Microsatellite Primers for Parkia biglobosa (Fabaceae: Mimosoideae) Reveal that a Single Plant Sires All Seeds Per Pod

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PRIMER NOTE

MICROSATELLITE PRIMERS FOR *PARKIA BIGLOBOSA* (FABACEAE: MIMOSOIDEAE) REVEAL THAT A SINGLE PLANT SIRES ALL SEEDS PER POD[1]

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- *Premise of the study:* Microsatellite primers were developed for an indigenous fruit tree, *Parkia biglobosa*, as a tool to study reproductive biology and population structure. Here we use the primers to determine the number of fathers per pod.
- *Methods and Results:* Microsatellite loci were enriched in a genomic sample and isolated using pyrosequencing. Eleven primer pairs were characterized in two populations of *P. biglobosa* in Burkina Faso (each with 40 trees). The number of alleles per locus ranged from eight to 15, and one locus had null alleles. We genotyped seeds from 24 open-pollinated pods. The genotypic profiles of seeds per pod suggest that all seeds are outcrossed and that only one pollen donor sires all ovules in a single fruit.
- *Conclusions:* Ten microsatellite markers were highly polymorphic. All seeds per pod of *P. biglobosa* were full siblings. The markers will be useful for reproductive and population genetic studies.

Key words: néré; *Parkia biglobosa*; paternity; pollen polyad; pollination; reproductive biology.

Parkia biglobosa (Jacq.) R. Br. ex G. Don (Fabaceae: Mimosoideae) is a common fruit tree species in the farmed parklands of West Africa. The pods are highly valued, due to their sweet fruit pulp and protein-rich seeds (Uwaegbute, 1996), but recent studies have shown a reduced regeneration of the species (Ouédraogo, 1995; Ræbild et al., 2012). Bats have been identified as pollinators of *P. biglobosa* since 1957 (Baker and Harris, 1957), and studies restricting access of bats to flower inflorescences have credited different species of bees as pollinators (Ouédraogo, 1995; Lassen et al., 2012). The mating system is reported to be predominantly cross-fertilization (Ouédraogo, 1995), although self-fertilization is possible (Ouédraogo, 1995; Lassen et al., 2012). *Parkia biglobosa* sheds pollen in polyads, each with 16 or 32 united pollen grains (Baker and Harris, 1957). The pod contains up to 24 seeds (Ouédraogo, 1995), and due to the small size of the stigma and the large polyads, it has been hypothesized that only one or two pollen donors sire all seeds in a single pod in *Parkia* spp. (Hopkins, 1984).

In the present paper, we present a set of microsatellite markers suitable for studies of population structure and reproduction. Allelic diversity and exclusion power based on the analysis of two farmed parkland populations in Burkina Faso are presented, and we use the markers to test the hypothesis of a single plant siring all seeds in a pod.

METHODS AND RESULTS

Leaf material of *P. biglobosa* was collected from mature trees in four populations in Burkina Faso, West Africa, and dried in silica gel for the development of primers (Appendix 1). Samples from two of these populations were used for testing the primers (40 trees near Pinyiri [syn. Kacheli], Pô [11°14′34.89″N, 1°8′1.73″W], and 40 trees near Tiba, Zitenga [12°42′26.26″N, 1°18′2.04″W]). Open-pollinated pods were collected from eight of the sampled trees in Pinyiri (three pods per tree), and the seeds were germinated in growth chambers at 25°C (day and night), with 12 h of daylight. The 24 collected pods contained a total of 396 seeds (mean: 16.5 seeds/pod ± 5.22 standard deviation [SD]). Forty-five seeds were considered aborted due to their black color, low weight (<0.02 g), and/or flat shape. The remaining 351 seeds (≥0.02 g and round shape) were sown, of which 336 seeds germinated (mean: 0.203 g/seed ± 0.053 SD); 15 seeds did not germinate (mean: 0.069 g/seed ± 0.054 SD). The germination percentage was 95.7 (±1.1% standard error [SE]). The seedlings were frozen at −18°C upon harvest. Total genomic DNA was extracted from the leaf material using the DNeasy Plant Mini Kit and the DNeasy 96 Plant Kit (QIAGEN, Hombrechtikon, Switzerland) following the manufacturer protocols.

