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# Identification and characterization of eighteen polymorphic microsatellite loci for a threatened freshwater fish, *Botia superciliaris*

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[Xiong F., Liu H.-Y., Duan X.-B. and Chen D.-Q. 2014 Identification and characterization of eighteen polymorphic microsatellite loci for a threatened freshwater fish, *Botia superciliaris*. *J. Genet*. **93**, e5-e7. Online only: http://www.ias.ac.in/jgenet/OnlineResources/93/e5.pdf]

## Introduction

*Botia superciliaris*, an endemic fish (Cobitidae) of China, is mainly distributed in the middle and upper reaches of the Yangtze river. It is an important freshwater species with potential commercial value. It lives in the bottom of the river with swift currents and spawns pelagic eggs (Yang and Ding 2010; Li *et al*. 2011). Relatively long rivers with continuous flow of water are necessary for its embryonic development and larval growth. However, with the construction of cascade hydropower stations within its distribution, *B. superciliaris* is suffering from severe threats to its survival (Yang and Ding 2010). In addition, due to overfishing and water pollution, natural resources of *B. superciliaris* have drastically decreased in recent years. Conservation and recovery of the genetic resources of this species should therefore be paid urgent attention (Heiner *et al*. 2011). For better planning of conservation efforts, it is necessary to understand its population genetic structure and genetic diversity. Unfortunately, very little is known about the population genetics of *B. superciliaris*, and the genetics studies thus far have focussed on mitochondrial markers (Liu *et al*. 2009).

Microsatellite markers are useful for answering population genetics and conservation genetics questions because of codominance and high allelic polymorphism (Lawson and Zhang 2006). Nevertheless, there are no microsatellite markers available for *B. superciliaris*. The development of microsatellites for this species and their application is therefore very important. So, here, to enhance the conservation and recovery of genetic resources of *B. superciliaris*, we report the development and characterization of 18 polymorphic microsatellite loci in this species.

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**Keywords.** microsatellites; Cobitidae; *Botia superciliaris*.

## Materials and methods

### *DNA extraction*

Thirty individuals of *B. superciliaris* were collected from Yibin (28°41.68′N, 104°31.43′E) section in the upper reaches of the Yangtze river. Fin tissue samples were stored in 95% ethanol until DNA extraction. The genomic DNA was extracted using the standard phenol–chloroform method with RNase treatment.

### *Microsatellite-enriched library construction*

Microsatellite-enriched library was constructed using the fast isolation by AFLP of sequences containing repeats (FIASCO) protocol (Zane *et al*. 2002). In brief, the mixed genomic DNA (about 100 ng) from four individuals was digested with *Mse*I restriction enzyme, then the adaptors (*Mse*I A: 5′-TAC TCA GGA CTC AT-3′ and *Mse*I B: 5′-GAC GAT GAG TCC TGA G-3′) were ligated into the end of DNA fragments. The linker-ligated DNA was amplified using adapter primer (*Mse*I N: 5′-GATGAGTCCTGAGTAA-3′) in a 20 $\mu$L reaction mixture. PCR conditions were 5 min at 94°C followed by 20 cycle of 1 min at 94°C, 1 min at 53°C, 1 min at 72°C, with a final extension at 72°C for 10 min. The products were then hybridized to biotin-labelled probes containing the repeat motif $(GT)_{13}$. The mixture was denatured for 10 min at 95°C, 30 min at 60°C, then slowly cooled to room temperature. The hybrids were captured with magnetic beads coated with streptavidin and was washed thrice with $TEN_{100}$, thrice with $TEN_{1000}$ and thrice with 0.2× SSC and 0.1% SDS. The captured fragments were eluted from the biotinylated probes by denaturing at 95°C with sterilized distilled water. The fragments were amplified and cloned into pGEM-T easy vector, and then transformed into *Escherichia coli* competent cell (Sambrook 2006). Clones were identified

