

## IS THE HUMAN-INFECTING *DIPHYLLOBOTHRIUM PACIFICUM* A VALID SPECIES OR JUST A SOUTH AMERICAN POPULATION OF THE HOLARCTIC FISH BROAD TAPEWORM, *D. LATUM*?

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**Abstract.** Using ITS2 gene sequences, the validity of the tapeworm *Diphyllobothrium pacificum* (Nybelin, 1931), infecting humans on the Pacific coast of South America and in Japan, was assessed. ITS2 sequences of this cestode differed markedly (sequence similarity 79.0–80.2%) from those of the most common human-infecting cestode, the broad fish tapeworm *Diphyllobothrium latum* (L.), as well as other four species of *Diphyllobothrium*, including potential human parasites (*D. cordatum*, *D. dendriticum*, and *D. lanceolatum*) and two species of *Spirometra* (sequence similarity 77.5–81.9%). Interspecific sequence similarity between all but one (*D. pacificum*) species was 86.1–99.6%, whereas individual isolates of *D. dendriticum* and *D. ditremum* exhibited intraspecific sequence similarity of 97.0–98.0% and 98.2–99.9%, respectively. Phylogenetic trees constructed from ITS2 sequences show a markedly distant position of *D. pacificum* from other species analyzed and also indicate the possible paraphyly of *Spirometra*.

### INTRODUCTION

Tapeworms of the genus *Diphyllobothrium* Cobbold, 1858 (Cestoda: Pseudophyllidea) are common intestinal parasites of birds and mammals. Some species have been reported to infect man, with estimated 20 million cases worldwide.<sup>1,2</sup> The most frequent human parasite is the broad fish tapeworm, *Diphyllobothrium latum* (L.), which occurs in some regions of North America (Alaska, Great Lakes), Europe, and Russia.<sup>2,3</sup> Another species, *Diphyllobothrium pacificum* (Nybelin, 1931) (syn. *Adenocephalus pacificum* Nybelin, 1931), is a parasite of fur seals and sea lions (Pinnipedia: Otariidae), originally described off the coast of Peru.<sup>4</sup> It has also been found in humans in Peru, Chile, Ecuador, and Japan,<sup>2,3,5–7</sup> where raw or undercooked fish are eaten.

Despite the fact that *D. pacificum* was differentiated from other *Diphyllobothrium* species by a number of morphological characters,<sup>4,5,8</sup> the validity and identity of this species found in humans was questioned because other species of the genus, such as *D. latum* and *D. dendriticum*, have been reported from fish and humans on the Pacific Coast of South America and in Brazil.<sup>2,5,9–15</sup> Therefore, a comparative study of several species of *Diphyllobothrium* and related genus *Spirometra* was carried out. Their phylogenetic relationships were assessed on the basis of the ITS2 gene sequences.

### MATERIALS AND METHODS

A total of 15 samples of six species of *Diphyllobothrium* and two species of *Spirometra* were sequenced. Adult tapeworms or plerocercoids were collected by the authors and other researchers (see the list of specimens sequenced) or obtained from the helminthological collections of the Natural History Museum in Vienna, Austria (Naturhistorisches Museum, Wien [NMW]) and the Parasitological Institute of the Russian Academy of Sciences, Moscow, Russia (previously

Helminthological Laboratory of the Academy of Sciences of the USSR [GELAN]).

