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Screening and characterization of novel polymorphic microsatellite markers from sea cucumber *Holothuria leucospilota*

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ABSTRACT. The sea cucumber *Holothuria leucospilota* has high medicinal value and rich nutritional edible value, and thus is a commercially important aquatic product in China. Microsatellite loci were developed and screened using a fast isolation protocol and amplified fragment length polymorphism of sequences containing repeats. In this study, 16 novel polymorphic microsatellite markers in *H. leucospilota* were identified, and the relevant genetic variability index was assessed using 30 individuals from a wild population. The polymorphic information content ranged from 0.183 to 0.668, and the number of alleles per locus varied from 3 to 5. The observed and expected heterozygosities were 0.0370-0.5000 and 0.0776-0.6250, respectively. With the exception of 3 loci (Y1-15, Y11-1, and Y28), the polymorphic loci were in Hardy-Weinberg equilibrium ($P > 0.003125$). These polymorphic microsatellite loci will contribute to studies of genetic diversity, the research of population structure, and the design of conservation strategies for *H. leucospilota*.

Key words: *Holothuria leucospilota*; Magnetic bead enrichment; Polymorphic microsatellite loci; Simple sequence repeat

INTRODUCTION

Holothuria leucospilota, commonly known as black sea cucumber, belongs to *Holothuria*, Holothuriidae, Aspidochirotida, Holothuroidea, Echinodermata (Li, 2010), and is widely distributed in the India-Western Pacific Region, Taiwan, Fujian, Guangdong, Guangxi, Hainan, and Xisha Islands in the south of China (Conand, 1998). Its edible and medicinal properties provide great economic benefits to people. However, in recent years, because of the destruction of habitats and other natural environments, as well as the overfishing of wild resources and continuous outbreak of popular diseases, the aquaculture and resource capacity of *H. leucospilota* have sharply declined. Thus, the organism has been listed as "Vulnerable" on the Red List of threatened animals in Singapore (Davison et al., 2008).

Currently, molecular markers are a practical and effective technology for developing conservation strategies and studying population genetic information. Microsatellite markers, which are powerful co-dominant genetic markers, have been widely applied to studies of population genetics, linkage analysis, and resource conservation in various marine organisms, including *Branchiostoma belcheri* Gray (Dai et al., 2013), *Fenneropenaeus penicillatus* (Shangguan et al., 2014), pen shell (Chen et al., 2012), and other animals and plants (Fopp-Bayat and Ciereszko, 2012), but few reports of molecular markers are available in *H. leucospilota*. Microsatellites, also known as simple sequence repeats (SSRs) or short tandem repeats, are repeating sequences of 1-6 base pairs (bp) of DNA (Schlötterer and Pemberton, 1998). The information regarding the genetic background of *H. leucospilota* remains limited. Therefore, screening polymorphic microsatellite loci in *H. leucospilota* is very important for the conservation of the *H. leucospilota* resource.

MATERIAL AND METHODS

Sample genomic DNA extraction

The microsatellite makers were developed using a modified fast isolation method with amplified fragment length polymorphism of sequences containing repeats (Zane et al., 2002). Thirty-two wild *H. leucospilota* individuals were collected along the coast of Hainan Province in the South China Sea in the spring of 2013. Genomic DNA was extracted and purified by using TIANamp Marine Animals DNA Kit (Tiangen Biotech, Beijing, China) and EZNA™ Cycle-Pure Kit (Omega Bio-Tek, Norcross, GA, USA), respectively, from the body wall of a single individual, which was selected from the 32 individuals and used in SSR primer development.

Microsatellite-enriched library construction

High-quality genomic DNA (20 μL 100 ng/μL), which was examined by 1% agarose gel electrophoresis and a superfine ultraviolet spectrophotometer, was digested with 1 μL *Tru*1I (*Mse*) (Fermentas, Vilnius, Lithuania) restriction enzyme in 25 μL at 37°C for 3 h. The digested products, ranging from 400 to 1200 bp, were ligated to *Mse*I adapter A (5'-GACGA TGAGTCCTGAG-3')/*Mse*I adapter B (5'-TACTCAGGACTCAT-3') by T4 DNA Ligase at 37°C for 3.5 h. The digestion-ligation fragments were denatured at 95°C for 5 min and immediately hybridized to the biotinylated oligonucleotide probes $(CT)_{15}$ and $(GT)_{15}$ at 64°C for 1 h. Next, the fragments containing microsatellite repeats (SSR) were captured and gathered

