

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

Manami Kanno,<sup>1</sup> Qi Li,<sup>2</sup> Akihiro Kijima<sup>1</sup>

<sup>1</sup>Integrated Center of Field Sciences, Graduate School of Agricultural Science, Tohoku University, Onagawa, Miyagi, 986-2242, Japan <sup>2</sup>Fisheries College, Ocean University of China, Qingdao, 266003, China

Received: 20 January 2004 / Accepted: 27 May 2004 / Online publication: 26 May 2005

#### 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 locigenetic 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 sandmuddy 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).

Correspondence to: Akihiro Kijima; E-mail: a-kijima@ mail.tains.tohoku.ac.jp

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).

#### 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  $(CT)_{12}$ , 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 greentype individual obtained from Onagawa Bay in Miyagi Prefecture, Japan, using phenol-cloroform extraction. Next, 50 µg of DNA was digested with 100 U each of AccII, AluI, DraI, HaeIII, HincII and RsaI, and 5  $\mu$ g 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 biotinated oligo probe, 5'-(CA)<sub>12</sub>GCTTGA-biotin and 5'-(CT)<sub>12</sub>GCTTGA-biotin, in  $3 \times SSC/0.1\%$  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  $3 \times SSC/0.1\%$  SDS at  $68^{\circ}$ C, and twice in  $6 \times SSC/0.1\%$  SDS at room temperature. Following purification, immobilized DNA fragments were eluted from the beads in 50  $\mu$ l of 0.15 M NaOH at room temperature for 20 minutes. PCR amplification was performed with an adaptor sequence primer (5'-CGGCGGCGGGATCC-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' supercompetent 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'-CGCCAGGGTTTTCCCAGTCACGAC-3') and reverse (5'-GAGCGGATAACAATTTCACACAGG-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- $\mu$ l volumes, containing 0.25 U of TaKaRa  $Taq^{TM}$ (TaKaRa),  $1 \times PCR$  buffer, 0.25 mM dNTP mix,  $1 \mu M$ 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  $Stichopus\ japonicus^a$ 

			T / T						
locus	Cloned repetitive	Primer seamence (5' to 3')	Annealing	Product size	No. of	Sample size (N)	Н	H	GenBank acc. no.
	aaranhaa	( o o o o o o o o o o o o o o o o o o o	(o) Juna	(Aa) asumi	arrorro	(11)	0++	211	
Psj 1828	$(CA)_8$	CAAACGCATACAATTACACA	59	171–199	11	32	0.77	0.85	AB106628
Psj 1856	$(CA)_6$	AAGATTGGTCCAAGCTTCCTT	65	192	1	34	0.00	0.00	AB106629
		TTACCGAGCACGACTGATGT							
Psj 2022	$(CA)_{13}$	TAAGAGACTTGAGCGGACAC AAAGTTGCATATTGGGTTCA	61	218–246	15	30	0.77	0.91	AB106630
Psj 2031	$(CA)_{15}$	( ') L	61	183–207	12	31	0.97	0.90	AB106631
Psi 2062	(CA) <sub>¢</sub>	AGIATTTGTTGGTAAGGTGTG	57	70–82	9	33	$0.37^{b}$	0.52	AB106632
	0/	GAACTTCCTTCTCT	ō	) )	<b>)</b>	)	) )		
Psj 2172	$(AT)_8$	TTAGAATATGATGCAACAGAA	55	162–174	5	34	0.47	0.46	AB106633
Psi 2212	(CA) <sub>7</sub>	CAACGACTTACTCCCCTACTC	55	168-170	2	31	0.10	0.20	AB106634
		GAAACAATATGGCACAAAAGA	)	)	I	(	)		
Psj 2270	$(CA)_6$	TCAAGTGCGGAGATAAAAGT	53	219–221	2	33	$0.03^{b}$	0.26	AB106635
0260 :00	140	TGTGTAGTTGTAGCCGAGATT	ri ri	105 903	L	2.4	730	0 65	A B 106626
FS/ 2300	1CA/6	TAGGTATTGATGCATTTAGGG	cc	607-661	n	, 10,	0.37	0.03	ABIU0030
Psj 2409	$(CA)_{11}(AT)_6$	GCAGGAGCATCTAAAATACAT	61	175–189	9	32	0.53	0.61	AB106637
		ATCGAACACACACACTTATC							
Psj 2463	$(CA)_8$	GCTGAAGGCAAAGGAATCT	57	234–256	∞	34	0.70	0.76	AB106638
Psj 2464	(CA) <sub>6</sub>	GTCTCATCAGCAATCCACCA	56	167–177	9	31	$0.53^{b}$	0.68	AB106639
		TCTCTCCCTACCCCAAAACAC							
Psj 2575	(CA)9	GCCTCGAGAGCTTATTCAATG	79	87–105	_	34	0.47	0.47	AB106640
Psj 2642	$(CA)_{13}$	ATAGTCCTGATTTTTGGTTTC	57	132–144	_	31	09.0	0.79	AB106641
Psj 2643	(CA) <sub>7</sub>		54	92–100	5	30	$0.33^{b}$	99.0	AB106642
Psj 2844	(CA) <sub>6</sub>	CAAAACGATAGGGACCATCTA	61	156–160	3	33	0.10	0.14	AB106643
Psj 2889	(CA) <sub>8</sub>	TIAACATTTTCIGCCACTTC CGAGACGTTTACTTCCACTG	59	204–224	10	32	0.80	98.0	AB106644
		AGAGGTTGCTGGCTTTACTC							
Psj 2969	$(CA)_8$	TTCCTGCCCTTACAAATAG	57	135–149	∞	30	0.57	0.80	AB106645
Psj 3072	$(CA)_{12}$	TGTTGCTGGTCGGTGTGTGTGTGTGTGTGTGTGTGTGTG	53	163–191	11	34	0.80	0.83	AB106646
Psj 3088	$(CA)_6$	CGTATTTACAAGCCCCCAACA CGCCTAGAAAGCAAGAAG	57	166–178	8	33	0.27	0.27	AB106647

