Short communication

Molecular analysis of larvae suggests the existence of a second species of *Sulcascaris* (Nematoda: Anisakidae: Anisakinae) in the Japanese moon scallop (*Ylistrum japonicum*) from Japanese waters

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Abstract

Herein, the morphological and genetic features of the larval specimens of anisakine nematode, isolated from *Ylistrum japonicum* (Pectinidae) collected from Japanese waters, were examined. Although the specimens were identified as members of the genus *Sulcascaris*, they were genetically divergent from *Sulcascaris sulcata*, which is currently the only member of the genus. The present study highlights the possibility that the *Sulcascaris* population inhabiting Japanese waters represents a unique taxonomic position within the genus *Sulcascaris*. Additionally, given that many pectinid scallops have been reported as intermediate host of *S. sulcata*, the present study implies that pectinid scallops may also represent the primary intermediate hosts for the present Japanese *Sulcascaris* species. Because *S. sulcata* infections can cause discoloration in the meat of scallops, regular monitoring of the prevalence and intensity of *Sulcascaris* infections is required to predict the impact of the infections on the market.

Keywords: Anisakinae; scallop; sea turtle; *Sulcascaris*

Sulcascaris sulcata (Rudolphi, 1819) (Anisakidae: Anisakinae) is an only species of the genus *Sulcascaris* Hartwich, 1957 that infects the gastrointestinal tract of sea turtles (*Caretta caretta*, *Chelonia mydas* and *Lepidochelys kempii*) [1]. The parasite has been recorded from the Mediterranean and Caribbean seas, and the Atlantic and Pacific Oceans [1, 2]. Infection occurs when the definitive host ingests mollusks (bivalves and gastropods) infected with fourth-stage larvae (L4) [1, 3]. A recent pathological study revealed that *S. sulcata* can cause ulcerous gastritis in *C. caretta* (loggerhead turtles) [4].

Japanese waters are the only area in the North Pacific Ocean where *Sulcascaris* nematode has been recorded [2] based on the L4 specimens from a gastropod host. Namely, they were "tentatively" identified as *S. sulcata*. Because larval anisakid nematode specimens often lack enough morphological features for species-specific identification [e.g., 5], the true taxonomic status of *Sulcascaris* species from Japanese waters should be clarified using genetic analysis of L4 specimens. To reveal the taxonomic status of Japanese *Sulcascaris* species, we morphologically and genetically studied larval specimens of the genus *Sulcascaris* collected from Japanese waters. We also discussed the potential impact of L4 infection on the seafood industry and consumer's risk.

A total of 15 nematode larvae were isolated from the adductor muscle of *Ylistrum japonicum* (Japanese moon scallop), which were collected from waters off the cost of Eguchi

Beach, Kagoshima Prefecture, Japan, during food inspections in 2020. Some of the obtained specimens were fixed in 70% ethyl alcohol, while others were fixed with a hot 5% solution of glycerin in 70% ethyl alcohol. To clear the nematode specimens, they were placed in 100% glycerin. The cleared specimens were observed with a light microscope (Eclipse Ni-U, Nikon, Japan). Some of the larval specimens examined in this study have been deposited in the Meguro Parasitological Museum Collections as vouchers (MPM21851, 21852). Identification was based on the morphological characteristics described in Lichtenfels et al. [6] and Berry and Cannon [3].

The morphometric values of the larval specimens are listed in Table 1. The bodies of specimens were long and slender and covered by the third-stage sheath. The specimens possessed three lips at the cephalic end, which were separated from the body by a postlabial groove, interlabia, a ventriculus and a short intestinal cecum. The opening of the excretory pore was located immediately behind the lips. A single pair of phasmids was present on both sides of the tail.

