MOLECULAR ANALYSIS OF JAPANESE *ANISAKIS SIMPLEX* WORMS

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Abstract. In this study, we used sequence and RFLP analysis of the ribosomal DNA internal transcribed spacer region to identify the sibling species of *Anisakis simplex* worms isolated in Japan as third stage larvae (L3) from fish and patients and as adults from marine mammals. Worms from North Pacific Ocean were identified as *A. simplex* s. str., while those from the southern Sea of Japan were *A. pegreffii.* Worms from patients were mainly identified as *A. simplex* s. str, even though they were obtained from southern Japan. Worms of the hybrid genotype were only detected in fish and marine mammals. We also demonstrated that our newly established RFLP method for mitochondrial *cox1* enables us to unambiguously classify members of *A. simplex*, including hybrid genotype worms, into *A. simplex* s. str. or *A. pegreffii*.

INTRODUCTION

In Japan, over 2,000 cases of human anisakiasis have been reported annually due to the high consumption of raw fish as sushi and sashimi. The nematode *Anisakis simplex* is the parasite most frequently associated with the disease. *A. simplex* is widespread worldwide with no obvious variation in morphology. However, sequencing and/or restriction fragment length polymorphism (RFLP) analysis of the ribosomal DNA internal transcribed spacer region [(rDNA ITS region; namely, 5.8S rDNA and flanking ITS regions (ITS1 and ITS2)] have demonstrated that *A. simplex* morphospecies comprises three sibling species: *A. pegreffii*, *A. simplex* sensu stricto and *A. simplex* C (Mattiucci and Nascetti, 2006). Parasites with hybrid genotype between *A. simplex* s. str. and *A. pegreffii* were also detected from waters around the Iberian Peninsula (Abollo *et al*, 2003; Martin-Sanchez *et al*, 2005). In this paper, we have applied molecular methods for sibling species-level identification of Japanese *A. simplex* worms isolated from fish, marine mammals and patients with anisakiasis.

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MATERIALS AND METHODS

Parasite materials

A. simplex worms were collected from fish as third stage larvae (L3) and marine mammalian hosts as adults, as well as from patients with anisakiasis as L3. Worms from fish and marine mammals were stored at -20 °C and those from human patients were stored in 80% ethanol at room temperature until analysis. Host animals, the geographical location of collection and numbers of worms examined are listed in Table 1. Species was confirmed by DNA sequencing and/or RFLP analysis as described below.

DNA amplification and sequencing

We extracted DNA samples from individual worms using QIAamp DNA Mini Kit (Qiagen k. k., Japan). The entire ITS region (ITS1, 5.8S rDNA and ITS2) and mitochondrial cytochrome C oxidase 1 (*cox1*) gene was amplified by PCR using primer pairs A and B (D Amelio *et al*, 2000) and JB3 and JB4.5 (Hu *et al*, 2001), respectively. PCR conditions

differed for frozen and ethanol-preserved worms and were the same as those described by Umehara *et al* (2006, 2007). Amplification products were separated on agarose gels and excised bands were sequenced using BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, USA) in an automated sequencer (ABI3100, Applied Biosystems). Sequence alignment and comparison were analyzed using GENETYX-WIN program (ver.7.0, Software Development, Japan).

RFLP analysis of PCR products

Amplicons of the entire ITS region were digested with *Hin*fI (D Amelio *et al*, 2000) and of mitochondrial *cox1* with *Sfc*I. The digested samples were then separated by electrophoresis in 3.0% (w/v) agarose gels.

RESULTS

Sequence and RFLP analysis of rDNA ITS region

Amplification of the rDNA ITS region produced a single band of about 950 bp for all samples. Digestion of PCR products by *Hin*fI produced three different RFLP patterns, corresponding to that of *A. simplex* s. str. (ca. 610 and 230 bp, Fig 1 lane 1), *A. pegreffii* (ca. 330, 280 and 230 bp, Fig 1 lane 2) or the hybrid genotype (ca. 610, 330, 280 and 230 bp, Fig 1 lane 3). The RFLP pattern produced by the hybrid genotype was identical to the combination of the RFLP patterns of *A. simplex* s. str. and *A. pegreffii*.

Sequences of the ITS amplicons were almost identical among samples; only two

Table 1
Identification of *A. simplex* at the sibling species-level.

