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Molecular Genetic Diversity in Bambara groundnut [*Vigna subterranea* (L.) Verdc] Assessed by Microsatellite Markers.

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Abstract
Bambara groundnut is a valuable leguminous crop with many landraces. A study was carried out to establish genetic diversity and phylogenetic relationship, among 33 Bambara groundnut accessions based on simple sequence repeat (SSR) markers. The nine microsatellite markers amplified a total of 27 alleles with a mean of 6.00 alleles per locus. Marker P 36 had the highest number of polymorphic bands while makers P131 and P68 were monomorphic. Genetic distance among the accessions based on Jaccard's similarity coefficient ranged from 0.84 to 1.00. Cluster analysis resolved the accessions into five major groups with subgroups. Each group had a combination of distinct accessions from different geographical origin. A substantial level of intra-accession polymorphism was obtained among the evaluated collection of Bambara groundnut. The significant genetic diversity observed can support the selection of appropriate parental genotypes for the improvement of Bambara groundnut through various breeding programmes.

Keywords: Accessions, Bambara groundnut, genetic diversity, microsatellite markers.

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Introduction
Variation exists in crops; it can be interspecific or intraspecific. Naturally, variation occur due to mutation and it is maintained over the years by processes of evolution and natural selection. The reality of natural variation in both wild and domesticated crops species is evident on existence of recognisable diverse forms of crop species, which is the basis for selection and for crop improvement. However not all recognisable variation in crops is genetic. Variations triggered by the influence of the environment occurs, in which differences observed in the expression of some characters among crop species were not intrinsic. On the other hand, variation in crops because of the action of gene(s) is genetic. Genes regulate structure (size, shape, colour), physiological processes and functions, adaptability, phenology, and expression of characters (1).

Conventionally assessment of genetic variability in crops is achieved through field screening in which morphological descriptors are used to discriminate between and within Species. This approach is popular among workers because it is easy to carry out and is relatively cost-effective (2-4). However, it has some limitations because the expression of some characters (especially polygenic) is environment specific. Therefore, the obtained result may not be reliable. Molecular characterisation on the other hand is the most reliable and effective technique to establish variations that exist in crop species. It uses molecular descriptors or markers that are not affected by the environment to discriminate among species at DNA level and can be used to screen the entire population at any stage of crop development. The use of molecular markers to establish genetic diversity has enabled breeders to identify the presence of allelic variations in the genes controlling different agronomic characters in crops (5), to differentiate between homozygote and heterozygote genotypes (6), and to manipulate important agronomic traits in crops (7).

Bambara groundnut is a leguminous food security crop. Its seed is a complete food and many forms of this crop has been reported by workers who made collections from major areas in west Africa eco-regions where this crop is grown (25). Unfortunately, the diversity reported by these workers where based on field morphological characterization of accessions. (8-11), There are few documented reports on assessment of diversity on Bambara groundnut based on molecular markers (12 13, 3, 14. 15), hence the need for this study whose primary target was to assess genetic diversity in Bambara groundnut based on microsatellite markers with a view to establishing phylogenetic relationship among the accessions.

Materials and method

The plant materials were 33 accessions of Bambara groundnut obtained from the germplasm collection maintained at the gene bank of the International Institute of Tropical Agriculture (IITA), Ibadan (**Table 1**).

DNA extraction

Genomic DNA was extracted using the modified minipreparation protocol described by Dellaporta *et al* (16). Approximately 200 mg (0.2 gm) of lyophilized leaf sample was ground into fine powder with the aid of Genogrinder 2000.

