Genetic differences among three species of the genus *Trichiurus* (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 (e-mail: kga303u@student.miyazaki-u.ac.jp)*
[2] *Division of Fisheries Sciences, Faculty of Agriculture, University of Miyazaki, 1-1 Gakuen-kibanadai-nishi, Miyazaki 889-2192, Japan*

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Abstract The mitochondrial DNA segment encoding the 16S ribosomal RNA (16S rRNA) gene sequence (ca. 600 bp) was compared among *Trichiurus* sp. 2 (sensu Nakabo, 2002) (obtained from various areas of Japan), *T. japonicus* Temminck and Schlegel (collected from various localities within Japan), and true *T. lepturus* Linnaeus (caught off the Atlantic coast of the United States and Brazil) of the family Trichiuridae using 10, 10, and 15 specimens, respectively. Based on phylogenetic analysis using a neighbor-joining (NJ) algorithm, the haplotypes of *Trichiurus* sp. 2, *T. japonicus*, and *T. lepturus* indicated three distinct monophyletic lineages, being supported by 100% bootstrap values with no haplotypes overlapping or sharing among the lineages. *Trichiurus* sp. 2, *T. japonicus*, and *T. lepturus* are genetically different from each other, suggesting that they are three distinct species.

Key words mtDNA · 16S rRNA gene · Genetic identification · Hairtails

In their worldwide taxonomic review of hairtails (family Trichiuridae), Nakamura and Parin (1993) recognized only *Trichiurus lepturus* Linnaeus (type locality: South Carolina, USA) as a valid, circumglobal species within larger congeners that can grow up to about 1.5 m in total length, although there have been differing taxonomic opinions on the composition of species (see Tucker, 1956; Nakabo, 1993; Li, 1992; Burhanuddin, 2003). Nakamura and Parin (1993) commented on *T. lepturus japonicus* Temminck and Schlegel (type locality: Nagasaki, Japan) as a presumably valid species with a note on two forms in *T. lepturus*, but many other taxonomists have treated *Trichiurus japonicus* as a valid species that is recognizable by morphological characters (e.g., Bleeker, 1854; Jordan et al., 1913; Fowler, 1936; Boeseman, 1947; Matsubara, 1955; Lee et al., 1977; Li, 1992; Nakabo, 2002).

According to Tucker (1956), the Atlantic specimens of the genus *Trichiurus* can be recognized as *T. lepturus* Linneaus, whereas the Indo-Pacific specimens are known as *Trichiurus haumela* Forsskål. Tokimura et al. (1995) and Yamada et al. (1995) reported specimens of *Trichiurus* with a yellowish-green dorsal fin from the Ryukyu Islands, the East China Sea, and coast of Kyushu Island, Japan. These specimens were subsequently called *Trichiurus* sp. 2 in Nakabo (2002). *Trichiurus* sp. 2 (sensu Nakabo, 2002) is widely recognized from the Indian Ocean to the western Pacific including the East Asian Shelf (Day, 1865; Li, 1992; Kimura and Matsuura, 2003; personal observation). Furthermore, *T. lepturus* from western Atlantic coast showed a dusky coloration of the dorsal fin (vs. yellow in *Trichiurus* sp. 2) (personal observation). The nomenclature among *Trichiurus* sp. 2, *T. japonicus*, and *T. lepturus* remains uncertain.

Trichiurus sp. 2 and *T. japonicus* are the most frequently caught hairtails in the East Asian Shelf (Nakabo, 2002) and the Indo-Pacific (personal observation), respectively. Most of the previous studies not only lacked comparative examination of any specimens of true *T. lepturus* from the western Atlantic and *Trichiurus* sp. 2 (hereafter *T.* sp. 2) from the Indo-Pacific (the latter is historically treated as *T. haumela* or *T. lepturus*) as well as *T. japonicus* but also failed to compare genetic data among them.

This study gives genetic data among *T.* sp. 2, *T. japonicus*, and *T. lepturus* based on their mitochondrially encoded 16S rRNA gene sequences. The genetic differences are compared and evaluated among these three species identified by morphological characters.

