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Research Article

Genetic relation in *Capcicum annum* [L.] cultivars through microsatellite markers: SSR and ISSR

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Abstract:
Capsicum annum [L.] is one of the most economically important vegetable crops in India. In order to assess the genetic relation, DNA from thirteen *capsicum* cultivars were screened using inter simple sequence repeat (ISSR) and microsatellite (SSR) markers. Five ISSR primers amplified 204 reproducible bands of which 139 were polymorphic. The percentage of polymorphic bands detected by ISSR was 100%. The highest polymorphic bands obtained by the use of primers UBC-809 (34) and UBC-66 (53). A total of 1-5 alleles were detected by six SSR primers, with an average of two alleles per primer. The number of alleles per locus ranged one (ssrCAMS-811) to five (ssrCAMS-142). The polymorphism information content (PIC) values ranged from 0.27 (ssrCAMS-405) to 0.67 (ssrCAMS - 142). This study reveals the great importance of guaranteeing the differentiation of chilli cultivars and the application for certification purposes.

Key words: *Capsicum*, genetic diversity, inter simple sequence repeat (ISSR), polymorphism, simple sequence repeat (SSR).

Introduction

Capsicum annuum L. is a dicotyledonous plant belonging to Solanaceae family. 27 species of chillies are known, of which five are domesticated and 22 wild species. Of the five domesticated species of pepper, *Capsicum annuum* is the most widely cultivated and is used as vegetable and spice. The other four species, *C. Chinese*, *C. baccatum*, *C. frutescens*, and *C. pubescens*, are used to produce spice or used as genetic resources for disease resistance genes. Pepper consists of 12 chromosome pairs with a variable genome size from 3,200 to 5,600 Mb (Pakozdi *et al.* 2002). *Capsicum* germplasm has a great diversity for morphological traits like fruit size, shape and colour. This diversity offer opportunities to develop unique cultivars for agronomic applications. Information on the genetic variation and phylogenetic relationships among the lines of the germplasm of a crop is the basic requirement in plant breeding. Genetic analysis in *Capsicum* species has been carried out using morphological, biochemical and molecular markers

Molecular marker for genetic variation in capsicum is studied in which Microsatellites (Simple Sequence Repeat) markers are ubiquitous, abundant and highly dispersed in eukaryotic genomes, with high variability at most loci. Length polymorphism occurs when PCR products from different alleles vary in length as a result of variation in the number of repeat units in an SSR. This variation in repeat number is caused by slippage during DNA replication or unequal crossing-over between sister chromatids. The main source of microsatellite polymorphisms is in the number of repetitions of these small tandem units that can be easily detected by amplification in polymerase chain reaction (PCR). The combination of data of several highly polymorphic microsatellite loci results in individual allelic profiles, enabling the discrimination of cultivars. ISSR markers have been employed in many species for fingerprinting and phylogenetic studies, gene tagging, and mapping. Inheritance of ISSR follows Mendelian rules as demonstrated in chickpea (Ratnaparkhe *et al*., 1998). It has been successfully used in *Capsicum* species by Kochieva *et al*. (2004).

Material and methods

Experimental material:

The experimental material consisted of 13 cultivars of *Capsicum annuum*. The cultivars and their origin are listed in Table No. 1.

DNA isolation from Indian chilli cultivars: Young and healthy leaves of thirteen chilli cultivars were collected from Main Vegetable Research Station, Anand Agricultural University, Anand. The leaves

were collected in polyethylene bags and stored at -20^{0}C for further use.
Genomic DNA was extracted from the leaves by Cetyl trimethyl ammonium bromide (CTAB) method (Zidani *et al*., 2005) with some modifications.

Microsatellite (SSR) analysis*:* PCR reactions for SSR (Table: 2) were carried out in a reaction volume of 25 µl using method given by Minamiyama *et al*. (2006) with modifications. Each SSR reaction during current study was performed in a 200 µl PCR tube. The reaction components were mixed by spinning. The amplification was carried out in Applied Biosystems thermal cycler, USA with the following PCR profile: 5 min initial denaturation at 94^{0}C, followed by 32 cycles consisting of 1 min at 94^{0}C for denaturation, 1 min at 50–62^{0}C (Δ T ^{0}C) for annealing and 2 min at 72^{0}C for extension and a final extension at 72^{0}C for 10 min. Samples were kept at hold at 4^{0}C after the final step.

