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Survey for the presence of ascaridoid larvae in the cinnamon flounder *Pseudorhombus cinnamoneus* (Temminck & Schlegel) (Pleuronectiformes: Paralichthyidae)

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ABSTRACT

The cinnamon flounder *Pseudorhombus cinnamoneus* is a frequently consumed marine fish in China. However, the occurrence of ascaridoid larvae in *P. cinnamoneus* remains unclear. In the present study, a total of 85 *P. cinnamoneus* caught from the Yellow Sea (off Shidao, 36°52′57″N, 122°26′42″E) in 2011, which is located between mainland China and the Korean Peninsula, was investigated for ascaridoid larval infection. Four ascaridoid larval types, including *Anisakis* type I of Berland (1961), *Hysterothylacium* type of Smith (1983), *Hysterothylacium* type HL of Guo et al. (2014) and *Raphidascaris* type of Zhao et al. (2016), were detected in this important food fish. These larval types were identified as *Anisakis pegreffii*, *Hysterothylacium aduncum*, *H. sinense* and *Raphidascaris lophii*, respectively, using PCR-based restriction fragment length polymorphism (PCR–RFLP) analysis and sequencing of the ITS region of nuclear ribosomal DNA (rDNA). The third-stage larvae of *H. sinense* are reported from Chinese waters for the first time. The prevalence of *H. sinense* was 100% and represents the predominant species of the ascaridoid larvae found in *P. cinnamoneus*. The prevalences of *A. pegreffii* and *H. aduncum* were 44.7% and 81.2%, respectively. Phylogenetic analyses based on ITS sequences were performed to elucidate the genetic relationships of these ascaridoid nematodes. The present study increases the knowledge and distribution of ascaridoid larvae in this area of Yellow Sea. The high prevalence of ascaridoid larvae in *P. cinnamoneus* shows that an assessment needs to be undertaken to assess the risk these parasites may pose to public health.

1. Introduction

Ascaridoid nematodes are important emerging zoonotic parasites, which are of interest to the broader community, including those involved in the food industry or public health. Some groups of Ascaridoidea, i.e. *Anisakis*, *Pseudoterranova*, *Contracaecum* and *Hysterothylacium*, are recognized as the parasites frequently associated with human anisakidosis, which is an important fish-borne zoonosis acquired by humans when consuming raw or undercooked fish infected with third-stage larvae (Hochberg and Hamer, 2010; Baird et al., 2014). In the coastal areas of China, consumption of raw or undercooked fish is a common practice, and human anisakidosis is therefore an important public health concern. To date, dozens of marine fish species in the Chinese waters have been investigated for ascaridoid nematodes infection in the last two decades by Sun et al. (1991), Luo (2001), Shih (2004), Shih et al. (2010), Du et al. (2010), Zhang et al. (2007, 2013), Li et al. (2007a,b, 2012, 2013, 2016a), Liu et al. (2013), Guo et al. (2014), Kong et al. (2015), Chen and Shih (2015) and Zhao et al. (2016). The results of these studies have improved our knowledge of the species composition, prevalence and distribution of ascaridoid nematodes in Chinese waters. It is imperative to determine what ascaridoid nematode species most frequently infects commercially important marine fishes and to what extent these marine fishes contribute to the epidemiology of anisakidosis in China, which has very important implications for forecasting possible future infections, and preventing or reducing the risk of human anisakidosis (Setyobudi et al., 2013; Pekmezci et al., 2014).

The cinnamon flounder *Pseudorhombus cinnamoneus* (Temminck & Schlegel) (Pleuronectiformes: Paralichthyidae) is a frequently consumed marine fish in China, mainly distributed in the Western Pacific, including the Chinese, Japanese and Philippine waters (Froese and Pauly, 2016). However, to date, no study has investigated the occurrence of ascaridoid larvae in this food fish. Thus the present study was aimed at the molecular identification of ascaridoid species present in *P. cinnamoneus* collected from Yellow Sea, China. The results will be useful to estimate the risk of human anisakidosis in future due to the consumption of this widely consumed fish species.

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Table 1
Infection information and samples selected for molecular analysis of ascaridoid third-stage larvae isolated from *Pseudorhombus cinnamoneus* (Temminck & Schlegel) (Pleuronectiformes: Paralichthyidae) in the Yellow Sea, China.

Larval types	Species	Site of infection	Prevalence (%) and intensity (mean)	Voucher specimens	No. of individuals for PCR-RFLP	No. of individuals for sequencing
Anisakis type I of Berland (1961)	*A. pegreffii*	Body cavity	44.7, 1–37 (4.1)	157	157	25
Hysterothylacium type of Smith (1983)	*H. aduncum*	Stomach	29.4, 1–4 (1.4)	36	36	6
Hysterothylacium type of Smith (1983)	*H. aduncum*	Body cavity	67.1, 1–9 (2.6)	151	151	20
Hysterothylacium type HL of Guo et al. (2014)	*H. sinense*	Stomach	87.1, 1–32 (5.8)	430	82	10
Hysterothylacium type HL of Guo et al. (2014)	*H. sinense*	Body cavity	95.3, 1–60 (13.0)	1053	218	17
Raphidascaris type of Zhao et al. (2016)	*R. lophii*	Stomach	1.2, 1 (1)	1	0	1

2. Materials and methods

2.1. Parasite collection

A total of 85 *P. cinnamoneus* (body length 15.2–22.6 cm, body weight 0.16–0.24 kg), caught by the commercial trawlers from the Yellow Sea [off Shidao (36°52′57″N, 122°26′42″E), Shandong Province, China], were dissected for infection with ascaridoid nematodes during 22–27 April 2011. The larvae isolated from the body cavity and visceral organs of the fish, were washed in physiological saline, and then fixed and stored in 80% ethanol. They were observed using light microscopy and identified to generic level or differentiated as different morphotypes based on the morphological characteristics, according to Berland (1961), Smith (1983), Shih (2004), Shamsi et al. (2011, 2013, 2015, 2016), Guo et al. (2014), Chen and Shih (2015). The morphometric data of the ascaridoid larvae were recorded and drawings/images were taken of the position of excretory pore and the morphology of digestive tract, head and tail. The prevalence (the percentage of fish infected with parasites) and intensity of infection (the numbers of parasites in infected hosts) were reported following the definitions of Bush et al. (1997).

2.2. DNA extraction and amplification

Samples selected for molecular analysis are presented in Table 1. DNA samples were extracted from individual worms using a Column Genomic DNA Isolation Kit (Shanghai Sangon, China) according to the manufacturer's instructions. DNA was eluted in buffer and kept at −20 °C until use. The ITS region was amplified by PCR using the primers A (forward: 5′-GTC GAA TTC GTA GGT GAA CCT GCG GAA GGA TCA-3′) and B (reverse: 5′-GCC GGA TCC GAA TCC TGG TTA GTT TCT TTT CCT-3′), which were designed by D'Amelio et al. (2000), and cycling conditions described previously (Li et al., 2012). PCR products were checked on GoldView-stained 1.5% agarose gels and purified with Column PCR Product Purification Kit (Shanghai Sangon, China).

2.3. RFLP analyses

Restriction enzymes *Hinf*I, *Taq*I and *Hha*I (Thermo Scientific, Waltham, MA, USA) were used in the RFLP analysis for differentiating the species of ascaridoid larvae, according to D'Amelio et al. (2000) and Cavallero et al. (2015). The PCR products were digested according to the manufacturer's recommendations. The digested samples were subjected to electrophoresis on 2% agarose gels and then photographed.

