Plant Archives Vol. 19 No. 2, 2019 pp. 3437-3445 **e-ISSN:2581-6063 (online), ISSN:0972-5210**

ASSESSMENT OF MOLECULAR DIVERSITY IN TOMATO GENOTYPES USING RAPD AND SSR MARKERS

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Abstract

Random Amplified Polymorphic DNA (RAPD) and Simple Sequence Repeats (SSR) analysis was carried out using five SSR markers and ten RAPD markers to assess the genetic diversity of tomato germplasm. Similarity coefficient of 18 genotypes using SSR markers ranged from 0.1 to 0.4. The clustering done using UPGMA was further confirmed by DARWIN (6.0 version). The genotypes LE - 150 and LE - 22 showed highest similarity as revealed by clustering using RAPD markers. Similarity coefficient of 18 genotypes using RAPD markers ranged from 0.22 to 0.33. The lowest genetic distance was (0.22) between genotypes LE - 6 and LE - 14, while, the highest genetic distance was (0.33) between varieties LE - 150 and LE - 22. The genetic diversity existing among the tomato genotypes *i.e.*, LE - 150 and LE - 22 (based on RAPD markers) and LE - 150 and LE - 22 (based on SSR markers) could be exploited through hybridization to recover the segregates possessing high yield potential with improved fruit quality characteristics. In RAPD and SSR markers detected medium locus polymorphism among the 18 tomato genotypes, indicating that both markers are of great utility for genetic diversity studies of tomatoes which can further be utilized in strengthening tomato breeding programmes.

Key Words: Molecular diversity, Polymorphism, RAPD, SSR Markers, Tomato

Introduction

Tomato (*Lyccopersicon esculentum* L.) is an annual crop and belongs to the family Solanaceae, which consists of approximately 100 genera and 2500 species, including several other plants of agronomic importance such as potato, eggplant, pepper, and tobacco (Olmstead *et al.,* 2008; Arumuganathan *et al.,* 1991). *Lycopersicon esculentum* has a relatively compact genome among the Solanaceae species, characterized by its diploid chromosome number (2n = 2x = 24). It is approximately 950 Mb in size, and is one of the most intensively genome among the Solanaceae species, characterized by its diploid chromosome number (2n = 2x = 24) and approximately 950 Mb in size, and is one of the most intensively characterized Solanaceae genomes. Successfully grown under a wide range of agro-climatic conditions, it is a warm season crop and requires a relatively long growing season to produce profitable yield. It is extensively used as salad as well as for culinary purposes. The fruit contains significant amounts of lycopene, beta-carotene, magnesium, iron, phosphorus, potassium, riboflavin, niacin, sodium and thiamine. It has antioxidant properties and potential beneficial health effects (Zhang *et al.,* 2009).

One of the primary needs of the crop industry is the estimation of genetic diversity between cultivated accessions for identification and breeding purposes. Molecular genetic diversity estimates are extremely useful for intellectual property protection, particularly in the determination of essential derivation. Molecular characterization is now the favoured means to quantify variation within large germplasm samples. New DNA sequencing and genotyping technologies provide the power to interrogate thousands to millions of diagnostic polymorphisms, across hundreds to thousands of genotypes, thus facilitating the analysis of genetic structure and providing a rationale basis to identify and select among the underlying lineages. Such approaches not only resolve genetic relationships at fine scale, but they also provide important measures of genetic

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divergence between and within the major genetic clusters that comprise crop germplasm. This provides another view of genetic diversity, which is very helpful in highlighting the role of hybridization in the overall crop evolution process (Glaszmann *et al.,* 2010).

The genetic diversity of tomato has been investigated in several studies using Random Amplified Polymorphic DNA (RAPDs) and Simple Sequence repeats (SSRs). With the exception of SSRs, limited information was obtained due to a lack of variability that was ascribed to the self pollinating nature of modern tomato cultivars combined with their narrow genetic base (Alvarez *et al.,* 2001). Microsatellite or simple sequence repeat (SSR) marker has been used in plant diversity analysis; the popularity of these markers is due to their ease of amplification by polymerase chain reaction (PCR), their co-dominant nature and their typically high levels of allelic diversity at different loci. There are numerous reports suggesting the usefulness of microsatellite markers for measuring the genetic variability in a wider taxonomic range (Li *et al.,* 2007; Kawka *et al.,* 2007; Chan *et al.,* 2008; Banhos *et al.,* 2008: Anandan *et al*., 2017).

It is widely accepted that SSR techniques are expensive if the sequence information for designing the primers has not yet been developed. However, for the tomato, the primer sets for SSR analysis have already been developed (Smulders *et al.,* 1997; Bredemeijer *et al.,* 1998; He *et al.,* 2003). Besides, SSR has recently produced highly informative genotyping sets in other crops, such as leafy brassicas (Celucia and villa *et al.,* 2009), among others. Furthermore, Jones *et al.,* (1997) and Wang *et al.,* (2011) indicated the reproducibility of SSRs in closely related species and cultivars. The present study was undertaken with the objective to study the genetic diversity of tomato cultivars using morphological traits and also to assess the molecular diversity using RAPD and SSR markers.

