



Molecular identification of *Anisakis* and *Hysterothylacium* larvae in marine fishes from the East China Sea and the Pacific coast of central Japan

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ABSTRACT

Anisakiasis is a human disease caused by the accidental ingestion of larvae belonging to the family Anisakidae. Three fish species, the small yellow croaker *Pseudosciaena polyactis*, the mackerel *Pneumatophorus japonicus* and the hairtail *Trichiurus haumela* are important source for food products in the East China Sea. The prevalence and the identification of Anisakidae larvae in these fishes will benefit the prevention and control of anisakiasis. In this study, fish samples were obtained from fish markets in the East China Sea and the Pacific coast of central Japan during April 2011 and July 2013. For species identification, the PCR-based restriction fragment length polymorphism (PCR–RFLP) analysis of the entire ITS region (ITS1, 5.8 S and ITS2) of nuclear ribosomal DNA (rDNA) was performed. In total, 2004 larvae were collected from 80 hairtail fish, 20 small yellow croaker, and 27 mackerel from the East China Sea and the Pacific coast of central Japan. High prevalence of Anisakidae larvae infection (116/122, 95.1%) was detected in the East China Sea. Seven species were identified belonging to the genera *Anisakis* (Nematoda: Anisakidae) and *Hysterothylacium* (Nematoda: Anisakidae). *Anisakis pegreffii* was the predominant species accounting for 84.8% of all larvae examined in East China Sea, while all Anisakidae larvae isolated from Japan were identified as *Anisakis simplex* sensu stricto (s.s.). In the East China Sea, *A. simplex* s.s. and *Anisakis typica* were 0.6% (4/619) and 1.5% (9/619) of the identified nematodes, respectively. Interestingly, one larva was identified as a recombinant genotype of *A. simplex* s.s. and *A. pegreffii*. In addition, four species of the genus *Hysterothylacium*, namely, *Hysterothylacium amoyense* (31/619, 5.0%), *Hysterothylacium aduncum* (10/619, 1.6%), *Hysterothylacium fabri* (21/619, 3.4%) and *Hysterothylacium* spp. (18/619, 2.9%) were also identified in the present study. This is a comprehensive epidemiological dataset for the family Anisakidae in the East China Sea. The identification of *A. typica*, recombinant genotype of *A. simplex* s.s. and *A. pegreffii*, *H. amoyense* and *H. fabri* is first reported in this area. The wide diversity and substantial geographical distributions of these nematodes will provide a foundation for future studies of Anisakidae family. The high prevalence of these nematodes in marine fishes off the East China Sea may pose considerable food safety problems, which is a potential cause of human anisakiasis.

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1. Introduction

Anisakidae Railliet & Henry, 1912 is a family of parasitic nematodes, which have life cycles involving invertebrates, cephalopods, fish and

marine mammals. Human anisakiasis is caused by infection with the larvae of the genera *Anisakis* Dujardin, 1845, *Pseudoterranova* Krabbe, 1878 and *Contracaecum* Railliet & Henry, 1913 (Sakanari and McKerrrow, 1989). Larvae belonging to the two latter genera are with weak invasive potential and case reports attributed to these two genera are rare (Macpherson, 2005). The parasites most frequently associated with the disease in humans comprise several species belonging to the genus *Anisakis*, including *Anisakis pegreffii* Campana-Rouget & Biocca, 1955, *Anisakis simplex* sensu stricto (s.s.) Rudolphi, 1809, *Anisakis berlandi* (Mattiucci et al., 2014), *Anisakis typica* Diesing, 1860 and *Anisakis ziphidarum* Paggi, Nascetti, Webb, Mattiucci, Cianchi & Bullini, 1988. Invasive anisakiasis occurs when the third-stage larvae attach to, embed in, or

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penetrate host tissues. The prevalence of anisakiasis is clearly related to eating habit of consuming raw, lightly cooked, or marinated seafood, such as Japanese sushi and sashimi, Dutch salted or smoked herring, Hawaiian lomi-lomi, and Latin American ceviche (Chai et al., 2005). This serious parasitic disease, which was first described by Van Thiel in 1960, has increased throughout the world in the last few decades. Recently, approximately 20,000 cases of anisakiasis were reported worldwide every year, over 90% are from Japan and most others in Spain, the Netherlands, Germany, Italy, Australia, South Korea and so on (Lee et al., 2014; Mattiucci et al., 2013; Pravettoni et al., 2012; Shibata et al., 2014; Yorimitsu et al., 2013). In China, there is only one case discovered by chance up to now (Qin et al., 2013), although more and more *Anisakis* species have been identified from marine fishes around Chinese waters (Du et al., 2010; Fang et al., 2010; Guo et al., 2014; Ma et al., 1997; Xu et al., 2014; Zhang et al., 2013; Zhang et al., 2007; Zhu et al., 2007), which may suggest that people lack awareness of *Anisakis* infection and lack the clinical diagnosis methods for *Anisakis* infection.

