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Note

Development and characterisation of 16 novel microsatellite markers for the brown alga *Sargassum fusiforme* (Harvey) Setchell

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ABSTRACT

Sargassum fusiforme (Harvey) Setchell (Sargassaceae, Phaeophyta) is an ecologically and economically important kelp species in East Asia. Restoration and responsible utilisation of *S. fusiforme* would be facilitated by the availability of an appropriate set of molecular markers. In the present study, we developed 16 microsatellite markers for *S. fusiforme*. A total of 99 different alleles were observed at the 16 microsatellite loci across 50 individual samples. The number of alleles per locus ranged from 4 to 10, with an average of 6.2 per locus. The observed and expected heterozygosities ranged from 0.483 to 0.833 and from 0.513 to 0.840, respectively. Only three of the loci deviated significantly from Hardy-Weinberg equilibrium after Bonferroni correction. No significant linkage disequilibrium was detected among the microsatellite loci. The obtained microsatellite markers will facilitate related research on *S. fusiforme*, such as ecological studies and genetic diversity assessments.

Keywords: Genetic diversity, Microsatellite, Polymorphism, *Sargassum fusiforme*

The large brown alga *Sargassum fusiforme* (Harvey) Setchell is widely distributed in the lower intertidal zones of the western North Pacific and is commercially cultivated as a food-stuff in South Korea, Japan and China (Hwang *et al*., 1994; Sun *et al*., 1996). This alga has important ecological functions both as a substantial contributor to marine primary production and as a seaweed bed habitat for spawning, nurseries and feeding of nearshore benthic marine organisms. However, the market demand for this alga is increasing because of its high commercial value in the nutrition and pharmaceutical industries (Pang *et al*., 2005; Choi *et al*., 2009). *S. fusiforme* is intensively cultivated in China and aquaculture of this alga has become a well developed industry in Zhejiang and Fujian provinces (Pang *et al*., 2005; 2006). However, over the last decade, natural resources of *S. fusiforme* have been severely damaged by overexploitation and ocean pollution. Along the coast of Zhejiang Province in the East China Sea, the floating biomass of *S. fusiforme*, which was once distributed widely in near-shore waters and the waters around islands, has been disappearing steadily since the end of the 1990s (Sun *et al*., 1996). Artificial breeding and seeding have also caused a serious decline in natural *S. fusiforme* biomass, even resulting in the "Isoyake" phenomenon (Pang *et al*., 2005; Yu *et al*., 2012). Therefore, the restoration and sustainable exploration of natural resources of *S. fusiforme* are of critical importance.

Molecular markers are important tools for population genetic studies, which could enhance the responsible exploitation of *Sargassum* species. In *S. fusiforme*, a variety of marker systems have been used for genetic analyses, including RAPD, ISSR, SCAP and AFLP (Park *et al*., 1998; Yu *et al*., 2012; Nan *et al*., 2015). However, although microsatellite markers are superior to the aforementioned markers for genetic analyses because of their high mutation rate and co-dominant Mendelian inheritance, no genomic microsatellite markers are available for *S. fusiforme*. In the present study, we developed and characterised 16 microsatellite markers for *S. fusiforme*. These new markers will facilitate future studies on the genetic diversity and structure of *S. fusiforme* populations and aid in the breeding programmes.

S. fusiforme was collected from lower intertidal rocks along the coastline of Dongtou, Zhejiang Province (120.80 E, 27.85 N). Sporophytes of *S. fusiforme*,

approximately 10 m apart, were randomly collected. Fifty individuals were randomly sampled and 2-3 lateral branches from each individual were collected and preserved at -80°C until use. Genomic DNA was extracted from the leaves of *S. fusiforme* by the modified cetyltrimethylammonium bromide (CTAB) method described by Shan and Pang (2009).

