

Genetic diversity among yellow maize with pro-vitamin A content

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Abstract: An improvement in the concentration of vitamin A in adapted yellow maize varieties grown in Africa may have a positive impact on the dietary intakes in regions where maize is a staple food. The present study was designed to identify divergent parents for developing new pro-vitamin A enriched maize lines. Ten Simple Sequence Repeats (SSR) markers were used to generate DNA profiles from thirteen yellow maize lines commonly grown across south western Nigeria from Institute of Agricultural Research and Training (IAR&T) and three high pro-vitamin A lines from International Institute of Tropical Agriculture (IITA), Ibadan, Nigeria. All the ten SSR markers showed 100% polymorphism with polymorphic information content that ranged from 0.28 to 0.71 on an average of 0.50. Genetic similarity coefficients among the 16 maize lines varied from 0.28 to 0.92 GS with an average of 0.63 GS. Four well defined groups were identified at 0.65 GS with an IITA line, PVA8, solely, formed a group. The study identified PVA8 and its most three distant relatives as potential divergent parents that could serve as important genetic resources for broadening the genetic base of the presently assessed maize collections and also to develop new maize lines with higher level of pro-vitamin A content.

Key words: genetic diversity, Pro Vitamin A, SSR markers, yellow maize.

Introduction

Maize, *Zea mays*, is a staple food for millions of people in sub-Saharan Africa and also an important source of carbohydrate, protein, iron, vitamins and minerals (Menkir *et al.*, 2008; IITA, 2010). Maize grain is used for three main purposes: as a staple food, as feed for livestock and poultry, and as a raw material for many industrial products. In Africa countries, 95% of maize grain production is used as human food

compared to other world regions that use most of its maize as animal feed (Muzhingi *et al.*, 2008; IITA, 2009).

In southern Africa, maize provides more than two-thirds of the daily energy intake (William, 2012). In tropical Africa, maize is mainly consumed as thick porridge ('ugali' in East Africa, 'sadza' in Zimbabwe). It can also be made into a thin porridge (known as 'uji' in East Africa, 'ogi' in Nigeria, and 'koko' in Ghana). The porridge is commonly eaten with cooked vegetables and meat (if available) by adults as a breakfast cereal and mainly used as a weaning food for infants (Longhurst, 1984; King, 1987; Victor, 2006; Nagai *et al.*, 2009). Maize grain malt can be prepared into local beer ('tella') and spiritual liquor ('arakie') in Ethiopia. In some part of Africa, it may be eaten fresh on the cob but is more commonly roasted into a popular snack called popcorn.

Plant foods are the major source of important nutrients and minerals including provitamin A for a vast population of the poor in Tropical Africa (Tawanda *et al.*, 2011). Deficiencies of vitamin A, iron, and zinc are observed to be widespread in sub-Saharan Africa, where their diets are mainly plant-based and intakes of animal products are low (Maziya-Dixon *et al.*, 2000). Heavy reliance on maize-based diets by significant large number of people in sub-Saharan Africa has led to vitamin A deficiency in this region (Menkir *et al.*, 2008). World Health Organization (WHO, 2002) also reported that many women and about 28-35% of children, in sub-Saharan Africa living on maize-based diets are vitamin A deficient. Deficiency in dietary vitamin A has been noted to cause eye diseases in 40 million children, each year and places 140 to 250 million at risk for health disorders (Carlos *et al.*, 2008). White maize grown and consumed in sub-Saharan Africa contains little or no provitamin A.

