Microsatellite Development for the Genus *Guibourtia* (Fabaceae, Caesalpinioideae) Reveals Diploid and Polyploid Species

Author(s): Felicien Tosso, Jean-Louis Doucet, Esra Kaymak, Kasso Daïnou, Jérôme Duminil and Olivier J. Hardy
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PRIMER NOTE

MICROSATELLITE DEVELOPMENT FOR THE GENUS *GUIBOURTIA* (FABACEAE, CAESALPINIOIDEAE) REVEALS DIPLOID AND POLYPLOID SPECIES[1]

FELICIEN TOSSO[2,7], JEAN-LOUIS DOUCET[2], ESRA KAYMAK[3], KASSO DAÏNOU[2,4], JÉRÔME DUMINIL[3,5,6], AND OLIVIER J. HARDY[3]

[2]Tropical Forestry, Management of Forest Resources, TERRA, Gembloux Agro-Bio Tech, University of Liège, 2 Passage des Déportés, B-5030 Gembloux, Belgium; [3]Evolutionary Biology and Ecology Unit, CP 160/12, Faculté des Sciences, Université Libre de Bruxelles, Av. F. D. Roosevelt 50, B-1050 Brussels, Belgium; [4]Nature+ asbl, Rue Provinciale 62, 1301 Wavre, Belgium; [5]Bioversity International, Forest Genetic Resources Programme, Sub-Regional Office for Central Africa, P.O. Box 2008, Messa, Yaoundé, Cameroon; and [6]Institut de Recherche pour le Développement, UMR-DIADE, BP 64501, 34394 Montpellier, France

- *Premise of the study:* Nuclear microsatellites (nSSRs) were designed for *Guibourtia tessmannii* (Fabaceae, Caesalpinioideae), a highly exploited African timber tree, to study population genetic structure and gene flow.
- *Methods and Results:* We developed 16 polymorphic nSSRs from a genomic library tested in three populations of *G. tessmannii* and two populations of *G. coleosperma*. These nSSRs display three to 14 alleles per locus (mean 8.94) in *G. tessmannii*. Cross-amplification tests in nine congeneric species demonstrated that the genus *Guibourtia* contains diploid and polyploid species. Flow cytometry results combined with nSSR profiles suggest that *G. tessmannii* is octoploid.
- *Conclusions:* nSSRs revealed that African *Guibourtia* species include both diploid and polyploid species. These markers will provide information on the mating system, patterns of gene flow, and genetic structure of African *Guibourtia* species.

Key words: Fabaceae; flow cytometry; *Guibourtia*; microsatellites; next-generation sequencing; polyploidy.

The African tree *Guibourtia tessmannii* (Harms) J. Léonard (Fabaceae, Caesalpinioideae) is a hermaphrodite rainforest species distributed from Cameroon to Gabon (Fougère-Danezan et al., 2007; Tosso et al., 2015). Known as "bubinga" or "kevazingo," it has high commercial and social value but is under significant threat due to illegal logging. The genus *Guibourtia* Benn. includes 13 African species distributed from Senegal to Mozambique in forest or savannah habitats. The genus was divided by Léonard (1949) into three main subgenera: (i) *Pseudocopaiva*: *G. tessmannii*, *G. pellegriniana* J. Léonard, *G. coleosperma* (Benth.) J. Léonard, *G. leonensis* J. Léonard; (ii) *Guibourtia*: *G. carrissoana* (M. A. Exell) J. Léonard, *G. copallifera* Benn., *G. demeusei* (Harms) J. Léonard, *G. sousae* J. Léonard; and (iii) *Gorskia*: *G. arnoldiana* (De Wild. & T. Durand) J. Léonard, *G. conjugata* (Bolle) J. Léonard, *G. dinklagei* (Harms) J. Léonard, *G. ehie* (A. Chev.) J. Léonard, *G. schliebenii* (Harms) J. Léonard. We developed polymorphic microsatellite markers for *G. tessmannii* and tested them on nine African congeneric species to verify species delimitation and document population genetic structure and gene flow patterns. Because microsatellite typing suggested that some species were polyploid, we used flow cytometry to compare the ploidy levels of two related species for which appropriate fresh material was available.