The larval specimens in this study were definitively identified as L4 of the genus *Sulcascaris*, since they possessed interlabia, a ventriculus, a short intestinal cecum, and a third-stage sheath [3, 6]. The present L4 specimens differed from the L4 specimens collected from *Hemifusus ternatanus* in Japanese waters by having shorter body lengths and widths [2]

66 (Table 1). The other measurements of the larval specimens were largely concordant with
67 those of L4 of *S. sulcata* isolated from other hosts (Table 1) [2, 6, 7].
68 We used four anisakine larvae identified morphologically as belonging to the genus
69 *Sulcascaris* for molecular analyses. Genomic DNA of nematode specimens was extracted
70 from the whole body of each individual using the DNeasy Blood and Tissue kit (QIAGEN,
71 Hilden, Germany). The partial sequences of 18S rDNA (18S) and cytochrome *c* oxidase
72 subunits II (COII) were amplified by polymerase chain reaction (PCR) using a TaKaRa Ex
73 Taq kit (Takara Bio Inc., Kusatsu, Japan) and a Tks Gflex DNA Polymerase kit (Takara Bio,
74 Otsu, Japan), respectively, and a T100 Thermal Cycler instrument (Bio-Rad Laboratories,
75 Hercules, CA, USA). The primer sets used for 18S PCR were 998F and 2646R [8], and those
76 for COII were 211Fmod [9] and 210R [10]. The PCR conditions were as follows: 35 cycles of
77 30 s at 95°C, 30 s at 51°C, and 1 min at 72°C for 18S; and 94°C for 1 min, followed by 30
78 cycles of 10 s at 98°C, 15 s at 60°C, and 30 s at 68°C for COII. The former condition was
79 initiated with an initial denaturation step at 95°C for 3 min, followed by a final extension at
80 72°C for 7 min. The PCR products were purified using ExoSAP-IT reagent (Thermo Fisher
81 Scientific, Waltham, MA, USA) and then sequenced directly using the primer sets used for
82 PCR (DNA sequencing service was provided by Eurofins Genomics K. K., Tokyo, Japan). We
83 also used 1813F and 1912R [8] for direct sequencing of 18S as internal primers. In total, one

84 and four DNA sequences of 18S and COII, respectively, were determined. All sequences were
85 deposited at the DNA Data Bank of Japan (18S: LC705336; COII: LC705332–LC705335).
86 The phylogenetic position of the Japanese *Sulcascaris* species and other related genera
87 were estimated based on 18S sequences using maximum likelihood (ML) and Bayesian
88 inference (BI). In addition to the newly determined sequence, the sequences of six species of
89 the Anisakinae retrieved from GenBank were *S. sulcata* (EF180080), *Anisakis simplex*
90 (MF072711), *Anisakis pegreffii* (MF072697), *Neoterranova caballeroi* (U94381),
91 *Neoterranova scoliodontis* (DQ442661), *Pseudoterranova decipiens* (U94380) and
92 *Porrocaecum depressum* (U94379) (Supplementary Table 1). These operational taxonomic
93 units were chosen to represent the anisakine genera. We also retrieved the 18S sequences of
94 two species of Contracaecinae from GenBank, *Contracaecum eudyptulae* (EF180072) and
95 *Contracaecum multipapillatum* (U94370) (Supplementary Table 1), and used them as
96 outgroups of the phylogenetic analysis. The sequences were edited using MEGA 5 [11] and
97 aligned using MAFFT (v. 7.490) (see https://mafft.cbrc.jp/alignment/software/) [12]. Regions
98 difficult to align due to alignment gaps were manually removed; therefore, the final sequences
99 yielded 1754 bp of aligned positions for 18S.
100 The best-fit model, GTR+I, was identified based on the corrected Akaike information
101 criterion using PartitionFinder v2.1.1 [13] with the ‘all’ algorithm. The ML tree was

102 calculated using IQ-Tree v2.1.3 [14] with nonparametric bootstrapping (BS) conducted with
103 1000 replicates. BI tree and Bayesian posterior probabilities (PP) were estimated using
104 MrBayes v3.2.7a [15]. Two independent runs for four Markov chains were conducted from 2
105 million generations, and the tree was sampled every 100 generations. The parameter estimates
106 and convergence were checked using Tracer v1.7.1 [16], and then the first 5001 trees were
107 discarded based on the results. Although we performed another phylogenetic analysis based
108 only on COII sequences (nematode species and accession numbers of the COII sequences
109 used for the analyses are listed in Supplementary Table 2), most of the support values of the
110 branches were weak (data not shown). Thus, we omitted this result from the discussion of the
111 study.

