Zoological Studies **45**(3): 419-427 (2006)

Zoological Studies

Genetic Differentiation of *Trichiurus japonicus* and *T. lepturus* (Perciformes: Trichiuridae) Based on Mitochondrial DNA Analysis

Anirban Chakraborty[1,*], Futoshi Aranishi[2], and Yukio Iwatsuki[2]

[1]*University of Miyazaki, The United Graduate School of Agricultural Sciences, Kagoshima University, 1-1 Gakuen-kibanadai-nishi, Miyazaki 889-2192, Japan*

[2]*Division of Fisheries Sciences, Faculty of Agriculture, University of Miyazaki, 1-1 Gakuen-kibanadai-nishi, Miyazaki 889-2192, Japan*

(Accepted August 26, 2005)

Anirban Chakraborty, Futoshi Aranishi, and Yukio Iwatsuki (2006) Genetic differentiation of *Trichiurus japonicus* and *T. lepturus* (Perciformes: Trichiuridae) based on mitochondrial DNA analysis. *Zoological Studies* **45**(3): 419-427. The taxonomic status of *Trichiurus japonicus* Temminck and Schlegel, 1844 as a valid species is still controversial, although it has long been considered to morphometrically and meristically differ from *T. lepturus* Linnaeus, 1758. A portion of the mitochondrial DNA encoding the 16S ribosomal RNA (16S rRNA) gene sequence was compared between *T. japonicus* (obtained from various parts of Japan) and *T. lepturus*, obtained from the Western Atlantic (around the type locality of *T. lepturus*) and Indo-Pacific waters. The intraspecific sequence divergences of the partial 16S rRNA gene (at ~600 bp) were calculated to be 0.6% - 1.0% and 0.2% -3.0% for *T. japonicus* and *T. lepturus*, respectively, while the interspecific divergence was much greater at 4.8% -7.0%. Phylogenetic analysis using a Neighbor-joining algorithm showed that the haplotypes of *T. japonicus* formed a separate cluster from both the Western Atlantic and Indo-Pacific populations of *T. lepturus* with no overlap or sharing between them. Furthermore, restriction digestion of unpurified PCR products with the *Hinf* I restriction enzyme generated reproducible species-specific restriction patterns showing 2 fragments (of 378 and 222 bp) in *T. lepturus* whereas no cleavage was observed in *T. japonicus*. This phylogenetic analysis study coupled with PCR- RFLP analysis confirms the validity of *T. japonicus* as a separate species (in accordance with previous morphometric classification) and rules out any synonymy with *T. lepturus*.
http://zoolstud.sinica.edu.tw/Journals/45.3/419.pdf

Key words: Trichiuridae, *Trichiurus japonicus*, 16S rRNA, PCR-RFLP, Genetic identification.

Trichiurus lepturus japonicus Temminck and Schlegel, 1844 was originally described as a subspecies from Nagasaki, Japan, but subsequent workers have long treated *T. japonicus* as a valid species distinctly different from *T. lepturus* Linneaus, 1758 based on morphometric and meristic characters (Bleeker 1854, 1879, Jordan et al. 1913, Fowler 1936, Lin 1936, Boeseman 1947, Matsubara 1955, Lee et al. 1977, Li 1992, Nakabo 2002). According to Tucker (1956), the Atlantic population of the genus *Trichiurus* is recognized as *T. lepturus* (Linneaus 1758), while the Indo-Pacific population is recognized as *T. haumela* (Forsskål 1775). However, in a recent worldwide review of the Trichiuridae for the Food and Agricultural Organization (FAO), Nakamura and Parin (1993) recognized only *T. lepturus* as a valid circum-global species with comments on *T. japonicus* as a presumably valid species. While *T. japonicus* has been recognized at the species level in contemporary publications (Li 1992, Nakabo 2002), not only are there any comments on some nominal species and an examination of type specimens of *Trichiurus*, but also no comparative genetic information has been provided between *T. japonicus* and specimens of *T. lepturus* from the Western Atlantic and Indo-Pacific regions. Accordingly, the taxonomic identity of *T.*

* To whom correspondence and reprint requests should be addressed. Tel: 81-985-58-7222. Fax: 81-985-58-2884.
E-mail: kga303u@student.miyazaki-u.ac.jp

japonicus remains uncertain, no proper classification of this species has been made.

Mitochondrial DNA analysis using conserved genes like 16S ribosomal RNA (rRNA) is a very useful tool for molecular taxonomic studies and is a frequently used marker in genetic studies (Allard et al. 1992, Milinkovitch et al. 1993) of terrestrial and marine vertebrates, especially at the genus and family levels. In addition, the 16S rRNA gene for which the substitution rate is 1/2 that of the protein-coding gene (Brown et al. 1982) is utilized to better identify species (Bourdy et al. 2003, Lam and Morton 2003). In this study, we analyzed partial sequences of the16S rRNA gene from morphologically identified *T. japonicus* and compared it to that of *T. lepturus* (obtained from both Western Atlantic and Indo-Pacific waters) in order to verify the validity of *T. japonicus* as a separate species. The study aims were to (1) to genetically identify *T. lepturus* and *T. japonicus* by targeting species specific nucleotide positions using RFLP analysis and (2) provide information on the mtDNA sequence polymorphism or variation.

MATERIALS AND METHODS

Fish samples

In this study, 10 samples of *T. japonicus* (obtained from 3 localities in southern Japan) and 63 samples of *T. lepturus* were used for the genetic analysis. *Trichiurus lepturus* tissues were obtained from 15 individual specimens from the Western Atlantic coast of the USA (around the type locality of *T. lepturus*) and Brazil, to constitute the Western Atlantic samples, while 48 samples of *T. lepturus* tissues were obtained from China (15), Indonesia (6), Oman (15), Pakistan (10) and Thailand (2), to constitute the Indo-Pacific samples. Details of the samples, indicating the collection dates and localities, are given in table 1 and figure 1.