Sources of parasites	Collection site[a]	Life cycle stage	No. of parasite identified[b] as		
			As	Ap	H
Fish					
Arabesque greenling	North	L3	20	0	0
Alaska pollack	North	L3	19	0	0
Chub mackerel	North	L3	16	0	0
Surf smelt	North	L3	10	0	0
Chub mackerel	South	L3	0	37	1 (Ap-type)
Marine mammal					
Minke whale	North	Adult	45	0	3 (2: As-type) (1: Ap-type)
Human					
5 patients	NJpn	L3	5	0	0
80 patients	SJpn	L3	94	1	0

[a] North = North Pacific Ocean; South = Southern Sea of Japan; N Jpn = Northern Japan; S Jpn = Southern Japan

[b] Identification for sibling species was based on rDNA RFLP patterns. For the hybrid genotype, mtDNA RFLP patterns were used.

As = *A. simplex* s. str.; Ap = *A. pegreffii*; H = Hybrid genotype

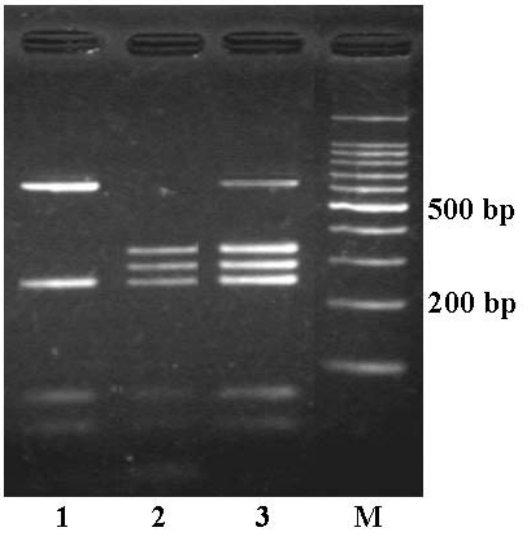

Fig 1- RFLP analysis of rDNA ITS amplicons from *A. simplex* s. str., *A. pegreffii* and hybrid genotype. Amplicons of *A. simplex* s. str. digested with *Hin*fI produced two bands (ca. 610 and 230 bp, lane 1), while those of *A. pegreffii* produced three bands (ca. 330, 280 and 230 bp, lane 2). Amplicons of hybrid genotype produced four bands (ca. 610, 330, 280 and 230 bp, lane 3). A 100-bp DNA ladder marker was used to estimate the size of the bands (lane M).

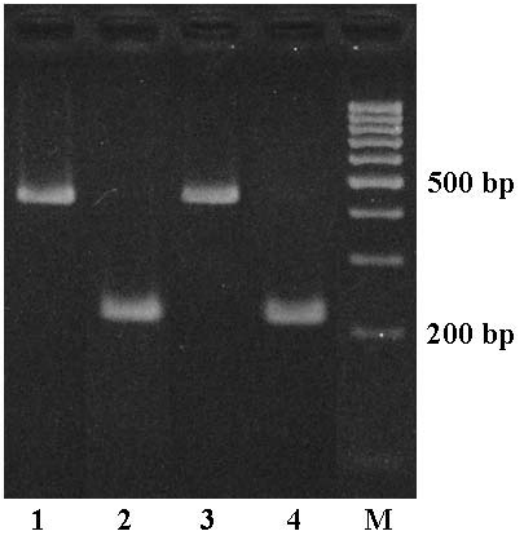

Fig 2- RFLP analysis of mitochondrial *cox1* amplicons from *A. simplex* s. str., *A. pegreffii* and hybrid genotype. Amplicons of *A. simplex* s. str. were not digested with *Sfc*I (ca. 440 bp, lane 1), whereas those of *A. pegreffii* produced a single digested band (ca. 220 bp, lane 2). Hybrid genotype amplicons were not digested (lane 3) or digested to produce two 200 bp bands (lane 4). A 100-bp DNA ladder marker was used to estimate the size of the bands (lane M).

thymine to cytosine transitions in the ITS1 region were observed between *A. simplex* s. str. and *A. pegreffii* (data not shown). Similarity searches of the GenBank/EMBL/DDBJ nucleotide database revealed that the sequences with two thymine bases and those with two cytosine bases were identical to sequence of *A. simplex* s. str. (GenBank/EMBL/DDBJ accession number: AB277822) and *A. pegreffii* (AB277823), respectively. Electropherograms of the hybrid genotype showed two double peaks at these transition sites (data not shown).

Sequence and RFLP analysis of mitochondrial *cox1*

PCR amplification of mitochondrial *cox1* produced a single band of about 440 bp for all samples. Sequence analysis revealed a high level of conservation among samples with base differences occurring at only seven sites between *A. simplex* s. str. and *A. pegreffii* (data not shown). At the amino acid level, no differences were observed between *A. simplex* s. str. and *A. pegreffii*.

