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Full Length Research Paper

Development of genomic tools for verification of hybrids and selfed progenies in cassava (*Manihot esculenta*)

Gerald Otti, Ayodele Fakoya, Ikpan Andrew and Melaku Gedil*

International Institute of Tropical Agriculture, Oyo Road PMB 5320, Ibadan Nigeria.

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Cross contamination arising from random natural processes and breeding errors is one of the prominent challenges to breeding in many crops including cassava (*Manihot esculenta*). This study attempts to identify molecular marker tools suitable for verifying the genetic purity of putative progenies of populations of cassava. It also proposes a rapid, high-throughput genomic DNA extraction protocol which is a modification of an existing extraction protocol adapted to the pace required for the DNA-based verification of often large population sizes. Three polymorphic simple sequence repeat (SSR) markers were selected from a total of 125 and used for genotyping three populations. The petiole color trait was also used to verify TMS 96/1089A X TME117 where the pink color of the male parent was dominant over the female's green color. The pace of genomic analysis of populations used in the study was enhanced using a modified , quicker DNA isolation protocol which slashed extraction time by 60%. SSRY153 identified six false progenies in the selfed line, 1M18. Segregation patterns of In combination, both markers identified 2 selfed individuals out of a total of 207. NS890 identified two false hprogenies resulting from selfing in the female parent of the cross TMS 30001 X TMS 96/1089A, out of a total of 93, The principles described for verification using each of the SSR markers applied in this study can be extended to other SSR markers with corresponding nature of polymorphism in any population of the crop. Increased attention would go to the development of more efficient markers such as single nucleotide polymorphisms (SNPs) and other gene-based markers using new and advanced genomics techniques for routine integration of such quality control step in the breeding scheme.

Key words: Cassava, simple sequence repeat (SSR), morphological trait, molecular markers, genomic DNA.

INTRODUCTION

Cassava, *Manihot esculenta*, is not only a staple food for hundreds of millions of people but also gradually becoming an industrial crop. Full exploitation of its huge potentials is however dependent on improvement to overcome production obstacles as well as meet specific consumer requirements. Breeding materials specific to agro-ecological regions are therefore being developed with focus on food, feed and agro-industrial use traits (Dixon et al., 2003), multiple disease and pest resistance, early vigor in plant growth, early root bulking, low cyanide and other favorable traits. The development of these materials involve extensive intercrossing of cassava clones with diverse traits to create superior genotypes combining multiple desirable traits followed by several

*Corresponding author. E-mail:m.gedil@cgiar.org. Tel: +1-201-633-6094-2703.

Abbreviations: **PCR**, polymerase chain reaction; **RFLP**, restriction fragment length polymorphism; **SSR**, simple sequence repeat; **SNPs**, single nucleotide polymorphisms.

stages of field evaluation and selection (Kawano, 2001). Such crosses, usually between different species of the *Manihot* genus (inter-specific cross) or within the *Manihot esculenta* species (intra-specific crosses) are subject to conditions capable of compromising cross accuracy. Naturally, cassava is subject to both cross-pollination (outcrossing) and self-pollination (selfing) depending on genotype, planting design and type of pollinating insect present (Jennings and Iglesias, 2002). Out-crossing is facilitated by the action of pollinating insects like wasps (*Polistes sp*) and honey bees (*Apis mellifera*), its major insect- pollinating agents in Africa and Latin America (Chavarriaga-Aguirre and Halsey, 2005) while selfing is made possible by the monoecious nature of the crop and by the fact that flowering on a single plant may last for more than two months (Jennings and Iglesias, 2002). In conventional cassava breeding practice, the contaminating effect of these rather random natural processes is checked by the adoption of controlled crossing which involves bagging female flowers before they open and subsequent pollination using specific pollens (Jennings and Iglesias, 2002; Chavarriaga-Aguirre and Halsey, 2005). Experience by cassava breeders has however shown a number of limitations inherent in this and indeed other methods of controlled crossing. For instance, absorption of contaminant pollens hanging on the surface of muslin pollen bags can be facilitated when the bags become wet after rainfall. Another window of contamination from controlled crossing (not restricted to cassava) comes from the possible erroneous admixture of foreign seeds during harvesting or of foreign pollens during fertilization (Bateman, 1947). In view of these limitations, it is evident that controlled crossing may not completely eliminate cross-contamination and thus may not yield an entirely pure hybrid population. The need therefore arises for the development of some means of verifying putative population hybrids. This study is therefore aimed at testing molecular tools for their applicability in rapid verification of the reliability of putative hybrid and selfed lines.

