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Development and characterization of microsatellite markers for *Dysoxylum binectariferum*, a medicinally important tree species in Western Ghats, India

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[Sumangala R. C., Mohana Kumara P., Shaanker R. U., Vasudeva R. and Ravikanth G. 2013 Development and characterization of microsatellite markers for *Dysoxylum binectariferum*, a medicinally important tree species in Western Ghats, India. *J. Genet*. **92**, e85–e88. Online only: http://www.ias.ac.in/jgenet/OnlineResources/92/e85.pdf]

Introduction

Dysoxylum binectariferum Hook.f (Meliaceae) is a medicinally important medium to large-sized tree species found in tropical and subtropical regions. This species is distributed in the Western Ghats of southern India and is well known for its wood (Rao *et al.* 1961). It has gained considerable interest in recent years because of the pharmacologically important compound rohitukine and its derivative flavopiridol. Rohitukine, a chromane alkaloid, is reported to possess anti-inflammatory, anti-fertility, anti-implantation, anti-cancer and immuno-modulatory properties (Naik *et al.* 1988; Carlson *et al.* 1996; Sedlacek *et al.* 1996; Mohanakumara *et al.* 2010). Dysobinin, the other chemical compound isolated from fruits of this tree is also reported to exhibit significant central nervous system depressant action and mild anti-inflammatory activity (Singh *et al.* 1976). The bark is also reported to be used for the treatment of leprosy and foul ulcers (Jain and DeFilipps 1991).

This species is threatened throughout its range in Western Ghats as a result of overexploitation and habitat destruction, which have reduced local population sizes and has led many populations to local extinction. In this study, we report the development of microsatellite markers and discuss the utility of these markers in addressing questions related to the population and conservation genetics of this species.

Materials and methods

Young leaves from *D. binectariferum* were collected from three populations in central Western Ghats, India, near Jog falls (14.228°N and 74.82°E), Heble (14.51°N and 74.60°E) and Teppara (14.55°N and 74.65°E). The genomic DNA was extracted using the CTAB method (Doyle and Doyle 1987). The extracted DNA was purified, dissolved in Tris-EDTA buffer and used for microsatellite development through the enrichment-subtractive hybridization protocol with minor modifications (Glenn and Schable 2005). The genomic DNA was digested with *Rsa*I (New England Biolabs, USA) and ligated to linker oligonucleotides SNX-F (5′-GTTTAAGGCCTAGCTAGC AGAATC) and SNX-R (5′-ATTCTGCTAGCTAGGCCTTA AACAAAA) using the rapid DNA ligation kit (Fermentas International, USA). Biotinylated oligonucleotides of selected repeats (microsatellite probes) were used for hybridizing with the DNA fragments and magnetically captured using dynabeads (Sigma-Aldrich, Bangalore, India). The captured DNA was washed, amplified and cloned in pTZ57R/T plasmid vector using Fermentas TA cloning kit. The positive colonies (white) were picked and subjected to PCR using the universal M13 forward and M13 reverse primers. The amplified PCR products were sequenced (ABI PRISM 3100 Genetic Analyzer Applied Biosystems, Chromous Biotech, Bangalore, India). Sequences that contained microsatellites were selected and the primers designed for the flanking sequences of the microsatellites by using web-based software Primer3 (http://primer3.wi.mit.edu/; Rozen

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Keywords. microsatellite; medicinal plant; cross-species amplification; Meliaceae; *Dysoxylum binectariferum*.

Table 1. Locus, primer sequence, repeat motif, allele size range, annealing temperature, GenBank ID and population genetic parameters for 11 microsatellites of *Dysoxylum binectariferum* in Jog population.

