

Paternity identification in *Spondias tuberosa* (Anacardiaceae: Sapindales) polycrosses using microsatellite loci

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ABSTRACT. The umbu tree (*Spondias tuberosa*) is endemic to the Brazilian semiarid region. This species is not cultivated and commercialized fruits are a result of extrativism; consequently little has been done to develop new and more productive cultivars. Hybrids of different accessions are a result of natural open pollination, so identifying putative male parents among progenies of umbu tree accessions is difficult. Eight accessions were pre-selected for larger fruit size and clones were established at Petrolina, PE. Female plants were randomly chosen among accessions of an established umbu tree experimental plot. Alleles of putative male parents were evaluated based on simple sequence repeat (SSR) loci. Of the 32 SSR loci evaluated, 10 showed polymorphism. Thirty-four alleles were identified, with an average of 3.4/locus. Polymorphism information content ranged from 0.195 to 0.744; the expected ranged from 0.233 to 0.825 and the observed heterozygosity ranged from 0.250 to 0.825. Accessions similarity ranged from 0.44 to 0.68, distributed into two groups: (I) common umbu, BGU30, BGU37, BGU44, BGU55 and BGU68, and (II) BGU 48 and BGU52. Hybrids and self-fertilized

plants were identified among BGU30 and BGU37 progenies; 17 out of 20 and 14 out of 44 hybrids were identified by SSR loci in the BGU30 and BGU37 progenies, respectively. The putative hybrids BGU30 × BGU48, BGU30 × BGU55, BGU30 × BGU44, BGU30 × BGU68, BGU37 × BGU44, BGU37 × BGU55 and BGU37 × BGU52 have potential for future evaluations, as they were more divergent in the dendrogram or had male parents with higher weight fruits. The 10 SSR loci were sufficient to identify a single parent in 12 of 20 BGU30 hybrids and all 14 BGU37 hybrids, demonstrating the potential of this procedure.

Key words: Umbu tree; Hybrids; SSR; Plant breeding

INTRODUCTION

The umbu tree (*Spondias tuberosa*) is an endemic species of the Brazilian semiarid region (Prado and Gibbs, 1993), with a canopy of numerous intertwined branches and a height ranging from 4 to 6 m. Its fruits are of the drupe type, with only one seed/fruit (Lima et al., 2018). The fruits weigh 4.5 to 96 g, are elliptical or rounded, and are rich in bioactive compounds, such as vitamin C, flavonoids, carotenoids, and phenolic compounds (Santos et al. 1999, Dantas-Junior, 2008). The umbu tree is a diploid species ($2n = 32$), predominantly allogamous, with an estimated outcrossing rate of 80% (Almeida et al., 2007; Santos and Gama, 2013).

Santos et al. (1999) reported umbu fruit with an average weight greater than 85 g, a value 4x greater than the average fruit weight of the species, 21 g. Assessments of cloned umbu tree accessions resulted in the registration of four clones, BGU-52, BGU-48 and BGU-68, enabling the commercial production of seedlings of the species (Ministério da Agricultura, Pecuária e Abastecimento, 2021). There is no information on crosses between umbu accessions that would allow the development of new cultivars with traits superior to those of the current genotypes of spontaneous occurrence in the Brazilian semiarid. The production of umbu fruits in 2019 was close to 9000 t, resulting from extractivism in some Northeastern Brazilian States, since agronomic cultivation is non-existent (Lima et al., 2018).

The technique of hand crossings is laborious, requiring intensive use of trained personnel. In allogamous species, polycrossing or open-pollination can be used without the need for hand pollinations, making it possible to form new genotypes among accessions present in a given area (Hittle, 1954). The polycross technique has been adopted in cassava (Fukuda and Iglesias, 2006) and in sugar cane (Xavier et al., 2014). In mango, also an allogamous species, Pinto et al. (1995) reported a hybridization success rate ranging from 0.79% to 8.2% by hand crossing. Adopting a scheme of polycrossing in mangoes, Santos and Lima Neto (2011) reported a hybridization success rate greater than 80%. There is no established protocol for hand crossing of the umbu tree, and studies on flower opening and pollen and stigmatic viability are limited, making hand crossing of umbu genotypes for allelic recombination still more difficult.

