의학석사 학위논문

Molecular differentiation of echinostomes occurring in Asia with description of *Echinostoma mekongi* n. sp. from humans in Cambodia

아시아 지역에 분포하는 극구흡충의 유전적 다양성 연구 및 캄보디아 환자에서 수집한 신종 메콩극구흡충 (*Echinostoma mekongi* n. sp.)의 보고

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ABSTRACT

Echinostomes (family Echinostomatidae) of the '37-collar-spined' or the '*revolutum*' group are recognized as zoonotic, food-borne intestinal trematodes that can cause echinostomiasis worldwide. Patients who suffer from echinostomiasis can complain severe epigastric or abdominal pain with diarrhea, easy fatigue, malnutrition and anemia. It is difficult to identify the species among the '37-collar-spined' members because a large number of morphological characters are very similar in their adult stages. To avoid the taxonomic confusion, molecular techniques have been used based on variable genetic markers. In total, 17 *Echinostoma* spp. adult specimens collected in Cambodia (13), Vietnam (2) and Thailand (2) were included in the study. Most specimens were assigned as *Echinostoma revolutum* based on morphological findings. For identification and phylogenetic study of the specimens, nested polymerase chain reaction (nested-PCR) was conducted using specific primers designed to amplify partial adenine dinucleotide dehydrogenase subunit 1 (ND1), cytochrome *c* oxidase subunit 1 (CO1) and internal transcribed spacer (ITS1-5.8S rRNA-ITS2) genes. The results indicated that here are no reference sequence of ND1, CO1 and ITS region of *revolutum* members in GenBank highly matched with the sequence which newly analyzed in this study. As shown by phylogenetic studies, the isolates from Cambodia, Vietnam and Thailand conducted a new monophyletic lineage. According to the comparisons based on the molecular data, the samples newly analyzed in this study are a distinctive species from other '37-collar-spined' members. In conclusion, we propose our echinostome

specimens from humans in Cambodia as a new species, *Echinostoma mekongi* n sp.

Key words: *Echinostoma mekongi* n sp., 37-collar-spined group, Human infection, Asia, Cambodia, ND1, CO1, ITS1-5.8S rRNA-ITS2

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INTRODUCTION

Echinostomes (family Echinostomatidae) of the '37-collar-spined' or the '*revolutum*' group are recognized as zoonotic, food-borne intestinal trematodes that cause echinostomiasis worldwide. The patients have echinostomiasis symptom can complain severe epigastric of abdominal pain with diarrhea, easy fatigue, malnutrition and anemia (Chai, 2009).

The echinostome eggs are laid with immature form, and hatch in about 3 weeks in the water environment (Chai, 2009). Miracidia invade the snail host, where they develop into mother rediae, daughter rediae, and cercariae. The mature cercariae which have well-developed tails and collar spines around the oral sucker may encyst within the body of their snail host. Or after escape the snail, they enter and encyst in other snails of the same of different species including bivalves, insects, tadpoles and frogs, fishes, or on vegetation as metacercariae form (Chai, 2009). Mammalians including human can be infected through ingestion of metacercariae encysted in the second intermediate host (Chai, 2009). Eating raw snails, clams, fishes, vegetation harboring metacercariae, or drinking water containing cercariae alive can be cause of the human echinostomiasis (Chai, 2009).

The species of the 37-collar-spined group of *Echinostoma* are characterized by the inter-specific morphological homogeneity of the life-cycle stages (Kostadinova and Gibson, 2000). There are currently numerous species defined as members of this group; *Echinostoma bolshewense*, *Echinostoma caproni, Echinostoma cinetorchis, Echinostoma echinatum, Echinostoma friedi, Echinostoma jurini, Echinostoma miyagawai,*

Echinostoma nasincovae, Echinostoma paraensei, Echinostoma parvocirrus, Echinostoma revolutum and *Echinostoma trivolvis* (Fried et al., 2004; Georgieva et al., 2013; Faltýnková et al., 2015). The taxonomic characters used to distinguish between species and genera of the echinotomes include the structure of the cicumoral disc, the number and arrangement of rows of uninterrupted crown spines, the number of collar spines as well as testicular characters (Miliotis and Bier, 2003). It is difficult if not impossible to differentiate among the eggs or immature stages of echinostome species using morphological characters. Moreover, a large number of morphologically very similar adult stages from many species exist due to a long history of inadequate descriptions, poor specific diagnoses and extensive synonymy (Kostadinova et al., 2003). To avoid this taxonomic confusion, molecular techniques have been used based on variable genetic markers.

Echinostomes distribute widely range from Asia and Oceania to Europe, the Americas and Australia. Substantial genetic variation was reported in *E. revolutum* based on isolates from Europe (Kostadinova et al., 2003), North America (Detwiler et al., 2010; Georgieva et al., 2013), Australia (Morgan and Blair 1998a) and Southeast Asia (Saijuntha et al. 2011a; Noikong et al., 2014; Nagataki et al., 2015). Recently, the intensive study using molecular analysis for 37-collar-spined echinostomes in Southeast Asia, revealed presence of 2 species: *E. revolutum* and *E. miyagawai*, in domestic ducks in Thailand and Lao PDR (Nagataki et al., 2015). About infection case of human in Southeast Asia, *E. revolutum* reported infecting humans from Thailand (Bhaibulaya et al., 1966), Lao PDR (Chai et al., 2012) and Cambodia (Sohn et al., 2011). However, previous studies are

based on the morphological diagnosis, there is need of molecular research to figure out the species human infecting echinostomes. The current investigation is the first diagnosis of ’37-collar-spined’ echinostomes originated from humans using DNA sequence variation in Southeast Asia.

