

PRIMER NOTE

Isolation and characterization of polymorphic microsatellite markers from *Primula nutans* (Primulaceae)

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Abstract

Seven polymorphic microsatellite loci were developed for a perennial seashore plant, *Primula nutans*. Degenerate oligonucleotide-primed (DOP)-polymerase chain reaction (PCR)-amplified DNA was ligated to TOPO TA vector and screened with radioactively labelled dinucleotide repeat probes. A sample of 378 individuals from Finland, Norway and Russia were used to characterize those loci, which exhibited two to four alleles per locus with observed heterozygosity of 0.003–0.229 and expected heterozygosity of 0.016–0.527. No linkage disequilibrium was found between these seven loci. These are the first microsatellite markers reported for *P. nutans*.

Keywords: microsatellite, *Primula nutans*, seashore meadow

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Primula nutans (Primulaceae) is a perennial rosette hemicryptophyte growing mainly on seashore meadows (Mäkinen & Mäkinen 1964). The species grows on low-lying clayey meadows mixed with sand and is often inundated both by the sea water and by flooded rivers. It prefers open habitats where its growth begins earlier than that of its competitors. It propagates by seeds and by runners that originate in the axils of the lower leaves (Mäkinen & Mäkinen 1964). Flowers are pollinated by insects, and seeds are dispersed mainly by water currents (Ulvinen 1997). *Primula nutans* is a member of *Primula sibirica* species group, which consists of seashore plant species that occur both on the shores of the Arctic Ocean and in the Baltic Sea (Euroala 1999). *Primula nutans* has a circumpolar distribution ranging from North America to Europe and Asia. The subspecies *P. nutans* ssp. *nutans* occurs in Asia and North America, whereas *P. nutans* ssp. *finmarchica* grows only in northern Europe and is divided into two varieties. Of these, *P. nutans* var. *finmarchica* occurs at the Arctic Ocean and *P. nutans* var. *jokelae* at the Bothnian Bay in Finland and Sweden and at the White Sea in Russia (Mäkinen & Mäkinen 1964).

The population number of the species has decreased in the last decades mainly due to the end of traditional land use and increased eutrophication (Ulvinen 1997; Pykälä 2000). According to the IUCN Red List criteria, the species

is classified as endangered in Finland (Rassi *et al.* 2001) and near threatened in Sweden (ArtDatabanken: <http://www.artdata.slu.se/redlist.htm>). Molecular markers are an effective tool for studying genetic variation, population structure and breeding system of the species, providing essential information for conservation efforts as well as studying species history after the latest glaciation. No previous microsatellite markers have been published, and no studies of genetic population structure have been made for *P. nutans*.

Genomic DNA was isolated with slightly modified cetyltrimethyl ammonium bromide (CTAB) method (Rogers & Bendich 1985) from 0.1 g of frozen or dried leaves of an individual plant. Genomic DNA (100 ng) was amplified using DOP (degenerate oligonucleotide-primed)-PCR (polymerase chain reaction) Master Kit (Roche Diagnostics), and the products were separated on 0.8% agarose gel in 0.5 × TBE buffer. DNA fragments of 650–1300 bp in size were extracted from agarose gel using the method by Glenn & Glenn (1994). DyNAzyme II polymerase (Finnzymes) was used to add 3' A-overhangs to DNA fragments (200 ng), which were cloned using TOPO TA Cloning Kit (Invitrogen™). Colony lifting and hybridization were performed using Hybond™-NX (Amersham Pharmacia Biotech) nylon membranes according to the instructions of the supplier. Hybridization temperature was 42 °C, and $\gamma^{32}\text{P}$ end-labelled dinucleotide oligos (AG)_{10'} (TC)_{10'} (GT)₁₀ and (CA)₁₀ were used as probes. Positive clones were

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sequenced using BigDye Terminator version 3.1 Cycle Sequencing Kit (Applied Biosystems) with M13 primers and analysed on ABI 3730 DNA Analyser (Applied Biosystems). Primers for 10 loci containing nine or more dinucleotide repeats were designed using PRIMER 3 (Rozen & Skaletsky 2000).

Microsatellite polymorphism was analysed by PCR with one fluorescently labelled primer and subsequent gel electrophoresis. Amplifications were performed in 10 µL reactions containing 10–20 ng template, 1.5 mM MgCl₂, 200 µM dNTPs, 0.5 µM primers and 0.4 U of DyNAzyme II (Finnzymes) in the buffer supplied with the enzyme. The PCR profile consisted of 94 °C for 5 min, 33 × (94 °C for 30 s, 60 °C for 30 s, 72 °C for 1 min) and 72 °C for 30 min. PCR products were run on ABI 3730 DNA Analyzer with GENEMAPPER 3.7 software (Applied Biosystems) using an internal size standard, GENESCAN 500 LIZ (Applied Biosystems). Primer sequences and empirically derived annealing temperatures are given in Table 1.

