Molecular characterization of a distinct bipartite Begomovirus *species infecting ivy gourd (*Coccinia grandis *L.) in Tamil Nadu, India*

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Virus Genes

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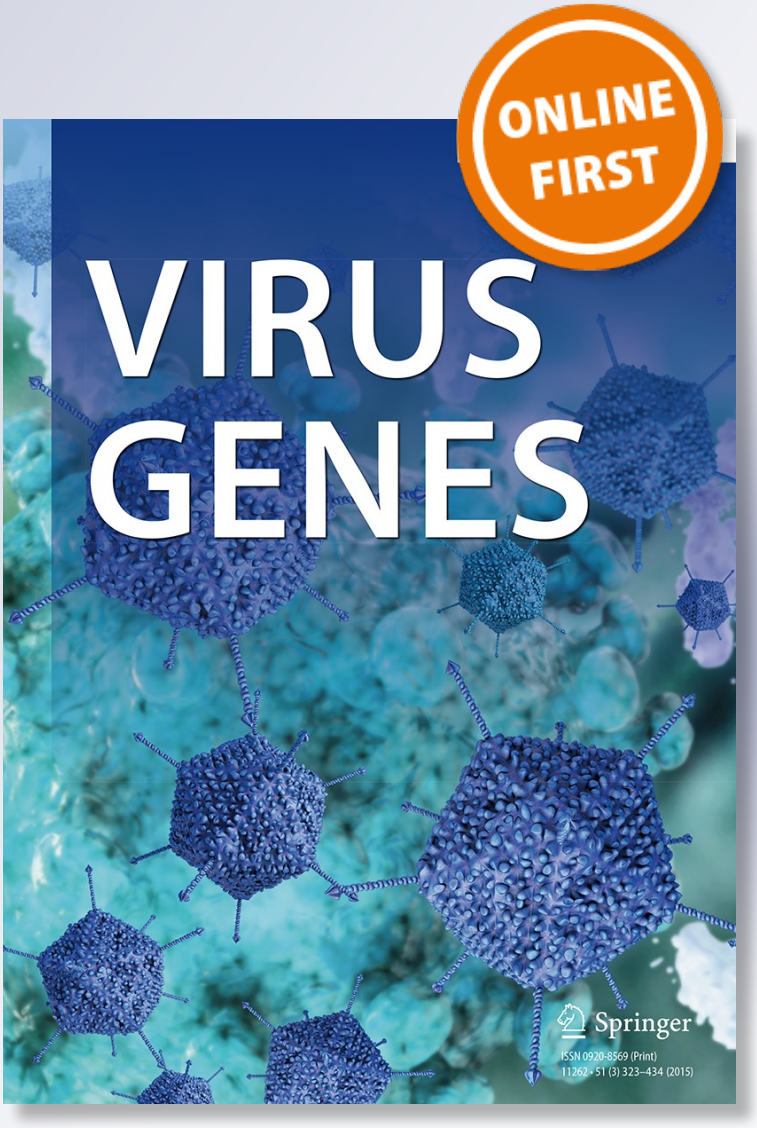

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Molecular characterization of a distinct bipartite *Begomovirus* species infecting ivy gourd (*Coccinia grandis* L.) in Tamil Nadu, India

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Abstract A distinct bipartite begomovirus was found to be associated with the mosaic disease on ivy gourd (*Coccinia grandis* L.) in Tamil Nadu, India. The complete DNA A and DNA B components were cloned by rolling circle amplification. Genome organization of this virus is found to be typical of Old World bipartite begomovirus. The association of betasatellite component with this virus is absent. The closest nucleotide identity of 73.4 % was seen with the *Loofa yellow mosaic virus* (LYMV-[VN]-AF509739) suggesting that it is a new virus species Coccinia mosaic virus (CoMoV-Ivy gourd [TN TDV Coc1]) and distantly related to the other known begomoviruses. The DNA B component shared a maximum identity of 55 % with that of *Tomato leaf curl New Delhi virus* (ToLCNDV). In the phylogenetic analysis, CoMoV-Ivy gourd form cluster separate from other begomoviruses. Recombination analysis showed that there was no recombination event in the genome. This is the distinct begomovirus infecting ivy gourd.

