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RESEARCH ARTICLE

Identification and molecular characterization of a begomovirus associated with yellow vein leaf curl disease of Kalmegh (*Andrographis paniculata*)

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Abstract

Kalmegh (*Andrographis paniculata*) is an important medicinal plant growing in the Southeast Asia. Recent initiatives have been undertaken by various agencies to promote its cultivation. Symptoms such as leaf curling, yellowing of vein and clearing were observed on Kalmegh plants in Bhubaneshwar, Odisha. To identify the causal virus, DNA extracted using the CTAB method and subjected to PCR amplification using primers targeting the coat protein gene, DNA-A and the associated betasatellite. The coat protein gene, DNA-A component and betasatellite were successfully amplified, cloned and sequenced. The sizes of the begomovirus DNA-A and betasatellite components were approximately 2.7 kb and 1.4 kb respectively. The nucleotide sequence shared highest identity (89 %) with *Andrographis* yellow vein leaf curl virus (AYVLCV) isolate-64 (KM359406). The associated betasatellite showed 95 % sequence similarity with AYVLC betasatellite (KC9672282, KM359409). The analysis identified three key structural elements: the satellite conserved region (SCR), an adenine (A)-rich region and the open reading frame (ORF) ßC1. The present investigation characterizes the leaf curl virus and the associated satellite DNA molecule, which will pave the way for further research for reliable diagnosis, epidemiology and durable management strategies.

Keywords: *Andrographis*; begomovirus; betasatellite; coat protein; monopartite

Introduction

Kalmegh (*Andrographis paniculata*), a member of the family Acanthaceae, is an annual herb renowned for its potent medicinal properties. It is indigenous to India and Sri Lanka and is extensively cultivated across Southern and Southeast Asia, where it has long been used in traditional medicine to treat a variety of infections and ailments. Although the entire plant is used for its preventive and curative properties, the leaves and roots are primarily employed in medicinal preparations due to their extremely bitter taste (1, 2). Kalmegh is a key component in 26 Ayurvedic formulations listed in the Indian Pharmacopoeia and serves as a principal ingredient in both Ayurvedic and Unani systems of medicine (1). Andrographolide, a diterpenoid lactone and the most prominent bioactive compound in Kalmegh, is primarily responsible for the herb's pharmacological effects and was notably used as a curative during the Indian flu epidemic of 1919; its highest concentration, reaching up to 2.39 %, is found in the leaves (1, 3-5).

Traditionally, most medicinal plants, including Kalmegh, have been growing in forests and barren lands; however, growing interest among farmers and constructive policy interventions have gradually shifted its sourcing from wild collection to commercial cultivation. Despite these efforts, profitable cultivation remains challenged by climatic variability and emerging abiotic and biotic stresses, which negatively impact both yield and quality (6). The identification and characterization of the emerging diseases and insects is instrumental in designing management strategies to alleviate the losses. Kalmegh plants growing in Bhubaneswar, located in eastern coastal region of India, exhibited virus-like symptoms including vein yellowing in young leaves, upward curling of older leaves, reduced leaf size and stunted plant growth. Similar symptomatology in Kalmegh plants were reported in Uttar Pradesh, northern part of India with 25-40 % disease incidence. The disease was associated with *Eclipta* yellow vein virus (EYVV) accompanied by a betasatellite DNA molecule, as well as *Catharanthus* yellow mosaic virus (CYMV) in association with AYVLC betasatellite DNA (7, 8). These viruses were identified as begomoviruses, belonging to the family Geminiviridae, which

represents the largest and most important group of viruses infecting agriculturally important crops. Bipartite begomoviruses possesses genomes composed of two components, DNA-A and DNA-B, each approximately 2.5 to 2.6 kb in size. While DNA-A is capable of independent replication and virion formation, DNA-B is essential for systemic infection. These two components share a ~200 base pair (bp) intergenic region, known as the "common region" (CR), which contains a conserved stem-loop structure and the 5′-TAATATTAC-3′ sequence, representing the viral origin of replication (v-ori). The current study aims to identify and molecularly characterize the begomovirus and associated components responsible for leaf curl symptoms in Kalmegh plants from Odisha.

