Short Communication

FIRST REPORT OF CHERRY TOMATO LEAF CURL VIRUS AND ASSOCIATED DNA SATELLITES INFESTING AN INVASIVE WEED IN PAKISTAN*

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SUMMARY

A complete begomovirus complex (including a begomovirus, beta- and alphasatellite) was amplified from an invasive and widespread weed, *Parthenium hysterophorus*, and obtained clones were sequenced. Results showed that isolated begomovirus is Cherry tomato leaf curl virus (CToLCV) sharing highest nucleotide (nt) sequence identity of 94.2% to an Indian isolate of CToLCV. The Alphasatellite isolated from this infection was found to be a recombinant showing the highest nt sequence identity (94.3%) to Tobacco curly shoot alphasatellite (TbCSA), whereas the isolated betasatellite shared the highest nt sequence identity (97.5%) to Papaya leaf curl betasatellite (PaLCuB). To the best of our knowledge this is a novel begomovirus disease complex infecting *P. hysterophorus* in Pakistan. Furthermore, this is the first report of CToLCV associated with DNA satellites and infecting a weed host in Indo-Pak subcontinent.

Keywords: *Parthenium hysterophorus*, begomovirus, alphasatellite, betasatellite.

Whitefly (*Bemisia tabaci*) transmitted begomoviruses (family *Geminiviridae*) having circular and single-stranded (~2.8kb) DNA genomes are among the most diverse and destructive plant pathogens worldwide. Till now *ca*. 300 species of begomoviruses have been identified (Brown *et al*., 2012; Adams *et al*., 2013). Begomoviruses are either bipartite, having two genome components DNA-A and DNA-B, or monopartite, having single genome component equivalent to DNA-A of bipartite begomoviruses (Brown *et al*., 2012). Six genes, involved in replication, encapsidation and pathogenicity, are transcribed on DNA-A of bipartite and/or monopartite begomoviruses; two in virion-sense and four in complementary-sense orientation (Rojas *et al*., 2005). The majority of monopartite begomoviruses are associated with *ca*. 1.4 kb ssDNA-satellite molecules referred to as alphasatellites and betasatellites. Betasatellites, encoding a single ORF in the complementary sense, are more numerous and dependent on helper virus for replication, encapsidation, *in planta* movement and transmission (Briddon and Stanley, 2006). Self-replicating alphasatellites, occasionally associated with begomovirus disease etiology, are also dependent on their helper virus for encapsidation, movement and insect transmission (Mansoor *et al*., 1999). Besides, a third type of DNA-satellite molecules have also been found recently, called deltasatellites (Fiallo-Olivé *et al*., 2016). From last three decades these viruses have been proliferated more by recombination, component capture, genetic drift, mutation, pseudo-recombination, vector (*B. tabaci*) proliferation and environmental changes, which purportedly help these viruses to overcome host plant resistance and extend their host range by infesting new hosts (Sattar *et al*., 2013; Kenyon *et al*., 2014). Moreover, perennial weeds have worsened the situation by providing a melting pot for evolution of diverse viruses through recombination (Mubin *et al*., 2012; Leke *et al*., 2012).

Parthenium hysterophorus (congress grass), an invasive weed of the family *Asteraceae*, has inadvertently proliferated across major cultivating and non-cultivating areas of Pakistan (Khan *et al*., 2014) causing 40-90% reduction in grain and forage yield (Tanveer *et al*., 2015). This weed serves as an alternate host for many insect pests of crop plants and a recombination reservoir for many plant viruses (Mubin *et al*., 2012; Shabbir, 2014). Several plant viruses have been reported from *P. hysterophorus* for example, *Tobacco streak virus* (Sharman *et al*., 2015), *Potato virus X*, *Potato virus Y* (Cordero, 1983), *Tomato leaf curl*

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Fig. 1. Typical *Parthenium hysterophorus* plants showing severe leaf curling **a),** as compared to the healthy plant **b)**.

virus (Govindappa *et al*., 2005) and *Tomato leaf curl Karnataka virus* (Zaffalon *et al*., 2012). In this study, viruses of a complete begomovirus complex have been isolated for the first time in Pakistan, from *P. hysterophorus*.

