E. paraulum* [1,18]. By comparison, the 2 lateral groups of vitelline follicles in *E. miyagawai* and *E. lindoense* merge near the posterior extremity [1].

Three phylogenetic trees based on ITS region, *cox*1, and *nad*1 sequences revealed a unique genetic lineage of *E. mekongi* distinguished from all other species of 37-collar-spined *Echinostoma* spp. (Figs. 3-5). Even in ITS region in which interspecific variation is not so remarkable, the sequence homologies between *E. mekongi* and other species such as *E. revolutum*, *E. robustum*, *E. caproni*, *E. trivolvis*, and *E. paraensei* were lower than 97.9% (Table 3). The *cox*1 sequences of *E. mekongi* revealed lower than 92.7% homologies in comparison with *E. revolutum* (Southeast Asian and American lineages; Nagataki et al. [5]) and 5 other species, and the *nad*1 sequences showed lower than 88.2% homologies compared with *E. revolutum* (Eurasian and American lineages; Nagataki et al. [5]), *E. miyagawai* (Eur-
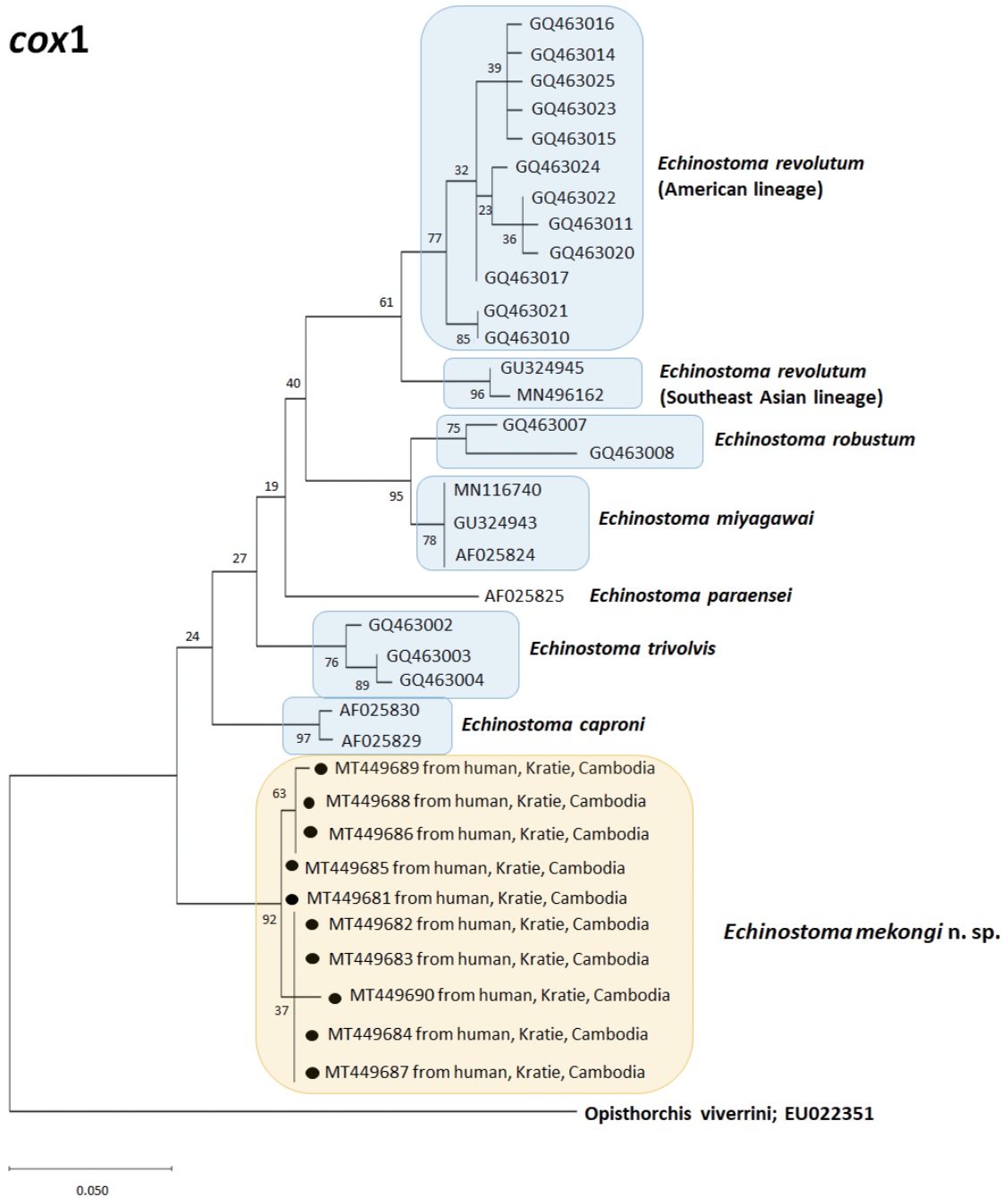
cox1