Microsatellite loci were isolated and cloned according to the protocol described by Yue *et al*. (2000). A partial genomic DNA library enriched for CA-repeats was constructed using an enrichment technique. DNA was digested with *Rsa*I and the resulting fragments were separated on a 1% agarose gel. Fragments sized 250-1000 bp were excised, purified and ligated to 21- and 25-mer oligo adaptors (20 pmol) (Fischer and Bachmann, 1998). CA-repeats in the ligated DNA were enriched using biotinylated probes $(CA)_{10}$ and streptavidin-coated magnetic beads (Dynal) and then the DNA containing microsatellites captured by the magnetic beads was eluted. The eluted fragments were amplified using 21-mers as primers and then ligated into the pGEM-T-vector (Promega) according to the manufacturer's protocol. The generated plasmids were transformed into *Escherichia coli* XL-blue 1 competent cells (Stratagene). DNA from the obtained colonies was sequenced in 5′ and 3′ directions with an ABI3100 sequencer (Applied Biosystems, Foster City, CA, USA) using BigDye chemistry (Applied Biosystems). Primers were designed for 16 microsatellites in the regions flanking the repeats using Primer 3.0 software (Rozen and Skaletsky, 2000).

PCR amplification was conducted in a 25 µl reaction mixture containing 10 pmol of each primer set, 100 µmol l^{-1} dNTPs, 10 mmol l^{-1} Tris-HCl (pH 8.3), 50 mmol l^{-1} KCl, 1.5 mmol l^{-1} $MgCl_2$, 1 U of Taq polymerase (Takara Biotechnology, Dalian, China) and approximately 40 ng of template DNA. The PCR profile consisted of an initial denaturation step at 94°C for 5 min, followed by 30 cycles of 94°C for 30 s, annealing for 45 s and 72°C for 1 min and a final elongation step at 72°C for 7 min. Amplification products were separated on a 6% denaturing polyacrylamide gel and visualised by silver staining to screen out polymorphic microsatellites for further genetic analyses. After optimisation, one primer of each pair used for the genetic analyses was labeled with a fluorescent dye (FAM, NED, PET or VIC) and the PCR amplification was conducted as described above. Following amplification, the PCR products were mixed with 5 µl of formamide HiDi and LIZ-500 size standard and heated at 95°C for 5 min. The fluorescently labeled microsatellite PCR products were analysed on an ABI3130xL DNA sequencer using GeneMapper 3.5 software (Applied Biosystems). The sizes of the PCR products were determined by comparison with the LIZ-500 size standard. Genotype data were exported as Microsoft Excel files for further analysis.

The number of alleles per locus (N_a), effective number of alleles (N_e), expected heterozygosity (H_e) and observed heterozygosity (H_o) were calculated using GenALEx 6 (Peakall and Smouse, 2006). Exact tests for Hardy-Weinberg equilibrium (HWE) of each sample for each locus and linkage disequilibrium (LD) between pairwise loci were tested by a Markov chain method (dememorisation = 10,000, batches = 1000 and iterations per batch = 10,000) using GENEPOP 4.0 (Rousset, 2008). The sequential Bonferroni technique was used to correct *p* values for multiple tests (Rice, 1989).

Based on the sequences of the microsatellite repeats, 50 putative loci were randomly selected for screening and primers were ordered for these microsatellites. In the first round of screening, denaturing PAGE showed that 25 of the 50 primer sets successfully amplified microsatellite DNA from *S. fusiforme*. Of these 25 microsatellites, 16 polymorphic microsatellites were selected and primers labeled with fluorescent dye were ordered to avoid invalid microsatellites. The allele size was 180-392 bp (Table 1). One wild *S. fusiforme* population containing 50 individuals was used to analyse the polymorphism of the 16 screened primer pairs. For each of the 16 loci, 45-50 of the *S. fusiforme* samples were successfully genotyped. A total of 99 different alleles were observed at the 16 microsatellite loci. The total number of alleles per locus in the tested population ranged from 4 to 10 (Table 1). The observed and expected heterozygosities ranged from 0.483 to 0.833 and 0.513 to 0.840 respectively. The number of alleles at each locus was similar to or higher than that at characterised microsatellite loci in other kelp species, such as *Laminaria digitata* (Liu *et al*., 2012), *Gracilaria tenuistipitata* (Song *et al*., 2014) and *Gambierdiscus caribbaeus* (Sassenhagen and Erdner, 2017).

The observed genotypic frequencies were tested for Hardy-Weinberg equilibrium. Only three of the loci (HM05, HM10, HM15) displayed heterozygosity levels that significantly deviated from HWE expectations after Bonferroni correction. No evidence of linkage disequilibrium (LD) was observed in the LD test for all pairs of loci in the population.

