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Ribosomal DNA variation in landraces of bambara groundnut

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The ribosomal DNA (rDNA) is a basic component of all eukaryotic chromosomes which codes for rRNA. The rDNA is made of tandemly repeated units of 18S, 5.8S, 28S, ITS and IGS. The 18S, 5.8S and 28S are coding regions while the ITS and IGS are non coding. The rDNA evolves in a concerted manner such that rDNA units within an individual are almost completely identical making it a useful tool in taxonomic studies. The rDNA marker is used to discriminate between species in the same or different genus. Bambara groundnut is an under-utilized legume that is highly nutritious and adapted to low input farming systems. Despite its potential, Bambara groundnut is still cultivated in the form of landraces, and no true varieties have been developed. Precise estimate of genetic variation and genetic relationship is *sine qua non* for the development of improved cultivars. About 48 individual seeds (3 each) from seven landraces of Bambara groundnut, 9 wild/cultivated species of other legumes (cowpea, mungbean and groundnut) were used in this study. Analysis of the rDNA gene unit shows genetic variation in the species of the genus *Vigna* and *Arachis*. The UPGMA dendrogram constructed separates the plant species in this study into three (3) main clusters. The landraces of Bambara groundnut were placed in two different clusters indicating variation of rDNA gene units between the landraces. The analysis of rDNA gene in this study reveals the presence of genetic variation between the landraces of Bambara groundnut which could be used by scientists and breeders in the production of improved cultivars.

Key words: Ribosomal DNA, Bambara groundnut, ITS, IGS, DNA variation.

INTRODUCTION

Bambara groundnut (*Vigna subterranea* (L) Verdc.) is an important leguminous crop of African origin. Bambara is a name of a tribe and district in Mali, which is apparently where this legume derives its name from (Rassel, 1960). Because it is widely cultivated in the whole of sub-Saharan Africa, the specific centre of origin of Bambara groundnut is still unclear. There are two botanical varieties of Bambara groundnut: *Vigna subterranea* var *subterranea* which is the cultivated type and *Vigna subterranea* var *spontanea* which is the wild type (Vijaykumar et al., 2011). Bambara groundnut is locally referred to as 'gurjiya' or 'kwaruru' by the Hausas' in Nigeria although each dialect has a unique local name for Bambara groundnut in Africa. The International Institute of Tropical Agriculture (IITA) in Ibadan, Nigeria, is the major repository of the genetic resources of Bambara groundnut in Africa. Bambara groundnut is the most important legume after cowpea (*Vigna unguiculata*) and groundnut (*Arachis hypogea*) (Howell et al., 1994). As a preferred food crop to many in Africa, it is a good

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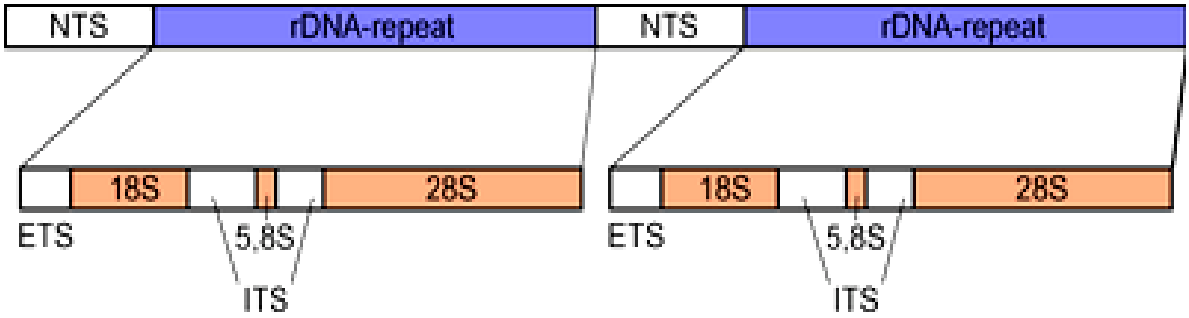

Figure 1. Ribosomal DNA cluster. Source: www.en.wikipedia.org/wiki/file:eucaryot_rdna.png.

supplement to cereal based diet and nutritionally superior to other grain legumes (Azam-Ali et al., 2001). Bambara groundnut is commonly consumed together with cereals because it contains sufficient amount of lysine which is lacking in cereals.

Ribosomal DNA (rDNA) is a component of all eukaryotic DNA. It is a conserved region that codes for ribosomal RNA (rRNA) which is involved in intra cellular protein synthesis (Kollipara et al., 1997). The ribosomal DNA consists of tandemly repeated units of 18S, 5.8S, 28S, intragenic spacer region (IGS) or non transcribed spacer (NTS) and internally transcribed spacer (ITS) as shown in Figure 1. The 18S, 5.8S and 28S regions are highly conserved in all eukaryotes unlike the ITS and IGS regions which are highly variable and non coding.

