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Molecular identification and sequence analysis of bipartite Begomovirus infecting Horsegram legume in India

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Abstract

The molecular diversity of Begomovirus infecting *Horsegram yellow Mosaic viruses* (HgYMV1 and HgYMV2), from two main horsegram growing farms near Bangalore, Karnataka State, South India was investigated. The viral DNA was amplified from horsegram plants exhibiting mild and severe symptoms by polymerase chain reaction, and complete genome of the HgYMV were identified by their sequence analysis. Isolates of HgYMV1 and HgYMV2 were found to be associated with severe symptom phenotype from HgYMV. HgYMV was most closely related to *Mungbean yellow mosaic indian viruse* (MYMIV) and *Mungbean yellow mosaic virus* (MYMV) at 81.8 to 84.8 % nucleotide identity, based on DNA-A and DNA-B component sequences. HgYMV was distantly related to *Dolicos yellow mosaic virus* from Asia (DoYMV-Ban and DoYMV-DB) and partially to Cowpea golden mosaic virus from Nigeria (CPGMV-[NG]) at 64 and 62 % DNA nucleotide identity. Analysis of the DNA-B sequence of HgYMV revealed a DNA-B component identical to those of *Bean golden yellow mosaic virus* BGYMV isolates described. Furthermore, the DNA-B component for extant BGYMV isolates and strains were also the closest relatives for the HgYMV1 DNA-B components at 48.7 % nucleotide identity. Therefore, HgYMV could be considered to be a new species of the genus Begomovirus (family Geminiviridae).

Keyword: Begomovirus, Horsegram, *yellow Mosaic viruses*, DNA sequencing.

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Introduction

Horsegram (*Macrotyloma uniflorum*) is a small pulse (legume) that produces seeds, and one of the lesser known beans. Horsegram is a slender, sub-erect annual herb with slightly twining, downy stems and branches. It can grow to a height of 30–40 cm. Leaves are trifoliate and flower is a pale yellow. Pod is linear and flattened, and contains 5-7 seeds. Seeds are small (3-6 mm), shining and colour ranges from light red, brown, and black or mottled (Purseglove, 1974; Neelam et al., 2014). Horsegram is an annual native to South Asia. It is regarded as a food of the poor, particularly in southern India. Presently, it is the fifth most widely cultivated grain legume in India (Fuller and Murphy, 2018). Horsegram is a good dietary source of protein, carbohydrates, dietary fibre, minerals, and vitamins (Sangita et al., 2004; Yadav et al., 2004; Prasad and Singh, 2015). Besides nutritional value, it has been linked to reduced risk of various diseases due to presence of phtyochemicals such as tannins and phytate (Kawsar et al., 2008; Sreerama et al., 2012, Prasad and Singh, 2015). These phtyochemicals are thought to have beneficial medicinal and nutraceutical properties (Muthukumara et al., 2014; Bhartiya et al., 2015).

Horsegram is affected by yellow mosaic virus during summer and rainy season. This incidence was correlated with the abundance of whitefly (*Bemisia tabac*), population in the field (Muniyappa and Veeresh, 1984). The symptoms include appearance of pale yellow discolouration on young leaves and as the disease progresses yellow mosaic mottling also appeared. Subsequently, the mottling enlarged and the entire leaves become bright yellow and eventually become completely bleached (Muniyappa and Veeresh, 1984). Begomoviruses are pathogens transmitted by whiteflies and have been known to occur more in the tropical and subtropical countries compared to the temperate region (Muniyappa and Veeresh, 1984). The whitefly- borne disease occurs on economically important crop plants such as horsegram, causing substantial yield losses every year in India (Muniyappa and Veeresh, 1984; Muniyappa and Veeresh, 1984; Abarshi et al., 2017).

