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Computational Characterization of *Begomovirus* Infecting Two Ornamental Plants: *Jasminum sambac* and *Millingtonia hortensis*

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ABSTRACT

Recombination plays a key role in the evolution of *Begomovirus* and may be contributing to the emergence of new species. With the development of computational recombination detection tools and an increasing number of available genome sequences, many studies have reported evidence of recombination. *Begomovirus* associated symptoms were observed in *Jasminum sambac* and *Millingtonia hortensis* plants growing in crop fields of Lakshmangarh, Rajasthan (India). Amplification of a PCR product was found up to the expected size (~550 bp). The PCR product was cloned and partially sequenced and it was utilized for *in silico* characterization. The *in silico* analysis suggested that interspecific recombination has resulted in significant diversity among *Begomovirus*.

Key words: Recombination, *Jasminum sambac*, *Millingtonia hortensis*, *in silico*, *Begomovirus*

INTRODUCTION

Jasminum sambac (Family Oleaceae) is a persistent shrub which reaches up to a height of 5 feet. The flowers are white in colour. *J. sambac*, probably originated in tropical India and Burma. The flowers are used in the manufacture of the perfumes and aromatizing. Peoples used to ornament their hairs and the neck in the form of collar by using the Liana form of this plant (Abdoul-Latif *et al*., 2010). *M. hortensis* belongs to the Family Bignoniaceae and is widely scattered in many part of India specially in the semi arid regions. *M. hortensis* is generally known as 'Akas neem' and also as the "Indian cork tree" (Kaushik and Saini, 2008). Therefore, molecular characterization and understanding of the genomic analysis of *J. sambac* and *M. hortensis* infecting *Begomovirus* is imperative for the pathogen diagnosis and disease management.

Geminiviruses members have circular single stranded DNA (ssDNA) genome that is remaining encapsidated in twinned quasi isometric particles (18×30 nm) (Moffat, 1999). On the basis of genome organization Geminiviridae family has been classified into four genera viz., *Mastrevirus, Curtovirus*, *Topocuvirus* and *Begomovirus* (Fauquet *et al*., 2005). *Begomovirus* is the only genus of the geminiviridae having bipartite with virus genes resident on two different circular ssDNA molecules (DNA A, DNA B) each of about 2.6-2.8 kb (Hanley-Bowdoin *et al*., 1999) or monopartite with all genes resident on one (DNA A-like) ssDNA of about 2.8 kb. *Begomovirus* is one of the largest genus of the family geminiviridae (Mansoor *et al*., 2003) and the vector white fly (*Bemisia tabaci*) is prevalent in the tropical and subtropical regions of the world

(Markham *et al*., 1994). Some monopartite begomoviruses are associated with beta satellites (DNAβ) which require begomoviruses for replication, encapsidation, insect transmission and movement in plants (Stanley *et al*., 2005). Increasing knowledge about its epidemiology, sequence diversity and biodiversity is highly important in order to implement preventative strategies.

Recombination has played and continues to play, a pivotal role in geminiviral evolution and may be contributing to the emergence of new forms of geminiviruses because the high frequency of mixed infections of begomoviruses provides an opportunity for the emergence of new viruses arising from recombination among strains and/or species (Harrison and Robinson, 1999; Power, 2002). The recombinants which have originated from two or more species sometimes reveal a new pathogenic phenotype which is often more virulent than the parents (Zhou *et al*., 1997). Hence, the object of this study is molecular, phylogenetic and in silico recombinational analysis of begomoviruses infecting two ornamental plants viz., *Jasminum sambac* and *Millingtonia hortensis*.

MATERIALS AND METHODS

Samples collection and DNA extraction: Survey for the epidemiology of *Begomovirus* was carried out in 2010-2011. *Begomovirus* associated symptoms were observed in *J. sambac* and *M. hortensis* plants growing in garden fields of Lakshmangarh, Rajasthan (India). To investigate the possibility Total DNA was extracted from leaves of plants with and without symptoms using CTAB (Cetyl Trimethyl Ammonium Bromide) method (Manen *et al*., 2005).

