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Genetic diversity analysis using SSR markers for high temperature tolerance in tomato (*Solanum lycopersicum* L.)

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Abstract

Molecular characterization of selected 22 tomato genotypes (*Solanum lycopersicum* L.) under high temperature stress condition was assessed using 25 simple sequence repeat (SSR) markers. Out of the 25 markers used 22 primers showed amplification, 15 SSRs were monomorphic and 7 were polymorphic. Polymorphism information content (PIC) value of the markers ranged from 0.58 to 0.65 with an average value of 0.6. The highest PIC value was shown by SSR 96(0.65). Phylogenetic tree was constructed based on Jaccard's similarity coefficient using the unweighted pair-group method with arithmetic mean (UPGMA) using NTSYSpc cluster analysis software. Cluster analysis divided the twenty-two genotypes into four clusters. SSR marker analysis and cluster analysis were able to differentiate the genotypes into; tolerant, moderately tolerant, susceptible and genotypes which show mixed characteristics. Genetic maps currently available in tomatoes have a limited number of SSR markers that are not uniformly distributed across the genome, so this study helps our fundamental understanding in identification of molecular markers linked to high temperature tolerance in tomato.

Keywords: Cluster analysis, molecular markers, polymorphism information content, phylogenetic tree, climate change

Introduction

Changing climate and growing food demand by increasing population have become two of the major challenges faced by humans in the last few years (Amrutha and Beena 2020). High temperature is a major abiotic stress affecting the growth, development, productivity and yield stability of several crops. The predicted increase of temperature causes a large threat for crop productivity and necessitates the importance of developing strategies to substantially progress food accessibility (Beena 2013). This is an outcome of the alteration of a number of basic molecular and physiological processes, such as protein folding, maintenance of membrane stability, photosynthesis and assimilate metabolism (Bokszczanin 2013). Although plants possess various strategies to make certain survival under elevated temperatures, even a small increase in temperature (1.5°C) can have a considerable impact on various stages of reproductive growth and negatively affecting fruit set and crop yield (Warland et al. 2006). Impact of high temperature stress on reproductive and grain filling stage was studied in rice by Beena et al. (2018).

Tomato (*Solanum lycopersicum* L.) is a widely grown vegetable crop belonging to the *Solanaceae* family. Tomatoes have a good potential to grow in both tropical and temperate regions of every part of the world. Although tomatoes have a good potential to grow under diverse environmental conditions, they experience numerous abiotic stresses in which high temperatures stress seems to be a crucial problem nowadays (Somraj et al. 2017). But high temperatures can negatively affect the vegetative and reproductive growth phases, resulting in up to 70% tomato harvest losses (Sato et al. 2004). For normal growth and fruit set in tomato, optimum day and night temperatures range from 20-24°C and 15-20°C, respectively (Amrutha and Beena 2020). There is an increase of 1-4°C in global mean temperature by the end of the 21st century (Driedonks et al. 2016). A temperature above this threshold can lead to serious deleterious effects such as flower abscission, decrease of pollen quality, abnormal growth and reduced fruit set. Tomato plants when exposed to a long heat stress with an average temperature of 34°C/19°C shows flower drop of 34% and decrease of fruit set upto 71% (Hazra et al. 2009). Since the response to heat stress is a very complex genetic trait, molecular markers targeting quantitative

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trait loci (QTLs) and genes involved in the heat tolerance might aid the selection of genotypes tolerant to high temperatures (Ruggieri et al. 2019).

Molecular markers are used to determine gene diversity in germplasm collections, and to classify varieties within the population. Genetic diversity analysis is the primary step to identify molecular markers linked to abiotic stress tolerance. It is often used to efficiently determine appropriate agronomic characteristics because they are based on plant genotypes and are thus independent of environmental fluctuations (He et al. 2003). Among the various available markers, the most widely used types are simple sequence repeats (SSRs) or microsatellites, SSRs are seen as an important tool for evaluating genetic diversity and characterizing germplasm due to their reproducibility, co-dominance in nature and adequate coverage of genomes (Zhou et al. 2015). Their high polymorphism, frequency and random distribution in the genome make microsatellites useful genetic markers for mapping and population and evolutionary studies, especially in species with low DNA variation rates. A preliminary study was conducted on the effect of high temperature on physiological, biochemical and yield parameters in 22 tomato accessions collected from NBPGR, Thrissur (Amrutha 2020). Keeping in view the above discussed facts, this study focused on the identification of molecular markers linked to high temperature tolerance in tomato using the previous data (Amrutha 2020) and to understand genetic variation among tomato genotypes using NTSYSpc software.

