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ORIGINAL ARTICLE

Molecular characterization of novel bipartite begomovirus associated with enation leaf disease of Garden croton (*Codiaeum variegatum* L.)

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Abstract Garden croton (*Codiaeum variegatum* L.) plants showing typical begomovirus symptoms of vein twisting, enation and curling were collected from different gardens at Varanasi, Uttar Pradesh state of India ranged from 20 to 30%. All the 10 ten (CR1-CR10) infected samples of garden croton resulted in expected amplicon of 1.2 Kb in PCR specific to begomoviruses. No amplification was obtained for betasatellite and alphasatellite specific primers. The complete genome sequence of DNA-A and DNA-B for two isolates (CR1 and CR2) was obtained through rolling cycle amplification (RCA) and comparisons were made with other begomoviruses using Sequence Demarcation Tool (SDT) which revealed that, DNA-A of two isolates, CR1 (Acc.No.: MW816855) and CR2 (Acc.No.: MW816856) showed maximum nucleotide (nt) identity of 85.7–85.9% with *Tomato leaf curl Karnataka virus,* which is below the threshold percentage of begomovirus species demarcation, hence considered as novel begomovirus and proposed the name *Garden croton enation leaf curl virus* (CroELCuV) [IN: Varanasi: Croton: 18]. Further, DNA-B these isolates shared maximum nt identity of 91.0–92.2% (DNA-B) with *Tomato leaf curl New Delhi virus*. Recombination and GC plot analysis showed that the recombination occured at in low GC content regions of DNA-A and DNA-B of the CroELCuV and are derived from the previously reported several begomoviruses. This is the first record of novel bipartite begomovirus associated with vein twisting, enation and leaf curling of disease of garden garden croton in India and world.

Keywords Garden croton · Begomovirus · Nucleotide sequence analysis · Recombination · *Tomato leaf curl Pune virus* (ToLCPuV) · *Cotton leaf curl Bangalore virus* (CLCuBaV) · *Chilli leaf curl virus* (ChiLCV) · *Papaya leaf curl virus* (PaLCuV) · *French bean leaf curl virus* (FbLCV) · *Cotton leaf curl Alabad virus* (CLCuAlV) · *Okra enation leaf curl virus* (OELCuV) · *Tomato leaf curl Kerala virus* (ToLCKeV)

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Introduction

Garden croton (*Codiaeum variegatum* L.) is an important plant grown ubiquitously indoor and outdoor for ornamental purpose across the world. It belongs to the family, *Euphorbiaceae*, which is a largest family of flowering plants comprising of about 500 genera and 5000 species [36]. Many species are used in the treatment of asthma,

leukemia, and other forms of cancer and also used as a laxative and diuretic across the globe [37]. Numerous species of ornamental garden croton are well-known for their coloured foliage in various combinations. Ornamental crotons are evergreen perennials, shrubs, and small trees, thus they improve the aesthetic value of gardens throughout the year. Decoction and extracts made from the root and leaves of garden croton are used to treat gastric ulcers and also taken as tea by Filipinos [12] and cure the various diseases [9, 18]. These are also known to produce valuable secondary metabolites [22, 27, 32]. Sap of the leaves mixed with coconut milk is used for control of syphilis. Apart from its many medicinal, pharmaceutical and ornamental uses, the plant is also a natural host for begomovirus [33].

The family, Geminiviridae has been divided into fourteen genera, *Begomovirus, Capulavirus, Citlodavirus, Curtovirus, Eragrovirus, Grablovirus, Maldovirus, Mastrevirus, Mulcrilevirus, Opunvirus, Topilevirus, Topocuvirus,* and *Turncurtovirus,* which includes more than 525 species [35, 40, 44, 46]. Of these, viruses belonging to the genus, *Begomovirus* are most economically important and threatening cultivation in many crops across the world. Based on the presence of one and two genomic components, they were divided into monopartite (single genome component known as homologue of the DNA-A bipartite begomoviruses) and bipartite (DNA-A and DNA-B) begomoviruse, respectively. DNA-A virion-sense strand encodes two open reading frames (ORFs) (AV1 and AV2) and complementary-sense strand encodes four (AC1, AC2, AC3 and AC4) and five ORFs (AC1, AC2, AC3 AC4 and AC5) in New World and Old World begomoviruses, respectively. DNA-B encodes two ORFs, BV1 and BC1 in virion-sense and complementary-sense strands, respectively. Both the genomic components (DNA-A and DNA-B) share ~200 nt common region (CR), which is present within the intergenic region, where replication origin is present. Majority of the begomoviruses are found to be associated with three types of DNA satellites known as betasatellites [3], alphasatellites [4], and deltasatellites [20].

Recently, two begomoviruses and betasatellite associated with garden croton were identified in Pakistan [1]. In India based on partial genome sequence (coat protein gene), it was showed that *Garden croton leaf curl virus* was associated with leaf curl disease [23, 24]. However, the exact identity of the DNA virus associated with severe twisting and curling disease of garden garden croton was not known due to non-availability of complete genome of the virus, which is required for classification and nomenclature of begomovirus. In addition to this, garden croton plant is vegetatively propagated and once planting material becomes infected with virus, it is difficult to remove from the stem cutting unless having proper diagnosis. In the current study novel bipartite begomovirus associated with severe twisting, enation and curling of garden croton plants from Uttar Pradesh was molecular characterized.

Materials and methods

DNA extraction, PCR amplification of partial genome, whole genome amplification by RCA and sequencing

Survey was undertaken at Varanasi, Uttar Pradesh, India in ten gardens, including ICAR-Indian Institute of Vegetable Research having garden croton plants for assessing the incidence of plants showing typical viral disease symptoms of twisting, enation and curling. Disease incidence was assessed by observing hundred plants in each garden and percent incidence was calculated by number infected plants divided by number of plants observed multiplied by hundred. One leaf sample from each garden croton (designated from CR1 to CR10) along with asymptomatic samples were collected from the ten gardens (Fig. 1a, b). Total genomic DNA was isolated from infected and healthy garden croton plants samples using CTAB method [9]. Begomovirus status in samples from garden croton plants was confirmed by PCR amplification of partial genome of the virus using begomovirus DNA A/homologue of DNA A (Forward primer 5′-GCTCCCTGAATGTTCGGATGGA-3′ and Reverse primer -GTTCTCRTCCATCCATATCTTAC-3′) with the expected amplicon of 1.2 Kb [41]. Similarly, PCR was carried out using begomovirus DNA B specific primers pair (Forward primer 5′-GCGTACTCWACGCGCTCA-GATTG-3′ and Reverse primer-5′ GTGTTTCACAGATTT CCT TACGCG-3′) [41] with the expected amplicon of 1.2 Kb. PCR amplification in both the cases was performed with 35 cycles of denaturation for 1 min at 94 °C, primer annealing at 55 °C for 45 s and primer extension for 1 min 30 s at 72 °C, with an initial denaturation at 94 °C for 3 min and a final extension for 10 min at 72 °C. Amplification was carried out in a Gene Amp PCR system 9700 (PE Applied Biosystems, Foster City, CA) thermocycler. All amplifications were performed in volume of 25 µl PCR mixture containing 2 µl 10 × PCR buffer, 100 ng DNA template, 1.5 U Taq DNA polymerase, 25 mM $MgCl_2$, 2 mM dNTPs and 20 pmol of each primer in a PCR tube. PCR products were electrophoresed (1 h at 80 V) on 0.8% agarose gels prepared using Tris–borate-EDTA buffer, pH 8. Gels were stained with ethidium bromide (10 mg/ml) and viewed in a Gel documentation system (Alpha Innotech, USA). Amplified PCR products (1.2 kbp) were purified, cloned into pTZ57R/T cloning vector as per protocol given by the manufacturer's (MBI Fermentas,