Materials and methods

Plant material

Eighteen tomato germplasm accessions (Table 1) belonging to the species of *Lycopersicon esculentum* L. were used in this study. A replicated experiment with 18 genotypes was conducted in Randomized Block Design (RBD) with three replications, in which 21 days old seedlings were transplanted in 2 rows of 5m length with a spacing of 45 × 90 cm. All the agronomic and plant protection techniques were adopted. In each genotype, five plants were selected for various observations while molecular markers were used to assess the prevalent diversity.

Sample Preparation

Newly expanded leaves (2g plant^{-1}) were collected from selected plants from each accession separately and dipped in liquid nitrogen. Total genomic DNA was isolated from leaf samples by using CTAB following the standard protocol of Doyle and Doyle (1990) with some modification. DNA was further purified by RNase digestion followed by extraction with phenol/chloroform/ iso-amyl alcohol and ethanol precipitation. The concentration of DNA was estimated spectrophotometrically and also by gel electrophoresis using 0.8 percent agarose with known concentrations of DNA. Random Amplified Polymorphic DNA (RAPD) and Simple Sequence Repeats (SSR) analysis was carried out using five SSR markers and ten RAPD markers were used to assess the genetic diversity of tomato germplasm table 2 and table 3.

PCR amplification

PCR was conducted in a thermo cycler (Mastercycler Personal, Eppendorf, USA). PCR amplifications were performed in a reaction volume of 10 µl containing 1 µl of genomic DNA (25 ng/µl) as template, 1.0 µl of each forward and reverse primers (10 ng/µl), 1 µl each dNTPs (10 mM), 0.5 unit of Taq DNA polymerase, 1.0 µl of 10X PCR buffer and rest milliQ water.

The amplification reaction consisted of an initial denaturation at 94°C for 4 min followed by 40 cycles of 1 min at 94°C, 1min at 50-62°C (depending on marker type), and 1 min at 72°C and finally terminated with an extension of 7 min at 72°C. List of SSR markers and their annealing temperatures are given in table 2. The amplified PCR products (10 µl) were resolved on 1.5% (w/v) agarose gels in 1X TBE buffer. The gel was stained with ethidium bromide as previously described. The size of the fragments was estimated using a 100bp ladder (Genei, Bangalore) as a size marker. The gel was run till the dye reached the end of the gel, then the gel images were photographed using Gel Documentation system (Vilber Lourmat, France). All the PCR reactions were repeated thrice to ensure reproducibility and reliability of the results.

Scoring of Bands

The SSR-PCR bands were examined under ultra violet transilluminator and photographed under gel documentation unit. The SSR bands were counted and scored as 1 for their presence or 0 for their absence. The sizes of the bands were estimated by using 100 bp standard marker. The presence and absence of bands in all genotypes for primers were used to generate Bi-nomial

Table 1: Genotypes of Tomato used for research study.

S. No.	Name of the Genotypes	Source
1	LE 6	Department.of Horticulture, TNAU, Coimbatore
2	LE 14	Department.of Horticulture, TNAU, Coimbatore
3	LE 115	Department.of Horticulture, TNAU, Coimbatore
4	LE 7	Department.of Horticulture ,TNAU, Coimbatore
5	LE 3	Department.of Horticulture, TNAU, Coimbatore
6	LE 104	Department.of Horticulture, TNAU, Coimbatore
7	LE 105	Department.of Horticulture, TNAU, Coimbatore
8	LE 23	Department.of Horticulture, TNAU, Coimbatore
9	LE 10	Department.of Horticulture, TNAU, Coimbatore
10	LE 355	Department.of Horticulture, TNAU, Coimbatore
11	LE 13	Department.of Horticulture, TNAU, Coimbatore
12	LE 118	Department.of Horticulture, TNAU, Coimbatore
13	LE 116	Department.of Horticulture, TNAU, Coimbatore
14	LE 19	Department.of Horticulture, TNAU, Coimbatore
15	LE 15	Department.of Horticulture, TNAU, Coimbatore
16	LE 11	Department.of Horticulture, TNAU, Coimbatore
17	LE 22	Department.of Horticulture, TNAU, Coimbatore
18	LE 150	Department.of Horticulture, TNAU, Coimbatore

Table 2: RAPD primers used for assessment of genetic diversity among tomato genotypes.

