SCIENTIFIC REPORTS

OPEN

Rapid identification of nine species of diphylobothriidean tapeworms by pyrosequencing

Tongjit Thanchomnang^{1,2}, Chairat Tantrawatpan^{2,3}, Pewpan M. Intapan^{2,4},
Oranuch Sanpool^{1,2,4}, Viraphong Lulitanond^{2,5}, Somjintana Tourtip¹, Hiroshi Yamasaki⁶ &
Wanchai Maleewong^{2,4}

Received: 20 July 2016

Accepted: 26 October 2016

Published: 17 November 2016

The identification of diphylobothriidean tapeworms (Cestoda: Diphylobothriidea) that infect humans and intermediate/paratenic hosts is extremely difficult due to their morphological similarities, particularly in the case of *Diphylobothrium* 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 diphylobothriidean species. This study, therefore, set out to establish a pyrosequencing method for differentiating among nine diphylobothriidean species, *Diphylobothrium dendriticum*, *Diphylobothrium ditremum*, *Diphylobothrium latum*, *Diphylobothrium nihonkaiense*, *Diphylobothrium 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 diphylobothriidean tapeworms. This method was found to be a reliable tool not only for species identification of diphylobothriids, but also for epidemiological studies of cestodiasis caused by diphylobothriidean tapeworms at public health units in endemic areas.

The order Diphylobothriidea (Platyhelminthes: Cestoda) is a large group of tapeworms that parasitize mammals, birds, amphibians and reptiles^{1,2}. The order also comprises several important genera of human parasites, such as *Diphylobothrium*, *Adenocephalus*, *Diplogonoporus* and *Spirometra*^{3–6}. Diphylobothriasis is a zoonosis of which adult-stage tapeworms of the genus *Diphylobothrium*, specifically *Diphylobothrium latum* and *D. nihonkaiense*, are the most frequent etiologic agents in humans^{3,4,7,8}. *Diphylobothrium dendriticum*⁶, *Adenocephalus pacificus* (syn. *Diphylobothrium pacificum*⁶), and *Diphylobothrium stemmacephalum*⁹ have rarely been detected in humans. The life cycle of the *Diphylobothrium* 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 proceroid larvae. When infected copepods are engulfed by a second intermediate host, the proceroid larva migrates to the musculature and the body cavity (mainly stomach, liver and other organs) and develops into a plerocercoid larva⁴. Humans become infected by eating raw or undercooked fish containing plerocercoids, which subsequently develop into adult tapeworms in the human intestine⁴. Symptoms of diphylobothriasis 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^{4,6}.

¹Faculty of Medicine, Mahasarakham University, Mahasarakham, 44000, Thailand. ²Research and Diagnostic Center for Emerging Infectious Diseases, Khon Kaen University, Khon Kaen, 40002, Thailand. ³Division of Cell Biology, Department of Preclinical Sciences, Faculty of Medicine, Thammasat University, Rangsit Campus, Pathum Thani, 12120, Thailand. ⁴Department of Parasitology, Faculty of Medicine, Khon Kaen University, Khon Kaen, 40002, Thailand. ⁵Department of Microbiology, Faculty of Medicine, Khon Kaen University, Khon Kaen, 40002, Thailand. ⁶Department of Parasitology, National Institute of Infectious Diseases, Tokyo, 162-8640, Japan. Correspondence and requests for materials should be addressed to W.M. (email: wanch_ma@kku.ac.th)

