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▣ ORIGINAL ARTICLE

Occurrence and Molecular Identification of *Anisakis* Dujardin, 1845 from Marine Fish in Southern Makassar Strait, Indonesia

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Abstract: *Anisakis* spp. (Nematoda: Anisakidae) parasitize a wide range of marine animals, mammals serving as the definitive host and different fish species as intermediate or paratenic hosts. In this study, 18 fish species were investigated for *Anisakis* infection. *Katsuwonus pelamis*, *Euthynnus affinis*, *Caranx* sp., and *Auxis thazard* were infected with high prevalence of *Anisakis* type I, while *Cephalopholis cyanostigma* and *Rastrelliger kanagurta* revealed low prevalence. The mean intensity of *Anisakis* larvae in *K. pelamis* and *A. thazard* was 49.7 and 5.6, respectively. A total of 73 *Anisakis* type I larvae collected from *K. pelamis* and *A. thazard* were all identified as *Anisakis typica* by PCR-RFLP analysis. Five specimens of *Anisakis* from *K. pelamis* and 15 specimens from *A. thazard* were sequenced using ITS1-5.8S-ITS2 region and 6 specimens from *A. thazard* and 4 specimens from *K. pelamis* were sequenced in mtDNA *cox2* region. Alignments of the samples in the ITS region showed 2 patterns of nucleotides. The first pattern (genotype) of *Anisakis* from *A. thazard* had 100% similarity with adult *A. typica* from dolphins from USA, whereas the second genotype from *A. thazard* and *K. pelamis* had 4 base pairs different in ITS1 region with adult *A. typica* from USA. In the mtDNA *cox2* regions, *Anisakis* type I specimens from *A. thazard* and *K. pelamis* showed similarity range from 94% to 99% with *A. typica* AB517571/DQ116427. The difference of 4 bp nucleotides in ITS1 regions and divergence into 2 subgroups in mtDNA *cox2* indicating the existence of *A. typica* sibling species in the Makassar Strait.

Key words: *Anisakis typica*, molecular identification, internal transcribed spacer, PCR-RFLP, Makassar Strait, Indonesia

INTRODUCTION

The Indonesian archipelago, which comprises of more than 17 thousands islands and coastal lines length of about 104,000 km, provides great natural resources for Indonesia economic growth in many regions, particularly the fishery sector. Total production from fisheries continues to grow from 8.2 million ton in 2007 to 12.4 million ton in 2011 [1]. Indonesia is also recognized as a center of marine organism diversity and is inhabited with abundant fish species. Fishery products, which are either traded as export commodities or for local consumption, have to be free from any zoonotic parasites, such as anisakid nematodes. Food safety has recently become a great concern for consumers. Zoonotic parasites in fish products are mainly caused by helminths, which utilize fish as the intermediate host, and marine mammals such as dolphins or whales as the final host [2-4]. Some helminths are transmittable and able to survive in the human digestive tract after consuming raw fish infected by larval helminths, causing significant clinical diseases, as well as allergic reactions [5-7]. Symptoms of anisakiasis include epigastric pain, nausea, vomiting, and diarrhea [8]. Among the helminth parasites, larval nematodes of the genus *Anisakis* are commonly found in the musculature or digestive tracts of many species of marine fish. *Anisakis simplex* is the most well-known zoonotic nematode which has been reported to cause anisakiasis of humans in many countries in Europe and Asia [8]. Though reported cases of anisakiasis in Indonesia are very rare, a study conducted by Uga et al. [9] using a seroepidemiological approach of inhabitants in East Java revealed that about 11% of samples were positive for *Anisakis* infection.

In previous studies, *Anisakis* spp. could only be categorized

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morphologically into *Anisakis* type I and type II, in which the former has a longer ventriculus and a mucron, while the latter has short ventriculus and no mucron [10]. Identification to the species level by microscopic examinations is usually unreliable due to undeveloped morphological characteristics of larval stage nematodes. However, accurate identification of *Anisakis* nematodes larvae is required for precise diagnosis of *Anisakis* infections in humans and fish and to improve food safety. In addition, they can be used as biological indicators in the study of stock discrimination of migratory fish [11-15]. Recent studies showed that molecular diagnostic techniques could be used to identify *Anisakis* to a species level. PCR-RFLP has been widely used to identify *Anisakis* spp. in different fish species [16-20]. Molecularly, the previous *Anisakis* type I is known to consist of 6 species (*A. ziphidarum*, *A. nascettii*, *A. typica*, and 3 sibling species of *A. simplex* complex, namely *A. simplex* (sensu stricto) (s.s.), *A. pegreffii*, and *A. simplex C*, whereas type II consists of 3 species (*A. paggiae*, *A. brevispiculata*, and *A. physeteris*) [21,22]. Recent studies in Japan showed that L3, L4, and adult of *A. simplex* (s.s) and *A. pegreffii* could be distinguished morphologically based on the ventricululs length, in which the former has a longer (0.90-1.50 mm) ventriculus than the latter (0.50-0.78 mm) [23].

Parasitological research on *Anisakis* spp. in Indonesia is relatively scarce. Research on *Anisakis* was previously reported from Seribu Islands, Jakarta from 3 species of fish: *Rastrelliger kanagurta*, *Decapterus russelii*, and *Sardinella sirm* [24]. The study has found 2 types of *Anisakis*, i.e., *Anisakis* type I and *Terranova* type B, where *Anisakis* type I predominated. A recent study conducted on several fish species of Balinese and Javanese waters has found 3 species of *Anisakis*; *A. typica*, *Anisakis* sp. 1, and *Anisakis* sp. 2 [25]. Another study of marine fish in Indonesia in Southern Coast of Kulon Progo, Yogyakarta, has also found 5 out of 11 fish species examined harbored *Anisakis* spp. [26]. However, from most studies conducted in Indonesia, except for that conducted by Palm et al. [25], identification of the *Anisakis* larva was solely based on morphology, making it unreliable for species identification.

The aim of the present study was to investigate the occurrence of *Anisakis* infection from some marine fish in the Southern Makassar Strait, and characterize them to species level using PCR-RFLP genetic analysis, the molecular keys described by D'Amelio et al. [27] and Pontes et al. [17], and sequencing of ITS-5.8S and mitochondrial cytochrome *c* oxidase subunit II (mtDNA *cox2*) regions.

