

Research Article

Genetic Diversity in Certain Genotypes of Chilli and Paprika as Revealed by RAPD and SSR Analysis

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Abstract: Genetic relationships between thirteen genotypes of chilli and paprika collected from different places of India were analyzed using twelve RAPD and nine SSR primers. Clustering based on the generated markers was conducted using NTSYS software's. The RAPD primers produced a total of 78 bands of which 65 were polymorphic with a mean of 5.41 polymorphic bands per primer while the SSR primers produced 28 SSR loci with a mean of 3.22 alleles per SSR locus. The similarity index matrix ranged from 0.36 to 0.91 (RAPD) and 0.117 to 0.9 (SSR) with mean of 0.62 and 0.39 respectively. Polymorphic Information Content (PIC) values of the SSR primers ranged from 0.00 to 0.75 with an average value of 0.546. The resulting RAPD and SSR dendrograms separated the cultivars with small and erect fruit position from the large and medium fruited cultivars with declining fruit position. Both RAPD and SSR markers showed genetic variability in the studied pepper genotypes and they are powerful tools for estimating genetic similarities and diversity.

Keywords: Dendrogram, polymorphism, randomly amplified polymorphic DNA, simple sequence repeats

INTRODUCTION

The Capsicum genus originated from tropical and humid zone of Central and Southern America and belongs to the Solanaceae family. Among the domesticated Capsicum species, pungent and non-pungent forms of *Capsicum annum* L. (pepper) are most popular and have a worldwide commercial distribution (Bosland and Votava, 2000). In India, hot pepper or chilli is an important commercial crop which is cultivated for vegetable, spice and value-added processed product. A wide range of variability is reported in the crop (Sreelathakumary and Rajamony, 2004).

Subjective classification based on morphological data has been reported to create confusion and difficulty in classifying the genus capsicum (Costa *et al.*, 2006). In addition, the level of polymorphism for morphological characteristics in genotypes is sometimes so limited that it is inadequate to allow varietal identification (Ilbi, 2003). In the past several years a range of molecular markers such as RFLPs, AFLP, RAPDs and SSRs have been used for the estimation of genetic diversity, genome mapping and gene tagging in the capsicum species and related crops. (Lefebvre *et al.*, 1995; Paran *et al.*, 1998; Rodrigue

et al., 1999; Thorup *et al.*, 2000; Lefebvre *et al.*, 2001; Minamiyama *et al.*, 2006; Portis *et al.*, 2007; Hanáček *et al.*, 2009; Parmar *et al.*, 2010).

RAPD (Randomly Amplified Polymorphic DNA) markers are not affected by the prevailing environmental conditions and present in all plant. They can identify a great number of polymorphisms that allow the distinction among accessions and the identification of possible duplicates (Ilbi, 2003). RAPDs are considered advantageous to analyze the genetic diversity of germplasms for the reason that they are simple, cheap and give quick results (Costa *et al.*, 2006). But they only identify dominant alleles and have reproducibility problem.

Recently, microsatellite markers have been applied to study phylogeny and construction of genetic maps in pepper. These microsatellites (Simple Sequence Repeats, SSRs) are having particular significance because of their co-dominance, which is lacking in RAPD markers and multiple allelic nature. Moreover, SSRs are abundant and hyper variable in almost all genomes examined to date (Powell *et al.*, 1996). A number of SSRs markers have been developed from genomic DNA, ESTs (Expression Sequence Tags) and cDNAs which can be used for the diversity analysis of

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Table 1: Details of chilli and paprika genotypes used in this study

