Original Research Article

Coat Protein characterization of Mungbean yellow mosaic virus infecting blackgram (*Vigna mungo* L. Hepper) in Tamil Nadu

ABSTRACT

The yellow mosaic disease is the main constraint for decreased yield in blackgram production. Coat protein gene of *mungbean yellow mosaic virus* (mymv) has been characterized and phylogenetic tree was constructed. The genomic dna from diseased plants was collected and it was tested for it's quality and quantity using a nanodrop. The isolated dna was subjected to polymerase chain reaction using begomovirus universal primers for preliminary confirmation of the virus. The samples amplified for universal primers were subjected for gene specific amplification using dna a and dna b primers. The dna a primers resulted in the amplification of the expected size of 1000bp amplicon. The dna fragments were sequenced and the sequence results of dna a were compared with other sequences available in ncbi and it showed higher similarity with *mungbean yellow mosaic virus* banglore coat protein gene (accession no: mk409376). The sequence was submitted in ncbi database and accession numbers were obtained. The phylogenetic tree was constructed using mega 11 software by comparing the selected strains with our isolate.

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Keywords: Blackgram, Mosaic, Coat protein, Characterization, Sequence, Phylogeny

1. INTRODUCTION

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BLACKGRAM (*VIGNA MUNGO* L. HEPPER) IS AN IMPORTANT PULSE USED IN INDIAN DIET, BECA
THE PROTEIN CONTENT OF BLACKGRAM IS THREE TIMES MORE THAN THAT OF THE CEREALS
BLACKGRAM IS USED AS A SUPPLEMENT OF THE CEREALS [17]. BLACKGRAM IS WIDELY GROWN IN COUNTRIES LIKE INDIA, BANGLADESH AND NEPAL [2]. IN INDIA, THE AREA UNDER BLACKGRAM IS 4.6 MILLION HECTARES. THE ANNUAL PRODUCTION OF BLACKGRAM IN INDIA IS 24.5LAKH TONNES WHICH CONTRIBUTES TO THE 23% OF TOTAL PULSE PRODUCTION IN INDIA. IN INDIA MADHYA PRADESH IS THE LARGEST PRODUCER OF BLACKGRAM FOLLOWED BY UTTAR PRADESH, RAJASTHAN AND MAHARASHTRA. IN SOUTHERN INDIA, TAMIL NADU IS ONE OF THE LEADING PRODUCERS OF BLACK GRAM WITH AN AREA OF 886.9 HECTARES, PRODUCING 365.3 TONNES ANNUALLY WITH THE PRODUCTIVITY OF 412 KG/HA. THE BLACKGRAM CROP IS GROWN DURING KHARIF AND RABI. DUE TO THE ABILITY OF THE CROP TO WITHSTAND ADVERSE ENVIRONMENTAL CONDITIONS, IT CAN BE GROWN AS RICE FALLOW CROP WITH MINIMAL CARE, WHICH IS A MOST COMMON PRACTICE BY FARMERS. BUT THERE ARE SEVERAL BIOTIC FACTORS THAT ARE INVOLVED IN HINDERING THE PRODUCTION AND YIELD OF BLACKGRAM WHICH ARE PESTS AND DISEASES, AMONG WHICH DISEASES LIKE YELLOW MOSAIC DISEASE [7], LEAF CRINCKLE VIRUS [10], CERCOSPORA LEAFSPOT [1], BACTERIAL LEAF SPOT AND POWDERY MILDEW [12] PLAY A MAJOR ROLE. YELLOW MOSAIC DISEASE IS DETECTED AS THE MAJOR CONSTRAINT FOR THE BLACKGRAM AND GREENGRAM PRODUCTION IN INDIA [19].

IN INDIA, YELLOW MOSAIC DISEASE IS FIRST REPORTED BY NARIANI IN 1960 IN INDIAN AGRICULTURAL RESEARCH INSTITUTE, LATER THE CAUSAL ORGANISM IS DIAGNOSED AS A VIRUS I.E. MUNGBEAN YELLOW MOSAIC VIRUS [9]. IN 1968, WILLIAMS ET AL HAS REPORTED THE

