Development and characterization of 33 SNP markers in a critically endangered *Hynobius amjiensis* using ddRAD sequencing

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Abstract

Hynobius amjiensis was considered as one of the 29 most threatened amphibian in China. To effectively conserve, manage and recover the population of this critically endangered species, 33 single nucleotide polymorphism (SNP) markers were developed using double digest restriction-site associated DNA (ddRAD) sequencing. The minor allele frequency per locus ranged from 0.0161 to 0.5000. The observed heterozygosity and expected heterozygosity varied from 0.0323 to 0.6667 (average 0.3303) and from 0.0317 to 0.5000 (average 0.2772), respectively. The inbreeding coefficient value ranged between − 0.3315 and 0.0000. No significant deviation from Hardy-Weinberg equlibrium ($P > 0.05$) were found in all loci. These novel SNPs will be helpful for the population genetic assessment and conservation of *H. amjiensis*.

Main Text

Anji salamander (*Hynobius amjiensis*) was an endemic species in China. Due to limited distribution and small population size, it was listed as a critically endangered species in the International Union for Conservation of Nature (IUCN) Red List of Threatened Species (Gu and Lau 2004). As one of the 29 most threatened amphibian in China (Zhao 1998), *H. amjiensis* attracted considerable attention. In recent years, a series of conservation activities which focused on this species were carried out. First, a specific National Natural Reserve was constructed to conserve the species in situ. Additionally, as a key species in national biodiversity survey, it was investigated annually to understand the distribution and population size extensively and deeply by the State Forestry Administration of China from 2012 (Yang et al. 2016). Finally, captive breeding and reintroduction were implemented to try to increase the population size from 2008. However, the basic researches, such as life history, ecology and especially population genetics, were fell far behind the conservation practice. Therefore, to formulate appropriate conservation strategies, it was urgent to perform population genetic studies on this species.

Single nucleotide polymorphism (SNP) had become the most effective molecular marker in conservation genomic researches since its wide genome distribution and high level of polymorphism (Liu et al. 2018). Here, 33 novel SNP loci were developed using double digest restriction-site associated DNA sequencing (ddRAD-seq) in *H. amjiensis.*

Thirty-four toe samples of *H. amjiensis* were collected from Qingliangfeng National Natural Reverve. Genomic DNA was extracted using a standard proteinase K and phenol-chloroform approach (Sambrook et al. 1989). DdRAD library was prepared and constructed for paired-end high-throughput sequencing on an Illumina NovaSeq 6000 (Illumina, USA) by Annoroad Gene Technology, China. A total of 77,016 putative SNPs were obtained, of which 100 SNPs were randomly selected for primer designing using software Primer 3 plus (Untergasser et al 2012).

PCR amplifications were performed in a total volume of 20 μL, containing forward and reverse primers at 10 μM of each primer, 10 μL 2×Taq Plus Master Mix (Vazyme Biotech, China), and 50-100 ng template DNA. The thermal cycling procedure was applied with an initial pre-denaturation step at 95 ℃ for 3 min, followed by 30 cycles at 95 ℃ for 15 s, the annealing temperature at 65 ℃ for 20 s, and elongation at 72 ℃ for 15 s; a final extension at 72 ℃ for 10 min. The amplicons for each sample were recycled and purified by 2.0 % agarose gel electrophoresis and verified using Sanger sequencing on ABI 3730XL (Thermo Fisher Scientific, US).

Several descriptive statistics were calculated. Specifically, the minor allele frequency (MAF) and P value representing the deviations from Hardy-Weinberg equilibrium (HWE) for each locus were tested using VCFtools (Danecek et al. 2011). The observed heterozygosity (H_O) and expected heterozygosity (H_E) were calculated using PLINK (Chang et al. 2015). The inbreeding coefficient index (F_{is}) was calculated using Genepop V4.7 (Rousset 2008).

