Molecular characterization of Cotton leaf curl Multan virus and DNA-satellites complex associated enation leaf curl and yellow vein mosaic disease of hollyhock

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Abstract

Hollyhock is one important decorative plant grown in garden beds in different region of the the world. The ornamental plant is susceptible to many diseases caused by diverse pathogens. Among these viral pathogens can cause enormous damage to the ornamental plant. The aim of the present study was identification of begomovirus and DNA sateelites is associated with the yellow vein mosaic and enation leaf curl disease complex of hollyhock. The hollyhock plants showing the typical begomovirus-like symptoms were collected from Pusa campus, New Delhi (India). To know the status of the begomovirus, the total DNA isolated from the infected hollyhock was subjected to PCR amplification using primers specific to the begomovirus. The partial (1.2 kb) genome sequencing of ten hollyhock samples indicates the associated of begomovirus (nucleotide identities is more 95% among themselves). Therefore three representative samples (H1, H2, H3) full-length genome (DNA-A, betasatellite and alphasatellite) was amplified through RCA method. The pairwise comparision of complete genome of the begomoviruses, betasatellites and alphasatellites using Sequence Demarcation Tool (SDT) showed highest nucleotide (nt) identity of 88.0 to 92.7% (DNA-A) with *Cotton leaf curl Multan virus*, 92.5–96.7% with *Ludwigia leaf distortion betasatellite* and 90.4 to 93. 2% % with *Ageratum enation alphasatellite*. Further recombinantion analysis showed that the begomoviruses and DNA satellites under study was recombinants of previously reported begomoviruses and DNA sattelites. This is the first report of *Cotton leaf curl Multan virus* and DNA satellites associated complex disease of hollyhock in India.

Introduction

The family *Geminiviridae* is divided into nine generas based on genome strcture, replication, vector transmission and host range (Zerbini et al. 2017), of these the begomoviruses are major group of plant viruses affecting the different crops in worldwide and are transmitted by complex morphological indistinguishable cryptic species of whiteflies (De Barro *et al*., 2011) in a persistent circulative and non-propagative manner. Further, based on the genome structure of the begomoviruses, were further classified into into Old and New world, where most of begomoviruses are belongs to the old world (OW) are monopartite with few bipartite viruses, while all New world (NW) begomoviruses are bipartite. The OW begomoviruses are more diverse as compared new world as it contain AV2 gene in OW, but it was absent in NW begomoviruses (Varsani *et al*., 2014). The genomes of the OW begomoviruses are single component (monopartite) or two-component (bipartite). The bipartite begomoviruses are encoding seven and eight proteins in DNA A and DNA B component, whereas monopartite begomoviruses are encode five or six proteins where all genes are present on the DNA-A component, while in bipartite begomoviruses CP and Rep gene are present on DNA-A component and gene for movement viral particle (MP and NSP) are present on DNA-B component (Nawaz-ul-Rehman and Fauquet, 2009).

Most of the OW begomoviruses, additionally a classes of circular ssDNA satellites (betasatellites and alphasatellites) are associated in their genome, whereas NW and few OW begomoviruses are associated deltasastellites (Zhou, 2013; Lozano *et al*., 2016). The betasatellites (ssDNA) are not a true satellites and play major role in symptoms modulation in their original hosts (Venkataravanappa *et al*., 2011).Where as

alphasatellites are ture satellites and play no role in symptom induction. Both the DNA satellites are depend on the helper virus for movement, replication and encapsidation (Briddon *et al*., 2006). Deltasatellites do not encode any proteins and in some cases to reduce begomovirus accumulation and symptom severity. These are still depend on helper begomovirus for replication and movement (Lozano *et al*., 2016).

Hollyhock (*Alcea rosea* L., family Malvaceae) is one important ornamental plant grown in garden beds in through out the world, including India. The ornamental hollyhock plant is originated from Asia and Europe, usually having very broad leaves, grown to increase the aesthetic value of the garden beds. Apart from its aesthetic values, the flowers are used for treatments of chest complaints and decoction is used to improve blood circulation (Sharma et al. 2011). The leaves and flowers are used for preventing and treating breathing disorders and digestive tract problems. Despite it many uses, the ornamental plant is a natural host for many viruses (Singh and Misra, 1971; Abdel-Salam et al. 1998, Bigarre et al. 2001; Choi et al., 2003; Menzel et al. 2010; Srivastava et al. 2014; Aswathappa et al 2020; Kumar et al. 2020) are causes severe damage to hollyhock. Therefore the current study was attempted to characterize begomovirus is associated with the yellow vein mosaic and enation leaf curl disease of hollyhock in India.

