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USE OF NUCLEAR AND MITOCHONDRIAL DNA PCR AND SEQUENCING FOR MOLECULAR IDENTIFICATION OF *DIPHYLLOBOTHRIUM* ISOLATES POTENTIALLY INFECTIVE FOR HUMANS

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Summary:

Tapeworms of the genus *Diphyllobothrium* (Cobold, 1858) are widely distributed all around the world and some of them are agents of human diphyllobothriasis. Approximately 50 species have been described within the *Diphyllobothrium* genus but only 13 are human pathogens. Species identification by using morphological criteria is very difficult. We determined the value of 18S ribosomal RNA gene, internal transcribed spacer (ITS) and cytochrome c oxidase subunit 1 gene (COI) sequences to differentiate between *Diphyllobothrium* isolates. Sequences from 18 isolates (larvae or adults) of *D. latum*, *D. nihonkaiense*, *D. ditremum*, *D. dentriticum* and *D. stemmacephalum* species were obtained. COI region sequences analysis was clearly more discriminative than those of the ITS1 and 18S rRNA and was a useful tool for identifying specimens.

KEY WORDS : *Diphyllobothrium*, genotyping, 18S rRNA, ITS, COI, PCR.

Tapeworms of the genus *Diphyllobothrium* (Cobbold, 1858) are widely distributed all around the world and some of them are agents of human diphyllobothriasis, one of the most important fish-borne zoonosis caused by a cestode parasite. Piscivorous birds and mammals are definitive hosts. The intermediate hosts include both freshwater and marine fish, mainly anadromous. Humans can contract this parasite by eating raw or partially cooked fish containing *Diphyllobothrium* plerocercoids. This cestode is widely distributed in the wildlife among fish, mammal and birds hosts that make a important reservoir (Chai *et al.*, 2005). To date, approximately 50 species have been described within the *Diphyllobothrium* genus but only 13 have been reported as human pathogens; the most frequent species being *D. latum, D. nihonkaiense* and *D. dendriticum* (Chai *et al.*, 2005; Dick *et al.*, 2001). Other species such as *D. stemmacephalum* and *D. ditremum* occur only in animals. In 1999, the worldwide prevalence of human diphyllobothriasis was estimated at 9 million cases (Crompton, 1999) and some reports suggested that this parasitosis could emerge or re-emerge in Europe (Dupouy-Camet & Peduzzi, 2004) and emerge in South-America and other countries where only sporadic cases had been reported so far (Sampaio *et al.*, 2005). The different species infective for humans can be distinguished by morpho-anatomical criteria (Andersen & Halvorsen, 1978; Andersen & Gibson, 1989) but, these criteria are relative as they vary with age, with the degree of development and physiological modifications (Dick & Poole, 1985) and finally need a skilful parasitologist to be performed. Biochemical techniques (isoenzymatic assay, RFLP or immuno-electrophoresis) have been used as alternatives to the traditional tools for species identification (Fukumoto *et al.*, 1988; De Vos & Dick, 1989; Matsuura *et al.*, 1992) but the development of molecular biology resulted in a better knowledge of the *Diphyllobothrium* genus. Since 1998, interesting results in the study of the *Diphyllobothrium* genus were provided by sequences analysis of the 18S ribosomal RNA, cytochrome *c* oxidase subunit 1 (COI) and NADH dehydrogenase subunit 3 genes and of the partial internal transcribed spacer (ITSα2) region (Mariaux, 1998; Olson & Caira, 1999; *Isobe et al.*, 1998; Ando *et al.*, 2001; Logan *et al.*, 2004). In this study, we analysed by PCR and sequencing two nuclear (18S rRNA, ITS) and one mitochondrial (COI) sequences from *Diphyllobothrium* isolates with the aim to discriminate and identify isolates of this genus.