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Keywords Bipartite begomovirus · Cucurbits virus · Whitefly · Rolling circle amplification

Introduction

Begomoviruses are single-stranded circular plant DNA viruses belonging to the family *Geminiviridae* and are characterized by twinned icosahedral virus particle of about 20 × 30 nm size. Begomoviruses are transmitted by the whitefly, *Bemisia tabaci*, and are a major threat to agricultural and horticultural crops causing significant yield loss throughout the world. Begomoviruses can either have a monopartite or a bipartite genomes. The bipartite genomes consist of two ssDNA molecules of ~2.7 kb referred to as DNA A and DNA B [1]. DNA A encodes pre-coat protein, and coat protein in virion strand and DNA replication-associated proteins are encoded in the complementary strand DNA. The DNA B encodes two proteins one in virion sense governing export of genomic DNA from nucleus and another in complementary sense strand DNA encoding movement protein [2].

Cucurbits are important vegetable crops rich in minerals and nutrients cultivated worldwide. These crops are found to be susceptible to the virus infection, and worldwide, fifty-nine viruses are recorded [3]. Ivy gourd (*Coccinia grandis* L. Voigt) native to tropical Africa and Asia belongs to the family Cucurbitaceae and is a vegetable crop grown perennially on trails in Tamil Nadu. The fruit yields of ivy gourd are low due to infection by viral diseases. Among these viral pathogens, begomoviruses play a major role. There are several begomoviruses reported in cucurbitaceous crops in India viz., *Tomato leaf curl New Delhi virus* [4, 5], *Squash leaf curl China virus* [6], *Pepper leaf curl Bangladesh virus* [7], *Mesta yellow vein virus* [8], *Bittergourd yellow mosaic*

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virus [9], *Indian cassava mosaic virus* [10], *Tomato leaf curl Palampur virus* [11], *Ageratum enation virus* [12], and *Pumpkin yellow mosaic virus* [13]. In this study, we have characterized a new bipartite begomovirus species associated with mosaic disease of ivy gourd, which is different from previously characterized bipartite begomoviruses. On the basis of nucleotide identity of DNA A component, it has been identified as a new begomovirus species. We have also established the phylogenetic relationship of DNA A and DNA B of the present species with different begomoviruses infecting cucurbitaceous hosts.

Materials and methods

Sample collection and DNA extraction

Coccinia leaves showing mosaic symptoms were collected from Tindivanam (Villupuram Dt), Tamil Nadu during June, 2013 (Fig. 1). The characteristic symptoms observed were mild mosaic mottling of leaves. Total nucleic acid was extracted from field samples of both healthy and infected leaves by cetyl-ammonium bromide method [14]. Begomovirus-specific primers, Deng 540 and Deng 541 [15], were used for the preliminary virus detection.

Rolling circle amplification and cloning

Rolling circle amplification (RCA) [16] was performed with 70 ng of total nucleic acid extracted from leaf samples using 10 units of ϕ29 DNA polymerase (Fermentas), 500 μM of exonuclease resistant random hexamer primers, and 0.1 unit of pyrophosphatase (Fermentas). RCA product was digested with different restriction endonucleases (*Bam*HI, *Eco*RI, *Hin*dIII, *Kpn*I, and *Xba*I). The ~2.7 kb fragments generated by *Xba*I and *Bam*HI digestion were purified and cloned in pUC18 vector. Recombinant clones containing apparently full-length genome fragments were screened and sequenced. The RCA products were also checked for the presence of a betasatellite component in a standard PCR reaction as described by Briddon et al. [17], using universal abutting primers β01 and β02.

