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Molecular characterization and identification of bhendi yellow vein mosaic virus with satellite DNAs infecting okra plants of district Mardan, Pakistan

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Abstract

Background: Bhendi or okra (*Abelmoschus esculentus*) is an important crop widely cultivated in the Indian subcontinent. The production of okra in tropical regions is constrained by several abiotic and biotic factors. Among biotic stresses, yellow vein mosaic disease (YVMD), transmitted by the whitefly (*Bemisia tabaci* Genn.), causes significant production losses.

Results: Leaves showing symptoms of YVMD were collected from okra plants in the district of Mardan, Pakistan, from which the total nucleic acid was extracted. It was found that the viral genome was 2739 bp in the length and had seven conserved open reading frames, comparable to those of monopartite begomovirus species from the Old World. Nucleotide sequence comparison revealed that the genome has 97.7% identity with bhendi yellow vein mosaic virus (BYVMV). Additionally, alpha- and betasatellite DNA components were amplified and sequenced. The alpha- and betasatellite DNA sequences were 1367 and 1346 nt in length, respectively. Sequence analysis revealed that the alpha- and betasatellite sequences shared 97.9 and 98.7% similarity with cotton leaf curl Multan alphasatellite DNA (CLCuMuA) and croton yellow vein mosaic betasatellite DNA (CroYVMB), respectively.

Conclusions: Based on the prevailing classification system, the isolate was identified as a variant of BYVMV, CLCuMuA, and CroYVB. Alphasatellite presence in the begomovirus betasatellite complex detected in the present study indicated a recent mobilization into the viral complex infecting okra in this region. The study findings may facilitate the design of new management strategies to protect this valuable crop against begomovirus infection.

Keywords: Okra, Yellow vein mosaic disease, Alphasatellite, Begomovirus, Betasatellite, Geminiviridae, Phylogenetic analysis

Background

Bhendi (*Abelmoschus esculentus* L.) and lady's finger are two popular names for okra (the Malvaceae family vegetable), which is widely cultivated across Pakistan most of the year. In Asia, *A. esculentus*, a species of *Abelmoschus*, is in a high demand because of its excellent nutritional contents. YVMD (yellow vein mosaic disease), the disease that reduces the quality and yield of okra plants, is the primary obstacle to okra production. BYVMV, the virus that causes YVMD, was first identified in Mumbai, India, in 1924 (Kulkarni 1924). It is a member of the Geminiviridae family's Begomovirus genus.

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Monopartite (equivalent of bipartite begomovirus DNA-A) and bipartite (two circular, single-strand DNA molecules designated as DNA-A or DNA-B) genomes are the two types of begomoviruses. Six proteins are encoded by DNA-A, whereas only two proteins are encoded by DNA-B; each of these genomes is 2.5–2.8 kb in size. Gene silencing regulates gene expression and suppresses the host defense by encoding proteins necessary for particle encapsidation (the coat protein [CP]), viral replication (the replication enhancement protein [REn] and the replication-associated protein [Rep]) and transcriptional activator protein [TrAP]. The C4 protein regulates symptom onset and gene silencing in begomoviruses from the Old World (OW). As for viral mobility and pathogenicity, DNA-A V2 protein is the key player. To transmit a virus intracellularly (through nuclear shuttle protein [NSP]) or intercellularly (by movement protein [MP]), the DNA-B component produces two proteins, BV1 and BC1 (Hanley-Bowdoin et al. 1999).

Satellite DNA components, including alphasatellites, betasatellites, and recently discovered deltasatellites, are widely found in Begomoviruses in the OW (Zhou 2013). Alphasatellite DNA encodes a single Rep protein, whereas betasatellite DNA encodes βC1 protein, an important determinant of pathogenicity and symptom development (Zhou 2013). Betasatellites and other viral satellites have recently been added to the Tolecusatellitidae family, which includes the genus *Betasatellite* and genus *Deltasatellite* (Adams et al. 2017). Although the nonanucleotide region inside the stem-loop structure has sequence similar to the helper virus, the alpha- and betasatellite DNA sequences are half the size of their helper begomovirus (about 1350 nucleotides).

