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GENETIC DIVERSITY ANALYSIS AND DNA FINGERPRINTING OF TOMATO BREEDING LINES USING SSR MARKERS

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ABSTRACT. There is a need to expand the information on genetic relationships between tomato (*Solanum lycopersicum* L.) lines to improve hybridization breeding. The genetic diversity and relationships among 24 tomato lines were evaluated by simple sequence repeat (SSR) markers. A total of 65 bands were generated with 15 SSR primers, of which 64 bands were polymorphic. The mean polymorphic information content was 0.356. There was a high degree of polymorphism between tomato cultivars. The mean marker index and heterozygosity were 0.045 and 0.454, respectively. Cluster analysis grouped cultivars into 6 main clusters. The cvs. Mo. H. P, 'C. C. Orange', and 'Marb' had the greatest genetic distance from other cultivars and is suitable for hybridization to achieve maximum variability for selection in segregating populations. The data can be used to select appropriate parents in tomato hybridization breeding.

Introduction

Various genetic bottlenecks resulting from self-pollination or artificial selection have occurred during the domestication of the cultivated tomato (*Solanum lycopersicum* L.), resulting in a loss of genetic diversity, especially within commercial cultivars (Foolad, Panthee, 2012).

Resistance, organoleptic properties, and variety have all been systematically favoured by domestication at the cost of high yield and efficiency. Extant diversity among tomato species may be a beneficial opportunity for enriching the genetic pool of planted tomatoes with marginalized alleles that could boost productivity and adaptability to challenges (Gur, Zamir 2004; Bai, 2017).

Despite attempts to improve tomato resistance by traditional and biotechnological breeding, findings have been insufficient due to the complexity of responses to different abiotic and biotic stresses. Landraces are diverse species of cultivated plants with specific eco-geographical backgrounds that have been adapted to local climatic conditions as well as conventional management and uses (Casanas *et al.*, 2017). Typically, landraces have evolved under natural and artificial selection in low-input agricultural systems and represent much of the lost diversity (Terzopoulos, Bebeli, 2010; Corrado *et al.*, 2014). Although pathogen resistance genes are usually absent from landraces, they may represent an important genetic diversity reservoir for traits like abiotic stress tolerance in plant breeding (Sacco *et al.*, 2015).

The phenotypic and molecular diversity of cultivated tomato has been investigated (Jin *et al.*, 2019; Kaur *et al.*, 2019). Parental lines for hybrid breeding are shown. Landraces' use in breeding is also limited by a lack of knowledge regarding phenotypic variation and genetic relationships between them, as well as high phenotyping costs (Corrado *et al.*, 2014). A subset of individuals reflecting the conserved diversity must be created in germplasm collections. Advances in whole-genome sequencing facilities, the abundance of several genomic databases, and the availability of a high-quality reference tomato genome sequence (The Tomato Genome Consortium, 2012), offer new possibilities for the development of highly informative molecular markers, overcoming some limitations associated with phenotypic selection. The low genetic diversity of cultivated tomatoes necessitates the use of modern molecular techniques for the discovery of markers able to detect minor variations within tomato germplasm (Foolad, Panthee, 2012).

Molecular markers have been used in tomato for the identification and characterization of numerous genes and Quantitative Trait Loci (QTL) linked to resistance to late blight, leaf mould or tomato spotted wilt virus (Kim *et al.*, 2017; Panthee *et al.*, 2017; Tseng *et al.*,

2016). The QTL analysis of fruit quality traits, including flavour and aroma (volatiles), firmness, vitamins (especially vitamin C and carotenoids) provide information into the genetic control of complex metabolic pathways that contribute to attributes for the market (Causse *et al.,* 2002; Sun *et al.,* 2012).

