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MOLECULAR DIVERSITY STUDY USING SSAP MARKERS IN PIGEONPEA (*CAJANUS CAJAN* L. MILLSP.)

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ABSTRACT

In the present study, based on *in silico* homology search SSAP markers were developed from one of the active *Cajanus cajan* retrotransposon element (*CcRT8*) and employed for molecular diversity study among 30 pigeonpea genotypes belonging to five different gene pools. Total four primer combinations were screened, which produced 156 bands of which 149 (95.50%) bands were polymorphic with an average of 37.2 polymorphic bands per primer combination. The average polymorphism information content (PIC) 0.26 and gene diversity (H^) 0.32 were found higher for primer combinations screened. These results indicated *CcRT8* element showed higher insertional polymorphism in the pigeonpea genome. The Jaccard coefficients ranged from 0.16 to 0.96 suggesting a broad genetic base across the gene pools. However, within cultivars coefficients ranged from 0.69-0.96 revealing a narrow genetic base and these results confirmed earlier findings. Cluster analysis showed grouping of genotypes mainly on the basis of gene pools used. The present study suggested two genetically distant genotypes BRG 3 and ICP 15701, which could be potential donors for resistance breeding and for introgressing of novel genes into pigeonpea cultivars. Here, we have successfully demonstrated *in silico* search based development of SSAP markers for studying molecular diversity among pigeonpea genotypes.

INTRODUCTION

Pigeonpea (*Cajanus cajan* L. Millsp.) is a most widely adapted food legume crops, cultivated throughout the semi-arid tropics and sub-tropics. India accounts for 90 per cent of the global production with an area 3.86 million hectare and production of 2.65 million tonnes (FAOSTAT, 2012). In spite of larger area under pigeonpea in India, the expected yield levels witnessed was very low due to various biotic and abiotic constraints. Wild relatives of pigeonpea are considered to be rich reservoir of genes for resistance to biotic and abiotic stresses (Sharma *et al.,* 2003). Introgression of these genes may be one of option in improving the yield, increasing production levels in cultivated pigeonpea (Singh, 2005). Therefore, knowledge about genetic diversity in germplasm is very useful for plant breeders. It supports their decision on the selection of cross combinations from large sets of parental genotypes and is always helpful for widening the genetic basis of a breeding program (Ganapathy *et al.,* 2011).

In order to study molecular diversity, various marker systems have been used in many plant species *viz.,* STMS in chickpea (Bharadwaj *et al.,* 2010), SSAP, AFLP and SSR in durum wheat (Mardi *et al.,* 2011), RAPD in chilli (Bahurupe *et al.,* 2013), and ISSR in chickpea (Pandey *et al.,* 2014). Among these techniques, *Ty1-copia* based sequence specific amplification polymorphism (SSAP) have been found extremely informative in many crops e.g. rye (Brbgoszewska *et al.,* 2012), faba bean (Quji *et al.,* 2012) and pea (Hamid *et al.,* 2012). Similarly, in pigeonpea various marker techniques have been used and SSAP was found more informative for phylogenetic analysis (Patil *et al.,* 2012).

SSAP technique employs a primer which is specific to the long terminal repeat (LTR) region of particular retrotransposon in combination with selective AFLP primer during second round of selective amplification (Waugh *et al.,* 1997). LTR-retrotransposons are broadly divided into the *Ty1-copia* and *Ty3-gypsy* groups. These are responsible for the vast differences in genome size and genome arrangements in various plant species (Bennetzen, 2000). The retrotransposon insertions are irreversible, high in copy numbers, well distributed throughout the genome and changes remain relatively fixed making them suitable candidates for analyzing genetic relationships.

Previously various PCR based strategies were employed for identifying novel retrotransposon sequences in un-sequenced plant genomes for development of SSAP markers (Pearce *et al.,* 1999). But, availability of whole genome sequence of a given crops in addition to well characterized retro elements from other related crops. *In silico* homology sequence search became an easy and efficient tool for identifying and characterizing novel retro elements for marker development. Zhao *et al.* (2009) used a similar approach *(in silico* homology searching) to identify novel retrotransposons in the *Botrytis cinerea* genome.

