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MOLECULAR ANALYSIS OF *PEDILANTHUS LEAF CURL VIRUS* AND ASSOCIATED SATELLITES INFECTING *Chenopodium album* IN PAKISTAN

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Whitefly-transmissible begomoviruses (Family-*Geminiviridae*) are the major pathogens of economically important crops and weeds. In 2004-05, during field surveys in Faisalabad, Pakistan, symptoms of leaf curling and leaf deformations were spotted on leaves of *Chenopodium album*. *Chenopodium album* is also known as Bathu (local name). This plant is commonly found in crop fields and around water channels. Fifteen plant leaves showing upward leaf curling were collected from different farmer's fields. Complete virus and associated satellite molecules were amplified through rolling circle amplification (RCA) followed by restriction digestion with suitable enzyme, cloning and sequencing. Basic local alignment search tool (BLAST) showed that leaf curling disease of *C. album* is associated with Pedilanthus leaf curl virus (PeLCV) along with two different types of betasatellite molecules i.e., Digera yellow vein betasatelite (DiYVB), Cotton leaf curl Multan betasatellite (CLCuMB) and one alphasatellite i.e., Ageratum enation alphasatellite (AEA). Sequence analysis showed that PeLCV is a new variant showing 95-98% sequence homology to PeLCV isolated from India and Pakistan. Associated betasatellites i.e., DiYVB and CLCuMB showed 96-99% sequence homology to satellites isolated from India and Pakistan. Ageratum enation alphasatellite associated with PeLCV was also a new combination and showed 99% sequence homology with other isolates from Pakistan. This is the first report of PeLCV and associated satellites infecting *C. album.* The data presented in this paper would help in understanding the diversity and etiology of begomoviruses in *C. album.*
Keywords: *Chenopodium album* L., pedilanthus leaf curl virus, digera yellow vein betasatellite, cotton leaf curl Multan betasatellite, ageratum enation alphasatellite, phylogenetic analysis.

INTRODUCTION

In tropical and subtropical regions of Pakistan, single stranded DNA (ssDNA) viruses i.e., begomoviruses often threaten field crop production (Hussain *et al*., 2005; Tahir and Haider, 2005; Mubin *et al*., 2009; Juarez *et al.*, 2013). These whitefly-transmitted viruses with genome size of 2.8 kb are encapsidated in twinned-icosahedral particles. The emergence of diverse and recombinant begomoviruses could be resulting from multiple and mixed infections of different species of viruses. Due to limitation and homogeneity of resistance sources, new strains of viruses are emerging day by day (Amrao *et al*., 2010). Weeds perform as alternate host for crop-infecting begomoviruses when main cropping season is not there. There are many reports of begomoviruses and associated satellites i.e., alphasatellite and betasatellite (Briddon *et al*., 2003; Briddon *et al*., 2004) infecting weeds, like *Sonchus arvensis* and *Alternanthera* (Guo and Zhou, 2005; Mubin *et al*., 2009; Mubin *et al*., 2010; Mubin *et al*., 2012). Betasatellites are pathogenicity determinant molecules of disease complex while the role of alphasatellites is still unclear. Betasatellites are 1300-1400 bp long having single coded protein i.e., betaC1 that is involved in pathogenicity; symptoms severity and can act as a suppressor of gene silencing. Alphasatellites are 1300-1400 bp long depending on the movement and transmission of begomoviruses to other host plants (Briddon *et al.*, 2004). Role of these molecules in disease etiology is still not clear, though these are emerging as the most diverse satellites in crop plants and weeds (Briddon *et al*., 2004; Fiallo-Olivé *et al*., 2012). The source of alphasatellites is also traced back in nanoviruses (another class of single-stranded DNA viruses) (Briddon *et al*., 2004). The genome of alphasatellites consists of origin of replication similar to nanoviruses, A-rich region and an alpha-Rep gene (Briddon *et al*., 2004). These are diverse in nature and do not exhibit any role in the development of disease symptoms though alpha Rep protein, reported to be viral suppressors of RNA silencing (Nawaz-ul-Rehman *et al*., 2010). *Chenopodium album* is a widely grown weed in Pakistan, India and China. The *C. album* belongs to family *Amaranthaceae* and is an annual herb. It is found in subtropical and tropical areas of the world. *Chenopodium album* can flourish in the wide-ranging ecological conditions, commonly found near the damp areas and crop fields. In Pakistan, *C. album* is found mostly in fields, around water channels and shows the symptoms of leaf curl disease. Pedilanthus leaf curl virus is a monopartite begomovirus, which is quite wide spread in Pakistan. There