MATERIALS AND METHODS

1. Specimens

In total, 19 *Echinostoma* spp. adult specimens collected in Cambodia, Vietnam, Thailand and Korea were included in the study (Table 1). Of them, 13 were isolated from human resided in Takeo and Kratie Province, Cambodia, 2 from experimental hamsters in Vietnam and 2 from domestic chicks in Thailand. These specimens were primarily assigned as *E. revolutum* based on the morphological findings. For comparison of genetic diversity between echinostomes, 2 worms from experimental rats in Korea were analyzed, which are identified as *Isthmiophora hortensis* with their morphological characters.

2. Morphometric examinations

The adult worms were examined under light microscopy and identified using the proper morphological characters (Kosupko, 1971 a, b, 1972; Nasincova 1986, 1991; Kostadinova et al., 2000a, b; Toledo et al., 2000; Faltýnková et al., 2015). The adult worms were investigated as permanent mounts in stains with acetocarmine. The type and voucher specimens are deposited in Helminthological Collections, Seoul National University College of Medicine, Seoul, Republic of Korea. Photomicrographs of the worms were taken with a digital camera, Olympus DP72, on an Olympus CKX41 microscope. Measurements were taken from digital images with the aid of CellSens Standard v1.5 image analysis software.

The following characters are used and all measurements are in

micrometers (Faltýnková et al., 2015): body length (BL), body width at level of intestinal bifurcation (BW1), body width at level of posterior border of ventral sucker (BW2), body width at mid-way between ventral sucker and ovary (maximum body width) (BW3), head collar length (CL), head collar width (CW), oral sucker length (OSL), oral sucker width (OSW), pre-pharynx length (PL), pharynx length (PHL), pharynx width (PHW), esophagus length (OL), cirrus-sac length (CSL), maximum cirrus-sac width (CSW), length of posterior portion of seminal vesicle (SVL1), width of posterior portion of seminal vesicle (SVW1), length of anterior portion of seminal vesicle (SVL2), width of anterior portion of seminal vesicle (SVW2), ventral sucker length (VSL), ventral sucker width (VSW), ovary length (OVL), ovary width (OVW), Mehlis' gland length (MEL), Mehlis' gland width (MEW), anterior testis length (ATL), anterior testis width (ATW), posterior testis length (PTL), posterior testis width (PTW), egg length (EL), egg width (EW), angle spine length (ASL), angle spine width (ASW), lateral spine length (LSL), lateral spine width (LSW), dorsal spine length (DSL), dorsal spine width (DSW), forebody length (FORE), distance from anterior extremity to intestinal bifurcation (ODIV), length of pre-ovarian region (OVAR), length of post-testicular region (TEND), pharynx to oral sucker width ratio (OSW/PHW), body width as a proportion of body length (BW1/BL, BW2/BL, BW3/BL), forebody as a proportion of body length (FO/BL), collar width as a proportion of maximum body width (CW/BW3), distance from anterior extremity to intestinal bifurcation as a proportion of body length (ODIV/BL), distance between ovary and posterior margin of ventral sucker as a proportion of body length (OVAR/BL), length

of post-testicular field as a proportion of body length (TEND/BL).

3. Molecular genetic examinations

For molecular analysis, the worms mounted were removed from the slide glasses using a slide heater or xylene. Genomic DNA was extracted using the Spin-Column Protocol of DNeasy® Blood & Tissue kit (QIAGEN, Hilden, Germany). Polymerase chain reaction (PCR) and nested-PCR were then conducted using specific primers designed to amplify partial adenine dinucleotide dehydrogenase subunit 1 (ND1), cytochrome *c* oxidase 1 (CO1) and internal transcribed spacer (ITS) region (ITS1-5.8s rRNA-ITS2) in echinostomes species; for ND1, JB11 and JB12 (Morgan and Blair, 1998), and subsequently followed by the second PCR of the inner region using EchND1/inF and EchND1/inR; JB3 and JB13 for CO1; BD1 and BD2 for ITS region (Table 1). PCR products were sequenced using BigDye® Terminator v3.1 cycle sequencing kit by ABI 3730XL DNA analyzer (Applied Biosystems, Foster City, CA, USA). The basic local alignment search tool (BLAST; http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used in evaluation of genetic identity of the samples. Using the Geneious® version 6.1.6 (Biometers Ltd., Auckland, New Zealand), we aligned the obtained sequences with GenBank reference ND1, CO1 and ITS1-5.8S rRNA-ITS2 region sequences of echinostome species. Phylogenetic information was assessed via maximum likelihood (ML) analyses of Kimura-2-parameter distances usig MEGA v6 (Tamura et al., 2013).

Table 1. Primer information used this study

Target gene	Name	Sequence (5′-3′)	Target size (bp)
ND1	JB11	AGATTCGTAAGGGGCCTAATA	530
	JB12	ACCACTAACTAATTCACTTTC	
	EchND1/inF	AGTTGATTGTCTTGGGTAGGAGT	357
	EchND1/inR	TCCTACCAACCACAAGCCAT	
CO1	JB3	TTTTTTGGGCATCCTGAGGTTTAT	257
	JB13	TCATGAAAACACCTTAATACC	
ITS region	BD1	GTCGTAACAAGGTTTCCGTA	1,000
	BD2	TATGCTTAAATTCAGCGGGT	

Table 2. Summary data for the specimens of *Echinostoma* spp. used for analyze of ND1, CO1 and ITS sequences

Sample no.	Location		Description	Host	
	Country	Province	Patient code	2nd intermediate host	Final host
1	Cambodia	Takeo	C2	-	Human
2	Cambodia	Takeo	32	-	Human
3	Cambodia	Kratie	62	-	Human
4	Cambodia	Kratie	149	-	Human
5	Cambodia	Kratie	149	-	Human
6	Cambodia	Kratie	149	-	Human
7	Cambodia	Kratie	1	-	Human
8	Cambodia	Kratie	1	-	Human
9	Cambodia	Kratie	62	-	Human
10	Cambodia	Kratie	62	-	Human
11	Cambodia	Kratie	62	-	Human
12	Cambodia	Kratie	62	-	Human
13	Cambodia	Pursat	-	-	Human
14	Vietnam	Nam Dinh	-	*Filopaludina* sp.	Experimental hamster
15	Vietnam	Nam Dinh	-	*Filopaludina* sp.	Experimental hamster
16	Thailand	Chiang Mai	-	-	Chick
17	Thailand	Chiang Mai	-	-	Chick
18	Korea	-	-	*Odontobutis interrupta*	Experimental rat
19	Korea	-	-	*Odontobutis interrupta*	Experimental rat

RESULTS

1. Taxonomic summary

Family Echinostomidae Looss 1899

Genus *Echinostoma* Rudolphi 1809

Echinostoma mekongi n. sp. (Figure 1)

Type host: human, *Homo sapiens*.

