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Brief Report
Successful cross-amplification of *Shorea* microsatellites reveals genetic variation in the tropical tree, *Shorea robusta* Gaertn.

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Sal (*Shorea robusta*) is a tree species with very good quality timber found across tropical south Asia. Due to over-exploitation, its genetic resources are highly depleted. Here we report for the first time on molecular markers in sal. Cross-species amplification of 27 *Shorea* microsatellites resulted in 24 of them harboring amplicons of expected sizes. Five microsatellites were moderate to highly polymorphic (N_a = 6–19). Observed and expected heterozygosities ranged from 0.49 to 0.77 and 0.52 to 0.89, respectively. These microsatellites are found to be adaptively neutral and are useful for population genetic studies in this species.

Sal (*Shorea robusta* Gaertn., Dipterocarpaceae) is one of the slow- to moderate-growing, dominant trees of dry-deciduous tropical forests in south Asia. Unlike other *Shorea* species, which are insect-pollinated (Momose et al. 1998), wind is the exclusive pollen vector in *S. robusta* (Alturi et al. 2004). The species is naturally found in Bhutan, Bangladesh, India and Nepal covering more than 12 million hectares (Tewari 1995) and is economically and ecologically important in that region. The wood of *S. robusta* is considered as the most suitable for railway sleepers, piles, beams and other load bearing constructions purposes. The tree when tapped yields resin which is burnt as incense during Hindu and Buddhist religious ceremonies. In many protected areas *S. robusta* is one of the most important tree species and thus essential for ecological conservation.

Due to heavy over-exploitation, fragmentation of the species' range is in an alarming state (Gautam and Devoe 2006) threatening the long-term maintenance of its genetic diversity and survival. In general, genetic knowledge of tropical forest species is more limited than in temperate or boreal forests (Finkeldey and Hattemer 2006). Genetic data from provenance trials are non-existent and marker-aided insights into this species are extremely limited. To the best of authors' knowledge, only isozymes have been used which indicate that the majority of genetic diversity is found within populations (Suoheimo et al. 1999).

Conservation and management of plant species, in addition to ecological information, requires a sound understanding of underlying genetic processes as well as variation within and among populations (Changtragoon 2001; Lee et al. 2006). In order to obtain such information highly sensitive techniques are required, which should be simple, informative and cost efficient. With recent developments in molecular biology, many options are now available to undertake such research. Microsatellites, or simple sequence repeats (SSRs), are a popular molecular marker for population genetic studies, including several tropical forest tree species (Lee et al. 2004; Mottura et al. 2005; Ouinsavi et al. 2006). These genetic markers compose of tandem repeats from one to six bases in length, which are arranged head-to-tail generally without interruption. In genomes of almost every organism so far studied microsatellites have been detected and seem to be distributed evenly throughout the genome (Hancock 1999). However, the development of these markers is expensive and time consuming. Successful cross-amplification of microsatellites in related species would save the development costs (including money and time) (Barbara et al. 2007) and would encourage to follow this approach by other studies.

Here we report for the first time the results of cross-amplification of microsatellites markers developed for other *Shorea* in nepalese populations of sal. Furthermore, the usefulness the markers for population genetic studies was assessed in a single natural population.

MATERIAL AND METHODS

To obtain an insight into the allelic richness of cross-species amplified microsatellites we used 30 individuals originating from 15 Nepalese well distributed populations (two adult trees from each population). To assess basic population genetic parameters we intentionally used an isolated, very small endemic population near to the city of Hetauda (27°23′47.8″N, 85°03′23.5″E), Nepal where we sampled 60 adult trees. Leaves were collected and dried in silica gel. DNA

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Table 1. *Cross-species amplifications of 27 microsatellites in Shorea robusta.*

Microsatellite	Repeat sequence	Annealing temperature (°C)	No. of alleles in sal trees (N = 30)
Sle 074a	$(CT)_{11}$	52	1
Sle 079	$(CT)_{11}$	52	1
Sle 105	$(GA)_{12}$	55	1
Sle 111a	$(GA)_{14}$	52	2
Sle 118	$(GA)_{16}$	52	2
Sle 216	T_{12} $(CT)_6$	54	Multiple bands
Sle 267	$(GA)_{17}$	55	9
Sle 271a	$(GA)_7$	52	1
Sle 280	$(CT)_7$	52	2
Sle 291a	$(GA)_8$	55	1
Sle 294	$(GA)_{12}$	52	2
Sle 303a	$(GA)_{12}$	55	6
Sle 384	$(CT)_{13}$	54	1
Sle 392	$(GA)_{11}$	52	2
Sle 465	$(CT)_{14}$	52	2
Sle 475	$(GA)_{10}$	52	1
Sle 562	$(GT)_8$	52	9
Sle 566	$(GA)_{13}$	52	11
Sle 605	$(GA)_{13}$	55	2
Shc 01	$(CT)_8(A)_{10}C$ T $(CA)_4CTA$	52	No amplification
Shc 04	$(CT)_{16}$	52	12
Shc 09	$(CT)_{12}$	54	2
Scd 1	$(TC)_{23}$	55	2
Scd 2	$(TC)_{14}$	54	1
Scd 4	$(TC)_{19}$	52	2
Scd 7	$(TC)_{15}$	54	Multiple bands
Scd 12	$(TC)_{19}$	62	2
Mean			2.85

extraction from these samples was carried out by using DNeasy Plant Minikit® (Qiagen) following the manufacturers protocol.