PCR amplification: PCR was performed using a pair of primers designed to the coat protein region of *Begomovirus* (Hallan, 1998). Forward primer sequence was GGRTTDGARGCATGHGTACATG (AC 1048) and reverse primer sequence was GCCYATRTAYAGRAAGCCMAG (AV 494). A typical PCR reaction contained about 100 ng DNA template, *Taq* 10x buffers (10 mM tris-HCl, pH 8.8, 50 mM KCl and 1% [v/v]) 25 mM $MgCl_2$, 200 μM of each dNTPs, 2 units of Taq DNA Polymerase, Nuclease free water and 10 pM of each primer. The PCR thermal profile were pre-PCR denaturation at 94°C for 120 sec followed by 35 cycles of denaturing at 94°C for 45 sec, annealing at 55°C for 60 sec and extension at 72°C for 60 sec and a final extension at 72°C for 5 min.

Cloning, sequencing and phylogenetic tree construction: PCR product of ~550 bp from infected *J. sambac* and *M. hortensis* samples was cloned and partially sequenced and has been deposited in NCBI GenBank having Accession No: JN998445 and JN998446, respectively. Homology sequence search was carried out through BLASTn using which Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 4.0 (Tamura *et al*., 2007).

RDP: To detect the possibility of recombination in *Geminivirus* isolates by using their sequence information 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 (Marwal *et al*., 2012).

RESULTS AND DISCUSSION

Begomovirus associated symptoms such as Leaf curl disease of *J. sambac* (Fig. 1) and yellow vein mosaic disease of *M. hortensis* (Fig. 2) was observed on several plants growing in the gardens of Lakshmangarh (Rajasthan) in Oct. 2011.

Fig. 1: Symptoms of leaf curl disease in *Jasminum sambac*

Fig. 2: Vein yellowing and mosaic symptoms on *Millingtonia hortensis*

Through PCR amplification product of the expected size (approximately 550 bp) was produced from all symptomatic samples of both *J. sambac* and *M. hortensis* but not from non-symptomatic samples. BLAST analysis was conducted with *Geminivirus* sequences available in the GenBank database (Altschul *et al*., 1997). According to ICTV guidelines the rule-of-thumb value of <89% nucleotide sequence identity threshold for DNA-A define an isolate as a distinct *Begomovirus* species (Fauquet *et al*., 2003; Brown *et al*., 2005). In the case of *J. sambac* the alignment process of begomoviral sequence revealed 96% identity each with Sonchus yellow mosaic virus isolate MP 2 coat protein gene, partial cds (JN000703) and Ageratum enation virus-Lucknow coat protein (AV1) gene, complete cds (DQ343286). Whereas, in the case of *M. hortensis* BLASTn analysis of begomoviral sequence showed 94% identity with Rose leaf curl virus clone RoLCuV-[PK, Fai, 06], complete genome (GQ478342) and 93% identity with Catharanthus yellow mosaic virus complete genome, clone KN5 (HE580235). Phylogenetic analysis of coat protein gene isolated from *J. sambac* (Fig. 3) and *M. hortensis* (Fig. 4) was done by using MEGA 4.0 showing the relationship with other closely related viruses.

