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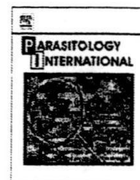

Short communication

Anomalous segmentation of *Diphyllobothrium nihonkaiense*☆

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ABSTRACT

An anomalous tapeworm with abnormal segmentation was obtained from a 6-year-old boy in Japan. The tapeworm consisted of proglottids with slanted anterior and posterior margins of proglottids and 4–6 sets of reproductive organs arranged between the margins. The morphology of the tapeworm did not correspond to any of the described cestodes. However, molecular identification based on nuclear and mitochondrial genes clearly showed the tapeworm was *Diphyllobothrium nihonkaiense*.

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Human diphyllobothriasis spreading worldwide, is a cestode zoonosis caused by eating raw or undercooked fish infected with larvae of *Diphyllobothrium* spp. (Diphyllobothriidae)[1,2]. There are more than 10 species known to cause human infection [1,3]. The main pathogenic organism is *Diphyllobothrium nihonkaiense* in Japan [4,5], while *Diphyllobothrium latum* is common in European countries [6]. *D. nihonkaiense* had been considered as the same species with *D. latum* until 1986, when Yamane et al. [7] differentiated the both species. Although those tapeworms are morphologically similar to each other, their second intermediate hosts are crucially different. *D. nihonkaiense* exploits anadromous Pacific salmons such as *Oncorhynchus masou masou* (masu salmon), *Oncorhynchus gorbuscha* (pink salmon) and *Oncorhynchus keta* (chum salmon), whereas *D. latum* exploits freshwater fish such as *Perca fluviatilis* (perch) and *Esox lucius* (pike). Another tapeworm *Diplogonoporus balaenopterae* belonging to the family Diphyllobothriidae also causes intestinal infections in Japan [8,9]. The most crucial morphological difference between the genera *Diphyllobothrium* and *Diplogonoporus* is the number of genitalia per segment. Except for the abnormal development of the strobila, *Diphyllobothrium* spp. have a single set of genitalia, whereas *Diplogonoporus* spp. have double sets.

Species identification of these tapeworms has been basically based on the morphology of larval and adult stages. However, morphological identification is less reliable, particularly in sibling species and atypical individuals. Therefore, molecular diagnostic tools using mitochondrial DNA (mtDNA) and nuclear DNA markers have been widely utilized for the identification of tapeworms [10–16]. Here we report a clinical case infected with an abnormal tapeworm, which was later identified as *D. nihonkaiense* by molecular diagnosis.

A 6-year-old boy, living in Oyama City, Tochigi Prefecture, shed a tapeworm for the first time on January 2008, and was referred to Jichi Medical University Hospital on September 3 but without specimen. No treatment was done at that time. Next time, he came on October 24, 2008 with a newly expelled worm specimen with 40 cm of length. He had not been claiming any subjective symptom other than releasing worms. Three weeks later, the patient was treated with praziquantel and laxative. We examined the patient's feces for 2 days and the tapeworm was only found in the feces obtained 24 h after the treatment. Because the tapeworm was considerably damaged and fragmented in much feces, the scolex part was unfortunately not found after all. Although the scolex was not found after the treatment, the whole tapeworm was considered to be released because the obtained mass of proglottids contained a narrow part close to the neck region.

The tapeworm consisted of only abnormal proglottids throughout the obtained strobila (Fig. 1A). Eggs found in the feces of the patient resembled the eggs of *Diphyllobothrium*. The worm obtained in October was fixed with 70% ethanol, and morphological observation and molecular diagnosis were conducted at Asahikawa Medical College. A part of the strobila was whole-mounted and stained with Semichon's acetic carmine. Some parts were embedded in paraffin, cut into 5 μm and stained with hematoxylin and eosin. Another part was refixed with 2.5% glutaraldehyde and used for scanning electron microscope (SEM) observation.

The width of the strobila was about 1 cm, and the maximum thickness was 650 μm (Fig. 1A, B). Margins of proglottids slanted to left both on the ventral and dorsal side. From the frontal view, 4–6 sets

☆ Nucleotide sequences determined in this report were deposited into DDBJ/EMBL/GenBank databases under the accession numbers: AB512013 (18 S rDNA), AB508837 (*cob*) and AB508838 (*cox1*).

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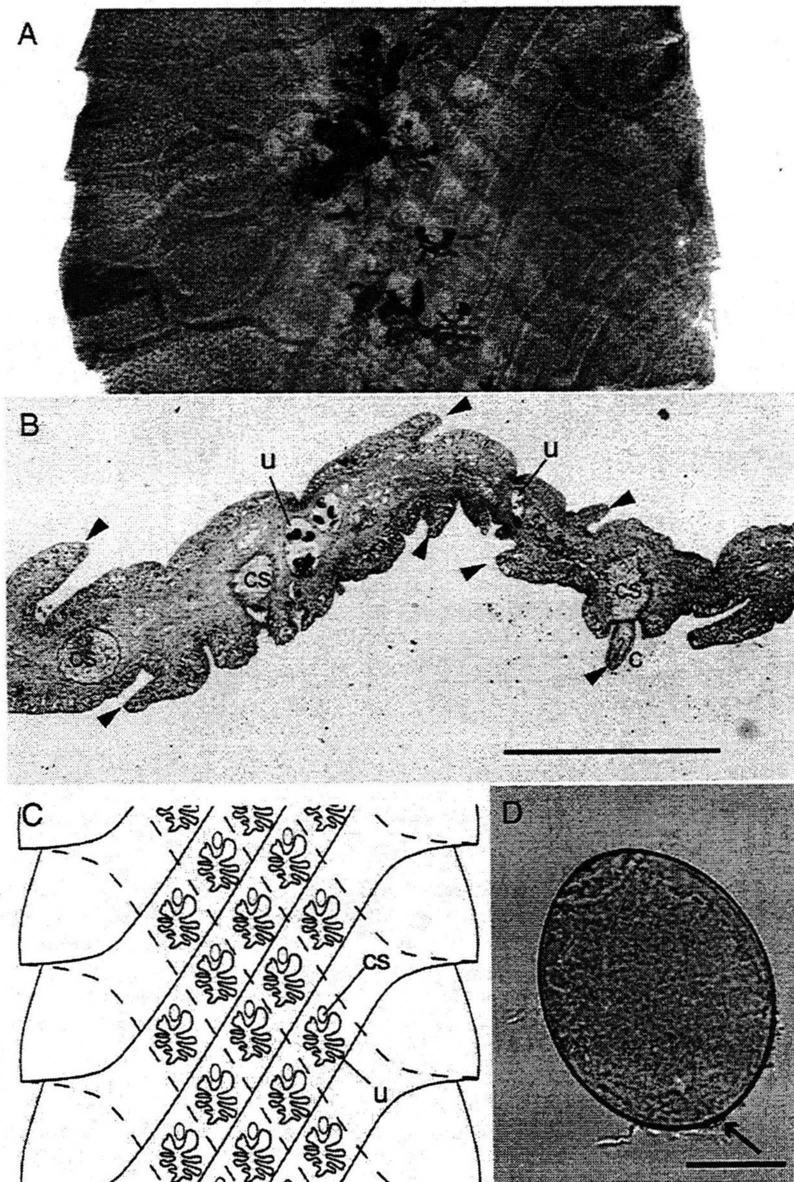

Fig. 1. The strobila and the egg obtained in the present case. (A) Whole-mounted specimen of the strobila with multiple reproductive organs. (B) Transverse section of the strobila stained with hematoxylin and eosin. Arrowheads show the margins. c: cirrus. cs: cirrus sac. u: uterus. Bar = 1 mm. (C) Schematic diagram of the strobila. Solid lines and broken lines show anterior and posterior margins of proglottidsr respectively. (D) The egg with an apical knob. Bar = 20 µm.

of reproductive organs were arranged between the anterior and posterior margins of proglottids (Fig. 1A, C). Some of the uteri contained eggs. SEM observation revealed the genital pores opened only on the ventral surface (data not shown). The eggs had an operculum at one end and an apical knob at the other. The size of the eggs ($n = 20$) were 67.6 (61.8–73.8) × 45.1 (42.4–48.1) µm (Fig. 1D).

The parasite DNA was extracted with DNeasy Blood & Tissue Kit (Qiagen), and the nuclear DNA fragment of 18S ribosomal RNA gene (18S rDNA) was amplified by polymerase chain reaction (PCR) using the universal eukaryotic primers ERIB1 (5′-acctggttgatcctgccag-3′) and ERIB10 (5′-cttccgcaggttcacctacgg-3′)[17]. The mtDNA fragments of cytochrome *c* oxidase subunit I (*cox1*) and cytochrome *b* (*cob*) were also amplified with two sets of primers; Diphyllo-Cox1-F/R (5′-tagactaagtgttttcaaaacacta-3′/5′-atagcatgatgcaaaagg-3′) and Diphyllo-Cob-F/R (5′-tgataggttatttaaactggc-3′/5′-tcaacagttgaaacaacca). All PCRs were performed in 20 µL volumes containing 0.5 units of Ex Taq Hot Start Version (TaKaRa), 0.2 mM of dNTP, 1 × Ex Taq Buffer with a final $MgCl_2$ concentration of 2.0 mM, 15 pmol of each primer and 1.0 µL of genomic DNA. Main thermal reactions were performed as follows; 35 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 60 s (*cox1* and *cob*), and 35 cycles of 95 °C for 1 min, 54 °C for 1 min and 72 °C for 2 min (18S rDNA). A BigDye™ Terminator v1.1 and a 310 DNA sequencer (Applied Biosystems) were used for the direct sequencing of the PCR products.

The partial sequence of 18S rDNA (2134 bp) and the complete sequences of *cob* (1107 bp) and *cox1* (1566 bp) were determined and compared with available sequences in the GenBank database. The 18S rDNA sequence was completely identical with that of *D. nihonkaiense* (AB374225). Both *cob* and *cox1* sequence showed more than 99.5% identities with those of *D. nihonkaiense* (EF420138), whereas the similarities of the *cox1* sequence with *D. dendriticum* (AM412738) and *D. latum* (FM209181) were 93.2% and 92.6%, respectively.

Morphologically, the most notable feature of the strange worm was the slanted anterior and posterior margins of proglottids. Because the margins of proglottids on the ventral and dorsal tegument were not

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parallel to each other, the tapeworm was considered not to have "segments" in a usual meaning. Another feature of the tapeworm was multiple sets of reproductive organs arranged between the anterior and posterior margins of proglottids. Although the worm was thought to be a member of the genus *Diphyllobothrium* or *Diplogonoporus* because of the egg morphology, there was no described species having such a strange strobila. While morphological observation did not identify the species, 18S rDNA, *cox1* and *cob* sequences of the parasite showed high similarity to that of *D. nihonkaiense*. 18S rDNA is rather conservative and unsuitable for interspecific characterization among some *Diphyllobothrium* species such as *D. latum*, *D. dendriticum* and *D. ditremum* [13,14]. On the other hand, *cox1* is more variable and can clearly discriminate those three species [13,14]. Intraspecific mtDNA sequence divergence in *D. nihonkaiense* is relatively high, around 2% in *cox1* and NADH dehydrogenase subunit 3 (*nad3*) [10,11]. The complete *cox1* sequence of the tapeworm obtained in the present study showed 99.7% similarity with *D. nihonkaiense*. Thus, molecular analysis clearly showed that the strange tapeworm was an anomalous *D. nihonkaiense*. Occurrence of abnormal progrottids of *D. nihonkaiense* was intensively examined with 140 specimens preserved in 17 universities and research institutes in Japan, and many types of abnormal forms were recorded [18]. They were divided into 12 major types including that with 2–4 sets of genital organs per segments and/or abnormal segmentation. Koga and Iwata [18] found that the occurrence of abnormal forms of *D. nihonkaiense* was rather common. However, none of the abnormal types in the previous report was similar to the present case. Most of the cases in the previous report consisted of both normal and abnormal segments, whereas the specimen in the present case was composed of only abnormal forms throughout the obtained strobila. The cause and the mechanism of the occurrence of the abnormal proglottids in the diphyllobothriid cestodes are still unknown. Koga and Iwata [18] speculated that the abnormal forms, such as double sets of genitalia in one segment, show the progress of subdivision of the mature progrottids. On the other hand, Anderson [19] obtained abnormally large and slender plerocercoids of *Diphyllobothrium dendriticum* from rainbow trout *Oncorhynchus mykiss* experimentally infected by the intraperitoneal injection of plerocercoids with the scolex removed. The author suggested the scolex or neck region control the growth and development of cestodes and the abnormal development would occur if those parts were removed. The abnormal segmentation in the present case was considered to have occurred not among the mature progrottids but in the scolex or neck region resulting from the failure of the regulation of developmental gene expression in early morphogenetic process, because the abnormal form appeared constantly in the specimen.