Materials And Methods

Virus source plants and vector transmission

Between 2018 and 2019, the hollyhock plants exhibiting symptoms of yellow vein mosaic, enation leaf curl, and vein netting were collected from Pusa campus, New Delhi, India (**Fig 1**). two asyptomatic samples was also collected in the same location. During collection it was also observed presence of a huge number of whiteflies an undersurface of the leaves indicates the infection of begomovirus. A part of samples used for transmission of disease by whiteflies (*Bemisia tabaci*), which are reared on cotton plants under controlled conditions similar to as the transmission described by Venkataravanappa et al (2017). The adult whiteflies were allowed to feed on the infected hollyhock plants for an acquisition access period of 24 h. After that, the whiteflies (ten number on each) were transferred to ten days old seedlings of hollyhock (ten) for an inoculation access period of 24h. After that insecticide (0.05% imidocloprid) were sprayed on the inoculated plants and maintained in an insect-proof net for symptom development. As there is a probability that natural infected plants may have contaminated by multiple viruses, the artificial transmission was repeated to check whether typical symptoms were expressed remain same or different. After repeated transmission, the symptom developemt remain same, its indicates absence of multiple infection in material. Plant tissues showing the symptoms and non-symptomatic tissues were used for analysis. The begomovirus was successfully transmitted from naturally infected to healthy hollyhock plants (10/10 plants), which developed similar symptoms at 20-25 days post-inoculation (dpi).

DNA extraction, Polymerase chain reaction, and sequence analysis

To asses the stauts of suspected begomovirus in the infected hollyhock plants, Total genomic DNA was extarcted from ten infected and two non-infected hollyhock leaf samples by using CTAB method (Doyle and Doyle 1990). The infection of begomovirus in hollyhock was screened through PCR assay using the begomovirus genome (DNA-A and DNA-B component) specific primers (Venkataravanappa et al. 2012, Rajos et al. 1993). Based on the partial genome sequencing (1.2kb fragment) of ten hollyhock samples (sample No. H1 to H10) indicates, these samples are associated with monopartite DNA virus species, closely related to *Cotton leaf curl Multan virus* (Nucleotide identity more than 94%). Therefore three plant samples (H1, H2, H3), which are showing distinct symptoms were used for complete genome amplification using rolling circle DNA amplification method (Venkataravanappa et al. 2016). Further to know the subgenomic components such as betasatellite and alphasatellite associated with infected hollyhock plants. Total gDNA isolated from the hollyhock plants was subjected to PCR assay using universal primer pair specific to betasatellite (Briddon et al. 2002) and alphasatellite (Kumar et al. 2010) as described earlier for sub-genomic components amplification in begomovirus infected crops so far. The RCA/PCR amplified products were cloned into pTZ57R ⁄T vector (Thermo Fisher Scientific) and the confirmed clones were sequenced in both orientations.

Analysis of viral genome sequences and recombination breakpoint events

The sequence similarity of the viral geneome and DNA satellites was checked at NCBI (www.ncbi.nlm .nih.gov) using Blastn. The begomoviruses (**Table S1**) and betasatellites (**Table S2**) and alphasatellites (**Table S3**) are showed maximum blast score were retrieved from the GenBank. The pair wise identity score between hollyhock infecting begomviruses and selected begomviruses was calculated by using SDT version 1.2 (Muhire et al. 2014). MEGA 7 software (Kumar et al. 2018) was used to draw the phylogeny using the maximum likelihood method with 1,000 bootstrapped replications by applying Kimura 2-parameter model test. Recombination in the viral geneome and DNA satellites associated with hollyhock was carried out using RDP4 (Martin et al. 2015) with default RDP settings.

Results

Genome organization of virus infecting hollyhock

The sequence data (DNA-A and DNA satellites) that were obtained from the three hollyhock isolates (H1, H2, H3) were assembled using different bioinformatics programs (Sea View, Bioedit and Clustal X2) and deposited in GenBank under accession number MN127817, MN127818, MN127819. The complete viral genomes of three hollyhock isolates (H1, H2, H3) were determined to be 2738 to 2750 nts long and genomic organization is analogous to other monopartite begomovirus from Old Word (OW) with potentially encoding five conserved ORFs: two ORFs: (V2 and V1), in plus strand and four ORFs (C1, C2 C3 C4) are present in minus strand of DNA component of the DNA virus. The plus strand and minus strand of DNA A were separated by an common region (CR), which contained highly conserved nonanucleotide (TAATATTAC), sequence nicked by the rolling-circle initiator protein (Rep protein) to initiate replication. The Rep protein (encoded by C1) has all conserved domains similar other

begomviruses as described by Vadivukarasi et al (2006), except the GRS motif (RFFDLISPTRSAHFHPNIQRAKS) in hollyhock infecting DNA virus is different.

