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GENETIC ANALYSIS OF ALTERNANTHERA YELLOW VEIN VIRUS (AYVV) INFECTING *Eclipta prostrata* PLANT IN PAKISTAN

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Whitefly-transmitted begomoviruses (Family *Geminiviridae*) are the major pathogens of food and fiber crops and are often associated with non-cultivated plants. In this study diversity of genomic components of begomoviruses associated with a weed host *Eclipta prostrata* (*E. prostrata*) and viral infectivity were investigated. *E. prostrata* is often found besides the water channels and agriculture fields in Pakistan, India, Vietnam and China. Plant leaves showing typical begomovirus symptoms i.e., vein yellowing, were collected from different areas of the Punjab. Amplification of the full-length viral molecules and associated satellite molecules was done through polymerase chain reaction (PCR) and rolling circle amplification (RCA) followed by cloning and sequencing. Basic local alignment search tool (BLAST) analysis showed that Alternanthera yellow vein virus (AlYVV) was associated with vein yellowing disease of *E. prsotrata* along with two different types of Alphasatellite molecules. Sequence analysis showed that AlYVV is a new strain showing 95-98% sequence homology to AlYVV isolated from India and China. Moreover this virus is a recombination free virus in all plant samples analyzed in this study. There was no betasatellite molecule found associated with vein yellowing disease of *E. prsotrata*. In the infectivity analysis, infectious molecules of the virus and associated alphasatellites were constructed. Infectious molecules of Tobacco leaf curl betasatellite (TbLCB) and Cotton leaf curl Multan betasatellite (CLCuMB) which were constructed earlier, were included in this assay together with AlYVV and its alphasatellites. Infectivity analysis showed that AlYVV is infectious to model host *Nicotiana benthamiana* and viral movement was confirmed through Southern blot analysis. When both alphasatellites and betasatellites were coinoculated with AlYVV, the movement of alphasatellites in systemic leaves was confirmed through PCR, however betasatellites were not detected in the systemic leaves. So AlYVV might be betasatellite independent virus not supporting its multiplication. The data presented in this paper would help in understanding the diversity and etiology of begomoviruses in weed plants.

Keywords: *Eclipta prostrata*, begomovirus, alphasatellites, infectivity analysis, phylogenetic analysis.

INTRODUCTION

Whitefly-transmitted geminiviruses (Family *Geminiviridae*; genus *Begomovirus*) with small single stranded DNA (ssDNA) genome (size varies from 2.6kb to 5.2kb) encapsidated in twinned-icosahedral particles were known to occur in the Indian subcontinent for a long time but have recently emerged as major pathogens on food and fiber crops (Hussain *et al*., 2005; Juarez *et al*., 2013; Mubin, Briddon, and Mansoor, 2009; Tahir and Haider, 2005). The most recent emergence of highly diverse begomoviruses could be resulting from multiple infections, recombination, component capture/exchange and emergence of whiteflies as major pest with extended host range. The sources of resistance are limited and are often prone to breakdown due to emergence of resistance breaking strains (Amrao *et al*., 2010). Weeds serve as alternative 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*., 2010a; Mubin *et al*., 2012). Betasatellites are pathogenicity determinant molecule of disease complex while the role of alphasatellites is still unclear. Alphasatellites are 1300-1400 bp long, depending on begomoviruses for movement and transmission to other plant hosts (Briddon *et al*., 2004). Role of these molecules in disease etiology is still not clear though emerging as the most diverse satellites in crop plants and weeds both from New World and Old World (Briddon *et al*., 2004; Fiallo-Olivé *et al*., 2012). The origin of alphasatellites is traced back to 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 Rep gene, known as alpha-rep and a A-rich region (Briddon *et al*., 2004). These molecules are diverse in nature and do not play a role in the development of disease symptoms though alpha Rep protein was found to be

suppressors of gene silencing (Nawaz-ul-Rehman *et al*., 2010).