Human diplogonoporiasis refers to a tapeworm infection caused by *Diplogonoporus balaenopterae* (syn. *Diplogonoporus grandis*¹⁰), which usually infects minke whale, sei whale and hump back whale¹¹ and has been found almost exclusively in Japan^{10,12,13}. The details of its life cycle are not clear, but various marine fish, such as raw whitebait, are suspected as potential sources of human infection^{14,15}. Human sparganosis is a harmful infectious 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 China¹⁶, Japan¹⁷, Korea^{18,19}, Taiwan²⁰ and Thailand²¹ and *Spirometra mansonioides* in the USA²². The latter is caused by the more pathogenic and disseminating *Sparganum proliferum*, which is reported as occurring only sporadically in Japan²³, Taiwan²⁴ and Thailand²⁵ 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^{21,28}. 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 intestine²⁹. 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 *Diphyllbothrium*, *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 diphyllbothriids³⁰. For these reasons, many molecular methods using different genetic markers have been developed for the identification of diphyllbothriid tapeworms: *cox1* for *Diphyllbothrium*^{31–34} and *S. erinaceieuropaei*^{16,35}, *sdhB* for *Sp. proliferum* and *S. erinaceieuropaei*³⁶, and *nad3* for *Sp. proliferum*⁵. These molecular methods are reliable and valuable tools for the identification of diphyllbothriidean 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-time³⁷. The method is principally 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 techniques³⁸. The method, an alternative method, has been used for species-specific discrimination of *Entamoeba* species³⁹ and for identification of various parasite taxa such as *Plasmodium*⁴⁰, *Trichinella* species⁴¹, *Paragonimus*⁴² and lymphatic filaria⁴³. Here we developed a pyrosequencing methodology that characterizes *cox1* gene amplicons for the identification of diphyllbothriidean tapeworms in humans and intermediate hosts.

Results

Based on sequence alignments of the *cox1* gene from the nine diphyllbothriid 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 diphyllbothriidean species is shown as pyrogram results (Fig. 2). Of the 41 nucleotides, 12 positions were sufficiently variable to discriminate among the diphyllbothriidean species (Tables 2 and 3). *Diphyllbothrium 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 diphyllbothriidean tapeworms generally depends on morphological classification, but morphological characteristics are not always decisive criteria, particularly in larval and immature worms, or inadequately preserved specimens. *Diphyllbothrium 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^{6,33,44}. For the differential detection of diphyllbothriidean tapeworms in diagnostic laboratories, rapid, specific and cost-effective tools for routine diagnosis of parasites have been developed^{46,8,33}. Molecular methods for differentiation among diphyllbothriideans are important in situations in which the morphology and epidemiological information are similar^{6,34,45,46}. Many molecular methods have been developed for the identification of diphyllbothriidean tapeworms, for example, multiplex PCR for *D. latum*, *D. dendriticum*, *D. nihonkaiense*, and *A. pacificus*³³, PCR for study of the taxonomic relationship between *Di. balaenopterae* and *Di. grandis*¹⁰, and PCR-restriction fragment length polymorphism for *Diphyllbothrium* and *Diplogonoporus* species⁸. 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 species³⁰. 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 pyrosequencing⁴⁷.

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
<i>D. dendriticum</i>	Plerocercoid	Glaucous gull (<i>Larus hyperboreus</i>)	Kansas, USA	2001
	Plerocercoid	Rainbow trout (<i>Oncorhynchus mykiss</i>)	Kelly Peterson Lake, Alaska, USA	2001
	Plerocercoid	<i>O. mykiss</i>	Lake Llanquihue, Los Lagos, Chile	2009
	Plerocercoid	<i>O. mykiss</i>	Lake Llanquihue, Los Lagos, Chile	2009
	Plerocercoid	<i>O. mykiss</i>	Lake Llanquihue, Los Lagos, Chile	2009
	Plerocercoid	<i>O. mykiss</i>	Lake Llanquihue, Los Lagos, Chile	2009
	Plerocercoid	<i>O. mykiss</i>	Lake Llanquihue, Los Lagos, Chile	2009
	Plerocercoid	<i>O. mykiss</i>	Lake Panguipulli, Los Rios, Chile	2014
<i>D. ditremum</i>	Plerocercoid	White char (<i>Salvelinus albus</i>)	Azabachye Lake, Kamchatka, Russia	1992
<i>D. latum</i>	Adult	Human	Santiago, Chile	2009
	Plerocercoid	Coho salmon (<i>Oncorhynchus kisutch</i>)	Lake Llanquihue, Los Lagos, Chile	2009
	Plerocercoid	<i>O. kisutch</i>	Lake Llanquihue, Los Lagos, Chile	2009
<i>D. nihonkaiense</i>	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
<i>D. stemmacephalum</i>	Adult	Human	Kanagawa, Japan	2015
<i>Di. balaenopterae</i>	Adult	Minke whale (<i>Balaenoptera acutorostrata</i>)	North Pacific Ocean	1997
	Adult	<i>B. acutorostrata</i>	North Pacific Ocean	1997
	Adult	<i>B. acutorostrata</i>	North Pacific Ocean	1997
<i>A. pacificus</i>	Adult	Human	Santiago, Chile	2000
<i>S. decipiens</i>	Plerocercoid	Japanese striped snake (<i>Elaphe quadrivirgata</i>)	Tokyo, Japan	2008
	Plerocercoid	<i>E. quadrivirgata</i>	Tokyo, Japan	2012
	Plerocercoid	<i>E. quadrivirgata</i>	Tokyo, Japan	2012
<i>Sp. proliferum</i>	Plerocercoid	Mouse (<i>Mus musculus</i>)	Origin is from Venezuela, maintained in Japan	2011