The pairwise comparison complete genome of three begomovirus isolates (H1, H2, H3) infecting hollyhock with other slected begomoviruses retrieved from the NCBI database using Sequence Demarcation Tool (SDT) (Muhire et al. 2014) (Table S). The analysis showed that the DNA-A sequences of three hollyhock isolates (H1, H2, H3) showed the highest nt identity of 88.0 to 92.7 % to CLCuMuV (JN678803) infecting cotton in India. The three hollyhock isolates H1, H2, H3 were also shared < 88 % nt identity with several other begomoviruses infecting different crops in India. This result also was supported by pairwise identity scores calculated between hollyhock isolates and other begomoviruses available NCBI database by using the Sequence Demarcation Tool (SDT) (Fig. 2b). Based on genome sequence comparisons and guidelines of ICTV Study group (Adams et al. 2017), the three hollyhock isolates (H1, H2, H3) are the distinct strain of *Cotton leaf curl Multan virus (CLCuMuV) for which* the additional descriptor [India: New Delhi: Hollyhock: 2018] is proposed.

Phylogenetic analysis was carriedout by comparing complete genome of three isolates (H1, H2, H3) obtained in the present study with 29 known begomoviruses infecting different crops available in NCBI database, and this showed that CLCuMuV -[IN:ND:Hol:18] is present in the same clade with CLCuMuV (JN678803) infecting cotton in India (Fig. 2a). Similarly, ORFs V1 and C2 of CLCuMuVinfecting hollyhock is shared the same clade with CLCuMuV (JN678803), ORF C1 shared the same clade with CLCuBaV (AY705380) and CLCuKoV (HF549182) and ORF C4 shared with CLCuKoV (HF549182) clade (data not shown). However, ORFs V1 shared a clade with BYVMV (AF241479) (data not shown). The analyses clearly showed that genome of CLCuMuV-[IN: ND: Hol: 18] associated hollyhock is probably arose as a result of recombination with other cotton infecting begomoviruses.

Attempts were made to detect DNA B component from infected hollyhock plants using primers specific DNA component as decribed ealier (Venkataravanappa et al. 2012, Rajos et al. 1993**)** resulted in no amplification was observed.

Genome organization of betasatellite

Most of the begomoviruses from OW are commonly associated with DNA satellites molecule, therefore PCR was performed with primers specific of DNA satellites (Briddon et al. 2002; Kumar et al. 2010). The resulted PCR amplicon of 1.2 to 1.3 kb in size products was amplified from the three hollyhock samples, indicating the association of DNA satellites with infected hollyhock plants.

The 1.3-kb size fragment of betasatellites amplified from the three hollyhock samples (H1β, H2β, and H3β) was cloned and sequenced. The length of complete genome sequence of three betasatellites (H1β, H2β, H3β) was determined to be ranged from 1363 to 1371bp in length and are submitted to NCBI database (under accession number MN127820, MN127821, MN127822) respectively. The sequences of three hollyhock samples (H1β, H2β, and H3β) have typical features of other betasatellites reported so far in different crops (Briddon et al. 2002; Venkataravanappa et al. 2011). The betasatellites characterized in

the part of the study is belong to the family *Tolecusatellitidae* and showed a maximum percent nt identity of 88.1 to 93.9% among themselves and 92.5-96.7% with *Ludwigia leaf distortion betasatellite* (LuLDB) isolates originating from the Indian subcontinents infecting okra and hibiscus (**Fig 3b**). Based proposed species demarcation threshold of 91% for betasatellites (Adams et al. 2017), The betasatellites identified in three hollyhock samples (H1β, H2β, and H3β) is an isolate of LuLDB. This result was also supportrd by Sequence Demacration tool, the three betasatellites isolated from hollyhock plants are close related to LuLDB.

A phylogenetic analysis of complete genome sequences of three betasatellites (H1β, H2β, H3β) isolated from hollyhock with selected betasatellites revaled that three betasatellites (H1β, H2β, H3β) are closely cluster with previous isolates of LuLDB originating from the Indian subcontinents infecting okra and hibiscus for which a full-length sequence is available in the databases (**Fig 3a**.)

