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ORIGINAL PAPER

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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

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Abstract The helminth fauna of the gastrointestinal tract and abdominal cavity of cutlass fish, *Trichiurus lepturus* L., off the Taiwanese coast of the north-western Pacific was investigated. The following helminths were found: (1) nematodes—*Anisakis simplex*, *Hysterothylacium aduncum*, *Porrocaecum decipiens*, *Raphidascaris trichiuri*; (2) digeneans—adult *Lecithochirium trichiuri*; and (3) cestodes—plerocercoids of *Proteocephalus* spp. The third-stage larvae of these four anisakid nematodes were characterized genetically using a molecular approach. The nuclear ribosomal DNA region spanning the first internal transcribed spacer (ITS-1), the 5.8S gene and the second internal transcribed spacer (ITS-2) was amplified and sequenced. Based on the sequence differences, a PCR-based restriction fragment length polymorphism method was established for the unequivocal delineation of the four species. Phylogenetic analysis showed that *H. aduncum* clustered with *P. decipiens*, whereas *A. simplex* was not closely related to these according to the nucleotide sequences of all rDNA.

Introduction

The cutlass fish, *Trichiurus lepturus* L., is a cosmopolitan coastal species which occurs throughout the tropical and temperate waters of the world between the latitudes 60°N and 45°S (Froese and Pauly 1997), including all Chinese seas (Liu 1996). World harvests are approximately 750,000 tonnes annually. China and Taiwan land about 80% of this catch (Claus 1995), and the cutlass fish is the most important commercial marine fish species in China and Taiwan in terms of weight caught (Luo 1991). Cutlass fish are voracious predators. A variety of prey is present in their diet, including pelagic and benthic species, i.e. large numbers of small fish, zooplanktonic and benthic crustaceans and cephalopods (Martins and Haimovici 1997). High parasitic helminth diversity should thus be predictable. Unfortunately, such information is largely lacking. During an inventory of the digenean fauna of marine fish in Taiwanese waters (Shih et al. 2003), many nematodes of the family Anisakidae at different developmental stages were recorded in *T. lepturus* with a nearly 100% prevalence.

The anisakids of some genera are transmissible to humans, in whom they can cause the significant clinical disease anisakiasis (Van Thiel 1962; Dick et al. 1991). The accurate identification of anisakid nematodes at any life cycle stage is central to the diagnosis of anisakid infections in humans and animals, and is therefore an important component in disease surveillance and control (Zhu et al. 1998a). As a consequence of the limitations in identifying larval stages using morphological characters (Olson et al. 1983; Fagerholm 1988; Dick et al. 1991), or when only part of a nematode is available for identification (Dick et al. 1991), the accurate diagnosis of anisakiasis in different host species has considerable obstacles. Recently, various studies have demonstrated that the internal transcribed spacers (ITS) of nuclear ribosomal DNA (rDNA) provide genetic markers for the accurate and effective identification of parasitic nematodes of the family Anisakidae and Ascarididae (Jacobs et al. 1997; Zhu et al. 1998a, 1998b). Polymerase chain reaction-based methods allowing the amplification of the specific parasite DNA from minute quantities of material have provided the appropriate DNA target sequence for the identification and delineation of many nematode species.

The aims of this study were to inventory the parasitic helminth fauna of *T. lepturus*, to characterize four morphologically well-defined anisakid L3 larvae harvested together from the digestive tracts of *T. lepturus* based on sequences of the first (ITS-1), second (ITS-2) internal transcribed spacers and the 5.8S gene, and to establish a

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Table 1 Parasitic helminths of the cutlass fish, *Trichiurus lepturus* L., from Taiwanese waters, with an indication of stage and location in the host

Parasites	Stage	Location in host
Nematoda		
Anisakis simplex	Larvae III	Stomach, free in abdominal cavity or encysted on mesenteries or liver
Hysterothylacium aduncum	Larvae III	Stomach
	Larvae IV	Stomach
	Adult	Stomach and intestine
Porrocaecum decipiens	Larvae III	Stomach, free in abdominal cavity
Raphidascaris trichiuri	Larvae III	Stomach
	Larvae IV	Stomach
	Adult	Stomach and intestine
Digenea		
Lecithochirium trichiuri	Adult	Intestine
Cestoda		
Proteocephalus spp.	Plerocerocoid	Intestine

PCR-based method for their species-specific identification utilizing genetic markers in the ITS sequences.

