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TECHNICAL NOTE

# Twenty-three microsatellite loci for *Styrax confusus* and *Styrax japonicus* (Styracaceae)

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**Abstract** From a genomic library enriched for AG repeats, 23 polymorphic microsatellite loci were isolated for *Styrax confusus* and *Styrax japonicus*. All the loci are polymorphic in *Styrax japonicus*. Analysis of 23 individuals from two populations revealed an average of 5.3 alleles per locus (range: 2–10), an average observed heterozygosity of 0.30 (range: 0–0.826) and an average expected heterozygosity of 0.66 (range: 0.198–0.888). Fourteen loci are polymorphic in *Styrax confusus*, showing 4–11 alleles per locus, an observed heterozygosity ranging from 0.087 to 0.870 and an expected heterozygosity ranging from 0.580 to 0.907. These polymorphic microsatellite loci provide useful molecular tools to study genetic variation and phylogeography of the two *Styrax* species across their ranges in eastern Asia.

**Keywords** *Styrax confusus* · *Styrax japonicus* · Microsatellite · Polymorphism · Genetic variation

The family Styracaceae contains 11 genera of woody plants, and has a widespread but disjunct distribution (Fritsch et al. 2001). Comprising over 80% of the total number of species in Styracaceae, *Styrax* distributes the whole range of the family (Fritsch et al. 2001). *Styrax* was frequently used to describe patterns and mechanisms of disjunct distribution (for example, Fritsch 1996; Xiang et al. 2004), and phylogeny and biogeography of the genus had been studied using isozymes, chloroplast genes, internal transcribed spacer region of nuclear ribosomal DNA and morphological traits (Fritsch 1996; 2001; Fritsch et al. 2001). Genetic variation may provide important information for biogeography. However, such information was very limited in *Styrax*. *S. confusus* and *S. japonicus* are common deciduous trees distributed in eastern Asia, and the latter is also planted for landscape aims. Microsatellites are powerful and effective tools for population genetic studies (Liu et al. 2008), and may provide important information for biogeography. We report here the isolation and characterization of 23 polymorphic microsatellite markers for the two *Styrax* species.

Due to difficulty in distinguishing *S. confusus* and *S. japonicus* without information of flower or fruit, we identified each leaf sample by sequencing internal transcribed spacer region of nuclear rDNA. Genomic DNA was extracted from dried leaves of *S. confusus* and *S. japonicus* as described by Fan et al. (2004). The ITS1, 5.8S, and ITS2 regions of the ribosomal DNA were amplified together using primers ITS-4 (5′-TCCTCCGCTTATTGATATGC-3′) and ITS-5 (5′-GGAAGGAGAAGTCGTAACAAGG-3′) (White et al. 1990). PCR products were visualized on 1% agarose gels before sequenced with ABI 3730 DNA Sequence Analyzer. After identifying the samples, a microsatellite-enriched library of *S. confusus* was constructed following the procedure of Liu et al. (2009). 903 positive clones were chosen and tested by PCR, which was performed in a 15 μl final volume (Liu et al. 2009) using $(AG)_{10}$ and M13+/M13 as primers, respectively. A total of 149 screened clones contained potential repeat motifs and were sequenced with ABI 3730 DNA Sequence Analyzer.

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**Table 1** Characteristics of 23 microsatellite loci developed for *Styrax confusus* and *Styrax japonicus*, including locus name, primer sequences, repeat motif, annealing temperature ($T_a$), size range, number of alleles ($N_A$), observed ($H_O$) and expected ($H_E$) heterozygosites, and accession number of Genbank

