Development of microsatellite markers for the resin-yielding, non-timber forest product species *Boswellia serrata* (Burseraceae)

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PREMISE OF THE STUDY: *Boswellia serrata* (Burseraceae) is an economically important aromatic, gum-resin-yielding, non-timber forest tree species. Microsatellite markers were developed for *B. serrata* for the first time to study genetic diversity and population structure.

METHODS AND RESULTS: A magnetic bead enrichment method was used to develop 16 microsatellite markers, of which 11 were polymorphic. The number of alleles per locus in the 60 individuals studied ranged from 3 to 10, and the levels of observed and expected heterozygosity ranged from 0.50 to 0.90 and 0.666 to 0.861, respectively. The primers successfully amplified in the congeneric species *B. ovalifoliolata*.

CONCLUSIONS: These microsatellite markers can be used to study the genetic variation and population structure of *B. serrata* and to provide crucial information on population and ecological issues for management and conservation of the species.

KEYWORDS: aromatic resin; *Boswellia serrata*; Burseraceae; microsatellites; non-timber forest product (NTFP) species.
METHODS AND RESULTS

Sample collection and DNA extraction

Fresh leaves of *B. serrata* were collected from three populations, Biligiri Ranganathaswamy Temple Tiger Reserve (BRT-TR), Male Mahadeshwara Hills Wildlife Sanctuary (MM Hills WLS), and Cauvery Wildlife Sanctuary (Cauvery WLS) of Western Ghats, India, to develop genetic markers and from one population of *B. ovalifoliolata*, from the Seshachalam foothills of Tirupati, Andhra Pradesh, India, to check cross-amplification (Appendix 1). Genomic DNA was extracted from leaf material using a cetyltrimethylammonium bromide (CTAB) method (Sambrook et al., 1989).

Microsatellite library construction and primer design

A microsatellite enrichment library was constructed using a magnetic bead hybridization method following Glenn and Schable (2005) with minor modifications. Total genomic DNA of one sample from BRT-TR was digested with the restriction enzymes *Rsa*I and *Xmn*I (New England Biolabs, Ipswich, Massachusetts, USA). Digested products were ligated to double-stranded SNX linkers using a rapid DNA ligation kit (Fermentas International, Thermo Fisher Scientific, Bangalore, India) and amplified with SNX primers. The amplified products were hybridized with 3′-biotinylated microsatellite probes, and hybridized probes were captured by streptavidin-coupled (M-280) Dynabeads (Invitrogen, Oslo, Norway). The captured fragments containing microsatellite repeats were enriched by amplification with SNX linker-specific primers. Enriched fragments were transformed into *E. coli* strain CB-5α with the pTZ5RT vector (Thermo Fisher Scientific, Bangalore, India). Recombinant clones were identified by colony PCR using M13 primers. The PCR fragments larger than 300 bp were sequenced using an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems, Waltham, Massachusetts, USA) at Chromous Biotech (Bangalore, India). After trimming vector and linker sequences, 216 nonredundant contig sequences were obtained, of which 68 contained microsatellite repeats. A total of 42 primer pairs were designed using the software Primer3Plus (Untergasser et al., 2007) with the following criteria: amplicon size 100–300 bp, annealing temperature 52–60°C, and GC content 40–60%.

Validation and evaluation of designed markers

Of the 42 primer pairs, 16 successfully amplified (Table 1) and were tested for polymorphism in 60 individuals of *B. serrata* and for cross-amplification in 10 individuals of *B. ovalifoliolata* (Table 2). PCR reactions were performed in a 15-μL reaction volume containing 10–20 ng of template DNA, 1 mM of dNTP mix, 1× polymerase buffer, 1 unit of *Taq* polymerase (all reagents from Bangalore Genei, India). Samples were amplified in a PCR machine (Eppendorf, Germany) using an initial denaturation step of 95°C for 15 min and followed by 35 cycles of 95°C for 10 s, 60°C for 10 s, and 72°C for 30 s, with a final extension step of 72°C for 5 min. Amplified products were visualized on a 3% agarose gel stained with ethidium bromide.

