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Development and characterization of 70 novel microsatellite markers for the sea cucumber (*Apostichopus japonicus*)

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ABSTRACT. The sea cucumber (*Apostichopus japonicus*) is an important item in Asian cuisine. It is currently produced through aquaculture, especially in China, after being overexploited in the wild in the 1990s. We isolated 70 novel polymorphic microsatellite loci using an enrichment-colony hybridization protocol. All loci were characterized in 48 individuals from a natural population in Rongcheng (Shandong, China) using genomic DNA isolated from muscle tissue. The number of alleles ranged from 2 to 17 (mean 7.0), and the observed and expected heterozygosities varied from 0.0010 to 1.0000 and from 0.2125 to 0.9477, respectively. Thirty-one of the 70 loci exhibited departure from Hardy-Weinberg equilibrium. These microsatellite markers should be useful resources for population genetic studies and

for molecular marker-assisted breeding of *A. japonicus*.

Key words: *Apostichopus japonicus*; Microsatellite; Polymorphic marker

INTRODUCTION

The sea cucumber, *Apostichopus japonicus*, is naturally distributed along the coast of China, Japan and Korea in the Western Pacific Ocean (Chen, 1990). This species is considered a delicacy in these countries. Due to strong consumer preferences, the demand for sea cucumber products increases insatiably. However, large demand has resulted in overexploitation and environmental deterioration. To satisfy the increasing demand and protect marine resources, the sea cucumber industry has developed rapidly in recent years. In an effort to increase the efficiency and profitability of aquaculture production systems, genome analysis on the sea cucumber has been carried out. To conduct genetic studies, molecular markers such as polymorphic microsatellites have been widely used as a principal tool. Although some microsatellite loci have been developed for *A. japonicus* (Kanno et al., 2005; Chen and Li, 2007; Zhan et al., 2007; Peng et al., 2009), more microsatellite loci are still needed to promote the implementation of genetic analysis and breeding programs. In this paper, we report the isolation and characterization of 70 novel microsatellite loci in *A. japonicus*.

MATERIAL AND METHODS

DNA extraction and enrichment for microsatellites

Genomic DNA was extracted from muscle tissue according to the protocol described by Zhan et al. (2007). Microsatellite markers were isolated by the enrichment-colony hybridization method reported by Fischer and Bachmann (1998) and Zhan et al. (2007) with some modifications. Approximately 10 µg extracted DNA was digested with 50 U *Alu*I restriction enzyme at 37°C for 6 h. The 400- to 1500-bp DNA fragments were selected with a 1% low melting temperature agarose gel. The 21-mer (5'-CTCTTGCTTGA ATTCGGACTA-3') and phosphorylated 25-mer (5'-pTAGTCCGAATTCAAGCAAGAG CACA-3') adaptors were ligated to the selected fragments using T_4 DNA ligase at 16°C for 12 h. The ligated fragments were amplified using a single adaptor (21-mer) as primers. The PCRs were performed as follows: 5 min at 94°C, and then 25 cycles of denaturation for 45 s at 94°C, annealing for 45 s at 55°C, and extension for 45 s at 72°C, and a final extension for 10 min at 72°C. The amplified products were enriched for microsatellite sequences through hybridization with $(AC)_{15}$, $(AG)_{15}$ or $(ACAG)_8$ oligonucleotides bound to nylon membranes. All hybridizations took place at 37°C for 12 h followed by washes of 58°C in 2X SSC, 1% SDS for three times. The quantity of captured DNA was increased by reamplification with 21-mer primer and rTaq DNA polymerase (denaturation for 5 min at 94°C; 25 cycles of 45 s at 94°C, 45 s at 55°C, and extension for 45 s at 72°C, and an additional extension for 1 h at 72°C). The PCR products were cloned into pMD 18-T vector (TaKaRa) and transformed into competent *Escherichia coli* DH5α cells. Then, the clones were rearranged in order on a new agar plate and transferred onto nylon membranes. The

blots were screened with digoxigenin labeled $(AC)_{15}$, $(AG)_{15}$ and $(ACAG)_8$ probes (Roche Applied Science), then, positive signals were generated using the DIG detection system (Roche Applied Science).