Recent advances made through sequencing of pigeonpea

whole genome resulted in a tremendous increase in genomic resources (Singh *et al.,* 2012; Varshney *et al.,* 2012). Therefore, in the present study we have demonstrated *in silico* homology searching based development of SSAP marker from one of the active pigeonpea *Ty1-copia* (*CcRT8*) element identified in the pigeonpea genome. Discussed their efficiency in revealing molecular diversity and relationships among 30 pigeonpea genotypes belonging to five different gene pools. The information generated will be helpful in selecting parents for introgressing useful genes into pigeonpea cultivars.

MATERIALS AND METHODS

Identification of novel LTR-retrotransposon

In silico homology sequence search was performed using BLASTn algorithm against pigeonpea whole genome shotgun sequences as per the method reported by Zhao *et al*. (2009) in *Botrytis cinera*. *Vigna radiata* L. Wilczek (GenBank Acc No AY684686.1), VRC-91 *copia* reverse transcriptase partial gene sequence was used as query sequence. Similar to the criteria as used to isolate paralogus copies of *copia* elements in grape vine genome (Moisy *et al.,* 2008). The pigeonpea contigs showing at least 80% query coverage and >70 % similarity with query were searched for presence of full length LTR-RT using LTR-FINDER *ver* 1.02 (Zhao and Wang, 2007).

Plant material and DNA extarction

The experimental material comprised of 30 pigeonpea genotypes belonging to five different gene pools *viz.,* primary gene pool (*Cajanus cajan*), secondary gene pool (*C. scarabaeiodes* and *C. albicans*), tertiary gene pool (*C. platycarpus*), quaternary gene pool (*Rhyncosia rothi* and *R. bracteata*) and other (*Flemingia macrophylla*) (Table 1). Genomic DNA were extracted from the fresh leaves of 15 days old seedlings using a CTAB protocol (Agbagwa *et al.,* 2012).

SSAP amplification

The sequence information on 5' LTR of newly identified pigeonpea *CcRT8 copia* element was used for designing LTR specific primer (Table 2). SSAP amplifications were carried out as per the protocol described in pigeonpea (Patil *et al*., 2012). The PCR amplification products were denatured and separated on 6% polyacrylamide gels. The bands were finally resolved using fast silver staining method as described by Benbouza *et al*. (2006).

Statistical analyses

The bands were scored as presence (1) or absence (0) for each SSAP primer profile. The pooled marker data so obtained were analyzed to generate pairwise Jaccard similarity coefficients (Jaccard, 1908) using NTSYS-pc *ver* 2.0. Similarity matrices thus generated were utilized to construct UPGMA dendrogram. The robustness of each dendrogram was evaluated by bootstrap analysis with 1000 times repeated sampling using WINBOOT (Yap and Nelson, 1996). PIC and average gene diversity (*H^*) of a given primer combinations were calculated using POWERMARKER program *ver* 3.0 (Liu and Muse, 2005).

RESULTS AND DISCUSSION

The present study was carried out in order to develop and utilize the SSAP markers based on *in silico* analysis in pigeonpea. Since, mung bean (*Vigna radiata* L. Wilczek) is phylogenetically more close to pigeonpea as a warm season legumes. Using mung bean VRC-91 *copia* reverse transcriptase partial gene sequence as a query, three full length pigeonpea *copia* elements *CcRT6*, *CcRT7* and *CcRT8* were identified in the pigeonpea genome. The structural characteristics of these elements were shown in table 3. Zhao *et al*. (2009) similarly identified novel retrotransposons in *Botrytis cinerea* based on *in silico* homology search. Contrast to this, Pearce *et al*. (1999) reported polymerase chain reaction (PCR)-based method to identify *Ty1* copia-LTR elements from un-sequenced genome of higher plants, and isolated novel LTR sequences from pea, broad bean, and norway spruce. Out of three *copia* elements identified in this study, only *CcRT8* showed perfect target site duplication (TTCT) near insertions with highest LTR sequence identity (98.9%) for 5' and 3' ends indicating the most active element. Moisy *et al*. (2008) also reported identification of active *Ty-1* copia

elements in the grapevine genome based on target site duplication and LTR sequence identity.