Fig. 1 Ornamental garden croton plant showing the symptoms of **a** enation with leaf curl **b** yellowing with enation leaf curl **c** enation at abaxial surface of the leaf

Germany) and transformed into *Escherichia coli* (DH5α) cells. Transformed plasmid was isolated using Qiagen plasmid miniprep kit (Qiagen, Hilder, Germany) and the recombinant inserts in the clones were confirmed by restriction digestion and colony PCR using specific primers mentioned above. The recombinant clones were sequenced in both orientations using automated DNA sequencing facility at Meduaxin Genomics India Pvt. Ltd, Bangalore, India. Obtained raw sequences from the ten garden croton samples were curated using different bioinformatics programs (Bioedit and Clustal X) and similarity search was performed at the NCBI database by using the BLASTn. Based on the sequence analysis, two samples (CR1 and CR2) were used for complete genome amplification (DNA-A and DNA-B) by RCA method [13, 42]. Resulting RCA products of both samples (CR1and CR2) were digested with *Bam*H1 (for DNA-A) and *Xba*I (for DNA-B) separately to obtain linear fragments of 2.8 kb in size each. The digested products were purified separately and cloned into pUC19 plasmid with respective sites as described by Venkataravanappa et al. [42]. Ligated products were transformed and positive clones were confirmed by PCR using begomovirus specific primers followed by restriction analysis. Confirmed clones were sequenced in both orientations. Sequence data obtained from CR1and CR2 isolates from garden croton were aligned into a circular contig by different omics programs and consensus sequences were submitted to NCBI GenBank for DNA-A under the accession number MW816855 and MW816856 and for DNA-B under accession number MW816857and MW816858, respectively**.**

Further, DNA satellites associated with infected garden croton plants samples were also amplified by PCR using specific primers (Forward primer 5′-GGTACCAC-TACGCTACGCAGCAGCC-3’ and Reverse primer 5′-GG TACCTACCCTC CCAGGGGTACAC-3′) for betasatellite and (Forward primer 5′AAGTGGGTCCTGGT TCT-3′ and Reverse primer 5′-TGTACAG GTCTCTGGC-3′) alphasatellite [5, 17]. Amplified PCR products were cloned into pTZ57 vector and sequenced as described above.

Genome analysis and detection of recombination events

Complete genome sequence of begomovirus isolates obtained in the present study was queried at NCBI using BLASTn (http://www.ncbi.nlm.nih.gov/). Sequences

closely related (> 90% nucleotide identity) with the garden croton isolates (CR1 and CR2) were retrieved from the NCBI database, aligned and pair wise identity scores for garden croton isolates (CR1 nad CR2) with GenBank begomoviruses sequences was calculated using Sequence Demarcation Tool(SDT) version 2.0 [26]. Neighbor-Joining method was used for constructing the phylogeny using MEGA X software [17]. Neigbour-Net analysis was carried out using SplitsTree version 4.11.3 with default settings to assess the phylogenic congruence in the garden croton isolates (CR1 and CR2) [15]. Possible recombination break point events were identified in genome of garden croton infecting begomovirus isolates with selected begomoviruses using Recombination Detection Program (RDP4) integrated with RDP, GENECOV, Bootscan, Max Chi, Chimara, Si Scan, 3Seq with default setting (p-value 0.05) [25]. The GC content in the genome of the begomovirus isolates (CR1 nad CR2) associated with garden croton was analyzed using per cent GC-plot graph generated through Artemis DNA plotter analysis tool v18.1.0. (http://www.sanger.ac.uk/Software/Artemis) [8]. Guanine-cytosine (GC) content refers to the proportion of guanine (G) and cytosine (C) in a given stretch fragment of the genome. Since, DNA-A of garden croton isolates (CR1and CR2) were having more homology (97% identical to each other) at genome sequence level, one of the DNA-A (GenBank accession no. MW816855) and DNA-B (GenBank accession no. MW816857) sequences was used for further analysis.

Results

Survey and detection of begomovirus

The garden croton plants at different gardens of Varanasi were exhibiting diverse kind of symptoms such as complete yellowing, leaf curling and twisting, enation and vein thickening and dwarfing of plants (Fig. 1a, b). Incidence of the garden croton plants showing these symptoms ranged from 20–30 percent. DNA extracted from one each infected garden croton samples collected from the ten gardens resulted in the expected PCR amplicon of 1.2 kb specific to the primers of begomovirus used for detection confirming the begomovirus infection in the samples. PCR amplification using DNA B specific also resulted in the expected amplicon of 1.2 kb indicating that the samples are associated with bipartite begomovirus. Partial genome sequences of begomovirus isolates (CR1 and CR10) obtained from the 1.2 kb PCR amplicons generated from ten garden croton samples showed maximum identity (85.9% nucleotide identity) with *Tomato leaf curl Karnataka virus* (MG758148) and had more than 97 percent identity among themselves. Two samples (CR1and CR2) were considered for complete genome amplification using RCA method to known the exact species of the begomovirus associated with garden croton plants showing the virus disease symptoms. The resulting RCA products of both samples (CR1and CR2) were digested with *Bam*H1 (DNA-A) and *Xba*I (DNA-B) separately to obtain linear fragments of 2.8 kb in size each. The digested product were purified separately and cloned into pUC19 plasmid with respective sites as described by Venkataravanappa et al. [43]. Ligated products were transformed and positive clones were confirmed by PCR using begomovirus specific primers followed by restriction analysis. Confirmed clones were sequenced in both orientations. Sequences data obtained for CR1and CR2 isolates from garden croton were aligned into a circular contig by different omics programs and consensus sequences were submitted NCBI GenBank under following accession number DNA-A of CR1 (MW816855) and CR2 (MW816856), DNA-B of CR1 (MW816857) and CR2 (MW816858). PCR detection of DNA satellites using specific primers described above in the infected garden croton samples was unsuccessful.

