Rapid identification of nine species of diphyllobothriidean tapeworms by pyrosequencing

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The identification of diphyllobothriidean tapeworms (Cestoda: Diphyllobothriidea) that infect humans and intermediate/paratenic hosts is extremely difficult due to their morphological similarities, particularly in the case of Diphyllobothrium and Spirometra species. A pyrosequencing method for the molecular identification of pathogenic agents has recently been developed, but as of yet there have been no reports of pyrosequencing approaches that are able to discriminate among diphyllobothriidean species. This study, therefore, set out to establish a pyrosequencing method for differentiating among nine diphyllobothriidean species, Diphyllobothrium dendriticum, Diphyllobothrium ditremum, Diphyllobothrium latum, Diphyllobothrium nihonkaiense, Diphyllobothrium stemmacephalum, Diplogonoporus balaenopterae, Adenocephalus pacificus, Spirometra decipiens and Sparganum proliferum, based on the mitochondrial cytochrome c oxidase subunit 1 (cox1) gene as a molecular marker. A region of 41 nucleotides in the cox1 gene served as a target, and variations in this region were used for identification using PCR plus pyrosequencing. This region contains nucleotide variations at 12 positions, which is enough for the identification of the selected nine species of diphyllobothriidean tapeworms. This method was found to be a reliable tool not only for species identification of diphyllobothriids, but also for epidemiological studies of cestodiasis caused by diphyllobothriidean tapeworms at public health units in endemic areas.

The order Diphyllobothriidea (Platyhelminthes: Cestoda) is a large group of tapeworms that parasitize mammals, birds, amphibians and reptiles. The order also comprises several important genera of human parasites, such as Diphyllobothrium, Adenocephalus, Diplogonoporus and Spirometra. Diphyllobothriasis is a zoonosis of which adult-stage tapeworms of the genus Diphyllobothrium, specifically Diphyllobothrium latum and D. nihonkaiense, are the most frequent etiologic agents in humans. Diphyllobothrium dendriticum, Adenocephalus pacificus (syn. Diphyllobothrium pacificum), and Diphyllobothrium stemmacephalum have rarely been detected in humans. The life cycle of the Diphyllobothrium tapeworm consists of intermediate hosts (copepods and fish) and definitive hosts (e.g., marine mammals, carnivores, humans and piscivorous birds). Once the eggs hatch in water, the ciliated coracidia are ingested by copepods and develop into procercoid larvae. When infected copepods are engulfed by a second intermediate host, the procercoid larva migrates to the musculature and the body cavity (mainly stomach, liver and other organs) and develops into a plerocercoid larva. Humans become infected by eating raw or undercooked fish containing plerocercoids, which subsequently develop into adult tapeworms in the human intestine. Symptoms of diphyllobothriasis include diarrhoea, loose bowels and light abdominal pain or discomfort. Most of the cases are asymptomatic. Heavy infections may cause intestinal obstruction, and motile proglottids can result in cholecystitis or cholangitis. Prolonged or severe D. latum infection can cause megaloblastic anaemia.

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Human diplophlobothriasis refers to a tapeworm infection caused by Diplogonoporus balaenopterae (syn. Diplogonoporus grandis)²⁰, which usually infects minke whale, sei whale and humpback whale¹¹ and has been found almost exclusively in Japan. Although the details of its life cycle are not clear, various marine fish, such as raw whitebait, are suspected as potential sources of human infection.¹⁰,¹¹ Human sparganosis is a harmful infective zoonosis caused by plerocercoid larvae (e.g., sparganum) belonging to the genus Spirometra or Sparganum. Sparganosis is classified into two forms, nonproliferative and proliferative. The former is caused by Spirometra erinaceieuropaei and Spirometra decipiens in China, Japan, Korea, Taiwan and Thailand and Spirometra mansonomoides in the USA.¹² The latter is caused by the more pathogenic and disseminating Sparganum proliferum, which is reported as occurring only sporadically in Japan, Taiwan and Thailand and very rarely in Paraguay, Venezuela, and the USA.²⁶,²⁷ Spirometra species require two intermediate hosts for the completion of their life cycle. Copepods serve as the first intermediate host for the procercoid larvae. Vertebrates (reptiles, amphibians, birds, and mammals) serve as the second intermediate and paratenic hosts for the plerocercoid larvae. Human infection occurs through the ingestion of water polluted with copepods carrying procercoids or through ingesting raw or undercooked meat such as frog, snake, or chicken infected with plerocercoids.¹³,¹⁴ The plerocercoid migrates predominantly to subcutaneous tissue, but it also affects the brain and eyes. Sparganum of Spirometra species can rarely develop into adult worms in the human intestine. The details of the Sp. proliferum life cycle are not clear and its adult stage is unknown.

