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Detection and molecular characterization of Begomovirus infecting some common weeds in West Bengal

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ABSTRACT

Weeds are often overlooked for their role as a potential carrier of several viruses. In our present study symptomatic weeds such as Croton sparsiflorus, Acalypha indica, Sida rhombifolia, and Ageratum conyzoides, were collected and detected through PCR by using universal and begomovirus specific primers. Begomovirus infecting Croton sparsiflorus and Acalypha indica showed a maximum similarity of 97.94% and 97.04% respectively to Jatropha leaf yellow mosaic virus (NC_038956), and begomovirus infecting Sida rhombifolia and Ageratum conyzoides showed maximum similarity of 96.84% with Cotton ileaf icurli Bangalore virus (KT390464.1), and Ageratum ienation ivirus isolate Gorakhpur (GQ268327) (95%) respectively. Further phylogenetic analysis and amino acid structure prediction were performed to determine the relationship with the existing begomovirus in the world, that showed variation in sequence diversity and expansion of population among the isolates infecting the weeds. The present outcomes revealed that weed species may play a significant role in harboring various begomoviruses and may play a crucial role in recombination with different begomoviruses.

Keywords: Characterization, diagnosis, phylogenesis, ssDNA virus, weeds

Weeds as opportunistic "colonizing species" or "pioneers of secondary succession" are inclined to assault by enormous plant viruses and that can be a potential reservoir of a countless number of plant viruses (Seal *et al*., 2006). Since the weed ecosystem has a wide hereditary base, the virus population may enslave to a distinct selection pressure compared to the main crops. Weeds play an important role as collateral as well as an alternate host of many viruses during the off-season and act as a pool or "Transmission bridge" amid cropping and non-cropping season (Salatti, 2002). The Geminiviridae family is one of the largest groups of plant viruses, got its unique name due to its distinctive virion morphology and contains ssDNA as their genomic material. The term 'Gemini' was derived from the Greek word "Geninus" which meant twins (Harrison *et al.,* 1977). The "twin" particles together are 30 × 20 nm in size (Bock *et al*., 1977). The protein subunits have an icosahedral symmetry with 22 pentamers and are *c*. 30 kDa in size, the bipartite genome having DNA-A and DNA-B of single-stranded circular DNA, measures each of 2.7-2.8 Kb (Stanley, 1983). According to the International Committee on Taxonomy of Viruses (ICTV) latest report on the taxonomy of the Geminiviridae, there are now nine genera collectively having >360 species. Viruses in the genus Begomovirus have mono- or bipartite genomes, while monopartite genomes are present in the genera Capulavirus, Eragrovirus, Curtovirus, Becurtovirus, Grablovirus, Turncurtovirus, Mastrevirus and Topocuvirus. Over the past few years, the importance of geminiviruses, especially begomoviruses which is the largest genera of the family, is increasing exponentially due to their fast ability to multiply by recombination, pseudore combination, and mutation. They are also whitefly-transmitted which are dramatically increasing with auxiliary increase of the associated plant diseases leading to many epidemic diseases. Many common weed species are reported not only to harbor these viruses, but, also to be infected, showing various symptoms for example chlorosis, yellowing, distortion, leaf curl and stunted growth (Tan *et al.*, 1995). There are many approaches for the detection and characterization of begomovirus in the uncultivated plants. However, in our present study visual identification of symptomatic weeds such as *Croton sparsiflorus*, *Acalypha indica*, *Ageratum conyzoides,* and *Sida rhombifolia* has been collected and detected through PCR by using universal primer and specific primers for the purpose towards characterization of begomovirus populations infecting the weeds in Kalyani, West Bengal, as a step towards evaluating their role as begomovirus repositories and species diversity.

MATERIALS AND METHODS

Collection of samples

A survey was conducted in the year 2018-19 in various regions of the crop fields, fallow lands, roadsides, and wasteland areas of Horticultural Research station and other experimental farms of Bidhan Chandra Krishi Viswa Vidyalaya (BCKV). Symptomatic leaf samples (*Croton sparsiflorus, Acalypha indica, Sida rhombifolia,* and *Ageratum conyzoides*) exhibiting characteristic

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begomovirus symptoms were collected then followed by storing it in -50æ%C for overnight. The extraction of DNA from the sample was followed by CTAB (Cetyl Trimethyl Ammonium Bromide) method with slight modification (Clark, 2009). PCR (Polymerase chain reaction) amplification was conducted by using begomovirus specific degenerate primers and gene-specific primers and electrophoresed to determine the type of virus infecting the weed species. The degenerate primer pair used in the study, SPG1/SPG2 was designed from the conserved region of sweet potato geminivirus (Li *et al*., 2004). Specific primer pairs, Aco1/Aco2 were designed from 'V1' gene of Ageratum yellow vein virus, complete genome (Acc.NC_004626) (Tan *et al*., 1995). Another set of primer pairs, AV2F/ AV2R comprising of AV2 (Precoat protein) gene were used for detection of ssDNA virus infection in *Sida rhombifolia* (Table.1). Each PCR reaction contained 2µl DNA, 2.5µL Taq buffer (10X) (Bioline, USA), Taq DNA polymerase (3U/µL) (Bioline, USA), 1µL dNTPs (2.5mM) (New England Biolab), 3µL$MgCl_2$(15mM) (Bioline,USA), 1µL Forward Primer(10mM), 1µL Reverse Primer(10mM) and molecular grade nuclease free water to make up a total volume of 25µL. PCR conditions involved denaturation at 94 °C for 30s and annealing at different temperatures speciûc for each primer pairs (Table.1) for 30 s with 34 number of cycles, Extension was carried out at 72 °C for 40 s with the ûnal extension for 10 min at 72 °C. The PCR products were run on 1 % (w/v) agarose gel in 1X TBE buffer and visualized under UV light using the Gel-Documentation System (3000x Vilber Lourmut).The PCR amplified products of *Acalypha indica, Croton sparsiflorus, Sida rhombifolia* were further purified using gel elution and purification kit (HIPURA™ PCR Product and Gel Purification Combo Kit) and cloning was done by using the pGEM T-easy vector (Promega, Madison, USA) by the method of Knoche and Kephart (1999) and then sent for DNA sequencing by Sanger dideoxy sequencing to **Chromous Biotech**, Bangalore. The consensus nucleotide sequences of the PCR products were blasted in the NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi) followed by annotation with the published nucleotides of the begomovirus by multiple alignments using the following software: BioEdit (7.2.5), Sequence scanner (1.0) Demarcation Tool (SDTv.1.2) (http://web.cbio.uct.ac.za), and submitted in genebank. After getting the Accession number, molecular evolutionary analyses and Phylogenetic analysis were carried out by using MEGA version X (Kumar *et al*., 2018). Secondary structure and 3-D structural prediction of the amino acid sequence of pre-coat protein 'AV2' was performed using software (Phyre v.2.0) (http://www.sbg.bio.ic.ac.uk/phyre2/).