Primer design and PCR amplification

Some of the positive clones, which were screened via PCR and 21-mer primer, were sequenced using the sequencing primer M13-47. After discarding redundant sequences and examining the length and GC content of the flanking regions, primers were designed using the primer premier 5 software (Premier Biosoft International). After optimization of the PCR parameter, primer pairs that produced clear amplifications of the expected size were selected for microsatellite polymorphism analysis. A total of 48 sea cucumber individuals from Rongcheng (Shandong, China) were sampled to test for polymorphisms. PCR amplifications were carried out in a 20-μL reaction mixture containing 1 U rTaq DNA polymerase (TaKaRa), 1X PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTP mix, 0.2 μM of each primer set, and 50-100 ng template DNA. PCR cycles were as follows: initial denaturation of 94°C for 5 min, followed by 35 cycles of 94°C for 30 s, primer specific annealing temperature for 30 s, 72°C for 30 s, and a final extension step at 72°C for 5 min. PCRs were performed in a thermal cycler (GeneAmp PCR System 9700, Perkin-Elmer ABI). The amplification products were separated by electrophoresis on a 12% nondenaturing polyacrylamide gel, stained with ethiduim bromide and visualized under UV light. Allele sizes were estimated using a 100-bp ladder molecular size standard (Invitrogen).

Data analysis

POPGENE32 (Yeh and Boyle, 1997) and ARLEQUIN softwares (Schneider et al., 2000) were used to calculate the number of alleles, observed (H_O) and expected (H_E) heterozygosities, Hardy-Weinberg equilibrium (HWE) and linkage disequilibrium, respectively. All results for multiple tests were corrected using Bonferroni's correction (Rice, 1989).

RESULTS AND DISCUSSION

Of the 1000 recombinant colonies screened, 723 gave a positive signal (72.3%). Plasmids from 300 positive clones randomly selected were sequenced, 295 contained at least one microsatellite (98.3%). After discarding redundant sequences and examining the length and GC content of the flanking regions, primers were designed for 121 sequences.

Of the 121 primer pairs, 70 loci showed clear and scorable amplification patterns, and were polymorphic in the 48 sea cucumber individuals tested. The number of alleles observed per polymorphic locus varied from 2 to 17 (mean 7.0). The H_O varied from 0.0010 to 1.0000, while H_E ranged from 0.2125 to 0.9477. Significant deviation from HWE ($P < 0.05$) was detected at 31 loci, which showed a significant heterozygote deficiency (Table 1). The presence of null alleles or population structure may be responsible for the deviations from HWE. Eight locus pairs displayed linkage disequilibrium after Bonferroni's correction. These polymorphic microsatellite markers developed in the present study will provide a useful tool for further genetic studies of *A. japonicus*.

Table 1. Characterization of 70 novel microsatellite markers for *Apostichopus japonicas*.

