Zoological Studies **48**(6): 835-849 (2009)

Zoological Studies

Speciation and Population Structure of Three *Trichiurus* Species Based on Mitochondrial DNA

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(Accepted February 9, 2009))

Kui-Ching Hsu, Nien-Tsu Shih, I-Hsun Ni, and Kwang-Tsao Shao (2009) Speciation and population structure of three *Trichiurus* species based on mitochondrial DNA. *Zoological Studies* **48**(6): 835-849. This study integrated most available genetic data of the "*Trichiurus lepturus* complex" and examined its species status and population structure using mitochondrial (mt) DNA sequences. Both the maximum-parsimony and Neighbor-joining distance trees supported 3 clear branches at 100% bootstrap value support. Due to a decisive genetic difference based on mtDNA cytochrome *b*, cytochrome oxidase subunit I, and 16S ribosomal RNA with the Kimura 2 parameter or maximum composite likelihood models, all historically confusing "*T. lepturus* complex" morphs were suggested to be 3 separate species: *T. japonicus*, *T. lepturus*, and *T.* sp. 2. This study suggested that *T. lepturus* from the Indo-Pacific in previous studies and *T.* sp. 2 are the same species. During the Miocene, these 3 *Trichiurus* species diverged from each other. According to the mtDNA phylogeographical patterns presented here, the vicariance events important to the speciation or structure of the "*T. lepturus* complex" were: (1) a warming event occurring around Japan during the middle Miocene, (2) cold water upwelling close to the tip of South Africa, (3) archipelagoes of the West Pacific, (4) the southeastern coast of Taiwan, (5) cyclical glacial periods, (6) the Isthmus of Panama, (7) the 2500 km long stretch of deep water separating the eastern and western Atlantic, and (8) freshwater plumes of the Amazon River. Based on current data, the East Indies Triangle of Indo-West Pacific was either a refuge or a colonization source for *T.* sp. 2.
http://zoolstud.sinica.edu.tw/Journals/48.6/835.pdf

Key words: *Trichiurus lepturus*, *Trichiurus japonicus*, *Trichiurus* sp. 2, Speciation, Phylogeography.

Hairtail fish (Trichiuridae) inhabit continental shelves and slopes of the world (Nelson 1994), and they are important commercial marine fishes (FAO 2004). In the family Trichiuridae, *Trichiurus lepturus* Linneaus, 1758 is considered to be a "variable species" (Tucker 1956). Nakamura and Parin (1993), based on morphological differences in pectoral and tooth patterns, estimated the existence of only 3 valid species: *T. lepturus* L., *T. auriga* Klunzinger, 1884, and *T. gangeticus* Gupta, 1966. The species *T. lepturus* was recognized as valid at all times by all ichthyologists, but the other 2 species were less discussed. *Trichiurus lepturus* (type locality: off South Carolina, USA), however, has a very broad geographical distribution and is known to cover tropical and temperate waters throughout the world. *Trichiurus auriga* and *T. gangeticus* have restricted geographical distributions; the former is distributed in Red Sea, Indian Ocean, and Timor, and the latter is confined to the east coast of India. However, because of its similar body appearance and silvery coloration as well as its unresolved taxonomy, until now, as many as 17 nominal species of the genus *Trichiurus* have been reported in the literature, but only 9 are valid species (Table 1) (Nakamura and Parin 1993, Wang et al. 1995, Eschmeyer 1998, Burhanuddin et al. 2002). In the taxonomic history of *Trichiurus*,

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most nominal species were considered synonyms of *T. lepturus*. In recent studies, 2 species groups within *Trichiurus* were generally recognized: the long-tailed hairtail, "*T. lepturus* complex," with the anal opening at a vertical position of the 38th-41st dorsal fin rays; and the short-tailed hairtail, "*T. russelli* complex," with the anal opening at a vertical position of the 34th-35th dorsal fin rays (Burhanuddin et al. 2002). Taxonomic identification within the "*T. lepturus* complex" has long been confusing.

In the northwestern Pacific, *T. japonicus* Temminck and Schlegel, 1844 used to be included in *T. lepturus* and was arguably treated as a junior synonym (Tucker 1956, Nakamura and Parin 1993, Nelson 1994). Nevertheless, *T. japonicus* from the East China and Japan Seas was reported to have higher numbers of dorsal fin rays and vertebrae (Lee et al. 1977, Nakabo 2000), and was recently regarded as a valid species after recent work by Chakraborty et al. (2006a) and Tzeng et al. (2007), who provided both traditional morphometric data and molecular data of mtDNA cytochrome (Cyt) *b* and 16S ribosomal (r) RNA genes. Wang et al. (1993) examined isozymes in samples over an extensive geographic range including the East and South China Seas and concluded that 3 species of *T. brevis* Wang and You, 1992, *T. haumela* Forsskål, 1775, and *T. nanhaiensis* Wang and Xu, 1992 exist. *Trichiurus brevis* is one of the short-tailed hairtails. *Trichiurus haumela* was considered by Wang et al. (1995) to be equivalent to *T. japonicus*; and *T. nanhaiensis* was described as a new species. Among them, *T. nanhaiensis* is less well known because the paper was written in Chinese and published in a Chinese journal; as a result, few ichthyologists challenged the validity of that species. A similar study was done by Chakraborty et al. (2006a), who treated their study materials of *Trichiurus* species as *T. lepturus* (Atlantic), *T. japonicus* (Pacific), and *Trichiurus* sp. 2 (Pacific). The last one was given no binominal name because the work lacked diagnostic morphological characters.

In Taiwan, Lee et al. (1977) proposed that *T. lepturus* and *T. japonicus* are 2 valid species based on the distinguishable external morphology of various body ratios. Subsequently, Lin and Shen (1986) and Lee et al. (1993) considered these 2 species to be the same based on minor differences in morphometric measurements and the high genetic identity shown in isozyme patterns. However, experienced hairtail fish anglers have voiced suspicions that more than 1 species within the "*T. lepturus* complex" occur in Taiwan. Recent studies based on a genetic analysis and growth patterns found that there are 3 species within the "*T. lepturus* complex" in Taiwanese waters but did not give them accurate nomination since there were no applicable diagnostic keys, and the authors treated their findings of 3 *Trichiurus* groups as *T. lepturus*, *T. nanhaiensis*, and *T.* cf. *nanhaiensis*

Table 1. Nomenclature history of *Trichiurus* species

Scientific name	Nomenclature	Valid names	Status
T. auriga	Klunzinger, 1884	*T. auriga*	rare
T. gangeticus	Gupta 1966	*T. gangeticus*	rare
T. lepturus	Linnaeus, 1758	*T. lepturus*	largehead
T. haumela	Forsskal, 1775	*T. lepturus*	largehead
T. nitens	Garman, 1899	*T. lepturus*	largehead
T. coxii	Ramsay and Ogilby, 1887	*T. lepturus*	largehead
T. malabaricus	Day, 1865	*T. lepturus*	largehead
T. argenteus	Shaw, 1803	*T. lepturus*	largehead
T. lajor	Bleeker, 1854	*T. lepturus*	largehead
T. japonicus	Temminck and Schlegel, 1844	*T. japonicus*	largehead
T. margarites	Li 1992	*T. nanhaiensis*	largehead
T. nanhaiensis	Wang and Xu 1992	*T. nanhaiensis*	largehead
T. australis	Chakraborty, Burhanuddin and Iwatsuki 2005	*T. australis*	short-tailed
T. minor	Li 1992	*T. brevis*	short-tailed
T. brevis	Wang and You 1992	*T. brevis*	short-tailed
T. nickolesis	Burhanuddin and Iwatsuki 2003	*T. nickolesis*	short-tailed
T. russelli	Dutt and Thankam 1967	*T. russelli*	short-tailed

(Shih 2004, Hsu et al. 2007). However, clear species identification for fishery purposes has been the subject of a major Food and Agriculture Organisation (FAO) program since the 1960s.

