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Assessment of phenotypic and genetic variation against pod borer among a subset of elite pigeonpea (*Cajanus cajan*) genotypes in Kenya

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Abstract

Molecular marker information supported by quality morphological data facilitates the choice of suitable parents for applied breeding. The main aim of this study was to assess genetic diversity among 55 cultivated yield elite pigeonpea lines using 21 simple sequence repeat (SSR) markers that are well distributed across the genome. Among the 55 pigeonpea genotypes, 16 medium duration were selected and evaluated in the field for response to pod borer resistance in varied agro-ecological zones of Kenya during long rains of April-October cropping season Twenty one primer pairs detected 80 alleles with a mean of 3.9 alleles per locus and polymorphism information content (PIC) ranging from 0.09 to 0.75 averaging to 0.39 suggesting a low genetic diversity. However, marker CcM1820 revealed the highest number of alleles (9) with a PIC value of 0.75. The genotype response to pod borer attack was significant ($P \leq 0.05$) with three genotypes (ICEAPs 01541, 01154-2 and 00902) revealing tolerance to pod borer. The markers based on Neighbor Joining, grouped the 55 genotypes into three main clusters based on parentage selection. Most genotypes developed from ICEAP 00068 as the maternal parent were grouped in Cluster I while cluster II comprised of improved genotypes and cluster III comprised genotypes developed from ICPL 87091 as maternal parent. The resistant genotypes identified in the field experiment were grouped in cluster I except ICEAP 00902 which grouped in cluster II. Future studies should focus on broadening genetic base by including more landraces and wild relatives to maximize selection and improve breeding work.

Keywords: Pod borer; genetic diversity; resistance; polymorphism information content; SSR markers; AMOVA.
Abbreviations: N_A _Total number of Alleles; C_A_Common alleles; R_A _Rare alleles; A_A_Abundant allele; S_A_ Specific allele; PIC_ Polymorphism information content.

Introduction

Pigeonpea [*Cajanus cajan* (L.) Millsp.] (2n=2x=22) is a legume crop majorly grown in semi-arid tropics (Saxena et al., 2002). It is the third most important grain legume worldwide and ranks second to field beans (*Phaseolus vulgaris*) in Kenya in both acreage and production. Globally, India is the main pigeonpea producer in Asia with 62.7% of total production while in Africa, it is grown in more than 33 countries with a high production from Malawi (237.210t), (FAOSTAT, 2015). In Kenya, it is grown mostly in Eastern regions mainly Machakos, Kitui and Makueni County under a total area of 184,500 ha with a production of 111,000MT. However, about 62% of total production of pigeonpea is marketed and the rest (38%) is locally consumed in the households. This has provided cash opportunities to farmers through its increased export potential. Pigeonpea is a drought tolerant crop that is able to give grain yield during dry spell. It is highly nutritive comprising of proteins, essential amino acids, vitamins and minerals making it the best solutions to protein-calorie malnutrition in the developing world and a source of dietary protein mainly in vegetarian based diets (Chitra et al., 1996) It is able to fix Nitrogen in the soil of about 40kg per season (Saxena et al., 2002) and access bound phosphorus in the soil due to presence of piscidic acid exudates that solubilize phosphorus in the rhizosphere (Rao et al., 2001). Pigeonpea is largely a self-pollinated crop though some are cross pollinated (20-30%). The outcrossing nature of pigeonpea depend on flower type, abundance of insect pollinators and weather conditions during flowering (Pando et al., 2011). It has a diploid number of chromosome 2n=2x=22 and genome size of 833.1 Mbp (Varshney et al., 2012). Insect pests are among the major constraints to pigeonpea production in Eastern Africa especially pod borer (*Helicoverpa armigera)*, pod fly (*Melanagromyza cholcosoma*) and sucking bug (*Clavigralla tomentosicollis*) (Minja et al., 1999) which substantially damage the crop and result to significant economic losses (Choudhary et al., 2013). Field evaluation of genotypes for response to insect pests can give breeders information on host plant resistance but is not sufficient in providing the extent of genetic diversity that is required in