The begomoviruses (family Geminiviridae, genus Begomovirus) are circular single-stranded plant DNA viruses, encapsidated in icosahedral geminate particles, and are 18 by 30 nm in size (de Almeida et al., 2013). The viral genome is characterized by one (monopartite) or two (bipartite) (DNA-A and DNA-B) segments each around 2.6 kb in length (Mansoor et al., 1993; Briddon and Markham, 2000; Briddon, 2003; Maruthi et al., 2005; Kumar et al., 2015). Members of the genus Begomovirus are transmitted by the whitefly *B. tabaci* and infect dicotyledonous plants such as Horsegram. Begomoviruses native to the New World (NW) have genomes consisting of two components, known as DNA-A and DNA-B. Although genetically distinct, bipartite begomoviruses have been identified in the Old World (OW). However, some of the emerging OW begomoviruses are monopartite, consisting of a component homologous to the DNA-A component of the bipartite viruses. Recently it has become clear that the majority of monopartite begomoviruses are associated with betasatellites that are important for infecting some hosts (Briddon and Stanley, 2006). Whitefly-transmitted viruses cause some of the most damaging and economically important diseases of crop plants, especially in tropical and subtropical regions (Samretwanich et al. 2000, Hidayat et al., 2008, Tiwari et al., 2013). This study reports the sequence of the Begomovirus causing Horsegram leaf curl in India. Its relationship with other Begomovirus causing leaf curl disease in legumes from other part of the world was analysed based on nucleotide sequence homology.

2.0 Materials and Methods

Sample collection and DNA extraction

The horsegram symptom bearing leaves were originally collected from infected plants in farms near Bangalore, Karnataka State of India. Leaves of Horsegram plant showing symptoms typical of yellow mosaic disease were collected from -80 °C freezer at Natural Resources Institute, University of Greenwich, United Kingdom. Total DNA was extracted from the dried plant samples by using a CTAB extraction buffer according to the manufacturer's instruction (Maruthi et al., 2002; Abarshi et al., 2010).

Virus detection using Degenerate and specific primers

DNA template for PCR was prepared from Leaves of infected Horsegram plants. Amplification of begomovirus genome was carried out using a pair of degenerate primers designed for the amplification of the DNA A and DNA B genomic components, Deng-A (5'TAATATTACCKGWKGVCCSC3') and Deng-B (5' TGGACYTTRCAWGGBCCTTCACA3') (Deng et al., 1994). To amplify DNA-A genomic component (2.8 kb), two consensus outwardly extending primers were designed in the satellite conserved region (SCR) (HYMV-A 1500F, **5'CTGCAG**TGATGTTGTCCCCKG3'; HYMV-A 1500R, 5' **CTGCAG**CTCAACTCAGGARTGG3'). For DNA-B component (2.7kb), outwardly extending primers (D_HYMV-B2200F, 5'**GAATTC**ATGAATAAATGGCCG3'; D_HYMV-B2200R, 5'**GAATTC**ATAATTCCCTTGTCATG3') were designed from the conserved region of DNA.

A master mix of the entire PCR components for a final volume of 25 µl was prepared in 1.5 ml eppendorf tube. 24 µl of master mix were dispensed into PCR tubes, and 1.0 µl of DNA template was added. A positive and

negative control was set up using 1.0 µl of DNA template (previously shown positive) and sterile distilled water (SDW), respectively. Finally the tubes was transferred to a thermal cycler (PCR machine) programmed as follows: Initial 10 cycles of: 94 °C for 1 min, 55 °C for 1 min and 72 °C for 1.2 min. Followed by 25 cycles of: 94 °C for 1 min, 52 °C for 1 min. 72 °C for 1.2 min. End with: 72 °C for 10 min. 20 °C Hold. The reaction mix is for the final volume of 25 l for each reaction/sample. PCR products were then analysed by electrophoresis in 1 % agarose gels in Tris-buffer EDTA.