Primer code	No. of scorable bandsper primer	No. of monor phic bands	No. of polym- orphic bands	Rangeof amplification
Rpi-1	16	9	7	600-700
Rpi-2	15	9	6	120-700
Rpi-3	14	13	1	400-600
Rpi-4	11	6	5	250-1000
Rpi-5	17	17	0	100-300
Rpi-6	9	5	4	300-400
Rpi-7	18	14	4	100-900
Rpi-8	18	18	0	550-800
Rpi-9	18	17	1	400-500
Rpi-10	11	6	5	650-700

data using excel sheet. Bands were marked as present only if the DNA amplification produced the fragment of a particular sequence and absent if the DNA amplification lacked that fragment. The banding patterns of all genotypes against each primer were compared. Bands present in one genotype and absent in another genotype, were regarded as variable and used to score for polymorphism. In order to check the informativeness and discriminatory power of SSR primers utilized in this study, certain parameters like polymorphism percentage, polymorphic information content and number of alleles were calculated. Percentage Polymorphism was calculated by dividing the polymorphic bands by the total number of scored bands. Polymorphism Information Content (PIC value) was calculated as proposed by Roldan-Ruiz *et al*., (2000).

Diversity Analysis

The collected data were aligned for the construction of cluster analysis and similarity matrix. The cluster analysis of 18 genotypes was constructed with the help of DARWIN software based on Unweighted paired group of arithmetic mean average (UPGMA). A tree like dendrogram was constructed using DARWIN (version 6.0) software (Perrier and Jacquemound-Collet, 2006). Genotypes were divided in various clusters, sub-cluster and sub-sub clusters based on genetic diversity among them and linkage distance was calculated.

Results and Discussion

Genetic divergence: Eighteen genotypes of tomato were grouped in the four clusters using clustering technique. The

Table 3: SSR primers used for assessment of genetic diversity among tomato genotypes.

Primer code	Primer sequence	Ta (°C)	Number of scorable bands per primer	No. of Monomorphic bands	No. of polymorphic bands	Exactam-plicon size(bp)
T-7	F:5'GTGGATTCACTTACCGTTACAAGTT-3' R:5'CATTCGTGGCATGAGATCAA-3'	55	15	11	7	100-700
T-57	F:5'GTGGACCATTTCAAGTTCAACA-3' R:5'TGAATGACATCCATCCATGA-3'	58	16	10	8	100-600
T-62	F:5'-GTGACCACATGAGATATCCAGA-3' R:5'-CAGTTGTCCATATTGTGTGGG-3'	58	18	0	0	200-500
T-70	F:5'-AACATGCGGAGAAAAATT-3' R:5'-GGAACACGTCCCAAAAATGT-3'	54	12	10	2	350-650
T-107	F:5'-GCACAAATAATTTTTCAAGACCAA-3' R:5'-AAAAACGGACATAGCTTTGTACT-3'	55	15	13	2	150-200

Table 4: Distribution of Tomato genotypes in different clusters based on D^2 analysis.

Cluster No.	Number of genotypes	List of the genotypes
I	10	LE-6, LE-14, LE-115, LE-7, LE-3, LE-
		104, LE-105, LE-23, LE355, LE-13.
II	2	LE-116, LE-19.
III	2	LE-15, LE-11.
IV	4	LE-10, LE-118, LE-22, LE-150.

Table 5: Parameters used for the evaluation of polymorphism in RAPD markers.

S. No.	Locus	PIC	Major allele frequency	No. of Alleles	Genetic Diver-sity	Heteroz-ygosity
1	Rpi-1	0.1411	0.9167	2.0000	0.1458	0.0556
2	Rpi-2	0.2106	0.8611	2.0000	0.2274	0.0556
3	Rpi-3	0.2859	0.7778	2.0000	0.3324	0.2222
4	Rpi-4	0.3680	0.5833	2.0000	0.4695	0.3889
5	Rpi-5	0.3742	0.5278	2.0000	0.4969	0.9444
6	Rpi-6	0.3742	0.5278	2.0000	0.4722	0.0556
7	Rpi-7	0.3742	0.9444	2.0000	0.0991	0.0000
8	Rpi-8	0.0994	0.5278	2.0000	0.4969	0.9444
9	Rpi-9	0.0994	0.9444	2.0000	0.0991	0.0000
10	Rpi-10	0.3047	0.7500	2.0000	0.3647	0.3889
Mean		0.2632	0.7361	2.0000	0.3204	0.3056

Table 6: Parameters used for the evaluation of polymorphism in SSR markers.

S. No.	Locus	PIC	Major allele frequency	No. of Alleles	Genetic Diver-sity	Heteroz-ygosity
1	T-7	0.3742	0.5278	2.0000	0.4905	0.7222
2	T-70	0.3742	0.5278	2.0000	0.4905	0.7222
3	T-107	0.3623	0.6111	2.0000	0.4640	0.5556
4	T-57	0.2392	0.8333	2.0000	0.2714	0.3333
5	T-62	0.0000	1.0000	1.0000	0.0000	0.0000
Mean		0.2700	.0.7000	0.7000	0.3433	0.4667

PIC: Polymorphism Information Content.

composition of different clusters are presented in table 4. All the 18 genotypes were resolved into as many as four clusters. Cluster analysis was conducted for morphological characters. Cluster I composed of 10 genotypes (LE-6, LE-14, LE-115, LE-7, LE-3, LE-104, LE-105, LE-23, LE-355, LE-13). Cluster IV compassed of four genotypes (LE-10, LE-13, LE-22, LE-150) and cluster III (LE-15, LE-11), cluster II (LE-116, LE-19) each composed of two genotypes. The cluster distance was maximum with cluster IV (20.54) and minimum with cluster II (4.79) was recorded. The maximum intra cluster distance was found between cluster III and IV. The minimum cluster distance was found between cluster II and III. The inter-cluster distance indicated wide range of variation among the clusters formed, and composed of genetically dissimilar genotypes. The minimum inter-cluster distance was found between cluster II, III and likely to have similar genotypes. The results of this study are in agreement with the results of Henarch (2015).