DNA extraction

Total DNA was extracted from muscle tissues using a DNeasy Tissue kit (QIAGEN K.K., Tokyo, Japan) according to the manufacturer's protocol.

Table 1. Specimens used for phylogenetic analysis, with date of collection and localities

Specimens	Localities/date of collection
Trichiurus japonicus	1) MUFS 22246, Miyazaki, Japan, 28th Oct. 2002
	2) MUFS 18237, Nagasaki, Japan,18th Oct. 1999
	3) MUFS 18240, Nagasaki, Japan,18th Oct. 1999
	4) MUFS 18242, Nagasaki, Japan,18th Oct. 1999
	5) MUFS 18245, Nagasaki, Japan,18th Oct. 1999
	6) MUFS 20254, Chiba, Japan, 20th, Aug. 2002
	7) MUFS 20255, Chiba, Japan, 20th, Aug. 2002
	8) MUFS 20256, Chiba, Japan, 20th, Aug. 2002
	9) MUFS 18397, Nagasaki, Japan,18th Oct. 1999
	10) MUFS 22093, Miyazaki, Japan, 7th Dec. 2002
Trichiurus lepturus From Western Atlantic	1) KU 1206, off Atlantic coast, USA, 12th Sept. 1994
	2) KU 1224, off Atlantic coast, USA, 12th Sept. 1994
	3) KU 1529, off Atlantic coast, USA, 10th Mar. 1995
	4) KU 3900, off Pascagoula, Gulf of Mexico, USA, 16th Nov. 2001
	5) KU 5078, off Brownsville, Gulf of Mexico, Texas, USA, 17th June 2002
	6-15) MUFS 01-10, off Brazil, South America, 28th Oct. 2004
Trichiurus lepturus From Indo-Pacific	1-15) MUFS 11-25, off Hainan Island, China, 16th Dec. 2003
	16-21) MUFS 26-31, off Jakarta, Indonesia, 13th Oct. 2003
	22-36) MUFS 32-46, off Muskat, Oman, 21st Oct. 2003
	37-46) MUFS 47-56, off Karachi, Pakistan, 24th Feb. 2004
	47-48) MUFS 57-58, off Phuket Island, Thailand, 1st Mar. 2004

MUFS, Miyazaki Univ. Fisheries Sciences, Japan; KU, Kansas Univ., USA.

In the case of *T. japonicus* both fresh and ethanol-preserved (99%) tissues were used while frozen preserved (-80°C) tissues were used for *T. lepturus*.

PCR amplification and sequencing

Polymerase chain reaction (PCR) amplification of the partial 16S rRNA gene (of ~ 600bp) was carried out using the following primers: L2510 (5'-GCCTGTTTAACAAAAACAT-3') and H3059 (5'-CGGTCTGAACTCAGATCACGT- 3') (Miya and Nishida 1996). The PCR was carried out in a 25 μl reaction volume containing 1x Gene *Taq* buffer (Wako, Japan), 5 μM of each dNTP (Wako, Tokyo, Japan), 0.40 μM of each primer, 0.125 μl of 5 U *Taq* polymerase (Wako) and 2 μl of a DNA template in a Techgene thermocycler (TC 312, Techne, Devon, UK). The thermal cycle profile was as follows: 94°C for 5 mins followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min with final extension for 5 min at 72°C. The PCR products were electrophoresed on a 1.0% agarose gel, stained with ethidium bromide (0.5 μg/ml), and visualized under a UV transilluminator in a Digi Doc-It System (UVP, BioImaging system, Upland, CA, USA).

Double-stranded PCR products were purified by Microcon 100 (Millipore, Bedford, MA, USA) and then used for direct cycle sequencing using a Big Dye Terminator v 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) following the manufacturer's instructions. Primers used were the same as those for PCR. The products were then analyzed on an ABI Prism 310 Genetic Analyzer (Applied Biosystems). Sequences obtained in this study were submitted to DDBJ (DNA Data Bank of Japan, Mishima, Japan) and can be accessed under the accession numbers AB197142-AB197149 and AB212875-AB212888.

Data analysis

The partial 16S rRNA sequences were edited using BioEdit (Hall 1999) and aligned with CLUSTAL W (Thompson et al. 1994) as implemented in the BioEdit program. The partial sequence of the 16S rRNA gene from *Eupleurogrammus muticus* (AY212325, Meng et al. unpubl. data) was used as an outgroup for the phylogenetic analysis. Pairwise evolutionary distances among the haplotypes were calculated following Kimura's 2-parameter (K2P) model (Kimura 1980) and were used to obtain a Neighbor-joining (NJ) phylogenetic tree (Saitou and Nei 1987) with MEGA version 2.1 (Kumar et al. 2001). Bootstrap values, indicating the robustness of the internal nodes of the NJ trees, were set at 1000 replications (Felsentein 1985).

