Legume Research, 42(1) 2019: 32-38
Print ISSN:0250-5371 / Online ISSN:0976-0571

AGRICULTURAL RESEARCH COMMUNICATION CENTRE
www.arccjournals.com/www.legumeresearch.in

Molecular dissection of genetic diversity in pigeonpea [*Cajanus cajan* (L) Millsp.] minicore collection

S. Muniswamy*, R. Lokesha[1], Rachit Saxena[2], B. Fakrudin, Kishan Patel[3] and Yamanura

Pulse Research Institute,
Agricultural Research Station, Gulbarga-585 104, Karnataka, India.

Received: 19-02-2017 Accepted: 12-01-2018 DOI: 10.18805/LR-3817

ABSTRACT

The present investigation was carried out using 191genotypes as mini core collections of pigeonpea along with 5 check varieties to know the genetic diversity at molecular level. Significant variation was observed by the way of analysis of variance for nine characters *viz.*, days to 50 % flowering, days to maturity, plant height, number of branches per plant, pod bearing length, number of pods per plant, number of seeds per pod, seed yield per plant and hundred seed weight. Molecular diversity using 18 polymorphic simple sequence repeat (SSR) markers divided genotypes into 15 clusters, of which ICP-11059 and AK-101 were solitary, indicating their distinctiveness among all genotypes. Similarly, BSMR-533, JKM-7, RVK-285, ICP-1126, ICP-348, ICP-6859 and ICP-7869 were found distinct among the genotypes. Geographical origin based diversity separated Indian and non Indian genotypes. The Un weighted Pair Group Method with Arithmetic mean (UPGMA) based dendrogram indicated distinctiveness of ICP-13633 and Bennur local, as they formed solitary cluster. The SSR marker CcM 602, as it could differentiate 4 genotypes at different base pair size can be used for identification and finger printing of genotypes.

Key words: *Cajanus cajan*, Genetic diversity, Minicore, Molecular characterization, Pigeonpea.

INTRODUCTION

Pigeonpea is cultivated in more than 25 tropical and sub-tropical countries, either as sole crop or as an inter crop with finger millet, sorghum, pearl millet, maize or even with short duration legumes. Globally, it is cultivated in a total area of 4.92 million ha (mha), with an annual production of 3.65 million tonnes (mt) and productivity is around 900 kg/ha. India has ~80 % of world acreage (3.90 mha) with a total production and productivity of 2.89 mt (~79 % of world acreage) and around 750 kg/ha respectively (http:// www.faostat.fao.org). It plays an important role in food security, balanced diet subsistence agriculture because of its diverse usage in food, fodder, soil conservation, integrated farming systems and symbiotic nitrogen fixation (Reddy *et al.*, 2005). Further, pigeonpea offers a rich source of variability in the form of wild species relatives, which could be used for bringing favourable alleles in cultivated gene pool for disease resistance, good agronomic traits, enhancing nutritional quality, identification and diversification of cytoplasmic base of Cytoplasmic Male Sterility (CMS) systems etc. Available genetic stock in pigeonpea have been characterized based on few agronomic traits and limited number of simple sequence repeat (SSR) markers previously and different types of subsets such as core collection, minicore collection and reference sets have been defined (Upadhyaya *et.al.*,2010). However, lack of sufficient characterization data and linkage drag have hindered the use of wild species in breeding programs.

Success of crop improvement program, in any crop *vis a vis* pigeonpea, depends on genetic diversity and extent of available variability, choice of parents for hybridization and selection. The existence of variability provides abundant chances to breeders to pick up the genotypes according to their necessities or type of breeding programme. Intriguingly, use of molecular markers will further help in identification of genotypes with accuracy and also been used to assess diversity. The concept of genetic distances is vital in many contexts and more so in differentiating well defined population (Arunachalam, 1981).

Among different kinds of molecular markers, SSR markers have proven as the markers of choice in practical breeding because of their abundance in genome wide distribution, reliable, reproducible and less cumbersome nature (Gupta and Varshney, 2000; Varshney *et al.*, 2005). Keeping above in view this study has undertaken detailed molecular and phenotypic characterization of pigeonpea minicore collection.

