RESEARCH NOTE

PCR-RFLP analysis of *cytochrome b* gene does not support *Coilia ectenes taihuensis* being a subspecies of *Coilia ectenes*

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Introduction

The current taxonomic status of *Coilia ectenes taihuensis* is unclear. One study claims that it has differentiated from *Coilia ectenes* to the level of subspecies, while two other studies refute this claim. In this study, we used RFLP analysis of cytochrome *b* gene to show that the differences between the two are not large enough to justify *C. ectenes taihuensis* being classified as a subspecies of *C. ectenes*.

Coilia fishes are small to moderate in size and China has four species of them (Zhang 2001). *C. ectenes* (Jordan and Seale 1905) is an anadromous fish which migrates from near ocean waters to fresh waters areas every year during the spawning season. Mature *C. ectenes* migrate upriver, and spawn in the lower and middle reaches of the Yangtze river. Some *C. ectenes* also some spawn in the lakes adjacent to the Yangtze river. *C. ectenes taihuensis* is an autochthonous group of *C. ectenes*. It has no migration habit and lives throughout its life in Taihu lake, which is the third largest freshwater lake in China (East China Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences and Shanghai Fisheries Research Institute 1990).

The taxonomic relationship between *C. ectenes* and *C. ectenes taihuensis* is still controversial. Yuan *et al*. (1976) considered *C. ectenes taihuensis* to be a subspecies of *C. ectenes* according to traditional meristic characters, and ecological and physiological differences. But isozyme and anatomical data of Liu (1995) and morphometric data of Cheng and Han (2004) suggest that the differences between the two have not risen to subspecies level.

Mitochondrial DNA (mtDNA) is extranuclear DNA that has proven to be a useful molecular marker for evolutionary studies in animal populations because of its predominantly maternal inheritance, relatively rapid base substitution rate, and lack of recombination (Avise *et al*. 1987). *cytochrome b* – a protein coding mitochondrial gene – is widely used in fish population and evolutionary genetic studies (Johns and Avise 1998; Xiao *et al*. 2001). Restriction fragment length polymorphism (RFLP) analysis of mtDNA has proved to be effective in distinguishing among three different species of catfish in the Arabian Gulf, two of which could hardly be differentiated based on morphological traits alone (Simsek *et al*. 1990).

In the present study, RFLP analysis of the *cytochrome b* gene was employed to assess the levels of genetic diversity within, and genetic differentiation between *C. ectenes* and *C. ectenes taihuensis*. Seven restriction enzymes were found to have at least one recognition site at this gene. Six different haplotypes were detected between the populations studied. About one to two restriction patterns of each enzyme were revealed. We found very low levels of genetic partitioning, with less than 0.1% of the total variation attributed to between population differences, and lack of genetic structure, thus supporting the view that the differences between *C. ectenes taihuensis* and *C. ectenes* have not risen to subspecies level.

Materials and methods

Sampling

Samples of *C. ectenes* were obtained from Yangtze river

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estuary, Shanghai (N31°37′, E121°80′), whereas those of *C. ectenes taihuensis* were collected from Taihu lake, Wuxi, Jiangsu province (N31°38′, E120°10′), respectively (figure 1). A total of 96 individuals (48 from each group) were sampled. All individuals were transferred in dry ice to the laboratory and stored at –80°C until used. Henceforth, based on their chinese names, *C. ectenes* and *C. ectenes taihuensis* are abbreviated as DJ and HJ, respectively.

DNA extraction, PCR amplification and restriction enzyme digestion

Total genomic DNA was extracted from muscle tissues. Muscle tissues were dissected, then digested by proteinase K overnight, followed by phenol-chloroform extraction and 100% ethanol precipitation (Sambrook *et al*. 1989). Extracted DNA was checked using 0.8% agarose gel electrophoresis, then diluted to appropriate concentration for PCR amplification.