Several types of molecular markers, including simple sequence repeat (SSR; microsatellites), amplified fragment length polymorphism (AFLP), sequence characterized amplified region (SCAR), and single nucleotide polymorphism (SNP), has been developed and extensively used for the genetic characterization of tomato germplasm collections and in Marker Assisted Selection (MAS) (Bauchet *et al.,* 2017). SSR markers have long been popular due to their high reproducibility, codominance, and polymorphism; however, SNP markers are becoming more popular due to their cost-effectiveness, precision, and suitability for large-scale genotyping and allelic determinations through technologies including High-Resolution Melting (HRM). The value of SSR (Benor *et al.,* 2008; Mazzucato *et al.,* 2008; Sardaro *et al.,* 2013) and SNP (Sacco *et al.,* 2015; Wang *et al.,* 2016) markers in the study of Solanum genetic variation and the genotyping of promising germplasm have been confirmed.

This study was undertaken to assess genetic diversity in 24 breeding lines of tomato using SSR markers to assist in parental selection for hybridization and to avoid the genetic similarities between hybrid pedigrees in future genetic improvement programs for tomato.

Material and methods

Seed of tomato (*Solanum lycopersicum* L.) breeding lines: 'Rose', 'S. G', 'Wis 55', 'Tas. Ch', 'Bran. 21', 'Glacier', 'Red P.t', 'San II', 'A. Pas.', 'German J.', 'Mo.', 'Nepal', 'Red Pear', 'Amish Pa.', 'Pi. Bee', 'B. B.', 'Fr.', 'Mo. H. P', 'C. C. Orange', 'Marb', 'T100S', 'T120S', T125S', T150S', were provided by the Tomato Genetics Resource Center (TGRC) of the University of California, Davis, USA (http://tgrc.ucdavis.edu). The experiment was conducted in a greenhouse at the horticultural gardens of the Department of Horticulture and Landscape Gardening, College of Agriculture, Diyala of University, Baqubah, Iraq. Ten seed/line were directly sown and germinated in 1 L plastic pots, on 10 January 2020, containing a growth medium composed of 45–50% composted pine bark, vermiculite, Canadian sphagnum, peat moss, perlite, and dolomitic limestone.

Plants were thinned to 5 per pot after emergence. Greenhouse temperature was 20–29 °C with a relative humidity of 75–90%. Light intensity was about 9678 lux. Pots were irrigated once every 2 days with 500 mL of distilled water. Each variety was replicated 3 times. No pesticides or additional fertilizer were used during the experiment.

Four weeks after emergence, leaf tissues were sampled from each plant and used to extract genomic DNA for molecular analyses. Genomic DNA extraction from leaves was according to a modified cetyl trimethyl ammonium bromide method (Hwang, Kim, 2000). About 0.5 g of fresh young leaves were powdered in liquid nitrogen. The leaf powder was transferred to a tube containing 0.6 mL of extraction buffer containing 1% of β-mercaptoethanol added just before use. The extract was incubated for 40 min at 60 °C with occasional swirling, mixed with an equal volume of chloroform: isoamyl alcohol (24:1; v:v), and centrifuged at 16,128 relative centrifugal force (rcf) for 10 min at 4 °C. The aqueous phase was transferred to a new tube and mixed with 2/3 volume of ice-cold isopropanol. The mixture was left at –20 °C for 30 min and again centrifuged at 16,128 rcf for 10 min at 4 °C. The pellet was washed with 70% ethanol and air-dried at room temperature for 20 min; the dried pellet was dissolved in 80 µL TrisEDTA (TE) buffer (Tris–hydrochloride buffer, pH 8.0, containing 1.0 mM ethylene diamine tetraacetic acid (EDTA) and stored at –20 °C.

The quality of total DNA was determined with 2% agarose gel electrophoresis and quantified by spectrophotometry. The concentration of extracted DNA for PCR was adjusted to 50 $ng \cdot \mu L^{-1}$. The PCR reaction for tomato lines was conducted using 15 SSR primers (Table 1). Amplification was carried out in 12.5 µL of reaction mix containing 1.5 µL of genomic DNA (50 $ng \cdot \mu L^{-1}$), 0.6 µL of the primer (10 µM), 1.25 µL of 10× reaction buffer, 1 unit of Taq DNA polymerase (5 $U \cdot \mu L^{-1}$), 0.25 mM of each dNTP, and 2.5 mM $MgCl_2$.