RFLP analysis

Restriction digestion of the PCR products was carried out in a 10μl reaction mixture containing 1x buffer R^+ (Fermentas, Hanover, MD, USA), 5 μl of unpurified PCR product and 5 units of *Hinf* I (Fermentas) at 37°C for 1 h. Five microlitres of the reactant was run on a 2.0% agarose gel and visualized as described above. Since there was no mutation within the *T. japonicus* and *T. lepturus* specimens with respect to the *Hinf* I recognition site, only the haplotypes of each species were used for the RFLP analysis. Five haplotypes of *T.*

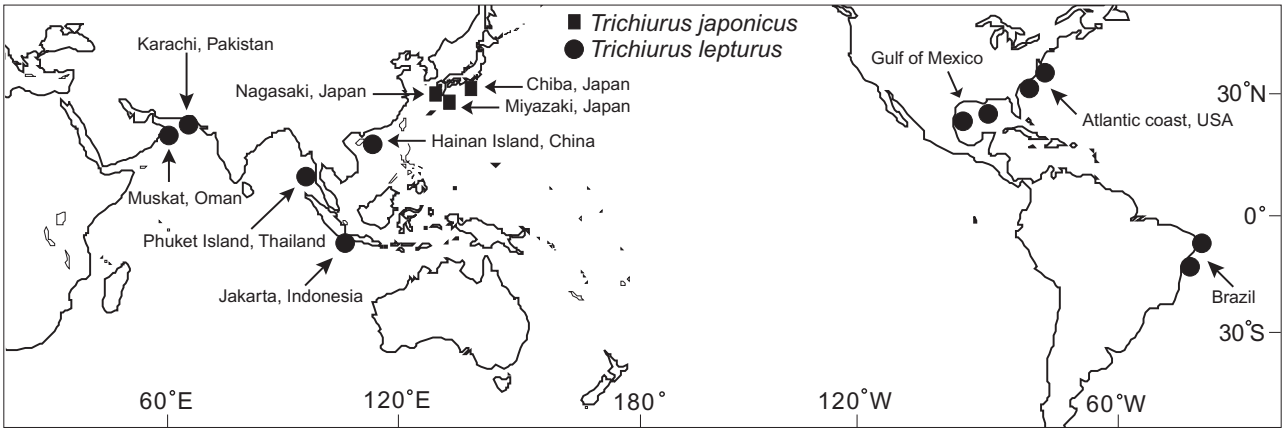

Fig. 1. Map indicating sampling locations of *Trichiurus japonicus* (■) and *T. lepturus* (●)

japonicus obtained from 3 different regions of Japan (Fig. 1) and 17 haplotypes of *T. lepturus* obtained from 3 different locations in the Western Atlantic and 5 locations in the Indo-Pacific (Fig. 1) were used for the RFLP analysis.

RESULTS

MtDNA sequence variation

The sequence analysis of the partial 16S rRNA gene (~600 bp) revealed a total of 22 haplotypes, 5 in *T. japonicus* (JH 1-5), and 17 in *T. lepturus* (LH1-17). Out of 17 haplotypes from *T. lepturus*, samples from the Western Atlantic revealed 3 haplotypes (LH 1-3) while the Indo-Pacific samples showed 14 haplotypes (LH 4-17). Haplotypes within *T. japonicus* differed in 1 to 5 nucleotide positions (2 C/T changes, 2 G/T changes and 1 C/G change), while the haplotypes from the Western Atlantic *T. lepturus* differed in only 2 nucleotide positions (two C/T changes). On the other hand, haplotypes of *T. lepturus* from the Indo-Pacific samples differed in 24 nucleotide positions (5 A/G changes, 5 A/T changes, 7 C/T changes, 3 A/C changes, 3 G/T changes, and 1 G/C change). In total, 55 variable nucleotide sites were found between haplotypes of *T. japonicus*, and *T. lepturus* from Western Atlantic and *T. lepturus* from Indo-Pacific (Fig. 2), 30 of which differed by transitional substitutions, 18 by transversional changes and 7 mutation sites exhibited multiple substitutions (Fig. 2). Transitional changes occurred more frequently than transversional changes as is typical of animal mitochondrial genomes (Li 1997).

Sequence divergence of the partial 16S rRNA gene between the two species (interspecific) was quite high at 4.8%-7.0% but within species (intraspecific) divergence values ranged from 0.60%-1.0% and 0.2%-3.0% for *T. japonicus* and *T. lepturus*, respectively. The K2P distance among haplotypes of *T. japonicus* ranged from 0.002 to 0.008 with an average of 0.003 ± 0.002, while in the case of *T. lepturus*, it ranged from 0.002 to 0.078 with an average of 0.029 ± 0.005. Between the 2 species, interspecific genetic distances ranged from 0.042 to 0.072 with a mean distance of 0.058 ± 0.010.

Phylogenetic analysis

The aligned sequences of the partial 16S rRNA gene from all haplotypes were used to construct a phylogenetic tree by the NJ method (Fig. 3). The NJ tree formed 2 major groups, one exclusively containing haplotypes of *T. lepturus* from the Indo-Pacific, supported by a high bootstrap value of 100%, while the other (with a bootstrap value of 80%) containing 2 subgroups, one with the haplotypes of *T. japonicus* (99%) and the other with the haplotypes of *T. lepturus* from the Western Atlantic (100%). Although the haplotypes of *T. japonicus*