Table 1: Passport data of the 33 Bambara groundnut accessions used for the study.

s/n	Accession name	Country of origin
1	TVSu-1483	Ghana
2	TVSu-1503	Nigeria
3	TVSu-1504	Nigeria
4	TVSu-1509	Nigeria
5	TVSu-1510	Nigeria
6	TVSu-1512	Nigeria
7	TVSu-1513	Nigeria
8	TVSu-1552	Nigeria
9	TVSu-1554	Nigeria
10	TVSu-1555	Nigeria
11	TVSu-1559	Nigeria
12	TVSu-1563	Nigeria
13	TVSu-1584	Nigeria
14	TVSu-1591	Togo
15	TVSu-1604	Togo
16	TVSu-1605	Togo
17	TVSu-1610	Togo
18	TVSu-1614	Togo
19	TVSu-1620	Togo
20	TVSu-1625	Togo
21	TVSu-1627	Togo
22	TVSu-1631	Togo
23	TVSu-1638	Mali
24	TVSu-1639	Mali
25	TVSu-1688	Togo
26	TVSu-1697	Togo
27	TVSu-1702	Togo
28	TVSu-1713	Zambia
29	TVSu-1766	Malawi
30	TVSu-1769	Malawi
31	TVSu-1788	Malawi
32	TVSu-1819	Cameroon
33	TVSu-1917	Cameroon

To each tube 700 µL of hot (65^{o}c) plant extraction buffer(PEB) [which contains 637.5 mL of double distilled water (ddH_20), 100 mL of 1M Tris-HCl (pH 8.0), 100 mL of 0.5 M ethylenediaminetetraacetic acid (EDTA) (pH 8.0), 100 mL of 5M $Nacl_2$ and 62.5mL of 20% sodium dodecylsulphate (SDS)] was added. One percent b-mercaptoethanol was added to the pre- warmed PEB just before use. The tubes were capped and inverted gently 6-7 times to mix the sample with buffer. The solution was incubated at 65°C in water bath for 20 minutes with occasional mixing to homogenize the samples. After 20 minutes, samples were removed from the water bath and uncapped. The tubes were allowed to cool at room temperature for 2 minutes. After which 500 µL of 5M of potassium acetate (CH_3COOK) was added to each tube and recapped. The tubes were mix inverted 6-7 times and incubated on ice for 20 minutes. After 20 minutes of incubation on ice, tubes were spun at 12,000 rpm for10 minutes at 4°C. The supernatant was transferred into new 1.5 mL eppendorf tubes using wider bore pipette tips (1000 µL) and making sure debris were not taken along with the supernatant. 700-µL chloroform isoamylalcohol was added to the supernatant and spun at 10,000 rpm for 10 minutes. The supernatant was transferred again to a new correspondingly labeled tubes and 700-µL ice-cold isopropanol was added to each tube and mixed by gently inverting the tubes 6-10 times. The tubes were allowed to stand undisturbed in a rack and stored in a freezer (-20°c) for at least 1 hour to precipitate the DNA. After 1-hour precipitation in the tubes, the DNA were centrifuged at 12,00 rpm for 10 minutes at 4°C. The supernatant in the freezer, was carefully discarded with great care to disallow the pellet from dislodging from the bottom of the tube. The tubes were allowed to drain inverted on clean paper towels for 1 hour. The DNA pellets were washed twice in 100µL, cold 70% ethanol for 20 minutes and air dried completely. After drying, 60µL of 1×TE [10mM Tris-HCL (pH 8.0), 1mM EDTA (Ph 8.0)] was added to the pellets, followed by 2µL of 10 ng/ml RNAse to remove the RNA. The DNA was then diluted to 10 ng/ul and then used for Polymerase Chain Reaction (PCR)

PCR Amplification

Nine SSR markers (Inqaba Biotech, South Africa) for Bambara groundnut was used to perform the PCR reactions and analysis for genetic diversity among the Bambara groundnut accessions. The amplification was performed in a 25 µL reaction volume containing 10X buffer, 1.6 µL of 25 mM of $Mgcl_2$, 2.0 of 5µ/µL Tag, 8.0µL sterile distilled water and 4.0 µL sample DNA using a PTC-200 Thermal cycle. The PCR reaction was carried out with the following protocol: initial denaturation at 94^{o}C for 5mins followed by 45 cycles of 30 secs at 94^{o}C, then 30mins at 65^{o}C and continues in that order. The resulting amplicons were loaded on 1.5% agarose.

