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Analysis of molecular diversity in tomato (*Solanum lycopersicum*) genotypes using SSR primers

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ABSTRACT

Tomato (*Solanum lycopersicum* L.) was sown during October 2010 at Department of Horticulture, Sardar Vallabhbhai Patel University of Agriculture and Technology, Meerut, for developing the nursery. After that the single plantlet of each variety was transferred and maintained into the separate pots for using conventional agronomic practices to keep the crop in good condition. Study of morphological and molecular diversity in landrace collections was carried out during 2010. Germplasm collections of all the 24 genotypes including determinate and indeterminate cultivars of tomato were screened with twenty SSR (simple sequence repeat) primers in order to determine genetic identities, genetic diversity and genetic relationships. On the basis of resolving power, primer A-8, A-13 and A-19 were most significant as they are able to recognize all 24 genotypes. The gene diversity was varied from 0.65 to 0.97 values with a mean diversity of 0.84. On an average, 54 scorable and reproducible alleles were amplified using all primers. Cluster analysis clearly showed the genetic diversity among the genotypes under study.

Key words: Alleles, Cluster analysis, Genetic diversity, Microsatellites, SSR primers, Tomato

Tomato (*Lycopersicum esculentum* Mill) is the most popular cultivated and versatile garden vegetable grown in the world (Manish Kumar *et al*. 2013). The traditional methodology of plant genetics, based on morphological marker and biochemical marker for assessing the genetic diversity with the advent of DNA marker technology in 1980s and 1990s, many limitations associated with morphological markers were overcome and genetic mapping entered a new exciting and progressive era. A DNA marker derived from a small region of DNA that shows sequence polymorphism between individuals within or between species (P Venkata Ramana Rao *et al.* 2013). DNA markers which are phenotypically neutral have allowed scanning of the whole genome in many crop plants including tomato (Williams *et al*. 1990). Recently, the development and use of PCR based markers have increased in tomato as these markers are generally more user friendly, cheaper, faster and less labor intensive. (Frary *et al*. 2005, Fulton *et al*. 1999, Huang *et al*. 2000).

The application of SSR technique to plants depends on the availability of suitable markers. SSRs are short (mostly 2–4 bp) tandem repeats of DNA sequences and are preferred molecular markers because of their properties of genetic co-dominance, high reproducibility and multi allelic variation (Asgedom *et al*. 2011). However, especially in crops like tomato where genetic diversity is limited (Kelly *et al*. 2010). For tomato, several primer sets for SSR analysis are available. The utility of technique in tomato has been shown by (Smulders *et al*. (1997), Bredemeijer *et al*. (2002), and Areshchenkiva and Jones *et al*. (1997) indicated the faithful reproducibility of SSRs being tested by a network of laboratory. Molecular marker for a broad range of application, such as genome mapping and molecular ecology and diversity studies (Gupt *et al*. 2010). The characterization of much diversified materials with molecular markers offers a unique opportunity to define significant marker trait association of biological and agronomic interest.

MATERIALS AND METHODS

Collection and maintenance of samples - Twenty four genotypes of *S. lycopersicum* were collected from IIVR, Varanasi (UP). The collected germplasm was sown in October 2010 for developing the nursery in Department of Horticulture, Sardar Vallabhbhai Patel University of Agriculture and Technology, Meerut, for using conventional agronomic practices to keep the crop in good condition.

Isolation and quantification of genomic DNA - After attaining the proper growth the fresh leaves of each variety ware collected and stored in (–8°C), deep freezer. The genomic DNA was isolated from various leaf samples following the method of Doyle and Doyle (1990) with some modifications as follows. 3 g fresh leaf tissue was taken and grounded to fine powder with a chilled mortar and pestle using liquid nitrogen and transferred to a