Molecular characterization: The same eighteen genotypes of tomato (*Lycopersicon esculentum* L.) were used to evaluate genetic diversity at the molecular level using SSR and RAPD primers. Genetic diversity/ relatedness among the genotypes was assessed on the basis of Polymorphic information content (PIC) value and percentage polymorphism. All five SSR primers used in the study were found to be polymorphic. A total of 10 alleles were found and out of which, 10 alleles were polymorphic with different product sizes. Similarly 10 RAPD primers used in the present study showed polymorphism. A total of 20 alleles were found and of which 20 alleles were polymorphic with different product sizes.

Polymorphism information content (PIC): Polymorphic information content (PIC Value) of each RAPD and SSR primer was calculated. In RAPD markers, the PIC value ranged from 0.0994 (RPi-9) and marker high PIC value was observed for RPi-5, RPi-6, Rpi-7 (0.3742). In SSR markers, the values ranged from 0.2393 (T-57) to 0.3742 (T-7) with an average of 0.2700 for SSR primers. The low PIC value was observed for T-62 (0.0000) for SSR markers and for RAPD markers, it was observed for RPi-1(0.1411). For RAPD primers, it ranged from 0.0994 (RPi-9) to 0.3742 (RPi-7) with an average of 0.2632. High PIC value for SSR marker was observed for SSR (T-7) 0.3742 and SSR 0.3742 (T-70) and for PIC values of all the genotypes are listed in the table 5 and table 6. Percentage polymorphism was calculated. 100 percent polymorphism has been observed for all the markers used in the study.

Clustering analysis done using RAPD markers: The genotypes were grouped into two main clusters, cluster A and cluster B. Cluster A was further divided into two sub clusters *i.e.* sub cluster A1 and sub cluster A2. Sub cluster A1 had 6 genotypes (LE-105, LE14, LE-3, LE-23, LE-13 and LE-355) and sub cluster A2 had 3 genotypes (LE-7, LE-115 and LE-104). Cluster B consisted of further two sub clusters B1 and B2. Sub cluster B1 had 7 genotypes (LE-11, LE-15, LE-10, LE-118, LE-6, LE-150 and LE-22.) while Sub cluster B2 had 2 genotypes (LE-19 and LE-116) in which LE-19 and LE-116 forms separate group each. The dendrogram showed genetic variation among the 18 genotypes of

Tomato. The similarity coefficients among 18 genotypes ranged from 0.05 to 0.25. Among all the pair-wise combinations, LE-150 showed the highest similarity index, while genotypes LE-16 and showed the lowest similarity index. The observations revealed similar genetic differentiation among 18 genotypes of tomato as revealed by UPGMA dendogram Fig. 1.

The genotypes were grouped into two main clusters, cluster A and cluster B. Cluster A was further divided into two sub clusters *i.e.* sub cluster A1 and sub cluster A2. Sub cluster A1 had 6 genotypes and A2 had 3 genotypes, where as cluster B consisted of two sub clusters B1 and B2. B1 had 7 genotypes and B2 had 2 genotypes. Dendrogram showed genetic variation among the 18 genotypes of tomato. Among 18 tomato genotypes, LE-150 and LE-22 showed highest similarity as revealed by clustering using RAPD markers. Similarity coefficient of 18 genotypes using RAPD markers ranged from 0.22 to 0.33. The lowest genetic distance (0.22) was found between genotypes LE-6 and LE-14, while, the highest genetic distance (0.33) was found between LE-150 and LE-22. The clustering done by using UPGMA was further confirmed by DARWIN (6.0 version). The observations revealed similar genetic differentiation among 18 genotypes of tomato as revealed by UPGMA dendogram. Similar results have been reported earlier by Sharifova *et al.,* (2013).

Cluster analysis done using SSR markers: The genotypes were grouped into three main clusters, cluster A, cluster B and cluster C. Cluster A, B and C were further divided into three sub clusters *i.e.* sub cluster A1, sub cluster A2, sub cluster B1 and sub cluster B2. Sub cluster A1 had 5 genotypes (LE-104, LE-115, LE-118, LE-6 and LE-11) and sub cluster A2 had 2 genotypes (LE-116 and LE-19), in which LE-116, and LE-19 formed separate sub group. Sub cluster B1 consisted of three genotypes (LE-23, LE-14, LE-355) and B2 had two genotypes (LE-23, LE-14) Fig. 2.