Materials and Methods

Samples and source of isolates

Kalmegh plants exhibiting typical virus-like symptoms were collected from the experimental farm of the All India Crop Research Project (AICRP) on Medicinal Aromatic Plants and Betelvine (MAP&B), Odisha University of Agriculture and Technology (OUAT), Bhubaneswar. The site is located at 20° 15' N latitude and 85° 52' E longitude, at an altitude of 25.5 m above mean sea level (MSL), approximately 40 km inland from the Bay of Bengal.

Screening of Kalmegh entries against yellow vein leaf curl disease

A total of 39 Kalmegh entries were evaluated for their resistance to yellow vein leaf curl disease at the experimental farm of the AICRP on MAP&B, Bhubaneshwar. Each entry was scored based on its disease reaction, as detailed in Table 1.

Nucleic acid isolation and PCR assays

Total plant DNA was extracted from 100 mg of symptomatic Kalmegh leaf tissue using the CTAB method (9), quantified using a NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific, USA) and stored at -20 °C until further use. DNA from 5 symptomatic plants was subjected to PCR amplification using universal primers for begomovirus coat protein primers, Tomato leaf curl virus-F (ToLCV-F) and ToLCV-R (Table 2) (10).

Each 25 µL PCR reaction contained 10× reaction buffer, 100 ng of plant DNA, 10 mM dNTPs, 25 mM $MgCl_2$, 1 U Taq DNA polymerase (Promega, UK), 200 ng of each primer and RNase-free water (Qiagen). PCR conditions were as follows: initial denaturation for 5 min at 94 °C, 30 sec for melting at 94 °C, 30 sec for annealing at 50 °C, 30 sec for synthesis at 72 °C and final extension for 10 min at 72 °C for 30 cycles. Samples that tested positive were further screened for the presence of betasatellite and alphasatellite DNA using specific primers (Table 2) (7, 11). The PCR mix was the same as for coat protein amplification. The PCR conditions for betasatellite were: initial denaturation for 5 min at 94 °C, 45 sec for melting at 94 °C, 1 min for annealing at 63 °C, 2 min for synthesis at 72 °C for 30 cycles and final extension at 72 °C for 10 min. For alpha-satellite detection, the conditions were: initial melting at 94 °C for 1 min, 35 cycles of annealing at 50 °C for 1 min and extension for 1.5 min at 72 °C. The amplified products (25 µL) were loaded on 1 % agarose gels and electrophoresed at 60 V for 1 hr. The gels were visualized under a UV transilluminator (Bio-Rad, USA).

Rolling circle amplification (RCA)

The RCA method was utilized to amplify the full-length genome of the virus using phi29 DNA polymerase (RCA, Templiphi, GE Healthcare, USA) from two symptomatic samples (12). The RCA products were then digested separately with three restriction enzymes-BamHI, HindIII and SacI-to generate linearized full-length genomic DNA. The resulting fragments were separated on a 1 % agarose gel.

Cloning and sequencing

The betasatellite fragments from Kalmegh, generated by digestion of RCA products, were inserted into the pUC18 vector and transformed into *Escherichia coli* (*E. coli*). Sequencing was performed using a commercial service. Betasatellite amplicons were purified with the Nucleospin® Gel and PCR Clean-up System (Macherey-

Table 1. Rating scale used for symptomatic screening of Kalmegh entries

Grade	Description	Reaction
1	No visible symptoms	Free
2	Small yellow specks with restricted spread covering 0.1-5 % area	Highly resistant (HR)
3	Mottling of leaves covering 6-10 % leaf area	Resistant (R)
4	Yellow mottling covering 11-15 % leaf area	Moderately resistant (MR)
5	Yellow mottling and discolouration of 15-20 % leaf area	Moderately susceptible (MS)
6	Yellow colouration of 21-30 % leaves and yellow pods	Susceptible (S)
7	Pronounced yellow mottling and discolouration of leaves and pods, reduction in leaf size and stunting of plants covering 30-50 % of foliage	Susceptible (S)
8	Severe yellow discoloration of leaves covering 50-75 % of foliage, stunting of plants and reduction in pod size	Highly susceptible (HS)
9	Severe yellowing of leaves covering above of foliage, stunting of plants and no pod formation	Highly susceptible (HS)

