Novel nuclear microsatellites in the endangered neotropical timber tree *Lecythis ampla* (Lecythidaceae)

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Abstract

For the first time, nuclear microsatellite (nSSRs) primers were developed in the endangered tree species *Lecythis ampla* (Lecythidaceae) as molecular tools. An enrichment protocol with genomic DNA libraries for nSSRs was used to identify candidate loci. A large number of candidate loci were identified. Consecutively population genetic parameters of these loci were tested in two available populations. Eventually 17 microsatellite loci have been identified that show no or only low evidence for linkage disequilibrium, deviations from Hardy-Weinberg expectations or high levels of null alleles. These markers are apt for future molecular population studies.

Key words: endangered species; genetic diversity; *Lecythis ampla*; Lecythidaceae; microsatellites.

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Tropical forests harbour a rich variety of tree species. Many of these species are rare and genetic knowledge is very limited. On the other side, current selective logging and increased fragmentation of tropical forest are certainly affecting the population structure and reproductive system of these tree species (e.g. Degen et al., 2006). Unfortunately, there is a general lack of information on the vast majority of these species. *Lecythis ampla* Miers is among these lesser studied species. It belongs to the Lecythidaceae, subfamily Lecythidoideae (Mori et al., 2007) which is restricted to the New World tropics. Molecular studies in this subfamily are very rare (but see Kanashiro et al., 1997; Lepsch-Cunha et al., 1999; Colpaert et al., 2005).

Lecythis ampla occurs throughout Central America along the Atlantic watershed, from southern Nicaragua down to Darien, Panama. The geographical range in South America encompasses the Magdalena River Valley and the Choco zone in Colombia, as well as the north coast of Ecuador. The species is a canopy to emergent tree found in primary lowland wet or moist forests from near sea level to 800 m altitude. Trees sometimes persist in pastures. Annual flowering occurs during the rainy season, May through July. The flower is hermaphroditic and pollinated mainly by bee species belonging to the genera *Xylocopa* and *Bombus* (Flores, 1994). The fruit is large but shows strong variation in size and form (20 to 30 cm long by 15 to 20 cm wide; Flores, 1994). It is a dry capsule, woody and ovoid or oblong. The fruit hangs upside down, so when the operculum ("fruit lid")

Table 1. – Primer sequences and repeat motifs for the 17 selected microsatellite loci developed for *L. ampla*.

Locus	Forward-Primer	Reverse-Primer	Ta (°C)	Repeat motif	GenBank accession no.
LAM-E1	GAAGTTACTGGCATCGGATTCTC	CATTCACAAAATGTGCGTGCGTG	58	$(CT)_n(CA)_n$	JX137045
LAM-H1	CCGACCACGAAAATGACATGTGG	GAGCTGACGGAAACTTGTGCA	58	$(GA)_n(GA)_n$	JX137046
LAM-E3	GGGCATCTAGCATTTTGGACTGG	TAGCTCCTATTGGCACCATTGAC	58	$(GT)_n(GA)_n$	JX137047
LAM-G4	GTGTAATGAACAGAAGGATAATG	CAACACCTTCCGGAGCATCAG	58	$(CA)_n(CT)_n$	JX137048
LAM-E5	GATACCCAGATCAGATCAAC	TCATAATCGCATCGAAGACCAC	58	$(GA)_n$	JX137049
LAM-C7	TCTGATAGGTGTTTGCAGGAAGC	TGCAGTAACCAACAACTAG	58	$(GT)_n(GA)_n$	JX137050
LAM-F7	GGAACACTCTAATCACCGGCAGC	CAAAGAAATGACATACGTGCA	58	$(CT)_n(CA)_n$	JX137051
LAM-F8	CAGACTGAATTACTGGTTGAGC	AGCTCCAGAGAGAAGTACCT	58	(GA)	JX137052
LAM-F3	GAGGCAGGAATTGGAGGCGTG	AATCCCCAACGTCAACACACC	58	(GA)	JX137053
LAM-C4	TGCTCCTGTCTAAATTGGCGGAA	TTAGGTGCAACCAGCATTTCC	58	$(CT)_n(GT)_n(GA)_n$	JX137054
LAM-G6	AACAGAGCTCCTGAGTGATGGA	TCACCAACATGCCCTCGAGGCTA	58	$(GT)_n$	JX137055
LAM-D1	TTTCCCACATTCTGCTTCCAC	GTTCAACTGTCCGGAATCCAC	58	(GA)	JX137056
LAM-E8	AGCATCTGCAGCAGCTTGGAGG	AATTTGCAGGTGAACGGATGTG	58	(GA)	JX137057
LAM-F1	CGTGCAACTATTAGTCCAGGTA	AGCCAGACCTCTGCATCGGCAA	58	(GA)	JX137058
LAM-D3	AGCCTCTGTAAATTTGGGGAGTC	TGATCCTAGTACAACAACGGCA	58	$(GT)_n$	JX137059
LAM-G8	GATTCCACAGGTCCATCTGAC	GCATGATGCTTCTGCCATGTA	58	$(CT)_n(CA)_n$	JX137060
LAM-D6	GAAGCTCAGAGTATATGCCGTCC	TTGCTTATCCCGACTGAGAACG	58	(GA)	JX137061

