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Research Article
Pollen Parameters Estimates and Molecular Characterisation of Selected Varieties of Soybean [*Glycine max* (L.) Merril]

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Abstract

Background and Objective: Soybean (*Glycine max*) is a very important leguminous crop known for its highly valued protein and oil. Genetic variability is the basic requirement for crop improvement, it provides a wider scope for selection. Selection through studies of pollen behaviours and molecular characterisation increases the frequency for favourable alleles which can be explored in a breeding programme for the crop. **Materials and Methods:** Nineteen varieties of soybeans were collected from the gene bank of the International Institute of Tropical Agriculture (IITA), Ibadan. The varieties were evaluated for their pollen parameters following standard procedures. **Results:** Eleven distinct genotypes obtained from the pollen studies were further evaluated for genetic diversity using a simple sequence repeat (SSR) molecular marker. Most of these varieties were seen to be highly fertile (26.70-84.60%). The highest sterility value was recorded in TGX-2010-11F with a value of 73.3% and the least value of 15.4% was recorded in TGX-2018-5E. TGX-2007-3F produced significantly ($p \leq 0.05$) highest pollen diameter (0.27 µ). Molecular diversity of 11 selected varieties from the initial twenty varieties using simple sequence repeat DNA marker generated 150-320 base pair with six primers. Genetic similarity among the varieties varies from 0.17-4.09 with an average gene diversity of 0.28. Clustered dendrogram of the 11 accessions revealed two major clades (TGX-1987-62F and TGX-2011-6F). **Conclusion:** The high genetic variability obtained for both pollen parameters and molecular characterisation indicate that soybean could be selected for those heritable traits and used as a tool for the improvement of the crop.

Key words: Genetic variability, soybean, genotypes, pollen behaviours, selection, SSR, DNA marker

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Soybean [*Glycine max* (L.) Merrill] is a member of the Papilionaceae family and is believed to have originated in North-Eastern China and distributed in Asia, USA, Brazil and Argentina[1]. The crop has a fairly wide range of climatic conditions for adaptation and is mostly cultivated on rain-fed land[1]. Soybean is aptly called "Golden bean" or "Miracle crop" of the 20th century and is one of the most important oilseed crops in the world[2] followed by cotton, sunflower canola, palm oil and peanut. It is also a very important crop for rotation with cereals like maize and sorghum because of biological nitrogen fixation that is important in improving soil fertility[3] and is considered a strategic crop in fighting the world's food shortage and malnutrition problems and most food aids to displaced and malnourished people are fortified with soybean[3]. Soybean is an important crop produced mainly in the guinea savannah zone of Nigeria[4].

Soybean is grown in many parts of the world and is a primary source of vegetable oil and protein used in food, feed and industrial applications[5]. Soybean was domesticated in the 11th century BC around Northeast China[6]. It may have been introduced to Africa in the 19th century by Chinese traders along the east coast of Africa[7]. Reports indicate that soybean was cultivated in Tanzania in 1907 and Malawi in 1909[7]. African countries with the largest area of production are Nigeria (650,000 ha), South Africa (245,000 ha), Uganda (147,000 ha), Malawi (79,480 ha) and Zimbabwe (69,900 ha)[8]. Nigeria is the largest producer of soybeans in Africa[9]. Soybeans were first introduced into Nigeria in 1908[10] but the first successful cultivation was in 1937 with the Malayan variety which was found suitable for commercial production in Benue State[11]. The producing areas of Central Nigeria have been responsible for a large proportion of the domestic requirement for this cheap source of plant protein. Today, soybean has made a successful incursion into the diet of many Nigerians, particularly children and nursing mothers.

The Nutritional value of soybean lies in protein (40-42%) and oil content (18-22%) thereby containing twice the protein of meat or poultry and containing all eight essential amino acids needed for childhood development[12]. In Nigerian markets, soybean costs about one-fifth as much as other forms of protein, including dairy and fish, are easier to store and transport. They also fix atmospheric nitrogen which reduces the need for farmers to purchase fertilizers. Soybean is also medicinal and is extremely useful for the treatment of malnutrition, particularly among children and fighting against diseases such as heart disease, cancer diabetes, high blood pressure, stroke, ulcer as well as loss of body mass among people living with HIV/AID[9]. Concerted efforts are greatly needed towards the improvement of this crop due to its high demand lately. Unfortunately, many factors, both biotic and abiotic, heavily hinder the production of this crop in Nigeria as a whole. Thus, there is a need to further assess the available landraces to exploit some hidden genetic variability for the improvement of the crop in the future.