Table 2. Primer sequences used for amplification of coat protein for DNA-A, betasatellite and alphasatellite

Primer	Target region	Sequence	Annealing temperature	Amplicon size
ToLCV FP	Coat protein	5'AAGATATGGATGGATGAGAAC 3'	50 °C	308 bp
ToLCV RP		5'ACATAATTATTAACCCTAACAA 3'		
β01	Betasatellite	5'GGTACCACTACGCTACGCAGCAGCC 3'	63 °C	1.4 kb
β02		5' GGTACCTACCCTCCCAGGGGTACAC 3'		
DNA 101	Alphasatellite	5' CTGCAGATAATGTAGCTTACCAG 3'	50 °C	1.4 kb
DNA 102		5' GATATGTGCACCTCCTAGACGTC 3'		

Nagel) and then cloned into a TA vector using a cloning kit (RBC TA Cloning Vector Kit, Taiwan), following the manufacturer's guidelines. Recombinant plasmids were screened by rapid lysis and blue/white colony screening was performed on indicator plates, followed by colony PCR using appropriate primer pairs. The selected clones were cultured and plasmid DNA was extracted and purified using the MDI Plasmid Isolation Kit (MDI Membrane Technology, Ambala Cantt, India). The purified plasmids were sequenced in both directions and the sequences were manually assembled.

BLAST analysis and phylogeny

To determine the identity and evolutionary relationship of the viral sequences, a BLASTN search was conducted using the National Center for Biotechnology Information (NCBI) nucleotide database (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The betasatellite sequence and the partial DNA-A sequence obtained from the infected Kalmegh samples were used as query sequences. The search parameters were set to retrieve the most closely related sequences based on nucleotide similarity scores, with default settings used for alignment algorithms and match/mismatch scoring.

The representative sequences of closely related begomoviruses and betasatellites identified through BLASTN were retrieved in FASTA format for comparative analysis. Multiple sequence alignment of the query sequences along with selected reference sequences was carried out using the Clustal W algorithm implemented in MEGA version 6.4. Manual editing was performed where necessary to optimize alignment accuracy. In order to infer phylogenetic relationships and assess the genetic relatedness between the Kalmegh isolates and other begomoviruses, phylogenetic trees were constructed using the Neighbor-Joining (NJ) method. Evolutionary distances were computed using the Kimura 2-parameter model and tree reliability was evaluated by bootstrap analysis with 1000 replicates. The final trees were visualized and annotated using MEGA 6.4, clearly indicating the clustering of the Kalmegh-associated sequences with other known begomoviruses and betasatellites (13).

Results

Symptomatology

In Bhubaneswar, Kalmegh plants exhibited typical virus-like symptoms including vein yellowing in younger leaves, upward curling of older leaves, vein clearing, chlorosis, reduced leaf size, poor inflorescence and stunted growth. These symptoms significantly reduced herb yield, with some cases leading to premature plant death (Fig. 1). The presence of these characteristic symptoms, coupled with the infestation of whiteflies (*Bemisia tabaci*) in infected fields and the absence of mechanical transmission, strongly suggested the involvement of a begomovirus. The disease incidence in the affected plants reached approximately 70 %.

Evaluation of Kalmegh entries against yellow vein leaf curl disease (YVLCD)

Among the 39 entries evaluated for their disease reaction, 13 entries, viz., Ak-7, 8, 11, 13, 14, 17, 19, 20, 21, 26, 27, 29 and 33 were completely free from viral symptoms. Three entries were resistant (R), 4 entries had moderate resistance (MR), while the remaining 26 entries were either susceptible (S), moderately susceptible (MS) or highly susceptible (HS) as described in Table 3.

