Gastric ulceration caused by genetically identified *Anisakis simplex* sensu stricto in a harbor porpoise from the Western Pacific stock

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ABSTRACT

The genus *Anisakis* is a well-known group of nematodes that parasitize cetaceans as the final host and cause mucosal damage to their stomach. However, little has been done to precisely identify the nematodes recovered from the final hosts, especially in the Western Pacific, because of taxonomic confusion about the discrimination of sibling species and the difficulties of obtaining specimens from cetaceans. We describe the results of genetic identification and histopathological observations of specimens recovered from an ulcerated lesion and stomach contents in the forestomach of a female harbor porpoise accidentally caught by a set net fishery in Usujiri, southern Hokkaido, Japan. All the specimens arbitrarily collected from the lesion and stomach contents were identified as *Anisakis simplex* sensu stricto according to their ITS rDNA sequences. The size of the ulcer was approximately 6.3 mm in diameter and it was infected with 119 individual nematodes, mostly consisting of L3 and L4 stage larvae (95.0%). Histological sections were characterized by a locally extensive ulcer with the parasites penetrating into the muscularis externa that caused a thickening of the surrounding mucosa.

Key words: harbor porpoise, *Anisakis simplex* sensu stricto, genetic identification, histopathology

Parasitic nematodes of the genus *Anisakis* have long been known to cause lesions in the stomachs of marine mammals including cetaceans [1]. The main symptom is the formation of ulcers due to an over-concentrated infection in one place [1–4], and the damages were sometimes thought to lead to host death in severe cases [e.g. 5–6]. However, identifying these nematodes has been a long-term problem especially in the Western Pacific, because of taxonomic confusion about discriminating sibling species in this genus [7–8]. Additionally, the complexity of infections, consisting not only of adults but also of third (L3) and fourth (L4) stage larvae [e.g. 2,9], make it difficult to identify the species morphologically.

As a result of taxonomic studies on the genus *Anisakis* based mainly on adult male morphology and DNA characters, the species previously identified as *A. simplex* A, B and C have been designated as *A. pegreffii*, *A. simplex* sensu stricto (s. s.) and *A. berlandi*, respectively [10–11]. Furthermore, larvae previously morphologically identified as *Anisakis* Type I have been genetically distinguished into *A. simplex* s. s., *A. pegreffii*, *A. berlandi*, *A. ziphidarum*, *A. nascettii*, and *A. typica*, and *Anisakis* Type II, III and IV has also been identified as *A. physeteris*, *A. brevispiculata* and *A. paggiae*, respectively [8].

Since Japan is the most prevalent area of human anisakiasis in the world [12], *Anisakis* infection in paratenic hosts (i.e. sources of human infection) has been intensively investigated [13]. However, determination of the final host has not been achieved for many years due to the difficulties of obtaining specimens from cetaceans [see 14]. Since the taxonomic confusion has currently been resolved, new examinations are required to verify the harmful species in this region and other sea areas [15].

During the course of dissecting a harbor porpoise, *Phocoena phocoena*, belonging to the Western Pacific stock [16], we found an *Anisakis* infection forming a cluster on the wall of its forestomach. Here, we report the results of histopathological observations together with genetic species identification.

The female host was accidentally caught by a coastal set net fishery in Usujiri (41°56′29″ N, 140°57′44″E) of southern Hokkaido, Japan, on 29 April 2020. After confirming its death, the host specimen was brought to a laboratory at Hokkaido University in Hakodate, where it was measured for body size and autopsied. The total length of the porpoise was 129.2 cm as recorded in the database of the Stranding Network Hokkaido (SNH 20019-1, http://kujira110.com/?p=3359). Some of the nematode specimens penetrating the wall of the forestomach were arbitrarily collected and preserved in a glass bottle filled with 99.5% ethanol. Most of the remaining individuals were removed along with the surrounding tissue and fixed with 10% formalin in a plastic bottle. In addition to these specimens, individuals in the stomach contents were also

recovered and preserved in a glass bottle filled with 99.5% ethanol. These were sent to Azabu University in Kanagawa Prefecture, where the following examinations were conducted.