The identification of Anisakidae nematodes at species level is essential for the determination of the spatial distribution and the evaluation of molecular epidemiology of the disease. Studies have demonstrated that the PCR-based restriction fragment length polymorphism (PCR–RFLP) analysis of the entire ITS region of rDNA can be employed effectively for the specific identification of anisakids (Abattouy et al., 2013; Pekmezci et al., 2014; Piras et al., 2014; Serracca et al., 2013b; Umehara et al., 2010). Anisakidae larvae have been reported in marine fishes and squids from the East China Sea in 1991 (Sun et al., 1991). However, accurate identification of these nematodes has not been conducted, and there is still scarce information on *Anisakis* infection in the small yellow croaker *Pseudosciaena polyactis*, the chub mackerel *Pneumatophorus japonicus* and the hairtail *Trichiurus haumela*, which are important food source in the East China Sea. In order to improve food safety and to raise awareness that anisakiasis should be considered in patients who have a habit of eating raw fish, we investigated the prevalence of *Anisakis* infection and identified Anisakidae larvae isolated from these fishes off the East China Sea and the Pacific coast of central Japan based on PCR–RFLP analysis.

2. Materials and methods

2.1. Sample collection

Anisakidae larvae were collected from the small yellow croaker, the mackerel and the hairtail obtained from fish markets in coast of the following 6 localities: Putuo, Shengsi, Xiangshan and Dongtou, China and Tokyo, Gifu, Japan during April 2011 and July 2013. The geographical coordinates of the sampling localities, the sampling date, the number of fresh fishes obtained from fish markets and the number of specimens analyzed from each fish species are showed in Fig. 2. These three species have been traditionally considered as a popular dish in China and Japan. Larvae were isolated from the visceral surface, body cavity and muscles of the fresh fishes. The visceral organs were separated and muscles were sliced and then carefully observed with the naked eye. All the detected larvae were isolated, counted and then fixed in 70% ethanol after being washed with 0.9% NaCl for further molecular characterization by PCR–RFLP. The investigation was performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the Zhejiang Academy of Medical sciences.

2.2. DNA extraction and amplification

Genome DNA from individual worms was extracted by DNeasy Blood and Tissue Kit (QIAGEN, China) according to the manufacturer's instructions. DNA was resuspended with 30 µl TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0). The concentration and purity of DNA was measured using NanoDrop 2000 (Thermo Scientific, USA). The entire ITS region (ITS1, 5.8 S and ITS2) was amplified using primers NC5 (5'-

GTAGGTGAACCTGCGGAAGGATCATT-3') and NC2 (5'-TTAGTTTCTTTTCTCCGCT-3') (Zhu et al., 1998). The PCR amplification contained 25 µl 2 × Ex Taq PreMix (1.25 U/25 µl TaKaRa Ex Taq Polymerase, 2 × 0.4 mM dATP, dCTP, dGTP and dTTP each, 2 × Ex Taq buffer, 4 mM MgCl₂), 1 µl of the 20 µM primers NC5 and NC2, 5 µl of extracted DNA, and distilled water up to a final volume of 50 µl. Reactions were cycled 30 times by denaturation at 94 °C for 1 min, followed by annealing at 56 °C for 30 s and an extension step at 72 °C for 50 s. A final extension was carried out at 72 °C for 7 min. The PCR products were analyzed by electrophoresis on a 2% agarose gel stained with GelRed™ (Biotium, China).

2.3. Digestion of PCR products with restriction enzyme

Restriction enzymes HinfI, TaqI and HhaI for rapid DNA digestion were used in the RFLP analysis (D'Amelio et al., 2000; Umehara et al., 2006). PCR products were digested according to the manufacturer's recommendations (Fermentas, China). Restriction endonuclease digestion was performed using 15 µl of the PCR product, 1 µl of FastDigest enzyme, 2 µl of 10 × FastDigest buffer and 2 µl of distilled water. The digestion with HinfI and HhaI was performed at 37 °C for 15 min, while TaqI at 65 °C for 15 min. 10 µl of the digested products was analyzed on 2% agarose gel stained with GelRed™ (Biotium, China).

2.4. Sequencing and analyses of sequence data

For further confirmation of the identified results using PCR–RFLP analysis, PCR products were excised from the agarose gels and were purified using MinElute Gel Extraction Kit (QIAGEN, China) according to the manufacturer's instruction. 30 ng per microliter of the purified PCR products were directly sequenced. The sequence of ITS region was aligned with the published sequences in GenBank database (NCBI) using Clustal method. The phylogenetic tree was constructed by MEGA (Bak et al., 2014; Tamura et al., 2013) for comparing the genetic relationship among other published sequences by the neighbor-joining criteria (Hall, 2013). The goodness-of-fit of each model to the *Anisakis* and *Hysterothylacium* ITS sequence data is measured by the Bayesian information criterion BIC, Schwarz 1978 and corrected Akaike information criterion AICc, Hurvich and Tsai 1989 (Tamura et al., 2011). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. Phylogenetic tree was rooted by the included two outgroup species, *Ascaris suum* Goeze, 1782 and *Toxocara canis* Werner 1782, as it was described previously by Vardic Smrzlic et al. (2012).