Site of infection: small intestine.

Intermediate host: Unknown.

Type locality: Kratie, Cambodia

Deposition of specimens: Helminthological Collections, Seoul National University College of Medicine, Seoul, Korea (no.1511-001, holotype; no. 1511-002, paratypes).

Ethymology: The specific name refer to the river (Mekong river) along which the new species was first discovered.

2. Morphological descriptions

Morphological description of adult worms is based on 26 acetocarmine stained specimens using light microscopy. The specimens used in this research revealed variations in their morphology although groups of worms were originated from the same patient. Most samples had *E. revolutum*-like shape with elongate-oval, dorso-ventrally flattened and short forebody and slightly extended wider hindbody from just posterior to the ventral sucker

(Figure 1-A, B, C, D; Table 3). However, several worms were quite small compared to other specimens and had a narrow body part on posterior to the ventral sucker (Figure 1-D; Table 3).

Head collar is re-informed, well developed and its width represents on average 42% of maximum body width. Collar spines are large and 37 in number; angle and lateral spines are in similar size and dorsal spines are smaller (Table 3). Testes represented 2 in number and located in third quarter of body. There are various morphology on the shape of testes among the isolates. Some of them have lobulated on both testes (Figure 1-A, C), some others lobulated on only one (Figure 1-D) and the others have 2 elongate-oval shaped testes (Figure 1-B, E).

3. Genetic identification analysis

The species identification was made after sequencing of the mtDNA ND1, CO1 and ITS region and aligned using the program Geneious v.6.1.1. The samples were undergone sequence analysis of the ND1 gene of 357 bp, CO1 of 240 bp, and ITS of 913 bp. The results indicated that there are no reference sequence highly matched with the sequence which newly analyzed in this study. Although we identified the worms as *E. revolutum* with the morphological characters, they revealed only 85.7-86.0% for ND1 and 90.1-91.2% for CO1 of similarity with *E. revolutum*, Asian isolate (GenBank accession no. KP455626 and KP455626, respectively). They also showed 97.4-97.8% similarity, with *E. revolutum* ITS region (GenBank accession no. KM520150, GQ463056). The value revealed quite high, because of low inter-specific variation of ITS region.

4. Phylogenetic analysis

High quality sequence data (ND1, CO1 and ITS region) was obtained for 19 adult worms. The phylogentic analyses of the ND1 (337 bp after trimming) sequences constructed from ML analyses showed similar topologies as did the CO1 (188 bp) sequences based on ML analyses. The sequences of the samples clustered with themselves with high boot strap value (Figure 2, 3 and 4). The isolates from humans in Cambodia conducted a new lineage including the isolates from Vietnam. The lineage of our samples appeared as a sister group to *E. paraulum* in ND1 phylogentic tree (Figure 2).

Table 3. Morphological characteristics of adults of *Echinostoma mekongi* n sp. obtained from human in Takeo and Kratie Provinces, Cambodia described in this study

Province	Takeo				Kratie						
Patient code	CT-2A		CT-32		62		149		1		60
Sample size	(n = 3)		(n = 3)		(n = 7)		(n = 9)		(n = 3)		(n = 1)
	Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range	
BL	10,255	9,790-10,502	14,052	12,346-14,995	10754	9,485-12,196	11,187	8,970-13,123	12,414	12,261-12,778	12,364
BW1	1,233	1,229-1,243	1,223	1,166-1,262	1001	774-1,293	1,058	965-1,259	984	782-1,107	1,145
BW2	1,938	1,897-1,971	2,214	2,097-2,319	1233	973-1,323	1,323	1,154-1,615	1,202	903-1,509	1,465
BW3	2,335	2,301-2,353	2,824	2,661-2,991	1900	1,336-2,208	2,074	1,794-2,503	1,651	1,488-1,856	1,747
CL	380	349-439	455	421-492	360	336-385	378	337-425	376	346-424	394
CW	589	550-624	681	635-749	515	468-603	572	496-649	575	546-602	613
OSL	229	219-226	283	273-299	229	199-263	231	211-259	227	220-238	258
OSW	285	256-311	308	268-349	229	201-251	253	200-297	245	220-288	274
PL	71	56-83	117	86-133	92	31-139	137	105-121	133	109-149	109
PHL	238	207-257	232	226-236	170	150-180	189	191-217	161	144-192	213
PHW	211	200-223	232	217-253	189	172-205	210	170-230	203	189-229	197
OL	545	533-565	793	597-952	475	333-596	661	466-783	619	576-664	589
CSL	441	429-451	512	491-528	427	321-564	391	262-498	353	303-416	451
CSW	205	186-215	258	250-270	233	190-274	267	222-326	231	218-243	253
SVL1	344	220-413	319	215-448	315	244-375	319	250-457	241	201-289	352
SVW1	138	95-193	146	107-168	133	100-159	165	119-216	128	113-150	147
SVL2	134	113-162	189	44-290	177	68-306	202	97-369	209	164-256	278
SVW2	60	58-63	109	42-195	115	94-141	147	60-290	110	73-152	144
VSL	685	635-678	749	726-775	560	517-611	650	604-694	571	516-645	589
VSW	678	629-721	765	703-802	558	520-595	607	532-648	513	448-588	560
OVL	246	212-303	321	304-356	235	187-252	248	224-393	288	258-302	221
OVW	334	327-344	438	425-445	279	252-313	301	279-437	324	302-351	264
MEL	318	302-310	403	368-468	443	321-811	385	328-572	446	391-521	588
MEW	607	511-659	900	859-947	668	516-932	619	550-847	562	504-629	682
ATL	733	704-790	833	805-868	684	536-800	592	470-729	708	703-713	662
ATW	597	560-636	825	739-972	585	475-674	570	406-809	579	516-704	487
PTL	755	715-781	813	687-905	754	670-828	653	489-803	659	490-784	747
PTW	581	487-645	784	745-827	566	522-656	545	287-884	574	509-675	512