Retrospective interview with the patient could not find any history of eating raw or undercooked salmons, except for a grilled rainbow trout farmed in the river in Japan. Rainbow trout has not been reported as the host of *D. nihonkaiense* so far. Besides, extensive fieldwork on masu salmon, *O. masou masou*, in Hokkaido, the north island of Japan, revealed that the juveniles in the river before migrating to the sea were not infected with *D. nihonkaiense*, whereas the adults that returned to the rivers had prevalences ranging from 0 to 50%. It strongly suggests that *O. masou masou* acquires the parasite during their migration to the ocean [20]. It is, therefore, highly unlikely that the grilled rainbow trout was the source of infection. The origin of the strange *D. nihonkaiense* remains unknown. Until recently, *D. nihonkaiense* was considered to distribute only around Japan and Far East Russia. However, clinical cases of *D. nihonkaiense* have been reported in France, Switzerland, Canada and New Zealand in the past 5 years [12,14,15,21,22]. The authors of these reports suspected that the cases were caused by eating Pacific salmons imported from Pacific coast of North America, except for the case of a Czech tourist who ate 5 species of Pacific salmons in Canada [14]. These case reports indicate that the expansion of *D. nihonkaiense* infection resulted from the worldwide transportation of fresh salmons, and a broader geographical distribution of the parasite than previously considered. However, *D. nihonkaiense* has not been confirmed from other than three species of Pacific salmons (*O. masou masou*, *O. gorbuscha*, and *O. keta*) in Japan and Far East Russia so far. There has been no report of *D. nihonkaiense* larvae from any imported/exported Pacific salmons. To clarify the geographical distribution and the host range of *D. nihonkaiense*, more extensive fieldwork on Pacific salmons, especially along the Pacific coast of North America, is needed.

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Genetic polymorphisms of *Echinococcus* tapeworms in China as determined by mitochondrial and nuclear DNA sequences ☆

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ABSTRACT

The genetic polymorphisms of *Echinococcus* spp. in the eastern Tibetan Plateau and the Xinjiang Uyghur Autonomous Region were evaluated by DNA sequencing analyses of genes for mitochondrial cytochrome c oxidase subunit 1 (*cox1*) and nuclear elongation factor-1 alpha (*ef1a*). We collected 68 isolates of *Echinococcus granulosus* sensu stricto (s.s.) from Xinjiang and 113 isolates of *E. granulosus* s. s., 49 isolates of *Echinococcus multilocularis* and 34 isolates of *Echinococcus shiquicus* from the Tibetan Plateau. The results of molecular identification by mitochondrial and nuclear markers were identical, suggesting the infrequency of introgressive hybridization. A considerable intraspecific variation was detected in mitochondrial *cox1* sequences. The parsimonious network of *cox1* haplotypes showed star-like features in *E. granulosus* s. s. and *E. multilocularis*, but a divergent feature in *E. shiquicus*. The *cox1* neutrality indexes computed by Tajima's *D* and Fu's Fs tests showed high negative values in *E. granulosus* s. s. and *E. multilocularis*, indicating significant deviations from neutrality. In contrast, the low positive values of both tests were obtained in *E. shiquicus*. These results suggest the following hypotheses: (i) recent founder effects arose in *E. granulosus* and *E. multilocularis* after introducing particular individuals into the endemic areas by anthropogenic movement or natural migration of host mammals, and (ii) the ancestor of *E. shiquicus* was segregated into the Tibetan Plateau by colonising alpine mammals and its mitochondrial locus has evolved without bottleneck effects.

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1. Introduction

Metacestodes of the dog tapeworm *Echinococcus granulosus* sensu stricto (s.s.) and the fox tapeworm *Echinococcus multilocularis* are highly pathogenic to humans, and cause cystic and alveolar echinococcoses, respectively. Humans become infected through oral ingestion of eggs derived from faeces of canine definitive hosts. Sheep are a main intermediate host for *E. granulosus* s. s., whereas arvicoline rodents serve as intermediate hosts for *E. multilocularis* (Eckert and Deplazes, 2004). Besides the main two species, *Echinococcus equinus*, *Echinococcus ortleppi*, *Echinococcus canadensis*, *Echinococcus felidis*, *Echinococcus shiquicus*, *Echinococcus vogeli* and *Echinococcus oligarthrus* have been regarded as valid by recent phylogenetic studies (Xiao et al., 2005; Nakao et al., 2007; Hüttner et al., 2008). However, the species status of *E. canadensis* is still debatable (Thompson, 2008).

In China, *E. granulosus* s. s. and *E. multilocularis* are widespread in western, northern and central parts of the country (Wang et al., 2008), and hyperendemic foci exist within pastoral areas of the eastern Tibetan Plateau (Schantz et al., 2003; Li et al., 2008) and the Xinjiang Uyghur Autonomous Region (Wang et al., 2001). Both endemic regions are geographically separated by the Kunlum mountains and the Taklamakan Desert. The alpine steppe of the Tibetan Plateau supports human pastoral activity for the raising of yak and sheep. In Xinjiang, nomads and semi-nomads keep

☆ Nucleotide sequence data reported in this paper are available in DDBJ/EMBL/GenBank databases under the Accession Nos. AB491414–AB491471.

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livestock on low-altitude grassland. This type of sheep husbandry system, including the use of pastoral dogs, is essential to maintain the synanthropic cycle of *E. granulosus* s. s. In contrast, the rodent fauna of grassland and the migration of foxes are key factors in establishing the endemic foci of *E. multilocularis* (Giraudoux et al., 2006). Human infections with *E. multilocularis* are extremely frequent in the Tibetan communities of Sichuan province (Craig, 2006), and the role of dogs in the communities is important to infections (Wang et al., 2006). Thus, the life-cycle of *E. multilocularis* is altered to be synanthropic in hyperendemic areas.

Species of *Echinococcus* prevailing in China have been clarified by molecular taxonomic studies using mtDNA markers (McManus et al., 1994; Zhang et al., 1998; Yang et al., 2005; Bart et al., 2006; Xiao et al., 2004, 2005, 2006; Ma et al., 2008; Li et al., 2008). The molecular identification of *Echinococcus* isolates from various origins showed the following host–parasite relationships in China: (i) domestic mammals (sheep, cattle, goats, yaks and dogs) for *E. granulosus* s. s., (ii) domestic mammals (camels, cattle and dogs) for *E. canadensis* (G6 genotype), (iii) wildlife (voles, hares, pikas, red foxes and Tibetan foxes) and domestic mammals (dogs) for *E. multilocularis* and (iv) wildlife (pikas and Tibetan foxes) for *E. shiquicus*. Human infections have been confirmed for all species except *E. shiquicus*. Interestingly, *E. canadensis* G6 has been found only in Xinjiang (Zhang et al., 1998) and *E. shiquicus* seems to be restricted in the Tibetan Plateau (Xiao et al., 2005). All of the epidemiological information provides a basis to consider the natural history of *Echinococcus* in China. Previous studies demonstrated that intraspecific mtDNA variations occurred in *E. granulosus* s. s. (Yang et al., 2005; Bart et al., 2006; Ma et al., 2008), *E. multilocularis* (Yang et al., 2005) and *E. shiquicus* (Xiao et al., 2005). However, the genetic populations of *Echinococcus* spp. in China have never been characterised in an evolutionary context.

In this study, the genetic diversities of *E. granulosus* s. s., *E. multilocularis* and *E. shiquicus* were explored by using mtDNA and nDNA markers. We sampled the isolates of *E. granulosus* s. s. from Xinjiang and the isolates of all three species from the eastern Tibetan Plateau. The main purpose of this study was to evaluate the population genetic structures of the three species. The resultant data and epidemiological information enabled us to suggest evolutionary hypotheses on how the parasites have spread in China.

2. Materials and methods

2.1. Isolates collected

An *Echinococcus* isolate was defined as a unilocular cyst or a separated alveolar cyst from an intermediate host. During the period from 2002 to 2007, the larval isolates of *Echinococcus* spp. from various hosts were collected in the eastern Tibetan Plateau (Qinghai and Sichuan provinces) and the Xinjiang Uyghur Autonomous Region. Table 1 summarises the number and origin of isolates examined in the two localities. For *E. granulosus* s. s., 113 isolates in the highlands and 68 isolates in the lowlands were treated as two separate populations. On the other hand, 49 isolates of *E. multilocularis* and 34 isolates of *E. shiquicus* were obtained only from the highlands. One isolate in the lowlands was identified as *E. canadensis* G6 genotype. In addition, 57 isolates of *E. granulosus* s. s. in Peru (Moro et al., 2009) served as a foreign control for population genetic analyses. The species identification of those isolates was validated by DNA sequencing described below.

2.2. PCR amplification and sequencing

The genomic DNA of each isolate was prepared from ethanol-preserved larval cysts by using a DNeasy blood and tissue kit (Qiagen), and used as a template for PCR. Partial fragments of a mitochondrial gene for cytochrome *c* oxidase subunit 1 (*cox1*) and a nuclear gene for elongation factor-1 alpha (*ef1a*) were amplified by PCR using specific primers reported previously (Nakao et al., 2000; Moro et al., 2009). The PCR mixture was prepared in a 25 µl final volume containing 1 µl template DNA, 200 µM of each dNTP, 0.2 µM of each primer, 0.5 U of Ex-*Taq* polymerase (Takara) and the manufacturer-supplied reaction buffer. Thermal reactions were performed for 35 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 60 s. Amplified DNA fragments were purified with QIAquick spin columns (Qiagen), and sequenced directly with a BigDye terminator cycle sequencing kit (Applied Biosystems). The resultant sequence ladders were read by an ABI PRISM 377 genetic analyzer (Applied Biosystems).

2.3. Data analysis

In each species of *Echinococcus*, multiple alignments in NEXUS format were made manually by editing the plain text files of nucleotide sequences. Amino acid sequences were inferred from the nucleotide sequences by echinoderm mitochondrial genetic code (Nakao et al., 2000; Telford et al., 2000) or standard genetic code. Percentage divergence values of nucleotide sequences were determined using the MEGA4 package (Tamura et al., 2007) using Kimura's two parameter model (Kimura, 1980) with a γ-shaped parameter ($a = 0.5$). The identification of haplotypes and the drawing of their networks were executed by TCS 1.2 software (Clement et al., 2000) using statistical parsimony (Templeton et al., 1992). The network estimation was run at a 95% connection limit.

