Moleular Technique confirms high apomixis level in Cassava bred clones

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Abstract

Apomixis genes have been transferred successfully to cassava by hybridizing it with the wild species, *M. glaziovii*. The interspecific hybrid of cassava and *M. glaziovii* was left for open pollination during subsequent three generations. Seven sibs and their 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* itself proved to be identical when examined by the same microsatellite *loci*. These evidences lead to the conclusion that apomixis do occur in wild cassava relatives and apparently has played an important role in *Manihot* speciation.

Introduction

Cassava is the most important staple crop in the humid tropics and a food for more than 800 millions people (FAO,2001). Apomixis is the asexual production of seed and apomictic plants are clones of their mothers. It preserves heterozigosity vigour 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 been extinct, and had been preserved for a long time. Apomixis beneficiates also international centers who export routinely their germplasm, because the destination country needs only to raise one plant and further propagate it vegetatively maintaining its superiority.

In the earlier work of the first author, apomixis was discovered in the wild cassava *Manihot glaziovii*. Since this species is characterized by resistance to both mealy bug and bacteria blight, it was planned to transfer genes of apomixis as well as resistance to mealy bug and bacteria blight by interspecific hybridization.

In 1996 hybrids between cassava and the wild species *M. glaziovii* were obtained by the first author. The detection of aposporic embryos formation by clearing method showed apomixis in these hybrids. Details of this method can be found elsewhere (NASSAR et al. 1997 ; 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 were examined for apomixis embrionically and all sibs but one showed apomixis and sterility. The only plant that showed fertility had its seeds grown to form a progeny of 22 sibs. These plants were examined embrionically 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 low probability of genetic identity (MORGANTE and OLIVIERI, 1993). Because of that, 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.

In this report we present the results of microsatellite screening of the plant clone 307 and its progeny, and of the original progenitor plant *M. glaziovii* to confirm the occurrence of apomixis.

MATERIAL AND METHODS

For the genetic analysis of the mother plants, clone 307 and *M. glaziovii*, and their progeny (seven sibs each), expanded leaves were collected and stored at –80ºC. Additionally, 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 for transferability to clone 307 and to *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. To test transferability the DNA from the mother plants (clone 307 and *M. glaziovii*) and two sibs from each progeny array were used. Additionally, the DNA from two individuals of *M. esculenta* was used as positive control. Microsatellite amplifications were performed in a 15 ml volume containing

0.3 mM of each primer, 1unit Taq DNA polymerase (Phoneutria, BR), 250 mM 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 of template DNA. Amplifications were performed using a Gene Amp PCR System 9700 (Applied Biosystems, CA) with the following conditions: 96°C for 2 min (1 cycle), 94°C for 1 min, 45 or 55°C for 1 min (according to each *locus*), 72°C for 1 min (30 cycles); and a final elongation of 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).

Those primers that amplified clear and interpretable products were used in our study. For this, the DNA of all individuals was amplified using the same PCR conditions presented above and fragments were visualize again 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).

For each *locus* number of alleles and expected and observed heterozygosities under Hardy-Weinberg equilibrium were estimated (NEI 1978). Other analysis of departure of Hardy-Weinberg, probability exclusion of the first and second parents and assignment test (MARSHALL et al. 1998) could not be performed because all the sibs, but one individual from the *M. glaziovii* progeny array, presented the same genotype as the mother plant. For the same reason, it was not possible to estimate the likelihood of observing at least n identical multilocus genotypes by simulation (STENBERG et al. 2003) Probability of genetic identity (PAETKAU et al. 1995), the probability of two random individuals displaying the same genotype, was obtained for each *loci* and overall *loci* in two manners. First, considering that the progeny array was obtained from a open pollinated population - without genetic drift and mutation allele frequency should be maintained between generations – if the first parents were heterozygous for all loci, than we expected that the frequency of all alleles in the population should be p=0.25. This method may represent a maximum value that should be obtained if all alleles presented the same frequency in the population (p=0.25). Second, in each generation mother plants were let for open pollination in a cassava living collection population; thus we estimated the probability using allele frequency obtained by ELIAS et al. (2001) from a genetic diversity study of 290 individuals from 29 varieties of *M. esculenta*. Although this method does not estimate the probability for the population of cassava used in the study, we obtained an expected value for the battery of loci developed for M. esculenta.

