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Phylogenetic relationship of coat protein genomic components of *Chilli leaf curl virus*

DP Sinha, S Saxena, M Singh and Shailesh K Tiwari

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Abstract: Chilli (*Capsicum annuum* L.) is one of the economically most important vegetable crops in India. Leaf curl disease in chilli, caused by begomovirus is a serious problem in northern India. The causal virus is transmitted to healthy plants by white flies (*Bemisia tabaci*). Infected plants exhibit typical curling of leaves, puckering, twisted petioles, and stunting. The confirmation of geminivirideae from leaf samples by Polymerase Chain Reaction (PCR) based preliminary detection of virus was done from the infected chilli samples by amplifying DNA fragments (approximately 550 bp) using begomovirus specific- coat protein (CP) gene primers. Further, PCR based detection conditions were optimized for chilli leaf curl virus using specific primers 5‘-AGAATTATGTCCAAGCGACCA-3‘ and 5‘-AAGC GTTGGGGATACACAAA-3‘. The 750 bp amplicon of CP gene was amplified, cloned and sequenced. Phylogenetic reconstructions of CP sequence with other begomovirus sequences retrieved from GenBank reveal close relationship among Indian and other asiatic isolates. This is the first report of the molecular characterization of coat protein sequence of chilli leaf curl virus and chilli plant is affected by radish leaf curl disease in India. The homology of the sequenced CP gene with other reported genes in GenBank database was 97% with other begomovirus coat protein gene.

Keywords: Begomovirus, *Capsicum annuum,* Chilli, Coat protein, PepLCV

Introduction

Members of the family Geminiviridae comprising of the genera *Mastrevirus*, *Curtovirus*, *Topocuvirus*, and *Begomovirus,* are plant pathogens with circular single-stranded DNA genomes encapsidated in twin particles. The genus *Begomovirus* is the largest genus of this family comprising of the whitefly transmitted geminiviruses that infect dicotyledonous plants. They are differentiated based upon host range, genome organization and insect vector specificity (Saunders *et al.,* 1991; Lazarowitz 1992). Most begomoviruses have a bipartite genome consisting of a DNA-A and DNA-B components. DNA-A encodes for genes responsible for viral replication, regulation of gene expression, suppression of gene silencing and particle encapsidation, while DNA-B encodes for proteins involved in viral movement, host range determination and symptom development (Lazarowitz 1992). Notable exceptions are the tomato (yellow) leaf curl viruses from the Near East, the Mediterranean basin, Australia, and India, whose genomes are monopartite (Lazarowitz 1992).

Begomovirus has been reported affecting a number of economically important food and fibre crops such as cassava, tomatoes, cucurbits, pepper, beans and cotton worldwide (Varma and Malathi, 2003). Chilli (*Capsicum annuum* L.) a member of *Solanaceae* is an important vegetable and spice crop of India. The occurrence of chilli leaf curl disease caused by *begomovirus*, namely pepper leaf curl virus (PepLCV) has been reported in India (Mishra *et al.,* 1963; Dhanraj and Seth, 1968; Raj *et al.,* 2005), United states (Stenger *et al.,* 1990) Nigeria (Alegbejo 1990) and several other countries such as Pakistan, Bangladesh and Indonesia (Fauquet and Stanley 2003). A plethora of new and non-characterized begomoviruses isolated from diverse locations world-wide necessitates the development of an accurate and simple methodology for their rapid and accurate identification. Consequently, DNA-based diagnostic approaches including polymerase chain reaction (PCR) amplification and DNA sequencing have complemented the detection, identification and classification of begomoviruses. To date, classification and phylogenetic relationships of begomoviruses have been based upon

D.P.Sinha, M. Singh and Shailesh K. Tiwari
Indian Institute of Vegetable Research, PB-01, P.O.-Jhakhini, Shahanshahpur, Varanasi, Uttar Pradesh, 221 305, India
S.Saxena
Department of Biotechnology, Babasaheb Bhimrao Ambedkar University, Lucknow, Uttar Pradesh, India

complete monopartite viral genomes or the component A of bipartite viruses, coat protein (CP) and/or replication association protein gene (Rep) trees (Rybicki, 1994; Padidam *et al.,* 1995; Brown *et al.,* 1999; 2000).

