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Morphological and Molecular Characterization of Pigeon Pea (Cajanus cajan (L.) Millsp.) For Improved Utilization

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Abstract

Background: *Pigeon pea (Cajanus cajan (L.) Millsp.) is a multipurpose short-lived perennial food legume that provides fodder and wood for small holders. However, the crop remains underutilized with limited research on its diversification and improvement. Understanding the genetic diversity in germplasm of a crop is an efficient mean for unveiling unique traits that could be exploited for enhancing genetic improvement and utilization. This study characterized pigeon pea accessions using morphological descriptors and molecular markers.*

Methods: *Twenty pigeon pea accessions were evaluated under field conditions in a randomized complete block replicated thrice. Data collected on agro-morphological descriptors, were used to characterize the accessions. Young leaf samples collected from each accession was used for DNA extraction following CTAB standard procedure. Genetic diversity was carried out using Diversity Arrays Technology Sequence Single Nucleotide Polymorphic (DArTseq SNP) markers. Data were analysed using PCA, cluster analysis, and ANOVA at α0.05.*

Results: *The accessions differed significantly for all morphological parameters except days to 75% maturity and seed thickness. The accessions grouped into three clusters based on the morphological traits. Out of 2934 SNPs discovered all over the pigeon pea reference genome, only 2532 SNPs were retained after filtering. They were distributed all over the eleven chromosomes of pigeon pea.*

Conclusion: *Morphological and molecular variability exists among the accessions. The variations in morphology of seed of pigeon pea accessions is a vital trait that could be selected for further improvement necessary for enhancing consumers' preference and utilization.*

Key Words: DArTseq SNP Markers, Genetic Diversity, Pigeon Pea

1. Introduction

Pigeon pea (*Cajanus cajan* (L.) Millsp.), a diploid (2n = 2x = 22) legume crop species is an annual or short-lived perennial food legume of the family *Fabaceae*. It is a pulse of nutritional importance, providing significant protein to human diet in less developed regions. It is also known as red gram or no-eye pea. It is a drought tolerant pulse and one of the major grain legumes grown in the tropics and subtropics for multiple uses. It is particularly suited for rain fed agriculture in semi-arid areas due to its deep taproot, heat tolerance and fast-growing habit [1]. It is the sixth most important grain legume crop grown in semi-arid tropics of Asia, Africa and the Caribbean under a wide variety of cropping systems [2]. According to India is the largest producer of pigeon pea followed by Myanmar in the world, while Malawi, Tanzania, Kenya and Uganda are the major producers in Africa [3].

In West Africa, pigeon pea is a minor crop, but it plays a key role in the subsistence of smallholders in Benin, Nigeria and Ghana, food security, nutritional balance and poverty-alleviation in sub-Saharan Africa [4-6]. The seed of pigeon pea is eaten as a green

vegetable, and is an important source of protein, vitamin B, carotene and ascorbic acid [7]. It is a major source of protein of about 20% of the world population [8]. The mature seed contains 18.8% protein, 53% starch, 2.3% fat, 6.6% crude fiber and 250.3 mg 100 g−1 minerals [9].

As a perennial shrub, pigeon pea has many advantages over annual legumes in that several harvests are possible and the capacity to contribute to enhancing soil fertility is much higher [10]. Pigeon pea has high biomass productivity, mainly used as fodder, and provides the most nutrient and moisture contributions to the soil [11,12]. Its deep taproot is able to extract nutrients such as phosphorus from the lower layers of the soil, and bring them to upper layers where they can be beneficial to the crop [13]. Owing to climate variability and the occurrence of prolonged drought, pigeon pea offers resilience to cropping systems and its cultivation is expected to expand to new areas [14]. Due to its ability to tolerate drought, it can be considered of utmost importance for food security in regions prone to rain failures [15].

In addition to being efficient in fixing nitrogen, pigeon pea rhizobia also present other biotechnological applications, such as biopolymer production and enzymatic activity [16,17]. Despite the potential benefits of pigeon pea, it is unfortunately considered an orphan crop in many countries in sub-Sahara Africa [18]. The national agricultural program study defined the crop as one of the nineteen neglected and underutilized priority crops that merit attention and support (Fiacre et al., 2018). The neglected status of pigeon pea affects its varietal diversity. In order to integrate its conservation and provide good parental line as strategies for increasing its utilization, it is necessary that more research works be done on its diversity.

Knowledge of the extent of genetic diversity in available germplasm promotes the efficient use of genetic variation, thereby facilitating their improvement Analyzing genetic relationships in species is important for revealing genetic diversity among different germplasms [19]. This explains why variability among cultivars provides useful information for successful breeding programs [20].

Various types of genetic markers for studying the genotypic diversity of plant germplasm, including pigeon pea, have been developed and used. The most widely used marker systems include single sequence repeat (SSR), single nucleotide polymorphism (SNP), amplified fragment length polymorphism (AFLP) and microarray-based diversity arrays technology (DArT) markers [21]. High throughput genotyping technologies such as genotyping by sequencing (GBS) are effective tools to detect abundant and highly reproducible SNPs and DArT markers [22]. Diversity array technology (DArT) markers have been confirmed to be beneficial for the investigation of wheat and other crops over the previous decade [23]. However, DArT markers have not been used to characterize pigeon pea. Considering the huge benefits of DArT markers over other genetic markers, using this marker to investigate molecular traits of pigeon pea could be useful in eliciting the genetic diversity among different accessions of the crops.

