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CHARACTERIZATION OF GROUNDNUT (*ARACHIS HYPOGEA* L.) GENOTYPES USING MOLECULAR AND MORPHOLOGICAL TECHNIQUES

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Abstract
Information on variation in groundnut genotypes is valuable for cultivation and breeding schemes. This study characterized 66 groundnut genotypes using molecular and morphological techniques. Fourteen SSR markers were used for molecular characterization. The marker profile, generated from scoring clear and unambiguous bands was used to calculate polymorphism information content (PIC) and resolving power (RP). The result revealed that, 12 polymorphic markers amplified a total of 30 bands, with 23 (76.6%) of the bands showing polymorphism. PIC of the markers ranged from 0.2 (TC4G10) to 0.9 (IPAHM395) while RP ranged from 0.5 (TC2D06) to 3.5 (TC4G10). The dendrogram based on marker profile generated two major clusters A and Band eight minor ones (AI, AII, AIII, AIV, AV, AVI, BI and BII). The morphological characterization revealed that, more than 82% of the total variation in the 12 characters was explained by the first eight principal components. The components were considered significant in the assessment of variability among the groundnut genotypes. The dendogram generated from morphological data also showed two major clusters, A and B, and four minor ones (AI, AII, AIII and BI). The clusters were consistent with clusters obtained from the molecular data suggesting that the two methods are complementary in assessing genetic diversity in groundnut.

Key words: Groundnut, Euclidean distance, Genotypes, Dendrogram, Clustering, SSR

Introduction
Characterization is the "recording and compilation of data on important characteristics or features which distinguish accessions within a species and enables an easy and quick discrimination among phenotypes" (Bioversity International, 2003). It is performed to identify varieties and to understand the genetic relationship between them (Laurentin, 2009). Characterization can be performed at any stage in the management process provided there are sufficient samples (Bioversity International, 2003) using morphological and molecular approaches (Laurentin, 2009).

Morphological characterization deals with variation in form and structure of the plant (Sattler and Rutishauser, 1997). Plant features such as height, number and type of tillers, number and size of leaves, type, size and color of flower, internode distance as well as length of roots are examples of morphological characters (Lutatenekwa *et*

al., 2020). These features are readily available and often irreplaceable (Singh *et al*., 2011). However, there are shortcomings in accuracy of identification of varieties as there are insufficient traits to characterize (Lutatenekwa *et al.*, 2020). Also, their manifestation, especially the quantitative traits, is significantly affected by environmental factors, age and system of cultivation (Laurentin, 2009; Rao, 2004) which make errors inevitable during scoring (Singh *et al*., 2011). Regardless of these shortcomings, morphological characteristics remain valuable for recognition of landraces to enhance selection and utilization till more refined techniques like molecular markers are affordable and easily accessible (Lutatenekwa *et al.*, 2020).

The use of molecular markers has become the most valuable means of evaluating inherent variation in plants (Bered *et al*., 2005) because of its ability to recognize and reveal valid differences between closely related species (Rocha *et al*., 2009). Laurentin (2009) revealed that, molecular markers have the capacity to assess genetic diversity and recognize duplicates in plants species. The use of molecular markers in characterization helps in identification of gaps and retrieval of relevant germplasm for breeding programmes, and results to an in-depth understanding of materials and their genetic variability (Bioversity International, 2007). They show high degree of non-tissue specific polymorphism, reduced environmental effect and simple pattern of inheritance (Dhall, 2011) in all plant species.

In groundnut, different molecular markers such as restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD), DNA amplified fingerprinting (DAF) and amplified fragment length polymorphism (AFLP) have been used for characterization. The result has been low level of diversity in cultivated species traced to evolutionary genetic bottleneck in the form of polyploidy and self-pollination (Subramanian *et al*., 2000), even though higher variation has been revealed in wild diploid *Arachis* species (Bravo *et al*., 2006). Simple sequence repeat (SSR) markers have been shown to be more informative and useful in genetic analysis and breeding programmes (Pandey *et al*., 2012). The markers are characterized by co-dominance of traits that are evenly distributed throughout the genome with high level of polymorphism. These attributes coupled with their small DNA or tissue requirements and inexpensive nature makes them valuable materials for assessment of heritable differences among plant materials (Dhall, 2011).

It has been reported that, morphological characteristics change in many species of plants in reaction to factors such as availability of light, depletion of organic matter as a result of their use by plants and compaction of soil due to human activities (Buckley *et al*., 1994). Mutation and cross pollination, even among self-pollinated plants, may cause changes in morphological features of plants species after several generations of cultivation. Therefore, there is need for characterization of groundnut varieties used over a long period of time for farming and breeding or research purposes. Thus, this research was carried out to assess the genetic diversity at morphological and molecular levels using simple sequence repeat (SSR) markers.

