



Eight polymorphic microsatellite markers for the spotted babylon, *Babylonia areolata* (Buccinidae)

Y. Wang, H. Lu, J. Zheng, K. Long, Y.H. Shi, Z.F. Gu and A.M. Wang

Key Laboratory of Tropical Biological Resources, Ministry of Education,
Hainan Provincial Key Laboratory of Tropical Hydrobiology and Biotechnology,
Marine Biology Experiment Teaching Demonstration Center,
Ocean College, Hainan University, Haikou, Hainan, China

Corresponding authors: A.M. Wang / Y. Wang
E-mail: aimwang@163.com / wy2005@163.com

Genet. Mol. Res. 10 (4): 3230-3235 (2011)
Received June 28, 2011
Accepted August 19, 2011
Published December 21, 2011
DOI <http://dx.doi.org/10.4238/2011.December.21.5>

ABSTRACT. The spotted babylon, *Babylonia areolata*, is one of the most extensively cultured marine mollusks in southeast Asia. Eight polymorphic microsatellite markers were developed for this species, from a microsatellite-enriched library. These markers, characterized in 32 individuals from a hatchery population, were polymorphic, with allele numbers ranging from 6 to 18 per locus, expected and observed heterozygosities ranging from 0.68 to 0.94 and 0.56 to 0.81, respectively. One locus (*HUBA09*) showed significant deviation from Hardy-Weinberg equilibrium, probably due to the presence of null alleles. These microsatellite loci should be useful for future population genetic studies and marker-assisted breeding in this species.

Key words: *Babylonia areolata*; Microsatellites; Population genetics; Aquaculture; Spotted babylon

INTRODUCTION

The spotted babylon *Babylonia areolata* (Link, 1807), also called the maculated ivory whelk, is a marine gastropod mollusc distributed in southeastern Asia. Its geographic distribution stretches from Ceylon and Nicobar Islands through the Gulf of Thailand, along Vietnamese and Chinese coasts to Taiwan (Altena and Gittenberger, 1981). It is mostly found on muddy and sandy littoral bottoms not exceeding 5-10 m in depth (Panichasuk, 1996). The spotted babylon is a popular model mollusc used in studies on heavy-metal toxicity and on the transmission of biological toxins (Chen and Chou, 1998; Supanopas et al., 2005), since it is a popular and nutritious seafood. In recent years, wild populations have rapidly declined due to fast-growing demand for consumption and to environmental pollution. The spotted babylon has been a commercially important aquaculture species in Thailand (Chaitanawisuti et al., 2002; Kritsanapuntu et al., 2006) and China (Liang et al., 2005). The individuals from Thailand have greater growth vigor than native ones in Hainan Island, so most hatchery populations in Hainan come from Thailand lines. However, little is known about the genetic structure and variation of this species, especially in hatchery lines (Hualkasin et al., 2008; Chen et al., 2011). Microsatellite markers are a powerful tool in population structure analyses, genetic linkage map construction (Reece et al., 2004), parentage assignment, and marker-assisted breeding (Wang et al., 2010b), owing to their codominant and highly polymorphic nature, as well as the reliability and testability in PCR and genotyping. To support population structure analyses and facilitate stock management, we developed a microsatellite-enriched library and characterized 8 polymorphic microsatellite markers for the spotted babylon.

MATERIAL AND METHODS

Samples and DNA extraction

Thirty-two *B. areolata* individuals were sampled from a hatchery population at Hainan Dingda Aquaculture Co. Ltd. (Wenchang, Hainan, China) in 2010 (coded Dd10). The population was the F₁ progeny of Thailand wild-caught broodstock that had been imported. DNA was extracted from the ethanol-preserved gastropod muscles using the Cell/Tissue Genomic DNA extraction kit (TianGen, Beijing, China).

Microsatellite-enriched library construction and sequencing

A microsatellite-enriched DNA library was constructed using a modified magnetic bead enrichment protocol as described by Wang et al. (2010a). Briefly, about 38 µg genomic DNA from three individuals was digested by the restriction enzyme *Mbo*I (Takara, Dalian, China). Fragments of 300-1000 bp, collected with the Gel DNA Extraction kit (TianGen), were ligated to double-strand *Mbo*I adapters by incubating with T4 DNA ligase (Takara) at 16°C overnight. Excess adapters were removed by washing with 0.1X TE buffer, pH 8.0, on an Ultrafree column (Pall, CA, USA). DNA fragments with adapters were amplified with linker B (one strand of *Mbo*I adapter) for 5 PCR cycles and purified using an Ultrafree column (Pall). The amplified products were denatured and then hybridized to biotin-labeled (CA)₁₂, (GA)₁₂, (ACA)₈, (AGA)₈, (GACA)₆, and (GATA)₆ oligonucleotides (mixed in advance at a ratio of

3:1:1:1:2:2, total 150 pmol) in 0.5X SSC at 68°C for 60 min. DNA fragments bound to these probes were captured with Streptavidin MagneSphere® Paramagnetic Particles (Promega, USA) and eluted with DNase-free water after being washed four times in 0.1X SSC at room temperature. The microsatellite-enriched eluate was amplified and purified as described. The amplified products were purified with an Ultrafree column (Pall), and cloned using the pGEM-T-easy system (Promega). Transformed colonies were screened for inserts by clone color and PCR test using M13 forward primer, nonbiotinylated microsatellite probes (noted earlier), and linker B as the primers. Positive clones were selected and sent to Sangon Biological Engineering Technology & Services Co., Ltd. (Shanghai, China) for sequencing.

