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Genomic characterization and Phylogenetic analysis of *Tomato leaf curl virus* (ToLCV) from Bangladesh

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Authors' contributions

This work was carried out in collaboration among all authors. Author MMH designed and performed the study, managed the literature searches, and wrote the first draft of the manuscript. Authors MBM and YS managed the analysis and supervised the study. All authors read and approved the final manuscript.

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ABSTRACT

Tomato leaf curl virus (ToLCV) has frequently emerged as a severe problem for tomato in the recent past, in the tropical and subtropical region of the world. We have cloned and sequenced two isolates of ToLCV responsible for the leaf curl disease of tomato in Bangladesh. Two betasatellite DNAs were associated with ToLCV, and complete nucleotide sequences were determined. The complete genome sequences of ToLCV-[BD:Mym:11] and ToLCV-[BD:Mym2:11] shared the highest nucleotide sequence identities at 95.04 and 92.38%, respectively, with Indian isolate of *Tomato leaf curl virus* - [India:Ranchi:2007] (ToLCV-[IN:Ran:07]) and *Tomato leaf curl Patna virus* - [India:Lucknow:2009] (ToLCPatV-[IN:PLuc:09]). Complete nucleotide sequence analysis of the two betasatellite clones, *tomato leaf curl betasatellite* – [BD:Mym:11] and *tomato leaf curl betasatellite* –

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[BD:Mym2:11] associated with (ToLCV-[BD:Mym:11] and (ToLCV-[BD:Mym2:11], when used in BLAST searches respectively revealed 95.51 and 86.43% identity with *Tomato leaf curl Bangladesh betasatellite* (ToLCBDB-[BD-Gaz-01]) and *Tomato leaf curl Patna betasatellite* (ToLCPaB-[IN-Pat-07]).

Keywords: Betasatellite; genomic characterization; phytogenetic tree; ToLCV.

1. INTRODUCTION

Tomato (*Lycopersicon esculentum* Mill.) is a highly nutritious, tasty and widely grown vegetable crop in Bangladesh due to its vitamins (A and C) and minerals contents. In Bangladesh, tomato is extensively grown in winter and to some area in summer. About 14396.03 ha of land were under tomato cultivation, producing 389000 tons of fresh fruit in the year 2016-17 [1]. The whole tomato cultivated area and production have increased progressively over the last few years. Still, the productivity is very low (6.46 t ha^{-1}) in comparison to the world average yield (26.29 t ha^{-1}) as per the Food and Agriculture Organization [2]. Diseases are considered to be the utmost constraint among the factors responsible for low yield of tomato. More than 200 diseases have been found in tomato, out of which 40 are caused by viruses [3]. *Tomato leaf curl virus disease* (ToLCVD) was first documented in Bangladesh only recently [4], although it is likely to have occurred earlier since the disease had been recorded in the neighboring areas of northern and central India since 1943 [5]. *Tomato leaf curl virus* (ToLCV) is the major limiting factor for tomato cultivation worldwide. This disease is caused by viral species of the genus Begomovirus, family *Geminiviridae*. During last two decades, the ToLCV been devastating, causing up to 100% economic loss in many tropical and subtropical regions including Bangladesh [6,7,8,9,10]. In tropical and subtropical areas ToLCV disease incidence is tremendous mainly because of higher activities of whitefly (*Bemisia tabaci*) [11]. In susceptible cultivars, ToLCV disease symptoms include yellowing, vein clearing, severe leaf curling, and stunting of the plant. If infection occurs at the seedling stage, plants become sterile causing up to 100% yield loss [12].

