

# Isolation and Characterization of Twenty Microsatellite Loci in Japanese Sea Cucumber (*Stichopus japonicus*)

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## Abstract

Twenty microsatellite markers were first developed from the Japanese sea cucumber *Stichopus japonicus* using an enrichment protocol. Of the 20 microsatellite loci, 19 loci were polymorphic in the population examined. At these polymorphic loci, the number of alleles per locus varied from 2 to 15, and the observed heterozygosities ranged from 0.03 to 0.97, which is considerably higher than those previously found for allozymes. The high variability of the microsatellite markers identified in this study will make them excellent tools for genetic analyses of *S. japonicus*.

**Key words:** sea cucumber *Stichopus japonicus* microsatellite loci genetic variability

## Introduction

The Japanese sea cucumber *Stichopus japonicus* is a common benthic detritus feeder of coastal sea habitats of East Asia, including Japan, China, Korea, and Far Eastern Russia. It is a popular fisheries resource, and artificial reproduction has begun in those countries. *S. japonicus* is composed of 3 distinct color groups, red, green, and black (Kanno and Kijima, 2002a), and color affects the price of the products. Generally, the red type is the most valuable. The red type usually inhabits gravel beds offshore, while the green and the black types inhabit the sand-muddy bottom inshore (Nishimura, 1995). Many other differences (spawning season, morphologic traits, etc.) have been reported between the red and the green types (Choe and Oshima, 1961).

Until recently, there had been no genetic analysis of this species, and the taxonomic position of these 3 color types had not been resolved. Kanno and Kijima (2002b) developed 10 allozyme markers for *S. japonicus* and clarified the genetic differences among the 3 color types using those markers (Kanno and Kijima, 2003). Allozyme analysis showed population differentiation between the red and the other color types and suggested the existence of reproductive isolation between them. However, no fixed allozyme differences were detected for the color types, and a small degree of hybridization could not be rejected without the availability of completely diagnostic nuclear genetic markers. In addition, enzyme activity is only detectable in fresh adult individuals. To reveal the genetic relationship among the color types, it is also necessary to examine the possibility of mating between the color types, and to search the genetic markers of each developmental phase that are associated with the color trait or the loci resulting in reproductive isolation.

Microsatellite analysis based on polymerase chain reaction (PCR) should offer the finest resolution for examining such genetic characterizations within species. Because microsatellites have high dispersal potential and polymorphism throughout nuclear DNA (Angers and Bernatchez, 1997), they would be available even at the pelagic larval stage (Bierne et al., 1998; Launey and Hedgecock, 2001), and they are not biased by a sex. Recently, 5 microsatellite markers were isolated from the same genus, *Parastichopus californicus*, and the authors reported their usefulness as genetic markers (Nelson et al., 2002). Here we provide a first report of 20 novel microsatellite loci that were isolated from *Stichopus japonicus* using a modified magnetic bead-hybridization selection protocol by Li et al. (2002).

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## Materials and Methods

### Isolation of Microsatellite-Containing DNA Fragment.

A genomic DNA library of *Stichopus japonicus* was built according to Li et al. (2002) using biotin-labeled microsatellite oligoprobes, (CA)<sub>12</sub> and (CT)<sub>12</sub>, and streptavidin-coated magnetic beads, with part of the enrichment procedure modified as in Carleton et al. (2002). Briefly, total DNA was extracted from longitudinal muscle of a single green-type individual obtained from Onagawa Bay in Miyagi Prefecture, Japan, using phenol-chloroform extraction. Next, 50 µg of DNA was digested with 100 U each of *AccII*, *AluI*, *DraI*, *HaeIII*, *HincII* and *RsaI*, and 5 µg of digested DNA was electrophoresed on a 2.5% NuSieve GTG agarose gel (FMC Bioproducts). Fragments ranging between 400 and 800 bp were excised and purified by Qiagen column (QIA quick Gel Extraction kit, Qiagen), and 1 µg of the fragments was ligated with 200 pmol of *EcoRI*-*NotI*-*BamHI* adapter (TaKaRa) using a DNA Ligation Kit (TaKaRa). For enrichment, the DNA was denatured and hybridized to a biotinylated oligo probe, 5'-(CA)<sub>12</sub>GCTTGA-biotin and 5'-(CT)<sub>12</sub>GCTTGA-biotin, in 3 × SSC/0.1% sodium dodecylsulfate (SDS) at 68°C for 1 hour (Carleton et al., 2002). The DNA hybridized to the probe was then captured with Streptavidin MagneSphere paramagnetic Particles (Promega) according to the manufacturer's protocol and washed twice in 6 × SSC/0.1% SDS at room temperature, twice in 3 × SSC/0.1% SDS at 68°C, and twice in 6 × SSC/0.1% SDS at room temperature. Following purification, immobilized DNA fragments were eluted from the beads in 50 µl of 0.15 M NaOH at room temperature for 20 minutes. PCR amplification was performed with an adaptor sequence primer (5'-CGGCGCCGCGGATCC-3') with initial denaturation of 95°C for 11 minutes, followed by 35 cycles of 94°C (1 minute), 63°C (30 seconds), and 72°C (1 minute). PCR products were purified using a QIA quick PCR purification kit (Qiagen).

