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Barcoding of ToLCV Resistant Tomato Germplasm in Bangladesh

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Key words: Barcoding, ToLCV resistant, Tomato, Germplasm

Abstract

The current study was carried out to confirm the existence of the ToLCV resistant genes (Ty-1 to Ty-5) in the germplasm using molecular markers and to identify at the genomic level following phylogenetic relationships analysis among some local tomato germplasm using DNA barcoding. Most of the tomato germplasm (Ten out of 14) contain the dominant Ty-genes as revealed by PCR analysis. "Barcoding" of the non-coding plastid *trn*H-*psb*A intergenic spacer region, three plastidal regions: *rbc*L, *rpo*B, *rpo*C1, spacer region of nuclear genome ITS and a mitochondrial region *mat*K were employed following PCR and sequence analysis of the germplasm. Among all the barcode genes, *rpo*B, *rbc*L, *trn*H-*psb*A and ITS were leading candidates for successful amplification and used for the identification of the germplasm as *S. lycopersicum* in multi-locus identification based on their sequences. Neighbor-Joining phylogenetic tree was constructed in which the germplasm were clustered into five main clades. The current study was successful to establish an efficient barcoding protocol for the correct identification of tomatoes and was capable of establishing elite gene source(s) for biotic stress resistance tomato varieties which would serve as potential donor plants in modern breeding programs.

Introduction

Tomato is an important vegetable of the Solanaceae, cultivated worldwide due to its good flavor and rich source of nutrients. It is also a well-known model species for studying fruit development and metabolite accumulation. The yield of tomato is getting low in recent years as tomato plant is susceptible to many biotic and abiotic stresses. Among all the diseases, tomato leaf curl disease (ToLCD) one is the most devastating one

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caused by tomato leaf curl viruses (ToLCVs). ToLCVs are the member of the genus *Begomovirus* belongs to Geminiviridae and transmitted by white fly (*Bemisia tabacci*). A socio-economic survey conducted in 2004 ranked TolCVs as the most devastating pathogen causing ToLCD of tomato (Chowda 2004). Epidemics of the virus associated with upsurge of white flies on tomato crops has been frequently reported with up to 100% yield losses. The tomato leaf curl disease was first documented in Bangladesh around two decades earlier (Green et al. 2001), although the disease was first reported from the northern region of India in 1948 (Vasudeva and Sam Raj 1948). Up to now, six ToLCV resistance/tolerance genes have been mapped, Ty-1 to Ty-6, from wild species and exploited for resistance breeding (Zamir et al. 1994, Hanson et al. 2006, Ji et al. 2007, Anbinder et al. 2009, Hutton et al. 2012). Most of these loci originated from accessions of *Solanum chilense*, *S. habrochaites* (Banerjee and Kalloo 1987, Kalloo and Banerjee 1990), *S. peruvianum* (Hutton et al. 2012, Levin et al. 2013). Though these Ty-genes were isolated from different wild accessions of tomato, they were naturally found in some tomato lines of Bangladesh confirmed by Ruman (2017) and Tabassum (2018). So, these Ty genes containing ToLCV resistant tomato germplasm required correct molecular identification.

To obtain tomato crops with these desired agronomical traits requires a good deel of understanding and management of tomato genetic resources diversity (Baucher and Causse 2012). A DNA barcode is an aid to taxonomic identification which uses a standard short genomic region that is universally present in target lineages and has sufficient sequence variation to discriminate among species (Hebert et al. 2003, Hebert et al. 2004, Savolainen et al. 2005, Hajibabaei et al. 2007). It refers to a sequence-based identification system that be constructed of one locus or several loci used together as a complementary unit (Kress and Erickson 2007). The most important characteristic features of a DNA barcode are its universality, specificity on variation and easiness on employment. So it is important to identify the local tomato germplasm at molecular level based on molecular markers. The importance of tomato DNA barcoding is underlained by the need for accurate species identification. An ideal DNA barcode should be present in all groups of land plants and it should be short (400 - 800 bp) showing sufficient sequence variation to distinguish between species and ease of application across a board range of taxa. The purpose of this study was to identify local tomato variety and to test the utility of DNA barcoding for the identification of closely related tomato varieties. To assess the identification, the non-coding plastid *trn*H-*psb*A intergenic spacer region, three plastidal regions: *rbc*L, *rpo*B, *rpo*C1, spacer region of nuclear genome ITS, and a mitochondrial region *mat*K were employed. The aim and objectives of the current work was to confirm the existence of the Ty gene/s conferring resistance against ToLCV using molecular markers leading to selection of potential donor parents for breeding programs and to identify the germplasm at molecular level by barcoding marker analysis and study genetic linkage among the germplasm.