In total, thirty-three SNP loci were identified from 33 primer pairs (Table 1). The minor allele frequency per locus ranged from 0.0161 to 0.5000. The observed heterozygosity and expected heterozygosity varied from 0.0323 to 0.6667 (average 0.3303) and from 0.0317 to 0.5000 (average 0.2772), respectively. The inbreeding coefficient value ranged between -0.3315 and 0.0000. No loci showed significant deviation from Hardy-Weinberg equilibrium (P > 0.05). These novel SNP markers will contribute to the understanding of population genetic structure and be useful for the population conservation of *H. amjiensis*.

Declarations

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This research was approved by the Ethics Committee of Zhejiang Normal University and it was conducted under Law of the People's Republic of China on the Protection of Wildlife (August 28, 2004).

Author contribution

All authors contributed to the study conception and design. Material preparation were performed by Yu Wang, Xia Kan and Shuangshuang Shan, data collection and analysis were performed by Xia Kan, Guoliang Wang, Lili Zhang, Xin Zhao, Guiming Liu, Chen Shao and Yu Wang. The first draft of the manuscript was written by Xia Kan. All authors read and approved the final manuscript, and consented to publish.

Data Availability

The datasets generated during and analysed during the current study are available from the corresponding author on reasonable request.

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Tables

Table 1 Characteristics of 33 SNP loci developed for *H.amjiensis*

Primer ID	Primer sequence (5′–3′)	Size (bp)	SNP position	SNP type	MAF	H_O	H_E	F_{is}	HWEP
Ham_snp01	F:CCAGAATTCACCCTGGGGAG R:GCCCTGTGAGATCTGTGCTG	106	54	A/C	0.0833	0.1667	0.1528	-0.0741	NS
Ham_snp02	F:CCAGAATTCACCCTGGGGAG R:GCCCTGTGAGATCTGTGCTG	127	43	T/G	0.0690	0.1379	0.1284	-0.0566	NS
Ham_snp03	F:CCAGAATTCACCCTGGGGAG R:GCCCTGTGAGATCTGTGCTG	104	65	C/T	0.0833	0.1667	0.1528	-0.0741	NS
Ham_snp04	F:CCAGAATTCACCCTGGGGAG R:GCCCTGTGAGATCTGTGCTG	125	96	T/G	0.2241	0.3793	0.3478	-0.0732	NS
Ham_snp05	F:CCAGAATTCACCCTGGGGAG R:GCCCTGTGAGATCTGTGCTG	119	46	C/T	0.5000	0.6667	0.5000	-0.3182	NS
Ham_snp06	F:CCAGAATTCACCCTGGGGAG R:GCCCTGTGAGATCTGTGCTG	105	51	A/C	0.0667	0.1333	0.1244	-0.0545	NS
Ham_snp07	F:GCTCTGCCACCCCATCTTC R:CCGCCAATGACATCAGGGTA	101	79	T/C	0.1333	0.2667	0.2311	-0.1373	NS
Ham_snp08	F:GTGGCAATAACCTCAGGGGA R:GATGCGGACCGATTGTTTGG	110	55	G/T	0.3621	0.5862	0.4620	-0.2526	NS
Ham_snp09	F:ATACAACAAGCCCAACACGC R:GCGTGTTGCTGGTTATTCCC	134	74	A/C	0.1833	0.3667	0.2994	-0.2083	NS
Ham_snp10	F:ACAAAAGGTGGGGGATGTCC R:GCCCTGTGAGATCTGTGCTG	119	55	C/T	0.1964	0.3929	0.3157	-0.2273	NS