| Locus | Primer sequences (5′–3′) | Repeat motif | Fluorescent label | *T*<sub>a</sub> (°C) | Allele size range (bp) | GenBank accession no. |
|-------|------------------------|--------------|------------------|---------------------|-----------------------|----------------------|
| BS6   | F: CTACGTATTGATGAGGCGGC | (GA)<sub>14</sub> | PET              | 60                  | 172–228               | MG811526             |
|       | R: GAGATCGTGATGAGGCGGC  |              |                  |                     |                       |                      |
| BS8   | F: CGCCCTACGTACCATGAC   | (CAAA)<sub>4</sub> (CAA)<sub>4</sub> | PET             | 52                  | 173–209               | MG811527             |
|       | R: CTGCCAGAGTATGAGAGGAA  |              |                  |                     |                       |                      |
| BS10* | F: ACCGCGGTGACACTCCTACC | (AAG)<sub>6</sub> | NED             | 60                  | 205                   | MG811542             |
|       | R: GAGATCTACCTGCCGAGAA  |              |                  |                     |                       |                      |
| BS11  | F: ACAAGAACCCACCCTCATCTC | (TC)<sub>15</sub> | NED             | 54                  | 172–206               | MG811528             |
|       | R: GTCTCGGTGGAGGTATGATG   |              |                  |                     |                       |                      |
| BS12* | F: ATGCGGTGATGATGATGAT  | (AG)<sub>9</sub> | HEX             | 58                  | 174                   | MG811537             |
|       | R: GCAGAATGCTGCTGATGAG  |              |                  |                     |                       |                      |
| BS13  | F: TCTCTTGAGCATAGGAATCT | (AG)<sub>12</sub> | 6-FAM           | 58                  | 258–296               | MG811529             |
|       | R: GTAGACCTCAGCACCTCAGCTG |              |                  |                     |                       |                      |
| BS14  | F: CGGCGCGAACCCTCATAA   | (AAG)<sub>8</sub> | 6-FAM           | 58                  | 183–208               | MG811530             |
|       | R: ATCAAGCGATGCTGTGCCC  |              |                  |                     |                       |                      |
| BS16  | F: GCCTTTCTATTTTCTCTTTGG | (CT)<sub>15</sub> | HEX             | 50                  | 118–138               | MG811531             |
|       | R: GCCTAGATGCGAATGCTGG  |              |                  |                     |                       |                      |
| BS18* | F: CAACAGGAGAGGACAGGATAT | (TC)<sub>15</sub> | 6-FAM           | 58                  | 198                   | MG811540             |
|       | R: TTAAGGCTTGCTAAGCAGAAGA  |              |                  |                     |                       |                      |
| BS19* | F: GGATCCAGCGCCCAGATACTC | (TG)<sub>12</sub> | HEX             | 56                  | 224                   | MG811541             |
|       | R: TCGAGACGCAGCAAGGAATGG  |              |                  |                     |                       |                      |
| BS21* | F: CAGCCCTCTCTCATGCGATA | (TGT)<sub>12</sub> | NED             | 52                  | 218                   | MG811539             |
|       | R: AAGTCGGTACCTACGCTGTG  |              |                  |                     |                       |                      |
| BS23  | F: CAGCATGCTGATGAGTTCTGCT | (CA)<sub>6</sub> | NED             | 52                  | 188–216               | MG811532             |
|       | R: CAGCGTCTACACAGAAAGAA  |              |                  |                     |                       |                      |
| BS25  | F: TCAAGCCTCTTGTTTGGTTG | (TC)<sub>15</sub> | 6-FAM           | 58                  | 206–252               | MG811533             |
|       | R: TGGAGGACACAGGAAAAGAGCA |              |                  |                     |                       |                      |
| BS28  | F: CCAGCTTTCCTCTTCTTCTCTT | (AAG)<sub>12</sub> | PET             | 58                  | 156–185               | MG811538             |
|       | R: TCTGCTACGAAATTCTCTCTCTCTCT |              |                  |                     |                       |                      |
| BS29  | F: CAGGTACGCCATGTTCTGCTG | (AG)<sub>6</sub> | HEX             | 52                  | 184–228               | MG811535             |
|       | R: CTCCAACATGCTCTCTCTCTC |              |                  |                     |                       |                      |
| BS32  | F: CTGCCAGGCTTACCAAAAAA | (TG)<sub>17</sub> | NED             | 60                  | 242–270               | MG811536             |

*Note: *<sup>T</sup><sub>a</sub> = annealing temperature. *Monomorphic locus.*
TABLE 2. Genetic analysis of 11 polymorphic microsatellite markers developed for *Boswellia serrata* and cross-amplification to *B. ovalifoliolata*.

| Locus | BRT-TR (N = 20) | MM Hills-WLS (N = 20) | Cauvery WLS (N = 20) |
|-------|----------------|-----------------------|---------------------|
|       | H_o            | H_e                   | H_o                 |
| BS6   | 7.05           | 0.75                  | 0.70                |
| BS8   | 3.05           | 0.55                  | 0.55                |
| BS14  | 5.07           | 0.70                  | 0.70                |
| BS16  | 7.05           | 0.75                  | 0.75                |
| BS23  | 10.00          | 0.80                  | 0.80                |
| BS25  | 6.00           | 0.75                  | 0.75                |
| BS28  | 6.00           | 0.65                  | 0.65                |
| BS29  | 10.00          | 0.85                  | 0.85                |
| BS32  | 10.00          | 0.70                  | 0.70                |

Mean: 0.718 (±0.075) 0.81 (±0.054) 0.782 (±0.071) 0.731 (±0.070) 0.783 (±0.087) 0.704 (±0.096) 0.805 (±0.066) 0.775 (±0.084) 0.722 (±0.057)

Note: A = number of individuals sampled; PIC = polymorphism information content.

