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Genetic diversity analysis and DNA fingerprinting of elite genetic stocks of tomato using SSR markers

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Abstract

Thirty genetic stocks of tomato (*Solanum lycopersicum* L.) were subjected to genetic diversity analysis and DNA fingerprinting using SSR markers. The stocks included male sterile lines (both pollen abortive and functional types); non-ripening mutants; disease (leaf curl virus, late blight and early blight) and nematode resistant lines; temperature stress (both low and high); and salt tolerant lines. Of the 25 primers used, 21 showed polymorphism and amplified 60 alleles with an average of 2.86 alleles per locus. Of these alleles, 21 were polymorphic and the rest were monomorphic. The PIC values for 21 primers obtained varied from 0.06 for SSR128 to 0.68 for SSR565, with an average PIC value to be 0.43. The greater number of repeat units and longer SSRs tend to have higher PIC values. Based on the PIC values and number of alleles amplified, the primer SSR565 was found to be more informative in the present set of genotypes. Similarity coefficient between any two genotypes estimated based on DNA amplification by SSR primers varied from 0.18 to 0.94. The lowest similarity coefficient (0.18) observed between genotypes belonging to the cultivated species *lycopersicum* and the wild species *pimpinellifolium* confirmed their differentiation at the species level. Many of the cultivated types were found to have fairly narrow genetic base. UPGMA revealed that SSR markers were helpful in differentiating the genotypes on the basis of horticultural and genetic factors. However, grouping of the 30 genetic stocks was independent of their geographic distribution. Based on the DNA fingerprints, it was possible to differentiate 23 of the 30 genotypes screened.

Key words: *Solanum lycopersicum*, genetic diversity, fingerprinting, molecular markers, SSRs

Introduction

Tomato (*Solanum lycopersicum* L.), one of the commercially important vegetable crops of the world, is a pre-eminent model system for genetic studies in plants. Germplasm resources are the most valuable and the basic raw materials to meet the current and future needs for genetic improvement of crops. Conventionally, genotype identification and genetic diversity in plants is based on phenotypic evaluation of morphological characteristics that demands collection of extensive data at different locations. Also, many of the traits having polygenic control are influenced by environment. Sometimes, the level of polymorphism for morphological characteristics in elite germplasm is too limited and inadequate to allow for genotype discrimination. Furthermore, with ever increasing rate at which new germplasm is acquired, there are increasing technical problems in achieving distinctness from the large number of genotypes that now make up the reference collections. On the other hand, molecular marker techniques have proved very useful in genotype identification, particularly when genetic variability is low among the cultivated types of the crop. These markers analyze genetic diversity at the DNA level, are independent of environment effects and are larger in numbers. Various molecular markers including isozymes, RFLPs (Restriction Fragment Length Polymorphism), RAPDs (Random Amplified Polymorphic DNA), SSRs (Simple Sequence Repeat) and SNPs (Single Nucleotide Polymorphism) are being used for germplasm characterization, parental verification in crosses, gene tagging for marker assisted breeding and gene cloning for use in transformation.

Earlier, efforts have been made at our centre to characterize and study genetic diversity among important genetic stocks of tomato using RAPD markers

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[1]. However, RAPDs exhibit low reproducibility and therefore, have limited utility in DNA fingerprinting and genetic diversity assessment. On the other hand, SSR markers have high reproducibility, higher level of polymorphism and have better use in molecular characterization and genetic diversity studies [2]. In recent years, SSRs have been used extensively for gene mapping in human, mouse and many crop plants; and for tagging genes in a variety of organisms [3, 4]. A number of polymorphic microsatellite markers generated from database sequences have been used successfully for genotyping tomato cultivars and germplasm accessions [5, 6]. These markers are especially suitable for a species like tomato, which has low levels of variation as detected by other types of markers [6].

Considering the wide range implications of SSRs, the present investigation was undertaken to assess genetic diversity/genetic relatedness among important genetic stocks of tomato and to characterize the stocks using SSR markers for the purpose of registration with the competent authority.

Material and methods

Experimental material

The experimental material comprised thirty genetic stocks of tomato. These include male sterile lines (both pollen abortive and functional types); non-ripening mutants; disease (leaf curl virus, late blight and early blight) and nematode resistant lines; temperature stress (both low and high) and salt tolerant lines. List of genotypes along with their source and specific attributes are given in Table 1. All the lines belong to the cultivated species *Solanum lycopersicum* L. except for L 3707 that belongs to *S. pimpinellifolium*.