Hence, this study characterized 20 pigeon pea accessions based on agro-morphological characters using Bioversity International descriptors for C. cajan. Also, the genetic diversity of 20 accessions of pigeon pea using Diversity Arrays Technology Sequence Single Nucleotide Polymorphic (DArTseq SNP) markers was assessed.

2. Methods

2.1 Location of Experimental Site

The field experiment was carried out at the International Institute of Tropical Agriculture (IITA), Ibadan (Latitude 7.49°N, longitude 3.89°E, altitude 239.34 m above sea level). The laboratory/ bench work on pigeon pea characterization was carried out at the Bioscience Center of the same Institute.

2.2 Genetic Materials Used For the Study

The selected twenty accessions of *Cajanus cajan* were collected from the Genetic Resources Centre (GRC) of IITA, Ibadan, Nigeria. The seed ID, place (State) of collection and country of origin of the evaluated accession are presented in Table 1. The accessions were selected for this study because they have not been characterized based on the traits considered in this study. Besides, the selected seeds were found in the major agro ecological zones in Nigeria.

2.3 Seed Preparation for Breaking Dormancy

All the seeds of the 20 pigeon pea accessions were scarified mechanically using a surgical blade to puncture/scratch the seed coat. This was done so as to break their dormancy, allowing water imbibition, and uniform germination because unscarified seeds take longer period to germinate. The scarified seeds were coated with Mancozeb, to prevent the seeds from fungal attack before seedling emergence.

2.4 Cultural Management Practices

The land used for the experiment was ploughed and harrowed. Seeds were sown in single row plots which were 4 m long. Intra- and inter-row spacing was 1.0 m and 1.0 m, respectively. Two seeds were sown per hole and later thinned to one plant per stand at two weeks after planting. The experiment was initially rain-fed, after which the plants were irrigated twice every week until 15 weeks after sowing. The experiment was laid out in a randomized complete block design in triplicates. Manual weeding was carried out as necessary to keep the fields free of weeds.

S/N	Accession	Seed source (State)	Agroecology
1	TCc-8869	Enugu	Moist semiarid
2	TCc-8870	Enugu	Moist semiarid
3	TCc-8871	Enugu	Moist semiarid
4	TCc-8872	Enugu	Moist semiarid
5	TCc-8873	Enugu	Moist semiarid
6	TCc-8874	Enugu	Moist semiarid
7	TCc-8875	Ebonyi	Humid forest
8	TCc-8876	Kaduna	Dry semiarid
9	TCc-8877	Jos	Mid-altitude
10	TCc-8878	Jos	Mid-altitude
11	TCc-8879	Kogi	Derived savannah
12	TCc-8880	Kogi	Derived savannah
13	TCc-8881	Kogi	Derived savannah
14	TCc-8882	Kogi	Derived savannah
15	TCc-8883	Ondo	Humid forest
16	TCc-8884	Oyo	Rainforest transition
17	TCc-8885	Oyo	Rainforest transition
18	TCc-8886	Oyo	Rainforest transition
19	TCc-8887	Oyo	Rainforest transition
20	TCc-8890	Oyo	Rainforest transition
Source: GRC, IITA, Ibadan.			

Table 1: Evaluated Cajanus cajan accessions collected from different locations and agroecological zones of Nigeria

2.5 Morphological Characterization of the Pigeon Pea Accessions
For morphological characterization, qualitative and quantitative characters were observed. These include number of branches, growth habit, stem colour, flower colour, pattern of streaks, leaflet shape, leaf hairiness, vigour at 50% flowering, seed eye width, seed shape, plant height, leaf size, days to 50% flowering, seeds per pod, pod length, and 100 seed weight.

2.6 Determination of Qualitative Traits

The growth habit of the different accessions was observed and described as erect and compact, semi-spreading, spreading or tailing. The number of branches which includes, primary, secondary and tertiary branches was determined for each accession at six weeks after sowing. The stem colour for each accession varied from green, sun red, purple and dark purple and this was scored at four weeks after sowing. A variety of leaflet shapes comprising lanceolate, narrow-elliptic, broad-elliptic, and obcordate was observed for all the accessions. The lower surface of five randomly selected leaves on each accession was observed by hand feeling for hairiness and described as either pubescent (with hair) or glabrous (without hair). The vigour at 50% flowering was scored for all the accessions at eight weeks after sowing and described as low, intermediate or high. The flowering was scored as determinate, semi-determinate, and indeterminate at five weeks after sowing. The pod color observed for the different accessions were green, purple, mixed, green and purple, and dark purple. The pod form observed for the different accessions was flat and cylindrical. Seed colour pattern of the different accessions was observed and described as plain, mottled, speckled, mottled and speckled, and ringed. The base flower colour of the different accessions was observed and described as ivory, light yellow, yellow, orange-yellow. The pattern of streaks on the dorsal side of the petal was observed on the different accessions and described as sparse streaks, medium streaks, dense streaks, uniform streaks.

2.7 Quantitative Traits

The plant height was measured with meter rule. This was done by measuring the stem from a surface above the ground to the apex of the topmost leaf. This was done for five randomly selected plants at 50% maturity and two weeks before the first harvest. The area of middle leaflet on a secondary branch was measured with a ruler.

Days to 50% flowering was taken as the date of sowing to when 50% of the plants had flowered and began to produce inflorescences. The duration of flowering was recorded as the number of days from first flowering to the end of flowering. Number of days from seedling emergence until 75% of the stands-initiated flower buds on the peduncles was observed and recorded.

Ten randomly selected pods from five randomly selected plants was collected and used to determine number of seeds per pod. The length of pod was measured with a tape rule. The 100 seed weight

was computed by weighing 100 seeds using a weighing balance and recorded in grams.

