Development and Characterization of Microsatellite Markers in the African Deciduous Tree Terminalia superba (Combretaceae)

Authors: Demenou, Boris B., Migliore, Jérémy, Tosso, Felicien, Kaymak, Esra, and Hardy, Olivier J.

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Primer Note

Development and characterization of microsatellite markers in the African deciduous tree *Terminalia superba* (Combretaceae)[1]

Boris B. Demenou[2,4], Jérémy Migliore[2], Felicien Tosso[3], Esra Kaymak[2], and Olivier J. Hardy[2]

[2]Evolutionary Biology and Ecology Unit, CP 160/12, Faculté des Sciences, Université Libre de Bruxelles, Av. F. D. Roosevelt 50, B-1050 Brussels, Belgium; and [3]Management of Forest Resources, Biosystem Engineering Department, Gembloux Agro-Bio Tech, Université de Liège, 2 Passage des Déportés, B-5030 Gembloux, Belgium

- *Premise of the study:* Microsatellites were designed and characterized in the African timber forest tree *Terminalia superba* (Combretaceae). Due to their high variability, these markers are suitable to investigate gene flow patterns and the structure of genetic diversity.
- *Methods and Results:* From a genomic library obtained by next-generation sequencing, seven monomorphic and 14 polymorphic microsatellite loci were developed. The polymorphic microsatellites displayed two to 27 alleles (mean 11.4; expected heterozygosity range 0.283–0.940, mean 0.736) in one population from southeastern Cameroon. Genotypes were typical of an outbreeding diploid species, although null alleles explain a significant heterozygote deficit in three loci. Cross-amplification in three congeneric species (*T. ivorensis*, *T. avicennioides*, and *T. mantaly*) failed, suggesting that *T. superba* is rather divergent.
- *Conclusions:* This set of newly developed microsatellite markers will be useful for assessing the genetic diversity, population structure, and demographic history of *T. superba* in tropical African forests.

Key words: Combretaceae; microsatellites; next-generation sequencing; rainforest history; *Terminalia superba*.

Terminalia superba Engl. & Diels (Combretaceae) is a Guineo-Congolese African tree naturally distributed from Sierra Leone to the Democratic Republic of the Congo. This pioneer tree is representative of tropical secondary semideciduous forests with an average annual rainfall exceeding 1500 mm and a dry season of less than four months (Orwa et al., 2009). Commonly called limba or fraké, *T. superba* has been planted for economic purposes in several other countries in Africa, the Americas, and Asia.

With its widespread distribution, *T. superba* is well suited for studying the response of the African rainforest to past climate changes, especially during the Pleistocene glacial oscillations, during which the rainforest underwent cycles of range contraction and expansion (Plana, 2004). Investigating the phylogeographic pattern, genetic diversity, and demographic history of natural populations requires the development of molecular markers with a high level of polymorphism, such as microsatellites (Frankham et al., 2004). However, to date, no microsatellite resources have been developed in *T. superba* to decipher its evolutionary history. In this paper, we describe the isolation and properties of 14 new polymorphic microsatellite markers, developed with a low-cost procedure and next-generation sequencing on *T. superba*. We also attempted cross-amplification in other *Terminalia* L. species, but without success.

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[4]Author for correspondence: bdemenou@ulb.ac.be

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METHODS AND RESULTS

***Microsatellite development*—**Using a cetyltrimethylammonium bromide (CTAB) method (Fu et al., 2005), total DNA was extracted from 30 mg of the leaf of one individual of *T. superba* (BoD597), from Ghana (Kakum National Park, collected in 2014) (Appendix 1). A DNA genomic library was then prepared, without enrichment, following the protocol of Mariac et al. (2014) and using the Illumina MiSeq platform (San Diego, California, USA) to generate 150-bp paired-end reads (Centre de coopération internationale en recherche agronomique pour le développement [CIRAD] facilities, Montpellier, France). A total of 472,566 reads were obtained, assembled by pair with PANDAseq (Masella et al., 2012), and then analyzed with the software QDD (Meglécz et al., 2014), which detected 7146 loci containing a microsatellite motif, of which 1397 loci had a microsatellite of at least 10 repeats and surrounded by flanking regions suitable to define pairs of PCR primers. From these, we selected 48 loci containing pure (i.e., not compound) microsatellites with at least 10 dinucleotide repeats, with primers situated at least 20 bp from the microsatellite region, and with PCR product length longer than 150 bp. We added to the 5′ end of the forward primer of each locus one of the four possible linkers (Q1–Q4) to label PCR products with distinct fluorochromes (FAM, NED, VIC, and PET; M13-like protocol [Micheneau et al., 2011]).