Population diversity indexes (number of haplotypes, haplotype diversity and nucleotide diversity) were calculated using DnaSP 4.5

Table 1
Number of *Echinococcus* isolates used for this study.

Species and localities	Origins of larval isolates						
	Human	Sheep	Yak	Rodent[a]	Hare[b]	Pika[c]	Total
Echinococcus granulosus							
Qinghai & Sichuan	37	57	19	0	0	0	113
Xinjiang	54	14	0	0	0	0	68
Total	91	71	19	0	0	0	181
Echinococcus multilocularis							
Qinghai & Sichuan	20	0	0	26	1	2	49
Echinococcus shiquicus							
Qinghai & Sichuan	0	0	0	0	0	34	34
Echinococcus canadensis (G6)							
Xinjiang	1	0	0	0	0	0	1

[a] *Microtus fuscus*, *Microtus limnophilus* and *Cricetulus kamensis*.
[b] *Lepus oiostolus*.
[c] *Ochotona curzoniae*.

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software (Rozas et al., 2003). The population genetics package Arlequin 3.1 (Excoffier et al., 2005) was employed to calculate the neutrality indexes of Tajima's *D* (Tajima, 1989) and Fu's Fs (Fu, 1997). The degree of gene flow between two populations was estimated using a pairwise fixation index (Fst) as determined by the Arlequin package. The three geographic populations of *E. granulosus* s. s. from Xinjiang, the eastern Tibetan Plateau and Peru (out of China) were used to compute the Fst values.

3. Results

3.1. Variations in nucleotide sequences

In our targeted regions of *Echinococcus* DNA, deletion or insertion mutations were not observed, even in different species. The total numbers of nucleotides examined were therefore stable in mitochondrial *cox1* (789 sites) and nuclear *ef1a* (656 sites). The *cox1* sequences could be amplified in all of the isolates of *E. granulosus* s. s. ($n = 181$), *E. multilocularis* ($n = 49$), *E. shiquicus* ($n = 34$) and *E. canadensis* G6 ($n = 1$). However, the PCR positive rate of *ef1a* was relatively lower than that of *cox1*, probably due to the low copy number of the nuclear gene. Each *Echinococcus* sp. retained the species-specific nucleotide sequences of *cox1* and *ef1a* (Table 2).

Considerable intraspecific variations were detected only in the mtDNA sequences of *cox1* (Table 1), indicating its primary use for population genetic analyses. Synonymous substitutions exceeded non-synonymous substitutions in the *cox1* sequences of *E. granulosus* s. s. and *E. shiquicus*. Out of all of the point mutations, 24 sites (49.0%) of *E. granulosus* s. s., one site (25.0%) of *E. multilocularis* and 11 sites (64.7%) of *E. shiquicus* were parsimony informative. Relatively to *E. shiquicus*, singleton substitutions were abundant in *E. granulosus* s. s. and *E. multilocularis*. The pairwise divergence of the *cox1* sequences was computed among individual isolates at an intraspecific level. The maximum values of the divergence were 0.9% in *E. granulosus* s. s., 0.3% in *E. multilocularis* and 1.5% in *E. shiquicus*. The autochthonous species *E. shiquicus* appeared to have the most variable mtDNA.

3.2. Haplotype networks

In *E. granulosus* s. s., 43 mtDNA haplotypes were found in 181 isolates from Xinjiang and the eastern Tibetan Plateau (Qinghai and Sichuan). To discern a genealogical relationship among the haplotypes, we constructed a statistical parsimony network. As shown in Fig. 1, each of the two regions possessed geographically specific haplotypes. The network, however, showed a star-like expansion, and one common haplotype (G01) occupied the centre of the network. The numbers of mutational steps between the common haplotype and the others ranged from one to five, and the frequency of the common haplotype was 53.6% in the population. A similar star-like network was observed in the Peruvian population of *E. granulosus* s. s. (Fig. 2A). Five mtDNA haplotypes were detected in the 57 Peruvian isolates (Moro et al., 2009), but the majority of the isolates (93.0%) belonged to the haplotype G01, which was the most common in the Chinese populations. A single-nucleotide variation was identified between members of the five haplotypes, three of which were geographically specific to Peru.

We detected five mtDNA haplotypes in 49 isolates of *E. multilocularis* from the eastern Tibetan Plateau. These were illustrated as a star-like network with one major haplotype (M01), which comprised 89.8% of the isolates examined (Fig. 2B). All variations found in the five haplotypes were single-nucleotide polymorphisms. As opposed to the convergent networks of *E. granulosus* s. s. and *E. multilocularis*, a divergent network was found in *E. shiquicus* (Fig. 2C). Although 10 mtDNA haplotypes were detected in 34 isolates of *E. shiquicus*, major haplotypes were absent in the population. The maximum number of mutational steps was 13 in the network of *E. shiquicus*.

Table 2
Number of nucleotide substitutions in mitochondrial cytochrome *c* oxidase subunit 1 (*cox1*) and nuclear elongation factor-1 alpha (*ef1a*) genes amplified from *Echinococcus* spp. in China.

Species	*cox1* (789)			*ef1a* (656)		
	n	*S*	NS	*n*	*S*	NS
Echinococcus granulosus	181	30	19	122	0	0
Echinococcus multilocularis	49	1	3	38	0	0
Echinococcus shiquicus	34	16	1	30	3	0

Number of the total nucleotide sites examined is shown in parentheses. Abbreviations are number of isolates examined (*n*), synonymous substitutions (*S*) and non-synonymous substitutions (NS).

3.3. Diversity and neutrality indexes

Diversity indexes for *Echinococcus* populations in each locality were calculated using the data set of *cox1* (Table 3). In the Chinese populations of *Echinococcus* spp., both values of haplotype and nucleotide diversities were the highest in *E. shiquicus* but the lowest in *E. multilocularis*. In the case of *E. granulosus* s. s., the Peruvian population showed the lowest values compared with the Chinese populations. The high levels of haplotype diversity were kept in the Chinese populations of *E. granulosus* s. s., but their nucleotide diversity was relatively low because of the richness of single-nucleotide substitutions.

Neutrality indexes calculated by Tajima's *D* and Fu's Fs tests are also shown in Table 3. The highly negative values were recorded in the Chinese populations of *E. granulosus* s. s. and *E. multilocularis*, indicating a significant deviation from neutrality. The Peruvian population of *E. granulosus* s. s. also showed significant negative values. By contrast, relatively low positive values were obtained in the population of *E. shiquicus*, which kept highly polymorphic mtDNA.

3.4. Fixation index for the populations of *E. granulosus* s. s

Using the data set of mtDNA, the values of the pairwise fixation index (Fst) were computed to estimate the degree of gene flow among three geographic populations of *E. granulosus* s. s in China and Peru (Table 4). Since one common haplotype existed predominantly in the three localities, the Fst values between the populations were very small, ranging from 0.036 to 0.009. These low values implied that the populations were not genetically differentiated from one another.

4. Discussion

Echinococcoses caused by *E. granulosus* s. s. and *E. multilocularis* lead to considerable social and economic losses in the endemic communities of the eastern Tibetan Plateau (Budke et al., 2005). Furthermore, *E. shiquicus* has been recently discovered in the plateau (Xiao et al., 2005). Information on the population genetic structures of these sympatric species is necessary to better understand the process of intra- and interspecific gene flows, and may provide a foundation for future epidemiological studies on the transmission dynamics of the parasites. In the present study, mtDNA revealed the basic structures of *Echinococcus* populations in the eastern Tibetan Plateau.

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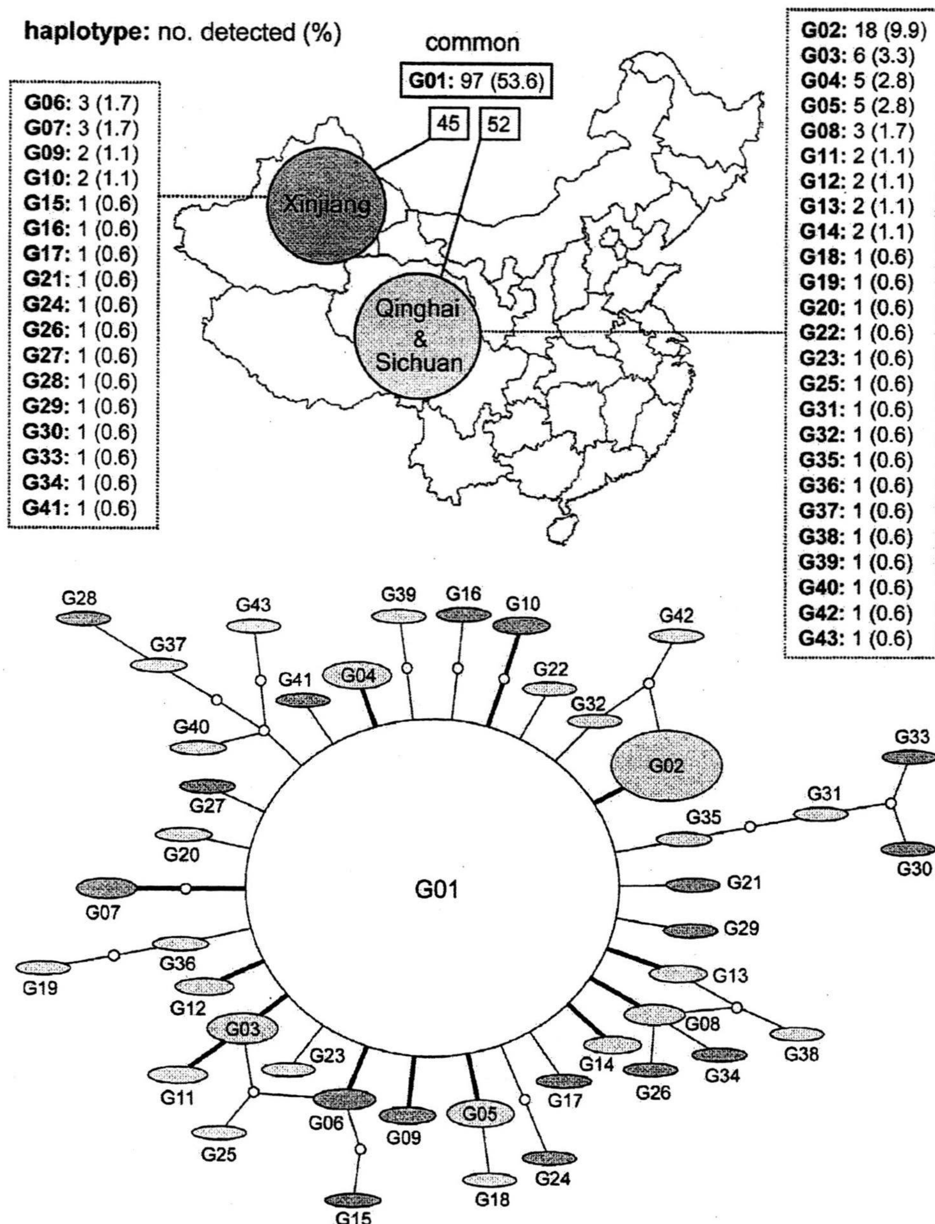

Fig. 1. Frequencies of mitochondrial cytochrome c oxidase subunit 1 (*cox1*) haplotypes in Chinese *Echinococcus granulosus* and their network based on statistical parsimony. In the network, the size of ovals indicates the frequency of the haplotypes. Small circles show hypothetical haplotypes. The haplotypes whose frequencies are more than 1% are connected with bold lines. Dark ovals represent the haplotypes found in Xinjiang, whereas the localities of grey ovals are Qinghai and Sichuan. A white oval shows the common haplotype.