For pathological observations, formalin-fixed samples were first photographed from dorsal and lateral views using a digital camera, followed by trimming of the nematode infection site and a distant normal stomach lining site. These blocks were dehydrated in a series of ascending concentrations of ethanol and xylene, embedded in paraffin and sectioned at a thickness of 10 μm in a direction transverse to the stomach wall with a microtome (Sakura Pteratome, CRM-440). The sections were subsequently mounted on a glass slide, stained with hematoxylin and eosin, dehydrated, and mounted in Canada balsam with a cover slip. The slides were observed with a light microscope (Olympus, BX51). The thicknesses of the mucosal layers (i.e. stratum corneum layer, and stratum spinosum and basale layers) were compared between the surrounding site (n = 15) and the normal site (n = 20) by constructing a linear model using R 3.5.3 (https://www.R-project.org/).

The nematode specimens kept in 99.5% ethanol were used for DNA extraction. A piece of each body was lysed in 20 μl of 0.02 N NaOH at 98°C for 30 min [17]. The targeted region for polymerase chain reaction (PCR) amplification was the internal transcribed spacer (ITS) region (ITS1-5.8S-ITS2) of ribosomal DNA (rDNA), and the PCR was conducted using the forward primer NC5 (5'- GTA GGT GAA CCT GCG GAA GGA TCA TT -3') and reverse primer NC2 (5'- TTA GTT TCT TTT CCT CCG CT -3') [18]. PCR was run for 35 cycles (94°C for 30 s, 53°C for 30 s, and 72°C for 60 s) in a total volume of 25 μl including 1 μM of each primer and 2 μl of the extracted template. PCR products were purified with the Wizard SV Gel and PCR Clean-Up System (Promega, Tokyo, Japan) and directly sequenced at Eurofin Genomics (Tokyo, Japan). The rDNA products were sequenced in both directions using the PCR primers. The obtained sequences were aligned and edited using MEGA-X software [19], and were compared with reference sequences available in the GenBank/EMBL/DDBJ databases using BLAST (Basic Local Alignment Search Tool). A phylogenetic tree was constructed based on the partial ITS rDNA sequences of specimens along with sequences of nine other related species retrieved from the GenBank [11]. Phylogenetic analysis was performed with MEGA-X software by the maximum likelihood method using the Kimura-two-parameter model with a discrete gamma distribution. The model was selected based on the lowest score of corrected Akaike's Information Criterion. Clades were assessed by bootstrap resampling with 1000 replicates.

The number of parasite individuals from the ulcer and stomach contents was counted

by discriminating their life stages from L3, L4 and subadult to adult [5,9], including the specimens used in the sequencing analysis. The total length of each undamaged individual was measured in millimeters following past research [20]. The life stage composition was compared between the specimens from the ulcer and those from the stomach contents, by fitting a generalized linear model into the number of individuals of each stage using R 3.5.3. A Poisson distribution was applied for the probability distribution of the response variable. The collected site (the ulcer = 0, the stomach content =1), life stage (adult = 0, subadult = 1, L4 = 3, L3 = 4) and those interactions were used as the categorical explanatory variables.

The lesion created by the nematodes was characterized by a locally extensive ulcer with parasite penetration, and a thickening of the surrounding stratified squamous epithelium (Fig. 1a–f). The ulcer was approximately 6.3 mm in diameter (Fig. 1b). Histologically, although the majority of the nematodes were found in the submucosa, some individuals were also seen in the muscularis externa (Fig. 1d). The mucosa surrounding the infection site was thickened compared to the normal sites, with the stratum corneum layer and the stratum spinosum and stratum basale layers being 1.8 and 4.4 times thicker on average ($p < 0.001$ in both cases), respectively (Fig. 1f).

The parasite life stages mainly consisted of L3 and L4 stage larvae in a total of 119 individuals in the ulcer and 339 individuals recovered from the stomach contents (Fig. 2a). In the ulcer, 95.0% of the specimens were larval stages compared to 92.0% in the stomach contents. In addition, one (0.8%) gravid female with eggs was found in the ulcer and 12 (5.8%) were among the specimens in the stomach contents. The fitted model indicated that there was a significant difference in the number of adults from the stomach contents being greater than that from the ulcer ($p = 0.002$) while the numbers of subadult and L4 stage from the former tended to be fewer than those from the latter ($p = 0.028$ and 0.016, respectively). Their body sizes ranged from 14.3–57.0 mm in the ulcer and 13.3–61.6 mm from the stomach contents (Fig. 2b).