| Locus name | Primer sequences (5′–3′) | Repeat motif | *Styrax japonicus* | | | | | *Styrax confusus* | | | | | Genbank accession number |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | $T_a$ (°C) | Size (bp) | $N_A$ | $H_O$ | $H_E$ | $T_a$ (°C) | Size (bp) | $N_A$ | $H_O$ | $H_E$ | |
| Styr 2 | F: TACTGACGAAACGGAGAAAG<br>R: ATGGAATATGAGCTGCTGTG | $(TG)_4(TA)_6\cdots(CT)_9\cdots(AG)_{18}$ | 58 | 278–378 | 6 | 0.476 | 0.726 | 59 | 286–318 | 7 | 0.870 | 0.828 | GU220028 |
| Styr 5 | F: ACACGCAACCCCATCTT<br>R: GGTCTTCGGTTCAAGTATCT | $(AG)_{16}$ | 63 | 356–392 | 6 | 0.435 | 0.761 | 64 | 346–374 | 4 | 0.304 | 0.580 | GU220029 |
| Styr 6 | F: AGACTGCCTTCTCCATTGT<br>R: GCGGAAATTCAATACTT | $(TC)_9$ | 54 | 132–146 | 4 | 0.318 | 0.745 | 59 | 140–148 | 5 | 0.087 | 0.732 | GU220030 |
| Styr 10 | F: TAACGGTGGGGATGACAATA<br>R: CCAACCAAACTCACAAATAC | $(AG)_{14}$ | 56 | 115–133 | 8 | 0.118 | 0.820 | 57 | 107–153 | 11 | 0.136 | 0.907 | GU220031 |
| Styr 14 | F: CCATAAAAGCTCAAGCTATCA<br>R: ATTCTTACCACCCCTGTCTCT | $(TC)_9$ | 53 | 112–126 | 4 | 0.409 | 0.711 | 51.5 | 112 | M | 0.000 | 0.000 | GU220032 |
| Styr 20 | F: CTTGTGTTCCATAATCTGTG<br>R: TAAAAGGGCACACACTCTAC | $(TC)_{17}$ | 60 | 169–189 | 5 | 0.318 | 0.555 | 61.5 | 177–201 | 6 | 0.522 | 0.808 | GU220033 |
| Styr 21 | F: AATAAAATGGCTGTCCCCTT<br>R: ATGGTTAGCACTGAGATGAT | $(CT)_{16}$ | 51.5 | 181–209 | 10 | 0.450 | 0.862 | 59 | 183–201 | P | – | – | GU220034 |
| Styr 22 | F: TTGACACACAACAACGCTAC<br>R: GACAACTATGGACGAAAACT | $(AG)_4\cdots(AG)_4\cdots(AG)_6$ | 62 | 215–229 | 3 | 0.130 | 0.484 | 63 | 231 | M | 0.000 | 0.000 | GU220035 |
| Styr 24 | F: CCAGACCCAACAACCTTACA<br>R: AGGAAGATCTGATAGTCA | $(TC)_4\cdots(TC)_6\cdots(TC)_{10}$ | 59 | 189–199 | 5 | 0.409 | 0.712 | 59 | 193–227 | 7 | 0.435 | 0.777 | GU220036 |
| Styr 25 | F: AGGGCTAAAGTTTCAGACGA<br>R: ATATTCCTATCATTGTGGC | $(TC)_8TG(TC)_6$ | 56 | 209–245 | 7 | 0.500 | 0.808 | 55.5 | 213–235 | P | – | – | GU220037 |
| Styr 26 | F: ATCGCAACATCCACATCCTA<br>R: GTAACAACCATTTCCACTG | $(TA)_4\cdots(TC)_4\cdots(TC)_9$ | 58 | 374–392 | 4 | 0.130 | 0.428 | 58 | 382–396 | P | – | – | GU220038 |
| Styr 30 | F: GAAGTGAGTGATAAATGTGC<br>R: AGTGTATCCACAGGGCAT | $(TC)_{23}$ | 63 | 201–223 | 5 | 0.091 | 0.720 | 63 | 189–261 | P | – | – | GU220039 |
| Styr 31 | F: TGGGATTAGAAGAATAGGTA<br>R: AACGCGTTCTTTATACATAT | $(GA)_7(GT)_9(GA)_{10}$ | 56 | 284–336 | 5 | 0.238 | 0.744 | 58 | 264–290 | 4 | 0.364 | 0.610 | GU220040 |
| Styr 32 | F: ATACAGATGAGGTTGGTCCC<br>R: GTCCCTAGCAGAGGTCAAAC | $(TC)_{11}$ | 65 | 156–198 | 9 | 0.826 | 0.888 | 64 | 150–172 | 8 | 0.478 | 0.862 | GU220041 |
| Styr 33 | F: TCCCAACAAACAAGCCTCTG<br>R: CAATGCCATCGGATACGACC | $(AG)_{13}$ | 65 | 107–117 | 6 | 0.545 | 0.759 | 65 | 105–115 | 6 | 0.174 | 0.726 | GU220042 |
| Styr 34 | F: GGTTTTATTGGACCGTTTGG<br>R: TGACTCATTCCCCGGACAGT | $(GA)_4\cdots(GA)_7$ | 61 | 143–159 | 5 | 0.087 | 0.721 | 65 | 147–161 | 6 | 0.304 | 0.679 | GU220043 |

