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Mitochondrial DNA divergence in populations of the tapeworm *Diphyllobothrium nihonkaiense* and its phylogenetic relationship with *Diphyllobothrium klebanovskii*

Naoki Arizono [a,*], Marina Shedko [b], Minoru Yamada [a], Ryuichi Uchikawa [a], Tatsuya Tegoshi [a], Kazutoshi Takeda [a], Katsumi Hashimoto [a]

[a] Department of Medical Zoology, Kyoto Prefectural University of Medicine, Kawaramachi-Hirokoji, Kyoto 602-8566, Japan
[b] Institute of Biology and Soil Science (IBSS), Far Eastern Branch of Russian Academy of Sciences (FEB RAS), 159 Stoletiya Street, Vladivostok 690022, Russian Federation

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ABSTRACT

Diphyllobothrium nihonkaiense [Y. Yamane, H. Kamo, G. Bylund, J.P. Wilkgren. *Diphyllobothrium nihonkaiense* sp. nov (Cestoda: Diphyllobothriidae)– revised identification of Japanese broad tapeworm. Shimane J Med Sci 1986;10:29–48.] and *Diphyllobothrium klebanovskii* [I.V. Muratov, P.S. Posokhov. Causative agent of human diphyllobothriasis – *Diphyllobothrium klebanovskii* sp. n. Parazitologiia. 1988;22:165–170.] are two major species of human diphyllobothriasis in Japan and Far East Russia, respectively, but their taxonomical relationship remains unclear. In this study, we analysed the DNA sequences of 16 clinical isolates of *D. nihonkaiense* from Japanese people, 3 isolates of *D. klebanovskii* from a bear in Kamchatka, and 4 clinical isolates of *D. klebanovskii* from native Udygeyci people in Russia, as well as 4 plerocercoids from *Oncorhynchus* spp. 18S rDNA and internal transcribed spacer 1 (ITS1) sequences from *D. nihonkaiense* and *D. klebanovskii* showed a high level of similarity, indicating synonymy of the two species. Analyses of mitochondrial DNA (mtDNA) sequence polymorphisms in the cox1 and nad3 genes of *D. nihonkaiense* (*D. klebanovskii*) revealed two deeply divergent lineages, A and B, with genetic distances (Kimura-2 parameter) of 0.018–0.022. Furthermore, the distinct monophyletic groupings of cox1 haplotypes corresponded to the distinct monophyletic groupings of nad3 haplotypes. The two lineages were neither distinguished by morphological features nor defined by the localities of the samples. These results suggest that the two morphologically cryptic lineages have diverged and coexisted over a long period of time.

1. Introduction

Diphyllobothriasis among Japanese was considered to be caused by infection with *Diphyllobothrium latum* until Yamane et al. revised the identification of the Japanese broad tapeworm and established the new species *D. nihonkaiense* [1]. In contrast to *D. latum*, which uses freshwater fish such as perch, char, and pike as the second intermediate host, *D. nihonkaiense* employs anadromous fish, *Oncorhynchus* spp., such as *O. masou* (masu salmon), *O. gorbuscha* (pink salmon), and *O. keta* (chum salmon) that migrate across the Northern Pacific including Okhotsk and Bering Sea as its second intermediate host [2–4]. *D. nihonkaiense* plerocercoids have been found in mature Pacific salmon migrating through the rivers or waters off the coast of Japan [5]. In Kyoto, Japan, 109 human cases were recorded between 1980 and 2002 [6], although the overall incidence throughout Japan has not been investigated. Although diphyllobothriasis nihonkaiense has been reported almost exclusively in Japan and Korea [7,8], several cases of human infection have also been recently found in France and Switzerland in persons who had consumed raw Pacific salmon (*Oncorhynchus* spp.) possibly imported from the Pacific coast of North America [9,10].

In Far East Russia, the most common species of human diphyllobothriasis is *D. klebanovskii* [11]. Plerocercoids of *D. klebanovskii* have been found in *O. keta* and *O. gorbuscha* [12], the same fish that harbour *D. nihonkaiense* plerocercoids. The incidence of human diphyllobothriasis klebanovskii in the early 1990s in Far East Russia was 10.4–68.7 cases per 100,000 population [12], and brown bears (infection rate 47.1 ± 8.6%) were considered to be the principal hosts [13].

Before the description and epidemiological surveillance of *D. klebanovskii* in Far East Russia, Rutkevich had described *D. giljacicum* and *D. luxi* from the native people in Sakhalin [14], but the second intermediate host was not recorded and these two species have not been fully recognized by later researchers. In northern communities bordering the Pacific, several additional diphyllobothriid species have been implicated in human infections including *D. latum*, *D. dendriticum*, *D. ursi* and *D. dalliae* [15–17]. Larvae of *D. dendriticum* are found predominantly in salmonid fish such as arctic char, salmon, trout, and whitefish. Larvae of *D. ursi* are found predominantly in the Pacific salmon *O. nerka*, and those of *D. dalliae* in Alaskan blackfish [17]. However, correct differential diagnosis of these species by using morphological features of adult cestodes alone is difficult because of a

* Corresponding author. Tel.: +81 75 2515325; fax: +81 75 2515328.
E-mail address: arizonon@koto.kpu-m.ac.jp (N. Arizono).

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wide range of variations in morphological characters and conservative morphological evolution in the genus *Diphyllobothrium* [15,16]. Recent studies have demonstrated complete mitochondrial genomes of *D. nihonkaiense* and *D. latum* [18–20] that have not only rendered species diagnosis more reliable but also provided a wealth of genetic markers that could be useful for identifying different life cycle stages and for investigating their population genetics, ecology and epidemiology.