Materials and methods

Parasite materials

Eighty specimens of *T. leptures* (preanal length range: 240–260 mm) were obtained from commercial catches in the coastal waters of Ilan, north-eastern Taiwan, between April and August 2003. Fresh specimens were placed on ice and transported to the laboratory. The study of cutlass fish parasites was conducted by examining the stomach, intestine and the body cavity. With practice, helminths may be seen with the naked eye. After washing in physiological saline, recovered digeneans and cestodes were flattened and fixed in 10% formalin. Whole mount flat worm specimens were prepared and stained with Semichon's carmine, dehydrated in an alcohol gradient series, and mounted in Permount balsam (Fisher Scientific, N.J., USA).

Nematode samples were fixed and stored in 70% alcohol. Fresh specimens were examined directly, but stored samples were cleared in glycerine before examination. Anisakid nematodes were identified to species and developmental stage based on the following morphological characters: (1) the shape and the presence of the boring tooth or three lips on the anterior end; (2) the shape of the tail and the presence of the mucron, the caudal spine or the cactus tail; (3) the position of the excretory pore; (4) the length and shape of the ventriculus; and (5) the presence, length and position of the anterior intestinal caecum and posterior ventricular appendix (Olson et al. 1983; Smith 1983; Køie 1993; Anderson 2000; Shih and Jeng 2002).

For scanning electron microscopy, the third-stage larvae of four anisakid species were re-fixed with 1% glutaraldehyde for 12 h and post-fixed with OsO_4 for 1 h. They were then dehydrated, critical point dried, and ion sputter-coated. Coated worms were observed under a scanning electron microscope (Hitachi-S2500, Japan) at 15 kV.

Genomic DNA isolation and polymerase chain reaction

Genomic DNA was isolated and purified from individual larvae using a genomic DNA extraction kit (Bilight, Taiwan) according to the instructions of the manufacture. For amplification of the rDNA region containing ITS-1, 5.8S gene, ITS-2, and ~70 bp of 28S rDNA gene, the previously described (Zhu et al. 1998a) primers NC5 (5′-GTA GGT GAA CCT GCG GAA GGA TCA TT-3′) and NC2 (5′-TTA GTT TCT TTT CCT CCG CT-3′) were used. Each reaction mixture (total volume of 50 µl) contained 2 µl of genomic DNA, 2 U Taq polymerase (Bioline, London, UK), dNTPs (250 µM each), and 100 pmol of each primer. Polymerase chain reactions (PCR) were performed in a thermocycler (Idaho Technology, USA) under the following conditions: after an initial denaturation at 95°C for 2 min; 30 cycles of 95°C for 1 min (denaturation), 55°C for 1 min (annealing), and 72°C for 1 min (extension) was employed; followed by a final extension at 72°C for 5 min. PCR products were checked on 2% agarose gels, stained with ethidium bromide and photographed upon transillumination with a Kodak digital camera (DC290 Zoom). A 100-bp ladder marker (Biotools, Madrid, Spain) was used to estimate the size of the PCR products.

DNA sequencing and sequence analysis

Both spacers and the 5.8S gene were sequenced in both directions from each PCR product using primers NC5 and NC2 and the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Calif., USA). The same cycling conditions employed for primary amplification of the rDNA region from genomic DNA were used for cycle-sequencing.

Sequences were aligned manually to maximize sequence similarity among species, with the 5′ and 3′ ends of the ITS-1, 5.8S and ITS-2 sequences being determined by comparison with those of three anisakid nematodes (Zhu et al. 1998a). Pairwise comparisons were made of the level of sequence differences (*D*) using the formula

