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Occurrence of *Anisakis* Larvae in Commercial Fish along the Northern Coast of Taiwan

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ABSTRACT

Anisakis larvae of three commercial fish species, including *Trichiurus lepturus*, *Scomber japonicus* and *Trachurus japonicus*, found in the ocean North of Taiwan, were examined. The morphology of the larvae was analyzed using light and scanning electron microscopes, the population dynamics (including prevalence, mean intensity and abundance) were studied and the molecular characteristics of the Anisakid nematodes were identified. The annual prevalence of the larvae in *T. lepturus*, *S. japonicus* and *T. japonicus* were 91, 39 and 89%, respectively. Using light microscopy and scanning electron microscopy demonstrated that the worms in the three species of fish showed no difference in morphological features. The Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) indicated that the larvae from the three species of fish belonged to a single species. However, using phylogenetic analysis in the molecular identification of ribosomal DNA (rDNA) domains ITS-1 (internal transcribed spacer 1) and ITS-2, the larvae in the three species of fish were not of a single species of *Anisakis*.

Key words: *Anisakis*, *Trichiurus lepturus*, *Scomber japonicus, Trachurus japonicus*

INTRODUCTION

Anisakid nematodes are marine parasites that cause allergic reactions and anisakiasis. Anisakid third-stage larvae (L3) have been reported widely in invertebrates, such as squid and in vertebrates, such as fish (Karl *et al*., 1994; Moreno-Ancillo *et al*., 1997). The larvae (L3) are transmitted from crustaceans to squid and fish via the food chain (Klimpel *et al*., 2004), then infect marine mammals and humans (Gardiner, 1990). The prevalence of Anisakid larvae was 100% for all three host species, including minke whales (*Balaenoptera acutorostrata*), porpoises (*Phocoena phocoena*) and long-finned pilot whales (*Globicephala melas*) in the North Atlantic (Ugland *et al*., 2004). All wild-caught Pacific salmon (*Oncorhynchus* spp.) were considered to have *Anisakis simplex* larvae present. The rate of prevalence is more than 75% in fresh U.S. commercial salmon (Myers, 1979). *A. simplex* worms were found in salmon throughout the North Atlantic (Beverley-Burton and Pippy, 1978) and detected as existing in 78-97% of smoked herring *(Clupea harengus*) obtained from a French supermarket (Lagoin, 1980). In Atlantic horse mackerel, *Anisakis* larvae, including *A. simplex s. str., A. pegreffii, A. physeteris, A. typica* and *Anisakis* spp., were found (Mattiucci *et al*., 2008). In the Mediterranean waters of coastal North Africa, several *Anisakis* sibling and morphospecies, such as *A. simplex s. str., A. pegreffii, A. physeteris, A. typica*

and *A. simplex s. str., A. pegreffii* hybrids, coexisted (Farjallah *et al*., 2008; Meloni *et al*., 2011). In Northern Japan and the sea areas in Korea, four *Anisakis* spp., including *A. simplex s. str., A. pegreffii, A. brevispiculata* and *Anisakis* spp. (Type II), were detected in marine fish. *A simplex s. str*. formed the majority of *Anisakis* spp. (Quiazon *et al*., 2009; Sohn *et al*., 2014). High prevalence of Anisakidae larvae infection was reported in fish from in the East China Sea (Kong *et al*., 2015). The specific identification of Anisakid larvae L3 was complex, because few morphological characteristics could be used for identification in the larval stage. The morphological features observed using Light Microscopy (LM) and Scanning Electron Microscopy (SEM) were the common characteristics used for larval identification (Pascual *et al*., 1999; Weerasooriya *et al*., 1986). Using recent molecular techniques, the assay with Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) amplification of the internal transcribed spacer 1 and 2 (ITS-1 and ITS-2) fragments of ribosomal DNA (rDNA) was used as a test for identifying the worm species (Kijewska *et al*., 2000; Pontes *et al*., 2005). Discrimination among the sibling species of *A. simplex* was performed using PCR-RFLP or sequencing analysis of domain ITS (Abe, 2008).