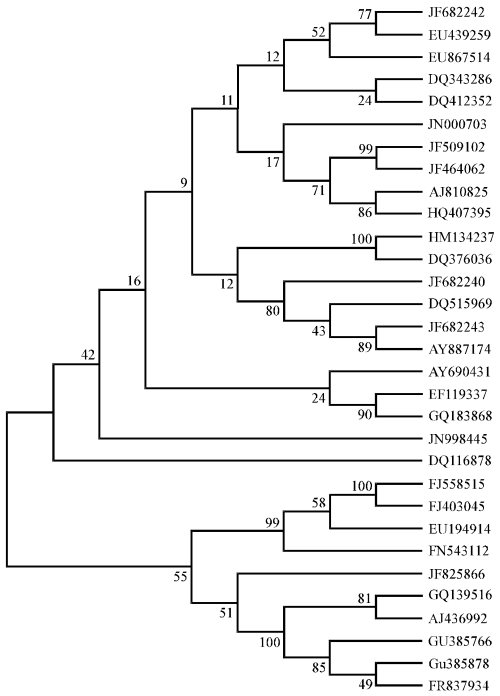

Fig. 3: Neighbor-Joining tree based on the partial sequence of coat protein gene (JN998445), of the virus isolated from *Jasminum sambac* and other *Begomovirus* sequences available in GenBank. JF682242: Ageratum enation virus isolate Lucknow coat protein (AV1) gene, complete cds. EU439259: Ageratum enation virus-Lucknow coat protein (AV1) gene, complete cds. EU867514: Ageratum enation virus-Gorakhpur AV1 gene, complete cds. DQ343286: Ageratum enation virus-Lucknow coat protein (AV1) gene, complete cds. GQ412352: Ageratum enation virus-Gorakhpur coat protein (AV1) gene, complete cds. JN000703: Sonchus yellow mosaic virus isolate MP 2 coat protein gene, partial cds. JF509102: Tobacco curly shoot virus isolate COB5 coat protein (AV1) gene, complete cds. JF461062: Tobacco curly shoot virus isolate Cob4 coat protein (AV1) gene, complete cds. AJ810825: Ageratum yellow vein virus-Pakistan V2 gene for coat protein. HQ407395: Tobacco curly shoot virus isolate WSF1 segment A, complete sequence. HM134237: Ageratum enation virus-B2 [India: Haryana: Papaya: 2009] coat protein gene, complete cds. DQ376036: Papaya leaf curl virus isolate PD coat protein gene, complete cds. JF682240: Ageratum enation virus isolate Lucknow coat protein (AV1) gene, complete cds. DQ515969: Ageratum enation virus-Lucknow coat protein (AV1) gene, complete cds. JF682243: Ageratum enation virus isolate Lucknow coat protein (AV1) gene, complete cds. AY887174: Calendulla yellow net virus segment DNA-A coat protein gene, complete cds. AY690431: Tomato leaf curl virus coat protein (AV1) gene, complete cds. EF119337: Crotalaria juncea *Begomovirus* coat protein (CP) gene, complete cds. GQ183868: Sunn hemp leaf distortion virus [India: Barrackpore3:2008] segment DNA-A, complete sequence. JN998445: Jasminum sambac leaf curl Lakshmangarh virus isolate LW coat protein gene, partial cds. DQ116878: Pepper leaf curl Pakistan virus isolate Khanewal 1 clone PC8 segment A, complete sequence. FJ558515: Chilli leaf curl virus coat protein (CP) gene, partial cds. FJ403045 Chilli leaf curl virus coat protein (AV1) gene, partial cds. EU194914: Radish leaf curl virus isolate Pusa Bihar, complete genome. FN543112: Croton yellow vein virus, complete genome, clone 1. JF825866: Tomato leaf curl Bangladesh virus isolate GUW-1 coat protein (AV1) gene, complete cds. GQ139516: Papaya leaf curl virus clone CPT coat protein gene, complete cds. AJ436992: Papaya leaf curl virus complete genome. GU385766: Cotton leaf curl virus isolate Lucknow movement/precoat protein (AV2) gene, partial cds; coat protein (AV1) gene, complete cds; and replication enhancer protein gene, partial cds. GU385878: Cotton leaf curl Kokhran virus clone jit-1 coat protein (CP) gene, complete cds. FR837934: Cotton leaf curl Burewala virus complete genome, clone MV14C

