

Molecular identification of *Anisakis simplex* sensu stricto and *Anisakis pegreffii* (Nematoda: Anisakidae) from fish and cetacean in Japanese waters

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Abstract

Parasites morphologically consistent with *Anisakis simplex* sensu lato collected from the coast of Japan and Western North Pacific Ocean were examined by PCR-RFLP of the ITS region (ITS1, 5.8 subunit rRNA gene and ITS2) and mtDNA *cox1*. The RFLP patterns of rDNA generated by *HinfI* and *HhaI* showed that 100% of the larvae collected from Hokkaido and 94% of adults collected from Western North Pacific Ocean were identified as *A. simplex* sensu stricto. On the other hand, 97% of the larvae collected from Fukuoka prefecture were identified as *A. pegreffii*. A hybrid genotype was found in adults in Western North Pacific Ocean and larva in Fukuoka prefecture. These findings revealed that *A. simplex* str. is primarily distributed in the North Pacific Ocean and *A. pegreffii* is primarily distributed in the southern Sea of Japan. RFLP analysis of mtDNA *cox1* showed different patterns between *A. simplex* s. str. and *A. pegreffii* after digestion with *HinfI*. This polymorphism obtained by RFLP analysis of mtDNA *cox1* proved the usefulness as new genetic markers to distinguish two sibling species.

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Keywords: *Anisakis simplex* sensu stricto; *Anisakis pegreffii*; Ribosomal DNA; Mitochondrial DNA *cox1*; RFLP

1. Introduction

Anisakis simplex in its larval stage is a common nematode parasite of marine fishes and squids. The adult parasites are widely distributed in marine mammals, living in different oceans and seas of the world [1].

Anisakiasis is an important fish borne zoonotic disease caused primarily by the nematodes of the genera *Anisakis* and *Pseudoterranova*. Anisakiasis was first recognized in the Netherlands in the mid-1950s. Since then, it has been recognized as a potential parasitic disease problem in areas of the world where people eat raw fish, including Japan [2]. *A. simplex* is most frequently

associated with this disease. Therefore, identification of *A. simplex* is crucial for proper diagnosis.

Over the last few years, analysis of the genetic structure of both adults and larvae of the morphospecies *A. simplex* by multilocus enzyme electrophoresis demonstrated three genetically differentiated species, namely *A. simplex* sensu stricto, *A. pegreffii*, and *A. simplex* C [3–7]. Furthermore, recent molecular techniques, PCR-RFLP analysis of the ITS (ITS-1 and ITS-2) and the 5.8 subunit rRNA gene have been successfully employed for the identification of these species [8–13]. Although *A. simplex* s. str. and *A. pegreffii* have been isolated in Japan [5,6,12,13], there are few studies that document distribution of these sibling species.

In this paper, we describe molecular identification of *A. simplex* sensu lato collected from the Japanese coast. This is the new record of *A. pegreffii* from southern Japan, and *A. simplex* s. str. is also newly recorded from two species of fish in Japanese waters.

Abbreviations: rDNA, ribosomal DNA; rRNA, ribosomal RNA; ITS, internal transcribed spacer; mtDNA, mitochondrial DNA; *cox1*, cytochrome c oxidase subunit 1; RFLP, restriction fragment length polymorphism.

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2. Materials and methods

2.1. Parasite materials

Third stage larvae (L3) and adults (A) were randomly collected from four paratenic hosts (fish) and one definitive host (marine mammal) caught in different areas during 1996–2003. Host, number, and geographic origin of samples are listed in Table 1 and Fig. 1. All fishes were collected from commercial catches and all minke whales were collected during the ‘Japanese Whale Research Program under Special Permit in the Western North Pacific’ (JARPN). The adult specimens were found to correspond to *A. simplex* s. lat., while the larvae to *Anisakis* sp. (Fig. 2) [14,15]. They were frozen and kept at –20 °C until use.

2.2. DNA extraction

DNA from individual worms was extracted by QIAamp DNA Mini Kit (Qiagen, Tokyo, Japan) according to the manufacturer’s instruction. DNA was eluted in elution buffer and kept at –20 °C until use.

