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VK Satya
National Pulses Research Centre, Vamban, Pudukottai, Tamil Nadu, India

D Alice
Department of Plant Pathology, Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu, India

Correspondence
VK Satya
National Pulses Research Centre, Vamban, Pudukottai, Tamil Nadu, India

Dolichos yellow mosaic virus from South India: Genetic characterization and evidence for recombination

VK Satya and D Alice

Abstract
The field survey was made in the *Dolichos* growing areas of Coimbatore district in Tamil Nadu and disease incidence was ranged from 86-90%. The symptoms observed are golden yellow specks sparsely distributed in leaf lamina; in severe case the entire leaf lamina become yellow and yellowing of pods. The viral genome was cloned using rolling circle amplification and sequenced. The sequence analysis revealed that *Dolichos yellow mosaic virus* (DoYMV) is causing the yellow mosaic disease in *Dolichos*. DoYMV-CBE1 showed 99% identity with DoYMV -Mysore (AJ968370) while DoYMV-CBE2 shared 100% identity with DoYMV-Bang1 (AM157412) and DoYMV-Bang2 (AM157413). The phylogenetic dendrogram showed the DoYMV is distinct from other legume yellow mosaic viruses. One additional N-glycosylation site (NLTH, coordinates 101-104) was identified in the coat protein of DoYMV. Statistically significant recombination events were detected in the replication enhancer protein and transcription activation protein. The genomic components were exchanged from *Croton yellow vein mosaic virus* and *Velvet bean severe mosaic virus* to DoYMV. From the results it is inferred that DoYMV is genetically different from other yellow mosaic viruses. The yellow discolouration of pods and seeds of infected plants were suggestive that the virus may be seed borne, which was investigated in the present study. The distribution of virus in various parts of the seeds of *Dolichos* plants naturally infected in the field was determined by polymerase chain reaction and sequencing. Nucleotide sequencing of the PCR amplicons from the seed parts revealed the presence of *Dolichos yellow mosaic virus* (DoYMV) in the embryonic axes of the seeds analyzed. The virus detected in embryonic axes of infected *Dolichos* seed revealed that DoYMV is seed borne in nature.

Keywords: *Dolichos*, begomovirus, recombination, seed borne

Introduction
Dolichos (*Lab lab purpureus*) is one of the most important leguminous vegetable crops in India. The major constraints for *Dolichos* cultivation are pests and diseases. Among diseases the yellow mosaic disease is a serious malady and causes significant yield reduction [1]. *Dolichos* yellow mosaic disease was first reported by Capoor and Varma [2]. Since 1950 the *Dolichos* yellow mosaic disease is a major constraint in the cultivation of this legume vegetable. Capoor and Varma (1950) reported that *Dolichos* yellow mosaic disease is transmitted only by whitefly *Bemisia tabaci* Genn. *Dolichos* yellow mosaic disease was not transmitted to lima bean or other legume species and it is biologically as well as antigenically distinct from other yellow mosaic viruses [2, 3]. The symptoms produced are bright golden speck on the leaf lamina followed by complete yellowing of leaves [4]. The disease was caused by the *Dolichos yellow mosaic virus* (DoYMV) [4] belonging to the genus *Begomovirus*; family *Geminiviridae* and consists of circular single stranded DNA genome encapsidated in twinned or geminate, icosahedral particles (18 × 30 nm size) [5]. The begomoviruses are either monopartite (DNA A component alone) or bipartite (DNA A and DNA B component). The DNA A component encodes for coat protein on the viral strand and replication initiator protein, transcription activator protein, replication enhancer protein on the complementary strand [6, 7]. The DNA B component encodes for nuclear shuttle protein in viral sense strand and the movement protein in the complementary sense strand. In recent years the yellow mosaic disease was severe in the *Dolichos* and pods turned to yellow colour resulting in yield reduction and reduction in market value in the district of Coimbatore, Tamil Nadu. From Tamil Nadu there was no report on characterization of yellow mosaic disease affecting *Dolichos*. Hence the present study was undertaken to understand and characterize the virus infecting *Dolichos* in the areas of Coimbatore district, Tamil Nadu.

Materials and Methods
Sample collection
Yellow mosaic disease affected *Dolichos* leaf samples and seed samples were collected from

farmer's field at Coimbatore district. The disease incidence and disease severity was scored for the yellow mosaic disease.

