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ISOLATION AND CHARACTERIZATION OF MICROSATELLITE LOCI IN *FIMBRISTYLIS SERICEA* (CYPERACEAE)¹

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- **Premise of the study:** We developed microsatellite markers for *Fimbristylis sericea*, a coastal herb found in sand dunes in Asia and Australia.
- **Methods and Results:** Twelve microsatellite loci were isolated, and the genetic variability within and among local populations was characterized. The number of alleles per locus was two to five with a mean of 3.5, total expected heterozygosity per locus was 0.069–0.645 with a mean of 0.336, and average expected heterozygosity within a population per locus was 0.051–0.230. Most of the loci deviated significantly from Hardy–Weinberg equilibrium.
- **Conclusions:** All 12 microsatellite loci were polymorphic within and among populations. These loci could be useful genetic markers for population genetic studies of *F. sericea* populations.

Key words: coastal plant; Cyperaceae; *Fimbristylis sericea*; microsatellite.

Fimbristylis sericea R. Br. (Cyperaceae) is a perennial herb that grows in sandy coastal areas of Asia and Australia. *Fimbristylis sericea* propagates asexually by short, thick rhizomes that produce clusters of ramets, which belong to the same genet. The genets do not spread over a wide range by rhizome formation, and they have a patchy distribution in their habitat. In the summer, some individuals form spikelets, where a single spikelet comprises 10–25 protogynous florets. The seeds are dispersed by gravity around the mother plants. Although the species depends primarily on sexual reproduction (Oka et al., 2009), the genets are long-lived and important for the maintenance of populations.

Coastal dunes are vulnerable to disturbance by human activities. Reduction and stabilization of sandy areas can lead to the loss of coastal vegetation (Shanmugam and Barnsley, 2002; Thompson and Schlacher, 2008). In Japan, the extinction risk for local populations of *F. sericea* is increasing because of habitat destruction and reduced population sizes. This species is listed in the local Red Data Book (Sawada et al., 2007). Thus, it is important to investigate the genetic structure of this species to facilitate its conservation. In the current study, we developed microsatellite markers and compound microsatellite markers to determine the genetic variability and genetic differentiation of *F. sericea*.

METHODS AND RESULTS

According to the method reported by Ohsako and Yamane (2007), we constructed a genomic library enriched for (CT)_n repeat sequences. Genomic DNA was isolated from leaves using a Wizard Genomic DNA Purification Kit (Promega Corporation, Madison, Wisconsin, USA) and digested with *Mbo*I.

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Approximately 1.5 µg of the digested DNA was electrophoresed on 1.0% agarose gels. Fragments with sizes ranging from 300 to 1000 bp were excised from the gel and purified using a QIAquick Gel Extraction Kit (QIAGEN, Valencia, California, USA). The DNA fragments were resolved in 12 µL of dH₂O. Two oligonucleotides, “F” (5′-TTGCTTACGCGTGGA-3′) and “R” (5′-GATCGA-GTCCACGCGTAAGCAA-3′), were used to prepare a linker by denaturation at 95°C for 5 min and cooling at room temperature (Nies and Reusch, 2004). The linker was ligated to the *Mbo*I-digested DNA fragments in a total volume of 21 µL, which contained 10 µL Ligation High (Toyobo, Osaka, Japan), 2 µM linker, and 6 µL of the DNA fragments (the DNA concentration was not measured). The ligated DNA fragments were amplified by PCR in a total reaction volume of 25 µL containing 2.5 unit TaKaRa *Ex Taq* DNA polymerase (TaKaRa Bio Inc., Otsu, Shiga, Japan), 10× *Ex Taq* Buffer, 200 µM dNTP mixture, 500 nM primer F, and 3 µL of the DNA fragments. The PCR cycling conditions were 94°C for 5 min; 30 cycles at 94°C for 15 s, 52°C for 15 s, 72°C for 40 s; and a final extension step at 72°C for 7 min. The fragments were precipitated with ethanol and resolved in 48 µL dH₂O. The DNA fragments were hybridized with 20 pmol of 5′-biotinylated (CT)₁₅ oligonucleotide at 70°C overnight in a 100-µL volume containing 10× saline sodium citrate (SSC) and 5% sodium dodecyl sulfate (SDS). The hybrid fragments were captured with 300 ng of streptavidin-coated magnetic beads (Dynabeads M-280; Dynal, Oslo, Norway) at 43°C for 4.5 h. The fragments on the magnetic beads were denatured at 95°C for 10 min in 50 µL of TE buffer. PCR was conducted to restore double-stranded DNA in a total reaction volume of 50 µL, which contained 2.5 unit *Ex Taq*, 10× *Ex Taq* Buffer, 200 µM dNTP mixture, 500 nM primer F, and 3 µL single-stranded DNA fragments. Thermal conditions of the PCR were 94°C for 5 min; 30 cycles at 94°C for 15 s, 52°C for 15 s, 72°C for 40 s; and a final extension step at 72°C for 7 min.

