Microsatellite Development and Flow Cytometry in the African Tree Genus Afzelia (Fabaceae, Caesalpinioideae) Reveal a Polyploid Complex

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Source: Applications in Plant Sciences, 3(1)

Published By: Botanical Society of America

URL: https://doi.org/10.3732/apps.1400097

Applications in Plant Sciences

Applications in Plant Sciences 2015 3(1): 1400097

Primer Note

Microsatellite development and flow cytometry in the African tree genus *Afzelia* (Fabaceae, Caesalpinioideae) reveal a polyploid complex[1]

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- *Premise of the study:* Microsatellites were developed in the vulnerable African rainforest tree *Afzelia bipindensis* to investigate gene flow patterns.
- *Methods and Results:* Using 454 GS-FLX technique, 16 primer sets were identified and optimized, leading to 11 polymorphic and readable markers displaying each six to 25 alleles in a population. Up to four alleles per individual were found in each of the loci, without evidence of fixed heterozygosity, suggesting an autotetraploid genome. Cross-amplification succeeded for all loci in the African rainforest species *A. pachyloba* and *A. bella*, which appeared tetraploid, and for most loci in the African woodland species *A. africana* and *A. quanzensis*, which appeared diploid, but failed in the Asian species *A. xylocarpa*. Flow cytometry confirmed the suspected differences in ploidy.
- *Conclusions:* African *Afzelia* species are diploid or tetraploid, a situation rarely documented in tropical trees. These newly developed microsatellites will help in the study of their mating system and gene flow patterns.

Key words: *Afzelia*; Caesalpinioideae; Fabaceae; microsatellites; next-generation sequencing; polyploidy; tropical timber tree.

The African tree *Afzelia bipindensis* Harms (Fabaceae, Caesalpinioideae) is a hermaphrodite Guineo-Congolese species of moist forests distributed from Ivory Coast to Angola and Zambia, up to 900 m elevation (Gérard and Louppe, 2011; Donkpegan et al., 2014). Commonly called "red doussié," it is registered as vulnerable on the International Union for the Conservation of Nature (IUCN) Red List, because it is substantially exploited for the international wood market. Other African *Afzelia* Sm. species include the rainforest species *A. bella* Harms, *A. pachyloba* Harms, and *A. parviflora* (Vahl) Hepper, and the dry forest and woodland species *A. africana* Sm. and *A. quanzensis* Welw. All of these species are of economic importance and undergo significant exploitation. Most of these species can occur in sympatry or in parapatry, and some of them are botanically similar. Therefore, thorough investigations of gene flow patterns within and among *Afzelia* populations are necessary for conservation and sustainable management purposes. To this end, we developed polymorphic microsatellite markers on *A. bipindensis* and tested them on other African and Asian congeneric species. Because the number of alleles found per individual suggested that some species are polyploid, flow cytometry was used to compare the ploidy levels of species for which fresh leaves were available.

[1]Manuscript received 6 October 2014; revision accepted 13 November 2014.

The authors thank the Fonds pour la Formation à la Recherche dans l'Industrie et l'Agriculture (FRIA, Belgium) and the Fonds de la Recherche Scientifique (F.R.S.-FNRS, grant T.0163.13) for financial support. We acknowledge Pallisco Ltd., Tran Thi Hoa, and Chulee La for sampling, and Rosalia Pineiro, Spencer Brown, Esra Kaymak, and Peter Thrall for their help.