METHODS AND RESULTS

***Microsatellite development*—**We extracted total DNA from 30 mg of dry leaf of *G. tessmannii* (FT0001; Appendix 1) using a cetyltrimethylammonium bromide (CTAB) method (Fu et al., 2005). We prepared a nonenriched DNA genomic library, following Mariac et al. (2014), and generated 150-bp-long paired-end reads on an Illumina MiSeq platform (San Diego, California, USA). We assembled the resulting 78,279 reads by pair with PANDAseq (Masella et al., 2012). Using the software QDD (Meglécz et al., 2014), we detected 2483 microsatellite loci. Of these, 149 had at least eight repeats and flanking regions appropriate to define pairs of PCR primers. We developed primers for 48 loci with at least eight di-, tri-, or tetranucleotide repeats and primer regions at least 20 bp distant from the microsatellite region. We added one of four possible linkers (Q1–Q4; Micheneau et al., 2011) to the 5′ end of the forward primer of each locus to label PCR products with fluorochromes FAM, NED, VIC, and PET (Table 1).

We tested 48 primer pairs using two samples of *G. tessmannii* (FT0002 and FT0003; Appendix 1). PCR reactions (total volume of 15 µL) used 1.5 µL of 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 Coral Load, 1 µL of template DNA (of ca. 10–50 ng/µL), and 9.27 µL of water. 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). We visualized PCR products stained with SYBR Safe (Invitrogen, Merelbeke, Belgium) on a 1% agarose gel. Forty-two loci amplified consistently.

We assessed polymorphism on seven *G. tessmannii* individuals from Cameroon and Gabon (Appendix 1). We used fluorescent labeling by PCR amplification in

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[7]Author for correspondence: dnftosso@ulg.ac.be

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TABLE 1. Characterization of 16 polymorphic and one monomorphic nuclear microsatellite loci isolated from *Guibourtia tessmannii*.

Primers[a,b]	Primer sequences (5′–3′)	Labeled primer[c]	Repeat motif	Allele range size (bp)	GenBank accession no.
R12-Seq10*	F: AGGACTTAAGAATGGTGATGCAA	Q1-6-FAM	$(AT)_{10}$	150–200	KX086193
	R: TTTGGCTTCCCTCTCTTCCT				
R12-Seq15*	F: CCTGATTGGAGTTACACCACC	Q1-6-FAM	$(AG)_{13}$	98–124	KX086194
	R: AGGACAAGCTTGAGCGACAT				
R12-Seq21*	F: TTTCATTCAAACAAACCGCA	Q2-NED	$(ATA)_{11}$	176–218	KX086197
	R: CTGACACACAAACACAGCCA				
R12-Seq35*	F: GACACTCCTCAGGTGGTTTCA	Q3-VIC	$(AAT)_{20}$	123–165	KX086204
	R: GAGGTTAGATTCCAACATGTGC				
R12-Seq29*	F: CCAAATTGCAGACGATGAAA	Q3-VIC	$(TCT)_{11}$	205–247	KX086201
	R: AATTCGGACTTGAAGTTGCAG				
R12-Seq08**	F: AACATGCATACTTTAACCGCAA	Q4-PET	$(TTTC)_9$	148–172	KX086191
	R: TTTCAATCAACACTTATCCTTGG				
R12-Seq06**	F: ATCTCCGCTTGTATCTGCGT	Q1-6-FAM	$(GA)_8$	187–203	KX086190
	R: AATCAAGCCTCCGTAAAGCA				
R12-Seq26**	F: CACAATACTAGAGCTGAAGAAACATGA	Q2-NED	$(TCT)_{13}$	153–186	KX086200
	R: CACGAGAAAGGGAGGAAATG				
R12-Seq34**	F: GACACTCCTCAGGTGGTTTCA	Q3-VIC	$(TAT)_{13}$	150–186	KX086203
	R: GAGGTTAGATTCCAACATGTGC				
R12-Seq16**	F: CCCATAATCAGCCTACAAACC	Q2-NED	$(AG)_{11}$	226–262	KX086195
	R: CAGATGAGGTAGACATTGTGGG				
R12-Seq09***	F: ACCTACGTTTGTGATTATGAATGG	Q1-6-FAM	$(GA)_8$	166–196	KX086192
	R: TTTGGGTGATCTTTATGCTTTC				
R12-Seq20***	F: AAATCCGGAGGAGAGGAAGA	Q2-NED	$(AG)_8$	194–218	KX086196
	R: CTGACTCTGGCTTGACCCAT				
R12-Seq22***	F: TTATGATGCGTGTCCCAAA	Q2-NED	$(TA)_{21}$	157–177	KX086198
	R: GAATTGAATGCAGGGAGGAC				
R12-Seq01****	F: CCTCATCATAACAATTCAAGTGC	Q1-6-FAM	$(AT)_{20}$	201–241	KX086189
	R: GATGCCATGACTCTGGCTAAA				
R12-Seq25****	F: CATAGACTTGGAGGGAGCCA	Q2-NED	$(GA)_9$	174–196	KX086199
	R: TTGCTTCCTTGTATCTTTAACAATTT				
R12-Seq31****	F: ATTCCTATCAGATGAACAGATTATCA	Q3-VIC	$(AT)_8$	221–245	KX086202
	R: AGCTTGTTGCAAATTGGATTG				
R12-Seq43**	F: GGCAGAATTTCCAGAAGCAA	Q4-PET	$(TA)_{23}$	143	KX086205
	R: ACACAACCTTCCTTTCCTGC				