Genomic DNA extraction and SSR analysis

The genomic DNA was extracted from fresh leaf tissues by bulking five plants per genotype, following CTAB method [7]. Quality and quantity of DNA was checked both by gel electrophoresis and spectrophotometer. A set of 25 microsatellite markers covering all the 12 linkage groups of tomato (Table 2) was selected. *In vitro* amplification using polymerase chain reaction (PCR) [8] was performed in 96 well microtiter plates in an Eppendorf Master Cycler^{TM}. Amplification of the DNA was performed with the following conditions: initial denaturation at 94°C for 4 minutes followed by 35 cycles of denaturation at 94°C for 1 minute, annealing at 50-67°C (depending upon the primer sequence) for 1 minute, elongation at 72°C for 1 minute and a final extension at 72°C for 7 minutes. After amplification 3 µl of 6X loading buffer was added to each of amplified product and mixed thoroughly. From this mixture, 10 µl of each sample was loaded in 2.5% superfine agarose gel (Amresco) prepared in 0.5X TBE buffer. PCR products were resolved by running gel at 5V/cm for 1-2 hour. The gels were visualized under UV light and photographed using photo documentation system (UVP Transilluminator).

The SSR allele sizes were determined depending on the position of bands relative to the ladder (Fermantas Gene Ruler 1 KB DNA ladder). Total number of alleles were recorded for each SSR marker in all the thirty genotypes by assigning allele number as 1, 2, 3, 4 and so on. The allele amplified in a particular set having highest molecular weight was numbered as allele 1. The amplified alleles were recorded as 1 (band present) or 0 (band absent) in a binary matrix. The polymorphic information content (PIC) values for all the primers were estimated using the formula:

$$PIC = 1 - \sum_{j=1}^{n} P_{ij}^2$$

where P_{ij} is the frequency of j^{th} allele in the i^{th} primer and summation extends over n patterns.

Estimation of genetic diversity/ similarity and cluster analysis

The SSR marker amplification profiles of thirty genotypes were used to estimate genetic diversity/ relatedness based on number of shared amplified bands. The presence or absence of a particular amplification product was used as an index of genetic diversity/ relatedness. Computer software package NTSYSpc. Version 2.02e [9] was used for estimation of genetic similarities among the lines using SIMQUAL module of NTSYS. The similarity matrix value based on Dice [10] coefficient of similarity was used to generate dendrogram. Clustering was done by UPGMA using SHAN module of NTSYSpc.

Results and discussion

Thirty important genetic stocks of tomato possessing special attributes have been characterized using 25 microsatellite markers. The results are reported and discussed under the following heads.

Variation in DNA amplification

Of the 25 primers screened, four primers *viz.*, SSR43,

Table 1. List of genotypes along with their source and salient characteristics

S.No.	Genotype	Source	Salient characteristics
1.	*ms-10*^{36} VF 36	TGRC, USA	Pollen sterile, *Verticillium* and *Fusarium* wilts resistant
2.	*ms-45* VFN 8	TGRC, USA	Pollen sterile, *Verticillium* and *Fusarium* wilts and nematode resistant
3.	*ms-16* Pritchard	TGRC, USA	Pollen sterile
4.	PNR-7	PAU, Ludhiana	Nematode resistant
5.	Edkawi	TGRC, USA	Salinity tolerant
6.	Hot Set	TGRC, USA	High temperature fruit setting
7.	EBR-6	TGRC, USA	Early blight resistant
8.	ps_2 San Pedro	PAU, Ludhiana	Functional male sterile
9.	Punjab Chhuhara	PAU, Ludhiana	High yielding, processing variety
10.	Cherry Tomato 2006	PAU, Ludhiana	Cherry tomato
11.	To LCVR 2	PAU, Ludhiana	Leaf Curl Virus resistant
12.	To LCVR 3	PAU, Ludhiana	Leaf Curl Virus resistant
13.	RM-4	PAU, Ludhiana	Non ripening mutant
14.	RM-3	PAU, Ludhiana	Non ripening mutant
15.	EC119197	NBPGR, New Delhi	Nematode resistant
16.	H-24	HAU, Hisar	Leaf curl virus resistant
17.	ps_2 L 3841	PAU, Ludhiana	Functional male sterile, early maturity
18.	ps_2 NS 101	PAU, Ludhiana	Functional male sterile, early maturity
19.	BL 1198	AVRDC, Taiwan	Large fruited
20.	L 3707	AVRDC, Taiwan	Late blight resistant
21.	IPA-3	Brazil	Firm fruited processing variety, Nematode resistant
22.	8-2-1-2-5	PAU, Ludhiana	Nematode resistant
23.	*rin* T-3	TGRC, USA	Non-ripening mutant
24.	*ip*NIL*VFN*-145	TGRC, USA	High TSS
25.	*ms-33* IPA-3	PAU, Ludhiana	Pollen sterile
26.	*ms-2* Pearson	TGRC, USA	Pollen sterile
27.	EBR-5	TGRC, USA	Early blight resistant
28.	UC82-B	TGRC, USA	Firm fruited, high lycopene
29	L3841	AVRDC, Taiwan	Early maturity
30.	58-11-1-1	PAU, Ludhiana	Leaf Curl Virus resistant

