Isolation of Nuclear Microsatellites in the African Timber Tree Lophira alata (Ochnaceae) and Cross-Amplification in L. lanceolata

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PRIMER NOTE

ISOLATION OF NUCLEAR MICROSATELLITES IN THE AFRICAN TIMBER TREE *LOPHIRA ALATA* (OCHNACEAE) AND CROSS-AMPLIFICATION IN *L. LANCEOLATA*[1]

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- *Premise of the study:* Microsatellite markers were isolated in the rainforest tree *Lophira alata* (Ochnaceae), an important timber tree from Central Africa, and cross-amplified on its savannah counterpart, *L. lanceolata*.
- *Methods and Results:* From a microsatellite-enriched library sequenced on a 454 GS FLX platform, 13 primer combinations were identified. Amplification was optimized in two multiplex reactions. The primers amplified di- and trinucelotide repeats, with two to seven alleles per locus. Eleven primers also amplified in *L. lanceolata*.
- *Conclusions:* Microsatellite primers developed for the genus *Lophira* displayed sufficient variation to investigate hybridization between congeneric species in the rainforest–savannah transition.

Key words: *Lophira*; next-generation sequencing; nSSR; Ochnaceae; tropical rainforests; universal primers.

Lophira Banks ex C. F. Gaertn. (Ochnaceae) is a genus of tropical trees endemic to Central and West Africa. Taxonomic treatments recognize two closely related species with similar morphology but well-differentiated habitats: a rainforest species, *L. alata* Banks ex C. F. Gaertn., and a savannah species, *L. lanceolata* Tiegh. ex Keay (Hutchinson and Dalziel, 1954; Aubreville, 1959; Bamps, 1970). The rainforest congener, *L. alata*, is a timber tree (azobé) of high economic value in Central Africa (Biwolé et al., 2012; Engone Obiang et al., 2012). Its timber is used for outdoor constructions such as harbors, hydraulic infrastructure, railways, and bridges (Palla et al., 2002). Despite its abundance in Cameroon, it is classified as a vulnerable species in the red list of the International Union for Conservation of Nature (IUCN, 2014) due to the massive logging pressure that currently affects its natural populations. In the contact zone between rainforest and savannah vegetation, the two species can be found in sympatry, and it has been hypothesized that hybridization may be occurring between them. In this study, we provide and characterize the first set of microsatellites for *L. alata* and test cross-amplification in two populations of *L. lanceolata*.

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METHODS AND RESULTS

A microsatellite-enriched library was generated by Genoscreen genomic platform (Lille, France) for one sample of *L. alata* from central Gabon (OH2473; Appendix 1) pooled with DNA of three other species, following the methods of Malausa et al. (2011) and Micheneau et al. (2011). One milligram of genomic DNA was digested with *Rsa*I, ligated to adapters, and amplified. PCR products were enriched with streptavidin-coated magnetic beads and 3′-biotinylated $(AG)_{10}$, $(AC)_{10}$, $(AAC)_8$, $(ACG)_8$, $(AGC)_8$, $(AAG)_8$, $(ACAT)_6$, $(ATCT)_6$ and subsequently amplified.