MATERIALS AND METHODS

Parasitological examinations

A total of 220 fish representing 18 species and 10 families were investigated for *Anisakis* larvae infection. Fish species, total length, and number of fish examined are given in Table 1. Fish were purchased from local markets located in Makassar, Takalar, and Barru regencies (Fig. 1). The fish were transported to the Laboratory of Fish Parasites and Diseases, Hasanuddin University for parasitological examinations. The length of fish was measured, body cavity was opened, and internal organs were placed on Petri dishes and examined for the presence of *Anisakis* larvae. *Anisakis* larvae were distinguished from other anisakids following of the identification protocols of Anderson [28]. *Anisakis* spp. were then categorized into type I or type II. *Anisakis* type I was characterized by the presence of boring tooth at the anterior end, ventriculus, and mucron at the posterior end, whereas type II has a boring tooth, ventriculus, and no mucron at the posterior end. Anisakid larvae were isolated from the visceral surface and body cavity of the fish. The larvae were observed under light and dissection microscope for morphological identification. All *Anisakis* larvae found were counted. Since the number of *Anisakis* larvae found from fish other than skipjack and frigate tuna was very few (mostly uninfected), *Anisakis* spp. from these fish were only morphologically identified into *Anisakis* type I or type II. Only larval *Anisakis* spp. isolated from skipjack tuna and frigate tuna were used for further molecular characterization.

The prevalence is defined as the percentage of fish infected by *Anisakis* larvae. The mean intensity is defined as the total number of *Anisakis* species found divided by the total number of fish infected [29].

DNA isolation and amplification

Anisakis spp. were fixed and stored in 70% ethanol. Genomic DNA from individual worms was extracted using a QIAamp DNA Mini Kit (Qiagen Inc., Hilden, Germany) following the manufacturer's tissue protocol. DNA was eluted with milliQ and stored at -20℃ before subsequent PCR amplification. Internal transcribed spacer (ITS) and mitochondrial cytochrome *c* oxidase subunit II (mtDNA *cox2*) regions were used for amplification and sequencing. The entire rDNA regions comprising ITS1, 5.8S, and ITS2 were amplified using previously described primers NC5 (5′-GTAGGTGAACCTGCGGAAGGATCATT-3′) and NC2 (5′-TTAGTTTCTTTTCCTCCGCT-3′) [30].

Table 1. Fish species, number examined, prevalence, and intensity of *Anisakis* type I infection

Fish species	Locality/Size	Date	Number of fish		Prevalence (%)	Intensity
			Examined	Infected		Mean intensity (range)
Caesionidae						
Fusilier *Caesio* sp.	Barru/25-32 cm	-/9/10	9	0	0	
Carangidae						
Giant trevally *Caranx* sp.	Makassar/64-67.5 cm	26/8/10	4	3	75	1 (1)
Indian scad *Decapterus russelii*	Makassar/23 -36.5 cm	26/8/10	20	0	0	
Longnose trevally *Carangoides* sp.	Barru/25-30 cm	-/9/10	9	0	0	
Clupeidae						
Goldstripe sardinella *Sardinella* sp.	Makassar	16/9/10	41	0	0	
Lutjanidae						
Snapper *Lutjanus* sp.	Barru/22-29 cm	-/9/10	8	0	0	
Priacanthidae						
Priacanthus sp.	Barru/28 cm	-/9/10	1	0	0	
Scaridae						
Parrot fish *Scarus* sp.	Barru/28 cm	-/9/10	1	0	0	
Scombridae						
Frigate tuna *Auxis thazard*	Takalar/19-25 cm	26/8/10	12	0	0	
	Makassar/33-41 cm	16,17,22/9/10	30	14	46.7	5.6 (1-19)
Indian mackerel *Rastrelliger kanagurta*	Makassar/20-30.5 cm	26/8/10	12	0	0	
	Takalar/20.2-27 cm	22/9/10	20	1	5	1
Mackerel tuna *Euthynnus affinis*	Makassar/52-56 cm	9/8/10	3	2	66.7	1 (1)
	Barru/51-52 cm	-/9/10	3	0	0	
Narrow-barred spanish *Scomberomorus commersoni*,	Makassar/38.5-45 cm	20/9/10	4	0	0	
Skipjack tuna *Katsuwonus pelamis*	Makassar/35-60 cm	26/8/10	13	12	92.3	49.7 (1-175)
Serranidae						
Grouper *Cephalopholis cyanostigma*	Makassar/22-25 cm	26/8/10	8	1	12.5	1
	Makassar/20-24 cm	16/9/10	10	0	0	
Grouper *Epinephelus fuscoguttatus*	Barru/26-29 cm	-/9/10	2	0	0	
Siganidae						
Barhead spinefoot *Siganus virgatus*	Barru	-/9/10	1	0	0	
Goldlined spinefoot *Siganus guttatus*	Makassar/23-32 cm	26/8/10	5	0	0	
Sphyraenidae						
Barracuda *Sphyraena* sp.	Barru/37-49 cm	-/9/10	4	0	0	

All PCR was performed in 20 µl which contain approximately dNTP 0.2 mM, primers 0.8 µM, *Taq* polymerase 0.02 U/µl and 10 x buffer PCR 1X, and 2 µl samples. Milli-Q was added to achieve the total PCR volume. Each PCR reaction was performed in a thermocycler iCycler (Bio-Rad, Hercules, California, USA) under the following conditions: after initial denaturation at 95℃ for 15 min, 30 cycles of 94℃ for 1 min (denaturation), 55℃ for 1 min (annealing), 72℃ for 1 min (extension), followed by a final extension at 72℃ for 5 min. The mtDNA *cox2* gene was amplified using the primers 210 (5′-CACCAACTCTTAAAATTATC-3′) and 211 (5′-TTTCTAGTTATATAGATTGRT-TYAT-3′) [31]. The PCR mixture was denatured at 94℃ for 3 min, followed by 35 cycles at 94℃ for 30 sec, 46℃ for 1 min, 72℃ for 1 min and 30 sec, followed by post-amplification at 72℃ for 10 min. The PCR products obtained were visualized in an SYBR green stained 1.5% agarose gel.

