



## Development of 18 microsatellite loci for the freshwater snail *Bellamya aeruginosa* (Mollusca, Gastropoda)

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**ABSTRACT.** Although it is a major freshwater gastropod species, genetic diversity of *Bellamya aeruginosa* was completely unknown. Eighteen microsatellite loci were isolated and characterized from (AC)<sub>15</sub>-enriched genomic libraries of the freshwater snail *B. aeruginosa*. Most of the 18 loci were successfully amplified and high polymorphic information content values were found, ranging from 0.244 to 0.792 (mean 0.541). The number of alleles per locus ranged from 5 to 13 (mean 8.8), the expected heterozygosity varied from 0.347 to 0.950 (mean 0.815) and the observed heterozygosity varied from 0.087 to 0.782 (mean 0.431). Eight loci showed significant deviation from Hardy-Weinberg equilibrium after Bonferroni's correction and no significant genotypic linkage disequilibrium was detected between most locus pairs, except for TXH79-TXH97 and TXH113-TXH121. These 18 polymorphic microsatellite loci should be useful for population genetics analysis and species identification of *Bellamya*.

**Key words:** *Bellamya aeruginosa*; Microsatellites; Polymorphism; Heterozygote deficiencies

## INTRODUCTION

As an important genus of freshwater gastropods, *Bellamya* plays a significant role in the ecosystem (Lamberti et al., 1989; Han et al., 2010) and also has high medicinal and nutritional value (Prabhakar and Roy, 2009; Li et al., 2010). However, its natural resources are under severe threat because of habitat loss and fragmentation. Many of the species of this genus have been named endangered species, for example, *Bellamya limnophila*, *B. lithophaga*, *B. manhungensis*, *B. papillapicala*, and *B. smith* [[http://www.baohu.Org/csis\\_search/search1.php](http://www.baohu.Org/csis_search/search1.php)]. However, *B. aeruginosa* is still widely distributed in many freshwater ecosystems and is frequently the dominant species, as in the past. *B. aeruginosa* is known to exhibit gonochorism and ovoviviparity; however, investigations on its resources and genetic characteristics are scarce. We characterized 18 microsatellite markers for studying their population structure and genetic signatures, which may provide a basis for further studies on the population genetics of *B. aeruginosa* and other *Bellamya* species.

## MATERIAL AND METHODS

Microsatellite enrichment and screening were performed using DNA from 1 individual according to the method (FIASCO) described by Zane et al. (2002), with some modifications. Total genomic DNA was extracted using traditional phenol-chloroform extraction protocols, as described by Liao et al. (2007). Isolation of microsatellite loci from an enriched library was performed as recommended by Wang et al. (2010), by using 5'-biotin-labeled microsatellite oligoprobes [AC]<sub>15</sub> and streptavidin MagneSphere paramagnetic particles (Promega). Capture fragments were ligated to pGEM-T Easy clone vector (Promega) and transformed into JM109 competent cells (Promega). We screened 453 clones, of which 321 afforded positive signals; the results were confirmed by PCR amplification using primer 1 (T7, SP6), primer 2 (T7, SSR01 ([AC]<sub>14</sub>N)), and primer 3 (SP6, SSR01); primers 2 and 3 were expected to yield amplified fragments that would be shorter than that yielded by primer 1.

Seventy positive clones were sequenced using an ABI PRISM 3730 sequencer. Sixty-five clones were sequenced successfully, and 57 sequences contained repeated motifs and flanking regions, allowing for the designing of PCR primers (designed using the Primer Premier 5.0 software). We chose 40 loci with a high number of repeated motifs, and 18 primer pairs were easily optimized and scored by independent PCR amplifications by using 45 unrelated individuals (collected from Taihu Lake, Yixing city, China) for each microsatellite locus. The PCRs (10 µL) were performed with 1X PCR buffer (100 mM Tris-HCl, pH 8.3), 500 mM KCl, 20-40 ng genomic DNA, 0.5 µM for each primer, 200 µM each dNTP, 1.5 mM MgCl<sub>2</sub>, and 0.25 U *Taq* DNA polymerase (TaKaRa). Amplification was performed using a TProfessional Thermocycler (Biometra) under the following conditions: 94°C for 5 min, 35 cycles at 94°C for 45 s, annealing temperature (46-57°C) for 30 s (Table 1), 72°C for 30 s, and a final extension at 72°C for 10 min. PCR products were separated on 10% denaturing polyacrylamide gel, and allele size was determined using Pbr322 DNA/*MspI* (TIANGEN) by silver staining. The number of alleles, observed heterozygosities ( $H_o$ ), and expected heterozygosities ( $H_e$ ) were estimated by POPGEN 32 (Yeh and Boyle 1997). The system was used to measure heterozygote deficiency or excess and also assess the deviation from Hardy-Weinberg equilibrium (HWE) and linkage disequilibrium between pairs of loci. Polymorphic information content (PIC) value was estimated using PowerMarker V3.25 (Liu and Muse 2005).