MATERIALS AND METHODS

SAMPLES

A total of 18 *Diphyllobothrium* specimens (larvae or adults) belonging to five species (*D. latum, D. nihonkaiense, D. dendriticum, D. ditremum* and *D. stemmacephalum*) were used (Table I) They were obtained from different geographic areas (Europe and South Korea) and from different hosts (fish, harbour porpoise and human) and identified by their

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Current identity	Stage	Countries of origin	Isolate	Donators	Host
D. latum	Adult	France (Leman lake)	AG	A. Gregory	Human
		France (Grenoble)	FD	H. Pelloux	
		France (Le Havre)	LF	L. Favennec	
	Larva	France (Leman lake)	JDC11.03	J. Dupouy-Camet	*Perca fluviatilis*
			JDC02.04	–	
			JDC04.04	–	
			JN02.05	J. Nicoulaud	
		Switzerland (Brienz)	ADB	A. De Bruyne	
		Italy (Maggiore lake)	BW1	R. Peduzzi & B. Wicht	
		–	BW2	–	
D. nihonkaiense	Adult	South Korea	KCH CSS PCH	J.Y Chai	Human
D. dendriticum	Larva	Norway (Fjellfrosvatn lake)	RK1 RK2	R. Knudsen	*Salvelinus alpinus*
D. ditremum	Larva	Norway (Fjellfrosvatn lake)	RK3 RK4	R. Knudsen	*Salvelinus alpinus*
D. stemmacephalum	Adult	Ukrain (Black sea)	SK	S. Krivokhrizin & A. Birkun	*Phocoena phocoena*

Table I. – Characteristics of the *Diphyllobothrium* specimens.

donators on morphological criteria. Another *Diphyllobothrium* specimen, previously described on the basis of mitochondrial sequences analysis (Yera *et al.*, 2006), was studied here for nuclear sequences. *Taenia saginata* proglottids obtained from a patient and *Proteocephalus* sp. isolates from Leman lake perchs were also analysed. All samples were stored in 60 % ethanol at – 20° C before analysis.

EXTRACTION OF DNA

DNA from the parasitic specimens were extracted by using QIAamp-DNA Mini kit with the tissue protocol (Qiagen, France) according to the manufacturer's instruction. The samples were incubated with ATL buffer and proteinase K for 1 h (larvae) or 4 h (proglottids).

AMPLIFICATION AND SEQUENCING

A partial region of the 18S rRNA gene was amplified using primers 81 and 83, designed for Eucestoda molecular phylogenetic study (Mariaux, 1998). The internal transcribed spacer regions (ITS1 and ITS2) including 5.8S rRNA gene were amplified using primers BD1 and BD2, originally designed for flukes analysis (Luton *et al.*, 1992). The cytochrome *c* oxidase subunit 1 gene (COI) was amplified by using JB3 and JB4.5 primers designed to analyse *Echinoccocus* sp. (Bowles *et al.*, 1992) and previously used to identify *Diphyllobothrium* species (Yera *et al.*, 2006). As for some specimens, no amplification was obtained, we designed new primers: JB6 (5'-GATAGTAAGGGTGTTGA-3') and JB5R (5'-CAAGTATCRTGCAAAATATTATCAAG-3') targeting a larger region. The PCR was performed with each primer pair in a 50 µl reaction volume, including 10 µl of DNA, in accordance with standard procedure for High Fidelity Platinium DNA polymerase (Invitrogen, France). After a denaturation step at 94° C for 7 min, amplification with 83 and 81 or BD1 and BD2 primers consisted of 38 cycles: 30 s at 94° C, 40 s at 55° C, 1 min 30 s at 68° C and a final elongation cycle of 10 min at 68° C. Amplification with JB3 and JB4.5 primer pair consisted of 41 cycles: 30 s at 94° C, 40 s at 50° C, 40 s at 68° C and a final elongation cycle of 10 min at 68° C. Ten microliters of sterile water was used as a negative control. PCR products were analysed after electrophoretic migration on 1 or 2 % agarose gel containing ethidium bromide. Then, they were purified by using QIAquick® PCR Purification Kit (Qiagen) according to the manufacturer's instruction.

