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GENETIC DIVERSITY IN BAMBARA GROUNDNUT (*Vigna subterranean*) AS REVEALED BY MOLECULAR WEIGHTS OF THE SEEDS' PROTEINS

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Abstract

Nine accessions of Bambara groundnut (*Vigna subterranea* (L.) Verdc.,syn. *Voandzeia subterranea* (L.) Thouars ex DC.) obtained from National Centre for Genetic Resources and Biotechnology (NACGRAB), Ibadan, Oyo state, were assessed for their genetic and phylogenetic relatedness through electrophoretic analysis of the seed proteins. 0.2g of the seeds were weighed and macerated with mortar and pestle in 0.2M phosphate buffer containing 0.133M of acid (NaH_2PO_4) and 0.067 of base (Na_2HPO_4) at pH 6.5. Protein characterization with standard marker revealed that the seeds of the nine accessions contained proteins (B.S.A, Oval Albumin, Pepsinogen, Trypsinogen and Lysozyme) with molecular weights ranging from 66kda and above, 45 – 65 kDa, 44 – 33 kda, 32-24 kDa and 23-14 kDa, respectively. The student T-test revealed that accessions B, C, E, F, H and I have molecular weights not significantly different from one another ($P<0.05$) while samples A, D and G showed significantly different values ($P>0.05$). All the accessions had at least two proteins and two major bands in common. The study revealed intra-specific similarities and genetic diversity in protein contents among the nine accessions of Bambara groundnut (*Vigna subterraranea (*L.) Verdc.

Keywords: Accessions; gel electrophoresis; intra-specific; germplasm; genetic diversity

INTRODUCTION

Genetic diversity deals with the total number of genetic characteristics in the genetic make-up of a species, it is different from genetic variability which explains the tendency of genetic characteristics to vary (*Wikipedia*). Grain legume is a major component of grain-based farming system in many parts of the world. It can be divided into two groups as major and minor species according to world economy and plant utility (Omitogun *et al.*,2001, Aremu *et al.*,2006) .The major ones are the industrial legumes such as soybean and groundnut which are extremely important in the world economy (Aykroyd and Doughty, 1969, Agbolade and Komolafe, 2016). Others among the list are common beans (*Phaseolus vulgaris*), Chicken pea (*Cicer arietanum*) and pea (*Pisum sativum*). The minor species have a broad spectrum of diversity across various regions worldwide; existing either as cultivated or wild species (Agbolade, 2016). Bambara groundnut (*Vigna subterranea* (L.) Verdc.) is a popular example of the minor legumes. Other examples in the minor species category include Kersting

groundnut (*Kerstingiella geocarpa*), Marama bean (*Tylosema esculentum*), Pigeon pea (*Cajanus cajan*), Rice bean (*Vigna angularies*), Mung bean (*Vigna mungo*) and few other ones.(Agbolade,2013 and 2016) Consumption of legumes was highly correlated with reduced mortality resulting from coronary heart diseases (Aremu *et al*.2006). Cultivation of Bambara groundnut (*Vigna subterranea* (L.) Verdc. syn. *Voandzeia subterranea* (L.) Thouars ex DC.) is largely practiced among the small holding farmers of the semi-arid regions of Africa where it is widely intercropped with cereals, root and tuber crops (Olukolu et al., 2012). The average yields of Bambara groundnut (650–850 kg ha^{-1}) are smaller than those of other legumes under favorable edaphoclimatic conditions(Stanton et al. 1966), but it out-yields other legumes under less favorable conditions, particularly under limited rainfall and on infertile soils (National Research Council, 2006; FAO, 2011).

According to Olukolu et al., (2012), Bambara groundnut is the third most important legume crop after peanut and cowpea; and it is a preferred food crop of many local people, as a good supplement to cereal-based diet and it is nutritionally superior to other legumes. Evaluation data on genetic diversity is a pre-requisite for genetic improvement in crop plants particularly in underutilized crops such as Bambara groundnut (Bamishaye and Adegbo,2011). Going by the available literature, a few numbers of studies on genetic diversity of Bambara groundnut are on record.Basu *et al.,(*2007) worked on genome mapping and molecular breeding of this Bambara groundnut; Collinson et al. (1997) found out the effects of soil moisture deficit on water relation capacity of Bambara groundnut. Amadou et al., (2001) and Massawe et al., (2002) used Random Amplified Polymorphic DNA (RAPD) and Amplified Fragment Length Polymorphism (AFLP) markers, respectively, to assess genetic diversity in this crop. Recently, Olukolu et al, (2012) evaluated the genetic diversity of a Bambara groundnut germplasm representing accessions from 25 African countries, maintained at the International Institute of Tropical Agriculture (IITA; Nigeria) was based on seed patterns, qualitative characters, quantitative traits and Diversity Arrays Technique (DArT) markers. Since protein is a direct product of gene action, the relationship of a group of specie or accession can be determined through electrophoresis which deals with proteins, the primary product of genes. Therefore any observed phenotypic expression in the banding pattern of proteins extracted from an organism is indicative of genetic variability among the studied taxa (Agbolade et al, 2013 and 2017). However, none of these studies has explored the use of proteins' molecular weights markers to delineate diversity among the various accessions of Bambara groundnut. Hence, this study has been designed to corroborate the existing data on the subject matter.

