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# A possible circulation of a dominant *Dibothriocephalus nihonkaiensis* haplotype in Japan revealed by molecular analysis of clinical tapeworm samples

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ABSTRACT

Human diphyllobothriasis, caused by *Dibothriocephalus nihonkaiensis,* is prevalent globally, especially in regions where raw fish is consumed. Recent molecular diagnostic techniques have made species identification of tapeworm parasites and the determination of genetic variations among parasite populations possible. However, only a few studies done over a decade ago, have reported on the genetic variation among *D. nihonkaiensis* in Japan. The present study employed PCR-based mitochondrial DNA analysis to specifically detect *D. nihonkaiensis* from archived clinical samples, and to determine any genetic variation that may exist among the Japanese broad tapeworms from patients of Kanagawa Prefecture, Japan. Target genes were amplified from DNA extracted from the ethanol- or formaldehyde-fixed samples by PCR. Further sequencing and comparative phylogenetic analyses based on mitochondrial COI and ND1 sequences were also performed. In our results, all PCR-amplified and sequenced samples were identified as *D. nihonkaiensis.* Analysis of COI sequences revealed two haplotype lineages. However, clustering of almost all COI (and ND1) sample sequences into one of the two haplotype clades, together with reference sequences from different countries worldwide, revealed a common haplotype among *D. nihonkaiensis* samples in our study. Our results suggest a possible presence of a dominant *D. nihonkaiensis* haplotype, with a global distribution circulating in Japan. Results from this study have the potential to improve the management of clinical cases and establish robust control measures to reduce the burden of human diphyllobothriasis in Japan.

## 1. Introduction

Human tapeworm infection has a global distribution with a relatively high prevalence, especially in Arctic regions, some parts of Europe, Asia, and the Americas [1–6]. In regions where raw fish is consumed, human diphyllobothriasis is among the common types of tapeworm infection caused by adult-stage fish tapeworms belonging to the genus, *Dibothriocephalus* [1,7,8]. Globally, diphyllobothriasis is estimated to affect 10–20 million people [7,9]. Humans are infected by ingesting raw or undercooked fish infected with larval plerocercoids [9]. Adult *Dibothriocephalus* tapeworms can grow to about 2–15 m in length in the human small intestine [7] and can lay millions of eggs, which are excreted in feces. The lifecycle of the diphyllobothriid tapeworm is completed in 2 intermediate hosts [9,10]. The first intermediate host in which the procercoid develops is probably brackish zooplanktonic copepods [11], which is usually consumed by the second intermediate hosts, Pacific salmonids, *Oncorhynchus* spp (mostly in the case of *Dib. nihonkaiensis*) and sometimes, freshwater fishes of the genus *Esox* in Europe and Russia (in the case of *Dib. latus*) [12–15]. In the second intermediate host, procercoids develop into plerocercoids, the larval form needed to infect the definitive host (e.g., humans). *Dibothriocephalus nihonkaiensis* infections are generally asymptomatic or induce relatively mild symptoms [16].

Some of the important causative agents of diphyllobothriasis include

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*Dib. dendriticus, Dib. latus, Dib. nihonkaiensis,* and *Adenocephalus pacificus,* especially in countries where fish consumption is expanding [17]. The causative species primarily associated with diphyllobothriasis in Europe, Russia, and South America is the broad fish tapeworm, *Dib. latus* (syn. *Diphyllobothrium latum)* [16,18,19]. However, in Japan, also popularly known for its custom of eating raw fish delicacies, the main causative species was later identified to be the Japanese broad tapeworm, *Dib. nihonkaiensis* (syn. *Diph. nihonkaiense),* after most cases, were initially misidentified as *Diph. latum* [10], suggesting a limitation in previous diagnostic methods of diphyllobothriasis.

Over the years, the diagnosis of diphyllobothriasis has been based on microscopical examinations of the morphological features of proglottids and fecal eggs. With this method, although species identification of the parasite is possible through histologic examination of the proglottids, parasites from patients who only discharge eggs cannot be conclusively identified to the species level [13,20]. Another problem is the misidentification of parasites, generally due to the lack of enough morphologically distinctive features, mostly among larval and immature worms or inadequately preserved specimens [9,21,22]. As a result, expert opinions on the validities of individual species and the taxonomic composition of the genus keep changing and remain undecided [9,23–26]. However, limitations with this approach have led to the recent rise in the use of advanced molecular based diagnoses, such as polymerase chain reaction (PCR) and sequencing of specific regions of mitochondrial DNA (mtDNA), including cytochrome oxidase subunit-1 (COI) and NADH dehydrogenase subunit 1(ND1) among others, in the management of diphyllobothriasis [2,6,7,9,13,27–32].

In Japan, not only have molecular methods using specific regions of several mitochondrial genes been used successfully to resolve the identity of *Dib. nihonkaiensis,* the major and frequent causative agent for human diphyllobothriasis [33], but also for investigations into the genetic diversity of *Dib. nihonkaiensis* [13,34,35]. Understanding genetic variations in parasite populations helps to elucidate parasite transmission patterns, identify new or exotic species, understand the epidemiology and etiology of the infection, to help in the development of effective control methods [36] Previous studies [13,35], on the genetic diversity of *Dib. nihonkaiensis* conducted *in* parts of Japan by phylogenetic analyses of specific regions of the COI gene revealed two major genetic lineages (i.e., A and B) using about 20 isolates from humans, bears, and Pacific salmon. However, these studies were conducted about a decade ago, within which possible changes or variations may likely occur. In addition, a recent study [34] focused on assessing the global genetic variability in mitochondrial COI sequences of *Dib. nihonkaiensis* by comparing 14 new isolates retrieved from 12 clinical patients in Japan, with all isolates available in the GenBank database, and identified a total of 48 haplotypes of three haplotype groups (Types A, B and C) among the dataset analyzed, as well as the presence of heterogenic *Dib. nihonkaiensis* infection in individuals. These data, however, suggest the need for more studies in other parts of Japan to ascertain the current genetic diversity among *Dib. nihonkaiensis* within Japan.