2.5. Statistical analysis

Statistical analyses were carried out using SPSS® Statistics software. The nonparametric Kruskal–Wallis H test was used to perform comparison of prevalence and mean intensity among four locations and three fish species in China (differences were considered significant at $P < 0.05$). A pairwise analysis of the mean intensity between locations in Japan was carried out using Mann–Whitney U test (differences were considered significant at $P < 0.05$).

3. Results and discussion

3.1. Investigation of Anisakidae larvae infection in marine fish from China and Japan

To date, artificial enzymatic digestion procedure by CODEX (STAN 244-2004: standard for salted Atlantic herring and salted sprat) is the recommended protocol for anisakids inspection. While visual examination is still widely adopted in the world for screening up to now (Bak et al., 2014; Jabbar et al., 2013; Pekmezci et al., 2014; Piras et al., 2014; Serracca et al., 2013a). In current study, visual inspection was adopted

for the detection of larvae in the flesh of the fishes from the East China Sea and the Pacific coast of central Japan.

High prevalence (116/122, 95.1%) of Anisakidae larvae infection was detected in marine fish off the East China Sea. No significant positive correlation was found between prevalence and locations (chi-square = 5.167, $P = 0.160$) and three fish species (chi-square = 0.866, $P = 0.649$) in China. Larvae were found coiled and encysted mostly on the external wall of intestine and the mesentery, with few specimens isolated from muscle fillets of the 80 hairtail fish, 20 small yellow croaker and 27 mackerel fish. In total, 2004 larvae were collected from 127 fish samples caught off the coast of 6 localities in China and Japan (Table 1). 1920 larvae collected in China were found mostly in body cavity surrounding the visceral organs (43.33%) and intestine, mesentery and gonad organs (55.61%). Only a few of Anisakidae larvae were found in the muscles (0.68%) and other organs (0.38%). While 84 larvae collected in Japan were found mostly in the muscles surrounding the visceral organs (96.43%) (Table 2). This investigation highlighted differences between the two sibling species indicating a greater tendency for *A. simplex* s.s. to migrate towards the muscle of fish than *A. pegreffii*. These differences to penetrate into the tissue of accidental hosts such as man and animals were also found in other studies (Arizono et al., 2012; Del Carmen Romero et al., 2013). The mean infection intensity of Anisakidae larvae from marine fishes in China and Japan was 17.60 ± 4.32 and 16.8 ± 4.64 respectively (Table 2), which was in accordance with those off the Yellow Sea in China (7.3) (Du et al., 2010) and those from Nagasaki in Japan (4.2) (Quiazon et al., 2011). The highest abundance in hairtail fish was 319 (Table 2). The mean infection intensity of Anisakidae larvae from marine fishes in China varied significantly among locations, showing low values in Shengsi and Dongtou and high values in Putuo and Xiangshan (chi-square = 30.508, $P < 0.001$). In Japan, there is no significant positive correlation between mean intensity and locations ($Z = 1.732$, $P = 0.083$).

3.2. Molecular identification and phylogenetic analyses of Anisakidae larvae

663 samples were randomly selected and identified by PCR–RFLP analysis (Table 1). Amplification of the entire ITS region produced a single band of about 1000 bp for all specimens. Eight different RFLP profile combinations were detected for all restriction endonucleases (Fig. 1), which were characteristic of *A. simplex* s.s., *A. pegreffii*, recombinant genotype of *A. simplex* s.s. and *A. pegreffii*, *A. typica*, *Hysterothylacium aduncum* Rudolphi, 1802, *Hysterothylacium fabri* Rudolphi, 1802, *Hysterothylacium muraenesoxi* Luo, 1999, *Hysterothylacium* spp. and *Hysterothylacium amoyense* Hsü, 1933, respectively. In RFLP, digestion

Table 2

Distribution and the mean intensity of nematodes in each infected fish.