Geminiviruses are characterized by twinned icosahedral shaped particles approximately 18 nm X 30 nm in size [13], which encapsidate single-stranded circular DNA (ssDNA) genomes. Based on the host range, insect vector, genome structure, organization and genome-wide pairwise sequence identities [14], geminiviruses are divided into nine genera (Becurtovirus, Begomovirus, Capulavirus, Curtovirus, Eragrovirus, Grablovirus, Mastrevirus, Topocuvirus and Turncurtovirus). Begomoviruses have a monopartite or bipartite genome, transmitted by the whitefly vector *Bemisia tabaci* and infect dicotyledonous plants. Most of the begomoviruses have a bipartite genome organization with two ssDNA components referred to as DNA-A and DNA-B, each with a length of 2.5–3 kb. Both DNA-A and DNA-B have an approximately 200 nucleotides long common region, which is highly conserved in both the components and contains repeated motifs (iterons) which are sequence-specific replication protein (Rep) binding sites [15]. The DNA-A component of the bipartite begomoviruses is involved in replication and production of virions, but requires the DNA B component for nuclear localization, systemic infection, host range determination and symptom expression [16]. However, a number of begomoviruses are monopartite having a single genomic DNA component which is a homolog of DNA-A, and encodes all the proteins required for replication, gene expression, whitefly transmission, and systemic infection [13].

The genomic characterization of ToLCV and the development of techniques like a virus isolation procedure, identification by PCR, etc, will allow developing resistant tomato line/variety and also the monitoring of the current prevalence of these begomoviruses in tomato in Bangladesh. Virus-resistant plants can be developed by using pathogen-derived resistance (PDR). PDR based on cross-protection and antisense inactivation has long been employed in the management of viral diseases in plants. In case of begomoviruses, expression of viral coat protein (CP), replicase and movement proteins has proven to be promising. This information can also be used to develop a virus resistance program against these begomoviruses using genetic engineering approaches.

A B

Fig. 1. Tomato leaf curl symptoms of tomato plants infected with A. ToLCV-[BD:Mym:11] B. ToLCV-[BD:Mym2:11] (Leaf curl symptoms are severe and trifoliate leaves exhibit asymmetry and look distorted)

2. MATERIALS AND METHODS

2.1 Virus Sources

Different leaf samples of tomato showing leaf curl symptoms were collected from various location of Mymensingh in Bangladesh (Fig. 1). The leaf samples were dried using self-indicating silica gel and stored at room temperature until use. The dry samples were carried to the plant virology laboratory, graduate school of science and technology, Niigata University, Japan, for DNA extraction and analysis of the viruses.

2.2 DNA Extraction

Nucleic acids were extracted from symptomatic leaves by a modified cetyl trimethylammonium bromide (CTAB) method [17]. Briefly, approximately 20 mg of dried tissues were transferred into a 1.5 mL eppendorf tube and homogenized with 500 µL extraction buffer containing 2 M NaCl, 25 mM EDTA, 100 mM Tris-HCl, 2% PVP and 2% Cetyl trimethyl ammonium bromide (CTAB) using mortar pestle. The total DNAs were extracted twice with chloroform:isoamyl alcohol (24:1) and pelleted with cold iso-propanol. The DNA pellets were washed twice with 70% ethanol and dried. Finally, the pellet was dissolved in 50 µL of TE buffer.

2.3 Polymerase Chain Reaction (PCR)

ToLCV was detected from diseased plants by PCR using degenerate and specific primers (Table 1). From the sequences amplified by degenerate primers, one abutting primer pair Tom V1/Tom R1, was designed and used to amplify the complete genomes of two virus isolates. For cloning of betasatellites, the samples were subjected to PCR using universal betasatellite-specific primers β01 and β02 [18]. The primers used in this study are listed in Table 1, and all amplifications were performed in a reaction mixture of 20 µL using PrimeStar Max enzyme according to the manufacturer's instructions (Takara Bio Inc., Japan).

2.4 Cloning, Sequencing and Sequence Analysis

The PCR products of 2 samples were cloned into T-vector pMD20 and used to transform *Escherichia coli* (JM109) following the manufacturer's instructions (Takara Bio Inc., Japan). DNA sequencing of one or two clones for each sample was carried out in both orientations by a commercial company (SolGent Co., Ltd., Korea). GENETYX Win. software package (GENETYX, Tokyo, Japan) ver. 12 used for the sequence data assemble and analysis. Viral genomic sequences were aligned, and pairwise

comparisons were carried out with the help of ClustalW software [19]. Phylogenetic trees were constructed using the neighbor-joining method calculated with the Molecular Evolutionary Genetics Analysis (MEGA) software, version 6 [20] (Tables 2-3).