Purified amplimers were automatically sequenced in both directions by using BigDye™ Terminator chemistry (Applera, France) using the four previously described primer pairs and supplemental primers which sequences are available on request.

PHYLOGENETIC CONSTRUCTIONS

Sequences obtained in this study were aligned with Clustal® W and minor corrections were made by using ChromasPro® 1.32 (Technelysium) and Bioedit® 5.0. Genetic distances were obtained from last alignments by using DNAdist® software. Phylogenetic constructions were generated with DNAmlk®. All the *Diphyllobothrium* sequences obtained in this study and some referent *Diphyllobothrium* sequences available in DDBJ/EMBL/GenBank databases were included in the phylo-

genetic analysis. *T. saginata*, *Proteocephalus* sp. and *Acanthocephalus* sp. sequences obtained in this study, by Nicoulaud *et al*. (2006) or in databases were used as outgroups. Bootstrap analysis was conducted by Seqboot® software. All the phylogenetic software were obtained from PHYLIP package (http://www.infobiogen.fr/).

RESULTS

Partial *Diphyllobothrium* 18S rRNA gene sequences were obtained from 1000 base pairs amplimers (bp) and were deposited in DDBJ/EMBL/GenBank databases under the following accession numbers DQ181941 to DQ181945 and DQ768152 to DQ768165. The alignment of these sequences showed a high identity (> 99 %) between *D. latum, D. nihonkaiense*, *D. dendriticum* and *D. ditremum*. *D. dendriticum* and *D. ditremum* had identical nucleic sequences. The *D. stemmacephalum* sequence was the most dissimilar and differed from the others by 2 to 3 %. The phylogenetic construction including referent *Diphyllobothrium* sequences showed a clear differentiation of *D. stemmacephalum* from other *Diphyllobothrium* species (Fig. 1). Partial *T. saginata* 18S rRNA sequence was obtained, deposited in databases (accession number DQ768166) and used as an outgroup together with already deposited *Acanthocephalus* sp. and *Proteocephalus* sp. sequences (Nicoulaud *et al*., 2006).

Complete *Diphyllobothrium* ITS1 and 5.8S rRNA gene sequences were obtained from 1100 bp amplimers and were deposited in databases (accession numbers DQ768167 to DQ768185). High identities (96 to 99 %) were found between *D. latum, D. nihonkaiense*, *D. dendriticum* and *D. ditremum*. The sequences of the two last species were identical. *D. stemmacephalum* showed again the most dissimilar sequence (10-11 %). The phylogenetic construction based on ITS1 sequences confirmed the separation of *D. stemmacephalum* from the others *Diphyllobothrium* species (Fig. 2). *Proteocephalus* sp. sequence was obtained, deposited in databases (DQ768186) and used as an outgroup with a referent *T. saginata* sequence.

Amplification of the partial COI gene was obtained with the JB3-JB4.5 primers from all samples except for