Thus, the aims of the present study were (i) to determine whether *D. nihonkaiense* and *D. klebanovskii*, the causative agents of human diphyllobothriasis in Japan and Far East Russia, are valid independent species by molecular genetic analyses, and (ii) to find genetic variations of the two species.

2. Materials and methods

2.1. Specimens

A total of 27 specimens were analysed in this study (Table 1). Sixteen *D. nihonkaiense* clinical isolates (Dn1–16) were collected in Kyoto, Japan (Fig. 1a) between 2000 and 2007. These specimens were passed naturally in the stool or discharged after administration of praziquantel and a purgative. Approximately one half of the patients had consumed raw salmon 2–3 months before they discharged strobila, while the other half of the patients did not remember eating salmon, although most of them habitually ate 'sashimi' dishes, which are normally composed of sea fish and often include salmon. Mature proglottids were examined by carmine staining and sectioning. Morphological features of the strobila and proglottids were compatible with those of *D. nihonkaiense*. Six of 16 isolates (Dn11–Dn16 in Table 1) had been immersed in neutral-buffered 4% formalin for 2–7 years before DNA was extracted, while other specimens had been stored in 75% alcohol. Segments of formalin-fixed isolates were washed in 70% alcohol for 2 weeks–2 months with repeated changes of alcohol, followed by immersion in 10 mM Tris–HCl buffer supplemented with 1 mM EDTA, pH8.0, for 48 h before DNA was extracted.

Fig. 1. Collection sites of specimens. a, Kyoto, Japan; b, Jintsu River, Japan; c, Settlement Agzu along Samarga river, about 80 km from the mouth of the river, Russia; d, Lake Azabachye along Kamchatka River, about 50 km from the mouth of the river, Kamchatka; e, Paratunka River, Kamchatka; f, Okhotsk Sea near Sakhalin.

Four clinical isolates of *D. klebanovskii* were collected by Dr. Mishakov from 'Udygeyci (Udege)' native people in the settlement of Agzu on the upper tributary of the river Samarga (Fig. 1c) in 1964 [21] and were kept

Table 1
Samples analysed in this study

Host	Isolate	Species[a]	Locality[b]	Year[c]	GenBank accession number			
					18S rDNA	ITS1	cox1	nad3
Human	Dn1	*Dn*	*a*	2006	AB374225	AB288368	AB288371	AB288374
	Dn2	*Dn*	*a*	2006		AB288369	AB288372	AB288375
	Dn3	*Dn*	*a*	2006		AB288370	AB288373	AB288376
	Dn4	*Dn*	*a*	2006		AB375018	AB374999	AB375005
	Dn5	*Dn*	*a*	2007		AB375171	AB375000	AB375006
	Dn6	*Dn*	*a*	2007		AB375172	AB375001	AB375007
	Dn7	*Dn*	*a*	2007		AB375173	AB375002	AB375008
	Dn8	*Dn*	*a*	2007		AB375174	AB375003	AB375009
	Dn9	*Dn*	*a*	2007		AB375175	AB375004	AB375010
	Dn10	*Dn*	*a*	2007				AB375011
	Dn11	*Dn*	*a*	2000				AB375012
	Dn12	*Dn*	*a*	2004				AB375013
	Dn13	*Dn*	*a*	2004				AB375014
	Dn14	*Dn*	*a*	2005				AB375015
	Dn15	*Dn*	*a*	2005				AB375016
	Dn16	*Dn*	*a*	2004				AB375017
	RH1	*Dk*	*c*	1964				AB375666
	RH2	*Dk*	*c*	1964				AB375667
	RH3	*Dk*	*c*	1964				AB375668
	RH4	*Dk*	*c*	1964				AB375669
Brown bear[d]	RB1	*Dk*	*d*	1991	AB374226	AB375657	AB375660	AB375663
	RB2	*Dk*	*d*	1991		AB375658	AB375661	AB375664
	RB3	*Dk*	*d*	1991	AB374227	AB375659	AB375662	AB375665
O. keta	Ok1	*Dk*	*f*	2001		AB375670	AB375672	AB375674
	Ok2	*Dk*	*e*	2001		AB375671	AB375673	AB375675
O. masou	Om1	*Dn*	*b*	1978				AB375676
	Om2	*Dn*	*b*	1978				AB375677

[a] Dn, *Diphyllobothrium nihonkaiense*; Dk, *D. klebanovskii*.
[b] a–f, refer to Fig. 1.
[c] The year samples were collected.
[d] *Ursus arctos piscator*.

in 70% alcohol in the Laboratory of Parasitology, Institute of Biology and Soil Science, Far Eastern Branch of Russian Academy of Science, Vladivostok. Epidemiological surveillance in Agzu carried out in 1964 showed that diphyllobothriid eggs were found in 30 out of 139 people and 14 out of 20 dogs [21]. The Udygeyci people's main occupations were hunting and fishery, and they caught *O. keta*, *O. masou*, *Salvelinus* spp., *Parahucho* spp., *Thymallus* spp., and *Brachymystax* spp. from local rivers and ate them raw or poorly salted [21]. Morphological studies conducted by the present authors showed that Mishakov's specimens were compatible with *D. klebanovskii*.

Diphyllobothriid tapeworms were also collected from 3 brown bears (*Ursus arctos piscator* Pucheran, 1855) at Lake Azabachye, Kamchatka (Fig. 1d) in 1991; 1 bear was found naturally dead on the coast of the lake and the other 2 bears were shot dead with official permission for academic research purposes. All three bears harboured diphyllobothriid tapeworms, with 5, 11 and 12 strobilae found in each. The length of the tapeworms was from 1.5 to 19.4 m. Morphological studies of these tapeworms involving histology and scanning electron microscopy led to species identification as *D. klebanovskii* [22]. Some strobilae from one bear were kept in 70% alcohol until the present genetic studies.