The challenge in identifying the male parent in polycrosses or natural crossings (Poehlman and Sleper, 1995) was overcome with the application of molecular markers, as

shown by Tew and Pan (2010) and Xavier et al., (2014), in sugar cane, by applying microsatellite loci. The use of molecular markers has improved the studies of population genetics among plant species, allowing, for example, the identification of hybrids in early stages of plant development (Grattapaglia and Ferreira, 1988). The application of phenotypic markers, such as flower color, is not feasible in species with a long juvenile period, such as the umbu tree, as it can take many years to begin flowering.

Our goal was to identify putative male parents in progenies of two umbu tree accessions obtained by open-pollination among eight umbu trees, aiming at determining whether this technique would be feasible to help organize a breeding program for this species.

MATERIAL AND METHODS

Plant material, DNA extraction and PCR protocol

The seeds were obtained from two open pollinated BGU30 and BGU37 female plants growing among six accessions and one additional common umbu (UC) tree, in an agronomic trial established at Embrapa Semiárido field (Figure 1), located in Petrolina, PE (latitude 09°09' S and longitude 40°22' W). The umbu trees presented synchronized flowering, which occurred at the end of the dry season (September to November) and fruit maturity at the end of the rainy season (February to March).

The accessions BGU30, BGU37, BGU44, BGU48, BGU52, BGU55 and BGU68 were collected in Afranio-PE, Uaua-BA, Anage-BA, America Dourada-BA, Parnamirim-PE, Lagoa Grande-PE and Lontra-MG, respectively, and they were pre-selected for greater fruit size. Scions of all accessions were collected from the pre-selected maternal plant and grafted to a common umbu rootstock, and cloned accessions were established in an experimental plot located at Petrolina, PE, Brazil. The accession BGU24 was included as border experimental control, and umbu trees of spontaneous growth were not observed within a radius of 500 m. BGUs 30 and 37 were chosen as female plants because they come from the semiárid depression, the most common landscape unit of spontaneous occurrence of umbu trees.

Samples of young and healthy leaves from 20 and 44 progenies of the female plants BGU30 and BGU 37, respectively, and six putative pollinator accessions were collected and stored at -80°C for DNA extraction. The DNA extraction was performed based on the CTAB 2x protocol, with some modifications, as described by Santos and Gama (2013). DNA quantification was obtained through comparative visual analysis of samples on 0.8% (w/v) agarose gel, stained with ethidium bromide and standardized at 10 ng μL^{-1} for SSR analysis.

The polymerase chain reaction (PCR) was run with 14 SSR loci developed by Aguilar-Bajaras et al. (2014) and 18 by Balbino et al. (2019) (Table 1). PCR protocols were adjusted to a final volume of 10 μL^{-1} , containing: 1x buffer solution, 2 mM of MgCl_2 , 0.22 μM of each dNTP, 0.4 μM of each primer (forward and reverse), one unit of Taq DNA polymerase and 10 ng of genomic DNA. The amplifications were performed in a thermocycler, with the program proposed by Aguilar-Bajaras et al. (2014). The program started with 15 min at 94°C, followed by 35 cycles at 94°C for 30 s, 58 or 60°C for 1 min, 72°C for 1 min and final extension at 72°C for 10 min. The PCR products were visualized in

a 6% polyacrylamide gel, stained with silver nitrate, as described by Santos and Gama (2013).

