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Novel polymorphic microsatellite markers for *Bellamya* and their application in population genetics of three species

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ABSTRACT. *Bellamya* is a widely distributed freshwater snail genus in China; however, its genetic diversity is completely unknown. Sixty-five novel microsatellite loci were isolated and characterized from a microsatellite-enriched library of *Bellamya aeruginosa* genomic DNA. Most of the 65 loci were successfully amplified. We found high polymorphic information content values for these loci, ranging from 0.235 to 0.892. There were 3 to 12 alleles per locus, and the H_E and H_O varied from 0.425 to 0.953 and 0.026 to 1.000, respectively. Fifteen loci deviated significantly from Hardy-Weinberg equilibrium after Bonferroni's correction. All 65 SSR markers were tested in an additional five *Bellamya* species, and 96.9% of the 325 locus/taxon combinations tested resulted in cross-species amplification. Seven polymorphic microsatellite markers were randomly selected for comparison among nine populations of three species. All populations had moderate to high genetic diversity. In genetic distance-based cluster analysis, the populations of *B. aeruginosa* and *B. dispiralis* formed species-based clusters, whereas populations of *B. angularia* did

not. The three examined *Bellamya* species could be differentiated using SSR markers. These microsatellite loci should be useful for genetic diversity analysis, analysis of phylogenetic relationship, and species delimitation of *Bellamya*.

Key words: *Bellamya*; Microsatellite markers; Genetic diversity; Population genetics

INTRODUCTION

Bellamya spp are freshwater snails that play a significant role in ecosystem services (Han et al., 2010). *Bellamya* are important pollution bioindicators for freshwater systems and represent an ideal system for the study of environmental toxicology (Chen et al., 2005; Li et al., 2012). These snails are also reported to be widespread invasive species in North American and African lakes (Sengupta et al., 2009; Solomon et al., 2010). Because *Bellamya* is a species-rich genus of primarily sessile freshwater snails that is widely distributed across Southeast Asia, India and Africa, it is a suitable model system for the study of population genetics and biological evolution (Sengupta et al., 2009; Schultheiβ et al., 2011; Van Bocxlaer and Hunt, 2013).

The *Bellamya* genus has a wide distribution across large parts of China. However, its natural habitats are under severe threat due to habitat fragmentation and loss. Many of the species of this genus have been named endangered species, including *B. limnophila*, *B. lithophaga*, *B. manhungensis*, *B. papillapicala*, and *B. smith* (Gu et al., 2012a).

Microsatellite markers are a useful marker system for the analysis of the population genetics of many snail species (Arthofer et al., 2011; Pálsson et al., 2014; Rivero-Wendt et al., 2014). Gu et al. (2012a,b) first developed SSR DNA markers for *B. aeruginosa*. In order to investigate the genetic diversity of *Bellamya* and to better understand their population genetics, it is important to develop additional polymorphic molecular markers. In this study, we isolated a total of 65 novel polymorphic SSR markers for *B. aeruginosa* and performed cross-species amplification in another five *Bellamya* species. Furthermore, seven polymorphic SSR markers isolated in this study were used to compare genetic characterizations and phylogenetic relationships of three *Bellamya* species from different locations.

MATERIAL AND METHODS

Sampling and DNA extraction

Bellamya populations were collected from different aquatorium in China. A total of 263 individuals were obtained from three local populations: *B. aeruginosa* were collected from Weihe River (p1, N = 30) in Xinxiang City, Liangzi Lake (p2, N = 29) in Wuhan City, and Danjiangkou Reservoir (p3, N = 31) in Nanyang City; *B. angularis* populations were obtained from Weihe River (p1', N = 29) in Xinxiang City, Banqiao Reservoir (p2', N = 30) in Zhumadian City, and Huaihe River (p3', N = 30) in Xinyang City; and *B. dispiralis* populations were collected from Weihe River (p1", N = 29), Huaihe River (p2", N = 29), and Danjiangkou Reservoir (p3", N = 26; Figure 1). Other three *Bellamya* species (*B. quadrata*, *B. purificata*, and *B. lapillorm*) were mostly collected from Liangzi Lake in Wuhan City.

Species were identified using the keys created by Zhang and Liu (1960). The adductor muscle tissue of *Bellamya* was preserved in absolute ethyl alcohol and stored at -20°C prior to DNA extraction. Genomic DNA was extracted using DNeasy® Blood and Tissue Kits (QIAGEN, Sangon Biotech, Shanghai Co., Ltd., China) following the manufacturer protocol and stored at -20°C until further use.

