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PRIMER NOTE

Development of polymorphic microsatellite markers in a perennial herbaceous plant, *Polygonum cuspidatum* (Polygonaceae)

M. MIWA,*† Z. ZHOU,* M. SHINONE,* E. MARUTA‡ and T. HOGETSU*

**Asian Natural Environmental Science Center, The University of Tokyo, Midori-cho 1-1-8, Nishitokyo, Tokyo 188–0002, Japan,*
‡Department of Biology, Faculty of Science, Toho University, Miyama 2-2-1, Funabashi, Chiba 274–8510, Japan

Abstract

Six single-locus, polymorphic microsatellite markers in a perennial herbaceous plant, *Polygonum cuspidatum*, were developed. Tests to amplify these six loci in another *Polygonum* species failed, suggesting that the six markers are specific to *P. cuspidatum*.

Keywords: gene flow, microsatellite markers, *Polygonum cuspidatum*, polymorphism, primary succession, reproduction

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A perennial herbaceous plant, *Polygonum cuspidatum*, is a typical pioneer plant species in the volcanic primary succession in Japan. This species is dioecious, and increases the population both by sexual and vegetative reproduction. The volcanic primary succession is in progress on the southeastern slope of Mt. Fuji, Japan, where *P. cuspidatum* forms a large number of patches at 1400–1700 m above sea level. It is thought that the patches play an important role for invasion of arboreous plants in this volcanic primary succession. However, the reproductive manners and gene flow of *P. cuspidatum* in this succession process have not been well understood. In the present study, single-locus, polymorphic microsatellite markers were developed to investigate reproductive structure of *P. cuspidatum* growing on the southeastern slope of Mt. Fuji.

We isolated microsatellite regions from total DNA of *P. cuspidatum* after an enrichment technique (Liam *et al.* 2000; Miwa *et al.* 2000; Zhou *et al.* 2001). Sampled *P. cuspidatum* leaves were dried with silica gel. The DNA was extracted from the dried leaves by a modified CTAB method (Zhou *et al.* 1999), and digested with a restriction enzyme (*Hin*dIII or *Eco*RI). The 5′ projecting ends of the DNA fragments were blunted by a DNA Blunting Kit (Takara Shuzo Co., Tokyo), and ligated with an *Eco*RI/*Not*I/*Bam*HI adaptor (Takara Shuzo Co.) by a DNA Ligation Kit version 1 (Takara Shuzo Co.). The adaptor-ligated fragments were amplified by polymerase chain reaction (PCR) using a primer designed on the basis of the sequence at their ends. An oligonucleotide combination of $(GCC)_{10}$, $(CAA)_{10}$ and $(CTG)_{10}$ or a combination of $(GC)_{15}$, $(GA)_{15}$ and $(GT)_{15}$ was attached to membrane filter and used for enrichment. Microsatellite-containing fragments were cloned into pT7Blue vectors using a pT7Blue Perfectly Blunt Cloning Kit (Novagen Co., WI, USA), and propagated in *Escherichia coli*, XL1-Blue MRF′ strain. Sequencing reactions were performed using a Texas Red T7 primer (Hitachi Instruments Service Co., Tokyo) and a Thermo Sequenase premixed cycle sequencing kit (Hitachi Instruments Service Co.) according to the manufacturer's instructions. Extension products were separated by a sequencer (SQ-5500, Hitachi Co., Tokyo). Primer pairs for amplification of the microsatellite regions were designed on the basis of the sequences flanking the obtained microsatellites.

To characterize each microsatellite locus, the regions in the DNA extracted from dried leaves of 36 *P. cuspidatum* individuals growing on the southeastern slope of Mt. Fuji were amplified by PCR using the designed primer pairs. The amplification was performed in 10 μL of a reaction mixture containing 10 ng DNA, 0.4 mM of each dNTP, 0.2 μM of each designed primer pair, 1 × GC buffer I (Takara Shuzo Co.; ingredients are unavailable) in which 2.5 mM of Mg^{2+} are included, and 0.5 U of LA *Taq* DNA polymerase (Takara Shuzo Co.), where the reverse primer was labelled with Texas Red, by a PCR Thermal cycler