Mitochondrial (mt)DNA analyses using conserved genes like 16S rRNA, Cyt *b*, or cytochrome oxidase subunit I (COI) have emerged as powerful approaches to answer questions of fish taxonomy, species identification, and population genetics (e.g., Hebert et al. 2003, Guo et al. 2005, Chen et al. 2007, Itoi et al. 2007). In the present study, we used mtDNA sequences that included COI, Cyt *b*, and 16S rRNA genes to study inter- and intraspecific variations. We attempted to use sequence data of the "*T. lepturus* complex" to address the following questions: (1) Are all of the current species valid?; (2) What biogeographic barriers are likely to have caused speciation between species?; (3) What degree of genetic structure exists within species and what can it tell us about the history of populations?; and (4) How do the observed phylogeographical patterns compare to those of other marine organisms?

MATERIALS AND METHODS

Species identification

The generic diagnosis of the genus *Trichiurus* followed Nakamura and Parin (1993). *Trichiurus* sp. 2 (hereafter *T.* sp. 2) was identified following Nakabo (2002), with confirmation of the yellow dorsal fin color when fresh and the bottom of the oral cavity being light-colored. The identification of *T. japonicus* followed Li (1992), Nakabo (2002), and Burhanuddin (2003), with the caudal peduncle length (mean 52% of the preanal length (PL)) longer than those of *T.* sp. 2 (mean 33% of the PL) and *T. lepturus* (mean 40% of the PL), the bottom of the oral cavity being dark-colored, and the ground color of the dorsal fin being whitish when fresh. *Trichiurus lepturus* is very similar to *T. japonicus*, and it was identified based on Burhanuddin (2003), with a whitish dorsal fin and a smaller caudal peduncle length than that of *T. japonicus*. To make our materials comparable to those of Chakraborty et al. (2006a), the sequences of 16S rRNA from samples of *T. japonicus* (AM779552-60), *T. lepturus* (AM779563-5), and *T.* sp. 2 (AM779561-2) were obtained, and it turned out that the corresponding materials were equivalent in the species identification.

Sampling and sequence analyses

Samples of the "*T. lepturus* complex" were obtained from longliners and trawlers operating off the Taiwanese coasts. Samples obtained were categorized into 4 hydrographic areas: the Taiwan Strait, Pacific Ocean, South China Sea, and East China Sea (Fig. 1A). Fresh specimens were placed on ice and transported to the laboratory. Total genomic DNA was extracted from a muscle sample of each fish using a standard phenol/chloroform method (Sambrook et al. 1989). Polymerase chain reaction (PCR) amplification of the partial Cyt b fragments subsequently used primers Glu (5'-CGAAGCTTGACTTGAArAACCA yCGTTG-3') and Cyt (5'-GGCAAATAGGAArTATC ATTC-3') (Hsu et al. 2007). Amplification of 16S rRNA was carried out using the following primers: L2510 (5'-GCCTGTTTAACA AAAACAT-3') and H3059 (5'-CGGTCTGAACTCAGATCACGT-3') (Miya and Nishida 1996). The COI gene was amplified using the universal primers for fish DNA barcoding: FishF1 (5'-TCAACCAACCACAAAGAC ATTGGCAC-3') and FishR1 (5'-TAGACTTCTGGG TGGCCAAACAATCA-3') (Ward et al. 2005). Each 100 μl of the PCR contained 10 ng template DNA, 10 μl 10x reaction buffer, 10 μl $MgCl_2$ (25 mM), 10 μl dNTP mix (10 mM), and 10 pmol of each primer. The reaction was programmed on an MJ Thermal Cycler (MJ Research, Inc. Ramsey, Minnesota, USA) as 1 cycle at 94°C for 4 min; 33 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 45 s; and a final extension at 72°C for 10 min. PCR products were purified by electrophoresis in a 1.0% agarose gel using 1x TAE buffer. The gel was stained with ethidium bromide, and the desired DNA band was cut and eluted using an agarose gel purification kit (QIAGEN, Valencia, CA, USA). Purified DNAs were sequenced on both strands on an Applied Biosystems 377 automated sequencer (division of Perkin-Elmer, Foster City, CA, USA).

Sequences of mtDNA haplotypes were deposited in GenBank with the following accession numbers: *T. lepturus*, AM910645-9, *T. japonicus*, AM910798-817, and *T.* sp. 2, AM921685-96 for Cyt *b*; *T. lepturus*, AM779563-5, *T. japonicus*, AM779552-60, and *T.* sp. 2, AM779561-2 for 16S rRNA; and *T. lepturus*, FM998057, *T. japonicus*, FM998055-6, and *T.* sp. 2, FM998058-9 for COI. In order to determine the species status of the "*T. lepturus* complex" in the world, this study also integrated genetic data from previous studies (accession nos.: DQ364146-50, AB197142-9, AB125746, AB198977-82, and

AB212875-88; Chakraborty et al. 2006a b, Chakraborty and Iwatsuki 2006, Tzeng et al. 2007). *Eupleurogrammus muticus* (16S rRNA: AY212325) and *Trichiusus brevis* (Cyt *b*: AM910640-4; 16S rRNA: DQ643037) obtained from GenBank were used as outgroups. The obtained sequences were edited and aligned using the BioEdit software (Hall 1999). First, we used COI as DNA barcodes to identify the fish species, so we followed the methods of DNA barcoding of Australia's fish species (Ward et al. 2005). 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 vers. 4 (Tamura et al. 2007). Second, we also used Cyt *b* as another molecular marker to identify the genetic diversity within the "*T. lepturus* complex". Pairwise genetic divergences among haplogroups were calculated following the K2P model, and these were used to obtain an NJ tree with a maximum composite likelihood distance model using MEGA 4.

Finally, we used 16S rRNA sequences to confirm the phylogroups, by displaying COI or Cyt *b*, and the genetic structure existing within species. DNA substitutions were determined to be the most appropriate models for the analyses by applying MODELTEST (vers. 3.06, Posada and Crandall 1998). The best model selected with the Akaike information criterion (AIC) was the GTR. Haplotype genealogies were generated by an NJ and maximum-parsimony (MP) analysis with DAMBA vers. 5.0.37 (Xia and Xie 2001) and MEGA 4. Bootstrapping was performed with 500 replicates. The number of mutations between DNA haplotypes via pairwise comparisons was calculated using MEGA 4. We also constructed

