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GENETIC VARIATION IN *TRICHIURUS LEPTURUS* (PERCIFORMES: TRICHIURIDAE) IN WATERS OFF TAIWAN: SEVERAL SPECIES OR COHORT CONTRIBUTION?

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***ABSTRACT.* –** The cutlassfish, *Trichiurus lepturus*, has a worldwide distribution and shows morphotypic variation among regions. We investigated genetic variation within and among populations of *T. lepturus* from Taiwanese waters. We surveyed 636 bp (base pairs) of mitochondrial DNA (mtDNA) cytochrome (cyt) *b* from 99 individuals collected at eight locations. Two different phylogenetic analysis software packages were utilized (MEGA version 2 and PHYLIP version 3.6a2), using neighbour-joining (NJ) and maximum likelihood (ML) methods. The resulting topologies were identical. Both methods suggest the same conclusion, revealing four deep lineages. The genetic divergences among these four lineages compared with *Trichiurus* species-level divergences were low. Hence, this study supports that the high genetic variations of *T. lepturus*, collected from different cohorts in waters off Taiwan, originated from distinct spawning events.

***KEY WORDS.* –** Cytochrome *b*, cutlassfish, population genetics, molecular evolution.

INTRODUCTION

The cutlassfish, *Trichiurus lepturus*, is an important commercial marine fish species and makes up about 10 - 20% of the total marine fish catch. World-wide harvests of the cutlassfish are estimated to be 750,000 tons annually and China accounts for 80% (600,000 tons) of the catch (Claus, 1995). After the original description of *T. lepturus* by Linnaeus (1758), 29 nominal species (15 valid species) were described. Most of which were synonymized under *T. lepturus* by the year 1993 (Food and Agriculture Organization of the United Nations, Nakamura & Parin, 1993). Wang et al. (1993) reported that the cutlassfish from Chinese coastal waters should be divided into three species. They include two new species, *T. nanhaiensis* Wang & Xu, 1992 in Wang et al. 1992 and *T. brevis* Wang & You, 1992 in Wang et al., 1992 and the existing *T. lepturus* Linnaeus, 1758. However, according to these papers, *T. nanhaiensis* was only distributed in the South China Sea, while *T. brevis* and *T. lepturus* were widely-distributed in Chinese coastal waters (Wang et al., 1992, 1993, 1994).

Systematic studies of the cutlassfish species in Taiwanese waters also displayed vague results. Lee (1977) classified the cutlassfish found in Taiwanese water into two species: 1) *T. japonicus*: thin, ribbon-like fish with pointed teeth and 2) *T. lepturus*: fat, ribbon-like fish with barbed teeth. Shao et al. (1978) examined muscle myogens by polyacrylamide disc electrophoresis and found that there was one major band difference between *T. lepturus* and *T. japonicus*. However, Lin & Shen (1986) reported that they were the same species, based on their covariance analysis of morphometric and meristic data.

We surveyed and identified the species diversity of *Trichiurus* in Taiwanese waters mostly by using the diagnostic key of Wang et al. (1992, 1993). From morphological, genetic and von Bertalanffy's growth model analyses results, we identified three *Trichiurus* species in Taiwan: a) *T. lepturus*; b) *T. nanhaiensis* and c) *T. cf. nanhaiensis* (unpublished data). However, we also found high genetic diversity within *T. lepturus*. In this study, we use mtDNA cytochrome (cyt) *b* gene sequences to investigate the patterns of genetic variability in order to understand the mechanism for this high genetic diversity within *T. lepturus* in Taiwan.

Table 1. Sample sizes, haplotype diversities and nucleotide diversity studied of natural populations of *Trichiurus lepturus* examined. mtDNA lineages correspond to the lineages resolved in the phylogenies shown in Fig. 2.