RESULTS AND DISCUSSION

From the field, symptomatic leaf samples such as *Croton sparsiflorus, Acalypha indica, Sida rhombifolia,* and *Ageratum conyzoides* showed typical symptoms for example yellowing, vein clearing, mosaic, and leaf curl (Fig.1). Previous studies revealed that many commonly grown weeds in India such as *Ageratum conyzoides, Solanum nigrum, Parthenium hysterophorus, Trigonella corniculata etc* showed above-mentioned symptoms and hence act as reservoir hosts of several begomoviruses (Raj *et al.,* 2011). The universal primer pair SPG1/SPG2 successfully amplified the band of size ~853bp (Fig.2A). The primer pairs, Aco1/Aco2 gave amplification of 432 bp (Fig.2B) from the infected sample *Ageratum conyzoides*, and AV2F/AV2R comprising of AV2 (Precoat protein) gene gave an amplicon of ~347 bp (Fig.2C), which further proved the infection of begomovirus in the above host.

Sequence analysis of the nucleotide sequences

The consensus nucleotide sequence of the partial gene of the virus isolate, derived from *Acalypha indica* (Accession No: MN006988), *Croton sparsiflorus* (Accession No: MN006989), and *Sida rhombifolia* (Accession No: MK986831), *Ageratum conyzoides* (Accession No: HF936708), showed highest homology of 97.94% with the genome sequences of Jatropha leaf yellow mosaic Katarniaghat virus segment DNA-A(NC_038956) (Fig.3A), 97.04% with Jatropha leaf yellow mosaic Katarniaghat virus segment DNA-A (NC_038956) (Fig.3C), 96.84% maximum nucleotide identity with Cotton leaf curl Bangalore virus isolate WOK48 segment DNA A (KT390464.1) (Fig.3E) and, 91.61% with Tobacco curly shoot virus isolate (>KM383752) (Fig.3G) respectively. Analysis of the sequence data of *Acalypha* and *Croton* begomovirus isolate covered partial sequences of 4 ORFs AC1, AC2, AC3, AC4 in the antisense direction and nucleotide sequence data of *Sida* begomovirus showed two ORF, AV1 and AV2 in the sense direction and in case of *Ageratum conyzoides* the nucleotide sequence showed one ORF AV1 in the sense direction. The present studies revealed that PCR techniques using degenerate primers could be used as a diagnostic tool to rapidly screen plants for begomovirus, also suggested by Chatterjee *et al.* (2007) and partial sequences confirmed the presence of Jatropha leaf yellow mosaic virus in *Acalypha indica* and *Croton sparsiflorus*, and Cotton leaf curl virus, Tobacco curly shoot virus in *Sida rhombifolia* and *Ageratum conyzoides* respectively.

Phylogenetic Analysis

The phylogenetic tree in the case of the nucleotide sequence from the sample *Acalypha indica* was followed

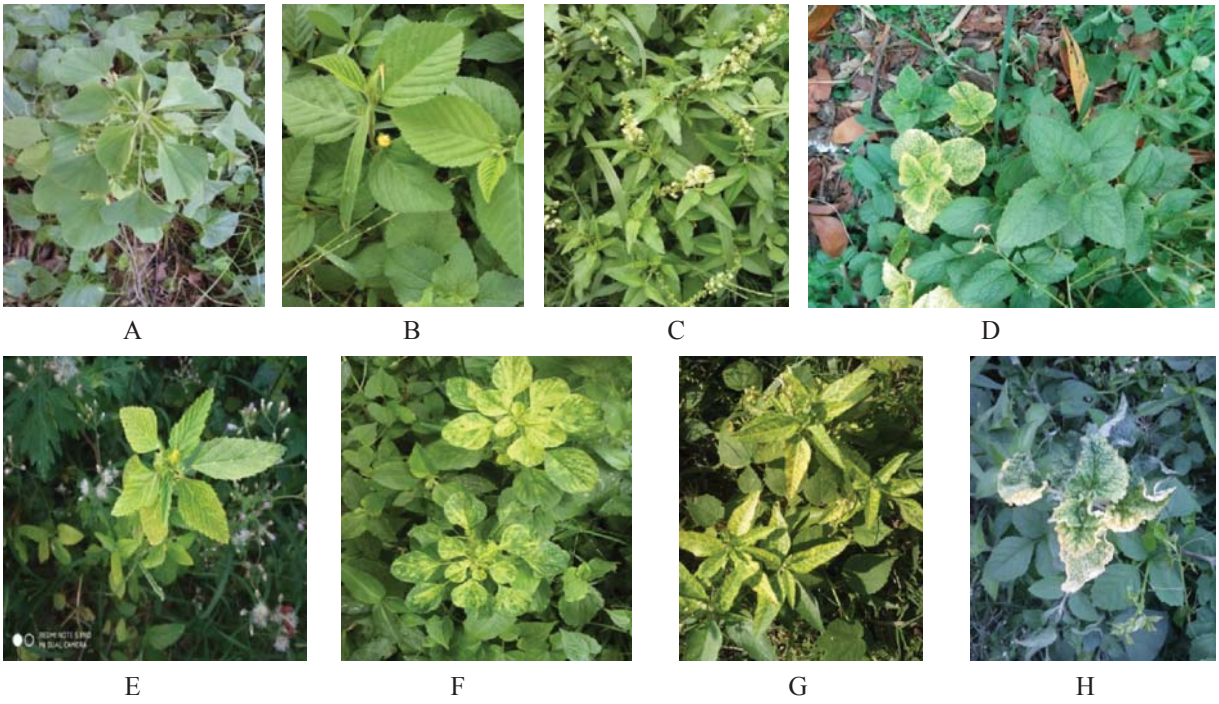

Fig. 1. **A**-*Acalypha indica*(-) ; **B**-*Sida rhombifolia*(-) ; **C**-*Croton sparsiflorus*(-) ; **D**-*Ageratum conyzoides*(+/); **E**-*Acalypha indica*(+) ; **F**-*Sida rhombifolia*(+) ; **G**-*Croton sparsiflorus*(+) ; **H**-*Ageratum conyzoides*(+/-) '+' indicating symptomatic plant; '-' indicating healthy plant