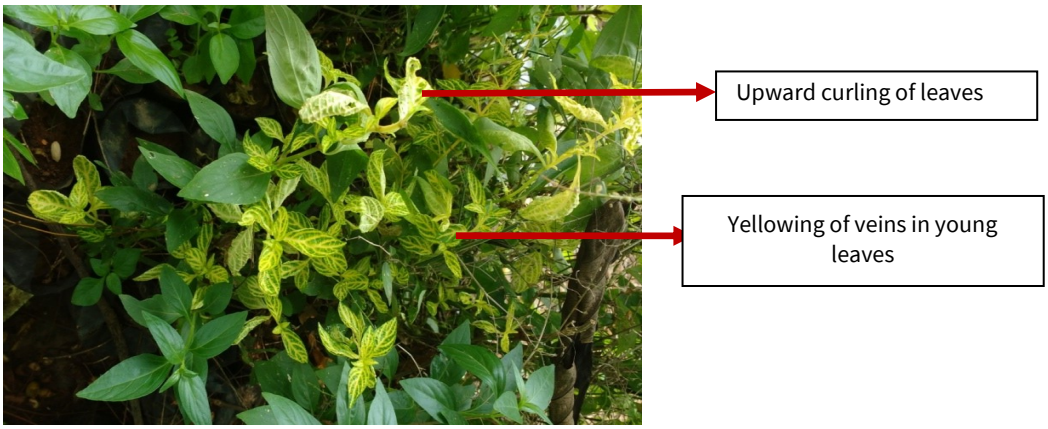


Fig. 1. Kalmegh plants showing typical symptoms of begomovirus infection.

Table 3. Reactions of Kalmegh entries against YVLCD

Sl No.	Entries	Grade	Sl No.	Entries	Grade	Sl No.	Entries	Grade
1	AK-1	9	14	AK-14	1	27	AK-27	1
2	AK-2	9	15	AK-15	7	28	AK-28	8
3	AK-3	5	16	AK-16	4	29	AK-29	1
4	AK-4	7	17	AK-17	1	30	AK-30	7
5	AK-5	8	18	AK-18	3	31	AK-31	4
6	AK-6	5	19	AK-19	1	32	AK-32	7
7	AK-7	1	20	AK-20	1	33	AK-33	1
8	AK-8	1	21	AK-21	1	34	AK-34	3
9	AK-9	4	22	AK-22	3	35	AK-35	8
10	AK-10	9	23	AK-23	6	36	AK-36	9
11	AK-11	1	24	AK-24	9	37	AK-37	9
12	AK-12	6	25	AK-25	9	38	AK-38	9
13	AK-13	1	26	AK-26	1	39	AK-39	4

AK: Anand Kalmegh

Isolation of genomic DNA

Genomic DNA was isolated from symptomatic leaves of 5 Kalmegh samples (K2, K3, K4, K5 and K6), yielding approximately 1200-2000 ng/µL of high-quality DNA. The integrity of the extracted DNA was further confirmed by 1 % agarose gel electrophoresis, which showed bright, distinct bands indicating good quality.

Confirmation of associated virus by PCR

A specific amplicon of 308 bp was observed in all 5 symptomatic samples of Kalmegh using ToLCV coat protein primers, confirming the presence of begomovirus (DNA-A) associated with yellow vein leaf curl disease (Fig. 2). A 1 kb DNA ladder (Fermentas) was used as a size marker to estimate the sizes of DNA bands. Upon this confirmation, genomic DNA from sample K4 was subjected to RCA for further identification of the virus. Restriction digestion of the RCA product using BamHI, HindIII and SacI enzymes yielded an amplicon of ~2.7 kb, further supporting the association of begomovirus DNA-A.