the field for improved productivity that results from selection. The extent of genetic diversity can be determined using molecular markers, which have recently been made available in pigeonpea (Odeny et al., 2009;Yang, et al., 2006 and Saxena et al., 2014). Simple sequence Repeat (SSR) markers, in particularly, have remained the markers of choice, especially in the developing world, as they are highly polymorphic, co-dominant, abundant in most species and are randomly distributed across the genome (Odeny et al., 2009).An understanding of the distribution of genetic diversity is essential for both utilization, production, improvement, promotion and conservation strategies (Songok et al., 2010). The present study therefore, seeks to assess genetic diversity using 21 SSR markers among 16 yield elite pigeonpea genotypes alongside 39 breeding lines from International Crops Research Institute for the Semi-Arid Tropics (ICRISAT).

Results and discussion

Phenotypic data on mechanisms of pod borer resistance

The field experiment revealed significant ($P \leq 0.05$) phenotypic differences among the pigeonpea genotypes in the study in terms of all the measured parameters (**Table 1**). The genotypes responded differently to the insect pest attack. Three genotypes (ICEAPs 01541, 01154-2 and 00902) recorded low damages in both pod and seed damage across the three sites in comparison to the resistant check (00850^{R}) and rated resistant to pod borer (**Table 1**).

The variations in incidence and severity of the pest attack among the pigeonpea genotypes may be attributed to host plant resistance. This may be contributed by presence of morphological traits or biochemical factors that influence selection or preference of pod borer among genotypes. Cheboi et al., 2016 reported potential yield among seven genotypes with low pod and seed damage. Similarly, Pod borer damage was high in Marigat (hotspot area) compared to other studied areas. This site is characterized by high temperatures and low rainfall hence a favorable environment for pod borer. Therefore, identification of tolerant pigeonpea genotypes is an immense opportunity for enhancing the production of pigeonpea by small scale farmers in Kenya who are characterized with limited resources. Such farmers would not be able to provide inputs like conventional pesticides and availability of tolerant lines could be advantageous since they will be able to maintain large plantations, use less agro- chemicals, higher quality seeds and less environmental pollution.

Allelic diversity

All the 21 SSR markers evaluated (**SupplementaryTable1**) were polymorphic with polymorphism information content (PIC) ranging between 0.09 and 0.75 (**Table2)**. Out of the 21 SSR markers, only 10 (48%) primer pairs were found to be highly polymorphic across the genotypes with polymorphism information content ranging from 0.44 to 0.75. However, 11(52%) of the markers showed low polymorphism with PIC values below 0.40 (**Table2**). The markers generated 80 alleles in total with a mean of 3.8 alleles per locus. Marker CcM1820 revealed the highest number of alleles (9) with a PIC value of 0.75.The markers generated PIC values ranging from 0.09 to 0.75 with an average of 0.39 (**Table2**) .The 21 SSR markers revealed 23 total number of rare alleles, 39 common alleles and 18 abundant alleles (**Table3**). Eight genotypes (ICEAPs 01181, 01154/2, 00936, 00902, 00554, 001150, ICPL 86012 and ICP 7035W) showed single allele (**Table3**).The study identified five primers (CcM0594, CcM1232, CcM0603, CcM2049 and CcM1506) recognizing specific alleles in the eight genotypes (**Table3**).

The generated mean numbers of 3.9 alleles per locus are similar to 3.10 reported by (Burns et al.,2001) in earlier diversity study on cultivated pigeonpea species based on 10 polymorphic markers. The low number of alleles and low number of alleles per locus indicate narrow genetic diversity among the pigeonpea genotypes studied which is also reported by Odeny et al. (2009) whose study revealed 110 total number of alleles with an average of 3.14 alleles per locus. These results are lower than 8 alleles reported by (Songok et al., 2010).The high number of alleles per locus recorded by Songok et al. (2010) might have been contributed by wild relatives and landraces included in the study which have been reported to have wide genetic diversity compared to -this study which were used elite lines that were selected specifically for yield. Odeny et al. (2009) used higher number of SSR markers (113) in her study which lead to the higher number of alleles.

