

Studies on Hybridization and Genetic Diversity in Cowpea (*Vigna Unguiculata* L)



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Abstract

Crossability of various accessions of cowpea on the basis of pollen development and fruit set was studied along with genetic diversity by SSR markers. There was a significant correlation between fruit set and percent viable pollen. In all selfings and the crosses, pollen germination increased from 2 hr to 6 hr after pollination. Pollen germination had a highly significant correlation with fruit set. On selfing, the fruit set was positively correlated with faster pollen tube growth whereas it was not so in the case of crosses. There was a positive correlation between pollen tube growth and fruit set. Thirty six primers were selected to assess genetic diversity of 36 accessions of cowpea and twenty seven primers were found to be polymorphic. The polymorphic primers generated a total of 72 alleles. The number of alleles ranged from 2 to 4 with an average of 2.66 per locus. Based on Euclidean similarity matrix and dendrogram constructed, two major clusters were revealed. Cluster I and Cluster II comprised of 14 and 22 accessions, respectively. M-5 was found to be more distinct from Kashi Gowri, EC- 559390 and EC- 9738; EC- 472260 and Sel- 16; EC- 30950 and PL- 2; EC- 472260 and EC- 9739. Sel- 16 and EC- 9739 were found to be very less distinct. The value of Euclidean similarity coefficient ranged from 0 to 3.61. PIC value obtained ranged from 0.09 – 0.68 with an average of 0.35. Based on PIC values, out of 27 primers, SSR 6314 was found to be highly informative.

Keywords: Crossability; *Vigna Unguiculata*; Pollen Tube; Fruit Set; SSR

Introduction

Cowpea (*Vigna unguiculata* L.) is a self-pollinated crop belonging to the family Fabaceae and is native to Central Africa. It is also known as the 'poor man's meat' because of its good protein quality and high nutritional value. Legumes are economically significant crops and rich source of nourishing diet for growing population [1,2]. Reported that the floral bud initiation and development is sensitive to photoperiod in many cowpea accessions, and in some genotypes the degree of photoperiod sensitivity (i.e. the extent of delay in flowering) is influenced by temperature [3]. Also reported that the productivity of cowpea (*Vigna unguiculata*) can be reduced substantially by hot weather during flowering.

The assessment of pollen viability is important in artificial pollination and breeding experiments [4,5] stated that for fertilization to occur, the pollen landing on the stigma must be viable i.e. which must be able to germinate, penetrate the stigma and style and reach the ovule during the effective pollination

period (EPP). For successful fertilization to occur there should be components of pollen performance which includes pollen produced in a flower, pollen morphological homogeneity, pollen germination, pollen tube growth and pollen competition [6-8] stated that the pollen tube growth and pollen germination are important research materials for morphological, physiological, biotechnological, ecological, evolutionary, and molecular studies. Intraspecific hybrids are an important source of variability in breeding programs. The knowledge of the intraspecific compatibility as well as of the direction of crossing is extremely important for success in hybrid breeding programs [9]. Genetic diversity analysis facilitates utilization of genetic resources for conservation of germplasm and the development of breeding programs [10]. Cowpea has a very narrow gene pool which limits development of new cowpea varieties by cross-breeding [11]. The knowledge of genetic diversity of cowpea germplasm is extremely essential for cowpea breeders to produce new cowpea varieties with higher yield and better

quality [12]. This paper reports pollen germination and pollen tube behavior in relation to crossability between various accessions of *Vigna unguiculata* L. and seed set and genetic diversity using SSR primers.

Materials and Methods

The 36 accessions used were Kashi Gowri, Kashi Kanchan, IC- 202826, EC- 30950, PL- 2, PL- 3, Kashi Nidhi, IC- 33922, Arka Garima, Kashi Unnati, PL- 4, PL- 1, PL- 5, EC- 472260, Sel- 16, IC- 559390, EC- 9738, IC- 3004, IC- 202865, EC- 9739, EC- 110598, Kashi Shyawal, IC- 332198, EC- 390221, EC- 15296, EC- 9736 and EC- 390211 and accessions of N- 1, PS- 1, CP- 7, HS, M- 1, M- 2, M- 3, M- 4 and M- 5 were collected from different locations of North Eastern region of India. Five parents were selected for crossability studies. They were used for one way crossing and so the total number of selfing and crossing were 15. For each cross at least ten to fifteen flowers were randomly selected from plants. Pollinations were performed from May to October, 2016 and care was taken to avoid any damage to the stigma. The styles were collected 2 to 6 hr after hand pollination and fixed immediately in 1:3 glacial acetic acid-ethyl alcohol for at least 24 hr and then preserved in 70 per cent alcohol till further use. For pollen germination and pollen tube growth observations, the pollinated flowers were gently rinsed in distilled water and pistils were separated from the flowers after which they were kept in a drop of 1N HCl for 10 minutes. They were again rinsed in distilled water and stained in 1 percent aniline blue [13,14]. The time required for staining was 10-20 seconds depending on the thickness of the style and the stage of penetration of the pollen tube in the stigma. After staining, the pistils were destained for 20-24 hr in a 1:1:1 mixture of 40 % acetic acid: orthophosphoric acid: distilled water. The pistils were then rinsed in distilled water and mounted in pure lactic acid and studied under the microscope. The pollen grains and pollen tubes stained deep blue.