Table 3. Continued

Province	Takeo				Kratie						
Patient code	CT-2A		CT-32		62		149		1		60
Sample size	(n = 3)		(n = 3)		(n = 7)		(n = 9)		(n = 3)		(n=1)
	Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range	
EL	112	98-131	117	107-130	113	94-125	120	95-136	121	100-136	113
EW	63	56-73	81	37-61	62	48-87	84	55-101	76	65-89	80
ASL	56	50-64	50	13-21	56	40-86	54	35-76	54	52-59	48
ASW	12	6-19	16	11-21	17	14-20	16	9-32	18	15-20	13
LSL	58	51-66	62	54-69	61	46-85	53	41-66	47	40-57	55
LSW	14	10-17	16	14-20	16	12-21	15	9-38	14	9-17	16
DSL	40	35-46	55	51-62	38	24-48	51	34-95	45	42-51	39
DSW	11	6-17	14	8-19	12	6-17	13	6-25	12	9-13	11
FORE	1,861	1,835-1,890	2,681	2,397-3,211	1,570	1,163-2,162	2,242	1,887-3,008	1,865	1,718-2,135	1,982
ODIV	1,306	1,234-1,347	1,742	1,508-1,969	1,372	1,080-1,883	1,508	1,255-1,708	1,390	1,376-1,400	1,425
OVAR	5,335	5,084-5,721	8,655	6,510-11,926	5,711	4,674-6,318	5,799	4,660-6,501	6,420	6,197-6,558	6,099
TEND	2,508	2,271-2,274	4,307	3,095-4,021	2,495	2,091-3,005	2,783	1,673-4,014	3,179	2,560-3,656	2,783
Sucker width ratio	1:2.38	1:2.20-2.50	0	1:2.26-2.62	1:2.45	1:2.15-2.78	1:2.43	1:2.71-2.80	1: 2.10	1:2.04-2.21	1:2.04
OSW/PHW	1.35	1.28-1.40	1	1.24-1.38	1.21	1.01-1.33	1.20	1.08-1.39	1.20	1.17-1.26	1.39
BW1/BL (%)	12.0	11.7-12.6	9	8.27-9.45	9.25	7.78-10.6	9.66	8.24-12.3	7.88	6.32-8.66	9.26
BW2/BL (%)	18.9	18.5-19.4	16	14.8-17.0	11.5	7.97-13.6	12.1	9.29-16.9	9.61	7.29-11.8	11.8
BW3/BL (%)	22.8	22.4-23.5	20	19.0-21.6	17.8	13.5-22.3	19.2	13.8-27.7	13.2	12.0-14.5	14.1
FO/BL (%)	18.2	17.5-19.0	19	16.4-21.4	14.6	9.53-18.5	20.7	14.5-31.7	15.0	13.4-17.2	16.0
CW/BW3 (%)	25.2	23.4-26.5	24	21.4-26.6	27.8	22.1-38.0	28.0	20.9-32.4	35.0	32.5-36.7	35.1
ODIV/BL (%)	12.7	12.6-12.8	12	11.8-13.1	12.9	9.67-19.1	13.8	9.67-17.8	11.1	10.8-11.4	11.5
OVAR/BL (%)	52.1	48.4-54.6	61	50.8-79.5	53.1	49.3-57.6	52.5	41.0-61.0	51.5	50.0-53.1	49.3
TEND/BL (%)	24.6	21.7-30.4	24	20.7-27.1	23.2	21.2-26.0	24.6	17.4-35.9	25.5	20.7-28.6	22.5

Table 4. Sequence comparison among *Echinostoma* spp. based on ND1, CO1 gene and ITS region; data reveal percent of similarity (%)

ND1		**CO1**		**ITS1-5.8S-ITS2**	
	Samples		**Samples**		**Samples**
Samples	99.4-99.8	**Samples**	99.6	**Samples**	100
E. revolutum (Eurasian lineage)	85.7-86.0	***E. revolutum*** (Southeast Asian lineage)	90.7-91.2	***E. revolutum*** (Europianlineage)	97.4
E. revolutum (American lineage)	86.3-86.6	***E. revolutum*** (American lineage)	89.4-89.9	***E. revolutum*** (American lineage)	97.8
E. miyagawai (Eurasian lineage)	86.0-86.3	***E. miyagawai***	90.0	***E. miyagawai***	98.1
E. miyagawai (Australian lineage)	87.4-87.7	***E. robustum***	89.4	***E. robustum***	97.9
E. robustum	87.4-87.7	***E. caproni***	92.6-92.7	***E. caproni***	97.0
E. paraulum	88.0-88.2	***E. trivolvis***	91.0	***E. trivolvis***	97.6
E. caproni	82.2-85.3				
E. trivolvis	83.5-83.8				
E. nasincovae	81.2-81.5				

