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SHORT NOTES

Development and Characterization of Fifteen Polymorphic Microsatellites for Extremely Endangered Salamander, *Echinotriton chinhaiensis*

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Abstract *Echinotriton chinhaiensis* is a critically endangered salamander and its distribution is restricted to Ningbo, Zhejiang Province of China. In this study, we developed and characterized fifteen polymorphic microsatellite loci for *E. chinhaiensis* from dinucleotide- and tetranucleotide- enriched library. The number of alleles ranges from 4 to 12 with an average of 7.27 alleles per locus. The observed and expected heterozygosities values were from 0.250 to 0.844 and 0.511 to 0.872 with an average value of 0.596 and 0.722; respectively. The polymorphic microsatellite loci described in this paper are useful in the further study on genetic diversity and gene flow, which would be helpful to formulate effective conservation strategies for the *E. chinhaiensis*.

Keywords *Echinotriton chinhaiensis*, polymorphic microsatellite loci, population genetics, conservation biology

Currently, amphibians are declining worldwide, among which many species are becoming extremely endangered (Houlahan *et al.*, 2000). Habitat loss, degradation, and fragmentation are probably the major causes contributing to the decline (Stuart *et al.*, 2004). Assessing the impact of such events requires information of population genetic diversity in a landscape scale. Molecular markers may be useful tools to detect the population structure and gene flow, and can provide useful message for enacting conservation strategies.

Echinotriton chinhaiensis (Urodela: Salamandridae) is one of the extremely endangered amphibians in the world. Its distribution is limited to the city of Ningbo, Zhejiang Province of China and the species was listed as Critically endangered in the IUCN Red List of Threatened Species since 2004 (Liu *et al.*, 2010). Although, a series of conservation measures have been undertaken to protect this species, for example, habitat restoration and artificial propagation, but the natural populations are still declining (Liu *et al.*, 2010). In order to find the mainspring of decline, we isolated and characterized 15 polymorphic microsatellite loci as molecular markers for *E. chinhaiensis*, which could be used in the conservation genetic studies in future.

Microsatellites were isolated from dinucleotide- and tetranucleotide- enriched library and constructed by using a modified method that was defined as fast isolation by AFLP of sequences containing repeats (FIASCO) protocol (Zane *et al.*, 2002). Genome DNA was extracted from two individuals of *E. chinhaiensis* (tail) with DNA extraction kit (TianGen, Inc.). About 4 μg DNA was restricted with *Mse I*, and DNA fractions of 200–1000 bp were recovered from 2% agarose gel by using gel column extraction kit (TianGen, Inc.). The retrieved DNA fragments were then ligated to the AFLP adaptors (5'-TACTCAGGACTCAT-3' /5'-GACGATGAGTCCTGAG-3') (Zane *et al.*, 2002). Subsequently, ligated fragments were hybridized with 5-biotinylated $(ATAG)_8$, $(AC)_{15}$ and $(AG)_{15}$ oligonucleotide probes and then captured by streptavidin-coated magnetic beads (Promega, Inc.). DNA fragments that had microsatellite motifs were isolated and amplified by PCR using Primer-N (5'-GATGAGTCCTGAGTAAN-3') (Zane *et al.*, 2002). The PCR products were first purified with DNA purified kit (Omega, Inc.) and then ligated into the plasmid pMD19-T Vector (Takara, Inc.), following the transformation into DH5α competent cells. Transformed cells grew at 37 °C for 12 h on LB/ampicilin/IPTG/ X-gal plates for blue/white selection. Positive colonies

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were screened by PCR amplification with 3 primers for checking the presence of microsatellite motifs. Clones that yielded two or more bands by PCR were regarded as ones containing microsatellite motifs fractions and were be sequenced on an automated ABI 3730 DNA sequencer. In total, 145 positive clones were obtained and sequenced, where microsatellite repeat motifs were found in 96 clones.

By using the software Premier 5.0, 70 pairs of primers were designed according to the flanking sequences. These primers were tested by temp-gradient PCR amplification. The primer pairs which could yield consistent specific PCR products were assessed by polymorphism examination. DNAs from 32 tail samples of *E. chinhaiensis*, which were collected from Ningbo in Zhejiang Province of China during 2010, were used as PCR templates to screen allelic polymorphism for each microsatellite loci. PCR was carried out in 25 μl reaction system, including 30–50 ng template DNA, 12.5 μl 2×PCR MasterMix (TianGen, Inc.), 0.5 μM of each primer (the forward primer was fluorescence-labelled with FAM, HEX or TAMRA). PCR reactions were performed in PTC-200 (Bio-Rad) using the following profile: 94 °C for 5 min, followed by 35 cycles of 30s at 94 °C, 30s at optimized annealing temperatures (*Ta*), and 1 min at 72 °C, then for a final extension at 72 °C for 10 min. PCR products were analyzed on a ABI PrismTM310 Genetic Analyzer. The Genetix software was used to determine the number of alleles (*Na*), observed (H_O) and expected heterozygosities (H_E) per locus. Micro-Checker version 2.2.3 was performed to assess null alleles (van Oosterhout *et al.*, 2004), and Genepop version 4.0 was used for tests of significant deviation from Hardy-Weinberg equilibrium (*HWE*) and linkage disequilibrius (*LD*).

