

Molecular characterization of a Begomovirus and Betasatellite infecting wild sunflower (*Helianthus* spp.) in India.

Susheel Kumar, Meraj Jaidi, Raj SK*

Plant Molecular Virology Laboratory, Council of Scientific and Industrial Research-National Botanical Research Institute, Lucknow, UP, India

Abstract

Wild sunflower (*Helianthus* spp.) plants exhibiting Begomovirus disease like symptoms: yellow vein net and leaf curl were observed growing in road side in Jaipur (Rajasthan), India. The causal virus was successfully transmitted on healthy seedlings of *Helianthus* spp. by whitefly (*Bemisia tabaci*) which induced similar symptoms to those of naturally infected plants that indicated presence of Begomovirus. The association of Begomovirus and betasatellite with these symptoms of *Helianthus* spp. was investigated by sequence analyses of viral DNA genome and satellite DNAs amplified by rolling circle amplification (RCA) and polymerase chain reaction (PCR), respectively. The highest sequence identities and closest phylogenetic relationships for the Begomovirus and betasatellite detected in *Helianthus* spp. were to *Ageratum enation* virus (AEV) and *Ageratum* leaf curl betasatellite (ALCB), respectively. These findings identified the virus and satellite DNAs infecting *Helianthus* spp. as AEV and ALCB, respectively. *Helianthus* spp. and Tobacco (*Nicotiana glutinosa*) seedlings infected with cloned AEV and ALCB by Agroinoculation developed yellow vein net and leaf curl symptoms, whereas plants infected with AEV alone or ALCB did not develop any symptom. The results show that this complex infects and causes disease in *Helianthus* spp. and Tobacco. *Helianthus* spp. is an invasive weed commonly found around agricultural fields and along roadsides in Rajasthan, India. These results also indicate that *Helianthus* spp. plants infected with AEV and associated betasatellite DNA may act as an alternate host (reservoir) for other economically important plants.

Keywords: *Ageratum enation* virus, *Ageratum* leaf curl betasatellite, Agroinfectivity assay, Wild sunflower, Yellow vein net and leaf curl disease.

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Introduction

Wild sunflower (*Helianthus* spp.) belongs to family *Asteraceae* which grows as weed in roadsides, fields, along desert washes and in grassy areas. This weed is drought-resistant and tolerant to various growth conditions. It causes the greatest harm in summer grain cereals because of the showy sunflower sprouts simultaneously with summer wheat, overtaking the latter in growth, drying and exhausting soil, which results in significant reduction in crop yield; mass weediness results in complete destruction of crops (http://www.agroatlas.ru/en/content/weeds/Helianthus_lenticularis/). It is also reported to be used for broadening the genetic base of cultivated sunflower in India and to introgress the desirable traits from wild sunflowers to cultivated sunflower [1].

Literature survey revealed that none of the plant virus has been reported on wild sunflower (*Helianthus* spp.) worldwide except an unpublished record of sequence data of *Tobacco curly shoot virus* (Accession HQ407395) and *Ageratum* yellow leaf curl betasatellite (Accession HQ407397) molecules infecting wild sunflower (*Helianthus* spp.) in India are available in GenBank database. *Tomato leaf curl Karnataka virus* and *Potato apical leaf curl betasatellite* associated with leaf curl disease of sunflower (*Helianthus annuus*) has been reported from Karnataka state of India [2]. Partial sequence data on *Ageratum enation virus* (AEV) (*Verbesina encelioides* yellow vein

Lakshmarhar virus) isolate (JN998449) and *Ageratum* leaf curl betasatellite (*Verbesina* yellow vein betasatellite) isolate (JQ693145) infecting wild sunflower (*Verbesina encelioides*) are also available from India in GenBank database. However, we report the natural occurrence of *Ageratum enation virus* (AEV) and *Ageratum* leaf curl betasatellite (ALCuB) infecting wild sunflower (*Helianthus* spp.) plants.

Viruses in the family Geminiviridae have circular single-stranded (ss) genomes encapsidated in twinned icosahedral (geminata) virions. Gemini viruses transmitted by the whitefly *Bemisia tabaci* (Gennadius) are in the genus *Begomovirus* and infect a wide range of crops including tomato [3,4]. Begomoviruses have either a monopartite or bipartite genome organization [5]. Monopartite Begomoviruses have genomes consisting of a single component that encode six genes: two in the virion sense, V1 (capsid protein) and V2 (pre-capsid protein); and four in the complementary sense, C1 (replication-associated protein), C2 (transcription activator protein), C3 (replication enhancer protein) and C4 (C4 protein). Monopartite Begomoviruses may associate ~1.4 kb (half the genome size of the helper Begomovirus) ssDNA molecules known as betasatellites and alphasatellites [6-8]. Betasatellites are symptom-modulating and are widespread across the Old World [9]. The $\beta C1$ gene encoded by the betasatellites has important roles in symptom induction, in suppression of transcriptional and post transcriptional gene silencing, and can affect jasmonic acid responsive genes [10].

Alphasatellites are self-replicating satellite-like molecules that are dependent on a helper virus for movement in and between plants. Alphasatellites occur with monopartite Begomoviruses and betasatellites in the Old World [4,11] and with bipartite Begomoviruses (but no betasatellites) in the New World [12]. The precise function of alphasatellites is unclear [10]. However, Nawaz-Ul-Rehman showed that alphasatellites may be involved in overcoming RNA-mediated host defense and attenuating symptoms of the helper virus [13]. The work of Nawaz-Ul-Rehman also showed that some alphasatellites suppress PTGS (host defense) [13].

