



Molecular Variability of Yellow Mosaic Virus (YMV) of Blackgram in Southern India

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ABSTRACT

Background: The yellow mosaic disease (YMD) creates a major hindrance and known to affect a number of grain leguminous crops in the Indian sub-continent. However, blackgram (*Vigna mungo* L.) is the most important grain legume crop which is affected widely by this disease. The disease is caused by a single stranded DNA containing begomovirus viz., *Yellow mosaic virus* (YMV) which is mainly transmitted through whitefly (*Bemisia tabaci* Genn.). Symptoms include severe patho-physiological alterations characterized by the presence of bright chlorotic yellow patches interspersed with green areas on leaf lamina. The present study was aimed to determine the variability among disease causing agents in six different geographical isolates representing four states viz. Andhra Pradesh, Karnataka, Odisha and Telangana states of southern India during the 2019 *rabi* season.

Methods: The variability of YMV associated with YMD of blackgram was studied based on molecular characterization of partial DNA-A coat protein gene with subsequent nucleotide sequencing and phylogenetic tree construction.

Result: The synthetic primers designed for the partial DNA-A segment forms a distinct viral gene specific PCR product. The band size corresponding to CP ~704 bp was obtained for MYMV, whereas CP ~500 bp gene band was obtained for MYMIV. Further, phylogenetic analysis based on partial DNA-A gene sequences of six isolates with other isolates from GenBank formed into two unique clusters viz., MYMV and MYMIV. Overall, our study confirming that the begomovirus causing YMD of blackgram in southern India is explored to be as strains of MYMV and MYMIV.

Key words: Blackgram, *Mungbean yellow mosaic virus* (MYMV), Variability, Yellow mosaic disease (YMD).

INTRODUCTION

Blackgram [*Vigna mungo* (L.) Hepper] (2n=22) is one of the important pulse crops of the tropics and sub tropics. It is the third major pulse crop after chickpea and pigeonpea cultivated in the Indian sub-continent. India is a major producer of blackgram with 3.06 million tonnes production harvested from area of 5.60 million hectares (Department of Agriculture and Cooperation, GOI, 2018-19). Despite large area under blackgram cultivation in India, the productivity is very low due to many pests (e.g.: pod borers, sucking pests etc.) and diseases (e.g.: Alternaria leaf spot, Cercospora leafspot, powdery mildew, viral diseases etc.). Among all the diseases, YMV is the major problem and has the most damaging effect on the productivity of blackgram crop across the Indian sub-continent with annual estimated yield loss up to 100% under favourable environmental conditions (Varma *et al.*, 1992; Varma and Malathi 2003; Sathees *et al.*, 2019).

Nariani (1960) was the first person ever to report the *Mungbean Yellow Mosaic Virus* (MYMV) disease of mungbean from the fields of IARI, New Delhi India. It is transmitted by whitefly (*Bemisia tabaci*) in a semi persistent manner. Symptoms are characterized by presence of bright yellow patches on leaves interspersed with green areas, complete yellowing and stunting of the plant. The infected leaves turn necrotic and the diseased plants usually mature later and had relatively few flowers and pods (Nene 1972;

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Singh *et al.*, 1980; Rathi *et al.*, 2002). As an obligate whitefly-borne pathogen, YMV has an extensive natural host-range that includes mungbean (*Vigna radiata*), blackgram (*V. mungo*), pigeon pea (*Cajanus cajan*), soybean (*Glycine max*), mothbean (*Vigna aconitifolia*) and common bean (*Phaseolus vulgaris*) causing yield loss of about \$300 million by upsetting the physiological machinery of the host. The disease in southern Asia is caused by four distinct begomoviruses collectively known as the yellow mosaic viruses (YMV); *Mungbean yellow mosaic virus* (MYMV), *Mungbean yellow mosaic India virus* (MYMIV), *Dolichos yellow mosaic virus*

(DoYMV), Lima bean Yellow Mosaic Virus (LYMV) (Bhagyashree *et al.*, 2017) and Horsegram yellow mosaic virus (HgYMV) (Qazi *et al.*, 2007).

