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Technical Notes

Characterization of nine novel microsatellite loci for the Venus clam (*Cyclina sinensis*)

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ABSTRACT. The Venus clam, *Cyclina sinensis*, is one of the most important bivalves in China marine aquaculture. Using $(CA)_{15}$-enriched genomic libraries of this species, nine novel polymorphic microsatellite loci were isolated and characterized. The mean number of observed alleles per locus was 16 (range 8-24). The observed and expected heterozygosity ranged from 0.119 to 0.872 and from 0.626 to 0.931, respectively. Three loci had significant departure from Hardy-Weinberg equilibrium and non-significant linkage disequilibrium was found among all nine loci. These highly informative microsatellite markers should be useful for population genetic analyses of *C. sinensis*.

Key words: Microsatellite; *Cyclina sinensis*; Genetic diversity; Polymorphism

INTRODUCTION

The Venus clam (*Cyclina sinensis*) is widely distributed throughout the seashores from the far eastern reaches of Russia, Japan, Korea, and China to Southeast Asia. As a high-valued marine shellfish species, the study of *C. sinensis* has centered on its morphology and anatomy (Yu and Zheng, 1995), fauna systematics (Xu, 1997) and reproductive habits (Wang et al., 2006; Shen et al., 2007). The population genetic diversity and genetic structure of the species had also been analyzed using amplified fragment length polymorphism (AFLP) markers (Zhao et al., 2007), ribosomal DNA gene internal transcribed spacer (ITS) sequences (Yuan et al., 2008), random amplified polymorphic DNA (RAPD) markers (Chen et al., 2004; Yao et al., 2005; Pan et al., 2005; Bai et al., 2008), and microsatellite DNA marker (Feng et al., 2010). Lack of sufficient and polymorphic molecular markers has limited development of molecular phylogeny, population structure and molecule-assisted selective breeding in this species. Thus, screening for polymorphic microsatellite or other molecular markers is necessary for analyzing genetic information in *C. sinensis*. In the present study, nine polymorphic microsatellite DNA markers were developed for *C. sinensis* to evaluate genetic variation in future studies.

MATERIAL AND METHODS

The samples of *C. sinensis* were collected from Zhoushan Island (Zhejiang Province, China). Genomic DNA was isolated from the adductor muscle of five *C. sinensis* individuals according to standard phenol-chloroform protocols. Then, extracted genomic DNA was mixed. The enriched library protocol for isolating CA repeat motifs was performed according to Hua et al. (2007) with some additional modifications. The mixed genomic DNA was digested with restriction enzyme *Mbo*I and the 300-800-bp fragments were selected on an agarose gel and recovered using a DNA purification kit (Tiangen). Fragments were then ligated to a blunt-end adapter (SAULA: 5'-GCGGTACCCGGGAAGCTTGG-3', SAULB: 5'-GATCCCAAGCTTCCCGGGTACCGC-3') with T4 DNA ligase (Takara). Using the linker sequences as specific primers, the ligation products were amplified and the amplified products were hybridized to a biotin-labeled dinucleotide repeat $(CA)_{15}$ probe at 50°C in sodium phosphate (0.5 M sodium phosphate, 0.5% SDS, pH 7.4) for 18 h. The hybridization mixture was incubated with VECTREX Avidin D (Vector Laboratories) at 37°C and washed four times with binding buffer at different temperatures to remove unbound fragments. Bound fragments were eluted with ddH_2O and recovered with PCR. Then, these targeted fragments were ligated with pMD19-T vector (Takara), which was then used to transform DH5α competent cells. Fifty-five positive clones were identified from 84 recombinant clones through PCR with SAULA as primers. They were sequenced with M13 universal primers (Invitrogen) and 35 sequences containing CA/TG repeats were selected for primer design using the PRIMER PREMIER 5.0 software (PREMIER Biosoft International). Through the gradient PCR, nine loci were amplified successfully and the optimal annealing temperature (T_a) was determined (Table 1).

To test the level of genetic polymorphism for these loci, we sampled 42 individuals from a natural population on Zhoushan Island (Zhejiang Province, China). A 15-µL reaction volume contained 50-100 ng genomic DNA, 0.2 µM forward primer (5' modified with FAM, HEX or TAMRA fluorescent dye), 0.2 µM reverse primer, 0.2 mM of each dNTP, 0.25 U Hotstar Taq DNA polymerase (Qiagen) and 1.5 mM $MgCl_2$. Typical PCR amplifications were performed with an initial denaturation at 95°C for 15 min, followed by 34 cycles at 94°C for 30 s, T_a (see

Table 1) for 30 s, 72°C for 30 s, and a further extension step of 72°C for 10 min. The PCR products were genotyped on an ABI 3730 sequencer (Applied Biosystems) and analyzed with the GeneMapper v4.0 software (Applied Biosystems). Observed and expected heterozygosity values, and tests for deviations from Hardy-Weinberg equilibrium (HWE) and linkage disequilibrium were performed using the GENEPOP 3.4 software (Raymond and Rousset, 1995).

