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Morphologic and Genetic Identification of *Diphyllobothrium nihonkaiense* in Korea

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Abstract: *Diphyllobothrium nihonkaiense* was first described by Yamane in 1986 but the taxonomical features have been obscure due to lack of critical morphologic criteria in its larval and adult stages. In Korea, this tapeworm had long been known as *Diphyllobothrium latum*. In this study, we observed 62 specimens collected from Korean residents and analyzed them by morphological features and nucleotide sequences of mitochondrial *cox1* gene as well as the ITS1 region. Adult tapeworms were examined after carmine or trichrome stain. Longitudinal sections of the gravid proglottids showed an obtuse angle of about 150 degree between the cirrus sac and seminal vesicle. This angle is known as a major differential point compared with that of *D. latum*. Nucleotide sequence differences between *D. latum* and the specimens from Koreans represented 17.3% in mitochondrial DNA *cox1* gene. Sequence divergence of ITS1 among 4 Korean isolates was 0.3% and similarity was 99.7% with *D. nihonkaiense* and *D. klebanovskii*. All of the Korean specimens analyzed in this study were identified as being *D. nihonkaiense* (n = 62). We propose its Korean name as "Dong-hae-gin-chon-chung" which means 'long tapeworm of the East Sea' for this newly analyzed diphyllobothriid tapeworm in Korea.

Key words: *Diphyllobothrium nihonkaiense*, *Diphyllobothrium latum*, genetic identification, distribution, Korea

INTRODUCTION

Cestodes of the genus *Diphyllobothrium* (Cobbold, 1858) are known to be widely distributed in northwestern Europe and Far-East Asia as a causative agent of diphyllobothriasis. Diphyllobothriasis is an intestinal parasitosis caused by the ingestion of raw freshwater fish containing infectious larvae of the genus *Diphyllobothrium*. Human diphyllobothriid cestodes have been reported so far as many as 18 species in the genus *Diphyllobothrium* (Yamane et al. 'Forum Cheju' in 1996, Japan). Among them, *D. latum* (Linnaeus, 1758), the broad fish tapeworm, is the most common human species whose life cycle is dependent on fish transport hosts. *D. latum* is mildly pathogenic in humans where it may cause pernicious anemia by absorbing large amounts of vitamin B_{12}. Human diphyllobothriasis in Korea was first reported in 1919 by Kojima and Ko, and was morphologically identified by the adult worm as *D. latum* [1]; *Diphyllobothrium yonagoense* [2] and *D. latum* parvum type [3] were also reported.

D. nihonkaiense was first described by Yamane [4] in 1986 in Japan. He described morphologic differences of *D. latum* in Japan from that in Finland in the adult worms, eggs, and plerocercoids and proposed reconsideration about the taxonomic status of *D. latum* in Japan. Since Japan and Korea share the East Sea which locates between the 2 countries, and the salmons return to both countries from the Pacific Ocean, we speculate the possibility of diphyllobothriid tapeworms of identical species present in both countries. The morphologic identification of *Diphyllobothrium* in Korea has been obscure because of lack of detailed observations. Therefore, a reliable taxonomic criterion is needed based on morphologic features combined with molecular data. Molecular approaches to differential identification of *D. latum* and *D. nihonkaiense* have been done by restriction fragment length polymorphisms (RFLP) of ribosomal DNA [5], sequence differences of mitochondrial cytochrome *c* oxidase I (*cox1*) gene [6,7] and differentiating molecular makers [8].

The present study focused on differences in the morphology,

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sequence variations in the mitochondrial *cox1*, and rDNA internal transcribed spacer I (ITS1) PCR as markers for identifying diphyllobothriid tapeworms in Korea.

MATERIALS AND METHODS

Specimens

A total of 68 *Diphyllobothrium* tapeworms (1982-2007) were analyzed in this study (Table 1). Six isolates of *D. latum* were collected from France (n = 1), Russia (n = 1), Spain (n = 1), and Switzerland (n = 3). Sixty-two isolates were originated from Korea. Among them, 57 collections were given from other laboratories in Korea, and the rest 5 were collected from infected Korean patients who passed proglottids naturally in the stool or after treatment with niclosamide or praziquantel with purgation using $MgSO_4$. These sample materials were divided each into 2 parts, which were then preserved in a -70℃ deep freezer and in 70% ethanol. Thirty-seven specimens were preserved in 10% formalin, 24 specimens were kept in 70% ethanol, and 7 specimens were kept between -20℃ and -70℃.

