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RESEARCH ARTICLE

Genetic diversity and the biogeographical process of *Acheilognathus macropterus* revealed by sequence variations of mitochondrial cytochrome *b* gene

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Abstract In this study, thirty-six individuals of *Acheilognathus macropterus* were collected from the Heilongjiang River, the Yangtze River, and the Nandujiang River. Partial mitochondrial cytochrome *b* gene region (636 base pair) was sequenced to these samples and 22 haplotypes were found. With *A. chankaensis* and *A. tokinensis* as outgroups, their relationships were analyzed. The *p*-distances were calculated with Mega software and a molecular phylogenetic tree was constructed using the neighbor-joining (NJ) method. The proportions of main morphological characters were compared as well. *P*-distances showed that the genetic differences in *A. macropterus* samples were far smaller than those between these samples and the outgroups. The molecular phylogenetic tree shows that samples with barbels and those without barbels were intermingled. There was no distinctive difference in proportions of morphological characteristics among them. These results suggested that samples with barbels and those without barbels (formally identified as *A. taenianalis*) are the same species; *A. taenianalis* is synonymous with *A. macropterus*. The thirty-six individuals were grouped into five clades and the positions of the samples in the clades were correspondingly grouped within their geographical distributions. Among the five clades, clades 1 and 5 included samples from the Heilongjiang River and Nandujiang River respectively. The samples from the Yangtze River scattered into clades 2, 3, and 4. There were distinctive genetic differences (>5%) among them. Interestingly, the distributions of the 21 samples in these three clades were not correlated to their geographical distributions. It is postulated that these genetic differences were due to the bitterlings' mating choice mechanism, the prozygotic isolation. The genetic differences between the fish from Nandujiang River and those from the mainland indicated that they were separated early. However, the small genetic differences among the samples and the positions of the fish from the Heilonjiang River in the molecular phylogenetic tree indicate that fish in Heilongjiang River might have dispersed from the Yangtze River to that area much later.

Keywords *Acheilognathus macropterus*, cytochrome *b* gene, species validity, genetic diversity, biogeographical process

1 Introduction

The subfamily Acheilognathinae, known as bitterlings, in Cyprinidae, Cypriniformes, has about 40 species or subspecies mainly distributed in Asia, with over 20 species in China and only one species in Europe (Lin, 1998). Bitterling fishes have an unusual spawning symbiosis with freshwater mussels. During the spawning season, female fish develop long ovipositors that they use to place their eggs into the mussels' gills through the mussels' exhalation siphon. The males release sperm into the mussels' inhalation siphon to fertilize the eggs. Larvae swim out of the mussels after 2–4 weeks' development. *Acheilognathus macropterus* was first described by Bleeker in 1871. It is widely distributed in China, for example, in Hainan Island, the Pearl River, the Yangtze River, the Yellow River, the Tumenjiang River and the Amur River. According to the classification of Lin (1998), all bitterlings with more than 15 branched dorsal fin rays and 12 branched anal fin rays should be labeled as *A. macropterus*. Whereas, Wu (1964) separated bitterling fishes with more than 15 branched dorsal fin rays and 13 branched anal fin rays into two species, *A. macropterus* (with barbels) and *A. taenianalis* (without barbels). The presence of barbels is the only difference between these two species. This classification was adopted by Lin (1998). Chen examined

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four holotypes of *A. taenianalis* preserved in the Natural History Museum in Britain and found that they have slightly different barbels. Thereby, Chen suggested that *A. taenianalis* and *A. macropterus* were the same species (Lin, 1998). In the present study, combined with morphological and molecular data, 30.56% of the samples with no barbels were used to test the validity of the *A. taenianalis* classification.

Recently, much effort has been devoted to studies on biogeographical differentiation of fish populations (Tang et al., 2003; Yang and Liu, 2003). It has been found that the low gene flow among different geographical populations may lead to high genetic differentiations among these populations and even to the formation of new species (Hardy et al., 2002). In the present study, the mitochondrial cytochrome *b* gene was used to study the effect of geographical isolation on *A. macropterus* genetic variations and the influence on the bitterlings' reproductive behavior as well.

