The Bioscan 10(2): 957-962, 2015 (Supplement on Genetics and Plant Breeding)
AN INTERNATIONAL QUARTERLY JOURNAL OF LIFE SCIENCES
www.thebioscan.in

ASSESSING GENETIC VARIATION OF PIGEONPEA [*CAJANUS CAJAN* (L.)] GENOTYPE USING RAPD AND ISSR MARKERS SYSTEMS

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KEYWORDS
Genetic diversity
ISSRs
RAPDs
Cajanus cajan

Received on :
10.02.2015

Accepted on :
25.05.2015

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ABSTRACT

Pigeonpea is an important food legume being as a key ingredient of the human diet and imperative component of sustainable agriculture /cropping system. In present investigation 42 pigeonpea genotypes were evaluated for genetic relationship analysis using 10 random amplified polymorphic DNAs (RAPDs) and 10 inter simple sequences repeats (ISSRs)markers.Ten RAPDs primers generated 43 bands, of which 39 were found to be polymorphic. While in case of ISSR, 45 were observed to be polymorphic of the total 52 markers screened. Concerning DNA polymorphism detection, RAPD markers were found to be more efficient than the ISSR, as the former detected 90.6% compared to 86.5% for ISSR markers. Polymorphic information content (PIC) value of ISSR (7.67) was higher as compared to RAPD (7.29). Furthermore, Jaccard's similarity coefficient obtained by pooled data revealed narrow range (0.71 to 0.93) among the studied genotypes. In the UPGMA based dendrogram the cluster analysis of RAPD and ISSR combined data revealed near similar clustering pattern. In this study we insight about the practical relevance of RAPD and ISSR based marker systems for understanding the genetic relationships in diverse pigeonpea genotypes.

INTRODUCTION

India is the largest producer and consumer of pulses in the world accounting for 25-28% of the global kitty (Parihar and Dixit, 2014). Being a primary source of dietary protein in the diet of the vast majority of population and mainstay of sustainable crop production, pulses continue to be play role of an important component of the semi-arid and subtropical farming systems. Pigeonpea [*Cajanus cajan* (L.); 2n = 22] is an important pulse crop of India after chickpea, its belongs to the genus *Cajanus,* subtribe *Cajaninae* and popularly known as red gram or Arhar or tur (Nagy *et al.,* 2013). It is the only food crop of the *Cajaninae* sub-tribe cultivated for multipurpose as food, feed and firewood (Dikshit *et al.,* 2014; Greilhuber and Obermayer 1998; Yadav *et al.,* 2014). Not with standing of being an important pulse crop of India pigeonpea has portrayed low yield potential (700 kg/ha) compared to other grain legumes over the years. Additionally, genetic improvement in production and productivity of most of the pulse crops remains very slow due to several constraints along with little attention paid by researchers. Never the less, in recent years Pigeonpea has become increasingly important because of its inherent ability to perform well under marginal input systems and to withstand a range of environmental stresses including drought. Despite the existence of substantial variability among pigeonpea landraces and varieties for various traits, no effective molecular breeding programme has been developed to facilitate its improvement.