Population	Abbreviation	n	h	Nucleotide Diversity (%)		Collection Date	Collection Methods	Cohort	Lineage
				π	θ				
Da Hsi	DH	9	1.00	3.45	3.82	Sep 2002	Bottom trawling	I	C
An Ping	AP	13	1.00	2.65	4.46	Nov 2002	Handline	II	D
Xin Da	XD	18	0.99	2.67	4.69	May 2003	Handline	III	B
Tung Kang	TK	13	0.94	1.09	1.47	May, Jun 2004	Bottom trawling	IV	A
Wu Chi	WC	6	0.87	1.70	2.14	May, Jul 2004	Bottom trawling	IV	A
Miao Li	ML	11	0.89	0.53	0.69	May, Jul 2004	Bottom trawling	IV	A
Nan Ao	NO	12	0.97	2.31	2.95	May 2004	Set net	IV	A
Hua Lian	HL	17	0.97	1.59	2.52	May 2004	Bottom trawling	IV	A
Total		**99**	**0.996**	**7.71**	**9.82**				

n = sample size; h = haplotype diversity.

MATERIALS AND METHODS

***Sample collection and DNA extraction.* –** A total of 99 *Trichiurus lepturus* specimens collected from eight fishing harbours in Taiwan (Fig. 1) were analyzed in this study (Table 1). The harbours (with their corresponding abbreviations) include Da Hsi (DH), Nan Ao (NO) and Hua Lian (HL) in the Northeastern to the mid-Eastern region, Tung Kang (TK), Xin Da (XD) and An Ping (AP) in the Southwestern region and Wu Chi (WC) and Miao Li (ML) in the mid-Western to the Northwestern region. Corresponding abbreviations for the eight harbours are also given in Table 1. Collection methods included commercial handlines, set nets and bottom trawls. Another species of cutlassfish, *T. nanhaiensis*, was used as the outgroup in our study. DNA was extracted from the muscle tissue of the specimens by standard phenol-chloroform methods (Duda & Palumbi, 1999).

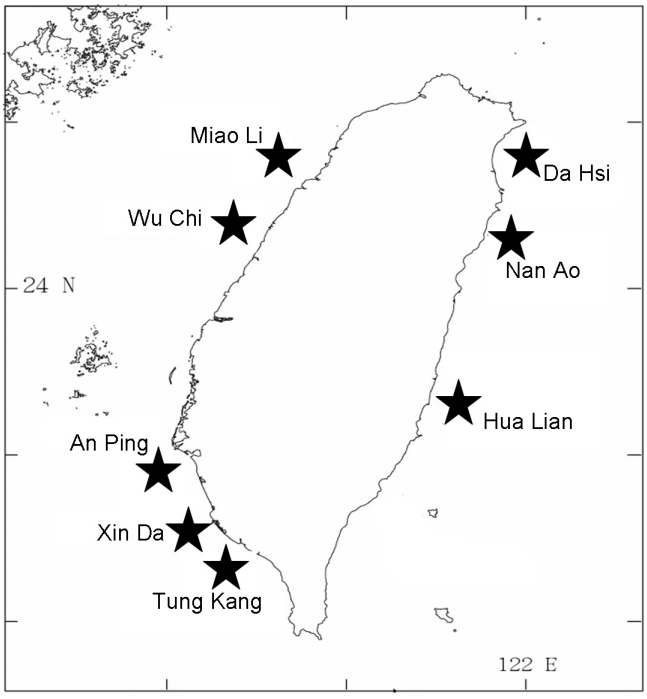

Fig. 1. Map of eight fishing harbours where samples of *Trichiurus lepturus* were collected.

***mtDNA analysis.* –** Mitochondrial DNA cytochrome (cyt) *b* fragments were subsequently amplified using primers designed by this study: Glu (5'-CGAAGCTTGACTTGAArAACCAyCGTTG-3') and Cyt (5'-GGCAAATAGGAArTATCATTC-3'). Each 100 μL PCR reaction contained: 10 ng template DNA, 10 μL 10× reaction buffer, 10 μL $MgCl_2$ (25 mM), 10 μL dNTP mixture (10 mM), 10 pmol of each primer (Glu and Cyt). The reaction was programmed on an MJ Thermal Cycler (MJ Research, Inc.) with an initial denaturation for 4 minutes at 94°C, followed by 33 cycles at 94°C for 30 seconds (denaturation), 53°C for 30 seconds (annealing), 72°C for 45 seconds (extension) and a final extension at 72°C for 10 minutes. The PCR products were purified using an agarose gel purification kit (QIAGEN, Valencia, USA). Both strands of the purified DNA were sequenced on an Applied Biosystems 377 automated sequencer (ABI division of Perkin-Elmer, Foster City, USA).