	[11111 1112222222 2222222222 2223333333 3333333444 44555] [2245700444 8890000122 2333333344 4480000112 2336669346 88011] [6926647467 2481489228 9012346823 5610689151 4380343373 89201]	Accession number	Locality	Number of specimens for each haplotype
JH 1	GGCGACTATC CACTCTTTAG CCCGCTAACC CCACTCTATG GCCTTTAAGC GGTGT	AB197142	Miyazaki,Japan	2
JH 2	. . . . . C . . . . . T	AB197143	Nagasaki,Japan	4
JH 3	. . . . . T . . . G	AB197144	Chiba,Japan	1
JH 4	. . . . . T	AB197145	Chiba, Japan	2
JH 5	. . . . . C . . . . . C . . . . . G T	AB197146	Miyazaki,Japan	1
LH 1	. . T . T . . T . A . C . G . C . . G . . . AAC . . TT ATT . AT . . . A AGTC . A . . . . T	AB197147	off Atlantic coast USA	3
LH 2	. . T . T . . T . A . C . G . C . . G . . . AAC . . T . ATT . AT . . . A AGTC . A . . . . T	AB197148	off Mexico and Brazil	4
LH 3	. . T . T . . T . A . C . G . C . . G . . . AACC . T . ATT . AT . . . A AGTC . A . . . . T	AB197149	off Brazil	8
LH 4	A . . A . . . C . T AGT . ACCA . A . . . AACC . . . . . CTCTCGCA . G . CA . . GA . T	AB212875	off Hainan Island,China	5
LH 5	AATAT . . C . T AGT . ACCA . A . . . AACC . . . . . CTCTCGCA . G . CA . . GA . ATA . .	AB212876	off Hainan Island,China	3
LH 6	A . . A . . . C . T AGT . ACCA . A . . . AACCT . . . . CTCTCGCA . G . CA . . GA . AT . . .	AB212877	off Hainan Island,China	3
LH 7	A . . . . A . . CT AGT . ACCA . A . . . AACC . T . . CTCTCGCA . G . CA . . GA . AT . . .	AB212878	off Hainan Island,China	4
LH 8	A . . A . T . C . T ACT . ACCA . A . . . AACC . . . . . CTCTCG . A . G . CA . . GA . AT . . .	AB212879	off Jakarta, Indonesia	3
LH 9	A . . A . T . C . T ACT . ACCA . A . . . AACC . . . . . CTCTCG . A . G . CA . . GA . AT . . .	AB212880	off Jakarta, Indonesia	3
LH 10	. . . . . AC . T ACT . ACCA . A . . . CACC . . . . . CTCTCG . A . G . CA . . G . . AT . . .	AB212881	off Muskat, Oman	4
LH 11	. . . . . T . C . T ACT . ACCA . A . . . CACC . . . . . CTCTCG . A . G . CA . . G . . A . . .	AB212882	off Muskat, Oman	3
LH 12	. . . . . C . T ACT . ACCA . A . . . CACC . . . . . CTCTCG . A . G . CA . . G . . AT . . .	AB212883	off Muskat, Oman	3
LH 13	. . . . . C . T ACT . ACCA . A . . . AACC . . . . . CTCTCG . A . G . CA . . G . . AT . T .	AB212884	off Muskat, Oman	5
LH 14	A . . A . T . C T ACT . ACCA . A TTTAACC . . . . . CTCTCG . A . G . CA . . GA . AT . . .	AB212885	off Karachi, Pakistan	4
LH 15	A . . A . T . C T ACT . ACCA . A TTTAACC . T . . CTCTCG . A . A . CA . CGA . AT . . .	AB212886	off Karachi, Pakistan	3
LH 16	A . . A . T . C . T ACT . ACCA . A TTTAACC . . . . CTCTCG . A . A . CA . . GA . AT . . .	AB212887	off Karachi, Pakistan	3
LH 17	A . . A . T . C . T ACT . ACCA . A . . . AAC . . . . . CTCTCG . A . G . CA . . GA . AT . . G	AB212888	off Phuket Island, Thailand	2

Fig. 2. Summary of nucleotide variations found in the partial 16S rRNA of *Trichiurus japonicus*, and *T. lepturus*. Only variable sites are shown. Haplotypes are named by letters referring to the species and a number. JH, *T. japonicus* haplotype; LH, *T. lepturus* haplotype. Dots indicate identity with the JH 1 haplotype sequence.

formed nested subclusters (with a bootstrap value of 64%), within each subgroup, the clustering of the haplotypes did not correspond to specific geographical locations. However, the cluster of *T. lepturus* from the Indo-Pacific formed 3 subgroups corresponding to specific geographic locations. Haplotypes from Indonesia (LH 8 and LH 9), Thailand (LH 17) and Pakistan (LH 14, LH 15 and LH 16) clustered in the same group (with a bootstrap value of 81%), with the haplotypes of Pakistan forming a separate cluster (50%), while the haplotypes from China (LH 4-7) and Oman (LH 10-13) formed separate subgroups with respective bootstrap values of 55% and 70%.

RFLP analysis

Comparisons of aligned sequences of the partial 16S rRNA gene between *T. japonicus* and *T. lepturus* showed a unique restriction site of the *Hinf* I enzyme that enabled the identification of these 2 species as indicated in figure 4. The restriction enzyme, *Hinf* I, produced 2 fragments of 378 and 222 bp from the PCR products of *T. lepturus*, whereas no restriction sites were present in *T. japonicus*. Hence, using *Hinf* I, 2 fragments and 1 fragment would be theoretically generated for *T. lepturus* and *T. japonicus*. Among the 22 haplotypes of *T. lepturus* and *T. japonicus*, no nucleotide mutation was observed at the recognized site of the *Hinf* I enzyme.

Direct digestion of the PCR products with *Hinf* I generated reproducible species-specific restriction patterns (Fig. 5). They corresponded to the expected profiles as described above, and 2 fragments of around 378 and 222 bp were found in *T. lepturus* while 1 uncut fragment (of ~600 bp) was seen in *T. japonicus*. In addition, *T. japonicus* samples obtained from 3 different regions of Japan and *T. lepturus* samples from 3 different locations in the Western Atlantic and 5 locations in the Indo-Pacific showed consistent banding patterns.

