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**DEVELOPMENT AND CHARACTERIZATION OF 25 EST-SSR MARKERS IN *PINUS SYLVESTRIS VAR. MONGOLICA* (PINACEAE)**

**PRIMER NOTE**

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**Key words:** expressed sequence tag; Pinaceae; *Pinus sylvestris var. mongolica*; polymorphism; primer pairs; transcriptome sequencing.

*Pinus sylvestris* L. var. *mongolica* Litv. (Pinaceae) is indigenous to the region north of the Greater Khingan Mountains and the Hulunbeier Grassland of the Inner Mongolia Autonomous Region (Zhu et al., 2006). This pine tree can live up to 150–200 yr and grow as tall as 15 m. It has strong cold resistance, enabling it to survive temperatures as low as –40°C, and is highly adaptable to various soil types with good growth on both barren and fertile land (Zhu et al., 2003). *Pinus sylvestris var. mongolica* is the best evergreen coniferous tree species for establishing windbreaks and providing sand fixation (Zhao et al., 2010). Due to these characteristics, it has economic and ecological benefits, and has been introduced and cultivated in many arid and semiarid regions of China, such as Chengde in Hebei Province (Zhao and Liu, 2007), Zhanggutai in Liaoning Province (Zeng et al., 2005), and Yulin in Shaanxi Province (Wang et al., 2009). This species originated from Honghuaerji in the Inner Mongolia Autonomous Region (Zhu et al., 2006). Molecular genetic studies have been few in number (Li et al., 2005), and no simple sequence repeats (SSRs) have been reported. To optimize the conservation and utilization of *P. sylvestris var. mongolica*, the development of expressed sequence tag (EST)–SSR markers is very useful for germplasm identification and research into the genetic diversity of this species.

Transcriptome sequencing is an efficient method for acquiring EST sequences. SSRs derived from EST sequences are more convenient and can be isolated with higher efficiency and at lower expense than genomic sequence SSRs (Wang et al., 2012). In a previous study, Niu et al. (2013) analyzed the evolution of genes in *Pinus* species and showed by clustering analysis that *P. sylvestris var. mongolica* is more closely related to *P. tabuliformis* Carrière than to three other *Pinus* species. Entries in PubMed (http://www.ncbi.nlm.nih.gov/pubmed) on Pinaceae SSRs account for only 0.42% of all entries related to SSRs to date (1 May 2013), and none of them focus on *P. sylvestris var. mongolica*. Because transcriptome sequence data are not available for *P. sylvestris var. mongolica*, we used the data available from *P. tabuliformis* to develop the markers described here. We developed and characterized 25 novel polymorphic EST-SSR markers for this species. These EST-SSR markers provide an important tool for the study of genetic diversity in *P. sylvestris var. mongolica*.

**METHODS AND RESULTS**

In total, 31,653 EST-SSR loci were identified in the transcriptome sequence data from the related species *P. tabuliformis* (SRA accession: SRA056887, http://www.ncbi.nlm.nih.gov/sra). The sequences were analyzed for potential SSRs using Simple Sequence Repeat Identification Tool (SSRIT) software (Tennaykh et al., 2001; http://www.graneme.org/db/markers/ssritool/). A set of 702 SSRs was identified that met a requirement for mono-, di-, tri-, tetra-, penta-, and hexanucleotide sequences with a minimum of 12, 6, 5, 5, 5, and 5 repeats, respectively. Among these, 175 SSRs were selected randomly for primer design, excluding the SSRs located at the loci termini. Primer pairs were designed using Primer Premier 5.0 software (PREMIER Biosoft International, Palo Alto, California, USA) (Wei et al., 2012) with the following criteria: primer lengths of 16–22 bp, GC content of 40–65%, annealing temperature (T_a) ranging from 40°C to 60°C, and a predicted PCR product size ranging from 100 to 500 bp.

Genomic DNA samples were isolated from the needles of 48 *P. sylvestris var. mongolica* plants using the advanced cetyltrimethylammonium bromide technique. 