The tropical adapted yellow maize varieties grown in Africa, naturally contain some levels of pro-vitamin A and non pro-vitamin A carotenoids (xanthophylls) with potential health benefits to humans, more importantly, for eye health (Nestel *et al.*, 2006; Muzhingi *et al.*, 2008). Though, the adapted yellow maize contains appreciable amount (2µg/g) of Pro Vitamin A but it is still considered insufficient and therefore, could not meet a significant proportion of daily human requirements (FAO, 1994). Furthermore, there is a tremendous variation in concentrations of provitamin A in yellow maize resulting in a pronounced genetic variability in their collections, which can be visualized as cream, butter, yellow, or orange endosperm. This genetic variability can be used in improvement of the concentration of provitamin A compounds in maize, and hence, have a positive impact on dietary intakes in areas where yellow maize is consumed (Menkir *et al.*, 2008; Muzhingi *et al.*, 2008). Bio-fortification of staple crops such as maize with high provitamin A carotenoids can help reduce micronutrient deficiencies, particularly in developing countries (Girum *et al.*, 2013). Application of molecular biology techniques complemented with conventional breeding will lead to the development of new pro-vitamin A-rich maize varieties (Babu *et al.*, 2013). This will translate into reduction in vitamin A deficiency and related diseases where maize is widely grown for consumption.

The use of genomics analysis tools in maize, such as association mapping studied have led to the identification of loci associated with provitamin A carotenoids (Jianbing *et al.*, 2011; Badu *et al.*, 2013; Girum *et al.*, 2013). DNA markers are tools for assessing the extent of genetic diversity present in maize germplasm and collections so as to identify genotypes useful for making crosses and to establish heterotic groups to develop yellow endosperm maize hybrids with high pro-vitamin A carotenoids (Russell *et al.*, 1997; Senior *et al.*, 1998). The development of DNA markers have led to accelerated genetic gain in maize breeding for increased provitamin A content (Menkir *et al.*, 2006; Adeyemo *et al.*, 2011; Babu *et al.*, 2013). Among the PCR-based markers that are available, simple sequence repeat (SSR) has been extensively used for genetic diversity assessment in maize (Smith *et al.*, 1997; Adetimirin *et al.*, 2008). SSR markers allow detection of polymorphisms at the DNA level which will facilitate the separation of yellow maize lines into well defined groups based on genetic distance estimates (Menkir *et al.*, 2004). Therefore, the present study was designed to assess the extent of genetic diversity and relationship among yellow maize lines selected across south western Nigeria with inclusion of some IITA lines with high level of pro vitamin A content using SSR markers. The study will be useful for the development of new improved maize lines in the current effort to increase pro-vitamin A content.

Materials and methods

Plant materials

Sixteen yellow endosperm maize lines of which thirteen yellow maize lines from the collection of IAR&T, Obafemi Awolowo University, Moor Plantation, Ibadan and three yellow maize lines with high pro-Vitamin A content obtained at IITA, Ibadan were used for the study (Table 1). Seeds of each yellow maize line were grown in the greenhouse at IAR&T. Five gram leaves of about two weeks old of each sample were harvested in ice, kept at -20 °C and later transported to IITA-Ibadan for DNA analyses.

Table 1 - Names and source of origin of the assessed 16 yellow maize lines.

NUMBER OF BRANCHING POINTS	SEEDLINGS (%)	AIR LAYERS (%)
1 st	82	100
2 nd	6	100
3 rd	0	85
4 th	0	37
5 th	0	4

DNA Extraction: DNA analyses were conducted in the Bioscience Center of the International Institute of Tropical Agriculture (IITA). DNA extraction was done by mini preparation as described by Dellaporta (1985). The quality of the extracted DNA samples were checked on 1.5% agarose gel via electrophoresis and visualized using stained-destained ethidium bromide method. Quantity and purity of DNA was determined by the use of Nano Drop spectrophotometer machine (ND-1000 Technologies, Wilmington, Delaware, USA) at $A_{260/280}$ absorbance. The ratio of $A_{260/280}$ absorbance obtained ranged between 1.80 and 2.0, indicating good quality DNAs.

