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Research Article

SSR Marker Aided Parental Polymorphic Survey for Rust Resistance in Cowpea [*Vigna unguiculata* (L.) Walp]

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Abstract:
Nine Cowpea (*Vigna unguiculata*) genotypes-six landraces and three cultivated varieties were selected for the study. Polymorphic survey was done by using 36 SSR primers and genetic distances among parental lines were calculated. Primers VM 1, VM 28, VM 36 and VM 68 were found to be polymorphic with two to four alleles per locus. Polymorphism percentage was 11.11. The total number of polymorphic alleles were 11 and number of alleles amplified per locus on an average was 2.75. Five primers produced heterozygous bands. Primer VM 36 produced unique band for GC-3, making it useful for marker assisted introgression studies. Nine genotypes clustered into seven groups, which showed correlation to their geographical origin and distinguished the cultivated varieties from the landraces. Dendrogram consisted of two major clusters diverged at 18.56 per cent dissimilarity level. The genotypes exhibited lower diversity at molecular level and higher diversity at phenotypic level.

Keywords: Cowpea, microsatellite, polymorphism, dendrogram, germplasm and breeding

Introduction

Cowpea (*Vigna unguiculata* L.) is most widely grown and highly esteemed grain legume in Africa and Asia. Cowpea suffers from several diseases and pests. Among them, rust causes serious damage and limits productivity. There is a need to incorporate the resistance genes to the popular varieties which are in demand.Conventional breeding approaches for transferring resistant genes to cultivated varieties is very slow. It needs to be assisted with molecular tools, that can enhance the breeding programme, a rapid and quick identification of resistant plants at earlier stages of growth itself. In this regard the use of SSR markers as a tool to detect polymorphism between the cultivated varieties and landraces of cowpea and to identify the extent of genetic variation with respect to quantitative traits and rust resistance reaction, provids insight into the diversity of crop varieties and their potential contributions. Utility of microsatellite markers for assessment of genetic diversity among cultivars and their wild relatives has been demonstrated in many crops including soybean, maize, wheat, rice and sorghum [Diouf and Hilu,2005 & Gupta and Varshney,2000].The usefulness of SSR markers in assessing the level of genetic diversity in wild and cultivated cowpeas in recent past was reported by many scientists[Li *et al*.,2001 & Uma *et al*.,2009]. In the present work, the genetic diversity as well as relationships and variation among nine cowpea genotypes including three cultivated varieties, were investigated using microsatellite markers.

Material and methods

Genotypes: Nine cowpea genotypes (KBC-2, GC-3, C-152, IC 68786, IC 243353, IC 219607, IC 202778, IC 259084 and IC 202784) representing different geographical regions and pedigree were used in this investigation (Table 1).The data collected on 10 quantitative traits viz., Days to 50 % flowering, Days to maturity, plant height, number of clusters per plant, number of pods per plant, pod length, number of seeds per pod, 100 seed weight, seed yield per plant and percent leaf area under rust incidence on nine genotypes were used for analysis (Table 3). The scoring for rust was done at vegetative, flowering and pod formation stages of crop growth period [Mayee and Datar, 1986]. Per cent leaf area under rust incidence was calculated in each of the genotype. The analysis of variance was carried out for all the 10 traits.

DNA Extraction: Young healthy leaves were pooled from 25 days old field grown cowpeas, washed free of dirt, mopped dry and powdered using liquid nitrogen. DNA was isolated by CTAB method [Sambrook *et al*., 2001].

SSR Primers: 36 microsatellite primer pairs were used in the present study. Their names, sequence and PCR reaction conditions are listed in Table 2. VM21 and VM22 were designed based on the sequence of cDNA of mung bean [*Vigna radiate* (L.)R.Wilgek] and moth bean [*Vigna accontifolia* (Jacqua Marechal)] respectively. The other 34 primer sets were isolated from cowpea genomic SSR's.

Polymerase chain reaction (PCR) and polyacrylamide gel electrophoresis:PCR reaction were carried out in an Eppendorf thermocycler.The PCR mixture consisted of 20 ng template DNA, 20 ng of each of the primers, 0.1 mM dNTPs, 1x PCR buffer (10mM Tris, P^H 8.0, 50 mM KCl, 18 mM $MgCl_2$ and 0.1 mg/ml gelatin) and 1 unit of *Taq* polymerase in a volume of 20 ml. Depending on the Tm of primers used, amplification was performed by the following "Touch down" PCR profile [Don *et al*., 1991].