In summary, we report here 16 microsatellite loci as the first set of molecular markers developed for *S. fusiforme*. These newly developed microsatellite markers will facilitate range-wide studies on the genetic diversity and structure of *S. fusiforme* populations and will be useful for effective recovery and management planning towards population augmentation and cultivation.

Table 1. Characteristics of 16 microsatellite markers developed for *Sargassum fusiforme*

Locus	Repeat motif	Primer sequence 5'–3'(label)	T_a (^{0}C)	Allele-size range	N	Na	Ho	He	P_{HW}	Gen Bank Acc. No.
HM01	$(TG)_{10}$	F:CTTGGTTTTAGGTATCCAGGCTCT R:TGCCCACTCACCTGTGTCATT	55	212-244	48	5.0	0.583	0.777	0.195	KY673702
HM02	$(CT)_{25}$	F:ACAGAACACATCCAAACAAAGG R:AACCAAAAGAAAGGAGCGAC	51	207-254	50	7.0	0.750	0.824	0.356	KY673703
HM03	$(GT)_9$	F:TGTCACACGCAGGGTAATG R:TCAGGCAGCCGCAGTAT	52	187-295	50	8.0	0.733	0.769	0.399	KY673704
HM04	$(GA)_{15}$	F:AAGACGGTATGCCAGGGTT R:GTTCAGGAGCAAAAAAATAAGAGA	51	228-306	45	6.0	0.607	0.744	0.043	KY673705
HM05	$(AG)_{13}$	F:GAAGAAGTGAGAGAAAAGGGG R:ATTATGTGAAAGTGCTGCGAG	53	191-207	50	7.0	0.733	0.757	0.014	KY673706
HM06	$(CT)_{10}$	F:CAAAGGTTAGCCCCAAATCTG R:CGCACAATAAGCAATCACATAC	55	201-219	46	4.0	0.483	0.513	0.166	KY673707
HM07	$(CA)_{10}$	F:GCACACCTGACCGTGAACA R:GAAGAGAACAACCTGGTAATGAACT	54	180-217	45	7.0	0.632	0.680	0.150	KY673708
HM08	$(GACA)_{23}$	F:AAATGTTTGGCTGTGCTATGA R:ATTGTGTGGCTGTTTTGTGTTA	51	236-306	50	6.0	0.542	0.700	0.082	KY673709
HM09	$(TC)_{26}$	F:TGCCTTTTGGAAATCAGCCT R:ACATCTCCCTAACAGTCGCATAATA	51	262-303	50	7.0	0.811	0.756	0.137	KY673710
HM10	$(AC)_{11}$	F:TCTTTGCTTTCTCTCGGTGA R:GGATGTGGTTTTGGTGTGTC	51	191-203	49	5.0	0.608	0.693	0.028	KY673711
HM11	$(TG)_{10}$	F:GCTCTGATACACAGCAGGACAA R:GGCAACACAAAAAAGGGG	51	182-253	50	6.0	0.533	0.672	0.818	KY673712
HM12	$(CA)_{17}$	F:ATGCCACCTCCTCCACAC R:TCCCCAAAAACACCTCTCA	54	226-295	50	7.0	0.715	0.730	0.079	KY673713
HM13	$(GT)_{19}$	F:GAGGAGGACGGGGGGAGCAGAA R:CCAGCCTAATGGCAACAAGGGAT	55	280-392	48	5.0	0.580	0.565	0.085	KY673714
HM14	$(CA)_9$	F:GCCCTCATTGACTGCTATCTGCT R:CCATTACAGTTTTAGCCTGAGCGT	55	181-272	50	10.0	0.825	0.840	0.102	KY673715
HM15	$(GT)_9$	F:GTGAAGAGAACAACCTGGTAATG R:GCACACCTGACCGTGAACA	53	186-298	50	7.0	0.617	0.795	0.001	KY673716
HM16	$(GT)_{11}$	F:TTGTCTGACTGTCTGTGCTTTTGTT R:GTTGACTAATGCGATGCTGGTTC	54	201-262	50	9.0	0.833	0.840	0.926	KY673717

*T*a - specific annealing temperature; *Na* - number of alleles; *Ho* - observed heterozygosity; *He* - expected heterozygosity; P_{HW} - probability of departure from Hardy - Weinberg equilibrium

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