Materials and Methods

Identification.—Generic diagnosis of the genus *Trichiurus* followed Nakamura and Parin (1993). *Trichiurus* sp. 2 was identified following Nakabo (2002) with confirmation of yellow dorsal fin color when fresh and bottom of oral cavity light colored. Identification of *T. japonicus* followed Li (1992) and Nakabo (2002), this species having longer caudal peduncle length [mean 52% of preanal length (PL), $n = 30$] than *T.* sp. 2 (mean 33% PL, $n = 22$) and *T. lepturus* (mean 40% PL, $n = 18$) from the western Atlantic (Burhanuddin, 2003), bottom of oral cavity dark colored, and ground color

Table 1. Collection data (species, localities, and date) and generic information of *Trichiurus* sp. 2, *T. japonicus*, and *T. lepturus*

Species	Localities	Haplotypes	Date (dd.mm.yyyy)	GenBank accession numbers	Catalogue number
Trichiurus sp. 2	Miyazaki, Japan	1	07.12.2002		MUFS 21660
	Miyazaki, Japan	1	17.09.1999	AB125746	MUFS 22242
	Okinawa, Japan	1	20.12.1999		MUFS 17756
	Okinawa, Japan	1	20.12.1999		MUFS 17757
	Okinawa, Japan	1	20.12.1999		MUFS 17758
	Okinawa, Japan	1	20.12.1999		MUFS 17759
	Miyazaki, Japan	2	17.09.1999		MUFS 22243
	Okinawa, Japan	2	11.11.2000	AB20990	MUFS 22247
	Okinawa, Japan	2	07.11.1999		MUFS 17760
	Miyazaki, Japan	2	07.04.1999		MUFS 17608
Trichiurus japonicus	Miyazaki, Japan	1	28.10.2002		MUFS 22246
	Nagasaki, Japan	1	18.10.1999	AB197142	MUFS 18240
	Nagasaki, Japan	2	18.10.1999		MUFS 18237
	Nagasaki, Japan	2	18.10.1999	AB197143	MUFS 18242
	Nagasaki, Japan	2	18.10.1999		MUFS 18245
	Nagasaki, Japan	2	18.10.1999		MUFS 18397
	Chiba, Japan	3	20.08.2002	AB197144	MUFS 20254
	Chiba, Japan	4	20.08.2002	AB197145	MUFS 20256
	Chiba, Japan	4	20.08.2002		MUFS 22093
	Miyazaki, Japan	5	07.12.2002	AB197146	MUFS 22093
Trichiurus lepturus	Off Atlantic coast, USA	1	12.09.1994		KU 1206
	Off Atlantic coast, USA	1	12.09.1994	AB197147	KU 1224
	Off Atlantic coast, USA	1	10.03.1995		KU 1529
	Off Pascagoula, Gulf of Mexico, USA	2	16.11.2001		KU 3900
	Off Brownsville, Gulf of Mexico, Texas, USA	2	17.06.2002	AB197148	KU 5078
	Off Brazil	2	28.10.2004		MUFS(T) 01
	Off Brazil	2	28.10.2004		MUFS(T) 02
	Off Brazil	3	28.10.2004		MUFS(T) 03
	Off Brazil	3	28.10.2004	AB197149	MUFS(T) 04
	Off Brazil	3	28.10.2004		MUFS(T) 05
	Off Brazil	3	28.10.2004		MUFS(T) 06
	Off Brazil	3	28.10.2004		MUFS(T) 07
	Off Brazil	3	28.10.2004		MUFS(T) 08
	Off Brazil	3	28.10.2004		MUFS(T) 09
	Off Brazil	3	28.10.2004		MUFS(T) 10

KU, Kansas University; MUFS, Miyazaki Univeristy Fisheries Science; MUFS (T), Miyazaki University Fisheries Science (tissue)

of dorsal fin whitish when fresh. *Trichiurus lepturus* was also identified based on Burhanuddin (2003), this species having a whitish dorsal fin and a smaller caudal peduncle length than *T. japonicus* [specimens from the type locality (South Carolina, North America) were also used].

Tissue samples.—Tissue samples of *T.* sp. 2 and *T. japonicus*, caught from various areas of Japan, were collected from 10 individual specimens each, and *T. lepturus* tissue samples were obtained from 15 individual specimens caught off the Atlantic coast of the United States and Brazil (Table 1).