The assessments of molecular diversity through DNA-based markers remain one of the basic strategies for understanding of amount and pattern of variation exists in pigeonpea germpalsm. A large number of molecular markers with polymorphism are required to assess genetic relationship and genetic diversity in reliable manner (Santalla *et al.,* 1998). However, assessment of genetic variability in different pigeonpea has been done using various molecular markers by different researchers (see Yadav *et al.,* 2014). Furthermore, use of molecular markers in genome analysis, mapping of agriculturally important traits and marker assisted selection (MAS) have been greatly advanced by the development of PCR based markers.Several issues pertaining to markers suitability, number of marker combinations to be employed and relevance of appropriate marker utility parameters need to be addressed to enable large-scale implementation of DNA markers. Different molecular marker systems have been evaluated for their efficiency in detecting polymorphism and assessing genetic diversity using various statistical parameters. Over the last 15 years, polymerase chain reaction technology has led to the development of two simple and quick techniques *viz.* random amplified polymorphic DNA (RAPD) and inter simple sequence repeat (ISSR). The former detects polymorphisms using a single primer of arbitrary nucleotide sequence while the latter permits detection of polymorphisms in inter-microsatellite. RAPD markers have been used for the identification of cultivars and for assessing the genetic diversity among the cultivars of several pulse crops (Skroch *et al.,* 1992; Hoey *et al.,* 1996; Lakhanpaul *et al.,* 2000). Similarly ISSR markers also have been applied successfully to elucidate the magnitude of genetic diversity at both inter and intra specific level in broad range of pulse crops (Ajibade *et al.,* 2000;

Souframanien *et al.*, 2004). The information regarding genetics of most of the important traits are meager and mapping strategies are lacking to enhance efficient selection of desirable lines. There is an urgent need to develop a robust set of polymorphic markers and eventually a linkage map. Keeping above mentioned fact in mind, this study was conceded with the overall objective of to examine and compare genetic diversity pattern among forty two pigeonpea diverse genotypes using RAPD and ISSR markers to generate the genomic information that would be helpful to accelerate molecular breeding in pigeonpea in coming years.

MATERIALS AND METHODS

Plant materials and extraction of genomic DNA

A comprehensive collection comprised of 42 diverse genotypes of pigeonpea obtained from geographical region of India received from crop research centre (Chirodi farm) of Sardar Vallabh Bhai Patel University of Agriculture andTechnology, Meerut (Table 1). Leaf samples from 60 days old plants (single plant for each genotype) were harvested for the purpose of isolation of genomic DNA, and stored at -80°C in deep freezer. Genomic DNA isolated and purified by CTAB method (Doyle and Doyle, 1990). The quality and quantity of isolated DNA was checked by spectrophotometer as well as by 0.8 % agarose gel electrophoresis. The size of amplified fragments was determined by using 100bp molecular weight standard DNA ladder, (MBI fragment, Lithuania). DNA fragments were visualized under ultraviolet light and photographed using Gel Doc photographic system for permanent

Analysis of genetic diversity of RAPD and ISSR markers

To determine the existing variation in experimental sample 10 RAPD and 10 ISSR primers were used. The banding pattern for individual genotypes were seen on gel documentation system and clearly resolved, unambiguous polymorphic band were scored visually for their presence or absence with each RAPD and ISSR markers. The scored were recorded based on presence (1) and absence (0) for banding pattern in each variety. The individual RAPD and ISSR profiles as well pooled data (RAPD + ISSR) were analysed for genetic diversity analysis. The Polymorphic Information Content (PIC) and Resolving Power (Rp) values for each marker were analysed as performed by Anderson *et al.* (1993) and Provost and Wilkinson (1999), respectively. Jaccard's similarity coefficient (Jaccard, 1908) was estimated from binary data and resulting similarity matrix was used for un- weighted pair group method with arithmetic mean (UPGMA)clustering method (Sneath and Sokal, 1973). The statistical analysis was carried out using NTSYS-pc software (version 2.02) (Rohlf, 1993).

RESULTS AND DISCUSSION

Development and use of molecular markers for the detection and exploitations on DNA polymorphism is one of the most significant development in the field of genomics.Knowledge about germplasm diversity and genetic relationships among breeding materials acts as invaluable information in crop improvement strategies.The present study aimed to understand the genetic diversity in pigeonpea using 10 RAPD and10 ISSR markers. 42 diverse accessions representing different geographical locations were studied for their genetic diversity at molecular level. The experimental results obtained are described here:

RAPD analysis

Total number of bands generated by 10 primers was 43 with an average of 4.3 bands per primer. Out of 43 amplified bands, 39 were polymorphic (90.60 %) with an average of 3.9 bands per primer. The percentage polymorphism exhibited range from 66-100 per cent. These RAPD results were in close conformity with those of earlier investigators (Yan *et al.*, 2007, Choudhary *et al.*, 2008, Kumar *et al.*, 2009, Waseem *et al.*, 2011, Dutta and Lal 2011). The maximum number of polymorphic bands (7 bands) was obtained using OPF-14F primer. The average PIC and RP values for RAPD primers were 0.72 and 3.3, respectively (Table 2).