Country	Organ	Distrubution (%)	MI ^a (per individual fish)	
			Mean $\pm$ SD	Range
China	Body cavity	43.33	7.63 ± 1.57	1–109
	Muscle	0.68	1.29 ± 0.29	1–3
	Intestine, mesentery and gonad	55.61	9.92 ± 2.79	1–206
	Others	0.38	1.0	
	Total	100	17.60 ± 4.32	1–319
Japan	Body cavity	2.38	2.0	
	Muscle	96.43	16.2 ± 4.19	8–29
	Intestine, mesentery and gonad	1.19	1.0	
	Total	100	16.8 ± 4.64	8–32

^a MI, mean intensity (mean $\pm$ standard deviation).

of the PCR products with HinfI produced different RFLP patterns (Fig. 1A, D, Pattern 1–Pattern 4), corresponding to *A. simplex* s.s. and *A. berlandi* (615 and 232 bp), or *A. pegreffii* (331, 284 and 232 bp), or recombinant genotype of *A. pegreffii* and *A. simplex* s.s. (615, 331, 284 and 232 bp), or *A. typica* (594 and 326 bp), respectively. For specimens showing pattern 1, we then digested the PCR products with HhaI and confirmed bands of 530 bp and 421 bp for the *A. simplex* s.s. other than *A. berlandi* (550, 300 and 130 bp) (D'Amelio et al., 2000; Murata et al., 2011). The PCR fragment of pattern 5 was not cut by HinfI, which was identified before as *Anisakis paggiae* Mattiucci, Nascetti, Dailey, Webb, Barros, Cianchi & Bullini, 2005 and *Anisakis brevispiculata* Dollfus, 1966. In present study, specimens with pattern 5 produced DNA fragment lengths after HhaI digestion (310, 270, 180 and 150 bp) (Fig. 1B, D) different from *A. paggiae* (520 and 410 bp) and *A. brevispiculata* (390, 330, and 190 bp) reported before (Murata et al., 2011). The electrophoresis of restriction fragments of *Hysterothylacium* with HinfI revealed a pattern of distinct bands between 400 and 700 bp (Fig. 1A, Patterns 6–8). Fragments of specimens with those patterns were subjected to digestion with HhaI and TaqI (Fig. 1B, C, Patterns 6–8). Results were produced which have not been observed previously, and their identities remain unknown by RFLP analysis. To further identify the taxonomy of the larvae, the nucleotide sequences of the ITS were determined. Specimens with patterns 5–8 were identified as *Hysterothylacium* spp., *H. aduncum*, *H. fabri* and *H. amoyense* by searching the nucleotide BLAST databases available from NCBI.

Distributions of *Anisakis* species were found to be locality-specific. The sequence data obtained combined with RFLP analyses of the 619 larvae (Table 1) confirms the widespread occurrence of *A. pegreffii* in

Table 1

Identification of nematodes from different fish species from the East China Sea and the Pacific coast of central Japan.

Locality	Date of collection	Fish species	Infection levels		Nematodes								
			Prevalence	MI ^a	Nematodes codes ^b	Identified/collected	Number and relative proportion of each species ^c						
							As	Ap	Hy	At	Hm	Ha	Hs
Putuo	Dec. 2011	<i>Trichiurus haumela</i>	17/18	20.9 ± 21.8	CA1-110	110/356	1	77	0	2	18	0	0
	Oct. 2012	<i>Trichiurus haumela</i>	6/6	6.8 ± 2.7	CB1-33	33/41	0	27	0	0	1	0	5
	Oct. 2012	<i>Pneumatophorus japonicus</i>	12/12	12.8 ± 10.3	CC1-145	145/154	1	143	1	0	0	0	0
	Oct. 2012	<i>Pseudosciaena polyactis</i>	20/20	20.1 ± 23.9	CD1-149	149/402	0	121	0	0	0	9	18
Xiangshan	Apr. 2012	<i>Trichiurus haumela</i>	24/25	30.1 ± 62.4	CE1-75	75/723	1	65	0	2	7	0	0
	May 2012	<i>Trichiurus haumela</i>	7/10	2.4 ± 1.6	CF1-17	17/17	1	9	0	0	3	1	0
	May 2012	<i>Pneumatophorus japonicus</i>	9/10	4.9 ± 3.3	CG1-43	43/44	0	42	0	0	1	0	0
	Nov. 2012	<i>Trichiurus haumela</i>	21/21	8.7 ± 6.9	CH1-47	47/183	0	41	0	5	1	0	0
Total in China			116/122 (95.1%)			619/1920	0.6%	84.8%	0.2%	1.5%	5.0%	1.6%	2.9%
Tokyo	Apr. 2011	<i>Pneumatophorus japonicus</i>	3/3	17.3 ± 6.3	JA1-15	15/52	15	0	0	0	0	0	0
	Jul. 2013	<i>Pneumatophorus japonicus</i>	2/2	16.0 ± 4.2	JB1-29	29/32	29	0	0	0	0	0	0
			5/5 (100%)			44/84	100%	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%

^a MI, mean intensity (mean $\pm$ standard deviation);

^b Numbers after the capital letters represent the number of identified specimens for each species, respectively.

^c As, *Anisakis simplex* s.s.; Ap, *Anisakis pegreffii*; Hy, Recombinant genotype of *Anisakis simplex* s.s. and *Anisakis pegreffii*; At, *Anisakis typica*; Hm, *Hysterothylacium amoyense*; Ha, *Hysterothylacium aduncum*; Hs, *Hysterothylacium* sp.; and Hf, *Hysterothylacium fabri*.