2 Materials and methods

2.1 Samples

Samples in this paper were collected from the Amur River, the Yangtze River and Hainan Island. All samples were preserved in 95% ethanol and deposited in the Institute of Hydrobiology, Chinese Academy of Science in Wuhan (Table 1).

2.2 DNA extraction, amplification and sequencing

Genomic DNA was extracted from the muscle by 100 μg mL^{-1} Proteinase K digestion. Using polymerase chain reaction (PCR), fragments containing the complete mtDNA cyt*b* gene were directly amplified with primer set L14724 and H15915 (Xiao et al., 2001). Polymerase chain reaction amplification

Table 1 Specimens and their localities in the present study

No.	Species	Specimen voucher	Origin	Drainage	Haplotype	Accession numbers
1	*A. macropterus**	IHB03120802	Heihe	HLJ	G	AY600903
2	*A. macropterus*	IHB03120803	Heihe	HLJ	I	AY600905
3	*A. macropterus**	IHB03120804	Heihe	HLJ	A	AY600883
4	*A. macropterus*	IHB03120807	Harbin	HLJ	E	AY600901
5	*A. macropterus*	IHB03120808	Harbin	HLJ	V	AY600918
6	*A. macropterus*	IHB03120810	Harbin	HLJ	A	AY600884
7	*A. macropterus*	IHB03120816	Fuyuan	HLJ	A	AY600885
8	*A. macropterus*	IHB03120817	Fuyuan	HLJ	A	AY600886
9	*A. macropterus*	IHB03120835	Fuyuan	HLJ	H	AY600904
10	*A. macropterus**	IHB03120821	Jiamusi	HLJ	B	AY600889
11	*A. macropterus**	IHB03120822	Jiamusi	HLJ	M	AY600909
12	*A. macropterus*	IHB03120823	Jiamusi	HLJ	A	AY600887
13	*A. macropterus*	IHB03120824	Jiamusi	HLJ	A	AY600888
14	*A. macropterus*	IHB03120827	Zhuaji	HLJ	F	AY600902
15	*A. macropterus**	IHB03120834	Hejiang	CJSY	J	AY600906
16	*A. macropterus*	IHB03120838	Hejiang	CJSY	K	AY600907
17	*A. macropterus*	IHB03120855	Hejiang	CJSY	L	AY600908
18	*A. macropterus**	IHB03120832	Hejiang	CJSY	B	AY600890
19	*A. macropterus**	IHB03120839	Rongchang	CJSY	B	AY600891
20	*A. macropterus**	IHB03120840	Yueyang	CJZY	R	AY600914
21	*A. macropterus*	IHB03120841	Yueyang	CJZY	C	AY600892
22	*A. macropterus*	IHB03120842	Yueyang	CJZY	D	AY600899
23	*A. macropterus*	IHB03120843	Yueyang	CJZY	C	AY600893
24	*A. macropterus*	IHB03120844	Yueyang	CJZY	C	AY600894
25	*A. macropterus*	IHB03120830	Yueyang	CJZY	D	AY600900
26	*A. macropterus*	IHB03120831	Yueyang	CJZY	S	AY600915
27	*A. macropterus*	IHB03120845	Jinkou	CJZY	C	AY600895
28	*A. macropterus*	IHB03120847	Jinkou	CJZY	C	AY600896
29	*A. macropterus**	IHB03120848	Jinkou	CJZY	C	AY600897
30	*A. macropterus**	IHB03120849	Jinkou	CJZY	T	AY600916
31	*A. macropterus**	IHB03120884	Wuhan	CJZY	N	AY600910
32	*A. macropterus*	IHB03120850	Hukou	CJXY	O	AY600911
33	*A. macropterus*	IHB03120851	Hukou	CJXY	Q	AY600913
34	*A. macropterus*	IHB03120852	Hukou	CJXY	P	AY600912
35	*A. macropterus*	IHB03120853	Hukou	CJXY	C	AY600898
36	*A. macropterus*	IHB03120829	Hainandao	NDJ	U	AY600917
37	*A. chankaensis*	IHB03080915	Duchang	CJXY	–	AY600881
38	*A. tokinensis*	IHB03080946	Huangshan	FCJ	–	AY600882

* indicates samples with barbels absent, A–V indicates different haplotype.

