ISSN 2347- 6893

Evidence of the association of begomovirus and its betasatellite with the yellow vein disease of an ornamental plant Calendula officinalis (pot marigold) in Rajasthan, India: Molecular, Sequence and Recombination analysis.

Avinash Marwal[1], Anurag Kumar Sahu[2], Rajarshi Kumar Gaur[3]
[1] Department of Science, Faculty of Arts, Science and Commerce, Mody Institute of Technology and Science
marwal_avinash@yahoo.co.in
[2]Department of Science, Faculty of Arts, Science and Commerce, Mody Institute of Technology and Science
anuragsahhu@gmail.com
[3]Department of Science, Faculty of Arts, Science and Commerce, Mody Institute of Technology and Science
Corresponding Author: gaurrajarshi@hotmail.com
Avinash Marwal and Anurag Kumar Sahu have contributed equally to the present research

ABSTRACT

Begomoviruses are major threats for various crops species throughout the globe, particularly in tropical and sub-tropical regions. Mutation, pseudorecombination and recombination are driving forces for the emergence and evolution of new begomoviruses. In light to that ornamental plants act as an alternate host of begomoviruses and its associated satellite molecules in the absence of main crop. Leaf yellow vein disease of Calendula officinalis plants was observed in Lakshmangarh city of Rajasthan province of India. Samples of Calendula officinalis leaves were collected from the gardens. An expected product of ~550 bp in size was amplified from extracts of symptomatic leaf samples with universal primers on the coat protein region of begomovirus. Moreover, betasatellite were also detected using betasatellite specific universal primers. The presence of begomoviruses was also confirmed by Dot blot hybridization using cloned DNA-A probe of Papaya leaf curl virus. We have identified and characterized the begomovirus and its associated betasatellite through molecular, sequence and recombination analysis. The betasatellite is identified as a new recombinant species, sharing nucleotide identity with other isolates reported from China, Pakistan, Bangladesh and Taiwan. The present study also suggests that the exchange of betasatellites with other begomoviruses would create a new disease complex posing a serious threat to agriculture crops and horticulture ornamental plants production.

Indexing terms/Keywords

Begomovirus, betasatellite, Calendula officinalis, ornamental plants, sequence and recombination analysis

Academic Discipline And Sub-Disciplines

Plant Molecular Biology

SUBJECT CLASSIFICATION

Virology and Bioinformatics

YPE (METHOD/APPROACH)

Research Article on Molecular, Sequence and Recombination analysis

Council for Innovative Research

Peer Review Research Publishing System

Journal: Journal of Advances in Biology

Vol. 1, No. 1

editor@cirworld.com

www.cirworld.com, member.cirworld.com

INTRODUCTION

Geminiviruses are a large family of plant viruses with circular, single-stranded DNA (ssDNA) genomes packaged within geminate particles [1, 2]. According to their genome organizations and biological properties the family Geminiviridae is divided into four genera i.e. Mastrevirus, Curtovirus, Topocuvirus, and Begomovirus [2, 3]. Members of the genus Begomovirus are transmitted by whiteflies (Bemisia tabaci) to dicotyledonous plants which mostly prevail in the tropical and sub tropical regions of the world [5, 6]. Some begomoviruses are associated with betasatellites, which affect the replication of their respective helper begomoviruses and alter the symptoms induced in some host plants [7, 8, 9]. Betasatellites require begomoviruses for replication, encapsidation, insect transmission, and movement in plants [10, 11].

Begomovirus are an outsized varied family of plant viruses [12] which infects an expansive assortment of plants such as ornamentals, weeds and crops and causes a noteworthy loss to Agriculture and Horticulture worldwide [13]. Ornamental plants are extensively scattered worldwide and have high environmental adaptability. Ornamentals are considered as a foundation of new viruses and reservoirs of unidentified economically imperative viruses but are often neglected during diversity study [14]. Many scientific reports have demonstrated that ornamental plants serve as reservoir or alternative hosts for begomovirus survival [15] and spread in the absence of the main crops [16]. Thus, there is a pressing need for additional information on the diversity and distribution of begomovirus in ornamental plants.

The beautiful plant Calendula officinalis has an aesthetic beauty of its bright yellow colored flowers (Figure 1) and is widely cultivated as an ornamental plant in India for the linings of the gardens. It is also used medicinally in Europe, China, US and India. It belongs to the family, Asteraceae, and is commonly known as Pot Marigold. The plant is native to Central and Southern Europe, Western Asia and the US. The plant is an annual, seldom biennial. It grows to between 30 and 50 cm

high [17]. The therapeutic properties of marigolds are anti-inflammatory, healing, antiseptic, skin ulcerations, eczema, conjunctivitis, antispasmodic, aperient, cholagogic, diaphoretic and vulnerary properties [18]. In the present investigation symptomology, molecular and computational biology approaches were done to characterize the begomovirus and its betasatellite component associated with leaf yellow vein disease of Calendula officinalis in India.

Fig 1: Healthy ornamental plants of Calendula officinalis exhibiting beautiful pattern of orange to bright yellow colored flowers growing near the vicinity of Guru Vashisht College, MITS campus.

Recombination has played, and continues to play, a pivotal role in geminiviral evolution and may be contributing to the emergence of new forms of geminiviruses because the high frequency of mixed infections of begomoviruses provides an opportunity for the emergence of new viruses arising from recombination among strains and / or species [19]. In some cases, the recombinants exhibited a new pathogenic phenotype which is often more virulent than the parents [20].

The specific recombination events including the recombination breaks and hot spots have not been reported so far in Calendula officinalis infecting begomovirus. It is also currently unknown as to whether the sequences in particular parts of the begomovirus genomes are exchangeable between different species and/or members of the same genus from different geographical locations. In addition, Northern India seems to be unusually rich in virus biodiversity; we investigated the extent of recombination events and examined their role in the evolution of virus in India and its neighboring countries.

MATERIALS AND METHODS

Virus Sources

Over the past few decades, there has been more interest in geminiviruses, especially begomoviruses, as many of the diseases they cause have now reached epidemic proportions [21]. Ornamental plants serve as an alternative host for begomovirus in gardens and may allow the transmission of begomovirus to other crop plants and this enhances the host range of this virus in different regions of the India. Keeping this view in mind surveys of different gardens were made during 2010 - 2011. Calendula officinalis plants were found with leaf yellow vein disease which symbolizes symptoms typical to begomovirus infection. To investigate the potential begomoviral infection, infected Calendula officinalis samples were collected.

Extraction of Total DNA

The leaf samples were cleaned, cut, rolled in a piece of tissue paper, and was stored at -20oC until DNA isolation. To begin with the molecular characterization total DNA was extracted from leaves of infected as well as healthy trees using

the Cetyl Trimethyl Ammonium Bromide (CTAB) method [22]. Samples of the total DNA product was analyzed by electrophoresis on a 1% or 2% agarose gel and the concentration was determined by using Nanodrop (Thermo Scientific).

Identification of begomovirus components by PCR

PCR was performed using a pair of degenerate primers specific to the coat protein region of begomovirus. The forward primer sequence was GGRTTDGARGCATGHGTACATG (AC 1048) and the reverse primer sequence was GCCYATRTAYAGRAAGCCMAG (AV 494). The primers are validated and have been used previously in screening of variety of begomovirus. Nearly 40 begomovirus were screened earlier such as Croton yellow vein mosaic virus (HQ631429), Croton yellow vein mosaic Hisar virus (JN000701), Datura leaf curl virus (JN000702), Vinca yellow vein virus (JQ693139), Cotton leaf curl virus (JQ693143) etc [23].

A typical PCR reaction contained about 100 ng DNA template, Taq 10 x buffers (10 mmol/L Tris-HCl, pH 8.8; 50 mmol/L KCl) 25mmol/L MgCl2, 200 µmol/L of each dNTPs, 2 units of Taq DNA Polymerase, Nuclease free water and 10 pmol/L of each primer. The PCR thermal profile was pre-PCR by denaturation at 94 °C for 120 s followed by 35 cycles of denaturing at 94 °C for 60 s, annealing at 55 °C for 60 s and extension at 72 °C for 60 s, and a final extension at 72 °C for 5 min. After amplification, 4 µl aliquot from each sample was electrophoresed in a 1 % agarose gel and visualized by staining with ethidium bromide and UV illumination [24].

For the detection of any DNA-B component in diseased Calendula officinalis plants, primer pair PCRc1 and PBLlv2040 were used [25, 26] having the same PCR condition and reaction as used in the case of begomovirus coat protein region. To test whether a satellite molecule was associated with the begomovirus, a universal primer pair specific for alphasatellite and betasatellite was also used to amplify the putative DNA [27, 28].

The PCR reaction for alphasatellite and betasatellite were the same as for DNA A, mentioned above in the manuscript. Whereas the PCR thermal profile was pre-PCR by denaturation at 94 °C for 120 s followed by 35 cycles of denaturing at 94 °C for 60 s, annealing at 68 °C for 60 s and extension at 72 °C for 60 s, and a final extension at 72 °C for 5 min [29, 30].

Cloning, Sequencing and Dot Blot Hybridization

The amplified PCR product was purified and cloned into Promega pGEMT vector system as per the manufacturer's instruction. The clones were sequenced and the details were submitted to NCBI. The presence of a begomovirus was further confirmed by Dot blot hybridization using Papaya leaf curl virus as a general probe for begomoviruses.

NCBI BLAST analysis and construction of Sequence Matrix

BLAST analysis was performed to reveal their closeness to other begomovirus sequence in the database. Nucleotide sequence and amino acid identities between begomovirus infecting Calendula officinalis and selected begomoviruses were analyzed by MatGAT software version 2.01.

Phylogenetic analysis

Based on the close sequence identity and the length of the sequences, begomovirus and betasatellite sequences of various begomoviruses were downloaded from Gen Bank with the accession numbers provided by the FASTA output and were fed into Molecular Evolutionary Genetics Analysis Program (MEGA) version 4.0 [31]. The sequence of begomovirus genomic components was aligned in Clustal-W sequence alignment program using IUB matrix for DNA alignments in the MEGA v.4.0 [32]. A phylogenetic tree was also generated by MEGA v.4.0 software by using the neighbor-joining method with 1,000 bootstrap replications. Boot strapping was applied to statistically validate the tree using NJ-plot [33]. This analysis clustered each one of the isolates with the other previously sequenced isolate of the respective species [34].

