

Molecular Characterization of *Tomato leaf curl Palampur virus* and Pepper leaf curl betasatellite Naturally Infecting Pumpkin (*Cucurbita moschata*) in India

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Abstract Pumpkin cultivation in India is affected by severe incidence of a yellow vein mosaic disease. *Tomato leaf curl New Delhi virus* and *Squash leaf curl China virus* are known to be associated with this disease in India. We were able to identify a third begomovirus—*Tomato leaf curl Palampur virus* (ToLCPMV), from pumpkin showing typical symptoms of the disease at Varanasi based on the sequence of complete DNA-A genome of the virus. The complete DNA-A sequence of the virus shared more than 99% sequence identity with other ToLCPMV isolates available in the GenBank and clustered with them in the phylogenetic analysis. This betasatellite amplified from the same infected sample has been identified as Pepper leaf curl betasatellite (PepLCB) which also infects chilli in India. There was 92% sequence identity between the two isolates. This is the first report of natural infection of ToLCPMV on pumpkin and association of PepLCB with yellow vein mosaic disease of pumpkin in India.

Keywords Begomovirus · ToLCPMV · Pumpkin · Betasatellite

Introduction

Geminiviruses are the largest group of plant viruses and cause significant damage to food and fiber crops and are increasingly being reported on new hosts [17, 23]. They have small twinned icosahedral virions which encapsidate single stranded DNA genomes. Family *Geminiviridae* is divided into four genera based on insect vector, host range and genome organization [5, 20]. Begomoviruses are whitefly transmitted geminiviruses which are mostly bipartite and possess two single stranded DNA components (DNA-A and DNA-B) of ~2.8 kb. Few of them have a single component homologous to DNA-A component of bipartite viruses [5, 20]. Large numbers of Old World begomoviruses have been identified to require satellites about half the size of a genomic component to induce symptoms in hosts from which they are isolated [2]. These are also known to be transreplicated by more than one begomovirus [11, 18]. Some begomovirus disease complexes are also known to be associated with another single stranded DNA molecule known as DNA-1 which has been renamed as alpha satellite [3, 10].

Pumpkin (*Cucurbita moschata*) cultivation in India is severely affected by a yellow vein mosaic disease [8, 14] associated with two begomoviruses, *Tomato leaf curl New Delhi virus* (ToLCNDV) and *Squash leaf curl China virus* (SLCCNV) [12, 19]. In South India 45–100% disease incidence is reported [13]. In this work, we report the association of a third begomovirus—*Tomato leaf curl Palampur virus* (ToLCPMV), and a betasatellite identified as Pepper leaf curl betasatellite (PepLCB) in pumpkin showing typical symptoms of the disease.

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Materials and Methods

Total DNA was extracted from young pumpkin plants (25 different plants) showing typical symptoms of vein yellowing, mosaic and crumpling symptoms on leaves, mosaic and distortion and reduction of size of fruits, using the method of Pich and Schubert [16] with some modifications. Primers were designed to specifically amplify DNA-A (FP1—5'-GTG GGG ATC CAT TAT TGC ACG-3'; RP1—5'-CCG GAT CCC ACA TGT TTG TAG A-3') from the corresponding conserved region of begomoviruses reported on pumpkin in NCBI website. The begomovirus positive DNA samples (all 25 samples) were also checked for the potential presence of a betasatellite component in a standard PCR reaction using betasatellite specific primers (FP2—5'-TAC GGG TAC CAC TAC GCT ACG CAG C-3'; RP2—5'-CGA CGG TAC CTA CCC TCC CAG GGG T-3') of *Tomato leaf curl virus*. The full-length amplicons of DNA-A (~2.8 kb) and Betasatellite (~1.3 kb) were cloned in Topo cloning vector (Invitrogen) and sequenced. Sequences were edited manually. The BLAST program at the NCBI website (<http://www.ncbi.nlm.nih.gov/>) [1] was used to find the related begomovirus sequences. The sequences showing the highest sequence identity in BLAST searches with the present isolate and other begomoviruses infecting pumpkin were taken for the analysis of DNA-A, and DNA- β . In order to simplify the phylogenetic reconstruction, closely related sequences of the same begomovirus species were skipped and only one representative sequence was taken for each begomovirus. Nucleotide and amino acid sequence homologies were analysed using BioEdit version 7.0 [6]. Multiple alignments of sequences were done using Clustal W program [22], and phylogenetic trees were constructed with MEGA 4.0 software [21] using neighbor joining methods. Statistical support for clades was estimated using 1,000 bootstrap iterations.