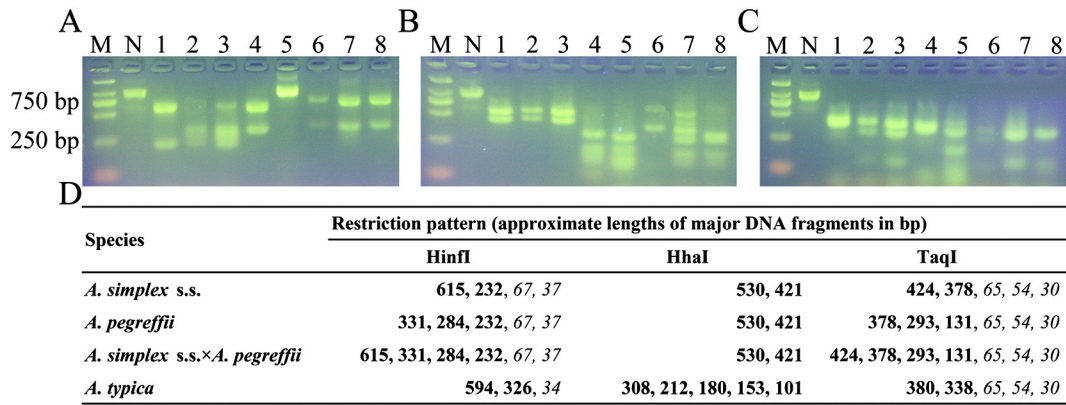


Fig. 1. PCR–RFLP analysis with Hinfi (A), HhaI (B), TaqI (C) of the ITS PCR products and fragment sizes (D) obtained for some of anisakids identified in this study. Fragments in bold could be visible in the gel, while fragments in italics might not be visible in the gel. M, the 2000 bp DNA ladder marker; N, ITS PCR products; Pattern 1, *A. simplex* s.s.; Pattern 2, *A. pegreffii*; Pattern 3, Recombinant genotype of *A. simplex* s.s. and *A. pegreffii*; Pattern 4, *A. typica*; Pattern 5, *Hysterothylacium* spp.; Pattern 6, *H. aduncum*; Pattern 7: *H. fabri*; and Pattern 8: *H. amoyense*.

different fish species (84.8%) from the East China Sea. This finding is in accordance with previous results from the Yellow Sea, where *A. pegreffii* is the dominant species (Du et al., 2010). The infection status of Anisakidae larvae in marine fish and cephalopods from the Bohai Sea showed that 81.8% of the larvae were *A. simplex* s.s. (Ma et al., 1997). In the South China Sea, *A. typica* was the predominant species accounting for 82% of all larvae examined (Zhang et al., 2013), and in Taiwan most larvae isolated from hairtail were *A. typica* (84%). The proportion of *A. typica* in all species examined from hairtail at Putuo (2/110, 1.8%), Xiangshan (2/75, 2.7%) and Dongtou (5/47, 10.6%) in this study rises from north to south off the East China Sea (Table 1), which relates to the finding that *A. typica* is mainly distributed in tropical and subtropical areas (Mattiucci and Nascetti, 2008). All of the nematodes larvae among 44 specimens collected from the Pacific coast of central Japan in present study were *A. simplex* s.s. (Table 1, Fig. 2), whereas *A. pegreffii* is the dominant species from the Sea of Japan to East China Sea side

according to the observations reported before (Quiazon et al., 2011; Umehara et al., 2006; Umehara et al., 2010).

Recombinant genotype *A. simplex* s.s. × *A. pegreffii* was first detected in the East China Sea. The presence of heterozygotes C/T at positions 278 and 294 (C in *A. pegreffii* and T in *A. simplex* s.s.) of the aligned sequence in recombinant genotypes was also confirmed by sequence analysis (Fig. 3). The two diagnostic nucleotide sites between the two sibling species were firstly found at heterozygote status in recombinant individuals by Abollo et al. (2003). The ITS sequence of recombinant genotypes *A. simplex* s.s. × *A. pegreffii* in China reported by Du, C and Shih was not available from the GenBank/EMBL/DBJ (Du et al., 2010; Shih et al., 2010). In addition, *A. simplex* s.s. (0.6%) was also identified in the present study, which suggested that the recombinant genotype could be the product of natural interspecific recombination.