Genomics tools have been used to verify crosses (Supawadee and Sompong, 2009; Terzic et al., 2006; Gomez et al., 2008). Tools utilized are mainly molecular markers capable of detecting false progenies in a hybrid or selfed line. The most desirable marker tools are those codo-minant and capable of showing homozygous polymorphic alleles in parents. Of the major DNA marker types, restriction fragment length polymorphism (RFLP) and simple sequence repeat (SSR) markers are codominant (Gomez et al., 2008). SSR markers are however more convenient as they require smaller quantities of genomic DNA (Powell et al., 1996), do not involve the use of radioisotopes (Gomez et al., 2008) and are amenable to automation using PCR machines (McCouch, 1997). SSR markers have been used for the verification of inter-specific crosses (Terzic et al., 2006) as well as verification of purity of intra-specific crosses in crops like cotton (Dongre and Parkhi, 2005) maize (Salgado et al., 2006) and rice (Tamilkumar et al., 2009). They can be used to identify selfed progenies in crosses (Gomez et al., 2008) as well as false hybrids in populations by observation of banding patterns not consistent with Mendelian segregation laws (Salgado et al., 2006). Polymorphism in SSR markers include cases in which a marker has one allele size per parent and where it has two allele sizes for one parent and a single allele size for the other parent. In the latter case, one allele is usually common in both parents. Though not ideal, SSR markers showing the latter kind of poly-morphism have been applied in hybrid verification (Asif, 2009). In this study, an attempt is made at testing the utility of both categories of SSR markers. For routine application of such tools in a breeding scheme, it is necessary to have a rapid and cost-effective DNA extraction system. We present a rapid, high-throughput genomic DNA extraction method necessary to enhance the pace of the verification process and save appreciable time. The laborious and time-consuming nature of most DNA extraction procedures does not allow for rapid molecular characterization of large numbers of samples. For instance, the average time required for isolating genomic DNA from 192 samples is up to a whole working day (approximately 8 h) using the method of Bhattacharjee et al. (2009) and up to two working days (approximately 16 h) using Dellaporta et al. (1983) excluding time for sample collection and preparation. We therefore describe a fast, high-throughput, SDS-based 96-well plate extraction method which can isolate good quality DNA in lesser time.

MATERIALS AND METHODS

DNA extraction

Approximately 1.5 g fresh, young cassava leaves was used for DNA extraction. The extraction buffer is composed of 100 mM Tris-HCl [pH 8], 500 mM NaCl, 50mM EDTA, 1.0% SDS, 2% PVP, 1% β-mercaptoethanol, and 0.05 mg/ml Proteinase K. Fresh young leaves were collected into pre-labelled 1.2 ml 8-strip tubes arranged in a 96-well plate and containing 2.4 mm steel grinding balls. Sample collection was done on ice. The 96 well-plate containing the strip tubes described above were placed in liquid nitrogen for about 2 min to freeze-dry samples. The tubes were covered and grinding was done at 1000 strokes/minute for 1 min using a GENOGRINDER 2000® instrument (BT and C Inc., New Jersey). Grinding was repeated after addition of 400 µl of extraction buffer to each sample. Samples were incubated at 65°C in a water bath and then centrifuged at 3500 rpm for 10 min. 300 µl ice-cold isopropanol (300 µl) was added to supernatant and mixed by repeated gentle inversion. Samples were centrifuged at 3500 rpm for 20 min and supernatant decanted carefully without disturbing the pellet. The