We chose the protein-coding gene *cox1* as a marker for *Echinococcus* mtDNA. In other organisms, the mitochondrial control region has been generally used to infer genealogical relationships. However, the corresponding mtDNA regions of *Echinococcus* spp. contain highly repetitive sequences, which are unsuitable for phylogenetic studies (Nakao et al., 2007). The *cox1* gene of *Echinococcus* spp. has already been shown to be a promising candidate for the classification of intra- and interspecific variants even in the short sequence (366 nucleotide sites) (Bowles et al., 1992). In this study, we determined the relatively long sequence of *cox1* (789 nucleotide sites), and detected a sufficient number of haplotypes to analyse the population genetic structures of each species. The haplotypes are only loosely correlated with the intraspecific genotypes of *E. granulosus s. s.* (G1, G2 and G3) and *E. multilocularis* (M1 and M2) defined by Bowles et al. (1992). The negative selection of the *cox1* for the purging of deleterious mutations has been demonstrated by the codon-based *Z*-test (Tamura et al., 2007) using the corresponding sequences of *E. granulosus s. s.*, *E. multilocularis*, *E. equinus*, *E. ortleppi*, *E. canadensis*, *E. felidis*, *E. vogeli* and *E. oligarthrus* (M. Nakao, unpublished data). The fragments of the nuclear gene *ef1a* were also sequenced in this study, but their species-specific sequences were not polymorphic at an intraspecific level. The results of species identification by the mitochondrial and nuclear markers were identical, suggesting the infrequency of introgressive hybridization among the sympatric species.

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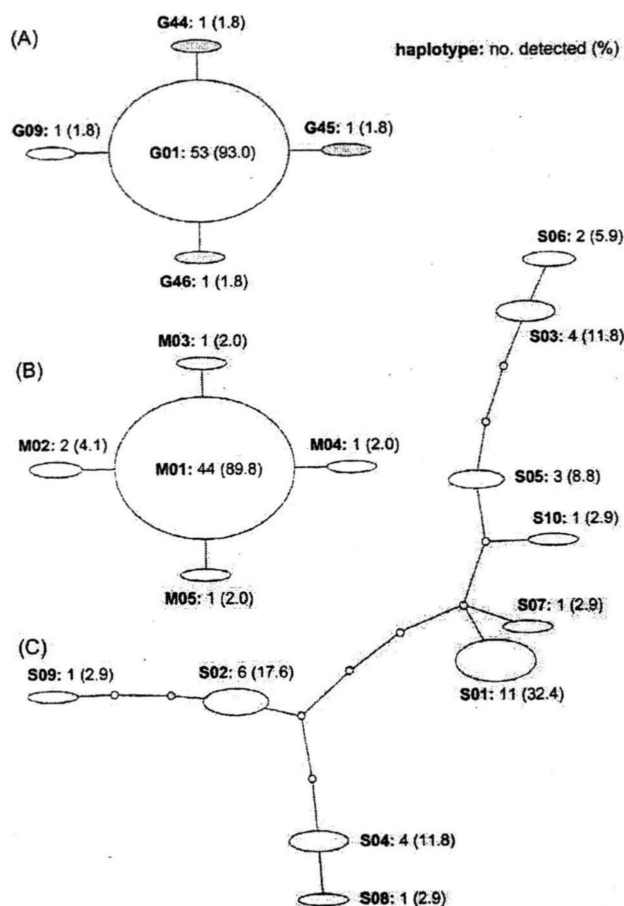

Fig. 2. The statistical parsimony networks of mitochondrial cytochrome *c* oxidase subunit 1 (*cox1*) haplotypes in *Echinococcus* spp. The size of ovals indicates the frequency of the haplotypes. Small circles show hypothetical haplotypes. (A) The Peruvian population of *Echinococcus granulosus* sensu stricto. Grey ovals represent the haplotypes specific to Peru. (B) The Tibetan population of *Echinococcus multilocularis*. (C) The Tibetan population of *Echinococcus shiquicus*.

The *cox1* haplotypes of *E. granulosus* s. s. found in this study did not show an apparent phylogeographic structuring in China. The parsimony network analysis revealed that the haplotypes exhibit a star-like expansion from a main founder haplotype, suggesting that the populations of eastern Tibet and Xinjiang are not fully differentiated from each other. It is noteworthy that the same founder was predominant in the Peruvian population. It seems unlikely that mutations of the founder haplotype are advantageous because the amino acid sequence deduced from the founder is the same as those from other minor haplotypes. The common genetic structure between geographically unrelated populations enables us to speculate that one particular lineage of *E. granulosus* s. s. is widespread globally. The genetic non-differentiation between the local populations is also demonstrated by extreme low values of the fixation index Fst. Furthermore, the significant negative values of the neutrality indexes Tajima's *D* and Fu's Fs suggest that bottleneck events might occur in the recent past. It is most likely that demographic expansions of the parasite occurred after introducing particular individuals into the endemic areas by anthropogenic movements of host mammals (sheep and dogs).

Table 4
Pairwise fixation index (Fst values) between *Echinococcus granulosus* sub-populations calculated from the nucleotide data set of mitochondrial cytochrome *c* oxidase subunit 1 (*cox1*) gene.

	1	2
1. Qinghai & Sichuan		
2. Xinjiang	0.031[a]	
3. Out of China (Peru)	0.036[a]	0.009

[a] Significant *P* values ($P < 0.01$).

It is assumed that *E. granulosus* s. s. was introduced into South America from Europe through livestock importation after the colonial period. The higher values of haplotype and nucleotide diversities in the Chinese populations of *E. granulosus* s. s. suggest that China historically preceded Peru in the time of initial founder introduction. One could speculate that bottleneck events might also occur in the ancestral population of *E. granulosus* s. s. during the colonisation of domestic sheep as an intermediate host. Archaeological and genetic evidence suggest that sheep were domesticated in the Ancient Near East (Pedrosa et al., 2005), but the genealogical survey of Chinese domestic sheep showed the possibility that additional domestication events occurred independently in other regions (Chen et al., 2006). The lack of archaeoparasitological data does not permit us to infer how and when the parasite invaded China. However, the Ancient Near East is one possible candidate for the cradle of *E. granulosus* s. s. The population genetic structures of *E. granulosus* s. s. should be compared in various endemic areas to clarify its ancestral origin and the process of its worldwide dispersal.

Our previous study has already indicated the rarity of mtDNA polymorphism in the Tibetan population of *E. multilocularis* (Xiao et al., 2005). The present study furthermore revealed that a particular *cox1* sequence was positioned as a basal haplotype, suggesting that a founder effect arose in the population. Our recent phylogeographic study on *E. multilocularis* showed that the worldwide iso-

Table 3
Diversity and neutrality indexes for *Echinococcus* populations calculated from the nucleotide data set of mitochondrial cytochrome c oxidase subunit 1 (*cox1*) gene.

Species and localities	Diversity				Neutrality	
	n	Hn	Hd ± S.D.	π ± S.D.	*D*	Fs
Echinococcus granulosus						
Qinghai & Sichuan	113	26	0.760 ± 0.038	0.0017 ± 0.0002	−2.323[a]	−26.023[a]
Xinjiang	68	18	0.562 ± 0.073	0.0015 ± 0.0003	−2.456[a]	−15.762[a]
Total (China)	181	43	0.702 ± 0.038	0.0017 ± 0.0002	−2.536[a]	−61.569[a]
Out of China (Peru)	57	5	0.137 ± 0.062	0.0002 ± 0.0001	−1.849[a]	−5.889[a]
Echinococcus multilocularis						
Qinghai & Sichuan	49	5	0.195 ± 0.075	0.0003 ± 0.0001	−1.765[a]	−4.788[a]
Echinococcus shiquicus						
Qinghai & Sichuan	34	10	0.847 ± 0.040	0.0055 ± 0.0004	0.164	0.258

Abbreviations are number of isolates examined (*n*), number of haplotypes (Hn), haplotype diversity (Hd), nucleotide diversity (π), Tajima's *D* (*D*) and Fu's Fs (Fs).
[a] Significant *P* values ($P < 0.01$).

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lates were classified into European, Asian and North American clades except the Inner Mongolia isolates from the corsac fox *Vulpes corsac* (Nakao et al., 2009). The geographic clustering indicates a possibility that genetic changes occurred in *E. multilocularis* after the fragmentation of the population during the Pleistocene ice ages. The red fox *Vulpes vulpes*, which has a flexible ability to adapt to various environments, extended its distributional range in the Holarctic region, and might play an essential role in introducing *E. multilocularis* into new areas. It seems likely that an epidemic of the parasite in the eastern Tibetan Plateau was initiated by natural migration of red foxes in the recent past.

The Tibetan indigenous species *E. shiquicus* showed a quite different pattern of population genetic structure when compared with *E. granulosus* s. s. and *E. multilocularis*. Statistical neutrality tests and haplotype network analyses suggest a possibility that the mitochondrial locus of *E. shiquicus* has evolved without bottleneck effects. Our previous report clarified that *E. shiquicus* utilises the Tibetan fox *Vulpes ferrilata* as a definitive host and the plateau pika *Ochotona curzoniae* as an intermediate host (Xiao et al., 2005). Both the autochthonous mammals are adapted to the high altitude steppe but do not survive in lowlands. We can therefore consider that *E. shiquicus* has been segregated in the plateau since the parasite's ancestor colonised the alpine mammals. The lasting geographic segregation seems to be a cause for extraordinary richness of polymorphism in Tibetan *E. shiquicus*.

Diploid organisms having a mixed sexual and asexual reproduction system show different patterns from theoretical population genetic models (Prugnolle et al., 2005). The adult tapeworms of *Echinococcus* are hermaphroditic, and self-fertilisation mainly occurs in the small intestine of canine definitive hosts (Haag et al., 1999). The larvae furthermore proliferate asexually in the viscera of intermediate hosts, and the clonal offspring develop into adults in a definitive host. The biphasic reproduction of *Echinococcus* spp. may strongly affect their population genetic structures and promote a very low genetic variability of nuclear loci. In this study we used a haploid maternally inherited mtDNA marker to examine the population genetic structure of *Echinococcus* because of the lack of appropriate nuclear markers. A panel of single-locus nuclear markers is required for further population genetic studies to elucidate the evolutionary backgrounds of *Echinococcus* worldwide.

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Evidence of hybridization between *Taenia saginata* and *Taenia asiatica*

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Hybridization

ABSTRACT

There has long been a debate as to the specific status of the cestode *Taenia asiatica*, with some people regarding it as a distinct species and some preferring to recognize it as a strain of *Taenia saginata*. The balance of current opinion seems to be that *T. asiatica* is a distinct species. In this study we performed an allelic analysis to explore the possibility of gene exchange between these closely related taxa. In total, 38 taeniid tapeworms were collected from humans living in many localities including Kanchanaburi Province, Thailand where the two species are sympatric. A mitochondrial DNA (mtDNA)-based multiplex PCR tentatively identified those parasites as *T. asiatica* ($n=20$) and *T. saginata* ($n=18$). Phylogenetic analyses of a mitochondrial cytochrome *c* oxidase subunit 1 (*cox1*) gene and two nuclear loci, for elongation factor-1 alpha (*ef1*) and ezrin-radixin-moesin (ERM)-like protein (*elp*), assigned all except two individual parasites to the species indicated by multiplex PCR. The two exceptional individuals, from Kanchanaburi Province, showed a discrepancy between the mtDNA and nuclear DNA phylogenies. In spite of their possession of sequences typical of the *T. saginata cox1* gene, both were homozygous at the *elp* locus for one of the alleles found in *T. asiatica*. At the *ef1* locus, one individual was homozygous for the allele found at high frequency in *T. asiatica* while the other was homozygous for the major allele in *T. saginata*. These findings are evidence of occasional hybridization between the two species, although the possibility of retention of ancestral polymorphism cannot be excluded.