Results from *in silico* homology search revealed pigeonpea *CcRT8* is the most active *Ty1-copia* element. This element may have contributed for higher sequence variation for pigeonpea genome. LTR sequence information of this element was further selected for development of SSAP markers. Moisy *et al*. (2008) observed SSAP insertion patterns of 8 active retrotransposon families on 10 *Vitis* accessions and found most of the scored bands are polymorphic, indicating these families have been active after speciation across the genus. Similarly, Brbgoszewska *et al*. (2012) developed SSAP markers based on novel novel *Ty1-copia* like element and used for genetic diversity study in rye (*Secale cereale* L.) inbred lines.

For SSAP analysis, total four primer combinations were

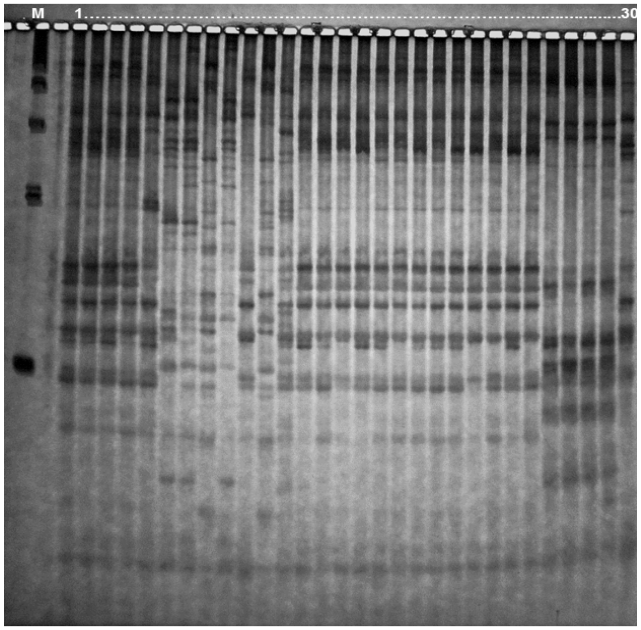

Figure 1: SSAP amplification profiles for 30 pigeonpea genotypes on 6% polyacrylamide gels using primer combination *CcRT8*-LTR/ *Eco*RI + CAG. (Where, Lanes: M = 100 bp ladder; 1-30 pigeonpea genotypes as mentioned in Table 1)

Table 1: Details of 30 pigeonpea genotypes used for genetic diversity study

Sl. No	Pigeonpea Genotype	Species	Source
1	BRG 3	*C. cajan*	Karnataka, India
2	ICP 8863	*C. cajan*	Maharashtra, India
3	ICP 7035	*C. cajan*	ICRISAT, AP, India
4	TTB 7	*C. cajan*	Karnataka, India
5	ICP 15770	*C. albicans*	India
6	ICP 15853	*R. rothi.*	ICRISAT, AP, India
7	BDNP 3	*R. rothi*	Maharashtra, India
8	ICP 817	*R. bracteata*	ICRISAT, AP, India
9	ICP 15890	*R. rothi*	ICRISAT, AP, India
10	BNG 1	*C. scarabaeoides*	Karnataka, India
11	ICP 15815	*R.bracteata*	ICRISAT, AP, India
12	BDNP 4	*C. albicans*	Maharashtra, India
13	HY 3C	*C. cajan*	Andra Pradesh, India
14	BRG 1	*C. cajan*	Karnataka, India
15	GRG 333	*C. cajan*	Karnataka, India
16	GT 101	*C. cajan*	Gujarat, India
17	BSMR 736	*C. cajan*	Maharashtra, India
18	WRP 1	*C. cajan*	Karnataka, India
19	IPA 8F	*C. cajan*	Kanpur, Uttar Pradesh
20	BRG 2	*C. cajan*	Karnataka, India
21	ICPL 87119	*C. cajan*	ICRISAT, AP, India
22	JKM 189	*C. cajan*	Madhya Pradesh, India
23	ICP 2376	*C. cajan*	ICRISAT, AP, India
24	GRG 811	*C. cajan*	Karnataka, India
25	TS3R	*C. cajan*	Karnataka, India
26	ICP 15701	*C. scarabaeoides*	ICRISAT, AP, India
27	ICP 15667	*C. platycarpus*	ICRISAT, AP, India
28	ICPW 71	*C. platycarpus*	Himachal Pradesh, India
29	ICPW 61	*C. platycarpus*	Uttar Pradesh, India
30	ICP 15799	*F. macrophylla*	ICRISAT, AP, India