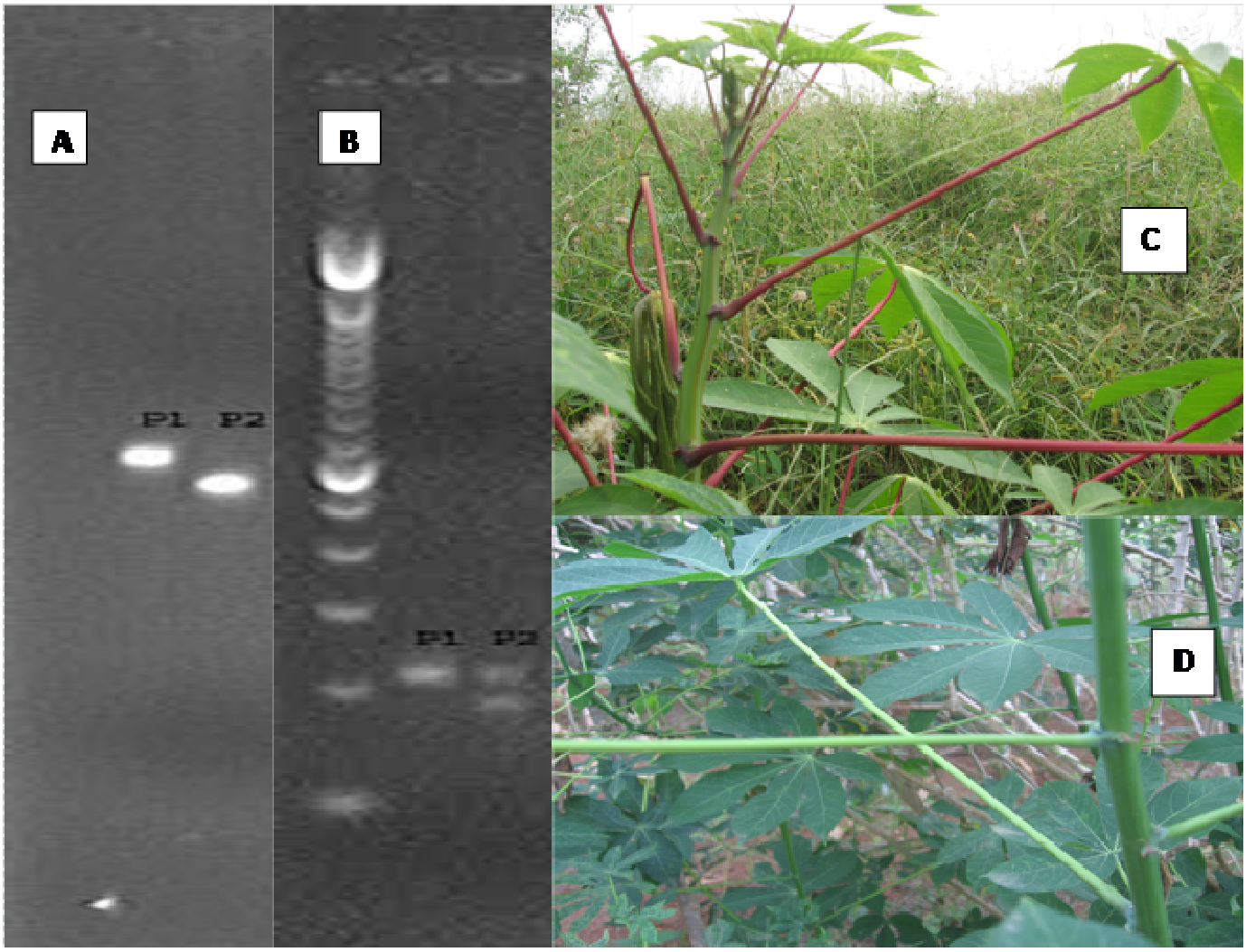

Figure 1. Marker and morphology-based polymorphisms of population parents. (A) Size polymorphism of NS158 between TMS 30001 (P1) and TMS 96/1089A (P2). (B) Size polymorphism of SSRY82 between TMS 96/1089A (P1) and TME117 (P2). (C) Pink petiole color of TME117. (D) Green petiole color of TMS 96/1089A.

samples can also be stored in – 80°C for 20 min before centrifugation. Precipitated pellet was washed with 300 µl of 70% ethanol. Dry pellets were recovered after centrifugation and re-suspended in 300 µl of ultra-pure water and then 3 µl of 10 mg/ml RNase A was added to each of the samples. Finally, samples were incubated for 10-15 min at 37°C.

DNA quantification and PCR amplification

The quality of a subset of 724 extracted DNA samples was examined by loading 2 µl of each sample on 0.8% agarose gel electrophoresis. Samples were randomly selected for accurate quantification using a NANODROP® spectrophotometer (Thermo Fisher Scientific Inc., Denver).

To verify the suitablility of the DNA for PCR, another set of 24 samples were randomly selected for PCR amplification .

Genotyping

Three populations of cassava were genotyped using SSR markers. These are the hybrid populations, TMS 30001 X TMS 96/1089A (93 progenies) and TMS 96/1089A X TME117 (207 progenies), and one selfed F1 line, 1M18 (47 F2 clones). Genotyping involved SSR marker amplification by polymerase chain reaction (PCR) and gel electrophoresis. Three SSR markers were selected from a total of one 125 based on their banding patterns in the population parents. For the hybrid populations, only markers showing size polymorphism between the parents were selected. Such polymorphic markers either had a single allele but different sizes in the two parents or heterozygous alleles in one parent but homozygous alleles in the other (Figure 1 A and B). In the latter case, one of the two alleles of the heterozygous parent is monomorphic and the other polymorphic to the only allele of the homozygous parent (Figure 1B).

