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

Jaejeun Cho¹, Bong-Kwang Jung¹, Tahee Chang¹, Woon-Mok Sohn², Muth Sinuon³, Jong-Yil Chai¹,*⁴

¹Institute of Parasitic Diseases, Korea Association of Health Promotion, Seoul 07649, Korea; ²Department of Parasitology and Tropical Medicine, and Institute of Health Sciences, Gyeongsang National University College of Medicine, Jinju 52277, Korea; ³National Center for Parasitology, Entomology and Malaria Control, Phnom Penh, Cambodia; ⁴Department 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, cox1 and nad1, 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. lindense Sandground & Bonne, 1940, E. luisreyi Maldonado et al., 2003, E. miyagawai Ishii, 1932, E. nasincovae Faltynková 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. lindense 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 & Lunachi, 1980, E. echinatum (Zeder, 1803) de Blainville, 1828, E. jarini (Skvortsov, 1924) Kanev, 1985, E. nudivulvatum Nasir, 1960, E. parvocirrus Nassi & Dupouy, 1988, E. pinnicudatum Nasir, 1961, E. ralli Yamaguti, 1934, and E. r. driguezi Hsu et al., 1968. Seven species among them, including E. revolutum, E. cinetorchis, E. echinatum (needs confirmation), E. lindense, 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 (cox1) and NADH dehydrogenase subunit 1 (nad1), 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 nad1 and Southeast Asian and American by cox1 analyses [5]. E. miyagawai also revealed 2 genetic lineages, Australian and Eurasian by nad1 analysis [5]. Other 37-collar-spined group deposited in GenBank, which are genetically distinct, include E. bolschewense, E. caproni, E. cinetorchis, E. deserticum, E. nasicercus, E. novaezelandense, 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 (cox1 and nad1) 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].

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. paralum [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 | Mean  | Range | Mean  | Range | Mean  | Range |
| Body length (BL) | 11.278 | 8,970-13,123 | 10.531 | 9,454-11,846 | 9.900 | 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* |
| 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* |
| 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* |
| Head collar width (CW) | 555 | 468-649 | 607 | 552-652 | 666 | 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* |
| Angle spine with (ASW) | 17 | 9-32 | 17 | 11-24 | 22 | 17-29 | 21 | 15-28 | 18* |
| Lateral spine length (LSL) | 55 | 40-85 | 79 | 64-91 | 76 | 67-87 | 101 | 85-120 | 62* |
| Lateral spine width (LSW) | 15 | 9-38 | 17 | 12-21 | 23 | 20-26 | 21 | 17-25 | 20* |
| Dorsal spine length (DSL) | 45 | 24-95 | 85 | 71-94 | 68 | 58-75 | 99 | 90-105 | 60* |
| Dorsal spine width (DSW) | 12 | 6-25 | 18 | 13-23 | 21 | 19-26 | 21 | 19-23 | 18* |
| Prepharynx length (PL) | 119 | 31-149 | 41 | 7-99 | 10 | 0-37 | 45 | 15-68 | 78* |
| 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 | 187-335 | 238 | 212-280 | 180-400 (diam.) |
| Esophagus length (ESL) | 586 | 333-783 | 408 | 318-569 | 585 | 542-665 | 408 | 265-546 | 519* |
| Cirrus sac length (CSL) | 401 | 262-564 | 552 | 410-735 | 506 | 469-598 | 413 | 318-531 | 300-500 |
| Cirrus sac width (CSW) | 249 | 190-326 | 316 | 243-403 | 300 | 281-355 | 281 | 205-326 | 300* |
| 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 | 665-785 | 731 | 705-766 | 600-1,380 (diam.) |
| Ovary length (OLV) | 248 | 187-393 | 349 | 288-394 | 283 | 243-355 | 150 | 114-182 | 300-500 (diam.) |
| Ovary width (OWV) | 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* |
| Mehlis' gland width (MEW) | 631 | 504-932 | 734 | 582-849 | 373 | 318-411 | 310 | 258-341 | 674* |
| Ant. testis length (ATL) | 645 | 407-800 | 788 | 629-932 | 564 | 430-692 | 384 | 341-455 | 1,011 (diam.)* |
| Ant. testis width (ATW) | 572 | 406-809 | 597 | 425-750 | 409 | 337-505 | 377 | 326-447 | 1,011 (diam.)* |
| Post. testis length (PTL) | 694 | 489-823 | 879 | 627-1,061 | 562 | 449-655 | 406 | 296-515 | 1,064* |
| Post. testis width (PTW) | 555 | 287-884 | 579 | 395-705 | 411 | 337-524 | 415 | 288-531 | 837* |
| 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* |
| 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* |