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abscises, the seeds are exposed. Fruits are dispersed by gravity, monkeys and bats. The timber is valuable and excellent for ship construction, tools and furniture. However, the species has not been introduced in reforestation programs. FLORES (1994) considers this species to be endangered in Costa Rica because it is under heavy logging pressure in the northern and Atlantic zones of the country though this species is not listed on the IUCN Red List (IUCN, 2010). As no nuclear DNA markers are at hand for this tree species, informative and reliable nuclear microsatellite as population genetic tools for *L. ampla* should be developed in this study.

DNA was extracted from leaf samples using a modified CTAB protocol following the procedure of DOYLE and DOYLE (1987), substituting chlorophorm for dichlormethane. DNA concentration was checked on a NanoDrop device. The development of specific nuclear microsatellite markers was performed via an enrichment protocol of nuclear DNA libraries. The procedure was as follows: DNA was extracted from 5 individual trees from separate populations. A one-step reaction for *Mse*I digestion and adaptor ligation followed by recovery PCR with adaptor primers was performed, according to the FIASCO protocol (fast isolation by AFLP of

Table 2. – Population genetic parameters for the 17 microsatellite loci developed for *L. ampla* in the two test populations.

Locus	Na	Ne	Ho	He	F	HWE deviation	Null allele freq.
Population El Roble							
LAM-E1	6	3.892	0.833	0.743	-0.121		0.000
LAM-H1	2	1.917	0.625	0.478	-0.307		0.540
LAM-E3	5	3.827	0.750	0.739	-0.015		0.010
LAM-G4	3	1.843	0.500	0.457	-0.093		0.000
LAM-E5	3	1.475	0.292	0.322	0.094		0.158
LAM-C7	7	4.283	0.792	0.766	-0.033		0.000
LAM-F7	7	4.380	0.583	0.772	0.244	***	0.128
LAM-F8	3	1.347	0.292	0.258	-0.131		0.000
LAM-F3	8	4.987	0.917	0.799	-0.147		0.000
LAM-C4	5	3.048	0.833	0.672	-0.240		0.000
LAM-G6	2	1.917	0.375	0.478	0.216		0.456
LAM-D1	4	1.407	0.333	0.289	-0.153		0.000
LAM-E8	4	1.538	0.375	0.350	-0.072		0.000
LAM-F1	4	2.356	0.458	0.576	0.204		0.118
LAM-D3	3	1.184	0.167	0.155	-0.073		0.000
LAM-G8	4	1.240	0.208	0.194	-0.076		0.000
LAM-D6	2	1.332	0.292	0.249	-0.171		0.000
Population La Selva							
LAM-E1	6	4.302	0.714	0.768	0.069		0.000
LAM-H1	2	1.936	0.545	0.483	-0.128		0.564
LAM-E3	5	3.195	0.818	0.687	-0.191		0.000
LAM-G4	3	1.689	0.455	0.408	-0.114		0.000
LAM-E5	4	1.803	0.500	0.445	-0.123		0.000
LAM-C7	10	6.769	0.864	0.852	-0.013		0.014
LAM-F7	9	4.914	0.818	0.796	-0.027		0.000
LAM-F8	2	1.308	0.273	0.236	-0.158		0.000
LAM-F3	8	4.545	0.773	0.780	0.009		0.023
LAM-C4	5	1.603	0.364	0.376	0.033		0.023
LAM-G6	2	1.766	0.364	0.434	0.162		0.369
LAM-D1	3	1.849	0.571	0.459	-0.244		0.000
LAM-E8	5	2.279	0.714	0.561	-0.273		0.000
LAM-F1	3	2.485	0.571	0.598	0.044		0.111
LAM-D3	3	1.385	0.227	0.278	0.182		0.085
LAM-G8	4	1.872	0.545	0.466	-0.171		0.034
LAM-D6	2	1.252	0.227	0.201	-0.128		0.000

Na = number of alleles detected; Ne = number of effective alleles; Ho = observed heterozygosity; He = expected heterozygosity; F = inbreeding index; *** = departure from Hardy-Weinberg expectations significant at the $p < 0.001$ level; null allele frequency estimated with MICRO-CHECKER.