First amplification tests were performed for each locus on three samples of *T. superba*: BoD459 (Togo), BoD556 (Ghana), and BoD713 (Nigeria) (Appendix 1). PCR reactions (15 µL) were performed using 1.5 µL buffer (10×), 0.6 µL $MgCl_2$ (25 mM), 0.45 µL dNTPs (10 mM each), 0.3 µL of each primer (0.2 µM), 0.08 µL TopTaq DNA Polymerase (5 U/µL; QIAGEN, Venlo, The

Netherlands), 1.5 μL of template DNA (of ca. 10–50 ng/μL), and H_2O. PCR conditions were: 94°C (4 min); 30 cycles of 94°C (30 s), 55°C (45 s), and 72°C (1 min); and a final extension at 72°C (10 min). PCR products were visualized on a 1% agarose gel and stained with SYBR Safe (Invitrogen, Merelbeke, Belgium). Forty-four loci amplified consistently. Their polymorphism was then assessed on seven individuals of *T. superba* from the Democratic Republic of the Congo, Gabon, Cameroon, Nigeria, Benin, Togo, and Ghana (Appendix 1). Fluorescent labeling was performed by PCR amplification in a total volume of 15 μL, combining: 0.3 μL of the reverse (0.2 μM) and 0.1 μL of the forward (0.07 μM) microsatellite primers with a Q1–Q4 universal sequence at the 5′ end, 0.3 μL of Q1–Q4 labeled primer (0.2 μM each), 3 μL of Type-it Microsatellite PCR Kit (QIAGEN), H_2O, and 1.5 μL of DNA. PCR program conditions were: 5-min initial denaturation at 95°C, followed by 30 cycles of denaturation/annealing/extension (95°C for 30 s, 55°C for 90 s, 72°C for 1 min), and a final elongation step at 60°C for 30 min. For each PCR product, 1.5 μL were directly added to 12 μL of Hi-Di Formamide (Life Technologies, Carlsbad, California, USA) and 0.3 μL of MapMarker 500 labeled with DY-632 (Eurogentec, Seraing, Belgium) and genotyped on an ABI3730 sequencer (Applied Biosystems, Lennik, The Netherlands).

We selected 14 polymorphic and readable loci (seven loci were monomorphic, 17 loci failed to amplify, and six loci were polymorphic but showed uninterpretable amplification patterns). The 14 polymorphic loci were combined in three multiplexed reactions (Table 1) using Multiplex Manager 1.0 software (Holleley and Geerts, 2009). Preliminary population genetic analyses were performed on 42 individuals of *T. superba* from southeastern Cameroon (Appendix 1). Multiplexed PCRs were carried out using the Type-it Microsatellite PCR Kit (QIAGEN) as follows: 7.5 μL of Multiplex Master Mix, 0.1 μL (0.07 μM) of forward primer and 0.3 μL (0.2 μM) of reverse primer labeled with Q-tailed fluorescent Q1–Q4, 0.3 μL (0.2 μM) of Q1–Q4 primer labeled with 6-FAM, NED, VIC, and PET, 3 μL of 5× Q-solution, H_2O, and 1.5 μL of DNA extract. Multiplex PCR conditions were identical to those above, with 27 cycles and annealing temperature of 56°C.