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The samples were collected from a single seed orchard in Qigou, Hebei Province (41°07'N, 118°27'E) and deposited at the National Engineering Laboratory for Forest Tree Breeding, China (NELFTB). All trees in the seed orchard derived from Honghuaerji in the Inner Mongolian Autonomous Region. PCR amplifications were performed in 20-μL volumes that included 50–80 ng of genomic DNA, 5 μM concentrations of each primer, and 10 μL 2× PCRMaster Mix consisting of 0.1 unit/μL Taq DNA polymerase, 4 mM MgCl₂, and 0.4 mM dNTP (Aidlab Co. Ltd., Beijing, China). The PCR reactions were performed in a Veriti Dx 96-well Thermal Cycler (Applied Biosystems, Foster City, California, USA) under the following conditions: initial denaturation at 95°C for 30 s, annealing for 45 s at the optimal temperature for each primer pair, and 72°C for 30 s, with a final extension of 10 min at 72°C (Table 1). PCR products were resolved on 6% polyacrylamide denaturing gels using an HT-CX01 gel sequencing cell (Hongtao Jiye Technology Development Co. Ltd., Beijing, China). SSR patterns were visualized by silver staining.

The SSR fragment sizes were estimated by comparison with DNA marker I (Aidlab Co. Ltd.).

One hundred seventy-five EST-SSR primer pairs were synthesized (Shanghai Sangon Co. Ltd., Beijing, China). Fifty-six primer pairs were identified that yielded stable, clear, and repeatable amplicons in *P. sylvestris var. mongolica*. The other primer pairs were unstable or gave no product. The 56 primers corresponded to 31 loci that were monomorphic (data not shown) and 25 loci that were polymorphic (Table 1). The polymorphic SSR loci were analyzed with POPGENE version 1.32 software (Yeh et al., 1999) for the number of alleles per locus (*A*), observed heterozygosity (*H*₀), expected heterozygosity (*H*ₑ), and fixation index (*F*_ₑₐₜ). Detailed data are shown in Table 2. The *A* values ranged from two to eight with a mean of 3.12. The *H*₀ and *H*ₑ values were 0.0435–0.8125 and 0.0430–0.7820 with averages of 0.3412 and 0.4027, respectively. The *F*_ₑₐₜ values ranged from –0.2877 to 0.6773 with an average of 0.1175. Hardy–Weinberg equilibrium (HWE) and linkage disequilibrium using Bonferroni correction were tested for every locus. The following loci deviated significantly (*P < 0.002*) from HWE:

### Table 1. Characteristics of 25 EST-SSRs developed in *Pinus sylvestris var. mongolica*.

| Locus     | Primer sequences (5′-3′) | Repeat motif                  | Expected size (bp) | *Tₐ* (°C) | GenBank accession no. |
|-----------|-------------------------|--------------------------------|---------------------|-----------|-----------------------|
| lw_isotig00542 | F: ACGAGACGACAATCAACAA | (T)₁₀                        | 257                 | 55        | KF501186              |
|           | R: GTGCACTTCTCAAGAATT   |                               |                     |           |                       |
| lw_isotig04204 | F: TCGCTTTGTTTGGTTTGTG   | (CGGCT)₅                    | 230                 | 55        | KF501187              |
|           | R: ATCTCGCCGCAATTTGGT   |                               |                     |           |                       |
| lw_isotig04600 | F: TCAAGGAAATGATGAAATG   | (CAG)₁₀                    | 305                 | 55        | KF501188              |
|           | R: AACCTGTTGTTGAACTGTTA  |                               |                     |           |                       |
| lw_isotig06440 | F: GGGCAGAGGGACATCG     | (AGGTTG)₅ (AGGCTG)₆           | 298                 | 55        | KF501189              |
|           | R: TGAGATCTTCGGGTCG     |                               |                     |           |                       |
| lw_isotig07383 | F: CAAAGAAGAAAGCTGCA    | (GAT)₆                      | 191                 | 55        | KF501190              |
|           | R: ATGCTGTACATCATCTGAC  |                               |                     |           |                       |
| lw_isotig10603 | F: CAAATGCTCTGCTCGCACC  | (CAG)₁₀                    | 196                 | 55        | KF501191              |
|           | R: CAAAAGAAAGAAGCTACAGA |                               |                     |           |                       |
| lw_isotig17679 | F: TTGTTGCCCCACTTGTGCCCC | (TATA)₆                   | 277                 | 55        | KF501192              |
|           | R: CAAACACCGCTCTTCTTAA  |                               |                     |           |                       |
| lw_isotig21953 | F: ATGGGTTGTTGAGGCCAA   | (ATGGG)₇                   | 208                 | 55        | KF501193              |
|           | R: ATGGCAGGCCACTGTTGCTCC |                           |                     |           |                       |
| lw_isotig26230 | F: GGGCATTACATAAGACCGG  | (TA)₁₀                      | 260                 | 55        | KF501194              |
|           | R: TGGGCTTCGAGCTGGATTA  |                               |                     |           |                       |
| lw_isotig27940 | F: GCAGAGCACAAACAAATTGAGCA | (TGGGA)₄                | 231                 | 55        | KF501195              |
|           | R: AGAATCTGCTGCGGCAATCTTC |                           |                     |           |                       |
| lw_isotig00080 | F: CGGCGAACAGTACGAGAG   | (CCG)₅                      | 177                 | 55        | KF501196              |
|           | R: TGGAGAAGTGAGAGGAGG    |                               |                     |           |                       |
| lw_isotig00081 | F: TGGGAGACGCTGCAGAGAGG | (CCG)₆                      | 290                 | 58        | KF501197              |
|           | R: TGGGAGACGCTGCAGAGAGG |                               |                     |           |                       |
| lw_isotig01420 | F: TCCGTGACCCTTATTACGT   | (CTG)₈                      | 174                 | 50        | KF501198              |
|           | R: CGTTTAAGTCTGTGACCT    |                               |                     |           |                       |
| lw_isotig02138 | F: ATGCCATCTTGCTCTCTCT  | (AG)₆                       | 124                 | 42        | KF501199              |
|           | R: TTCTGTGACATACCTCCTC   |                               |                     |           |                       |
| lw_isotig02347 | F: CTGGCTCTTGCTGCTGCC   | (TG)₇                      | 198                 | 50        | KF501200              |
|           | R: GCTATTTGCTGCACTTCC    |                               |                     |           |                       |
| lw_isotig03088 | F: CATTTGTTGGACATCTTGT   | (GA)₆                      | 235                 | 45        | KF501201              |
|           | R: TTGATGAGTACGTGTGCG    |                               |                     |           |                       |
| lw_isotig04931 | F: TAGACCCCTACACAAACT    | (AC)₆                       | 132                 | 40        | KF501202              |
|           | R: ACAACACGGATACAAAT    |                               |                     |           |                       |
| lw_isotig02842 | F: GTGATGGTGTGTTGGTGTGA  | (AGA)₃                      | 229                 | 55        | KF501203              |
|           | R: TCTCTTGTTGAGAGTGGTG   |                               |                     |           |                       |
| lw_isotig04195 | F: GAGATCACCCGAAACACAAAA | (GAG)₅                    | 189                 | 55        | KF501204              |
|           | R: TACAGTGCCCCGAAACACAT  |                               |                     |           |                       |
| lw_isotig04306 | F: GCCATTCTTTTTCTCTCTCCT  | (TCC)₉                      | 196                 | 55        | KF501205              |
|           | R: GCTGCTTCTGCTCTCTCTCT  |                               |                     |           |                       |
| lw_isotig05123 | F: TGGTGCTTTCTGAATTTCTAA | (GAG)₆                    | 166                 | 55        | KF501206              |
|           | R: ATGAAGTGAGGAGAAAGGG    |                               |                     |           |                       |
| lw_isotig06215 | F: TCAGGTTGGTACCTCTTCTCC | (CAA)₅                      | 275                 | 55        | KF501207              |
|           | R: TGGCGACATCTCCAGTCTTT  |                               |                     |           |                       |
| lw_isotig11166 | F: ACACACCTAGCTGCTCAATT  | (TA)₁₀                      | 137                 | 55        | KF501208              |
|           | R: AGTCCACCCCTCTGCTGTACA |                               |                     |           |                       |
| lw_isotig12667 | F: CCAAGGGGAAAAAGGAATGA  | (CA)₆                       | 199                 | 55        | KF501209              |
|           | R: TTGTGACGAGGACGAGAGAG  |                               |                     |           |                       |
| lw_isotig20215 | F: AGAGGTGACGCGCACTCAGAGA| (TA)₁₀                      | 186                 | 55        | KF501210              |