Alternanthera is a widely grown weed in India, Pakistan and China. Alternanthera was infected by a new species of begomovirus, namely Alternanthera yellow vein virus (AlYVV) in Hainan province of China during 2004 (Guo and Zhou, 2005). Since then AlYVV has been reported from *Eclipta prostrata* and several other weeds in different Asian countries (Mubin *et al*., 2010a). AlYVV seems to be widespread begomovirus in Pakistan, India and China. However, none of the hosts have shown it to be associated with DNA-B component, suggesting it as a monopartite begomovirus. In Pakistan, AlYVV has been found to be associated with multiple satellites, like Ageratum yellow vein betasatellite and potato leaf curl alphasatellite (Mubin *et al*., 2010a). But these isolates were identified from *Sonchus arvensis* weed. While in China, no such satellites have been reported from Eclipta or Alternanthera plants. Similarly, in Vietnam also, there are two reports of AlYVV infecting *Zinnia elegance* and *E. prostrata*. Incase of *Z. elegance* AlYVV is associated with AlYVB while in case of *E. prostrata* no satellite was found. (Ha *et al*., 2008). The *Eclipta prostrata* belongs to family *Asteraceae*, and is an annual herb. It is found both in subtropical and tropical regions of the world. *E. prostrata* is able to grow and thrive in the varied geographical conditions, mainly found around the wet crop fields and moist places. It widely grows almost in all the continents except Antarctica (Holm *et al*., 1977).

In Pakistan *Eclipta prostrata* is found mostly around water channels, having vein yellowing disease symptoms. Objective of this study is to uncover the component viruses, possibility of variation in the disease complex and understand the etiology of these begomoviruses.

During a survey conducted in 2013-14, natural occurrence of vein yellowing disease was observed on *E. prostrata*. We analyzed *E. prostrata* plants for the possible presence of components of begomovirus disease complex producing vein-yellowing symptoms. To our surprise, there were diverse alphasatellites associated with AlYVV in the region while no betasatellites were detected from eclipta plants analyzed. Infectivity analysis also showed that AlYVV might be a betasatellite independent virus, not supporting betasatellite replication in model host *N. benthamiana*. This data will help in understanding that how weeds can play a vital role in speedy evolution of begomoviruses and how understanding the diversity of begomoviruses in weed hosts could help in devising control strategies against begomoviruses.

MATERIALS AND METHODS

***Virus sources, viral DNA detection and cloning using PCR and rolling circle amplification*:** *Eclipta prostrata* symptomatic plants showing vein yellowing were collected from different areas of Punjab from 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. Total DNA was extracted from leaf samples by CTAB method described by (Doyle and Doyle, 1990). Total DNA extracted from infected leaves of *E. prostrata* was subjected to rolling circle amplification (RCA) using ϕ29 DNA polymerase (Blanco *et al*., 1989). The RCA product of *E. prostrata* DNA was restricted using different restriction enzymes i.e., *Sal*I, *Bgl*II, *Hin*dIII, *Kpn*I and *Eco*RI. Fragments of sizes 2.8kb and 1.4kb, which could be begomovirus and associated satellites, were generated by restriction with *Eco*RI and *Hin*dIII enzymes. The restricted product was gel eluted and cloned into the pTZ57RT vector (Fermentas) and completely sequenced to generate clone names pVirol126-2.8, pVirol127-1.4 and pVirol128-1.4.

***Sequence analysis and recombination detection*:** Begomoviruses were completely sequenced by the dideoxynucleotide chain termination sequencing on an automated DNA sequencer (Sanger *et al*., 1977). Sequences were assembled and analyzed by the Lasergene DNA analysis package (v8; DNAStar Inc., Madison, WI, USA). Pairwise comparisons for sequence similarities were produced using the MegAlign program of the Lasergene package. Phylogenetic trees were generated, first by aligning the molecules using CLUSTAL-W, followed by Neighbor joint method of phylogenetic tree construction in MEGA7 program. The GenBank accession numbers for AlYVV isolated from Pakistan are: KX906694, KX906695, KX906696 and KX906697, respectively. The other viral sequences were downloaded from GenBank and virus abbreviations are used as described by ICTV. After the sequence confirmation, detailed recombination analysis was conducted for both the viral molecules and satellites. Prior to recombination analysis the sequences were aligned by CLUSTAL-W in MEGA7 DNA analysis software (Kumar *et al*., 2016) followed by recombination detection through RDP4 program (Martin *et al*., 2015).