The rest 5 treated patients, all from Chungbuk Province in Korea, were addressed as follows. The code number G1213 was a 44-year-old man living in Cheongju City. He had eaten many unknown kinds of sea fishes raw and brought with an apolysed 1 m long strobila without scolex in 1998. G1215 was a 42 year-old woman living in Jochiwon, who had eaten sea fishes, brought an apolysed 130 cm long strobila without scolex, and a 5 m long strobila was collected after treatment in 1997. G1214 was a 42-year-old man living in Cheongju City, who he had eaten salmons and other sea fishes, brought an apolysed 2 m long strobila without scolex in 1996. G1623 was a 41-year-old man living in Eumseong. He had eaten 'masou salmon' and brought an apolysed 1 m long strobila, and a 5 m strobila was discharged after treatment in 2007. G1621 was a 65-year-old man living in Cheongwon. He also had eaten 'masou salmon' and a 6 m long strobila was collected after treatment in 2007.

Morphologic analysis

Ten or more gravid proglottids were longitudinally disrupted with a dissecting needle and fresh eggs were collected from terminal proglottids in saline and were prepared for scanning electron microscopy. After drying with critical point drier (CPD) and gold coating, specimens were observed by SEM (Hitachi S-570, Tokyo, Japan). The tapeworms were pressed and fixed in alcohol-formalin-acetic acid (AFA) for carmine stain. Some part of the specimen was used in hematoxylin and eosin (H-E) and trichrome stain after longitudinal sections for observation of the cirrus pouch, seminal vesicle, uterus, and uterine pore.

Genetic analysis

DNA extraction

Total genomic DNA was extracted from a single specimen that was chopped into small pieces and then the DNeasy Tissue Kit (Qiagen, Valencia, California, USA) was used according to manufacturer's instructions. The specimens were crushed in liquid nitrogen, soaked in TE buffer for 3-5 hr, and then digested for 30 min in DNA extraction buffer (100 mM NaCl, 50 mM EDTA [pH8.0], 50 mM Tris base/HCl [pH 8.0], 50 mM EDTA, 10% SDS, and 20 mg/ml proteinase K) at 56℃. After the incubation was continued in a hexadecyltrimethylammonium bromide (CTAB)/NaCl solution for 3 hr at 65℃, the cellular debris was removed and the genomic DNA was extracted using the phenol/chloroform extraction protocol. DNA was then precipitated in 3 M sodium acetate (pH 5.2) and 2 volumes of 95% ethanol were added, after which the DNA was dried. The pellet was subsequently dissolved in 50 μl TE buffer.

PCR

18S small subunit nuclear ribosomal DNA (18S rDNA), ribosomal DNA ITS1, and mitochondrial *cox1* gene were amplified by PCR. PCR were performed in a reaction mixture of 50 μl with 0.01 μg/μl of genomic DNA, 10 × PCR buffer (20 mM Mg^+), 10 mM dNTP mixture, 10 *p*moles of each primer, and 2.5 U/μl *Taq* DNA polymerase (High Fidelity PCR system, Roche, Mannheim, Germany). PCRs were performed in a GeneAmp PCR System 9700 (Applied Biosystems, Langen, Germany) and involved 1 cycle of initial denaturation at 94℃ for 3 min, followed by 35 cycles of denaturation (94℃ for 30 sec), annealing (52℃ for 30 sec for ITS1), and extension (72℃ for 1 min), with a final extension at 72℃ for 10 min. The PCR primers used were 5′-AACAAGGTTTCC GTAGGTG-3′ and 5′-AGCAGTCTGCGATTCACATT-3′ for ITS1 which yielded a 534 bp product including 18S rDNA and 5.8S rDNA, and 84C (5′-TGATTTTTTGGCCACCCCGAA AGTATA-3′) and 85C (5′-TGACATTACATAGTGGAAGTGAGCTAC-3′), which yield a 348-bp product and designed from *D. nihonkaiense* [6]. These primers were used in PCR, which employed 35 cycles of 94℃ for 20 sec, 46℃ for 40 sec, 72℃ for 1 min, and incubation at 72℃ for 4 min. This resulted in 370 bp DNA fragments, which were isolated on a 1.0% agarose gel, excised under long-wave UV light, and extracted