Genetic variability is the basic requirement for crop improvement as this provides a wider scope for selection[13]. Thus the effectiveness of selection is dependent upon the nature, extent and magnitude of genetic variability present in the material and the extent to which it is heritable. The success of any crop improvement depends on the nature and magnitude of genetic variability present in the crop along with an in-depth understanding of the underlying gene action and genetic architecture of traits related to yield. The knowledge of the nature and magnitude of genetic variability is of immense value for planning an efficient breeding programme to improve the yield potentials of genotypes[13]. The role of genetic diversity in crop improvement programmes has been emphasized by Sujata and Basavaraja[14]. Genetic divergence among parents is essential since the crossing programme involving diverse parents is likely to produce high heterotic effects and also more variability could be expected in the segregating generations.

Despite the economic importance of soybean as the source of oil and other pulses, the crop has been facing some challenges of agronomic inferiority, particularly among accessions compared to commercial cultivars. Inadequate knowledge of the scope, nature of the alleles and their relationship with those already introgressed into commercial cultivars have been reported to be limiting its genetic diversity[15]. In Nigeria, the genetic diversity of this crop has not been fully studied and poorly understood, as well as improper documentation of research finding for exploitation in its breeding programme, thus narrowing its genetic base. Major focus on genetic diversity on soybean has been on random amplified polymorphic DNA and restriction fragment length polymorphism, limited works have been done using simple sequence repeat marker to determine genetic variability among the accessions. Proper attention is yet to be given to pollen characteristics, such as pollen viability, as an important trait in the characterisation of soybean genotypes. It is on this premise that this research is aimed at evaluating the genetic variation among selected varieties of soybean for effective selection, utilisation and improvement.

MATERIALS AND METHODS

Study area: Field experiments were carried out at the Experimental Garden of the Department of Plant Biology,

Federal University of Technology Minna, Niger State, Nigeria. Geographically, Minna is located in the North-Central geographical zone of Nigeria. Found within latitude 9°37'N and longitude 6°33'E. The area has a tropical climate with mean annual temperature, relative humidity and rainfall of 30.20°C, 61.00% and 1334.00 mm, respectively. The climate presents two distinct seasons, a rainy season between May and October and a dry season between November and April. The vegetation in the area is typically grass-dominated in Savannah with scattered trees. The experiment took place between May and August, 2020.

Pollen grain viability test: A pollen grain viability test was carried out by collecting ten matured flowers whose anthers have not dehisced from each accession. The pollen grains of each flower were dusted on a clean glass slide one after another and stained with 2% aceto-carmine solution. Each slide was carefully covered with a coverslip and observed under the light microscope. Properly stained pollen grains containing nuclei were regarded as being viable and those that appeared empty and/or slightly stained were considered as non-viable[16]. Percentage of pollen grain viability was calculated as the proportion of the grains that absorbed the stained to the total count using the equation below:

$$\text{Pollen viability } (\%) = \frac{\text{Number of stained pollen grains}}{\text{Total number of pollen grains}} \times 100$$

$$\text{Pollen sterility } (\%) = \frac{\text{Number of unstained pollen grains}}{\text{Total number of pollen grains}} \times 100$$

Molecular characterization using simple sequence repeat molecular markers: Molecular analysis of the selected samples of the soybean based on superiority in morphological traits was done at the Genetic Engineering Laboratory, International Institute of Tropical Agriculture (IITA) Ibadan. The accessions of soybean were subjected to DNA extraction using a modified procedure of[17]. Approximately 100 mg of young leaf from each sample was ground in 1000 µL of Dellaporta extraction buffer separately. The extracted mix was collected in a sterile Eppendorf tube and 40 µL of 20% SDS was then added. The samples were vortex and incubated at 65°C for 10 min. At room temperature, 160 µL of 5 M potassium acetate was added vortexed and centrifuged at 10000 g for 10 min. The supernatant was collected in another Eppendorf tube and 400 µL of cold isopropanol was added gently and kept at -20°C for 60 min. Centrifugation was done at 13000 g for 10 min to precipitate the DNA after which the supernatant was gently decanted and ensured that the pellet was not disturbed. DNA was then washed with 500 µL of 70% ethanol by centrifuging at 10000 g for 10 min. Ethanol was decanted and DNA air-dried at room temperature until no trace of ethanol was seen in the tube. Pellet was then re-suspended in 50 µL of Tris EDTA buffer to preserve and suspend the DNA.