**Table 1.** Characteristics of 18 microsatellite loci in *B. aeruginosa*.

Locus	Primer sequence (5'-3')	Ta (°C)	Repeat motif	$N_A$	Size range (bp)	$H_O$	$H_E$	PIC	HWE P value	Genbank accession No.
TXH8	F: CCAGATAGAGCGATGACCAC R: AAGCAATGTACCCGAGAACC	56	(TG) <sub>40</sub>	12	132-180	0.309	0.613	0.695	0.129	JN555764
TXH12	F: AGGCCTCAGCTTGAATCCCTA R: CGGCTCCCATTTTGACATTG	55	(CA) <sub>19</sub>	7	189-225	0.782	0.841	0.482	0.002*	JN555765
TXH18	F: TGTGACTAAGTGTGTTGTCATG R: CTACCAAGGTGCTTGGTGTG	53	(GCGT) <sub>8</sub> (GT) <sub>36</sub>	11	105-174	0.286	0.668	0.544	0.003	JN555768
TXH21	F: CACGAACAGTGAATAGGACTGG R: TGCATGTGTTCTATGTTGTTGC	52	(AC) <sub>26</sub>	9	196-214	0.573	0.828	0.287	0.078	JN555771
TXH26	F: TATCCTCCCCTGACTGAACTGG R: CCGTGAATCCCGACACCACAAC	57	(CA) <sub>30</sub>	10	260-304	0.433	0.805	0.265	0.053	JN555774
TXH28	F: GGTGATTCAAGGCTCGTCA R: ACCTAATGACAGCGCCGATA	53	(GC) <sub>7</sub> ACATG(CA) <sub>8</sub> T (AC) <sub>7</sub> (TC) <sub>7</sub> ACT(CA) <sub>9</sub>	5	101-157	0.225	0.608	0.381	0.001*	JN555775
TXH30	F: CACATAGAAGGTCACACGT R: GAATTCAAAACCTCAGACAACGG	51	(CA) <sub>30</sub>	8	273-307	0.233	0.347	0.513	0.004	JN555777
TXH36	F: CGCTTTCTCAATGATGACAGTC R: CGAGACATATGAGGCTGAAGG	55	(GT) <sub>10</sub> (GCGT) <sub>2</sub> (GT) <sub>20</sub> A (GT) <sub>8</sub>	6	179-209	0.087	0.750	0.605	0.001*	JN555782
TXH65	F: TGTGACTAAGTGTGTTGTCATG R: CTACCAAGGTGCTTGGTGTG	52	(AC) <sub>12</sub>	11	190-226	0.748	0.865	0.441	0.069	JN580068
TXH79	F: TCCTGATTCAAGGACGTTGTGC R: GACTGGTGCTGGCTGTAGTGC	55	(GT) <sub>43</sub>	8	273-410	0.613	0.839	0.718	0.000*	JN555804
TXH88	F: GGTTACCAAAGCAGAGGGGTG R: TGCAAGCTCCAGTGGTGACG	57	(TG) <sub>35</sub>	7	118-134	0.425	0.784	0.744	0.000*	JN555810
TXH91	F: ATATCAACAAGGCGCCCGC R: TCTCAACGCACGCATCCCTAC	55	(CA) <sub>27</sub>	13	102-135	0.763	0.950	0.728	0.034	JN555811
TXH94	F: TAAATGACTTCAAGTGCCAC R: ACCATCCATTTAGCCTACACAC	55	(GT) <sub>17</sub> A(TG) <sub>6</sub> TA(TG) <sub>11</sub>	7	115-138	0.529	0.869	0.244	0.456	JN555813
TXH97	F: GTACGTGCACGCCTAGATTTC R: TATTAGTACACCCACTCACTCTC	54	(TG) <sub>47</sub>	11	107-157	0.446	0.902	0.382	0.374	JN555814
TXH98	F: GACTGCTGCCGATCAACACC R: GTCTAGCCATCTCAGCATGTC	55	(GTG) <sub>3</sub> (TG) <sub>12</sub> A(GT) <sub>35</sub>	5	170-260	0.335	0.879	0.657	0.000*	JN555815
TXH101	F: TACAAATGAAGTGTGAAAATC R: CAAGACTCATTTCTCCAAGACA	46	(TG) <sub>11</sub> A(GT) <sub>26</sub>	13	113-163	0.182	0.826	0.792	0.000*	JN555818
TXH113	F: CAAGCATGGATGCAGAACTC R: CTCGTTGGTCCGATACAACC	53	(GT) <sub>27</sub>	7	174-248	0.529	0.896	0.674	0.000*	JN555824
TXH121	F: TACAGTGAGATAGGGAATCAGC R: ATAGTGGGTAGCGTCCTTG	54	(TG) <sub>40</sub>	9	145-206	0.264	0.491	0.587	0.036	JN555829
Mean				8.8		0.431	0.815	0.541		