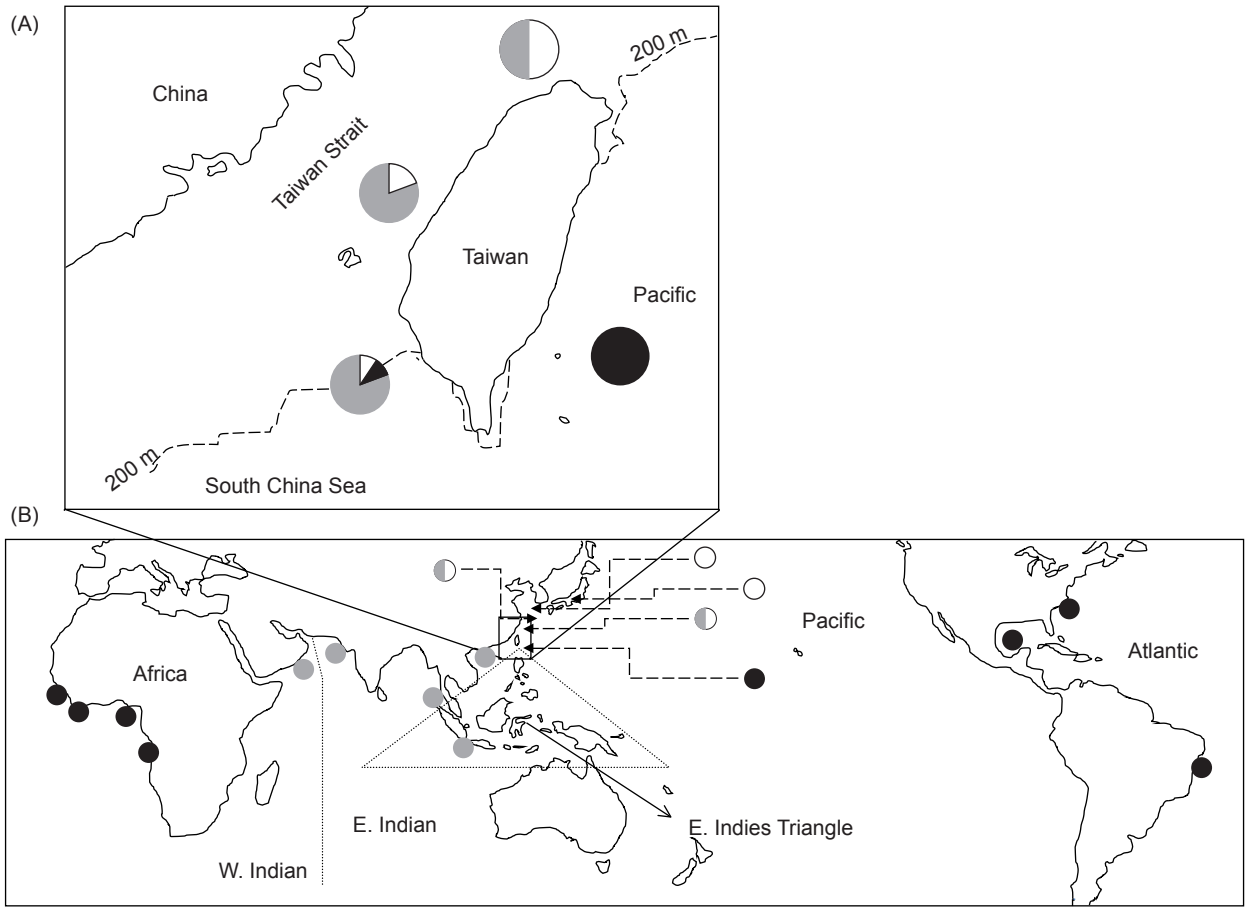


Fig. 1. (A) Map illustrating the locations of samples identified as *Trichiurus* sp. 2 (gray), *T. japonicus* (white), and *T. lepturus* (black), and their frequencies in Taiwanese waters. *Trichiurus lepturus* is distributed in southern and eastern Taiwan, *T. japonicus* is distributed in northern and western Taiwan, and *T.* sp. 2 is distributed in western and southern Taiwan. (B) After molecular identification, *T. lepturus* is distributed on the west coast of Africa, western Atlantic coast, and West Pacific, *T. japonicus* is distributed in various parts of Japan and Taiwan, and *T.* sp. 2 is distributed in Indo-Pacific waters.

a haplotype network with the aid of MINSPNET (Excoffier and Smouse 1994) as it seemed to give similar or better results. The genetic structures of species and populations were tested using an analysis of molecular variance (AMOVA) approach in Arlequin (vers. 2.000; Schneider et al. 2000). In order to compare our data with previous studies, we calculated pairwise genetic divergences among haplogroups calculated following the K2P and maximum composite likelihood models.

The MDIV program (Nielsen and Wakeley 2001) was employed to estimate and test different phylogeographical scenarios. MDIV uses a Markov chain Monte Carlo (MCMC) method within a Bayesian framework to estimate the posterior distribution of theta ($\theta = 2N_e u$), the migration rate per generation ($M = N_e m$; m, migration rate), and the divergence time between populations (equations adjusted for mtDNA). The program also estimated the expected time to the most recent common ancestor (T_{MRCA}) for all sequences in the samples. Five runs were performed with 5×10^6 cycles each for the MCMC and a burn-in time of 10% as recommended by the program manual.

RESULTS

Phylogenetic analyses

In Taiwan, 16 specimens were used to identify species by COI, and 5 haplotypes were detected. The sequence analysis of the partial COI gene (642 bp) revealed a total of 73 variable nucleotide sites; all of which were parsimoniously informative. The NJ phylogenetic analyses with the K2P model revealed a phylogeny that was consistent with 3 reciprocally monophyletic species lineages, i.e., *T. lepturus*, *T. japonicus*, and *T.* sp. 2 (Fig. 2A). The pairwise sequence divergences between COI sequences of *Trichiurus* species ranged 0.115-0.135, with an average of 0.127 (Table 2); whereas the divergences within species ranged 0.000-0.003. In the Cyt *b* data, 54 specimens were used, and 37 haplotypes were detected. The sequence analysis of the partial Cyt *b* gene (606 bp) revealed a total of 203 variable nucleotide sites, 166 of which were parsimoniously informative. The NJ phylogenetic analyses with the maximum composite likelihood model revealed a phylogeny that was also consistent with 3 reciprocally monophyletic lineages in Taiwanese waters, i.e., *T. lepturus*, *T. japonicus*, and *T.* sp. 2 (Fig. 2B). The sequence divergence of Cyt *b* within *T.* sp. 2 ranged 0.007-0.019; and the divergences within *T. japonicus* and *T. lepturus* ranged 0.005-0.027 and 0.001-0.010, respectively. Sequence divergences with the K2P model among the 3 species, on the other hand, were significantly high; at 0.183 between *T.* sp. 2 and *T. japonicus* and 0.181 between *T.* sp. 2 and *T. lepturus* (Table 2). The interspecific divergences, estimated using the maximum composite likelihood model, were also similar to that estimated with the K2P model (Table 2).

In order to determine species status within the "*T. lepturus* complex" in the world, this study also integrated genetic data from previous studies (Chakraborty et al. 2006a b, Chakraborty and Iwatsuki 2006, Tzeng et al. 2007) and this study. Totally, 100 specimens were used, and 87 haplotypes were detected. The sequence analysis of the partial 16S rRNA gene (411 bp) revealed a total of 89 variable nucleotide sites, 44 of which were parsimoniously informative. Both the MP (with a CI excluding the uninformative characters of 0.80 and an RI of 0.95) and NJ (with the K2P and GTR models) phylogenetic analyses were based on 16S rRNA (Figs. 3, 4). The phylogenetic trees of 16S rRNA clearly indicated that *T.* sp. 2, *T. japonicus*, and *T. lepturus* are reciprocally monophyletic groups (Figs. 3, 4). *Trichiurus lepturus* populations from the Indo-Pacific (Chakraborty et al. 2006b) were clustered into *T.* sp. 2. *Trichiurus lepturus* populations from West Africa and the West Atlantic (Chakraborty and Iwatsuki 2006) were clustered into *T. lepturus* in Taiwanese waters (Fig. 4). The sequence divergence of 16S rRNA within *T.* sp. 2 ranged 0.001-0.010; whereas the ranges were 0.000-0.001 and 0.001-0.012 within *T. japonicus* and *T. lepturus*, respectively. However, the sequence divergence among the 3 species was significantly high at 0.055 between *T.* sp. 2 and *T. japonicus* and 0.067 between *T.* sp. 2 and *T. lepturus* (Table 2). The current data suggest that *T. lepturus* from the Indo-Pacific (Chakraborty et al. 2006b) and *T.* sp. 2 are the same species; and *T. lepturus* from the West Atlantic (Chakraborty et al. 2006a b), West Africa (Chakraborty and Iwatsuki 2006), and Pacific are the same species. Finally, we suggest that *T. lepturus* occurs in the Pacific and Atlantic, *T.* sp. 2 in the Indian Ocean, East China Sea, and South China Sea (Indo-Pacific), and *T. japonicus* in the Japan Sea and East China Sea (Fig. 1B).