***Construction of infectious clones for viruses and alphasatellites*:** Infectious clones of both begomovirus and alphasatellite (Ghulam *et al.,* 2017) were generated in pGreen0029 binary vector (Hellens *et al.,* 2000). For DNA-A component, full-length AlYVV, 2.8kb molecule from clone pVirol126-2.8 was circularized by self-ligation and then subjected to RCA. Then through partial restriction of RCA product using *Eco*R1 restriction enzyme, a fragment of 5.6kb was generated, which was cloned in pTZ57RT first and after confirmation of full dimeric molecule it was shifted in pGreen0029 vector i.e., (pVirol129-5.6).

For alphasatellite TYLCCNA^{Ecl} infectious molecule, full-length alphasatellite molecule i.e., pVirol127-1.4 originally cloned at *Hin*dIII restriction site was digested with *Hin*dIII and *Bgl*II producing one fragment with 200 bp and other with 1200bp with origin of replication. The fragment of 1200bp with origin of replication was ligated with full-length

Table 1. Layout of the infectivity experiment: Representing the inoculation of plants with AlYVV alone and in combinations with alphasatellites and betasatellites.

No. of plants	Virus	Alphasatellite-1	Alphasatellite-2	Symptoms	Southern blot analysis
30 plants (10+10+10) +10 control	(AlYVV)	None		25 plants showed symptoms, Control plants were symptomless	All Plants positive for AlYVV
30 plants (10+10+10)+ 15 control	(AlYVV)	AlYVV + EcYVA	AlYVV + TYLCCNA^{Ecl}	9+8+8 showed symptoms, Control plants were symptomless	All Plants positive for AlYVV+PCR positive for EcYVA and TYLCCNA^{Ecl}
30 plants (10+10+10)+15 controls	(AlYVV)	AlYVV + Tobacco leaf curl betasatellite (TbLCB)	AlYVV + Cotton leaf curl Multan betasatellite (CLCuMB)	8+8+7 showed symptoms, Control plants were symptomless	All Plants positive for AlYVV+PCR negative for betasatellite

alphasatellite molecule pVirol127-1.4. After confirmation the infectious molecule was shifted to pGreen0029 vector to generate pVirol130-2.6. For second alphasatellite EcYVA, which was originally cloned at *Hin*dIII. Restriction with *Hin*dIII and *Sal*I produced two fragments of 850 bp and 575 bp. The fragment with 575bp contains origin of replication as confirmed through sequencing. This fragment was ligated with full-length molecule in pVirol128-1.4. After confirmation of dimeric molecule, the whole construct was shifted to pGreen0029 vector i.e., pVirol131-1.9. Infectious molecules of tobacco leaf curl betasatellite (TbLCB) and Cotton leaf curl Multan betasatellite (CLCuMB) were already present in the lab i.e., pVirol90-1.8 and pVirol188-2.0.