Agarose gel electrophoresis of the PCR product

1.5% agarose was prepared, which was microwaved to dissolve the agarose and cooled down (56^{0}C). The gel

Table 2. Description of the SSRs markers used in this study

Marker	Forward primer	Reverse primer	Gene bank ID
P36	5¹-AAAATTGGAGAAAGGGGTTTTT-3¹	5¹-GATTTCGCCATATCCCCATC-3¹	GQ411715.1
P56	5¹-GCAATGGGTTCGTCGATACT-3¹	5¹-GCTCGATGCTTTTTGTTTCC-3¹	EU717407.1
P57	5¹-GGGAAACAAAAAGCATCGAG-3¹	5¹-CGCTACCCCAAAATACCAAA-3¹	EU717407.1
P61	5¹-GTCAGAGGCGAATTGAAAGC-3¹	5¹-AGGTCTTCCCGTTCCTTCAT-3¹	EU717373.1
P63	5¹-ATGAAGGAACGGGAAGACCT-3¹	5¹-CCTAAGGGCATATCGGTTGA-3¹	EU717373.1
P68	5¹-CAAGTCCCTCTATCCCCAAA-3¹	5¹-CAAGTCCCTCTATCCCCAAA-3¹	EU717348.1
P71	5¹-GTGTTGGGTTCAAAGCTGGT-3¹	5¹-CATCGGTCCACACAGTTGTC-3¹	EU717266.1
P131	5¹-CAAAGCCATTGCTGAAGACA-3¹	5¹-GGATGCTACACCGTTCGATT-3¹	HB823749.1
P184	5¹-GCCAGAGACTCTCACGTTCC-3¹	5¹-TGCATGGTCCCTGTTGTAGA-3¹	HB465729.1

was poured into the gel tray that was prefixed with comb. The gel tray was immersed into an electrophoresis tank containing 0.5 XTBE buffer.

The comb in the gel was removed to expose the wells formed. The amplified DNA (10 µL) was loaded into the wells of the gel with the aid of a pipette. A standard DNA molecular size marker (1 kb DNA lambda) was also loaded as a check. The gel ran for 2 hours at 150V and 0.5mAmp. After electrophoresis, the gel was silver stained. Thereafter the gel was destained in distilled water for 10 minutes. and the DNA bands in the gel was observed under UV (Ultra violet) lamp and photographed using a digital camera.

Each accession was scored (1) for presence and (0) for absence of polymorphic band for each primer. The band scoring data was used to calculate genetic similarity based on Jaccard's similarity coefficients (17) as follows:

$$GSij = a/(a+b+c),$$

Where,

GSij is the similarity between two accession i and j;
a is the number of bands present in both I and j;
b is the number of bands present in I but absent in j; and
c is the number of bands present in j and absent in i.

In addition, the Jaccard's similarity coefficient was used to perform cluster analysis based on the Unweighted Pair Group with Arithmetic mean (UPGMA) and in constructing a dendrogram. The computer program NTSYS pc version 2.1 (18) was used for these analyses. Furthermore, the genetic diversity, allele frequency and polymorphic information content (PIC) were computed using PowerMarker (Version 3.25).

Results and discussion

Polymorphism in several genes controls all phenotypic variations within a species. In this respect, the assessment of genetic variability within crop species using molecular markers is of great importance to plant breeders (15, 19). The molecular analysis of genetic diversity in the evaluated accessions of Bambara groundnut as determined by the SSR markers amplified a total of 27 alleles. The PIC values, which is a measure of the allelic diversity of SSRs, ranged from a minimum of 0.001 to a maximum of 0.617 with a mean of 0.419. Seemingly the obtained PIC mean was relatively high when compared to the report from Basu *et al* (20), but however relatively lower than the report of Mohammed (21). Apparently the SSR makers used in this study were not *Vigna subterranea* specific but *Vigna unguiculata*. Concisely maker P36 had the highest Marker Index (MI) which is a measure of efficiency to detect polymorphism. Invariably maker P36 was more genetically efficient in distinguishing the phenotypical similarity that exist between the Bambara groundnut accessions. On the other hand, markers P131 and P68 had monomorphic phenotype. These two markers recorded the least number of alleles per locus. A similar result has been reported by other workers (14, 15, 22, 19).

