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Genetic diversity of wild and cultivated genotypes of pigeonpea through RAPD and SSR markers

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Abstract

Eight wild and four cultivated pigeonpea genotypes were subjected to RAPD and microsatellite analysis, with 40 primers each. Out of these, eight RAPD and five SSR primers were found polymorphic. RAPD primers showed 100% polymorphism and produced a total of 517 DNA fragments, whereas SSR primers produced 67 fragments and they too showed 100% polymorphism. The RAPD markers revealed highest similarity co-efficient of 0.93 (GT-100 and ICPL-87), whereas the highest similarity co-efficient obtained with SSR markers was 1.00 (GTH-1 and GT-100). Average PIC value obtained with RAPD and SSR were 0.90 and 0.18, respectively. The arithmetic mean heterozygosity and marker index were 0.90 and 22.47 respectively with RAPD marker, whereas the corresponding values for SSR markers were 0.18 and 33.66. Moreover, the four wild genotypes (*Cajanus scarabaeoides*, *Rhyncosia rufescence, Cajanus cajanifolius* and *Rhyncosia canna*) and the four cultivars (GTH-1, GT-100, ICPL-87 and GT-1) grouped distinctly in the same subgroups of the dendrograms obtained with both RAPD and SSR analysis. Therefore, the findings of SSR supplement and validate the results obtained with RAPD analysis.

Key words

Cluster analysis, Fingerprinting, Microsatellites, Random primers, Redgram

Introduction

Pigeonpea [*Cajanus cajan* (L.) Millsp.] is an economically important grain legume belonging to the *Cajaninae* subtribe of tribe *Phaseoleae* (Young *et al*., 2003). The genus *Cajanus* comprises of 32 species. However, pigeonpea is the only cultivated food crop of the *Cajaninae* subtribe and has a diploid genome comprised of 11 pairs of chromosomes (2n = 22) (Greilhuber and Obermayer, 1998). Wild relatives of cultivated plants are known to possess many agronomically important traits including resistance to pests and diseases (Reddy *et al*., 1996), which may be useful in improving the performance of pigeonpea cultivars. Genetic diversity plays an instrumental role in plant breeding programme in testing the genetic resources for productivity, quality parameters and stress tolerance. Field trials are normally time consuming, therefore, molecular markers and DNA technology are used to assess diversity in the gene pool to identify genes of interest and to develop a set of markers for screening of progeny and germplasm (Linda *et al.,* 2009).

Random amplified polymorphic DNA (RAPD) markers have been frequently used in plant genetics research despite having the disadvantages of poor reproducibility and not being associated with gene regions (Welsh *et al.,* 1990; Williams *et al.,* 1990). Although, being a multilocus marker (Karp *et al.,* 1997), RAPD represent the simplest and fastest detection technology and have been successfully employed for the determination of intraspecies genetic diversity in several grain legumes (Sadeghi and Cheghamirza, 2012).

SSR is also known as microsatellites or simple tandem repeats (STR) due to the presence of tandemly repeated units of short nucleotide motifs, 1–6 bp long. Such regions are reported to occur frequently and randomly throughout the genomes of the eukaryotes and show extensive variation (Jarne and Lagoda, 1996). SSR markers have received considerable attention and are probably the current marker system of choice for marker based genetic analysis and marker assisted selection in plant breeding (Powell *et al.,* 1996). However, very limited SSRs have

been previously reported in pigeonpea and very few of them were polymorphic in pigeonpea germplasm (Odeny *et al.,* 2009; Saxena *et al.,* 2010). SSR are considered as one of the most reliable and highly reproducible molecular markers and are now widely recognized as the foundation for many framework linkage maps (Song *et al.,* 2004). SSR have played a critical role even in merging disparate linkage maps, since they define specific locations in the genome unambiguously (Odeny *et al.,* 2009).

The reliability of the quantification of genetic diversity using only one type of marker has normally been questioned as compared to the analysis with different markers. Therefore, the present investigation was carried out to see the efficiency of RAPD and SSR markers for analysis of genetic diversity among 12 diverse pigeonpea genotypes (eight wild and four cultivated); and to evaluate how well these two types of markers discriminated the pigeonpea genotypes under study.