Cloning and Sequencing of DNA from HYgMV Isolate

The complete genome (DNA-A & DNA-B) of HgYMV isolate infecting horsegram, as a product of PCR amplification, was cloned into pGEM-T vector and sequenced at the MRC Gene service, Cambridge, UK. Selected DNA clone was then sequenced by the dideoxy nucleotide chain termination method. The present of inserts was check by PCR using the primers (See Table 1).

Two independent clone of each virus isolate were sequenced to ensure sequence identity and reliability. Minor discrepancies were corrected manually using the software package DNAStar. Phylogenetic analyses were carried out to generate parsimonious tree via phylogenetic analysis using parsimony (PAUP) 4.0 version 10 beta for Mac (Sinauer Association, Sunderland, MA; 42) with bootstrapping for 100 replicates. Sequence of HgYMV was compared with sequence of selected begomoviruses obtained from the database.

Table 1: Primers used for detection of HgYMV begomovirus in horsegram

Primer name	Primer sequence (5' – 3')	Annealing site on DNA -A	Size of PCR products	Primer specificity	Reference
Virus genome amplification					
Deng A	TAATATTACCKGWKGVCCSC	1	530 bp	Degenerate, begomovirus	(Deng et al., 1994).
Deng B	TGGACYTTRCAWGGBCCTTCACA				
PAL1c1960	ACNGGNAARACNATGTGGGC	1960	1.4 kb	Degenerate, begomovirus	(Rojas et al., 1993)
PAL1v720					
HYMV -A 1500F	**CTGCAG** TGATGTTGTCCCCKG	1500	2.8 kb	HgYMV	This study
HYMV -A 1500R	**CTGCAG** CTCAACTCAGGARTGG	1500			
D_HYMV -B2200F	**GAATTC** ATGAATAAATGGCCG	2200	2.7 kb	HgYMV	This study
D_HYMV -B2200R	**GAATTC** ATAATTCCCTTGTCATG	2200			
Sequencing primers					
T7	TAATACGACTCACTATAGG	On the plasmid		Plasmid	
Sp6	GATTTAGGTGACACTATAG	On the plasmid		Plasmid	
FYMV -A2200F	AT **ACGCGT** CGTTTGCAGA	2200		HgYMV	This study
FYMV -A600R	TTCCGACACGATGAGTGA	600		HgYMV	This study
LYMV -B1350R	GACTCTATATCACAAACACAATC	1350		HgYMV	This study
YLBMV -B400F	CATGTGTAATGGTTTTGCC	400		HgYMV	This study

Sequencing the complete genome of HgYMV1 and HgYMV2 isolates

The present of inserts was check by PCR using the restriction enzymes (EcoRI and PStI). Two clones were sequenced for every PCR product and each clone was sequenced bidirectionally to ensure sequence identity and reliability. Sequences obtained were highly reproducible. The sequence used in this analyses, were aligned using the software package DNAStar. Phylogenetic of virus sequence of HgYMV begomovirus sequences were compared with those sequences of selected reference begomoviruses sequence from the gene bank. The sequences were aligned using the cluster method and the dendograms.

Results

Total DNA extraction from virus infected leaves

Total DNAs were extracted from infected leaves of horsegram. Upon optimisation we have obtained higher yields of clean DNA from horsegram leaves by using modified CTAB-based DNA extraction procedure. The amplification in PCR using Deng et al. (1994) primers indicated the successful DNA extraction and PCR amplification as shown in Figure 1. These results confirmed the infection of horsegram by a begomovirus.