Recombination Detection

Recombination between divergent genomes is believed to be a major mechanism by which diversity amongst viruses is generated [35]. To detect the possibility of recombination in begomovirus and betasatellite, Recombination Detection Program (RDP) was utilized, which is based on a pair wise scanning approach. It usually runs under Windows 95/98/NT/XP/VISTA/7 and couples a high degree of analysis automation with an interactive and detailed graphical user interface [36]. Using various recombination detection methods the conclusion of recombination studies were evaluated [37, 38]. The recombination breakpoint could be identified by using Recombination detection program [RDP], GENECONV, Maximum-Chi, BOOTSCAN, CHIMAERA, and 3SEQ methods. All these methods were implemented in RDP v.3.44 [39]. Due to large amount of data here we are focusing on the results obtained from RDP method only.

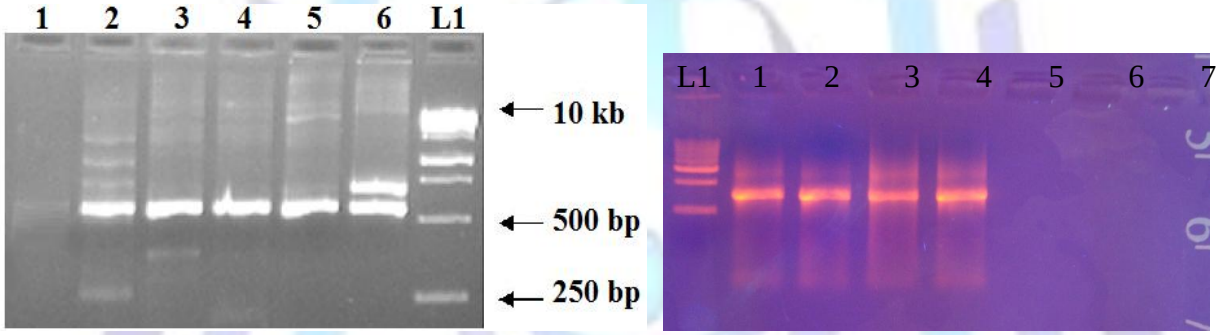
RESULTS AND DISCUSSION

During the survey for begomovirus infection in 2010 - 2011, yellowing of leaf vein disease typical of begomovirus symptoms were observed in Calendula officinalis (Figure 2). To investigate the potential begomoviral infection, healthy as well as infected Calendula officinalis leaf samples were collected from the location Latitude: 27N 80' 27.58 and Longitude: 75E 03' 48.79" (GVC, FASC, Lakshmangarh, Sikar district of Rajasthan province of India). Considerable time and expense is required for complete genome sequences. However full length coat protein gene sequences are accepted by the International Committee on the Taxonomy of Viruses (ICTV) for the provisional classification of begomoviruses when complete genome sequences are unavailable [40].

ISSN 2347- 6893

Fig 2: Natural begomovirus infection in Calendula officinalis exhibiting yellow vein disease in leaf resulting in flowerless plants, thus affecting the aesthetic beauty of ornamental plant.

Positive PCR reaction confirmed the begomovirus infection in Calendula officinalis. PCR product of complete coat protein region of DNA-A having an expected size (~550 bp) was obtained from infected plants but not from symptomless samples (Figure 3a). Further we also found betasatellite component (~650 bp) associated with Calendula officinalis (Figure 3b). Both the PCR products were suitably cloned into pGEM-T vector and sequenced, having Accession number as JN998443 (Calendula officinalis yellow vein Lakshmangarh virus) and JQ693147 (Calendula yellow vein betasatellite) respectively. In our investigation we haven't found any DNA-B and alphasatellite molecules associated with the begomovirus.



(a) **(b)**

Fig 3: (a) Agarose gel electrophoresis of the PCR products amplified from DNA extracts prepared from infected Calendula officinalis plants confirming positive results of begomovirus, whereas Lane 1 stands for healthy plant, L 1 represents the 250 bp Ladder. (b) PCR amplification of betasatellite isolated from Calendula officinalis collected from Lakshmangarh, India. 1 % agarose gel of PCR amplification products obtained with universal satellite primer. L 5-7 represents healthy plant; L 1 represents the 500 bp Ladder.

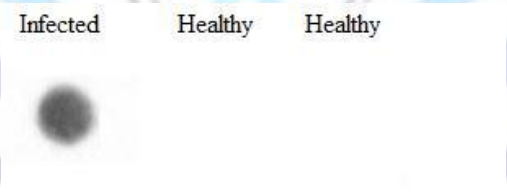


Fig 4: Dot blot hybridization confirming positive signal from the infected samples of Calendula officinalis.

The positive PCR reaction showed the presence of begomovirus. For further confirmation of begomovirus infectivity, Dot blot hybridization was performed. In Dot blot hybridization technique all samples from symptomatic plants hybridized with the probe, whereas samples extracted from non - symptomatic plants did not show positive results (Figure 4). Hybridization of Papaya leaf curl virus probe with the DNA fragment on the filter membrane further indicated that this fragment contained DNA sequence complementary to the probe. The strong signal showed that the virus in Calendula officinalis had some homology with the Papaya leaf curl virus.

During nucleotide BLAST analysis, the begomovirus revealed highest nucleotide sequence identities of 99 % with Cyamopsis tetragonoloba leaf curl Sikar virus (JN998448) which infecting an important palatable crop Cyamopsis tetragonoloba and were reported from India. It further showed moderate sequence identity of 95 – 97% with Tomato leaf curl virus (AY690431) and Ageratum enation virus (KC795968), both infecting tomato crops in India and revealed lower sequence identity of 90 – 92 % with Papaya leaf curl virus (AJ436992) contaminating fiber crop cotton in Pakistan and Chili leaf curl Pakistan virus (HM587709) causing disease in Capsicum annuum plants in China.

The begomovirus sequenced from Calendula officinalis have a putative conserved domain of the geminivirus coat protein family which encodes for coat protein of 174 amino acids having a molecular weight of 20.43 kDa (protein id="AEY68276.1") (Table 1). Even with protein BLAST analysis, the begomovirus showed highest amino acid sequence identities of 99 % with Ageratum enation virus (CCV01318) infecting Sonchus sp. in India; 98% amino acid sequence identities with Croton yellow vein mosaic virus (CBK44091) causing disease in Alcea rosea in Pakistan and Euphorbia leaf curl virus (AGF41099) distorting Passiflora edulis in Taiwan.

Table 1. Positions and coding capacity of predicted genes for the coat protein gene of begomovirus and its betasatellite molecule isolated from Calendula officinalis.

Components	Description	ORF	Strand	Frame	Start codon (nucleotide coordinates)	Stop codon (nucleotide coordinates)	Predicted size (no. of amino acids)	Predicted molecular weight (kDa)
Begomovirus	Coat protein	AV1	Sense strand	1st frame (+)	1	225	174	20.43
DNA-β	Symptoms inducing protein	C 1	Complement strand	3rd frame (–)	466	645	59	6.86

Whereas the betasatellite had highest nucleotide sequence identities of 94 % with Ageratum leaf curl betasatellite (KC589700) infecting Tagetes patula in India. The betasatellite infecting Calendula officinalis in India showed moderate sequence identity of 87 – 91 % with Digera arvensis yellow vein disease-associated DNA betasatellite (AM494977) infecting Digera arvensis plants in Pakistan; Okra leaf curl disease associated betasatellite (AJ316031) causing disease symptoms in Hibiscus esculentis reported from Pakistan and Zinnia leaf curl disease associated DNA beta molecule (AJ316041) distorting Zinnia elegans plants in Pakistan.

It further confirmed lower sequence identity of 80 – 82 % with Tomato leaf curl virus-associated DNA beta (AJ542489) reported from Bangladesh and Chilli leaf curl betasatellite (FJ515274) reported from Pakistan, both the virus were found infecting tomato crops in their respective countries.

Pairwise BLAST is used to calculate similarity, but its limitations are that only two sequences may be analyzed at one time and percent similarity/identity is based on local alignment – not global alignment. MegAlign, which comes with the DNASTAR package (DNASTAR, Inc.), also generates similarity matrices, but it is quite expensive and not available as a stand-alone product. MatGAT (Matrix Global Alignment Tool) is a simple, easy to use similarity/identity matrix generator that calculates the similarity and identity between every pair of sequences in a given data set without requiring pre-alignment of the data.

The program performs a series of pairwise alignments using the Myers and Miller global alignment algorithm, calculates similarity and identity, and then places the results in a distance matrix [41]. The nucleotides (Figure 5) and amino acids (Figure 6) sequence analyses of the coat protein gene of begomovirus isolated from Calendula officinalis and its associated betasatellite (Figure 7) exhibited sequence similarity with the reference begomovirus isolates reported from Pakistan, China, Bangladesh, and Taiwan.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1. JN998443		99.4	65.8	18.4	18.4	65.6	65.5	18.4	18.4	65.4	65.4	18.3	18.4	18.3	95.2	18.2	90.5	94.3	63.7	17.9
2. JN998448	99.4		65.9	18.5	18.5	65.8	65.6	18.4	18.4	65.5	65.5	18.4	18.4	18.3	95.8	18.4	90.9	94.9	63.8	17.9
3. AY690431	65.8	65.9		27.4	27.5	98.1	97.7	27.4	27.3	97.7	97.4	27.4	27.5	27.4	64.7	27.2	67.7	64.4	96.0	27.0
4. KC795968	18.4	18.5	27.4		95.8	27.7	27.7	94.8	89.9	27.7	27.6	95.5	96.2	95.3	18.3	86.1	19.3	18.4	27.1	87.9
5. JX436473	18.4	18.5	27.5	95.9		27.8	27.8	97.5	92.7	27.7	27.6	98.0	94.0	97.9	18.3	86.3	19.3	18.4	27.1	88.1
6. JN896941	65.6	65.8	98.1	27.7	27.8		99.4	27.5	27.5	99.1	98.7	27.5	27.9	27.5	65.0	27.7	68.2	65.2	96.6	27.1
7. JF682240	65.5	65.6	97.7	27.7	27.8	99.4		27.5	27.5	99.0	98.8	27.5	27.9	27.5	65.1	27.7	68.2	65.3	96.5	27.2
8. JQ911767	18.4	18.4	27.4	95.0	97.5	27.5	27.5		91.4	27.5	27.6	97.4	93.2	97.3	18.1	85.6	19.1	18.1	27.0	88.0
9. HE861940	18.4	18.4	27.3	92.7	95.4	27.5	27.5	94.0		27.4	27.4	93.3	89.1	93.2	18.2	81.1	19.1	18.2	27.0	83.4
10. JN896943	65.4	65.5	97.7	27.7	27.7	99.1	99.0	27.5	27.4		98.6	27.5	27.7	27.4	65.0	27.6	68.2	65.2	96.2	27.1
11. JF682243	65.4	65.5	97.4	27.6	27.6	98.7	98.8	27.6	27.4	98.6		27.4	27.7	27.4	64.7	27.5	67.7	64.8	96.0	27.0
12. FN794201	18.3	18.4	27.4	95.6	98.0	27.5	27.5	97.4	96.0	27.5	27.4		93.0	99.6	18.2	86.0	19.2	18.2	27.1	88.1
13. FJ177031	18.4	18.4	27.5	96.4	94.3	27.9	27.9	93.5	91.8	27.7	27.7	93.3		92.9	18.3	85.3	19.3	18.4	27.1	87.5
14. FN543099	18.3	18.3	27.4	95.5	97.9	27.5	27.5	97.3	95.9	27.4	27.4	99.6	93.2		18.2	86.1	19.2	18.2	27.0	88.0
15. JN998445	95.4	95.8	64.7	18.3	18.3	65.0	65.1	18.1	18.2	65.0	64.7	18.2	18.3	18.2		18.3	91.8	96.2	64.3	18.2
16. JN135233	18.2	18.4	27.2	86.7	87.0	27.7	27.7	86.3	84.2	27.6	27.5	86.7	86.0	86.7	18.3		19.4	18.6	27.2	84.6
17. JN000703	90.5	90.9	67.7	19.3	19.3	68.2	68.2	19.1	19.1	68.2	67.7	19.2	19.3	19.2	91.8	19.4		93.3	67.7	19.3
18. JN998449	94.3	94.9	64.5	18.4	18.4	65.2	65.4	18.1	18.2	65.2	64.9	18.2	18.4	18.2	96.2	18.6	93.4		65.3	18.6
19. JF461062	63.7	63.8	96.0	27.1	27.1	96.6	96.5	27.0	27.0	96.2	96.0	27.1	27.1	27.0	64.3	27.2	67.7	65.4		27.4
20. AJ971266	17.9	17.9	27.0	88.1	88.3	27.1	27.2	88.3	85.9	27.1	27.0	88.3	87.8	88.3	18.2	85.3	19.3	18.6	27.4	