SSR48, SSR76 and SSR218 did not show any polymorphism, hence were not considered for further analysis. Twenty-one primers were thus used for final analysis on the basis of easily scoreable amplified bands. The number of bands amplified, as resolved in 2.5% superfine agarose gel, by each of the 21 primers ranged from 2 to 5 (Table 3). DNA amplification profiles for markers SSR4 and SSR111 are presented in Fig. 1. A total of 60 alleles with an average of 2.86 alleles per locus were amplified in 30 genotypes. Maximum of five alleles were observed for primer SSR565 followed by primer SSR111 amplifying four alleles. Most of the primers viz. SSR92, SSR448, SSR57, SSR13, SSR590, SSR108, SSR244, SSR340, SSR70, SSR4, SSR606, SSR46 and SSR20 amplified three alleles. Two alleles were amplified by SSR22, SSR593, SSR128, SSR38, SSR136 and SSR124. Bredemeizer *et al.* [6] screened more than 500 European tomato cultivars with 20 SSR loci that amplified on an average 4.6 alleles per locus. He *et al*. [11] used 158 SSR primers to screen a set of

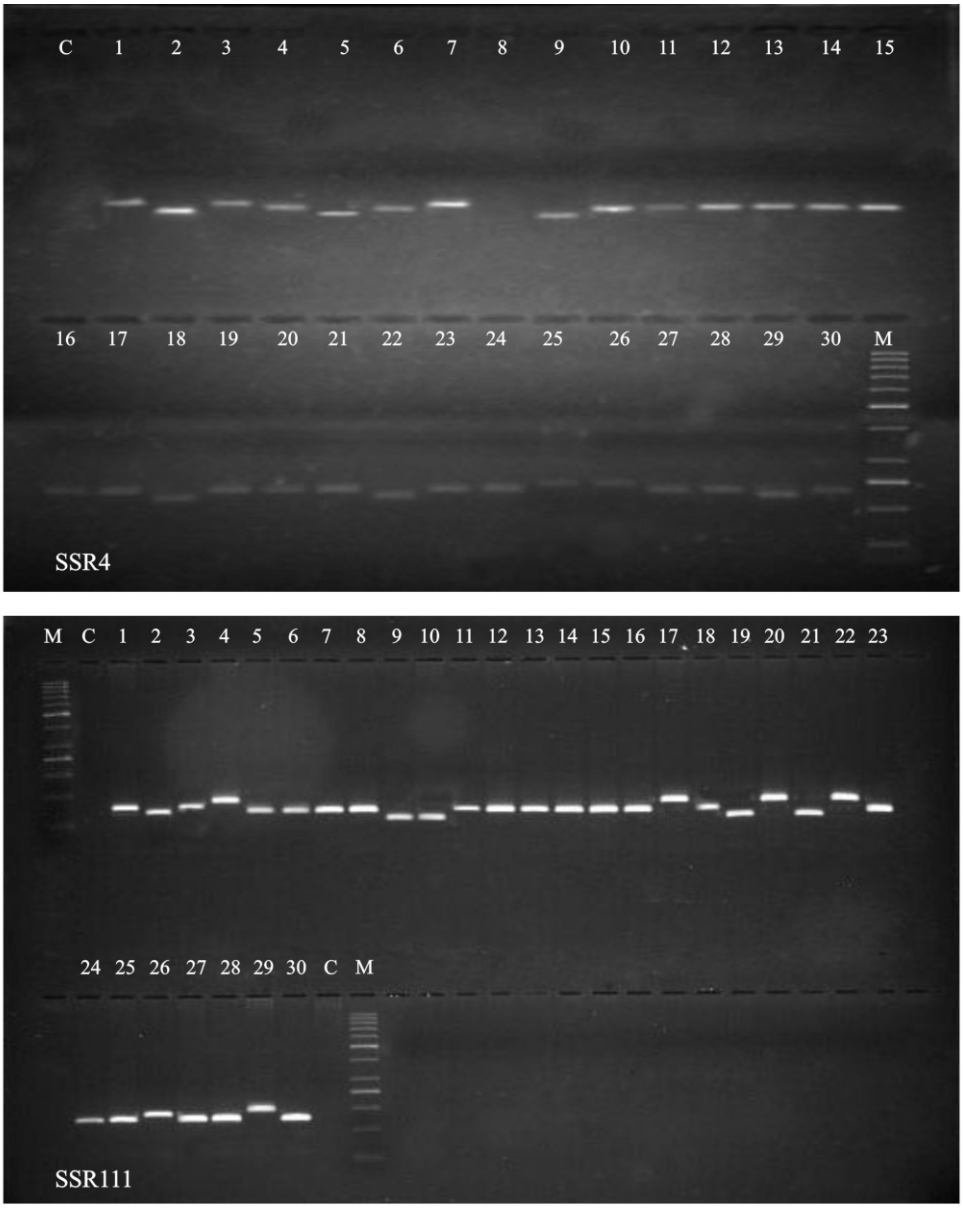

Fig. 1. Amplification profile of 30 genotypes of tomato with SSR4 and SSR111. M: Marker 1 Kb ladder; C: Negative control; Lanes 1-30: Tomato genotypes

19 diverse cultivars, out of which 129 showed amplification. Of these, 65 were polymorphic producing an average of 2.7 alleles per locus. Frary *et al.* [13] reported that 109 SSRs identified 172 different alleles in the cultivated species of tomato, thus giving an average of 1.6 alleles per locus. Caguiat *et al.* [12] used 27 SSR primers in tomato and found that only two were polymorphic.

The PIC values for 21 polymorphic markers, obtained in the present study, varied from 0.06 for SSR128 to 0.68 for SSR565, with an average PIC value for the 21 markers to be 0.43 (Table 2). Lower PIC values results from the closely related genotypes and the vice-versa. Ni *et al.* [14] observed a relationship between the number of alleles detected at a locus and maximum number of simple repeats within targeted microsatellite locus. They observed that, larger the maximum repeat number in the microsatellite locus, larger would be the number of alleles detected. However, the same was not true in the present study. For example, primer SSR70 having 20 (AT) repeats amplified 3 alleles, and SSR46 having 14 (AT) repeats also amplified same number of alleles. Frary *et al.* [13] reported that number of repeats

Table 2. Primers, their chromosome locations, SSR motif, number of alleles amplified and PIC values of microsatellite markers used

