Efficiency of DNA Marker Systems in Discriminating *Cajanus cajan* (L.) Millsp. and its Wild Relatives

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Two multilocus markers, *viz.*, RAPD (25 primers), ISSR (11 primers) and one single locus marker, *viz.*, SSR (10 primer pairs) were used to assess the genetic diversity among 32 genotypes of pigeonpea and to find the efficient marker system to discriminate the genotypes at molecular level. Diversity analysis was done with the help of statistics such as marker index, mean marker index, polymorphism information content, principal component analysis and dendrogram. The random primers, *viz*., OPG06, OPG15, OPG03 and the ISSR primers, *viz*., UBC842, UBC876, and UBC857 are considered to be efficient in discriminating the genotypes. The RAPD, ISSR and SSR markers clustered the genotypes into 10, 12 and 7 clusters, respectively. Among the three marker systems used for the analysis, ISSR markers revealed a higher amount of divergence among the genotypes by producing 215 markers, 99.53 per cent polymorphism, and an average of 19.54 markers than SSR and RAPD. The present study clearly elucidated the superiority of ISSR markers for genetic diversity studies over RAPD and SSR markers.

Key Words: Pigeonpea, Polymorphism, Wild relatives

Introduction

Evaluation of germplasm in terms of diversity and relatedness is an important component of plant genetic resource management. Also the estimation of genetic diversity between different genotypes is the first and foremost process in any plant breeding program. Lynch (1988) proposed that the technique of DNA fingerprinting could be employed for the estimation of relatedness. Earlier, morphological and isozyme markers were used to assess the relatedness between the cultivated and their wild relatives, but these markers had the environmental influences.

The recently developed molecular marker technology has opened up new vistas for the assessment of genetic relatedness among genotypes at molecular level. DNA based markers have the obvious advantage of sampling the genome directly. PCR-based markers system like RAPD can be used for DNA fingerprinting of genotypes. Fingerprinting genomes using arbitrary primers was first reported by Welsh and McClelland (1990). RAPD markers have been successfully used by several earlier workers in various leguminous crops to determine genetic diversity, *viz.*, soybean (Abdelnoor *et al*., 1995), *Pisum* (Hoey *et al*., 1996), pigeonpea (Ratnaparkhe *et al.*, 1995). RAPD and RFLP analyses have been used to arrive at the phylogenetic relationship among the cultivated and wild species of pigeonpea (Ratnaparkhe *et al*., 1995).

Microsatellites are recently gaining importance over RFLP, RAPD, since they display considerable polymorphism due to variation in number of repeat units. This technique has been used for DNA fingerprinting and variety identification in many crops like rice (Yang *et al*., 1994; Olufowote *et al*., 1997), wheat (Roder *et al*., 1995; Gupta and Varshney, 2000), pearlmillet (Chowdari *et al*., 1998) and pigeonpea (Burns *et al*., 2001). ISSR markers have been successfully used to estimate the extent of genetic diversity at inter- and intra-specific level in a wide range of crop species, which include rice (Joshi *et al*., 2000), wheat (Nagaoka and Ogihara, 1997), Vigna (Ajibade *et al*., 2000). DNA polymorphism in blackgram mutants was identified with ISSR markers (Souframanien *et al*., 2002). With the above background two multilocus markers, *viz.*, RAPD (25 primers), ISSR (11 primers) and one single locus marker, *viz.*, SSR (10 primer pairs) were used in the present study to assess the genetic diversity among 32 genotypes of pigeonpea and its wild relatives and to find the efficiency of the different marker systems to discriminate the genotypes at molecular level.

Materials and Methods

The experiments were conducted at Centre for Plant Molecular Biology, Tamil Nadu Agricultural University during 2001-2003. The seeds were obtained from the Pulses Breeding Station, Centre for Plant Breeding and

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Plant Material and DNA Extraction

Thirty two pigeonpea genotypes, which included 23 cultivated species and nine wild relatives, were subjected to diversity analysis. The seeds of all cultivated genotypes were obtained from Pulse Breeding Station, TNAU, Coimbatore (Table 1) and the wild relatives were obtained from International Crops Research Institute for the Semi-Arid Tropics, (ICRISAT), Patencheru, India. DNA from the thirty two genotypes was extracted following Gawal and Jarret (1991) with slight modifications.