Polymorphic Information Content (PIC)*:* The PIC value for each locus of SSR was calculated on the basis of allele frequency.

$$PIC_i=1-\sum P_{ij}^2$$

Where P_{ij} is the frequency of j^{th} allele for marker i, and summation extends over n alleles. This referred to as heterozygosity and gene diversity.

ISSR analysis: PCR reactions for ISSR (Table: 3) were carried out in a reaction volume of 25 µl using method given by Josiah *et al*. (2008) with modifications. The reaction components were mixed by spinning. The amplification was carried out in Bio-Rad and ABI thermocycler, USA with the touchdown PCR conditions: one cycle of 94^{0}C for 5 min; 12 cycles of 94^{0}C for 1 min, 64^{0}C for 1 min, decreasing by 1^{0}C per cycle, and 72^{0}C for 2 min; 32 cycles of 92^{0}C for 1 min, 53^{0}C for 1 min, and 72^{0}C for 2 min; a final extension at 72^0 C for 10 min.

Data Scoring of SSR and ISSR markers*:* The fingerprints generated by all the different primers were scored as present (1) or absent (0) to compile a binary matrix which was then subjected to cluster analysis.

Data Analysis and Interpretation*:* For calculating the similarity between the accessions and dendrogram construction the data generated by SSR loci were analyzed with the software NTSYSpc version 2.20f. The data obtained by scoring the SSR profiles with different primers individually as well as collectively were subjected to the construction of similarity matrix using Jaccard's coefficient. The similarity values were used for cluster analysis. Sequential agglomerative hierarchical non-overlapping (SAHN) clustering was done using unweighted pair group method with arithmetic averages (UPGMA) method.

Result and discussion:
Microsatellite (SSR) markers are PCR-based markers that have been developed in many plant species; they have the advantage of being multiallelic, highly polymorphic and co-dominant. A total of six SSR primers were used to fingerprint 13 genotypes of *Capsicum annuum* (Minamiyama *et al*., 2006). Six of these primers succeeded to produce polymorphic as well as monomorphic alleles when applied to the thirteen chilli cultivars. The results (Table: 4, Plates: 1 to 3) presented in this chapter are according to the amplification profiles of different markers described below:

1. **CAMS−142** : The marker CAMS−142 produced a total of 5 alleles ranging from 251−269 bp. The five alleles were designated as 'A', 'B', 'C', 'D' and 'E'. Allele 'A' (269) was observed in Jwala while allele 'B' (234 bp) was observed in G-4, GVC-121, LCA-436 and GVC-101. Six genotypes showed allele 'C' (260 bp) viz. AVNPC-131, S-49, GVC-111, RHRC Pendent, Punjab Gucchedar and Kumthi whereas only Phule Jyoti displayed allele 'D' (256 bp) and Reshampatti displayed allele 'E' (251 bp). The PIC value was found to be 0.67.

2. **CAMS-153**: CAMS-153 produced a total of four alleles. The size of these alleles ranged from 225 to 265 bp. The alleles were designated as 'A', 'B', 'C', and 'D'. Allele 'A' (265 bp) was observed in AVNPC-131, while allele 'B' (234 bp) was observed in GVC-111 and Punjab Gucchedar. The genotypes GVC-121, LCA-436, GVC-101, RHRC Pendent and Kumthi displayed allele 'C' (230 bp) and allele 'D' (225 bp) was observed in genotype G-4, AVNPC-131, S-49, Jwala, Phule Jyoti and Reshampatti. The PIC value for this marker was found to be 0.66.

3. **CAMS-405**: Total three alleles ranging in size from 97 to 107 bp were produced by the primer CAMS-405. The three alleles were designated as 'A', 'B' and 'C'. Allele 'A' (107bp) was observed in AVNPC-131. Allele 'B' (104 bp) was observed in G-4 while allele 'C' (97 bp) was observed in S-49, Jwala, GVC-121, LCA-436, GVC-101, GVC-111, RHRC Pendent, Punjab Gucchedar, Kumthi, Phule Jyoti and Reshampatti. The PIC value for marker CAMS- 405 was 0.27.