Table 1. Sequences of primers used in this study

Primer	Sequences (5′-3′) [a]
Primers used for ToLCV cloning and sequencing	
Tom V1	G*GGGCCC*CCATGAAYTCYTT
Tom R1	ACGGTCAGGGAAA*CCCGGG*
T1 Seq R1	GCCAGCTTCTTGCTGGTTAT
T1 Seq V1	GGGAAAGTGCCTCTTCTT
T2 Seq R1	GACCACCTGTAACCGTTGC
T2 Seq V1	GTGAAGATGAGGTTCCCCA
TYLC-V1	TATAATCATTTCCACGCCCG
TYLC-R1	TCKSCTGGTCGCTTCGACATA
TYLC-V2	GATAGTGGGAATTCCSCCTT
TYLC-R2	TTCCTCTGCAATCCAGGACC
Primers used for checking DNAβ	
β01	*GGTACC*ACTACGCTACGCAGCAGCC
β02	*GGTACC*TACCCTCCCAGG GGTACAC
Beta F1	GTGGCCATATATCAGGATATATGG
Beta R11	TCAACTCCACTTCCTATGAT
Primers used for checking DNAα	
αF	TGGTTYTATWCACGTGGHGG
αR	ARAWGATAGTKCKRTCATCTG

[a] *B=C or T or G, K=G or T, R=A or G, S=C or G, V=A or C or G, W=A or T, Y=C or T*
Restriction endonuclease sites are underlined and in italic type

Table 2. Acronyms and accession numbers of ToLCV sequences used in comparative and phylogenetic analyses

Species	Acronym	Accession number
Tomato leaf curl Bangladesh virus - [Bangladesh:BD2]	ToLCBV-[BD:BD2]	AF188481
Tomato leaf curl virus - [India:Dhanbad:2008]	ToLCV-[IN:Dha:08]	EU573714
Tomato leaf curl virus - [Nepal:Panchkhal:2000]	ToLCV-[NP:Pan:00]	AY234383
Tomato leaf curl virus - [India:Mirzapur:1999]	ToLCV-[IN:Mir:99]	AF449999
Tomato leaf curl virus - [India:Vadodara:1999]	ToLCV-[IN:Vad:99]	AF413671
Tomato leaf curl virus - Bangalore [India:Punjab:Mentha:2007]	ToLCV-Ban[IN:Pun:Me:07]	FJ514798
Tomato leaf curl Kerala virus - [India:Kerala 5:2007]	ToLCKeV-[IN:Ker5:07]	EU910140
Tomato leaf curl Laos virus - [Laos]	ToLCLV-[LA]	AF195782
Tomato leaf curl virus - [India:New Delhi:CTS:2006]	ToLCV-[IN:ND:06]	DQ629101
Tomato leaf curl Patna virus - [India:Lucknow:2009]	ToLCPatV-[IN:PLuc:09]	GU253915
Tomato leaf curl Patna virus- [Jatropha:Lucknow:2010]	ToLCPatV-[Ja:Lu:10]	HQ848381
Tomato leaf curl virus - [India:Ranchi:2007]	ToLCV-[IN:Ran:07]	GQ994095

** Source: DNA data bank of Japan*

Table 3. Acronyms and accession numbers of *Tomato leaf curl betasatellite* sequences used in comparative and phylogenetic analyses

Species	Acronym	Accession number
Tomato leaf curl Bangladesh betasatellite	ToLCBDB-[BD-Gaz-01]	AJ542489
Tomato leaf curl Bangalore betasatellite	ToLCBaB-[IN-Ban-03]	AY428768
Tomato leaf curl betasatellite	ToLCB-[PK-RYK-97]	AJ316036
Tomato leaf curl China betasatellite	ToLCCNB-[CN-Gx14-02]	AJ704609
Tomato leaf curl Java betasatellite	ToLCJaB	AB100306
Tomato leaf curl Joydebpur betasatellite	ToLCJoB-[BD-Gaz-05]	AJ966244
Tomato leaf curl Karnataka betasatellite	ToLCKaB	AY754813
Tomato leaf curl Laos betasatellite	ToLCLAB-[LA-Sav-01]	AJ542491
Tomato leaf curl Maharastra betasatellite	ToLCMaB	AY838894
Tomato leaf curl Patna betasatellite	ToLCPaB-[IN-Pat-07]	EU862324
Tomato leaf curl Philippines betasatellite	ToLCPHB-[PH-Lag1-06]	AB308071
Tomato leaf curl Ranchi betasatellite	ToLCRaB	GQ994096

