

1 **Development of polymorphic microsatellite loci in the endangered tree species**  
2 ***Dysoxylum malabaricum***

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19 Keywords: Microsatellite, conservation genetics, *Dysoxylum malabaricum*, endangered species, Western  
20 Ghats

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28 Running title: Microsatellite loci in *Dysoxylum malabaricum*  
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**Abstract**

*Dysoxylum malabaricum* Bedd. (Meliaceae) is an economically important tree species occurring in the Western Ghats, a mega-diversity hotspot in southern India. In this paper, we report the development of fifteen microsatellite markers for *D. malabaricum*. The microsatellite primers screened had 2-9 alleles per locus and the observed and expected heterozygosity ranged from 0.07 to 1.00 and 0.07 to 0.9 respectively. Seven microsatellites cross amplified in the related species *Dysoxylum binectariferum* and showed good polymorphism. These are the first microsatellites described for *D. malabaricum* and they will be used to study population structure and genetic diversity.

1 *Dysoxylum malabaricum* Bedd. (white cedar, Meliaceae ) is an economically important tree species in the  
2 Western Ghats, one of India's megadiversity hotspots. *D. malabaricum* is restricted to the evergreen and semi-  
3 evergreen forests of the Western Ghats where it is harvested mainly for its cedar scented wood (Khan 2007).  
4 Apart from the logging, another threat towards the species is the habitat destruction by conversion of forests into  
5 agricultural land (Vasudeva *et al*, 2003). As a consequence, the distribution of *D. malabaricum* is fragmented  
6 with an imminent threat to reducing the gene flow between and across populations and thereby lowering levels of  
7 genetic diversity. In this paper, we report the development of fifteen microsatellite markers and discuss the utility  
8 of these markers in addressing questions related to the population genetics of this species.

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10 The plant material of *Dysoxylum malabaricum* and its closely related species, *Dysoxylum binectariferum*, was  
11 collected from north Western Ghats (latitude 13<sup>0</sup>N-14<sup>0</sup>N and longitude 74<sup>0</sup>E-75<sup>0</sup>E) in southern India. Genomic  
12 DNA was extracted from fresh leaves of *D. malabaricum* and *D. binectariferum* using a CTAB (cetyltrimethyl  
13 ammonium bromide) extraction method (Sambrook *et al*. 1989). The DNA of *D. malabaricum* was used to  
14 construct a genomic library selected for high proportions of repeat inserts. This was done by digesting the  
15 genomic DNA with MboI restriction enzyme (Fermentas) and size-selecting fragments of 300-1500 base pair  
16 lengths, using the streptavidin-biotin bench version (Fleisher & Loew, 1995; Hamilton & Fleisher, personal  
17 communication) where magnetic beads with biotin-labelled (GT)<sub>13</sub>, (CT)<sub>13</sub>, (AAC)<sub>8</sub>, (AAG)<sub>8</sub>, (ACT)<sub>8</sub> and  
18 (ATC)<sub>8</sub> oligonucleotides were used for the enrichment step. The fragments were ligated into pGEM®-T Easy  
19 vector (Promega) and transformed into competent *Escherichia coli* (JM109) cells with the plasmids, all according  
20 to the manufacturer's protocol. The *E. coli* colonies were grown on agar plates prepared with IPTG and X-gal  
21 and the true transformants distinguished by their white colour were selected.

22  
23 Transformed colonies were screened for positive clones i.e. clones containing repeat arrays, by running PCR:s  
24 with universal M13 primers plus the respective oligonucleotide repeat as a primer (Invitrogen) and analysing the  
25 bands on their agarose gel image. Positive clones were then sequenced in order to characterize the repeat arrays.  
26 Colonies were prepared for sequencing using the DYEnamic™ ET Dye Terminator Kit (GE Healthcare) and  
27 M13 primers. The product was sequenced on a MegaBACE 1000. Out of the 359 clones screened, 106 contained  
28 microsatellite inserts. Primers were designed for the 38 unique microsatellites found in *D. malabaricum* using the  
29 web-based software Primer3 (Rozen & Skaletsky 2000).

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31 Polymorphism studies were carried out on the microsatellite loci using two different methods. A total of 27  
32 individuals of *D. malabaricum*, sampled from three populations were tested for the first 16 loci. For the  
33 remaining 22 loci, fifteen individuals from three populations were tested. DNA was amplified by running a PCR  
34 on a 25µL reaction consisting of 30ng of DNA, 1mM of each dNTP, 1X PCR reaction buffer (Bangalore Genei,  
35 India), containing 1.5 mM MgCl<sub>2</sub>, 1unit Taq polymerase (Bangalore Genei, India) and 5 pico moles of primers.