Table 1. Diphyllobothriid specimens analyzed in this study collected between the years 1982 and 2007

Code	Locality	Sex/age	Year	Molecular identification	GenBank
G1213	Korea (Cheongju)	M/44	1998	*D. nihonkaiense*	
G1214	Korea (Cheongju)	M/42	1996	*D. nihonkaiense*	EF420138
G1215	Korea (Jochiwon)	F42	1997	*D. nihonkaiense*	
G1223	Korea (Chuncheon)	M/41	1998	*D. nihonkaiense*	
G1224	Korea (Chuncheon)	M/43	1996	*D. nihonkaiense*	
G1225	Korea (Yangyang)	F/6	1996	*D. nihonkaiense*	
G1226	Korea (Yangyang)	M/37	1996	*D. nihonkaiense*	
G1227	Korea (Yangyang)	M	1998	*D. nihonkaiense*	
G1228	Korea (Yangyang)	M/61	1998	*D. nihonkaiense*	
G1229	Korea (Yangyang)	M	–	*D. nihonkaiense*	
G1230	Korea (Yangyang)	M	1995	*D. nihonkaiense*	
G1262	Korea (Seoul)	F/13	1988	*D. nihonkaiense*	
G1263	Korea (Seoul)	M/41	1987	*D. nihonkaiense*	
G1264	Korea (Seoul)	M	1987	*D. nihonkaiense*	
G1358	Korea (Seoul)	M/38	2001	*D. nihonkaiense*	
G1359	Korea (Seoul)	F/38	1996	*D. nihonkaiense*	
G1360	Korea (Seoul)	M/52	1991	*D. nihonkaiense*	
G1361	Korea (Seoul)	M/43	1987	*D. nihonkaiense*	
G1362	Korea (Seoul)	M/31	1987	*D. nihonkaiense*	
G1363	Korea (Seoul)	M/38	2001	*D. nihonkaiense*	
G1364	Korea (Seoul)	M/25	1987	*D. nihonkaiense*	
G1365	Korea (Seoul)	M/59	2002	*D. nihonkaiense*	
G1366	Korea (Seoul)	M	1988	*D. nihonkaiense*	
G1367	Korea (Seoul)	M/45	2000	*D. nihonkaiense*	
G1368	Korea (Seoul)	F/53	1983	*D. nihonkaiense*	
G1369	Korea (Hoengseong)	M/21	1994	*D. nihonkaiense*	
G1370	Korea (Hoengseong)	M/23	1997	*D. nihonkaiense*	
G1371	Korea (Wando)	M/64	1982	*D. nihonkaiense*	
G1372	Korea (Wando)	F/40	1985	*D. nihonkaiense*	
G1373	Korea (Seoul)	M/30	1996	*D. nihonkaiense*	
G1374	Korea (Seoul)	–	–	*D. nihonkaiense*	
G1375	Korea (Seoul)	M/43	1987	*D. nihonkaiense*	
G1376	Korea (Seoul)	M/41	1995	*D. nihonkaiense*	
G1377	Korea (Seoul)	–	1982	*D. nihonkaiense*	
G1378	Korea (Seoul)	M/40	1994	*D. nihonkaiense*	
G1379	Korea (Seoul)	M/30	1986	*D. nihonkaiense*	
G1380	Korea (Seoul)	F/37	1999	*D. nihonkaiense*	
G1381	Korea (Seoul)	M/27	1987	*D. nihonkaiense*	
G1382	Korea (Seoul)	M45	2000	*D. nihonkaiense*	
G1383	Korea (Seoul)	M/50	1999	*D. nihonkaiense*	
G1384	Korea (Gunpo)	M/43	2001	*D. nihonkaiense*	
G1385	Korea (Goheung)	M/23	1983	*D. nihonkaiense*	
G1386	Korea (Seoul)	M/36	1984	*D. nihonkaiense*	
G1387	Korea (Seoul)	M/20	1986	*D. nihonkaiense*	
G1388	Korea (Hwasun)	M/33	1999	*D. nihonkaiense*	
G1389	Korea (Hwasun)	F/53	–	*D. nihonkaiense*	
G1390	Korea (Seoul)	–	–	*D. nihonkaiense*	
G1391	Korea (Hwasun)	M/27	1987	*D. nihonkaiense*	
G1397	Korea (Chuncheon)	–	–	*D. nihonkaiense*	
G1463	Korea (Seoul)	F/29	2004	*D. nihonkaiense*	
G1464	Korea (Seoul)	F/29	2004	*D. nihonkaiense*	
G1465	Korea (Seoul)	M/50	2004	*D. nihonkaiense*	
G1466	Korea (Seoul)	M/58	1996	*D. nihonkaiense*	
G1533	Korea (Wando)	M/64	1982	*D. nihonkaiense*	
G1577	Korea (Seoul)	M/35	1995	*D. nihonkaiense*	