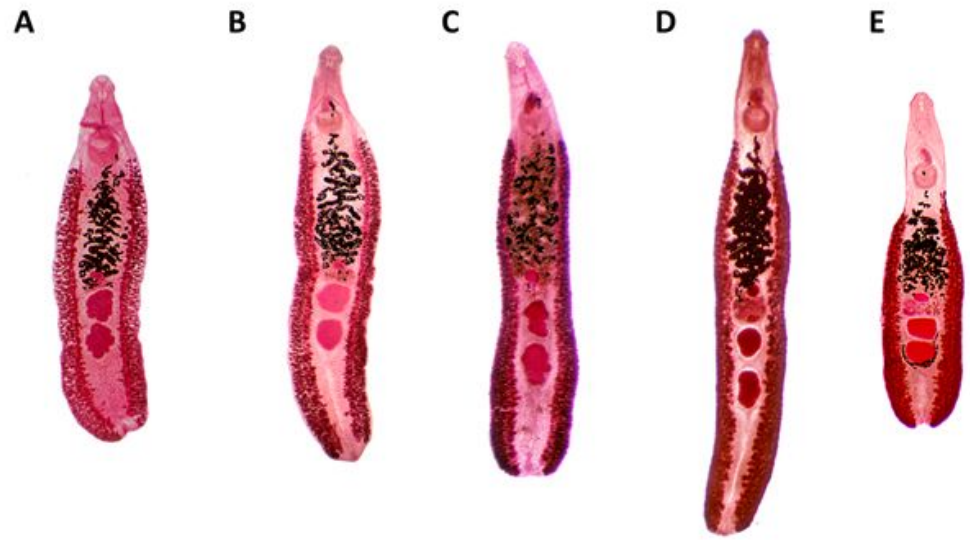

Figure 1. *Echinostoma mekongi* n. sp. specimens recovered from humans in Takeo and Kratie Province, Cambodia. They reveal various morphological characters in size, shape, and location of testes. (A) A echinostome from a patient numbered C2 in Takeo Province, which have lobulated testes. (B) A trematode from a patient numbered 32 in Takeo Province with un-lobulated testes, (C) A worm from the patient numbered 62 in Kratie Province, (D) and (E) a specimen from a patient labeled 149 in Kratie Province.

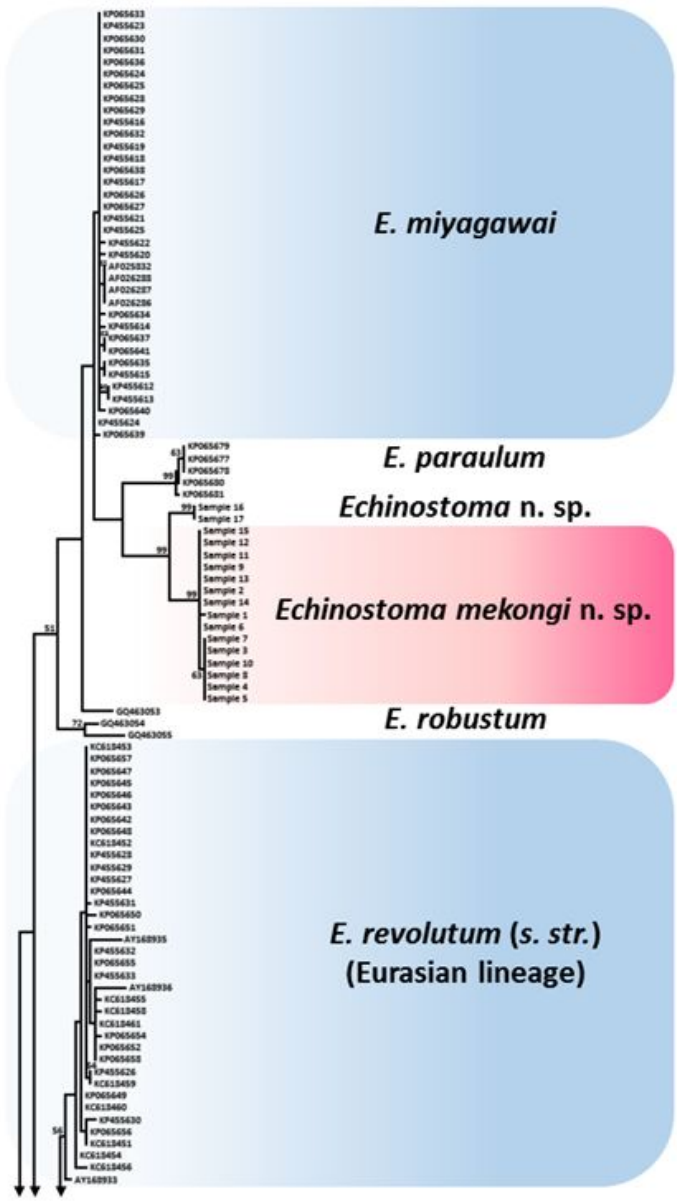

Figure 2. A Maximum Likelihood (ML) phylogenetic tree constructed based on 337 bp of ND1 sequences of *Echinostoma* spp. from Cambodia, Vietnam and Thailand. The other samples as well as related species available in GenBank were included.

