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Breeding cassava for apomixis

Nagib M.A. Nassar[1] and Rosane G. Collevatti[2]

[1]Departamento de Genética e Morfologia, Universidade de Brasília, Brasília, DF, Brasil
[2]Pós-Graduação em Ciências Genômicas e Biotecnologia, Universidade Católica de Brasília, Brasília, DF, Brasil
Corresponding author: N.M.A. Nassar
E-mail: nagnassa@rudah.com.br

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ABSTRACT. Apomixis genes have been successfully transferred to cassava (*Manihot esculenta*) by hybridizing it with the wild species, *M. glaziovii*. The interspecific hybrid of cassava and *M. glaziovii* was exposed to open pollination during three subsequent generations. Seven sibs and the maternal progenitor of the fourth generation were genotyped using five microsatellite loci previously developed for cassava. All sibs were identical with each other and with their maternal progenitor. Sibs from *M. glaziovii* proved to be identical when examined by the same microsatellite loci. This evidence leads to the conclusion that apomixis does occur in wild-cassava relatives and apparently has played an important role in *Manihot* speciation. This is the first report of nearly 100% apomixis.

Key words: Apomixis, Molecular marker, Satellite technique

INTRODUCTION

Cassava (*Manihot esculenta*) is the most important staple crop in the humid tropics and is food for more than 800 million people (FAO, 2001). Apomixis is the asexual production of seed and apomictic plants are clones of their mothers. It preserves heterozygosity vigor and maintains superior varieties without genetic segregation. In cassava, propagation is normally by cuttings which accumulate bacterial germs and virus year after year, causing deterioration of productivity. The use of seeds in reproduction of this crop may avoid contamination. Thus, an advantage of introducing apomixis in the cultivated cassava is that it will assure preservation of superior clones in place of their extinction since new emerging stems through apomixis will be free from viral and bacteria germs. If apomixis was found or had been introduced into the excellent Brazilian clones like guaxupe and vassourinha, they would not have become extinct, and could have been preserved for a long time. Apomixis also benefits international centers that routinely export their germplasm, because the destination country needs only to raise one plant and further propagate it vegetatively, maintaining its superiority.

Through our program of surveying apomixis in *Manihot* species it was discovered in the wild-cassava *Manihot glaziovii*. Since this species has resistance to both mealy bug and bacteria blight, it was planned to use interspecific hybridization to transfer genes for apomixis as well as resistance to mealy bug and bacterium blight.

Hybrids between cassava and the wild-species *M. glaziovii* were obtained in 1995 by Nassar (1995). The detection of aposporic embryo formation by clearing method showed apomixis in these hybrids. Details of this method can be found elsewhere (Nassar et al., 1998b, 2000; Nassar, 2001). The hybrid, however, formed fibrous roots, which impedes its utilization for human consumption. It was left for open pollination among a population of cassava. Its progeny was examined embryonically; all sibs but one showed apomixis and sterility. The only plant that was fertile had its seeds grown to form a progeny of 22 sibs. These plants were examined embryonically by the first author in 2000. One plant out of them proved to be aposporic (clone 307). It was left for open pollination, formed fruit, and seven plants from its progeny were raised in 2002 for genetic analysis to confirm apomixis.

Microsatellite simple sequence repeats (SSR) are co-dominant and multiallelic markers that usually display high levels of polymorphism and expected heterozygosity, and a low probability of genetic identity (Morgante and Olivieri, 1993). Consequently, microsatellites are one of the most powerful molecular markers to understand detailed patterns of parentage composition and for individual discrimination in clone identification (e.g., Dayanandan et al., 1998; Rita et al., 2002; Bekkaoui et al., 2003; James et al., 2003; Wyman et al., 2003). Additionally, microsatellite primers may be transferred between closely related species because of the homology of flanking regions of simple sequence repeats.

We used microsatellite screening of cassava clone 307 and its progeny, and of the original progenitor plant *M. glaziovii*, to determine if there was apomixis.

MATERIAL AND METHODS

For the genetic analysis of the mother plants, a cassava clone namely 307 (see Appendix) obtained by the first author through his program of cassava breeding (Nassar and Santos, 2002) and *M. glaziovii*, and their progeny (seven sibs each), expanded leaves were collected

and stored at -80ºC. Additionally, leaf samples from two individuals of *M. esculenta* were collected for amplification control. Genomic DNA was extracted following a standard CTAB procedure (Doyle and Doyle, 1987).