2.4. Sequencing of the ITS region

The PCR products were excised from the agarose gels and sequenced using a DyeDeoxy Terminator Cycle Sequencing Kit (v.2, Applied Biosystems, California, USA) and an automated sequencer (ABI-PRISM

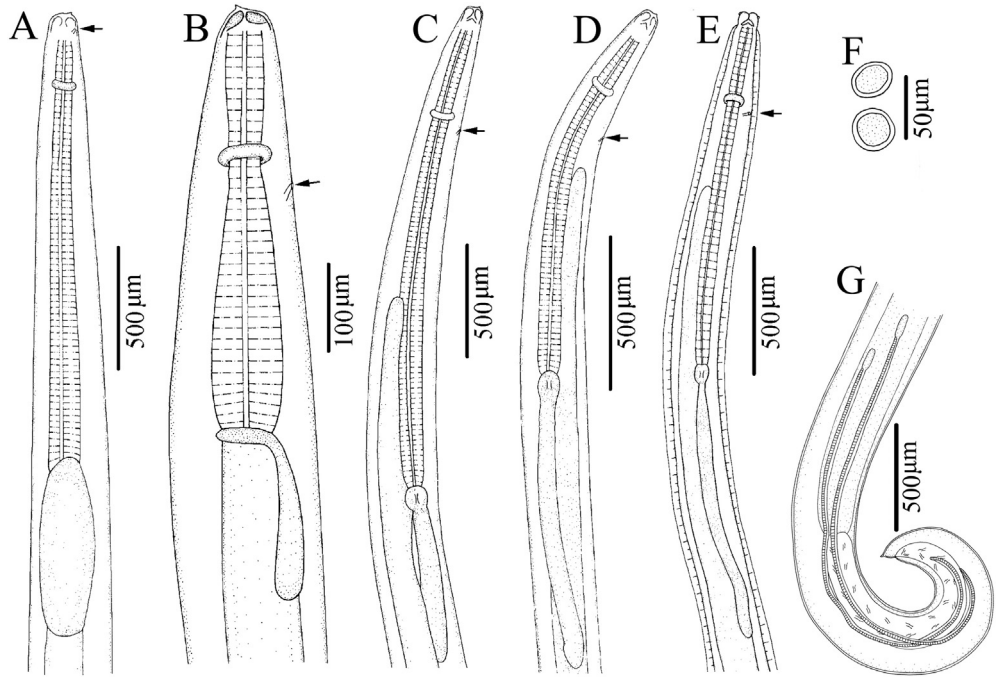

Fig. 1. Line drawings of the anterior ends of ascaridoid third-stage larvae (excretory pore arrowed) isolated from *P. cinnamoneus* collected from the Yellow Sea, China and paratype of *Hysterothylacium sinense*. A, *Anisakis* type I of Berland (1961); B, *Raphidascaris* type of Zhao et al. (2016); C, *Hysterothylacium* type of Smith (1983); D, *Hysterothylacium* type HL of Guo et al. (2014); E, *Hysterothylacium sinense*; F, eggs of *H. sinense*; G, posterior end of *H. sinense*.

377). Sequencing for each sample was carried out for both strands. Sequences were aligned using ClustalW2 (Thompson et al., 1994) and adjusted manually. The newly-generated sequences were compared (using the algorithm BLASTn) with those available in the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm. nih.gov).

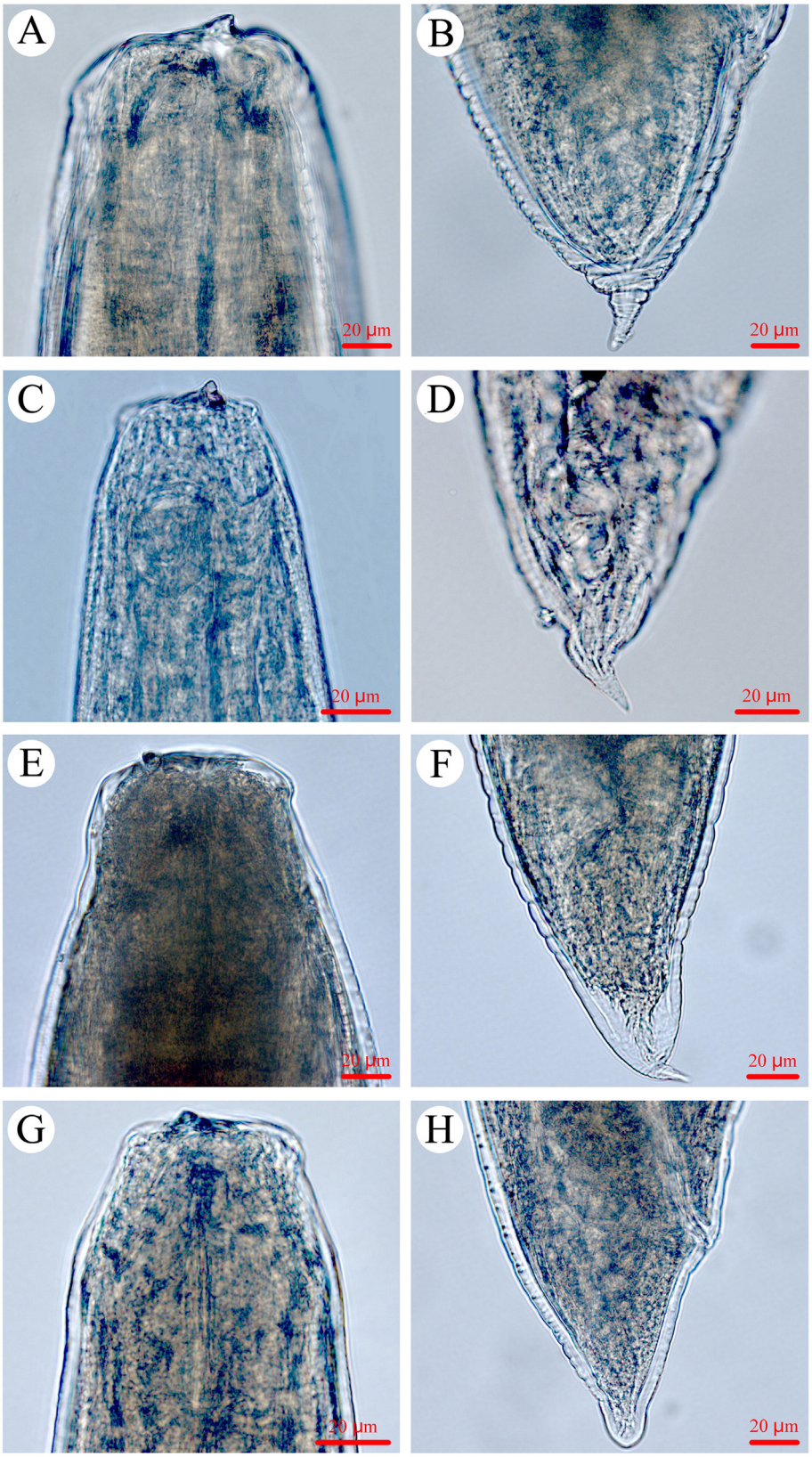

Fig. 2. Photomicrographs of the cephalic ends and tails of ascaridoid third-stage larvae isolated from *P. cinnamoneus* collected from the Yellow Sea, China. A, B, *Anisakis* type I of Berland (1961); C, D, *Raphidascaris* type of Zhao et al. (2016); E, F, *Hysterothylacium* type of Smith (1983); G, H, *Hysterothylacium* type HL of Guo et al. (2014).

Table 2
Morphometrics of ascaridoid larval types isolated from *Pseudorhombus cinnamoneus* and paratypes of *Hysterothylacium sinense* (measurements in mm). *Abbreviations*: BL–length of body, OL–length of oesophagus, VL–length of ventriculus, VW–width of ventriculus, ICL–length of intestinal caecum, VAL–length of ventricular appendix, EC–distance of excretory pore to cephalic end, SL–length of spicules, ES–size of eggs, TL–length of tail.

Characteristics	*Anisakis* type I of Berland (1961)	*Hysterothylacium* type of Smith (1983)	*Hysterothylacium* type of Guo et al. (2014)	*Raphidascaris* type of Zhao et al. (2016)	*Hysterothylacium sinense*
BL	14.3–24.2	11.1–26.0	11.2–19.2	4.60	23.1–37.1
OL	1.54–1.99	1.15–2.44	1.04–1.73	0.48	2.04–3.45
VL	0.45–0.77	0.059–0.14	0.059–0.12	0.02	0.097–0.15
VW	0.15–0.27	0.059–0.12	0.059–0.12	0.069	0.087–0.15
ICL	–	0.47–1.09	0.54–1.20	–	1.26–2.33
VAL	–	0.53–1.28	0.47–1.54	0.21	1.07–2.43
EC	At base of lip	0.34–0.71	0.22–0.57	0.22	0.49–0.74
SL	–	–	–	–	1.65–2.32
EZ	–	–	–	–	0.036–0.042 × 0.036–0.042
TL	0.089–0.14	0.15–0.25	0.089–0.21	0.089	0.078–0.12
OL/BL (%)	7.8–10.8	8.3–10.9	7.8–12.4	10.3	7.2–10.3
ICL/VAL	–	1:0.79–1.45	1:0.68–1.37	–	1:0.80–1.08
ICL/OL (%)	–	37.2–52.4	52.4–75.9	–	61.0–71.0

2.5. Phylogenetic analyses

The phylogenetic tree was constructed using MEGA 6 (http://www.megasoftware.net/) for elucidating the genetic relationships of the anisakid larvae obtained herein. The nucleotide sequences were aligned using ClustalW2 (Thompson et al., 1994), edited manually and tested with MEGA 6 model test to find the best DNA model to construct the phylogenetic trees. Phylogenetic analyses with other known ascaridoid nematodes were conducted using both Neighbor Joining (NJ) and Maximum-likelihood (ML) analyses for both loci. Evolutionary relationships were calculated using the Kimura two-parameter model. *Ascaris lumbricoides* was chosen as the outgroup. Reliabilities for both NJ and ML trees were tested using 1000 bootstrap replications (Felsenstein, 1985) and bootstrap values exceeding 70 were considered well supported (Hillis and Bull, 1993).