DNA isolation and PCR amplification

Total genomic DNA was isolated from young leaves and seeds of *Dolichos* showing yellow mosaic symptoms using the GEM-CTAB method [8] using 2 per cent β - mercapto ethanol. The purified genomic DNA was amplified by polymerase chain reaction (PCR) using universal Begomovirus-specific primers PALIc1960 and PALIr772 [9]. The reaction was programmed with initial denaturation at 94°C for 2 min, followed by 30 cycles of 94°C for 1 min, 55°C for 2 min, 72°C for 3 min, and a final extension step at 72°C for 10 min. The PCR amplicons was run on the 1.5% agarose gel and viewed under alpha imager (Alpha Innotech, USA). The size of the amplicons were determined with 1 kb DNA ladder.

Cloning of viral genome

PCR positive samples for begomovirus were subjected to rolling circle amplification (RCA) using Ø29 DNA polymerase[10]. 500 ng of rolling circle amplified DNA was subjected to digestion with different endonucleases (*Bam* HI, *Eco* RI, *Hin*d III, *Pst* I, *Kpn* I, and *Xba* I) to identify the unique restriction sites which can be used for cloning. The full length viral DNA was cloned into pUC18 vector and the recombinant clones were identified through restriction digestion of miniprep.

Sequencing and sequence analysis

Sequences of the recombinant plasmids were determined at Xceleris, Ahmedabad. Nucleotide similarity searches were performed using BLAST at NCBI (www.ncbi.nlm.nih). Nucleotide sequence alignments were done with ClustalW using Bio Edit 7.0.9.0 and phylogenetic analyses (neighbor-joining trees with 1,000 bootstrap replicates) with Mega 5.0 and subsequently dendrograms were prepared. The recombination events were determined with RDP4 [11] using following methods RDP, GENECONV, Maxchi, Bootscan, SiScan and Chimaera methods using selected begomovirus species. Default RDP4 settings with a 0.05 *P*-value cutoff with standard Bonferroni correction for multiple testing were used throughout. Both internal and external references were allowed, with parental cutoff of 70-100 per cent and a window size of 30. Open reading frames (ORFs) were confirmed by GENERUNNER. In addition, complete sequence analysis for each open reading frame (ORF) and intergenic region (IR) was also done for the closest virus species.

Results

Disease incidence and symptoms

The percent disease incidence was ranged from 86-90%. The affected plants showing bright golden yellow patches initially and later turned into complete yellowing of leaves. In severe case the beans turned into yellow colour (Fig.1).

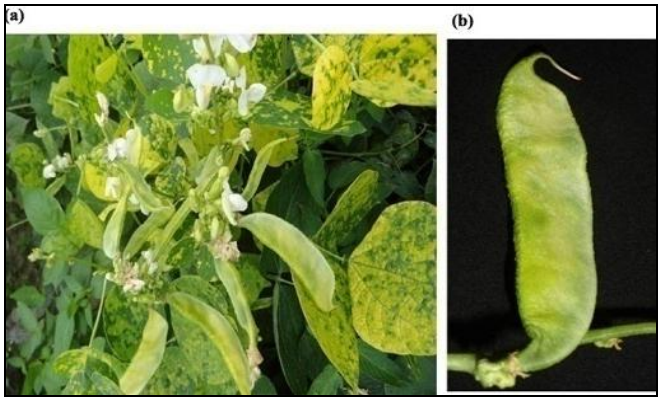

Fig 1: Yellow mosaic disease in *Dolichos* (a); yellowing of pods (b)

PCR amplification and cloning

The genomic DNA from leaves and embryonic axes of seed was amplified through PCR using degenerate primers PAR1v772/ PAL1c1960. The expected amplification of ~1.1 kb was observed in 23 leaves samples (92%) out of 25 samples tested and 12 seed samples (48%) out of 25 samples tested. This result confirmed the presence of a begomovirus in leaf and embryonic axes of seed. Then RCA was performed from PCR positive samples of leaf and embryonic axes of seed and restricted with different endonucleases, *Hind*III, *Bam*HI, *Cla*I and *Pst*I. Among these *Pst*I yielded 2.7 kb fragment, which was chosen for the cloning of viral DNA. The 2.7 kb fragment was purified using PCI (phenol: chloroform: isoamyl alcohol) method and ligated with linearized pUC18 vector. Recombinant clones were screened and two clones, DC1 from leaf and DC5 from embryonic axes of seed were selected and sequenced.