Anisakid larvae infestation of fresh commercial fish from Taiwan remains indistinct. In this work, the infection of *Anisakis* larvae was surveyed in three commercial fish commonly found in Northern Taiwan waters. Data on the prevalence, mean intensity, abundance, morphological features (LM and SEM) and molecular studies in Anisakid worms in these commercial fish were provided in this study for primary surveying of the infestation and species constitution of the worms.

MATERIALS AND METHODS

Six fish specimens from each of *Trichiurus lepturus* (Linnaea 1758), *Scomber japonicus* (Houttuyn 1782) and *Trachurus japonicus* (Temminck and Schlegel 1844) were collected from a selected fisherman at monthly intervals from the ocean around Keelung Islet, Northern Taiwan (25.1 N- 25.5 N, 121.6 E-122.0 E). Immediately on capture, the live fish were placed in a marine water tank and sent to the laboratory. Each fish was weighed and measured for preanal length (distance between the tip of the upper jaw to the anus) and total length (distance from the tip of the snout to the tip of the longer lobe of the caudal fin). All organs were examined for Anisakine nematodes by using a magnifier with illumination. The prevalence, mean intensity and abundance of Anisakid were studied. The data was statistically analyzed with Scheffe's test.

Morphological features: The worm specimens were washed, stored in a saline solution (0.9% NaCl) at 4°C overnight and then examined or fixed. The live larvae were examined under a light microscope. Additional larvae were primarily fixed with 2.5% glutaraldehyde and 1.6% paraformaldehyde in a 0.1 M phosphate buffer (pH 7.2). After being washed with a phosphate buffer, the specimens were refixed with osmium tetroxide, washed again with a phosphate buffer, dehydrated in an ethanol series, critical-point-dried in CO_2 by using a critical-point dryer (HCP-Z, Hitachi) and coated in a sputter coater (E101, Hitachi). The larvae were examined using an SEM (S-2400, Hitachi) operated at 15 kV.

rDNA cloning and sequence: Anisakid DNAs were isolated from the worms by using a blood-and-tissue genomic mini-kit (Viogene), as Volgelstein and Gillespie (1979) described. Internal Transcribed Spacer (ITS) domains were extracted from the worms from *T. lepturus, S. japonicus*

and *T. japonicus*. For cloning, the first and second internal transcribed spacers (ITS-1, ITS-2) of ribosomal DNA (rDNA) were individually amplified by Polymerase Chain Reaction (PCR) with primers NC5 (forward: 5'-GTAGGTGAACCTGCGGAAGGATCATT-3') and NC2 (reverse: 5'-TTAGTTTCTTTTCCTCCGCT-3') (Zhu *et al*., 2000). In summary, the transcription was conducted using deoxynucleoside triphosphate (dNTP) (10 mM) 2 μL, primer NC5 (25 mM) 2 μL, primer NC2 (25 mM) 2 μL, $MgCl_2$ (25 mM) 6 μL, 10×buffer (Mg free) 5 μL, H_2O 30 μL, Tag (5 U μL^{-1}) 1 μL and genomic DNA (8 μg μL^{-1}) 2 μL as a template. A 50 μL aliquot of the reaction mixture was performed using the following conditions: 94°C for 5 min, 60°C for 30 sec and 72°C for 90 sec in the first cycle; 94°C for 30 sec, 60°C for 30 sec and 72°C for 30 sec in the second reaction with 30 cycles and the final cycle 94°C for 30 sec, 60°C for 30 sec and 72°C for 5 min.

The ITS domains were extracted from five worms in the three species of fish by using PCR. Two domains, named TL1 and TL2, were separated from the two worms in *T. lepturus*; two domains, named SJ1 and SJ2, from the two worms in *S. japonicus*; and one domain, named TJ1, from a worm in *T. japonicus*.