Materials and Methods

Plant Materials

The plants materials used for the study were sixty-six (66) groundnut genotypes (supplementary material) obtained from International Crop Research Institute for Semi-Arid Tropics (ICRISAT) Kano, Nigeria, by the Molecular Biology Laboratory, Federal University of Agriculture, Makurdi.

Molecular Characterization Extraction of DNA Using Fast Technology Application (FTA) Cards

The sixty-six (66) genotypes of groundnut were sown in plastic pots in the screen house of the Laboratory. The leaf tissue of one-

month old seedlings was used to obtain genomic DNA of the groundnut genotypes under study using Fast Technology Application (FTA) plant saver cards.

The young leaf removed from the groundnut seedlings was placed on the FTA card and covered with paraffin paper. Pressure was applied gently using a pestle until sufficient plant material was absorbed by the card. The FTA cards containing the plant materials were allowed to air dry for at least one hour before placing in a paper pouch and stored in a desiccator at ambient temperature. The DNA was prepared for polymerase chain reaction (PCR) as explained by Omoigui *et al*. (2011).

PCR Analysis

Amplification of the extracted DNA was done in 25µl reaction mixture which contained 2mm FTA DNA disc with DNA sample, 18µl of molecular grade water, 2.5µl each of balanced dNTPs mix and 10 x PCR buffer, 0.05µl of Taq polymerase and 1µl each of forward and reverse primers (table 1) produced by Integrated DNA Technologies, Coralville, IA. The PCR reactions were performed on a heated lid thermal cycle (Cycle thermocycler from Bio-Rad) operated as follows: 35 cycles of denaturation at 94ºC for a period of 30 seconds followed by annealing temperature at 57.5ºC for a period of 30 seconds and extension at 72ºC for 2 minutes. In accordance with Omoigui *et al*. (2011), a final extension cycle of 10 minutes at 72ºC was added to ensure the completion of the final amplification products.

Table 1: Simple Sequence Repeat (SSR) Markers used for the study and their sequences

S/NO	MARKER	SSR MOTIF	FORWARD, REVERSE PRIMER SEQUENCE (5'-3')
1	GA101		CACGACGTTGTAAAACGACTGAAATGATGCA ACCACACAT, AAGGGAAAGTAAAACCATGCAA
2	GA5		CACGACGTTGTAAAACGACAAACTTGGACGT TGGCTTTGT, TTGATCCAGAACCTGAAAGGA
3	GM2156	(TTC)13	CGTGTCCTTGTTCTCTTCTCTG, TGGAGGAATGAAGGAAGGAAT
4	GM2165	TC	CTACGCGCATCGCATAATATC, CAACCAGCTCCTTCTCTACCC
5	GM632		TTCAATCATTTCACGTGTCAATC, AGTGCTAGGAGCCAGCAATTT
6	IPAHM23	(CA)17(TA)3	GTGTCTTTTCGTTCGCGATT, CGACTCTTAGGGTGGATTATAGTAAGA
7	IPAHM254	(GA)5(GA)20(GA)4	TGAAATGGTGGCTTGCAATA, TGCTGAGTCGCTCATTCTCT
8	IPAHM395	(GA)14	CAGAGTCAATGGCAGCGTAG, TCCTTCCCTCATCTAAAACCAA
9	RN34A10	(AT)6	CCCATTTTGGACCCCTCAAATA, GAGCAATAGTGACCTTGAATTGTTG
10	SEQ19E9	(TAA)14	ACTGCTTGCTCTCTTCCTCG, TTCCACCTATAAAATCAATGGTGA
11	SEQ3A06	(GA)35	TGCATCAGCAAGCTACATACG, GCGATTCACCATCAATCTCA
12	SEQ4G2	(GA)9	TCAACTTTGGCTGCTTCCTT, TCAACCGTTTTTCACTTCCA
13	TC2D06	(AG)30	AGGGGGAGTCAAAGGAAAGA, TCACGATCCCTTCTCCTTCA
14	TC4G10	(GA)20(GA)5(GT)5	TTCGGTCATGTTTGTCCAGA, CTCGAGTGCTCACCCTTCAT

Analysis of PCR Products

Horizontal gel electrophoresis system (GALILEO biosciences) was used to electrophorese 10µl of the final PCR product on a 2% agarose gel stained with ethidium bromide. The gel was run at 120 voltages in 1 x Tris acetic acid (TAE) buffer (45 mmol L-1 glacial acetic acid, 0.5 mmol l-1 ethylenediaminetetra acetic acid (EDTA), (pH, 8.4) for approximately 1h 30 min. DNA molecular ladder of 1 kb was loaded in the first well for determination of band sizes of PCR products. The ethidium bromide-stained gel was visualized on a Benchtop UV Transilluminator (M-26V) and photographed with a digital camera for scoring.

