

Application of SSR markers for genetic purity testing of some commercial hybrid maize (*Zea mays* L.) and their parental inbreds.

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Abstract:

Simple Sequence Repeat (SSR) primers were used to conduct DNA analysis of two commonly grown F₁ hybrids in Nigeria collected from two sources alongside with their inbred parents. The objective of the study was to evaluate the usefulness of SSR primers to determine the genetic purity of the commercial hybrids and their inbred parents. DNA extraction was done according to Dallaporta method with some modification before six SSR primers that showed polymorphic amplifications were used for Polymerase Chain Reaction (PCR) using TDSSR-1 protocol. Alleles/loci (bands) were scored as present (1) or absent (0). Polymorphic information content (PIC) of the SSR primers was assessed with the use of Power Marker software while genetic diversity was estimated using PAST analytical software. Mathematical equation was developed from the genetic distances matrix to determine the genetic purity status of the genotypes. Results from the study showed that SSR primers are powerful biotechnological tool capable of detecting genetic purity status of Nigerian maize hybrids. Genetic purity level of inbred lines ranged between 91.3% and 98.7% while the hybrids ranged between 81.3% and 95%. Inclusion of DNA analysis of seeds using SSR primers to determine genetic purity of maize seed is recommended.

Keywords: Maize, Genetic purity, DNA analysis, SSR primers.

Introduction:

Maize accounts for between 30 and 60% of food calories and dietary proteins in developing countries, especially in foods used for weaning infants. The grain is processed into several local dishes and consumed by both young and old (Showemimo *et al*, 2007). It has made a major contribution to the GDP of Nigeria. In the industrial sector, it is used in the production of flour, livestock feed, processed food (such as cornflakes) and beverages, sweeteners for soft drinks, pharmaceuticals, and malt for brewing beer. In Nigeria, several breeding projects had led to evolution of improved crop varieties in the past five decades. For instance, about 40 inbred lines of maize had been released by public agricultural research institutions to commercial seed companies for hybrid seed production (NACGRAB, 2004). Recent achievements by breeders in the development and release of superior varieties of maize with higher yield potentials and better resistance to insect pests and diseases has played a major role in increasing maize production in Nigeria (Iken and Amusa, 2004). To meet varied and diversified uses of maize, five hybrids and fifty-four open pollinated varieties have also been registered and released into Nigeria seed industry (NACGRAB, 2004). Hybrid seed production of maize involves crossing highly homogeneous inbred lines produced by several generations of selfing to produce heterogeneous hybrids. During hybrid seed production, much effort is always directed towards managing the process to ensure maximum kernel set and high levels of genetic purity (Fonseca *et al*, 2003) but it has not been possible to predict the level of out-crossing resulting from adventitious pollen entering the seed field (Mark, 2009). The genetic quality of seed is influenced by agronomic practices and the ecological characteristics of the site where the seed is planted. In the field production of maize hybrid seeds, the main source of genetic contamination is the self-pollination of the female parent due to the incomplete removal of its tassel. This contamination

reduces the genetic and physiological quality of the seeds that consequently decreases the crop productivity (Kalinka *et al.* 2006). Presently in Nigeria, 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 and have very little precision. There are however, good numbers of high precision technologies being used for crop breeding. Among these are electrophoresis (Tkachuk & Mellish, 1980) and use of molecular markers (Perry 2004 and Muhammad *et al.*, 2008) which saves time and resources. The objective of this research work is therefore to explore the use of SSR primers of DNA analysis to test the genetic purity of two commercial hybrid maize varieties and their inbred parents used in seed industries in Nigeria.

Materials and Methods:

Two F₁ hybrids and their inbred parents (4) sourced from International Institute of Tropical Agriculture (IITA) and two F₁ hybrids sourced from Premier Seed Ltd (a private seed company) were used for this experiment (Table 1). DNA analysis of the varieties were conducted by randomly selecting five seeds per genotype from the seed lot and planted into a one kg plastic container filled with sieved top soil in the screen house of IITA for young shoots to emerge for DNA extraction. DNA extraction was done according to Dallaporta method with some modification. About 0.3 g of freshly harvested leaves from each seedling of three out of the five planted were grinded over liquid nitrogen in 2.0ml eppendorf tube before 480 µl extraction buffer was added and incubated at 65°C for 20 minutes. They were allowed to cool. Thereafter, 250 µl of cold Potassium Acetate 5M was added and mixed by inversion before placing it on ice chips for 20 minutes. It was then centrifuged for 10 minutes at 12,000 rpm. The supernatant was carefully collected into new labeled tubes where 450 µl of ice cold Iso-propanol