The dendrogram based on RAPD markers grouped the 42 genotypes into two main clusters I and clusters II. The main cluster I and II both further divided into two sub-clusters (I and II). Sub-cluster I was divided only Ia has only one genotype PantA-291. The sub cluster II was divided into two clusters IIa and IIb. The cluster IIa had five varieties (ICP-1088, ICP-16169, ICP-9120, ICP-10269 and ICP-7133) while IIb had three varieties (ICP-10977, ICP-1321 and ICP-11916). The cluster II had again two sub-clusters (III and IV). Sub-cluster III had only one genotype ICP-10976 while, sub-cluster IV (32 genotypes) which were further divided into two sub clusters (IVa and IVb), IVa had four ICP-8861, ICP-7627, ICP-10231, ICP-1149 and IVb had 28 genotypes, which divided into clusters (V and VI), Cluster V has 1 genotype ICP-1090 and cluster VI has 27 genotype.Cluster VI divided into two sub clusters (VIa and VIb), cluster VIa had 16 and cluster whereas VIb had11 genotypes (Fig. 1).

Most of the primers produced amplicons below 1 kb range, though a few amplicons crossed 1.0 kb range *viz* OPF-13F, OPD-08F, OPK-11F, OPF-14F, the amplified fragments covered a narrow range for some primers from 350-800 bp,while for other it was much higher (350 to 1300).The amplification of variable number of fragments by different primers is a phenomenon commonly reported by various research groups (Choudhury *et al.*, 2007; Malviya and Yadav, 2010).

ISSR analysis

Ten ISSR primers showed the ability to provide robust, complete and polymorphic fingerprints among 42 genotypes of pigeonpea. As a result, 52 bands were detected with an average of 5.2 bands per primer. Out of which 7 were monomorphic and 45 were polymorphic. The number of polymorphic bands ranged from 2 to 8. Percentage polymorphism ranged from 50 per cent (ISSR-2F) to a maximum of 100 per cent (ISSR-3F, ISSR-4F, ISSR-5F, ISSR-6F, ISSR-7F), with an average of 86.50 per cent polymorphism (Table 3).

The average PIC and Rp values for ISSR primers were 0.76, and 3.7 respectively. In earlier studies, di-nucleotide based ISSR primers have been used in genotyping of pigeonpea with high reproducibility and sufficient polymorphism (Godwin *et al.*, 1997, Rajesh *et al.*, 2002, Reddy *et al.*, 2002, Sudupak,