3. Results

3.1. Detection of ascaridoid larvae in P. cinnamoneus

A total of 1828 ascaridoid larvae were isolated from *P. cinnamoneus*. *Anisakis* third-stage larvae were identified morphologically as *Anisakis* larval type I of Berland (1961) (Figs. 1A, 2A, B). The prevalence and mean intensity of *Anisakis* type I larvae were 44.7% and 4.1, respectively. Only one *Raphidascaris* third-stage larva belonging to the *Raphidascaris* larval type of Zhao et al. (2016) was isolated (Figs. 1B, 2C, D). The other third-stage larvae all belonged to the genus *Hysterothylacium*, represented by two different larval types, the *Hysterothylacium* larval type of Smith (1983) (81.2% of fish infected with a mean intensity of 2.7 nematodes per fish) (Figs. 1C, 2E, F) and the *Hysterothylacium* larval type HL of Guo et al. (2014)[1] (100% of fish infected with a mean intensity of 17.4 nematodes per fish) (Figs. 1D, 2G, H). The morphometric data of the four ascaridoid larval types and the paratypes of *H. sinense* are provided in Table 2.

3.2. PCR-RFLP analyses

644 samples were selected and identified by PCR-RFLP analyses (Table 1). Amplification of the ITS region produced a single band of about 1000 bp for all specimens. In RFLP, digestion of the PCR products with *Hinf*I produced three different RFLP profiles, corresponding with *Anisakis* larval type I of Berland (1961) (ca. 350, 300 and 260 bp), the *Hysterothylacium* larval type HL of Guo et al. (2014) (ca. 1100 bp) and the *Hysterothylacium* larval type of Smith (1983) (ca. 710 and 380 bp). Using *Hha*I produced three different RFLP profiles, corresponding with the *Anisakis* larval type I of Berland (1961) (ca. 530 and 430 bp), the *Hysterothylacium* larval type HL of Guo et al. (2014) (ca. 300, 220, 170, 150 and 140 bp) and the *Hysterothylacium* larval type of Smith (1983) (ca. 350, 280, 190 and 150 bp). Using *Rsa*I produced three different RFLP profiles, corresponding with the *Anisakis* larval type I of Berland (1961) (ca. 550 and 310 bp), the *Hysterothylacium* larval type HL of Guo et al. (2014) (ca. 1100 bp) and the *Hysterothylacium* larval type of Smith (1983) (ca. 620 and 230 bp) (Fig. 3).

3.3. Sequence analyses

The ITS region was sequenced for 25 third-stage larvae of *Anisakis* type I of Berland (1961), 26 third-stage larvae of the *Hysterothylacium* type of Smith (1983), 27 third-stage larvae of the *Hysterothylacium* type HL of Guo et al. (2014) and 1 third-stage larva of the *Raphidascaris* type of Zhao et al. (2016) (approximately 10% of the samples of each larval type, except for the third-stage larva of *Raphidascaris*) (Table 1). However, no intraspecific nucleotide differences were detected in the ITS sequences between these individuals of each species. Three ITS sequences of the paratypes of *H. sinense* obtained herein, also displayed no intraspecific nucleotide variability. There are over 150 ITS sequences of *A. pegreffii* registered in GenBank, and pairwise comparison between our present third-stage larvae of *Anisakis* type I of Berland (1961) and the ITS sequences of *A. pegreffii* registered in GenBank displayed no nucleotide differences (AY821738, AY821740, AY821745, EU624343, KJ011486, JQ900763, JQ934869, JQ934867, JQ934871, KP301519, KF032066) (Nadler et al., 2005; Quiazon et al., 2009; Smrzlić et al., 2012; Setyobudi et al., 2013; Mattiucci et al., 2013; Sohn et al., 2014) to 0.11% nucleotide differences (KJ011495–KJ011498, JN005756) (Hermida et al., 2012; Sohn et al., 2014). There is only one ITS sequence of *H. sinense* (KX084795) registered in GenBank, and pairwise comparison between the present third-stage larvae of the *Hysterothylacium* type HL of Guo et al. (2014) and the ITS sequence of *H. sinense* (KX084795) showed 100% identity. There are over 100 ITS sequences of *H. aduncum* registered in GenBank, and pairwise comparison between our third-stage larvae of the *Hysterothylacium* type of Smith (1983) and the ITS sequences of *H. aduncum* registered in GenBank displayed between 0 (KF736937) (Guo et al., 2014) and 0.50% (KT852542) (Klapper et al., 2016) nucleotide differences. There are 27 ITS sequences of *R. lophii* registered in GenBank and pairwise comparison between our third-stage larva of *Raphidascaris* and the ITS sequences of *R. lophii* registered in GenBank displayed from 0 (KP262039, KP326520–KP326531, KP326533–KP326538, KP419720) (Xu et al., 2012; Li et al., 2016a; Zhao et al., 2016) to 0.30% (JF809816, KP326532) (Xu et al., 2012; Zhao et al., 2016) nucleotide differences. Consequently, we considered that the four species of ascaridoid larvae

[1] *Hysterothylacium* larval type HL of Guo et al. (2014) represents the larval type identified as third-stage larvae of *H. liparis* in Guo et al. (2014).