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doi:10.3732/apps.1400097

METHODS AND RESULTS

***Microsatellite development*—**A DNA bank enriched in microsatellite markers was developed from an equimolar mix of genomic DNA extracts from four species, including *A. bipindensis*, using the 454 GS-FLX platform (454 Life Sciences, a Roche Company, Branford, Connecticut, USA) according to the protocols of Malausa et al. (2011) and Micheneau et al. (2011). The software QDD (Meglécz et al., 2010) detected 635 loci containing a microsatellite formed by at least five repeats, and surrounded by flanking regions adequate to define PCR primers. From these, we selected 71 loci containing perfect (i.e., not compound) microsatellites with at least nine dinucleotide (or trinucleotide for two loci) repeats and with primers situated at least 20 bp from the microsatellite region. We also considered the primers of eight microsatellite loci defined on *A. xylocarpa* (Kurz) Craib, an Asian species (Pakkad et al., 2009). Following Micheneau et al. (2011) for ordering primers (71 + 8 = 79 pairs), we added to the 5′ end of the forward primer of each locus one of four possible linkers (Q1–Q4), which allowed the PCR products to be labeled with a fluorochrome ("M13" protocol). These pairs of primers were tested individually on *A. bipindensis* samples using the following PCR conditions: a reaction volume of 15 µL containing 1.5 µL of buffer (10×), 0.6 µL of $MgCl_2$ (25 mM), 0.45 µL of dNTPs (10 mM each), 0.3 µL of each primer (0.2 mM), 0.08 µL of DNA polymerase TOPTAQ 5 U/µL (QIAGEN, Venlo, The Netherlands), 1.5 µL of Coral Load, H_2O, and 1.5 µL of DNA extract. The amplification reactions were carried out using the PTC-200 Thermal Cycler (Bio-Rad Laboratories, Hercules, California, USA) using the following program: 4-min initial denaturation at 94°C, followed by 30 cycles of denaturation/annealing/extension (94°C for 30 s, 52°C or 55°C for 45 s, 72°C for 1 min). The final elongation step was at 72°C for 10 min. The PCR products were visualized on a 1% agarose gel and stained with SYBR

Safe (Invitrogen, Merelbeke, Belgium). DNA extracts were obtained using the NucleoSpin Plant Kit (Macherey-Nagel, Düren, Germany).

Two of the eight loci developed for *A. xylocarpa* amplified on *A. bipindensis* samples but resulted in illegible multipeak band patterns, so that none were kept. For the new microsatellite bank, 16 primer pairs amplified consistently on *A. bipindensis* samples and were tested again to assess their levels of polymorphism in seven individuals of *A. bipindensis* from Cameroon, Gabon, and the Republic of Congo. Fluorescent labeling was performed via amplification with: the reverse primer, the forward primer with a Q1–Q4 universal sequence at the 5′ end, and a Q1–Q4 primer labeled with 6-FAM, NED, VIC, and PET, respectively (Schuelke, 2000; Micheneau et al., 2011). PCR conditions were identical to those we mentioned before, but the Q-tailed forward primer was used at one-third the amount (0.7 µM) of the reverse and fluorescently labeled primers (2 µM). PCR conditions were: 94°C (4 min); followed by 25 cycles each at 94°C (30 s), 55°C (45 s), 72°C (1 min); 10 cycles each at 94°C (30 s), 53°C (45 s), 72°C (45 s); and a final extension at 72°C for 10 min.

After excluding loci that did not amplify or were unreadable, we selected 11 polymorphic loci. They were combined in two multiplexed reactions (Table 1) using Multiplex Manager 1.0 software (Holleley and Geerts, 2009). Preliminary population genetics analyses were performed on 42 individuals from an *A. bipindensis* population from eastern 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 mM) of forward primer and 0.15 µL (0.1 mM) of reverse primer labeled by Q-tailed fluorescent Q1 to Q4, H_2O, and 1.5 µL of DNA extract. Multiplex PCR programs consisted of: 95°C (5 min); followed by 20 cycles at 95°C (30 s), 57°C (90 s), 72°C (30 s); eight cycles at 94°C (30 s), 53°C (45 s), 72°C (45 s); and a final extension step at 60°C (30 min). For each PCR product, 0.8 mL was directly added to 12 µL of Hi-Di Formamide (Life Technologies, Carlsbad, California, USA) and 0.3 µL of Radian DYE size standard (Eurogentec, Seraing, Belgium) and genotyped on an ABI 3730 sequencer (Applied Biosystems, Lennik, The Netherlands). Results were analyzed using two programs: Peak Scanner version 1.0 (Applied Biosystems) and GeneMapper version 3.7 (Applied Biosystems). Some read errors were corrected manually.