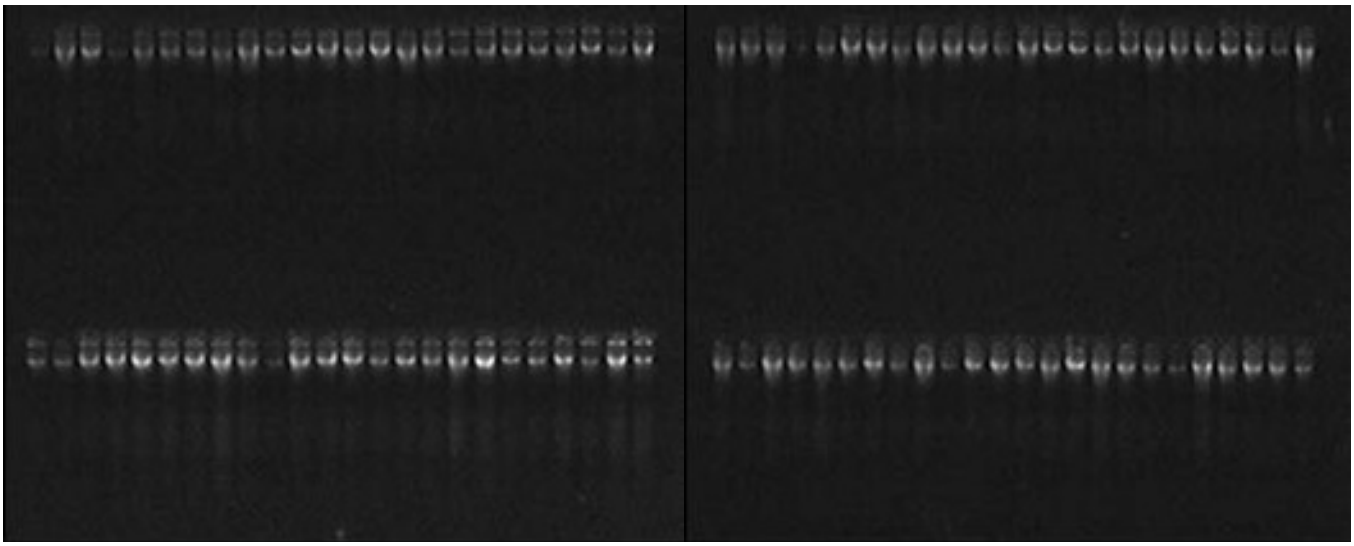

Figure 2. Quality of extracted DNA for 96 samples of 1089A X TME117 electrophoresed on 0.8% Agarose gel.

For the selfed line 1M18, a marker with two alleles (maximum number of alleles found on the parent for all screened markers) was selected. Forward and reverse primers of the selected three markers were used for PCR amplification in the populations for which they were selected.

PCR amplification involved preparation of a 25 µl PCR mix which included 14.8 µl ultra-pure H_2O, 2.5 µl 10x Buffer, 1.5 µl of 50 mM $MgCl_2$ at a final concentration of 3mM, 1.0 µl DMSO, 1 µl each of 5 µM forward and reverse primers at a final concentration of 0.2 µM, 2 µl of 2.5 mM dNTPs at a final concentration of 0.2 mM, 100 ng DNA template and 1 unit Taq polymerase. PCR amplification was achieved using a touch-down profile involving an initial denaturation step at 94°C for 2 min followed by 35 cycles of amplification with denaturation at 93°C for 15 s, annealing at 65°C for 20 s which is reduced at 1°C per cycle for the first 9 cycles, and extension at 72°C for 30 s. The final extension step was at 72°C for 5 min. 4 µl of PCR products were loaded on 2% Super Fine Resolution (SFR) Agarose and run at 90V for 4 h alongside 100 bp standard size marker. Gel banding pattern on gel stained with ethidium bromide was viewed in a UV minidark room.

Morphological characterization

Putative progenies of TMS 96/1089A X TME117 were assessed for petiole color. The parents had different petiole colors, green for the female and pink for the male (Figure 1 C and D). Morphological scores were taken according to standard descriptions of morphological traits (Fukuda, 1998).

RESULTS

DNA extraction

Three populations of cassava were subjected to molecular marker-based hybrid verification assay with the aim of identifying false hybrids or selfs. To enhance the pace of genomic analysis and drastically reduce the time required to verify a population, a high-throughput, rapid DNA extraction method was developed in parallel. The average extraction time per person was 3 h for 192 samples. This represents more than 60% reduction in the time required to extract the same and lesser number of samples using the Bhattarchajee et al. (2009) and Dellaporta et al. (1983) methods respectively. Major timesaving aspects of the protocol are the absence of a manual sample-grinding step of Dellaporta et al. (1983) and the introduction of Proteinase K in the extraction buffer. The truncated steps of extraction, notwithstanding, extracted DNA was of good quality (Figure 2), with A_{260}/A_{280} absorbance ratio values ranging between 1.8 and 2.0 and of reasonable yield, an average of about 1.2 µg/µl for the three populations in a total volume of 100µl (Table 2). Furthermore, extracted DNA had less shearing suggesting its suitablity to a wide array of application (Figure 2).