2.8 Molecular Characterization of Pigeon Pea

Molecular characterization was done using young tender leaves from three weeks old plants harvested for DNA extraction.

3. DNA Extraction

3.1 Collection of Leaf Samples, Isolation and Quantification of DNA for PCR

To optimize DNA quality and concentration, young and healthy leaves were collected from 15 days old seedlings from each of the accessions. The DNA was extracted using the modified Cetyl Trimethylammonium Bromide (CTAB) extraction protocol as described by Tel-Zur et al. (1999). Twenty milligrams (20 mg) of fresh leaf tissue was collected from each accession by putting steel balls and approximately 100 mg of lyophilized tissues into 2 mL eppendorf tubes and covered. The lyophilized leaf samples were ground into fine powder using Genogrinder -2000 for 30-40 seconds at 1500 revolutions per minute (rpm). The CTAB extraction buffer (800 µl) was added after the supernatant was removed. The tubes were incubated at 65°C for 30 minutes followed by intermittent inversion every 10 minutes to homogenize the sample in a homogenizer. Tubes containing the mixture were removed and allowed to cool for 3 minutes, thereafter, 600 µl of chloroform: Isoamylalchol (24:1) was added to the samples. The mixtures were centrifuged at 10,000 rpm for 10 minutes and then the supernatants were transferred into newly labelled tubes (this step was carried out twice). A 400 µl of ice-cold isopropanol was added to the supernatant, mixed gently for about 1 minute and kept in -20°C blast freezer to precipitate for 90 minutes. The tubes were removed from the freezer and left to thaw for 30 minutes at 10,000 rpm. The supernatant was carefully decanted and DNA pellets washed with 70% cold ethanol and air dried in the fume hood until the ethanol evaporated completely. The pellets were re-suspended in 95 µL low salt TE (Tris-EDTA) buffer and 5µl of RNase per sample.

3.2 Determination of Isolated DNA quality

The following steps were involved in ascertaining the quality of the extracted DNA. The quality of the extracted DNA was checked using 1% agarose gel. One gram of agarose tablet was dissolved in 100 ml of 0.5X Tris borate EDTA (TBE) buffer in a conical flask. The conical flask was placed in a microwave and allowed to boil, thereafter cooled at 60°C. Then, 5 µl ethidium bromide was added to the mixture and gently swirled to mix. The solution was poured into a sealed gel casting unit on a gel casting plate. The comb was placed at the top of the gel and allowed to solidify at room temperature. After solidification, the comb was removed carefully and the gel plate (along with gel) was placed in the gel electrophoresis tank. In each well, the DNA samples along with the gel loading dye was loaded carefully. The gel was viewed under UV light (332 nm) and the quality of DNA were detected.
The concentrations of the extracted DNA samples were determined using Nanodrop spectrophotometer (Thermo Fisher Scientific) at 260/280 nm wavelength. Subsequently, high quality DNA of each sample was diluted to 50 ng/µl, isolated and shipped to Diversity Array Technology, Pty LTD, Canberra, Australia for whole genome sequence using Diversity Array Technology Sequence (DArTseq) Single Nucleotide Polymorphic (SNP) markers. The DNA was processed using Genotyping By Sequencing(GBS) platform which combines Diversity Arrays Technology (DArT) with a next-generation sequencing technique called DArTseq™ described by to determine the genetic relatedness across the 20 pigeon pea accessions. The discovered DArTseq SNP markers were aligned on pigeon pea reference genome *Cajanus cajan* (Phytozome 2018, Version 1.0).

3.3 Data Collection and Statistical Analysis

Data on the morphological characters were collected from five representative plants per accession and their means were recorded for all observations. The qualitative and quantitative traits were measured using the International Board for Plant Genetic Resources (IBPGR, ICRISAT 1993) pigeon pea descriptors. Morphological and quantitative data were analysed using descriptive statistics and Analysis of Variance (ANOVA) with PROC. GLM procedure of SAS Statistical Analysis System (SAS 2010, Version 9.4). Significant means were separated using Duncan's Multiple Range Test (DMRT) at $p<0.05$. Principal Component Analysis (PCA) was used to determine the overall variance accounted for by the evaluated parameters and also to check for elementary pattern of relationship using the PRINCOMP procedure (SAS, 2010). Principal components with Eigen values ≤ 0.20 were selected. Cluster analysis was constructed to group the accessions into various cluster groups using the CLUSTER procedure of SAS.

4. Results

4.1 Morphological Characterization of the Twenty Pigeon Pea Accessions

Morphological variations in the 20 pigeon pea accessions using seven qualitative descriptors is presented in Table 2. The semi-spreading growth pattern had a greater presence among the pigeon pea accessions. Thirteen accessions constituting 65% of the total pigeon pea assessed had semi-spreading growth pattern, while the rest had spreading growth pattern. The pattern of streaks varied across the evaluated accessions. Eight accessions had medium streak pattern, while six had dense streak pattern, four had sparse pattern and only two accessions displayed uniform pattern of streaks (Plate 2).

It was observed that nine accessions had yellow base flower, while eight had orange yellow base flower whereas, accessions TCc-8878, TCc-8883 and TCc-8887 had light yellow base flower. With regards to flowering pattern, 75% of the accessions showed semi-determinate flower, while four accessions: TCc-8877, TCc-8885, TCc-886 and TCc-8890 had indeterminate flower and only TCc 8880 showed determinate flowering.

All the accessions, had plain seed colour except TCc-8887 which had mottled and speckled seed colour. Thirteen accessions showed white base seed colour, six had light brown base seed colour, while only TCc-8887 displayed grey base seed colour. The colour of the pods also vary from green to purple. Seven of the accessions had green pods, while 13 accessions had combination of green and purple pods as shown in Table 2.