Locus	Primer sequence 5'–3'	Repeat unit	Allele size range (bp)	T_a (°C)	GenBank ID	Jog population (22)		
						A	H_o	H_e
DY1	F: ATTCTTGGGTGAACAGTGAC R: GTGTCAGGCTTTCAAATGAT	$(CACCGA)_3$	353–366	55	JQ996634	4	1.00000	0.71459
DY2	F: CAGATCGTTGGTGAAGGA R: TATAAAGAGGTGGCATGGAC	$(AG)_2GA(AG)_5A(AG)_6$	350–410	59	JQ996635	5	0.86364	0.69767
DY4	F: CAACACATTCAAAGCGGGTA R: CCGTTGGATTCTAAGGCTGA	$(ATT)A(ATT)_4$	232	58	JQ996637	1	Monomorphic	
DY5	F: TCGGAAGTTTTCAATGTTTT R: GCCTCCGTAAACTATGATTG	$(CT)_5C(CT)_6TC(CT)$	332–359	58	JQ996638	6	0.95455	0.69767
DY6	F: AATCACTTGTCCAATGACACT R: GCAGAATCCATAAGCATAGC	$(GT)AT(GT)_{13}$	240–290	45	JQ996639	6	0.81818	0.70085
Dysmal 02	F: TTCACCACTCTAACTTTACAAGCAC R: CTAGGGTGCGGCATTCTG	$(CA)_7...(GA)_{25}$	130–170	50	GF102000	5	1.0000	0.79493
Dysmal 07	F: TCGAGTAATGAAGTAGTCGATGAAG R: GATCGTCGGCAAATTACACC	$(TTG)_7$	85–110	56	GF102002	4	0.40909	0.60148
Dysmal 09	F: AGATTCTGGGGTCGGAAAAG R: TCCCTTTCACATTCCCAAAG	$(GAA)_9$	224–236	56	GF102003	4	0.81818	0.69662
Dysmal 13	F: CCAGCAAAAGATTAGCGACAG R: CGAGGAAGAATGTCATGGTC	$(GT)_{27}$	151–183	56	GF102004	6	0.13636	0.61099
Dysmal 18	F: TTCTCACTGCCTTGCAGAAG R: GGATGCATCACTGGTTCTGG	$(AAG)_8$	90–200	56	GF102007	6	0.27273	0.39852
Dysmal 22	F: GATCAAGACGCAAGGATTTTC R: CGGTGCTTAGATAATTTGTTCG	$(CA)_{13}$	90–130	56	GF102008	5	0.90909	0.64693

Locus; F, forward; R, reverse; primer sequence; repeat motif; allele size range; T_a, annealing temperature; GenBank accession number; *A*, number of alleles; H_o, observed heterozygosity; H_e, expected heterozygosity.
Note: DY1, DY4, DY5, Dysmal09, Dysmal13 were labelled with FAM, PET, VIC, FAM and NED fluorescent dyes respectively for the forward primers.

Table 2. Allele number, observed and expected heterozygosity of two populations (Heble and Teppara) in *D. binectariferum*.

	Heble population ($n = 15$)			Teppara population ($n = 27$)		
Locus	A	H_o	H_e	A	H_o	H_e
DY1	6	1.00	0.7015	8	1.000	0.669
DY4	1	0.000	0.000	2	0.000	0.073
DY5	5	0.875	0.817	8	0.75	0.853
Dysmal 09	4	0.643	0.717	5	0.704	0.725
Dysmal 13	4	0.077	0.588	6	0.24	0.469

A, number of alleles; n, sample size; allele size range (bp), H_o, observed heterozygosity; H_e, expected heterozygosity.

and Skaletsky 2000). Of the 96 positive clones, 75 clones were sequenced. Of these, 36 sequences had microsatellite repeats of adequate size and primers were designed for 10 microsatellite inserts.

