Chinese Journal of Oceanology and Limnology
Vol. 28 No. 4, P. 795-801, 2010
DOI: 10.1007/s00343-010-9093-3

Genetics and phylogeny of genus *Coilia* in China based on AFLP markers*

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Received Jun. 4, 2009; revision accepted Oct. 23, 2009

Abstract The taxonomy of *Coilia* has been extensively studied in China, and yet phylogenetic relationships among component taxa remain controversial. We used a PCR-based fingerprinting technique, amplified fragment length polymorphism (AFLP) to characterize and identify all four species of *Coilia* in China. We examined the genetic relationships of the four species of *Coilia* and a subspecies of *Coilia nasus* with AFLP. A total of 180 AFLP loci were generated from six primer combinations, of which 76.11% were polymorphic. The mean genetic distance between pairs of taxa ranged from 0.047 to 0.596. The neighbor-joining tree and UPGMA dendrogram resolved the investigated species into three separate lineages: (1) *C. mystus*, (2) *C. grayii* and (3) *C. brachygnathus*, *C. nasus*, and *C. nasus taihuensis*. Phylogenetic analysis of the AFLP data is inconsistent with current morphological taxonomic systems. The AFLP data indicated a close relationship among *C. brachygnathus*, *C. nasus taihuensis*, and *C. nasus*. Therefore, the two species described under *Coilia* (*C. brachygnathus* and *C. nasus taihuensis*) are treated as synonyms of *C. nasus*.

Keyword: *Coilia*; phylogeny; fingerprint; species identification; AFLP

1 INTRODUCTION

Fish of the genus *Coilia* occur only in the Indo-West Pacific Ocean and comprise 13 species (Wongratana, 1980). They inhabit marine and coastal areas, often frequenting estuaries and tolerate low salinities (Whitehead et al., 1988). According to the FAO Fisheries Synopsis, at least four species (comprising *C. brachygnathus*, *C. nasus*, *C. grayii*, and *C. mystus*) are distributed along the coast or rivers in China, with *C. brachygnathus* living exclusively in freshwater environments (Whitehead et al., 1988). However, the problematic taxonomy of the genus *Coilia* in China warrants further research.

Historically, the taxonomy and nomenclature of *C. brachygnathus* has been greatly debated, in part because of the similar morphological and ecological traits shared by *C. nasus* (Yuan et al., 1980; Whitehead et al., 1988; Zhang, 2001). *C. brachygnathus* is confined to the Yangtze River system, but is not found in the sea. Wongratana (1980) believed *C. brachygnathus* to be *C. nasus*, but the latter has a long maxilla (extending well beyond gill cover), while the maxilla in *C. brachygnathus* was described as at most reaching the gill cover in adults as well as juveniles, and Yangtze River specimens can be distinguished from sympatric *C. nasus* on the basis of this characteristic. More recently, *C. brachygnathus*, supported by molecular data of complete mitochondrial control region gene sequences, was considered as synonymous with *C. nasus* (Tang et al., 2007). In addition, *C. nasus taihuensis* inhabiting Taihu Lake was proposed for a subspecies of *C. nasus* based on traditional meristic characters, ecological conditions and physiological differences (Yuan et al.,

* Supported by the Special Research Foundation for Public Welfare Marine Program (No. 200905019-1), and the National Natural Science Foundation of China (Nos. 30770354, 40676086).

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1976). Although the taxonomic position of *C. nasus taihuensis* has gone through morphological (Yuan et al., 1976, 1980; Liu, 1995; Cheng et al., 2004) and mitochondrial DNA analyses (Cheng et al., 2005; Tang et al., 2007), the validity of its subspecies status remains ambiguous. Further genetic research using more variable molecular markers might help to elucidate whether *C. brachygnathus* is a synonym of *C. nasus* or not and whether *C. nasus taihuensis* is one distinct subspecies of *C. nasus*, or merely a separated local population of *C. nasus*.