S/N	Accession	Growth habit	Pattern of streaks	Base flower colour	Flowering pattern	Seed colour	Base seed colour	Pod colour
1	TCc-8869	Semi-spreading	Medium	Yellow	Semi-determinate	Plain	Light brown	Green
2	TCc-8870	Spreading	Dense	Orange yellow	Semi-determinate	Plain	White	Mixed (G&P)
3	TCc-8871	Semi-spreading	Medium	Yellow	Semi-determinate	Plain	White	Green
4	TCc-8872	Semi-spreading	Dense	Yellow	Semi-determinate	Plain	Light brown	Mixed (G&P)
5	TCc-8873	Semi-spreading	Dense	Orange yellow	Semi-determinate	Plain	Light brown	Mixed (G&P)
6	TCc-8874	Semi-spreading	Sparse	Yellow	Semi-determinate	Plain	White	Green
7	TCc-8875	Semi-spreading	Sparse	Yellow	Semi-determinate	Plain	White	Green
8	TCc-8876	Semi-spreading	Medium	Yellow	Semi-determinate	Plain	White	Green
9	TCc-8877	Semi-spreading	Medium	Yellow	Indeterminate	Plain	Light brown	Green
10	TCc-8878	Semi-spreading	Sparse	Light yellow	Semi-determinate	Plain	White	Mixed (G&P)
11	TCc-8879	Semi-spreading	Medium	Orange yellow	Semi-determinate	Plain	Light brown	Mixed (G&P)
12	TCc-8880	Spreading	Medium	Orange yellow	Determinate	Plain	Light brown	Mixed (G&P)
13	TCc-8881	Semi-spreading	Uniform	Orange yellow	Semi-determinate	Plain	White	Mixed (G&P)
14	TCc-8882	Spreading	Sparse	Orange yellow	Semi-determinate	Plain	White	Green
15	TCc-8883	Spreading	Medium	Light yellow	Semi-determinate	Plain	White	Green
16	TCc-8884	Spreading	Dense	Orange yellow	Semi-determinate	Plain	White	Mixed (G&P)
17	TCc-8885	Spreading	Dense	Orange Yellow	Indeterminate	Plain	White	Mixed (G&P)
18	TCc-8886	Semi-spreading	Dense	Yellow	Indeterminate	Plain	White	Mixed (G&P)
19	TCc-8887	Semi-spreading	Medium	Light Yellow	Semi-determinate	Mottled & speckled	Grey	Mixed (G&P)
20	TCc-8890	Spreading	Uniform	Yellow	Indeterminate	Plain	White	Mixed (G&P)
G&P = Green and purple								

Table 2: Morphological variations among twenty accessions of Cajanus cajan

4.2 Quantitative Characters Analysis for Pigeon Pea Accessions

The mean squares from analysis of variance of selected quantitative parameters of 20 accessions of pigeon pea are presented in Table 3. The accessions differed significantly for all parameters except the days to 75% maturity and seed thickness.

(a) Dense **(b) Uniform**

Plate 1. Variation in pattern of streaks of pigeon pea flowers

Source of variation	Df	Days to 50% Flowering	Duration of Flowering	Days to 75% Maturity	PL (cm)	Pod Width	Seed Length	Seed Width	Seed Thickness	Stem Thickness	Leaf Size	100 SW	PH (cm)
Accession	19	364.08*	374.64*	337.06ns	1.36**	1.47**	0.20**	0.64**	0.14ns	4.12*	120.67**	3.33*	8.14**
Rep	2	99.05ns	105.35ns	547.82ns	1.79**	0.20ns	0.02ns	0.12ns	0.16ns	25.9**	49.84ns	2.22ns	0.97ns
Error	38	170.82	183.75	247.38	0.19	0.38	0.04	0.08	0.09	2.3	23.71	1.09	1.7
PL = Pod length, SW = Seed weight, PH = Plant height, * and ** significant at p = 0.05 and p = 0.01 level of significance respectively.													

Table 3: Mean square from analysis of variance of twelve morphological traits evaluated among 20 pigeon pea accessions

4.3 Contribution of Various Morphological Components to Variation in Different Accessions of C. cajan

The principal component analysis and the percentage contribution of each component to the total variation is indicated Table 4. The first four principal components explained 88% of the total variation, cumulatively. The first principal component axis (PC1) with the Eigen value 5.43 contributed 45% of the total variability, while PC2 and PC3 contributed 24% and 13% of the total variability, respectively. The Eigen values ranged from 5.43 in PC1 to 0.81 in PC4. Eigen values equal to or greater than 0.2 were identified as the logical cut-off points where each selected trait made an important contribution towards the PC axis. In the first principal component, days to 75% maturity, pod length, pod width, seed width, stem thickness, leaf size, 100 seed weight and plant height all had a positive loading on PC1. Duration of flowering and seed width had positive contribution to the variation in the PC2. Contributing negatively to the variation on PC2 were the days to 50% flowering, days to 75% maturity and seed length. The positive contributors to the loading on the PC3 are seed length, seed thickness and 100 seed weight, the remaining values which is pod width contributed negatively to the third principal component. The fourth component accounted for 7.0% of the total variation in the population. Days to 75% maturity, pod width, seed length, seed thickness and stem thickness all contributed positively to the variation in PC4.

