MICROSATELLITE PRIMERS FOR PARKIA BIGLOBOSA
(FABACEAE: MIMOSOIDEAE) REVEAL THAT A SINGLE PLANT SIRES ALL SEEDS PER POD¹

KRISTIN MARIE LASSEN²,4, ERIK DAHL KJER², MOUSSA OUÉDRAOGO³, AND LENE ROSTGAARD NIELSEN²

¹Department of Geosciences and Natural Resource Management, Faculty of Science, University of Copenhagen, Rolighedsvej 23, 1958 Frederiksberg C, Denmark; and ²Centre National de Semences Forestières, Route de Kaya, 01 BP 2682 Ouagadougou, Burkina Faso

• 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 shed pollen in Parkia 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 (20.02 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 Malassa et al. (2011). Total genomic DNA was mechanically fragmented and enriched for AG, AC, AAC, AAG, AGG, ACG, and ATCT repeat motifs. Enriched...
Locus Primer sequences (5′-3′) Repeat motif Allele size range (bp) a Fluorescent label b GenBank accession no.
PbL02 F: CGAATAAGAACCTCGGACAAA (GA) 17 180–205 NED KJ475533 R: ATCCGGGTGTCTGTTACC
PbL03 F: TATGATTTCAATTCATCTTCGAG (GA) 17 90–138 6-FAM KJ475534 R: TCCGATCTGGATCAATGACG
PbL04 F: GAAAGCTTGAGTTAGTTGA (CA) 17 161–181 VIC KJ475535 R: GAAAAGGGAGGATGGTTA
PbL05 F: GAATCGAGAGACCTCTTAGGT (AC) 17 183–259 PET KJ475536 R: GCCGCTTGTCTTCTTCTTGA
PbL09 F: TGAGGTATTGTGTGCTTTTACAACACA (AG) 18 126–170 6-FAM KJ475537 R: GCAAGAACAACTACAATACAGA
PbL11 F: TATCGGCAGTGAGATGTCAG (CCT) 18 170–206 PET KJ475538 R: ACGAGGAGATTAGCTCAG
PbL12 F: ATCTAGGCTGACATCAGAATAGTG (TG) 18 108–137 VIC KJ475539 R: GACGATTTCTGATTAGAAAGTCAG
PbL15 F: CCAGAAGACGACAACATCAT (CT) 18 120–140 PET KJ475540 R: GGCAGTTCTTCATTAGGAAGTCTG
PbL18 F: ATCTCTCAAGAAGCTCGACAAC (CA) 18 84–135 NED KJ475541 R: TGCATTCTTATTCTTATTTGTGC
PbL21 F: TGTGGCTTTGGTTTGTCTTG (CA) 18 269–305 VIC KJ475542 R: CCCCCCTGCAAGATTTGGCC
PbL22 F: TGGGAATAGGATGATGTTTTG (AC) 22 164–218 6-FAM KJ475543 R: GAAGAGGACGGAGTCATCA

a All primers were run at an annealing temperature of 55°C.
b Fluorescent tags used to label the 5′ ends of the forward primers.

Table 1. Description of 11 microsatellite loci for Parkia biglobosa developed from 10 samples from Burkina Faso.

| Locus | Primer sequences | Repeat motif | Allele size range (bp) | Fluorescent label | GenBank accession no. |
|-------|----------------|--------------|-----------------------|------------------|----------------------|
| PbL02 | F: CGAATAAGAACCTCGGACAAA | (GA) 17 | 180–205 | NED | KJ475533 |
| PbL03 | F: TATGATTTCAATTCATCTTCGAG | (GA) 17 | 90–138 | 6-FAM | KJ475534 |
| PbL04 | F: GAAAGCTTGAGTTAGTTGA | (CA) 17 | 161–181 | VIC | KJ475535 |
| PbL05 | F: GAATCGAGAGACCTCTTAGGT | (AC) 17 | 183–259 | PET | KJ475536 |
| PbL09 | F: TGAGGTATTGTGTGCTTTTACAACACA | (AG) 18 | 126–170 | 6-FAM | KJ475537 |
| PbL11 | F: TATCGGCAGTGAGATGTCAG | (CCT) 18 | 170–206 | PET | KJ475538 |
| PbL12 | F: ATCTAGGCTGACATCAGAATAGTG | (TG) 18 | 108–137 | VIC | KJ475539 |
| PbL15 | F: CCAGAAGACGACAACATCAT | (CT) 18 | 120–140 | PET | KJ475540 |
| PbL18 | F: ATCTCTCAAGAAGCTCGACAAC | (CA) 18 | 84–135 | NED | KJ475541 |
| PbL21 | F: TGTGGCTTTGGTTTGTCTTG | (CA) 18 | 269–305 | VIC | KJ475542 |
| PbL22 | F: TGGGAATAGGATGATGTTTTG | (AC) 22 | 164–218 | 6-FAM | KJ475543 |