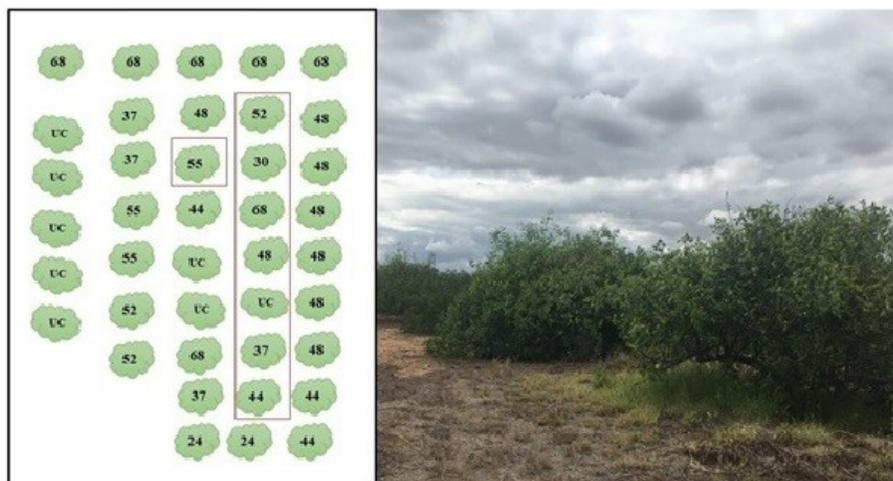


Figure 1. Field photo and representative aerial field disposal of umbu tree accessions: common umbu (UC), BGU30, BGU37, BGU44, BGU48, BGU52, BGU55 and BGU68. BGU30 and BGU37 = female plants. Spacing among plants: 10 m x 10 m.

Analysis of genetic diversity among accessions and progeny paternity

The genetic diversity analysis was estimated with support of the NTSYSpc program (Rohlf, 2009), based on the Jaccard index similarity matrix. The distance for each accession was generated using the UPGMA (unweighted pair group method with arithmetic mean) grouping method. The adjustment of the dendrogram was tested using cophenetic correlation or the correlation among the real distances and those represented graphically. The allelic frequency expected and observed heterozygosity, and polymorphism information content (PIC) of the SSR loci were estimated with the Cervus 3.0 program (Kalinowski et al. 2007).

The paternity analysis was performed based on the presence or absence of alleles of male origin in the progeny, according to the procedure adopted by Santos et al. (2013) for mangoes. The genotype of each progeny was compared with the genotype of putative male parents, excluding a male parent that does not share allele in a given progeny: male plant with alleles 3 and 4 is excluded from progeny paternity with alleles 1 and 2, for example. In this analysis, information of the set of 10 SSR loci was considered, and the absence of a single allele of a given SSR was enough to exclude progeny paternity.

RESULTS

Parameters of SSR and genetic diversity among accessions

Of the 32 loci of SSRs evaluated, 10 loci showed polymorphism and amplicons that were easy to visualize and define in 6% polyacrylamide gels, stained with silver nitrate. The total number of alleles analyzed was 34, with an average of 3.4/locus, ranging from 2 to

6, with a higher frequency of three alleles/locus (Table 1). The PIC ranged from 0.195 to 0.744, with SPRO4 and SSR14 being the most informative (Table 1). The expected heterozygosity (He) ranged from 0.233 to 0.825 and the observed (Ho) from 0.250 to 0.825 (Table 1). The SSR loci with the highest number of alleles, SSR04 and SSR14, also showed higher values of PIC, He, and Ho (Table 1).

The co-phenetic correlation was 0.86, indicating consistent grouping in the dendrogram obtained based on the Jaccard distances of the seven umbu accessions and one UC plant (Figure 2). The accessions showed similarity from 0.44 to 0.68, with the most dissimilarities among the common umbu (UC) and BGU48 and BGU52 and the most similar were BGU37 and BGU55 accessions (Figure 2). Two groups were formed in the dendrogram, at the cut-off point of 0.45: group I: common umbu (UC), BGU30, BGU37, BGU44, BGU55 and BGU68, and group II: BGU 48 and BGU52. Group I was formed by 75% of the genotyped accessions.

Table 1. Primers sequences (forward - F, reverse - R), annealing temperatures (T_m), number of alleles (NA), polymorphic information content (PIC), expected (He) and observed heterozygosity (Ho) of *Spondias tuberosa* SSR loci.