1. *Diphyllobothrium cordatum* (Leuckart, 1863)—adult from the intestine of walrus (*Odobenus rosmarus*), Chukotka, Russia (fixed with formaldehyde) (GELAN; sample 04/27).
2. *Diphyllobothrium dendriticum* (Nitzsch, 1824)—plerocercoid from the body cavity of whitefish (*Coregonus lavaretus*), Loch Lomond, Scotland, UK, 12.9. 2002 (R. Kuchta; sample 02/29).
- 3–4. *D. dendriticum*—plerocercoids from the body cavity of whitefish (*C. lavaretus*), Loch Lomond, Scotland, 12.8.2004 (A.P. Shinn; samples 05/11 and 05/12f).
5. *Diphyllobothrium ditremum* (Creplin, 1825)—plerocercoid from the mesenteries of marine Atlantic salmon (*Salmo salar*), Isle of Arran, Scotland, UK, 23.3. 2001 (T. Scholz and R. Kuchta; sample 01/11).
6. *D. ditremum*—plerocercoid from the mesenteries of freshwater Atlantic salmon (*S. salar*), Loch Arkaig, Scotland, UK, 23.3. 2001 (T. Scholz and R. Kuchta; sample 01/12).
7. *D. ditremum*—plerocercoid from the mesenteries of brown trout (*Salmo trutta*), Loch Earn, Scotland, UK, 13.9. 2002 (R. Kuchta; sample No. 02/31).
8. *D. ditremum*—plerocercoid from the mesenteries of charr (*Salvelinus alpinus*), Loch Doyne, Scotland, UK, 12.9. 2002 (R. Kuchta; sample 02/32).
9. *D. ditremum*—plerocercoid from the mesenteries of rainbow trout (*Oncorhynchus mykiss*), Loch Leven, Scotland, 27.8.2004 (A.P. Shinn; sample 05/9).
10. *Diphyllobothrium lanceolatum* (Krabbe, 1865)—adult from the intestine of bearded seal (*Erignathus barbatus*), Jan Mayen (Wilesek; NMW Coll. 2687; sample 05/6).
11. *Diphyllobothrium latum* (Linnaeus, 1758)—plerocercoid from the mesenteries of burbot (*Lota lota*), Rybinsk water reservoir, Yaroslavl Region, Russia, VI. 2005 (L.G. Poddubnaya; sample 5/X).
12. *D. latum*—adult segregated from the intestine of man infected with plerocercoids from salmonid fish, Canada, 29.9.2005 (O. Ditrich; sample 05/60).

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13. *Diphyllobothrium pacificum* (Nybelin, 1931)—adult segregated from the intestine of man after anthelmintic treatment, Lima, Peru, 1.3.2005 (Cysticercosis Working Group in Peru; sample 05/16).
14. *Spirometra decipiens* (Diesing, 1850)—adult from the intestine of puma (*Felis concolor*), Brazil (Natterer; NMW 2682; sample 05/2).
15. *Spirometra folium* (Diesing, 1850) (*species inquirenda*<sup>16</sup>)—adult from the intestine of mongoose (*Herpestes lucurus*), Sannar, Sudan (Kotschy; NMW 2616; sample 05/3).

As the outgroup, the diphyllbothriid cestode *Schistocephalus solidus* (Müller, 1776), a common parasite of fish-eating birds and also reported infecting humans in Alaska,<sup>1</sup> was used (sequence AY549509).

Total DNA was extracted from 0.5 cm of strobila using the QIAamp Tissue Kit (Qiagen, Valencia, CA). To amplify ITS2 rDNA, the primers Proteo1/Proteo2 were used.<sup>17</sup> The PCR products were cloned into pGEM-T Easy (Promega, Madison, WI) and sequenced in both directions using T7 and SP6 primers. DNA sequencing was performed on the 310 ABI PRISM automated sequencer (PE Biosystems, Foster City, CA) using the GenomeLab DTCS-Quick Start Kit (Beckman, Fullerton, CA). The sequences were deposited in GeneBank under accession nos. DQ386120–DQ386135.

The sequences were aligned using the Megalign program (DNASTar, Nevada City, CA), and their similarity was determined using its Martinez/Needleman-Wunsch method (Table 1). The alignment was corrected manually using the Bioedit program.<sup>18</sup> The alignment is available from the first author on request (andrea@paru.cas.cz). The phylogenetic analysis and calculation of nodal support (MP, maximum parsimony; ME, minimum evolution and bootstrap) were performed using PAUP\* version 4.0b10.<sup>19</sup> MP analysis was performed by heuristic search (TBR) with 1,000 replicates of random sequence addition under the assumption of Tv/Ts ratio 1:1 and 1:2. Gaps were treated as missing data. Bootstrap support (1,000 replicates) was calculated for Tv/Ts 1:1. The distance method ME was performed using model Jukes-Cantor, bootstrap support was calculated for 1,000 replicates.