Amplification of DNA from the same plant with betasatellite-specific primers produced a 1.4 kb amplicon, indicating the presence of a betasatellite DNA component in all symptomatic samples (Fig. 3). Sequencing of the RCA clone containing the DNA-A fragment yielded 963 bp, which was submitted to GenBank under accession number MT281376. Sequence analysis showed highest similarity (89 %) with AYVLCV isolate A-64 (KM359406) and more than 80 % identity with other begomoviruses. The betasatellite sequence was 1369 bp and was deposited in GenBank under accession number MT408028. It shared 95 % identity with the betasatellite of AYVLCV (KC967282, KM359409) and more than 80 % identity with other begomovirus-associated betasatellites. These results confirm the etiological role of begomovirus DNA-A and its associated betasatellite in the manifestation of yellow vein leaf curl symptoms in Kalmegh.

Sequence and phylogenetic analysis

The phylogenetic analysis of the partial DNA-A sequence revealed its clustering with *Pedilanthus* leaf curl virus (PeLCV), Chilli leaf curl virus (CLCV) and Papaya leaf curl virus (PaLCV), showing a maximum sequence similarity of 89 %. This partial DNA-A sequence also demonstrated a distinct identity from the AYVLCV isolated from Northern India, despite being from the same host (Fig. 4). Conversely, it showed very low identity with other leaf crumple, leaf curl and mosaic viruses reported from different parts of India and some parts of Asia like Sri Lanka (Fig. 5). The phylogenetic analysis of the betasatellite sequence of the AYVLCV showed maximum similarity of 95 % with the AYVLC betasatellite isolate SS-05 (KR779821). Furthermore, this betasatellite sequence formed a cluster with AYVLC betasatellite clone bt-2 (KC967282), AYVLC betasatellite clone bt-8 (KM359409) and AYVLC betasatellite clone bt-6 (KM359407) (Fig. 6). The sequence exhibited lower similarity with other betasatellites from different leaf curl and yellow mosaic viruses and the least similarity was observed with the Tomato yellow leaf curl China virus satellite (GU058338).

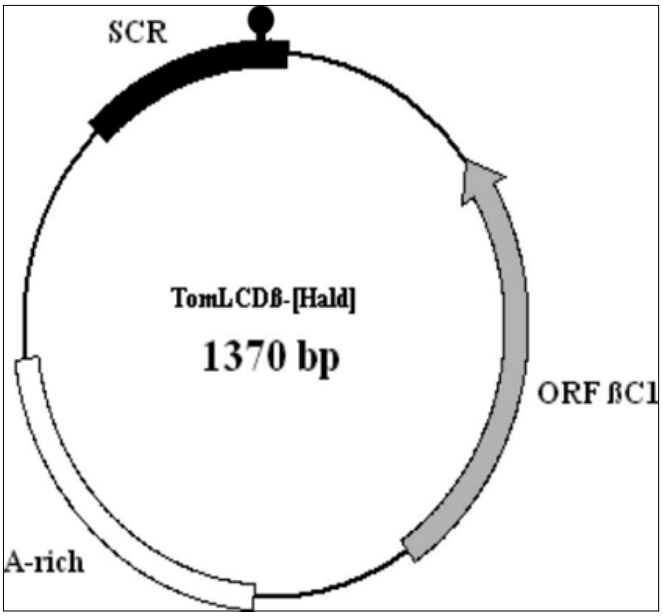
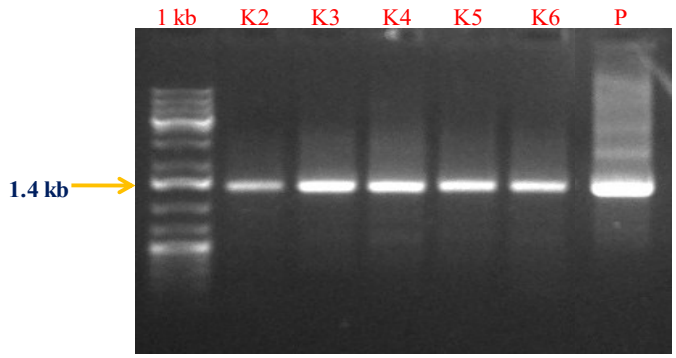
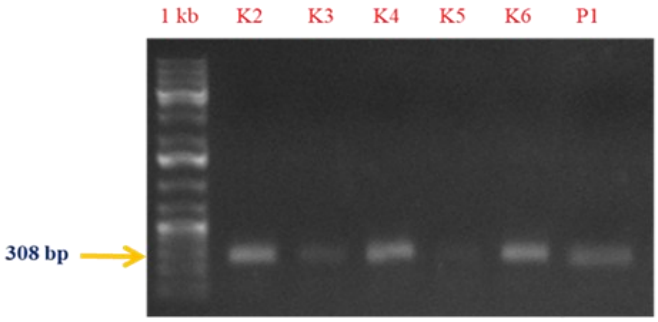