MATERIALS AND METHODS

Materials and Seeds Acquisition: Tris-base, tris-HCl, ethanol, phosphoric acid and coomassie Brilliant blue G-250 were purchased from Sigma Chemical Company, St. Louis, MO, USA. Bovine Serum Albumin (BSA), Standard proteins as contained in Sigma Molecular Weight Markers Calibration Kit for SDS polyacrylamide gel electrophoresis (Daltons Mark VII-L, Molecular Weight Marker Range 14,000-70,000) were purchased from Sigma Chemical Company, St. Louis, MO, USA. Seeds of the nine Bambara groundnut (*Vigna subterranea* (L.) Verdc.) accessions were obtained from the Genetic Resources Unit of the International Institute of Tropical Agriculture (IITA), Ibadan, Oyo-State, Nigeria.

Sample Preparation: Two grains of each sample was macerated using a sterile mortar and pestle; it was soaked in 10 ml of 0.2M phosphate buffer, PH 6.5 and left in the refrigerator for 24 hours. The samples were then centrifuged to obtain a supernatant and the protein concentrations were also determined using Bradford method. The supernatants obtained were used for the SDSPAGE.

Protein Determination: Protein concentration was determined by the method of Bradford, as contained in Weber and Osborn (1976), using Bovine Serum Albumin (BSA) as the standard, whereby the protein absorbance was interpolated from the standard curve. The reaction mixture consists of 10 µl of the sample solutions and 1.0ml of Bradford reagent (Weber and Osborn, 1976). The absorbance was read at 595nm. Mathematically, according to a standard protein graph, at every optical density (O.D) of 0.4, the protein concentration is 5.0mg/ml.

Therefore, at 0.4 = 5.0mg/ml
$O.D_{new} = X$
$5.0mg/ml \times O.D_{new} = 0.4X$
X=O.Dnew/*5.0mg/ml/0.4
X=0.4Dnew*12.5

Preparation of Bradford Reagent: 0.1g of coomassie brilliant blue 6-250 was weighed and dissolved in 50ml of 99% ethanol and made up to 1000ml using 100ml of 85% (w/v) of phosphoric acid. The mixture was then filtered to give a homogenize mixture with minimal or no impurities.

Electrophoresis on SDS-PAGE
The molecular weight ranges of the samples were determined by SDS polyacrylamide gel electrophoresis as described by Weber and Osborn (1976). Standard proteins were as contained in Sigma Molecular Weight Markers Calibration Kit for SDS polyacrylamide gel electrophoresis (Daltons Mark VII-L, Molecular Weight Marker Range 14,000-70,000). The vial was reconstituted in 1 ml of sample buffer, mixed properly and placed in a boiling water bath for 2 min. A 10 µl aliquot was applied to a gel. The preparation of enzyme sample, running conditions, staining and destaining were as described earlier by Weber and Osborn (1976). The relative mobility was calculated using the following expression:

$$R_f = \frac{\text{Distance of protein migration}}{\text{Length of gel after staining}} \times \frac{\text{Length of gel before staining}}{\text{Distance of dye migration}}$$

R_f values of the standards were then plotted against the logarithms of their molecular weights. The molecular weight of the enzyme preparations was then interpolated from the curve.

Data Analysis: The molecular weight values obtained were subjected to student t-test to evaluate the significance level of differences among the various accessions studied.