DNA extraction, PCR amplification, and sequencing.—Total DNA was extracted using a DNeasy Tissue Kit (Qiagen, Tokyo, Japan) from muscle tissues preserved in 99.5% ethanol, according to the manufacturer's protocols. The partial 16S rRNA gene was amplified using the following primers: L2510 (5′-GCCTGTTTA ACAAAAACAT-3′) and H3059 (5′-CGGTCTGAACTCAGATCACGT-3′) (Miya and Nishida, 1996). PCR amplification was carried out in a 25-µl reaction volume containing 1× Taq DNA polymerase reaction buffer (Bioneer, Daejeon, Korea), 5 µM each dNTP (Bioneer), 0.40 µM of each primer, 0.125 U *Taq* DNA polymerase (Bioneer), and 1 µl DNA template in a Techgene thermocycler (TC 312; Techne, Devon, UK). The thermal cycling profile was as follows: 94°C for 5 min 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 a final extension for 5 min at 72°C. The polymerase chain

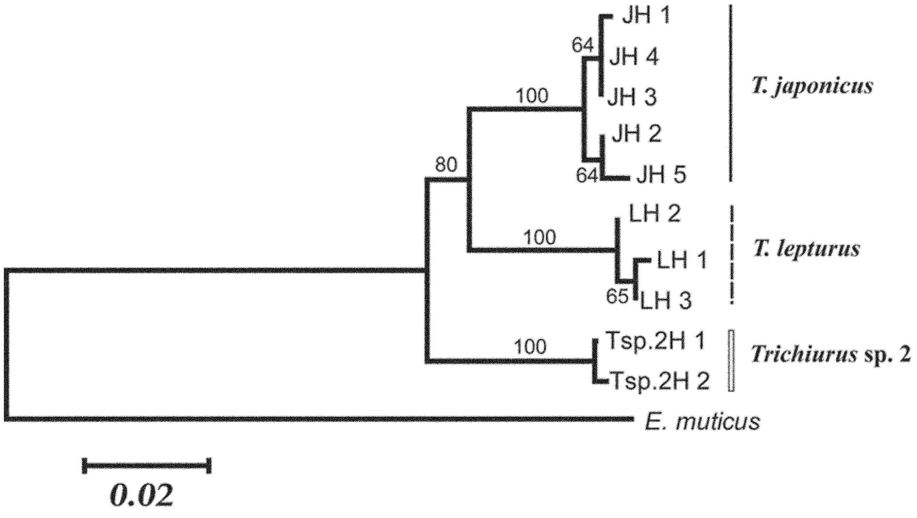

Fig. 1. Neighbor-joining phylogenetic tree based on the partial 16S rRNA gene for the haplotypes of *Trichiurus* sp. 2, *T. japonicus*, and *T. lepturus* with *Eupleurogrammus muticus* (Trichiuridae) as outgroup taxa. *Numbers above branches* indicate bootstrap values based on 1000 replications (>50% values are indicated). *JH1–5*, *T. japonicus* haplotypes 1–5; *LH 1–3*, *T. lepturus* haplotypes 1–3; *Tsp2H1–2*, *Trichiurus* sp. 2 haplotypes 1–2

reaction (PCR) products were separated by electrophoresis on a 1.0% agarose gel, stained with ethidium bromide (0.5 µg/ml), and visualized under a UV transilluminator.

Double-stranded DNA products were purified by Microcon 100 (Millipore, Bedford, MA, USA), and their nucleotide sequences were determined by direct sequencing using a Big Dye Terminator v 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) in a PRISM 310 Genetic Analyzer (Applied Biosystems) following manufacturer's instructions. Primers used were the same as those for PCR amplification. Sequences are available from DDBJ/EMBL/GenBank under the accession numbers AB125746, AB197142–46, AB197147–49, AB200989–90, and AB201821.