Table 1. Diphyllbothriid samples tested by pyrosequencing.

sequences short and medium-length DNA fragments (approximate size of 25–50 bp)⁴⁸, whereas the SNP mode searches only for single base variations. Pyrosequencing has been used in various applications such as differentiation of *Rickettsia* species in hard tick samples⁴⁹ and detection of benzimidazole-resistance-associated β -tubulin SNPs in cattle nematodes⁵⁰. It is also suitable for identifying, screening and/or predicting resistant strains of bacteria for antibiotic drugs⁵¹. Here, we report on a new tool for rapid identification of nine diphyllbothriidean tapeworms using pyrosequencing. This method can identify *D. dendriticum*, *D. ditremum*, *D. latum*, *D. nihonkaiense*, *D. stemmacephalum*, *Di. balaenopterae*, *A. pacificus*, *S. decipiens* and *Sp. proliferum* (Table 1) at the species level. As another advantage of pyrosequencing, short PCR products are sufficient for this analysis. Thus, DNA from 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 *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 these 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 diphyllbothriidean 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^{5,52}.

Materials and Methods

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

Genomic DNA samples from non-diphyllbothriid parasites were used for the evaluation of primer specificity. These included samples from trematodes (*Opisthorchis viverrini*, *Clonorchis sinensis*, *Fasciola gigantica*,

Figure 1. Multiple sequence alignments for indicating primer annealing positions and diagnostic target region. Nucleotide alignment of the mitochondrial *cox1* gene derived from *D. dendriticum*, *D. ditremum*, *D. latum*, *D. nihonkaiense*, *D. stemmacephalum*, *Di. balaenopterae*, *A. pacificus*, *S. decipiens* and *Sp. proliferum*. The solid arrows indicate the position of Psedo_F (forward primer) and biotinylated Psedo_R (reverse primer). The broken arrow indicates Psedo_S (sequencing primer). The boxed region shows the position (881–921) of the target area used for species-level identification. Dots indicate identical nucleotides between sequences. AM712906 (*D. latum*) is the reference sequence for nucleotide positions.

Species (Accession No.)	Nucleotide positions											
	882	885	888	889	891	897	903	906	909	912	918	921
<i>Diphyllobothrium dendriticum</i> (JN152995, AB374223, KC812048, AM412738)	A	A/C	T	T	A	A	G	T	T	C	T	T
<i>Diphyllobothrium ditremum</i> (DQ768196, FM209182)	G	G	T	T	A	A	G	C	T	T	T	A
<i>Diphyllobothrium latum</i> (AM712906*, AB269325)	G	G	T	T	A	A	G	T	T	T	T	A
<i>Diphyllobothrium nihonkaiense</i> (AB544064, AM412559)	A	G	T	T	A	A	G	T	T	C	T	G
<i>Diphyllobothrium stemmacephalum</i> (JQ268543, DQ768191)	A	A	T	T	A	A	G	T	T	T	G	A
<i>Diplogonoporus balaenopterae</i> (AB355624)	G	A	T	T	A	G	A	T	T	T	G	A
<i>Adenocephalus pacificus</i> (AB548652, AB548653, AB548654)	G	A	G	C	T/C	T	G	A	C	T	T	G/T
<i>Spirometra decipiens</i> (KJ599679)	T	G	G	T	G	G	T	T	T	C	T	T
<i>Sparganum proliferum</i> (AB015753)	T	G	G	T	G	G	T	T	G	T	T	A