## RESULTS

The lengths of the ITS2 sequences of 15 samples analyzed varied from 661 bp in *Diphyllobothrium latum* (sample 05/60)

to 708 bp in *D. pacificum* (sample 05/16). Sequences of all but one species exhibited high similarity (86.1–99.6%; Table 1). *D. pacificum* was markedly distinct (Figures 1 and 2) and its ITS2 sequence similarity with other taxa was only 77.5–81.9%. From *D. latum*, the most common human-infecting species of the genus, the former taxon differs in almost 20% of base pairs (sequence similarity only 79.0–80.2%). Three other species of *Diphyllobothrium*, occasionally reported from man (i.e., *D. cordatum*, *D. dendriticum*, and *D. lanceolatum*), also differed from *D. pacificum* in as many as 20.4%, 18.1–21.5%, and 18.1% base pairs, respectively (Table 1).

Two species currently placed to *Spirometra*, namely *S. decipiens* and *S. folium*, were split among species of *Diphyllobothrium* (Figures 1 and 2), thus indicating possible paraphyly of the genus. Intraspecific differences were smaller than those found between species. Sequence similarity of three *D. dendriticum* samples, including plerocercoids collected simultaneously in two whitefish from the same locality, was 97.0–98.0%, whereas that among five isolates of *D. ditremum* was 98.2–99.9% only.

## DISCUSSION

These data strongly support the validity of *D. pacificum* as a species markedly distant from species of *Diphyllobothrium* reported from humans (i.e., *D. latum*, *D. cordatum*, *D. dendriticum*, and *D. lanceolatum*). The very low values of sequence similarity of *D. pacificum* with other taxa sequenced even indicate that *Adenocephalus*, in which the species was originally placed,<sup>4</sup> should still be retained as a valid genus.<sup>8</sup>

However, taxonomic conclusions cannot be made on the basis of these data because sequences of only one gene were used. In addition, the taxonomic importance of the morphological features used to distinguish *Adenocephalus* from other diphyllbothriids (i.e., the presence of numerous unicellular gland cells within the scolex [Drüsenzellen] and separate openings of the male [cirrus-sac] and female [vagina] genital pores [versus a common opening of both genital pores in other diphyllbothriid genera])<sup>4</sup> requires the critical evaluation of comparative material.

Samples of *D. dendriticum* and *D. ditremum* did not form monophyletic clades and were split among other species of *Diphyllobothrium* and *Spirometra* (Figures 1 and 2). The ME analysis provided more resolved relationships among indi-

TABLE 1  
Similarity of ITS2 sequences of *Diphyllobothrium* species from different hosts and localities

	04/27	02/29	05/11	05/12f	01/11	01/12	02/31	02/32	05/9	05/6	05/x	05/60	05/16	05/2	05/3
<i>D. cordatum</i> 04/27	—														
<i>D. dendriticum</i> 02/29	90.3	—													
<i>D. dendriticum</i> 05/11	90.7	98.0	—												
<i>D. dendriticum</i> 05/12f	89.7	97.0	97.0	—											
<i>D. ditremum</i> 01/11	90.4	99.6	98.1	97.1	—										
<i>D. ditremum</i> 01/12	90.4	99.6	98.1	97.1	99.7	—									
<i>D. ditremum</i> 02/31	90.7	98.2	99.3	97.1	98.2	98.2	—								
<i>D. ditremum</i> 02/32	90.8	98.4	99.6	97.4	98.5	98.5	99.7	—							
<i>D. ditremum</i> 05/9	90.7	98.2	99.4	97.2	98.4	98.4	99.6	99.9	—						
<i>D. lanceolatum</i> 05/6	90.6	99.4	97.9	97.0	99.6	99.6	98.1	98.4	98.2	—					
<i>D. latum</i> 05/x	89.9	95.6	96.4	94.5	95.7	95.7	96.7	96.9	96.7	95.7	—				
<i>D. latum</i> 05/60	86.1	91.6	92.4	90.6	91.8	91.8	92.7	92.8	92.7	91.7	93.6	—			
<i>D. pacificum</i> 05/16	79.6	81.9	81.2	78.5	81.8	81.8	81.3	81.3	81.2	81.9	80.2	79.0	—		
<i>Spirometra decipiens</i> 05/2	90.3	99.4	97.9	97.0	99.6	99.6	98.1	98.4	98.2	99.4	95.6	91.6	81.8	—	
<i>S. folium</i> 05/3	99.0	90.4	90.8	90.0	90.5	90.5	90.8	90.9	90.8	90.7	90.1	86.2	77.5	90.4	—