Cross-amplification was also tested on three congeneric species (Appendix 1), including another African forest tree species (*T. ivorensis* A. Chev.,

Table 1. Characterization of 14 polymorphic and seven monomorphic nuclear microsatellite loci isolated from *Terminalia superba*.[a]

Locus	Primer sequences (5′–3′)	Fluorescent label	Repeat motif	Allele size range (bp)	GenBank accession no.
Multiplex 1					
TS-08	F: TGTAAAACGACGGCCAGTTTGACAAGTGTTGCAGGAGA	Q1-6-FAM	$(CT)_{36}$	179–248	KT222788
	R: GTTTGACTGGGATGGCTGAG				
TS-25	F: TAGGAGTGCAGCAAGCATCGTTTGAAGTGGGAGACCAT	Q2-NED	$(AC)_{15}$	281–301	KT222793
	R: TTCCCTCTTGATTCTTCCTAGA				
TS-30	F: CACTGCTTAGAGCGATGCAGATCACCCGAGATCACCC	Q3-VIC	$(GT)_{17}$	202–302	KT222795
	R: TTCTCTTCCAACTGGTCCTCTT				
TS-37	F: CTAGTTATTGCTCAGCGGTGTGGTGGGTGCTTGAATTG	Q4-PET	$(GA)_{15}$	177–250	KT222798
	R: TAGCAGCTAAGGAAGCTGGG				
Multiplex 2					
TS-5	F: TGTAAAACGACGGCCAGTGGGCATTCAGGGACTACCTT	Q1-6-FAM	$(GA)_{24}$	174–272	KT222787
	R: ACCACTCAGCCACCACTACC				
TS-12	F: TGTAAAACGACGGCCAGTTCACGGACGAGAAATCTCAA	Q1-6-FAM	$(AG)_{13}$	308–310	KT222789
	R: GCATCATTGGACAAGGAAGAA				
TS-17	F: TAGGAGTGCAGCAAGCATGGCCCATATCGAATGAGTTT	Q2-NED	$(TA)_{14}$	157–213	KT222790
	R: GTTCATCGAACATGGGCTG				
TS-28	F: CACTGCTTAGAGCGATGCTAACGAGCAGCAACCATGTC	Q3-VIC	$(AT)_{10}$	181–197	KT222794
	R: TGCACATCAAAGACCCATCT				
TS-42	F: CTAGTTATTGCTCAGCGGTTCCCATGATTAGCTGTCATCC	Q4-PET	$(TA)_{10}$	214–220	KT222799
	R: TGAGTCCAGTAGTTTAGGAGTGGT				
Multiplex 3					
TS-22	F: TAGGAGTGCAGCAAGCATCGAGCTTAGCACTTTCCTCC	Q2-NED	$(GA)_{10}$	224–240	KT222791
	R: GCTCAGTTCGTTTGCACCTT				
TS-24	F: TAGGAGTGCAGCAAGCATATCACCTCGCCTCACTGTCT	Q2-NED	$(AC)_{12}$	303–321	KT222792
	R: TTTACCTTCCGCTACACGAA				
TS-32	F: CACTGCTTAGAGCGATGCCAAGTTTGATTCCTGCCCTT	Q3-VIC	$(AG)_{16}$	222–254	KT222796
	R: ATCCCGTTTGGTTGTTTGAA				
TS-34	F: CACTGCTTAGAGCGATGCACCTCAATGGGTGGAATTGA	Q3-VIC	$(AG)_{23}$	243–286	KT222797
	R: GCTCTCCTCGCCTGAGTAAA				
TS-44	F: CTAGTTATTGCTCAGCGGTCCATATTCAACTTCAAGCTCCAC	Q4-PET	$(TC)_{16}$	196–271	KT222800
	R: AAGCTAGGAGCTTCGTCGG				
Monomorphic loci					
TS-03	F: TGTAAAACGACGGCCAGTTTGGTGAAATTGAGAGCCAA	Q1-6-FAM	$(TA)_{10}$	167	KT250550
	R: GCATATGAACATAAGGTGTTAGGG				
TS-06	F: TGTAAAACGACGGCCAGTCAAAGACCCTTATTTCTAGTCCTCA	Q1-6-FAM	$(AT)_{10}$	187	KT250551
	R: TACTTCGCCCAATTCCACCT				
TS-10	F: TGTAAAACGACGGCCAGTTTGTTATGGTTATCCTTTCGGG	Q1-6-FAM	$(TG)_{10}$	225	KT250552
	R: TGCTCGCATGTCATACAAATG				
TS-20	F: TAGGAGTGCAGCAAGCATCGATTAGTGGGCAAGGGTTA	Q2-NED	$(AT)_{12}$	205	KT250553
	R: TCTGATGCTTGTGTTCGCTC				
TS-33	F: CACTGCTTAGAGCGATGCGCATTCATTTCTCTACCTTGAGC	Q3-VIC	$(AT)_{12}$	218	KT250554
	R: AGACCTGCTCGCTCCATAAA				
TS-43	F: CTAGTTATTGCTCAGCGGTCGCACCAAATTTCACTCCTT	Q4-PET	$(TC)_{17}$	228	KT250555
	R: TCATTAAGTCCATCTCACGGC				
TS-47	F: CTAGTTATTGCTCAGCGGTTGAAGGAGGAAGAAGATGAATG	Q4-PET	$(GA)_{21}$	373	KT250556
	R: CAGTTGCAATGTTTCACGAT				

