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SHORT COMMUNICATION

Identification of anisakid nematodes with zoonotic potential from Europe and China by single-strand conformation polymorphism analysis of nuclear ribosomal DNA

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Abstract Using genetic markers defined previously in the second internal transcribed spacer (ITS-2) of nuclear ribosomal DNA (rDNA), isotopic, and non-isotopic polymerase-chain-reaction-coupled single-strand conformation polymorphism (SSCP) were utilized to identify each of three anisakid species [*Anisakis simplex* (*s.l.*), *Contracaecum osculatum* (*s.l.*), and *Hysterothylacium aduncum*] from different host species and geographical locations in Poland and Sweden. While subtle microheterogeneity was observed within each of *Anisakis simplex* (*s.l.*) and *H. aduncum*, distinct SSCP profiles were displayed for each of the three species, allowing identification and differentiation of the three taxa. Subsequent sequencing of the ITS-1 and ITS-2 rDNA revealed that *A. simplex* (*s.l.*) represented *Anisakis simplex* s.s. and *Contracaecum osculatum* (*s.l.*) represented *C. osculatum* C. Application of the non-isotopic SSCP assay of ITS-2 to larval anisakid samples from different hosts and geographical locations in China revealed three distinct SSCP profiles, one of which was consistent with that of *A. simplex* (*s.l.*), and the other two had different SSCP profiles from that of *C. osculatum* C and *H. aduncum*. Sequencing of the ITS-1 and ITS-2 rDNA for representative Chinese anisakid samples examined revealed three anisakid species in China, i.e., *Anisakis typica*, *Anisakis pegreffii*, and *Hysterothylacium* sp. These molecular tools will be useful for identification and investigation of the ecology of anisakid nematodes in China and elsewhere.

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Anisakids are common parasites of fish, mammals, and birds (Adams et al. 1997; Shih 2004; Farjallah et al. 2006). Larval anisakids of some genera can infect humans and cause significant clinical diseases in a number of countries (Adams et al. 1997; McCarthy and Moore 2000; Couture et al. 2003; Pellegrini et al. 2005). The accurate identification and differentiation of anisakid nematodes at any life cycle stage is central for studying the life cycles, transmission patterns, and ecology of anisakids and is also important for the diagnosis of anisakid infections in humans and animals. However, there remain difficulties in the accurate diagnosis of anisakiasis in different host species, mainly as a consequence of limitations in identifying larval stages using morphological characters (Olson et al. 1983; Fagerholm 1988; Dick et al. 1991) or when only small portions of worms are available for identification (Dick et al. 1991).

Previous studies have demonstrated that the internal transcribed spacers (ITS-1 and/or ITS-2) of nuclear ribosomal DNA (rDNA) provide genetic markers for the accurate identification of anisakid nematodes by polymerase

chain reaction (PCR)-based approaches (e.g., Zhu et al. 1998, 2002; D'Amelio et al. 2000; Shih 2004; Li et al. 2005). In particular, our preliminary study (Zhu et al. 1998) demonstrated that isotopic PCR-linked single-strand conformation polymorphism (PCR-SSCP) was useful for characterizing ten larval samples representing *Anisakis simplex* (*s.l.*), *Contracaecum osculatum* (*s.l.*), and *Hysterothylacium aduncum* from Poland. But the sample number (n=10) in that study was small, and the samples were from limited hosts and geographical localities.

Extending this preliminary investigation, the objectives of the present study were to establish, using ITS-2 rDNA as genetic markers, isotopic and non-isotopic PCR-SSCP for the specific detection of anisakid nematodes from different hosts and geographical locations in Europe and then to apply these tools to determine the specific status of anisakid samples from various hosts and geographical localities in China.

Larval anisakids were collected from different fish species from Europe and mainland China (Table 1). Individual anisakid larvae were repeatedly washed in physiological saline (pH 7.3) and identified to morph-species (for the ones from Europe, n=281) or generic level (for those from mainland China, n=25) based on the host and tissue from which they were derived, the geographical origin of the host/parasite, and the morphology of the parasite (Nascetti et al. 1986, 1993; Orecchia et al. 1994; Mattiucci et al. 1997).