In the present study, we applied genetic analysis to re-evaluate the identities of *Dib. nihonkaiensis* from archived clinical tapeworm samples, to determine the genetic variation of the Japanese broad tapeworms from patients in the Kanagawa Prefecture of Japan. We, therefore, report the successful identification of a possibly dominant and globally distributed *Dib. nihonkaiensis* haplotype circulating in Japan, and discuss its implications for the effective management of clinical cases.

## 2. Methods

### 2.1. Parasite specimens

This study involved a total of 57 preserved tapeworm specimens from patients collected from hospitals in Kanagawa Prefecture between 1979 and 2020, and stored in the Parasitology and Tropical Medicine Department at the Kitasato University School of Medicine in Sagamihara, Kanagawa Prefecture of Japan. No clear morphological data were archived on these specimens, and species diagnosis appeared to have been estimated by observation of mature proglottids under a stereomicroscope, after which they were preserved in formaldehyde or ethanol.

### 2.2. DNA extraction

Before DNA extraction, parasites kept in formaldehyde or ethanol were washed with and subsequently re-hydrated in phosphate-buffered saline (PBS) for several days. Total DNA was then extracted from the proglottids of each sample using the DNeasy® Blood and Tissue Kit (Qiagen, Hilden, Germany), according to the manufacturer's protocol with the following modifications:

(i) the samples were incubated in lysis buffer overnight to ensure complete lysis of the samples, as a result of hardening due to long storage in formaldehyde or ethanol, and
(ii) the volume of the elution buffer was reduced to 50 µl to ensure high DNA yield since the quality of the sample may have been compromised due to the long storage in formaldehyde or ethanol.

The concentrations of the eluted DNA samples were also measured by ultraviolet light absorbance using the NanoDrop™ One Microvolume UV–Vis Spectrophotometer (Thermo Scientific, Rockford, IL, USA).

### 2.3. Polymerase chain reaction (PCR)

PCRs were performed to amplify a 420 bp region of *Dib. nihonkaiensis* COI with species-specific primers BW3 and BW4.5 [4] and a 950 bp region of *Dib. nihonkaiensis* ND1 using species-specific primers designed in this study by primer 3 (v. 0.4.0) [37,38], which were purchased from Eurofins Genomics K.K., Tokyo, Japan. Primer sequences are shown in Table 1. A PCR positive control was established using DNA extracted from a fresh tapeworm sample brought to our laboratory, while water served as a negative (no template) control. The reaction mixtures for PCR were prepared using the KOD One™ PCR Master Mix (Toyobo, Japan), and band amplification was done using a Bio-Rad T100™ Thermal Cycler, with the following cycling conditions; initial denaturation at 98 °C for 1 min, followed by 45 cycles of denaturation at 98 °C for 10 s, annealing at either 56 °C (COI) or 57 °C (ND1) for 5 s and extension at 68 °C for either 6 s (COI) or 5 s (ND1), with a final extension performed at 68 °C for 2 mins. The PCR products were separated by a 1.5% agarose gel electrophoresis and stained with ethidium bromide. PCR products were then purified from the gel with Viogene Gel/PCR DNA isolation system (VIOGENE BIOTEK, TAIWAN) according to the manufacturer's protocol.

### 2.4. Sequencing analysis

Sequencing analysis was performed on amplified fragments of the COI and ND1 genes. The purified DNA isolates were directly sequenced with primer sets purchased from Eurofins Genomics K.K., Tokyo, Japan (Table 1) using Sanger technology by an independent external company (Eurofins Genomics K.K., Tokyo, Japan).

### 2.5. Species identification and phylogenetic tree construction

The sequences obtained were validated using BioEdit Sequence Alignment Editor version 7.2.5 ((https://bioedit.software.informer.com/7.2/) from the chromatogram, confirmed sequences were subjected to a BLAST Search using the National Center for Biotechnology Information's (NCBI) nucleotide BLAST (BLASTN) program webtool (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch). Partial sequences of the COI (260 bp) and ND1 (442 bp) genes without primer regions were used as BLAST queries for *Dib. nihonkaiensis* species

**Table 1**
List of primers.

| Primer | Position | Sequence (5′ to 3′) | Tm (°C) | Target Size | Reference |
|---|---|---|---|---|---|
| BW3* | Forward | TTTTTTGGCCACCCCGAAGTATAT | 57.1 | 420 bp | [4] |
| BW4.5* | Reverse | TAGTGACATTACATAGTGGAAGTG | 55.3 | | |
| ND1 | Forward | GGGACCCAATAAGGTTGGCA | 58.4 | 950 bp | This study |
| ND1 | Reverse | AACCTGCAAAGCAGCCCTAT | 56.3 | | |
| ND1(for sequencing) | Forward | TGTTAGGTGCTATGCGTTCTG | 56.5 | – | This study |
| ND1(for sequencing) | Reverse | TAAAGATCACACCCCTCCCC | 58.4 | – | This study |

* Same primers were used to sequence the COI amplified products.

identification. Using these sequences, further multiple sequence alignment was performed with reference sequences retrieved from GenBank for *Dib. nihonkaiensis* (Table 2), using MAFFT web program (https://mafft.cbrc.jp/alignment/server/). Phylogenetic analyses were then run for both COI and ND1 sequences obtained under the maximum likelihood criterion in MEGA v.11 (Penn State University, PA, USA). The analysis was run under the HKY + I model with bootstrap resampling of 2000 replicates for the COI gene. The sequences of the COI genes of *Dib. latus, Ligula intestinalis* and *Diphyllobothrium balaenopterae (syn. Diplogonoporus balaenopterae)*, three species belonging to the family Diphyllobothriidae and close to *Dib. nihonkaiensis*, were used as out-groups. A similar analysis was also run under the TN93 + G + I model with bootstrap resampling of 2000 replicates for the ND1 gene. The sequences of the ND1 genes of *Dib. latus, Ligula intestinalis* and *Ligula interrupta (syn. Digramma interrupta)*, also belonging to the family Diphyllobothriidae and close to *Dib. nihonkaiensis*, were used as out-groups. The newly generated sequences of the COI and ND1 genes were submitted to the GenBank database under the accession numbers in Table 3.