[a]Annealing temperature for all reactions was 56°C. The linkers (Q1, Q2, Q3, Q4) attached to the forward primers are underlined.

TABLE 2. Genetic properties of 14 polymorphic nuclear microsatellite loci developed in *Terminalia superba* for 42 individuals sampled in southeastern Cameroon.

Locus	*N*	*A*	H_o	H_e	*F*	*r*
Multiplex 1						
TS-08	40	19	0.850	0.913	0.069	0.022 ± 0.055
TS-25	40	9	0.575	0.586	0.019	0.000 ± 0.000
TS-30	40	17	0.780	0.861	0.094	0.033 ± 0.049
TS-37	41	27	0.902	0.940	0.040	0.010 ± 0.046
Multiplex 2						
TS-5	32	15	0.375	0.822	0.544*	0.369 ± 0.093
TS-12	41	2	0.415	0.493	0.158	0.042 ± 0.062
TS-17	42	10	0.595	0.627	0.050	0.013 ± 0.052
TS-28	42	12	0.738	0.841	0.123*	0.055 ± 0.056
TS-42	35	4	0.143	0.570	0.749*	0.407 ± 0.066
Multiplex 3						
TS-22	41	6	0.659	0.723	0.089	0.043 ± 0.047
TS-24	30	5	0.267	0.283	0.058	0.000 ± 0.000
TS-32	38	9	0.579	0.753	0.231	0.090 ± 0.058
TS-34	31	17	0.806	0.895	0.099	0.037 ± 0.057
TS-44	28	10	0.393	0.807	0.513*	0.320 ± 0.068

Note: *A* = number of alleles; *F* = fixation index; H_e = expected heterozygosity; H_o = observed heterozygosity; *N* = number of individuals successfully genotyped on 42 individuals; *r* = frequency of null alleles.

* Significant deviation from Hardy–Weinberg equilibrium ($P < 0.001$).

n = 7), an African savanna tree (*T. avicennioides* Guill. & Perr., *n* = 2), and a Madagascar native tree (*T. mantaly* H. Perrier, *n* = 2), but all of these tests failed (no amplification), indicating that our microsatellites are probably not transferable.

***Microsatellite marker data analysis*—**Genotypes were analyzed with GeneMapper version 3.7 (Applied Biosystems). Microsatellite profiles were typical of a diploid species, displaying at most two alleles per individual and locus. For each of the 14 loci, allele size range, number of alleles (*A*) per locus, observed (H_o) and expected (H_e) heterozygosities, inbreeding coefficient (*F*), and null allele frequencies (*r*) were calculated with INEst 1.0 (Chybicki and Burczyk, 2009). Deviation from Hardy–Weinberg equilibrium (HWE) was tested for each locus with SPAGeDi (Hardy and Vekemans, 2002), and linkage disequilibrium tests were performed with GENEPOP 4.1 (Rousset, 2008).

The southeastern Cameroon population of *T. superba* revealed a high degree of polymorphism, with the number of alleles per locus ranging from two to 27 (mean of 11.43 alleles per locus; Table 2). Regarding the indices of heterozygosity, H_o ranged between 0.143 and 0.902 (H_o was higher than 0.5 for nine of 14 loci) and H_e ranged between 0.283 and 0.940 (Table 2). Four loci (TS-5, TS-28, TS-42, and TS-44) significantly departed from HWE (Table 2), generally due to the presence of null alleles or a large amount of missing data. After accounting for the effect of null alleles, INEst inferred a global inbreeding coefficient ($F = 0.026 \pm 0.089$) not significantly different from zero, indicative of an outbreeding species. Significant linkage disequilibrium was detected between four pairs of loci ($P < 0.05$) after Bonferroni correction (TS-17 and TS-22, TS-37 and TS-25, TS-28 and TS-32, TS-42 and TS-32).