Materials and Methods

Plant materials: This research work was carried out in the Department of Plant Physiology, College of Agriculture, Vellayani, Kerala Agricultural University. The study includes 22 genetically diverse genotypes of tomato which were collected from NBPGR, Thrissur, India (Table 1).

Extraction of genomic DNA and SSR analysis: DNA was extracted from the young and healthy leaves by CTAB method (Murray and Thompson 1980). The genomic DNA isolated from 22 tomato varieties were analyzed and confirmed by agarose gel electrophoresis. The quality and quantity of DNA was determined. A set of 25 SSR markers were used to screen the genotypes (Table 2). The markers associated with high temperature are; SSR-270, SSR-75, SSR-134, SSR-356, SSR-605, SSR-13, SSR-115, SSR 13, SSR 115, SSR 47, SSR 276, SSR 304, SSR 63, SSR 19, SSR 293, SSR 96 Wen et al. (2019) and their sequences were obtained from the Sol Genomics Network (SGN, http://solgenomics.net/) database. PCR reaction was

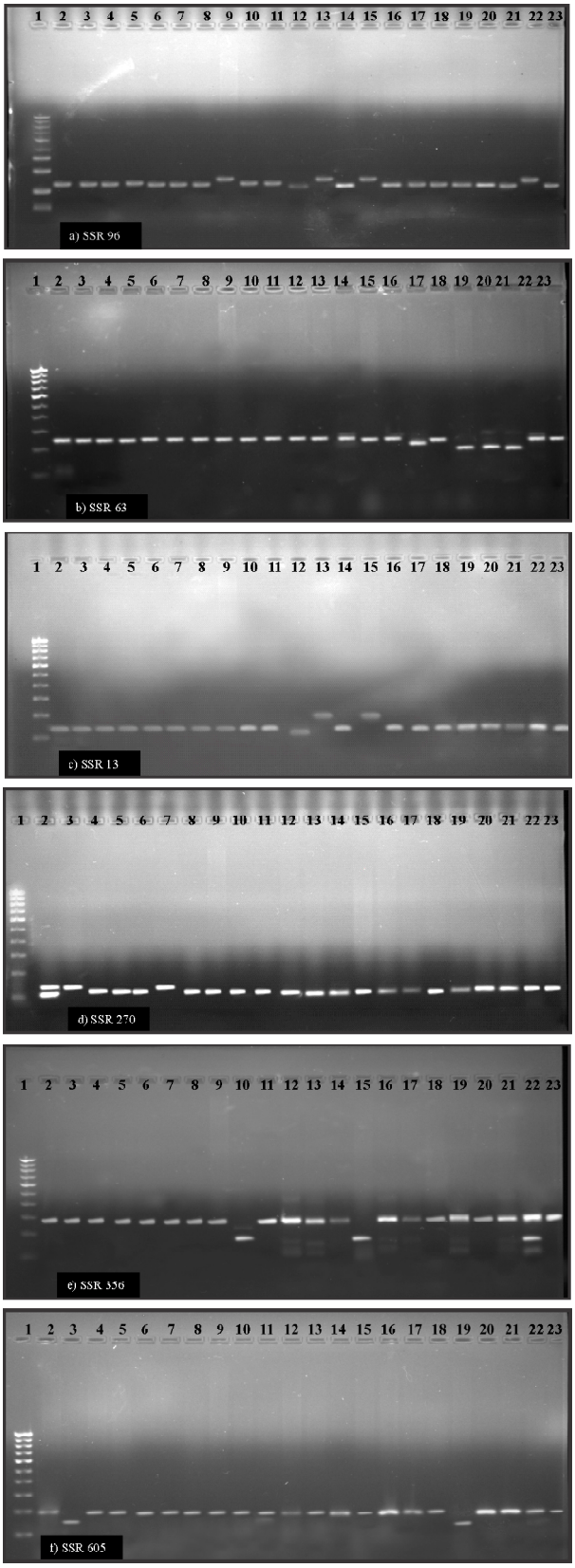

Figure 1: Amplification profile of 22 genotypes with a) SSR 96, b) SSR 63, c) SSR 13, d) SSR 270, e) SSR 356, f)SSR 605. 1-Marker ladder (100bp). Lane 2-23 correspond to tomato genotypes in the same order of Table 1.

Table 1: List of tomato genotypes along with their source.