Fig 5: Coat protein gene nucleotide sequence identities (bottom left highlighted in yellow) and similarities (top right highlighted in blue) expressed in percentage for the begomovirus associated with yellow vein disease of

Calendula officinalis in Rajasthan province of India and selected previously characterized begomoviruses. Accession numbers of the Begomoviruses used for the analyses and their origin are given as follows:- Calendula officinalis yellow vein Lakshmangarh virus (JN998443: INDIA), Cyamopsis tetragonoloba leaf curl Sikar virus (JN998448: INDIA), Tomato leaf curl virus (AY690431: INDIA), Ageratum enation virus (KC795968: INDIA), Ageratum enation virus (JX436473: INDIA), Ageratum enation virus (JN896941: INDIA), Ageratum enation virus (JF682240: INDIA), Ageratum enation virus (JQ911767: INDIA), Ageratum enation virus (HE861940: INDIA), Ageratum enation virus (JN896943: INDIA), Ageratum enation virus (JF682243: INDIA), Ageratum enation virus (FN794201: INDIA), Ageratum enation virus (FJ177031: INDIA), Ageratum enation virus (FN543099: INDIA), Jasminum sambac leaf curl Lakshmangarh virus (JN998445: INDIA), Papaya leaf curl virus (JN135233: INDIA), Sonchus yellow mosaic virus (JN000703: INDIA), Verbesina encelioides yellow vein Lakshmangarh virus (JN998449: INDIA), Tobacco curly shoot virus (JF461062: INDIA), Tobacco curly shoot virus (AJ971266: China).

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
1. AEY68275		97.2	95.0	67.2	66.4	66.8	97.7	66.4	66.4	66.4	66.4	98.3	66.0	87.6	93.4	96.0	66.0	65.6	66.0
2. CCV01318	98.3		97.2	68.0	68.0	68.4	97.2	68.0	67.6	68.0	68.0	96.6	67.6	89.6	94.0	94.4	67.2	67.2	67.2
3. ACL34425	96.1	97.8		68.8	69.5	69.1	94.4	68.8	68.4	69.5	68.8	93.9	68.4	90.2	94.0	92.2	68.0	69.1	69.1
4. AAV98394	67.6	68.4	69.5		97.7	98.8	66.0	98.0	97.7	98.0	98.0	66.4	96.5	72.7	68.0	64.5	93.8	96.5	94.5
5. AEK48288	68.0	68.8	69.9	99.2		98.0	66.0	98.0	97.7	98.8	97.3	65.6	96.5	72.7	66.8	64.5	93.4	97.3	94.9
6. AFQ23196	67.6	68.4	69.5	99.6	99.6		66.4	98.4	98.0	98.4	98.0	66.0	96.9	73.0	67.6	64.8	94.9	96.9	95.3
7. AEY68282	98.9	97.7	95.6	66.8	67.2	66.8		66.8	66.8	66.0	66.0	97.1	67.2	88.5	91.8	96.0	66.4	66.8	66.4
8. AEA76797	67.6	68.4	69.5	99.2	99.2	99.6	66.8		99.6	98.4	97.3	65.6	97.7	73.4	67.2	65.2	94.9	98.0	94.9
9. AGA60266	67.6	68.4	69.5	99.2	99.2	99.6	66.8	100.0		98.0	96.9	65.6	97.3	73.8	67.2	65.2	95.3	97.7	95.3
10. CBK44091	67.6	68.4	69.5	99.6	99.6	100.0	66.8	99.6	99.6		97.3	65.6	96.9	72.7	67.2	64.5	93.8	98.0	95.7
11. AEV76902	67.6	68.4	69.5	99.6	99.6	100.0	66.8	99.6	99.6	100.0		65.6	95.7	72.7	67.2	64.5	93.0	95.7	93.8
12. AEY68277	98.3	97.2	95.0	66.4	66.8	66.4	98.3	66.4	66.4	66.4	66.4		65.2	87.0	92.3	94.3	65.2	64.8	65.2
13. AFB83455	67.2	68.0	69.1	98.4	98.4	98.8	67.2	98.4	98.4	98.8	98.8	66.0		72.3	66.8	64.8	93.0	96.9	93.8
14. ADD52430	89.1	90.6	92.2	73.8	74.2	73.8	88.5	73.8	73.8	73.8	73.8	88.0	73.0		87.6	87.0	74.2	72.7	72.7
15. AEH04543	94.0	94.5	95.1	68.4	68.4	68.4	92.9	68.4	68.4	68.4	68.4	92.3	68.0	89.6		89.6	66.8	66.8	67.2
16. AEY68278	99.4	97.7	95.6	67.2	67.6	67.2	98.3	67.2	67.2	67.2	67.2	97.7	66.8	89.6	93.4		65.6	64.5	64.1
17. AGF41099	67.2	68.0	69.1	95.7	95.7	96.1	66.4	96.5	96.5	96.1	96.1	66.0	94.9	74.2	68.0	67.6		93.4	92.2
18. CBE70775	67.2	68.0	69.5	98.4	98.4	98.8	67.2	99.2	99.2	98.8	98.8	66.0	98.4	73.4	68.4	66.8	95.7		96.9
19. CAM58876	67.2	68.0	69.5	97.7	97.7	98.0	67.2	98.4	98.4	98.0	98.0	66.0	97.7	73.4	68.4	66.8	94.9	99.2	

Fig 6: Coat protein amino acid sequence identities (bottom left highlighted in yellow) and similarities (top right highlighted in blue) expressed in percentage for the begomovirus coat protein associated with yellow vein disease of Calendula officinalis in Rajasthan province of India and selected previously characterized begomoviruses. Accession numbers of the Begomoviruses used for the analyses and their origin are given as follows:- Calendula officinalis yellow vein Lakshmangarh virus (AEY68275: INDIA), Ageratum enation virus (CCV01318: INDIA), Chilli leaf curl virus (ACL34425: INDIA), Tomato leaf curl virus (AAV98394: INDIA), Radish leaf curl virus (AEK48288: INDIA), Tobacco curly shoot virus (AFQ23196: INDIA), Spinacia oleracea leaf curl Bhatinda virus (AEY68282: INDIA), Chilli leaf curl Kanpur virus (AEA76797: INDIA), Pepper leaf curl Bangladesh virus (AGA60266: INDIA), Croton yellow vein mosaic virus (CBK44091: PAKISTAN), Papaya leaf curl virus (AEV76902: INDIA), Jasminum sambac leaf curl Lakshmangarh virus (AEY68277: INDIA), Cotton leaf curl Burewala virus (AFB83455: INDIA), Duranta leaf curl virus (ADD52430: INDIA), Sonchus yellow mosaic virus (AEH04543: INDIA), Millingtonia hortensis yellow vein mosaic Lakshmangarh virus (AEY68278: INDIA), Euphorbia leaf curl virus (AGF41099: TAIWAN), Cotton leaf curl Shadadpur virus (CBE70775: INDIA), Cotton leaf curl Rajasthan virus (CAM58876: INDIA).