Table 2. Variable *cox1* nucleotide positions used for the differentiation of diphyllobothriidean tapeworms. *AM712906 is the reference sequence for nucleotide positions.

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 left over 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* (syn. *D. pacificus*); and *S. decipiens*, and *Sp. proliferum* (see Table 2 for GenBank accession nos.) The following

Figure 2. Theoretical pyrograms and pyrograms of control plasmids or analytical sample raw reads of 9 diphyllbothriid 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) *Di. 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 diphyllbothriidean tapeworms.

primers were designed from *A. pacificus* sequence (GenBank accession no. AB548654): our primer sets (Pseudo_F; 5'-TTTGGGTAGTGTGTGTGGG-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'-GGGGTCATCATATGTTTA-3', corresponding to positions 863–880) were designed using PyroMark Q96ID software version 2.0 (Biotage, Uppsala, Sweden) (Fig. 1). *Diphyllbothrium 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 diphyllbothriid specimens (Table 1) were extracted using a DNeasy Blood & Tissue kit (Qiagen, Hilden, Germany). In addition, positive control plasmids for all diphyllbothriidean 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 reports⁵³. 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 diphyllbothriidean DNA. The total PCR reaction volume was 25 µl and included 2 µl of the DNA template, 2.5 µl of 10× PCR buffer (Roche Applied Science, Mannheim, Germany), 0.2 mM of dNTP, 2 mM MgCl₂, 0.2 µM of Pseudo_F primer, 0.2 µM 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

Genus species	<i>D. dendriticum</i>		<i>D. ditremum</i>		<i>D. latum</i>		<i>D. nihonkaiense</i>		<i>D. stemmacephalum</i>		<i>Di. balaenopterae</i>		<i>A. pacificus</i>		<i>S. decipiens</i>		<i>Sp. proliferum</i>	
	*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
<i>D. dendriticum</i>		—		—		—		—		—		—		—		—		—
<i>D. ditremum</i>	5	A882G, A/C885G, T906C, C912T, T921A		—		—		—		—		—		—		—		—
<i>D. latum</i>	4	A882G, A/C885G, C912T, T921A	1	C906T		—		—		—		—		—		—		—
<i>D. nihonkaiense</i>	2	A/C885G, T921G	4	G882A, C906T, T912C, A921G	3	G882A, T912C, A921G		—		—		—		—		—		—
<i>D. stemmacephalum</i>	3	C912T, T918G, T921A	4	G882A, G885A, C906T, T918G	3	G882A, G885A, T918G	4	G885A, C912T, T918G, G921A		—		—		—		—		—
<i>Di. balaenopterae</i>	6	A882G, A897G, G903A, C912T, T918G, T921A	5	G885A, A897G, G903A, C906T, T918G	4	G885A, A897G, G903A, T918G	7	A882G, G885A, A897G, G903A, C912T, T918G, G921A	3	A882G, A897G, G903A		—		—		—		—
<i>A. pacificus</i>	8	A882G, T888G, T889C, A891T/C, A897T, T906A, T909C, C912T	8	G885A, T888G, T889C, A891T/C, A897T, T906A, T909C, A921G/T	8	G885A, T888G, T889C, A891T/C, A897T, T906A, T909C, A921G/T	9	A882G, G885A, T888G, T889C, A891T/C, A897T, T906A, T909C, C912T	9	A882G, T888G, T889C, A891T/C, A897T, T906A, T909C, G918T, A921G/T	9	T888G, T889C, A891T/C, G897T, A903G, T906A, T909C, G918T, A921G/T		—		—		—
<i>S. decipiens</i>	6	A882T, A/C885G, T888G, A891G, A897G, G903T	8	G882T, T888G, A891G, A897G, G903T, C906T, T912C, A921T	7	G882T, T888G, A891G, A897G, G903T, T912C, A921T	6	A882T, T888G, A891G, A897G, G903T, G921T	9	A882T, A885G, T888G, A891G, A897G, G903T, T912C, G918T, A921T	8	G882T, A885G, T888G, A891G, A903T, T912C, G918T, A921T	9	G882T, A885G, C889T, T/C891G, T897G, G903T, A906T, C909T, T912C		—		—
<i>Sp. proliferum</i>	9	A882T, A/C885G, T888G, A891G, A897G, G903T, T909G, C912T, T921A	7	G882T, T888G, A891G, A897G, G903T, C906T, T909G	6	G882T, T888G, A891G, A897G, G903T, T909G	8	A882T, T888G, A891G, A897G, G903T, T909G, C912T, G921A	8	A882T, A885G, T888G, A891G, A897G, G903T, T909G, G918T	7	G882T, A885G, T888G, A891G, A903T, T909G, G918T	9	G882T, A885G, C889T, T/C891G, T897G, G903T, A906T, C909G, G/T921A	3	T909G, C912T, T921A		—