Identification and molecular characterization of begomoviruses and associated satellite DNA infecting okra were the primary focuses of this research. This information will be used to develop novel management strategies to protect this essential crop from begomovirus infection.

Methods

Collection of okra samples

Okra plant samples were collected during a survey of an okra farm in the district of Mardan, Pakistan. Five plants exhibiting symptoms such as yellow veins and yellow mosaic-like patterns were identified, and 2 to 4 young symptomatic leaves from each suspected plant were collected from different areas and placed in zip bags. The samples were kept at −80 °C until needed. Along with leaf samples, viruliferous whiteflies (*Bemisia tabaci* (Genn.), the vector of begomovirus) were also collected and preserved in absolute ethanol. Some viruliferous whiteflies were collected from infected plants and transferred to healthy okra plants. New inoculated plants were observed daily for symptom development.

DNA extraction

Frozen leaf tissue (100 mg) was used to extract the total nucleic acid utilizing the CTAB (cetyltrimethylammonium bromide) method (Mansoor et al. 2000). After resuspension in TE buffer or nuclease-free water, the DNA was kept cold at a temperature of −20 °C. DNA concentration was quantified using NanoDrop spectrophotometer, and DNA integrity was assessed on 1% agarose gel. Initially, a set of degenerate primers WTGF (5'-GATTGTACGCGTCCDCCT TTAATTT GAAYBGG-3') and WTGR (5'-TANACG CGTGGC TTCKRTACATGGCCTDT-3') were used to identify the begomovirus DNA-A component, covering the 1.5 kb region starting from the *Rep* gene to the *CP* gene, including the long intergenic region (LIR) (Mansoor et al. 2000). Primers CPF and CPR were used to detect the approximately 700 bp region of the *CP* gene (Haider 1996). PCR products were directly sequenced in both orientations.

Whole genome amplification, cloning, and sequencing

To amplify the whole genome of the virus, the TempliPhi DNA Amplification Kit was used to perform rolling circle amplification (RCA) on the total DNA according to the manufacturer's recommendations. The amplified RCA products were digested with restriction endonucleases, and fragments that totaled about ~ 2.8 kbp were directly cloned into the pGEM-3Zf + plasmid vector. The primer sets AlphaF/R and Beta01/02 were used for amplification of the associated alpha- and betasatellite DNA, respectively, following standard procedure (Zia-ur-Rehman et al. 2013). The PCR amplicons were cloned into the pTZ57R/T plasmid vector using the InsTAcloneTM PCR Cloning Kit (Fermentas Life Sciences, Waltham, MA, USA). Full-length clones were sequenced in their entirety in both orientations by Macrogen Inc. (Seoul, Korea).

Sequence comparisons and phylogenetic analysis

The complete nucleotide sequences of the DNA-A component and alpha- and betasatellite DNA were done utilizing the BLASTn algorithm. The ORF finder tool was utilized to locate ORFs (open reading frames) in either the virion- or complementary-sense DNA strands. The pairwise identity scores for full-length sequences of the begomovirus genome and associated satellite DNA were calculated using the MUSCLE algorithm implemented in the Species Demarcation Tool (SDT), as previously described (Muhire et al. 2014). Phylogenetic trees were

constructed using the maximum likelihood (ML) method in MEGA 6.0 software and viewed using TreeView (Tamura et al. 2013).

Results

Symptomatic okra plants collected in the study displayed vein yellowing and yellow mosaic-like pattern (Fig. 1). The samples were checked for the presence of begomovirus and its associated satellite DNA by PCR since these symptoms are characteristic of YVMD infection. All the five symptomatic samples revealed begomovirus infection, evident through amplification of the partial DNA-A genome and alpha- and betasatellite DNA, whereas no amplification was obtained for the DNA-B component. The sequences gained from all five infected samples were 100% identical; therefore, only one sample was taken for the full genome amplification.