Restriction enzyme *Sfc*I was selected for RFLP analysis based on the sequence differences between *A. simplex* s. str. and *A. pegreffii*. A single undigested 440 bp band was obtained for *A. simplex* s. str. (Fig. 2, lane 1), while digested 220 bp for *A. pegreffii* (Fig 2, lane 2). Amplicons from hybrid genotype were either not digested (Fig 2, lane 3, *A. simplex* s. str.-type) or digested to produce two 220 bp bands band (Fig 2, lane 4, *A. pegreffii*-type).

Infection rates of sibling species of *A. simplex* in Japan

The identification of the *A. simplex* worms at the sibling species-level is summarized in Table 1. L3 larvae from fish collected in North Pacific Ocean were all identified as *A. simplex*

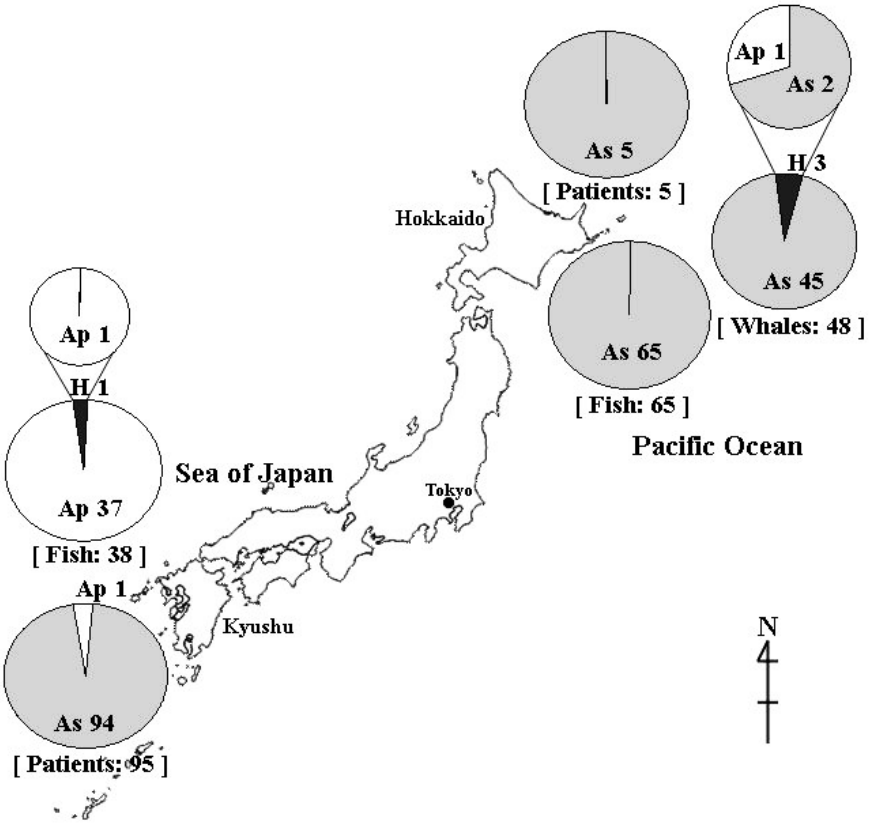

Fig 3- Distribution in Japan of *A. simplex* s. str. (As), *A. pegreffii* (Ap) and hybrid genotype (H) isolated from fish, marine mammals and patients with anisakiasis. Large pie charts show the results of identification of *A. simplex* based on analysis of the rDNA ITS region. Small pie charts show the results of typing of the hybrid genotype based on analysis of mitochondrial *cox1*. Numbers in square brackets and after the abbreviations of the species (As, Ap and H) represent the numbers of worms examined and identified, respectively.

s. str. However, almost all L3 larvae from fish captured in the southern Sea of Japan were identified as *A. pegreffii*, while only one L3 larva was identified as the hybrid genotype. However, the latter's genotype was determined to be *A. pegreffii*-type by RFLP analysis of mitochondrial *cox1*.

Adult worms from marine mammals in North Pacific Ocean were identified as *A. simplex* s. str. or the hybrid genotype. Of the three hybrid genotype worms, two were determined to be *A. simplex* s. str.-type and one *A. pegreffii*-type by RFLP analysis of mitochondrial *cox1*.

Five larvae from 5 patients in northern Japan were all identified as *A. simplex* s. str. In contrast, 94 of 95 larvae from 80 patients in southern Japan were identified as *A. simplex* s. str., while the remaining single larva was identified as *A. pegreffii*.

Mixed infections with the hybrid genotype and either *A. simplex* s. str. or *A. pegreffii* were found in marine mammals collected from North Pacific Ocean and in fish from southern Sea of Japan. However, no example of mixed infection with both *A. simplex* s. str. and *A. pegreffii* in a single host was detected.