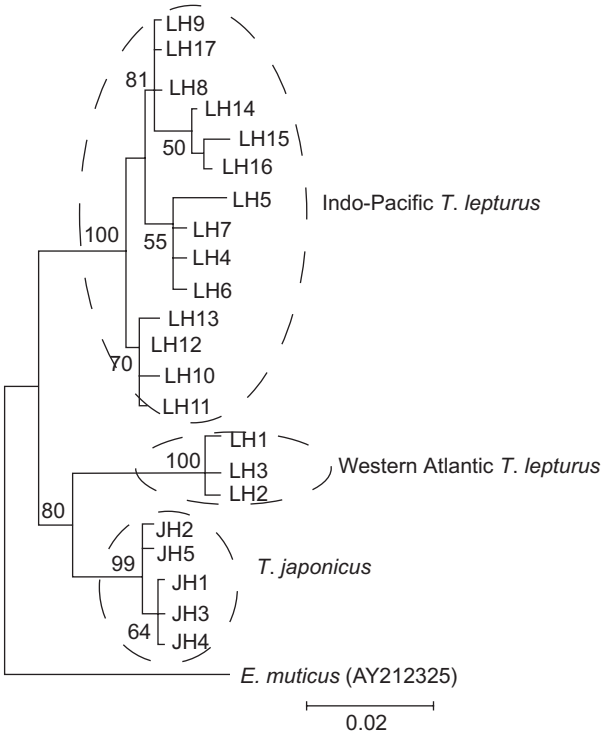

Fig. 3. Neighbor-joining phylogenetic tree based on the partial 16S rRNA gene for the haplotypes of *Trichiurus japonicus* and *T. lepturus* with *Eupleurogrammus muticus* (Trichiuridae) as the outgroup taxon. Numbers above the branches indicate bootstrap values based on 1000 replications. Only values of >50% values are indicated. JH1, 2, 3, 4, 5: *T. japonicus* haplotypes 1to 5; LH 1-17: *T. lepturus* haplotypes 1 to17.

DISCUSSION

This is the first comprehensive study of mtDNA sequences of *T. japonicus* to address its taxonomic status in comparison with *T. lepturus*. The present results obtained from the partial sequences of the 16S rRNA gene and practical PCR-RFLP analysis indicate that *T. japonicus* and *T. lepturus* are genetically distinct species thus eliminating any doubt as to the synonymy of these 2 species as suggested in the most recent review by the FAO for the family Trichiuridae (Nakamura and Parin 1993).

Although the FAO review (Nakamura and Parin 1993) recognized only *T. lepturus* Linnaeus, 1758 (type locality: South Carolina, North America) as a valid circum-global species within larger congeners which grow to around 1.5 m in total length, the review was not based on sufficient global specimens including type specimens. Moreover, different taxonomic ideas of this species also appeared in the literature before and after the FAO review (Tucker 1956, Nakabo 1993, Li 1992, Burhanuddin 2003, Burhanuddin et al. 2002). Hence the taxonomic classification of *T. lepturus* and *T. japonicus* has remained controversial to the present.

Morphometric studies have shown that *T. japonicus* varies from *T. lepturus* in having a smaller head (Boeseman 1947, Li 1992) and longer caudal peduncle length (Temminck and Schlegel 1844, Burhanuddin 2003), and these are sufficiently different to consider them as separate species in addition to other subtle morphological and meristic

differences. Our study also shows that *T. japonicus* and *T. lepturus* exhibit significant differences of 4.8%-7.0% in the 16S rRNA genes between them considering the conserved nature of 16S rRNA among species (Brown et al. 1982). The degree of sequence divergence between these 2 species is comparable to those of other well-accepted and valid fish species. Among the species of *Trachurus*, for example, the interspecific 16S rRNA divergence between *Trachurus mediterraneus* and *T. trachurus* was found to be 1.46% and that between *T. mediterraneus* and *T. pictatus* was 1.83% (Karaiskou et al. 2003). According to Mabuchi et al. (2003), the 16S rRNA sequence divergence between the cardinal fishes (Apogonidae), *Apogon cynosoma* and *A. properuptus*, was found to be around 5.4%.

The present phylogenetic analysis revealed 2 major clusters, one exclusively containing the haplotypes of the Indo-Pacific *T. lepturus* with the other containing 2 subgroups, one with haplotypes of *T. japonicus* and the other with the haplotypes of *T. lepturus* from the Western Atlantic with no sharing or overlap of the haplotypes. The fact that no haplotypes were shared among the species suggests an interruption of gene flow for an efficient number of generations. Lineage sorting of the haplotypes of *T. lepturus* from the Indo-Pacific and Western Atlantic into separate clusters indicates that *T. lepturus* populations from 2 geographic locations are genetically distinct. In this study, we saw that the ranges of sequence divergences and K2P distances among haplotypes of *T. lepturus* were quite high (0.2% -3.0% and 0.002-0.078, respectively). However, those within the Western Atlantic haplotypes alone were only 0.2% -0.8% and 0.000-0.002, respectively. On the other hand, those between Indo-Pacific and Western Atlantic *T. lepturus* were 5.9% - 6.9% and 0.058-0.078, respectively. Previous morphological studies also showed that the Indo-Pacific population of *T. lepturus* (historically treated as *T. haumela* Forsskål, 1775) differed from the Western Atlantic population based on the yellow coloration of the dorsal fin in the former and the absence of such coloration in the latter (Day 1865, Tokimura et al. 1995, Yamada et al. 1995, Nakabo 2002, Kimura and Matsuura 2003). In this study, we noted that