Table 1: The details of forty two varieties/genotypes of pigeonpea

S. No.	Variety	Accession no.	Origin/Source	Special features
1	Pant a 291	UPAS 120 × KPBR 802-1	Pantnagar	Tolerant to Phytophtora
2	Azad	Bahar × KPBR 80-1	Kanpur	
3	Manak	T21 × Upas 120	Hissar	Semi spreading
4	Upas-120	Selection from P4768	Pantnagar	Fusarium resistant
5	Amar	Selection from Bahar	Kanpur	SMD resistant
6	AL201	AL16 × LP200		
7	ICP10976	IC-SMR-Sel70;ICP70-S1X VIII NDT	ICRISAT	Resistance to SMD
8	ICP14751	ICPL88047;Icpx-800275-SWB8-SWP5-SWIX-SWP6-SW	ICRISAT	Resistance to wilt
9	ICP-934	P 3203;PI 396427	INDIA	Resistance to blight
10	ICP-1090	P4682/1;brownseed 16-1	INDIA	Resistance to blight
11	ICP-15045	ICPL90097;ICPX810202 I4B-W7-WB-B-B	ICRISAT	Resistance to wilt
12	ICP-8258	PLA367	INDIA	blight resistant
13	ICP-1150	P240-129-2	INDIA	Resistance to blight
14	ICP-8151	ANM 504	INDIA	Resistance to blight
15	ICP-1123	P984-40-1;parbhanilocal	INDIA	Resistance to blight
16	ICP-16335	ICPL90006	ICRISAT	Early dwarf
17	ICP-14988	ICPL83063;74243-B-B-S30X-SWIX-5NDTSWIX-WBX	ICRISAT	Resistant to wilt
18	ICP-15018	ICPL88013,ICPX 820005-HBX-H1-H1-HB-HB	ICRISAT	Resistant to early wilt
19	ICP-13205	4745-2-E8-SEB	ICRISAT	Tolerant to pod borer
20	ICP-10978	IC-SMR-Sel.95;ICP95-1-S2X VII NDT	ICRISAT	Resistant to SMD
21	ICP-11962	PPE-36-2	ICRISAT	Tolerant to pod borer
22	ICP-14622	ICPL333;ICP8863-B	ICRISAT	Resistant to Wilt
23	ICP1088	P4654;T-6-72	INDIA	Resistant to Blight
24	ICP-10222	PI394530;P707	INDIA	Resistant to SMD
25	ICP-16169	ICPL90020	ICRISAT	Medium dwarf
26	ICP-10977	IC-SMR-Sel.85;ICP85-1-1-5-S1 X III NDT	ICRISAT	Resistant to SMD
27	ICP-1321	P739;PI 394551	INDIA	Resistance to blight
28	ICP-11916	PR 5193	INDIA	Resistance to pod borer
29	ICP-10269	PI394961;P1272	INDIA	Wilt resistant
30	ICP-15011	ICPL88003,ICPX810060-HB-SBX-H1-HB-HB-HB	ICRISAT	Early
31	ICP-10979	IC-SMR-Sel.457;ICP457-3-S2X VI NDT	ICRISAT	Resistant to SMD
32	ICP-9010	LJR 118C	INDIA	Intermediate flowering
33	ICP-9008	LJR 118A	INDIA	Intermediate flowering
34	ICP-14760	ICPL89048;ICPX-810203-F4B-W6-WB	ICRISAT	Resistant to wilt
35	ICP-13207	ICP 909-E3-5EB	ICRISAT	Tolerant to pod borer
36	ICP-7133	EC109882;Sel XIII	SRILANKA	Medium dwarf
37	ICP-9120	JM 2397;Nsukka-C	NIGERIA	Wilt resistant
38	ICP-10980	IC-SMR-Sel.504;ICP504-1-4SXVI NDT	ICRISAT	Resistant to SMD
39	ICP-8861	ICWR-4;7035-S34X-W29XB	ICRISAT	Wilt resistant
40	ICP-7627	L-28	INDIA	Medium dwarf
41	ICP-10231	PI 394559 ;P749	INDIA	Resistant to SMD
42	ICP-1149	P 240-129-1	INDIA	Resistant to blight

Table 2: RAPD Primer code, molecular wt, Polymorphic bands, PIC (Polymorphism Information Content) and resolving power of 10 RAPD Primers

S.No.	Primer	Molecular weight range (bp)	Polymorpic bands	Pic value	Resolving power
1	OPC-07F	100- 650	5	0.5344	6.5238
2	OPF-13F	400- 1200	6	0.8271	3.666
3	OPF-06F	350- 650	4	0.6493	4.7142
4	OPJ-08F	700-800	1	0.4898	1.4285
5	OPD-08F	400- 1100	4	0.9141	2.1904
6	OPK-11F	300- 1100	6	0.7340	5.2857
7	OPJ-13F	300- 500	2	0.6628	2.2857
8	OPF-14F	400- 1300	7	0.8987	3.6666
9	OPC-15F	400- 800	2	0.9509	0.7142
10	OPF-17F	350- 550	2	0.6279	2.9047
Total			39	7.289	33.380

2004).