Plerocercoids were isolated from the body musculature of *O. keta* caught in Paratunka River, Kamchatka (Fig. 1e) and the Sea of Okhotsk near Sakhalin (Fig. 1f) in 2001. These were identified as *D. klebanovskii* by experimental infection in hamsters. Two of the plerocercoid specimens were kept in 70% alcohol until the present genetic studies. Plerocercoids were also collected from the body musculature of *O. masou* caught in Jintsu River, Japan (Fig. 1b) in 1978. Two of the plerocercoids from *O. masou* had been stained and embedded in resin. After dissolving the resin by immersing the slides in xylene for 2 weeks, plerocercoids detached from the glass slides were washed in 70% alcohol for 2 weeks with repeated changes of alcohol before DNA was extracted.

2.2. DNA extraction, PCR, DNA sequencing and analyses

DNA was extracted using a QIAmp DNA Mini Kit (Qiagen GmbH, Germany) according to the manufacturer's instructions. 18S small subunit nuclear ribosomal DNA (18S rDNA), ribosomal DNA internal transcribed spacer (ITS)1, the mitochondrial cox1 gene for cytochrome oxidase subunit 1, and the mitochondrial nad3 gene for NADH dehydrogenase subunit 3 were targeted for PCR amplification. Amplification was performed using Takara Taq polymerase with the appropriate number of cycles of denaturation (30 s at 94C), annealing (30 s at 62 °C for 18S rDNA and ITS1; 30 s at 52 °C for cox1 and nad3) and extension (30 s at 72C). 18S rDNA was amplified in 4 overlapping fragments using forward and reverse primers as described elsewhere [23]. Other primers used were: 5′-AACAAGGTTTCCGTAGGTGA-3′ and 5′-AGCAGTCTGCGATTCACATT-3′ for ITS1, which yielded a 649-bp product including 18S rDNA and 5.8 S rDNA; 5′-TTGATCGTAAATTTGGTTC-3′ and 5′-AAAGAACCTATTGAACAAAG-3′ for cox1, which yielded a 748-bp product; and 5′-AACTTTGTGTTTCATTGGTA-3′ and 5′-GACAATAAGTTATTAGCAGT-3′ for nad3, which yielded a 475-bp product. The PCR amplification products were directly sequenced on both strands using the Big-Dye Terminator Cycle Sequencing Ready Reaction Kit version 3.1 (Applied Biosystems, Langen, Germany) and an Applied Biosystems 3730XL DNA sequencer. The nucleotide sequences of 18S rDNA, ITS1, cox1 and nad3 determined in this study were deposited in DNA databases with the accession numbers indicated in Table 1. Nucleotide sequences were aligned using ClustalW software, and phylogenetic analyses were conducted using MEGA version 3.1 software [24]. The sequences of 18S rDNA and 5.8S rDNA were excluded from ITS1 analyses. For cox1 analyses, sequences corresponding to positions 652–1362 of the *D. nihonkaiense* cox1 gene (AB015755) were used, and for nad3 analyses, the full 357-bp nad3 sequence was employed.

3. Results

3.1. Morphological features of D. nihonkaiense and D. klebanovskii

The morphology of *D. nihonkaiense* and D. *klebanovskii* is described based on 7 clinical isolates from Japan and 3 isolates from bears caught in Kamchatka (Fig. 2).

Strobilae of *D. nihonkaiense* (*Dn*) obtained by administration of praziquantel and a purgative were 2–8 m long. The length of *D. klebanovskii* (*Dk*) from brown bears was from 1.5 to 19.4 m. In both species, the strobila is so thin that the uterine loops could be seen from outside. The scolex was spatulate, measuring 1.8–3.6 (av. 2.5) mm long by 0.63–1.38 (av. 0.94) mm thick in *Dn* and 3.0–3.4 (av. 3.2) by 1.4–1.6 (av. 1.5) mm in *Dk*. The neck was long and strobila widened posteriorly with margins slightly serrated in both *Dn* and *Dk*. The genital primordium was first visible 7 to 25 cm posterior to the scolex in the 124th to 800th segment in *Dn*, and 7 to 13 cm posterior to the scolex in the114th to 190th segment in *Dk*. The width and length of gravid segments were 6.0–11.2 mm by 1.2–3.6 mm in *Dn*, and 6.2–12.0 mm by 1.4–3.8 mm in *Dk*. The cirrus sac in sagittal sections was oval, 425–875 μm in length by 275–525 μm in width in *Dn*, and 473–700 by 387–522 μm in *Dk*. The genital pore was situated ventrally on the midline in the anterior one-fifth to one-third of segments in *Dn*, and one-seventh to one-fourth of segments in *Dk*. The uterine pore was on the midline, 137–498 and 140–249 μm posterior to the genital pore in *Dn* and *Dk*, respectively. The seminal vesicle was thick-walled, round to elliptical, and 299–473 μm in length by 174–349 μm in diameter in *Dn*, and 300–500 by 200–280 μm in *Dk*. The seminal vesicle connected to the postero-ventral part of the cirrus sac. The uterus consisted of 4 to 6 loops on each side in *Dn*, and 4 to 11 loops in *Dk*. Anterior-most loops often extended beyond the genital pore in both *Dn* and *Dk*. The ovary was reticulate, situated near the posterior