PIC values measure the usefulness of each marker in distinguishing one individual from another. This study revealed PIC values ranging from 0.09 to 0.75 with Marker CcM1820 revealing the highest number of alleles (9) with higher PIC value of 0.75.The polymorphism is attributed by number of alleles present at a locus and their frequency of distribution (Songok et al., 2010).The larger number of rare alleles reported in this study is basically due to the natural outcrossing nature of pigeonpea hence difficult to maintain homozygosity among pigeonpea populations. Five improved genotypes revealed specific alleles showing their uniqueness in their performance. The five primers that identified the specific alleles are of great importance in establishing unknown traits.

Genetic relationships in pigeonpea

The dendrogram based on weighted Neighbor Joining grouped the genotypes into three main distinct clusters (cluster I, II and III) **Figure 1**. The clustering was based on parental selection. Thirty two genotypes clustered in Cluster I while 14 genotypes grouped in cluster II and 9 in cluster III. Most of the improved genotypes clustered in cluster II while genotypes developed from crossing with ICEAP 87091 were grouped in cluster III and cluster I constituted genotypes developed from a cross with ICEAP 00068 (**Figure 1**).

The cluster number is higher than two clusters reported earlier by Songok et al. (2010) based on agro climatic regions and state of origin using RAPD markers and SSR markers respectively. The consistency of these genotypes clustering in the three clusters explains the viability of genes being passed from one generation to another. It also explains the high contribution of the female parent in genetic makeup of progenies. The clustering is very important in future pigeonpea breeding program through selection of diverse parental lines.

Table 1. Table of means for % pod damage, % seed damage and resistance rating for pod borer among 16 pigeonpea genotypes in the three study sites during April-October 2014 cropping season.

% Pod damage				% Seed damage			
Genotype	Marigat	Koibatek	Fluorspar	Marigat	Koibatek	Fluorspar	Rating
ICEAP 01147	11.5h	0d	31a	30.2f-h	2.5b-d	2.7df	S
ICEAP 01179	24.2b-d	0d	14.9c	31.1fg	2.3c-e	2.8d--f	S
ICEAP 1147-1	21.3c-f	1.1c	12.6c	38.7cd	1.8e-g	0.0f	S
ICEAP 01159	39.3a	0d	0.0d	57.7a	0.7jk	1.8d-f	S
ICEAP 00554	22.1c-e	0d	0.0d	35.3e	2.2ce	4.9cd	S
ICEAP 01541	16.1e-g	0d	0.0d	26.9i	1.6gh	1.1d-f	R
ICEAP 00540	21c-f	0d	22b	39.8c	1.6gh	1.8d-f	S
CEAP 00911	19.3d-g	2.5b	0.0d	27.4g-i	1.7fg	11.9a	S
ICEAP 00902	15.8e-g	0d	0.0d	29.9f-h	0.9ij	0.0f	R
ICEAP 01150	22.3c-e	0d	0.0d	42.7c	1.1h-j	2.9d-f	S
ICEAP 00068	20.3d-f	0d	0.0d	40c	2.7bc	11.4ab	S
ICEAP 00557	28.6bc	0d	0.0d	33.5ef	1.4h-j	3.9c-e	S
KAT 60/8^{S}	25bcd	3.4a	29.7a	55.8a	4.6a	7.6bc	S
ICEAP 00850^{R}	15.2e-g	0d	0.0d	33.7ef	1.3h-j	0.7ef	R
ICEAP 0979-1	30.5b	0d	30.9a	47.1b	3b	1.1d-f	S
ICEAP 1154-2	13.7gh	0d	0.0d	27.5hi	0.2k	0.0f	R
Genotype	**	**	**	***	***	***	
Grand mean	21.6	0.4	11.6	37.2	1.9	3.6	
CV%	21.7	34.1	14.8	6.4	16.2	65.5	
LSD	7.83	0.25	2.8	4	0.5	3.9	

Key: Means followed by a different letter within a column are significantly different ($P \leq .05$) where $P \leq .05$=*; $P \leq .01$=** and $P \leq .001$=*** as analyzed by least significant different test (lsd). Genotypes with superscript (S and R) are susceptible and resistant checks. NS=not significant; CV= coefficient variation and LSD- least significant difference.