Results and Discussion

The complete sequence of DNA-A of begomovirus isolated from pumpkin was 2756 nt long. In a BLAST search, the complete sequence of DNA-A showed 99% nucleotide sequence identity with *Tomato leaf curl Palampur virus*. The DNA-A component of this isolate has a typical Old World bipartite begomovirus genome organisation encoding six ORFs—two (AV1, AV2) in the virion sense and four (AC1, AC2, AC3 and AC4) in viral complementary sense (online resource 1). There was a 273 nt long intergenic region (IR) separating the ORFs which carried the non-nucleotide sequence (TAATATTAC) in a conserved inverted repeat with the potential to form a stem loop

structure as in case of other Geminiviruses. All the ORFs showed more than 96% nucleotide sequence identity with ToLCPMV ruling out any possibility of recombination. The sequence was submitted to the GenBank (Accession No. FJ931537). The nucleotide and amino acid sequences of proteins encoded by DNA-A showed highest similarities to, ToLCPMV (Accession No. AM884015), ToLCNDV (Accession No. AM286434) and SqLCCNV (Accession No. DQ026296). Based on species demarcation threshold of 89% for begomoviruses [5], the virus isolated from pumpkin is an isolate of ToLCPMV for which we propose the isolate descriptor ToLCPMV-[India: Varanasi: Pumpkin:2008] (-[In: Var: Pum: 08]). Phylogenetic tree showed that the DNA-A of this begomovirus forms a cluster with other ToLCPMV (AM884015; EU547682) isolates (Fig. 1).

The complete sequence of betasatellite of begomovirus isolated from pumpkin was 1361nt long. It showed all the typical features of betasatellites [2], consisting of a single ORF in complimentary sense (β C1) and a region of sequence rich in adenine. The sequence was submitted to the GenBank (Accession No. GQ330541). The β C1 encoded protein comprised 120 aa and the adenine rich region extended from 717 to 989. The complete sequence of betasatellite showed 92% nucleotide sequence identity with PepLCV satellite betasatellite in BLAST analysis. The percent nucleotide identity of the complete betasatellite and amino acid identity of encoded protein with the respective sequences of different betasatellites used for analysis are listed in online resource 2. In a phylogenetic analysis based on complete sequence of betasatellite, the sequence of present isolate clustered with that of PepLCB (Accession No. EF190215). Based on the species demarcation threshold of 78% nucleotide sequence identity [4] the isolate from pumpkin at Varanasi is an isolate of PeLCB for which we propose the isolate descriptor PeLCB-[In: Var: Pum: 08] (Fig. 2).

Infection by multiple viruses and betasatellites is increasing the complexity of pumpkin yellow vein mosaic disease. ToLCNDV is the third begomovirus reported to be associated with this disease in India. ToLCPMV is a recently identified [9] unclassified begomovirus. It is reported only on tomato, cucumber (*Cucumis sativus* L.) and melon (*Cucumis melo*) [9, 7]. This is the first report of ToLCPMV infecting pumpkin. We have been able to detect mixed infection of ToLCPMV, ToLCNDV (Accession No: GQ225737) and SLCCNV (Accession No: GQ225733) from pumpkin plants showing yellow vein mosaic symptoms from same field in Varanasi. It is believed that ToLCPMV evolved in a cucurbit host infected with ToLCNDV and another yet unidentified cucurbit infecting begomovirus [9, 7]. Our finding adds strength to this hypothesis. The sequence of betasatellite amplified from the same sample was 92% identical to that of PepLCB,