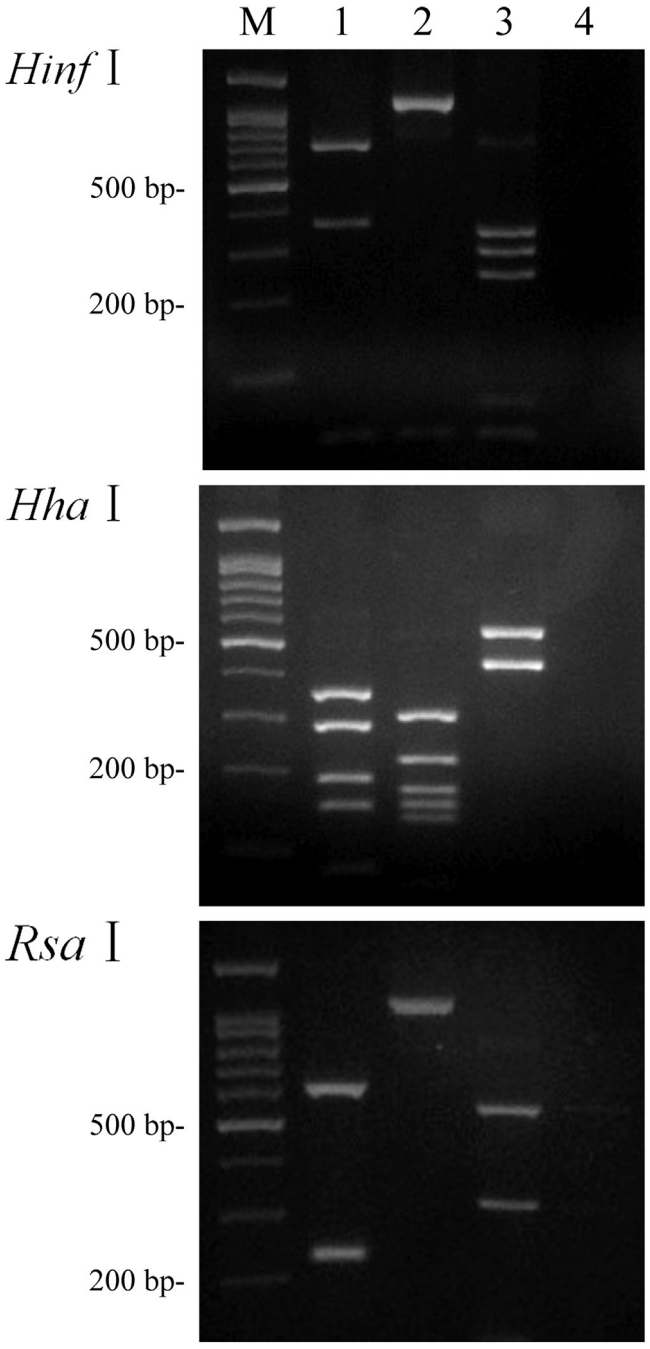

Fig. 3. PCR-RFLP analyses of ITS rDNA obtained for *Anisakis* and *Hysterothylacium* larval individuals with *Hinf*I, *Hha*I and *Rsa*I (the profiles for each restriction endonuclease represent *Hysterothylacium* type of Smith (1983) (lane 1), *Hysterothylacium* type HL of Guo et al. (2014) (lane 2), *Anisakis* type I of Berland (1961) (lane 3), negative control (lane 4), M refers to the 100 bp ladder).

in our study belong to *A. pegreffii* [=*Anisakis* type I of Berland (1961)], *H. aduncum* [=*Hysterothylacium* type of Smith (1983)], *H. sinense* [=*Hysterothylacium* type HL of Guo et al. (2014)] and *R. lophii* [*Raphidascaris* type of Zhao et al. (2016)], respectively. The ITS sequences of the different individuals of each species obtained herein only represent one genotype, thus we uploaded only one ITS sequence for each species. The ITS sequences of these four species of ascaridoid larvae obtained herein are deposited in the GenBank database (http://www.ncbi.nlm. nih.gov) under accession number *H. aduncum* (KX110074), *H. sinense* (KX110078), *A. pegreffii* (KX110076) and *R. lophii* (KX110077).

3.4. Phylogenetic analyses

Phylogenetic analyses showed that the NJ and ML trees were similar in topology, and both revealed that the representatives of ascaridoid nematodes included were divided into two distinct clades (Figs. 4, 5). Clade I included the species of *Hysterothylacium*, *Raphidascaris* and *Raphidascaroides* (representing Raphidascarididae). Clade II included the species of *Anisakis*, *Pseudoterranova* and *Contracaecum* (representing Anisakidae) (Figs. 4, 5). The identities of genotypes obtained in this study are also indicated by the phylograms: the genotype KX110076, constituted a monophyletic group representing *A. pegreffii* (KF032066); the genotype KX110077 is clustered with the previously reported sequence representing *R. lophii* (KP262039); the genotype KX110078 is grouped together with *H. sinense* (KX084795); the genotype KX110074 grouped with the published sequence representing *H. aduncum* (KF736937) (Figs. 4, 5).

4. Discussion

Ascaridoid larvae have been recorded in a wide range of marine fishes worldwide, and third-stage larvae of many ascaridoid species are known to be the etiological agents for human anisakidosis. The specific identification of ascaridoid larvae in the frequently consumed marine fishes is crucial for studying their epidemiology and forecasting possible future infections. However, it is nearly impossible for the accurate identification of ascaridoid larvae to the species level only using morphological characterization (Li et al., 2012; Chen and Shih, 2015). In recent years, many studies have proved that PCR-based restriction fragment length polymorphism (PCR–RFLP) analysis of the internal transcribed spacer (ITS) of nuclear ribosomal DNA (rDNA) provides a simple and practical approach for the specific delimitation of both distantly and closely related ascaridoid species (D'Amelio et al., 2000; Du et al., 2010; Espineira et al., 2010; Setyobudi et al., 2013; Chen and Shih, 2015; Kong et al., 2015; Cavallero et al., 2015). To date, PCR-RFLP has become one of the most common molecular methods for large-scale studies involving nematode species identification. However, it contains a number of limitations. A major disadvantage of PCR-RFLP is that there is no guarantee that all species will give unique restriction patterns. An unknown sample containing a species that has not yet been analyzed with PCR-RFLP could be falsely identified if its restriction profile matches that of a previously studied species (Sotelo et al., 2001; Rasmussen and Morrissey, 2008). Consequently, the combination of PCR-RFLP and sequencing of the ITS target region has been widely recommended for accurately differentiating and identifying ascaridoid larvae (Umehara et al., 2010; Du et al., 2010; Smrzlić et al., 2012; Setyobudi et al., 2013; Zhang et al., 2013; Pekmezci et al., 2014; Cavallero et al., 2015; Chen and Shih, 2015; Kong et al., 2015). In the present study, the ITS region of the *Anisakis* and *Hysterothylacium* larvae isolated from *P. cinnamoneus* was digested with 3 restriction enzymes *Hinf*I, *Taq*I and *Hha*I. The restriction profiles of the *Anisakis* larval type I of Berland (1961) and the *Hysterothylacium* larval type of Smith (1983) were consistent with *A. pegreffii* and *H. aduncum* in previous studies (D'Amelio et al., 2000; Du et al., 2010; Espineira et al., 2010; Setyobudi et al., 2013; Chen and Shih, 2015; Kong et al., 2015; Cavallero et al., 2015). However, there has been no report of the PCR–RFLP patterns of *H. sinense* [=the *Hysterothylacium* larval type HL of Guo et al. (2014)] obtained by digestion of ITS amplicons with the restriction enzymes *Hinf*I, *Taq*I and *Hha*I. The results of our PCR-RFLP analyses indicated that the restriction enzymes *Hinf*I, *Taq*I and *Hha*I are able to distinguish *H. sinense* from *H. aduncum* by the fragment lengths and patterns. The diagnostic profiles of *H. sinense* based on restriction enzymes (*Hinf*I, *Taq*I and *Hha*I) could be used as taxonomic criteria for the rapid and accurate identification of this species.

Most ascaridoid larvae (78.3%) isolated from *P. cinnamoneus* were *H. sinense*, the adults of which have been reported from several marine fishes in the Yellow Sea, China by Li et al. (2007a). This is the first report of the third-stage larvae of *H. sinense* from Chinese waters. There is only