Genotyping and morphological assessment

Isolated genomic DNA showed good amplification upon genotyping. This allowed for clear and unambiguous identification of true or false hybrids. Three informative SSR markers out of a 125 markers were selected for screening three populations for the hybrid status of their putative progenies. These were SSRY82, SSRY153 and N890 used for the purpose of verifying populations B, C and A, respectively. Primer sequences of the above markers are presented in Table 1. True hybrids were identified their by possession of alleles of both parents.

Table 1. Primer sequences of markers used to genotype the three populations.

ID	Population	Population size	Marker	Forward primer sequence of marker	Reverse primer sequence of marker
A	TMS 30001 X TMS 96/1089A	93	NS890	TAAATTGGGGGTTCTTGCTC	TGCTTACTCTTTGATTCCACG
B	TMS 96/1089A X TME117	207	SSRY82	TGTGACAATTTTCAGATAGCTTCA	CACCATCGGCATTAAACTTTG
C	1M18	47	SSRY153	TTCCAGAAAGACTTCCGTTCA	CTCAACTACTGCACTGCACTC

Table 2. Concentration and absorbance ratio values of randomly selected putative genotypes of three populations of cassava.

Putative population genotypes	A_{260}/A_{280}	Concentration (ng/ µl)
1M18		
1m18-1	1.96	1264
1m18-3	1.98	1544
1m18-10	1.96	792
1m18-15	1.9	1903
30001 X 1089A		
30001x1089a_1	1.84	1993
30001x1089a_6	1.87	1170
30001x1089a_12	1.85	436
30001x1089a_21	1.87	922
1089A X TME117		
1089axTme117_97	1.94	1140
1089axTme117_102	1.95	861
1089axTme117_121	1.92	528
1089axTme117_128	1.95	441

For the selfed population C, only genotypes possessing either of the parental alleles, are considered true (Figure 5). The marker NS890 showed a single fragment of different size in each of 30001 and 1089A (Figures 1 and 3). It identified 2 false genotypes which resulted from selfing of the female parent (Table 3). Population B was verified using SSRY82, a polymorphic marker showing two allele-sizes in TME117 and one allele-size in 1089A (Figures 2 and 4). Morphological assessment of petiole color in the progenies showed that 203 individuals representing 98.1% of the total of 207 assessed were pink and of different pinkish green shades. However only four individuals (approximately 2% of the total) were green.

Population C was genotyped using SSRY153. The marker has two allele sizes in the parent (Figure 5). It identified 6 false progenies in 41 putative progenies of 1 M. The banding patterns of these six progenies were strikingly dissimilar to that of the parent, each having a unique allele not found in the parent (Figure 5).

DISCUSSION

Molecular marker-based verification of putative population hybrids of cassava can be integrated into germplasm enhancement pipeline, with the availability of rapid and cost-effective DNA extraction protocol used in this study. Over 60% reduction in extraction time would imply a 30% reduction in the overall time between DNA extraction and genotyping of 192 samples, if genotyping alone takes a full working day (8 h). The reduction in time required to verify any average (100 - 200) population makes it