RESULTS AND DISCUSSION

The relationship of a group of a group of specie/ accession can be determined directly through electrophoresis, which deals with proteins that are the primary product of gene actions. Hence any similarities and differences observed in the banding patterns of protein extract of organisms is an indication of genetic similarities and differences. Despite the similarities observed in the banding patterns, a close examination revealed that differences still abound among the Bambara groundnut accessions. These similarities are expected and the differences noticed are understandable because both 'nature' and 'nurture' determine the phenotype of an organism. The differences observed might be due to the impact of the environment (nurture). It follows quite logically that different accession belonging to the same species are expected to be more phylogenetically related.

The result of electrophoresis of crude protein from the nine (9) of the Bambara groundnut accessions studied are shown in Plate 1. Table 2 shows the list of the accessions used for the electrophoresis and the protein concentration observed for each accession. Table 3 shows characterization of protein based on their molecular weight. The result of electrophoresis shows that some of the accessions are quite dissimilar both in terms of number and intensity of the bands while some of other one shows a certain degree of relatedness; this is similar to the observation made by (Agbolade *et al*., 2013). Table 2 shows the result for determination of protein at optical density (O.D) 595nm which gives the concentration of protein in each accession. BN_1 has the highest protein concentration while BN2 has the lowest. Similarities and differences in protein composition of the accessions are represented in table 3 which characterized the proteins based on their molecular weights. According to a standard protein graph (Weber and Osborn, 1976) the result shows that all accessions have a high degree of BSA present in their bands with molecular weight ranging from 66 KiloDaltons and above.

Table 1. Result for Determination of Protein at Optical Density (O.D) of 595Nm

S/N	Protein concentration (mg/ml)
BN1	0.066
BN2	0.053
BN3	0.065
BN4	0.060
BN5	0.062
BN6	0.062
BN7	0.063
BN8	0.061
BN9	0.061

Sample A with sample number NGB 01494^{k5} and sample identification (BN_1) with average protein concentration of 0.066 at optical density 595nm with length of gel 7.1 which contains total bands of 6 of which the first two (2) fall under Bovine Serum Albumin with RF value of 0.042 and 0.085 respectively and molecular weight of 66kilo Dalton and above and band 3 falls under Oval Albumin with RF value of .324 and molecular weight 45-65kilo

Daltons, bands 4 and 5 fall under protein Pepsinogen with RF values of 0.451 and 0.507 and molecular weights of 33-44kiloDaltons, the last band, band 6 falls under protein Trypsinogen with RF value of 0.648 and molecular weight of 24-32kiloDaltons, but lack the presence of lysozymes.

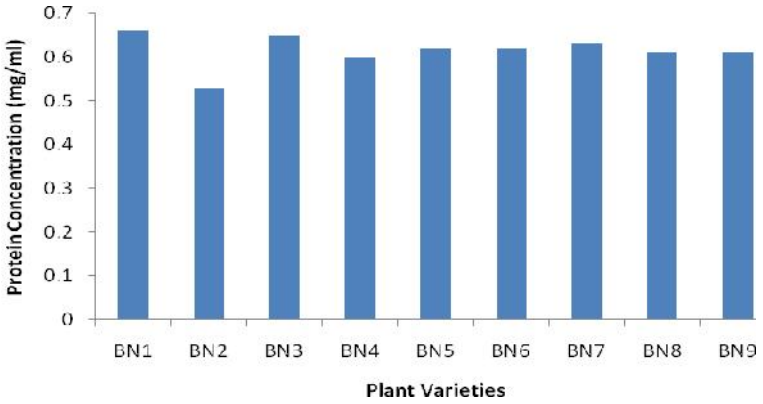


Fig 1. Standard protein graph.

Sample B with sample number NGB 01497^{K5} and sample identification (BN_2) with average protein concentration of 0.053 at optical density of 595nm with the length of gel 7.3 which consist of 6 bands, 3 thick bands and 3 thin bands, in which two (2) of these fall under Bovine Serum Albumin with RF values of 0.041 and 0.082 and molecular weights of 66kiloDaltons and above, and band 3 falls under the Oval Albumin of RF value of 0.288 and molecular weight of 45-66kilodaltons, bands 4 and 5 fall under the protein Pepsinogen with RF values 0.438 and 0.506 with molecular weights of 33-44kiloDalto ns and the last band, band 6 falls under the protein Trypsinogen with RF value of 0.644, molecular weight 24-33kilodalton also lacks the lysozyme.

