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Author(s)	Katahira, Hirotaka; Matsuda, Ayaka; Banzai, Azusa; Eguchi, Yuya; Matsuishi, Takashi F.
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1 **Gastric ulceration caused by genetically identified *Anisakis simplex***
2 **sensu stricto in a harbor porpoise from the Western Pacific stock**

3
4 Hiroataka Katahira^{a,*} Ayaka Matsuda^{b,**}, Azusa Banzai^a, Yuya Eguchi^a, Takashi F.
5 Matsuishi^b

6
7 ^a *Department of Environmental Science, School of Life and Environmental Science, Azabu*
8 *University, 1-17-71 Fuchinobe, Chuo-ku, Sagamihara, Kanagawa 252-5201, Japan*

9 ^b *Faculty of Fisheries Sciences, Hokkaido University, 3-1-1 Minato, Hakodate, Hokkaido,*
10 *Japan*

11 *Corresponding author.

12 E-mail address: katahira@azabu-u.ac.jp

13 **Equally contributed author.

14
15 **ABSTRACT**

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

30
31 **Key words:** harbor porpoise, *Anisakis simplex* sensu stricto, genetic identification,
32 histopathology

34 Parasitic nematodes of the genus *Anisakis* have long been known to cause lesions in
35 the stomachs of marine mammals including cetaceans [1]. The main symptom is the
36 formation of ulcers due to an over-concentrated infection in one place [1–4], and the
37 damages were sometimes thought to lead to host death in severe cases [e.g. 5–6]. However,
38 identifying these nematodes has been a long-term problem especially in the Western
39 Pacific, because of taxonomic confusion about discriminating sibling species in this genus
40 [7–8]. Additionally, the complexity of infections, consisting not only of adults but also of
41 third (L3) and fourth (L4) stage larvae [e.g. 2,9], make it difficult to identify the species
42 morphologically.

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

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

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

60 The female host was accidentally caught by a coastal set net fishery in Usujiri
61 (41°56'29" N, 140°57'44"E) of southern Hokkaido, Japan, on 29 April 2020. After
62 confirming its death, the host specimen was brought to a laboratory at Hokkaido
63 University in Hakodate, where it was measured for body size and autopsied. The total
64 length of the porpoise was 129.2 cm as recorded in the database of the Stranding Network
65 Hokkaido (SNH 20019-1, <http://kujira110.com/?p=3359>). Some of the nematode
66 specimens penetrating the wall of the forestomach were arbitrarily collected and
67 preserved in a glass bottle filled with 99.5% ethanol. Most of the remaining individuals
68 were removed along with the surrounding tissue and fixed with 10% formalin in a plastic
69 bottle. In addition to these specimens, individuals in the stomach contents were also

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

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

85 The nematode specimens kept in 99.5% ethanol were used for DNA extraction. A piece
86 of each body was lysed in 20 μl of 0.02 N NaOH at 98°C for 30 min [17]. The targeted
87 region for polymerase chain reaction (PCR) amplification was the internal transcribed
88 spacer (ITS) region (ITS1-5.8S-ITS2) of ribosomal DNA (rDNA), and the PCR was
89 conducted using the forward primer NC5 (5'- GTA GGT GAA CCT GCG GAA GGA
90 TCA TT -3') and reverse primer NC2 (5'- TTA GTT TCT TTT CCT CCG CT -3') [18].
91 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
92 volume of 25 μl including 1 μM of each primer and 2 μl of the extracted template. PCR
93 products were purified with the Wizard SV Gel and PCR Clean-Up System (Promega,
94 Tokyo, Japan) and directly sequenced at Eurofin Genomics (Tokyo, Japan). The rDNA
95 products were sequenced in both directions using the PCR primers. The obtained
96 sequences were aligned and edited using MEGA-X software [19], and were compared
97 with reference sequences available in the GenBank/EMBL/DDBJ databases using
98 BLAST (Basic Local Alignment Search Tool). A phylogenetic tree was constructed based
99 on the partial ITS rDNA sequences of specimens along with sequences of nine other
100 related species retrieved from the GenBank [11]. Phylogenetic analysis was performed
101 with MEGA-X software by the maximum likelihood method using the Kimura-two-
102 parameter model with a discrete gamma distribution. The model was selected based on
103 the lowest score of corrected Akaike's Information Criterion. Clades were assessed by
104 bootstrap resampling with 1000 replicates.

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

106 by discriminating their life stages from L3, L4 and subadult to adult [5,9], including the
107 specimens used in the sequencing analysis. The total length of each undamaged individual
108 was measured in millimeters following past research [20]. The life stage composition was
109 compared between the specimens from the ulcer and those from the stomach contents, by
110 fitting a generalized linear model into the number of individuals of each stage using R
111 3.5.3. A Poisson distribution was applied for the probability distribution of the response
112 variable. The collected site (the ulcer = 0, the stomach content =1), life stage (adult = 0,
113 subadult = 1, L4 = 3, L3 = 4) and those interactions were used as the categorical
114 explanatory variables.