In total, 27 microsatellites were selected based on high number of alleles and simplicity of their repeats, i.e. 19 from *S. leprosula* (Lee et al. 2004), five from *S. cordifolia* (Stacy et al. 2001) and three from *S. curtisii* (Ujino et al. 1998). PCR was performed in 15 µl reaction mixture, which consisted of approximately 5 ng of template DNA, 50 mM KCl, 20 mM Tris-HCl (pH 8.0), 1.5 mM $MgCl_2$, 0.2 µM of each primer, 0.2 mM of each dNTP, and 0.5 U of Platinum^R Taq DNA polymerase (Invitrogen). The amplification was carried out by using the PTC-200 gradient cycler (MJ Research). The PCR conditions were as following: an initial denaturing step of 3 min at 94°C, 33 cycles of 94°C for 1 min, 50–62°C annealing temperature for 30 s, and 72°C for 1 min, followed by 8 min at 72°C for final extension step. PCR fragments were separated on CEQ™ 8000 Genetic Analysis System (Beckmann and Coulter). To confirm the presence of microsatellites, two to four different homozygous alleles per microsatellite were PCR amplified and purified using Qiaquick PCR purification kit (Qiagen). Sequencing was carried out using ABI 3770 (Applied Biosystems) following the ABI BigDye terminator cycle sequencing kit (Applied Biosystems). The microsatellites that contained more than two alleles in the initial screening panel were selected for further analysis.

Population genetic parameters (i.e. number of alleles per locus (N_a), effective number of alleles (N_e), observed heteroygosity (H_o), expected heterozygosity (H_e) and fixation index (F_{is})) were estimated using GENALEX6 (Peakall and Smouse 2006). Tests for Hardy-Weinberg expectations (HWE; exact probabilities) at each microsatellite and linkage disequilibrium between loci (LD; exact probabilities) were performed using genepop (ver. 3.4: Raymond and Rousset 1995). Presence of null alleles was tested using micro-checker (ver. 2.2.3: Van Oosterhout et al. 2004) while neutrality test was carried out using arlequin (ver. 3.1: Excoffier et al. 2005).

RESULTS AND DISCUSSION

Results for the initial screening panel are presented in Table 1. Twenty-four (90%) microsatellites resulted into amplicons of expected sizes. Two markers amplified multiple bands, for one no amplification was detected. Eleven microsatellites were biallelic and

Table 2. *Population genetic parameters of five selected microsatellites in an isolated natural population of Shorea robusta: number of samples (N), number of alleles (N_a), effective number of alleles (N_e), observed heterozygosity (H_o), expected heterozygosity (H_e), fixation index (F_{is}).*

Microsatellite	N	N_a	N_e	H_o	H_e	F_{is}
Sle-267	59	9	2.07	0.49	0.52	0.05
Sle-303	60	6	2.26	0.58	0.56	−0.04
Sle-566$^{\varphi}$	59	15	7.89	0.77	0.87	0.12
Sle-562	56	10	3.60	0.65	0.72	0.10*
Shc-04	60	19	7.97	0.67	0.88	0.23*
Mean		11.80	4.76	0.63	0.71	0.09

$^{\varphi}$ new primers were designed for the microsatellite.
* significant departure from Hardy-Weinberg expectation ($P < 0.05$).

five were moderate to highly variable resulting in six to 13 alleles. This 90% success rate is within the range but at the upper end of cross-species transferability of microsatellites in other tropical tree species, e.g. 71–100% in *Prosopis* (Mottura et al. 2005); 29–100% in *Macaranga* (Guicking et al. 2006) and 90% in *Milicia* (Ouinsavi et al. 2006). Population genetic parameters of the five most polymorphic microstatellites are shown in Table 2. Scoring the amplicons of Sle 566 was not reliable. Therefore, new primers (F(dye3): TTAATGCTTGCCCCCTGTAG, R: GCAGAGAT TGAAACAGAAG) were designed using the genebank sequence (accession no: AJ616890) of the microsatellite. The total number of alleles per microsatellite ranged from six to 19 with an average value of 11.8, and the average effective number of alleles was 4.76. The observed and expected heterozygosities ranged from 0.49 to 0.77 and 0.52 to 0.89, respectively. All microsatellites were neutral ($P > 0.05$). Two of them (Sle 566 and Sch 04) significantly ($P < 0.05$) deviated from HWE. Such deviation from HWE may be due to the presence of null alleles (*P*-values for excess of homozygotes in most allele size classes = < 0.01). Significant linkage disequilibrium was found between Sle 566 and Shc 04 ($P < 0.05$). The observed homology of the allele sequences in each microsatellite confirmed the interpretation of alleles as product of single locus. Although the five microsatellites markers may not be sufficient for assessing genetic diversity across broad spatial scales, they could be useful molecular tools for population genetic studies at population level of sal.

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