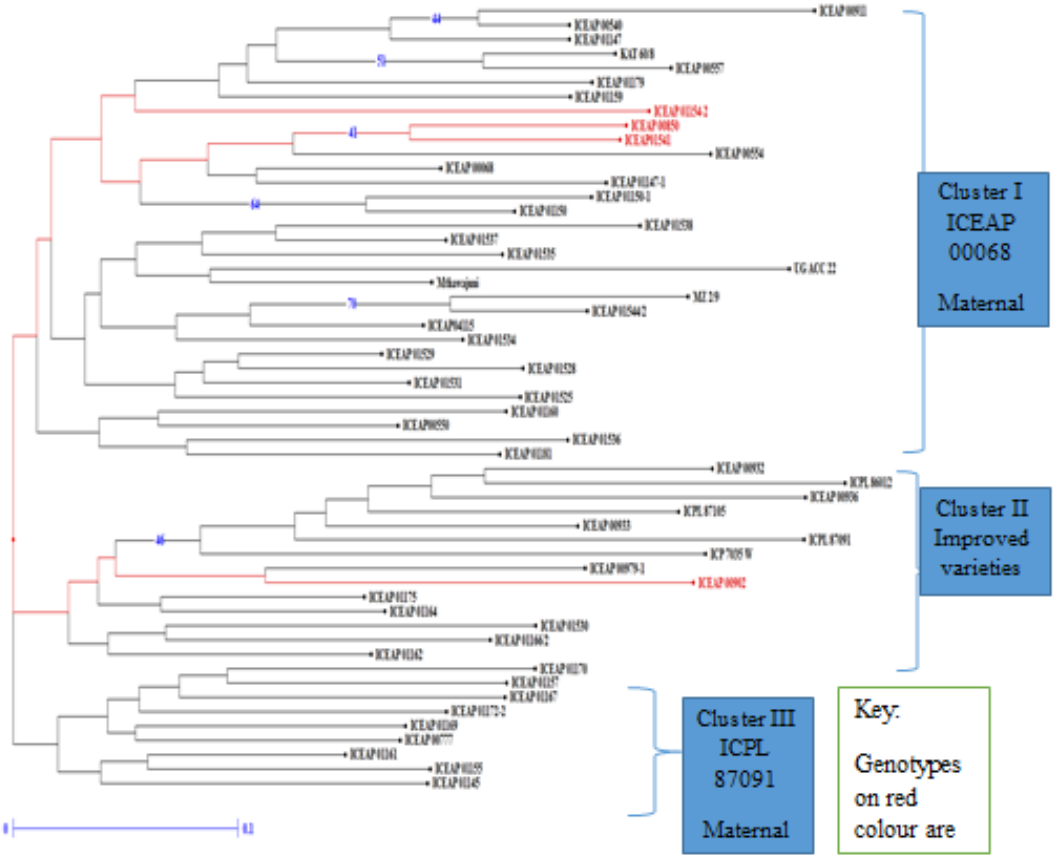

Fig 1. Dendrogram of 55 pigeonpea genotypes showing different clusters based on parentage selection as revealed by 21 SSR markers.

Table 2. Number of alleles, gene diversity and Polymorphism information content among 55 pigeonpea genotypes as revealed by 21 SSR markers.