Since the eggs, larvae and adult tapeworms of the genera Diphyllobothrium, Adenocephalus and Spirometra are morphologically similar, their identification in human tissues is exceedingly difficult and requires special expertise. Only one study has successfully used morphometric and ultrastructural (surface morphology) egg features to distinguish among the 8 species of diphyllobothrids.²⁰ For these reasons, many molecular methods using different genetic markers have been developed for the identification of diphyllobothriid tapeworms: cox1 for Diphyllobothrium²¹–²³ and S. erinaceieuropaei,²⁶,²⁷ sdhB for Sp. proliferum and S. erinaceieuropaei,²⁸,²⁹ and nad3 for Sp. proliferum. These molecular methods are reliable and valuable tools for the identification of diphyllobothriidean tapeworms. Pyrosequencing is a DNA sequencing method that utilizes enzyme-coupled reactions and bioluminescence to monitor the pyrophosphate (PPi) release accompanying nucleotide incorporation using the synthesis of short nucleotide fragments that are directed by the sequence in real-time.³⁷ The method is principled on the real-time monitoring of 4 enzymes during DNA synthesis by luminescence using a step that results in a detectable light signal upon nucleotide incorporation. The detection is based on the PPi released when a nucleotide is incorporated into the DNA strand. The signal can be measured according to the number of bases added. This method can be used for mutation detection and single-nucleotide polymorphism (SNP) genotyping of large samples of screening material and high-throughput DNA analysis techniques.³⁸ The method, an alternative method, has been used for species-specific discrimination of Entamoeba species and for identification of various parasite taxa such as Plasmodium, Trichinella species, and lymphatic filaria. Here we developed a pyrosequencing methodology that characterizes cox1 gene amplicons for the identification of diphyllobothriidean tapeworms in humans and intermediate hosts.

**Results**

Based on sequence alignments of the cox1 gene from the nine diphyllobothriid species shown in Table 1, the 41 nucleotides following the 3' end of the sequencing primer presented high accuracy and were used as the target region for species identification (Fig. 1 and Table 2). The nucleotide sequence pattern in the target region of each diphyllobothriidean species is shown as pyrogram results (Fig. 2). Of the 41 nucleotides, 12 positions were sufficiently variable to discriminate among the diphyllobothriidean species (Tables 2 and 3). Diphyllobothrium dendriticum and A. pacificus were found to show intra-specific variation (Table 2). The pyrosequencing reproducibility was confirmed for the amplicons by Sanger sequencing conducted at First BASE Laboratories Sdn Bhd (Selangor, Malaysia) using the BigDye terminator v3.1 cycle-sequencing kit (Applied Biosystems (ABI), Carlsbad, CA), and both strands were directly sequenced using the PCR primers as sequencing primers (Model 310 or 3100, ABI), which yielded identical sequence data. No PCR products could be obtained from DNA samples of other parasites, human leukocytes or uninfected fish, proving the high specificity of the PCR primers.