(Continued to the next page)
**Korean J Parasitol** Vol. 58, No. 4: 431-443, August 2020

**Molecular analyses (ITS region, cox1, and nad1)**

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 cox1 and nad1 [16] genes in echinostomes. The primers for ITS gene were BD1 and BD2 [19], and those for cox1 were JB3 and JB13 [16]. The primers for nad1 were JB1 1 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 (Biemers Ltd., Auckland, New Zealand), we aligned the obtained sequences with GenBank reference ITS, cox1, and nad1 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)

| 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                   | 5,876             | 4,461             | 3,081             | 3,043            | 6,740a           |
| Range                  | 4,674-6,558       | 3,663             | 2,824-3,329       | 2,739-3,855      | 3,370a           |
| OVAR                   | 1.21              | 1.29              | 1.07              | 1.41             | 1.27             |
| Mean                   | 1.01-1.39         | 1.15-1.54         | 0.78-1.11         | 1.24-1.58        | 1.56             |
| Range                  | 9.62-12.32        | 11.42-12.5        | 9.70-10.9         | 18.9-24.7        | 15.6             |
| TEND                   | 11.5              | 15.4              | 10.3              | 20.7             | 15.6             |
| Mean                   | 7.29-16.9         | 13.8-17.4         | 9.7-10.9          | 18.9-24.7        | 15.6             |
| Range                  | 7.0-14.4          | 10.9-16.7         | 23.9              | 21.5-26.9        | 20.2             |
| OSW/PHW                | 17.6              | 17.3              | 13.1              | 19.3             | 14.8             |
| Mean                   | 12.0-27.7         | 15.2-19.5         | 12.4-13.6         | 15.5-26.3        | 14.8             |
| Range                  | 12.0-20.4         | 10.9-19.5         | 28.4              | 15.5-26.3        | 14.8             |
| BWH/BL (%)             | 29.3              | 33.6              | 50.4              | 14.1             | 17.0             |
| Mean                   | 20.9-38.0         | 28.3-38.3         | 46.2-56.9         | 13.4-14.9        | 14.5-23.7        |
| Range                  | 9.8-31.7          | 8.3-11.2          | 48.4              | 11.2-12.5        | 14.5-23.7        |
| OVID/BL (%)            | 13.0              | 11.8              | 11.8              | 17.0             | 28.4             |
| Mean                   | 9.67-19.1         | 8.3-11.2          | 11.2-12.5         | 14.5-23.7        | 14.5-23.7        |
| Range                  | 10.9-14.8         | 15.2-19.5         | 24.7-49.7         | 13.4-14.9        | 14.5-23.7        |
| OVAR/BL (%)            | 52.4              | 42.5              | 48.6              | 17.0             | 29.6             |
| Mean                   | 41.0-61.0         | 39.1-46.2         | 47.7-49.7         | 14.5-23.7        | 14.5-23.7        |
| Range                  | 34.6              | 31.4-37.4         | 30.1-31.9         | 22.0-35.4        | 25.9             |

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].

*Estimated from the original drawing of the worm [23].