**Table 1** continued

| Locus name | Primer sequences (5′–3′) | Repeat motif | *Styrax japonicus* | | | | | *Styrax confusus* | | | | | Genbank accession number |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | $T_a$ (°C) | Size (bp) | $N_A$ | $H_O$ | $H_E$ | $T_a$ (°C) | Size (bp) | $N_A$ | $H_O$ | $H_E$ | |
| Styr 40 | F: GTGGGGAGTGGAAAGTTGTT<br>R: AGTGCAGATAGAGATCATCA | $(TC)_{22}$ | 62 | 118–128 | 2 | 0.217 | 0.198 | 65 | 122–164 | 9 | 0.391 | 0.835 | GU220044 |
| Styr 42 | F: GAAGCGCATAAAATCACACG<br>R: CCAAACAGGGACAGGAAACC | $(TC)_{10}$ | 68 | 222–238 | 7 | 0.227 | 0.674 | 68 | 222–248 | 6 | 0.478 | 0.767 | GU220045 |
| Styr 43 | F: CAAGAAGACTAAGAAGAACA<br>R: AGCATCTCTTACTCAATTTC | $(AG)_4\cdots(AG)_{18}$ | 58 | 150–168 | 4 | 0.261 | 0.506 | 57.5 | 152–178 | 4 | 0.391 | 0.697 | GU220046 |
| Styr 46 | F: GGTTTATCCTCATTCCTCGC<br>R: CTTTGGCTCTGGTATGGTCT | $(AG)_7$ | 63 | 255–263 | 3 | 0.087 | 0.518 | – | – | – | – | – | GU220047 |
| Styr 49 | F: ATGAGATGCCAAAACACAAA<br>R: AAGTTTCCTTCACGCAATAA | $(CT)_{10}CCCTCT(CA)_{10}$ | 55 | 114–202 | 10 | 0.227 | 0.839 | 56 | 134–168 | 11 | 0.348 | 0.907 | GU220048 |
| Styr 51 | F: GATGACAAAGTACCAGAAC<br>R: TTTATTGTGGAAAGATGCTC | $(AG)_5$ | 55 | 150–152 | 2 | 0.391 | 0.507 | 62 | 150 | M | 0.000 | 0.000 | GU220049 |
| Styr 52 | F: CAAGCTCTTCCATCACCACC<br>R: AAAAGCATGAACGTCGCAAT | $(TC)_8TT(TC)_6$ | 61 | 103–105 | 2 | 0.000 | 0.394 | 66 | 103–131 | P | – | – | GU220050 |

"P" indicates polymorphic locus which was difficult to interpret, and "M" indicates monomorphic locus

Forty-six of the cloned sequences were used to design locus specific primers using program PRIMER 5.0 ( http://www.premierbiosoft.com). Variability at the loci was tested with 23 *S. confusus* individuals and 23 *S. japonicus* individuals. The PCR was performed in 20 μl volumes, which included 50 ng genomic DNA, 2.0 μl 10× PCR buffer, 1.875 mM $MgCl_2$, 0.15 mM each dNTPs, 0.05 μM of each primer, 1 U of DNA Taq polymerase (Sangon). The thermal profile for PCR amplification was 94°C for 3 min, followed by 30 cycles of 94°C for 30 s, a primer-specific annealing temperature for 30 s (Table 1), 72°C for 30 s, ending with a single extension of 72°C for 7 min. PCR products were visualized on 1% agarose gels and then resolved on 8% polyacrylamide denaturing gel and visualized by silver staining using pUC19 DNA/*Msp I* (*Hpa II*) (Fermentas) as the ladder.

Finally, we found 23 polymorphic loci in *S. japonicus* from the 46 primer pairs (Table 1). Among the other primers, 6 pairs produced polymorphic products but difficult to interpret, and 17 had no amplified products. The number of alleles per locus ranged from 2 to 10, with an average of 5.30. We calculated observed ($H_O$) and expected ($H_E$) heterozygosities with software GENEPOP v4.0 (Rousset 2008). Hardy–Weinberg equilibrium and linkage disequilibrium were also tested by GENEPOP v4.0 (Rousset 2008) followed by the sequential Bonferroni correction (Rice 1989). The $H_O$ and $H_E$ ranged from 0 to 0.826 and 0.198 to 0.888, respectively. There were 14 loci significantly biased from Hardy–Weinberg equilibrium (Table 1), and no locus pair exhibited significant linkage disequilibrium.

Fourteen loci were found to be polymorphic in *S. confusus* (Table 1). Among the other primers, 5 were monomorphic, 10 were polymorphic but difficult to interpret, and 17 had no amplified products. The number of alleles per locus ranged from 4 to 11, with an average of 6.71. 13 loci was significantly biased from Hardy–Weinberg equilibrium (Table 1), and loci *Sty2* and *Sty32* exhibited significant linkage disequilibrium ($P < 0.001$). $H_O$ and $H_E$ ranged from 0.087 to 0.870 and 0.580 to 0.907, respectively.

The 23 polymorphic microsatellite loci are reliable genetic markers and will be useful for studying the genetic variation and phylogeography of the two *Styrax* species across their ranges in eastern Asia.

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