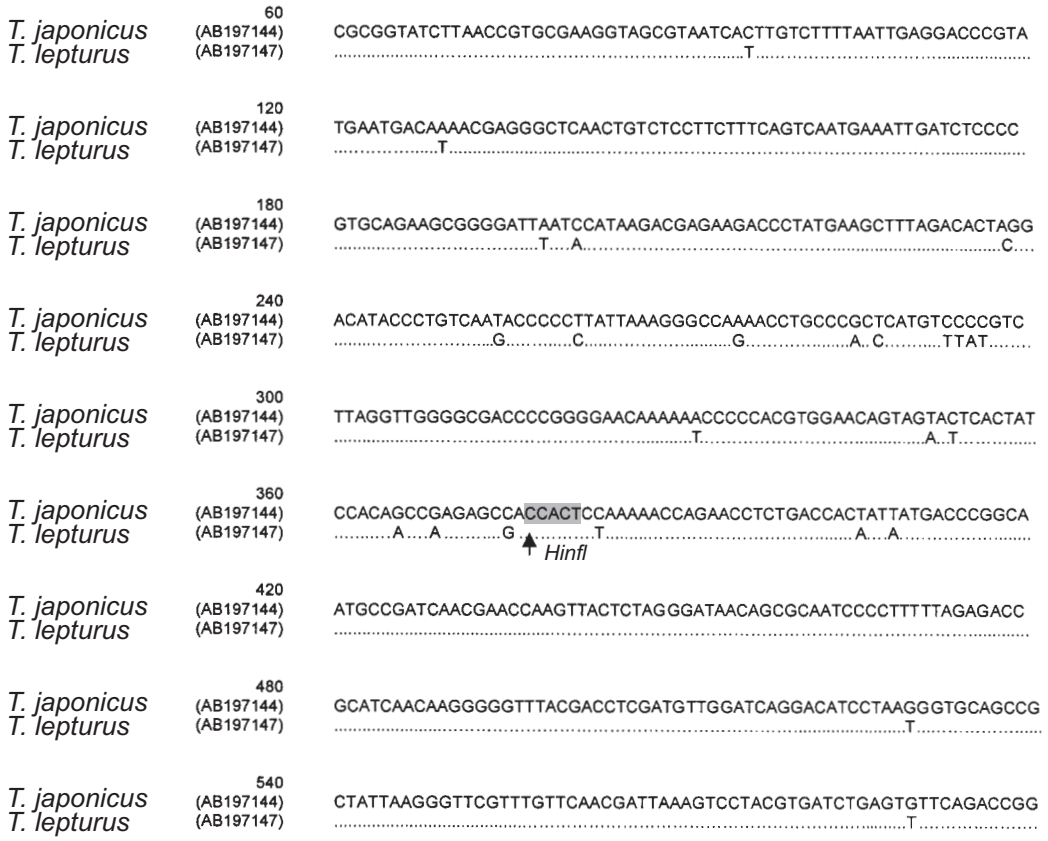

Fig. 4. CLUSTAL W alignment of the partial DNA sequences of the mitochondrial 16S rRNA gene from *Trichiurus japonicus* and *T. lepturus*. The *Hinf* I restriction site is shadowed and shown in a box. Dots indicate identity with the 1st sequence.

the samples of *T. lepturus* obtained from China, Indonesia, Oman, Pakistan, and Thailand had the yellow coloration of the dorsal fin, while samples from the Western Atlantic coast showed a dusky, not yellow, coloration. The genetic results obtained in this study clearly show that the 2 regional populations of *T. lepturus* (Western Atlantic and Indo-Pacific) are represented by at least 2 species. The clustering of the *T. japonicus* lineage with *T. lepturus* haplotypes from the Western Atlantic (Fig. 3) indicates a closer relationship of *T. japonicus* to the Western Atlantic population of *T. lepturus* than to the Indo-Pacific population. Morphologically, *T. japonicus* is most similar to *T. lepturus* from the Western Atlantic (by the absence of the yellow dorsal fin) and other meristic characters when compared to *T. lepturus* from the Indo-Pacific.

The PCR-RFLP method has been used for species identification of many closely related species of fish (Chow et al. 1993, Quinteiro et al. 1998, Aranishi 2005a, b, Aranishi et al. 2005a b). Likewise in this study, RFLP analysis revealed that *T. japonicus* and *T. lepturus* have different restriction patterns as evident from the species – specific restriction pattern obtained for each species (Fig. 5). Haplotypes of *T. lepturus* from both the Western Atlantic and Indo-Pacific possessed the restriction site for *Hinf* I, while those of *T. japonicus* did not posses the *Hinf* I site. Thus, PCR-RFLP analysis using *Hinf* I restriction enzymes easily and effectively identified the 2 species with no need to conduct sequencing or phylogenetic analysis.

The database shows 3 partial 16S rRNA sequences (AY216492, AY216493 and AY216494) which were submitted as *T. lepturus* haplotypes (Meng et al., unpubl. data). However, sequence alignment (BLAST) of these 3 sequences and comparison with our sequences obtained in this study showed a 99% homology with *T. japonicus* haplotypes, while the extent of homology with the Indo-Pacific *T. lepturus* and Western Atlantic *T. lepturus* was 94%. In addition, the restriction patterns for these 3 submitted sequences (AY216492, AY216493 and AY216494) were identical to those of *T. japonicus* used in this study (by the absence of the *Hinf* I site). The high homology and identical restriction patterns of these 3 submitted sequences with *T. japonicus* sequences obtained in this study proves that these submitted sequences are indeed from *T. japonicus*, and not from *T. lepturus*, although there is no information on their collection localities. In reviewing the recorded distribution of *T. japonicus*, Lin (1936) as well as our current observations found that *T. japonicus* inhabits Chinese and Japanese coastal waters and is not known to live beyond the Asiatic continental shelf or southward to the Indian Ocean.