Generally, the 9 SSR markers (**Table 2)** showed the availability of a substantial level of polymorphism among the Bambara groundnut lines as revealed by both genetic distance and cluster analysis. The accessions were grouped into five groups based on Jaccard Neighbor-joining dendrogram. Each group had subgroups comprised of distinct genotypes from different eco-regions. This implies that variations among individual genotypes was mainly responsible for the observed genetic variation among the Bambara groundnut lines and not variations established between specific accession groups. Generally, reasonable intra-accessions polymorphism was observed in the cluster analysis of the accessions. In previous studies some workers reported extensive genetic diversity (12, 15), while others observed considerable genetic diversity (13, 3), yet some others reported a low range of genetic diversity (23) in Bambara groundnut. Further in this study, divergent genotype was revealed by the cluster analysis using Unweighted Pair-Group Method with Arithmetic Average (UPGMA). TVSU 1554 from Nigeria was the only accession found in Group 5 among the five groups of the cluster analysis. Invariably this accession was dissimilar from other accessions evaluated. Divergent genotype(s) usually have good breeding value which can be used for crop improvement, in both direct selection and as parents for making crosses.

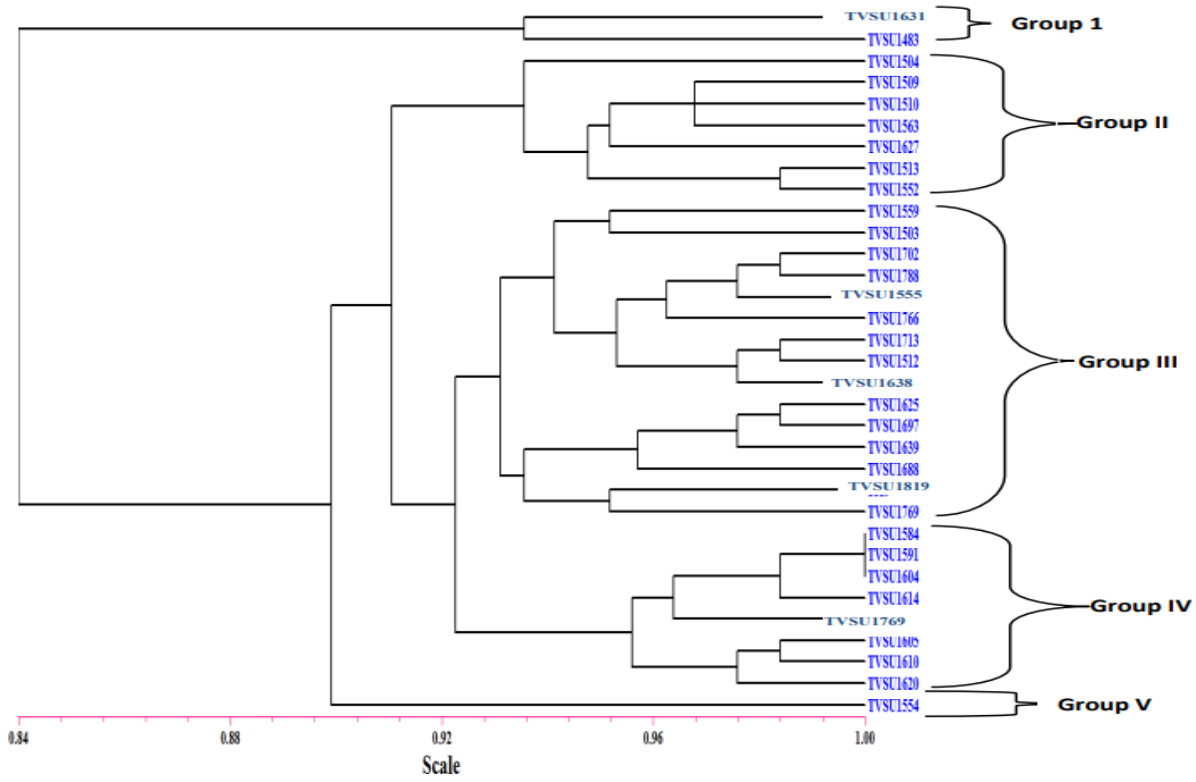

Figure.1: Molecular dendrogram analysis of 33 accession of Bambara groundnut with unweighted pair group method with arithmetic method (UPGMA).

The genetic distance from the molecular dendrogram analysis based on Jaccard's similarity coefficient ranged from 0.84 to 1.00 (**Figure 1**).

This implies that at 100% level of similarity, all the accessions were distinct from each other, while at 84% level of similarity all the accessions clustered to form a single accession. Invariably it means that each accession had at least one neighbour with more than 84% similarity. However, at 94% level of similarity, the accessions were grouped into five clusters.

