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GENETIC VARIABILITY ANALYSIS OF TOMATO VARIETIES USING RAPD MARKERS UNDER HEAT STRESS

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Abstract

Heat stress threatens agricultural and food security by reducing yield and causing plant death. The use of intra-genetic diversity in heat stress responses is a potential avenue for harnessing tolerance to climate change circumstances. This study investigated the genetic diversity of 12 tomato genotypes in Bangladesh, focusing on their suitability for summer cultivation using seven Random Amplified Polymorphic DNA (RAPD) markers. The banding patterns obtained from the PCR results were used to identify genetic differences between individuals. The RAPD markers were found to show their effectiveness in discriminating the studied lines through the computation of average Polymorphism Information Content (PIC) values, particularly focusing on dominant markers with PIC values ranging from 0 to 0.5, emphasizing the informative nature of the employed primers. Cluster analysis grouped several genotypes with possible common ancestry. Overall, BARI Hybrid Tomato 4, BARI Hybrid Tomato 8, A12, D13, and RHS1 emerged as promising candidates for further genetic research and breeding programs, particularly in the context of heat-stress conditions during summer tomato cultivation in Bangladesh.

Introduction

Temperature is vital for plant growth and development, with each genotype having an ideal range. Deviations from this range create temperature stress, notably heat stress when temperatures exceed optimal levels. Heat stress impacts plant survival, growth, development, and physiological processes, with reproductive functions, like flower loss, pollen death, and reduced fruit formation, being especially vulnerable. The ability of plants to thrive in high temperatures is known as heat tolerance (Kamel *et al.* 2010, Golam *et al.* 2012). Tomatoes, being highly nutritious and widely consumed, rank as the second most popular vegetable crop globally after potatoes (Golam *et al.* 2012, El-Mansy *et al.* 2021). The ideal temperature range for tomato growth is 18-25°C, with nighttime temperatures between 10-20°C, as per the FAO database. However, significant temperature fluctuations between day and night can negatively impact crop productivity. Temperatures above 25°C, coupled with high humidity and strong winds, result in reduced yields. Furthermore, low sunlight and high humidity at nighttime temperatures exceeding 20°C can lead to excessive vegetative growth but hinder fruit development (Kamel *et al*. 2010, Golam *et al.* 2012, El-Mansy *et al.* 2021).

In Bangladesh, tomatoes are primarily grown in winter due to favorable ambient temperatures (Mehraj *et al.* 2014). To meet increasing demand, the Bangladesh Agricultural Research Institute (BARI) introduced off-season tomato cultivation using polytunnels and developed various hybrids (Hajong *et al.* 2018). Dinajpur district leads in tomato production with 25,911 MT, followed by Rajshahi with 22,222 MT, and Mymensingh contributed over 6,800 MT in 2015 (BBS 2019). However, challenges include low acclimatization to the tropical monsoon environment and complicated agronomic practices, with high labor costs and the use of growth regulators (Rahman and Acharjee 2020). Bangladesh faces limitations in tomato production due to challenges in

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adapting to hot, humid climates and a lack of heat-tolerant varieties (Mehraj *et al.* 2014). Conventional breeding efforts have been employed to understand the genetic basis of heat stress tolerance (Wahid *et al.* 2007, Golam *et al.* 2012), but these methods are time-consuming and costly, with limited success in identifying key genomic regions for complex traits like heat stress tolerance (Golam *et al.* 2012).

To overcome the limitations in tomato production in Bangladesh genetic diversity assessment and marker-assisted breeding are called for (Mehraj *et al.* 2014). Random Amplified Polymorphic DNA (RAPD) markers are valuable tools for identifying genetic variations and polymorphisms in crop species, aiding in breeding efforts (Collard *et al*. 2005, Babu *et al.* 2021). These markers do not require prior DNA sequence information, making them versatile for genetic analysis (Premkrishnan and Arunachalam 2012). The RAPD primers, typically 8 to 15 nucleotides long with 50-80% GC content, amplify DNA sections randomly via PCR, creating complex patterns useful for genetic diversity assessment. This approach facilitates the study of genotypic differences and does not rely on sequence data, offering a simple and rapid method for genetic analysis (Premkrishnan and Arunachalam 2012).