Note* *C- Cajanus*; *R-Rhyncosia*; *F-Flamingia*; AP-Andra Pradesh

Table 2: Details of adaptor and primer sequences used for SSAP analysis

Type	Primer/adapter name	Sequence
SSAP primer	*CcRT8*-LTR	5'-GTGCTGGTGGCCTTTTCTCC-3'
SSAP adapters	Double stranded *Mse*I adapters	5'-GACGATGAGTCCTGAG-3' 5'-TACTCAGGACTCAT-3'
	Double stranded *Eco*RI adapters	5'-CTCGTAGACTGCGTAC-3' 5'-AATTGTACGCAGTC -3'
Primers used for pre-amplification	E + C (*Eco*RI adapter specific primer)	5'-GACTGCGTACAATTCC-3'
	M + G (*Mse*I adapter specific primer)	5'-GATGAGTCCTGAGTAAG-3'
Primers used for selective amplification	*Eco*RI + CAG	5'-GACTGCGTACAATTCCAG-3'
	*Eco*RI + CAT	5'-GACTGCGTACAATTCCAT-3'
	*Mse*I + GCC	5'-GATGAGTCCTGAGTAAGCC-3'
	*Mse*I + GTG	5'-GATGAGTCCTGAGTAAGTG-3'

Table 3: Structural features for homologous *Ty1-copia* elements identified in the pigeonpea genome

Query sequence used	Pigeonpea contigs with RT	Name of RT	Size of RT(bp)	Length 5'-LTR/3'-LTR (bp)	TSD 5′-3'	LTR identity (%)
Vigna radiata	AFSP01000619.1	*CcRT6*	2007	108/112	-	93.8
(AY684686.1)	AFSP01034138.1	*CcRT7*	5245	496/499	-	90.2
	AFSP01018343.1	*CcRT8*	4965	174/175	TTCT	98.9

Note* RT- Retrotransposons; LTR- Long terminal repeat; TSD-Target site duplication; bp- base pairs.

screened on 30 pigeonpea genotypes (Fig 1). A total of 156 bands were scored, out of which 149 (95.5%) bands were polymorphic (Table 4). The number of scorable bands produced by each primer combinations ranged from 33 to 45, with an average of 39 bands per primer combination. The percent of polymorphism was ranged from 93.3% to 97.6%, with an average of 95.5% per primer combination. Two SSAP primers *CcRT8*-LTR/*Eco*RI + CAG and *CcRT8*-LTR/ *Eco*RI + CAT produced the highest numbers of bands (45 and 42, respectively), followed by *CcRT8*-LTR/*Mse*I + GCC and *CcRT8*-LTR/*Mse*I + GTG (36 and 33 bands, respectively). Overall, higher polymorphism rate 95.5% was observed for SSAP primers screened. Similarly, Patil *et al.* (2012) reported 90.19 % polymorphism for SSAP markers developed in pigeonpea based on *Panzee* retrotransposon. The higher polymorphism

Table 4: Degree of polymorphism and informativeness as revealed by SSAP primer combinations

Sl. No	Primer combinations	TNB	NPB	%P	PIC	Average genediversity (*H*ˆ)
1	*CcRT8*-LTR/*Eco*RI + CAG	45	42	93.3	0.23	0.29
2	*CcRT8*-LTR/ *Eco*RI + CAT	42	41	97.6	0.26	0.32
3	*CcRT8*-LTR/ *Mse*I + GCC	36	35	97.2	0.26	0.33
4	*CcRT8*-LTR/ *Mse*I + GTG	33	31	93.9	0.28	0.35
	Total	156	149	-	-	0.29
	Minimum	33	31	93.3	0.23	0.29
	Maximum	45	42	97.6	0.28	0.35
	Average	39	37.2	95.5	0.26	0.32

Note* TNB-Total number of bands produced; NPB- Number of polymorphic bands: % P- Percent polymorphism; PIC-Polymorphism information content