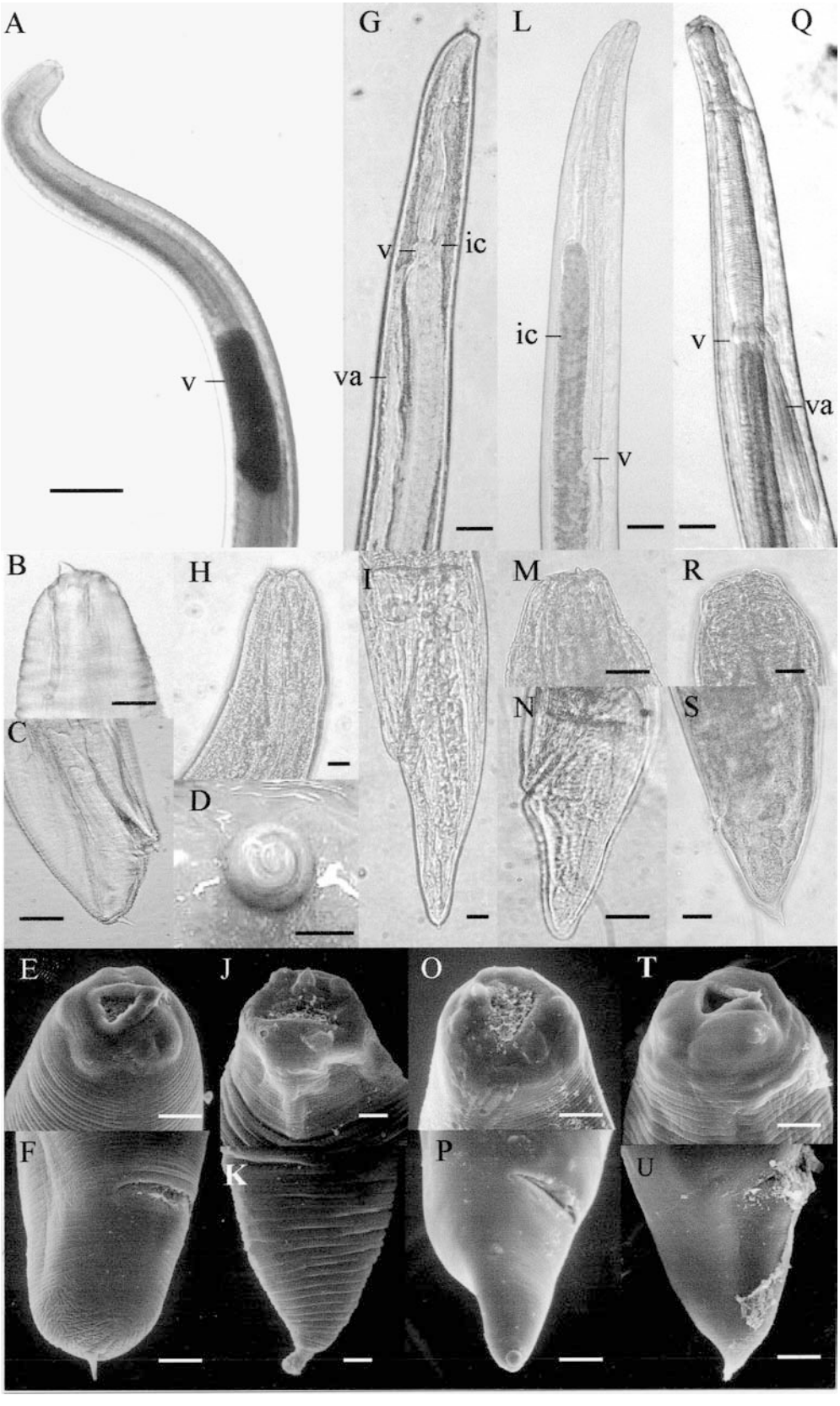

Fig. 1A–U Morphology of third stage larvae of four anisakid nematodes from cutlass fish. **A–F** *Anisakis simplex*; **G–K** *Hysterothylacium aduncum*; **L–P** *Porrocaecum decipiens*; **Q–U** *Raphidascaris trichiuri*. **A**, **G**, **L**, **Q** Anterior ends showing variations in the esophagi. **B**, **E**, **H**, **J**, **M**, **O**, **R**, **T** Anterior extremities showing single boring tooth of each larva. **C**, **F**, **I**, **K**, **N**, **P**, **S**, **U** Posterior extremities showing the mucron or spine or simply blunt end. **D** Encysted *A. simplex* located on mesentery. Micrographs are taken from a stereomicroscope **A**, **D** or phase contrast microscope **B**, **C**, **G–I**, **L–N**, **Q–S**. SEM photos of the anterior and posterior extremities are also shown **E**, **F**, **J**, **K**, **O**, **P**, **T**, **U**. *ic* Intestinal caecum,*v* ventriculus, *va* ventricular appendage. *Scale bars* = 2 mm in **D**, 0.4 mm in **A**, 0.2 mm in **Q**, 0.1 mm in **G**, 0.02 mm in **B**, **C**, **E**, **F**, **L–P**, **T**, **U**, 0.01 mm in **H–K**, **R**, **S**

$D = 1-(M/L)$ (Chilton et al. 1995), where M is the number of alignment positions at which the two species have a base in common, and L is the total number of alignment positions over which the two species are compared. These comparisons were conducted independently for the sequence of each spacer and the 5.8S

Table 2 Lengths (in bp) and G + C content (in %) of the first internal transcribed spacer (ITS-1), 5.8S and second internal transcribed spacer (ITS-2) rDNA sequences of the third stage larvae of *A. simplex*, *H. aduncum*, *P. decipiens* and *R. trichiuri*

	ITS-1		5.8S		ITS-2	
Species	Length	G + C	Length	G + C	Length	G + C
A. simplex	348	50.9	157	51.6	355	47.3
H. aduncum	443	52.8	157	51.0	372	51.3
P. decipiens	440	52.5	157	51.0	366	51.1
R. trichiuri	468	56.6	157	51.0	356	52.0

gene, and combined the sequence data for all three regions.