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centrifuge tube containing 15 ml of pre-warmed (60ºC) CTAB-extraction buffer. The sample was mixed well by inverting the tubes several times and incubated for 1 hr in a shaking water bath at 65ºC. An equal volume of chloroform: isoamylalcohol (24:1) was added to the tube and mixed gently for 15 minutes. The tubes were centrifuged in cooling centrifuge at 8000 rpm for 10 minutes at room temperature. After centrifugation, aqueous phase was transferred to a fresh tube and again extracted with an equal volume of chloroform : isoamylalcohol (24:1). Aqueous layer was transferred to the fresh tube and 1.5 ml of NaCl (5 M) was added and mixed gently. Treatment with 5 µl of RNase (10 mg/ml) and carrying out incubation for 30 minutes at room temperature. For precipitating DNA, 0.6 volume of chilled isopropanol was added to the tube and incubated for 2 hr. The tubes were then centrifuged at 10 000 rpm for 10 minutes. The supernatant was discarded and 5 ml of 70% ethanol was added for washing. The tubes were centrifuged at 7500 rpm for 10 minutes. The supernatant was discarded and pellet was air dried and dissolved in 100 µl of TE buffer.

The genomic DNA dissolved in the TE buffer was taken for quantified by recording A260 nm in a UV-VIS spectrophotometer reference was set against TE buffer as blank and absorbance was recorded at 260 and 280 nm. The ratio of A260/A280 provided an estimate of purity of nucleic acid. The ratio of OD260 / OD280 should be 1.8-2.0 for pure DNA preparation. The concentration of DNA in µ gm/ml of the sample was calculated using the extinction coefficient of DNA, i e 1 OD unit at 260 nm=50µg/ml of double stranded DNA.

PCR reaction – A set of 20 SSR primers (Table 1) was used to study the genetic diversity of tomato. DNA amplification reaction for SSR primers were performed a total volume of 10 µl an end concentration 2.5 mM each of the dNTPs, 1 U/µ Taq polymerase enzyme, 25 ng DNA template and 1 µl of reverse primer and 1 µl of forward primer, 10 ng primer in Taq polymerase assay buffer (1X) (10 X buffer contains 100mM Tris Cloride, 500 mM KCl, 15mM $MgCl_2$ and 0.1% gelatin). Amplification reaction was carried out in a long – gene Thermal cycler with the following thermal profile – 1 cycle of 4 min. at 94°C (Initial denaturation) followed by 45 cycles of 15 sec at 94°C (denaturation), 45 sec (primer annealing at temp according to the primer sequence) and 90 sec at 72°C (primer elongation) and finally a cycle of 4 min at 72°C (final extension). Amplified PCR Product were separated on 1.5% (w/v) agarose gel in 1X TBE (10X TBE buffer contained

Fig 1 At various stages the plants are fully developed

Table 1 The polymorphic SSR primers with their corresponding gene diversity and resolving power