Genomic DNA was isolated from individual nematodes (n=306) by sodium dodecyl sulphate/proteinase K treatment, column-purified (Wizard™ DNA Clean-Up, Promega). PCR was used to amplify the ITS-2 (plus ~120 bp of primer flanking sequences) with primers XZ1 (forward: 5′-ATTGCGCCATCGGGTTCATTCC-3′) and NC2 (reverse: 5′-TTAGTTTCTTTTCCTCCGCT-3′; Zhu et al. 2002). PCR reactions (in a volume of 25 μl) were performed in 10 mM Tris–HCl, pH 8.4, 50 mM KCl, 3 mM $MgCl_2$, 250 μM each of dNTP, 50 pmol of each primer (end-labeled with γ-^{33}P-ATP, Amersham Biosciences, or unlabeled) and 2 U *Taq* polymerase (Takara) in a thermocycler (Biometra) under the following conditions: 94°C for 5 min (initial denaturation), followed by 30 cycles of 94°C, 30 s (denaturation), 55°C, 30 s (annealing), 72°C, 30 s (extension), and a final extension of 72°C for 5 min. An aliquot (4 μl) of each PCR product was examined on a 1% agarose gel, stained with ethidium bromide, and photographed using a gel documentation system (UVItec).

Isotopic SSCP analysis of ITS-2 rDNA was carried out essentially as described previously (Zhu et al. 2002; Li et al. 2005). The conditions for electrophoresis (20 W for 7 h at 20°C) were standardized for optimal resolution of bands (Li et al. 2005). Gels were subsequently dried on to blotting paper and exposed to X-ray film (Super RX, Fuji). The non-isotopic ITS-2 amplicons were subjected to SSCP analysis as described previously (Li et al. 2006). For the sequencing of the complete ITS-1 and ITS-2 rDNA, the rDNA region spanning the ITS-1, 5.8S rDNA, and ITS-2 was amplified from representative anisakid samples by PCR using primers NC5 (forward: 5′-GTAGGT

Table 1 DNA samples of anisakid nematodes used in this study and the geographical origins of their fish hosts

Species	Sample code	Host species	Geographical origin
Samples from Europe			
Anisakis simplex (*s.l.*)	Achvl1-50	*Clupea harengus*	Vistula Lagoon, Baltic sea, Poland
Anisakis simplex (*s.l.*)	Achpb51-100	*Clupea harengus*	Pomeranian Bay, Baltic sea, Poland
Anisakis simplex (*s.l.*)	Achsf101-130	*Clupea harengus*	Slupsk Furrow, Baltic sea, Poland
Anisakis simplex (*s.l.*)	Achs131-160	*Clupea harengus*	Skagerrak (Danish Straits), Sweden
Anisakis simplex (*s.l.*)	Achgb161-220	*Clupea harengus*	Gdansk Bay, Baltic sea, Poland
Anisakis simplex (*s.l.*)	Apfbb221-225	*Platychthis flesus*	Bornholm Basin, Baltic sea, Poland
Contracaecum osculatum (*s.l.*)	Cosc1-40	*Gadus morhua*	Bornholm Basin, Baltic sea, Poland
Contracaecum osculatum (*s.l.*)	Cosc41	*Gadus morhua*	Gdansk Basin, Baltic sea, Poland
Hysterothylacium aduncum	Hadu1-7	*Zoarces viviparus*	Gdansk Bay, Baltic sea, Poland
Hysterothylacium aduncum	Hadu8-13	*Platychthis flesus*	Slupsk Furrow, Baltic sea, Poland
Hysterothylacium aduncum	Hadu14-15	*Platichthys flesus*	Gdansk Bay, Baltic sea, Poland
Samples from China			
Anisakis sp.	ASSCD1, ASSCD2	*Selar crumenophthalmus*	Daya Bay, Guangdong, China
Anisakis sp.	PS1, PS2	*Nemipterus virgatus*	Daya Bay, Guangdong, China
Anisakis sp.	ASP5-1, ASP5-2	*Nemipterus bathybius*	Daya Bay, Guangdong, China
Anisakis sp.	ASNVD1-ASNVD9	*Nemipterus virgatus*	Daya Bay, Guangdong, China
Anisakis sp.	ASP2-1	*Astroconger myriaster*	Weihai, Shandong, China
Anisakis sp.	ASP4-1, ASP4-2	*Hemiptripterus villosus*	Dalian Bay, Liaoning, China
Hysterothylacium sp.	HS1, HS2	*Astroconger myriaster*	Weihai, Shandong, China
Hysterothylacium sp.	ASP3-1, ASP3-2	*Pseudosciaena polyactis*	Dalian Bay, Liaoning, China
Hysterothylacium sp.	CSPP1-CSPP3	*Pseudosciaena polyactis*	Weihai, Shandong, China

GAACCTGCGGAAGGATCATT-3′; Zhu et al. 2002) and NC2 under the same conditions as for amplification of the ITS-2. PCR products were purified, cloned, and then sequenced. The 5′ and 3′ ends of the anisakid ITS-1 and ITS-2 sequences were determined by comparison with previously published anisakid ITS sequences (Zhu et al. 1998, 2002; Li et al. 2005), and then the ITS-1 and ITS-2 sequences were aligned and compared with relevant sequences available in the GenBank™.

