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Identification and characterization of microsatellite markers from the tropical sea cucumber, *Stichopus horrens* (Selenka)

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ABSTRACT. Tropical commercial sea cucumber *Stichopus horrens* is extensively distributed throughout the tropical Indo-Pacific region, and wild stocks have been severely depleted over the past decade. In this study, we used the microsatellite enrichment library of *S. horrens* to identify and characterize 13 microsatellite loci, including 11 polymorphic loci and 2 monomorphic loci. Among the 11 polymorphic loci, the number of alleles was 3-8. The observed and expected heterozygosity varied from 0.1364 to 0.8966 and from 0.1653 to 0.7551, respectively. Additionally, all 11 polymorphic loci showed moderate and high polymorphism with the polymorphism information content (0.271-0.7311). A total of 9 polymorphic loci were in Hardy-Weinberg equilibrium, except for 2 loci (adjusted P = 0.004545). Linkage disequilibrium was not detected in any pairs of polymorphic loci. The present study will be useful for studying genetic structure, population conservation, and breeding of wild *S. horrens*; moreover, our results contribute to the phylogeny and evolutionary research of Holothuroidea.

Key words: Fast isolation method with amplified fragment length polymorphism of sequences containing repeats; Microsatellite markers; *Stichopus horrens*; Tropical commercial sea cucumber

INTRODUCTION

The tropical sea cucumber, *Stichopus horrens*, Selenka, 1867 (Echinodermata: Holothuroidea), is widely distributed in the west Pacific from Malaysia to Society Islands, around French Polynesia, and from Southern Japan and Hawaii to New Caledonia (Schoppe, 2000; Massin, et al., 2002; Hearn and Pinillos, 2006). With its greyish background color and its very large conical, dorsal papillae, particularly the only species with tack-like tables among *Stichopus* species, *S. horrens* is easy to identify among *Stichopus* species (Massin, et al., 2002). *Stichopus horrens* typically roosts in shallow sea areas within 15 m, hides itself under coral and rocks, and is nocturnal (Schoppe, 2000).

As a commercial sea cucumber in China, *S. horrens* is harvested for traditional medicine and delicacies, and is generally consumed worldwide (Baine and Choo, 1999; Chen, 2003; Rasolofonirina et al., 2004). However, over-fishing and habitat destruction have severely depleted the wild resources of *S. horrens*. Microsatellites, or simple sequence repeats (SSRs), are stretches of DNA consisting of tandemly repeated short units of 1-6 base pairs (bp) in length and are ubiquitous in prokaryotes and eukaryotes (Beckmann and Weber, 1992; Chistiakov, 2006). The microsatellite marker is popular in the field of population genetics research as the second generation of molecular marker technology. Additionally, genetic studies of *S. horrens* are rather limited, except for 16microsatellite loci developed by Yuan et al. (2012). The demand for sea cucumbers in China and abroad is increasing, and thus it is urgent to take immediate measures to maintain the natural population and implement sustainable practices in southern China, such as closed seasons/areas, captivity, aquaculture, and breeding (Yuan, 2012; Hu, 2013).

In this study, we conducted genetic characterization of 13 novel microsatellite loci, including 11 polymorphic loci and 2 monomorphic loci. Our results provide new basic information for genetic studies of *S. horrens*, such as genomic structure, population diversity, phylogenetic analyses, and cross-amplification among related species.

MATERIAL AND METHODS

Specimen collection and genomic DNA extraction

Thirty-five wild adult *S. horrens* individuals were collected in Sanya, Hainan Province, China. After dissection, each body wall of the samples were soaked in 100% alcohol, transported to the laboratory, and stored at -20°C. High-quality genomic DNA was extracted from the body wall of 35 samples using the TIANamp Marine Animals DNA Kit (Tiangen Biotech, Beijing, China), followed by purification with EZNA™ Cycle-Pure Kit (Omega Bio-Tek, Norcross, GA, USA), and then examined by 1% agarose gel electrophoresis and a superfine ultraviolet spectrophotometer.