The domains were analyzed using Restriction Fragment Length Polymorphism (RFLP) in a 15 μL aliquot of rDNA 10 μL, 10x reaction buffer 1.5 μL, H_2O 3 μL and restriction enzymes (0.5 μL), including *EcoR* I, *Hha* I, *Rsa* I, *Hinf* I and *Taq*I in 37°C for 4 h. The products from RFLP were analyzed using 2% agarose. The PCR products were quantified using agarose gel electrophoresis and sequenced using an ABI PRISM 377-96 DNA sequencer with an ABI PRISM Bigdye Terminator Cycle Sequencing Ready Reaction Kit at Mission Biotech, Taipei. The ITS domains and its homology from the different worms were compared using Genome Net (http://clustalw.genome.jp/) to examine the Anisakid groups and phylogenesis.

RESULTS

Morphological features: Among the three examined species of fish, *T. lepturus* was the most substantial in the preanal length, ca. 269 mm, followed by *S. japonicus* and *T. japonicus*, ca. 175 mm and ca. 122 mm, respectively (Fig. 1). A distinct stomach was observed in the worm body of *Anisakis* spp. by using light microscopy (Fig. 2). The average worm lengths in the three species

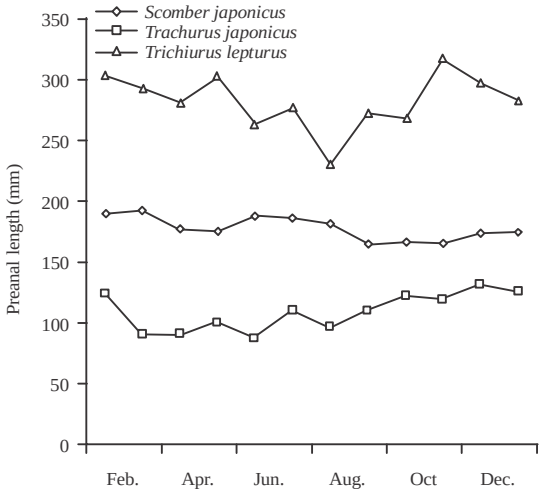

Fig. 1: Monthly preanal length of *Scomber japonicus*, *Trichiurus lepturus* and *Trachurus japonicus*

Fig. 2: Light micrograph of *Anisakis* larva L3 with ventriculus (arrow)

Table 1: Annual infection of *Anisakis* worms in *Scomber japonicus*, *Trachurus japonicus* and *Trichiurus lepturus*

Parameters	*Scomber japonicus*	*Trachurus japonicus*	*Trichiurus lepturus*
Prevalence (%)	91	39	89
Mean intensity (worms)	39	2	62
Abundance (worms)	62	3	80
Worm length (mm)	18.7±5.2[a]	14.9±6.2[b]	18.9±3.5[a]

*Means with the same letter are not significantly different at p<0.05 with Scheffe's test

of fish, *T. lepturus*, *S. japonicus* and *T. japonicas*, were 18.9, 18.7 and 15.5 mm, respectively (Table 1). The worms were found in the three species on the external wall of the stomach, on the gonads and in the mantle cavity. Two forms of worm, including active-wriggling and static-coiling, were found. During scanning electron microscopy examination, striation was observed along the entire body length of the worms (Fig. 3a). The worms exhibited trilobed dorsal lips and bilobed ventrolateral lips around the mouth. Each lip exhibited indistinct papilla. A boring tooth appeared in the mouth anteroventrally projecting (Fig. 3b). Between the ventrolateral lips, an excretory pore opened from a single excretory duct as a transverse slit (Fig. 3c). The postanal tail was round and with a terminal mucron (Fig. 3d). The worms from the three species of fish showed the same morphological features when light microscopy and scanning electron microscopy were used.

Occurrence: The prevalence of *Anisakis* larvae was the highest (91%) in *S. japonicus*, followed by 89% in *T. lepturus* and 39% in *T. japonicus*. The annual mean intensity (62 worms) and the abundance (80 worms) of the *Anisakis* in *T. lepturus* were the highest among the three species of fish (Table 1). The prevalence in *T. japonicus* was low in spring season (April to June) and no infection in fall (August to October). No infection was found in the *T. lepturus* collected in August (Table 2), although the prevalence in *S. japonicus* was 100%. Monthly mean intensities and abundances of the larvae in *S. japonicus* and *T. lepturus* were higher in the period from February to July than in other months (Fig. 4a-b).