The high variability of the non coding regions (ITS and IGS) has been attributed to insertion, deletion and gene mutations. And it is this variation that is used to discriminate between individuals of the same or different species (Kollipara et al., 1997). For more than two decades now, the rDNA has been used as a powerful tool in comparative evolutionary and phylogenetic studies (Hillis and Davis, 1986; Mindell and Honeycutt, 1990; Wesson et al., 1992; Schlotterer et al., 1994; Vogler and DeSalle, 1994; Tang et al., 1996).

At present, there are no improved varieties of Bambara groundnut, as such the crop is still cultivated in the form of landraces in semi-arid regions where rainfall is limiting and environmental conditions for sustainable growth are harsh and unsuitable. A landrace is a combination of different genotypes with the ability to withstand biotic and abiotic stress and still produce good yields under low-input farming system (Zeven, 1998). Ever since Bambara groundnut was discovered in native West Africa, so many attempts have been made to characterize its complete germplasm in order to provide useful information that would facilitate the development of improved cultivars. Up till date, the complete germplasm of Bambara groundnut is yet to be fully exploited and no true variety has been produced.

The use of rDNA marker in this particular study is aimed at resolving molecular phylogeny between landraces of Bambara groundnut and some of the wild and cultivated species within the genus *Vigna* and *Arachis*. A similar study on tomato revealed the crop to be a close relative of potato and so taxonomists have placed both tomato and potato in the same genus *Solanum*, hence the change of name of tomato from *Lycopersicon esculentum* to *Solanum lycopersicon* (Doyle and Gaut, 2000). The information obtained from model crops through molecular phylogeny can thus be applied to the under-utilized crops in a bid to improve them.

The proposed study aims to evaluate genetic variation based on the occurrence of distinct rDNA within the landraces of Bambara groundnut which will thus, facilitate the appropriate selection of superior germplasms/genotypes for the breeding of true varieties of Bambara groundnut.

MATERIALS AND METHODS

About 48 individual seeds (3 each) from 7 landraces of Bambara groundnut and 9 wild/cultivated species of other legumes (cowpea, groundnut and mungbean) were obtained from the AAR-UNMC biotechnology research centre. The seeds were originally collected from Nigeria, Namibia, Botswana and Malaysia. The list of the seed material used is shown in Table 1. Finn peat soil contained in plastic pots (20 cm diameter) was used as a planting medium.

DNA extraction

Leaves were harvested from 3 weeks old plants (Table 1) and used for DNA extraction. The harvested leaves were ground to fine powder in liquid nitrogen. The fine powder was poured into 50 ml falcon tubes and DNA was then extracted using an optimized protocol developed at the AAR-UNMC biotechnology research centre. The protocol used was modified from previous protocol described by Dellaporta et al. (1983). To further confirm the integrity of the DNA extracted, 0.8% agarose gel electrophoresis was carried out. The DNA extracted was used as template for the PCR reactions.

Polymerase chain reaction

In order to optimize the primer conditions, a gradient PCR was carried out where all the primers were subjected to different annealing temperatures to select the best (optimum) primer annealing temperature.

The ranges of temperature used were between 50 – 60°C for the

Table 1. The list of plant accessions used, their status, origin and lanes they appeared on the gel pictures.

Plant	Status	Origin	Lane
V subterranea (Kano I)	Landrace	Nigeria	1
V subterranea (Kano II)	Landrace	Nigeria	2
V subterranea (Kano III)	Landrace	Nigeria	3
V subterranea (Malaysia Red)	Landrace	Malaysia	4
V subterranea (Getso)	Landrace	Nigeria	5
V subterranea (Namibia I)	Landrace	Namibia	6
V subterranea (Botswana I)	Landrace	Botswana	7
V radiata	Wild	Ghana	8
V mungo	Wild	India	9
V membranacea	Wild	Ethiopia	10
V lasiocarpa	Wild	Brazil	11
V racemosa	Wild	Ghana	12
V trilobata	Wild	Belgium	13
V unguiculata (Cowpea)	Cultivated	Nigeria	14
Arachis hypogea (Groundnut)	Cultivated	Malaysia	15
V radiata (Mungbean)	Cultivated	Malaysia	16

18S, IGS, ITS I and ITS II primers. The best annealing temperatures observed were selected and included in the main PCR amplification conditions. Similarly, for every reaction set (for each primer), a negative control (containing all PCR components except DNA template) was included. PCR amplification was carried out in a total reaction volume of 25 µl. The concentration of primer used was 0.2 µM and the amount of DNA added is 25 ng. The PCR amplification conditions used were: 94°C for 4 min (initial denaturation); 35 cycles of 94°C (denaturation) for 1 min, 55°C (primer annealing) for 1 min, 72°C (for amplification) for 1 min, and a final extension period of 10 min at 72°C.