Fig. 4. A phylogenetic tree of *Echinostoma mekongi* n. sp. and other 37-collar-spined '*E. revolutum* group' constructed based on 184 bp of mitochondrial *cox*1 sequences.

asian and Australian lineages; Nagataki et al. [5]), and 11 other species deposited in GenBank. Therefore, *E. mekongi* is considered a new species morphologically and molecularly distinct from the pre-existing 37-collar-spined *Echinostoma* spp. reported around the world.

DISCUSSION

Kanev [4] reported that the species of '*E. revolutum* group' cannot be morphologically identified only by adult flukes but can be more clearly discriminated by the morphology of larval forms, in particular, the cercariae, and host-parasite relation-
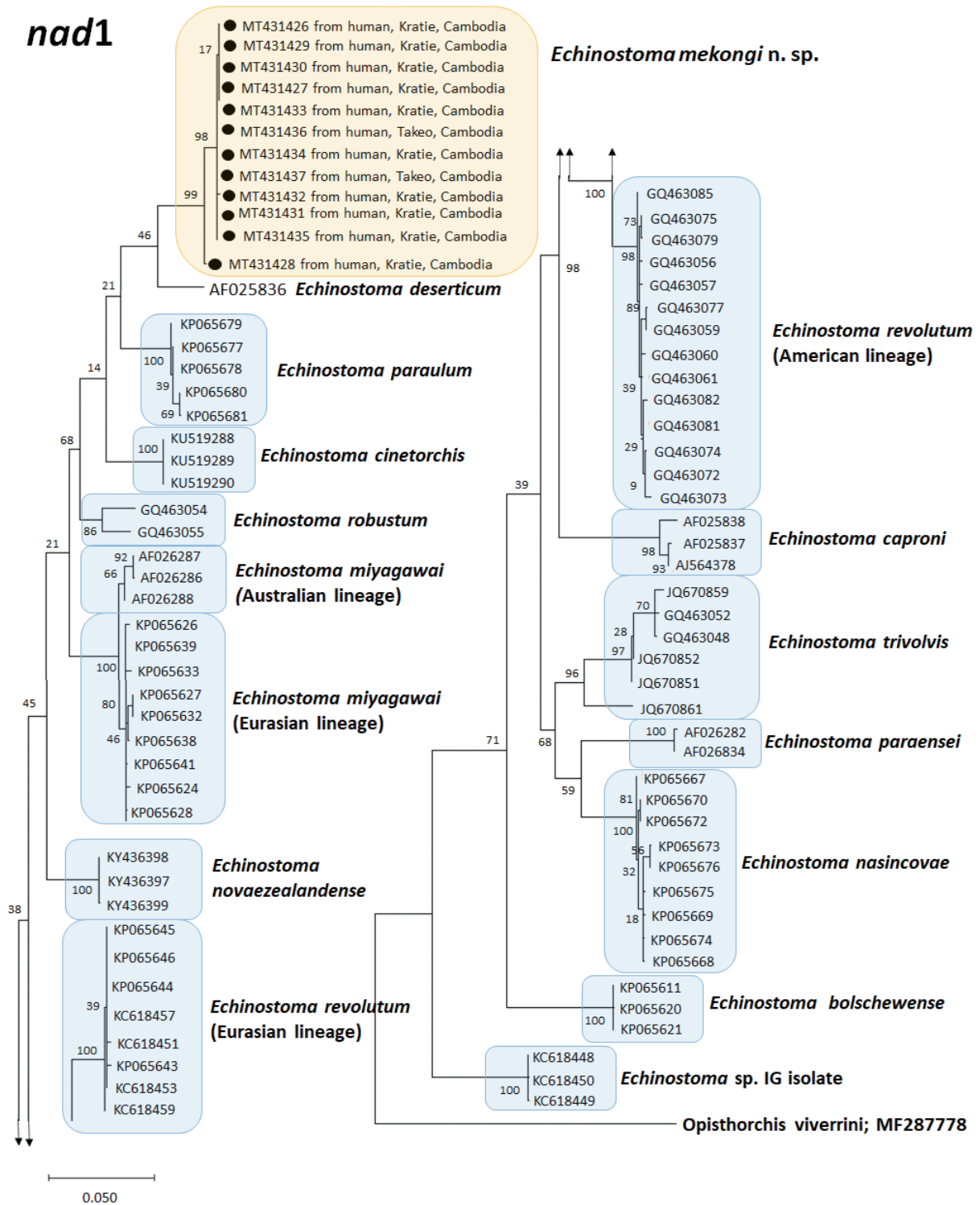
Fig. 5. A phylogenetic tree of *Echinostoma mekongi* n. sp. and other 37-collar-spined '*E. revolutum* group' constructed based on 472 bp of mitochondrial *nad*1 sequences.