Using a pair of labeled primers, the ITS-2 was amplified from 281 anisakid nematodes from Europe. On agarose gels, the ITS-2 PCR products represented single bands and were approximately 430–480 bp in size (not shown). Then, the 281 isotopic ITS-2 amplicons were subjected to SSCP analysis, and Fig. 1 is a representative SSCP gel showing sequence variation in the ITS-2 among amplicons of anisakids from Europe. With the exception of slight microheterogeneity within a species, SSCP analyses of all the ITS-2 amplicons displayed three distinct banding profiles, which allowed the identification of all anisakid samples examined into three groups, corresponding to the three morpho-species examined (Fig. 1, cf., Table 1).

While isotopic SSCP proved a useful method for the identification of anisakid samples, it requires the use of radioisotopes and is relatively costly. Therefore, a non-isotopic SSCP approach was then established to identify anisakid samples. Figure 2 is a representative non-isotopic SSCP gel showing sequence variation in the ITS-2 among amplicons of anisakids from Poland and Sweden. While

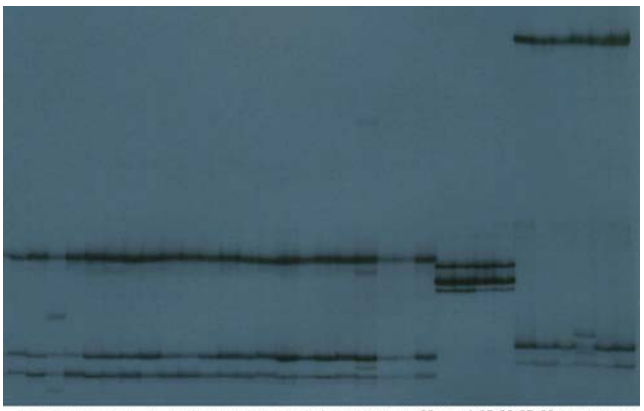

Fig. 1 Identification of anisakid nematodes from Europe by isotopic SSCP analysis of ITS-2 rDNA. Lanes *1–22* represent samples of *A. simplex* (*s.l.*) (samples Achvl5, Achvl7, Achvl23, Achvl27, Achvl28, Achvl29, Achpb69, Achpb75, Achpb78, Achpb80, Achsf105, Achsf108, Achsf111, Achs146, Achs153, Achs154, Achgb165, Achgb166, Achgb168, Apfbb221, Apfbb222, and Agmbb226, respectively, cf. Table 1). Lanes *23–26* represent samples of *C. osculatum* (*s.l.*) (samples Cosc16, Cosc17, Cosc18, and Cosc41, respectively, cf. Table 1). Lanes *27–32* represent samples of *H. aduncum* (samples Hadu3, Hadu4, Hadu8, Hadu11, Hadu10, and Hadu14, respectively, cf. Table 1)

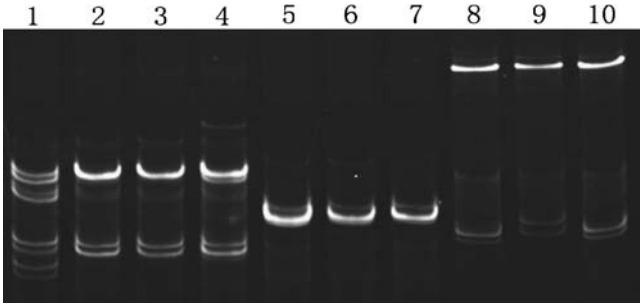

Fig. 2 Identification of anisakid nematodes from Europe by non-isotopic SSCP analysis of ITS-2 rDNA. Lanes *1–4* represent samples of *A. simplex* (*s.l.*) (samples Achvl23, Achpb54, Achs153, and Achgb168, respectively, cf. Table 1). Lanes *5–7* represent samples of *C. osculatum* (*s.l.*) (samples Cosc4, Cosc17, and Cosc33, respectively, cf. Table 1). Lanes *8–10* represent samples of *H. aduncum* (samples Hadu3, Hadu11, and Hadu13, respectively, cf. Table 1)

there were subtle variation in the SSCP profiles within *A. simplex* (*s.l.*) and *H. aduncum*, non-isotopic SSCP analyses displayed three distinct banding patterns, allowing the unequivocal differentiation of the three morpho-species examined (Fig. 2).

Then, the established non-isotopic SSCP approach was applied to determine the identity of 25 anisakid samples from different hosts and geographical locations in China (cf. Table 1). SSCP analysis of ITS-2 rDNA exhibited three distinct banding profiles (Fig. 3), one of which was consistent with that of *A. simplex* (*s.l.*), and the other two were different from that of *C. osculatum* (*s.l.*) and *H. aduncum*, demonstrating the existence of three different species of anisakid nematodes.