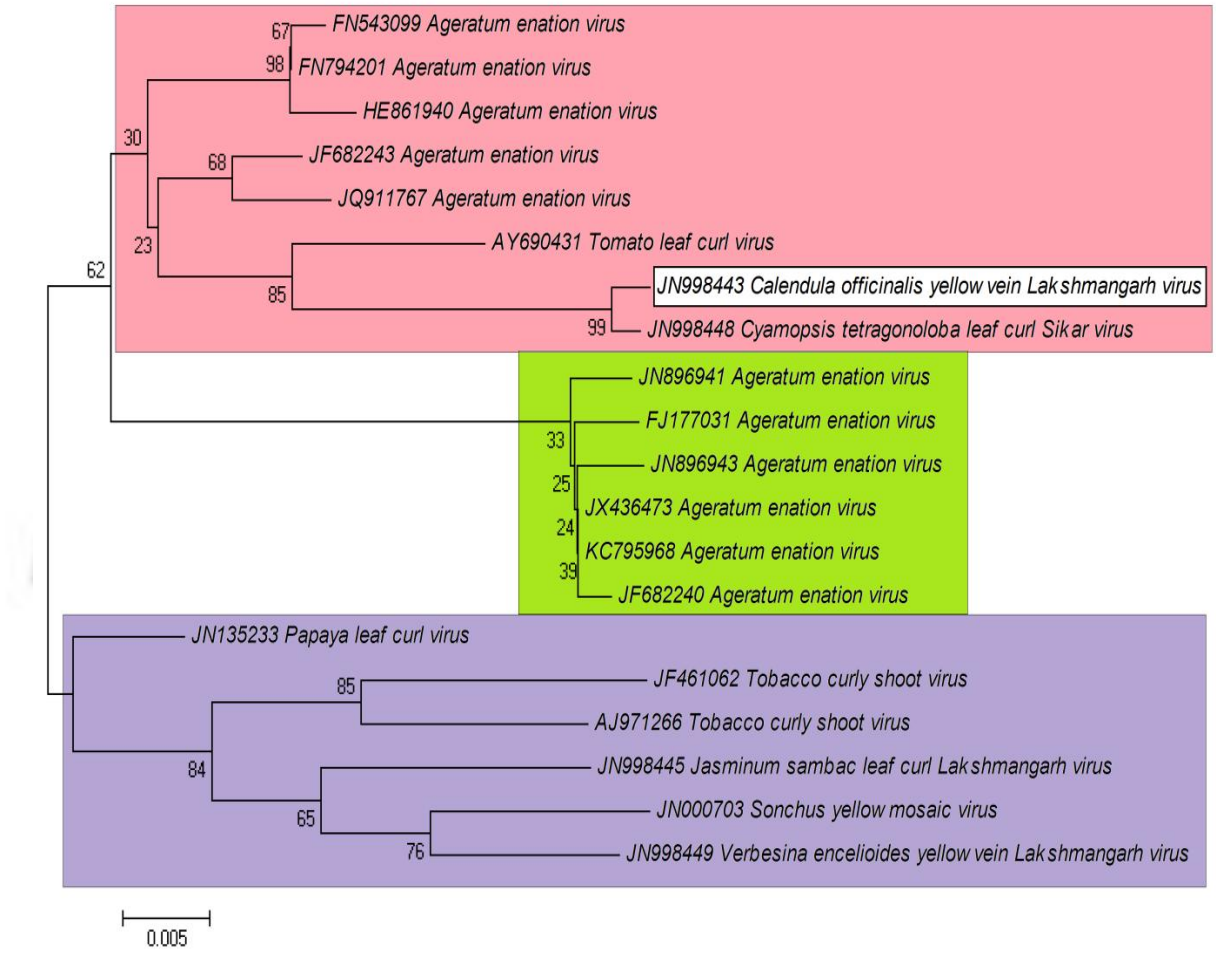
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
1. JQ693147		41.1	45.0	37.8	50.7	39.3	38.2	38.7	38.5	38.2	45.8	36.9	35.9	35.6	36.3	35.8	36.2	35.1	35.8
2. KC589700	41.1		33.2	85.1	54.4	91.1	85.0	85.2	85.9	85.4	65.8	71.8	71.2	70.8	71.4	63.4	71.3	63.6	63.3
3. JQ693150	51.3	33.2		26.3	39.4	27.6	26.9	27.4	27.3	27.2	33.7	26.3	25.4	25.9	26.1	27.0	25.8	26.1	26.4
4. FN432358	37.8	86.5	26.4		61.5	90.4	94.7	92.3	93.0	92.9	69.1	72.1	72.1	71.4	73.1	64.3	73.2	63.6	63.5
5. AJ316042	51.5	55.6	39.7	61.6		59.2	60.8	61.4	61.4	60.0	74.1	49.0	48.6	46.8	47.7	49.2	47.9	48.6	48.5
6. HQ407397	39.6	93.2	27.9	90.6	59.4		90.7	90.6	91.4	90.9	69.6	71.0	71.2	70.9	71.4	63.5	71.5	63.4	62.8
7. AJ316031	38.3	86.3	27.0	94.7	60.8	90.7		95.0	95.7	96.2	69.9	71.8	71.9	71.7	72.9	63.4	73.0	63.7	63.6
8. AJ316041	38.8	87.2	27.5	93.0	61.5	91.1	95.1		99.0	97.4	69.3	71.1	71.0	70.7	71.5	63.6	71.6	63.2	63.1
9. AJ316028	38.6	87.9	27.5	93.7	61.5	91.8	95.7	99.2		98.1	69.4	71.5	71.5	71.3	71.9	63.7	72.0	63.2	63.3
10. AJ316027	38.3	87.1	27.4	93.6	60.0	91.1	96.2	97.5	98.1		68.9	71.5	71.2	70.8	71.8	63.9	71.9	63.5	63.4
11. KC305086	45.8	66.8	33.8	72.0	74.3	70.4	70.2	71.9	71.9	71.4		59.9	58.8	58.2	59.9	56.9	59.9	56.1	56.6
12. HM007107	37.0	73.3	26.4	74.1	49.5	73.4	74.9	74.1	74.5	74.6	60.6		79.9	79.1	83.7	64.8	83.5	64.8	65.6
13. EU847239	35.9	72.4	25.4	74.0	48.8	73.5	74.8	73.7	74.2	74.0	59.3	80.9		93.0	81.3	63.8	81.1	62.4	64.8
14. AY728263	35.6	72.4	26.0	73.2	47.8	72.8	74.0	73.0	73.6	73.0	58.4	80.2	93.2		80.8	63.9	80.8	61.8	63.2
15. AM279662	36.3	72.4	26.1	75.1	49.1	73.4	75.9	74.2	74.8	74.7	60.6	84.7	82.0	81.7		64.3	99.6	63.4	64.7
16. AM410551	35.9	65.8	27.0	67.4	49.4	66.6	67.0	67.5	67.6	67.8	57.7	66.8	66.1	66.4	66.1		64.5	90.5	90.0
17. AM279663	36.2	72.3	25.8	75.3	49.3	73.6	76.1	74.4	75.0	74.8	60.6	84.5	81.9	81.6	99.6	66.4		63.5	64.8
18. JN831448	35.1	66.3	26.1	66.9	49.1	66.3	67.0	66.5	66.5	66.8	56.8	67.6	65.1	64.3	65.9	90.8	66.1		87.6
19. JN663874	35.9	65.3	26.4	66.5	48.8	65.5	67.1	66.5	66.6	66.8	57.3	67.6	66.9	65.1	66.6	90.6	66.8	88.2	

Fig 7: Betasatellite nucleotide sequence identities (bottom left highlighted in yellow) and similarities (top right highlighted in blue) expressed in percentage associated with yellow vein disease of Calendula officinalis in Rajasthan province of India and selected previously characterized Betasatellites. Accession numbers of the

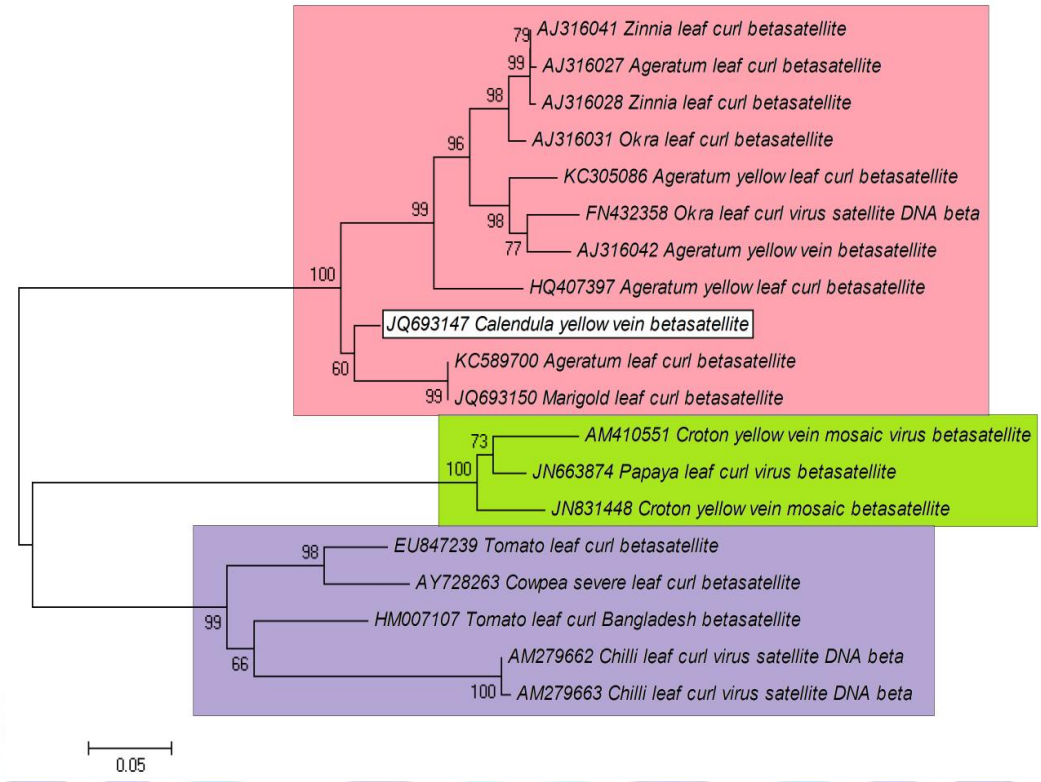
Betasatellites used for the analyses and their origin are given as follows:- Calendula yellow vein betasatellite (JQ693147: INDIA), Ageratum leaf curl betasatellite (KC589700: INDIA), Marigold leaf curl betasatellite (JQ693150: INDIA), Okra leaf curl virus satellite DNA beta (FN432358: PAKISTAN), Ageratum yellow vein disease associated sequence (AJ316042: INDIA), Ageratum yellow leaf curl betasatellite (HQ407397: INDIA), Okra leaf curl disease associated sequence, putative C1 gene (AJ316031: PAKISTAN), Zinnia leaf curl disease associated sequence defective DNA beta molecule (AJ316041: PAKISTAN), Zinnia leaf curl disease associated sequence putative C1 gene (AJ316028: PAKISTAN), Ageratum leaf curl disease associated sequence putative C1 gene (AJ316027: PAKISTAN), Ageratum yellow leaf curl betasatellite (KC305086: INDIA), Tomato leaf curl Bangladesh betasatellite (HM007107: INDIA), Tomato leaf curl betasatellite (EU847239: INDIA), Cowpea severe leaf curl-associated DNA beta (AY728263: INDIA), Chilli leaf curl virus satellite DNA beta (AM279662: PAKISTAN), Croton yellow vein mosaic virus-associated DNA beta (AM410551: PAKISTAN), Chilli leaf curl virus satellite DNA beta (AM279663: PAKISTAN), Croton yellow vein mosaic betasatellite (JN831448: INDIA), Papaya leaf curl virus betasatellite (JN663874: INDIA).