The 4 primer pairs used to analyze the rDNA segments of Bambara groundnut and other species in the genus *Vigna* and *Arachis* in this study were synthesized by Bioline Marker Corporations, North Korea. The primer pair sequences are as follows: 18S (Forward: NS7 - 5' GAG GCA ATA ACA GGT CTG TGA TGC 3', Reverse : R635 - 5' GGT CCG TGT TTC AAG ACG G 3') (Diaz et al., 2000), 5S-IGS (Forward: VR5SL - 5 CCATCAGAACTCCGCAGTTA 3', Reverse: VR5SR - 5' GGATCCGGTGCATTAGTGCT 3') (Vijaykumar et al., 2011), ITS I: Forward - 5' AAGTCGTAACAAGGTTTCCGTAG 3', Reverse - 5' AAAGACTCGATGGTTCACG 3' (Souframanien et al., 2003) and ITS II: Forward – 5' TAGCGAAATGCGATA CTTGGT 3', Reverse - 5' GTTAGTTTCTTTTCCTCC 3') (Souframanien et al., 2003).

On completing the amplification of rDNA segments of the isolated genomic DNA, all the products were separated on a 1.5% agarose gel. One microliter (1µl) of SYBR safe (Invitrogen Oregon, USA) was added to the gel prior to casting. The PCR product was mixed with 6x loading dye (Fermentas life science, Canada) in a ratio of 5:1. Tris-borate-EDTA (TBE) buffer was used to cast as well as run the gels. The gels were run at 80V for 90 min.

Data analysis

Gel results from agarose electrophoresis were scored as (1) for the presence or (0) for the absence of bands. Each band size was treated independently. The scored data was prepared on a Microsoft excel spreadsheet and imported into the multivariate statistical (software) program (MSSP). The data consists of seven variables and sixteen samples and each size of amplified band fragment was treated as a variable. The data was then analyzed using average genetic distance and Bray-Curtis similarity coefficient. The similarity matrix obtained was used to construct the dendrogram based on UPGMA (Un-weighted Pair Group method with Arithmetic Averaging) clustering method. The diversity indices were also computed based on Shannon's diversity index ($\log_{10}$) without data transposition using the MSSP. Evolution in plant species is the basis on which the UPGMA algorithm analyses molecular data (Olukolu et al., 2011).

The % polymorphism of the rDNA markers were also calculated using the formula:

$$\frac{\text{Total number of polymorphic bands X 100}}{\text{Total number of bands}}$$

Table 2. Percentage polymorphism of the four primer pairs.

Primer	Number of bands	Polymorphism (%)
18S	12	16%
ITS I	14	14.2%
ITS II	9	33.3%
IGS	34	58%
Total bands	69	

RESULTS

The percentage polymorphisms of all the four primer pairs used are shown in Table 2. Of all the four primers used, IGS had the highest polymorphism (58%) followed by ITS II (33%). The dendrogram of the seven landraces of Bambara groundnut is shown in Figure 6 while the dendrogram of the 15 plant species in the genus *Vigna*