112 Both ML and BI phylogenetic inference based on the 18S DNA sequence dataset yielded
113 almost identical topologies; therefore, the BI tree with BI and ML support values is shown in
114 Fig. 1. The monophyly of Japanese *Sulcascaris* sp. + Mediterranean *S. sulcata* was strongly
115 supported by both analyses (PP = 1.0; BP = 100%).

116 We also calculated the net genetic distances between Japanese (LC705332–LC705335)
117 and Mediterranean (MN991205–MN991209, MN713883–MN713921) populations of
118 *Sulcascaris* nematodes. The calculation was conducted with MEGA 5 using the 501 bp of
119 COII sequences based on Kimura's two-parameter model. The net genetic distance value

between the Japanese and Mediterranean *Sulcascaris* populations was 9.6%.

Phylogenetic analysis based on the 18S sequences suggested close affinity between the Japanese *Sulcascaris* species and Mediterranean *S. sulcata*. On the other hand, remarkable net genetic distances (9.6%) of COII sequences between the Japanese *Sulcascaris* species and Mediterranean *S. sulcata* populations were detected. This value exceeded the COII genetic distance among three sibling species of the *Anisakis simplex* complex (*A. simplex* s. s., *A. pegreffii* and *A. berlandi*) (4.5%–6.1%) [17]. Thus, the present L4 specimens likely represent an undescribed species of the genus *Sulcascaris*.

This is the first record of *Sulcascaris* species from *Y. japonicum* (Pectinidae). Since nine species of pectinid scallops have been reported as intermediate hosts of *S. sulcata* from the waters of Australia, the United States and Brazil, and the Mediterranean Sea [1, 18], pectinid scallops may also serve as the primary intermediate hosts for the present *Sulcascaris* species. The western coast of the Satsuma Peninsula, which includes the sampling site of the L4 specimens, is one of the great-scale spawning grounds of loggerhead turtles in the northern Pacific Ocean, and a number of female loggerhead turtles visit the coast to lay eggs during May–August from their foraging areas [19]. Thus, infection of bivalve and gastropod mollusks by the larvae of *Sulcascaris* sp. may be caused by the intakes of their eggs deposited with feces of the female loggerhead turtles, similar

to *S. sulcata* [1, 3, 20]. *Ylistrum japonicum* inhabits sandy sea bottoms at depths of 10–50 meters, similar to *H. ternatanus* [21, 22]. Thus, bivalve and gastropod mollusks inhabiting the same habitat would be potential hosts of the larval *Sulcascaris* sp. Given that female loggerhead turtles do not feed during internesting periods [23], they may not intake the infected molluskan hosts. Thus, transmission to the female loggerhead turtles from molluskan intermediate hosts may not occur in this area.

The genus *Ylistrum* is currently composed of two species, *Y. japonicum* and *Y. balloti* [24]. *Ylistrum balloti* has also been reported as an intermediate host of *S. sulcata* in Australia [1]. These two scallop species are important food sources in Japan and Australia, and raw scallops or raw adductor meats have high commercial value. Recently, unprecedented numbers of *S. sulcata* infections in the adductor muscle of captured sea scallops were reported in the United States [25], and the infected adductor muscles exhibited brownish lesions. Because the lesions may decrease the market acceptance of the meats, regular monitoring of the prevalence and intensity of *Sulcascaris* infections is required in these countries to predict the impact of the infections on the market. Although no impact of the infections on human health has been reported, a possible risk of an allergic reaction induced by the consumption of dead larvae for consumers is also noted [20]. Future studies regarding the human immunological responses on the proteins derived from *Sulcascaris* species are

also required.

Declaration of Competing Interest

The authors declare that they have no conflict of interest with the contents of this article.

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References

[1] M. Santoro, E. Marchiori, M. Palomba, B.D. Uberti, F. Marcer, S. Mattiucci, The Mediterranean mussel (*Mytilus galloprovincialis*) as intermediate host for the anisakid *Sulcascaris sulcata* (Nematoda), a pathogen parasite of the Mediterranean loggerhead turtle (*Caretta caretta*), Pathogens 9 (2020) 118. https://doi.org/10.3390/pathogens9020118.

[2] M. Machida, K. Yoshida, Larval anisakine nematode, *Sulcascaris sulcata*, from Japanese

174 marine mollusc, Jpn. J. Parasitol. 33 (1984) 365–368.