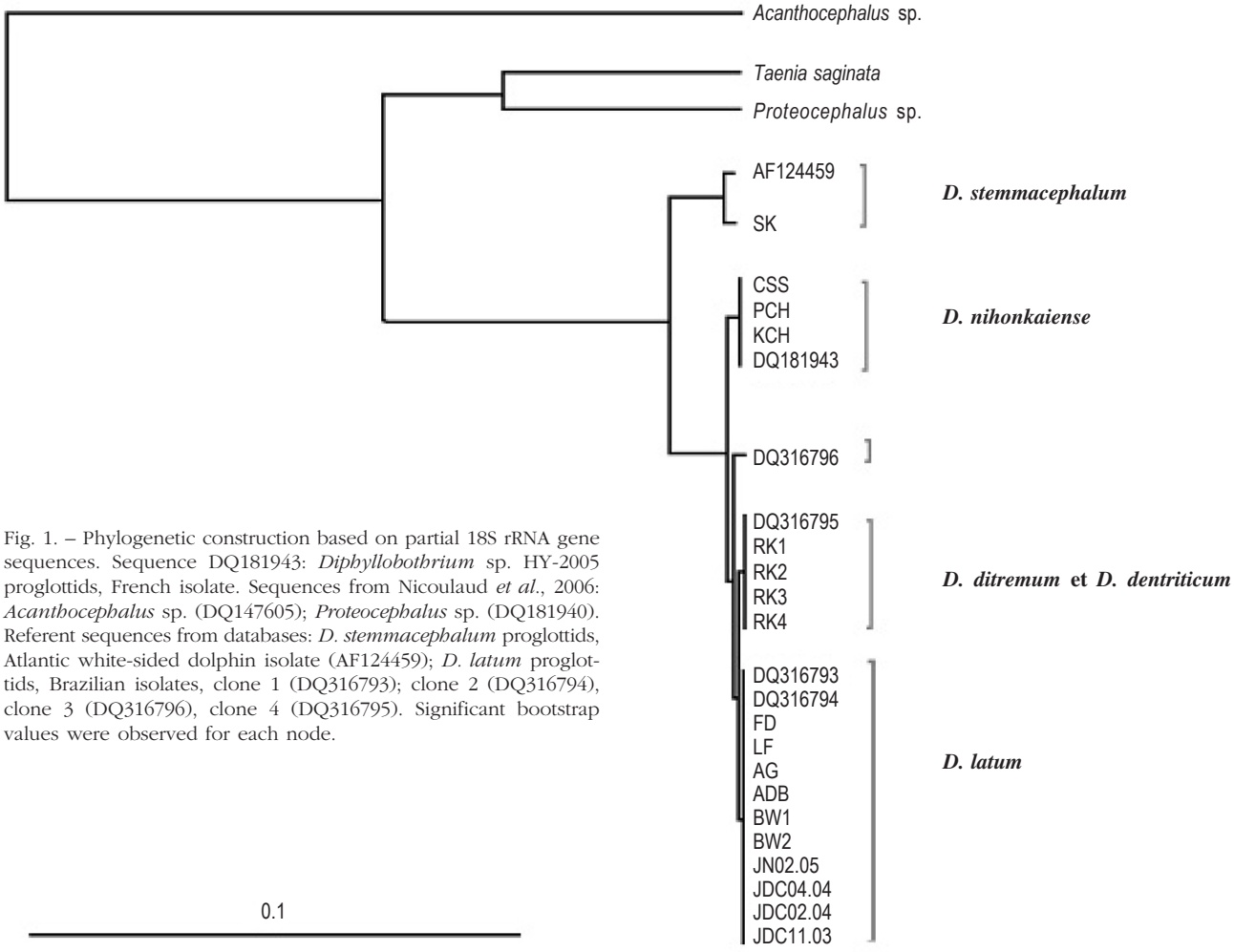

Fig. 1. – Phylogenetic construction based on partial 18S rRNA gene sequences. Sequence DQ181943: *Diphyllobothrium* sp. HY-2005 proglottids, French isolate. Sequences from Nicoulaud *et al*., 2006: *Acanthocephalus* sp. (DQ147605); *Proteocephalus* sp. (DQ181940). Referent sequences from databases: *D. stemmacephalum* proglottids, Atlantic white-sided dolphin isolate (AF124459); *D. latum* proglottids, Brazilian isolates, clone 1 (DQ316793); clone 2 (DQ316794), clone 3 (DQ316796), clone 4 (DQ316795). Significant bootstrap values were observed for each node.