The nucleotide sequence of all three regions was compared among these anisakids. A boot-strapped tree was generated with the MEGA software (version 2.0). Distance analysis was implemented with a heuristic algorithm. The tree was generated with tree-bisection-reconnection. The data were resampled by 1,000 bootstrap replicates to determine the confidence index within the phylogenetic tree.

Polymerase chain reaction linked restriction fragment length polymorphism

For polymerase chain reaction linked restriction fragment length polymorphism (PCR-RFLP), the amplified DNA fragments (15 μl) were digested directly with 20 units (2 μl) of *Taq*I restriction enzyme (MBI Fermentas, USA) at 65°C for 16 h. Restriction fragments were separated electrophoretically on 2.5% agarose gel and visualized by staining with ethidium bromide. A 100-bp ladder marker (Biotools) was used as a DNA marker.

Results

Six parasite species were found in *T. lepturus* from Taiwanese waters (Table 1). All of these are reported for the first time from this host, providing new host and locality records for: *Anisakis simplex*, *Hysterothylacium aduncum*, *Porrocaecum decipiens* and *Raphidascaris trichiuri* (Nematoda: Anisakidae), *Lecithochirium trichiuri* (Digenea: Hemiuridae) and *Proteocephalus* spp. (Cestoda: Proteocephalidae). Among these four anisakids, three developmental stages including L3, L4 larvae and adults of *H. aduncum* and *R. trichiuri* were harbored in the gastrointestinal tracts of cutlass fish. For *A. simplex* and *P. decipiens*, only their L3 larvae were present either in the stomach or abdominal cavity of the fish. Except for free *A. simplex* L3, encysted L3 were located mainly on mesentery tissue. Each parasitized cutlass fish usually harbored more than two anisakid species. Based on the morphological characters mentioned above, nematodes collected from the GI tracts were identified to species and developmental stage. Morphologically well-defined L3 larvae of the four anisakid species illustrated in Fig. 1 were used for the following studies.

The ITS-1, 5.8S and ITS-2 rDNA sequences were determined for two individual L3 of each anisakid. The characteristics of the sequences for each species are shown in Table 2. The lengths of the ITS-1 and ITS-2 sequences ranged from 348 to 468 bp and 355 to 372 bp, respectively, whereas the 5.8S sequence was 157 bp for all four species. The G + C contents for the three regions of rDNA for all species ranged from 47.3% to 56.6%.

The alignment of the ITS-1, 5.8S and ITS-2 consensus sequences representing *A. simplex*, *H. aduncum*, *P. decipiens* and *R. trichiuri* is shown in Fig. 2. No polymorphism was detected in these sequences. Pairwise comparisons of the ITS-1, 5.8S and ITS-2 sequences are shown in Table 3. Sequence differences between species for the ITS-1 (25.2–52.7%) and ITS-2 (52.3–71.9%) were greater than for the 5.8S gene (0.6–3.8%), except between *H. aduncum* and *P. decipiens*, which was only 1.0% for all rDNA.

Restriction maps were constructed for the ITS-1, 5.8S and ITS-2 sequences of these four species, and several sites existed for a number of common restriction endonucleases, e.g., *Hae*III, *Rsa*I, and *Taq*I (Fig. 3).

Comparison of undigested NC5-NC2 products revealed that *H. aduncum* could be distinguished from the other three species on agarose gels based on a larger product (1,030 bp). But PCR products of the other three species could not be clearly distinguished from each other because the sizes of the products were all between 900 and 1,000 bp, and only slight differences appeared (Fig. 4A). As expected from the restriction maps, PCR-RFLP analysis of NC5-NC2 PCR products with *Taq*I allowed the unequivocal delineation of samples representing the four species (Fig. 4B).

Digestion with *Taq*I produced three fragments of 380 bp, 340 bp and 100 bp for *A. simplex*. Three fragments of 320 bp, 170 bp and 120 bp were detected for *H. aduncum*. Three fragments of 310 bp, 250 bp and 120 bp were resolved for *P. decipiens,* and two bands of 350 bp and 150 bp were found for *R. trichiuri*, the larger of which was more evident since it was composed of two fragments with almost the same size, as expected from the restriction map.