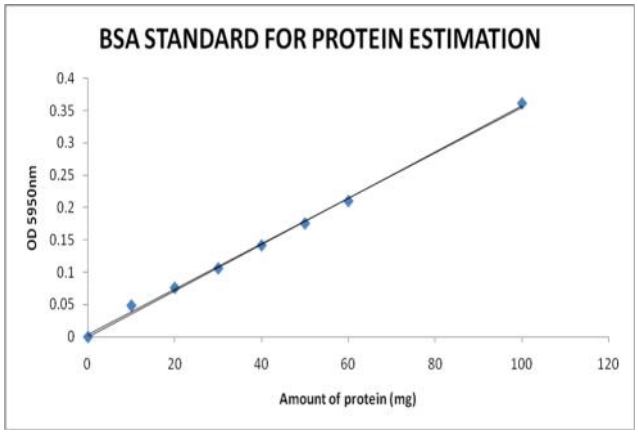
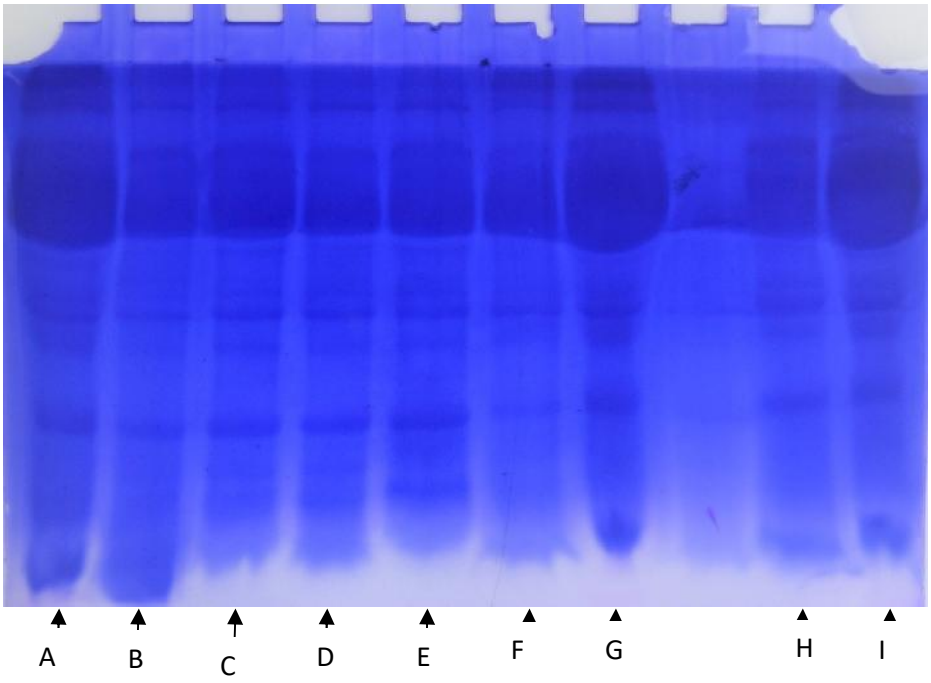


Fig 2. BSA Standard for protein estimation.

Sample C with sample number NGB 01499[K5] and sample identification (BN_3) with average protein concentration of 0.065 at optical density of 595nm with gel length 7.0 has a total number of 7 bands, 5thick bands and 2 thin bands. Two (2) of these bands fall under Bovine Serum Albumin with RF value of 0.057 and 0.086 and molecular weight of 66kilodaltons and above, band 3 falls under Oval Albumin of RF value of 0.314 and molecular weight of 45-65kilodalton, band 4 and 5 falls under the protein Pepsinogen with RF value of 0.457 and 0.514 and molecular weight of 33-44kilodalton while bands 6 and 7 fall under protein lysozyme with RF value of 0.657 and molecular weight 14-23kilodalton, no band was indicated under Trypsinogen.

Sample D with sample number NGB 01501[K5] and sample identification BN_4 with an average protein concentration of 0.060 at optical density 595nm with length of gel 6.8, consisting of 8 bands with 6 thick bands and 2 thin bands, two (2) of which fall under Bovine Serum Albumin with RF value 0.044 and 0.074 and molecular weight of 66kilodalton and above. Band 3 occurs in the region of 45-65kilodalton regarded as Oval Albumin with RF value of 0.309, bands 4 and 5 fall in the region of protein Pepsinogen with RF value of 0.426 and 0.470 and molecular weight of 33-44kilodalton, while bands 6,7 and 8 falls under the protein lysozyme with RF value of 0.676, 0.750 and 0.809 respectively with molecular weight 14-23kilodalton. This accession of all has the highest crude protein concentration.



