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Morphological and molecular diversity analyses of high biomass sorghum (*Sorghum bicolor* L. Moench)

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Abstract

Smart crop sorghum [*Sorghum bicolor* L. Moench] is the 5th most significant grain crop grown around the globe in marginal land supplementing feedstuff, biofuel and fodder apart from food. Sorghum is considered as an ideal candidate for bioenergy production due to its small genome size, drought tolerance, increased production and potential as biofuel crop. For biomass studies Sorghum germplasm has a higher genetic diversity at molecular and morphological levels so markers assisted breeding has broad applications. To identify genomic diversity of sorghum, SSR markers can be used in breeding techniques. In current research we used morphological characters and molecular markers for evaluating the sorghum genotypes and potential of sorghum to produce a higher yield of biomass. The phenotypic data of diverse germplasm of sorghum for four traits including plant height, stem thickness, number of nodes and internodal length was recorded. PCA analysis specified highly significant differences among all the traits. These traits showed positive correlation and thus used for efficient screening of best performing sorghum accessions. The molecular screening of selected sorghum genotypes was done by using ten SSR primers. The number of alleles per locus ranged from 1 to 2 and the allelic polymorphism information content (PIC) value ranged from 0 to 0.3750 for the Xtxp101. The results revealed that sorghum genotypes differed for morphological characters and this diversity was also indicated by SSR markers. In breeding programs these genomic groups of sorghum may be used for development of efficient energy and high biomass of sorghum.

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Introduction

Sorghum

Sorghum is the earliest multipurpose annual grass (Fernanda *et al.*, 2014). It is the 5th most significant cereal crop worldwide. As an effect of increasing food and fuel values globally, smart crops such as sorghum has emerged as a main choice for nourishment, fuel and feedstuff. It is mainly developed for nutrition, feed, dietary fiber and biofuel. Sorghum has a small genome size (~730 Mb) due to which it is preferable over other C-4 crops (Paterson *et al.*, 2009). It is preferably grown in arid and semi-arid areas due to its hot and stress tolerance ability. Sorghum is a saccharine plant which is most efficient accumulator for biomass, providing fuel and food from starch and has likely used as a cellulosic crop.

Origin and domestication

Sorghum is an earliest crop plant which belongs to family *Gramineae*. Its sub family and tribes are Panicoideae and Andropogoneae respectively (Hitchcock, 1971). The crop name is derived from the Italian word which is "sorgo" and come out from Latin word "Syricum" which means the granule in Syria. It is judged that due to the reason of greater diversity of sorghum in Ethiopia and Sudan, sorghum production is very noticeable in Central Africa (Damon, 1962).Major species of grass as well as millet, maize, sugarcane and sorghum area until assumed to share conventional ancestor rice concerning about sixty million ago (Doebley, 1990). Hence, sorghum allocates ordinary features with millet, maize and sugarcane. Sugarcane and sorghum have a close similarity which resulted as divide species about 6 million years back (Al-Janbi *et al.*, 1997).Sorghum genus has the great potential for development because it is extremely diverse in nature (Assar*et al.*, 2005). Sorghum is consider to be launched to Asia, especially in India and China as a consequence of human migration in 1049 B.C (Kimber, 2000). Sorghum is categorized into five vital races named as caudatum, bicolor, durra, kafir and guinea and ten intermediary races (durra-bicolor, caudatum-bicolor, guinea-bicolor, durra-caudatum, guinea-caudatum, kafir-bicolor, guinea-durra, kafir-caudatum, guinea-kafir and durra-kafir) (Harlan and De Wet, 1972) based on panicle form and spikeletmorphology distributed on various geographical regions. These races are consider to be initiated as a consequence of disruptive selection and migration (Doggett, 1965). Durra types are typically originate in African region with no information of their background (De AlencarFigueiredo *et al.*, 2008). The Bicolor race is typically originated in Africa and Asia and explained by open and long inflorescences including the granule at maturity. Kafir is originate in Africa and illustrated by balanced grains with glumes shorter than the granule. Guinea is easily recognized by long wide-open glumes revealing granules at maturity. Granules of the race Caudatum are irregular with pointed bae, turtleback and short glumes.

Cross breed cultivars originate in different geographical regions. Sorghum is greatly adaptable to trivial lands as well as liberal to abiotic stress mainly hot and drought climatic regions than other crop plants (Berenji and Dahlberg, 2004).

Production of sorghum in Pakistan

In Pakistan, the area under sorghum is about 0.41mha with the fodder production of 6.31 ton (Khan *et al.*, 2008). There is a summer fodder shortage in Pakistan and during this time, sorghum is only available fodder to the cattle. In 2015-16, sorghum production was 161,000 tones on an area of 274,000 hectares. As compared to 115,000 tones over an area of 195,000 hectares in 2014/15, depicting a 40 percentage higher sorghum production over the last year (Survey, 2015-2016).

In this regard, nations are devoting greatly in additional energy assets, including biofuels from various sets of food stocks (Paterson *et al.*, 2009). Wide genetic diversity exists in sorghum (Hariprasanna and Patil, 2015). The crop is preferred over maize for biofuel production due to its higher biomass content, high stem sugar substances and additional nutrient use ability (Bekele *et al.*, 2013).Sweet sorghum has sweet juicy stem, producing about 13.2 metric tons of sugars per hectare under

marginal field conditions (Murray *et al.*, 2009). It needs smaller amount of water to produce higher fermentable sugar levels in contrast with corn (Reddy *et al.*, 2013).Nearly4000 genotypes of sweet sorghum are prevalent globally thriving under numerous climatic regions (Ritter *et al.*,2008).

World sorghum production

The United States Department of Agriculture reported that the production of sorghum in 2017 was 12199 thousand metric tons and is expected to increase by 9242 thousand metric tons in 2018.

Importance of plant biomass

It has been known for the last ten years that plant biomass is a renewable and good energy source. More popularity is due to its low cost and least emission of carbon dioxide. Around the world plant biomass contributes 13% in total energy resources. It plays a central role in economy of world. Moreover, the plant biomass usage can make the farmer's income better and it can also improve their ecological and social status (Xiong*et al.*, 2008). The shortage of energy and its safety have considered as a major issue globally. Furthermore, the glasshouse gases are continually damage the atmosphere by the burning of fossil fuels. Global warming and change in atmosphere caused by these air pollutants that are produced in glasshouse through glasshouse gasses. So, dealing with these two main issues eco-friendlier and economically better energy assets have driven considerable attention for usage of plants to produce energy from their biomass. Now a day's plant biomass is used for the production of biofuel and to fulfill energy requirements without damaging the environment. Therefore, sorghum is an important crop that is used for the production of energy and biofuel from its biomass. It has the capability to grow in hot climate and in stress conditions. Various traits of sorghum have been grown on a large area for the production of biofuel and to fulfill energy requirements globally.

Assessment of genetic diversity of sorghum by using microsatellite markers

Morphological markers have been frequently used to screen germplasm collections. However, these markers are significantly influenced by ecological factors (Ayana *et al.*, 2000). Phenotypic characters facilitate the differentiation of germplasm at the species level, however such a method is less reliable (Dhaliwal *et al.*, 2009). Molecular markers, however, offer a reliable tool for germplasm screening and selection. These markers give enhanced mapping resolution in less time (Yu andBuckler, 2006). Presently, different molecular markers have been used, the choice of each marker is based on features like cost-effectiveness, greater yield, research purpose and awareness about the genotype of interest (Ji *et al.*,2004). Among these, microsatellites or Simple Sequence Repeats (SSR) have extensive use in DNA finger printing studies of crop as these are co-dominant, give high outputand are not radioisotope dependent (Massiaggia and Grattapaglia, 2006).

Molecular markers are based on polymorphism of DNA sequences. Different techniques are used to detect the DNA sequences i.e. microarray and southern blotting and PCR. The variations among different organisms can be detected by the changes in the sequence of DNA. To study the evolutionary history, genetic relationship and phylogenetic studies, different molecular markers can be used. These molecular markers are based on the Mendelian framework and agronomic characters of any crop can be improved by using these markers. These markers become a reliable source and are used to improve sorghum by using various breeding techniques.

Molecular markers are proved to be very useful for the improvement of breeding techniques. Various molecular markers are being used to determine the verity among different traits of sorghum.

These markers include SNPs, SSRs, AFLPs, RFLP and microsatellites. Genetic fingerprints are used to detect different genomic traits. To select our desired plant having desired characters, various microsatellite markers have been frequently used. These markers are proved to be very helpful for plant breeders to select the plant of interest having desired characters.

Materials and methods

The molecular and morphological analysis were carried out to assess the genetic diversity of various sorghum accessions in Somatic Cell Genetics Lab and Plant Molecular Biology Lab, Centre of Agricultural Biochemistry and Biotechnology (CABB), University of Agriculture, Faisalabad, during 2017-2018.

Genetic material

In current research thirteen hundred lines of sorghum were sown for genetic diversity analysis. These various genotypes were kindly given by Agriculture Research Services, United States Department of Agriculture (ARS-USDA), USA.

Experimental site and growth conditions

A total of thirteen hundred lines of sorghum were sown for the assessment of genetic diversity. We used Randomized Complete Block Design (RCBD) for the evaluation of three replications of sorghum genotypes and the research was conducted at the fields of University of Agriculture Faisalabad. The recorded monthly temperature and rainfall of 2017 during cultivation of sorghum is graphically shown in Fig. 3 and 4 individually.

Plant material and cultivation

The proposed research was explore USDA high biomass sorghum collections. The sorghum genotypes were screened on the basis of their biomass related traits (given below). The selected genotypes were further assessed for the presence of variations and similarities among the genotypes with the help of SSRs. The genotypes were planted at Directorate of Farms, UAF. Sowing design was RCBD having three repeats. Dibbler method was used for sowing with two seeds of each genotype per hole. Fifteen plants were planted in a line. The morphological data were collected from three random plants per genotype per replication. The best performing plants were selected from each genotype per replication. Phenotypic characters were recorded as emergence percentage (%), days to 50% flowering (days), plant height (cm), number of leaves per plant (n), leaf area (cm), fresh biomass (g), dry biomass (g), and days to maturity (n).

