Assessment of seed genetic purity of hybrid maize variety UH6303 in southern highlands of Tanzania by random amplified polymorphic DNA (RAPD) markers

Bakari Amiri Mrutu
Tanzania Official Seed Certification Institute (TOSCI), Tanzania.

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One of the main features that give high quality to seeds is its genetic purity. Up to date, seed certification for genetic purity using molecular markers has not yet employed in seed certification system in Tanzania. This work was carried out to assess the seed genetic purity of hybrid maize UH6303 using random amplified polymorphic DNA markers. Hybrids UH6303 and their parental lines (47 genotypes) were used in this study. DNA extracted from young leaves and PCR were conducted using seventeen primers. In total, 134 bands produced and all 134 bands were polymorphic. Genetic similarity ranged from 0.09 to 0.99. Cluster analysis based on Jaccard’s similarity coefficient grouped the genotypes into two major clusters; one cluster contained 42 genotypes and the other cluster contained five genotypes. Two dimensional PCA grouped the genotypes into two major clusters. The varieties which were considered to be genetically identical were found to be different.

Key words: Maize, seed genetic purity, random amplified polymorphic DNA (RAPD) markers.

INTRODUCTION

Tanzania has established the formal seed sub-sector since 1973 which include breeding, multiplication, processing, certification and distribution of seeds. Crop variety development in Tanzania is mainly undertaken by the public sector through the zonal Agricultural Research Institutes (ARIs). Private sectors are also responsible for development of their own varieties. Variety testing for release, quality control in seed production and regulation of the seed market are all handled by the Tanzanian Official Seed Certification Institute (TOSCI).

In Tanzania, responsibility for foundation seed production has been passed to the Agricultural Seeds Agency (ASA). Certified seed production is carried out by both public and private sectors. The public sector concentrates much on less commercial crops such as millet, rice and cowpeas. The private sector concentrates on local hybrid and Open Pollinated Varieties (OPV) maize and limited amounts of a few commercial crops; sunflower, sesame, sorghum, beans and vegetables. Production by the private sector is primarily through contract farmers. There is also a Quality Declared Seeds (QDS) system in place which permits local production and sale of seeds.
mainly of non-commercial crops with relatively light regulation. Approval is given by TOSCI trained and authorized inspectors at the district level and sales of QDS are restricted to the same ward for quality control. QDS accounts for relatively small amounts of seed in the seed sector.

Distribution and sales of seeds are effected through the agro-dealer network in the country. Good quality seed is a crucial and the cheapest input in crop production, and key to agriculture development. Crops status largely depends on the seed materials used for sowing provided other conditions of weather and nutrients are kept optimum. It is estimated that good quality seeds of improved varieties can contribute about 20 to 25% increase in yield (Abdelmonem, 2000; Beshir, 2011; Macha et al., 2011).

Poor access to quality seeds by farmers is a major crop production constraint for a long time in Tanzania. This is due to insufficiency of seeds and poor management during seed production which limits seed systems capable to sustain farmers’ needs for good quality seeds (Lyimo, 2006). When buying seed, farmer can inspect it for physical impurities and may be able to detect varietal mixtures in some cases, but it is not possible to know the genetic potential until when the crops are growing in their fields. In the absence of other information, farmers are unlikely to pay a premium for high quality seed without being sure that the quality is actually higher. Sellers are sometimes tempted to sell lower quality seed knowing that farmers cannot distinguish between the two. In practice, farmers make use of other sources of information: They rely on their experience with the seed in previous years, they may infer seed quality from the packaging and physical purity, they may decide based on the reputation of the vendor or the seed producer, or they may ask other farmers about their experiences with the seed. None of these strategies is ideal, however, and information remains a problem (Matlon and Minot, 2007).

During the quality control of seeds, most of the quality components are tested. Unfortunately, the genetic component has not received greater attention, although it determines the genetic quality of the materials (Thompson and Bergersen, 1980). Genetic purity ensures that the seed is of the true variety under certification, and that there is no mixture from other varieties or other crops.