are several published reports of PeLCV infecting four different hosts i.e., *Pedilanthus tithymaloides* (Tahir *et al*., 2009), *Glycine max* (Ilyas *et al*., 2010), *Sesbania bispinosa* (Zaidi *et al*., 2016) and *Raphanus sativus* (Ismail *et al.*, 2017). In all cases, PeLCV was associated with tobacco leaf curl betasatellite (TbLCB) (Tahir *et al*., 2009), thus continuously expanding its host range (Zaidi *et al*., 2016; Ismail *et al*., 2017). Main purpose of this study is to understand the biodiversity of begomoviruses associated with *C. album.* During a survey conducted in 2004-05, natural occurrence of leaf curl disease was observed on *C. album.* We analyzed *C. album* plants for the possible presence of components of begomovirus disease complex producing leaf-curling symptoms. To our surprise, there were diverse satellites i.e., Digera yellow vein betasatelite (DiYVB), Cotton leaf curl Multan betasatellite (CLCuMB) and Ageratum enation alphasatellite (AEA) associated with PeLCV in the plants. This data will help to understand the importance of weeds as alternate hosts in boosting up the evolution rate of begomoviruses. Information about the diversity of begomoviruses in weed hosts could assist in developing control strategies against begomoviruses.

MATERIALS AND METHODS

***Virus sources*:** Fifteen *Chenopodium album* symptomatic plants showing leaf curling were collected from different areas of Faisalabad in the farmer's fields. The asymptomatic leaves were taken as negative control. Young leaves were collected, labeled and transported on ice to lab and stored at -80°C. Cetyl trimethyl ammonium bromide (CTAB) method was applied to isolate DNA from leaves described by (Doyle,1990).

***Cloning and Sequencing of viral molecule*s:** Total DNA obtained from leaves was preceded for rolling circle amplification (RCA) using ϕ29 DNA polymerase (Blanco *et al*., 1989). The RCA product of *C. album* DNA was restricted using different restriction enzymes i.e., *Sal*I, *Bgl*II, *Hin*dIII, *Kpn*I and *Eco*RI. Fragments of sizes 2.8 kb and 1.4 kb, which could be begomovirus and associated satellites, were generated by restriction with *Sac*I enzyme. The restricted product was gel eluted and cloned into the pTZ57R vector at *Sac*I restriction site (Fermentas) and four clones were completely sequenced to generate clone names pVirol152-2.8, pVirol153-1.4, pVirol154-1.4 and pVirol155-1.4.

***Sequence assembly and analysis*:** Begomovirus and associated satellites were completely sequenced by the dideoxynucleotide chain termination DNA sequencing method on an automated sequencer (Sanger *et al*., 1977). DNA analysis (laser gene package by v8; DNAStar Inc., Madison, WI, USA) was used for the sequence alignment and analysis. The sequences were assembled by contig assemblies integrated in SeqMan module of DNA-STAR. Sequence wise matching for homology was done by applying the MegAlign module of the Lasergene program. Phylogenies were analyzed by aligning the sequences first by applying CLUSTAL-W, and by Neighbor joining method of phylogeny building in MEGA7 software. The GenBank accession numbers for the virus and satellites isolated in this study are: KY937947, KY937948, KY937949and KY937950, respectively. The other viral sequences were retrieved from GenBank and virus abbreviations were used according to ICTV rules (Zerbini *et al*., 2017).

***SDT and Phylogenetic analysis*:** Sequences were aligned using molecular evolutionary genetic analysis (MEGA7) (Tamura *et al.*, 2013) by applying the CLUSTAL-W program (Thompson *et al*., 1994) and dendrogram was constructed using neighbor-joining program with 1000 bootstrap replication values. Due to prerequisite of begomovirus taxonomy (Brown *et al*., 2015), further confirmation of virus was performed using Multiple Sequence Comparison by Log-Expectation (MUSCLE) alignment in species demarcation tool (SDT).