Fig. 1 Mosaic symptoms on ivy gourd leaf

Sequence analysis

Full-length sequence of the selected clones was determined from Xcelris Labs Pvt. Ltd, Ahmedabad, India. Nucleotide similarity searches were performed by BLAST at NCBI (www.ncbi.nlm.nih). The names and accession numbers of begomovirus sequences used for comparison are given in Supplementary Table 1. Multiple sequence alignment was done using Clustal W (www.ebi.ac.uk) followed by phylogenetic analysis using MEGA 6.0 (www.megasoftware.net), and phylogenetic trees were constructed with the maximum likelihood algorithm, bootstrapped with 1000 replicates. Further recombination analysis to identify recombinants and recombination break points was carried out using RDP3 [18] with RDP, GENECOV, Bootscan, Max Chi, Chimara, Siscan, and 3Seq methods with a cutoff value of $P = 0.05$.

Results

Detection and molecular characterization

PCR was carried out with the universal degenerate primers (Deng 540/541), and of twenty symptomatic samples analyzed, all the twenty samples were positive and revealed ~510 bp fragment of DNA A component. No such amplification was observed in symptom-free samples. Nucleotide sequence analysis of this fragment revealed 78 % identity towards the coat protein region of the DNA A component of *Tomato leaf curl New Delhi virus*.

Upon running an aliquot of 1 μl of RCA product on the 0.8 % agarose gel, high molecular weight bands were obtained which confirmed the amplification of circular DNA. Aliquots of 2 μl of RCA product were subjected to the digestion with different endonucleases. A single fragment of ~2700 nt was obtained in the samples digested with *Xba*I and *Bam*HI. Both *Xba*I and *Bam*HI digested products were purified, and 2.7 kb DNA was cloned at the respective endonuclease site in pUC18. Recombinant plasmids were screened, sequenced, and analyzed.

Identity of the virus

Complete nucleotide sequence of *Xba*I clone was determined in both orientations. The total length of the cloned component was 2749nt and was identified to be a DNA A component (KM244719). The three clones of *Xba*I shared

99.8 % nucleotide sequence identity between themselves. In a BLAST search, the complete DNA sequence showed less than 79 % identity with any previously reported begomoviruses. Maximum nucleotide identity observed was only 78 % with *Loofa yellow mosaic virus* (LYMV-[VN]-AF509739) reported from Vietnam causing yellow mosaic disease of *Luffa acutangula.* Complete nucleotide sequence of *Bam*HI clone was determined in both orientations. The total length of the cloned component was 2691 nt and was identified to be a DNA B component (KM244718). The clones of *Bam*HI exhibited 99 % identity among three sequenced clones and shared identity with the DNA B component of begomoviruses. A maximum sequence identity of 76 % was obtained with the DNA B component of ToLCNDV (FN432357) from Pakistan on weed host, *Sonchus arvensis*.

Based on the threshold value of 91 % set for the begomovirus demarcation by the Geminivirus Taxonomy study group, ICTV [19], the present isolate of begomovirus infecting ivy gourd is considered as a new species for which the name Coccinia mosaic virus (CoMoV-Ivy gourd [TN TDV Coc1]) is proposed.

Genome organization and phylogenetic analysis of DNA A

Analysis of sequences shows that the genome organization was similar to the Old World bipartite begomoviruses encoding six ORF's, two (AV1 and AV2) in the virion sense and four (AC1, AC2, AC3 and AC4) in complementary sense (Supplementary Fig. 1a). The ORFs are separated by the intergeneric region (IR) of about 285nt length that contains a sequence of TAATATTAC in the stem loop structure, observed in geminiviruses.

When predicted amino acid sequences of inferred protein of CoMoV were compared with other begomoviruses, ORF AV1 was showing identity of 93.7 % with ToLCKV (KF551585) and ORF AV2 sharing maximum identity of 75.0 % towards *Squash leaf curl Philippines virus* (EU487041). Similarly ORF AC1 shared highest similarity with *Croton yellow vein virus* (FN543112) with 81.2 %, ORF AC2 with 61.4 % identity towards *Squash leaf curl Philippines virus* (EU487041), ORF AC3 with 60.7 % identity towards *Cotton leaf curl Multan virus* (AY765256), and ORF AC4 with 75.5 % identity towards *Pepper leaf curl Bangladesh virus* (HM007097). Upon comparison of complete DNA A sequence with other begomoviruses, it was found to exhibit 73.4 % identity with *Loofa yellow mosaic virus* (AF509739) (Supplementary Table 2). The intergeneric region between the ORFs shared a maximum identity (63 %) towards *Clerodendron yellow mosaic virus* (EF408037).