** Source: DNA data bank of Japan*

3. RESULTS AND DISCUSSION

Infected tomato plants showing severe leaf curl symptoms were observed in fields during a survey carried out in Mymensingh, Bangladesh (Fig. 1). To identify the ToLCV in the infected plants, total DNA was isolated from the leaf samples of infected plants as well as from healthy tomato plants (as a negative control). PCR was performed with the isolated DNA. Two tomato samples assigned as tomato-1 and tomato-2 were positive with degenerate primers (TYLC-V1/ TYLC-R1, TYLC-V2/ TYLC-R1). The 1.3 kb PCR products of the two isolates were then cloned and sequenced using PCR primers. BLAST searches showed tomato-1 and tomato-2 shared more than 97% nucleotide sequence identities with ToLCV-[IN:Ran:07] and ToLCPatV-[IN:PLuc:09].

Using the primer pair Tom V1/Tom R1, the full-length DNA clones of two ToLCV isolates, namely, ToLCV-[BD:Mym:11] (tomato-1) and ToLCV-[BD:Mym2:11] (tomato-2) were amplified and fully sequenced. In the initial screening, the primer pairs were used to amplify genomic DNA A of ToYLCV and ToLCV; however, we did not find any ToYLCV sequence in the amplified DNA fragments. Our attempts to amplify sub-genomic components from the tomato samples using primer pairs targeting the betasatellite [18] were successful.

The complete genomes of ToLCV-[BD:Mym:11] and ToLCV-[BD:Mym2:11] are comprised of 2763 nt and 2751 nt respectively and their genome organizations are identical to those of the previously characterized Old World monopartite begomoviruses. The two genomes contain six open reading frames (ORFs) of which two ORFs V1 and V2 are in the virion sense and the other four ORFs, i.e., C1, C2, C3 and C4, are in complementary sense (Fig. 2). The IR sequences (between the start codons of the C1 and V1 ORF) of the virus isolates are approximately 300 nt and have the characteristic geminivirus stem-loop structure with the conserved nonanucleotide sequence TAATATTAC, the Rep high-affinity binding site, and the C1 TATA box. The conserved nonanucleotide sequence TAATATTAC is present within the replication origin of almost all geminiviruses. The complete genome sequences of ToLCV-[BD:Mym:11] and ToLCV-[BD:Mym2:11] shared the highest nucleotide sequence identities at 95.04 and 92.38%, respectively, with Indian isolate of ToLCV-[IN:Ran:07] and ToLCPatV-[IN:PLuc:09] (Table 4).

Complete nucleotide sequence analysis of two betasatellite clones associated with ToLCV-[BD:Mym:11] and ToLCV-[BD:Mym2:11], respectively in BLAST search revealed 95.51 and 86.43% identity with ToLCBDB-[BD-Gaz-01] and ToLCPaB-[IN-Pat-07], respectively. The identity observed is higher than 78% identity kept as the threshold value for demarcation of betasatellite species. The name *tomato leaf curl betasatellite* – [BD:Mym:11] and *tomato leaf curl betasatellite* – [BD:Mym2:11] are proposed for the new betasatellites characterized in this study. The complete nucleotide sequence determined to be 1371 and 1350 nt long for *tomato leaf curl betasatellite* – [BD:Mym:11] and *tomato leaf curl betasatellite* – [BD:Mym2:11], respectively. The complete nucleotide sequence of betasatellites in the present study was compared with other betasatellites available in the GenBank database (Table 5).

