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Datura Leaf Curl Betastellite Associated for the First Time with *Datura Inoxia* Leaf Curl Syndrome in India.

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ABSTRACT

Begomovirus is one of the largest group of *Bemisia tabaci* transmitted plant viruses (family *Geminiviridae*) containing single-stranded circular DNA that encapsidated in geminate particles and prevalent in the tropical and subtropical regions of the world. In the present report we identified a begomovirus infecting *Datura inoxia*, a common toxic weed found in India annually. PCR product of complete betasatellite having an expected size 1.04 kb was obtained from infected plants samples. The PCR products were suitably cloned into pGEM-T vector and sequenced, having Accession number as JQ693149 (*Datura leaf curl betasatellite*). The betasatellite had showed highest nucleotide sequence identities of 93 % with *Croton yellow vein mosaic betasatellite* (HM143908). Rcombination analysis with RDP v.3.44 clearly indicates the portion of recombinant fragment of betasatellite infecting *Datura inoxia* is contributed from the two viruses prevailing at different geographical region, undoubtedly pointing towards the betasatellite evolution.

Indexing terms/Keywords

Geminiviridae, Begomovirus, *Datura inoxia*, Betasatellites, RDP.

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INTRODUCTION

Begomovirus is one of the largest group of *Bemisia tabaci*-transmitted plant viruses (family *Geminiviridae*) containing single-stranded circular DNA encapsidated in geminate particles and prevalent in the tropical and subtropical regions of the world [1] zones and infect a wide range of plants including crops, ornamental plants and weeds [2]. New world begomoviruses genomes consist of two ssDNA components, known as DNA A and DNA B, each 2.6-2.8 kb in size. Although a small number of bipartite begomoviruses occur in the Old World, the majority are monopartite, having genomes consisting of only a homolog of the DNA A components of the bipartite viruses the majority are monopartite and associate with additional ssDNA molecules [3].

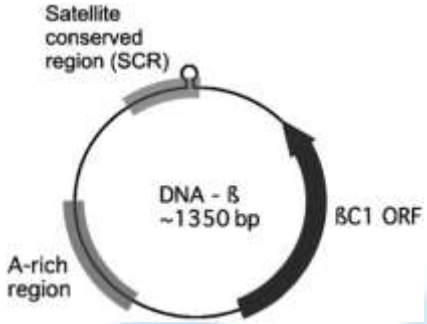

Figure 1: Genome organisation of a representative DNA-β satellite (1.4 kb) showing the key conserved domains: the adenine (A)-rich region (function unknown, 160–280 nt), the satellite conserved region (SCR) containing the origin of replication and nanonucleotide sequence associated with rolling circle replication (200 nt) and the complementary (C)-sense ORF, βC1 (pathogenicity determinants, 354 nt).

Sometimes there is also satellite like molecules associated with begomovirus components. They are designated as betasatellite and alphasatellite [4]. The beta satellites (DNA β) are large group of highly diverse ssDNA satellites that are approximately half (~1350 nt) the size of their helper begomoviruses and associate with monopartite begomoviruses [5].

The betasatellite ranges from 1.3 – 1.4 kb in size (Figure 1) that encodes a protein known as βC1, a region of sequence rich in adenine residues (A-rich). All betasatellites contains a conserved region at its 3` end, a predicted hairpin structure with similarity to the origin of virion-strand DNA replication of geminiviruses [6]. Weeds may infect crops and those weeds may serve as a reservoir for crop-infecting geminiviruses [7]. In the present report we identified a begomovirus infecting *Datura inoxia*, a common hallucinogenic alkaloid containing toxic weed found in India annually.

MATERIALS AND METHODS

Virus Sources

Datura inoxia plants were found with leaf curling disease in Rajasthan province of India. To investigate the potential betasatellite infection, infected *Datura inoxia* samples were collected.

Extraction of Total DNA

The leaf samples were cleaned, cut, rolled in a piece of tissue paper, and was stored at -20^{o}C until DNA isolation. To begin with the molecular characterization total DNA was extracted from leaves of infected as well as healthy weeds using the Cetyl Trimethyl Ammonium Bromide (CTAB) method. Samples of the total DNA product was analyzed by electrophoresis on a 1% or 2% agarose gel and the concentration was determined by using Nanodrop (Thermo Scientific).