Leaf samples from five *P. hysterophorus* plants showing leaf curl symptoms (Fig. 1) were collected in December, 2013, from the vicinity of University of the Punjab, Lahore (31.29° N, 74.17° E), Pakistan and subjected to DNA extraction (Doyle and Doyle, 1990) and rolling circle amplification (RCA) using phi29 DNA polymerase (ThermoScientific) (Haible *et al*., 2006). In total reaction volume *ca*. 100 ng of total genomic DNA was mixed with 3 µl 10 mM dNTPs, 1 µl of 50 µM random hexamer primers, 2 µl of 10X phi29 DNA polymerase reaction buffer and nuclease free water to make the volume up to 18 µl. The mixture was incubated at 95ºC for 3 min and snap cooled at ice. After adding 0.75 µl phi29 DNA polymerase, 0.2 µl of pyrophasphatase and 1.05 µl nuclease free water, the reaction was run for 18 h at 28°C. The reaction was stopped by incubating at 65°C for 10 min. The diluted RCA product was exploited in PCR to amplify whole begomovirus complex.

Table 1. Nucleotide sequences of primers used to detect and amplify begomoviral genome and associated DNA-satellites in *P. hysterophorus* from Pakistan.

Primers	Primer Sequences	PCR product
AC1048	GGRTTDGARGCATGHGTACATG	Coat Protein
AV494	GCCYATRTAYAGRAAGCCMAG	
Begomo-F	ACGCGTGCCGTGCTGCTGCTGCCCCCA	CToLCV
Begomo-R	ACGCGTATGGGCTGYCGAAGTTSAGACG	
Beta01	GGTACCACTACGCATCGCAGCAGCC	PaLCB
Beta02	GGTACCTACCCTCCCAGGGGTACAC	
ParA-F	AAGAATCACACGAACAGTGTC	CToLCV
ParA-R	GGTCTTGATGTTCTCATCCA	
ParB-F	GTTCTGAATTCATATGATCAT	PaLCB
ParB-R	GAATCTTCTGAATTCATATGA	
DNA101	CTGCAGATAATGATGTAGCTTACCAG	TbCSA
DNA102	CTGCAGATCCTCCACGTGTATAG	

Initially, coat protein (CP) was amplified by using degenerate PCR primers (AC1048/AV494) (Wyatt and Brown, 1996). The obtained CP sequences were used to design an abutting primer pair ParA-F/ParA-R (Table 1) to amplify full-length begomovirus genome. The designed primers (ParA-F/ParA-R) and the universal primers (Begomo-F/Begomo-R; Table 1) (Briddon and Markham, 1995) were employed independently to amplify full-length begomovirus genome. Subsequently, betasatellite and alphasatellite were amplified using the primers ParBF/ParBR (Table 1) and DNA101/DNA102 (Bull *et al*., 2003), respectively. All PCR products were cloned into the cloning vector pTZ57R/T (InsTAclone PCR cloning kit, Thermoscientific) and sequenced completely from First BASE Laboratories Sdn Bhd, Malaysia. The obtained sequences were analyzed using BLASTn and ORF finder tools (NCBI webpages). The nucleotide (nt) sequence identities were determined by Species Demarcation Tool (SDT) (Muhire *et al*., 2014), whilst phylogenetic dendrograms were constructed in Mega7 software (Tamura *et al*., 2013) using Neighbour-joining (N-J) algorithm. Recombination Detection Program (RDP4) was used for recombination analysis of the obtained begomovirus and the alphasatellite sequences (Martin *et al*., 2015). Six different algorithms available in RDP4, including BootScan (Martin *et al*., 2005), Chimera (Posada and Crandall, 2002), GENECONV (Padidam *et al*., 1999), MaxChi (Maynard Smith, 1992), RDP (Martin and Rybicki, 2000) and SiScan (Gibbs *et al*., 2000), were used with highest acceptable P-value set at 1×10^{-5}, to predict any putative recombination breakpoint.