Genotypes	Geographical origin	Characteristic
Chilli type		
CA 97	Tamil Nadu, India	Medium fruit, erect fruit position
CCH	Tamil Nadu, India	Medium fruit, up right growth
CF 1	Tamil Nadu, India	Small fruit, erect fruit position
CO 2	Tamil Nadu, India	Bold fruit, prostate growth
K1	Tamil Nadu, India	Large fruit, up right growth
CA 110	Tamil Nadu, India	Medium fruit, prostate growth, yellow anther
Kashi annol	Uttar Pradesh, India	Medium fruit, prostate growth
Paprika type		
Bayadagi-kaddi	Karnataka, India	Large fruit, up right growth
KT-PL-19	Himachal Pradesh, India	Large fruit, up right growth
KT-PL 18	Himachal Pradesh, India	Large fruit, up right growth
ARKA ABHIR	Karnataka, India	Large fruit, up right growth
VSH		Large fruit, up right growth, orange fruit
V1		Medium fruit, up right growth, yellow fruit

Table 2: Primers sequences with their polymorphic percentage obtained from the twelve RAPD markers

Primer code (RAPD)	Sequence (5'-3')	Total bands	Polymorphic bands	Polymorphic percentage (%)
OPA 1	CAGGCCCTTC	8	8	100
OPA 2	TGCCGAGCTG	6	3	50
OPB 3	CATCCCCCTG	5	4	80
OPB 6	TGCTCTGCCC	4	4	100
OPF 9	CCAAGCTTCC	5	5	100
OPF 11	TTGGTACCCC	9	6	66.66
OPF 14	TGCTGCAGGT	5	5	100
OPF 15	CCAGTACTCC	6	6	100
OPF 16	GGAGTACTGG	9	7	77.77
OPF 18	TTCCCGGGTT	10	8	80
OPF 19	CCTTAGACC	5	5	100
OPF 20	GGTCTAGAGG	6	4	66.66

pepper genetic resources (Minamiyama *et al.*, 2006; Portis *et al.*, 2007).

The basis and the pattern of variations that are presented in genetic resources have considerable role in the success of plant breeding programs. Therefore, this investigation was undertaken with the objective of estimating the genetic diversity presented in chilli and paprika accessions/varieties using RAPD and SSR markers.

MATERIALS AND METHODS

Plant materials: Seeds of thirteen varieties and accessions of chilli and paprika collected from different places of India were obtained from Horticultural College and Research Institute, Tamil Nadu Agricultural University, India (Table 1). The genotypes were grown in pots placed in a 35% shade net under the regular agronomic practices.

DNA extraction: Healthy and fully expanded leaves (1 g) were collected from four or five plant for each accession. Bulk samples were powdered using mortar and pestle in presence of liquid nitrogen. DNA extractions were performed according to the procedure of De Masi *et al.* (2006) with some modification.

In brief, the powder was suspended in 10 mL of CTAB lysis buffer consisting of 1.4 M NaCl, 2% w/v Cetyltrimethyl Ammonium Bromide (CTAB), 100 mM Tris HCl pH 8.0, 20 mM EDTA, 1% PVP, 1.4% v/v 2-mercaptoethanol and incubated at 65°C for 30 min. The

samples were then treated by equal volume of chloroform: isoamyl alcohol (24:1) and centrifuged at 10,000 rpm for 10 min. This step was repeated once more and a volume of cold isopropanol was added to the aqueous phase and centrifuged at 10,000 rpm for 10 min.

The collected precipitate was washed with 20 mL of 70% ethanol and air dried. After drying the pellet was dissolved in 3 mL double-distilled water and treated with RNaseA (15 µL) at 37°C for 30 min. Then, 5 M sodium chloride (1/10 volumes) and cold ethanol (3 volumes), were added to the solution and centrifuged at 10,000 rpm for 20 min. Finally, after washing the recovered pellets with 20 mL of 70% ethanol, the DNA was dissolved in double distilled water. Integrity and size of genomic DNA was evaluated on Gel electrophoresis.