CONCLUSIONS

We developed the first set of microsatellite markers for the Guineo-Congolese Combretaceae species *T. superba*. These 14 microsatellite markers display a high level of polymorphism at the intrapopulation scale. These markers will help to estimate a reliable and informative panel of genetic diversity parameters for the reconstruction of the population history of African rainforests, still rarely conducted in tropical timber tree species.

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Appendix 1. Locality information for the samples used in this study.[a]

Species	Sample no.	Country	Locality	Latitude	Longitude
Terminalia superba	BoD597[b]	Ghana	Kakum National Park	5.34833	−1.38444
	BoD459[c]	Togo	Agou Nyogbo	6.87204	0.72110
	BoD556[c] (BRLU herbarium sample *Demenou 63*)	Ghana	Sagyimasi	6.20716	−0.52927
	BoD713[c]	Nigeria	Ondo state	7.11568	4.80733
	MP309[d]	DRC	Luki	−5.62133	13,10709
	GiD1654[d]	Gabon	Région du Mayumbe	−3.40982	11.41850
	NB669[d]	Cameroon	UFA 09-024	2.32537	10.4336
	BoD721[d]	Nigeria	Bolorunduro, Owena Forest	7.2161	5.01921
	BOD335[d]	Benin	Pobè	6.98891	2.66840
	BoD476[d]	Togo	Tomégbé	7.41984	0.60108
	AD815, AD816, AD817, AD818, AD819, AD820, AD821, AD822, AD823, AD824, AD825, AD826, AD827, AD828, AD829, AD830, AD831, AD832, AD833, AD834, AD835, AD836, AD837[e]	Cameroon	Mindourou	3.27	14.34
	JFG0468, OH3472, OH3473[e]	Cameroon	Barrière Mbang	3.45230	14.07680
	JFG0504, CM5, OH3778[e]	Cameroon	Carrefour Bali	3.76350	14.81470
	JFG0406[e]	Cameroon	Forêt communautaire de Mbol	3.44230	14.62310
	JMR0022, JMR0029[e]	Cameroon	SFID-Mbang 10.038	3.82336	13.97913
	OH3781[e]	Cameroon	UFA 09.021	3.52333	14.49806
	JFG0306, JFG0313, JFG0348, JFG0374, JFG0453[e]	Cameroon	UFA 10.026 Alpicam	3.55030	14.63600
	OH3780[e]	Cameroon	UFA 10.044-Pallisco	3.69722	13.49667
	JFG0401, JFG0472[e]	Cameroon	UFA 10.052 Decolvenaere	3.76800	14.56470
	RP0132, RP0278, RP0334[e]	Cameroon	UFA 10031, Boumba	3.19004	14.25405
	NB779, NB814, NB800, OH3468, OH3469[e]	Cameroon	UFA Lorema (SFID)	3.31335	13.02998
Terminalia avicennioides	BoD387, BoD388	Benin	Manigri	8.90456	1.77053
Terminalia ivorensis	NB398, NB407, NB411, NB412	Cameroon	UFA 11.005 Wijma	5.49082	8.95034
	NB439	Cameroon	Village d'Ogurang	5.48998	8.95359
	NB457	Cameroon		5.58650	9.05916
	PE15	Liberia	Nimba	7.48599	−8.63453
	OH3197	Ghana	near Yegue	8.2959	0.60137
Terminalia mantaly	BoD00467	Togo	Kpalimé	6.91005	0.63224
	BoD00470	Togo	Badou	7.58249	0.61276

Note: DRC = Democratic Republic of the Congo.

[a] Herbarium sample of individual BoD556 (*Demenou 63*) is available at the Herbarium of the Université Libre de Bruxelles (BRLU), Brussels, Belgium. Leaf or cambium silica gel samples for all other samples are deposited at BRLU (silica gel collection of Dr. Olivier Hardy).

[b] Individual used for DNA bank enriched in microsatellite markers.

[c] Individuals used for first amplification test.

[d] Individuals used for testing the polymorphism of loci.

[e] Individuals from southeastern Cameroon used to compute population genetic polymorphism.