Materials and Methods

Seeds of the tomato (*Solanum lycopersicum*) germplasm were collected by the courtesy of Plant Genetic Resource Centre (PGRC), as well as Horticulture Research Division, Bangladesh Agricultural Research Institute, Gazipur and also from different sources through personal communication. The germplasm collected from PGRC are BD10122, BD10123, BD10124, BD10125, BD7755, BD7761, Local Khustia-1 and from Horticulture Research Division are BARI-8 and BARI-14. The personally collected seeds were named as RHS-1, RHA-2, MNIBW, MNIBB and MNID etc. The germplasm were grown during Rabi season, 2018-19 and maintained in the field of Botanical garden, Department of Botany, University of Dhaka. The genomic DNA was isolated from 40 - 50 mg of fresh young leaves from each individual sample using Roti Prep Genomic DNA Mini Kit following a modified protocol as supplied with the kit. Concentration of isolated DNA was measured through comparison with standard DNA bands on agarose gel electrophoresis and by estimating the absorbance of DNA using a spectrophotometer (BioDrop µLITE) at 260 nm.

To check the presence or absence of six ToLCV resistant genes, screenig of the genotypes were made with six major begomovirus resistant genes, Ty-1 to Ty-5. The genomic DNA of the fourteen tomato germplasm were subjected to 25µL PCR reaction with 1.0 µL each of the forward and reverse primers of respective Ty genes (Ty-1 – Ty 5) followed by the method described in Zamir et al. (1994), Hanson et al. (2006), Ii et al. (2007), Anbinder et al. (2009), Hutton et al. (2012) using Go Taq G2 Green Master mix.

For molecular identification, the six plant DNA barcodes, *rbc*L, *trn*H-*psb*A, *rpo*C1, *rpo*B, ITS and *mat*K were amplified in 25 µl reaction volume, using Go Taq G2 Green Master mix, 1.0 µl each primers and 70 ng DNA template. PCR amplification was performed on a Thermal Cycler (Applied Biosystem). PCR condition were used following condition reported by Caprari et al. (2017). The success of PCR amplification was verified by subjecting 10 µl of the PCR product to 1% agarose gel electrophoresis in TAE buffer and visualized under an UV trans-illuminator. The PCR products were purified using alcohol precipitation method. Purified PCR products were sent to MC Lab (USA) and sequenced in both directions with the same primers used for PCR.

Sequences for each region were viewed and edited using BioEdit version 7.0. Then, the edited sequences were aligned by ClustalW in MEGA7. Bootstrap values were calculated over 1000 replications. The barcode sequences were queried against GenBank database (NCBI) using Nucleotide BLAST algorithm BOLDSYSTEMS in order to confirm the barcode gene markers along with their locus in tomato germplasm.

Results and Discussion

The morphological appearance of the germplasm were related to ToLCV resistance. All of the six Ty-genes were present in BD10122 and the morphological appearance was good including fruit production. Bd10123, BD10125, BD7755, Local khustia-1, RHS-1,

RHS-2, MNIBB and MNID possessed more than one of the dominant Ty-genes and they were morphologically stable in the field condition (Fig. 1), and the production was also good. But though the MNIBW possessed good number of Ty-genes it did not show good field performance, which indicated its more time requirement to be adapted in the new environmental condition.