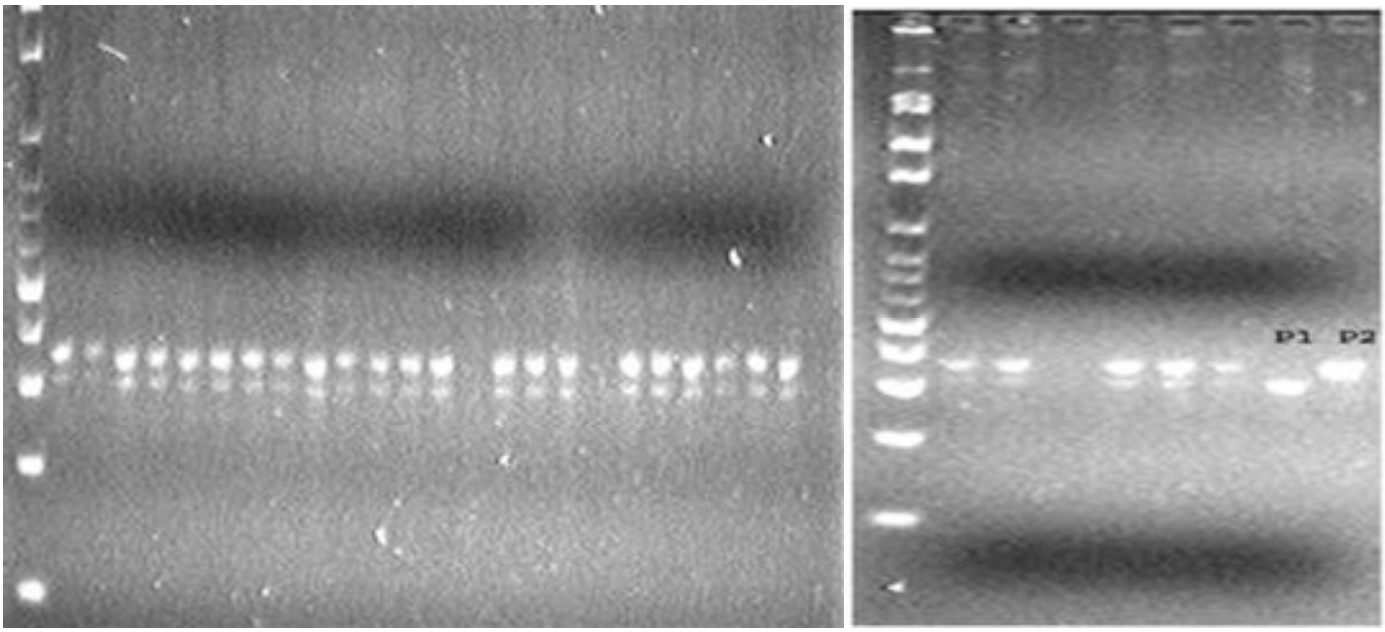

Figure 3. Banding patterns of NS890 in parents and putative progenies of 30001X1089A. Both parents are homozygous with alleles of different sizes. P1 is 30001 and P2 is 1089A. All progenies shown are true.

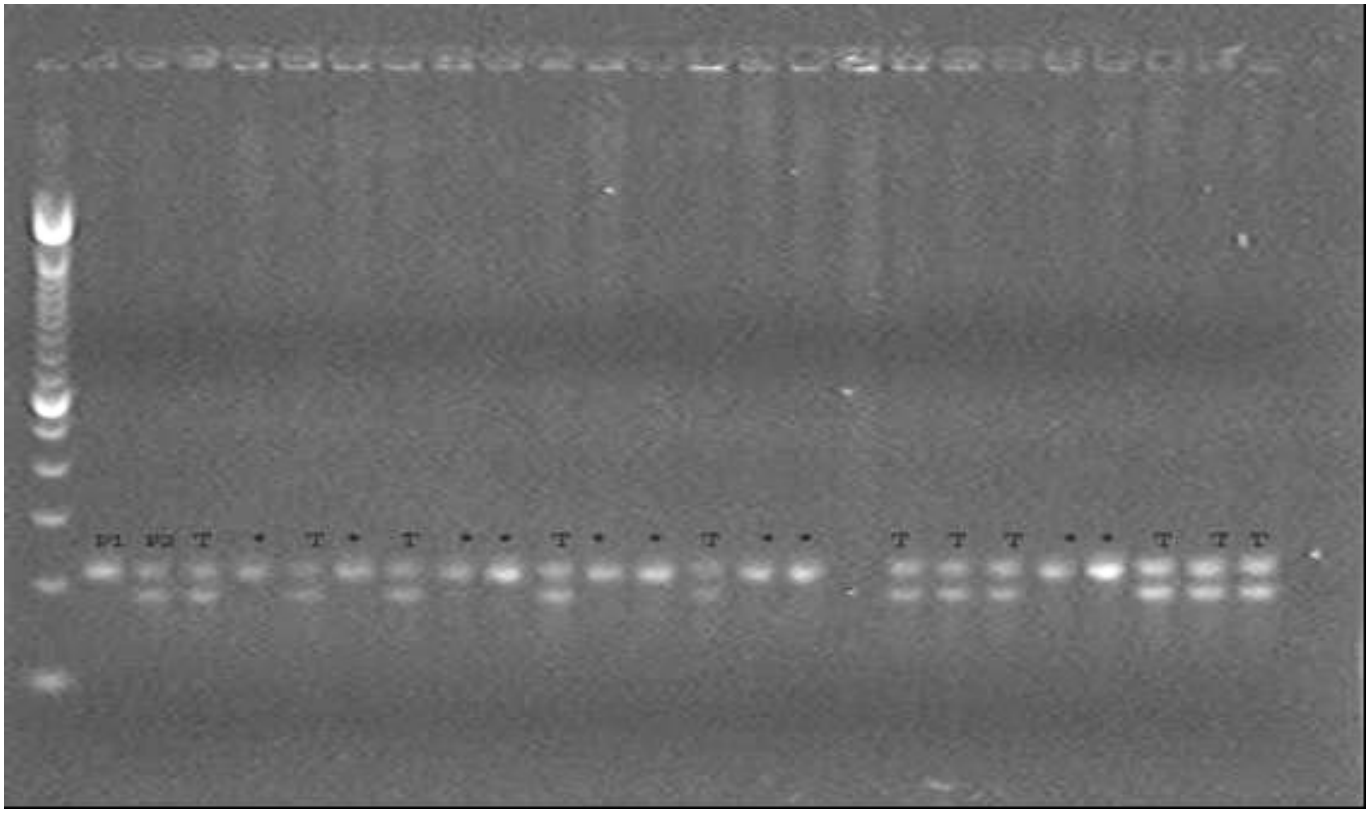

Figure 4. Banding patterns of SSRY82 in parents and putative progenies of 1089AXTME117. P1 is homozygous and P2 is heterozygous. P1 is 1089A and P2 is TME117. Bands labeled T are true whereas bands labeled * are unclassifiable using this marker.