Locus	Primer sequence (5'-3')	T_a (°C)	Repeat motifs	Range	N_A	H_O/H_E	Accession No.
1S45	F: CAGTGATGACATTATATTGGGC	56	$(TG)_{11}$	157-183	9	0.2917	JF692822
	R: TAGGAAGCGTCCTGTAGTTGC					0.8262*	
1S46	F: TTGAAGTACATACACTTTGCC	50	$(CA)_{31}$	120-158	9	0.6429	JF692823
	R: ATTACTCCTAAATTGAGTCCC					0.8889	
1S55	F: CATGCTATGATAAGTCCTCCTG	56	$(TG)_8$	254-308	6	0.5455	JF692826
	R: AAGATGAATGCCAATTCCCG					0.8425*	
3S08	F: TTGAGTACACAAAGCAAGCG	45	$(CA)_{14}$	129-161	10	0.4167	JF692848
	R: GGACTAAAATGGTGAGTAGGAC					0.8892*	
3S14	F: GGAAACCATCATGTTAATGC	58	$(TC)_7$	231-265	7	0.1739	JF692851
	R: ATCAACACTGCCAATTTGTG					0.7072*	
3S16	F: TTATCCTCATCCATACCGTC	58	$(AG)_{10}GTG(GA)_{22}$	255-291	5	0.8261	JF692852
	R: AAGTTATCTGTTCACCGTCG					0.7923	
3S17	F: CTGTGTTTGAAGAGCAAAGT	45	$(AG)_{26}$	177-203	8	0.8260	JF692853
	R: CATTCCATCTAACCCAGTATC					0.8029	
3S18	F: GCACTACCCACGACATATAAG	62	$(AC)_7 \ldots (CA)_{14}$	207-229	3	0.9583	JF692854
	R: ATCAGCAACCACCAAGCAAG					0.6658*	
3S22	F: GCTTATCATAGATGTCAGTTTGC	58	$(TG)_9TATGG\ (GT)_{10}$	188-224	8	0.4348	JF692856
	R: CAATCTGTGATTTCATAGGTGC					0.7101*	
3S24	F: CAACAGGTAAAGAATGAAATG	62	$(GT)_8$	135-167	7	0.2273	JF692857
	R: ATTAGCAAGTAAACGTCTGC					0.8383*	
3S29	F: AAAGGAACCTATGCAGTCAGG	62	$(GT)_7$	175-211	8	0.4545	JF692860
	R: GTGGATGGTTTTGCACATTG					0.8784*	
3S33	F: ACCAGTTAACAAGTTAAGATGGC	50	$(CA)_{27}$	166-176	3	0.2777	JF692861
	R: CATTTCGGTTACAAGCACCTC					0.2125	
3S34	F: CAGAATTGACGAGATACGATAAG	50	$(GT)_{26}$	233-251	4	0.7778	JF692862
	R: CAGAAATGGACCAGGAAACC					0.6937	
4S02	F: AAGCGTAACAGAAAGAACAC	56	$(CA)_{12}$	144-186	13	0.9167	JF692827
	R: CCTCACCAGTAACACAGAATC					0.9131	
4S05	F: CCAATGCTTTACTTGATACAACC	48	$(GT)_{13}(GA)_{29}$	196-226	4	0.2632	JF692830
	R: CATGCACTTTAACAGTCCTCAG					0.3926	
4S08	F: GCATGTTAGACATTATGGTTCAG	56	$(GT)_{22}$	180-262	11	0.9583	JF692831
	R: GTACCCAAGCAAGTGTCACAG					0.9113*	
4S23	F: CCTATCCATTTTCCAAGTGTC	56	$(TG)_{11}$	167-191	11	0.5417	JF692884
	R: AAGCCCTGAAATCATGTCAAG					0.8892	
AJ04	F: TTACAACTCTTCTCCCCCTC	62	$(GA)_{14}$	232-256	9	0.1818	JF692788
	R: TCGTATATCCCTGTTCTCCTC					0.8721*	
AJ06	F: TATTGTAGGAAGGGTAAGTCG	62	$(GCAC)_4$	240-252	3	0.0010	JF692789
	R: CTGAGTGTTGAATTTCTGGC					0.5643*	
AJ08	F: CTACATCTTATGCAACCCTGC	58	$(CT)_{23}(GT)_{31}$	199-293	8	0.5238	JF692791
	R: GGAACTCATTGTTTTGCTTACC					0.5761	
AJ09	F: AATATGTTAGAGAAGGTCCTCC	60	$(CTGT)_{12}$	190-246	12	0.4706	JF692792
	R: GTAAGACAGACACATACAAAGG					0.8930	
AJ10	F: CAGAGGTTTCCTATTGTTAAGTG	56	$(AGAC)_{21}(AGAT)_6$	243-271	3	0.3750	JF692793
	R: CGTTCTGTTGTCTGACATTTC					0.5667	
AJ13	F: CCAACACTGGAGTCTAACGAG	62	$(CTTCT)_3$	243-313	11	0.3750	JF692796
	R: GATAACACAGTGACAAGCAAGG					0.8342*	
AJ14	F: GATAGACGGATAGATGGATAG	62	$(GACA)_{12}$	302-362	11	0.6667	JF692797
	R: CGATACAGTGTTACAAACAAGC					0.9111	
AJ17	F: CAAAGTGCTCTATTTCCCTCC	60	$(CTGT)_{12}$	122-206	17	0.5417	JF692798
	R: TTCCTGCTTTTCAAACCACC					0.9477	
AJ19	F: TGCTCCTTCTGTTTCTACCTTC	60	$(CAGA)_{14}$	328-384	10	0.5417	JF692799
	R: GGCTCCCCCATTTTTATCAC					0.8537	
AJ28	F: GATGATTCTGATACACATACCC	60	$(AGAC)_{11}(CGAC)_{17}$	264-320	7	0.4000	JF692802
	R: CATCTGAAGTCAAAGCCGAG					0.5359	
BH02	F: CAAATACTCTATATGTTCTGAGGG	50	$(TC)_{32}$	257-259	2	0.2222	JF692832
	R: TAAGAAATGGTCAGTGCTGGT					0.3660	
BH021	F: GAATGTCTGACTCATTTGTGC	48	$(GTT)_4$	183-261	9	0.6923	JF692833
	R: AACTGACAGTCTTGTTTGACG					0.8738	
BH05	F: CAACCTATTAACTCGTTATGGC	62	$(TG)_{10}$	132-180	10	0.7083	JF692834
	R: ATCCTCTACTCGACAGTCTCC					0.8821	
BJ43	F: TGTTCCTCGTAGTTAATAGCC	56	$(AC)_{12}$	181-235	11	0.6087	JF692803
	R: CAGCAACCTTTTCCTGTGTAT					0.8870	

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Table 1. Continued.