Of these MYMIV and MYMV are most important as it infect large number of legumes in India (Fauquet *et al.*, 2003). MYMIV is more predominant in northern, central and eastern regions of India (Usharani *et al.*, 2004), whereas MYMV occurs in peninsular and southern region of India (Karthikeyan *et al.*, 2004; Girish and Usha 2005; Haq *et al.*, 2011). Polymerase chain reaction with coat protein gene-specific primers (RHA-F and AC abut) primers are used to detect MYMV in YMV infected blackgram which specifically amplifies a 900 bp product (Reddy *et al.*, 2015). Viruses of the family Geminiviridae have characteristic twinned quasi-icosahedral particles (18×30 nm) that encapsulated the circular single-stranded DNA (Stanley *et al.*, 2005). The YMV's are included in the begomovirus genus, being transmitted by the whitefly (*Bemisia tabaci*) and having bipartite genomes. DNA-A encodes proteins required mainly for encapsidation, replication and transcription regulation, whereas DNA-B for intra- and intercellular protein movement. The DNA-A and DNA-B components of begomoviruses have highly conserved intergenic common region (CR) which comprises stem-loop structure with the loop containing the invariant nano-nucleotide motif (TAATATTAC) that characterizes the origin of viral replication strand. MYMIV and MYMV isolates share only 82% sequence identity between them in the whole of DNA-A component justifying their separation into distinct species. Full length coat protein gene sequences are accepted by International Committee on Taxonomy of Viruses (ICTV) for provisional classification of begomoviruses and it is useful for species/ strain level identification (Fauquet *et al.*, 2008).

Therefore, associations of YMV strains in blackgram was reported in southern India (Karthikeyan *et al.*, 2004; Haq *et al.*, 2011), a study was conducted to investigate and characterize coat protein gene of YMV infecting blackgram and to confirm that *Blackgram yellow mosaic virus* (BGYMV) from southern Indian states is an isolate or variant of *Mungbean yellow mosaic virus* (MYMV) or *Mungbean Yellow Mosaic India Virus* (MYMIV) with the aid of specific CP gene primers.

MATERIALS AND METHODS

Selection of locations and sample collection

The experiment was conducted during *rabi* season of 2019 at Molecular Biology Laboratory, Department of Plant

Pathology, Agricultural College, Bapatla, ANGRAU, Andhra Pradesh. Information regarding main blackgram growing locations in southern India was obtained from different Agricultural Research Stations (ARS), State Agricultural Universities (SAU's) and Krishi Vignan Kendra (KVK's) across four states viz, Odisha andhra Pradesh, Karnataka and Telangana of southern India and samples were collected to study the variability. A total of six blackgram samples showing typical yellow mosaic symptoms were collected from six different regions of the southern India and placed in zip-lock plastic bags and transported in cold packs to Agricultural college Bapatla, snap-frozen in liquid nitrogen and stored in a -80°C freezer until further use. Three samples from Andhra Pradesh *i.e.*, from Tirupati, Bapatla and Lam farm and other three samples each in different states viz., Bengaluru (Karnataka), Bhubaneswar (Odisha) and Madhira (Telangana) were collected. Samples from healthy plants were collected as controls in all places. The samples were named as Tirupathi isolate, Bapatla isolate, Lam farm isolate, Bengaluru isolate (Karnataka), Bhubaneswar isolate (Odisha) and Madhira isolate (Telangana).

Total DNA extraction

The total genomic DNA from leaf tissues of healthy (control sample) and YMV-infected blackgram samples were extracted from leaves by the modified CTAB method (Murray and Thompson 1980). Infected plant material (0.5g) was ground in a pre-sterilized pestle and mortar with liquid nitrogen (-196°) until a fine powder was obtained and transferred to sterile Eppendorf tube of 1.5 ml. To this, 1ml of pre-heated (65°C) extraction buffer (100 mMTris (pH 8.0), 1.4 M NaCl, 50 mM EDTA, 2% CTAB, 1% PVP and 0.1% β-Mercaptoethanol) were added and incubated for 1 hour in water bath at 65°C. The extraction mixture in tubes was centrifuged at 10,000 rpm for 10 min at room temperature (27°C) and the supernatant was collected into Eppendorf tubes. To this, equal volumes of chloroform and Isoamyl alcohol (24:1) were added and incubated at room temperature for 10-20 min. Then these tubes were centrifuged at 10,000 rpm at 4°C for 10 min, separated the supernatant into sterile Eppendorf tube of 1.5 ml and added 0.1 volume of 3M sodium acetate (pH 4.8) and 0.6 volume of ice-cold isopropanol were added and then incubated at -20°C for overnight. After incubation, the tubes were taken out and centrifuged at 10,000 rpm at 4°C for 20 min to pelletize the DNA. Supernatant was discarded and the DNA pellet was washed three times with 70% DNA alcohol mixture was centrifuged at 5,000 rpm at 4°C for 5 min, supernatant

Table 1: List of oligonucleotide primers used for amplification of YMV.