using streptavidin-coated magnetic sphere particles (Promega, Madison, WI, USA), and the noncaptured and loose DNA fragments were washed away using an eluent. The recovered DNA fragments were amplified using *Mse*I adapter A, and the polymerase chain reaction (PCR) products were purified using the EZNA™ Cycle-Pure Kit (Omega Bio-Tek) to remove the extra dNTPs and adaptors. Next, 2 μL purified products were ligated to 0.5 μL PMD19-T (TaKaRa, Shiga, Japan) at 16°C for 8 h and then transformed into *Escherichia coli* DH5α cells (Tiangen) for further selection on ampicillin plates. Positive clones were amplified using M13 general primers and PCR products were separated on 1% agarose gels. The fragments ranging from 400 to 1000 bp were selected for sequencing by Life Technologies (Carlsbad, CA, USA).

Primer design and gene polymorphism test

Microsatellite sequences meeting the requirements were examined using SSRhunter1.3 (Li and Wan, 2005). Microsatellite amplification primers were designed using Primer Premier 5.0 (Clarke and Gorley, 2001). Thirty genomic DNA samples extracted and selected from the above 32 wild individuals from Hainan, China, were analyzed for polymorphisms using microsatellite markers. The PCR was conducted in a volume of 10 μL containing genomic 100 ng/μL DNA, 10X Dream Taq Buffer, 10 mM dNTPs, 5 U/μL Dream Taq DNA polymerase (Fermentas), 10 μM forward primer, and 10 μM reverse primer. The PCR was performed as follows: 1 cycle of denaturation at 94°C for 4 min; 30 cycles at 94°C for 40 s, at the optimal annealing temperature for 40 s (Table 1), and at 72°C for 1 min; final extension at 72°C for 15 min; and storage at 8°C. The PCR products were separated using polyacrylamide gels in a Sequi-Gen Sequencing Cell (Bio-Rad, Hercules, CA, USA); these included a 10-bp DNA ladder (Invitrogen, Carlsbad, CA, USA) that were visualized by silver staining.

Genetic data analysis

Finally, the basic genetic information index of the polymorphic microsatellite loci, such as the number of alleles per locus, polymorphism information content, observed heterozygosities, and the expected heterozygosities were calculated and estimated using the CERVUS 3.0 software (version 3.0), POPGENE 32 (version 1.32) (Yeh et al., 2000), and MICRO-CHECKER (Van Oosterhout et al., 2004).

RESULTS AND DISCUSSION

Using M13 general primers for amplification, 100 fragments (400-1000 bp) of 384 positive monoclonal clones were selected for sequencing. Ninety fragments were successfully sequenced, and 42 microsatellite sequences meeting the requirements were examined. Thirty-two pairs of microsatellite amplification primers were designed. The enrichment rate of positive clones (26.04%) was clearly higher than that for *Penaeus monodon* Fabricius (10.5%). Additionally, the gain efficiency of microsatellites (42%) was much greater than in *Fenneropenaeus chinensis* (7.16%), but significantly less than in *Portunus trituberculatus* (87.5%) and shrimp scallops (98.53%). These observations may have been related to species diversity; the speed, power, and times of the elution; and the freshness of streptavidin-coated magnetic sphere particles during the microsatellite enrichment process.

Sixteen polymorphic microsatellite loci were screened, and the basic genetic information of these primers is presented in Table 1.

Table 1. Basic genetic information of 16 microsatellite primers in *Holothuria leucospilota* (sample size = 30 individuals).