By assessing genetic diversity using RAPD markers under heat-stress conditions, researchers aim to enhance tomato varieties' resilience to challenging environments (Hailu and Asfere 2020). The technique's simplicity, minimal DNA requirement, and ability to detect polymorphisms make RAPD analysis a valuable tool for breeding programs (Nadeem *et al.* 2018). Therefore, the study was aimed to evaluate genetic diversity among 12 tomato varieties, focusing on phenotypic traits and polymorphisms related to heat stress tolerance. This approach can aid in developing heat-resistant tomato cultivars to address the upcoming challenges posed by Bangladesh's climatic conditions.

Materials and Methods

Seeds from twelve tomato varieties, comprising hybrids, local cultivars, wild, and exotic types, were collected from various sources for RAPD profiling. The sources included the Plant Genetic Resources Center (PGRC); Horticulture and vegetable research center of Bangladesh Agricultural Research Institute (BARI) for four tomato accessions and two varieties, namely BD10124 (A2, A12, A17), BD10122 (B14), Local Khustia1 (D13, D14), BD7755 (H10), BARI Hybrid Tomato 4 and BARI Hybrid Tomato 8; while two wild varieties, RHS1 and RHS2, were collected from remote locations in Bangladesh by Professor Dr. Rakha Hari Sarker (RHS) Dept. of Botany, University of Dhaka. Additionally, seeds for the variety named Delicious were obtained by Professor Dr. Mohammad Nurul Islam (Department of Botany, University of Dhaka).

The experimental tomato plants were cultivated and maintained within the controlled greenhouse facility at the Department of Botany, University of Dhaka. For the controlled lines, the optimum room temperature was 22 to 25°C during the daytime and nighttime, the average temperature was maintained at a range of 18 to 22°C. To induce heat acclimation, the growth chamber was equipped with an effective insulation system, ensuring that daytime temperatures remained within the range of 28 to 36°C, typically spanning approximately 8 hours. During nighttime, the average temperature was maintained at a range of 22 to 25°C. The relative humidity of the room was kept between 80-85%. Caring for the plants involved a regimen of watering twice daily, in the morning and afternoon, and periodic weeding to maintain a healthy growth environment. Additionally, a monthly application of NPK (Nitrogen-Phosphorus-Potassium) fertilizers, coupled with bio-fertilizers, was implemented to enhance plant yield and overall productivity. This cultivation protocol was designed to create optimal conditions for heat

acclimation and rigorous growth of the experimental tomato plants within the greenhouse (established with the financial support from BCCT, GoB).

Young and healthy tomato plant leaves at their mature stage were carefully collected for the extraction of genomic DNA. To ensure the purity of the DNA, the leaves underwent a thorough cleansing process involving successive washes with distilled water and ethanol. Subsequently, the cleaned leaves were gently dried on fresh tissue paper and promptly flash-frozen using liquid nitrogen. Genomic DNA extraction was carried out using the modified CTAB (cetyltrimethylammonium bromide) procedure, a method known for yielding high-quality plant DNA (Aboul-Maaty and Oraby 2019). The extracted DNA was quantified and assessed for purity using a spectrophotometer (Bio Drop Resolution) by measuring its absorbance at 260 nm. The resulting DNA solution was diluted to a final concentration of 50 ng/µl using water and then stored at -20°C for subsequent molecular analysis, maintaining the integrity of the genetic material for scientific investigations.

The RAPD analysis was conducted using seven decamer random primers sourced from Operon Technology (Table 1). Genomic DNA was combined with GoTaq^{R} G2 Green Master Mix, a pre-mixed solution containing GoTaq^{R} G2 DNA Polymerase, dNTPs, MgCl2, and reaction buffers, designed for efficient DNA amplification via PCR. The GoTaq^{R} G2 Green Master Mix contained blue and yellow dyes, enabling real-time tracking of the reactions during electrophoresis. Notably, the GoTaq^{R} G2 DNA Polymerase featured 5'→3' exonuclease activity. Each amplification reaction had a total volume of 50 µl, comprising 25 µl of GoTaq^{R} G2 Green Master Mix, 22 µl of dH_2O, 2 µl of RAPD Primer, and 1 µl of genomic DNA. PCR amplification was carried out using a thermal cycler (Applied Biosystem) under the following conditions: Initial denaturation at 94°C for 5 mins, followed by denaturation at 94°C for 45 seconds, annealing at 34°C for 30 seconds, elongation at 72°C for 3 mins, final elongation for 7 mins, and a holding step at 4°C for until the samples were removed from the machine. This cycle (step 2 to 4) was repeated 42 times.