*Corresponding author's e-mail: muniswamygpb@gmail.com
[1]Department of Genetics and Plant Breeding, College of Agriculture, Raichur-584 102, Karnataka, India.
[2] International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, Hyderabad-502 324,Telangana, India.
[3]University Horticulture Sciences, GKVK, Bangalore-560 065, Karnataka, India.

MATERIALS AND METHODS

Plant material and experiment design: The plant material comprised of 196 *Cajanus* accessions representing pigeonpea minicore collection (191 accessions) and five check varieties [ICPL 87119 (ASHA), WRP-1, TS 3R, BSMR-736 and ICP 8863 (Maruti)]. Out of 196 *Cajanus* accessions, 167 accessions were received from National Initiative on Climate Resilient Agriculture (NICRA) network project and 29 accessions were local collections maintained at ARS, Gulbarga. The experiment was carried out in Lattice Design (14 × 14) with two replications during 2013-2014. Each accession was sown in 2 rows of 4 meter length with a spacing of 90 cm and 30 cm between row to row and plant to plant respectively. Sowing was undertaken by hand dibbling @ two seeds per hill. Thinning was carried out at 30 days after sowing to retain only one healthy seedling per hill. Intercultural and plant protection measures were adopted as per the package of practices recommended by the University of Agricultural Sciences, Raichur and Dharwad (Anonymous, 2014). The observations on nine phenotypic characters *viz.*, days to 50 % flowering, days to maturity, plant height, number of branches per plant, pod bearing length, number of pods per plant, number of seeds per pod, seed yield per plant and hundred seed weight were recorded on five randomly selected plants in each accession.

Genomic DNA isolation and SSRs: Genomic DNA was isolated from two to three young leaves of *Cajanus* accessions following a procedure mentioned in Cuc *et al*. (2008). A total of 20 SSR markers available in public domain (Saxena *et al.,* 2010a, b and Metkar *et al.,* 2010*)* were used for genotyping (Table 1).

Polymerase chain reactions (PCRs): PCR for amplification of SSR loci were performed in a 10 µl reaction volume using 1.0 µl of 10X PCR buffer, 0.4 µl of 25 mM $MgCl_2$, 1.2 µl of 2mM dNTPs, 0.5 µl of 10 pM/µl forward primer, 0.5 µl of 10 pM/µl reverse primer (MWG-Biotech AG, Bangalore, India) 0.3 µl of Taq polymerase (Bioline, London, UK), 2.0 µl (5ng/µl) of template DNA ,0.6 µl dye (either Pet, Ned,Vic or Fam) and 3.5 µl millipore water in 96-well micro titre plate (AB gene, Rockford, IL, USA) using thermal cycler GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA, USA). A touch down PCR programme was used to amplify the DNA: initial denaturation was for 5 min at 95°C followed by 5 cycles of denaturation for 20 sec at 94°C, annealing for 20 sec at 60 °C (the annealing temperature for each cycle being reduced by 1°C per cycle) and extension for 30 sec at 72°C. Subsequently, 35 cycles of denaturation at 94°C for 20 sec followed by annealing for 20 sec at 56°C and extension for 30 sec at 72°C and 20 min of final extension at 72°C. PCR products were checked for amplification on agarose gel.

Capillary electrophoresis: For post-PCR multiplexing 1.5 µl PCR product of each Fam, Vic, Ned and Pet labeled amplified SSRs were pooled (according to above mentioned criteria) and mixed with 7 µl Hi-Di formamide (Applied Biosystems, USA) 0.2 µl of the Liz-500 size standard (Applied Biosystems, USA) and 2.8 µl distilled water. The pooled PCR amplicons were denatured at 95°C for 5 min.

Table 1: Details of the polymorphic SSR markers used across minicore genotypes of pigeonpea.