The similarity coefficients among 18 genotypes ranged from 0.1 to 0.5. Among all the pair-wise combinations, LE-19, LE-16and LE-6 showed the highest similarity index (0.5), while genotypes (LE-13, LE-150, LE-3, LE-22) and LE15 (0.01) showed the lowest similarity index. Scored data were used for the estimation of Jaccard's similarity coefficient using DARWIN version 6.0 package to compute pair-wise Jaccard's similarity coefficient.

The Jaccard similarity coefficients among 18 genotypes ranged from 0.0100 to 0.3742. The lowest genetic distance (0.01) was noted between genotypes

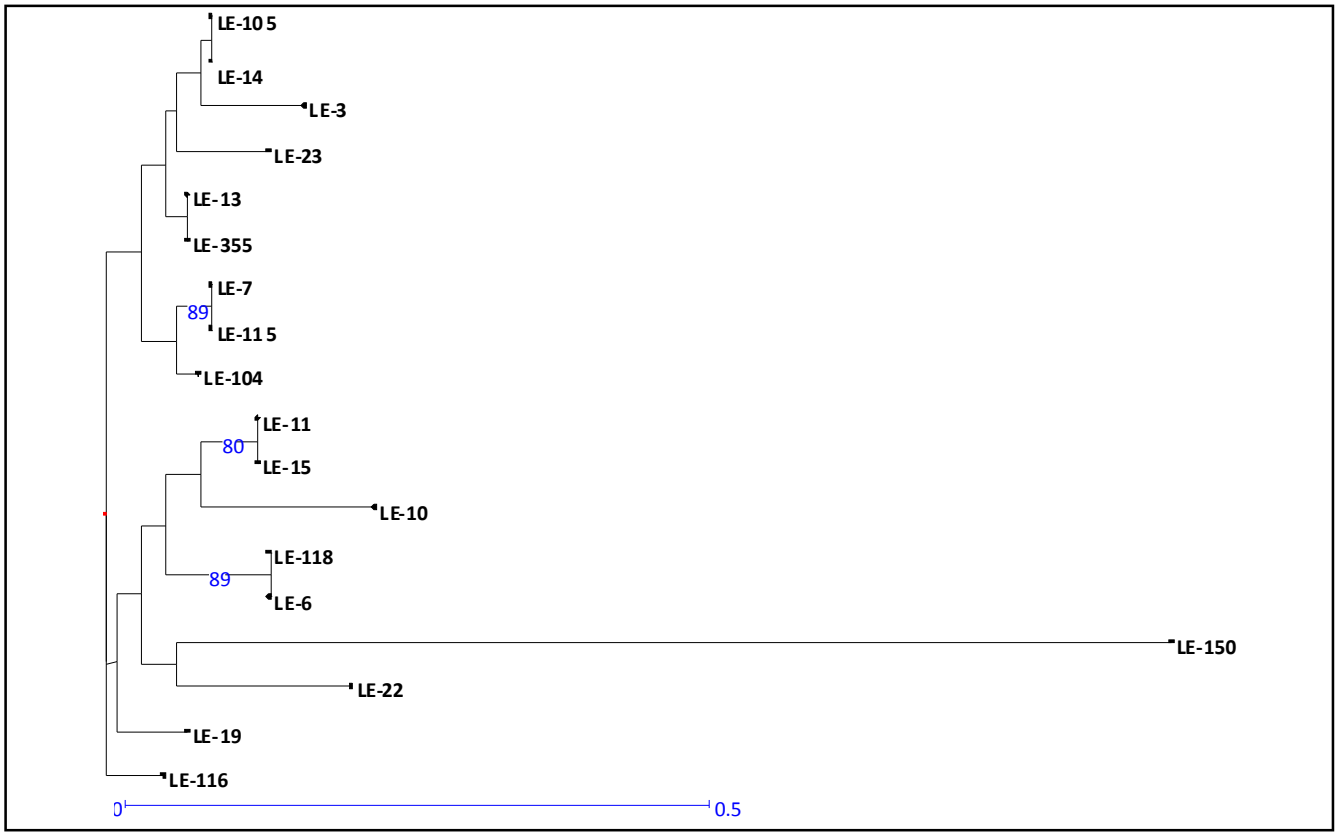

Fig. 1: Distribution of 18 tomato genotypes into two main clusters-RAPD Marker.

LE-6 and LE-14, while, the highest genetic distance (0.49) was found between varieties LE-150 and LE-22. Among all the pair-wise combinations, LE-150 and LE-22 showed the highest similarity index, while genotypes LE-6 and LE-14 showed the lowest similarity index. The dendrogram revealed a peculiar picture showing the different clusters of different genotypes. The genotypes were grouped into two main clusters, cluster A and cluster B. Among 18 tomato genotypes, LE-150 and LE-22 showed highest similarity as revealed by clustering using SSR markers. Similarity coefficient of 18 genotypes using SSR markers ranged from 0.1 to 0.4. The clustering done using UPGMA was further confirmed by DARWIN (6.0 version). The observations revealed similar genetic differentiation among 18 genotypes of tomato as revealed by UPGMA dendrogram.