**Discussion**

The taxonomy of the diphyllobothriidean tapeworms generally depends on morphological classification, but morphological characteristics are not always decisive criteria, particularly in larval and immature worms, or inadequately preserved specimens. Diphyllobothrium dendriticum, D. latum, D. nihonkaiense, A. pacificus and Spirometra species are of medical importance and have caused an emerging public health problem, especially in countries in which fish consumption is expanding and new cooking habits involving raw or uncooked processing are gaining ground.³⁳,³⁴ For the differential detection of diphyllobothriid tapeworms in diagnostic laboratories, rapid, specific and cost-effective tools for routine diagnosis of parasites have been developed.³⁵–³⁷ Molecular methods for differentiation among diphyllobothriideans are important in situations in which the morphology and epidemiological information are similar.³⁴,³⁵,³⁶ Many molecular methods have been developed for the identification of diphyllobothriidean tapeworms, for example, multiplex PCR for D. latum, D. dendriticum, D. nihonkaiense, and A. pacificus,³⁵,³⁶ PCR for study of the taxonomic relationship between Di. balaenopterae and Di. grandis,¹⁰ and PCR-restriction fragment length polymorphism for Diphyllobothrium and Diplonoporus species.³⁸ However, the limitation with these methods is that analyzing the results requires gel electrophoresis, which is imprecise, has restricted throughput and can only be used to differentiate up to eight species.³⁹ Moreover, the differentiation among D. ditremum, Di. balaenopterae, S. decipiens and Sp. proliferum has not been evaluated. This prompted us to seek out a new tool for elucidation, namely pyrosequencing.⁴⁰ The pyrosequencing operating system is divided into two modes, sequence analysis (SQA) and SNP. These are obtainable for handling, and each mode can detect sequenced nucleotides in a different manner. The SQA mode
| Species          | Stage    | Host                          | Locality             | Year of isolation |
|------------------|----------|-------------------------------|----------------------|-------------------|
| D. dendriticum   | Plerocercoid | Clausous gull (Larus hyperboreus) | Kansas, USA          | 2001              |
|                  | Plerocercoid | Rainbow trout (Oncorhynchus mykiss) | Kelly Peterson Lake, Alaska, USA | 2001              |
|                  | Plerocercoid | O. mykiss                     | Lake Llanquihue, Los Lagos, Chile | 2009              |
|                  | Plerocercoid | O. mykiss                     | Lake Llanquihue, Los Lagos, Chile | 2009              |
|                  | Plerocercoid | O. mykiss                     | Lake Llanquihue, Los Lagos, Chile | 2009              |
|                  | Plerocercoid | O. mykiss                     | Lake Llanquihue, Los Lagos, Chile | 2009              |
|                  | Plerocercoid | O. mykiss                     | Lake Llanquihue, Los Lagos, Chile | 2009              |
|                  | Plerocercoid | O. mykiss                     | Lake Llanquihue, Los Lagos, Chile | 2009              |
|                  | Plerocercoid | O. mykiss                     | Lake Llanquihue, Los Lagos, Chile | 2009              |
|                  | Plerocercoid | O. mykiss                     | Lake Llanquihue, Los Lagos, Chile | 2009              |
|                  | Plerocercoid | O. mykiss                     | Lake Llanquihue, Los Lagos, Chile | 2009              |
|                  | Plerocercoid | O. mykiss                     | Lake Panguipulli, Los Rios, Chile | 2014              |
| D. ditremum      | Plerocercoid | White char (Salvelinus albus)  | Azabache Lake, Kamchatka, Russia | 1992              |
| D. latum         | Adult     | Human                         | Santiago, Chile      | 2009              |
|                  | Plerocercoid | Coho salmon (Oncorhynchus kisutch) | Lake Llanquihue, Los Lagos, Chile | 2009              |
|                  | Plerocercoid | O. kisutch                   | Lake Llanquihue, Los Lagos, Chile | 2009              |
| D. nihonkaiense  | Adult     | Human                         | Tokyo, Japan         | 2008              |
|                  | Adult     | Human                         | Tokyo, Japan         | 2008              |
|                  | Adult     | Human                         | Tokyo, Japan         | 2008              |
|                  | Adult     | Human                         | Tochigi, Japan       | 2008              |
|                  | Adult     | Human                         | Hamamatsu, Japan     | 2008              |
|                  | Adult     | Human                         | Tokyo, Japan         | 2009              |
|                  | Adult     | Human                         | Hamamatsu, Japan     | 2010              |
|                  | Adult     | Human                         | Nagasaki, Japan      | 2010              |
| D. stemmacephalum| Adult     | Human                         | Kanagawa, Japan      | 2015              |
| Di. balaenopterae| Adult     | Minke whale (Balaenoptera acutorostrata) | North Pacific Ocean | 1997              |
|                  | Adult     | B. acutorostrata              | North Pacific Ocean  | 1997              |
| A. pacificus     | Adult     | Human                         | Santiago, Chile      | 2000              |
| S. decipiens     | Plerocercoid | Japanese striped snake (Elaphe quadrivirgata) | Tokyo, Japan         | 2008              |
|                  | Plerocercoid | E. quadrivirgata              | Tokyo, Japan         | 2012              |
| Sp. proliferum   | Plerocercoid | Mouse (Mus musculus)          | Origin is from Venezuela, maintained in Japan | 2011              |

Table 1. Diphyllobothriid samples tested by pyrosequencing.

sequences short and medium-length DNA fragments (approximate size of 25–50 bp)\(^46\), whereas the SNP mode searches only for single base variations. Pyrosequencing has been used in various applications such as differentiation of \textit{Rickettsia} species in hard tick samples\(^49\) and detection of benzimidazole-resistance-associated \(\beta\)-tubulin SNPs in cattle nematodes\(^50\). It is also suitable for identifying, screening and/or predicting resistant strains of bacteria for antibiotic drugs\(^51\). Here, we report on a new tool for rapid identification of nine diphyllobothriidean tapeworms using pyrosequencing. This method can identify formalin-fixed samples that has degraded due to formalin fixation is suitable for pyrosequencing. In this study, we found that pyrosequencing was able to identify formalin-fixed \textit{A. pacificus} (Fig. 2G).