SSR Marker	Expected size of PCR product	Observed size range (bp)	Major allele freq.	Allele No.	PIC
CcM 008	182	150-200	0.57	2	0.67
CcM 021	280	270-300	0.49	3	0.73
CcM 047	160	160-180	0.84	2	0.27
CcM 0121	273	250-300	0.67	2	0.59
CcM 0126	218	200-270	0.63	2	0.60
CcM 0133	176	160-210	0.76	3	0.40
CcM 0171	157	100-250	0.94	2	0.12
CcM 0181	278	270-300	0.75	2	0.42
CcM 0185	232	230-250	0.65	2	0.55
CcM 0257	241	230-300	0.74	2	0.40
CcM 0262	203	200-250	0.77	3	0.38
CcM 0268	210	200-250	0.79	3	0.36
CcM 0494	117	100-150	0.71	2	0.48
CcM 0588	266	250-300	0.93	3	0.13
CcM 0602	216	200-250	0.40	4	0.73
CcM 0698	188	150-200	0.75	3	0.42
CcM 0844	224	200-250	0.82	2	0.29
CcM 0195	223	200-250	0.73	2	0.45
Maximum	-	-	0.94	4	0.73
Minimum	-	-	0.40	2	0.12
Mean	-	-	0.72	2.44	0.44

Dissimilarity Maximum Value: 1.0
Dissimilarity Minimum Value: 0.1
Euclidian index value: 0.49

and cooled immediately on ice and size separated by capillary electrophoresis using an ABI Prism 3730 DNA analyser (Applied Biosystems, Inc.). Raw data produced from the ABI 3730 DNA analyser were analyzed using Genemapper® software version 4.0 (Applied Biosystems, USA) and fragment size was scored in base pairs based on the relative migration of the internal size standard, LIZ 500.

Statistical analysis: The analysis of variance was carried out for all characters individually using MSTAT-C.

Genetic parameters namely major allele frequency and polymorphic information content (PIC) value were estimated using following formula (Anderson *et al.*1993):

$$PIC = 1 - \sum_{i=1}^{k} P_i^2$$

Where k is the total number of alleles detected for a given marker locus and *Pi* is the frequency of the i^{th} allele in the accessions analyzed. Phylogenetic tree was constructed using the Neighbourhood-joining algorithm using DARwin 5.0 (Perrier *et al.*, 2003).

RESULTS AND DISCUSSION

The mean sum of squares (MSS) due to genotypes were highly significant for all the nine characters under investigation. Significant differences among genotypes is prerequisite for further statistical analysis. The details are presented in Table 2.

Molecular diversity in minicore collection: A total of 20 SSR markers were used for estimating molecular diversity across *Cajanus* accessions. Out of 20 SSR markers, 18 SSRs were found polymorphic across 196 *Cajanus* accessions. Polymorphic SSRs provided a total of 44 alleles with an average of 2.44 alleles per marker. Allele numbers detected by polymorphic SSRs varied from 2 in 11 markers to 4 (CcM 0602). The PIC value for these markers ranged from 0.12 (CcM 0171) to 0.73 (CcM 0602), with an average of 0.44 per marker. While major allele frequency at the polymorphic SSR loci ranged from 0.40 (CcM 0602) to 0.94 (CcM 0171 (Table 1). The results thus suggest the suitability of the selected markers for reliably ascertaining genetic diversity in the minicore collection.

The cluster analysis based on Un Weighted Pair Group method with Arithmetic mean (UPGMA), revealed 15 main clusters (Fig.1). Cluster analysis indicated dissimilarity minimum and maximum value of 0.1 and 1.0 respectively, with Euclidian index value of 0.499. It indicates wide variation is present among the studied genotypes, so that variability can be created by crossing these genetically distant accessions. It is very interesting that, out of 15 main clusters, two cluster were having only one genotype each (AK 101 and ICP 11059). It signifies the distinctiveness of these two genotypes among 196 accessions. The genotype AK 101 genetically distinct from others, it is having desirable characters like early maturity (137 days), hence, it can serve as good parent for crossing programme. Similarly, ICP-11059 is also a genetically distant parent for hybridisation. Sub cluster analysis revealed that the genotypes *viz.*, BSMR-533(27), JKM-7(60), RVK-285(88), ICP-1126(109), ICP-348(150), ICP-6859(168), ICP-7869(179) were distinct among 196 genotypes and these can be used as genetically distant parent for hybridisation [value in parenthesis indicates genotype number of Fig 1].