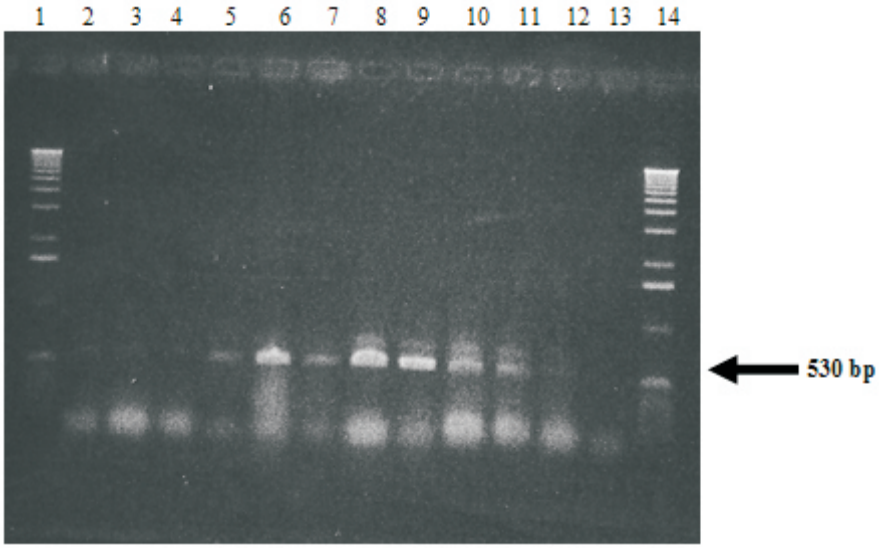

Figure 1 Gel photograph of PCR products generated using degenerate primer, Deng et al. (1994) primers showing successful detection of begomovirus in different crop plants. Lane 1: ACMV (*African cassava mosaic virus*) +ve control, Lane 2: LYMV (diluted), Lane 3: FYMV (diluted), Lane 4: HgYMV (diluted), Lane 5: HgYMV (diluted), Lane 6: LYMV, Lane 7: FYMV, Lane 8-12: HgYMV, Lane 13: negative, Lane 1 and 14: are the sizes of 1 Kb marker as shown on the left and right of the figure.

Comparison of genome sequence and the construction of phylogenies based on DNA-A sequences

The dendogram based on partial DNA-A sequences of HgYMV infecting horsegram with other begomoviruses transmitted by whitefly grouped the viruses into Old World and New world categories (Figure 2). The Old World group consisted of six major clusters and the new World consists of single cluster. Phylogenetic analysis (Most parsimony analysis) for HgYMV1 and HgYMV2 with HgYMV DNA-A component from the database indicated that the isolates shared 95.7 to 98.4% nucleotide identities with its closest relatives, which includes the previously described isolates and strains of HgYMV (Table 2). This result suggested that based on the >90% nucleotide identities, HgYMV1 and HgYMV2 are two isolates of previously described HgYMV species.

HgYMV1 and HgYMV2 isolates from horsegram were similar to MYMIV and MYMV isolates at 81.8 to 84.8% (DNA-A component), indicating that the isolates are same viral species with common ancestors, and hence MYMV was used in most of the nucleotide analyses. Phylogenetic analyses for the DNA-A component for HgYMV1 and HgYMV2 indicated that the isolates were 64% similar to DoYMV from Asia (DoYMV-Ban and DoYMV-DB) (Table 2). The percentage divergences of 62% were shared between HgYMV 1solates and CPGMV [NG].