ITS domain analysis: Using a PCR-RFLP assay, 720 and 520 bp were demonstrated after the domains were treated using *EcoR*I; 800 and 590 bp with *Hha*I; 480 bp and 430 bp with *Hinf*I; 550, 300 and 220 bp with *Rsa*I and 440 and 430 bp with *Tag*I (Fig. 5). Using a PCR

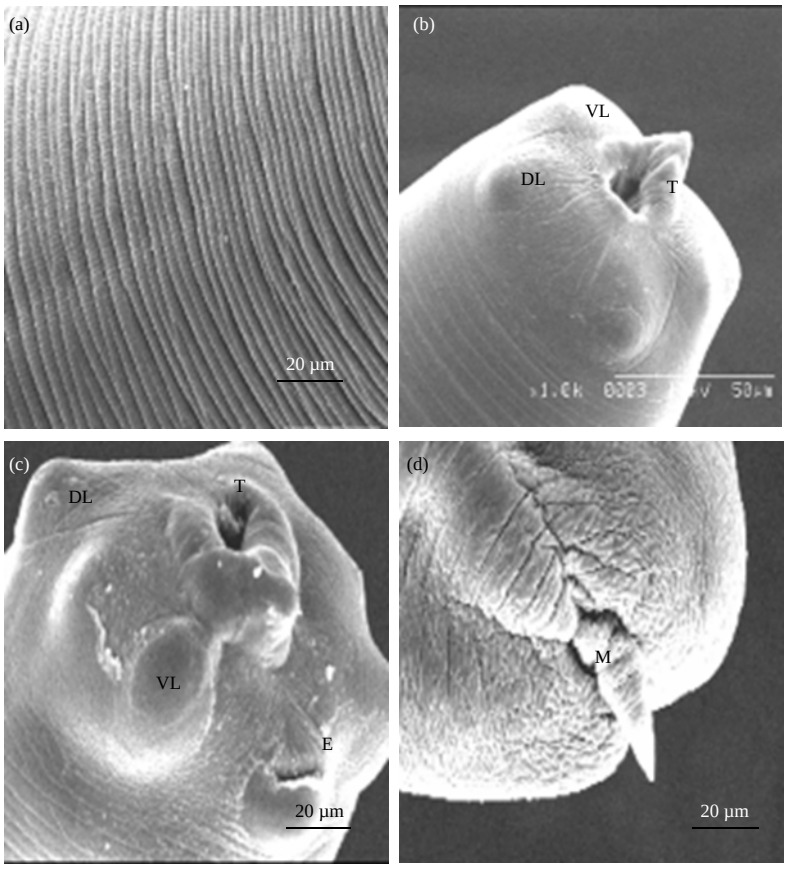

Fig. 3(a-d): Scanning electron micrographs of *Anisakis* worms (a) Skin with transverse striation, (b and c) Anterior extremity and (d) Posterior end, DL: Dorsal lip, VL: Ventrolateral lip, E: Excretory pore, T: Tooth, M: Mucron

Table 2: Monthly prevalence of *Anisakis* worms in *Scomber japonicus*, *Trachurus japonicus* and *Trichiurus lepturus*

	Prevalence (%)		
	Scomber japonicus	*Trachurus japonicus*	*Trichiurus lepturus*
Feb	80	75	100
Mar	80	37.5	100
Apr	100	33	100
May	100	50	100
Jun	80	17	100
Jul	100	67	100
Aug	100	0	0
Sep	100	0	85
Oct	67	0	100
Nov	100	83	100
Dec	100	83	100
Jan	83	17	83
Mean	91	39	89

*Prevalence = fish infected/fish examined

procedure, ITS domains TL1, TL2, SJ1, SJ2 and TJ1 were obtained, demonstrating 898, 898, 909, 907 and 832 nt, respectively (Fig. 6). There was a marked similarity of the domain sequences between TL1 and TL2 (99%) and between SJ1 and SJ2 (98%). The domain TJ1 sequence

