Assessment of Genetic Variability of Bambara Groundnut (*Vigna subterranea* (L.) Verdc.) Accessions Using Morphological Traits and Molecular Markers

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Abstract

The efficiency of breeding can be improved by employing a number of genetic diversity measures of the crop germplasm. Genetic diversity and relatedness of 24 Bambara groundnut accessions (*Vigna subterranea* L. Verdc.) were estimated using 36 morphological characters, 201 Diversity Array Technology (DArT) and 65 simple sequence repeat (SSR) markers to assess the relationships between the material and also between the three marker types. These accessions were selected to represent the diversity within cultivated Bambara groundnut germplasm. A broader group of 119 Bambara groundnut accessions (87 from International Institute of Tropical Agriculture and 37 from University of Nottingham stocks; three seeds per accession) were planted in an unheated glasshouse at University of Nottingham in May, 2008. Data on the variation for morphological and agronomic traits were recorded following the IPGRI descriptors (IITA, BAMNET (2000). Samples were also analysed with microsatellite markers which have been characterised in this project. In addition, DNA extracted from the 119 accessions was sent for DArT analysis. This study has demonstrated the utility of the three marker types for genetic diversity and relatedness analysis. The genetic distance (GD) among the landraces and the first two axes for the principal component analysis (PCA) were obtained. The highest average genetic distance (GD) was obtained using morphological marker (0.55) followed by DArT (0.43) and SSR marker (0.25). The estimated values of the genetic relationship between all the markers were significantly correlated at ($P<0.01$). The markers provided consistent information as they produced relatively similar patterns of clustering, but DArT markers produced a cluster which grouped landraces based on their areas of origin. SSR markers have the advantage of being co-dominant and were employed to investigate pure line selection of Bambara groundnut. The results revealed significant reduction in the levels of heterogeneity from the 1st season of selection to the 3rd season, with no signs of residual heterozygosity suggesting that these lines (81-Acc-385TZA, 84-Acc 696ZMB, 88-AHM 753NAM, S19-3NAM and Bots1-BWA) have been effectively selected as pure lines. The application of SSR markers in this study further demonstrates their effectiveness as an approach suitable for Marker Assisted Selection in Bambara groundnut breeding.

INTRODUCTION

Bambara groundnut (*Vigna subterranea* L. Verdc.) is an indigenous African crop grown and consumed in most parts of Sub-Saharan Africa and it is a cheap source of protein for the majority of the rural population (Linneman and Azam-Ali, 1993). The crop is also valued for its drought tolerance and resistance to pests and diseases. Despite its potential as a promising crop for alleviating poverty and hunger, the crop has no established cultivars. Farmers are using landraces, which are usually low yielding (Zeven, 1998). The development of new cultivars could potentially increase the yields of Bambara

groundnut. The efficiency of breeding can be improved by employing various genetic diversity measures of crop germplasm, such as the use of morphological, biochemical and molecular markers. Morphological markers are the standard and usually the first step in germplasm characterisation, especially in underutilised crop species such as Bambara groundnut (Azam-Ali et al., 2001). A number of authors (Goli et al., 1995; Karikari, 2004; Ntundu et al., 2006; Ouedraogo et al., 2008) have described the morphological variation present in Bambara groundnut landraces. Molecular markers assist breeders in developing new cultivars since they can be employed to thoroughly assess the genetic diversity in crops and estimate the level of heterozygosity.