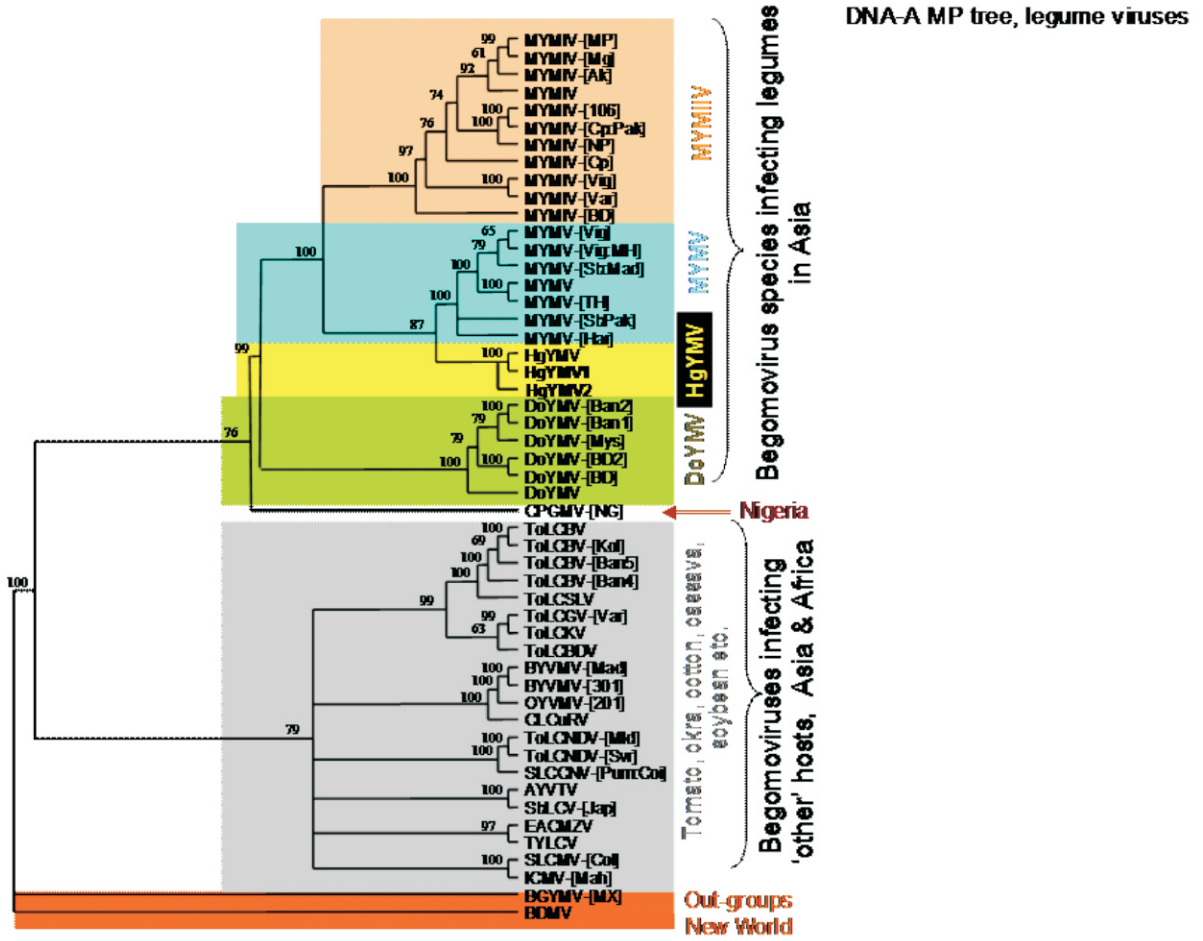

Figure 2 Parsimonious tree showing the relationship of HgYMV with other begomoviruses based on complete DNA-A nucleotide sequences. Vertical distances are arbitrary and the horizontal distances are proportional to mutational changes in the sequences. Numbers at nodes indicate percent bootstrap scores using 100 replicates.

Table 2 Percent nucleotide identities for the complete DNA-A genome and genes of Indian HgYMV with selected begomoviruses.