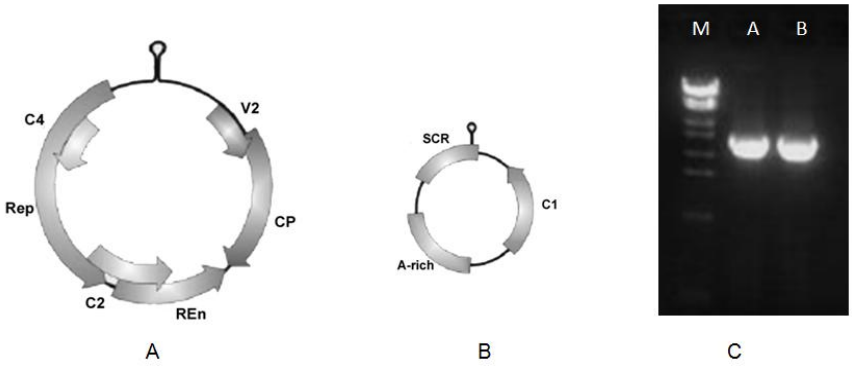

Fig. 2. Genomic organization of ToLCV A. (ToLCV-[BD:Mym:11] and ToLCV-[BD:Mym2:11]), B. Tomato leaf curl betasatellite (ToLCMyB and ToLCMy2B) and C. Full length ToLCV genome amplified by PCR (M-DNA marker (2.7 kb and Takara Bio Inc., Japan), A - ToLCV-[BD:Mym:11] and B - ToLCV-[BD:Mym2:11])

Table 4. Nucleotide sequence identity of ToLCV isolates with other begomoviruses and comparison of ORF-wise amino acid sequence identity. Highest values are indicated in bold

Begomovirus	ToLCV-[BD:Mym:11]			ToLCV-[BD:Mym2:11]		
	Full genome	CP	Rep	Full genome	CP	Rep
ToLCBV-[BD:BD2]	89.16	**98.82**	88.00	77.90	83.65	81.63
ToLCV-[IN:Dha:08]	84.38	80.46	90.25	75.68	79.76	81.11
ToLCV-[NP:Pan:00]	84.56	80.46	90.77	75.85	79.76	82.20
ToLCV-[IN:Mir:99]	84.34	80.46	90.83	75.52	79.76	81.11
ToLCV-[IN:Vad:99]	84.70	80.46	91.69	75.73	79.76	81.11
ToLCV-Ban[IN:Pun:Me:07]	88.14	96.48	90.85	77.52	84.04	81.35
ToLCKeV-[IN:Ker5:07]	82.66	96.48	86.00	80.52	83.65	87.70
ToLCLV-[LA]	75.33	84.04	80.97	85.09	92.99	**92.50**
ToLCV-[IN:ND:06]	84.31	80.46	89.68	75.78	79.37	80.00
ToLCPatV-[IN:PLuc:09]	75.17	80.15	74.92	**92.38**	**94.55**	88.33
ToLCV-[IN:Ran:07]	**95.04**	98.04	**94.85**	76.79	82.87	80.50

Table 5. Nucleotide sequence identity of ToLCVB isolates with other begomoviruses betasatellites and comparison of ORF-wise amino acid sequence identity. Highest values are indicated in bold

Betasatellite	ToLCMyB		ToLCMy2B	
	Full genome	C1	Full genome	C1
ToLCBDB-[BD-Gaz-01]	**95.51**	**95.76**	66.45	**100**
ToLCBaB-[IN-Ban-03]	63.75	62.71	60.64	**100**
ToLCB-[PK-RYK-97]	78.61	75.42	65.49	**100**
ToLCCNB-[CN-Gx14-02]	59.29	66.66	59.63	50.00
ToLCJaB	61.63	53.38	60.21	57.62
ToLCJoB-[BD-Gaz-05]	67.02	64.40	77.74	77.67
ToLCKaB	72.63	73.72	64.91	**100**
ToLCLAB-[LA-Sav-01]	58.14	49.13	58.76	48.71
ToLCMaB	64.44	63.55	59.72	**100**
ToLCPaB-[IN-Pat-07]	66.25	61.86	**86.43**	87.28
ToLCPHB-[PH-Lag1-06]	60.26	56.14	60.25	55.93
ToLCRaB	80.45	81.35	67.31	64.00