HLJ: Amur; CJSY: upper reaches of Yangtze River; CJZY: middle reaches of Yangtze River; CJXY: lower reaches of Yangtze River; NDJ: Nadujiang River; FCJ: Fuchunjiang River.

was performed in 60 µl total volume, and the reaction system contained 10 x *Taq* buffer 6 µL, dNTPs 3 µL (2.5 mM), primers 3 µL + 3 µL (10 pM), *Taq* polymerase 0.3 µL (1.5U), DNA template 3–5 µL. Then, dd H_2O was added to make a total volume of 60 µL. The amplification was performed with an initial denaturation at 95°C for 3 min followed by 35 cycles at 94°C for 30 s, 54°C for 30 s, 72°C for 1 min. and a final extension at 72°C for 5 mins. Polymerase chain reaction products were purified and sequenced by commercial companies. All sequences are available from GenBank (Table 1).

2.3 Sequence analysis

Primer L14724 was used for sequencing. Sequences were aligned by Clustal X (Thompson et al., 1997). The first 636 bp from the starting code ATG (the position of first amino acid in peptide chain polymerizing) were retained for analysis. *A. chankaensis* and *A. tokinensis* were used as controls. The sequence variations and phylogenetic analyses with neighbor-joining (NJ) method were performed using Mega 2.0 (Kumar et al., 2001). 1000 bootstrap replicates were performed in NJ analyses.

2.4 Morphological analyses

The following characters were measured for all samples: body length, body depth, caudal peduncle length, caudal peduncle depth, head length and snout length. The proportions of body length/body depth, caudal peduncle length/caudal peduncle depth and head length/snout length were calculated as well.

3 Results

3.1 Sequence variations

For the 636bp sequences, neither insertions nor deletions were found. 107 sites were found to be variables of which 57 were parsimony informative. The average nucleotide composition for all the sequences were A = 27%, T = 29.9%, C = 27.1% and G = 16%. The content of A + T (56.9%) was higher than that of C + G (43.1%). Significant base composition biases of lower G content were observed in the sequence, which is in agreement with that of other vertebrate (Hochachka and Mommsen, 1993). The average sequence divergence between outgroup and all *A. macropterus* samples were estimated using *p*-distance (Table 2). The sequence divergence between the samples with barbels and the outgroups varied from 11.03% to 16.08%, the mean sequence divergence among all samples was 4.20%, and that of the samples between *A. chankaensis* and *A. tokinensis* were 14.64% and 12.81%. As to the samples without barbels, the sequence divergence was from 11.03% to 16.11%, 3.21%, 14.16% and 12.65%, respectively. The mean sequence divergence between the samples with barbels and those without barbels was 3.86%, which was far smaller than that between the two outgroup species (15.59%).

3.2 Morphological analysis

Analysis to morphological character of all samples in this study showed that, in the samples with barbels, the proportion of body length/body depth varied from 2.02 to 2.62. The proportion of caudal peduncle length/caudal peduncle depth was from 1.00 to 1.75 and that of head length/snout length from 2.88 to 3.80. While in the samples without barbels, the range was from 2.05 to 2.50, from 0.86 to 1.86 and from 3.00 to 3.60, respectively. There was almost an overlap between these two types and this supports that there were no significant differences between samples with barbels and those without barbels in morphological characters.