Genome organization of alphasatellite associated with the disease

For detection of alphasatellites, total gDNA of infected hollyhock plant was amplified by PCR using universal primers specific for alphasatellites (Kumar et al. 2010**)**. The resulted PCR amplicon of 1.2 kb in size product was amplified in three samples were sequenced. The sequence analysis of three alphasatellites (H1D1, H2D1, H3D1) isolated from hollyhock was determined to be1364 to 1371 bp in length and submitted to NCBI database (under accession number MN127823, MN127824, MN127825). The alphasatellites sequences have similar characterstic other alphasatellites reported so far in many crops (Briddon et al. 2004), containing a single large ORF encoding a replication associated protein (Rep) in sense orienation (Briddon et al. 2004). The two alphasatellites (H1D1, H2D1) shared maximum nt identity of 90.4 to 93.2% with *Ageratum enation alphasatellite* (AEA) (HG518790, FR772085, HE599396) infecting cotton, okra and hollyhock (Fig 4b). Based on proposed species demarcation threshold for alphasatellite (Briddon et al. 2018), the alphasatellite identified here is an isolate of AEA infecting okra, hollyhock and cotton are belongs the family *Alphasatellitidae*, subfamily *Geminialphasatellitinae* and genus *Colecusatellite*. This results was also supported phylogeny analysis showed that two alphasatellites (H1D1, H2D1) isolated from hollyhock are more homology with several isolates AEA (HG518790 and FR772085) infecting okra and hollyhock, where as the alphasatellite (H3D1) is closely cluster with AEA (HE599396) infecting cotton respectively. This result was also supportrd by Sequence Demacration tool, the three alphasatellite isolated from hollyhock plants are close related to AEA (Fig 4a)

Recombination

The contrary of nt identity and phylogenetic relationship of CLCuMuV-[IN: ND: Hol: 18] associated hollyhock understudy with *Cotton leaf curl Multan virus* (CLCuMuV), *Cotton leaf curl Kokhran virus* (CLCuKoV), *Cotton leaf curl Alabad virus* (CLCuAlV), *Cotton leaf curl Bangalore virus* (CLCuBaV), *Papaya leaf curl virus* (PaLCuV), *Tomato leaf curl Bangalore virus* (ToLCBaV), *Tobacco curly shoot virus* (TbCSV) and *Bhendi yellow vein mosaic virus* (BYVMV) isolates indicated the possibility of recombination in its genomes with above viruses. Therefore, further analysis was carried out with the RDP4 with default settings, to identify recombination break points (Martin et al. 2015). RDP analysis showed that the

evidence of intra and inter specific recombination in CLCuMuV isolates (H1, H2, H3) with most of the coding and non-coding regions of DNA-A is derived from different begomoviruses includes CLCuMuV, CLCuKoV, CLCuAlV, CLCuBaV, PaLCuV, ToLCBaV, TbCSV and BYVMV as major and minor parents (**Fig 2b, Table S 2**). Similar RDP analysis was done for betasatellites (H1β, H2β, H3β) revealed evidence of intra and inter-specific recombination, suggestive that most of betasatellites DNA descended from LuLDB infecting okra and hibiscus crops (**Fig 3b, Table S 2**). Further, RDP analysis for alphasatellites (H1D1, H2D1, H3D1) indicates, exchange of gene fragment from *Sunflower leaf curl Karnataka alphasatellite* (SLCuKaA), *Ageratum yellow vein India alphasatellite* (AYVIA) and *Ageratum enation alphasatellite* (AEA) as donor parents infecting okra, hollyhock and cotton (**Fig 4b, Table S 2**).

Discussion

Hollyhock (*Alcea rosea* Linn.) is an important winter ornamental plant grown in different garden beds to incease the asthetic value in many places through out India. The plant is not only ornamental but it also a good source of medicines. In the present study hollyhock plants (ten samples) showing the yellow vein mosaic, enation leaf curl and vein netting symptoms are confirmed with PCR diagnostic and viral genome sequencing, indcaites the disease was associated with *CLCuMaV* infecting cotton in the Indian subcontinent. *CLCuMaV* was first identifed in 1967 in the Multan district of Pakistan, on scattered cottin (*Gossypium hirsutum)* plants (Hussain, 1975, Hussain and Mahmood, 1998, Thakur, 2002), form their it has spread rapidly other cotton-growing areas of Pakistan and Indian subcontinent. The virus (*CLCuMaV*) not only infecting the cotton, it will also spread to the other horticutlural crops (Mansoor et al. 2000; Mansoor et al. 2003; Hussain et al. 2004). All begomviruses associated with cotton leaf curl disease were identified from India are infected with cotton, okra, and tomato. The infection of cotton viruses both malvaceous and non-malvaceous hosts may be high inoculum load of cotton viruses and their strains and morphological indistinguishable whitefly cryptic species. Hollyhock is malvaceous plant, which is commonly grown ornamental plant in the garden as well as pots in front of houses. Due to existing of morphological indistinguishable whitefly cryptic species complex in India, the virus may be spreading the cotton viruses into hollyhock. Delhi is near the state of Punjab a place heavily cotton-growing area is likely whitefly vector, carrying the virus and transmits to hollyhock. Similarly the litratured also showed that the diverse viruses are infecting ornamental plants in worldwide (He et al. 2009; Ilyas et al. 2013), but information regarding begomovirus infections in ornamental plants was very scanty (Marwal et al. 2013b). This may be due to the study of the ornamental crops viruses are neglected or are not taken into consideration while carrying out surveys and begomovirus studies.