PCR-RFLP

PCR products of about 965 bp amplified with primers NC5 and NC2 were used for PCR-RFLP analysis to identify *Anisakis* spp. following D'Amelio et al. [27] and Pontes et al. [17]. Three individual restriction enzymes (*Taq I*, *Hinf I*, and *Cfo I*) were used. The PCR products were digested following the manufacturer's recommendation. Briefly, amplicons of 8 µl were mixed with 10 x reaction buffer, 0.5% BSA (only for *Taq I*), and digested with restriction enzymes *Taq I* (10 U/µl, Takara) at 65℃ for 3-4 hr, and with *Hinf I* (10 U/µl, Roche) and *Cfo I* (10 U/µl, Roche) at 37℃ for 3-4 hr. Milli Q was added to reach a final

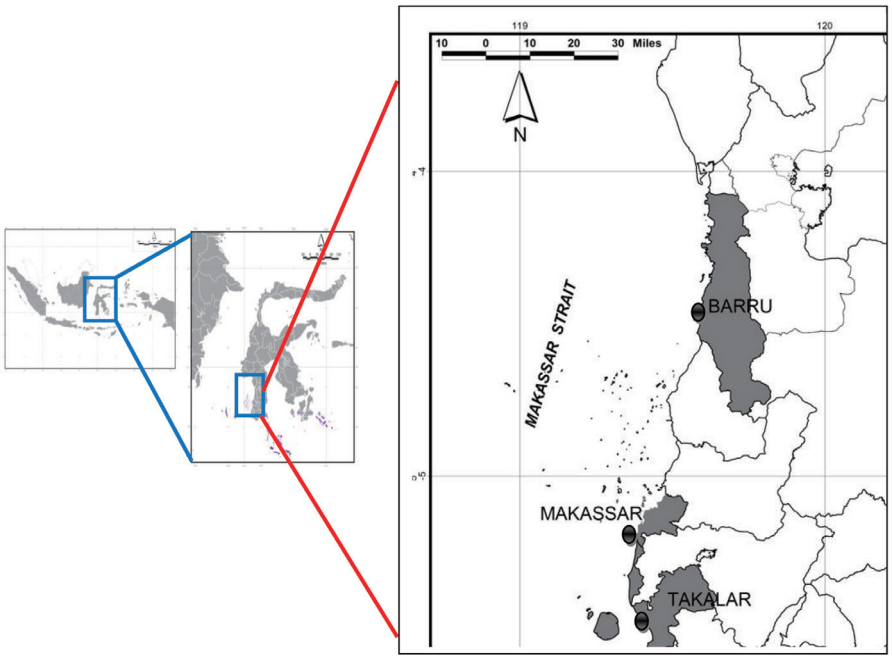

Fig. 1. Sampling sites of marine fish at the Southern Makassar Strait: Barru, Makassar, and Takalar Regencies, Indonesia.

volume of 20 µl. The digested samples were then separated by electrophoresis using 1.5% agarose gel at 100 V for 40 min, stained with SYBR green, and photographed. Their size was estimated using 100 bp ladder marker (Takara).

Sequencing

Twenty specimens, 5 from *K. pelamis* and 15 from *A. thazard* were molecularly identified using PCR-sequencing in ITS1-5.8S-ITS2. In addition, 10 *Anisakis* samples (6 from *A. thazard* and 4 from *K. pelamis*) were sequenced in mtDNA *cox2* region. PCR products were purified using a PCR purification kit (Qiagen) and used directly in sequencing reactions. A 100 bp or 1 kb ladder marker (Takara) was used to estimate the size of PCR products. Afterwards, a total volume of 14 µl containing 6.4 *p*mol primer and 10 to 40 ng DNA was prepared and sent to Operon Biotechnologies Company (Tokyo, Japan) for sequencing. Milli-Q was added when necessary for DNA dilution to meet the concentration of DNA required. Both spacers (ITS1 and ITS2) and the 5.8S gene were sequenced in both directions from each PCR product, using the same primers as above (NC5 and NC2), NC13 (forward; 5′-ATCGATGAAGAACGCAGC-3′), NC13R (reverse; 5′-GCTGCGTTCTTCATCGAT-3′), and XZ1R (reverse; 5′-GGAATGAACCCGATGGCGCAAT-3′). Both forward (primer 210) and reverse (primer 211) directions of mtDNA *cox2* region was sequenced using the same primer as used for PCR amplification.

Alignment and phylogenetic analysis

The forward and reverse sequences of ITS (ITS1, 5.8S, and ITS2) and mtDNA *cox2* regions were assembled and edited using Bioedit Allignment Sequence Editor Ver. 7.0.5.3. They were compared manually with the original chromatograms when necessary. The obtained sequences were aligned with previously characterized sequences of *Anisakis* spp. registered in GenBank, using CLUSTAL X Version 2.1 Multiple Sequence Alignments [32]. Phylogenetic and molecular evolutionary analyses were conducted using *MEGA* version 5 [33]. Maximum likelihood tree was constructed for ITS-5.8S using *Pseudoterranova decipiens* as an outgroup and neighbour-joining tree for mtDNA *cox2* using *H. reliquens* as an outgroup. ITS-5.8S and mtDNA *cox2* gene sequences were deposited in GenBank under accession no. KC928261 to KC928272.

RESULTS

Prevalence and intensity of *Anisakis* larvae

Morphologically, all *Anisakis* larvae found were identified as *Anisakis* type I. Among the fish examined, *K. pelamis*, *E. affinis*, *Caranx* sp., and *A. thazard* were infected at a high prevalence and high intensity, while other fish (*C. cyanostigma* and *R. kanagurta*) were infected with a low prevalence and low intensity (Table 1). The remainders were not infected. The parasites were mainly found on the surface of the viscera such as the liver and

Table 2. PCR-RFLP patterns of *Anisakis* species [36] and the present samples using Taq I, Hinf I or Cfo I/Hha I restriction endonucleases

Anisakis species	Enzymes		
	Taq I	*Hinf I*	*Cfo I/HhaI*
Anisakis simplex s.s.	430, 400, 100	620, 250, 80	550, 430
A. pegreffii	400, 320, 150	370, 300, 250	550, 430
A. ziphidarum	330, 300, 140	370, 320, 290	550, 430
A. typica	400, 350	620, 350	320, 240, 180, 160
Present sample	400, 350	620, 350	320, 240, 180, 160

intestines, and no *Anisakis* larvae were found in the muscle. For identification, the parasites were cleaned and the sheath removed. *Anisakis* larvae can be distinguished from other anisakid larvae such as members of *Pseudoterranova*, *Hysterothylacium*, and *Contracaecum* based on the shape of the ventriculus, which is clearly visible under a stereomicroscope. The prevalence of *Anisakis* type I in *K. pelamis* was 92.3%, and mean intensity was 49.7 parasites/fish. The highest number was 175 parasites in an individual fish. The prevalence of *Anisakis* type I larvae in *A. thazard* (33-41 cm in total length) reached 46.7% with a mean intensity of infection of 5.6 parasites/fish. However, none of *A. thazard* of smaller size (19-25 cm in total length) were infected out of 12 fish examined. The other fish with a high prevalence of *Anisakis* type I were *Caranx* sp. (75%) and *E. affinis* (66.7%) (Table 1).