### Cloning and Sequencing of Microsatellite Loci.

The purified PCR products were digested with *NotI*, ligated into pBluescriptII SK<sup>+</sup> vector (Stratagene), and transformed into XL1-Blue MRF' super-competent cells (Stratagene) as outlined by the manufacturer. Positive white colonies were picked and used as template in the PCR with 2 vector primers (T<sub>3</sub> and T<sub>7</sub>) and the nonbiotin-labeled (CA)<sub>12</sub> and (CT)<sub>12</sub> primer: initial denaturation of 95°C for 11 minutes followed by 35 cycles of 94°C (1 minute), 57°C (1 minute), and 72°C (1 minute). PCR products were checked on 2% agarose gels, and inserts producing 2 or more bands were considered to contain a

microsatellite locus. Positive clones were grown, purified, and then double sequenced on a DSQ-2000L DNA sequencer (Shimadzu) using a ThermoSequenase cycle sequencing kit (Amersham) and universal forward (5'-CGCCAGGGTTTTCCAGTCACGAC-3') and reverse (5'-GAGCGGATAACAATTTACACAGG-3') primers, respectively. Primer designs were based on sequence flanking the microsatellite motifs using the OLIGO software package (National Bioscience).

### Assessment of Polymorphism in Microsatellite Loci.

Genetic variability was analyzed on 30 to 34 green type individuals of *S. japonicus* originating from Onagawa Bay in Japan. Total DNA of *S. japonicus* was extracted from longitudinal muscle by the phenol/chloroform method. PCRs were performed in 10-µl volumes, containing 0.25 U of TaKaRa *Taq*<sup>TM</sup> (TaKaRa), 1 × PCR buffer, 0.25 mM dNTP mix, 1 µM forward FITC-labeled primer, and reverse unlabeled primer, and about 0.01 µg template DNA. Thermal cycling conditions were as follows: 1 cycle of 94°C for 3 minutes; 7 cycles of 94°C for 1 minute, annealing temperature (given in Table 1) for 30 seconds, 72°C for 30 seconds; 33 cycles of 90°C for 30 seconds, annealing temperature for 30 seconds, 72°C for 30 seconds; and 1 cycle of 72°C for 5 minutes. Following PCR, an equal volume of deionized formamide (containing 1% wt/vol Blue dextran, and 0.1 mM EDTA) was added to each sample, denatured for 10 minutes at 95°C, and size-fractionated via 6% denaturing polyacrylamide gel on a DSQ-2000L DNA sequencer (Shimadzu).

**Statistical Analysis.** Number of alleles per locus, expected and observed heterozygosities, and  $\chi^2$  tests of deviation from Hardy-Weinberg expectations (HWE) were calculated using GENEPOP Version 3.1d (Raymond and Rousset, 1995).