Genomic DNA from cowpea leaves was isolated using CTAB method of [15]. Young, actively growing leaves of 5-10 days old plants were collected and used for DNA extraction. The

quantification of DNA were done by staining DNA with ethidium bromide after electrophoresis in 0.8% agarose gel at 80 V for 1 hr in TBE buffer (0.04 M Tris borate, 0.001 M EDTA, pH 8.0) using known DNA concentration standards. Molecular weights of bands were estimated by using 100 bp for SSR. The homology of bands was based on the distance of migration in the gel. SSR amplicons obtained from each entry were resolved as multiple and a single band, respectively on the agarose system and the pair-wise correlation matrix was developed on the normalized data set to find out the relationship among 36 accessions of cowpea. Bands were marked: presence of a band was scored as one (1) and absence of a band as zero (0). Very faint bands were not considered for final scoring. After scoring, the Polymorphism Information Content (PIC) was calculated. The SSR data was subjected to Paleontological Statistics, PAST v3.15 software [16] to construct a Dendrogram by hierarchical cluster analysis based on Ward’s method (Ward, 1963) using the Euclidean similarity matrix [17] as cited by [18].

Results and Discussion

Pollen Fertility

The pollen fertility of all the accessions under this study revealed that the maximum fertile, stained pollen was recorded in Kashi Kanchan (96.59 ± 0.23) whereas the lowest percentage of fertile pollen recorded was in IC- 202836 (88.21 ± 0.87) (Table 1). However, the average percent pollen fertility did not show too much difference among the accessions [19] stated that in cowpea, high night temperature (33/30° C) increased the occurrence of small and shrunken pollen leading to zero pod set compared with low night temperature (33/20° C) (Figures 1-3).

Table 1: Pollen fertility in the parents in cowpea.

Parents	Pollen fertility (%)	Fruit set (%)
Kashi Kanchan	96.59 ± 0.23	91.30
Kashi Gowri	95.95 ± 0.48	88.23
PL- 2	93.32 ± 0.13	87.87
EC- 30950	94.14 ± 0.97	86.60
IC- 202826	88.21 ± 0.87	84.00

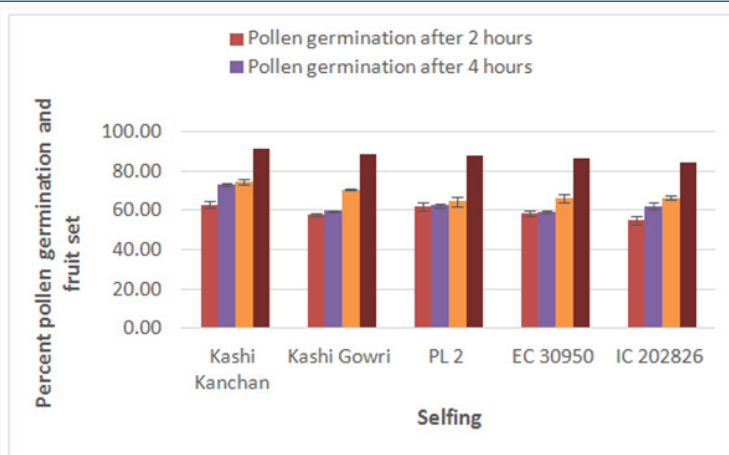


Figure 1: Pollen germination at different time intervals and percent fruit set in selfing.