The relationship between these anisakid sequences was examined in a phylogenetic analysis (Fig. 5). *H. aduncum* and *P. decipiens* were in the same group with a bootstrap value of 100. *R. trichiuri* was grouped with these two species, but *A. simplex* was not closely

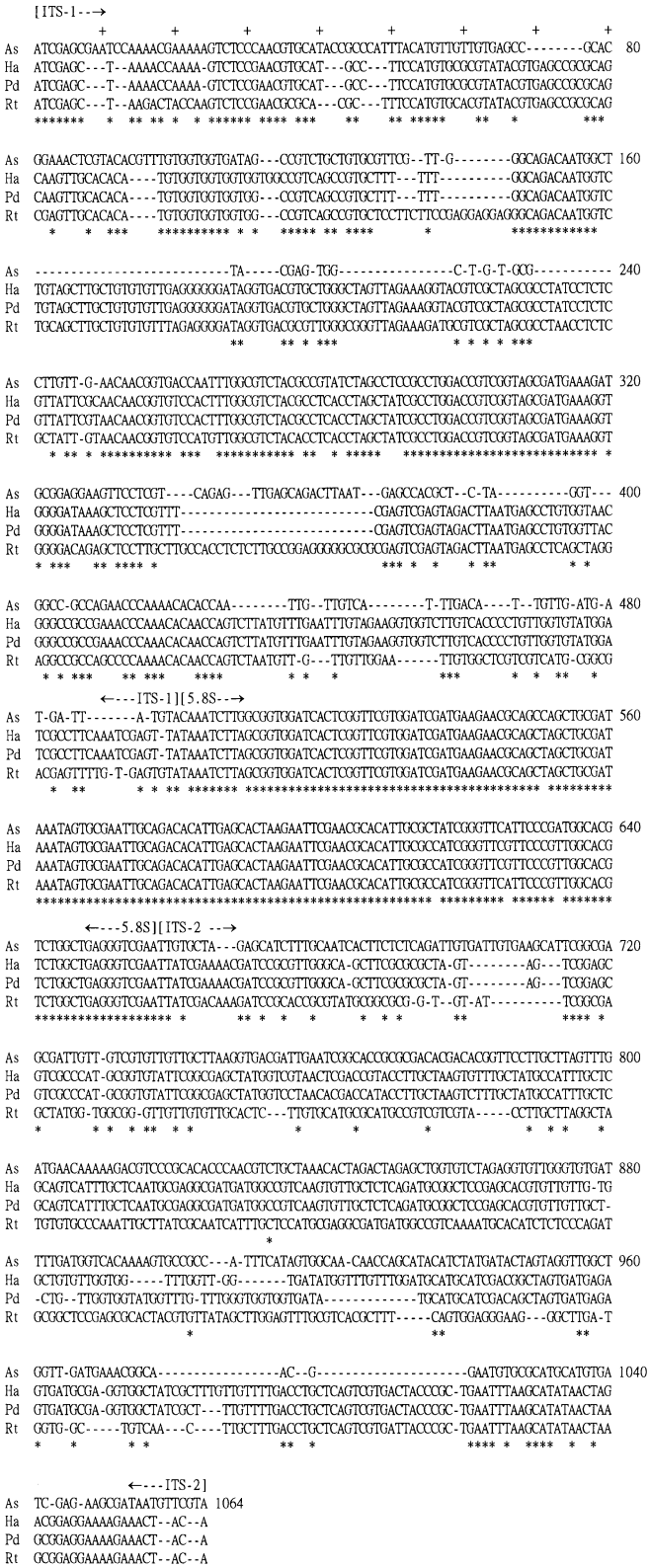

Fig. 2 Alignment of the first internal transcribed spacer (*ITS-1*), 5.8S and second internal transcribed spacer (*ITS-2*) consensus sequences for third stage larvae of *A. simplex* (*As*), *H. aduncum* (*Ha*), *P. decipiens* (*Pd*), and *R. trichiuri* (*Rt*). The *numbers* refer to alignment positions, and *asterisks* indicate nucleotide similarity among the four species

related to *H. aduncum*, *P. decipiens* or *R. trichiuri* according to the whole rDNA sequences.

Discussion

In addition to large numbers of small fish found in *T. lepturus*, zooplanktonic euphausiids and cyclopoid copepods were the most abundant items in its diet (data not shown). Since these organisms could harbor the early larval stages of nematodes, digeneans, and cestodes, a diverse endoparasite fauna was found in *T. lepturus*. Among these endoparasites, four nematodes of the family Anisakidae, namely *A. simplex*, *H. aduncum*, *P. decipiens* and *R. trichiuri*, were the most abundant species. Further, nematode L3 larvae, especially larval *A. simplex*, were apparently the most abundant endoparasites in the cutlass fish examined.