Data analysis.— The partial 16S rRNA sequences were edited in BioEdit (Hall, 1999) and aligned with CLUSTAL W (Thompson et al., 1994) as implemented in BioEdit. The partial sequence of the 16S rRNA gene from a trichiurid *Eupleurogrammus muticus* (AY212325) was used as an outgroup for phylogenetic analysis. Pairwise evolutionary distance among the haplotypes was calculated following Kimura's two-parameter (K2P) model (Kimura, 1980) and used to obtain a neighbor-joining (NJ) phylogenetic tree (Saitou and Nei, 1987) with MEGA 2.1 (Kumar et al., 2001). To verify the robustness of the internal nodes in the NJ trees, bootstrap analysis was carried out using 1000 pseudoreplications (Felsenstein, 1985).

Results and Discussion

The sequence analysis of the partial 16S rRNA gene (570 bp) revealed a total of 42 variable nucleotide sites, 36 of which were phylogenetically informative. Among the 42 variable sites found between the haplotypes of *T.* sp. 2, *T. japonicus*, and *T. lepturus*, 25 differed by transitional substitutions and 13 by transversional changes, with 4 mutation sites exhibiting multiple substitutions. The 10 specimens of *T.* sp. 2 exhibited two haplotypes differing only in one nucleotide position (C/T change), whereas in *T. japonicus* five haplotypes were found among the 10 specimens that differed in one to five nucleotide positions (two C/T changes, two G/T changes, and one C/G change). The 15 specimens of *T. lepturus* exhibited three haplotypes differing in only two nucleotide positions (two C/T changes).

The percentage of sequence divergence among *T.* sp. 2 ranged between 0.2% and 0.6%, whereas in *T. japonicus* and *T. lepturus* the range was 0.6%–1.0% and 0.2%–0.8%, respectively. However, among the three species, the percentage of sequence divergence was significantly high, at 5.5%–6.1% between *T.* sp. 2 and *T. japonicus* and 6.1%–6.5% between *T.* sp. 2 and *T. lepturus*. The degree of sequence divergence among the three species was comparable to those of congeneric species in other fish group. Among the species of *Trachurus* (Carangidae), interspecific 16S rRNA divergence of *Trachurus mediterraneus* to *Trachurus pictatus* and *Trachurus trachurus* was found to be 1.83% and 1.46%, respectively (Karaiskuo et al., 2003). According to Mabuchi et al. (2003), the 16S rRNA sequence divergence between *Apogon cyanosoma* and *Apogon properuptus* of cardinal fishes (Apogonidae) was found to be about 5.4%.

The NJ tree showed three major lineages with strong bootstrap values (100%), indicating that *T.* sp. 2, *T. japonicus*, and *T. lepturus* are reciprocally monophyletic (Fig. 1). Although the haplotypes of *T. japonicus* and *T. lepturus* formed nested subclusters within each lineage, the clustering of the haplotypes did not correspond to specific geographical locations. The lineage sorting of the haplotypes (without any sharing or overlapping) of each species into separate clusters is further evidence of the genetic divergence among the three species. The clustering of the *T. japonicus* lineage with the *T. lepturus* lineage indicated the closer relationship of the two species when compared to *T.* sp. 2 (see Fig. 1); this is also evident from the fact that the mean genetic distance among the haplotypes of *T. japonicus* and *T. lepturus* was found to be 0.027 ± 0.005 (vs. 0.029 ± 0.005 in *T. japonicus* and *T.* sp. 2). The NJ tree topology of the three species was also in accordance with their previous morphological characters. *Trichiurus japonicus* is very similar to *T. lepturus* in having a whitish dorsal fin while fresh, whereas *T.* sp. 2 is

distinctly different from both in having a yellowish-green dorsal fin along with other morphometric and meristic characters.

In conclusion, the genetic results together with the morphological differences indicated that *T.* sp. 2, *T. japonicus*, and *T. lepturus* were probably three distinct species. *Trichiurus japonicus* appears to be restricted on the East Asian Shelf, inhabiting the Chinese and Japanese coastal waters including Taiwan (Lin, 1963; personal observation) and sympatrically distributed with *T.* sp. 2, the latter probably being reproductively isolated from the former. The high sequence divergence between *T.* sp. 2 and *T. lepturus* (6.1%–6.5%) when compared to that between *T.* sp. 2 and *T. japonicus* (5.5%–6.1%), together with distinguishable coloration of dorsal fin and distribution pattern, could suggest that *T. lepturus* is geographically isolated from both *T.* sp. 2 and *T. japonicus*, representing a separate allopatric species.

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