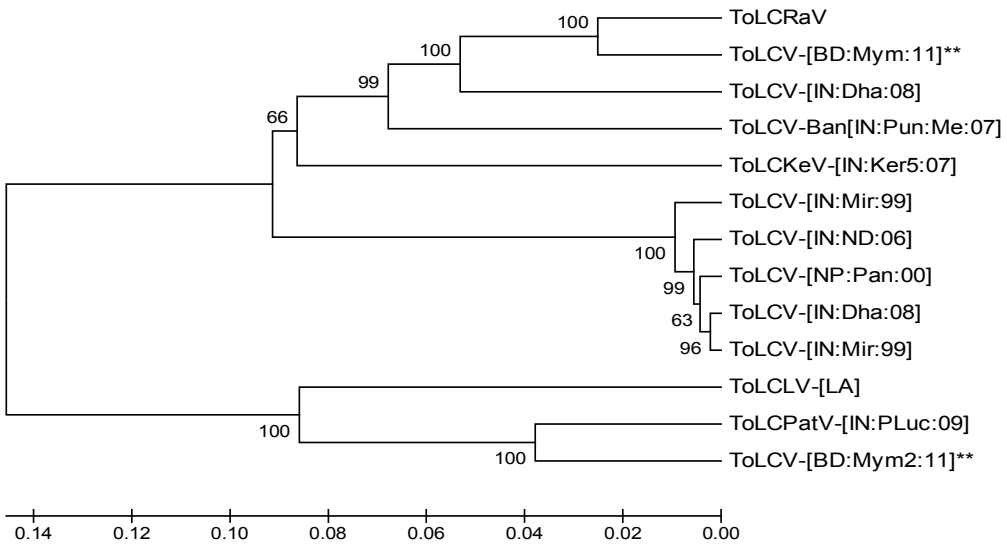

Fig. 3. Phylogenetic analysis of ToLCV and other ToLCVs based on the alignment of complete nucleotide sequences of viral genome. Phylogenetic Analyses were performed with MEGA (Molecular Evolutionary Genetics Analysis), version 5.2. Dendrograms were constructed by the neighbor-joining method. Bootstrap values (1.000 replicates) are given at the branch nodes. The scale at the base of the diagrams is the pairwise distance expressed as percentage dissimilarity. For abbreviations of virus names, see Table 2

An A-rich region is found between 706 to 979 nt in approximately 51 to 53% of the betasatellite DNA sequences. The A-rich region is present in both betasatellites. The betasatellites encode a single gene named as βC1 in the complementary strand. It is 369 bp long, encodes for a protein of ~14 kDa. Comparison of the amino acid sequence of the βC1 protein of *tomato leaf curl betasatellite* – [BD:Mym:11] (ToLCMyB) and *tomato leaf curl betasatellite* – [BD:Mym2:11] (ToLCMy2B) (Fig. 2) clearly shows that the βC1 protein of tomato betasatellites in the current work are nearly identical in their amino acid composition with other ToLCBs (Table 5).

Phylogenetic tree analysis on the basis of nucleotide sequence of complete genomes of the ToLCV-[BD:Mym:11] and ToLCV-[BD:Mym2:11] with the sequences of other selected begomoviruses revealed that the ToLCV-[BD:Mym:11] isolate has close relationship with ToLCV-[IN:Ran:07] and that the ToLCV-[BD:Mym2:11] isolate has close relationship with ToLCPatV-[IN:PLuc:09] (Fig. 3). Complete nucleotide sequences of the betasatellites in the present study were compared with other betasatellites deposited in the GenBank database and the phylogenetic tree was constructed (Fig. 4). Betasatellites (two from this study) branch out separately from each other.

Leaf curl and leaf distortion symptoms are observed in tomato plants in farmers´ fields of Bangladesh. The leaf curl symptoms are severe and trifoliate leaves exhibit asymmetry and look distorted. The present investigation was taken to find out whether any begomoviruses may be the cause of the crinkling symptoms or if any betasatellite components are involved. The results revealed that ToLCV-[BD:Mym:11] and ToLCV-[BD:Mym2:11] were associated with ToLCMyB and ToLCMy2B, respectively. The complete genomes of ToLCV-[BD:Mym:11] and ToLCV-[BD:Mym2:11] are comprised of 2763 nt and 2751 nt respectively and their genome organizations are identical to those of the previously characterized Old World monopartite begomoviruses.