Locus	Sequence (5' - 3')	T _m (°C)	NA	PIC	HE	HO
SSR04 [#]	F:CGCTAGTTGTCATTCGCGG R:GCTTAACCTCTGGAAAGTCGC	62	6	0.744	0.825	0.625
SSR08 [#]	F:GCAGCAGCCATTTGTGAAC R:CACGTGTTCCAGTTATGATTTG	60	3	0.427	0.508	0.625
SSR14 [#]	F:ACACCAACGTTTGC GGAG R:TCTAGGTAGACAGCGACAAATC	62	5	0.636	0.742	0.875
SSR18 [#]	F:TCTATTTGCGTCCAGGTATTTTC R:GAATGGGCACGTTCTTTGG	62	3	0.511	0.633	0.750
TUB78*	F:TGCTCTGCCTTCCAACATGT R:GTACGTGAGGGACAATGGGG	59	3	0.427	0.508	0.625
TUB84*	F:CACCTCTACGTTACTGCCA R:TCAAACCTGGATTACAGGCATGC	59	2	0.337	0.458	0.625
TUB93*	F:AGCCTTTTTGAGTCACATGCA R:ACACTGATGGTACGTGAACAA	60	2	0.195	0.233	0.250
TUB94*	F:TGTCTGAGGATCGAGACGAGA R:GCACGCGCTTACTTATGTTGG	60	3	0.482	0.575	0.750
TUB98*	F:AGCGGAAAAGAATGATGAAGGC R:GTTGGCTTCTTTCTTGC GGCC	60	3	0.427	0.508	0.625
TUB103*	F:GGAGCAGTGAAACCCTGAA R:GTCCAGTTCGCCGTAAGAA	60	4	0.387	0.442	0.500

[#] Aguilar-Bajaras et al. (2014); *Balbino et al. (2019).

Paternity analysis

The allelic data of the 10 SSRs made it possible to identify hybrid and self-fertilized plants, mainly in the BGU30 progenies (Table 2). Male parents BGU52 and BGU48 have exclusive alleles, alleles 5 and 6 of locus SPO4, respectively, facilitating the exclusion or inclusion of paternity (Table 2). The female plants, BGU30 and BGU37, were homozygous for 4 and 5 loci, respectively, while among pollinator parents, BGU44 and BGU48 showed greater heterozygosity, 80% (8/10) (Table 2).

Among the BGU30 20 progenies, male parents were identified in 17 of them, two self-fertilized progenies and only one with unknown male parent (Table 3). BGU37 was the parent with the highest number of progenies, seven, and BGUs 68, 44 and 48, all clones

with fruits greater than 85g, each one with a progeny (Table 3). Of the 20 progenies that constituted the population, 17 were the result of cross-pollination, that is, 85% of the total population. Progenies 7, 9, 12 14 and 17 have more than one likely parent, while 12 progenies have only one. BGU37 and BGU55 were parents of seven and six progenies, respectively (Table 3).

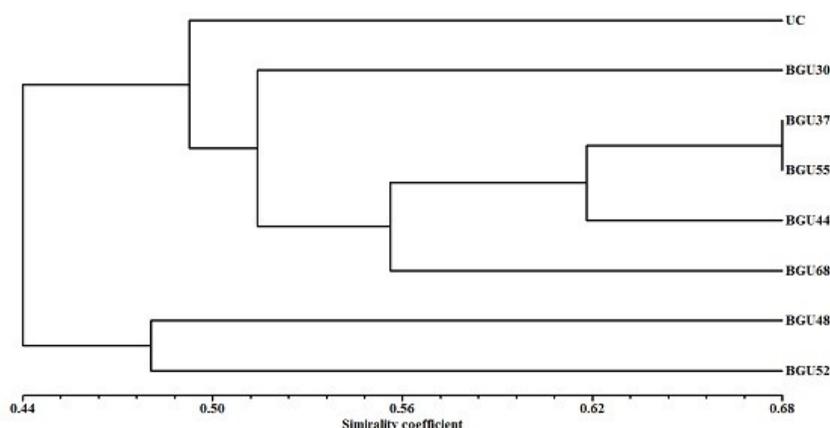


Figure 2. UPGMA dendrogram of the Jaccard coefficient among eight *Spondias tuberosa* accessions genotyped with 10 microsatellite loci. Cophenetic correlation = 0.85 (UC=common umbu tree).

