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# Isolation and characterization of microsatellite loci in *Santalum lanceolatum* and *Santalum leptocladum* (Santalaceae)[1]

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- *Premise of the study:* Microsatellite primers were developed for the first time in the native Australian sandalwood species *Santalum lanceolatum*.
- *Methods and Results:* Using an enrichment cloning protocol, five novel polymorphic codominant loci were developed and characterized in *S. lanceolatum* and *S. leptocladum*. In addition to these, three existing microsatellite loci from other sandalwood species were successfully amplified and characterized for *S. lanceolatum* and *S. leptocladum*. Among the eight loci, allelic diversity ranged from 4 to 29.
- *Conclusions:* Primers will be useful for studies of clonality, genetic diversity and spatial genetic structure in wild populations. When coupled with other molecular techniques will help investigate the relationship between *S. lanceolatum* and *S. leptocladum*, species of commercial and conservation interest.

**Key words:** Sandalwood; *Santalum*; microsatellite; cross-species amplification.

*Santalum*, or sandalwood, are hemiparasitic trees, with many species highly valued for their heartwood oils, resulting in widespread exploitation of the genus in the wild. One species has been recently classed as extinct, and of the remaining 15, one classed as endangered and two as vulnerable (IUCN, 2010). The detrimental impacts of harvesting being observed among many of the remaining unlisted species. Northern sandalwood, *S. lanceolatum* (R.Br.), is a wide-spread Australian species harvested for oil since the 1800s. Recently, *S. lanceolatum sensu lato* was revised taxonomically and a southern segregate species, *S. leptocladum*, defined (Harbaugh, 2007). Here we report on the development of five novel microsatellite loci for use in *S. lanceolatum* and *S. leptocladum* and the characterization of three microsatellite loci designed for *S. insulare* (Lhuillier et al., 2006) and *S. austrocaledonicum* (Bottin et al., 2005) for use in *S. lanceolatum* and *S. leptocladum.*

## METHODS AND RESULTS

A genomic DNA library enriched for simple sequence repeats was constructed following a modified version of Glenn and Schable (2005), in which the Hamilton SNX linker system was used (Hamilton et al., 1999). Nuclear genomic DNA was used from multiple individuals in library construction. Amplified SNX linker-ligated DNA fragments were ligated into plasmid DNA (pMOSBlue, GE Healthcare, Uppsala, Sweden) and then ligated products were transformed into pMOSBlue competent cells (GE Healthcare) and plasmid inserts were PCR amplified with T7 and U19 vector primers. PCR products were visualized and fragment length estimated using gel electrophoresis. Of the 1440 clones, 215 contained PCR products greater then 300 bp in length, which were then gel extracted (Epoch Biolabs, Sugar Land, TX, USA) and sequenced using DYEnamic ET Dye Terminator sequencing chemistry (GE Healthcare) and then base-called using a MegaBACE 1000 system (GE Healthcare). Few of the sequences obtained from clones yielded SSRs, and of those only 15 were suitable for primer design, which was performed in Primer 3 (www.frodo.wi.mit.edu). Primers were obtained from commercial providers (GeneWorks [Adelaide, SA] and Invitrogen [Carlsbad, CA, USA], Table 1) with the forward primer of each pair synthesized with an M13 tail (5′-CACCACGTTGTAAAACGAC-3′) to facilitate easy automated screening (Boutin-Ganache et al., 2001). Primers were tested against eight *S. lanceolatum* individuals representing a large geographic region. Five were found to display biallelic heterozygosity and polymorphism. Supplementary to these, 16 loci designed for the congeners *S. austrocaledonicum* (Bottin et al., 2005) and *S. insulare* (Lhuillier et al., 2006) were tested for amplification in *S. lanceolatum*, with three successfully amplifying polymorphic codominant loci.