T. lepturus plays different roles in the life cycle of each helminth listed in Table 1, based on the presence of the different developmental stages of worms recovered. For nematodes, the L3 larvae of *A. simplex* and *P. decipiens* were present either in the stomach or the abdominal cavity (both free and encysted for *A. simplex*), indicating that *T. lepturus* could act as the transport or paratenic host for these nematodes, as moulting from L3 to L4 larvae does not occur in this fish (Smith 1983; Bristow and Berland 1992). In addition, *H. aduncum* and *R. trichiuri* were present in the stomach and intestine and were found in an advanced developmental or even reproductive stage in the intestine, indicating that *T. lepturus* could act as their definitive host (Køie 1993; Shih and Jeng 2002). As for the platyhelminths, *T. lepturus* could act as the definitive host for both *L. trichiuri* and *Proteocephalus* spp. because adult digeneans and fully developed plerocercoids were present in its intestine. In terms of the zoonotic potential of anisakidosis, the presence of *A. simplex* and *P. decipiens* in *T. lepturus* should be carefully monitored in fish product processing. Although encysted infective larvae were found only on the mesenteries or livers in the present study, the possibility of location in the musculature should not be neglected. Either contamination from the preparation of cutlass fish or the direct ingestion raw or undercooked fish in sushi or sashimi are the prime routes of human infection.

The accurate identification and differentiation of the species of nematodes based on their morphological characters is neither easy nor always reliable. In addition to limitations in identifying larval stages or only small portions of the nematodes available, when fresh samples are not obtainable fixation and preservation procedures could reduce the clarity of their internal structures too substantially for them to be recognized. Various studies by Chilton and his colleagues over the last decade have demonstrated that the ITS of rDNA provide genetic markers for the accurate identification of parasitic nematodes. For instance, they demonstrated that sequences of the ITS-1 and/or ITS-2 can be used

Table 3 Pair-wise comparisons of the sequence differences (%) among the third stage larvae of *A. simplex*, *H. aduncum*, *P. decipiens* and *R. trichiuri* for ITS-1, 5.8S, and ITS-2 sequences and for all rDNA regions combined

rDNA region	Species	*A. simplex*	*H. aduncum*	*P. decipiens*
ITS-1	*H. aduncum*	47.2	-	1.1
	P. decipiens	48.1	1.1	-
	R. trichiuri	52.7	26.1	25.2
5.8S	*H. aduncum*	3.8	-	0
	P. decipiens	3.8	0	-
	R. trichiuri	3.2	0.6	0.6
ITS-2	*H. aduncum*	71.9	-	9.6
	P. decipiens	71.5	9.6	-
	R. trichiuri	66.8	56.3	52.3
ITS-1, 5.8S and ITS-2	*H. aduncum*	53.1	-	1.0
	P. decipiens	52.1	1.0	-
	R. trichiuri	54.3	34.0	33.1

effectively for the differentiation of nematodes within the following superfamilies or families: (1) Ancylostomatidae—*Necator americanus* from Africa and Malaysia (Romstad et al. 1998); (2) Ascarididae—*Toxocara canis*, *T. cati* and/or *T. leonina* (Jacobs et al. 1997; Zhu et al. 1998b); (3) Metastrongyloidea—*Metastrongylus* spp. (Conole et al. 1999); (4) Molineoidea—*Nematodirus* spp. (Audebert et al. 2000); (5) Strongylidae—cryptic species within *Cylicostephanus minutus* (Hung et al. 1999), *Hypodontus macropi* complex (Chilton et al. 1995); (6) Trichostrongylidae—*Haemonchus placei* and *H. contortus* (Stevenson et al. 1995), five species within *Trichostrongylus* (Hoste et al. 1995).