Horizontal gel electrophoresis was performed using a 1.5% agarose gel containing 0.5 µg/ml ethidium bromide in TAE buffer, with electrophoresis conducted at 50 volts for 1.5 hrs. To visualize the DNA banding pattern post-electrophoresis, the gel was carefully transferred to the Bio-Rad Gel Documentation System (omni-DOC, Cleaver Scientific LTD, USA).

The present research endeavor, RAPD analysis was conducted to assess genetic diversity among the tomato specimens. Each amplified fragment or band generated through RAPD primers was treated as an individual unit character. The determination of the molecular weight of these fragments was accomplished using Image J software, a process that involved comparing the migration distances of these fragments with known-size segments from a 1.0 kb DNA ladder, post-electrophoresis. The presence or absence of each distinct band referred to as RAPD markers, was visually assessed and recorded as either "1" or "0." The outcomes of the RAPD analysis, utilizing a range of primers, were amalgamated to generate a comprehensive data matrix. To investigate the genetic relationships among the individual tomato specimens, an unweighted pair group method with arithmetic mean (UPGMA)-based cluster analysis was executed. This analysis yielded a UPGMA-SAHN clustering dendrogram, as proposed by (Kriege *et al.* 2014) and presented in the study (Fig. 2).

Furthermore, a genetic similarity assessment among the experimental tomato lines was conducted using the Simple Matching Coefficient (SMC) based on the presence or absence of similar and dissimilar RAPD bands. This analysis was performed using the NTSYSpc 2.10e software, enabling the creation of a similarity matrix. Additionally, the genetic distance matrix

between the experimental tomato lines was calculated based on the method of Nei (1972), again utilizing the NTSYSpc 2.10e software.

Results and Discussion

In the greenhouse chamber, the lines maintained at optimum temperature exhibited significantly higher flower settings compared to the lines maintained under high-temperature heat stressed conditions. In the heat stressed conditions, BARI Hybrid Tomato 4 and BARI Hybrid Tomato 8 followed by A2, A17 and RHS1 displayed notably higher flower and fruit setting values. In contrast, H10, D14, BD10122, Delicious, and RHS2 rarely set flowers and fruits under heat stress conditions (Fig. 1). These findings highlight the differential performance of tomato varieties in response to different temperatures, with BARI Hybrid Tomato 4, BARI Hybrid Tomato 8 demonstrating resilience and suitability for high-temperature cultivation.

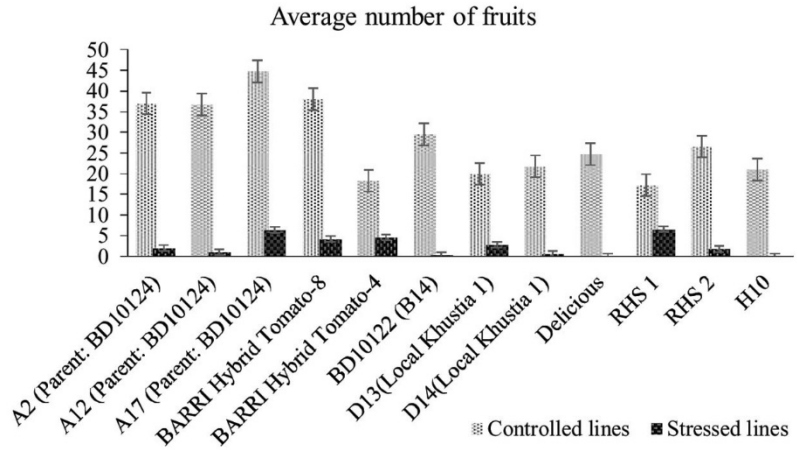

Fig 1. Mean number of fruits in Controlled condition and Heat-stressed condition.