Population structure and genetic diversity

Within the *T. lepturus* lineage, 3 sub-lineages were identified which corresponded to 3 geographic regions: I in Taiwan (Pacific), II in the West Atlantic, and III of West Africa (Fig. 4). The sequence divergences based on 16S rRNA within these 3 sub-lineages ranged 0.001-0.003; among the 3 sub-lineages, the ranges were 0.013 between West Africa and the West Atlantic, 0.015 between the West Atlantic and the Pacific, and 0.016 between West Africa and the Pacific (Table 2). The topology of the minimum spanning network of *T.* sp. 2 exhibited significant geographical structuring of 4 mtDNA clades. Clade I contained 3 populations belonging to regions in the East and South China Seas (Pacific), which included Hainan I. (China), Taiwan, and Japan. Clade II was restricted to the East Indies Triangle; clade III was restricted to the East Indian Ocean; and clade IV was restricted to the West Indian Ocean (Fig. 5A). The East Indies Triangle (clade

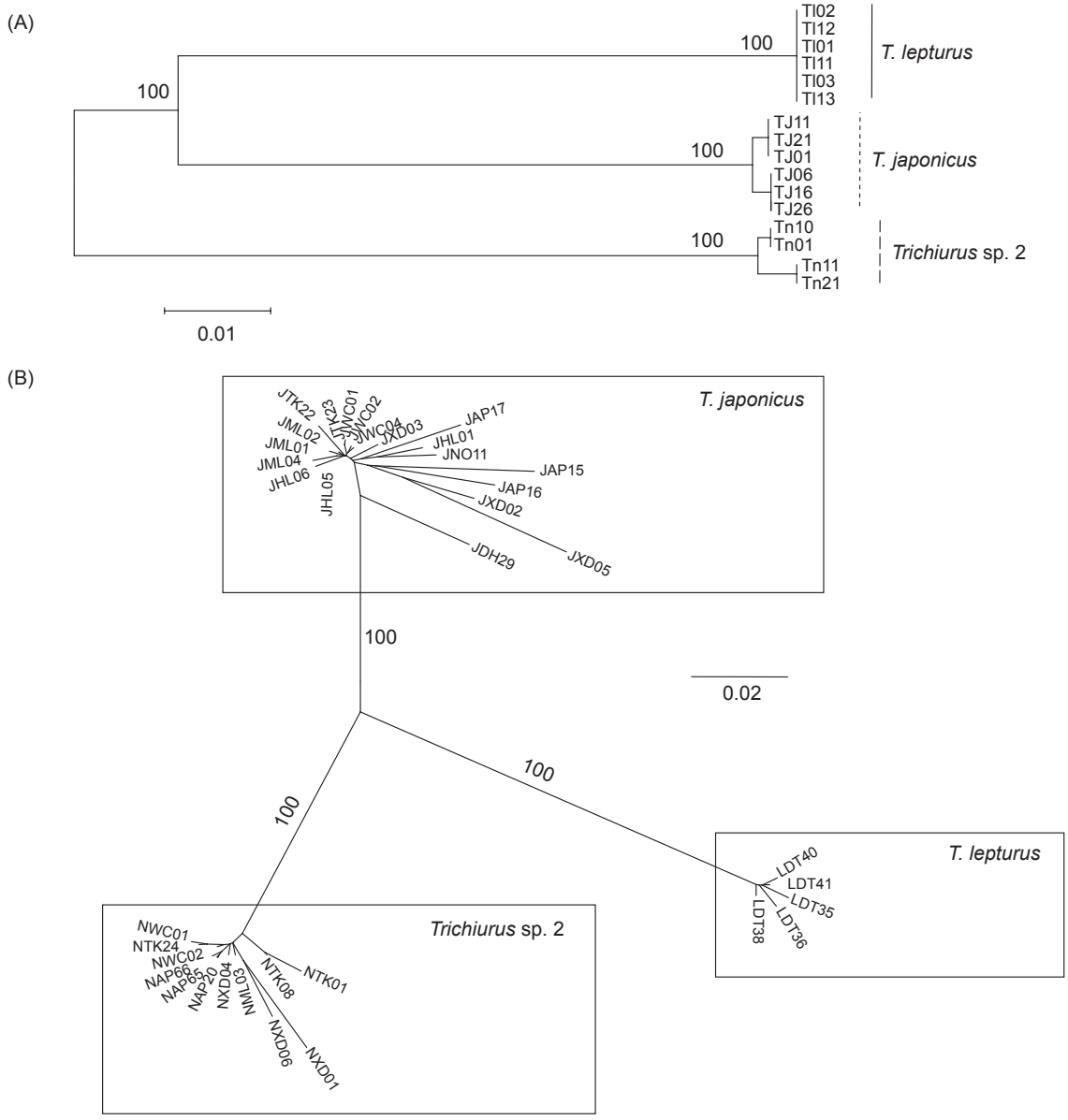


Fig. 2. Trees generated from Neighbor-joining (NJ) method with the Kimura 2-parameter (K2P) model based on cytochrome oxidase subunit I (COI) sequences (A) and based on cyclooxygenase (Cyt) *b* sequences with maximum composite likelihood distance (B). Numbers on the major branches are percentages of bootstrap values obtained with 500 replicates.

II) formed an interior clade linked to the other 3 clades. Based on current data, *T.* sp. 2 uses the East Indies Triangle of the Indo-West Pacific as either a refuge or a source of colonization. The AMOVA indicated that the regional structuring of the 4 clades explained much of the total genetic variance. The large sequence divergences among clades indicated that they have been isolated from each another for a considerable amount of time. Sequence divergences within these 4 clades ranged 0.001-0.003, whereas among the 4 clades, the ranges were 0.017 between the West Indian and East Indian Oceans, 0.007 between the West Indian Ocean and the East Indies Triangle, and 0.012 between the West Indian and West Pacific Oceans (Table 2).

The hierarchical analyses of sequence differences with the AMOVA indicated significant differences among species (F_{ST} = 0.974). The results of the AMOVA also found that a substantial proportion of molecular variance was attributable to differences among populations (F_{ST} = 0.814 for *T.* sp. 2; F_{ST} = 0.930 for *T. lepturus*; and F_{ST} = 0.630 for *T. japonicus*) and to differences among populations within regions (F_{SC} = 0.470 for *T.* sp. 2; F_{SC} = 0.520 for *T. lepturus*; and F_{SC} = 0.560 for *T. japonicus*). The relative contribution of differences among regions was small for *T. japonicus* (F_{CT} = 0.160), but large for *T.* sp. 2 and *T. lepturus* (F_{CT} = 0.648 and 0.854, respectively).