The whole DNA-A and cloned alpha- and betasatellite DNA were used to identify species by cloning and sequencing. DNA-A comprised 2739 nucleotides (nt) and matched 96–100% sequence similarity with isolates of BYVMV. Thus, the virus collected from the infected okra plants was identified as an isolate of bhendi yellow vein mosaic virus (BYVMV) designated as BYVMV [PK: Mrd:Okra:OM1:17] (Table 1). The DNA-A sequence was deposited in GenBank under accession number MH252996. Analysis of the OM1 sequence indicated that it possessed typical features of the DNA-A component of begomoviruses obtained from the OW. The sequence contained five predicted genes, two in the virion-sense strand (encoding CP and V2 proteins) and five in the complementary-sense strand (encoding Rep, REn, TrAP, C4 and C5 proteins). These genes diverged from the non-coding LIR with a predicted hairpin structure containing the non-nucleotide sequence (TAATATTAC) as part of the loop that originates geminivirus DNA replication. For DNA-B component, if associated in the complex, all sequenced clones show non-representing molecule regarding DNA-B component. This elucidates the presence of monopartite begomovirus infecting okra plants solely.

The OM2 sequence contained 1346 nt and possessed typical betasatellite features, including a region rich in adenine (A) residues (51% A; coordinates 651 to 980), a sequence conserved in all betasatellites known as the satellite conserved region (SCR; coordinates 1238–14) and a single conserved gene (βC1) in the complementary-sense strand with the capacity to encode a protein of 118 amino

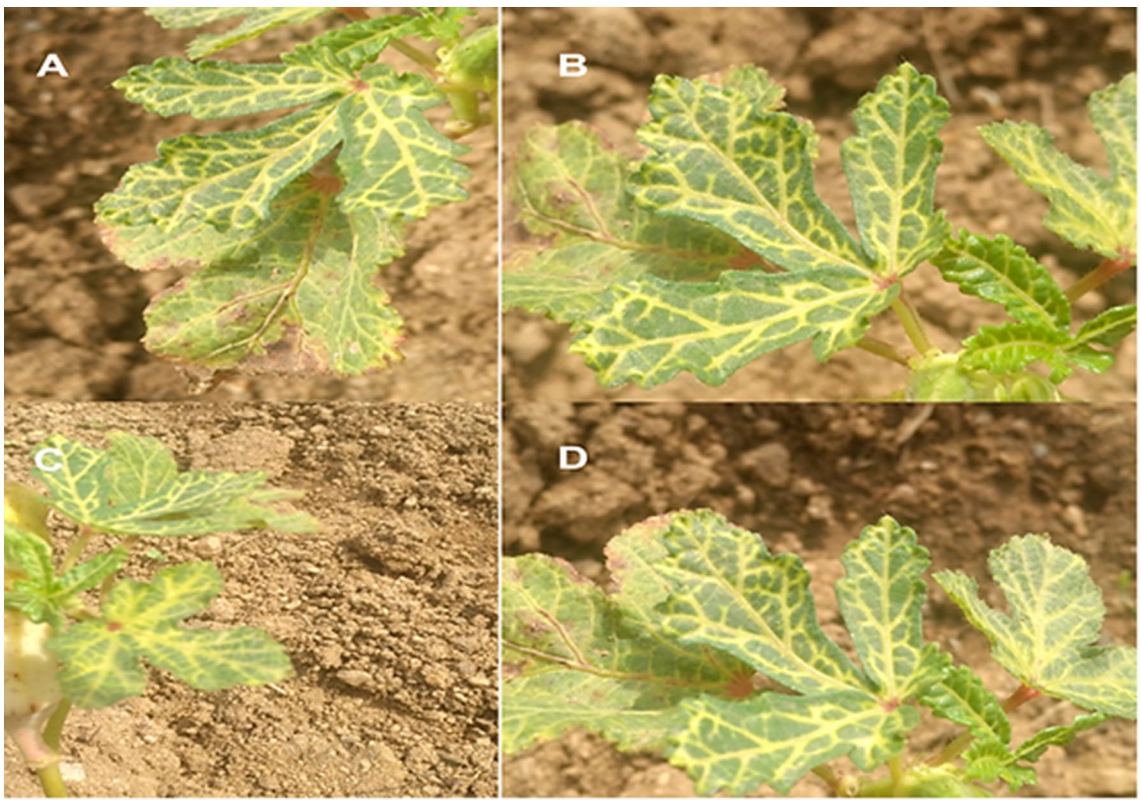

Fig. 1 Infected okra plants collected from okra farm in district Mardan, showing yellow vein and yellow mosaic pattern (**A**–**D**)