PCR profile consisting of 18 cycles of 94 ^oC for 1 minute(denaturing) and 72^oC for 1 minute(Extension). Annealing temperature (30 s) was reduced in every cycle from 64 to 55^oC at the rate of 0.5^oC per cycle. The PCR reaction continued for 30 additional cycle at 94^oC for 1min; 55^oC for 1min and 72 ^oC for 1min.The reaction ended with a 10 min extension at 72^oC. The amplified products were electrophoretically resolved on 9% poly acryl amide in 1X TAE buffer.

Gel scoring and data analysis:Each amplified loci were considered as a unit character and was scored as '0' and '1' for different levels of amplification obtained for each SSR markers. Genetic distances among breeding lines were calculated using score data with unweighted pair group average method in STATISTICA software.

Results and discussion

In the present study nine genotypes were analyzed for quantitative traits and rust resistance reaction. The genotypes differ significantly for days to 50 % flowering, Days to maturity, plant height, number of pods per plant, pod length,100 seed weight, seed yield per plant and per cent leaf area under rust incidence (Table 3).Genetic variation for all the quantitative traits and rust resistance reaction was observed in the cultivated varieties and land races. It may be due to diverse genotypes included in the present study representing all three growth types and different distinct morphological characters. They were also from different geographical origin within India (Table 1).Per cent leaf area under rust incidence varied from 1 to 53,among the parental lines, C-152 was highly susceptible with a score of 7 and other two cultivated varieties KBC-2 and GC-3 were moderately resistant and rest of six landraces were resistant to rust disease with a score of 1 and less than 1 per cent leaf area under rust incidence. Landraces IC 219607 and IC 202778 recorded maximum number of pods per plant, seed weight and seed yield per plant.

The phenotypic variation observed in any plant is often mismatching and may poorly reflect actual level of genotypic variation. By applying molecular techniques, the better understanding of genetic variation has been successfully achieved in many species. Phenotypically, cowpea is highly variable and influenced by the environment easily. However little is known about its variation at DNA level. In the present study, the selected genotypes were analysed for genetic variation for rust resistance using 36 SSR primers and some primers produced polymorphic bands with 11.11 per cent polymorphism. Out of 36 primers, 12 were polymorphic and 4 primers showed very distinct polymorphic bands. SSR primers VM 1, VM 28,VM 36 and VM 68 produced polymorphic bands with two to four alleles per locus (Fig.1). Maximum number of alleles amplified per primer pair was four in the present study. Number of alleles amplified per SSR primer pair was varied from 3-25 for rice, 11-26 for soybean, 3-16 for wheat and 2-23 for maize. Earlier studies in cowpea [Diouf and Hilu,2005 & Li *et al*.,2001] reported up to seven and nine alleles, respectively per SSR primer pair. This difference in number of alleles may be due to difference in the genotypes or varieties used and difference in the concentration of polyacrylamide gel. Low percentage of polymorphism and lesser number of polymorphic alleles indicate that microsatellite aided polymorphism is low in cowpea. Twelve per cent polymorphism for SSR primers was observed in cowpea on PAGE [Diouf and Hilu, 2005]. Low level of microsatellite polymorphism in cowpea was reported in earlier findings [Diouf and Hilu, 2005 & Li *et al*.,2001]. They attributed the low level of microsatellite polymorphism to relatively low genetic diversity of cowpea compared to other crops. It has been suggested that cowpea was domesticated only once [Ogunkanmi *et al*.,2008]. The low level of genetic diversity may be due to single domestication of cowpea[Li *et al*.,2001]. Some studies [Diouf and Hilu, 2005 & Ba *et al*.,2004] also indicated that genetic bottleneck induced by domestication as the

probable reason for low genetic diversity in cowpea. Legumes which are domesticated twice like common bean, showed high level of microsatellite polymorphism compared to cowpea [Blair *et al*.,2006]. In the present study SSR marker VM 36 produced unique band for GC-3. This primer can be used for marker assisted breeding programmes using GC- 3 as one of the parent.