1 PCR conditions were 94°C for 3 minutes, 35 cycles of 94°C for 1 minute, annealing temperature for 1 minute  
2 and 72°C for 1 minute. A final elongation step of 72°C for 10 minutes was added.

3  
4 In the 16 first loci, the PCR products were separated by a polyacrylamide electrophoresis (PAGE) using a 12%  
5 polyacrylamide gel consisting of 12 mL of 40% acrylamide solution, 8 mL of 5xTBE buffer and ddH<sub>2</sub>O added to  
6 a final volume of 22 mL. Right before casting the gel, 250 µL of 10 % ammonium persulphate and 40 µL of  
7 TEMED were added. The gel was run at 150 V for about 12-13 h. After electrophoresis, the gels were silver  
8 stained as described by Creste *et al.* (2001) and thereafter the number of alleles was scored.

9  
10 In the remaining 22 loci, the forward primers were labeled with fluorescent dyes (Operon Technologies) and the  
11 PCR products were genotyped on an ABI 3100 with the help of the ROX-500 Size Standard (Applied  
12 Biosystems) which includes the dyes NED, HEX, FAM and VIC. Out of the 38 primers, fifteen showed  
13 polymorphism in *D. malabaricum* (Table 1). Over all the 15 loci, the number of alleles per locus ranged from 2  
14 to 9, with an average of 4.73 alleles per locus. The allele frequencies varied among populations. Observed  
15 heterozygosity over all the loci ranged between 0.07-1.00 with a mean of 0.51 and the expected heterozygosity  
16 ranged between 0.07-0.90 with an overall mean of 0.53. Tests for linkage disequilibrium and deviation from  
17 Hardy-Weinberg equilibrium were carried out using GENEPOP 3.4 (Rousset 2007) and the results were non-  
18 significant. These markers could thus be used to study the population structure and genetic diversity within and  
19 between existing populations.

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21 Another closely related species, *Dysoxylum binectariferum* was checked for cross amplification using the first 16  
22 primers. In *D. binectariferum*, 29 individuals from three different populations were tested for each locus. In *D.*  
23 *binectariferum*, Seven primers proved most useful for *D. binectariferum* after cross amplification. The number  
24 of alleles ranged between two and nine. These primers are shown in Table 2. When tested for linkage  
25 disequilibrium we found some association between loci D267 and D312 and between D267 and D618 ( $p < 0.05$ ).  
26 The test for deviation from Hardy-Weinberg equilibrium was significant for all loci ( $p < 0.05$ ). However, there  
27 does not seem to be any overall deficiency or excess of heterozygotes as the Weir & Cockerham estimation of the  
28  $F_{IS}$  values in the same test were roughly equally positive and negative.

### 30 **Acknowledgements**

31  
32 The work was supported by grants from SIDA, Sweden to Martin Lascoux and R. Uma Shaanker. The work was  
33 also supported in part by grants from the Department of Biotechnology, Government of India. Cooperation of the  
34 Karnataka State Forest Department for enabling field visit and collection of plant tissue is acknowledged.