Molecular analysis

Phenotypically screened sorghums will be analyzed at molecular level with SSRs. DNA extraction will be performed using fresh leaves of selected sorghum plants following Khan *et al.* (2004). DNA samples will be checked for quality by electrophoresis and quantity by spectrophotometer and used for further analysis.

Sample collection

Selected genotypes of sorghum were planted in sand for the collection of seeds. After fifteen days of seedling leaves were collected for the extraction of DNA.

DNA extraction

In current research CTAB method was used for the extraction of DNA from leaves (Khan *et al.*, 2004) with a little bit modification. Water bath was used to heat up 2x CTAB solution at 65°C. Fresh leaves were taken and washed by using distilled water. After washing the leaves were grind into small pieces with the help of autoclaved pastel mortars. For grinding and to a make a fine paste of leaves about 500500ul 2X CTAB was mixed with leaf tissues. After grinding the fine paste was shifted to 1.5ml eppendorf tubes. After that the collected fine paste was placed in water bath for 40 minutes at 65°C for incubation. During incubation the fine paste was slightly mixed by inverting eppendorf tubes after each ten minutes. After incubation, five hundred micro-litter chloroform/isoamyl alcohol (24:1) was added in the mixture present in the tube and mixed it slightly to form a suspension. Than the tubes were centrifuged at 12000 rpm for ten minutes to make a supernatant. The upper liquid phase contained the DNA and it was moved to a new 1.5ml eppendorf tube. After that 500ul ice chilled isopropanol was added to each tube, and the tubes were shaken slightly for a few minutes to precipitate the DNA. To get a fine DNA pallet again centrifuged the tubes at maximum rpm. 250-450ul of seventy percent ethanol was added into pallet for washing and the supernatant was removed. After removing supernatant the pellet was again

centrifuged for one minute at 12000 rpm to get a fine pellet of DNA and allowed it to dry. 100µl of distilled water was used to suspend the pallet. The quantity of water required to dissolve DNA is directly proportional to the pellet size.

RNase treatment protocol

For the purification of DNA samples the samples of DNA were then exposed to RNase by using following procedure (Khan *et al.* 2004). Incubated the mixture of 20µl of DNA solution and 1µl of stock RNase at 37°C for 1 hr.Then the mixture of RNase and DNA solution was treated with chloroform isoamyl alcohol (24:1) and mixed it gently by overturning the tubes or two to three times. The obtained Samples then subjected to centrifugation for ten minutes at maximum rpm. The solution was established in 2 coatings. The above coating was shifted to a new 1.5 ml eppendorf tube and lasting solution was castoff. For the precipitation of DNA added 250- 500µl cold isopropanol in supernatant. With the help of centrifugation the precipitated DNA was screened out at room temperature. Centrifugation speed was set to be 12000 rpm for five minutes. Seventy percent ethanol was used to Wash the DNA pellet, dry it with air and liquefy in 100µl d_3H_2O.To check the DNA quality, 0.8% agarose gel was used for the gel electrophoresis of Genomic DNA.The stock solution was preserved in -20°C for upcoming usage. After the validation of DNA existence, the quantification principles for the samples of DNA were note down with the help of Nanodrop spectrophotometer (Thermo Scientific, Japan).

Primers selection for sorghum genotyping

A total of 30 unlinked SSR markers were selected from database (http://sat.cirad.fr/sat/sorghum _SSR_kit/data/msat_char.html)data base and used for diversity analysis.

PCR amplification protocol

All PCR reactions were performed with a negative control missing template DNA. The PCR profile for each SSR was same as reported. Working DNA (for PCR reactions) of each genotype was made by diluting stock DNA to a concentration of 25 ng/µl with DEPC water. Each PCR consisted of 96 reactions but PCR master mixture was made for 100 reactions to avoid shortage.

The volume of each PCR reaction was 20µl; therefore, 17 µl of master mix was pipetted in each well of PCR plate containing 1µl of template DNA. The composition of master mixture was as follow:

Optimization of polymerase chain reaction was performed to estimate annealing temperature for primers. Two annealing temperatures (55 and 60°C) were found optimum for proper amplification. Complete PCR profile for SSR markers is given below in Table 4.

Results

The proposed study was carried out with the objective to characterize the diverse germplasm of sorghum. Different morphological parameters of high biomass sorghum were assessed. About fifty sorghum genotypes were studied to screen out the best performing genotypes. Statistical tools were used to analyze the various traits of sorghum related to biomass for the recorded data.

The study showed significant differences among different genotypes for all observed traits (plant height, stem thickness, number of nodes and Internodal length). For each morphological trait, response of sorghum genotypes is described below:

Plant height (cm)

The mean values of plant height in sorghum genotypes ranged from 106.67 to 365.00 cm with an average of 264 cm. Sorghum genotype 13 had the highest plant height 365.00 cm followed by sorghum genotypes 40 and 36 with 351.67 and 348.33 cm respectively. Whereas the lowest plant height recorded for genotype 28 was 106.67 cm followed by genotype number 29 and 50 with 150.00 and 171.00 cm respectively. The ANOVA showed highly significant results for plant height among 50 various germplasm of sorghum (Table 5 and Table 6).

Table 1. Worldwide production of sorghum.

Countries	Production (1000 MT)	Area Harvested (1000 HA)
Argentina	3600.00	760.00
Australia	2100.00	630.00
Brazil	1650.00	632.00
Burkina Faso	1950.00	1850.00
Cameroon	1200.00	750.00
Chad	1150.00	1100.00
China	3500.00	780.00
Ethiopia	3800.00	1800.00
India	5300.00	5000.00
Mexico	6600.00	1450.00
Nigeria	6550.00	5350.00
Niger	4200.00	3700.00
Sudan	5600.00	8000.00
United States	12750.00	2042.00
Zimbabwe	430.00	170.00
Others	7150.00	5855.00

Source: USDA (Estimated Year: 2017).

Table 2. SSR analysis applied on selected genotypes.

Sr.#	Accessions	Sr.#	Accessions
1	PI474655	16	PI526890
2	PI475735	17	PI543158
3	PI 514220	18	PI562971
4	PI514249	19	PI563006
5	PI515688	20	PI563464
6	PI524691	21	PI569090
7	PI525007	22	PI569444
8	PI525689	23	PI585679
9	PI525917	24	PI525999
10	PI525937	25	PI937761
11	PI526012	26	PI585316
12	PI526020	27	PI585705
13	PI526022	28	PI54484
14	PI526028	29	PI550635
15	P1526113	30	PI550810

Stem thickness (mm)

The mean values for stem thickness ranged from 4.23 to 26.12 mm in the germplasm of sorghum with an average of 17 mm.

The maximum stem thickness (26.12 mm) was observed in sorghum genotype 4 followed by followed by genotype 10 and 6 with 24.54 and 23.24 mm respectively. While the minimum stem thickness (4.23 mm) was recorded for genotype 28 followed by genotype 25 with 5.61 mm. The statistical analysis exposed highly significant results for stem thickness among all germplasm of sorghum (Table 7 and Table 8).

Number of nodes per plant (n)

The mean values for number of nodes per plant ranged from 7.66 to 25.33 in the germplasm of sorghum with an average of 17.

Table 3. Optimized concentrations of PCR ingredients for SSR analysis.

Reagents	Quantity 1x (µl)
Template DNA 30ng/µl	3
Distilled water	1.8
Taq polymerase buffer (10X)	2
$MgCl_2$ (25mM)	3
dNTPs (10mM)	5
Forward Primer	2.5
Reverse Primer	2.5
Taq polymerase	0.2
Total	20µl

Table 4. PCR Profile of SSR primers for sorghum varieties.

No. of Cycles	Step	Temperature	Time
1x	Initial denaturation	94°C	5 min
35x	Denaturation	94°C	45 sec
-	Annealing	55°C and 60°C	1 min
-	Extension	72°C	1min
1x	Final extension	72°C	10min

Table 5. Plant height values of sorghum germplasm.

Sorghum Genotypes	Plant height (Mean)	Sorghum genotypes	Plant height (Mean)	Sorghum genotypes	Plant height (Mean)
1	265.00	18	265	35	190.00
2	258.33	19	308.33	36	348.33
3	231.67	20	326.67	37	248.33
4	271.67	21	286.67	38	251.67
5	305.00	22	303.33	39	234.00
6	328.33	23	278.33	40	351.67
7	325.00	24	293.33	41	180.00
8	295.00	25	171.67	42	184.00
9	255.00	26	236.67	43	344.00
10	288.33	27	340.00	44	355.00
11	298.33	28	106.67	45	190.00
12	176.67	29	150.00	46	201.67
13	365.00	30	323.33	47	246.67
14	246.67	31	308.67	48	310.33
15	255.00	32	253.33	49	204.00
16	291.67	33	256.67	50	171.00
17	255.00	34	320.00		

The maximum number of nodes per plant (25.33) was observed in sorghum genotype 43 followed by genotype 35 and 27 with 25.00 and 24.33 respectively.

Whereas the minimum number of nodes per plant (7.66) was recorded for genotype 28 followed by genotype 29 with 9.00 number of nodes.

The ANOVA showed highly significant results for number of nodes per plant among all germplasm of sorghum (Table 9 and Table 10).

Table 6. ANOVA for plant height.