Then, the ITS-1 and ITS-2 rDNA of seven representative anisakid samples (Achs153, Achvl23, Achgb168, Hadu3, Hadu11, Cosc17, cf. Table 1) from Europe were sequenced, and the sequences were deposited in the GenBank™ under accession numbers AJ937669–AJ937674. The sequencing results supported the findings achieved by SSCP analyses. The two *H. aduncum* samples had identical ITS-1 and ITS-2 sequences, and the three *A. simplex* (*s.l.*) samples also had

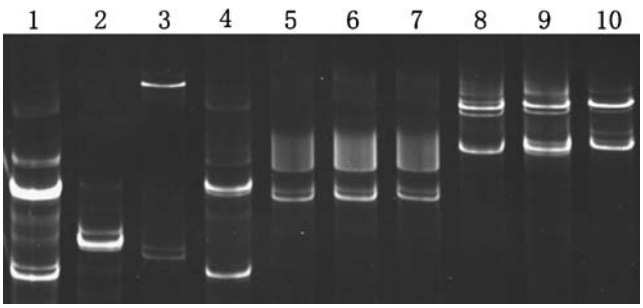

Fig. 3 Identification of anisakid nematodes from China by non-isotopic SSCP analysis of ITS-2 rDNA using *A. simplex* (*s.l.*) (*lane 1*, sample Achs153), *C. osculatum* (*s.l.*) (*lane 2*, sample Cosc17), and *H. aduncum* (*lane 3*, sample Hadu3) for comparison. Lanes *4–10* represent samples ASP2-1,HS1,ASP3-1,CSPP1,PS1,ASP5-1, and ASSCD2 (cf. Table 1), respectively

identical consensus sequences, with only one polymorphic position in the ITS-1 (sequence position 148, C for Achgb168, and T for Achs153 and Achvl23). The consensus ITS sequences of samples Achs153, Achvl23, and Achgb168 were identical to that of *A. simplex* s.s. (i.e. *A. simplex* B, accession nos. AJ225065 and AJ225066), sample Cosc17 had identical sequences to that of *C. osculatum* C (accession nos. AJ225062 and AJ225064), and samples Hadu3 and Hadu11 had identical sequences to that of *H. aduncum* (accession nos. AJ225068 and AJ225069), respectively, demonstrating that the anisakid samples from Poland and Sweden examined represent three species, namely, *A. simplex* s.s., *C. osculatum* C, and *H. aduncum*.

For anisakid samples from China, the ITS-1 and ITS-2 rDNA of seven representative samples (ASSCD2, PS1, ASP5-1, HS1, ASP3-1, CSPP1, and ASP2-1, cf. Table 1) were sequenced. Samples ASSCD2, PS1, and ASP5-1 had identical ITS-1 and ITS-2 sequences (accession no. AM706345), which had 96.9 and 100% identity with that of *A. typica* (accession no. AY826724), respectively, indicating that it represents *A. typica*. Samples HS1, ASP3-1, and CSPP1 also had identical ITS-1 and ITS-2 sequences (accession no. AM706344), which had 79.6 and 47.5% identity with that of *H. bidentatum* (accession no. AY603539), indicating that it may represent a member of the genus *Hysterothylacium*. Sample ASP2-1 had unique ITS sequences (accession no. AM706346). While the ITS-2 sequence of sample ASP2-1 was identical to that of *Anisakis pegreffii* (accession nos. AY826720, AB196671, and AB277823) and *A. simplex* s.s. (accession no. AJ225066), its ITS-1 sequence was more similar to that of *A. pegreffii* (99.2%, accession nos. AY826720, AB196671, and AB277823) than to that of *A. simplex* s.s. (98.5%, accession no. AJ225065), indicating that it represents *A. pegreffii*.

The present study proved that ITS sequences provide ideal genetic markers, and both isotopic and non-isotopic PCR-SSCP approaches are reliable techniques for the identification and differentiation of anisakids. The infection of anisakid nematodes in marine fish off the Chinese coasts was quite common, with up to 100% of infection rate in some fish (e.g., Tang et al. 2001). Given the difficulty in the accurate identification of anisakid nematodes using morphological approach, the isotopic and non-isotopic SSCP approaches, in particular the non-isotopic one, provide useful alternative approaches for the unequivocal identification and differentiation of anisakids. These findings and the molecular approaches taken should have important implications for studying the ecology, epidemiology, and population genetics of these anisakid nematodes and for the diagnosis of their infections in various hosts.

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