Although complete genome sequences are desirable for identification and classification of geminiviruses, this requires considerable time and expense. Full-length CP-gene sequences (Idris *et al.,* 1998; Paximadis *et al.,* 1998) are accepted by the International Committee on the Taxonomy of Viruses (ICTV) for the provisional classification of begomoviruses when complete genome (monopartite virus) or A component (bipartite virus) sequences are unavailable (Mayo *et al.,* 1998).

CP-genes have traditionally proven useful for plant virus identification and classification (Idris *et al.,* 1998). At the 5'end of the begomoviral CP-gene 200 nucleotides (nt) are highly variable and this region is an informative region for predicting taxonomic relationships within the genus *Begomovirus* (Mayo *et al.,* 1998); Padidam et al., 1995). We examined the utility of this region of CP-gene to identify over 39 field isolates from representative crops, worldwide for phylogenetic analysis. Such analyses (BLAST results) using CP sequence data, should simplify begomovirus characterization.

Materials and Methods

Materials

Diseased leaf samples were collected from infected plants showing symptoms of leaf curl, vein yellowing or stunting from farm of Indian Institute of Vegetable Research, Varanasi. The young infected leaves exhibiting distinct symptoms of curling of leaves were collected from infected plant, stored in plastic bags and carried to the lab for further study.

Viral DNA isolation

For plant DNA isolation protocol by Doyle and Doyle (1990) used with minor modification. Further, viral DNA was separated from total plant DNA by modifying alkaline lysis plasmid isolation protocol of Birnboim and Doly (1979). For this fresh lysis solution (0.2N NaOH, 1% SDS) (1:2 v/v) was added to the total plant DNA. Mixture was gently mixed and kept at room temperature for 10 min, followed by 15 min on ice. Ice cold solution of 3M NaoAc, pH 4.8 was mixed on ice, centrifuged at 15,000 rpm for 30 min at 4ºC, supernatant was collected and equal volume phenol:chloroform:isoamyle alcohol (25:24:1) was added and centrifuged at 13000 rpm for 10 min at 20ºC. Aqueous phase was collected and 2.5 volume of ethanol was mixed gently and kept at -20ºC for one hour. The precipitate was centrifuged at high speed, washed twice with 70% ehanol, air dried, dissolved in minimal amount of sterile double distilled water and stored at -20ºC.

Detection of geminiviral nuclic acid

PCR amplification was carried out according to Kumar et al. (2006) with minor modifications. Amplification was carried out in 25 ìl reaction volume containing 50 ng genomic DNA, 2.5 ìl PCR buffer (MBI Fermentas, Hanover, USA), 200 ìM dNTPs (Bangalore Genei, Bangalore, India), 1.5U *Taq* DNA polymerase (MBI Fermentas) and 0.4 ìM primer using a thermal cycler (Biorad, USA). The first amplification cycle consisted of 94°C for 4 min, at Tm 55°C for 1 min and at 72°C for 1 min. This was followed by 36 cycles with 1 min at 94°C, 1 min at 55°C and 1 min and 72°C; the final extension was allowed for 10 min at 72°C. The amplified DNA fragments were resolved through electrophoresis in 1.5% agarose gel prepared in TBE buffer (54.0 g Tris-base, 27.5 g boric acid, 0.5 M EDTA (pH 8.0) in 1000 ml) and visualized in a gel documentation system (AlfaImager 2200, Alfa Innotech Corporation, California). The 1 kb DNA ladder (MBI Fermentas) was used as molecular size marker. The amplification was repeated 2 to 3 times to ensure the reproducibility and consistency of the amplification product.