Primer Code	Sequence	% of GC	Tm	Total no of bands	Polymorphic bands	Genetic diversity	Resolving power
A-1	GTTATGGATTCACTTACCGCAAGTT (F) CATTCGTGGCATGAGATCAA (R)	40% 45%	53.0ºC 52.0ºC	2	2	0.651	1.82
A-2	GGTAATCAATTTTGAAGCTAAAAGC (F) TGGGAAGAACTCAAGTCAAAAA (R)	32% 37%	51.5ºC 52.4ºC	3	3	0.899	1.25
A-3	GTC AACTAGCGCTCCAATCT (F) AAAGGGTTGTGGGAATTGTG (R)	53% 45%	57.0ºC 53.5ºC	3	3	0.882	1.86
A-4	GAATTAGAGGGTTTGTGATACCG (F) AAAAAGCTTCCTGGCTAAGAAAT (R)	44% 35%	53.2ºC 53.1ºC	3	3	0.824	0.78
A-5	GAGAGAGAGTATGTATGTGCATTTCC (F) TGAAAATTTGTGGTGTTGACG (R)	48% 40%	55.0ºC 52.0ºC	2	2	0.75	1.55
A-6	GTGAGGGAGTGGGATTCAAAC (F) AATTAGGGGATACGGGATCG (R)	43% 50%	55.6ºC 53.7ºC	3	3	0.849	1.83
A-7	GTTGGAGTGCAATATTTGGGT (F) TTCGCTTAGGACAAGAATGACTT (R)	43% 40%	54.2ºC 53.6ºC	3	3	0.889	1.66
A-8	GTGAAGAGATGGGGTTGTGAA (F) TCTGTTTTGAAGGGAAGAAGATG (R)	48% 40%	54.8ºC 53.0ºC	2	2	0.768	2.66
A-9	GCAATTTGATGAGAATTTCGG (F) TTTGTTAAAGTGTGTTAGCCGTTT (R)	43% 34%	52.3ºC 53.7ºC	2	2	0.674	1.91
A-10	GTGACCACATGAGACATATCCAGA (F) CAGTTGTCCATATTGTGTGGG (R)	46% 48%	56.1ºC 53.7ºC	3	3	0.792	2.29
A-11	AACATGCGGGAGAAAAATTG (F) GGAACACGTCCCAAAAATGT (R)	40% 45%	51.8ºC 53.7ºC	3	3	0.88	2.00
A-12	GTGAGGATTTTGATTTGTCTGAAG (F) TGGGTTAGTTCTCCCTCAGG (R)	38% 50%	52.5ºC 56.2ºC	3	3	0.974	1.83
A-13	GTGCGGATAACTTTAAGGGACTG (F) CCTCTCAAAATCCTTGCCAC (R)	48% 48%	55.6ºC 53.8ºC	3	3	0.894	2.72
A-14	GTGAAAAATCACAAAACTTTGAAAA (F) CGAAAACACCCTCCATGTTAAA (R)	30% 38%	50.3ºC 53.2ºC	3	3	0.876	1.99
A-15	CCAAAACCCAAGGAATAGCA (F) GGGAGATAAAACCAAAATGAGAAGA(R)	43% 38%	53.0ºC 52.8ºC	3	3	0.942	1.11
A-16	GCACAAATAAATTTTCAAGACCAA (F) AAAAAACGGACATGTAGCTTTGTAC (R)	30% 36%	51.4ºC 55.3ºC	3	3	0.854	1.99
A-17	TTGCGCTTGTTGAGTGAAGC (F) TGTCGTTGCCTTTGATTCAGG (R)	50% 48%	55.7ºC 56.0ºC	3	3	0.889	1.99
A-18	TTGTGTTTGCCGCGCCAGTGGAT (F) GCGCCTTCCATTGAAGCCAAGTAT (R)	53% 50%	60.9ºC 60.0ºC	3	3	0.866	2.73
A-19	ATTATTACATTGAGGGACAAG (F) TCATCAGACAAGACTCAACTCATC (R)	34% 40%	54.2ºC 54.3ºC	3	3	0.84	3.82
A-20	CAGAGAGCTGTTGGAATCGGTGG (F) CATTCTTTATCCTGTAACAAATTGT (R)	57% 30%	59.4ºC 53.6ºC	4	4	0.927	2.14

108g Tris base, 9.3g EDTA and 55g boric acid in 1 litre double distilled water, pH 8.3). Electrophoresis was performed at 100 volt for 2 hr. then the gel was visualized and photographed for further analysis. A low range DNA ruler was used as a molecular size marker. The reproducibility of the amplification products was checked twice for each polymorphic primer (Ezekiel C N *et al.* 2011).

In order to assess the ability of primer to resolve the different varieties the resolving power (Rp) for each primer was calculated following Prevost and Wilkinson's (1999) method. It is very useful for selecting primers that can distinguish maximal number of accessions. Resolving power (Rp) of a primer is Rp=Ib (band information) is calculated as 1-[2 X (0.5-p)], p being the proportion of the 15 accessions containing the bands and Gene Diversity is calculated as 1-Σ Pi^2 (Anderson *et al.* 1993).

The bands were scored as present (1) or absent (0) for each DNA sample with the all 20 primer. Amplification was performed twice and only reproducible amplifications products were included in the data analysis. Similarity