RAPD Analysis

DNA from the 32 genotypes was amplified using a set of 25 arbitrary oligonucleotide decamer primers obtained from Operon Technologies, USA. Amplification reaction were in volume of 15 ml containing 25-50 ng of genomic DNA, 0.5 mM of primer, 10 mM of dNTPs (2.5 mM each), 50 mM KCl, 10 mM Tris Hcl, (pH8.3), 2.5 mM $MgCl_2$, 0.01% gelatin, 0.15 units of *Taq* polymerase (Bangalore Genei Pvt. Ltd., Bangalore). Amplification were performed in PTC thermal cycler (MJ Research Inc., USA) programmed for an initial denaturation of 94°C for 4 min, 45 cycles of 1 min. denaturation at 94°C, 1 min annealing at 37°C, and extension at 72°C for 2 min, followed by final extension for 5 min at 72°C and then at 4°C for storage. PCR amplified products (10 ml) were subject to electrophoresis in a 1.2% agarose gel in 1xTBE buffer at 50 V for 5 h using Broviga^{TM} standard submarine gel electrophoresis unit.

ISSR Analysis

A total of 11 primers were used for the study obtained from University of British Columbia (UBC) was used. PCR amplification was carried out in 15 ml reaction containing 25 ng of genomic DNA, 1 mM of primer, 10 mM of dNTPs (2.5 mM each), 50 mM KCl, 10 mM Tris Hcl (pH8.3), 2.5 mM $MgCl_2$, 0.01% gelatin, 0.15 units of *Taq* polymerase (Bangalore Genei Pvt. Ltd., Bangalore). Amplification was done using a PTC thermal cycler (MJ Research Inc., USA) programmed for initial denaturation at 94°C for 5 min, 45 cycles of 1 min denaturation at 94°C, 1 min annealing at 37°C, 2 min extension at 72°C, final extension of 5 min at 72°C and then at 4°C for storage. The PCR products (5 ml) were run in a 4% denaturing polyacrylamide gel electrophoresis (PAGE) at a constant current of 80 watts for 3 hours and 45 minutes and resolved by silver staining (Panaud *et al*., 1996).

Table 1. Level of polymorphism detected by RAPD, ISSR and SSR analysis across 32 *Cajanus cajan* and its wild relatives

Parameters	Type of marker		
	RAPD	ISSR	SSR
Genotypes screened	32	32	32
Number of primers used	25	11	10
Total number of markers	249	215	95
Range of markers across primers	6-17	8-26	4-13
Average number of markers	9.96	19.54	9.50
Number of monomorphic markers	1	1	0
Number of polymorphic markers	248	214	95

SSR Analysis

A total of 10 pigeonpea specific SSR primer pairs reported by Burns *et al*. (2001) were synthesized by Sigma-Genosys, USA and used for the present the study. PCR amplification was carried out in 15 ml reaction containing 25 ng of genomic DNA, 0.4 mM of primer, dNTPs (2.5 mM each), 50 mM KCl, 10 mM Tris Hcl, (pH 8.3), 2.5 mM $MgCl_2$, 0.01% gelatin, 0.15 units of *Taq* polymerase (Bangalore Genei Pvt. Ltd., Bangalore). Amplification was done using a PTC thermal cycler (MJ Research Inc., USA) programmed for initial denaturation at 94°C for 3 min, 27 cycles of 30 sec denaturation at 94°C, followed by annealing for 30 sec (temperature vary according to the primer), extension at 72°C for 1 min, final extension for 3 min. at 72°C and then at 4°C for storage. The PCR products (5 ml) were run in a 4% denaturing polyacrylamide gel electrophoresis (PAGE) at a constant current of 60 watts for 1 h and 15 minutes and resolved by silver staining procedure (Panaud *et al*., 1996).

DATA Analysis

Clearly resolved, unambiguous polymorphic band were scored visually for their presence or absence with each primer. The scores were obtained in the form of a matrix with '1' and '0', which indicate the presence and absence of bands in each variety, respectively. Polymorphism information content (PIC) was calculated for the SSR primer pairs based on the formula $PIC = 1- Sfi^2$, where *fi* is the frequency of the i^{th} allele (Smith *et al*., 1997). Marker index was calculated for RAPD and ISSR markers (multilocus), in order to characterize the capacity of each primer to reveal or detect polymorphic loci among the genotypes. It was calculated based on the formula $MI=1-Spi^2$, where *pi* is the frequency of the i^{th} allele, (Ghislain *et al*., 2002). Mean marker index was calculated

for RAPD and ISSR markers (multilocus markers), based on the formula $MMI = (1/N)\ (1\text{-}Spi^2)$, where *pi* is the frequency of the i^{th} allele and *N* is the total number of markers that a primer produced.