RESULTS

***Cloning and sequencing of viral molecules from Chenopodium album*:** Leaves from 15 *Chenopodium album* plants showing typical symptoms of begomovirus infection (Figure 1A) were collected from different farmers' fields of cotton of the Punjab province in 2004-05. At that time crops like cotton, sugarcane and different vegetables were present in the field. At every place, *C. album* plants were found to be showing similar symptoms of leaf curling (Figure 1A). Rolling circle amplification (Blanco *et al*., 1989) was used to amplify all circular ssDNA molecules from infected samples of *C. album.* Restriction with *SacI* enzyme yielded 2.8 kb and 1.4 kb fragments i.e., size of begomovirus and associated satellites. These fragments were cloned in pTZ57R cloning vector and then completely sequenced. Sequencing of these cloned fragments confirmed the presence of begomovirus and associated satellites.

***Sequence and Phylogenetic analysis of PeLCV*:** Total of around 25 DNA-A molecules of size 2.8 kb were cloned from fifteen plant samples collected from different farmers' fields and partial sequencing showed that all molecules represent maximum sequence similarity to Pedilanthus leaf curl virus (PeLCV). These results showed that only PeLCV might be present in all infected *C. album* samples. One molecule was completely sequenced and analyzed. Diagnostic primers for DNA-B (Rojas *et al*., 1993) were also used to find out its presence in infected samples but there was no PCR amplification for any of these samples. These viruses isolated from *C. album* showed the highest homology with the already reported PeLCV (PeLCV-[PK:Isd:Capsicum:14]-KY978406) and PeLCV-[PK:Fsd:Glycinemax:15]-KX671562, although samples were taken from distant places (Figure 2). Phylogenetic analysis showed that sequences of the PeLCV had a higher

Figure 1. *Chenopodium album* plant A) Symptomatic plant showing leaf curling symptoms; B) Asymptomatic

level of nucleotides similarities with the viruses found from Pakistan and India PeLCV-[PK:Isd:Capsicum:14]-KY978406,PeLCV-[PK:Mul:04]-AM712436andRaLCV-[IN:Carrot:16]-KX168427 (Fig. 2). The DNA-B component of Tomato leaf curl New Delhi virus (ToLCNDVB-IN:ND:Luffa:10-HM989846) was used as out group.

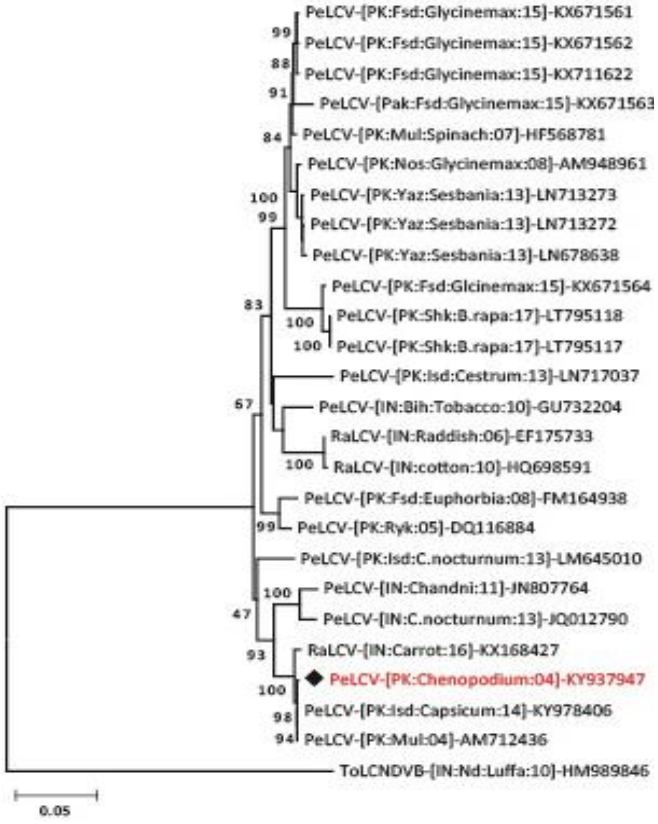

Figure 2. Phylogenetic dendrogram based upon selected complete sequences of PeLCV. Begomovirus sequences used for comparison were downloaded from GenBank. The database accession number in each case is given. The viral sequence associated with leaf curl disease of *Chenopodium album* is indicated by shapes and red color.