PCR-RFLP patterns

Amplification of entire ITS and 5.8S regions of all specimens of *Anisakis* produced a PCR product of about 960 bp. The PCR products were digested using 3 different restriction enzymes, *Taq I*, *Hinf I*, and *Cfo I*. In PCR-RFLP, all specimens digested with *Taq I*, *Hinf I*, and *Cfo I* indicated that the samples belong to *A. typica* (Table 2). Based on the RFLP analyses of 73 *Anisakis* type I specimens from *K. pelamis* (40 specimens) and *A. thazard* (33 specimens) were all (100%) identified as *A. typica* (Table 2).

Sequencing of entire ITS region and mtDNA *cox2*

Sequencing of entire ITS and 5.8 S regions was performed for 5 samples of *Anisakis* type I from *K. pelamis* and 15 from *A. thazard*. Using the primer in the ITS and 5.8S region approximately 950 bp nucleotides were generated. Nucleotide sequences from all specimens were analyzed using the software Bioedit, and were manually compared with chromatogram when necessary. No variation in the nucleotide sequences was found in *Anisakis* from *K. pelamis*, whereas samples from *A. thazard* showed 2 nucleotide sequence patterns. They differed in 4 base pairs in ITS1 region. The first nucleotide pattern was only recorded from *A. thazard*, whereas the second one was found from both *K. pelamis* and *A. thazard*. The first pattern (genotype) showed 100% similarity with adult *A. typica* reported from dolphins in USA and high similarity with the Brazilian *A. typica* from dolphins, which only differed in 3 deletions in ITS1. Whereas the second pattern (genotype) has 4 base pairs difference in ITS1 with *A. typica* from USA, but 100% similarity with Indonesian *A. typica* EU346093 from fish *Auxis rochei rochei*, 4 base pairs different with *A. typica* EU346092 and 2 base pairs different with *A. typica* EU346091. A phylogenetic tree using maximum likelihood showed that *A. typica* found in the present study were in the same clade with other *A. typica* published in GenBank (Figs. 2, 3). Sequences of 10 samples of *Anisakis* using the primer in mtDNA *cox2* region produced about 600 bp nucleotides. Pair distances of the alignment of mtDNA *cox2* showed 94-99% similarity of present samples with *A. typica* AB517571 from *Scomber japonicus*, 93-98% with *A. typica* AB517572 from *S. japonicus*, and 94-100% with adult *A. typica* DQ116427 from dolphins (Table 3). The phylogenetic tree of mtDNA *cox2* region showed that all samples were in the same cluster with *A. typica* but produced broad divergence consisting of 2 subgroups (Fig. 4). The first subgroup showed 96% to 100% similarity with the known *A. typica*, whereas the second one has 93-95% similarity with the nematode (Table 3).

DISCUSSION

The present study provides molecular identification of *Anisakis* from *K. pelamis* and *A. thazard* using PCR-RFLP and sequencing of ITS-5.8S and mtDNA *cox2* regions. This is the first record of molecular identification of *Anisakis* type I from fish of eastern part of Indonesia. The first molecular identification of *Anisakis*, which consisted of 3 different genotypes, namely, *A. typica*, *Anisakis* sp. 1, and *Anisakis* sp. 2, was reported from Balinese and Javanese waters [25]. In the present study, based

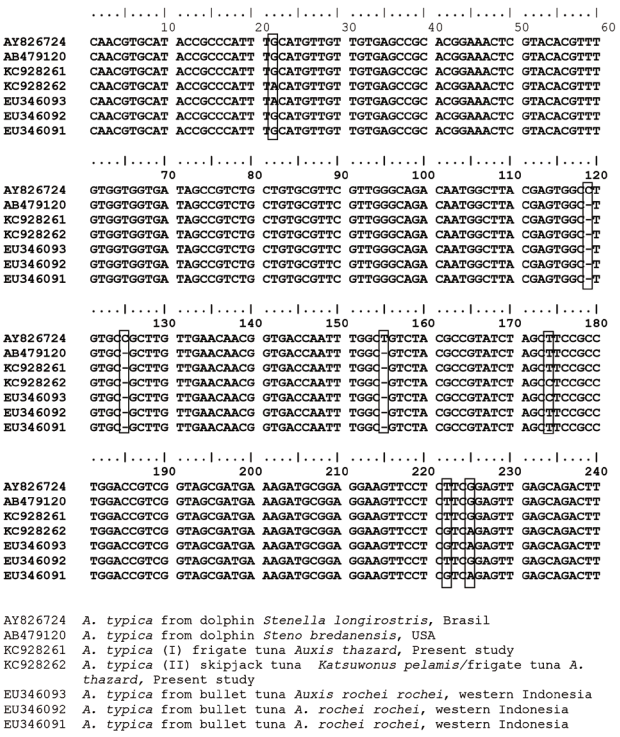

Fig. 2. Comparison of ITS1 sequences of *Anisakis typica* larvae in the present study. KC928261 *A. typica* (I), KC928262 *A. typica* (II), and *A. typica* larva from the bullet tuna from western Indonesia (GenBank accession no. EU346093, EU346092, and EU346091, respectively) were compared with adult specimens from dolphins from Brazil (AY826724) and USA (AB479120). Square shows differences of nucleotides in the ITS1 regions among *A. typica*.