[a] * = Multiplex Mix 1, ** = Mix 2, *** = Mix 3, **** = Mix 4.

[b] Optimal annealing temperature was 60°C for all loci.

[c] Q1 = TGTAAAACGACGGCCAGT (Schuelke, 2000); Q2 = TAGGAGTGCAGCAAGCAT; Q3 = CACTGCTTAGAGCGATGC; Q4 = CTAGTTATTGCTCAGCGGT (Q2–Q4, after Culley et al., 2008).

a total volume of 15 µL, combining 0.15 µL of the reverse and 0.1 µL of the forward (0.2 µM for both) microsatellite primers, 0.15 µL of Q1–Q4 labeled primers (0.2 µM each), 3 µL of Type-it Microsatellite PCR Kit (QIAGEN), H_2O, and 1 µL of DNA. PCR conditions were: 5-min initial denaturation at 95°C; followed by 30 cycles of (95°C for 30 s, 60°C for 90 s, 72°C for 1 min) and 10 cycles of (95°C for 30s, 55°C for 45 s, 72°C for 60 s, 72°C for 1 min); and a final elongation step at 60°C for 30 min. We mixed 1.1 µL of each PCR product with 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). The preparation was genotyped on an ABI3730 sequencer (Applied Biosystems, Lennik, The Netherlands).

After excluding loci that did not amplify consistently or were unreadable, we combined 16 polymorphic loci (one locus [R12-Seq43] was monomorphic) in four multiplexed reactions (Table 1) using Multiplex Manager 1.0 software (Holleley and Geerts, 2009). Preliminary population genetic analyses were performed on three populations of *G. tessmannii* (35–58 individuals per population; Table 2 and Appendix 1). Multiplexed PCRs were as above except that 3 µL of the 5× Q-solution of the Type-it Microsatellite PCR Kit was added. The individuals of *G. tessmannii* studied revealed a high degree of polymorphism, with more than two alleles per individual, suggesting a polyploid genome (Table 2).

***Microsatellite marker data analysis in* G. tessmannii *and* G. coleosperma—** The three populations of *G. tessmannii* (Table 2 and Appendix 1) had three to 14 alleles per locus (mean 8.94 alleles per locus, Table 2). Single-locus genotypes had one to eight alleles (2.35 ± 0.94 alleles per locus) and no fixed heterozygosity, suggesting an autopolyploid.