***Inoculation of plants and detection of viral molecules*:** The binary vectors pVirol129-5.6, pVirol130-2.6, pVirol131-1.9 and pVirol90-1.8 and pVirol188-2.0 were transformed into *Agrobacterium tumefaciens* strain GV3101 by freeze-thaw method (Reyes *et al*., 2010). Later, agro-infiltration was performed by injecting 0.2ml of culture into *Nicotiana benthamiana* leaves as described previously (Ma *et al*., 2012). The different inoculation combinations are presented in Table 1. Total genomic DNA was isolated from the systemically infected plant tissue at 16 or 20 dpi using CTAB method (Doyle and Doyle, 1990). Alphasatellite molecules were detected by PCR with the universal primer pairs (Briddon *et al*., 2004). For Southern blot hybridization analyses, total genomic DNA (500ng) was equally loaded in each lane and separated on 1.5% agarose gel. The DNA was later transferred onto nylon membrane, as recommended by manufacturer (Hybond N+; Amersham). The betasatellites replication was confirmed by PCR reaction through universal betasatellite primers (Briddon *et al.,* 2002). The probe was prepared for the coat protein gene of AlYVV, with biotin Decalabel DNA labeling kit (K0651, Thermo scientific). Southern hybridization was performed by standard methods (Van Embden *et al*., 1993).

RESULTS

***Symptomatology*:** Leaves from *Eclipta prostrata* weed showing typical symptoms of begomovirus infection were collected from different districts of Northern and Southern parts of the Punjab province in 2013 and 2014. In all these areas there were cultivated crops like cotton, rice, sugarcane and vegetable crops present in the field. This weed is commonly found in the fields as well as around the water channels. At every place *E. prostrata* plants were found to be showing similar symptoms of vein yellowing. Vein yellowing was so conspicuous that the infected plants showed clear distinction from the non-infected ones (Fig. 1).

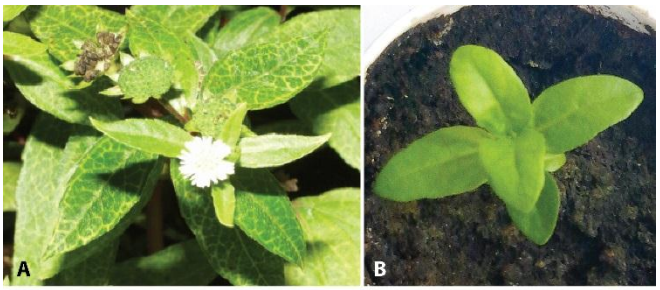

Figure 1. *Eclipta prostrata* plant: A) Symptomatic plant showing vein yellowing symptoms; B) Asymptomatic plant

***Cloning and molecular characterization of a begomovirus species and a new alphasatellite species infecting the Eclipta prostrate*:** Rolling circle amplification (Blanco *et al.,* 1989) was used to amplify all circular ssDNA molecules from infected samples of *E. prostrata*. Restriction with *Eco*RI enzyme yielded 2.8kb fragment i.e., size of begomovirus cloned in pTZ57RT (Fig. 2A). While restriction with *Hind*III yielded 1.4kb fragment. The restriction digestion with *EcoRI* and *Pst*I enzyme confirmed the 2.8kb sized bands in the gel (Fig. 2B). Sequencing of 2.8kb sized bands confirmed the presence of begomovirus. We were unable to amplify,

betasatellites by using the same dilution as a template in PCR reaction.

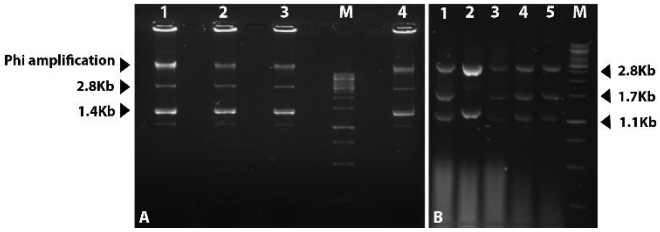

Figure 2. Amplification using Phi29 DNA polymerase, restriction and cloning of viral molecules associated with vein yellowing disease of *Eclipta prostrata*: A) Amplified RCA product and restriction of RCA product resulted in 2.8kb and 1.4kb fragments using *Eco*RI and *Hind*III; B) Confirmation of cloning of virus 2.8kb in pTZ57R/T vector using *Eco*RI and *Pst*I.