A total of 853 bp were determined from the sequencing of the targeted region of 11 randomly selected specimens. No variation was found within the seven individuals from the ulcer (one subadult, five L4 larvae and one L3 larva) and the four individuals from the stomach contents (one adult female, one L4 larva and two L3 larvae), indicating a single haplotype. The representative data were registered in the GenBank (LC589664) and used in the subsequent analysis. A BLAST search of the targeted region showed a 100% sequence match between our specimens and specimens of *A. simplex* (e.g. JX535521). In the constructed phylogenetic tree, our sequence data was also located on the same branch as known specimens of *A. simplex* s. s. (Fig. 2c), indicating that our

specimens were identical to this species.

Morphologically identified nematodes of *A. simplex* B have been reported as a causative species of gastric ulcers in harbor porpoises from the Scottish coast [2]. Other reports based on morphological identification have also been published sporadically from the same host species [e.g. 9,21], but infections by *A. simplex* s. s. have been limited to the northeastern Pacific stock [22], and subsequently from stocks in the North Sea, Baltic Sea and North Atlantic [23]. In Japanese waters, there is no published information on *Anisakis* spp. from harbor porpoises. Kagei et al. [24] only referred to *A. simplex* sensu lato from the same host, but it had been caught in the Bering Sea. The present report is therefore the first confirmation of *A. simplex* s. s. infecting the Western Pacific stock of the harbor porpoise around Japan.

The preferred host of *A. simplex* in the Northwestern Pacific has long been considered to be common minke whales, *Balaenoptera acutorostrata* [25]. In addition to the offshore whale host, harbor porpoises may serve as a supplementary host to complete the life cycle of this nematode parasite in coastal areas, because this mammal feeds on a variety of nearshore fishes that potentially serve as hosts for the third stage larvae [26]. This is suggested by the presence of a few gravid females in our study, even though their body length appears to be shorter than those from the offshore whale hosts [25]. If harbor porpoises are a host reservoir in coastal areas, they might affect the parasite population size and dynamics that relate to the risk of human anisakiasis.

The harmfulness to the stomach wall has historically been thought to diminish with nematode growth [1,5], although various life stages can be found from ulcers of dolphins and porpoises [1–2,5, see also 9]. The third stage larvae tend to penetrate deeply into the mucosa before moulting to L4, whereas adults are normally attaching superficially to the stomach wall [1,5]. The nematode composition found from the ulcer where L3 larvae are the most dominant followed by L4 may reflect the dependence on the wall. Attachment to the wall and apparently using the ulcer for nutrient intake, provides not only a proximate benefit for growth, but also provides an ultimate benefit to stay in the suitable habitat and not be discharged from the host until reaching maturity. In any case, the infection is an undesired cost to the host, because the resulting damage can extend to the muscularis externa and induce host responses such as thickening of the surrounding tissues.

The infection site, ulcer positions, can be found not only in the forestomach but also in the main stomach and rarely in the pyloric stomach depending on the host species [e.g. 5]. Further investigations are needed to determine whether these differences in the infection site and their harmfulness are due to the host's vulnerability and/or reflect the

parasite-specific features such as host specificity and infection loads. In order to effectively conduct these studies, interdisciplinary collaborations especially between marine mammologists and parasitologists will be essential.

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Figure captions

Fig. 1. The gastric ulcer found in a setnet-bycatch female harbor porpoise from off Usujiri, southern Hokkaido, Japan. (a) Dorsal view of the ulcer, (b) lateral view of (a), (c) section of the infected site including the surrounding tissue; arrows indicate borders between the stratum corneum and stratum spinosum, and between the stratum basale and lamina propria, (d) high-magnification view of the anterior end of the nematode penetrating the muscularis externa, indicated by the dotted box in (c), (e) section of a normal site, (f) boxplots of the thickness in the stratum corneum layer (Sc) and the stratum spinosum and stratum basale layers (Ss and sb) compared between the sections of the surrounding site (n =15) and those of the normal site (n = 20). Scale bars indicate 1 cm in (a) and (b), 2.5 mm in (c), 0.2 mm in (d) and 1 mm in (e).

Fig. 2. Profile of the infecting individuals from the ulcer and stomach contents in the female harbor porpoise from off Usujiri, southern Hokkaido, Japan. (a) Component proportion of each developmental stage recovered from the ulcer (upper) and stomach

286 contents (bottom); the number of individuals accompanied by the percentage in
287 parenthesis, (b) boxplot of the body length recovered from the ulcer (upper) and stomach
288 contents (bottom); the number of undamaged individuals examined is presented near each
289 box; the inset shows a photo of eggs from a gravid female (scale bar 10 μm), (c) a
290 phylogenetic tree estimated by the maximum likelihood method based on ITS rDNA
291 sequences and the Kimura-two-parameter model with a discrete gamma distribution.
292 Bootstrap values (> 60) are shown on branches.