SSR PCR protocol and bands separation: Six polymorphic SSR markers were used for genotyping of the selected soybeans genotypes (Table 1). The PCR reaction was optimized at 15 µL. Touch-down PCR protocol was followed, which consist of an initial denaturation at 94°C for 5 min followed by 9 cycles of denaturation of 94°C for 15 sec, Annealing of 65°C for 20 sec and extension of 72°C for 30 sec followed by a 25 cycles denaturation of 94°C for 15 sec, Annealing of 55°C for 20 sec and extension of 72°C 30 sec then a final extension of 72°C for 7 min.

Band separation: The separation of bands as produced by each primer was done in a 1.5% agarose gel. The suspension was boiled in a microwave for 5 min. Molten agarose was allowed to cool to 60°C and stained with 3 µL of 0.5 g mL^{-1} ethidium bromide (which absorbs invisible UV light and transmits the energy as visible orange light). A comb was inserted into the slots of the casting tray and the molten agarose was poured into the tray. The gel was allowed to solidify for 20 min to form the wells. Prepared buffer was poured into the gel tank to barely submerge the gel. Seven µL of each PCR product and loaded into the wells after the 100 bp DNA ladder was loaded into well 1. The gel was electrophoresed at 120 V for 45 min visualized by ultraviolet trans-illumination and photographed. The sizes of the PCR products were estimated by comparison with the

Table 1: Description of the SSR Primers used for molecular characterisation

Primers	Primer sequence (forward)	Primer sequence (Reverse)
Satt 569	GCGCAAATTGCTTCACGCATCCAAAT	GCGGCCTACTATAGTGAAGGGTATA
Satt 572	GCGGAGCATGTAAATCCAGCCTATTGA	GCGGGCTAACTTATGTTACTAAACAAT
Satt 294	GCGGGTCAAATGCAAATTATTTTT	GCGCTCAGTGTGAAAGTTGTTTCTAT
Satt 288	GCGGGGTGATTTAGTGTTTGACACCT	GCGCTTATAATTAAGAGCAAAAGAAG
Satt 222	GCGTGTTTTGTGAAAATAATAATTAAAGATG	GCGCCACAAGTAACTAATGTAATAGGTGTT
Satt 194	GGGCCCAACTGATATTTAATTGTAA	GCGCTTTGTGTTCCGATTTTGAT

mobility of a 100 bp molecular weight ladder that was run alongside experimental samples in the gel.

Data analysis: Data obtained was pooled for analysis, Analysis of Variance (ANOVA) was carried out to test for significant difference among the varieties for the pollen diameters, Duncan Multiple Range Test (DMRT) was used to separate the mean where there was a difference. A simple percentage was used to compare the estimate of some of the parameters like pollen sterility, pollen fertility etc. All value was considered significant at $p \leq 0.05$. Binary data was generated for each primer set using 1 and 0 for the presence and absence of positive amplification at a particular band size, respectively. The generated binary data was used to create a data matrix which was analyzed using the Power Marker V2.35 software for genetic diversity parameters such as major allele frequency, gene diversity and polymorphic information content. The genetic relationship among treated samples was also estimated by constructing a dendrogram through an unweighted pair group method with arithmetic means [UPGMA] using the mega6 software and genetic distance where computed also using the mega6 software.

RESULTS

Pollen parameters estimates: Table 2 shows that TGX-2018-5E has the highest pollen viability value of 84.6 and the least viability of 26.7% as seen in accession TGX-2010-11F. Most of these accessions were seen to be highly fertile. The highest sterility value was recorded in TGX- 2010-11F with a value of 73.3% and the least value of 15.4% was recorded in TGX- 2018-5E. Photomicrographs of both fertile and non-fertile pollens are shown in Fig. 1a-b. For pollen diameter, the result shows significant differences among accessions at a 5% level of significance with accession TGX-2007-3F having the highest value of 0.27 and accession TGX-1989-19F having the least value of 0.21. However, there were no significant difference in accessions TGX-2018-5E, TGX-1988-5F, TGX-1485-1D, TGX-2025-8E, TGX-2010-11F, TGX-2027-1E, TGX-2023-4E, TGX-2017-6E, TGX-1987-62F, TGX-2004-9F, TGX-2011-6F, TGX-2016-4E, TGX-2008-4F, TGX-1488-2E and TGX-2019-1E (Table 2).