***Sequence and Phylogenetic analysis of CLCuMB*:** Total of around 50 molecules of size 1.4 kb were cloned from fifteen plant samples and completely sequenced. Basic local search alignment tool (BLAST) analysis showed 12 molecules as cotton leaf curl Multan betasatellite (CLCuMB) having 95-99% sequence homology with already reported CLCuMB sequences and 17 molecules as Digera yellow vein betasatelite (DiYVB) having 97-99% sequence homology with DiYVB, already reported from India and Pakistan. One molecule had a defective DiYVB with 766bp length. These results showed that two different betasatellites are present in association with PeLCV in all infected samples though these are new variants of already reported betasatellites. In the phylogenetic tree, Cotton leaf curl Multan betasatellite i.e., CLCuMB clustered with the betasatellites from India and Pakistan i.e.(CLCuMB-[PK:Okara:cotton]-HF549188) and CLCuMB-[IN:Cotton:2009]-JF502380 (Figure 3A). Second betasatellite i.e., DiYVB clustered with another species of betasatellites from India and Pakistan (DiYVB-[IN: Carrot: 17] - KY661758), (DiYVB - [PK: Tobacco:09]-GQ478344) and (DiYVB - [IN: Sik: Rosa: 13] - KF584009) (Figure 3A). Ageratum enation alphasatellite molecule (AEA - [PK: Lhr: 17] - LT716987) of similar length was used as out group.

***Sequence and Phylogenetic analysis of AEA*:** The 21 clones of 1.4 kb size (out of 50) from 15 infected plants showed maximum sequence homology (99%) to Ageratum enation alphasatellite (AEA-[PK:Raj:Croton:12]-HG417078). These results showed that only one type of alphasatellite is associated with PeLCV in *C. Album.* In the phylogenetic tree, AEA clustered with the alphasatellites isolated from Pakistan AEA-[PK:Raj:Croton:12]-HG417075, AEA-[PK: Jam: Croton: 12]- HG417076, OLCuA - [PK: Fsd: Croton: 14]-LT674455 (Figure 3B). The cotton leaf curl Multan Betasatellite (CLCuMB-[PK:Fsd:Cotton:15]-LN908793) of similar length was used as an out group.