The pyrosequencing technique developed in this study is a high-throughput tool that can be completed within 4 hours (excluding the DNA extraction period) and can achieve results from 96 samples simultaneously. The approximate estimated cost is $5 per test. However, the cost can vary depending upon the commercial origin of there agents used. In addition to its medical applications, this pyrosequencing technique can be useful for identification of larvae isolated from copepods, eggs, and adults among different taxa that share similar morphology. Pyrosequencing is a promising alternative high-throughput method for molecular genotyping of nine species of diphyllobothriidean tapeworms and can have important implications for epidemiological studies. This not only applies to endemic countries but also non-endemic areas in which travellers coming from endemic areas bring in the parasites\(^52\).

Materials and Methods

**Parasite materials.** Specimens of diphyllobothriidean tapeworms (larval and adult stages) were collected from intermediate and definitive hosts from different geographical localities (Table 1). A total of 29 diphyllobothriidean tapeworm specimens, including \textit{D. dendriticum}, \textit{D. ditremum}, \textit{D. latum}, \textit{D. nihonkaiense}, \textit{D. stemmacephalum}, \textit{Di. balaenopterae}, \textit{A. pacificus}, \textit{S. decipiens} and \textit{Sp. proliferum} were examined (Table 1). The diphyllobothriid specimens examined in this study were fixed in 70% ethanol, with the exception of \textit{A. pacificus}, which was fixed in 10% formalin.

Genomic DNA samples from non-diphyllobothriid parasites were used for the evaluation of primer specificity. These included samples from trematodes (\textit{Opisthorchis viverrini}, \textit{Clonorchis sinensis}, \textit{Fasciola gigantica},...
Paragonimus heterotremus, Schistosoma japonicum, Schistosoma mekongi, Haplorchis taichui, Stellantchasmus sp., Centrocestus sp., Echinostoma malayanum, intestinal lecithodendriid flukes), nematodes (Trichuris trichiura, hookworms, Strongyloides stercoralis, Ascaris lumbricoides, Capillaria philippinensis, Trichostrongylus sp.) and protozoa (Isospora belli, Giardia duodenalis). DNA samples from uninfected fish and human leukocytes were also analyzed.

Ethical Approval. All methods in the study protocol were approved and were performed in accordance with the relevant guidelines and regulations of the ethical approval in the Ethics Committee in the National Institute of Infectious Diseases, Tokyo, Japan (No. 177). No experiment involving human subjects was used. All parasites were derived from leftover specimens (informed consent was obtained from all subjects and the data were analyzed anonymously) or from specimens bought at markets. We confirmed that the process did not involve endangered or protected species.

Primer design. Primer design was based on nucleotide alignment of cox1 from D. dendriticum, D. ditremum, D. latum, D. nihonkaiense, D. stemmacephalum, Di. balaenopterae, A. pacificus, S. decipiens and Sp. proliferum (see Table 2 for GenBank accession nos.) The following
primers were designed from *A. pacificus* sequence (GenBank accession no. AB548654): our primer sets (Pseudo_F; 5′-TTTGGGTAGTGTTGTGGG-3′, corresponding to positions 846–865 and biotinylated Pseudo_R; biotin-5′-GGCTCACGTAAAGAAACACGACT-3′, corresponding to positions 1010-988) and Pseudo_S sequencing primer (5′-GGGGTCATCATATGTTA-3′, corresponding to positions 863–880) were designed using PyroMark Q96ID software version 2.0 (Biotage, Uppsala, Sweden) (Fig. 1).

*Diphyllobothrium latum* (GenBank accession no. AM712906) is the reference sequence that was used to determine the nucleotide positions.

DNA extraction, plasmid preparation, DNA amplification by polymerase chain reaction (PCR) and pyrosequencing. Genomic DNA samples from 29 diphyllobothrid species were produced by ligation of each species-specific amplicon into a pGEM-T easy vector (Promega, WI), as has been described previously in other reports. Each inserted gene was Sanger sequenced in both directions and the resulting sequences were identical to the gene sequences from which the primers were designed.