RESULTS AND DISCUSSION

Details for the newly developed nine novel microsatellite loci and variability measures across 42 individuals are summarized in Table 1.

Table 1. Characterization of nine microsatellite loci developed for *Cyclina sinensis*.

Locus	GeneBank accession No.	Repeat motif	Primer sequences (5'-3')	T_a (°C)	N_A	Size range (bp)	H_O/H_E	P
C7C	GU980135	$(TG)_8TT(TG)_5CG(TG)_{13}$	F: GATTCTGTGGACTTTGGCTA R: TAAAACTGTGCGGTCTTACA	55	15	248-296	0.250/0.864	0.0026*
C16D	GU980136	$(CA)_{28}$	F: CCACATACATCGCAAACAA R: ACAATACAGACGACGAGGAC	55	15	161-225	0.667/0.626	1.0000
C25A	GU980137	$(TG)_{84}$	F: GCGTGTCTGGACACCACT R: ATCTGACCTCCGCACAAT	46	13	168-350	0.514/0.818	0.0052*
C35B	GU980138	$(TG)_{22}(CGTG)_2TT(TG)_5$	F: CACAATACAGACGACCAG R: ACAATGAATCCACTTTTC	48	24	251-371	0.452/0.931	1.0000
C45B	GU980139	$(TG)_9TA\ (TG)_{11}$	F: GCAGACAATACAGACGAC R: CACAATGAATCCACTTTT	53	17	251-303	0.350/0.901	0.2322
CD19B	GU980140	$(TG)_{18}(TT)_2GTCAGAACG$ $(TG)_2GTTCAACCC(GT)_{11}$	F: TTGGGAAGAACCGTTACTA R: ATCACTGAATTTCGATTACATA	55	21	136-250	0.452/0.741	1.0000
CM32A	GU980141	$(TG)_{67}$	F: AGCCGTGTTGTCGTTGTA R: AATGTCCCAGTAGTCTTGC	50	8	89-247	0.738/0.746	0.1976
CR13A	GU980142	$(TG)_{21}$	F: GCTTTGAAATGATGCCTGA R: ATACCCATTCACGAACGC	48	16	132-170	0.872/0.915	0.9299
CR27A	GU980143	$(CA)_2GGT(TG)_{18}$	F: TTGACAATCCAATACTCTACC R: GTCCTACTCTTCCAAACCT	55	13	161-203	0.119/0.849	0.0000*

T_a = annealing temperature; N_A = number of alleles; H_O/H_E = observed and expected heterozygosities, respectively. *Indicates significant deviation from Hardy-Weinberg equilibrium after Bonferroni's correction for multiple comparisons ($P < 0.0056$).

In total, nine of 35 amplicons from the microsatellite-enriched genomic libraries were successfully amplified and shown to be polymorphic in *C. sinensis*. The remaining 26 loci were monomorphic and failed to amplify. The 9 sequences containing microsatellite loci were deposited in GenBank (GU980135-GU980143). No similarity was found between the nine microsatellites and the published sequences in GenBank. The observed numbers of alleles per locus ranged from 8 to 24 (mean 16). Observed heterozygosity ranged from 0.119 to 0.872 and expected heterozygosity ranged from 0.626 to 0.931. Three loci (C7C, C25A and CR27A) deviated from HWE expectations in the sampled population after Bonferroni's correction (adjusted P value = 0.0056); the remaining six loci conformed to HWE. C7C, C25A and CR27A deviated from HWE possibly due to the presence of null alleles or the existence of subpopulations. Null alleles were found in six loci (C7C, C25A, CR27A, CD19B, C45B, and C35B) and stuttering errors were found in one locus (CR27A) using Micro-Checker (Van Oosterhout et al., 2004) (Bonferroni's correction), but no evidence of allelic dropout was found in any of the loci (Bonferroni's correction). All pairwise tests for linkage disequilibrium among the nine loci were non-significant. These polymorphic microsatellite loci in *C. sinensis* will be useful in studies of conservation genetics and population genetic structure of *C. sinensis* as well as other species of this genus.

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