Materials and Methods

The cultivated and wild genotypes of pigeonpea were obtained from the Centre of Excellence on Pulses, S. D. Agricultural University, Sardarkrushinagar, Gujarat (India). These genotypes were utilized for evaluation of the efficiency of RAPD and SSR markers for diversity analysis of pigeonpea.

DNA extraction : Total DNA was extracted from young leaves of the seedlings by CTAB method as described by Doyle and Doyle (1990) with some modification. Quality of DNA was tested by running DNA on 0.8% agarose gel at a constant voltage of 60V using 0.5X TBE buffer for 1 hr and the gel image was visualized under UV light and gel photographs were scanned through Gel Documentation system (Alpha Innotech Corporation, USA). The pure DNA samples thus obtained were diluted to the concentration of 30 ng μl^{-1} and used for further genetic analysis.

RAPD and SSR markers and gel electrophoresis : The genomic DNA was amplified using random primers OPA-07, OPA-09, OPA-10, OPA-11, OPA-12, OPA-14, OPA-16 and OPA-19 for RAPD; whereas primers CCB1, CCtta001, CCat001, CCtta003 and CCtta005 were used for SSR analysis. The RAPD-PCR reactions were carried out in final volume of 25µl, having 1X PCR buffer containing one units of Taq DNA polymerase, 0.2mM of dNTPs, 20 ng RAPD primer and 30ng of genomic DNA. Amplification was achieved in a master cycler gradient (Eppendrof) programmed for initial denaturation (4 min at 94 ºC) followed by 40 cycles, composed of denaturation (94 ºC for 1 min), annealing (37ºC for 1 min), extension (72ºC for 2 min) and a final extension of 5 min at 72ºC and subsequent cooling at 4ºC temperature. PCR amplification products were size- separated along with a molecular weight marker (50bp, Bangalore genei, India) by standard horizontal gel electrophoresis in 1.6% agarose gel (stained with 0.1% ethidium bromide) for 2.5hr at 70 volts. The gels were photographed using Gel Documentation system (Alpha Innotech Corporation, USA) and the amplification product size was evaluated using software quantity one (Biorad, USA).

However, PCR conditions used for SSR amplification consisted of initial denaturation (94 ºC for 4 minutes) followed by 40 cycles of denaturation (94 ºC for 1 min), annealing (56°C for 1 min), extension (72 ºC for 2 min), and a final extension of 7 min at 72 ºC. The amplified products obtained in PCR were separated by agarose gel (2%) electrophoresis and the gel were photographed and documented as described earlier.

The amplification products were scored separately for each primer, using binary code 1 and 0 respectively for the presence and absence of bands corresponding to each cultivar. The data were maintained in the spreadsheet format for further analysis and were subsequently analyzed using NTSYSpc version 2.02 (Rohlf, 1994). Similarity coefficients were calculated using Jaccard's similarity coefficient (Jaccard, 1908) by SIMQUAL function and cluster analysis was performed by agglomerative technique using the UPGMA (Un-weighted Pair Group Method with Arithmetic Mean) and relationships between the pigeonpea genotypes were represented in the form of dendrograms. Support for clusters obtained was evaluated by bootstrap analysis, using the software package 'WINBOOT' developed at IRRI (Yap and Nelson, 1996).