was added and thoroughly mixed before placing it on ice chips for 1hour. The solution was then centrifuged at 12,000rpm for 15minutes; the supernatant was carefully discarded without dislodging the DNA pellets. The pellets were air-dried by inversion of the tubes on paper towel for 35 minutes. It was re-suspended in 200 µl of low salt TE + 3 µl 10mg/ml RNase and incubated at 37°C for 30minutes, 200ul of Chloroform was added and mixed by inversion for about 5min, later centrifuged at 12,000rpm for 10min, the clear supernatant was collected into a new eppendorf tube, then 15ul of 2M Sodium Acetate and 300ul cold Absolute Ethanol were added. The samples were kept at -20°C for 1hr, later centrifuged at 14,000rpm for 10min, the samples were washed with 70% Ethanol and air dried for 30min. It was re-suspended in 100µl sterile distilled water and stored in -20°C. Extracted DNA was tested for concentration and quality on 1% w/v agarose gel stained with ethidium bromide. The gel was run at 90V for 30 minutes in 1% running buffer. Forty one Simple Sequence Repeat (SSR) primer sets provided by International Institute of Tropical Agriculture, Ibadan were screened with the maize sample DNA before six that showed polymorphic amplifications (Table 2) were used for Polymerase Chain Reaction (PCR) using TDSSR-1 protocol. The PCR Products were electrophoretically separated on 2% agarose gels and visualized after staining with Ethidium bromide using Uvitech gel documentation system. For statistical analysis, each individual band was considered as a single locus/allele. Alleles/loci (bands) were scored as present (1) or absent (0). Polymorphic information content (PIC) of the SSR primers was assessed with the use of Power Marker software while genetic diversity was estimated using PAleontological Statistics (PAST) analytical software. To calculate the percentage genetic purity of the genotypes, mathematical equation was developed from the genetic distances matrix as follows:

$$Genetic\ purity(\%) = 100 - \frac{\sum x_1}{n}$$

Where, x_1 = values of genetic distances between the tested seeds and n = no of observations .

Results and Discussion:

The amplification profile of the maize genotypes using six SSR primers sets showed alleles of different molecular weight. All the SSR primers were able to detect genetic diversity which is defined as the probability that two randomly chosen alleles from the population are different among the maize genotypes (Liu and Muse, 2005). Information regarding the SSR primers and their informative ability is presented in Table 2. Average genetic diversity and polymorphic information content (PIC) recorded by the six primers was 0.592 and 0.512 respectively. The highest genetic diversity (0.673) and PIC (0.613) was recorded by primer phi96100 while primer phi123 recorded the lowest genetic diversity (0.484) and PIC (0.367). Similar PIC values have been recorded by other researchers in genetic diversity experiments of maize. Legesse *et al* (2007) recorded average PIC of 0.58 on 56 inbred lines of maize while Banisetti *et al* (2012) reported average PIC value of 0.49 on 22 maize genotypes. Also, Choukan and Warburton, (2005) has reported average gene diversity of 0.58 in 36 early maturing Iranian maize inbred lines. Since the genetic diversity and PIC values recorded in these studies are within those recorded for genetic diversity studies in maize, these SSR primers are considered useful for genetic purity analysis of maize varieties.

Genetic distance (GD) indices computed by 0/1 method using the six SSR primers are presented in Table 3. Average genetic distances among the seeds of each genotype are rectangular enclosed and ranged from 0% (between seed 2 and 3 of 1368, 9071 and KU1414SR) to 2.6% (between seed 1 and 3 of Oba Super 2 IITA). Highest genetic distance recorded among the genotypes was 2.6%, thus they can be considered genetically similar. Research finding using SSR for seed purity

is few, but SSR primers used for diversity studies has recorded genetic distance range of 0-53% among 14 genotypes of maize using four SSR primers (Sedafet *al*2010).