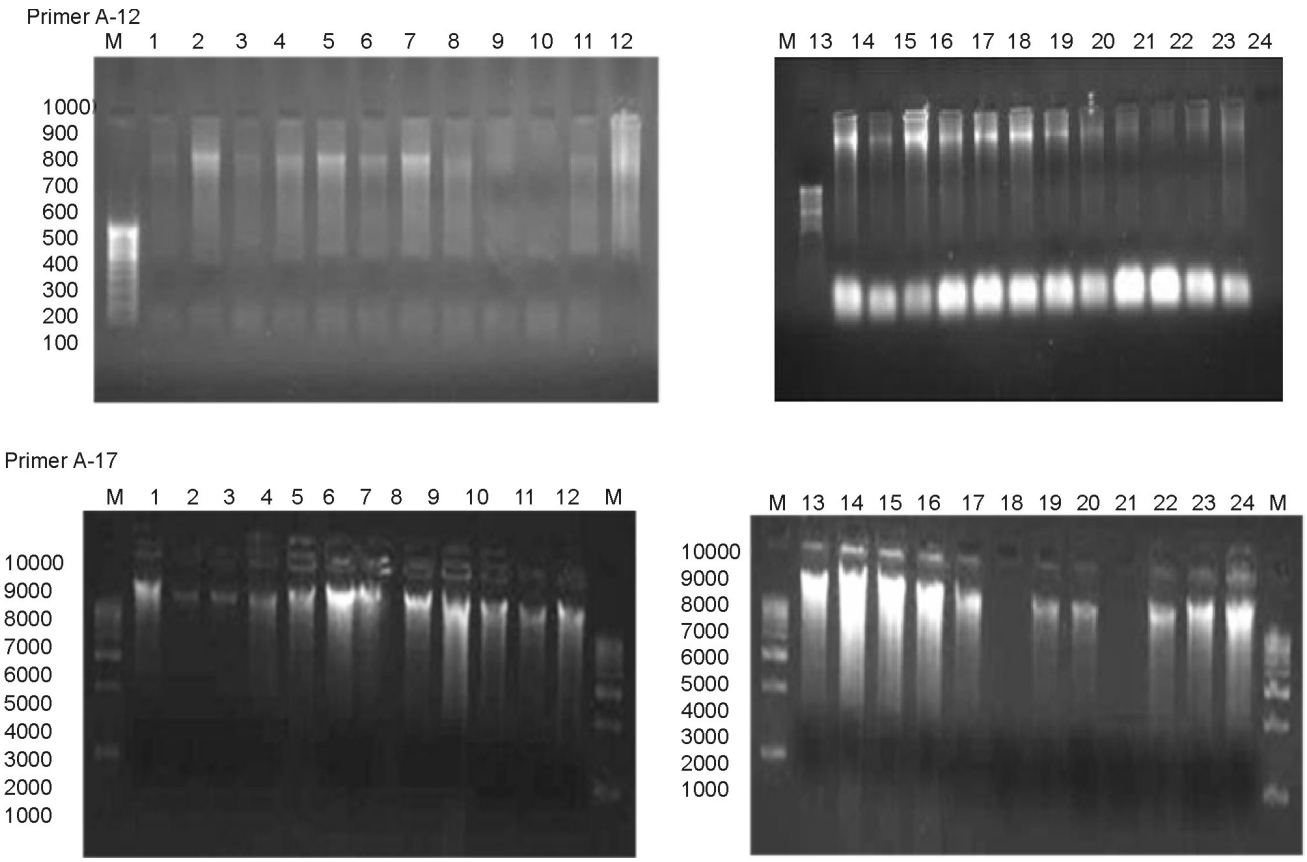

Fig 2 Amplification profiling of 24 genotypes of tomato using SSR primers

matrix using the similarity coefficient of Jaccard (1908) was constructed from the whole RAPD data. Pairwise distances between DNA accessions were calculated and analysed using the Unweighted Pair Group Method Arithmetic average (UPGMA) (Sneath and Sokal 1973). Clusters were analyzed using the computer program NTSYS-PC, version 2.11s (Rohlf 2000).