The PCR reactions were conducted in a thermal cycler, model AG (Eppendorf, Germany). Amplifications were conducted with an initial denaturation of 95 °C for 3 min, followed by 40 cycles of denaturation at 95 °C for 1 min, annealing at 35 °C for 1 min, and extension at 72 °C for 1 min; followed by 1 cycle of a final extension at 72 °C for 10 mins. The PCR products were separated by electrophoresis on 2% agarose gel. Gene ruler 100 bp DNA ladder plus SM0321 (Fermentas, Lithuania) was used as the standard to determine the size of polymorphic fragments. The DNA fragments were visualized by staining the gel with ethidium bromide and images documented using Gel Doc (Vilber Lourmat, France).

Group method with arithmetic averages (UPGMA). Combined analysis was performed using the dendrogram and Jaccard's coefficient using NTSYS software (Rohlf, 1998). Polymorphism Information Content (PIC) was calculated according to Roldan *et al.* (2000); PIC refers to the value of a marker for detecting polymorphism within a population. Depending on the number of detectable alleles, and distribution of their frequency, it provides an estimate of the discriminating power of the marker.

The SSR polymorphisms in the tomato accessions were measured in terms of numbers of alleles, gene diversity, and PIC using the Power Marker software ver. 3.23 (Liu, Muse, 2005).

Table 1. Primers used with annealing temperature and amplicon band size

Marker identifier	Primer sequence (5' → 3')	Annealing temperature (°C)	Amplicon band size
SSR47	TCC TCA AGA AAT GAA GCTCTG A CCT TGG AGA TAA CAA CCA CAA	58.4	200–1000
SSR63	CCA CAA ACA ATT CCA TCT CA GCT TCC GCC ATA CTG ATA CG	54.3	200–1200
SSR111	TTC TTC CCT TCC ATC AGT TCT TTT GCT GCT ATA CTG CTG ACA	57.4	200–1000
SSR248	GCA TTC GCT GTA GCT CGT TT GGG AGC TTC ATC ATA GTA ACG	59.4	150–700
SSR304	TCC TCC GGT TGT TAC TCC AC TTA GCA CTT CCA CCG ATT CC	60.5	125–500
SSR603	GAA GGG ACA ATT CAC AGA GTT TG CCT TCA ACT TCA CCA CCA CC	61.1	150–700
T-57	GTG GAC CAT TTC AAG TTC AAC A TGA ATG ACA TCC ATC CAT GA	58.4	125–800
TG12-13	GAA AGA GGT AA ATC GCG GGT CCT TTA CGA TTT CGC CTA CG	59.4	200–300
SLM-6-7	CAA TTG AAG ATT GGG GCT TT AGC AGC TCA CCT CAC GTT TT	54.3	225–1000
STI-0012	GAA GCG ACT TCC AAA ATC AGA AAA GGG AGG AAT AGA AAC CAA AA	57.4	200–1200
TMS-42	AGA ATT TTT TCA TGA AAT TGT CC TAT TGC GTT CCA CTC CCT CT	54.0	100–450
TMS-9	TTG GTA ATT TAT GTT CGG GA TTG AGC CAA TTG ATT AAT AAG TT	54.0	125–1000
AI486387.1	ACG CTT GGC TGC CTC GGA AAC TTT ATT ATT GCC ACG TAG TCA TGA	60.7	300–400
STI003	ACC AAT CCA CCA TGT CAA TGC CTC ATG GAT GGT GTC ATT GG	58.4	125–300
Le-tat002	ACG CTT GGC TGC CTC GGA AAC TTT ATT ATT GCC ACG TAG TCA TGA	62.2	100–1000

Results

The PCR amplification using SSR primers resulted in the generation of reproducible amplification products.