Sequence analysis

The sequences of DC1 and DC5 were analysed in BLAST search programme (www.ncbi.nlm.nih) and DC1 clone showed 99% identity with DoYMV -Mysore (AJ968370) while DC5 clone shared 100% identity with DoYMV-Bang1 (AM157412) and DoYMV-Bang2 (AM157413). Hence the virus isolates infecting *Dolichos* in Tamil Nadu were identified as *Dolichos yellow mosaic virus* according to the geminivirus taxanomy study group 91% nucleotide percent identity as threshold level (www.ictvonline.org.in) and the isolates are named as DoYMV-CBE1 and DoYMV-CBE2 and submitted to GenBank database under the accession numbers KP784661 and KP784662 respectively.

Genome organization

The length of the genome of DoYMV-CBE1 and DoYMV-CBE2 was 2760 and 2762 nt respectively. Genome organization consists of the two open reading frames, ORF AV1 (~30 kDa) and ORF AV2 (11 kDa) on the virion sense strand and four open reading frames, ORF AC1 (41.2 kDa), ORF AC2 (15.9 kDa), ORF AC3 (16 kDa) and ORF AC4 (9.1 kDa) on the complementary strand were observed. The details of the coding region are mentioned in the Table 1.

Table 1: Details of the coding region of the DoYMV clones from Coimbatore

Clone ID	Coding Region Nucleotide co ordinates					
	ORF AV1 (CP)	ORF AV2 (Pre-CP)	ORF AC1 (Rep)	ORF AC2 (TrAP)	ORF AC3 (REn)	ORF AC4 (PTGS)
DoYMV-CBE1	283-1056	123-410	2633-1536	1630-1202	1482-1072	2479-2234
DoYMV-CBE2	283-1056	123-410	2634-1537	1631-1203	1483-1073	2480-2235

Comparison with other begomoviruses

The comparison of the complete nucleotide sequences of the DNA A component of DoYMV isolates of present study were compared with other selected begomoviruses (Table 2). The results indicated that the DoYMV under this study shared 93-100% identity with other DoYMV isolates. The nucleotide similarity with other begomoviruses was 60-63% identity with yellow mosaic viruses infecting grain legumes, 58% identity with *Indian cassava mosaic virus* (ICMV), *Srilankan cassava mosaic virus* (SLCMV), tomato leaf curl viruses; 57-58% identity with cotton leaf curl viruses and 54% identity with *Bean golden mosaic virus*.

Table 2: Percentage identity in complete nucleotide sequence of DNA A component of Do YMV isolates and other selected begomoviruses.

Virus isolate	DoYMV-CBE1	DoYMV-CBE2
DoYMV-CBE1	100	97
DoYMV-CBE2	97	100
DoYMV-Mysore (AJ968370)	99	98
DoYMV-Bang2 (AM157413)	97	100
DoYMV-Bang1(AM157412)	97	100
DoYMV-ND (AY309241)	94	94
DoYMV-JD18 (JX315325)	93	93
MYMV-Vam DQ400848	61	61
MYMV-Vig (Mad) AJ132575	61	61
MYMV-Sb (Mad) AJ421642	61	61
MYMV (KH PP-03) AY271892	62	62
MYMIV-Mg (BD) AF314145	61	61
MYMIV-Mg (Np) AY271895	62	62
MYMIV-Mg (Pak) AJ512495	61	61
MYMIV-Bg3 AF126406	60	60
HgYMV-[IN Coi] AJ627904	62	62
HgYMV-Ban AM932427	62	62
HgYMV-Fb AM932425	62	62
HgYMV-Lb AM932429	62	62
RhYMIV-HM777509	62	61
RhYMV-AM999981	63	63
VBSMV-FN543425	62	62
KuMV-Yg3 FJ539014	62	62
KuMV-VN DQ641690	63	62
ICMV-Mah AY730035	58	58
SLCMV-Col AJ314737	58	58
ToLCNDV-Svr U15015	58	58
ToLCBV-Ban5 AF295401	58	58
ToLCNDV-Svr U15015	58	58
CLCuMV-Raj AF363011	57	57
CLCuMV-His[PK:Mul] AJ496461	57	57
BGYMV D0201	54	54
PaLCuCNV-Age AJ876548	59	59
PaLCuCNV-Tom DQ641700	59	59
BYVMV-Mad AF241479	57	57
CYVMV-Del-Cr JN817516	62	62
CYVMV-Bang-Cr2 JN831446	61	61

Comparison of the predicted amino acid sequence of the DoYMV isolates of present study and other isolates indicated that ORF AV1 shared 90-100% identity between themselves; ORF AC1, AC2, AC3 and AC4 shared 96-100% identity; but ORF AV2 shared 98-100% identity with one set of isolates, DoYMV -Mysore (AJ968370), DoYMV-Bang1 (AM157412) and DoYMV-Bang2 (AM157413) whereas 83-86% identity only with DoYMV (AY309241) and DoYMV-JD18 (JX315325).