A number of molecular markers have been employed in Bambara groundnut for genetic diversity assessment, such as RAPDs (Amadou et al., 2001), AFLP (Massawe et al., 2002; Ntundu et al., 2003), SSR markers (Basu et al., 2007) and, recently, DArT markers and morphological markers have been used (Olukolu et al., 2011). However, a thorough molecular analysis of genetic variation in Bambara groundnut landraces in which a comparison is made with morphological markers has not yet been performed. In common bean (*Phaseolus vulgaris*), Perseguini et al. (2010) found AFLP to provide better resolution in cluster analysis compared to SSR markers, in their analysis of 60 genotypes using 65 SSR markers and 20 ALFP primer combinations. Tantasawat et al. (2010) compared the efficiency of SSR markers, ISSR markers and morphological markers in assessing the genetic diversity of 28 yard-long bean accessions (*Vigna unguiculata* spp. *sequipedalis*) and found ISSR markers to be more efficient with a cophenetic correlation matrix of 0.76, followed by morphological markers (0.38) and SSR (0.23). The comparison of markers in terms of their efficiency and reliability has been investigated in a number of crops. DArT are dominant markers and thus have the disadvantage that they cannot differentiate heterozygous loci from homozygous, but have the advantage of high locus specificity, due to their detection by hybridisation (Jaccoud et al., 2001). While SSR markers have the advantage of being co-dominant, highly polymorphic and widely distributed in the genome (Tang et al., 2006) but require substantial sequence information to generate.

Bambara groundnut is a predominantly self-pollinating crop so we would expect it to exist as non-identical inbred lines, although the previous lack of co-dominant markers has prevented a formal assessment of heterozygosity within Bambara groundnut genotypes. Low levels of heterozygosity among Bambara groundnut landraces suggests that selection based on single seed descent could yield pure lines – essentially unselected cultivars (Basu et al., 2007b), although this initial study was based on a limited number of SSR markers. Pure line selection could be a rapid and effective way for Bambara groundnut improvement especially given that artificial hybridisation is difficult in this species (Suwaprasert et al., 2006; Oyiga et al., 2010). Wigglesworth (1996) selected six lines from Bambara groundnut landraces and reported a relatively high average number of 250 pods per plant. Singrun and Schenkel (2003) employed 10 primer combinations of AFLP and one heterologous SSR primer pair to investigate the intra-landrace variety using 10 to 15 individual samples drawn from 10 Bambara groundnut landraces. All of the landraces had more than one genotype present. An initial study on the comparison of S19-3 and UniSwaRed using SSR markers showed S19-3 to be genetically narrower (Mayes et al., 2009), but still to have line-to-line genetic variation. The importance of a comparison of different marker systems is to assist in making informed decisions as to which marker is best to use in germplasm characterisation and plant breeding. Such markers can then aid with the selection of germplasm for breeding, quality control within breeding programmes and, potentially, direct selection via Marker Assisted Selection (MAS).

The aim of this study is to compare the use of morphological markers, DArT and SSR in assessing the genetic diversity of 24 Bambara groundnut landraces and to investigate employing SSR markers for pure line selection of Bambara groundnut.

MATERIALS AND METHODS

Plant Materials and DNA Extraction

A total of 119 Bambara groundnut accessions (87 from the International Institute of Tropical Agriculture and 37 from University of Nottingham stocks) were planted in an unheated glasshouse at the University of Nottingham in May, 2008. A subset of 34 landraces was studied for variation of morphological and agronomic traits following the IPGRI descriptors (IITA, BAMNET, 2000). Samples were also analysed with a subset of the microsatellite markers which were characterised in this project. In addition, DNA extracted using the GenElute Plant Genomic DNA kit (Sigma Aldrich) were sent for DArT analysis. In addition, a total of 24 landraces, that were previously selected from 17 clusters identified from the analysis of 223 Bambara groundnut landraces with 10 AFLP primer pairs of enzyme *Eco*RI/M*SE*I and one heterologous SSR (Singrun and Schenkel, 2003), were analysed. These are expected to represent the diversity of the known races in African Bambara groundnut.

The procedures for generating DArT markers, screening for polymorphism and genotyping was done by Diversity Arrays Pty Ltd., Yarralumla, Australia, following the methods described in Jaccourd et al. (2001b).