DNA-A genome component organization in begomovirus associated with enation leaf disease of garden croton

Sequences of the two isolates, CR1 and CR2 (accession number MW816855-56) were 2758 nucleotides in length and displayed similar genomic structure to other Old Word (OW) begomovirus, which codes six conserved ORFs: two ORFs: AV2 (144–500 position/codes for 118) and AV1 (304–1074 position/codes for 256) in plus strand (sense) and AC3 (1071–1475 position/codes for 134aa), AC2 (1216–1620 position/codes for 134aa), AC1 (1523–2605 position/codes for 360aa) and AC4 (2161–2457 position/codes for 98aa) in minus (antisense) strand of DNA-A component. The plus and minus strands were separated by an untranslated intergenic region (IR) from nucleotides 2606 to 2758nts and 1 to 143nts, contains the origin of replication (ori) and highly conserved nonanucleotide (TAATATTAC) sequence. Within the IR, an iteron sequence, GACCC close to the TATA box, is presumably the Rep promoter (13, **2**) share more homology with iterons identified in DNA A so far. Rep gene (encoded by C1) of CR1and CR2 isolates contained all conserved motifs as described so for in many begomoviruses [38], except new GRS motif (RFFDLVSPTRSAHFHPNIQGAKS), which is required for viral infection and initiation of rolling-circle replication during geminivirus infection. The central GRS residues are present, which are highly conserved in the Rep proteins of begomoviruses, curtoviruses, and topocuviruses but not mastreviruses, were assumed to be acquired later in

geminivirus evolution during their adaptation to dicotyledonous plants [29]

Pair wise comparison of complete DNA-A sequence revealed that CR1 and CR2 have 97% nucleotide identity among them. These two isolates shared maximum nt identity of 85.7–85.9% % with *Tomato leaf curl Karnataka virus* (MG758148) and 82.9 to 83.2% nt identity with *Tomato leaf curl Kerala virus* (EU910141) infecting tomato and less than 83% nt identity with other begomovirus infecting different crops in India (Table 1). Based on the criteria used for begomoviruses species demarcation (91% nt identity) [44], the begomovirus characterized here should constitute a new species. We propose them to be named as *Garden croton enation leaf curl virus* (CroELCuV) [IN: Varanasi: Croton: 18].

Table 1 Pair wise percent nucleotide sequence identities for comparisons between the complete genome sequences (DNA-A) and intergenic regions (IR) of the virus isolates from ornamental croton obtained here with selected other begomoviruses available in the databases

Begomovirus species[#]	Accession No	CR1		CR2	
		Genome	IR	Genome	IR
PeLCuV	MF278788	81.0	60.8	81.3	60.8
PeLCuV	MF278789	81.0	60.8	81.3	60.8
PaLCuV	MF278787	74.9	52.6	75.8	52.6
ToLCKV	MG758148	85.9	71.1	85.7	71.1
ToLCPuV	AY754814	76.3	63.4	76.3	63.4
ToLCRaV	DQ339117	78.3	66.0	78.5	66.0
ToLCBaV-C	AF165098	76.6	56.2	76.5	56.2
ToLCKeV	EU910141	82.9	74.3	83.2	74.3
ToLCPatV	EU862323	75.3	58.2	75.5	58.2
ToLCNDV	U15015	71.9	52.1	72.0	52.1
ToLCPalV	AM884015	70.9	51.4	70.8	51.4
ToLCBV	AF188481	78.7	62.6	79.0	62.6
PaLCuV-Tob	HM143914	78.3	61.5	78.5	61.5
AEV-IN	JX436472	79.9	54.4	80.1	54.4
TbCSV	JN387045	78.0	57.5	78.1	57.5
ChiLCV	JQ654460	79.3	67.8	79.7	67.8
RaLCuV	GU732204	82.4	75.9	82.7	75.9
CLCuKoV-Bu	HM461862	77.4	52.5	77.7	52.5
CLCuBaV	AY705380	81.8	77.7	82.3	77.7
CLCuAlV	AJ002452	70.1	48.0	70.7	48.0
CLCuMuV	GQ220850	77.9	61.4	78.3	61.4
OELCuV	GU111999	75.7	65.6	76.2	65.6
BYVMV	AF241479	76.0	59.2	76.7	59.2
FbLCV	JQ866297	75.8	67.4	76.3	67.4
MYMIV	AF481865	60.9	36.0	61.4	36.0
PeLCV-Sb	AM948961	82.2	68.1	82.5	68.1

IR Intergenic region

[#]Definition for acronyms as per the Brown et al. [7], For each column the highest value is underlined

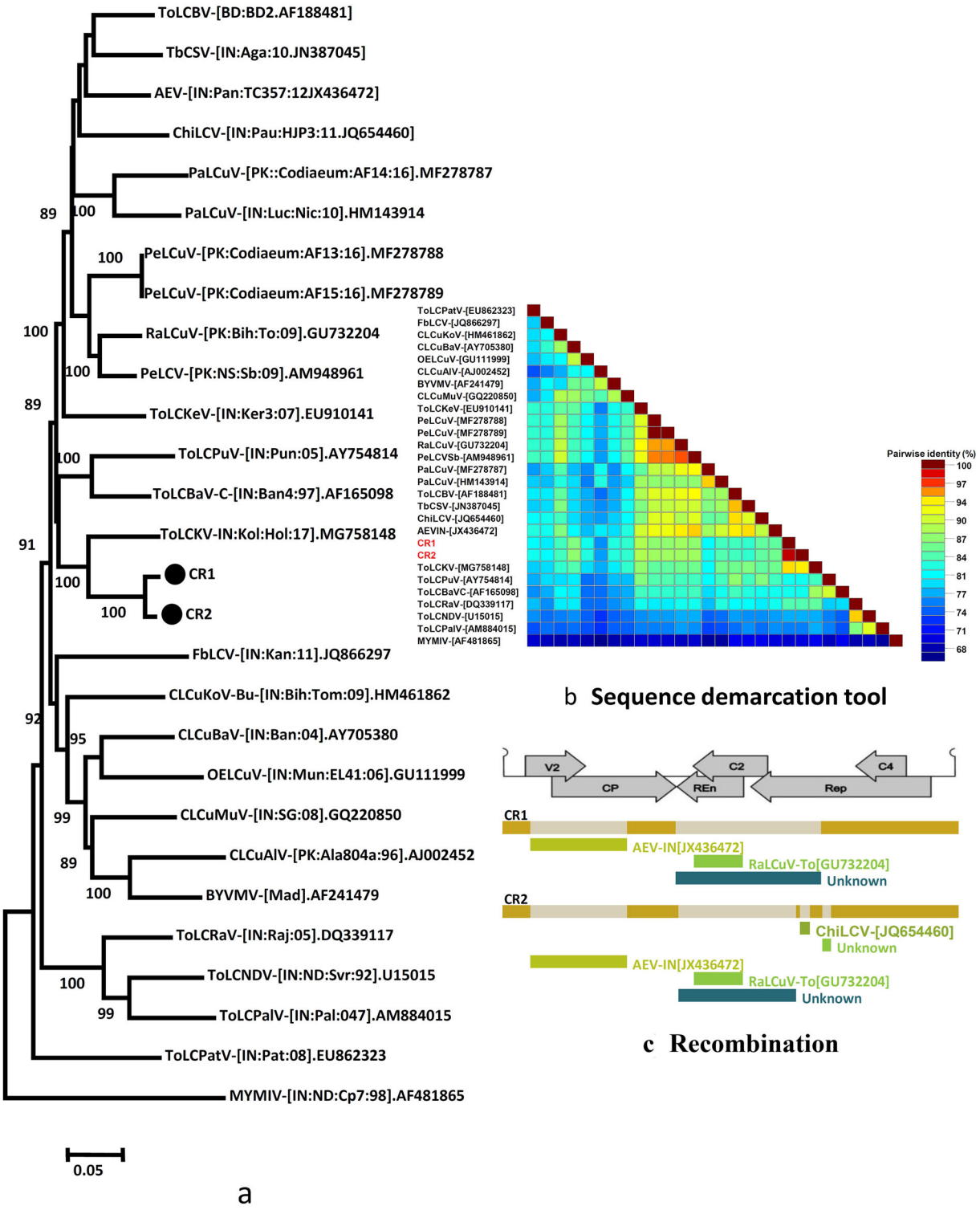
To determine the evolutionary relationship of CR1 and CR2 isolates, other selected 28 begomoviruses sequences were obtained from database, the phylogeny was constructed using the Neighbor-joining (NJ) method employed in the MEGA X software with 1000 bootstrap replications. The CR1 and CR2 isolates are separately clustered clearly indicating that they are distinct and different from the other begomoviruses reported so far. This result is also well supported by Sequence Demarcation Tool (SDT) (Fig. 2a, b).

