



Development and characterization of polymorphic microsatellite loci in the sea cucumber *Holothuria leucospilota*

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ABSTRACT. *Holothuria leucospilota* is a tropical holothurian species that is widely distributed in the tropical and sub-tropical India-Western Pacific Region. Eight polymorphic microsatellite loci were developed from *H. leucospilota* by using the protocol fast isolation by amplified fragment length polymorphism of sequences containing repeats and tested in 30 individuals from Hainan Island in China. The number of alleles was 2-6 and polymorphism information content ranged from 0.371-0.694. The levels of expected and observed heterozygosities varied from 0.3913-0.6701 and from 0.1154-0.7000, respectively. No significant linkage disequilibrium was detected for any pairwise combination of loci. Only loci YZHS1-42 deviated from Hardy-Weinberg equilibrium. These polymorphic microsatellite loci may be useful for germplasm conservation of *H. leucospilota*.

Key words: Germplasm conservation; *Holothuria leucospilota*; Microsatellite; Sea cucumber

INTRODUCTION

Holothuria leucospilota, commonly known as black sea cucumber, is a tropical holothurian species that is widely distributed in shallow reef areas of the tropical and sub-tropical India-Western Pacific Region (Conand, 1998). Similarly to abalone shells or shark fins, sea cucumber is a commercially important resource for coastal populations. However, because of overfishing, the sea cucumber population has sharply declined in recent years, leading to the listing of *H. leucospilota* as 'vulnerable' on the Red List of Threatened Animals in Singapore (Davison et al., 2008). Studying the distribution of genetic diversity of natural stocks using molecular markers is important for developing conservation strategies, but no reports are available regarding molecular marker isolation in *H. leucospilota*. Microsatellites, also known as simple sequence repeats or short tandem repeats, are repeating sequences of 1-6 bp DNA (Schlötterer and Pemberton, 1998). They are powerful co-dominant genetic markers that have been applied in population genetic studies, linkage analysis, and germplasm conservation (Fopp-Bayat and Ciereszko, 2012). No microsatellite sequences are available in GenBank for *H. leucospilota*. Thus, screening for polymorphic microsatellite loci in *H. leucospilota* is very important for conserving *H. leucospilota* resources.

MATERIAL AND METHODS

We report 8 polymorphic microsatellite loci from *H. leucospilota* identified using the protocol fast isolation by amplified fragment length polymorphism of sequences containing repeats (FIASCO) (Zane et al., 2002). Genomic DNA was extracted from the tube-foot tissues using the Genomic DNA Extraction kit (TIANGEN, Beijing, China). The DNA was digested by the restriction enzyme *Mse*I (Fermentas, Vilnius, Lithuania) for 3.5 h at 65°C, and the digested fragments were ligated to the *Mse*I adaptor A (5'-GACGATGAGTCCTGAG-3')/*Mse*I adaptor B (5'-TACTCAGGACTCAT-3') using T4 DNA ligase (Fermentas) over night at 22°C. Linker ligated DNA was hybridized to the biotinylated probes (CT)₁₅ and (GT)₁₅, then captured with streptavidin-coated magnetic sphere particles (Promega, Madison, WI, USA) and non-annealed DNA was washed away. The recovered DNA fragments were amplified using an *Mse*I primer (5'-GACGATGAGTCCTGAG-3') and purified by GenClean Cycle Pure Kit (Omega Bio-Tek, Norcross, GA, USA) to remove the extra adaptors and dNTPs. The purified products were ligated into the pMD19-T vector (Takara) and then transformed into *Escherichia coli* competent DH5 α cells (TIANGEN). The transformants were selected on LB agar plates containing ampicillin. Recombinant clones were amplified using universal M13 primers and the polymerase chain reaction (PCR) products were visualized on 1% agarose gels. One hundred and thirty-nine positive clones in the size range of 500-1000 bp were selected for sequencing by Majorbio Company (Shanghai, China), and 33 pairs of primers were designed using Primer Premier 5.0 (Premier Biosoft, Palo Alto, CA, USA).