The eastern Cameroon population of *A. bipindensis* revealed a high degree of polymorphism, with the number of alleles per locus ranging from six to 25 (average of 14.9 alleles per locus; Table 2). We noticed that the number of alleles per individual and locus ranged from one to four, suggesting a tetraploid genome. Because single-locus genotypes did not show fixed heterozygosity at any locus, *A. bipindensis* would be a priori an autotetraploid.

Cross-amplification in congeneric species and ploidy determination— All of the 11 polymorphic loci successfully amplified on *A. bella* ($N = 5$) and *A. pachyloba* ($N = 5$), and seven amplified in *A. africana* ($N = 39$) and *A. quanzensis* ($N = 19$) (see Appendix 1). In *A. bella* and *A. pachyloba*, all of these loci were polymorphic and the observed genotypes presented up to four alleles per locus and individual, indicating that these species are probably tetraploid. By contrast, *A. africana* and *A. quanzensis* individuals never displayed more than two alleles per locus, suggesting diploid genomes.

To confirm whether *Afzelia* species have different ploidy levels, we relied on flow cytometry, a convenient and rapid method for estimating the size of the nuclear genome. The fresh material used was obtained through seeds from five species collected in southwestern Cameroon for *A. bipindensis*, northwestern Cameroon for *A. bella*, northeastern Benin for *A. africana*, northern Zimbabwe for *A. quanzensis*, and northern Thailand for *A. xylocarpa* (Appendix 1). These seeds were planted in a greenhouse so that fresh leaves could be collected for our analyses. Flow cytometry was carried out using Ploidy Analyser equipment (Partec GmbH, Münster, Germany). Nuclear suspensions of leaf cells were obtained from 1-cm^2 pieces of fresh leaves lacerated with a razor blade in a buffer solution using the CyStain UV Precise P kit (Partec GmbH), following the manufacturer's protocol. This kit uses DAPI (4′,6-diamidino-2-phenylindole, dilactate), a blue fluorescent stain that preferentially binds double-stranded DNA with A-T nucleotides. We used as internal standard either pea (*Pisum sativum* L. 'Express Long' [genome size 2C value = 8.37 pg, 49.5% GC]) or tomato (*Solanum lycopersicum* L. 'Montfavet 63-5' [2C = 1.99 pg, 40.0% GC; Marie and Brown, 1993]). Each sample was first analyzed alone to identify its fluorescence intensity profile and then analyzed with an internal standard to measure their ratio of mean cell fluorescence intensities, which is approximately proportional to their C-value ratio, at least if the GC contents of species are similar.

Our genome size estimates (2C values) ranged from 4.9 to 5.3 pg in *A. africana* ($n = 3$), 5.0 pg in *A. quanzensis* ($n = 1$), 4.3 to 4.4 pg in *A. xylocarpa* ($n = 2$), 9.9 pg in *A. bipindensis* ($n = 3$), and 8.5 to 9.6 pg in *A. bella* ($n = 2$). For the same species, estimates were usually a bit larger using pea as an internal

TABLE 1. Characterization of 11 polymorphic nuclear microsatellite loci isolated from *Afzelia bipindensis* and tests of cross-amplification in polymorphic loci.