Source	DF	SS	MSS	F-value	P-value
Repication	2	8933	4466.5		
Lines	49	521074	10634.2	5.40**	0.0000
Error	98	192986	1969.2		
Total	149	722993			

DF: Degree of Freedom, SS: Sum of Square, MSS: Mean Sum Square, F- value: Calculated value, P-value: Tabulated value. Scale used to measure the significance of results:P>0.05: Non-Significant, P≤0.05: Significant, P<0.01: **Highly SignificantGrand Mean 264.62, CV 16.77.

Table 7. Stem thickness values of sorghum germplasm.

Sorghum genotypes	Stem thickness (Mean)	Sorghum genotypes	Stem thickness (Mean)	Sorghum Genotypes	Stem thickness (Mean)
1	17.500	18	16.767	35	20.300
2	14.800	19	15.767	36	17.178
3	19.444	20	21.189	37	17.756
4	26.122	21	18.367	38	10.522
5	19.822	22	20.178	39	15.456
6	23.244	23	10.822	40	21.000
7	19.978	24	11.700	41	9.600
8	21.833	25	5.611	42	9.789
9	20.289	26	10.067	43	20.333
10	24.544	27	12.722	44	22.722
11	20.733	28	4.233	45	16.844
12	13.956	29	17.233	46	17.367
13	13.056	30	22.711	47	13.400
14	21.900	31	16.733	48	18.244
15	21.033	32	20.244	49	14.933
16	19.400	33	16.933	50	19.211
17	13.511	34	16.889		

Table 8. ANOVA for stem thickness.

Source	DF	SS	MSS	F-value	P-value
Replication	2	24.33	12.1673		
Lines	49	3251.50	66.3572	5.59**	0.0000
Error	98	1163.33	11.8707		
Total	149	4439.17			

DF: Degree of Freedom, SS: Sum of Square, MSS: Mean Sum Square, F- value: Calculated value, P-value: Tabulated value. Scale used to measure the significance of results:P>0.05: Non-Significant, P≤0.05: Significant, P<0.01: **Highly Significant, Grand Mean 17.080, CV 20.17.

Internodal length (cm)

The mean values of Internodal length in sorghum genotypes ranged from 4.77 to 20.55 cm with an average of 14 cm. Sorghum genotype 19 had the highest Internodal length 20.55 cm followed by sorghum genotype 40 and 47 with 20.44 and 20.11 cm respectively. Whereas the lowest Internodal length recorded for genotype 28 was 4.77 cm followed by genotype number 45 and 12 with 6.77 and 7.88 cm respectively. The statistical analysis showed highly

significant results for Internodal length among all germplasm of sorghum (Table 11 and Table 12.

Principle component analysis of morphological traits of sorghum genotypes

The morphological data of 50 sorghum genotypes were examined to correlate the genetic variability and similarity among them using XLSTAT software. The statistical summary of PC analysis showed maximum, minimum and mean values with standard deviation among different traits. The minimum and maximum values of plant height were ranged from 106.67 to 365.00 while mean plant height was 265.02.

Similarly, stem thickness mean was 17.08 with minimum and maximum values ranged from 4.23 and 26.12 and other traits showed in (Table 13).

Table 9. Number of nodes per plant for sorghum germplasm.

Sorghum genotypes	Number of nodes (Mean)	Sorghum genotypes	Number of nodes (Mean)	Sorghum genotypes	Number of nodes (Mean)
1	16.333	18	15.333	35	25.000
2	15.000	19	20.667	36	23.000
3	12.333	20	23.000	37	18.000
4	18.000	21	19.000	38	13.333
5	21.333	22	22.333	39	15.333
6	23.000	23	18.000	40	22.667
7	19.000	24	20.667	41	10.000
8	15.000	25	13.000	42	10.000
9	17.333	26	16.667	43	25.333
10	18.667	27	24.333	44	21.667
11	17.000	28	7.667	45	17.667
12	11.000	29	9.000	46	17.333
13	18.000	30	18.333	47	10.333
14	17.000	31	19.333	48	18.333
15	16.333	32	17.333	49	13.000
16	19.333	33	21.000	50	10.667
17	16.667	34	16.000		

Table 10. ANOVA for number of nodes per plant.

Source	DF	SS	MSS	F-value	P-value
Replication	2	10.45	5.2267		
Lines	49	2752.43	56.1720	5.50**	0.0000
Error	98	1000.21	10.2063		
Total	149	3763.09			

Table 11. Internodal length per plant for sorghum germplasm.

Sorghum genotypes	Internodal length (Mean)	Sorghum genotypes	Internodal length (Mean)	Sorghum Genotypes	Internodal length (Mean)
1	17.444	18	16.111	35	10.778
2	18	19	20.556	36	16.778
3	16.556	20	19.333	37	10.333
4	16.111	21	17.778	38	13.556
5	18.556	22	15.778	39	12.778
6	16.778	23	17.444	40	20.444
7	16.778	24	14.778	41	13.667
8	17.333	25	9.222	42	11.556
9	18.778	26	14.667	43	13.444
10	19	27	17.222	44	14
11	19.333	28	4.778	45	6.778
12	7.889	29	8.333	46	10.444
13	19.778	30	18.889	47	20.111
14	11.556	31	16.778	48	14.889
15	16.667	32	13.333	49	10.778
16	19.556	33	10	50	8.111
17	16.222	34	14.778		

The principal components that showed Eigen values greater than one were considered as highly significant. This principal component analysis indicated that most significant components accounted for about 66% of the total variation among traits in sorghum cultivars. The first principle component with 66% of total variation was strongly associated with plant height and its components. Other components accounted for 17, 12 and 3% of variation between traits and were entitled as stem thickness, number of nodes and internodal length respectively. The highly related traits exhibiting eigen values and variability in PC1, PC2, PC3 and PC4 as shown in table 14.

Table 12. ANOVA for Internodal length of each plant.

Source	DF	SS	MSS	F-value	P-value
Replication	2	20.65	10.3230		
Lines	49	2330.26	46.5563	7.97**	0.0000
Error	98	584.98	5.9692		
Total	149	2935.89			

DF: Degree of Freedom, SS: Sum of Square, MSS: Mean Sum Square, F- value: Calculated value, P-value: Tabulated value. Scale used to measure the significance of results:P>0.05: Non-Significant, P≤0.05: Significant, P<0.01: **Highly Significant, Grand Mean 14.891, CV 16.41.

Table 13. Summary Statistics of morphological traits of sorghum genotypes.

Variable	Observations	Minimum	Maximum	Mean	Std. deviation
Plant height	50	106.670	365.000	265.020	60.084
Stem thickness	50	4.233	26.122	17.080	4.703
Number of nodes	50	7.667	25.333	17.293	4.327
Internodal length	50	4.778	20.556	14.891	3.981

Table 14. Eigen values of principle components.

	PC1	PC2	PC3	PC4
Eigenvalue	2.650	0.707	0.517	0.126
Variability (%)	66.259	17.672	12.919	3.150

Table 15. Correlation between variables and principal components.

Variables	PC1	PC2	PC3	PC4
Plant height	0.934	-0.209	0.105	0.272
Stem thickness	0.684	0.629	-0.368	0.009
Number of nodes	0.828	0.178	0.507	-0.158
Internodal length	0.790	-0.485	-0.336	-0.164

The PCA analysis indicated that only one principal components out of four, exhibited more than one Eigen value and thus showed more variability with the rest of principal components.

The maximum variation (66%) was observed in PC1 with Eigen value 2.650 which then reduced gradually.

Correlation analysis

The correlation analysis may help to identify the effective traits for screening of the best performing genotypes. Correlation for four observed morphological traits like (plant height, stem thickness, number of nodes and internodal length) as shown in the given table (Table 15).

Table 16. Correlation coefficient between morphological characters.

Variables	Plant height	Stem thickness	Number of nodes	Internodal length
Plant height	1			
Stem thickness	0.471	1		
Number of nodes	0.746	0.491	1	

Table 17. Nei's genetic identity and genetic distance among genotype's

1	****	1.0000	0.9487	0.8367	0.9487	0.8944	0.9487	0.9487	0.9487	0.8944
2	0.0000	****	0.9487	0.8367	0.9487	0.8944	0.9487	0.9487	0.9487	0.8944
3	0.0527	0.0527	****	0.8819	1.0000	0.8250	0.8889	0.8889	0.8889	0.8250
4	0.1783	0.1783	0.1257	****	0.8819	0.8018	0.8819	0.8819	0.8819	0.8018
5	0.0527	0.0527	0.0000	0.1257	****	0.8250	0.8889	0.8889	0.8889	0.8250
6	0.1116	0.1116	0.1924	0.2209	0.1924	****	0.9428	0.9428	0.8250	0.8750
7	0.0527	0.0527	0.1178	0.1257	0.1178	0.0589	****	1.0000	0.8889	0.9428
8	0.0527	0.0527	0.1178	0.1257	0.1178	0.0589	0.0000	****	0.8889	0.9428
9	0.0527	0.0527	0.1178	0.1257	0.1178	0.1924	0.1178	0.1178	****	0.8250
10	0.1116	0.1116	0.1924	0.2209	0.1924	0.1335	0.0589	0.0589	0.1924	****

Table 18. Summary of marker diversity.

Markers	Major Allele Frequency	Allele No.	Gene Diversity	Heterozygosity	PIC*
SB3996	1.0000	1.0000	0.0000	0.0000	0.0000
Xcup13	0.8000	2.0000	0.3200	0.0000	0.2688
Xcup02	1.0000	1.0000	0.0000	0.0000	0.0000
Xtxp141	1.0000	1.0000	0.0000	0.0000	0.0000
Xtxp 090	0.9000	2.0000	0.1800	0.0000	0.1638
Xtxp101	0.5000	2.0000	0.5000	0.0000	0.3750
Xtxp274	0.7000	2.0000	0.4200	0.0000	0.3318
Xcp66	1.0000	1.0000	0.0000	0.0000	0.0000
Xcup1	0.8000	2.0000	0.3200	0.0000	0.2688
msbCIR262	0.9000	2.0000	0.1800	0.0000	0.1638
Mean	0.8429	1.5714	0.2029	0.0000	0.1628

The analysis showed that plant height was highly related trait to screen out best sorghum genotypes which can be used for further breeding related programs.