Analysis with 9 SSR primers identified a total of 65 reproducible fragments in the tomato cultivars (Table 2). Most bands were produced by SSR63 and the lowest number of bands was obtained by TG 12–13, and A1486387.1.

The numbers of polymorphic bands ranged from 1 for TG 12–13 to 7 for SSR63. Most SSR primers produced 100% polymorphism, the exception was for TG12-13 primers (50%). Of the 15 SSR markers, the overall PIC value ranged from 0.290889 (TG12-13) to 0.497149 (AI486387.1) with an average of 0.3561703. A higher marker index value occurred for AI486387.1 (0.55875) compared to SSR47 (0.003549). The marker index is a feature of a marker that elucidates the discriminatory power of a marker and was calculated for all the markers.

The maximum heterozygosity values of the AI486387.1 codominant marker were 0.55875 in comparison to SSR47 (0.360725).

The genetic similarity matrix, based on Jaccard's similarity coefficients, indicated that the cultivars were genetically similar (Table 3). The highest genetic similarity was related to the 'C. C. Orange' vs. 'Wis 55' or 'San II' or 'Glacier', and 'Mo. H. P' vs. 'San II' and 'Glacier', followed by 'C. C. Orange', vs. 'S. G', and 'C. C. Orange' vs. 'Tas. Ch'. The lowest similarity was for 'San II' vs. 'Red P.t' followed by 'Mo.' vs. 'German J.' (Table 3). In hybridization, crossing cultivars with greater genetic distances are expected to produce more heterosis and desirable recombinants in segregating generations. The average genetic similarity (Table 3) indicated the existence of high levels of diversity among genotypes.

Table 2. Data on primer polymorphism in the diversity of tomato genotypes

Marker identifier	No. of bands	No. of polymorphic bands	Polymorphism %	Polymorphic information content	Marker index	Heterozygosity
SSR47	3	3	100	0.295664	0.003549	0.360725
SSR63	7	7	100	0.336086	0.005513	0.427438
SSR111	5	5	100	0.351488	0.006635	0.455000
SSR248	4	4	100	0.361526	0.012131	0.473741
SSR304	4	4	100	0.366056	0.011935	0.482422
SSR603	4	4	100	0.369627	0.011682	0.489366
T-57	5	5	100	0.373258	0.009482	0.496528
TG12-13	2	1	50	0.290889	0.011347	0.353299
SLM-6-7	5	5	100	0.323648	0.004794	0.406111
STI-0012	6	6	100	0.362349	0.007702	0.475309
TMS-42	4	4	100	0.361526	0.012131	0.473741
TMS-9	5	5	100	0.374374	0.008971	0.498750
AI486387.1	2	2	100	0.497149	0.558750	0.558750
STI003	3	3	100	0.304688	0.011719	0.375000
Le-tat002	6	6	100	0.374227	0.009808	0.498457