Trichiurus lepturus has been considered a single species (Tucker 1956, Nakamura and Parin 1993) with a very broad geographic distribution covering the Indo-Pacific, the Eastern Pacific, the West Atlantic, and the West Africa although there are different views as discussed above. Further

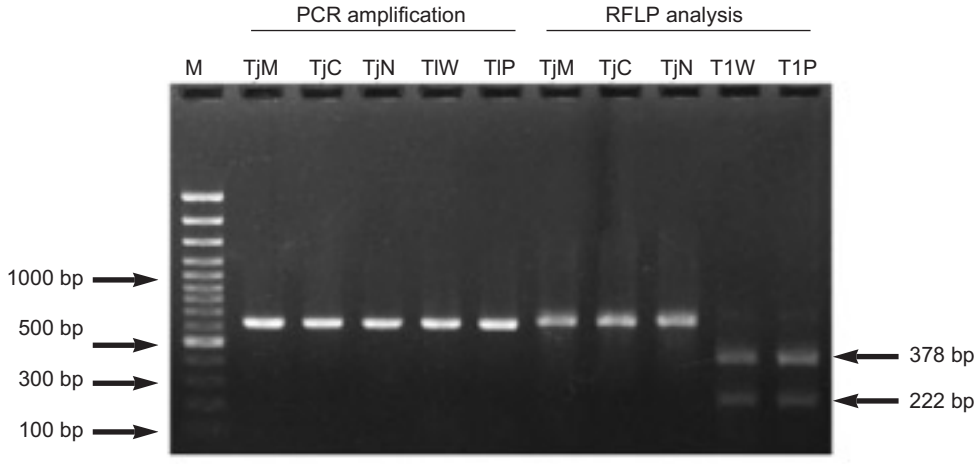

Fig. 5. PCR-RFLP analysis of the mitochondrial 16S rRNA gene from *Trichiurus japonicus* and *T. lepturus*. Lane M, molecular weight marker (100-bp ladder); TjM, *T. japonicus* from Meitsu; TjC, *T. japonicus* from Chiba; TjN, *T. japonicus* from Nagasaki; TlW, *T. lepturus* from the Western Atlantic coast; TlP, *T. lepturus* from the Indo-Pacific. PCR amplification and RFLP analysis refer to the PCR products and those after *Hinf* I digestion, respectively.

studies, using morphological and meristic data are required in order to achieve a correct taxonomic classification of the Atlantic and Indo-Pacific populations of *T. lepturus*. However, in this study, *T. japonicus* was at least confirmed to be genetically distinct from both the Western Atlantic and Indo-Pacific populations of *T. lepturus*, and proven to be a valid species.

Acknowledgments: We are most grateful to Andrew C. Bentley and Edward O. Wiley (Biodiversity Research Center and Natural History Museum, Univ. of Kansas, Lawrence, KS, USA) and J. K. Dooley (Department of Biology, Adelphi Univ., Garden city, NY, USA) for providing tissue samples of true Western Atlantic *Trichiurus lepturus* specimens. We also thank T. Yoshino (Univ. of Ryukyus, Naha, Japan) and S. Kimura (Mie Univ., Mie, Japan) for providing important references and K. Hidaka (Univ. of Miyazaki, Miyazaki, Japan) for help with specimen collection. This study was financially supported in parts by the Sasakawa Scientific Research Grant from the Japan Science Society awarded to the first author and by the Ministry of Education, Science, Sports and Culture, Japan (no.16570079) to the 3rd author.

REFERENCES

Allard MW, MM Miyamoto, L Jareche, F Kraus, MR Tennant. 1992. DNA systematics and evolution of the artiodactyl family Bovidae. Proc. Natl. Acad. Sci. USA **89:** 3972-3976.

Aranishi F. 2005a. PCR-RFLP analysis of nuclear nontranscribed spacer for mackerel species identification. J. Agric. Food Chem. **53:** 508-511.

Aranishi F. 2005b. Rapid PCR-RFLP method for discrimination of imported mackerel and domestic mackerel. Mar. Biotechnol. (in press)

Aranishi F, T Okimoto, S Izumi. 2005a. Identification of gadoid species (Pieces, Gadidae) by PCR-RFLP analysis. J. Appl. Genet. **46:** 69-73.

Aranishi F, T Okimoto, M Okhubo, S Izumi. 2005b. Molecular identification of commercial spicy Pollack roe products by PCR-RFLP analysis. J. Food. Sci. **70:** 235-238.

Bleeker P. 1854. Nieuwe nalezingen op de ichthyologie van Japan. Verh. Batav. Genootsch. Kunst. Wet. **37:** 1-132.

Bleeker P. 1879. Sur quelques especes inedites ou peu connues de poissons de Chine appartenant au museum de Hambourg. Versl. Akad. Amsterdam **84:** 1-17.

Boeseman M. 1947. Revision of the fishes collected by Burger and von Siebold in Japan. Zoologisch. Meded. **28:** 1-242.

Bourdy P, S Heurtebise, S Lapegue. 2003. Mitochondrial and nuclear DNA sequence variation of presumed *Crassostrea gigas* and *Crassostrea angulata* specimens: a new oyster species in Hong Kong? Aquaculture **228:**15-25.

Brown WM, EM Prager, A Wang, AC Wilson. 1982. Mitochondrial DNA sequences of primates: tempo and mode of evolution. J. Mol. Evol. **18:** 225-239.

Burhanuddin AI. 2003. Review of the family Trichiuridae without a forked caudal fin (Perciformes). PhD dissertation, The United Graduate School of Agricultural Sciences, Kagoshima Univ., Kagoshima, Japan.

Burhauddin AI, Y Iwatsuki, T Yoshino, S Kimura. 2002. Small and valid species of *Trichiurus brevis* Wang and You, 1992 and *T. russelli* Dutt and Thankam, 1966, defined as the "*T. russelli* complex" (Perciformes: Trichiuridae). Ichthyol. Res. **49:** 211-223.