Cluster diagram (Fig.2) constructed using 12 polymorphic markers identified two major clusters. First major cluster comprised IC 219607 and IC 243353. Both of these genotypes were collected from Andhra Pradesh. Genotypic similarity between both the genotypes may be due to same geographical origin. Clustering according to the geographical location from where they were collected was reported using SSR markers in cowpea[Uma *et al*.,2009]. Other major cluster was diverged to two sub-clusters. One sub-cluster contains IC 202784, IC 259084, IC 68786 and IC 202778. This sub-cluster divided into two individual clusters, containing genotypes IC 202784 and IC 259084 in one cluster and genotypes IC 202778 and IC 68786 in another. Markers used in the present study were not able to differentiate between IC 202784 and IC 259084 as well as IC 68786 and IC 202778. Another sub-cluster comprised GC-3, C-152 and KBC-2. Thus markers were able to differentiate cultivated varieties from landraces. Grouping together of domesticated accessions was reported in Cowpea[Ogunkanmi *et al*.,2008 & Ba *et al*.,2004]. With RAPD markers in cowpea. In addition, the separation of wild and domesticated cowpea gene pools was observed with isozyme data also [Pasquet, 1991]. Geographical origin of GC-3 is Gujarath and C-152 is a selection from Iran material. Grouping together of cultivated varieties in the same cluster irrespective of their geographical origin indicates the genetic uniformity produced through artificial selection. The two potential landraces IC 219607 and IC 202778 can be used as donors in introgression of rust resistant genes to popular cultivated variety C-152.

The present work indicates moderate to high level of genetic variation among cultivated and landraces of Cowpea genotypes with respect to quantitative traits and rust reaction and moderate level of variation was observed at DNA level, which otherwise showed a low level of polymorphism in different earlier studies [Li *et al*.,2001 & Uma *et al*.,2009].Therefore SSR markers serve as a basis for future work on tagging of disease resistance and agronomic traits, and construction of linkage map in cultivated Cowpea. This should be taken into account for the development of breeding programme.

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Table .1 Salient features of parental genotypes of cowpea

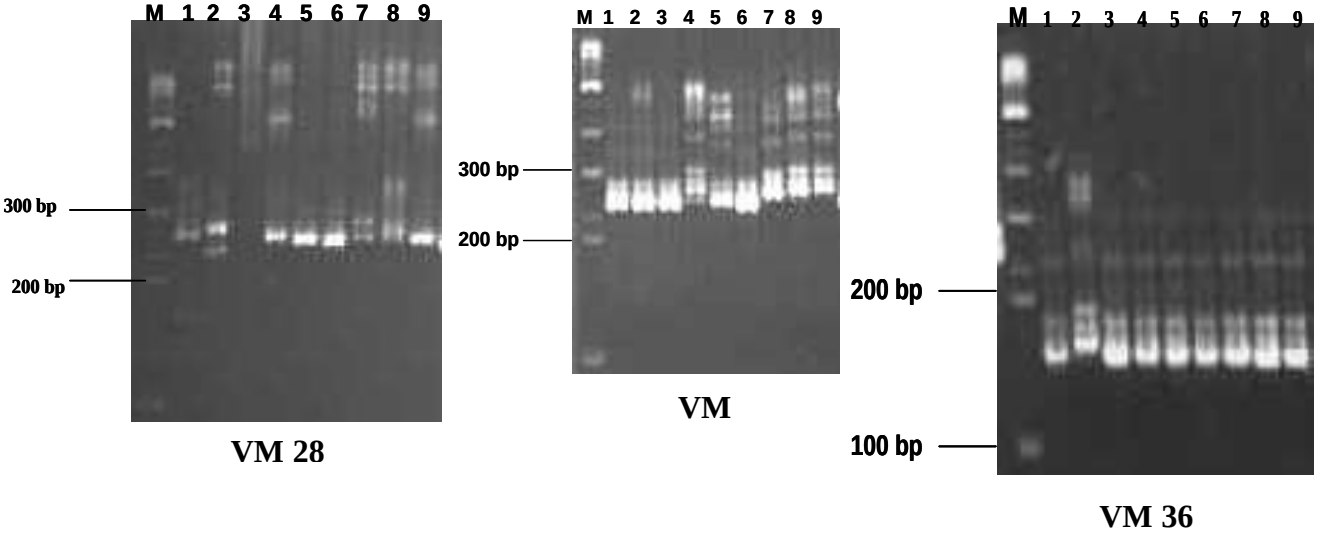
Parental genotype	Origin and Pedigree	Plant habit	Leaf type	Pod type	Seed type	
					Colour	Size
C-152	Selection from germplasm collection (Iran material from IARI)	Semi spreading Indeterminate	Medium green Ovate	Straight Green Seeds closely spread	Brown	Medium
KBC-2	Mutant of V-16	Spreading, Indeterminate	Green, Oval	Curved,seeds Closely packed	Browm	Medium
GC-3	Gujarat cowpea	Semi prostrate	Medium, Green	Short, green, Seeds closely spread	Cream	Medium
IC243353	Landrace, Andra Pradesh	Determinate, Semi spreading	Medium, Light green	Medium, Green, Seeds closely spread	Brown	Medium
IC219607	Landrace, Andra Pradesh	Semi determinate, Semi erect	Medium, Green,Trifoliate, Narrow lobed leaflets	-	Cream	Medium
IC202778	Landrace, Himachal Pradesh	Determinate, Semi spreading	Light green, Large	Long, Light green, Seeds loosely spread	Brown	Large
IC259084	Cultivar, IARI	Determinate, Semi erect	Medium, Light green	Medium, Light green, Seeds closely spread	Cream	Medium
IC68786	Collection from NBPGR	Semi erect	Light green, Red pigment at petiole ends	Small, Light green	Cream	Medium
IC202784	Landrace, South Goa	Semi erect	Large, Green	Short, Light yellow pods, Red colour pod petiole	Brown	Large