The aa sequences of respective ORFs of CR1 and CR2 were comparison with selected 28 begomoviruses isolates from NCBI GenBank revealed that CR1 and CR2 isolates had maximum amino acid identity in the of coat protein (CP) region with *Tomato leaf curl Rajasthan virus* (ToLCRaV, DQ339117) infecting tomato, pre-coat (AV2) and REn (C3) region with *Tomato leaf curl Karnataka virus* (ToLCKV, MG758148) infecting tomato, Rep (C1) region with *Cotton leaf curl Bangalore virus* (CLCuBaV, AY705380), TrAP (C2) region with ToLCKV (MG758148), *Tomato leaf curl Pune virus* (ToLCPuV, AY754814) and C4 region with PeLCV (*Pedilanthus leaf curl virus,* AM948961). In IR regions of these isolates had more homology with IRs of CLCuBaV infecting cotton and okra (Table 2). The length of intergenic region (IR) in CR1 and CR2 isolates is about 296 nts and showed more homology with bipartite begomovirus reported so for.

DNA-B genome component organization in begomovirus associated with enation leaf disease of garden garden croton

The complete nucleotide sequence of DNA-B genome component of CR1 and CR2 isolates had similar genomic structure of other bipartite begomoviruses reported so far. DNA B of CR1 and CR2 isolates encodes two ORFs, BV1 (movement protein) and BC1 (nuclear shuttle protein) in sense and antisense strand, respectively. These two genes of CR1 isolate shared maximum amino acid sequence identity with *Tomato leaf curl New Delhi virus* (ToLCNDV, HM989846) infecting luffa. However, in case of CR2 isolate BV1 and BC1 genes share maximum amino acid nucleic acid identity with *Tomato leaf curl New Delhi virus* (ToLCNDV) infecting luffa (HM989846) and infecting okra (HQ586007), respectively (Table 3).

The length of DNAB nt sequence of CR1 and CR2 was 2697 and 2698 nts, respectively and submitted to GenBank under accession number (Acc.No. MW816857 and MW816858). Comparison of DNA-B sequences of CR1 and CR2 isolates with selected begomoviruses indicated that these isolates shared the maximum nt identity with


ToLCBV-[BD:BD2.AF188481]
TbCSV-[IN:Aga:10.JN387045]
AEV-[IN:Pan:TC357:12JX436472]
ChiLCV-[IN:Pau:HJP3:11.JQ654460]
PaLCuV-[PK::Codiaeum:AF14:16].MF278787
PaLCuV-[IN:Luc:Nic:10].HM143914
PeLCuV-[PK:Codiaeum:AF13:16].MF278788
PeLCuV-[PK:Codiaeum:AF15:16].MF278789
RaLCuV-[PK:Bih:To:09].GU732204
PeLCV-[PK:NS:Sb:09].AM948961
ToLCKeV-[IN:Ker3:07].EU910141
ToLCPuV-[IN:Pun:05].AY754814
ToLCBaV-C-[IN:Ban4:97].AF165098
ToLCKV-IN:Kol:Hol:17].MG758148
CR1
CR2
FbLCV-[IN:Kan:11].JQ866297
CLCuKoV-Bu-[IN:Bih:Tom:09].HM461862
CLCuBaV-[IN:Ban:04].AY705380
OELCuV-[IN:Mun:EL41:06].GU111999
CLCuMuV-[IN:SG:08].GQ220850
CLCuAlV-[PK:Ala804a:96].AJ002452
BYVMV-[Mad].AF241479
ToLCRaV-[IN:Raj:05].DQ339117
ToLCNDV-[IN:ND:Svr:92].U15015
ToLCPalV-[IN:Pal:047].AM884015
ToLCPatV-[IN:Pat:08].EU862323
MYMIV-[IN:ND:Cp7:98].AF481865
0.05
a
ToLCPatV-[EU862323]
FbLCV-[JQ866297]
CLCuKoV-[HM461862]
CLCuBaV-[AY705380]
OELCuV-[GU111999]
CLCuAlV-[AJ002452]
BYVMV-[AF241479]
CLCuMuV-[GQ220850]
ToLCKeV-[EU910141]
PeLCuV-[MF278788]
PeLCuV-[MF278789]
RaLCuV-[GU732204]
PeLCVSb-[AM948961]
PaLCuV-[MF278787]
PaLCuV-[HM143914]
ToLCBV-[AF188481]
TbCSV-[JN387045]
ChiLCV-[JQ654460]
AEVIN-[JX436472]
CR1
CR2
ToLCKV-[MG758148]
ToLCPuV-[AY754814]
ToLCBaVC-[AF165098]
ToLCRaV-[DQ339117]
ToLCNDV-[U15015]
ToLCPalV-[AM884015]
MYMIV-[AF481865]
Pairwise identity (%)
100
97
94
90
87
84
81
77
74
71
68
b Sequence demarcation tool
V2
C2
C4
CP
REn
Rep
CR1
AEV-IN[JX436472]
RaLCuV-To[GU732204]
Unknown
CR2
ChiLCV-[JQ654460]
Unknown
AEV-IN[JX436472]
RaLCuV-To[GU732204]
Unknown
c Recombination