RESULTS AND DISCUSSION

Collection and maintenance of tomato germplasm – All the twenty four diverse genotypes of *S. lycopersicum* viz. Arka vikas, FLA-7421, PDT-3-1, TLBR- 5, DVRT-1, Selection-7, Nandi, Pusa Rohini, Meghalaya Local, Sikkim Local, Pusa Gaurav, Arkha Abha, TLBR-3, Castal Rock, K.S-17, Money Maker, Palam Pink, IIHR-2200, IIHR-2201, H-24, H-86, FLA-7171, Tura Local and Agata -30 were grown for agronomically practices of all the plants (like germination, flowering, fruiting and ripening) were maintained and grown in the pots (Fig 1). The resistant varieties take an average 4 days for germination, whereas the hybrid varieties take 5 days for germination. In all the genotypes Nandi is resistant towards leaf curl virus, Arka Abha is susceptible for yellow leaf curl virus, Tura local and Sikkim local are tolerant to bacterial wilt, while Selection-7, Meghalaya local and Arkha Abha are resistant to bacterial wilt. Pusa Rohini and Arkha Vikash are hybrid varieties.

Isolation and estimation of genomic DNA – The genomic DNA from all the diverse genotypes of *S. lycopersicum* was isolated and Conc. of DNA samples (Table 2) using the CTAB method. The isolated DNA was quantified using 0.8% agarose gel. For estimating the size of total genomic DNA the standard molecular weight marker of 1Kb was loaded at both ends. In reference to the known molecular weight of different bands of 1Kb ladder the molecular weight of tomato germplasm was found to be more than 10000 Kb and varied in different genotypes.

Molecular profiling – The genetic diversity of all the genotypes of tomato, using SSR primer were used to amplify the genomic DNA of tomato. Out of 20 SSR primers used, 16 (80%) primers resulted in polymorphic, scorable and reproducible results whereas 4 primers did not amplify any of the tomato genotypes. The following two reasons may be attributed for the non-amplification of primers (i) the appropriate complementary microsatellite sequence may occur infrequently in *S. lycopersicum* genome; or (ii) the corresponding microsatellite sites may be distantly located in *S. lycopersicum* DNA in such a way that no amplification occurred. All of them were found to be polymorphic hence considered for genetic diversity analysis. The number of polymorphic primers and fragments generated were not in similar range for all genotypes.

Gene diversity and resolving power

Gene diversity was calculated for each primer, which

Table 2 Purity and concentration of DNA isolated of tomato genotypes

Genotypes	A_{260}	A_{280}	$A_{260/280}$	Conc (µg/ml)
Arka vikas	0.098	0.079	1.72	48
FLA-7421	0.230	0.186	1.79	80
PDT-3-1	1.008	0.582	1.89	38
TLBR-5	0.152	0.125	1.66	44
DVRT-1	0.234	0.174	1.73	47
Selection -7	0.728	0.661	1.70	43
Nandi	0.432	0.422	1.24	50
Pusa Rohini	0.117	0.098	1.69	240
Meghalaya Local	0.043	0.020	1.27	43
Sikkim Local	0.095	0.058	1.70	275
Pusa Gaurav	0.095	0.057	1.70	58
Arkha Abha	0.093	0.058	1.69	43
TLBR-3	0.095	0.079	1.71	46
Castal Rock	0.020	0.014	1.24	163
K.S-17	0.082	0.051	1.90	14
Money Maker	0.290	0.212	1.70	51
Palam Pink	0.230	0.186	1.80	47
IIHR-2200	0.153	0.122	1.68	46
IIHR-2201	0.153	0.125	1.67	44
H-24	0.090	0.083	1.78	49
H-86	0.020	0.022	1.16	205
FLA-7171	0.674	0.047	1.80	41
Tura Local	0.789	0.759	2.06	50
Agata-30	1.008	0.582	1.89	55

varied from 0.65 to 0.97 values with a mean diversity of 0.84. Resolving power of the 20 SSR primers (Table 1) ranged from 0.78 to 3.82 with an average 1.91. Based on resolving power and the ability of primers to differentiate all accessions, the primers 10, 17, 22 and 23 were found most informative with resolving power 2.664, 2.724, 2.734 and 3.824 respectively. In this study, the SSR primers were not able to recognize all 24 genotypes and so could not be segregated on the basis of their ability to diagnose genotypes. Nevertheless, these could be ranked according to their Rp values under the reasonable premise that primers with higher Rp value have a greater capacity to separate different genotypes (Prevost and Wilkinson 1999).