Variables	PC1	PC2	PC3	PC4
Days to 50% flowering	0.15	-0.52	-0.17	0.02
Duration of flowering	-0.14	0.54	0.15	0.01
Days to 75% maturity	0.21	-0.44	-0.07	0.28
Pod length	0.39	0.05	-0.19	0.06
Pod width	0.34	0.18	-0.21	0.26
Seed length	0.15	-0.27	0.41	0.41
Seed width	0.34	0.27	-0.15	0.14
Seed thickness	0.07	0.03	0.68	0.42
Stem thickness (mm)	0.26	0.14	0.05	0.69
Leaf size (cm2)	0.40	0.11	-0.06	0.01
100 Seed weight	0.33	-0.05	0.45	0.06
Plant height	0.41	0.12	0.03	0.07
Eigen values	5.43	2.82	1.55	0.81
Proportion of variance	0.45	0.24	0.13	0.07
Cumulative proportion	0.45	0.69	0.82	0.88
PC- Principal Component, only loadings with values ≥ 0.20 on the PC axes are presented				

Table 4: Eigen vectors and Eigen values showing contribution of the first five principal component axes to the variation in cowpea accessions

4.4 Genetic Relatedness of 20 Accessions of Pigeon Pea Based on Agro-Morphological Characters

The quantitative phanerogam obtained from cluster analysis using Ward technique on the 20 accessions of pigeon pea was based on similarities of some morphological traits among the accessions (Figure 4.1). Three clusters described the clustering of the accessions at an R-squared distance of 0.9936 similarity index. Cluster 1 comprised six accessions: TCc 8862, TCc 8871, TCc 8883, TCc 8885, TCc 8872, and TCc 8890. The second cluster had seven accessions, TCc 8870, TCc 8869, TCc 8880, TCc 8875, TCc 8881, TCc 8874, and TCc 8878. Seven accessions including TCc 8887, TCc 8877, TCc 8879, TCc 8876, TCc 8873, TCc 8884, and TCc 8886 cluster together in the third category.

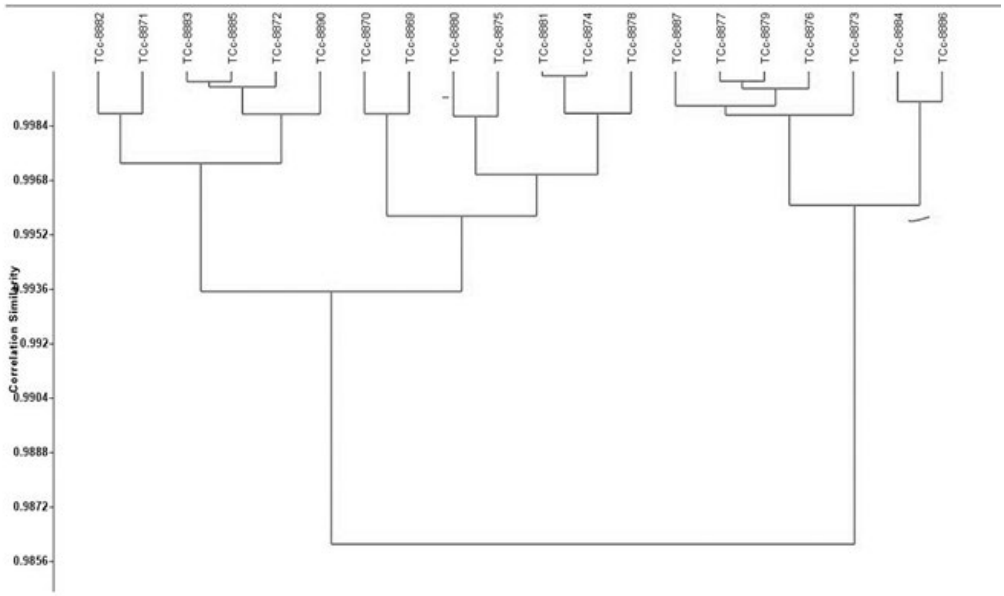

Figure 1: Dendrogram produced from Ward's minimum variance cluster analysis showing genetic relatedness among pigeon pea accessions based on agro-morphological characters

4.5 Pearson Correlation Coefficients of Agro-Morphological Traits of 20 Accessions of Pigeon Pea

Pearson correlation coefficients between plant characters evaluated among twenty accessions of pigeon pea showed that the number of days to flowering was strongly and negatively correlated with days to 50% flowering (Table 5). The days to 75% maturity was strongly and positively associated with days to 50% flowering but negatively correlated with days to flowering. Also, pod length was negatively correlated with days to 50% flowering. Also, pod width was strongly and positively associated with pod length. Seed width was strongly and positively correlated with pod length and pod width. Seed thickness positively correlated with seed length, while stem thickness was also positively correlated with pod width and seed width. Leaf size also showed a strong and positive correlation with pod length, pod width, seed width and stem thickness. Weight of 100 seeds also showed a strong and positive association with pod length, pod width, seed length, seed width, seed thickness and leaf size. Plant height was also strongly and positively correlated with pod length, pod width, seed length, seed width, seed thickness, stem thickness, leaf size and 100 seed weight.