Molecular characterisation of selected soybean genotypes: Table 3 shows that only three out of the six primers of SSR markers produced a total number of 12 repeatable and scorable polymorphic bands. DNA fragments ranged from 150-320 bp (Fig. 2). In Fig. 2a, Primer satt 288 was fragmented at 200 bp, in Fig. 2b, Primer 294 was fragmented at a little above 300 bp while in Fig. 2c, Primer 222 fragmented at about 200 bp. The number of alleles per locus was within 1-3 with an average of 2.0. Although Satt 596, Satt 572 and Satt 194 did not produce any polymorphic band, Satt 294, Satt 288 and Satt 222 were highly polymorphic, each producing three different alleles. Out of the six markers, Satt 294, Satt and Satt 222 were considered suitable markers for detecting genetic diversity among individuals each having

Table 2: Pollen viability and diameter of the soybean accessions

	Pollen parameters					
Varieties	Total pollen counts	No. of fertile pollens	No. of sterile pollen	Pollen viability (%)	Pollen sterility (%)	Pollen diameter (μ)
TGX-2018-5E	130.00	110.00	20.00	84.60	15.40	0.24 ± 0.00^{bc}
TGX-2023-4E	190.00	110.00	80.00	57.90	42.10	0.24 ± 0.00^{bc}
TGX-1988-5F	200.00	150.00	50.00	75.00	25.00	0.24 ± 0.00^{bc}
TGX-1485-ID	230.00	190.00	40.00	82.60	17.40	0.25 ± 0.00^{d}
TGX-2017-6E	90.00	70.00	20.00	77.80	22.30	0.24 ± 0.01^{bc}
TGX-2019-IE	110.00	80.00	30.00	72.70	27.30	0.24 ± 0.00^{bc}
TGX-2025-8E	170.00	120.00	50.00	70.60	26.30	0.24 ± 0.00^{bc}
TGX-1448-2E	21.00	16.00	50.00	76.20	23.80	0.25 ± 0.00^{d}
TGX-2008-4F	50.00	30.00	20.00	60.00	40.00	0.25 ± 0.00^{d}
TGX-2016-4E	170.00	140.00	30.00	82.40	18.80	0.25 ± 0.00^{d}
TGX-2010-11F	300.00	80.00	220.00	26.70	73.3	0.24 ± 0.00^{bc}
TGX-2027-IE	220.00	160.00	60.00	72.70	27.3	0.24 ± 0.00^{bc}
TGX-2009-11F	130.00	100.00	30.00	76.90	23.10	0.23 ± 0.01^{b}
TGX-1989-19F	110.00	50.00	60.00	45.50	54.54	0.21 ± 0.00^{a}
TGX-1987-62F	80.00	50.00	30.00	62.50	37.5	0.24 ± 0.01^{bc}
TGX-2007-3F	150.00	100.00	50.00	66.70	33.30	0.27 ± 0.01^{e}
TGX-2004-9F	100.00	70.00	30.00	70.00	30.0	0.24 ± 0.00^{bc}
TGX-2022-4E	120.00	80.00	40.00	66.70	33.40	0.25 ± 0.01^{d}
TGX-2011-6F	210.00	150.00	60.00	71.43	28.57	0.25 ± 0.00^{d}

Values under pollen diameter are Mean±S.E of means and values followed by the same letter(s) on the column do not significantly as tested by DMRT

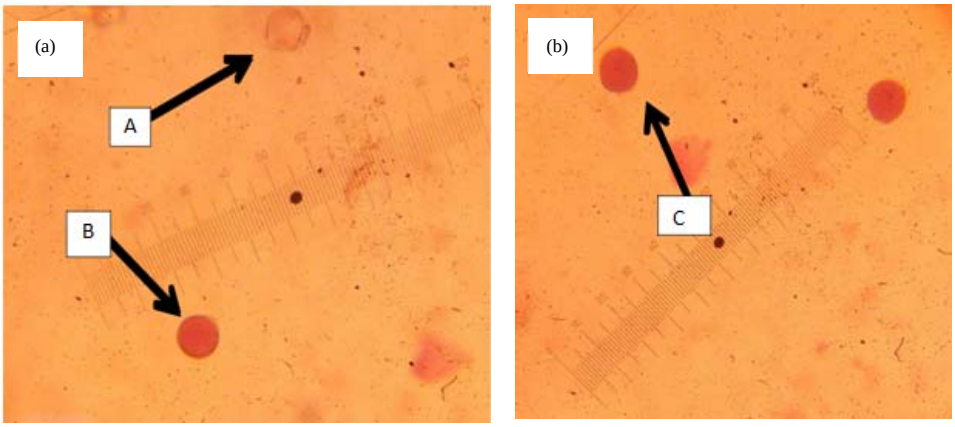

Fig. 1(a,b): Photomicrographs of both fertile and non-fertile pollens, (a) Black arrow on A points to non-fertile pollen, the black arrow on B points to fertile pollen and (b) Black arrow on C also points to fertile pollen