3.3 Phylogenetic relationships

The molecular phylogenetic tree (Fig. 1) shows that samples with barbels and those without barbels were intermingled and the species of the controls were clearly separated. Therefore, the relationships of the samples with barbels and those without barbels belong to the same species. Among the 36 samples, 22 haplotypes (some were shared by samples with barbels and those without barbels) were found (Table 1). The 36 samples were grouped into five clades, and the positions of the samples in the clades corresponded to their geographical distributions. Among the five clades, clades 1 and 5 included samples from the Amur River and the Nandujiang River, respectively. The samples from the Yangtze River were scattered into clades 2–4. The sequence divergence of intra-clades were shown in Table 2 (all low than 1%) and those of inter-clades were shown in Table 3.

4 Discussion

4.1 Validity of *A. taenianalis*

The sequence divergence between samples with barbels and those without barbels (3.86%) was far smaller than that between the two species of controls (15.59%) and those between these samples and the controls (> 10.00%). The low sequence divergence compared with the controls and the same morphological characters indicated that *A. macropterus* and *A. taenianalis* were the same species which was supported by the results of molecular phylogenetic analysis. In the phylogenetic tree, the samples with and without barbels were intermingled and did not form their own monophyly. Therefore, *A. taenianalis* (1873) and *A. macropterus* belong to the same species. Since *A. macropterus* (1871) was described earlier than *A. taenianalis* (1873), *A. macropterus* should be the valid species.