Locus	Primer sequence (5'-3')	T_a(°C)	Repeat motifs	Range	N_A	H_O/H_E	Accession No.
FS01	F: CACGTTTTGTTGACCTCACC	48	$(TG)_{33}$	192-194	2	0.5000	JF692804
	R: TTTATCCCATCACACGAAGC					0.5303	
FS07	F: ATGGCAGTTTGGATCGAGTG	52	$(TG)_{29}$	311-323	2	0.3529	JF692805
	R: CGCAGCAACATCAAAAGTTC					0.2995	
FS08	F: TACCTCATGGACAAGATTCG	48	$(GT)_{26}$	221-249	7	0.6364	JF692806
	R: ACACCAGTGTGTTCTCTCAAG					0.6883	
HC506	F: AATAGAACGACTGAACCAAGG	50	$(GA)_{25}(GT)_{14}$	157-171	9	0.4783	JF692784
	R: GAAAGTATCAAAAGTAGCACACG					0.3961*	
HD303	F: CATTTGGCTCGACTGACGAC	62	$(TG)_{12}$	199-243	8	0.2381	JF692863
	R: TAGGTCAAGGGTCAATAAAGG					0.7422*	
HD307	F: TGCTTCTTTGCGAGTTCCTG	62	$(CACG)_4(CA)_{15}(TACA)_{14}$	277-295	4	0.6250	JF692866
	R: GGATGAGATGTAGGGACCAAT					0.7250*	
HD308	F: GAGATACCGTGTTTACAAGCC	45	$(CA)_9CG(CA)_9$	211-257	9	1.0000	JF692867
	R: GTCAGGACACCTATATGTTTCG					0.8992	
HD406	F: CTGACATGGATGTCCACCAG	62	$(GT)_{25}$	229-263	11	0.3333	JF692870
	R: ATCATGTTTACAAGCGAGGC					0.8483*	
HD407	F: GTTCCATTACCGTCCATTGC	62	$(TG)_8$	198-222	7	0.7500	JF692871
	R: GTTGGCACGTTGGAGGTTAC					0.8271*	
HD409	F: ATTCTGGTTGAGTTGGTTCG	62	$(CA)_{12}$ ... $(CA)_9$	172-298	9	0.6667	JF692872
	R: AGATTGGAGTCTACGGTATGG					0.8785	
HD411	F: CAGAGGCTCGGTAAGTAATG	62	$(TG)_7$ ... $(GT)_9$	236-252	4	0.3750	JF692873
	R: TCTATCTATGCAGTCTAGTGGC					0.7145*	
HD417	F: CACTTCAAAGAACAAACGACAG	50	$(TC)_{12}$	238-244	3	0.3128	JF692876
	R: CATGAGTTGTTCGTCCTTGA					0.3943	
HD419	F: CACCCAGCAAGTTTGGACATC	58	$(CA)_9CG(CA)_9$	118-128	3	0.5000	JF692877
	R: GCACACGCCCGTATGTTTC					0.4140	
HD420	F: ACATGATCTTTCACCACTGCC	62	$(AC)_{14}$	166-170	3	0.8235	JF692878
	R: TCCTTGAATCTTTGTGATGCTG					0.6471*	
HD422	F: CTCACTCGGCTTTACTTCCT	45	$(TC)_{22}$	141-151	4	0.5500	JF692880
	R: CAAACATACAAGTCCATGTCC					0.4487	
HD602	F: CTTCTACTCAATGTGGTAATCC	62	$(CA)_{19}$	240-254	5	0.5101	JF692881
	R: CTGTTCATGTTATCTTGGGC					0.8094*	
HD604	F: AACATGGCAGAATTCAACGAC	45	$(TG)_{24}$	202-218	6	0.2857	JF692882
	R: GCACACCACTTACTCAAAACAC					0.6876*	
HD608	F: TGTGACAATCAACAACAACC	55	$(CA)_{17}CGCAT(AC)_{10}$	154-172	8	0.3529	JF692883
	R: TTCATACTAATGGGAAAGGG					0.8217*	
HS16	F: CTAACCGCTTCACTAGGCTTTG	62	$(GA)_8TGG(GA)_3$	265-307	4	0.4167	JF692785
	R: CGTCATGTTTCATTGTCATCG					0.5955*	
HS38	F: AGGGATTGCCTAATCATTATG	60	$(GT)_2ATGTCT(GT)_5$	214-248	7	0.0909	JF692786
	R: CACTTGATCTGAGTATTCTGC					0.8223	
PS205	F: GACTACTATAGCAGTGACCTCG	56	$(CA)_{12}$	144-160	3	0.1818	JF692836
	R: GTTTACATCTATCCATGTGATTG					0.5032	
PS245	F: ACTTAACCACACACCAGAAATG	52	$(GT)_{11}AC(GT)_7$	158-206	7	0.7391	JF692838
	R: ACCAATCACACTGGCACAAG					0.6715*	
PS440	F: AAATACAACCCTGGGAAGCC	62	$(GA)_{19}$	123-207	4	0.6667	JF692839
	R: CACCCGAAGACGAAATGAAC					0.7316*	
PS445	F: CACTTATTTTCTCACCAGACACG	45	$(AG)_{10}ATG(GA)_{24}$	229-273	5	0.6364	JF692841
	R: CGCAACTCAACAAGTTGTGC					0.7219	
PS457	F: GGGGATGAGATGTAGGGACC	54	$(TATG)_{15}(TG)_{14}$	324-413	6	0.4118	JF692842
	R: ACACGAGCTTGCATACCTGC					0.7241*	
PS462	F: TCAGTTCCTTATATCTCGTTCT	45	$(TC)_{22}$	165-189	4	0.8000	JF692844
	R: AATTCATTTACGGGCAGGTC					0.7000*	
PS470	F: CAGCCATCTCATTTTGTTCTAC	62	$(TG)_8$ … $(GT)_7$	259-299	5	0.2914	JF692845
	R: CAGATAATACCACTTTACACCG					0.4848*	
PS474	F: CAATCAGATGCGATTTAGAGAC	62	$(CT)_{15}$ … $(CT)_{10}$	267-348	7	0.3913	JF692846
	R: ATACCCTTCCTATCCTATCCAG					0.6947*	
QS06	F: AAATACAACCCTGGGAAGCC	62	$(GA)_{22}$	160-230	4	0.6667	JF692807
	R: AGCCGTAGACGAATTGAACC					0.5151	
WS68	F: GTCCGAATTAGAACATAGAGAC	48	$(AG)_{29}$	189-243	6	0.2667	JF692835
	R: AGCCCAAATTGAACGAATAG					0.7770*	
XS03	F: ATACAACACCCTGACATAGCG	62	$(GT)_{10}...(TG)_{10}$	271-303	10	0.5217	JF692819
	R: TGAGATAGAGCGTACCCAAG					0.8386	