Table 2. Genotyping of eight parents (PAR) and some progenies of BGU37 and BGU30, with hybrid (H) identification and putative male parent (PMP), in a polycross scheme for *Spondias tuberosa* *.

PAR	SSR loci genotyping										H	PMP
	SPO4	SPO8	SPO14	SPO18	TUB78	TUB84	TUB93	TUB94	TUB98	TUB103		
UC	12	33	11	11	13	12	11	22	11	34		
30	32	33	23	11	23	12	11	12	13	33		
37	22	13	23	12	33	11	11	23	11	23		
44	42	23	23	12	13	11	12	23	12	33		
48	51	23	42	23	33	12	11	12	12	23		
52	66	13	13	23	13	12	11	12	13	33		
55	12	13	12	12	23	11	12	23	11	33		
68	41	33	23	12	33	12	11	22	12	13		
BGU37: Progenies												
8	22	13	12	11	13	11	11	23	11	23	H	UC
9	22	11	12	12	33	11	11	12	12	23	H	ND
10	12	33	12	12	33	12	11	23	11	24	H	UC
20	12	13	13	12	33	12	11	22	11	34	H	UC
21	22	11	12	12	33	12	11	13	13	23	H	ND
22	22	33	33	11	33	11	12	23	00	23	H	44
30	22	13	12	11	23	11	11	23	11	23	H	55
BGU30: Progenies												
1	35	33	23	12	33	11	11	22	13	33	H	48
3	31	33	23	12	23	11	12	12	13	33	H	55
4	31	33	12	11	12	22	11	12	11	34	H	UC
5	22	33	12	11	12	22	11	22	13	34	H	UC
6	12	33	33	12	23	11	11	22	13	13	H	68
7	32	33	23	12	23	12	11	12	13	33	H	37,44,55
11	31	13	23	12	23	12	12	11	33	33	H	ND
16	22	33	23	12	23	11	11	22	11	33		SF

*Partial data. ND=not defined. SF=self-fertilization.

In the BGU37 progenies, male parents were identified in 14 of them, one self-fertilized (Table 4) and 29 progenies with unknown male parent, indicating a 32% outcrossing rate (14/44). The large number of progenies without a defined male parent can be attributed to the location of the maternal plant BGU 37, close to BGU24 (Figure 1), that was not included in the present genotyping, with great possibility of also being a progenitor of BGU37 progenies. The common umbu (UC) is the putative male parent of nine progenies, and BGUs 30, 48 and 68 were not putative parents of the BGU37 progenies (Table 4). All BGU37 hybrids have only one putative parent (Table 4).

Table 3. Progenies of the female plant BGU30 and putative male parent, in a polycross scheme of *Spondias tuberosa* accessions.

Progeny	Putative male parent	Progeny	Putative male parent
1	BGU48	11	Not defined
2	Self-fertilization	12	BGU37, BGU44, BGU55
3	BGU55	13	BGU37
4	UC	14	BGU44, BGU55
5	UC	15	BGU37
6	BGU68	16	Self-fertilization
7	BGU37, BGU44, BGU55	17	BGU37, BGU55
8	BGU44	18	BGU37
9	UC, BGU55	19	BGU37
10	BGU37	20	BGU37

Table 4. Progenies of the female plant BGU37 and putative male parent, in a polycross scheme of *Spondias tuberosa* accessions.