Band Scoring and Analysis

The presence and absence of bands on the SSR markers was designated with '1' and '0' respectively. This generated the marker profile of the groundnut genotypes under study used as follows:

Percentage polymorphism was calculated using the equation:

$$\%\ Polymorphism = \frac{Polymorphic\ bands}{Total\ Number\ of\ bands}\ x\ 100$$

On the other hand, Polymorphism Information Content (PIC) for each polymorphic marker was calculated as $\text{PIC(Codominant markers)} = 1 - \sum_{i=1}^{k} \text{Pi}^2$ as explained by De Reik *et al.* (2001), where P = frequency of present allele. The mean PIC was the sum of all polymorphic bands divided by the number of polymorphic markers (Suvendu *et al.*, 2009). Resolving power for each primer was calculated as RP = $\sum 1_b$ where $\sum 1_b = 1 - [2$ x(0.5-p] and P = proportion of genotypes containing the band (Prevost and Wilkinson *et al.*, 1999). Clustering was also performed from the generated marker profile using SPSS (version 23).

Morphological Characterization

Experimental Site and Design

Field experiment for morphological characterization was established at the University Farm behind College of Agronomy, Federal University of Agriculture, Makurdi between April to July 2022. The field was arranged in a randomized complete block design with three replications comprising of 66 entries each. Each replication was divided into plots of four ridges, each 3m long. One plant was maintained per hill at spacing of 0.75m inter row and 0.2m intra row. The field was kept weed free by hand pulling till the plants matured.

Data Collection

A total of twelve (12) morphological characteristics comprising of eight qualitative (discontinuous) and four quantitative (continuous) traits were evaluated. Number of branches and seeds per plant were evaluated per plot at harvest using five randomly sampled plants. Pod and fodder weight per plant were also recorded at harvest as an average weight of pods and fodder respectively from five middle row plants randomly sampled per plot.

Stem hairiness was measured as: scarce, abundant, and very abundant while stem pigmentation and susceptibility to diseases were measured as presence or absence of pigmentation and traces of diseases from five middle row plants randomly sampled per plot. Flower color was measured as yellow or yellow with patches while leaf color was measured as green/yellow green or dark/very dark respectively. Growth habit, on the other hand was scored as procumbent, decumbent, and erect. Other qualitative traits such as pod constriction and pod beak were evaluated

after harvest as absent, slight, moderate, deep and very deep as well as absent, slight, moderate, prominent and very prominent respectively. The data generated from these evaluations was subjected to clustering analysis using SPSS (version 23).

Results and Discussion

Analysis of SSR markers on groundnut genotypes

From the fourteen (14) markers used for the study, two (2) primers (GM 2156 and GM 632) failed to show polymorphism while twelve (12) primers showed polymorphism (table 2). The twelve polymorphic markers amplified a total of 30 bands with 23, representing 76.6% of the bands showing polymorphism with an average of 1.6 bands per marker. Percentage polymorphism ranged from 50 (IPAHM23) to 100 (GA 101, GM 2156, IPAHM 254, IPAHM 395, RH34A10, SEQ19E9, SEQ3A06, SEQ4G2 and TC4G10). The percentage polymorphism revealed in this study is higher than the previous study by Dughdugh *et al*. (2021) (66.7%), Gautami *et al*. (2009) (57%), Li *et al*. (2011) (42.47%), Shoba *et al*. (2010) (24%) and Bosamia *et al*. (2015) (10.66%). This confirms the high level of SSR primers informativeness earlier reported in groundnut germplasm by Nadaf *et al*. (2019) and Pandy *et al* (2012). Kanyika *et al*. (2015) reported a higher percentage polymorphism of 94.2% across groundnut varieties using 799 markers.

