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Molecular characterization of *Anisakis pegreffii* larvae in Pacific cod in Japan

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Abstract

It is now recognized that the morphospecies *Anisakis simplex* is not a single species but a complex composed of three sibling species, *A. simplex* sensu stricto, *A. pegreffii* and *A. simplex* C. In Japan, *A. simplex*-like larvae have been isolated from a variety of fish and humans, but the larvae collected have been identified as *A. simplex* by only light microscopy. Therefore, the epidemiology of the *A. simplex* complex, composed of three sibling species, is still unclear in Japan. In the present study, 26 *A. simplex*-like larval isolates were obtained from two Pacific cod landed in Hokkaido, Japan, and examined genetically by PCR–RFLP and direct sequencing of the ITS region of rDNA. Among the 26 isolates, 24 were identified as *A. simplex* sensu stricto, the other two as *A. pegreffii*. The present study is the first to confirm the distribution of *A. pegreffii* in Japan, and to detect *A. pegreffii* larvae in Pacific cod.

Introduction

Anisakis simplex is a parasitic nematode infecting fish and mammals. The definitive hosts of this parasite are marine mammals such as seals, whales and dolphins, whilst the intermediate or paratenic hosts are pelagic fish or squid. It is well recognized that human infection known as anisakiasis occurs through the ingestion of raw or undercooked fish. Cases of anisakiasis are predominantly reported in Japan, Italy, Spain, The Netherlands and North America, and regional eating habits such as the consumption of raw fish (e.g. sashimi and sushi in Japan, and raw marinated fish in European countries facing the Mediterranean Sea) are recognized as a factor in the high prevalence of anisakiasis (Sakanari & McKerrow, 1989).

In Japan, 'The Food Sanitation Law Enforcement Regulation' was partly amended at the end of 1999. Anisakiasis was newly added to the causative agents of food poisoning, and a physician must now notify a nearby health centre within 24 h following diagnosis (National Institute of Infectious Diseases and Tuberculosis and Infectious Diseases Control Division, Ministry of Health, Labour and Welfare, 2004).

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It is now recognized that the morphospecies *A. simplex* is not a single species but a complex of three sibling species, *A. simplex* sensu stricto, *A. pegreffii* and *A. simplex* C, differing in their genetic structure and ecological traits (Mattiucci *et al*., 1986, 1997; Nascetti *et al*., 1986; Orecchia *et al*., 1986; Paggi *et al*., 1998b). In Japan, *A. simplex*-like larvae have been isolated from a variety of fish and humans, but the larvae collected have been identified as *A. simplex* using only light microscopy. Therefore, the epidemiology of the *A. simplex* complex is still unclear in Japan. In the present study, *A. simplex*-like larvae from fish landed in northern Japan were analysed using molecular techniques, and the distribution of *A. pegreffii* determined for the first time in Japan.

Materials and methods

Twenty-six whitish nematodes about 15–20 mm long, suspected to be *Anisakis* spp. larvae, were collected from commercial raw Pacific cod (*Gadus macrocephalus*). One nematode sample was collected from a slice of fish packed for sale (identified as AC-1), and the other 25 were from the visceral cavity, and the external wall of the stomach of another raw fish sample (identified as AC-2 to AC-26). Both fish were landed in Hokkaido, northern Japan, the latter in eastern Hokkaido facing the Pacific Ocean. All isolates were washed three times with phosphate-buffered

saline (PBS) and fixed in 70% ethanol for 24 h. After fixing, each isolate was cut at the centre using a clean razor and, for morphological examination, the anterior part of the body including the oesophagus and ventriculus was cleared in lactophenol and identified morphologically (Koyama *et al.*, 1969). For genetic analysis, the posterior part was suspended in 200 μl of PBS and subjected to freezing and thawing three times. Subsequently, the suspension was boiled for 10 min, and the DNA extracted and purified using a QIAamp DNA Mini Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions.

A polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP)-based analysis of the internal transcribed spacer (ITS) region of rDNA with the restriction enzyme *Hin*fI was performed to identify the isolates genetically, because this method had been reported to successfully discriminate among *Anisakis* spp. including the *A. simplex* complex (D'Amelio *et al.*, 2000; Abollo *et al.*, 2003). The entire ITS (ITS1, 5.8S rDNA and ITS2) was amplified using the forward primer NC5 and the reverse primer NC2 (Zhu *et al.*, 1998). This primer pair produced an approximately 950-bp product. PCR amplification was performed in a volume of 50 μl containing 1X PCR buffer, 2 mM $MgCl_2$, 250 μM of each dNTP, 0.5 μM of each primer, 1.25 units of Ex Taq DNA polymerase (TAKARA Shuzo Co. Ltd, Otsu, Japan), and 5 μl of the DNA sample. The PCR buffer and dNTP mixture appended to Ex Taq DNA polymerase were used. Reactions were performed on a GeneAmp PCR System 9700 thermocycler (Perkin-Elmer, Foster City, California) using the same conditions reported previously (Zhu *et al.*, 1998). The PCR products were purified using a QIAquick Gel Extraction kit (QIAGEN GmbH, Hilden, Germany). Digestion of the purified products using *Hin*fI (TAKARA Shuzo Co. Ltd, Otsu, Japan) was performed following the manufacturer's directions. Digested products were subjected to electrophoretic separation using 3% agarose gels, stained with ethidium bromide, and visualized on a UV transilluminator. Recently, it has been shown that there were 2 bp substitutions between *A. simplex* sensu stricto and *A. pegreffii* within the sequences of the ITS region amplified with NC5 and NC2 (Abollo *et al.*, 2003). When isolates showing the same RFLP pattern as *A. pegreffii* were found, the ITS region of these isolates was sequenced to confirm the sequence differences as mentioned above. Purified PCR products were sequenced using an ABI PRISM BigDye Terminator Cycle Sequencing FS Ready Reaction kit (Perkin-Elmer Corp., USA) on an automated sequencer (ABI PRISM 310 model; Perkin-Elmer Corp., USA). The products were sequenced in both directions using NC5 and NC2. Nucleotide sequences of the samples were aligned with Clustal-X (version 1.63b).

Results and Discussion

All isolates examined were identified as *A. simplex* larvae based on the morphology of the ventriculus. The RFLP patterns of 24 isolates (AC-3 to AC-26) were the same, and comprised two visible fragments (approximately 620 bp and 220 bp) (fig. 1). This pattern corresponded to that of *A. simplex* sensu stricto reported previously (D'Amelio *et al.*, 2000; Abollo *et al.*, 2003). However, the RFLP pattern of the isolates AC-1 and AC-2 originating from a different cod were different and three fragments (approximately 330 bp, 280 bp and 220 bp) were clearly found (fig. 1). This pattern corresponded to that of *A. pegreffii* reported previously (D'Amelio *et al.*, 2000; Abollo *et al.*, 2003).

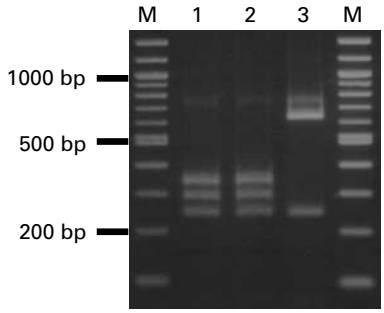

Fig. 1. Restriction patterns obtained using *Hin*fI in the ITS region of rDNA amplified with NC5 and NC2. Lanes: M, molecular marker (100 bp ladder); 1, AC-1 from a slice of raw cod packed for sale; 2 and 3, AC-2 and AC-3 from the visceral cavity and external stomach wall of a second raw cod sample. The restriction patterns of isolates AC-1 and AC-2 were identical, and composed of three visible fragments (approximately 330 bp, 280 bp and 220 bp). In contrast, the pattern of AC-3 was different from that of AC-1 and AC-2, and had two visible fragments (approximately 620 bp and 220 bp). The RFLP patterns of the other 23 isolates were the same as that of AC-3.

The isolate AC-3 as representative of the 24 isolates that showed the *A. simplex* sensu stricto-like pattern, in addition to AC-1 and AC-2 were sequenced in the ITS region to confirm the differences. A 907-bp sequence excluding the forward and reverse primer regions were obtained in the three isolates sequenced. The sequences of ITS of isolates AC-1, AC-2 and AC-3 were deposited in the DNA Data Bank of Japan (DDBJ: http://www.ddbj.nig.ac.jp/) under accession numbers AB196670, AB196671 and AB196672, respectively. The sequences of AC-1 and AC-2 were identical, but differed by 2 bp from that of AC-3 (fig. 2). These differences were found at positions 280 and 296 (C in AC-1 and AC-2 was T in AC-3), and corresponded to the sequence differences between *A. pegreffii* and *A. simplex* sensu stricto reported previously (Abollo *et al.*, 2003).

In Japan, the identification of anisakid larvae from fish and humans has been performed under light microscopy only, and it has not been recognized that *A. simplex* is a complex composed of three sibling species, *A. simplex* sensu stricto, *A. pegreffii* and *A. simplex* C. Although Mattiucci *et al.* (1997) and Paggi *et al.* (1998a) genetically analysed *A. simplex* larvae from walleye pollock, *Theragra chalcogramma*, landed in Hokkaido, the larvae were identified as *A. simplex* sensu stricto. On the basis of the present results, the isolates AC-1 and AC-2 were identified as *A. pegreffii*, thereby confirming the distribution of *A. pegreffii* in Japan for the first time. *Anisakis pegreffii* has been recovered from three squid, 26 fish and three mammal species (Mattiucci *et al.*, 1997), but has not been recovered from the Pacific cod *Gadus macrocephalus*.