Pooled SSR analysis: A total of 79 bands were produced by 6 SSR primers with an average frequency of 13.1 bands per primer. Three (CAMS-

398, CAMS-806 and CAMS-811) of the six primers produced monomorphic profiles. Amplified fragments varied in size from 97 bp, with primer CAMS-405, to 269 bp, with primer CAMS-142. Out of 79 fragments, 40 fragments were polymorphic resulting in 50% polymorphism among the thirteen genotypes. The mean PIC value for SSR markers was found to be 0.53 and the highest and lowest PIC value were 0.67 (CAMS-142) and 0.00 (CAMS-398, CAMS-806, CAMS-811) respectively.

Similarity indices based on Jaccard's similarity coefficient of the chilli genotypes using 6 SSR markers ranged from 0.000 to 1.000 with a mean of 0.5 (Table: 6). Genetically most diverse genotypes were GVC-111 and G-4, RHRC Pendent and G-4, GVC-121 and S-49, LCA-436 and S-49, GVC-101 and S-49, GVC-111 and Jwala, RHRC Pendent and Jwala, GVC-111 and GVC-121, GVC-111 and LCA-436, GVC-111 and GVC-101, RHRC Pendent and G-4, RHRC Pendent and Jwala, Phule Jyoti and GVC-111, Phule Jyoti and RHRC Pendent, Reshampatti and GVC-111 and Reshampatti and RHRC Pendent (0.000) whereas genetically most similar genotypes were LCA-436 and GVC-121, GVC-101 and GVC-121 and GVC-101 and LCA-436 (1.000). The cluster analysis of SSR markers separated the chilli genotypes into two major clusters (Fig No.1). The first major cluster was divided into two minor clusters; the first minor cluster was further divided into two sub-clusters. The first sub-cluster separated G-4 and Jwala from GVC-121, LCA-436 and GVC-101. The second minor cluster was divided into two sub-clusters; the first one contained AVNPC-131, S-49, RHRC Pendent and Kumthi while the second sub-cluster contained GVC-111 and Punjab Gucchedar. Whereas the second major cluster separated Phule Jyoti and Reshampatti from other cultivars. The Polymorphism showed by the SSR primers used in the present study were low, therefore, more number of SSR primers have to be used to obtain high levels of polymorphism information and unique band profiling. The SSR analysis of 13 chilli cultivars was in accordance with the results obtained by Minamiyama *et al*., 2006. They found lower polymorphism among *C. annuum* lines under study; whereas Lee *et al*. (2004) successfully developed and used SSR markers for differentiating Capsicum species. Among 10 SSR primers studied, three primers were found to be useful in fingerprinting of some genotypes of the present study. Fingerprinting of AVNPC-131 can be done by primer CAMS-153 and CAMS-405. Fingerprinting of other cultivars can be done by using more number of primers. A unique band of 265 bp size produced by the primer CAMS-153 can be used to identify AVNPC-131. This band was absent in rest of the twelve genotypes. The primer CAMS-405 generated a band unique to AVNPC-131 (207 bp). This fingerprinting makes identification and characterization of genotype very easy and further it will be of greater help in background selections during back cross breeding programs.

Inter Simple Sequence Repeat (ISSR) analysis:
Genomic DNA was amplified using ISSR markers to obtain unique fingerprints. A total of nine ISSR primers were screened, out of which five markers were selected giving clear and reproducible bands. The results obtained are as given in table 5 and plates: 4 to 8 and presented under different sub-heads for convenience followed by an overall result of ISSR analysis.

(1) **UBC-809** : Primer UBC-809 produced 38 scorable bands with their size ranging from 468-1677 bp. All the 34 loci were polymorphic giving 100% polymorphism for this primer. The PIC value for this primer was found to be 0.96.(2) **UBC-823** : A total of 26 scorable bands were produced by this primer. The size of bands ranged from 274-1962 bp. All fourteen loci were polymorphic resulting in 100 % polymorphism for this primer. The PIC value for this primer was 0.76. (3)**UBC-827** : The number of scorable bands produced by primer UBC-827 was 32. The size of these bands ranged from 414-2014 bp. All the 16 loci were polymorphic with 100% polymorphism. The Polymorphic Information Content (PIC) value for primer UBC-827 was found to be 0.82. (4) **UBC-828** : UBC-828 amplified 25 scorable bands. Amplified products ranged from 424-2207 bp in size. This primer showed 100% polymorphism with all the 22 loci being polymorphic. The Polymorphic Information Content (PIC) value for primer UBC-828 was found to be 0.95. (5) **UBC-886** :Primer UBC-886 generated total 83 scorable bands. The size of these bands ranged from 192-2629 bp. All the 53 loci were polymorphic resulting in 100% polymorphism. The Polymorphic Information Content (PIC) value for primer OPB-17 was found to be 0.95.