SSR Analysis: Ten SSR markers were chosen based on repeat units and bin location distributed uniformly throughout the maize genome (Table 2). The primers were diluted to a working concentration of 5 μ M and then stored at -20 °C. The ten SSR markers were used for the amplification of DNA sequences from the 16 yellow maize line samples based on Polymerase chain reaction (PCR) technique using a thermal cycler (GeneAmp PCR system 9700, Applied Biosystems, USA). The polymerase chain reaction conditions was as described by Senior *et al* (1998) with some modifications, using variable “touchdown” method as required by each primer and described as follows: DNA denature at 93 °C for 20 sec, annealing temperatures ranged between 65 °C-55 °C for 35 sec, extension at 72 °C for 45 sec at 9 cycles followed by 24 cycles of 93 °C for 20 sec, 55 °C for 35 sec, 72 °C for 45sec and final extension at 72 °C for 5 min. The amplified PCR products were separated on 2% (w/v) metaphor-agarose gel and stained with ethidium bromide solution. The gel was viewed and photographed under UV light attached to a gel documentation system (Bio-Rad, Hercules, CA). DNA profiles of amplified fragments with clear and polymorphic SSR profiles (bands) were scored as presence (1) or absence (0) of a DNA band, respectively, with the aids of white illuminating light.

Analysis of SSR Profiles: The bands scored were assembled into a binary data matrix and analyzed using Numerical Taxonomy System of Statistic v.2.0j (NTSYS, Roulgh, 2000). Genetic diversity parameters such as number of alleles (A), average number of alleles (n), allelic frequency (p), rate of polymorphism (Pj), proportion of polymorphic loci (p), effective number of alleles (Ae), and polymorphic information content, PIC (h) were based on manual calculations in excel sheet. Genetic similarity (GS) between pair of yellow maize lines were calculated for the combination of data from 10 SSR primers pair by selecting similarity for Qualitative Analysis (SIMQUAL) using methods of Jaccard (1908) $GS = 2N_{ij} / (N_i + N_j)$, where N_{ij} is the number of SSR alleles common to maize lines I and j, while N_i and N_j are the total numbers of SSR alleles observed for maize lines I and j, respectively. The similarity data matrix was then used for data cluster analysis and the cluster analysis generated a dendrogram. Principal coordinate analysis (PCoA) was used to generate a scatter plot among the 16 of maize lines.

Table 2 - Name, repeat types and bin number of Ten SSR primers used for the molecular characterization of the 16 yellow maize lines.

NAME OF PRIMERS	REPEAT TYPE	BIN NUMBER	FORWARD PRIMERS	REVERSED PRIMERS
PHI 015	AAC	8.08	ACGCTGCATTCAATTACCGGAAG	GCAACGTACCGTACCTTTCCGA
PHI 034	CCT	7.02	TAGCGACAGGATGGCCTCTTCT	GGGGAGCACGCCTTCGTTCT
PHI 041	AGCC	10.02	TTGGCTCCCAGCGCCGCAAA	GATCCAGAGCGATTTGACGGCA
PHI 046	GCAC	3.08	ATCTCGCGAACGTGTGCAGATTCT	TCGATCTTTCCCGAACTCTGAC
PHI 053	ATAC	3.05	CTGCCTCTCAGATTGAGAGATTGAC	AACCCAACGTACTCCGGCAG
PHI 056	GCC	1.01	ACTTGCTTGCCTGCCGTTAC	CGCACACCACTTCCAGAA
PHI 059	CCA	10.02	AAGCTAATTAAGGCCGTCATCCC	TCCGTGTACTCGCGGACTC
PHI 064	ATCC	1.11	CCGAATTGAAATAGCTGCGAGAACCT	ACAATGAACGGTGGTTATCAACACGC
PHI 072	AAAC	4.01	ACCGTGCATGATTAATTTCTCCAGCCTT	GACAGCGCGAAATGGATTGAACT
PHI 076	GAGCGG	4.11	TTCTTCGCGGCTTCAATTTGACC	GCATCAGACCCGCAGAGTC

Results and discussion

Level of Polymorphisms Detected by the Ten SSR Markers Assessed among the 16 Yellow Maize Lines

The ten SSR loci isolated a total number of 25 distinct and scorable alleles (a DNA band represents an allele) among the 16 yellow maize lines, ranging from two (phi015, phi046, phi053, phi072 and phi076 primers) to three alleles (phi034, phi041, phi056, phi059 and phi064 primers) with an average of 2.5 alleles per locus.

Table 3 - Allelic frequency (q), number of alleles, number of effective alleles (Ae), and PIC value of the ten SSR markers overall the 16 assessed yellow maize inbred lines cultivated in Nigeria.