The Jaccard's pair wise similarity coefficient values for ISSR ranged from 0.70 to 0.98. The 42 genotypes were clustered into two groups, group 1 and group 2 respectively. Group 1 divided into two clusters (I and II) cluster I has one variety ICP-9010 while cluster II with 41(5 and 36) genotype. Group II

Table 3: ISSR Primer code, molecular wt, Polymorphic bands, PIC (Polymorphism Information Content) and resolving power of 10 ISSR Primers

S.No	Primer	Molecular weight range (bp)	Polymorphic bands	Pic value	Resolving power
1	ISSR-1F	400- 750	2	0.6566	3.2856
2	ISSR-2F	350- 1100	2	0.4726	4.9047
3	ISSR-3F	300- 1200	5	0.9130	2.1428
4	ISSR-4F	300- 900	6	0.7736	4.0952
5	ISSR-5F	350- 1300	8	0.7937	5.7142
6	ISSR-6F	500- 1000	3	0.9121	1.3809
7	ISSR-7F	300- 700	3	0.9822	0.66667
8	ISSR-8F	300- 1300	6	0.7339	5.5238
9	ISSR-9F	350- 1300	6	0.7066	6.000
10	ISSR-10F	300- 1200	4	0.7287	3.8095
Total			45	7.673	37.5233

Table 4: Comparative of RAPD & ISSR markers of 42 pigeonpea populations

Components	RAPD	ISSR
Number of primers used	10	10
Total number of polymorphic bands	39	45
Total number of bands	43	52
Average number of bands/primer	4.3	5.2
Average number of polymorphic bands/ primer	3.9	4.5
Average PIC value	0.728	0.767
Average resolving power of 10 primers	3.330	3.750

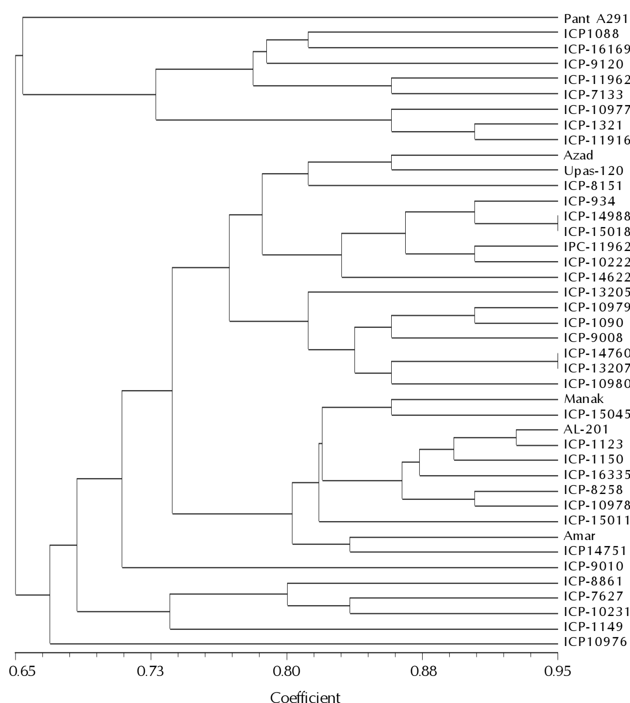

Figure 1: Dendrogram showing clustering of 42 pigeonpea construced using NTSYS based on jaccard's similariy coeffcient obtainded from RAPD analysis