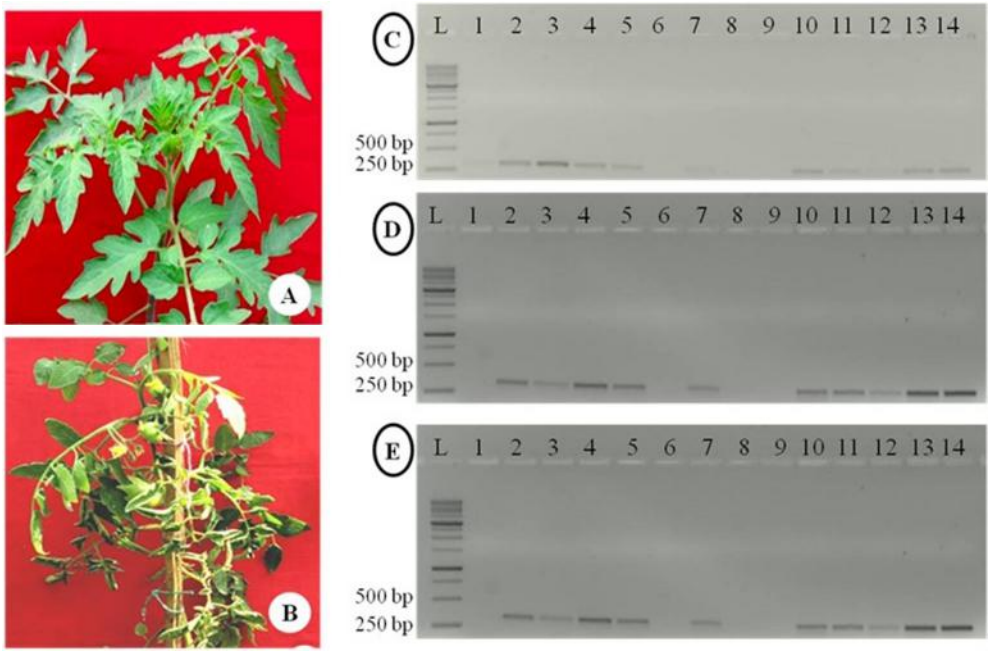

Fig. 1. (A-E) Molecular analysis of the ToLCV resistant tomato germplasm. A. A healthy BD10122 tomato plant bearing dominant Ty-genes. B. An unhealthy plant with leaf curl disease lacking Ty-genes. (C-E)- Representative gel pictures of the results of electrophoresis on 1% agarose gel of PCR products obtained with Ty- genes. Lane L DNA ladder. C. PCR products of Ty-3a gene. Except lanes 6, 8 and 9 which were BD7761, BARI-8 and BARI-14 all other germplasm showed positive amplification at ~300bp. D. PCR products of Ty-4 gene. Lanes 1-5, 7, 10-14 which are Bd10123, BD10125, BD10122, BD7755, Local khustia-1, RHS-1, RHS-2, MNIBW, MNIBB, MNID showed positive band at ~ 250bp - 350 bp. E. PCR products of Ty-5 gene. Lanes 1-5, 7, 10-14 showed positive band at ~ 180 bp which are BD10124, BD10123, BD10125, BD10122, BD7755, Local khustia-1, RHS-1, RHS-2, MNIBW, MNIBB and MNID.

The results of this study showed that, the Ty-containing germplasm showed lower disease symptoms. Nevertheless, there were distinct differences between the germplasm bearing majority Ty genes performing the best. However, in the long term, germplasm with only a single resistance gene would seem undesirable – a resistance based on a single gene (a single mechanism) being, at least in theory, less durable than multiple genes. The use of molecular markers linked to these genes for resistance is a tool which can be used efficiently in molecular plant breeding for the indirect selection of quantitative resistance and for accelerating transfer of resistant gene from different sources into a single cultivar by gene pyramiding through marker assisted selection

(MAS). The identification of the reported markers such as Ty-1, Ty-2, Ty-3, Ty-3a, Ty-4 and Ty-5 linked to ToLCV resistance in the local germplasm of tomato has been reported recently (Ruman 2017 and Tabssum 2018). In their study, molecular marker assisted identification of potential donor parent was carried out in selected local tomato germplasm with the goal of improving cultivars with enhanced and durable resistance against ToLCV. They have identified some potential lines carrying Ty genes linked to ToLCV resistance but this is yet to confirm at the genomic level.

For identification of ToLCV resistant germplasm, barcoding was conducted. All the barcodes are not equally efficient to identify the germplasm. They varied in their rate of PCR amplification (Fig. 2), sequencing success and aligned sequence length (Table 1). *rpo*B had highest rate of PCR amplification and *mat*K showed lowest or no PCR amplification. But all the successfully amplified barcodes had 100% sequence success. The sequences characteristics of the five regions are presented in (Table 1). The locus *matK*, which has been shown to be quite variable in numerous phylogenetic studies (Shaw et al. 2005, Soltis et al. 2001) had the lowest amplification success (0%) of all loci tested in this study.