	BGYMV-[Mx]	CPGMV-[Nig]	DoYMV-[BD]	DoYMV	DoYMV-[Mys]	HgYMV	**HgYMV1**	**HgYMV2**	MYMIV	MYMIV-[Har]	MYMI-[Vig]	MYMIV-[Mg]	MYMV	MYMV-[106]	MYMV-[SbMad]	MYMV-[SbPak]	MYMV-[TH]	MYMV-[Vig]	MYMV-[VigMah]	ToLCBDV	ToLCBV
BGYMV-[Mx]																					
CPGMV-[Nig]	58.8																				
DoYMV-[BD]	59.1	59.5																			
DoYMV	58.9	59.7	94.9																		
DoYMV-[Mys]	59.9	59.6	95.7	93.5																	
HgYMV	60.8	61.9	63.9	63.8	64.1																
HgYMV1	**60.6**	**62.0**	**64.0**	**63.9**	**64.3**	**98.4**															
HgYMV2	**60.8**	**62.0**	**63.9**	**63.8**	**64.1**	**95.7**	**95.2**														
MYMIV	59.6	62.4	63.4	62.8	63.6	81.5	**81.9**	**80.4**													
MYMIV-[Har]	61.5	62.0	64.3	64.3	64.3	84.4	**84.6**	**83.2**	80.8												
MYMI-[Vig]	59.5	62.7	64.3	63.9	64.8	82.2	**82.5**	**81.1**	95.7	81.5											
MYMIV-[Mg]	59.6	63.0	64.2	63.8	64.6	82.1	**82.2**	**81.3**	95.3	81.5	95.6										
MYMV	61.8	61.7	64.0	64.0	64.0	84.4	**84.3**	**82.8**	80.8	93.5	81.1	81.0									
MYMV-[106]	59.2	62.4	63.8	63.5	64.4	81.7	**82.0**	**80.9**	95.5	80.6	97.3	95.2	80.6								
MYMV-[SbMad]	61.2	60.9	63.7	64.0	63.4	82.1	**81.9**	**81.2**	78.8	89.8	79.2	78.9	92.6	78.4							
MYMV-[SbPak]	61.9	62.2	63.9	64.2	63.9	84.3	**84.4**	**83.0**	81.2	94.6	81.5	81.4	94.5	80.7	90.7						
MYMV-[TH]	61.8	61.8	64.1	64.1	64.0	84.4	**84.2**	**82.8**	80.7	93.4	81.1	81.1	99.9	80.6	92.5	94.4					
MYMV-[Vig]	61.1	61.3	63.8	64.1	63.7	84.6	**84.6**	**82.6**	81.0	93.3	81.3	81.1	96.3	80.8	93.7	94.2	96.3				
MYMV[VigMah]	61.4	61.1	64.1	64.2	63.8	84.9	**84.8**	**83.0**	81.3	93.2	81.6	81.4	96.3	80.9	93.9	94.3	96.3	98.2			
ToLCBDV	63.4	63.3	62.8	62.4	62.9	66.6	**66.5**	**66.6**	64.5	64.9	64.7	64.7	65.5	64.3	65.8	65.4	65.6	65.4	65.8		
ToLCBV	63.9	63.6	61.6	61.7	61.6	65.2	**65.5**	**65.5**	64.9	65.3	64.8	64.8	65.5	64.5	65.6	65.5	65.6	65.5	65.6	81.3	
ToLCNDV-[Svr]	62.6	62.8	62.0	62.1	62.1	63.2	**63.1**	**63.4**	62.5	63.7	62.5	62.6	63.8	62.4	64.7	63.5	63.7	63.4	63.7	73.2	71.6
TYLCV	63.6	63.0	62.8	62.7	63.1	65.6	**65.8**	**65.6**	64.4	65.0	64.9	64.7	65.2	64.6	65.0	65.3	65.3	65.5	65.7	75.0	73.8

Comparison of genome sequence and the construction of phylogenies based on DNA-B

The dendogram based on partial DNA-B sequences of HgYMV infecting horsegram with other begomoviruses transmitted by whitefly grouped the viruses into Old World and New world categories (Figure 3). The Old Would group consisted of five major clusters and the new World consists of single cluster. A comparison of nucleotide distances for DNA-B component of HgYMV1 indicated that it was closest to previously described HgYMV at 70% nucleotide identity. However, HgYMV1 shared nucleotide identity with MYMV-[Cp], MYMV-BB31, MYMV-[Mg] at 90.8, 94.8 and 94.4%, respectively. A low nucleotide identity of 48.7% was shown between HgYMV1 and BGYMV-[Mx]. The percent divergence of 44.5% was shared between strain of HgYMV and TLCNDV-Svr, indicating large genomic variation between the strains (Table 3).