ships. In the cercariae, the numbers of penetration gland-cell outlets and paraesophageal gland-cell outlets, flame cell patterns, and the presence or absence as well as the number of tail fin-folds are important criteria for species differentiation [4,24]. However, an integrated taxonomic approach, linking morphological and molecular evidence, was also important in assessing the species diversity within the '*E. revolutum*' group from Europe [18]. In the present study, this integrated approach was extremely useful for establishing a new species (*E. mekongi*) from Asia, particularly in the absence of larval stage information, including the larval morphology and intermediate hosts.

The new species was found from riparian people along the Mekong River. Most of the patients were young females at the age of the 10s-20s, except one who was a 37-year-old female. They recalled that they had eaten various kinds of snails (species unknown) purchased from local markets which were stated to have been caught around the river. Snails (gastropods and bivalves) have been reported to be the first and/or second intermediate host for most of the '*E. revolutum* group' [1,4]. However, the larval stages, including the rediae, cercariae, and metacercariae, of *E. mekongi* have not yet been discovered, and the source of infection in our patients remains to be determined. The patients complained of abdominal discomfort, indigestion, and other mild gastrointestinal symptoms, although there is no evidence whether these symptoms were directly related to *E. mekongi* infection or not. However, it is referable that in *Isthmiophora hortensis* (under the name *Echinostoma hortense*) infection, the patients complained of severe epigastric discomfort with ulcerative lesions in the stomach or the duodenum, and living worms were detected near the ulcerative lesions through gastroduodenal endoscopy [2,3]. Moreover, in *Artyfechinostomum malayanum* (under the name *Artyfechinostomum mehrai*) infection, a fatal human case was reported in India in whom marked malnutrition and anemia were found and several hundred worms were collected at autopsy [25].