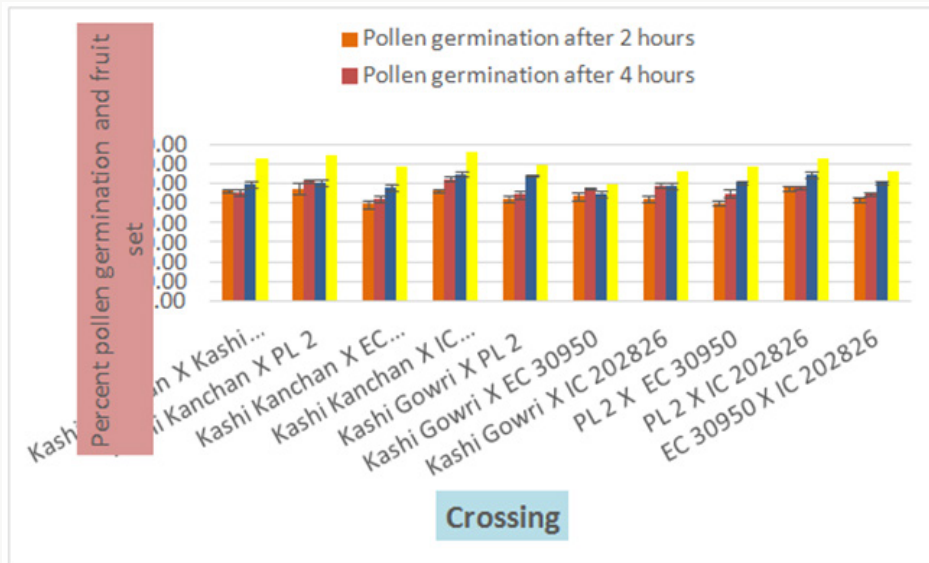


Figure 2: Pollen germination at different time intervals and percent fruit set in crosses.

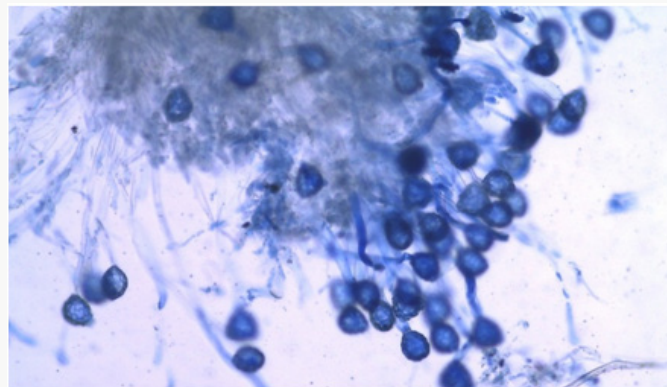


Figure 3: Pollen germination in Kashi Kanchan on selfing, after 4 hr of pollination (10 X).

Pollen Germination

Maximum pollen germination after 2 hr of pollination was observed in selfing of Kashi Kanchan (62.93 %) and the least pollen germination was recorded in cross of Kashi Kanchan and EC- 30950 (49.47 %). The results show that there was a constant increase in pollen germination from 2 hr to 6 hr in all the parents. After 2 hr of pollination, on selfing, maximum pollen germination was recorded in Kashi Kanchan (62.93 %) followed by PL- 2 (61.97 %), EC- 30950 (58.31 %), Kashi Gowri (57.98 %), and IC- 202826 (55.21 %). After 4 hr of pollination on selfing, maximum pollen germination was recorded in Kashi Kanchan (73.18 %) and the least was recorded in EC- 30950 (59.06%). After 6 hr of pollination on selfing, maximum pollen germination was recorded in Kashi Kanchan (74.52 %) and the least was recorded in PL- 2 (64.47 %).

In crosses between the parents, the maximum pollen germination was recorded in PL- 2 X IC- 202826 (57.88 %) after 2 hr of pollination and the least was found in Kashi Kanchan X EC- 30950 (49.47 %). After 4 hr of pollination, maximum pollen germination was recorded in Kashi Kanchan X IC- 202826 (62.40

%). The least germination was recorded in Kashi Kanchan X EC- 30950 (52.40%). After 6 hr of pollination, maximum pollen germination was recorded in Kashi Kanchan X IC- 202826 (64.97%) and the least was recorded in Kashi Gowri X EC- 30950 (54.96%). It was observed that pollen germination had significant correlation with fruit set. Similar results have been reported by [20,21] in wild accessions of chillies and tomato, respectively.

Pollen Tube Growth

The emergence of pollen tubes on the stigma and the style was seen after different time intervals on selfing and in crosses. Pollen fall on the stigma of each individual flower was variable because the amount of pollen grains differ during the hand pollination and it also depends on how many will stick on the stigma. The length of pollen tubes was measured (μm) at three different time intervals on selfing as well as in crosses. Length of pollen tube was recorded after staining with 1 % aniline blue stain. At 2 hr after pollination, a number of pollen grains had germinated and the tubes penetrated into the stigma hairs. Some of the pollen tubes had just started their growth in the hairs and a few had already entered the

transmitting tissue of the style. After 2 hr of selfing the maximum pollen tube growth was observed in Kashi Kanchan (86.90 μm) and the minimum in IC- 202826 (80.19 μm). Pollen tubes can act cooperatively during growth in the style to increase fertilization success [22] and they can also compete with each other to fertilize the available egg cells [23].