Results

From the 14 microsatellite loci tested five (GA12, GA13, GA16, GA21, GA126) and six loci (GA12, GA13, GA16, GA21, GA126 and GA131) amplified for 307 and for M. glaziovii, respectively. The two individuals of M. esculenta (positive control) presented clear amplification for all *loci*. For the five loci used for clone 307 and its progeny array, both individuals of *M. esculenta* presented the same genotype as them. For GA131 both presented the genotype 98/116 bp, different from M. glaziovii (116/116).

All *loci* presented just one allele for the mother plant 307 and its progeny (Table 1). Thus, observed and expected heterozygosity and probability of exclusion of first and second parents were equal to zero. For *M. glaziovii* and its progeny all *loci,* but GA21 and GA126 that presented two alleles, presented just one allele (Table 2). For GA21 just one individual was heterozygous (GF25) and observed and expected heterozygosity were equal to 0.125. For GA126 all individuals were heterozygous and observed and expected heterozigosity were 1.000 and 0.533, respectively. Hence, 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 frequency for all alleles in all *loci* (p=0.25), was *I*=0.1094 for each *loci*, and combined probability was $IC=1.56527.10^{-5}$, for the five *loci* used for 307 and its progeny array, and $IC=1.71202.10^{-6}$, for the six *loci* used for *M. glaziovii* and its progeny array. Considering the allele frequency obtained by ELIAS et al. (2001), the genetic identity could be determined only for four *loci*: GA12 (*I*=0.2833), GA21 (*I*=0.4129), GA126 (*I*=0.1437) and GA131 (*I*=0.1684), resulting in a combined probability of $IC=2.85124.10^{-3}$.

DISCUSSION

Our results indicate that all the progeny from the clone 307 may be the outcome of apomixis because all sibs presented the same genotype as the mother for the five *loci* (Table 1, Fig 1). Apomixis can be inferred for *M. glaziovii* either, since only one sib presented a different genotype from the mother plant (Table 2, Fig. 1), as a result of cross pollination.

Considering that each progeny were submitted to open pollination in each generation, the low number of alleles may be the outcome of the low diversity of the original population used in the control breeding, homozygous genotype of the parental used in the beginning of the breeding program, genetic drift during the breeding research, or the occurrence of apomixis.

The *loci* used in this work presented medium to high number of alleles (five to 15) in CHAVARRIAGA-AGUIRRE et al (1998) and on ELIAS et al. (2001) (four to nine alleles). 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 in a maximum value of $1.56527.10^{-5}$ and $1.711202.10^{-6}$.

The genetic uniformity of *M. glaziovii* ant its progeny detected by microsatellite showed the apomictic nature of the maternal plant. It seems that apomixis has played an important role in the whole *Manihot* genus speciation. Apparently, polyploidy have been providing the wide genetic variability of the genus and apomixis maintained the genotypes that may be favoured in certain niches. The facultative apomixis may keep the genetic variability through sexual reproduction. These new genotypes may undergo another cycle of speciation in 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 limited to inflorescences size, petiole attachment and bracteole width. These species do hybridise in our living collection creating intermedian types.