With designation of our flukes as a new species of '*E. revolutum* group', it is interesting to consider its possible wider geographical distribution from Kratie and Takeo Province to other provinces of Cambodia as well as to other Indochina countries, including Vietnam, Lao PDR, and Thailand. In addition, the specific diagnosis of 37-collar-spined echinostomes reported previously from Indochina peninsula needs reconfirmation. For example, in Cambodia, human infections with *E. ilocanum* were reported among inhabitants in Oddar Meanchey Province [26] and human infections with *E. revolutum* were reported among schoolchildren in Pursat Province [27]. The morphological diagnosis of *E. ilocanum* seems to be of no problem because the worms characteristically had 49-51 collar spines [26]. However, the diagnosis of *E. revolutum* was based only on worm morphology [27] and needs further verification using molecular analyses.

In Vietnam, at least 3 papers reported the presence of 37-collar-spined *Echinostoma* spp. [28-30]. Two were reports of *E. cinetorchis* infection in dogs in Nghe An Province [28] and in chickens and ducks in Nam Dinh Province [29]. The first paper shows a figure of *E. cinetorchis* [28] but based on the figure we consider that the diagnosis had not been properly given for them. The second paper does not show any figure of *E. cinetorchis* nor any description of the worms [29]; thus, we cannot assure the specific diagnosis. The third paper was on the partial life cycle of *E. revolutum*, with the metacercariae found in *Filopaludina* snails in Nam Dinh Province and adults obtained from experimentally infected hamsters [30]. Because the adult worms are morphologically difficult to distinguish from those of *E. mekongi*, further confirmation of the species using molecular methods seems to be needed.

In Lao PDR, very few papers were published regarding the existence of '*E. revolutum* group'. One was the report of human *E. revolutum* infections who were co-infected with *A. malayanum* and others [17]. Other echinostome species reported from Lao PDR included *Echinochasmus japonicus* [31], *Echinostoma macrorchis* [32], *Echinostoma ilocanum* [33], *Echinostoma aegyptica* [34], and *Echinochasmus caninus* [35]. The diagnosis of all these echinostomes was based on the morphology of adult flukes. Among them, the diagnosis of *E. revolutum* [17] remains to be reconfirmed through molecular studies.

In Thailand, many papers have reported human and animal (ducks) infections with *E. revolutum* [2,5,13,14,36-39]. Among them, molecular data were provided by Saijuntha and co-workers [13,38], Noikong et al. [14], Nagataki et al. [5], and Buddhachat and Chontananarth [39]. Regarding the isolate of *E. revolutum* by Saijuntha et al. [38] which exhibited close affinity to the European isolate studied by Morgan and Blair [12,16], Georgieva et al. [9] pointed out that it was shown to represent *E. miyagawai* rather than *E. revolutum*. In addition, the molecular data presented by Noikong et al. [14] were not deposited in GenBank, so there is no evidence to confirm the diagnosis of *E. trivolvis/revolutum*-like clade among their samples [9]. However, Nagataki et al. [5] demonstrated the presence of 2 species

of '*E. revolutum* group' in Thailand and Lao PDR, which included *E. revolutum* and *E. miyagawai*. Moreover, from the phylogenetic tree they established, 2 separate genetic lineages of *E. revolutum* were found, namely, the Southeast Asian and American lineages by analysis of *cox*1 or the Eurasian and American lineages based on *nad*1 sequences. Using DNA barcoding conjugated with high-resolution melting analysis, Buddhachat and Chontananarth [39] further classified *E. revolutum* genetic lineages into 3 based on *nad*1 sequences, i.e., the Asian, Eurasian, and American lineages. However, the existence of *E. mekongi* in Thailand and Lao PDR remains to be determined.

In conclusion, we discovered a new species of '*E. revolutum* group' from human infections in Kratie and Takeo provinces, Cambodia, i.e., *E. mekongi* n. sp. This is a new echinostome fauna in Cambodia and the first report of a human-infecting *Echinostoma* sp. diagnosed by molecular analyses in Southeast Asia. Several previous reports on the existence of *E. revolutum* in Indochina countries need re-validation through molecular analyses.

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CONFLICT OF INTEREST

We have no conflict of interest related to this study.

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