Molecular genetic studies can be very powerful for investigating the species relationship and resolving taxonomic uncertainties. Amplified fragment length polymorphism (AFLP) analysis (Vos et al., 1995) is a PCR-based, multilocus fingerprinting technique that combines the strength and overcomes the weakness of PCR-RFLP and RAPD. AFLP analysis has been widely applied to study species, strain and hybrid identification, gene mapping, linkage, and genetic diversity of species and populations in a wide variety of organisms, including fish and shrimp (Liu et al., 2004; Wang et al., 2004; Kakehi et al., 2005; Kassam et al., 2005; Gwo et al., 2008). The aims of the present study are: (1) to produce a phylogenetic hypothesis for the genus *Coilia* in China using AFLP markers. To accomplish this we sampled a total of nine populations from the main distributions of the genus *Coilia* in China (and one population of *C. nasus* from Shiota River in Japan). These include four species and one subspecies of *C. nasus* (two with multiple populations; Fig.1 and Table 1) from the genus *Coilia* in China reported in the FAO Fisheries Synopsis; and (2) to test whether molecular data supports the current systematics of the genus *Coilia*, especially the phylogenetic relationship among *C. brachygnathus*, *C. nasus* and *C. nasus taihuensis*.

2 MATERIALS AND METHODS

2.1 Sample collection and DNA extraction

A total of 25 samples were collected from nine localities (Table 1). Voucher specimens of these species were deposited in the Ocean University of China. The samples were preserved in 95% ethanol or frozen for DNA extraction. Total DNA was isolated from muscle tissue by proteinase K digestion followed by a standard phenol-chloroform method. DNA concentration was measured with a UV spectrophotometer. The quality of extracted DNA was assessed by 1.0% agarose gel electrophoresis with ethidium bromide.

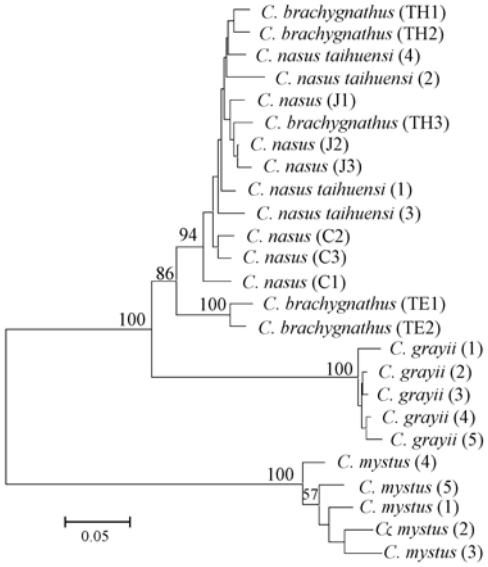

Fig.1 Neighbor-joining tree based on the Nei's genetic distance obtained from AFLP fingerprinting of the species of *Coilia*

Bootstrap values >50 in 200 replicates are shown

2.2 AFLP reactions

The AFLP protocol was carried out as described by Vos et al (1995). DNA templates for AFLP reactions

Table 1 Locations of sampling and sample sizes included in the study

Species	Locations of sampling	Number of specimens
C. grayii (Richardson, 1845)	Pearl River estuary	5
C. brachygnathus (Krayenberg & Pappenheim, 1908)	Tianezhou (Hubei Province), Taihu Lake	2 (TE1-TE2) 3 (TH1-TH3)
C. nasus (Schlegel, 1846)	Shiota River (Japan) Nantong (Jiangsu Province) Dongying (Shandong Province) Zhenjiang (Jiangsu Province)	3 (J1-J3) 1 (C1) 1 (C2) 1 (C3)
C. mystus (Linnaeus, 1758)	Xiamen (Fujian Province)	5
C. nasus taihuensis (Yuan, 1976)	Wuxi (Jiangsu Province)	4