Identification of betasatellite by PCR

To test for the presence of satellite molecule, a universal primer pair specific for betasatellite was used to amplify the putative DNA [8]. A typical PCR reaction contained about 100 ng DNA template, Taq 10 x buffers (10 mmol/L Tris-HCl, pH 8.8; 50 mmol/L KCl) 25mmol/L $MgCl_2$, 200 µmol/L of each dNTPs, 2 units of Taq DNA Polymerase, Nuclease free water and 10 pmol/L of each primer. The PCR thermal profile was pre-PCR by denaturation at 94 °C for 120 s followed by 35 cycles of denaturing at 94 °C for 60 s, annealing at 68 °C for 60 s and extension at 72 °C for 60 s, and a final extension at 72 °C for 5 min. After amplification, 4 µl aliquot from each sample was electrophoresed in a 1 % agarose gel and visualized by staining with ethidium bromide and UV illumination [9].

Cloning and Sequencing

The amplified PCR product was purified and cloned into Promega pGEMT vector system as per the manufacturer's instruction. The clones were sequenced and the details were submitted to NCBI. BLAST analysis was performed to reveal their closeness to other betasatellite sequence in the database.

Phylogenetic analysis

Based on the close sequence identity and the length of the sequences, betasatellite sequences of various begomoviruses were downloaded from Gen Bank with the accession numbers provided by the FASTA output and were fed into Molecular

Evolutionary Genetics Analysis Program (MEGA) version 4.0. The sequence of begomovirus genomic components was aligned in Clustal-W sequence alignment program using IUB matrix for DNA alignments in the MEGA v.4.0.

A phylogenetic tree was also generated by MEGA v.4.0 software by using the neighbor-joining method with 1,000 bootstrap replications. Boot strapping was applied to statistically validate the tree using NJ-plot. This analysis clustered each one of the isolates with the other previously sequenced isolate of the respective species.

Recombination Detection

Recombination between divergent genomes is believed to be a major mechanism by which diversity amongst viruses is generated. To detect the possibility of recombination in begomovirus and betasatellite, Recombination Detection Program (RDP) was utilized, which is based on a pair wise scanning approach. It usually runs under Windows 95/98/NT/XP/VISTA/7 and couples a high degree of analysis automation with an interactive and detailed graphical user interface.

Using various recombination detection methods the conclusion of recombination studies were evaluated. The recombination breakpoint could be identified by using Recombination detection program [RDP], GENECONV, Maximum-Chi, BOOTSCAN, CHIMAERA, and 3SEQ methods. All these methods were implemented in RDP v.3.44. Due to large amount of data here we are focusing on the results obtained from RDP method only.

GC Profile

GC-Profile provides a quantitative and qualitative view of genome organization. Based on the obtained results, the relationships between the G+C content and other genomic features, such as distributions of genes, can be analyzed in a perceivable manner. GC-Profile is freely available at the website http://tubic.tju.edu.cn/GC-Profile/. In addition, precompiled binaries, together with examples and documentation, can also be freely downloaded for a local execution [10]

RESULTS AND DISCUSSION

We have earlier reported begomovirus infection in *Datura inoxia* [11]. Here in this report we identified a betasatellite associated with leaf curl disease of *Datura inoxia*. Positive PCR reaction confirmed the betasatellite presence in *Datura inoxia*. PCR product of complete betasatellite having an expected size 1.04 kb was obtained from infected plants but not from symptomless samples. The PCR products were suitably cloned into pGEM-T vector and sequenced, having Accession number as JQ693149 (*Datura leaf curl betasatellite*).

The betasatellite had showed highest nucleotide sequence identities of 93 % with *Croton yellow vein mosaic betasatellite* (HM143908) infecting *Carica papaya* in India. The betasatellite revealed moderate sequence identity of 87 – 91 % with *Croton yellow vein mosaic betasatellite* (AM410551) infecting croton plants in Pakistan; *Tomato leaf curl Joydebpur betasatellite* (JF681133) found infecting *Capsicum annuum* in India.

It further confirmed lower sequence identity of 78 – 82 % with *Croton yellow vein mosaic betasatellite* (GU111995) reported from India found in association with *Abelmoschus esculentus* and *Tomato leaf curl Patna betasatellite* (HQ180394) reported from India and were found infecting *Nicotiana tabacum*.

Phylogenetic tree of the obtained sequences was generated by MEGA 4.0 software by using the neighbor-joining method with 1,000 bootstrap replications. Neighbor-Joining (NJ) analysis was carried out using Maximum Composite Likelihood model with uniform rates among the sites, the 1000 bootstraps replicates were used to evaluate the significance of generated tree. In the Neighbor-Joining tree, the β satellite of begomovirus infecting *Datura inoxia* were placed within the tree thus inferring evolutionary history. Names of related viruses were summarized in Figure 2.