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sequences containing repeats) of ZANE et al. (2002) with the modifications of ARTHOFER et al. (2005). The PCR products were enriched for microsatellite sequences by hybridization to biotinylated $(AC)_8$ and $(GA)_8$ oligonucleotides and subsequent capture with Streptavidine MagneSphere paramagnetic particles (PMPs, Promega). PMPs were washed at high stringency (four times 0.1 × SSC). Enriched DNA was thermally eluted to sterile water. Another recovery PCR was performed as described above. PCR products were purified with the QIAquick PCR purification kit (QIAGEN), cloned into the pGEM-T vector (Promega) and used for transformation of JM109 competent *Escherichia coli* cells. One hundred white colonies were inoculated onto masterplates and transferred to Nylon membranes (Roche 1 699 075) as suggested by the manufacturer. After overnight hybridization at 60 °C with (digoxigenin) DIG-labelled simple sequence repeat oligoprobes and subsequent washing steps, screening was performed with the DIG Luminescent Detection Kit (Roche). Nintey-six positive colonies were transferred to liquid culture and plasmid DNA was extracted by alkaline lysis (SAMBROOK and RUSSELL, 2001). Plasmid DNA yielding a product in a PCR pre-test with $(AC)_8$ and $(GA)_8$ oligonucleotides as microsatellite specific primers combined with one of the vector primers was completely sequenced for determination of repeat regions. Twenty-seven out of 64 sequences containing microsatellites were selected for subsequent primer design. Primers were developed using the software FASTPCR 3.7.1 (KALENDAR, 2006). Amplifications for population screening were performed in 15 µL reaction volume containing 1 × reaction buffer, 0.2 mM dNTPs, 0.2 µm of each primer, 0.6 units of Taq polymerase (peqlab), 60 ng DNA and 11.58 µL ddH_2O. Cycling conditions were 94 °C for 30 s, 58 °C for 1 min, 72 °C for 45 s for 35 cycles with an initial denaturation step at 94 °C for 5 min and a final extension step at 72 °C for 10 min. Amplifications were successful for 23 loci. For these loci a modified forward primer was developed, where an M13 motif (CACGACGTTGTAAAACGAC; OETTING et al. 1995) was added to the primer sequence complementary to a third dye-labelled primer (WellRed oligos, Sigma) used in the PCR, which facilitated the visualization of PCR products on a CEQ8000 sequencer (Beckman-Coulter). Resulting fragments were run on the CEQ 8000 using a 400 internal size standard. Alleles were scored by hand.

The DNA of a total of 46 individual trees of *L. ampla* from 2 populations (El Roble: 10.52 N, 84.11 W; N = 24; La Selva: 10.41 N, 84.05 W; N = 22) was extracted and genotyped for the 23 loci. Observed and expected heterozygosities, and deviations from Hardy-Weinberg expectations (HWE) and from linkage equilibrium were calculated using GENALEX v3.4 (PEAKALL and SMOUSE, 2006) and GENEPOP version 3.4 (RAYMOND and ROUSSET, 1995). Exclusion probabilities were calculated in CERVUS (MARSHALL et al., 1998). Seven of the 23 loci tested were discarded from further analysis due to low allele number or deviations from HWE. All other loci were polymorphic in both populations and the observed number of alleles ranged from two to ten (*Table 1* and *2*). Expected heterozygosity ranged from 0.155 to 0.852. One locus (F7) showed significant deviation from HWE in population 1; however, did conform to HWE in the other population and thus was retained for further use in the species. Following Bonferroni correction, no linkage disequilibrium was observed for any pair of loci. Null allele occurrence was generally low (*Tab. 2*), but was high in the loci G6 and H1 in both populations. The combined exclusion probability calculated in CERVUS for the first parent was 0.978, for the second parent 0.999, and 1.000 for a parent pair.

The markers developed can certainly be applied for future population genetic studies including gene flow and mating system parameters in *L. ampla*.

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