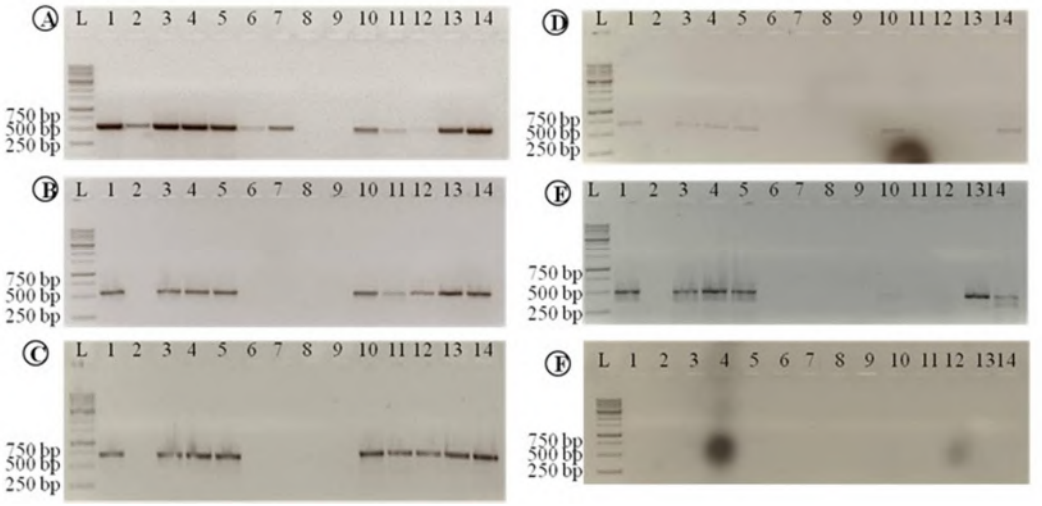

Fig. 2A-F. Results of electrophoresis on 1% agarose gel of PCR products obtained with barcode genes. Lane L is DNA ladder. A. PCR product of *rpoB* gene. Except lanes 8, 9 and 12 which are BARI-8, BARI-14 and MNIBW, rest of the germplasm showed positive band at ~ 550 bp. B. PCR products of *rpoC1* gene showing amplification on lanes 1,3-5, 10-14 in germplasm BD10124, BD10125, BD10122, BD7755, RHS-1, RHS-2, MNIBW, MNIBB, MNID. Positive bands are seen at ~500 -550 bp. C.PCR products of ITS gene. Lanes 1, 3-5, 10-14 showed positive bands at ~700 bp which indicates the germplasm BD10124, BD10125, BD10122, BD7755, RHS-1, RHS-2, MNIBW, MNIBB and MNID. D.PCR products of *rbcL* gene. Lanes 1, 3-5, 10-11, 14 showing positive band at ~500-700 bp. The positive germplasm are BD10124, BD10125, BD10122, BD7755, RHS-1, RHS-2, MNID. E. PCR product of *trn*H-*psb*A gene. Lanes 1, 3-5, 13-14 which are BD10124, BD10125, BD10122, BD7755, MNIBB, MNID. Positive bands are a ~ 500 bp. F. PCR products of *matK* gene. There was no positive result for none of the germplasm.

Each barcode sequence was compared againest the NCBI database through BLAST search and the BOLD systems. All sequences of the *rbc*L, *rpo*B, ITS and *trn*H-*psb*A loci identified the tomato germplasm as *Solanum lycopersicum* with 99 or 100% identity. Sequences of *rpo*C1 identified the tomato germplasm as *Solanum violaceimarmoratum* with 99 or 100% identity. Caprari et al. (2017) did barcode sequence of local tomato plant from north- western Romania. They used four barcode genes *trn*H-*psb*A, *rbc*L, *rpo*B and *rpo*C1 and got 100% success in PCR for all the loci. So, in multilocus identification system the tomato germplasm were successfully identified as *Solanum lycopersicum* (Table 2).

Table 1. Characteristis of each single barcodes.

Marker	PCR success (%)	Sequencing success (%)	Average aligned length (bp)
*rpo*B	78.6	100	444.25
ITS	64.3	100	556.22
*rpo*C1	64.3	100	467.125
*rbc*L	50	100	601.5
*trn*H-*psb*A	42.9	100	442.83
*mat*K	0	0	0