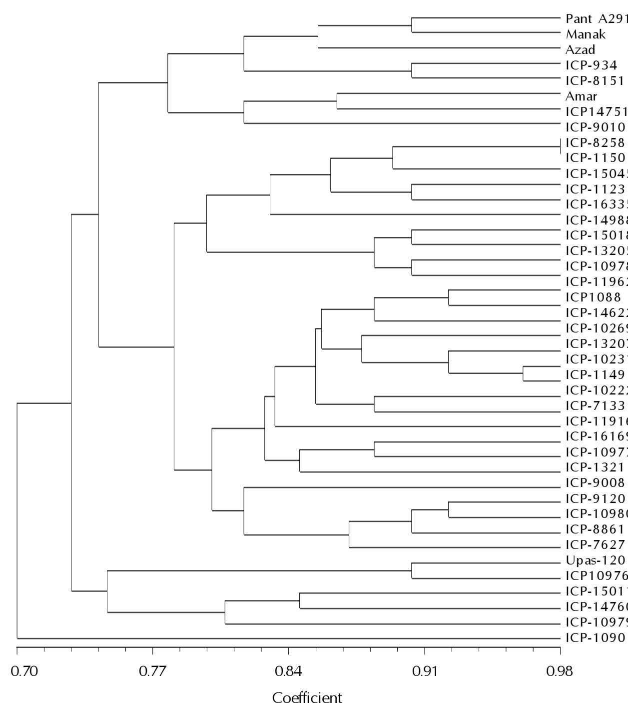

Figure 2: Dendrogram showing clustering of 42 pigeonpea construced using NTSYS based on jaccard's similarity coeffcient dotained feom ISSR analysis

was further divided into two sub clusters (cluster III and IV). Cluster III is divided into IIIa and IIIb while cluster IV into IVa and IVb. Cluster IIIa has three varieties PANTA-291, MANAK, AZAD and IIIb has five varieties ICP-934, ICP-8151, AMAR, ICP-14751 and AL-201. Cluster IVa has ten genotypes namely ICP-1090, ICP-8258, ICP-1150, ICP-15045, ICP-1123, ICP-16335, ICP-14968, ICP-15018, ICP-13205 and ICP-10978, while cluster IVb has 18 genotypes. Based on dendogram genotypes ICP1090 and, ICP8258 were 100 per cent similar. Maximum varieties of ICP were grouped in a same cluster indicating presence of common base pair sequence repeats (Fig. 2).

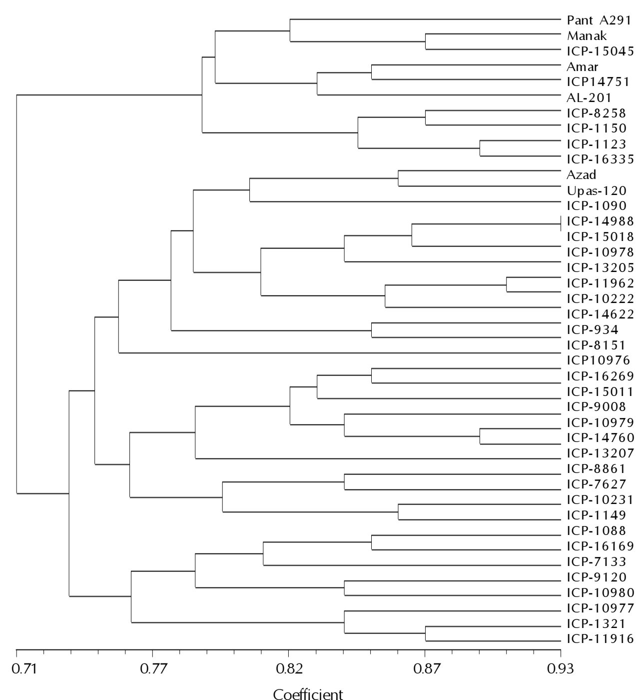

Figure 3: Dendrogram showing clustering of 42 pigeonpea varieties constructed using NTSYS based on jaccard's similarity coeffcient dotained feom Combined (RAPD and ISSR) analysis