Ham_snp11	F:CTAGGGCCGGAGATGAGAGA R:CCTTGGAAATGGAGGGCTGT	146	61	C/T	0.1667	0.3333	0.2778	-0.1837	NS
Ham_snp12	F:CTAGGGCCGGAGATGAGAGA R:CCTTGGAAATGGAGGGCTGT	126	43	T/C	0.1786	0.3571	0.2934	-0.2000	NS
Ham_snp13	F:CTAGGGCCGGAGATGAGAGA R:CCTTGGAAATGGAGGGCTGT	112	85	T/A	0.2667	0.4000	0.3911	-0.0058	NS
Ham_snp14	F:TGGCGCGTTACCTGATAGAC R:GGACACATCGATCCGTCTCA	104	62	G/T	0.3667	0.5333	0.4644	-0.1317	NS
Ham_snp15	F:GGATAGGTGCTCTAAGCCGG R:AGACTCTACTGGCCCAGGAT	116	59	A/G	0.0179	0.0357	0.0351	0.0000	NS
Ham_snp16	F:CCCCTCCTTTTCCCAACCTC R:AGAGTCCCGGGAAGAGGAAT	103	53	A/C	0.0167	0.0333	0.0328	0.0000	NS
Ham_snp17	F:AATCAGGAGCCGCACATGTG R:AAATGCGTTCGCTCTCCTCC	101	36	C/A	0.1667	0.3333	0.2778	-0.1837	NS
Ham_snp18	F:TAGATGATGGTGCGCAGTGG R:GACCCATCGATCCGTCTCAA	119	36	T/C	0.0167	0.0333	0.0328	0.0000	NS
Ham_snp19	F:CACGCTGTACAAAGTGCACC R:CCAACACCCTCAGCACCATA	111	67	A/G	0.1500	0.3000	0.2550	-0.1600	NS
Ham_snp20	F:GGGTAAATTGACCGCGCTTT R:CGACGACATCTGGAACCCTG	120	80	T/C	0.1452	0.2903	0.2482	-0.1538	NS
Ham_snp21	F:GTCCCGCGGGTCTTCAAA R:CAGCCTTCCCTCACTCCTTG	104	33	T/C	0.1071	0.2143	0.1913	-0.1020	NS
Ham_snp22	F:TGGCAATCAGAGAACACGCT R:GGGCAATAGGCTGAAGCTGA	101	76	T/C	0.2258	0.3871	0.3496	-0.0909	NS
Ham_snp23	F:GGCCCTTTAAGAGAGCAGCA R:GGTAGGGAGCATGGACATGG	102	29	A/G	0.0161	0.0323	0.0317	0.0000	NS
Ham_snp24	F:CACGTCGTGGATGAACGGG R:GCACATCAGACTGCGCCA	101	21	T/A	0.4500	0.5667	0.4950	-0.1281	NS
Ham_snp25	F:CCGAGACGCTTACTTCCACA R:ACTCTGGGAGCAGCATTGAG	123	53	G/A	0.2241	0.4483	0.3478	-0.2727	NS
Ham_snp26	F:ATCCTCCGGGGGTAAGACC R:ACGACAATGTTCCTGGGGTC	127	53	A/C	0.3571	0.5000	0.4592	-0.0708	NS
Ham_snp27	F:ATCCTCCGGGGGTAAGACC R:ACGACAATGTTCCTGGGGTC	105	31	A/G	0.1333	0.2667	0.2311	-0.1373	NS
Ham_snp28	F:ATCCTCCGGGGGTAAGACC R:ACGACAATGTTCCTGGGGTC	104	23	A/G	0.2500	0.5000	0.3750	-0.3182	NS
Ham_snp29	F:ATCCTCCGGGGGTAAGACC R:ACGACAATGTTCCTGGGGTC	109	57	T/G	0.1250	0.2500	0.2188	-0.1250	NS
Ham_snp30	F:ATCCTCCGGGGGTAAGACC R:ACGACAATGTTCCTGGGGTC	110	68	A/C	0.1250	0.2500	0.2188	-0.1250	NS
Ham_snp31	F:ATCCTCCGGGGGTAAGACC R:ACGACAATGTTCCTGGGGTC	123	65	C/T	0.1786	0.3571	0.2934	-0.2000	NS
Ham_snp32	F:ATCCTCCGGGGGTAAGACC R:ACGACAATGTTCCTGGGGTC	106	48	G/A	0.3929	0.6429	0.4770	-0.3315	NS
Ham_snp33	F:ATCCTCCGGGGGTAAGACC R:ACGACAATGTTCCTGGGGTC	104	39	T/C	0.3214	0.5714	0.4362	-0.2934	NS

MAF: minor allele frequency, HO: observed heterozygosity, HE: expected heterozygosity, Fis: inbreeding coefficient, HWEP: results for Hardy–Weinberg equilibrium test, NS: non-significant