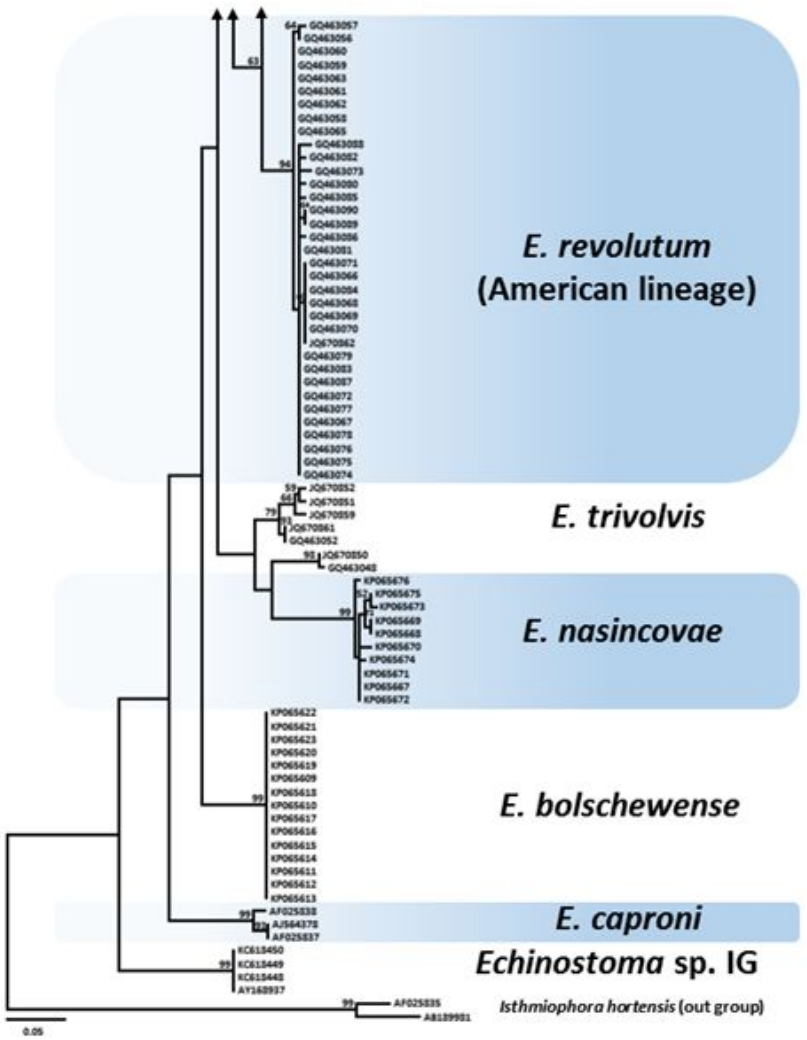

Figure 2. Continued

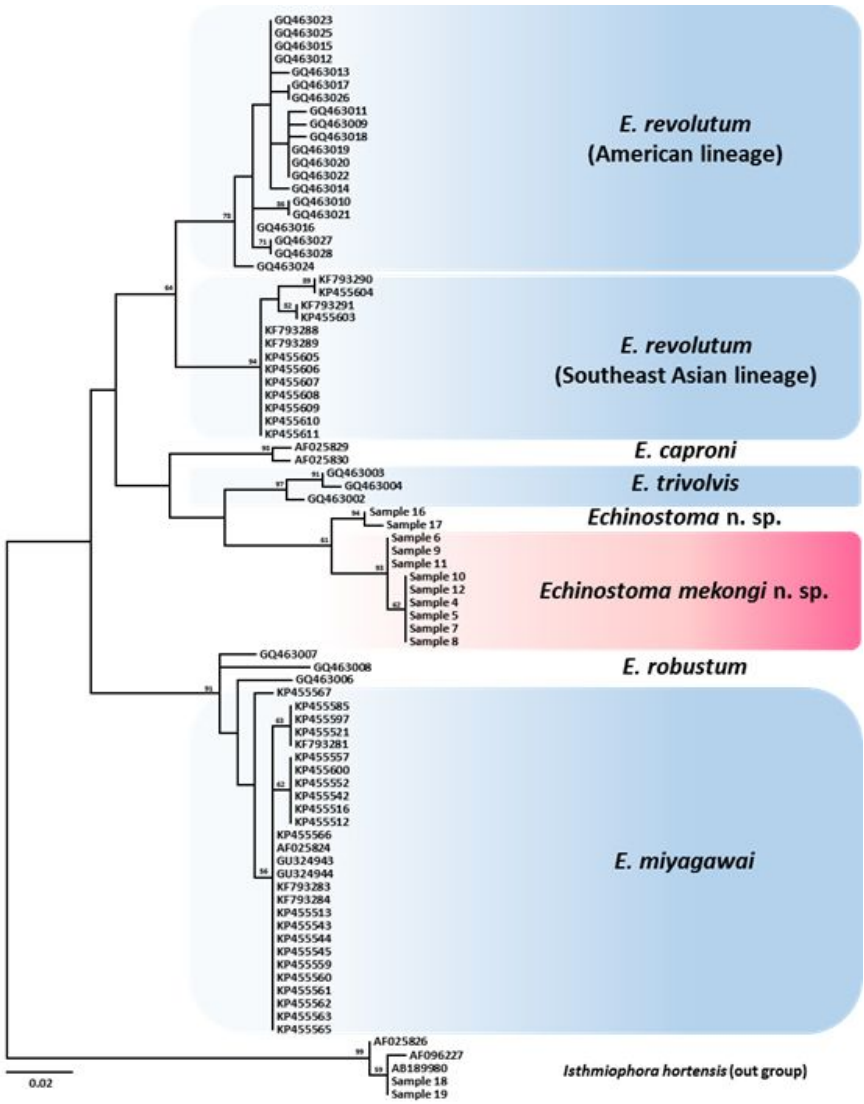

Figure 3. A Maximum Likelihood (ML) phylogenetic tree constructed based on 188 bp of CO1 sequences of *Echinostoma* spp. from Cambodia, Vietnam and Thailand. Thus, samples of *Isthmiophora hortensis* isolated in Korea examined and included as an out group. The other samples as well as related species available in GenBank were included.