A total of 14,207 reads were generated on a 454 GS FLX sequencer (454 Life Sciences, a Roche Company, Branford, Connecticut, USA). Using QDD (Méglecz et al., 2010), the Genoscreen genomic platform identified 1505 loci containing microsatellite motifs. Subsequently, primer sets were designed on 376 of the loci (multiple primer pairs per locus up to a total of 2615) giving 80–500-bp PCR products, with more than five repeats and with 50–64°C annealing temperature. Sixty-eight primer combinations, corresponding to loci with the longest di-, tri-, and tetranucleotide repeats and with a minimum distance of 10 bp from the microsatellite, were selected. The amplification of the 68 primer pairs was tested on seven geographically separated individuals of *L. alata.* Fluorescent labeling of primers was performed using three primers per locus: a reverse primer, a forward primer with a universal linker sequence (Q1, Q2, Q3, or Q4) at the 5′ end, and a third primer consisting of the same universal sequence Q1, Q2, Q3, or Q4, labeled with 6-FAM, NED, VIC, or PET, respectively (Schuelke, 2000; Micheneau et al., 2011). PCR amplifications were performed in 1.5 µL buffer (10×), 0.6 µL $MgCl_2$ (25 mM), 0.45 µL dNPTs (10 mM each), forward primer at 0.07 µM, dye-labeled and reverse primers at 0.2 µM, 0.08 µL *Taq* polymerase (TopTaq DNA Polymerase, 5 U/µL [QIAGEN, Valencia, California, USA]), 1.5 µL of template DNA (of ca. 10–50 ng/µL), and H_2O up to a final volume of 15 µL. Amplifications were performed as follows: 94°C (4 min); 25 cycles of 94°C (30 s), 55°C (45 s), 72°C (1 min); followed by 10 cycles each of 94°C (30 s), 53°C (45 s), 72°C (45 s); and a final extension

at 72°C for 10 min. PCR products (0.8 µL) were separated on an ABI 3730 sequencer (Applied Biosystems, Lennik, Belgium) with 12 µL of HiDi and 0.3 µL of GeneScan 500 LIZ Size Standard (Applied Biosystems). Thirteen primer combinations exhibiting robust amplification, no stutter peaks, and polymorphism at the intraspecific level were selected (Table 1).

Multiplex PCR containing six (LML11) and seven (LMK13) microsatellites were set up, taking care to avoid complementary primers and markers with identical dye and amplicon size in the same multiplex with the help of Multiplex Manager (Holleley and Geerts, 2009). Fluorescent labeling of primers was performed using three primers per locus: a reverse primer, a forward primer with one universal sequence (Q1, Q2, Q3 or Q4) at the 5′ end, and a third primer consisting of the same universal sequence Q1, Q2, Q3, or Q4, labeled with 6-FAM, NED, VIC, or PET, respectively (Schuelke, 2000; Micheneau et al., 2011). Type-it Microsatellite PCR Kit (QIAGEN) was used for amplification in a final volume of 14.5 µL containing: 1 µL DNA (approx. 5–20 ng), 7.5 µL Type-it Multiplex Mix, 0.1 µL of forward primers (final concentration of 0.07 µM), 0.15 µL of the reverse primers and labeled primers Q1, Q2, Q3, and Q4 (final concentration of 0.1 µM for each), and H_2O. The PCR profile was: 95°C (5 min); followed by 20 cycles of 95°C (30 s), 57°C (180 s) for LMK13 or 60°C (90 s) for LML11, 72°C (30 s); followed by eight cycles of 94°C (30 s), 53°C (45 s), 72°C (45 s); and a final extension at 60°C (30 min). PCR products were run on an ABI 3730 (Applied Biosystems) with 12 µL of HiDi and 0.3 µL of GeneScan 500 LIZ Size Standard (Applied Biosystems). The resulting electropherograms were automatically scored with GeneMapper 3.7 (Applied Biosystems) and manually corrected. Individual genotypes exhibited one or two alleles per microsatellite locus, as expected for diploid organisms.

To characterize the 13 microsatellite loci (Table 2), 33 individuals from a *L. alata* population in northwestern Cameroon (Pallisco) were genotyped. Allele sizes, number of alleles per locus, expected (H_e) and observed (H_o) heterozygosity, and inbreeding coefficient (F_{IS}) were estimated with SPAGeDi 1.4 (Hardy and Vekemans, 2002). Linkage disequilibrium (LD) between each pair of loci was tested with GENEPOP 4.1.4 (Raymond and Rousset, 1995). Deviation from Hardy–Weinberg equilibrium (HWE) was tested for each locus using a χ^2 test with GENEPOP, and permutation tests (999 permutations of alleles among individuals) were conducted with SPAGeDI. Transferability of the isolated primers to the savannah congener was tested in 26 individuals from two Cameroonian populations of *L. lanceolata* (Yong and Bango). The number of private alleles was calculated for each species.