We used the technique reported by Lian et al. (2006) to construct a DNA library enriched with a compound microsatellite motif. The total DNA was digested with the blunt-end restriction enzyme *Eco*RV. The digested fragments were ligated with specific blunt adapters (a 48-mer 5′-GTAATACGACTCA-CTATAGGGCAGCGTGGTCGACGGCCCGGGCTGGT-3′ and an 8-mer 5′-ACCAGCCC-NH₂-3′) using Ligation High (Toyobo). Subsequently, fragments flanked by a microsatellite at one end were amplified from the *Eco*RV library using each compound microsatellite primer [(AC)₆(AG)₅, (TC)₆(AC)₅, (AG)₆(TG)₅, or (CT)₆(CA)₅] and an adapter primer AP2 (5′-CTATAGGGC-ACGCGTGGT-3′).

The DNA fragments containing (CT)_n or the compound microsatellite motif were cloned into the pGEM-T Easy Vector (Promega Corporation) and transformed into DH5α-competent cells (Toyobo). Next, the clones were

TABLE 1. Characteristics of 12 microsatellite loci developed in *Fimbristylis sericea*.

Locus	Primer sequences (5′–3′)	Repeat motif	Allele size range (bp)	T_a (°C)	GenBank accession no.
Fse33	F: GCGGTATAAGTCGCGAAGTC R: TCTCATCTTTCGCGAGAACCT	(GA) ₁₄	220–224	52	AB914726
Fse47	F: CGTTAGTGATGCAGAGATGAGC R: AGAGGTGGAGCAGAAAACGA	(GA) ₂₄	218–226	52	AB914727
Fse1-1	F: ACACACACACACAGAGAGAGAG R: GCGAAGATTAATGCACTAACCAGA	(AC) ₆ (AG) ₁₅	218–224	52	AB914728
Fse1-5	F: ACACACACACACAGAGAGAGAG R: GCGAGTAGCCTTTGCGGTTCTAG	(AC) ₆ (AG) ₁₄ (GT) ₄	264–270	52	AB914729
Fse1-18	F: ACACACACACACAGAGAGAGAG R: GCAGTGCTAGGCAATTAAGCGTCG	(AC) ₆ (AG) ₁₃	265–275	52	AB914730
Fse2-22	F: ACACACACACACAGAGAGAGAG R: TTAACACATAACCCTCTACCACCC	(AC) ₆ (AG) ₁₄	227–235	52	AB914731
Fse3-70	F: AGAGAGAGAGAGTGTGTGTGTG R: CTCATAAACAGTCGTGCCTTGACC	(AG) ₆ (TG) ₅	228–230	52	AB914732
Fse4-5	F: CTCTCTCTCTCTCACACACACA R: AATTCAGTGTCAAACCTCCACCTG	(CT) ₆ (CA) ₈	172–176	52	AB914733
Fse4-19	F: CTCTCTCTCTCTCACACACACA R: GAAGCATGGAGGAGGTGAGTGAC	(CT) ₆ (CA) ₅	165–170	52	AB914734
Fse4-25	F: CTCTCTCTCTCTCACACACACA R: GCTGGCCGTTTGGTTATATCAGG	(CT) ₆ (CA) ₁₈	132–148	52	AB914735
Fse4-29	F: CTCTCTCTCTCTCACACACACA R: CCAGATCTGACAAGTAGTCTCTTA	(CT) ₆ (CA) ₉	144–147	52	AB914736
Fse4-31	F: CTCTCTCTCTCTCACACACACA R: GGATAATCAGGTACATGTGCAGTC	(CT) ₆ (CA) ₁₆	161–177	52	AB914737

Note: T_a = annealing temperature for PCR amplification.

sequenced using an ABI 3730xL DNA sequencer (Applied Biosystems, Foster City, California, USA). Out of 96 (CT)_n clones and 162 compound microsatellite clones sequenced, 50 (CT)_n clones and 48 compound microsatellite clones were useful for designing primers. For the (CT)_n clones, the primers were designed using Primer3 (Rozen and Skaletsky, 2000). For the compound microsatellite clones, Oligo Calculator (Buehler, 1997) was used to calculate the melting temperature (T_m). PCR amplification was performed in a total volume of 10 μ L, which contained 0.125 unit Blend Taq-Plus Polymerase (Toyobo), 10 $\times$ buffer for Blend Taq, 200 μ M of each dNTP, 500 nM of each of the primers, and approximately 10 ng of total DNA. The thermal conditions were as follows: 94°C for 5 min; 30 cycles at 94°C for 20 s, 52°C for 20 s, 72°C for 40 s; and a final extension step at 72°C for 7 min. PCR with 12 primer pairs amplified fragments with the expected sizes (Table 1). The fragment sizes were measured using a CEQ8800 system (Beckman Coulter, Fullerton, California, USA) after PCR using the forward primer labeled with a fluorescent dye (i.e., D2, D3, or D4).