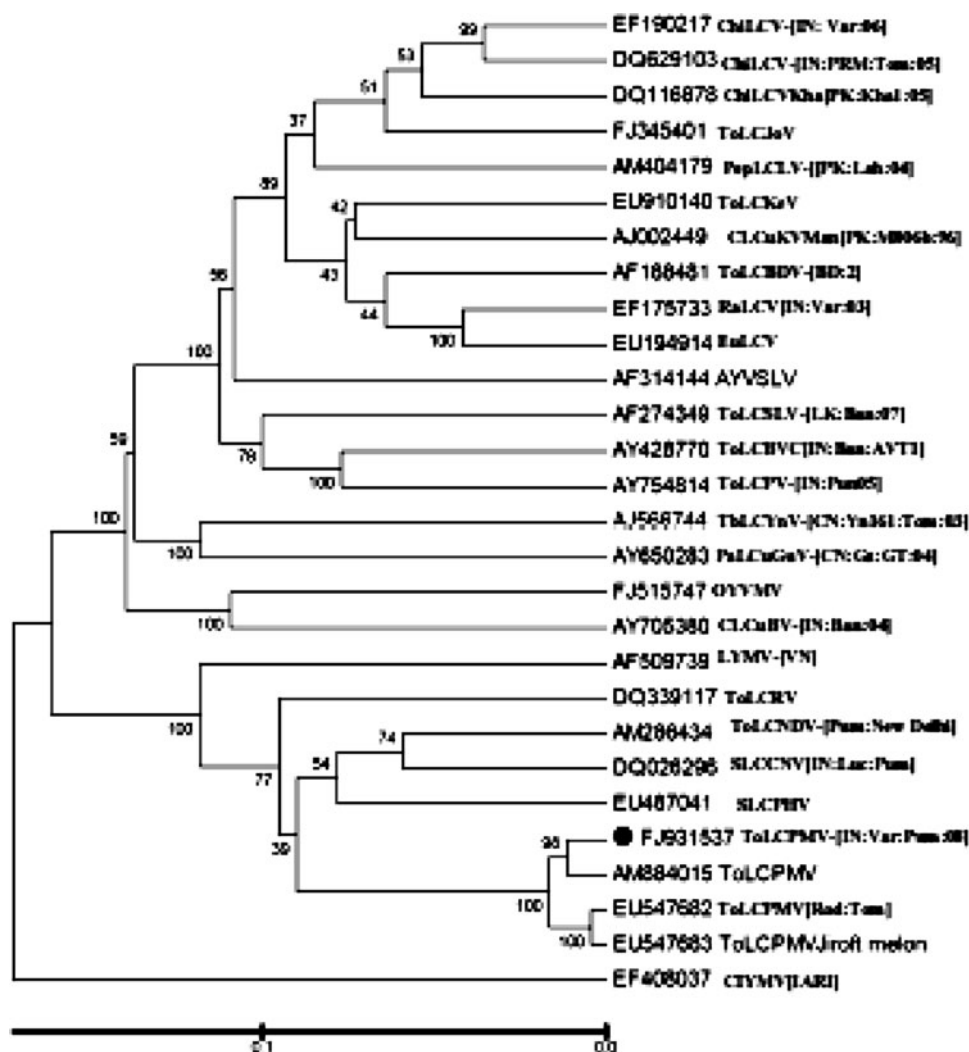


Fig. 1 Phylogenetic tree DNA-A of selected begomoviruses. The tree was constructed using MEGA 4.1 at 1,000 bootstrap values. The numbers at the nodes indicate bootstrap values (%). The begomovirus acronyms used are: *Ageratum yellow vein Sri Lanka virus* (AYVSLV), *Bhendi yellow vein Delhi virus* (OYVMV), *Chilli leaf curl virus- India* [India: PRM: Tomato: 2005] (ChiLCVIN[IN:PRM:Tom:05]), *Chilli leaf curl virus- India* [India:Varanasi:2006] (ChiLCV-[IN:Var:06]), *Chilli leaf curl virus-Khanewal* [Pakistan:Khanewal1:2004] (ChiLCV[Kha][PK:Kha1:05]), *Clerodendron yellow mosaic virus isolate* [IARI] (CIYMV [IARI]), *Cotton leaf curl Bangalore virus*-[India:Bangalore:2004] (CLCuBV-[IN:Ban:04]), *Cotton leaf curl Kokhran virus* -Manisal [Pakistan:Manisal806b:1996] (CLCuKV-Man[PK:M806b:96]), *Euphorbia leaf curl virus* [Pusa (EuLCV), *Luffa*

yellow mosaic virus (LYMV), *Pepper leaf curl Lahore virus* (PepLCLV), *Papaya leaf curl Guangdong virus* (PaLCuGuV), *Radish leaf curl virus* (RaLCV), *Squash leaf curl China virus*-[India: Lucknow: Pumpkin] (SLCCNV[IN:Luc:Pum]), *Squash leaf curl Philippines virus* (SLCPHV), *Tobacco leaf curl Yunnan virus* (TbLCYnV), *Tomato leaf curl Bangalore virus* (ToLCBV), *Tomato leaf curl Bangladesh