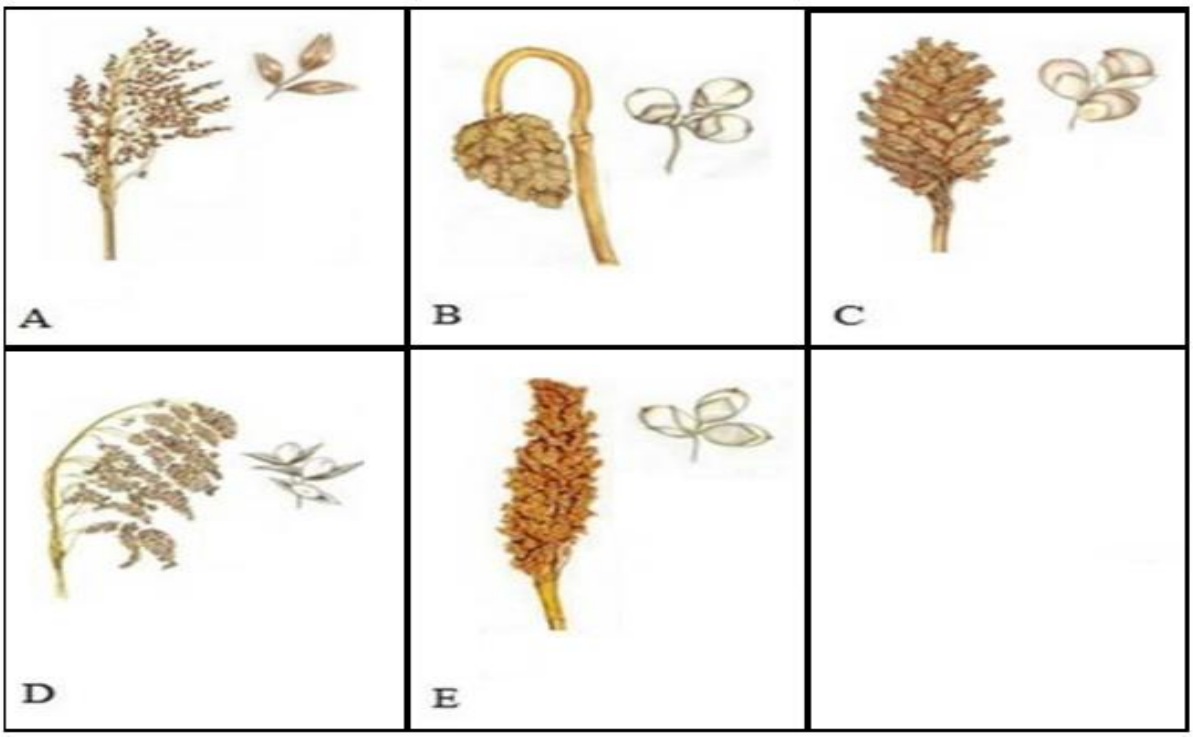

Fig. 1. Sorghum 5 basic races; A= bicolor, B= caudatum, C= durra, D= huinea and E= Kafir.

Scree and score plot

Scree plot explained the graphical representation of total variance associated with each principle components and thus showed the significance of the parameters. The score plot analysis was carried out for 50 sorghum genotypes based on four parameters. The more significant variables are those parameters which were dispersed away from the central point in the coordinate system as compared to those plotted near the central point. The most variable characters in coordinates system was plant height (Figs 9 and 10).

Fig. 2. Sorghum field at the University of Agriculture, Faisalabad.

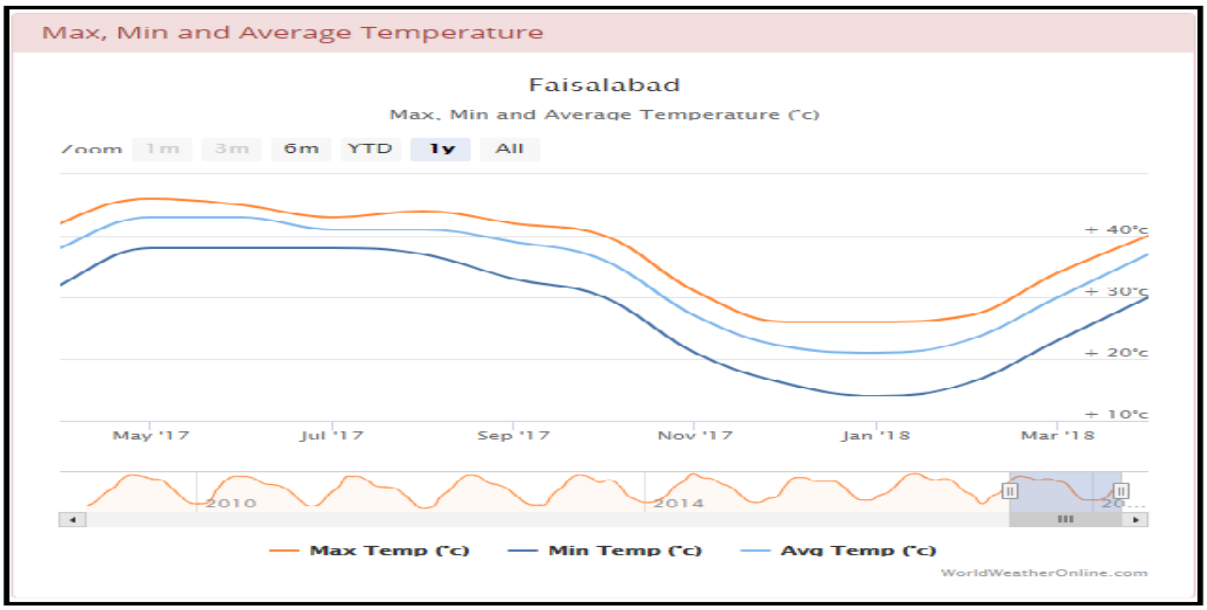

Fig. 3. Recorded average temperature of 2017.

Bi plot analysis visualized the relationships between and among the traits of sorghum genotypes. It explained the total variation when the correlation coefficient between any two traits can be approximated by the cosine of the angle between their vectors.

Two traits are positively correlated and showed higher significance when the angle is < 90°, negatively correlated when the angle is >90° and independent when the angle is 90°. This analysis also explained that traits with longer vectors are more reliable to the treatment combinations; traits with shorter vectors are less responsive to the genotypes and those located at the biplot origin are not responsive at all. It revealed that plant height is positively correlated with all the other traits (Fig. 11).

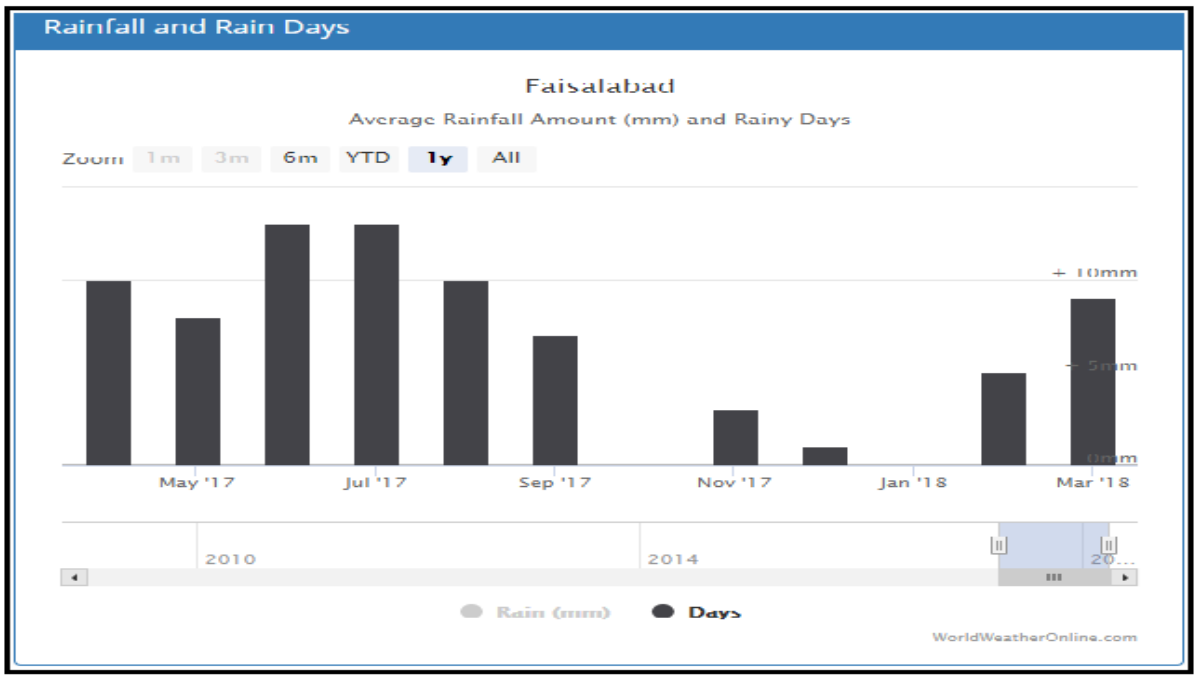

Fig. 4. Recorded Average Rainfall during (2017).