The identical genotype of plant 307 and its progeny confirms their apomitic nature. The fact that all sibs investigated may be the outcome of apomixis showed the high level that the apomixis reached in this clone. The first author documented apomixis in the progeny of cassava hybrids with *M. dichotoma* (NASSAR 1995, NASSAR et al. 1998a, 1998b) and in cassava hybrids with *M. neusana* (NASSAR et al. 2000, NASSAR 2001), but it was detected only on less than 1% of the progeny,

confirmed by RAPD technique. In a further work of the same author, he could selected plants for a higher level of apomixis that reached 13% (NASSAR and SANTOS , 2002). This is the first report where apomixis is as high as almost 100%. 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 hybridisation 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 clearly that recurrent selection has resulted in a very significant increase in apomixis level to point making the present clone a very practical for use in cassava production, particularly because of its high root productivity which reached 6-7 kg per plant.

Some authors have questioned if obligate apomixis exists (ASKAR, 1979), but certainly a very high level of apomxis has been reached in this work. The presence of apomixis in the first generation of hybridisation, and its absence on the following two generations, when plants were left for open pollination, shows clearly two genetic facts with respect of apomixis inheritance in cassava: first, apomixis is determined by recessive alleles; second, it is controlled by more than one pair of genes, probably located in different chromosomes. One of the most important consequences of our results is the possibility of mapping and characterising the genes of apomixis by molecular markers and isolating it in the future.

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APPENDIX:

Description of the clone 307

The plant is 2 m. height, roots from seed are swollen (not a tap root), the shape is ovate with 20-30cm, color is dark brown, stipules scars raised and prominent. Leaves are palmately lobed with seven lobes. Leaves lobe obovate with 8-10 cm; its margin are entire, petioles about 15 cm, attached basally to the lamina, frequently 5 lobed, lamina dark green with red petioles, petioles 10 cm long, young foliage at stem apices green. Inflorescence a panicle about 6 cm long, bracts and bracteoles are caduceus. Pistillate flowers green, staminate flowers green with stamins almost sterile having 10% of pollen viability measured by carmin stain. Fruit winged, dark green. Seeds are caranculate and elongate and high brown in color.

Table 1. Genotype of the clone 307 and its progeny (seven sibs), based on five microsatellite *loci* transferred from *M. esculenta*. 307/M – clone 307 – mother plant; 307/2 to 307/9 – progeny; allele size in base pair.

Individual	***GA12***	***GA13***	**GA16**	**GA21**	**GA126**
307/M	140/140	140/140	104/104	114/114	180/180
307/2	140/140	140/140	104/104	114/114	180/180
307/3	140/140	140/140	104/104	114/114	180/180
307/4	140/140	140/140	104/104	114/114	180/180
307/5	140/140	140/140	104/104	114/114	180/180
307/6	140/140	140/140	104/104	114/114	180/180
307/8	140/140	140/140	104/104	114/114	180/180
307/9	140/140	140/140	104/104	114/114	180/180

Table 2. Genotype of *M. glaziovii* and its progeny (seven sibs), based on six microsatellite *loci* transferred from *M. esculenta*. G/M – *M. glaziovii* mother plant; G/24 to G/32 – progeny; allele size in base pair.

Individual	**GA12**	**GA13**	**GA16**	**GA21**	**GA126**	**GA131**
G/M	140/140	140/140	104/104	114/114	176/206	116/116
G/24	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
G/26	140/140	140/140	104/104	114/114	176/206	116/116
G/29	140/140	140/140	104/104	114/114	176/206	116/116
G/30	140/140	140/140	104/104	114/114	176/206	116/116
G/31	140/140	140/140	104/104	114/114	176/206	116/116
G/32	140/140	140/140	104/104	114/114	176/206	116/116

Figure Legends

Fig. 1. Genotype of the clone 307 and *M. glaziovii*, and their progeny for *locus* GA21, visualized in silver-stained denaturing polyacrylamide gel. First and last lanes are 10 bp DNA ladder standard (Invitrogen). Second and third lanes are positive controls (*M. esculenta*). Fourth to eleventh lanes are the hybrid and its progeny array. Twenty eighth to thirty fifth lanes are *M. glaziovii* and its progeny.