

1 Short communication

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

(Table 1). The other measurements of the larval specimens were largely concordant with those of L4 of *S. sulcata* isolated from other hosts (Table 1) [2, 6, 7].

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

and four DNA sequences of 18S and COII, respectively, were determined. All sequences were deposited at the DNA Data Bank of Japan (18S: LC705336; COII: LC705332–LC705335).

The phylogenetic position of the Japanese *Sulcascaris* species and other related genera were estimated based on 18S sequences using maximum likelihood (ML) and Bayesian inference (BI). In addition to the newly determined sequence, the sequences of six species of the Anisakinae retrieved from GenBank were *S. sulcata* (EF180080), *Anisakis simplex* (MF072711), *Anisakis pegreffii* (MF072697), *Neoterranova caballeroi* (U94381), *Neoterranova scoliodontis* (DQ442661), *Pseudoterranova decipiens* (U94380) and *Porrocaecum depressum* (U94379) (Supplementary Table 1). These operational taxonomic units were chosen to represent the anisakine genera. We also retrieved the 18S sequences of two species of Contracaecinae from GenBank, *Contracaecum eudyptulae* (EF180072) and *Contracaecum multipapillatum* (U94370) (Supplementary Table 1), and used them as outgroups of the phylogenetic analysis. The sequences were edited using MEGA 5 [11] and aligned using MAFFT (v. 7.490) (see <https://mafft.cbrc.jp/alignment/software/>) [12]. Regions difficult to align due to alignment gaps were manually removed; therefore, the final sequences yielded 1754 bp of aligned positions for 18S.

The best-fit model, GTR+I, was identified based on the corrected Akaike information criterion using PartitionFinder v2.1.1 [13] with the ‘all’ algorithm. The ML tree was

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

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

We also calculated the net genetic distances between Japanese (LC705332–LC705335) and Mediterranean (MN991205–MN991209, MN713883–MN713921) populations of *Sulcascaris* nematodes. The calculation was conducted with MEGA 5 using the 501 bp of 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.

Acknowledgments

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257 **Figure caption**

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

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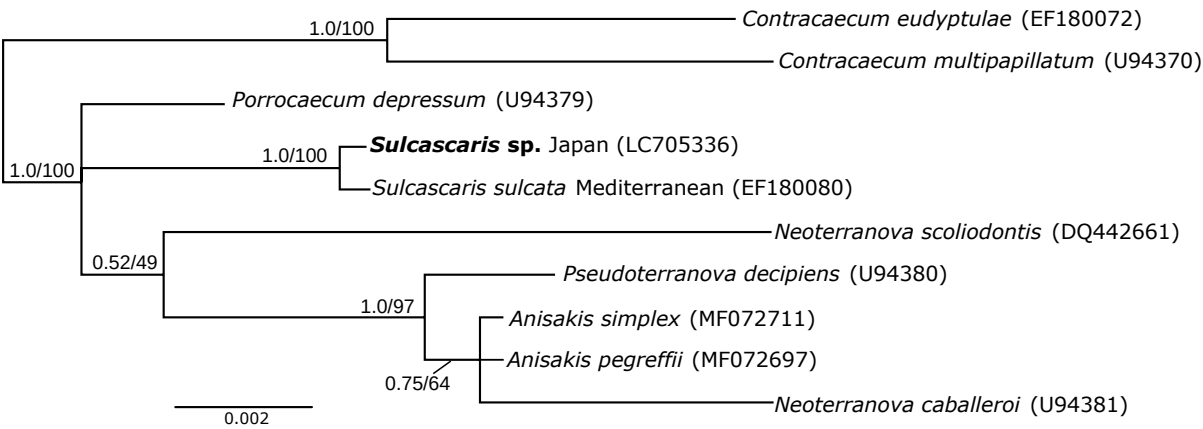
Table 1

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	<i>Ylistrum japonicum</i>	<i>Hemifusus ternatanus</i>	<i>Chelonia mydas</i>	<i>Caretta caretta</i>	<i>Ylistrum balloti</i>	<i>Spisula solidissima</i>	<i>Argopecten gibbus</i>
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 ventriculus-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) (<i>N</i> = 13)	110–150	50–160	50–150	50–140	21–147	42–97
Rectum length	133–244 (187) (<i>N</i> = 13)	215–235	-	-	-	101–323	176–201
Distance from tail end to phasmid	68–177 (111) (<i>N</i> = 10)	120–148	100–140	90–130	80–170	76–147	97–122
Tail length	161–259 (207) (<i>N</i> = 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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269 Fig. 1. A Bayesian phylogenetic tree for anisakine nematodes based on the nuclear 18S
270 marker. The numbers on branches represent Bayesian posterior probabilities and bootstrap
271 values for maximum likelihood.