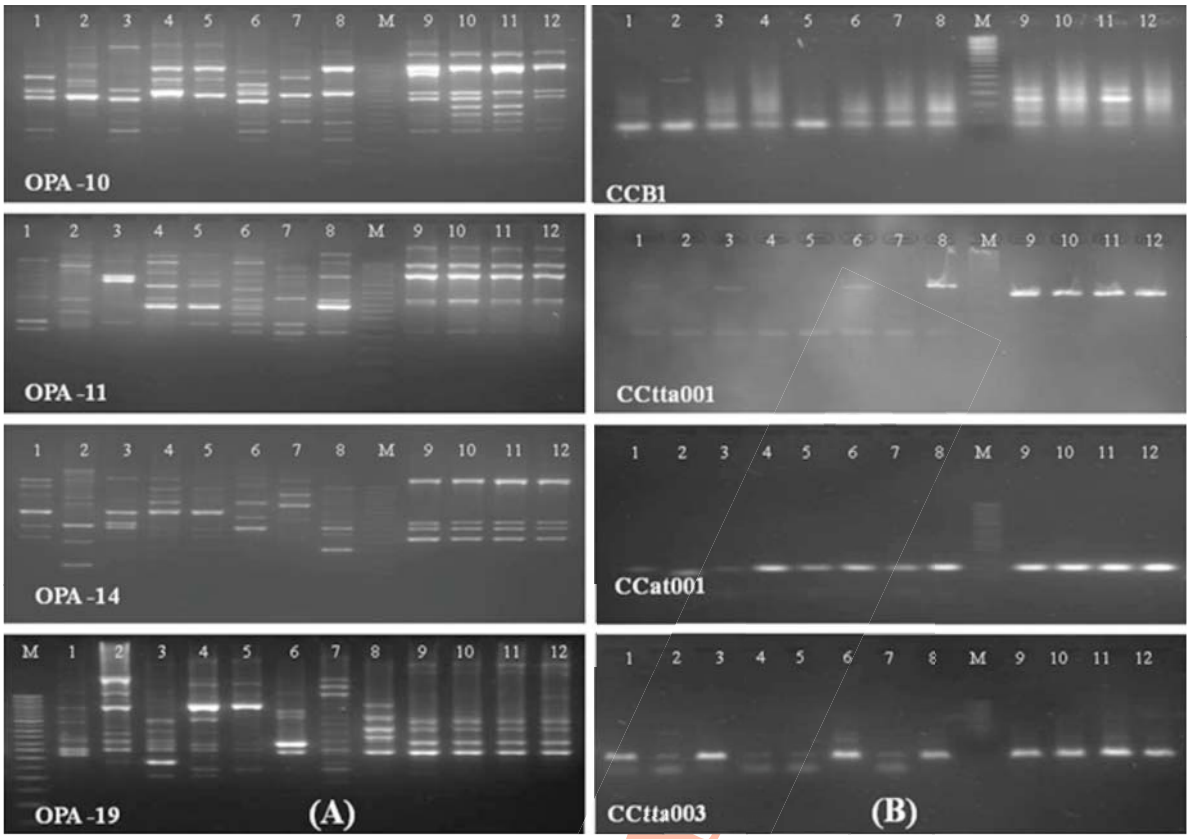
Data collection and analysis : The expected heterozygosity (H_n), arithmetic mean heterozygosity (H_{av}) and marker index (MI) were calculated using the methodology of Nei (1973) and Powell *et al.* (1996). The expected heterozygosity (H_n) for a molecular marker was calculated as: $H_n = 1-\sum Pi^2$ (where, pi is the allele frequency of the i^{th} allele). The arithmetic mean heterozygosity, H_{av} was calculated for each marker class as: $H_{av} = \sum H_n/n$ (where, n is the number of markers or loci analyzed). However, Marker index (MI) was calculated as: $MI = E(H_{av})p$ (where, E is the effective multiplex ratio; and $E=n\beta$, where β is the fraction of polymorphic markers or loci).

Polymorphism percentage of the obtained bands was calculated by using the formula: Polymorphism% = (No. of polymorphic bands/Total bands)X100. The Polymorphism information content (PIC) value for each band was calculated by the formula as: $1 - (p^2 - q^2)$ (where, p= band frequency and q = no band frequency *i.e.* absence of band). However, average PIC for a primer was calculated as: $(1-p^2-q^2)/n$ (where, n=total number of bands amplified per primer).