We assessed the polymorphisms in 64 individuals from eight populations of *F. sericea*. Eight individuals were collected from each of the following populations in Japan: Arahama, Niigata Prefecture (37°24′58.3″N, 138°35′05.4″E);

Hamazume, Kyoto Prefecture (35°39′24.9″N, 134°57′14.4″E); Hakoishi, Kyoto Prefecture (35°39′02.8″N, 134°56′23.9″E); Ajigaura, Ibaraki Prefecture (36°24′04.6″N, 140°36′35.2″E); Fukude, Shizuoka Prefecture (34°39′49.4″N, 137°52′54.3″E); Shiratsuka, Mie Prefecture (34°46′10.9″N, 136°32′29.4″E); Irino, Kochi Prefecture (33°01′42.8″N, 133°01′22.8″E); and Ryujin, Oita Prefecture (33°30′41.6″N, 131°43′51.6″E). Voucher specimens are unavailable because only a small amount of samples were collected to limit negative impact of sampling on maintenance of the local populations. We used GenAlEx 6.5 (Peakall and Smouse, 2006, 2012) to estimate the genetic diversity. The number of alleles per locus in the total sample and within populations was 2–5 and 1–3, with mean values of 3.5 and 1.4, respectively (Table 2). The total expected heterozygosity per locus (H_t) was 0.069–0.645, with a mean value of 0.336. The average observed heterozygosity within a population per locus (H_o) was 0.000–0.016. The average expected heterozygosity within a population per locus (H_e) was 0.051–0.230. We determined the deviation from Hardy–Weinberg equilibrium (HWE) and the linkage disequilibrium (LD) between all pairs of polymorphic loci using GENEPOP 4.2 (Rousset, 2008). Exact tests of the deviation from HWE detected significant excesses for homozygotes at 12 loci ($P < 0.05$), suggesting a high level of inbreeding. Significant LD was found for two pairs

TABLE 2. Genetic variability of each locus within eight natural populations of *Fimbristylis sericea*.

Locus	Total samples		Within population			Populations deviating from HWE ($P < 0.05$)
	A	H_t	A (mean)	H_o (mean)	H_e (mean)	
Fse33	3	0.253	1–3 (1.3)	0.000	0.000–0.594 (0.130)	Shiratsuka, Ryujin
Fse47	4	0.151	1–3 (1.5)	0.000	0.000–0.406 (0.128)	Hamazume, Hakoishi
Fse1-1	4	0.549	1–2 (1.3)	0.000	0.000–0.469 (0.121)	Arahama
Fse1-5	4	0.536	1–3 (1.7)	0.000	0.000–0.594 (0.203)	Arahama, Irino
Fse1-18	5	0.645	1–3 (1.8)	0.000–0.125 (0.016)	0.000–0.490 (0.230)	Fukude, Irino, Ryujin
Fse2-22	4	0.312	1–2 (1.5)	0.000–0.125 (0.016)	0.000–0.492 (0.194)	Fukude, Shiratsuka, Irino
Fse3-70	2	0.069	1–2 (1.1)	0.000	0.000–0.408 (0.051)	Ryujin
Fse4-5	3	0.402	1–2 (1.6)	0.000	0.000–0.375 (0.167)	Hamazume
Fse4-19	3	0.451	1–2 (1.5)	0.000–0.125 (0.016)	0.000–0.305 (0.123)	—
Fse4-25	4	0.202	1–2 (1.2)	0.000	0.000–0.469 (0.145)	Ajigaura, Fukude
Fse4-29	3	0.215	1–2 (1.2)	0.000	0.000–0.490 (0.120)	Arahama, Hakoishi
Fse4-31	4	0.243	1–3 (1.3)	0.000	0.000–0.612 (0.139)	Ajigaura, Fukude
Mean	3.5	0.336	1.4	0.004	0.146	

Note: A = number of alleles; H_e = expected heterozygosity within population; H_o = observed heterozygosity; H_t = total expected heterozygosity; HWE = Hardy–Weinberg equilibrium.

of polymorphic loci: Fse47 and Fse4-5 from Hamazume and Fse2-22 and Fse4-25 from Fukude ($P < 0.05$).

CONCLUSIONS

In this study, we developed 12 microsatellite markers for *F. sericea*, which were polymorphic among and within populations. These markers will be useful for surveying the genetic structure of local populations and the spatial genetic structure within local populations of this species. Genetic information could be obtained using these markers to facilitate the conservation of local populations. These markers could also be used to estimate the mating system of *F. sericea*.

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