Table 3. The nucleotide patterns used to differentiate diphyllbothriidean tapeworms by the pyrosequencing technique. *Nos dif. is the number of nucleotide differences.

using 1.5% agarose gel electrophoresis. After PCR amplification, biotinylated PCR products were added into 96-well plates and processed as described elsewhere^{40,42}. The pyrosequencing system included DNA polymerase I, ATP sulfurylase, luciferase and apyrase. Pyrosequencing is a DNA sequencing technique that is based on the detection of released PPi during DNA synthesis. In a cascade of enzymatic reactions, visible light is generated that is proportional to the number of incorporated nucleotides. The cascade starts with a nucleic acid polymerization reaction in which inorganic PPi is released as a result of nucleotide incorporation by polymerase. The released PPi 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^{37,53}. In cases which the target sequence showed up to four homopolymers, the readout was analyzed manually to ensure accuracy.

References

- Schmidt, G. D. In *CRC handbook of tapeworm identification* (CRC Press Inc, 1986).
- 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).
- Chai, J. Y., Murrell, K. D. & Lymbery, A. J. Fish-borne parasitic zoonoses: status and issues. *Int J Parasitol* **35**, 1233–1254 (2005).
- Scholz, T., Garcia, H. H., Kuchta, R. & Wicht, B. Update on the human broad tapeworm (genus *Diphyllbothrium*), including clinical relevance. *Clin Microbiol Rev* **22**, 146–160 (2009).
- Schauer, F. *et al.* Travel-acquired subcutaneous *Sparganum proliferum* infection diagnosed by molecular methods. *Br J Dermatol* **170**, 741–743 (2014).
- Kuchta, R., Scholz, T., Brabec, J. & Wicht, B. *Diphyllbothrium*, *Diplogonoporus*, and *Spirometra* in *Biology of Foodborne Parasites* (eds Xiao, L., Ryan, U. & Feng, Y.) 299–326 (CRC Press Taylor & Francis group, 2015).
- Dick, T. A., Nelson, P. A. & Choudhury, A. Diphyllbothriasis: update on human cases, foci, patterns and sources of human infections and future considerations. *Southeast Asian J Trop Med Public Health* **32**, 59–76 (2001).
- Yamasaki, H., Tsubokawa, D., Mercado, R. & Kuramochi, T. A simple method for identifying the diphyllbothriids based on mitochondrial DNA analysis in *Parasitological Research: Materials and Methods* (ed. Takamiya, S.) 47–49 (Sankeisha, 2014).
- Yamasaki, H. *et al.* First confirmed human case of *Diphyllbothrium stemmacephalum* infection and molecular verification of the synonymy of *Diphyllbothrium yonagoense* with *D. stemmacephalum* (Cestoda: Diphyllbothriidae). *Parasitol Int* **65**, 412–442 (2016).
- Yamasaki, H., Ohmae, H. & Kuramochi, T. Complete mitochondrial genomes of *Diplogonoporus balaenopterae* and *Diplogonoporus grandis* (Cestoda: Diphyllbothriidae) and clarification of their taxonomic relationships. *Parasitol Int* **61**, 260–266 (2012).
- Kamo, H. *Guide to identification of diphyllbothriid cestodes* (ed. Hirai, K.) 1–146 (Gendaiikaku, 1999). (In Japanese).
- Kamo, H. *Cestodes in Progress of Medical Parasitology in Japan*. Vol. 8 (eds Otsuru, M., Kamegai, S. & Hayashi, S.) 235–243 (Meguro Parasitological Museum Press, 2003).