The microsatellite primers were developed by GenoScreen (Lille, France) from 10 samples of total genomic DNA (Appendix 1). One microgram of genomic DNA was used for development of microsatellite libraries through 454 GS-FLX Titanium pyrosequencing (Roche Applied Sciences, Meylan, France) of enriched DNA libraries as described by Malausa et al. (2011). Total genomic DNA was mechanically fragmented and enriched for AG, AC, AAC, AAG, AGG, ACG, ACAT, and ATCT repeat motifs. Enriched

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TABLE 1. Description of 11 microsatellite loci for *Parkia biglobosa* developed from 10 samples from Burkina Faso.[a]

Locus	Primer sequences (5′–3′)	Repeat motif	Allele size range (bp)[b]	Fluorescent label[c]	GenBank accession no.
PbL02	F: CGAAATAAGAACTCGGACCAAA	$(GA)_{17}$	180–205	NED	KJ475533
	R: ATGCCGTGTTCTGTTTCACC				
PbL03	F: TTCGATTCAATTCAACTTGCAG	$(GA)_{17}$	90–138	6-FAM	KJ475534
	R: TCGGTGCTAGCAATATCAGC				
PbL04	F: GAAGCCTTGGAATGAAGTTGA	$(CA)_{17}$	161–181	VIC	KJ475535
	R: GAAAACGGAAGGCATGGTTA				
PbL05	F: GAATCAGAGAAGCCCTTAGGTT	$(AC)_{17}$	183–259	PET	KJ475536
	R: GCCGCTTGTTTTCTTGTGA				
PbL09	F: TGACGTATTTGAGTGTCTTTTACACA	$(AG)_{18}$	126–170	6-FAM	KJ475537
	R: GCAGAAAATCACAAATGCAGA				
PbL11	F: TCACGGCAATAGAACTCATCA	$(CCT)_{18}$	170–206	PET	KJ475538
	R: ACGGAGCAGGATGAAGTCAG				
PbL12	F: ATCTAGGCATCCATGCAAATG	$(TG)_{19}$	108–137	VIC	KJ475539
	R: CAGAAGTTGTTAGACAATTATGGTGA				
PbL15	F: CCCCAAGAGCAACAACTCAT	$(CT)_{19}$	120–140	PET	KJ475540
	R: GGCAGTGTTCTATAGGAAATAGTCG				
PbL18	F: ATTCATCAACAAGTCTGCCAA	$(CA)_{19}$	84–135	NED	KJ475541
	R: TGCATTTCTTACTTTTATTTGTGC				
PbL21	F: TGTTGCTTTTGCTTTTGCTG	$(CA)_{21}$	269–305	VIC	KJ475542
	R: CCCTCTGCAGAATTGAGTCC				
PbL22	F: TTGCGAATAGGATATGGGTTG	$(AC)_{22}$	164–218	6-FAM	KJ475543
	R: GAAGACGACAGGCTGCTACA				

[a] All primers were run at an annealing temperature of 55°C.

[b] Based on 40 samples from Pinyiri (syn. Kacheli), Pô (11°14′34.89″N, 1°8′1.73″W), and 40 samples from Tiba, Zitenga (12°42′26.26″N, 1°18′2.04″W), Burkina Faso.

[c] Fluorescent tags used to label the 5′ ends of the forward primers.

fragments were subsequently amplified. PCR products were purified, quantified, and GS-FLX libraries were then produced following the manufacturer's protocols and sequenced on a GS-FLX PicoTiterPlate (Roche Applied Sciences). The pyrosequenced data were processed with QDD version 1 software (Meglécz et al., 2010). Hereafter, the QDD version 1 software comprising the program Primer3 version 1.1.4 (Rozen and Skaletsky, 2000) was used for designing PCR primers from a unique flanking region of the microsatellite repeats. A total of 63,571 sequences (raw data) were generated. The number of generated sequences containing microsatellite motifs was 12,261 and the number of unique loci was 1319.

From these sequences with unique loci, a total of 24 primer pairs were tested that had a PCR product at the expected size and in a sufficient amount to perform the polymorphism analysis. The 24 PCR primer pairs were amplified using a GeneAmp 9700 thermal cycler (Applied Biosystems, Lille, France) as 25-µL reactions containing 1.5 mM $MgCl_2$, 0.4 µM of each primer, 240 µM dNTPs, 1 unit FastStart *Taq* DNA polymerase (Roche Applied Sciences), and approximately 20 ng genomic DNA template. The PCR program consisted of denaturation at 95°C for 3 min, followed by 40 cycles made up of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min. The final step was a prolonged extension at 72°C for 10 min. The sizes of the amplified products were controlled and the polymorphic state of each tested microsatellite was estimated on a Fragment Analyzer automated capillary electrophoresis system (Advanced Analytical Technologies, Ames, Iowa, USA), resulting in 13 validated primer pairs. These 13 primer pairs were tested for polymorphism by GenoScreen on 30 samples of *P. biglobosa* from four sites in Burkina Faso (Appendix 1). Finally, the resulting 11 polymorphic primer pairs were labeled using the fluorochromes 6-FAM, VIC, NED, and PET (Table 1), and characterized by testing 40 samples from Pinyiri and 40 samples from Tiba (see Table 1 for the size range of alleles and Table 2 for number of alleles and observed heterozygosity).