**Table 1.** Characterization of 18 polymorphic microsatellite loci of *B. superciliaris*.

| Locus | Accession no. | Repeat motif | Primer (5'–3') | $N_a$ | Size range (bp) | $H_o$ | $H_e$ | $P$ | PIC |
|---|---|---|---|---|---|---|---|---|---|
| BS16 | JQ911676 | $(TG)_{21}$ | F: TGCTAATGAATGCAACCATATTT<br>R: GGGAGTTCAGGGATAATTGC | 5 | 195–238 | 0.9264 | 0.7589 | 0.1726 | 0.695 |
| BS23 | JQ911677 | $(CA)_8(CACG)_6$ | F: AAGGGAGGTTTTGTCAGAT<br>R: CGCATAAGCAGAGTGGCAGT | 6 | 173–230 | 1.0000 | 0.8067 | 0.6981 | 0.758 |
| BS34 | JQ911678 | $(TG)_{22}$ | F: GAGAAGGCAGGCAGAAGACA<br>R: GACAAGCATCTCAGGAAGAGAG | 5 | 146–226 | 0.9325 | 0.7943 | 0.4218 | 0.741 |
| BS37 | JQ911679 | $(GT)_{34}(TC)_{15}$ | F: CTGTCGCTCCAACACACAGT<br>R: CCAGCTGACATGAGCAGAGA | 6 | 220–262 | 0.9724 | 0.8138 | 0.5828 | 0.767 |
| BS38 | JQ911680 | $(GT)_{14}(GT)_7$ | F: CACTGCGAACTATAAGCCCTCT<br>R: CATCGAACCCAAGTTTGACC | 6 | 236–272 | 0.7369 | 0.7429 | 0.6845 | 0.685 |
| BS39 | JQ911681 | $(GT)_{25}$ | F: TGGTCAAGCCTCTTACTGCT<br>R: GGATGTTCCAGCACACCTGA | 8 | 145–228 | 0.8637 | 0.8608 | 0.3268 | 0.823 |
| BS49 | JQ911682 | $(AC)_8(CA)_5$ | F: GGCTGGACACATAGACATTGC<br>R: GTGCCAAACCATGTCAACAG | 3 | 300–326 | 0.3333 | 0.2979 | 0.7031 | 0.272 |
| BS50 | JQ911683 | $(TG)_7(GT)_{31}$ | F: TGCAGATGTGTCATTTGGAC<br>R: GATGAAGGAGAACGCCAAAG | 4 | 196–254 | 0.4167 | 0.3608 | 0.8796 | 0.328 |
| BS52 | JQ911684 | $(AC)_{33}$ | F: GCTTGGGAGAGAGAATGGTG<br>R: TTCGGCAAGTCTGAAAGGTT | 6 | 175–253 | 1.0000 | 0.7819 | 0.4736 | 0.730 |
| BS54 | JQ911685 | $(CA)_{20}(TC)_5$ | F: CGGAGAGCTACGAAACTGCT<br>R: CAGAAATCCCTCTTCCAGTCC | 2 | 242–264 | 0.7500 | 0.4787 | 0.5817 | 0.359 |
| BS62 | JQ911686 | $(TG)_{10}$ | F: CATGTGAAGATGCAGAAATG<br>R: CACCTGAAACCTCCTGCTC | 4 | 153–177 | 0.9583 | 0.7580 | 0.0220 | 0.694 |
| BS65 | JQ911687 | $(TG)_7(TG)_{24}$ | F: AACTGGTGTGTTCTGACAGTGTG<br>R: TGATAAGAGCCCGAACAACC | 8 | 176–224 | 1.0000 | 0.8590 | 0.7456 | 0.824 |
| BS66 | JQ911688 | $(TG)_7$ | F: CAAAGGAGAGGAAGGACAGG<br>R: GCGATCTCTGTATGTGAAAGCA | 5 | 117–169 | 0.7917 | 0.5754 | 0.2304 | 0.510 |
| BS80 | JQ911689 | $(TG)_6$ | F: TTCTGGTGCTGTTTGTCCTTT<br>R: TGTGCAGACTGGTCCTTTCA | 2 | 165–179 | 0.4583 | 0.3608 | 0.0821 | 0.291 |
| BS97 | JQ911690 | $(AC)_{15}(AC)_7$ | F: TGCAGTCCATTCAAAGGTGA<br>R: ATTCAATGCCACTCGGTAGG | 4 | 302–322 | 1.0000 | 0.7021 | 0.2351 | 0.630 |
| BS103 | JQ911691 | $(CA)_6$ | F: TGGCATAAAGGATTCTGTGTCA<br>R: AACTGCGGATCTGTGCTTGT | 3 | 110–124 | 0.9583 | 0.5293 | 0.0002 | 0.404 |
| BS117 | JQ911692 | $(GT)_6(TG)_8$ | F: CAGTGAGGGATGGAACACTAGA<br>R: TGAAATCATGCTATGCCACCT | 4 | 271–295 | 0.8333 | 0.6055 | 0.0193 | 0.545 |
| BS118 | JQ911693 | $(AC)_6$ | F: ACATCACCAGCCTTCAGCTT<br>R: TGGCCTCAGAAATGTGTTCA | 3 | 127–139 | 0.9583 | 0.5293 | 0.0002 | 0.404 |