*Note: Tₐ = annealing temperature.*

http://www.bioone.org/loi/apps
Table 2. Allelic diversity of 25 polymorphic EST-SSR loci in *Pinus sylvestris var. mongolica*.

| Locus               | A | $H_s$  | $H_e$  | $F_{is}$ |
|---------------------|---|--------|--------|----------|
| lw_isotig00542      | 2 | 0.4348 | 0.4816 | 0.0873   |
| lw_isotig04204      | 2 | 0.4375 | 0.3454 | 0.2800   |
| lw_isotig04600      | 3 | 0.1053 | 0.1021 | -0.0447  |
| lw_isotig06440      | 3 | 0.2083 | 0.1932 | -0.0897  |
| lw_isotig07383      | 3 | 0.3696 | 0.4728 | 0.2097   |
| lw_isotig10603      | 2 | 0.4375 | 0.4086 | -0.0821  |
| lw_isotig11679      | 3 | 0.4375 | 0.4432 | 0.0025   |
| lw_isotig21953      | 7 | 0.6250 | 0.7820 | 0.1924   |
| lw_isotig26230      | 3 | 0.3958 | 0.4629 | 0.1560   |
| lw_isotig27940      | 3 | 0.1778 | 0.5571 | 0.6773   |
| lw_isotig00080      | 2 | 0.2979 | 0.2919 | -0.0313  |
| lw_isotig00081      | 2 | 0.2128 | 0.2276 | 0.0553   |
| lw_isotig01420      | 3 | 0.7872 | 0.6179 | -0.2877  |
| lw_isotig02138      | 2 | 0.0571 | 0.1590 | 0.6354   |
| lw_isotig02347      | 2 | 0.0435 | 0.0430 | -0.0222  |
| lw_isotig03088      | 2 | 0.4545 | 0.5057 | 0.0909   |
| lw_isotig04931      | 4 | 0.4348 | 0.6530 | 0.3078   |
| lw_isotig02842      | 2 | 0.1250 | 0.3789 | 0.6667   |
| lw_isotig04195      | 4 | 0.4468 | 0.4221 | -0.0699  |
| lw_isotig04306      | 3 | 0.5000 | 0.4781 | 0.0569   |
| lw_isotig05123      | 2 | 0.1458 | 0.1366 | -0.0787  |
| lw_isotig06215      | 2 | 0.0625 | 0.0612 | -0.0323  |
| lw_isotig11166      | 5 | 0.2917 | 0.6252 | 0.5286   |
| lw_isotig12667      | 2 | 0.2292 | 0.4998 | 0.5366   |
| lw_isotig20215      | 8 | 0.8125 | 0.7371 | -0.1140  |
| Average             | 3.12| 0.3412 | 0.4027 | 0.1175   |

Note: $A$ = number of alleles; $F_{is}$ = fixation index; $H_e$ = expected heterozygosity; $H_s$ = observed heterozygosity.

CONCLUSIONS

Very few SSR markers for *P. sylvestris var. mongolica* have previously been reported. Here we have developed 25 novel EST-SSR polymorphic markers for this species. The 25 markers provide an efficient tool for investigating population genetic diversity in different environments, as well as illuminating infraspecific phylogeography, mating systems, and gene flow in different populations. These new EST-SSRs will facilitate studies on molecular breeding, genetic improvement, and conservation of *P. sylvestris var. mongolica*.