The data obtained by scoring the RAPD, ISSR and SSR profiles of different primers were subject to cluster analysis. Similarity matrix was constructed suing Jaccard's coefficient and the similarity values were used for sequential agglomerative hierarchial non-overlapping (SAHN) clustering based on unweighted pair group method with arithmetic averages (UPGMA) using NTSYSpc version 2.0 (Rohlf, 1998).

Results and Discussion

Several studies were made to test the efficiency of these markers in terms of informativeness in exploring the genetic variability among the genotypes. Comparison between RFLP and PCR based methods in pea (Lu *et al*., 1996), among RFLP, RAPD, AFLP and SSR markers in soybean (Powell *et al*., 1996), between RAPD and RFLP markers in *Theobroma cacao* (Lerceteau *et al*., 1997), among ISSR, RFLP and RAPD markers in wheat (Nagaoka and Ogihara, 1997), between RAPD and SSR markers in kiwifruit (Palombi and Damiano, 2002), between RAPD and ISSR in blackgram (Souframanien *et al*., 2002), between RAPD and SSR markers in rice (Ravi *et al*., 2003) have been reported so far. In the present investigation, two multilocus marker systems, *viz*., RAPD and ISSR and one single locus marker system, *viz*., SSR were evaluated for their efficiency in revealing the genetic diversity among the genotypes studied.

The major advantage of the PCR based marker systems is the generation of fragments from both single and multilocus. In the present study, 25 RAPD, 11 ISSR and 10 SSR primers were used to assess the genetic diversity and relatedness among a set of 32 genotypes in pigeonpea. All the primers produced high level of polymorphism (~100 per cent). This is because of highly divergent genotypes (cultivars and wild relatives) were included in the study. Maciel *et al*. (2001) also reported such a high level of polymorphism among population consisting of both cultivated and wild relatives.

Level of polymorphism detected by RAPD, ISSR and SSR analysis across 32 pigeonpea genotypes is presented in Table 1. The average number of fragments amplified by RAPD primers among the genotypes was 9.96 with a range of 6 to 17. Ratnaparkhe *et al.* (1995) reported an average of eight markers per primer. However, Maciel *et al*. (2001) reported the generation of RAPD fragments ranging from 7 to 31 in common beans. Such a high variation in the number of fragments produced by these arbitrary primers may be attributed to the differences in the binding sites through out genome of the genotypes included. ISSR primers generated 8 to 26 markers with average of 19.54 markers and SSR primer pairs generated 4 to 13 alleles and average of 9.5 alleles. Earlier, generation of 4 to 12 markers in Vigna (Ajibade *et al*., 2000) and 8 markers in *Phaseolus vulgaris* (Galvan *et al*., 2003) by ISSR primers and generation of 2-5 alleles with SSR primers in pigeonpea (Burns *et al*., 2001) and 5.3 alleles in common bean (Metais *et al*., 2002) were reported. Generation of higher number of markers or alleles in the present study can be attributed to the higher amount of genetic diversity among cultivars and wild relatives of pigeonpea.

Marker index (MI) reveals the amount of information that can be obtained from a particular primer. In the present study, marker index for RAPD and ISSR was in the range of 3.701 to 11.671 and 5.300 to 31.722, respectively. The higher MI value indicates the more informativness of the primer. Hence, the random primers, *viz*., OPG06, OPG15, OPG03 and the ISSR primers, *viz*., UBC842, UBC876, and UBC857 are considered to be worth in future studies in the field of taxonomical and genetic resource management. Among these two multilocus marker system, ISSR primers had higher index values compared to RAPD suggesting their superiority in generating polymorphism among the genotypes than RAPD primers as reported by Galvan *et al.* (2003). PIC also provides the estimate of discriminatory power of a locus in the case of SSR marker system by taking into account not only the number of alleles expressed, but also the relative frequency of those alleles. The PIC values of the 10 SSR ranged from 0.844 to 0.982 with a mean of 0.921 whereas a wide range of PIC values (0.12 to 0.72) with a mean of 0.44 was reported by Metais *et al*. (2002) in common beans. This contradictory situation of obtaining a higher mean and range in the present study may be attributed to the wide diversity among the cultivars and their relatives.