Morphological and Molecular characterization: Genetic diversity could well assessed at the phenotypic, genotypic, physiochemical and molecular level which is helpful for the selection of diverse parents for hybridization. In the present study, five microsatellite markers and ten RAPD markers were used to assess the genetic diversity of tomato germplasm. In this study, while assessing the genetic diversity using SSR markers, the number of alleles detected varied from one to two alleles per locus with an average PIC value of 0.2632 for RAPD, and 1.00-2.00 alleles per locus with average PIC value of 0.2700 for SSR markers.

A total of 20 alleles were observed in RAPD and 9 in SSR markers. PIC value among the genotypes varied from 0.000 (T-62) to 0.3742 (T-70). PIC value was recorded up to 0.2700 for all the five SSR markers. These results are in agreement with the observations of Jamil *et al.,* (2013). Korir *et al*., (2014) also reported similar results while studying the PIC of genomic SSRs and EST-SSR markers in various tomato lines.

The genetic diversity has been assessed using RAPD marker. The number of alleles detected varied from 1.00 to 2.00. The average number of polymorphic alleles per marker was 2.00 with an average PIC of 0.2632. The results of this study are in close conformity with the results of Sharifova *et al*., (2013).

Percentage polymorphism, number of alleles per locus and PIC values depicted the level of genetic divergence. A total of 20 alleles were produced, PIC value among the genotypes varied from 0.1411 (Rpi-1) to 0.3742 (Rpi-7) with an average of 2.00 allele per primer. The results are in conformity with the findings of Dhaliwal *et al.,* (2009), Thamir *et al*., (2014) and Zargar *et al.,*

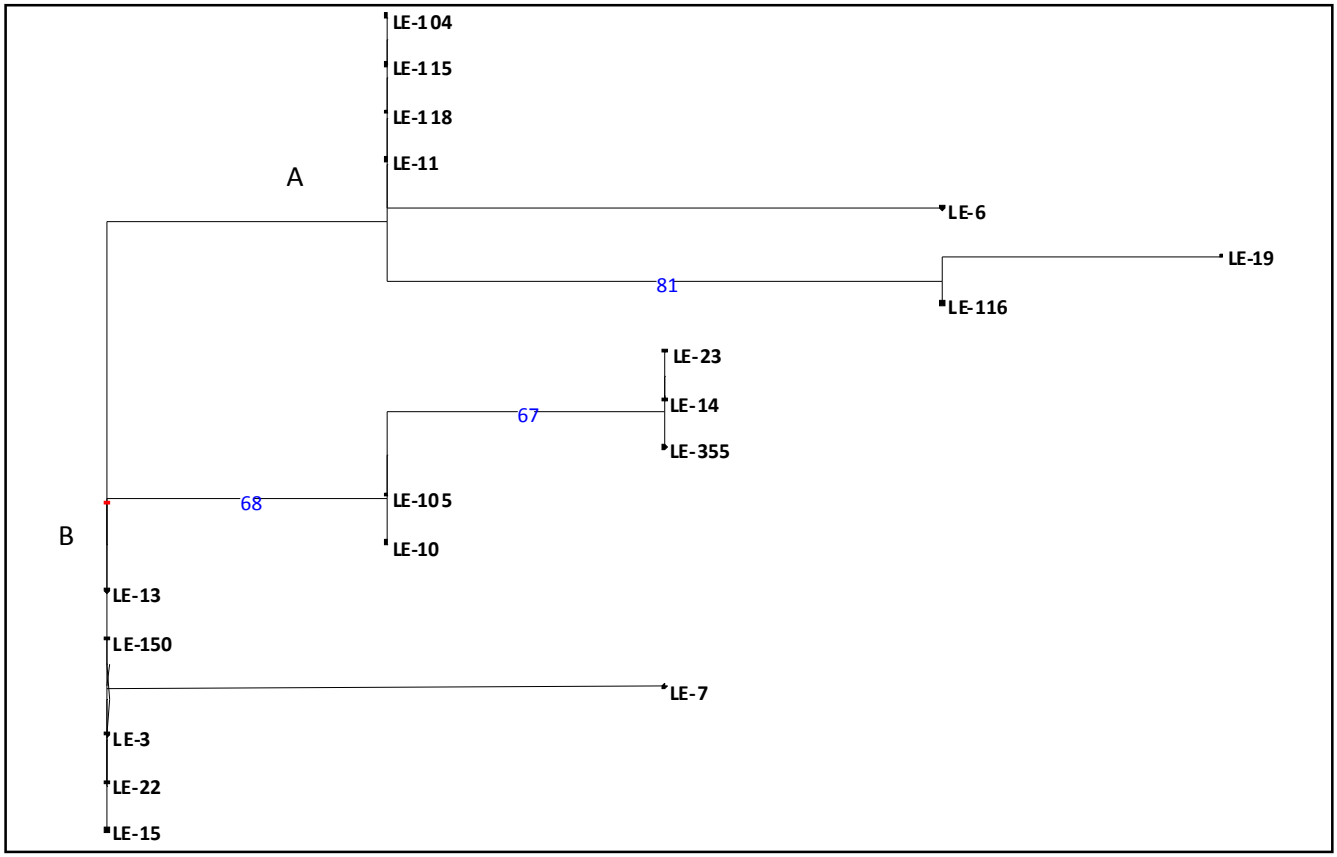

Fig. 2: Cluster analysis of tomato based on UPGMA using SSR marker.