(Continued to the next page)

Table 1. *(Continued from the previous page)*

Code	Locality	Sex/age	Year	Molecular identification	GenBank
G1588	Korea (Daegu)	–	2001	*D. nihonkaiense*	
G1589	Korea (Daegu)	–	1998	*D. nihonkaiense*	
G1590	Korea (Daegu)	–	2000	*D. nihonkaiense*	
G1620	Korea (Jincheon)	M/40	2007	*D. nihonkaiense*	
G1621	Korea (Cheongwon)	M/65	2007	*D. nihonkaiense*	
G1623	Korea (Eumseong)	M/41	2007	*D. nihonkaiense*	
G1683	Korea (Seoul)	–	–	*D. nihonkaiense*	
G1245	Swiss (Geneva)	–	–	*D. latum*	
G1304	France	–	–	*D. latum*	
G1460	Swiss (Geneva)	M/38	2004	*D. nihonkaiense*	
G1461	Russia	F/31	2004	*D. latum*	DQ985706
G1462	Swiss (Geneva)	F/36	2004	*D. latum*	
G1551	Spain	–	2004	*D. nihonkaiense*	

–: unknown.

using a QIAquick PCR purification kit (Qiagen Co.).

DNA sequencing and analyses

The purified PCR-amplified fragments of ITS1 were then separately cloned. Ligation of the fragments was performed overnight at 15℃ using the pGEM-T easy vector kit (Promega, Madison, Wisconsin, USA). The ligates were transformed into the DH5α cell line. Plasmid DNA was then purified by using a QIAprep spin miniprep kit (Qiagen). The primer walking method was employed to obtain overlapping sequences for each of the amplified fragments. Cyclic sequencing from both ends of the fragments was performed by using a Big-Dye Terminator sequencing kit (Applied Biosystems, Foster City, California, USA) and the reaction products were electrophoresed on an automated DNA sequencer (model 3730KL, Applied Biosystems).

The sequences were assembled and aligned by using CLUSTAL X multiple alignment program [9] and the Bioedit program version 5.0.6 (BIOSOFT, Ferguson, Missouri, USA). The sequencing regions were identified by comparing them using BLAST searches with those of Platyhelminthes that had been deposited in the GenBank database. The molecular identification of *Diphyllobothrium* tapeworm specimens was based on the similarity of nucleotide sequences of *cox1* gene and ITS1 region, and phylogenetic relationships with those of *D. nihonkaiense* (GenBank accession number EF420138), *D. latum* (Genbank accession number DQ985706). Phylogenetic analyses were determined by the neighbor-joining (NJ), maximum-parsimony (MP), and minimum-evolution (ME) methods using the Mega 3.1 program [11]. NJ analysis was performed using a distance matrix calculated using the Kimura 2 parameter method. Bootstrap analysis was performed with 3,000 replications.

RESULTS

Morphologic characteristics of *D. nihonkaiense*

Morphologic observation was based on 5 specimens collected from the Korean people; G1213-5, G1261, and G1623. Whitish-yellow adult tapeworms were 1-5 m long with longitudinal central line of genital pores. The widest gravid proglottids measured 11 mm. The uterine structure showed typical diphyllobothriid tapeworm's feature showing rosette formation swirling 5-7 loops in carmine-stained specimens (Fig. 1A). Average size of eggs was 55.5 ($\pm$ 1.0) $\times$ 40.5 ($\pm$ 1.5) μm and the ratio of length and width was 1.37 ($\pm$ 0.06) μm (n = 20). The eggshell showed shallow pits on the smooth surface in SEM (Fig. 1B). The ovary was renal shape and located at the posterior side. Testes were follicular and the ovaries were dumbbell shape. Longitudinal sections of the gravid proglottids showed an obtuse angle of about 150 degree between the cirrus sac and seminal vesicle, which looked different from *D. latum* after H-E or trichrome stain (Fig. 1C, D). The uterine and genital pores were separated on the midline with 150-300 μm. The uterine pore opened slightly posterior to the genital pore. The genital pore was located ventral on the middle at 1/3 of the proglottids. The cirrus sacs in sagittal sections were 430-480 μm in length and 275-310 μm in width. The seminal vesicles were round to elliptical and 300-420 in length by 170-300 μm in diameter.