The fruit yield of BARI Hybrid Tomato 4 and BARI Hybrid Tomato 8 was lower compared to previous studies (Ali *et al.* 2014, Hossain *et al.* 2017, Sanjida *et al.* 2020, Quamruzzaman *et al.* 2023) conducted under both controlled and high-temperature conditions (Fig. 1). Additionally, previous research (Jannat *et al.* 2020) reported favorable winter season production for Local Khustia 1, RHS1, and RHS2. These findings indicate variations in fruit yield among different tomato varieties under varying environmental conditions.

In the present study, seven decamer random primers were employed to assess genetic polymorphism across twelve tomato genotypes, and all primers produced distinct polymorphic patterns (Table 1). Among these, primers C02, C15, and P06 yielded the highest number of unique polymorphic bands, while primer C08 resulted in the lowest count (3).

The primer P06 generated a total of 40 bands, of which 9 were monomorphic and the remaining bands exhibited polymorphism. Notably, 8 unique polymorphic bands were identified in various tomato genotypes, including RHS1 (1211 bp), A12 (888 bp), A2 (707 bp), RHS 2 (663 bp), Delicious (360 bp), A17 (347 bp and 314 bp), and BARI Hybrid Tomato 4 (500 bp). These findings suggest the potential presence of RAPD markers associated with heat resistance traits, particularly the 0.5 kb fragment linked to high yield, which was exclusively found in BARI

Hybrid Tomato 4. While the exact band sizes were not determined in other lines, the presence of 552 bp and 495 bp bands in BARI Hybrid Tomato 8, A12, and D13 (Local Khustia 1) indicates the possible flanking of heat-tolerance markers in these genomic regions (Lin *et al*. 2006, Comlekcioglu *et al*. 2010).

Table 1. RAPD Primers linked to heat-tolerance and heat-susceptibility found in different literatures (Lin *et al.* 2006, Kamel *et al.* 2010, Golam *et al.* 2012).

RAPD Primer	Sequence (5′ – 3′)	Total number of bands	Mono-morphic bands	Polymorphic bands	Unique polymorphic bands	% of polymorphisms	Average PIC value
P06	5’-AGCCAGCGAA-3’	40	9	31	8	77.50	0.276
Z13	5’-GACTAAGCCC-3’	6	4	2	4	66.67	0.294
A16	5’-GTGAGGCGTC-3’	60	27	33	7	55	0.215
C02	5’-GACGGATCAG-3’	41	0	41	10	100	0.234
C08	5'-TGCGTGCTTG-3'	59	28	31	3	52.54	0.363
C14	5'-GTGGGCTGAC-3'	41	0	41	7	100	0.282
C15	5’-GACTAAGCCC-3’	38	0	38	9	100	0.288

Primer A16 revealed seven unique polymorphic bands in different genotypes, including Delicious (848 bp), BD10122 (767 bp), A12 (700 bp), RHS1 (688 bp), RHS2 (589 bp), B14 (448 bp), and A12 (500 bp). Although the 100 bp band observed in the resistant parent in a prior study (Kamel *et al*. 2010) was not found in the experimental varieties.

In the case of primer Z13, a total of six distinct polymorphic bands were detected in the samples H10, B14, A17, and A12. The bands exhibit a lighter shade. Two distinct polymorphic bands were detected in the H10 sample, measuring 1491 base pairs, and in the B14 (BD10124) sample, measuring 410 base pairs. The region responsible for heat-tolerance in genotypes is amplified by Primer Z13, resulting in a 500 bp product (Kamel *et al*. 2010). However, the experimental types did not yield any band sizes in the vicinity of 500 base pairs.