Marker	Major.Allele.Frequency	Allele No.	Gene Diversity	Heterozygosity	PIC
CcM0047	0.53	2	0.50	0.00	0.37
CcM0444	0.95	2	0.10	0.00	0.09
CcM0594	0.80	3	0.33	0.11	0.28
CcM0602	0.72	4	0.45	0.15	0.41
CcM0603	0.57	4	0.56	0.18	0.48
CcM1014	0.63	5	0.55	0.16	0.51
CcM1139	0.52	3	0.55	0.20	0.44
CcM1232	0.82	3	0.31	0.00	0.27
CcM1348	0.46	8	0.70	0.02	0.61
CcM1373	0.38	4	0.68	0.00	0.62
CcM1447	0.53	3	0.54	0.22	0.43
CcM1493	0.87	2	0.22	0.00	0.19
CcM1506	0.72	4	0.43	0.09	0.37
CcM1582	0.71	4	0.44	0.00	0.38
CcM1598	0.86	3	0.24	0.02	0.22
CcM1611	0.52	2	0.50	0.05	0.37
CcM1820	0.33	9	0.79	0.11	0.75
CcM1825	0.87	3	0.22	0.00	0.21
CcM2044	0.68	4	0.49	0.05	0.44
CcM2049	0.45	5	0.69	0.00	0.64
CcM2332	0.95	3	0.10	0.00	0.10
Mean	0.66	3.8	0.45	0.06	0.39
Total	13.9	80	9.37	1.36	8.27

Table 3. Description of number of total, rare, common, abundant and single alleles based on 21 SSR markers.

Marker	N_A	R_A (<5%)	C_A (5-50%)	A_A (>50%)	S_A
CcM0047	2	0	0	2	
CcM0444	2	0	1	1	
CcM0594	3	1	1	1	43
CcM0602	4	1	2	1	
CcM0603	4	1	2	1	26
CcM1014	5	2	2	1	
CcM1139	3	0	3	0	
CcM1232	3	1	1	1	19 &21
CcM1348	8	5	3	0	
CcM1373	4	0	4	0	
CcM1447	3	0	2	1	
CcM1493	2	0	1	1	
CcM1506	4	2	1	1	8, 21 & 22
CcM1582	4	2	1	1	
CcM1598	3	0	2	1	
CcM1611	2	0	1	1	
CcM1820	9	4	4	1	
CcM1825	3	1	1	1	
CcM2044	4	0	3	1	
CcM2049	5	1	4	0	4 & 9
CcM2332	3	2	0	1	
Mean	3.8	1.09	1.85	0.85	
Total	80	23	39	18	

Key; N_A .Total number of Alleles; C_A-Common alleles; R_A -Rare alleles, A_A-Abundant allele; S_A- Specific allele in particular genotypes.
Names of the genotypes are found in Supplementary Table 1.

Table 4. Analysis of molecular variance (AMOVA) within and among populations.

Source of variation	Sum of squares	Variance components	Percentage variation	P value
Among populations	61.088	0.64943	12.99503	0.05
Among individuals within populations	416.749	3.66629	73.36190	
Within individuals	37.50	0.68182	13.64307	
Total	515.336	4.99754		

Table 5. Summary of computation among pigeonpea populations.

STATISTICS	Population I	Population II	Population III	Mean	Sd.
No. of gene copies	48	42	20	36.67	14.7
No. of gene loci	21	21	21	21.00	0.00
No. of usable loci	21	21	21	21.00	0.00
No. of polymorphic loci	21	16	16	17.67	2.89
H	0.81	0.59	0.55	0.65	0.13
Obs .Het.	0.03	0.09	0.17	0.09	0.12
Exp. Het.	0.45	0.49	0.47	0.47	0.17
No. of alleles per locus	3.2	2.8	2.2	2.7	0.50

Genetic diversity within and among pigeonpea populations

The three clusters generated based on the parental selection were treated as different populations. Results for analysis of molecular variance (AMOVA) revealed significant (P<0.05) low genetic variation among the three populations with percentage variation of 12.9%. However, high variation among individual genotypes within the populations with a variation of 73% was shown. On the other hand, a low significant (P<0.05) variation within the 55 individuals was observed at 13.6% variation (**Table 4**).