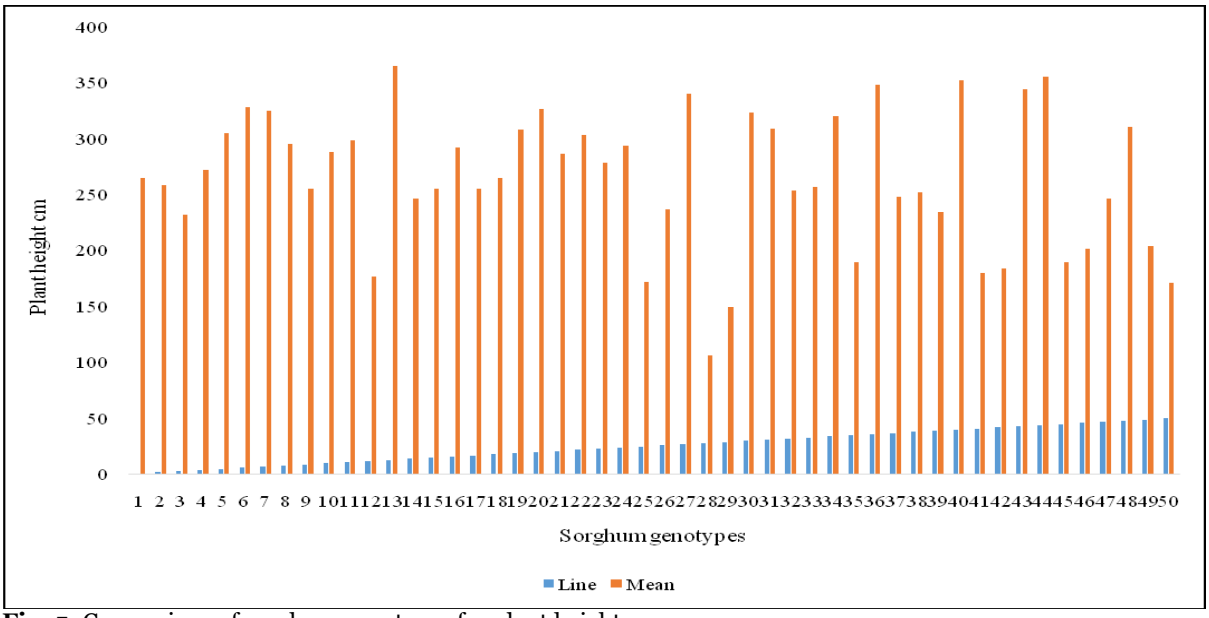

Fig. 5. Comparison of sorghum genotypes for plant height.

Molecular screening of selected sorghum accessions using SSR primers

On the basis of plant height, top 20 heighted sorghum genotypes were assessed to examine the variability among these by using molecular markers. The seeds of these sorghum genotypes were germinated in sand and leaves were harvested from seedling stage for genomic DNA extraction.

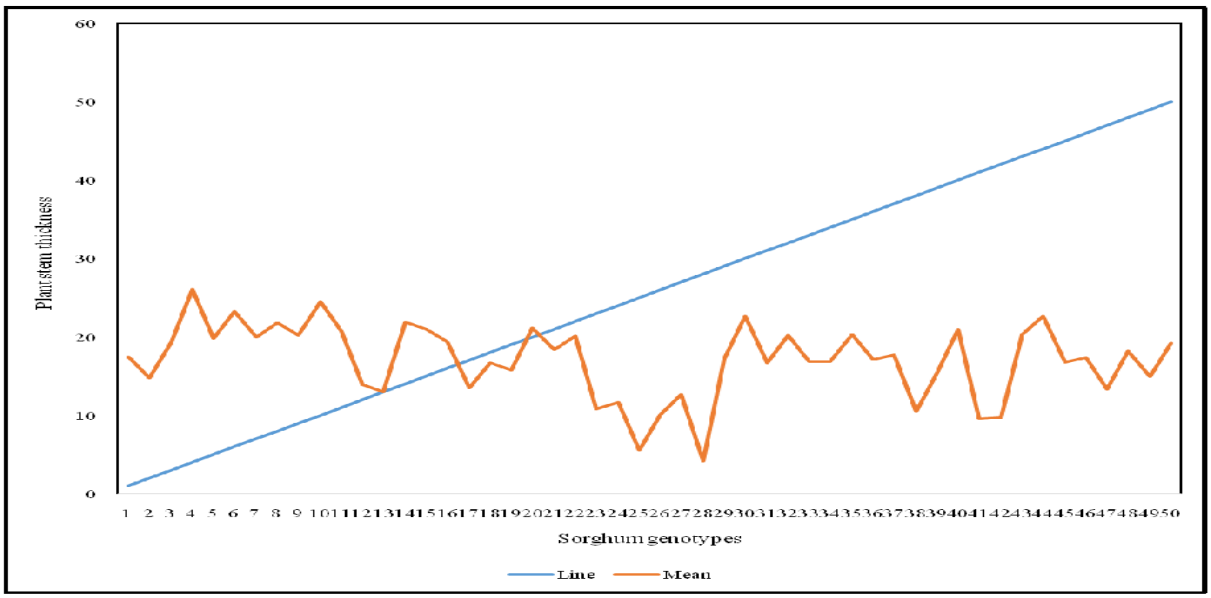

Fig. 6. Comparison of sorghum genotypes for plant stem thickness.

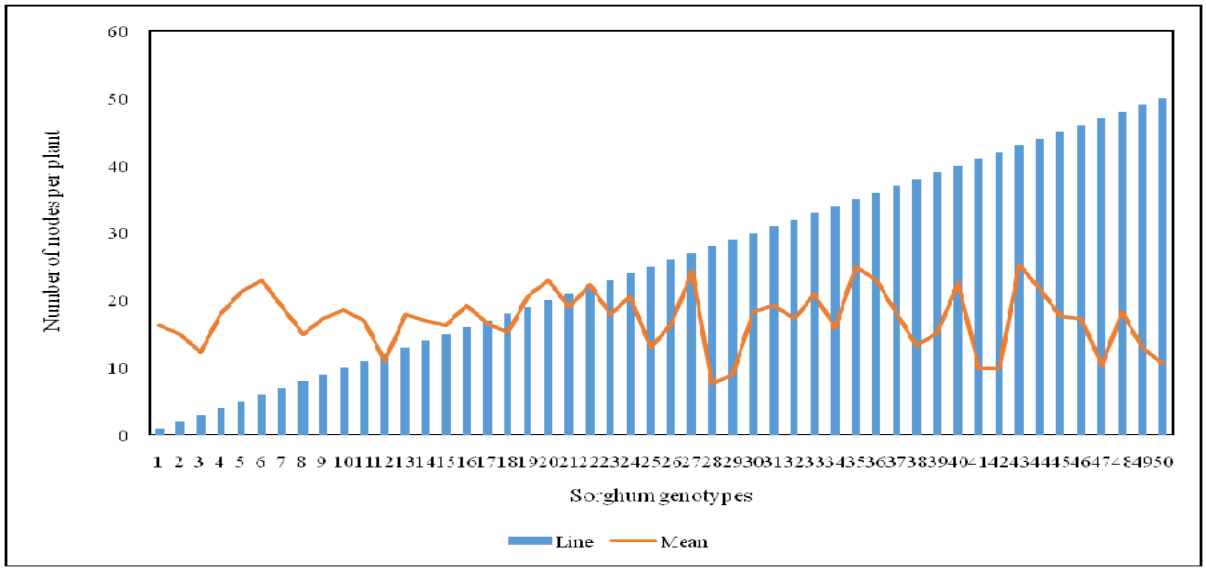

Fig. 7. Comparison of sorghum genotypes for number of nodes per plant.

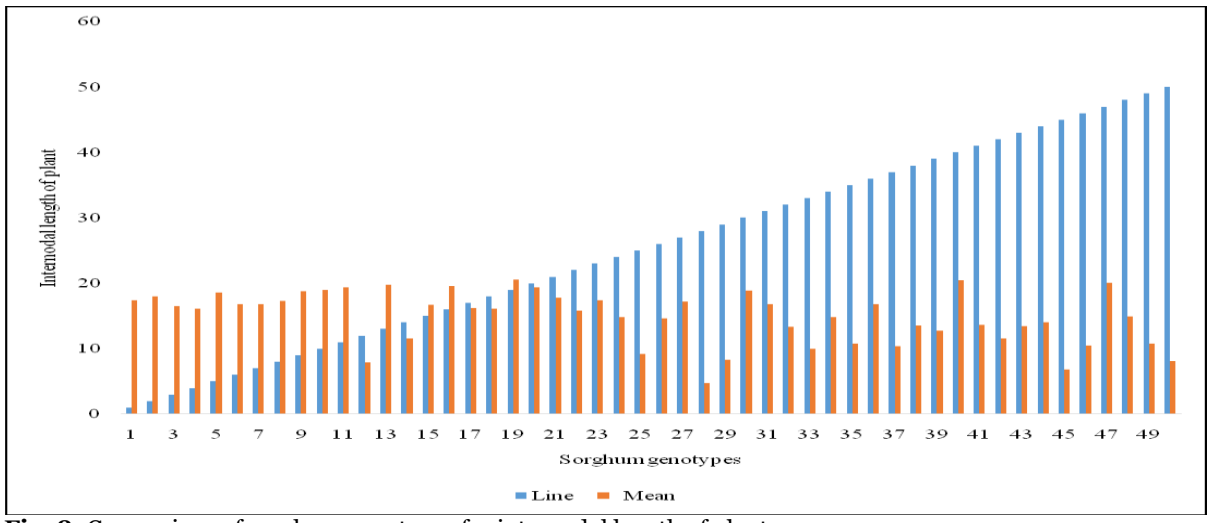

Fig. 8. Comparison of sorghum genotypes for internodal length of plant.

DNA concentration was computed by using Nano drop method. The ten best promising sorghum genotypes having higher DNA concentration *viz.* L-1, L-3, L-6, L-8, L-13, L-14, L-15, L-23, L-28, L-29 were evaluated and the dilutions were prepared for amplification of these DNA samples.