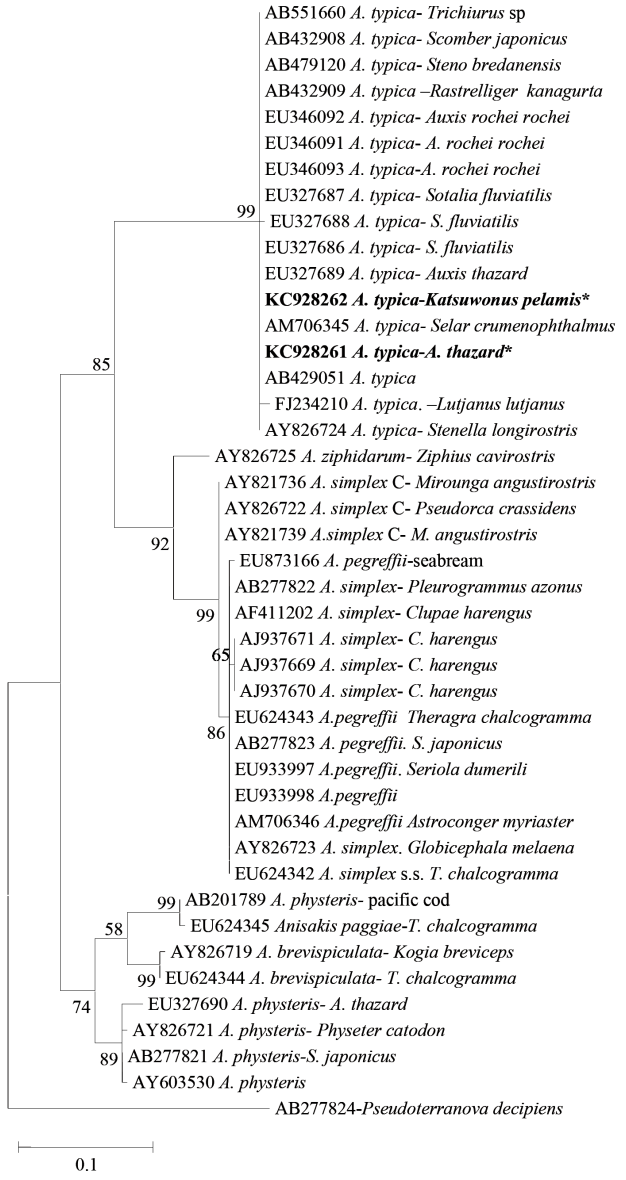

Fig. 3. Phylogenetic tree of *Anisakis* spp. including the present samples (KC928262 *A. typica* from *Katsuwonus pelamis* and KC928261 *A. typica* from *Auxis thazard*) based on ITS-5.8S-ITS2 gene sequences. Asterisks represent present samples. Maximum likelihood tree was constructed using MEGA version 5.1 [33], drawn using Nearest-Neighbor-Interchange (NNI) with Kimura 2-parameter model and 1,000 boostrap number with complete deletion. Percentages ≥50% are shown at the internal nodes.

on PCR-RFLP and sequencing, *Anisakis* larvae were identified as *A. typica*. PCR-RFLP was further used to detect proportion of *Anisakis* spp. in fish, and the results showed that from 73 *Anisakis* type I specimens collected from *K. pelamis* (40 specimens) and *A. thazard* (33 specimens), 100% belonged to *A. typica*. From 20 samples of *Anisakis* type I sequenced, 2 different patterns, or genotypes, were noted and further identified as *A. typica*. The 2 patterns differed in 4 base pairs in the ITS1 region. The first pattern showed 100% similarity with adult *A. typica* (AB479120) reported from dolphins in USA and high similarity with the Brazilian *A. typica* (AY826724) from dolphins, whereas the second genotype has 4 base pairs different in ITS1 with *A. typica* from USA (AB479120), and 100% similarity with Indonesian *A. typica* EU346093 from *Auxis rochei rochei*. The first genotype was only found in *A. thazard*, whereas the second one was found in both *K. pelamis* and *A. thazard*.

Palm et al. [25] proposed that difference in 4 bp of nucleotide in ITS1 region may indicate the occurrence of *A. typica* sibling species. Therefore, the present finding suggests that *A. typica* sibling species may occur in *K. pelamis* and *A. thazard*. Phylogenetic trees from the mtDNA *cox2* region also showed a cluster within *A. typica*, and 2 subgroups were noted. The first subgroup consists of all samples from *A. thazard* and *A. typica* DQ116427, AB517571, and AB517572. The second subgroup consists of 4 samples from *K. pelamis* and 1 sample from *A.*

Table 3. Similarity of nucleotide sequences among *A. typica* including the present *Anisakis* based on mtDNA *cox2* region

Present samples	*1-C*	*3-C*	*4-C*	*6-T*	*7-T*	*8-T*	*9-T*	*59-T*	*69-T*	*2-C*	AB517572 *A. typica*	AB517571 *A. typica*	DQ116427 *A. typica*
1-C		98	98	95	95	98	95	95	95	98	93	94	94
3-C			99	95	94	99	95	95	95	99	94	94	95
4-C				95	95	99	95	95	95	99	93	95	95
6-T					99	95	99	98	99	95	98	99	100
7-T						95	98	97	99	95	98	98	99
8-T							95	95	95	99	94	95	95
9-T								97	99	95	97	98	99
59-T									97	95	96	97	97
69-T										95	97	99	99
2-C											94	95	95

Note: 1-C to 4-C means sample codes of *Anisakis typica* from *Katsuwonus pelamis*, and 6-T to 69-T means sample codes of *A. typica* from *Auxis thazard*.

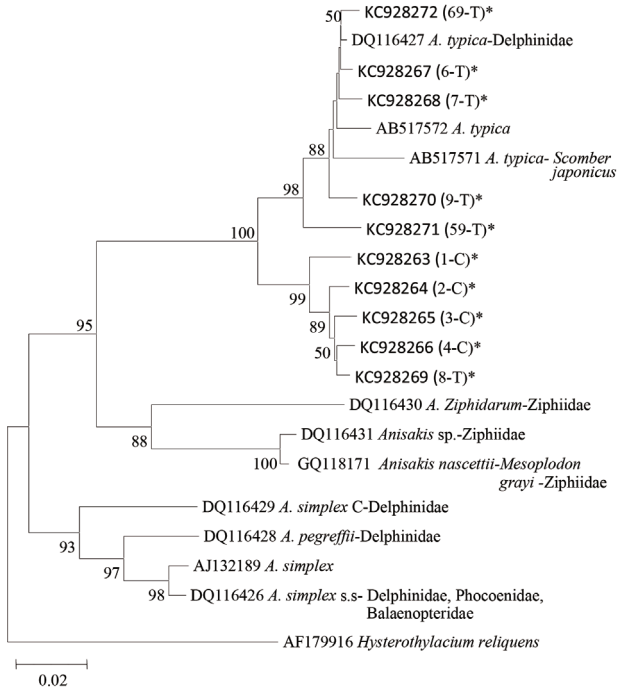

Fig. 4. Phylogenetic tree of *Anisakis* species from the present study (KC928263 to KC928272) and other *Anisakis* spp. based on mtDNA *cox2* gene sequences. Asterisks represent present samples. Neighbour joining tree was constructed using MEGA version 5.1 [33], drawn using Maximum composite likelihood Model and 1,000 boostrap number with complete deletion. Percentages ≥50% are shown at the internal nodes. Sample codes were presented in Table 3.

thazard. The first subgroup of the present samples showed 96-100% similarity with the known *A. typica*, while the second subgroup had 93-95% similarity. The second subgroup forms another cluster which separate them from the known *A. typica* and this may also indicate the presence of *A. typica* sibling species. A previous study in Papua New Guinean waters also showed a similar pattern with the present study in which genetic divergence occurred within *A. typica* clade [34]. Though a study in other nematode taxa showed that sequence differences of about 10-20% were interspecific, and differences of about 7% were regarded as conspecific [35], the present study, as proposed by Palm et al. [25], indicates the presence of *A. typica* sibling species in *K. pelamis* and *A. thazard*, whereas *A. typica* was recorded only from *A. thazard*.