***SDT analysis*:** Pairwise identity scores were calculated with species demarcation tool (S.D.T.) after aligning the

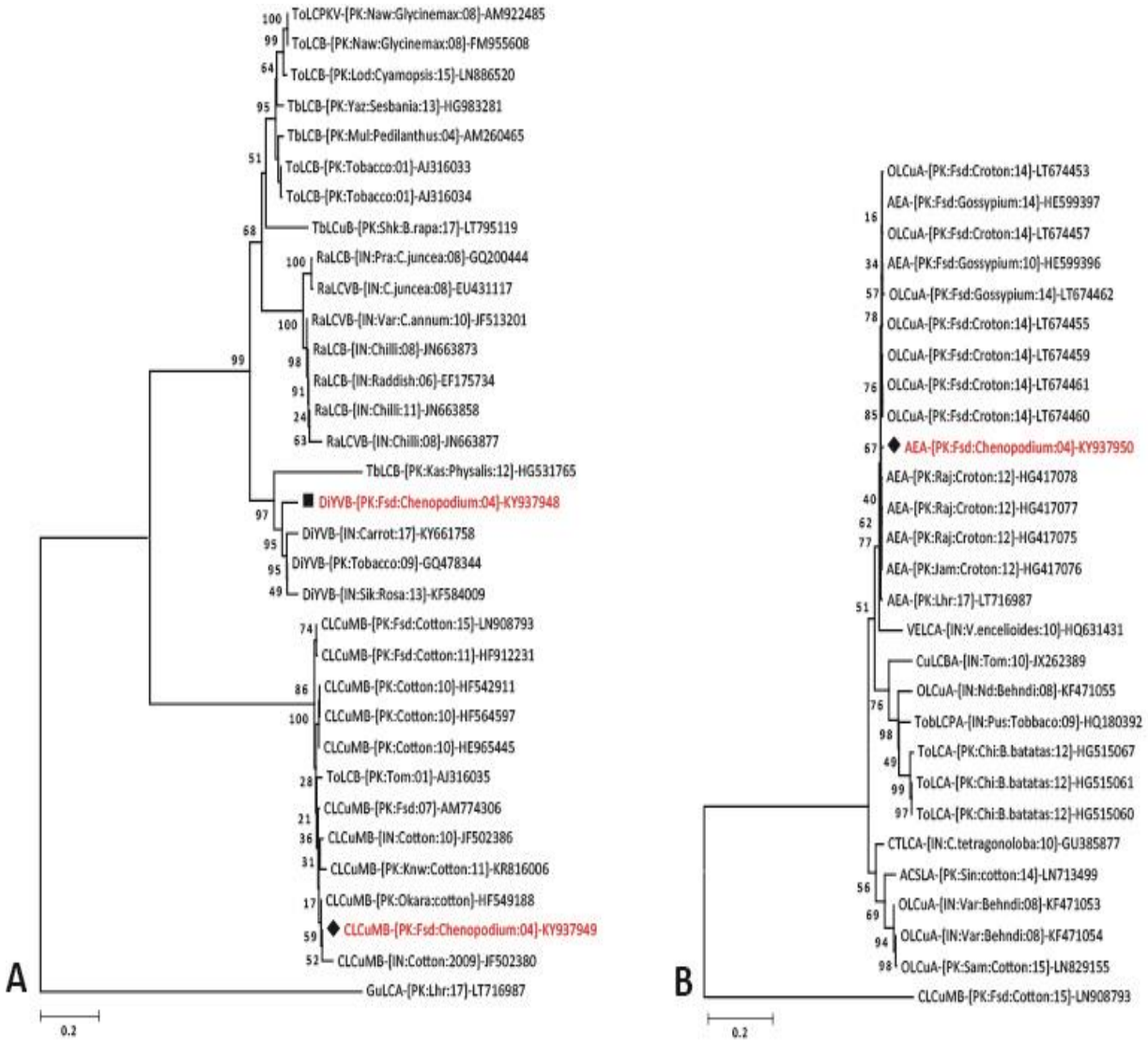

Figure 3. Phylogenetic dendrogram based upon selected complete sequences of A) DiYVB and CLCuMV; and B) AEA. Satellite sequences used for comparison were downloaded from GenBank. The database accession number in each case is given. The sequences of satellite molecules associated with leaf curl disease of *Chenopodiumalbum,* are indicated by shapes and red color.

sequences with previously available begomovirus sequences using MUSCLE (Martin *et al*., 2010).

Virus isolated in this study i.e., PeLCV-[PK:Chenopodium:04]-KY937947 shared 98% nucleotide identity with isolate i.e., PeLCV-[PK:Fsd:Glycinemax:15]-KX671562 and 97% nucleotide identity, with isolates from Islamabad and Faisalabad i.e., PeLCV-[PK:Isd:Capsicum:14]-KY978406 and PeLCV-[PK:Fsd:Glycinemax:15]-KX711622 respectively (Figure 4). Digera yellow vein betasatellite isolated from *C. album* i.e., DiYVB-[PK:Fsd:Chenopodium:04]-KY937948 shared 97-98% nucleotide identity with Indian and Pakistani isolates DiYVB-[PK:Tobacco:09]-GQ478344 and DiYVB-[IN:Carrot:17]-KY661758 and 94% nucleotide identity with Pakistan isolate TbLCB-[PK:Kas:Physalis:12]-HG531765. Second betasatellite i.e., CLCuMB - [PK: Fsd: Chenopodium: 04] - KY937949 showed 98% nucleotide identity with isolates, CLCuMB - [PK: Okara: Cotton] - HF549188 and CLCuMB - [PK: Fsd: 07] - AM774306 (Figure 5A). Alphasatellite isolated in this study i.e., AEA-[PK:Fsd:Chenopodium:04]-KY937950 showed 98% nucleotide identity with AEA-[PK:Raj:Croton:12]-HG417075 and AEA-[PK:Jam:Croton:12]-HG417076 (Figure 5B). Nucleotide identity of all five components was more than 97% with the most closely related begomoviruses that is above the threshold for species demarcation. This