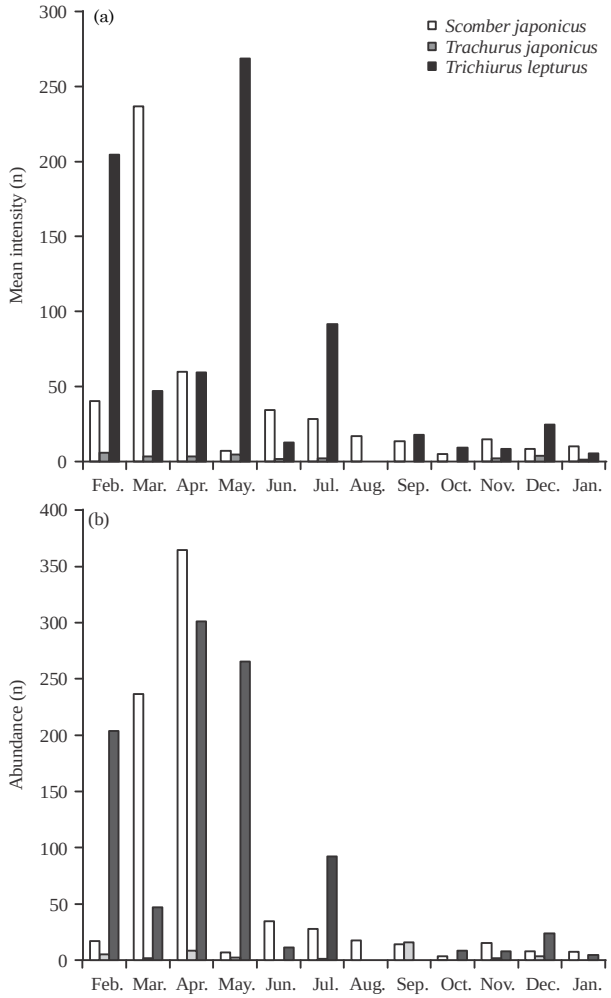

Fig. 4(a-b): Monthly infections of *Anisakis* worms in *Scomber japonicus*, *Trichiurus lepturus* and *Trachurus japonicus* (a) Mean intensity and (b) Mean abundance

Table 3: Homologous comparison (%) of the internal transcribed spacers of ribosomal DNA of *Anisakis* worms from *Trichiurus lepturus* (domains: TL1 and TL2), *Scomber japonicus*, (domains: SJ1 and SJ2) and *Trachurus japonicus* (domain: TJ1) with *Anisakis simplex, Hysterothylacium aduncum* and *Contracaecum osculatum*

Correlation parameters	*A. simplex*	*H. aduncum*	*C. osculatum*	TL1	TL2	SJ1	SJ2	TJ1
A. simplex	100.00	52.85	49.71	19.45	18.37	70.03	69.49	20.18
H. aduncum		100.00	53.20	15.17	14.77	44.69	44.01	21.02
C. osculatum			100.00	15.95	15.57	47.00	46.33	20.18
TL1				100.00	99.0	13.96	15.07	55.22
TL2					100.00	13.97	15.08	55.23
SJ1						100.00	97.78	19.57
SJ2							100.00	15.20
TJ1								100.00

1: The ITS domains and its homology from the different worms were compared using Genome Net (http://clustalw.genome.jp/). 2: ITS domains of *Anisakis simplex, Hysterothylacium aduncum* and *Contracaecum osculatum* cited from (Zhu *et al*., 2000)

was significantly different from domains TL1, TL2, SJ1 and ST2 (Table 3). Domains SJ1 and SJ2 exhibited a 70% homology to the domain of *A. simplex* and had a 44-47% similarity to that of