Cloning and DNA sequencing

The PCR amplification product of approximately 750bp was excised from the agarose gel and the DNA was eluted using a gel extraction kit (Stratagene, USA). The DNA product thus, obtained was again amplified using the CP specific forward and reverse primers 5‘-AGAATTATGTCCAAGCGACCA-3‘ and 5‘-AAGCGTTGGGGATACACAAA-3‘. The viral gene was ligated into pGEMT vector and ligation mix was used for transformation of *E. coli* (DH5-á) competent cells as per the method of Mandel and Higa (1970). The transformants were identified on a Luria agar plate containing X-gal/IPTG and ampicillin (100 µl/ml). Plasmid was isolated from the transformed cells as per the procedure of Birnboim and Dolly (1979) and the insert showing amplification of CP gene was sequenced (3130 Genetic Analyzer, Applied Biosystems, California). The viral ORF AV1 was identified from the partial sequence using NCBI Blast search (Altschul *et al.,* 1997) and analysed using the software MEGA Ver. 4.1 beta (Kumar *et al.,* 2008). Accession number was obtained for the CP nucleotide sequence and the sequence was deposited in GenBank.

Multiple sequence alignment

References sequences

Begomovirus sequences used in comparative analyses were obtained from GenBank (Table 1). For full length A or monopartite genome sequences, when necessary, the nucleotide in the first position was reset so that the A of T/A nick site junction in the invariant geminivirus "loop" nonamer sequence TAATATT/AC was the first nucleotide of the sequence (Lazarowitz, *et al.,* 1992). CP sequences were excised immediately 5' of the ATG start codon for the CP gene and continuing to the end of the ORF as predicted by SeqEd program (Seaview USA). Core CP sequences were obtained using the amino acid sequence to identify the residues flanking the ends of the core region in all known begomoviruses. CP sequences were then truncated upstream from the second base in the arginine codon, which is the first base (nt1) in the priming site targeted by core PCR primers. Finally, sequences were truncated to remove degeneracy imposed by use of the degenerate PCR primers, ultimately yielding 533 and 536 nt sequences for New and Old World viruses, respectively. The

Table 1: Begomovirus name and their GenBank accession number

GenBank Accession	Name	Host	Country	Acronym
AB267834	Pepper yellow leaf curl Indonesia virus	Tomato, *Ageratum*	Indonesia	PepYLCIV
AF149227	Pepper golden mosaic virus	Pepper	Costa Rica	PepGMV
AF314531	Pepper leaf curl Bangladesh virus	Pepper	Bangladesh: Bogra	PepLCBV
AM691548	Pepper yellow vein Mali virus	*Capsium*	China: Fuzhou, Fujian	PepYVMV
AY044162	Pepper huasteco yellow vein virus	-	Sinaloa	PepHYVV
AY502935	Pepper yellow vein Mali virus	-	West Africa- Mali	PepYVMV
AY928512	Pepper golden mosaic virus	-	USA: Texas	PepGMV
AY928514	Pepper golden mosaic virus	-	USA	PepGMV
AY928516	Pepper golden mosaic virus	-	USA	PepGMV
DQ083764	Pepper yellow leaf curl Indonesia virus	Pepper	Indonesia: Bogor, Java	PepYLCIV
DQ083765	Pepper yellow leaf curl Indonesia virus	Tomato	Indonesia: Bogor, Java	PepYLCIV
DQ116877	Chili leaf curl Pakistan virus	Chilli	Pakistan: Multan	ChiLCPV
DQ116881	Pepper leaf curl Bangladesh virus	Chili	Pakistan: Khanewal	PepLCBV
NC001359	Pepper huasteco yellow vein virus	-	Sinaloa	PepHYVV
NC004101	Pepper golden mosaic virus	-	Mexico, Southern United States	PepGMV
NC004192	Pepper leaf curl Bangladesh virus	Chili	Bangladesh: Bogra	PepLCBV
NC005347	Pepper yellow vein Mali virus	Tomato	Mali	PepYVMV
NC008283	Pepper yellow leaf curl Indonesia virus	Tomato, *Ageratum*	Indonesia	PepYLCIV
U57457	Pepper golden mosaic virus	-	Mexico, Southern United States	PepGMV
X70418	Pepper huasteco yellow vein virus	-	Mexico	PepHYVV
NC000882	Pepper leaf curl virus	-		PepLCV
EF190217	Pepper leaf curl virus isolate Varanasi	Pepper	India: Varanasi	PepLCV
DQ343285	Pepper leaf curl virus	Soybean	India	PepLCV
AM051090	Bell pepper leaf curl virus	Bell Pepper	Pakistan: Lahore	BPepLCV
Y18056	Pepper leaf curl virus	*Capsicum annum*		PepLCV
AF414287	Pepper leaf curl virus	Chili	Malaysia: Klang	PepLCV
AF134484	Pepper leaf curl virus	-		PepLCV
FJ403045	Chilli leaf curl virus	*Capsicum annuum*	India: Varanasi	ChiLCV
NC004628	Chilli leaf curl virus	Tomato and chili	Pakistan: Multan, Punjab	ChiLCV
EU939533	Chilli leaf curl virus	*Capsicum annuum*	India	ChiLCV
FM210476	Chilli leaf curl virus	*Capsicum spp*	India: Himachal Pradesh	ChiLCV
AM773441	Chilli leaf curl virus	Chilli	India: Northern India	ChiLCV
AF336806	Chilli leaf curl virus	Pepper	Pakistan: Multan, Punjab	ChiLCV
AY883570	Tomato leaf curl New Delhi virus-Chilli pepper	*Capsicum annuum*	India- Lucknow	ToLCNDV
DQ114477	Chilli leaf curl virus	Chilli pepper	Pakistan	ChiLCV
FJ968525	Pepper leaf curl virus	Chilli pepper	India	PepLCV
HQ698591	Radish leaf curl virus	*Capsicum annuum*	India	RaLCV
HM991146	Tomato leaf curl Joydebpur virus	*Capsicum annuum*	India	ToLCJV

