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TECHNICAL NOTE

Isolation and characterization of microsatellite DNA primers in *Juniperus przewalskii* Kom (Cupressaceae)

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Abstract *Juniperus przewalskii* (Cupressaceae) is a dominant tree species endemic to the northeast Qinghai-Tibetan Plateau. This species plays an important role in maintaining the arid ecosystem in this region. However, natural distributions of this species have been declined. In order to develop effective conservation methods, it is important to know the distribution of the genetic diversity within and among populations. In this study, we developed nine new microsatellite loci for this species. We used the combining biotin capture method to enrich AG/CT/AC/GGT microsatellites. The polymorphisms of each locus were further assessed in 12 individuals from four geographically distant populations. The number of alleles per locus varied from three to six and expected heterozygosity ranged from 0.58 to 0.70. These loci together provide a useful tool to investigate the genetic diversity of this species. In addition, all markers have been crossly checked in the other four congeneric species.

Keywords *Juniperus przewalskii* · Microsatellite markers · Genetic diversity

Juniperus przewalskii Kom (Cupressaceae) is a dominant tree species of the 'island forests' scattered among the alpine meadow ecosystem in the northeast Qinghai-Tibetan Plateau (Zhang et al. 2005). The forest stands of this species plays an important role in maintaining the arid ecosystem in the plateau high altitude region. However, because of the overexploitation, the natural distributions of this species have been declined and it is necessary to conserve and artificially cultivate this spices (Zhang et al. 2005). It is important to characterize the genetic diversity of this species before developing conservation and cultivation methods. In this paper, we describe development of nine polymorphic microsatellite loci in *J. przewalskii*.

The total genomic DNA was extracted from the silica gel dried needle tissues using the cetyltrimethyl ammonium bromide (CTAB) method (Doyle and Doyle 1987). We isolated microsatellite regions after the enrichment technique following the protocol suggested by Korfanta et al. (2002) and Hauswaldt and Glenn (2003). About 500 ng genomic DNA was digested into approximately 500 bp fragments with a restriction enzyme *Rsa*I (NEB) and then ligated to SuperSNX24 double-stranded adaptors (mixation of equal volumes of equal molar amounts of SuperSNX24-F: 5′-GTTTAAGGCCTAGCTAGCAGAATC-3′ + SuperSNX24 + 4P-R: 5′-GATTCTGCTAGCTAGGCCTTAA ACAAAA-3′). For enrichment, the ligation products were hybridized with an oligonucleotide combination of 5′-biotinylated probes, $(AG)_{15}$, $(CT)_{15}$, $(AC)_{15}$ and $(GGT)_{12}$. The hybridization in the 50 µl solution (2 × SSC, 1 µmol/l probe and 10 µl ligation products) was 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 and then washed by the solution I (2 × SSC, 0.1% SDS) and solution II (1 × SSC, 0.1% SDS). The captured DNA was

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recovered by polymerase chain reactions (PCR) with SuperSNX-F (5′-GTTTAAGGCCTAGCTAGCAGAATC-3′) and PCR products were purified with TIANquick Midi Purification Kit (TIANGEN). These fragments enriched with microsatellite loci were cloned using pMD18-T vector (Takara) and propagated in the Top10 strain of *E. coli*. Positive colonies were amplified using M13 forward and reverse primers. PCR products of 300–600 bp were sequenced using 3130xl Genetic Analyzer. The sequences containing motifs repeating more than 5 times were regarded as microsatellites. A total of 31 sequences were identified out of 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, 12 individuals from four distantly populations (Qilian, Tianzhu, Maqin, and Luqu) (Zhang et al. 2005) were selected for genotyping. PCR amplifications were performed in 20 μl of a reaction mixture containing 0.3 mM of each dNTP, 0.3 μM of each primer, 2 μl *Taq* buffer, 0.5 unit of *Taq* polymerase (Takara) and 20 ng of genomic DNA using PTC-200 thermal cycle (MJ Research). The amplifications used an initial denaturation of 5 min at 94°C, followed by 35 cycles of denaturation for 40 s at 94°C, annealing for 40 s at 48–54°C and 90 s at 72°C, and a final extension of 4 min at 72°C. PCR products were initially checked for PCR amplification on 2.0% agarose gels. The successful PCR products were further resolved on 6.5% polyacrylamide denaturing gel using a 10 bp DNA ladder (Invitrogen) as the reference and visualized by silver staining. We calculated observed and expected heterozygosity (H_o and H_E) using GENEPOP version 3.4 (http://wbiomed.curtin.edu.au/genepop/) (Raymond and Rousset 1995). In total, 22 out of 31 sequences comprised a single-locus and the other nine loci showed polymorphic banding patterns (Table 1). These loci had 3 to 6 alleles per locus and the observed heterozygosity and expected heterozygosity ranged from 0.39 to 0.50 and from 0.58 to 0.70, 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 size of the PCR products of these alleles exceeded 20 bp in most of nine microsatellite loci and the alleles were sequenced and verified to be the target sequences.