For the Anisakidae with zoonotic potential occurring in important aquatic organisms, including various fish and squid species which could act as intermediate or paratenic hosts, the larval stages of *A. simplex*, *H. aduncum* and *Contracaecum osculatum* were delineated using PCR-RFLP and PCR-SSCP (single-strand conformation polymorphism) based on the sequence differences in their ITS-1, 5.8S and ITS-2 rDNA (Zhu et al. 1998a). In the present study, a nuclear rDNA region encompassing ITS-1, 5.8S, and ITS-2 was amplified using PCR with the previously described (Zhu et al. 1998a) primers from four larval anisakid nematodes collected from *T. lepturus*. The lengths of the PCR products of *A. simplex* and *H. aduncum* were ~960 and ~1,030 bp, respectively, and were identical to those reported previously (Zhu et al. 1998a). The other two anisakids, *P. decipiens* and *R. trichiuri*, were first genetically characterized using this molecular approach and the lengths of their PCR products fell within the range of 960–1,030 bp. Because the size range of their PCR products was too narrow to be easily differentiated using electrophoresis, RFLP analysis based on the sequence differences in their rDNA was carried out.

It has been shown that nucleotide differences in the ITS sequences among anisakid species were significantly greater than those within a species, and interspecific differences in the 5.8S sequence were considerably lower than in the ITS, which is not unexpected because this rRNA gene is relatively conserved in nematodes (Zhu et al. 1998a). Similarly, the present study demonstrated that nucleotide differences in the 5.8S sequences among *A. simplex*, *H. aduncum*, *P. decipiens* and *R. trichiuri* were also highly conserved; interspecific differences in 5.8S were 0–3.8%. Except for minor differences (1.0% of all rDNA) between *H. aduncum* and *P. decipiens*, differences in the ITS-1 sequences (25.2–52.7%) and the ITS-2

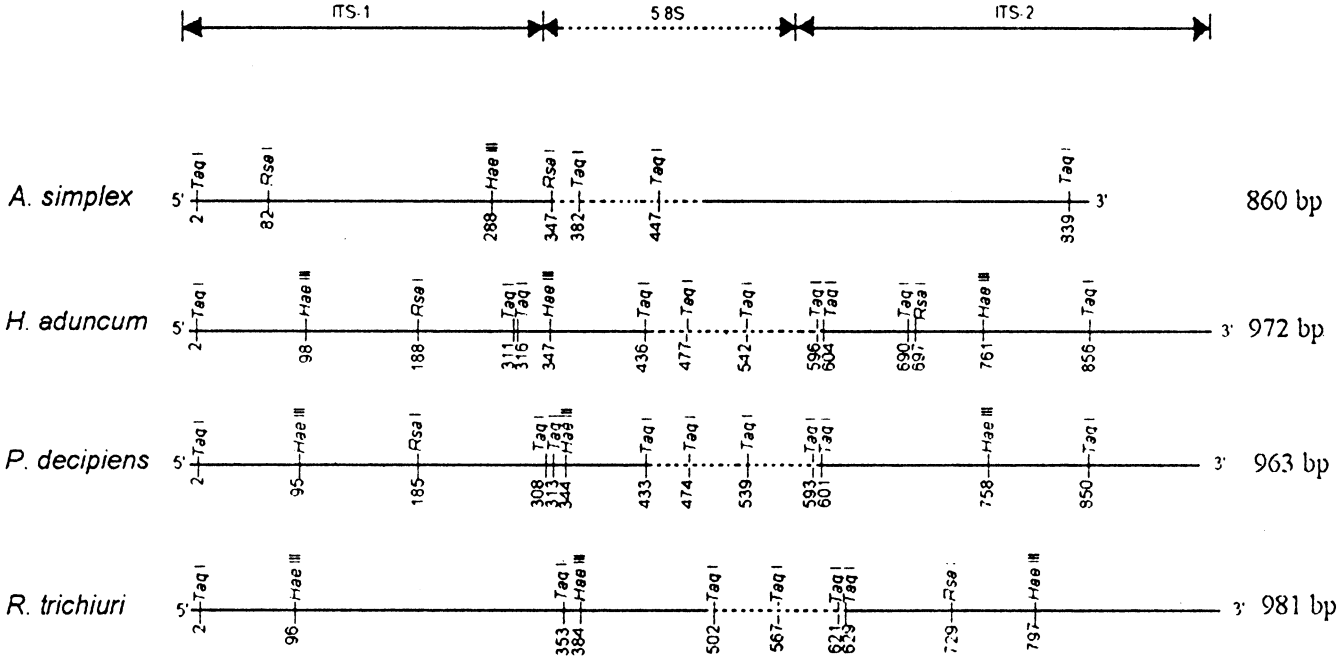

Fig. 3 Restriction maps of the ITS-1, 5.8S and ITS-2 rDNA sequences of the third stage larvae of *A. simplex*, *H. aduncum*, *P. decipiens*, and *R. trichiuri*. The *numbers* representing endonuclease restriction sites are sequence positions. These sequence positions are distinct from the alignment positions in Fig. 2