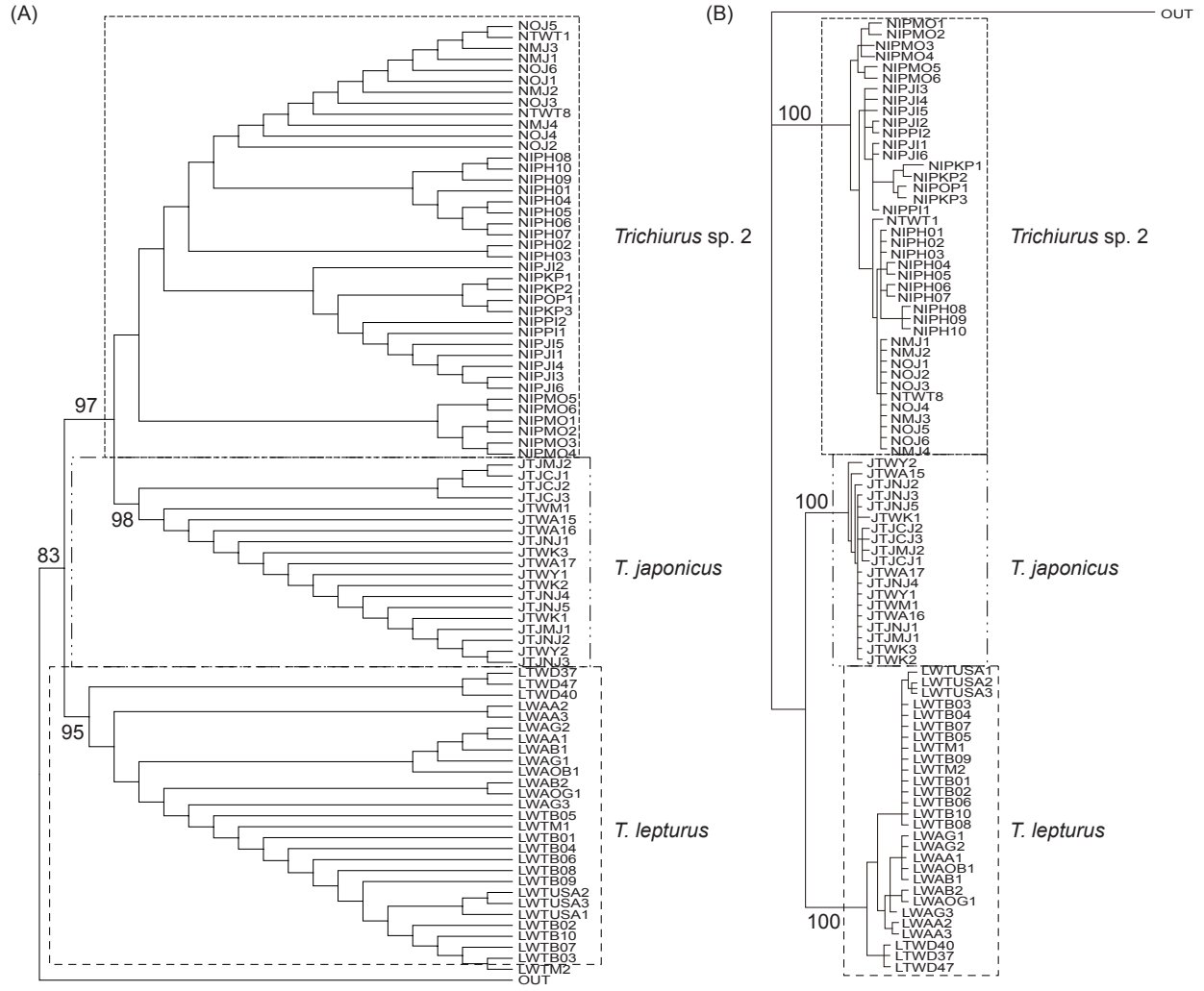


Fig. 3. (A) Phylogenetic tree produced by the maximum-parsimony (MP) analysis using MEGA 4 (CI excluding the uninformative characters = 0.80, RI = 0.95) and (B) generated from Neighbor-joining method with the GTR model using DAMBE based on partial 16S rRNA with *Eupleurogrammus muticus* (Trichiuridae) as the outgroup taxon. Numbers above the branches indicate bootstrap values based on 500 replicates.

Coalescence analyses and divergence times

The mtDNA Cyt *b* and 16S rRNA evolutionary rates in *Trichiurus* were not estimated because there was no calibration point. Therefore, estimates of *T* were converted to real time, assuming a range of neutral mutation rate estimates for fishes of 0.91% and 0.23% sequence divergence per 10^6 yr for Cyt *b* and 16S rRNA, respectively (Bermingham et al. 1997, Martin and Bermingham 1998, Alves-Gomes 1999, He et al. 2004, Guo et al. 2005, Tzeng et al. 2007). Based on the coalescent estimates of divergence times among species, the gene divergence (when haplotypes first began to differentiate) took place between (15.23 and 9.88) × 10^6 yr before the present (Ma BP). The 3 species became isolated from each other between 11.57 and 8.83 Ma BP. Among the 4 disjoined clades (populations) in the network within *T.* sp. 2, gene divergence took place between 5.05 and 1.90 Ma BP, and they became isolated from each other between 4.32 and 1.28 Ma BP. Within *T. lepturus*, gene divergence took place between 2.97 and 2.74 Ma BP, and they became isolated from each other between 3.23 and 2.26 Ma BP (Table 3). The large disparity in estimates of T_{MRCA} (gene divergence) versus population divergence (*T*) suggest that gene flow had begun to obscure the phylogeographical structure among populations (Table 3).

DISCUSSION

Taxonomic status

The 1st aim of this study was to examine the species status of the "*T. lepturus* complex." Systematists attempt to describe variations among taxa as well as historical relationships among them. Johns and Avise (1998) calculated and compared levels of Cyt *b* sequence divergence with the K2P