Multiplex PCRs were done with the QIAGEN Multiplex PCR kit, according to the manufacturer instructions and with two mixes (Mix 1 = PbL03, PbL04, PbL05, PbL15, PbL18, PbL22; and Mix 2 = PbL02, PbL09, PbL11, PbL12, PbL21), using a GeneAmp 2700 Thermal Cycler (Applied Biosystems, Foster City, California, USA) or a Bio-Rad C1000 Thermal Cycler (Bio-Rad Laboratories, Copenhagen, Denmark). Each reaction mixture (10 µL) was made up of 1 µL extracted DNA, 5 µL of 2× Multiplex PCR Master Mix (QIAGEN), 1 µL of primer mix (final concentration of 1.0 µM for each forward and reverse primer, except primers in locus PbL22 on 0.6 µM), and 3 µL RNase-free water (QIAGEN). The PCR program consisted of denaturation at 95°C for 10 min; 40 three-step cycles consisting of denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min; and a final extension at 72°C for 10 min. PCR products were diluted 1 : 200 (primer mix 1) or 1 : 300 (primer mix 2). Fragment analyses were done with capillary electrophoresis on an ABI 3130xl Genetic Analyzer (Applied Biosystems) with GeneScan 500 LIZ as internal size standard. Finally, the allele sizes were determined using the software GeneMapper version 4.0 (Applied Biosystems).

Each locus was checked for null alleles using MICRO-CHECKER version 2.2.3 (van Oosterhout et al., 2004). Number of alleles per locus and heterozygosity (observed and expected) were calculated using GenAlEx

TABLE 2. Genetic properties of 11 microsatellite loci for *Parkia biglobosa* tested on 40 samples from Pinyiri (syn. Kacheli), Pô (11°14′34.89″N, 1°8′1.73″W), and 40 samples from Tiba, Zitenga (12°42′26.26″N, 1°18′2.04″W), Burkina Faso.

	Tiba, Zitenga, Oubritenga (n = 40)					Pinyiri, Pô, Nahouri (n = 40)				
Locus	n	A	H_o	H_e	HWE	n	A	H_o	H_e	HWE
PbL02	40	9	0.750	0.743	Yes	40	9	0.650	0.649	Yes
PbL03	40	13	0.875	0.860	Yes	40	12	0.950	0.837	Yes
PbL04	40	9	0.825	0.781	Yes	40	10	0.875	0.811	Yes
PbL05	40	13	0.925	0.831	Yes	40	9	0.900	0.811	Yes
PbL09	40	15	0.800	0.884	Yes	40	13	0.875	0.874	Yes
PbL11	40	10	0.700	0.674	Yes	40	9	0.650	0.657	Yes
PbL12	40	12	0.800	0.862	Yes	40	10	0.850	0.817	Yes
PbL15	40	9	0.675	0.643	Yes	40	8	0.625	0.670	Yes
PbL18[a]	36	15	0.389	0.894	No	35	14	0.429	0.884	No
PbL21	40	12	0.875	0.830	Yes	40	13	0.850	0.824	Yes
PbL22	40	15	0.825	0.813	Yes	40	15	0.950	0.874	Yes

Note: A = number of observed alleles per locus; H_e = expected heterozygosity (GenAlEx version 6.501); H_o = observed heterozygosity; HWE = Hardy–Weinberg equilibrium (GENEPOP version 4.2); n = number of individuals genotyped.

[a] This marker has 10–12% missing data and shows signs of a null allele ($P < 0.001$, Bonferroni analysis by MICRO-CHECKER version 2.2.3).

TABLE 3. Number of germinated seeds per pod, cumulative number per pod of alleles per locus present in the offspring, but not present in the mother tree, and number of loci per pod with alleles differing from the mother tree in a total of 336 offspring from 24 pods.