Nucleotide sequences were aligned with the program CLUSTALX version 1.81 (Thompson et al., 1997) and then verified manually. Neighbour-joining (NJ) and maximum likelihood (ML) analyses were used for phylogenetic tree construction with *Trichiurus nanhaiensis* (caught off Dai Tong, Southeast Taiwan) as the outgroup taxon. The analyses were performed using the Molecular Evolutionary Genetics Analysis program (MEGA) version 2 (Kumar et al., 2001) and PHYLIP version 3.6a2 (Felsenstein, 1993) program packages. The NJ and ML trees were constructed according to Tamura & Nei's (1993) substitution model, which recovers slightly biased branch lengths of lineages (Håstad & Björklund, 1998). We tested the confidence of reconstructed clades by bootstrapping (Felsenstein, 1985) with 1,000 replicates using unweighted characters. The nodes with bootstrap values greater than 70, as a rule of thumb, were significantly supported with 95% probability (Hillis & Bull, 1993). Nucleotide compositions and genetic distance values were determined with MEGA version 2. Genetic diversity within populations was estimated by haplotype and nucleotide diversities (Nei, 1987) by using DnaSP version 3.99 (Rozas & Rozas, 1999).

Table 2. Matrix of pairwise F_{ST} values among the *Trichiurus lepturus* populations in Taiwanese waters based on mtDNA cytochrome *b* sequence data.

Population		DH	AP	XD	TK	WC	ML	NO	HL
Da Hsi	(DH)	-	-	-	-	-	-	-	-
An Ping	(AP)	0.635	-	-	-	-	-	-	-
Xin Da	(XD)	0.640	0.693	-	-	-	-	-	-
Tung Kang	(TK)	0.630	0.795	0.791	-	-	-	-	-
Wu Chi	(WC)	0.805	0.817	0.801	0.847	-	-	-	-
Miao Li	(ML)	0.857	0.870	0.865	0.921	0.074	-	-	-
Nan Ao	(NO)	0.699	0.751	0.772	0.728	0.828	0.887	-	-
Hua Lian	(HL)	0.673	0.729	0.744	0.649	0.816	0.892	0.640	-

Table 3. Tamura-Nei's genetic distances (Tamura & Nei, 1993) of the major *Trichiurus lepturus* lineages (from Fig. 2A & 2B) and other *Trichiurus* species based on cytochrome *b* mtDNA sequences.

Species	*Trichiurus lepturus*				*Trichiurus nanhaiensis*	*Trichiurus cf. nanhaiensis*	*Trichiurus brevis*
	A	B	C	D			
Trichiurus lepturus	-	-	-	-	0.215	0.215	0.243
T. lepturus A	-	0.101	0.099	0.102	-	-	-
T. lepturus B	-	-	0.087	0.089	-	-	-
T. lepturus C	-	-	-	0.087	-	-	-
T. lepturus D	-	-	-	-	-	-	-
Trichiurus nanhaiensis	-	-	-	-	-	0.210	0.257
Trichiurus cf. nanhaiensis	-	-	-	-	-	-	0.280
Trichiurus brevis	-	-	-	-	-	-	-

