2012 International Conference on Environment, Chemistry and Biology
IPCBEE vol.49 (2012) © (2012) IACSIT Press, Singapore
DOI: 10.7763/IPCBEE. 2012. V49. 6

Genetic Diversity of *Botia Superciliaris* Populations in the Upper Reaches of the Yangtze River Revealed by ISSR Markers

Hongyan Liu[1], Fei Xiong[1+], Xinbin Duan[2] and Daqing Chen[2]

[1]College of Life Science, Jianghan University, Wuhan 430056, China
[2]Yangtze River Fisheries Research Institute, Wuhan 430223, China

Abstract. *Botia superciliaris*, an important freshwater species endemic to China, has undergone a severe decline in numbers due to human impacts. We studied its genetic diversity of four *Botia superciliaris* populations (CS, ZY, YB and WJ) in the upper reaches of the Yangtze River. Inter-simple sequence repeat (ISSR) analysis was performed to evaluate genetic diversity of 99 individuals. Genetic diversity varied within populations with the percentage of polymorphic bands (*PPB*) values ranging from 44.59% to 55.41%, indicating a high level of genetic diversity in the four populations. The ZY and YB populations were the most closely related, for the smallest genetic distance (0.0215) between the two populations, whereas, the distance between CS and WJ populations was the biggest (0.0405), suggesting the greatest divergence existed in the two populations. UPMGA cluster was consistent with the distance results. AMOVA analysis reflected that mostly variation occurred within populations. The coefficient of population differentiation (*Fst*) was 0.1111, indicating a moderate genetic differentiation among samples from the four populations. Pairwise *Fst* showed that the differentiations between the populations from WJ and CS, WJ and ZY were the biggest, differentiations between populations from ZY and YB was the smallest. The results showed that the gene interchange between populations of ZY and YB occurred more frequently, and gene flow between the populations of WJ and CS, WJ and ZY occurred less than the other populations. Our research might provide reliable information for population conservation, recovery and management of this species in the Yangtze River.

Keywords: *Botia superciliaris*, ISSR, genetic diversity, the upper reaches of the Yangtze River

1. Introduction

Botia superciliaris, a teleost species belonging to Cobitidae, is an important freshwater species with ornamental and potentially commercial value because of its vivid color and good taste. It mainly distributes in the middle and upper reaches of the Yangtze River and its tributaries, and is endemic to China[1]. It is often found near gravel and rock crevice and habitats in the middle and the bottom of the rivers and the streams with swift currents, and feeds on aquatic insects, benthic invertebrates and decomposed fish body[2]. When rivers flood, these fish usually swim upstream. Spawning occurs from April to July, and eggs and developing larvae drift with the currents[3]. Thus, relatively long rivers with continuous flow are necessary for embryonic development and larval growth[4]. However, this requirement in their natural history conflicts with construction of cascade hydropower stations in the middle and upper reaches of theYangtze River. With the construction of cascade hydropower stations within its distribution, *Botia superciliaris* is suffering from severe threats to its survival. In additional of over-fishing, water pollution, the resource of this species has seriously declined. It is urgent to conserve and recover the populations through appropriate management.

Knowledge of the population genetic diversity is a basic prerequisite for rational conservation, exploitation and management, and also for correct interpretation of ecological investigations[5]. Therefore, it is necessary to study the genetic diversity of natural populations for conservation and recovery of this species.

[+] Corresponding author. Tel.: + 86-02784230422;
E-mail address: xf9603@163.com

However, very little information has been published on population genetics of *B. superciliaris*. There was only our former study that investigated the genetic diversity of this species using mtDNA control region[6]. We found that haplotype diversity of this species was high and there was weak significant genetic differentiation among the populations. However, there were difference degrees of divergence in different markers due to distinct mutation rates, selective pressures, ploidy levels and inheritance[7]. Therefore, it is necessary to integrate other molecular markers data to analysis the genetic diversity of *B. superciliaris*.

In recent years, inter-simple sequence repeats (ISSR) have been successfully employed to reveal genetic variation[8-10]. As a dominant marker, ISSR has a few advantages over other markers. ISSR primers anneal directly to simple sequence repeats and the sequences that ISSR target are abundant throughout the eukaryotic genome and evolve rapidly, In addition, ISSR amplification does not require prior knowledge of DNA background information[11]. In this study, ISSR markers were performed for further analysis on genetic diversity of this species from the level of nuclear genome with increasing sample sizes and enlarging the sample ranges. It will provide reliable information for population conservation, recovery and management of this species in the Yangtze River.