Tomato genotype	Source
Manuprabha	Kerala Agricultural University (KAU), Thrissur
Akshaya	KAU
Pusa Ruby	Indian Agricultural Research Institute (IARI), Delhi
IC 45	Indian Institute of Horticultural Research (IIHR), Bengaluru
Nandi	University of Agricultural Sciences (UAS),Bangalore
IIHR 2200	IIHR
IIHR 26372	IIHR
Palam Pride	Himachal Pradesh Agricultural University(HPAU), Palampu
PKM 1	Tamil Nadu Agricultural University (TNAU), Tamil Nadu
Manulakshmi	KAU
Arka Sambrat	IIHR
Arka Rakshak	IIHR
Arka Vikas	IIHR
Pusa Rohini	IARI
Arka Alok	IIHR
Sakthi	KAU
Vaibhav	Asian Vegetable Research and Development Center (AVRDC), Hyderabad
Vellayani Vijay	KAU
Anagha	KAU
Kashi Vishesh	ICAR- Indian Institute of Vegetable Research (ICAR-IIVR), Uttar Pradesh
Arka Saurabh	IIHR
Arka Abha	IIHR

performed in a 20µl reaction mixture which consisted of genomic DNA (25ng/µl) - 2.0µl, 10X Taq assay buffer A - 2.0µl, dNTPs mix (10mm each) - 1.5µl, Taq DNA polymerase (1U) - 0.3µl, Forward primer (10pM) - 0.75µl., Reverse primer (10pM) - 0.75µl, Autoclaved distilled water - 12.7µl**.** PCR reaction was carried out using Master Cycler gradient 5331-Eppendorf version 2.30.31-09, Germany. The thermal cycling was carried out with the following programme; Initial denaturation – 94°C for 3 minutes, Denaturation – 94°C for 1 minute, Primer annealing – 53°C to 55°C for 1 minute, Primer extension – 72°C for 1 minute, Final extention – 72°C for 5 minute, Incubation – 4°C for infinity to hold the sample. The PCR products were separated on agarose gel. The documented SSR profiles were carefully examined for the polymorphism in banding pattern between the tomato genotypes.

PIC value and cluster analysis: The sizes of the SSR alleles were determined by band position relative to the DNA ladder. The total number of alleles for each microsatellite marker was reported in all the genotypes under analysis, by giving the number to amplified alleles as 0 for absence and 1 for allele presence. Polymorphic information content values of primers were calculated using the formula:

Table 2: List of primers along with their sequence, chromosome number and expected product size

S. No	Chro-mosome	Primer	Sequence	Expected product size
1	1	SSR 134	F: CCCTCTTGCC TAAACATCCA R:CGTTGCGAATTCAGATT AGTTG	171
2	2	SSR75	F:CCATCTATTATCTTCTCT CCAACAC R:GGTCCCAACTCGGTACA CAC	155
3	2	SSR 356	F:ACCATCGAGGCTGCATA AAG R:AACCATCCACTGCCTCA ATC	259
4	2	SSR 605	F:TGGCCGGCTTCTAGAAAT AA R:TGAAATCACCCGTGACC TTT	196
5	1	SSR 270	F:AGCTCAAGGCTTCTGTTG GA R:AACCACCTCAGGCACTT CAT	231
6	2	SSR 96	F:GGGTTATCAATGATGCA ATGG R:CCTTTATGTCAGCCGGTG TT	222
7	6	SSR 47	F:TCCTCAAGAAATGAAGC TCTGA R:CCTTGGAGATAACAACC ACAA	191
8	7	SSR 276	F:CTCCGGCAAGAGTGAAC ATT R:CGACGGAGTACTTCGCA TTT	148
9	7	SSR 304	F:TCCTCCGGTTGTTACTCC AC R:TTAGCACTTCCACCGATT CC	186
10	8	SSR 63	F:CCACAAACAATTCCATCT CA R:GCTTCCGCCATACTGATA CG	250
11	10	SSR 4	F:TTCTTCGGAGACGAAGG GTA R: CCTTCAATCCTCC AGATCCA	166
12	5	SSR 13	F:GGGTCACATACACTCAT ACTAAGGA R:CAAATCGCGACATGTGT AAGA	104
13	5	SSR 115	F:CACCCTTTATTCAGATTC CTCT R:ATTGAGGGTATGCAACA GCC	211
14	9	SSR 19	F:CCGTTACCTTGGTCCATC AC R:GGGAGATGCCACATCAC ATA	188
15	4	SSR 293	F:GCAAAGAGCTCGATCTC CAA R:TTCAGTTACTGGCCTTCG CT	129