115 The lesion created by the nematodes was characterized by a locally extensive ulcer
116 with parasite penetration, and a thickening of the surrounding stratified squamous
117 epithelium (Fig. 1a–f). The ulcer was approximately 6.3 mm in diameter (Fig. 1b).
118 Histologically, although the majority of the nematodes were found in the submucosa,
119 some individuals were also seen in the muscularis externa (Fig. 1d). The mucosa
120 surrounding the infection site was thickened compared to the normal sites, with the
121 stratum corneum layer and the stratum spinosum and stratum basale layers being 1.8 and
122 4.4 times thicker on average ($p < 0.001$ in both cases), respectively (Fig. 1f).

123 The parasite life stages mainly consisted of L3 and L4 stage larvae in a total of 119
124 individuals in the ulcer and 339 individuals recovered from the stomach contents (Fig.
125 2a). In the ulcer, 95.0% of the specimens were larval stages compared to 92.0% in the
126 stomach contents. In addition, one (0.8%) gravid female with eggs was found in the ulcer
127 and 12 (5.8%) were among the specimens in the stomach contents. The fitted model
128 indicated that there was a significant difference in the number of adults from the stomach
129 contents being greater than that from the ulcer ($p = 0.002$) while the numbers of subadult
130 and L4 stage from the former tended to be fewer than those from the latter ($p = 0.028$ and
131 0.016 , respectively). Their body sizes ranged from 14.3–57.0 mm in the ulcer and 13.3–
132 61.6 mm from the stomach contents (Fig. 2b).

133 A total of 853 bp were determined from the sequencing of the targeted region of 11
134 randomly selected specimens. No variation was found within the seven individuals from
135 the ulcer (one subadult, five L4 larvae and one L3 larva) and the four individuals from
136 the stomach contents (one adult female, one L4 larva and two L3 larvae), indicating a
137 single haplotype. The representative data were registered in the GenBank (LC589664)
138 and used in the subsequent analysis. A BLAST search of the targeted region showed a
139 100% sequence match between our specimens and specimens of *A. simplex* (e.g.
140 JX535521). In the constructed phylogenetic tree, our sequence data was also located on
141 the same branch as known specimens of *A. simplex* s. s. (Fig. 2c), indicating that our

142 specimens were identical to this species.

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

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

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

174 The infection site, ulcer positions, can be found not only in the forestomach but also in
175 the main stomach and rarely in the pyloric stomach depending on the host species [e.g.
176 5]. Further investigations are needed to determine whether these differences in the
177 infection site and their harmfulness are due to the host's vulnerability and/or reflect the

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

181

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183

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190

191 **References**

192

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269

270 **Figure captions**

271

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

282

283 Fig. 2. Profile of the infecting individuals from the ulcer and stomach contents in the
 284 female harbor porpoise from off Usujiri, southern Hokkaido, Japan. (a) Component
 285 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