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http://www.bioone.org/loi/apps
| Locus            | BLAST top hit accession no. | BLAST top hit description [organism] | E-value | GO_ID                          | Putative gene function                                                                 |
|------------------|-----------------------------|--------------------------------------|---------|-------------------------------|----------------------------------------------------------------------------------------|
| lw_isotig00542   | None                        | None                                 | None    | None                          | None                                                                                   |
| lw_isotig04204   | ABK21059.1                  | Unknown [Picea sitchensis]           | 2E-64   | None                          | None                                                                                   |
| lw_isotig04600   | XP_002273895                | PREDICTED: uncharacterized protein   | 2E-47   | None                          | None                                                                                   |
| lw_isotig06440   | None                        | None                                 | None    | None                          | None                                                                                   |
| lw_isotig07383   | XP_004154913                | PREDICTED: protein RCC2-like         | 1E-25   | None                          | None                                                                                   |
| lw_isotig10603   | None                        | None                                 | None    | None                          | None                                                                                   |
| lw_isotig17679   | None                        | None                                 | None    | None                          | None                                                                                   |
| lw_isotig21953   | ADE76095.1                  | Unknown [Picea sitchensis]           | 7E-12   | None                          | None                                                                                   |
| lw_isotig26230   | None                        | None                                 | None    | None                          | None                                                                                   |
| lw_isotig27940   | None                        | None                                 | None    | None                          | None                                                                                   |
| lw_isotig00080   | ABA54143.1                  | Putative glycine-rich protein [Picea glauca] | 1E-11   | GO:0046872, GO:0008270, GO:0006355, GO:0009055, GO:0005506, GO:0016491, GO:0006536 | Metal ion binding, Regulation of transcription, DNA-dependent, Electron carrier activity, Metal ion binding, DNA-dependent, Electron carrier activity, Metal ion binding |
| lw_isotig00081   | ABA54143.1                  | Putative glycine-rich protein [Picea glauca] | 1E-11   | None                          | None                                                                                   |
| lw_isotig01420   | ACN39897.1                  | Unknown [Picea sitchensis]           | 2E-157  | GO:0055114, GO:0046872, GO:0020037, GO:0016705, GO:0016491, GO:0006536, GO:0005506, GO:0046872 | Oxidoreductase activity, Oxidation-reduction process, Metal ion binding, Heme binding, Oxidoreductase activity, Electron carrier activity, Metal ion binding, Electron carrier activity, Metal ion binding |
| lw_isotig02138   | XP_002971210.1              | Hypothetical protein SELMODRAFT_171829 [Selaginella moellendorfii] | 3E-107  | None                          | None                                                                                   |
| lw_isotig02347   | XP_002990606.1              | Hypothetical protein SELMODRAFT_448108 [Selaginella moellendorfii] | 2E-57   | None                          | None                                                                                   |
| lw_isotig03088   | XP_002266814.1              | PREDICTED: CCA-adding enzyme [Vitis vinifera] | 1E-118  | GO:0016779, GO:0006396, GO:0006536, GO:0003723 | Nucleotidyltransferase activity, RNA processing, RNA binding |
| lw_isotig04931   | ABR16534.1                  | Unknown [Picea sitchensis]           | 2E-143  | None                          | None                                                                                   |
| lw_isotig02842   | ADE76527.1                  | Unknown [Picea sitchensis]           | 0       | None                          | None                                                                                   |
| lw_isotig04195   | ABK21301.1                  | Unknown [Picea sitchensis]           | 7E-80   | None                          | None                                                                                   |
| lw_isotig04306   | ABR17562.1                  | Unknown [Picea sitchensis]           | 1E-79   | None                          | None                                                                                   |
| lw_isotig05123   | ABK22664.1                  | Unknown [Picea sitchensis]           | 6E-165  | None                          | None                                                                                   |
| lw_isotig06215   | ABQ51222.1                  | R2R3-MYB transcription factor MYB6 [Picea glauca] | 1E-127  | GO:0006355, GO:0005634, GO:0009055, GO:0005506, GO:0006536 | Regulation of transcription, Nucleus, Electron carrier activity, Metal ion binding, Electron carrier activity, Metal ion binding |
| lw_isotig11166   | ABK23767.1                  | Unknown [Picea sitchensis]           | 2E-23   | None                          | None                                                                                   |
| lw_isotig12667   | None                        | None                                 | None    | None                          | None                                                                                   |
| lw_isotig20215   | DAA51826.1                  | TPA: hypothetical protein ZEA'MMB73_014853 [Zea mays] | 2E-14   | GO:0005506, GO:0005506, GO:0005506, GO:0016491, GO:0009055, GO:0005506 | Iron binding, Iron binding, Iron binding, Oxidoreductase activity, Electron carrier activity, Iron binding |

Note: GO_ID = Gene Ontology ID.