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

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# Isolation of nuclear microsatellites in the African timber tree Lophira alata (Ochnaceae) and cross-amplification in $\operatorname{L}$. 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

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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.

## 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 RsaI, ligated to adapters, and amplified. PCR products were enriched with streptavidin-coated magnetic beads and $3^{\prime}$-biotinylated $(\mathrm{AG})_{10},(\mathrm{AC})_{10},(\mathrm{AAC})_{8},(\mathrm{ACG})_{8},(\mathrm{AGC})_{8},(\mathrm{AAG})_{8},(\mathrm{ACAT})_{6}$, $(\mathrm{ATCT})_{6}$ 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 $50-64^{\circ} \mathrm{C}$ 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 ( $\mathrm{Q} 1, \mathrm{Q} 2, \mathrm{Q} 3$, or Q 4$)$ at the $5^{\prime}$ end, and a third primer consisting of the same universal sequence $\mathrm{Q} 1, \mathrm{Q} 2, \mathrm{Q} 3$, or Q 4 , labeled with 6-FAM, NED, VIC, or PET, respectively (Schuelke, 2000; Micheneau et al., 2011). PCR amplifications were performed in $1.5 \mu \mathrm{~L}$ buffer ( $10 \times$ ), $0.6 \mu \mathrm{~L} \mathrm{MgCl}_{2}(25 \mathrm{mM}), 0.45 \mu \mathrm{~L}$ dNPTs ( 10 mM each), forward primer at $0.07 \mu \mathrm{M}$, dye-labeled and reverse primers at $0.2 \mu \mathrm{M}, 0.08 \mu \mathrm{~L}$ Taq polymerase (TopTaq DNA Polymerase, $5 \mathrm{U} / \mu \mathrm{L}$ [QIAGEN, Valencia, California, USA]), $1.5 \mu \mathrm{~L}$ of template DNA (of ca. $10-50 \mathrm{ng} / \mu \mathrm{L}$ ), and $\mathrm{H}_{2} \mathrm{O}$ up to a final volume of $15 \mu \mathrm{~L}$. Amplifications were performed as follows: $94^{\circ} \mathrm{C}$ $(4 \mathrm{~min}) ; 25$ cycles of $94^{\circ} \mathrm{C}(30 \mathrm{~s}), 55^{\circ} \mathrm{C}(45 \mathrm{~s}), 72^{\circ} \mathrm{C}(1 \mathrm{~min})$; followed by 10 cycles each of $94^{\circ} \mathrm{C}(30 \mathrm{~s}), 53^{\circ} \mathrm{C}(45 \mathrm{~s}), 72^{\circ} \mathrm{C}(45 \mathrm{~s})$; and a final extension
at $72^{\circ} \mathrm{C}$ for 10 min . PCR products $(0.8 \mu \mathrm{~L})$ were separated on an ABI 3730 sequencer (Applied Biosystems, Lennik, Belgium) with $12 \mu \mathrm{~L}$ of HiDi and $0.3 \mu \mathrm{~L}$ 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 ( $\mathrm{Q} 1, \mathrm{Q} 2, \mathrm{Q} 3$ or Q 4$)$ at the $5^{\prime}$ end, and a third primer consisting of the same universal sequence $\mathrm{Q} 1, \mathrm{Q} 2, \mathrm{Q} 3$, 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 $14.5 \mu \mathrm{~L}$ containing: $1 \mu \mathrm{~L}$ DNA (approx. $5-20 \mathrm{ng}$ ), $7.5 \mu \mathrm{~L}$ Type-it Multiplex Mix, $0.1 \mu \mathrm{~L}$ of forward primers (final concentration of $0.07 \mu \mathrm{M}), 0.15 \mu \mathrm{~L}$ of the reverse primers and labeled primers Q1, Q2, Q3, and Q4 (final concentration of $0.1 \mu \mathrm{M}$ for each), and $\mathrm{H}_{2} \mathrm{O}$. The PCR profile was: $95^{\circ} \mathrm{C}(5 \mathrm{~min})$; followed by 20 cycles of $95^{\circ} \mathrm{C}(30 \mathrm{~s})$, $57^{\circ} \mathrm{C}(180 \mathrm{~s})$ for LMK13 or $60^{\circ} \mathrm{C}(90 \mathrm{~s})$ for LML11, $72^{\circ} \mathrm{C}(30 \mathrm{~s})$; followed by eight cycles of $94^{\circ} \mathrm{C}(30 \mathrm{~s}), 53^{\circ} \mathrm{C}(45 \mathrm{~s}), 72^{\circ} \mathrm{C}(45 \mathrm{~s})$; and a final extension at $60^{\circ} \mathrm{C}$ ( 30 min ). PCR products were run on an ABI 3730 (Applied Biosystems) with $12 \mu \mathrm{~L}$ of HiDi and $0.3 \mu \mathrm{~L}$ 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 $\left(H_{\mathrm{e}}\right)$ and observed ( $H_{\mathrm{o}}$ ) heterozygosity, and inbreeding coefficient ( $F_{\text {IS }}$ ) 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 $\chi^{2}$ 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. $H_{\mathrm{e}}$ varied between 0.24 and 0.75 , and $F_{\text {IS }}$ between -0.14 and 0.85 . Three loci (P36, P44, P47) significantly deviated from $\operatorname{HWE}\left(P<0.01\right.$ in both $\chi^{2}$ 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 ( $5^{\prime}-3^{\prime}$ ) | Fluorescent label ${ }^{\text {a }}$ | Repeat motif | $T_{\mathrm{a}}\left({ }^{\circ} \mathrm{C}\right)$ | GenBank accession no. |
| :---: | :---: | :---: | :---: | :---: | :---: |
| LML11 |  |  |  |  |  |
| P12 | F: TTCCTCACCTTCTCCGTCAC | Q4-NED | $(\mathrm{CAC})_{10}$ | 60 | KP666122 |
|  | R: TGAGAGTTTGGTTGGTGGTG |  |  |  |  |
| P24 | F: CACCGAAGCATCTTCCTCTC | Q4-NED | $(\mathrm{CT})_{8}$ | 60 | KP666123 |
|  | R: TACCCAATTCAGCACGTCAA |  |  |  |  |
| P31 | F: CACATGCACACGCACTTGTA | Q3-VIC | $(\mathrm{TTC})_{8}$ | 60 | KP666124 |
|  | R: TGAGATCCACGGTAAGGTGA |  |  |  |  |
| P44 | F: CTCCTTTCTCTTTCGTTTGTCCT | Q2-NED | $(\mathrm{AC})_{10}$ | 60 | KP666125 |
|  | R: TGATTCGATCTTCGGGTTTC |  |  |  |  |
| P53 | F: CCCTTGGAAACCAAACTCAA | Q1-6-FAM | $(\mathrm{AC})_{9}$ | 60 | KP666126 |
|  | R: GCAACATTATGAAGAAGGCAAA |  |  |  |  |
| P66 | F: ATTGGACAAGCCTCAACTCG | Q2-NED | $(\mathrm{AC})_{12}$ | 60 | KP666127 |
|  | R: GGGGTCGATAATCAAGGAGG |  |  |  |  |
| LMK13 |  |  |  |  |  |
| P18 | F: TGCAGTGCAAATCATTCAA | Q2-NED | $(\mathrm{TTG})_{7}$ | 57 | KP666128 |
|  | R: ATTCAATCACAACCAACGCA |  |  |  |  |
| P34 | F: CCCTCTGTTGTCACCTCCAT | Q2-NED | (TTC) ${ }_{9}$ | 57 | KP666129 |
|  | R: GGAGCAAGAGGAAGCAAATG |  |  |  |  |
| P36 | F: GACTTAAATAGAAACGTCAATAATAGCA | Q1-6-FAM | $(\mathrm{ACA})_{12}$ | 57 | KP666130 |
|  | R: GGTTTGTTGAGTTCAATCATTGTT |  |  |  |  |
| P40 | F: AAACAGGTTTGTGTCCCCTTT | Q4-NED | $(\mathrm{GTT})_{7}$ | 57 | KP666131 |
|  | R: CGAAGGGAGAATTGGAACC |  |  |  |  |
| P47 | F: GGGGTACCACCATTTGGAGT | Q1-6-FAM | $(\mathrm{AC})_{7}$ | 57 | KP666132 |
|  | R: TTCCTATTTGTAGGACTTGGAGTT |  |  |  |  |
| P51 | F: CATGTGCAGACATGCCATACT | Q3-VIC | $(\mathrm{TG})_{7}$ | 57 | KP666133 |
|  | R: ATTTGTAGGGCCTCAACCAG |  |  |  |  |
| P62 | F: CCTCTTGCTCCTCCAAATCA | Q2-NED | $(\mathrm{TC})_{8}$ | 57 | KP666134 |
|  | R: TTGAAACATGACTTAAATGGATGTG |  |  |  |  |