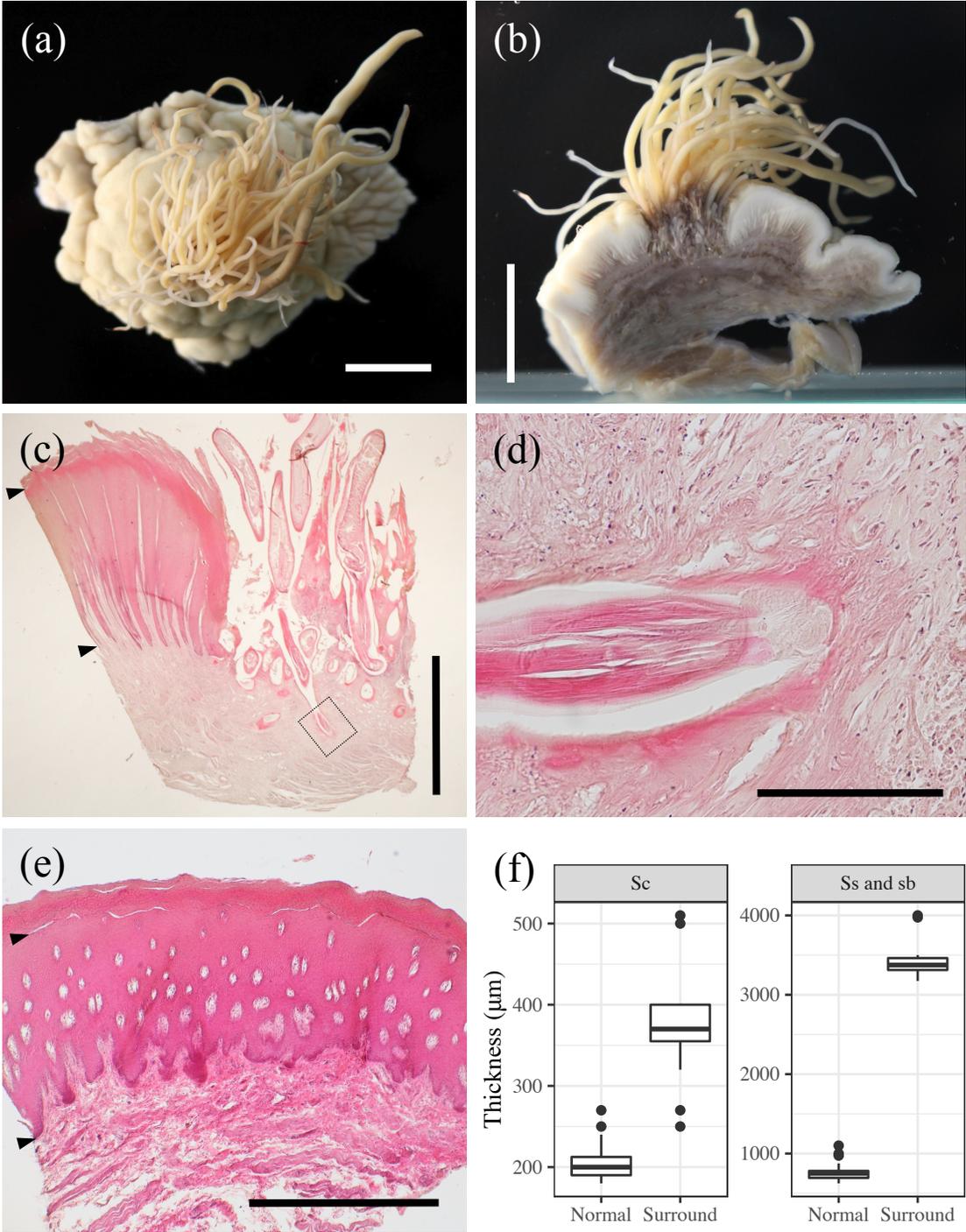
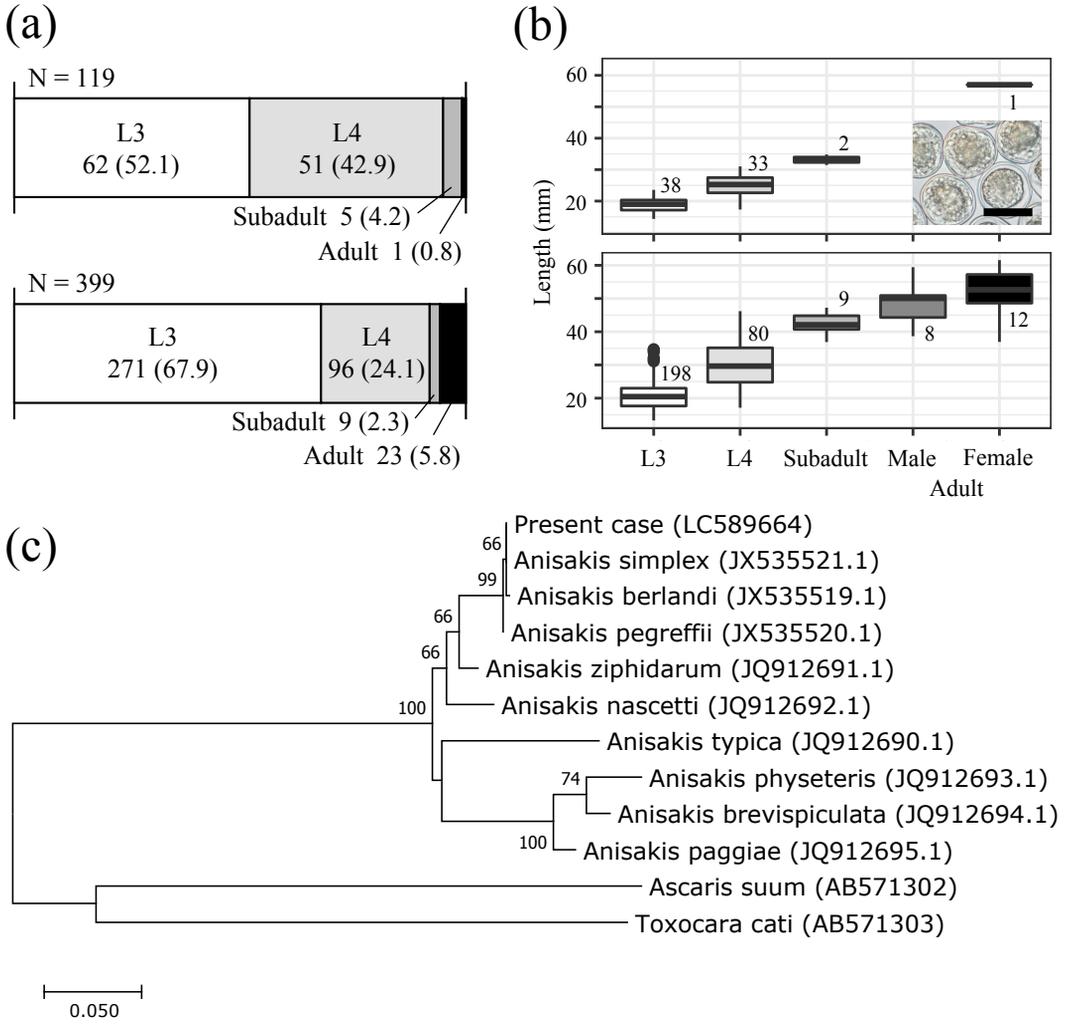


Fig. 2



Graphical abstract