For *G. coleosperma*, the diploid species in which cross-amplification was the most successful (see below), we considered two populations (Table 2). For each of the 10 amplifiable loci, we calculated allele size range, number of alleles (A) per locus, observed (H_o) and expected (H_e) heterozygosity, inbreeding coefficient (F), and null allele frequency (r) 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). Loci exhibited one to 14 alleles (mean 4.5) with H_o (mean ± SE) of 0.28 ± 0.09 and H_e of 0.41 ± 0.11 for the Democratic Republic of Congo (DRC) population and one to 10 alleles (mean 3.67) with H_o of 0.17 ± 0.05 and H_e of 0.36 ± 0.10 for the Namibia population. Significant deviation from HWE was observed in at least one population for four primer pairs. Loci R12-Seq20 and R12-Seq22 for the DRC population exhibited a significant deficit of heterozygotes due to the presence of null alleles (Table 2).

***Flow cytometry—**We used flow cytometry to confirm the ploidy level of *G. tessmannii* and compare its genome size with *G. coleosperma*. We used fresh material from seeds collected in central Gabon (*G. tessmannii*) and northern Namibia (*G. coleosperma*) (Appendix 1). From 1 cm^2 pieces of fresh leaves, we obtained suspensions of leaf cell nuclei by chopping them in a buffer solution using the CyStain UV Precise P Kit (Partec GmbH, Münster, Germany) with DAPI (4′,6-diamidino-2-phenylindole, dilactate). We ran samples with Ploidy Analyser equipment (Partec GmbH). We used tomato as an internal standard (*Solanum lycopersicum* L. "Montfavet 63-5" [2C = 1.99 pg, 40.0% GC; Marie and Brown, 1993]). Under the assumption that the GC content of our samples and the standard were similar, the genome size of *G. coleosperma* ranged from 3.20 to 3.70 pg (N = 3) and *G. tessmannii* from 11.87 to 15.78 pg (N = 3). Although these estimates should be considered with caution in the absence of information on the GC content, the genome size of *G. tessmannii* is nearly four times larger than that of *G. coleosperma*. Because the latter species displays microsatellite profiles typical of diploids, the flow cytometry results confirm that *G. tessmannii* is an octoploid species.

TABLE 2. Results of initial primer screening of 17 nuclear microsatellite loci developed in *Guibourtia tessmannii* (three populations) and 10 that cross-amplified in *G. coleosperma* (two populations).

	G. tessmannii (octoploid)								*G. coleosperma* (diploid)											
	Gabon (Makokou; $N = 35$)			Gabon (Bambidie; $N = 58$)			Cameroon (Ma'an; $N = 38$)		DRC (Dilolo; $N = 20$)						Namibia (Rundu; $N = 13$)					
Locus	A	A_{range}	A_{ind}	A	A_{range}	A_{ind}	A	A_{range}	A_{ind}	N	A	H_o	H_e	F^a	r	N	A	H_o	H_e	F^a	r
R12-Seq15	4	1–3	1.13	11	1–3	1.5	6	1–3	1.51	20	4	0.11	0.26	0.31	0.09 ± 0.08	12	2	0.08	0.08	0***	0.11 ± 0.09
R12-Seq10	14	1–5	2.34	18	1–4	2.02	20	1–6	2.13	18	14	0.67	0.91	0.23	0.07 ± 0.05	13	10	0.62	0.92	0.33	0.07 ± 0.05
R12-Seq35	6	1–4	1.85	13	1–5	2.69	8	1–3	2.12	20	4	0.5	0.71	0.12	0.06 ± 0.05	13	4	0.46	0.7	0.35	0.10 ± 0.07
R12-Seq08	6	1–4	2.45	5	1–3	1.28	5	1–3	1.89	20	2	0	0.09	1*	0.11 ± 0.09	13	1	—	—	—	0.12 ± 0.10
R12-Seq26	7	1–5	2.56	11	1–4	1.91	9	1–3	1.65	20	1	—	—	—	0.12 ± 0.09	13	1	—	—	—	0.13 ± 0.10
R12-Seq34	7	1–6	4.48	5	1–4	2	5	2–3	2.08	20	7	0.67	0.67	0	0.05 ± 0.04	13	5	0.15	0.73	0.79	0.20 ± 0.11
R12-Seq16	14	1–8	5.38	16	2–8	4.72	13	2–8	4.22	19	3	0.5	0.39	–0.07	0.05 ± 0.04	13	1	—	—	—	0.12 ± 0.12
R12-Seq09	10	1–5	2.95	12	2–6	3.55	12	2–6	4.06	19	2	0.06	0.11	0.49	0.29 ± 0.09	12	2	0	0.51	1	0.72 ± 0.11
R12-Seq20	9	1–5	2.63	5	1–3	1.51	2	1–2	1.46	20	3	0	0.3	1**	0.20 ± 0.36	12	—	—	—	—	1 ± 0.00
R12-Seq22	3	1–3	1.42	9	1–3	1.22	4	1	1	20	6	0.34	0.77	0.49***	0.16 ± 0.07	13	7	0.23	0.87	0.74	0.29 ± 0.11
R12-Seq01	11	1–6	2.83	14	1–7	2.98	17	1–7	2.94	—	—	—	—	—	—	—	—	—	—	—	—
R12-Seq25	7	1–4	2.03	7	1–4	2.25	7	1–4	2.26	—	—	—	—	—	—	—	—	—	—	—	—
R12-Seq31	13	1–5	2.31	10	1–4	2.26	7	1–6	2.88	—	—	—	—	—	—	—	—	—	—	—	—
R12-Seq21	10	1–4	2.44	12	1–3	2.07	11	1–3	1.68	—	—	—	—	—	—	—	—	—	—	—	—
R12-Seq29	6	1–4	2.16	4	1–3	1.76	10	1–3	1.94	—	—	—	—	—	—	—	—	—	—	—	—
R12-Seq06	5	1–3	2.03	4	1–4	2.14	5	1–3	2.11	—	—	—	—	—	—	—	—	—	—	—	—
R12-Seq43	1	1	1	1	1	1	1	1	1	—	—	—	—	—	—	—	—	—	—	—	—