The betasatellites and alphastellies are found associated with OW mono and bipartite begomoviruses in many crops (Briddon and Stanley 2006, Rouhibakhsh and Malathi 2005; Venkataravanappa et al. 2019a, b). In the present the hollyhock samples are associated with Ludwigia leaf distortion betasatellite, which is also identified in begomviruses infecting different crops in India (Das et al. 2008; Roy et al. 2009; Srivastava et al. 2014). Phylogenetic analysis showed that betasatellite associated hollyhock is close clustering of the Ludwigia leaf distortion betasatellite infecting begomviruses in solanaceous crops. This clearly indicates the possible spread of the betasatellite across geographic regions among them. The

study also show that betasatellites does not contain iteron sequences, therefore still it depend helper virus for its replication (Kon et al. 2007; Saunders et al. 2000; Briddon et al. 2001).

The alphasatellites (H1D1, H2D1, H3D1) associated with hollyhock were identified in present study were closely related to the AEA infecting okra and hollyhock (Serfraz et al. 2015) and cotton respectively (Siddiqui et al. 2016). Which are similar feature of other alphastellites identified in many crops (Briddon et al. 2004; Venkataravanappa et al. 2019a, b). The alphasatellites are somewhat larger than betasatellites play major role in attenuated the disease symptoms and are involved in the maintenance of low level of betasatellite accumulation in the plant (Wu and Zhou 2005). It is also showed that suppress RNAi pathway in begomovirus disease complexes (Nawaz-ul-Rehman et al. 2010).

Recombination is one of the key factor for evolution and creating genetic diversity in begomoviruses (Prasanna at al. 2010; Venkataravanappa et al. 2014). The results showed that recombination has similarly led to the formation of a distinct strain of CLCuMuV and its satellites occurring in India. The recombination analysis suggested that CLCuMuV isolates have obtained at least some of its sequences from the previously reported begomoviruses, betsatellites and alphasatelites from Indian subcontinents infecting different crops. The overall the results of the recombination and phylogenetic analysis suggested that CLCuMuV and its satellites are evolved from different monopartite begomoviruses (CLCuMuV, CLCuKoV, CLCuAlV, CLCuBaV, PaLCuV, ToLCBaV, TbCSV, BYVMV), betastellites (LuLDB), and alphasatellies(SLCuKaA, AYVIA and AEA) reported previously in India on different crops.

The present study clear indicating that the cotton leaf curl Multan virus is expanding its host range by infecting hollyhock to other malvaceous hosts is a serious threat for cultivation of many ornamental and horticulture crop plants. There is a need more comprehensive survey to identify possible spread the begomovirus infections in the country to assess their losses in crop plants. This will form the basis of our future investigations.

Declarations

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Figures

Figure 1

Holly hock plants showing (a) mild yellow mosaic (b) complete yellow vein mosaic, (b) leaf curl symptoms under natural conditions