Genetic purity of each genotype calculated from the standard genetic distance indices is presented in Table 4. The result showed that the inbred parents of Oba Super 1 (1368 and 9007) recorded higher purity of 97.3% and 98.7% while inbred parents of Oba Super 2 (4001 and KU-1414SR) purity were 96% and 91.3% respectively. The result clearly confirmed the fact that the purity level of the inbred parents determines the purity of the hybrids because Oba Super 1 that has higher purity level of inbred parents' recorded higher purity than Oba Super 2 from both sources despite the fact that the inbred parents used for this experiment was only sourced from IITA. The clustered dendrogram based on genetic distance matrix and obtained with unweighted pair group method arithmetic (UPGMA) average displayed two main groups (Fig 1). Most of the hybrid seeds collected from IITA were clustered together in group one. Also, one sample each of hybrid seed from Premier Seed Ltd (Oba Super 2) and its inbred parent (KU-1414-SR) clustered into same group. Relationships among seed samples clustered into group two is clearly subdivided into another five sub groups (A-E). Each cluster formed several small groups and revealed the genetic divergence within the genotypes and among the seed samples. All the tested samples of Oba Super-1 (Premier), 4001 and 1368 were clustered as group A, B and C respectively indicating close relationship and high purity level. Similarly, samples of 9071 were clustered together in group D with one of the samples of Oba Super-1 (IITA). The last group comprises of two samples each of Oba Super-2 from Premier Seed Ltd and its inbred parent (KU1414SR). This result revealed a clear demarcation between most of the genotype indicating differentiation of the inbred lines into groups due to genetic diversity and also the hybrids according to their source. The broad genetic diversity detected within and among the seed

samples demonstrates the genetic purity that exists among the tested samples as well as the potentials of using SSR primers for genetic purity analysis in maize.

Conclusion.

This study has shown that SSR primers are powerful biotechnological tool capable of detecting genetic purity status of Nigerian maize hybrids. It is also proved from this study, as many other studies have done, that purity level of inbred parents is a determinant of the purity level expected from the hybrid. It can be concluded that the inclusion of DNA analysis using SSR primers to determine genetic purity of maize seed is recommended, though more numbers of seed will be required for more precision.

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Table 1: List of the hybrid maize varieties, their inbred parents and sources.

Genotypes	Type	Kernel Colour
Oba Super 1 (IITA)	Experimental hybrid	White
Oba Super 2 (IITA)	Experimental Hybrid	Yellow
Oba Super 1 (Premier Seed Ltd)	Commercial hybrid	White
Oba Super2 (Premier Seed Ltd)	Commercial hybrid.	Yellow
1368 (IITA)	Inbred line of Oba Super 1	White
9071 (IITA)	Inbred line of Oba Super 1	White
4001 (IITA)	Inbred line of Oba Super 2	Yellow
KU 1414SR (IITA)	Inbred line of Oba Super 2	Yellow

Seed source in parenthesis.

Table 2:

SSR Primers	Sequence	Major Alleles	Genetic diversity	PIC
phi072	F- ACCGTGCATGATTAATTTCTCCAGCCTT R- GACAGCGCGCAAATGGATTGAACT	0.457	0.642	0.589
phi109642	F-CTCTCTTTCCTTCCGACTTTCC R-GAGCGAGCGAGAGAGATCG	0.500	0.616	0.542
phi123	F-GGAGACGAGGTGCTACTTCTTCAA R-TGTGGCTGAGGCTAGGAATCTC	0.591	0.484	0.367
phi423796	F- CACTACTCGATCTGAACCACCA R- CGCTCTGTGAATTTGCTAGCTC	0.553	0.495	0.372
phi96100	F- AGGAGGACCCCAACTCCTG R- TTGCACGAGCCATCGTAT	0.444	0.673	0.613
umc1136	F- CTCTCGTCTCATCACCTTTCCCT R- CTGCATACAGACATCCAACCAAAG	0.526	0.644	0.597
Mean		0.512	0.592	0.510

Table 3: Standard genetic distances indices (D) among and between the inbred parents and the hybrids