were generated by restriction digestion and ligation. Initially, 1μl DNA was digested with 5U *Eco*RI and *Tru*1I (Fermentas Life Sciences) for 3 h at 37°C in a reaction mixture containing 1×Tango Buffer, followed by 75°C for 20 min to denature the enzymes. To perform preamplification, 5 μl digested DNA fragments were ligated with 2.5 pmol of *Eco*RI and 25 pmol *Mse*I adaptors for 16 h at room temperature, in a reaction mixture containing the Ligase 1×Buffer and 0.3U T4 DNA Ligase. Preamplification was performed with the adenine and cytosine, respectively, as the first selective nucleotide on the *Eco*RI and *Mse*I linkers. This amplification was confirmed by electrophoresis on a 1% agarose gel. The preamplification product mixtures were diluted 10-fold with distilled water and used as templates for the second amplification reaction. The second amplification primer combinations selected to generate the AFLP markers included *Eco*RI-AAC/*Mse*I-CAC, *Eco*RI-AAG/*Mse*I-CAG, *Eco*RI-ACG/*Mse*I-CTC, *Eco*RI-AAG/*Mse*I-CTC, *Eco*RI-AAC/*Mse*I-CTC, *Eco*RI-ACC/*Mse*I-CTC.

2.3 Gel electrophoresis and silver staining

The PCR products were mixed with 10 μl AFLP Loading Buffer (99% formamide, 10 mmol/L EDTA, 0.05% bromophenol and 0.05% xylene cyanol). The product mixtures were denatured and concentrated at 95°C for 5 min, and quickly cooled down in an ice bath after denaturation. Then, PCR reactions were separated on a 6% denaturing polyacrylamide gel using a Bio-Rad Sequi-Gen GT DNA sequencing cell (38×50 cm) run for 2 h at 80 W, following a pre-electrophoresis of 30 min at 80 W.

Silver staining procedures were derived and modified from Merril et al. (1979). After electrophoresis, we added a step to fix the gel for 30 min in 10% acetic acid followed by silver staining. The gel was stained with a mixture of 0.2% silver nitrate and 0.007% benzene sulphonic acid for 30 min followed by rinsing with distilled water.

2.4 Data analysis

Only the recognizable AFLP markers were scored. The binary matrix was built up from AFLP patterns, attributing "1" to the presence and "0" to the absence. We calculated the total number of bands amplified and the number of polymorphic bands. Nei et al. (1979) coefficient of genetic similarity between two individuals was estimated as the ratio of the double of matching markers over the sum of markers displayed by each one of the individuals. Genetic distance was calculated as 1 minus the coefficient of genetic similarity. The AFLP binary matrices were converted to Nei's genetic distance (Nei et al., 1979) matrices using FreeTree (Hampl et al., 2001). The phenetic analyses of phylogenetic relationships were based on UPGMA and neighbor-joining methods. The UPGMA and neighbor-joining trees were constructed from these distance matrices by the software Mega 3.0 (Kumar et al., 2004) and bootstrapped for 200 replicates in PAUP 4.0 (Swofford, 2001).

To make an analysis of AMOVA and calculate the coefficient of gene differentiation (*G*st), which could be used as short-term genetic distances (Nei, 1972, 1973; Reynolds et al., 1983; Slatkin, 1991), we used the software Arlequin 3.1 (Excoffier et al., 2006).