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1. Introduction

The family Taeniidae consists of only two genera, *Taenia* and *Echinococcus*. These distinctive tapeworms mature in carnivorous mammals worldwide and several species occur in humans, sometimes causing severe disease. There have been many studies on the morphology and genetics of members of these genera, yet there is still debate about the number of species in each genus and the best means of distinguishing them. In *Echinococcus*, molecular studies based largely on mitochondrial but partially on nuclear genes support the recognition of several distinct species [1–6]. Similar studies on members of *Taenia* [7] have also used mitochondrial sequences to aid recognition of species. Under the biological species concept, distinct species are not expected to exchange genes, or to do so very rarely [8]. Evidence for hybridization is often found by study of nuclear loci and comparison of these with mitochondrial data. Introgression (the infiltration of genes from the gene pool of one species into that of another) can be inferred from the finding of mitochondrial sequences typical of one species in an organism with the nuclear alleles of another. Alternatively, hybridization can be demonstrated by the presence of nuclear alleles in a single individual that are typical of more than one species. Evidence of gene exchange between species of *Echinococcus* has been noted [9,10], thus potentially rekindling the debate about species boundaries in this genus. No such study has explored the question of gene exchange between species in the genus *Taenia*. In this paper, we provide the first evidence that this can occur.

Three human *Taenia* species are found in the Asia-Pacific region: *Taenia solium* (pork tapeworm), *Taenia saginata* (beef tapeworm) and *Taenia asiatica* [11]. The larval stages of these *Taenia* species have been identified as *Cysticercus cellulosae*, *Cysticercus bovis* and *Cysticercus viscerotropica*, respectively [12]. Humans may harbor adult worms after consuming raw or under-cooked pork, beef or viscera of swine, respectively, contaminated with metacestodes of these species. It is important to discriminate among these three taxa, since ingestion of *T. solium* eggs by humans may result in neurocysticercosis, a serious public health problem in many areas worldwide [11,13]. *T. saginata* and *T. asiatica* are morphologically very similar, as are their mitochondrial DNA sequences [14]. On the other hand, the two taxa clearly differ in biological features including host specificity and organotropism [12]. Mitochondrial DNA (mtDNA) sequence data are

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frequently used to distinguish between these species, but nuclear gene markers have not been used previously. In this study, genotyping at two nuclear loci was carried out to explore the possibility of gene exchange between *T. saginata* and *T. asiatica* in a locality where they are sympatric.

2. Materials and methods

2.1. Parasite samples

Adult tapeworms, which were morphologically similar to *T. saginata*, were collected from humans. In the end, 38 samples from 11 nations (Brazil, Ecuador, Ethiopia, Japan, South Korea, Philippines, China, Taiwan, Cambodia, Thailand and Indonesia) were used in this study. Of these, 15 samples were collected from small villages in Kanchanaburi Province, Thailand. These areas are unique, since the three species of human *Taenia* were identified as sympatrically endemic during surveys in 2002–2005 [16].

2.2. DNA preparation

Genomic DNA was individually extracted from mature or immature proglottids using a QIAamp DNA Mini Kit or a DNeasy tissue kit (QIAGEN, Germany) in accordance with the manufacturer's instructions, and then used as a template for polymerase chain reaction (PCR).

2.3. Multiplex PCR for Taenia species identification

Multiplex PCR based on the mitochondrial cytochrome *c* oxidase subunit 1 (*cox1*) gene is an easy method for identification of human taeniid cestodes [16–18]. Samples were first screened by this method for the tentative identification of species. According to the results, the code "Tasi" (*T. asiatica)* or "Tsag" (*T. saginata*) was added to the sample ID. It is important to note that this code refers to the identification from the mitochondrial genome.

2.4. DNA sequencing and data analysis

Multiplex PCR yields products of taxon-specific lengths that can be visualized in a gel. For finer genetic discrimination, the complete sequence of the *cox1* gene was obtained for each individual. Partial sequences of two nuclear genes, elongation factor-1-alpha (*ef1*) and ezrin/radixin/moesin-like protein (*elp*), were also obtained. Primer pairs listed in Table 1 were used for the PCR amplification and sequencing of those genes.

PCR was carried out in 15 µl reaction mixtures containing 1 µl template, 200 µM of each dNTP, 0.2 µM of each primer, 0.3 U of *Ex Taq* polymerase (TaKaRa, Japan) and the manufacturer-supplied reaction buffer. Thermal cycling was performed for 35 cycles of denaturation (94°C for 30 s), annealing (66°C: *cox1*, 60°C: *ef1*, 65°C: *elp*, for 30 s), and extension (72°C for 80–90 s). The PCR products were purified using MinElute PCR Purification Kits (QIAGEN). Direct sequencing was performed with a Dye Terminator Cycle Sequencing Kit and an ABI PRISM 3100 Generic Analyzer (Applied Biosystems, USA). At least two independent PCR products were used for sequencing. Samples that could not be directly sequenced were subjected to cloning using a TOPO TA Cloning Kit (invitrogen, USA), and more than ten clones were sequenced per sample.

DNA sequences were aligned using the CLUSTAL W computer program [19]. Phylogenetic trees were constructed by the neighbor-joining (NJ) method [20] using the MEGA4.0 computer program [21]. Evolutionary distances were computed using the Maximum Composite Likelihood Method [22]. Each of the phylogenetic trees was evaluated using a bootstrap test based on 1000 resamplings [23]. Sequences of *Taenia solium* from Kanchanaburi Province were used as outgroups (AB066487 for *cox1*, AB505027 for *ef1* and AB505025 for *elp*) to indicate the location of the root of the ingroup. For presentation purposes, the long branch leading to the outgroup is not shown for any tree.

Table 1
Primer pairs used for PCR.

Target genes	Primer names	Sequences (5′ – 3′)
cox1 (mt DNA)	Tsag_cox1/F	GAGGAAATTGTGAAGTTACTGCTA
	Tsag_cox1/R	ATGATGCAAAAGGCAAATAAACCT
ef1 (nuclear DNA)	Tae_ef1/F4	TGTGGTGGAATCGATAAAAGG
	Tae_ef1/R4	TCGATCTCATGTCACGAACG
elp (nuclear DNA)	Tsag_elp/F	CGTATGGAGAATGAACAGAAACTG
	Tsag_elp/R	CTGTGCATCGTGTTTCTCACGCAT

cox1: cytochrome *c* oxidase subunit 1.
ef1: elongation factor-1-alpha.
elp: ezrin/radixin/moesin-like protein.

3. Results

The mtDNA-based multiplex PCR tentatively assigned our samples to *T. asiatica* ($n=20$) or *T. saginata* ($n=18$). The phylogenetic tree inferred from complete mitochondrial *cox1* gene sequences (1620 bp) clearly identified two main and rather uniform clusters, agreeing with the results of the multiplex PCR (Fig. 1a). Although pairwise differences between "Tasi" and "Tsag" occurred at approximately 70 sites in the *cox1* gene, variations within each cluster were very small. In the case of the nuclear genes, introns were included in the fragments sequenced. The *ef1* gene fragment was 1095–1096 bp in length (60–61 bp introns and 1035 bp exons) and the *elp* gene sequences consisted of 1162–1164 bp (902–904 bp intron and 260 bp exons).

At the *ef1* locus, we found three alleles (*ef1A*, *ef1B* and *ef1C* – Fig. 1b). Two of these alleles were identified in "Tasi" (*ef1A*, *ef1B*), differing from each other at only a single site in an exon. Most of the samples were homozygous at this locus, but TasiA190 and TasiA210 were heterozygous. We found no variation in "Tsag" sequences (*ef1C*), with the exception of sample TsagA199, which was homozygous for an allele (*ef1A*) found at high frequency in "Tasi". The *ef1C* sequence differed from the other two alleles at 6–8 sites. Four alleles were found at the *elp* locus (*elpA*, *elpB*, *elpC* and *elpD* – Fig. 1c). The first two of these occurred in "Tasi" (*elpA*, *elpB*) and three in "Tsag" (*elpA*, *elpC*, *elpD*). Allele *elpA* thus occurred in both "Tasi" and "Tsag". Alleles *elpA* and *elpB* differed at 1–2 sites, as did *elpC* and *elpD*. Differences between these two pairs of alleles occurred at 6–7 sites. Samples TasiT010 and TsagA201 were heterozygous at this locus. Samples TsagA199 and TsagT017 were homozygous for *elpA*, the major allele found in "Tasi" (Fig. 1c).

For comparison, *ef1* and *elp* genes of *T. solium* both differ from their orthologs in *T. asiatica* and *T. saginata* at 47–50 sites (data not shown). This highlights the close relationship between the last two taxa. The mtDNA classification of all samples and their genotypes at the two nuclear loci are summarized in Table 2.

Nucleotide sequences from *Taenia* species in this study have been deposited into DDBJ/EMBL/GenBank databases under accession numbers AB465211–AB465248 for the *cox1* gene, AB462851–AB462890 for the *ef1* gene and AB462811–AB462850 for the *elp* gene.

4. Discussion

We examined the nucleotide sequences of one mitochondrial gene (*cox1*) and of alleles at two nuclear loci (*ef1* and *elp*) from taeniid worms, which had been tentatively identified as *T. asiatica* or *T. saginata* using multiplex PCR. Phylogenetic analyses of a mitochondrial gene and

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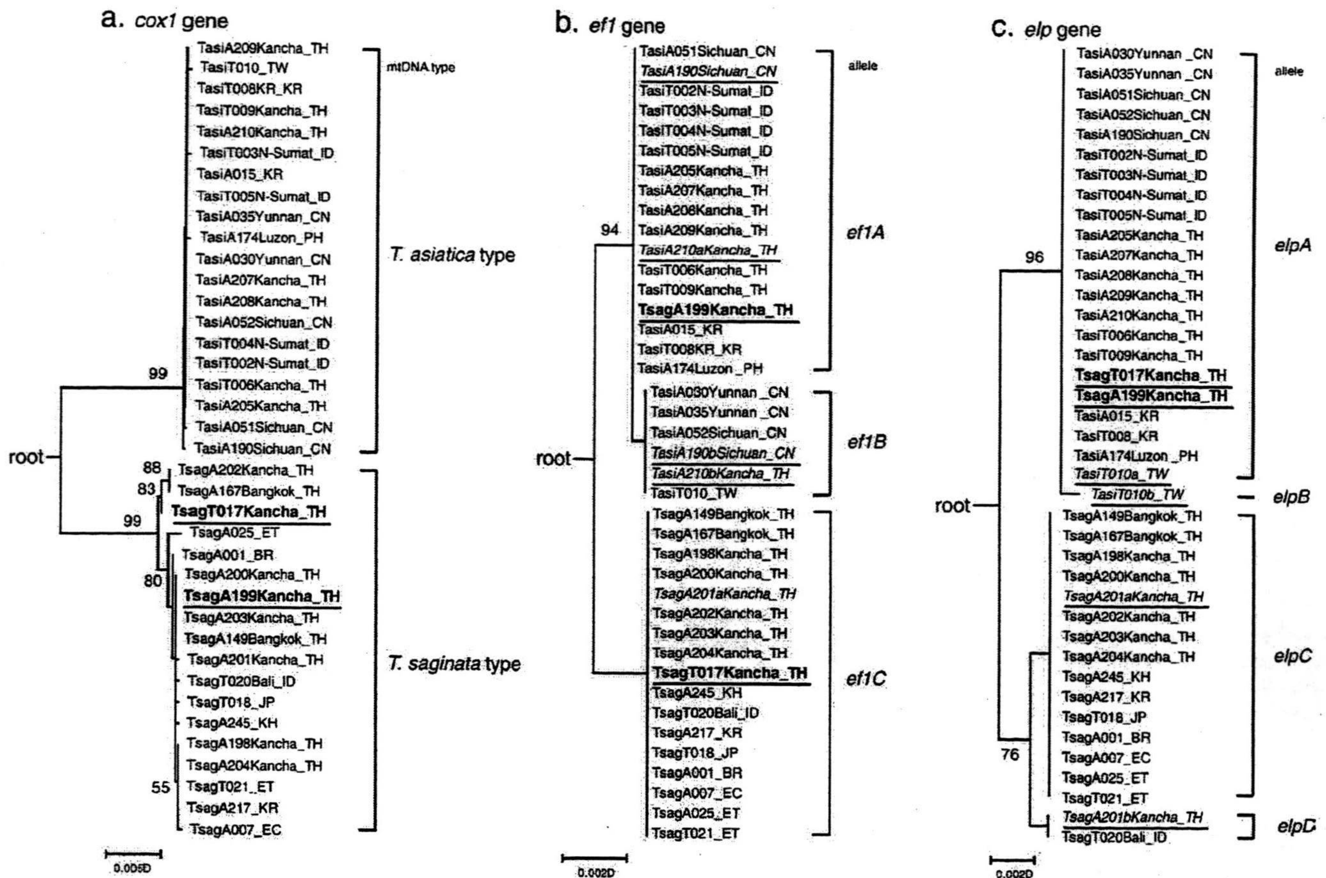