Ta = annealing temperature of primer pairs;  $N_A$  = number of alleles;  $H_O$  = observed heterozygosity;  $H_E$  = expected heterozygosity. Significant deviation from HWE is indicated with asterisks ( $P < 0.003$ , Bonferroni's correction).

## RESULTS AND DISCUSSION

All the 18 primer pairs showed very efficient polymorphism in the population of *B. aeruginosa*. The number of alleles and  $H_E$  and  $H_O$  are presented in Table 1. Allele number per locus ranged from 5 to 13, with a mean of 8.8. The  $H_E$  and  $H_O$  values were 0.347-0.950 and 0.087 to 0.782, with an average of 0.815 and 0.431, respectively. All the 18 loci were amplified successfully and had high PIC values ranging from 0.244 to 0.792 (average, 0.541), thereby indicating that the 18 microsatellite loci represent efficient tools for studies on population genetics of *B. aeruginosa*.

Significant heterozygote deficiencies were found in most loci, except for TXH-65 ( $F_{IS} = -0.019$ ). The deficiencies were frequently explained by inbreeding, null alleles, population substructure, bottleneck effects, Wahlund effects, or low gene flow (Zouros, 1987; Charbonnel et al., 2002; Fauvelot et al., 2009). However, in many mollusks, the null allele was the most likely reason for heterozygote deficiency (Kennington et al., 2008;

Nicot et al., 2009; Xiao et al., 2011). Large allele dropout and evidence of stuttering were absent, but using Bonferroni's confidence interval, we determined that null alleles were present in most loci, except TXH66 and TXH113, in the studied population (Van Oosterhout et al., 2004). Eight loci showed significant deviation from the HWE after Bonferroni's correction ( $P < 0.003$ ) (Table 1), which would be closely related to the significant heterozygote deficiencies detected at all loci, especially, the existence of null alleles. In addition, no significant genotypic linkage disequilibrium was detected between most of the locus pairs ( $P < 0.003$ , Bonferroni's correction), except for 2 pairs (TXH79-TXH97 and TXH113-SSR121). Certainly, all these findings should be verified in further research.

We performed a forward investigation on the cross-species amplification of the 18 loci, 13 of which were amplified successfully and had high polymorphism in *B. purificata* and *B. quadrata*. The 5 loci that were not amplified were TXH12, TXH26, TXH30, TXH36, and TXH101; however, they showed successful amplification in *B. angularia* and *B. lapillorm* and moderate polymorphism.

In conclusion, these 18 polymorphic microsatellite loci isolated from *B. aeruginosa* will be important for further characterizing the population genetics and for identification of the species of this poorly studied genus. Genetic analysis of the species of the genus *Bellamya* may provide a strong basis for the development of conservation programs for the vulnerable and threatened species in *Bellamya*.

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