175 [3] G.N. Berry, L.R.G. Cannon, The life history of *Sulcascaris sulcata* (Nematoda:

176 Ascaridoidea), a parasite of marine molluscs and turtles, Int. J. Parasitol. 11 (1981) 43–54.

177 https://doi.org/10.1016/0020-7519(81)90024-2.

178 [4] M. Santoro, E. Marchiori, D. Iaccarino, B. degli Uberti, R. Cassini, F. Di Nocera, A.

179 Cerrone, G. Galiero, F. Marcer, Epidemiology of *Sulcascaris sulcata* (Nematoda: Anisakidae)

180 ulcerous gastritis in the Mediterranean loggerhead sea turtle (*Caretta caretta*), Parasitol. Res.

181 118 (2019) 1457–1463. https://doi.org/10.1007/s00436-019-06283-0.

182 [5] J. Suzuki, R. Murata, A review of anisakiasis and *Anisakis* larvae in Japan: from the

183 prevalence and risk of *Anisakis* infections to the identification of *Anisakis* larvae, Ann. Rep.

184 Tokyo Metr. Inst. Pub. Health. 62 (2011) 13–24.

185 [6] J.R. Lichtenfels, J.W. Bier, P.A. Madden, Larval anisakid (*Sulcascaris*) nematodes from

186 Atlantic molluscs with marine turtles as definitive hosts, Trans. Amer. Micros. Soc. 97 (1978)

187 199–207. https://doi.org/10.2307/3225593.

188 [7] J.F.A. Sprent, Ascaridoid nematodes of amphibians and reptiles: *Sulcascaris*, J. Helminth.

189 51 (1977) 379–387. https://doi.org/10.1017/S0022149X00007744.

190 [8] M. Holterman, A. van der Wurff, S. van der Elsen, H. van Megen, T. Bongers, O.

191 Holovachov, J. Bakker, J. Helder, Phylum-wide analysis of SSU rDNA reveals deep

phylogenetic relationships among nematodes and accelerated evolution toward crown clades, Mol. Biol. Evol. 23 (2006) 1792–1800. https://doi.org/10.1093/molbev/msl044.

[9] T. Takano, T. Iwaki, T. Waki, R, Murata, J. Suzuki, Y. Kodo, K. Kobayashi, K. Ogawa, Species composition and infection levels of *Anisakis* (Nematoda: Anisakidae) in the skipjack tuna *Katsuwonus pelamis* (Linnaeus) in the Northwest Pacific, Parasitol. Res. 120 (2021) 1605–1615. https://doi.org/10.1007/s00436-021-07144-5.

[10] S.A. Nadler, D.S.S. Hudspeth, Phylogeny of the Ascaridoidea (Nematoda: Ascaridida) based on three genes and morphology: hypothesis of structural and sequence evolution, J. Parasitol. 86 (2000) 380–393. https://doi.org/10.1645/0022-3395(2000)086[0380:POTANA]2.0.CO;2.

[11] K. Tamura, D. Peterson, N. Peterson, G. Stecher, M. Nei, S. Kumar, MEGA5: molecular evolutionary genetic analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods, Mol. Biol. Evol. 28 (2011) 2731–2739. https://doi.org/10.1093/molbev/msr121.

[12] K. Katoh, D.M. Standley, MAFFT multiple sequence alignment software version 7: improvements in performance and usability, Mol. Biol. Evol. 30 (2013) 772–780. https://doi.org/10.1093/molbev/mst010.

[13] R. Lanfear, P.B. Frandsen, A.M. Wright, T. Senfeld, B. Calcott, PartitionFinder 2: New

methods for selecting partitioned models of evolution for molecular and morphological phylogenetic analyses, Mol. Biol. Evol. 34 (2017) 772–773. https://doi.org/10.1093/molbev/msw260.

[14] B.Q. Minh, H.A. Schmidt, O. Chernomor, D. Schrempf, M.D. Woodhams, A. von Haeseler, R. Lanfear, IQ-TREE 2: New models and efficient methods for phylogenetic inference in the genomic era, Mol. Biol. Evol. 37 (2020) 1530–1534. https://doi.org/10.1093/molbev/msaa015.