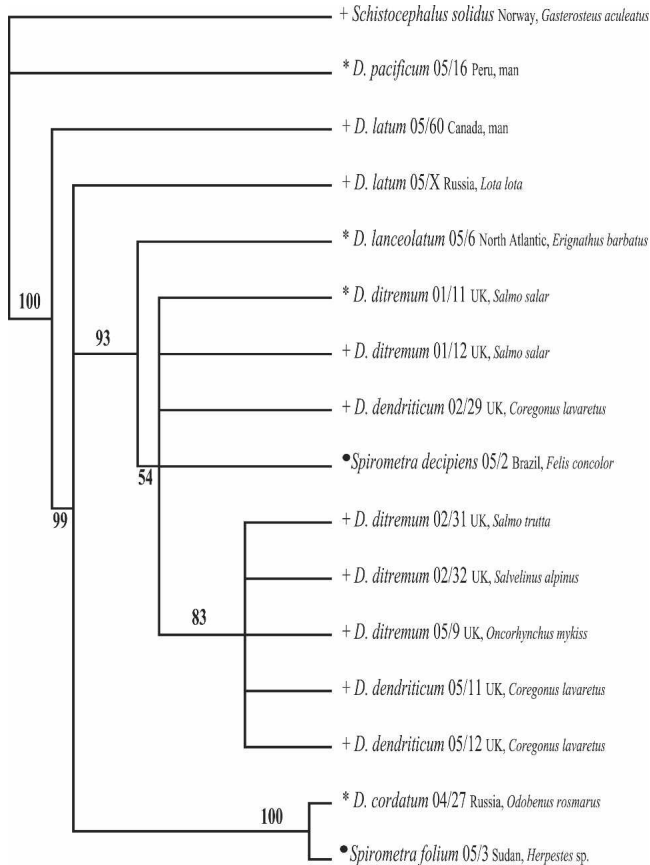


FIGURE 1. Strict consensus of 150 trees based on the MP analysis of ITS2 rDNA sequences under the assumptions of Tv/Ts ratio 1:1 (774 characters, 62 parsimony-informative characters, TL = 349, CI = 0.96). The numbers at the nodes show bootstrap values. The tree is identical to the topology based on Tv/Ts ratio 1:2. \*Marine species; +freshwater species; •terrestrial species.

vidual species, but did not contradict the less resolved topology of the MP analysis. Using single-strand conformation polymorphism-based analysis inferred from partial sequences of the cytochrome *c* oxidase subunit 1 gene (*pcox1*), marked intraspecific variation among isolates of *Spirometra erinacei* from definitive (dogs, foxes, cat) and intermediate (snakes, frogs) hosts was also found.<sup>20</sup>

Placement of two cestodes of *Spirometra* among species of *Diphyllobothrium* may indicate that the former genus is paraphyletic or that the two taxa sequenced actually belong to *Diphyllobothrium*. The systematics and taxonomic status of *Spirometra* have been controversial for a long time,<sup>21–23</sup> and it is obvious that more representative species sampling is necessary to make more definitive conclusions of the validity of this genus and its relationships to other diphyllobothriid genera. Another source of confusion may be related to the incorrect identification of the taxa studied, because a number of species of both genera have been established only on the basis of negligible or questionable morphological differences.