Two full-length begomovirus clone sequences (pTParA2 and pTParA10) were deposited in the GenBank (accession nos. LN906594, LN906593). The genome size of each resultant sequence was 2748 and 2749 nt in length, respectively with genome organization similar to the Old World (OW) monopartite begomoviruses. Both clones were 99% identical to each other and shared maximum nt sequence identity at 94.2% with Cherry tomato leaf curl

Table 2. Pairwise percent (%) nucleotide sequence comparison of full-length genome of pTParA2 and pTParAa10 identified from *P. hysterophorus* with selected begomoviruses from the GenBank database using MUSCLE alignment in Sequence Demarcation Tool (SDT).

Accession	Abbreviation	Host species	Country	Year	Length (bp)	% identity
DQ629102	CToLCV	Cherry Tomato	India	2006	2751	**94.2**
HM143914	PaLCuV	*Nicotiana glutinosa*	India	2010	2746	92.1
HM851186	TLCKV	*Lycopersicon esculentum*	India	2009	2760	90.3
FM955601	PaLCuV	*Rhynchosia capitata*	Pakistan	2007	2754	90.3
JX678965	SLCV	Sunflower	India	2011	2761	90.2
HM007094	TLCKV	Chilli	India	2008	2760	90.1
FN678906	PaLCuV	*Alcea rosea*	Pakistan	2006	2752	90.0
U38239	TLCV	*Lycopersicon esculentum*	India	1995	2759	89.8
AJ436992	PaLCuV	Cotton	Pakistan	2003	2753	89.6
AJ507777	PaLCuV	*Croton bonplandianum*	India	2002	2757	89.5
FN543112	PaLCuV	*Croton glandulosus*	Pakistan	2006	2744	89.0
GQ200446	PaLCV	*Crotalaria juncea*	India	2008	2738	88.8
HM007096	PepLCBV	Chilli	India	2008	2754	88.8
GQ200448	PaLCV	*Crotalaria juncea*	India	2008	2738	88.7
JX524173	PepLCBV	Chilli	India	2008	2754	88.5
JN135233	PaLCV	*Amaranthus cruentus*	India	2011	2746	88.4
FJ593629	PaLCV	Radish	India	2009	2759	88.3

The highest percentage of nt sequence identity of pTParA2 and pTParA10 is underlined and bold.

virus (CToLCV) (DQ629102) (Pandey *et al*., 2010) and 92.1% with *Papaya leaf curl virus* (PaLCuV) (HM143914) identified from tomato and *Nicotiana glutinosa* in India, respectively (Table 2). Thus, following the guidelines of the International Committee for the Taxonomy of Viruses (ICTV), which fix species demarcations limits at a genome nucleotide sequence identity <91% (Brown *et al*., 2015), both identified clones are new isolates of an already existing species of CToLCV. In phylogenetic analysis both begomovirus isolates formed a well-supported clade (98% bootstrap value) with CToLCV (DQ629102) (Fig. 2A). RDP4 analysis predicted three putative recombination break points in both isolates at nt coordinates 1372-1455, 2107-2716, 2571-2679 and 550-1079, respectively with PaLCuV and CToLCV as major parents with highest P-values 6.47×10^{-09}, 6.02×10^{-08}, 2.22×10^{-06} and 5.46×10^{-09}, respectively (**Supplementary Table 1a**).