Phylogenetic analysis based on the betasatellite isolated from *Datura inoxi*and other selected sequences indicates that the isolate cluster with the isolates of virus reported from India and Pakistan. This evidence enlightens the prevalence of begomovirus from neighboring countries, into India thus inferring evolutionary history. An expected consequence of this scenario would be recombination which plays an important role for the evolution of new begomovirus strains in India and these new strains are responsible for heavy loss of new host variety.

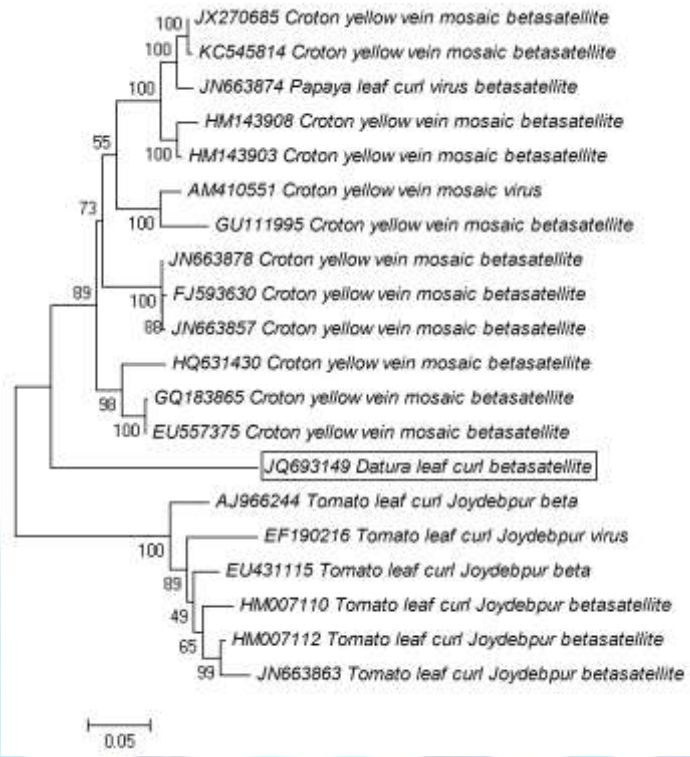

Figure 2. Phylogenetic dendrograms based on alignments of selected betasatellite sequences. Betasatellite sequences used for comparison are mentioned in the tree. Vertical branches are arbitrary, horizontal branches are proportional to calculated mutation distances. Values at nodes indicate percentage bootstrap values (1000 replicates).

Recombination is a major mechanism in virus evolution, allowing viruses to evolve more quickly by providing immediate direct access to many more areas of a sequence space than are accessible by mutation alone. Recombination analysis was performed using Recombination Detection Program (RDP v.3.44) on Windows operating system, which detects and analyses recombination points in a set of aligned DNA sequence.

The betasatellite (JQ693149) and its homologous were subjected to recombination analyses using RDP method used to drive automated recombination scan and the manual checking of automated analysis results. Analysis was allowed by employing Bonferroni correction with confidence greater than 95% (P value 0.05). In RDP analysis, the length of the window was set to 10 variable sites, and the step size was set to one nucleotide. P values were estimated by randomizing the alignment 1,000 times.

Recombination positions were observed in the betasatellite isolated from *Datura inoxia*. The schematic sequence display is where the results of automated recombination scans are presented and it is the part of the program that is used to drive the manual checking of automated analysis results (Figure 3).

The colored rectangles correspond to sequence fragments, thus representing the recombinant, major and minor parents in a graphical representation of a sequence fragments that have been potentially derived through recombination from a sequence resembling the one named to the right of the rectangle.

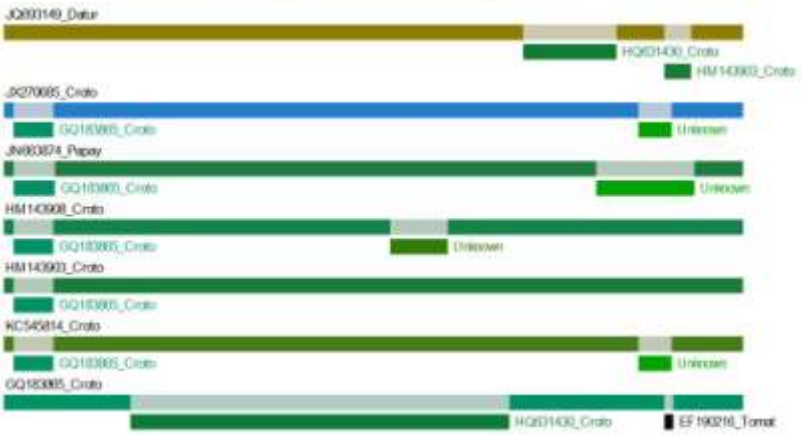

Figure 3: Diagram of the schematic sequence display representing the RDP recombination map of the recombinant fragments for the *Datura leaf curl betasatellite* (JQ693149). Each color/pattern represents a sequence specific of a virus. The virus genome organization is represented under the diagram, positioning the different viral genes named according to the begomovirus convention.