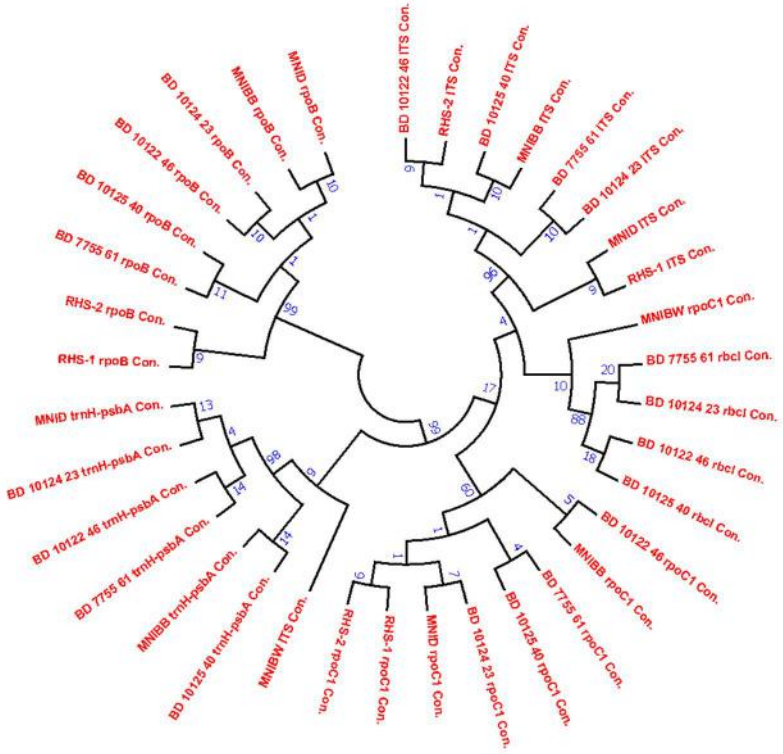

Fig. 3. Bootstrap consensus tree generated by Neighbor-joining method for *ITS*, *rpoB*, *rpoC1*, *rbcL*, *trn*H-*psb*A and *matK* sequences obtained for tomato germplasm studied here. Evolutionary analyses were conducted in MEGA 7.0. Numbers below the branches are bootstrap values expressed as percentage of 1000 replicates.

Table 2. Multi-locus identification of the germplasm based on sequence analysis of barcode genes.

Sl. no.	Name of tomato germplasm and plant number	Sequence similarity found with the *Solanum* sp. (Accession no., % similarity & Query coverage) in NCBI BLAST search					Remarks
		ITS	*rpo*B	*rpo*C1	*rbc*L	*trn*H-*psb*A	
1.	BD10124_23	*S. lycopersicum* (MH573911.1/ KF668233.1 and 5 others; 100%; 100%)	*S. lycopersicum* (KY887588.1/ KY887587.1; 99.43%; 100%)	*S. violaceimarmoratum*, (NC041634.1 99.79%, 100%); *S. verrucosum* (NC041633.1, 99.79%, 100%); *S. tarijense* (NC041632.1, 99.79%, 100%); *S. spegazzinii* (NC041631.1, 99.79%, 100%)	*S. lycopersicum* (MH722375.1/ MH588526.1and 2 others; 99.80%; 100%)	*S. lycopersicum* (KY887587.1/ AM087200.3 and 4 others; 97.10%; 99%)	*S. lycopersicum*
2.	BD10122_46	*S. lycopersicum* (KF668233.1 and 5 others; 100%; 100%)	*S. lycopersicum* (KY887588.1/ KY887587.1; 99.80%; 99%)	*S. violaceimarmoratum* (NC041634.1, 100%, 100%); *S. verrucosum* (NC041632.1, 100%, 100%);*S. tarijense* (NC041631.1, 100%, 100%); *S. spegazzinii* (NC041630.1, 100%, 100%)	*S. lycopersicum* (CPO23757.1/ KY887588.1 and 4 others; 98.31%; 99%)	*S. lycopersicum* (AM087200.1/ KP117024.1 and 2 others; 95.73%, 95.53%; 100%)	*S. lycopersicum*
3.	BD10125_40	*S. lycopersicum* (KF668233.1 and 5 others, 100%, 100%)	*S. lycopersicum* (KY887588.1/ KY887587.1; 99.80%; 99.04%)	*S. violaceimarmoratum* (NC041634.1, 98.53%%, 100%); *S. verrucosum* (NC041632.1, 98.53%%, 100%); *S. tarijense* (NC041631.1, 98.53%%, 100%); *S. spegazzinii* (NC041630.1, 98.53%%, 100%)	*S. lycopersicum* (MH722375.1 and 2 others;	*S. lycopersicum* (KP117024.1 and 4 others; 98.67%; 100%)	*S. lycopersicum*