Hysterothylacium was 12.8% (79/619) of the identified nematodes from the East China Sea, including *H. aduncum* (10/619, 1.6%),

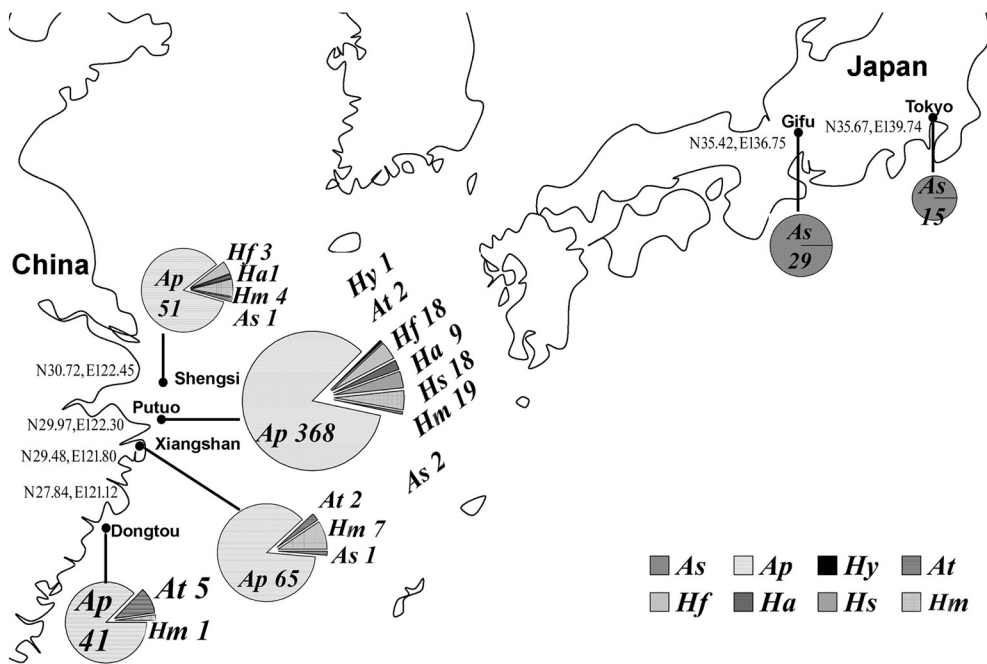


Fig. 2. Distribution of anisakid isolated from different fish species from the East China Sea and the Pacific coast of central Japan. The numbers before the localities represent the geographical coordinates, N means northern latitude and E means east longitude; the numbers after the abbreviations of the species represent the number of identified specimens for each species, respectively; *A. simplex* s.s. (As), *A. pegreffii* (Ap), the recombinant genotype of *A. simplex* s.s. and *A. pegreffii* (Hy), *A. typica* (At), *H. aduncum* (Ha), *Hysterothylacium* spp. (Hs), *H. fabri* (Hf) and *H. amoyense* (Hm).