Locus[a]	Primer sequences (5′–3′)	Labeled primer[b]	Repeat motif	T_a (°C)	Successful cross-amplification[c]	GenBank accession no.
R9-48†	F: TTCCACCAGAGAACAATCACA	Q4-PET	$(AC)_{12}$	57	*A. pach*, *A. bel*, *A. afr*, *A. qua*	KP076432
	R: AAGGAAAGACAATGTTAGACTGGA					
R9-01‡	F: CACTCTTTCTTCCTTTTCAACCA	Q1-6-FAM	$(CT)_{11}$	57	*A. pach*, *A. bel*, *A. afr*, *A. qua*	KP076434
	R: AAGATTGAAGCTTGAAAATTCACC					
R9-07‡	F: GCAAAGACACGCAAACTGAA	Q4-PET	$(AG)_{10}$	57	*A. pach*, *A. bel*, *A. afr*, *A. qua*	KP076426
	R: TTCGATAAACCGATCCATGTC					
R9-19†	F: TGTGATAGGAAGTGAGATTGCG	Q2-NED	$(CT)_{10}$	57	*A. pach*, *A. bel*, *A. afr*, *A. qua*	KP076435
	R: TGTATAAGACAAAGACCCACCTT					
R9-51‡	F: CAAATGCTCAAATGACTAACCA	Q2-NED	$(AC)_{13}$	57	*A. pach*, *A. bel*	KP076436
	R: GCAATTCAATGATGTCTTGCTT					
R9-73‡	F: CAGTCACACCTGCTTTCAGC	Q1-6-FAM	$(CT)_9$	57	*A. pach*, *A. bel*	KP076430
	R: CCAACAATCAAATCACTCATCG					
R9-65†	F: CGAAAGTAAGAACCGTGCAA	Q1-6-FAM	$(CA)_9$	57	*A. pach*, *A. bel*, *A. afr*, *A. qua*	KP076431
	R: AACAGGTAATGAAAGCAAAGGG					
R9-61†	F: TCAAGGGATGACTTGGCTTT	Q2-NED	$(TC)_{10}$	57	*A. pach*, *A. bel*, *A. afr*, *A. qua*	KP076424
	R: GAGAAAGACAGCTTAATTTTACCCC					
R9-54‡	F: TCCCTTACCAAATTTGTGAACAT	Q4-PET	$(GA)_{11}$	57	*A. pach*, *A. bel*	KP076425
	R: CCCGTTGGTTCAGTAGCAAT					
R9-53‡	F: TTAATCGAGCTTAGTCGAGC	Q3-VIC	$(TC)_{10}$	57	*A. pach*, *A. bel*	KP076433
	R: GACTCAAGAGAAGCAAGCTAAGA					
R9-60‡	F: CTTTCCCTCCTTTCTTTCAA	Q2-NED	$(TC)_9$	57	*A. pach*, *A. bel*, *A. afr*, *A. qua*	KP076427
	R: GTTATGCTAGACATTTAATCCC					

Note: *A. afr* = *A. africana*; *A. bel* = *A. bella*; *A. pach* = *A. pachyloba*; *A. qua* = *A. quanzensis*; T_a = annealing temperature.

[a] † = Mix 1, ‡ = Mix 2.

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

[c] All loci are polymorphic for these species except R9-48, R9-07, and R9-60, which are monomorphic for *A. quanzensis*, and R9-19 which is monomorphic for *A. africana*, considering these samples.

TABLE 2. Characterization of polymorphism of nuclear microsatellite loci in a sample of 42 individuals of *Afzelia bipindensis* from eastern Cameroon and 39 individuals of *A. africana* from northern Benin.