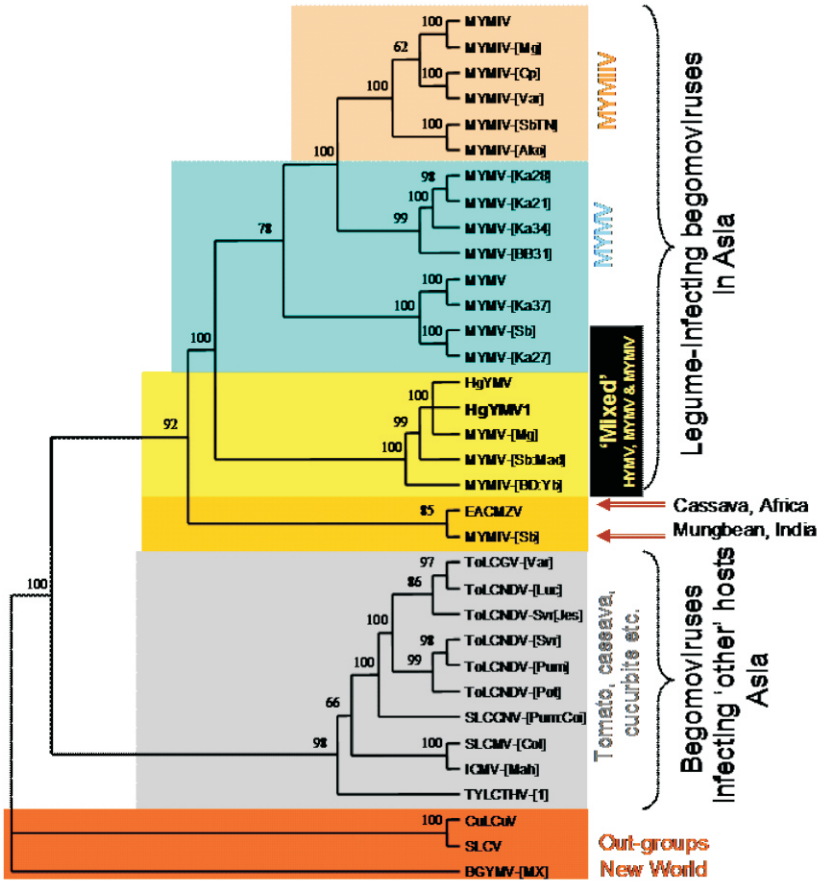

Figure 3 Parsimonious tree showing the relationship of HgYMV1 with other begomoviruses based on complete DNA-B nucleotide sequences.Vertical distances are arbitrary and the horizontal distances are proportional to mutational changes in the sequences. Numbers at nodes indicate percent bootstrap scores using 100 replicates.

Table 3: Percent nucleotide identities for the complete DNA-B genome and genes of Indian HgYMV with selected begomoviruses