RAPD analysis: The thirteen DNA samples were analyzed by 12 arbitrary oligodeoxynucleotide primers (10-mer) selected from those that had reproducible amplification patterns in RAPD PCR test (Table 2). The optimized PCR assay was done in a 25 µL reaction volume containing 2.5 mM MgCl₂, 200 µM of dNTP mix, 20 pmol of single RAPD primer, 50 ng of pepper genomic DNA as template and 0.3 U Taq DNA polymerase in PCR reaction buffer (Genei, Bangalore, India).

BIORAD (BioRad Laboratories, Inc. USA) with heated lid was programmed with an initial incubation at 94°C for 4 min followed by 45 amplification cycles

made of three steps: denaturation at 94°C for 1 min, annealing at 36°C for 1 min and elongation at 72°C for 1½ min. The cycles were ended with final elongation at 72°C for 10 min.

SSR analysis: Nine SSR markers described previously for pepper were used (Lee *et al.*, 2004; Minamiyama *et al.*, 2006). The 25 µL-reaction mixture for PCR contained: 50 ng template DNA, 1 U Taq polymerase 0.2 µM of forward primer, 0.2 µM of reverse primer and 0.2 mM dNTPs all in PCR reaction buffer (Genei, Bangalore India).

PCR amplification was done in the BIORAD thermal cycler (Bio-Rad Laboratories, Inc. USA) set with initial denaturation step at 94°C for 3 min followed by 35 cycles of 94°C for 1 min, 55°C for 1 minute and 72°C for 2 min. The reaction was terminated by a final extension step at 72°C for 10 min.

Gel electrophoresis: The RAPD and SSR amplicons were separated by 1X TBE buffered electrophoresis on 2% w/v agarose gel, containing ethidium bromide (0.5 µg/mL). RAPD amplicons were run for 2 h at 70 V, while the SSR amplicons were run at 120 V for 1½ h. The resultant bands were visualized under UV light and digitized by imaging System (G: BOX, SYNGENE, UK). Size of amplicons was estimated using a 100 bp DNA ladder as molecular weight standard (Genei, Bangalore).

Data analysis: Reproducible and bright RAPD and SSR fragments were scored in each genotype as “1” if present or as “0” if absent. The matrices were entered into the NTSYS program (Numerical Taxonomy System, version 2.02, Rohlf, 1998) and used to calculate the pair wise similarity among the genotypes according to Jaccard’s similarity coefficient:

$$S_{ij} = \frac{a_{ij}}{a_{ij} + b_i + c_j}$$

where

S_{ij} : The measurement of the genetic similarity between individuals i and j

a : The number of polymorphic bands present in both individuals

b_i : The number of bands present in i and absent in j

c_j : The number of bands present in j and absent in i

The Similarity coefficients were used to construct a dendrogram using the UPGMA (unweighted pair group method with arithmetic average) and the SHAN (sequential, hierarchical and nested clustering) procedures in the NTSYS program.

Polymorphic information content: Polymorphism Information Content (PIC) value was estimated using the equation given by Smith *et al.* (1997):

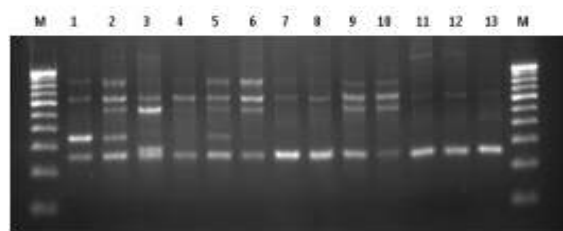


Fig. 1: PCR amplification pattern obtained from thirteen capsicum genotypes using RAPD primer OPF-20
Lane 1: CA97; 2: CCH; 3: CF1; 4: CO2; 5: K1; 6: CA110; 7: Kashi anmol; 8: Arka abhir; 9: ktpl 19; 10: ktpl 18; 11: Byadagikaddi; 12: V1; 13: VSH; M: Molecular size marker (100 bp ladder, Genei link)