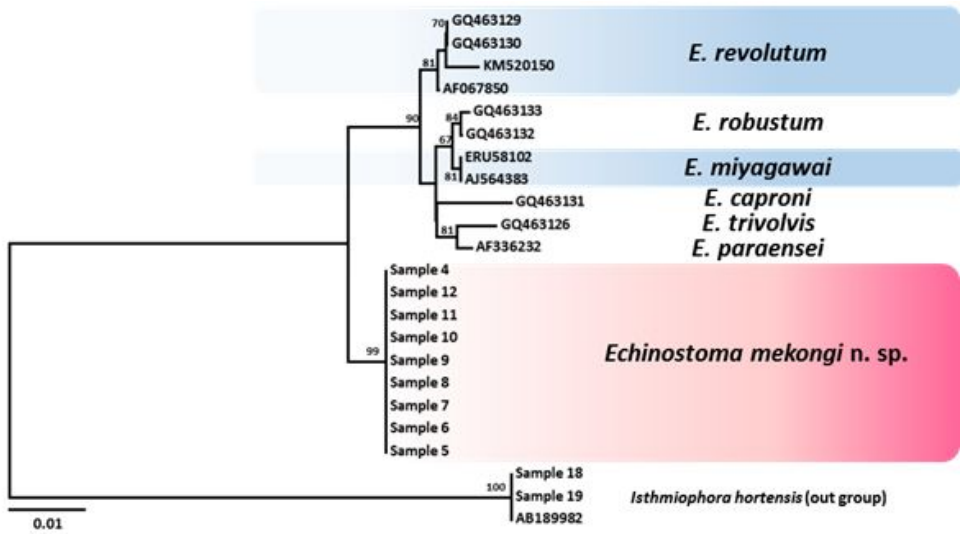

Figure 4. A Maximum Likelihood (ML) phylogenetic tree constructed based on 1,000bp of ITS1-5.8S rRNA-ITS2 sequences of *Echinostoma* spp. from Cambodia, Vietnam and Thailand. Thus, samples of *Isthmiophora hortensis* isolated in Korea examined and included as an out group. The other samples as well as related species available in GenBank were included

DISCUSSION

The taxonomy and species identification based on morphological characters of '*revolutum*' group have been debatable due to both the interspecific homogeneity of character on their adult stages and the poor differential diagnoses of newly established taxa. Beaver (1937) first suggested that only the type-species of *Echinostoma* is valid, placed 9 species (*Distoma echinatum, Echinostoma miyagawai, E. cinetorchis, E. armigerum, E. coalitum, E. mendax, E. paraulum,* and *E. limicoli*) in synonymy and regarded additional 11 species as '*syn. inq.*'. Kanev and colleagues (1994; 1995a, b) developed the *revolutum* group to 5 species (*E. revolutum, E. trivolvis, E. caproni, E. jurini,* and *E. echinatum*) based on a single morphological character of their larval stages, the specificity toward the first intermediate host, their ability to infect avian or mammalian hosts and their geographical range on a global scale. After that, based on molecular data, *E. revolutum* was recorded in Australia (Morgan and Blair, 1998) and North America (Sorensen et al., 1997; Detwiler et al., 2010; 2012), *E. paraensei* was revalidated and recorded in Australia and South America (Morgan and Blair, 1998), and unidentified species were discovered in New Zealand, North America and Europe (Morgan and Blair, 1998; Sorensen et al., 1998; Detwiler et al., 2010; 2012; Georgieva et al., 2013). Moreover, a number of species, *E. bolschewense, E. friedi, E. spiniferum, E. miyagawai, E. deserticum* and *E. luisreyi,* were described based on experimental completion of the life-cycles (Kostadinova et al., 2000a; 2000b; Našincová, 1991; 1992; Toledo et al., 2000; Kechemir et al., 2002;

Maldonado et al., 2003). Recently, Georgieva et al. (2014) and Faltýnková et al. (2015) reported that *E. bolschewense, E. miyagawai, E. revolutum, E. paraulum* and *E. nasincovae* were valid using molecular markers and provided keys European *revolutum* group based on morphological charaters.

To avoid taxonomic misperception, it is increasingly important to not only employ molecular methods, but also select proper markers (Kostadinova et al., 2003). The first molecular study on the 37-collar-spined group found very low levels (1.1-3.7%) of interspecific sequence variation of the nuclear rDNA ITS sequences (Morgan and Blair, 1995). After that, Morgan and Blair (1988a, b) reported interspecific sequence divergence of ND1 within the '*revolutum*' group of 12.3-13.8% (Morgan and Blair, 1998a) and 9.6-30.8% (Morgan and Blair, 1998b). They suggested that ND1 is diverging significantly faster that CO1 and ITS gene regions and thus appears to be the most informative region. The mitochondrial ND1 gene have been considered as a best molecular marker for researching relationships among the 37-collar-spined species (Morgan and Blair, 1995). In Asia, CO1 gene and ITS region were also used to investigate variability of echinostomes in Southeast Asia (Saijuntha et al., 2011a; Noikong et al., 2014). In 2015, Nagataki et al. (2015) primarily employed ND1 to analyze Asian echinostomes. In this study, we applied the markers, ND1, CO1 and ITS genes, to investigate relationship between Asian *Echinostoma* spp. ND1 and CO1 were shown to be as proper markers in our results with moderate intraspecific diversity. Nonetheless, the echinosotmes in current inquiry showed high similarity between *Echinostoma* spp. in ITS region because of their high conserved sequence.

Previous analyses of the echinostomes in 37-collar-spined group using molecular method have verified that several cryptic lineage exist in this group in Europe, North America and Southeast Asia (Kostadinova et al., 2003; Nagataki et al., 2015). The taxonomic status and systematics of the group, including *E. revolutum* and other related species, in Southeast Asia have been unclear. The recent research, the intensive investigation of 37-collar-spined echinostomes in Southeast Asia, has provided strong evidence that cryptic species, *E. revolutum* and *E. miyagawai*, exist in free-grazing ducks in Thailand and Lao PDR (Nagataki et al., 2015). They also suggested the possibility that other cryptic species of this group can distribute in Southeast Asia. This study give purpose to suggest new information about diversity of *revolutum* group and existence of *E. mekongi* as an cryptic species in Southeast Asia.