Two universal primers, L14724 and H15915 (Xiao *et al*. 2001), were used to amplify the whole mtDNA *cytochrome b* gene. Amplification reaction mixtures consisted of 100 ng DNA template, 0.2 mmol/l dNTPs, 1.0 μmol/l primers each, 4.0 mmol/l $MgCl_2$, 5.0 μl 10× reaction buffer, 2 U Taq polymerase, with sterilized water added to make up the final volume to 50 μl. Amplifications were performed under the following conditions: initial denaturation at 94°C for 4 min, followed by 35 thermal cycles of denaturation at 94°C for 50 s, annealing at 55°C for 1 min, and extension at 72°C for 1 min 30 s.The final extension was performed at 72°C for 7 min. PCR products were visualized on 1% agarose gels buffered with Tris-Acetate-EDTA (TAE), stained with ethidium bromide, and visualized under UV light.

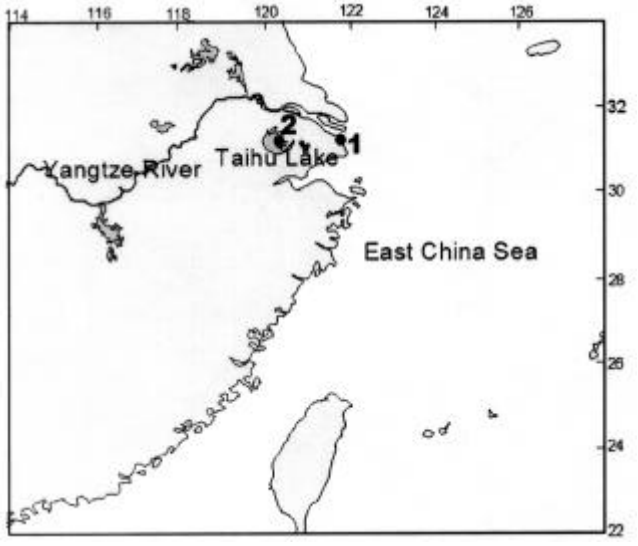

Figure 1. Locations of sampling sites: (1) Yangtze river estuary, Shanghai, and (2) Taihu lake, Wuxi, Jiangsu province.

Amplified mtDNA *cytochrome b* gene from five individuals of each population were digested with 16 restriction endonucleases in order to check the presence of recognition sites. The informative restriction endonucleases were then applied to 48 individuals from each population. The informative restriction endonucleases used were: *Dde*I, *Hae*III, *Hha*I, *Hin*fI, *Rsa*I, *Taq*I and *Xho*I. Digestion was performed for 4–12 h, under conditions specified by enzyme manufacturers (New England Biolabs). The digested fragments were separated by 2.0% horizontal agarose gel electrophoresis. Gels were stained with ethidium bromide and visualized under UV light. The size of DNA fragments were compared to the PCR marker (Takara DL2000) run on the same gel.

Data analysis

For each enzyme, variable restriction patterns were alphabetically designated as they were encountered. The presence or absence of restriction sites were inferred for each enzyme from completely additive fragment patterns, and composite haplotypes were assigned to each individual. The composite haplotype data and the restriction site matrix were used for a number of analyses using the REAP software package (McElroy *et al*. 1992). The following measures of genetic diversity were calculated: within-population diversity was estimated by haplotype diversity *h* (Nei and Tajima 1981) and nucleotide diversity *p* (Nei and Tajima 1981), whereas between-population diversity was estimated as net nucleotide sequence divergence *d* (Nei and Tajima 1981).

The extent of genetic differentiation between populations was estimated using the fixation index F_{ST} (Wright 1951). A pairwise population comparison following the methodology of Raymond and Rousset (1995) using analysis of molecular variance (AMOVA) was performed, using ARLEQUIN 2.0 (Schneider *et al*. 2000) to partition variance components attributable to (1) variance between populations; and (2) variance among individuals within populations, in order to evaluate hypothesized patterns of spatial genetic structure.

Results and discussion

A 1300 bp fragment was consistently amplified from these two populations. Of the seven restriction enzymes screened, variants were inferred to be the result of either a gain or a loss of a restriction site. A total of 27 restriction sites were inferred, of which four sites were variable (table 1).

Between two and eight fragment variants were detected for each restriction enzyme. Combining haplotype designations for the seven polymorphic restriction enzymes surveyed revealed six composite haplotypes among the 98 individuals analysed (table 2). Composite haplotype1 (type 1) was very abundant, representing 88.5% of

the individuals analyzed. The remaining five haplotypes occurred at low frequencies (<5% of total sample size), and type 4 was observed only within the HJ population.