The pattern of clustering of the Bambara groundnut accessions in groups with the Unweighted Pair Group with Arithmetic mean (UPGMA) and Jaccard's Neighbour-joining dendrogram was similar (**Figure 2**). Accessions were clustered in the same group based on genetic similarity and not on sources of collection or eco-region of origin. There were five heterogenic groups in the two-cluster analysis. The reason why accessions from the same geographical area could not form a distinct cluster was because they were genetically dissimilar. Each cluster, therefore, contained accessions with similar genetic characters. For example, group one of the Unweighted Pair Group with Arithmetic mean (UPGMA) grouping was the smallest group. It had two accessions, and both were collected from different geographical areas as can be seen in their identification numbers; TVSU 1483 was from Ghana, while TVSU 1631 from Togo.

However, both were clustered together in group one, implying that the two accessions were duplicates or closely similar, and not two different accessions from two different localities as indicated in their identification numbers and places of collections. Another outstanding example was observed in group four. The accessions clustered in this group were TVSU 1584, TVSU 1591 and TVSU 1604. Accession TVSU 1584 was collected from Nigeria, while TVSU 1591 and TSVU 1604 were from Togo. A detailed analysis of this result surprisingly showed that these three accessions, that were collected from different geographical areas, were the most genetically similar lines among the evaluated Bambara groundnut collections. Similarly, in group 1 of Jaccard Neighbour-joining (JNJ) dendrogram, a comparable association was substantiated between TVSU 1697 and TVSU 1627 (from Togo) and TVSU 1503 (from Nigeria).

These complex linkages suggest the possibility that these accessions were related. They either had similar genes or where from a common origin but were given different identification numbers. Apparently, these results showed the potentials of the SSR markers in detecting differences and establishing the extent of genetic relatedness in existence among the evaluated genetic materials. It also emphasizes the superiority of SSR markers in classifying collections of Bambara groundnut more precisely, as against the use of morphological markers in germplasm characterization (19).