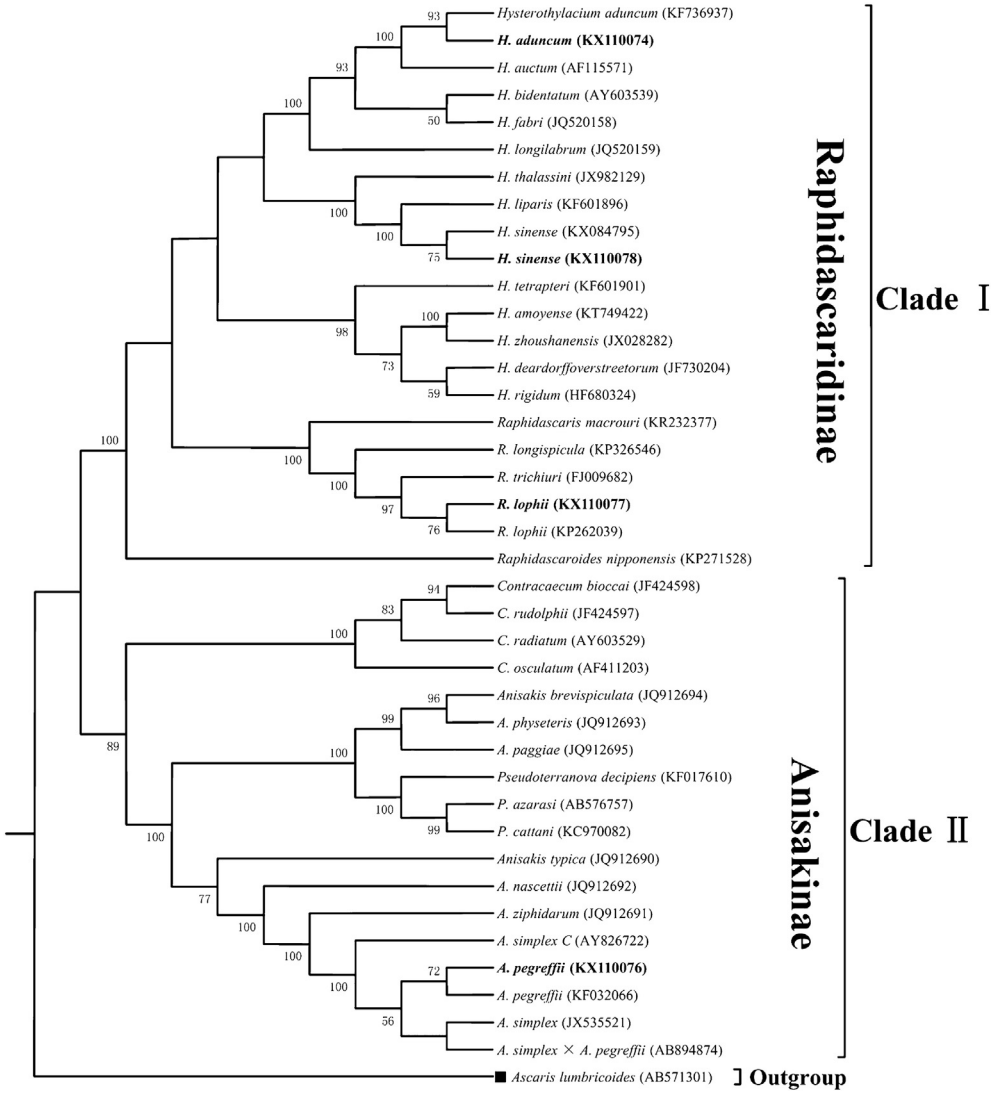

Fig. 4. Neighbor-Joining (NJ) tree revealing phylogenetic relationships of four species of *Anisakis*, *Hysterothylacium* and *Raphidascaris* isolated in this study (shown in bold) and anisakid species registered in GenBank based on ITS rDNA sequences. *Ascaris lumbricoides* was chosen as the outgroup. Values above nodes correspond to bootstrap percentages above 50%.

one ITS sequence of *H. sinense* registered in GenBank, which was based on paratype of this species by the corresponding author of the present study. In order to further ascertain the systematic status of the third-stage larvae of the *Hysterothylacium* type HL of Guo et al. (2014) and to test whether there are intraspecific nucleotide differences in the ITS region in *H. sinense*, three additional paratypes of *H. sinense* were characterized by sequencing and analyzing the ITS sequence. Three ITS sequences of the paratypes of *H. sinense* obtained herein (KX817293–KX817295) showed no intraspecific nucleotide variability. A comparison of these three ITS sequences of *H. sinense* with that available in GenBank displayed 100% identity. There were no nucleotide differences detected in the ITS sequences between the third-stage larvae of the *Hysterothylacium* type HL of Guo et al. (2014) collected from *P. cinnamoneus* in the present study and the paratypes of *H. sinense*. Because many significant taxonomic morphological features are not fully developed in the third-stage larvae, it is unreliable and unpractical to identify ascaridoid larvae to species-level based on morphological characters. However, the relative length of the intestinal caecum to the oesophagus and the ratio of the intestinal caecum to the ventricular appendix of the third-stage larvae of the *Hysterothylacium* type HL of Guo et al. (2014) are similar to that of the adults of *H. sinense* (Fig. 1E, Table 2). Moreover, they occurred in the same geographical area. Therefore, it is reasonable to consider our present larval specimens to be conspecific with *H. sinense*. However, we only detected the third-stage larvae of *H. sinense* in *P. cinnamoneus* (we did not find adults), which possibly indicates that *P. cinnamoneus* serves as an intermediate or paratenic host for *H. sinense*. To date, our knowledge of the phylogeny of the genus *Hysterothylacium* is fragmentary. The genetic relationships of *H. sinense* and the other *Hysterothylacium* species remain unclear. The phylogenetic analyses presented here revealed a sister relationship of *H. sinense* with *H. liparis*, with very high supported value (Figs. 4, 5).

Anisakis pegreffii and *H. aduncum* were the subdominant species of ascaridoid larvae found in *P. cinnamoneus*, but both detected frequently in marine fishes in the Northwest Pacific (Shih, 2004; Quiazon et al., 2011; Umehara et al., 2006, 2010; Zhang et al., 2007; Du et al., 2010; Li et al., 2007b, 2013; Guo et al., 2014; Chen and Shih, 2015). To date, most cases of human anisakidosis in Japan are known to be caused by *A. simplex* (*s. s.*) (D'Amelio et al., 1999; Macpherson, 2005, Umehara et al., 2007; Quiazon et al., 2011; Audicana and Kennedy, 2008; Suzuki et al., 2010). Suzuki et al. (2010) and Quiazon et al. (2011) stated that *A. pegreffii* has a lower capacity to penetrate fish musculature compared to *A. simplex* (*s. s.*). However, *A. pegreffii* is also capable of penetrating fish muscle and causing lesions in rats and human anisakidosis (Fumarola et al., 2009; Romero et al., 2013). Recent molecular studies

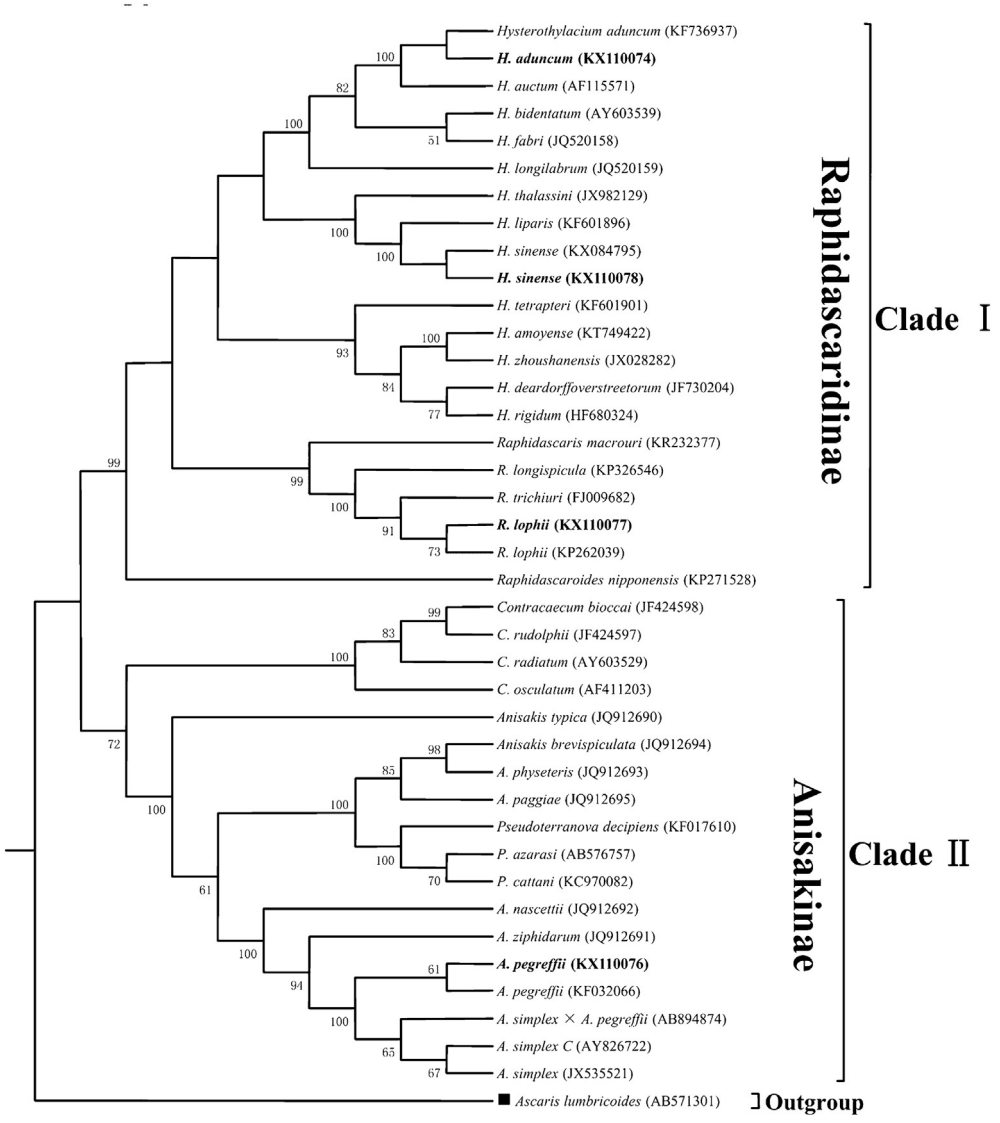

Fig. 5. Maximum likelihood (ML) tree revealing phylogenetic relationships of four species of *Anisakis*, *Hysterothylacium* and *Raphidascaris* isolated in this study (shown in bold) and anisakid species registered in GenBank based on ITS rDNA sequences. *Ascaris lumbricoides* was chosen as the outgroup. Values above nodes correspond to bootstrap percentages above 50%.