Polymorphism information content (PIC) among the polymorphic primers ranged from 0.2 for TC4G10 to 0.9 for IPAHM395. The mean PIC value was 0.6 with PIC values above 0.5 realized in 9 (64%) of primers analyzed. The PIC values obtained in this study were relatively high compared to Nadaf *et al*. (2019) that reported PIC range of 0.08-0.83 with a mean of 0.57. Also, Shoba *et al*. (2010) reported PIC range of 0.17 to 0.63 with a mean of 0.41 where only 1(5.8%) of the primers showed PIC value above 0.5. Cuc *et al*. (2008) reported PIC range of 0.12 to 0.75 with a mean of 0.46 where 15.7% of SSR primers revealed PIC values above 0.5. A lower range of PIC values (0.13 to 0.36) was reported by Gautami *et al*. (2009) with a mean of 0.25 and no PIC value of 0.5 and above. This is an indication that, the SSR primers used in this study are highly informative. The highest PIC value of 0.9 and mean PIC of 0.6 in this study signify the occurrence of abundant variations in the genotypes under study that could be relevant to improvement. The range of resolving power (0.5 to 3.1) for polymorphic primers in the study is comparable with Dughdugh *et al*. (2021) where a range of 1.22-2.19 and a mean of value of 1.7 were obtained.

Table 2: PIC and RP Values of Polymorphic SSR Markers among 66 Groundnut Genotypes

S/NO	PRIMER	TOTAL BANDS	POLYMORPHIC BANDS	PERCENTAGE POLYMORPHISM	PIC	RP
1	GA101	3	3	100.0	0.6	2.1
2	GA5	3	2	66.7	0.3	2.3
3	GM2156	1	0	0.0	0.0	0.0
4	GM2165	2	2	100.0	0.5	1.7
5	GM632	2	0	0.0	0.0	0.0
6	IPAHM23	2	1	50.0	0.8	0.9
7	IPAHM254	1	1	100.0	0.7	1.2
8	IPAHM395	1	1	100.0	0.9	0.7
9	RN34A10	3	3	100.0	0.8	1.2
10	SEQ19E9	2	2	100.0	0.6	1.8
11	SEQ3A06	2	2	100.0	0.6	1.8
12	SEQ4G2	1	1	100.0	0.8	0.8
13	TC2D06	1	1	100.0	0.9	0.5
14	TC4G10	6	4	66.7	0.2	3.1
	Total	**30**	**23**		**7.7**	**18.1**
	Mean	**2.1**	**1.6**	**76.7**	**0.6**	**1.5**

PIC = Polymorphic information content. RP = Resolving power

Squared Euclidean Distance was used to produce two major clusters (labelled A and B) and eight minor ones with "A" producing six minor clusters (AI – AVI) and "B" producing two minor clusters (BI – BII) as shown in figure 1. Clusters BI and AIII with 14 and 12 groundnut genotypes respectively contained the largest numbers of groundnut genotypes followed by AI, AV and AVI with 10, 10 and 9 genotypes respectively. Clusters BII, AII and AIV were least with 5, 4 and 2 groundnut genotypes respectively. Samba *et al*. (2019) and Mofokeng *et al*. (2021) reported similar clustering patterns in molecular diversity studies of groundnut genotypes.