Primer name	Nucleotide sequence (5'→3')	Product size (bp)	Target molecule	Annealing temp. (°C)
MYMV - CP- F	GCGGAATTACGATACCGCC	704	Coat protein	54.9
MYMV - CP- R	GATGCATGAGTACATGCC			
MYMIV - CP- F	GGTCCCCTGATGTCCCTCGTG	500	Coat protein	55.0
MYMIV - CP- R	ATGCGTTCTCAGTATGGTTCT			

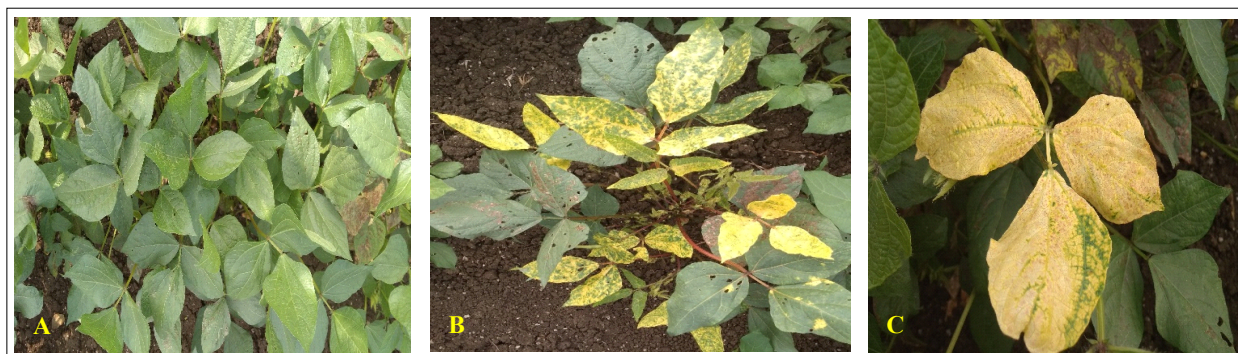


Fig 1: Symptoms of yellow mosaic virus on blackgram. A- Healthy leaves, B- Mosaic and partial chlorosis of leaves and C- Complete chlorosis of leaves.

was discarded, pellet was air dried and dissolved in 50 µl of sterile distilled water. The DNA samples were quantified using Nanodrop spectrophotometer (ND1000, USA).

Polymerase chain reaction and gel electrophoresis

Polymerase chain reaction (PCR) was performed in 25 µl of reaction mixture using 10X PCR reaction buffer, 25 mM of $MgCl_2$, 10 mM of each dNTPs, 10 mM of each primer, 5 U/µl of Taq DNA polymerase (Thermo Scientific, Lithuania) and 100 ng of DNA template. The PCR amplification was performed in a Thermocycler machine (Bio-RAD T100, USA). The amplified PCR products were separated on 2% agarose gel in 1X TAE buffer at 110V by using gel electrophoresis. Specific primers used for amplification of YMV and their sequence were presented in (Table 1). The composition of the reaction mixture (25 µl) used in polymerase chain reaction (PCR) for DNA amplification of respective isolate are given in (Table 2). The migration pattern of the DNA fragments in the gel was noted by using gel documentation system (Omni Doc, Cleaver Scientific, UK) in an auto exposure mode.

Nucleotide Sequencing and construction of phylogenetic tree

DNA amplicons were excised from agarose gel, eluted, purified and sequenced by automated DNA Sanger's dideoxy chain-termination sequencing facility (Eurofin Genomics India Pvt. Ltd., Bangalore). Sequence assembling was analysed by using the software programme BioEdit version 7.0.5 (Hall, 1999). Later, the nucleotide homology searches for a partial sequence of the DNA-A segment of YMV isolates were done with the BLASTN sequence analysis of the NCBI. Phylogenetic tree was constructed from aligned sequences by using the neighbour-joining method in MEGA X (Molecular Evolutionary Genetics Analysis) software version 10.0 (Tamura *et al.*, 2007) based on partial DNA-A segment coat protein gene sequences with 30 others known begomovirus causing diseases in pulses and other crops, downloaded from NCBI GenBank database (www.ncbi.org). Robustness of trees was determined by bootstrap sampling of multiple sequence alignment with 500 replications.



Fig 2: PCR amplification of partial DNA-A segment of Mungbean Yellow Mosaic Virus (MYMV) and Mungbean Yellow Mosaic Indian Virus (MYMIV) infecting Blackgram in Southern India. Lane M: 100bp DNA ladder, Lane 1: MYMIV-Bhubaneswar, Lane 2: MYMIV-Guntur, Lane 3: MYMIV-Bapatla, Lane 4: MYMIV-Madhira, Lane 5: MYMIV-Bengaluru, Lane 6: MYMV-Tirupathi and Lane H: Healthy sample.