3 RESULTS

This study established AFLP fingerprints for 25 individuals from four species of *Coilia*: *C. brachygnathus*, *C. nasus*, *C. mystus*, *C. grayii* and a subspecies of *C. nasus*, *C. nasus taihuensis*. The six pairs of primer sets yielded a total 180 AFLP loci, based on the size of AFLP markers in all specimens (size range 80–550 bp). The average percentage of polymorphic bands was 76.11% (Table 2). The number of AFLP markers for each species varied

Table 2 Primer combination, the number of AFLP bands, and the percentage of polymorphic bands resulting from AFLP analysis of *Coilia* in China

Primer combinations	No. of total loci	No. of total polymorphic loci	No. of polymorphic loci/ Percentage of polymorphism				
			C. grayii	*C. brachygnathus*	*C. nasus*	*C. mystus*	*C. nasus taihuensis*
E-AAC/M-CAC	48	41(85.42)	2(6.45)	16(48.48)	9(29.03)	5(21.74)	9(29.03)
E-AAG/M-CAG	48	35(72.92)	3(9.09)	5(15.63)	2(6.67)	3(11.11)	1(3.33)
E-ACG/M-CTC	16	13(81.25)	1(12.50)	6(50.00)	5(41.67)	5(50.00)	4(36.36)
E-AAG/M-CTC	24	14(58.33)	2(10.53)	2(11.76)	0(0.00)	3(17.75)	1(5.88)
E-AAC/M-CTC	24	17(70.83)	0(0.00)	3(18.75)	0(0.00)	3(18.75)	0(0.00)
E-ACC/M-CTC	20	17(85.00)	3(21.43)	2(15.38)	5(33.33)	4(33.33)	2(15.38)
Total	180	137(76.11)	11(9.17)	34(37.64)	21(17.50)	23(21.90)	17(14.53)

from 105 (in *C. mystus*) to 123 (in *C. brachygnathus*). However, only 43 markers were fixed and 137 markers were not. In addition, there were 18 and 16 species-specific markers in *C. grayii* and *C. mystus*, respectively. By comparison, more intraspecific polymorphic markers were observed for specimens of *C. brachygnathus*, yet there were few polymorphic markers in specimens of *C. grayii*: 34 and 11 polymorphic markers for *C. brachygnathus* and *C. grayii*, respectively. The number of polymorphic markers for other species was 17 for *C. nasus taihuensis*, 21 for *C. nasus*, and 23 for *C. mystus* (Table 3).

The genetic distances and the coefficients of gene differentiations (*G*st) are given in Table 4. The average intraspecific genetic distance ranged from 0.022 (between individuals of *C. grayii*) to 0.087 (between individuals of *C. brachygnathus*). The average interspecific genetic distance among *C. brachygnathus*, *C. nasus*, and *C. nasus taihuensis* varied from 0.047 to 0.077. This was significantly lower than the interspecific genetic distance between these three species and the species of *C. grayii* and *C. mystus* (0.251–0.499). The highest interspecific genetic distance (0.596) was obtained between *C. grayii* and *C. mystus*, and the lowest interspecific genetic distance (0.047) was between *C. nasus* and *C. nasus taihuensis*. The *G*st value among *C. brachygnathus*, *C. nasus*, and *C. nasus taihuensis* ranged from 0.081 to 0.123, while the *G*st value between these species and the species of *C. grayii* and *C. mystus* ranged from 0.783 to 0.870. The highest *G*st value (0.912) was obtained between *C. grayii* and *C. mystus*, and the lowest *G*st value (0.081) was found between *C. nasus* and *C. nasus taihuensis*.

Table 3 Number of scored markers and polymorphisms resulting from AFLP analysis of *Coilia* in China

	C. grayii	*C. brachygnathus*	*C. nasus*	*C. mystus*	*C. nasus taihuensis*
Number of individuals studied	5	5	6	5	4
Total number of AFLP markers	120	123	120	105	117
Number of polymorphic markers	11	34	21	23	17
Number of species-specific markers	18	0	0	16	0

Table 4 Genetic distances between species (below diagonal) and coefficient of gene differentiations (*G*st) (above diagonal) among Chinese *Coilia*

Species	*C. grayii*	*C. brachygnathus*	*C. nasus*	*C. mystus*	*C. nasus taihuensis*
C. grayii		0.783	0.856	0.912	0.867
C. brachygnathus	0.272		0.090	0.812	0.123
C. nasus	0.251	0.069		0.870	0.081
C. mystus	0.596	0.485	0.477		0.868
C. nasus taihuensis	0.268	0.077	0.047	0.499	