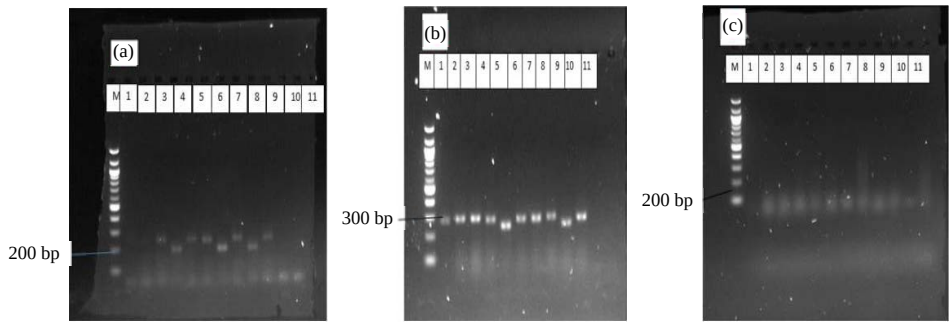

Fig. 2(a-c): SSR amplification of the DNA primers, (a) Primer satt 288, (b) Primer 294 and (c) Primer 222

Table 3: Data matrix of SSR-PCR primers for the selected soybean genotypes

596	572	294	288	194	222
0	1	1/0	0/0	0	1/0
0	1	1/0	0/0	0	0/1
0	1	1/0	1/0	0	1/1
0	1	1/0	0/1	0	1/0
0	1	0/1	1/0	0	1/1
0	1	1/0	1/0	0	1/0
0	1	1/0	0/1	0	0/1
0	1	1/0	1/0	0	1/0
0	1	0/0	0/1	0	1/0
0	1	1/0	1/0	0	1/0
0	1	0/0	0/0	0	0/1

high gene diversity values (Table 3). The highest and lowest gene diversity values were determined as 0.6446 and 0.4298 for Satt 288 and Satt 294, respectively with an average of 0.2782. Polymorphic information content (PIC) values were within average ranging from 0.3855 to 0.5721 with an average of 0.2473 (Table 4).

The evolutionary relationship inferred using the Neighbor-Joining method shows the optimal tree with the sum of branch length of 1.63685646. Based on the phylogenetic dendrogram as presented in Fig. 3, the tree generated consisted of 2 major clades with each claude consisting of branches. The major claude has 3 branches with TGX-2016-4E clustering with TGX-2017-6E and TGX-2019-1E clustering with TGX-1987-62F. The major branch on the other hand consists of a close cluster consisting of TGX-2007-3F and TGX-2010-11F and both related to TGX-1448-2E. The second claude consists of a close cluster between TGX-2023-4E and TGX-2018-5E which are both closely related to TGX-2011-6F and TGX-2022-4E (Fig. 3).

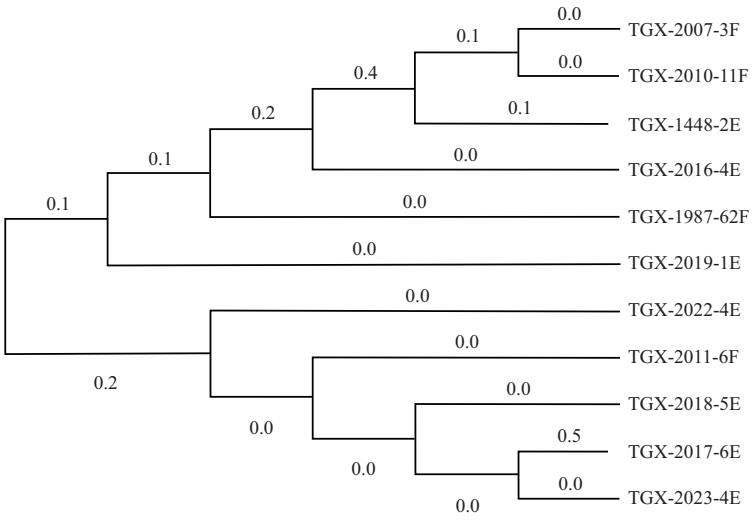

Fig. 3: Dendrogram based on UPGMA analysis of genetic dissimilarity of soybean genotypes, showing the relationship among them