**Table 2**
Reference sequences used in the COI and ND1 sequence alignment and phylogenetic analysis.

| Organism | GenBank accession no. | Origin |
|---|---|---|
| Sequence alignment | | |
| *Dib. nihonkaiensis* | AB015755.1 | Japan |
| *Dib. nihonkaiensis* | MK975239.1 | France |
| *Spirometra erinaceieuropaei* | AB015754.1 | Japan |
| *Diph. ditremum* | FM209182.1 | Scotland |
| *Ligula intestinalis* | AF153910.1 | China |
| *Dib. nihonkaiensis* | AB375662.1 | Japan |
| *Diph. ursi* | AB605762.1 | Japan |
| Phylogenetic analysis (COI) | | |
| *Dib. nihonkaiensis* | AB015755.1 | Japan |
| *Dib. nihonkaiensis* | AB288371.2 | Japan |
| *Dib. nihonkaiensis* | AB288372.2 | Japan |
| *Dib. nihonkaiensis* | AB375003.1 | Japan |
| *Dib. nihonkaiensis* | AB375004.1 | Japan |
| *Dib. nihonkaiensis* | AB573405.1 | Japan |
| *Dib. nihonkaiensis* | AM412559.2 | Switzerland |
| *Dib. nihonkaiensis* | LC070678.1 | China |
| *Dib. nihonkaiensis* | DQ768190.1 | South Korea |
| *Dib. nihonkaiensis* | AM412560.2 | Switzerland |
| *Dib. nihonkaiensis* | MK070864.1 | France |
| *Dib. nihonkaiensis* | KU984425.1 | China |
| *Dib. nihonkaiensis* | MK070860.1 | France |
| *Dib. nihonkaiensis* | JQ245471.1 | Russia |
| *Dib. latus* | AB269325.1 | Japan |
| *Ligula intestinalis* | MF671696.1 | United Kingdom |
| *Diphyllobothrium balaenopterae* | AB822370.1 | Japan |
| Phylogenetic analysis (ND1) | | |
| *Dib. nihonkaiensis* | EF420138.1 | Korea |
| *Dib. nihonkaiensis* | AB268585.1 | Japan |
| *Dib. nihonkaiensis* | HQ423295.1 | Canada |
| *Dib. nihonkaiensis* | HQ423296.1 | Canada |
| *Dib. nihonkaiensis* | MK975240.1 | France |
| *Dib. latus* | AB269325.1 | Japan |
| *Ligula intestinalis* | MW602520.1 | United Kingdom |
| *Ligula interrupta* | AF524044.1 | China |

## 3. Results

### 3.1. Species name on the labels of archived samples

The majority of archived clinical tapeworm samples were described as *Diph. nihonkaiense /latum* excreted from patients with diphyllobothriasis (Fig. 1). Of the 57 samples collected, 39% (22/57) were indicated as *Diph. nihonkaiense,* while 13% (7/57) were shown as *Diph. latum* (Table 3). The identity of 37% (21/57) of the stored tapeworm samples did not appear to be sufficiently evaluated to fully resolve to the species level (Table 3). Thirteen percent (13%) (7/57) of the samples were shown as *Taenia saginata* or *Diplogonoporus grandis* (Table 3).

### 3.2. Molecular identification

To completely resolve the species identification of *Dib. nihonkaiensis*, PCR and sequence analysis of the COI and ND1 genes were performed in all 57 clinical tapeworm samples. Representative images of electrophoretic gels showing COI and ND1 DNA bands are shown in Fig. 2. No bands were observed for 21 samples. Therefore, sequencing of purified amplicons was only done for the other 36 samples with successful amplification of the COI gene. From the BLAST analysis, the DNA sequences of all 36 samples exhibited high homology (>97%) with other *Dib. nihonkaiensis* sequences stored in GenBank, as presented in Table 3. This conclusively confirms the identification of the samples as *Dib. nihonkaiensis*. These results also showed that the species identity of 86% (31/36) of the archived samples, which were initially either misdiagnosed or could not completely be identified, were successfully resolved by the molecular method, suggesting the usefulness of the molecular identification method in differentiating *Dib. nihonkaiensis*. PCR based on the ND1 gene was done to confirm the identities of *Dib. nihonkaiensis* in 25 (out of 36) archived samples, as shown by COI PCR (Table 3). The GenBank accession numbers of the COI and ND1 sequences of the archived samples are listed in Table 3. The COI sequences of 3 samples showed three ambiguous sites within the sequences, while the ND1 sequences of 18 samples also showed 23 ambiguous sites. These samples with ambiguous sites within the sequence region selected for phylogenetic analyses were excluded from further analyses.

### 3.3. Phylogenetic analyses

Phylogenetic trees generated by the maximum likelihood method were used in constructing dendrograms for *Dib. nihonkaiensis* using the COI and ND1 genes. The HKY + 1 and TN93 + G + I models were used in constructing the COI and ND1 dendrograms, respectively (Figs. 3 and 4). The COI gene was accurately sequenced in more samples (32/35) than the ND1 gene (7/25). Both dendrograms of clinical tapeworm specimens in this study, using both genes confirmed all samples to be identical to *Dib. nihonkaiensis* isolates previously reported in Japan. However, phylogenetic analyses based on COI gene, revealed two haplotype lineages (Fig. 3) among the *Dib. nihonkaiensis* samples analyzed. It was also observed that about 91% (29/32) of the samples were grouped in clade A (Fig. 3), while just about 9% were grouped in clade B, suggesting the