Sequence divergence of mitochondrial cox1 and ITS1 of human diphyllobothriid tapeworms

The *cox1* sequences (335 bp) of 62 Korean isolates showed 99% similarity to reference sequences of the Japanese origin *D. nihonkaiense* (GenBank No. AB015755) and 83.7% similarity

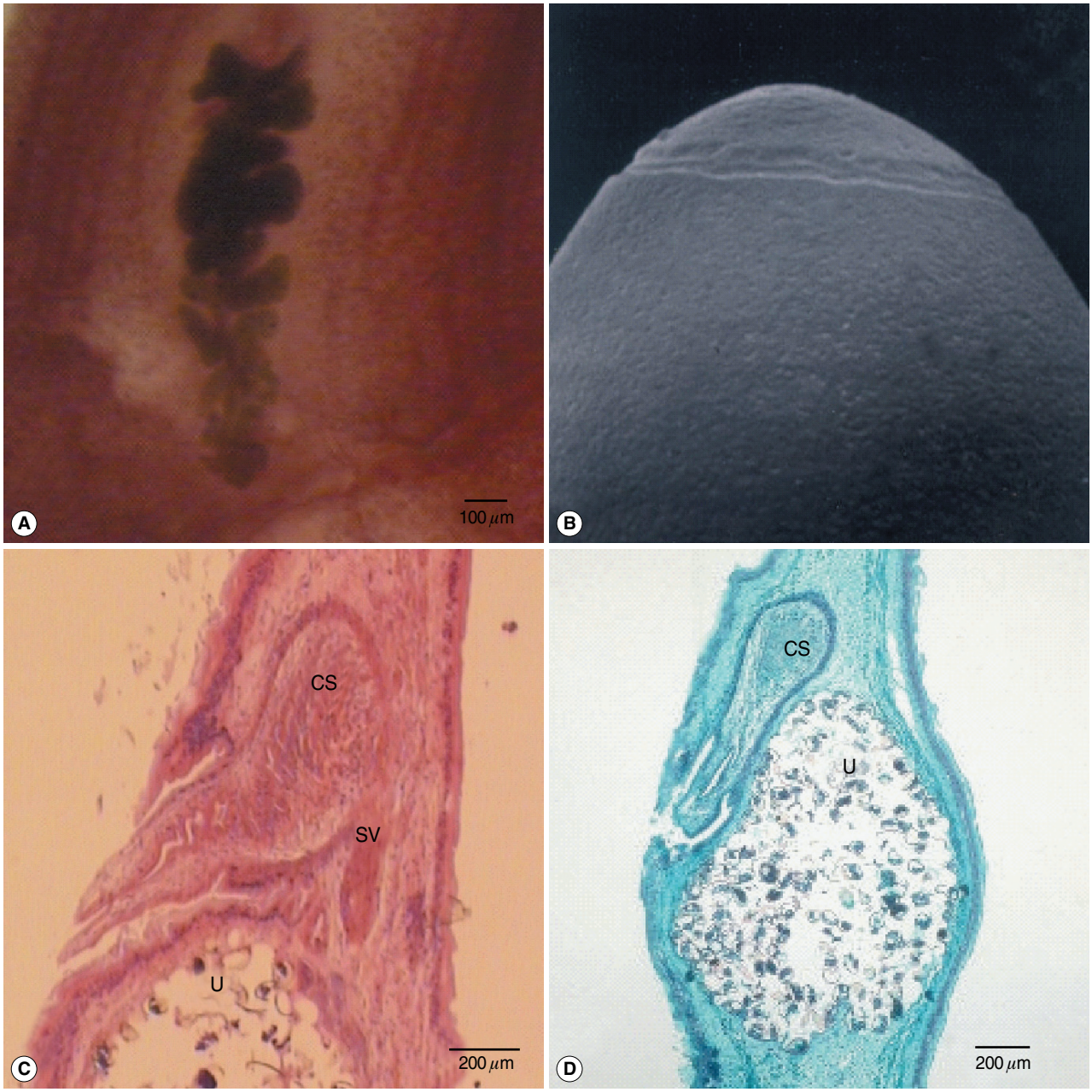

Fig. 1. Gravid proglottids and an egg of *Diphyllobothrium nihonkaiense*. (A) Whole mounted specimens of a proglottid showing the uterus and cirrus sac (× 25), (B) A SEM photo of the eggshell showing shallow pits and opercular structure (× 3,000), (C, D) Longitudinal sections of a mature proglottid showing the cirrus sac (CS), seminal vesicle (SV), and uterus (U) (C: H-E stain, D: trichrome stain).

with reference sequences of the Russian origin *D. latum* (G1461; GenBank No. DQ985706). Phylogenetic analysis of the mitochondrial DNA *cox1* (mtDNA *cox1*) sequences for a total 62 isolates identified *Spirometra* sp. as basal to the *D. nihonkaiense-D. latum* clade. The mtDNA *cox1* sequences of *D. nihonkaiense* and *D. latum* differed by 17.3% based on Kimura's 2-parameter model. Trees topology using various analytical methods (NJ, MP, and ME) generated very high confidence values (bootstrap values of 100%, 99%, and 100% in NJ, MP and ME, respectively) for 2 major branches representing each of *D. nihonkaiense* and *D. latum* (Fig. 2). The ITS1 sequences of Spain (G1551) and Switzerland (G1460) presented 100% similarity (525 bp) to the Japanese reference sequences of *D. nihonkaiense* (GenBank No. AB375175), and other 4 Korean isolates (G1213-5 and

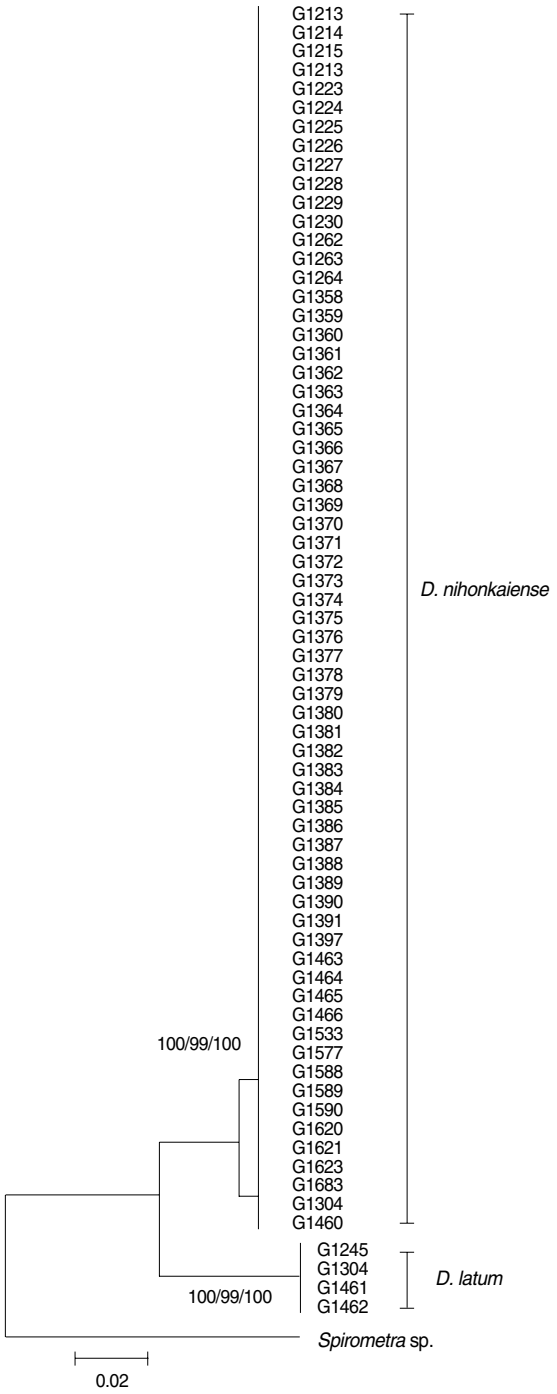

Fig. 2. A phylogenetic tree of diphyllobothriid tapeworms based on partial *cox1* sequences inferred from neighbor-joining (NJ) analysis. Numbers on branches indicate the bootstrap supporting values based on the 3,000 replicates. There were 335 bps corresponding to positions 735-1107 of the *cox1* gene.