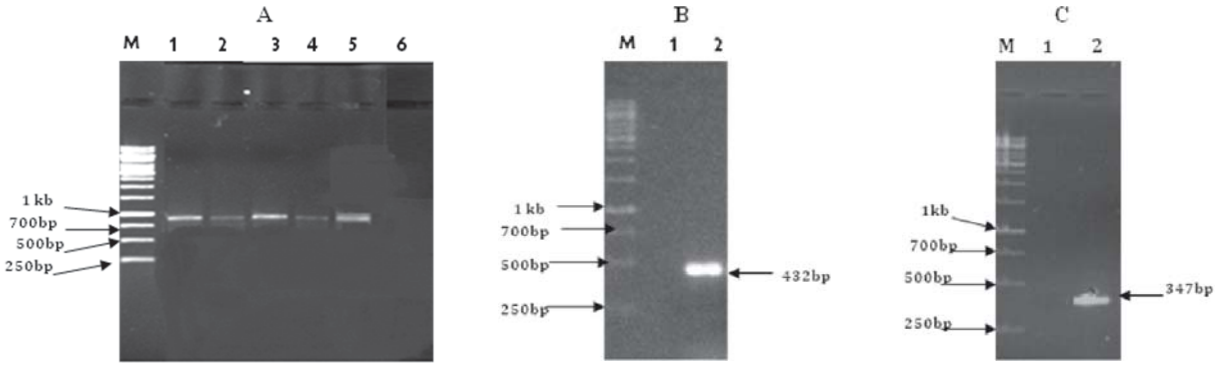

Fig. 2. Agarose gel electrophoresis of the PCR-amplified products

(A) with degenerate primers(SPGF/SPGR)(~853bp size) and (B) with cp specific primers(ACO1/ ACO2)(~432bp size). (C) with precoat protein specific primers(AV2F/AV2R)(~347bp).Lane M represents 1 kb DNA ladder, Lane 1 (B and C- asymptomatic healthy samples , lane 1-4(A) symptomatic samples , lane 5(A)-Positive control sample ,lane 6(A)-asymptomatic healthy sample

Table1: Primer pairs used for the detection of begomovirus and respective annealing temperature and amplicon produced by polymerase chain reaction (PCR)

Primer Name	Orientation	Sequence	Band size(bp)	Annealing temperature	Reference
SPG1	Sense	5'CCCKGTGCGWRAATCCAT3'	853	59^{0}C	Li *et al.*,2004
SPG2	Antisense	5'ATCCVAAYWTYCAGGGAGCTA3'	-	-	
Aco1	Sense	5' CTCACAACGCTCCCTCAAAG3'	432	56^{0}C	Tan *et al.*,1995
Aco2	Antisense	5' GACCTTACATGGGCCTTCAC3'	-	-	
AV2F	Sense	5'GCATCTGTGGTTCTTGCAGG3'	347	55^{0}C	-
AV2R	Antisense	5'CCTTTGAGTCCACAAGCCAA3'	-	-	

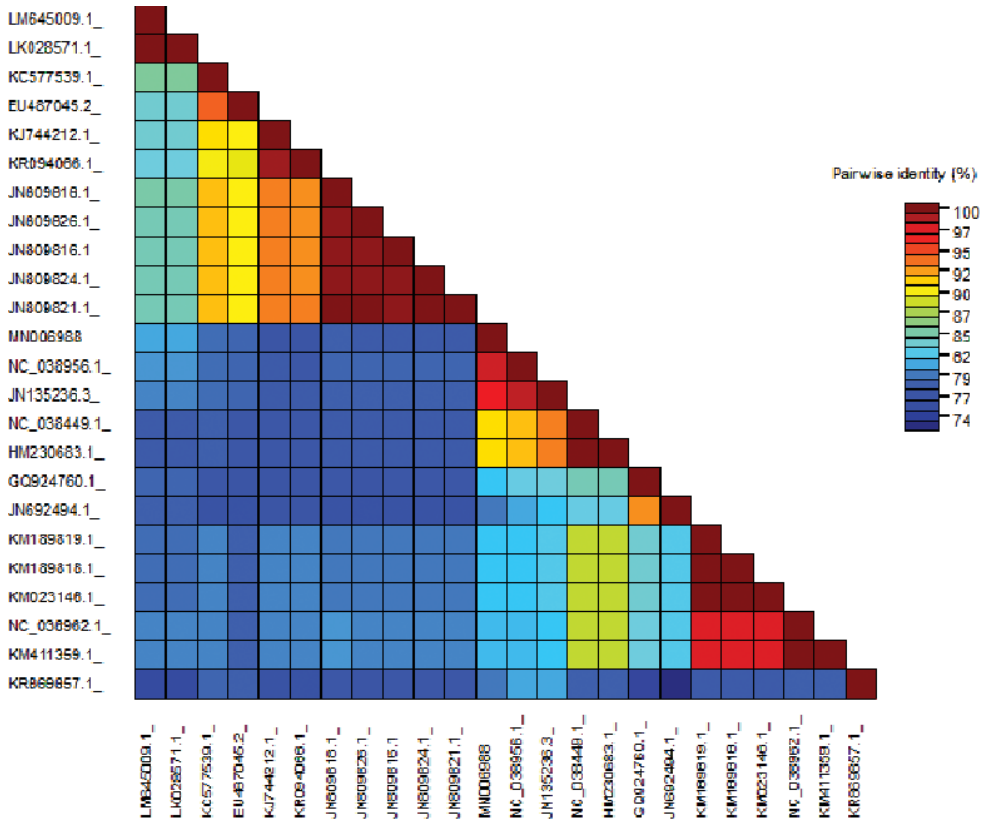

Fig.3 Phylogenetic analysis (Neighbor-Joining method) and Sequence diversity analysis using Sequence Demarcation Tool version 1.2 (SDTv1.2) of MN006988, MN006989, MK986831, and HF936708. Pairwise identity is shown from blue (minimum) to red (maximum) matrix which represents percentage pairwise genome scores in nucleotide identity.The name of viruses taken for analysis is given in the phylogenetic analysis.