Table 1 Percentage nucleotide sequence identity between the complete sequences of okra infecting begomovirus isolate OM1 with selected begomoviruses DNA-A in the database

Isolates	AYVVG (5)	PepYVMLV (4)	RhYMV (3)	MYMIV (4)	MeYVMV (3)	MaYMV (5)	BYNMV (9)	OM1
OM1	77.3–77.6	73.5–74.0	67.6–67.8	67.5–67.8	81.3–82.5	80.8–81.3	**96.5–97.7**	100
BYNMV	77.1–77.9	73.7–74.2	67.3–69.0	66.9–68.4	79.2–84.8	80.1–81.5	100	
MaYMV	77.5–78.9	73.4–74.4	68.0–68.4	66.8–68.2	82.0–83.4	100		
MeYVMV	76.0–77.2	72.8–74.1	67.6–67.9	66.4–68.3	100			
MYMIV	67.6–68.8	66.4–68.4	75.6–75.9	100				
RhYMV	69.2–69.5	68.8–69.4	100					
PepYVMLV	75.5–76.8	100						
AYVVG	100							

Sequences were selected of the most similar species from the blast comparison

acids in length (coordinates 577 to 221) (Briddon et al. 2003). Comparison of the OM2 sequence with closely related sequences using the BLASTn algorithm revealed that it was highly similar to croton yellow vein mosaic betasatellite (CrYVMB) (Table 2). Subsequent alignment of the OM2 sequence with the nucleotide sequences of all available CrYVMB isolates (51 in total) using CLUSTAL V revealed 98.7% sequence identity to a CrYVMB isolate originating from Punjab, Pakistan (AM410551). The obtained results indicated that OM2 was similarly a CrYVMB isolate, based on new criteria for classifying betasatellites (Briddon et al. 2004). The OM2 sequence was deposited in GenBank under accession number MH252994 and was named CrYVMB-[PK:Mrd:OM2:17].

The OM3 sequence contained 1367 nt and possessed typical alphasatellite features, including an A-rich region (52% A; coordinates 1107–1262), a predicted hairpin-loop structure with the nonanucleotide sequence TAG TATTAC forming part of the loop (seen in nanoviruses; family Nanoviridae), and a single conserved gene in the virion-sense strand (coordinates 77–1024) encoding a Rep protein of 315 amino acids in length (Briddon et al. 2004). Comparison of the OM3 sequence with available full-length alphasatellite sequences revealed that it shared 99.3% sequence identity with cotton leaf curl Multan alphasatellite (CLCuMA) [PK:Fsd:C2A3:13] (HG934786) (Table 3). Thus, the OM3 sequence was named CLCuMA-[PK:Mrd:OM3:17] and deposited in GenBank under accession number MH252995. For further confirmation of alphasatellite, nucleic acid from the viruliferous whitefly was obtained and subjected to PCR using specific primers for alphasatellite. The same isolate was identified which substantiates its association with the complex.

A phylogenetic tree was constructed using the full-length DNA-A sequence identified in the study, revealing that the most similar DNA-A sequences in the databases were encompassed in separated clades, indicating species confirmation. Along with this, other begomoviruses producing yellow vein or mosaic-like pattern were also selected (Fig. 2A). Furthermore, the alpha- and betasatellite DNA sequences aligned with similar satellite sequences for viruses in the databases that infect okra and other economically valuable crops in the surrounding

Table 2 Percentage nucleotide sequence identity between the complete sequences of betasatellite isolate OM2 with selected betasatellites in the database