(2014).

Catur Herison (2018) studied genetic diversity of 27 tomato accessions by morphological and molecular markers and found that the tomato accessions could be grouped into 5 major groups with 70 % genetic similarity levels and Cherry, Kudamati 1 and Lombok 3 were the farthest genetic distant. Ghaffar Kiani and Mohammad Siahchehreh (2017) studied genetic diversity and relationships among 12 tomato varieties with Inter Simple Sequence Repeat (ISSR) markers and found that 69 bands were generated with 9 ISSR primers, among which 53 bands were polymorphic (65.2%) and the mean polymorphism index content was 0.29.

Therefore, it is inferred that genetic diversity exists among the tomato genotypes at genotypic level. The genotypes LE-19 and LE-9 (based on SSR markers) and LE-150 and LE-22 (based on RAPD markers) could well be exploited through hybridization to recover the genotypes with high yield potential along with improved fruit quality characteristics. Genetic diversity analysis at molecular level by SSR markers that cover the whole genome would be helpful to identify the diverse parents.

High, medium, and low locus polymorphism is defined as PIC 0.2392 > PIC > 0.25 and PIC < 0.3742, respectively. Therefore, in our study, SSR and RAPD markers detected medium locus polymorphism among the 18 tomato genotypes, indicating that both markers are of great utility for genetic diversity studies of tomatoes which can be used in strengthening tomato breeding programmes.