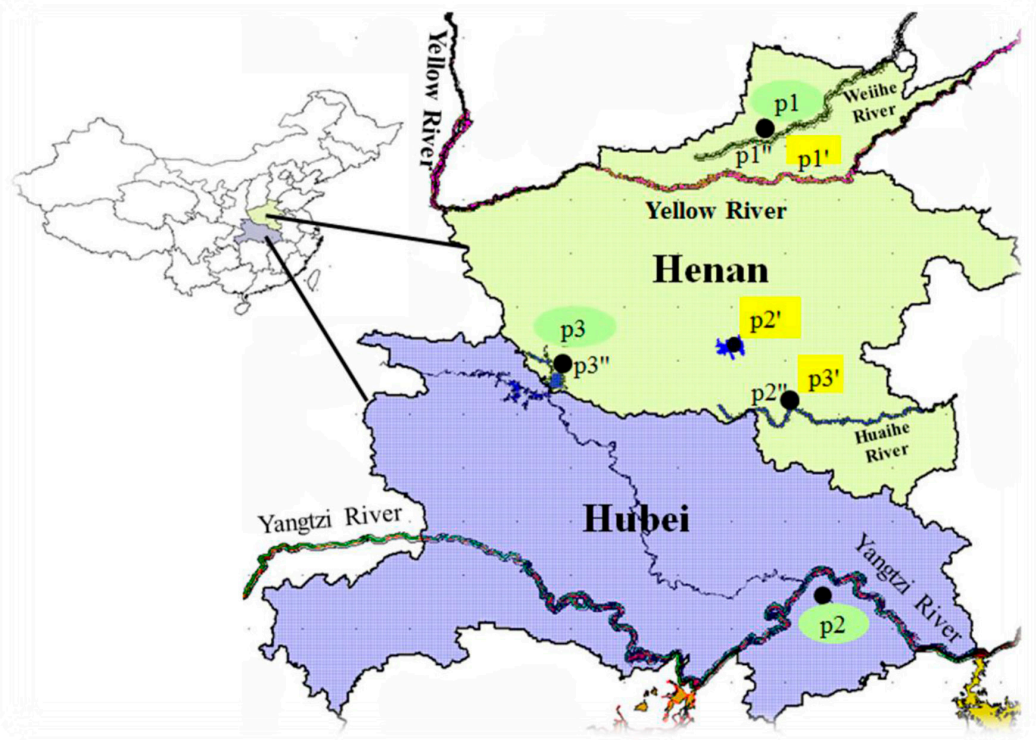

Figure 1. Map of sampling locations in lakes, rivers and reservoirs in Henan and Hubei Province in China.

Development of microsatellite markers

Microsatellite loci were isolated from an enriched library according to the hybridization-based capture method described by Zane et al. (2002) and recommended by Gu et al. (2012a,b). High-quality genomic DNA from individual *B. aeruginosa* was fragmented using the restriction enzyme *Mse*I (BioLabs, USA), and then double-stranded DNA linkers specifically designed for this application (5'-GACGATGAGTCCTGAG-3' and 5'-TACTCAGGACTCAT-3') were added into the ends. The PCR products were size-selected to preferentially obtain small fragments (300-800 bp), which were subsequently hybridized to a mixture of 5'-biotinylated oligoprobes: $(TC)_{20}$, $(CAG)_{15}$, $(ATG)_{15}$, $(CCT)_{8}$, and $(GATA)_{8}$. The oligoprobes were then captured by streptavidin MagneSphere paramagnetic particles (Promega). The enriched DNA fragments were amplified, cloned and sequenced. Finally, SSRs were screened using the SSRHUNTER program (Li and Wan, 2005). A minimum repeat

length criterion of 20 bp was used for selection. Primers flanking SSRs were designed using the PRIMER PREMIER 5.0 program (PREMIER Biosoft International, USA).

Characterization of microsatellite loci

PCR (10 μL) was performed with 1X PCR buffer (100 mM Tris-HCl, pH 8.3), 500 mM KCl, 20 ng genomic DNA, 0.5 μM of each primer, 200 μM of each dNTP, 1.5 mM $MgCl_2$, and 0.25 U Taq DNA polymerase (TaKaRa, China). Amplification was performed using a TProfessional Thermocycler (Biometra) under the following conditions: initial denaturation at 94°C for 4 min, followed by 30 cycles of denaturation at 94°C for 45 s, annealing at 50°-57°C for 30 s (Table 1), and extension at 72°C for 30 s. PCR products were separated on a 10% denaturing polyacrylamide gel, and allele size was determined using pBR322 DNA/*Msp*I (TIANGEN) and silver staining. All SSR markers were screened using 36 unrelated individuals (*B. aeruginosa*, sampled from Nansihu Lake, in Jining City).

Cross-species amplification was carried out to investigate the versatility and polymorphism of the isolated SSR loci in *B. quadrata*, *B. purificata*, *B. angularia*, *B. dispiralis*, and *B. lapillorm*. Amplification products were visualized on 1.5% agarose gels, and fragments with similar sizes as those observed in the source species were considered to be successful cross-species amplification. A total of 263 individuals from three *Bellamya* species (*B. aeruginosa*, *B. angularia* and *B. dispiralis*, 3 populations for each species) were genotyped using the seven polymorphic SSR loci isolated and characterized in this study. Forward primers were 5'-labeled with HEX, ROX, or TAMRA. The sizes of the fluorescently labeled PCR products were estimated according to an internal size marker (GeneScan_500 LIZ) on an ABI PRISM® 3700 sequencer (Applied Biosystems, Foster City, CA, USA) and analyzed using STRAND v.2.3.48 (UC Davis Veterinary Genetics Laboratory, http://www.vgl.ucdavis.edu/informatics/strand.php).

Data analysis

The resulting data was first inspected for unexpected mutation steps, large gaps in data, unusually sized alleles and the presence of null alleles using MICRO-CHECKER v. 2.2.3 (Van Oosterhout et al., 2004). The number of alleles (N_A), the expected (H_E) and observed heterozygosities (H_O), *F*-statistics, inbreeding coefficients (F_{IS}; Weir and Cockerham, 1984), and genetic distances were estimated using MICROSATELLITE ANALYZER (MSA) v. 3.15 (Dieringer and Schlötterer, 2003).