**Table 3**
Summary of optical and molecular identification data of archived tapeworm samples.

| No. | Sample ID | Year Collected | The descriptions on the label | Molecular Identification | Phylogenetic Analysis COI | Phylogenetic Analysis ND1 | Gene Accession No. COI | Gene Accession No. ND1 |
|---|---|---|---|---|---|---|---|---|
| 1 | 050202 | 2005 | *Diph. nihonkaiense /latum* | *Dib. nihonkaiensis* | ◆ | Δ | LC765117 | – |
| 2 | 051110 | 2005 | *Diph. nihonkaiense* | *Dib. nihonkaiensis* | ◆ | Δ | LC765116 | LC765149 |
| 3 | 070124 | 2007 | *Diph. nihonkaiense /latum* | *Dib. nihonkaiensis* | ◆ | Δ | LC765115 | – |
| 4 | 081219 | 2008 | *Diph. nihonkaiense /latum* | *Dib. nihonkaiensis* | ◆ | Δ | LC765128 | LC765147 |
| 5 | 100407 | 2010 | *Diph. nihonkaiense /latum* | *Dib. nihonkaiensis* | ◆ | Δ | LC765127 | LC765152 |
| 6 | 100707 | 2010 | *Diph. nihonkaiense /latum* | *Dib. nihonkaiensis* | ◆ | Δ | LC765126 | LC765153 |
| 7 | 180611 | 2018 | *Diplogonoporus grandis* | *Dib. nihonkaiensis* | ◆ | Δ | LC765135 | LC765146 |
| 8 | 180620 | 2018 | *Diph. nihonkaiense /latum* | *Dib. nihonkaiensis* | ◆ | ▲ | LC765125 | LC765139 |
| 9 | 190416 | 2019 | *Diph. nihonkaiense /latum* | *Dib. nihonkaiensis* | ◊ | Δ | LC765132 | – |
| 10 | 200413 | 2020 | *Diph. nihonkaiense /latum* | *Dib. nihonkaiensis* | ◆ | Δ | LC765118 | – |
| 11 | 200615 | 2020 | *Diph. nihonkaiense* | *Dib. nihonkaiensis* | ◆ | Δ | LC765124 | LC765154 |
| 12 | 79018 | 1979 | *Diph. latum* | *Dib. nihonkaiensis* | ◆ | ▲ | LC765130 | LC765140 |
| 13 | 850522 | 1985 | *Diph. latum* | *Dib. nihonkaiensis* | ◆ | Δ | LC765137 | – |
| 14 | 851025 | 1985 | *Diph. nihonkaiense /latum* | *Dib. nihonkaiensis* | ◆ | Δ | LC765123 | LC765155 |
| 15 | 900523 | 1990 | *Diph. latum* | *Dib. nihonkaiensis* | ◆ | Δ | LC765114 | LC765148 |
| 16 | 900815 | 1990 | *Diph. latum* | *Dib. nihonkaiensis* | ◆ | Δ | LC765136 | LC765156 |
| 17 | 930630 | 1993 | *Diph. latum* | *Dib. nihonkaiensis* | ◆ | Δ | LC765131 | LC765157 |
| 18 | 931008 | 1993 | *Diph. nihonkaiense* | *Dib. nihonkaiensis* | ◊ | Δ | LC765134 | LC765158 |
| 19 | 931018 | 1993 | *Diph. nihonkaiense /latum* | *Dib. nihonkaiensis* | ◆ | Δ | LC765113 | LC765159 |
| 20 | 940613 | 1994 | *Diph. nihonkaiense /latum* | *Dib. nihonkaiensis* | ◆ | Δ | LC765112 | – |
| 21 | 940719 | 1994 | *Diph. nihonkaiense /latum* | *Dib. nihonkaiensis* | ◆ | ▲ | LC765111 | LC765141 |
| 22 | 960527 | 1996 | *Diph. nihonkaiense /latum* | *Dib. nihonkaiensis* | ◆ | Δ | LC765110 | – |
| 23 | 960821 | 1996 | *Dipl. grandis* | *Dib. nihonkaiensis* | ◆ | Δ | LC765109 | – |
| 24 | 980114 | 1998 | *Diph. nihonkaiense* | *Dib. nihonkaiensis* | ◆ | Δ | LC765108 | LC765160 |
| 25 | 980323 | 1998 | *Diph. latum* | *Dib. nihonkaiensis* | ◊ | Δ | LC765133 | LC765161 |
| 26 | 980616 | 1998 | *Diph. latum* | *Dib. nihonkaiensis* | ◆ | ▲ | LC765107 | LC765142 |
| 27 | 990323 | 1999 | *Diph. nihonkaiense /latum* | *Dib. nihonkaiensis* | ◆ | ▲ | LC765106 | LC765143 |
| 28 | 1005258 | 2010 | *Diph. nihonkaiense /latum* | *Dib. nihonkaiensis* | ◆ | Δ | LC765122 | – |
| 29 | 100406A | 2010 | *Diph. nihonkaiense /latum* | *Dib. nihonkaiensis* | ◆ | Δ | LC765105 | LC765162 |
| 30 | 100406B | 2010 | *Diph. nihonkaiense /latum* | *Dib. nihonkaiensis* | ◆ | Δ | LC765121 | – |
| 31 | 100427A | 2010 | *Diph. nihonkaiense /latum* | *Dib. nihonkaiensis* | ◆ | ▲ | LC765120 | LC765144 |
| 32 | 100427B | 2010 | *Diph. nihonkaiense /latum* | *Dib. nihonkaiensis* | ◆ | ▲ | LC765119 | LC765138 |
| 33 | 110422A | 2011 | *Diph. nihonkaiense /latum* | *Dib. nihonkaiensis* | ◆ | Δ | LC765129 | LC765145 |
| 34 | 110422B | 2011 | *Diph. nihonkaiense* | *Dib. nihonkaiensis* | ◆ | Δ | LC765104 | – |
| 35 | 9004XXA | 1990 | *Diph. nihonkaiense /latum* | *Dib. nihonkaiensis* | ◆ | Δ | LC765103 | LC765151 |
| 36 | 2104 | 2020 | *Diph. nihonkaiense /latum* | *Dib. nihonkaiensis* | ◊ | Δ | – | LC765150 |
| 37 | 70828 | 2007 | *Diph. nihonkaiense* | ◈ | ◊ | Δ | – | – |
| 38 | 160719 | 2016 | *Taenia saginata* | ◈ | ◊ | Δ | – | – |
| 39 | 820331 | 1982 | *Diph. nihonkaiense* | ◈ | ◊ | Δ | – | – |
| 40 | 100806A | 2010 | *Diph. nihonkaiense* | ◈ | ◊ | Δ | – | – |
| 41 | 941214A | 1994 | *T. saginata* | ◈ | ◊ | Δ | – | – |
| 42 | 941214B | 1994 | *T. saginata* | ◈ | ◊ | Δ | – | – |
| 43 | 70221 | 2007 | *Diph. nihonkaiense* | ◈ | ◊ | Δ | – | – |
| 44 | 90501 | 2009 | *Diph. nihonkaiense* | ◈ | ◊ | Δ | – | – |
| 45 | 100203 | 2010 | *Diph. nihonkaiense* | ◈ | ◊ | Δ | – | – |
| 46 | 941107 | 1994 | *T. saginata* | ◈ | ◊ | Δ | – | – |
| 47 | 950621 | 1995 | *Diph. nihonkaiense* | ◈ | ◊ | Δ | – | – |
| 48 | 951226 | 1995 | *Diph. nihonkaiense* | ◈ | ◊ | Δ | – | – |
| 49 | 970214 | 1997 | *Diph. nihonkaiense* | ◈ | ◊ | Δ | – | – |
| 50 | 050216A | 2005 | *Diph. nihonkaiense* | ◈ | ◊ | Δ | – | – |
| 51 | 7 | Unknown | *Diph. nihonkaiense* | ◈ | ◊ | Δ | – | – |