Genetic similarity matrix and cluster analysis - Total 20 primers amplified 57 allels across the 24 genotypes. The entire fragments generated in this way were polymorphic. Thus, the higher percent polymorphism suggests that the SSR procedure is a viable approach for the examination of genetic diversity of *S. lycopersicum*. The number of amplified allels observed ranged from minimum 2 to maximum 4 with primer-1/8/9 and primer-20 respectively (Table 3). SSR primers were scored for presence (1) and absence (0) across all tomato genotypes for each primer. The pair wise genetic similarities among all pairs of samples were estimated with Jaccard's coefficient (Jaccard 1908). The statistical analysis was carried out by using NTSYS-PC software (version 2.11s) (Rohlf 2000). In order to group genotypes into discrete clusters a dendrogram was constructed by employing UPGMA method (Sneath and Sokal 1973). Distance matrix expressed as similarity coefficient a dendrogram was generated by the UPGMA method. Similarity value for all the genotypes ranged from 0.38 to 0.88 (Table 1).

The lowest similarity displayed by Agata-30 and DVRT-1 in genotype analysis, samples collected from Sikkim loacal- Nandi, Agatha-30-Sikkim Local, H-24 - IIHR2200 and FLA-7171-Nandi displayed the greatest genetic similarity, with a similarity coefficient value of 0.88 (Table 1). The resultant dendrogram grouped of all genotypes into two main distinct clusters with two genotypes, viz. Arkha Abha and H-86 did not grouped in any major cluster and placed outside the major clusters at one end of the dendrogram. The cluster 1 grouped 8 genotypes, viz. Arka vikas, FLA-7421, PDT-3-1, TLBR-5, DVRT-1, Money maker Palam pink and TLBR-3. The maximum genetic similarity within group was exhibited by PDT-3-1, TLBR-5, Money maker and Palam pink. Whereas the minimum genetic similarity within this group was showed by Arka vikas and TLBR-3. The main cluster 2 was subdivided into three sub clusters. The sub cluster 1 grouped 7 genotypes, viz. Selection-7, Nandi, Sikkim local, Pusa rohini, Pusa Gaurav, FLA-7171 and Agata 30. The maximum genetic similarity within this subcluster was exhibited by Nandi which is resistant to leaf curl virus and Sikkim local which is tolerant to Bacterial wilt. The sub cluster 2 grouped 7 genotypes, viz. Castal rock, Tura local, IIHR-2200, H-24, IIHR-2201, KS-17 and Meghalaya local. The genotypes IIHR-2201 and H-24 showed the maximum genetic similarity 0.89. The genotypes Agata-30 and DVRT-1 showed significant genetic diversity with a coefficient value of 0.38. This showed that climatic conditions and physical parameters may affect the plant genome as the plant is adapted and these changes are inherited through genome to the next generation (Ilic Z S *et al.* 2013). The range of genetic diversity values broadly indicates the degree of heterogeneity or homogeneity in different genotypes of the plant species (Goswami and Ranade 1999). Cluster analysis clearly indicates that human intervention, which makes partitioning and distribution of variability complex is cited as reason for the grouping of samples to one cluster suitable for different regions (Kumar M *et al*. 2013, Thakaew U *et al.* 2011). The present study suggests that SSR is appropriate for analysis of genetic variability in closely related genotypes. Moreover, SSR could able to amplify the different loci of all the 24 genotypes.

CONLUSION

To access the genetic diversity of 24 genotypes of *S. lycopersicum*, 16 primers gave amplification and all resulted in polymorphic, scorable and reproducible results. The total of 16 primers amplified 57 alleles across the 24 genotypes. On the basis of resolving power, primer 8, 13 and 19 were most significant as they are able to recognize all 24 genotypes and so could be segregated on the basis of their ability to