For phylogenetic analysis, complete sequences of distinct begomoviruses showing similarity with the Coccinia mosaic virus in a BLAST search were retrieved from the database. Representatives of closely related sequences of begomoviruses were selected for phylogenetic tree construction. Phylogenetic tree showed that the CoMoV occupies distinct node is well supported at 46 % of bootstrap value (Fig. 2a).

Genome organization and phylogenetic analysis of DNA B

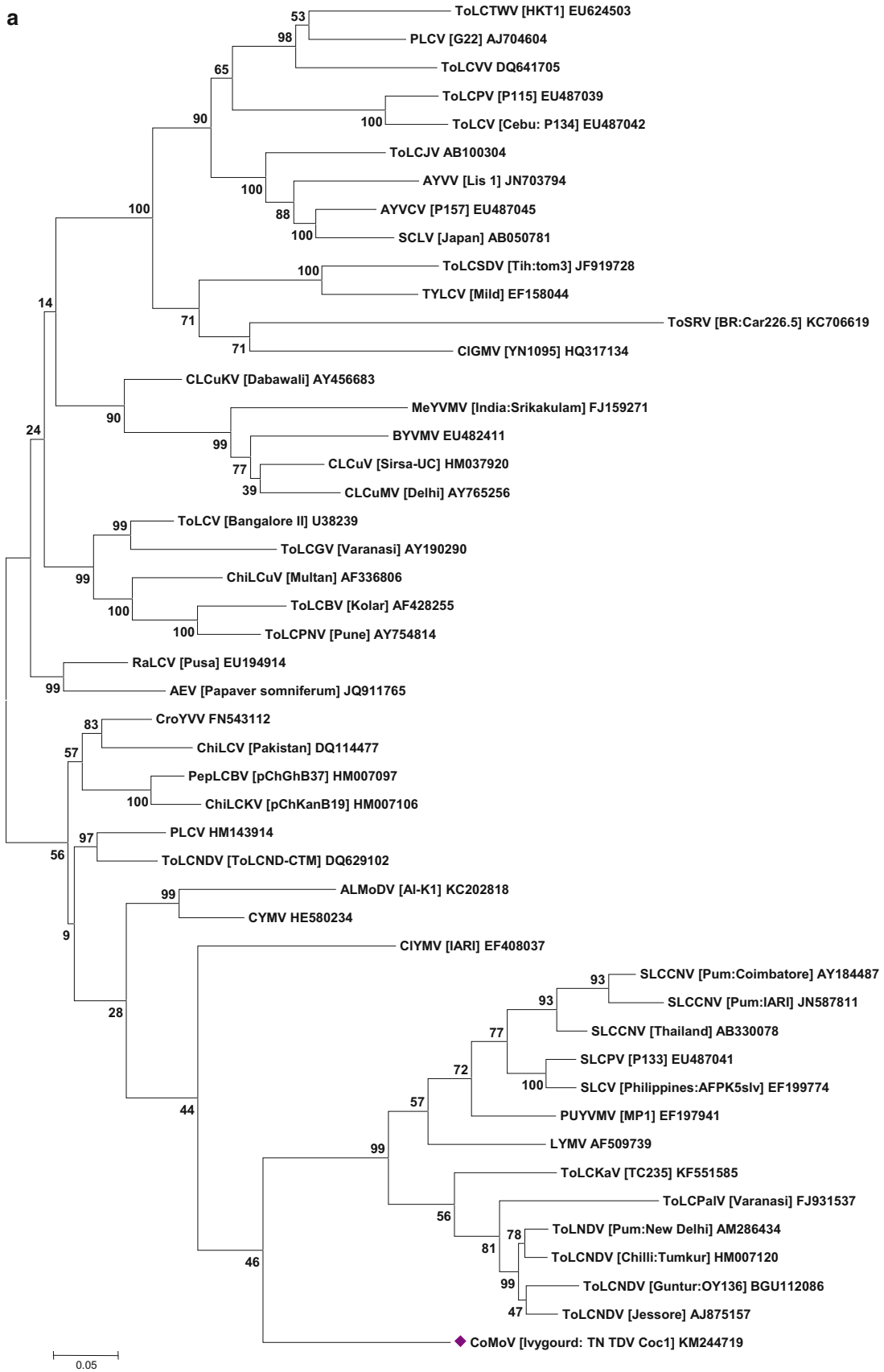
Analysis of sequences shows two ORFs, one in virion sense (BV1) and other (BC1) in complementary sense (Supplementary Fig. 1b). Complete nucleotide sequence of DNA B shared only 53 % identity towards the DNA B of ToLCNDV (AM286435). The BV1-encoded protein with 278 amino acids in *Bam*HI clone shares 52.1 % identity towards ToLCPMV (KC456162). BC1 encoding protein with 283 amino acids shares identity of 77.4 % ToLCNDV (AM286435, DQ020490, AY150304, EF043233, AY150 304, and AB330080) in *Bam*HI clone (Supplementary Table 3). The common region of DNA A extended from nt 2592 to 34 and that of DNA B from 2535 to 34 both of which share 90 % sequence identity with each other. Phylogenetic analysis of DNA B revealed that CoMoV occupies the distinct branch with other begomoviruses (Fig. 2b).