Samples: A, B, C, D, E, F, G, H, I.
KEY: A to I represent samples BN1 to BN9

Sample E with sample number NGB 01245 and sample identification BN^5 with average protein concentration of 0.062 at optical density 595nm with length of gel 6.6 consist of 7 bands in total, 5 thick bands and 2 thin bands. 2 of these bands fall under Bovine Serum Albumin with RF value of 0.045 and 0.091 and molecular weight of 66 kilodalton and above. Band 3 occurs in the region of 45-65kilodalton regarded as Oval Albumin with RF value 0.318 and bands 4 and 5 occur in the region of protein Pepsinogen with RF value 0.469 and 0.530 molecular weight of 33-44 kilodalton. Bands 6 and 7 occur under the protein Lysozyme with RF value 0.682 and 0.818, respectively. Bands 1, 2, and 3 have the highest concentration of crude protein.

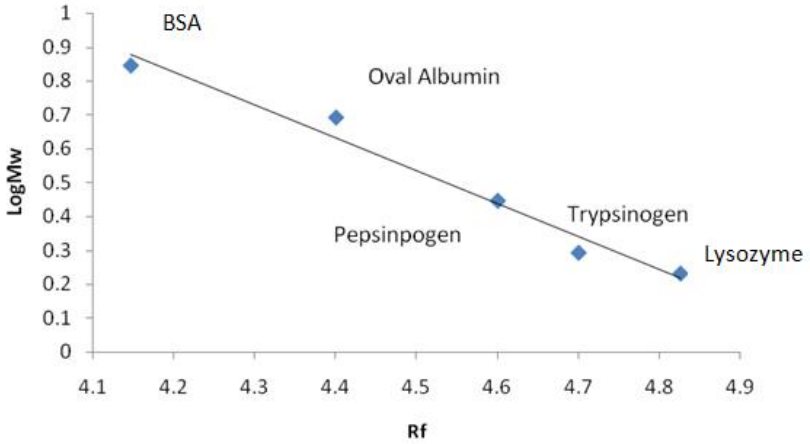


Fig 2. Log M_w versus R_f Graph for the Estimation of Proteins' Molecular Weights.

Sample G with sample number NGB 01311^{K5} and sample identification of BN_7 with the average protein concentration of 0.063 at optical density 595nm with gel length 6.9 has a total band of 6 of which 4 are thick and 2 are thin. The highest concentration of crude protein is found in bands 1, 2, and 3. Two (2) of these bands occur in the Bovine Serum Albumin region with RF value of 0.029 and 0.072 and molecular weight of 66 kilodalton and above. Band 3 occurs in the protein Oval Albumin region with molecular weight of 45-65 kilodalton and RF value of 0.333. Bands 4 and 5 occur in the region of protein Pepsinogen with molecular weight of 33-44kilodalton and RF value of 0.449 and 0.493, respectively. Band 6 falls under the region of protein Trypsinogen with RF value 0.623 and molecular weight of 24-32kilodalton, no band indicated the presence of Lysozyme in the sample.

Sample H with sample number NGB 01496^{K5} and sample identification BN_8 with the average protein concentration of 0.061 at optical density 595nm with gel length of 6.7 has a total bands of 6, with 4 thick bands and 2 thin bands. The highest concentration of crude protein is found in band 1, 2 and 3. Two of these bands occur in the region of the Bovine Serum Albumin protein with RF value of 0.029 and 0.075 with molecular weight of 66kilodalton and above. Band 3 with RF value of 0.313 and molecular weight of 45-65 kilodalton occurs in the region Oval Albumin. Bands 4 and 5 with RF value 0.463 and 0.507, respectively occurs in the region protein Pepsinogen of molecular weight 33-44kilodalton while the last

band occur in the region of the protein Trypsinogen with RF value of 0.642 and molecular weight of 24-32kilodalton, also no bound was found under Lysozyme. Table 2 below shows the characterization of the seed proteins. The proteins were characterized based on the molecular weight of the bands in comparison to the standard gel.