GENEPOP v 4.0.10 (Rousset, 2008) was used to measure heterozygote deficiency or excess, and to assess the significance of deviations from Hardy-Weinberg equilibrium (HWE). P values were corrected for multiple comparisons by applying a sequential Bonferroni's correction (Rice 1989). ARLEQUIN v3.5 (Excoffier et al., 2005) was used to calculate pairwise F_{ST} (coefficient of genetic differentiation) values and test their significance by bootstrapping analysis (1000 replicates) in order to evaluate genetic differentiation between populations. We used chord distance to construct an UPGMA (unweighted pair group method with arithmatic mean) dendrogram of *Bellamya* populations, based on a D_C matrix (Cavalli-Sforza and Edwards, 1967) generated by MICROSATELLITE ANALYZER (Dieringer and Schlötterer, 2003) with 1000 distance matrices from resampled data sets bootstrapped over loci, using the MEGA v 6.0 software (Tamura et al., 2013).

RESULTS

Polymorphic microsatellite loci and cross-amplification

A total of 1000 putative recombinant clones were selected from the mixture-enriched library and sequenced. Of these, 909 clones contained unique repeated motifs (SSRs, GenBank accession Nos. JX018235-JX018346, JX018881-JX019072, JX019101-JX019198, JX019213-JX019326, JX019413-JX019502, JX019926-JX020033, JX020164-JX020222, and JX020313-JX020456) and 213 unique clones were used to design PCR primers.

Among the 213 loci examined, 65 (30.05% of the designed primers) showed polymorphism in the 36 *B. aeruginosa* individuals tested (Table 1). The N_A per locus ranged from 3 to 12. The H_O and H_E per locus varied from 0.026 to 1.000 and 0.425 to 0.953, respectively. All 65 of the primer pairs, indicated very efficient polymorphism in the examined *B. aeruginosa* population, with high polymorphic information content (PIC) values that ranged from 0.235 to 0.892. Fifteen SSR loci deviated significantly from HWE ($P < 0.00077$) after Bonferroni's correction (Table 1). Analysis using MICRO-CHECKER revealed no evidence of scoring errors due to stuttering or large allele dropout. However, the presence of null alleles was suggested for all loci except GC91, CCT238, ATG146, and GATA328.

Forward research on cross-species amplification was carried out using an additional five species (**Table S1**). All 65 loci (100%) were amplified successfully in *B. quadrata* and *B. purificata*, whereas 63 (96.92%), 62 (95.38%) and 60 (92.31%) were successfully amplified in *B. angularia*, *B. dispiralis* and *B. lapillorm*, respectively.

Population genetic variation

A total of 263 *Bellamya* individuals collected from nine populations of three species were genotyped using seven SSR loci isolated in this study (Table 2). All seven of the SSR loci we screened were highly polymorphic in most of populations (Table 2), and the lowest PIC value was 0.477. Among the three species and nine populations, the lowest mean N_A per locus (6.429) was observed in the p1' population, whereas the highest (8.143) were both found in the p1 and p2' populations.

H_E and H_O varied from 0.759 to 0.834 and 0.583 to 0.763, respectively (Table 2), among the nine populations. The lowest H_E and H_O were in the p3' population, the highest H_E was in the p3" population, and the highest H_O was in p1. As shown in Table 2, H_O was consistently lower than H_E, indicating that heterozygote deficiency is common in *Bellamya*. GENEPOP results confirmed this and all populations showed deviations from HWE at multiple loci ($P < 0.05$). Average inbreeding coefficients ranged from 0.071 to 0.299 (Table 2). MICRO-CHECKER revealed no evidence of scoring errors due to stuttering or large allele dropout. However, the presence of null alleles was suggested for all loci except for GATA328.

Population genetic differentiation

Most of the pairwise F_{ST} were significant ($P < 0.01$; Table 3), suggesting that all of the populations were significantly different from one another. The F_{ST} ranged from 0.070 to 0.182 (Table 3). The highest value of F_{ST} was obtained for the comparison between the p1" and p2 populations, whereas the lowest values of F_{ST} was obtained for the comparison between the p2" and p3' populations.

Table 1. Characteristics of 64 polymorphic microsatellite loci in *Bellamya aeruginosa* in a sample of 40 individuals.