Results and Discussion

In the present investigation, twelve pigeonpea genotypes including eight wild and four cultivated were subjected to amplification by RAPD and SSR primers to assess genetic diversity (Fig.1). The banding pattern thus obtained by both RAPD and SSR primers clearly distinguished cultivars into different clusters showing diversity.

Out of 40 primers tested in RAPD analysis, only eight showed 100% polymorphic bands. Pooled RAPD analysis of



Lane 1: *Cajanus scarabaeoides* Lane 5-*Rhyncosia rothi* Lane 9: GTH-1Lane 2-*Rhyncosia bracteata* Lane 6-*Rhyncosia canna* Lane 10: GT-100 Lane 3-*Cajanus cajanifolius* Lane 7: *Rhyncosia minima* Lane 11: ICPL-87 Lane 4-*Cajanus platycarpus* Lane 8 : *Rhyncosia rufescence* Lane 12: GT-1

Fig.1 : RAPD (A) and SSR (B) profiles of the wild and cultivated genotypes of pigeonpea with selected primers

these eight arbitrary oligonucleotide primers generated a total sum of 517 scorable bands ranging from 65 bp (by OPA 12) to 3230 bp (by OPA 19) with 150 loci. Among them all 150 loci were found polymorphic showing 100% polymorphism (Table 1). Therefore, an average of 64.62 bands were amplified per RAPD primer. The maximum number of 89 amplified products was obtained with primer OPA 19 followed by, 84 bands by OPA 10 and 82 bands by OPA 11; whereas the minimum number of bands were observed with OPA 12 (28). The PIC value for primers was found to be 0.98; whereas the arithmetic mean heterozygosity and the marker index were 0.90 and 22.47, respectively. However, Choudhury *et al*. (2008) observed an arithmetic mean heterozygosity of 0.48 and maker index (MI) as 5.027 in a population of 24 *Cajanus cajan* cultivars using 76 RAPD primers.

A high number of bands per primer showed the suitability of primers used in the present study for diversity analysis of pigeonpea cultivars. Choudhury *et al*. (2008) found polymorphism ranging from 9.1 to 100 % and only 10.47 bands per primer, whereas at Lohithaswa *et al.* (2003) observed 63.46% polymorphism while studying genetic diversity among pigeonpea cultivars, using RAPD primers. Malviya *et al*. (2010) observed that nine out of seventeen RAPD primers depicted more than 80 % polymorphism in 17 cultivars of pigeonpea

Clustering pattern of dendrogram generated using pooled molecular data of RAPD primers indicated two clusters having a similarity coefficient of 0.14. The first and major cluster was again divided into two sub-clusters; the first of which contained majority of the genotypes (wild and cultivated) while the second sub cluster contained only *Rhyncosia minima* (Fig.2). It was quite interesting to observe that all the four cultivated genotypes (GTH-1, GT-100, ICPL-87 and GT-1) were grouped closely in the same branch of dendrogram, showing similarity of about 0.30 with the wild genotypes in the same sub cluster. However, the second cluster comprised only of wild genotypes of pigeonpea namely, *Rhyncosia bracteata, Cajanus platyacarpus* and *Rhyncosia rothi*. Overall, the highest similarity coefficient (0.93) was observed between the cultivated genotypes, GT-100 and ICPL–87. Among wild genotypes, *Cajanus scarabaeoides* and *Cajanus cajanifolius* showed highest similarity coefficient of 0.37. The present study revealed high level of diversity among

Table 1 : List of primers showing polymorphic amplification in RAPD analysis

Primer	Primer sequence (5'—3')	GC content %	No. of bands	Total Loci	Polymorphic Loci	Percentage Polymorphism	PIC value	Molecular weight range (bp)
OPA-07	GAAACGGGTG	60	63	21	21	100	0.92	147-1778
OPA-09	GGGTAACGCC	70	74	23	23	100	0.93	111-3204
OPA-10	GTGATCGCAG	60	84	22	22	100	0.93	289-1511
OPA-11	CAATCGCCGT	60	82	23	23	100	0.93	112-2435
OPA-12	TCGGCGATAG	60	28	8	8	100	0.78	65-1850
OPA-14	TCTGTGCTGG	60	60	20	20	100	0.91	73-3607
OPA-16	AGCCAGCGAA	60	37	11	11	100	0.86	212-1891
OPA19	CAAACGTCGG	60	89	22	22	100	0.94	173-3230
Total			**517**	**150**	**150**	**100**	**7.2**	**-**