$N_a$, number of alleles; $H_o$, observed heterozygosity; $H_e$, expected heterozygosity; $P$, exact $P$ value for the HWE test; PIC, polymorphism information content.

through PCR using M13 primers. Positive clones were sequenced on an ABI 3730 automatic sequencer (ABI Biosystem, Foster City, USA).

### *PCR amplification and genotyping*

Ninety positive clones were sequenced and 77 sequences contained repeat motifs. Sixty-five pairs of primers were designed using the online software PRIMER ver. 3 (Rozen and Skaletsky 2000). All the designed loci were tested for polymorphism with 30 individuals. PCR amplifications were performed in 10 $\mu$L reaction mixture which contain 1× PCR buffer, 0.2 mM each dNTP, 2.0 mM $MgCl_2$, 0.4 mM each primer, 0.5 U *Taq* polymerase and about 20 ng template DNA. Thermal cycling conditions were: 94°C for 2 min, followed by 35 cycles of 94°C for 30 s, 58°C for 30 s, 72°C for 40 s, and a final extension at 72°C for 10 min. PCR products were electrophoresed on 8% nondenaturing polyacrylamide gel and visualized by silver staining.

### *Data analysis*

Allele sizes were estimated according to 10-bp ladder molecular size standard. The number of alleles, observed heterozygosity, expected heterozygosity and agreement with Hardy–Weinberg equilibrium (HWE) were calculated using PopGene ver. 1.2 (Raymond and Rousset 1995). Polymorphism information content (PIC) was estimated by PIC-CALC ver. 0.6. All results for multiple tests were subjected to Bonferroni correction.

## Results and discussion

In 65 designed microsatellite loci, 31 loci produced blurred bands, 16 loci were monomorphic and 18 loci were polymorphic in 30 individuals of *B. superciliaris*. The loci of 18 polymorphic microsatellites were deposited in GenBank (JQ911676–JQ911693) and the characteristics are listed in table 1. The number of alleles ranged from 2 to 8. Observed and expected heterozygosity ranged from 0.3333 to 1.000 and from 0.2979 to 0.8608, respectively. These polymorphic loci exhibited relatively high observed heterozygosity. Two loci (BS103 and BS118) significantly deviated from HWE after Bonferroni correction (adjusted $P< 0.004$). The PIC ranged from 0.272 to 0.824. Twelve loci were highly informative (PIC > 0.5), six were moderately informative (0.25 < PIC < 0.5) (Botstein *et al*. 1980). The results suggest that the 18 microsatellite loci developed in this study are mostly highly polymorphic. These polymorphic microsatellite markers can be useful for analyses of population genetics and conservation genetics on *B. superciliaris* and other related Cobitidae species, which will provide a powerful tool for studying correct methods of appropriate management and rational recovery of *B. superciliaris*.

**Acknowledgements**

This work was supported by the National Natural Science Foundation of China (51109091, 51310105036), Hubei Provincial Department of Education (B2013149), Special Fund for Agro-scientific Research in the Public Interest of China (200903048), Fund of China Three Gorges Corporation (0799527). We thank Luo-xin Xia for technical assistance.

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Received 25 October 2013, in revised form 25 November 2013; accepted 28 November 2013
Published on the Web: 8 April 2014