13. Arizono, N. *et al.* Diplogonoporiasis in Japan: genetic analyses of five clinical isolates. *Parasitol Int* **57**, 212–216 (2008).
14. Kino, H., Hori, W., Kobayashi, H., Nakamura, N. & Nagasawa, K. A mass occurrence of human infection with *Diplogonoporus grandis* (Cestoda: Diphyllbothriidae) in Shizuoka Prefecture, central Japan. *Parasitol Int* **51**, 73–79 (2002).
15. Kawai, S. *et al.* A case of cestode infection caused by *Diplogonoporus balaenopterae* most likely due to the consumption of raw whitebait. *Dokkyo J Med Scis* **40**, 189–192 (2013). (In Japanese).
16. Liu, W. *et al.* Sequence variability in three mitochondrial DNA regions of *Spirometra erinacei* spargana of human and animal health significance. *J Helminthol* **86**, 271–275 (2012).
17. Yoshikawa, M., Ouji, Y., Nishiofuku, M., Ishizaka, S. & Nawa, Y. Sparganosis cases reported in Japan in the recent decade, 2000–2009. *Clin Parasitol* **21**, 33–36 (2010). (In Japanese).
18. Jeon, H. K. *et al.* Human infections with *Spirometra decipiens* plerocercoids identified by morphologic and genetic analyses in Korea. *Korean J Parasitol* **53**, 299–305 (2015).
19. Eom, K. S. *et al.* Mitochondrial genome sequences of *Spirometra erinacei* and *S. decipiens* (Cestoidea: Diphyllbothriidae). *Korean J Parasitol* **53**, 455–463 (2015).
20. Ooi, H. K., Chang, S. L., Huang, C. C., Kawakami, Y. & Uchida, A. Survey of *Spirometra erinacei* in frogs in Taiwan and its experimental infection in cats. *J Helminthol* **74**, 173–176 (2000).
21. Anantaphruti, M. T., Nawa, Y. & Vanvanitchai, Y. Human sparganosis in Thailand: an overview. *Acta Trop* **118**, 171–176 (2011).
22. Bowman, D. D., Hendrix, C. M., Lindsay, D. S. & Barr, S. C. In *Feline Clinical Parasitology* (Wiley-Blackwell, 2002).
23. Nakamura, T., Hara, M., Matsuoka, M., Kawabata, M. & Tsuji, M. Human proliferative sparganosis. A new Japanese case. *Am J Clin Pathol* **94**, 224–228 (1990).
24. Lo, Y. K. *et al.* Spinal cord proliferative sparganosis in Taiwan: a case report. *Neurosurgery* **21**, 235–238 (1987).
25. Laovachirasuan, P., Mootsikapun, P., Maleewong, W. & Intapan, P. M. Intraosseous proliferative sparganosis presenting as a pathological fracture a case report and review of the literature. *Southeast Asian J Trop Med Public Health* **46**, 558–563 (2015).
26. Meric, R. *et al.* Disseminated infection caused by *Sparganum proliferum* in an AIDS patient. *Histopathology* **56**, 824–828 (2010).
27. Tappe, D. *et al.* Case report: molecular diagnosis of subcutaneous *Spirometra erinacei* sparganosis in a Japanese immigrant. *Am J Trop Med Hyg* **88**, 198–202 (2013).
28. Liu, Q., Li, M. W., Wang, Z. D., Zhao, G. H. & Zhu, X. Q. Human sparganosis, a neglected food borne zoonosis. *Lancet Infect Dis* **15**, 1226–1235 (2015).
29. Yamaura, H. *et al.* Two cases of *Spirometra erinacei* infection and one of sparganosis mansoni in three people who ate the same snake (*Elaphe quadrigata*). *Clin Parasitol* **13**, 159–163 (2003). (In Japanese).
30. Leštinová, K., Soldánová, M., Scholz, T. & Kuchta, R. Eggs as a suitable tool for species diagnosis of causative agents of human diphyllbothriosis (Cestoda). *PLoS Negl Trop Dis* **10**, e0004721 (2016).
31. Wicht, B., de Marval, F., Gottstein, B. & Peduzzi, R. Imported diphyllbothriasis in Switzerland: molecular evidence of *Diphyllbothrium dendriticum* (Nitsch, 1824). *Parasitol Res* **102**, 201–204 (2008).
32. Wicht, B., Scholz, T., Peduzzi, R. & Kuchta, R. First record of human infection with the tapeworm *Diphyllbothrium nihonkaiense* in North America. *Am J Trop Med Hyg* **78**, 235–238 (2008).
33. Wicht, B. *et al.* Multiplex PCR for differential identification of broad tapeworms (Cestoda: Diphyllbothrium) infecting humans. *J Clin Microbiol* **48**, 3111–3116 (2010).