Chromosome	Primers	SSR Motif	No. of alleles amplified	PIC value
1	SSR 92	$(CT)_{11}$	3	0.42
2	SSR 448	$(CT)_{14}$	3	0.53
	SSR 57	$(AAC)_6$	3	0.34
3	SSR 111	$(TC)_6$ $(TCTG)_6$	4	0.52
	SSR 22	$(AT)_{11}$	2	0.12
4	SSR 43	$(TAC)_7$	1	0.0
	SSR 593	$(TAC)_7$	2	0.23
5	SSR 13	$(AAG)_6$	3	0.64
	SSR 590	$(TC)_6$ $(AC)_4$	3	0.65
6	SSR 48	$(CAC)_7$	1	0.0
	SSR 128	$(CAG)_6$ $(CAA)_3$	2	0.06
7	SSR 108	$(ACTCTC)_2$ $(TC)_6$	3	0.50
	SSR 565	$(GGGGAT)_3$ $(GAT)_3$ $(GAG)_2$ $(AAC)_3$ $(CAG)_7$	5	0.68
8	SSR 244	$(TA)_{14}$	3	0.42
	SSR 38	(TCT)8	2	0.23
9	SSR 340	$(GTTGA)_2$ $(GA)_7$	3	0.34
	SSR 70	$(AT)_{20}$	3	0.57
10	SSR 4	$(CGG)_7$	3	0.54
	SSR 606	$(CGG)_7$	3	0.56
	SSR 218	$(TCA)_7$	1	0.0
11	SSR 136	$(CAG)_7$	2	0.28
	SSR 76	$(CGG)_7$	1	0.0
	SSR 46	$(AT)_{14}$	3	0.38
12	SSR 20	$(GAA)_8$	3	0.56
	SSR 124	$(CACC)_2$	2	0.28
	Total		60[a]	
	Average		2.86[a]	0.43[a]

[a]Primers that did not show polymorphism were not included for estimating total and average value

and total length of the SSRs had significant effects on PIC values. A greater number of repeat unit and longer SSRs tend to have higher PIC values. This was also observed in the present study. The primer SSR565, for example, had the motif $(GGGGAT)_3$ $(GAT)_3$ $(GAG)_2$ $(AAC)_3$ $(CAG)_7$ and it amplified maximum number of alleles i.e. 5 and also had the highest PIC value of 0.68. SSR70 had maximum number of di-nucleotide repeats with motif $(AT)_{20}$ and also had comparatively higher PIC value of 0.57. In the present set of genotypes, primer SSR565 was, therefore, found to be more informative than other primers as it amplified maximum number of alleles and had highest PIC value.

Similarity coefficient and cluster analysis

Similarity coefficient between any two genotypes estimated based on DNA amplification by SSR primers varied from 0.18 to 0.94. The vast range of similarity coefficient between any two genotypes indicated the presence of wide genetic variability among the genetic stocks studied. The lowest similarity coefficient was observed for two pairs of genotypes viz. L3707 with *rin* T-3 and ms_2Pearson. This confirmed the differentiation of the genotypes at the species level as L 3707 belonged

to the wild species *pimpinellifolium* and the rest of the genotypes belonged to the cultivated species *lycopersicum*. Highest similarity coefficient was observed between *ms45*VFN8 and *ms16* Pritchard, both genotypes having been introduced from TGRC, USA. Highest similarity coefficient of 0.55 between genotypes belonging to species *lycopersicum* with the genotype of *pimpinellifolium* was observed between L 3841 and L 3707. The similarity coefficient between any two pollen sterile lines ranged from 0.70 (*ms16* Pritchard and *ms2* Pearson) to 0.94 (*ms45* VFN 8 with *ms16* Pritchard). Among the functional male sterile lines, similarity coefficient ranged from 0.68 between *ps2* L3841 and *ps2* NS101 to 0.89 between *ps2* San Pedro and *ps2* NS101. The range of similarity co-efficient between non-ripening mutants ranged from 0.57 between *rin* T3 and RM 3 to 0.66 between RM 3 and RM 4. The latter two non-ripening mutant lines had been derived from the same cross. Similarity coefficient between all the tomato leaf curl virus resistant stocks *viz*., ToLCVR 2, ToLCVR 3, 58-11-1-1 and H24 ranged from 0.60 (ToLCVR 3 and 58-11-1-1) to 0.85 (ToLCVR 2 and ToLCVR 3). The latter two have the common parentage.

Based on similarity coefficients, clustering was done using Unweighted Pair Group Method based on Arithmetic Average (UPGMA). Genetic relationships among the 30 genotypes are presented in the form of dendrogram (Fig. 2). The small fruited genotype 'L3707' belonging to wild species *pimpinellifolium*, grouped separately from all other genotypes belonging to the cultivated species *lycopersicum* at 34% similarity level. The cultivated tomato genotypes, however, showed more than 50% genetic similarity. These genotypes formed two groups and separated at 51% similarity level. The first group consists of five genotypes, while the second major group consists of twenty-four genotypes (Fig. 2).

