Diphyllobothrium stemmacephalum (Cestoda: Diphyllobothriidae) found from a harbor porpoise in northern Japan, with comments on a geographical gap with human infection cases in southern Japan

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ABSTRACT

Even though the cetacean tapeworm *Diphyllobothrium stemmacephalum* occurs in both cold and warm waters, human infections and final host occurrences have been confined to temperate areas in and near Japan. We recently obtained a strobila of this cestode that was excreted from a harbor porpoise accidentally caught offshore of Hokkaido of northern Japan. Genetic analysis of 28S rDNA and *cox1* genes confirmed that the cestode was *D. stemmacephalum*. Our finding sets the northernmost record of *D. stemmacephalum* in the western Pacific, suggesting that the risk of human infections by this parasite in northern Japan deserves further attention.

Keywords: harbor porpoise; Cestoda; *Diphyllobothrium stemmacephalum*; genetic identification; distribution

Diphyllobothrium stemmacephalum (Cestoda: Diphyllobothriidae) is a zoonotic tapeworm. Human infections occur by eating raw marine fish, but the causative fishes remain to be determined [1]. The patients infected with this parasite have been sporadically reported from the lower latitudes of Japan and Korea [1]. Human infections are generally asymptomatic, but sometimes mild symptoms such as abdominal discomfort, light diarrhea and nausea have been reported [1]. A taxonomic review has resulted in the specimens previously identified as *Diphyllobothrium yonagoense*, which was first recovered from a patient of Tottori Prefecture in western Japan [2], being synonymized with *D. stemmacephalum* (morphologically in [3,4] and molecularly in [1]).

This cestode has been found from marine mammals such as those of the Phocoenidae and Delphinidae in the northern Atlantic, Baltic Sea, Black Sea and the Gulf of Mexico regardless of whether or not they are cold or warm areas [5,6]. Contrary to this, however, the distribution of *D. stemmacephalum* seems to be limited to the temperate areas in Japanese and Korean waters (Fig. 1a) [1]. Three odontocete species are known to have been infected with the tapeworm, which are the Risso's dolphin, *Grampus griseus*, from off Shimane Prefecture in the southern part of the Sea of Japan [7], the short-beaked common dolphin, *Delphinus delphis,* from off Mie Prefecture in western Japan facing the Pacific Ocean [8], and the Pacific white-sided dolphin, *Lagenorhynchus obliquidens*, in the Toba Aquarium of Mie Prefecture [9].

We recently observed a cestode strobila identified as *D. stemmacephalum* from the harbor porpoise, *Phocoena phocoena*, in Hokkaido of northern Japan. This report provides its genetic profiles and updates information about the northern limit of this parasite's distribution around Japan. The possible causes of the geographical gap with the known human infection areas are also considered.

The male porpoise host was accidentally caught by a coastal large set net fishery targeting tuna, squid, and salmon off Hakodate (42°01′03″N, 140°52′50″E) of southern Hokkaido on 10 May 2017 (SNH17013-2, https://www.gbif.org/occurrence/3319849495) [10]. The body length of the porpoise was 120.5 cm, indicating that it was immature. The porpoise was cared for in a tank (ca. 6.0 m in diameter with a water depth of about 110-120 cm) temporarily set up at the Usujiri Fisheries Station of Hokkaido University, and was released two days later, after confirming the progress of its recovery.

During its recovery period, the strobila was observed hanging down from the anus of the porpoise (Fig. 1b). That part of the worm (about 49 cm in length) then dropped off spontaneously. It was scooped up with a net, preserved in a glass bottle filled with 99.5% ethanol, and then it was sent to Azabu University in Kanagawa Prefecture, where the following examinations were conducted.