Amplification conditions for the loci Lanc07, Lanc08, Lanc09 and mSiCIR33 were a 15-μL reaction containing 1.005 nmol Tris-HCL, 240 ρmol $(NH_4)_2SO_4$, 15 ρmol $MgCl_2$, 0.5 ρmol of the M13 tailed forward primer, 7.0 ρmol of the reverse, 7.0 ρmol of M13 primer labeled with either TET, FAM or HEX, 2.0 ρmol dNTP, 0.24 ng of BSA, 0.5 units of *Taq* DNA polymerase and 2.5 ng genomic DNA. Conditions of the PCR for the loci Lanc03, Lanc10, MSaCIRE09, and mSACIRH10 were the same except for $MgCl_2$ (45.0 ρmol), dNTP (4.0 ρmol), and BSA (0.2 ng).

Thermocycler conditions for Loci Lanc03, Lanc07, Lanc08, Lanc09, and mSiCIR33 were 95°C denaturation for 5 min, 30 s at 94°C, 15 s at 65°C (decreasing 1.5°C per cycle), then 30 s at 72°C for 15 cycles, followed by 30 s at 94°C, 15 s at 48°C and 30 s at 72°C for 24 cycles with a final extension at 72°C for 5 min. For Lanc10, mSaCIRE09, and mSACIRH10 the program was altered to have 12 cycles (decreasing 1°C per cycle) in the first block and 53°C annealing in the second block of 24 cycles.

Once optimized loci were screened across 240 samples from 10 populations of *S. lanceolatum s.l.,* comprised of 9 populations of *S. lanceolatum s.s.* and one *S. leptocladum* in northeastern Australia. Actual population locations and specific place names are not published here as many samples were collected under permit and an agreement to keep such information confidential, with regards to intellectual property considerations. The locations of the 10 populations stretched over 1000 km, and population sampling sizes ranged from 10 to 31 with an average of 24 individuals collected. Genotypes were verified with genetic analysis software Fragment Profiler version 1.2 (GE Healthcare).

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TABLE 1. Characteristics of eight nuclear microsatellite loci (five novel and three cross-species amplified), for use in *Santlaum lanceolatum* and *Santalum leptocladum*. Shown for each pair are the forward and reverse sequences, repeat type in cloned fragment, size range (bp) in individuals tested, annealing temperature (Ta) and GenBank accession number.

| Locus | Sequence | Repeat Motif | Size Range (bp) | Ta (°C) | GenBank Accession No. |
|---|---|---|---|---|---|
| Lanc03 | F:*AGAAAAAACTTTTCAGTTCC<br>R:CTCGTGATTAGGGAGCAAGC | $(GTTT)_4$ | 130–154 | 58°C | HM448038 |
| Lanc07 | F:*AAACCCCTTCTCCTCCCATT<br>R:CCGATATTCCCCCATTTCTT | $(TC)_4(CT)_{10}$ | 156–182 | 59°C | HM448039 |
| Lanc08 | F:*AAGTGATCACAGCTCCGGTTA<br>R:GGGGTCGTTGCATCTATTTC | $(GA)_8$ | 134–140 | 59°C | HM448040 |
| Lanc09 | F:*ATGAGAGCGAGAGGGAGACA<br>R:GTCCACTCCTCACCAAAACC | $(AG)_{15}$ | 165–217 | 59°C | HM448041 |
| Lanc10 | F:*TTTGCTGTCACATTTGGATTG<br>R:TCCTCTTGGTGGAAGTAGCC | $(TC)_{21}$ | 136–203 | 59°C | HM448042 |
| **mSiCIR33 | F:*GAAGTTGAAGTTGTTGATGC<br>R:AAATGAGAGACCTGAGTGAAG | $(TC)_{19}$ | 222–282 | 58°C | AM113978 |
| **mSaCIRE09 | F:*GGAAAGGGTTGACAGGAAGAAAA<br>R:TGCGAGTGAGTGGGAAAAGTAGA | $(CT)_{16}$ | 153–183 | 59°C | AJ831397 |
| **mSaCIRH10 | F:*AAGCCCGATAACGAGAAAAGAAA<br>R:ATGAATAGGGATGGCGAGAGGAT | $(GA)_{27}$ | 219–239 | 59°C | AJ831403 |