In the *L. alata* population, all 13 microsatellite loci were polymorphic (Table 2), with the number of alleles per locus ranging from two to seven. H_e varied between 0.24 and 0.75, and F_{IS} between −0.14 and 0.85. Three loci (P36, P44, P47) significantly deviated from HWE ($P < 0.01$ in both χ^2 and permutation tests) due to homozygosity excess (Table 2). Significant LD ($P < 0.01$) was found between loci P18 and P34. LD at $P < 0.05$ was detected between four pairs of loci (P62 and P40, P62 and P51, P18 and P31, and P51 and P31). Eleven microsatellite markers exhibited robust amplification in the two populations of *L. lanceolata* studied (Table 2), where one to eight alleles per locus were retrieved. All loci were polymorphic between the two *L. lanceolata* populations. P24 was monomorphic in the Bango population, whereas P12 and P40 were monomorphic in the Yong population. All loci except for three (P53, P34, and P47) exhibited private alleles for *L. alata*, whereas all but one (P24) exhibited specific alleles for *L. lanceolata*.

CONCLUSIONS

The 13 nuclear microsatellite loci optimized showed sufficient levels of variation to estimate genetic diversity levels in *L. alata* and *L. lanceolata*. These markers will be an important tool to assess how the Pleistocene climatic oscillations have shaped the genetic structure of the two species in Central African rainforests. In addition, the finding of species-specific alleles may help detect ongoing interspecific gene flow in the rainforest–savannah contact zone, where the two species can be found in sympatry.

Table 1. Description of 13 newly developed microsatellite loci in *Lophira alata* in two multiplex reactions LML11 and LMK13.

Locus	Primer sequences (5′–3′)	Fluorescent label[a]	Repeat motif	T_a (°C)	GenBank accession no.
LML11					
P12	F: TTCCTCACCTTCTCCGTCAC	Q4-NED	$(CAC)_{10}$	60	KP666122
	R: TGAGAGTTTGGTTGGTGGTG				
P24	F: CACCGAAGCATCTTCCTCTC	Q4-NED	$(CT)_8$	60	KP666123
	R: TACCCAATTCAGCACGTCAA				
P31	F: CACATGCACACGCACTTGTA	Q3-VIC	$(TTC)_8$	60	KP666124
	R: TGAGATCCACGGTAAGGTGA				
P44	F: CTCCTTTCTCTTTCGTTTGTCCT	Q2-NED	$(AC)_{10}$	60	KP666125
	R: TGATTCGATCTTCGGGTTTC				
P53	F: CCCTTGGAAACCAAACTCAA	Q1-6-FAM	$(AC)_9$	60	KP666126
	R: GCAACATTATGAAGAAGGCAAA				
P66	F: ATTGGACAAGCCTCAACTCG	Q2-NED	$(AC)_{12}$	60	KP666127
	R: GGGGTCGATAATCAAGGAGG				
LMK13					
P18	F: TGCAGTGCAAATCATTCAA	Q2-NED	$(TTG)_7$	57	KP666128
	R: ATTCAATCACAACCAACGCA				
P34	F: CCCTCTGTTGTCACCTCCAT	Q2-NED	$(TTC)_9$	57	KP666129
	R: GGAGCAAGAGGAAGCAAATG				
P36	F: GACTTAAATAGAAACGTCAATAATAGCA	Q1-6-FAM	$(ACA)_{12}$	57	KP666130
	R: GGTTTGTTGAGTTCAATCATTGTT				
P40	F: AAACAGGTTTGTGTCCCCTTT	Q4-NED	$(GTT)_7$	57	KP666131
	R: CGAAGGGAGAATTGGAACC				
P47	F: GGGGTACCACCATTTGGAGT	Q1-6-FAM	$(AC)_7$	57	KP666132
	R: TTCCTATTTGTAGGACTTGGAGTT				
P51	F: CATGTGCAGACATGCCATACT	Q3-VIC	$(TG)_7$	57	KP666133
	R: ATTTGTAGGGCCTCAACCAG				
P62	F: CCTCTTGCTCCTCCAAATCA	Q2-NED	$(TC)_8$	57	KP666134
	R: TTGAAACATGACTTAAATGGATGTG				

Note: T_a = annealing temperature.