virus* (ToLCBDV), *Tomato leaf curl Joydebpur virus* (ToLCJoV), *Tomato leaf curl Kerala virus* (ToLCKeV), *Tomato leaf curl New Delhi virus* (ToLCVND), *Tomato leaf curl Palampur virus*-[Roodan:tomato] (ToLCPMV Rod: tom), *Tomato leaf curl Palampur virus* (ToLCPMV), *Tomato leaf curl Pune virus* (ToLCPV), *Tomato leaf curl Rajasthan virus* (ToLCRV), *Tomato leaf curl Sri Lanka virus* (ToLCSLV)

which is well above the established threshold of 78% [4] for designating new betasatellite species. Thus this betasatellite is a strain of PeLCVB has been designated as PeLCVB-[In: Var: Pum: 08]. Chilli grown in the nearby fields of Varanasi shows severe infection by *Pepper leaf curl virus*. PeLCB would have come through whiteflies from the nearby chilli crop that showed severe leaf curl symptoms. Betasatellites are associated with majority of

monopartite begomoviruses [15] ToLCPMV is not known to have an associated betasatellite [9, 7]. Due to the limited number of clones sequenced or using a restricted primer rather than the universal primer for the detection of DNA-A of begomoviruses, we were unable to detect the presence of any monopartite begomovirus from the same sample. It is possible that in addition to the three bipartite begomoviruses already reported there are other monopartite