◄**Fig. 2** Phylogenetic tree showing the relationships of the **a** DNA-A component (CroELCuV, *MW816855-56*) of begomovirus (CroELCuV) associated with garden croton plant with selected begomoviruses DNA-A sequences. Trees were drawn using MEGA7 tool, using Neighbor-joining method. **b** Pairwise identity scores for the DNA-A of CroELCuV obtained comparing with other begomoviruses sequences using Sequence Demarcation Tool (**b**). Putative recombination events of begomovirus (*MW816855-56*) associated with garden croton plant was identified by RDP analysis (**c**). A genomic map of begomovirus and arrangement of genes along with their coding direction nucleotide scale (1–2758). Acronyms of begomoviruses were given as *Ageratum enation virus* (AEV) and *Chilli leaf curl virus* (ChiLCV), Radish *leaf curl virus* (RaLCuV). Indeterminate sequence origin indicated as "unknown". Recombination position in the genome of the begomovirus indicated as a box below, at the top of the diagram. Details of the sequences used for this study are listed in Table S1. Abbreviations indicate, IR-Intergenic region, AV2-Pre-coat protein, CP/AV1- Coat protein, Rep/AC1-Replication-associated protein REn/AC3- Replication enhancer protein, TrAP/AC2 -Transcriptional activator protein

ToLCNDV (91.0–92.2%) infecting pumpkin and okra (AM286435, HQ586007) in India (Table 3). This result is well supported both phylogenetic tree and SDT analysis showing that CR1 and CR2 isolates closely clustering with ToLCNDV infecting cucurbits and tomato (Fig. 3a, b). The intergenic region (IR) of CR1 and CR2 isolates shared 91.9% nt identity with ToLCNDV (AM286435) infecting pumpkin. The length of the IR in these isolates is about 312 nucleotides, which is also having more homology with previously reported ToLCNDV isolates available in the database.

Recombination

RDP analysis showed that most part of the DNA-A sequence (IR, TrAP, REn, REP, AC4 and V2, CP) might have derived from several previously reported

Table 2 Pair wise percent amino acid sequence identities of genes encoded by DNA A components of CR1And CR2 isolates of begomivirus isolates associated with garden croton obtained by comparing with genes of closely related begomoviruses selected from the databases

Begomovirus species[#]	Accession No	CP(AV1)		AV2		Rep(AC1)		TrAP (C2)		REn (C3)		C4	
		CR1	CR2	CR1	CR2	CR1	CR2	CR1	CR2	CR1	CR2	CR1	CR2
PeLCuV	MF278788	94.9	94.9	79.1	79.1	80.5	81.7	70.1	73.1	70.1	73.8	68.6	69.6
PeLCuV	MF278789	94.9	94.9	79.1	79.1	80.5	81.7	70.1	73.1	70.1	73.8	68.6	69.6
PaLCuV	MF278787	94.5	94.5	88.1	88.1	71.1	72.2	60.7	71.8	67.9	72.3	35.7	35.7
ToLCKV	MG758148	97.2	97.2	*95.7*	*95.7*	85.0	85.3	*77.6*	*75.3*	*80.5*	*81.3*	45.4	44.4
ToLCPuV	AY754814	91.7	91.7	69.4	69.4	82.2	82.8	*77.6*	*75.3*	64.9	68.6	48.4	47.4
ToLCRaV	DQ339117	*98.0*	*98.0*	92.3	92.3	77.5	78.1	51.4	53.7	58.0	58.0	40.8	41.8
ToLCBaV-C	AF165098	87.9	87.9	69.4	69.4	81.9	81.7	67.1	68.6	64.9	67.1	49.4	48.4
ToLCKeV	EU910141	92.9	92.9	90.6	90.6	85.2	86.3	68.6	70.8	64.9	69.4	75.5	76.5
ToLCPatV	EU862323	80.9	80.9	75.6	75.6	81.7	82.5	59.2	59.2	66.4	67.9	75.4	76.4
ToLCNDV	U15015	92.5	92.5	69.4	69.4	77.5	77.5	51.0	51.7	60.2	60.2	38.7	37.7
ToLCPalV	AM884015	90.2	90.2	66.1	66.1	74.3	74.3	51.0	51.0	61.7	62.5	37.7	36.7
ToLCBV	AF188481	95.3	95.3	94.9	94.9	81.4	82.5	63.4	65.6	63.4	66.4	50.5	49.4
PaLCuV-Tob	HM143914	97.6	97.6	93.2	93.2	70.6	71.7	66.4	67.9	65.6	67.9	33.6	33.6
AEV-IN	JX436472	95.7	95.7	94.9	94.9	83.3	84.7	68.6	67.1	67.9	70.1	60.2	61.2
TbCSV	JN387045	92.9	92.9	92.3	92.3	81.7	82.8	63.4	63.4	66.4	67.9	41.4	40.4
ChiLCV	JQ654460	95.7	95.7	80.5	80.5	82.8	84.4	67.1	70.8	67.9	70.8	46.4	45.4
RaLCuV	GU732204	95.7	95.7	90.6	90.6	75.5	76.6	67.9	70.1	66.4	70.8	74.7	75.7
CLCuKoV-Bu	HM461862	95.3	95.3	92.3	92.3	82.6	83.7	58.2	61.9	67.9	68.6	66.6	65.6
CLCuBaV	AY705380	93.3	93.3	67.7	67.7	*87.8*	*89.7*	52.0	54.6	61.1	61.9	74.5	75.4
CLCuAlV	AJ002452	94.1	94.1	66.9	66.9	69.6	71.3	50.6	54.0	65.6	66.4	36.7	36.7
CLCuMuV	GQ220850	93.7	93.7	58.4	58.4	84.2	85.3	51.3	55.3	68.6	69.4	65.6	64.7
OELCuV	GU111999	77.7	77.7	64.4	64.4	86.1	87.2	49.3	53.3	66.4	65.6	68.6	69.6
BYVMV	AF241479	93.7	93.7	66.1	66.1	82.0	83.7	53.1	57.3	67.1	67.9	76.4	77.4
FbLCV	JQ866297	91.0	91.0	66.3	66.3	83.4	84.5	58.9	65.6	61.1	63.4	72.5	73.5
MYMIV	AF481865	75.0	75.0	36.9	36.9	70.4	71.2	45.5	46.3	41.7	39.5	41.4	41.4
PeLCV-Sb	AM948961	96.0	96.0	93.2	93.2	87.5	89.4	69.4	72.3	69.4	73.8	*76.5*	*77.5*