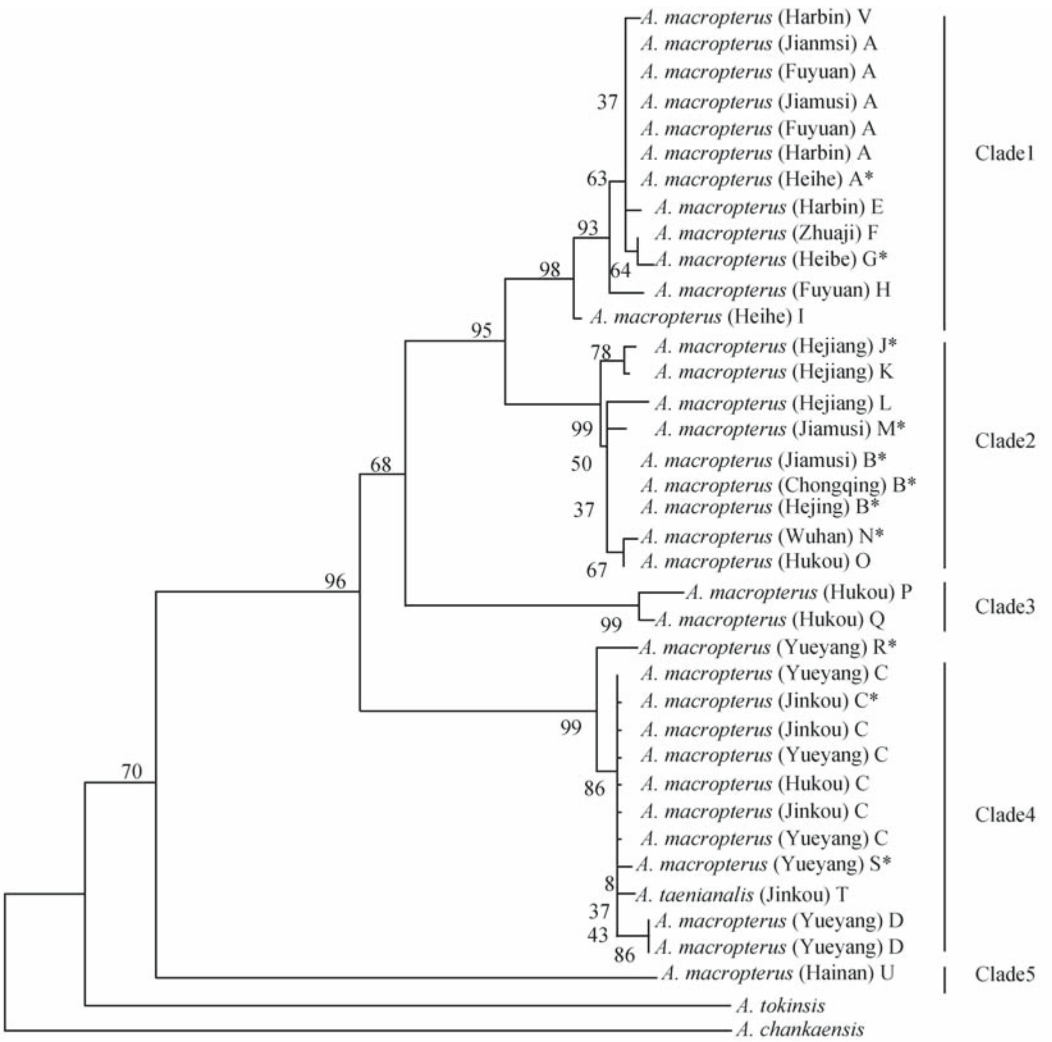

Fig. 1 The molecular phylogenetic tree of all *A. macropterus* samples (numbers indicate bootstrap values of 1000 replicates, symbols of asterisk and A–V are as in Table 1)

4.2 Factors affecting the genetic variations of *A. macropterus*

The molecular phylogenetic tree and sequence divergence among clades showed that all 21 samples from Yangtze River were grouped into three clades (clades 2–4) supported by a high bootstrap value (99%). There were distinctive genetic differences ($>5\%$) among them. Interestingly enough, the distributions of the 21 samples in these three clades were not correlated to their geographical distributions. It was suggested that these genetic differences might be due to the bitterlings' mating choice mechanism, the prozygotic isolation. The reproductive methods of cyprinid fishes are mainly through aggregative mating and external fertilization. Therefore, genetic divergence is difficult to achieve because of the high gene flow in different groups. However, for bitterling fishes, they have a mating choice mechanism in reproduction. In reproduction season, the males have red coloration to attract females and are territorial in behavior. It is very possible that different standards of mate choices exist in *A. macropterus* individuals and those with the same standard may group into one clutch. In one clutch, the male would defend the mussel to prevent the other males from sneaking in while the female laid the eggs, which restricts the gene flow between different clutches and leads to genetic divergence among them.

4.3 Biogeographical process of *A. macropterus*

Based on results from the molecular phylogenetic tree and Table 2, distinctive genetic differences existed between fishes from the Hainan River and those of clade 5. It was suggested that these distinctive genetic differences were due to the very low gene flow caused by the prolonged geographic isolation (Liu, 1998) between the fishes from the continental drainages and those from Hainan Island. Further studies should be conducted to test this hypothesis.

In the molecular phylogenetic tree, samples from the Amur River were located at the top of the clades. It is possible that the fishes in the Heilongjiang River might have dispersed

Table 2 Percentage of sequence variations of the cytochrome *b* gene between all haplotypes and control samples (%)