Chow S, ME Clarke, PJ Walsh. 1993. PCR-RFLP analysis of thirteen western Atlantic snappers (subfamily Lutjaninae): a simple method for species and stock identification. Fish. Bull. **30:** 207-224.

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

Forsskål P. 1775. Descriptions animalium, avium, amphibiorum, piscium, insectorum, vermium, quae in itinere orientali observavit. Hauniae.

Fowler HW. 1936. A Synopsis of the Fishes of China. Hong Kong: Hong Kong Naturalist.

Hall TA. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucleic Acids Symp. Ser. **41:** 95-98.

Jordan DS, S Tanaka, JO Snyder. 1913. A catalogue of the fishes of Japan. J. Coll. Sci. Imp. Univ. Tokyo **33:** 1-497.

Karaiskuo N, AP Apostolidis, A Triantafyllidis, A Kouvatsi, C Triantaphyllidis. 2003. Genetic identification and phylogeny of three species of the genus *Trachurus* based on mitochondrial DNA analysis. Mar. Biotechnol. **5:** 493-504.

Kimura M. 1980. A simple method for estimating evolutionary rates of base substitutions through comparative studies of DNA sequences. J. Mol. Evol. **16:** 111-120.

Kimura S, K Matsuura, eds. 2003. Fishes of Bitung, northern tip of Sulawesi, Indonesia. Tokyo: Ocean Research Institute, Univ. of Tokyo.

Kumara S, K Tamura, IB Jakobsen, M Nei. 2001. MEGA 2: Molecular Evolutionary Genetics Analysis software. Bioinformatics **17:** 1244-1245.

Lam K, B Morton. 2003. Mitochondrial DNA and morphological identification of a new species of *Crassostrea* (Bivalvia: Ostreidae) cultured for centuries in the Pearl River Delta, Hong Kong, China. Aquaculture **228:** 1-13.

Lee SC, KH Chang, WL Wu, HC Yang. 1977. Formosan ribbonfishes (Perciformes: Trichiuridae). Bull. Inst. Zool. Acad. Sinica. **16:** 77- 84.

Li CS. 1992. Hairtail fishes from Chinese coastal waters (Trichiuridae). Mar. Sci. Acad. Sinica. **4:** 212-218.

Li WH. 1997. Molecular Evolution. Sunderland MA, Sinauer Associates.

Linnaeus C. 1758. Systema Naturae 10th Edition, Holmiae. Systema Nat. **1:** 1-824.

Lin SY. 1936. Notes on hairtails and eels of China. Bull. Chekian Prov. Fish. Exp. Sta. **2:** 1-16.

Lin X, S Xiaomin. 1986. Preliminary research on species indentification of largehead hairtail in the Dong Hai Sea and Yellow Sea. J. Fish. China **10:** 339-350.

Mabuchi K, N Okuda, T Kokita, M Nishida. 2003. Genetic comparison of two color-morphs of *Apogon properuptus* from southern Japan. Ichthyol. Res. **50:** 293-296.

Matsubara K. 1955. Fish morphology and hierarchy. Part 1. Tokyo: Ishizaki Shoten.

Milinkovitch MC, G Orti, A Meyer. 1993. Revised phylogeny of

whates suggested by mitochondrial ribosomal DNA sequences. Nature **361:** 346-348.

Miya M, M Nishida. 1996. Molecular phylogenetic perspective on the evolution of the deep-sea fish genus *Cyclothone* (Stomiiformes: Gonostomatidae). Ichthyol. Res. **43:** 375-398.

Nakabo T. 2002. Family Trichiuridae. *In* T Nakabo, ed. Fishes of Japan with pictorial keys to the species. Tokyo: Tokai Univ. Press, pp. 1344-1345.

Nakamura I, NV Parin. 1993. FAO Species Catalogue. Vol. 15. Snake mackerels and cutlassfishes of the world (families Gempylidae and Trichiuridae). An annotated and illustrated catalogue of the snake mackerels, snoeks, escolars, gemfishes, sackfishes, domine, oilfish, cutlassfishes, scabbardfishes, hairtails and frostfishes known to date. Rome: Food and Agricultural Organization.

Quinteiro J, CG Sotelo, H Rehbein, SE Pryde, RI Perez-Martin, M Rey-Mendez, IM Mackie. 1998. Use of mtDNA direct polymerase chain reaction (PCR) sequencing and PCR-restriction fragment length polymorphism methodologies in species identification of canned tuna. J. Agric. Food. Chem. **46:** 1662-1669.

Saitou N, M Nei. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. **4:** 406-425.

Temminck C, J Schlegel. 1844. Pisces. *In* P.F.Siebold, Fauna Japonica, sive description animalium, quae in itinere per Japoniam suscepto, annis 1823-30 collegit, notis, observationsbus et adunbrationibus illustravit P.F. Siebold. Lugduni Vatavorum, Lugduni Batavorum, p. 325.

Thompson JD, DG Higgins, TJ Gibson. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. Nucleic Acid. Res. **22:** 4673-4680.

Tokimura M, U Yamada, T Irie. 1995. Comments on taxonomy and distributions of the species of *Trichiurus* in the East China and Yellow Seas and adjacent waters. Seikaku Suisan Kenkyusho News **80:** 12-14. (in Japanese)

Tucker DW. 1956. Studies on trichiuroid fishes-3. A preliminary revision of the family Trichiuridae. Bull. Br. Mus. Nat. Hist. Zool. **4:** 73-130.

Yamada U, S Shirai, T Irie, M Tokimura, S Deng, Y Zheng, CS Li, YU Kim, YS Kim. 1995. Names and illustrations of fishes from the East China Sea and the Yellow Sea. Tokyo: Overseas Fishery Cooperation Foundation, pp. xi+288. (in Japanese, Chinese, and Korean)