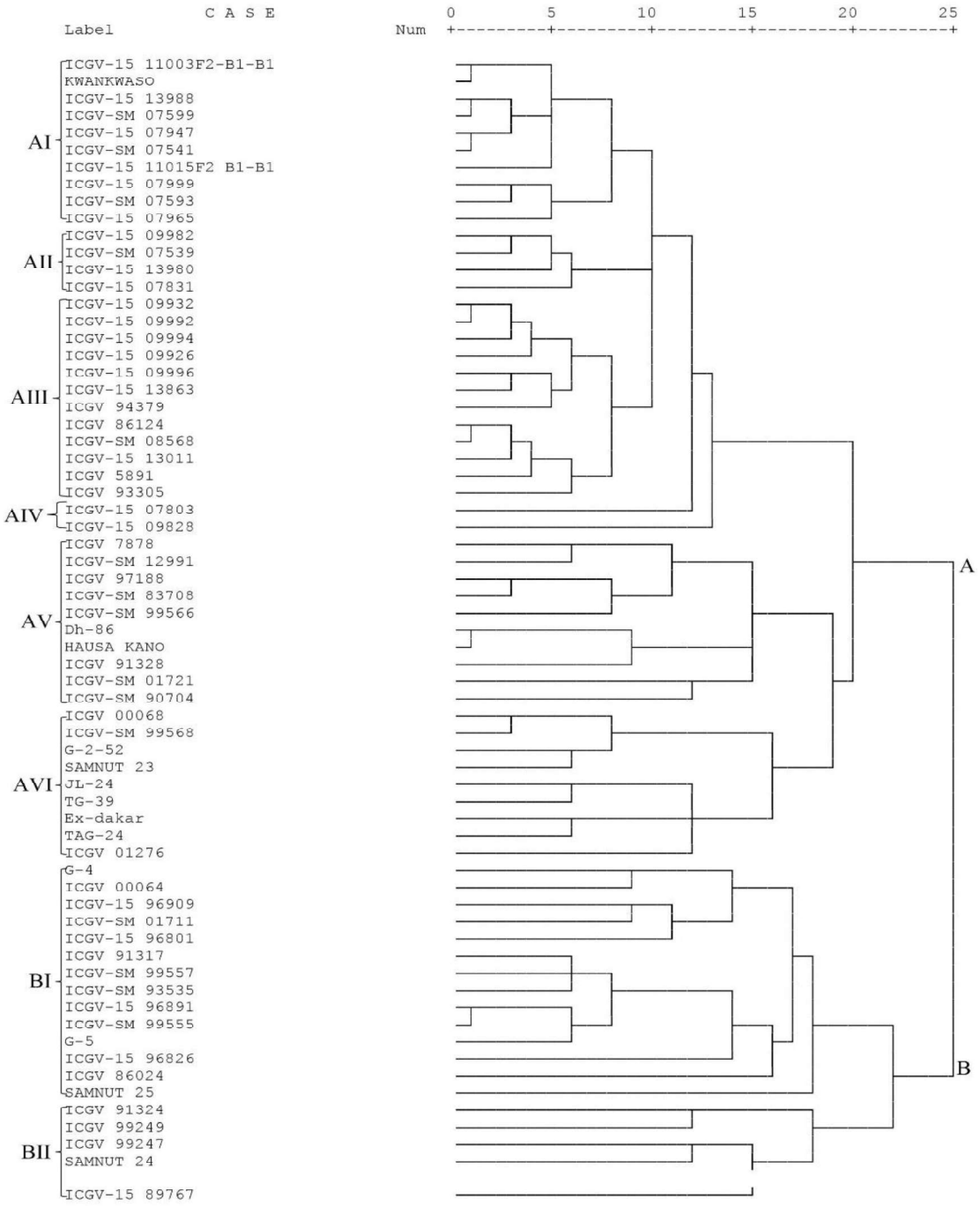

Figure 1: Dendrogram showing clustering in 66 groundnut genotypes based on molecular characters

Analysis of morphological characteristics

Principal component analysis for morphological data revealed six components (PC I, PC II, PC III, PC IV, PC V and PC VI) with eigenvalues greater than one (1) (table 3) while the rest of the principal components have eigenvalues greater than 0.5. These components in accordance with the findings of Hair *et al*. (1998) are valuable in assessment of variability in the genotypes under study. The first eight (8) principal components elucidated more than 82% of the total variation among the 66 groundnut varieties under study. Specifically, the first principal component (PC I) which elucidated 14.6% of the total variation was positively associated with fodder weight per plant,

susceptibility, and pod weight per plant while it was negatively associated with growth habit, leaf color, stem hairiness, number of seeds per plant and number of branches per plant. The second principal component (PC II), which explained 13% of the total variation, was positively associated with number of branches per plant; stem pigmentation, pod weight per plant, stem hairiness and leaf color while it was negatively associated with flower color, number of seeds per plant, fodder weight per plant, pod constriction and susceptibility. The variation attributed by the first two principal components (PC I and PC II) is low compared to Hassen *et al*. (2006) where the first two components accounted for more than 80% of the total variation in *Indigofera* species. This could be due to the difference in crop plant and the large number of groundnut varieties used as well as the spread of variability among the study materials. The result is comparable to Shegro *et al*. (2013) where the first two principal components accounted for 23.84% and 19.57% respectively.

In the third principal component, positive coefficients were observed in pod constriction (0.436), pod peak (0.365), stem hairiness (0.291), susceptibility (0.201) and pod weight per plant (0.053) while negative coefficients were observed in stem pigmentation, leaf color, fodder weight per plant, number of seeds per plant and number of branches per plant contributed negatively. The fourth principal component which explained 10.2% of the total variation produced positive coefficients under pod constriction (0.565), number of branches per plant (0.362), leaf color (0.33), susceptibility (0.198) and flower color (0.079). Pod weight per plant, stem hairiness, number of seeds per plant, growth habit, pod beak and fodder weight per plant with -0.507, -0.279, -0.180, -0.149, -0.062 and -0.059 respectively contributed negatively to the fourth principal component. The fifth principal component produced its largest coefficient value in flower color (0.416) while pod weight per plant (-0.302) recorded the highest negative contribution. Stem hairiness with 0.547 and 0.121 had the highest positive contributions to the variations observed in principal components VI and VII respectively. On the other hand, pod beak and susceptibility with coefficients -0.581 and -0.615 respectively produced the highest negative variations observed in principal components six and seven (PCVI and VII). Flower color with 0.572 and number of seeds per plant with -0.689 produced the highest positive and negative contributions respectively in principal component eight (PCVIII). According to Jonah *et al*. (2014), traits are inter correlated to some extent and so, not all principal components are relevant to concisely review data. In this study therefore, the first eight principal components have accounted for up to 82% of total variation observed in the groundnut genotypes under study.

