



## **Development and Characterization of 25 EST-SSR markers in *Pinus sylvestris* var. *mongolica* (Pinaceae)**

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Source: Applications in Plant Sciences, 2(1)

Published By: Botanical Society of America

URL: <https://doi.org/10.3732/apps.1300057>

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## DEVELOPMENT AND CHARACTERIZATION OF 25 EST-SSR MARKERS IN *PINUS SYLVESTRIS* VAR. *MONGOLICA* (PINACEAE)<sup>1</sup>

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- **Premise of the study:** A set of novel expressed sequence tag (EST) microsatellite markers was developed in *Pinus sylvestris* var. *mongolica* to promote further genetic studies in this species.
- **Methods and Results:** One hundred seventy-five EST-simple sequence repeat (SSR) primers were designed and synthesized for 31,653 isotigs based on *P. tabuliformis* EST sequences. The primer pairs were used to identify 25 polymorphic loci in 48 individuals. The number of alleles ranged from two to eight with observed and expected heterozygosity values of 0.0435 to 0.8125 and 0.0430 to 0.7820, respectively.
- **Conclusions:** These new polymorphic EST-SSR markers will be useful for assessing genetic diversity, molecular breeding and genetic improvement, and conservation of *P. sylvestris* var. *mongolica*.

**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  $-40^{\circ}\text{C}$ , 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 (Temnykh et al., 2001; <http://www.gramene.org/db/markers/ssrtool/>). 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  $40^{\circ}\text{C}$  to  $60^{\circ}\text{C}$ , 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

<sup>1</sup>Manuscript received 25 July 2013; revision accepted 22 September 2013.

This work was supported by the National Natural Science Foundation of China (grant no. 31370657).