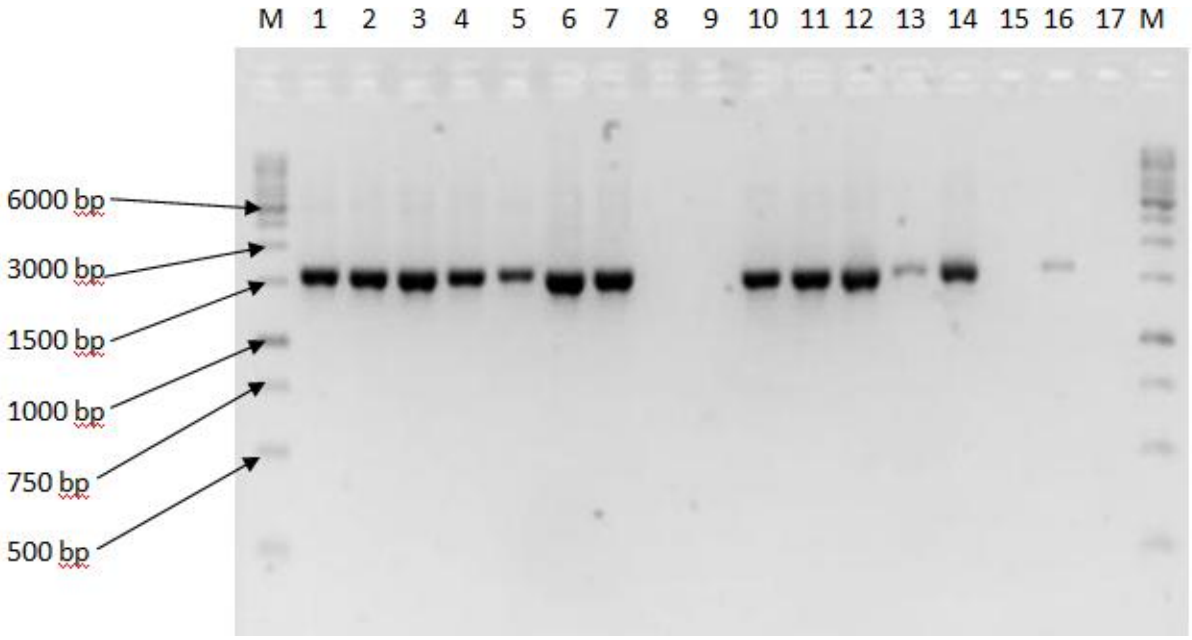

Figure 2. Gel picture of the PCR amplified 18S fragments. Lanes 1 – 7 correspond to the landraces of Bambara groundnut while lanes 8 – 13 correspond to the wild species listed in Table 1. Lane 14 – *V unguiculata*, lane 15 – *A hypogea*, lane 16 – *V radiata*, lane 17 – negative control while M represents the DNA ladder.

and groundnut (genus *Arachis*) is shown in Figure 7.

The dendrogram in Figure 6 separates the landraces of Bambara groundnut into two main clusters. Botswana I, Namibia I, Malaysia red, getso and Kano III formed the first cluster. The first cluster has two subclusters with Namibia I, Botswana I and Malaysian red forming the first subcluster while Kano III and getso formed the second subcluster. The second cluster has only Kano I and Kano II. Kano II formed the first subcluster while Kano I formed the second cluster.

The dendrogram, as shown in Figure 7 separates the landraces of Bambara groundnut used in this study into three (3) main clusters. The first cluster consists of cultivated mungbean, wild mungbean and wild *Vigna mungo* with an average genetic distance of (0.60). The cultivated mungbean formed the first subcluster while the wild mungbean and *Vigna mungo* formed the second subcluster. Similarly, all the cultivated landraces of Bambara groundnut except Kano I and Kano II formed the second cluster with an average genetic distance of (0.38). The Bambara groundnut landraces Botswana I, Namibia I and Malaysia red were very similar (bands produced by these landraces were consistent) and in addition to wild *Vigna membranaceae* they formed the first subcluster. Getso and Kano III formed the second subcluster.

The third cluster was formed by cowpea, groundnut, Kano I, Kano II, wild species of *Vigna racemosa*, *Vigna lasiocarpa* and *Vigna trilobata* with an average genetic distance of (0.58). In the third cluster, cowpea alone formed the first subcluster while the wild species of *Vigna racemosa* and *Vigna lasiocarpa* formed the second subcluster. The third subcluster was formed by groundnut and wild *Vigna trilobata* while the fourth subcluster was formed by Kano I and Kano II.

DISCUSSION

There is no sequence length variation in the 18S region of all landraces of Bambara groundnut (lanes 1 – 7) which produced only 1500 bp fragments (Figure 2). This confirms the fact that the 18S region of rDNA is highly conserved since it codes for 18S rRNA.

The result obtained in Figure 3 suggests that the ITS regions of Bambara groundnut landraces (lanes 1 – 7) are highly conserved because they have the same sequence length of ITS I region (350 bp). However, the result in Figure 4 suggests the ITS II region to be more diverged than the ITS I region as revealed by the varieties and pattern of band sizes produced in the landraces of Bambara groundnut. All landraces produced bands (400 bp) except Kano II (lane 2) and Kano III (lane 3) from Nigeria. This difference between the landraces of Bambara groundnut could be due to geographic origin as reported in previous studies (Massawe et al., 2002).

Similarly, the results in Figure 5 indicates that the sequence length of IGS varies between landraces of Bambara groundnut, wild species of *Vigna*, cultivated species of cowpea, mungbean and peanut which makes it a powerful tool in phylogenetic studies. This justifies its wide use in resolving taxonomic differences (Singh et al., 2008).