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
(1) V																								
(2) A	0.0016																							
(3) E	0.0031	0.0016																						
(4) F	0.0031	0.0016	0.0031																					
(5) G	0.0047	0.0031	0.0047	0.0016																				
(6) H	0.0063	0.0047	0.0063	0.0063	0.0079																			
(7) I	0.0079	0.0063	0.0079	0.0079	0.0094	0.0079																		
(8) J	0.0283	0.0267	0.0283	0.0283	0.0299	0.0283	0.0204																	
(9) K	0.0267	0.0252	0.0267	0.0267	0.0283	0.0267	0.0189	0.0016																
(10) L	0.0299	0.0283	0.0299	0.0267	0.0283	0.0299	0.022	0.0079	0.0094															
(11) M	0.0267	0.0252	0.0267	0.0267	0.0283	0.0267	0.0189	0.0047	0.0063	0.0063														
(12) B	0.0252	0.0236	0.0252	0.0252	0.0267	0.0252	0.0173	0.0031	0.0047	0.0047	0.0016													
(13) N	0.0283	0.0267	0.0283	0.0283	0.0299	0.0283	0.0204	0.0063	0.0079	0.0079	0.0047	0.0031												
(14) O	0.0267	0.0252	0.0267	0.0267	0.0283	0.0267	0.0189	0.0047	0.0063	0.0063	0.0031	0.0016	0.0016											
(15) P	0.0519	0.0503	0.0519	0.0519	0.0503	0.0519	0.0472	0.0519	0.0503	0.0535	0.0503	0.0487	0.0519	0.0503										
(16) Q	0.0487	0.0472	0.0487	0.0487	0.0472	0.0487	0.044	0.0487	0.0472	0.0503	0.0472	0.0456	0.0487	0.0472	0.0063									
(17) R	0.0566	0.055	0.0535	0.0566	0.055	0.0566	0.0519	0.0566	0.0582	0.0582	0.0519	0.0535	0.0566	0.055	0.055	0.0519								
(18) C	0.0535	0.0519	0.0503	0.0535	0.0519	0.0535	0.0487	0.0566	0.0582	0.0582	0.0519	0.0535	0.0566	0.055	0.055	0.0519	0.0063							
(19) S	0.055	0.0535	0.0519	0.055	0.0535	0.055	0.0503	0.0582	0.0597	0.0597	0.0535	0.055	0.0582	0.0566	0.0566	0.0535	0.0079	0.0016						
(20) T	0.055	0.0535	0.0519	0.055	0.0535	0.055	0.0503	0.0582	0.0597	0.0597	0.0535	0.055	0.0582	0.0566	0.0566	0.0535	0.0079	0.0016	0.0031					
(21) D	0.0566	0.055	0.0535	0.0566	0.055	0.0566	0.0519	0.0597	0.0613	0.0613	0.055	0.0566	0.0597	0.0582	0.0582	0.055	0.0094	0.0031	0.0047	0.0047				
(22) U	0.0975	0.0959	0.0975	0.0975	0.0959	0.0975	0.0928	0.0943	0.0959	0.0959	0.0896	0.0912	0.0928	0.0928	0.1006	0.1006	0.0991	0.0928	0.0943	0.0943	0.0959			
(23) *A. tokinensis*	0.1242	0.1226	0.1211	0.1211	0.1226	0.1242	0.1164	0.1195	0.1211	0.1179	0.1148	0.1164	0.1148	0.1148	0.1321	0.1289	0.1022	0.1006	0.1022	0.1022	0.1038	0.1195		
(24) *A. chankaensis*	0.1226	0.1211	0.1226	0.1226	0.1242	0.1258	0.1211	0.1211	0.1226	0.1226	0.1195	0.1179	0.1195	0.1195	0.1274	0.1336	0.1384	0.1384	0.1399	0.1399	0.1384	0.1274	0.1368	

Symbols of A–V are as in Table 1

Table 3 Average genetic distances of the cytochrome *b* gene between clades

	Clade 1	Clade 2	Clade 3	Clade 4	Clade 5	*A. tokinensis*	*A. chankaensis*
Clade 1							
Clade 2	0.0262						
Clade 3	0.0516	0.0514					
Clade 4	0.0558	0.0592	0.0573				
Clade 5	0.1059	0.1017	0.1114	0.1032			
A. tokinensis	0.1375	0.1306	0.1485	0.1114	0.1339		
A. chankaensis	0.1377	0.1346	0.1487	0.1593	0.1448	0.1559	

from the Yangtze River to there during the glacial period. Noticeably, one sample from Jamusi in clade 2 shared one haplotype with those from Chongqing and Hejiang and a very low sequence divergence was found among this haplotype and the related ones. It is possible that artificial introduction may have occurred between the Yangtze River and the Heilongjiang River recently.

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