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Table 1. Continued.

Locus	Primer sequence (5'-3')	T_a(°C)	Repeat motifs	Range	N_A	H_O/H_E	Accession No.
XS05	F: TGAGGAGGAAGACGATCTATC	56	$(CA)_{12}$	131-185	9	0.7778	JF692820
	R: ATTCGGACTATATCACCCCAT					0.8746	
XS06	F: GTTCATCTACTCACTATGGGC	62	$(CA)_{13}T(CA)_{10}$	184-256	10	0.5714	JF692808
	R: ATACTGTCACATCAAGCACG					0.9101	
XS19	F: ATGGAGCAACTGTGTGTCAAG	62	$(CA)_{14}$	147-179	8	0.6250	JF692812
	R: GCCAAAGGCAGGTATTGTAAC					0.7961	
XS20	F: GATAGCACAAGCCAAGCGTC	62	$(CA)_{28}$	122-238	14	0.6250	JF692813
	R: GCCACAGAGAGGAGTTATTCAG					0.9415	
XS25	F: CCCATGAACATGCTAACAGAAG	62	$(GT)_8A(TG)_{11}$	244-278	13	0.6818	JF692814
	R: ACCCAAGCAAGTGTCACAGAC					0.8890	
XS32	F: TATTTGTGCAGGATCGAGGC	56	$(GT)_{15}$	121-177	8	0.7692	JF692816
	R: ATAGGTGGAGGAAGCATTGG					0.8800	
XS33	F: CGTGTGCGTGTAAATGTTTG	60	$(CT)_{25}$	186-248	14	0.7143	JF692817
	R: GAGTGTGTTGTGGTCTCAGTC					0.9471	
XS34	F: GGCTATTGTGGCTGTGAAGTC	60	$(TG)_{59}$	251-287	5	0.6250	JF692818
	R: AGTGGAGTCATTGAAAGGCG					0.6750	

T_a = annealing temperature; Range = size range of alleles; N_A = number of alleles; H_O = observed heterozygosity; H_E = expected heterozygosity. *Indicates significant deviation from HWE after Bonferroni's correction ($P < 0.01$).

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