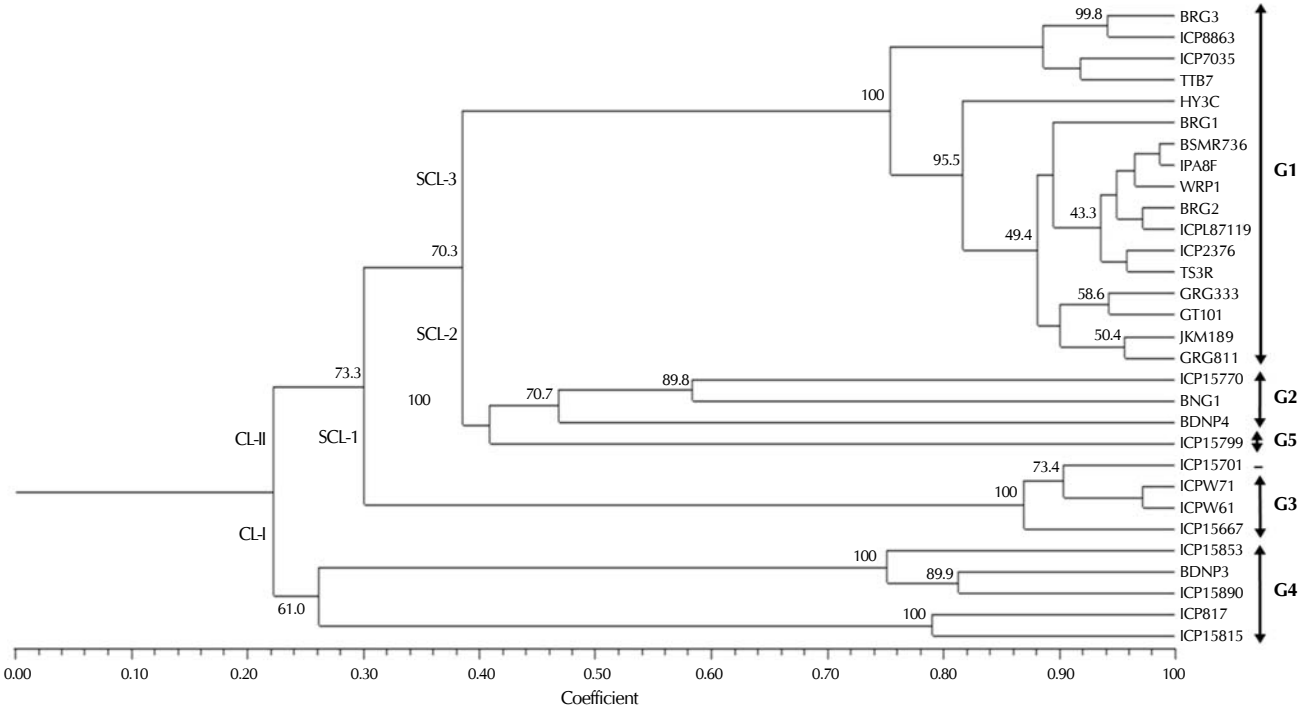

Figure 2: Dendrogram showing clustering pattern for 30 pigeonpea genotypes based on SSAP data (Where, G1-genotypes belongs to primary gene pool; G2-secondary gene pool; G3-tertiary gene pool; G4 quaternary gene pool and G5- others)

rate observed in our study confirmed the active role of *CcRT8* in pigeonpea genome variations.

The PIC values ranged from 0.23 (*CcRT8*-LTR/*Eco*RI + CAG) to 0.28 (*CcRT8*-LTR/*Mse*I + GTG), with an average of 0.26 per primer combination. These results were supported by average PIC of 0.21 as observed for SSAP markers in pigeonpea (Patil *et al.,* 2012). The average gene diversity of a given primer combination (*H*ˆ) was ranged from 0.29 (*CcRT8*-LTR/ *Eco*RI + CAG) to 0.35 (*CcRT8*-LTR/*Mse*I + GTG), with a mean of 0.32 per primer combination. These results suggested SSAP is a best marker of choice for genetic diversity studies. Using small number of SSAP primers, it is possible to generate abundant genetic information with sufficient precision and at reasonable cost analysis (Ouji *et al.*, 2012).