Fig. 4. Structural features of DNA-β associated with AYVLCV.



K2- Kalmegh DNA2 K3-Kalmegh DNA3
K4-Kalmegh DNA4 K5-Kalmegh DNA5
K6-Kalmegh DNA6 P1- Positive control

Fig. 2. PCR amplification of coat protein gene using ToLCV coat protein primers.



K2- Kalmegh DNA2 K3-Kalmegh DNA3
K4-Kalmegh DNA4 K5-Kalmegh DNA5
K6-Kalmegh DNA6 P1- Positive control

Fig. 3. PCR amplification of DNA-β using universal DNA-β-specific primers.

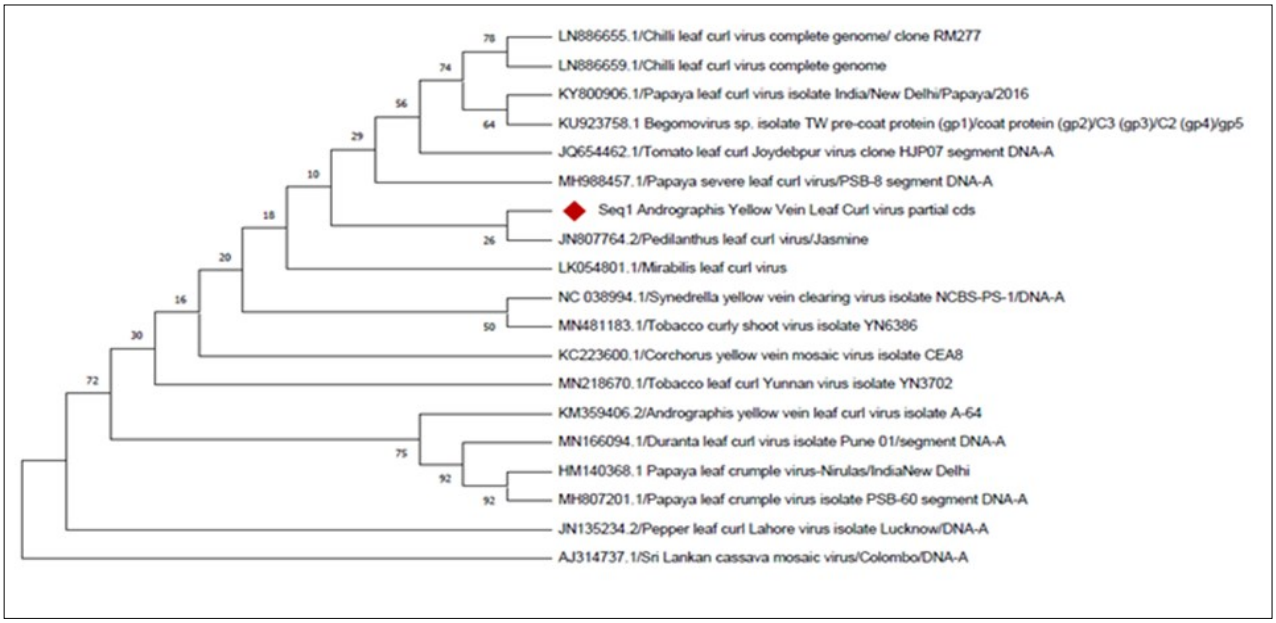


Fig. 5. Phylogenetic tree based on the nucleotide sequence of selected partial DNA-A, constructed using the NJ method in Clustal X and visualized in TreeView v1.6.5. Bootstrap values (100 replicates) are shown at the nodes. The DNA-A sequence associated with YVLCV used in this study is marked in red.