Genetic purity test is done to verify any deviation from genuineness of the variety during its multiplication. For certification, genetic purity test is compulsory for all foundation and certified seeds. Higher genetic purity is an essential prerequisite for the commercialization of any seed variety (Macha et al., 2011). During the seed multiplication process it needs to be assured after each multiplication step, that the identity and the purity of that variety is kept and that the multiplied seed has the potential to produce a healthy plant. Determination of genetic purity of F1-hybrid seeds is a quality control requirement in the production of hybrid maize seeds, to avoid unacceptable contamination with self-inbred (sib) seeds (Ballester and de Vicente, 1998). This is assured through a seed certification programme.

Currently in Tanzania, seed certification for purity and variety distinctness is based on morphological evaluation of seeds and growing plants. These evaluation methods often involve field inspection, which are rigorous, resource intensive, time consuming, subject to bias in giving phenotypic estimation and have very little precision. These phenotypic values have a high environmental component that may easily lead to a wrong estimation creating confusion and probably accelerating the deterioration of the quality of materials evaluated on field. DNA markers have proven to be efficient in evaluation and selection of plant material because, these markers are not affected by the environment as morphological markers (Sundaram et al., 2008).

The present study was focused on assessing the seeds genetic purity of hybrid maize variety UH6303 using random amplified polymorphic DNA (RAPD) markers. This method in combination with morphological method may assist in seed quality assessment in Tanzania.

MATERIALS AND METHODS

A total of 47 seed samples of maize UH6303 were collected from Southern Highland of Tanzania and used in this study. Among these 47 seed samples; 2 samples were the inbred lines (parents) collected from Uyole Agriculture research institute and 45 samples were hybrids collected along the seed supply chain from seed producers to seed sellers/agro-dealers in Southern Highland of Tanzania. The seed samples were grouped into four populations: Population 1 contained 2 inbred samples (parents) from the breeder, population 2 contained 16 hybrid seed samples from seed producer/ multiplier, population 3 contained 15 hybrid samples from seed whole sellers/distributors and population 4 contained 14 hybrid samples from seed retailer (Table 1). The breeder seeds were used as a reference to verify the genetic purity of the seeds in the rest of the groups along the seed supply chain.

Total genomic DNA was extracted from young leaves according to Dellaporta et al. (1983) protocol with minor modification as stated by Ogunkammi et al. (2008). The young leaves were ground in 500 µl of plant extraction buffer then transferred to new eppendorf tube and 40 µl of 20% SDS was added to the tube and mixed thoroughly by vortexing briefly. The samples were incubated in water bath at 65°C for 30 min and 200 µl of 3 M sodium acetate was added to each tube and mixed thoroughly by vortexing followed by incubation in ice for 20 min.

The samples were centrifuged at 14000 rpm for 10 min at room temperature and the supernatant was removed and transferred to new eppendorf tube. Equal volume of Chloroform:Isoamyl alcohol (24:1) was added and mixed gently by inverting the tube followed by centrifugation at 14000 rpm for 10 min at room temperature in a microfuge.

The upper aqueous layer was transferred to a new 1.5 µl eppendorf tube. An equal volume of cold isopropanol (pre-chilled in -20°C freezer) was added and mixed thoroughly by gently inverting the tube and incubated at -20°C for 30 min. The samples were centrifuged at 14000 rpm for 10 min at room temperature. The DNA pellet was washed by adding 500 µl of 70% ethanol and centrifuged
Table 1. List of maize inbred lines and hybrids used in the study.