The major group having twenty-four genotypes formed six sub-groups and had genotypes from different geographical regions *viz*., twelve from India, ten from USA and one each from Brazil and AVRDC, Taiwan. The smaller group included three genotypes developed at PAU, Ludhiana and one each from AVRDC, Taiwan and TGRC, USA. Out of the five male sterile genotypes included in the study, four genotypes, except *ms2* Pearson, clustered in one sub-group at 80% similarity level. All these five genotypes were similar at overall 63% similarity level. This similarity could be attributed to the fact that different *ms* genes have been incorporated into the genetic background of the commercial types having most of the desirable horticultural traits for their direct exploitation through

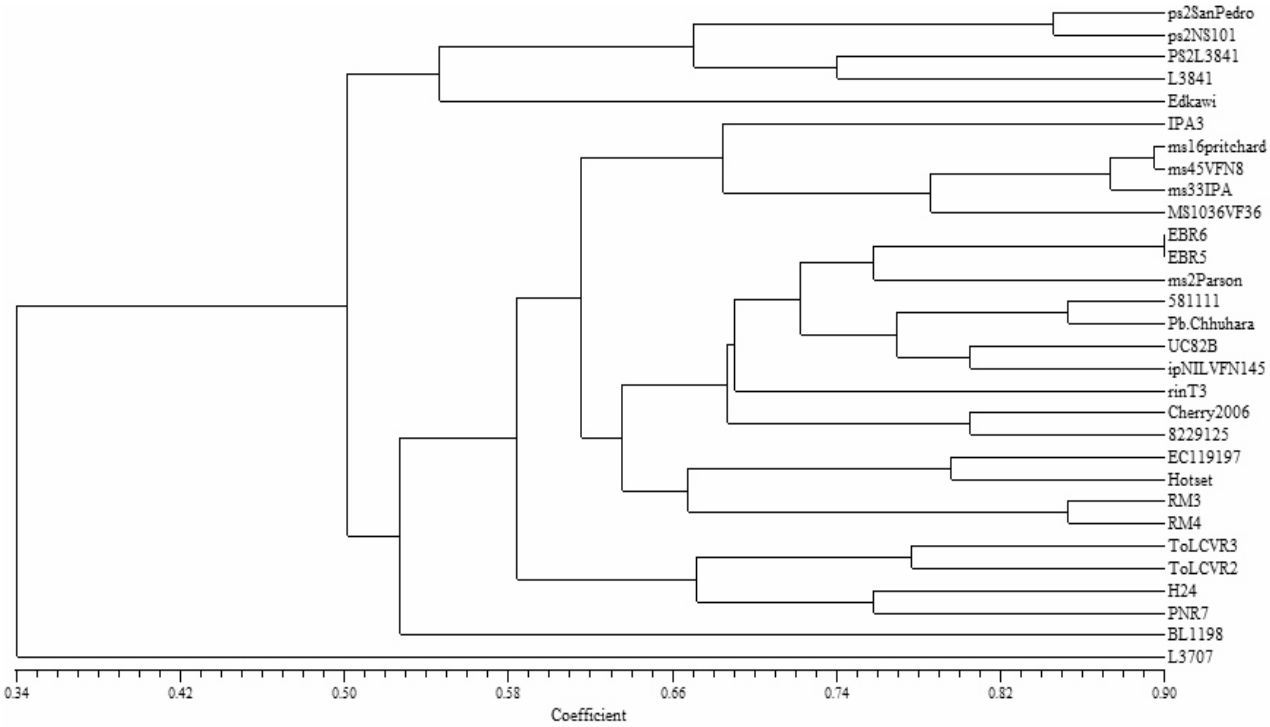

Fig. 2. Dendrogram showing genetic relationship of 30 genotypes of tomato

Table 3. SSR primers that could fingerprint tomato genotypes uniquely

S.No.	Genotype	Distinguishing markers
1.	IPA-3	SSR340
2.	*ps*$_2$ L3841	SSR136, SSR593, SSR606
3.	BL 1198	SSR22, SSR46, SSR136, SSR606
4.	ToLCVR-3	SSR92, SSR565
5.	H-24	SSR92, SSR565
6.	L3841	SSR590, SSR593
7.	*ms-16* Pritchard	SSR13
8.	EBR-6	SSR4, SSR38, SSR244
9.	Cherry 2006	SSR124, SSR 565, SSR590, SSR606
10.	EC 119197	SSR590
11.	*ms-45* VFN8	SSR340
12.	UC82-B	SSR13, SSR606
13.	Hot Set	SSR590
14.	*ip*NIL*VFN*145	SSR565
15.	ToLCVR-2	SSR108, SSR244
16.	8-2-1-2-5	SSR13
17.	*rin* T-3	SSR448, SSR565, SSR606
18.	RM-3	SSR4, SSR13, SSR111, SSR565
19.	EBR-5	SSR38, SSR590
20.	*ps*$_2$ NS101	SSR38, SSR111, SSR244, SSR565
21.	RM-4	SSR448, SSR565
22.	L3707	SSR4, SSR22, SSR70, SSR124, SSR128, SSR340, SSR565
23.	*ms*$_2$ Pearson	SSR38, SSR340, SSR606

heterosis breeding. The pollen sterile genotypes *viz*., *ms16* Pritchard and *ms45* VFN8, introduced from the USA, revealed maximum similarity (94%). The functional male sterile lines *viz*., *ps*$_2$ San Pedro, *ps*$_2$ NS101and *ps*$_2$ L3841 developed locally clustered in one sub-group at 74% similarity level. To develop these stocks, the functional male sterile gene '*ps*$_2$' was derived from the same donor parent through backcrossing that allows transfer of some genetic material along with the target gene(s). Two tomato leaf curl virus resistant stocks *viz*., ToLCVR-2 and ToLCVR-3, having same pedigree, were clustered into one sub-group at 85% similarity level. Non-ripening mutants RM-3 and RM-4, both from PAU Ludhiana, India, were placed in same sub-group at 86% similarity level, while the other non-ripening mutant, *rin* T-3 from USA, clustered with these two genotypes at 57% similarity level.

DNA Fingerprinting