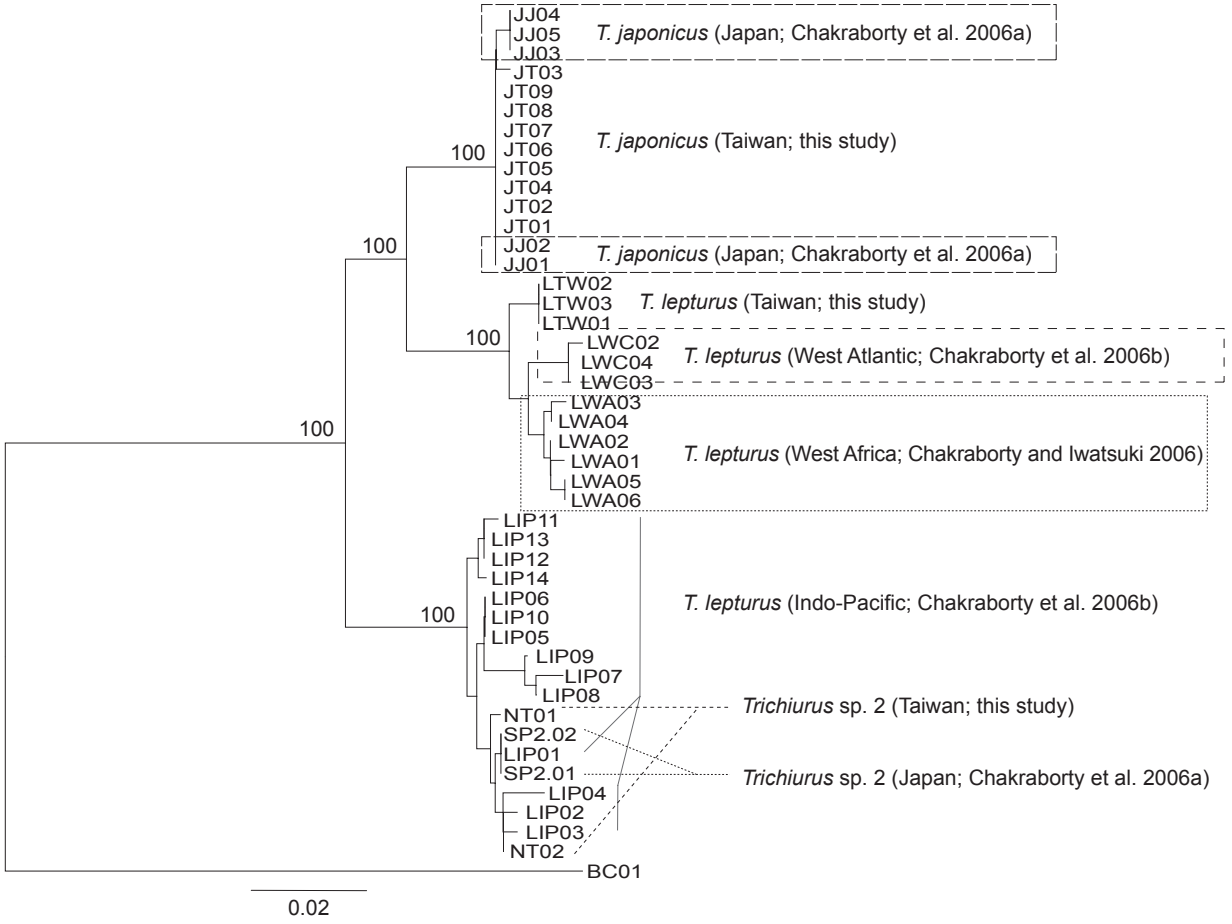


Fig. 4. Tree generated from the Neighbor-joining method with Kimura 2 parameter (K2P) model based on partial 16S rRNA with *Trichiurus brevis* as the outgroup taxon. Numbers above the branches indicate bootstrap values based on 500 replications.

model between sister species, congeneric species, and confamilial genera within and across major vertebrate taxonomic classes. The divergence between species among 81 confamilial fish genera averaged 0.120 (Johns and Avise 1998). Ward et al. (2005) and Hsu and Shao (2007) calculated the level of COI sequence divergence with the K2P model within species, genera, families, orders, and classes from Australian and Taiwan marine fishes, respectively, and suggested that the divergence between species within a genus averaged 0.099. In the present study, 3 major mtDNA clades, corresponding to the nominal species of *T.* sp. 2, *T. japonicus*, and *T. lepturus*, were discovered. The interspecific divergences of COI were 0.115-0.135 and those based on Cyt *b* with the K2P and maximum composite likelihood models were 0.168-0.183 and 0.165-0.183, respectively (Table 2). Moreover, sequence divergences of 16S rRNA among the 3 *Trichiurus* species were 0.044-0.067 (Table 2). The degree of sequence divergence among the 3 species was comparable to those of congeneric species in other fish groups. Among the species of *Trachurus* (Carangidae), the interspecific 16S rRNA divergences of *Tra. mediterraneus* to *Tra. pictatus* and *Tra. trachurus* was found to be 0.018 and 0.015, respectively (Karaiskou et al. 2003). The 16S rRNA sequence divergence between *Apogon cyanosoma* and *A. properuptus* (Apogonidae) was found to be about 0.054 (Mabuchi et al. 2003). According to the interspecific sequence divergences based on the COI, Cyt *b*, and 16S rRNA described above, the divergences estimated in this study were much larger than the estimates of intraspecific divergence in other species. Therefore, these large sequence divergences and well-supported phylogeny suggest that the 3 major clades within the "*T. lepturus* complex" are 3 distinct species: *T.* sp. 2, *T. japonicus*, and *T. lepturus*.

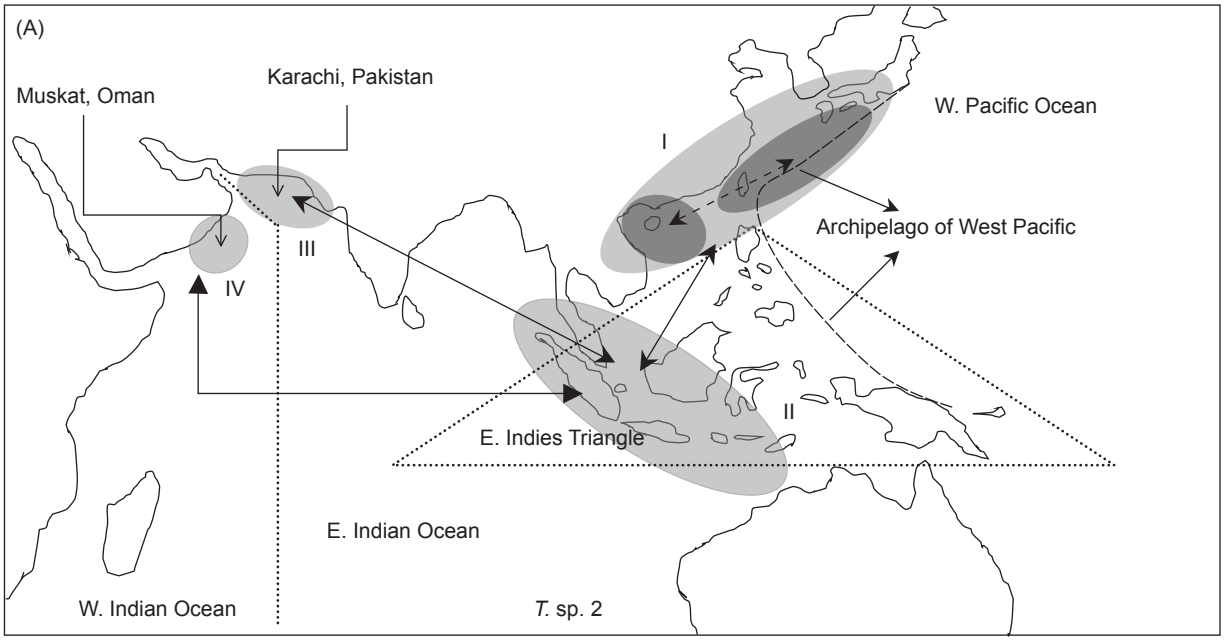
Chakraborty et al. (2006b) suggested that since haplotypes of *T. lepturus* from the Indo-Pacific and West Atlantic were in separate clusters, the *T. lepturus* populations from these 2 geographic locations were genetically distinct. The genetic results obtained by Chakraborty et al. (2006b) clearly showed that the 2 regional populations of *T. lepturus* (West Atlantic and Indo-Pacific) were represented by at least 2 species. Likewise, Chakraborty and Iwatsuki (2006) examined genetic variations among species from various localities and indicated that there were 3 major clades within the "*T. lepturus* complex". The Atlantic clade was comprised of 2 separate subclades containing haplotypes of West Africa and the West Atlantic, whereas the Indo-Pacific clade and *T. japonicus* formed separate lineages. Chakraborty and Iwatsuki (2006) suggested that the morphotypes of *T. lepturus* obtained from the West African coast were genetically distinct and probably represented a separate species. The low sequence divergence between the West African and West Atlantic types

Table 2. Samples size (for cytochrome (Cyt) *b*, 16S rRNA, and cytochrome oxidase subunit I (COI), in parentheses) and average sequence divergence with the Kimura 2 parameter (K2P) model (bold) and maximum composite likelihood model among *Trichiurus* species or populations for Cyt *b* (above the diagonal), 16S rRNA (below the diagonal), and COI (above the diagonal, in italics)