In case of crosses, the maximum pollen tube growth was observed in Kashi Kanchan X PL- 2 (77.11 μm) and the minimum was observed in Kashi Gowri X IC- 202826 (51.16 μm). After 4 hr of pollination, there was a greater differentiation and elongation in the pollen tubes. Some had just started to elongate and all intermediate

stages were observed. On selfing, maximum pollen tube growth was observed in Kashi Gowri (174.91 μm) and the least in EC- 30950 (167.53 μm) at 4 hr after pollination. In crosses, maximum pollen tube growth was observed in Kashi Kanchan X PL- 2 (154.32 μm) as at 2 hr after pollination and the least in Kashi Gowri (116.16 μm). On selfing, maximum pollen tube growth after 6 hr of pollination was observed in Kashi Kanchan (288.34 μm) and the minimum was observed in EC- 30950 (273.70 μm). In crosses, maximum pollen tube growth was observed in Kashi Kanchan X Kashi Gowri (243.52 μm) and minimum pollen tube growth was observed in Kashi Kanchan X EC- 30950 (220.07 μm). So, the results were quite similar at 2 hr, 4 hr and 6 hr after pollination (Figures 4-6).

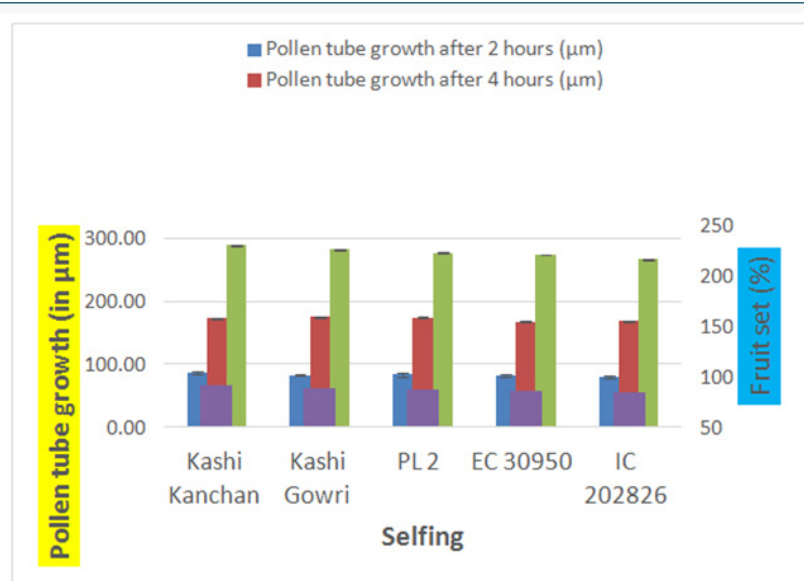


Figure 4: Pollen tube growth at different time intervals and percent fruit set in selfing.

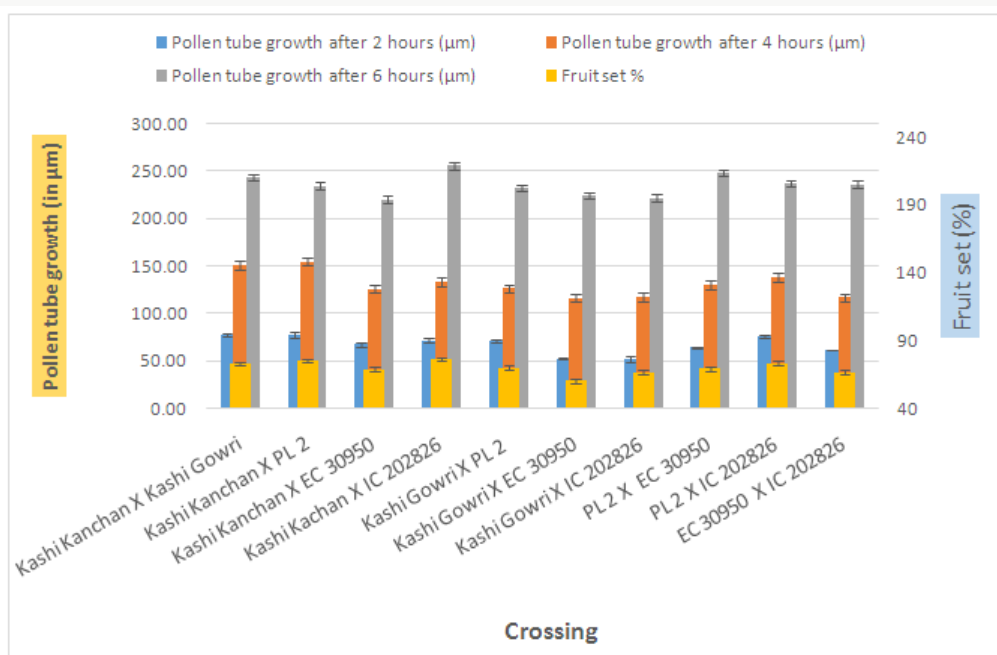


Figure 5: Pollen tube growth at different time intervals and percent fruit set in crosses