In this communication we report the molecular characterization of a Begomovirus (AEV) together with Ageratum leaf curl betasatellite (ALCuB) infecting wild sunflower (*Helianthus spp.*) plants and causing yellow vein net and leaf curl symptoms. We further confirmed that the cloned DNAs of AEV and ALCuB cause yellow vein net and leaf curl disease in *Helianthus spp.*

Materials and Methods

Sample collection and whitefly transmission

Leaf samples were collected from five diseased plants showing yellow vein net and leaf curl symptoms and a healthy plant of wild sunflower (*Helianthus spp.*) growing near road side in Jaipur (Rajasthan), India during December 2015. The whitefly transmission test was attempted using B biotype whiteflies (*Bemisia tabaci*). The adult *B. tabaci*, starved for 2 h, were allowed to feed on infected *Helianthus spp.* for a 24-h acquisition period, then transferred onto healthy *Helianthus spp.* seedlings (Ten *B. tabaci* per seedling) and allowed a 24-h inoculation access period. The inoculated plants were maintained in insect proof glass house for about 35 days post inoculation for symptom development if any.

DNA isolation, nucleic acid spot hybridization (NASH) assays and PCR

The total DNA was isolated from 100 mg leaf tissue of five naturally infected as well as a healthy and whitefly transmitted *Helianthus spp.* plants using a DNeasy Plant Mini Kit (Qiagen GmbH, Hilden, Germany). For detection of Begomovirus infection, nucleic acid spot hybridization assays (NASH) was done using a radiolabelled probe generated from a Begomovirus clone [14].

Further, the PCR was performed with Begomovirus specific degenerate primers, PALIv1978 and PARIc496 following the described conditions [15]. All these samples were also checked for the presence of a Begomovirus DNA B component with the primers BC1F and BC1R [16] and betasatellite DNA with primers Beta 01 and Beta 02 [7] following the recommended conditions. All PCR products were analyzed by electrophoresis in 1.0% agarose gels with the 1 kb DNA ladder (Thermo Fisher Scientific Inc., Pittsburgh, USA) as size markers.

Full length viral genome amplification, cloning and sequencing of amplicons

The full-length Begomovirus genome was amplified from the DNA isolated from fifteen symptomatic samples by the rolling circle amplification (RCA) method using an Illustra TempliPhi Amplification kit (GE Healthcare, Buckinghamshire, UK). The resulting products were digested with BamHI, XbaI, DraI and

XhoI and analyzed by electrophoresis in 1.5% agarose gels to identify enzymes to clone full length genomic DNA. Putative full-length (~2.7 kb) amplicons from three randomly selected samples were cloned into the pCAMBIA1300 vector digested with the appropriate enzyme. The cloned Begomovirus genome (~2.7 kb) and ~1.3 kb DNA fragments obtained by PCR using and betasatellite-specific primers were sequenced and deposited in GenBank database under accession numbers KY089033 and KY089034, respectively.

Sequence analyses

The sequences were compared with those publicly available in the NCBI sequence database by BLASTn analysis for sequence identities. The open reading frames (ORFs) located in the genome and their predicted amino acid sequences were analyzed with the ORF finder (www.ncbi.nlm.nih.gov/projects/gorf/) and ExPasy (<http://www.expasy.org/resources/search/keywords:translation>) tools, respectively. The species demarcation tool (SDT, Muhire et al. 2014) was used with default parameters to obtain the specific nucleotide sequence identity of Begomovirus, alphasatellite and betasatellites identified here with respective sequences of DNA molecules. The amino acid identity of protein coding ORFs of these molecules were obtained by the *DiAlign* tool (<http://www.genomatix.de/cgi-bin/dialign/dialign.pl>). The evolutionary history was inferred using the Maximum Parsimony method and the tree was obtained using the Close-Neighbor-Interchange algorithm in MEGA v6.1 program [17].

Construct preparation and Agro-infectivity assays

Constructs for the infectivity of the Begomovirus were produced by digesting the clone with *Bam*HI and *Hind*III and ligating the resulting 2,607 bp fragment into the pCAMBIA1300 vector. Then, the full-length genomic 2746 bp *Bam*HI fragment was ligated at the *Bam*HI site in the sense orientation. For preparation of infectious clone of the betasatellite, the RCA amplified DNAs were partially digested with *Sma*I and *Eco*RI, respectively which produced ~1.4 kb monomer and ~2.8 kb dimer. The dimer of ~2.8 kb recovered from both the digestions were eluted independently and ligated at respective cloning site of pCAMBIA1300 vector. The constructs for the infectivity of the Begomovirus and betasatellite will henceforth be referred to as SKJA-Agro and SKJB-Agro respectively. pCAMBIA1300 constructs were mobilized into *Agrobacterium tumefaciens* (GV3101) and Agroinoculated by the syringe infiltration method (in combination or separately) into leaf lamina of *Helianthus spp.* and *Nicotiana glutinosa* seedlings at the 4 to 6 leaf stage. The infiltrated plants were kept in glasshouse conditions (natural illumination with 25°C ± 2°C temperature) and examined regularly for appearance of symptoms. The experiments were repeated three times with ten plants of each species for each inoculum.

Results

Disease symptoms and virus transmission

The golden yellow mosaic disease was noticed on a number of wild sunflower (*Helianthus sp.*) plants growing in road side in Jaipur (Rajasthan), India during December 2015 with the disease incidence about 60% (Figure1a). The infected plants exhibited golden yellows accompanied with yellow vein net on

leaf and leaf curl symptoms (Figure 1b and 1c) as compared to healthy (Figure 1d) ones. The disease symptoms in these plants were similar to those in other plants induced by Begomoviruses.