Table 2. Characterisation of proteins based on their molecular weights

Samples	Total Bands	BSA (66 and Above)	Oval Albumin (45-65)	Pepsinogen (33-44)	Trypsinogen (24-32)	Lysozyme (14-23)
A	6	2	1	2	1	0
B	6	2	1	2	1	0
C	7	2	1	2	0	2
D	8	2	1	2	0	2
E	7	2	1	2	0	2
F	6	2	1	1	2	0
G	6	2	1	2	1	0
H	6	2	1	2	1	0
I	5	1	1	2	1	0

Sample F with sample number NGB 01489^{K5} and sample identification of BN_6 with average protein concentration of 0.062 at optical density of 595nm with gel length of 6.8, which consist of 6 clear bands, with 4 thick bands and 2 thin bands. Two (2) bands are found under the protein Bovine Serum Albumin with molecular weight 66kilodalton and above and RF value of 0.029 and 0.072 respectively, band 3 has a RF value of 0.333 and occurs in the region of Oval Albumin of molecular weight 45-65kilodalton. Band 4 with RF value of 0.449 and molecular weight of 33-44kilodalton is found under Pepsinogen, while bands 5 and 6 occur in the region of protein Trypsinogen with molecular weight of 24-32kilodalton with RF value of 0.493 and 0.623, respectively. Bands 1 and 3 have the highest crude protein concentration and no band was found under Lysozyme.

Sample I with sample number NGB 01495^{K5} and sample identification of BN_9 with the average protein concentration 0.061 at optical density 595nm with gel length 7.0 which has a total band of 5, 3 thick and 2 thin bands. Band 1 occurs in the BSA region with molecular weight of 66kilodalton and above and RF value of 0.071. Band 2 occurs in the Oval Albumin region with molecular weight of 45-65kilodalton and RF value of 0.314. Band 3 and 4 with RF values 0.428 and 0.471 occur in the region of protein Pepsinogen with molecular weights of 33-44kilodalton while the last band with RF value of 0.600 falls under the Trypsinogen protein with molecular weight of 24-32 kilodalton, no band again was noticed under Lysozyme. The proteins characterized in these samples are of great importance to science and humanity. BSA is a protein with high negative charge, it binds water, salts, fatty acids, vitamins, hormones and carries these bound component between tissues and cells. It is also an effective scavenger removing toxic substance, also a stabilizer for other soluble proteins. Oval Albumin works effectively as selective insulin binding agent. Its potential applications are used in the area of oral drug delivery and also as

functional food supplement. Pepsinogen is a product of pepsin in an active precursor form by the chief cell. It helps in digestion of protein, while Trypsinogen, a precursor of trypsin functions as protein activation at appropriate. Lastly, Lysozyme according to Philips Mechanism is used as binding agent (Okpuzor et al., 2010, Agbolade et al., 2017).

It's worthwhile to emphasize that ultimately and logically differences in electrophoresis mobility of protein fractions from two sources are of greater importance for taxonomic purposes than the similarities of mobility. The possibility of two dissimilar proteins having identical electrophoretic mobility is known (Grant et al., 1988; Agbolade et al., 2013), yet the assumption is made that bands derived from two accession that migrate the same distance in Bambara groundnut are considered to be produced by gene(s) common to both accessions. The student T-test revealed that accessions B, C, E, F, H and I have molecular weights not significantly different from one another ($p<0.05$).

The result of the electrophoretic binding pattern of the studied accession of Bambara groundnut revealed some diagnostic characteristics that could be used for taxonomic decision. Similarities and differences observed in this work agreed with the studies of Grant et al., 1988 who adopted protein electrophoresis in evolutionary systematics; Massawe et al. 2002; Modini et al. 2006 and Agbolade et al. 2013 and 2017, who at both studies employed comparative electrophoretic protein banding pattern of different species and accessions in establishing relation among various taxa.

CONCLUSION

The essence of diversity studies in crop species is to reveal variation and its pattern within the crop's germplasm. Products of such assessments become materials(s) for crop improvement. The similarities and differences that occur in this protein profile of all accessions of Bambara groundnut are indicative of genetic protein content and thus may be useful in the taxonomic delimitation of the different accessions belonging to this specie. Furthermore, the electrophoresis of the seed proteins appeared to demonstrate close relationship and distinctiveness of the different accessions put into consideration and could therefore be important in genetic delimitation. Despite the fact that they portrayed similar phylogenic characteristics, there are still some little differences and similarities in the protein concentration. The seeds as revealed to be highly nutritious can be used as an excellent supplement in helping to achieve a balanced diet and overcome malnutrition especially among children. Extracts like boiled seeds, ethanol, boiled immature seeds are used to treat various ailments such as anemia, diarrhea, ulcers, gonorrhea, impotence and colon cancer. The current report clearly revealed diversity and phylogeny studies on the nine accessions of Bambara groundnut. Values recorded are representative of the genetic variants and overlaps and within the accessions of this taxon, and may serve as future reference for other studies in this field.

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