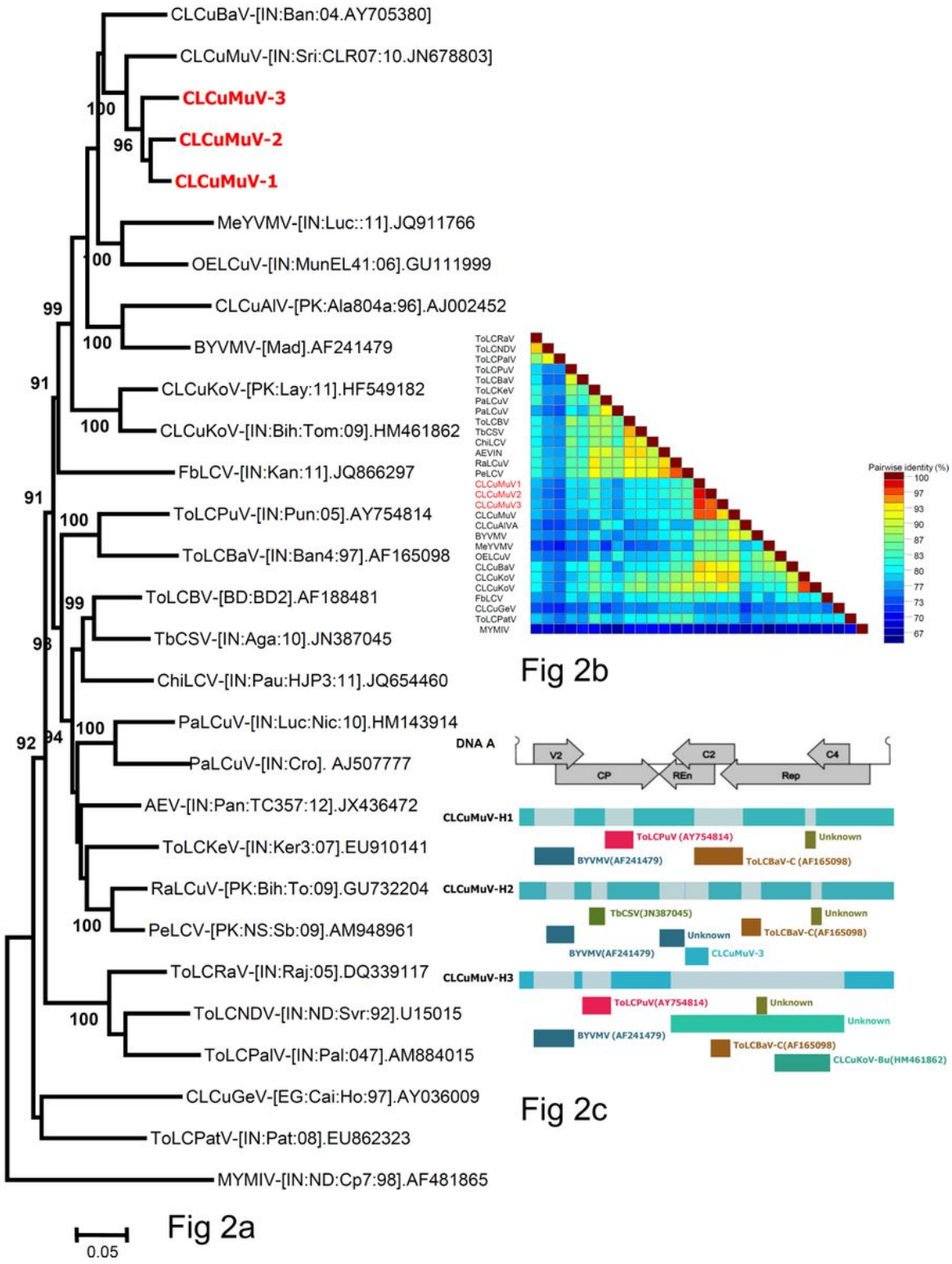

Figure 2

Phylogenetic tree showing relationships of the begomovirus (CLCuMuV) under study (MN127823, MN127824, MN127825) (a) and selected begomoviruses. The phylogenetic trees were constructed employing the MEGA7 tool, using the neighbor-joining method with 1000 bootstrap replicates. The two dimensional color-coded matrix of pairwise identity scores of the begomovirus (b) under study were obtained using Species Demarcation Tool. Pictorial depiction of the genomic map, putative

recombination events identified by RDP analysis (c) of the CLCuMuV isolates under study (MN127823, MN127824, MN127825). A genomic map of CLCuMuV is presented, showing the arrangement of genes along with their coding direction and a nucleotide scale (1 to 2750). The Begomoviruses acronyms given are Bhendi yellow vein mosaic virus (BYVMV), Tomato leaf curl Pune virus (ToLCPuV), Tomato leaf curl Bangalore virus – C (ToLCBaV-C). Tobacco curly shoot virus (TbCSV), Cotton leaf curl Multan virus (CLCuMuV), Cotton leaf curl Kokhran virus – Burewala (CLCuKoV-Bu). Sequence of indeterminate origin is indicated as “unknown”. The box below at the top of the diagram indicates the approximate position recombination is occurring in the genome of the begomovirus. The accessions and their details used for this study are listed in Supplementary Table 1