Pooled ISSR analysis: A total of 204 clear and reproducible bands were amplified from 13 genotypes using the 5 selected ISSR primers, all of which were polymorphic. The number of bands varied from 25, with UBC-828, to 83, with primer UBC-886 with fragment size ranging from 192-2629 bp. Mean PIC was found to be 0.88. The similarity coefficient values determined using Jaccard's coefficient based on five ISSR markers ranged from 0.029 to 0.333 (Table: 7) showing a close

relationship between GVC-111 and GVC-101 (0.333) and least genetic similarity between Reshampatti and Kumthi (0.029) genotypes. The mean similarity index was found to be 0.181 indicating that high level of diversity exists among the genotypes. The cluster analysis of 13 genotypes based on ISSR markers separated the genotypes into two major clusters (Fig. 2). The first major cluster was further divided into two minor clusters, the first minor cluster consisted of G-4 and Jwala; whereas second minor cluster divided AVNPC-131 from S-49, GVC-121, GVC-101, GVC-111, RHRC Pendent, LCA-436, Phule Jyoti, Punjab Gucchedar and Reshampatti which formed further sub-clusters. The second major cluster consisted only Kumthi. The results obtained are in agreement with those obtained by Kumar *et al*. (2001). They successfully used ISSR and FISSR (Fluoresecent ISSR) for DNA profiling of chilli. ISSR has also been used by Kochieva *et al*. (2004) along with AFLP and RAPD for determining genetic variation and phylogenetic relationships within the genus Capsicum. A number of unique bands were obtained upon amplification of genomic DNA of 13 genotypes using ISSR primers. In the present investigation, ISSR markers were found to be reproducible, and thus, very useful for fingerprinting of thirteen genotypes.

Conclusion: Microsatellite based markers are powerful tools for describing genetic dis/similarities and diversity among the studied capsicum genotypes. the observed genetic relationship and diversity among capsicum genotypes helpful for current and future breeding programme in order to select genetically distinct parents.

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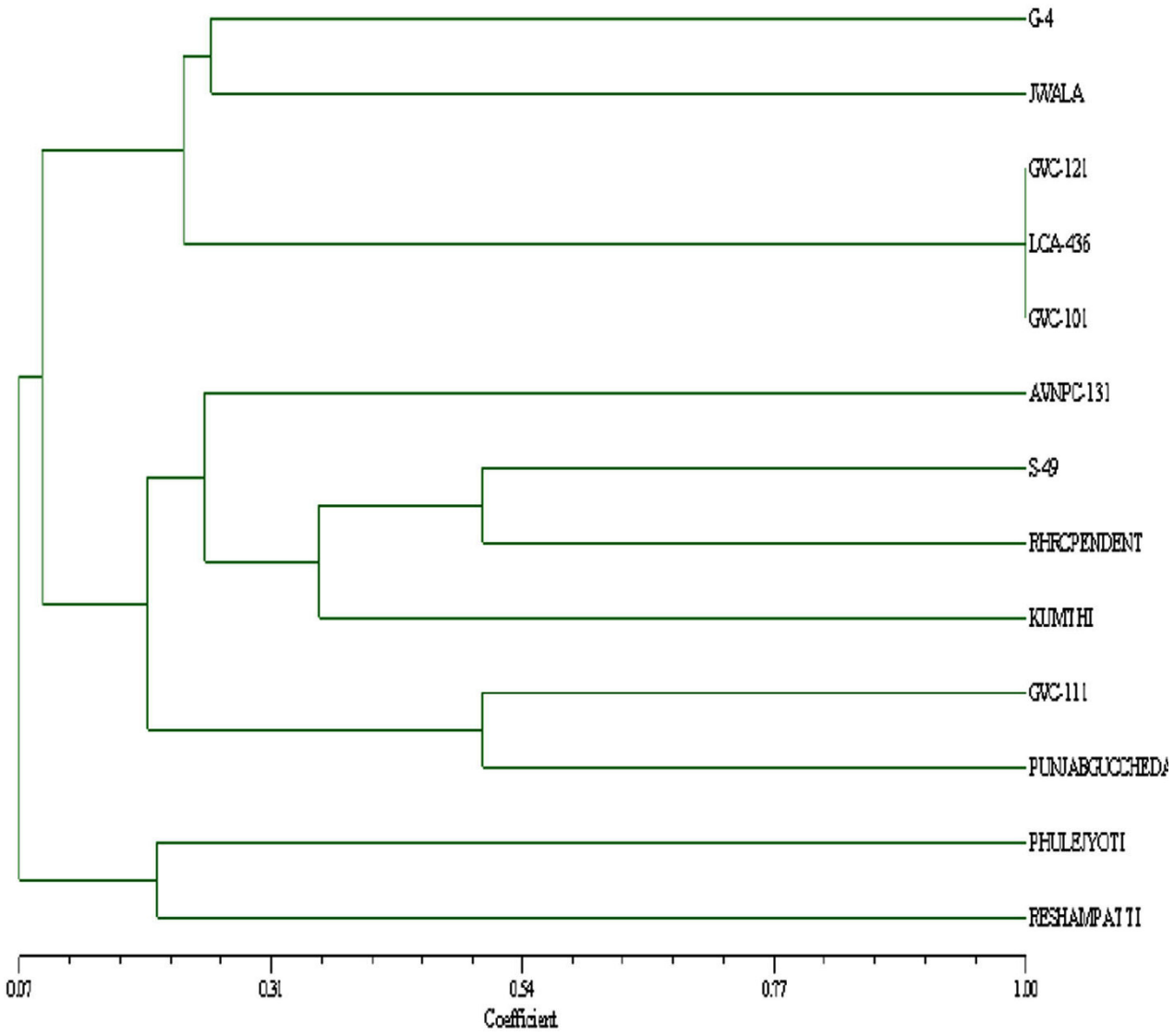