 $^aH_0:$  indicates observed heterozygosity,  $H_e,\,$  expected heterozygosity  $^bP<0.01$ 

(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  $(CT)_n$ . 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;  $H_0 = 0.26$ ;  $H_e = 0.27$ ) 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.

#### References

- 1. Angers B, Bernatchez L (1997) Complex evolution of a salmonid microsatellite locus and its consequences in inferring allelic divergence from size information. Mol Biol Evol 143, 230–238
- 2. Bierne N, Launey S, Naciri-Graven Y, Bonhomme F (1998) Early effect of inbreeding as revealed by microsatellite analyses on *Ostrea edulis* larvae. Genetics 1484, 1893–1906
- 3. Callen DF, Thompson AD, Shen Y, Phillips HA, Richards RI, Mulley JC, Sutherland GR (1993) Incidence and origin of 'null' alleles in the (AC)<sub>n</sub> microsatellite markers. Am J Hum Genet 52, 922–927
- 4. Carleton KL, Streelman JT, Lee B-Y, Garnhart N, Kidd M, Kocher TD (2002) Rapid isolation of CA microsatellites from the tilapia genome. Anim Genet 33, 140–144
- Choe S, Oshima Y (1961) On the morphological and ecological differences between two commercial forms, 'green' and 'red', of the Japanese common sea cucumber, Stichopus japonicus SELENKA. Nippon Suisan Gakkaishi 27, 97–105
- 6. Gullberg A, Tegelstrom H, Olsson M (1997) Microsatellite in the sand lizard (*Lacerta agilis*): description, variation, inheritance, and applicability. Biochem Genet 35, 281–295
- Jones AG, Stockwell CA, Walker D, Avise JC (1998)
   The molecular basis of the microsatellite null allele from the white sand pupfish. J Hered 89, 339–342
- 8. Kanno M, Kijima A (2002a) Quantitative and qualitative evaluation on the color variation of the Japanese sea cucumber *Stichopus japonicus*. Suisanzoshoku 50, 63–69
- Kanno M, Kijima A (2002b) High genetic variability of isozymes in Japanese sea cucumber Stichopus japonicus. Fish Genet Breed Sci 31, 7–12
- 10. Kanno M, Kijima A (2003) Genetic differentiation among three color variants of Japanese sea cucumber *Stichopus japonicus*. Fish Sci 694, 806–812
- 11. Launey S, Hedgecock D (2001) High genetic load in the Pacific oyster *Crassostrea gigas*. Genetics 159, 255–265
- Li Q, Park C, Kijima A (2002) Isolation and characterization of microsatellite loci in the Pacific abalone, Haliotis discus hannai. J Shellfish Res 212, 811–815

- 13. Li Q, Park C, Kobayashi T, Kijima A (2003a) Inheritance of microsatellite DNA markers in the Pacific abalone *Haliotis discus hannai*. Mar Biotechnol 5, 331–338
- Li Q, Park C, Kijima A (2003b) Allelic transmission of microsatellites and application to kinship analysis in newly hatched Pacific abalone larvae. Fish Sci 69, 881– 887
- 15. McGoldrick DJ, Hedgecock D, English LJ, Baoprasertkul P, Ward RD (2000) The transmission of microsatellite alleles in Australian and North American stocks of the Pacific oyster (*Crassostrea* gigas) selection and null alleles. J Shellfish Res 19, 779–788
- 16. Nelson J, Cooper G, Garner T, Schnupf P (2002) Polymorphic markers for the sea cucumber *Parastichopus californicus*. Mol Ecol Notes 2, 233–235
- 17. Nishimura S (1995) Guide to Seashore Animals of Japan with Color Pictures and Keys, Vol 2. (Osaka, Japan: Hoikusya)
- Raymond M, Rousset F (1995) Population genetics software for exact tests and ecumenicism. J Hered 86, 248–249
- 19. Tam YK, Kornfield I (1996) Characterization of microsatellite markers in *Homarus* (Crustacea, Decapoda). Mol Mar Biol Biotechnol 5, 230–238
- 20. Zane L, Bargelloni L, Patarnello T (2002) Strategies for microsatellite isolation: a review. Mol Ecol 11, 1–16