***Sequence and phylogenetic analysis of AlYVV*:** Total of around 75 DNA A molecules of size 2.8 were cloned from fifteen plant samples collected from different regions of Punjab and partial sequencing showed that all molecules show maximum sequence similarity to Alternanthera Yellow Vein Virus (AlYVV). Then 4 molecules were completely sequenced. These completely sequenced molecules also showed that only AlYVV is present in all infected samples. Diagnostic primers for DNA B (Rojas *et al.*, 1993) were also used to find out its presence in infected samples. There was no hint of the presence of DNA B in any of these samples. These viruses isolated from *E. prostrata* showed the highest homology with the already reported AlYVV, although samples were taken from distant places of Punjab province (Fig 3). In the phylogenetic tree it was evident that sequences of the AlYVV had a high level of sequence similarity with the viruses isolated from India and China (AlYVV-IN:13:LN795903, AlYVV-IN:13:KT717678 and AlYVV-CN:LC1:06EF544603) (Fig. 3). The phylogenetic tree also showed that this conservation in the sequence of AlYVV was not only consistent with respect to the geographical positions rather it was also consistent over the period of time. The sequence of the virus isolated from Pakistan in 2009 had 98% to 100% similarity level with each other. Similarly, the full length AlYVV isolated from China in 2006 had least difference in the nucleotide sequence and had a higher level of homology with each other and these viruses were seemed to be the strains of each other without having considerable difference over a period of 8 to 10 years.

Higher level of sequence similarity also revealed that apparently no recombination of AlYVV occurred with any other begomovirus.

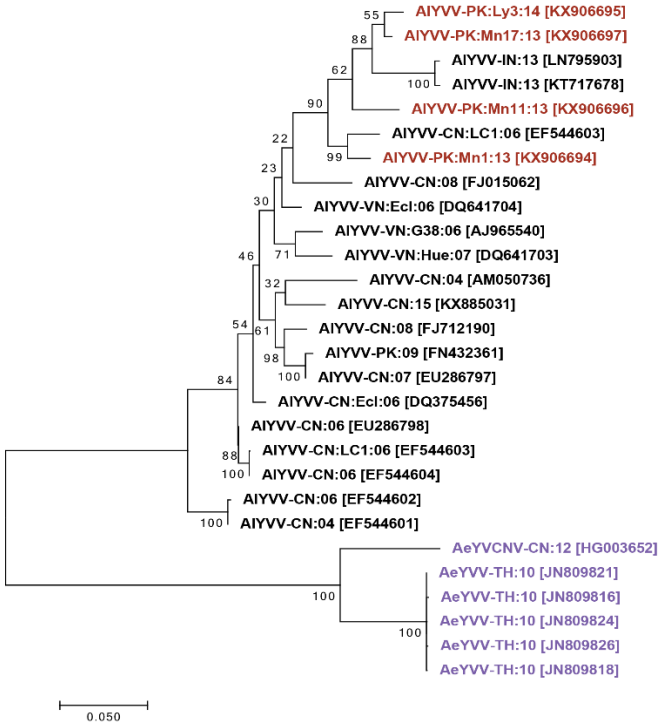

Figure 3. Phylogenetic dendrogram based upon selected complete sequences of AlYVV. Begomovirus sequences used for comparison were downloaded from GenBank. The database accession number in each case is given. The sequences associated with Alternanthera yellow vein disease are indicated by a red color and sequences associated with Ageratum yellow vein virus (the most closely related virus of AlYVV) are indicated by magenta color.

***Infectivity analysis*:** Fresh cultures of *Agrobacterium tumefaciens* cells transformed with the binary vectors harboring infectious molecules for alphasatellites, betasatellites and AlYVV were grown under standard conditions. Prepared inoculum from these cultures was used for inoculation of the *Nicotiana benthamiana* plants. Infected plants showed symptoms at 12-14 dpi (Fig. 4A). Healthy *N. benthamiana* plants with 4 to 5 leaves grown under controlled conditions of 16/8 hours light period and 25C temperature were used for inoculation.