The cluster analysis based on SSAP markers depicted a well resolved relationships among 30 pigeonpea genotypes with high bootstrap values ranging from 43.3% to 100% (Fig. 2). Similarly, grouping of 21 pigeonpea genotypes with higher bootstrap support (42% to 100%) were noticed using SSAP markers (Patil *et al.,* 2012). SSAP analysis is not only useful in differentiating genotypes, but also attaches bootstrap confidence values to the branching patterns (Bousios *et al.,* 2007). In the dendrogram, two major clusters were observed CL-I and CL-II. CL-I constituted all the genotypes belonging to quaternary gene pool (*R. rothi* and *R. bracteata*). CL-II further divided into three sub-clusters (SCL-1, 2 & 3). SCL-1 comprised all the genotypes belongs to tertiary gene pool (*C. platycarpus*) with one exception (*C. scarabaeoides*). SCL-2 included all the genotypes of secondary gene pool (*C. scarabaeiodes* and *C. albicans*) with one exception (*F. macrophylla*). SCL-3 constituted all the genotypes of primary gene pool (*C. cajan*). In the dendrogram, all pigeonpea cultivars were distinguished from the wild genotypes as a separate sub-cluster. The grouping of genotypes observed was mainly on the basis of gene pools used. These results indicated common *CcRT8* insertions regions shared by members of each gene pools.

The overall genetic distances in terms of Jcccard similarity coefficients ranged from 0.16 to 0.96 (Suppl Table I), revealing a broad genetic base across 30 genotypes. Patil *et al.* (2014) also reported broad genetic base across 22 pigeonpea

Supplementary Table I: Jacccard similarity matrix as obtained based on SSAP marker data

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
BRG	1.00																													
ICP8863	0.94	1.00																												
ICP 7035	0.89	0.87	1.00																											
TTB 7	0.88	0.91	0.92	1.00																										
ICP 15770	0.45	0.44	0.47	0.47	1.00																									
ICP15853	0.24	0.24	0.23	0.24	0.21	1.00																								
BDNP 3	0.26	0.25	0.25	0.25	0.23	0.80	1.00																							
ICP 817	0.23	0.24	0.24	0.25	0.27	0.29	0.32	1.00																						
ICP 15890	0.23	0.23	0.23	0.23	0.23	0.71	0.81	0.27	1.00																					
BNG 1	0.43	0.43	0.42	0.44	0.58	0.26	0.29	0.28	0.25	1.00																				
ICP 15815	0.21	0.22	0.23	0.24	0.24	0.24	0.25	0.79	0.20	0.24	1.00																			
BDNP 4	0.36	0.36	0.36	0.38	0.49	0.23	0.25	0.23	0.22	0.44	0.23	1.00																		
HY 3C	0.70	0.70	0.71	0.70	0.40	0.21	0.22	0.20	0.24	0.39	0.18	0.36	1.00																	
BRG 1	0.72	0.74	0.75	0.76	0.42	0.19	0.21	0.19	0.20	0.38	0.18	0.40	0.81	1.00																
GRG 333	0.70	072	0.75	0.72	0.39	0.21	0.21	0.20	0.21	0.38	0.19	0.34	0.86	0.83	1.00															
GT 101	0.72	0.74	0.77	0.74	0.41	0.21	0.23	0.22	0.22	0.37	0.21	0.37	0.86	0.84	0.94	1.00														
BSMR 736	0.75	0.78	0.81	0.78	0.43	0.20	0.21	0.21	0.20	0.38	0.20	0.38	0.83	0.90	0.90	0.93	1.00													
WRP 1	0.75	0.78	0.79	0.78	0.41	0.20	0.20	0.21	0.19	0.38	0.20	0.38	0.80	0.90	0.90	0.90	0.97	1.00												
IPA 8F	0.76	0.79	0.82	0.79	0.42	0.20	0.21	0.21	0.20	0.37	0.21	0.37	0.81	0.92	0.89	0.92	0.99	0.96	1.00											
BRG2	0.76	0.79	0.80	0.79	0.42	0.20	0.21	0.21	0.20	0.36	0.21	0.39	0.81	0.92	0.86	0.92	0.96	0.96	0.97	100										
ICPL 87119	076	0.79	0.77	0.76	0.43	0.19	0.20	0.21	0.20	0.37	0.21	0.38	0.79	0.84	0.89	0.93	0.93	0.93	0.94	0.97	1.00									
JKM 189	0.69	0.71	0.74	0.71	0.38	0.21	0.20	0.19	0.20	0.35	0.18	0.33	0.79	0.87	0.87	0.90	0.89	0.89	0.88	0.88	0.85	1.00								
ICP 2376	0.77	0.78	0.81	078	0.43	0.21	0.22	0.22	0.21	0.38	0.20	0.38	0.78	0.88	0.85	0.88	0.92	0.95	0.93	0.96	0.93	0.86	1.00							
GRG 811	0.72	0.74	0.77	0.74	0.40	0.20	0.21	0.20	0.21	0.35	0.19	0.35	0.84	0.84	0.89	0.94	0.93	0.90	0.92	0.92	0.89	0.96	0.88	1.00						
TS 3R	0.74	0.75	0.78	0.75	0.42	0.19	0.20	0.20	0.20	0.37	0.19	0.39	0.81	0.86	0.89	0.89	0.93	0.96	0.92	0.94	0.92	0.88	0.96	0.89	1.00					
ICP 15701	0.35	0.36	0.33	0.35	0.32	0.30	0.29	0.20	0.27	0.31	0.17	0.35	0.29	0.29	0.29	0.30	0.31	0.31	0.30	0.30	0.30	0.29	0.30	0.31	0.29	1.00				
ICP 15667	0.30	0.30	0.28	0.29	0.31	0.33	0.30	0.17	0.28	0.28	0.16	0.32	0.24	0.24	0.24	0.25	0.26	0.25	0.25	0.25	0.25	0.24	0.26	0.24	0.24	0.86	1.00			
ICPW 71	0.35	0.35	0.35	0.32	0.34	0.32	0.31	0.28	0.19	0.27	0.32	0.16	0.35	0.27	0.27	0.27	0.28	0.30	0.30	0.29	0.29	0.28	0.28	0.29	0.28	0.90	0.87	1.00		
ICPW 61	0.36	0.35	0.32	0.34	0.33	0.32	0.29	0.19	0.28	0.31	0.18	0.35	0.28	0.28	0.28	0.29	0.30	0.30	0.29	0.29	0.29	0.28	0.29	0.30	0.28	0.90	0.88	0.97	1.00	
ICP 15799	0.42	0.41	0.40	0.41	0.40	0.30	0.30	0.19	0.30	0.44	0.16	0.39	0.36	0.37	0.36	0.35	0.35	0.35	0.34	0.34	0.35	0.32	0.35	0.35	0.36	036	0.34	0.40	0.40	1.00