	D50F	DF	D75M	PdL	PdWth	SdL	SdW	SdTh	StTh	LS	100SW	PH
D50F	1											
DF	-0.98**	1										
D75M	0.67**	-0.67**	1									
PdL	0.26*	-0.21ns	0.16 ns	1								
PdWd	-0.03 ns	0.10 ns	0.15 ns	0.66**	1							
SdL	0.18 ns	-0.21 ns	0.11 ns	0.12 ns	-0.03 ns	1						
SdW	-0.12 ns	0.15 ns	0.02 ns	0.65**	0.71**	0.04 ns	1					
SdTh	-0.20 ns	0.19 ns	-0.16 ns	-0.01 ns	-0.05 ns	0.30*	0.03 ns	1				
StTh	-0.09 ns	0.10 ns	-0.09 ns	0.14 ns	0.28*	0.17 ns	0.31*	0.19 ns	1			
LS	0.16 ns	-0.13 ns	0.18 ns	0.70**	0.61**	0.15 ns	0.64**	0.15 ns	0.44**	1		
100SW	0.17 ns	-0.15 ns	0.20 ns	0.38**	0.26*	0.43**	0.29*	0.32*	0.14 ns	0.56**	1	
PH	0.12 ns	-0.09 ns	0.14 ns	0.66**	0.60**	0.23*	0.65**	0.23*	0.58**	0.98**	0.63**	1

*, ** Significant differences at p = 0.05 and 0.01 levels of probability respectively; ns not significant
D50F=Days to 50% flowering; DF= Duration of Flowering; D75M= Days to 75% maturity; PdBL=Pod length, PdWth= Pod width; SDL= Seed length; SDW= Seed width; SDTH= Seed thickness; STMTH= Stem thickness; LFSZ= Leaf size; 100SW= 100 seed weight; PH= Plant height

Table 5: Pearson's correlation coefficient of some agro-morphological traits of 20 accessions of pigeon pea

5. Molecular Characterization of Twenty Pigeon Pea Accessions

5.1 SNP Markers Discovery by Genotyping by Sequencing (GBS)

A total of 2934 SNPs were discovered all over the pigeon pea reference genome Cajanus cajan v1.0. These SNPs were filtered based on call rate ≥ 80% and marker reproducibility ≥ 95%. After further filtering which was based on minor allele frequency (MAF) ≤ 1% and missing data (SNP loci) ≤ 20%, 2532 SNPs which accounts for less than 20% of the total SNPs discovered were retained all over the 11 chromosomes of pigeon pea. Minor allele frequency ranged from 0.02 to 0.50 (Fig. 2). Overall, heterozygosity was 2.67% and ranged from 0.02% to 0.25% in the accessions (Fig. 3).

5.2 Genetic Diversity of the Pigeon Pea Accessions

A total of 2532 filtered SNPs which distinguish the 20 pigeon pea accessions were used to generate a cladogram using Unweighted Pair Group Method with Arithmetic Mean (UPGMA) method. The accessions varied at a genetic distance of 0.01 Nei-genetic distances. The color of branches indicates accessions corresponding to different geographical regions. Clade colors namely red, blue, green, aqua blue, pink, black and orange represent Enugu, Oyo, Kogi, Jos, Ondo, Kaduna and Ebonyi, respectively. The pigeon pea accessions were clustered in three distinct groups according to their geographical area with the presence of South-eastern part of Nigeria (Enugu), a moist semiarid agroecology in group one. The second cluster consists of accessions from the southern part of Nigeria (Oyo), a rainforest humid savannah transition zone and the third cluster consists of accessions from the moist semiarid (TCc-8874), humid forest (TCc-8875), dry semiarid (TCc-8876), mid-altitude (TCc-8878 and TCc-8877), derived savanna (TCc-8879 - TCc-8882) and humid forest (TCc-8883) as indicated in Fig. 5. The cladogram was produced using the Identity-by-state (IBS) matrix among the 20 accessions.

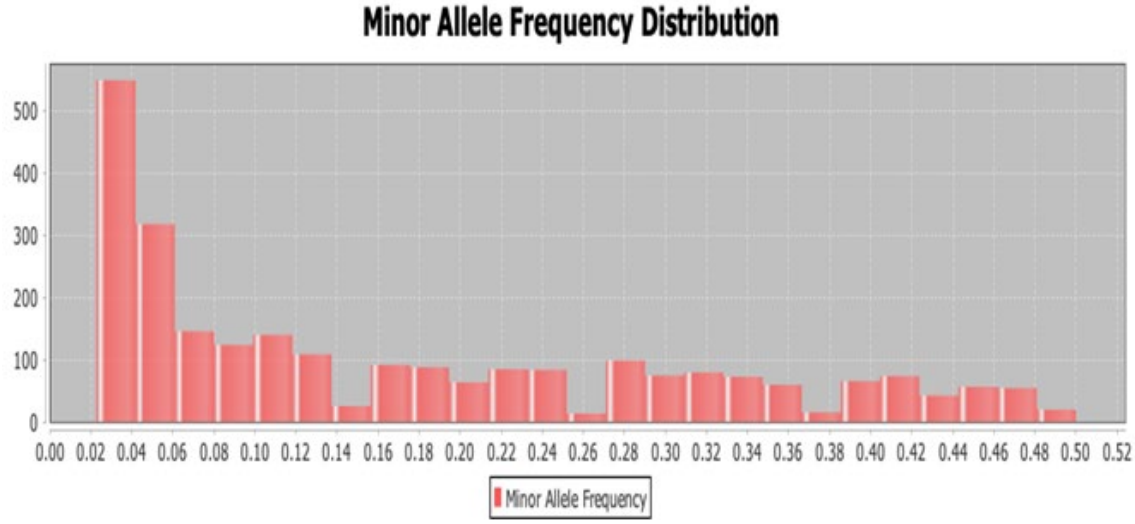

Figure 2: Distribution of the minor allele frequency in 2934 SNPs derived over pigeon pea reference genome from DArTseq high deep sequencing