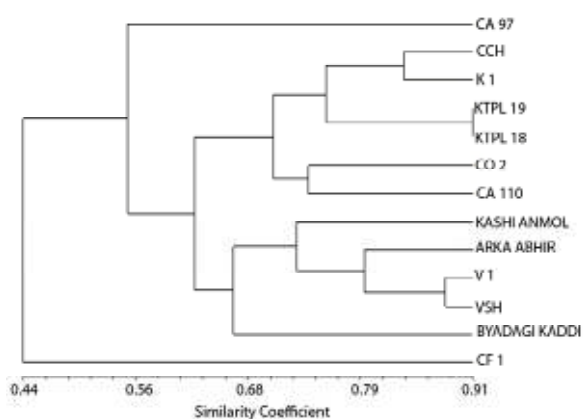


Fig. 2: Dendrogram of thirteen pepper accessions and varieties using UPGMA cluster analysis

$$PIC = 1 - \sum_i^n f_i^2 \quad i = 1$$

where, f_i^2 is the frequency of the i^{th} allele.

RESULTS

The 12 RAPD primers used in the study amplified a total of 78 bands and 65 polymorphic bands (Table 2). The number of polymorphic bands for each primer varied from 3 (OPB 6) to 8 (OPA 1, OPF 18) with an average of 5.41 bands per primer. The primers OPA 1, OPB6, OPF 9, OPF14 OPF15 and OPF19 were the most polymorphic among all primers tested, generating 100% polymorphism while the primers (OPA 2) showed the lowest 50% polymorphism. The distribution pattern of RAPD markers across the thirteen pepper varieties is shown in (Fig. 1).

The similarity coefficient ranged from 0.36 to 0.91 with a mean value of 0.60. Pair wise similarity between (Byadagi Kaddi and CF1) and (CA97 and CF1) showed the highest genetic diversity with the value of 0.36 and 0.41 similarity coefficients, respectively (Table 3). Whereas, the highest similarity value of 0.91 was found between KT PL 19 and KT PL 18. The dendrogram generated based on RAPD data resulted in four major clusters (Fig. 2). One genotype (CA 97) with

Table 3: Similarity matrix of chillies and paprika generated by jaccard's similarity index based on RAPD and SSR markers

	CA 97	CCH	CF 1	CO 2	K 1	CA 110	Kashi Anmol	Arka Abhir	KT-PL-19	KT-PL-18	Bayadagi Kaddi	V-1	VSH
CA 97	1.00												
CCH	0.62 ¹ (0.22) ²	1.00											
CF 1	0.41 (0.31)	0.49 (0.31)	1.00										
CO 2	0.51 (0.43)	0.64 (0.4)	0.43 (0.31)	1.00									
K 1	0.56 (0.36)	0.84 (0.54)	0.51 (0.17)	0.70 (0.58)	1.00								
CA 110	0.57 (0.36)	0.68 (0.54)	0.45 (0.17)	0.74 (0.36)	0.77 (0.38)	1.00							
Kashi Anmol	0.56 (0.44)	0.62 (0.44)	0.42 (0.50)	0.57 (0.26)	0.67 (0.44)	0.62 (0.57)	1.00						
Arkaabhir	0.56 (0.29)	0.67 (0.44)	0.44 (0.21)	0.57 (0.29)	0.70 (0.50)	0.64 (0.50)	0.74 (0.66)	1.00					
KT-PL-19	0.51 (0.46)	0.72 (0.54)	0.48 (0.17)	0.69 (0.46)	0.86 (0.50)	0.72 (0.50)	0.66 (0.57)	0.69 (0.40)	1.00				
KT-PL-18	0.47 (0.20)	0.68 (0.26)	0.44 (0.12)	0.74 (0.20)	0.78 (0.21)	0.68 (0.21)	0.61 (0.31)	0.61 (0.25)	0.91 (0.55)	1.00			
Bayadagi Kaddi	0.49 (0.28)	0.57 (0.46)	0.36 (0.19)	0.52 (0.20)	0.56 (0.31)	0.60 (0.55)	0.63 (0.40)	0.66 (0.54)	0.55 (0.42)	0.50 (0.23)	1.00		
V-1	0.57 (0.33)	0.62 (0.61)	0.46 (0.23)	0.61 (0.43)	0.67 (0.46)	0.68 (0.90)	0.72 (0.53)	0.78 (0.57)	0.66 (0.46)	0.62 (0.20)	0.67 (0.50)	1.00	
VSH	0.63 (0.5)	0.69 (0.46)	0.48 (0.29)	0.59 (0.50)	0.65 (0.43)	0.63 (0.33)	0.73 (0.5)	0.82 (0.43)	0.64 (0.54)	0.64 (0.46)	0.68 (0.36)	0.83 (0.40)	1.00