Fig. 1

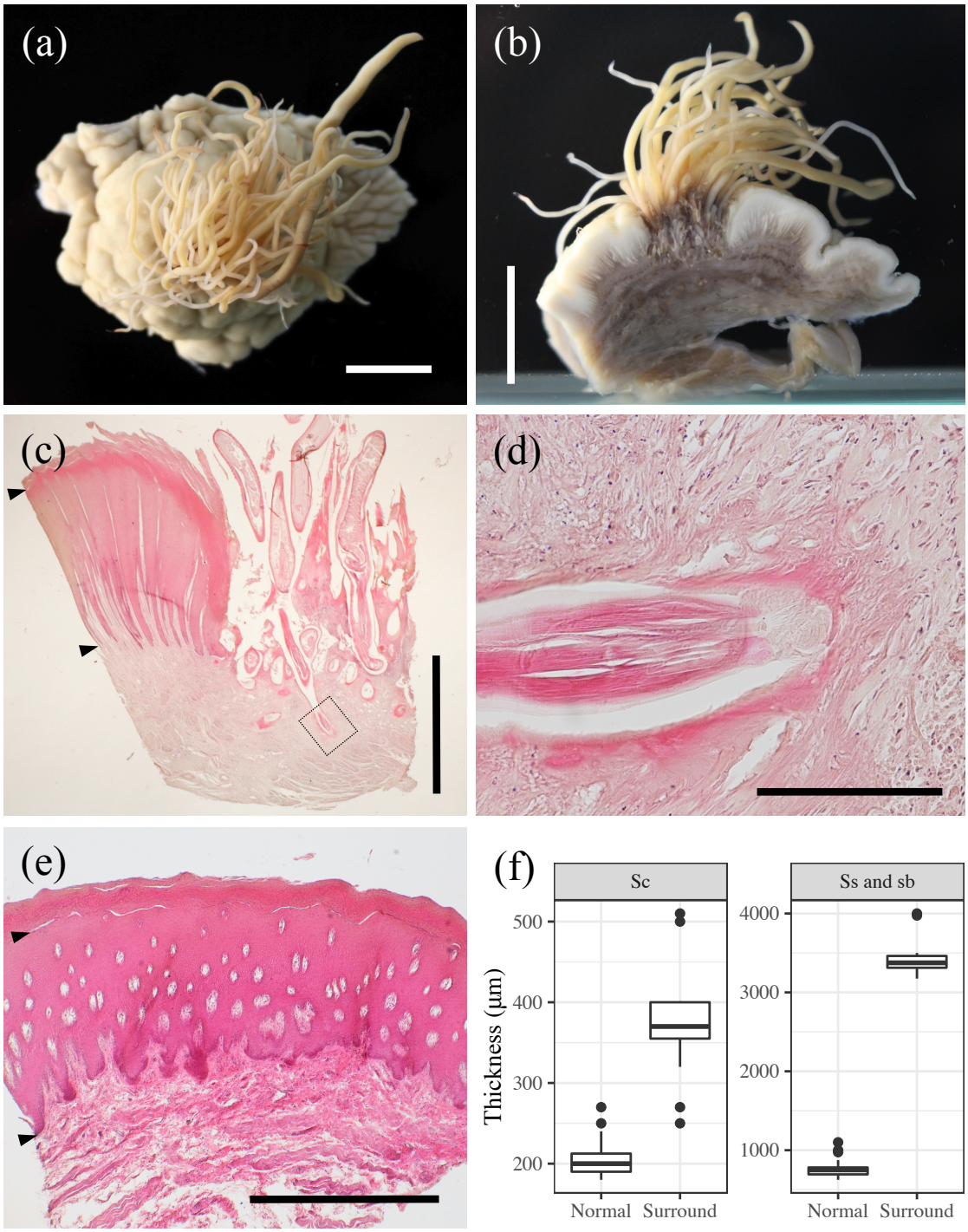
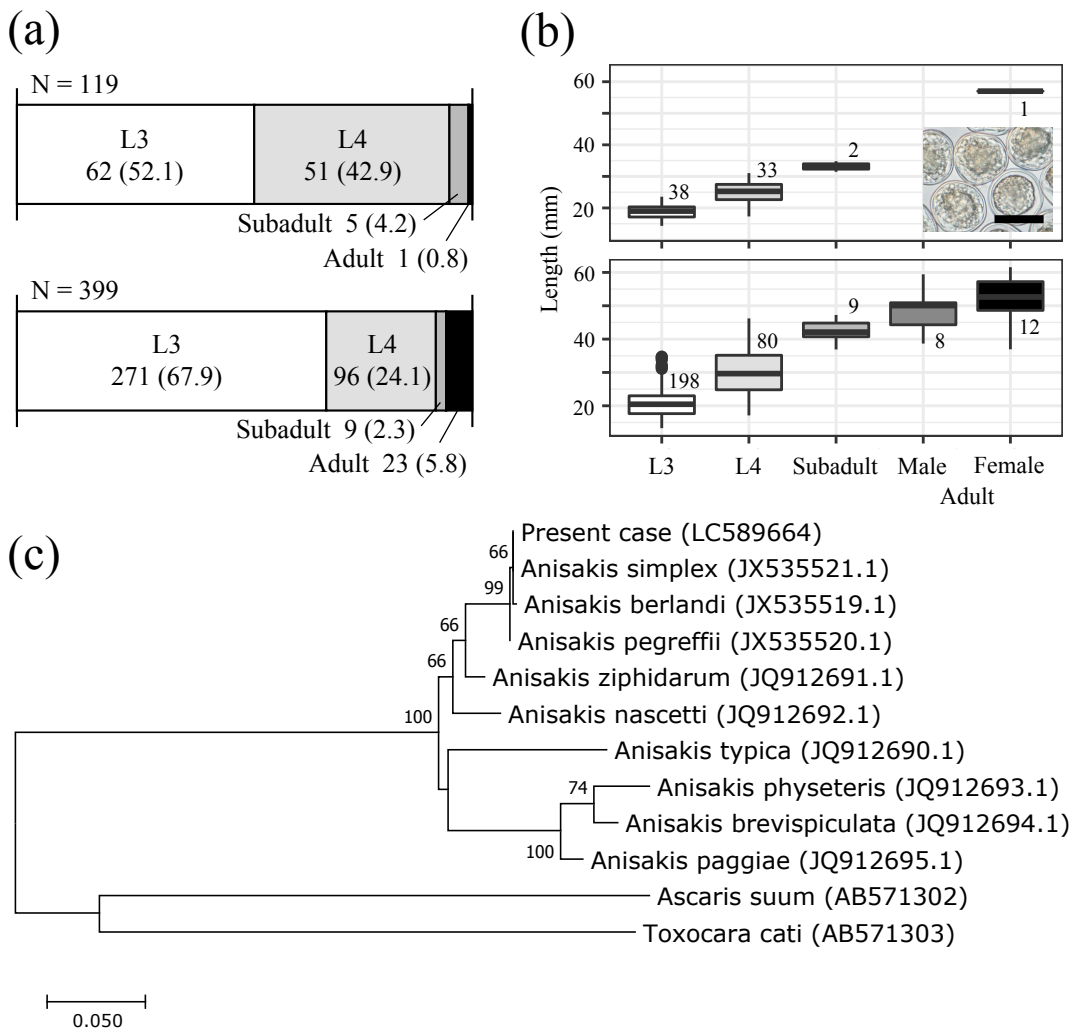

(a)
(b)
(c)
(d)
(e)
(f)
Sc
Ss and sb
Thickness (μm)
500
400
300
200
4000
3000
2000
1000
Normal
Surround
Normal
Surround

Fig. 2


(a)
N = 119
L3
62 (52.1)
L4
51 (42.9)
Subadult 5 (4.2)
Adult 1 (0.8)
N = 399
L3
271 (67.9)
L4
96 (24.1)
Subadult 9 (2.3)
Adult 23 (5.8)
(b)
Length (mm)
60
40
20
38
33
2
1
198
80
9
8
12
L3
L4
Subadult
Male
Female
Adult
(c)
Present case (LC589664)
Anisakis simplex (JX535521.1)
Anisakis berlandi (JX535519.1)
Anisakis pegreffii (JX535520.1)
Anisakis ziphidarum (JQ912691.1)
Anisakis nascetti (JQ912692.1)
Anisakis typica (JQ912690.1)
Anisakis physeteris (JQ912693.1)
Anisakis brevispiculata (JQ912694.1)
Anisakis paggiae (JQ912695.1)
Ascaris suum (AB571302)
Toxocara cati (AB571303)
66
99
66
66
100
74
100
0.050

Graphical abstract