Phylogenetic tree of the obtained sequences was generated by MEGA 4.0 software by using the neighbor-joining method with 1,000 bootstrap replications. Neighbor-Joining (NJ) analysis was carried out using Maximum Composite Likelihood model with uniform rates among the sites, the 1000 bootstraps replicates were used to evaluate the significance of generated tree. In the Neighbor-Joining tree, the coat protein gene and the β satellite of begomovirus infecting Calendula officinalis were each placed within the tree thus inferring evolutionary history. Names of related viruses were summarized in Figure 8a and Figure 8b. Phylogenetic analysis based on the coat protein sequence of begomovirus and betasatellite isolated from Calendula officinalis and other selected sequences indicates that the isolate cluster with the isolates of virus reported from India, Pakistan, China, Bangladesh and Taiwan.

This evidence enlightens the prevalence of begomovirus from neighboring countries, into India thus inferring evolutionary history. An expected consequence of this scenario would be recombination which plays an important role for the evolution of new begomovirus strains in India and these new strains are responsible for heavy loss of new host variety.



(a)



(b)

Fig 8: Phylogenetic dendrograms based on alignments of selected begomovirus (a) and betasatellite (b) sequences. Begomovirus sequences used for comparison are mentioned in the respective trees. Vertical branches are arbitrary, horizontal branches are proportional to calculated mutation distances. Values at nodes indicate percentage bootstrap values (1000 replicates).

The CP gene sequence analysis of the begomovirus infecting Calendula officinalis in India with related isolates from the database sequences were carried out. The conserved region for cp is boxed in blue. The so-called "variable region" is boxed in purple. The T rich region is boxed in red and the repeating units are boxed in green. Virus sequence from Calendula officinalis are written at the top (Figure 9). The accession numbers of the sequences from GenBank are indicated on the left. The significance of these accession numbers are as follows: Calendula officinalis yellow vein Lakshmangarh virus (JN998443: INDIA), Cyamopsis tetragonoloba leaf curl Sikar virus (JN998448: INDIA), Tomato leaf curl virus (AY690431: INDIA), Ageratum enation virus (JN896941: INDIA). All of these sequences were multiple aligned using ClustalW program.

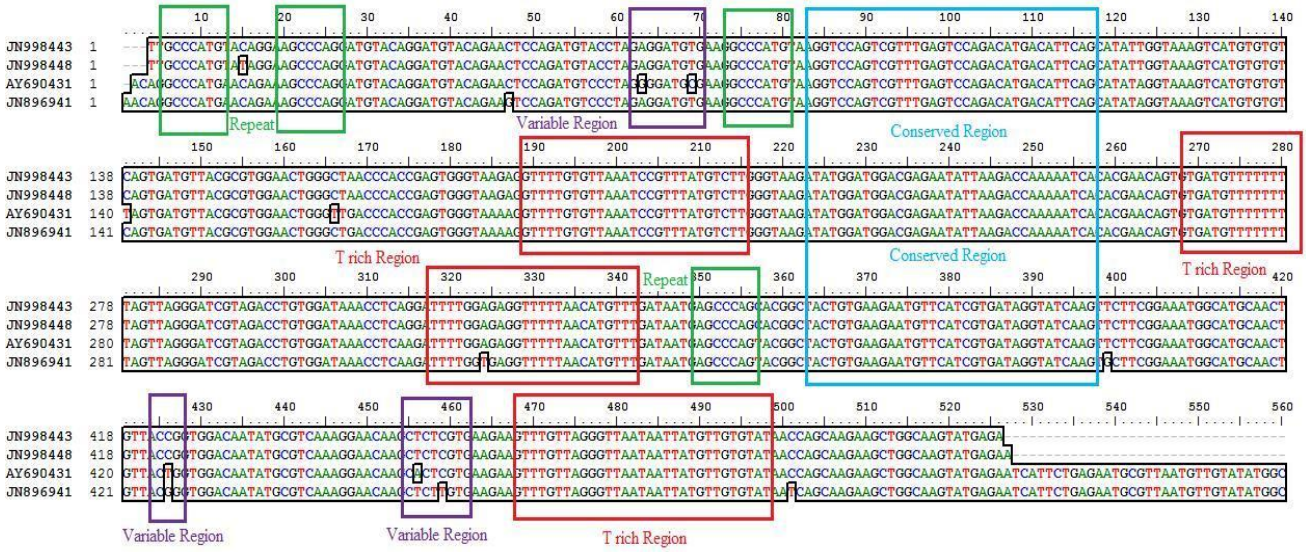


Fig 9: Alignment of coat protein region nucleotide sequences of begomovirus isolated from Calendula officinalis.

Entropy power explains the sequence mutates by the function of entropy plot. As shown in Figure 10 and Figure 12, that there are several peaks which contains high variable regions. So entropy power can be used to screen the variable region.

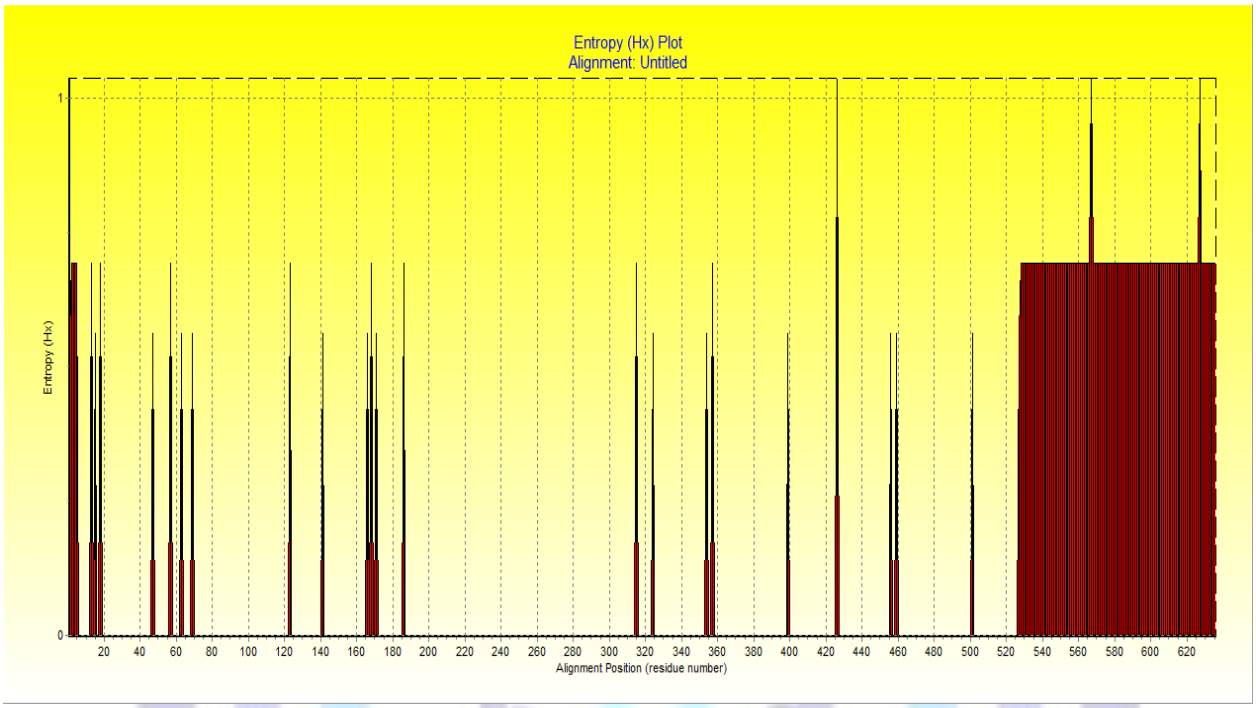


Fig 10: The Entropy power result of begomovirus from the alignment of coat protein nucleotide region.

Similarly sequence analysis of betasatellite with related isolates from the database sequences were also carried out. The conserved region is boxed in blue. The so-called "variable region" is boxed in purple. The A rich region is boxed in red. Betasatellite sequence from Calendula officinalis are written at the top. The accession numbers of the sequences from GenBank are indicated on the left (Figure 11). The significance of these accession numbers are as follows: Calendula yellow vein betasatellite (JQ693147: INDIA), Ageratum leaf curl betasatellite (KC589700: INDIA), Okra leaf curl virus satellite DNA beta (FN432358: PAKISTAN), Zinnia leaf curl disease associated sequence defective DNA beta molecule (AJ316041: PAKISTAN). Sequence analyses of the betasatellite revealed that there is a divergence due to nucleotide substitution, deletion and insertion.

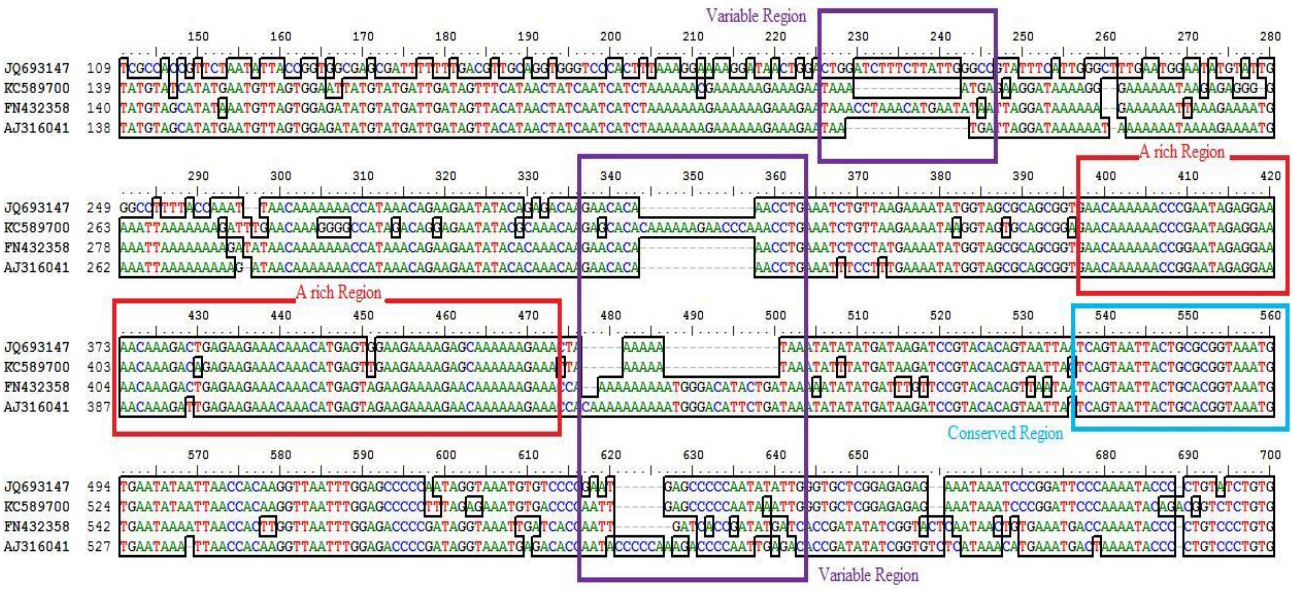


Fig 11: Alignment of betasatellite nucleotide sequences infecting Calendula officinalis in India with related isolates from the database sequences.