16	10	SSR 248	F:GCATTCGCTGTAGCTCGTTT R:GGGAGCTTCATCATAGTAACG	249
17	12	SSR 124	F:TCAATCCATCACACCTTGGA R: GAGGAAGAAGACCACGCAAA	131
18	9	SSR 70	F:TTTAGGGTGTCTGTGGGTCC R:GGAGTGCGCAGAGGATAGAG	120
19	3	SSR 111	F:TTCTTCCCTTCCATCAGTTCT R:TTTGCTGCTATACTGCTGACA	188
20	12	SSR 20	F:GAGGACGACAACAACAACGA R:GACATGCCACTTAGATCCACAA	157
21	5	SSR 602	F:GGGTCACATACACTCATACTAAGGA R:GGCAATCATAGCCACTTGGT	299
22	4	SSR 450	F:AATGAAGAACCATTCCGCAC R:ACATGAGCCCAATGAACCTC	265
23	1	SSR 341	F:TTCTCTGTGGGTGGCAAT R:AAGCCCCGAATCTGGTAGC	292
24	2	SSR 331	F:CGCCTATCGATACCACCACT R:ATGATCCGTTGGTTCGC	178
25	11	SSR80	F: GGCAAATGTCAAAGGATTGG R: AGGGTCATGTTCTTGATTGTCA	180

$$PIC = 1 - \sum (Pi)2$$

P_i depicts the proportion of samples carrying the i^{th} allele.

The binary data generated for all the varieties for the polymorphic markers was entered in the NTedit program of NTSYSpc version 2.10 software. Using the SHAN module for cluster analysis the similarity matrix was used to produce dendrogram. Clustering was done by UPGMA using NTSYS-pc version 2.10 software. The individuals are only clustered in the software, screening and selection are not possible. Perhaps the most common use of NTSYSpc is to perform different types of agglomerative cluster analysis of some kind of matrix of similarity or dissimilarity (Rohlf 1998).

Results and Discussion

Molecular characterization of twenty-two tomato genotypes were done using twenty five SSR primers. The results were discussed under the following headings:

Polymorphism by SSR markers: In the present study agarose gels containing PCR products of SSR primers were carefully visualized for analysing variations among 22 genotypes. The DNA was good in quality and quantity, free from proteins and RNA contamination. Out of the 25 primers, 3 primers SSR 80, SSR 331, SSR 341 didn't show any amplification hence not used for further analysis. Out of twenty two, fifteen SSR markers, SSR450, SSR 602, SSR20, SSR111, SSR70, SSR 124, SSR 293, SSR 19, SSR115, SSR 304, SSR 276, SSR 47, SSR 75, SSR 134 and SSR-4 amplified and shown monomorphic banding patterns, hence they were not considered for further analysis. Seven markers were used for final analysis. The polymorphic markers for temperature tolerance were SSR 96, showed polymorphic band with size ~ 222bp, SSR 63 with polymorphic bands of size ~ 250bp, SSR 13 with polymorphic bands of size ~ 104 bp, SSR 270 with polymorphic bands of size ~ 231bp, SSR 356 with polymorphic bands of size ~ 259bp, SSR 605 with polymorphic bands of size ~ 196 bp (Figure 1 a, b, c, d, e, f).

Almost similar product size was given by Frary et al. (2015) for SSR 96 (~ 222bp), SSR 605 (~ 196bp), SSR 111 (~188bp), SSR 450 (~ 265bp), SSR 70 (~ 120bp), SSR 13 (~ 104bp), SSR 47 (~ 191bp), SSR 248(~ 249bp), SSR 124 (~ 131bp), SSR 4(~ 166bp), SSR 19 (~ 188bp), SSR 270 (~ 231bp). Wen et al. (2019) conducted a study in 516 tomato genotypes using SSR markers and stated that 146 were polymorphic between the two parental lines with 28.25% polymorphism. Quantitative trait locus (QTL) for heat tolerance; qCC-2-2 was identified on chromosome 2 with SSR96 flanking marker by traditional QTL analysis and identified PSII (photosystem II) maximum photochemical quantitative efficiency (Fv/Fm) was linked to two QTLs on chromosomes 5 and 12 with SSR13 as flanking marker. In this study, SSR 13 was identified as a polymorphic marker with a maximum of five alleles amplified. It was also confirmed by (Dhaliwal et al. 2011) in a study using thirty genetic tomato stocks and analysed the genetic diversity and DNA fingerprinting using SSR markers. A total of 60 alleles were amplified in 30 genotypes, with an average of 2.8 alleles per locus. In the present study distinct polymorphism for high temperature tolerance between high temperature tolerant and susceptible varieties was shown by SSR 63 and SSR 96.