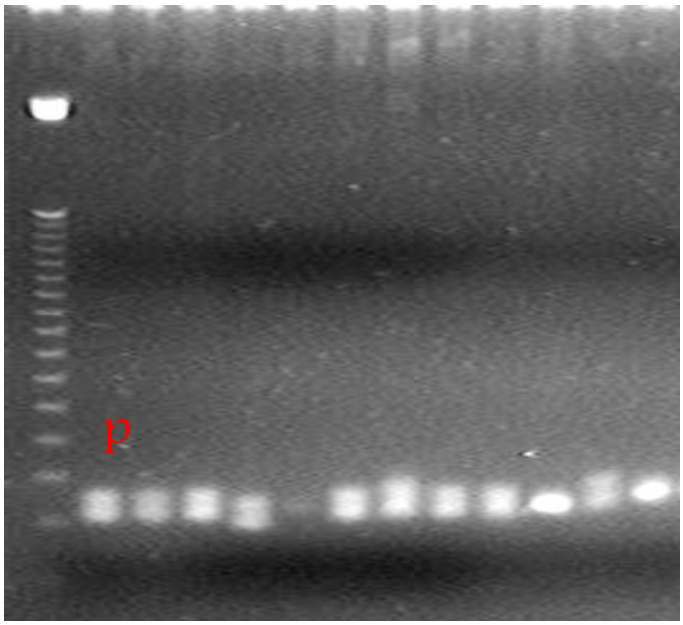

Figure 5. Segregation pattern of SSRY153 in select progenies of 1M18. Parent (P) is heterozygous. False progenies are indicated by the arrow pointers.

Table 3. Quantitative hybrid verification data of three populations based on three microsatellite markers.

Population	size	marker	Number of true	Number of false	Number of selfs
30001X1089A	93	NS890	91	-	2
1089AXTME117	207	SSRY82 and petiole color	207	-	2
1M18	41	SSRY153	35	6	-

feasible to use this method as quality control step particularly when combined with fragment analysis by capillary electrophoresis on ABI DNA analyzer. Furthermore, the quality and quantity of extracted DNA were reasonably good (average quantity of 1.2µg/µl and $A_{260}/A_{280\ ratio}$ between 1.8 and 2.0) and comparable to the quantity and quality parameters reported by Bhattacharjee et al. (2009). As expected, all three markers utilized in the study showed good amplification after PCR using the extracted DNA as template. NS890 showed a single allele of different sizesin each of the parents, 30001 and 1089A and polymorphic between them. Markers showing this form of polymorphism are expected to be codominant in all true hybrid progenies (Asif et al., 2009; Tamilkumar et al., 2009). Its ability to distinguish both parents (Figure 1) and codominantly segregate in the 30001 X TME117 populations (Figure 3) would ensure easy identification of false hybrids and progenies resulting from selfing of the female parent, hence its applicability as a veritable tool for hybrid verification. Any putative progeny bearing only one of the two expected codominant alleles is considered false hybrid, or selfed progeny if its allele corresponds to the female allele (Figure 5). Putative hybrids producing any other allele apart from the expected ones are also considered as false hybrids.

Using SSRY82, all heterozygous putative progenies of 1089A X TME117 can be described as true hybrids because they contain the unique male allele (Figure 4). However, the homozygote progenies are not easily classified because they possess a single- allele which is common to both parents. This allele in progenies could result from selfing of the female parent or from its segregation into true progenies from both parents since the allele is found in both parents. This inability to identify selfs makes SSRY8, insufficient in itself as a marker tool for verification of population crosses. However, in combination with characterization of petiole color, selfed