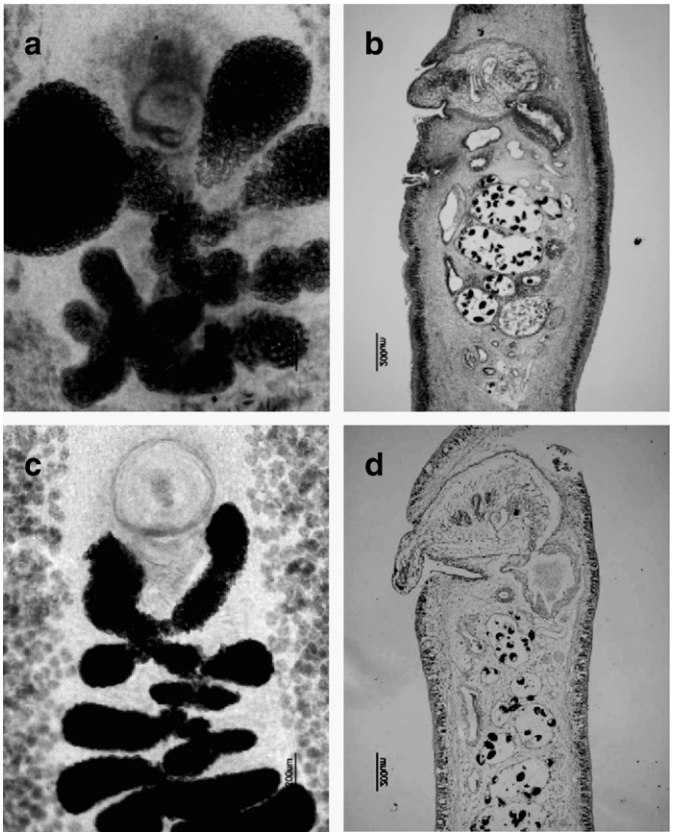

Fig. 2. *Diphyllobothrium nihonkaiense* (Dn3) (a, b) and *Diphyllobothrium klebanovskii* (RB2) (c, d). Whole mount of gravid segment (a, c) was stained with carmine. Sagittal sections (b, d) were stained with haematoxylin and eosin. Bars indicate 200 μm.

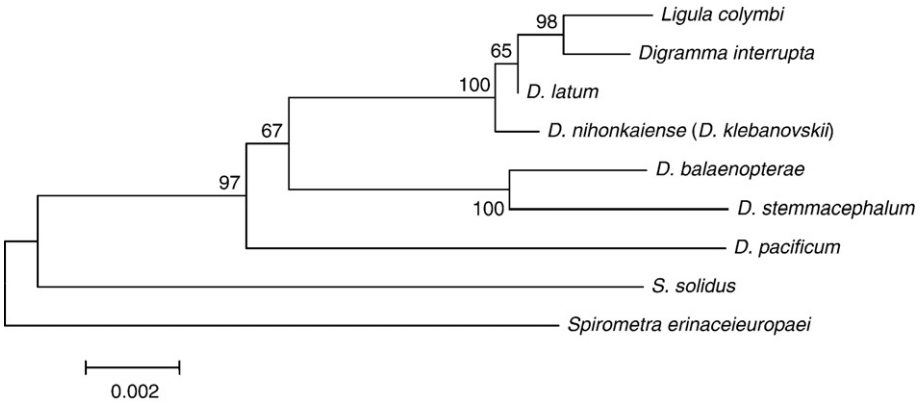

Fig. 3. Phylogenetic analyses of *Diphyllobothrium nihonkaiense* and *Diphyllobothrium klebanovskii* based on 18S rDNA sequences. The evolutionary history was inferred using the Neighbor-Joining method. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Kimura 2-parameter method (substitutions included transitions and transversions, pattern among lineages assumed homogeneous, and the rate variation among sites uniform) and are in the units of the number of base substitutions per site. There were a total of 2119 positions in the final dataset. Bootstrap values (percentage of 1000 replicates) are shown next to the branches. Sequences analysed were: *Ligula colymbi* (DQ925312), *Digramma interrupta* (DQ925308), *Diphyllobothrium latum* (DQ925309), *D. stemmacephalum* (AF124459), *D. pacificum* (DQ925310), *Diplogonoporus balaenopterae* (AB353272), *Spirometra erinaceieuropaei* (D64072), and *Schistocephalus solidus* (AF124460).

margin of the segment, with two lobes connected by a narrow isthmus in both *Dn* and *Dk*. Testes were in the lateral field and did not merge ahead of the genital pore. Testes measured 100–160 μm and 80–170 μm in maximal diameter in *Dn* and *Dk*, respectively. Intrauterine eggs measured 55.0–65.0 (av. 60.6) by 32.5–43.8 (av. 38.8) μm in *Dn*, and 56.3–65.0 (av. 59.7) by 37.5–42.5 (av. 39.1) μm in *Dk*.

All of these characters of adult cestodes corresponded with the original descriptions of *D. nihonkaiense* and *D. klebanovskii*, which were similar and measurements widely overlapped [1,11]. Thus, in the present study, species identification of *D. nihonkaiense* and *D. klebanovskii* was tentatively made based on geographical ranges; Japan for *D. nihonkaiense* [1], and Amur basin, Kamchatka, Sakhalin, Primorski Krai and Chukotka for *D. klebanovskii* [11]. Species identification of plerocercoids from *O. keta* was carried out based on adult worms obtained by experimental infection in golden hamsters. Plerocercoids from *O. masou* were also identified by experimental infection in dogs.