Correspondence: M. Miwa. †Present address: Center for Environmental Science in Saitama, Kamitanadare 914, Kisai Town, Kita-Saitama-gun, Saitama 347-0115, Japan. Fax + 81 480 70 2031; E-mail: m-miwa@kankyou.pref.saitama.jp

Table 1 Primer pairs for amplification of six polymorphic microsatellite regions in *Polygonum cuspidatum* and some characteristics of the microsatellite loci

Microsatellite locus	Primer pair sequence (5′ → 3′)	Repeat type†	Size range (bp)	T_a(°C)‡	Allele number	H_O§	H_E¶
Pcu 1 (AB055129*)	F: ACGTAAAACACATATATGCAGTG R: TCATGTCTTACGCATACAATTAC	$(AT)_2(GT)_{35}$	96–205	53	28	0.89	0.94
Pcu 2 (AB055130)	F: AATGGTCTGAGAGCCGCCG R: GGCAGCTTTCAACTGGAGAGG	$(GGC)_5$	83–121	60	5	0.42	0.36
Pcu 3 (AB055131)	F: CGCTCAACCTAAGAGCACTAG R: TGGAGTTGACATTATCACCGTC	$(CCG)_3(CCACCG)_2$ $(CCACGG)(CCG)_4$	82–95	57	4	0.44	0.45
Pcu 4 (AB055132)	F: GATGGCTAGCGAAACAACAC R: CTTCTAGGTAGTTTCAGTGG	$(CA)_{10}(CG)(CA)_{32}$	155–219	54	18	0.78	0.93
Pcu 5 (AB055133)	F: TGCATATTATCGTCTGTTTC R: GACATGTTATTCAAGTCTTATC	$(CA)_{22}$	73–117	51	14	0.63	0.87
Pcu 6 (AB055134)	F: GGTCAAGACTTTGAGATGTGTATG R: CTCAACTGTTCAATAATACCCC	$(CA)_{39}$	44–136	51	22	0.46	0.95

*DDBJ/EMBL/GenBank accession number for the cloned sequences.
†Sequenced microsatellite repeat type.
‡Annealing temperature for PCR amplification.
§Observed heterozygosity (n = 36 individuals).
¶Expected heterozygosity (n = 36 individuals).

(TP3000, Takara Shuzo Co.). The reaction schedule was as follows: (i) 29 cycles at 94 °C for 1 min, annealing temperature shown in Table 1 for 1 min, 72 °C for 1 min; (ii) one cycle at 94 °C for 1 min, annealing for 1 min and 72 °C for 5 min. The PCR products labelled with Texas Red were denatured by heating and separated by the sequencer on a sequencing gel [6% Long Ranger (FMC BioProducts Co., ME, USA), 6.1 M urea and 1.2 × TBE] in 0.6 × TBE. The allele sizes were estimated by a computer software FRAGLYS version 2 (Hitachi Electronics Engineering Co., Tokyo). Observed and expected heterozygosities at each microsatellite locus were calculated after Nei's equation (Nei 1987).

The sequences of 16 microsatellite loci in DNA of *P. cuspidatum* were determined. Six out of 16 loci showed single-locus, polymorphic banding patterns (Table 1). These polymorphic loci had 4–28 alleles per locus and expected heterozygosities ranging from 0.36 to 0.95. Observed heterozygosities in most loci showed relative consistency with the expected ones. However, observed genotype frequencies at four loci, i.e. Pcu 1, Pcu 4, Pcu 5 and Pcu 6, differed significantly from those expected under Hardy–Weinberg equilibrium ($P < 0.05$) (Nei 1987), indicating that these loci may have null alleles. Two out of 16 loci were not polymorphic and at another eight loci, multiple bands were amplified by the designed primer pairs (data not shown).

P. weyrichii var. *alpinum* also grows as a pioneer plant species on the southeastern slope of Mt. Fuji. We investigated whether the six single-locus, polymorphic microsatellite markers in *P. cuspidatum* are amplified by PCR using total DNA extracted from a *P. weyrichii* var. *alpinum* individual. However, no clear bands were amplified by the designed primer pairs. This suggests that the six microsatellite markers are specific to *P. cuspidatum*.

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