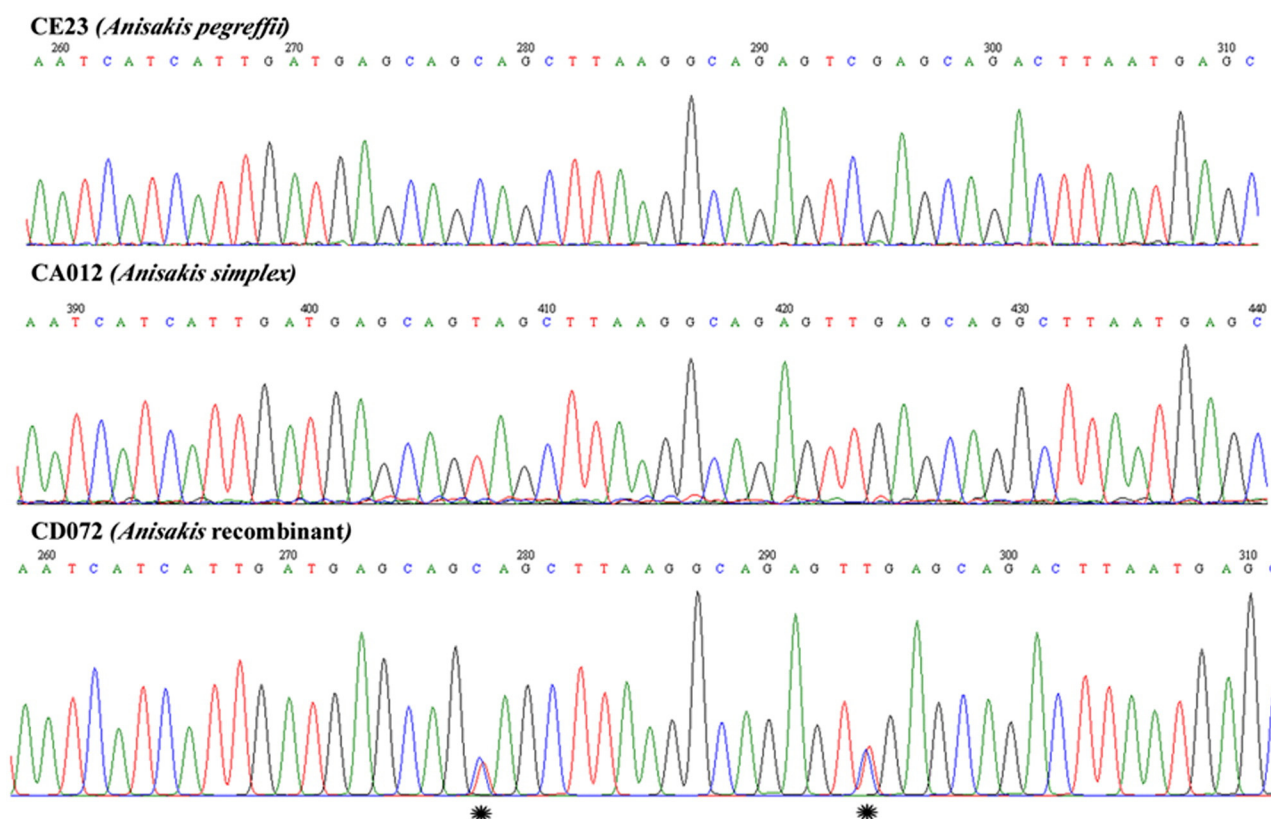


Fig. 3. Electropherograms of the part ITS region sequences of homozygote and heterozygote *Anisakis* individual. The numbers refer to nucleotide positions, the “*” indicates heterozygotes C/T at position 278 and 294 of the aligned sequence of the isolate strain CE23 (*A. pegreffii*), CA012 (*A. simplex* s.s.) and CD072 (the recombinant genotype of the sibling species) in present study.

Hysterothylacium spp. (18/619, 2.9%), *H. fabri* (21/619, 3.4%) and *H. amoyense* (31/619, 5.0%) in this study. *H. fabri*, which presents in many Mediterranean fish species (Martin-Sanchez et al., 2003), was first detected in the East China Sea. This species is a complex comprising at least three sibling species with little host specificity (Martin-Sanchez et al., 2003). In this study, *H. fabri* was collected and identified from the hairtail and the small yellow croaker. *H. amoyense* (*Contracaecum muranesoxi* has been synonymized as *H. amoyense*) (Knoff et al., 2012; Li et al., 2008), which was found only in the China–Taiwan Strait to date (Fang et al., 2010), was also identified in the East China Sea with 5% relative abundances. In Chinese waters, *Hysterothylacium* genus larvae have been found from the marine fish in the Bohai and Yellow Sea, the East China Sea and the South China Sea (Guo et al., 2014; Li et al., 2012; Li et al., 2013; Shih, 2004; Xu et al., 2014; Zhu et al., 2007). Larvae of *Hysterothylacium* are common parasites of marine, estuarine and freshwater fishes worldwide. Moreover, species of this genus have been reported to cause human anisakiasis (Yagi et al., 1996).

NJ tree was constructed using ITS sequence data of identified species and a number of reference species. Based on the Bayesian information criterion BIC, Schwarz 1978 and corrected Akaike information criterion AICc, Hurvich and Tsai 1989 values for each model (Table S1) (Tamura et al., 2011), we chose the best evolutive model K2 + I for the phylogenetic analysis in MEGA6 (Kimura, 1980; Tamura et al., 2013). The resulting tree revealed that Anisakidae larvae were divided into two distinct clades, *Anisakis* and *Hysterothylacium* genera (Fig. 4). *Anisakis* included two different types in the present study, clade I and clade II, which was previously identified by other authors (Hermida et al., 2012; Mattiucci and Nascetti, 2008). In our study, the diversity of Anisakidae larvae isolated was remarkable (seven species). *A. simplex*

s.s., *A. pegreffii* and *A. typica* belong to clade I of *Anisakis*. *H. aduncum*, *Hysterothylacium* spp., *H. fabri* and *H. amoyense*, which are clustered into one group, belong to *Hysterothylacium* genus.

The presence of Anisakidae larvae in examined fish from the East China Sea could have significant implications for public health in China. *A. simplex* s.s. and *A. pegreffii* have been identified as two major agents of human anisakiasis (Mattiucci et al., 2011; Umehara et al., 2007). In Japan, *A. simplex* s.s. is the most important etiological agent as reported that 84 out of the 85 patients (98.8%) were infected with *A. simplex* s.s. while one patient (1.2%) infected with *A. pegreffii* (Umehara et al., 2007). Recently, 57 larvae collected from 44 patients with anisakiasis from Middle Eastern Japan were analyzed retrospectively and nearly all larvae were *A. simplex* s.s. (99%) while one larva displayed a hybrid genotype (Arai et al., 2014). *A. simplex* s.s. larvae have the potential to survive acidic gastric juice to some extent and penetrate the stomach, small intestine, or colon in infected humans (Arizono et al., 2012). In the East China Sea, *A. pegreffii* was the predominant species while others occurred with low prevalence of infection. The finding is in accordance with previous results for the Yellow Sea and distinct from the results for the Bohai Sea, where *A. simplex* s.s. had a high prevalence in different marine fish (Du et al., 2010; Ma et al., 1997). However, there is only one case reported in China so far (Qin et al., 2013), which suggests the possibility of missed diagnosis in China. *Anisakis* spp. infection should be carefully monitored when consuming raw, lightly cooked, or marinated seafood, because there is a high risk of anisakiasis in humans in China, particularly for the people live near the Bohai Sea. Epidemiological studies of anisakiasis should be conducted to attract enough attention for preventing anisakiasis and *Anisakis*-induced allergy in China. Health care workers should be especially alert for signs of anisakiasis for suspected patients.