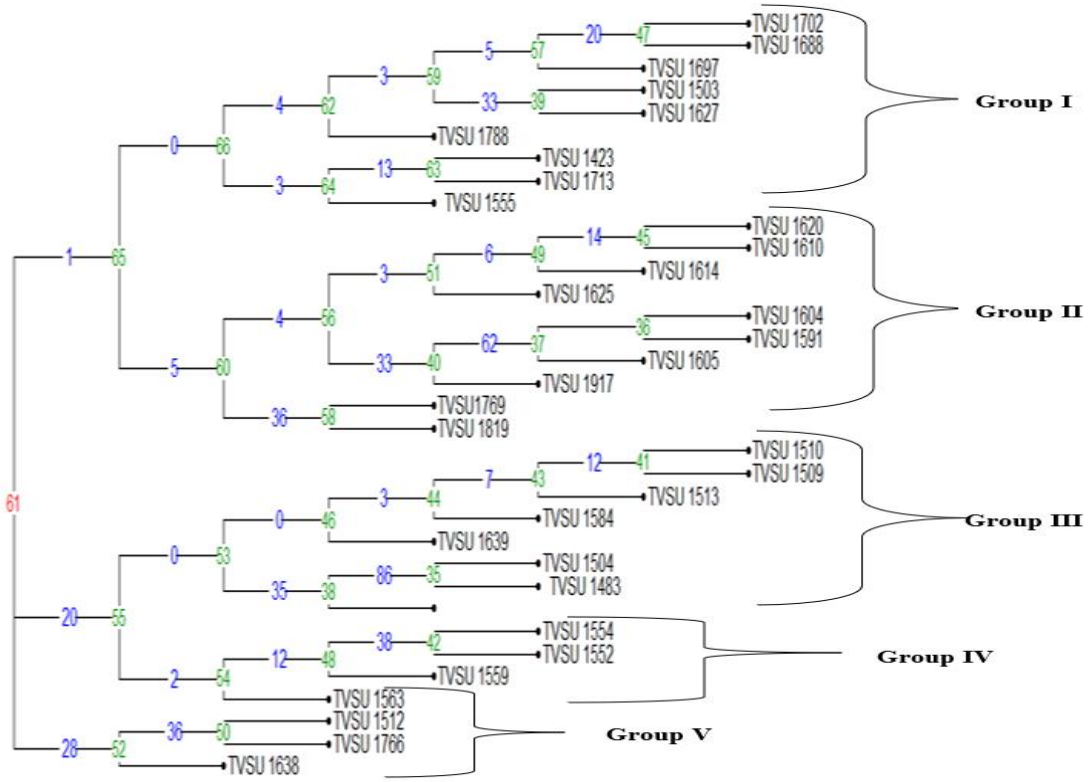

Figure 2: Jaccard Neighbour-joining dendrogram illustrating genetic diversity and relationships among 33 Bambara groundnut accessions.

Clustering of accessions of Bambara groundnut collected from different geographical areas in the same group may have arisen due to duplication of genotypes caused by high rate of seed exchange between farmers from diverse ethnic and agro-geographical areas. In fact, there was a report from a previous study on a similar trend of association between Bambara groundnut accessions collected from different geographical areas, and in that study, it was concluded that these accessions were either related, or the genotypes were the same (22).

A detailed examination of the result from the dendrogram showed that accessions from Nigeria were more dispersed among the clusters of accessions than accessions from other African countries. These accessions were found in four out of the five genetic groups. This observation may have some implication on the origin of Bambara groundnut, in that it supports the report of earlier studies on the origin of this crop; Nigeria may have been a regional centre of diversity of Bambara groundnut (24), which other studies confirmed by the existence of genuinely wild state of the crop in this area (25, 26).

Conclusion

Molecular analysis of genetic diversity of 33 accessions of Bambara groundnut based on SSR maker was reported. Genetic distances result and the cluster analysis revealed high level of polymorphism, indicating the existence of a wide range of genetic diversity among the accessions. This result can facilitate selection of appropriate genotypes for the development of improved lines of Bambara groundnut through various breeding programs. This study has contributed to broadening the genetic base of Bambara groundnut. The usefulness of this report in the effective utilisation, management, and conservation of Bambara groundnut germplasm is undoubtable.

Author's contribution

MIU conceptualized the research proposal and supervised the research activities. NCO performed the lab works, scoring, data analysis and interpretation. NCO and GOC wrote the first draft of the paper. All authors read and approved the final manuscript.

Competing Interests

No competing interests were disclosed.

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