Polymorphism information content: The degree of polymorphism is usually calculated in quantitative terms by two distinct quantities, one is called heterozygousness, and its unbiased estimator and

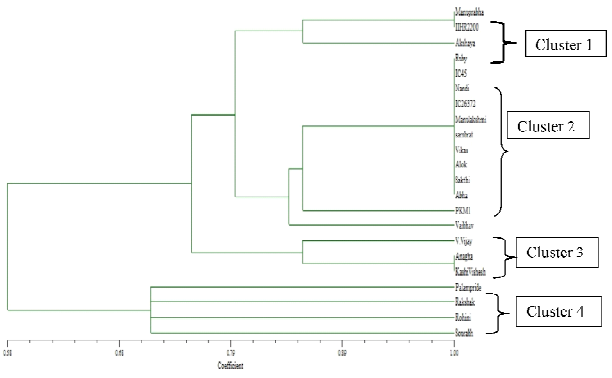

Figure 2: Dendrogram showing the genetic relationship of 22 tomato genotypes built using the method UPGMA and based on SSR. The scale at the bottom is Jaccard's coefficient of similarity.

variance formula. Polymorphic information content (PIC) value was calculated (Table 3). In the present study, totally 25 SSR primers were used across twenty-two tomato accessions for PIC value detection in tomato. The PIC values for polymorphic markers ranged from 0 to 0.65. The primers which showed highest PIC values were SSR96 (0.65) followed by SSR63 and SSR 248 (0.612). A slightly higher PIC value was given by Kwon et al. (2009) in a study using thirty-three pairs of SSR primers by screening 63 varieties of tomatoes. With 33 SSR markers, a total of 132 polymorphic amplified fragments were collected. The average information content for polymorphism PIC ranged from 0.210 to 0.880 and PIC values was identified as SSR13 (0.642) and SSR 248(0.748). Similar results were reported by He et al. (2003), where 129 pairs produced the expected DNA fragments in their PCR products, and 65 pairs produced PIC value ranging from 0.09 to 0.67. Kumar et al. (2016) conducted a study to determine the genetic variation of 19 tomato genotypes starts with the screening of polymorphic microsatellite markers. A total of 261 Polymorphic amplified fragments were obtained from the 11 polymorphic SSR markers identified. The average PIC value ranged from 0.979 (SSR-110) to 0.995 (SSR-253) and SSR-63 displayed a PIC value of 0.994.

Cluster analysis: NTSYS produces the dendrograms that are quickly visualised. Of the most common current clustering programs, NTSYS is the most efficient and flexible. This provides a broad range of approaches and strategies for clustering similarities. For the biologist, NTSYS is superior due to its easily produced dendrogram and other features, such as the coefficient of cophenetic correlation, which are explicitly designed for biological use. The user manual is also in the numerical

Table 3: Different SSR primers used for detecting polymorphism among 22 tomato genotypes and their PIC values

Primer	PIC value
SSR 63	0.62
SSR 13	0.58
SSR 248	0.62
SSR 270	0.58
SSR 356	0.58
SSR 605	0.58
SSR 96	0.65

taxonomy language, and the logic of the whole system is well suited to biological problems. The phylogenetic tree was constructed through NTSYSpc cluster analysis software using UPGMA (Un-weighted pair group method with arithmetic mean). UPGMA cluster analysis of genetic similarity matrix resulted in the dendrogram and further divided into four major clusters. Physiolgical, biochemical and yield parameters of these varieties were obtained from the Department of Plant Physiology, College of Agriculture, Vellayani, Kerala Agricultural University (Amrutha 2020). Cluster 1 consists of three varieties; Akshaya, IIHR 2200, Manuprabha. They categorized as moderately tolerant varieties based on physiological, biochemical and yield data under high temperature condition (Amrutha 2020). They showed various parameters in the range of pollen viability-45-50%, leaf membrane thermostability-40-50%, starch content <200 mg/g, photosynthetic rate-17-19 µmol CO_2 $m^{-2}sec^{-1}$, stomatal conductance-55-65mmol H_2O m^{-2} sec^{-1}, chlorophyll florescence-0.6-0.8 (Fv/Fm ratio) and yield-10-30 g /plant.