Fig.3A

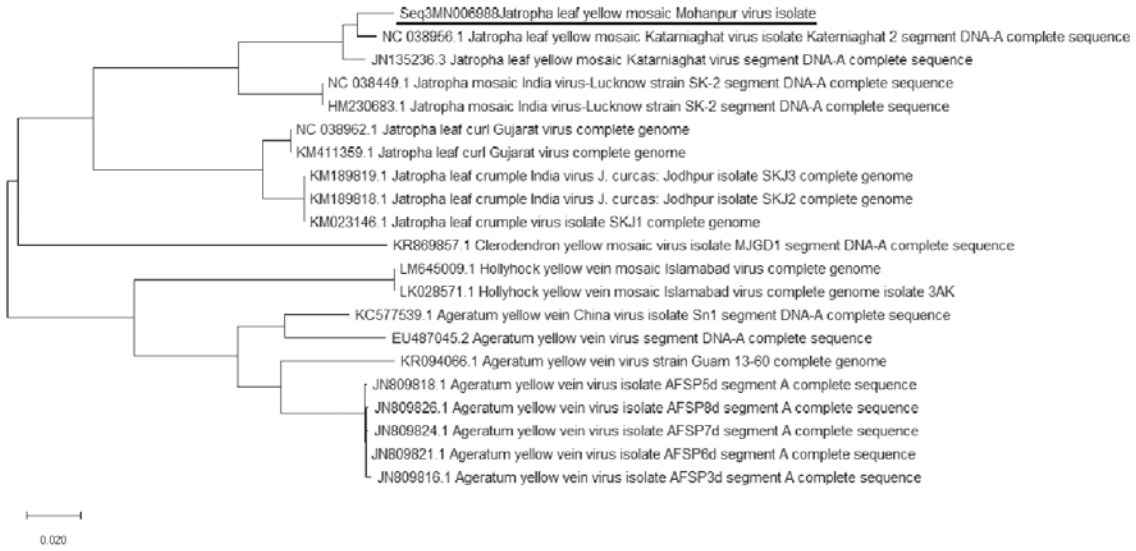

Fig.3 B

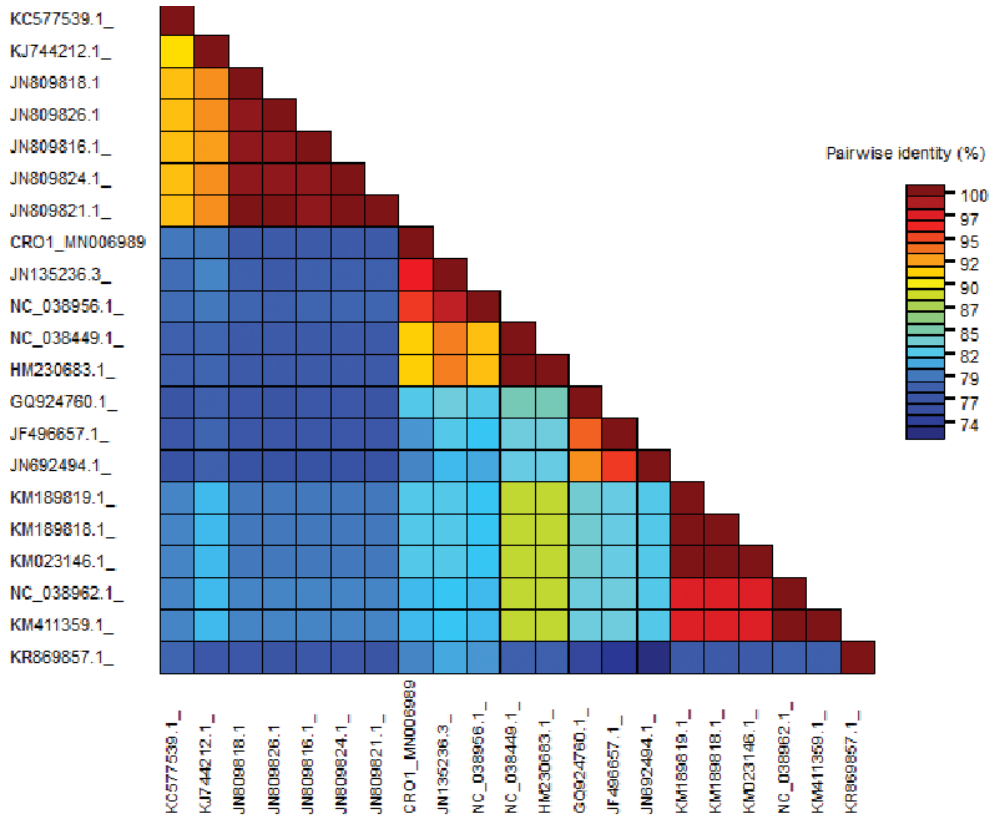
KC577539.1_
KJ744212.1_
JN809818.1
JN809826.1
JN809816.1_
JN809824.1_
JN809821.1_
CRO1_MN006989
JN135236.3_
NC_038956.1_
NC_038449.1_
HM230683.1_
GQ924760.1_
JF496657.1_
JN692494.1_
KM189819.1_
KM189818.1_
KM023146.1_
NC_038962.1_
KM411359.1_
KR869857.1_
Pairwise identity (%)
100
97
95
92
90
87
85
82
79
77
74