Genetic Relationships between Pigeonpea Cultivars and Wild Relatives

Cluster analysis was used to group the genotypes to construct dendrogram based on RAPD (Fig. 1), ISSR (Fig. 2) and SSR (Fig. 3) data. The three marker systems

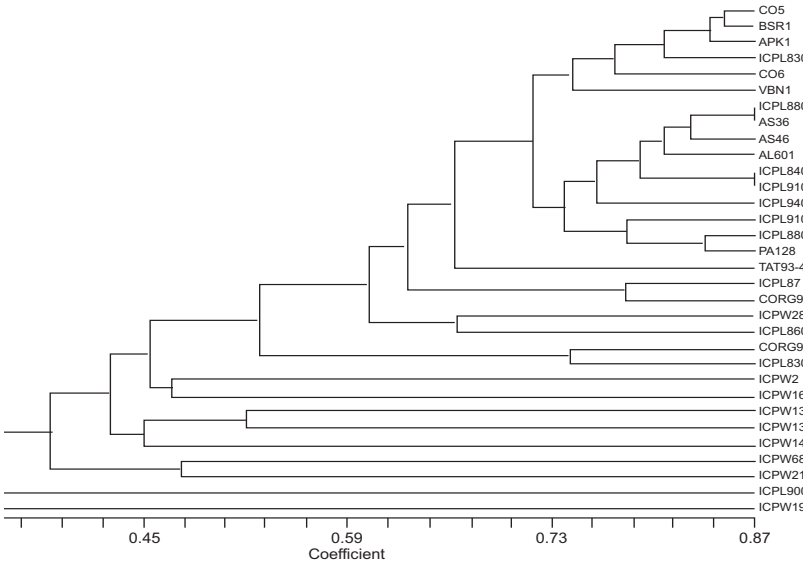

Fig. 1: Dendrogram of 32 genotypes of pigeonpea and its wild relatives based on 249 RAPD markers

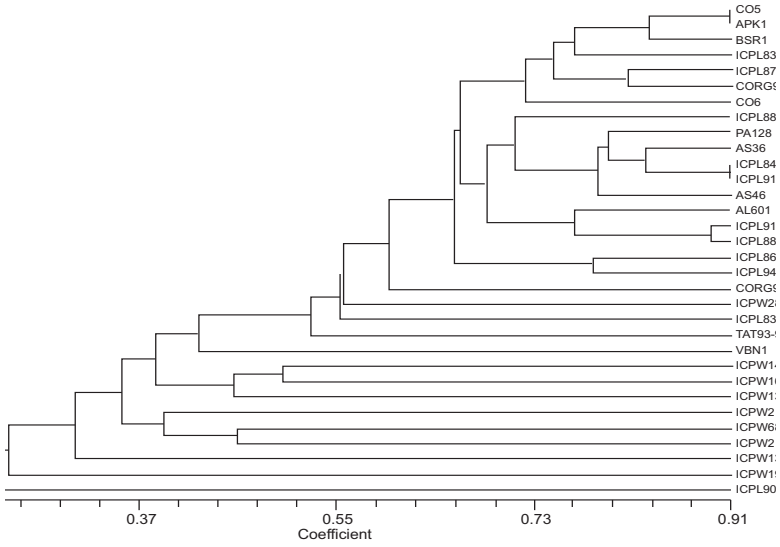

Fig. 2: Dendrogram of 32 genotypes of pigeonpea and its wild relatives based on 215 ISSR markers

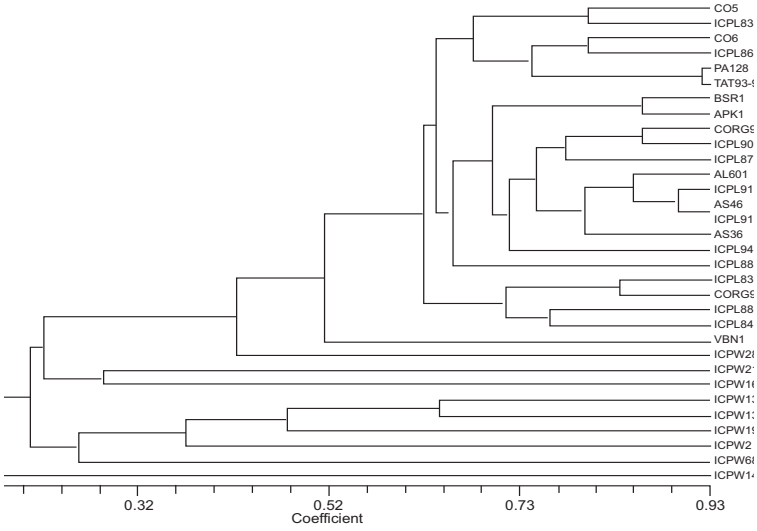

Fig. 3: Dendrogram of 32 genotypes of pigeonpea and its wild relatives based on 95 SSR markers