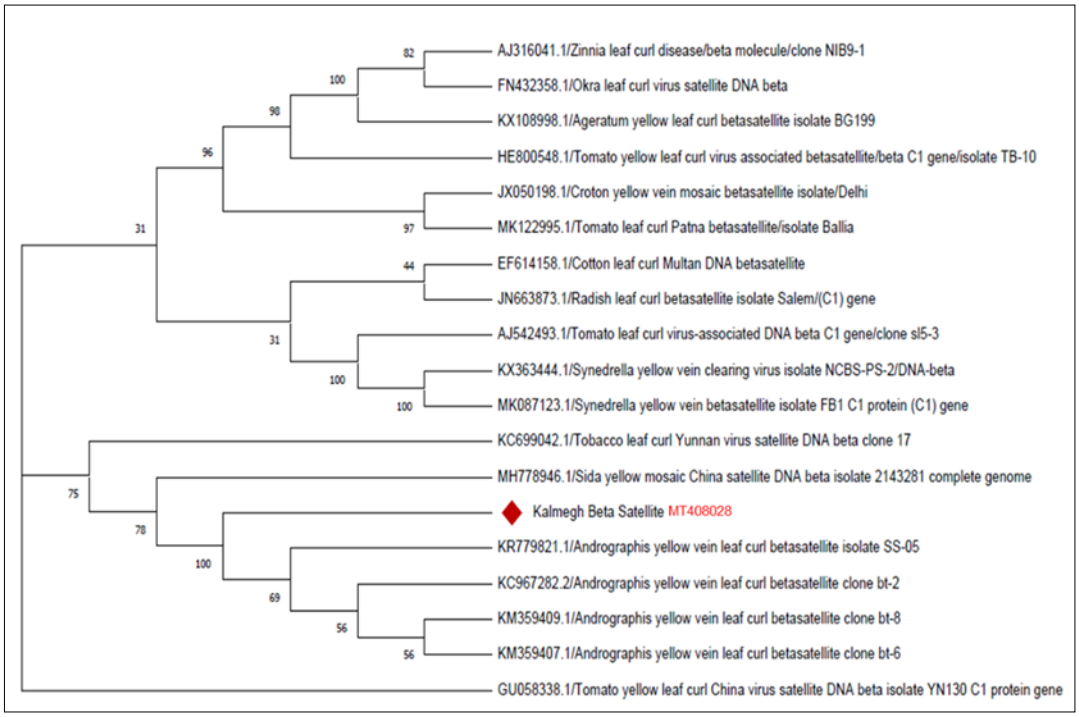


Fig. 6. Phylogenetic tree based on the nucleotide sequence of selected betasatellite, constructed using the NJ method in Clustal X and visualized with TreeView v1.6.5. Bootstrap values (100 replicates) are indicated at the nodes. Red mark indicates the betasatellite associated with YVLCV used in this study.

Discussion

Kalmegh, commonly known as the "King of Bitters", is a valued medicinal herb in the Indian Ayurvedic system. Recent interventions have successfully restored this herb's place in the Indian market, leading to increasing demand. However, its medicinal properties and overall production can be affected by systemic pathogens like viruses. In the present study, 39 Kalmegh germplasm lines from Advanced Varietal Trial-II (AVT-II) trials under the AICRP on Medicinal and Aromatic Plants were screened for natural infection by viral pathogens. Thirteen germplasms were identified as virus-free and 3 other exhibited resistances are the potential candidates not only for further cultivation on a largescale but also for taking up investigations into their resistance mechanisms. Among the infected plants, 5 symptomatic plants were evaluated for the presence of viruses and specifically for the confirmation of begomovirus based on their coat protein, alpha satellite and beta satellite regions. Another begomovirus, EYW, has also been detected in Kalmegh, often found in association with a same betasatellite (8).