[15] F. Ronquist, M. Teslenko, P. van der Mark, D.L. Ayres, A. Darling, S. Höhna, B. Larget, L. Liu, M.A. Suchard, J.P. Huelsenbeck, MrBayes 3.2: Efficient Bayesian phylogenetic inference and model choice across a large model space, Syst. Biol. 61 (2012) 539–542. https://doi.org/10.1093/sysbio/sys029.

[16] A. Rambaut, A.J. Drummond, D. Xie, G. Baele, M.A. Suchard, Posterior summarization in Bayesian phylogenetics using Tracer 1.7, Syst. Biol. 67 (2018) 901–904. https://doi.org/10.1093/sysbio/syy032.

[17] A. Valentini, S. Mattiucci, P. Bondanelli, S.C. Webb, A.A. Mignucci-Giannone, M.M.Colom-Llavina, G. Nascetti, Genetic relationship among *Anisakis* species (Nematoda: Anisakidae) inferred from mitochondrial *COX2* sequences, and comparison with allozyme data, J. Parasitol. 92 (2006) 156–166. https://doi.org/10.1645/GE-3504.1.

228 [18] F. Marcer, F. Tosi, G. Franzo, A. Vetri, S. Ravagnan, M. Santoro, E. Marchiori, Updates
229 on ecology ad life cycle of *Sulcascaris sulcata* (Nematoda: Anisakidae) in Mediterranean
230 grounds: molecular identification of larval infecting edible scallops, Front. Vet. Sci. 7 (2020)
231 64. https://doi.org/10.3389/fvets.2020.00064.
232 [19] Herpetological Society of Japan (ed.), Amphibians and Reptiles of Japan, Sunrise
233 Publishing, Hikone, 2021.
234 [20] M. Santoro, M. Palomba, M.V. Modica, Larvae of *Sulcascaris sulcata* (Nematoda:
235 Anisakidae), a parasite of sea turtles, infect the edible purple dye murex *Bolinus brandaris* in
236 the Tyrrhenian Sea, Food Control 132 (2022) 108547.
237 https://doi.org/10.1016/j.foodcont.2021.108547.
238 [21] I. Hayami, Family Pectinidae, in: T. Okunani (ed.), Marine Mollusks in Japan, second
239 ed., Tokai University Press, Hiratsuka, 2017, pp. 534–541, 1190–1197.
240 [22] T. Okutani, Family Melongenidae, in: T. Okunani (ed.), Marine Mollusks in Japan,
241 second ed., Tokai University Press, Hiratsuka, 2017, pp. 274, 940.
242 [23] H. Tanaka, K. Sato, Y. Matsuzawa, W. Sakamoto, Y. Naito, K. Kyroyanagi, Analysis of
243 possibility of feeding of loggerhead turtles during interesting periods based on stomach
244 temperature measurements, Nippon Suisan Gakkaishi 61 (1995) 339–345.
245 https://doi.org/10.2331/suisan.61.339.

246 [24] G. Mynhedt, A. Alejandrino, L. Puslenik, J. Corrales, J.M. Serb, Shell convergence
247 masks biological diversity in gliding scallops: description of *Ylistrum* n. gen. (Pectinidae)
248 from Indo-Pacific Ocean, J. Mollus. Stud. 80 (2014) 400–411.
249 https://doi.org/10.1093/mollus/eyu038.
250 [25] D. Rudder, S. Roman, R.A. Fisher, D. Bushek, D. Munroe, E.A. Bochenek, E. McGurk,
251 S. Borsetti, An investigation into the scallop parasite outbreak on the mid-atlantic shelf:
252 transmission pathway, spatio-temporal variation of infection and consequences to
253 marketability: final report, Marine resource report No. 2019-02, Virginia Institute of Marine
254 Science, William and Mary, 2019. https://doi.org/10.25773/m6xq-nq36.
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Figure caption

Fig. 1. A Bayesian phylogenetic tree for anisakine nematodes based on the nuclear 18S marker. The numbers on branches represent Bayesian posterior probabilities and bootstrap values for maximum likelihood.

Table 1

Morphometric values of L4 specimens of *Sulcascaris* species from Japanese waters. Measurements are shown as the range, followed by the mean in parentheses. All measurements are shown in micrometers (μm) unless otherwise noted.