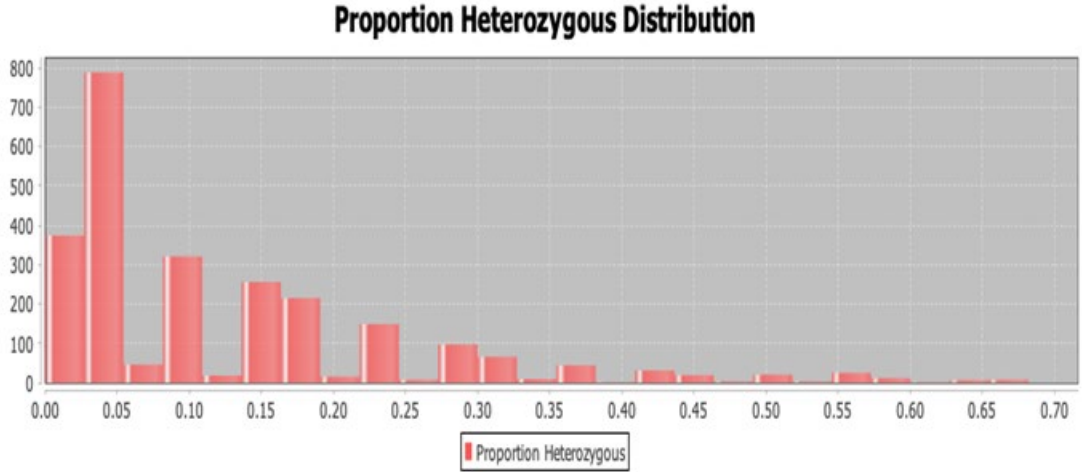

Figure 3: Proportion of heterozygosity among the 2934 filtered SNPs in 20 pigeon pea accessions, derived from DArT high deep sequencing.

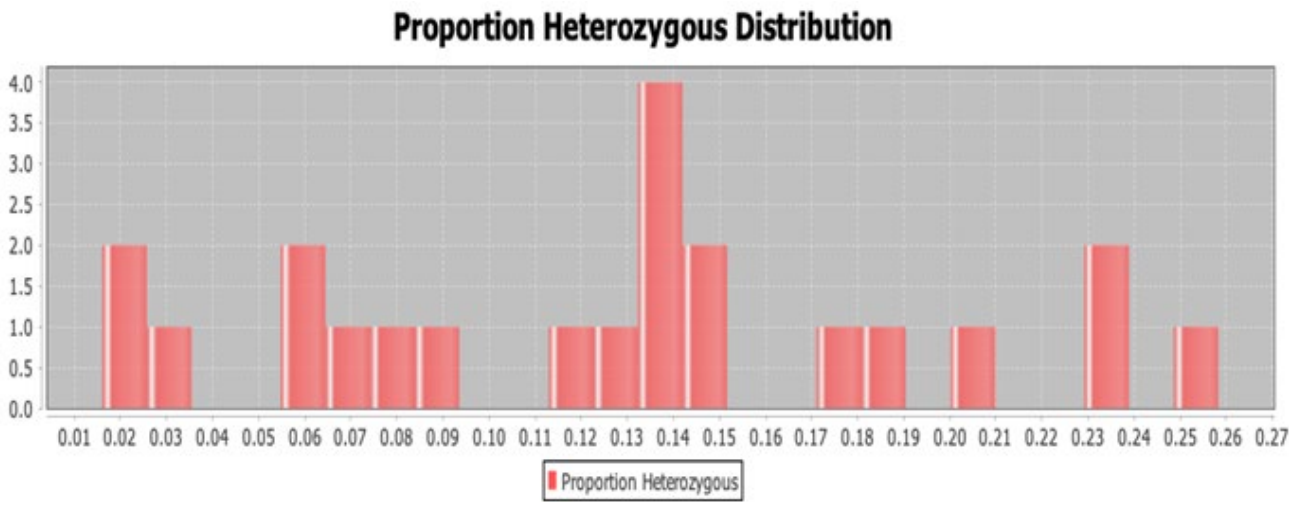

Figure 4: Distribution of overall heterozygosity distribution among the 20 pigeon pea accessions, derived from DArT high deep sequencing.