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
1	0	0.04	0.04	0.09	0.11	0.19	0.13	0.11	0.11	0.07	0.09	0.09	0.09	0.07	0.09	0.13	0.15	0.17	0.11	0.20	0.11	0.13	0.11	0.11
2		0.00	0.00	0.09	0.11	0.22	0.09	0.11	0.07	0.07	0.09	0.09	0.09	0.11	0.06	0.13	0.15	0.20	0.11	0.24	0.07	0.17	0.07	0.07
3			0.00	0.09	0.11	0.22	0.09	0.11	0.07	0.07	0.09	0.09	0.09	0.11	0.06	0.13	0.15	0.20	0.11	0.24	0.07	0.17	0.07	0.07
4				0.00	0.06	0.17	0.15	0.13	0.13	0.09	0.11	0.11	0.11	0.13	0.11	0.11	0.13	0.15	0.13	0.19	0.17	0.15	0.17	0.17
5					0.00	0.11	0.13	0.11	0.15	0.15	0.17	0.17	0.09	0.11	0.09	0.17	0.11	0.09	0.19	0.13	0.19	0.09	0.19	0.19
6						0.00	0.20	0.15	0.22	0.22	0.24	0.24	0.17	0.15	0.17	0.24	0.11	0.06	0.26	0.02	0.26	0.13	0.26	0.26
7							0.00	0.06	0.02	0.09	0.11	0.11	0.11	0.13	0.07	0.11	0.20	0.22	0.13	0.22	0.09	0.15	0.09	0.09
8								0.00	0.07	0.11	0.13	0.13	0.09	0.11	0.09	0.13	0.15	0.17	0.11	0.17	0.11	0.13	0.11	0.11
9									0.00	0.07	0.09	0.09	0.09	0.11	0.06	0.09	0.19	0.24	0.11	0.24	0.07	0.17	0.07	0.07
10										0.00	0.02	0.02	0.09	0.11	0.09	0.06	0.19	0.24	0.07	0.24	0.11	0.17	0.11	0.11
11											0.00	0.00	0.07	0.09	0.11	0.04	0.20	0.26	0.09	0.26	0.13	0.19	0.13	0.13
12												0.00	0.07	0.09	0.11	0.04	0.20	0.26	0.09	0.26	0.13	0.19	0.13	0.13
13													0.00	0.02	0.04	0.11	0.13	0.19	0.17	0.19	0.17	0.15	0.17	0.17
14														0.00	0.06	0.13	0.15	0.17	0.19	0.17	0.19	0.13	0.19	0.19
15															0.00	0.15	0.13	0.19	0.17	0.19	0.13	0.15	0.13	0.13
16																0.00	0.24	0.26	0.09	0.26	0.13	0.19	0.13	0.13
17																	0.00	0.06	0.15	0.09	0.15	0.13	0.15	0.15
18																		0.00	0.20	0.04	0.20	0.07	0.20	0.20
19																			0.00	0.24	0.04	0.13	0.04	0.04
20																				0.00	0.24	0.11	0.24	0.24
21																					0.00	0.13	0.00	0.00
22																						0.00	0.13	0.13
23																							0.00	0.00
24																								0.00

Legend: 1-3=1368;4-6=Oba Super 1(IITA);7-9=Oba Super1(Premier seed);10-12=9071;13-15=4001;16-18=Oba Super 2(IITA);19-21=Oba super-2(Premier seed);
22-24=KU-1414SR

Table 4: Genetic Purity (%) calculated from the Genetic distance indices

Genotype	Genetic distance (%) between			Average genetic distance between the genotypes $(\frac{\sum abc}{3})$ (d)	Calculated genetic purity (%) $(100 - \frac{\sum abc}{3})$
	Seed 1 and 2 (a)	Seed 1 and 3 (b)	Seed 2 and 3 (c)		
1368	4	4	0	2.67	97.3
Oba Super 1 (IITA)	6	17	11	11.33	88.7
Oba Super 1 (Premier Seed)	6	2	7	5.00	95.0
9007	2	2	0	1.33	98.7
4001	2	4	6	4.00	96.0
Oba Super 2 (IITA)	24	26	6	18.67	81.3
Oba Super 2 (Premier Seed)	24	4	24	17.33	82.7
KU 1414SR	13	13	0	8.67	91.3