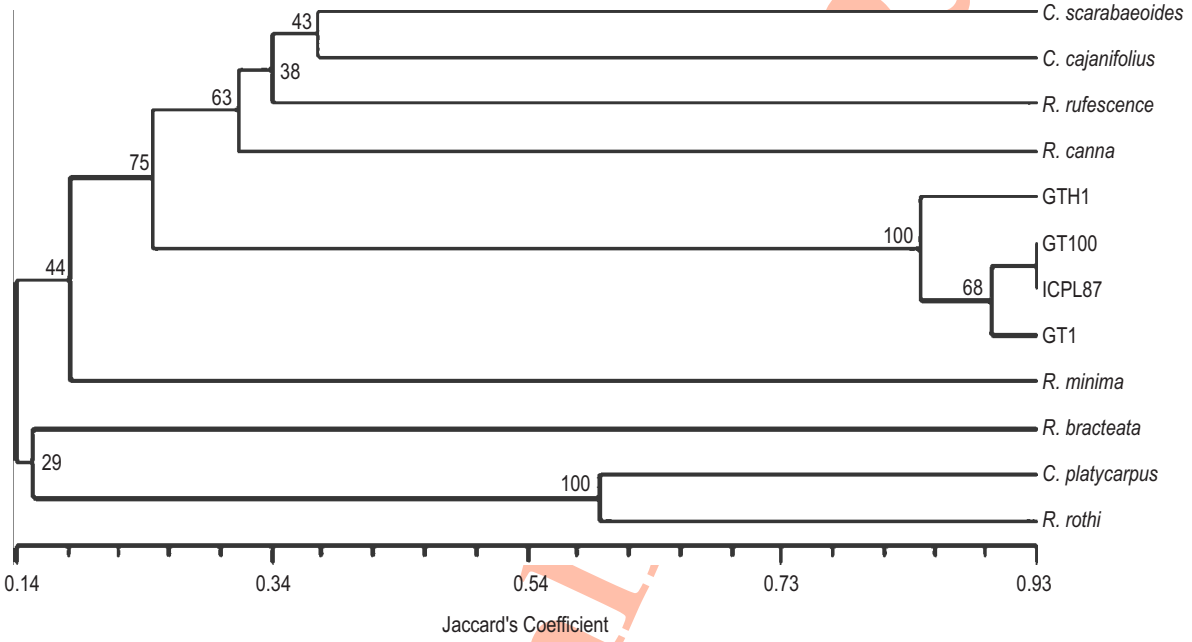


Fig. 2 : UPGMA cluster analysis showing the diversity among the pigeonpea genotypes produced by RAPD primers

wild pigeonpea genotypes as compared to cultivated genotypes. This may be due to the presence of narrow genetic base of the cultivated genotypes which serves as a serious impediment in the breeding program of pigeon pea (Souframanien *et al.,* 2003).

RAPD is an effective tool to evaluate and reveal molecular diversity in crop plants (Ratnaparkhe *et al.,* 1995). A wide variation observed in the Jaccard similarity coefficients (range 0.07 to 0.93) shows greater diversity among the genotypes under study. The bootstrap analysis was done to evaluate the degree of support for various clusters within the dendrogram. It was noted that clusters, subclusters and sub-groups within the dendrogram were supported by the respective high bootstrap values. In the present study, the banding patterns pertaining to different genotypes were obtained which helped in clustering of pigeonpea genotypes. Although, all the cultivated genotypes were grouped in the same sub cluster, the wild types of genotypes were scattered throughout the dendrogram. This shows that diversity among the cultivated genotypes was less as compared to the wild type. Low level of diversity among cultivated pigeonpea as compared to the wild relatives was also reported by Ratnaparkhe *et al.* (1995), who studied 10 cultivars and five wild type pigeonpea genotypes using 16 polymorphic RAPD primers.