¹: Similarity coefficients obtained from RAPD markers; ²: Similarity coefficients obtained from SSR markers

Table 4: Primers sequences with their Polymorphism Information Content (PIC) values from SSR markers examined in the thirteen pepper varieties/accessions

Primer code (SSR)	Sequence (5'-3')	Repeat motifs	Number of alleles	PIC
CAMS-117	F: TTGTGGAGGAAACAAGCAAA R: CCTCAGCCCAGGAGACATAA	(tg) ₂₁ (ta) ₃	2	0.26
CAMS-405	F: TTCTTGGGTCCCACACTTC R: AGGTTGAAAGGAGGGCAATA	(tc) ₁₈	2	0.43
CAMS-864	F: CTGTTGTGGAAGAAGAGGACA R: GCTTCTTTTCAACCTCCTCCT	(aga) ₃₂	6	0.75
GPMS 113	F: GCACAAGTCAATCCAAACGA R: CAAAAAGATGATGATGGATGAGA	(at) ₂₀ (ag) ₁₈	5	0.66
GPMS 161	F: CGAAATCCAATAAACGAGTGAAG R: CCTGTGTGAACAAGTTTTTCAGG	(aat) ₂₅	5	0.72
GPMS 197	F: GCAGAGAAAATAAAATTCTCGG R: CAATGGAAATTTTCATCGACG	(ga) ₃ (tat) ₁₆	3	0.53
EPMS 303	F: AAAACTCCAACTACCCCTGG R: TTAAGCGTAGCGCTGTGTGTC	(ta) ₂₅	2	0.48
EPMS 418	F: ATCTTCTTCTATTCTCCCTTC R: TGCTCAGCATTACGACGTC	(ca) ₁₀	1	0
CAMS-327	F: GCATCTAAGTCTACGCCCTTG R: AAAGCCTTTGGCAATGAACA	(tc) ₇	3	0.40

erected fruit position grouped in the first cluster. The second and third clusters showed to form the largest clusters containing 84.6% of the population studied. The second cluster contains four chilli genotypes (CCH, CO2, K1 and CA110) and two paprika varieties (KT PL 19 and KT PL 18). Five genotypes including one chilli variety (Kashi Anmol) and four paprika (Arka Abhir, Bayadagi Kaddi, V1 and VSH) types were grouped in the third cluster. The accession CF1 with erect fruit position made the fourth cluster.

Nine SSR primer pairs were used to study the genetic diversity or/and similarity of the thirteen pepper varieties. Of these primer pairs, 8 (88.88%) revealed polymorphism in cultivars of the chillies and paprika.

The average number of alleles per primer pairs was 3.22, ranging from 1 to 6. The PIC values obtained from the SSR primers ranged from 0.00 to 0.75 with a mean value of 0.546. The primer CAMS-864 was found to be the most informative locus for fingerprinting and differentiating the pepper genotypes (Table 4). The distribution pattern of alleles per SSR locus for SSR1 across the thirteen pepper varieties is shown in Fig. 3.