Isolates	RhYMB (2)	CLCuGeB (3)	PaLCuB (3)	ToLCB (3)	EpYVB (3)	CLCuMB (4)	BYVMV (4)	AYVCNB (4)	CrYVMB (6)	OM2
OM2	66.6–67.1	63.0–64.1	72.6–73.0	68.9–70.2	63.2–63.9	67.4–68.3	63.1–65.5	66.7–67.2	96.5–98.7	100
CrYVMB	65.7–67.4	62.3–64.2	70.7–72.2	68.6–70.2	62.0–64.1	66.3–67.5	61.6–66.5	66.1–69.2	100	
AYVCNB	63.2–65.0	63.5–65.9	67.4–71.3	67.1–69.0	66.4–67.1	65.0–66.9	64.6–66.9	100		
BYVMV	64.-65.5	66.3–68.8	64.2–68.0	64.0–66.2	61.9–63.9	68.6–69.9	100			
CLCuMuB	68.0–69.5	65.2–66.9	67.5–68.7	65.6–68.7	62.0–65.2	100				
EpYVB	62.6–63.3	62.6–63.9	63.7–65.4	63.1–66.8	100					
ToLCB	66.0–67.1	63.0–66.0	71.0–72.9	100						
PaLCuB	67.4–68.5	63.0–64.7	100							
CLCuGeB	60.9–62.0	100								
RhYMB	100									

Sequences were selected of the most similar species from the blast comparison

Table 3 Percentage nucleotide sequence identity between the complete sequences of alphasatellite isolate OM3 with selected alphasatellites in the database

Isolates	TbLCPuA-2	GDaSA-4	MeMA-3	HLCuA-2	SiYVA-4	CaMMGA-3	MalYVMA-2	MalYVA-3	CLCuMA-8	OM3
OM3	84.5–84.7	81.2–81.8	86.3–86.4	81.2–82.5	80.5–80.6	76.8–76.9	81.0–81.2	77.5–78.0	93.2–97.9	100
CLCuMA	84.2–86.8	81.7–82.8	86.1–87.5	82.1–83.1	78.8–81.9	76.1–77.7	81.0–82.1	78.1–80.8	100	
MalYVA	80.3–80.6	80.2–81.5	80.2–80.7	79.0–80.4	83.5–84.3	73.5–74.2	85.7–86.2	100		
MalYVMA	80.3–80.9	81.7–82.0	81.2–81.4	79.4–81.6	82.2–82.6	73.8–74.2	100			
CaMMGA	77.1–77.5	74.4–76.0	75.4–75.8	76.8–77.1	76.1–76.5	100				
SiYVA	84.2–84.5	82.1–82.3	83.3–84.1	84.7–86.3	100					
HLCuA	85.9–89.7	78.1–80.7	82.2–83.4	100						
MeYMA	82.1–82.2	82.1–82.4	100							
GDaSA	79.7–79.9	100								
TbLCPuA	100									

Sequences were selected of the most similar species from the blast comparison

area. High shared identity with the available sequences in the databases indicated coherence of different clades with species cutoff demarcation for alpha- and betasatellites (Fig. 2B, C).

Discussion

Bemisia tabaci (Genn.), the whitefly species that transmits begomoviruses, has been found throughout the globe. These infections affect various crops, medicinal plants, ornamental plants and weeds, resulting in significant losses every year (Silva et al. 2012). Begomovirus infections have been found in a broad variety of hosts, with weeds and ornamental plants providing reservoirs for begomoviruses, acting as "mixing bowls" for recombination events (Silva et al. 2012). Begomoviruses have also been reported in medicinal plants, such as ashwagandha (*Withania somnifera*) (Baghel et al. 2012).

Due to exclusive transmission by the whiteflies, each begomovirus has been isolated from various hosts and each host serves as a reservoir for many begomoviruses, clarifying a bilateral transmission phenomenon. Indeed, okra has been reported as a natural reservoir for BYVMV, OELCuV, OLCV, OMoV, OYCrV, OYVMV, and an alternative host for CLCuAlV, CLCuBaV, CLCuGeV, CLCuMuV, ToLCNDV, ToLCGV, and SiMMV (Shih et al. 2009). The high incidence of cotton-infecting begomoviruses in okra suggests that plants from the same Malvaceae family may be susceptible to infection, explaining why many regions in the Indian subcontinent have hedges or barriers around cotton farms to protect against begomovirus transmission.