2. Materials and Methods

2.1. Sample Collection and DNA Extraction

The fish materials used in this study was sampled a total of 99 individuals from four locations in the upper reaches of the Yangtze River during May 2007 and June 2010. The four sample locations were Chishuihe tributary (CS), Zhuyangxi section (ZY), Yibin section (YB), and Wujiang tributary (WJ). CS is an important tributary for diversity conservation of fishes, which is maintained natural conditions well and not still exploitted for hydropower stations. ZY and YB are both in the main stream, which are in natural reserves area of nationally rare and endemic fishes. WJ is polluted seriously and many hydropower stations are constructed on it. The numbers of four populations were 34 individuals from CS, 35 individuals from ZY, 20 individuals from YB and 10 individuals from WJ respectively. Fin tissues were collected from each individual and immediately stored in 95% alcohol. Genomic DNA was extracted from 5 cm^2 fin tissues following the standard phenol-chloroform protocol. The concentration and purity of acquired genomic DNA was determined by BioPhotometer plus. The concentration of genomic DNA was diluted to 20ng/μl.

2.2. ISSR-PCR Amplification

A total of 36 primers (microsatellite set 9, university of British Columbia) were screened on four randomly DNA of individuals for PCR, and 8 primers that could produce reproducible bands were selected for further ISSR analysis (Table 1). PCR reactions were carried out in a volume of 25μl, containing 10×Taq Buffer 2.5μl, 0.4mM primer, 2.0mM Mg^{2+}, 0.2mM of each dNTP, 1U Taq polymerase and about 20ng DNA template. Reactions were carried out on a PTC-100TM thermocycler (MJ Research, Inc). PCR conditions were as follows: initial denaturation at 94℃ for 2 min, followed by 35 cycles of 94℃ for 45 s, 53℃ for 45 s, 72℃ for 90s, and a final extension at 72℃ for 5 min. Amplification products were separated on 1.5% agarose gels, stained with ethidium bromide, visualized under UV light, and photographed by GEL imaging analysis system. Molecular sizes were estimated with a DL2000 DNA ladder.

2.3. Data Analysis

ISSR bands amplified by PCR were scored as present (1) or absent (0) for each DNA sample, and transformed into a 0/1 binary character matrix. POPGENE1.31[12] was used to calculate some parameters of genetic diversity, including the percentage of polymorphic bands (*PPB*), Nei's gene diversity (*H*), Shannon information index (*I*), Nei'S genetic distance (*D*). Nei'S genetic distances were subsequently applied to construct a UPGMA dendogram with MEGA4.0.[13]

Population structure was evaluated using the analysis of molecular variance (AMOVA 1.55)[14]. The overall molecular variance was partitioned into components corresponding to the divergence within and among populations. Input files for the program were generated using AMOVA-PREP. Significance tests were made after 1000 permutations. The gene flow estimates were derived using the equation Nm= [(1/*Fst*) – 1]/4.

Table 1 Name and sequence of eight effective primers used in the study

Primers name	Sequence(5'--3')
817	CAC ACA CAC ACA CAC AA
834	AGA GAG AGA GAG AGA GYT
840	GAG AGA GAG AGA GAG AYT
845	CTC TCT CTC TCT CTC TRG
846	CAC ACA CAC ACA CAC ART
855	ACA CAC ACA CAC ACA CYT
857	ACA CAC ACA CAC ACA CYG
861	ACC ACC ACC ACC ACC ACC

Note: Y= (C/T), R= (A/G)

3. Results

3.1. ISSR Polymorphism

Thirty-six ISSR primers were screened on four randomly DNA of individuals. Eight primers that produced clear and reproducible fragments were selected for further analysis (Table 1). The eight primers generated 74 bands (an average of 9.25 bands per primer) ranging in size from 200 to 2 000 bp. Genetic diversity varied in populations with the percentage of polymorphic bands (*PPB*) values ranging from 44.59% (ZY and WJ) to 55.41% (CS). The Nei's gene diversity (*H*) and the Shannon information index (*I*) showed the same trend of genetic variation in populations (Table 2). As a whole, the CS population exhibited the highest level of variability (the *PPB*, *H* and *I* was 55.41%, 0.1572 and 0.2469, respectively), whereas the WJ population exhibited the lowest level of variability (the *PPB*, *H* and *I* was 44.59%, 0.1328 and 0.2069, respectively).