4.	BD7755_61	*S. lycopersicum* (KC213743.1 and 5 others, 99.84%, 100%)	*S. lycopersicum* (KY887588.1/ KY887587.1 and 4 others; 99.01%; 99%)	*S. violaceimarmoratum* (NC041634.1, 98.53%%, 100%); *S. verrucosum* (NC041632.1, 98.53%%, 100%); *S. tarijense* (NC041631.1, 98.53%%, 100%); *S. spegazzinii* (NC041630.1, 98.53%, 100%)	*S. lycopersicum* (MH722375.1 and 3 others; 99.84%; 99%)	*S. lycopersicum* (AM087200.1 and 2 others; 96.40%; 100%)	*S. lycopersicum*
5.	MNIBW	*S. violaceimarmoratum* (NC041634.1, 100%, 100%); *S. verrucosum* (NC041633.1, 100%, 100%); *S. tarijense* (NC041632.1, 100%, 100%); *S. spegazzinii* (NC041631.1, 100%, 100%)	No PCR amplification				Molecular identification failed
6.	MNIBB	*S. lycopersicum* (MH573911.1 and 4 others; 100%; 100%)	*S. lycopersicum* (KY887588.1 and 2 others; 99.61%; 100%)	*S. violaceimarmoratum* (NC041634.1, 99.81%%, 100%); *S. verrucosum* (NC041632.1, 99.81%%, 100%); *S. tarijense*(NC041631.1, 99.81%%, 100%); *S. spegazzinii* (NC041630.1, 99.81%, 100%)	No PCR amplification	*S. lycopersicum* (KY887587.1 and 4 others; 99.75%; 99%)	*S. lycopersicum*

7.	MNID	*S. lycopersicum* (AC246968.1 and 4 others; 99.85%; 99%)	*S. lycopersicum* (KY887588.1 and 2 others; 98.84%; 100%)	*S. lycopersicum* (EU293359.1; 99.26%; 100%) *S. violaceimarmoratum* NC041634.1, 99.26%%, 100%); *S. verrucosum* (NC041632.1, 99.26%%, 100%); *S. tarijense* (NC041631.1, 99.26%%, 100%); *S. spegazzinii* (NC041630.1, 99.26%, 100%)	No PCR amplification	*S. lycopersicum* (KY887588.1 and 4 others; 98.67%; 100%)	*S. lycopersicum*
8.	RHS-1	*S. lycopersicum* (KF668233.1 and 4 others; 99.85%; 98%)	*S. lycopersicum* (KY887588.1 and 2 others; 100%; 100%)	*S. violaceimarmoratum* NC041634.1, 99.81%, 100%); *S. verrucosum* (NC041632.1, 99.81%, 100%); *S. tarijense* (NC041631.1, 99.81%, 100%); *S. spegazzinii* (NC041630.1, 99.81%, 100%)	No PCR amplification	No PCR amplification	*S. lycopersicum*
9.	RHS-2	*S. lycopersicum* (KF668233.1and 3 others; 99.56%; 100%)	*S. lycopersicum* (KY887588.1 and 2 others; 100%; 100%)	*S. violaceimarmoratum* (NC041634.1, 97.74%, 93%); *S. verrucosum* (NC041632.1, 97.74%, 93%); *S. tarijense* (NC041631.1, 97.74%, 93%); *S. spegazzinii* (NC041630.1, 97.74%, 93%)	No PCR amplification	No PCR amplification	*S. lycopersicum*

The phylognetic tree obtained by Neighbor-joining bootstrap consensus method based on combined data analysis showed linkage among the germplasm in this study. The phylogenetic relationships among the tomato germplasm based on their sequence obtained with ITS, *rpo*B, *rpo*C1, *rbc*L, *trn*H-*psb*A and *mat*K barcode genes. In the neighbor-joining tree, bootstrap values for each node were estimated by 1000 replications. The evolutionary tree has been estimated from DNA sequence. The genotypes were clustered in five main clades. Because of multi-loci tests bias, the bootstrap values were trustable more than 95% (Fig. 3).

It can be concluded that, in the complex situation that prevails in Bangladesh and globally, the Ty genes are useful in countering ToLCV. Nevertheless, there were distinct differences between the germplasm bearing most Ty genes performing the best in field condition. The germplasm carrying Ty-genes were successfully identified by sequence based identification system as *S. lycopersicum*. Genetic sequences obtained in the context of DNA barcoding had also been used to create phylogenetic trees for use in phylogenetic community analysis. The identified germplasm further could be treated as donor parent of elite resistance gene source.

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