For pure line selections, seed derived from single plants were selected from 34 lines and planted in the field at Botswana College of Agriculture (Botswana) in the 2008-2009 season. To investigate the intra-landrace diversity, 7 individuals of the best five lines 81-Acc-385TZA, 84-Acc696ZMB, 88-AHM753NAM, S19-3NAM and Bots1-BWA, which produced higher pod numbers per plant and shoot dry weight, were selected for molecular analysis. Seeds from the same individual lines were planted in a growth room in a drought response experiment in 2010 season at the University of Nottingham, making this the third season of selection of these lines. The effects of single plant selection on the genetic diversity based on selection during three cropping seasons is inferred, based on the mean and ranges of genetic distance estimates using Popgene version 1.31 (Yeh and Boyle, 1997).

Microsatellite Development

A microsatellite-enriched genomic library was constructed based on the method of Edwards et al. (1996) with some modifications, and polymerase chain reaction (PCR) protocols are given in Basu et al. (2007c). Rather than cloning, a mixture of enriched libraries was pyrosequenced (Roche 454). Microsatellite primers were designed using PRIMER 3 (Rozen and Skaletsky, 2000) and were labelled using a three-primer tagged reaction (Schuelke, 2000). 75 primer pairs that were designed were screened and optimised for annealing temperature for 45 to 60°C via an annealing temperature gradient (Hybaid PCR Express). A total of 65 microsatellites that produced clear amplification products were selected for further use. Individual samples of 24 Bambara groundnut landraces representing genetic variability within the known germplasm were also characterised with the 65 microsatellites.

Morphological Traits

Twenty five quantitative characters (days to emergence; days to flowering; leaf number per plant; plant canopy size; middle leaflet length; leaflet width; leaf area; plant height; internode length; petiole length; petiole-internode ratio; petiolule; peduncle length; number of stems; days to maturity; shoot dry weight; number of pods per plant; pod dry weight; pod width; pod length; number of seed per plant; seed length; seed width; shelling percentage and seed weight per plant) were recorded in plants grown in 2008 in a glasshouse at the Sutton Bonington Campus, University of Nottingham, UK and in 2009 in a field experiment at the Botswana College of Agriculture, Gaborone, Botswana. Phenotypic data was collected based on Bambara groundnut descriptors (IPGRI, 2000). Eleven qualitative characters were recorded (leaf colour at emergence; pod texture; testa colour; eye pattern; stem hairiness; testa pattern; pod colour; pod shape; plant growth

habit; leaflet colour; stem petiole colour). To reduce the effects of scale differences, quantitative characters were standardised using Genstat version 13.0 (Updahyaya, 2003). The standardised values were used to perform cluster analysis and principal component analysis. The qualitative characters were recorded as binary data, with the absence of a trait as 0 and the presence of a trait as 1. For the estimates of genetic distance on Popgene, quantitative data were converted to binary data; the genotypes that were significantly different were scored 1 and those not significantly different were scored 0.

Data Analysis

Genetic distances, cluster analysis, principal component analysis and Mantel tests were performed on the data. Pair-wise comparisons were estimated on Jaccard's similarity coefficient for each marker type. Morphological-, DArT- and SSR-based dendrograms were constructed using the UPGMA method of cluster analysis. The goodness of fit (matrix correlation) for genotypes to each cluster was estimated using cophenetic tests in NTSYS-pc software, version 2.1 (Rohlf, 2000). Cophenetic correlation is a procedure for evaluating hierarchical cluster techniques by comparing the input data for either similarity or dissimilarity matrices with the output hierarchy (Holgersson, 1975). The similarities between markers were calculated based on the correlation between the similarity matrices for each marker type (morphological marker vs. SSR; morphological vs. DArT; SSR vs. DArT) and were tested using a Mantel's test in the NTSYS software. The genetic distance between genotypes was estimated based on POPGENE version1.31 (Yeh and Boyle, 1997), while the principal components analysis was calculated using MVSP (Kovach, 2006).