For registration of cultivars and genetic stocks for granting Plant Breeder's Rights under the criteria of distinctness, uniformity and stability (DUS), the breeders need to describe their varieties and genetic stocks. DNA fingerprinting is a powerful tool as it provides sharply defined and repeatable genotypic descriptors than characterizing these based on morphological characteristics. DNA fingerprinting of 30 tomato genotypes having different horticultural and genetic traits was carried out using 25 SSR markers, of which 21 were found to be polymorphic. A total of 60 alleles were amplified in all 30 genotypes. The results revealed that each primer exhibited a specific banding pattern and it was possible to differentiate 23 of the 30 genotypes used in the present study. The remaining seven genotypes could not be distinguished on the basis of SSR markers used in this study. Allele SSR565 was amplified by the most genotypes (eight), followed by SSR606 by six and SSR590 by 5 genotypes. With primers SSR70 and SSR128, the first allele was present only in the *pimpinellifolium* genotype L3707 and absent in all other genotypes. Therefore, this primer was able to differentiate the wild relative of tomato from the cultivated types. Likewise, there were some alleles which were specific to few genotypes (Fig. 1). SSR46 was specific to BL 1198, a large fruited beef tomato; and SSR108 was specific to ToLCVR 2, a tomato leaf curl virus resistant genotype. SSR92 amplified in ToLCVR 3 and H24, the other two ToLCV resistant genotypes. SSR 539 was amplified by L 3841 and *ps2* L 3841, an isogenic line of L 3841. The primer SSR565 differentiated all the three non-ripening mutants i.e. RM-3, RM-4 and *rin* T-3 from other genotypes. The primer SSR38 was specific to both the early blight resistant genotypes EBR-5 and EBR-6. Likewise, specific fingerprints generated by each of the 20 primers are listed in Table 3.

It is concluded that the SSR analysis proved helpful for estimating the magnitude of genetic diversity at molecular level and establishing genetic relatedness among genetic stocks evaluated. Cluster analysis revealed that the genotypes were not grouped as per their geographic distributions indicating that geographic distribution may not be the true index of genetic diversity in tomato. The recent trends toward breeding for a specific plant and fruit type seem to have brought about considerable genetic uniformity among the modern

cultivars. SSR markers clearly differentiated the genotypes classified on the basis of horticultural and genetic factors. On the basis of banding pattern, SSRs were effectively used for molecular characterization of tomato genetic stocks used in this study.

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