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Complete mitochondrial genomes of *Diplogonoporus balaenopterae* and *Diplogonoporus grandis* (Cestoda: Diphyllobothriidae) and clarification of their taxonomic relationships ☆

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ABSTRACT

Although the diplogonadic human tapeworm, *Diplogonoporus grandis*, has long been considered to be a synonym of the whale tapeworm, *Diplogonoporus balaenopterae*, the identity of the both species at the complete mitochondrial genomes and nuclear DNA levels has been not sufficiently undertaken to date. In the present study, to clarify the taxonomic relationships between *D. balaenopterae* and *D. grandis* at the molecular level, the complete mitochondrial genomes of both species were sequenced and compared. In addition, the genetic variation in the mitochondrial cytochrome *c* oxidase subunit 1 gene (*cox1*) and the nuclear internal transcribed spacer-1 (ITS-1) region of the ribosomal RNA gene were examined. The complete mitochondrial genomes of *D. balaenopterae* and *D. grandis* consisted of 13,724 bp and 13,725 bp, respectively. These mitochondrial genomes contained 12 protein-coding, 22 transfer RNA and 2 ribosomal RNA genes and two longer non-coding regions. Except for *Hymenolepis diminuta*, the genomic organization in both species was essentially identical to that in other cestode genomes examined to date. However, differences were observed between *Diplogonoporus* and *Diphyllobothrium* species in abbreviated stop codons, sequences and the number of repeat units in the 2nd non-coding regions. The genetic differences observed in the mitochondrial genomes, *cox1* and ITS-1 regions of both species were considered typical of intraspecific variation. In conclusion, *D. balaenopterae* is a taxonomically valid species and *D. grandis* is a junior synonym of *D. balaenopterae* based on the zoological nomenclature. Further, molecular-phylogenetic analysis confirmed that *D. balaenopterae* is more closely related to *Diphyllobothrium stemmacephalum*, the type-species of the genus *Diphyllobothrium*, and the taxonomical validity of the genera *Diplogonoporus* and *Diphyllobothrium* was also discussed.

1. Introduction

Broad tapeworms belonging to the genus *Diplogonoporus* Lönnberg, 1892, are characterized by having a double set of genitalia in a single proglottid, distinguishing them from *Tetragonoporus* Skriabin, 1961, *Hexagonoporus* Gubanov in Delyamure, 1955, and *Polygonoporus* Skriabin, 1967, which all possess multiple gonads in a single proglottid, and many members of *Diphyllobothrium* Cobbold, 1858, which usually have only one set of gonads per proglottid [1]. *Diplogonoporus balaenopterae* Lönnberg, 1892, infects the small intestine of whales, such as the minke whale (*Balaenoptera acutorostrata*, Balaenopteridae, Cetacea), sei whale (*Balaenoptera borealis*, Balaenopteridae, Cetacea) and humpback whale (*Megaptera novaeangliae*, Balaenopteridae, Cetacea) [1]. In contrast, *Diplogonoporus grandis* Lühe, 1899, which causes diplogonoporiasis in humans, has been regarded as a synonym of *D. balaenopterae*, based on adult tapeworm morphology [2–5], characteristics of larval coracidia and procercoids [6,7], and protein profiles [8]. Nevertheless, *D. grandis* has been referred to as the causative agent of human diplogonoporiasis, especially in Japan [9,10].

Diplogonoporiasis cases in humans have been found almost exclusively in Japan where more than 200 cases have been reported over the last 100 years [11]. Outside Japan, a total of 3 cases have been reported in Chile [12], Korea [13] and Spain [14]. In the latter two cases, the etiologic agents were identified as *D. balaenopterae* based on proglottid morphology. While the complete life cycles of these tapeworms have not yet been elucidated, Japanese anchovy or "shirasu" (*Engraulis japonica*, Engraulidae, Clupeiformes), Japanese sardine

Abbreviations: *atp6*, ATPase subunit 6 gene; *cob*, cytochrome *b* gene; *cox1–cox3*, cytochrome *c* oxidase subunits 1–3 genes; *nad1–nad6*, NADH dehydrogenase subunits 1–6 genes; *nad4L*, NADH dehydrogenase 4 large subunit gene; *rnl*, ribosomal RNA large subunit gene; *rns*, ribosomal RNA small subunit gene; *trn*, transfer RNA genes; PCR, polymerase chain reaction.