Fourteen microsatellite loci previously developed for *M. esculenta* (Chavarriaga-Aguirre et al., 1998) were tested on clone 307 and *M. glaziovii* to perform the genetic analysis: GAGG-5, GA-12, GA-6, GA-21, GA-57, GA-126, GA-127, GA-131, GA-134, GA-136, GA-140, GA-161. The DNA from the mother plants (clone 307 and *M. glaziovii*) and two sibs from each progeny array were used. The DNA from two individuals of *M. esculenta* was used as a positive control. Microsatellite amplifications were performed in a 15-µLvolume containing 0.3 µM of each primer, 1 unit Taq DNA polymerase (Phoneutria, Brazil), 250 µM of each dNTP, 1X reaction buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$), 0.25 mg BSA, and 10.0 ng template DNA. Amplifications were performed using a Gene Amp PCR System 9700 (Applied Biosystems, CA, USA), under the following conditions: 96°C for 2 min (1 cycle), 94°C for 1 min, 45 or 55°C for 1 min (depending on the locus), 72°C for 1 min (30 cycles), and a final elongation at 72°C for 10 min (1 cycle). The amplified products were separated on 4% denaturing polyacrylamide gels stained with silver nitrate (Bassam et al., 1991) and sized by comparison to a 10-bp DNA ladder standard (Invitrogen, MD, USA). Those primers that amplified clear and interpretable products were used in our study.

The number of alleles for each locus and the expected and observed heterozygosities under Hardy-Weinberg equilibrium were estimated (Nei, 1978). The probability of genetic identity (Paetkau et al., 1995) was obtained in two manners, for each locus and for the loci overall. First, considering that the progeny array was obtained from an open-pollinated population, without genetic drift, the mutation allele frequency should be maintained between generations, as the first parents were heterozygous for all loci. Consequently, we expected that the frequency of all alleles in the population would be P = 0.25. This method may give a maximum value that should be obtained if all alleles presented the same frequency in the population (P = 0.25). Second, the mother plants were exposed to open pollination in a cassava living-collection population; thus, we estimated the probability using allele frequencies obtained by Elias et al. (2001) from a genetic diversity study of 290 individuals of 29 *M. esculenta* varieties. Although this method does not estimate the probability for the population of cassava used in our study, we obtained an expected value for the battery of loci developed for *M. esculenta*.

RESULTS

Among the 14 microsatellite loci tested five (GA-12, GA-13, GA-16, GA-21, GA-126) amplified for both clone 307 and *M. glaziovii*, and one (GA-131) amplified only for *M. glaziovii*. The two *M. esculenta* plants (positive control) presented clear amplification for all loci. Both samples of *M. esculenta* presented the same genotype as clone 307 and its progeny array for the five loci examined in this clone. Both *M. esculenta* and clone 307 presented the genotype 98/116 bp for GA-131, which was different for *M. glaziovii* (116/116).

All loci presented just one allele for the clone 307 mother plant and its progeny (Table 1, Figure 1). For *M. glaziovii* and its progeny all loci presented just one allele, except for GA-21 and GA-126, which presented two alleles (Table 1). Just one individual (GF-25) was heterozygous for GA-21, and observed and expected heterozygosity were equal to 0.125. All individuals were heterozygous for GA-126, and observed and expected heterozygosity were 1.000 and

0.533, respectively. Hence, the probability of exclusion of first and second parents for the battery of loci was very low (0.131008 and 0.232318, respectively). The probability of genetic identity (*I*), considering equal frequencies for all alleles at all loci (P = 0.25), was *I* = 0.1094 for each locus, and the combined probability was *IC* = 1.56527 x 10^{-5}, for the five loci used for 307 and its progeny array, and *IC* = 1.71202 x 10^{-6}, for the six loci used for *M. glaziovii* and its progeny array. Given the allele frequency obtained by Elias et al. (2001), the genetic identity could be determined only for four loci: GA-12 (*I* = 0.2833), GA-21 (*I* = 0.4129), GA-126 (*I* = 0.1437), and GA-131 (*I* = 0.1684), resulting in a combined probability of IC = 2.85124 x 10^{-3}.

Table 1. Genotypes of cassava clone 307 and its progeny (seven sibs), and *Manihot glaziovii* and its progeny (seven sibs), based on six microsatellite loci transferred from *M. esculenta*. Allele size is given in base pairs.

Individuals	Microsatellite loci					
	GA-12	GA-13	GA-16	GA-21	GA-126	GA-131
307/M, 307/2-9	140/140	140/140	104/104	114/114	180/180	-
G/M, G/24, G/26, G/29-32	140/140	140/140	104/104	114/114	176/206	116/116
G/25	140/140	140/140	104/104	110/114	176/206	116/116

307/M = clone 307 - mother plant; 307/2 to 307/9 = clone progeny; G/M - *M. glaziovii* mother plant; G/24 to G/32 = *M. glaziovii* mother plant progeny.