Panguluri *et al.*, (2006) observed low polymorphism among cultivated pigeonpea and very high polymorphism between cultivated and wild relatives. Sarika and Anand (2013), Neha and Dinesh (2010), Yadav *et al.* (2012) studied genetic diversity using RAPD markers. Songok and Serah (2013), Singh *et al.* (2013) and Saxena (2010a) used simple sequence repeats for diversity analysis. Wasike *et al.* (2005) observed that East African pigeonpea cultivars are less diverse than Indian cultivars. Yang *et al.* (2006) noticed low diversity among cultivated types, however, he noticed high diversity among wild species and between wild and cultivated species. Odeny *et al.* (2007) observed less allelic variation with in cultivated species than across wild species.

Geographical diversity based on origin: Genetic diversity arises due to geographical separation or due to genetic barriers to crossability. Hence, a total 26 genotypes comprised of thirteen each of the genotypes originated outside India (foreign) and local origin (genotypes of ARS, Gulbarga) were analysed for diversity at molecular level.

Table 2: Analysis of variance for yield and yield attributing characters in minicore collection of pigeonpea.

Source of variation	d.f	DFF	DM	PH	Br/plant	PBL	NPPP	NSPP	Yd/ pnt	100 SW
Replications	1	18.0	117.92	426.52	107.4	2217.0	5412.0	0.43	404.70	0.003
Genotypes	195	328.06**	337.45**	439.6**	44.7**	114.03**	2080.6**	0.43**	126.2**	3.51**
Blocks	26	0.40	1.07	29.39	6.35	21.1	72.4	0.104	5.55	0.015
Error (Intra block)	169	1.50	2.09	38.74	10.34	19.6	142.9	0.09	8.03	0.032
Total	391	164.41	169.57	239.0	27.49	72.4	1118	0.264	67.85	1.76

**=>Significant at P=0.01

DFF: Days to fifty per cent flowering
DM: Days to maturity
PH: Plant height
Br/plant: Branches per plant
PBL: Pod bearing length
NPPP: Number of pods per plant
NSPP: Number of seeds per pod
Yd/pnt: Seed yield per plant
SW: Seed weight
d.f: degrees of freedom