2.3. Amplification of rDNA and mtDNA *cox1* regions

PCR was conducted using a mixed solution of extracted DNA as a template (3 µl) and 27 µl reaction mixture containing 0.75 units of *TaKaRa EX Taq*, 3 µl of 10× PCR buffer (100 mM Tris-HCl pH 8.3, 500 mM KCl, 15 mM MgCl₂), 0.17 mM dNTP mixture (Takara Bio Inc., Shiga, Japan) and 0.3 µM each primer. A region of rDNA (ITS1, 5.8 subunit rRNA gene and ITS2) was amplified using primers A (5′-GTC GAA TTC GTA GGT GAA CCT GCG GAA GGA TCA-3′) and B (5′-GCC GGA TCC GAA TCC TGG TTA GTT TCT TTT CCT-3′). Detailed methodology has been reported previously [8]. A portion of the mtDNA *cox1* was amplified using the primers JB3 (5′-TTT TTT GGG CAT CCT GAG GTT TAT-3′) and JB4.5 (5′-TAA AGA AAG AAC ATA ATG AAA ATG-3′). Amplification was carried out using the above described method [16] except that annealing was done at 49 °C for

30 s. Reactions were carried out using a RoboCycler Gradient 96 with Hot Top (Stratagene Japan K.K., Tokyo, Japan). PCR products were separated by electrophoresis in a 1.0% Seakem GTG agarose gel (Cambrex Bio Science Rockland, Inc., Rockland, Maine) in Tris-Borate-EDTA buffer and visualized by illumination with shortwave ultraviolet light after ethidium bromide staining.

2.4. Digestion of PCR products with restriction enzyme

The restriction enzymes, *Hinf*I and *Hha*I, were used in RFLP analysis of rDNA for the identification of the members of *A. simplex* complex according to genetic key of D’Amelio *et al.* [8]. On the other hand, mtDNA *cox1* were analyzed by seven restriction enzymes: *Hinf*I, *Hha*I, *Rsa*I, *Alu*I, *Taq*I, *Eco*RI, and *Hae*III. PCR products were digested according to the manufacturer’s recommendations. The digested products were analyzed by electrophoresis on a 3.0% Seakem GTG agarose gel (Cambrex) and visualized by illumination with shortwave ultraviolet light after ethidium bromide staining.

3. Results

3.1. PCR-RFLP analysis of rDNA

Digestion of the DNA region spanning the ITS-1, ITS-2, and the 5.8 subunit rRNA (approximately 1000 bp in length) with *Hinf*I produced three different patterns, while digestion with *Hha*I produced a single pattern. RFLP patterns of all specimens corresponded either to that of *A. simplex* s. str., *A. pegreffii* or a hybrid genotype reported previously. Digestion with *Hinf*I produced two strong bands and a weak band (620 bp, 250 bp and 80 bp, respectively) in *A. simplex* s. str., while *A. pegreffii* showed three bands of 370, 300 and 250 bp. Furthermore, the hybrid genotype showed four fragments, corresponding to all the bands observed in *A. simplex* s. str. and in *A. pegreffii*. On the other hand, digestion with *Hha*I produced two fragments of 550 bp and 430 bp in all genotypes (Fig. 3).

3.2. PCR-RFLP analysis of mtDNA *cox1*

The amplification of the mtDNA *cox1* produced a fragment of 490 bp. Of the seven restriction enzymes tested, one enzyme (*Hinf*I) cleaved the target sequence in all samples under study, while six enzymes did not. Different RFLP patterns were produced between *A. simplex* s. str. and *A. pegreffii* after digestion with *Hinf*I. *A. simplex* s. str. showed two bands of approximately 270 bp and 210 bp, while *A. pegreffii* showed two strong bands of approximately 270 bp and 150 bp and a weak band of approximately 80 bp. Hybrid genotype produced each identical RFLP pattern. A total of four hybrid genotypes were analyzed. Three of them showed the RFLP pattern of *A. simplex* s. str.. One hybrid genotype showed the RFLP pattern of *A. pegreffii* (Fig. 4). The RFLPs in mtDNA *cox1* have provided

Table 1
Collection data of *A. simplex* s. lat. adults and *Anisakis* sp. larvae

Host species	No. of parasites tested	Life stage	Collected location
<i>Pleurogrammus azonus</i>	20	L3 ^a	Hokkaido
<i>Theragra chalcogramma</i>	19	L3	Hokkaido
<i>Scomber japonicus</i>	16	L3	Hokkaido
<i>Hypomesus pretiosus japonicus</i>	10	L3	Hokkaido
<i>Scomber japonicus</i>	38	L3	Fukuoka prefecture
<i>Balaenoptera acutorostrata</i>	48	A ^b	Western North Pacific Ocean ^c

^a Third stage larvae.