3.2. Phylogenetic relationship between D. nihonkaiense and D. klebanovskii

To determine the phylogenetic relationship between *D. nihonkaiense* and *D. klebanovskii*, 18S rDNA and the ITS1 region were sequenced. 18S rDNA sequences (2171 bp) from *D. nihonkaiense* isolated from a Kyoto resident (Dn1) and 2 *D. klebanovskii* isolates from a brown bear from Kamchatka (RB1and 3) were 100% identical. ITS1 sequences (553 bp) of 9 isolates of *D. nihonkaiense* from Kyoto residents and 3 isolates of *D. klebanovskii* from the brown bear also showed a high level of similarity. In seven of 12 isolates, the ITS1 sequences were 100% identical to those of *D. nihonkaiense* reported in DNA databases (DQ768182), while the other 5 isolates (Dn5, Dn6, Dn8, RB1 and RB2) showed a single base substitution (G→A) at position 19 from the 5′ end of ITS1. This single nucleotide substitution was not restricted to isolates from Kyoto residents or to isolates from the bear. ITS1 sequences of plerocercoid isolates from *O. keta* from Kamchatka and Okhotsk were also identical to those of *D. nihonkaiense*. These results clearly show the synonymy of the two species.

Phylogenetic analyses based on 18S rDNA showed that the isolates of *D. nihonkaiense* (*D. klebanovskii*) formed a monophyletic group with *D. latum*, *Ligula colymbi* and *Digramma interrupta* (Fig. 3). Phylogenetic analyses based on ITS1 sequences also showed a close relationship of *D. nihonkaiense* (*D. klebanovskii*) with *D. latum* (DQ768174), *D. dendriticum* (DQ768177), *Ligula* sp. (AF385760) and *Digramma* sp. (AF354291) with genetic distances (Kimura-2 parameter) of 0.014, 0.020, 0.031 and 0.033, respectively.

Table 2
Polymorphic sites of cox1 (711 bp) and nad3 (357 bp) sequences in 14 isolates of *Diphyllobothrium nihonkaiense* and *D. klebanovskii*

Isolate	cox1																						nad3																
	15	30	33	66	69	72	144	174	222	294	319	372	417	462	483	531	534	543	561	633	636	657	20	21	22	34	38	45	47	57	99	168	180	184	204	292	315	321
	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	N	S	S	N	N	N	N	N	N	N	S	S	S	S	S	S	S	S	S
Dn1	T	T	C	C	C	C	T	T	T	G	C	A	C	T	C	T	C	G	A	C	G	T	G	T	G	T	T	G	T	G	T	C	T	T	C	T	A	A
Dn2	·	·	·	T	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	C	·	·	·	C	·	·	·	·	·	·	·	·	·	·
Dn3	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	C	·	·	·	C	·	·	·	·	·	·	·	·	·	·
Dn4	·	C	·	·	·	·	·	·	·	·	·	G	·	·	·	·	·	·	·	·	·	·	·	C	·	·	·	C	·	·	·	·	·	·	·	·	·	·
Dn5	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	C	·	·	·	·	·	·	·	·	·	·
Dn6	·	·	·	T	·	·	·	·	·	·	T	·	·	·	·	·	·	·	·	·	·	·	·	C	·	·	·	G	·	·	·	·	·	·	·	·	·	·
Dn7	·	·	T	·	·	·	·	·	C	·	·	·	·	·	·	·	·	·	·	·	·	·	·	C	·	·	·	C	·	·	·	·	·	·	·	·	·	·
Ok1	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	·	C	·	·	·	·	·	·	·	·	·	·
Ok2	·	·	·	T	·	·	·	·	·	·	·	·		·	·	·	·	·	·	·	·	·	·	·	·	C	·	C	·	·	·	·	·	·	·	·	·	·
RB3	C	C	·	·	·	A	·	·	·	A	T	·	·	·	·	·	·	·	G	T	·	·	·	·	·	·	·	C	·	·	·	T	·	·	T	C	·	·
Dn8	·	·	T	·	T	·	C	·	·	A	·	·	·	·	T	C	T	A	·	T	·	C	T	·	A	·	C	C	·	·	G	·	C	·	·	·	G	G
Dn9	·	·	T	·	T	·	·	·	·	A	·	·	·	·	T	C	T	A	·	T	T	C	·	·	·	·	C	C	·	A	G	·	C	C	·	·	G	G
RB1	·	·	T	·	T	·	·	·	·	A	·	·	·	·	T	C	T	A	·	T	·	C	·	·	·	·	C	C	C	·	G	·	C	·	·	·	G	G
RB2	·	·	T	·	T	·	·	C	·	A	·	·	·	C	T	C	T	A	·	T	·	C	·	·	·	·	C	C	·	·	G	·	C	·	·	·	G	G

Dots indicate a match to the Dn1 sequence. Shaded residues indicate those that are strictly discriminative between lineage A and lineage B (Fig. 4c). Numbers in the second line represent the position from the 5′ end of cox1 and nad3, respectively. S and N in the third line indicate synonymous and nonsynonymous substitutions, respectively.