MYMIV - [Akola]															
BGYMV -[Mx]	48.5														
MYMV	69.8	48.0													
HgYMV	70.2	48.0	97.4												
HgYMV1	**93.6**	**48.7**	**69.9**	**70.1**											
MYMV -Cp	90.5	48.8	69.5	69.9	**90.8**										
MYMV -BB31	94.3	48.9	69.7	70.0	**94.8**	90.4									
MYMV -Mg	94.0	48.6	69.3	69.6	**94.4**	89.8	99.5								
MYMIV	72.1	46.9	69.2	69.2	**71.8**	72.4	72.3	71.7							
MYMV -Ka21	89.6	49.1	70.0	70.4	**89.8**	92.5	89.7	89.1	72.8						
MYMV -Ka27	71.8	46.7	69.5	69.6	**71.5**	72.2	72.2	71.7	95.0	72.7					
MYMV -Ka28	89.6	48.8	70.2	70.6	**89.8**	92.6	89.6	89.1	73.2	98.2	72.7				
MYMV -Ka34	89.5	48.7	69.9	70.1	**89.6**	92.4	89.3	88.9	73.2	97.1	73.1	96.8			
MYMV - SbMad	70.1	47.8	95.9	96.8	**70.0**	69.8	69.9	69.4	69.2	70.3	69.5	70.4	70.1		
MYIV -SbTN	97.1	48.6	69.7	70.0	**93.6**	90.2	94.3	94.0	72.3	89.1	71.7	89.2	89.2	70.0	
TLCNDV -Svr	44.9	42.3	43.8	44.0	**44.5**	44.4	44.7	44.4	43.3	44.3	43.3	44.5	44.2	44.0	44.6

Discussion

HgYMV were detected successfully using degenerate primers Deng A/B in the symptomatic plant leaves samples. The presence of these viruses (HgYMV1 and HgYMV2) was confirmed by PCR. The PCR diagnostic results were further confirmed by sequencing of virus genomes. The analysis of DNA-A and DNA-B sequences and construction of phylogenies indicated that HgYMV of this study belonged to the genus *Begomovirus* of family *Geminiviridae* and they are Old World species.

The genomes for two previously uncharacterized bean-infecting begomoviruses from the India, HgYMV1 and HgYMV2, have been cloned and completely characterized. Full-length infectious clones of DNA-A and DNA-B component for HgYMV1 and HgYMV2 were each cloned from symptomatic horsegram plant. Analysis of their genome sequences revealed that they are strains of begomovirus. The genome organisation of (HgYMV1 and HgYMV2) reported in this study was similar to that of a typical bipartite genome reported by previous studies (Abarshi et al., 2017; Rienzie et al., 2016; Abarshi et al., 2017).

The percent nucleotide identities and divergences for the partial sequences of DNA-A of HgYMV1 and HgYMV2 infecting horsegram to those of reference begomoviruses were compared (see Table 2). There are some isolates of begomoviruses sharing >90% nucleotide identities to each other. The percentage divergences in the range of 60-84% were shared between HgYMV and other begomoviruses. The high percent divergence of 60.6 and 60.8 were shared between (HgYMV1 and HgYMV2) and BGYMV-[Mx] strain of New World. This study was in agreement with the previous findings that showed relatedness of HgYMV1/ HgYMV2 to HYMV (Muniyappa et al., 1987), Corchorus yellow vein virus (CoYVV) (Ha et al., 2008), HgYMV-[LK:09] (Monger et al., 2010),

MYMV[33], MYMIV (Manjunatha et al., 2015; Rienzie et al., 2016) and HgYMV-Lb (Abarshi et al., 2017).

The percent nucleotide identities and divergences for the partial sequences of DNA-B of HgYMV1 infecting horsegram to those of reference begomoviruses were compared (see Table 3). There are some isolates of begomoviruses (such as MYMV-[Cp], MYMV-BB31, MYMV-[Mg]) sharing >90% nucleotide identities to HgYMV1. The high percent divergence of 44.5 was shared between HgYMV1 and TLCNDV-Sv. Surprisingly, HgYMV was closest to previously described HgYMV at 70% nucleotide identity. This indicated that the genomic variation is primarily correlated with geographical location rather than their host range.

Conclusion

In this study only HgYMV1 and HgYMV2 from India were isolated and characterised. This would open the possibilities for epidemiological studies and testing the infectivity of the Virus. The relationship of HgYMV and other begomoviruses in different geographical regions are generated in sequencing studies. The nucleotide identities and arrangement of the genes, however, indicated that both the HgYMV and some viruses from the Old World belong to the genus *Begomovirus* of Family *Germiniviridae*.

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