Conversely, primers C02, C08, C14, and C15 exhibited five molecular markers solely in the susceptible F2 bulk, with sizes of 500 bp and 1500 bp for C02, 550 bp for C08, 400 bp for C14, and 650 bp for C15 (Kamel *et al.* 2010). In the present study, primer C02 produced a total of 41 bands, with 10 unique polymorphic bands detected in various genotypes, including A17 (1216 bp), B14 (888 bp), A2 (809 bp, 760 bp), D14 (731 bp), A2 (695 bp), BHT 8 (435 bp and 393 bp), Delicious (539 bp), H10 (469 bp), and D13 (258 bp). Furthermore, primer C08 revealed unique polymorphic bands in BHT8 (1305 bp and 571 bp) and A2 (439 bp). Although the susceptible locus is typically 550 bp (Kamel *et al.* 2010), this size was not observed in any of the lines. Instead, a 512 bp locus was detected in A2, B14, Delicious, RHS2, and H10. These findings shed light on the genetic variations associated with heat susceptibility in tomato genotypes (Kamel *et al*. 2010).

Primer C14 yielded unique polymorphic bands in various genotypes, including A2, A17, B14, and D14, with susceptible locus size reported at 450 bp in a prior study (Kamel *et al.* 2010). However, the exact 450 bp locus was not found in the experimental lines, but a 512 bp locus was observed in A2, B14, Delicious, RHS2, and H10 (Fig. 3). Under the heat-stress condition, the number of flowerings and the number of fruit settings were also very low for these lines (Fig. 1). Lastly, primer C15 produced unique polymorphic bands in various genotypes, including A2, A17, B14, D14, RHS1, and H10. While the susceptible fragment for C15 reported in (Kamel *et al.*

2010) was 650 bp, the exact size was not found in the experimental tomato lines. However, a 633 bp DNA fragment was discovered in B14, which also exhibited reduced flowerings and fruit settings under heat-stress conditions.

In order to evaluate the effectiveness of the markers in differentiating the studied lines, average PIC (Polymorphism information content) values were calculated as PIC $_{(\text{dominant marker})}$ = 2f (1-f), where f= frequency of present allele (De Riek *et al.* 2001). PIC values ranged from (0-0.5) for dominant markers. The highest value for PIC (0.363) was revealed by C08 which is aligned with the SCoT marker in the study of (EL-Mansy *et al.* 2021). The observed increased levels of polymorphic information content (PIC) following the analysis of RAPD markers provide confirmation that the primers utilized in this study were highly informative. The authors of (Meng *et al.* 2010) reported a higher average PIC value (0.687) compared to the findings of the present investigation. In contrast, Shahlaei *et al.* (2014) reported a PIC value (0.088) that was lower than the PIC values computed in the present study. The percentage of polymorphisms and the number of polymorphic bands were greater than the study of (Lin *et al.* 2006, Kamel *et al.* 2010). In the study of (Ezekiel *et al.* 2011, Hassan *et al.* 2013) the size of the amplification RAPD products varied from 200 bp to 3100 bp and 196 bp to 1790 bp, whereas the total numbers of scored bands were 74 and 180, respectively, which aligned with the present results.

The RAPD data analysis resulted in the generation of a dendrogram, as depicted in Fig. 2, which revealed the presence of five primary clusters. This cluster analysis effectively distinguished all twelve tomato lines from one another, grouping similar genotypes within the same cluster and separating distinct genotypes into different clusters.

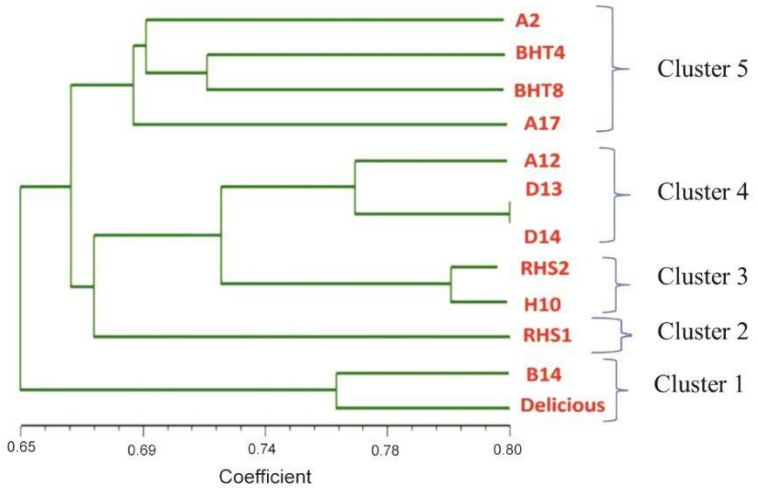

Fig 2. A dendrogram based on Nei's (1972) genetic distance summarizing the data on differentiation among 12 varieties, according to RAPD analysis.