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1 **Table 1.** Locus, repeat motif, fluorescent dye label, primer sequence, number of individuals genotyped (n), annealing temperature (T) and product sizes for  
 2 fifteen microsatellites in *Dysoxylum malabaricum*. Observed and expected heterozygosity, ( $H_O$ ) and ( $H_E$ ) and GenBank accession numbers are also given.  
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Locus	Repeat motif	Label	Primer (5'-3')	n	T (°C)	Size (bp)	No. of alleles	$H_O$	$H_E$	GenBank accession number
D146	(AAC) <sub>7</sub>	N/A	L: CGAGAAAATTTCAACTGGGTCT R: TGGGGTTTGATTCTGACGAT	27	61	200-220	2	0.15	0.14	GF102010
D182	(AAC) <sub>10</sub>	N/A	L: ACGTGGCTGTGGAGAGAAAC R: GAAATCCGAAACGAGACGAA	27	62	156-164	2	0.63	0.50	GF101997
D250	(AAC) <sub>8</sub>	N/A	L: GCAAGTTTCAAATCCGGTTG R: CTTGCAAACCTTGCTGCTG	27	62	234-246	2	0.19	0.17	GF102011
D429	(AAG) <sub>12</sub>	N/A	L: CGGAGGAGTCAGTCCCAGT R: TCGGTGCACCTCTACAATGA	27	61	238-246	2	0.07	0.07	GF101998
Dysmal 01	(TC) <sub>22</sub> (AC) <sub>12</sub>	FAM	L: TGATGACAAATGAAAAGGGTTG R: AGCATCGTTTACGCCATTTT	15	50	210-239	7	0.67	0.79	GF101999
Dysmal 02	(CA) <sub>7</sub> ...(GA) <sub>25</sub>	NED	L: TTCACCACTCTAACTTTACAAGCAC R: CTAGGGTGCGGCATTCTG	15	50	149-180	9	0.80	0.85	GF102000
Dysmal 03	(TC) <sub>16</sub>	FAM	L: TTAGATACTCACACTACAAACACACAG R: AAAGACGACAGAACACAGAGC	15	50	60-79	5	0.73	0.64	GF102001
Dysmal 07	(TTG) <sub>7</sub>	HEX	L: TCGAGTAATGAAGTAGTCGATGAAG R: GATCGTCGGCAAATTACACC	15	56	84-90	2	0.13	0.13	GF102002
Dysmal 09	(GAA) <sub>9</sub>	FAM	L: AGATTCTGGGGTCGGAAAAG R: TCCCTTTCACATTCCCAAAG	15	56	209-235	5	0.73	0.64	GF102003
Dysmal 13	(GT) <sub>27</sub>	HEX	L: CCAGCAAAGATTAGCGACAG R: CGAGGAAGAATGTCATGGTC	15	56	196-239	8	0.60	0.75	GF102004
Dysmal 14	(CT) <sub>13</sub> (CA) <sub>12</sub>	NED	L: CATTGAGAGCAGAGGTAAGTTTG R: CCTCACCATCAATCTGCAAC	15	56	159-183	7	0.60	0.84	GF102005
Dysmal 17	(GA) <sub>26</sub>	NED	L: TCTGCAGCCTGCCATATTAG R: TTCCCTTAATGAAAGAAATATGAAG	15	50	100-152	8	1.00	0.90	GF102006
Dysmal 18	(AAG) <sub>8</sub>	FAM	L: TTCTCACTGCCTTGCAAG R: GGATGCATCACTGGTTCTGG	15	56	90-96	2	0.20	0.24	GF102007
Dysmal 22	(CA) <sub>13</sub>	FAM	L: GATCAAGACGCAAGGATTTTC R: CGGTGCTTAGATAATTTGTTTCG	15	56	91-97	3	0.40	0.36	GF102008
Dysmal 26	(CT) <sub>15</sub> (TG) <sub>8</sub>	VIC	L: TGCTTATGGCATGATTCCTTC R: GAGCAATGAGCAGGGTTTG	15	56	214-234	7	0.80	0.87	GF102009

1 **Table 2.** The seven primers that cross amplified successfully in *Dysoxylum binectariferum*.  
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Locus	Repeat motif	Primer (5'-3')	n	T (°C)	Size (bp)	No. of alleles	GenBank accession number
D182	(AAC) <sub>10</sub>	L: ACGTGGCTGTGGAGAGAAAC R: GAAATCCGAAACGAGACGAA	55	62	158-172	8	GF101997
D250	(AAC) <sub>8</sub>	L: GCAAGTTTCAAATCCGGTTG R: CTTGCAAACCTTTGCTGCTG	29	62	245-302	8	GF102011
D267	(AAC) <sub>10</sub>	L: AGAAATCCGAAACGAGACGA R: AGGCTGTGGAGAGAAACCAA	55	61	160-178	9	GF102015
D312	(ATG) <sub>11</sub>	L: GAACTCTTCCTCGTGGCACT R: CAGGAAATCCGCTTTCTTCTT	55	62	220-260	6	GF102016
D471	(AAG) <sub>13</sub>	L: ACAGGAAAGTCAACTGCTTGG R: TGCTTTATAGTAGTAATTTGGGAATG	29	59	230-240	2	GF102012
D615	(AG) <sub>8</sub>	L: TCGAGAGAGCATTTTGAGAGTG R: TATCGGAATAAACCCGACAA	29	60	160-170	3	GF102013
D618	(AG) <sub>12</sub>	L: GATCGGAGGCAGTAGAGAGG R: CGGATATGTGTCGGAATGAA	55	61	155-165	6	GF102014

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