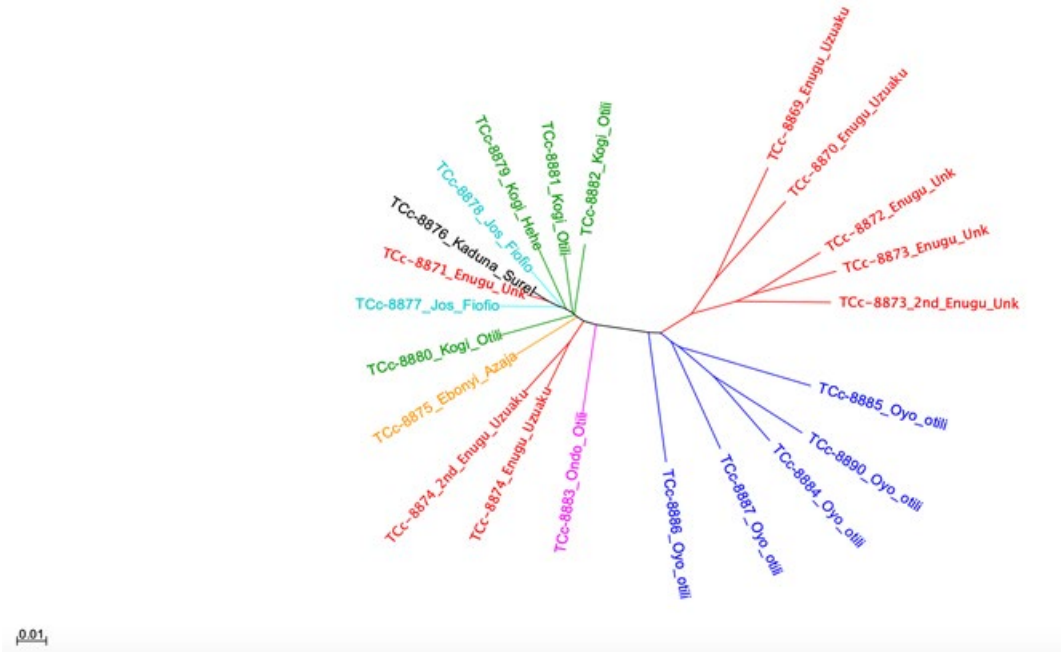

Figure 5: Unrooted phylogenetic tree of the 20 pigeon pea accessions. The cladogram was produced using the unweighted UPGMA method among the accessions. The color of branches indicates accessions corresponding to different geographical regions. Clade colors namely red, blue, green, aqua blue, pink, black and orange represent Enugu, Oyo, Kogi, Jos, Ondo, Kaduna and Ebonyi, respectively.

6. Discussion

Determining the genetic diversity of any crop species is a suitable precursor for its improvement. Several studies have been conducted previously for estimating genetic diversity in pigeon pea. Various techniques such as morphological variability (Upadhyaya et al., 2009); , biochemical characterization and molecular marker analysis have been used in the pigeon pea diversity assessment [24,25]. Although morphological characterization is susceptible to environmental effects, it is however the first step in the description and classification of germplasms [26].

In recent years, molecular markers have attained great significance in evaluating plant material. Scientists have invented numerous marker systems depending upon their need and material to be handled to explore the hidden information stored in the DNA as variation in nucleotide sequences. The significant relevance

of using these markers is that they are devoid of environmental interaction. The present study revealed a wide range of variation in qualitative traits as well as in quantitative variables of C. cajan. With significant means squares being recorded for seed width, leaf size, plant height, pod length and seed length, this makes these traits important in estimating genetic variability among the accessions. Study on the basis of days to 50% flowering made it possible to categorize the genotypes into three groups like, early, medium and late flowering. Earlier reports by have also exhibited the presence of variation for different quantitative characters in pigeon pea germplasm accessions [27,28].

Growth habit like branching pattern was a very important parameter for varietal identification and genetic diversity studies evaluated 28 accessions for 23 descriptors and observed leaf pubescence in all accessions [29,30]. This is similar to our observation in the present study as all the accessions have leaf pubescence. The semi-determinate flowering pattern displayed by most of the accessions implies that the crop is more of semi-determinate than indeterminate [31,32]. Our observation contradicts that of who reported that majority of pigeon pea from different parts of the world have determinate flowering pattern. Determinate flowering patter is more desirable because of its importance in determining time of scheduling different cultural activities. It is also imperative in mechanizing the operation such as harvesting and processing. In this regard, TCc-8880 an accession with determinate flowering pattern could be selected for further improvement. Further improvement of days to 75% maturity and seed thickness appeared to have reached its peak as the coefficient of variations for these parameters were very narrow and the similarity in these parameters. Besides, these parameters were very similar in all the accessions.

Clustering methods assemble accessions into groups based on similarities (low genetic distances) among the accessions on some morphological traits that were assessed. Cluster analysis also explained the nature and level of genetic diversity among the cultivars in the dendrogram. Seed indices like pod length, pod width, seed width and 100 seed weight exhibit distinct and substantial contribution to variations observed in the pigeon pea accessions from Nigeria. Similarly, growth parameters such as days to 50% flowering, days to 75% maturity, stem thickness and leaf size are important variables that determines the variability in different pigeon pea accessions. Reported that variation existed in both qualitative and quantitative traits of pigeon peas accession from India. Three clusters described the clustering of the accessions at an R-squared distance of 0.9936 similarity index thus implying existence of considerable genotypic differences among the evaluated accessions. This may be linked to diversity in the origin as well as environmental conditions. This view was also shared by the study observed genetic variation among the accessions evaluated. The seeds of the various accessions evaluated showed qualitative variability in seed eye colour, seed colour pattern, base seed colour and quantitatively in seed weight and length. This indicates that genetic variability exists among the accessions and it also revealed that the variations in the seed's morphology of the pigeon pea accessions could determine consumer's preference and could be exploited for selection of the crop.

7. Conclusions

The level of genetic variability and relationship detected in this study could further be utilized in prospecting for highly diverse accessions which could be of paramount significance for improving the food and nutrition quality of pigeon pea. The information generated on the attributes of pigeon pea will certainly pave ways for prospecting for this important underutilized food crop for its quality traits. Information from this study is expected to facilitate development of improved cultivars of pigeon pea and help elevate its importance for nutritional security and sustained livelihoods through a directed breeding programme.

Ethics Approval and Consent to Participate

Not applicable.

Availability of Data and Material

The set of data used in this study has not been published elsewhere either partly or in its entirety. The data sets used and/or analysed during the current study are available from the corresponding author on request.

Competing Interests

The authors declare that there are no competing interests. The authors have no relevant financial or non-financial interest to disclose.

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Authors' Contributions

Ojo, T. Bolatito prepared the proposal, conducted the field experimentation, collected and analysed the data. Adeniyi O. Togun designed the experiment and supervised the first author, Michael Abberton conceived the research idea and provided research support for the first author, while, Olaniyi Oyatomi and Oyeyemi A. Dada assisted in field experimentation, experimental design, data analysis and revised the manuscript. All the authors contributed equally and approved the final manuscript.

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