Pod ID	No. of seeds per pod	Loci in *Parkia biglobosa*										No. of loci with foreign alleles
		PbL02	PbL03	PbL04	PbL05	PbL09	PbL11	PbL12	PbL15	PbL21	PbL22	
P10-T0-1	20	0	0	1	1	2	1	2	0	2	2	7
P10-T0-5	11	1	1	2	2	2	2	1	0	2	2	9
P10-T0-9	20	0	1	2	2	1	1	1	1	2	1	9
P14-T0-2	21	1	1	1	1	1	1	2	1	1	2	10
P14-T0-7	14	1	2	1	2	0	2	2	2	1	2	9
P14-T0-9	16	0	2	1	1	1	1	1	1	2	0	8
P22-T0-3	17	2	2	2	1	1	2	2	1	1	2	10
P22-T0-6	15	2	1	1	0	1	2	1	2	2	2	9
P22-T0-9	10	2	2	2	1	1	2	2	1	1	2	10
P33-T0-2	21	0	2	0	2	1	1	1	0	2	2	7
P33-T0-3	21	2	1	1	1	1	1	2	1	2	2	10
P33-T0-8	8	2	1	1	2	2	2	0	1	2	2	9
P76-T0-2	18	1	0	1	2	2	1	2	0	1	2	8
P76-T0-8	8	1	1	2	1	2	1	1	1	1	2	10
P76-T0-9	5	1	2	2	1	2	1	1	0	1	2	9
P90-T0-2	10	0	0	2	2	2	2	2	0	2	2	7
P90-T0-4	16	2	1	1	0	1	2	2	0	1	2	8
P90-T0-5	23	1	2	2	1	2	2	2	0	1	2	9
P92-T0-1	3	0	2	0	1	1	2	1	1	2	0	7
P92-T0-4	3	1	2	2	1	2	1	1	1	2	1	10
P92-T0-8	14	1	2	2	1	2	2	2	1	2	2	10
P93-T0-3	11	1	1	2	1	1	1	2	0	2	2	9
P93-T0-5	15	1	0	0	1	2	1	2	0	2	2	7
P93-T0-8	16	1	1	2	1	1	1	2	0	2	2	9

version 6.501 (Peakall and Smouse, 2006, 2012), and GENEPOP version 4.2 (Rousset, 2008) was used to check for genotypic linkage disequilibrium (LD, D') and deviations from Hardy–Weinberg equilibrium (HWE). All markers were highly polymorphic (Table 2). Genotypic linkage disequilibrium was seen in two pairs of loci in the Tiba population and in three pairs of loci in the Pinyiri population; however, none were significant when adjusted with table-wide sequential Bonferroni corrections (Rice, 1989) with Holm's method. One of the markers, PbL18, had 10–12% missing data, and genotypic frequencies deviated significantly from HWE with the likely presence of a null allele ($P < 0.001$, Bonferroni analyses as implemented in MICRO-CHECKER version 2.2.3). The genotypic frequencies in the 10 other markers did not differ significantly from Hardy–Weinberg expectations (Table 2). Only these 10 markers were used in the following study to detect the number of pollen donors per pod, where we analyzed at least nine loci per offspring. The combined probability of exclusion (P_E) with one parent known (Jamieson and Taylor, 1997) was calculated using GenAlEx version 6.501 (Peakall and Smouse, 2006, 2012), resulting in 0.997 and 0.998 for nine and 10 markers, respectively, and showing that the efficiency of excluding a false father (pollen donor) is 99.7% and 99.8%, respectively. To find the number of pollen donors per pod, we counted for each pod the number of alleles per locus present in the offspring that was not present in the mother tree. This number can in principle vary from 0 to the number of seeds per tested pod (if all seeds are sired by different pollen donors), but any value higher than 2 in any of the 10 tested loci would reveal presence of more than one pollen donor per pod. However, the analysis revealed a maximum of two alleles (foreign to the mother tree) per locus for all pods (Table 3), which corresponds to the hypothesis that all seeds in a pod of *P. biglobosa* are being sired by only one father each. Because all germinated seeds had alleles different from the mother tree in at least seven out of 10 loci, all of the pods must have been cross-pollinated.

CONCLUSIONS

Ten of the 11 microsatellite markers presented here have proven to be highly polymorphic and easy to genotype. The remaining marker contained null alleles and was excluded from the pollen donor analysis. Regarding the mating system of *P. biglobosa*, we found that all seeds in a pod were likely to have been sired by a single pollen donor. Furthermore, we found that all germinated seeds were cross-pollinated. We conclude that the markers are well-suited for studies of population genetics and reproductive biology of *P. biglobosa*.

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Appendix 1. Information on samples of *Parkia biglobosa* used in this study.

Voucher specimen accession no.[a]	Collection locality[b]	Geographic coordinates	No. of individuals in the herbarium	No. of individuals used for development of primers	No. of individuals used for testing primers for polymorphism	No. of individuals used for characterizing the primers
Pbg-001-MO	Tiba, Zitenga, Oubritenga	12°42′26.26″N, 1°18′2.04″W	3	3	11	40
Pbg-002-MO	Loumbila, Loumbila, Oubritenga	12°28′54.17″N, 1°24′28.97″W	1	1	2	0
Pbg-003-MO	Boulbi, Komsilga, Kadiogo	12°13′38.71″N, 1°31′59.53″W	3	1	2	0
Pbg-004-MO	Pinyiri, Pô, Nahouri	11°14′34.89″N, 1°8′1.73″W	2	5	15	40

Note: MO = Moussa Ouédraogo, collector.

[a]Vouchers are deposited in the Herbarium at the Centre National de Semences Forestières, Route de Kaya, 01 BP 2682 Ouagadougou, Burkina Faso.

[b]Village, department, and province in Burkina Faso.