Locus	GenBank	Primer sequence (5'-3')	Repeat motif	Ta (°C)	N_A (size range, bp)	H_O	H_E	PIC	P_{HWE}
GC36	JX018235	F: GGCAACAAAATCACATCAGTAAC R: AGAAAGAGGGGTAACTTGAAATC	$(TC)_{21}$	53	11 (146-224)	0.619	0.785	0.769	0.251
GC43	JX018241	F: CTTCTGAGGTGACGCCTGATAA R: GAGACAAATAAAACCAAGCATGAG	$(TC)_{28}$	53	4 (220-298)	0.783	0.841	0.237	0.023
GC44	JX018242	F:ATGAGATGAGAAAACTCAATTGCA R: GCGTGAAAGATGAAGACCAAAAC	$(TG)_8(AG)_{17}$	53	5 (178-252)	0.386	0.544	0.455	0.027
GC71	JX018263	F: CCGAGAAGATCCAGATTTTCA R: GATACATCACGGGTCTTTGG	$(AG)_{12}$	52	9 (148-202)	0.828	0.756	0.775	0.005
GC91	JX018276	F: TGCTGTCGTCATTATTTTCC R: TGCCGTTTTATTGTCTACCT	$(AG)_5AA(AG)_{20}$	52	12 (184-272)	0.673	0.841	0.698	0.000*
GC149	JX018306	F: CCGAAATCTGATGCCCTTATGTAT R: AATACGGCTACGGCAACAACC	$(AG)_{24}GGG(AG)_7$	55	9 (176-256)	0.729	0.667	0.623	0.012
GC151	JX018308	F: TCCAGGAACATTACACAACCATT R: TGAGATGACAACCTACTATACCGC	$(TC)_{33}$	52	12 (195-268)	1.000	0.816	0.790	0.002
GC199	JX018334	F: CGATGGTGCCAAGAAGTTATGAC R: CCTTTATCTCCCACCTCGTTTCT	$(AG)_{21}$	55	6 (167-229)	0.413	0.579	0.433	0.038
GC205	JX018337	F: CCGTCAACACACTGTCACACTA R: ACAATCATGCCCACTCGTATC	$(GA)_{29}$	52	7 (176-252)	1.000	0.653	0.551	0.000*
GC231	JX018346	F: TTGAATACAGGCGATAGGTTTG R: GACAACGAAAGAGGTGTGCTG	$(TC)_{13}(AC)_{21}$	52	4 (221-302)	0.653	0.466	0.423	0.018
TC09	JX018881	F: ACAAGCGAGTGTTGTGCATTCT R: TATCTTCCAATGTTGCTCGGTG	$(AG)_{32}AA(AG)_7$	55	5 (204-266)	0.759	0.881	0.541	0.156
TC21	JX018890	F: TACAATGCCAGTCGTAGGAAGC R: CTAAACAGAAACCGAGCCAAGC	$(AG)_{28}$	55	14 (147-261)	0.419	0.796	0.670	0.027
TC33	JX018896	F: ACTTCCCGCTGAACTAATAACA R: ATACTAACAAAACTGAGATGCTGG	$(TG)_{18}(AG)_{24}$	50	11 (202-293)	0.536	0.802	0.752	0.000*
TC34	JX018897	F: GTGAGAAGATGTGAGGGTCCAA R: CTGGTGCTGATATTTACTCTGTGG	$(TC)_{11}(AC)_6$	55	4 (136-184)	0.814	0.759	0.667	0.233
TC45	JX018906	F: GACGCACTGGGGTAGACAAAAGA R: CAAGTCACGTTGAAAACTGGGGA	$(GA)_{21}$	57	3 (239-252)	1.000	0.869	0.762	0.006
TC47	JX018908	F: CGATTGATGCTTACCTGATTTACT R: GCTGACGGAAGATACAAGATGC	$(GA)_{20}$	55	5 (164-228)	0.567	0.726	0.713	0.012
TC71	JX018920	F: GGACTGAAGGTGGAGGATAAATG R: CAGCGTAGCTCATTCTGTGGAT	$(TC)_{20}$	55	14 (148-268)	1.000	0.856	0.790	0.002
TC86	JX018931	F: AATGGTGGTAGGCTTCGTTCTT R: CTCACGCATGACTGACTCAAATG	$(GA)_{24}$	55	5 (158-198)	0.462	0.767	0.235	0.000*
TC94	JX018937	F: GTGCGTTTTGGAATAAATCTG R: CTGGAAATGAGATGTTCAGTTAC	$(GA)_{28}$	50	9 (180-246)	0.767	0.706	0.526	0.056
TC98	JX018940	F: TAGCGATAAGAACACGCAGCAT R: CTTTCTTCCCTTCTTCCTGTTGA	$(AG)_{16}GG(AG)_9$	55	11 (192-288)	0.805	0.896	0.762	0.224
TC99	JX018941	F: ATACGGGTAAAATAAGAAAACG R: GGATTGTACTGAGGAAAACAGA	$(GA)_{26}$	50	13 (138-216)	0.874	0.912	0.681	0.016
TC101	JX018943	F: CACGGAGATTTGCATGACAGAG R: TTAGTTTCTTCAACCCTCCAACAG	$(AC)_{16}$	55	12 (176-249)	0.825	0.894	0.780	0.127
TC119	JX018957	F: ATATCTTTTCAGGCTTTGTGGG R: TGGTTTGATTTGAGCACTTCGT	$(AG)_{25}$	52	10 (145-214)	0.412	0.668	0.593	0.000*
TC206	JX019015	F: CAAAGCGAAATAAATGTGACTTGTG R: GTGGTTCATACAGAGCGGCAGT	$(TC)_{15}AC(TC)_{16}$	55	3 (146-225)	0.892	0.718	0.772	0.123
TC225	JX019028	F: ATAGCCAGCATCAAACGGAAT R: AAAGCAACCAGGATACAACAGC	$(AG)_{21}TA(AG)_8$	52	15 (116-211)	0.712	0.953	0.749	0.000*
TC298	JX019072	F: CTCCAAAGACTGTTACTGCTACGA R: CACACAAACTAGGTAAGGGGACAT	$(AG)_{29}$	57	7 (156-252)	0.758	0.708	0.690	0.189
AAT26	JX019101	F: TTCTTTCAACTCCGTTTGGTCC R: CTGCTCGCTTTCTATCGCTTCT	$(AG)_{29}TCA(GT)_{12}$	55	4 (234-264)	0.026	0.615	0.254	0.000*
AAT43	JX019111	F: CGCTTACCTATGTGCTTGCTG R: GATAATCGAGATTTGTATGCCTTGT	$(AG)_{26}$	55	14 (288-428)	0.776	0.925	0.732	0.178
AAT116	JX019148	F: CAGTTTAGTTAGTATGGGTGGGAAGA R: ATGTGAGGTTGGGCAAGCGTAG	$(AG)_{22}$	57	5 (184-274)	0.308	0.490	0.431	0.058
AAT123	JX019155	F: GCTAATGACATCCACAGCAGG R: TTTCTATAAAATGGGGCAACC	$(TC)_{21}$	52	3 (268-306)	0.467	0.609	0.580	0.113
AAT140	JX019165	F: CTAAGTATTGTATGCCCTCGTT R: ATATTTTGTTGTCCTAGTGCTG	$(TC)_{13}$	55	6 (208-258)	0.672	0.879	0.559	0.000*
AAT143	JX019167	F: GAGATGGGTCCGCATTGATTC R: CACGCCACCACTTAGCATTTAG	$(TC)_{27}$	57	4 (228-248)	0.719	0.825	0.790	0.062