[#]Definition for acronyms of the viruses as per the Brown et al. [7], For each column the highest value is in italic

Table 3 Pair wise percent amino acid sequence identities of genes encoded by DNA B components of CR1 And CR2 isolates of begomivirus isolates associated with garden croton obtained by comparing with genes of closely related begomoviruses selected from the databases

Begomoviruses	Accession No	CR1				CR2			
		Genome[a]	IR[a]	BV1[b]	BC1[b]	Genome[a]	IR[a]	BV1[b]	BC1[b]
ToLCNDV-Pot	KC874498	88.9	85.8	93.6	97.5	88.0	85.8	92.5	92.5
ToLCNDV-OK	HQ586007	90.2	89.3	95.5	96.4	*91.0*	89.6	94.4	*95.0*
ToLCNDV-ND	DQ020490	89.0	88.5	89.9	96.4	88.2	88.9	91.0	90.7
ToLCNDV-Chi	JN663848	91.2	88.3	96.6	92.8	90.2	88.3	95.4	87.1
ToLCNDV-Svr	U15017	91.2	87.7	96.4	92.8	90.1	87.7	95.3	87.1
ToLCNDV-Cuc	KC545813	91.5	89.6	96.2	97.5	90.2	89.6	95.1	91.8
ToLCNDV-ND	DQ169057	91.8	89.6	96.5	97.5	90.5	89.6	95.2	91.8
ToLCNDV-luf	HM989846	91.6	89.2	*96.6*	*97.8*	90.3	89.9	*95.5*	92.1
ToLCNDV-Pum	AM286435	*92.2*	*91.9*	93.2	99.6	90.8	*91.9*	92.9	91.2
ToLCNDV-AG	JN208137	91.5	89.6	94.0	99.2	90.1	89.6	93.2	92.8
ToLCPaV-Tom	KC456162	76.6	70.4	77.6	91.4	75.8	70.7	77.2	87.1
ToLCPaV-Cuc	FJ660427	75.5	80.0	77.2	92.1	74.3	78.2	76.8	87.1
ToLCPaV-Mel	EU547681	74.6	76.1	77.2	90.0	73.3	74.7	76.8	85.7
SLCCNV- Pum	JN624306	73.8	75.6	72.7	91.4	73.1	75.6	71.6	86.4
MYMIV-Cp7	AF503580	59.0	57.6	29.6	44.8	58.8	57.6	30.0	47.5

IR Intergenic region

[#]Definition for acronyms of the viruses as per the Brown et al. [7], For each column the highest value is in italic

begomoviruses, which includes AEV, RaLCuV ToLCPuV, CLCuBaV, ChiLCV, PaLCuV, FbLCV, CLCuAlV, OELCuV, ToLCBV and ToLCKeV (All viruses Acronymonous has been given Table S1) as the major as well as minor donor parents (Table 4, Fig. 2c). Similarly, in case DNA-B component, most part of movement protein (ORFBV1) might have derived from ToLCNDV infecting pumpkin. Out of nine methods implemented in RDP4, five methods strongly supported recombinant origin of the DNA-A component, whereas in DNA-B component, recombinant origin was strongly supported all methods (Table 4, Fig. 3c).

GC plot analysis

The GC analysis for the two current virus isolates for both DNA-A and DNA-B sequences was done using Artemis DNA plotter version 18.1.0 (Fig. 4). Both the isolates, showed varied GC content at different stretches of the DNA-A nucleotide sequence. However, between these isolates there is no variation with respect to the GC content across the genome, except in one stretch, which fall in the overlapping sequence of genome encoding Rep (AC1) and TrAP (AC2) genes. All the genes have stretches of GC rich and GC low region in the viral genome, except IR region, which is completely below average GC content. However, the position these regions within the genes varied. Similarly, the GC content analysis was carried for DNA-B nucleotide sequence of the current viral isolates, which indicated there is no difference in the GC composition between the two isolates across the genome sequence, except in one stretch of MP (BC1). Further, when GC content within the gene was analyzed, both MP (BC1) and NSP coded by DNA-B were shown to have high GC content, where as other regions had below average GC content.

Discussion

Garden croton is one of the most popular ornamental as well as medicinal plant grown very well in the tropics. It is commonly called garden croton or Joseph's coat due to its beautiful variegated leaves, which are often either leathery or shiny varying greatly in shape and colour. Garden croton is cultivated as either landscape plants or containerized foliage plants considering its medicinal properties and aesthetic value and prone to many biotic stresses. Literature survey showed that garden croton is infected with different begomoviruses and betasatellites [23, 24]. Survey for the incidence of garden croton plants showing begomovirus like symptoms and subsequent PCR detection using begomovirus specific primers in the infected samples revealed ubiquitous presence of begomovirus in all gardens surveyed. Analysis of begomovirus genome sequences obtained from the infected garden croton plants showed that the begomovirus isolates associated with garden croton had maximum nt identity with ToLCKV (85.9%) and