SSR primers from CC series (SAF Labs, Mumbai, India) were used to analyze genetic diversity among 12 pigeonpea genotypes in the present study. However, out of 40 SSR primers, only five were found polymorphic in the selected pigeonpea genotypes. Microsatellite primers produced a total sum of 12 alleles. The maximum amplified allele size of 250 bp was generated by the primer CCtta-005; whereas the least amplified allele size of 119 bp by the marker CCtta001. A maximum of three alleles were recorded for primers CCtta-003 and CCtta-005, while primers CCB-1, CCat-001 and CCtta-001 produced two alleles each, which were

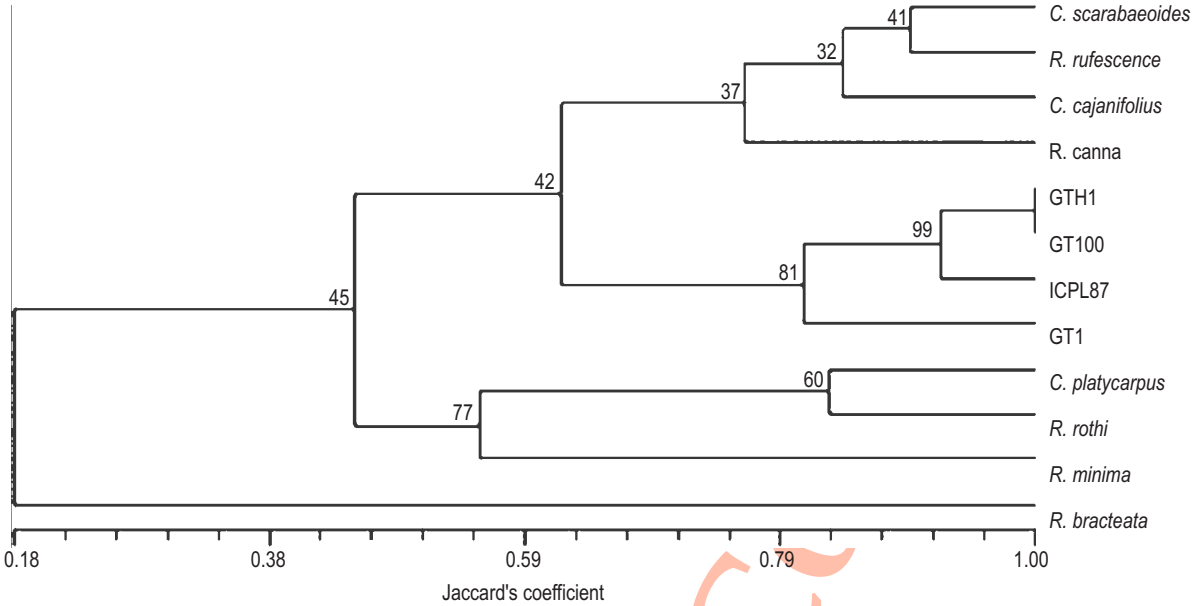


Fig.3 : UPGMA based clustering analysis of wild and cultivated pigeonpea genotypes obtained from SSR analysis

Table 2 : List of primers showing polymorphic amplification in SSR analysis

Primer name	Primer sequence (5'—3')	GC content %	No. of bands amplified	Molecular weight range (bp)	Difference in bp	Total no. alleles	PIC value
CCB1-FP	AAGGGTTGTATCTCCGCGTG	5545	8	110-206	96	2	0.25
CCB1-RP	GCAAAGCAGCAATCATTTCG						
CCtta001-FP	TTCTGGATCCCTTTCATTTTTCT	3647	18	101-222	121	2	0.25
CCtta001-RP	GACACCCTTCTACCCCATAA						
CCat001-FP	CTTCCCCCAACTAAGATCCAGTT	5037	12	71-119	48	2	0.076
CCat001-RP	CGTTCTCTTTAATTGACTTGC						
CCtta003-FP	CCAAGAAAAGGTGCTCCAAGTT	4745	15	87-187	100	3	0.14
CCtta003-RP	TGCTTCTTTTCTCGCTTGC						
CCtta005-FP	TCTTCCATTGCATGGTGTTG	4240	14	156-250	94	3	0.19
CCtta005-RP	CATGATATGAGATGATGACGA						