Three loci showed nonspecific amplification (PN1, PN8) or were monomorphic (PN7). The seven polymorphic loci were scored using 378 samples from 15 populations from Norway, Finland and Russia, consisting of both *P. nutans* var. *finmarchica* and *P. nutans* var. *jokelae*. Amplification success varied among loci; locus PN10 did not amplify correctly in 137 samples. The number of alleles was low, ranging from two to four (Table 1). Online version of the software GENEPOP (<http://wbimed.curtin.edu.au/genepop/>) was used in estimating observed (H_O) and expected (H_E) heterozygosities and linkage disequilibrium. As shown in Table 1, H_O among *P. nutans* var. *jokelae* varied from 0.004 to 0.367 and H_E from 0.013 to 0.491, and among *P. nutans* var. *finmarchica*, H_O varied from 0.007 to 0.958 and H_E from 0.007 to 0.595. Among pooled sample, H_O ranged from 0.003 to 0.229 and H_E from 0.016 to 0.527. All loci and populations were individually tested for significant deviations from Hardy–Weinberg equilibrium with conservative Bonferroni correction (Quinn & Keough 2002). We found significant ($P < 0.007$) heterozygote deficiency in locus PN10 in one and excess in two populations. At loci PN4 and PN6, there was a significant deficiency in one population. No linkage disequilibrium was detected for any pairwise combination of loci after conservative Bonferroni correction ($P < 0.007$). The microsatellite loci reported here are currently being used in analyses of genetic structure and variation of *P. nutans* populations.

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Table 1 Characteristics of seven microsatellite loci in *Primula nutans* var. *jokelae*. Observed (H_O) and expected (H_E) heterozygosities were calculated for pooled sample (378 individuals from 15 populations from Finland, Norway and Russia) and for the both varieties *P. nutans* var. *finmarchica* ($N = 143$) and *P. nutans* var. *jokelae* ($N = 235$)

| Locus | Primer sequences (5'–3') | Repeat motif | T_a (°C) | No. of alleles | Allele size range (bp) | <i>P. nutans</i> var. <i>jokelae</i> | | <i>P. nutans</i> var. <i>finmarchica</i> | | Pooled sample | | Accession no. |
|-------|--|---|------------|----------------|------------------------|--------------------------------------|-------|--|-------|---------------|-------|---------------|
| | | | | | | H_O | H_E | H_O | H_E | H_O | H_E | |
| PN2 | F: †TCGAGCGAATCAGGATAAATG R: ACCGGAGAATGATCAAAATG | (AC) ₁₀ (AT) ₉ | 60 | 2 | 198–200 | 0.004 | 0.013 | NA | NA | 0.003 | 0.476 | DQ200870 |
| PN3 | F: †TTTTTCACAATCAGTATGTGGG R: †GTGACAAAAGTGAAAAGACACC | (AG) ₉ | 60 | 3 | 148–152 | 0.022 | 0.026 | NA | NA | 0.014 | 0.016 | DQ200871 |
| PN4 | F: †CCCTCCCTACCCTTTGGTTTC R: CTCGGCAGGGGAGAGAGAG | (TC) ₈ CC(TC) ₈ | 60 | 3 | 98–102 | 0.367 | 0.491 | 0.007 | 0.035 | 0.229 | 0.398 | DQ200872 |
| PN5 | F: †TGATAAAGCTGCCAGAAAATG R: TGGAGTTTCTCGTATGGAGG | (AT) ₁₃ | 60 | 3 | 144–148 | 0.013 | 0.081 | NA | NA | 0.008 | 0.487 | DQ200873 |
| PN6 | F: †AGAGAGGGATATTTAGATCGCG R: CCCAACACACATTTATCTTCTCC | (GA) ₉ | 60 | 4 | 81–87 | 0.090 | 0.095 | 0.063 | 0.137 | 0.080 | 0.527 | DQ200874 |
| PN9 | F: †AGGTACGGCGGAGAGTGTTC R: TCAATGGGGTTTCTTACCC | (AG) ₉ | 60 | 3 | 175–181 | 0.215 | 0.250 | 0.007 | 0.007 | 0.136 | 0.167 | DQ200875 |
| PN10 | F: †GGGATGTGTATCTTTAC R: ACCCTAACCCCTAGACCTAACAC | (CT) ₇ CCCC(CT) ₂ CG(CT) ₈ | 60 | 4 | 144–158 | 0.005 | 0.057 | 0.958 | 0.595 | 0.199 | 0.248 | DQ200876 |

*fluorescent label FAM in primer; †fluorescent label Yakima yellow in primer; T_a (°C), annealing temperature; NA, not available.

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