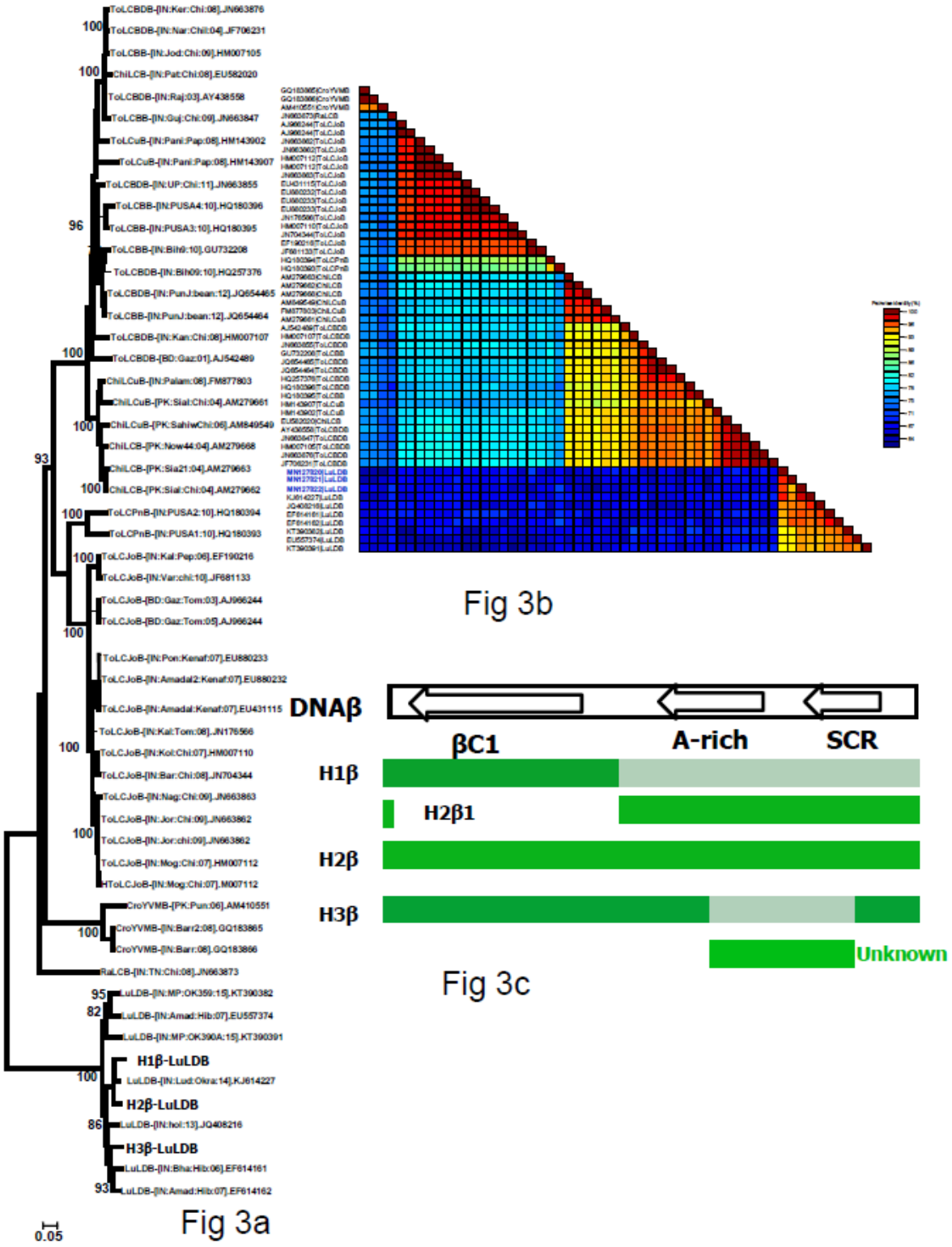

Figure 3

Phylogenetic tree showing relationships of betasatellites (MN127820, MN127821, MN127822) (a) associated with yellow vein mosaic and enation leaf disease hollyhock and selected satellites. The phylogenetic trees were constructed employing the MEGA7 tool, using the neighbor-joining method with 1000 bootstrap replicates. The two dimensional color-coded matrix of pairwise identity scores of the betasatellite (b) molecule under study were obtained using Species Demarcation Tool. Pictorial depiction

genomic map of betasallites under study and their putative recombination events were identified by RDP analysis (c). A genomic map of betasatellite is presented, showing the arrangement of gene along with their coding direction and a nucleotide scale (1 to 1359). Sequence of indeterminate origin is indicated as “unknown”. The accessions and their details used for this study are listed in Supplementary Table 1