Comparative (RAPD and ISSR) analysis

In the combined analysis, similar clustering pattern were gained as was found in analysis using individual primers.RAPD markers were more efficient than the ISSR assay with regards to polymorphism detection, as they detected 90.6 per cent as compared to 86.5 per cent for ISSR markers. Similar results were reported by Gupta *et al.* (2008) and Dutta *et al.* (2010). The average number of polymorphic bands per primer and total number of polymorphic bands were more in ISSR (4.5 and 45 respectively) than RAPD (3.9 and 39 respectively). Similar results were obtained in several other studied involving pigeonpea (Datta *et al.,* 2010; Hemalatha and Shanmugasundaram 2010).

Comparison of PIC values for the two DNA marker systems indicated that the range of PIC values for RAPD primers was 0.48 (OPJ-08F) to 0.95 (OPC15 F) with an average of 0.73 whereas the range of PIC values for ISSR primers was 0.47 (ISSR 2F) to 0.98 (ISSR 7F) with an average of 0.76. Moreover, higher average and wider range of PIC values of ISSR marker is not unexpected, as the primers detected a large number of loci and their might be variation in the length of ISSR loci and distance between them Among the two DNA marker techniques, ISSR had the highest average Rp (3.7), followed by RAPD (3.3). Based on ISSR marker system, the similarity index values ranged from 0.58 to 0.98 while that based on RAPD markers ranged from 0.51 to 0.95(Table 4).

Comparative analyses in pigeonpea using RAPD and ISSR primers have been successfully used by limited researchers (Yadav *et al.*, 2014). The dendrogram obtained from the cluster analysis of RAPD and ISSR combined data gave near similar clustering pattern. The main cluster divides into two groups (I and II). Group I divide into two clusters (cluster I and cluster II), cluster I into Ia and Ib while cluster II into IIa and IIb.Group II divided into two sub clusters (cluster III and cluster IV) Cluster III Contain 13 genotypes. Cluster IV has 19 genotypes. Cluster III divided IIIa and IIIb and cluster IV into IVa and IVb. Cluster IIIa has only one genotype ICP-10976 and cluster IIIb has 12 genotypes. Cluster IVa has 11 genotypes ICP-10269,ICP-15011, ICP-9008, ICP-10979, ICP-14760, ICP-13207, ICP-9010, ICP-8861, ICP-76227, ICP-10231,ICP-1149.Cluster IVb has 8 genotypes,ICP-1088,IPC16169,ICP-7133,ICP-9120,ICP-10980,ICP-10977,ICP-1321and ICP-11916(Fig-3).In comparative analysis of RAPD and ISSR primers only ICP-14968 and ICP-15018 show maximum similarity.

To some extent, there was a consensus between the RAPD and ISSR based grouping of the 42 genotypes.The differences found among the dendrograms generated by RAPDs and ISSRs could be partially explained by the different number of PCR products analyzed, marker sampling error and/or the level of polymorphism detected, reinforcing again the importance of the number of loci and their coverage of the overall genome(Yadav *et al.,* 2014, Gupta *et al.,* 2008).

In summary, present results indicate there is ample amount of genetic variability existing among pigeonpeagenotypes studied.In addition, some differences between the two marker systems could be detected like PIC values higher for ISSRs than for RAPDs, when different genotypes were compared. Similarly a number of total polymorphic fragments higher for ISSRs than RAPDs.However, further investigations are required to verify such kind of preliminary observations. Furthermore, the close correspondence between the genetic similarity matrices of RAPD and ISSR revealed that the two marker systems could be effectively used individually or in combinations in estimation of genetic diversity in pigeonpea. Therefore, results of the present study can be seen as additional information for future researches aiming at defining the level of intra- and inter-specific genetic diversity. These studies have given important clues in understanding genotype relationship, which may further assist in developing and planning molecular breeding strategies.

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