About 4-6 whiteflies (*Bemisia tabaci*) per plant were also observed on *Helianthus* spp. plants (with and without symptoms). Based on presence of a whitefly population and typical Begomovirus-like (yellow vein net and leaf curl) symptoms, the causal agent was suspected to be a Begomovirus. Therefore, a whitefly transmission test was attempted onto healthy *Helianthus* spp. seedlings using *B. tabaci*. As a result, the causal pathogen was successfully transmitted from naturally infected to healthy *Helianthus* spp. seedlings which developed similar yellow vein net and leaf curl symptoms (5/5 plants) at 25-30 days post inoculation (dpi), suggesting Koch's postulates.

Begomovirus detection by NASH assays and PCR

NASH resulted in positive signals of hybridization with a Begomovirus probe in all 5/5 samples of naturally infected as well as in all 2/2 whitefly-transmitted *Helianthus* spp. plants, similar to a positive control, while no signals of hybridization was obtained in a healthy plant (Figure 2). These results indicated the presence of Begomovirus.

The Begomovirus infection was further confirmed by PCR performed using Begomovirus specific degenerate primers (PALiv1978 and PARIc496, [15], which resulted in amplification

of ~1.2 kb DNA fragments from all 5/5 samples of naturally infected and 2/2 whitefly transmitted plants of *Helianthus* sp. suggesting Begomovirus infection, whereas no fragment was amplified from a non-symptomatic plant (Figure 3). All samples were also assessed for the presence of a DNA-B component by PCR with specific primers. Because no DNA fragments were amplified from any of seven samples, this suggested the Begomovirus was monopartite.

Characterization of the Begomovirus associated with *Helianthus* spp.

RCA products obtained from five naturally infected symptomatic leaf samples were digested with BamHI, XbaI, DraI and XhoI. The expected size ~2.7 kb DNA fragment was observed in all five samples following digestion with BamHI only. The ~2.7 kb fragment from three positive samples were cloned and sequenced. The sequences of three isolates were identical to each other; therefore, the 2746 nucleotide sequence of one isolate (from *Helianthus* spp.) was deposited in the GenBank database under accession number (KY089033). Analysis of the sequence revealed that the Begomovirus characterized here contains six open reading frames: V1 and V2 in the virion sense and C1, C2, C3 and C4 in the complementary sense and an intergenic region. The Begomovirus isolated from *Helianthus* spp was designated as SKJA isolate.

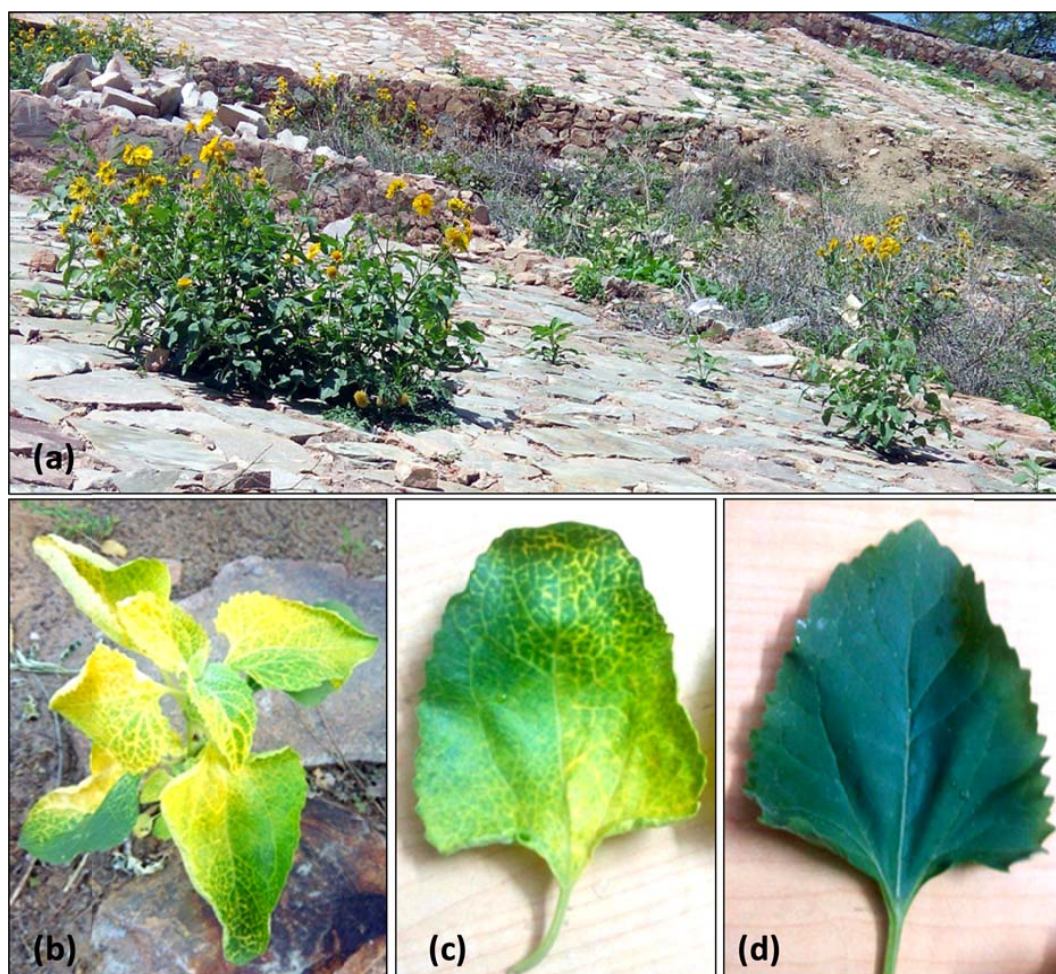


Figure 1. Naturally infected wild sunflower (*Helianthus* sp.) plants growing in baron land at Jaipur, Rajasthan (a). A close view of a diseased plant showing symptoms of severe yellow vein net and leaf curl (b) and a diseased leaf (c) as compared to healthy plant (d).