| S/No | Seed group     | Seed category | Seed source       | Sample ID* |
|------|----------------|---------------|-------------------|------------|
| 1    | Population 1   | Inbred (Female) | Uyole ARI       | A-98       |
| 2    | Inbred (Male)  |               | Uyole ARI       | A-99       |
| 3    | Population 2   | F1 Hybrid     | Seed producer   | B-72       |
| 4    | F1 Hybrid      |               | Seed producer   | B-73       |
| 5    | F1 Hybrid      |               | Seed producer   | B-75       |
| 6    | F1 Hybrid      |               | Seed producer   | B-77       |
| 7    | F1 Hybrid      |               | Seed producer   | B-78       |
| 8    | F1 Hybrid      |               | Seed producer   | B-79       |
| 9    | F1 Hybrid      |               | Seed producer   | B-81       |
| 10   | F1 Hybrid      |               | Seed producer   | B-83       |
| 11   | F1 Hybrid      |               | Seed producer   | B-84       |
| 12   | F1 Hybrid      |               | Seed producer   | B-85       |
| 13   | F1 Hybrid      |               | Seed producer   | B-102      |
| 14   | F1 Hybrid      |               | Seed producer   | B-103      |
| 15   | F1 Hybrid      |               | Seed producer   | B-105      |
| 16   | F1 Hybrid      |               | Seed producer   | B-107      |
| 17   | F1 Hybrid      |               | Seed producer   | B-109      |
| 18   | F1 Hybrid      |               | Seed producer   | B-110      |
| 19   | Population 3   | F1 Hybrid     | Seed whole seller| C-1       |
| 20   | F1 Hybrid      |               | Seed whole seller| C-3       |
| 21   | F1 Hybrid      |               | Seed whole seller| C-4       |
| 22   | F1 Hybrid      |               | Seed whole seller| C-5       |
| 23   | F1 Hybrid      |               | Seed whole seller| C-6       |
| 24   | F1 Hybrid      |               | Seed whole seller| C-7       |
| 25   | F1 Hybrid      |               | Seed whole seller| C-8       |
| 26   | F1 Hybrid      |               | Seed whole seller| C-10      |
| 27   | F1 Hybrid      |               | Seed whole seller| C-23      |
| 28   | F1 Hybrid      |               | Seed whole seller| C-24      |
| 29   | F1 Hybrid      |               | Seed whole seller| C-25      |
| 30   | F1 Hybrid      |               | Seed whole seller| C-45      |
| 31   | F1 Hybrid      |               | Seed whole seller| C-46      |
| 32   | F1 Hybrid      |               | Seed whole seller| C-51      |
| 33   | F1 Hybrid      |               | Seed whole seller| C-52      |
| 34   | Population 4   | F1 Hybrid     | Seed retailers   | D-11       |
| 35   | F1 Hybrid      |               | Seed retailers   | D-13       |
| 36   | F1 Hybrid      |               | Seed retailers   | D-15       |
| 37   | F1 Hybrid      |               | Seed retailers   | D-16       |
| 38   | F1 Hybrid      |               | Seed retailers   | D-19       |
| 39   | F1 Hybrid      |               | Seed retailers   | D-21       |
| 40   | F1 Hybrid      |               | Seed retailers   | D-22       |
| 41   | F1 Hybrid      |               | Seed retailers   | D-26       |
| 42   | F1 Hybrid      |               | Seed retailers   | D-27       |
| 43   | F1 Hybrid      |               | Seed retailers   | D-28       |
| 44   | F1 Hybrid      |               | Seed retailers   | D-30       |
| 45   | F1 Hybrid      |               | Seed retailers   | D-37       |
| 46   | F1 Hybrid      |               | Seed retailers   | D-42       |
| 47   | F1 Hybrid      |               | Seed retailers   | D-53       |

*The sample ID used is only for the purpose of this research. *The sample ID with alphabet initials are the same as sample ID without alphanets; e.g. A-98 is the same as 98.
at 14000 rpm for 5 min at room temperature. This step was repeated twice. The DNA pellet was air dried for 20 to 60 min by leaving the tubes open on its side in fume hood. The DNA pellet was dissolov in 50 μl sterile distilled water overnight. A 2.5 μl of RNase was added to the dissolved DNA and incubated at 37°C for 30 min and the DNA was stored at 4°C.

The concentration of DNA was measured by using NanoDrop2000 (Thermo Scientific™ NanoDrop 2000) and the final DNA concentration was adjusted to 50 ng/ml. The quality of the DNA was checked on 1% w/v agarose gel by electrophoresis at 100V for 30 min followed by staining with ethidium bromide and visualized under UV Trans-illuminator (BioDoc-It™ Imaging System).