of clinical isolates have shown that in Europe, *A. pegreffii* is most frequently associated with human anisakidosis (D'Amelio et al., 1999; Paggi et al., 2001; Fumarola et al., 2009; Mattiucci et al., 2011, 2013). According to Mattiucci and Nascetti (2006, 2008), *A. pegreffii* is mainly distributed in the Mediterranean Sea, the South Atlantic and the Austral Region between 35° N and 55° S. However, many previously published studies (Zhang et al., 2007; Du et al., 2010; Li et al., 2013; Setyobudi et al., 2013; Guo et al., 2014; Chen and Shih, 2015; Kong et al., 2015), have indicated that *A. pegreffii* is also widely distributed in the Yellow Sea, the East China Sea and the Taiwan Strait and it appears to be the predominant *Anisakis* species in these areas. Indeed, *A. pegreffii* seems to be the most important potential etiological agent for human anisakidosis in Mainland China, in spite of the fact that the causative pathogen of the case of human anisakidosis reported in Mainland China has not been precisely identified (Qin et al., 2013). There are over 150 ITS sequences of *A. pegreffii* registered in GenBank, most of which are identical to our ITS data of third-stage larvae of *Anisakis* type I of Berland (1961), i.e. (AY821738, AY821740, AY821745, EU624343, KJ011486, JQ900763, JQ934869, JQ934867, JQ934871, KP301519, KF032066) (Nadler et al., 2005; Quiazon et al., 2009; Smrzlić et al., 2012; Setyobudi et al., 2013; Mattiucci et al., 2013; Sohn et al., 2014). Only a few ITS sequences of *A. pegreffii* registered in GenBank showed a very low level of nucleotide variability (0.11% nucleotide differences), i.e. (KJ011495–KJ011498, JN005756) (Hermida et al., 2012; Sohn et al., 2014). Thus we considered our third-stage larvae of *Anisakis* type I of Berland (1961) to be conspecific with *A. pegreffii*. Many previous phylogenetic studies based on different ribosomal and mitochondrial target markers, have proved that *A. pegreffii* and *A. simplex* have very close relationship (Mattiucci and Nascetti, 2006; Smrzlić et al., 2012; Borges et al., 2012; Zhang et al., 2013; Koinaria et al., 2013; Setyobudi et al., 2013; Kong et al., 2015). The present phylogenetic analyses based on ITS data also revealed the sister relationship between *A. pegreffii* and *A. simplex* with high branch supports (Figs. 5).

Hysterothylacium aduncum is a common parasite in various marine, estuarine and freshwater fishes worldwide (Li et al., 2016b). Yagi et al. (1996) reported a case of human anisakidosis caused by this species. There are over 100 ITS sequences of *H. aduncum* registered in GenBank. However, pairwise comparison between our specimens of the *Hysterothylacium* type of Smith (1983) and the ITS sequences of *H. aduncum* registered in GenBank displayed very low level of nucleotide differences (0–0.50%), which should be considered as intraspecific nucleotide variability. Therefore, we considered that these larvae of the *Hysterothylacium* type of Smith (1983) collected from *P. cinnamoneus*

should belong to *H. aduncum*, *H. aduncum* seems to be a colder water ascaridoid species. Adroher et al. (1991) demonstrated that the optimal temperature for the survival of *H. aduncum* in vitro was 16 °C and that this nematode survived for only a few hours at 37 °C. The present results of phylogenetic analyses based on ITS data supported the hypothesis that *H. aduncum* is closely related to *H. auctum*, which is consistent with the recent study by Zhao et al. (2016). The present finding of a heavy infection of *Hysterothylacium* larvae in *P. cinnamoneus*, suggests that we need to attach increased importance to this group of parasites.

The cinnamon flounder *P. cinnamoneus* is a demersal fish that eats benthic crustaceans and small fishes (Amaoka and Hensley, 2001) and may therefore become infected with *A. pegreffii* and *H. aduncum* larvae by consuming these invertebrates and vertebrates, which are intermediate or paratenic hosts of these nematodes (Smith, 1983; Koie, 2001; Klimpel et al., 2004). However, the life cycles of *H. sinense* and *R. lophii* are still unclear. We speculate that the oceanic life cycle of these two species may be similar to the life cycle patterns of *H. aduncum*. The high level of infection of ascaridoid larvae showed that *P. cinnamoneus* serves as a suitable intermediate/paratenic host for these anisakids, especially for *H. sinense* (prevalence 100%; mean intensity 17.4). Guo et al. (2014) surveyed the occurrence of ascaridoid nematodes in Tanaka's snailfish, *Liparis tanakae* (Gilbert & Burke) (Scorpaeniformes: Liparidae) in the Yellow Sea and East China Sea, and demonstrated that *L. tanakae* was heavily infected with four species of ascaridoid nematodes *H. liparis*, *H. fabri*, *A. pegreffii* and *H. aduncum* (total prevalence 100% and mean intensity 82.3 nematodes per fish) and that the prevalence of *A. pegreffii* and *H. aduncum* was 10.0% and 100%, respectively. Although *P. cinnamoneus* and *L. tanakae* have the similar feeding habits and geographical distributions (Yamada et al., 1995; Froese and Pauly, 2016), the species composition and the level of infection of ascaridoid species are different to those we found in *P. cinnamoneus*.

Although it is also common to eat raw or undercooked seafood dishes in China, especially in the coastal areas, people generally lack awareness of human anisakidosis and the risk of anisakidosis is underestimated. The data obtained herein increases the knowledge of the species and distribution of ascaridoid larvae in this area of the Yellow Sea. The high level of ascaridoid larvae infection in *P. cinnamoneus* indicates that an assessment needs to be undertaken to assess the risk these parasites may pose to public health. More extensive investigations of the ascaridoid nematodes infecting a variety of food fish species in Chinese waters will be necessary to clarify the epidemiology of anisakidosis in China.

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References

Adroher, F.J., Valero, A., Wolff, M., Ruiz-Valero, J., 1991. Mantenimiento y ecdisis in vitro de *Hysterothylacium aduncum*. ICASEP I, Valencia, Spain, 1–5 July, p. 124.

Amaoka, K., Hensley, D.A., 2001. Paralichthyidae. Sand flounders. In: Carpenter, K.E., Niem, V. (Eds.), FAO Species Identification Guide for Fishery Purposes. The Living Marine Resources of the Western Central PacificBony Fishes Part 4 (Labridae to Latimeriidae), Estuarine Crocodiles 6. FAO, Rome, pp. 3842–3862.

Audicana, M.T., Kennedy, M.W., 2008. *Anisakis simplex*: from obscure infectious worm to inducer of immune hypersensitivity. Clin. Microbiol. Rev. 21, 360–379.

Baird, F.J., Gasser, R.B., Jabbar, A., Lopata, A.L., 2014. Foodborne anisakiasis and allergy. Mol. Cell. Probes 28, 167–174.

Berland, B., 1961. Nematodes from some Norwegian marine fishes. Sarsia 2, 1–50.

Borges, J.N., Cunha, L.F.G., Santos, H.L.C., Monteiro-Neto, C., Santos, C.P., 2012. Morphological and molecular diagnosis of anisakid nematode larvae from cutlassfish (*Trichiurus lepturus*) off the coast of Rio de Janeiro, Brazil. PLoS ONE 7, 1–14.

Bush, A.O., Lafferty, K.D., Lotz, J.M., Shostak, A.W., 1997. Parasitology meets ecology on its own terms: Margolis et al. revisited. J. Parasitol. 83, 575–583.

Cavallero, S., Magnaboscob, C., Civettini, M., Boffo, L., Mingarelli, G., Buratti, P., Giovanardi, O., Fortuna, C.M., Arcangeli, G., 2015. Survey of *Anisakis* sp. and *Hysterothylacium* sp. in sardines and anchovies from the North Adriatic Sea. Int. J. Food Microbiol. 200, 18–21.