The *cox1* gene fragments (165 bp) were PCR amplified from the diphyllobothrid DNA. The total PCR reaction volume was 25μl and included 2μl of the DNA template, 2.5μl of 10× PCR buffer (Roche Applied Science, Mannheim, Germany), 0.2 mM of dNTP, 2 mM MgCl2, 0.2μM of Pseudo_F primer, 0.2μM of biotinylated Pseudo_R primer and 0.625 U of FastStart High Fidelity Enzyme Blend (Roche Applied Science). PCR was performed at 94 °C for 5 min followed by 35 cycles of denaturation at 95 °C for 30 sec, annealing at 48 °C for 30 sec, and extension at 72 °C for 30 sec, followed by a final extension at 72 °C for 10 min. PCR products were visualized [Figure 2](#). Theoretical pyrogram patterns (top of each panel) and representative raw data of control plasmids or analytical samples from pyrosequencing (bottom of each panel) are shown. The actual sequence obtained by pyrosequencing is displayed below the panels following “Seq”. The Y-axis shows the level of fluorescence emitted by the incorporation of a nucleotide base, and the X-axis shows total number of bases added at that point in time; G, C, T, A, nucleotide bases. The underlined letters indicate the nucleotides used for identification of diphyllobothridian tapeworms.

![Figure 2](image-url)

*Figure 2. Theoretical pyrograms and pyrograms of control plasmids or analytical sample raw reads of 9 diphyllobothrid species.* Pyrograms of the 41-base fragment of the *cox1* gene from *D. dendriticum* (A) *D. ditremum* (B) *D. latum* (C) *D. nihonkaiense* (D) *D. stemmacephalum* (E) *D. balaenopterae* (F) *A. pacificus* (G) *S. decipiens* (H) and *Sp. proliferum* (I). Theoretical pyrogram patterns (top of each panel) and representative raw data of control plasmids or analytical samples from pyrosequencing (bottom of each panel) are shown. The actual sequence obtained by pyrosequencing is displayed below the panels following “Seq”. The Y-axis shows the level of fluorescence emitted by the incorporation of a nucleotide base, and the X-axis shows total number of bases added at that point in time; G, C, T, A, nucleotide bases. The underlined letters indicate the nucleotides used for identification of diphyllobothridian tapeworms.
Table 3. The nucleotide patterns used to differentiate diphyllobothriidean tapeworms by the pyrosequencing technique. *Nos dif. is the number of nucleotide differences.

| Genus species | D. dendriticum | D. ditremum | D. latum | D. nihonkaiense | D. stemmacephalum | D. balaenopterae | A. pacificus | S. decipiens | Sp. proliferum |
|---------------|----------------|-------------|----------|-----------------|------------------|----------------|-------------|-------------|--------------|
|               | nos dif. positions | nos dif. positions | nos dif. positions | nos dif. positions | nos dif. positions | nos dif. positions | nos dif. positions | nos dif. positions | nos dif. positions |
| D. dendriticum | — | — | — | — | — | — | — | — | — |
| D. ditremum    | 5 | A882G, A/C885G, T906C, C912T, T921A | — | — | — | — | — | — | — |
| D. latum       | 4 | A882G, A/C885G, C912T, T921A | 1 | C906T | — | — | — | — | — |
| D. nihonkaiense| 2 | A/C885G, T921G | 4 | G882A, C906T, T912C, A891G | — | — | — | — | — |
| D. stemmacephalum| 3 | C912T, T918G, T921A | 4 | G882A, C906G, T914G | — | — | — | — | — |
| D. balaenopterae| 6 | A882G, A897G, G903A, C912T, T918G, T921A | 4 | G882A, C906G, T914G | 7 | A882G, A897G, G903A, C912T, T918G, T921A | 3 | A882G, A897G, G903A | — | — |
| A. pacificus    | 8 | A882G, T918G, T919C, G921A | 8 | G882A, T918G, T919C | 9 | A882G, A897G, G903A | 8 | A882G, T918G, T919C | — | — |
| S. decipiens   | 6 | A882T, A/C885G, T988G, A919G, A897G, G903T | 7 | G882T, T988G, A919G, A897G, G903T, T912C, A921T | 8 | G882T, T988G, A919G, A897G, G903T, T912C, A921T | 9 | A882T, A895G, C889G, A919G, A897G, G903T, T912C, G918T, A921T | — | — |
| Sp. proliferum | 9 | A882T, A/C885G, T888G, A919G, A897G, G903T, T909G, C912T, T921A | 7 | G882T, T888G, A919G, A897G, G903T, T909G | 8 | G882T, T888G, A919G, A897G, G903T, T909G, C912T, A921T | 7 | G882T, A895G, C889G, A919G, A897G, G903T, T909G, G918T | 3 | T909G, C912T, T921A | — | — |