Molecular differentiation of *Anisakis* spp. using PCR-RFLP has been successfully used [16-20,27,35-38]. In the present study, digestion of 73 samples with restriction enzyme *Taq I*, *Hinf I*, and *Cfo I* indicated that the samples were *A. typica*, and suggested a predominance of *A. typica* in the eastern parts of Indonesia. This high abundance of *A. typica* in the present study, and the report of *A. typica* in the previous study in Balinese and Javanese waters [25], as well as a recent report from Papua New Guinea [34] support the previous findings that this species was more abundant in temperate and tropical waters [39]. Mattiucci et al. [39] recorded larvae of *A. typica* from *A. thazard* and *Thunnus thynnus* from Brazilian Atlantic Ocean, *Scomber japonicus* and *Trachurus picturatus* from Madeira Atlantic Ocean, *E. affinis*, *S. commerson*, *Sarda orientalis*, and *Coryphaena hippurus* from Somali Coast, and *Merluccius merluccius* from the Mediteranian Sea off Cyprus and off Crete. In Indonesia, more than 34 species of marine fish have been reported to harbor *Anisakis* spp. [25]. Recent reports of *Anisakis* infection added 3 more new fish genera to harbor *Anisakis* spp. Two genera were reported from the Southern coast of Kulon Progo; *Parupeneus* sp. (Mullidae) and *Terapon jarbua* (Terapontidae) [26], and 1 genus in the present study, *C. cyanostigma* (Serranidae), is a new

record of *Anisakis* infection. At present, based on molecular studies, 9 species of *Anisakis* are known, namely *A. simplex* s. s., *A. pegreffii*, *A. simplex C*, *A. typica*, *A. ziphidarum*, *A. nascettii*, *A. physeteris*, *A. brevispiculata*, and *A. paggiae* [21,22].

A. typica has been reported from numerous marine fish worldwide. The parasite was reported from marine fish in Korea, Japan, China, Portugal, Taiwan, Brazil, Western Indonesia, Morocco, Papua New Guinea, Adriatic Sea of Croatia, Mauritania, and some countries at Mediterranean Sea [20,25,34-37, 40-47]. The existence of *A. typica* from the Portuguese coast may have extended the distribution of this parasite to cold water. However, the infection level of the parasite was very low. Therefore, it might be possible that the fish may accidently infected through the food chain originating from warm waters. Marques et al. [45] stated that the Portuguese coast is a transition between North-Eastern Atlantic warmer temperate and cold temperate regions so that it might provide an area of species overlap and hence could promote accidental infection.

High prevalence of *Anisakis* was found in migratory fish, *K. pelamis* and *A. thazard*. High prevalence of *Anisakis* infection was also reported from some marine fish in southern coast of Kulon Progo, Yogyakarta [26]. The prevalence of *Anisakis* sp. infection was generally higher in bigger fish than in smaller ones [19,25,48]. The same result was found in this study that small *A. thazard* were not infected with *Anisakis*, while bigger ones were infected with the prevalence of 47% and the mean intensity of 5.6. This result might be caused by accumulation of the parasites in the big fish due to a long period of infection. Previous reports on *Anisakis* infection in Indonesian waters showed high prevalence of infection with the parasite in some species of marine fish. Hadidjaja et al. [24] reported that the prevalence of *Anisakis* type I larvae in *Rastrelliger kanagurta*, *Decapterus russeli*, and *Sardinella sirm* was 49.3%, 50.3%, and 40.9%, respectively, whereas in the present study no *Anisakis* infection was found in *D. russeli* and only 5% infection in *R. kanagurta*. The difference in the prevalence of infection was also noticed at different locations by Palm et al. [25], and they suggested that the high prevalence of *Anisakis* infection at Northern Balinese coast was due to the high abundance of dolphins, as the final host for *Anisakis*, in that area. A previous study on the ecology of *Pseudoterranova decipiens* in Antarctic waters showed that a high prevalence of infection was in accordance with a high abundance of final hosts as well as intermediate hosts in the area [49].

Anisakiasis has been reported from several countries such as Japan, Korea, and some countries in Europe. *Anisakis* may infect humans and causes anisakiasis after consuming raw infected fish or other marine organisms that function as intermediate hosts. The first report of anisakiasis was from a patient in the Netherlands who had gastrointestinal problems due to *A. simplex* infection. Most cases of anisakiasis in Europe and Japan have been reported to be caused by *Anisakis* type I, particularly *A. simplex* [4]. However, anisakiasis due to *A. pegreffii infection* has also been reported from humans in Italy [50,51]. *A. simplex* might penetrate and migrate to fish muscle [52], which may explain the higher cases of anisakiasis due to *A. simplex* infection. Anisakiasis due to *A. typica* has not been reported. Umehara et al. [20] reported that *A. typica* so far has only received limited attention and is not widely recognized, thus its zoonotic impact has not been well documented. However, Palm et al. [25] reported that *A. typica* was not only found on the surface of gastrointestinal tract but it might also penetrate muscle of fish. In the present study, though not common, an *Anisakis* larva was observed to migrate into the musculature, indicating that the parasite has the potential to infect humans through consumption of uncooked food. In Indonesia, reports about anisakiasis in humans were suggested by Uga et al. [9] using a seroepidemiological approach of inhabitants in East Java and revealed that about 11% of samples who visited hospital showed positive results for *Anisakis* antibodies. The species of *Anisakis* spp. was not determined, but it might be possible that the parasite was *A. typica* since this species is widely distributed in tropical waters, compared to the well known causative agent of anisakiasis by *A. simplex* and *A. pegreffii* which have limited geographical distribution in cold waters. However, it might also be possible that *Anisakis* spp. could be from imported raw materials. Yoshinaga et al. [53] reported the presence of *A. pegreffii* in amberjack *Seriola dumerili* imported from China to Japan as mariculture seedlings.

In conclusion, based on PCR-RFLP and sequencing, all the *Anisakis* examined were *A. typica*, indicating the predominance of this species in the Southern Makassar Strait, Indonesia. Sequencing and phylogenetic tree analyses of *Anisakis* type I in ITS1-5.8S-ITS2 and mtDNA *cox2* regions showed that the present samples were in the same cluster as *A. typica* published in GenBank. However, differences of 4 bp in nucleotides in ITS1 region and broad divergence consisting of 2 subgroups in the mtDNA *cox2* of *Anisakis* from *K. pelamis* and *A. thazard* indicated the existence of *A. typica* sibling species in that area.