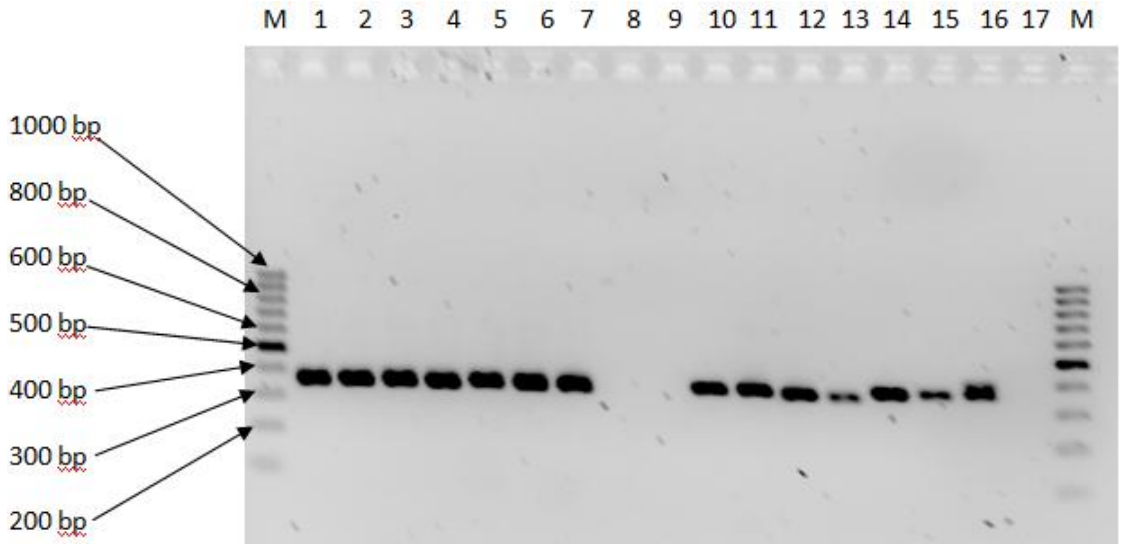

Figure 3. Gel picture of the PCR amplified ITS I fragments. Lanes 1 – 7 correspond to the landraces while lanes 8 – 13 correspond to the wild species listed in Table 1. Lane 14 – *V unguiculata*, lane 15 – *A hypogea*, lane 16 – *V radiata*, lane 17 – negative control while M represents the DNA ladder.

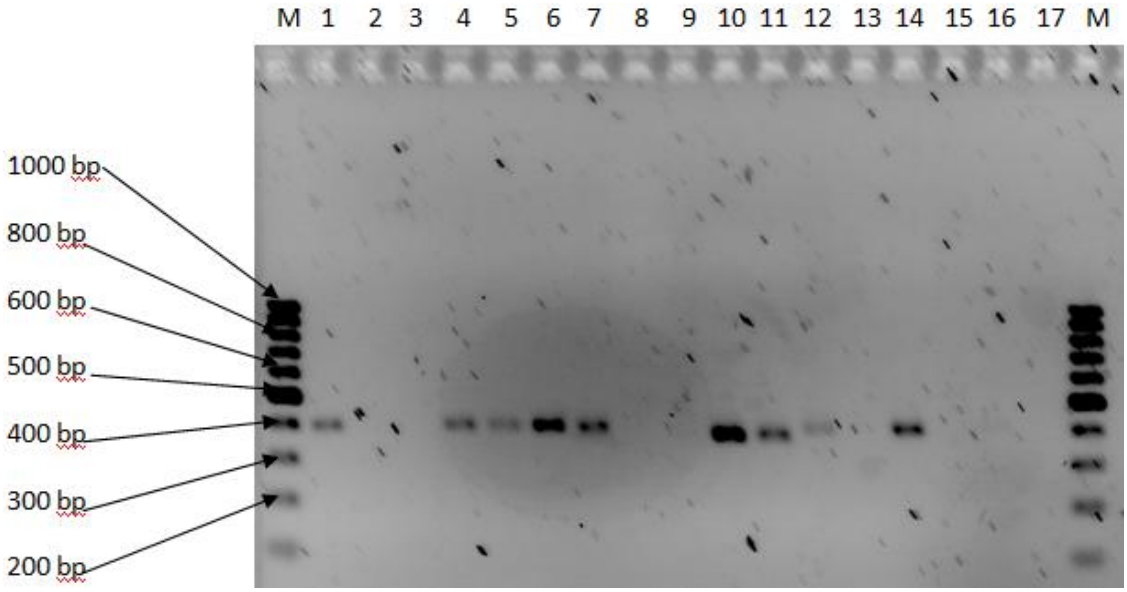

Figure 4. Gel picture of the PCR amplified ITS II fragments. Lanes 1 – 7 correspond to the landraces while lanes 8 – 13 correspond to the wild species listed in Table 1. Lane 14 – *V unguiculata*, lane 15 – *A hypogea*, lane 16 – *V radiata*, lane 17 – negative control while M represents the DNA ladder.

Phylogenetic analysis

The taxonomy (classification) of plant species including those within the genus *Vigna* has been a subject of controversy over the years. So many wild crop species are yet to be identified and this can be a limitation to breeding since wild species of plants are known to contain large repositories of genetic information. Similarly the genetic improvement of under-utilized legumes such as Bambara groundnut also relies on the identification and characterization of wild species within the genus *Vigna*. In a bid to prevent the wild type species from going extinct, phylogenetic studies enables the identification and preservation of the wild plant species. Crosses made between wild and cultivated species could produce new and improved cultivars of Bambara groundnut.