Species	Locus	Allele size range (bp)	*A*	H_o	H_e	F[a]	$H_e(Cd)$	$F(Cd)$[a]
A. bipindensis	R9-48	151–170	14	0.54	0.80	0.32***	0.75	0.27***
	R9-01	84–132	18	0.56	0.84	0.34***	0.79	0.29***
	R9-07	102–129	14	0.51	0.73	0.30***	0.68	0.25***
	R9-19	111–150	14	0.54	0.60	0.10***	0.56	0.04***
	R9-51	154–172	11	0.60	0.84	0.29***	0.79	0.23***
	R9-73	223–243	8	0.22	0.30	0.28***	0.28	0.22***
	R9-65	210–218	6	0.50	0.69	0.28***	0.64	0.22***
	R9-54	193–239	25	0.53	0.83	0.37***	0.78	0.32***
	R9-61	188–270	20	0.70	0.91	0.23***	0.85	0.17***
	R9-60	185–228	21	0.79	0.83	0.04***	0.77	–0.02***
	R9-53	182–225	13	0.35	0.87	0.59***	0.81	0.57***
A. africana	R9-48	150–166	5	0.51	0.76	0.33*		
	R9-01	86–151	13	0.87	0.87	–0.01		
	R9-07	105–128	5	0.1	0.1	–0.02		
	R9-19	88–140	1	0	0			
	R9-65	199–226	9	0.72	0.78	0.08		
	R9-61	195–204	4	0.57	0.73	0.22*		
	R9-60	174–214	11	0.62	0.8	0.23*		

Note: A = number of alleles; F = fixation index; $F(Cd)$ = fixation index assuming chromatid segregation; H_e = expected heterozygosity (assuming chromosome segregation in the tetraploid *A. bipindensis*); $H_e(Cd)$ = expected heterozygosity assuming chromatid segregation; H_o = observed heterozygosity.

[a] For F and $F(Cd)$, significance of deviation from Hardy–Weinberg equilibrium: $*P < 0.05$, $***P < 0.001$.

standard rather than tomato. Although these 2C estimates should be considered with caution given that the GC content of *Afzelia* species is unknown, their relative values clearly show that the genome sizes of both *A. bipindensis* and *A. bella* are nearly double the genome sizes of *A. africana*, *A. quanzensis*, and *A. xylocarpa*. This was confirmed when analyzing together *A. bipindensis*, *A. bella*, and *A. africana* in the flow cytometer, which then displayed two peaks with a ratio of fluorescence intensity of 1.9. Hence, together with the microsatellite profiles, the flow cytometry results confirm that *Afzelia* forms a polyploid complex, with *A. africana*, *A. quanzensis*, and *A. xylocarpa* being diploid while *A. bipindensis*, *A. bella*, and *A. pachyloba* are tetraploid.

***Population genetics parameters in the tetraploid* A. bipindensis *and the diploid* A. africana—**For *A. bipindensis*, we considered the 42 individuals from a population from eastern Cameroon (see above; Appendix 1) and calculated heterozygosities assuming autopolyploidy, using the software AUTOTET (Thrall and Young, 2000). In autotetraploids, multiple levels of heterozygosities can occur at a single locus because a genotype can contain one to four alleles, and a genotype with just two or three alleles can present different allelic dosages (1 : 3, 2 : 2, or 3 : 1 for two alleles; 1 : 1 : 2, 1 : 2 : 1 or 2 : 1 : 1 for three alleles). Defined as the probability that a random pair of gene copies bears different alleles within individuals, the observed heterozygosity is 0 for a genotype AAAA, 0.5 for AAAB, 0.667 for AABB, 0.833 for AABC, and 1 for ABCD. The allelic dosage could be estimated based on the ratios between peak intensities following Esselink et al. (2004). The expected heterozygosity (H_e) under random mating was computed separately under either random chromosome segregation (*RCeS*) or random chromatid segregation (*RCdS*), which assumes maximum double reduction ($\alpha = 1/7$; Wricke and Weber, 1986). Fixation indices (F) were calculated as $1 - (H_o/H_e)$. Departure from Hardy–Weinberg equilibrium (HWE) was tested with χ^2 goodness-of-fit tests for observed to expected genotype frequencies, under either *RCeS* or *RCdS*. The χ^2 test is known to give suspect results when expected frequencies of some genotypic classes are low. Consequently, we pooled into the same class all alleles except the most common one.