Continued on next page

Table 1. Continued.

Locus	GenBank	Primer sequence (5'-3')	Repeat motif	Ta (°C)	N_A (size range, bp)	H_O	H_E	PIC	P_{HWE}
AAT159	JX019177	F: ACAGTGAGGGAGTAATCAAGGG R: TTTCTACTGGGGATAAGGGTCA	$(GA)_{16}$	55	5 (186-276)	0.562	0.649	0.542	0.003
AAT160	JX019178	F: CACTACGAGATTTGGTGGGTTA R: GATTTGAAACAAGTTAGGTTACCG	$(AG)_{14}$	55	11 (148-226)	0.751	0.819	0.716	0.013
AAT166	JX019182	F: GATAGTAGATGGGTTACGGCAAT R: CCATTTTATTCATCTCATACACTCAC	$(AG)_{32}$	55	9 (155-228)	0.575	0.749	0.655	0.006
AAT169	JX019185	F: CAAAGCCCATCAACATATCAGTCG R: GCGTGCCAGTAGGACATCAAAG	$(CT)_{14}(CA)_{23}$	57	6 (217-272)	0.376	0.589	0.458	0.000*
AAT186	JX019198	F: AGGGTCTGAGTGTTGATGTGCC R: GCATTCACCATTTCACGCTTACT	$(TG)_{12}TA…GG(GA)_{27}$	55	7 (264-292)	0.617	0.756	0.697	0.042
CCT04	JX019213	F: GAAAACGGGGCACTTTGGAATG R: GGCAAAACCGCACCAGCAACTA	$(GA)_{24}$	57	12 (196-277)	0.572	0.714	0.696	0.054
CCT56	JX019242	F: CCAGCAGTTATCATGTATGTCATTG R: AGGGGTCACTTTTACTAGGAGTTCA	$(GA)_{10}AA(GA)_6$	57	8 (125-198)	0.615	0.779	0.657	0.006
CCT77	JX019254	F: GTATGGGCTGAGGAGTCTAACAA R: AACAGCGATCCACTAATAAGCAG	$(TC)_{15}(AC)_8$	55	7 (170-246)	0.649	0.815	0.713	0.000*
CCT99	JX019261	F: GCGTCGTTAGAGAGCCCTAATCTACC R: GCAGCGAACTTCCAATCATCCA	$(AG)_{14}$	57	4 (148-190)	0.358	0.425	0.258	0.008
CCT142	JX019284	F: AAATAGCCAAACATTCCAAACGC R: GTCCGTTGATGTCCATGTCACTAAA	$(AG)_{19}$	55	6 (155-192)	0.559	0.739	0.655	0.072
CCT158	JX019290	F: CCTAAAACAAGCCCTGGGTGGAT R: CTGCGGGGTCATAGTTCAACATT	$(GA)_{29}$	57	8 (181-224)	0.917	0.799	0.763	0.072
CCT238	JX019326	F: ACATAAGTGCTTGCGATAGTGCG R: ATCCCAGTGTCCTCAACCTAAACG	$(TG)_{16}$	57	8 (110-168)	0.646	0.860	0.733	0.000*
CAG41	JX019413	F: TTTGCTGCGTTTACTCGTCCTG R: CTCGTTCTTGGGCTGGGTGTT	$(TCG)_8$	55	4 (231-249)	0.667	0.718	0.657	0.439
CAG52	JX019420	F: GAGGAAGGACAGACGGGAACTAC R: TGTGTCCCCACTAATTTCTTTGC	$(CT)_{12}$	55	6 (148-196)	0.568	0.672	0.571	0.007
CAG84	JX019444	F: TCTTGGGCTGGGTGTTTCATC R: CCTCCTTTGTTTATTCCCTTCTTTA	$(CAG)_7$	55	3 (255-276)	0.572	0.728	0.256	0.075
CAG131	JX019475	F: TTTGCTGCGTTTACTCGTCCTG R: TCTTGGGCTGGGTGTTTCATCT	$(TGC)_8$	55	4 (225-251)	0.296	0.668	0.592	0.003
