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TECHNICAL NOTE

Isolation of polymorphic microsatellite markers from *Przewalskia tangutica* (Solanaceae)

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Abstract Microsatellite-containing regions were isolated and characterized in *Przewalskia tangutica* Maxim (Solanaceae), an endemic and endangered species to the Qinghai-Tibetan Plateau of China. An enrichment protocol yielded 200 positive clones. We designed primers to amplify 29 unique microsatellites, 12 of which amplified cleanly and were polymorphic. A survey of 17 individuals showed that these loci are highly variable with the number of alleles ranging from 3 to 12, and expected heterozygosity ranged from 0.2929 to 0.4947. Those markers will be useful for studies of population structure and intraspecific variation in *P. tangutica*.

Keywords *Przewalskia tangutica* · Microsatellite markers · Genetic diversity

Przewalskia tangutica, the only species in this monotypic genus of the tribe Hyoscyameae (Solanaceae), is mainly distributed in sandy and gritty grasslands of the Qinghai-Tibetan Plateau at altitudes ranging from 3,000 to 4,000 m. Because of its medicinal importance (associated with high biologically active nicotine and tropane alkaloid contents) (Pei and Li 1982; Wang et al. 2002), the species has been subjected to extensive collection and the size of most populations has decreased greatly; some have even disappeared (Yang 1991). Except for its phylogenetic and taxonomic relationships (Yang et al. 2002), this species has been poorly studied. The genetic diversity within and between populations undoubtedly is essential for designing conservation grams. In this study, we aimed to develop microsatellite primers for this endangered species.

We used DNeasy^{TM} Tissue Kit (Qiagen) to extract the total genomic DNA from the silica gel using dried leaves. The microsatellite regions were isolated following Zhang et al. (2007). About 500 ng genomic DNA was digested into approximately 500 bp fragments with restriction enzymes *Rsa*I (NEB) and *Xmn*I (NEB), then ligated to SuperSNX24 double-stranded adaptors (mixation of equal volumes of equal molar amounts of SuperSNX24-F: 5′-GTTTAAGG CCTAGCTAGCAGAATC-3′ + SuperSNX24 + 4P-R: 5′-GATTCTGCTAGCTAGGCCTTAAACAAAA-3′). For the further enrichment, the ligation products were hybridized with an oligonucleotide combination of 5′-biotinylated probes: $(AG)_{15}$, $(CT)_{15}$, $(AC)_{15}$, $(GT)_{15}$, $(CG)_{15}$, and $(CCA)_{15}$. The hybridization in the 50 μl solution (2 × SSC, 1 μmol/l probe and 10 μl ligation products) following the protocol: 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 and then washed by the solution I (2× SSC, 0.1% SDS) and solution II (1× SSC, 0.1% SDS). The captured DNA was recovered by polymerase chain reactions (PCR) with SuperSNX-F (5′-GTTTAAGGCCTAGCTAG CAGAATC-3′) and PCR product was purified with TIANquick Midi Purification Kit (TIANGEN). These fragments enriched with microsatellite loci were cloned using pMD18-T vector (TakaRa) and transformed into the *E. coli* competent cell (JM109, TakaRa). Positive colonies were amplified using BcaBEST^{TM} Sequencing Primers RV-M and M13-47. PCR products of 300–600 bp were

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sequenced using 3130xl Genetic Analyzer. The sequences containing motifs repeating more than 5 times were regarded as microsatellites. A total of 29 sequences were identified among the sequenced 200 sequences and primer pairs for amplification of the microsatellite regions were designed using the Primer 5.0 (Clarke and Gorley 2001).

In order to check polymorphisms of the identified microsatellite loci, 17 individuals from distantly distributed populations were selected for test. The PCR reactions were performed in 25 μl reaction mixtures with 10–40 ng template DNA, containing 19 μl of sterile double distilled water; 2.5 μl of 10 × Taq polymerase reaction buffer; 1 μl each of the primers; 1 unit TaqDNA polymerase. The amplifications used an initial denaturation of 5 min at 94°C, and then followed by 38 cycles of 94°C for 40 s, annealing for 40 s at 50–55°C, 72°C for 45 s plus a final extension of 72°C for 10 min. PCR products were initially checked for PCR amplification on 2.0% agarose gels. The successful PCR products were further separated through 6.5% polyacrylamide denaturing gels. Allele sizes were compared with a 50 bp DNA ladder (TakaRa) and visualized by silver staining.