Fig. 3C

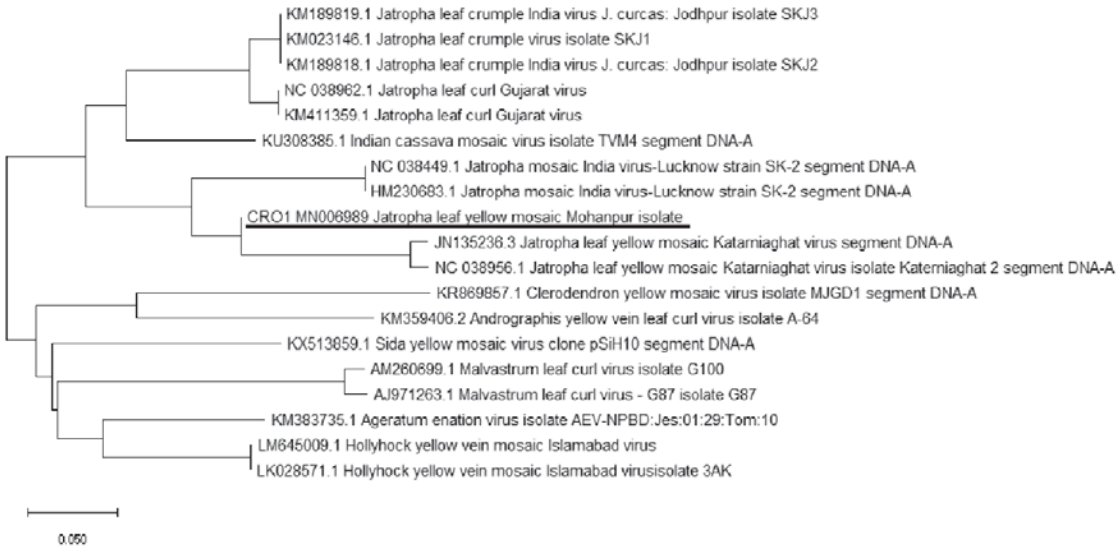
KM189819.1 Jatropha leaf crumple India virus J. curcas: Jodhpur isolate SKJ3
KM023146.1 Jatropha leaf crumple virus isolate SKJ1
KM189818.1 Jatropha leaf crumple India virus J. curcas: Jodhpur isolate SKJ2
NC 038962.1 Jatropha leaf curl Gujarat virus
KM411359.1 Jatropha leaf curl Gujarat virus
KU308385.1 Indian cassava mosaic virus isolate TVM4 segment DNA-A
NC 038449.1 Jatropha mosaic India virus-Lucknow strain SK-2 segment DNA-A
HM230683.1 Jatropha mosaic India virus-Lucknow strain SK-2 segment DNA-A
CRO1 MN006989 Jatropha leaf yellow mosaic Mohanpur isolate
JN135236.3 Jatropha leaf yellow mosaic Katarniaghat virus segment DNA-A
NC 038956.1 Jatropha leaf yellow mosaic Katarniaghat virus isolate Katerniaghat 2 segment DNA-A
KR869857.1 Clerodendron yellow mosaic virus isolate MJGD1 segment DNA-A
KM359406.2 Andrographis yellow vein leaf curl virus isolate A-64
KX513659.1 Sida yellow mosaic virus clone pSiH10 segment DNA-A
AM260699.1 Malvastrum leaf curl virus isolate G100
AJ971263.1 Malvastrum leaf curl virus - G87 isolate G87
KM383735.1 Ageratum enation virus isolate AEV-NPBD:Jes:01:29:Tom:10
LM645009.1 Hollyhock yellow vein mosaic Islamabad virus
LK028571.1 Hollyhock yellow vein mosaic Islamabad virusisolate 3AK
0.050

Fig. 3D

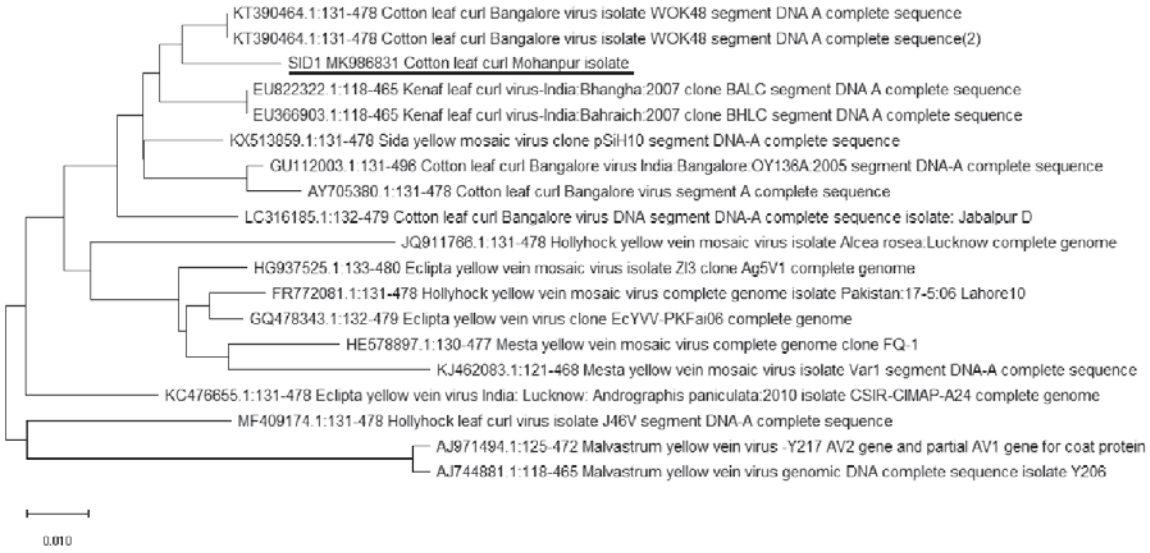
KT390464.1:131-478 Cotton leaf curl Bangalore virus isolate WOK48 segment DNA A complete sequence
KT390464.1:131-478 Cotton leaf curl Bangalore virus isolate WOK48 segment DNA A complete sequence(2)
SID1 MK986831 Cotton leaf curl Mohanpur isolate
EU822322.1:118-465 Kenaf leaf curl virus-India:Bhangha:2007 clone BALC segment DNA A complete sequence
EU366903.1:118-465 Kenaf leaf curl virus-India:Bahraich:2007 clone BHLC segment DNA A complete sequence
KX513859.1:131-478 Sida yellow mosaic virus clone pSiH10 segment DNA-A complete sequence
GU112003.1:131-498 Cotton leaf curl Bangalore virus India Bangalore:OY136A:2005 segment DNA-A complete sequence
AY705380.1:131-478 Cotton leaf curl Bangalore virus segment A complete sequence
LC316185.1:132-479 Cotton leaf curl Bangalore virus DNA segment DNA-A complete sequence isolate: Jabalpur D
JQ911766.1:131-478 Hollyhock yellow vein mosaic virus isolate Alcea rosea:Lucknow complete genome
HG937525.1:133-480 Eclipta yellow vein mosaic virus isolate ZI3 clone Ag5V1 complete genome
FR772081.1:131-478 Hollyhock yellow vein mosaic virus complete genome isolate Pakistan:17-5:06 Lahore10
GQ478343.1:132-479 Eclipta yellow vein virus clone EcYVV-PKFai06 complete genome
HE578897.1:130-477 Mesta yellow vein mosaic virus complete genome clone FQ-1
KJ462083.1:121-468 Mesta yellow vein mosaic virus isolate Var1 segment DNA-A complete sequence
KC476655.1:131-478 Eclipta yellow vein virus India: Lucknow: Andrographis paniculata:2010 isolate CSIR-CIMAP-A24 complete genome
MF409174.1:131-478 Hollyhock leaf curl virus isolate J46V segment DNA-A complete sequence
AJ971494.1:125-472 Malvastrum yellow vein virus -Y217 AV2 gene and partial AV1 gene for coat protein
AJ744881.1:118-465 Malvastrum yellow vein virus genomic DNA complete sequence isolate Y206
0.010