OCCURRENCE OF MYMV IN BLACKGRAM [20]. YELLOW MOSAIC DISEASE CAUSED BY BEGOMOVIRUS BELONGING TO FAMILY GEMINIVIRIDAE. THE BEGOMOVIRUS ARE THE SSDNA VIRUSES THAT ARE TRANSMITTED IN A NON-PROPAGATIVE PERSISTENT AND CIRCULATIVE MANNER BY THE WHITE FLIES (*BEMISIA TABACI*) [6] [8] [9] [13]. THIS DISEASE IS MAINLY OCCURRED BY FOUR DISTINCT BEGOMOVIRUSES THEY ARE, MUNGBEAN YELLOW MOSAIC VIRUS(MYMV), HORSEGRAM YELLOW MOSAIC VIRUS (HGYMV), DOLICHOS YELLOW MOSAIC VIRUS (DOYMV) AND MUNGBEAN YELLOW MOSAIC INDIA VIRUS (MYMIV). BUT MYMV AND MYMIV ARE OF MOST IMPORTANCE AS THEY CAUSE SEVERE YIELD LOSSES IN GRAIN PULSES ESPECIALLY IN GREENGRAM AND BLACKGRAM [5]. THE SYMPTOMS OF MYMV APPEARS MOSTLY ON LEAVES AND ALSO IN PODS AND SEEDS. THE VISIBLE SYMPTOMS ON LEAVES WILL START AS YELLOW SPOTS WHICH LATER TURN INTO THE NOTABLE YELLOW AND GREEN ALTERATION IN MOSAIC PATTERN IN LATER STAGES IT LEADS TO NECROSIS AND DEATH OF THE LEAVES. THE PODS PRODUCED BY THE DISEASED PLANT ARE MISSHAPEN AND SMALL COMPARED TO HEALTHY PODS. THE DISEASE WILL USUALLY SPREAD FROM PODS TO SEEDS. THE DISEASED SEEDS CAN BE VISUALLY SEPARATED FROM THE HEALTHY SEEDS AS THEY ARE ILL FILLED, NOT IN A REGULAR SHAPE AND IN SOME CASES YELLOW DISCOLORATION IS ALSO SEEN ON INFECTED SEEDS.
MYMV IS A BEGOMOVIRUS CONTAINING BIPARTITE GENOME IMPLYING THAT IT CONTAINS DNA A AND DNA B AS IT'S GENOMETHE DNA A CONTAINING THE COAT PROTEIN GENE IS RESPONSIBLE FOR SYMPTOM EXPRESSION AND VECTOR TRANSMISSION. [3][4]

2. MATERIAL AND METHODS

2.1 COLLECTION OF DISEASED SAMPLES:

THE SAMPLES WERE COLLECTED FROM THE PLANTS SHOWING TYPICAL AND PROMINENT SYMPTOMS OF YELLOW MOSAIC DISEASE WHICH INCLUDE YELLOW COLOR PATCHES ALTERNATING WITH GREEN COLOR SHOWING MOSAIC PATTERN. THOUGH THE MATURE LEAVES ARE SHOWING PROMINENT AND NOTICEABLE SYMPTOMS, THE SAMPLES ARE COLLECTED FROM YOUNG LEAVES I.E., EMERGING AND EXPANDING LEAVES BECAUSE THE CONCENTRATION OF VIRUS WILL BE MORE IN THE DEVELOPING MERISTEMATIC TISSUES. THE DISEASED SAMPLES WERE COLLECTED FROM THE EXPERIMENTAL FIELDS OF DEPARTMENT OF PULSES, TAMIL NADU AGRICULTURAL UNIVERSITY, COIMBATORE.

2.2 PLANT GENOMIC DNA ISOLATION:

AS THE VIRUS UNDERGOES ITS REPLICATION IN THE NUCLEUS OF THE PLANT CELL, THE TOTAL GENOMIC DNA OF THE PLANT IS ISOLATED USING THE GEM- CTAB METHOD WITH SLIGHT MODIFICATIONS. GEM-CTAB METHOD IS A DNA ISOLATION METHOD SPECIFICALLY MODIFIED FOR THE DETECTION OF BEGOMOVIRUSES IN LEGUMES [16]. THE BUFFER CONTAINING 100MM TRIS-HCL, 10MM EDTA, 2.0M NACL, AND 2% CTAB IS PREPARED AND AUTOCLAVED AFTER ADJUSTING THE P^H TO 8.0. B MERCAPTOETHANOL IS ADDED PRIOR TO THE ISOLATION PROCEDURE. TO 100MG OF LEAF SAMPLE TAKEN IN PRE CHILLED PESTLE AND MORTAR, 1ML OF THE BUFFER IS ADDED, TRANSFERRED INTO 1.5ML EPPENDORF TUBE AND KEPT FOR INCUBATION AT @65°C FOR ONE HOUR. DURING INCUBATION THE TUBES WERE INTERMITTENTLY MIXED TO KEEP THE BUFFER AND PLANT SAMPLE HOMOGENOUS. AFTER INCUBATION, 0.8 VOLUMES OF CHLOROFORM: ISO- AMYL ALCOHOL IS ADDED TO THE TUBES AND MIXED GENTLY BY INVERSION UNTIL THE FORMATION OF EMULSION. THE TUBES ARE CENTRIFUGED AT 10,000RPM FOR 10 MIN AT 4°C IN A REFRIGERATED CENTRIFUGE. THE CHLOROFORM: ISO- AMYL ALCOHOL IS REPEATED WHEN THE PHENOL CONTENT IN THE SAMPLE IS MORE. THE SUPERNATANT/ THE UPPER AQUEOUS LAYER IS COLLECTED INTO A NEW TUBE. 0.6 VOLUMES OF ICE COLD ISOPROPANOL WAS ADDED TO THE TUBES AND INCUBATED AT 4°C OVERNIGHT. AFTER OVERNIGHT INCUBATION, THE TUBES ARE CENTRIFUGED AT 10,000 RPM FOR 10 MIN TO OBTAIN THE NUCLEIC ACID PELLET. THE OBTAINED PELLET IS WASHED TWICE WITH 70% ETHANOL. AFTER ETHANOL WASH THE TUBES ARE KEPT OPEN AT ROOM TEMPERATURE FOR THE ETHANOL TO EVAPORATE COMPLETELY FROM THE DNA. 50µL OF DOUBLE STERILE DISTILLED WATER IS ADDED TO DISSOLVE THE PELLET. THE ISOLATED DNA ARE STORED AT -20°C FOR FURTHER USE.