A DNA sample was extracted by lysing a piece of the parasite specimen in 100 μL of Tail lysis buffer (Nacalai Tesque, Inc., Kyoto, Japan) with 2 μL of Proteinase K (Qiagen K. K., Tokyo, Japan, 20mg/ml) at 56℃ overnight. The extracted DNA was purified using Ethachinmate following the manufacturer's instructions (NIPPON GENE, Tokyo, Japan). The

targeted genes for polymerase chain reaction (PCR) amplification were nuclear 28S ribosomal DNA (28S rDNA) and mitochondrial cytochrome *c* oxidase gene subunit I genes (*cox1*). The PCR was conducted by applying the primer sets LSU-5 and 1500R [11], and Cox1Forward and Cox1Reverse [5]. The PCRs were run for 30 cycles (94℃ for 30 s, 52℃ for 30 s and 72℃ for 90 s) for 28S rDNA and 35 cycles (95°C for 30 s, 50°C for 30 s, and 72°C for 90 s) for *cox1* in a total volume of 25 μL. PCR products were purified with the Wizard SV Gel and PCR Clean-Up System (Promega, Tokyo, Japan) and directly sequenced at Eurofins Genomics (Tokyo, Japan). The 28S rDNA and *cox1* products were sequenced in both directions using the PCR primers and internal primers: 300F and ECD2 for the former [11]: and cox1F9845-9864 (5′-TAGCTGCTGCTATTACAATG-3′) and cox1R10077-10096 (5′-ACTACCAAGACAAACAATG-3′) for the latter. The obtained sequences were aligned and edited using MEGA-X software [12], and were compared with reference sequences available in the GenBank/EMBL/DDBJ databases using BLAST (Basic Local Alignment Search Tool). Phylogenetic trees were constructed based on the partial 28S rDNA and the complete *cox1* sequences of specimens along with sequences of other related species retrieved from the GenBank [5,13]. Phylogenetic analysis was performed with MEGA-X software by the maximum likelihood method. Clades were assessed by bootstrap resampling with 1000 replicates.

A total of 1411 and 1566 bp were determined from the sequencing of the targeted regions of 28S rDNA and *cox1*, respectively. These sequence data were registered in the GenBank as LC644720 and LC644653 for 28S rDNA and *cox1*, respectively. BLAST searches for both targeted regions showed 100% sequence matches between our specimen and known *D. stemmacephalum* (e.g. KY552825.1 and AP017648.1). Phylogenetic analysis also supported that the species is *D. stemmacephalum* (Fig. 2a and 2b).

The previous records of human infections suggest that *D. stemmacephalum* is mainly distributed off the southern coast of Japan between 25°N and 35°N (Fig. 1a). The locality reported in the present study represents the new northernmost record of the species in the western Pacific.

The Risso's dolphin and the short-beaked common dolphin are southern species commonly occurring in warm-sea environments and are known to be final hosts [14–16]. The Pacific white-sided dolphin is the other known host, and it is a migratory species that moves between the cold waters (i.e., the northern region around Hokkaido) and warm waters (i.e., off the southern coast of Japan), with individuals overwintering off Fukuoka and Yamaguchi Prefectures that are located in the southern the Sea of Japan and also off the Kii Peninsula along the Pacific coast of Japan, respectively [17–19]. Although the feeding habits of these cetaceans have not yet been clarified, some of their prey fishes are expected to serve as a second intermediate host for *D. stemmacephalum*, which may have been transmitted to humans by eating them raw.

The harbor porpoise that is newly recognized as the host of *D. stemmacephalum* in this report,

is a northern species that does not appear to inhabit southern or western areas (i.e., off Honshu and off western Hokkaido) with sea surface temperatures exceeding 12-16℃ in summer [20,21]. Some individuals are known to migrate south from Hokkaido to Honshu as sea surface temperatures decrease in winter [20]. The southward limits of harbor porpoises are off Niigata Prefecture (i.e. 39°N) in the Sea of Japan and off Ibaraki Prefecture (i.e. 36°N) along the Pacific coast of Japan; but in an exceptional case, one individual was found in a more southwest area off Taiji of Wakayama Prefecture [20]. Therefore, it seems possible that parasite infections of harbor porpoises occur during those periods of southward migrations.

Alternatively, the recent global warming may have resulted in the parasite being more easily transported to the north by migrations or habitat expansions of fish intermediate hosts [e.g. 22,23]. Because the harbor porpoise is known to be an opportunistic generalist predator that uses any available prey at each time and place [24], this potential host might become infected with *D. stemmacephalum* around Hokkaido as a result of rising water temperatures.