3.3. Sequence divergence of mitochondrial DNA cox1 and nad3 genes of *D. nihonkaiense* and *D. klebanovskii*

To investigate the genetic diversity of *D. nihonkaiense* (*D. klebanovskii*), we analysed mitochondrial DNA (mtDNA) cox1 sequences in 14 isolates:

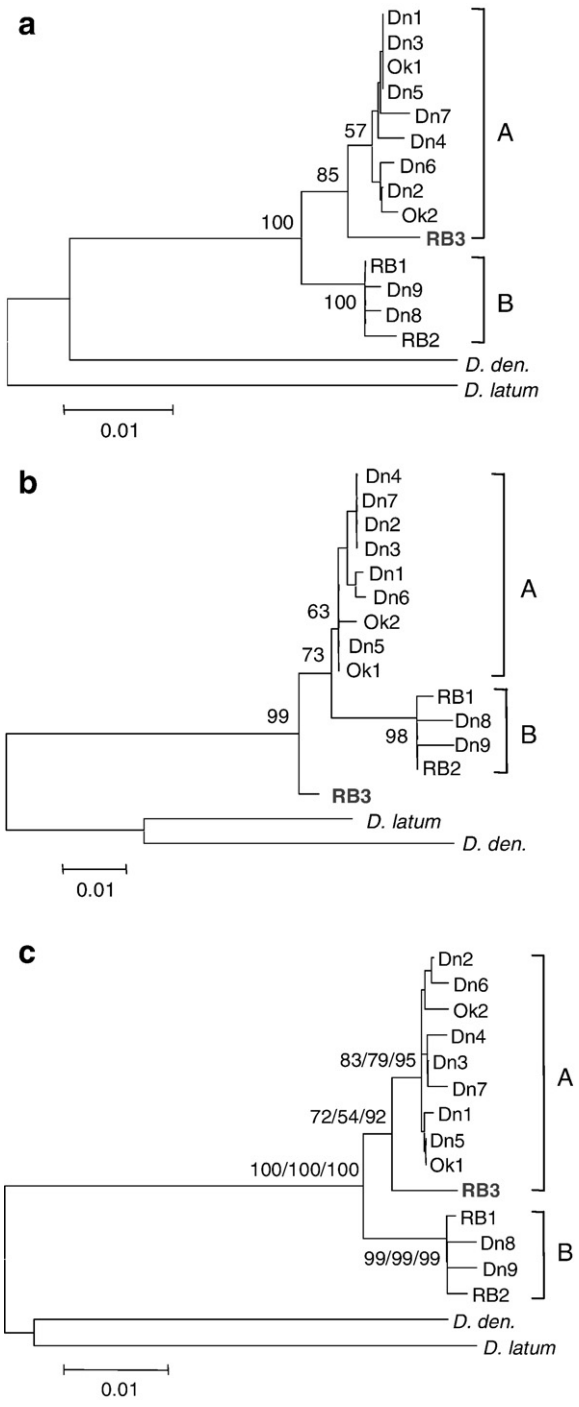

Fig. 4. Polymorphism of mtDNA cox1 and nad3 genes in *D. nihonkaiense* and *D. klebanovskii* isolates. The evolutionary history was inferred using the Neighbor-Joining method. The evolutionary distances were computed using the Kimura 2-parameter method and are in the units of the number of base substitutions per site. a, Phylogenetic tree based on cox1 gene sequences (number of sites: 711). b, Phylogenetic tree based on nad3 gene sequence (number of sites: 357). c, Phylogenetic tree based on the combined data set of cox1 and nad3 gene sequences (number of sites: 1068). Bootstrap values (percentage of 1000 replicates) are shown next to the branches. In c, three sets of bootstrap values by the Neighbor-Joining, Minimum Evolution, and UPGMA methods are shown from left to right.

9 from residents in Kyoto, 3 from the brown bear, and 2 from *O. keta*. Cox1 sequences of the 14 isolates were well separated from *D. dendriticum* (DQ768194) and *D. latum* (DQ768201) with genetic distances (Kimura-2 parameter) of 0.062–0.070 and 0.075–0.078, respectively. The cox1 sequences of *D. nihonkaiense* (*D. klebanovskii*) showed 22 variable sites, including 21 synonymous substitutions (Table 2). Phylogenetic trees generated by the Neighbour-Joining (NJ) method revealed two major lineages designated as A and B (Fig. 4a). The Minimum Evolution (ME) and UPGMA methods resulted in the same topology tree.

Nad3 sequences were also analysed for the same isolates analysed for cox1. Nad3 sequences were well separated from *D. dendriticum* (AB374224) and *D. latum* (AY973606) with genetic distances (Kimura-2 parameter) of 0.117–0.142 and 0.104–0.121, respectively. The nad3 sequences (357 bp) showed 16 polymorphic sites with 7 sites of nonsynonymous substitutions (Table 2). A phylogenetic tree based on nad3 sequences also revealed two major lineages (Fig. 4b). Except for the topology of isolate RB3, the nad3 sequence divergence followed the same pattern as in cox1. As expected, the combined cox1 and nad3 sequence data set also resulted in a similar tree (Fig. 4c). In the combined sequence data set, there were 11 fixed segregating sites between the two lineages of which only one site was nonsynonymous substitution (Table 2). The valid status of lineage B as a monophyletic group was well supported by high bootstrap values (99/99/99 by the NJ, ME, and UPGMA methods, respectively), and that of lineage A (except RB3) by slightly lower bootstrap values (83/79/95 by the NJ, ME, and UPGMA methods, respectively).

Additional 13 isolates were subjected only to nad3 sequence analysis, because PCR amplification of cox1 and ITS1 was unsuccessful. These isolates included 4 from the Udygeyci people in the settlement of Agzu that had been collected more than 50 years ago (RH1–RH4), 7 from Kyoto residents collected between 2000 and 2007, and 2 isolates of *D. nihonkaiense* plerocercoids from *O. masou* (Om1 and 2) collected 29 years ago. Six of 7 isolates from Kyoto residents had been immersed in neutral-buffered formalin for 2–7 years. Nad3 sequences of these isolates were also grouped into either lineage A or B. The translated amino acid sequences of nad3 gave rise to comparable lineages A and B, where isolate RB3 was grouped in lineage A. Assuming that RB3 belongs to lineage A, the frequencies of each lineage in a total of 27 isolates were 66.6% and 33.3%, for A and B, respectively. Of these isolates, 3 of 4 from Udygeyci people, 10 of 16 from Japanese people, and 1 of 3 from the bear belonged to lineage A, and the rest to lineage B.