The current taxonomic status between DJ and HJ is controversial (Yuan *et al*. 1976; Liu 1995; Cheng and Han 2004). Yuan *et al*. (1976) classified HJ as a subspecies based on meristic, ecological and physiological differences. In general, fishes are quite prone to exhibit environmentally induced morphological variation (Allendorf *et al*. 1987). Compared to morphological characters, DNA data and isozyme data are, therefore, a relatively stable marker. The net nucleotide divergence between DJ and HJ was 0.00063, which is within the geographical level of 0 to 0.05 (Nei 1975). The AMOVA analysis revealed that that variation attributed to between-population haplotype frequency differences was very low (< 0.1%), with almost all of the variation (99.9%) found within populations. The variation between population only contribute 0.06% to their total differences and F_{ST} value (0.00063) was not significant ($P = 0.38$), suggesting these two populations are not genetically diverged. The test for population differentiation (Raymond and Rousset 1995) gave a P value of 0.84475 ± 0.009, indicating that the composite haplotypes were distributed randomly. So, our results support the view (Liu 1995) that the differences between DJ and HJ are still within geographical population and have not risen to subspecies level.

Haplotype diversity (h) within each population was 0.1613 (DJ) and 0.2713 (HJ), respectively (table 2), with an average of 0.2163. Nucleotide diversity (p) was 0.6173% (DJ) and 1.3298% (HJ), respectively, with an overall average of 0.9736%.

Nucleotide diversity (p) indicates the mean number of differences between all pairs of haplotypes in each population, and is, therefore, a genetic diversity index of a population. It is difficult to imagine why the p value of DJ is less than that of the HJ population. Intuitively, one would expect the HJ population, being founded by a small number of fish from the ancestral DJ population, to show lower genetic diversity. Taihu Lake is the third largest freshwater lake in China, and currently serves more than 33 million people in this area for drinking water, flood control, shipping, industiral, agricultural and human waste disposal, fishing, aquaculture and farming (Chang 1996; Chang and Liu 1996). Perhaps this has resulted in a greatly variable environment, thus promoting the maintenance of higher levels of genetic diversity in the HJ population. The Yangtze river estuary is a good fishing ground and the DJ population has decreased sharply over the last 25 years or so, suggesting a possible

Table 1. Fragment size estimates (in base pairs) of all fragment patterns observed on mtDNA *cytb* gene between the two populations studied.

*Dde*I			*Hae*III		*Hha*I			*Hinf*I			*Rsa*I			*Taq*I		*Xho*I	
	A	B		A		A	B		A	B		A		A	B		A
630		–	950	–	800		–	600	–	–	440	–	950		–	900	–
350	–		230	–	500	– × 2	–	390		–	380	–	600	–		350	–
280	– × 2	–	70	–	300	–		220	–		150	–	350	– × 2	–	50	–
120	–	–	50	–				190	–	–	120	–					
110	–	–						170	–		100	–					
80	–	–						70	–	–	80	–					
50	–	–						50	–	–	30	–					
30	–	–															

Table 2. Composite genotypes (haplotype), haplotype frequencies, haplotype diversity including standard error, nucleotide diversity (%) and sample size of the two populations (DJ and HJ) studied.

	*Dde*I	*Hae*III	*Hha*I	*Hinf*I	*Rsa*I	*Taq*I	*Xho*I	DJ	HJ
Type1	A	A	A	A	A	A	A	44	41
Type2	A	A	A	A	A	B	A	1	2
Type3	A	A	A	B	A	A	A	1	1
Type4	A	A	A	B	A	B	A	0	2
Type5	A	A	B	A	A	A	A	1	1
Type6	B	A	A	A	A	A	A	1	1
				Haplotype diversity				0.1613	0.2713
				Standard error				0.0715	0.0838
				Nucleotide diversity(%)				0.6173	1.3298
				N				48	48

cause for reduction in genetic diversity in the DJ population. Further studies on other loci may be needed to clarify this issue.

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