CAG178	JX019502	F: CATGACATAACACCCCTACCCTT R: AATTTGGTATCTTGGAATCTGACG	$(AC)_{26}(CAG)_5(CAC)_5$	55	6 (142-217)	0.681	0.792	0.753	0.0819
ATG48	JX019926	F: CGAAGTGAGATAAATGCAGAATAA R: TCCCTGAATACCAGTGAAAAGA	$(GAT)_8$	52	5 (162-198)	0.625	0.795	0.650	0.006
ATG79	JX019947	F: TGAGCCTGACTGACTACGGTGAC R: CGCAGCATGTAGAAGACGAAAG	$(TCA)_7$	57	4 (175-225)	0.096	0.658	0.458	0.000*
ATG97	JX019956	F: CCTGCGGGCTGGTGAGAACT R: TCGTCTACAGCAATAATGATGTTGATG	$(CAT)_{10}$	57	5 (157-185)	0.713	0.920	0.732	0.035
ATG142	JX019983	F: ATGCCGATTATTCTGATTCTGG R: ACGCAAGTTTCATTCATGTATGTC	$(GAT)_{10}$	52	9(212-248)	0.875	0.808	0.773	0.513
ATG146	JX019986	F: GCTCTGTCCAGCAAGAAACTAG R: ATAGACATCAGTCCGACAAAGC	$(TGG)_6(TGA)_{16}TGG(TGA)_{27}$	55	5 (256-358)	0.894	0.857	0.630	0.290
ATG166	JX020001	F: CAGCATAATCTGCCCTAAGTCCC R: AATATCCACTTCTCGCAGCCTCT	$(ATG)_9$	57	4 (169-198)	0.746	0.824	0.459	0.059
ATG182	JX020010	F: TGGCTAGTACCCATTGAAAGAA R: AACATCAGCATCACTAACACCC	$(ATG)_9$	52	7 (217-248)	0.589	0.725	0.667	0.036
ATG224	JX020033	F: GTTCTTGGGCTGGGTGTTTC R: TGCTGCGTTTACTCGTCCTG	$(AGC)_8$	57	6 (235-284)	1.000	0.685	0.549	0.000*
GACA41	JX020164	F: GTTCAACTTCCCGACCTCCTCA R: GCCCACCTTCAGACATCCCA	$(GTT)_{10}$	57	5 (104-136)	0.557	0.676	0.579	0.853
GACA165	JX020209	F: GCATCAGTGATTACCAAAGCAAAATG R: CGGTCAACGGAAAGTTTGGGTC	$(TGG)_6(TGA)_{44}$	57	5 (361-416)	0.318	0.745	0.605	0.000*
GACA208	JX020214	F: ATGAGAAGGAGCAGGAAGGCG R: TGGACCTCTGCCATCGTCTTTA	$(ATG)_{20}$	57	4 (199-228)	0.618	0.673	0.596	0.562
GACA226	JX020222	F: CCAACTTTCATAGTCACATTCACCCAC R: ACCGTCGTAGCCATCACAGCATT	$(ACG)_5(ATG)_8(TGA)_6$	57	5 (179-216)	0.956	0.749	0.766	0.074
GATA115	JX020313	F: ACTTCGGCTGCTTCTGTACTCAT R: TCTCCCTTTCTTGGTTTTCTTTTC	$(GT)_5(GA)_9GTGTG(TGTC)_6$	55	3 (181-220)	0.317	0.426	0.363	0.054
GATA160	JX020340	F: TGTGTATTTATCAGCGTATTTGTGT R: GTATGTGAGTGCCAGTTGGTAGT	$(AGAC)_7$	52	9 (268-384)	0.972	0.819	0.892	0.156
GATA170	JX020348	F: CGACAAGTAACCGCTTCAATCA R: GTTCTGGCATCCTCAGGCTATG	$(GT)_{13}(GTGC)_8$	55	6 (169-269)	0.973	0.765	0.716	0.558
GATA328	JX020456	F: CCTGCGTCAATTTAAAACCATAG R: GGGTAGGTAGGTGGGTAAGTGAG	$(GTCT)_8…(TCTA)_8$	55	7 (192-344)	0.646	0.889	0.766	0.000*