Fig. 1. Neighbor-joining phylogenetic trees of the mitochondrial cytochrome *c* oxidase subunit 1 gene (1a: *cox1*), nuclear genes for elongation factor-1-alpha (1b: *ef1*), and ezrin/radixin/moesin-like protein (1c: *elp*). Samples in italic type represent heterozygotes that displayed two alleles. Samples in bold type showed contradictions in the phylogeny between the mitochondrial gene and one or both of the nuclear genes. Numbers on the nodes represent bootstrap values. Each scale bar represents the evolutionary distances. The number after the species code (e.g. A030 or T002) identifies the sample ID used in the Asahikawa Medical College or Tottori University. Each sample code is followed by a locality name (absent from some) and country name (abbreviated). Abbreviations of country names are as follow: BR, Brazil; CN, China; EC, Ecuador; ET, Ethiopia; ID, Indonesia; JP, Japan; KH, Cambodia; KR, South Korea; PH, Philippines; TH, Thailand; TW, Taiwan. See the text for abbreviations of mitochondrial types and alleles.

two nuclear genes yielded trees consisting of two rather uniform clusters in each case. We regard the two clusters as corresponding to *T. asiatica* and *T. saginata*. The considerable difference between the mitochondrial lineages indicates a long period of separation between *T. asiatica* and *T. saginata*. Nuclear alleles *ef1A*, *ef1B*, *elpA* and *elpB* all occurred in *T. asiatica* even where it was not sympatric with *T. saginata*. We therefore regard these alleles as originating with that species. Similarly, *ef1C*, *elpC* and *elpD* were found in individuals of *T. saginata* from parts of the world where *T. asiatica* is not known. Regardless of the species identified, most individuals are homozygous at the nuclear loci. This is to be expected because taeniids are primarily self-fertilizers [24,25], a mating system that will lead to increase homozygosity. Some outcrossing has been demonstrated in *Echinococcus* species and should partially counter the trend towards increasing homozygosity [9]. The presence of a few heterozygous individuals in our study suggests that outcrossing also occurs in *Taenia* species. As shown in Table 2, these individuals are the samples TasiA210 and TasiA190 (heterozygous at *ef1*), TasiT010 (heterozygous at *elp*) and TsagA201 (heterozygous at *elp*).

Table 2
Samples used, their geographical origins and genotypes[a].

Samples	mtDNA type	Genotype at *ef1* locus	Genotype at *elp* locus
14 samples[b]	*T. asiatica* type	*ef1A*/*ef1A*	*elpA*/*elpA*
3 samples[b]		*ef1B*/*ef1B*	*elpA*/*elpA*
TasiA190 Sichuan, China		*ef1A*/*ef1B*	*elpA*/*elpA*
TasiA210 Kanchanaburi, Thailand		*ef1A*/*ef1B*	*elpA*/*elpA*
TasiT010 Taiwan		*ef1B*/*ef1B*	*elpA*/*elpB*
14 samples[b]	*T. saginata* type	*ef1C*/*ef1C*	*elpC*/*elpC*
TsagT020 Bali, Indonesia		*ef1C*/*ef1C*	*elpD*/*elpD*
TsagA201 Kanchanaburi, Thailand		*ef1C*/*ef1C*	*elpC*/*elpD*
TsagT017 Kanchanaburi, Thailand		*ef1C*/*ef1C*	*elpA*/*elpA*
TsagA199 Kanchanaburi, Thailand		*ef1A*/*ef1A*	*elpA*/*elpA*

[a] See the text for abbreviations of mitochondrial haplotypes and alleles. The number after the species code (e.g. A190 or T010) identifies the sample ID used in the Asahikawa Medical College or Tottori University.
[b] See Fig. 1 for further details of geographical origins of samples.

Two individuals with mitochondrial genomes of the *T. saginata* type possessed at least some alleles typical of *T. asiatica*, suggesting a hybrid origin. TsagA199 has alleles typical of *T. asiatica* at both nuclear loci and TsagT017 displayed such allele at one locus (Table 2). Given the genotypes we observed in our other samples, neither of these individuals could have been an F1 hybrid because they were homozygous at both nuclear loci. Assuming descent from a hybrid ancestor, the observed genotypes could have arisen in two different ways. The presence of a mitochondrial genome of one species in the nuclear environment of another suggests mitochondrial introgression – past hybridization followed by backcrossing into the

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paternal species and eventual dilution and loss of alleles inherited from the maternal species. It is possible that TsagA199 is a case of mitochondrial introgression. If so, additional nuclear loci in this individual should prove to be from *T. asiatica*. Alternatively, genotypes seen in both TsagA199 and TsagT017 could have arisen from a hybrid ancestor by selfing. The F1 hybrid would have been heterozygous at both loci. Its haploid gametes, however, following self-fertilization, could produce zygotes with the observed genotypes, each with a frequency of one-sixteenth in the next generation (F2). Further generations of selfing would eventually produce worms fixed at every locus for an allele from one parent species or the other. Testing between these two scenarios will simply require genotyping of additional nuclear loci in "pure" *T. saginata* and *T. asiatica* and in worms of supposed hybrid origin. The first scenario (mitochondrial introgression) requires many generations of backcrossing, unlikely in a taxon that predominantly self-fertilizes. The second scenario is therefore the more plausible.

In many animal taxa, hybrids are sterile. That is not the case for at least one of the worms studied here. When severe combined immunodeficiency (SCID) mice were infected with the eggs from TsagT017, mature cysticerci developed [26], demonstrating that the eggs had been fertilized and were viable. In addition, two of the cysticerci were homozygous at the *elp* locus and also at the cathepsin L-like cysteine peptidase locus, with each possessing the major allele found in *T. asiatica* [26]. In the second scenario above, the likelihood of a single egg from the F1 hybrid (F2) having such a genotype is also one-sixteenth. Thus, the hybrid-derived offspring observed in this study may not be from the F2 generation but from later generations.

Since hybrid-derived worms have been found only in Kanchanaburi Province, Thailand, where the two species are sympatrically endemic now, it is highly likely that hybridization between *T. asiatica* and *T. saginata* is an event in progress. However, it may not be very common: we observed hybrid-derived offspring in 13.3% of samples (2 cases/15 samples collected from Kanchanaburi). The difference of intermediate hosts utilized by *T. asiatica* and *T. saginata* may reduce the opportunity of a simultaneous infection with these two species. In fact, we have never found a mixed infection with *T. asiatica* and *T. saginata* in Kanchanaburi, although *T. asiatica* and *T. solium* were simultaneously found in humans because pig is the common intermediate host for both parasites [16].

Although we propose occasional crossing between *T. asiatica* and *T. saginata* as an explanation for our observations, it is also possible that ancestral polymorphism has been retained in what are actually two reproductively isolated species [9]. We argue that this is unlikely because self-fertilization by taeniid tapeworms will lead to rapid loss of some alleles and fixation of others. In addition, sequences of *ef1A* and *elpA* in "TsagA199" or "TsagT017" were identical with those in "Tasi", in introns as well as exons. Complete identity, especially in introns, would not be expected if there had been no gene exchange for long periods of time. Nevertheless, the possibility of retention of ancestral polymorphism cannot be completely dismissed by the present data.

When proglottids or eggs of taeniid cestodes are detected in human feces, the accurate identification of species is now dependent on specific PCR or the sequencing analysis of mtDNA [17,18,27]. The species of intermediate host is then inferred from the taeniid species identified. The occurrence of gene exchange between *T. saginata* and *T. asiatica* indicates that the intermediate host inferred from analysis of the mtDNA may not always be the correct one. In addition, the intermediate host cannot be identified from analysis of nuclear genes such as *ef1* and *elp* alone. Unless the gene or genes that regulate host specificity can be identified, the intermediate host should not be deduced based solely on DNA analysis in sympatric endemic areas. The inability to identify intermediate hosts accurately continues to be a significant epidemiological problem. It is necessary now to examine the genotypes of cysticerci from intermediate hosts (cattle and pigs) in sympatric endemic areas.

The results of this study strongly suggest that reproductive isolation is still incomplete between *T. saginata* and *T. asiatica*, and that hybrid breakdown does not occur. Although *T. asiatica* is still able to hybridize with *T. saginata*, each taxon has its own biological identity [12,15]. Biomedical researchers need more pragmatic approaches that can be understood by non-specialists [25]. At the very least, medical or veterinary researchers should not equate *T. asiatica* with *T. saginata*. Further population genetic studies are necessary to better understand the close relationship between these species. In particular, the finding of F1 hybrids is required to exclude the possibility of ancestral polymorphism.

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Inter- and intra-specific characterization of tapeworms of the genus *Diphyllobothrium* (Cestoda: Diphyllobothriidea) from Switzerland, using nuclear and mitochondrial DNA targets

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ABSTRACT

Human diphyllobothriosis is caused by at least 14 species of cestodes belonging to the genus *Diphyllobothrium*. Molecular analysis by sequencing of nuclear and mitochondrial targets identifies some species at inter- and intra-specific level, and helps to reconstruct their phylogenetic relationships. Nevertheless, the suitability of further molecular targets deserves to be widened, and the comparison of samples of different geographical origin could allow their intra-specific characterization, which could also be useful for epidemiological purposes. In this study, we investigated inter- and intra-specific variability among tapeworms of the genus *Diphyllobothrium*, with focus on *Diphyllobothrium latum*, originated from Switzerland. Samples were analyzed by comparing the sequences of two nuclear and two mitochondrial DNA targets. We analyzed 27 samples belonging to 4 species (*D. latum*, *Diphyllobothrium nihonkaiense*, *Diphyllobothrium dendriticum* and *Diphyllobothrium ditremum*), 15 of which isolated from clinical cases (adults and eggs), 2 from wild canines, and 2 from fish of Swiss lakes (plerocercoid larvae); 8 samples of homologous species from other geographic origins were also sequenced and compared with the Swiss ones. Sequences of partial small subunit ribosomal RNA (18S rRNA) gene and partial internal transcribed spacers 1 and 2 (ITS1-2) were not useful even in inter-specific identification, whereas sequences of complete cytochrome *c* oxidase subunit 1 (*cox1*) and cytochrome b (*cob*) genes allowed us to assess inter- and intra-specific variations among the samples. *Cox1* and *cob* could differentiate 3 and 5 haplotypes within the species *D. latum*. The results are discussed in the light of the anamneses provided by part of the patients.