		T. lepturus			*Trichiurus* sp. 2	
	T. japonicus	W. Africa	Pacific	E. Indian	Indies Triangle	Pacific
T. japonicus (23/21/6)		**0.168 ± 0.017** 0.165 ± 0.016 ***0.115 ± 0.032***	**0.183 ± 0.016** 0.183 ± 0.017 ***0.132 ± 0.036***			
T. lepturus (10/32/4)	**0.044 ± 0.011** **0.045 ± 0.011**		**0.181 ± 0.018** 0.180 ± 0.017 ***0.135 ± 0.037***			
W. Atlantic		**0.013 ± 0.005**	**0.015 ± 0.006**			
W. Africa			**0.016 ± 0.006**			
Trichiurus sp. 2 (21/47/6)	**0.055 ± 0.013** 0.055 ± 0.012	**0.067 ± 0.014** 0.068 ± 0.014				
W. Indian				**0.017 ± 0.006**	**0.007 ± 0.004**	**0.012 ± 0.005**
E. Indian					**0.010 ± 0.004**	**0.017 ± 0.006**
Indies Triangle						**0.007 ± 0.004**

indicated that there was a much closer relationship between them when compared to that between *T. japonicus* and the Indo-Pacific morphotypes. The closer relationship was probably due to geographical proximity and a relatively short time since the 2 types (West Africa and West Atlantic) diverged from a common ancestor.

When the genetic data from all of the studies were reexamined, we found that the small sequence divergence between West Africa and the West Atlantic indicated that these 2 subclades should not be elevated to species, but rather geographic populations, and the populations from the Indo-Pacific *T. lepturus* (Chakraborty et



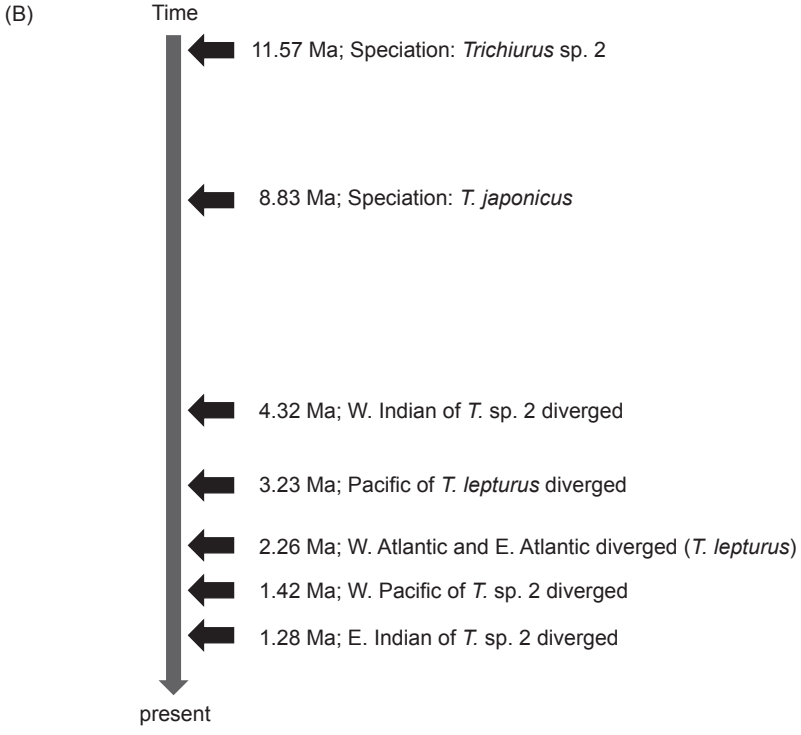


Fig. 5. (A) *Trichiurus* sp. 2 16S rRNA nested clade design and its geographical distribution. (B) Time of vicariance events are given.

al., 2006b) were clustered into *T.* sp. 2 (Fig. 4). The low sequence divergence within this clade indicated a much-closer relationship between them when compared to that of *T. japonicus* or *T. lepturus* from the Atlantic and Taiwan. Therefore, *T. lepturus* from the Indo-Pacific in previous studies and *T.* sp. 2, herein, are the same species. Nonetheless, Tucker (1956) recognized the Atlantic population of the genus *Trichiurus* as *T. lepturus* Linneaus, 1758; while the Indo-Pacific population was recognized as *T. haumela* Forsskål, 1775. Another species, *T. malabaricus* Day, 1865, was also described from the Indo-Pacific but was subsequently considered a synonym of *T. haumela* (Day, 1876). In addition, *T. nanhaiensis* Wang and Xu, 1992, was described from the South China Sea, but this species is less well known. In this study, we suggest that the Indo-Pacific population significantly differed from *T. lepturus* and *T. japonicus*, and is synonymous with *T.* sp. 2. However, the nomenclature of the Indo-Pacific population has remained uncertain. Herein, we recognize the Indo-Pacific population as *T.* sp. 2, following Chakraborty et al. (2006b) and suggest that only 3 species, *T. japonicus*, *T. lepturus*, and *T.* sp. 2, are valid within the "*Trichiurus lepturus* complex."

Table 3. Pairwise estimates of N_{ef} (because the mutation rate is the same for each population, differences in θ correspond to differences in N_{ef} for each pair of populations), migration rates ($M = N_{ef}m$), time since divergence (T), and time to the most recent common ancestor (T_{MRCA}) based on analysis of mtDNA sequence data using MDIV

	Trichiurus sp. 2				*T. lepturus*		
		W. Indian	Indies Triangle	Pacific		W. Atlantic	Pacific
T. japonicus	N_{ef} = 1.71x10^6 M = 0.010 T = 10.68Ma T_{MRCA} = 12.27Ma				N_{ef} = 1.15x10^6 M = 0.006 T = 8.83Ma T_{MRCA} = 9.88Ma		
Trichiurus sp. 2					N_{ef} = 2.26x10^6 M = 0.002 T = 11.57Ma T_{MRCA} = 15.23Ma		
E. Indian		N_{ef} = 8.26x10^5 M = 0.006 T = 2.23Ma T_{MRCA} = 3.6Ma	N_{ef} = 4.05x10^5 M = 0.010 T = 1.28Ma T_{MRCA} = 1.90Ma	N_{ef} = 9.21x10^5 M = 0.018 T = 3.00Ma T_{MRCA} = 3.83Ma			
Indies Triangle		N_{ef} = 2.63x10^5 M = 0.008 T = 4.32Ma T_{MRCA} =5.05Ma		N_{ef} = 5.26x10^5 M = 0.012 T = 1.42Ma T_{MRCA} = 2.16Ma			
Pacific		N_{ef} = 6.37x10^5 M = 0.006 T = 1.48Ma T_{MRCA} = 2.50Ma					
T. lepturus							
W. Africa						N_{ef} = 4.95x10^5 M = 0.010 T = 2.26Ma T_{MRCA} = 2.74Ma	N_{ef} = 5.11x10^5 M = 0.004 T = 2.52Ma T_{MRCA} = 2.97Ma
Pacific						N_{ef} = 3.32x10^5 M = 0.002 T = 3.23Ma T_{MRCA} = 2.77Ma	