Sequence divergences between lineages A and B are summarised in Table 3. The variations within lineages were rather small and similar (around 0.004) for each lineage. In contrast, the divergence between lineages was fairly high with genetic distances of 0.0175 for the combined cox1 and nad3 data set and 0.0214 for nad3.

4. Discussion

4.1. *D. nihonkaiense* and *D. klebanovskii* are indistinguishable by morphology

Original descriptions of *D. nihonkaiense* and *D. klebanovskii* are similar and morphological measurements widely overlap, opening the question of whether these are two different species [25]. Nevertheless, Muratov and Posokhov [11] pointed out a difference in adult cestodes: the genital primordium of *D. klebanovskii* from humans first appears in the 234th to 1197th (av. 693th) segment [11], while that of *D. nihonkaiense* experimentally infected in hamsters appears in the 117th to 120th (av. 119th) segment [1]. In the present study, the genital primordium of *D. nihonkaiense* from humans was first visible in the 124th to 800th segment, indicating that this character is not useful for the differentiation of the two species. Other morphological features of *D. nihonkaiense* and *D. klebanovskii* adult worms also failed to disclose any significant differences between the two species. The number of uterine loops has been regarded as an important character in differentiating species of *Diphyllobothrium*. In the present materials, this number ranged from

Table 3
Estimates of evolutionary divergence over sequence pairs within and between lineages A and B of *D. nihonkaiense* (*D. klebanovskii*)

	cox1+nad3				nad3			
	n[a]	No of site[b]	No variable[c]	Average diversity[d] (SE)	*n*[a]	No of site[b]	No variable[c]	Average diversity[d] (SE)
Within[e] [A+B]	14	1068	38	0.0101 (0.0019)	27	351	16	0.0120 (0.0035)
Within[e] [A]	10	1068	18	0.0044 (0.0011)	18	351	6	0.0034 (0.0017)
Within[e] [B]	4	1068	9	0.0042 (0.0014)	9	351	5	0.0065 (0.0030)
Between[f] [A] and [B]		1068		0.0175 (0.0039)		351		0.0214 (0.0069)
Between[f] Dl and [A+B]		1068		0.0880 (0.0098)		351		0.1041 (0.0179)
Between[f] Dd and [A+B]		1068		0.0853 (0.0095)		351		0.1200 (0.0198)

The number of base substitutions per site from averaging over all sequence pairs within and between groups is shown. Standard error estimate(s) are shown in parenthesis and were obtained by a bootstrap procedure (500 replicates). Codon positions included were 1st+2nd+3rd. Analyses were conducted using the Kimura 2-parameter method. Dl, *D. latum*; Dd, *D. dendriticum*.

[a] Number of isolates.
[b] Number of sites analysed.
[c] Number of variable sites.
[d] Genetic distance.
[e] Within-group divergence.
[f] Between-groups divergence.

4 to 6 in *D. nihonkaiense* and 4 to 11 in *D. klebanovskii*. The large number of lateral loops found in some of *D. klebanovskii* segments (up to 11) may show a wide range of variation, possibly resulting from differences in the number of eggs present and the state of contraction of the segment. Eggs in the uterine loops were also similar in size between *D. nihonkaiense* and *D. klebanovskii*, measuring approximately 60 by 39 μm. These findings clearly show that the morphology of *D. nihonkaiense* and *D. klebanovskii* is indistinguishable.

On the other hand, intrauterine eggs of *D. nihonkaiense* (*D. klebanovskii*) in the present materials were smaller than those of *D. latum* (av. 67 by 46 μm), and the genital pore and uterine pore were more narrowly separated in *D. nihonkaiense* (*D. klebanovskii*) (137–498 μm) than in *D. latum* (260–1, 240 μm) [16]. However, these and other measurements of *D. nihonkaiense* at least partly overlapped those of *D. latum*, indicating that morphological features of the adult cestode alone do not permit accurate differential diagnosis of *D. nihonkaiense* (*D. klebanovskii*) and *D. latum*.

4.2. Taxonomic relationship between D. nihonkaiense and D. klebanovskii

The present findings that 18S rDNA sequences of *D. nihonkaiense* and *D. klebanovskii* were 100% identical and that the ITS1, cox1, and nad3 gene sequences exhibited high levels of similarity (over 99.8%, 98.6% and 98.8%, respectively) clearly indicate that the two species are synonymous. This result is not surprising considering the geographical closeness of the two species and the exploitation of common second intermediate hosts, *Oncorhynchus* spp. The first intermediate host of *D. nihonkaiense* has been suggested to be the fresh water zooplanktonic copepod *Cyclops sternuus* based on experimental infection of coracidium larvae to cultured copepods [26], whereas the natural final hosts of *D. nihonkaiense* has been unknown. The present molecular studies confirmed for the first time that brown bears in the Far East serve as a definitive host of *D. nihonkaiense*, suggesting the contributions of terrestrial mammals and freshwater organisms in perpetuating the life cycle of *D. nihonkaiense*.

In1937, Rutkevich described *D. giljacicum* and *D. luxi* from native Evenki and Yakuts (Iakuts) peoples of Sakhalin [14]. The morphological descriptions of these two species are similar to those of *D. nihonkaiense* and *D. klebanovskii*. Later, *D. giljacicum* and *D. luxi* were listed as synonymous to *D. latum* [27]. However, Dovgalev and Valovaya [28] reviewed literatures and stated that '*D. latum* is absent from the basins of rivers of the Pacific coast of Russia; *D. luxi* is considered as a valid species, with *D. giljacicum*, *D. nihonkaiense* and *D. klebanovskii* as its junior synonyms'. In the present study, we could not obtain specimens of *D. giljacicum* or *D. luxi*, and were not able to determine their relationships to *D. nihonkaiense* and *D. klebanovskii*.