RESULTS AND DISCUSSION

The 65 SSR markers had an average polymorphic information content (PIC) of 0.46 among the 24 Bambara groundnut landraces. This was relatively higher when compared to the 201 DArT markers which detected an average PIC of 0.35 from the same genotypes (data not shown). The genetic diversity shown by the three marker types in the selected 24 markers was reasonably high (Table 1). The polymorphism detected by both markers was in the range estimated in the earlier studies. Basu et al. (2007) using 10 SSR markers detected an average PIC=0.46 when analysing 18 Bambara groundnut genotypes. In common bean (*Phaseolus vulgaris*), Buso et al. (2006) observed a PIC=0.56 among a set of 85 selected genotypes. Genetic diversity for the cultivated and wild relatives of pigeon pea using DArT markers recorded an average PIC of 0.34 (Yang et al., 2006).

Higher genetic variability was revealed by morphological markers, with genetic distance (GD) ranging from 0.140-1.10 and an average of 0.55, followed by DArT with an average of 0.43 and a range of 0.07 to 0.770. While SSR markers revealed the least differences among the 24 landraces. All the three markers showed clearer clustering as revealed by the Pairwise comparisons based on Jacccard similarity coefficients estimates. However, the DArT markers were more efficient in detecting clear differences among the 24 landraces with a wide range difference of 1.09, followed by morphological marker with 0.93, and displaying the least differences was the SSR marker at 0.52. The differences in genetic distance estimated by markers had been attributed to the extent of distribution of genome coverage by markers and their evolutionary different properties and individual loci used for analysis (Geleta et al., 2004).

The principal component analysis using a combination of the first two axes shows that DArT markers (35.81%), and morphological markers (32.41%) were providing a higher total variation among the 24 Bambara groundnut landraces, with SSR (25.43%) accounting for less genetic variation. Even though morphological data showed more variation, molecular data could also be used to predict the phenotypic diversity in crops and avoid a lot of field work (Motier et al., 2005). Morphological data will also have a higher environmental dependency than DNA-based markers, which should have negligible genotypic, developmental or tissue effects.

The estimated values of the genetic relationship between all the markers show a

significant correlation (P<0.01) (Table 2). That was not surprising since the cophenetic matrix (goodness of fit) for all the markers was significant at P<0.01, with morphological markers (0.82), DArT (0.97) and SSR (0.84). Raman et al. (2008) recorded a similar cophenetic coefficient of 0.97 for DArT markers in a set of 94 genotypes of *Lupinus albus* L. In a similar study, Stodart et al. (2006) observed a strong positive correlation (r=0.84) between DArT and SSR markers when using 256 DArT markers and 63 SSR markers on 44 accessions of bread wheat (*Triticum aestivum* L.). Mantovani et al. (2008) found a coefficient correlation between the genetic distance matrices of DArT and SSR (r=0.68) among a set of 31 accessions of wheat using 1,315 DArT markers and 103 SSR markers, which also showed an agreement between the two markers.

The dendrograms from the UPGMA cluster analysis for the morphological, DArT and SSR markers are recorded in Figures 1, 2 and 3, respectively. The morphological markers revealed two major clusters with landraces separated mainly on the characters which contribute more variation in Bambara groundnut such as leaf area, shoot dry weight, and number of pods per plant. Landraces clustered together are more likely to have a similar performance in a specific environment. Cluster 2 consists of mostly landraces from Southern Africa with the exception of landraces, Tvsu 610 from Nigeria, DodR from Tanzania and Ramayana from Indonesia, which also perform equally well in Southern Africa, perhaps arguing that they have been introduced by farmers from other regions.