Amplification was carried out in Eppendorf thermocycler in 20 μL reactions containing 2 μL of 20–30 ng DNA, 2 μL of dNTPs (1 mM), 0.9 μL of each primer (5 pmole), buffer 2.0 μL (10×), *Taq* DNA polymerase 0.2 μL (Chromous Biotech, Bangalore, India) and 12 μL of water. The PCR conditions for all loci was carried out at initial denaturation for 94°C for 2 min and 35 cycles of denaturation for 50 s at 94°C, annealing temperature for 1 min, elongation for 1 min 30 s at 72°C followed by final elongation for 72°C at 10 min. After initial screening, five primer pairs that showed polymorphism were retained for further analysis. The motif of these five primers include three interrupted dinucleotide repeats, one trinucleotide repeat and one hexanucleotide repeat. Another six pairs of primers from the closely related species *Dysoxylum malabaricum* (Hemmilä *et al.* 2010) were used for cross amplification. In total, we screened 11 primers with 22 adult individuals from Jog population of this species (table 1).

The genotyping was carried out in ABI PRISM 3130 Genetic analyzer by labelling one of the primer with fluorescent dye. In our study, we used FAM (blue colour, 6-carboxyfluorescine), VIC and NED (green and yellow colour, respectively, proprietary to Applied Biosystems). Thus, the 5′ end of the forward primer of DY1 (6-FAM) and DY5 (VIC) from *D. binectariferum* and Dysmal09 (6-FAM) and Dysmal13 (NED) from *D. malabaricum* were labelled with fluorescent dyes and individuals were genotyped using ABI PRISM 3130 Genetic analyser (Applied Biosystems).

The electropherograms were analysed using Gene Scan 3.7 and Genotyper 3.7 software packages (Applied Biosystem). The genotyping of samples for the remaining six primers were performed using polyacrylamide gel electrophoresis. The aliquots of 5 μL of PCR products were electrophoresed along with a 20 bp ladder on 12% polyacrylamide gel. The gel was run at 100 V for about 12–13 h. After the electrophoresis, the gels were silver stained as described by Creste *et al.* (2001) for visualization of DNA fragments. We also assessed genetic diversity for the other two populations namely Heble ($n = 15$) and Teppara ($n = 27$) with labelled primers.

Results and discussion

Twenty-two adult individuals of *D. binectariferum* from Jog populations were genotyped at 11 loci to assess the genetic variability. The number of alleles, allele range, expected and observed heterozygosity (H_e and H_o) at each microsatellite loci were calculated using Arlequin ver. 3.1 (www.cmpg.unibe.ch/software/arlequin 3) and are presented in table 1. The number of alleles per locus ranged from 4 to 6. The observed and expected heterozygosity ranged from 0.136 to 1.00 and 0.398 to 0.794, respectively (table 1). The exact tests for departure from Hardy–Weinberg equilibrium (HWE) were performed using Markov-chain random walk algorithm (Guo and Thompson 1992) using the programme Arlequin 3.1 (Excoffier *et al.* 2005, table 1). Two loci (Dy6 and Dysmal09) showed deviation from HWE ($P < 0.05$). We also performed an exact test for genotypic linkage disequilibrium between loci based on 3600 permutations using the FSTAT ver. 2.9.3 (Goudet 1995). No evidence of allelic dropout was found in any of the loci by analysis using the program Micro-Checker (www.microchecker.hull.ac.ak) with Bonferroni correction (Van *et al.* 2004). Even after multiple tests correction of the probability value ($P > 0.0013$), none of the loci showed significant linkage disequilibrium. The samples from two other populations (Heble and Teppara) were genotyped for assessing the genetic variability using only fluorescent labelled primers (table 2). The number of alleles per locus ranged from 1 to 6 and 2 to 8 for each population respectively. The observed and expected heterozygosity ranged from 0.00 to 1.00 and 0.00 to 0.817 for Heble population and 0.00 to 100 and 0.073 to 0.853 for Teppara population (table 2). The locus DY4 which was monomorphic in the initial screening, however showed two alleles in Teppara population. The microsatellite markers reported in

this paper are invaluable for researchers to study population genetic structure of *D. binectariferum* and to formulate sound conservation and management programmes.

Acknowledgement

This work was funded by the Department of Biotechnology (DBT), Government of India.

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Received 23 April 2013, in revised form 8 May 2013; accepted 10 May 2013
Published on the Web: 11 October 2013