Chen, H.-Y., Shih, H.-H., 2015. Occurrence and prevalence of fish-borne *Anisakis* larvae in the spotted mackerel *Scomber australasicus* from Taiwanese waters. Acta Trop. 145, 61–67.

D'Amelio, S., Mathiopoulos, K.D., Brandonisio, O., Lucarelli, G., Doronzo, F., Paggi, L., 1999. Diagnosis of a case of gastric anisakidosis by PCR-based restriction fragment length polymorphism analysis. Parassitologia 41, 591–593.

D'Amelio, S., Mathiopoulos, K.D., Santos, C.P., Pugachev, O.N., Webb, S.C., Picanco, M., Paggi, L., 2000. 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. 30, 223–226.

Du, C.-X., Zhang, L.-P., Shi, M.-Q., Ming, Z., Hu, M., Gasser, R.B., 2010. Elucidating the identity of *Anisakis* larvae from a broad range of marine fishes from the Yellow Sea, China, using a combined electrophoretic-sequencing approach. Electrophoresis 31, 654–658.

Espineira, M., Herrero, B., Vieites, J.M., Santaclara, F.J., 2010. Detection and identification of anisakids in seafood by fragment length polymorphism analysis and PCR–RFLP of ITS-1 region. Food Control 21, 1051–1060.

Felsenstein, J., 1985. Confidence limits on phylogenies: an approach using the bootstrap. Evolution 39, 783–791.

Froese, R., Pauly, D., 2016. FishBase. World Wide Web electronic publication. http://www.fishbase.org (version 01/2016).

Fumarola, L., Monno, R., Ierardi, E., Rizzo, G., Giannelli, G., Lalle, M., Pozio, E., 2009. *Anisakis pegreffi* etiological agent of gastric infections in two Italian women. Foodborne Pathog. Dis. 6, 1157–1159.

Guo, Y.-N., Xu, Z., Zhang, L.-P., Hu, Y.-H., Li, L., 2014. Occurrence of *Hysterothylacium* and *Anisakis* nematodes (Ascaridida: Ascaridoidea) in the tanaka's snailfish *Liparis tanakae* (Gilbert & Burke) (Scorpaeniformes: Liparidae). Parasitol. Res. 113, 1289–1300.

Hermida, M., Mota, R., Pacheco, C.C., Santos, C.L., Cruz, C., Saraiva, A., Tamagnini, P., 2012. Infection levels and diversity of anisakid nematodes in blackspot seabream, *Pagellus bogaraveo*, from Portuguese waters. Parasitol. Res. 110, 1919–1928.

Hillis, D., Bull, M.J.J., 1993. An empirical test of bootstrapping as a method for assessing confidence in phylogenetic analysis. Syst. Biol. 42, 182–192.

Hochberg, N.S., Hamer, D.H., 2010. Anisakidosis: perils of the deep. Clin. Infect. Dis. 51, 806–812.

Klapper, R., Kochmann, J., O'Hara, R.B., Karl, H., Kuhn, T., 2016. Parasites as biological tags for stock discrimination of beaked redfish (*Sebastes mentella*): parasite infra-communities vs. resolution of cytochrome markers. PLoS ONE 11, E0153964.

Klimpel, S., Palm, H.W., Ruckert, S., Piatkowski, U., 2004. The life cycle of *Anisakis simplex* in the Norwegian Deep (northern North Sea). Parasitol. Res. 94, 1–9.

Koie, M., 2001. Experimental infections of copepods and sticklebacks *Gasterosteus aculeatus* with ensheathed and large third-stage larvae of *Anisakis simplex* (Nematoda, Ascaridoidea, Anisakidae). Parasitol. Res. 87, 32–36.

Koinaria, M., Karlb, S., Elliot, A., Ryana, U., Lymberyc, A.J., 2013. Identification of *Anisakis* species (Nematoda: Anisakidae) in marine fish hosts from Papua New Guinea. Vet. Parasitol. 193, 126–133.

Kong, Q.-M., Fan, L.-F., Zhang, J.-H., Akao, N., Dong, K.-W., Lou, D., Ding, J.-Z., Tong, Q.-B., Zheng, B., Chen, R., Ohta, N., Lu, S.-H., 2015. Molecular identification of *Anisakis* and *Hysterothylacium* larvae in marine fishes from the East China Sea and the Pacific coast of central Japan. Int. J. Food Microbiol. 199, 1–7.

Li, L., An, R.-Y., Zhang, L.-P., 2007a. A new species of *Hysterothylacium* (Nematoda: Anisakidae) from marine fishes from Yellow Sea, China, with a key to the species of the genus *Hysterothylacium*. Zootaxa 1614, 43–52.

Li, L., Xu, Z., Zhang, L.-P., 2007b. Investigation on the Nematode of *Hysterothylacium aduncum* (Anisakidae) from Bohai Sea and Yellow Sea in China. Chin. J. Parasitol. Parasit. Dis. 25, 364–367.

Li, L., Liu, Y.-Y., Zhang, L.-P., 2012. Morphological and molecular identification of *Hysterothylacium longilabrum* sp. nov. and larvae of different stages (Nematoda: Anisakidae) from marine fishes in the South China Sea. Parasitol. Res. 111, 767–777.

Li, J., Guo, J.-N., Zhou, J.-B., Shi, W., Li, W.-W., Fang, F., Huang, W.-Y., 2013. Preliminary investigation of *Anisakis* sp. third stage larvae infection of *Pneumatophorus japonicus* from the Yellow Sea area, China. Chin. J. Food Hyg. 25, 56–61.

Li, L., Zhao, W.-T., Guo, Y.-N., Zhang, L.-P., 2016a. Nematode parasites infection in the starry batfish *Halieutaea stellata* (Vahl) (Lophiiformes: Ogcocephalidae) from the East and South China Sea. J. Fish Dis. 39, 515–529.

Li, L., Gibson, D.I., Zhang, L.-P., 2016b. An annotated catalogue of the ascaridoid nematode parasites of Chinese vertebrates. Syst. Parasitol. 93, 1–35.

Liu, Y.-Y., Xu, Z., Zhang, L.-P., Li, L., 2013. Redescription and genetic characterization of *Hysterothylacium thalassini* Bruce, 1990 (Nematoda: Anisakidae) from marine fishes in the South China Sea. J. Parasitol. 99, 655–661.