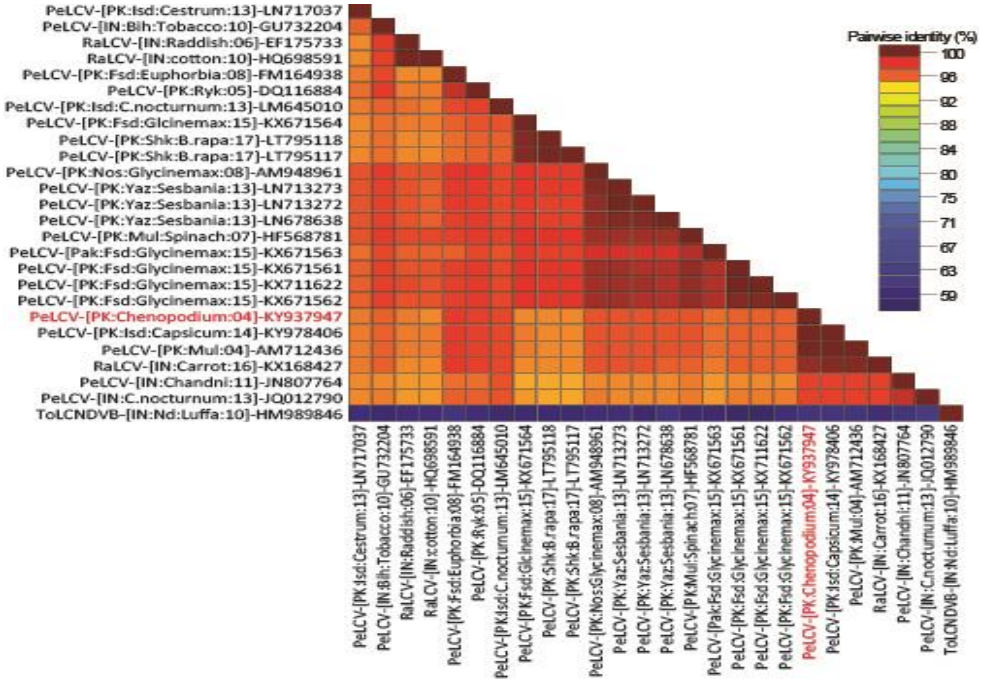

Figure 4. Color-encoded matrix of pairwise sequence homology concluded from alignments of complete begomovirus i.e., PeLCV. The matrix consists of three colors (red, green and blue) differentiating two break values showing the strain (93-94%, brown-red) and the species (90-91%, yellow-green) demarcation thresholds of begomoviruses. Identities were calculated with SDT v. 1.2 (Martin *et al*., 2010)