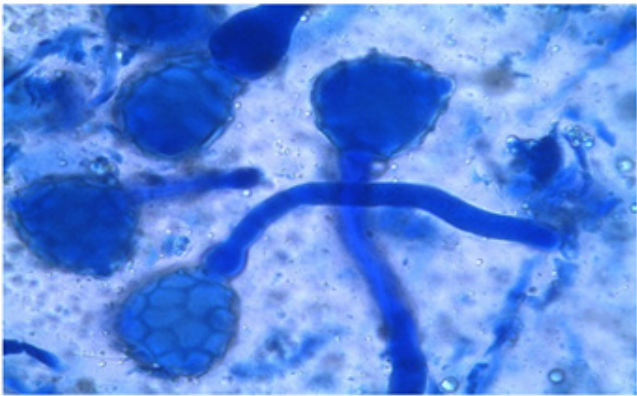


Figure 6: Pollen tube growth in Kashi Kanchan on selfing, after 4 hr of pollination (40 X).

Fruit Set

The maximum fruit set was obtained in selfing as compared to crosses. Among the selfing, the maximum fruit set was obtained in Kashi Kanchan (91.3 %) and the least in IC- 202826 (84 %). When the parents were crossed, the maximum fruit set was obtained in Kashi Kanchan X IC- 202826 (76.19 %) and the least fruit set was obtained in Kashi Gowri X EC- 30950 (60 %). In general, fruit set was less in crosses. The results indicated that cowpea flowers are self-fertile, and crosses in cowpea make good cross compatibility, which makes the successful formation of fruit set. This is similar to the results of Elmobarak (2005). This also reveals that crossing between the accessions was successful, as normal pod set was observed [24]. Reported that out crossing in flowering plants avoids the deleterious effects of inbreeding depression and promotes heterozygosity, genetic variability, and genetic exchange, which in turn leads to the long-term survival and adaptation of a species (Table 2) (Figures 7-10).



Figure 8: Fruit set of Kashi Gowri after selfing



Figure 9: Fruit set of EC- 30950 X Kashi Kanchan.



Figure 7: Fruit set of Kashi Kanchan after selfing



Figure 10: Fruit set of IC- 202826 X EC- 30950

Table 2: Pollen germination, pollen tube growth and percent fruit set on selfing and in crosses.

Sl. No.	Parents/Crosses	Pollen germination after 6 hr (%)	Pollen tube growth after 6 hr (μm)	Fruit set (%)
1	Kashi Kanchan	74.52 \pm 1.08	288.34 \pm 0.76	91.30
2	Kashi Gowri	70.41 \pm 0.37	281.83 \pm 0.89	88.23
3	PL- 2	64.47 \pm 2.25	276.54 \pm 0.80	87.87
4	EC- 30950	66.03 \pm 1.90	273.70 \pm 0.87	86.60
5	IC- 202826	66.22 \pm 1.08	266.00 \pm 0.95	84.00
6	Kashi Kanchan X Kashi Gowri	59.42 \pm 1.65	243.52 \pm 1.83	72.70
7	Kashi Kanchan X PL- 2	60.50 \pm 1.33	234.25 \pm 2.23	75.00
8	Kashi Kanchan X EC- 30950	57.99 \pm 1.78	220.07 \pm 0.90	68.75
9	Kashi Kanchan X IC- 202826	64.97 \pm 1.18	255.31 \pm 1.43	76.19
10	Kashi Gowri X PL- 2	64.28 \pm 0.44	231.96 \pm 0.97	70.00
11	Kashi Gowri X EC- 30950	54.96 \pm 1.60	224.46 \pm 1.33	60.00
12	Kashi Gowri X IC- 202826	58.85 \pm 1.65	221.69 \pm 0.91	66.60
13	PL- 2 X EC- 30950	60.59 \pm 0.67	247.88 \pm 1.25	69.23
14	PL- 2 X IC- 202826	64.52 \pm 1.45	237.03 \pm 1.71	73.30
15	EC- 30950 X IC- 202826	60.68 \pm 0.55	235.71 \pm 0.86	66.60