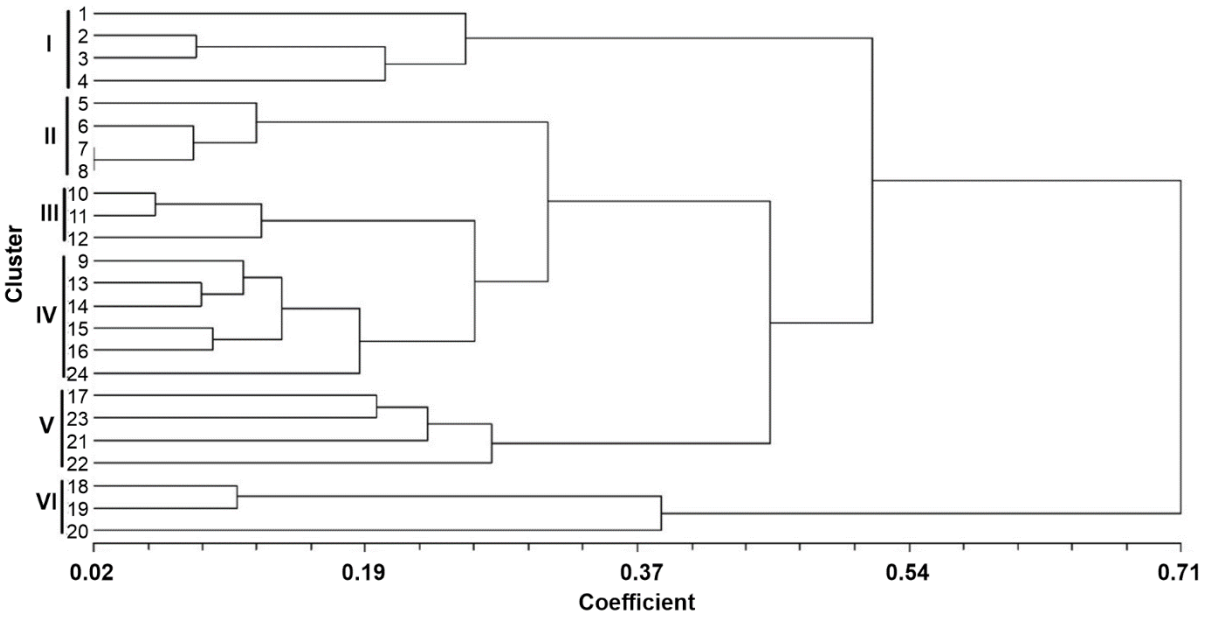
Cluster analysis, based on similarity matrix coefficients using UPGMA, grouped the cultivars into 6 main clusters (Fig. 1). According to the cluster, cvs. 'Rose', 'S. G', 'Wis 55' and 'Tas. Ch' were placed in the same group and cvs. 'Bran. 21', 'Glacier', 'Red P.t' and 'San II' were placed in another group (Fig. 1). The cvs. 'German J.', 'Mo.' and 'Nepal' were placed alone in a separate category and cvs. 'A. Pas.', 'Red Pear', 'Amish Pa.', 'Pi. Bee', 'B. B.', and 'T150S' were placed in

another group. The cvs. 'Fr., T125S', 'T100S', and 'T120S' were placed in the fifth group, and cvs. 'Mo. H. P', 'C. C. Orange', and 'Marb' were placed in a sixth group. Divergent genotypes may have good breeding value. Genotypes in the same cluster may represent members of a single heterotic group.