Primer design and genotyping

After removing the sequences of linkers and T vector, DNA sequences were searched against each other and against 9 spotted babylon microsatellite sequences from GenBank (accessed June 19, 2011) using Vector NTI Advance 11.0.0 (<http://www.invitrogen.com>) to check for duplicates. The MISA software (<http://pgrc.ipk-gatersleben.de/misa/>) was used to screen for sequences containing at least 6 di-, 5 tri-, 5 tetra-, 4 penta-, and 3 hexa-, hepta-, and octa-nucleotide repeats. Good sequences with sufficient flanking regions were selected for primer design with Primer3 (http://biotools.umassmed.edu/bioapps/primer3_www.cgi). An M13 (-21) universal leading sequence (5'-TGTAACAACGACGGCCAGT-3') was added to the 5' end of each forward primer listed in Table 1 (Schuelke, 2000) and primers were synthesized by Sangon Biological Engineering Technology & Services Co., Ltd.

All primer pairs were tested for PCR amplification in the 32 individuals of the Dd10 population. PCR was conducted in a 10- μ L solution containing about 50 ng template DNA, and reagents (TianGen) as follows: 1X reaction buffer containing 20 mM Tris-HCl, pH 8.4, 20 mM KCl and 10 mM $(\text{NH}_4)_2\text{SO}_4$, 1.2 mM MgCl_2 , 0.2 mM of each dNTP, 0.4 U Taq polymerase, 0.2 pmol M13 (-21) tailed forward primer, 0.8 pmol reverse primer, and 0.8 pmol M13 (-21) primer labeled with fluorescent dyes (FAM, VIC, NED, or PET; Applied Biosystems, Foster City, CA, USA). PCR was conducted on a Master Cycler Gradient (Eppendorf, Germany) with the following steps: 94°C for 4 min followed by 34 cycles of 94°C for 30 s, annealing (temperatures indicated in Table 1) for 45 s, 72°C for 45 s, followed by 10 cycles of 94°C for 30 s, 53°C for 45 s, and 72°C for 45 s, and a final extension at 72°C for 10 min. Amplified products were detected and sized on an ABI 3130xl Prism Genetic Analyzer (Applied Biosystems) using GS-500LIZ (Applied Biosystems) as the size standard. Allele scoring was performed with GeneMapper v3.5 (Applied Biosystems). Samples failing to amplify the first time were reamplified once.

Statistical analysis

The MICRO-CHECKER 2.2.1 software (van Oosterhout et al., 2004) was used for identifying possible null alleles (1000 randomizations). GENEPOP on the web (<http://genepop.curtin.edu.au/>) was used to identify deviations from Hardy-Weinberg equilibrium (HWE) for each locus as well as for linkage disequilibrium (LD) between all pairs of loci (exact tests, 1000 iterations). The ARLEQUIN 3.0 software (Excoffier et al., 2005) was used to calculate observed (H_o) and expected (H_e) heterozygosities. All tests were corrected for multiple comparisons by Bonferroni's correction (Rice, 1989).

RESULTS

Microsatellite sequences

One hundred and twenty-eight positive clones were sequenced, producing a total of 59,252 bp of DNA sequences. Analysis with MISA identified 118 microsatellite-containing sequences, corresponding to an enrichment efficiency of 97.5%. The 118 microsatellite-containing sequences harbored 406 microsatellites with di- (85.5%), tri- (4.2%), tetra- (6.2%), penta- (0.7%), hexa- (1.7%), and heptarepeats (1.7%). Ninety (76.3%) sequences contained more than one microsatellite. Most of these sequence contained clustered and complex repeated structures, while only 56 sequences had sufficient flanking regions for primer designing.

Polymorphism and heterozygosity

Eight primer pairs were successfully amplified and genotyped in 32 individuals from the Dd10 population (Table 1). The 8 microsatellite loci were polymorphic with allele numbers ranging from 6 at *HUBA13* to 18 at *HUBA08* (mean: 13.6 ± 3.4). The H_E and H_O varied from 0.68 at *HUBA13* to 0.94 at *HUBA08* (mean: 0.85 ± 0.09), 0.56 at *HUBA13* to 0.81 at *HUBA17* and 22 (mean: 0.70 ± 0.09), respectively (Table 1). No linkage disequilibrium was detected among all the loci at $P > 0.05$ (after Bonferroni's correction). One locus (*HUBA09*) showed significant ($P < 0.05$ after Bonferroni's correction) deviation from HWE. *HUBA09* and two other loci (*HUBA08* and *15*) showed signs of null alleles as indicated by Micro-Checker (1000 randomizations) (Table 1), suggesting that the presence of null alleles was the main cause for the deviation from HWE. Samples that failed to amplify were rare (cf., Table 1), indicating that null homozygotes were not common.