☆ Nucleotide sequences of the *D. balaenopterae* and *D. grandis* mitochondrial genomes reported in the present paper are deposited at the DDBJ/GenBank databases under accession numbers AB425839 and AB425840, respectively. AB355622–AB355626, AB355628, AB355629, AB474567, and AB474568 are the accession numbers for the *cox1* gene and AB449346–AB449356, AB474569, and AB474570 are the accession numbers for the ITS-1 regions.

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Table 1
Diplogonoporus isolates examined in the present study.

Sample no.	Year collected	Locality collected (latitude/longitude)	Host animals	DDBJ/GenBank accession numbers	
				cox1	ITS-1
No. 1	1997	Western North Pacific Ocean (N37/E160)	*Balaenoptera acutorostrata* (minke whale)	AB355622	AB449346
No. 2[a]	1997	Western North Pacific Ocean (N37/E163)	*B. acutorostrata*	AB355623	AB449347
No. 3	1997	Western North Pacific Ocean (N39/E161)	*B. acutorostrata*	AB355624	AB449342
No. 4	1997	Western North Pacific Ocean (N39/E161)	*B. acutorostrata*	AB355625	AB449349–AB449352
No. 5	1997	Western North Pacific Ocean (N39/E158)	*B. acutorostrata*	AB355626	AB449353
No. 6	2002	Western North Pacific Ocean	*Balaenoptera borealis* (sei whale)	AB474567	AB474569
No. 7	2002	Western North Pacific Ocean	*B. borealis*	AB474568	AB474570
No. 8[a]	2004	Tokyo, Japan	*Homo sapiens* (58-year-old Japanese man)	AB355628	AB449354
No. 9	2006	Hamamatsu, Shizuoka, Japan	*H. sapiens* (58-year-old Japanese man)	AB355629	AB449355, AB499356

[a] *Diplogonoporus* isolates used for the complete mitochondrial genome analysis.

(*Sardinops melanostictus*, Clupeidae, Clupeiformes) [9] and skipjack tuna (*Katsuwonus pelamis*, Scombridae, Perciformes) have been suspected to be the most likely sources of infection in humans [10].

To assess the phylogenetic relationships among eucestodes, genes such as the ribosomal RNA large subunit (28S rRNA) and small subunit (12S rRNA) genes [15–20], *cox1* and *nad3*[21], elongation factor-1 alpha gene [16], the internal transcribed spacer (ITS) regions [22,23] and the 18S rRNA gene [19,24] have been used. With regard to *Diplogonoporus* isolates, preliminary DNA analysis using *cox1* recently supported the assignment of *D. grandis* as a synonym of *D. balaenopterae* [25,26]. Genetic analysis of *Diplogonoporus* isolates from clinical cases has recently revealed a close relationship between *Diplogonoporus* and *Diphyllobothrium stemmacephalum*[24].

Thus, in the present study, the complete mitochondrial genomes of both species were sequenced and compared in order to clarify the molecular–taxonomic relationship between *D. balaenopterae* and *D. grandis*. In addition, the genetic variation within the *cox1* and ITS-1 regions was examined using nine *Diplogonoporus* isolates obtained from whales and humans. The need for a revision of the taxonomic affiliation of the genera *Diplogonoporus* and *Diphyllobothrium* is also discussed.

2. Materials and methods

2.1. Diplogonoporus tapeworms examined in the present study

The *Diplogonoporus* tapeworm specimens examined in this study are listed in Table 1. Minke whale and sei whale were taken in the Western North Pacific Ocean with special permission from The Institute of Cetacean Research, Japan. The seven mature *Diplogonoporus* tapeworms collected from the small intestines of the whales were identified as *D. balaenopterae* based on morphological characters (Nos. 1–7 in Table 1). Two diplogonadic tapeworms, one immature tapeworm lacking a scolex (No. 8) and a mature tapeworm with a scolex (No. 9), were obtained from two Japanese patients; these samples were identified morphologically as *D. grandis*. The tapeworms were rinsed thoroughly in phosphate-buffered saline after collection and preserved in 80% ethanol, except for two specimens (Nos. 6 and 9) which were fixed in 10% formalin.