DArT and SSR markers are showing a similar pattern, as each produced 3 clusters. The 201 DArT markers separated samples particularly based on their areas of origin (Fig. 2). DodC and DodR from Tanzania in the cluster 1 grouped together, while cluster 2 consists of landraces originally from Southern Africa, with the exception of Ramayana 'currently' from Indonesia. This could also reflect the genetic relation of this Indonesian landrace with the Southern Africa landraces and a potential place of origin from Africa. The third cluster consists of landraces only from East Africa. As for the SSR markers, the dendrogram was produced from 65 SSR markers and identified three major groups. The clustering is not based on the areas of origin of landrace, since both clusters show some mixtures of landraces from other regions. However, like the morphological markers, the clusters consist of many landraces which do come from the same region.

Applications of Microsatellites in Pure Line Selection

Twelve microsatellites employed in the molecular analysis of the five landraces in the first seasons of selection revealed an average genetic distance of 0.40 based on Nei's genetic distance estimates. The lowest residual heterozygosity was recorded in lines 88-AHM753NAM at 0.222, while the highest was recorded in line 81-Acc385TZA at 0.751 (Table 3). Variability within Bambara groundnut has been reported before in landraces, and has been attributed to the mixing of seeds during planting, especially those of same colour (Massawe et al., 2005; Mayes et al., 2009) although even within landraces, there are still differences between lines. As a breeding strategy for inbreeding crops like Bambara groundnut, it is advantageous to obtain pure homozygous lines with good attributes. As expected, in the second and third round of selection pure lines were selected through single plants (Table 3). There was no observed or expected heterozygosity in the second and third round of selection. These data strongly suggest these genotypes are now relatively pure lines.

CONCLUSIONS

The study has demonstrated the utility of the three marker types to provide information on genetic diversity and relatedness among the 24 Bambara groundnut landraces, since both markers recorded a significant correlation at P<0.01. The markers provided consistent information as they produced similar dendrograms, but DArT markers produced a more robust cluster which grouped landraces based on their areas of origin and a higher principal component analysis. However, it was the morphological markers which revealed a higher genetic diversity among the landraces with a larger

average genetic distance and range, based on Nei's (1972) genetic distance, which suggests that the morphological markers can still be useful in the genetic diversity analysis of Bambara groundnut despite that they are affected by environmental factors. Using SSR markers for pure line selection of Bambara groundnut, it was noted that the level of heterozygosity was significantly reduced in the second round of selection, with no signs of residual heterozygosity in the third cycle of selection. The application of SSR markers in this study demonstrates their effectiveness as a technique for Marker Assisted Selection in Bambara groundnut.

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Tables

Table 1. The estimates of genetic distance, pairwise range and principle component analysis among the 24 Bambara groundnut landraces.

Marker type	Number of markers used	Genetic distance estimates			
		Average	Range[a]	Range[b]	PCA[c]
Morphological	36	0.55	0.63-1.56	0.14-1.11	32.41
DArT	201	0.43	0.26-1.35	0.07-0.77	35.81
SSR	65	0.25	0.74-1.26	0.08-0.39	25.43

[a] Jaccard genetic distance; [b] Nei's 1972 genetic distance on Popgene; [c] PCA % accumulation for first two axes.

Table 2. The Pearson's correlation for the average Nei's 1972 genetic distance for the three markers, morphological, DArT and SSR, for the 24 Bambara groundnut landraces.

	DArT	Morphology	SSR
DArT			
Morphology	0.703**		
SSR	0.582**	0.681**	

** Significant at (P<0.01).

Table 3. Mean and range of the genetic distances for three different selection cycles of Bambara groundnut from single seed descent estimated based on 12 microsatellite markers using Popgene version1.31 (Yeh and Boyle, 1997).