SSR LOCI	ALLELIC FREQUENCY (Q)			NO OF ALLELE (A)	PIC VALUE
Phi015	0.59	0.41	N/A	2	0.48
Phi034	0.19	0.31	0.44	3	0.68
Phi041	0.03	0.06	0.84	3	0.28
Phi046	0.56	0.38	N/A	2	0.54
Phi053	0.19	0.63	N/A	2	0.57
Phi056	0.44	0.31	0.06	3	0.71
Phi059	0.44	0.44	0.13	3	0.60
Phi064	0.44	0.47	0.03	3	0.59
Phi072	0.53	0.41	N/A	2	0.55
Phi076	0.25	0.69	N/A	2	0.46
Mean				2.5	0.55

The number of alleles isolated by the ten SSR primers among the 16 maize lines ranged from 4 alleles, for maize line PVA 8, to 18 for line Ado-Ekiti. Only SSR phi041 (allele 1) and SSR phi064 (allele 3) had a rare allele at a $P_j = q \leq 0.05$ (Table 3). The proportion of polymorphic loci (P) is calculated as 100%, based on the formula: $PIC = 1 - \sum p_i^2$, where P_{ij} is the allele frequency of the j th allele for the i th marker summed over numbers of alleles. Number of effective allele ($A_e = 1/\sum P_i^2$) in the overall 16 maize lines ranged between 1.40 and 3.41 with an average of 2.34, over all loci, where P_i^2 is the frequency of the i th allele in a locus. PIC value ranged from 0.28 to 0.71 with an average of 0.55 (Table 3). The ten SSR markers used in the present study were able to detect good levels of polymorphism and gene diversity among the selected 16 yellow maize lines and are comparable to values previously obtained with SSR markers in maize collections. The total (25) and average number of alleles (2.5) obtained in the present study was fewer to values obtained in previous similar studies in maize (Senior *et al.*, 1998; Hoxha *et al.*, 2004; Xia *et al.*, 2004).

It is noteworthy that the number of detected alleles varied with sample size and number of SSR markers employed, which could possibly explain for the differences in the numbers of isolated alleles in the different studies. Therefore, the least number of alleles obtained in the present, might be based on the smaller sample size of maize lines and SSR markers evaluated when compared to the other previous studies. In addition, number of alleles generated was also observed to be highly influenced by the DNA fragment separation technique with agarose gel having the least number as reported in the present study while ABI genetic analyzer had highest (Xia *et al.*, 2004). Nonetheless, the present study obtained comparable high polymorphic information content, despite its lower sample size and fewer number of alleles when compared to previous studies with higher sample sizes and allele numbers. For example, in a study of 155 maize lines with a total of 584 alleles, PIC ranged from 0.13 to 0.81 with an average of 0.60 and in another study of 94 maize with 365 alleles, PIC ranged from 0.17 – 0.92 with an average of 0.59 (Xia, *et al.*, 2004; Senior *et al.*, 1998). Therefore, the present study further supports the effectiveness of SSR markers in detection of polymorphism and estimation of genetic diversity in maize.

Genetic relationships among the 16 yellow maize lines based on ten SSR markers

Similarity coefficient estimates among all pairs of the 16 assessed yellow maize lines obtained from NTSYS package, as a measure of genetic diversity, varied from 0.11 to 0.95 GS with an average of 0.50 GS, which indicates a moderate genetic diversity among the assessed 16 yellow maize lines. The result is comparable to the results of Senior *et al.* (1998), who reported a lesser genetic diversity with a range of 0.21 to 0.90 GS with an average of 0.59 GS (GD = 0.41) among 94 inbred lines of maize using 70 SSR markers and that of Adeyemo *et al.* (2011) with a GD ranging between

