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TECHNICAL NOTE

Development of 10 microsatellite loci for *Rheum tanguticum* (Polygonaceae)

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Abstract Rhubarb is an important Traditional Chinese Medicine. However, the wild resource has been declining. In order to design appropriate conservation methods for the official species across their natural distributions, it is important to characterize their genetic diversity. Here, we describe the development of 10 new microsatellite loci for AC/TG/CCA in *Rheum tanguticum*. The microsatellites were enriched using the combined biotin capture method. The polymorphism of each locus was further assessed in 12 individuals from four geographically distinct populations of this species. The number of alleles ranged from three to seven and the expected heterozygosity ranged from 0.53 to 0.73. All markers have been checked in the other three species in the genus and two of them together comprise the official medicinal rhubarb resource with *R. tanguticum*. These microsatellite markers could provide a useful tool for genetic and conservation studies of the rhubarb species.

Keywords Rhubarb · *Rheum tanguticum* · Microsatellite markers · Genetic diversity · Conservation

Rhubarb (*Rheum*) is known, in China, as the 'lord' or 'king of herbs'. It is a very important Traditional Chinese Medicine, commonly used for the treatment of constipation, diarrhea, jaundice, etc (Yang et al. 2001). Three closely related species (*R. palmatum* L., *R. tanguticum* Maxim. ex Balf. and *R. officinale* Baill) are officially regarded as medicinal plants in China (Wang et al. 2005). Because of excessive exploitation, the wild sources of these species decrease annually and they are now classified as endangered (Yang et al. 2001). To design effective conservation and recovery strategies, it is necessary to have available basic genetic information about these species. In this paper we describe the development of 10 polymorphic microsatellite loci in *R. tanguticum*. These are potential tools for investigating the genetic structures of rhubarb.

Genomic DNA was extracted from the dried leaves by a modified cetyltrimethyl ammonium bromide (CTAB) method (Zhou et al. 1999). About 500 ng genomic DNA was completely digested with a restriction enzyme *Rsa*I (NEB), and ligated to SuperSNX linkers as described by Hauswaldt and Glenn (2003). For enrichment, the ligation products were hybridized with an oligonucleotide combination of 5′-biotinylated probes $(AC)_{15}$, $(TG)_{15}$ and $(CCA)_{10}$ in 50 µl hybridization solution (2 × SSC, 1µmol/l probe and 10 µl ligation products) as follows: an initial 5 min at 95°C, then a rapid cooling to 70°C, followed by 0.2°C incremental decreases every 5 s for 99 cycles, and maintenance at 50°C for 10 min; then decreases of 0.5°C every 5 s for 20 cycles, and finally rapid cooling to 15°C. The DNA hybridized to the probe was captured by streptavidin-coated magnetic beads at 37°C for 1 h, followed by two washing steps; each including two washes with washing solution I (2× SSC, 0.1% SDS) for 2 min at room temperature and four washes with washing solution II (1× SSC, 0.1% SDS) for 2 min at 40°C, 50°C, 45°C, 45°C in turn. Captured DNA was recovered by polymerase chain PCR with SuperSNX-f (5′-GTTTAAGGCCTAGCTAG-CAGAATC-3′) as follows: 2 min at 94°C; 30 cycles of

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Table 1 Characteristics of 10 polymorphic microsatellite loci for *Rheum tanguticum*

Locus	Repeat	Primers sequence (5′-3′)	Ta (°C)	N	Size range (bp)	Number of alleles	Ho	H_E	GenBank Accession number
Rta001	$(TG)_9$	F:GTATGCTATTATTGTGGTTGGAC	50	12	133–155	5	0.39	0.57	EF571580
		R:CAGCGGAATCATGAATTAGTAAC							
Rta 002	$(AC)_{12}$	F:GAATCACAAACAAAGCTTACCC	52	12	208–234	3	0.45	0.64	EF571579
		R:CATATGTTGCTTGTATGTATGGC							
Rta 003	$(TCG)_5$	F:AAAGCCATCCAAATCGAAGC	50	12	81–115	3	0.47	0.70	EF571578
		R:CTACAGAGGCAAGACAATCAAC							
Rta 004	$(TCG)_8$	F:AAGCGTGTGGTGTGCTGAGAG	48	12	173–206	4	0.44	0.64	EF571577
		R:CACAGTTTGAACCATTTAAACAC							
Rta 005	$(CCA)_5$–$(CCA)_7$	F:CCGAAGTCCAAGTAGGGGTCC	54	12	172–202	6	0.32	0.73	EF571576
		R;CACCAAACCCACTTCAACCAC							
Rta006	$(AC)_{12}$	F:CAGCGTAATCACGACTTAGAAC	52	12	69–93	4	0.39	0.57	EF571575
		R;GAGTGTGTATGACGTGTTGATG							
Rta007	$(TGG)_5$	F:GGGTAGTCCCCTTTGAGGTTGTAG	52	12	173–197	7	0.36	0.53	EF571574
		R:TGCATGCCTGCAGGTCGACG							
Rta 008	$(AC)_{6}$-$(AC)_5$-$(AC)_4(CA)_4$	F:AGCAGAATCAATTCACGTTCAC	47	12	221–265	5	0.41	0.60	EF571573
		R:CAATATGTGCTTAGATTTGGC							
Rta009	(GT)-$_4(TG)_9$-$(TG)_4$-$(TG)_4$	F:TTGAGGCATTGCGTGTGAGC	52	12	217–249	7	0.39	0.57	EF571572
		R:ACACAATCCTTTGTCTCATATGC							
Rta010	$(AC)_{12}$	F:GAGCTCGGTACCCGGGGATC	52	12	88–124	5	0.44	0.64	EF571571
		R:TGCAGGTCGACGATTTTTAAGGC							