The betasatellite component could not be amplified using the beta01 and beta 02 in PCR amplification in both RCA product and total DNA extracted from infected tissue. Also in the recombination analysis using RDP3 tool, no recombination events were observed.

Discussion

Begomoviruses cause major threat to productivity of both agricultural and horticultural crops with an estimated yield loss as high as 100 % [20–22]. Begomoviruses have very high mutation and recombination rates leading to newer species evolution. Mosaic diseases caused by newer begomoviruses are increasing in various crops by recombination of viruses due to mixed infection, association of satellite molecules, and polyphagous nature of the whitefly vector [22–24].

Cucurbits are the important vegetable crops extensively grown in Indian subcontinent. The cultivation of cucurbits are mostly observed with high incidence of viral diseases. Bipartite begomoviruses infecting cucurbits in India are *Tomato leaf curl NewDelhi virus*, *Squash leaf curl China virus* (synonyms, *Pumpkin yellow vein mosaic virus*), and *Tomato leaf curl Palampur virus* on cucurbits such as



◀**Fig. 2** Phylogenetic tree of complete nucleotide sequences of DNA A (**a**) and DNA B (**b**) of Coccinia mosaic virus (TN TDV Coc 1 isolate) with other related begomoviruses. This tree was generated using Maximum Likelihood method in MEGA 6. The bootstrap consensus tree was inferred from 1000 replicates

pumpkin, bitter gourd, bottle gourd, ash gourd sponge gourd, ridge gourd, chayote, cucumber, ivy gourd, etc. [11, 25, 26]. We describe here the identification and characterization of a new bipartite begomovirus species on ivy gourd (*C. grandis*) for the first time. The cloning of begomovirus components associated with mosaic disease of ivy gourd (TN TDV CoC1) resulted in DNA A and DNA B from ivy gourd. The comparison of complete DNA A clearly established the presence of a new begomovirus species on ivy gourd which shared <91 % nucleotide identity in DNA A with the already reported begomovirus sequences in the GenBank database. According to the ICTV species demarcation criteria for begomoviruses, the virus isolate under this study is considered as a new species, and Coccinia mosaic virus has been proposed with the acronym (CoMoV-Ivy gourd [TN TDV Coc1]). Mosaic diseases were reported from many cucurbitaceous vegetable crops and cucurbitaceous weed plants in the Indian subcontinent and throughout the world. Singh et al. [6] had reported that a bipartite begomovirus, *Squash leaf curl China virus* from Varanasi, India (SLCCNV-IN[IN:Var:-Pum]), is being associated with the yellow mosaic disease of pumpkin. Typical yellow mosaic and curling of leaves were observed on the sponge gourd (*Luffa cylindrica*) in Uttar Pradesh, and it was found to be associated with ToLCNDV [27]. Similarly Tiwari et al. [28] reported that ToLCNDV causes yellow mosaic associated with slight curling of leaves in bitter gourd from Uttar Pradesh, India.