34. Mercado, R. *et al.* Molecular identification of the *Diphyllbothrium* species causing diphyllbothriasis in Chilean patients. *Parasitol Res* **106**, 995–1000 (2010).
35. Boonyasiri, A. *et al.* Sparganosis presenting as cauda equina syndrome with molecular identification of the parasite in tissue sections. *Korean J Parasitol* **51**, 739–742 (2013).
36. Miyadera, H. *et al.* Phylogenetic identification of *Sparganum proliferum* as a pseudophyllidean cestode by the sequence analyses on mitochondrial COI and nuclear sdhB genes. *Parasitol Int* **50**, 93–104 (2001).
37. Ronaghi, M. Pyrosequencing sheds light on DNA sequencing. *Genome Res* **11**, 3–11 (2001).
38. Ahmadian, A., Ehn, M. & Hober, S. Pyrosequencing: history, biochemistry and future. *Clin Chim Acta* **363**, 83–94 (2006).
39. Stensvold, C. R. *et al.* Identification and delineation of members of the *Entamoeba* complex by pyrosequencing. *Mol Cell Probes* **24**, 403–406 (2010).
40. Lulitanond, V. *et al.* Molecular markers for detection and differentiation of *Plasmodium falciparum* and *Plasmodium vivax* in human blood samples by pyrosequencing. *J Clin Microbiol* **50**, 1455–1457 (2012).
41. Sadaow, L. *et al.* Molecular differentiation of *Trichinella spiralis*, *T. pseudospiralis*, *T. papuae* and *T. zimbabwensis* by pyrosequencing. *J Helminthol* **89**, 118–123 (2013).
42. Tantrawatpan, C. *et al.* Molecular identification of *Paragonimus* species by DNA pyrosequencing technology. *Parasitol Int* **62**, 341–345 (2013).
43. Sanpool, O. *et al.* Pyrosequencing using SL and 5S rRNA as molecular markers for identifying zoonotic filarial nematodes in blood samples and mosquitoes. *Vector Borne Zoonotic Dis* **16**, 326–333 (2016).
44. Wicht, B. *et al.* Inter- and intra-specific characterization of tapeworms of the genus *Diphyllbothrium* (Cestoda: Diphyllbothriidae) from Switzerland, using nuclear and mitochondrial DNA targets. *Parasitol Int* **59**, 35–39 (2010).
45. Jongthawin, J. *et al.* Molecular evidence of *Spirometra erinacei* infection in snakes *Ptyas korros* from Lao PDR and Thailand and frogs *Hoplobatrachus rugulosus* from Myanmar. *Southeast Asian J Trop Med Public Health* **45**, 1271–1278 (2014).
46. Chen, S. *et al.* Molecular detection of *Diphyllbothrium nihonkaiense* in humans, China. *Emerg Infect Dis* **20**, 315–318 (2014).
47. Novais, R. C. & Thorstenson, Y. R. The evolution of pyrosequencing[®] for microbiology: From genes to genomes. *J Microbiol Methods* **86**, 1–7 (2011).
48. Tantrawatpan, C. *et al.* Development of a PCR assay and pyrosequencing for identification of important human fish-borne trematodes and its potential use for detection in fecal specimens. *Parasit Vectors* **7**, 88 (2014).
49. May, K. & Strube, C. Prevalence of Rickettsiales (*Anaplasma phagocytophilum* and *Rickettsia* spp.) in hard ticks (*Ixodes ricinus*) in the city of Hamburg, Germany. *Parasitol Res* **113**, 2169–2175 (2014).
50. Ademola, I. O., Krücken, J., Ramünke, S., Demeler, J. & von Samson-Himmelstjerna, G. Absence of detectable benzimidazole-resistance associated alleles in *Haemonchus placei* in cattle in Nigeria revealed by pyrosequencing of β -tubulin isotype 1. *Parasitol Res* **114**, 1997–2001 (2015).
51. Zheng, R. *et al.* Pyrosequencing for rapid detection of tuberculosis resistance in clinical isolates and sputum samples from re-treatment pulmonary tuberculosis patients. *BMC Infect Dis* **14**, 200 (2014).
52. de Marval, F., Gottstein, B., Weber, M. & Wicht, B. Imported diphyllbothriasis in Switzerland: molecular methods to define a clinical case of *Diphyllbothrium* infection as *Diphyllbothrium dendriticum*, August 2010. *Euro Surveill* **18**, pii:20355 (2013).
53. Thanchomnang, T. *et al.* Rapid molecular identification of human taeniid cestodes by pyrosequencing approach. *PLoS One* **9**, e100611 (2014).