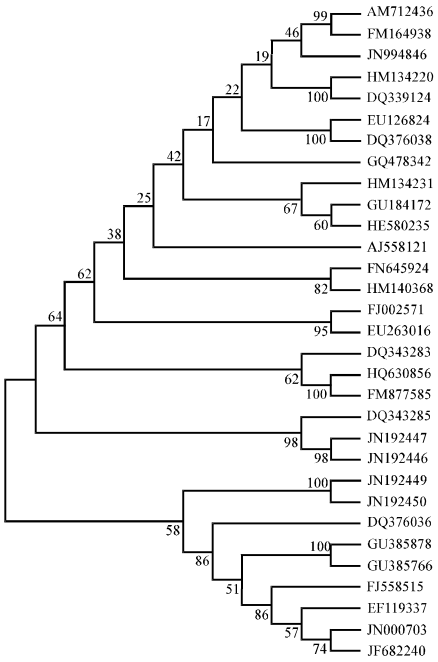

Fig. 4: Neighbor-Joining tree based on the partial sequence of coat protein gene (JN998446), of the virus isolated from *Millingtonia hortensis* and other *Begomovirus* sequences available in GenBank. AM712436: Pedilanthus leaf curl virus-Pedilanthus [Pakistan: Multan: 2004] complete genome. FM164938: Tomato leaf curl Pakistan virus, DNA A, complete genome. JN998446: Millingtonia hortensis yellow vein mosaic Lakshmangarh virus isolate L8 coat protein gene, partial cds. HM134220: Pedilanthus leaf curl virus-HD [India:New Delhi: Papaya 2007] coat protein gene, complete cds. DQ339124: Whitefly-transmitted Indian *Begomovirus* from Tabernaemontana divaricata coat protein (AV1) gene, complete cds. EU126824: Papaya leaf curl virus from Carica papaya cv. Coimbatore-2 coat protein gene, complete cds. DQ376038: Papaya leaf curl virus isolate Oad coat protein gene, complete cds. GQ478342: Rose leaf curl virus clone RoLCuV-[PK, Fai, 06], complete genome. HM134231: Pedilanthus leaf curl virus-Naj 1[India:New Delhi:Papaya:2008] coat protein gene, complete cds. GU184172: Duranta leaf curl virus isolate Ludhiana coat protein (AV1) gene, partial cds. HE580235: Catharanthus yellow mosaic virus complete genome, clone KN5. AJ558121: Euphorbia leaf curl virus-[G35] DNA A, complete sequence, isolate G35. FN645924: Tomato leaf curl Karnataka virus partial AV1 gene for coat protein, clone 6-PCR A1. HM140368: Papaya leaf crumple virus-Nirulas [India:New Delhi:Papaya:2007], complete genome. FJ002571: Tomato leaf curl virus isolate MS-6 coat protein (AV1) gene, complete cds. EU263016: Tomato leaf curl virus isolate MV3 coat protein (AV1) gene, complete cds. DQ343283: Cotton leaf curl Kokhran virus from soybean coat protein gene, complete cds. HQ630856: Papaya leaf curl virus isolate Lucknow coat protein (CP) gene, complete cds. FM877858: Chilli leaf curl India virus segment A, complete genome. DQ343285: Pepper leaf curl virus from soybean coat protein gene, complete cds. JN192447: Pepper leaf curl virus isolate Gorakhpur coat protein gene, partial cds. JN192446: Pepper leaf curl virus isolate Maharajganj coat protein gene, partial cds. JN192449: Pepper leaf curl virus isolate Mirzapur coat protein gene, partial cds. JN192450: Pepper leaf curl virus isolate Mirzapur pre-coat protein (AV2) gene, complete cds. DQ376036: Papaya leaf curl virus isolate PD coat protein gene, complete cds. GU385878: Cotton leaf curl Kokhran virus clone jit-1 coat protein (CP) gene, complete cds. GU385766: Cotton leaf curl virus isolate Lucknow movement/precoat protein (AV2) gene, partial cds; coat protein (AV1) gene, complete cds; and replication enhancer protein gene, partial cds. FJ558515: Chilli leaf curl virus coat protein (CP) gene, partial cds. EF119337: Crotalaria juncea *Begomovirus* coat protein (CP) gene, complete cds. JN000703: Sonchus yellow mosaic virus isolate MP 2 coat protein gene, partial cds. JF682240: Ageratum enation virus isolate Lucknow coat protein (AV1) gene, complete CDs

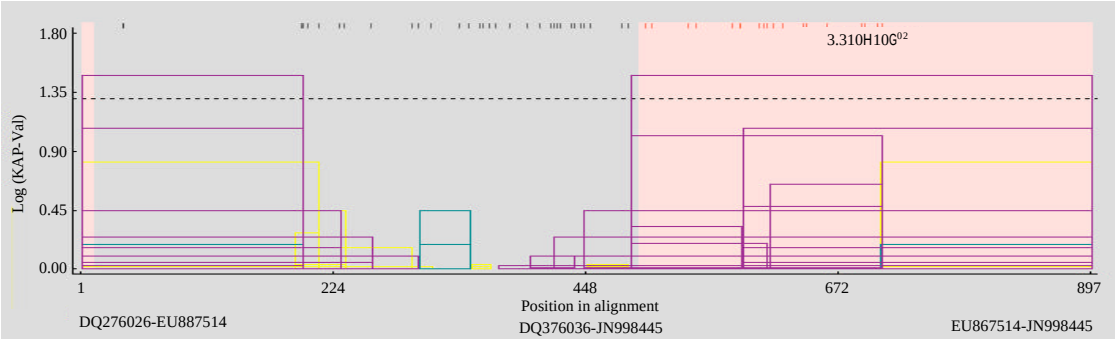

Fig. 5: The GENECONV plot of high scoring fragment (EU867514) in recombinant virus infecting *J. sambac* (JN998445) for coat protein gene. Major parent was DQ376036 and minor parent was EU867514. The pairwise identity plot have major parent: minor parent plot (DQ376036: EU867514; yellow), major parent: recombinant plot (DQ376036: JN998445; blue) and minor parent: recombinant plot (EU867514: JN998445; purple)