Table 2: Composition of the PCR reaction mixture (25µl).

Component	Volume
10X PCR reaction buffer (Thermo Scientific, Lithuania)	3µl
25 mM $MgCl_2$ (Thermo Scientific, Lithuania)	3µl
10 mM dNTPs (Thermo Scientific, Lithuania)	0.5µl
10 mM Primer (Eurofins Genomics, Bangalore)	0.5µl
5U Taq polymerase (Thermo Scientific, Lithuania)	0.2µl
Template DNA	500ng
Molecular grade water (Q.S.)	25µl

RESULTS AND DISCUSSION

Symptoms of yellow mosaic virus disease in blackgram

YMV infected plants were identified based on visual symptoms such as small yellow round spots on early emerged leaves to yellow mosaic patches alternate with green colour leaf lamina on older leaves. Severely infected plants showed reduced leaf size with stunted growth and smaller number of pods showing yellow discolorations (Fig 1).

Phylogenetic analysis of partial DNA-A nucleotide sequences of MYMV and MYMIV

DNA was extracted from the infected blackgram samples

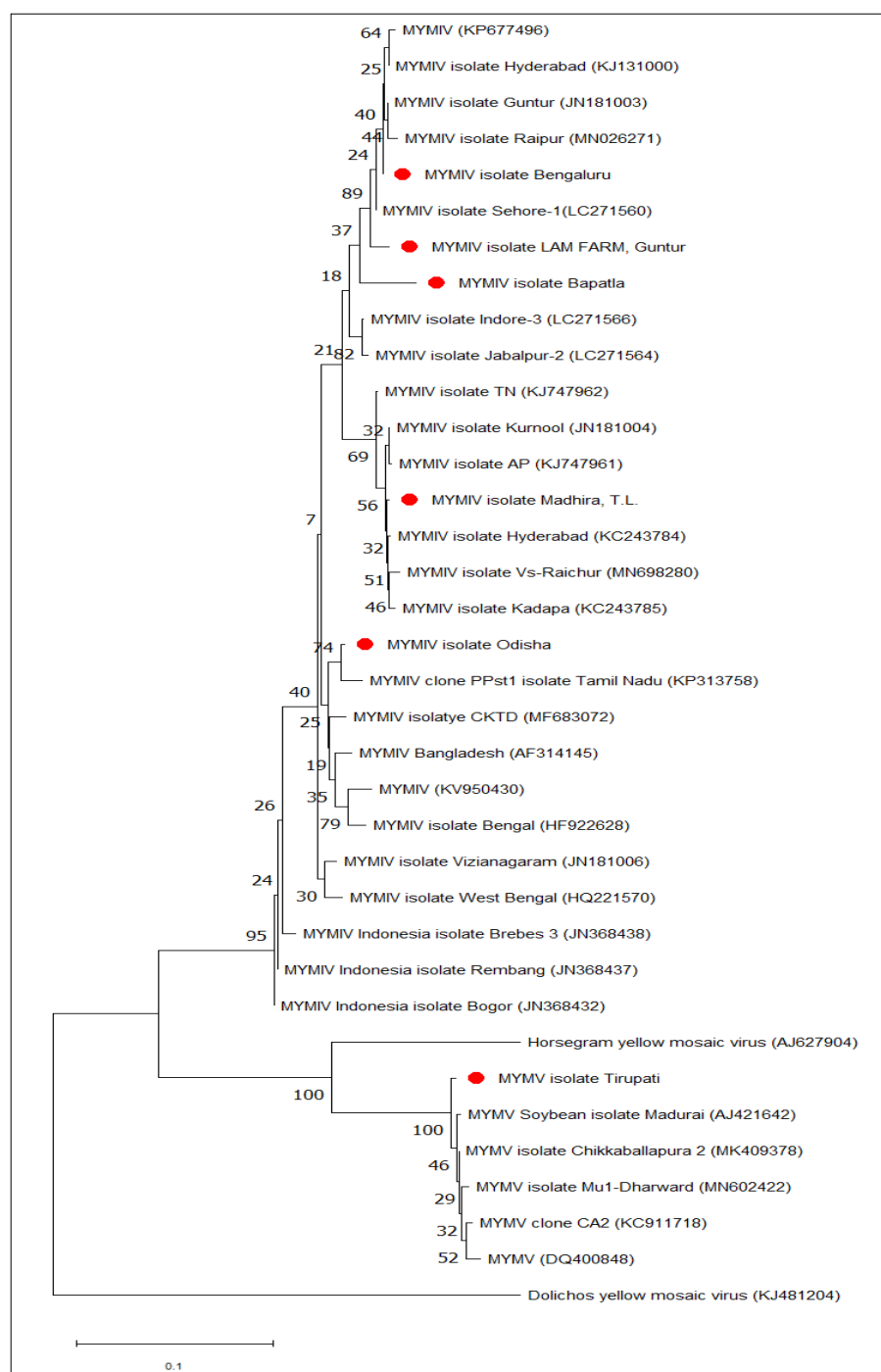


Fig 3: Phylogenetic tree of the coat protein gene sequence *Yellow mosaic virus* (YMV) isolates from Southern parts of India with other Gemini virus sequences.