genotypes using AFLP-RGA markers. The high polymorphism noticed may be due to unique insertions of *CcRT8 copia* elements across wild genotypes. But, within cultivars similarity values ranged from 0.69-0.96 indicating a narrow genetic base (27% variation), may be due to sharing of many common insertions. It was also reflected from dendrogram that all cultivars are grouped at 0.74 similarity. Results were in agreement with narrow genetic base as reported within pigeonpea cultivars compared to wild accessions using RFLP (Nadimpalli *et al.,* 1992), RAPD (Ratnaparkhe *et al.,* 1995), AFLP (Punguluri *et al.,* 2006), DArT (Yang *et al.,* 2006) and SSR (Datta *et al.,* 2013).

Based on estimated genetic similarity matrix within primary gene pool, lower similarity (0.69) was observed between cultivars BRG 3 and JKM 189 indicating genetically diverse parents. The higher similarity (0.99) was observed between IPA 8F and BSMR 736 indicating genetically similar parents. BRG 3 is known to be sterility mosaic disease resistant genotype and can be used as a best donor parents for SMD resistance beeding in pigeonpea. Since, the genotypes of secondary gene pools can be easily crossed with primary gene pool. The lower similarity values (0.29-0.31) was observed between ICP 15701 (*C. scarabaeoides*) and 13 cultivars (HY 3C, BRG1, GRG 333, GT 101, BSMR 736, WRP 1, IPA 8F, BRG 2, ICPL 87119, JKM 189, ICP 2376, GRG 811 and TS 3R). Therefore, ICP 15701 can be used as best donor parents to broaden genetic base and to introgress useful genes into cultivars.

In summary, we have successfully demonstrated *in silico* search based development of SSAP markers in pigeonpea. This strategy has greater potential in increasing the number of markers for linkage analysis in pigeonpea. SSAP could be one of the best marker of choice for pigeonpea crop improvement.

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