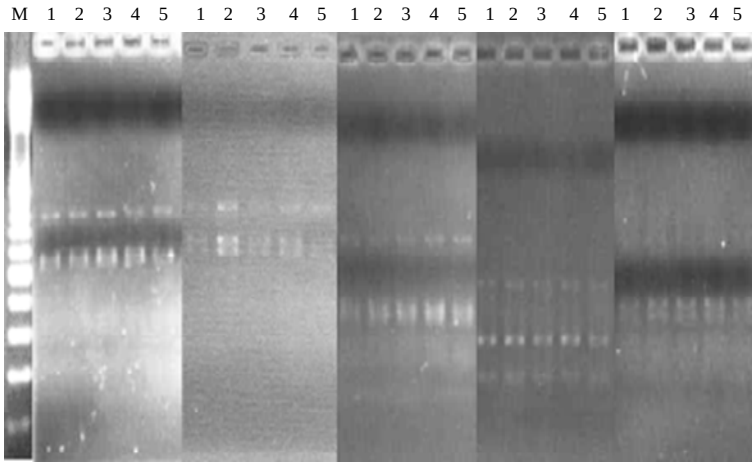

Fig. 5: Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) analysis of *Anisakis* worms from *Trichiurus lepturus* (Lane 1: TL1 and Line 2: TL2), *Scomber japonicus*, (Lane 3: SJ1 and Line 4: SJ2) and *Trachurus japonicus* (Lane 5: TJ1). Five restriction enzymes including *Eco*RI, *Hha*I, *Hinf*I, *Rsa*I and *Taq*I were used. M, molecular weight marker (100 bp interval)

H. aduncum and *C. osculatum*. Phylogenetic analysis revealed the various groups found in the five worms from the three species of fish.

DISCUSSION

Morphological features: A significant difference was found in length among the worms from the three species of fish. *T. japonicus* was the shortest among the three (Fig. 1), containing the shortest worms (Table 1). Mean intensity and abundance in *T. lepturus* and *S. japonicus* were considerably higher than in *T. japonicus* (Table 2). In the three species of fish, larval infection had a positive correlation with fish size. As in a single species, an increase in parasitization with increasing host length was reported in European hake (Valero *et al*., 2006). The larval burden was strongly related to host length in the species, common blue scad mackerel (Manfredi *et al*., 2000). However, among various larger fish showed greater degrees of worm infection and had larger worms than smaller fish.

Two forms of the worms, including active-wriggling and static-coiling, were found in the three species on the external wall of the stomach, on the gonads and in the mantle cavity. The great majority of *Anisakis* spp. larvae were located in the cavity, only a minor part in the muscle (Piras *et al*., 2014).

Anisakis had a marked stomach (Fig. 2), a boring tooth in the mouth, an excretory pore between the ventrolateral lips and a mucron in the posterior end (Fig. 3c-d), but no caeca. The larval specimens from the three species of fish had no morphological difference when examined using light microscopy and scanning electron microscopy.

Occurrence: Infections of *Anisakis* larvae in *T. lepturus* and *S. japonicus* were considerable, approximately 90 %, although the infection in *T. japonicus* was only 39 %. The mean intensity and abundance were extreme in *T. lepturus* and *S. japonicus* (Table 1). The prevalence of Anisakidae larvae infection was 55.4% in *S. japonicus* caught off Korea (Bak *et al*., 2014). *Scomber japonicus*