RESULTS

A total of 636 bp of the cyt *b* mitochondrial gene were amplified successfully for all the 99 *T. lepturus* individuals sampled, resulting in 82 unique haplotypes defined by 231 variable sites. Nucleotide sequences in the cyt *b* of *T. lepturus* are A + T rich (55.3%), which is similar to many other fishes (Johns & Avise, 1998). No significant deviations from the expected results under neutral conditions were identified when all haplotypes are analyzed together (Fu, 1996; D = – 2.15, $P > 0.05$; F = – 1.83, $P > 0.10$; Tajima, 1983; D = – 0.71, $P > 0.10$). Haplotype diversity within populations ranged from 0.87 to 1.00 (mean 0.95) (Table 1). The intra-population nucleotide diversity was high in populations of DH (3.45%) and low in populations of ML (0.53%) (Table 1). When we analyzed the total diversity, the haplotype diversity (h = 0.996) and nucleotide diversity (π = 7.71%; θ = 9.82%) (Table 1) were higher than those in other fishes (Johns & Avise 1998). Geographical divisions assessed by DnaSP (Table 2) indicated higher genetic diversity among populations (with F_{ST} ranging from 0.630 to 0.921), with the exception of the diversity between the WC and ML populations (0.074). This result confirms the low levels of genetic exchanges among the populations in Taiwan.

The individual trees constructed by the two phylogenetic methods (NJ and ML) were almost identical. The NJ and ML trees (Fig. 2) constructed based on the complete data set of all haplotypes resulted in a well-constructed phylogeny, defined by 168 phylogenetically informative sites. Our phylogenetic analysis identified four mtDNA lineages within *T. lepturus*. These four *T. lepturus* mtDNA lineages were supported by high bootstrap values (Fig. 2): 1) lineage A (included all individuals collected in 2004); 2) lineage B (found in XD in Southwestern Taiwan in 2003); 3) lineage C (collected from DH in Northeastern Taiwan in 2002) and 4) lineage D (found in AP in Southwestern Taiwan in 2002) (Table 1). The average genetic distance among the major *T. lepturus* mtDNA lineages (A - D) was considerably high (0.096; range = 0.087 - 0.102) (Table 3). These results indicate that the genetic variation within the *T. lepturus* populations in Taiwan is high.

DISCUSSION

Two different methods of analysis and the consistent homology shown by phylogenetic trees inferred strongly support an evolutionary history of Taiwan *Trichiurus lepturus* where at least four distinct lineages are present. We next set out to determine: 1) whether there was only one or several species in this taxon; 2) whether genetic divergences could be used to separate these four lineages into different species since it is difficult to differentiate them morphologically and 3) whether the taxonomic ranks of these taxa are consistent with the sequence divergence estimates if the taxa were distinguished based on genetic divergence.

Avise & Aquadro (1982) summarized mean genetic distances between congeneric species and confamilial genera across the major vertebrate classes. Johns & Avise (1998) also calculated and compared the levels of genetic distance between sister species, congeneric species and confamilial genera within and across the major vertebrate taxonomic

classes. There were some salient trends that emerged from these summaries. In this study, all estimates of the genetic divergence among lineages (d = 0.087 - 0.102), were lower than the estimates for species level. For example, divergences among congeneric fish species average 0.120 for cyt *b* sequences (Johns & Avise, 1998).

However, measures of genetic divergence with mtDNA cyt *b* and allozymes do not always agree with taxonomic rank in some vertebrate groups. Ferguson (2002) commented that the use of genetic distances to infer separate species was not parsimonious, as its theoretical foundations were not well established and it could not be applied over a wide range of plants and animals. In addition, he suggested that genetic divergence fails to identify separate species. Nevertheless, although universal criteria cannot be applied over a wide range of taxa, we consider that a criterion measured from congeneric species can be applied to identify population or species level within this genus. We calculated the Tamura & Nei's (1993) genetic distances of four *Trichiurus* species based on cyt *b* mtDNA sequences (Table 3). The genetic distances among species based on cyt *b* sequences (d = 0.210 - 0.282), were higher than the criterion estimated by Johns & Avise (1998). Moreover, Chakraborty et al. (2006) described the genetic differences among three species of the genus *Trichiurus* based on mtDNA 16S rRNA analysis, to be between 0.044 and 0.066. The levels of intra-specific genetic diversity for cyt *b* and 16S rRNA genes in *Trichiurus* species are considered typical as they were similar to the results in the study by Tinti & Piccinetti (2000). In this study, they examined the molecular systematics of Atlanto-Mediterranean *Solea* species through sequence analysis of two mtDNA genes (16S rRNA and cyt *b*) and analysis at the generic level of differentiation and they obtained similar results (d = 0.164 - 0.272 for cyt *b* and 0.042 - 0.103 for 16S rRNA).