Table 3. Similarity coefficient matrix of tomato cultivars using SSR markers

Variety	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
2	0.2307																						
3	0.2571	0.0847																					
4	0.2812	0.1698	0.2413																				
5	0.2584	0.3589	0.2771	0.3246																			
6	0.3571	0.4520	0.3589	0.4166	0.1134																		
7	0.2727	0.4026	0.3170	0.3947	0.1287	0.0833																	
8	0.2954	0.4026	0.3170	0.3947	0.1287	0.0833	0.02																
9	0.3580	0.5714	0.5466	0.5652	0.2766	0.2584	0.2258	0.2258															
10	0.5873	0.7692	0.7193	0.8039	0.3947	0.3802	0.4133	0.4133	0.2941														
11	0.5937	0.7735	0.7241	0.8076	0.3766	0.3888	0.3947	0.4210	0.3043	0.0588													
12	0.5625	0.7358	0.6896	0.7692	0.4026	0.3888	0.3684	0.3947	0.2463	0.1372	0.1153												
13	0.3589	0.5522	0.5277	0.5757	0.2527	0.2558	0.2000	0.2000	0.1084	0.2307	0.2121	0.2121											
14	0.3246	0.5151	0.5211	0.5076	0.2666	0.2941	0.2359	0.2359	0.1219	0.3437	0.3230	0.3230	0.0886										
15	0.3600	0.5625	0.5362	0.5873	0.3181	0.3253	0.2413	0.2643	0.1750	0.2580	0.2381	0.2381	0.1168	0.1315									
16	0.4594	0.6190	0.5882	0.6451	0.3103	0.3170	0.3023	0.3023	0.1645	0.2131	0.1935	0.1935	0.1052	0.1466	0.0958								
17	0.4386	0.5652	0.6078	0.5555	0.5428	0.5692	0.4782	0.5072	0.4193	0.4545	0.4666	0.4222	0.3559	0.3448	0.3214	0.3454							
18	0.7674	0.8125	0.8378	0.8064	0.8214	0.8823	0.8181	0.8545	0.7916	0.7333	0.7419	0.6774	0.7777	0.7727	0.7619	0.7561	0.5833						
19	0.8095	0.8709	0.8888	0.8666	0.8545	0.8800	0.8518	0.8888	0.8297	0.7241	0.7333	0.7333	0.8181	0.8139	0.8048	0.8000	0.6521	0.1111					
20	0.5833	0.7297	0.7619	0.7222	0.6721	0.7142	0.6666	0.7000	0.6603	0.5428	0.5555	0.5000	0.6000	0.5918	0.5744	0.5652	0.4482	0.3333	0.4285				
21	0.4098	0.5200	0.5636	0.5102	0.4594	0.4202	0.3972	0.4246	0.3636	0.3750	0.3877	0.3877	0.2698	0.2903	0.3333	0.3220	0.2381	0.7142	0.7037	0.4545			
22	0.5294	0.5500	0.6000	0.5384	0.6562	0.6949	0.6190	0.6507	0.5714	0.6315	0.6410	0.5384	0.5094	0.5000	0.4800	0.4693	0.2500	0.4444	0.5294	0.3913	0.3333		
23	0.5254	0.5416	0.5849	0.5319	0.5555	0.5522	0.4929	0.5211	0.4375	0.4782	0.4893	0.4468	0.3770	0.4000	0.3448	0.2982	0.2000	0.6153	0.6800	0.5483	0.2272	0.2352	
24	0.3600	0.5625	0.5652	0.5555	0.2954	0.3012	0.2413	0.2413	0.2250	0.2903	0.3015	0.3015	0.1428	0.1842	0.1891	0.2054	0.3214	0.7619	0.8048	0.5744	0.3000	0.4800	0.3103

1 = 'Rose', 2 = 'S. G', 3 = 'Wis 55', 4 = 'Tas. Ch', 5 = 'Bran. 21', 6 = 'Glacier', 7 = 'Red P.t', 8 = 'San II', 9 = 'A. Pas', 10 = 'German J', 11 = 'Mo.', 12 = 'Nepal', 13 = 'Red Pear', 14 = 'Amish Pa', 15 = 'Pi. Bee', 16 = 'B. B.', 17 = 'Fr.', 18 = 'Mo. H. P', 19 = 'C. C. Orange', 20 = 'Marb', 21 = 'T100S', 22 = 'T120S', 23 = 'T125S' and 24 = 'T150S'.



Numbers 1 to 24 are 'Rose', 'S. G', 'Wis 55', 'Tas. Ch', 'Bran. 21', 'Glacier', 'Red P.t', 'San II', 'A. Pas.', 'German J.', 'Mo.', 'Nepal', 'Red Pear', 'Amish Pa.', 'Pi. Bee', 'B. B.', 'Fr.', 'Mo. H. P', 'C. C. Orange', 'Marb', 'T100S', 'T120S', 'T125S' and 'T150S' respectively.

Figure 1. Cluster analysis of tomato cultivars using SSR data

Discussion

Sufficient knowledge from the genetic diversity of a crop for the selection of parental materials is essential to maximize genetic improvement. More accurate, and complete, descriptions of genotypes, and genetic diversity patterns, can help determine breeding strategies and facilitate the introgression of diverse germplasm into the current commercial tomato genetic base (Tsivelikas *et al*., 2009).