^b Adult worms.

^c 35–52° North latitude, 140–170° East longitude.

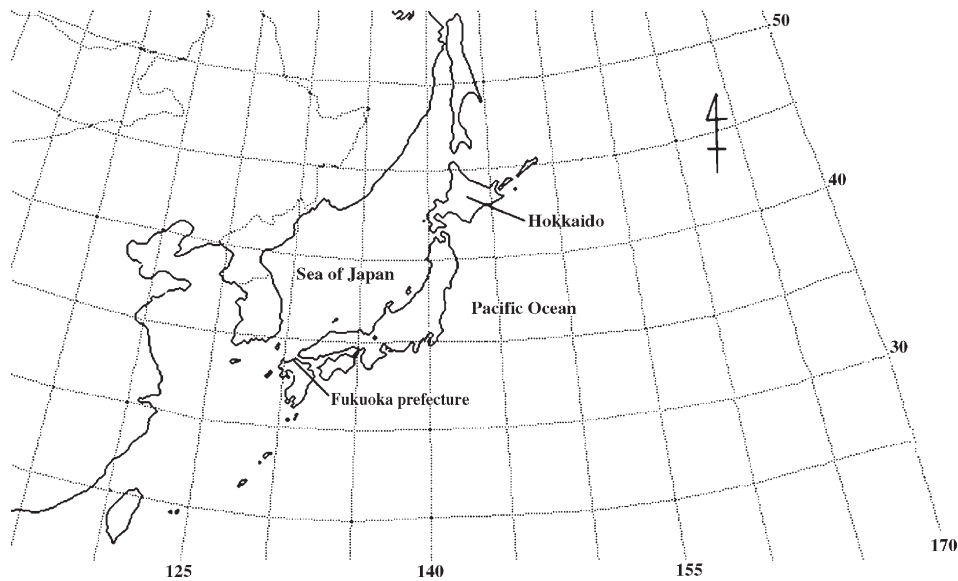


Fig. 1. The collection sites of paratenic hosts and a definitive host in Japanese coastal waters.

new genetic markers for identification of *A. simplex* s. str. and *A. pegreffii*.

3.3. The distribution of *A. simplex* complex in Japanese waters

Sixty five out of sixty five (100%) of the larvae collected from Hokkaido and the forty five out of forty eight (94%)

adult worms collected from the Western North Pacific Ocean were identified as *A. simplex* s. str. On the other hand, thirty seven out of thirty eight (97%) larvae collected from Fukuoka prefecture were identified as *A. pegreffii*. Three out of forty eight (6%) adult worms from the Western North Pacific Ocean and one out of thirty eight (3%) larva collected from Fukuoka prefecture were hybrid genotypes