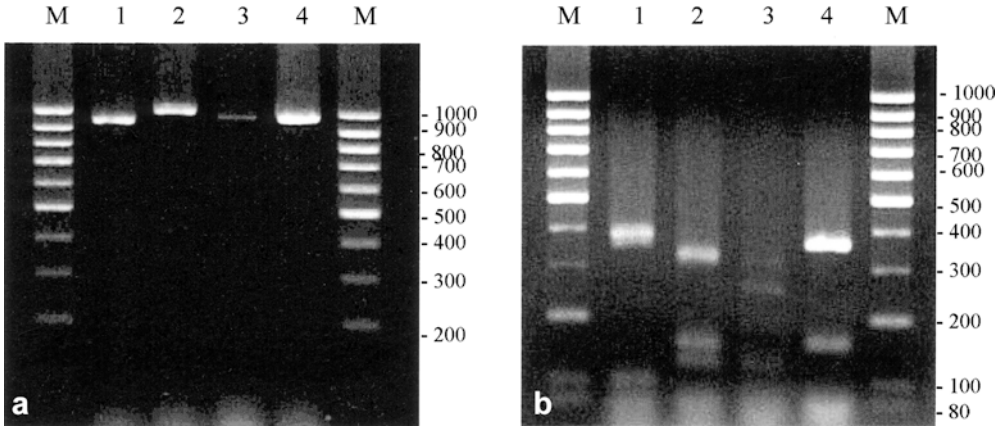

Fig. 4a, b Molecular delineation of the third stage larvae of *A. simplex* (*lane 1*), *H. aduncum* (*lane 2*), *P. decipiens* (*lane 3*), and *R. trichiuri* (*lane 4*) by PCR-linked restriction fragment length polymorphism of NC5-NC2 PCR products (representing ITS-1, 5.8S gene and ITS-2) using a restriction enzyme *Taq*I. **a** Products of PCR amplification of the rDNA fragment. **b** Restriction fragment length polymorphism of the PCR-amplified DNA fragments digested with *Taq*I. M DNA molecular marker (100-bp ladder)

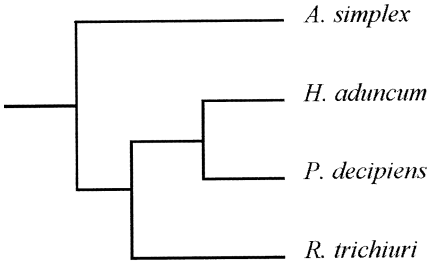

Fig. 5 Phylogenetic analysis of the nucleotide sequences of the ITS-1, 5.8S and ITS-2 rDNA sequences of the third-stage larvae of *A. simplex*, *H. aduncum*, *P. decipiens*, and *R. trichiuri* collected from the stomach of the cutlass fish, *Trichiurus lepturus*. Bootstrapped tree generated by MEGA analysis. Branches are supported by over 70% of the bootstrap replicates

sequences (52.3–71.9%) among *A. simplex*, *H. aduncum*/*P. decipiens* and *R. trichiuri* were significantly greater those that in 5.8S. In addition, the sequence differences in ITS-2 were greater than in ITS-1. For the first time, a phylogenetic analysis was applied to examine the relationship of the anisakid nematodes according to the sequences of the whole rDNA. Among the four nematodes harvested from cutlass fish, *H. aduncum* and *P. decipiens* were in the same group since there was only a 1.0% difference between all of their rDNA, but *A. simplex* was more widely separated. The phylogenetic relationships between Taiwanese anisakid nematodes inferred from rDNA sequence data will be studied further.

The rDNA sequence data also provided the basis for the construction of restriction maps for the four species of anisakid nematodes. Based on these restriction maps, a PCR-RFLP method was established using a restriction enzyme *Taq*I, which has been declared to be the most useful enzyme for the identification of species of marine and freshwater Ascaridoidea (Kijewska et al. 2002). The observation of restriction profiles unique to each species suggests an unequivocal delineation of the four species in the present study.

In conclusion, the parasitic helminth fauna of *T. lepturus* including four nematodes, one digenean and one cestode was investigated. The characterization of the L3 larvae of *A. simplex*, *H. aduncum*, *P. decipiens* and *R. trichiuri* by their ITS rDNA sequences allowed for the definition of genetic markers for their unequivocal identification. Using these markers, the PCR-based RFLP approach was established for the rapid identification of each of the four species. For the first time, a phylogenetic analysis was used to examine the relationships of the anisakid nematodes inferred from rDNA sequence data, and *H. aduncum* and *P. decipiens* were clustered in one group.

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