Microsatellite-enriched library construction

The process of microsatellite-enriched library construction was based on a modified fast isolation method with amplified fragment length polymorphism of sequences containing repeats (Zane et al., 2002). First, 20 µL 100 ng/µL satisfactory genomic DNA mixed with 4 randomly selected samples was digested into 300-1200-bp fragments with 1 µL Tru1I (*Mse*I) (Thermo Scientific, Waltham, MA, USA) restriction enzyme in 25 µL at 65°C for 3 h and 80°C for

10 min. The digested products with sticky ends were linked to pretreated adapters (adapter A5'-GACGATGAGTCCTGAG-3' and adapter B5'-TACTCAGGACTCAT-3') by T4 DNA Ligase (Thermo Scientific) at 37°C for 3.5 h. Following denaturation of the adapter-ligated products, the library was hybridized to (CT)15 and (GT)15 probes labeled with biotin at 61°C for 1 h. Subsequently, the target fragments containing microsatellite SSRs were captured using streptavidin-coated magnetic beads (Promega Corporation, Madison, WI, USA). After elution, the highly purified SSR products were amplified by polymerase chain reaction (PCR) using the above adapter A. Finally, 4 µL microsatellite enriched library was ligated into 1 µL PMD19-Tvector (TaKaRa, Shiga, Japan) at 16°C for 3.5 h, and then the recombinant plasmids were introduced into *Escherichia coli* DH5α strains (Tiangen Biotech) for further selection on LuriaBertani agar medium containing 100 µg/mL ampicillin. Positive monoclones were randomly obtained, amplified using M13 general primers, and evaluated by 1% agarose gelselectrophoresis. The selected fragments of 400-1000 bp were sequenced by Life Technologies (Carlsbad, CA, USA).

Microsatellite sequence discovery and primer screening

After the vector and adapters were removed from the sequenced results, sequences containing a perfect microsatellite motif of at least 5 repetitions for any microsatellite motif of 1-6 bp were selected using SSR hunter 1.3 software for further analyses (Li and Wan, 2005). Next, microsatellite amplification-specific primers were designed from applicable flanking sequences using Primer Premier 5.0. The optimum annealing temperature of different primers was screened by gradient temperature PCR and a mixture of DNA template.

DNA amplification and genotyping

Some microsatellite markers were tested for polymorphisms using a panel of 30 genomic DNA samples selected from the above 35 wild individuals after preliminary screening of all primers. The protocol for PCR was conducted in a 10-µL volume: 100 ng/µL template genomic DNA, 10X Dream Taq Buffer, 10 mM dNTPs, 5 U/µL Dream Taq DNA polymerase, and 10 µM forward and reverse primers. PCR was performed under the following cyclic conditions: 95°C for 5 min; followed by 35 cycles of 94°C for 40 s, annealing temperature (Table 1) for 40 s and 72°C for 1 min; final extension at 72°C for 20 min. The PCR products were separated using 6% polyacrylamide gels in a vertical Sequi-Gen Sequencing Cell (Bio-Rad, Hercules, CA, USA) at a constant temperature of 50°-55°C. Additionally, 10-bp DNA ladder (Invitrogen, Carlsbad, CA, USA) was used as a size standard. Finally, the gels were visualized by silver staining and documented by counting the alleles.

Microsatellite data analysis

Null alleles and scoring errors of all SSR loci were assessed using MICRO-CHECKER ver. 2.2.3 (Van Oosterhout et al., 2004). POPGENE 32 (version 1.32) (Yeh et al., 2000) was used to evaluate genotypic linkage disequilibrium, Hardy-Weinberg equilibrium, observed heterozygosities, and expected heterozygosities. Significance values were adjusted for multiple comparisons using Bonferroni corrections when necessary (Rice, 1989). The number of alleles per locus and the polymorphism information content were estimated using CERVUS 3.0 (version 3.0).

Table 1. Basic genetic information of 13 microsatellite loci in *S. horrens* (N = 30 individuals).