The Similarity coefficients based on SSR markers ranged from 0.117 to 0.9 with mean value of 0.39 (Table 3). The dendrogram grouped the 13 genotypes into three main clusters (Fig. 4). The first cluster further divided into two sub clusters. The first sub cluster contains four genotypes (CA 97, CO2, VSH and K1)

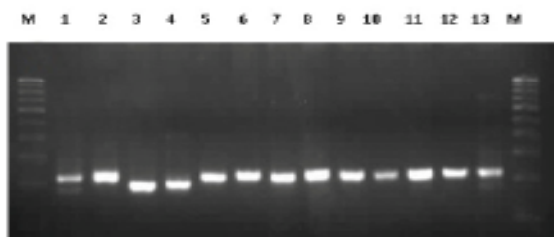


Fig. 3: PCR amplification pattern obtained from thirteen capsicum genotypes using SSR primer CAMS-117
Lane 1: CA97; 2: CCH; 3: CF1; 4: CO2; 5: K1; 6: CA110; 7: Kashi anmol; 8: Arka abhir; 9: KT PL 19; 10: KT PL 18; 11: Byadagi kaddi; 12: V1; 13: VSH; M: Molecular size marker (100 bp ladder, Gene link)

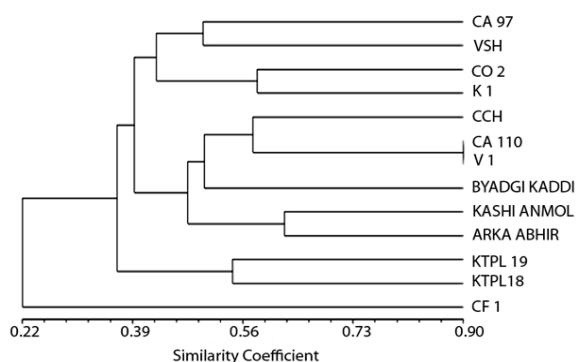


Fig. 4: Dendrogram of thirteen pepper accessions and varieties using UPGMA cluster analysis based on SSR markers

and the second sub cluster contained six genotypes (Arka Abhir, Byadagi kaddi, Kashi Anmol, V1, CA110 and CCH). The two paprika varieties KTPL19 and KTPL18 were grouped in the second cluster while only one cultivar CF1 was found to make the third major cluster.

DISCUSSION

Understanding genetic variability's presented among cultivars or accessions have practical application in protecting genotypes, conservation and widening the genetic basis of varieties (Charcosset *et al.*, 1998). The polymorphism revealed by RAPD primers used in the study is in consistence with previous reports where an average of 4.96 and 6.4 polymorphic bands/primer was detected in pepper genotypes (Rodriguez *et al.*, 1999; Litoriya *et al.*, 2009). However, the proportion of polymorphic fragments was comparatively lower than those earlier reports as observed on an average of 11.2 polymorphic bands per primer using tomato varieties (Abd El-Hady *et al.*, 2010).

The classification obtained with RAPD markers is generally consistent with the varietal group, that is, genotypes with similar fruit and growth characteristics

generally clustered together and those with different fruit characteristics are clustered apart.

The cluster analysis based on RAPD revealed that the thirteen genotypes could be classified into four major groups (Fig. 2). The first cluster containing CA97 from the *Capsicum annum* group with medium and erect fruit position was distantly separated from other *Capsicum annum* genotypes which are having pendent fruit position. The second and the third cluster comprise genotypes of medium to large sized fruits with declining fruit position. The same pattern of variation was found in the previous study that the cultivar with medium and erect fruits formed a more divergent group than the large fruited cultivar and similarly the medium and long fruited cultivar were closely related to each other due to their declining fruit position (Sitthiwong *et al.*, 2005).

One subgroup from the second cluster comprises the two paprika varieties KT PL 19 and KT PL18 which are believed to come from the same geographical place of origin. Similarly, Arka Abhir and Byadagi Kaddi coming from the same place (Karnataka) were placed in the third cluster. This grouping further strengthened by previous findings (Agrama and Tuinstra, 2003; Gupta *et al.*, 2008) that DNA based data can be used for studying the genetic relationship among different genotype of a species based on place of origin.