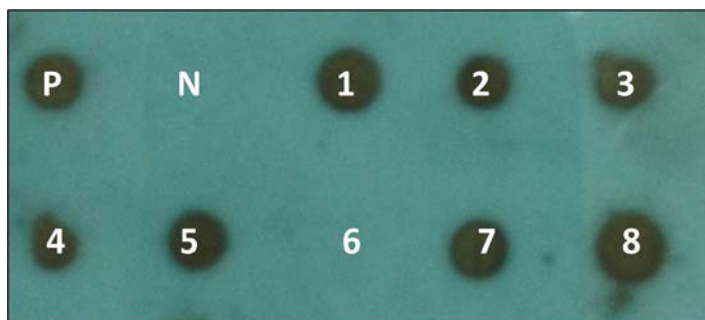


Figure 2. Nucleic acid spot hybridization (NASH) assay of the DNAs isolated from five naturally infected (1-5), two whitefly transmitted leaf samples (7-8) and a healthy sample (6) of *Helianthus sp.* plants with a radiolabelled probe generated from a Begomovirus clone [15]. NASH assays showed positive signals of hybridization in all samples of naturally infected (1-5) as well as whitefly transmitted (7-8) plants but not in a healthy (6) *Helianthus spp* plant. P=Begomoviral cloned DNA blotted as a positive control and N=genomic DNA isolated from a leaf sample of healthy tobacco plant as negative control.

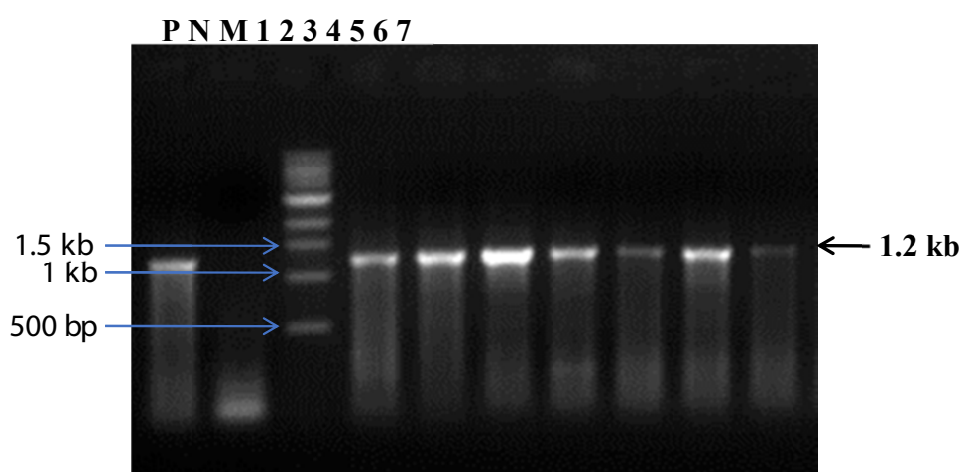


Figure 3. Gel image for detection of Begomovirus by PCR using Begomovirus specific degenerate primers, PALIv1978 and PARIc496, Rojas et al. 1993) showing 1.2 Kb band in all five naturally infected (lane 1-5) and two whitefly transmitted (lane 6-7) *Helianthus sp.* plants similar to a positive control (begomoviral cloned DNA, lane P) but not in a negative control (DNA isolated from a healthy sample, lane N). M=1 Kb DNA ladder as marker.

During BLASTn analysis, the virus sequence (KY089033) isolated from *Helianthus spp.* (SKJA isolate) shared 95% to 98% with complete genome of *Ageratum enation virus* (AEV) isolates: AM698011 (*Ageratum conyzoides*-Pakistan); KC795968 (*Solanum lycopersicum*-India); AM701770 (*Brassica rapa*-Pakistan); AJ437618 (*Ageratum conyzoides*-Nepal); GQ268327 (*Trichosanthes dioica*-India); FJ177031 (*Cleome gynandra*-India); EU867513 (*Amaranthus cruentus*-India). It also shared 92% to 93% identities with AEV isolates: KP195264 (*Solanum lycopersicum*-India); KJ488991 (*Ageratum houstonianum*-India); FN794201 (*Crassocephalum crepidioides*-India) [18]; KM262822 (*Calendula officinalis*-India); KJ488990 (*Ageratum houstonianum*-India); JQ911767 (*Ageratum sp.* (ornamental)-India); JX436472 (*Solanum lycopersicum*-India). While identities were scored as 88% to 89% with *Tobacco curly shoot virus* isolates: HQ407395 (*Helianthus spp.*- India); KM383757 (*Solanum lycopersicum*-Bangladesh); GU001879 and AJ420318 (pepper-China) [19] and 87% with *Tomato leaf curl Bangladesh virus* isolates: KM383762, KM383765 and KM383759 (*Solanum lycopersicum*-Bangladesh).

Pairwise sequence comparison of nucleotide sequence of the DNA genome and amino acid sequences of various ORFs of the SKJA isolate with respective sequences of various isolates of AEV (appeared in BLASTn) was also performed (Table 1). The

SKJA isolate shared the highest nucleotide sequence identity (98.3%) with three isolates of AEV (KM262822, GQ268327 and JF682242) reported from India and between 95.7% and 97.5% nucleotide sequence identity with other AEV isolates reported from India and Pakistan (Table 1). The coat protein (VI) gene of the virus isolate also showed 98.4% to 100% amino acid sequence identities with respective sequences of various isolates of AEV considered for study. The other genes: VI, CI, C2, C3 and C4 of the virus isolate under study shared 46.8% to 88.6%, 75.3% to 93.1%, 88.1% to 92.5%, 88.1% to 100% and 52.6% to 100%, respectively (Table 1).