## Results

Out of 1170 white colonies screened, 218 clones initially showed positive by the above PCR-based technique. Of these, 191 clones were sequenced, and 95 loci contained microsatellite arrays with primarily 2-bp repeat motifs, some of which were in combination with other 2-bp or 4-bp repeat motifs. Primers were developed for 51 loci that exhibited unique sequence regions flanking the microsatellite array. Twenty primer sets yielded variable profiles consisting of 1 or 2 bands (Table 1). Nineteen loci, with the exception of *Psj 1856*, were all polymorphic. The number of alleles per locus ranged from 2 to 15 (mean, 6.9), and the mean observed and expected heterozygosities were calculated as 0.56

**Table 1. Characterization of 20 Microsatellite Loci Isolated from *Stichopus japonicus*<sup>a</sup>**

Locus	Cloned repetitive sequence	Primer sequence (5' to 3')	Annealing temp. (°C)	Product size range (bp)	No. of alleles	Sample size (N)	H <sub>o</sub>	H <sub>e</sub>	GenBank acc. no.
<i>Psj</i> 1828	(CA) <sub>8</sub>	CAAACGCATACAAATTACACA CGATCGATAGTCTCAATC	59	171–199	11	32	0.77	0.85	AB106628
<i>Psj</i> 1856	(CA) <sub>6</sub>	AAGATGGTCCAAGCTTCCTT TTACCGAGCACGACTGATGT	65	192	1	34	0.00	0.00	AB106629
<i>Psj</i> 2022	(CA) <sub>13</sub>	TAAAGACTTGAGCGGACAC AAAGTTGCATATGGGTTC	61	218–246	15	30	0.77	0.91	AB106630
<i>Psj</i> 2031	(CA) <sub>15</sub>	ACTAAAAGTCAATGGACACCC AATCATAGCCATTTTCTGT	61	183–207	12	31	0.97	0.90	AB106631
<i>Psj</i> 2062	(CA) <sub>6</sub>	AGTATTTGGTAAGGTGTG GAAC TTCCTTCATTTCTCT	57	70–82	6	33	0.37 <sup>b</sup>	0.52	AB106632
<i>Psj</i> 2172	(AT) <sub>8</sub>	TTAGAATATGATGCAACAGAA GATACCGTGATAATTGGTTT	55	162–174	5	34	0.47	0.46	AB106633
<i>Psj</i> 2212	(CA) <sub>7</sub>	CAACGACTTACTCCCTACTC GAAACAATATGGCACAAGA	55	168–170	2	31	0.10	0.20	AB106634
<i>Psj</i> 2270	(CA) <sub>6</sub>	TCAAGTGGGAGATAAAAGT TGTGTAGTTGTAGCCGAGATT	53	219–221	2	33	0.03 <sup>b</sup>	0.26	AB106635
<i>Psj</i> 2368	(CA) <sub>6</sub>	GCTAGTCGTGGACTTCTC TAGGTAATTGATGCATTTAGGG	55	195–203	5	34	0.57	0.65	AB106636
<i>Psj</i> 2409	(CA) <sub>11</sub> (AT) <sub>6</sub>	GCAGGAGGATCTAAAATACAT ATCGAACACAAACACACTTATC	61	175–189	6	32	0.53	0.61	AB106637
<i>Psj</i> 2463	(CA) <sub>8</sub>	GCTGAAGGCAAAAGGAATCT GTAGCAAATGTGGCAAAGGAT	57	234–256	8	34	0.70	0.76	AB106638
<i>Psj</i> 2464	(CA) <sub>6</sub>	GTCTCATCAGCAATCCACCA TCTCTCCCTACCCCAAAACAC	56	167–177	6	31	0.53 <sup>b</sup>	0.68	AB106639
<i>Psj</i> 2575	(CA) <sub>9</sub>	GCCTCGAGAGCTTTATCAATG GCTCGCTTGGAGAGTAAACAC	62	87–105	7	34	0.47	0.47	AB106640
<i>Psj</i> 2642	(CA) <sub>13</sub>	ATAGTCCCTGATTTTTGGTTTC GAGATACCCGTGTTTACAAGC	57	132–144	7	31	0.60	0.79	AB106641
<i>Psj</i> 2643	(CA) <sub>7</sub>	TAAATTTTCGAGCTTTTGAT TACGGTATTTACAAGCAAAGT	54	92–100	5	30	0.33 <sup>b</sup>	0.66	AB106642
<i>Psj</i> 2844	(CA) <sub>6</sub>	CAAAACGATAGGGACCATCTA TTAACATTTCTGCCCACTTC	61	156–160	3	33	0.10	0.14	AB106643
<i>Psj</i> 2889	(CA) <sub>8</sub>	CGAGACGTTTACTTCCACTG AGAGGTTGCTGGCTTTACTC	59	204–224	10	32	0.80	0.86	AB106644
<i>Psj</i> 2969	(CA) <sub>8</sub>	TTCTGCCCCTTACAAAATAG GCAGCAGAATGATGAGTGTG	57	135–149	8	30	0.57	0.80	AB106645
<i>Psj</i> 3072	(CA) <sub>12</sub>	TGTTGCTGGTCGGAGCTACTG AAGATTGGGGCAGGTGATGT	53	163–191	11	34	0.80	0.83	AB106646
<i>Psj</i> 3088	(CA) <sub>6</sub>	CGTATTTACAAGCCCCCAACA GGCGTAGAAAAGCAAGGAAAG	57	166–178	3	33	0.27	0.27	AB106647