Interestingly, putting the variety CO2 and the accession line CA110 in one subgroup may be explained by their common prostrate growth habit though they have different fruit characteristics. Finally the most genetically divergent line CF 1 which belongs to the *Capsicum frutescens* species having small and erect fruit position was grouped in the fourth cluster. This result is in agreement with the previous works reported on capsicum species (Rodriguez *et al.*, 1999; Sitthiwong *et al.*, 2005).

The wide variation in genetic similarity coefficient among the different genotypes reflected a relatively high level of polymorphism at the DNA level. The highest similarity (0.91) was found between KTPL 19 and KTPL 18 (Table 4). The reason was probably due to that both KTPL 19 and KTPL 18 belong to *C. annum* and originated from the same place. Rodriguez *et al.* (1999) and Ince *et al.* (2009) reported the large genetic variation presented among different groups of capsicum species. Likewise, Bydaggi kaddi and CF1 which belongs to different group of capsicum species showed the least similarity coefficient (0.36) displaying wide genetic difference between the *Capsicum annum* and *Capsicum frutescens*.

SSR markers are considered more reliable because of their ability to produce highly consistent profiles (McCouch *et al.*, 1997). The twenty-eight polymorphic SSR markers clearly differentiated the 13 chilli and paprika varieties/accession.

The average numbers of alleles per SSR primer pairs were in agreement with earlier works reported in pepper with the mean value of 3.5 alleles/locus (Hanáček *et al.*, 2009). In addition, the average PIC values of 0.546 from this study were similar to the PIC values for pepper (0.529) and Brassica (0.55) (Kwon *et al.*, 2005; Tonguç and Griffiths, 2004).

Similar to the RAPD clustering pattern the SSR markers put the accession CF1 in one single major cluster from the large fruited types of *Capsicum annuum* accessions (Fig. 4). These genetic relationships between the accessions were in good agreement with the previous reports on pepper genotypes (Portis *et al.*, 2007). Moreover the two paprika varieties (KT PL19 and KTPL 18) which were grouped together using the RAPD primer were again clustered in one major cluster based on the SSR markers. This consistency between RAPD and SSR in clustering genotypes into the same group was shown by closer placement of Arka Abhir and Kashi Anmol in both dendrograms. In contrary, the cultivar CA97 with medium and erect fruit position was closely clustered with the variety CO2 with small and bold type fruit. This may be explained by that mostly SSR markers measures genetic variation mainly in non-coding sequences which possibly do not have a major impact on the morphology of genotype (Kwon *et al.*, 2005).

Interestingly, the clusters were also found to contain genotypes from both the pungent (Chilli) and the non pungent (Paprika) types. This indicates that capsaicinoid profiles are not good taxonomic indicators for the capsicum species (Zewdie and Bosland, 2001).

The pairwise similarity analysis of the SSR markers produced an index values ranging from 0.117 to 0.9 with a lesser mean value of 0.39 when compared to RAPD (0.6) mean value. Moreover, most of the similarity index values from the SSR analysis stretched out in the range between 0.117 and 0.6 when compared to RAPD analysis where most of the similarity index values found between 0.4 and 0.85. This indicates that SSR markers have a different polymorphic capability which resulted in wider genetic associations than displayed by the RAPD markers (Powell *et al.*, 1996).

CONCLUSION

Both RAPD and SSR markers showed genetic variability and are found to be powerful tools for estimating genetic similarities and diversity in the studied pepper genotypes. The genetic relationships presented among the genotypes are helpful for future breeding programs through selection of genetically diverse parents. Hence, the cultivar CF 1 can be used as genetically diverse parent for future hybridization program.

ACKNOWLEDGMENT

This work was supported in part by Cadbury and NAIP schemes, Tamil Nadu agricultural university, India.

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