To determine the phylogenetic relationship of the SKJA isolate with other close Begomoviruses, phylogeny was done using the MEGA v6.1 program. The SKJA isolate showed closest relationship with isolates of *Ageratum enation virus* (AEV): KJ488990, KJ488991 (*Ageratum houstonianum*) and JQ911767 (*Ageratum sp.*, ornamental) reported from India and close relationships with other AEV isolates considered for this study (Figure 4). Based on high sequence identity and close phylogenetic relationships, and Begomovirus species demarcation criteria of >91% identity recommended by the ICTV Geminiviridae study group (Brown et al. 2015), the Begomovirus isolate SKJA was identified as an isolate of AEV for which the isolate descriptor AEV-[In:Lko:Hel:16] is proposed.

Table 1. Sequence identities of *Ageratum enation virus* of *Helianthus* sp. with other selected Begomovirus genomes at nucleotide (nt) and their ORFs at amino acid (aa) levels.

Accessions	Virus	Host	Location	DNA genome	V2	V1	C3	C2	C1	C4
KM262822	AEV	<i>Calendula officinalis</i>	India	98.3	46.8	98.8	89.5	91.0	92.2	95.3
GQ268327	AEV	<i>Amaranthus cruentus</i>	India	98.3	87.8	98.8	88.8	90.3	91.4	82.4
JF682242	AEV	<i>Solanum lycopersicum</i>	India	98.3	85.3	97.3	88.1	89.6	92.2	82.1
KP195264	AEV	<i>Solanum lycopersicum</i>	India	97.5	85.1	99.2	88.1	88.1	92.2	84.7
KJ488991	AEV	<i>Brassica rapa</i>	India	97.5	85.8	98.8	100.0	92.5	92.2	92.9
KC795968	AEV	<i>Solanum lycopersicum</i>	India	97.5	86.4	100.0	90.3	92.5	75.3	52.6
JX436472	AEV	<i>Ageratum</i> sp.	India	97.5	85.7	99.2	100.0	92.5	92.8	83.5
JQ911767	AEV	<i>Ageratum houstonianum</i>	India	97.5	86.0	99.2	90.3	92.5	92.2	96.5
KJ488990	AEV	<i>Ageratum houstonianum</i>	India	97.5	86.1	99.2	89.6	92.5	92.2	96.5
KJ488991	AEV	<i>Brassica rapa</i>	India	97.5	85.8	98.8	100.0	92.5	92.2	92.9
FJ177031	AEV	<i>Cleome gynandra</i>	India	97.4	88.1	98.8	92.5	89.6	92.5	81.2
AM698011	AEV	<i>Ageratum conyzoides</i>	Pakistan	97.4	86.4	98.4	88.1	88.8	90.6	82.4
JQ911765	AEV	<i>Papaver somniferum</i>	India	97.4	85.1	98.8	88.6	89.6	92.0	81.2
FN794201	AEV	<i>Crassocephalum crepidioides</i>	India	96.7	85.5	99.2	90.3	91.1	93.1	94.1
AJ437618	AEV	<i>Ageratum conyzoides</i>	India	96.6	87.0	99.2	100.0	88.1	92.2	80.0
EU867513	AEV	<i>Amaranthus hypochondriacus</i>	India	96.6	88.6	98.4	88.1	89.6	91.4	81.2
AM701770	AEV	<i>Ageratum conyzoides</i>	India	95.7	87.2	98.4	100.0	90.3	92.0	100.0

AEV=*Ageratum enation virus*

Characterization of satellite molecules associated with *Helianthus* sp.

Because monopartite Begomoviruses are reported to be associated with satellites, PCR analyses with betasatellite-specific primers were performed with three DNA samples of infected *Helianthus* spp. (from which the Begomovirus genome was amplified). The primers directed the amplification of ~1.4 kb DNA fragments from all three infected samples which indicated the association of a betasatellite with AEV in *Helianthus* sp. The ~1.4 kb betasatellite components obtained from SKJ1 sample were sequenced and deposited in GenBank under accession numbers: KY089034 (SKJB-betasatellite). The betasatellite associated with Begomovirus isolated from *Helianthus* spp was designated as SKJB-betasatellite isolate. Sequence analysis of the SKJB isolate revealed 1366 nucleotides that encode for a conserved β C1 ORF in the complementary-sense orientation as well as the satellite conserved region (SCR) and an A-rich region.

BLASTn analysis showed that the isolate SKJB (KY089034) showed highest 97% sequence identity with *Ageratum* leaf curl betasatellite (ALCB) isolates: JQ408218 (*Ageratum* sp.), KR922823 (*Calendula officinalis*), KR922821 (*C. officinalis*), KR922822 (*C. officinalis*) reported from India. While the identities were 90% to 94% with other ALCB isolates: KX108998 (Fenugreek), AM412239 (*Sonchus oleraceus*), AM698010 (*Ageratum conyzoides*), AM701771 (*Brassica rapa*), AM494977 (*Digera arvensis*), F728868 (*Ageratum conyzoides*), JF728869 (*Daucus carota*), KJ028213 (*Solanum nigrum*), KC305084 (wheat), KC305085 (wheat), HM143915 (*Nicotiana glutinosa*), KJ948106 (*Papaver somniferum*), JX512904 (*Amaranthus hypochondriacus*), JQ710745 (*Amaranthus cruentus*) and KU376492 (*Calotropis procera*) reported from India and Pakistan.