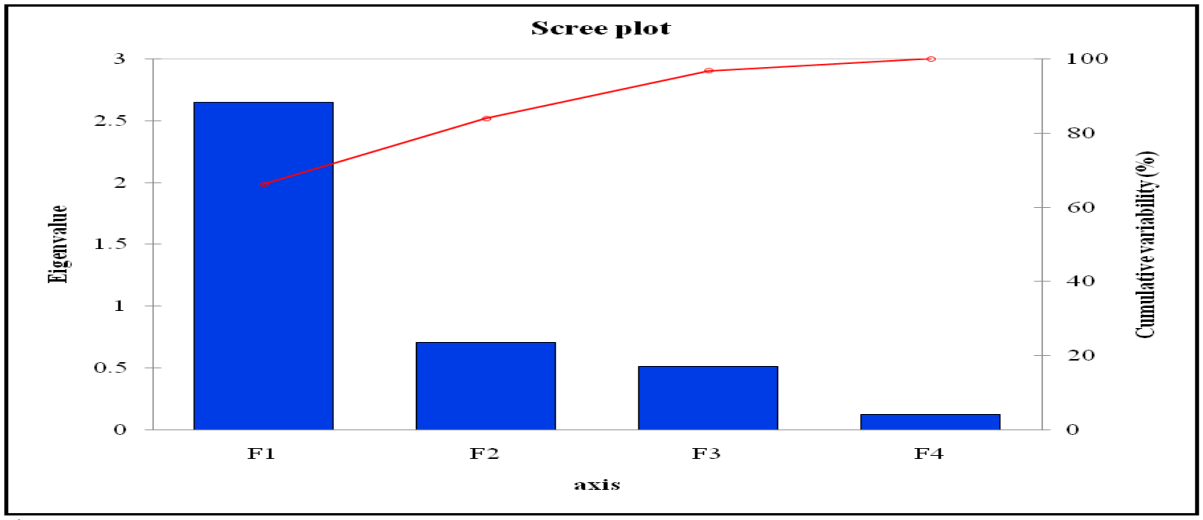

Fig. 9. Scree plot between numbers of principle components and eigenvalue for sorghum genotypes.

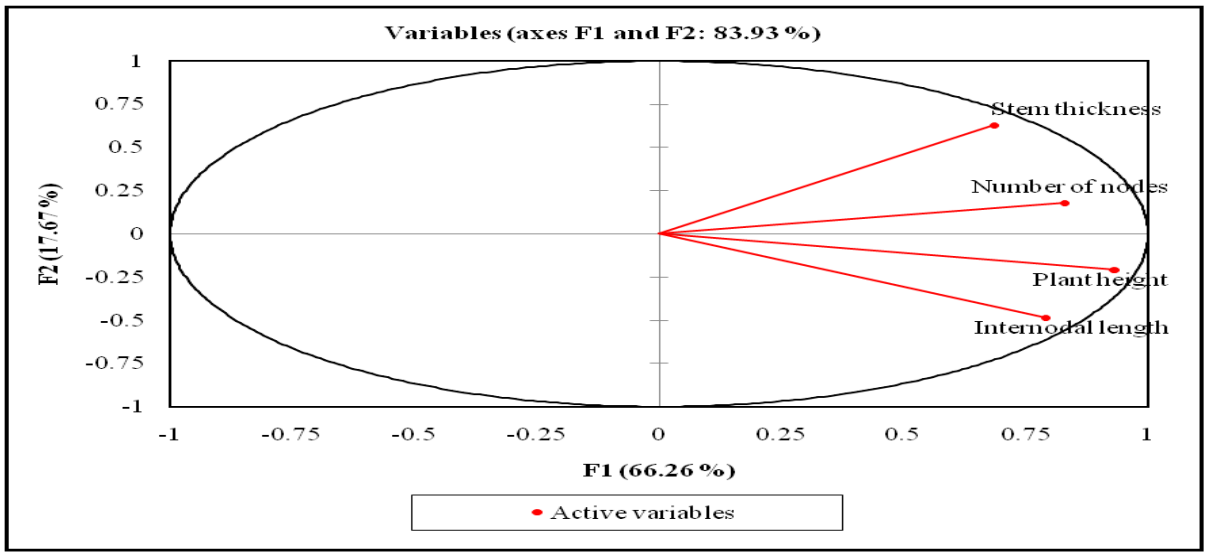

Fig. 10. Score plot of ordination variables.

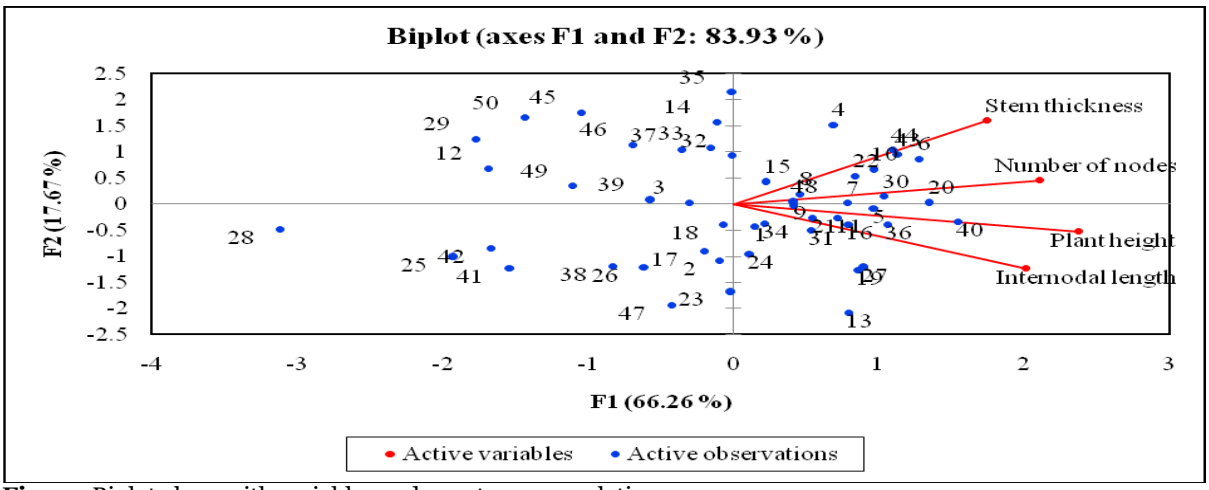

Fig. 11. Biplot along with variables and genotypes cumulative.

Ten SSR primers mSbCIR262, SB3996, Xcup13, Xcup02, Xtxp141, Xtxp 090, Xcup17, Xcup66, Xtxp101 and Xtxp274 used for molecular screening of ten selected sorghum genotypes. The amplification of PCR product of ten selected genomic DNA samples with SSR primers is as follow:

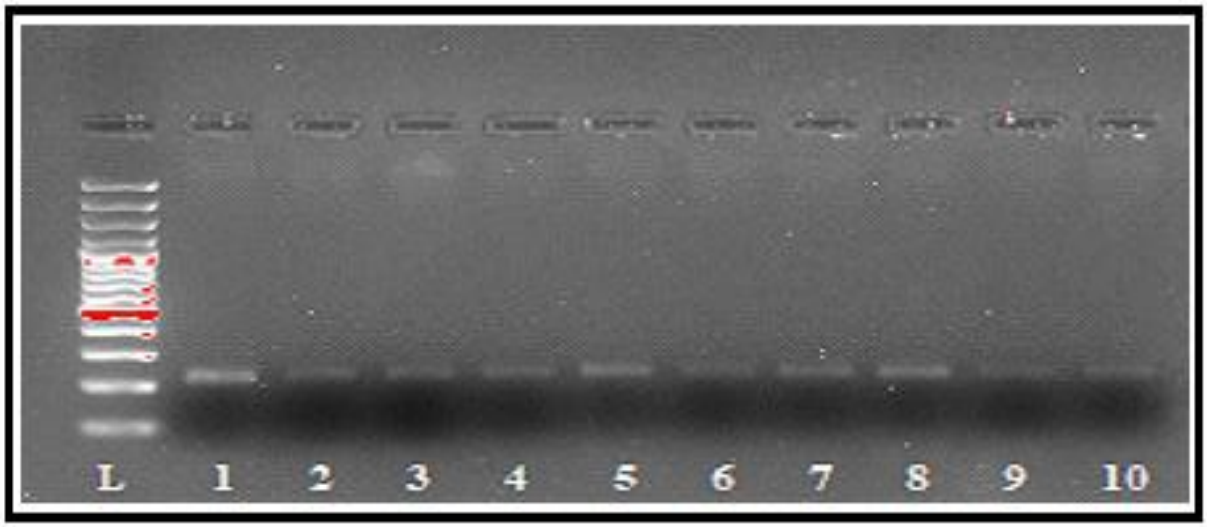

Fig. 12. PCR product of SSR primer SB3996; 1-10 (Sorghum samples); L (Ladder).