Complete nucleotide sequence comparisons of the two betasatellite clones from (ToLCV-[BD:Mym:11] and (ToLCV-[BD:Mym2:11] using BLAST searches revealed 95.51 and 86.43% identity with ToLCBDB-[BD-Gaz-01] and ToLCPaB-[IN-Pat-07], respectively. If the betasatellite is present, it is well known that it augments the viral pathogenicity. It contributes to helping viral DNA accumulation as suggested by Guo et al. [21] and symptom severity in the host plant as observed by Jyothsna et al. [22] and Jose and Usha [23]. All the betasatellite

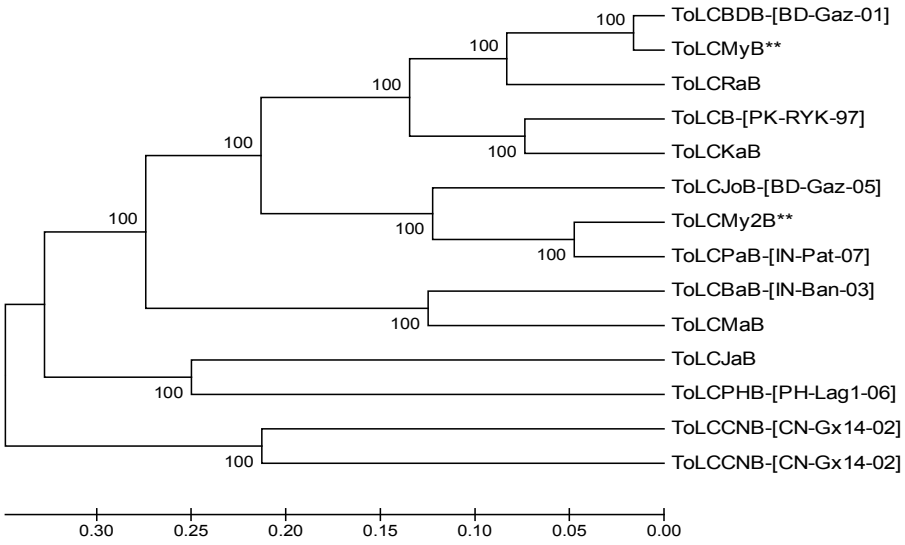

Fig. 4. Phylogenetic analysis of ToLCB and other begomoviruses betasatellites based on the alignment of complete nucleotide sequences of betasatellites. Phylogenetic Analyses were performed with MEGA (Molecular Evolutionary Genetics Analysis), version 5.2. Dendrograms were constructed by the neighbor-joining method. Bootstrap values (1.000 replicates) are given at the branch nodes. The scale at the base of the diagrams is the pairwise distance expressed as percentage dissimilarity. For abbreviations of virus names, see Table 3

molecules under study showed several characteristic features in common with other betasatellites reported from other crops. The sequence of ToLCV shows the typical arrangement of betasatellites [24, 25], consisting of a single conserved (between distinct betasatellites) gene in the complementary-sense known as βC1, a region of sequence rich in adenine and a sequence highly conserved between all betasatellites known as the satellite conserved region (SCR). The βC1 protein encoded by the betasatellite has been shown to be a PTGS suppressor, capable of knocking out RNAi defense of plants [26, 27]. It is suggested that βC1 may even alter the environment of the cell creating an advantageous atmosphere for the replication of the virus [24]. The position and size of βC1 were found to be conserved in all the betasatellites in this study.

4. CONCLUSION

The complete genomes of ToLCV and betasatellite were cloned and sequenced. The complete genome sequences of ToLCV-[BD:Mym:11] and ToLCV-[BD:Mym2:11] shared the highest nucleotide sequence identities at 95.04 and 92.38%, respectively, with Indian isolate of ToLCV-[IN:Ran:07] and ToLCPatV-[IN:PLuc:09]. It appears that this ToLCV-[BD:Mym:11] and ToLCV-[BD:Mym2:11] is a variant of ubiquitous ToLCV-[IN:Ran:07] and ToLCPatV-[IN:PLuc:09], respectively. Complete nucleotide sequence analysis of two betasatellite clones from ToLCV-[BD:Mym:11] and ToLCV-[BD:Mym2:11] in BLAST search revealed 95.51 and 86.43% identity with ToLCBDB-[BD-Gaz-01] and ToLCPaB-[IN-Pat-07], respectively. Begomoviruses and satellites from Africa, Asia and Mediterranean Region have been separated for a long time and thus are more distant from each other, but they can still support and interact with each other. The interactions between helper viruses and betasatellites are much more complex.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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