Fig. 3E

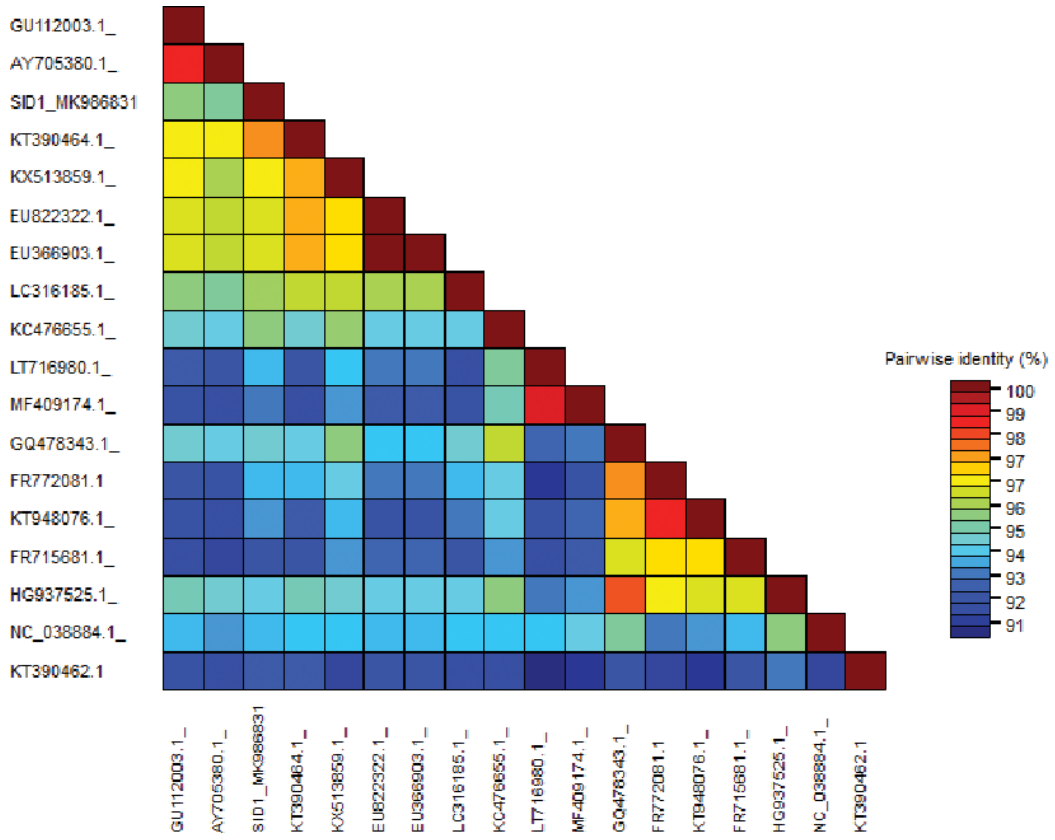
GU112003.1_
AY705380.1_
SID1_MK986831
KT390464.1_
KX513859.1_
EU822322.1_
EU366903.1_
LC316185.1_
KC476655.1_
LT716980.1_
MF409174.1_
GQ478343.1_
FR772081.1
KT948076.1_
FR715681.1_
HG937525.1_
NC_038884.1_
KT390462.1
Pairwise identity (%)
100
99
98
97
97
96
95
94
93
92
91