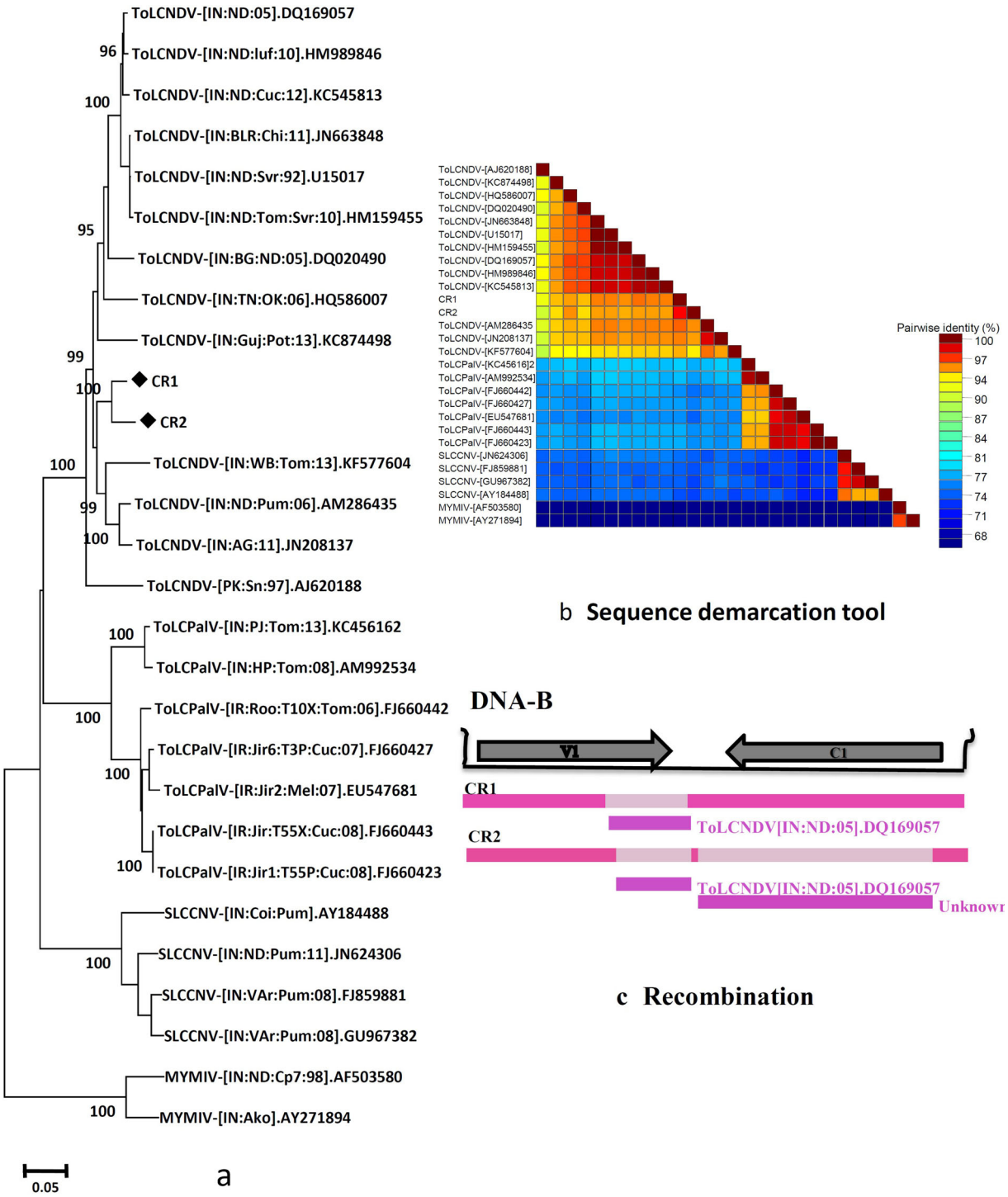


Fig. 3 Phylogenetic tree showing the relationships of the **a** DNA-B component (*MW816857-58*) of begomovirus (CroELCuV) associated with garden croton plant with selected begomoviruses. Trees were drawn using MEGA7 toolusing Neighbor-joining method. **b** Pair wise identity scores for the DNA-B of CroELCuV obtained comparing with other begomoviruses sequences using Sequence Demarcation Tool (**b**). Putative recombination events of begomovirus (*MW816857-58*) associated with garden croton plant was identified by RDP analysis (**c**). A genomic map of begomovirus and arrangement of genes along with their coding direction nucleotide scale (1–2698). Acronym of begomovirus was given as *Tomato leaf curl New Delhi virus* (ToLCNDV). Indeterminate sequence origin indicated as "unknown". The recombination position in the genome of the begomovirus indicated as a box below, at the top of the diagram. Details of the sequences used for this study are listed in Table S1. Abbreviations indicates; V1: movement protein and C1:nuclear shuttle protein

Table 4 Breakpoint analysis of CR1 and CR2 isolates of CroELCuV associated with garden croton and their putative parental sequences

	Position of Recombination	Break point begin-end	Major Parent	Minor parent	*P*-Values					
					RDP	GENECOV	Max Chi	Chimera	Si Scan	3Seq
DNA A										
CR1	IR, AV2	133–542	ToLCPuV-[IN:Pun:05].AY754814	AEV-[IN:Pan:TC357:12]JX436472	1.32×10^{-3}	2.66×10^{-3}	8.81×10^{-7}	5.937×10^{-5}	3.76×10^{-16}	2.69×10^{-6}
	AV1	921–1095			3.46×10^{-2}	NS	2.607×10^{-5}	9.93×10^{-1}	1.67×10^{-9}	NS
	AV1	836–1453	CLCuAlV-[PK:Ala804a:96].AJ002452	RaLCuV-To[PK:Bih:To:09].GU732204	2.885×10^{-4}	NS	1.23×10^{-4}	4.53×10^{-4}	4.25×10^{-4}	8.23×10^{-6}
			ToLCBV-[BD:BD2].AF188481	FbLCV-[IN:Kan:11].JQ866297						
CR2	IR, AV2	133–542	ToLCPuV-[IN:Pun:05].AY754814	AEV-[IN:Pan:TC357:12]JX436472	1.32×10^{-3}	2.66×10^{-3}	8.81×10^{-7}	5.937×10^{-5}	3.76×10^{-16}	2.69×10^{-6}
	AV1	921–1095			3.46×10^{-2}	NS	2.607×10^{-5}	9.93×10^{-1}	1.67×10^{-9}	NS
	AV1, AC3	849–1422	CLCuAlV-[PK:Ala804a:96].AJ002452	RaLCuV-To[PK:Bih:To:09].GU732204	2.885×10^{-4}	NS	1.23×10^{-4}	4.53×10^{-4}	4.25×10^{-4}	8.23×10^{-6}
	AC3, AC2	1438–1488		FbLCV-[IN:Kan:11].JQ866297	1.68×10^{-14}	3.677×10^{-17}	9.32×10^{-5}	2.94×10^{-2}	NS	8.97×10^{-7}
	AC2, AC1	1547–1594	ToLCBV-[BD:BD2].AF188481	ChiLCV-[IN:Pau:HJP3:11]JQ654460	4.047×10^{-6}	1.43×10^{-6}	2.73×10^{-4}	NS	NS	7.17×10^{-5}
			CR1							
			CR1	RaLCuV-To[PK:Bih:To:09].GU732204						
DNA B										
CR1	BV1	733–1160	ToLCNDV-[IN:ND:Pum:06].AM286435	ToLCNDV[IN:ND:05].DQ169057	1.933×10^{-10}	$1.11 \times \times 10^{-4}$	3.83×10^{-7}	3.34×10^{-6}	1.78×10^{-13}	4.64×10^{-7}
CR2	BV1	770–1160	ToLCNDV-[IN:ND:Pum:06].AM286435	ToLCNDV[IN:ND:05].DQ169057	1.933×10^{-10}	1.1×10^{-14}	3.83×10^{-7}	3.34×10^{-6}	1.78×10^{-13}	4.64×10^{-7}
	BC2	1184–2394		ToLCNDV-[IN:ND:Pum:06].AM286435	1.69×10^{-7}	–	3.86×10^{-20}	2.99×10^{-7}	4.89×10^{-5}	8.122×10^{-17}
			CR1							