Table 3: Eigenvector coefficient of 12 morphological traits for the first nine principal components with Eigenvalue, proportion and cumulative percentages of the total variance

Characteristics	**Principal Component**							
	I	**II**	**III**	**IV**	**V**	**VI**	**VII**	**VIII**
Stem hairiness	-0.281	0.041	0.291	-0.279	0.31	0.547	0.121	0.098
Stem pigmentation	0.017	0.457	-0.416	0.015	0.343	-0.133	0.036	-0.206
Flower color	-0.12	-0.468	-0.131	0.079	0.416	-0.097	0.051	0.572
Leaf color	-0.326	0.034	-0.399	0.33	-0.184	-0.121	-0.481	0.13
Pod beak	0.207	0.182	0.365	-0.062	0.385	-0.581	-0.029	-0.106
Pod constriction	0.057	-0.173	0.436	0.565	-0.238	0.118	-0.035	-0.249
Growth habit	-0.570	0.071	0.171	-0.149	-0.001	-0.108	-0.300	-0.002
Number of seeds per plant	-0.203	-0.396	-0.116	-0.180	0.275	0.078	-0.251	-0.689
Number of branches per plant	-0.033	0.470	-0.069	0.362	0.251	0.442	0.009	-0.002
Susceptibility	0.353	-0.052	0.201	0.198	0.378	0.132	-0.615	0.132
Pod weight per plant	0.266	0.194	0.053	-0.507	-0.302	0.147	-0.465	0.157
Fodder weight per plant	0.437	-0.290	-0.390	-0.059	0.036	0.234	0.022	-0.118
Eigenvalue	**1.7462**	**1.5651**	**1.2694**	**1.2241**	**1.1939**	**1.0669**	**0.9688**	**0.8286**
Individual Percentage	**14.60**	**13.00**	**10.60**	**10.20**	**9.90**	**8.90**	**8.10**	**6.90**
Cumulative percentage	**14.60**	**27.60**	**38.20**	**48.40**	**58.30**	**67.20**	**75.30**	**82.20**

Squared Euclidean Distance was used to generate two major clusters (labelled A and B) and four minor ones with "A" producing three minor clusters (labelled AI, AII and AIII) and "B" producing one minor cluster (labelled BI) as shown in figure 2. Each minor cluster contained varying numbers of groundnut genotypes with cluster AI, AII and AIII containing 17, 21 and 20 groundnut genotypes respectively while minor cluster BI contained 8 groundnut genotypes. Similar clustering patterns were reported by Boyorbor *et al*. (2010), Garba *et al*. (2015) and Yol *et al*. (2018) in morphological diversity studies in groundnut germplasm as well as Valombola *et al*. (2019) in morphological diversity studies in Bambara groundnut.