The RDP plot of JQ693149 sequence conservation displayed a graphical overview of the sequence alignment that also indicates the portion of the alignment. Within the sequence part of the display, individual nucleotides are color coded according to their degree of conservation. When a recombination event selected, the 'toggle sequence display' button can be used to highlight nucleotide polymorphisms that contribute to the recombination signals depicted in the plot display. As per the schematic sequence display two recombination break point positions were identified in the betasatellite by the RDP and GENECONV method. The first evidence is given in Figure 4 where breakpoint begin from 781th [position 1018 in alignments] position and ending breakpoint ends at 935th [position 1203 in alignments] position. Approximate p-value for this region was 1.897 x 10^{-12}.

The major parent was identified as Croton yellow vein mosaic betasatellite (KC545814) identified in New Delhi, India and were found infecting *Cucumis sativus*. Whereas the minor parent was Croton yellow vein mosaic betasatellite (HQ631430) found infecting wild *Calotropis procera* in Rajasthan, India. This clearly indicates that this portion of recombinant fragment of betasatellite infecting *Datura inoxia* is contributed from the two viruses prevailing at different geographical region, undoubtedly pointing towards the betasatellite evolution.

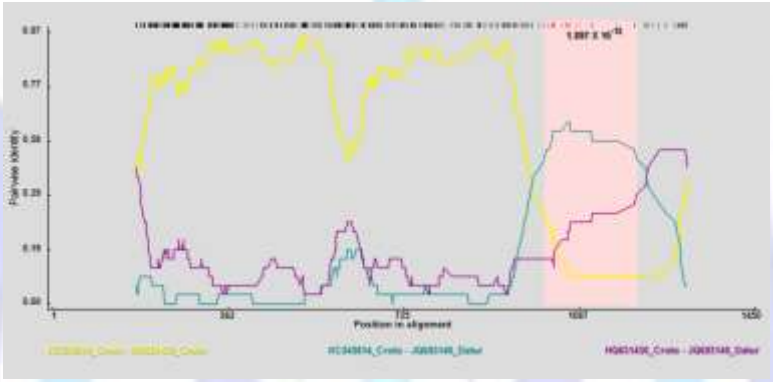

Figure 4: An RDP pairwise identity plot for the piece of sequence from the major parent (KC545814_Croto) Uppermost bares indicating positions of informative sites; pink region indicates breakpoint positions suggested by the RDP software method. The pairwise identity plot have major parent: minor parent plot (KC545814_ Croto: HQ631430_ Croto; yellow), major parent: recombinant plot (KC545814_ Croto: JQ693149_Datur; dark blue) and minor parent: recombinant plot (HQ631430_Croto: JQ693149_ Datur; purple).

The second recombination identified by GENECONV method was detected downstream of the first recombinant sequence where breakpoint begin from 1021th [position 1295 in alignments] position and ending breakpoint ends at 1042th [position 1347 in alignments] position (Figure 5). Approximate p-value for this region was 2.166 x 10^{-03}. Here the contribution of major parent in the GENECONV plot was by *Croton yellow vein mosaic betasatellite* (GQ183865) infecting *Crotalaria juncea* West Bengal, India. The minor parent was identified as Croton yellow vein mosaic betasatellite (HM143903) infecting *Carica papaya* Haryana, India.