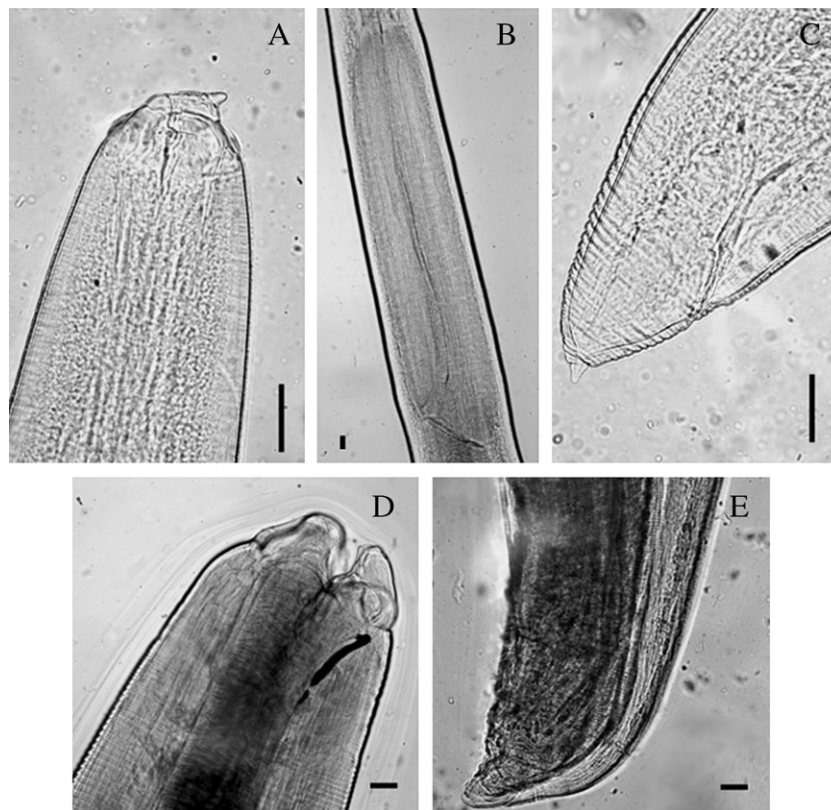


Fig. 2. A–C; Morphology of third stage larvae of *Anisakis* sp. A, cephalic end; B, ventricular part; C, caudal end. Bar: 50 μ m. D–E; Morphology of adult male of *A. simplex* s. lat. D, cephalic end; E, caudal end. Bar: 100 μ m.

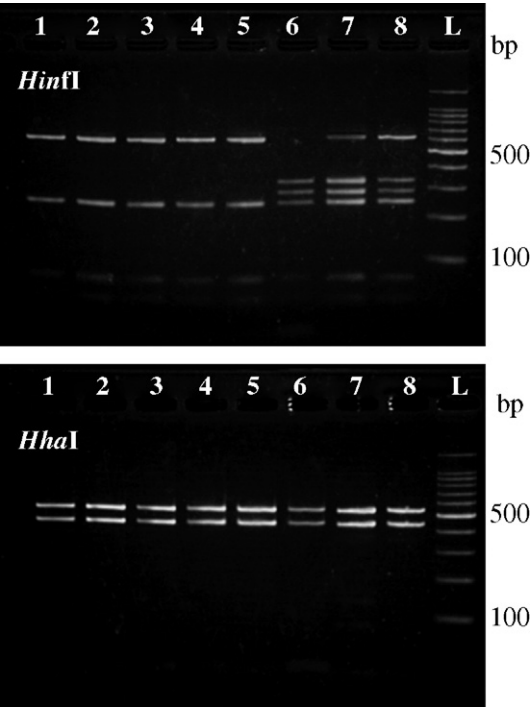


Fig. 3. PCR-RFLP analysis for the identification of *A. simplex* complex. Lane 1–5, *A. simplex* s. str. (1–4, from Hokkaido; 5, from Western North Pacific Ocean); Lane 6, *A. pegreffii* (from Fukuoka prefecture); Lane 7 and 8; Hybrid genotype. (7, from Fukuoka prefecture; 8, from Western North Pacific Ocean). L: 100 bp ladder.

(Table 2 and Fig. 5). Mixed infection of *A. simplex* s. str. and *A. pegreffii* in both fishes and minke whales was not detected in this study.

4. Discussion

The rDNA gene is an important multigene family. One unit of rDNA consists of three genes encoding rRNA separated by ITS. The ITS region does not encode any product, permitting it to evolve at a faster rate than the ribosomal coding regions. The level of variation in this

Table 2

Genotypes obtained by RFLP analysis for the 151 isolates

No. of parasites	Collected location	Species ^a
65(L3 ^b)	Hokkaido	<i>A. simplex</i> s. str.
37(L3)	Fukuoka prefecture	<i>A. pegreffii</i>
1(L3)	Fukuoka prefecture	Hybrid genotype
45(A ^c)	Western North Pacific Ocean	<i>A. simplex</i> s. str.
3(A)	Western North Pacific Ocean	Hybrid genotype

^a Species are based on rDNA RFLP patterns.
^b Third stage larvae.
^c Adult worms.

region makes it suitable for detecting genetic variation within species. In this study, our results support the usefulness of RFLP analysis of rDNA for identification of *A. simplex* complex. On the other hand, mtDNA polymorphism has been successfully employed as a marker for population and phylogenetic studies. The RFLPs in mtDNA were found between *A. simplex* s. str. and *A. pegreffii* in the present study. This result raised the possibility of new genetic markers for *A. simplex* complex.