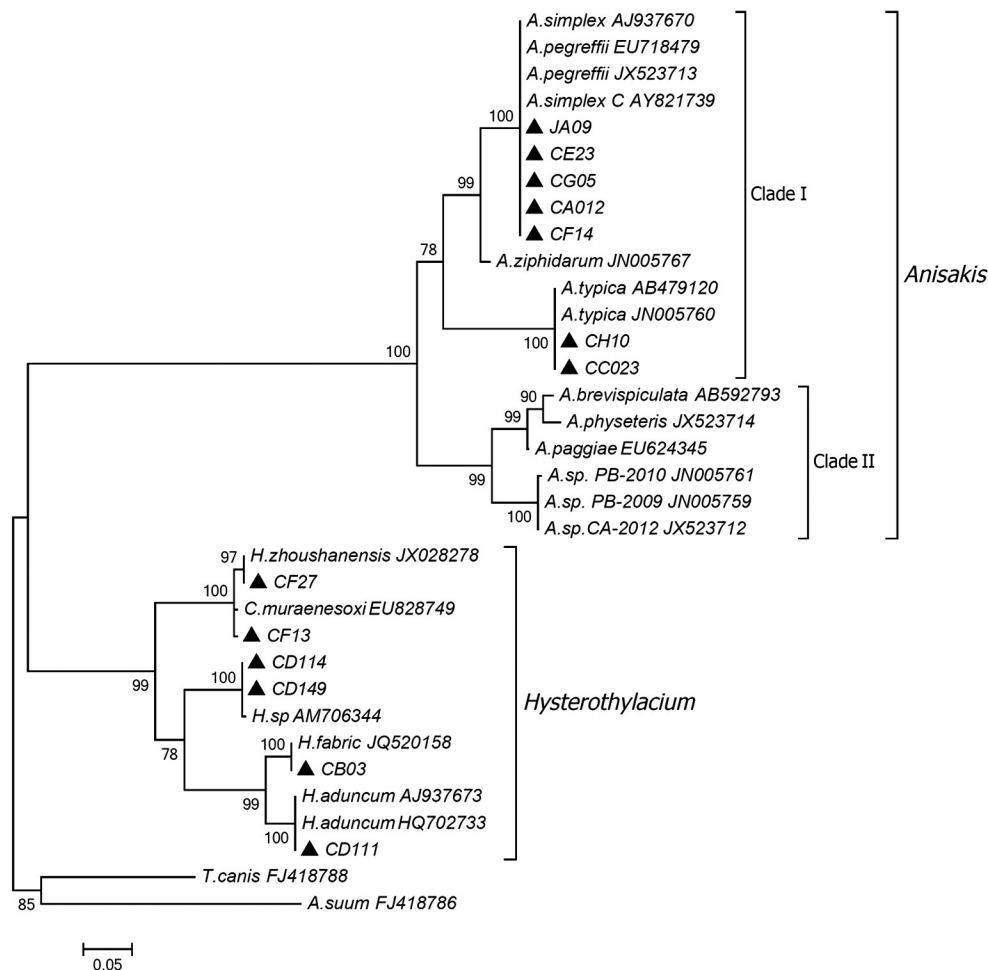


Fig. 4. Molecular phylogenetic tree showing the genetic relationship among nematode species based on rDNA ITS sequences. ▲ represents nematode species isolated in present study. The tree was constructed using the neighbor-joining criteria by MEGA5.0. *A.suum* and *T. canis* as outgroup. Values beside nodes correspond to bootstrap percentages above 50% are shown.

4. Conclusion

This study investigated Anisakidae larvae infection in marine fishes of the small yellow croaker, the mackerel and the hairtail fishes off the East China Sea. A striking finding was the high prevalence, intensity of infection and a wide diversity of anisakid nematodes in this study. In conclusion, seven species belonging to two genus were detected using PCR-RFLP and sequence analyses of the ITS marker. And the study suggests that the genus *Anisakis* has substantial geographical distributions. In the East China Sea, *A. pegreffii* was the predominant species. This is a comprehensive epidemiological dataset for the family Anisakidae in the East China Sea and the first report of *A. typica*, recombinant genotype of *A. simplex* s.s. and *A. pegreffii*, *H. fabri* and *H. amoyense* in this area. These findings provide important basic information on Anisakidae nematodes in this region and have significant implications for the ecology, epidemiology and population genetics of Anisakidae nematodes.

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