After inoculation of 30 *N. benthamiana* plants with only AlYVV, leaf-curling symptoms were produced at 12 dpi in 25 plants. Upward curling of leaves and somewhat stunted growth was observed (Fig. 4A). The replication and movement of virus in systemic leaves was also checked with Southern blot hybridization (Fig 4B). For this purpose CP was used as a probe. Purified PCR product of the CP was used as +ve control while DNA isolated from a plant inoculated with simple *A. tumefaciens* was used as –ve control.

In next experiment AlYVV was coinoculated with EcYVA in 30 plants. This time symptoms were produced after 14 dpi but the symptoms were comparable to the symptoms produced in case of AlYVV alone (Fig. 4A). AlYVV was also coinoculated with the TYLCCNA^{Ecl} (30 plants). Again the symptoms appeared after 14 dpi but the produced symptoms were delayed and milder as compared to those produced when plants were inoculated with the DNA A alone (Fig. 4A). Table 1 enlists the details of the experiment.

We checked the movement and presence of Alphasatellites (Fig 5) through PCR in plants, which showed negative results in southern blot analysis (data not shown). We were able to amplify EcYVA in 6 plants out of 10 plants and TYLCCNA^{Ecl} from 5 plants out of 10 plants (Fig. 5). Co-inoculation of betasatellites with AlYVV produced symptoms similar to AlYVV alone and subsequently we were unable to detect betasatellites in systemic leaves through PCR. It means in infectivity experiments, AlYVV might have supported both Alphasatellites but virus titre was too low to be detected through southern blot analysis, while AlYVV didn't support betasatellites at all. All these experiments were repeated thrice to validate the results.

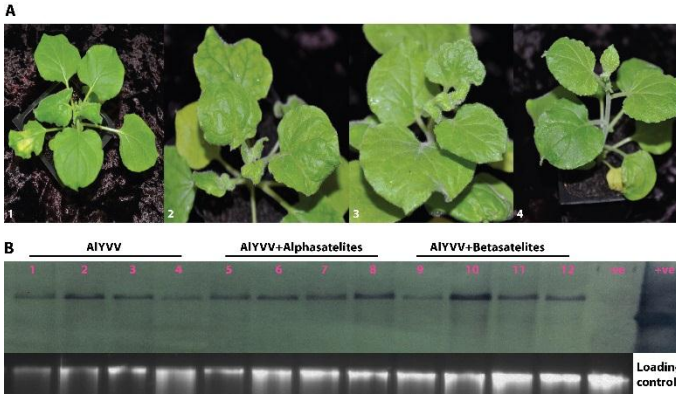

Figure 4. Infectivity analysis: A) Agro-infiltration of ALYVV and associated satellites on *N. benthamiana*, 1) plant infected with the AlYVV alone showing leaf curling, 2) Plant inoculated with AlYVV+ EcYVA showing same symptoms as 1. Plant inoculated with AlYVV+ TYLCCNA^{ecl} (not shown here) produced similar symptoms, 3) Plant inoculated with AlYVV+CLCuMB showing same symptoms as 1 and 2. Plant inoculates with AlYVV+TbLCB (not shown here) produced similar symptoms, 4) Plant inoculated with *A. tumefaciens* only i.e., negative control; (B) Detection of ALYVV through Southern blot analysis using CP as probe in systemic leaves of *N. benthamiana* plants, (lane 1-4) agro infiltrated with infectious molecules of AlYVV, associated alphasatellites EcYVA (lane 5-6) and TYLCCNA^{Ecl} (lane 7-8) and betasatellites CLCuMB (lane 9-10) TbLCB (lane 11-12). Purified PCR product of CP was used as positive control. Second last lane from left is –ve control and last lane is +ve control.