clearly distinguished the cultivars and wild forms into different clusters as expected since they were morphologically and genetically well differentiated. This is in accordance with studies in lima bean (Fofana *et al*., 1997). It is interesting to note that these markers were able to further subdivide the 23 cultivars into five, seven and three clusters by RAPD, ISSR and SSR markers, respectively, which revealed the existence of sufficient amount of genetic variability available among these cultivars that could be exploited further. Archak S *et al*. (2002) reported that the cultivars derived from a common parent always tend to cluster together. Villand *et al*. (1998) observed the grouping of both wild and cultivated tomatoes in a single cluster and concluded that this might be due to the introgression of genes from the wild relatives into cultivated genotypes. In the present investigation, based on RAPD markers, ICPW28, a wild accession belongs to *Cajanus cajanifolius* and a cultivated line, ICPL86020 formed a single cluster due to their genetic relatedness. This type of relatedness was established only by the RAPD marker system, because RAPD scans the whole genome, whereas SSR and ISSR (to some extent) search for specific sites in the genome. Such information on the relatedness between the cultivated line and wild relatives would enable the breeders to introgress the favourable genes from the wild relatives through conventional breeding without any cytological barrier.

The wild relatives taken for the study belong to three different genera, *viz., Cajanus, Rhyncosia and Flemingia*. The accessions belonging to the genus *Cajanus* were grouped together which is in accordance with the results of Sivaramakrishnan *et al*. (2002). In the present study, larger amount of genetic diversity was revealed among wild relatives by all the three marker systems and the reduced genetic variability among the cultivars might be due to genetic erosion over a long period of breeding (Brown, 1978).

Among the nine wild accessions studied, five belong to secondary, two belong to tertiary and two belong to unknown origin of gene pool (Van der Maeson, 1990). These wild accessions belonging to the same gene pool were discriminated into 3-4 clusters by different marker systems revealing sufficient amount of genetic diversity among them. The clustering among the wild relatives was according to their gene pool origin classified based on crossability, evolution of form and chemotaxonomy. All the clusters had different species belonging to the same genera except in one cluster where included two different genera, *i.e., Cajanus platycarpus* and *Rhyncosia aurea* which belong to tertiary gene pool were grouped together by ISSR marker system. Galvan *et al*. (2003) also reported that the ISSR markers had the ability to differentiate the common bean genotypes based on gene pool origin, *i.e.,* Mesoamerican origin and Andean origin. Among the wild species, both accessions belonging to Australian origin and Indian origin clustered together suggesting the movement gene pool form the centre of origin to other regions.

For overall comparison of all the three marker systems used in the present study, the different parameters, *viz*., mean number of markers generated, level of polymorphism, marker index, PIC values, clustering pattern and the mean intercluster distances are summerised hereunder:

From Table 2 it is evident that among the two multilocus markers, *viz*., RAPD and ISSR, ISSR was the most efficient based on all the parameters studied. Even though the mean similarity index was slightly higher in RAPD than ISSR, ISSR was able to generate more markers with less number of primers than RAPD.

Ajibade *et al*. (2000) and Galvan *et al*. (2003) also concluded that ISSR would be a better tool than RAPD for phylogenetic studies. When comparing the SSR with RAPD and ISSR based on the information on the level of polymorphism and the PIC value, SSR was more efficient in generation of polymorphism among the genotypes and high informativness (PIC), but they were less efficient in discriminating closely related genotypes/ cultivars. Hence it can be stated that even though SSR markers are highly polymorphic, for genetic diversity

Table 2. Comparison of different parameters among the three markers

Marker system	Mean number of markers	Level of polymorphism %	Mean similarity index	Mean marker index (PIC)	Number of clusters	Average intercluster disatnce
RAPD (25)	9.96	99.60	0.535	0.655	10	0.489
ISSR (11)	19.54	99.53	0.478	0.751	12	0.582
SSR (10)	9.50	100.00	0.458	0.921	7	0.598

(Figures in the parenthesis are number of primers used)

studies generation of more number of markers is of prime importance. Since SSR are more loci specific, they can be used for mapping studies (Wu and Tanksley, 1993). ISSR markers are multilocus markers and produce a higher percentage of polymorphism (Blair *et al*., 1999; Ajibade *et al*., 2000; Martin and Sanchez-Yelamo, 2000; Galvan *et al*., 2003) and reveal relationship between genotypes both at intra-specific and inter-specific levels (Huang and Sun, 2000).

The present study, clearly established the utility of molecular markers like RAPD and ISSR in assessing the genetic diversity among the cultivars and their wild relatives to identify the best parental lines for further breeding programmes and for effective management of genetic resources.

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