ISSN 2347- 6893

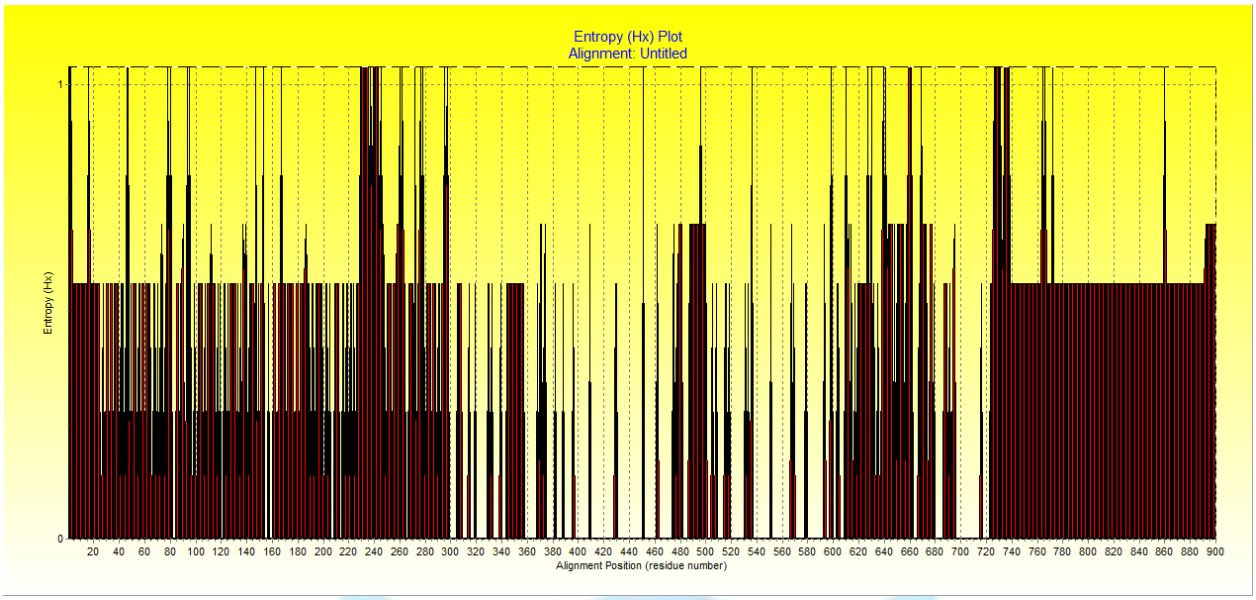


Fig 12: The Entropy power result of betasatellite from the alignment of nucleotide sequence.

This pattern of variation typically occurs due to point mutations in different isolates of a betasatellite often causing genetic drifts. Comparative nucleotide homology of betasatellite sequences indicates that, the genes have different evolutionary origin in the begomovirus infecting Calendula officinalis. The plausible reason for this difference could be the genetic recombination between begomoviruses [42].

Recombination is a major mechanism in virus evolution, allowing viruses to evolve more quickly by providing immediate direct access to many more areas of a sequence space than are accessible by mutation alone. Recombination analysis was performed using Recombination Detection Program (RDP v.3.44) on Windows operating system, which detects and analyses recombination points in a set of aligned DNA sequence. Since the coat protein gene is highly conserved therefore possibility of recombination is quite rare. The betasatellite (JQ693147) and its homologous were subjected to recombination analyses using RDP method used to drive automated recombination scan and the manual checking of automated analysis results. Analysis was allowed by employing Bonferroni correction with confidence greater than 95% (P value 0.05). In RDP analysis, the length of the window was set to 10 variable sites, and the step size was set to one nucleotide. P values were estimated by randomizing the alignment 1,000 times.

Recombination positions were observed in the betasatellite isolated from Calendula officinalis (Figure 13). The schematic sequence display is where the results of automated recombination scans are presented and it is the part of the program that is used to drive the manual checking of automated analysis results. The colored rectangles correspond to sequence fragments, thus representing the recombinant, major and minor parents in a graphical representation of a sequence fragments that have been potentially derived through recombination from a sequence resembling the one named to the right of the rectangle.

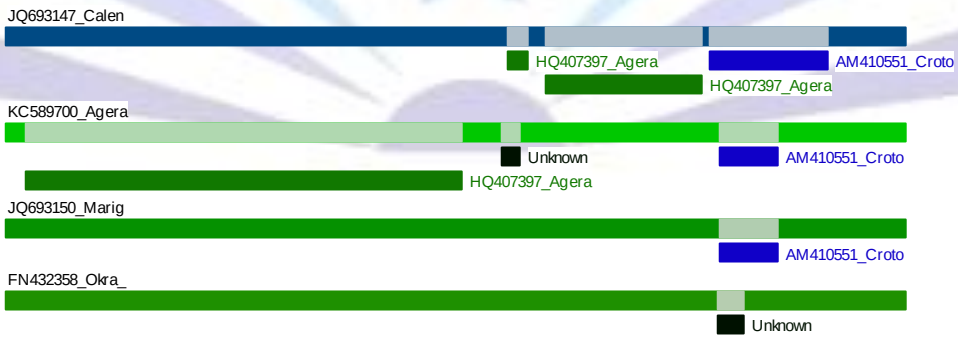


Fig 13: Diagram of the schematic sequence display representing the RDP recombination map of the recombinant fragments for the Calendula yellow vein betasatellite (JQ693147). Each color/pattern represents a sequence specific of a virus. The virus genome organization is represented under the diagram, positioning the different viral genes named according to the begomovirus convention.

The RDP plot of JQ693147 sequence conservation displayed a graphical overview of the sequence alignment that also indicates the portion of the alignment. Within the sequence part of the display, individual nucleotides are color coded according to their degree of conservation. When a recombination event selected, the 'toggle sequence display' button can be used to highlight nucleotide polymorphisms that contribute to the recombination signals depicted in the plot display. As per the schematic sequence display three recombination break point positions were identified in the betasatellite by the RDP method. The first evidence is given in Figure 14 where breakpoint begin from 260th [position 841 in alignments]

position and ending breakpoint ends at 295th [position 879 in alignments] position. Approximate p-value for this region was 6.914 x 10^{-04}. It suggested recombination in a small fragment of the sequence.

The major parent was identified as Zinnia leaf curl disease associated betasatellite (AJ316028) identified in Pakistan and were found infecting Zinnia elegans. Whereas the minor parent was Ageratum yellow leaf curl betasatellite (HQ407397) found infecting wild sunflower in India. This clearly indicates that this portion of recombinant fragment of betasatellite infecting Calendula officinalis is contributed from the two viruses prevailing at different geographical region, undoubtedly pointing towards the betasatellite evolution.

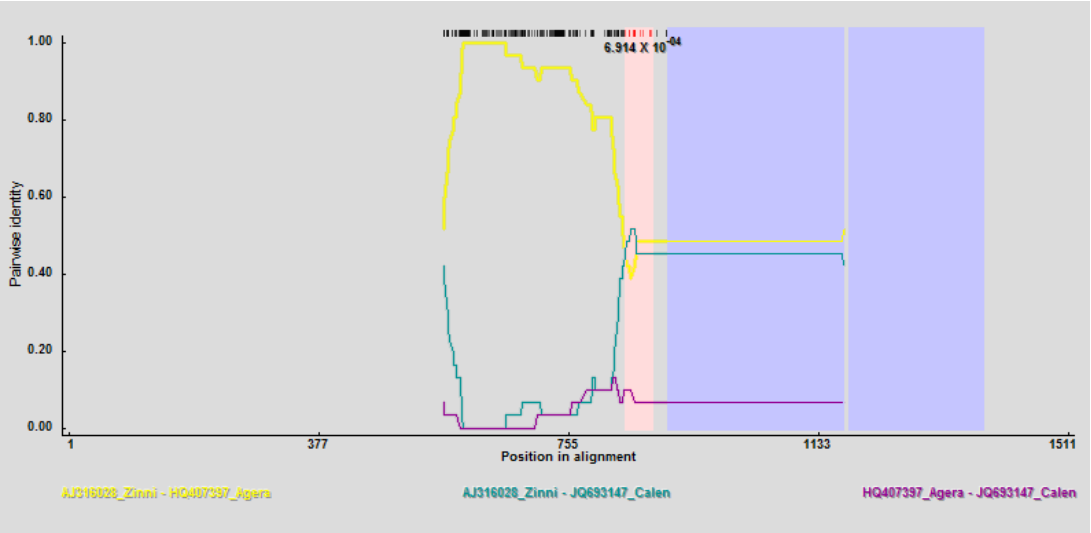


Fig 14: An RDP pairwise identity plot for the piece of sequence from the major parent (AJ316028_Zinnia) Uppermost bares indicating positions of informative sites; pink region indicates breakpoint positions suggested by the RDP software method. The pairwise identity plot have major parent: minor parent plot (AJ316028_Zinnia: HQ407397_Agera; yellow), major parent: recombinant plot (AJ316028_Zinnia: JQ693147_Calen; dark blue) and minor parent: recombinant plot (HQ407397_Agera: JQ693147_Calen; purple).

RDP looks for regions within a sequence alignment in which sequence pairs are sufficiently similar to suspect that they may have arisen through recombination. The second recombination was detected downstream of the first recombinant sequence where breakpoint begin from 322nd [position 906 in alignments] position and ending breakpoint ends at 513th [position 1168 in alignments] position (Figure 15). Approximate p-value for this region was 6.050 x 10^{-06}. Here the contribution of major parent in the RDP plot was by Okra leaf curl virus satellite DNA beta (FN432358) infecting Sonchus arvensis in Pakistan.

The minor parent was identified as Ageratum yellow leaf curl betasatellite (HQ407397) infecting wild sunflower in India. In both the recombinant events the minor parent was found to be same. Recombination positions were recognized by only RDP method and other methods such as GENECONV, Bootscan, Maxchi and Chimera were not found suitable for recombination analysis because of lowest recombination breakpoint detection accuracy.