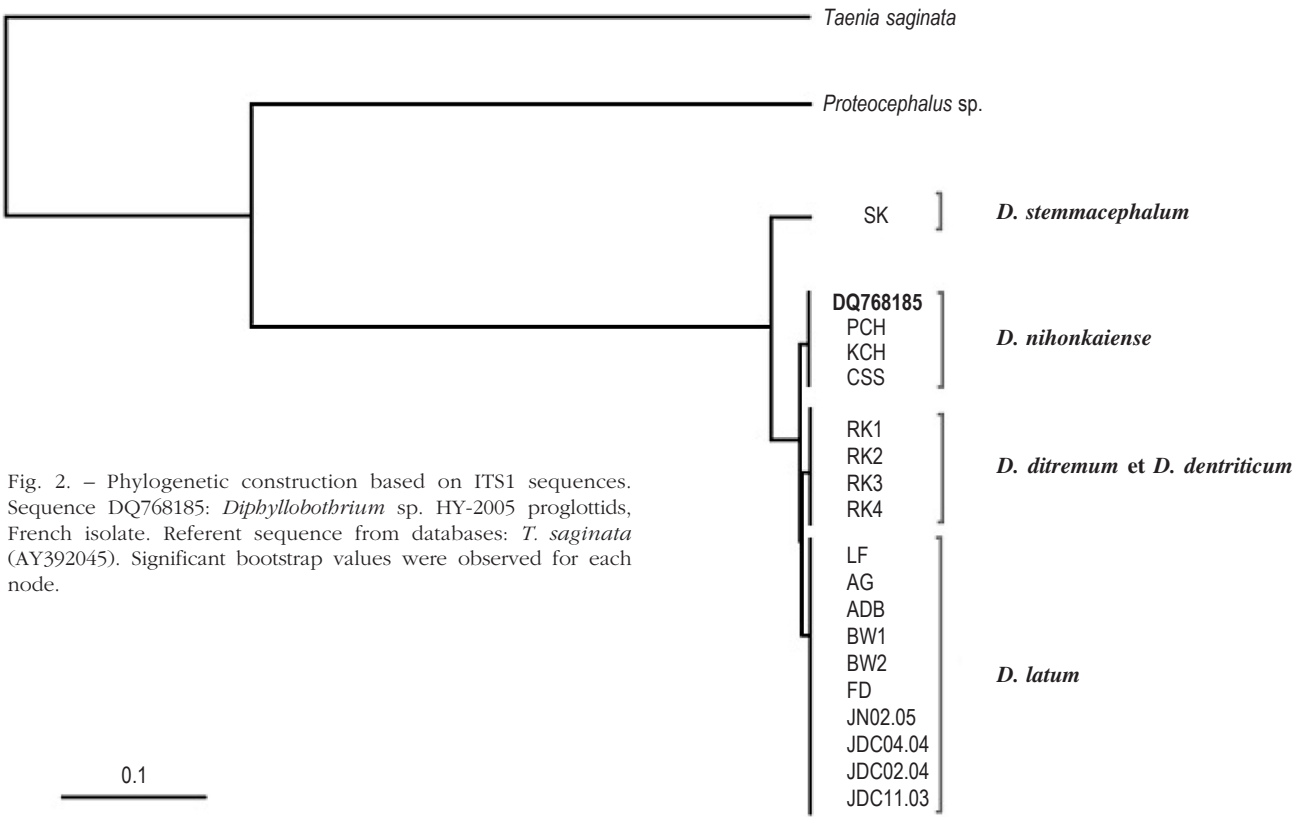

Fig. 2. – Phylogenetic construction based on ITS1 sequences. Sequence DQ768185: *Diphyllobothrium* sp. HY-2005 proglottids, French isolate. Referent sequence from databases: *T. saginata* (AY392045). Significant bootstrap values were observed for each node.

D. stemmacephalum. For this last species, DNA amplification was possible when the JB5R-JB6 primers were used. Amplimers sizes were 440 bp (JB3-JB4.5) and 650 bp (JB5R-JB6). Sequences were then determined and deposited in databases under the following accession numbers DQ768188 to DQ768191 and DQ768193 to DQ768206. These sequences were aligned on a region of 380 bp. *D. latum, D. nihonkaiense* and *D. dendriticum* showed 92 to 94 % identities. *D. ditremum,* showing 89 to 90 % identities with the previous last species, was clearly different from *D. dendriticum*. *D. stemmacephalum* differed from the others by 13 to 14 %. The phylogenetic construction including referent sequences allowed the differentiation of five genetic *Diphyllobothrium* groups (Fig. 3). Partial *T. saginata* and *Proteocephalus* sp. COI sequences, obtained in this study (accession number DQ768207 and DQ768192) were used as outgroups.