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doi:10.3732/apps.1300057

(CTAB) method (Porebski et al., 1997). The samples were collected from a single seed orchard in Qigou, Hebei Province (41°0'13"N, 118°27'38"E) and deposited at the National Engineering Laboratory for Forest Tree Breeding, China (NELFTB). All trees in the seed orchard derived from Honghuerji in the Inner Mongolia Autonomous Region. PCR amplifications were performed in 20- $\mu$ L volumes that included 50–80 ng of genomic DNA, 5  $\mu$ M concentrations of each primer, and 10  $\mu$ L 2 $\times$  PCRMaster Mix consisting of 0.1 unit/ $\mu$ L *Taq* DNA polymerase, 4 mM MgCl<sub>2</sub>, 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 5 min followed by 35 cycles 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 POP-GENE version 1.32 software (Yeh et al., 1999) for the number of alleles per locus (*A*), observed heterozygosity (*H<sub>o</sub>*), expected heterozygosity (*H<sub>e</sub>*), and fixation index (*F<sub>IS</sub>*). Detailed data are shown in Table 2. The *A* values ranged from two to eight with a mean of 3.12. The *H<sub>o</sub>* and *H<sub>e</sub>* values were 0.0435–0.8125 and 0.0430–0.7820 with averages of 0.3412 and 0.4027, respectively. The *F<sub>IS</sub>* 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) | <i>T<sub>a</sub></i> (°C) | GenBank accession no. |
|----------------|---|---|--------------------|---------------------------|-----------------------|
| lw_isotig00542 | F: AACAGGAGCATATCAATCAA<br>R: GTGGCATTCTACAAGCAATT    | (T) <sub>40</sub>                           | 257                | 55                        | KF501186              |
| lw_isotig04204 | F: CTCCGTTTGGGTTGTGTTTG<br>R: ATCCTTGGCCGAGATTGT      | (CGGCT) <sub>5</sub>                        | 230                | 55                        | KF501187              |
| lw_isotig04600 | F: TCAGGGAAAATGTAGGAAAATG<br>R: AATCTGTTGTGTGGGACTTGA | (CAG) <sub>10</sub>                         | 305                | 55                        | KF501188              |
| lw_isotig06440 | F: GGGACAAGGGACATCG<br>R: TGGAGACTTCGGGTGC            | (AGGTTG) <sub>5</sub> (AGGCTG) <sub>6</sub> | 298                | 55                        | KF501189              |
| lw_isotig07383 | F: CAAAACAAAAACAGTCTGCA<br>R: ATCGTCATCATCATCGTCAC    | (GAT) <sub>8</sub>                          | 191                | 55                        | KF501190              |
| lw_isotig10603 | F: CAAAATCGTCTACTTCTCCCCC<br>R: CAAAGCAAAGAACTCCAACGA | (CAG) <sub>7</sub>                          | 196                | 55                        | KF501191              |
| lw_isotig17679 | F: TTGTTTGCCACATTGTTGCC<br>R: CAAACCACCGCTGCTTCTAA    | (TTAA) <sub>5</sub>                         | 277                | 55                        | KF501192              |
| lw_isotig21953 | F: ATGGTGTGTTTGAAGCGGAA<br>R: ATTGCAGCCACTGGTGTCTT    | (ATGGG) <sub>7</sub>                        | 208                | 55                        | KF501193              |
| lw_isotig26230 | F: GGGCATTACATAAACACGGG<br>R: TGCCCTTGAGCATTGATTA     | (TA) <sub>10</sub>                          | 260                | 55                        | KF501194              |
| lw_isotig27940 | F: GCAGGCAACACAAAAGTGACA<br>R: AGCAATCGAGTGGCAAATCTTC | (TGGA) <sub>5</sub>                         | 231                | 55                        | KF501195              |
| lw_isotig00080 | F: CGGGCAAAAATGACCGAAG<br>R: TGGAGGAGGTAGAGGGGG       | (CCG) <sub>6</sub>                          | 177                | 55                        | KF501196              |
| lw_isotig00081 | F: TGCGGAAGGCGTAGTAG<br>R: TGGAGGAGGTAGAGGGGG         | (CCG) <sub>6</sub>                          | 290                | 58                        | KF501197              |
| lw_isotig01420 | F: TCCGTGACCCTATTACGT<br>R: CGATTAGTTGCTTGCCCTT       | (CTG) <sub>5</sub>                          | 174                | 50                        | KF501198              |
| lw_isotig02138 | F: ATGCATCTTGCTCCTCTCT<br>R: TTCTGATTCACACTCCC        | (AG) <sub>6</sub>                           | 124                | 42                        | KF501199              |
| lw_isotig02347 | F: CTCGTCCTTCTGTCCGC<br>R: GCTATTGCTCCACTTGCC         | (TG) <sub>7</sub>                           | 198                | 50                        | KF501200              |
| lw_isotig03088 | F: CATTGGTTGACTTTGTT<br>R: TTGTAGTGAGATCTGTGC         | (GA) <sub>6</sub>                           | 235                | 45                        | KF501201              |
| lw_isotig04931 | F: TAGACCTCATCACAAACT<br>R: ACAAAAACGAATACAAAT        | (AC) <sub>6</sub>                           | 132                | 40                        | KF501202              |
| lw_isotig02842 | F: GTGATGGTGTGGTGGCTGTA<br>R: TCCTTTGTGGGAGATTGGTG    | (AGA) <sub>5</sub>                          | 229                | 55                        | KF501203              |
| lw_isotig04195 | F: GAGATCACCGAAAACAACAAAA<br>R: TACAAGTCCCAGCAAACAAT  | (GAG) <sub>5</sub>                          | 189                | 55                        | KF501204              |
| lw_isotig04306 | F: GCCATTTTTTCTTCTCTCCT<br>R: GGTGGTTTCTGAATTTCTAA    | (TCC) <sub>7</sub>                          | 196                | 55                        | KF501205              |
| lw_isotig05123 | F: TGTGCGTATAGGAGGTGGAG<br>R: ATGAAAGGTGACAAAGCGGT    | (GAG) <sub>6</sub>                          | 166                | 55                        | KF501206              |
| lw_isotig06215 | F: TCAGGTGCTTACCCCTTTTC<br>R: TGGCAGCTATTCCAGTCTTT    | (CAA) <sub>5</sub>                          | 275                | 55                        | KF501207              |
| lw_isotig11166 | F: ACACACACTGAGCTCCAATTT<br>R: AGTCCCACCTCTGCTGATACA  | (TA) <sub>7</sub>                           | 137                | 55                        | KF501208              |
| lw_isotig12667 | F: CCAAGGTGAAAAGGAAATGA<br>R: TTCTGACAGGGAGCGACTGA    | (CA) <sub>6</sub>                           | 199                | 55                        | KF501209              |
| lw_isotig20215 | F: AGAGGTGATCGCAGTCAAAGA<br>R: TTCAAAAAGACCAACCCTAG   | (TA) <sub>7</sub>                           | 186                | 55                        | KF501210              |

Note: *T<sub>a</sub>* = annealing temperature.

TABLE 2. Allelic diversity of 25 polymorphic EST-SSR loci in *Pinus sylvestris* var. *mongolica*.

| Locus          | A    | $H_o$  | $H_e$  | $F_{IS}$ |
|----------------|------|--------|--------|----------|
| 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_isotig17679 | 3    | 0.4375 | 0.4432 | 0.0025   |
| lw_isotig21953 | 7    | 0.6250 | 0.7820 | 0.1924   |
| lw_isotig26230 | 3    | 0.3958 | 0.4629 | 0.1360   |
| lw_isotig27940 | 3    | 0.1778 | 0.5571 | 0.6773   |
| lw_isotig00080 | 3    | 0.2979 | 0.2919 | -0.0313  |
| lw_isotig00081 | 3    | 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 | 0.6350 | 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;  $H_o$  = observed heterozygosity.

lw\_isotig27940, lw\_isotig02138, lw\_isotig04931, lw\_isotig02842, lw\_isotig11166, and lw\_isotig12667. No linkage disequilibrium ( $P < 0.002$ ) was detected among any loci.