In this study, the average genetic distance within single cluster of the dendrogram (Figure 7) was uniform because

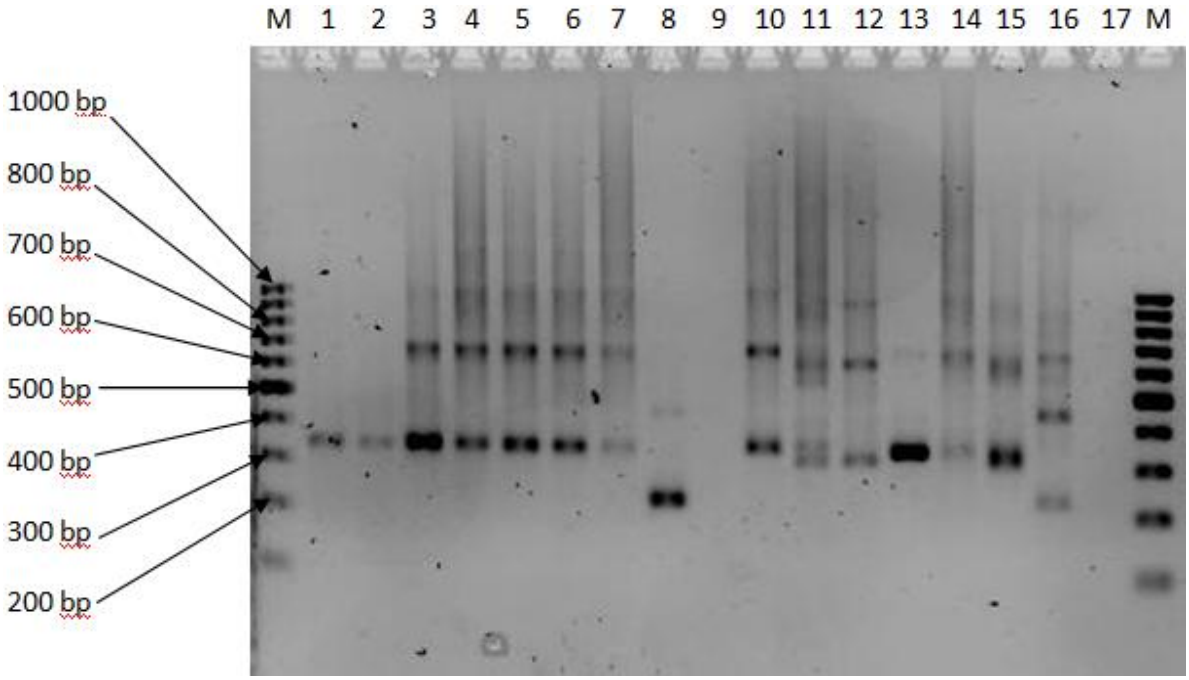

Figure 5. Gel picture of the PCR amplified IGS fragments. Lanes 1 – 7 correspond to the landraces while lanes 8 – 13 correspond to the wild species listed in Table 1. Lane 14 – *V unguiculata*, lane 15 – *A hypogea*, lane 16 – *V radiata*, lane 17 – negative control while M represents the DNA ladder.

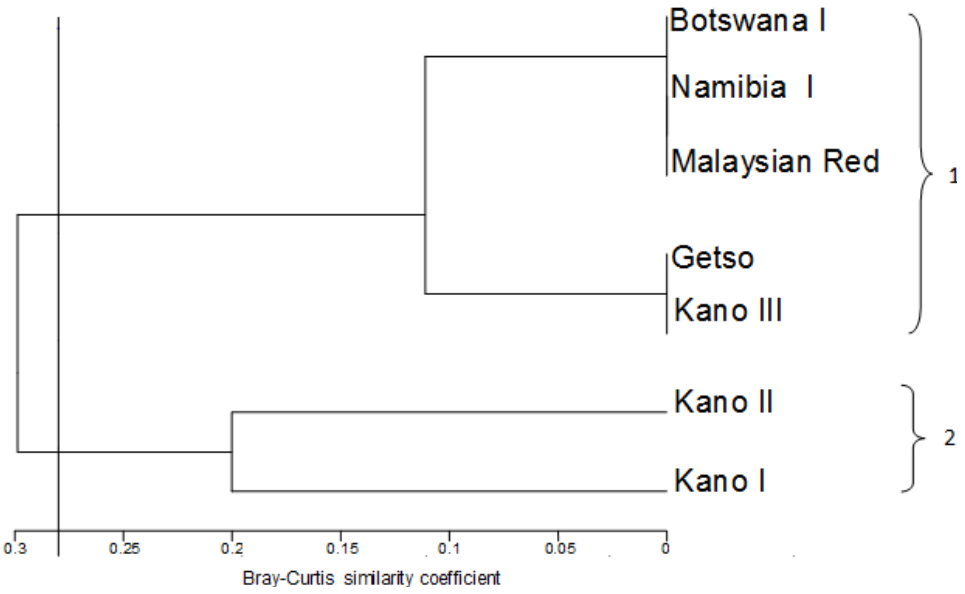

Figure 6. UPGMA dendrogram of the seven landraces of Bambara groundnut based on Bray-Curtis similarity coefficient.

the plants in the cluster have similar sequence length of rDNA genes based on the (UPGMA) clustering analysis. The classification of organisms through clustering algorithms based on average genetic distance has been described as a reliable tool in inferring phylogenetic relationship (Oosthuizen et al., 2006). Usually, low genetic distances signify close relationship while high genetic distances signify distant relationship (Oosthuizen et al., 2006). The average genetic distance of the 15 species in the genus *Vigna* is 0.52. This value is slightly more than the average genetic distance expected (0.40) for species within the same genus (Grant et al., 1988).