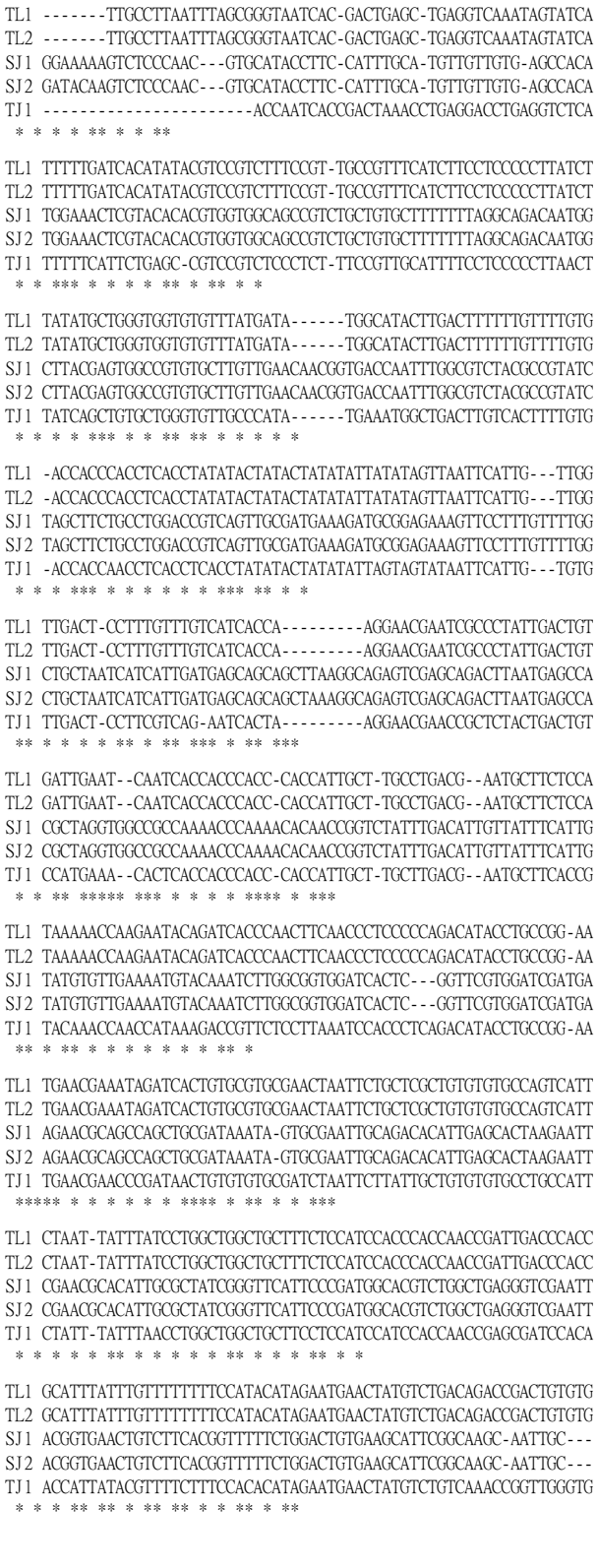

Fig. 6: Continue

Fig. 6: Comparison in the internal transcribed spacers (ITS domains) of ribosomal DNA of *Anisakis* worms from *Trichiurus lepturus* (TL1 and TL2), *Scomber japonicus*, (SJ1 and SJ2) and *Trachurus japonicus* (TJ1)

and *T. lepturus* survives on small fish, squid and crustaceans. Anisakid third-stage larvae (L3) were found widely in squid (Pascual *et al*., 1999). All squid likely carry and transmit anisakines (Guerra *et al*., 1993). Sticklebacks *Gasterosteus aculeatus*, were naturally infected with larvae of *A. simplex* in brackish coastal waters through eating either a crustacean host or third-stage larvae (L3) from fish (Koie, 2001). Pelagic and mesopelagic fish and invertebrates were considered intermediate or paratenic hosts of Anisakid larvae L3 (Klimpel *et al*., 2007). L2 larvae in small fish, squid and crustaceans developed into L3 worms. L3 larvae infected *T. lepturus* and *S. japonicus* when the fish consumed small fish, squid and crustaceans. The study found a notably higher prevalence of Anisakidae larvae infection in *T. lepturus* and *S. japonicus* along the northern coast of Taiwan.