The sequence analysis identified 3 key structural features: the SCR, an adenine (A)-rich region and the ORF βC1. The SCR, which is highly conserved (~98 %) among DNA-β molecules of Old World begomoviruses, contains essential elements such as the putative iteron sequence 'GCTACGC'. This sequence facilitates binding of the Replicase (Rep) protein encoded by DNA-A, enabling initiation of replication via the rolling-circle mechanism starting at the nonanucleotide 'TAATATTAC'. The A-rich region acts as a buffering

segment, thought to be important for the encapsidation of the molecule into virions (14). Additionally, the genome analysis revealed a single ORF (positions 217-573 nt) encoding the βC1 protein consisting of 118 amino acids, a hallmark feature conserved across all betasatellites.

Functionally, the βC1 protein plays a critical role in the pathogenicity of the virus complex. In monopartite begomoviruses, βC1 is essential for establishing infection and symptom development by suppressing host gene silencing mechanisms and disrupting plant defense pathways (15-20). In bipartite begomoviruses, although not essential for replication, βC1 enhances disease severity, promotes viral DNA accumulation and increases transmission efficiency by the whitefly vector (21-24). These structural and functional insights strongly support the conclusion that the identified betasatellite, through its conserved βC1 gene and regulatory elements, is intricately involved in the manifestation and severity of yellow vein leaf curl symptoms observed in infected Kalmegh plants.

Alphasatellites, often found in begomovirus disease complexes, are self-replicating satellite molecules that encode a replication-associated protein (Rep). PCR amplification using universal primers did not detect alphasatellite in any of the infected Kalmegh samples, indicating that the virus involved is likely monopartite. The EYVV associated with a betasatellite in Kalmegh also lacked alphasatellite (7). Similar to betasatellite, alphasatellite molecules encode a single replication-associated protein (Rep), which enables their autonomous replication. Although alphasatellites are typically not essential for disease development or maintenance, they have, in some cases, been shown to alleviate disease symptoms (25-27). On the other hand, alphasatellites have been reported to play a role in overcoming host defense mechanisms, potentially through their gene silencing suppression activity (26). Interestingly, the beta sequence identities were found to differ from those of other *Andrographis* leaf curl sequences in the database, contrary to the partial sequences of DNA-A. This difference could be attributed to the unavailability of a complete DNA-A sequence in existing genomic databases.

The experimental results indicate that the begomovirus causing yellow vein leaf curl in Kalmegh in the Odisha region of India is associated with a DNA-β or a betasatellite (1.4 kb). The sequence information revealed from this study can be utilized in diagnostics for identification and epidemiological research purposes. The Kalmegh entries with resistant response to screening against the said virus can also be used for durable Kalmegh resistance breeding programmes.

Conclusion

This study presents the first report of a begomovirus associated with YVLCD in Kalmegh from Odisha, India. Molecular characterization confirmed the presence of begomovirus DNA-A and betasatellite molecules, with phylogenetic analysis identifying it as closely related to AYVLCV. The virus was successfully transmitted through whiteflies under controlled conditions. Additionally, a screening of 39 Kalmegh germplasm lines led to the identification of resistant genotypes. These findings provide valuable insights for future disease management and resistant variety development in Kalmegh.

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Authors' contributions

SS conducted molecular studies and contributed to manuscript drafting. SK conceived the study and coordinated the work. SRK performed molecular studies. VRB framed the study design. PD assisted in molecular studies. BS, SSB, and SCS conducted screening and field studies. CR contributed to data analysis. HKS performed sequence alignment and assisted in manuscript drafting. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest: The authors declare that they have no competing interest in the publication

Ethical issues: None

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