G1621) also presented 100% identity to that of *D. nihonkaiense*. The analyses of *cox1* and ITS1 sequences identified all 62 Korean specimens as *D. nihonkaiense*.

DISCUSSION

Diphyllobothriid tapeworm infections have been reported in 43 cases in South Korea since 1971, including *D. latum* parvum type and *D. yonagoense* [11]. The major symptoms were gastrointestinal discomfort, except in a case of a child reported in 1983 by Joo and colleagues who showed microcytic hypochromic anemia (25th annual meeting of The Korean Society for Parasitology in Seoul). The suspected sources of infection of diphyllobothriid tapeworms in Korea were salmon, mullet, perch, and trout, but there have been no reports on plerocercoid infections from these fish intermediate hosts. The main infective source of *D. nihonkaiense* is now known to be *Oncorhynchus masou*, *O. keta*, and *Hucho perryi* (Salmoniidae) in Japan [6]. These species migrate around the Okhotsk, Bering, and Pacific Ocean. The salmons return back to the East Sea which is located between Korea and Japan; that is the reason why we consider *D. nihonkaiense* is shared by the 2 countries. The prevalence of diphyllobothriasis among Koreans was considered to be caused mainly by infection with *D. latum* until the address of *D. nihonkaiense* by Eom and colleagues in 2001 (35th annual meeting of The Korean Society for Parasitology at Kwangju, Korea). Characterization of the complete mitochondrial genome of *D. nihonkaiense* was also reported by Kim et al. in 2007 [8]. More recently, revised identification of *D. nihonkaiense* Yamane et al., 1986 and *D. klebanovskii* Muratov and Posokhov, 1988 [12] has been studied, and they were considered as the same species according to Arizono et al. in 2009 [13].

D. nihonkaiense was first identified by Yamane et al. in 1986 [4] with establishment of distinct characteristics of this parasite, such as an angle of the axis of the cirrus sac and seminal vesicle, of which morphology was first addressed by Kamo in 1978 [14]. Morphologic differentiation of human-infecting diphyllobothriid tapeworms is based on the features like the pit shape of egg shells, genital atrium openings, the angle between the long axis of the cirrus sac and seminal vesicle. The average size of *D. nihonkaiense* eggs was 55.5 ($\pm$ 1.0) $\times$ 40.5 ($\pm$ 1.5) μm which is smaller than that of *D. latum* [7,15]. The egg shells of *D. nihonkaiense* exhibit shallower pits distributing on the smooth surface. The genital pore and uterine pore were closer in *D. nihonkaiense* (150-300 μm) than in *D. latum* (260-1,240 μm) [15]. The angle between the long axis of the cirrus sac and seminal vesicle was sharper in *D. nihonkaiense* than those of *D. latum*. Nevertheless, species differentiation between *D. latum* and *D. nihonkaiense* is not clear sometimes due to their morphological

similarities.

Recently, the taxonomic status of diphyllobothriid tapeworm infections was questioned because only *D. latum* was reported in Korea. Consequently, it was necessary to clarify the distribution of these tapeworms in this country. Only 15 specimens could be examined on the morphological basis; the rest 47 specimens could not afford it. Most of them were preserved in 10% formalin or improper for morphologic examinations. The *cox1* sequences of 62 specimens showed 2 polymorphic sites with 2 non-synonymous substitution in *D. nihonkaiense* Korean isolates. The overall sequence difference in the full mitochondrial *cox1* gene between *D. nihonkaiense* and *D. latum* was 7.7%, whereas the full mitochondrial genome differed by 10.1% [8]. The sequence divergence of ITS1 among 4 Korean isolates was 0.3% and the similarity was 99.7% with *D. nihonkaiense* (GenBank No. AB375175) and *D. klebanovskii* (GenBank No. AB375657-AB375671). These results clearly indicate that *D. nihonkaiense* is a dominant species distributing in Korea without exception in all specimens examined (n = 62).

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