Table 2. List of SSR primers used, their sequence and annealing temperature

Primer name	Forward sequence (5' to 3')	Reverse sequence (5' to 3')	Annealing temperature (^{o}C)
VM 1	CACCCGTGATTGCTTGTTG	GTCCCCTCCCTCCCACTG	66.10
VM 2	GTAAGGTTTGGAAGAGCAAAGAG	GGCTATATCCATCCCTCACT	60.50
VM 4	AGTAAATCACCCGCACGATCG	AGGGGAAATGGAGAGGAGGAT	66.90
VM 5	AGC GAC GGC AAC AAC GAT	TTC CCT GCA ACA AAA ATA CA	63.70
VM 6	GAGGAGCCATATGAAGTGAAAAT	TCGGCCAGCAACAGATGC	65.40
VM 7	CGCTGGGGGTGGCTTAT	AATTCGACTTTCTGTTTACTTG	61.30
VM 8	TGGGATGCTGCAAAGACAC	GAAAACCGATGCCAAATAG	62.20
VM 9	ACCGCACCCGATTTATTTCAT	ATCAGCAGACAGGCAAGACCA	66.60
VM 10	TCCCACTCACTAAAATAACCAACC	GGATGCTGGCGGCGGAAGG	69.90
VM 11	C GG GAA TTA ACG GAG TCA CC	CCC AGA GGC CGC TAT TAC AC	65.00
VM 16	TCCTCGTCCATCTTCACCTCA	CAAGCACCGCATTAAAGTCAAG	66.20
VM 18	AGCCGTGCACGAAATGAT	TGGCCTCTACAACAACACTCT	62.60
VM 20	GGGGACCAATCGTTTCGTTC	ATCCAAGATTCGGACACTATTCAA	65.90
VM 21	TAGCAACTGTCTAAGCCTCA	CCAACTTAACCATCACTCAC	57.40
VM 22	GCG GGT AGT GTA TAC AAT TTG	GTA CTG TTC CAT GGA AGA TCT	57.80
VM 23	AGACATGTGGGCGCATCTG	AGACGCGTGGTACCCATGTT	66.70
VM 24	TCAACAACACCTAGGAGCCAA	ATCGTGACCTAGTGCCCACC	65.30
VM 27	GTCCAAAGCAAATGAGTCAA	TGAATGACAATGAGGGTGC	61.20
VM 28	GAATGAGAGAAGTTACGGTG	GAGCACGATAATATTTGGAG	56.30
VM 30	CTCTTTCGCGTTCCACACTT	GCAATGGGTTGTGGTCTGTG	65.30
VM 31	CGC TCT TCG TTG ATG GTT ATG	GTG TTC TAG AGG GTG TGA TGG TA	60.00
VM 32	GAAAAAGGGAGGAACAAGCACAAC	AGCGAAAACACGGAACTGAAATC	67.30
VM 33	GCACGAGATCTGGTGCTCCTT	CAGCGAGCGCGAACC	67.00
VM 34	AGCTCCCCTAACCTGAAT	TAACCCAATAATAAGACACATA	55.30
VM 35	GG CAA TAG AATAATGGAAAGTGT	ATG GCT GAA ATA GGT GTC TGA	59.55
VM 36	ACT TTC TGT TTT ACT CGA CAA CTC	GTC GCT GGG GGT GGC TTA TT	64.25
VM 37	TGT CCG CGT TCT ATA AAT CAG C	CGA GGA TGA AGT AAC AGA TGA TC	63.10
VM 38	AATGGGAAAAGAAAGGGAAGC	TCGTGGCATGCAGTGTCAG	65.80
VM 39	GAT GGT TGT AAT GGG AGA GTC	AAA AGG ATG AAA TTA GGA GAG CA	60.75
VM 40	TATTACGAGAGGCTATTTATTGCA	CTCTAACACCTCAAGTTAGTGATC	59.00
VM 68	CAA GGC ATG GAA AGA AGT AAG AT	TCG AAG CAA CAA ATG GTC ACA C	63.70
VM 69	CAAAGCATTGGGCCCTTGT	GGCTTTGGGACCTCCTTTCC	67.40
VM 70	AAA ATC GGG GAA GGA AAC C	GAA GGC AAA ATA CAT GGA GTC AC	63.40
VM 71	TCG TGG CAG AGA ATC AAA GAC AC	TGG GTG GAG GCA AAA ACA AAA C	68.10
VM 72	TGCTGAAGTGAACAATCGC	CCTTCTCCAACAACTCTAC	58.10
VM 73	CGGCGTGATTTGGGGAAGAAG	CTAGTAACGGCCGCCAGTGTCCTG	64.00