0.007 to 0.59 GD with an average of 0.49GD among 38 inbred lines of yellow maize. However, a higher average genetic distance that varied between 0.34 and 0.92 with an average of 0.68 was obtained for all pairs of backcross derived line of yellow maize using AFLP's marker (Menkir *et al.*, 2006). The two identified closest relative lines were Ado-Ekiti and Ado-Ekiti-5-y at 0.11 GD, an indication that they share many common alleles in their genome, which could be pointing towards a common genetic origin or parentage. The most distant pairs of relatives were PVA8 and Abeokuta-3-y, and PVA8 and Ado-Ekiti-5-y at a similarity coefficient of 0.95 GD, followed by PVA 8 and Ado-Ekiti at 0.90 GD (Table 4). These three pairs of most distant relatives are identified as potential divergent parents that could be used for provitamin A improvement in the yellow maize collection of IAR&T-Nigeria. Such similar studies for the identification of divergent parents for crop improvement including essential nutrients in maize and many other food crops have been carried out.

Genetic distance as a measure of genetic diversity is a useful tool for estimating relatedness that could be exploited in genetic improvement programs in maize. Menkir *et al.* (2006) was able to estimate genetic diversity among yellow maize lines derived from adapted and exotic backcrosses for broadening the genetic base of tropical maize. Genetic distance among 105 indigenous maize lines was assessed for identification of parents to develop heterotic hybrids in maize (Ganesan *et al.*, 2010). Adeyemo *et al.* (2011) diversified 38 tropical yellow endosperm maize inbred lines into two well defined DNA groups to facilitate the selection of parental lines to develop new lines with enhanced provitamin A content. Genetic diversity is of great value to assist breeders in parental line selection and breeding system design as pointed out by Yanli Lu *et al.* (2009). The genetic dissimilarity matrix obtained was used to generate a dendrogram for visual relationships among the 16 yellow maize lines using un-weighted pair group method of arithmetic (UPGMA) in the NTSYS package.

The 16 maize lines were clustered into four well defined groups at similarity coefficient of 0.65 GS as shown in the dendrogram with a frequency occurrence of 8, 74, 30 and 18 % in 1000 bootstrapped structures. The resulted dendrogram is considered as the best structure over consensus structure because it was inferred from the initial data with each edge receiving a bootstrap value ranging between 0 and 100, corresponding to its occurrence frequency in 1000 bootstrapped structures (Fig. 1). The dispersion of the 1000 bootstrapped structures around the dendrogram has an edge distance of 0.73 with a confidence interval of 0.93 at 0.95.

Nine yellow maize lines obtained from IAR&T were in group one; group two consisted of two yellow maize lines, also from IAR&T collections, Abeokuta-1-y and Ago-y1-2-4; group three had two lines from IAR&T with inclusion of two IITA accessions, PVA 12 and PVA 2; while the fourth group consisted solely of a line from IITA, PVA 8. The dendrogram revealed PVA 8 as the most distant relative to the rest of 15 maize lines in agreement with the obtained GD values in the dissimilarity matrix (Table 4).

Table 4 - Genetic Distance among the all pairs of 16 yellow maize inbred lines based on 10 SSR markers as estimated by Darwin software.

	IREE4-2-Y	ADO-EKITI	IREE4-1Y	IREE4-5-Y	IREE4-4-Y	KILA-4-Y	ADO-EKITI-5-Y	ADO-EKITI-1-Y	KILA-1-Y	ABEOKU-5-Y	ABEOKU-1-Y	AGO-Y1-2-4	IJERU-IGBO	ABEOKU-3-Y	PVA2	PVA8
ADO-EKITI	0.42															
IREE4-1Y	0.47	0.47														
IREE4-5-Y	0.29	0.25	0.44													
KILA-4-Y	0.27	0.32	0.44	0.37												
ADO-EKITI-5-Y	0.44	0.11	0.41	0.35	0.24											
ADO-EKITI-1-Y	0.56	0.32	0.44	0.45	0.53	0.33										
KILA-1-Y	0.41	0.35	0.38	0.32	0.39	0.37	0.39		0.42							
ABEOKU-5-Y	0.31	0.17	0.47	0.22	0.47	0.28	0.39	0.56	0.50	0.65						
ABEOKU-1-Y	0.59	0.57	0.47	0.62	0.47	0.53	0.58	0.61	0.56	0.56	0.23					
AGO-Y1-2-4	0.47	0.62	0.53	0.53	0.61	0.47	0.45	0.55	0.50	0.50	0.50	0.56				
IJERU-IGBO	0.50	0.43	0.63	0.50	0.57	0.50	0.62	0.71	0.53	0.60	0.53	0.50	0.28	0.47		
ABEOKU-3-Y	0.60	0.30	0.58	0.59	0.83	0.88	0.95	0.80	0.81	0.88	0.77	0.75	0.88	0.95	0.79	
PVA2	0.44	0.59	0.85	0.53	0.45	0.45	0.43	0.45	0.32	0.48	0.47	0.60	0.32	0.35	0.50	0.83
PVA8	0.77	0.90														
PVA12	0.55	0.33														
Max	0.95															
Average	0.50															