The pairwise sequence identity of the complete SKJB-betasatellite sequence (KY089034) revealed highest 97.4% nucleotide identities with *Ageratum* leaf curl betasatellite

(ALCB) sequences (KR922821, KR922822, KR922823, JQ408218) and 91.8% to 94.3% identities with other ALCB. It also showed 91.7% to 91.8% identities with *Ageratum* yellow leaf curl betasatellite (AYLCB) isolates (Table 2). The β C1 ORF of the SKJB-betasatellite showed 78.90% to 79.70% amino acid sequence identities with corresponding ORF of ALCB isolates (Table 2). The phylogenetic analysis showed the close relationships of SKJB-betasatellite with isolates of ALCB and these isolates clustered together in a monophyletic group (Figure 5). Because of the high sequence identity and close relationships with ALCB, the SKJB-betasatellite was identified as isolate of ALCB for which isolate descriptor ALCB-[In:Lko:Hel:16] is proposed.

Agro-infectivity assays

The infectivity assay was conducted with Agroinfectious clones of AEV-(In:Lko:Hel:16) and ALCB-(In:Lko:Hel:16) DNAs. *Helianthus* sp. and *N. glutinosa* plants agroinoculated with the AEV+ALCB DNAs developed prominent yellow vein and leaf curl symptoms respectively, by 35 dpi (Table 3). The symptoms that developed in experimentally infected *Helianthus* sp. plants were indistinguishable from the symptoms observed in *Helianthus* sp. plants in the field. Plants inoculated with AEV-A alone also developed mild symptoms, whereas no symptoms were observed when plants were inoculated with ALVB alone or mock (Table 3). These results satisfy Koch's postulates for AEV with ALCB causing leaf curl disease of *Helianthus* sp.

The presence and/or absence of AEV+ALCB DNAs in the leaves developing after inoculation of both *Helianthus* sp. and *N. glutinosa* plants were assessed by PCR with the respective specific primers. The AEV DNA was detected in newly emerged leaves of plants agroinoculated with AEV alone as well as in combinations with AEV+ALCB DNAs was also detected in *Helianthus* sp. and *N. glutinosa* plants (Table 3). These results indicated that AEV can replicate and move independently or in combinations with AEV+ALCB in inoculated plants.