Fig. 3F

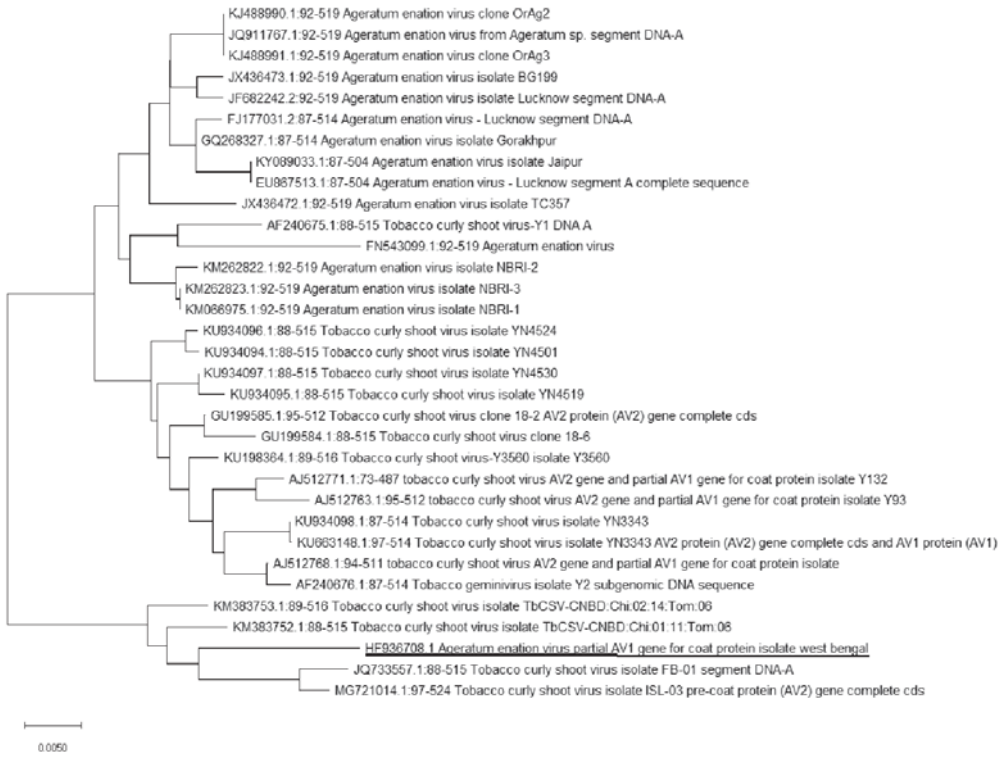
KJ488990.1:92-519 Ageratum enation virus clone OrAg2
JQ911767.1:92-519 Ageratum enation virus from Ageratum sp. segment DNA-A
KJ488991.1:92-519 Ageratum enation virus clone OrAg3
JX436473.1:92-519 Ageratum enation virus isolate BG199
JF682242.2:92-519 Ageratum enation virus isolate Lucknow segment DNA-A
FJ177031.2:87-514 Ageratum enation virus - Lucknow segment DNA-A
GQ268327.1:87-514 Ageratum enation virus isolate Gorakhpur
KY089033.1:87-504 Ageratum enation virus isolate Jaipur
EU867513.1:87-504 Ageratum enation virus - Lucknow segment A complete sequence
JX436472.1:92-519 Ageratum enation virus isolate TC357
AF240675.1:88-515 Tobacco curly shoot virus-Y1 DNA A
FN543099.1:92-519 Ageratum enation virus
KM262822.1:92-519 Ageratum enation virus isolate NBRI-2
KM262823.1:92-519 Ageratum enation virus isolate NBRI-3
KM066975.1:92-519 Ageratum enation virus isolate NBRI-1
KU934096.1:88-515 Tobacco curly shoot virus isolate YN4524
KU934094.1:88-515 Tobacco curly shoot virus isolate YN4501
KU934097.1:88-515 Tobacco curly shoot virus isolate YN4530
KU934095.1:88-515 Tobacco curly shoot virus isolate YN4519
GU199585.1:95-512 Tobacco curly shoot virus clone 18-2 AV2 protein (AV2) gene complete cds
GU199584.1:88-515 Tobacco curly shoot virus clone 18-6
KU198364.1:89-516 Tobacco curly shoot virus-Y3560 isolate Y3560
AJ512771.1:73-487 tobacco curly shoot virus AV2 gene and partial AV1 gene for coat protein isolate Y132
AJ512763.1:95-512 tobacco curly shoot virus AV2 gene and partial AV1 gene for coat protein isolate Y93
KU934098.1:87-514 Tobacco curly shoot virus isolate YN3343
KU663148.1:97-514 Tobacco curly shoot virus isolate YN3343 AV2 protein (AV2) gene complete cds and AV1 protein (AV1)
AJ512768.1:94-511 tobacco curly shoot virus AV2 gene and partial AV1 gene for coat protein isolate
AF240676.1:87-514 Tobacco geminivirus isolate Y2 subgenomic DNA sequence
KM383753.1:89-516 Tobacco curly shoot virus isolate TbCSV-CNBD:Chi:02:14:Tom:06
KM383752.1:88-515 Tobacco curly shoot virus isolate TbCSV-CNBD:Chi:01:11:Tom:06
HF936708.1 Ageratum enation virus partial AV1 gene for coat protein isolate west bengal
JQ733557.1:88-515 Tobacco curly shoot virus isolate FB-01 segment DNA-A
MG721014.1:97-524 Tobacco curly shoot virus isolate ISL-03 pre-coat protein (AV2) gene complete cds
0.0050

Fig. 3G

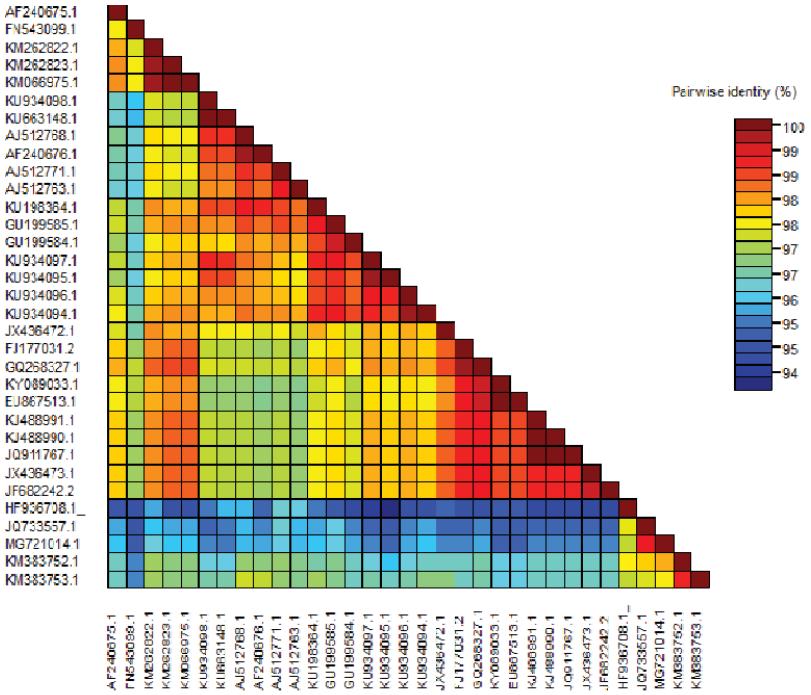
Pairwise identity (%)
100
99
99
98
98
97
97
96
95
95
94

Fig. 3H

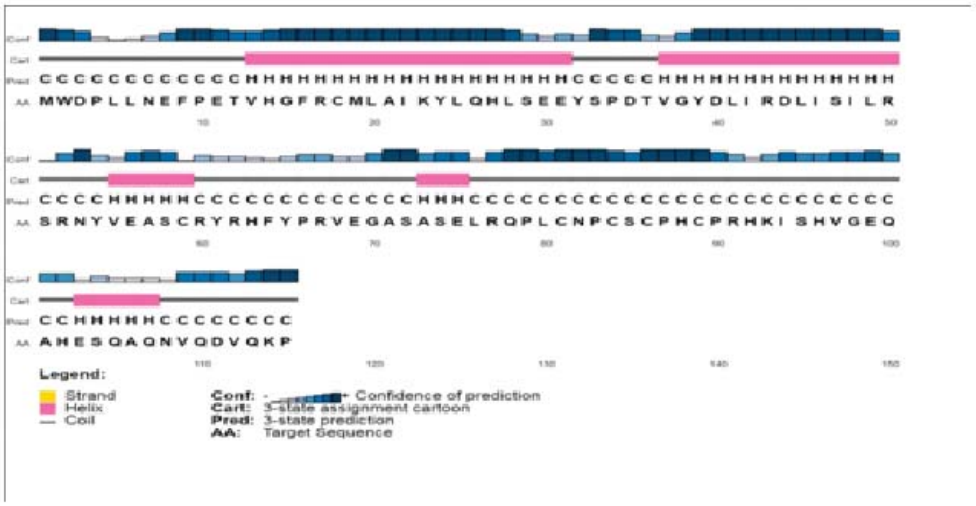

Fig.4 (A) : Secondary structure of AV2 protein and its predicted helical domains.