Table 2. Continued
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 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-

---

**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.

**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.
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. MT409010-409019) and 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 comprised of sequences of 7 species of 37-collar-spined Echinostoma group, including our samples (E. mehongi 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. mehongi 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 coxl (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 nad1 (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. mehongi 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 nad1 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).
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. cinetorchis, 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
group spines [21], whereas the new species has 5 end group spines. *E. robustum* could be distinguished from *E. mekongi* in the morphology of testses, globular or slightly lobulated, and vitellaria not merging post-testicularly in the new species and irregularly lobed and horizontally extended testses 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, cox1, and nad1 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. paraensai* were lower than 97.9% (Table 3). The cox1 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 nad1 sequences showed lower than 88.2% homologies compared with *E. revolutum* (Eurasian and American lineages; Nagataki et al. [5]), *E. miyagawai* (Eur-
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.

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 *nad1* 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 mehraii) 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. cinetorhisch 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. cinetorhisch [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. cinetorhisch 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 Filoputalina 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 Echinococchasmus japonicus [31], Echinostoma macrocrisch [32], Echinostoma ilocanum [33], Echinostoma aegyptica [34], and Echinococchasmus 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 cox1 or the Eurasian and American lineages based on nad1 sequences. Using DNA barcoding conjugated with high-resolution melting analysis, Buddhachat and Chontananarth [39] further classified E. revolutum genetic lineages into 3 based on nad1 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.

ACKNOWLEDGMENTS

We are grateful to the staff of National Center for Parasitology, Entomology and Malaria Control, Phnom Penh, Cambodia, and Provincial Health Department, Kratie and Takeo Province, Cambodia who helped this study. We also thank the staff of Korea Association of Health Promotion, Seoul, Korea who performed fecal examinations of people in Kratie and Takeo Province, Cambodia. We also appreciate Hyejoo Shin, Institute of Parasitic Diseases, Korea Association of Health Promotion, Seoul, Korea for her help in line drawing of worm figures.

CONFLICT OF INTEREST

We have no conflict of interest related to this study.

REFERENCES

1. Chai JY, Cho J, Chang T, Jung BK, Sohn WM. Taxonomy of Echinostoma revolutum and 37-collar-spined Echinostoma spp.: a historical review. Korean J Parasitol 2020; 58: 343-371.
2. Chai JY. Human Intestinal Flukes. Chapter 2. Echinostomes. The Netherlands. Springer Nature B.V. 2019, pp 169-343.
3. Chai JY. Echinostomes in humans. In Fried B, Toledo R eds, The Biology of Echinostomes. New York, USA. Springer. 2009, pp 147-183.
4. Kanem J. Life-cycle, delimitation and redescription of Echinostoma revolutum (Froelich, 1802) (Trematoda: Echinostomatidae). Syst Parasitol 1994; 28: 125-144.
5. Nagataki M, Tantrawatpan C, Agatsuma T, Sugita T, Duenngai K, Sithithaworn P, Andrews RH, Petney TN, Saijuntha W. Mitochondrial DNA sequences of 37 collar-spined echinostomes (Digenea: Echinostomatidae) in Thailand and Lao PDR reveals presence of two species: Echinostoma revolutum and E. miyagawai. Infect Genet Evol 2015; 35: 56-62.
6. Georgieva S, Blasco-Costa I, Kostadinova A. Molecular characterization of four echinostomes (Digenea: Echinostomatidae) from birds in New Zealand, with descriptions of Echinostoma novaezelandense n. sp. and Echinoparyphium poulini n. sp. Syst Parasitol 2017; 94: 477-497.
7. Kostadinova A, Herniou EA, Barrett J, Littlewood DTJ. Phylogenetic relationships of Echinostoma Rudolphi, 1809 (Digenea: Echinostomatidae) and related genera re-assessed via DNA and morphological analyses. Syst Parasitol 2003; 54: 159-176.
8. Georgieva S, Selbach C, Faltynková A, Soldánová M, Sures B, Skinniss K, Kostadinova A. New cryptic species of the ‘revolutum’ group of Echinostoma (Digenea: Echinostomatidae) revealed by molecular and morphological data. Parasit Vectors 2013; 6: 64.
9. Georgieva S, Faltynková A, Brown R, Blasco-Costa I, Soldánová M, Šítko J, Scholz T, Kostadinova A. Echinostoma ‘revolutum’ (Digenea: Echinostomatidae) species complex revisited: species delimitation based on novel molecular and morphological data gathered in Europe. Parasit Vectors 2014; 7: 520.
10. Detwiler JT, Bos DH, Minchella DJ. Revealing the secret lives of cryptic species: examining the phylogenetic relationships of echinostome parasites in North America. Mol Phylogenet Evol 2010; 55: 611-620.
11. Detwiler JT, Zajac AM, Minchella DJ, Belden LD. Revealing cryptic parasite diversity in a definitive host: echinostomes in muskrats. J Parasitol 2012; 98: 1148-1155.
12. Morgan JA, Blair D. Mitochondrial ND1 gene sequences used to identify echinostome isolates from Australia and New Zealand. Int J Parasitol 1998; 28: 493-502.
13. Saijuntha W, Sithithaworn P, Duenngai K, Kiatsopit N, Andrews RH, Petney TN. Genetic variation and relationships of four species of medically important echinostomes (Trematoda: Echinostomatidae) in South-East Asia. Infect Genet Evol 2011; 11: 375-381.
14. Noikong W, Wongsaawad C, Chai JY, Saenphet S, Trudgett A. Molecular analysis of echinostome metacercariae from their second intermediate host found in a localised geographic region reveals genetic heterogeneity and possible cryptic speciation. PLoS Negl Trop Dis 2014; 8: e2778.
15. Mohanta UK, Watanabe T, Anisuzzaman, Ohari Y, Itagaki T. Characterization of Echinostoma revolutum and Echinostoma robustum from ducks in Bangladesh based on morphology, nuclear ribosomal ITS2 and mitochondrial nad1 sequences. Parasitol Int 2019; 69: 1-7.
16. Morgan JA, Blair D. Relative merits of nuclear ribosomal internal
transcribed spacers and mitochondrial CO1 and ND1 genes for distinguishing among *Echinostoma* species (Trematoda). Parasitology 1998; 116: 289-297.