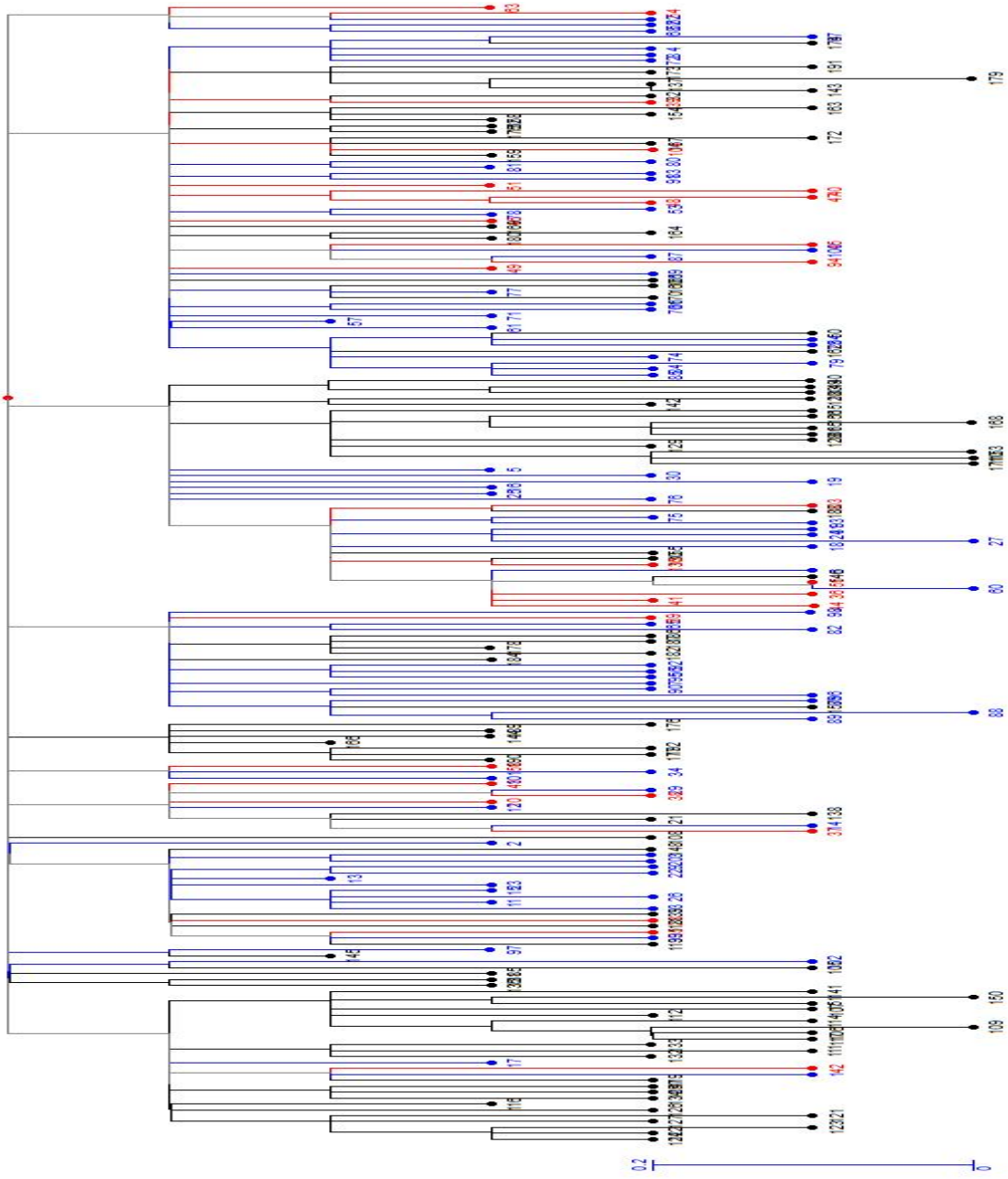

Fig 1: Dendrogram showing diversity among minicore collection of pigeonpea.
Legend: Red = Gulbarga genotypes, Pink = Kanpur genotypes, Black = ICRISAT genotypes.

The clusters analysis based on UPGMA revealed 7 main clusters with dissimilarity minimum and maximum value of 0.3 and 1.2 respectively having Euclidian index value was 0.619. It indicates the high diversity in the selected genotypes. Among the 7 main clusters, two clusters were solitary having one genotype each (ICP 13633 and Bennur local) (Fig 2). The accession ICP 13633 has originated in Nigeria, exhibited distinctiveness among 26 genotypes. Similarly, Bennur local has originated in India (Karnataka), having desirable characters like early maturity, drought tolerance *etc*. Two group of independent clusters of local and foreign origin were formed indicating genetic diversity due to geographical separation, except for the two out of thirteen genotypes *viz.*, ICP 14116 (origin; Jamaica) and ICP 7148 (origin; Srilanka), which were clustered with local accessions. The values of genetic distance followed

Table 3: Unique SSRs identified through capillary electrophoresis.

Marker	Genotypes	Unique base bairs observed (bp)
CcM 0602	AL-201	281 and 284
	ASHA	278
	RVK 283	237
	RVK 284	210