DISCUSSION

The aim of this study was to use molecular targets to identify parasites of the *Diphyllobotrium* genus. The 18S rRNA region showed less divergence (2-3 %) than the COI region (13-14 %). The 18S rRNA gene has already been described as a very conserved genomic target (Hillis & Dixon, 1991). Nevertheless, sequencing of this gene could discriminate *D. latum, D. nihonkaiense* and *D. stemmacephalum* but not *D. ditremum* and *D. dendriticum*. Similar results were found with ITS1, though the ITS1 sequences showed more variability than those of the 18S rRNA gene. A recent study using ITS2 sequences showed that *D. pacificum* was clearly different from other members of the genus and that ITS2 could not discriminate between *D. ditremum* and *D. dendriticum* (Skerikova *et al.*, 2006). COI gene analysis provided the most discriminative sequences between the species including the differentiation between *D. dendriticum* and *D. ditremum*. Mitochondrial sequences are known to be more polymorphic than the 18S rRNA and ITS1 nuclear sequences (Brown *et al.*, 1979). *D. dendriticum* and *D. ditremum* could be differentiated only by using the mitochondrial sequences. Both species are sharing the same geographic areas (circumpolar), the same intermediate (coregonids) and definitive hosts (fish eating birds and mammals). But, they differ as *D. dendriticum* is a human pathogen. Amino acids COI region alignments show that lysine at position 329 was changed to asparagin in *D. ditremum* sequences. Could this modification be related to the difference of pathogenicity between *D. dendriticum* and *D. ditremum*? Might they be two different variants of the same species? Our study also confirm the COI differences between *D. nihonkaiense* and *D. latum*