<sup>a</sup>H<sub>o</sub>: indicates observed heterozygosity; H<sub>e</sub>, expected heterozygosity<sup>b</sup>P < 0.01

(range, 0.03-0.97) and 0.61 (range, 0.14-0.91), respectively. Significant deviations from HWE for the observed heterozygosities were observed in 4 loci ( $P < 0.01$ ) as shown in Table 1.

### Discussion

The first microsatellite markers have been identified and characterized for the Japanese sea cucumber, *S. japonicus*. We created microsatellite libraries enriched for CA and CT repeat sequences following the protocol by Li et al. (2002) with the modification described in Carleton et al. (2002). With the above protocol for *S. japonicus*, the percentage of positive clones containing microsatellite repeats of 8.1% (95 of 1170) was lower than the values of 13.1% for abalone (Li et al., 2002) and 96% for tilapia (Carleton et al., 2002). Almost all 95 microsatellite sequences contained (CA)<sub>n</sub> but no (CT)<sub>n</sub> repeat. Such bias of the repeat motif in the libraries may represent the different absolute numbers of different repeats in the sea cucumber genome or may reflect the effects of different hybridization and washing conditions on (CT)<sub>n</sub>. Details about the repeat preference and the effect of hybridization condition have not been extensively investigated (Zane et al., 2002).

Nineteen of 20 new microsatellites from *S. japonicus* were polymorphic, showing 2 to 15 alleles per locus, and observed heterozygosities ranged from 0.03 to 0.97. The genetic variability is high in comparison with that of 10 allozyme loci (average, 3.7 alleles per locus;  $H_o = 0.26$ ;  $H_e = 0.27$ ) calculated on the same samples ( $n = 84$ ) of *S. japonicus* from Onagawa Bay reported by Kanno and Kijima (2002b). The high variation in length found here is similar to that found in *P. californicus*, for which the number of alleles per locus varied from 3 to 14, and the observed heterozygosities ranged from 0.2 to 0.9 (Nelson et al., 2002).

Departure from HWE was observed in 4 loci (*Psj* 2062, *Psj* 2270, *Psj* 2464, *Psj* 2643) because of homozygote excess. In allozyme analysis, no deviation from HWE was observed for the same samples (Kanno and Kijima, 2002b); therefore, the deviation observed here would be caused by the characteristics of microsatellite markers, such as large allele "dropout" artifacts in the PCR amplification process, small sample size, presence of null alleles, or size homoplasy (Li et al., 2002). Of these, the existence of null alleles is the most probable cause of differences in findings, as null alleles have been reported in studies of numerous organisms, including mammals (Callen et al., 1993), lizards (Gullberg et al., 1997), fishes (Jones et al., 1998), crustaceans (Tam and Kornfield, 1996), oysters (McGoldrick et

al., 2000), and abalones (Li et al., 2003a, 2003b). Certainly, to determine if these results are statistically reliable, it is necessary to examine more samples and investigate microsatellite transmission by controlled crosses.

Nevertheless, the high variability of the microsatellite markers identified in this study will make them useful genetic markers for various analyses of *S. japonicus*. Further studies are in progress using these microsatellite markers to investigate the genetic structure of *S. japonicus* and to detect reproductive isolation among color types through mating experiments.

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