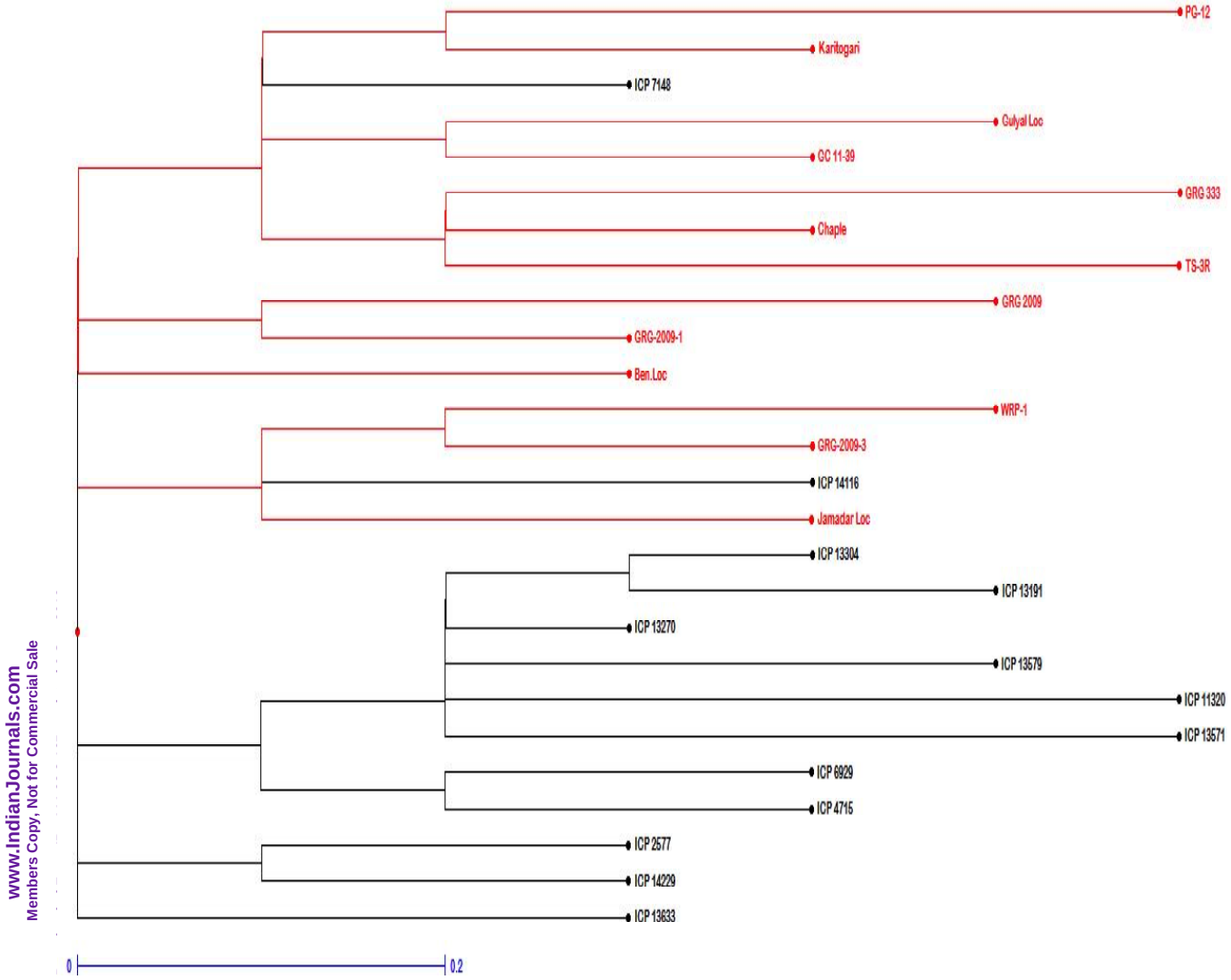

Fig 2: Dendrogram generated using 13 each of Indian and non-Indian origin Pigeonpea.
Legend: Red = Indian origin , Black = Non Indian origin

geographical proximity. The results are in agreement with Songok *et al.* (2010), who studied germplasm from Kenya, Tanzania, Uganda and India. Upadhyaya *et al.* (2007) studied pigeonpea genotypes collected in different elevations in Kenya and observed that elevation of collection site is important in determining variance. In contrast, Katiyar *et al.* (2004) evaluated 221 accessions of early maturing pigeonpea genotypes (*Cajanus cajan*) of diverse eco-geographical origin and observed that diversity was not related to geographical diversity.

Unique SSR obtained through capillary electrophoresis: The marker CcM 602 was highly polymorphic (PIC = 0.73) and could able to distinguish 4 genotypes among 196 genotypes *viz.*, AL 201, ASHA, RVK 283 and RVK 284 at different base pairs (Fig.3a and b). Hence, the marker CcM 602 can be used for identification and finger printing of these genotypes. The details are presented in Table 3.

Saxena *et al.*(2010) used SSR marker by capillary gel electrophoresis for purity assessment of hybrid ICPH 2438 and characterisation of hybrid parents. Ventriventhan *et al.* (2012) used SSR markers through capillary gel electrophoresis for studying genetic relationship among races of core collection in pearl millet. Metkar *et al.* (2010) characterised CMS lines of pigeonpea at molecular level.