Two sibling species of *A. simplex* complex were confirmed in Japanese coastal waters. Results from restriction enzyme digestion with *HinfI* and *HhaI* revealed that *A. simplex* s. str. was detected from the north area (Hokkaido and Western North Pacific Ocean), while *A. pegreffii* was detected from the south area (Fukuoka prefecture). In the previous study, these two sibling species were detected from *Theragra chalcogramma*, and *Gadus macrocephalus* collected in northern Japan, especially Hokkaido [5,6,12]. We report here for the first time the distribution of *A. pegreffii* in southern Japan. Furthermore, we detected *A. simplex* s. str. from *Pleurogrammus azonus* and *Hypomesus pretiosus japonicus*. We report two new host species for *A. simplex* s. str. in this study. Interestingly, although we detected two sibling species in *Scomber japonicus* captured from different area, no mixed

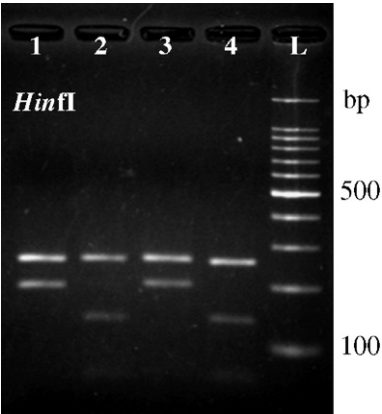


Fig. 4. RFLP patterns obtained by digestion of mtDNA *cox1* with restriction enzyme *HinfI*. Lane1, *A. simplex* s. str.; Lane2, *A. pegreffii*; Lane3 and 4; Hybrid genotype. L: 100 bp ladder.

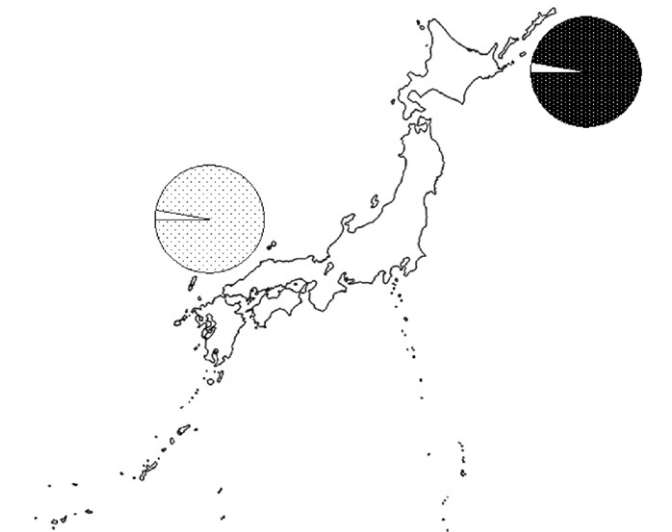


Fig. 5. Distribution of *A. simplex* complex in Japanese waters. (■, *A. simplex* s. str.; □, *A. pegreffii*; ◐, Hybrid genotype).

infection was observed. It is conceivable that difference in the proportion of two sibling species in *S. japonicus* is due to captures of two stocks of chub mackerel, the Tsushima Current stock and the Pacific stock.

Recently, the existence of a hybrid genotype was reported as the result of natural interspecific hybridization between *A. simplex* s. str. and *A. pegreffii* [10,11]. However, there has been no report of the existence of a hybrid genotype at the adult stage, they speculated that the fitness of a hybrid form is low. We detected three hybrid genotypes at adult stage from *Balaenoptera acutorostrata* captured during a survey in 1996. Therefore, this is the first record of the adult worms of hybrid genotype.

In this paper, we provide new information on distribution and host of *A. simplex* complex in Japanese waters. These findings give fundamental epidemiological and biological data on the *A. simplex* s. str. and *A. pegreffii*.