using 1.5% agarose gel electrophoresis. After PCR amplification, biotinylated PCR products were added into determining the exact length of a homopolymer can be a problem for this technique. In cases which the target and generate light. Because the added nucleotide is known, the sequence of the template can be determined. Light is subsequently converted to ATP by ATP sulfurylase, which provides the energy to luciferase to oxidize luciferin and generate light. Because the added nucleotide is known, the sequence of the template can be determined. Light is only generated when a newly added nucleotide is complementary to the next unpaired base in the template strand. The intensity of light is proportional to the number of sequential identical bases in a homopolymer, but determining the exact length of a homopolymer can be a problem for this technique. In cases where the target sequence showed up to four homopolymers, the readout was analyzed manually to ensure accuracy.

References
1. Schmidt, G. D. In CRC handbook of tapeworm identification (CRC Press Inc, 1986).
2. Bray, R. A., Jones, A. & Andersen, K. I. Order Pseudophyllidea Carus, 1863 in Keys to the Cestode Parasites of Vertebrates (eds Khalil, L. F. & Bray, R. A.) 205–247 (CAB International, 1994).
3. Chai, J. Y., Murrell, K. D. & Lymbery, A. J. Fish-borne parasitic zoonoses: status and issues. Int J Parasitol 35, 1233–1254 (2005).
4. Scholz, T., Garcia, H. H., Kuchta, K. I. & Wicht, B. Update on the human broad tapeworm (genus Diphyllobothrium), including clinical relevance. Clin Microbiol Rev 22, 146–160 (2009).
5. Schauer, E. et al. Travel-acquired subcutaneous Sparganum proliferum infection diagnosed by molecular methods. Br J Dermatol 170, 741–743 (2014).
6. Kuchta, R., Scholz, T., Brabec, J. & Wicht, B. Diphyllobothrium, Diplonoporus, and Spironemella in Biology of Foodborne Parasites (eds Xiao, L., Ryan, U. & Feng, Y.) 299–326 (CRC Press Taylor & Francis group, 2015).
7. Dick, T. A., Nelson, P. A. & Choudhury, A. Diphyllobothriasis: update on human cases, foci, patterns and sources of human infections and future considerations. Southeast Asian J Trop Med Public Health 32, 79–86 (2001).
8. Yamasaki, H., Tsumakura, D., Mercado, R. & Kuramochi, T. A simple method for identifying the diphyllobothriids based on mitochondrial DNA analysis in Parasitological Research: Materials and Methods (ed. Takamiya, S.) 47–49 (Sankeisha, 2014).
9. Yamasaki, H. et al. First confirmed human case of Diphyllobothrium stemmacephalum infection and molecular verification of the synonymy of Diphyllobothrium yonagoense with D. stemmacephalum (Cestoda: Diphyllobothriidae). Parasitol Int 65, 412–442 (2016).
10. Yamasaki, H., Ohmae, H. & Kuramochi, T. Complete mitochondrial genomes of Diplonoporus balaenopterae and Diplonoporus grandis (Cestoda: Diphyllobothriidae) and clarification of their taxonomic relationships. Parasitol Int 61, 260–266 (2012).
11. Kamo, H. Guide to identification of diphyllobothrid cestodes (ed. Hirai, K.) 1–146 (Gendaikakaku, 1999). (In Japanese.)
12. Kamo, H. Cestodes in Progress of Medical Parasitology in Japan. Vol. 8 (eds Otsura, M., Kamegai, S. & Hayashi, S.) 235–243 (Meguro Parasitological Museum Press, 2003).
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**Author Contributions**

T.T., P.M.I., H.Y. and W.M. conceived and designed the experiments; C.T., O.S. and S.T. performed the experiments; T.T., V.L. and W.M. analyzed the data; P.M.I., H.Y. and W.M. contributed reagents/materials/analysis tools; T.T., H.Y. and W.M. wrote the paper and reviewed the manuscript.

**Additional Information**

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