Population genetic diversity parameters, i.e., observed heterozygosity ($H_o$), expected heterozygosity ($H_e$), number of alleles per locus ($A$), effective number of alleles ($A_e$), Shannon's information index, and the probability of deviations from Hardy–Weinberg equilibrium were estimated using GenAlEx 6.5 (Peakall and Smouse, 2012). Polymorphism information content (PIC) and probability of identity (PI) were calculated by CERVUS 3.0 (Kalinowski et al., 2007).

Of the 16 successfully amplified primers, 11 were found to be polymorphic, of which eight had dinucleotide, two had trinucleotide, and one had compound repeat motifs (Table 1). Significant differences were found in allele frequencies between the analyzed populations. $A$ ranged from three to 10, and levels of $H_o$ and $H_e$ of each population ranged from 0.50 to 0.90 and 0.66 to 0.861 (Table 2), respectively. Shannon's information index values ranged from 1.098 to 2.072 and PIC ranged from 0.592 to 0.845 (Table 2), indicating that the markers designed are highly polymorphic (PIC > 0.5) and informative. The PI value is low for many loci, with the combined PI value of 2.081E-0015 confirming their applicability for population genetic studies. Significant deviations ($P$ < 0.05 and $P$ < 0.01) from Hardy–Weinberg equilibrium were detected for two markers (BS11 and BS25) but were not consistent across the populations. In *B. ovalifoliolata*, $A$ ranged from three to nine, $H_o$ ranged from 0.5 to 0.9, and $H_e$ ranged from 0.66 to 0.83 (Table 2). Raw genotyping data for both species are available in Appendix S1.

CONCLUSIONS

In this study, 16 microsatellite markers were developed specifically for *B. serrata* and 11 of these showed considerable polymorphism in all three studied populations. These markers will be useful for studying genetic diversity, gene flow, population structure, and inbreeding. The resulting information will help in developing appropriate strategies for sustainable utilization and conservation of this important resin-yielding tree. The allelic overlap and intrageneric amplification of these microsatellite markers indicate a close relationship between *B. serrata* and *B. ovalifoliolata*, which needs support from further investigation.

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Bangalore, India), and 0.2 μM of each primer. The PCR profile was as follows: an initial denaturation at 95°C for 5 min; followed by 35 cycles of 95°C for 30 s, 50–60°C for 30 s, and 72°C for 30 s; and a final extension at 72°C for 10 min. Amplicons were analyzed on an ABI 3730 Genetic Analyzer (Applied Biosystems) at Chromous Biotech (Bangalore, India) and scored using GeneMapper version 3.2 (Applied Biosystems) software.
Mahadeshwara Hills Wildlife Sanctuary and Cauvery Wildlife Sanctuary (PCCF/[WL]/E2/CR/22/2014-15).

**DATA ACCESSIBILITY**

Sequence information for the developed primers has been deposited to the National Center for Biotechnology Information (NCBI); GenBank accession numbers are provided in Table 1. Raw genotyping data for both species are available in Appendix S1.

**SUPPORTING INFORMATION**

Additional Supporting Information may be found online in the supporting information tab for this article.

**APPENDIX S1.** Genotyping data for *Boswellia serrata* and *B. ovalifoliolata*.

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**APPENDIX 1.** Voucher information for *Boswellia* species used in this study.

| Species                  | Voucher specimen accession no. | Collection locality (Code)                                      | Geographic coordinates | n  |
|-------------------------|---------------------------------|-----------------------------------------------------------------|------------------------|----|
| *Boswellia serrata*     | ATREEBs5925a                    | Billigiri Rangaswamy Temple Tiger Reserve (BRT-TR)              | 11°59′38″N, 77°8′26″E   | 20 |
| *B. serrata*            | ATREEBs5925b                    | Mahadehwara Hills Wildlife Sanctuary (MM Hills WLS)             | 12°1′50″2″N, 77°35′16″E | 20 |
| *B. serrata*            | ATREEBs5925c                    | Cauvery Wildlife Sanctuary (Cauvery WLS)                        | 12°10′12″N, 77°32′34″E  | 20 |
| *B. ovalifoliolata*     | ATREEBo5926a                    | Seshachalam Hills                                              | 14°19′59″99″N, 78°15′0″E| 10 |

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Note: n = number of individuals sampled.

*One sample per population is deposited at the ATREE Herbarium, Bangalore, India.*