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References

- [1] Nagasawa K. The life cycle of *Anisakis simplex*: a review. In: Ishikura H, Kikuchi K, editors. Intestinal Anisakiasis in Japan. Infected fish, sero-immunological diagnosis, and prevention. Tokyo: Springer-Verlag; 1990. p. 31–40.
- [2] Thomas CO, Lawrence RA. *Anisakis* and *Pseudoterranova* species. Parasites in human tissues. Chicago: American Society of Clinical Pathologists press; 1995. p. 104–9.
- [3] Nascetti G, Paggi L, Orecchia P, Mattiucci S, Bullini L. Two sibling species within *Anisakis simplex* (Ascaridida: Anisakidae). Parasitologia 1983;25: 239–41.
- [4] Nascetti G, Paggi L, Orecchia P, Smith JW, Mattiucci S, Bullini L. Electrophoretic studies on the *Anisakis simplex* complex (Ascaridida: Anisakidae) from the Mediterranean and North-East Atlantic. Int J Parasitol 1986;16:633–40.
- [5] Mattiucci S, Nascetti G, Cianchi R, Paggi L, Arduino P, Margolis L, et al. Genetic and ecological data on the *Anisakis simplex* complex, with the evidence for a new species (Nematoda, Ascaridoidea, Anisakidae). J Parasitol 1997;83:401–16.
- [6] Mattiucci S, Paggi L, Nascetti G, Ishikura H, Kikuchi K, Sato N, et al. Allozyme and morphological identification of *Anisakis*, *Contracaecum* and *Pseudoterranova* from Japanese waters (Nematoda, Ascaridoidea). Syst Parasitol 1998;40:81–92.
- [7] Mattiucci S, Abaunza P, Ramadori L, Nascetti G. Genetic identification of *Anisakis* larvae in European lake from Atlantic and Mediterranean waters for stock recognition. J Fish Biol 2004;65:495–510.
- [8] D. Amelio S, Mathiopoulou KD, Santos CP, Pugachev ON, Webb SC, Picanco M, et al. Genetic markers in ribosomal DNA for the identification of members of the genus *Anisakis* (Nematoda : Ascaridoidea) defined by polymerase chain reaction-based restriction fragment length polymorphism. Int J Parasitol 2000;30:223–6.
- [9] Abollo E, D. Amelio S, Pascual S. Fitness of the marine parasitic nematode *Anisakis simplex* s. str. in temperate waters of the NE Atlantic. Dis Aquat Organ 2001;45:131–9.
- [10] Abollo E, Paggi L, Pascual S, D. Amelio S. Occurrence of recombinant genotypes of *Anisakis simplex* s. s. and *Anisakis pegreffii* (Nematoda: Anisakidae) in an area of sympatry. Infect Genet Evol 2003;3:175–81.
- [11] Martin-Sanchez J, Artacho-Reinoso ME, Diaz-Gavilan M, Valero-Lopez A. Structure of *Anisakis simplex* s. l. populations in a region sympatric for *A. pegreffii* and *A. simplex* s. s. Absence of reproductive isolation between both species. Mol Biochem Parasitol 2005;141:155–62.
- [12] Abe N, Ohya N, Yanagiguchi R. Molecular characterization of *Anisakis pegreffii* larvae in pacific cod in Japan. J Helminthol 2005;79:303–6.
- [13] Abe N, Tominaga K, Kimata I. Usefulness of PCR-restriction fragment length polymorphism analysis of the internal transcribed spacer region of rDNA for identification of *Anisakis simplex* complex. Jpn J Infect Dis 2006;59:60–2.
- [14] Yamaguti S. Studies on the helminth fauna of Japan. Part 41. Mammalian nematodes. III. Kyoto: Published by the author, 1942: 35.
- [15] Ishii Y, Fujino T, Weerasooriya MV. Morphology of *Anisakis* larvae. In: Ishikura H, Namiki M, editors. Gastric Anisakiasis in Japan. Epidemiology, diagnosis, treatment. Tokyo: Springer-Verlag; 1989. p. 19–29.
- [16] Hu M, D. Amelio S, Zhu X, Paggi L, Gasser R. Mutation scanning for sequence variation in three mitochondrial DNA regions for members of the *Contracaecum osculatum* (Nematoda: Ascaridoidea) complex. Electrophoresis 2001;22:1069–75.