Also in the recombination analysis with RDP3, genome of this virus does not show any recombination events between known begomoviruses taken under this study. This indicates that the present isolate is found to be entirely new virus found associated with the ivy gourd in Tamil Nadu. The phylogenetic dendrogram clearly showed, CoMoV is distinct from other begomoviruses of Indian subcontinent and rest of the world infecting crop plants. Generally, begomoviruses of common geographical origin are clustered together [29] due to conserved motiff in coat protein region, which is required for recognition and transmission of the virus by a whitefly genotype [30] prevalent in that region. But the new virus species forms a separate group away from the ToLCNDV and SLCCNV. Similarly in the phylogenetic analysis, several workers reported formation of separate cluster by the distinct

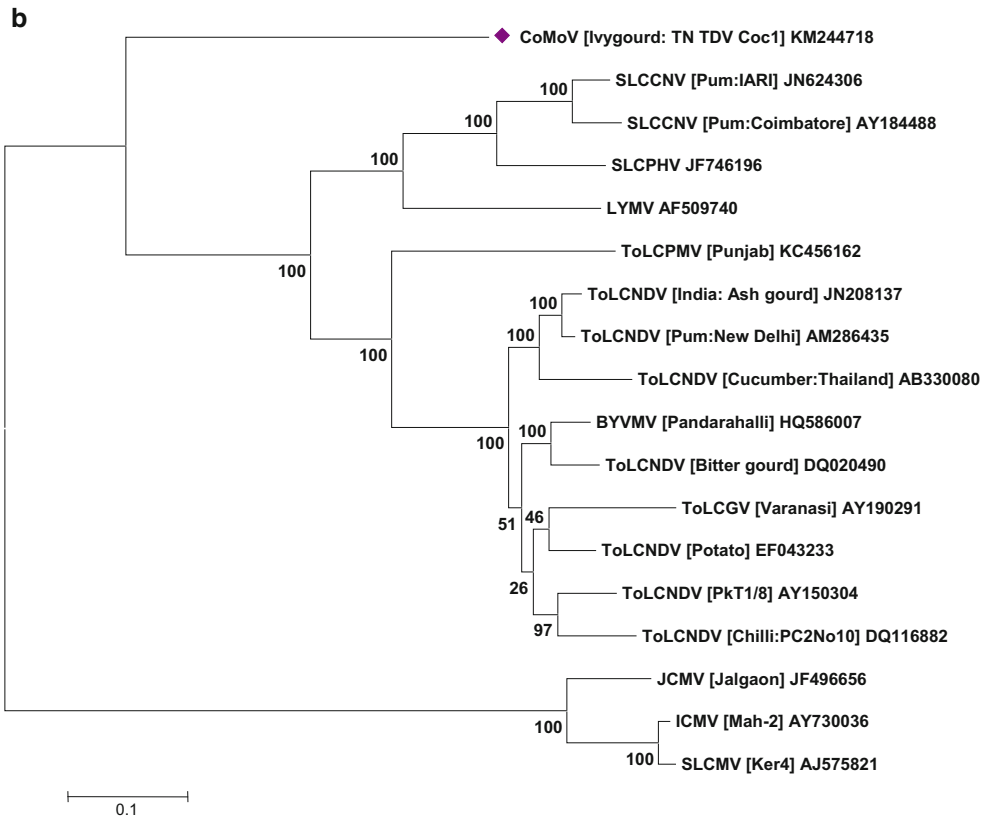


Fig. 2 continued

begomoviruses in bipartite begomovirus associated with bhendi yellow vein mosaic disease [31], bipartite *Tomato leaf curl Palampur virus* (ToLCPalV) on cucumber and cantaloupe from Iran associated with yellow mosaic disease [32], etc.