5'-proximal 200 nucleotides of the CP gene of reference geminiviruses were obtained by truncating the CP ORF to achieve the specified fragment, as described (Padidam *et al.,* 1995).

BLAST analysis

Sequences of CP of begomovirus were well-surveyed in database (available in GenBank) including the begomoviral field isolates investigated in our laboratory during the years were used in the study. The BLAST program was linked to the begomovirus CP database and to GenBank, enabling the user to input the CP sequence of an 'unknown' isolate and receive a diagnostic report that provides begomovirus identification, or a closest match as an 'output'. BLAST searches of the CP database can be carried out at the site and yield an output that contains a list of the highest scores and percent sequence identities. The closest match to a taxon in the database with the highest sequence homology is also considered.

Sequence alignment and phylogenetic analysis

For distance analysis, begomovirus sequences were aligned using the Clustal W program (MEGA 4).The default values for gaps and gap length penalties were used. For parsimony analysis, sequences were aligned using Clustal W in MegAlign, (MEGA 4 software), and a single most parsimonious tree was sought using the branch-swapping options in Phylogenetic Analysis. Bootstrap values were based on 1000 replicates using the > 60% majority rule.

Results

Phylogenetic analysis

Genetic distances for all CP fragment were in general agreement, and were nearly equally useful for making accurate predictions with respect to accession, though a few minor in congruencies were seen with respect to several viruses that grouped together differently for different region (Padidam *et al.,* 1995).

Relationships predicted by the CP tree (Fig. 1) indicates six well-supported Old World begomoviral lineages *viz*: (1) a large group of viruses from Southeast Asia (99% bootstrap), (2) a small group from the Indian subcontinent (99%), (3) a clade containing viruses from the Mediterranean-Middle East-Africa. (4) a small group from Indonesia, and (5) a small group from Malasia. (6) all New World begomovirus separated in single group. Three distinct sub groups were seen within the NewWorld cluster: (i) Pepper huasteco yellow vein virus from Sinloa, (ii) Pepper golden mosaic virus from texas,