Population I reveals greater diversity followed by population II and finally Population III. The high variation in population I is attributed to large number of gene copies, number of alleles per locus and number of polymorphic loci revealed in the study (**Table 5**). In natural populations, heterozygosity is an important parameter for measuring genetic variation as it tells about the evolutionary structures of a population (Songok et al., 2010). The low variations observed within individuals and populations may be attributed to the low observed heterozygosity which results to low genetic variability due to parental selection for yield improvement. Heterozygosity measures the genetic variation at a locus which is also contributed by number of alleles at a locus and number of polymorphic loci.

The high variations revealed among individuals within populations (**Table 4)** might have been contributed by differences in number of gene copies, number of alleles per locus and number of polymorphic loci. Population I was mostly occupied by landraces and some improved varieties; therefore, much of the variations observed here were due to higher number of alleles revealed in this population compared to the other two populations.

High levels of inbreeding reduce heterozygosity hence reducing genetic variability. This was revealed within the 55 pigeonpea individuals which are yield elites selected based on yield performance, farmers taste and preference. These genotypes were developed by making a cross between two lines with desirable agronomic traits and maintaining population within the population to maintain integrity of the genotypes. High levels of inbreeding have been reported in pigeonpea (Ratnaparkhe et al., 1995)

Materials and Methods

Composition of the study

The study comprises of two experiments namely, field, and laboratory experiments. Phenotypic data collected from the field experiment was used to back-up molecular data from the laboratory.

Field experiment

Plant material and study sites

Sixteen medium duration pigeonpea genotypes sourced from ICRISAT were selected among 55 yield elite lines and evaluated for resistance to pod borer (*Helicoverpa armigera*) during long rains of April- October 2014 cropping season (**Supplementary Table 1**). Two among the 16 genotypes ICEAP 00850 (resistant) and KAT 60/8 (susceptible) are commercial varieties and were used as checks. The genotypes were planted at Kenya Agricultural and Livestock Research organization (KALRO) Marigat, Agricultural Training College (ATC) Koibatek and Fluorspar-Chepsirei. KALRO is located at an altitude of 1067meters above sea level, 0°28'0" N and 36°1'0" E with an annual rainfall of 654mm. ATC Koibatek is situated 1°35'S, 36 °66'E at an elevation of 1890 meters a.s.l with an annual rainfall of 767mm while Fluorspar is located at an altitude of 1200 m with mean annual rainfall of 400-800 mm.

Experimental design, data collection and analysis

The experiment was laid out in a randomized complete block design replicated three times with five rows per each plot. Each experimental plot measured 4m by 3m and the genotypes were randomly assigned to entire plots in each block with in the replication. All genotypes were sown in five

rows with inter row spacing of 75 cm and intra spacing of 25 cm. Five plants per plot were randomly tagged for evaluation. Data was collected on % pod damage by pod borer on green pods and dry seed. The severity of the pod borer damage was rated based on 1-9 resistance scale where 1= highly resistant, 6 = check and 9 = highly susceptible**.** Data was analyzed statistically using SAS version 9.1 and subjected to analysis of variance (ANOVA) with the means of treatments separated using Fishers' least significant difference test (lsd).

Laboratory experiment

DNA extraction

Fifty five pigeonpea elite lines selected based on yield performance were obtained from ICRISAT Nairobi. The molecular assessment was conducted at the International Crops Research Institute for the Semi-Arid Tropics- ICRISAT laboratory- Nairobi. Three seeds of each of the 55 genotypes were planted in root trainers measuring 402 *600cm filled with soil sourced from Karura forest in Nairobi. After germination, thinning was done in order to maintain two plants per pot per genotype. Fresh leaf tissues weighing 100-150gwere harvested from two weeks-old seedlings and used for total genomic DNA extraction using CTAB protocol (Mace et al., 2003).The quantity and quality of the DNA samples was checked using the spectrophotometer at absorbance ratio 260nm:280nm and running on 0.8% (w/v) agarose gel stained with 5ul/100ml Gel Red $^{(R)}$ (Biotium inc. USA) across a standard λ DNA. Then the genomic DNA was diluted to a concentration of 20ng/µl for polymerase chain reaction (PCR).