Note: $T_{\mathrm{a}}=$ annealing temperature.
${ }^{\text {a }}$ 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. ${ }^{\text {a,b }}$

|  | Lophira alata (Pallisco, $N=33$ ) |  |  |  |  |  |  |  | Lophira lanceolata |  |  |  |  |
| :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: |
|  |  |  |  |  |  |  |  |  | Yong ( $N=10$ ) |  | Bango ( $N=16$ ) |  | Private |
|  | Allele size range (bp) | A | $H_{\text {e }}$ | $H_{\text {o }}$ | $F_{\text {IS }}$ | $\chi^{2}$ | Perm $P$ value | Private | Allele size range (bp) | A | Allele size range (bp) | A |  |
| 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 |

[^1]
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Appendix 1. Voucher information for Lophira populations characterized in this study.

| Voucher no. | Species | Collection locality ${ }^{\text {a }}$ | Collection date ${ }^{\text {b }}$ | Collector | Latitude ( ${ }^{\circ} \mathrm{N}$ ) | Longitude ( ${ }^{\circ} \mathrm{E}$ ) |
| :---: | :---: | :---: | :---: | :---: | :---: | :---: |
| 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 ${ }^{\text {c }}$ | L. alata | Savanne-Okondja | 15/10/09 | Hardy O.J. | -0.654 | 13.675 |


${ }^{\mathrm{b}}$ Collection date (day/month/year).
${ }^{\mathrm{c}}$ Seedling grown by Precious Woods Gabon, Compagnie Equatoriale des Bois S.A. The geographical coordinates correspond to the mother plant and are approximate.


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[^1]:     specific alleles.
    ${ }^{\mathrm{b}}$ See Appendix 1 for population locality information.