A similarity matrix that quantifies the pairwise similarity or dissimilarity between data points. This matrix can be based on various metrics such as Euclidean distance, or correlation coefficients. The results from the similarity matrix based on correlation coefficient, as presented in Table 2, corroborated the outcomes of the cluster analysis. In Cluster 1, the tomato lines Delicious and B14 (BD10122) were found, both of which exhibited the lowest mean values for flower and fruit numbers and 77% similarity was found (Table 2). The variety RHS1 occupied a distinct

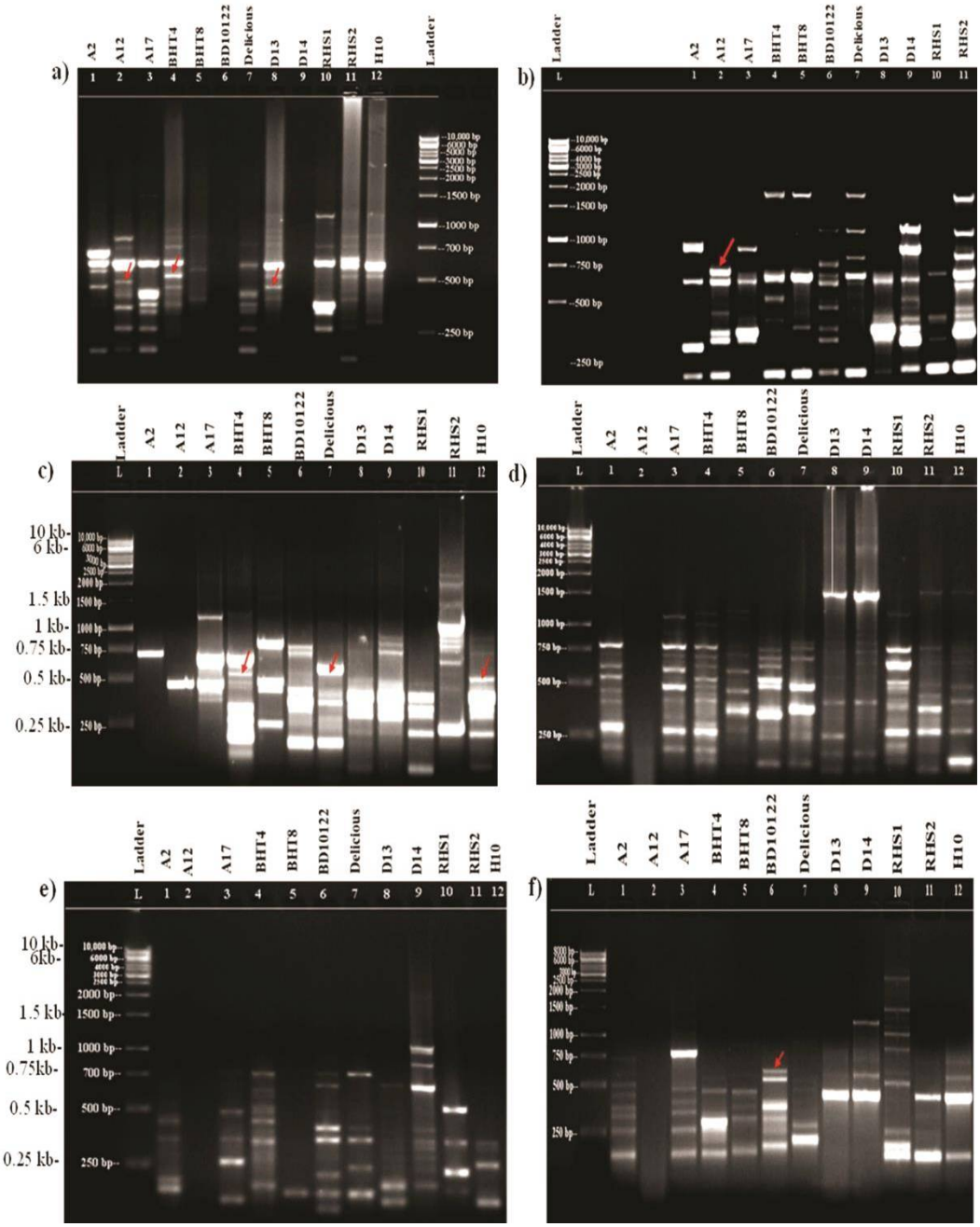

Fig 3. Amplification patterns of RAPD primers were obtained from twelve tomato varieties. (a) Primer P06 (b) primer A16 (c) Primer C02 (d) Primer C08 (e) Primer C14 (f) Primer C15. L-10 kb DNA Ladder; Lane 1-A2; Lane 2-A12; Lane 3-A17; Lane 4-BHT 4; Lane 5-BHT 8; Lane 6-BD10122; Lane 7-Delicious; Lane 8-D13; Lane 9-D14; Lane 10-RHS1; Lane 11-RHS 2; Lane 12-H10. Red arrows showing the unique band and polymorphic loci.

position in a separate cluster. Cluster 3 comprised RHS2 and H10, while Cluster 4 included A12, D13, and D14. Finally, Cluster 5 encompassed A2, BHT4, BHT8, and A17. Notably, the highest similarity, at 82%, was observed between D13 and D14, both of which belong to the Local Khustia 1 varieties. A12 exhibited the highest similarity with D13, measuring 71%, while its similarity with D14 was lower, at 57%. Both BHT4 AND BHT8 (BARI Hybrid tomato 4 and BARI Hybrid tomato 8) were released from Bangladesh Agricultural Research Institute and the similarity percentage between these two varieties was 71% denoting the common ancestor. Both from the dendrogram and similarity matrix index analysis (Fig. 2 and Table 2) it was found that RHS2 and H10 maintained a similarity ratio of 81%.

Table 2. Genetic Similarity Index based on simple matching coefficient (SMC).