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1. Introduction

Human diphyllobothriosis is a fish-borne zoonosis distributed world-wide and it is transmitted by cestodes belonging to the genus *Diphyllobothrium*. The life cycle of these tapeworms involves two intermediate hosts (zooplankton and some marine and freshwater fish species), and piscivorous mammals and birds as definitive hosts. As many as 14 of the 50 species of *Diphyllobothrium* so far described are known to infect humans [1,2]. *Diphyllobothrium latum* and *Diphyllobothrium nihonkaiense* being the most frequent ones.

Molecular (and, to a lesser extent, biochemical) tools represent the most reliable methods for species identification, because of the huge variability of the morphological characters among *Dipyhllobothrium* taxa. During the last decade, the sequences of the small subunit ribosomal RNA (18S rRNA) gene [3–5], the internal transcribed spacer (ITS) regions [6,7], the NADH dehydrogenase subunit 3 [8] and the cytochrome *c* oxidase subunit 1 (*cox1*) genes [9] were used to assess the phylogenetic relationships of *Diphyllobothrium* species, but these have not yet been adequately defined. Genetic analyses of clinical isolates of *Diplogonoporus* tapeworms recently raised a question about the validity of this genus, which consistently clustered with *Diphyllobothrium stemmacephalum* [10]. These studies, as well as the recent sequencing of complete mitochondrial genomes of *D. latum* and *D. nihonkaiense* [11–13], indicate that mitochondrial targets appear to be more discriminative than nuclear ones, especially at intra-specific level.

To our knowledge, very few studies have been carried out to investigate the genetic variation within a *Diphyllobothrium* species, by sampling and comparing sequences obtained from a number of samples originated from the same region [14]. Nevertheless, such a population analysis could be of primary importance in the epidemiological survey of human parasites. The intra-specific characterization of adults and eggs

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isolated from patients could link the infections to their sources (fish), thus allowing a better control of the disease, which recently showed a resurgence in some well-developed countries, especially in Swiss, Italian and French sub-alpine areas [15], Japan and Far East Russia [16].

The major aim of this study was to identify and discriminate samples of *Diphyllobothrium* spp. originated from Switzerland, by sequencing different nuclear and mitochondrial DNA targets, and to compare them with homologous data of samples collected abroad, to investigate the existence of geographical relationships. The sequences of the 18S rRNA gene, ITS1 and 2 regions, *cox1* and cytochrome *b* (*cob*) genes were analysed by constructing phylogenetic trees, to assess the degrees of discrimination at inter- and intra-specific level.

2. Materials and methods

2.1. Samples

Nineteen samples of *Diphyllobothrium* spp. originated from Switzerland and 8 samples of different human pathogen species originated from other countries were selected for a genetic comparison (Table 1).

Swiss samples consisted of 14 segments of adult tapeworms and 1 faecal sample containing eggs (all from humans), 1 segment from fox (*Vulpes vulpes*; wild cycle), 1 segment from domestic dog, and 2 plerocercoid larvae from perch (*Perca fluviatilis*). Part of these specimens had been already studied on the basis of partial 18S rRNA and partial *cox1* genes sequences [17,18].

Samples identified abroad consisted of adults (1 from an Italian and 2 from Finnish patients) and plerocercoids (1 from Lake Como, Italy and 1 from Lake Peipsi, Estonia) of *D. latum*, as well as plerocercoids of *Diphyllobothrium dendriticum* (1 from Lake Peipsi) and *Diphyllobothrium ditremum* (1 from Finland and 1 from Scotland).

2.2. Molecular analysis

DNA from adults, plerocercoids and eggs was extracted following the Tissue Protocol of the QIAamp DNA Minikit (Qiagen, Germany). Polymerase chain reaction (PCR) was performed following the protocol of Taq PCR Master Mix Kit (Qiagen, Germany), with primers specifically designed for the amplification of the 18S rRNA gene [3], the ITS1 and ITS2 regions, including the 5.8S rRNA gene [19], *cox1* (F2Dnihcox: 5′-TAT CAA ATT AAG TTA AGT AGA CTA-3′ and R2Dnihcox: 5′-CAA AAG ACT GAA TAT TAA ATT CA-3′) and *cob* (F2Dnihcob: 5′-GTT TTA CTG ATA GGT TAT TTA AAC-3′ and R2Dnihcob: 5′-CAA TTT AAA AAA CGA GTT AAA GAT-3′). DNA was tested in a 50 µl reaction volume with final concentration of 1.5 mM of magnesium chloride, 200 µM of each dNTP, 0.3 µM of each primer and 2.5 units of Taq DNA Polymerase. The amplification cycles of 18S rRNA and ITS regions were carried out according to the literature [3,19]. As to *cox1* and *cob*, 35 cycles of amplification (denaturation at 94 °C for 15 s, annealing at 56 °C for 30 s and extension at 72 °C for 1 min) were performed after initial denaturation at 94 °C for 1 min, and followed by terminal extension at 72 °C for 4 min. Amplicons were detected by 0.8% agarose gel electrophoresis containing ethidium bromide for DNA staining. PCR products purified by NucleoSpin® Extract II kit (Macherey-Nagel, Germany) were used as templates for direct sequencing. Sequencing was performed with the BigDye® Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems, USA), and ABI PRISM® 310 Genetic Analyzer (Perkin Elmer, USA).

Sequences were visualised by electropherograms and corrected both manually and using the software EditSeq™ (DNASTAR Inc.). Pairwise and multiple alignments were performed by CLUSTAL® W method [20], with the software MegAlign™ (DNASTAR Inc.). Phylogenetic trees [Minimum Evolution (ME) and Neighbor-Joining (NJ) methods; Kimura-2 parameters; bootstrap test for 1000 replicates] and genetic distances (within group and between groups average calculations; Kimura-2 parameters) were obtained with MEGA version 4.0 [21]. Reference sequences were selected from public available data banks (EMBL-EBI Databank and GenBank). References for the 18S rRNA tree were DQ925309 (plerocercoid from *Gymnocephalus cernuus*, Russia), DQ316796 (adult from Brazilian patient) for *D. latum*; AB374225 (adult from Japan) for *D. nihonkaiense*; AF124459 for *D. stemmacephalum*; *Spirometra erinaceieuropaei* (D64072) was used as outgroup. References for the ITS1-ITS2 tree were DQ768176 (plerocercoid from Lake Brienz, Switzerland) for *D. latum*; AB288369 (adult from Japanese patient) for *D. nihonkaiense*; DQ386121 (plerocercoid from *Coregonus lavaretus*, Scotland) for *D. dendriticum*; DQ768179 (plerocercoid from *Salvelinus alpinus*, Norway) for *D. ditremum*; and DQ768181 for *D. stemmacephalum*. References for

Table 1
Data on the samples analyzed in this study. P: proglottids; E: eggs; L: plerocercoid larva.

ID	Host	Species (parasite)	Origin	Year	Ref
1-P	Human	*D. latum*	Geneva, Switzerland	2004	[17]
2-P	Human	*D. latum*	Geneva, Switzerland	2005	[17]
3-P	Human	*D. latum*	Geneva, Switzerland	2006	[17]
4-P	Human	*D. latum*	St Gallen, Switzerland	2006	This study
5-P	Human	*D. latum*	Geneva, Switzerland	2006	[17]
6-P	Human	*D. latum*	Vaud, Switzerland	2006	This study
7-P	Human	*D. latum*	Vaud, Switzerland	2007	This study
8-P	Human	*D. latum*	Geneva, Switzerland	2007	This study
9-P	Human	*D. latum*	Valais, Switzerland	2007	This study
10-P	Human	*D. latum*	Vaud, Switzerland	2007	This study
11-P	Human	*D. latum*	Geneva, Switzerland	2007	This study
12-P	Human	*D. latum*	Geneva, Switzerland	2008	This study
13-P	Dog	*D. latum*	Bern, Switzerland	2002	This study
14-P	Fox	*D. latum*	Grisons, Switzerland	2002	This study
15-P	Human	*D. latum*	Lombardy, Italy	2006	This study
16-P	Human	*D. latum*	Finland (?)	2007	This study
17-P	Human	*D. latum*	Kotka, Finland	2004	This study
18-P	Human	*D. nihonkaiense*	Geneva, Switzerland	2006	[17]
19-E	Human	*D. nihonkaiense*	Geneva, Switzerland	2005	[17]
20-P	Human	*D. dendriticum*	Bern, Switzerland	2006	[18]
21-L	*Perca fluviatilis*	*D. latum*	Lake Maggiore	2008	This study
22-L	*Perca fluviatilis*	*D. latum*	Lake Geneva	2007	This study
23-L	*Perca fluviatilis*	*D. latum*	Lake Como, Italy	2006	This study
24-L	Fish	*D. latum*	Lake Peipsi, Estonia	2004–2006	This study
25-L	*Coregonus lavaretus*	*D. dendriticum*	Lake Peipsi, Estonia	2004	This study
26-L	*Coregonus widegreni*	*D. ditremum*	Oulu, Finland	2006	This study
27-L	*Salvelinus alpinus*	*D. ditremum*	Loch Lomond, Scotland	2002	This study

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the *cox1* and *cob* trees were DQ985706 (adult from Russian patient) and AB269325 (adult from another Russian patient) for *D. latum*; AB268585 (adult from Japanese patient) and EF420138 (adult from Korean patient) for *D. nihonkaiense*; and *S. erinaceieuropaei* (D64072) as outgroup.

3. Results

For all genes and the ITS regions, the ME and NJ methods resulted in the same topology trees.