The intermediate fish hosts exploited by *D. stemmacephalum* are still uncertain, but have been assumed from the diets of the patients who frequently eat raw fish. The consumed fish include the skipjack tuna (*Katsuwonus pelamis*), the Japanese Spanish mackerel (*Scomberomorus niphonius*), tunas (*Thunnus* spp.), and the Japanese amberjack (*Seriola quinqueradiata*) [25]. In addition to these highly migratory fishes, other hosts may be resident species such as mackerels (*Rastrelliger* spp.), the silver-stripe round herring (*Spratelloides gracilis*), and the striped beakfish (*Oplegnathus fasciatus*), which are mainly distributed and consumed in the southern regions [26]. Regardless of the species that caused the infection, the present finding may suggest that the risk of human infection by this parasite by consuming fishes caught from the northern cold-water areas needs to be considered, and it may be necessary to monitor possible fish hosts species.

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225 **Figure captions**
226
227 Fig. 1. Human infection records around Japan and the *Diphyllobothrium stemmacephalum*
228 parasite specimen of the present study. (a) Localities of human infections reported in previous
229 studies (orange closed-circles) [1] and the locality reported in the present study (blue closed-
230 circle). (b) A video frame-capture (see the video clip in Appendix S1) of the sheltered harbor
231 porpoise in the holding tank, showing the cestode strobila being excreted.
232
233 Fig. 2. The phylogenetic relationships of *Diphyllobothrium stemmacephalum* reported in this
234 study and related species. (a) A phylogenetic tree estimated by the maximum likelihood method
235 based on 28S rDNA sequences using K2P+G. *Spirometra erinaceieuropaei* was used as an out-
236 group. (b) A phylogenetic tree estimated by the maximum likelihood method based on *cox1*
237 sequences using GTR+G+I. *Spirometra mansoni* was used as an out-group. Bootstrap values
238 (> 90) are shown on branches.