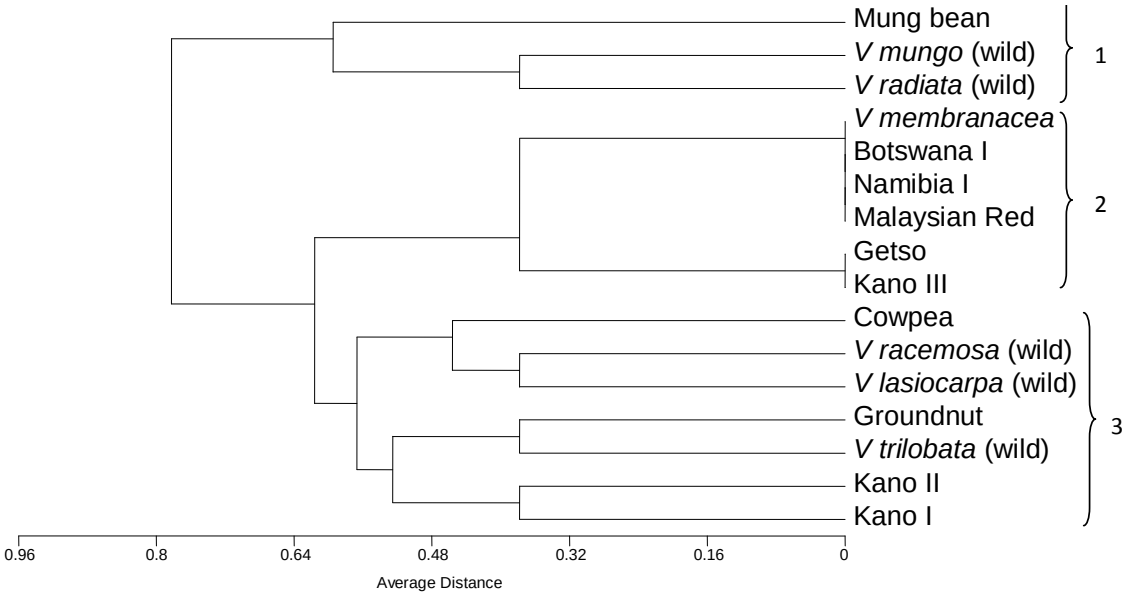

Figure 7. UPGMA dendrogram of the 15 species of the genus *Vigna* and groundnut (genus *Arachis*) based on their average genetic distance.

One important aspect of this study is that the seven (7) landraces of Bambara groundnut were placed in two separate clusters (Figures 6 and 7) implying that there is variation of rDNA gene units between the landraces. Geographic origin may be responsible for the non inclusion of the Kano I and Kano II in the second cluster (Figure 7) because they were originally collected from Kano state, Nigeria. Previous studies using RAPD marker indicates that landraces of Bambara groundnut are related to their place of origin (Massawe et al., 2002).

The landrace variation could be responsible for the inconsistent yields in Bambara groundnut. One of the goals of plant breeding is to develop pure lines of a crop that would produce uniform yield under low input farming systems. A recent study by Singrun and Sheckel (2004) indicates that landraces of Bambara groundnut from the Southern parts of Africa have between 3 – 8 different genotypes. The different genotypes are believed to confer agronomic traits such as the ability of Bambara groundnut to withstand low input farming and still produce significant yields. The task ahead lies in identifying the high yielding genotypes of Bambara groundnut and using them to make crosses that would produce improved and uniform yields in Bambara groundnut. Even though creation of pure lines in Bambara groundnut could have its own limitations, it will ensure consistency in yields obtained. One of the drawbacks of pure line development is that it could be wiped out in the case of disease outbreak because of its genetic uniformity. However, development of multiple in-bred lines could help integrate many traits in a single cultivar and overcome the limitations associated with the production of pure lines in harsh environments. Notwithstanding, achieving this goal largely depends on the identification of sufficient variation within the germplasms of Bambara groundnut.

The fact that the sequence length of rDNA genes in landraces of Bambara groundnut from Nigeria varied considerably could be due to the enormous genetic diversity of the landraces in West Africa. It is a common knowledge that the evaluation of genetic diversity is *sine qua non* for the improvement of under-utilized crop species because rich germplasms (high genetic diversity) provides surplus raw material for the production of superior varieties of crops.