Locus ID	Primer sequences (5'-3')	T_A (°C)	Repeat motif	Allele size (bp)	N_A	PIC	H_O	H_E	GenBank accession No.
Y1-11	F: TCATATAAATTTTATAAGGACA R: GTAAGAGTAGAGGGATAGGGAG	40	$(AC)_{21}G(CT)_{11}(CA)_{12}CG(CT)_{18}$	170-180	3	0.576	0.2609	0.4991	KM880033
Y1-15*	F: ACTGTGAGGTTACTTTGCGTGCT R: GCTTTGTTCAGAATCTTTGTGCT	40.8	$(CA)_{25}(CT)_{18}$	205-220	5	0.328	0.0741	0.2051	KM880035
Y2-7	F: AATATGGGGCATCTATGTACC R: AGGAAATCAAAATAAGGCAAC	55	$(AC)_{18}N\ (TC)_8\ N\ (CT)_{25}$	310-330	5	0.609	0.4167	0.5148	KM880030
Y2-8	F: AGAGTGGAGTGTAGAGAGCAG R: ACTTTGAATTAGTTGTGGGAG	51.8	$(AG)_{27}$	170-180	4	0.612	0.3571	0.6244	KM880037
Y11-1*	F: TGTAAAATAAAATGTAGAGCGAG R: ATAGAGGACTGTTGAGTATGAGC	40.8	$(CT)_{19}$	95-105	3	0.247	0.0370	0.1049	KM880038
Y11-2	F: ATGGTATGGTCCCTCAATAAAT R: GGATAGCACTGAACAAACACTG	46.5	$(GT)_{13}$	110-120	5	0.183	0.2000	0.1872	KM880044
Y67-2	F: ATGAATAAACGATCAGAGA R: ACTAGAAAAGGAGACAACC	40	$(GA)_6$	230-250	4	0.558	0.3077	0.4919	KM880031
Y67-3	F: AGAGAGGAAGAGCGTGA R: AGTGAACTGCTTGTGGT	40	$(GT)_{19}$	186-198	4	0.454	0.4815	0.3861	KM880032
Y3	F: TGTATGCTTATTGTTCGTCTT R: AAATTGCTGTACAAATGTGAG	46.5	$(GT)_{29}$	155-160	3	0.531	0.0800	0.4770	KM880041
Y5	F: ATCAAAAATATTCCATTAG R: ACAGTACATCAGTCCAGTG	46.5	$(GT)_{35}$	155-165	3	0.412	0.3571	0.4082	KM880043
Y16	F: AAGCAGTCAATAAGTTCAT R: ACAGAGTTTACAAGCAGTG	46.5	$(AG)_{18}N(GA)_7\ (GT)_{32}$	170-182	4	0.295	0.0800	0.0776	KM880042
Y21	F: AGAAGGAGAGACAAACGGAT R: TCGCAAAGTTAGAAAGGAGT	60	$(AAAG)_3$	190-205	3	0.388	0.1538	0.2604	KM880039
Y28*	F: CACCTCTAAGATAGAGCCACAA R: ACATCACTCACACCATGAAAAT	40	$(GT)_{21}GG(GT)_{24}$	270-310	4	0.668	0.0833	0.6250	KM880034
Y31	F: TGCAGTCATTACAGCAAATTAGATC R: TACATTCATTGAGAAGGAAAAGAGG	40	$(AC)_{23}G(CA)_{10}$	170-180	3	0.347	0.1250	0.1172	KM880029
Y48	F: AATCAAGGAAAATGTGCCACTAACC R: GCTTCAAAAAATATATGACCGGGAG	40.8	G_{13}	115-120	3	0.356	0.5000	0.3994	KM880040
Y78	F: TAAACCCTCTTTTCCGGTACA R: AGCTCTCCGCTTAGATAGTGC	40.8	$(AG)_{19}\ (TG)_{29}$	180-190	3	0.526	0.2857	0.3628	KM880036

T_A = annealing temperature; N_A = number of polymorphic alleles per locus; PIC = polymorphic information content; H_O = observed heterozygosity; H_E = expected heterozygosity. *Significant deviations of locus from Hardy-Weinberg equilibrium after Bonferroni correction ($P < 0.003125$).

The number of alleles per locus ranged from 3 to 5, and polymorphism information content varied from 0.183 to 0.668. Seven of the 16 newly developed and screened microsatellite loci were considered to be highly polymorphic according to the judgment standard (polymorphism information content >0.5) (Botstein et al., 1980), and may be useful for further genetic studies in *H. leucospilota*, such as pedigree analysis, genetic diversity, construction of genetic linkage maps, and marker-assisted selection breeding studies. Thirteen polymorphic loci were in Hardy-Weinberg equilibrium and there was no genotypic linkage disequilibrium in the population tested after Bonferroni's correction (adjusted P = 0.003125), with the exception of 3 loci (Y1-15, Y11-1 and Y28), which may result from the present of null alleles, natural selection, mutation, for the migration of species.

Observed and expected heterozygosities were 0.0370-0.5000 and 0.0776-0.6250, respectively, which were used to estimate the degree of population genetic differentiation. Dumb alleles, inbreeding, natural selection, the Wahlund effect, and other factors likely caused the observed heterozygosity to be less than the expected heterozygosity. The 16 novel polymorphic microsatellite loci presented here may be useful for further population studies and cultivation of *H. leucospilota*.

Conflicts of interest

The authors declare no conflict of interest.

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