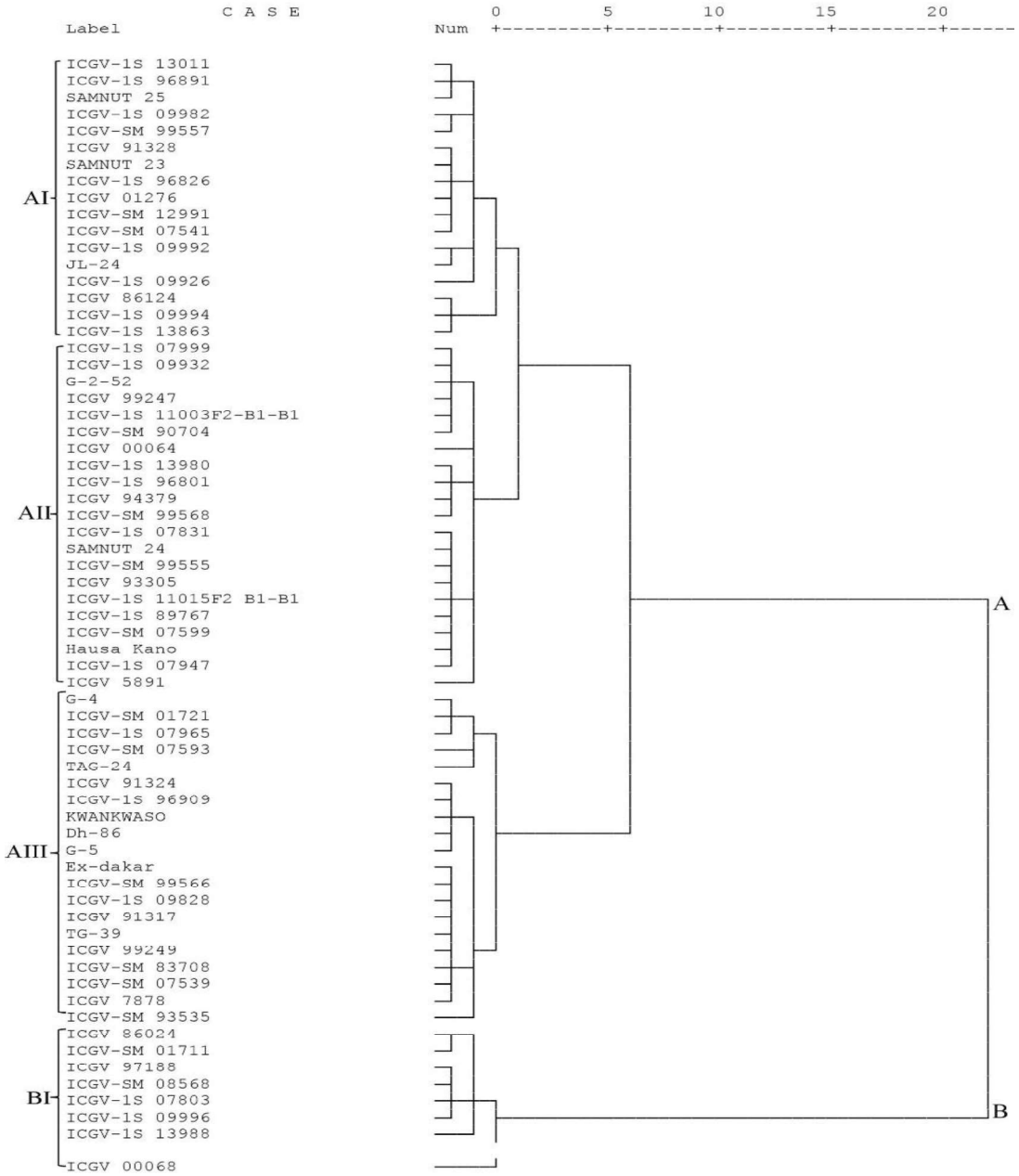

Figure 2: Dendrogram showing clustering in 66 groundnut genotypes based on morphological characters

Figure 3 is the graphical representation of the loading coefficients (weights) of the principal component analysis (PCA) of twelve (12) morphological characters of groundnut. In the loading plot, the x-axis represents the first principal component (PC1), which accounted for 14.60% variability and the y-axis represents the second principal component (PC2), which accounted for 13.00% (Table 3). The plot shows that number of seeds per pod and flower colour had the strongest influence on PC1. Fodder weight per plant also had an influence on PC1. Therefore, PC1 can be interpreted as being related to grain and fodder yield. Growth habit had the strongest influence on PC2.Stem hairiness and leaf colour also had an influence on PC2. Therefore, PC2 can be interpreted as a measure of growth.

The above results indicate that number of seeds per pod, flower colour, fodder weight per plant, growth habit, Stem hairiness and leaf colour were responsible for diversity among the groundnut varieties evaluated. These morphological features which comprised both qualitative and quantitative traits are the most important for differentiating between groundnut varieties as similarly stated by Mubia *et al.* (2020).