Fig .No. 01 Dendrogram of genetic relationship among 13 *Capsicum annum* cultivars based on SSR markers.

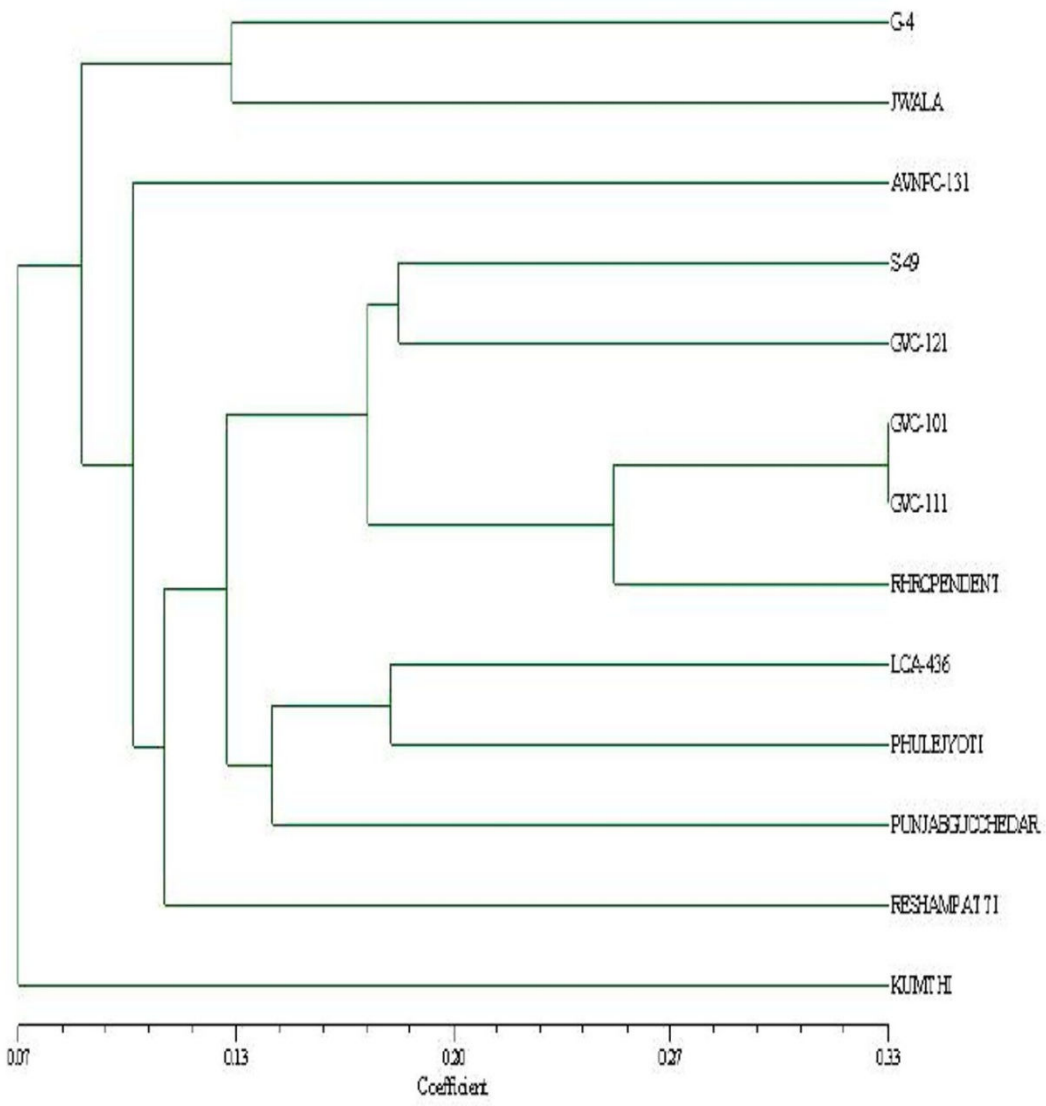

Fig .No. 2 Dendrogram of genetic relationship among 13 *Capsicum annum* cultivars based on ISSR markers.

Table: 1 List of cultivars studied and their origin.

Sr. No.	Cultivars	Origin
1.	G-4	Andhra Pradesh
2.	AVNPC-131	Anand (Gujarat)
3.	S-49	Anand (Gujarat)
4.	Jwala	I.A.R.I. (New Delhi)
5.	GVC-121	Anand (Gujarat)
6.	LCA-436	Lam (Andhra Pradesh)
7.	GVC-101	Anand (Gujarat)
8.	GVC-111	Anand (Gujarat)
9.	RHRC Pendent	Rahuri (Maharashtra)
10.	Punjab Gucchedar	P.A.U. (Punjab)
11.	Kumthi	Local
12.	Phule Jyoti	Rahuri (Maharashtra)
13.	Reshampatti	Jamnagar (Gujarat)

Table: 2 List of SSR primers used in the study with respective repeat motif and expected product size (Minamiyama *et al*., 2006)

SSR marker	Repeat motif	F/R	Primer sequence (5'——>3')	Expected product size (bp)