Fig. 1

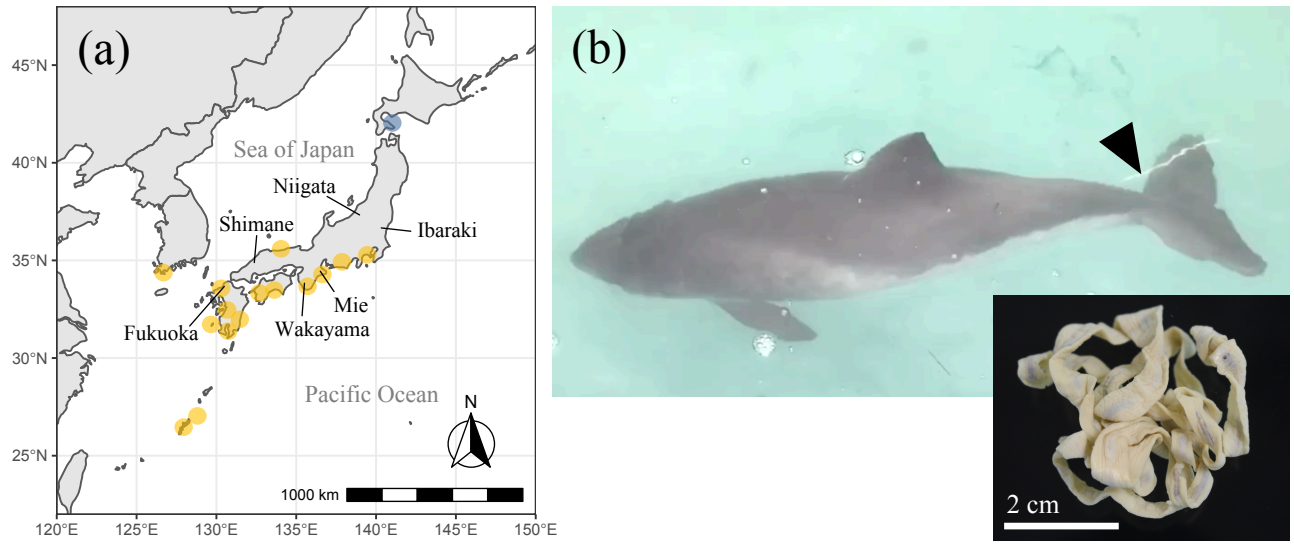
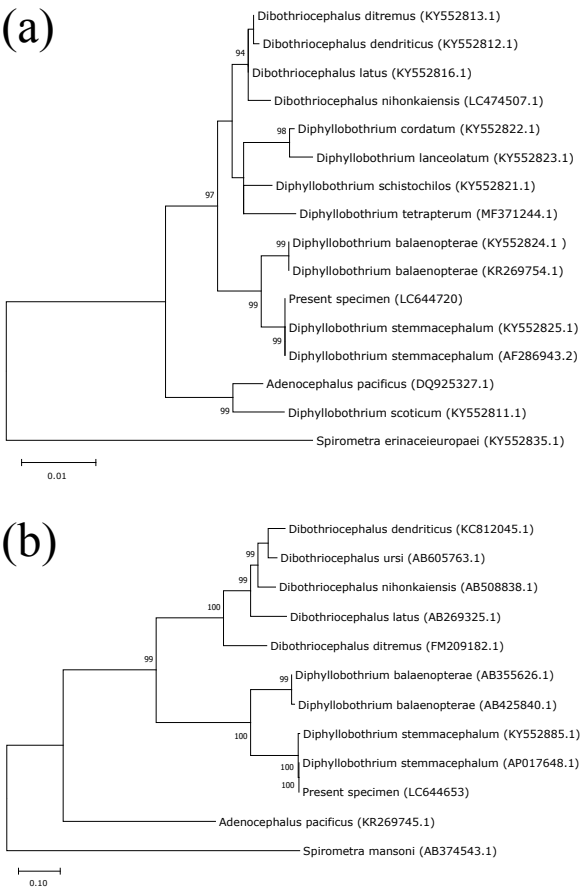

(a)
45°N
40°N
35°N
30°N
25°N
120°E
125°E
130°E
135°E
140°E
145°E
150°E
Sea of Japan
Niigata
Shimane
Ibaraki
Mie
Wakayama
Fukuoka
Pacific Ocean
N
1000 km
(b)
2 cm

Fig. 2


(a)
Dibothriocephalus ditremus (KY552813.1)
Dibothriocephalus dendriticus (KY552812.1)
94
Dibothriocephalus latus (KY552816.1)
Dibothriocephalus nihonkaiensis (LC474507.1)
98
Diphyllobothrium cordatum (KY552822.1)
Diphyllobothrium lanceolatum (KY552823.1)
Diphyllobothrium schistochilos (KY552821.1)
97
Diphyllobothrium tetrapterum (MF371244.1)
99
Diphyllobothrium balaenopterae (KY552824.1)
Diphyllobothrium balaenopterae (KR269754.1)
99
Present specimen (LC644720)
Diphyllobothrium stemmacephalum (KY552825.1)
99
Diphyllobothrium stemmacephalum (AF286943.2)
Adenocephalus pacificus (DQ925327.1)
99
Diphyllobothrium scoticum (KY552811.1)
Spirometra erinaceieuropaei (KY552835.1)
0.01
(b)
Dibothriocephalus dendriticus (KC812045.1)
99
Dibothriocephalus ursi (AB605763.1)
99
Dibothriocephalus nihonkaiensis (AB508838.1)
100
Dibothriocephalus latus (AB269325.1)
Dibothriocephalus ditremus (FM209182.1)
99
99
Diphyllobothrium balaenopterae (AB355626.1)
Diphyllobothrium balaenopterae (AB425840.1)
100
Diphyllobothrium stemmacephalum (KY552885.1)
100
Diphyllobothrium stemmacephalum (AP017648.1)
100
Present specimen (LC644653)
Adenocephalus pacificus (KR269745.1)
Spirometra mansoni (AB374543.1)
0.10

Graphical abstract