Cluster 2 includes 11 varieties; Nandi, Vaibhav, Pusa Ruby, Manulakshmi, Arka Alok, Sakthi, IIHR 26372, Arka Vikas, Arka Abha, IC 45, Arka Sambrat- They categorized as tolerant varieties based on physiological, biochemical and yield data under high temperature condition (Amrutha, 2020). They showed various parameters in the range of pollen viability-50-70%, leaf membrane thermostability-60-70%, starch content-190-230 mg/g, photosynthetic rate-17-22µmol CO_2 m^{-2} sec^{-1}, stomatal conductance-47-68mmol H_2O $m^{-2}sec^{-1}$, chlorophyll florescence-0.6-0.8 (Fv/Fm ratio) and yield-50-60 g /plant. Cluster 3 consists of three varieties: Kashi Vishesh, Anagha, Vellayani Vijay. This is a mixed cluster as per physiological, biochemical and yield data.

Cluster 4 includes four varieties: Arka Saurabh, Pusa Rohini, Palam Pride, Arka Rakshak. They were categorized as susceptible varieties under high temperature condition based on physiological, biochemical and yield data under high temperature condition (Amrutha 2020). They showed various

parameters in the range of pollen viability-44-45%, leaf membrane thermostability-25-30%, starch content- 90-110 mg/g, photosynthetic rate-13-16µmol CO_2 m^{-2} sec^{-1}, stomatal conductance-30-37mmol H_2O $m^{-2}sec^{-1}$, chlorophyll florescence-0.4-0.5 (Fv/Fm ratio) and fruit set was very poor (Figure 2). Cluster analysis based on Jaccard's coefficient of similarity using the unweighted pair-group method with arithmetic mean (UPGMA) in tomato based on the SSR markers was also done by Kwon et al. (2009) and He et al. (2003).

Conclusion

Genetic diversity analysis using SSR markers classified 22 tomato varieties into four clusters. These clusters were analyzed for physiological, biochemical and yield data under high temperature condition and categorized into tolerant, moderately tolerant, mixed and susceptible group. Validation of SSR markers for high temperature stress tolerance in tomato revealed that since high temperature is quantitative in nature single marker can't differentiate the genotypes into tolerant or susceptible clusters. Phenotypic evaluation of a greater number of tomato germplasm collection for high temperature tolerance and its quality parameters are needed.

सारांश

टमाटर (*सोलेनम लाइकोपर्सिकम* एल.) की चयनित 22 प्रभेदों को उच्च तापमान प्रतिबल दशा के तहत आण्विक लक्षण का वर्णन 25 सरल अनुक्रम दोहराने (एस.एम.आर) मार्कर का उपयोग करके मूल्यांकन किया गया। कुल इस्तेमाल किए गये 25 मार्करों में से 22 प्राइमरों ने प्रवर्धन दिखाया जिनमें 15 एस.एस.आर. मोनोमोर्फिक और 7 पॉलीमॉर्फिक थे। पॉलिमर की सूचना सामग्री (पी.आइ.सी.) मूल्य के आधार पर मार्करों का मान 0.58 से लेकर 0.65 तक 0.6 के औसत मूल्य के साथ है। उच्चतम पी.आई.सी. मूल्य एस.एस.आर–96 (0. 65) द्वारा स्पष्ट हुआ। एन.टी.एस.वाई.एस. पी.सी. क्लस्टर विश्लेषण सॉपटवेयर का उपयोग अंकगणित माध्य (यू.पी.जी.एम.ए.) के साथ अनवेदित युग्म–समूह पद्धति का उपयोग करते हुए जैकार्ड के समानता गुणांक के आधार पर फ्लोजेनेटिक ट्री का निर्माण किया गया। क्लस्टर विश्लेषण में 22 प्रभेदों को चार समूहों में विभाजित किया गया। एस.एस.आर. मार्कर विश्लेषण और क्लस्टर विश्लेषण सहिष्णु, मध्यम सहिष्णु, अति संवेदनशील और मिश्रित विशेषताओं वाले प्रभेदों को अलग करने में सक्षम थे। वर्तमान में टमाटर के उपलब्ध उपलब्ध मानचित्रों में सीमित संख्या में एस.एस.आर. मार्कर होते हैं जो समान रूप से जीनोम में वितरित नहीं होते हैं। अतः यह अध्ययन टमाटर में उच्च तापमान सहिष्णुता से जुड़े आण्विक मार्करों की पहचान में हमारी मूलभूत समझ में मदद करता है।

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