Progeny	Putative male parent	Progeny	Putative male parent
1	Not defined	23	Not defined
2	Not defined	24	Not defined
3	Not defined	25	Not defined
4	UC	26	Not defined
5	Not defined	27	Not defined
6	Not defined	28	UC
7	Not defined	29	Not defined
8	UC	30	55
9	Not defined	31	Not defined
10	UC	32	Not defined
11	Not defined	33	Not defined
12	Not defined	34	Not defined
13	UC	35	52
14	UC	36	Not defined
15	UC	37	52
16	UC	38	Not defined
17	Not defined	39	30
18	Not defined	40	Not defined
19	Not defined	41	Self-fertilization
20	UC	42	Not defined
21	Not defined	43	Not defined
22	44	44	Not defined

DISCUSSION

The umbu tree is the endemic species in the Brazilian semiarid region with the greatest potential for integrating agronomic cultivation systems. It has a propagation protocol and

registered cultivars for clonal production, can be managed under without irrigation, and has great acceptance and use of fruits for consumption in natural or semi-industrialized products. One of the biggest challenges for the advancement of umbu tree domestication is the development of new genotypes, by recombination of superior clones, which could result in new cultivars. We used a polycross strategy to obtain and identify hybrids, by monitoring with SSR loci, aiming at the development of new umbu tree cultivars.

The diversity parameter estimates, such as PIC and heterozygosity among loci, are in line with estimates from other studies with *Spondias*. Silva et al. (2017) also reported PIC values above 0.25 for most ISSR markers applied to estimate genetic diversity among *S. mombim*. Balbino et al. (2019) also reported expected and observed heterozygosity values ranging from 0.10 to 0.75 and 0.10 to 0.75, respectively, when estimating the genetic diversity among *S. tuberosa*. Gois et al. (2009) also reported values of observed heterozygosity higher than expected among *S. lutea*. Heterozygosity is an important parameter for genetic studies, because when at high levels, it indicates an increasing possibility to obtain new genotypic combinations (Sebbenn et al., 2000).

The co-phenetic correlation coefficient of 85% is close to that reported by Lima et al. (2011), in *S. mombim* and by Santos et al. (2008), in *S. tuberosa*. Co-phenetic correlation >85% indicates a good fit in the UPGMA dendrogram (Sokal and Rohlf, 1962). Dendrogram is an additional parameter for the selection of accessions to be crossed, by selecting those with greater divergence. The similarity coefficient ranged from 0.44 to 0.68 among the eight parents and indicates great diversity and the possibility of obtaining genotypes superior to parents. Similarity values close to the present study were estimated by Santana et al. (2011), in *Spondias* sp.

Despite the pioneering application of SSR loci to identify male parents in polycross in umbu tree, in other species this strategy have been applied. Nandakaumar et al. (2000) reported the use of SSR loci to identify parents in 11 rice hybrids. Tew and Pan (2010) also reported the use of SSR loci to identify parents among sugar cane hybrids. Santos and Lima Neto (2011) identified mangoes hybrids by applying SSR loci. The outcrossing rate estimated in the BGU30 progenies, 90%, is close to that estimated for *S. tuberosa*, 81%, by Santos et al. (2013). The lowest outcrossing rate in the BGU37 progenies, 32%, may occur due the close position of BGU24, not included in the present genotyping.

The putative hybrids BGU30 × BGU48 and BGU30 × BGU55 have great potential for future evaluations, as they were divergent in the dendrogram, forming different groups, while hybrids BGU30 × BGU44 and BGU30 × BGU68 also have potential because male parents have higher weight fruits, 85g and 96g, respectively. The putative hybrids BGU37 × BGU44, BGU37 × BGU55 and BGU37 × BGU52 also have the potential for future evaluations, as their parents belong to different groups or have considerable fruit weight. UC was the major male parent among BGU37 hybrids, which have a low potential for recovery of superior segregating genotypes.

The hybrids resulting from these polycrosses are of great interest for breeding and are new for the umbu tree. The 10 SSR loci were sufficient to identify a single parent in 12 BGU30 hybrids (60%) and all 14 BGU37 hybrids, signaling the potential of this genotyping in polycrosses to determine paternity. In situations where the hybrids had more than one parent, additional polymorphic SSR loci should be applied.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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