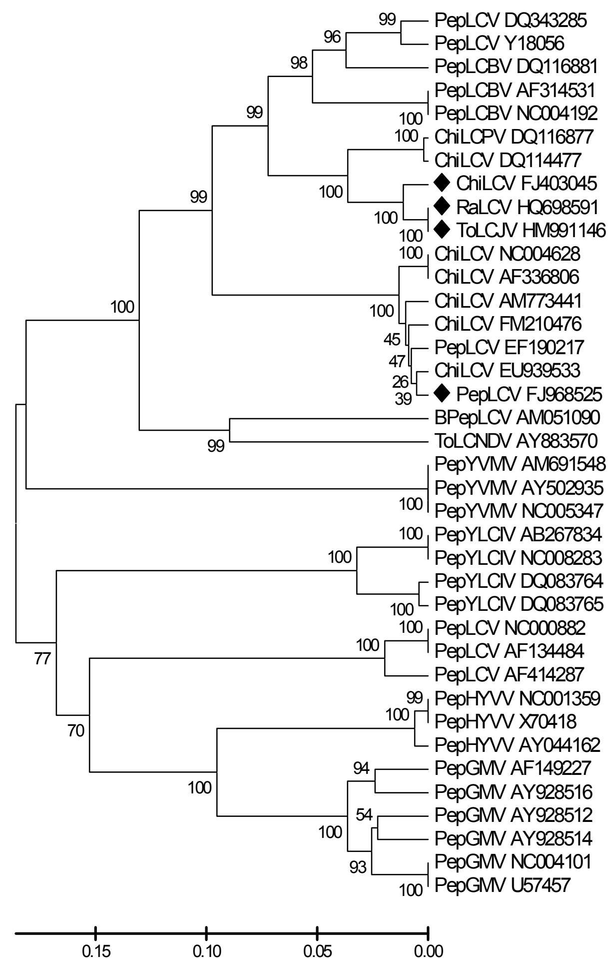

Fig: 1 Phylogenetic tree based on selected sequences of coat protein gene of chilli leaf curl virus (Begomoviruses). The tree was constructed using MEGA 4.1 at 1000 bootstrap values. (♦) marked indicates the accessions obtained from the farm and sequences at IIVR, Varanasi.

USA and (iii) a clade containing only Pepper golden mosaic virus from Mexico.

The phylogenetic tree revealed congruent relationships with the CP cluster, with some exceptions. Among Old World begomoviruses, those from Pakistan were separated from Indian species creating two groups and Bangladesh also separated in single group. However, one group comprised of two taxa members of a small polytomy containing begomoviruses from India and Pakistan. This is the first report of the molecular characterization of coat protein sequence of chilli leaf curl virus and chilli plant is affected by radish leaf curl disease in India.

For the New World lineage, the phylogenetic tree revealed a single, polytomy and clusters of strains with the exception of the clustering of Pepper huasteco yellow vein virus from Mexico and Sinaloa. Interestingly, trees generated for viral genomic, CP reference sequences

(Table 1) are all in general agreement in that Old World viruses are strikingly delimited with a basis in geographic location of origin, whereas, Caribbean region and Central American viruses tend to overlap geographically with North American begomoviruses. Furthermore, the closest relatives of some viruses from Central America, and Mexico appear to be from South America (Padidam *et al.,* 1995).

Distance and parsimony trees predicted for geminivirus classification using CP (Padidam *et al.,* 1995) was in good agreement with classification of begomoviruses into Eastern or Western lineages. However, in this tree, three of the nine New World species, and thirty Old World viruses were separated from the pairs or clusters of taxa that were otherwise defined by core CP or CP sequences (Padidam *et al.,* 1995). Our results are consistent with the narrow host range and geographical location. In contrast, the pepper and tomato infecting isolates, BPepLCV and ToLCNDN were most closely related isolates from Pakistan and India. In Old World virus Pepper leaf curl virus from Malasia is close to the New World virus.