Speciation and genetic population structures

The distribution patterns of the "*T. lepturus* complex" suggest their dispersal, biogeography, and speciation. Based on distribution patterns, we found 3 biogeographic barriers which might have promoted speciation. First, the barrier between *T. lepturus* and *T.* sp. 2 or between *T. lepturus* and *T. japonicus* is inferred to lie along the archipelagos of the West Pacific which are located at the west rim of the Pacific Ocean and are composed of the Kuril I., Japan, the Ryukyu I., Taiwan, the Philippines, and New Guinea (Fig. 5A). The archipelagos of the West Pacific have frequently been identified as a boundary of the distributions of marine organisms (Blum 1989, Williams and Reid 2004). We consider that the archipelagos of the West Pacific blocked the east-west dispersal of populations on either side (Figs. 1B, 5A). Second, the barrier between *T. japonicus* and *T.* sp. 2 was inferred to lie along the southeastern coast of Taiwan. In a previous study (Blum 1989), the differentiation and distribution of *Chaetodon xanthurus* (Chaetodontidae) and its adjacent relatives were explained by actions of this barrier. Third, cold water upwelling close to the tip of South Africa blocks gene flow between the Indian and Atlantic Oceans. The upwelling first appeared in the Miocene (Dister-Haass and Schrader 1979, Siesser 1980), intensified in the late Pliocene, and assumed its present weaker and fluctuating levels at the Plio-Pleistocene border (Shannon 1985, Marlow et al. 2000). It has been an effective barrier since at least the Pliocene for sea urchins of *Eudicaris* (Lessios et al. 1999), *Echinometra* (McCartney et al. 2000), and *Tripneustes* (Lessios et al. 2003), as well as for bonefishes (Colborn et al. 2001). We, therefore, suggest that the combined actions of these 3 barriers assisted the "*T. lepturus* complex" in speciation. This result also suggests that the 3 species were separated during the late Miocene (ca. 8.83-11.57 Ma BP).

The genetic analyses of *T. lepturus* revealed that the West Atlantic, West Africa, and the Pacific populations were reciprocally monophyletic, and the genetic divergences suggested restricted gene flow among them. This genetic isolation was likely triggered by the isolation of biogeographic realms. According to the mtDNA phylogeny presented herein, the barriers important to the phylogenetic separation in *T. lepturus* were (1) the Isthmus of Panama and (2) the 2500 km long stretch of deep water separating the eastern from the western Atlantic. The Isthmus of Panama was completed in the Pliocene, 3.1 Ma BP, and split the ranges of a large number of previously continuous marine populations (Lessios 1998). The Atlantic and Pacific clades of *T. lepturus* were presumed to have split by the original Pliocene completion of the isthmus (Fig. 5B, Table 3). The split in the Atlantic between sampled populations on the American and African coasts occurred in the late Pliocene. Presumably these separations of Atlantic populations are related to the 2500 km long stretch of deep water. The separation of populations of *T. lepturus* sampled from Brazil and the Gulf of Mexico is even more recent, dating to approximately 0.52 Ma BP. The barrier most likely consists of inhospitable habitat created by freshwater plumes of the Amazon River discharge (Muller-Karger et al. 1988). This stretch of muddy habitat and low-salinity water was proven to be a surprisingly strong barrier to gene flow.

Based on the NCA analysis results, the East Indies Triangle of the Indo-Pacific was either a refuge or a colonization source for *T.* sp. 2. The East Indies Triangle, a relatively small part of the Indo-Pacific Ocean, is unique in terms of its species diversity and evolutionary importance. Within this area, the species diversity of major groups of marine organisms such as fishes, corals, echinoderms, and mollusks is extremely high; and this is considered to be related to their mode of speciation. Speciation events may be concentrated at the periphery of the region (as suggested by center-of-accumulation models of the diversity focus; Jokiel and Martinelli 1992), in the species-rich center (the center-of-origin model; Briggs 1999), or scattered across the region (Bellwood and Wainwright 2002). In this study, the NCA analysis suggested that *T.* sp. 2 used the East Indies Triangle of the Indo-West Pacific as an origin and gradually moved outward (Fig. 5A). The present study supports an earlier suggestion (Briggs 1966 2003 2005, Mora et al. 2003, Williams and Reid 2004) that the East Indies Triangle represents a center of evolutionary origin.

Phylogeographical reconstructions of *T.* sp. 2 populations based on mtDNA allowed us to identify 4 clades. The large divergence among clades indicated that they have been isolated from each another for a considerable amount of time (Tables 2, 3). Within the Indo-Pacific, falls in sea levels associated with glacial maxima resulted in massive losses of shallow inner reefs and lagoons. This may have exposed large areas of continental shelves and reduced habitats to narrow fringes; on the other hand, it may have increased habitats

in some areas by exposing submerged islands. All these habitat losses and increased/ reduced circulations contributed to the isolation between adjacent regions. There is evidence that climatic oscillations during the Pliocene and Pleistocene were important causes of marine speciation (Palumbi 1997, Benzie 1999). Molecular phylogenetic studies of closely related clades also showed that sister clades diverged in the last (1-5) × 10^6 yr (McCartney et al. 2000, Williams 2000, Lessios et al. 2001, Harrison 2004). Based on the 16S rRNA estimation, the separation of these clades of *T.* sp. 2 occurred in the Pliocene (4.32 Ma BP) and early Pleistocene (1.42-1.28 Ma BP) (Fig. 5B). These results suggest that the lowering of sea levels during glaciations may have occurred many times producing different barriers which caused population divergence.

Trichiurus japonicus is distributed in the northwestern Pacific (Fig. 1B). It is very similar to *T. lepturus* in having a whitish dorsal fin when fresh, whereas *T.* sp. 2 distinctly differs from both in having a yellowish-green dorsal fin along with other morphometric and meristic characters. Additionally, our data supported previous analyses of *T. japonicus* being genetically and geographically distinct from, but sister to, *T. lepturus*. The northwestern Pacific has a unique tectonic and geographical history with several marginal seas separating Asia from the Pacific Ocean. In the high latitudes around Japan in the northwestern Pacific, a warming event occurred in the middle Miocene and was subsequently followed by global cooling (Ennyu 2003). The warming process might have driven a northward dispersal of the "*T. lepturus* complex" from the tropical south to temperate waters around Japan. Then the global cooling process drove the "*T. lepturus* complex" to its approximate current distribution and probably also drove relict populations to local deeper waters on the continental slope. During periods of low sea levels, the northerly population might have been isolated, and speciation subsequently occurred (Liu et al. 2007). Based on the molecular data, *T. japonicus* originated around 8.83 Ma BP. This scenario matches the subsequent speciation of *T. japonicus* as known by its current distribution from subtropical to temperate waters.

CONCLUSIONS

From the present mtDNA sequences and previous morphological and molecular analyses (e.g., Lee et al. 1977, Nakabo 2000, Chakraborty and Iwatsuki 2006, Chakraborty et al. 2006a b, Tzeng et al. 2007), 3 taxonomic species of the "*T. lepturus* complex" should be considered: *T. japonicus* (northwestern Pacific), *T. lepturus* (Atlantic and Pacific), and *T.* sp. 2 (Indian and West Pacific) (Fig. 1B). *Trichiurus lepturus* from the Indo-Pacific in previous studies (Chakraborty and Iwatsuki 2006, Chakraborty et al. 2006 b) and *T.* sp. 2 are the same species. Although Chakraborty et al. (2007) developed methods to identify species of *Trichiurus*, *T.* sp. 2 still lacks identifying characters. Consequently, additional nomenclature work is needed in order to develop the morphological adjustment for *T.* sp. 2. In addition, there should be future work on revising the few known species, e.g., *T. nanhaiensis* and *T. haumela*, to resolve the taxonomy of the Indo-Pacific type of *Trichiurus*.

Acknowledgments: This research was supported by grants from Academia Sinica. The authors wish to thank members of the Laboratory of Fish Ecology and Evolution, Biodiversity Research Center, Academia Sinica, Taipei, Taiwan for sampling work. Part of this work was carried out using the resources of the Computational Biology Service Unit at Cornell University (Ithaca, NY) which is partially funded by Microsoft Corporation (Redmond, WA). Two anonymous reviewers provided valuable comments on the manuscript.

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