Locus	Primer sequences (5'-3')	Ta (°C)	Repeat motif	Allele size (bp)	N_A	PIC	H_O	H_E	GenBank No.
sh1*	F: ATACAAGCACACAGTCAGACACAT R: TTTAGTTTGATAGTACCACGGAGC	47.0	$(AC)_{28}$	128-138	4	0.600	0.1429	0.5091	KR653147
sh2	F: GTAGAAAATCTGGAAAGCAACA R: CGGTAACCTTAGTCGTATGACA	40.0	$(TTTA)_3T_{11}$	230-240	3	0.309	0.2857	0.2449	KR653148
sh3*	F: ACGAACTGGTTTTGCTGCG R: TGGCTATATCCATTGGCGG	47.0	$(AG)_{25}$	400-410	3	0.545	0.1364	0.4163	KR653149
sh4	F: GCAGCAAAGCATAGCCAACA R: GTCTCTCTACCGCCACCCAC	40.0	$(AG)_{46}$	158-165	4	0.580	0.6842	0.4668	KR653150
sh5	F: GCTGTGACTTGTGATGAG R: GATACTGTGTAGGTGTGC	51.8	T_{12}	195-205	3	0.554	0.6957	0.4537	KR653151
sh6	F: AAAGTTATGACAGCGGGTGAT R: CAGCAGTATGTAGTCCGTGAA	42.0	$(AG)_{31}$	155-160	3	0.584	0.5455	0.4959	KR653152
sh7	F: CAACAAAGTAAAAAGGAGGTGGGG R: CGGAAAAAGTGGCTTATGTCAGAG	45.0	T_{15}	210-220	3	0.382	0.4643	0.3565	KR653153
sh8	F: GAAAAACGGAAAGGAGAAGAG R: CGTACACCTATGTATGCGGAC	53.0	$(AG)_{29}(TG)_{23}$	180-190	3	0.412	0.1818	0.1653	KR653154
sh9	F: CCACCGACACAGGAACAAA R: CGCAGGTCAAATCAGCAAA	48.6	$(AC)_{48}AT(A_C)_5$	270-280	3	0.476	0.5769	0.4105	KR653155
sh10	F: TGTGTGTAGGCTATGTGAAA R: AACGAGATAAATGCGTGAAG	42.0	C_{12}	160-180	8	0.734	0.8966	0.7551	KR653156
sh11	F: GAGGAAGAAAAAACAAAAGAA R: AAAACAGCAATAATAGAACAT	47.0	$(TG)_5$	100-105	3	0.271	0.1429	0.1913	KR653157
sh12	F: CTCCTTAGTGAAAGTTATGACAGCG R: ACACTTGAGGGTATATAGCCAGGTC	46.5	$(AG)_{41}$	189	-	-	-	-	KR653158
sh13	F: TGAAACAAGCACACTGTAATCA R: GAGCAATAACTGTAAAGCGGTA	40.8	$(AC)_{51}$	363	-	-	-	-	KR653159

Locus name, primer sequences (F: forward, R: reverse), annealing temperature (Ta), repeat motif, allele size, number of polymorphic alleles per locus (N_A), polymorphic information content (PIC), observed heterozygosity (H_O), expected heterozygosity (H_E), and GenBank accession Nos. are shown. *Significant deviations of locus from Hardy-Weinberg equilibrium after Bonferroni correction ($P < 0.004545$).

RESULTS AND DISCUSSION

From the microsatellite enrichment library of *S. horrens*, 110 positive clones were randomly selected for sequencing, and 80 microsatellite sequences were obtained; the positive rate of cloning was 72.72%. As described by Zane et al. (2002), wide differences exist in the efficiency of microsatellite enrichment, ranging from 50% (*Passera lagia*) to 95% (*Sparus aurata*) based on the fast isolation method with amplified fragment length polymorphism of sequences containing repeats method. Most frequently encountered repeat motifs were di-nucleotides (77%), followed by mononucleotides (23%) and tetra-nucleotides (2%). Interestingly, mononucleotide contigs were frequently observed, and the core base was repeated more than 10 times, such as for loci sh5, sh7, and sh10. These results may be related to the gene structure of different species, the type of probe, and the restriction enzyme. Thirteen microsatellite loci, including 11 polymorphic loci and 2 monomorphic loci, were isolated from the synthetic 43 pairs of specific primers after polymorphism testing.

All genetic data from the 11 polymorphic loci were analyzed and evaluated. The results showed that the number of polymorphic alleles per locus was 3-8 (total 40 alleles, mean 3.6). Observed heterozygosity (0.1364-0.8966) was significantly lower than expected heterozygosity (0.1653-0.7551), which may be related to the Wahlund effect (Li et al., 2004), natural selection, and null alleles, particularly inbreeding. In addition, of the 11 polymorphicloci, 6 loci were in high polymorphism with polymorphic information content >0.5 according to the judgment standard (Botstein et al., 1980). Finally, 9 polymorphic loci were in Hardy-Weinberg equilibrium after

application of Bonferroni correction (adjusted P = 0.004545), except for 2 loci (sh1, sh3); no genotypiclinkage disequilibrium was observed. No genotyping error among the loci was detected.

In this study, we present a set of novel microsatellite markers in the sea cucumber *S. horrens*. This basic information will be used in our further studies of population and conservation genetic studies of *S. horrens*. Furthermore, the results will be useful for preserving the aquaculture of *S. horrens* and for enriching the phylogenetic analyses of the holothurians, which can also be applied to echinoderms.

Conflicts of interest

The authors declare no conflict of interest.

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