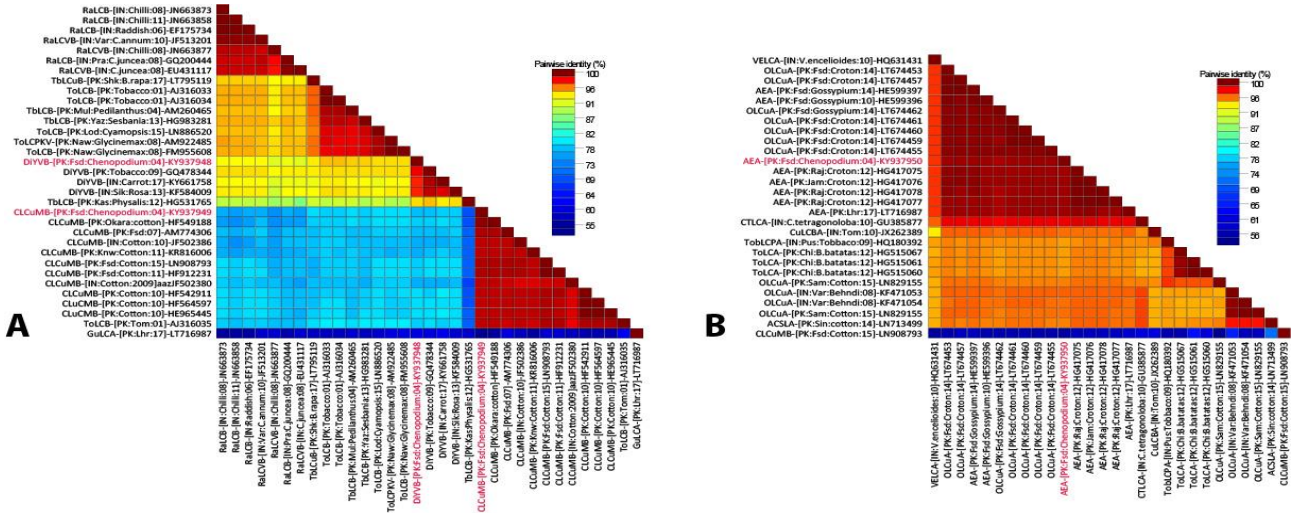

Figure 5. Color-encoded matrix of pairwise sequence homology concluded from alignments of complete begomovirus associated betasatellites i.e., DiYVB and CLCuMB (Figure 5A); and alphasatellite i.e., AEA (Figure 5B) and selected satellite sequences from databank. The matrix consists of three colors (red, green and blue) differentiating two break values showing the strain (91%, yellow-green, for betasatellites; 90% yellow-green, for Alphasatellites) and the species (78%, blue-green, for betasatellites; 88% blue-green, for alphasatellites) demarcation thresholds. Identities were calculated with SDT v. 1.2 (Martin *et al*., 2010)

shows that the begomoviruses infect *C. album* in Pakistan, are isolates instead of new species.

DISCUSSION

The viruses belonging to genus Begomovirus are increasingly spreading to the weeds and cultivated crops (Mansoor *et al*., 2006). These begomoviruses are associated with satellites in India, Pakistan, China and several other

Asian countries (Mansoor *et al*., 2006; Mubin *et al*., 2009; Mubin *et al*., 2012). Previously, these satellites were only known from Old World, but due to efficient cloning techniques, they have also been reported from New World (Fiallo-Olive *et al*., 2012). During offseason when the host crop is absent, these begomoviruses hibernate on alternate hosts. Mostly these alternate hosts are weeds present in the vicinity of crop fields. Weeds are preferable alternate hosts at that time because the whole year they can be found in field. So, these weeds can act as a giant reservoir of viruses which give rise to diversification of viruses. The implementation of efficient control strategies for these viruses seems difficult due to incomplete information about their diversity. Information is lacking about the viruses' species infecting both crop and non-crop hosts. Pakistan is home for begomoviruses due to high disease prevalence as well as having a diverse group of viruses infecting *Zinnia elegans*, *Solanum nigrum*, *Ageratum conyzoides* (Haider *et al*., 2007), *Duranta erecta* (Iram *et al*., 2005), *Capsicum annum*, *Solanum lycopersicum* (Mansoor *et al*., 1997; Hussain *et al*., 2004) *Croton bonplandianus* (Amin *et al*., 2002)**,** *Abelmoschus esculentus* (Mansoor *et al*., 2001), *Citrullus lanatus* (Mansoor *et al*., 2001), *Raphanus sativus* (Mansoor *et al*., 2000), *Vigna aconitifolia* (Qazi *et al*., 2006) *Vigna radiate* (Hameed and Robinson, 2004), *Eclipta prostrata* (Haider *et al*., 2005; Murtaza *et al*., 2018) *Carica papaya* (Nadeem *et al*., 1997) *Digera arvensis* L. *Sonchus arvensis, Xanthium strumarium* (Mubin *et al*., 2009; Mubin *et al*., 2012) and *Rosa arvensis* (Khatri *et al*., 2014).

In this study, genetic biodiversity and phylogenetic analysis of begomoviruses in naturally infected *Chenopodium album* are investigated. *Chenopodium album* is a natural weed that is widely found in Asia. The diversity of begomoviruses infecting *C. album* is not known so far. Our results showed that PeLCV having two different types of betasatellite molecules i.e., Digera yellow vein betasatelite (DiYVB), Cotton leaf curl Multan betasatellite (CLCuMB) and an alphasatellite, ageratum enation alphasatellite (AEA) had association with the leaf curl disease of *C. album.* BLASTn analysis of begomovirus genome (2757 bp) revealed 98% nucleotide sequence homology with *Pedilanthus leaf curl virus* isolated from *Capsicum annum* (PeLCV-[PK:Isd:Capsicum:14]-KY978406). This confirms that begomovirus isolated from *C. album* are an isolate of PeLCV. Similarly, associated betasatellites i.e., DiYVB and CLCuMB showed 96-99% sequence homology to satellites isolated from India and Pakistan. Ageratum Enation alphasatellite (AEA) associated with PeLCV was also new variant and showed 99% sequence homology with AEA from Pakistan. This begomovirus disease complex infecting *C. album* is quite interesting as PeLCV has never been reported in association with such diverse satellites.