The fact that begomovirus-associated satellites are capable to promiscuity replication is an intriguing feature of these viruses. Alpha- and betasatellites may interact with a variety of helper begomoviruses (Zhou 2013). Although a helper begomovirus is required for replication, betasatellites can be transreplicated by diverse non-cognate helper begomoviruses, including both monopartite and bipartite forms (Mansoor et al. 2006). For example, experimental data indicated that betasatellite DNA can be transreplicated by beet curly top virus (BCTV), a curtovirus (family Geminiviridae) that is not known to generally correlate with DNA satellites, with betasatellites complementing the host defense suppression mechanisms of this non-cognate helper virus (Patil and Fauquet 2010). Despite the observed replicative promiscuity, phylogenetic analysis indicates that betasatellites can be grouped according to the host from which they were originally isolated, suggesting that adaptation of betasatellites to their cognate helper begomoviruses for replication results from coevolution (Briddon et al. 2003).

Alphasatellites may be relatively mobile since they do not seem to have a preference for certain helper begomoviruses (Briddon et al. 2004). The widespread transmission of these molecules in hosts may be facilitated by OW bipartite begomoviruses which are not usually correlated with alphasatellites (Briddon et al. 2004). For a long time, scientists believed that DNA satellites could only be found in the OW. Satellite DNA related to begomovirus has expanded in recent years, as several alphasatellites have been attributed to new-world begomoviruses (Paprotka et al. 2010). The BCTV leafhopper vector was able to transmit ageratum yellow vein virus-associated alphasatellite DNA in laboratory studies, suggesting that alphasatellite DNA had been transencapsidated (Saunders et al. 2002).

CroYVB and CLCuMuA, DNA satellites of the monopartite begomovirus BYVMV, were found to infect okra according to the results. Natural monopartite begomovirus–betasatellite complexes infect dicots