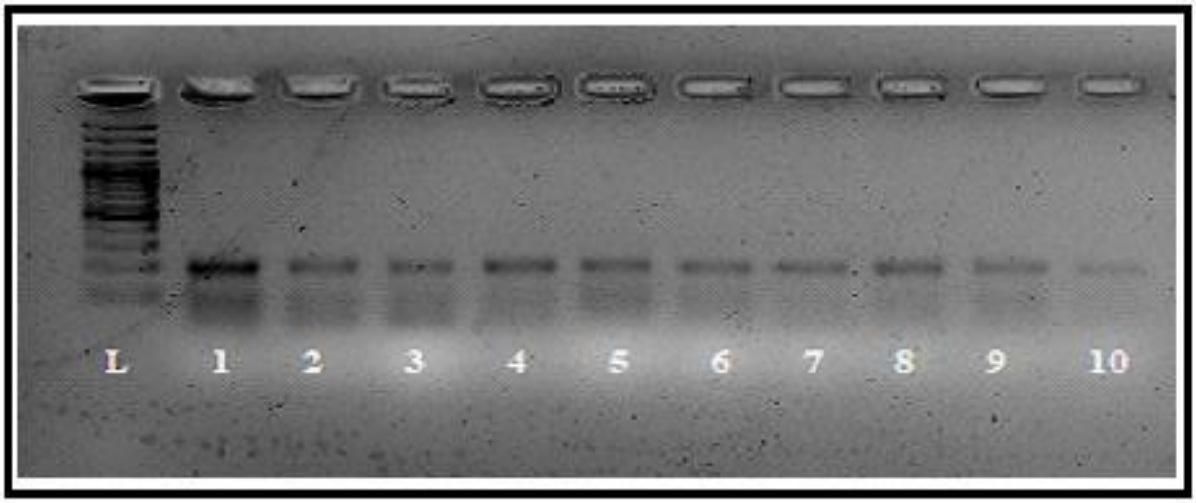

Fig. 13. PCR product of SSR primer Xtxp090; 1-10 (Sorghum samples); L (Ladder.

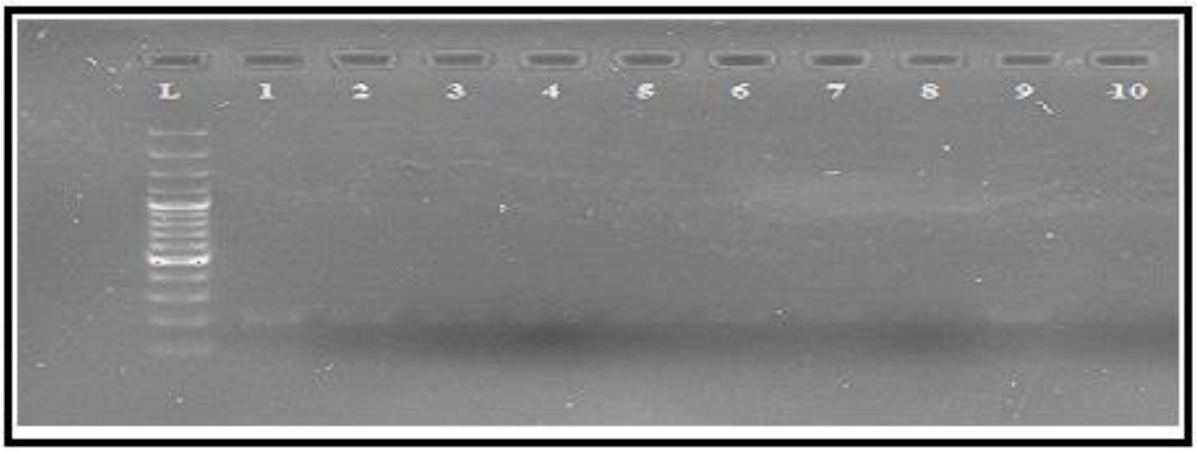

Fig. 14. PCR product of SSR primer Xtxp101; 1-10 (Sorghum samples); L (Ladder).

Population genetic analysis

SSR primers were used to detect genetic polymorphism at DNA level in ten selected sorghum genotypes. Only the scorable bands were included in the analysis. Every single band was considered as a singlelocus/allele for all the genetic analyses. The monomorphic alleles were identified by SSR markers.

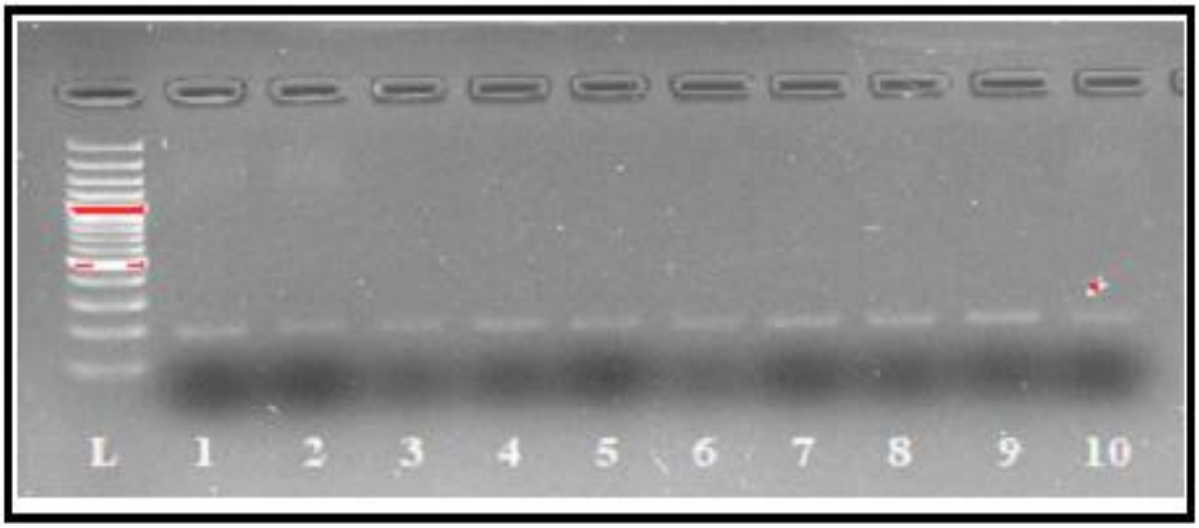

Fig. 15. PCR product of SSR primer Xtxp141; 1-10 (Sorghum samples); L (Ladder).

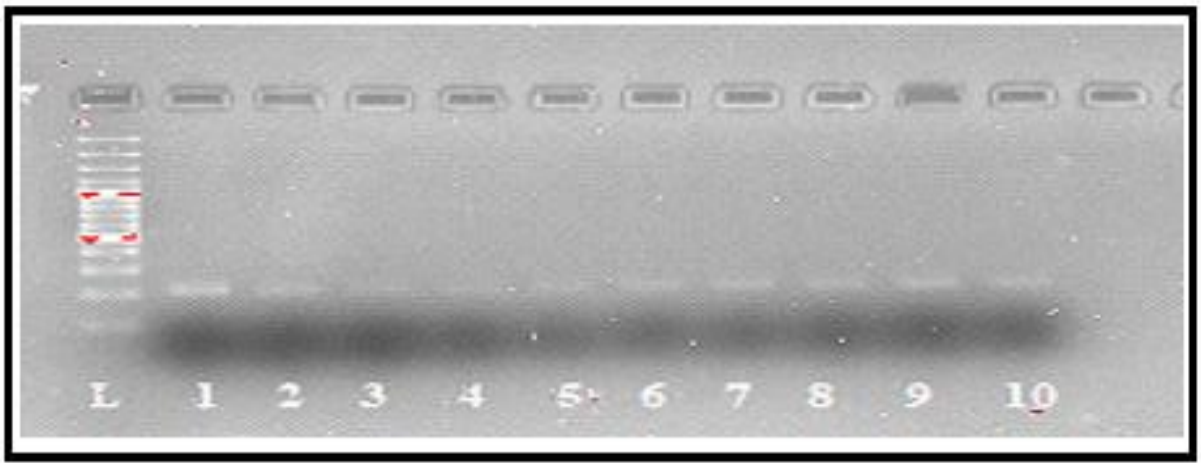

Fig. 16. PCR product of SSR primer Xtxp274; 1-10 (Sorghum samples); L (Ladder).

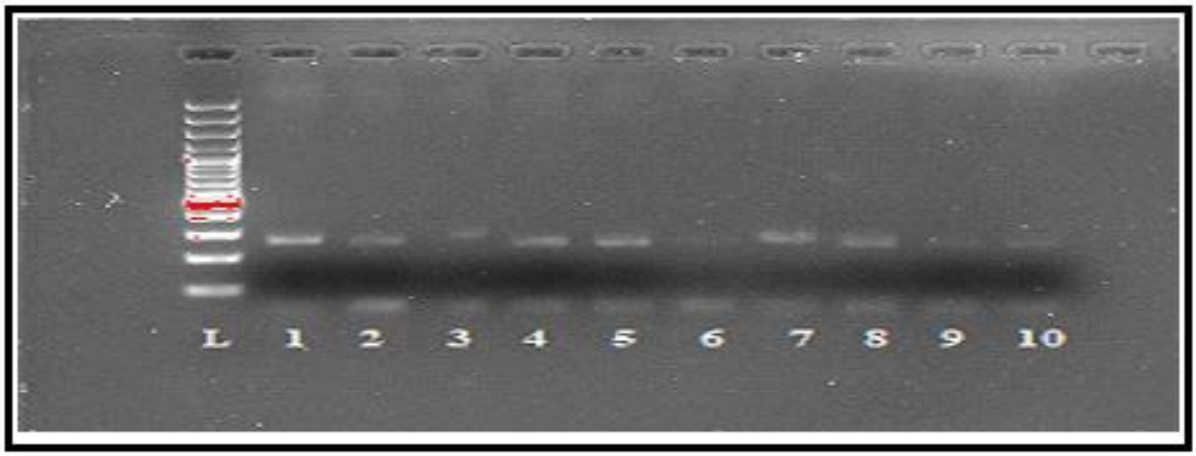

Fig. 17. PCR product of SSR primer msbCIR262; 1-10 (Sorghum samples); L (Ladder).

Confirmation of the polymorphism

To check genetic diversity among selected sorghum genotypes, 10 SSR primers were used. Total number of bands per primer and number of bands per sorghum genotype varied. Some primers showed close genetic relationship among the sorghum genotype and named as monomorphic bands while primers who exhibited polymorphism showed higher genetic variability between genotypes.