Table 1: The different recombination detection methods available in RDP3

Method	Estimates regions	Estimates breakpoints	Identifies recombinants	p-Value calculation	References
RDP method	+	+	+	Binomial distribution	Martin and Rybicki (2000)
GENECONV	+	+	+	Blast-Like Karlin-Altschul and Permutation	Padidam *et al.* (1999)
BOOTSCAN	+	+	+	Bootstrapping , binomial distribution and χ^2	Salminen *et al.* (1995)
Maximum	+/-	+	+	χ^2 and Permutation	Smith (1992)
CHIMAERA	+/-	+	+	$\chi^2$and Permutation	Posada and Crandall (2001)

+: Absolute chance of program to identify recombinants, estimate breakpoints and estimate regions through RDP, GENECONV, BOOTSCAN, Maximum χ^2 and CHIMAERA methods. +/-: Half the chance of program to estimates regions through Maximum χ^2 and CHIMAERA methods, RDP3: Instruction manual at http://darwin.uvigo.es/rdp/rdp.html, (Marwal *et al.*, 2012)

Using various recombination detection method the conclusion of recombination studies are evaluated (Posada and Crandall, 2001; Posada, 2002). The recombination breakpoint could be identified by using Recombination Detection Program [RDP], GENECONV, Maximum-Chi, BOOTSCAN, CHIMAERA and 3SEQ methods (Table 1). All these methods were implemented in RDP v.3.44 (Martin *et al.*, 2005).

Gene Conversion detecting program (GENECONV) (Padidam *et al.*, 1999; Sawyer, 1989) looks for regions within a sequence alignment in which sequence pairs are sufficiently similar to suspect that they may have arisen through recombination (Fig. 5). This method used for triplet scanning (used in exploratory analyses) is identical to that used for pair scanning (used in manual analyses) except that instead of analyzing the entire alignment the triplet scan splits the alignment up into every possible alignment of three sequences and analyses each of these alignments separately. The major parent was found to be Papaya leaf curl virus isolate PD coat protein gene, complete cds (DQ376036) and minor parent was found to be Ageratum enation virus-Gorakhpur AV1 gene, complete cds (EU867514). The approximate p-value was 3.310×10^{-2}. In this case the left and right bounds of the pink region indicate breakpoint positions suggested by

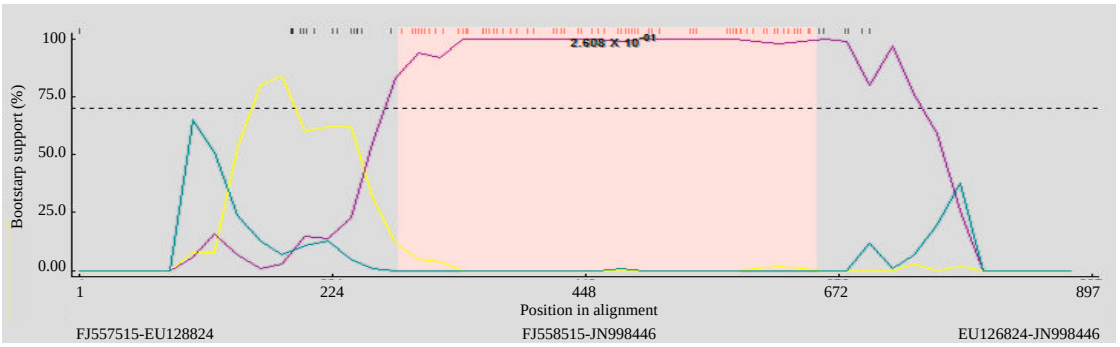

Fig. 6: A BootScan pairwise identity plot of *Begomovirus* infecting *M. hortensis* for the gene region encoding for coat protein [JN998446]. Uppermost bares indicating positions of informative sites, pink region indicates breakpoint positions suggested by the BootScan method. The pairwise identity plot had major parent: minor parent plot (FJ558515: EU126824; yellow), major parent: recombinant plot (FJ558515: JN998446; blue) and minor parent: recombinant plot (EU126824: JN998446; purple)

the GENECONV method. The breakpoint begins from position 0th [position 10 in alignment] in alignment and ending breakpoint at position 306th [position 495 in alignment] in alignment of JN998445.