Primer selection, amplification and optimization

Forty SSR primer pair that were well distributed across the 11 chromosomes of pigeonpea were selected from a consensus map and from these, 21 primers were selected for this study (Supplementary Table 2) based on genome position, repeat size, reported polymorphism and number alleles (Bohra et al., 2012). The forward primer for each of the SSR makers were labeled at the 5' end of the oligonucleotide using florescent dyes to enable detection by the automated sequencer ABI 3730 genetic analyzers (Applied Biosystems). PCR reaction was performed in 10 µl final volume in a mixture containing 2 µl 5x My Taq reaction buffer (5Mm dNTPs, 15Mm $MgCl_2$, stabilizers and enhancers), 0.9 µl dye, 0.6 µl forward primer, 1.5 µl reverse primer, 0.04 µl My Taq polymerase, 2.96 µl double distilled water and 10ng/µl of template DNA. The primers were optimized using touch down PCR amplification procedure. The reaction had initial denaturation step at 95^0c for 5 min, followed by 1 cycle of 95^0c for 1 min, 8 cycles of 60^0c for 30 sec and 28 cycles of52^0c for 45 sec. The final extension step was at 72^0c for 12 min and a holding temperature of 15^0c. Successful amplification of products was confirmed on 2% (w/v) agarose gels stained with 5ul/100ml Gel Red $^{(R)}$ (Biotium inc. USA) across 100bp molecular size ladder.

Fragment analysis

Fragment analysis was performed on the ABI 3730 sequencer machine. Peak sizing and calling of alleles based on highest relative fluorescent unit (RFU) was done using Gene Mapper ver. 4.0 software (Applied Biosystems).Power Marker ver. 3.25 program was used to generate summary statistics such as allele number, gene diversity, polymorphic information content (PIC), heterozygosity and number of major alleles. Polymorphic information content was calculated using the formula;

$$PIC = 1 - \sum_{i=0}^{k} p_i^2 - \sum_{i=1}^{k-1} \sum_{j=i+1}^{k} 2\, p_i^2 p_j^2$$

Where, p_i and p_j are the frequencies of alleles *i* and *j*, respectively.

Phylogenetic analysis

DARwin software was used to generate phylogenetic information by calculating dissimilarity using the formulae below;

$dij = 1 - \frac{1}{L}\sum_{l=1}^{L} m1/\pi$.

Where *dij*is the dissimilarity between units *i* and *j*, *L* is the number of loci, π is the ploidy and *ml* is the number of matching alleles for locus *l*. The software was also used to display dendrogram using the dissimilarity matrix calculated by simple matching. The distances were computed for microsatellite data and trees constructed using the neighbor- joining method. The strength of the clusters was evaluated by bootstrap analysis using the PowerMarker software. Genetic distance between the populations was estimated by F_{ST} statistics based on (Wright, 1951) method;

$F_{ST} = (F_{IT} - F_{IS}) / (1\text{-}F_{IS})$

Where,

F_{ST} is the fixation index describing the correlation of genes of different individuals in the same population;

F_{IS} is the inbreeding coefficient, describing the correlation of genes within individuals in the population;

F_{IT} is the overall inbreeding coefficient, describing the correlation of genes within individuals relative to the total population.

Based on the clusters, variation among the populations was analyzed by analysis of molecular variance (AMOVA) using Arlequin version 3.11software. Pair-wise relatedness among the populations was calculated as genetic distances.

Conclusion

The study revealed narrow genetic diversity among the 55 pigeonpea genotypes. This is indicated by the low number of alleles and the three clusters generated. To maximize selection, future breeding strategies should focus on broadening genetic base by including wild relatives and land races in the study. SSR markers revealing greater than 4 numbers of alleles per locus can be used for future genotyping and diversity studies since they are able to separate the genotypes efficiently.

The three identified genotypes with potential tolerance to pod borer can be utilized as sources of resistance and can be explored and used in breeding programs for development of resistant lines in Kenya.

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