(*continued on next page*)

**Table 3** (*continued*)

| No. | Sample ID | Year Collected | The descriptions on the label | Molecular Identification | Phylogenetic Analysis COI | Phylogenetic Analysis ND1 | Gene Accession No. COI | Gene Accession No. ND1 |
|---|---|---|---|---|---|---|---|---|
| 52 | 50119 | 2005 | *Diph. nihonkaiense* | ◈ | ◊ | Δ | – | – |
| 53 | 880629 | 1988 | *Diph. nihonkaiense* | ◈ | ◊ | Δ | – | – |
| 54 | 880903 | 1988 | *T. saginata* | ◈ | ◊ | Δ | – | – |
| 55 | 890815 | 1989 | *Diph. nihonkaiense* | ◈ | ◊ | Δ | – | – |
| 56 | 900313 | 1990 | *Diph. nihonkaiense* | ◈ | ◊ | Δ | – | – |
| 57 | 960904 | 1996 | *Diph. nihonkaiense* | ◈ | ◊ | Δ | – | – |

◈: Samples with unsuccessful molecular identification.
Samples included (◆) and samples not included (◊) in phylogenetic analysis COI.
Samples included (▲) and samples not included (Δ) in phylogenetic analysis ND1.

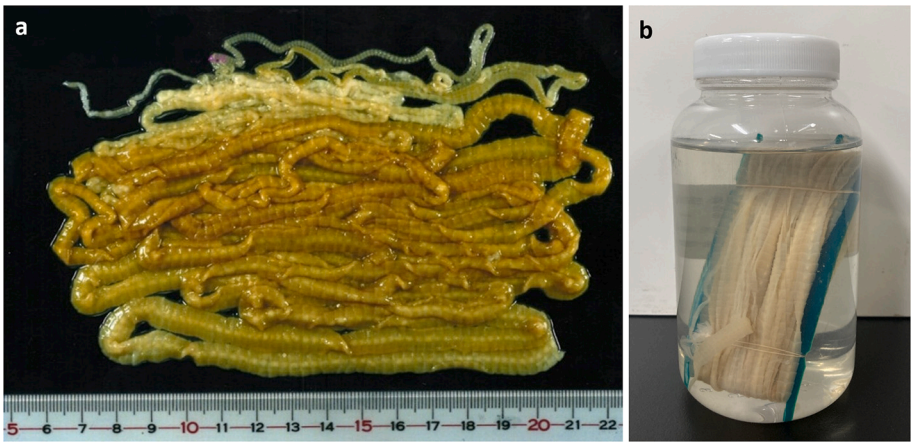


**Fig. 1.** Archived tapeworm sample. (a) an adult tapeworm expelled from a patient in Japan, 2010 at the time of optical identification. The length of each gap on the measuring rule is 1 mm. (b) Preservation of archived tapeworm sample in a bottle of formaldehyde or ethanol.

presence of a possible dominant haplotype in Japan. This haplotype was evidently observed to be identical to haplotypes reported in China, South Korea, Russia, and France, suggesting that it is also distributed in countries around the world. Additionally, phylogenetic analyses based on ND1 gene, revealed a monophyletic grouping of the 7 samples analyzed in this study, together with the reference sequences obtained from Genbank (Fig. 4), suggesting a common haplotype which also appears to be globally distributed. Altogether, the above results suggest the possible presence of a dominant and globally distributed *Dib. nihonkaiensis* haplotype circulating in Japan.

## 4. Discussion

Recent molecular methods have been useful in the complete differential identification of tapeworms, including parasites of the *Dibothriocephalus* genus [2,4,17,28–30,32,39–42]. Additionally, they have also made it possible to determine variations in the genetic composition of these parasites populations around the world, including Japan [13,34,35], Understanding the genetic diversity of parasites however, helps to elucidate parasite transmission patterns for the development of effective and appropriate control measures [36]. However, only a few studies, mostly conducted over a decade ago, have reported the genetic diversity of *Dib. nihonkaiensis,* the major causative agent of human diphyllobothriasis around the world, including Japan [13,34,35], despite the extensive reports on other parasitic worm species, including tapeworm parasites of the *Taenia* genus, in different geographical areas [36,43–48].