On a whole, CoMoV is found to be associated with different begomovirus in its different part of its genome. In DNA A, gene sequences had similarities with already available gene sequences viz., AV1 with ToLCKV (93.7 %), AV2 with SLCCNV (75 %), AC1 with *Croton yellow vein virus* (81.2 %), AC2 with SLCPV (61.4 %), AC3 with *Cotton leaf curl Multan virus* (60.7 %), and AC4 with *Pepper leaf curl Bangladesh virus* (75.5 %). *Bam*HI clones of DNA B sharing amino acid identity from 52.1 to 53.1 % towards ToLCPalV at BV1 and 77.4–77.8 % towards ToLCNDV at BC1. This further confirms, CoMoV as an entirely new begomovirus associated with the mosaic disease of ivy gourd. Sequences in the stem loop regions on both DNA A and DNA B are exactly same for this virus. Also, nucleotide sequences in the common region are sharing 91 % identity between DNA A and DNA B component.

We failed to detect the association of betasatellite component, suggesting that mosaic disease in ivy gourd is caused by Old World *Begomovirus* with bipartite genomes lacking betasatellite. This perennial crop (ivy gourd) may act as a reservoir for the mobilization of this virus to other cucurbitaceous crops nearby in future. Identification of this new virus in cucurbitaceous crop suggests that many other new viruses may be associated in the Indian subcontinent. Therefore, further investigation on analysis of occurrence, distribution, and diversity of begomoviruses in cucurbits crops is needed.

In conclusion, molecular characterization of this new begomovirus species with RCA can be utilized to develop the infectious clone by constructing dimers and multimers of this virus genome. This will be helpful in proving the pathogenicity of this new virus and also helps in screening of resistance source against this particular virus among different cucurbitaceous crops.