BootScan was used to identify the parental origins of sequence blocs (Salminen *et al*., 1995) within suspected recombinant sequence JN998446 (Fig. 6). In its original implementation BootScan involved: (1) Construction of an alignment containing a potentially recombinant sequence and a set of (non-recombinant) reference sequences. (2) Moving a window of set length along the alignment, a set number of nucleotides at a time and calculating a bootstrapped Neighbour Joining tree for each window. (3) Plotting the relative bootstrap support for nearest neighbour groupings of the potentially recombinant sequence with each of the reference sequences at each window position.

The major parent found was Chilli leaf curl virus Coat Protein (CP) gene, partial cds (FJ558515) and the minor was Papaya leaf curl virus from *Carica papaya* cv. Coimbatore-2 coat protein gene, complete cds (EU126824). The piece of sequence from major parent (FJ558515) that breakpoint begin from 95th [position 292 in alignment] position and ending breakpoint at 465th [position 652 in alignment] position of JN998446. Approximate p-value for this region was 2.608×10^{-1}.

Recombination: Major mechanism involved 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 positions in virus infecting Jasminum sambac leaf curl Lakshmangarh virus (JN998445) and in Millingtonia hortensis yellow vein mosaic Lakshmangarh virus (JN998446) were identified (Fig. 7, 8).

The colored rectangles represent sequence fragments from major and minor parents. This is where the results of automated recombination scans were presented and it was the

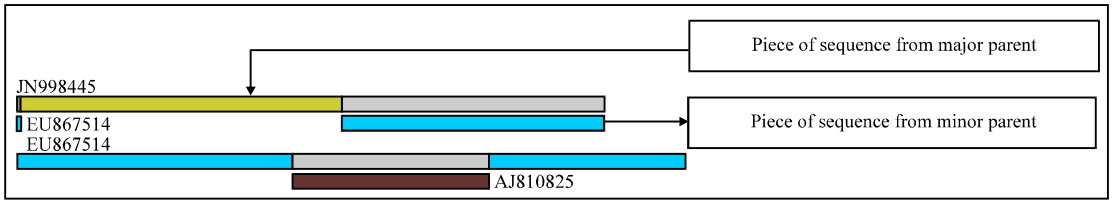

Fig. 7: The schematic sequence display. The coloured rectangles represent sequence fragments representing the recombinant (JN998445), major parent (DQ376036) and minor parent (EU867514)

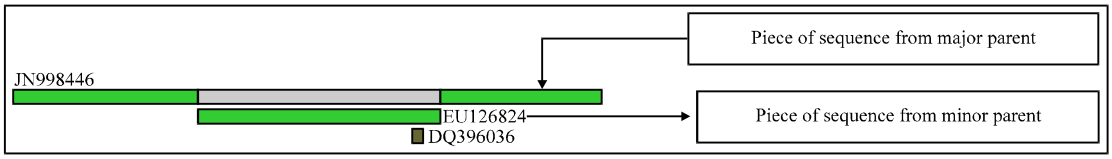

Fig. 8: The schematic sequence display. The coloured rectangles represent sequence fragments representing the recombinant (JN998446), major parent (FJ558515) and minor parent (EU126824)

part of the program that was used to drive the manual checking of automated analysis results. The coloured rectangles represent sequence fragments of major and minor parent.

CONCLUSION

Geminiviruses cause a variety of symptoms in host plant species and are spreading easily due to a high rate of recombination and pseudorecombination events that contribute in the evolution of new viral species. For the coming out of new begomoviral diseases. Inter-specific recombination has a better involvement. *J. sambac* has shown 96% identity with Sonchus yellow mosaic virus isolate MP 2 coat protein gene, partial cds (JN000703) and *M. hortensis* showed 94% identity with Rose leaf curl virus clone RoLCuV-[PK,Fai,06], complete genome (GQ478342). *J. sambac* and *M. hortensis* plant showed typical begomoviral symptoms in leaves which was identified and confirmed through PCR using coat protein gene specific primers and BLAST analysis. Uses of computational recombination detection tools such as RDP, have demonstrated the evidence of recombination in a wide range of the available genome sequences of *Begomovirus*. This study could be used to understand the role of recombination and pseudorecombination in evolution of new *Begomovirus* species and genetic diversity.

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