Twenty eight RAPD primers (Ascefra LLC, USA) were screened using two individuals of the variety UH6303 from each population. Seventeen primers produced clear and polymorphic band patterns and they were selected for further analysis (Table 2).

PCR was carried out in a total volume of 25 μl by mixing 10 mMTris-HCl (pH 8.6), 1xTaqpolymerase buffer, 50 mM KCl, 2 mM MgCl₂, 0.27mM of each dNTPs, 0.8 mM primer, 1 U Taq DNA polymerase (Thermo Scientific) and 50 ng/μl template DNA.

The PCR amplification program was started by initial denaturation temperature at 94°C for 5 min followed by 42 cycles denaturation temperature at 94°C for 1 min, annealing at 38°C for 1 min, and extension at 72°C for 2 min. After that, the temperature was lowered to 4°C. Electrophoresis was done at 100 V for 75 min. and the bands were visualized under UV Trans-illuminator (BioDoc-It™ Imaging System) and photographed as shown in Figure 1.

The polymorphisms observed between the parents are used as markers for hybrid identification. Comparing the RAPD banding patterns of parents with respective hybrids, genuine hybrids were confirmed. The clear bands in each variety were observed and recorded as '0' (absent) or '1' (present) values. Band patterns ('0', '1' matrix) were tabulated for individual primers separately and the data were pooled to obtain a combined matrix for the genotypes. The NTSYS-pc software program version 2.1 (Rohlf, 2000), was used to compute the binary data ('0', '1') and evaluate the genetic associations between genotypes. Pair-wise comparisons were made between samples based on Jaccard similarity coefficient and visualized by cluster analysis, Unweighted Pair-Group Method with Arithmetic Averages (UPGMA) and illustrated in a phylogram. A principal coordinate analysis was performed based on Jaccards' similarity for all individuals and a plot was generated using the first two principal coordinates.

The bootstrap procedure was applied to calculate variance of the genetic similarities obtained from the markers and, thus, to verify the consistency of the obtained phylogram. The variance coefficient was obtained from 1,000 bootstrap random draws using the DBOOT program (Coelho, 2001). Analysis of molecular Variance (AMOVA) was carried out by grouping the genotypes into four populations. Population 1 contained inbred lines (parents), population 2 contained seed samples from seed producer (multiplier), and population 3 contained seed samples from seed suppliers (whole sellers) while population 4 contained seed samples from seed retailers.

RESULTS

In total, 17 primers amplified 134 fragments in the parents and hybrids in range of 250 to 5000 bp. All these 134 fragments produced were polymorphic. On average, 7.88 fragments per primer were observed with maximum of 12 and minimum of 6 fragments.

Based on the presence or absence of bands in each hybrid and their respective parents, three categories of bands have been distinguished. First category included bands common in hybrids and both of its parents (Type 1 marker) and bands common in cross and its female parent (Type 3 marker) or its male parent (Type 4 marker). The bands of marker Types 1, 3 and 4 are good markers to confirm that the cross is of its respective parents. In addition, bands of Type 4 marker are especially important markers to identify the true cross. Based on this categorization, 114 bands out of 134 bands observed with 17 RAPD primers fall under type 1. The band 2100 bp produced by primer OPP-05 were present only in A-98 (female) and missing in A-99 (male) thus classified as type 3 marker. Second category included bands found in either or both parents but not shared with the cross. These were bands of Type 2 markers, which were monomorphic for parents but absent in cross. Bands of Type 5 marker are expressed only in female and bands of Type 6 expressed only in male parent. Based on this categorization, the bands 2800 bp in primer OPP-03, bands 3000, 2300, 1800, 900, 750 and 250 bp in primer OPP-07, the band 2800 bp in OPP-09, the band 3000 bp in OPP-10, the band 7000 bp in OPK-01, the band 3200 bp in OPK-04, the band 2800 bp in OPK-08, the band 5000 bp in OPK-10, the band 3000 bp in OPO-04 and the band 2600 bp in OPO-08 falls in bands Type 2 because they were observed only in both parents and not in F1 hybrids. Third category included non-parental bands, which expressed only in crosses/hybrids, such cross-specific bands are useful for the identification of specific crosses. Sample 109 in primer OPJ-05 produced bands 4000, 3000 and 1700 bp which were not observed in either male or female parent.