Most previous studies targeted echinostomes of *revolutum* group isolated from birds and mammalians other than humans. In Asia, for example, earlier molecular examination of the 37-collar-spined group have targeted echinostomes from fresh water snails and domestic ducks, not humans (Saijuntha et al., 2011; Noikong et al., 2014; Nagataki et al., 2015). Some cases of human infections with 37-collar-spined echinostomes have been reported in Southeast Asia. *E. revolutum* has been identified from humans in Thailand (Bhaibulaya et al., 1966), Lao PDR (Chai et al., 2012) and Cambodia (Sohn et al., 2011). Furthermore, human infection cases of *Echinostoma cinetorchis* were reported by Lee et al. (1988), Ryang et al. (1986) and Jung et al. (2014). However, the previous reports are based on the morphology of adult worms or eggs. This current investigation is not

only morphological analysis but also the first molecular diagnosis of 37-collar-spined echinostomes originated from humans using DNA sequence variation in Asia.

Some of our specimens closely resemble *E. revolutum* morphologically; the number of collar spines, elongated and dorso-ventrally flattened body, with maximum width at level of proximal part of uterus, and number and location of testes (Table 3). However, our samples revealed identical nucleotide sequence from other 37-collar-spined species (Table 4). Furthermore, our sequences constructed a clade newly but were included the '*revolutum*' group (Figure 2, 3, 4). In conclusion, there is a new species of 37-collar-spined echinostomes infecting humans in Cambodia and domestic chicks in Thailand and freshwater snails in Vietnam with *E. mekongi* n sp. Comprehensive investigations of the genetic variation and the relationships between populations and species of 37-collar-spined echinostomes in Southeast Asia need to be continued using larger sample sizes covering the full extent of species across their whole range in endemic regions including different species of animal hosts.

Our samples have morphological diversity although some of them were originated from the same patient and shared identical genetic information (Figure 1, Table 3). This may be due to differences of developmental stage in the adult worms. In case of *E. caproni*, their adult worms presented significant morphometric differences in relation to worm ages in experimental rodents (Toledo et al., 2004). Furthermore, other previous studies have indicated age-related morphometric changes in *Echinostoma* spp. (Odaibo et al., 1988, 1989; Isaacson et al., 1989; Fried et

al., 1997; Kostadinova et al., 2000; Toledo et al., 2003). Adult worms from Cambodia in this study recovered after treatment of praziquatel and underwent purgation with magnesium salts, and that might affect expulsion of various stage of the worms. The biology and morphology of each life cycle stage should also be investigated to confirm the validity of the present new 37-collar-spined echinostome species, *E. mekongi* n. sp.

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국문 초록

극구흡충(echinostomes)은 극구흡충과에 속하는 흡충류로, 길이 1 cm 남짓한 크기에 두흡반 주위에 두관이 있고 주변으로 두극(collar spine)이 있다. 극구흡충은 전세계적으로 약 355종이 보고되어 있는데, 종감별시 두극의 모양, 배열, 크기, 개수로 형태학적 분류한다. 그 중 '37-collar-spined group'은 두극의 개수가 37개인 극구흡충들로, 형태적으로 매우 유사하여 종을 분류하는데 논란이 있었다. 이를 극복하기 위해 형태학적 특성뿐 아니라 분자유전학적 연구가 활발히 진행되고 있다. 그러나 아시아 여러 국가에 극구흡충이 분포함에도 유전적 다양성 연구는 아직까지 미미하여, 아시아권에 분포하는 극구흡충의 유전적 다양성을 연구하고자 하였다. 그 중 외선극구흡충(*Echinostoma revolutum*)과 형태학적으로 유사하나 조류가 아닌 인체에서 발견된 극구흡충을 메콩극구흡충(*Echinostoma mekongi* n. sp.)으로 명명하고 신종으로 보고하고자 한다. 이 연구에서는 캄보디아의 감염 환자에서 나온 극구흡충(*Echinostoma* spp.) 성충 13마리, 베트남에서 실험감염으로 얻은 극구흡충(*Echinostoma* spp.) 성충 2마리, 태국의 닭에서 얻은 극구흡충 2마리를 사용 하였다. 각각의 성충에서 genomic DNA 를 추출하고, ND1과 CO1, ITS 유전자를 택하여 PCR 과 nested-PCR 법으로 증폭하고 염기서열을 분석하였다. 캄보디아 인체 유래 극구흡충과 베트남에서 실험감염으로 얻은 극구흡충이 서로 유사도가 99.4-100% (ND1), 99.6-100% (CO1), 100% (ITS) 으로 이들이 하나의 종임을 확인하였다. 또한 이들은 "37-collar-spined group" 중 외선극구흡충 (88.4-87.7%), 물오리극구흡충 (*Echinostoma miyagawai*) (87.4-87.6%), *Echinostoma friedi* (87.1-87.7%), *Echinostoma paraulum*

(86.7-88.8%), *Echinostoma robustum* (87.7-88.8%), *Echinostoma caproni* (82.2-85.5%) 와 상이한 ND1 유전자 일치도를 보여 어느 종에도 속하지 않음을 확인하였다. 태국의 닭 유래 극구흡충은 인체 유래 극구흡충과 93.4-93.3% (ND1)의 유사도를 보여 별개의 종으로 판단하였다. ND1, CO1, ITS 계통수에서도 높은 bootstrap value 를 보이며 하나의 그룹을 이루어 새로운 종임을 확인할 수 있었다. 이 연구에서는 캄보디아와 베트남에서 유래된 37개의 두극을 가진 극구흡충을 신종으로 확인하고 메콩극구흡충 (*Echinostoma mekongi* n. sp.) 으로 명명하고자 한다.

주제어 : 메콩극구흡충, *Echinostoma mekongi* n sp., 인체감염, 캄보디아, CO1, ND1, ITS1-5.8s rRNA-ITS2