Cowpea which is also believed to have originated from West Africa was also grouped in the same cluster as Kano I and Kano II supporting the classification of both cowpea and Bambara groundnut in the subgenera *Vigna*. This is due to the presence of paralogous genes in both cowpea and Bambara groundnut. However, none of the landraces were grouped together with mungbean, another legume from the genus *Vigna*. But interestingly, the cultivated and wild mungbean (*V radiata*) were clustered together thus corroborating previous claim that the wild mungbean is the progenitor of the present day cultivated mungbean. Only both of them (wild and cultivated mungbean) produced 250 bp fragments within the IGS region and were grouped together in the first main cluster. This also implies that the cultivated mungbean still contain genes homologous to its wild counterpart even after going through evolution. Mungbean is an important legume consumed and cultivated majorly in Asia. It can also be improved by making intra-specific crosses with its wild counterpart.

The dendrogram (Figure 7) also reveals a close relationship between *Vigna radiata* and *Vigna mungo* which

corroborates similar findings of Kaga et al. (1996) and Goel et al. (2001). Both *V radiata* and *V mungo* are in the subgenera *Ceratotropis* also known as Asian genus *Vigna* and their germplasms are useful for making crosses within the species of *Vigna*.

The spacer regions (IGS and ITS) exhibited more variability in this study and has helped in analyzing the phylogenetic relationship in different species of the genus *Vigna*. This variability is an important asset to the taxonomist where it is used in resolving taxonomic differences in plants (Penteado et al., 1996). Phylogenetic relationship does not refer to the similarity between species but the relative times in the past when species shared common ancestors. However, the variability within the spacer regions could be due to gene flow where genes are exchanged between species through natural and artificial means (Nepolo et al., 2010). This makes gene flow to be an invaluable source of genetic variation within a particular species as seen in the landraces of Bambara groundnut and the other species of *Vigna* used in this study. As such, the rDNA marker can be associated with specific traits of interest, and thus facilitate breeding true varieties of Bambara groundnut.

Conclusion

One of the goals of this study is to explore the level of genetic variation in Bambara groundnut available to plant breeders for genetic improvement purposes. Analysis of the non coding regions (ITS and IGS) reveal the presence of genetic variation between the landraces of Bambara groundnut as well as in other species of the genus *Vigna* and *Arachis* in this study. This study supports the classification of Bambara groundnut and cowpea in the same subgenera *Vigna* in contrast to previous report by Phansak et al. (2005) where Bambara groundnut and cowpea were placed in the subgenera *ceratotropis* and *Vigna* respectively. The close relationship between Bambara groundnut and other *Vigna* species observed in this study justifies the change of name of Bambara groundnut from *Voandzeia subterranea* to *Vigna subterranea*. The average genetic distance (0.52) obtained is also within the range expected for species within the same genus and family. Analysis of the rDNA genes support claims that wild cowpea and mungbean are progenytors of the present day cultivated cowpea and mungbean respectively. Similarly, the analysis of the rDNA genes reveals the presence of homologous genes in *Vigna radiata* and *Vigna mungo* corroborating previous studies by Kaga et al. (1996) and Goel et al. (2001).

The variation revealed between the landraces of Bambara groundnut in this study makes the rDNA gene an invaluable tool in molecular phylogeny. The genetic variation obtained in this study adds to and further our understanding of Bambara groundnut germplasm and could help facilitate the production of ideal Bambara groundnut types with useful agronomic traits that would meet consumer preferences. Estimation of genetic variability based on the rDNA genes in plants as done in this study is very imperative because, species in the genus *Vigna* belong to the family of legumes known for their ability to fix nitrogen. As such, they have tremendous roles to play in maintaining soil fertility and sustaining global agriculture.

Future studies

This particular study adds to the list of studies that confirms the usefulness of rDNA in molecular phylogeny. The rDNA gene analysis should be extended to other crops whose origin and genetic relationship is still unclear. Because of the enormous potential of Bambara groundnut, future studies should be focused on assessing genetic variability in all of the existing landraces of Bambara groundnut distributed across Africa. This would help in providing sufficient genetic variation that is required for the production of improved cultivars of Bambara groundnut.

Legumes play significant roles in agriculture especially in low input farming systems where they are intercropped with cereals. Being cheap source of proteins, they complement cereals (and other foods) in feeding a large population of over 7 billion people that live in the world today. The main focus should be on improving the production of these cheap food products and also unraveling the mystery behind the genes that help in feeding the world.

Biotechnology offers one of the best tools of improving under-utilized crop species and so requires a concerted effort by researchers, local farmers and funding agencies. Promotion is equally important in making these under-utilized (including Bambara groundnut) crops reach their potentials.

ACKNOWLEDGEMENT

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