NS Non significance

Intergenic region (IR) and genes are indicated as coat protein (CP), replication-associated protein (Rep), transcriptional activator protein (TrAP), and replication enhancer (REn), Movement protein (BV1), Nuclear shuttle protein (BC1). The products encoded by ORFs V2 and C4 have yet to be named

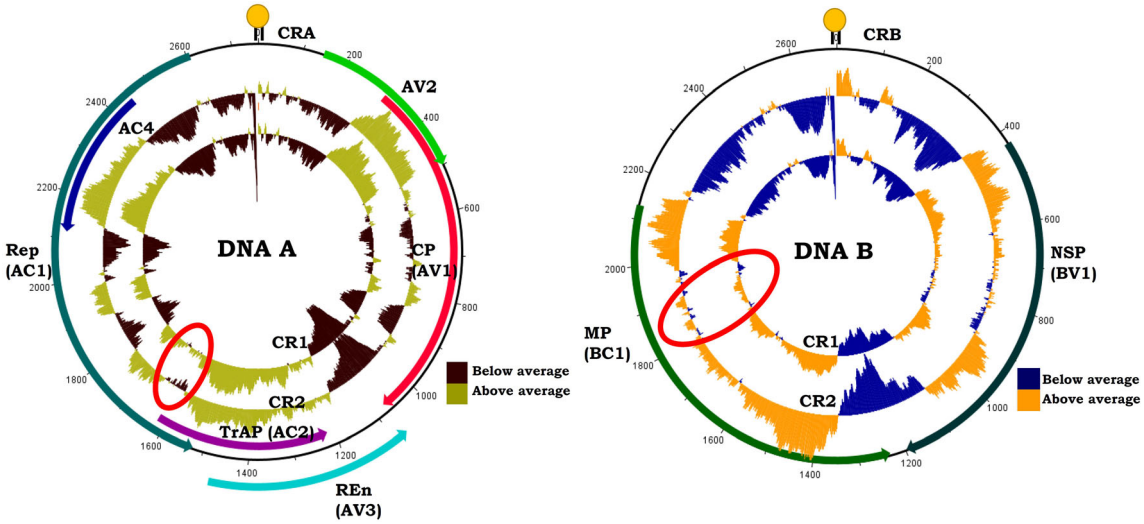


Fig. 4 GC (Guanine-cytosine) content analysis in the genome of CR1 and CR2 isolates of CroELCuV from the garden croton. Outermost ring represents the nucleotide position in DNA-A (**a**) and DNA-B (**b**) of the viral genome. Inner coloured arrows represents respective genes (AV2, AV1) and AC1, AC2, AC3 and AC4 encoded by the DNA-A and MP and NSP gene encoded by DNA-B component of CroELCuV, innermost circle and bar represent the GC-plot with above average and below average GC content of the genome with window size of 80 and step size 1 showing the highest and lowest possible regions of recombination, respectively. Red circle indicates difference in GC content among two isolates. This analysis was performed using Artemis DNA plotter version 18.1.0, (http://www.sanger.ac.uk/Software/Artemis)

ToLCKeV (85.7%), which is below the species demarcation threshold for begomoviruses [44]. Therefore, these two isolates in the current study are considered as a new bipartite begomovirus virus species, for which the name *Garden croton enation leaf curl virus* (CroELCuV) is proposed. Phylogenetic analyses of viral genome further supported the above results with virus isolates forming a distinct clade, which is closely related to ToLCKV.

Recombination is a one of the important factor for evolution of different begomoviruses in which two parental viruses exchange the genetic information to an offspring, or recombinant virus. Unlike with sexual reproduction, recombination involves the transfer DNA fragments between the members of distantly related species of viruses [25], which create novel recombinant genomes with adaptive advantages leading to expansion of its host range and emergence of new diseases [20, 42]. Recombination might have played major role in origin of CroELCuV infecting ornamental croton. The evidence showed that major portion of the DNA-A and DNA-B component of garden croton (CR-1 and CR-2) isolates are obtained from distinct viruses which includes TbCSV, ToLCPuV, CLCuBaV, ChiLCV, PaLCuV, FbLCV, CLCuAlV, OELCuV, ToLCNDV and ToLCKeV as the major donor and minor parents leading to the evolution of new *Enation leaf curl virus* infecting garden croton in Indian subcontinent. Recombination between the species and within species of begomovirus is important prime factor for emergence of novel virus species and adapted to the new hosts in agricultural system [11, 19].

Low GC region in DNA-A and betasatellite of the begomoviruses are potential recombination sites facilitating the evolution of virus has been well documented in many viruses infecting plants and animals [34, 45]. Sequence with high GC content has more stability due to triple hydrogen bond, stacking interactions between the bases and also linked with topology and orientation of DNA strands [31, 45]. Higher the number of bonds between bases in a DNA strand, generally they required more energy to break the strand. Similarly, the high GC content was found intergenic region of herpes simplex virus (HSV) genome, linked with possible role in the viral evolution and pathogenesis [6].

In both DNA-A and DNA-B of two isolates GC content and recombination analysis showed that possible recombination break points were observed in the regions of below average GC content. The possible recombination break points occurred in below average GC content regions of DNA-A are in IR region (133-542nt recombination occur), coat protein (921-1095nt), replication-associated protein (1547–1594 nts), transcriptional activator protein and replication enhancer (1438–1488 nts) (Table 4). In the DNA-B two break points identified in the regions which also falls into below average GC content regions, movement protein (1184–2394 nts) and nuclear shuttle protein (733–1160 nts). These analysis suggests that begomovirus associated with enation leaf curl disease of garden croton

can undergone recombination in its genome region having below average GC content in both DNA-A and DNA-B components. Similar results were reported showing the region having low GC content as potential recombination site in plant infecting begomovirus [45] and human adenovirus, which may facilitate virus molecules to evolve and allow a species to increase its host range in new environmental niche [34].

Prevalence of enation leaf curl disease on garden croton may drastically affect its utilization as planting material for landscape or gardening in India. The disease may spread in the country, mainly through infected propagative materials of ornamental garden croton as well as whitefly as vector. The other major concern is exchange of ornamental germplasm resources, without checking the planting material may result in spilling and expansion of begomoviruses host range [39].

Identification of new bipartite begomovirus infecting garden croton necessitating the need for more comprehensive study to identify possible spread of virus in the country and come out with intervention for containing its spread. The PCR based technique described in here may be suitable tool for searching the disease free planting material for the utilization in landscape or gardening and pharmaceutical industry in India. This is the first record of novel bipartite begomovirus associated with enation leaf curl disease in garden croton plants in India and world.

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Data availability All data analyzed during this study are included in this article and its supplementary information files.

Declarations

Conflict of interest All the authors declare that they have no conflict of interest.

Human or animal rights This article does not contain any studies with human or animal subjects performed by any of the authors.

Ethical approval This article does not contain any studies with human participants performed by any of the authors.

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