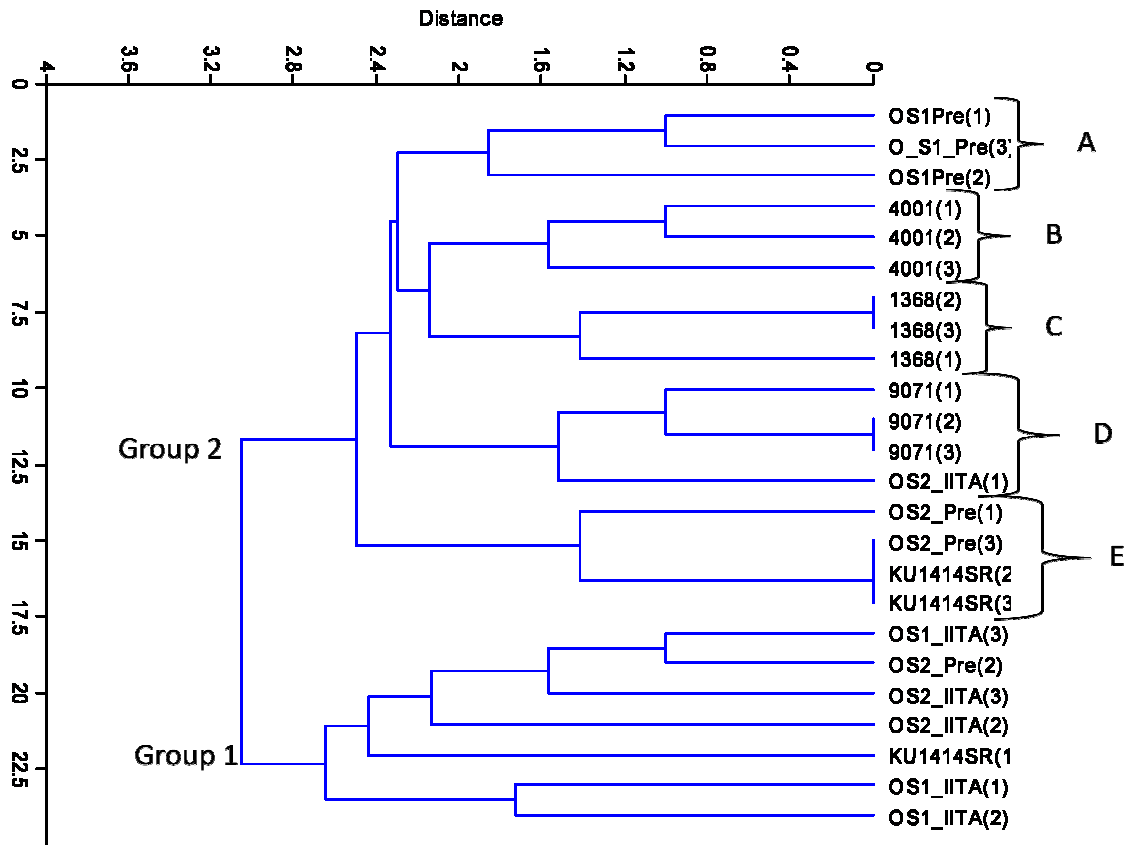


Figure 1: Dendrogram of the hybrids along with their inbred parents developed from SSR data using PAST software

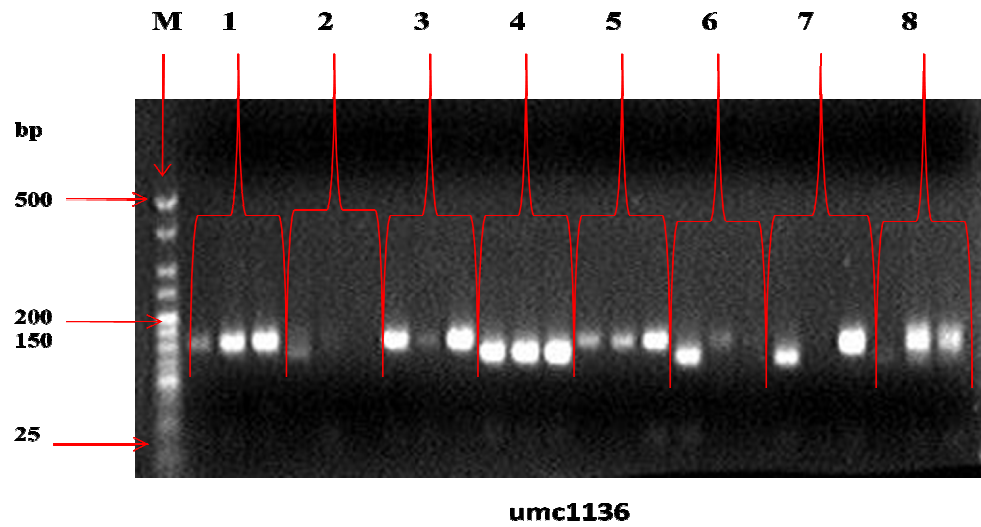


Figure 2: PCR amplification products generated using umc1136 primer.

Legend: Lane M: Bioline hyper v ladder100lane DNA marker; 1 = 1368; 2 = Oba Super 1 (IITA); 3=Oba Super 1(Premier); 4= 9071; 5=4001;6=Oba Super 2(IITA); 7= Oba super 2 (Premier seed); 8=KU1414SR