17. Chai JY, Sohn WM, Yong TS, Eom KS, Min DY, Hoang EH, Phammasack B, Issisngnmy B, Rim HJ. Echinostome flukes recovered from humans in Khammouane Province, Lao PDR. Korean J Parasitol 2012; 50: 269-272.

18. Faltýnková A, Georgieva S, Soldánová M, Kostadinova A. A re-assessment of species diversity within the ‘revolution’ group of *Echinostoma* Rudolphi, 1809 (Digenea: Echinostomatidae) in Europe. Syst Parasitol 2015; 90: 1-25.

19. Luton K, Walker D, Blair D. Comparisons of ribosomal internal transcribed spacers from two congeneric species of flukes (Platyhelminthes: Trematoda: Digenea). Mol Biochem Parasitol 1992; 56: 323-328.

20. Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Mol Biol Evol 2013; 30: 2725-2729.

21. Yamaguti S. Studies on the helminth fauna of Japan. Part 5. Avian trematodes, II. Jpn J Zool 1934; 5: 542-583.

22. Yamaguti S. Studies on the helminth fauna of Japan. Part 5. Trematodes of birds, III. Jpn J Zool 1935; 6: 159-182.

23. Sandground JH, Bonne C. *Echinostoma limaense* n. sp., a new parasite of man in the Celebes with an account of its life history and epidemiology. Am J Trop Med Hyg 1940; 20: 511-536.

24. Kanev I, Dimitrov V, Radev V, Fried B. Redescription of *Eutrompa* (Echinostomatidae) from man and the introduction of a new species. J Parasitol 1978; 64: 599-600.

25. Reddy DB, Ranganaykamma I, Venkataratnam D. *Artystechinostoma mehrai* infestation in man. J Trop Med Hyg 1964; 67: 58-59.

26. Sohn WM, Kim HJ, Yong TS, Jeong HG, Kim JK, Kang AR, Kim MR, Park JM, Ji SH, Sinuon M, Socheat D, Chai JY. *Echinostoma revolutum* infection in Oddar Meanchey Province, Cambodia. Korean J Parasitol 2011; 49: 187-190.