All 33 pairs of primers were validated in 30 individuals of *H. leucospilota* collected from Hainan Island, China. PCR amplification was performed in a 10- μ L reaction volume containing 50 ng genomic DNA, 10X Taq buffer, 2 mM MgCl₂, 0.25 U Taq DNA Polymerase (Fermentas), 0.2 mM dNTP, and 0.4 μ M primer of a gradient thermal cycler (Bio-Rad, Hercules, CA, USA) was used for the amplification reaction. PCR was carried out under the following conditions: 95°C for 5 min, 32 cycles at 95°C for 30 s, at annealing temperature (Table 1) for 30 s, 72°C for 1 min, 72°C for 10 min, and then stored at 4°C. Amplified products were resolved on 6% denaturing polyacrylamide gels in a Sequi-Gen Sequencing Cell (Bio-Rad).

Table 1. Eight polymorphic microsatellite loci for *Holothuria leucospilota*.

Locus ID	Gene Bank	Primer sequence (5'-3')	Repeat motif	Ta (°C)	N_A	PIC	H_O	H_E
YZHS1-7	KF741213	F: CAGACGGGAACATACCAT R: GAAACCGCACCTGACACTC	(GACAAG) ₃	49	4	0.479	0.5000	0.3913
YZHS1-35	KF741214	F: ATCCCAGCAITCTTTGTG R: CAGAGTAGCTCGCAITTC	(CT) ₁₉	49	3	0.396	0.5517	0.4518
YZHS1-42	KF741215	F: AACCGAGACCGAACCACAA R: CTGCCCTACAGAAAAGTAATAACGAG	(CT) ₂₇	57	4	0.575	0.1154	0.5214*
YZHS2-6	KF741216	F: GCGACTGTTCATAGACCATA R: CACATCTGACACCCCACTT	(AG) ₁₈ (TG) ₁₁	45	4	0.535	0.5357	0.5230
YZHS2-15	KF741217	F: TAACGGAGGGGAGGGTCA R: GGGAGGAGGGGAGGAAAAG	(GT) ₃₇	50	6	0.694	0.6154	0.6701
YZHS2-17	KF741218	F: TATAATTTGGTCGGGAACG R: AGTAAAGGGGCAGCATCA	(GT) ₁₈	53	3	0.482	0.6292	0.4753
YZHS2-47	KF741219	F: CGAAACCCACCGAAACTG R: AACCGAGACCGAACCACA	(GA) ₃₂	45	2	0.371	0.3333	0.4911
YZHS2-55	KF741220	F: AGACACCAAACAAGAACTCC R: AGGCACTGAAGGGTAAAAT	(GA) ₄₅ (GT) ₂₈	42	2	0.375	0.7000	0.4994

Ta = annealing temperature; PIC = polymorphism information content; N_A = number of alleles per locus; H_O = observed heterozygosity; H_E = expected heterozygosity. *Significant deviations of locus from Hardy-Weinberg equilibrium after Bonferroni's correction ($P < 0.00625$).

RESULTS AND DISCUSSION

We successfully amplified 8 polymorphic loci (Table 1). The number of alleles per locus, polymorphic information content and observed and expected heterozygosities were calculated using POPGEN32 (version 1.32) (Yeh et al., 2000) and CERVUS 3.0 (version 2.2.3) softwares. Deviations from Hardy-Weinberg equilibrium and genotypic linkage disequilibrium were tested using POPGENE 32 (version 1.32).

The number of alleles ranged from 2-6. The polymorphism information content ranged from 0.371-0.694. The levels of expected and observed heterozygosities varied from 0.3913-0.6701, and from 0.1154-0.7000, respectively. No significant linkage disequilibrium was detected for any pairwise combination of loci. Most loci were in Hardy-Weinberg equilibrium ($P > 0.05$), except locus YZHS1-42, even after sequential Bonferroni's correction ($P < 0.00625$), which may be attributed to the limited number of samples tested.

These polymorphic loci represent the first microsatellites isolated for *H. leucospilota*, increasing the available molecular markers for this species. These loci will be useful for studying genetic diversity, population structure, and conservation strategy design.

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