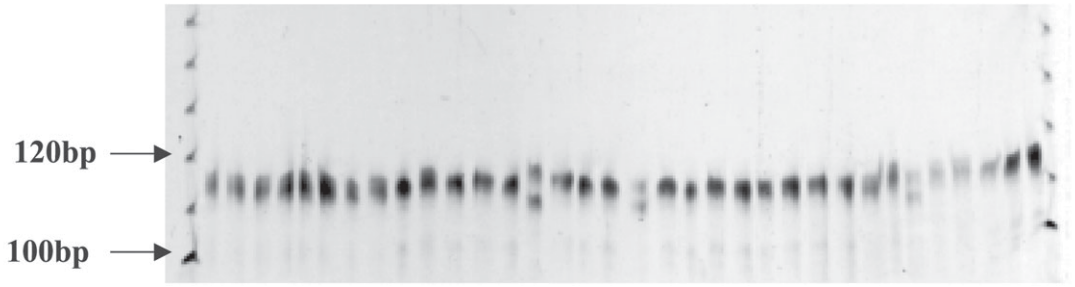

Figure 1. Genotype of cassava clone 307 and *Manihot glaziovii*, and their progeny for locus GA21, visualized in silver-stained denaturing polyacrylamide gel. First and last lanes are a 10-bp DNA ladder standard (Invitrogen). Second and third lanes are positive controls (*M. esculenta*). Fourth to 11th lanes are the hybrid and its progeny array; 28th to 35th lanes are *M. glaziovii* and its progeny.

DISCUSSION

All the progeny from clone 307 may be the outcome of apomixis, because all sibs presented the same genotype as the mother for the five loci (Table 1, Figure 1). Apomixis can be inferred for *M. glaziovii*, since only one sib presented a different genotype from the mother plant (Table 1, Figure 1), as a result of cross-pollination.

Considering that the progeny were submitted to open pollination in each generation, the low number of alleles may be due to the low diversity of the original population used in the controlled breeding (selection) program, the homozygous genotype of the parental used to start

the breeding program, genetic drift during the breeding research, or the occurrence of apomixis.

The loci used in this work have a medium to high number of alleles (5-15, according to Chavarriaga-Aguirre et al., 1998; 4-9, according to Elias et al., 2001). The battery of loci used displayed a medium power of individual distinction, showed by the probability of genetic identity. This may be the result of the breeding design, resulting maximum values of 1.56527×10^{-5} and 1.711202×10^{-6}.

The genetic uniformity of *M. glaziovii* and its progeny detected by microsatellite analysis showed the apomictic nature of the maternal plant. It seems that apomixis has played an important role in *Manihot* speciation. Apparently, polyploidy provided the wide genetic variability of the genus and apomixis maintained the genotypes that are favored in certain niches. Facultative apomixis may maintain genetic variability through sexual reproduction. These new genotypes may undergo another cycle of speciation under new environmental conditions. Rogers and Appan (1973) defined seven species in the subgenus *Glazioviannae*, to which *M. glaziovii* belongs. The differences among these species are very narrow and are limited to inflorescence size, petiole attachment and bracteole width. These species do hybridize in our living collection, creating intermediate types.

The identical genotype of plant 307 and its progeny confirms their apomitic nature. The finding that all sibs investigated are probably the outcome of apomixis showed the high level that apomixis has reached in this clone. Apomixis has been documented in the progeny of cassava hybrids with *M. dichotoma* (Nassar, 1995; Nassar et al., 1998a,b) and with *M. neusana* (Nassar et al., 2000; Nassar, 2001), but it was detected only in less than 1% of the progeny, which was confirmed by RAPD technique. Later, after selection, plants with 13% apomixis were found (Nassar and Santos, 2002). Genes controlling different levels of apomixis have been found in wild relatives of cultivated crops, such as in *Panicum maximum* (Warmke, 1954). Several F2 genotypes, which resulted from interspecific hybridization of cassava with wild relatives in our living collection, have shown strong evidence of apomixis and abundant fruitfulness, accompanied by sterility. In this case, it is clear that recurrent selection has resulted in a very significant increase in apomixis level, to a point making the 307 clone very practical for use in cassava production.

Some authors have questioned if obligate apomixis exists (Askor, 1979). Here we have demonstrated a very high level of apomixis. The finding of apomixis in the first generation of hybridization, and its absence in the following two generations, when plants were exposed to open pollination, clearly shows two genetic facts concerning apomixis inheritance in cassava. First, apomixis is determined by recessive alleles. Second, it is controlled by more than one pair of genes, probably located on different chromosomes. One of the most important consequences of our findings is the possibility of mapping and characterizing the genes of apomixis with molecular markers, and of isolating it in the future.

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APPENDIX: DESCRIPTION OF CLONE 307

The plant is 2 m in height; roots from seed are swollen (not a tap root); the root shape is ovate, 20-30 cm long; root color is dark brown; stipule scars raised and prominent. Leaves are palmately lobed with seven lobes. Leaf lobes obovate, 8-10 cm long; the margins are entire; petioles about 15 cm, attached basally to the lamina, frequently five-lobed; lamina dark green with red petioles; petioles 10 cm long; young foliage at stem apices green. The inflorescence is a panicle about 6 cm long; bracts and bracteoles are caduceus. Pistillate flowers green; staminate flowers green with almost sterile stamens, with 10% pollen viability (measured by carmin staining). The fruit is winged and dark green. Seeds are carunculate, elongate, and dark brown in color.