Pedilanthus leaf curl virus (PeLCV) was reported first time in association with TbLCB from *Pedilanthus tithymaloides* (Tahir *et al*., 2009). After that Tahir *et al*.,(2017), reported that PeLCV had association with Cotton leaf curl Multan Betasatellite (Shahdadpur strain), which infects Spinach (*Soinacia oleracea*). Catharanthus yellow mosaic virus (CYMV) was found to be infecting *Catharanthus roseus*, which is recombinant of PeLCV and croton yellow mosaic virus (CrYMV) (Ilyas *et al*., 2013). Pedilanthus leaf curl virus (PeLCV) was also found to be associated with the leaf curling disease in *Sesbania bispinosa* along with Tobacco leaf curl betasatellite (TbLCB) (Zaidi *et al*., 2016). Phylogenetic analysis of begomovirus based on the alignment of the complete nucleotide sequences of selected PeLCV sequences are available in the databases (Fig. 2). In phylogenetic dendrogram, the DNA-A isolated from *C. album* clustered with viruses isolated from *Capsicum annum* and carrot i.e., PeLCV-[PK:Isd:Capsicum:14]-KY978406, PeLCV-[PK:Mul:04]-AM712436 and PeLCV-[IN:Carrot:16]-KX168427. According to SDT analysis, isolates from *C. album* showed maximum homology (97-99%) with the isolate from *Glycine max* PeLCV-[PK:Fsd:Glycinemax:15]-KX671562 and PeLCV-[PK:Isd:Capsicum:14]-KY978406 (Fig. 4).

The infected sample analyzed in this study was very interesting, as the begomovirus disease complex was associated with two different species of betasatellites i.e., Digera yellow vein betasatellite and cotton leaf curl Multan betasatellite. Both the betasatellites share 76% of sequence homology. It is also evident from the phylogenetic tree representing betasatellites make different clusters. Previously, from Pakistan, the PeLCV was only found in combination with TbLCB (Zaidi *et al*., 2016), however, from India, PeLCV is known to be associated with Digera yellow vein betasatellite, Tomato leaf curl Patna betasatellite and Tobacco leaf curl betasatellite (Kumar *et al*., 2013). It is possible that with the change in host, the betasatellite association with PeLCV was also changed. Further infectivity analysis with different betasatellites will prove the disease severity level in different hosts. From the phylogenetic tree, it is obvious that PeLCV shows huge variation among different isolates infecting different plant hosts. Indeed, it is the only virus with a broad host range in India and Pakistan. Interestingly, both the isolates of RaLCV were identified from India, while PeLCV was initially isolated from Pakistan. This shows the frequent movement of viruses between both the countries. Alphasatellites isolated from *C. album* were similar to okra leaf curl alphasatellite or ageratum enation alphasatellites. These alphasatellites were isolated from croton, okra or cotton plants from Pakistan (Fig. 3B). Although, OLCuA is a recent introduction of alphasatellites on cotton, Guar and okra plants, but *C. album* and croton are weed plants, which grow in Indian-subcontinent from centuries. So, it is possible that OLCuA can spread from *C. album*, *Croton bonplandianus* (croton) or other such weeds to cultivated plants recently.

However, experimental evidences may further prove this hypothesis. Interestingly, all the OLCuA isolates are reported from Pakistan, but its cognate DNA-A component i.e, PeLCV, is found both in Pakistan and India. This shows that DNA-A component's migration across the borders is not necessarily linked with alphasatellites.

***Conclusion*:** *Chenopodium album* is a natural host of Pedilanthus leaf curl virus (a monopartite begomovirus) found with a variety of betasatellites and alphasatellites. As a matter of fact, this is a weed host and remains in fields for all year along. These plants can act as safe reservoir for the begomovirus disease complex in off-season and these viruses might be transferred through white fly to crop plants. So, farmers should eradicate this weed from in and around of their fields.

***Author's contributions*:** MM and MSN conceived and designed the experiments. MM, MSN, AAK analyzed the data and wrote the manuscript. SI and MM performed the experiments. All authors read and approved the final manuscript.

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***Competing interests*:** The authors declare that they have no competing interests.

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