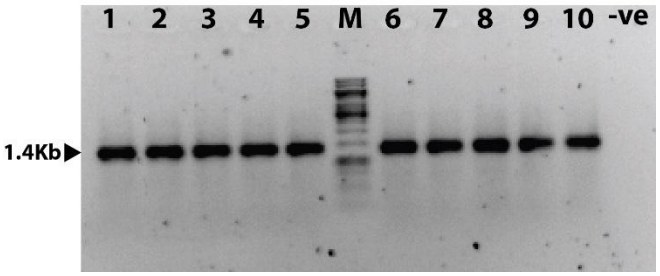

Figure 5. PCR amplification from systemic leaves: Lane 1 to 4, confirmation of alphasatellite EcYVA replication through PCR, M is the marker. Lane 5 to 9 is confirmation of alphasatellite TYLCCNA^{Ecl}, 10 is +ve control and next lane is -ve control.

DISCUSSION

Begomoviruses, infecting crop plants are economically important. Begomoviruses are one of the rapidly emerging groups of plant viruses, which can be attributed to various factors, including increased insect vector populations, presence of alternative hosts and/or rapid manifestation of insect vectors. These begomoviruses hibernate on alternate hosts when the main crop is not present in the field. These alternate hosts are usually weeds found naturally inside or around the crop fields. Higher diversity of economically important viruses is found in these weeds. But unfortunately these reservoirs are often neglected due to less/no economic importance. One of the major obstacles to implement efficient virus control strategies is our incomplete knowledge of genetic diversity and populations of begomoviruses within a distinct area in all crops and non-crop hosts. Pakistan is home for begomoviruses and there is a high disease incidence as well as high diversity of viruses infecting *Zinnia elegans*, *Solanum nigrum*, *Ageratum conyzoides* (Haider *et al*., 2007), *Duranta erecta* (Iram *et al*., 2005), *Chili pepper*, *Tomato* (Hussain *et al*., 2004; Shih *et al*., 2003) (Mansoor *et al*., 1997) *Croton bonplandianus* (Amin *et al*., 2002) Okra, Watermelon and Radish (Mansoor *et al*., 2001; Mansoor *et al*., 2000a; Mansoor *et al*., 2000b), *Vigna aconitifolia* (Qazi *et al*., 2006) Mungbean (Hameed and Robinson, 2004; Bashir *et al*., 2006; Hameed and Robinson, 2004), *Eclipta prostrata* (Haider *et al*., 2005) papaya (Nadeem *et al*., 1997) and *Digera arvensis* L. *Sonchus arvensis* and *Xanthium strumarium* (Mubin *et al*., 2012; Mubin *et al*., 2009).

OW begomoviruses infecting economically important field crops are believed to be originated from the weeds usually grow in or around the fields of these crops (Ndunguru *et al*., 2005; Al-Saleh *et al*., 2014), *E. prostrata* is also harboring a complex of viral disease components, including begomovirus molecules and its associated satellite molecules. Resistance breaking begomoviruses originated from the weed hosts like

Cotton Leaf Curl Burewala Virus (CLCuBuV), which is a recombinant molecule sharing genome segments from *Cotton Leaf Curl Multan Virus* (CLCuMuV) and *Cotton leaf Curl Khokhran Virus* (CLCuKV) isolated by (Mubin *et al*., 2012) from a weed known as *X. strumarium L. found inside and around the cotton fields.*

In this study, the geographical distribution, genetic biodiversity and infectivity analysis of begomoviruses in naturally infected *E. prostarata* were investigated. Eclipta is widely grown in Asia as a natural weed. The viruses infecting Eclipta are already known. However, the diversity of viruses infecting eclipta from Pakistan and infectivity analysis is not reported. Therefore, in this study we conducted a survey to isolate begomoviruses and their satellites from Eclipta plant.

We selected the Punjab province of Pakistan for isolation of viruses. Although, Eclipta is happy to grow, where there is standing water for a longer period of time around the water channels, Eclipta plants growing in the dry areas of Punjab were also found. This shows its wide adaptability in different regions of Punjab. Throughout the Punjab province, there were only one kind of symptoms i.e., vein yellowing in the leaves.