The clone pTPar-al2 (LN906596) comprised of 1386 nt in length and showed 94.3% nt sequence identity with Tobacco curly shoot alphasatellite (TbCSA) (HQ407396) identified from sunflower in India. The N-J phylogenetic dendogram grouped pTPar-al2 into a well-supported clade with TbCSA separated from other TbCSA isolates (Fig. 2B). Moreover, two recombination events were observed for pTPar-al2: first at nt coordinates 810-1334 with Chili leaf curl alphasatellite (ChLCA) as major parent while Tomato yellow leaf curl Yunnan alphasatellite (TYLCYnA) as minor parent whereas, second event occurred at nt position 204-461 with TYLCYnA as major parent and an unknown minor parent, respectively (**Supplementary Table 1b**). These results were corroborated by multiple algorithms of RDP4 with highest P-values as 6.25×10^{-18} and 1.26×10^{-11}, respectively. Thus, following species demarcation >83% and phylogenetic analysis the pTPar-al2 is named as a new isolate of TbCSA: TbCSA-[PK:pTPar-al2:Par:15], identified from Pakistan.

The clone pTParB1 (LN906595) was 1362 nt in length and shared maximum nt sequence identity at 97.5% with Papaya leaf curl betasatellite (PaLCuB) (DQ118862), reported from India. In phylogenetic tree, pTParB1 clustered well with PaLCuB (DQ118862) (Fig. 2C). Thus pTParB1 is a new isolate of PaLCuB identified from *P. hysterophorus* in Pakistan.

Although, CToLCV has recently been reported from tomato crop in India (Pandey *et al*., 2010), to the best of our knowledge this is the first report where, CToLCV along with DNA-satellites has been isolated from *P. hysterophorus* in Pakistan. Our results highlight the capacity of *P. hysterophorus* to accept crop infecting begomoviruses and associated DNA-satellites thus, could serve as an alternate host for begomoviruses. The outcomes of these results also highlight the ability of CToLCV to trans-replicate and support non-cognate DNA-satellites. The capability of *P. hysterophorus* as a perennial, invasive and alternate host of begomoviruses can limit the controlling strategies, which are currently employed to control these viruses in elite cultivars. Consequently such a situation would make the story much more complex in future.

Note added in proof

During the final review stages of this manuscript, a recently published paper by Kumar *et al.* (2016) was brought to our attention, reporting a similar begomovirus disease complex infecting *P. hysterophorus* in India. The begomovirus disease complex was reported as *Tomato leaf curl virus* (ToLCV), Ageratum yellow vein India alphasatellite (AYVIA) and Papaya leaf curl betasatellite (PaLCuB). We have compared the reported sequences with the sequences