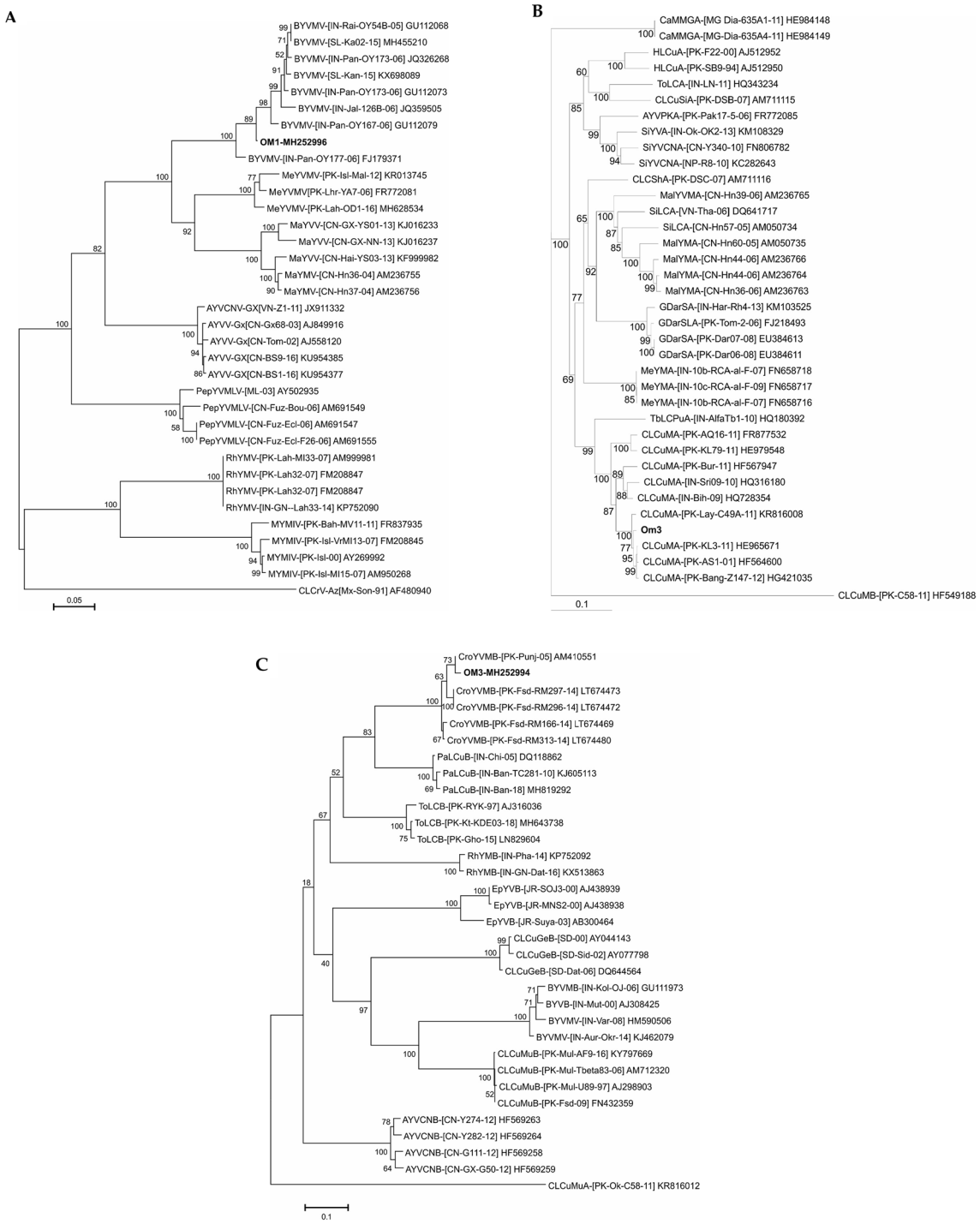

Fig. 2 Phylogenetic trees of BYVMV (OM1), CroYVMB (OM2) and CLCuMuA (OM3). **A** Maximum likelihood phylogenetic tree of BYVMV was constructed based on MUSCLE alignment of selected begomovirus sequences from GenBank and sequences obtained here. Cotton leaf crumple virus (AF480940) sequence was used as an outgroup to validate the tree. **B** Phylogenetic tree of CroYVMB was generated by MUSCLE alignment using related betasatellite sequences from GenBank and sequence obtained here. Cotton leaf curl Multan alphasatellite (KR816012) was taken as outgroup. **C** Maximum likelihood (ML) phylogenetic tree was constructed based on MUSCLE alignments of CLCuMuA (OM3) with the complete nucleotide sequences of selected alphasatellite genomes available in the databases. The tree was arbitrarily rooted on the sequence of cotton leaf curl Multan betasatellite (HF549188), as outgroup. The numbers at nodes represent percentage bootstrap confidence scores (1000 replicates)

in the OW, but an alphasatellite–begomovirus complex has not been previously reported to infect okra in this region. The presence of an alphasatellite with the begomovirus–betasatellite complex identified in the current study may be due to vector transmission.

Conclusions

Monitoring begomovirus–satellite complexes infecting field crops and native vegetation is vital to enable rapid and efficient control. Interactions between various begomoviruses and satellite DNA may lead to the creation of new devastating plant pathogens. In this study, analyzing leaves from okra plants suspected of YVMD infection extended our knowledge of diversity of the virus and acquiring new association/complexes with satellite DNA. To our knowledge, this is the first report of alphasatellite CLCuMuA with BYVMV-CroYVB complex infecting okra in Pakistan.

Abbreviations

YVMD: Yellow vein mosaic disease; CLCuMuA: Cotton leaf curl Multan alphasatellite DNA; CroYVMB: Croton yellow vein mosaic betasatellite DNA; BYVMV: Bhendi yellow vein mosaic virus; BCTV: Beet curly top virus; CTAB: Cetyltrimethylammonium bromide.

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Author contributions

MNS and HR performed conceptualization and methodology; MA, MQ and MA contributed to software and validation; MNS, HR, MA, MQ, MA, and SW were responsible for investigation and resources; MA, MQ, MA and SW contributed to data curation and software; MNS and HR provided resources and performed formal analysis; MNS, HR, MA, MQ, MA and SW performed writing—original draft preparation; MA, SW, AB, MMH, AES, SH and MME reviewed and edited the manuscript; SW, AB and MMH were involved in funding acquisition. All authors read and approved the final manuscript.

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Availability of data and materials

All data and materials are available.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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