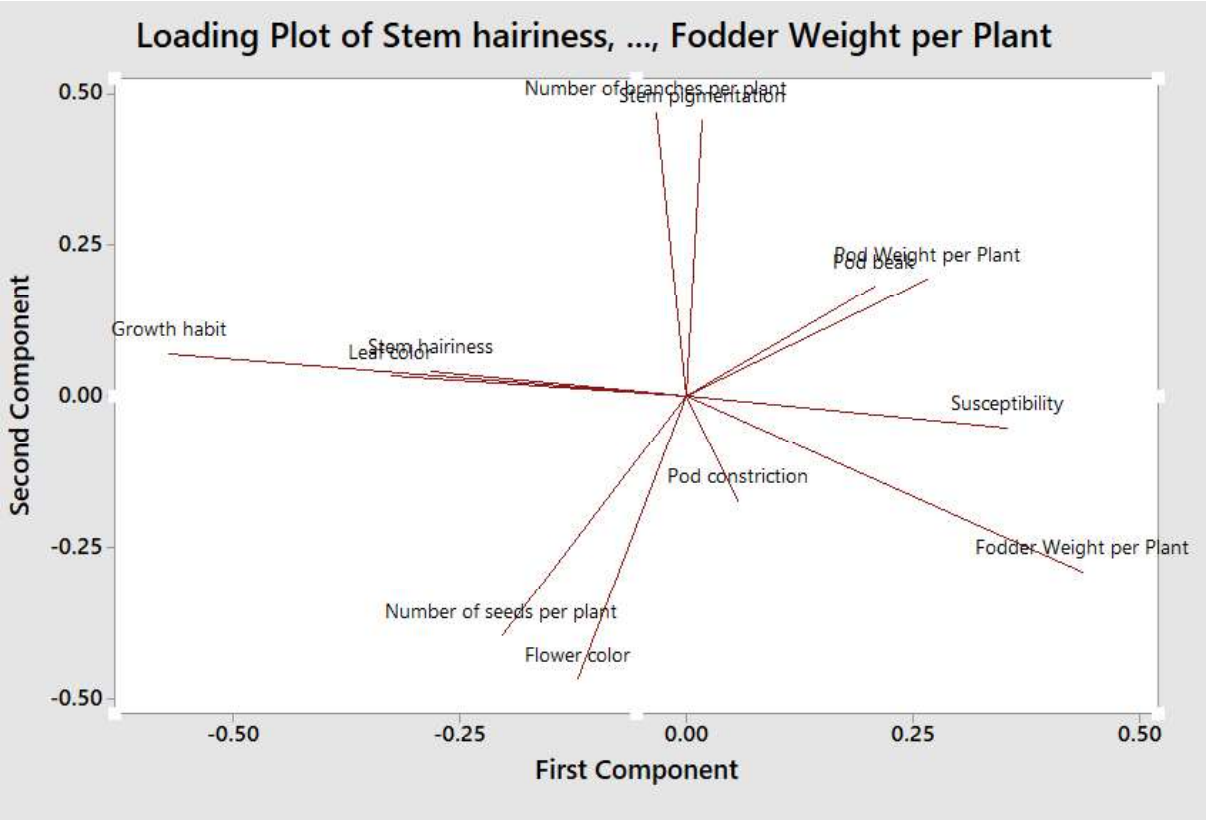

Figure 3: Loading plot showing group of correlated morphological characters.

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Appendix 1

Groundnut genotypes used for the study.

S/NO	GENOTYPE	S/NO	GENOTYPE	S/NO	GENOTYPE
1	Dh-86	23	ICGV-15 07831	45	ICGV-SM 01711
2	Ex-dakar	24	ICGV-15 07947	46	ICGV-SM 01721
3	G-2-52	25	ICGV-15 07965	47	ICGV-SM 07539
4	G-4	26	ICGV-15 07999	48	ICGV-SM 07541
5	G-5	27	ICGV-15 09828	49	ICGV-SM 07593
6	HAUSA KANO	28	ICGV-15 09926	50	ICGV-SM 07599
7	ICGV 00064	29	ICGV-15 09932	51	ICGV-SM 08568
8	ICGV 00068	30	ICGV-15 09982	52	ICGV-SM 12991
9	ICGV 01276	31	ICGV-15 09992	53	ICGV-SM 83708
10	ICGV 5891	32	ICGV-15 09994	54	ICGV-SM 90704
11	ICGV 7878	33	ICGV-15 09996	55	ICGV-SM 93535
12	ICGV 86024	34	ICGV-15 11003F2-B1-B1	56	ICGV-SM 99555
13	ICGV 86124	35	ICGV-15 11015F2 B1-B1	57	ICGV-SM 99557
14	ICGV 91317	36	ICGV-15 13011	58	ICGV-SM 99566
15	ICGV 91324	37	ICGV-15 13863	59	ICGV-SM 99568
16	ICGV 91328	38	ICGV-15 13980	60	JL-24
17	ICGV 93305	39	ICGV-15 13988	61	KWANKWASO
18	ICGV 94379	40	ICGV-15 89767	62	SAMNUT 23
19	ICGV 97188	41	ICGV-15 96801	63	SAMNUT 24
20	ICGV 99247	42	ICGV-15 96826	64	SAMNUT 25
21	ICGV 99249	43	ICGV-15 96891	65	TAG-24
22	ICGV-15 07803	44	ICGV-15 96909	66	TG-39