[a]Fluorescent label on forward primer. Q1 = TGTAAAACGACGGCCAGT (Schuelke, 2000), Q2 = TAGGAGTGCAGCAAGCAT, Q3 = CACTGCTTAGAGCGATGC, Q4 = CTAGTTATTGCTCAGCGGT (Q2–Q4, after Culley et al., 2008).

TABLE 2. Genetic properties of the 13 newly developed, polymorphic microsatellite loci in *Lophira alata* and cross-amplification results in two populations of *L. lanceolata*.[a,b]

	Lophira lanceolata												
	Lophira alata (Pallisco, $N = 33$)							Yong ($N = 10$)		Bango ($N = 16$)		
	Allele size range (bp)	A	H_e	H_o	F_{IS}	χ^2	Perm P value	Private	Allele size range (bp)	A	Allele size range (bp)	A	Private
LML11													
P12	105–119	3	0.31	0.30	0.03	1.000	0.812	2	113	1	105–122	3	2
P24	147–151	3	0.28	0.24	0.13	0.036	0.464	1	147–151	2	151	1	
P31	153–176	6	0.69	0.73	−0.05	0.296	0.672	4	151–158	2	151–158	3	1
P44	170–178	3	0.26	0.09	0.64	**0.006**	**0.005**	3	—	—	—	—	—
P53	202–204	2	0.33	0.34	−0.05	1.000	0.857	—	196–210	5	202–212	5	4
P66	271–283	6	0.28	0.28	0.01	0.207	0.811	4	269–272	3	267–272	4	2
LMK13													
P18	130–137	5	0.70	0.51	0.27	0.097	0.018	1	123–137	4	123–140	5	2
P34	162–165	2	0.39	0.27	0.30	0.159	0.110	—	160–165	3	160–165	3	1
P36	148–170	7	0.75	0.16	0.78	**0.000**	**0.000**	7	—	—	—	—	—
P40	171–181	3	0.24	0.27	−0.13	1.000	0.555	1	171	1	171–179	3	1
P47	180–184	3	0.24	0.04	0.85	**0.000**	**0.000**	—	180–203	7	180–213	8	7
P51	192–196	3	0.25	0.21	0.14	0.468	0.384	2	191–194	3	189–193	3	3
P62	255–260	4	0.66	0.75	−0.14	0.391	0.269	3	253–255	2	253–256	3	2

Note: A = number of alleles; F_{IS} = inbreeding coefficient; H_e = expected heterozygosity; H_o = observed heterozygosity; N = number of individuals.

[a] Tests for deviation from HWE: χ^2 = P value for χ^2 test; perm P value = P value for permutation of alleles among individuals (values $P < 0.01$ are set boldface); private = number of species-specific alleles.

[b] See Appendix 1 for population locality information.

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Appendix 1. Voucher information for *Lophira* populations characterized in this study.