Table 4: SSR molecular parameters detected by different DNA primers

Markers	Major allele frequency	Sample size	Allele No.	Availability	Gene diversity	PIC
Satt 596	1.0000	11.0000	1.0000	1.0000	0.0000	0.0000
Satt 572	1.0000	11.0000	1.0000	1.0000	0.0000	0.0000
Satt 294	0.7273	11.0000	3.0000	1.0000	0.4298	0.3855
Satt 288	0.4545	11.0000	3.0000	1.0000	0.6446	0.5721
Satt 194	1.0000	11.0000	1.0000	1.0000	0.0000	0.0000
Satt 222	0.5455	11.0000	3.0000	1.0000	0.5950	0.5262
Mean	0.7879	11.0000	2.0000	1.0000	0.2782	0.2473

PIC: Polymorphic information content

DISCUSSION

High fertility and viability observed in this study buttress the fact that they are a good source of hybridization for crop improvement. Filho *et al.*[18] also reported similar findings in tomatoes. The pollen diameter from this study ranges from 0.21-0.27. This result shows that the characters are fairly uniform within the accessions. This result is in contrast to the work of Filho *et al.*[18] who reported a range of 1.93-2.57. These variations could be attributed to the difference in a sample. According to Animasaun *et al.*[19] utilization of the knowledge of pollen viability as a selection criterion for high yield could provide vital information for an effective breeding programme.

SSR markers are efficient for measuring genetic diversity and relatedness as well as identifying varieties of soybean[20]. The low mean polymorphic information content (PIC) of 0.25 obtained for all the SSR markers used indicated the ineffectiveness of the markers used on the selected soybean accessions. Ahmad *et al.*[21] reported that marker is effective if the PIC value is higher than 0.5. The range of polymorphic information content 0.38-0.57 recorded in the present study is slightly similar to the work of Sudeshina *et al.*[22], who reported PIC range from 0.4-0.9 on 40 genotypes using 34 primers, similarly, Ghosh *et al.*[23] also reported arranged of 0.1-0.4 for 32 genotypes using 10 primers. The slight variations could be attributed to the number of genotypes and primers used. The high number of alleles and high polymorphism is very important for correct estimation of genetic diversity and effectiveness of marker development[24]. The allele number in this study ranges from 1-3 with an average of 2.0 indicate the highly specific and reproducible nature of the SSR markers used and also showing that genetic variability exists among the studied soybean samples. Koutu *et al.*[25] reported an allele range of 2-6 with an average of 4.0. Similarly, Kumar *et al.*[26], Singh *et al.*[27] and Bisen *et al.*[28] also had a range of 2.0- 6.0 with an average of 4.0.

The gene diversity obtained in this present study (0.0-0.6) signifies high genetic diversity among the chosen genotypes. This result is following the findings of Ghosh *et al.*[23] 0.0-0.63 and Chauhan *et al.*[29]. The clustering of the genotypes into two major clades indicates that most of them are similar in their genetic constitution and geographical origin. Thus, implying that some of the variations observed among the genotypes

could have arisen due to environmental and or edaphic factors which manifested in the phenotypic differences observed in the crop. Similarly, distance-related genotypes could be the best candidates for hybridization rather than closely related genotypes.

CONCLUSION

The high pollen viability observed in genotypes TGX-2018-5E, TGX-1485-1D and TGX-2016-4E could result in high seed yield, thus, they can serve as a source of a good candidate for hybridization for higher yield. The high polymorphic information content (0.53 and 0.57) produced by primers 222 and 288, respectively indicates their reliability and effectiveness in the determination of diversity among soybean accessions. This further buttressed the variation detected by agro morphological parameters.

SIGNIFICANCE STATEMENT

The study established that a high level of genetic variability occurred among soybean genotypes. The range of pollen diameters of the soybean genotypes from 0.21-0.27 µ as well as the variation in their pollen fertility and sterility further established this variation. Similarly, the SSR molecular further buttress the genetic variability that exists among them by grouping the genotypes in separate clusters. Thus, existing of genetic variability in this crop is a veritable tool for the improvement of the crop in the study area.

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