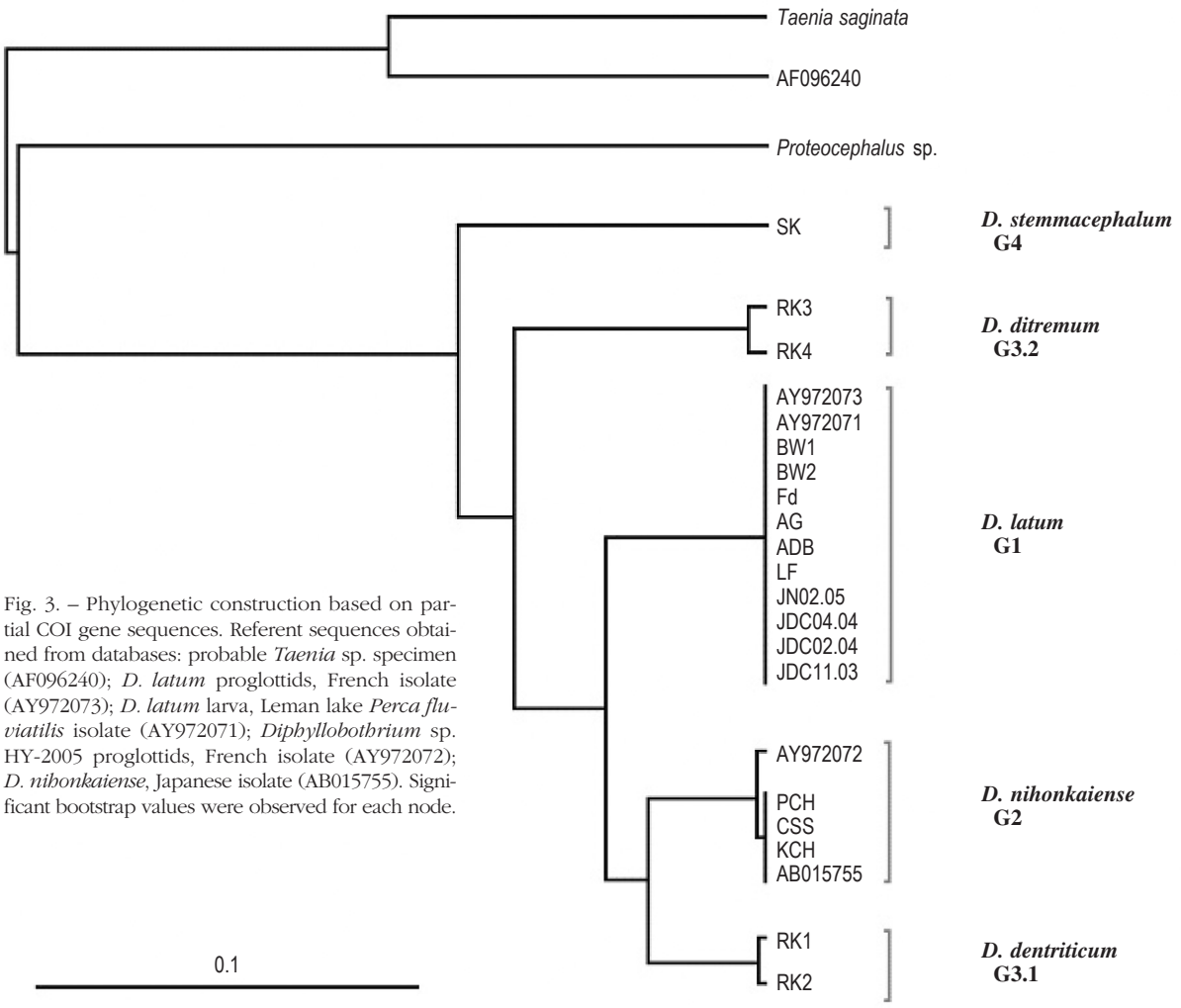

Fig. 3. – Phylogenetic construction based on partial COI gene sequences. Referent sequences obtained from databases: probable *Taenia* sp. specimen (AF096240); *D. latum* proglottids, French isolate (AY972073); *D. latum* larva, Leman lake *Perca fluviatilis* isolate (AY972071); *Diphyllobothrium* sp. HY-2005 proglottids, French isolate (AY972072); *D. nihonkaiense*, Japanese isolate (AB015755). Significant bootstrap values were observed for each node.

(Isobe *et al.*, 1998) and, in addition, found high similarities between *D. nihonkaiense* Korean isolates and one from databases. However, we observed slight differences with the sequence of a specimen obtained from a French patient who had eaten a Pacific salmon caught in Canada (Yera *et al.*, 2006). The sequences obtained from *D. latum* for the three different targets were identical between them and to those from the databases; except for two isolates from Brazil (DQ316795 and DQ316796) with different 18S rRNA sequences and which might be variant of *D. latum*. The ITS1 and COI sequences from these Brazilian isolates should be analyzed.

The growing popularity of raw fish dish has probably led to an increase of diphyllobothriasis in Europe and elsewhere (Chai *et al.*, 2005; Dick *et al.*, 2001; Sampaio *et al.*, 2005). Imported Pacific salmons or travel in endemic countries have led to the occurrence of cases of *D. nihonkaiense* or *D. dendriticum* in France and Switzerland (Yera *et al.*, 2006; Wicht *et al.*, 2007; Wicht *et al.*, 2008). In our study, the most discriminative of the three targets used for the identification of *Diphyllobothrium* specimens, was the cytochrome *c* oxidase subunit 1 gene and this target could be very useful in the identification of further specimens and in a precise classification of the species described in humans. This approach could also be used to identify the different larvae or worms *(Proteocephalus* sp., *Acanthocephalus* sp.) which can be found in the usual hosts of *Diphyllobothrium* (Nicoulaud *et al.*, 2006). Moreover, the recent total determination of the mitochondrial genomes of *D. latum* and *D. nihonkaiense* may also provide tools to study intraspecific variations (Nakao *et al.*, 2007).

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