In the present study, we re-examined 57 archived tapeworm samples using conventional PCR and sequencing methods based on *Dib. nihonkaiensis* COI and ND1 genes to detect *Dib. nihonkaiensis* from the archived samples for the determination of any current genetic variation that may exist among *Dib. nihonkaiensis* parasites in Japan. Of the 57 samples analyzed by PCR, no amplified PCR products were observed for 21 samples. This could either be attributed to the fact that those samples were of different species and *Dib. nihonkaiensis*-specific primers could not amplify target genes as expected, or the fact that the preserved samples were stored in formaldehyde for a prolonged period. Formalin-based fixatives are recognized to cause DNA damage, and could have contributed to the unsuccessful gene amplication [9]. Consequently, it is often discouraged to use traditional sample storage methods for long-term storage intended for subsequent DNA extraction and PCR, as these methods can degrade DNA over time and potentially lead to false or unreliable PCR results. Another plausible reason for unsuccessful amplification could be the possibility that the samples were already damaged at the point of reception, especially where specimens were provided by the patients themselves, who mostly have little or no knowledge and required skills in preserving the integrity of retrieved samples, as have been the case in some instances. The results of the other 36 samples, however, demonstrated an accurate resolution of the identities of tapeworm samples as *Dib. nihonkaiensis*, showing the importance of molecular methods in identifying clinical *Dibothriocephalus* samples at the species level. The success story of these methods is much attributed to the characterization of the complete mitochondrial genomes of *Dib. latus* and *Dib. nihonkaiensis*, which provided essential information for the parasite's identification [49–51] This led to the selection of the COI gene as an appropriate target for species identification of human broad tapeworms, mainly because of its higher mutation rate than that of other nuclear genes, which although have been used to determine the relationship among taxa [9,21,52], cannot be used to distinguish between all *Dibothriocephalus* species routinely [20]. Consequently, in our study, identification of *Dib. nihonkaiensis* tapeworms was done based on the COI gene. PCR and sequencing based on the COI gene appeared to be about 86% more efficient in the resolution of the identity of *Dib. nihonkaiensis*, as the identity of 31 (out of 36) optically estimated samples were correctly resolved by the molecular approach, which is consistent with other studies reported

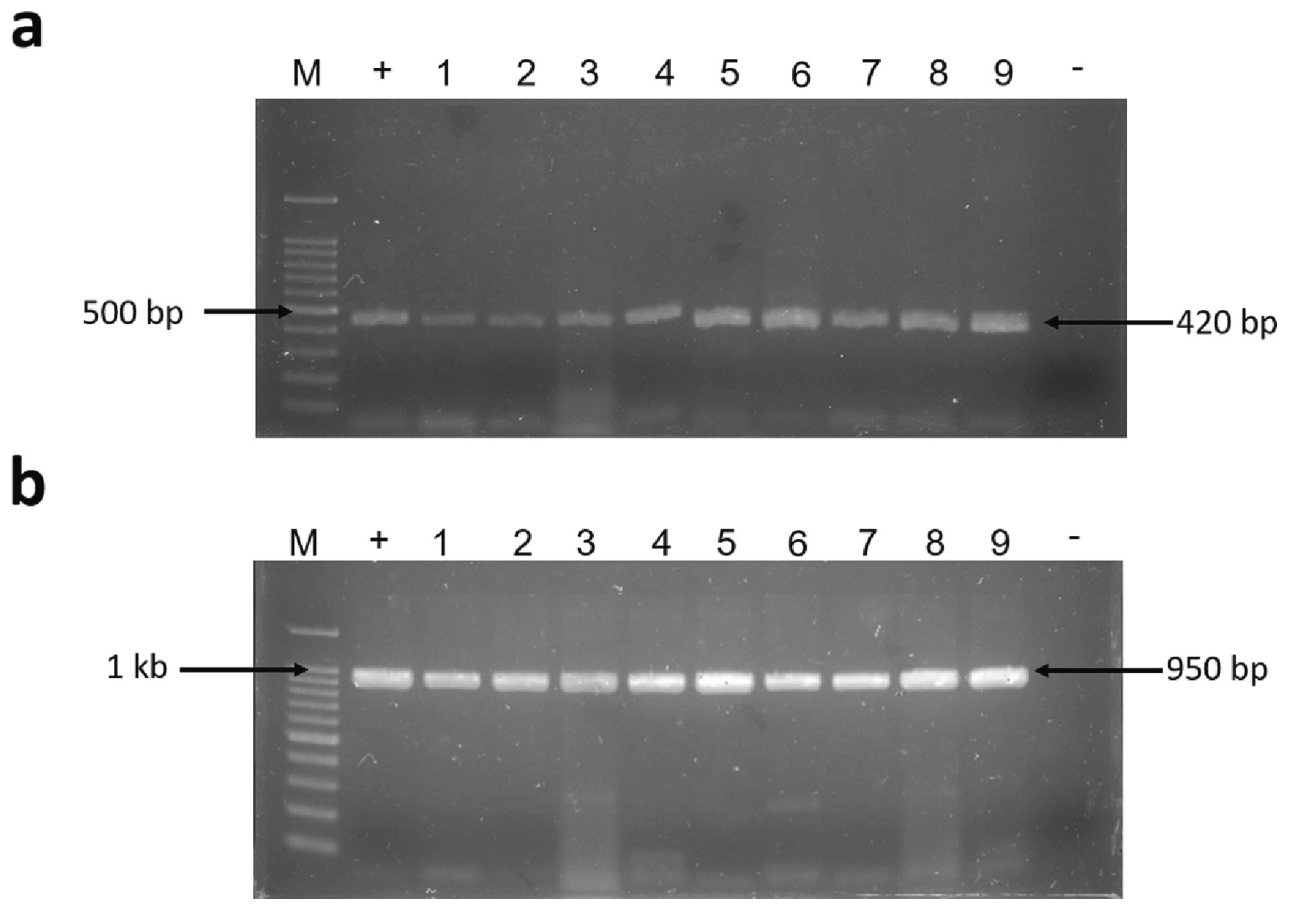


**Fig. 2.** DNA amplification of archived tapeworm samples. Representative images of agarose gel electrophoresis, showing amplified PCR bands for (a) COI gene (420 bp) and (b) ND1 gene (950 bp). Lane M: 100 bp DNA ladder, lane +: positive control, lanes 1–9: archived tapeworm samples and lane -: no template control.