Correlation Studies in Crosses of *Vigna Unguiculata* L

Correlation studies on pollen germination recorded non-significant values for percent viable pollen. However, pollen germination after 4 hr of pollination was highly correlated with pollen germination after 2 hr of pollination and pollen germination after 6 hr of pollination had a highly significant correlation with pollen germination after 2 hr and 4 hr of pollination, respectively. Pollen tube growth after 6 hr of pollination recorded significant correlation with percent viable pollen. Pollen tube growth after 2 hr of pollination was observed to be significantly correlated with pollen germination after 4 hr of pollination and highly correlated with pollen germination after 2 hr and 6 hr of pollination. Pollen

tube growth after 4 hr of pollination was highly significant with pollen germination after 2 hr, 4 hr and 6 hr of pollination, and also with pollen tube growth after 2 hr of pollination. Pollen tube growth after 6 hr of pollination correlated with percent pollen fertility at 5 % level of significance and highly correlated with pollen germination after 2 hr, 4 hr and 6 hr of pollination and also with pollen tube growth after 2 hr and 4 hr of pollination. Fruit set had highly significant correlation with pollen germination (0.794, 0.687 and 0.872). A positive correlation with pollen tube growth was seen which was highly significant at 1 % level of significance (0.903, 0.947 and 0.939). At 5 % level of significance, positive correlation was seen between fruit set and percent viable pollen (0.892) (Table 3).

Table 3: Correlation studies for various characters in crosses of *Vigna unguiculata* L

Characters	Percent viable pollen	PG after 2 hr (%)	PG after 4 hr (%)	PG after 6 hr (%)	PTG after 2 hr (μm)	PTG after 4 hr (μm)	PTG after 6 hr (μm)	Pod set (%)
Percent viable pollen	1							
PG after 2 hr (%)	0.6827	1						
PG after 4 hr (%)	0.3240	0.798**	1					
PG after 6 hr (%)	0.6368	0.681**	0.704**	1				
PTG after 2 hr (μm)	0.7896	0.759**	0.523*	0.783**	1			
PTG after 4 hr (μm)	0.5544	0.795**	0.612**	0.731**	0.914**	1		
PTG after 6 hr (μm)	0.922*	0.752**	0.678**	0.857**	0.802**	0.878**	1	
Pod set (%)	0.892*	0.794**	0.687**	0.872**	0.903**	0.947**	0.939**	1

PG= pollen germination; PTG= pollen tube growth.

Note: *denotes significant at 5 % level of significance, ** denotes significant at 1 % level of significance.

SSR Analysis

Thirty six primers were used for genetic diversity analysis and twenty seven primers were found to be polymorphic. The polymorphic primers generated a total of 72 alleles. The data obtained from SSR analysis were subjected to Euclidean similarity analysis to find out the relationship among the accessions analyzed.

The value of Euclidean similarity coefficient ranged from 0 to 3.61. Based on Euclidean similarity matrix it was found that the lowest similarity coefficient was found between M-5 and Kashi Gowri (3.61) whereas EC- 559390 and EC- 9738; EC- 472260 and Sel- 16; EC- 30950 and PL- 2; EC- 472260 and EC- 9739; and Sel- 16 and EC- 9739 showed the highest similarity coefficient (0) (Table 4).

Table 4: Forward and reverse primer sequence for polymorphic SSR primers.