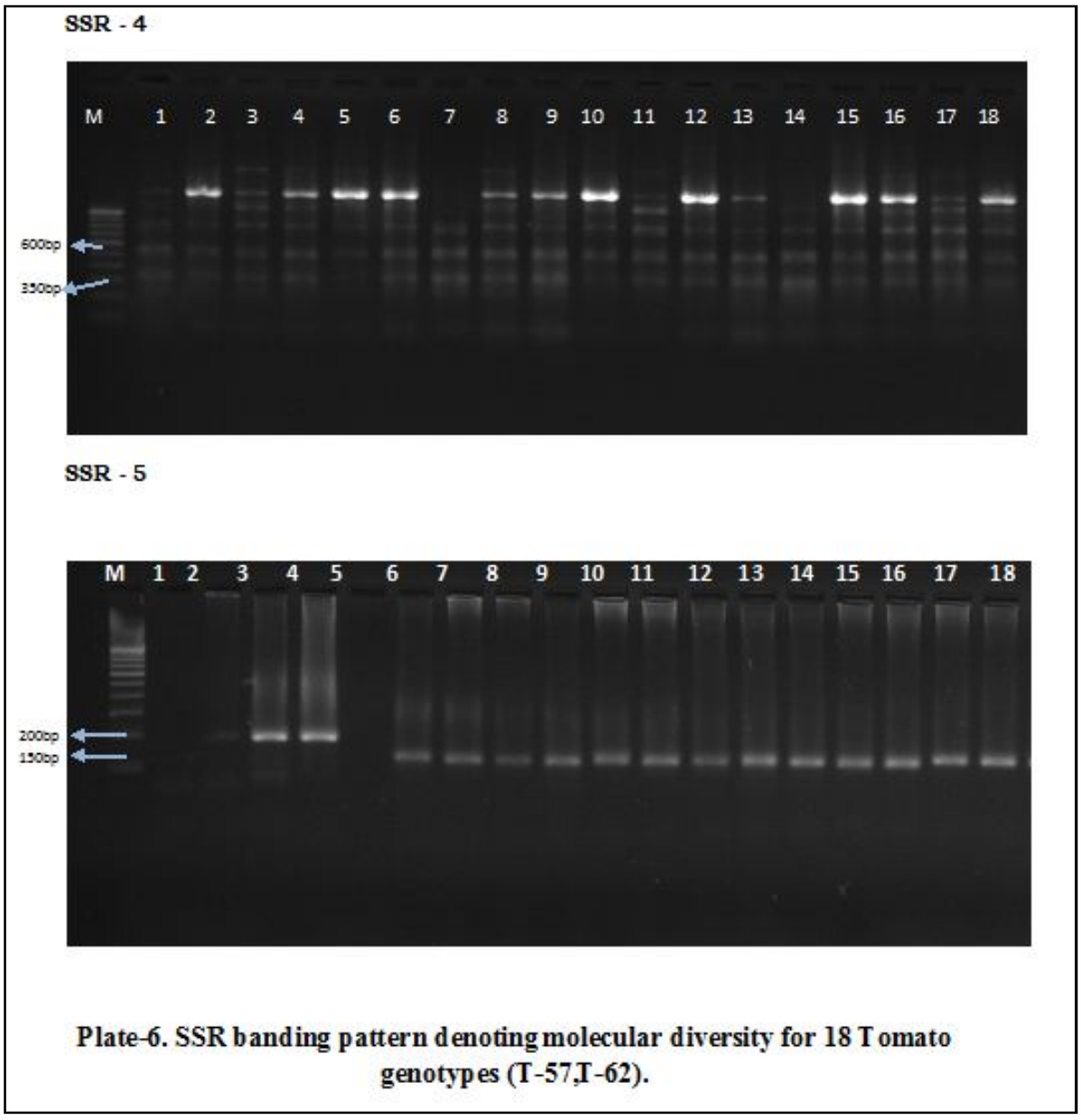

Plate-6. SSR banding pattern denoting molecular diversity for 18 Tomato genotypes (T-57,T-62).

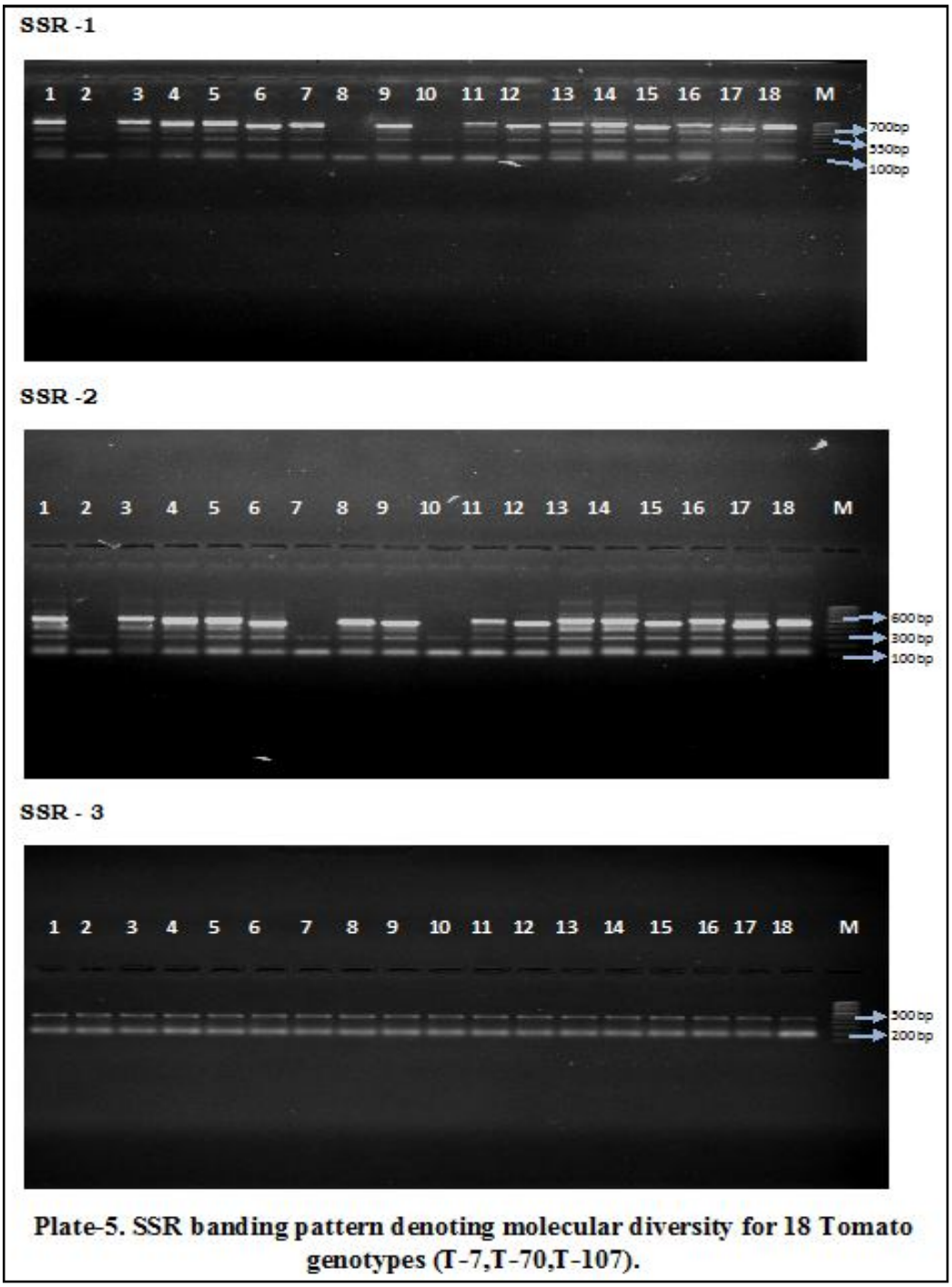

Plate-5. SSR banding pattern denoting molecular diversity for 18 Tomato genotypes (T-7,T-70,T-107).

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