Luo, D.-M., 2001. Notes on nematodes of fishes from Taiwan Strait. II. (Nematoda: Spiruridea; Ascarididea). Acta Zootaxon. Sinica 26, 281–288.
Macpherson, C.N., 2005. Human behaviour and the epidemiology of parasitic zoonoses. Int. J. Parasitol. 35, 1319–1331.
Mattiucci, S., Nascetti, G., 2006. Molecular systematics, phylogeny and ecology of anisakid nematodes of the genus *Anisakis* Dujardin, 1845: an update. Parasite 13, 99–113.
Mattiucci, S., Nascetti, G., 2008. Advances and trends in the molecular systematics of anisakid nematodes, with implications for their evolutionary ecology and host-parasite co-evolutionary processes. Adv. Parasitol. 66, 47–148.
Mattiucci, S., Paoletti, M., Borrini, F., Palumbo, M., Palmieri, R.M., Gomes, V., Casati, A., Nascetti, G., 2011. First molecular identification of the zoonotic parasite *Anisakis pegreffii* (Nematoda: Anisakidae) in a paraffin-embedded granuloma taken from a case of human intestinal anisakiasis in Italy. BMC Infect. Dis. 11, 82.
Mattiucci, S., Fazii, P., De Rosa, A., Paoletti, M., Megna, A.S., Glielmo, A., De Angelis, M., Costa, A., Meucci, C., Calvaruso, V., Sorrentini, I., Palma, G., Bruschi, F., Nascetti, G., 2013. Anisakiasis and gastroallergic reactions associated with *Anisakis pegreffii* infection, Italy. Emerg. Infect. Dis. 19, 496–499.
Nadler, S.A., D'Amelio, S., Dailey, M.D., Paggi, L., Siu, S., Sakanari, J.A., 2005. Molecular phylogenetics and diagnosis of *Anisakis*, *Pseudoterranova*, and *Contracaecum* from northern Pacific marine mammals. J. Parasitol. 91, 1413–1429.
Pekmezci, G.-Z., Onuk, E.-E., Bolukbas, C.-S., Yardimci, B., Gurler, A.-T., Acici, M., Umur, S., 2014. Molecular identification of *Anisakis* species (Nematoda: Anisakidae) from marine fishes collected in Turkish waters. Vet. Parasitol. 201, 82–94.
Paggi, L., Mattiucci, S., D'Amelio, S., 2001. Allozyme and PCR-RFLP markers in anisakid nematodes, aethiological agents of human anisakidosis. Parassitologia 43, 21–27.
Qin, Y., Zhao, Y., Ren, Y., Zheng, L., Dai, X., Li, Y., Mao, W., Cui, Y., 2013. Anisakiasis in China: the first clinical case report. Foodborne Pathog. Dis. 10, 472–474.
Quiazon, K.M., Yoshinaga, T., Santos, M.D., Ogawa, K., 2009. Identification of larval *Anisakis* spp. (Nematoda: Anisakidae) in Alaska pollock (*Theragra chalcogramma*) in northern Japan using morphological and molecular markers. J. Parasitol. 95, 1227–1232.
Quiazon, K.M.A., Yoshinaga, T., Ogawa, K., 2011. Experimental challenge of *Anisakis simplex* sensu stricto and *Anisakis pegreffii* (Nematoda: Anisakidae) in rainbow trout and olive flounder. Parasitol. Int. 60, 126–131.
Rasmussen, R.S., Morrissey, M.T., 2008. DNA-based methods for the identification of commercial fish and seafood species. Compr. Rev. Food Sci. Food Saf. 7, 280–295.
Romero, M.C., Valero, A., Navarro-Moll, M.C., Martín-Sánchez, J., 2013. Experimental comparison of pathogenic potential of two sibling species *Anisakis simplex* s. s. and *Anisakis pegreffii* in Wistar rat. Tropical Med. Int. Health 18, 979–984.
Setyobudi, E., Jeon, C.H., Choi, K., Lee, S.I., Lee, C.I., Kim, J.-H., 2013. Molecular identification of anisakid nematodes third stage larvae isolated from common squid (*Todarodes pacificus*) in Korea. Ocean Sci. J. 48, 197–205.
Shamsi, S., Eisenbarth, A., Saptarshi, S., Beveridge, I., Gasser, R.B., Lopata, A.L., 2011. Occurrence and abundance of anisakid nematode larvae in five species of fish from southern Australian waters. Parasitol. Res. 108, 927–934.
Shamsi, S., Gasser, R.B., Beveridge, I., 2013. Description and genetic characterisation of *Hysterothylacium* (Nematoda: Raphidascarididae) larvae parasitic in Australian marine fishes. Parasitol. Int. 62, 320–328.
Shamsi, S., Poupa, A., Justine, J.-L., 2015. Characterisation of ascaridoid larvae from marine fish off New Caledonia, with description of new *Hysterothylacium* larval types XIII and XIV. Parasitol. Int. 64, 397–404.
Shamsi, S., Ghadam, M., Suthar, J., Mousavi, H.E., Soltani, M., Mirzargar, S., 2016. Occurrence of ascaridoid nematodes in selected edible fish from the Persian Gulf and description of *Hysterothylacium* larval type XV and *Hysterothylacium persicum* n. sp. (Nematoda: Raphidascarididae). Int. J. Food Microbiol. 236, 65–73.
Shih, H.-H., 2004. Parasitic helminth fauna of the cutlass fish, *Trichiurus lepturus* L., and the differentiation of four anisakid nematode third-stage larvae by nuclear ribosomal DNA sequences. Parasitol. Res. 93, 188–195.
Shih, H.-H., Ku, C.-C., Wang, C.-S., 2010. *Anisakis simplex* (Nematoda: Anisakidae) third-stage larval infections of marine cage cultured cobia, *Rachycentron canadum* L., in Taiwan. Vet. Parasitol. 171, 277–285.
Smith, J.W., 1983. Larval *Anisakis simplex* (Rudolphi, 1809, det. Krabbe, 1878) and larval *Hysterothylacium* sp. (Nematoda: Ascaridoidea) in euphausiids (Crustacea: Malacostraca) in the North-East Atlantic and northern North Sea. J. Helminthol. 57, 167–177.
Smrzlić, I.V., Valic, D., Kapetanovic, D., Kurtovic, B., Teskeredzic, E., 2012. Molecular characterisation of Anisakidae larvae from fish in Adriatic Sea. Parasitol. Res. 111, 2385–2391.
Sohn, W.M., Kang, J.M., Na, B.K., 2014. Molecular analysis of *Anisakis* type I larvae in marine fish from three different sea areas in Korea. Korean J. Parasitol. 52, 383–389.
Sotelo, C.G., Calo-Mata, P., Chapela, M.J., Perez-Martin, R.I., Rehbein, H., Hold, G.L., Russell, V.J., Pryde, S., Quinteiro, J., Izquierdo, M., Rey-Mendez, M., Rosa, C., Santos, A.T., 2001. Identification of flatfish (Pleuronectiforme) species using DNA-based techniques. J. Agric. Food Chem. 49, 4562–4569.
Sun, S.-Z., Koyama, T., Kagei, N., 1991. Anisakidae larvae found in marine fishes and squids from the Gulf of Tongking, the East China Sea and the Yellow Sea. Jpn. J. Med. Sci. Biol. 44, 99–108.
Suzuki, J., Murata, R., Hosaka, M., Araki, J., 2010. Risk factors for human *Anisakis* infection and association between the geographic origins of *Scomber japonicus* and anisakid nematodes. Int. J. Food Microbiol. 31, 88–93.
Thompson, J.D., Higgins, D.G., Gibson, T.J., 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22, 4673–4680.
Umehara, A., Kawakami, Y., Araki, J., Uchida, A., 2006. Molecular identification of *Anisakis simplex* sensu stricto and *Anisakis pegreffii* (Nematoda: Anisakidae) from fish and cetacean in Japanese waters. Parasitol. Int. 55, 267–271.
Umehara, A., Kawakami, Y., Araki, J., Uchida, A., 2007. Molecular identification of the etiological agent of the human anisakiasis in Japan. Parasitol. Int. 56, 211–215.
Umehara, A., Kawakami, Y., Ooi, H.K., Uchida, A., Ohmae, H., Sugiyama, H., 2010. Molecular identification of *Anisakis* type I larvae isolated from hairtail fish off the coasts of Taiwan and Japan. Int. J. Food Microbiol. 143, 161–165.
Xu, Z., Zhang, L.-P., Liu, B.-C., Li, L., 2012. Morphological and molecular characterization of *Raphidascaris (Ichthyascaris) lophii* (Wu, 1949) (Nematoda, Anisakidae) from marine fishes from China, with a key to the species of the subgenus *Ichthyascaris*. Acta Parasitol. 57, 316–322.
Yagi, K., Nagasawa, K., Ishikura, H., Nakagawa, A., Sato, N., Kikuchi, K., Ishikura, H., 1996. Female worm *Hysterothylacium aduncum* excreted from human: a case report. Jpn. J. Parasitol. 45, 12–23.
Yamada, U., Shirai, S., Irie, T., Tokimura, M., Deng, S., Zheng, Y., Li, C., Kim, Y.U., Kim, Y.S., 1995. Names and Illustrations of Fishes from the East China Sea and the Yellow Sea. Overseas Fishery Cooperation Foundation, Tokyo, Japan (288 p).
Zhao, W.-T., Lü, L., Chen, H.-X., Yang, Y., Zhang, L.-P., Li, L., 2016. Ascaridoid parasites infecting in the frequently consumed marine fishes in the coastal area of China: a preliminary investigation. Parasitol. Int. 65, 87–98.
Zhang, L.-P., Hu, M., Shamsi, S., Beveridge, I., Li, H.-M., Xu, Z., Li, L., Cantacessi, C., Gasser, R.B., 2007. The specific identification of anisakid larvae from fishes from the Yellow Sea, China, using mutation scanning-coupled sequence analysis of nuclear ribosomal DNA. Mol. Cell. Probes 21, 386–390.
Zhang, L.-P., Du, X.-J., An, R.-Y., Li, L., Gasser, R.B., 2013. Identification and genetic characterization of *Anisakis* larvae from marine fishes in the South China Sea, using an electrophoretic-guided approach. Electrophoresis 34, 888–894.