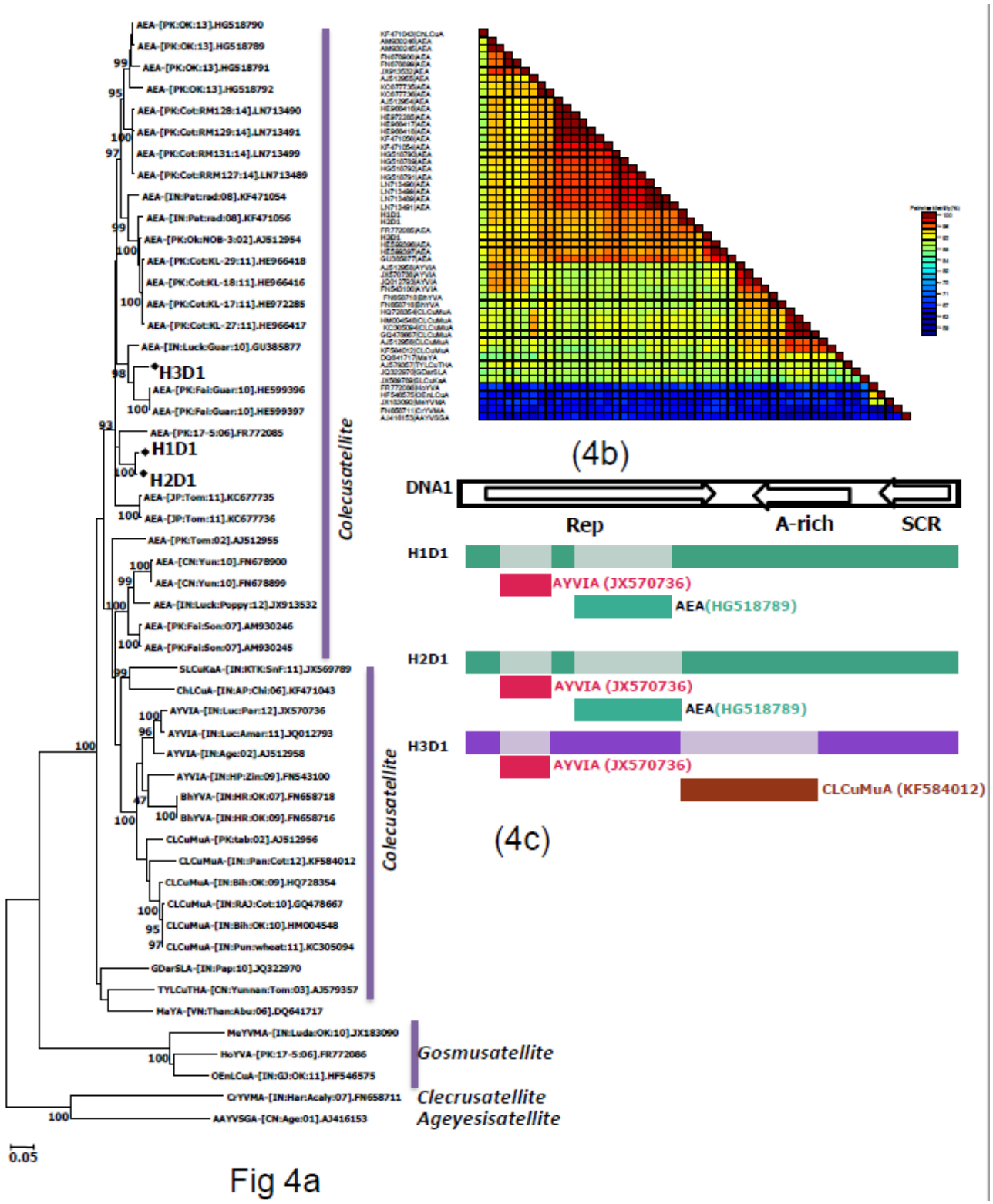

Fig 4a

Figure 4

Phylogenetic tree showing relationships of alphasatellites (MN127823, MN127824, MN127825) (a) associated with yellow vein mosaic and enation leaf disease hollyhock and selected satellites. The phylogenetic trees were constructed employing the MEGA7 tool, using the neighbor-joining method with 1000 bootstrap replicates. The two dimensional color-coded matrix of pairwise identity scores of the betasatellite (b) molecule under study were obtained using Species Demarcation Tool. Pictorial depiction genomic map of alphasallites under study and their putative recombination events were identified by RDP analysis (c). A genomic map of alphasatellite is presented, showing the arrangement of gene along with their coding direction and a nucleotide scale (1 to 1371). The alphasatellite acronyms given are Ageratum yellow vein India alphasatellite (AYVIA) and Ageratum enation alphasatellite (AEA), Cotton leaf curl Multan alphasatellite (CLCuMuA). The accessions and their details used for this study are listed in Supplementary Table 1

Supplementary Files

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- SupplementaryTable13.doc
- SupplementaryTable4.docx