Protein binding motif in coat protein and Rep was compared between the *Mungbean yellow mosaic virus* (MYMV) and DoYMV. The results indicated that ATP/GTP bindibg site, protein kinase phosphorylation site, casein kinase phosphorylation site and N-glycosylation site in DoYMV differed from MYMV (Table 3). DoYMV and MYMV differ in their ATP/GTP binding site identified in AC1 by two amino acids. One additional N-glycosylation site (NLTH, coordinates 101-104) was identified in the AV1 of DoYMV.

Table 3: Comparison of protein binding motifs between DoYMV and MYMV

ORF	Protein motif	Common motif between MYMV and DoYMV	Motifs only in DoYMV
AC1 (Coat protein)	ATP/GTP binding site	-	GDSRTGKT
	Protein kinase phosphorylation site	SAR, TGK, SYK	SDK, SWR, SPK, SLK
	Casein kinase II phosphorylation site	SYKE	SKEE, TADD, SSSD, SNLE, SLKE, STGEE, TQND
AV1(Rep)	N-glycosylation site	NHTN, NHTE	NLTH
	Protein kinase phosphorylation site	TNR, THR, TVK, TLK	SKR, TPR, SVR, TSRR, TIK
	Casein kinase II phosphorylation site	-	TPMD

Phylogenetic dendrogram based on complete nucleotide sequences of DoYMV isolates and other selected begomoviruses revealed that the DoYMV isolates formed separate major clusters and deviated from other begomoviruses. Within these DoYMV isolates, three sub clusters were formed, first sub cluster containing DoYMV-Mysore and present isolate, DoYMV-CBE1, second sub cluster containing DoYMV-Bang1, DoYMV-Bang2 and the other isolate, DoYMV-CBE2 from the current study and third sub cluster contains DoYMV (AY309241) and DoYMV-JD18 (JX315325) (Fig. 2).

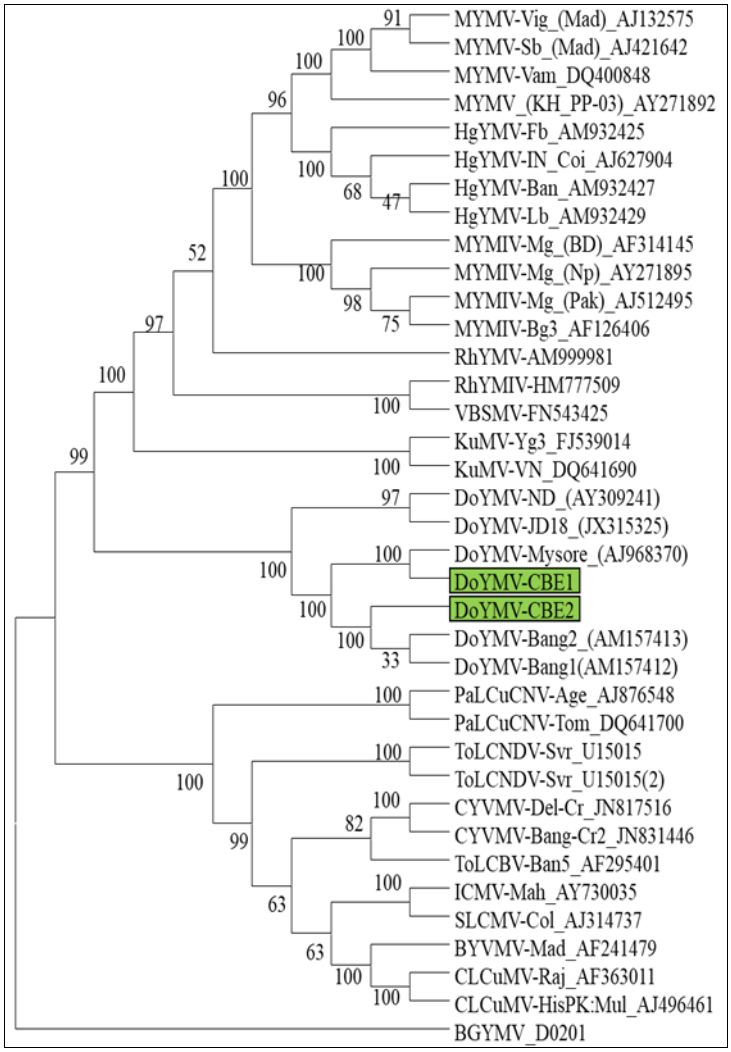

Fig 2: Phylogenetic dendrogram based on complete nucleotide sequences of DNA A component of DoYMV isolates with other selected begomoviruses. Vertical branches are arbitrary, horizontal branches are proportional to mutation distances. Values at nodes indicate percentage bootstrap values (1000 replicates). Isolates of present study are highlighted.