Table 3. Phenotypic characters of parental lines of Cowpea

Genotypes	Days Flowering	Days to maturity	Plant height (cm)	Number of clusters	Number of pods per plant	Pod length (cm)	Seeds per pod	Test weight (g)	Single plant yield (g)	% Area affected	Rust Score
C-152	60	78	39.00	11	17	14.67	12	11.00	22.45	43	7
KBC-2	67	90	31.20	11	15	17.22	15	9.50	26.84	15	3
GC-3	63	79	42.30	12	15	15.36	14	10.00	25.40	20	3
IC 243353	62	90	45.50	13	15	19.74	14	13.00	23.97	2	1
IC 219607	63	74	43.40	15	23	15.63	11	9.50	27.00	4	1
IC 202778	65	91	51.20	10	15	19.12	13	13.00	14.24	4	1
IC 259084	59	75	40.10	13	15	16.12	12	8.50	16.41	1	1
IC 68786	62	81	41.50	12	17	13.87	12	9.00	20.00	3	1
IC 202784	52	79	39.40	11	15	11.07	11	12.30	20.24	3	1



M: Molecular weighted marker (100 bp)
1:KBC-2; 2: GC-3; 3: C-152; 4: IC 68786; 5:IC 243353; 6: IC 219607; 7: IC 202778; 8: IC259084; 9: IC 202784

Figure1: DNA amplified products of VM 28, VM 36 and VM 68 for parental lines resolved on PAGE

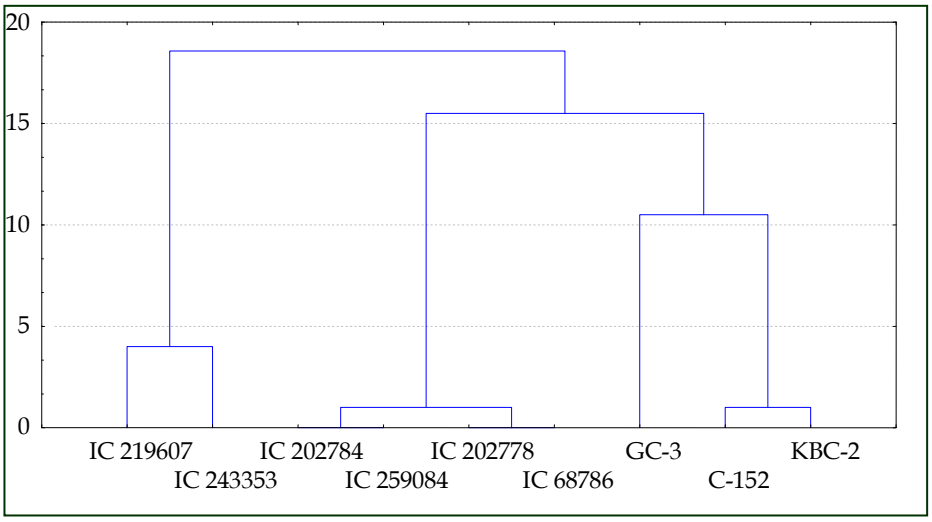


Figure 2: Molecular dendrogram of parental genotypes constructed using SSR primers