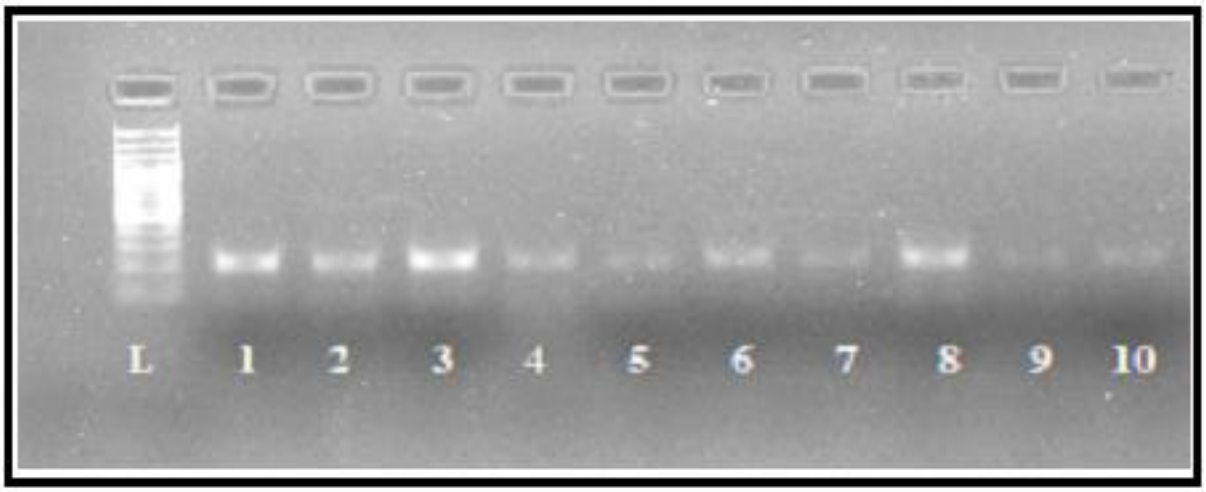

Fig. 18. PCR product of SSR primer Xcup02; 1-10 (Sorghum samples); L (Ladder).

Fig. 19. PCR product of SSR primer Xcup13; 1-10 (Sorghum samples); L (Ladder).

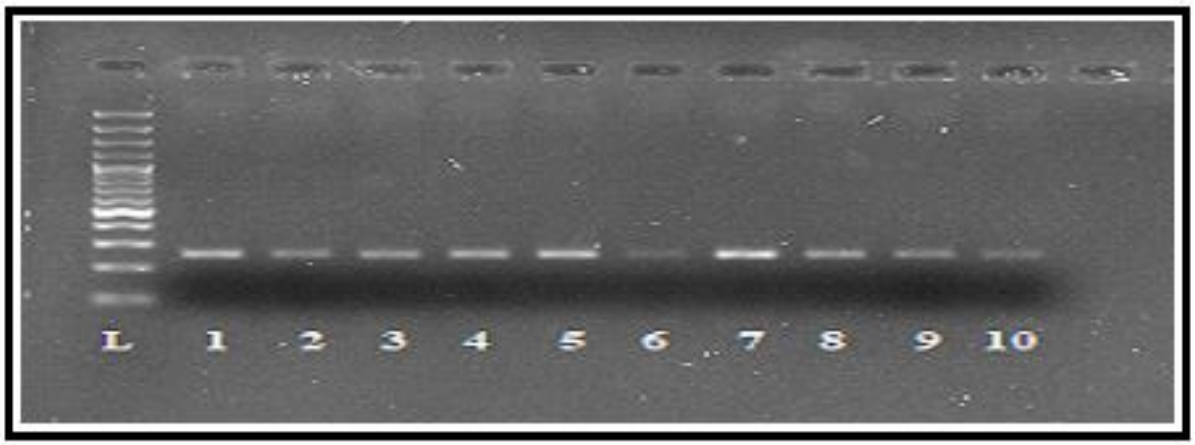

Fig. 20. PCR product of SSR primer Xcup17; 1-10 (Sorghum samples); L (Ladder).

Genetic similarity between sorghum genotypes

To check the genetic similarities among sorghum genotypes, Popgen (version 1.31) software was used. Nei's UPGMA method was applied to check the genetic association among genotypes (Table 17).

Marker diversity

PowerMarker version 3.25 software was used to check the marker diversity for ten selected sorghum genotypes which were studied for ten SSR primer loci. The number of allele was ranged from 1 to 2.00 alleles per locus with an average of 1.57 alleles per locus. On the other hand, genetic diversity was ranged from 0 to 0.50 with the mean of 0.20. Similarly, polymorphism information content (PIC value) was estimated from 0 to 0.3750 with mean of 0.1628. The allelic frequency of primer was ranged from 0.5 to 1.0 and showed 0.8429 mean value.

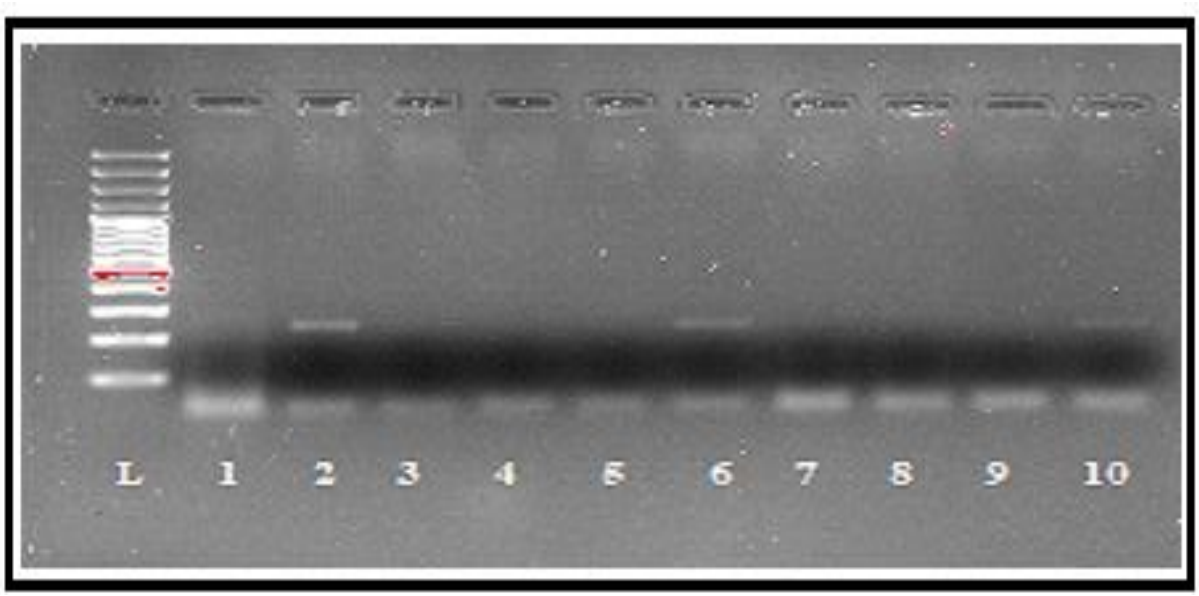

Fig. 21. PCR product of SSR primer Xcup66; 1-10 (Sorghum samples); L (Ladder).

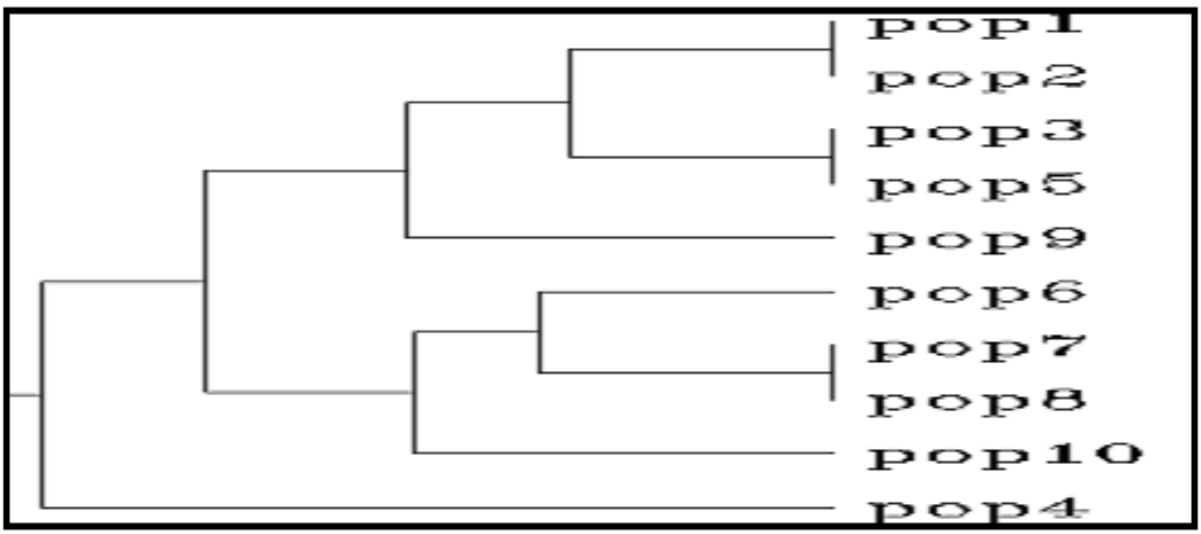

Fig. 22. Dendrogram of 10 sorghum genotypes based on UPGMA analysis.

Genetic relationship among sorghum accessions

Genetic relationship among sorghum accessions was identified by Nei's Unweighted Paired Group of Arithmetic Means Average (UPGMA) method using popgen software (1.32). The UPGMA-based dendrogram grouped genotypes into 3 clusters. Sorghum genotypes 1, 2, 3, 5 and 9 have genetic similarity and present in same cluster I while

sorghum genotype 6, 7, 8 and 10 have similar genetic diversity and present in cluster II. Most genetic difference is seen for 1 and 4. These two genotypes had greater genetic distance to each other. Line 4 had most diverse genome and grouped in single cluster. Dendrogram exhibits the genetic diversity (Fig.22).

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