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

YEZA H.*, NICOULAUD J.* & DUPOUY-CAMET J.*

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. dendriticum 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.

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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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 _Echinococcus_ 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’-GATAGTAAGGGGTGGTA-3’) and JB5R (5’-CAAGTATGCTGCAAATATATCATCGA-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 Platinum 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-

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<th>Isolate</th>
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Table I. – Characteristics of the _Diphyllobothrium_ specimens.
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

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**Diagram:**

- **Acanthocephalus sp.**
  - *Taenia saginata*
  - *Proteocephalus* sp.
  - *D. stemmacephalum*
  - *D. nihonkaiense*
  - *D. diremum et D. dendriticum*
  - *D. latum*

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.
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. stemmacephalum 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 Diphyllobothrium 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.
(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 I 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). More over, 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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