[4,5,16,29,42]. For instance, it was reported that mitochondrial COI gene analysis provides the most discriminative means for the identification of the species in genus *Dibothriocephalus*, especially between *Dib. nihonkaiensis* and *Dib. latus* [20]. Similarly, in a study in Korea, most of the previous diphyllobothriasis cases were thought to be caused by *Dib. latus* (syn. *Diph. latum)*; however, they were re-confirmed as *Dib. nihonkaiensis* (syn. *Diph. nihonkaiense)* infections [9]. Additional PCR test based on ND1 successfully identified all 25 samples tested in our study as *Dib. nihonkaiensis*, suggesting confirmation of COI PCR results. Altogether, the identification of all tested samples as *Dib. nihonkaiensis* strongly supports recent observations that *Dib. nihonkaiensis* diphyllobothriasis, which is mainly associated with the consumption of raw Pacific salmon, is the most frequently occurring foodborne parasitic infection in Japan, representing 86% of causative agents encountered [16]. *Dib. nihonkaiensis* diphyllobothriasis is reported to occur widely (40/47 prefectures) throughout Japan, with the highest cases reported in the northern regions and along the coastal regions of the Sea of Japan from 1979 through the 1990s, especially where salmon are caught and consumed locally [53], and recently, in the populous cities of Tokyo and Saitama, within the Kanto region of Japan due to their high consumption of raw Pacific salmon since the 1990s [16]. Therefore, the high prevalence *Dib. nihonkaiensis* identified in this study is not surprising, as the Kanagawa prefecture also lies within the Kanto region, close to Tokyo and Saitama.

Phylogenetic analysis of COI sequences in our study, revealed two haplotype clades, A and B, among *Dib. nihonkaiensis* species identified (Fig. 3). This observation is consistent with reports by Arizono et al, when *Dib. nihonkaiensis* clinical human and animal isolates, collected from Kyoto and Jintu river, Japan; Lake Azabachye and Paratunka River, Kamchatka, Russia; and Okhotsk Sea near Sakhalin, Russia between 2000 and 2007 [35], revealed two divergent mtDNA lineages among *Dib. nihonkaiensis* species identified by the analyses of COI and NADH dehydrogenase subunit 3 (ND3) sequences. Actually, isolates identified as Type A (Dn1, Dn2) and Type B (Dn8, Dn9) in the previous studies by Arizono et al. [35] exhibit similar clustering in the present analysis (Fig. 3), confirming previous reports and suggesting the accuracy of our analysis.

However, the observation of about 91% of identified *Dib. nihonkaiensis* samples clustered in one of the two haplotype clades observed (Fig. 3, Clade A), suggest the circulation of a possible dominant haplotype of *Dib. nihonkaiensis* in Japan. Again, this observation was seemingly the case in previous studies by Arizono et al. [35] and Suzuki et al. [13], however has become more pronounced in our study due to the relatively bigger sample size in this study. In addition, the monophyletic grouping of all 7 samples used in the phylogenetic analysis of ND1 sequences in this study, also supports the notion of a possible presence of a dominant *Dib. nihonkaiensis* haplotype in Japan, as observed in the analysis of COI sequences. Moreover, the clustering of samples with reference sequences from different countries around the world (Japan, China, South Korea, and Russia), Europe (France, Switzerland), and North America (Canada) in both COI and ND1 phylogenetic analysis (Figs. 3 and 4), suggests that this possibly dominant haplotype circulating in Japan, appears be distributed across countries worldwide. This observation is consistent with reports that *Dib. nihonkaiensis* infection is no longer restricted to East Asia and the North Pacific coast of North America due to the high rate of spread through the globalization of trade and increased commerce with salmon, as well as increased consumption of raw fish by tourists and travelers in endemic areas such as Japan [9,16]. As a matter of fact, cases of *Dib. nihonkaiensis* tapeworm infections are on the rise in Europe, where the pathogen was previously