ITS domain analysis: The PCR amplification of ITS1 and ITS2 regions, followed by RFLP, was used to distinguish species of the genera *Anisakis* and *Pseudoterranova* (La Rosa *et al*., 2006). When examined using PCR-RFLP assay, the larvae from the three species of fish had the same pattern in gel electrophoresis (Fig. 7). The morphological characteristics and PCR-RFLP assay

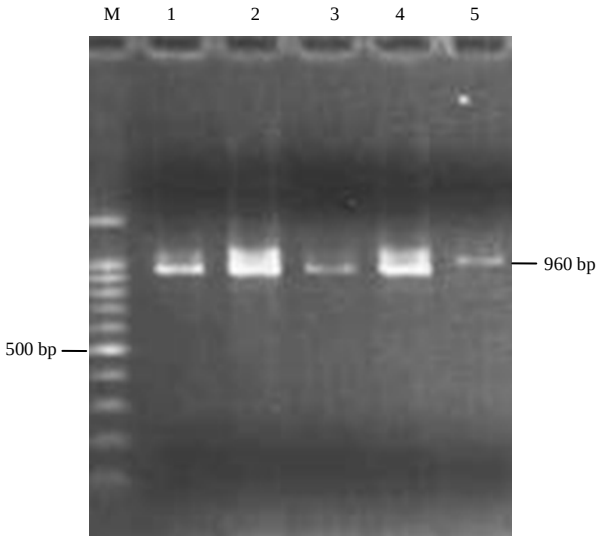

Fig. 7: Polymerase Chain Reaction (PCR) gel electrophoresis of genomic DNA of *Anisakis* worms from *Trichiurus lepturus* (Lane 1: TL1 and Line 2: TL2), *Scomber japonicus*, (Lane 3: SJ1 and Line 4: SJ2) and *Trachurus japonicus* (Lane 5: TJ1). M: Molecular weight marker (100 bp interval)

showed that the larvae in the three species of fish constituted one genus of *Anisakis*. The molecular assay was used for identification of *A. simplex* (Kijewska *et al*., 2000). In this work, sequences of rDNA fragment ITS-1 and ITS-2 were used for identifying Anisakid larvae from the three species of fish. Larvae from the same fish showed high homologous ITS domains. The domains TL1 and TL2, had the same insert of 898 bp. The domains SJ1 and SJ2 had the insert of 909 and 907 bp, respectively (Fig. 6). The sequence identity between TL1 and TL2 was 99% and that between SJ1 and SJ2 was 97%. The results suggested that the larvae in the same fish constituted a single species. The ITS insert showed that the larvae from the three species of fish were various groups or subspecies. *Anisakis* was not a single species but a complex composed of several sibling species: *A. brevispiculata*, *A. pegreffii*, *A. physeteris*, *A. simplex* s. str., *A. simplex* C, *A. typica* and *Anisakis* spp. (Type II) (Abe, 2008; Chen *et al*., 2008; Farjallah *et al*., 2008; Mattiucci *et al*., 2008; Quiazon *et al*., 2009). The diversity of the *Anisakis* species along the North African coasts of the Mediterranean Sea indicated that several *Anisakis* sibling and morphospecies coexisted (Farjallah *et al*., 2008). In Japan, *S. japonicus* was infected by *A. simplex* and *A. pegreffii* larvae, together with a few larvae of other anisakid species (Arizono *et al*., 2012). *Anisakis typica*, recombinant genotype of *A. simplex* s. s. and *A. pegreffii*, *H. amoyense* and *H. fabri* were identified in East China Sea (Kong *et al*., 2015). The domains SJ1 and SJ2 of the larvae in *S. japonicus* were 70% homologous to that of *A. simplex* (Table 3). The domains TL1, TL2 and TJ1 of the larvae in *T. lepturus* and *T. japonicus* were minimally homologous (ca. 20%) to that of *A. simplex*. The domain TJ1 had a 55% homology to domains TL1 and TL2. Phylogenetic analysis demonstrated that the larvae from *T. lepturus T. japonicus* and *S. japonicus* constituted various groups. This finding supports the hypothesis of host-parasite coevolutionary relationships suggested for *Anisakis* spp. and their cetacean hosts (Mattiucci *et al*., 2009). The fish are possibly infected by different groups of the worm found in its various prey. The worms found in the three species of fish in the ocean around Keelung Islet in Northern Taiwan, might be different subspecies

or sibling species instead of a single species of *Anisakis*. Further studies are necessary for assessing the diversity of larval anisakid nematodes.

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