2.2. DNA extraction, PCR amplification and DNA sequencing

DNA was extracted from the ethanol-fixed proglottids using a DNeasy Blood & Tissue kit (Qiagen, Germany) according to the manufacturer's instructions. The formalin-fixed specimens were embedded in paraffin and DNA was efficiently extracted from the unstained, 10 μm-thick sections using a DEXPAT kit (Takara Bio Inc., Japan) as described previously [26,27]. For analysis of the complete mitochondrial genome, DNA was extracted from two representative tapeworms fixed in ethanol (Nos. 2 and 8 in Table 1). Amplification of the mitochondrial genomes was performed using 13 primer pairs (Table 2) designed based on the mitochondrial genomes of *Diphyllobothrium nihonkaiense* (AB268585) and *Diphyllobothrium latum* (AB269325). The PCR consisted of an initial denaturation step of 98 °C for 30 s, followed by 35 cycles of 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 90 s, with a final extension cycle of 72 °C for 5 min. Samples were amplified in a final reaction volume of 50 μL with *Ex Taq* DNA polymerase (Hot Start version, Takara Bio Inc., Japan).

For the polymorphism analysis of the *cox1* and ITS-1 regions of the ethanol-fixed samples (Nos. 1, 3–5 and 7), primer pairs P1/P2 and P28/P31 were designed based on the nucleotide sequences of the *cox1* and ITS-1 regions in *Diplogonoporus* and *Diphyllobothrium*, respectively (Table 3). For the formalin-fixed samples (Nos. 6 and 9), short and overlapping DNA fragments were amplified using primer pairs P3–P27, and P28/P29 and P30/P31 (Table 3) for the *cox1* and the ITS-1 regions, respectively. High fidelity *KOD FX* DNA polymerase (Toyobo, Japan) was occasionally used for DNA samples extracted from formalin-fixed materials. PCR performed using *KOD FX* DNA polymerase employed an initial denaturation step of 94 °C for 15 min, followed by 35 cycles of 94 °C for 30 s, 58 °C for 30 s, 72 °C for 60 s and a final cycle of 72 °C for 5 min.

Amplicons confirmed by agarose gel or capillary electrophoresis (HAD-GT12, *e*Gene Inc., LA) were purified using a NucleoSpin Extract II kit (Macherey-Nagel, Germany) and used as templates for direct DNA sequencing. Samples for DNA sequencing were prepared using

Table 2
Oligonucleotide primers used for amplification of mitochondrial genomes.

Primer pairs	Nucleotide sequences (5′ to 3′)
P1 atp6/Fl	ATGATCTTCAGTGGTTATTCAAGTT
P2 nad1/R25	CACCTGTTAAAAACATAAAAATCAT
P3 trnA/F	ACAGAATACTGGGTTTGCGTCTCAG
P4 nad3/R60	AAATGATATGACTATAAACAACJAA
P5 nad3/F1	ATGTTAGCTTATTTTTTGGTGG
P6 rnL/R250	CTATACACATTTACITGTCTCCTC
P7 trnT/F38	CAGGGGTGGGTTTACTCTTGGGCCT
P8 trnC/R25	TACTAAGACCAAAGGCAATAGACTT
P9 rnL/F451	CATATTATAAATTTTATATGTAGG
Pl0 trnC/R25	TACTAAGACCAAGGCAATAGACTT
P11 rnL/F880	TGAGGTGAGTTAAGACCGGCGTGAG
P12 rnS/R245	ATTTCACCTACTCTTACCTTTACCT
P13 trnC/F4O	GTGAATATTGTTTATTCTAGGCTTT
P14 cox2/R25	CGTAGTACAGCAAAGAAAATTTCAT
P15 rnS/F570	GTAACAAGGTAGCCCAGATGAATC
P16 trnE/R25	TTATGCTCCAATACAACAAAACAGG
P17 cox2/F525	GGTGGGTCACEGTTATATGCCIATA
P18 nad/R761	CAAGTGGATATGGCAACTATCTTCT
P19 nad5/F565	CTACCCCTGTTAGTTCTTTAGTACA
P20 cox3/R205	TACCAAAGGCTAAAACITCIAAG
P21 trnG/F40	GTGGGGATCTAATGGTTTTAGATAA
P22 trnH/R25	GCCAGTTTAAATAACCTATCAGTAA
P23 cox3/F444	GGTTCTAGATTTTATGCTAGTTGT
P24 nad4/R 325	ACAGAGGTAACATGGATAGCTCATA
P25 nad4/F1	ATGAGAGTGTACAAAATTATTAGAT
P26 atp/R25	AATCTTGAATAACCATAAAGATCAT