Table 1. Primer sequences and characteristics of 8 *Babylonia areolata* microsatellite markers.

Locus	GenBank acc. No.	Repeat motif [§]	Primer sequences (5'-3')	Ta (°C)	N	N _A	AS	H _E	H _O	P _{HWE}
<i>HUBA08</i> [*]	FJ595005	(GA) _n (GACA) _N	F:agcagcctaacatacgcacac R:atfgaattgcagttggggaac	62	29	18	166-236	0.94	0.66	0.101
<i>HUBA09</i> [*]	FJ595006	(TG) _N (AG) _N	F:agagttgaggactggtggag R:tgctaaaaccttatgcagtg	63	29	14	152-198	0.90	0.63	0.000
<i>HUBA13</i>	FJ595010	(TTG) _N	F:gtttaccacgcgtaactttgc R:taaatcaacctcccctacce	62	32	6	274-299	0.68	0.56	0.225
<i>HUBA15</i> [*]	FJ595012	(TG) _N	F:tcaccggttctaacccttg R:tttaagcaaccgagccaaac	62	29	12	309-359	0.90	0.63	0.183
<i>HUBA17</i>	FJ595014	(AC) _N	F:cgcacgttcgattgtaagac R:gttttcccagaggtgttc	62	32	16	295-341	0.91	0.81	0.134
<i>HUBA20</i>	FJ595017	(TTG) _N (TG) _N (TC) _N	F:ctggagttggttctctctac R:ttcaaatgccaaggtagagg	62	30	14	324-397	0.81	0.78	0.029
<i>HUBA21</i>	FJ595018	(GA) _n	F:gtcactgctggaatgaaaaag R:ttcctcaacaccagcttc	62	30	11	372-410	0.80	0.72	0.273
<i>HUBA22</i>	FJ595019	(CA) _N	F:ggcaacaaaactttcactcg R:cttcattgctctctccac	62	32	14	205-241	0.89	0.81	0.058

[§]Repeat motif: N = pure; n = interrupted. Value in bold represents significant probability estimates after correction for multiple tests (initial $\alpha < 0.05/8 = 0.00625$). ^{*}Loci showing evidence of null alleles suggested by Micro-Checker. N = number of individuals analyzed; N_A = number of alleles; AS = allele size range in base pairs; H_O and H_E = observed and expected heterozygosities; Ta = annealing temperature; P_{HWE} = probability of Hardy-Weinberg equilibrium.

DISCUSSION

There is probably a high frequency of microsatellite sequences in *B. areolata* genomes, since we obtained an enrichment efficiency of 97.5% (118 of 121) in this study, which is much higher than that obtained in four other molluscan species, whose microsatellite markers were developed in parallel in our laboratory using the same protocol and reagents: 63.3% for *Spisula solidissima* (Wang et al., 2009b), 53.2% for *Pinctada maxima* (Wang et al., 2009a), 59.4% for *Mercenaria mercenaria* (Wang et al., 2010a), and 58.5% for *Haliotis diversicolor* (Wang et al., 2011). In addition, we found clustered and complex repeated structures in the babylon genome. Extraordinarily, there were 76 microsatellite sequences (64.4%, 76 of 118) containing very long and complex microsatellites to have sufficient flanking regions for primer designing, and such high frequency was also observed by other researchers in *B. areolata* (Chen et al., 2009) and in other whelk, e.g., *Buccinum undatum* (Weetman et al., 2005). These results suggested that the *B. areolata* genome may be a good model to study the evolution of repetitive DNA sequences.

One locus (*HUBA09*) deviating from HWE and two other loci (*HUBA08* and *15*) may be influenced by one or more null alleles in the hatchery sample. This observation indicates that using *HUBA09* for population genetic analyses that assume HWE may prove to be problematic, e.g., false homozygotes resulting from underestimation of heterozygotes (Pemberton et al., 1995), overestimation of F_{ST} and some reduction in the percentage of correctly assigned individuals in parentage assignment (Carlsson, 2008). Therefore, caution must to be taken when these markers are used.

The 8 polymorphic microsatellite markers developed here are a useful addition to the collection of other molecular markers that are now available for *B. areolata*, including the cytochrome oxidase subunit (CO) gene and the internal transcribed spacer 1 (ITS1) (Hualkasin et al., 2008), other microsatellite markers (Chen et al., 2009), and AFLP (Chen et al., 2011). They will prove valuable for future population genetic studies and in the tracking of hatchery strains in this species.

ACKNOWLEDGMENTS

Research supported by the National Basic Research Program of China (973 Program, #2009CB126005), the National Natural Science Foundation of China (#40866003 and #31060354), and the Natural Science Foundation of Hainan (#80616).

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