According to the taxonomic rank described above, all the estimates of the genetic divergence between lineages (d = 0.087 - 0.101), were lower than those of *Trichiurus* species-level divergences (d = 0.210 - 0.282). Furthermore, according to Shih (2004), no morphological and growth pattern difference could be observed within the Taiwanese populations *T. lepturus*. Thus, we suggest that there is only one species within *T. lepturus*.

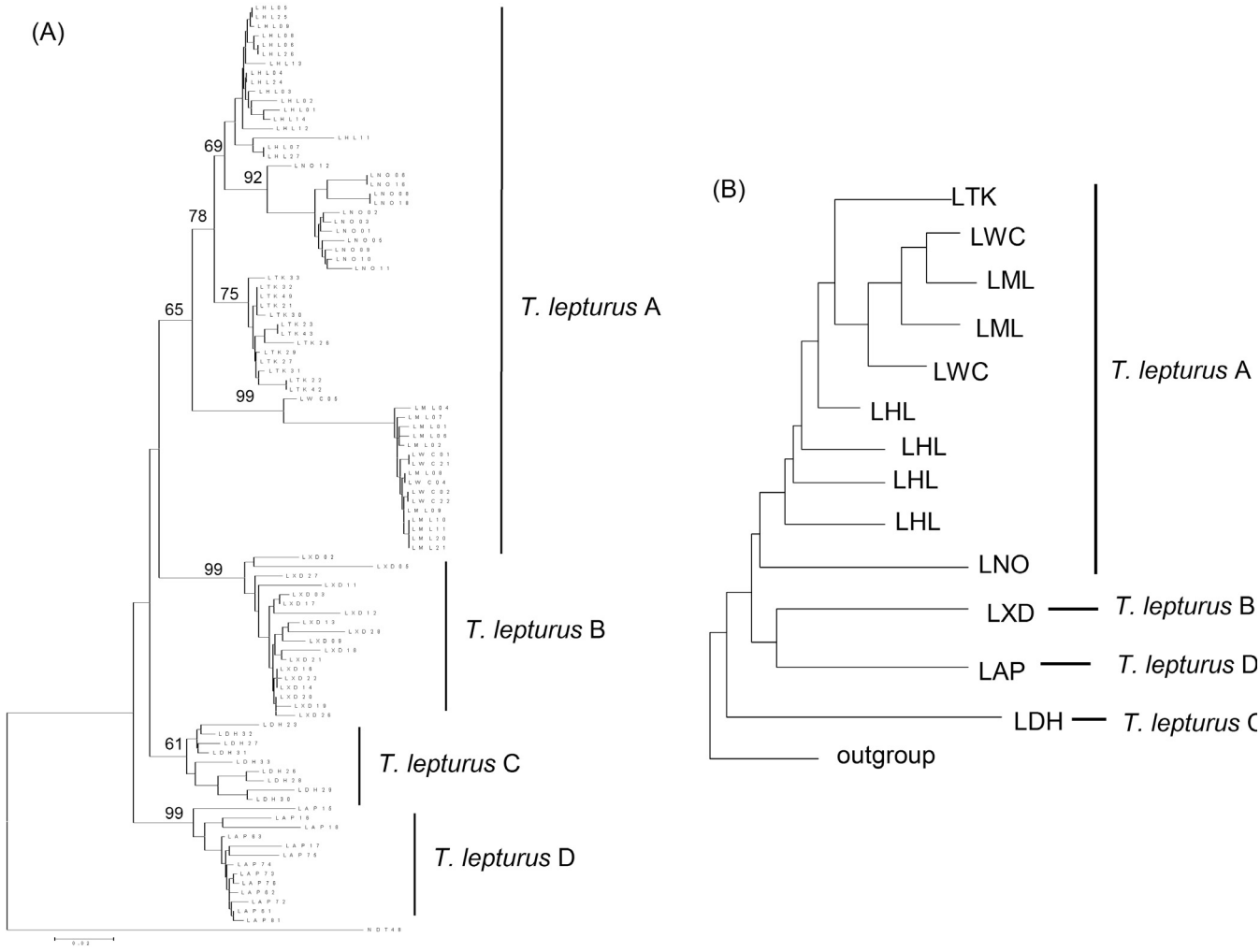

Fig. 2. Two tree topologies constructed from the inferred sequence variation of mtDNA *Trichiurus lepturus* with *Trichiurus nanhaiensis* (caught off the Southeast Taiwan, Dai Tong) as outgroup taxa. A) neighbour-joining (NJ) tree, B) maximum likelihood (ML). Numbers at nodes indicate the bootstrap values for 1,000 replicates. L = *T. lepturus*; N = *T. nanhaiensis*; and the two other characters are the abbreviations of the fishing harbours where the specimens where collected (see Table 1 for the list of abbreviations).

However, the mechanisms that cause such high genetic diversity within *T. lepturus* are still unknown. Such genetic diversities were similar to the previous study of temporal genetic change in the marine fish *Diplodus sargus* (Lenfant & Planes, 2002). Marine species with high fecundity and high larval mortality are susceptible to a large variance in reproductive success. Variance in reproductive success influences genetic diversity through its effects on effective population size (Flowers et al., 2002). In some studies (Ruzzante et al., 1996; Planes & Lenfant, 2002), the results suggested that the population genetic structure of a marine species was partially determined by spatial-temporal larval settlement and recruitment variations. The fundamental result from these studies was the high genotypic differentiation among cohorts. Further analysis of the data was done to determine if the high genetic variation within *T. lepturus* was due to the cohort contribution.