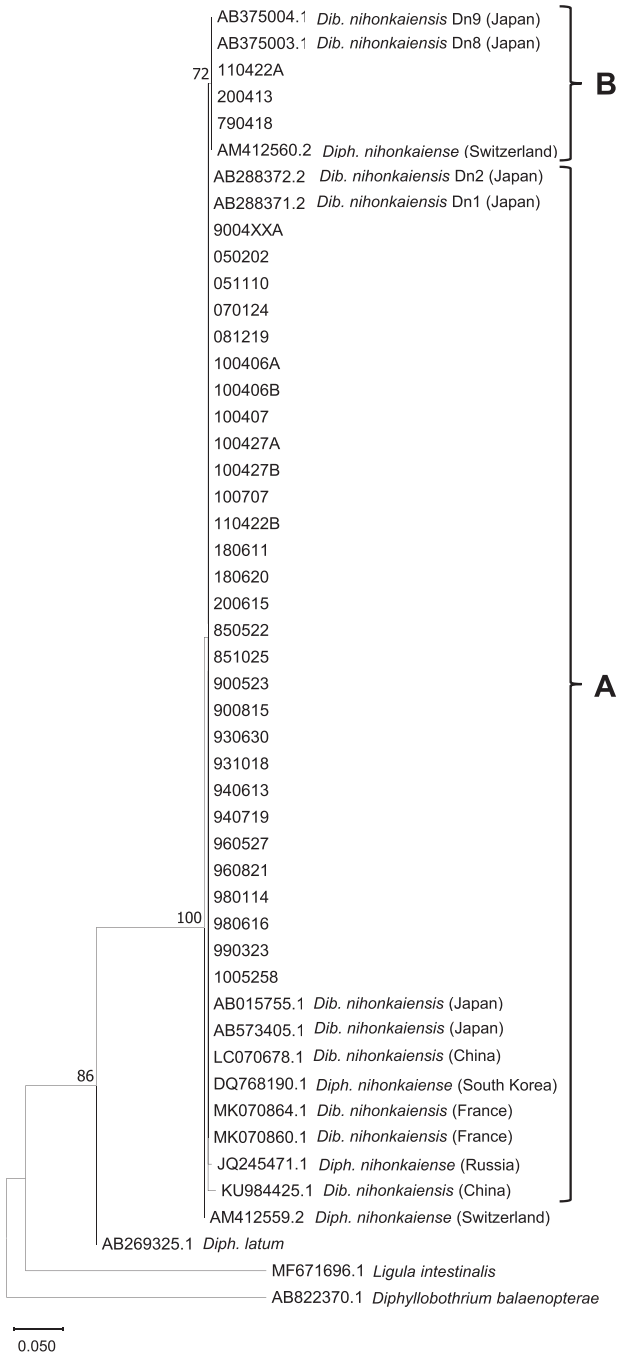


**Fig. 3.** Molecular Phylogenetic analysis based on mtDNA COI sequences for exploring the relationships among other *Dib. nihonkaiensis* strains. Phylogenetic trees were inferred by the maximum likelihood method, based on COI obtained from *Dib. nihonkaiensis* archived samples. GenBank accession numbers of all the reference sequences used to construct the trees are indicated. The numbers at the branches indicate the bootstrap values for 2000 replicates (only bootstrap scores higher than 50% are shown). The scale bar represents the distance in substitutions per nucleotide.

absent [1,4,5,29,54,55]. Although the current study establishes the possibility of the existence of a dominant haplotype in Japan, further studies are required to confirm this observation with a larger sample size from different regions across Japan.

The treatment of human diphyllobothriasis cases is by the use of praziquantel, which seems to have a broad spectrum of activity against most of the causative agents of diphyllobothriasis, especially against *Dib.*

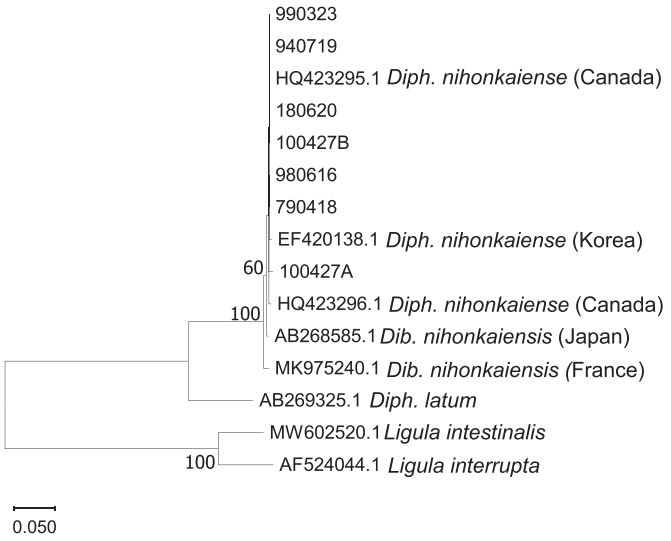


**Fig. 4.** Molecular Phylogenetic analysis based on mtDNA ND1 sequences for exploring the relationships among other *Dib. nihonkaiensis* strains. Phylogenetic trees were inferred by the maximum likelihood method, based on ND1 obtained from *Dib. nihonkaisensis* archived samples. GenBank accession numbers of all the reference sequences used to construct the trees are indicated. The numbers at the branches indicate the bootstrap values for 2000 replicates (only bootstrap scores higher than 50% are shown). The scale bar represents the distance in substitutions per nucleotide.

*nihonkaiensis* which appears to be more sensitive to praziquantel [56–58]. Therefore, treatment of the infection does not seem to pose a severe problem. On the other hand, a generally low awareness of the infection risk when eating raw or undercooked fish may be the problem needed to be addressed. While most people in Japan are not aware of the risk for *Dib. nihonkaiensis* infection associated with the consumption of raw salmon or the risks for infections with other cestodes, others who are aware still go ahead to take the risk in consuming it [9,16]. It is therefore important to adequately disseminate information regarding parasitic infections and warnings of the potential risks associated with these infections to all stakeholders in the food chain value, in addition to the institution of a more stringent inspection protocol of fish products before they are transported throughout the globe.

## 5. Conclusion

The present study reveals the limitations in the morphological methods of characterizing *Dibothriocephalus* parasites, and demonstrates the importance of mtDNA-based molecular analysis in resolving the species identities of these parasites. The study also revealed the possible presence of a dominant and globally distributed haplotype of *Dib. nihonkaiensis* circulating in Japan. Based on this data, it is important to replicate this study in different regions of Japan using a larger sample size to strongly establish the genetic relationship of *Dib. nihonkaiensis* across Japan. Finally, integrating molecular methods in the routine identification of clinical parasites will be useful to improve the management of clinical cases and establish robust control measures to reduce the burden of human diphyllobothriasis in Japan.

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## CRediT authorship contribution statement

**Danielle Ladzekpo:** Conceptualization, Methodology, Software, Formal analysis, Investigation, Data curation, Writing – original draft, Visualization. **Kofi Dadzie Kwofie:** Methodology, Software, Validation, Formal analysis, Investigation, Writing – original draft, Visualization. **Hayato Kawada:** Writing – review & editing. **Fusako Mikami:** Writing – review & editing. **Naotoshi Tsuji:** Resources, Writing – review & editing, Project administration, Funding acquisition. **Shiroh Iwanaga:** Writing – review & editing. **Samuel Kweku Dadzie:** Writing - review & editing. **Takeshi Hatta:** Conceptualization, Methodology, Software, Validation, Investigation, Writing – review & editing. **Tomoko Ishino:** Supervision.

## Declaration of Competing Interest

None.

## Data availability

All data generated or analyzed during this study are included in this published article.

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