The final step was a prolonged extension at 72°C for 1 min.

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.

| Locus | n | A | Hn | Hs | HWE | n | A | Hn | Hs | 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 | 10 | 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 | 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 |

Notes: A = number of observed alleles per locus; Hn = expected heterozygosity (GenAIEx version 6.501); Hs = observed heterozygosity; HWE = Hardy–Weinberg equilibrium (GENEPOP version 4.2); n = number of individuals genotyped.

* 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).

http://www.bioone.org/loi/apps
The pods must have been cross-pollinated. Alleles different from the mother tree in at least seven out of 10 loci, all of which are being sired by only one father each. Because all germinated seeds had the mother of two alleles (foreign to the mother tree) per locus for all pods (Table 3), 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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### 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 | PbL02 | PbL03 | PbL04 | PbL05 | PbL09 | PbL11 | PbL12 | PbL15 | PbL21 | PbL22 | No. of loci with foreign alleles |
|----------|----------------------|-------|-------|-------|-------|-------|-------|-------|-------|-------|-------|---------------------------------|
| P10-T0-1 | 20                   | 0     | 0     | 1     | 1     | 2     | 1     | 2     | 0     | 2     | 2     | 7                              |
| P10-T0-5 | 11                   | 1     | 1     | 2     | 2     | 2     | 1     | 0     | 2     | 2     | 9                              |
| P10-T0-9 | 20                   | 0     | 1     | 2     | 2     | 1     | 1     | 1     | 2     | 1     | 9                              |
| P14-T0-2 | 21                   | 1     | 1     | 1     | 1     | 1     | 1     | 2     | 1     | 1     | 10                             |
| P14-T0-7 | 14                   | 0     | 2     | 1     | 1     | 1     | 1     | 1     | 2     | 0     | 8                              |
| P14-T0-9 | 16                   | 0     | 2     | 1     | 1     | 1     | 1     | 1     | 2     | 0     | 8                              |
| P22-T0-3 | 17                   | 2     | 2     | 1     | 1     | 2     | 2     | 1     | 1     | 2     | 10                             |
| P22-T0-6 | 15                   | 2     | 1     | 1     | 0     | 1     | 2     | 1     | 2     | 2     | 9                              |
| P22-T0-9 | 10                   | 2     | 2     | 2     | 1     | 1     | 2     | 2     | 1     | 1     | 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     | 2     | 1     | 1     | 2     | 2     | 0     | 1     | 2     | 9                              |
| P33-T0-9 | 18                   | 0     | 1     | 1     | 2     | 1     | 2     | 1     | 0     | 1     | 2     | 8                              |
| P33-T0-10| 21                   | 1     | 2     | 1     | 1     | 1     | 1     | 2     | 1     | 1     | 2     | 10                             |
| P33-T0-11| 5                    | 1     | 2     | 1     | 2     | 1     | 1     | 0     | 1     | 2     | 9                              |
| P90-T0-2 | 10                   | 0     | 0     | 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     | 0     | 1     | 2     | 9                              |
| P92-T0-1 | 3                    | 0     | 2     | 0     | 1     | 2     | 1     | 1     | 2     | 0     | 7                              |
| P92-T0-4 | 3                    | 1     | 2     | 2     | 1     | 2     | 2     | 1     | 2     | 2     | 10                             |
| P93-T0-3 | 11                   | 1     | 2     | 2     | 1     | 2     | 2     | 1     | 2     | 2     | 10                             |
| 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, Δ′) 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 Pinjyri 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 (P3) 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*.
**APPENDIX 1. Information on samples of *Parkia biglobosa* used in this study.**

| Voucher specimen accession no.a | Collection localityb | 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.