The term "cohort" normally refers to a year class. If we take the *T. lepturus* populations sampled in the same year as a cohort, we can assign all samples into three cohorts: 1) 2002 (AP and DH); 2) 2003 (XD) and 3) 2004 (HL, ML, NO, TK and WC). These three cohorts correspond with the four mtDNA lineages: 1) 2002 (lineage C and D); 2) 2003 (lineage B) and C) 2004 (lineage A). Shih (2004) reported the spawning period and reproductive cycle evidence based on the temporal profile of Relative Gonadal Index / Gonosomatic Index, macroscopic examination of ovaries and microscopic examination of whole oocytes. Shih (2004) observed that the spawning periods derived from the results of the three different profiles are very similar, that *T. lepturus* may have two spawning seasons in one year. Consequently, we consider that the populations from a year class might originate from different spawning groups, hence forming different cohorts. For example, the samples collected from Southern Taiwan (AP, 2002) and Northern Taiwan (DH, 2002) might have originated from different spawning groups and thus, we define these two groups as different cohorts. We therefore re-assigned all samples into four cohorts based on the collection data and the results of phylogenetic analysis: 1) I = September 2002, Northeast Taiwan (lineage C); 2) II = November 2002, Southern Taiwan (lineage D); 3) III = May 2003, Southern Taiwan (lineage B) and 4) IV = samples collected in 2004 (lineage A) (Table 1).

The presence of four heterogeneous groups contributed to the spatial and temporal genetic composition within *T. lepturus* from Taiwanese waters. Consequently, we suggested that the high genetic diversity of *T. lepturus* was the contribution of different cohorts as well. However, the existence of significant genetic differences between cohorts remains unknown. A previous study on genetic composition of a larval cod, *Gadus morhua* (Ruzzante et al., 1996) showed the larval cohort to have greater genetic similarity to adult cod sampled two years later suggesting that the genetic composition of cod remains stable over time. In light of this, the *T. lepturus* populations in Taiwanese waters would need to be further monitored and studied in order to understand the links between genetic structure and population recruitment.

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