Notwithstanding taxonomic problems still existing in this group of pseudophyllidean cestodes, this study seems to provide convincing evidence that *D. pacificum* is a valid species that may have evolved as a parasite of otariid seals, long before human populations colonized the Pacific coast of South America.<sup>5</sup> Results of this study also support the as-

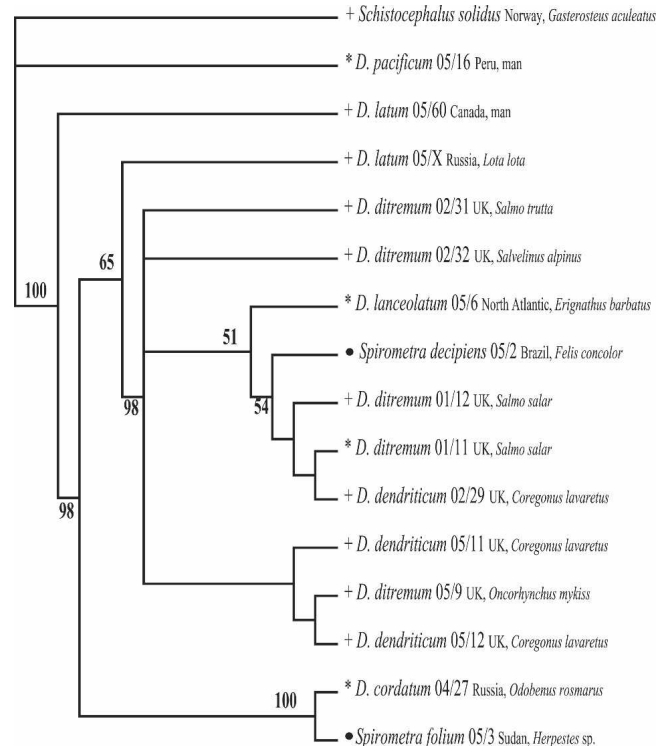


FIGURE 2. Strict consensus of three trees based on the ME analysis of ITS2 rDNA sequences (ME score = 0.57). The numbers at the nodes show bootstrap values. \*Marine species; +freshwater species; •terrestrial species.

sumption that the source of human infections with *D. pacificum* is sea fish, whereas human-infecting taxa of *Diphyllobothrium* widely distributed in the northern hemisphere, such as *D. latum* and *D. dendriticum*, are transmitted by freshwater fishes.<sup>10,11,13,14</sup>

Received February 14, 2006. Accepted for publication April 5, 2006.

Acknowledgments: The authors thank the following persons, who kindly provided them specimens for this study: Oleg Ditrich, Luboš Pialek, Larisa G. Poddubnaya, Ekaterina N. Protasova, Helmut Sattmann, Andy P. Shinn, and members of the Cysticercosis Working Group, Lima, Peru. Support of the Embassy of Peru in Prague and that of the Czech Republic in Lima, Peru, is also greatly appreciated. The authors also thank Václav Hypša for valuable advice concerning phylogenetic analyses.

Financial support: This study was financially supported by the Grant Agency of the Czech Republic (Projects 524/04/0342 and 524/03/H133) and research projects of the Institute of Parasitology, AS CR (Z60220518 and LC522) and the research project of the Faculty of Biological Sciences USB (NSM 6007665801). Stays of two of the authors (R.K. and T.S.) at the Institute of Aquaculture, University of Stirling, Scotland, were supported by the Access to Research Infrastructure Action of the Improving Human Potential Programme of the European Community (contract HPRI-CT-2001-00180), and their expeditions to Peru were realized on the basis of the joint project between the Institute of Parasitology, AS CR, and the Regional Government of Loreto (GOREL), Iquitos.

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