In the Pacific coast of North America, *D. ursi* has been isolated from brown bears (*Ursus arctos middendorffi*) in Kodiak Island, Alaska [15], black bears (*Ursus americanus*) and humans in Alaska [16], humans in North Vancouver, British Columbia [29] and black bears in Quebec [30]. *D. ursi* is a very large tapeworm that measures up to 11.7 m [15]. However, some *D. klebanovskii* isolates from Kamchatka brown bears were even longer: the longest tapeworm measured 19.4 m before fixation. One major difference between *D. ursi* and *D. nihonkaiense* (*D. klebanovskii*) is present in their plerocercoid stage: plerocercoids of *D. ursi* encyst on the serosa of the stomach of *O. nerka* [15] and occasionally in *O. kisutch* [16], while plerocercoids of *D. nihonkaiense* and *D. klebanovskii* have been found in the body musculature of *O. masou*, *O. gorbuscha* and *O. keta* [2–4,12]. The taxonomic relationship of *D. ursi* and *D. nihonkaiense* (*D. klebanovskii*) remains to be elucidated.

4.3. Mitochondrial DNA divergence in D. nihonkaiense (D. klebanovskii)

Mitochondrial DNA (mtDNA) evolves more rapidly than nuclear DNA, and often shows higher levels of intraspecific polymorphism [31]. Phylogenetic tree analyses based on the cox1 and nad3 sequences of *D. nihonkaiense* (*D. klebanovskii*) revealed two deeply divergent mtDNA lineages with genetic distances (Kimura-2 parameter) of 0.0175 for the combined data set of cox1 and nad3 and 0.0214 for nad3 (Table 3). These levels of divergence are smaller than those reported for congeneric species, but are well above the usual intraspecific values [32,33]. Furthermore, the distinct monophyletic groupings of cox1 haplotypes corresponded to the distinct monophyletic groupings of nad3 haplotypes. It is known that in closely related organisms that have recently diverged from a common ancestor, the phylogenetic relationship derived from different molecular markers often shows discordant patterns [34]. Thus, it appears that *D. nihonkaiense* (*D. klebanovskii*) is composed of at least two silent subsets that have diverged far enough in the past for complete lineage sorting to have occurred.

A growing number of recent genetic studies have identified morphologically cryptic species occurring in sympatry [35–41]. Several mechanisms have been proposed for diversification in sympatry, such as niche partitioning, feeding preferences [36,40], ancestral host switches during the evolution of the mutualism [37], and allopatric speciation and subsequent range shifts into sympatry [38]. The presence of two divergent lineages may be explained by demographic factors such as the geographic distribution of the population. In this respect, Japanese isolates do not necessarily provide accurate information because most of the Japanese patients lived in cities and probably caught the parasite by consuming wild Pacific salmon not only from Japanese rivers, but also from the Northern Pacific or imported from Far East Russia (or possibly Alaska). In contrast, the native Udygeyci people are thought to have only consumed locally caught wild salmon. The finding that 3 of 4 Udygeyci isolates belonged to lineage A and the other to lineage B indicate that lineage A and B definitely coexist in the same locality. Furthermore, bear

isolates from Kamchatka showed that the two lineages could even coexist in one individual host. Since *D. nihonkaiense* (*D. klebanovskii*) has only been found in the Northern Pacific rim of the Palaearctic and isolates in the present study came from a fairly wide range of the region, it is unlikely that hidden localities exist in the Palaearctic where lineage A or B is isolated. The evolutionary history for the two cryptic lineages of *D. nihonkaiense* (*D. klebanovskii*) remains to be elucidated.

It has been reported that partial gene fragments could be amplified from formalin-fixed tissues, even though formalin causes DNA fragmentation [42]. The present specimens included 6 that had been fixed in formalin for 2–7 years. PCR of these specimens and of specimens preserved in alcohol for more than 40 years amplified nad3 successfully, but failed to amplify ITS1 and cox1. In the present study gravid proglottids were employed for DNA retrieval. We speculate that DNA of the eggs in the uteri might have been less degraded by formalin than other tissues and was the major source of DNA. The unsuccessful amplification of ITS1 and cox1 seems to be at least partly due to the longer targeted sequences (649 bp and 748 bp for ITS1 and cox1, respectively) than that of nad3 (475 bp). However, nad3 amplification was not always successful: DNA fragments extracted from 5 other isolates of *D. nihonkaiense* which were preserved in formalin for half a year to 4 years did not yield amplification, suggesting that varying collection and storage conditions may give rise to prolonged or reduced DNA survival.

Taken together, the present findings show 1) the synonymy of *D. nihonkaiense* and *D. klebanovskii,* and 2) the coexistence of two mtDNA sequence lineages in the population of *D. nihonkaiense* (*D. klebanovskii*). There have been a few human cases of *D. nihonkaiense* acquisition after consumption of salmon (*O. keta*) possibly imported from Alaska or Canada [9,10]. These cases suggest that the geographical distribution of *D. nihonkaiense* (*D. klebanovskii*) extends to the Nearctic Northern Pacific rim. Molecular genetic studies of *Diphyllobothrium* spp. from this area might clarify the evolutionary history of *D. nihonkaiense* (*D. klebanovskii*) and its closely related species.

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