Table 3 Pair wise similarity co-efficient matrix of 24 tomato genotypes

	Arka vikas	FLA-7421	PDT-3-1	TLBR-5	DVRT-1	Selection-7	Nandi	Pusa Rohini	Meghalaya Local	Sikkim Local	Pusa Gaurav	Arkha Abha	TLBR-3	Castal Rock	K.S-17	Money Maker	Palam Pink	IIHR-2200	IIHR-2201	H-24	H-86	FLA-7171	Tura Local	Agata-30
Arka Vikas	0.1																							
FLA-7421	0.61	1																						
PDT-3-1	0.68	0.75	1																					
TLBR -5	0.68	0.75	0.77	1																				
DVRT-1	0.63	0.75	0.72	0.72	1																			
Selection -7	0.63	0.61	0.59	0.77	0.5	1																		
Nandi	0.56	0.68	0.61	0.65	0.43	0.84	1																	
PusaRohini	0.59	0.7	0.63	0.72	0.54	0.81	0.84	1																
Meghalaya local	0.54	0.56	0.54	0.59	0.4	0.68	0.65	0.72	1															
Sikkim local	0.59	0.75	0.59	0.63	0.5	0.86	0.88	0.86	0.63	1														
PusaGaurav	0.63	0.65	0.63	0.77	0.54	0.81	0.79	0.86	0.59	0.81	1													
ArkhaAbha	0.61	0.59	0.61	0.56	0.56	0.61	0.63	0.56	0.47	0.65	0.7	1												
TLBR-3	0.63	0.65	0.63	0.72	0.63	0.77	0.61	0.63	0.63	0.68	0.68	0.61	1											
Castal Rock	0.5	0.61	0.54	0.63	0.5	0.72	0.65	0.81	0.68	0.77	0.68	0.47	0.63	1										
K.S-17	0.52	0.72	0.52	0.56	0.56	0.56	0.59	0.75	0.65	0.7	0.61	0.5	0.56	0.79	1									
Money Maker	0.59	0.65	0.72	0.77	0.59	0.68	0.61	0.54	0.5	0.65	0.66	0.7	0.77	0.59	0.52	1								
Palam Pink	0.59	0.65	0.72	0.72	0.72	0.5	0.47	0.59	0.54	0.54	0.63	0.61	0.59	0.63	0.7	0.77	1							
IIHR-2200	0.56	0.63	0.52	0.7	0.52	0.75	0.63	0.7	0.52	0.75	0.75	0.5	0.56	0.79	0.68	0.7	0.65	1						
IIHR-2201	0.65	0.63	0.56	0.7	0.52	0.75	0.63	0.7	0.52	0.7	0.75	0.59	0.61	0.7	0.63	0.75	0.61	0.86	1					
H-24	0.59	0.65	0.59	0.72	0.59	0.72	0.61	0.77	0.63	0.72	0.72	0.52	0.63	0.86	0.79	0.72	0.77	0.88	0.84	1				
H-86	0.56	0.54	0.61	0.61	0.61	0.61	0.59	0.75	0.52	0.61	0.79	0.72	0.56	0.61	0.54	0.52	0.56	0.59	0.59	0.65	1			
FLA-7171	0.5	0.65	0.63	0.63	0.4	0.77	0.88	0.81	0.63	0.81	0.77	0.61	0.59	0.68	0.61	0.59	0.5	0.65	0.61	0.63	0.65	1		
Tura Local	0.47	0.68	0.61	0.7	0.56	0.75	0.77	0.75	0.61	0.79	0.7	0.5	0.65	0.79	0.63	0.7	0.65	0.77	0.72	0.75	0.5	75	1	
Agata-30	0.56	0.63	0.52	0.52	0.38	0.75	0.81	0.75	0.65	0.88	0.7	0.54	0.56	0.7	0.63	0.56	0.47	0.68	0.63	0.65	0.5	0.7	0.72	1

diagnose genotypes. The estimating gene diversity was found most suitable for the purpose as they showed significant values. Cluster analysis clearly showed that some genotype is closely related while some as significantly distinct. The study did not show the separation of individuals on the basis of their response towards leaf curl virus infection. The statistical analysis was carried out the pair wise genetic similarities among all pairs of samples were estimated with Jaccard's coefficient. The genotypes IIHR-2201 and H-24 showed the maximum genetic similarity 0.89, and the genotypes Agata-30 and DVRT-1 showed significant genetic diversity with a coefficient value of 0.38. In order to group genotypes into discrete clusters a dendrogram was constructed by employing UPGMA method. The resultant dendrogram grouped the 24 genotypes into two main distinct clusters. The two genotypes, viz. Arkha Abha and H-86 did not grouped in any major cluster.

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