The genetic structures of the three species (*C. brachygnathus*, *C. nasus*, and *C. nasus taihuensis*, were investigated by AMOVA. Only 9.90% of the genetic variation ($P>0.05$, not significant) was found among species, whereas 90.10% of the variation was within species. If *C. brachygnathus* and *C. nasus taihuensis* were regarded as populations of *C. nasus* to investigate the genetic structures of the three species, *C. nasus*, *C. grayii* and *C. mystus*, most of the variance (83.88%, $P=0.00$) was found among species and a small amount (16.12%) within species. In the neighbor-joining tree and UPGMA dendrogram (Figs.1 and 2), three groups were formed: (1) *C. mystus*, (2) *C. grayii* and (3) *C. brachygnathus*, *C. nasus*, and *C. nasus taihuensis*.

4 DISCUSSION

AFLPs have proven to be useful in a variety of evolutionary and taxonomic genetic studies (Dragoo, 2003; Techaprasan et al., 2008; Gwo et al., 2008). The species-specific patterns produced by AFLP can be used for the identification of closely related species (Zhang et al., 2004). AFLPs were employed in this study primarily to further assess the taxonomy and the evolutionary relationship of *Coilia* species in China. There were a total of 180 AFLP loci based on the size of the AFLP markers, and the number of AFLP markers for each species varied from 105 to 123, but only 43 markers were fixed to all the species. In addition, many specific markers among *C. grayii*, *C. mystus* and other *Coilia* species were detected.

There were 18 species-specific markers in *C. grayii* and 16 specific markers in *C. mystus*. This interspecific heterogeneity is sufficient to distinguish *C. grayii* and *C. mystus* from other *Coilia* species.

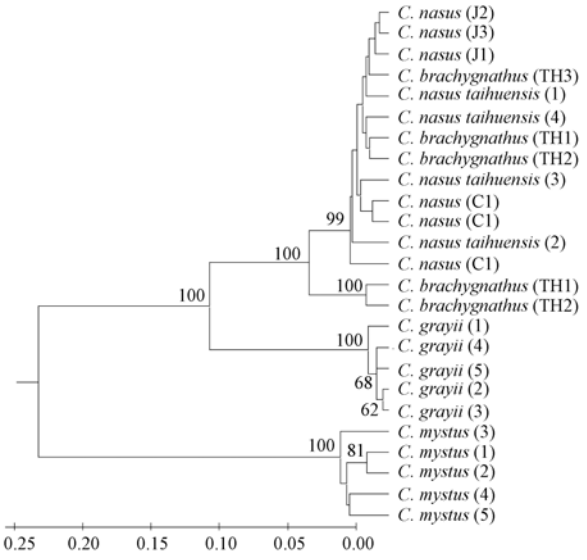

Fig.2 Neighbor-joining tree based on the Nei's genetic distance obtained from AFLP fingerprinting of the species of *Coilia*

Bootstrap values >50 in 200 replicates are shown

The relatively high levels of genetic divergence among *Coilia* species (0.047–0.596) observed in this study are comparatively greater than values of mitochondrial control region sequences (0.011–0.098, Tang et al., 2007). This is probably due to the large number of polymorphic loci revealed by AFLP, which increases the ability to examine interspecific genetic differences of these taxa (Techaprasan et al., 2008).