progenies were identified. Petiole color difference exists between the parents, 1089A and TME117, where the former has green petiole and the latter has a pink petiole. Segregation of this trait in the population, 1089A X TME117 showed that the pink color trait of the male parent is more predominantly expressed, with 203 out of the 207 progenies being either pink or pink-green leaving the green petiole trait of the female as the recessive trait. The pink-green shades seen in some progenies suggest the possibility of a polygenic control of the petiole color trait in cassava. The segregation ratio of the progeny phenotypes is closest to the phenotype segregation expected of a cross involving polygenic genes. Results of morphological assessment of the 207 progenies of 1089A X TME117 showed that 124 progenies were pink, 79 pink-green and 4 green. This implies a ratio of 1.5:1.0:0.05 for pink, pink-green and green respectively. Given the recessive inheritance of the green petiole color trait of the female parent, any cross involving a single heterozygous or homozygous pink color gene for the male parent would be significantly different from the segregation ratio obtained in the study. The former would imply 50% green petiole progenies while the latter would imply 100% pink colored heterozygous progenies strongly indicating that the petiole color trait may not be polygenic. The segregation of 1089A X TME117 predominantly into pink and shades of pink indicates a polygenic controlled petiole color trait in which the purple color allele is dominant over the green color allele. The ratio suggests that the pink color allele is not only dominant but also outnumbers the green color alleles in the pink petiole parent. Ideally,in polyhybrid crosses between such a parent and a recessive parent as seen in the 1089A X TME117 population, the recessive allele is not expressed. Hence the observed expression of the recessive green color in two genotypes is anomalous and can be attributed to cross contamination. The expression of shades of pink petiole phenotype and the observed segregation ratios suggests that some of the petiole color genes may be heterozygous. The possible heterozygosity of some constituent genes of the pink color petiole trait is supported by an evidence of high variability of the morphological characteristics of cassava (Alves, 2002) owing to the heterozygous nature of the crop (Raghu, 2007). Only F1 progenies with pink and different shades of the pinkish green morphology should be expected in a cross between these parents. However, four progenies with green morphology were identified in this study.

These 4 green genotypes were therefore not considered true hybrids but being of same morphology as the female parent, are more likely selves. Molecular markers with same kind of polymorphism as SSRY82 are only useful in combination with morphological traits, like the petiole color trait. In this scenario, the marker is used to distinguish between true and false hybrids, identifying the true hybrids while the morphological trait would be useful to support the marker in identifying likely selfs. SSRY82 identified 103 out of the two 207 total putative progenies genotyped, as true. These are the heterozyotes possessing both the female allele and the unique male allele. The remaining 94 genotypes possessing only the single allele shared by both parents (gel picture in Figure 5) could not be confidently classified as either true hybrids or products of selfing. Meanwhile, four genotypes had green petiole morphology and are therefore considered false morphologically. It is expected that these four genotypes should be among the ninety-four genotypes unclassifiable using SSRY82. However, only two of the four were among these ninety-four. The remaining two were genotyped as true. Only the two green petiole-genotypes not identified as true by SSRY82 can be confidently regarded as selfs. The other two individuals genotypically identified as true by the same marker cannot however be confidently regarded as selfs despite having the green petiole morphology. Instead, they are regarded as true hybrids in line with SSRY82-based designation, thus placing the marker in higher order of precedence over morphological trait for determining hybrid status in such cases of disparity. This higher precedence of SSRY82 can be explained by the fact that molecular markers (genotypes) are not subject to environmental influence unlike morphological traits (phenotypes) which are subject to environmental influence. From the fore-going, only two genotypes representing 0.97% of the 207 genotypes of 1089A X TME117 population can be regarded as false. This implies that 99% of genotypes in the population were identified as true hybrids following this joint assessment of marker and morphological trait segregation.

Any SSR marker showing same pattern of banding as SSRY82 can be used with any male-dominant morphological trait to verify the hybrid status of crosses of cassava according to the method described above.

With respect to verification of selfing in the 1M18 selfed line, two alleles were identified in the parent while the forty one putative progenies had a total of three alleles. The third allele completely alien to the 1M18 parent was identified in six putative progenies. Since all true progenies are expected to possess any one of the two parental alleles (segregating at a probability level of 0.25 for each of two possible homozygous categories and 0.5 for the heterozygous progenies according to Mendelian inheritance principles) these six individuals are considered as false progenies.

The dearth of information on the inheritance of various morphological traits of the Manihot species was a major challenge. The availability of this knowledge is expected to enhance further studies at identifying marker tools for

verification of selfed lines, intra-specific and inter-specific crosses. Therefore, it is imperative to study the genetics of easily scorable morphological markers in *Manihot* species.

Conclusion

Microsatellite markers can provide a fast and convenient means of accurate selection of true progenies of population crosses of cassava. In this study, we attempted to present a simple but useful molecular tool for early detection of false hybrids to prevent propagation in subsequent generation. In line with global trend in genomics research, abundant genomic resources such as SNPs and other markers are being developed for cassava using gene discovery, transcriptomics and other advanced techniques for use in genome selection and association study. These resources are expected to enhance efficient and more accurate selection of true progenies of population crosses. While morphological markers are not as reliable, they can be useful in hybrid verification in cassava if they are qualitative and their inheritance is dominant and well-studied.

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