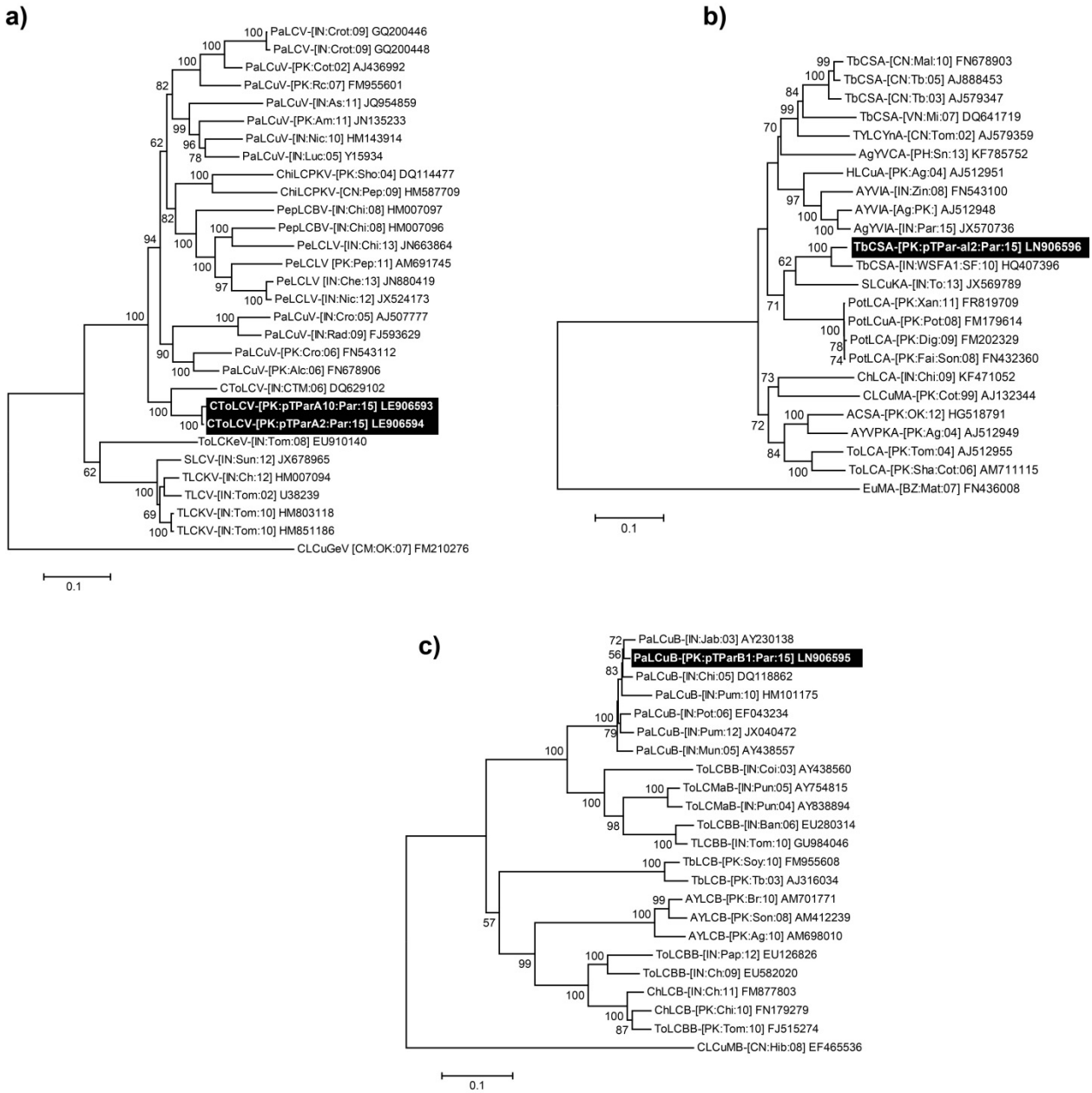

Fig. 2. Phylogenetic analysis based on complete nt sequences of begomovirus **(A)**, alphasatellite **(B)** and betasatellite **(C)** isolates, using neighbor joining algorithm in MEGA7. All the isolates identified from Pakistan in this study are shown in bold white text on black background. Horizontal lines are representing nt substitutions per site. Numeric values at branch nodes are representing percent bootstrap values higher than 60 percent (1000 replicates). All isolates used for comparison are represented by their respective accession numbers in the trees. All abbreviations for begomovirus, alpha- and betasatellite isolates are according to Brown *et al.* (2012) and Briddon *et al.* (2012), respectively.

described in this paper using SDT analysis. We have found that ToLCV shared 89.5-89.8% nt sequence identity with CToLCV, AYVIA shared 78.5% nt sequence identity with TbCSA, and PaLCuB shared 93.5% nt sequence identity with PaLCuB reported in this paper. Thus following ICTV guidelines for species demarcation >91%, the begomovirus isolates reported in this paper are new isolates of CToLCV, which are quite distinct from ToLCV reported by Kumar *et al.* (2016). Moreover, the phylogenetic analysis grouped CToLCV and ToLCV in two well supported separate clades (data not shown). The TbCSA and AYVIA are also found to be new isolates of two distinct species, which are grouped in two well supported separate clades in the phylogenetic analysis (data not shown). However, PaLCuB reported by Kumar *et al.* (2016) and PaLCuB reported in this paper are representing two different isolates of the same species.

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