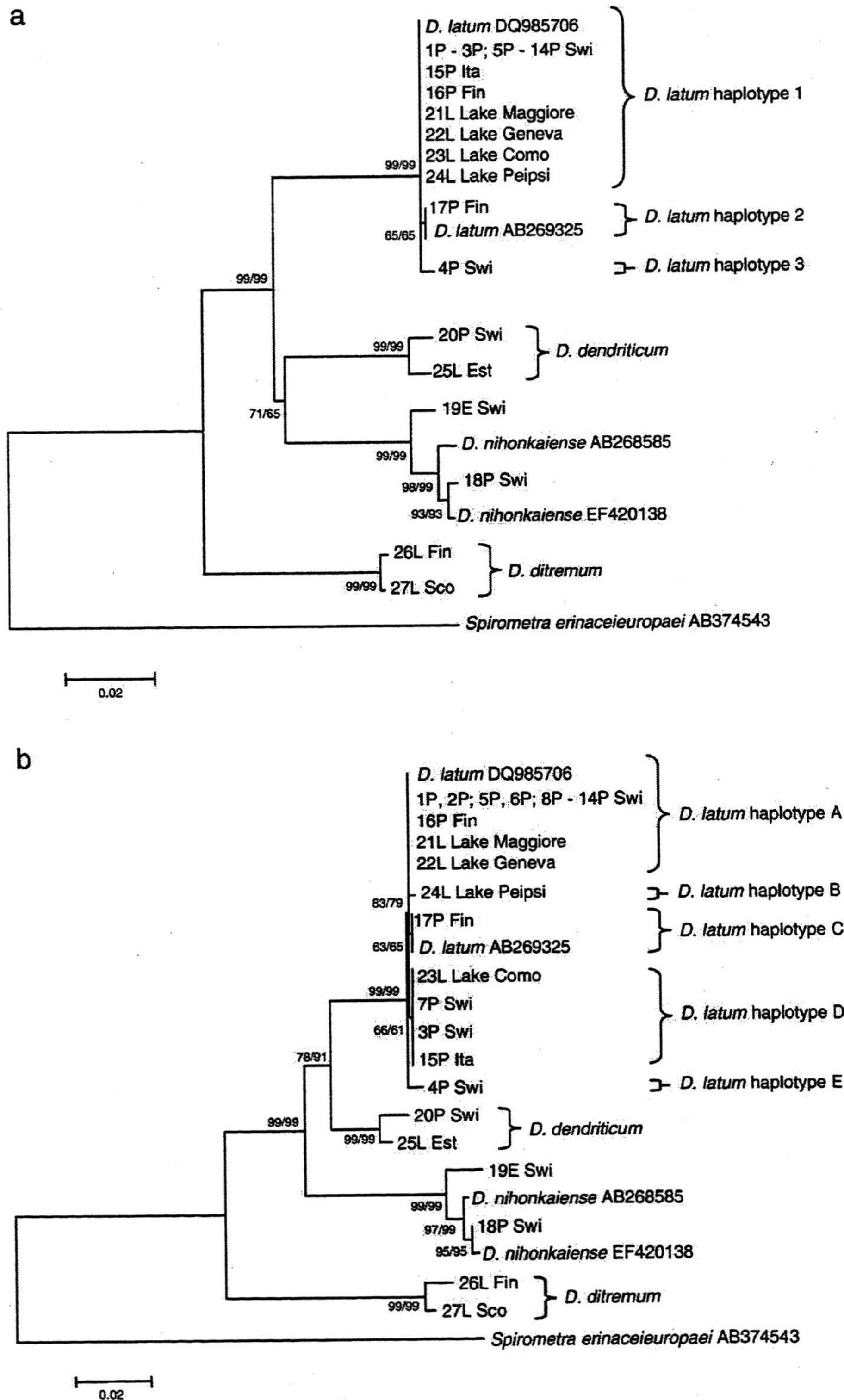

Fig. 1. Neighbor-Joining trees based on (a) 1566 bp fragment of complete *cox1* gene, and (b) 1107 bp fragment of complete *cob* gene. Kimura-2 parameters, bootstrap values > 50 shown on nodes (ME/NJ).

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The phylogenetic tree built on concatamers of partial 18S rRNA gene sequences was not discriminative for *Diphyllobothrium* species, with *D. dendriticum* (20-P and 25-L) and *D. ditremum* (26-L and 27-L) appearing in the same cluster (data not shown). Estimates of the average evolutionary divergence over sequence pairs within groups, based on the pairwise analysis of 3 sequences in the cluster *D. dendriticum*-*D. ditremum*, showed a genetic distance of 0.0031 ± 0.0014. Nevertheless, the genetic distance between the 2 sequences of *D. latum* was 0.0009, indicating some intra-specific variation (one nucleotide substitution). The 3 sequences of *D. nihonkaiense* were identical.

Results of partial ITS1–ITS2 regions alignments also showed a low discrimination between *D. dendriticum* and *D. ditremum*, both in the same cluster (data not shown). The average evolutionary divergence of the sequences in this group was low, with 99.74% similarity ($d = 0.0026 \pm 0.0014$). Similarity within 21 sequences of *D. latum* was 100%, and 99.83% between 2 sequences of *D. nihonkaiense* ($d = 0.0017$).

The tree based on complete *cox1* gene sequences (Fig. 1a) showed to be more discriminative both at inter- and at intra-specific level, indicating the existence of 3 haplotypes of *D. latum* (indicated as haplotype 1, 2 and 3 in the figure). The estimate average evolutionary divergence among their sequences showed 99.83% similarity ($d = 0.0017 \pm 0.0008$). *D. dendriticum* (2 haplotypes: 20-P and 25-L) was separated from *D. ditremum* (2 haplotypes: 26-L and 27-L). Similarity within each species was respectively 98.97% ($d = 0.0103$) and 99.68% ($d = 0.0032$). The cluster grouping 4 sequences of *D. nihonkaiense* showed a high intra-specific variation, with 4 haplotypes. Similarity within these sequences was 98.88% ($d = 0.0112 \pm 0.0020$).

The tree based on complete *cob* gene sequences (Fig. 1b) allowed distinguishing 5 haplotypes among 21 sequences of *D. latum* (indicated as haplotype A, B, C, D and E in the figure). The estimate of the average evolutionary divergence within them showed 99.64% similarity ($d = 0.0036 \pm 0.0011$). *D. dendriticum* (20-P and 25-L) and *D. ditremum* (26-L and 27-L) formed two separate clusters, with an average evolutionary divergence showing 98.81% ($d = 0.0119$) and 98.9% ($d = 0.0110$) similarity. Four sequences of *D. nihonkaiense* represented 4 haplotypes, with 98.92% similarity ($d = 0.0108 \pm 0.0023$).

The estimates of evolutionary divergence over sequence pairs between species were calculated for each region sequenced and are reported in Table 2.

4. Discussion

The sequences of 18S rRNA and ITS1–ITS2 regions are unsuitable for the inter-specific characterization between *D. dendriticum* and *D. ditremum*, as well as for the intra-specific polymorphism within the other species analyzed (*D. latum* and *D. nihonkaiense*). Similar results were obtained by Škeříková et al. [7] for ITS2 and by Yéra et al. [22] for ITS1 and 18S rRNA. The aim of these studies, however, was not to analyze intra-specific variations among samples with the same geographic origin. In our study, only the partial ITS1–ITS2 sequences of *D. nihonkaiense* showed some degree of variability, and allowed differentiating 2 groups. For *D. latum*, the reference sequence DQ316796 (18S rRNA of an isolate from a Brazilian patient) was also slightly different from other samples. This might represent a variant, but this specimen should be further analysed using other genes, to confirm whether there is a major genetic difference between European and South American *D. latum*.

Mitochondrial genes such as *cox1* and *cob* allowed both inter- and intra-specific discrimination. In particular, the results of the analysis of Swiss samples of *D. latum* showed a clustering of 3 (*cox1*) to 5 (*cob*) different haplotypes. The high polymorphism of mitochondrial DNA is well known among the cestodes, whilst ribosomal RNA genes are more conserved targets [23,24]. In the present study, such intra-specific variability is reported for the first time for this species, usually regarded as very homogeneous [22]. Nevertheless, the genetic intra-specific diversity of *D. latum* in Europe is lower than the diversity within *D. nihonkaiense*, *D. dendriticum* and *D. ditremum*, for which each sample represented a distinct haplotype. The majority of the *D. latum* samples analysed exhibited identical sequences, suggesting that one mtDNA lineage prevails on the others, which are rare to encounter.

Based on the collected data, we tried to elucidate what the similar haplotypes of *D. latum* had in common. We considered geographic origins and intermediate hosts of the parasites.

In *cox1* and *cob* trees, the sample 4-P appeared to be different from all the others. It was an adult tapeworm isolated from a 53-years-old, self-declaring symptomless man, who ate mainly pike (*Esox lucius*). Being the only anamnesis reporting the consumption of this fish, the particularity of the sample could be linked to the intermediate host, perhaps to its origin. The patient declared to regularly eat pike in restaurants in Southern Switzerland, where this species is only sporadically caught by local fishermen. Pike served in restaurants is probably imported from other countries — especially from Eastern Europe. Since molecular analysis of 75 plerocercoid larvae of *D. latum* sampled in Southern Switzerland showed that they are genetically identical (partial 18S rRNA, partial *cox1*, ITS1-2 regions; [25]), it is possible that this patient was infected from an imported parasite.

In both trees, the sequence of sample 17-P (adult from Southern Finland) was identical with the reference sequence AB269325 (adult isolated from Russia). According to the anamnestic data, the Finnish patient was infected by eating a typical preparation made with local

Table 2
Genetic distances (below the diagonal) with standard errors (SE; above the diagonal) between *Diphyllobothrium* species, based on the analysis of partial 18S rRNA, ITS1–ITS2, *cox1* and *cob* gene sequences (Kimura-2 parameters, complete deletion option).

Distance	SE	*D. latum*	*D. nihonkaiense*	*D. dendriticum*	*D. ditremum*
D. latum	18S rRNA	–	[0.0019]	[0.0012]	[0.0012]
	ITS1–ITS2	–	[0.0061]	[0.0044]	[0.0044]
	cox1	–	[0.0077]	[0.0077]	[0.0095]
	cob	–	[0.0087]	[0.0062]	[0.0108]
D. nihonkaiense	18S rRNA	0.0038	–	[0.0020]	[0.0019]
	ITS1–ITS2	0.0196	–	[0.0058]	[0.0058]
	cox1	0.0757	–	[0.0078]	[0.0099]
	cob	0.0808	–	[0.0088]	[0.0119]
D. dendriticum	18S rRNA	0.0024	0.0052	–	[0.0010]
	ITS1–ITS2	0.0112	0.0183	–	[0.0010]
	cox1	0.0713	0.0717	–	[0.0095]
	cob	0.0438	0.0803	–	[0.0095]
D. ditremum	18S rRNA	0.0019	0.0047	0.0024	–
	ITS1–ITS2	0.0117	0.0189	0.0017	–
	cox1	0.0931	0.1018	0.0956	–
	cob	0.1190	0.1380	0.1067	–

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fish in Kotka, a city close to the Russian border. The two countries being confining, this clustering could be related to a geographical proximity. Sample 16-P did not appear in this cluster. According to the anamnesis, it was isolated from a patient living in Southern Finland (unknown locality), who often consumed different kinds of raw fish (such as pike, salmon and rainbow trout from various origins). There is no evidence that this parasite was locally acquired and, due to the possibility to have been imported, we cannot draw any conclusion about its phylogenetic position.

According to the *cob* tree, a cluster with 4 samples (23-L, 7-P, 3-P and 15-P) differentiated from the other sequences. 23-L was the only plerocercoid isolated from fish (perch) of Lake Como (Northern Italy). Interestingly, the patient 15-P was infected after the consumption of perch bought in Northern Italy. Patient 3-P declared eating fish in many countries, including Italy. No data were available for patient 7-P.

The plerocercoid larva 24-L also represented a distinct haplotype. It was the only sample of *D. latum* originated from Estonia.

Our results suggest the existence of a link between different haplotypes of *D. latum* and their geographic origin. Nevertheless, the number of samples collected abroad was too low, and the information on some specimens too scant, to allow a detailed phylogeographic analysis in confirmation of this hypothesis. In the future, these and further mtDNA targets deserve to be investigated on more consistent pools of samples from various ensured provenances. Such a link would be of primary importance from an epidemiological perspective, in particular concerning human pathogen species of *Diphyllobothrium*, because it could help trace the origin of the fishes acting as infective sources.

Our study has some limitations. First of all, suitable reference sequences were not always included in all phylogenetic trees, because they were not available in public databases, as was the case for 18S rRNA, *cox1* and *cob* of *D. ditremum* and *D. dendriticum*. Finally, some hypotheses about the relationships linking some specimens were made according to the anamneses provided by the patients, which could be clearly biased by their memories and interpretations.

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Note: The nucleotide sequence data reported in this paper are available in the GenBank™, EMBL and DDBJ databases under the accession numbers AM408316, AM410601, AM412559, AM412560, AM412738, AM412739, AM412740, AM712620, AM712906, AM778553, AM778554, FM204787, FM204789, FM209180, FM209181, FM209182, AB522608, AB522609, AB522610, AB522611, AB522612, AB522613, AB522614, AB522615, AB522616, AB522617, and AB522618.

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