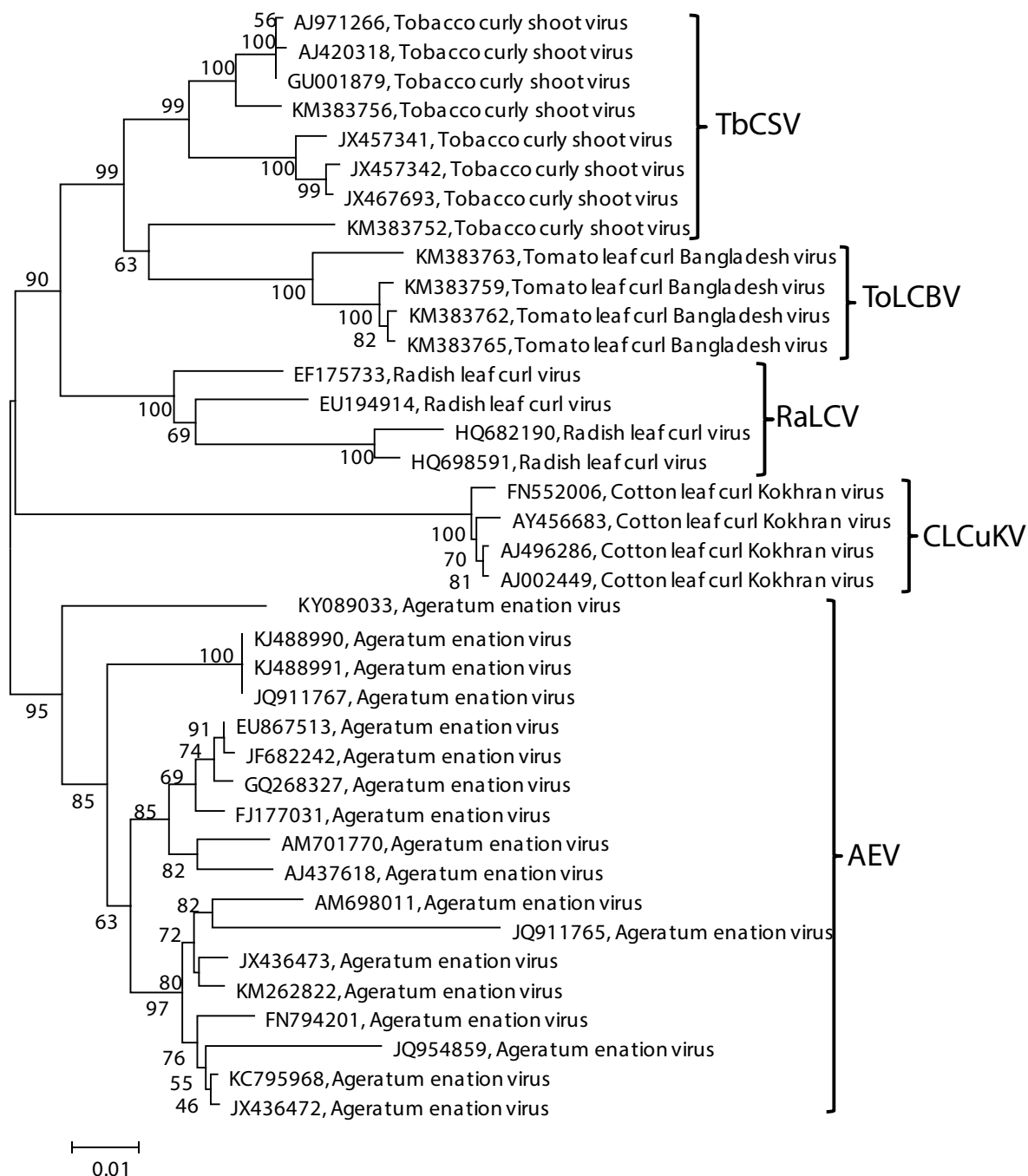


Figure 4. Phylogenetic relationships of Begomovirus genome under study (KY089033) with Ageratum enation virus (AEV), Tobacco curly shoot virus (TbCSV), Tomato leaf curl Bangladesh virus (ToLCBV), Radish leaf curl virus (RaLCV), Cotton leaf curl Kokhran virus (CLCuKV) selected based on BLASTn analysis was determined by NJ method within MEGA v6.1 program with 1000 bootstrap replicates. AEV strains showing close relationships with isolate under study (indicated by filled black square).

Discussion

The Begomovirus species AEV was first established in 2003, when the presently applicable species demarcation criteria for the genus Begomovirus were established, based upon the sequence of the Nepal isolate (AJ437618), which is the type isolate of the species [5]. Since this time AEV has been shown to be widespread across northern India and to infect a number of distinct hosts [7]. The occurrence of AgEV has been reported on *Amaranthus cruentus* [14], *Cleome gynandra* [20], *Zinnia* sp. [21], *Trichosanthes dioica* [22], *Daucus carota* [23] and *Solanum lycopersicum* [24] from India and *Brassica rapa* (AM701770), *Ageratum conyzoides* (AM698011), *Sonchus*

oleraceus (AM261836) from Pakistan Tahir et al. [25] are reported in literature. Tahir et al. also showed that AEV has two distinct strains, and to occur across a large geographic area [25].

It is evident from the literature that AEV has a large natural host range which includes species from the families *Brassicaceae* (turnip), *Asteraceae* (*A. conyzoides*, *Z. elegans*, *C. crepidioides*, *Tagetes patula* [17] and *S. oleraceus*), *Amaranthaceae* (*A. cruentus*), *Cucurbitaceae* (*T. dioica*), *Apiaceae* (carrot) and *Cleomaceae* (*C. gynandra*). This indicates that AEV is predominantly a weed infecting Begomovirus, although it apparently has the capacity to occasionally infect crop species and is reported to be causing losses of the minor grain crop *A. cruentus* [20] and carrot.

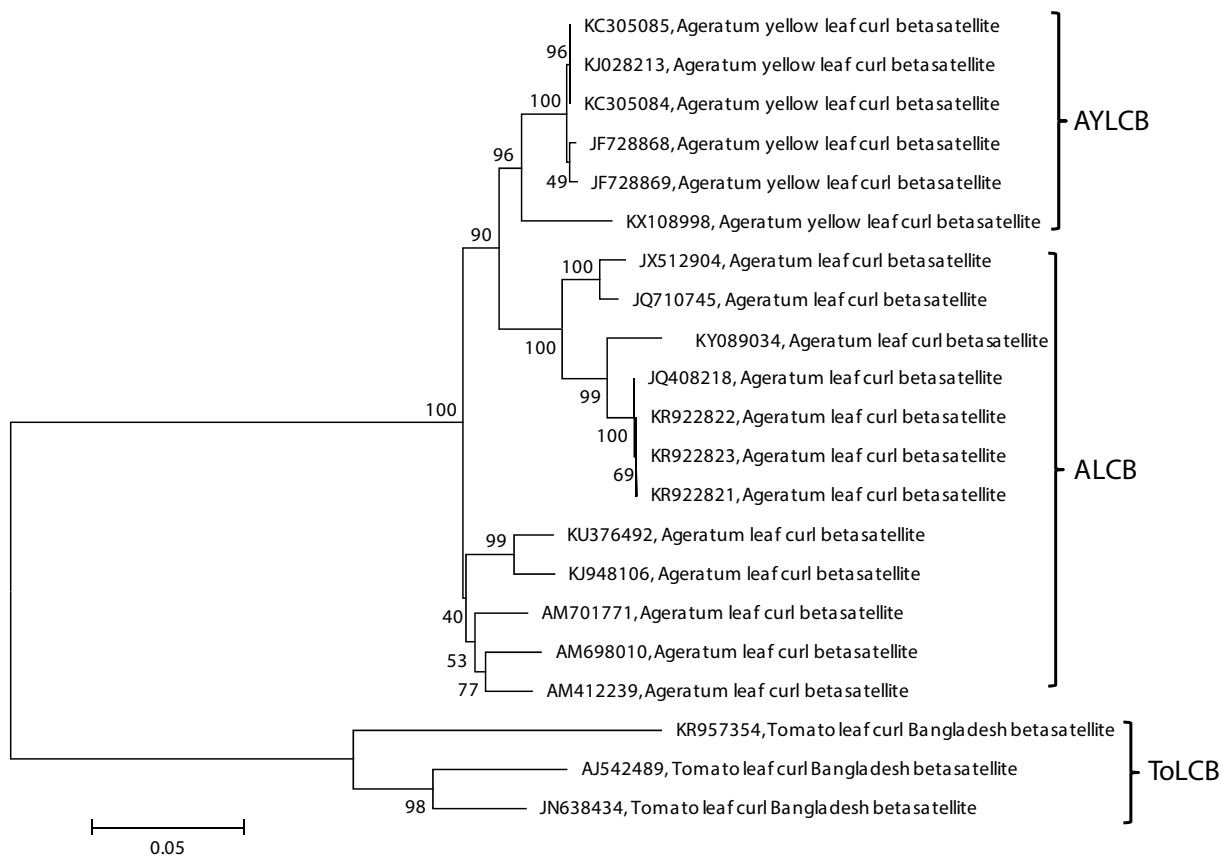


Figure 5. Phylogenetic relationships of nucleotide sequences of DNA-beta genome (KY089034) with *Ageratum* leaf curl Betasatellite (ALCB), *Ageratum* yellow leaf curl Betasatellite (AYLCB), *Tomato* leaf curl Bangladesh Betasatellite (ToLCB) determined by NJ method within MEGA v6.1 program with 1000 bootstrap replicates.