CONCLUSION

The genotypes AK 101 and ICP-11059 were found genetically distant and agronomically superior parents for hybridisation. Sub cluster analysis revealed that the genotypes *viz.*, BSMR-533, JKM-7, RVK-285, ICP-1126, ICP-348, ICP-6859, ICP-7869 were distinct among 196 genotypes and these can be used as genetically distant parent for hybridisation. The marker CcM 602 can be used for identification and finger printing of genotypes *viz.*, AL 201, ASHA, RVK 283 and RVK 284.

Fig 3a: Snapshot showing electropherogram of allele size difference between genotypes AL- 201 and ASHA by the marker CcM 602.

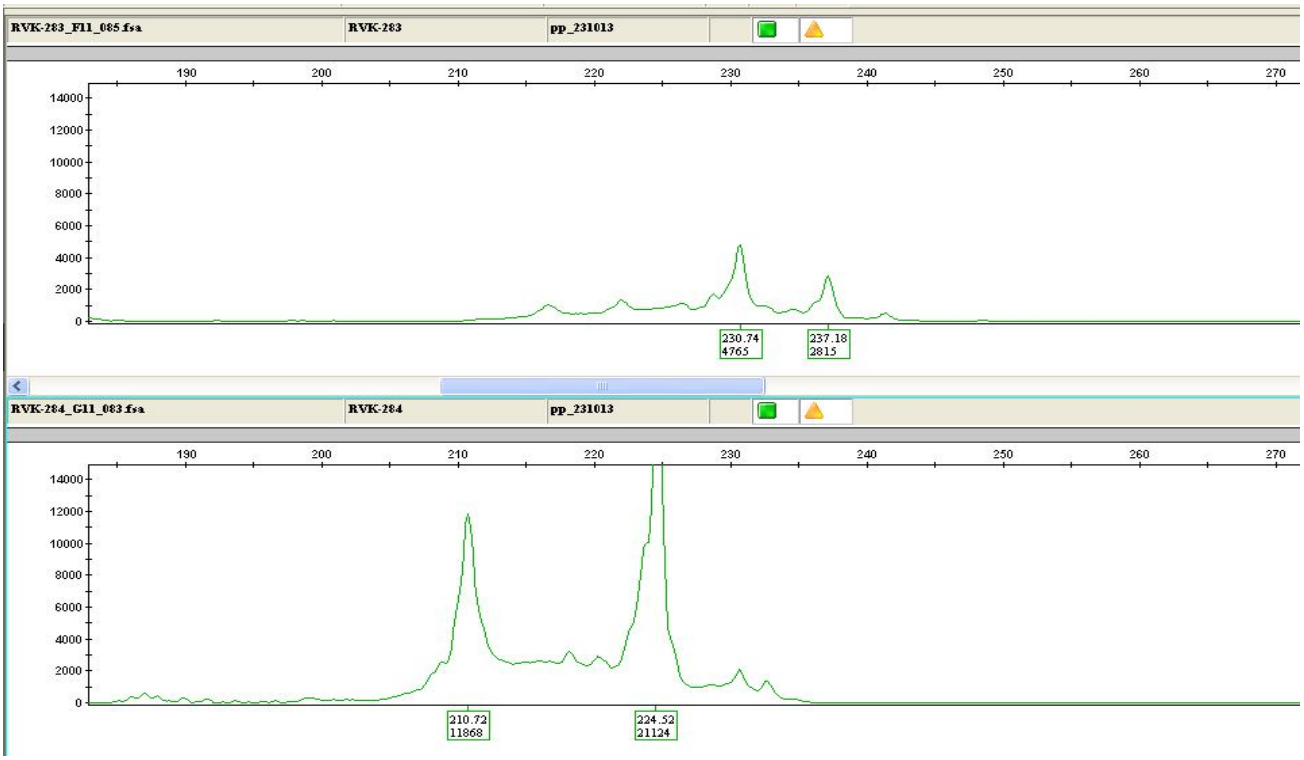

Fig 3b: Snapshot showing electropherogram of allele size difference between genotypes RVK 283 and RVK 284 by the marker CcM 602.

ACKNOWLEDGEMENT

The laboratory work on molecular diversity was carried out at Dept.of Biotechnology, UAS, Dharwad and Centre of Excellence in Genomics at ICRISAT, Patancheru, Hyderabad. (Under the guidance of Dr.Rajeev K. Varshney). The seed material was obtained from NICRA net work project.

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