Table 2 Genetic diversity parameters in the populations of *Botia superciliaris*

	Number of polymorphic loci	*PPB*	*H*	*I*
CS	41	55.41	0.1572	0.2469
ZY	33	44.59	0.1392	0.2170
YB	40	54.05	0.1411	0.2225
WJ	33	44.59	0.1328	0.2069

3.2. Genetic Distance and Cluster Analysis

Genetic distance and genetic similarity were showed in Table 3. Genetic distance ranged from 0.0215 to 0.0405. The distance between YB and ZY populations (0.0215) was the smallest, indicating that the two populations were the most closely related. The distance between CS and WJ populations was the biggest (0.0405), suggesting the greatest divergence existed in the two populations.

Table 3 Genetic distance (below diagonal) and genetic similarity indices (above diagonal) among the four populations of *Botia superciliaris*

	1	2	3	4
CS		0.9698	0.9664	0.9595
ZY	0.0302		0.9785	0.9606
YB	0.0336	0.0215		0.9645
WJ	0.0405	0.0394	0.0355	

UPMGA cluster was consistent with the distance results (Fig. 1). It showed that ZY and YB populations were firstly aggregated a branch, which was then aggregated with CS population, Finally, WJ population attached with the three populations. The cluster analysis indicated that ZY and YB populations were most closely related, WJ population was the least genetic similarity with other populations.

3.3. Genetic Structure of Populations

The *Fst* value among four populations calculated by AMOVA analysis was 0.1111 (Table 4), indicating that there were 11.11% genetic variations among the populations and 88.89% within the populations. The

results reflected that most genetic variations occurred within the populations. Analysis of *Fst* on the four populations showed that there was significant differentiation among the populations ($P < 0.001$).

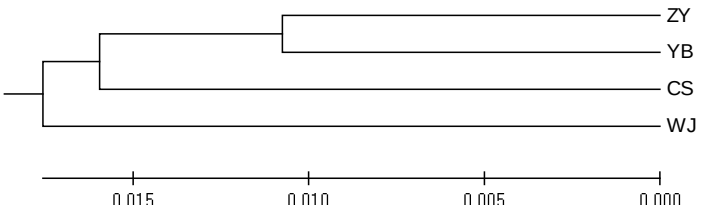

Fig. 1 UPGMA cluster analysis of *Botia superciliaris* based on Nei's genetic distances of ISSR data

Table 4 Analysis of molecular variance (AMOVA) among the populations of Botia superciliaris

Source of variation	d. f	Sum of squares	Variance component	Variance percentage(%)	*P*-value
Among population	3	53.6749	0.69158	11.11	< 0.001
Within population	73	403.9096	5.53301	88.89	< 0.001

Significance tests after 1000 random permutations

Pairwise *Fst* showed that the differentiations between populations from WJ and CS, WJ and ZY were the biggest, differentiations between populations from ZY and YB was the smallest (Table 5). The gene flow ranged from a low of 2.05 between populations from WJ and CS, WJ and ZY to a high of 3.96 between populations from ZY and YB (Table 5). It can be seen that the gene interchange between ZY and YB populations occurred more frequently than among other populations.

Table 5 Genetic differentiation (below diagonal) and gene flow (above diagonal) between paired populations of *Botia superciliaris*

	1	2	3	4
CS		2.65	2.63	2.05
ZY	0.0863		3.96	2.05
YB	0.0868	0.0594		3.47
WJ	0.1085	0.1085	0.0671	

4. Discussion

In this study, the *PPB* values ranged from 44.59% to 55.41% within four populations. Compared with species previously reported[15-18], the *PPB* of *B. superciliaris* was high, which showed that the genetic diversity of *B. superciliaris* was in high level. The results were consistent with our previously results from mtDNA markers[6].

Environmental change may play an important role in genetic variation[19]. The difference of genetic diversity exhibited in different population could be associated with life and ecological characteristics. The level of genetic diversity in CS population was the highest among the four populations. CS River is one of tributaries that keep natural state very well, with less disturbance by human activities and without constructing hydropower projects on it. Therefore, CS River is the region which having the richest fish diversity in the upper reaches of the Yangtze River. It preserves the germplasm pool of fishes well. The analysis on *B. superciliaris* genetic diversity confirmed the view very well. The level of genetic diversity in WJ population was the lowest among the four populations. At present, WJ tributary is seriously polluted and built many hydropower projects[20]. Owing to hydrological environment deteriorating in WJ tributary, the population size of *B. superciliar* is sharply decreased. Relatively small population may be one reason of the lowest genetic diversity. When sampling, we found that the distribution range and the number of *B. superciliaris* population was small in WJ tributary. The small effective individuals of this population could possibly result in loss of genetic diversity in WJ tributary.