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CONFLICT OF INTEREST

There is no conflict of interest related with this study.

REFERENCES

1. Ministry of Marine Affair and Fisheries of Indonesia. Marine and fisheries in figures. Center for data statistics and information. 2011, p 1-120.
2. Mattiucci S, Nascetti G, Dailey M, Webb SC, Barros NB, Cianchi R, Bullini L. Evidence for a new species of *Anisakis* (Dujardin, 1845): morphological description and genetic relationships between congeners (Nematoda: Anisakidae). Syst Parasitol 2005; 61: 157-171.
3. Sluiters JF. *Anisakis* sp. larvae in the stomachs of herring (*Clupea harengus* L.). Z Parasitenkd 1974; 44: 279-288.
4. Smith JW, Wooten R. *Anisakis* and anisakiasis. Adv Parasitol 1978; 16: 93-163.
5. Foti C, Nettis E, Cassano N, DI Mundo I, Vena GA. Acute allergic reactions to *Anisakis simplex* after ingestion of anchovies. Acta Derm Venereol 2002; 82: 12-123.
6. Noh JH, Kim BJ, Kim SM, Ock MS, Park MI, Goo JY. A case of acute gastric anisakiasis provoking severe clinical problems by multiple infection. Korean J Parasitol 2003; 41: 97-100.
7. Rosales JM, Mascaró C, Fernandez C, Luque F, Moreno MS, Parras L, Cosano A, Muñoz JR. Acute intestinal anisakiasis in Spain: a fourth-stage *Anisakis simplex* larva. Mem Inst Oswaldo Cruz 1999; 94: 823-826.
8. Sakanari JA, Mckerrow JH. Anisakiasis. Clin Microbiol Rev 1989; 2: 278-284.
9. Uga S, Ono K, Katokan N, Hasan H. Seroepidemiology of five major zoonotic parasite infections in inhabitants of Sidoarjo, East Java, Indonesia. Southeast Asian J Trop Med Public Health 1996; 2: 556-561.
10. Berland B. Nematodes from some Norwegian marine fishes. Sarsia 1961; 2: 1-50.
11. Chenoweth JF, McGladdery SE, Sindermann CJ, Sawyer TK, Bier JW. An investigation into the usefulness of parasites as tags for herring (*Clupea harengus*) stocks in the Western North Atlantic, with emphasis on use of the larval nematode *Anisakis simplex*. J Northw Atl Fish Sci 1986; 7: 25-33.
12. Mattiucci S, Abaunza P, Ramadori L, Nascetti G. Genetic identification of *Anisakis* larvae in European hake from Atlantic and Mediterranean waters for stock recognition. J Fish Biol 2004; 65: 495-510.
13. Mattiucci S, Abaunza P, Damiano S, Garcia A, Santos MN, Nascetti G. Distribution of *Anisakis* larvae, identified by genetic markers, and their use for stock characterization of demersal and pelagic fish from European water: an update. J Helminthol 2007; 81: 117-127.
14. Mattiucci S, Nascetti G. Advances and trends in the molecular systematics of anisakid nematodes, with implications for their evolutionary ecology and host-parasite co-evolutionary processes. Adv Parasitol 2008; 66: 47-148.
15. Podolska M, Horbowy J, Wyszynski M. Discrimination of Baltic herring populations with respect to *Anisakis simplex* larva infection. J Fish Biol 2006; 68: 1241-1256.
16. Abe N, Tominaga K, Kimata I. Usefulness of PCR-restriction fragment length polymorphism analysis of the internal transcribed spacer region of rDNA for identification of *Anisakis simplex* Complex. Jpn J Infect Dis 2006; 59: 60-62.
17. Pontes T, D'Amelio S, Costa G, Paggi L. Molecular characterization of larval anisakid nematodes from marine fishes of Madeira by a PCR-based approach, with evidence for a new species. J Parasitol 2005; 91: 1430-1434.
18. Quiazon KMA, Yoshinaga T, Santos MD, Ogawa K. Identification of larval *Anisakis* spp. (Nematoda: Anisakidae) in Alaska pollock (*Theragra chalcogramma*) in northern Japan using morphological and molecular markers. J Parasitol 2009; 95: 1227-1232.
19. Setyobudi E, Jeon CH, Lee CH, Seong KB, Kim JH. Occurrence and identification of *Anisakis* spp. (Nematoda: Anisakidae) isolated from chum salmon (*Oncorhynchus keta*) in Korea. Parasitol Res 2011; 108: 585-592.
20. Umehara A, Kawakami Y, Ooi HK, Uchida A, Ohmae H, Sugiyama H. Molecular identification of *Anisakis* type I larvae isolated from hairtail fish off the coasts of Taiwan and Japan. Int J Food Microbiol 2010; 143: 161-165.
21. Mattiucci S, Farina V, Campbell N, MacKenzie K, Ramosd P, Pinto AL, Abaunza P, Nascetti G. *Anisakis* spp. larvae (Nematoda: Anisakidae) from Atlantic horse mackerel: their genetic identification and use as biological tags for host stock characterization. Fish Res 2008; 89: 146-151.
22. Mattiucci S, Paoletti M, Webb SC. *Anisakis nascettii* n. sp. (Nematoda: Anisakidae) from beaked whales of the southern hemisphere: morphological description, genetic relationships between congeners and ecological data. Syst Parasitol 2009; 74: 199-217.
23. Quiazon KMA, Yoshinaga T, Ogawa K, Yukami R. Morphological differences between larvae and in vitro-cultured adults of *Anisakis simplex* (sensu stricto) and *Anisakis pegreffii* (Nematoda: Anisakidae). Parasitol Int 2008; 57: 483-489.
24. Hadidjaja P, Ilahude HD, Mahfudin B, Malikusworo H. Larvae