N_A = observed number of alleles; H_O = observed heterozygosity; H_E = expected heterozygosity; Ta = annealing temperature; PIC = polymorphic information content. *Indicates significant deviation from Hardy-Weinberg equilibrium (HWE) after Bonferroni's correction (adjusted P value = 0.00077).

Table 2. Genetic variability of seven microsatellite loci in nine populations of three *Bellamya* species (each 3 populations).

Locus/Parameters	*B. aeruginosa*			*B. angularis*			*B. dispiralis*		
	p1 (N = 30)	p2 (N = 29)	p3 (N = 31)	p1' (N = 29)	p2' (N = 30)	p3' (N = 30)	p1'' (N = 29)	p2'' (N = 29)	p3'' (N = 26)
ATG142									
N_A	6	6	7	4	5	9	5	7	6
H_O	0.800	0.621	0.807	0.552	0.567	0.667	0.724	0.633	0.654
H_E	0.796	0.656	0.717	0.678	0.783	0.773	0.758	0.841	0.797
PIC	0.751	0.602	0.664	0.609	0.733	0.738	0.705	0.804	0.748
F_{IS}	0.014	0.046	0.135	0.181	0.272	0.131	0.037	0.242	0.173
P_{HWE}	0.609	0.460	0.939	0.320	0.011*	0.009**	0.435	0.002**	0.098
ATG146									
N_A	10	9	9	4	8	7	6	8	8
H_O	0.893	0.724	0.867	0.552	0.400	0.483	0.897	0.821	1.000
H_E	0.875	0.856	0.855	0.733	0.856	0.828	0.788	0.842	0.878
PIC	0.843	0.823	0.822	0.668	0.823	0.788	0.742	0.804	0.845
F_{IS}	0.030	0.148	0.022	0.242	0.531	0.414	0.149	0.015	0.152
P_{HWE}	0.542	0.050*	0.664	0.009**	0.000**	0.013*	0.711	0.114	0.871
CAG41									
N_A	5	8	7	7	8	6	6	5	6
H_O	0.767	0.393	0.345	0.379	0.433	0.567	0.483	0.536	0.423
H_E	0.755	0.841	0.794	0.823	0.799	0.523	0.803	0.762	0.805
PIC	0.701	0.805	0.750	0.782	0.758	0.477	0.759	0.711	0.757
F_{IS}	0.024	0.531	0.564	0.537	0.455	0.093	0.396	0.292	0.472
P_{HWE}	0.688	0.000**	0.000**	0.000**	0.000**	0.133	0.005**	0.001**	0.000**
CAG178									
N_A	7	7	6	7	9	4	7	6	4
H_O	0.586	0.793	0.774	0.586	0.733	0.167	0.621	0.933	0.423
H_E	0.756	0.836	0.760	0.861	0.860	0.586	0.710	0.800	0.737
PIC	0.710	0.798	0.706	0.826	0.828	0.528	0.670	0.755	0.670
F_{IS}	0.219	0.043	0.027	0.315	0.141	0.715	0.119	0.178	0.423
P_{HWE}	0.001**	0.502	0.667	0.000**	0.007**	0.000**	0.049*	0.934	0.003**
CCT238									
N_A	9	5	9	6	8	11	6	4	7
H_O	0.600	0.483	0.677	0.621	0.467	0.633	0.690	0.600	0.731
H_E	0.814	0.785	0.842	0.600	0.829	0.899	0.811	0.561	0.857
PIC	0.773	0.732	0.807	0.542	0.790	0.873	0.768	0.512	0.821
F_{IS}	0.258	0.381	0.191	0.045	0.434	0.292	0.144	0.079	0.141
P_{HWE}	0.032*	0.003**	0.008**	0.580	0.000**	0.000**	0.003**	0.423	0.017*
GATA328									
N_A	10	8	7	7	8	7	8	8	8
H_O	0.767	1.000	0.871	0.897	0.633	0.667	0.593	0.833	0.769
H_E	0.859	0.854	0.817	0.786	0.838	0.829	0.851	0.850	0.877
PIC	0.827	0.819	0.774	0.747	0.802	0.791	0.815	0.817	0.844
F_{IS}	0.101	0.183	0.075	0.152	0.240	0.191	0.299	0.012	0.115
P_{HWE}	0.213	0.873	0.804	0.836	0.002**	0.001**	0.000**	0.359	0.238
TC298									
N_A	10	10	11	10	11	9	8	8	10
H_O	0.931	0.964	0.742	0.724	0.900	0.900	0.517	0.655	0.958
H_E	0.884	0.890	0.857	0.870	0.903	0.873	0.775	0.857	0.887
PIC	0.855	0.862	0.828	0.839	0.878	0.842	0.735	0.823	0.855
F_{IS}	0.063	0.094	0.128	0.161	0.005	0.040	0.329	0.231	0.092
P_{HWE}	0.891	0.969	0.144	0.013*	0.654	0.772	0.004**	0.033*	0.834
Multilocus									
Mean N_A	8.143	7.571	8.000	6.429	8.143	7.571	6.571	6.571	7.000
mean H_O	0.763	0.711	0.726	0.616	0.590	0.583	0.646	0.716	0.708
mean H_E	0.820	0.817	0.806	0.764	0.838	0.759	0.785	0.787	0.834
mean PIC	0.780	0.777	0.764	0.716	0.802	0.720	0.742	0.747	0.792
F_{IS}	0.071	0.130	0.097	0.197	0.299	0.233	0.178	0.097	0.156
P_{HWE}	0.036*	0.002**	0.001**	0.000**	0.000**	0.000**	0.000**	0.001**	0.000**

N_A = number of alleles; H_O = observed heterozygosity; H_E = expected heterozygosity; PIC = polymorphic information content; P_{HWE} = Hardy-Weinberg probability test (*P < 0.05, **P < 0.01); F_{IS} = fixation indices; p1 = Weihe River; p2 = Liangzi Lake; p3 = Danjiangkou Reservoir; p1' = Weihe River; p2' = Banqiao Reservoir; p3' = Huaihe River; p1'' = Weihe River; p2'' = Huaihe River; p3'' = Danjiangkou Reservoir.

Table 3. Pairwise F_{ST} values (below the diagonal) and D_{CE} distance (above the diagonal) between nine *Bellamya* populations in China based on eight microsatellite loci.

Populations	p1	p2	p3	p1'	p2'	p3'	p1"	p2"	p3"
p1	-	0.466	0.454	0.744	0.791	0.820	0.805	0.784	0.769
p2	0.078	-	0.430	0.762	0.812	0.775	0.813	0.791	0.791
p3	0.076	0.080	-	0.794	0.767	0.759	0.796	0.768	0.755
p1'	0.147	0.156	0.169	-	0.756	0.735	0.696	0.677	0.711
p2'	0.143	0.157	0.146	0.161	-	0.817	0.768	0.735	0.690
p3'	0.148	0.139	0.143	0.123	0.157	-	0.715	0.628	0.707
p1"	0.171	0.182	0.180	0.126	0.175	0.130	-	0.456	0.606
p2"	0.126	0.134	0.135	0.101	0.122	0.070	0.073	-	0.491
p3"	0.161	0.163	0.158	0.169	0.128	0.132	0.161	0.098	-

Figure 2 shows the UPGMA dendrogram of *Bellamya* populations based on *DC* distance. Three *B. aeruginosa* populations (p1, p2, and p3) clustered together to form group A and three *B. dispiralis* populations (p1", p2", and p3") clustered together to form group B. *B. angularia* did not cluster together.