To identify potential functions of the 25 SSR-associated unigenes, the sequences were aligned with the GenBank database using the BLASTX program (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) (Yang et al., 2012). The E-value was limited to  $0-1.0E^{-5}$ . Gene Ontology (GO) was also used to predict functions of the unigenes (<http://geneontology.org/>). Eighteen sequences were found to have potential functions by BLASTX or GO analysis. These sequences showed significant homology to protein sequences from *Picea sitchensis* (Bong.) Carrière, *Picea glauca* Voss, *Selaginella moellendorffii* Hieron., *Vitis vinifera* L., *Cucumis sativus* L., and *Zea mays* L. The potential functions were mainly related to ionic bonding, oxidation-reduction processes, and feedback regulation (Table 3).

## 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 in-fraspecific 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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TABLE 3. Potential functions of the SSR-associated sequences in *Pinus sylvestris* var. *mongolica*.

| 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 [ <i>Picea sitchensis</i> ]  | 2E-64   | None   | None   |
| lw_isotig04600 | XP_002273895                | PREDICTED: uncharacterized protein LOC100267221 [ <i>Vitis vinifera</i> ]    | 2E-47   | None   | None   |
| lw_isotig06440 | None                        | None   | None    | None   | None   |
| lw_isotig07383 | XP_004154913                | PREDICTED: protein RCC2-like [ <i>Cucumis sativus</i> ]                      | 1E-25   | None   | None   |
| lw_isotig10603 | None                        | None   | None    | None   | None   |
| lw_isotig17679 | None                        | None   | None    | None   | None   |
| lw_isotig21953 | ADE76095.1                  | Unknown [ <i>Picea sitchensis</i> ]  | 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 [ <i>Picea glauca</i> ]                        | 1E-11   | GO:0046872<br>GO:0008270<br>GO:0006355<br>GO:0003676   | Metal ion binding<br>Zinc ion binding<br>Regulation of transcription, DNA-dependent<br>Nucleic acid binding  |
| lw_isotig00081 | ABA54143.1                  | Putative glycine-rich protein [ <i>Picea glauca</i> ]                        | 1E-11   | None   | None   |
| lw_isotig01420 | ACN39897.1                  | Unknown [ <i>Picea sitchensis</i> ]  | 2E-157  | GO:0055114<br>GO:0046872<br>GO:0020037<br>GO:0016705<br>GO:0016491<br>GO:0009055<br>GO:0005506                             | Oxidation-reduction process<br>Metal ion binding<br>Heme binding<br>Oxidoreductase activity, acting on paired donors, with incorporation or reduction of molecular oxygen<br>Oxidoreductase activity<br>Electron carrier activity<br>Iron ion binding                                |
| lw_isotig02138 | XP_002971210.1              | Hypothetical protein SELMODRAFT_171829 [ <i>Selaginella moellendorffii</i> ] | 3E-107  | GO:0046872   | Metal ion binding  |
| lw_isotig02347 | XP_002990606.1              | Hypothetical protein SELMODRAFT_448108 [ <i>Selaginella moellendorffii</i> ] | 2E-57   | None   | None   |
| lw_isotig03088 | XP_002266814.1              | PREDICTED: CCA-adding enzyme [ <i>Vitis vinifera</i> ]                       | 1E-118  | GO:0016779<br>GO:0006396<br>GO:0003723   | Nucleotidyltransferase activity<br>RNA processing<br>RNA binding   |
| lw_isotig04931 | ABR16534.1                  | Unknown [ <i>Picea sitchensis</i> ]  | 2E-143  | None   | None   |
| lw_isotig02842 | ADE76527.1                  | Unknown [ <i>Picea sitchensis</i> ]  | 0       | None   | None   |
| lw_isotig04195 | ABK21301.1                  | Unknown [ <i>Picea sitchensis</i> ]  | 7E-80   | None   | None   |
| lw_isotig04306 | ABR17562.1                  | Unknown [ <i>Picea sitchensis</i> ]  | 1E-79   | None   | None   |
| lw_isotig05123 | ABK22664.1                  | Unknown [ <i>Picea sitchensis</i> ]  | 6E-165  | None   | None   |
| lw_isotig06215 | ABQ51222.1                  | R2R3-MYB transcription factor MYB6 [ <i>Picea glauca</i> ]                   | 1E-127  | GO:0006355<br>GO:0005634   | Regulation of transcription, DNA-dependent<br>Nucleus  |
| lw_isotig11166 | ABK23767.1                  | Unknown [ <i>Picea sitchensis</i> ]  | 2E-23   | GO:0009055   | Electron carrier activity  |
| lw_isotig12667 | None                        | None   | None    | None   | None   |
| lw_isotig20215 | DAA51826.1                  | TPA: hypothetical protein ZEAMMB73_014853 [ <i>Zea mays</i> ]                | 2E-14   | GO:0055114<br>GO:0051536<br>GO:0050660<br>GO:0046872<br>GO:0016614<br>GO:0016491<br>GO:0009055<br>GO:0005506<br>GO:0003824 | Oxidation-reduction process<br>Iron-sulfur cluster binding<br>Flavin adenine dinucleotide binding<br>Metal ion binding<br>Oxidoreductase activity, acting on CH-OH group of donors<br>Oxidoreductase activity<br>Electron carrier activity<br>Iron ion binding<br>Catalytic activity |

Note: GO\_ID = Gene Ontology ID.