Sl. No.	Code	Sequence (5' - 3')	Tm (°C)	Allele
1	SSR-6550	F: GGTGACATTATATCTTCTG R: AAGTTACACCAATGCCAAAAAC	50.9 61.6	3
2	SSR-6553	F: ACTTTGTGACAATAGTGCTAC R: AAGGATTCTCAGATGATTAA	53.4 52.7	2
3	SSR-6556	F: GGTTACTAGCTAATTTTATT R: ATGAACCAGGTCTAATATGA	46.6 53.4	2
4	SSR-6577	F: GACATACAACATTTGAACATA R: AAGGAGGTAGATTAAGATGAG	52.3 63.3	3
5	SSR-6581	F: GAATTCCTCATCAACAAGTT R: GTGTTCCAGCTTTACTTTACTAG	55.6 52	2
6	SSR-6627	F: GGCCACTGTTGCAGAGC R: CATAAGAAGTAACAAGCAAAGATCC	64.8 61.9	2
7	SSR-6634	F: CGGGGGGCTGTGGG R: CATGCAAAGTCGAGGGC	71.8 63.2	3
8	SSR-6636	F: CCACAAATCTTCGAACACC R: GTAGTTTATATTTTCATTCACTTATGG	61.9 57.1	2
9	SSR-6652	F: CAAAATTCACGGTCACC R: CGGGAAGTGGAGGTAGCGCG	61.4 70.2	3
10	SSR-6676	F: CTTTCAGAGGATGCAGCC R: GACCACCTCCTTGCCTTTG	59.6 64.7	3
11	SSR-6206	F: AGGCATGCATTCATCTTTCC R: GCAGTCATAACCCAAAACAA	63.9 63.5	2
12	SSR-6209	F: AAACAAGATAACTCTAAGGCAGAACAA R: ACGGTGGAAGGTTAACTGGT	63.1 63.4	2
13	SSR-6243	F: GTAGGGAGTTGGCCACGATA R: CAACCGATGTAAAAAGTGGACA	63.8 63.5	3
14	SSR-6251	F: CCAAGAAAGGCCACTAGCAG R: GACGTTGAGCAGGGAACTC	63.8 63.8	3
15	SSR-6258	F: GGTTCCTAGTTGGGAAGGAA R: ATTATGCCATGGAGGGTTCA	63 64	3
16	SSR-6255	F: TGTTCACCTTGAATAGTATCAT R: TTGCAGGTCACCACTCTCTG	61.3 64.3	3
17	SSR-6257	F: TGCTTTTGTAAGGGTGGAA R: ACTTGACGGAACAGCAGAT	63.2 63.7	3
18	SSR-6281	F: GCATCAATTTGAGCGAGGAT R: GAGTGACATTTCCGCTCTT	64 64.2	3
19	SSR-6284	F: GAAAGGAAGGATTATGGGATA R: GGCAAATAGCGGGTAGAGT	61.7 64.1	2
20	SSR-6291	F: TCATGAGTTCCACACACCAA R: CCTTCGTATGTATGTGGCTACTG	64.1 62.3	2

21	SSR-6292	F :AAGGGTGCCTGGTAGAGGA R :GCTCACTTTGTGCATGTTCC	63.7 63.4	2
22	SSR-6302	F : TGGAGGCATAAAAATGACACCT R : AAGCTGATTGTGGAACCATTG	64.3 63.8	3
23	SSR-6314	F : TGGAGGCATAAAAATGACACCT R : TGAAGCTGATTGTGGAACCAT	64.3 64.4	4
24	SSR-6323	F : CAAAGGGTCATCAGGATTGG R : TTTAAGCAGCCAAGCAGTTGT	64.2 63.7	4
25	SSR-6876	F : GGAATTGAAATTGATCTAATG R : GTATTTAAGTGGCTTATGAGGTTG	55.2 59.5	2
26	SSR-6906	F : GGACATTTAGGATTGGGTGG R : CAAGAATGTCTGAACTAATATGC	62.9 58.4	2
27	SSR-6540	F :GGACATTTAGGATTGGGTGG R : CCATAGGTTAACTTATTGTACTC	62.9 54.6	3

From the dendrogram generated using Paleontological Statistics, PAST v3.15 software, the genotypes were grouped into two major clusters. The first major cluster was divided into I A and I B sub- clusters. The second major cluster II was divided into II A and II B sub- clusters. The sub- cluster I A consisted of EC-559390 and EC-9738 which are highly similar to each other. Sub- cluster I B was divided into two small sub- clusters I B I and I B II. Five accessions were grouped into sub-cluster I B I and Kashi Kanchan appears to be the most distinct from all the other in sub- cluster I B I. I B II sub- cluster comprise of 7 accessions and EC 472260 and Sel-16 are highly similar to each other. Cluster II was divided into two II A and II B sub- clusters. II A comprises of four accessions and Kashi Gowri appears to be most distinct from all the others in sub- cluster II A and EC- 30950 and PL- 2 are highly similar to each other. Cluster II B again was further divided into II B I and II B II

sub- clusters. Sub- cluster II B I comprises of 5 accessions and M-1 appears to be the most distinct from all the others in sub- cluster II B I. Thirteen accessions are grouped in small sub- cluster II B II and in this accessions, Kashi Shyawal and EC-390221 appear to be more distinct.

For an effective breeding programme, information concerning the extent and nature of genetic diversity within a crop species is essential [25]. Accurate assessment of genetic variability is important for the preservation and utilization of germplasm resources (Huaqiang et al., 2012). The knowledge of the genetic diversity available within the local and regional germplasm collections can enhance the overall effectiveness of cowpea improvement programs (Hegde and Mishra, 2009). The use of genetic diversity in breeding program is important to decrease crop vulnerability to abiotic and biotic stresses [26].