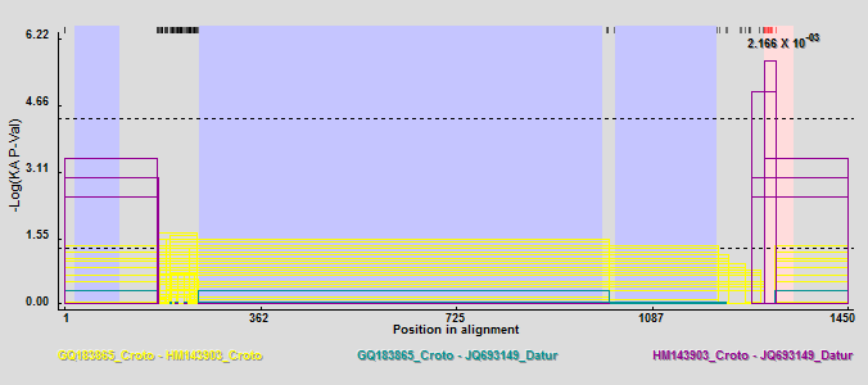

Figure 5: An GENECONV pairwise identity plot for the piece of sequence from the major parent (GQ183865_Croto) Uppermost bares indicating positions of informative sites; pink region indicates breakpoint positions suggested by the RDP software method. The pairwise identity plot have major parent: minor parent plot (GQ183865_ Croto: HM143903_ Croto; yellow), major parent: recombinant plot (GQ183865_ Croto: JQ693149_Datur; dark blue) and minor parent: recombinant plot (HM143903_Croto: JQ693149_ Datur; purple).

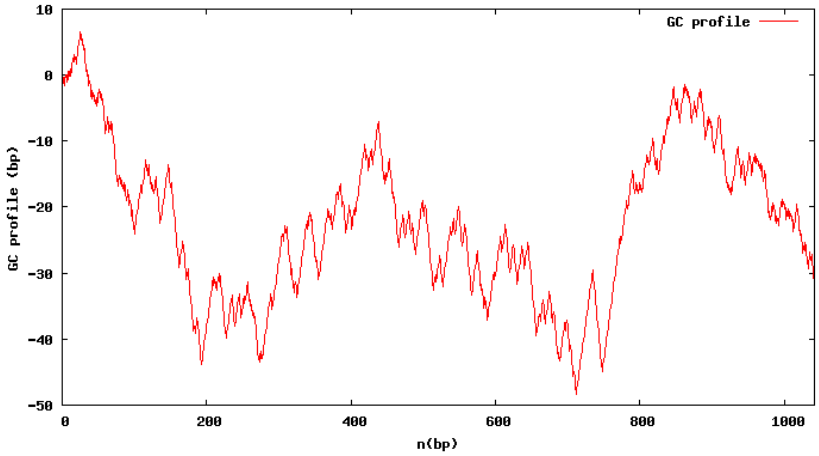

Fig 6: GC profile of JQ693149 (Datura leaf curl betasatellite).

GC-Profile implements a new segmentation algorithm based on the quadratic divergence, and integrates a windowless method for the G + C content computation, known as the cumulative GC profile. The integration of cumulative GC profile with the coordinates of segmentation points leads to a clear graphical representation of the G + C content variation along a genome or chromosome and enables us to establish the relationships between the G + C content and other genomic features [13].

There is a desert like region in an up and down distribution (Figure 6), which was calculated in complete sequence of *Datura leaf curl betasatellite* (JQ693149). It is also shown that the obtained segmentation points have clear biological implications. Two segmented boundaries were detected. There is an abrupt decrease of the density of GC profile at the first boundary below zero ranging from 100 to 350 bp. Second boundary has a poorest negative region (G + C) between 420 to 790 bp.The overall GC content of the DNA-A sequence was calculated as 42.42 %.

CONCLUSION

The present study extended our knowledge of the diversity of betasatellites associated with begomovirus disease of *Datura inoxia* in Rajasthan, Inda. We have earlier reported begomovirus infection in *Datura inoxia*. The betasatellite (JQ693149) had showed highest nucleotide sequence identities of 93 % with *Croton yellow vein mosaic betasatellite* (HM143908) infecting *Carica papaya* in India. Recombination analysis indicates that this portion of recombinant fragment of betasatellite undoubtedly pointing towards the betasatellite evolution. The result suggested that the virus in *D. inoxia* samples belonged to the genus begomovirus. To the best of our knowledge, this is the first report of begomovirus betasatellite associated with leaf curl of *D. inoxia*.

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Author' biography with Photo

Dr Rajneesh Prajapat

Dr. Rajneesh Prajapat has completed his under the supervision of Dr R K Gaur on the project entitled "In Silico molecular characterization of Begomovirus in Rajasthan, India. He graduated from M.D.S University, Ajmer, Rajasthan (India) in 2003 and did M.Sc Biotechnology from Universiry of Rajasthan, Jaipur, Rajasthan (India) in 2005 and obtains M.Phil in Environmental Biotechnology from Department of Environmental studies, M.D.S University, Ajmer, Rajasthan (India) in 2006. He has 30 publications and published 3 books on Bioinformatics and Virology.

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