Based on the 134 different bands produced by the 17 RAPD markers, it has been observed that about 85% of bands are in category one; Type 1, 1% of bands are in category one; Type 3, 12% of bands are in category 2; Type 2 while 2% of bands are in third category (data not shown).

Jaccard’s similarity coefficients ranged from 0.09 to 0.99. The lowest coefficient (0.09) was between genotype C-5 and B-77 while the highest coefficient was 0.99 between the genotype A-98 and A-99.

The cluster tree based on similarity coefficients from UPGMA revealed that the genotypes have been grouped into two major groups. The first group consisted of 42 genotypes while the second group consisted of 5 genotypes. The first group consisted of 42 genotypes have been subdivided into 2 clusters. These two clusters were joined together at about 0.34 genetic similarity levels while the two groups observed to be independent from each other (Figure 2).

Genotypes which are in group 1, first cluster are genotypes A-98 and A-99, B-72, C-7, A-110, B-81, B-83, C-3, C-4, D-19, D-21, D-22, C-23, C-1, C-10, B-107, B-73, B-84, D-30, D-15, C-5, C-52, D-16, D-26, D-27, C-8, D-37, B-109, B-79, B-105, B-102, B-103, D-42, D-53, D-11, D-13, D-28, C-24, C-25, B-75, and B-78, while the second cluster contained genotype C-46. The second
Table 2. RAPD primers used, sequences, band sizes, number of bands amplified per primer and percentage of polymorphism in maize genotypes.

| S/No | Primer name | Sequence | Size of fragments in base pairs | Total no. of bands | No. of polymorphic bands | Percent polymorphism |
|------|-------------|----------|-------------------------------|-------------------|-------------------------|---------------------|
|      |             |          | Largest | Smallest |                           |                     |                     |
| 1    | OPP-01      | GTAGCCTCC | 2100    | 430      | 8                        | 8                   | 100                 |
| 2    | OPP-03      | CGCATACGCC | 2800    | 400      | 8                        | 8                   | 100                 |
| 3    | OPP-05      | CCCGGTAAC | 2600    | 400      | 7                        | 7                   | 100                 |
| 4    | OPP-06      | GTGGGCTGAC | 2300    | 600      | 6                        | 6                   | 100                 |
| 5    | OPP-07      | GTCACTGCCA | 3000    | 250      | 12                       | 12                  | 100                 |
| 6    | OPP-09      | GTGGTCGCA | 2800    | 500      | 6                        | 6                   | 100                 |
| 7    | OPP-10      | TCCGCCTAC | 3000    | 400      | 9                        | 9                   | 100                 |
| 8    | OPK-01      | CATTCGAGCC | 7000    | 400      | 10                       | 10                  | 100                 |
| 9    | OPK-04      | CCAGCTAGG | 3200    | 400      | 6                        | 6                   | 100                 |
| 10   | OPK-05      | CGGCCAAAC | 2800    | 450      | 7                        | 7                   | 100                 |
| 11   | OPK-08      | AGCGAGCAAG | 2800    | 250      | 8                        | 8                   | 100                 |
| 12   | OPK-10      | GAACCTGGG | 5000    | 350      | 9                        | 9                   | 100                 |
| 13   | OPO-04      | AGTACGGTC | 3000    | 250      | 8                        | 8                   | 100                 |
| 14   | OPO-06      | CCCAGTCACT | 3500   | 600      | 7                        | 7                   | 100                 |
| 15   | OPJ-05      | CCAAGCGGAAG | 4000  | 250      | 10                       | 10                  | 100                 |
| 16   | OPJ-07      | CTCCATGGGG | 3000    | 630      | 7                        | 7                   | 100                 |
| 17   | OPJ-08      | CCTCTCGACA | 2600    | 700      | 6                        | 6                   | 100                 |
|      | Total       |          | 134     | 134      |                           |                     | 100                 |
|      | Average     |          | 7.88    | 7.88     |                           |                     | 100                 |