*Forward primer synthesized with an M13 tail of 5′-CACCACGTTGTAAAACGAC-3′
**First described in Bottin et al. (2005) and Lhuillier et al. (2006)

TABLE 2. Results of initial primer screening on samples from 10 populations of *Santalum lanceolatum s.l.*. Shown for each locus are the number of individuals data were obtained from (N), number of alleles ($N_a$). For the 9 populations of *Santalum lanceolatum s.s,* average observed and expected heterozygosity (Ave. $H_o$ and Ave. $H_e$) and the range of values ($H_o$ and $H_e$) are displayed. For the population comprised of *Santalum leptocladum* individuals, single heterozygosity values are given.

| | *S. lanceolatum* | | | | | | *S. leptocladum* | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Locus | N | $N_a$ | $H_o$ | Ave. $H_o$ | $H_e$ | Ave. $H_e$ | N | $N_a$ | $H_o$ | $H_e$ |
| Lanc03 | 167 | 9 | 0–0.56 | 0.17 | 0.05–0.66 | 0.37 | 8 | 2 | 0.13 | 0.11 |
| Lanc07 | 210 | 10 | 0.18–0.9 | 0.55 | 0.36–0.76 | 0.66 | 21 | 6 | 0.52 | 0.81 |
| Lanc08 | 209 | 4 | 0.03–1 | 0.57 | 0.03–0.65 | 0.51 | 21 | 4 | 0.24 | 0.40 |
| Lanc09 | 195 | 18 | 0.1–1 | 0.57 | 0.55–0.82 | 0.78 | 16 | 8 | 0.38 | 0.75 |
| Lanc10 | 165 | 29 | 0–0.93 | 0.53 | 0.4–0.87 | 0.8 | 22 | 9 | 0.77 | 0.81 |
| mSiCIR33 | 190 | 27 | 0.17–0.92 | 0.63 | 0.32–0.85 | 0.77 | 20 | 11 | 0.65 | 0.59 |
| mSaCIRE09 | 213 | 16 | 0.37–1 | 0.6 | 0.38–0.84 | 0.76 | 20 | 8 | 0.86 | 0.83 |
| mSaCIRH10 | 213 | 10 | 0.38–0.84 | 0.26 | 0.08–0.79 | 0.56 | 21 | 6 | 0.76 | 0.61 |

All loci were found to be polymorphic and codominant. All markers worked equally well on both species, under identical reaction conditions. Genetic diversity parameters were estimated in GenAlEx version 6 (Peakall and Smouse, 2006). The number of alleles ranged from 4 to 29 (Tables 1 and 2) with mean alleles per locus of 15.34. Average heterozygosity [$H_o$ ($H_e$)] ranged from 0.17 (0.37) to 0.63 (0.8). Significant deviations from Hardy-Weinberg Equilibrium (HWE) were detected within two populations for all loci ($p < 0.05$). These same two popultions also showed significant linkage disequilibrium ($P < 0.05$, using Fisher's exact test in GENEPOP (Raymond and Rousset, 1995)).

## CONCLUSIONS

Significant ($p < 0.05$) deviations from HWE and linkage disequilibrium ($p < 0.05$) in two of the 10 populations was attributed to these two populations containing the highest frequency of identical genotypes, or displaying high levels of clonality. These markers will be a valuable tool for investigating genetic diversity, clonality and spatial genetic structuring of wild populations of two sandalwood species, *S. lanceolatum* and *S. leptocladum*. Additionally, they can be used to assist in understanding the relationship between these two species, aid in conservation strategies and commercial plantation development.

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