The ZY and YB regions were both on the main stream and had no barrier, thus gene interchange of the two populations could occur more frequently than other populations. The WJ population was isolated from other populations for many hydropower barriers, which lessened the opportunity contacting with other populations and limited the gene flow. So the WJ population emerged the most genetic divergence. Geographic barrier may be an important reason that caused genetic differentiation.

The *Fst* value of *B. superciliaris* populations was 0.0291 based on mtDNA control region markers[6], indicating a weak significant genetic differentiation. The *Fst* value of the four populations inferred from the ISSR (0.1111) in this study was higher than that from mtDNA control region analysis, indicating a moderate level of genetic differentiation at nuclear genome level. The estimates of the genetic differentiation slightly differed between the two molecular makers. The data suggested that a gender biased dispersal of genetic structure occurred when the results inferred from genomic markers was compared with mtDNA markers in this species. Therefore, several molecular markers data should be integrated to analysis the genetic variations of *B. superciliaris*.

Genetic divergence also existed in some endemic fishes in different tributary of the upper reaches of the Yangtze River in previous researches. For instance, three populations of Prenant's schizothoracin (*Fst* =0.1276)[21]and six populations of Procypris rabaudi (*Fst* =0.0657) [22] in the upper reaches of the Yangtze River had a moderate degree of genetic divergence. And that some dispersed species in the Yangtze River, such as *Cyprinus carpio*, *Mylopharyngodon piceus*, *Hypophthalmichthys molitrix*, *Aristichthys nobilis* also had a significant genetic differentiation [23]. In general, Fishes that were resident type or weak swimming ability or can complete life cycle in part regions would generate genetic differentiation because of limited gene flow. Divergence of *B. superciliaris* populations may be related with its distinct life biological traits, such as low dispersing ability, adapting to rapid current in the river course, the same river migratory.

In recent years, some investigators considered that genetic diversity was vital to a species for surviving and persisting[24]. The other investigators believed that ecological factors, such as habitat fragment and variation of water environmental condition were direct reason that caused recession of a species[25]. In fact, genetic diversity, life history traits and ecological factors may all exert effect on surviving and persisting of a species. Researches on fishes of the Yangtze River in recent years showed that construction of Dam and variation of water environmental condition were important factors that caused recession of the fishes in the Yangtze River. Most river sections of the Yangtze River changed from river habitat to reservoir habitat attributing to building Dam[26]. As a fish adapting to rapid currents, the population size of *B. superciliaris* had sharply declined. Moreover some process in life cycle also underwent negative influence as results of the water environment changes, including deterioration of spawning sites, no enough distance for egg floating and offspring failure to hatch.

There were high level genetic diversity and significant population differentiation of *B. superciliaris* in this study. The upper reaches of the Yangtze River have been heavily affected by anthropogenic activity, which has the potential to reduce genetic diversity of *B. superciliaris* in the future. Conservation of the genetic resource of *B. superciliaris* should pay the urgent attention so that *B. superciliaris* can be managed appropriately and recovery rationally.

5. Acknowledgements

This work was supported by the National Natural Science Foundation of China (51109091, 51249004), Special Fund for Agro-scientific Research in the Public Interest of China (200903048) and the Fund of Wuhan Bureau of Education (2010060). We thank Di Di Wu, Ling Yan Shi for technical assistance.

6. References

[1] R. Ding. *Sichuan fish volunteers*. Sichuan Sci. Technol. Press. 1994.

[2] T. Zhao, J. Liu. The Initial study of the otolith, age and growth of *Botia superciliaris* in the Jiangjin Segment of Yangtze River. *Freshwater Fisheries*. 2008, 38 (5): 46-50.

[3] M. Yang, X. Ding. Study on the reproductive biology of *Botia superciliaris*. *J. Hydroecology*. 2010, 3 (2): 38-41.

[4] X. Li, D. Huang, W. Xie, et al. Current status of spawning grounds of fish with pelagic eggs in the middle reaches of Hanjiang River. *J. Dalian fisheries university.* 2006, 21 (2): 105-111.

[5] X. Yuan, Y. Wu, B. Hong, et al. Mitochondrial DNA diversity of *Glyptothorax zanaensis* populations in Nu River, *China*. *Environ. Bio. Fish.* 2012, 93 (1): 137-142.

[6] H. Liu, D. Chen, S. Liu, et al. Genetic diversity of *Botia supercilia* ris in the upper Yangtze River. *Freshwater Fisheries.* 2009, 39: 8-13.

[7] J. Campos, D. Posada, M. Paloma. Genetic variation at MHC, mitochondrial and microsatellite loci in isolated populations of Brown trout (*Salmo trutta*). *Conserv. Genet.* 2006, 7: 515-530.