Overall, the 11 microsatellite loci were highly polymorphic, with a mean $H_e \pm$ SE of 0.75 ± 0.17 under *RCeS* and 0.70 ± 0.16 under *RCdS*, and a mean $H_o \pm$ SE of 0.53 ± 0.15 (Table 2). They showed a large number of rare alleles, with an average of 44% of alleles with a frequency <2%. These results are concordant with the results of Truong et al. (2005; on the tetraploid birch, *Betula pubescens* subsp. *tortuosa*) and with the relative conservancy of the tetrasomic genome, in which rare alleles are eliminated much more slowly than under strict disomic inheritance and allelic richness is usually higher compared with related diploids (Bever and Felber, 1992). In total, all polymorphic loci significantly deviated from HWE because of heterozygote deficiency, even under *RCdS*. Further work is needed to test whether heterozygote deficiency results from the mating system (e.g., high selfing) and/or null alleles.

For *A. africana*, we considered 39 individuals from a population in Benin (1.55586°N, 9.26674°E; Appendix 1). Seven microsatellites (i.e., R9-48, R9-01, R9-07, R9-19, R9-65, R9-61, and R9-60) amplified on all individuals. Observed and expected heterozygosities, as well as the fixation index, were calculated for each locus with SPAGeDi (Hardy and Vekemans, 2002).

The seven loci exhibited from one to 13 alleles per locus (mean = 6.8) with $H_o \pm$ SE $= 0.48 \pm 0.14$ and $H_e \pm$ SE $= 0.58 \pm 0.15$. Only locus R9-19 was monomorphic (but some polymorphism was detected in other *A. africana* individuals, results not shown). Locus R9-48 exhibited significant deficit of heterozygotes (Table 2).

CONCLUSIONS

By developing microsatellite markers in the vulnerable (per the IUCN Red List) timber tree *A. bipindensis*, we found that the species is tetraploid (possibly a selfing autotetraploid). Tetraploidy was also found in other congeneric African rainforest species by successful cross-amplification of the microsatellites and flow cytometry, while the African woodland species *A. africana* and *A. quanzensis* appear diploid and only a subset of the microsatellite markers are transferable to them. To the best of our knowledge, polyploidy had never been documented in the genus *Afzelia*, and rarely in tropical timber tree species. The microsatellite loci will be useful for assessing the mating system, gene flow, and evolutionary relationships of *Afzelia* species, and will provide essential information for their conservation.

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

Species	*n*	Voucher no.	Collection locality	Geographic coordinates
Afzelia bipindensis[b]	1	OH2474	Bambidie, eastern Gabon	0.755449°S, 12.969804°E
Afzelia bipindensis[c]	42	AD336–AD338, AD340–AD346, AD254–AD269, AD349, AD350, AD352–AD357, AD367, AD368, AD372, AD374–AD377, AD455, AD660	Mindourou, eastern Cameroon	3.2924°N, 14.343339°E
Afzelia africana[c]	32	AD61–AD99	Bassila, central Benin	1.55586°N, 9.26674°E
Afzelia bella[c]	5	AD437, AD438, AD440–AD442	Mamfé, northwestern Cameroon	5.75515°N, 9.540129°E
Afzelia pachyloba[c]	5	AD411, AD413, AD417, AD419, AD420	Campo-Ma'an, southwestern Cameroon	2.249193°N, 10.469494°E
Afzelia quanzensis[c]	19	AD513–AD531	Gede and Kwale, eastern Kenya	3.30317°S, 39.98346°E
Afzelia bipindensis[d]	3	AD443	Campo-Ma'an, southwestern Cameroon	5.745634°N, 9.538056°E
Afzelia africana[d]	3	AD195	Djona, northeastern Benin	2.992373°N, 11.50940°E
Afzelia bella[d]	2	AD426	Mamfé, northwestern Cameroon	5.75515°N, 9.540129°E
Afzelia quanzensis[d]	1	JLD1658	Victoria Falls, northern Zimbabwe	17.92217°S, 025.84743°E
Afzelia xylocarpa[d]	2	CL1	Khon Kaen, northern Thailand	16.462389°N, 102.81989°E

Note: *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 enriched in microsatellite markers.

[c] Individuals used for cross amplification.

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