Distribution of *A. simplex* sibling species in Japan

The distribution in Japan of *A. simplex* identified at the sibling species-level is shown in Fig 3. Almost all worms from fish were

classified into one of the two sibling species corresponding to the geographical location from which the samples were obtained; worms from north Pacific Ocean were all identified as *A. simplex* s. str., while almost all worms from the southern Sea of Japan were *A. pegreffii*. In contrast, worms from patients were mainly identified as *A. simplex* s. str. even though they were obtained from southern Japan where *A. pegreffii* predominates in samples isolated from fish.

DISCUSSION

A. simplex worms occurring worldwide show no obvious variation in morphology. However, Nascettii *et al* (1983, 1986) divided *A. simplex* worms isolated from Mediterranean Sea and North Atlantic Ocean into two sibling species, *A. pegreffii* and *A. simplex* s. str., respectively, based on difference observed in isozyme electrophoretic patterns. In addition to these two sibling species, Mattiucci *et al* (1997) differentiated some *A. simplex* worms showing another electrophoretic pattern as *A. simplex* C. Consequently, *A. simplex* worms have come to be regarded as a complex composed of three sibling species, namely, *A. pegreffii*, *A. simplex* s. str. and *A. simplex* C.

This classification has been generally accepted because the three sibling species were also unequivocally discriminated based on nuclear rDNA sequence differences (D Amelio *et al*, 2000). In this study, we applied molecular methods for sibling species-level identification of Japanese *A. simplex* worms and confirmed the usefulness of RFLP analysis of rDNA for this purpose. Thus Japanese *A. simplex* worms were classified into *A. simplex* s. str., *A. pegreffii* or the hybrid genotype.

It is well known that mitochondrial DNA (mtDNA) evolves at a faster rate than nuclear DNA and is useful for differentiating cryptic species (Blouin, 2002). Therefore, we used mitochondrial *cox1* in developing an RFLP method that is capable of classifying *A. simplex* into sibling species. Initially, the enzyme *Hin*fI was used for this discrimination (Umehara *et al*, 2006), but based on the detection of intraspecific variations at the recognition sites of *Hin*fI in mitochondrial *cox1* sequence in one of 12 *A. pegreffii* worms examined, we selected another enzyme, *Sfc*I, that discriminates between *A. simplex* s. str. and *A. pegreffii*. By sequence analysis, we confirmed that there were no intraspecific variations at the *Sfc*I recognition site in the sequences of 39 worms examined so far (20 *A. simplex* s. str. and 19 *A. pegreffii* worms).

The hybrid genotype worms were clearly classified into either *A. simplex* s. str.-type or *A. pegreffii*-type by the RFLP method employing *Sfc*I. Since the vast majority of mitochondrial genomes are inherited uniparentally from the female parent, the results obtained with the hybrid genotype worms were as predicted. The mitochondrial genome is effectively haploid (Moore, 1995) and, thus, may be advantageous for study of the evolutionary history of *A. simplex*.

The results of this study showed that *A. simplex* s. str. is primarily distributed in fish and marine mammals in North Pacific Ocean and *A. pegreffii* is predominantly distributed in fish in the southern Sea of Japan. However, worms from patients were identified as *A. simplex* s. str. even though they were obtained from southern Japan where *A. pegreffii* is the predominant species in fish. The reason for this discrepancy between the predominant sibling species in fish and in patients remains unresolved. Studies are now in progress to identify the fish species that is responsible for human infection. This information is crucial for initiating prevention measures against human anisakiasis, especially in southern Japan.

REFERENCES

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.

Blouin MS. Molecular prospecting for cryptic species of nematodes: mitochondrial DNA versus internal transcribed spacer. *Int J Parasitol* 2002;32:527-31.

D Amelio S, Mathiopoulos KD, Santos CP *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.

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.

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.

Mattiucci S, Nascetti G, Cianchi R, *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.

Mattiucci S, Nascetti G. Molecular systematics, phylogeny and ecology of anisakid nematodes of the genus *Anisakis* Dujardin, 1845: an update. *Parasite* 2006;13:99-113.

Moore WS. Inferring phylogenies from mtDNA variation: mitochondrial-gene trees versus nuclear-gene trees. *Evolution* 1955; 49:718-26.

Nascetti G, Paggi L, Orecchia P, Mattiucci S, Bullni L. Two sibling species within *Anisakis simplex* (Ascaridida: Anisakidae). *Parassitologia* 1983;25:239-41.

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.

Umehara A, Kawakami Y, Araki J, Matui T, Uchida A. Molecular identification of *Anisakis simplex* sensu stricto and *Anisakis pegreffii* (Nematoda: Anisakidae) from fish and cetacean in Japanese waters. *Parasitol Int* 2006;55:267-71.

Umehara A, Kawakami Y, Araki J, Uchida A. Molecular identification of the etiological agent of the human anisakiasis in Japan. *Parasitol Int* 2007;56:211-15.