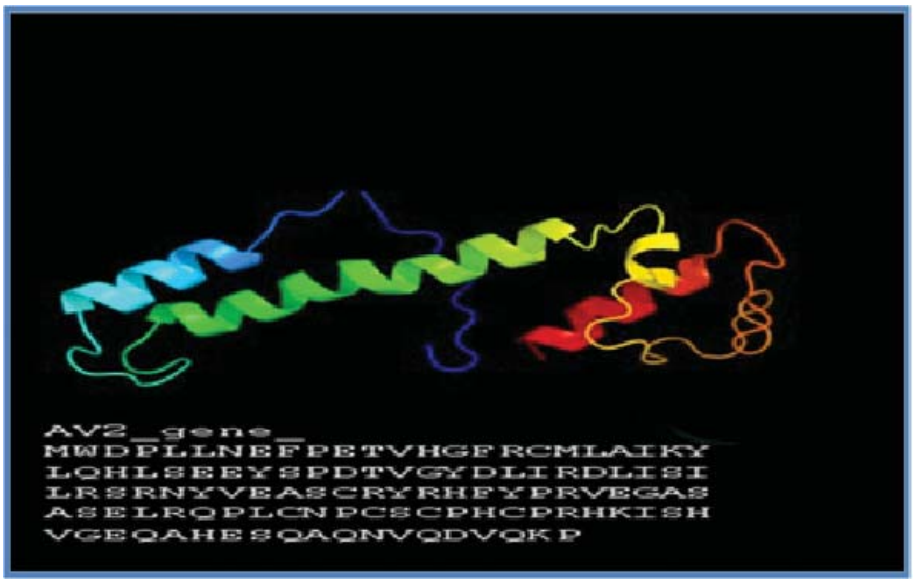

Fig.4 (B) : Amino Acid sequence of AV2 full CDS and its predicted 3D protein structure.

by the Neighbor-joining method (Saitou, N. and Nei, M., 1987) and showed occurrence of two major clusters in the phylogenetic tree (Fig.3B). All the Jatropha infecting begomovirus falls within one large cluster and the partial gene sequence (MN006988) under study was found within same cluster. Similarly, phylogenetic analysis in case of the nucleotide sequence from the sample *Croton sparsiflorus* showed that all the begomovirus infecting Euphorbiaceae family belongs to one major cluster and other weeds infecting begomovirus belongs to another major cluster (Fig.3D). The partial gene sequence (MN006989) under study deviated from the sister clade of Jatropha leaf yellow mosaic Katarniaghat virus segment DNA-A (NC_038956.1) and Jatropha leaf yellow mosaic Katarniaghat virus isolate Katerniaghat 2 segment DNA-A (NC_038956.1) but falls under the same cluster group. Phylogenetic analysis of the nucleotide sequence from the sample *Sida rhombifolia* showed that there are two major clusters within the tree (Fig.3F). The nucleotide sequence (MK986831) under study falls within the sister cluster of Cotton leaf curl Bangalore virus isolate WOK48 (KT390464.1) and Kenaf leaf curl virus [India: Bhangha: 2007] (EU822322.1), thus deviating from the cluster of weed infecting begomovirus. Phylogenetic analysis from the sample *Ageratum conyzoides* (HF936708) showed that there are two major clusters and the begomovirus isolate under study falls within the sister clade of Tobacco Curly Shoot Virus isolates (Fig.3H).

Amino acid and structure prediction

The Amino acid sequence of the AV2 protein (Precoat protein) of the isolate from *Sida rhombifolia* was annotated with the published amino acid sequences of AV2 protein, using the software (Phyre v.2.0) (http://www.sbg.bio.ic.ac.uk/phyre2/). The result showed maximum hits with other geminivirus AV2 protein, with coverage of 116 Amino acid sequences of AV2 protein by using software (HMMR Biosequence Analysis using profile hidden Markov Models). The analysis predicted maximum identity with other AV2 proteins and similarities. The present findings on the Geminivirus AV2 protein gave a detailed insight of the AV2 protein structural characteristics with the Cotton Leaf Curl Mohanpur isolate infecting *Sida rhombifolia,* showing five helices and rest partly coiled.(Fig.4 A,B).

Jatropha leaf yellow mosaic virus is commonly associated with *Jatropha curcas* (Srivastava, *et al.*,2015) but there has also been a report of Jatropha mosaic India virus isolates Katarniaghat segment DNA-A (JN135236) in association with *Acalypha indica* in North Bengal (Saha *et al.,* 2013). There is also previous evidence of Croton yellow vein mosaic virus (CYVMV) in association with *Croton bonplandianum* and other species (Pramesh *et al.,* 2013). Report of the association of *Sida* infecting begomovirus with *Sida rhombifolia* (Tavares *et al*., 2012), and Goat weed (*Ageratum conyzoides*) which is known to harbor several begomoviruses such as Ageratum enation virus and Ageratum leaf curl virus (Huang and Zhou, 2006) has already been reported in various part of the world. Henceforth the present investigation truly supports the relationship of Tobacco curly shoot virus isolate with the Goat weed (*Ageratum conyzoides*) (Asteraceae) and Jatropha leaf yellow mosaic virus isolate (MN006988, MN006989) with the weeds *Acalypha indica* and *Croton sparsiflorus* which belongs to Euphorbiaceae family, thus confirming their common association under this family. Again Cotton leaf curl is devastating disease worldwide infecting cotton plants which belongs to the Malvaceae family, thus confirming positive association of these begomoviruses with other plants under the same family.

However, it is concluded these weed species are abundantly grown in the crop fields and other areas. *Acalypha indica* and *Croton sparsiflorus* harboring Jatropha leaf yellow mosaic Mohanpur isolates (MN006988) and *Jatropha leaf yellow mosaic Mohanpur isolate* (MN006989) respectively, and *Sida rhombifolia ,Ageratum conyzoides* harbouring Cotton leaf curl Mohanpur isolate (MK986831) and Tobacco Curly Shoot Virus isolates (HF936708) respectively, can play an important role as alternate host and unique source of overwintering begomoviruses. The present result reflected that *Sida rhombifolia* under the Malvaceae family could play an important source of Cotton Leaf curl virus for further spread and recombination as well. Hence more detailed analysis is required to confirm their role in association and recombination.

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