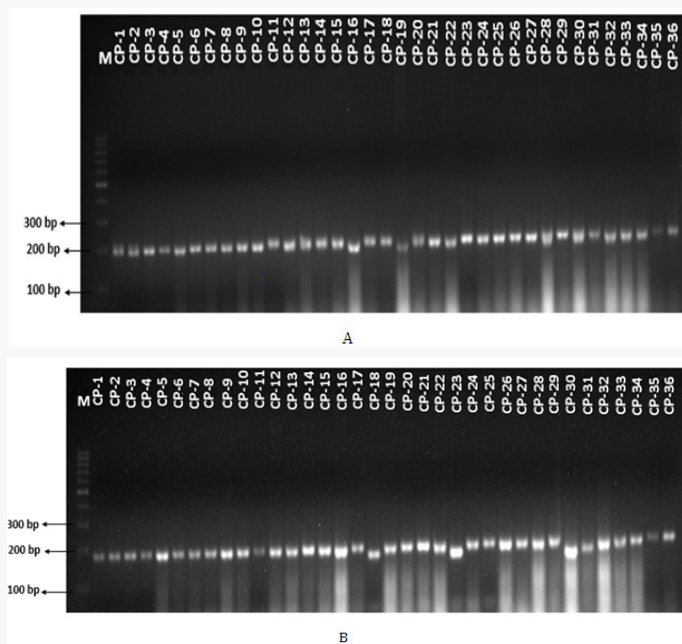


Figure 11: (A) and (B). SSR profiles generated by SSR 6257 and SSR 6314 primers, respectively on 36 accessions of *Vigna unguiculata*. Number 1-36 refers to genotypes, M indicates 100 bp ladder.

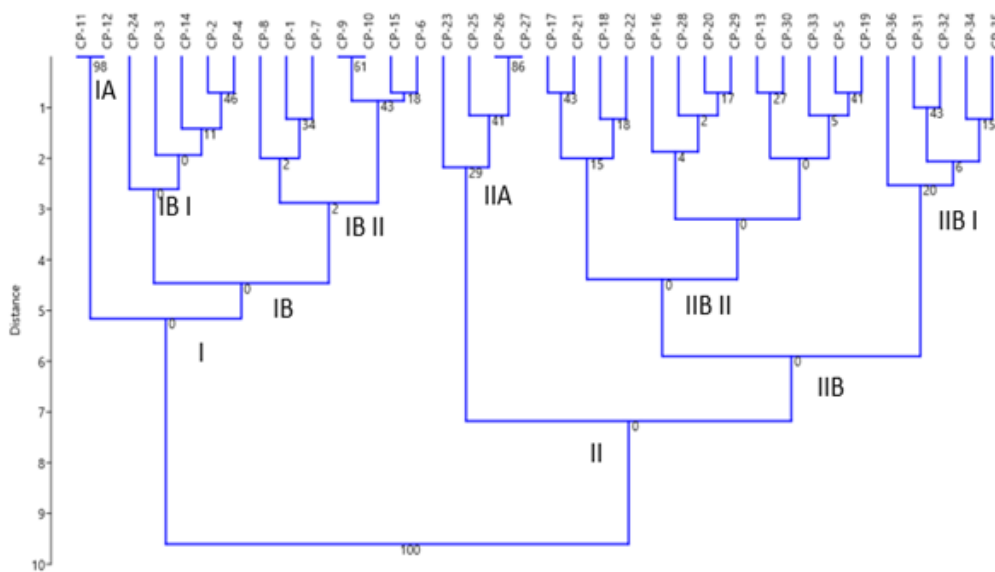


Figure 12: Dendrogram generated using Ward's method showing relationship between 36 accessions of cowpea.

In the present study genetic diversity among 36 accessions of cowpea was assessed by using 36 SSR markers (Figures 11 A & 11B). A total of 72 alleles were detected using 27 polymorphic SSR loci with the mean value of 2.66 per locus. The alleles number was lesser than that reported by [27] who detected 249 alleles with the mean value of 5.08 per locus. The polymorphism information content varied from 0.09 to 0.68 with an average of 0.35. This was similar to that reported by [28] who analyzed genetic diversity in 141 accessions of cowpea using 25 SSR markers. They found PIC value ranging from 0.07 to 0.66 with an average of 0.38. Based on PIC values, out of 27 primers, SSR 6314 was found to be highly informative. When compared with the study of [27] the allele's number was lesser which may be due to the use of more number of primers and the PIC value found in the study was more than the PIC value found by [27]. The Euclidean similarity coefficient for all 36 accessions ranged from 0 to 3.61. However, some of the accessions showed similarity value of 0. This may be due to lesser number of SSR primers used in the present study [29-32]. Cluster analysis performed based on Euclidean similarity matrix generated two major clusters. Cluster I and cluster II comprised of 14 and 22 accessions, respectively. Cluster I was divided into I A and I B sub clusters. The sub cluster II B was further divided into two small clusters. Cluster II was also divided into II A and II B sub clusters. The sub cluster II B was further divided into two small clusters (Figure 12).

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