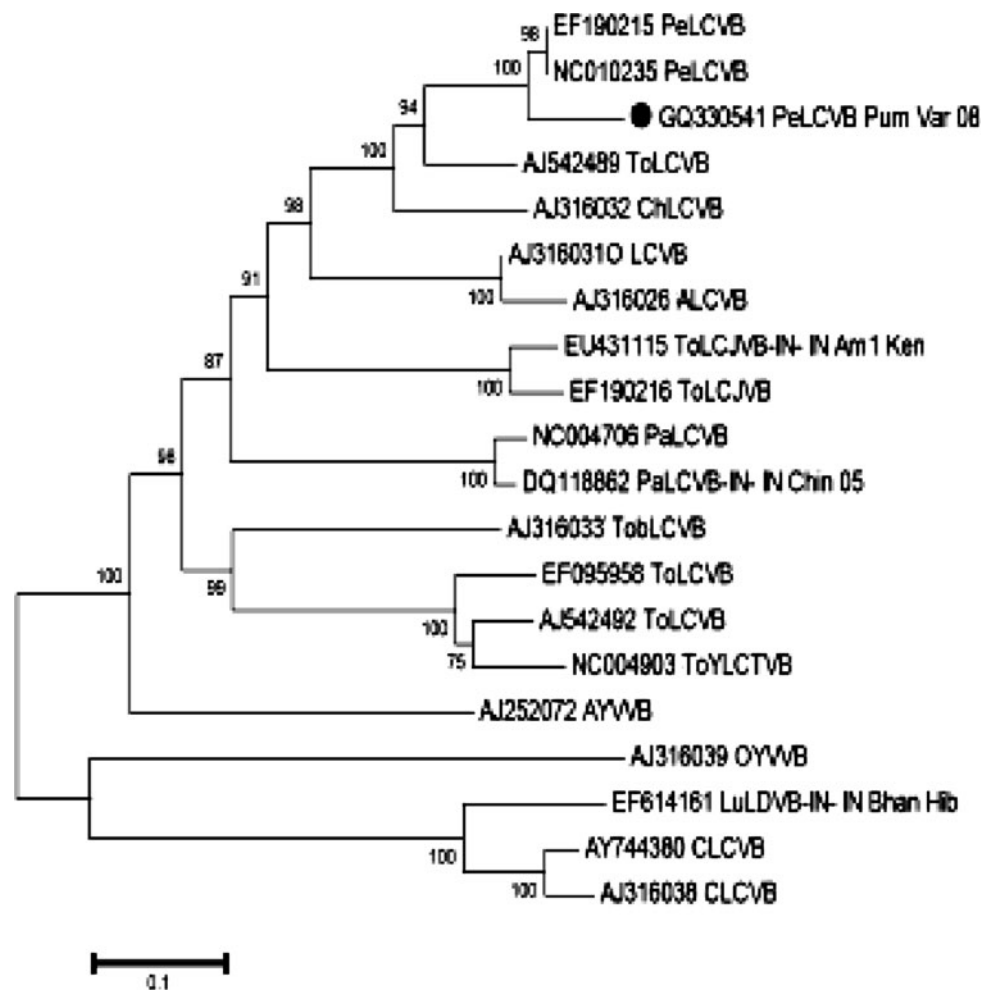


Fig. 2 Phylogenetic tree based on selected sequences of betasatellites of begomoviruses. The tree was constructed using MEGA 4.1 at 1,000 bootstrap values. The betasatellite acronyms used are: Ageratum leaf curl betasatellite (ALCB), Ageratum yellow vein betasatellite (AYVVB), Chilli leaf curl betasatellite (ChLCB), Cotton leaf curl betasatellite (CLCB), Ludwigia leaf distortion betasatellite [India:Bhangha:Hibiscus:2006] (LuLDB [IN:Bhan:Hib:06]), Okra leaf curl betasatellite (OLCB), Okra yellow vein betasatellite

(OYVVB), Papaya leaf curl betasatellite (PaLCB), Papaya leaf curl betasatellite [India:Chinthapalli:2005] (PaLCB [IN:Chinth:05]), Pepper leaf curl betasatellite (PeLCB), Tobacco leaf curl betasatellite (TobLCB), Tomato leaf curl betasatellite (ToLCB), Tomato leaf curl Joyedebpur betasatellite (ToLCJB), Tomato leaf curl Joyedebpur virus (ToLCJB), Tomato leaf curl Joyedebpur betasatellite [India:Amadalavalasa1:Kenaf:2007] (ToLCJB-IN-[IN:Am:Ken:07]), Tomato yellow leaf curl Thailand betasatellite (ToYLCTB)

begomoviruses associated with pumpkin yellow vein mosaic disease. Infectivity studies using cloned components of genome of individual viruses and satellites would bring to light the possible synergistic effect of the components in enhancing the losses.

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