Recombination analysis of DoYMV isolates

Recombination analysis was done for the DoYMV isolates using RDP4 to know whether any recombination or exchange of genomic components within the isolates of DoYMV or between the isolates of begomoviruses has occurred. The results indicated that recombination was observed in the coding region of TrAP and REn protein. The genome was transferred between the nucleotide 1342 to 1538 from *Croton yellow vein mosaic virus* (CYVMV: Bang: Cr2 JN831446) to DoYMV-CBE1 and from *Velvet bean severe mosaic virus* (VBSMV; FN543425) to DoYMV-CBE2 between the nucleotide 1051 to 1368 (Fig. 3).

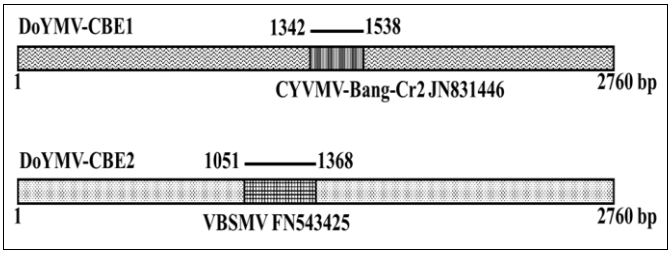

Fig 3: Recombination events detected in DoYMV across the DNA A genome of begomoviruses. CYVMV- Croton yellow vein mosaic virus, VBSMV- Velvet bean severe mosaic virus

Discussion

Dolichos yellow mosaic disease is the major challenge in the *Dolichos* cultivation. The yield loss due to DoYMV was 80% and if the plants get infected at early stage, the plants become stunted and produced less number of branches and beans [4, 12]. The field survey was made in the *Dolichos* growing areas of Coimbatore district and disease incidence was ranged from 86-90%. The symptoms observed are golden yellow specks sparsely distributed in leaf lamina; in severe case the entire leaf lamina become yellow and yellowing of pods. The characterization of DoYMV was first reported by Maruthi *et al* [4]. The sequence analysis of the present study revealed that DoYMV-CBE1 showed 99% identity with DoYMV -Mysore (AJ968370) while DoYMV-CBE2 shared 100% identity with DoYMV-Bang1 (AM157412) and DoYMV-Bang2 (AM157413). Swanson and coworkers reported that DoYMV was biologically as well as genetically distinct from other yellow mosaic viruses [3]. DoYMV was not transmitted to other legume hosts and it is highly specific to *Dolichos*[2]. In the present study the phylogenetic dendrogram showed the DoYMV is distinct from other legume yellow mosaic viruses and the protein binding motifs also different from other yellow mosaic viruses. Because of this reason the DoYMV is highly specific to *Dolichos*. The multiple alignments of predicted amino acid sequence clearly showed that coat protein, replication initiator protein, replication enhancer protein and transcription activation protein was highly conserved among the isolates. *ori* was a hot spot for recombination during replication of the circular single-stranded DNA of phage M13 [13] and reported that *ori* was a site of recombination in *African cassava mosaic virus*[14]. Recombination events were detected in the IR, Rep gene of MYMIV isolate and such recombination may facilitate the virus to recognize and replicate DNA B molecules with divergent common regions[15]. In the present case, statistically significant recombination events were detected in the replication enhancer protein and transcription activation protein. The genomic components were exchanged from *Croton yellow vein mosaic virus* and *Velvet bean severe mosaic virus* to DoYMV.

The DoYMV is considered as very distinct and genetically isolated begomovirus, as the host range of this virus is limited even within leguminous species. However, the recombination events involving begomovirus infecting other hosts as shown in the present study indicate the possibility of this virus occurring in mixed infection. In a phylogenetic analysis too, the virus is well separated from both yellow mosaic and other Indian begomoviruses. Whether DoYMV originated independently much earlier than other viruses will have to be looked into to understand the interaction between the viruses.

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