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References

1. J. Stanley, D.M. Bisaro, R.W. Briddon, J.K. Brown, C.M. Fauquet, B.D. Harrison, in *Virus Taxonomy: VIII Report of the International Committee on Taxonomy of Viruses*, ed. by C.M. Fauquet, M.A. Mayo, J. Maniloff, U. Desselberger, L.A. Ball (Elsevier Academic Press, London, 2005), pp. 301–326
2. M.S. Nawaz-ul-Rehman, C.M. Fauquet, FEBS Lett. **583**(12), 1825–1832 (2009)
3. H. Lecoq, C. Desbiez, Adv. Virus Res. **84**, 67–126 (2012)
4. S.S. Sohrab, B. Mandal, A. Ali, A. Varma, Indian J. Virol. **21**, 56–63 (2010)
5. K. Nagendran, S. Mohankumar, S.K. Manoranjitham, G. Karthikeyan, Trends Biosci. **7**(23), 3925–3931 (2014)
6. A.K. Singh, K.K. Mishra, B. Chattopadhyay, S. Chakraborty, Virus Genes **39**, 35–370 (2009)
7. S.K. Raj, S.K. Snehi, M.S. Khan, A.K. Tiwari, G.P. Rao, Australas. Plant Dis. Notes **5**, 14–16 (2010)
8. J.A. Khan, M.R. Siddiqui, B.P. Singh, Plant Dis. **86**, 328 (2002)
9. N. Rajinimala, R. Rabindran, M. Ramiah, A. Kamlakannan, Acta Phytopathol. Hun. **40**, 23–30 (2005)
10. N. Rajinimala, R. Rabindran, Australas. Plant Dis. Notes. (2007). doi:10.1071/DN07033
11. J. Namrata, R.K. Saritha, D. Datta, M. Singh, R.S. Dubey, A.B. Rai, M. Rai, Indian J. Virol. **21**(2), 128–132 (2010)
12. S.K. Raj, S.K. Snehi, M.S. Khan, A.K. Tiwari, G.P. Rao, Phytoparasitica. **39**, 497–502 (2011)
13. V. Muniyappa, M.N. Maruthi, C.R. Babitha, J. Colvin, R.W. Briddon, K.T. Rangaswamy, Ann. Appl. Biol. **142**, 323–331 (2003)
14. J.J. Doyle, J.L. Doyle, Focus **12**, 13–15 (1990)
15. D. Deng, P.F. McGrath, D.J. Robinson, B.D. Harrison, Ann. Appl. Biol. **125**, 327–336 (1994)
16. D. Haible, S. Kober, H. Jeske, J. Virol. Methods **135**, 9–16 (2006)
17. R.W. Briddon, S. Bull, I. Amin, A. Idris, S. Mansoor, I. Bedford, P. Dhawan, N. Rishi, Virology **312**, 106–1213 (2003)
18. D.P. Martin, P. Lemey, M. Lott, V. Moulton, D. Posada, P. Lefeuvre, Bioinformatics **26**, 2462–2463 (2010)
19. J.K. Brown, F.M. Zerbini, J. Navas-Castillo, E. Moriones, R. Ramos-Sobrinho, J.C. Silva, E. Fiallo-Olivé, R.W. Briddon, C. Hernández-Zepeda, A. Idris, V.G. Malathi, D.P. Martin, R. Rivera-Bustamante, S. Ueda, A. Varsani, Arch. Virol. **160**(6), 1593–1619 (2015)
20. I. Dasgupta, V.G. Malathi, S.K. Mukherjee, Curr. Sci. India. **84**, 341–354 (2003)
21. K.B. Pun, S. Doraiswamy, Indian Phytopath. **52**, 221–223 (1999)
22. A. Varma, V.G. Malathi, Ann. Appl. Biol. **142**, 145–164 (2003)
23. M. Padidam, S. Sawyer, C.M. Fauquet, Virology **265**, 218–225 (1999)
24. S. Paul, R. Ghosh, S. Chaudhuri, S.K. Ghosh, A. Roy, J. Plant Path. **91**(3), 637–647 (2009)
25. S.K. Raj, S.K. Snehi, M.S. Khan, R. Singh, A.K. Tiwari, G.P. Rao, in *Recent trends in Plant Virology. Biological, Molecular Studies and Management of Begomoviruses Affecting Cucurbitaceous Crops in India*, ed. by G.P. Rao, V.K. Baranwal, B. Monadal, N. Rishi (Studium Press LLC, New Delhi, 2012), pp. 135–155
26. R. Singh, S.K. Raj, V. Prasad, J. Phytopath. **156**, 222–228 (2008)
27. A.K. Tiwari, S.K. Snehi, R. Singh, S.K. Raj, G.P. Rao, P.K. Sharma, Arch. Phytopathol. Pfl. **45**, 62–72 (2012)
28. A.K. Tiwari, P.K. Sharma, M.S. Khan, S.K. Snehi, S.K. Raj, G.P. Rao, Med. Plants. **2**, 117–123 (2010)
29. R.W. Briddon, B.L. Patil, B. Bagewadi, M.S. Nawaz-ul-Rehman, C.M. Fauquet, BMC Evol Biol. **10**, 97 (2010)
30. P. Höfer, I.D. Bedford, P.G. Markham, H. Jeske, T. Frischmuth, Virology **236**(2), 288–295 (1997)
31. V. Venkataravanappa, C.N. Lakshminarayana Reddy, S. Jalali, M. Krishna Reddy, Virus Genes **44**, 522–535 (2012)
32. J. Heydarnejad, A. Mozaffari, H. Massumi, R. Fazeli, A.J.A. Gray, S. Meredith, F. Lakay, D.N. Shepherd, D.P. Martin, A. Varsani, Arch. Virol. **154**, 1015–1018 (2009)