Studies using mitochondrial control region sequences have led to conclusions regarding the species identification and relationship among the four species and the subspecies of *Coilia* in China (Tang et al., 2007). Our conclusions, using an AFLP approach, are consistent with those from genetic analyses of the mitochondrial control region sequences of *Coilia* in China. The AFLP data indicated a close relationship among *C. brachygnathus*, *C. nasus taihuensis*, and *C. nasus*. The average intraspecific genetic distance within *C. brachygnathus* (0.087), *C. nasus* (0.041), and *C. nasus taihuensis* (0.044) approximates to their interspecific genetic distance (0.047–0.077). The *G*st values among *C. brachygnathus*, *C. nasus*, and *C. nasus taihuensis* ranged from 0.081–0.123 and are significantly lower than those obtained when any of these three *Coilia* were compared to *C. grayii* and *C. mystus* (0.783–0.870). The result of AMOVA detected no significant differences among *C. brachygnathus*, *C. nasus*, and *C. nasus taihuensis*. The AFLP data also showed strong support (86% bootstrap in NJ and 100% bootstrap in UPGMA) for clustering of *C. brachygnathus*, *C. nasus*, and *C. nasus taihuensis*. Our results are, however, at odds with the morphological revision by Yuan et al. (1980) and Whitehead et al. (1988), as they support reciprocal monophyletism for the two putative species, *C. brachygnathus* and *C. nasus*. Instead *C. brachygnathus* and *C. nasus taihuensis* are embedded within *C. nasus*. Based on these data, we share the same opinion expressed by Tang et al. (2007), that *C. brachygnathus* is not a single species of *Coilia*, and *C. nasus taihuensis* should not be regarded as a subspecies *C. nasus*. As a priority, *C. brachygnathus* and *C. nasus taihuensis* should therefore be synonymized with *C. nasus* (see Table 1 for species authorities).

Kreyenberg et al. (1908) originally referred to *C. brachygnathus* based on a specimen of Tungting Lake. The main discriminating feature from *C. nasus* is that the individuals of *C. brachygnathus* have short maxilla and live in freshwater. However, due to the discovery of resident populations of *C. nasus* in Chaohu Lake and Taihu Lake, the morphological differentiation that distinguishes *C. brachygnathus* from *C. nasus* is uncertain on the basis of the length of maxilla. In particular, the lengths of the maxilla differ significantly in maturing migratory fishes of *C. nasus* sampled from the estuary of Yangtze River in recent years (Tang et al., 2007). Thus, the length of the maxilla in *C. nasus* is not a static feature. *C. nasus taihuensis* was considered as a subspecies of *C. nasus* based on the differences in morphological, behavioral and ecological aspects in comparison with *C. nasus* (Yuan et al., 1976). However, this subspecies of *C. nasus* has been disputed by many scientists (Liu, 1995; Cheng et al., 2004a; Cheng et al., 2004b; Cheng et al., 2005; Tang et al., 2007). In general, morphological characters are known to be particularly prone to environmental differences (Allendorf et al., 1987). Many morphological traits originated from the acclimation in nature, which could be susceptible to homoplasy through natural selection (Hufford et al., 1996; Rüber et al., 1999; Takahashi et al., 2007). Therefore, we speculate that the morphological differences among *C. nasus*, *C. brachygnathus*, and *C. nasus taihuensis* are likely to be associated with natural selection.

In conclusion, although AFLP is not commonly

used in phylogenetic studies, it has been successfully used for identifying species origins of morphologically similar taxa and confirmed the species status of taxa, including clades of Lake Malawi cichlids (Albertson et al., 1999; Kassam et al., 2005), a species flock of African electric fish (Sullivan et al., 2004), and four subspecies of cherry salmon (Gwo et al., 2008). This method can be directly applied to detect the genetic relationships and phylogenies among the genus of *Coilia* in China. We conclude that, with appropriate precautions, AFLP is informative in distinguishing these phylogenetic relationships. Our results once again highlight the potential power of AFLP to resolve complex phylogenies among closely related species.

5 ACKNOWLEDGMENTS

We gratefully thank YOKOGAWA Koji, DOU Cunyin, WANG Yuting, and CHEN Yancui for collecting the specimens of *Coilia*. We are also grateful to YING Yiping for his cooperation during the experiment. Thanks ANENE Nwafili Sylvanus for his checking of the writing English.

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