Ta, annealing temperature of primer pair; N, number of individuals genotyped; Ho: Observed heterozygosity; H_E, Expected heterozygosity

94°C for 20 s, 60°C for 20 s, 72°C for 90 s; followed by 5 min at 72°C. The PCR products, after purification using a TIANquick Mini Purification Kit (TIANGEN), were cloned into a pMD18-T vector (Takara) according to the manufacturer's instructions, and propagated in the Top10 strain of *E. coli*. Positive clones were harvested and cultured. Plasmid DNA was extracted using a U-gene Plasmid Moni Kit (U-gene). Each clone, 300–700 bp long as determined by PCR using universal M13 primers, was sequenced to verify the presence of microsatellites and to characterize flanking regions for primer design using a 3130xl Genetic Analyzer. The sequences containing motifs repeating more than five times were regarded as microsatellites.

A total of 26 sequences were found to contain simple sequence repeats. Primer pairs for amplification of the microsatellite regions were designed on the basis of the sequences flanking them using the Primer 5.0 (Clarke and Gorley 2001). To analyze the genetic polymorphism of the identified microsatellite loci, 12 individuals collected from four wild populations were used for genotyping. The amplification was performed in 20 μl of a reaction mixture containing 10 ng of genomic DNA, 0.3 mM of each dNTP, 0.3 μM of each primer, 2 μl *Taq* buffer and 0.5 unit of *Taq* polymerase (Takara) using a PTC-200 thermal cycler (MJ Research). Amplifications used an initial denaturation of 3 min at 94°C, followed by 35 cycles of denaturation for 40 s at 94°C, annealing for 40 s at 47–52°C and 90 s at

Table 2 Cross-species amplification of *Rheum tanguticum* microsatellites in other species *R. palmatum*, *R. officinale* and *R. pumilum*

Species	Locus									
	Rta25	Rta23	Rta19	Rta17	Rta16	Rta12	Rta8	Rta7	Rta5	Rta2
R. palmatum	+	+	–	+	+	+	+	+	+	+
R. officinale	+	–	+	+	–	+	+	±	+	+
R. pumilum	+	–	+	–	+	–	+	–	+	–

Two individuals of each species were screened. +, Expected size band amplification; ±, Unexpected size band amplification; –, no amplification

72°C, and a final extension of 4 min at 72°C. The PCR products were resolved on 6.5% polyacrylamide denaturing gel and visualized by silver staining. The band size was determined using a 10 bp DNA ladder (Invitrogen) as the reference.

Preliminary population genetics analyses were performed using GENEPOP version 3.4 (http://wbiomed.curtin.edu.au/genepop/) (Raymond and Rousset 1995). In total, 16 out of the 26 sequences comprised a single-locus, and the other 10 produced polymorphic banding patterns (Table 1). These 10 polymorphic loci had three to seven alleles per locus and the observed heterozygosity ranged from 0.32 to 0.47. For each locus, the expected heterozygosity was always significantly higher than the observed heterozygosity ($P < 0.05$). No significant genotypic disequilibrium was detected for any pair of loci. As shown in Table 1, the size of the PCR products of these alleles exceeded 20 bp in most loci. The alleles were sequenced and it was confirmed that they were the target sequence.

Cross-priming tests were performed in the other three species, *R. palmatum*, *R. officinale* and *R. pumilum*, using two individuals of each species (Table 2). Nine loci were successfully amplified in *R. palmatum*, seven in *R. officinale* and five in *R. pumilum*. These polymorphic microsatellite loci could be useful for assessing the genetic structure of the wild populations of the three official species (*R. tanguticum, R. palmatum* and *R. officinale*).

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