by modified CTAB method and the DNA quantity was measured by taking absorption at 260 nm in Nanodrop spectrophotometer and the concentration was found to be ~1.7 µg/µl. Two CP primers specific to *MYMV* and *MYMIV*, for reproducible amplification of virus in the blackgram infected sample are used in this present work. All the six isolates viz., Tirupathi isolate, Bapatla isolate, Lam farm isolate, Bengaluru isolate (Karnataka), Bhubaneswar isolate

(Odisha) and Madhira isolate (Telangana) were amplified by PCR using CP gene specific primers (Table 1). Results showed that five isolates viz., Bapatla isolate, Lam farm isolate, Bengaluru isolate (Karnataka), Bhubaneswar isolate (Odisha) and Madhira isolate (Telangana) were successfully amplified with MYMIV-CP-500 bp primer, indicating the fields in these areas are infected with *Mungbean yellow mosaic Indian virus* (MYMIV) strain,

whereas Tirupati isolate was successfully amplified with *MYMV*-CP-704 bp primer, indicating the fields in these areas are infected with *Mungbean yellow mosaic virus* (*MYMV*) strain (Fig 2).

The synthetic primers designed for the partial DNA-A segment coat protein gene of *MYMV* and *MYMIV* amplified a specific product of 704 bp and 500 bp respectively (Fig 2). The partial nucleotide sequences of the DNA-A segment of *MYMV* and *MYMIV* isolates along with available corresponding DNA-A sequences of yellow mosaic virus's (*YMV*'s) infecting pulses when subjected to the phylogenetic analysis (Fig 3) formed two distinct clusters. The DNA-A sequence of *MYMV* Tirupati isolate clustered with other available corresponding sequences for DNA-A of *MYMV* isolates. It also showed more relationship with DNA-A sequence of *Horsegram yellow mosaic virus* (*HYMV*) than other *YMV*'s sequences. While DNA-A sequences of *MYMIV* isolates from Bengaluru, Guntur, Bapatla and Madhira clustered together with isolates Hyderabad, Guntur, Raipur, Sehore, Raichur, Jabalpur, Indore, Karnool and Kadapa. Whereas, isolate, Bhubaneswar clustered along with isolates Tamil Nadu, Bangladesh and Bengal. The coat protein gene is the most highly conserved gene in the family *Geminiviridae*. It is the sole structural protein of begomoviruses and has been shown to play a determinative role in the transmission of these viruses (Sharma *et al.*, 2005). CP gene has traditionally useful for identification and classification of plant viruses (Mayo and Pringle, 1997). The International Committee on Taxonomy of Viruses (ICTV) accepts the classification of begomoviruses based on CP gene sequences, when full length sequences are not available (Rybicki, 1998). Reddy *et al.* (2015) observed six isolates causing YMD of blackgram in Andhra Pradesh are closely related to *MYMIV* (old world gemini viruses) than *MYMV* (New world gemini viruses). The *YMV* infecting blackgram and greengram in Telangana and AP is closely related to *MYMIV* rather than *MYMV* or other begomoviruses was reported by Banu (2014). Therefore, the present results with partial coat protein gene sequence alignment and construction of phylogenetic tree using CP gene sequences had clearly revealed that *YMV* infecting the blackgram crop is not an identical strain in southern India.

CONCLUSION

The present study showed that *YMV* infecting blackgram in different southern Indian regions is not a same strain. Our results showed that isolates collected from Bangalore, Madhira, Odisha, Bapatla and Lam farm were amplified with *MYMIV* specific primers and represents the *MYMIV* strain of virus, whereas the isolate collected from Tirupati was amplified with *MYMV* specific primer and represents the *MYMV* strain of virus. The partial nucleotide sequences of the DNA-A segment of *MYMV* and *MYMIV* isolates along with available corresponding DNA-A sequences of yellow mosaic virus's (*YMV*'s) infecting pulses when subjected to the phylogenetic analysis formed two distinct clusters.

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