Molecular markers, which assess genome sequence composition, enable the detection of differences in the genetic information of genotypes and utilize genetic variability for breeding.

The SSRs have been employed to assess genetic diversity within germplasm. In self-pollinating species such as tomato, genetic diversity mainly depends on domestication history and pool size of accessions (Mazzucato *et al.*, 2008). Tomato is generally considered to present low genetic diversity. Landraces

and local populations of tomatoes are thought to have more genetic and phenotypic diversity than commercial cultivars (Park *et al.*, 2004; Mazzucato *et al.*, 2008).

Exploring genetic diversity among tomato accessions is important to breeding and germplasm management (Kanjariya *et al.*, 2017; Gonias *et al.*, 2019). To investigate genetic relationships between the 24 tomato accessions (modern cultivars), 15 SSR loci were selected that have been previously reported to be highly informative in distinguishing tomato genotypes.

Although several different tomato landraces are grown, in the Middle East, exhibiting phenotypic variation, few experiments have been conducted to genetically classify the collections or differentiate them from commercial cultivars. Iraq tomato landraces are believed to have grown in semi-arid environments with low inputs, which may be useful genetic material in productive agricultural systems. Emerging evidence suggests that some of these landraces have high nutritional value. These microsatellites have been verified to be highly polymorphic and able to discriminate different patterns.

The efficiency of a molecular marker system in distinguishing genotypes depends largely upon the polymorphism it can discover (Castellana *et al.*, 2020; Gbadamosi *et al.*, 2020). Based on high polymorphism information content (PIC) values, marker index, and the number of bands (Table 2) the SSR markers used were informative in the assessment of the genetic diversity of tomato accessions. The high PIC values indicate all primers were informative and can be related to high genetic variation among accessions, with similar results previously reported for tomato (Ronga *et al.*, 2018). The variation may have been contributed by gene flow, natural hybridization, propagation by seed and human selection (Choudhary *et al.*, 2018; Gbadamosi *et al.*, 2020).

The heterozygosity and marker index measurements display the distribution and number of alleles (bands) within the genotypes. Bands scored in most genotypes would possess optimal discriminatory power, and with an increase in the number of bands, the heterozygosity of a particular primer pair will be increased (Mazzucato *et al.*, 2008; Ronga *et al.*, 2018; Castellana *et al.*, 2020). Primers with the highest PIC, marker index, and heterozygosity values (AI486387.1) were generally most effective in distinguishing between accessions and could be further used in genetic diversity studies.

Considering time and cost savings, the SSR can differentiate and characterize cultivars useful in tomato breeding. Depending on objectives, potential lines to be selected from different clusters as parents in a hybridization program may be based on genetic distance. The clustering pattern can be used for parent selection for cross-combinations likely to generate the highest possible variability for economic characters.

Conclusions

The highly polymorphic nature of the SSRs used in this analysis was shown, and the existence of a high degree of genetic variation among tomato cultivars was clearly shown.

The discovery of genetic similarities between tomato cultivars makes for more effective germplasm management and use. The results of the SSR analysis showed that each genotype could be distinguished from the others, that the primers were appropriate for tomato germplasm evaluation, and that the SSR marker method was reliable and efficient for identifying tomato cultivars and clonal identification.

The use of well-known divergent genotypes as crossing parents could boost the amount of diversity in a segregating population, which could be beneficial in a tomato development programme.

In the current climate-change scenario, which threatens tomato development, the studied materials could serve as a possible source of genes responsible for widespread adaptation. It may also mean that the observed variety could be exploited by developing a strong crossing programme to produce hybrid cultivars that combine high yield, efficiency, and climate change resilience. The current fingerprint data may be used to build a DNA reference database for the molecular identification of the cultivars in the future breeding program.

Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Author contributions

GH – writing a manuscript;
GH, MA – acquisition of data;
GH, AA, MA, NM – analysis and interpretation of data;
GH, MA, AA – critical revision and approve the final manuscript

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