Selected lines	N	Genetic distance estimates									
		First cycle			Second cycle				Third cycle		
		Mean	Ho-He	Range	N	Mean	Ho-He	N	Mean	Ho-He	Range
81-Acc385TZA	3	0.751	0.000-0.356	0.287-1.049	7	0.000	0.000	6	0.000	0.000	0.000
84-Acc696ZMB	3	0.314	0.000-0.222	0.206-0.403	4	0.000	0.000	6	0.000	0.000	0.000
88-AHM753NAM	3	0.222	0.000-0.267	0.198-0.248	7	0.000	0.000	6	0.000	0.000	0.000
S19-3NAM	3	0.347	0.000-0.311	0.305-0.405	7	0.000	0.000	6	0.000	0.000	0.000
Bots1-BWA	3	0.389	0.000-0.311	0.311-0.431	7	0.000	0.000	6	0.000	0.000	0.000

N=Number of individual sample.

Figures

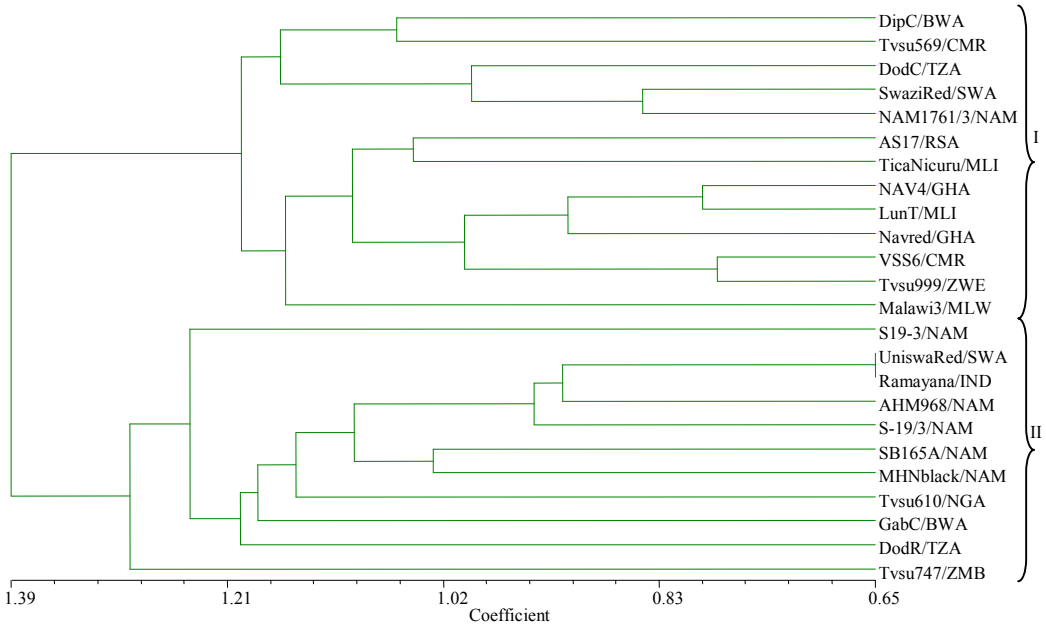

Fig. 1. A dendrogram of 24 Bambara groundnut landraces showing a (UPGMA) cluster analysis based on 36 morphological markers.