27. Sohn WM, Chai JY, Yong TS, Eom KS, Yoon CJ, Sinuon M, Socheat D, Lee SH. *Echinostoma revolutum* infection in children, Pursat Province, Cambodia. Emerg Infect Dis 2011; 17: 117-119.

28. Anh NT, Phuong NT, Murrell KD, Johansen MV, Dalsgaard A, Thu LT, Chi TTK, Thamsborg SM. Animal reservoir hosts and fish-borne zoonotic trematode infections on fish farms, Vietnam. Emerg Infect Dis 2009; 15: 540-546.

29. Anh NT, Madsen H, Dalsgaard A, Phuong NT, Thanh DTH, Murrell KD. Poultry as reservoir hosts for fishborne zoonotic trematodes in Vietnamese fish farms. Vet Parasitol 2010; 169: 391-394.

30. Chai JY, Sohn WM, Na BK, De NV. *Echinostoma revolutum*: metacercariae in *Filopaludina* snails from Nam Dinh Province, Vietnam, and adults from experimental hamsters. Korean J Parasitol 2011; 49: 449-455.

31. Sayasone S, Tesana S, Ulzinger J, Hatz C, Akkhavong K, Odermatt P. Rare human infection with the trematode *Echinocotus japonicus* in Lao PDR. Parasitol Int 2009; 58: 106-109.

32. Sohn WM, Chai JY, Na BK, Yong TS, Eom KS, Park H, Min DY, Rim HJ. *Echinostoma mekongi* in Lao PDR: metacercariae in *G. pangopaludina* snails and adults from experimentally infected animals. Korean J Parasitol 2013; 51: 191-196.

33. Chai JY, Sohn WM, Cho J, Eom KS, Yong TS, Hoang EH, Phammasack B, Ississngnmy B, Rim HJ. *Echinostoma ilocanum* infection in two residents of Savannakhet Province, Lao PDR. Korean J Parasitol 2018; 56: 77-81.

34. Chai JY, Jung BK, Chang T, Shin H, Sohn WM, Yong TS, Kim Min DY, Phommasack B, Issisngnmy B, Rim HJ. *Echinostoma aegyptica* (Trematoda: Echinostomatidae) infection in five riparian people in Savannakhet Province, Lao PDR. Korean J Parasitol 2020; 58: 67-72.

35. Chai JY, Chang T, Jung BK, Shin H, Sohn WM, Eom KS, Yong TS, Min DY, Phommasack B, Issisngnmy B, Rim HJ. *Echinocotus caninus* n. comb. (Trematoda: Echinostomatidae) infection in eleven riparian people in Khammouane Province, Lao PDR. Korean J Parasitol 2019; 57: 451-456.

36. Bhaiyalaya M, Harinasuta C, Tritanchatra S. The finding of *Echinostoma revolutum* infection in man in Thailand. J Med Assoc Thailand 1966; 49: 83.

37. Radomyos P, Radomyos B, Tungtrongchit A. Multi-infection with helminths in adults from northeast Thailand as determined by post-treatment fecal examination of adult worms. Trop Med Parasitol 1994; 45: 133-135.

38. Saijuntha W, Tantrawatpan C, Sithithaworn P, Andrews RH, Petney TN. Genetic characterization of *Echinostoma revolutum* and *Echinoparyphium recurvatum* (Trematoda: Echinostomatidae) in Thailand and phylogenetic relationships with other isolates inferred from ITS1 sequence. Parasitol Res 2011; 108: 751-755.

39. Buddhachat K, Chontananarth T. Is species identification of *Echinostoma mekongi* n. sp. from people in Cambodia 443 valid with high-resolution melting analysis? Parasitol Res 2019; 118: 1799-1810.

40. World Health Organization. Basic Laboratory Methods in Medical Parasitology. Geneva, Switzerland. World Health Organization. 1991, pp 26.