Sequence analysis of begomovirus and associated satellites from all the samples collected from the different districts revealed that the full length viral molecules (~ 2.8kb) from all different localities were found to be highly conserved and level of sequence homology was 92 to 98%. The homology difference was observed as a point mutation throughout the genome as compared to recombination.

To our surprise, we were unable to find a single betasatellite from these samples. Therefore, we applied Universal primers based PCR. We used both the genomic DNA dilutions and RCA dilutions to amplify any detectable level of betasatellite. But surprisingly, there was no amplification of betasatellite. This showed that Alternanthera yellow vein virus is not associated with betasatellites in E. prostrata plants. Similar results were found by (Ha et al., 2008) from Eclipta prostrata as no betasatellite was found to be associated with AlYVV. (Guo and Zhou, 2005; Ha et al., 2008; Mubin et al., 2010a) showed the association of AlYVV with betasatellite but in different host i.e., Alternanthera philoxeroide from China, Zinnia elegance from Vietnam and Sonchus arvensis from Pakistan. Similarly *AlYVV was isolated from* Sonchus arvensis, along with alphasatellites and betasatellites (Mubin et al., 2010b). This gives an indication that for AlYVV to interact with betasatellite there is a need of suitable hosts. Or in other words Eclipta plants do not support betasatellites replication. There are some reports where AlYVV was found to be infected different hosts but no association with betasatellites (Nawaz-ul-Rehman et al., Unpublished data).

Role of alphasatellites in the etiology of AlYVV is unclear. In a series of experiments it was postulated that the alphasatellite are autonomous in sense of replication but depends on helper virus for their encapsidation and spread through insect to other plants and even within the plant while it has, apparently no role in the symptom modulation or it have no role in the systemic disease spread (Briddon et al., 2004). Infectivity of dimeric molecules for AlYVV alone and in combination with two different alphasatellites showed that perhaps alphasatellites have no role in disease symptoms development in N. benthamiana plants. We observed similar symptoms in plants inoculated alone with the AlYVV or AlYVV in combination with alphasatellites. There was no symptom severity in case of alphasatellites. We were able to detect AlYVV through Southern blot analysis but not the alphasatellites. *We were able to amplify EcYVA and TYLCCNA^{Ecl} using PCR (Fig 5). It means AlYVV might have supported both Alphasatellites but virus titre was too low to be detected through southern blot analysis.* The attempts were made to inoculate original host through Agrobacterium tumefaciens but we were unable to get any infection (results not shown). Indeed, the agrobacterium produced severe necrosis on the patch of inoculation, but no systemic movement or symptoms could be reproduced in the inoculated plants.

***Conclusions*:** There are many interesting findings presented here. Usually in Old World, begomoviruses are associated with betasatellites but in this study no associated betasatellite was found, though association with multiple alphasatellites was found. Alternanthera yellow vein virus was found to be recombination free across the infected samples collected from Punjab, which again is an interesting observation. The association of different alphasatellites with same helper monopartite begomovirus was found. Infectious molecules were made, infectivity analysis was performed and viral movement was checked by Southern blot analysis. Regarding these discoveries, the future directions are to search for pathogenicity determinant responsible for vein yellowing in this disease complex. The aim of this study was to understand the begomovirus complex responsible for the disease in *E. prostrata*. Findings of these studies were very interesting and novel in this regard that such extensive work on *E. prostrata* has not been reported so far. *E. prostrata* proved to be very important reservoir for begomoviruses. So there is always a chance that whitefly feed on different weed hosts and mix up these components. Crop plants showed specificity to specific viruses and components and thus only specific viruses and their components propagated in crop plants. Weeds act as reservoir of these viral components and also as recombination vessels where when all the components are present together there would be a greater chance of recombination.

***Authors Contribution*:** GM performed all the experiments. MM, MSN and LA were involved in design of the experiments and data analysis. GM and MM wrote the paper while MM, MSN and LA critically reviewed the manuscript.

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