Michalczyk et al. (2006) reported five microsatellite loci from another congeneric species, *J. communis* L. We checked the polymorphisms of these loci. Two out of five failed to amplify and only one (JC032) of the remaining three loci (JC031, JC032 and JC035) showed polymorphic banding patterns (Table 1). The observed heterozygosity and the expected heterozygosity are 0.50 and 0.69, respectively. As pointed out repeatedly, the different species even within the same genus may have different microsatellite loci (Primmer et al. 1996).

Table 1 Characteristics of 10 polymorphic microsatellite loci for *Juniperus przewalskii* (nine are firstly developed here and Jc032 was reported by Michalczyk et al. (2006))

Locus	Primers sequence (5′-3′)	Repeat	T_a (°C)	N	Size range (bp)	No. alleles	H_o	H_E	GenBank accession no.
Jp01	F:AAGGCCTACCTAGCAGAATCAC R:ACTCACTATAGGGCGAATTGGG	$(GA)_6$	50	12	117–129	3	0.40	0.64	EF656603
Jp02	F:CAACAATGGAAGGACAGTCAC R:GGGGGTCCTTTAGAGATTAAG	$(AG)_9$	50	12	138–155	4	0.42	0.61	EF656604
Jp03	F:AGGCCAAATCACTTGAGTATAAC R:CCTACATGAGTTCCTTCTACACC	$(AG)_{12}$	50	12	140–172	4	0.44	0.63	EF656605
Jp04	F:CTCTCAAGTTCTCTTTCTTCCTC R:TAAAACGACGGCCAGTGCC	$(CT)_5$-$(TC)_6$-$(CT)_5$	52	12	234–251	4	0.41	0.60	EF656606
Jp05	F:CGGGGATCCTCTAGAGATTTTTAAG R:CTGCAGGTCGACGATTGTTTAAG	$(CA)_{24}$-$(TC)_{11}$-$(CA)_{16}$	54	12	327–367	6	0.41	0.70	EF656607
Jp06	F:CTGGCAGCGTATGCAACAATAC R:TGCAGGTCGACGATTTTTAAGG	$(GGC)_7$-$(GGT)_5$	50	12	236–264	5	0.45	0.62	EF656608
Jp07	F:CATCCTCTTCAGTTAGGGTCC R:GATTTAGTGGCACCTACATGAG	$(AG)_5$	54	12	179–208	4	0.50	0.68	EF656609
Jp08	F:AGCAGAATCAATTCACGTTCAC R:CAATATGTGCTTAGATTTGGC	$(CT)_{11}$	52	12	164–189	5	0.47	0.66	EF6566010
Jp09	F:GCTAAATAGGCAATGGCAGG R:GTGCTTTCTTATGGATACTTACCCC	$(AC)_{16}$	48	12	163–176	3	0.39	0.58	EF6566011
Jc032	F:ACATTGCAAATATGGGGTAA R:TTGATGAGTTGTTGAGTTATTAAG	$(AC)_{14}$-$(ATC)_8$	48	12	160–183	4	0.50	0.69	DQ192496

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

Table 2 Cross-species amplification of *J. przewalskii* and *J. communis* microsatellites in other species: *J. saltuaria*, *J. microsperma*, *J. tibetica*, and *J. convallium*

Species	Locus													
	Jp01	Jp02	Jp03	Jp04	Jp05	Jp06	Jp07	Jp08	Jp09	Jc032	Jc016	Jc031	Jc035	Jc037
J. przewalskii	+	+	+	+	+	+	+	+	+	+	−	+	+	−
J. saltuaria	−	+	−	−	−	+	+	+	−	−	−	−	−	−
J. microsperma	+	−	+	+	+	−	+	+	+	+	−	−	+	−
J. tibetica	−	−	+	+	+	−	+	+	−	+	−	−	+	−
J. convallium	+	−	+	−	+	−	+	+	+	−	−	+	−	−

Three individuals of each species were screened. +, expected size band amplification; −, no amplification. (Jc032, Jc016, Jc031, Jc035, and Jc037 were developed for *J. communis* by Michalczyk et al. 2006)

We further performed cross-priming tests (including nine loci developed here and five previously reported by Michalczyk et al. 2006) in the other four congeneric species, i.e., *J. saltuaria*, *J. microsperma*, *J. tibetica*, and *convallium* using three individuals of each species (Table 2). Four loci were successfully amplified in *J. saltuaria*, seven in *J. microsperma*, five in *J. tibetica* and six in *convallium*.

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