	A2	A12	A17	BHT4	BHT8	B14	Delicious	D13	D14	RHS1	RHS2	H10
A2	1.0											
A12	0.71	1.0										
A17	0.68	0.74	1.0									
BHT4	0.69	0.65	0.7	1.0								
BHT8	0.7	0.65	0.69	0.71	1.0							
B14	0.61	0.71	0.57	0.59	0.65	1.0						
Delicious	0.65	0.72	0.65	0.66	0.73	0.77	1.0					
D13	0.71	0.8	0.70	0.72	0.72	0.64	0.68	1.0				
D14	0.57	0.75	0.57	0.63	0.64	0.59	0.61	0.82	1.0			
RHS1	0.63	0.69	0.67	0.7	0.62	0.6	0.65	0.68	0.59	1.0		
RHS2	0.67	0.65	0.67	0.71	0.73	0.65	0.72	0.75	0.70	0.76	1.0	
H10	1.35	0.71	0.63	0.68	0.64	0.6	0.7	0.8	0.72	0.66	0.81	1.0

In conclusion, Tomato farming in Bangladesh faces challenges related to low yield potential and decreased fruit settings due to seasonal variations. Marker-assisted selection (MAS) offers a promising strategy for overcoming these limitations by utilizing molecular markers to evaluate genetic variability independently of environmental factors. This approach provides plant breeders with genetic markers for specific economic traits, reducing the dependence on environmental conditions. Furthermore, the diversity in phenotypic traits identified in this study allows for the selection of potential parental lines that can be instrumental in addressing the changing climatic challenges in Bangladesh. Notably, unique polymorphic DNA band sizes were observed in A12, D13, BARRI Hybrid Tomato 4, and BARRI Hybrid Tomato 8. Additionally, A12, A17, RHS1, BARRI Hybrid Tomato 4, and BARRI Hybrid Tomato 8 exhibited fruit-setting capabilities under high-temperature conditions. Overall, the RAPD primer P06, in conjunction with these identified lines, holds significant potential for accelerating tomato breeding programs in Bangladesh, offering a promising avenue to enhance tomato yield and resilience in the face of changing climate conditions.

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