Figure 1. RAPD gel profile with fragments generated by primer OPP-05 in 47 genotypes of maize. The numbers listed above refer to the collection listed in Table 1. M is 1kb plus DNA size marker (Thermo Scientific).

The same pattern was observed based on factorial analysis computed using DARWin software that the genotypes with identity 6, 45, 51, 77 and 85 were clustered separately from the other genotypes and they are independent from one another (Figure 4). The other genotypes; 46, 75 and 78 observed to have a slight deviation from the other genotypes in the group which they all belongs to. This is in agreement with the results observed in the phylogram in Figures 2 and 3.

DISCUSSION

The present study indicates that RAPD marker is very effective in identifying the hybrids due to high rate of polymorphism (100%) it has produced. According to the
Figure 2. Phylogram of 47 maize genotypes constructed using UPGMA method based on Jaccard similarity coefficient from RAPD data by NTSYSpc 2.1 software.

Figure 3. Phylogram of 47 maize genotypes constructed using UPGMA method based on Jaccard similarity coefficient from RAPD data by DARWin software. *The genotypes used are the same though the alphabets (A, B, C and D) put before numbers in genotype ID have omitted.
phylogram, two distinct clusters were formed based on similarity coefficients with UPGMA. The two inbreds A-98 and A-99 were clustered in one group with the other 40 genotypes. As the inbreds were used as a comparator, it clearly indicates that the 40 hybrids which have clustered together with the inbreds are the true hybrids resulting from the cross between A-98 and A-99.

The hybrids B-77, B-85, C-6, C-45 and C-51 have clustered differently from the parents responsible for the variety development. This indicates that these hybrids are not a true hybrids resulting from the cross between A-98 and A-99. The genetically pure seeds should cluster with the parents indicating that they are related. With this observation, the hybrids B-77, B-85, C-6, C-45 and C-51 seem to be originated from other inbreds thus they are observed to be different varieties.

The genotypes B-77 and B-85 belong to population 2, have been collected from the seed multiplier (seed producer) while the genotypes C-6, C-45 and C-51 belonging to population 3, have been collected from the seed suppliers (Table 1). Based on these findings, there were no genotype from population 4 (from seed retailers) which were suspected to be not a true hybrid resulting from the cross between A-98 and A-99. This has also been proved by the phylogram where 40 hybrids (85%) have grouped together forming one cluster with the parents.

The percentage of markers not shared in the studied hybrids (crosses) was ranged from 11% in primer OPK-01 to 50% in primer OPP-07. This situation has also reported in some findings by other people in other different crops. Mehetre et al. (2004) reported 39.1% bands not shared in crosses for cotton while Hussein et al. (2013) reported the percentage ranging from 2.53 to 21.25% form some wheat crosses. Chung et al. (2000), cited by Husein et al. (2013) reported a range 38.0 to 52.6% of markers not shared in the cross in Chrysanthemum.

The percentage of non-parental bands (4000, 3000 and 1700 bp) of 30% observed by the hybrid B-109 in primer OPJ-05 implies the big possibilities of contamination in that sample. The contamination may be during seed
multiplication due poor isolation in the fields. The other possibility is that these bands may be generated due to recombination or mutation through meiosis process during hybridization (Williams et al., 1990).

Based on the findings of the present study, it can be concluded that clustering of the genotypes into groups based on their similarity coefficients using UPGMA can be able to identify the true hybrids and the off-types. From this study, it has been observed that even the genotypes which were considered to be genetically identical were found to be different. Identification of the hybrids and the parents may in turn support quality control of the hybrids under multiplication.

Conflict of Interest

The authors have not declared any conflict of interest.

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