CAMS−142	(ta)3...(ac)7...(ac)12a (ta)8	F R	GAGCGCTTAAGTGGTCATAGG CTACAACGCCCCAAAACAAT	241
CAMS−153	(ta)7(tg)14cg(tg)6	F R	TGCACAAATATGAATCCCAAGA AAGTCAGCAAACACATCTGACAA	243
CAMS−398	(ag)22	F R	ATGGTCCATGGTCAGCAGAT GGGCAGAACAGTGGATGATT	165
CAMS−405	(tc)18	F R	TTCTTGGGTCCCACACTTTC AGGTTGAAAGGAGGGCAATA	241
CAMS−806	(aga)19	F R	TGTCACAAGTGTCAAGGTAGGAG CCCCAAAAATTTTCCCTCAT	227
CAMS−811	(aag)3...(gaa)3... (gaa)7	F R	GAAGAAACGAAGGATGAACAAAA CCTGTTTCCTCTTCCTCAGC	260

Table: 3 List of ISSR markers used and their sequence information

Sr. No.	Primer locus	Sequence (5'——>3')
1	UBC 809	AGA GAG AGA GAG AGA GG
2	UBC 823	TCT CTC TCT CTC TCT CC
3	UBC 827	ACA CAC ACA CAC ACA CG
4	UBC828	TGT GTG TGT GTG TGT GA
5	UBC886	VDV CTC TCT CTC TCT CT

Table: 4 Annealing temperature, total bands, polymorphic loci, total loci, total alleles, product size and percent polymorphism revealed by SSR markers