Host	*Ylistrum japonicum*	*Hemifusus ternatanus*	*Chelonia mydas*	*Caretta caretta*	*Ylistrum balloti*	*Spisula solidissima*	*Argopecten gibbus*
Locality	Japan	Japan	-	-	Australia	Atlantic	Atlantic
Body length (mm)	23.6–31.5 (26.5)	34.3–41.5	29–32	19–33	15–44	8.31–45	11.6–23.7
Maximum body width	382–485 (428)	520–620	360–500	400–470	260–750	168–752	294–390
Body width at ventricullus-intestine junction	294–435 (360)	-	300–400	340–470	220–580	-	-
Labia length	71–112 (91) (*N* = 14)	107–128	90–100	100–130	80–190	50–126	46–71
Interlabia length	44–59 (52) (*N* = 14)	77–87	60–80	70–90	50–110	29–84	28–46
Distance from cephalic end to nerve ring	197–455 (379) (*N* = 13)	286–342	430–520	370–550	340–670	248–660	227–382
Distance from cephalic end to excretory pore	72–143 (103) (*N* = 9)	-	-	-	-	-	-

Esophagus length (mm)	1.7–2.6 (2.2)	2.2–2.6	3.3–4.9	2.5–3.3	1.9–4	1.03–3.58	1.52–2.38
Esophagus width	118–164 (137)	130–140	-	-	-	82–231	96–147
Ventriculus length	396–534 (438)	420–450	480–660	360–510	340–700	218–580	252–403
Ventriculus width	128–228 (183)	130–150	-	-	-	101–300	130–227
Intestinal cecum length	88–192 (133) (*N* = 13)	110–150	50–160	50–150	50–140	21–147	42–97
Rectum length	133–244 (187) (*N* = 13)	215–235	-	-	-	101–323	176–201
Distance from tail end to phasmid	68–177 (111) (*N* = 10)	120–148	100–140	90–130	80–170	76–147	97–122
Tail length	161–259 (207) (*N* = 14)	240–270	200–220	130–190	120–300	127–222	126–206
Reference	This study (MPM21851, 21852)	[2]	[7]	[7]	[7]	[6]	[6]

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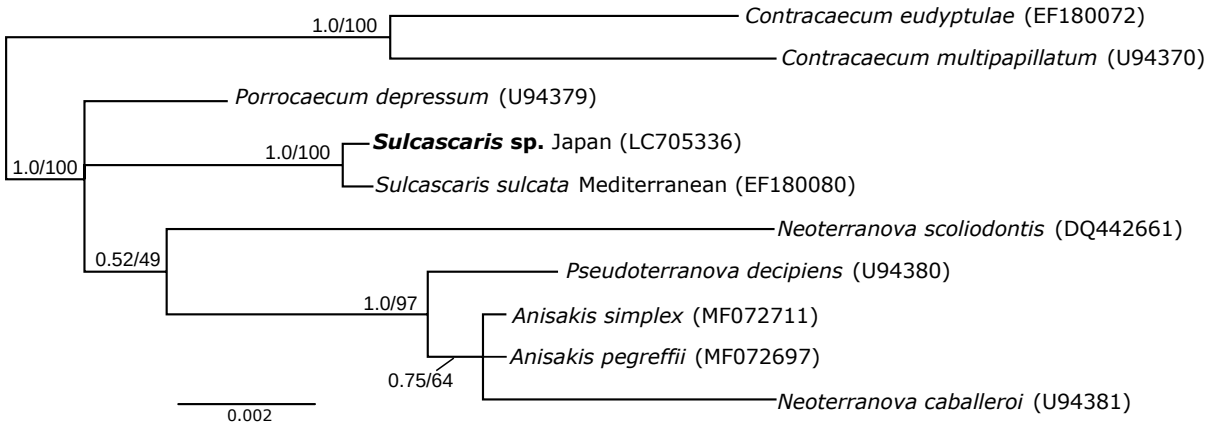

Fig. 1. A Bayesian phylogenetic tree for anisakine nematodes based on the nuclear 18S marker. The numbers on branches represent Bayesian posterior probabilities and bootstrap values for maximum likelihood.