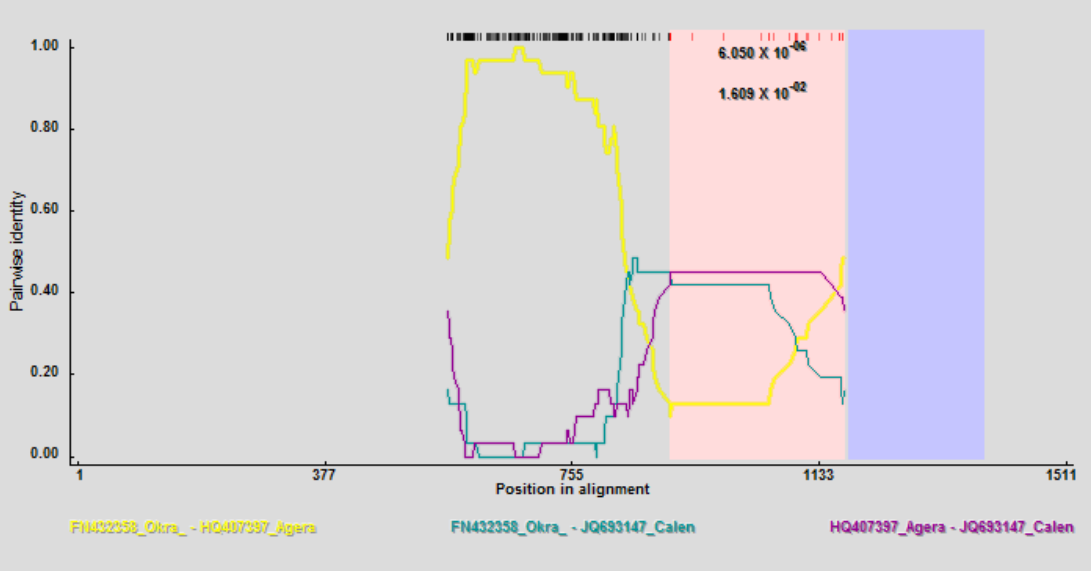


Fig 15: An RDP pairwise identity plot for the piece of sequence from major parent (FN432358_Okra) Uppermost bares indicating positions of informative sites; pink region indicates breakpoint positions suggested by the RDP

software method. The pairwise identity plot have major parent: minor parent plot (FN432358_Okra: HQ407397_Agera; yellow), major parent: recombinant plot (FN432358_Okra: JQ693147_Calen; dark blue) and minor parent: recombinant plot (HQ876467_Agera: JQ693147_Calen; purple).

Finally the third recombination event was discovered at the downstream of the second recombination event in the beastsatellite sequence, where breakpoint begin from 524th [position 1179 in alignments] position and ending breakpoint ends at 647th [position 1380 in alignments] position. Approximate p-value for this region was 2.821 x 10^{-14}. In this case the major parent was identified as Ageratum yellow leaf curl betasatellite (KC305086) causing disease in wheat and was reported from India (Figure 16). The minor parent in the RDP plot was found to be Croton yellow vein mosaic virus betasatellite (AM410551) reported from Pakistan infecting Croton sp. The extraordinary finding is this case is that the betasatellite (KC305086) infecting wheat was found in association with a Mastrevirus. Mastrevirus is another genera of Geminiviridae family, thus highlights the evolution of sequence fragment from a different virus.

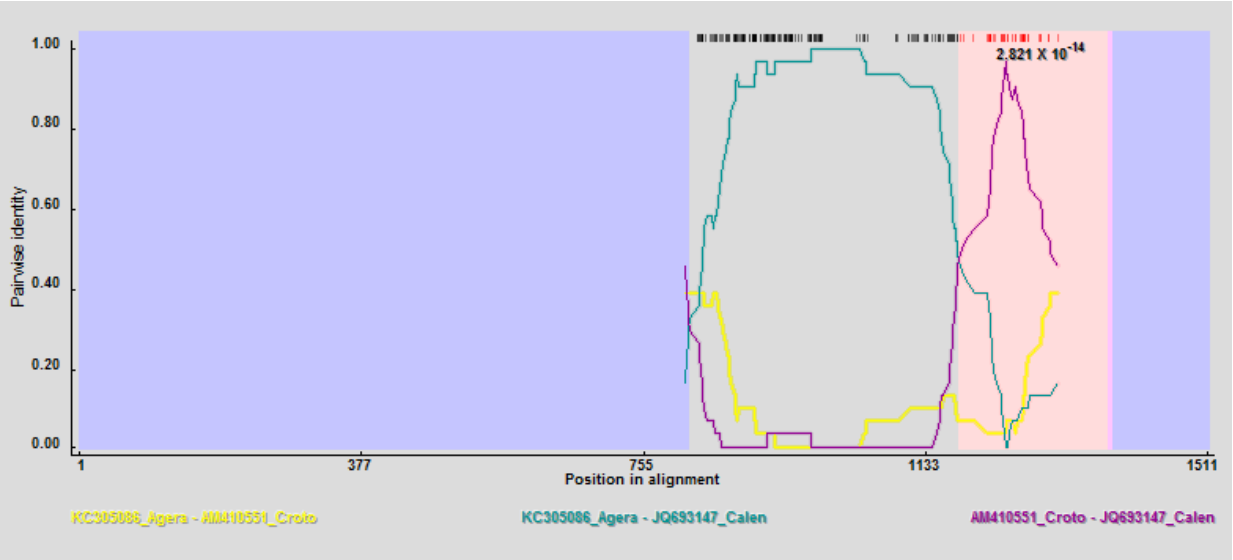


Fig 16: An RDP pairwise identity plot for the piece of sequence from major parent (KC305086_Agera) Uppermost bares indicating positions of informative sites; pink region indicates breakpoint positions suggested by the RDP software method. The pairwise identity plot have major parent: minor parent plot (KC305086_Agera: AM410551_Croto; yellow), major parent: recombinant plot (KC305086_Agera: JQ693147_Calen; dark blue) and minor parent: recombinant plot (AM410551_Croto: JQ693147_Calen; purple).

Recombination sites have been reported in both the DNA and RNA viruses [43, 44]. Presumably, the different pathotypes could simultaneously infect a host cell and exchange genetic materials through recombination. The recombination observed between geographically separated isolates probably represents older events, which may have occurred before their present separation. Movement of vectors and/or infected plant materials could be another factor for the gene flow between the widely separated locations [45].

A recombination may result in significant changes in the biological properties of virus isolates with the ability to adopt and sustain in different environmental conditions. Perhaps this is the first report of recombination in betasatellite infecting Calendula officinalis in India, which would provide significant information for understanding the diversity and evolution of begomoviruses in India.

CONCLUSIONS

In conclusion, we have established the existence of yellow vein disease of Calendula officinalis in India is caused by association of begomovirus and its betasatellite. This study supported evidence that virus associated with this disease was identified through biological and molecular characters as well as bioinformatics analysis. Moreover this is the first evidence for the detection of recombination events in betasatellite associated with the yellow vein disease of an ornamental plant Calendula officinalis (pot marigold) in Rajasthan province of India. The betasatellite can be replicated by other begomovirus species which increases the possibility of recombination and reassortment events. This could lead to evolution of new recombinant viruses or begomovirus complexes with different biological properties. It is also possible that exchange of betasatellites could extend the virus host range thereby emergence of new diseases in cultivated crop plants and other ornamental plants. One factor favoring the spread of begomoviruses among these plants is that many dicotyledonous species in India are hosts of whiteflies of the B. tabaci complex, which are the known or likely vectors of all the viruses.

The genetic diversity and evaluation of entropy power were viewed against the phylogenetic background which highlights the variable region where possibility of recombination increases. The increasing occurrence of begomoviruses in India demands efforts to study their diversity in order to anticipate and monitor outbreaks as well as to understand the evolutionary forces driving the emergence of novel begomoviruses in ornamental species previously unaffected by these pathogens. Our results provide much new information on these topics. Thus, this identification represents the possibility of a serious threat to other economically important ornamental and horticulture crop plants and there is a need for a more comprehensive study which will be focused on identification of possible further begomoviruses infection in the country in order to assess the contribution each makes to losses with a view to devising control strategies. Moreover advanced molecular techniques such RNAi will be used for development of transgenic plant resistance to begomovirus. This will form the basis of our future investigations. Results of these techniques effectively applied for disease management, crop protection and development of quarantine strategies at state and national level in India.

ACKNOWLEDGMENTS

The authors would like to acknowledge a vote of thanks to Department of Biotechnology (DBT project No. BT/PR13129/GBD/27/197/2009) and Department of Science and Technology (DST project no SR/FT/LS-042/2009), India for their financial support.

REFERENCES

[1] Cui, X., Tao, X., Xie, Y., Fauquet, C. M., and Zhou, X. 2004. A DNAβ associated with Tomato yellow leaf curl China virus is required for symptom induction. J. Virol 78: 13966-13974.

[2] Moffat, A. S. 1999. Geminiviruses emerge as serious crop threat. Science 286: 1835.

[3] Fauquet, C. M., Bisaro, D. M., Briddon, R. W., Brown, J. K., and Harrison, B. D. 2003. Virology division news: Revision of taxonomic criteria for species demarcation in the family Geminiviridae and an updated list of begomovirus species. Archives of Virology 148: 405-421.

[4] Saunders, K., Bedford, I. D., Briddon, R. W., Markham, P. G., Wong, S. M., and Stanley, J. 2000. A unique virus complex causes Ageratum yellow vein disease. Proc. Natl. Acad. Sci 97: 6890-6895.

[5] Hanley-Bowdoin, L., Settlage, S. B., Orozco, B. M., Nagar, S., and Robertson, D. 1999. Geminiviruses: Models for plant DNA replication, transcription and cell cycle regulation. Critical Review in Plant Science 18: 71-106.

[6] Markham, P. G., Bedford, S. L., and Pinner, M. S. 1994. The transmission of geminivirus by Bemisia tabaci. Pesticide Science 42: 123-128.

[7] Briddon, R. W, and Markham, P. G. 2001. Complementation of bipartite begomovirus movement functions by topocuviruses and curtoviruses. Arch. Virol 146: 1811-1819.

[8] Jose, J., and Usha, R. 2003. Bhendi yellow vein mosaic disease in India is caused by association of a DNAβ satellite with a begomovirus. Virology 305: 310-317.

[9] Li, Z., Xie, Y., and Zhou, X. 2005. Tobacco curly shoot virus DNAβ is not necessary for infection but intensifies symptoms in a host-dependent manner. Phytopathol 95: 902-908.