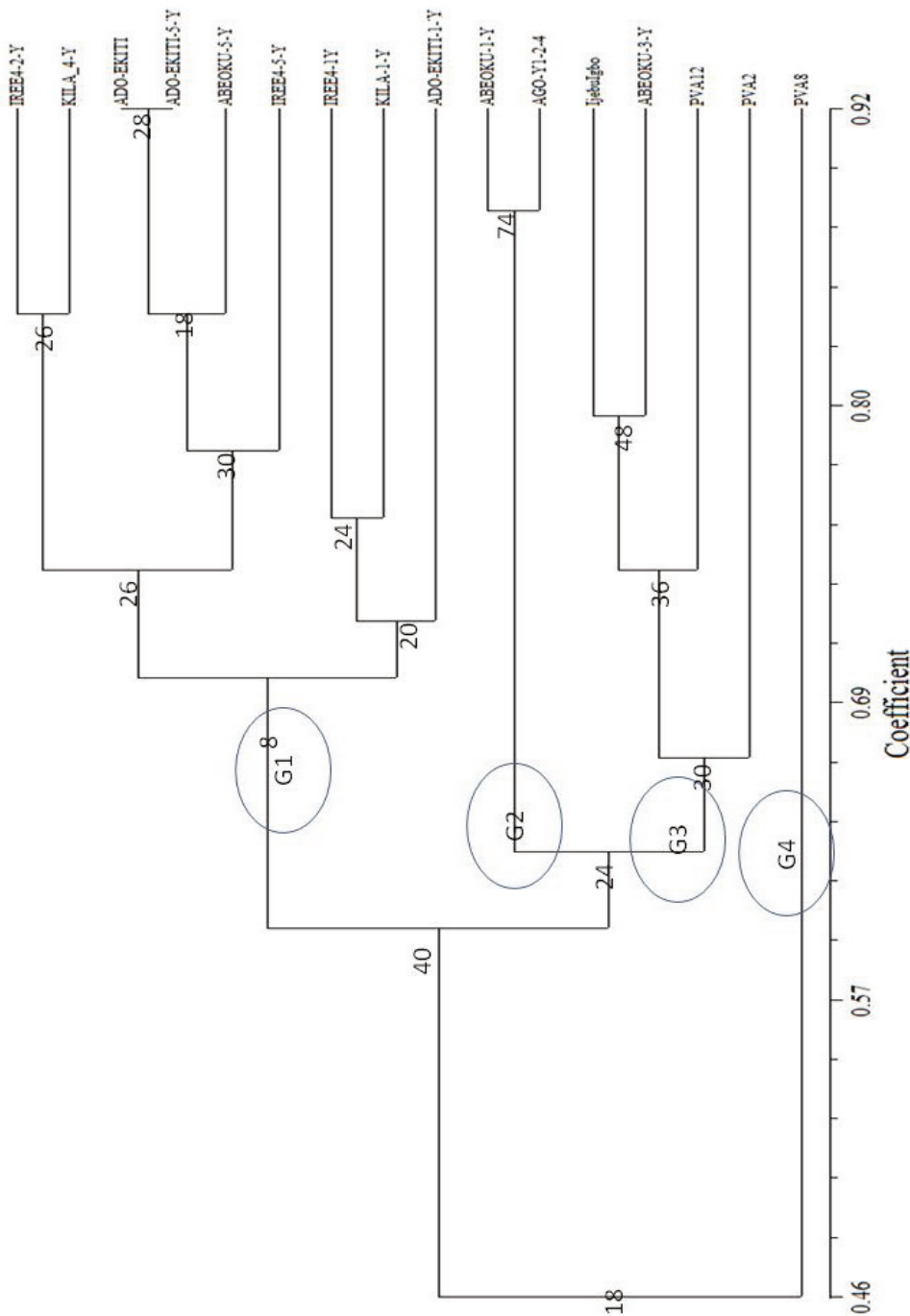


Figure 1 - Dendrogram of 16 yellow maize lines using 10 SSR markers
The values given at the edges in the structure are the bootstrapping value in percent indicating the frequency occurrence of the edges in 1000 bootstrapped dendrograms.

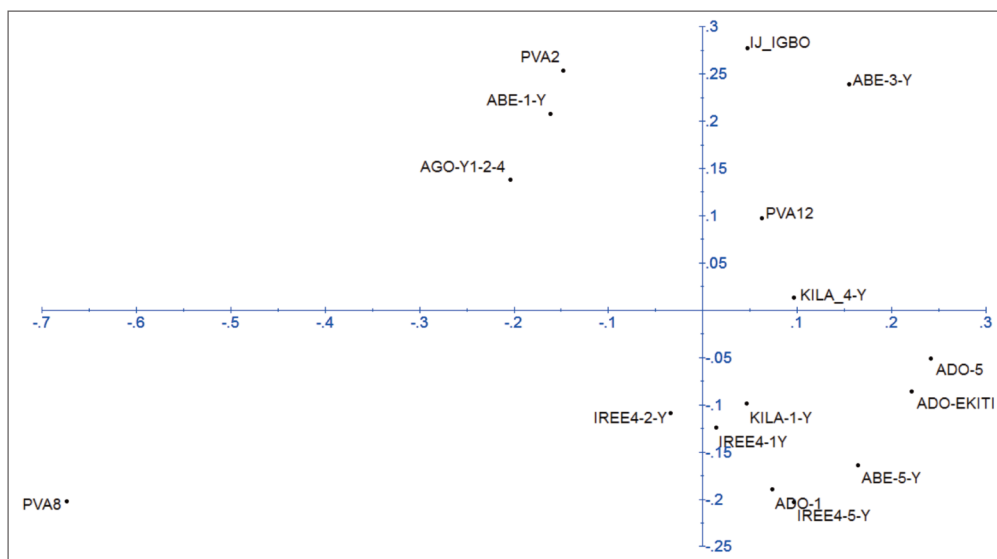


Figure 2 - A scatter diagram based on genetic distances among 16 yellow maize inbred lines assessed with 10 SSR markers.

Principal coordinate analysis (PCoA) gave the proportion of first three principal components (PCo) that contributed largely to the variation observed among the 16 yellow maize lines as 30.95, 19.43 and 12.52%, respectively. A plot of PCo1 versus PCo2 generated a scatter diagram that distributed the 16 yellow maize lines into four quadrants in accordance to the groupings generated in dendrogram with NTSYS but with slight disparities. Line PVA 8 solely was an outlier at lower left quadrant of the scatter diagram in consistence with the visual relationships observed in the NTSYS dendrogram (Figure 2). Therefore, the groupings obtained from the PCoA analyses and the dendrogram using NTSYS package, identified maize line PVA8 as the most distant relative and a potential divergent parent.

Because of the aforementioned results, maize line, PVA 8, could serve as an important genetic resource for broadening the genetic base of the presently assessed IAR&T collections. In addition, PVA 8 and its most distant three pair of relatives can as well be used as parental lines to develop new lines with higher level of pro-vitamin A (Adeyemo *et al.*, 2011).

Conclusion

The SSR markers used in the present study were able to detect genetic variation and estimate genetic diversity among the assessed 16 yellow maize lines. In addition, they also characterized the 16 maize lines into well defined groups and identify

potential genetic resources for breeding improvement in the assessed collection. The present study revealed IITA PVA8 yellow maize as a genetic resource material for pro-Vitamin A improvement breeding programs in maize.

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