also lowest in the present investigation. The highest PIC value (0.25) was recorded for SSR marker CCB-1 and CCat-001; whereas the least value (0.076) was obtained with marker CCtta001 (Table 2). The arithmetic mean heterozygosity and the marker index for SSR markers were 0.18 and 33.66, respectively.

The dendrogram generated using the pooled molecular data of SSR primers indicated two clusters with a similarity coefficient of 0.18. The first cluster was divided into two sub clusters and included all the genotypes except *Rhyncosia bracteata*, which was the lone member in the second cluster. The first sub cluster of the first major cluster included 4 wild genotypes (*Cajanus scarabaeoides*, *Rhyncosia rufescence, Cajanus cajanifolius* and *Rhyncosia canna*) in a sub-group and 4 cultivated (GTH-1, GT-100, ICPL-87 and GT-1) one in the other sub-group. Maximum similarity of 100% was observed between the cultivar GTH-1 and GT-100 (Fig. 3).

The SSR analysis of pigeonpea genotypes under study showed considerable diversity among the wild relatives. As microsatellite or SSR markers are highly polymorphic, reproducible, co-dominant in nature and distributed throughout the genome, they have become ideal an marker system for genetic analysis and breeding application. In case of pigeonpea, however, only few hundreds of SSR markers have been reported in literature (Singh *et al.,* 2013; Saxena *et al.,* 2010).

Odeny *et al*. (2007) reported twenty polymorphic SSR primers in fifteen cultivated and nine wild pigeonpea relatives and obtained a polymorphic information content value ranging from 0.17 to 0.80, whereas Saxena *et al*. (2010) reported thirteen SSR primers to be polymorphic amongst the cultivated and wild pigeonpea genotype representing six *Cajanus* species and the PIC value for these markers ranged from 0.05 to 0.55. They reported the highest genetic similarity (98%) between the cultivar

ICP 7543 and ICP 14144. However, the present study showed PIC value ranging from 0.78 to 0.94 for RAPD and 0.076 to 0.25 for SSR study. High polymorphic results indicated wide genetic base in pigeonpea accessions and genetic diversity may be due their characteristics, wide distribution, amplification protocol used/selection of suitable primers.

Though great advancement has taken place in marker technology, still RAPD is quite convenient to apply provided, the problem of reproducibility is minimized. Nevertheless, the diversity obtained with the RAPD primers must be validated with other makers (Malviya *et al.,* 2010). The present analysis using pigeonpea genotypes showed that both RAPD and SSR techniques may provide consistent data, and thereby, can be used to study genetic diversity in pigeonpea, showing concordant values of genetic diversity. With minor fluctuations, the four wild genotypes (*Cajanus scarabaeoides*, *Rhyncosia rufescence, Cajanus cajanifolius* and *Rhyncosia canna*) and the four cultivars (GTH-1, GT-100, ICPL-87 and GT-1) were present distinctly in the same subgroups both in the dendrograms obtained with RAPD and SSR analysis. Hence, it can be assumed that the results of diversity analysis with SSR markers validate the finding of RAPD analysis. This led us to generate an opinion that RAPD markers can be considered as effective as SSR markers. Therefore, the option to analyse pigeonpea genetic diversity using RAPD markers help the plant breeders in genetic breeding programmes, and hence, can be considered as an excellent strategy for marker assisted plant breeding. On practical grounds OPA19, OPA10 and OPA11 primers, for instance which produced the greatest number of bands and which showed the greatest potential to discriminate polymorphic DNA segments, can be recommended for future analysis of the pigeonpea genome using RAPD markers.

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