Note: — = not applicable; A = number of alleles; A_{ind} = mean number of alleles per individual; A_{range} = range of number of alleles per individual; DRC = Democratic Republic of Congo; F = fixation index; H_e = expected heterozygosity; H_o = observed heterozygosity; N = number of individuals sampled; r = null allele frequency.

[a] Significance of deviation from Hardy–Weinberg equilibrium: $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

Cross-amplification in congeneric species and ploidy determination— Among the 17 loci selected from *G. tessmannii*, a majority successfully amplified in two other species from the subgenus *Pseudocopaiva* (Table 3). Less than six loci amplified in the other species, most of which belong to other subgenera (Table 3). In *G. pellegriniana*, all loci were polymorphic and the genotypes showed up to eight alleles per individual and locus, suggesting an octoploid genome. By contrast, in the other species individuals did not display more than two alleles per locus, suggesting diploid genomes.

CONCLUSIONS

We developed 16 polymorphic microsatellite markers in *G. tessmannii* that amplified to varying degrees in nine congeneric species. The microsatellites and flow cytometry results showed for the first time that the genus *Guibourtia* includes diploid and polyploid species. These markers will be useful to assess the mating system and genetic structure of *Guibourtia* species.

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Table 3. Results of cross-amplification (allele size ranges) of microsatellite loci isolated from *Guibourtia tessmannii* and tested in nine additional taxa.

	Subg. *Pseudocopaiva*			Subg. *Guibourtia*			Subg. *Gorskia*		
Species	*G. pellegriniana* (*N* = 14)	*G. leonensis* (*N* = 3)	*G. coleosperma* (*N* = 33)	*G. carrissoana* (*N* = 2)	*G. copallifera* (*N* = 7)	*G. demeusei* (*N* = 9)	*G. arnoldiana* (*N* = 2)	*G. ehie* (*N* = 20)	*G. dinklagei* (*N* = 1)
R12-Seq10	156–170	148–150	148–186	—	—	—	—	—	—
R12-Seq15	108–124	—	108–122	—	—	—	—	—	—
R12-Seq21	182–212	—	—	—	—	—	—	—	—
R12-Seq35	129–159	141–150	136–154	—	—	—	—	—	—
R12-Seq29	199–223	—	—	—	—	217	—	—	—
R12-Seq08	136–168	—	152–156	142–148	—	—	—	136–208	—
R12-Seq06	194–198	194	—	—	—	—	—	—	—
R12-Seq26	156–180	158–160	158*	—	—	158	—	138–198	—
R12-Seq34	150–174	150	150–160	150	—	—	—	150–174	154
R12-Seq16	226–250	202	224–266	236	226	228–252	232	232–252	—
R12-Seq09	200	—	168–170	—	—	—	—	—	—
R12-Seq20	205	—	203–223	—	203–205	203–207	—	—	—
R12-Seq22	169–173	—	168–172	—	164–172	162–166	—	—	—
R12-Seq01	205–231	—	—	—	—	—	—	—	—
R12-Seq25	180–190	—	—	—	—	—	—	180–192	—
R12-Seq31	221–231	—	—	—	—	—	—	—	—
R12-Seq43	143*	143*	—	—	—	—	—	143*	—