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AC-1  ATCGAGCGAA  TCCAAAACGA  ACGAAAAAGT  CTCCCAACGT  GCATACCTTC  CATTTGCATG  TTGTTGTGAG  CCACATGGAA  80
AC-2  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........
AC-3  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........
AC-1  ACTCGTACAC  ACGTGGTGGC  AGCCGTCTGC  TGTGCTTTTT  TTAGGCAGAC  AATGGCTTAC  GAGTGGCCGT  GTGCTTGTTG  160
AC-2  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........
AC-3  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........
AC-1  AACAACGGTG  ACCAATTTGG  CGTCTACGCC  GTATCTAGCT  TCTGCCTGGA  CCGTCAGTTG  CGATGAAAGA  TGCGGAGAAA  240
AC-2  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........
AC-3  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........
AC-1  GTTCCTTTGT  TTTGGCTGCT  AATCATCATT  GATGAGCAGC  AGCTTAAGGC  AGAGTCGAGC  AGACTTAATG  AGCCACGCTA  320
AC-2  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........
AC-3  ..........  ..........  ..........  .........T  ..........  .....T....  ..........  ..........
AC-1  GGTGGCCGCC  AAAACCCAAA  ACACAACCGG  TCTATTTGAC  ATTGTTATTT  CATTGTATGT  GTTGAAAATG  TACAAATCTT  400
AC-2  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........
AC-3  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........
AC-1  GGCGGTGGAT  CACTCGGTTC  GTGGATCGAT  GAAGAACGCA  GCCAGCTGCG  ATAAATAGTG  CGAATTGCAG  ACACATTGAG  480
AC-2  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........
AC-3  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........
AC-1  CACTAAGAAT  TCGAACGCAC  ATTGCGCTAT  CGGGTTCATT  CCCGATGGCA  CGTCTGGCTG  AGGGTCGAAT  TACGGTGAAC  560
AC-2  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........
AC-3  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........
AC-1  TGTCTTCACG  GTTTTTCTGG  ACTGTGAAGC  ATTCGGCAAG  CAATTGCTGT  TGTGTTGTTG  GTGATTCTAT  CATGGACAAT  640
AC-2  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........
AC-3  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........
AC-1  ATGACGAGCG  GTTCCTTGCT  TAGTGATGAC  AAAAGAAGAC  GTCAACACCG  AATCTACTAT  ACTACTAATA  CTAGTATATA  720
AC-2  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........
AC-3  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........
AC-1  GGTGAGGTGC  TTTTGGTGGT  CACAAAAGTG  ACAAGTATGC  CATTTCATAG  GGGCAACAAC  CAGCATACGT  GATAAGTTGG  800
AC-2  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........
AC-3  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........
AC-1  CTGGTTGATG  AAACGGCAAC  GGAATGACGG  ACGTCTATGT  GATCAAAAAT  GATACTATTT  GACCTCAGCT  CAGTCGTGAT  880
AC-2  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........
AC-3  ..........  ..........  ..........  ..........  ..........  ..........  ..........  ..........
AC-1  TACCCGCTGA  ATTTAAGCAT  ATAATTA     907
AC-2  ..........  ..........  .......
AC-3  ..........  ..........  .......
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Fig. 2. Alignment of the ITS region (except for the primer regions) of the rDNA amplified with primers NC5 and NC2 for the isolates AC-1, AC-2 and AC-3. Dots indicate bases that are identical to AC-1. Sequences of AC-1 and AC-2 are identical. Sites differing between AC-1, AC-2 and AC-3 are boldfaced.

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Therefore, the detection of *A. pegreffii* larvae in Pacific cod is also a first record.

In 1999, *A. pegreffii* infection in humans was first confirmed by the PCR–RFLP analysis of rDNA of larvae from an Italian patient (D'Amelio *et al.*, 1999). In Japan, the annual number of cases of anisakiasis has been estimated to be over 1000 (Oshima & Kliks, 1987), but almost all larvae from patients have been identified as *A. simplex* based only on morphological examination. Therefore, it appears that cases involving *A. pegreffii* might be occurring in Japan. In the present study, only one of 25 *A. simplex*-like larvae collected from a Pacific cod was identified as *A. pegreffii*, and it is likely that *A. pegreffii* is not very well adapted to live in Pacific cod. In Italy, it is speculated that cases involving *A. pegreffii* might be the most dominant of all the cases of anisakiasis, because fish species, especially the anchovy, *Engraulis encrasicholus*, which has high commercial value and is used for raw marinade, is frequently infected with *A. pegreffii* larvae in Italian seas (Mattiucci *et al.*, 1997). In Japan, surveys of *Anisakis* infection in Japanese anchovies, *Engraulis japonica*, have been performed, but again the *A. simplex* larvae were only identified by light microscopy (Kato *et al.*, 1992; Uchida *et al.*, 1998). Therefore, it is still unclear whether the *A. simplex* larvae found in Japanese anchovies are *A. pegreffii* or not. Further genetic analysis of larvae from humans and fish is required to clarify the epidemiology and ecology of the *Anisakis simplex* complex in Japan.

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