of Anisakidae in marine fish of coastal waters near Jakarta, Indonesia. Am J Trop Med Hyg 1978; 27: 51-54.
25. Palm HW, Damriyasa IM, Linda, Oka IBM. Molecular genotyping of *Anisakis* Dujardin, 1845 (Nematoda: Ascaridoidea: Anisakidae) larvae from marine fish of Balinese and Javanese waters, Indonesia. Helminthologia 2008; 45: 3-12.
26. Setyobudi E, Soeparno, Helmiati S. Infection of *Anisakis* sp. larvae in some marine fishes from the southern coast of Kulon Progo, Yogyakarta. Biodiversitas 2011; 12: 34-37.
27. D'Amelio S, Mathiopoulos KD, Santos CP, Pugachev ON, Webb SC, Picanço M, Paggi L. Genetic markers in ribosomal DNA for the identification of members of the genus *Anisakis* (Nematoda: Ascaridoidea) defined by polymerase-chain-reaction-based restriction fragment length polymorphism. Int J Parasitol 2000; 30: 223-226.
28. Anderson RC. Nematode parasites of vertebrates. Their development and transmission, 2nd ed. Wallingford, UK. CABI Publishing International. 2000, p 1-650.
29. Bush AO, Lafferty KD, Lotz JM, Shostak AW. Parasitology meets ecology on its own terms: Margolis et al. revisited. J Parasitol 1997; 83: 575-583.
30. Zhu XQ, D'Amelio S, Paggi L, Gasser RB. Assessing sequence variation in the internal transcribed spacers of ribosomal DNA within and among members of the *Contracaecum osculatum* complex (Nematoda: Ascaridoidea: Anisakidae). Parasitol Res 2000; 86: 677-683.
31. Nadler SA, Hudspeth DSS. Phylogeny of the Ascaridoidea (Nematoda: Ascaridida) based on three genes and morphology: hypotheses of structural and sequence evolution. J Parasitol 2000; 86: 380-393.
32. Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG. The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res 1997; 25: 4876-4882.
33. Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol Biol Evol 2011; 28: 2731-2739.
34. Koinari M, Karl S, Elliot A, Ryan U, Lymbery AJ. Identification of *Anisakis* species (Nematoda: Anisakidae) in marine fish hosts from Papua New Guinea. Vet Parasitol 2012; 193: 126-133.
35. Blouin MS, Yowell CA, Courtney CH, Dame JB. Substitution bias, rapid saturation, and the use of mtDNA for nematode systematics. Mol Biol Evol 1998; 15: 1719-1727.
36. Farjallah S, Busi M, Mahjoub MO, Slimane BB, Paggi L, Said K, D'Amelio S. Molecular characterization of larval anisakid nematodes from marine fishes off the Moroccan and Mauritanian coasts. Parasitol Int 2008; 57: 430-436.
37. Farjallah S, Slimane BB, Busi M, Paggi L, Amor N, Blel H, Said K, D'Amelio S.. Occurrence and molecular identification of *Anisakis* spp. from the North African coasts of Mediterranean Sea. Parasitol Res 2008; 102: 371-379.
38. Szostakowska B, Myjak P, Kur J. Identification of anisakid nematodes from the Southern Baltic Sea using PCR-based methods. Mol Cell Probes 2002; 16: 111-118.
39. Mattiucci S, Paggi L, Nascetti G, Portes Santos C, Costa G, Di Beneditto AP, Ramos R, Argyrou M, Cianchi R, Bullini L. Genetic markers in the study of *Anisakis typica* (Diesing, 1860): larval identification and genetic relationships with other species of *Anisakis* Dujardin, 1845 (Nematoda: Anisakidae). Syst Parasitol 2002; 51: 159-170.
40. Chai JY, Chu YM, Sohn WM, Lee SH. Larval anisakids collected from the Yellow Corvina in Korea. Korean J Parasitol 1986; 24: 1-11.
41. Chen Q, Yu HQ, Lun ZR, Chen XG, Song HQ, Lin RQ, Zhu XQ. Specific PCR assays for the identification of common anisakid nematodes with zoonotic potential. Parasitol Res 2008; 104: 79-84.
42. Costa G, Pontes T, Mattiucci S, D'Amelio S. The occurrence and infection dynamics of *Anisakis* larvae in the black-scabbard fish, *Aphanopus carbo*, chub mackerel, *Scomber japonicus*, and oceanic horse mackerel, *Trachurus picturatus* from Madeira, Portugal. J Helminthol 2003; 77: 163-166.
43. Iniquez AM, Santos CP, Vicente ACP. Genetic characterization of *Anisakis typica* and *Anisakis physeteris* from marine mammals and fish from the Atlantic Ocean off Brazil. Vet Parasitol 2009; 165: 350-356.
44. Lee MH, Cheon DS, Choi C. Molecular genotyping of *Anisakis* species from Korean sea fish by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). Food Contr 2009; 20: 623-626.
45. Marques JF, Cabral HN, Busi M, D'Amelio S. Molecular identification of *Anisakis* species from Pleuronectiformes off the Portuguese coast. J Helminthol 2006; 80: 47-51.
46. Smrzlic IV, Valic D, Kapetanovic D, Kurtovic B, Teskeredzic. Molecular characterisation of Anisakidae larvae from fish in Adriatic Sea. Parasitol Res 2012; 111: 2385-2391.
47. Zhu XQ, Podolska M, Liu JS, Yu HQ, Chen HH, Lin ZX, Luo CB, Song HQ, Lin RQ. Identification of anisakid nematodes with zoonotic potential from Europe and China by single-strand conformation polymorphism analysis of nuclear ribosomal DNA. Parasitol Res 2007; 101: 1703-1707.
48. Costa G, Madeira A, Pontes T, D'Amélio S. Anisakid nematodes of the blackspot seabream, *Pagellus bogaraveo*, from Madeiran waters, Portugal. Acta Parasitol 2004; 49: 156-161.
49. Palm HW. Ecology of *Pseudoterranova decipiens* (Krabbe, 1878) (Nematoda: Anisakidae) from Antarctic waters. Parasitol Res 1999; 85: 638-646.
50. Fumarola L, Monno R, Lerardi E, Rizzo G, Giannelli G, Lalle M, Pozio E. *Anisakis pegreffii* etiological agent of gastric infections in two Italian women. Foodborne Pathog Dis 2009; 6: 1157-1159.
51. Mattiucci S, Paoletti M, Borrini F, Palumbo M, Palmieri RM, Gomes V, Casati A, Nascetti G. 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 2011; 11: 82.

52. Quiazon KMA, Yoshinaga T, Ogawa K. Experimental challenge of *Anisakis simplex* sensu stricto and *Anisakis pegreffii* (Nematoda: Anisakidae) in rainbow trout and olive flounder. Parasitol Int 2011; 60: 126-131.

53. Yoshinaga T, Kinami R, Hall KA, Ogawa K. A preliminary study on the infection of anisakid larvae in juvenile greater amberjack *Seriola dumerili* imported from China to Japan as mariculture seedlings. Fish Pathol 2006; 41: 123-126.