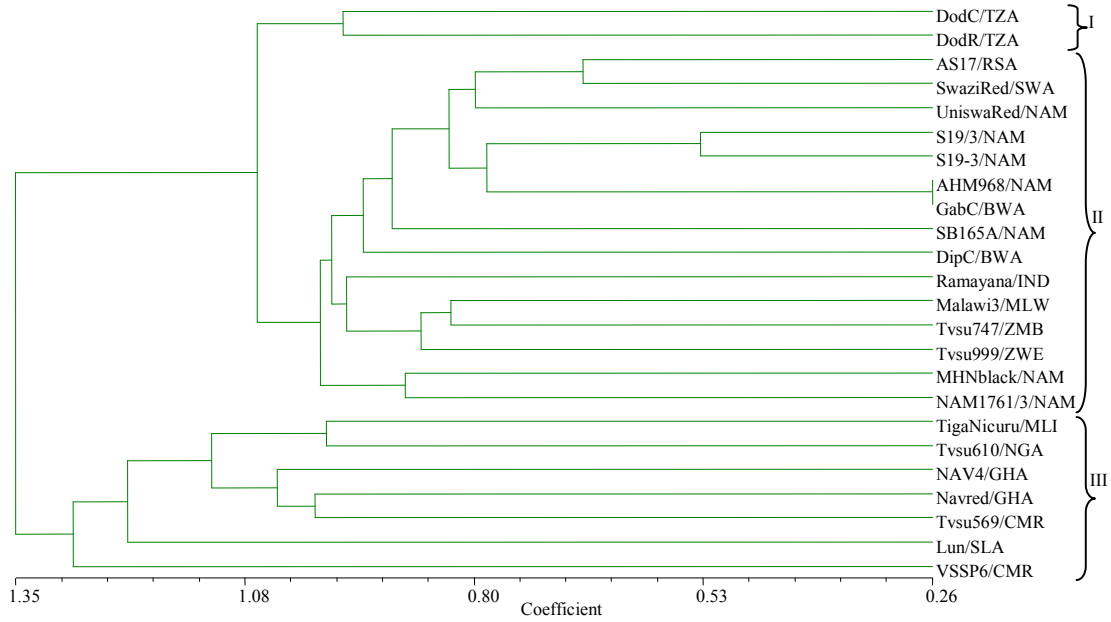

Fig. 2. A dendrogram of the 24 Bambara groundnut landraces showing a (UPGMA) cluster analysis based on 201 DArT markers.

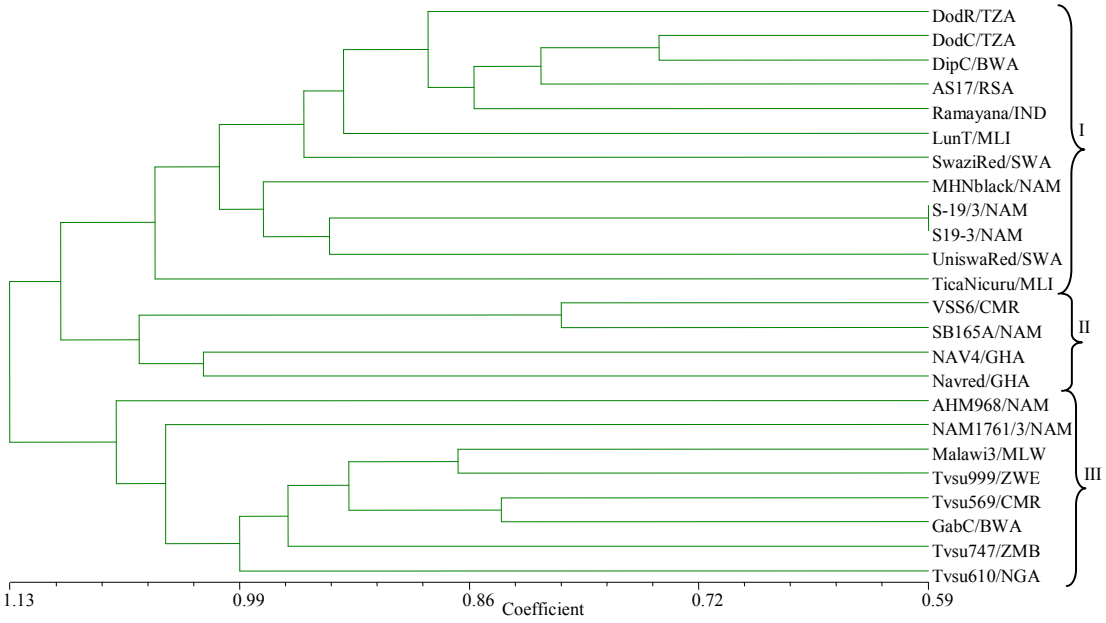

Fig. 3. A dendrogram of the 24 Bambara groundnut landraces showing a (UPGMA) cluster analysis based on 65 SSR markers.