Allelic/genotypic frequencies were analyzed using GENEPOP version 3.4 (http://wbiomed.curtin.edu.au/genepop/) (Raymond and Rousset 1995) to estimate observed and expected heterozygosity (H_o and H_E). Twelve loci of the 29 sequences showed polymorphic banding patterns and the other 17 comprised a single locus (Table 1). These loci had 3–12 alleles per locus and the observed heterozygosity and expected heterozygosity ranged from 0.1652 to 0.3183 and from 0.2929 to 0.4947, respectively. For each locus, the expected heterozygosity was always significantly bigger than the observed heterozygosity ($P < 0.05$). No significant genotypic disequilibrium was detected for any pair of loci. As shown in Table 1, the sizes of the PCR products of these alleles range exceeding 20 bp in most of 12 microsatellite loci and the alleles were sequenced and verified to be the target sequence.

The developed 12 loci in this study display successful amplification, reliable scoring and highly polymorphism

Table 1 Characteristics of 12 polymorphic microsatellite loci for *P. tangutica*

Locus	Primers sequence (5′-3′)	Repeat motif	T_a (°C)	N	size range (bp)	No. of alleles	H_o	H_E	GenBank accession no.
Pt01	F:CTGACTTTGCCTGAACG R:GAATCCTCCAGCCAGTA	$(GA)_5$	50	17	82–107	6	0.1652	0.2929	EU123857
Pt02	F:ACAAGAAAGAGGGGATAGA R:CCACCACCACGCTCTT	$(AG)_{17}$	50	17	127–163	5	0.3183	0.4947	EU123858
Pt03	F: TAGCAGAATCACTTGGTC R: TTCACAGGGATACAAACAG	$(GT)_8$- $(TG)_5$	52	17	125–205	12	0.3115	0.4874	EU123859
Pt04	F:CAGCCCCAAAGAACAA R:GGGCTGCTTCAACTACAC	$(GT)_{10}$	50	16	130–142	4	0.2714	0.4237	EU123860
Pt05	F: CCCTTCCAAGTCACAAA R: ATCGTCGTGAAACAAACAT	$(TC)_8$	50	17	105–125	3	0.2241	0.3742	EU123861
Pt06	F: ACAATGTTTTGCGATAGAC R: CACCAGTAGTGATTATGCT	$(AC)_8$	52	17	142–187	6	0.1661	0.2980	EU123862
Pt07	F: AACTACGCTCTCTGATCCT R: TTCTTGGTTCACTGTTCTC	$(CCA)_5$	55	17	110–126	5	0.2161	0.3325	EU123863
Pt08	F: TCTTCCTGCAACTTATACAC R: CAGGAAACAGCTATGACC	$(TC)_8$	55	15	102–132	8	0.2549	0.4125	EU123864
Pt09	F: GGCCTAGCTAGCAGAATC R: TCTTAACAGAGCAACCATG	$(TG)_5$	50	17	182–196	4	0.2549	0.4181	EU123865
Pt10	F: GCTGCTGACATAGTTCCG R: GTCAATAGAGTTTATTGCA	$(TC)_8(CT)_5(CT)_9(TC)_9$	55	17	209–176	10	0.2780	0.4086	EU123866
Pt11	F: CCTACTGTCGCAACCC R: AATGACGACGAGGTGC	$(AG)_5$	50	15	95–110	6	0.2618	0.4116	EU123867
Pt12	F: GAATCGTTTCCACAATACA R: AAATGACCTGTCCAAGAG	$(TC)_{10}$	50	17	140–172	9	0.2360	0.3950	EU123868

The number of repeats is based on the sequence of the cloned alleles.

T_a, annealing temperature; N, number of individuals genotyped; H_o, observed heterozygosity; H_E, expected heterozygosity

levels. This set of loci will be useful in the studies of population genetics of *P. tangutica*.

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