*Monomorphic locus.

Appendix 1. Voucher and locality information for the samples used in this study.[a]

Species	*n*	Voucher no.	Country	Latitude	Longitude
Guibourtia tessmannii (Harms) J. Léonard[b]	1	FT0001	Gabon	1.4286	11.5886
Guibourtia tessmannii[c]	3	FT0002, FT0635–FT0636	Cameroon	2.2236	10.3793
Guibourtia tessmannii[c]	4	FT0003, FT0800–FT0802	Gabon	−0.3802	12.5649
Guibourtia tessmannii[d]	35	FT0540–FT0545, FT0572–FT0600	Gabon	0.36	13.10
Guibourtia tessmannii[d]	58	FT0800–FT0849, FT0851–FT0856, FT0900–FT0902	Gabon	0.76	12.9
Guibourtia tessmannii[d]	38	FT0605–FT0636, OH4675, OH4679, OH4682, OH4683, OH4684, OH4685	Cameroon	2.37	10.63
Guibourtia pellegriniana J. Léonard[d]	14	FT0641–FT0654	Gabon	−2.53	9.77
Guibourtia coleosperma (Benth.) J. Léonard[d]	20	FT0698–FT0717	DRC	−10.48	22.45
Guibourtia coleosperma[d]	13	FT0021–FT0024, FT0028–FT0031, FT0722–FT0726	Namibia	−18.05	19.62
Guibourtia leonensis J. Léonard[d]	3	BR0000013186371[f], BR0000013186401[f], BR0000013186388[f]	Liberia	7.66	−10.02
Guibourtia demeusei (Harms) J. Léonard[d]	9	FT0873–FT0879, OH3245, BR0000009459977[f]	DRC	−0.88	18.12
Guibourtia ehie (A. Chev.) J. Léonard[d]	10	FT0335–FT0344	Ivory Coast	6,28	−3,68
Guibourtia ehie[d]	10	FT0163–FT0172	Ghana	7,02	−2,05
Guibourtia carrissoana (M. A. Exell) J. Léonard[d]	2	BR0000013186210[f], BR0000013186418[f]	Angola	−8.83	13.25
Guibourtia copallifera Benn.[d]	7	FT0880–FT0886	Burkina-Faso	9.95	−4.67
Guibourtia arnoldiana (De Wild. & T. Durand) J. Léonard[d]	2	FT0638, GID2040	Gabon	−3.4098	11.4185
Guibourtia dinklagei (Harms) J. Léonard[d]	1	BR0000013186265[f]	Liberia	6.23084	−9.81249
Guibourtia tessmannii[e]	3	FT0007, FT006, FT008	Gabon	−0.42	12.58
Guibourtia coleosperma[e]	3	FT0020, FT0024, FT0028	Namibia	−17.99	24.09

Note: DRC = Democratic Republic of Congo; *n* = number of individuals.

[a] Vouchers are deposited at the Herbarium of the Université Libre de Bruxelles, Belgium (BRLU), silica gel collection of Dr. Olivier Hardy.

[b] Individual used for DNA bank.

[c] Individual used for tests of amplification and polymorphism.

[d] Individuals used for cross-amplification.

[e] Individuals used for flow cytometry (code for the mother tree).

[f] Codes of specimens from which samples were collected in Botanic Garden Meise (BR), Belgium.