[10] Stanley, J., Bisaro, D. M., Briddon, R. W., Brown, J. K., Fauquet, C. M. el at., 2005. Geminiviridae. In Virus Taxonomy. VIIIth Report of the International Committee on Taxonomy of Viruses. pp. 301–326. Edited by Fauquet, CM., Mayo, MA., Maniloff, J., Desselberger, U and Ball, LA. London: Elsevier/Academic Press.

[11] Zhou, X., Xie, Y., Tao, X., Zhang, Z., Li, Z., and Fauquet, C. M. 2003. Characterization of DNAβ associated with begomoviruses in China and evidence for co-evolution with their cognate viral DNA-A. Journal of General Virology 84: 237-247.

[12] Mansoor, S., Briddon, R. W., Zafar, Y., and Stanley, J. 2003. Geminivirus disease complexes: An emerging threat. Trends Plant Sci 8: 128-134.

[13] Lima, A. T., Sobrinho, R. R., González-Aguilera, J., Rocha, C. S., Silva, S. J., Xavier, C. A., Silva, F. N., Duffy, S., and Zerbini, F.M. 2013. Synonymous site variation due to recombination explains higher genetic variability in begomovirus populations infecting non-cultivated hosts. J Gen Virol 94: 418–31.

[14] Urbino, C., Gutiérrez, S., Antolik, A., Bouazza, N., Doumayrou, J., Granier, M., Martin, D. P., and Peterschmitt, M. 2013. Within-host dynamics of the emergence of tomato yellow leaf curl virus recombinants. PLoS ONE. 2013; 8: e58375. doi:10.1371/journal.pone.0058375.

[15] Raj, S. K., Khan, M. S., Snehi, S. K., Kumar, S., and Khan, A. A. 2007. Natural occurrence of a Begomovirus on Dimorphotheca sinuate in India. Australasian Plant Disease Notes 2: 25–26.

[16] Ilyas, M., Nawaz, K., Shafiq, M., Haider, M. S., and Shahid, A. A. 2013. Complete nucleotide sequences of two begomoviruses infecting Madagascar periwinkle (Catharanthus roseus) from Pakistan. Arch Virol 158: 505–10.

[17] Muley, B. P., Khadabadi, S. S., and Banarase, N.B. 2009. Phytochemical Constituents and Pharmacological Activities of Calendula officinalis Linn (Asteraceae): A Review. Tropical Journal of Pharmaceutical Research 8: 455-465.

[18] Butnariu, M., and Coradini, C. Z. 2012. Evaluation of Biologically Active Compounds from Calendula officinalis Flowers using Spectrophotometry. Butnariu and Coradini Chemistry Central Journal 6:35.

[19] Harrison, B. D., and Robinson, D. J. 1999. Natural genomic and antigenic variation in whitefly-transmitted geminiviruses (begomoviruses). Annual Review of Phytopathology 37: 369-398.

[20] Zhou, X., Liu, Y., Calvert, L., Munoz, C., Otim-Nape, G. W., el at., 1997. Evidence that DNA-A of a geminivirus associated with severe cassava mosaic disease in Uganda has arisen by interspecific recombination. Journal of General Virology 78: 2101-2111.

[21] Brown, J. K., and Czosnek, H. 2002. Whitefly transmission of plant viruses. Advances in Botanical Research 36: 65-100.

[22] Manen, J. F., Sinitsyna, O., Aeschbach, L., Markov, A. V., and Sinitsyn, A. 2005. A fully automatable enzymatic method for DNA extraction from plant tissues. BMC Plant Biol 5: 3–23.

[23] Marwal, A., Sahu, A., Prajapat, R., and Gaur, R. K. 2012. First report of association of begomovirus with the leaf curl disease of a common weed, Datura inoxia. Indian J Virol 23: 3–84.

[24] Marwal, A., Sahu, A., Sharma, P., and Gaur, R. K. 2013a. Molecular Characterizations of Two Begomoviruses Infecting Vinca rosea and Raphanus sativus in India. Virologica sinica 28: 53–56.

[25] Rojas, M. R., Gilbertson, R. L., Russell, D. R., and Maxwell, D. P. 1993. Use of degenerate primers in the polymerase chain reaction to detect whitefly-transmitted geminiviruses. Plant Disease 77: 340-347.

[26] Bela-ong, D. B., and Bajet, N. B. 2007. Molecular Detection of Whitefly-Transmissible Geminiviruses (Family Geminiviridae, Genus begomovirus) in the Philippines. Philippine Journal of Science 136: 87-101.

[27] Briddon, R. W., Bull, S. E., Mansoor, S., Amin, I., and Markham, P. G. 2002. Universal primers for the PCR-mediated amplification of DNA α; a molecule associated with some monopartite begomoviruses. Mol Biotech 20:315–318.

[28] Bull, S. E., Briddon, R. W., and Markham, P. G. 2003. Universal primers for the PCR-mediated amplification of DNA 1: a satellite-like molecule associated with begomovirus-DNA β complexes. Mol Biotech 23:83–86.

[29] Marwal, A., Sahu, A., Prajapat, R., and Gaur, R. K. 2013b. First report of Begomovirus infecting two ornamental plants: Jasminum sambac and Millingtonia hortensis. Indian Phytopathology 66: 115 – 116.

[30] Marwal, A., Sahu, A., Choudhary, D. K., and Gaur, R.K. 2013c. Complete nucleotide sequence of a begomovirus associated with satellites molecules infecting a new host Tagetes patula in India. Virus Genes DOI: 10.1007/s11262-013-0914-2.

[31] Thompson, J. D., Higgins, D. G., and Gibson, T. J. 1994. CLUSTAL-W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. Nucleic Acids Research 22: 4673-4680.

[32] Kumar, S., Dudley, J., Nei, M., and Tamura, K. 2008. MEGA: A biologist-centric software for evolutionary analysis of DNA and protein sequences. Briefings in Bioinformatics 9: 299-306.

[33] Perrière, G., and Gouy, M. 1996. WWW-Query: an on-line retrieval system for biological sequence banks. Biochimie 78: 364–369.

[34] Saitou, N., and Nei, M. 1987. The neighbor-joining method: A new method for reconstructing phylogenetic trees. Mol. Biol. Evol 4: 406-425.

[35] Robertson, D. L., Haha, B. H., and Sharp, P. H. 1995. Recombination in AIDS virus. J. Mol. Evol 40: 249-259.

[36] Marwal, A., Prajapat, R., Sahu, A., and Gaur, R. K. 2011. In Silico Recombination analysis: A Study for Geminivurs Host Mobility. Asian Journal of Biological Science 5: 1-8.

[37] Posada, D., and Crandall, K. A. 2001. Evaluation of methods for detecting recombination from DNA sequences: Computer simulations. Proceedings of the National Academy of Science. USA 98: 13757-13762.

[38] Posada, D. 2002. Evaluation of methods for detecting recombination from DNA sequences: empirical data. Molecular Biology and Evolution 19: 708-717.

[39] Martin, D. P., Posada, D., Crandall, K. A., and Williamson, C. 2005. A modified BOOTSCAN algorithm for automated identification of recombinant sequences and recombination breakpoints. AIDS Research and Human Retroviruses 21: 98-102.

[40] Mayo, M. A., and Pringle, C. R. 1998. Virus taxonomy--1997. J Gen Virol 79: 649-657.

[41] Campanella, J. J., Bitincka, L., and Smalley, J. 2003. MatGAT: An application that generates similarity/identity matrices using protein or DNA sequences. BMC Bioinformatics 4:29.

[42] Zhou, X., Liu, Y., Robinson, D. J., and Harrison, B. D. 1998. Four DNA-A variants among Pakistani isolates of cotton leaf curl virus and their affinities to DNA-A of geminivirus isolates from okra. J. Gen. Virol 79: 915-923.

[43] Prasanna, H. C., and Rai, M. 2007. Detection and frequency of recombination in tomato-infecting begomoviruses of South and Southeast Asia. Virol. J 4: 111-120.

[44] Gagarinova, A. G., Babu, M., Strömvik, M. V., and Wang, A. 2008. Recombination analysis of Soybean mosaic virus full-length genome sequences reveals evidence of RNA recombination between distinct pathotypes.Virol. J 4: 111-120.

[45] Rojas, A., Kvarnheden, A., Marcenaro, D., and Valkonen, J. P. T. 2005. Sequence characterization of Tomato leaf curl Sinaloa virus and Tomato severe leaf curl virus: phylogeny of New World begomoviruses and detection of recombination. Archives of Virology 150: 1281-1299.

Author' biography with Photo

Avinash Marwal and Anurag Kumar Sahu

Avinash Marwal and Anurag Kumar Sahu are PhD Scholars (enrolled in 2010) at Mody Institute of Technology and Science, Lakshmangarh, Rajasthan (India) in a DBT funded project (DBT project No. BT/PR13129/GBD/27/197/2009) and DST funded project (DST project no SR/FT/LS-042/2009) respectively, under the supervision of Dr R. K. Gaur, Principal Investigator. Both has 30 international publications in reputed journals and has command over various molecular, biotechnological and bioinformatics tools and techniques. His core interests are Homology Modeling, Molecular Docking, Biological Database development, In Silico drug designing, Plant Molecular Biology and Development of Transgenic Plants.

Dr Rakarshi Kumar Gaur

Dr. Rajarshi Kumar Gaur is presently working as Head and Associate Professor, Department of Science, Mody Institute of Technology and Science (Deemed University), Lakshmangarh, Sikar, Rajasthan. He did his Ph.D on molecular characterization of sugarcane viruses of India. He had partially characterized three sugarcane virus viz., sugarcane mosaic virus, sugarcane streak mosaic virus and sugarcane yellow luteovirus. He received MASHAV fellowship in 2004 of Israel government for his post doctoral studies and joined The Volcani Centre, Israel and then shifted to Ben Gurion University, Negev, Israel. In 2007 he received the Visiting Scientist Fellowship from Swedish Institute Fellowship, Sweden for one year to work in the The Umeå University, Umeå, Sweden. He is also a recipient of ICGEB, Italy Post Doctoral fellowship in 2008. He worked on development of marker-free transgenic plant against cucumber viruses. He has made significant contributions on sugarcane viruses and published 80 national/international papers and presented near about 50 papers in the national and international conferences. He was awarded as Fellow of International Society of Biotechnology, Fellow of Madhaswami Educational Trust and Fellow of International Consortium of Contemporary Biologist. He has also visited Thailand, New Zealand, London and Italy for the sake of attending the conference/workshop. Recently, he received two projects on begomovirus from Department of Biotechnology, Government of India and Department of Science and Technology, India, New Delhi. Presently, he is working on the characterization of geminiviruses and RNAi technology.