Primer	Annealing Temperaure (^{0}C)	Total Bands	No. of Polymorphic Loci	Total No. of Loci	Total No. of Alleles	Amplified Product Range (bp)	Percent Polymo -rphism (%)	PIC Value
CAMS-142	**58**	**13**	**1**	**1**	**5**	**251-269**	**100**	**0.67**
CAMS-153	**62**	**14**	**1**	**1**	**4**	**225-265**	**100**	**0.66**
CAMS-405	**58**	**13**	**1**	**1**	**3**	**97-107**	**100**	**0.27**
CAMS-398	**62**	**13**	**0**	**1**	**1**	**130**	**0**	**0.00**
CAMS-806	**60**	**13**	**0**	**1**	**1**	**241**	**0**	**0.00**
CAMS-811	**58**	**13**	**0**	**1**	**1**	**144**	**0**	**0.00**

Table 5: Total Scorable Bands, Polymorphic loci, Total loci, Product size, Percentage Polymorphism revealed by ISSR analysis

Primer	Total Scorable Bands	No. of Polymorphic Loci	Total No. of Loci	Amplified Product Range (bp)	Percent Polymorphism (%)	PIC Value
UBC 809	38	34	34	468-1677	100	0.96
UBC 823	26	14	14	274-1962	100	0.76
UBC 827	32	16	16	414-2014	100	0.82
UBC 828	25	22	22	424-2207	100	0.95
UBC 886	83	53	53	192-2629	100	0.95
POOLED	204	139	139	192-2629	100	0.88

Table 6: Similarity matrix for 13 genotypes in *Capsicum annuum* based on SSR markers determined using Jaccard's coefficient

	G-4	AVNPC-131	S-49	JWALA	GVC-121	LCA-436	GVC-101	GVC-111	RHRC PENDENT	PUNJAB GUCCHEDAR	KUMTHI	PHULE JYOTI	RESHAMPATTI
G-4	1.00												
AVNPC-131	0.40	1.00											
S-49	0.20	0.40	1.00										
JWALA	0.50	0.40	0.20	1.00									
GVC-121	0.50	0.17	0.00	0.20	1.00								
LCA-436	0.50	0.17	0.00	0.20	1.00	1.00							
GVC-101	0.50	0.17	0.00	0.20	1.00	1.00	1.00						
GVC-111	0.00	0.17	0.50	0.00	0.00	0.00	0.00	1.00					
RHRC PENDENT	0.00	0.17	0.20	0.00	0.20	0.20	0.20	0.20	1.00				
PUNJAB GUCCHEDAR	0.20	0.40	0.20	0.20	0.20	0.20	0.20	0.50	0.20	1.00			
KUMTHI	0.20	0.40	0.20	0.20	0.50	0.50	0.50	0.20	0.50	0.50	1.00		
PHULE JYOTI	0.50	0.40	0.20	0.50	0.20	0.20	0.20	0.00	0.00	0.20	0.20	1.00	
RESHAMPATTI	0.50	0.40	0.20	0.50	0.20	0.20	0.20	0.00	0.00	0.20	0.20	0.50	1.00

Table 7: Similarity matrix for 13 genotypes of *Capsicum annuum* based on ISSR markers determined using Jaccard's coefficient

	G-4	AVNPC-131	S-49	JWALA	GVC-121	LCA-436	GVC-101	GVC-111	RHRC PENDENT	PUNJAB GUCCHEDAR	KUMTHI	PHULE JYOTI	RESHAMPATTI
G-4	1.00												
AVNPC-131	0.06	1.00											
S-49	0.09	0.16	1.00										
JWALA	0.13	0.06	0.10	1.00									
GVC-121	0.07	0.10	0.18	0.08	1.00								
LCA-436	0.09	0.04	0.10	0.11	0.07	1.00							
GVC-101	0.10	0.14	0.19	0.12	0.18	0.13	1.00						
GVC-111	0.10	0.13	0.18	0.11	0.17	0.18	0.33	1.00					
RHRC PENDENT	0.09	0.09	0.17	0.11	0.16	0.11	0.21	0.29	1.00				
PUNJAB GUCCHEDAR	0.07	0.10	0.15	0.08	0.11	0.13	0.18	0.17	0.12	1.00			
KUMTHI	0.05	0.05	0.05	0.06	0.04	0.17	0.07	0.06	0.06	0.07	1.00		
PHULE JYOTI	0.08	0.08	0.18	0.10	0.09	0.18	0.17	0.15	0.09	0.17	0.11	1.00	
RESHAMPATTI	0.08	0.08	0.11	0.07	0.17	0.05	0.15	0.14	0.09	0.09	0.03	0.11	1.00