Acknowledgements

The authors thank Drs. Toshiaki Kuramochi (Japan), Naoki Arizono (Japan), Ruben Mercado (Chile), and Roman Kuchta (Czech Republic) for providing specimens of *Sp. proliferum* and *Di. balaenopterae*, *D. ditremum*, *A. pacificus* and *D. latum*, and *D. dendriticum* from the USA, respectively. This study was supported by a TRF

Senior Research Scholar Grant, Thailand Research Fund grant number RTA5880001; the Higher Education Research Promotion and National Research University Project of Thailand, Office of the Higher Education Commission, Thailand, through the Health Cluster (SHeP-GMS). O.S. was supported by Scholarship under the Post-Doctoral Training Program from Research Affairs and Graduate School, Khon Kaen University (58101). This work was also supported in part by Grants-in Aid for Scientific Research from the Ministry of Health, Labour and Welfare, Japan (H25-Shinko-Ippan-014) and Research Program on Emerging and Re-emerging Infectious Diseases from the Japan Agency for Medical Research and Development (Kansensho Jitsuyouka-Ippan, 15fk0108025h0502) to H.Y.

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

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Thanchomnang, T. *et al.* Rapid identification of nine species of diphylobothriidean tapeworms by pyrosequencing. *Sci. Rep.* **6**, 37228; doi: 10.1038/srep37228 (2016).

Publisher's note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

This work is licensed under a Creative Commons Attribution 4.0 International License. The images or other third party material in this article are included in the article's Creative Commons license, unless indicated otherwise in the credit line; if the material is not included under the Creative Commons license, users will need to obtain permission from the license holder to reproduce the material. To view a copy of this license, visit <http://creativecommons.org/licenses/by/4.0/>

© The Author(s) 2016