Discussion

The CP gene is the most highly conserved gene in the family Geminiviridae (Wyatt and Brown, 1996). This gene sequence, which effectively predicts discrete strains, species, and taxonomic lineages of begomoviruses, has been accepted by the ICTV as a desirable marker for virus identity when a full-length genomic sequence is not available (Rybicki, 1994). The utility of the CP sequence for these purposes is likely possible because the CP sequence more optimally averages variable and conserved regions to predict close to accuracy more in line with the extent of sequence variation and conservation across the entire genome. However, conserved, sequences suitable for 'universal' PCR amplification and sequencing of the begomovirus CP gene are not readily available.

The core CP region spans seven of the eight most highly conserved amino acid motifs in the CP, and is characterized by stretches of nearly identical sequences interspersed with variable bases in all members of the genus begomovirus. These features facilitated use of CP as a molecular epidemiological tool, which dramatically simplifies begomovirus detection and their preliminary identification. Sequence identities predicted by the core CP and begomovirus clusters comprise of apparent lineages of related species. Relationships between taxa predicted by the core CP sequence relationships are similar to those in trees reconstructed from complete CP sequences. This is seen presumably because of the collective neutralization contributed to the full CP sequence by the highly variable 200 nucleotide fragment along with the highly conserved region near the 5' and 3' ends of the CP gene, both of which are absent in the core CP fragment. The core CP sequence appears, nonetheless, to contain sufficiently conserved and variable regions that virtually mimic the overall composition of the complete CP sequence.

In contrast, the highly variable 5' end of the CP gene sequence (200 nt) is proposed as a reliable molecular marker for predicting begomovirus identity (Padidam *et al.,* 1995) and seems best suited for revealing differences between closely related strains, owing to its characteristic ability to vary, apparently in an independent manner with respect to the remainder of the gene. It stands to reason that sole reliance on such a highly variable region may confuse relationships that are otherwise revealed by conserved regions. The disadvantage to both the 5' end and CP as marker sequences is that they represent only a fraction of the genome. Consequently, neither molecular marker is capable of detecting reassortants comprising a non-cognate DNA B component, a recombination event outside of the marker sequence nor a relevant mutation not revealed within the sequence. This caveat naturally places a requirement on the 'implementers'. The recognition of a possible 'new strain' or 'new virus', irrespective of the mechanism involved, requires a concomitant observation of phenotypic variation. Clearly, follow-up studies are then warranted to establish precise identity and relationships to other begomoviruses, and the molecular basis for observed differences. Alignment of CP sequences and tree reconstruction permits a prediction of relatedness among begomoviruses that is highly similar to the outcome from alignment of begomoviral coat protein gene sequences. Begomoviruses are highly similar (92-100%) typically form discrete clusters on the tree, while those with a single genotype are positioned as unique taxa in a polytomy. This indicates that the CP region is sufficient to provide a simple, rapid, and reliable method for begomovirus identification and classification as the manageable size of the CP amplicon provides sufficient viral sequence obtained in a single sequence reaction. Here, we demonstrated the utility of the CP region for predicting the identities of the begomovirus relative and other members of the same subcluster, and often the probable identification of a particular virus or strain. These conclusions can be visualized numerically in the similarity distance matrix generated in the analysis for which representative comparisons are provided in clades.

A large CP-core sequence database has been established containing sequences from many sources, including both laboratory-authenticated and field isolates representative of a wide geographical and host range. Findings of the study demonstrate that the core CP database is useful for rapid begomovirus detection and identification. Sequence alignments of begomoviruses indicate that tree topologies and virus clusters predicted by the complete CP gene sequences and CP-core sequences are nearly indistinguishable. The availability of universal PCR primers for amplification, the ease and availability of automated sequencing, and the establishment of an interactive web-based database makes it possible to rapidly identify a begomovirus sample via the Genbank database. Knowledge of the identity and distribution of particular virus genotypes affecting a chilli crop form a cornerstone of successful disease resistance programs. Ultimately, knowledge of virus identity and distribution of begomoviruses will permit rationale decisions as to prioritize for disease resistance efforts, and identify isolates to be selected when assessing cultivar responses to begomovirus infection.

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