[8] B. Bornet, E. Antoine, M. Bardouil, et al. ISSR as new markers for genetic characterization and evaluation of relationships among phytoplankton. *J. Appl. Phycol.* 2004, 16: 285-290.

[9] C. Mattioni, M. Casasoli, M. Gonzalez, et al. Comparison of ISSR and RAPD markers to characterize three Chilean *Nothofagus* species. *Theor. Appl. Genet.* 2002, 104: 1064-1070.

[10] J. Li, G. Wang, Z. Bai. Genetic diversity of freshwater pearl mussel (*Hyriopsis cumingii*) in populations from the five largest lakes in China revealed by inter-simple sequence repeat (ISSR). *Aquacult. Int.* 2009, 17: 323-330.

[11] E. Esselman, J. Li, D. Crawford, et al. Clonal diversity in the rare Calamagrostis porterissp. insperata (Poaceae): comparative results for allozymes and random amplified polymorphic DNA (RAPD) and inter simple sequence repeat (ISSR) markers. *Mol. Ecol.* 1999, 8: 443-451.

[12] F. Yeh, R. Yang, T. Boyle. (1999) POPGENE version 1.3.1. Microsoft Window-bases Freeware for Population Genetic Analysis.Available: (www.ualberta.ca/~fyeh/) University of Alberta and the Centre for International Forestry Research

[13] K. Tamura, J. Dudley, M. Nei, et al. MEGA4: Molecular evolutionary genetics analysis (MEGA) software version 4.0. *Mol. Biol. Evol.* 2007, 24: 1596-1599.

[14] M. Miller. AMOVA-PREP, a program for preparation of AMOVA input files from dominant-marker raw data, Release 1.01. *Department of Biological Science*, Northern Arizona University, Flagstaff, AZ. 1998.

[15] Y. Liu, S. Chen, J. Li, et al. Genetic diversity in three Japanese flounder (*Paralichthys olivaceus*) populations revealed by ISSR markers. *Aquaculture.* 2006, 255: 565–572.

[16] D. Li, G. Lin, J. Zhang, et al. Preliminary analysis on genetic diversity of two finless porpoise (*Neophocaena phocaenoides*) populations with ISSR marker. *J. Nanchang University.* 2005, 29: 546-550.

[17] J. Xu, Q. Zhang, Z. Wang, et al. ISSR analysis of wild and farmed stone flounder (*Kareius bicoloratus*) stocks. *Acta Oceanologica Sinica.* 2009, 31: 128-134.

[18] Y. Zhang, Z. Hu, Z. Zhou, et al. Population genetic structure of *Coilia ectenes* sampled from Yangtze River estuary revealed by RAPD-PCR and ISSR-PCR markers. *J. Shanghai Fisheries University.*2006, 15 (4): 390-397.

[19] K. Scribner, M. Wooten, M. Smith, et al. Variation in life history and genetic traits of Hawaiian mosquitofish populations. *J. Evol. Biol.* 1992, 5: 267-288.

[20] H. Liu, Q. Liu, J. Mao. Study on relationship between water resources, water quality changes and reservoir construction in Wujiang Basin. *Water Res. Protec.* 2010, 26: 18-22.

[21] J. Song, Z. Song, B. Yue, et al. Assessing genetic diversity of wild populations of Prenant's schizothoracin, *Schizothorax prenanti*, using AFLP markers. *Environ. Biol. Fish.* 2006, 77: 79-86.

[22] J. Song. Population genetic diversity of rock carp (*Procypris rabaudi*) based on AFLP makers and mitochondrial DNA control region. *Master Thesis, Sichuan University*. 2006.

[23] G. Lu, S. Li, L. Bernatchez. Mitochondrial DNA diversity, population structure and Conserv. Genet. of four native carps within the Yangtze River, China. *Can. J. Fish. Aquat. Sci.* 1997, 54: 47-58.

[24] R. Frankham, J. Ballou, D. Briscoe. *Introduction to Conservation Genetics*. Cambridge University Press, Cambridge. 2002.

[25] C. Wang, X. Yu, J. Tong. Microsatellite diversity and population genetic structure of redfin culter (*Culter erythropterus*) in fragmented lakes of the Yangtze River. *Hydrobiologia.* 2007, 586: 321-329.

[26] W. Cao. Analysis on construction of natural natural reserves of endemic fishes in the upper reaches of Yangtze River. *Res. Environ. Yangtze Basin*. 2000, 2: 131-132.