Voucher no.	Species	Collection locality[a]	Collection date[b]	Collector	Latitude (°N)	Longitude (°E)
AS0055–59, AS0065–69	*L. lanceolata*	Yong	25/02/13	Biwole A.	6.21	11.57
AS0099	*L. lanceolata*	Bango	26/02/13	Biwole A.	6.76	11.84
AS0100, AS102–105	*L. lanceolata*	Bango	26/02/13	Biwole A.	6.73	11.87
AS0101	*L. lanceolata*	Bango	26/02/13	Biwole A.	6.73	11.88
AS0120	*L. lanceolata*	Bango	26/02/13	Biwole A.	6.72	11.97
AS0121	*L. lanceolata*	Bango	26/02/13	Biwole A.	6.72	11.98
AS0124	*L. lanceolata*	Bango	26/02/13	Biwole A.	6.71	11.99
AS0128–129	*L. lanceolata*	Bango	26/02/13	Biwole A.	6.67	12.04
AS0130	*L. lanceolata*	Bango	26/02/13	Biwole A.	6.67	12.05
AS0135–137	*L. lanceolata*	Bango	26/02/13	Biwole A.	6.66	12.06
AB0007	*L. alata*	Pallisco	08/08/12	Biwole A.	5.529	9.012
AB0008	*L. alata*	Pallisco	08/08/12	Biwole A.	5.497	9.060
AB0023	*L. alata*	Pallisco	08/08/12	Biwole A.	5.526	9.011
AB0024	*L. alata*	Pallisco	08/08/12	Biwole A.	5.493	9.069
AB0025	*L. alata*	Pallisco	08/08/12	Biwole A.	5.496	9.059
AB0026	*L. alata*	Pallisco	08/08/12	Biwole A.	5.492	9.068
AB0027	*L. alata*	Pallisco	08/08/12	Biwole A.	5.494	9.068
AB0028	*L. alata*	Pallisco	08/08/12	Biwole A.	5.495	9.065
AB0033	*L. alata*	Pallisco	08/08/12	Biwole A.	5.495	8.952
AB0034	*L. alata*	Pallisco	08/08/12	Biwole A.	5.495	8.953
AB0035	*L. alata*	Pallisco	08/08/12	Biwole A.	5.494	8.948
AB0036	*L. alata*	Pallisco	08/08/12	Biwole A.	5.495	8.953
AB0037	*L. alata*	Pallisco	08/08/12	Biwole A.	5.493	8.948
AB0038	*L. alata*	Pallisco	08/08/12	Biwole A.	5.487	8.947
AB0039	*L. alata*	Pallisco	08/08/12	Biwole A.	5.490	8.948
AB0040	*L. alata*	Pallisco	08/08/12	Biwole A.	5.492	8.950
AB0041	*L. alata*	Pallisco	08/08/12	Biwole A.	5.494	8.949
AB0042	*L. alata*	Pallisco	08/08/12	Biwole A.	5.490	8.950
AB0043	*L. alata*	Pallisco	08/08/12	Biwole A.	5.488	8.943
AB0044	*L. alata*	Pallisco	08/08/12	Biwole A.	5.495	8.952
AB0045	*L. alata*	Pallisco	08/08/12	Biwole A.	5.487	8.946
AB0046	*L. alata*	Pallisco	08/08/12	Biwole A.	5.495	8.951
AB0047	*L. alata*	Pallisco	08/08/12	Biwole A.	5.489	8.949
AB0048	*L. alata*	Pallisco	08/08/12	Biwole A.	5.493	8.949
AB0049	*L. alata*	Pallisco	08/08/12	Biwole A.	5.488	8.949
AB0051	*L. alata*	Pallisco	08/08/12	Biwole A.	5.488	8.947
NB0395	*L. alata*	Pallisco	08/08/12	Biwole A.	5.492	8.950
NB0401	*L. alata*	Pallisco	08/08/12	Biwole A.	5.554	9.053
NB0405	*L. alata*	Pallisco	08/08/12	Biwole A.	5.493	8.949
NB0408	*L. alata*	Pallisco	08/08/12	Biwole A.	5.491	8.952
NB0410	*L. alata*	Pallisco	08/08/12	Biwole A.	5.491	8.952
NB0413	*L. alata*	Pallisco	08/08/12	Biwole A.	5.489	8.949
OH2473[c]	*L. alata*	Savanne-Okondja	15/10/09	Hardy O.J.	–0.654	13.675

[a] All collections are located in Cameroon except for OH2473 from central Gabon.

[b] Collection date (day/month/year).

[c] Seedling grown by Precious Woods Gabon, Compagnie Equatoriale des Bois S.A. The geographical coordinates correspond to the mother plant and are approximate.