Table 2: Percent sequence identities of *Ageratum* leaf curl betasatellite molecule of *Helianthus* sp. with other selected betasatellite at complete nucleotide (nt) and their beta C1 ORF at amino acid (aa) levels.

Accessions	Betasatellite	Percent sequence identity at	
		Complete nucleotide	beta C1 amino acid
KR922822	<i>Ageratum</i> leaf curl beta satellite	97.4	79.70
KR922821	<i>Ageratum</i> leaf curl beta satellite	97.4	79.70
KR922823	<i>Ageratum</i> leaf curl beta satellite	97.4	79.70
JQ408218	<i>Ageratum</i> leaf curl betasatellite	97.4	79.70
KU376492	<i>Ageratum</i> leaf curl betasatellite	94.3	78.90
JQ710745	<i>Ageratum</i> leaf curl betasatellite	93.7	80.25
JX512904	<i>Ageratum</i> leaf curl betasatellite	93.6	80.50
KJ948106	<i>Ageratum</i> leaf curl betasatellite	91.9	78.90
HM143915	<i>Ageratum</i> leaf curl betasatellite	91.8	78.90
KC305085	<i>Ageratum</i> yellow leaf curl betasatellite	91.8	82.10
KC305084	<i>Ageratum</i> yellow leaf curl betasatellite	91.7	82.10
KJ028213	<i>Ageratum</i> yellow leaf curl betasatellite	91.8	82.10

Table 3. Infectivity assay using seedlings of *Helianthus* spp. and *Nicotiana glutinosa* and agroinfectious clones of AEV-[In:Lko:Hel:16] and ALCB-[In:Lko:Hel:16] DNAs abbreviated as DNA-A and DNA-β respectively.

Assay plant	Agroinfectious clone/s	Symptoms (by 35 dpi)	Symptomatic/inoculated plants	Detection of*	
				DNA-A	DNA-β
<i>Helianthus</i> sp.	DNA-A	Mild leaf curl	9/10	9/10	0/10
	DNA-A + DNA-β	Severe leaf curl	10/10	10/10	10/10
	DNA-β	No symptoms	0/10	0/10	0/10
<i>N. glutinosa</i>	DNA-A	Mild yellowing	8/10	8/10	0/10
	DNA-A + DNA-β	Yellowing & leaf curl	9/10	9/10	9/10
	DNA-β	No symptoms	0/10	0/10	0/10

*Fourth systemically infected leaf (from the top) was used for PCR detection. Amplifications during PCR were obtained using degenerate primers of begomovirus, betasatellite and alphasatellite DNAs.

Kumar et al., Tahir et al. and Marwal et al. suggested that the common partner of AEV is the betasatellite species AYLCB [25-27]. For none of the other AEV sequences available in the databases has the associated betasatellite been identified. Of the other AYLCB isolates available in the databases, only for two have the helper Begomovirus been identified *Tobacco curly shoot virus* isolated from wild sunflower (*Helianthus sp.*) originating from India (for HQ407397) and *Alternanthera yellow vein virus* (AIYVV) in *Sonchus arvensis* originating from Pakistan (for FN432358) [26]. It is interesting to note that the AYLCB from *S. arvensis* was identified in co-infection with a second betasatellite, Cotton leaf curl Multan betasatellite (CLCuMB). The identification of YLCB with several distinct Begomoviruses suggests that, as has been shown for other betasatellites [28-31], this has the capacity to be trans-replicated and maintained by more than one Begomovirus [25].

Tahir et al. also suggested that the availability of infectious AEV and AYLCB clones that induce distinct symptoms in common hosts opens up the possibility of investigating the molecular basis for these differences by, for example, exchanging betasatellite components. Although initially it was isolated from weeds, they also showed that now it is crossing into cultivated species and could become a more significant problem for agriculture in the future [25].

In India, *Verbesina encelioides* yellow vein Lakshmangarh virus (JN998449) and *Verbesina* yellow vein betasatellite (JQ693145) have been reported to associated with yellow vein disease of *Verbesina encelioides*. However, to the best of our knowledge, the present study is the first report on the molecular characterization of AEV and *Ageratum* leaf curl betasatellite (ALCB) associated with yellow vein net and leaf curl disease in wild sunflower (*Helianthus spp.*). Since wild sunflower (*Helianthus spp.*) is found grows as an invasive weed along with roadside, agricultural fields or/and in baron lands of Rajasthan, India, therefore, it may act as a reservoir host for AEV and ALCB, and become a threat to cultivation of sunflower or other commercially cultivated plants. Moreover, the first report of AEV and ALCB betasatellite on a new weed host i.e. wild sunflower (*Helianthus spp.*) is a novelty.

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***Correspondence to:**

Raj SK
 Plant Molecular Virology Laboratory
 Council of Scientific and Industrial Research
 National Botanical Research Institute,
 Lucknow 226001, UP
 India
 Tel: 091-522-2297950
 Fax: 091-522-2205836
 E-mail: skraj2@rediffmail.com