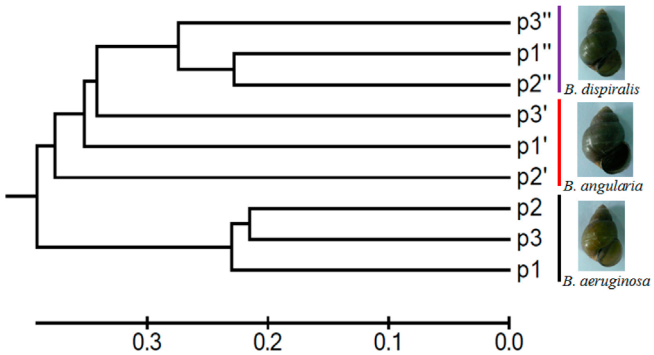

Figure 2. UPGMA dendrogram of *Bellamya* populations based on a matrix of *DC* distance. Three *B. aeruginosa* populations (p1, p2, p3) were clustered into in A and *B. dispiralis* (p1", p2", p3") were clustered into B, but not *B. angularia*.

DISCUSSION

SSR markers have been widely used as highly effective DNA markers in the study of molluscan population genetics (Arthofer et al., 2011; Pálsson et al., 2014). In this study, 65 novel microsatellite loci were isolated and some of them were used to analyze the population genetics of *Bellamya* spp in China. In the present study, 30.05% of the designed primers were polymorphic: a lower percentage than in two previous studies (45.0 and 48.4%; Gu et al., 2012a,b). This difference may have been caused by the use of a different sampling strategy and enriched DNA. Although significant heterozygote deficiencies were suggested by the significant deviation of 15 of the examined loci from HWE, these markers can still be used for studies of population genetics if analytical methods are employed to correct for the presence of null alleles (Park et al., 2012). The high levels of polymorphism and heterozygosity observed suggest that the 65 loci examined in this study would be a valuable tool for examination of the population genetics and genetic evolution of *Bellamya*.

As expected, due to the close taxonomical relationship between *B. aeruginosa* and *B. quadrata*, *B. purificata*, *B. angularia*, *B. dispiralis*, and *B. lapillorm*, we found a high level of cross-species amplification across these species. The high level of cross-species amplification reflected conservatism of the microsatellite markers and indicated that the markers may be useful for a wide range of studies, including evolutionary, species identification, conservation, and management studies of *Bellamya*, or even Viviparidae.

Seven randomly selected polymorphic SSR markers were successfully used to conduct a preliminary population genetics analysis for nine populations of three *Bellamya* species.

All of the population genetic parameters indicated strong genetic diversity and clear genetic differentiation between populations. We found a high level of genetic diversity within all nine examined *Bellamya* populations. However, most species of freshwater snails have low levels of genetic diversity, which is mainly attributed to high selfing rates and demographic instability (Jarne, 1995; Feher et al., 2012; Coutellec et al., 2013; Nguema et al., 2013). Demographic instability is generally accompanied by large fluctuations in population size and bottlenecks. Campbell et al. (2010) suggested that instability of snail habitats in Senegal (associated with the construction of the Diama dam) may explain the lack of genetic diversity in those populations. The high level of genetic diversity observed for *Bellamya* species likely results from their wide distribution and high degree of adaptation to freshwater environments.

Heterozygosity deficits were observed in most populations, and were accompanied by significant departure from HWE (Table 2). The departure from HWE is likely associated with the presence of null alleles (Selkoe and Toonen, 2006), which are common in invertebrates (Launey and Hedgecock, 2001). Inbreeding within populations also contributes to significant departures from HWE in invertebrates (Dailianis et al., 2011). This is especially true for freshwater snails with poor mobility, as indicated by high F_{IS} estimates for *B. pfeifferi* populations (0.49-1.00; Nguema et al., 2013). Moderate to high F_{IS} values were also found in this study, especially for *B. angularis* populations (0.197- 0.299).

Whitlock (2011) suggested that F_{ST} provides a better understanding of the evolution and demographic processes that lead to interpopulation differentiation. The F_{ST} values in the present study suggested a low to moderate level of genetic differentiation ($0.1 < F_{ST} < 0.2$) among populations. However, populations of the same *Bellamya* species had relatively low F_{ST} values in comparison to populations from different species. This result was supported by the constructed UPGMA dendrogram, which indicated the relationships between *Bellamya* populations. *B. aeruginosa* and *B. dispiralis* populations clustered by species. Although *B. angularia* populations did not form a monophyletic group, the microsatellite loci used in the present study are valuable tools for analysis of genetic diversity, phylogenetic relationships, and species delimitation of *Bellamya*.

In this study, 65 novel polymorphic SSR markers for *B. aeruginosa* were isolated and cross-amplified in five additional *Bellamya* species. In addition, seven polymorphic SSR markers developed in this study were used to examine the genetic characterization and genetic relationships of nine populations of three *Bellamya* species. These novel SSR loci represent a valuable tool for further studies of the diversity and species delimitation of *Bellamya*.

Conflicts of interest

The authors declare no conflict of interest.

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Supplementary material

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