A total of fifteen pairs of primers could yield consistent specific PCR products with polymorphic alleles. The sequences of primers, repeat motifs, *Ta*, PCR product size, number of alleles, and heterozygosity for these loci were shown in Table 1. The number of alleles per locus (*Na*) ranged from 4 to 12 with an average of 7.27. The

Table 1 Primer sequences and summary polymorphism information for 15 microsatellite loci characterized from *Echinotriton chinhaiensis* (n = 32)

Locus	Primer sequence (5'-3')	Repeat motif	*Ta* (°C)	Size (bp)	*Na*	H_O/H_E	Accession No.
CIBEC-01	F: CAGATGTAAGACAGCACCCT R: TGCCCACGCCTAAAT	$(CA)_{19}$	58	198–212	5	0.250/0.511	JF411826
CIBEC-02	F: ATGCCACATTGGACAGAC R: GAGACGACCTGCTAGATGA	$(AGAT)_{15}$	57	317–341	7	0.625/0.695	JF411827
CIBEC-03	F: CGCTCAAAAGAAAATGG R: TTAGCAATGGTTAGCAGTAG	$(ATCT)_{13}$	55	256–272	6	0.438/0.651	JF411828
CIBEC-04	F: GGTTACTGCTGCTTTGC R: CGATTGAATGAACTTGGA	$(GT)_{15}$	58.6	130–166	10	0.719/0.745	JF411829
CIBEC-05	F: AAGAGTTGCACCCCTATG R: GATGGTATGTGGCTGTCC	$(GATA)_{14}$	60.5	114–126	4	0.531/0.570	JF411830
CIBEC-06	F: ATGAAAGGCTGAGTGG R: GTGACATCCGATAGCAC	$(GT)_{16}$	60	259–295	12	0.344/0.678	JF411831
CIBEC-07	F: GATGTTGCTCAATCCTTCC R: GCCAGTAACCACCAGTATC	$(TG)_{14}$	60.5	141–159	9	0.594/0.714	JF411832
CIBEC-08	F: CTACAGCCTCACCTTCTG R: TAATGCTCTGGACGTTTC	$(TCTA)_{15}$	57	208–228	8	0.750/0.781	JF411833
CIBEC-09	F: CAGCGGTAGGAAGTGCTAT R: GGTTGCATGAGCAGATAC	$(CTAT)_{10}$	57	270–294	7	0.500/0.709	JF411834
CIBEC-10	F: GTGGGTAGCCTGGACAA R: ACCGAACAAGAAATGGG	$(AC)_{10}(TC)_{15}$	61	304–346	10	0.844/0.872	JF714877
CIBEC-11	F: ACATGGTGGGACATACACT R: GAAGCAGGATTGATGAGAT	$(TC)_5N(TC)_5N(TC)_{10}$	61	258–282	7	0.719/0.777	JF714878
CIBEC-12	F: AGATGGTCTACAAGCAAATG R: TCGGACTGATAGGAAAGG	$(AG)_{12}$	61	246–260	4	0.406/0.677	JF714879
CIBEC-13	F: CTCCTCTCCCAACCCAAT R: AAGGACATAGGACCAAGATC	$(TC)_{20}$	63.5	196–118	7	0.781/0.866	JF714880
CIBEC-14	F: GAGTTGCACCCCTATG R: TGGTATGTGGCTGTCC	$(ATAG)_{12}$	60	101–133	6	0.688/0.772	JF714881
CIBEC-15	F: TTTACCAATCCAAAGAAGC R: TGCAGAAATTATACGCAAT	$(TC)_{13}N_3(TC)_{23}$	59.3	304–328	7	0.750/0.805	JF714882

Ta Annealing temperature, *Na* Number of alleles, H_E Expected heterozygosity, H_O Observed heterozygosity

observed and expected heterozygosities (H_O and H_E) ranged from 0.250 to 0.844 and from 0.511 to 0.872, with the respective average of 0.596 and 0.722. No significant linkage disequilibrium was observed among any microsatellite loci. However, six loci (CIBEC-01, CIBEC-02, CIBEC-03, CIBEC-06, CIBEC-09 and CIBEC-12) showed significant deviations from Hardy-Weinberg equilibrium ($P < 0.01$). The null allele was probably existed in the loci CIBEC-12. In summary, the fifteen polymorphic microsatellite loci described in this study are expected to be useful in the further studies of genetic diversity and gene flow, which would be helpful to provide conservation strategies for the *E. chinhaiensis*.

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