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2.2.1 QUALITY AND QUANTITY CHECK OF ISOLATED DNA:

THE ISOLATED PLANT DNA ARE CHECKED BY GEL ELECTROPHORESIS BY MAKING THEM TO RUN ON 0.8% AGAROSE GEL AFTER STAINING WITH ETHIDIUM BROMIDE. 6X DNA LOADING DYE IS ADDED TO THE DNA TO MAKE THEM VISUALIZED UNDER UV LIGHT.

THE CONCENTRATION OF THE DNA IS MEASURED BY USING NANODROP. THE ABSORBANCE OF THE SAMPLE DNA PLACED IN NANOMETER AT 220NM OF WAVELENGTH HAS GIVEN THE CONCENTRATION OF THE DNA AND ALSO THE LEVEL OF PROTEIN CONTAMINATION IN DNA.

2.2.2 DILUTION OF GENOMIC DNA:

THE DNA OF ALL THE SAMPLES ARE CONTAING MORE THAN 1000NG OF DNA PER 1µL OF ISOLATED DNA. THE REQUIRED CONCERTATION OF DNA FOR EFFECTIVE POLYMERASE CHAIN REACTION IS 50-100NG/ µL. SO, IN ORDER TO REDUCE THE CONCENTRATION OF DNA, 1 µL OF RAW DNA IS ADDED TO 9 µL OF NUCLEASE FREE WATER.

2.3 PCR BASED DETECTION USING UNIVERSAL PRIMERS:

FOR THE PRELIMINARY CONFIRMATION OF THE ISOLATED DNA FOR THE PRESENCE OF BEGOMOVIRUS, THE DNA IS SUBJECTED TO POLYMERASE CHAIN REACTI0N USING UNIVERSAL DEGENERATIVE PRIMERS OF BEGOMOVIRUS I.E., PALR772 AND PALC1960 (ROJAS *ET AL*., 1993).THE SEQUENCE OF THE PRIMER SET IS PALR772-GGNAARATHTGGGATGGA AND PALC1960-ACNGGNAARACNATGTGGGC .THE REACTION MIXTURE WAS ADDED TO STERILE PCR TUBE CONTAINING 10µL OF TAQ DNA POLYMERASE, OPTIMIZED BUFFER, AND DNTP MIXTURE IN A READY-TO-USE MASTER MIX (2X TAKARA EMERALAMP GT PCR MASTERMIX), 1µL OF EACH FORWARD PRIMER REVERSE PRIMERS AND TEMPLATE DNA, THE FINAL VOLUME IS ADJUSTED TO 20µL USING DOUBLE STERILE DISTILLED WATER. THE TEMPERATURE PROFILE FOLLOWED WAS INITIAL DENATURATION AT 94°C FOR 2 MINUTES FOLLOWED BY 35 CYCLES OF DENATURATION AT 95°C FOR 1 MINUTE, ANNEALING AT 55°C FOR 2 MINUTES AND ELONGATION AT 72°C FOR 3 MINUTES, THE FINAL EXTENSION STEP WAS CARRIED OUT AT 72°C FOR 10 MINUTES. THE PCR PRODUCT WAS KEPT IN HOLD AT 4°C. THE POLYMERASE CHAIN REACTION IS CARRIED OUT IN THE MASTERCYCLER® NEXUS GRADIENT THERMAL CYCLER. THE PRIMER SET IS EXPECTED TO AMPLIFY THE DNA FRAGMENT OF 1100BP.

AFTER THE PCR REACTION THE SAMPLES ARE MADE TO RUN ON 1.2% AGAROSE GEL USING 1X TAE BUFFER AFTER STAINING THE GEL WITH ETHIDIUM BROMIDE. THE GEL IS IMAGED UNDER AN ALPHA IMAGER TO KNOW THE AMPLIFICATION OF THE SAMPLE TO THE DESIRED SIZE TO CONFIRM THE PRESENCE OF THE VIRUS.

2.4 AMPLIFICATION USING GENE SPECIFIC PRIMERS:

BY THE CONFIRMATION OF THE PRESENCE OF BEGOMOVIRUS IN THE SAMPLE, WE USED THE MYMV SPECIFIC PRIMERS TO AMPLIFIES DNA A OF MYMV. THE FORWARD PRIMER OF ATG GG (T/G) TCC GTT GTA TGC TTG SEQUENCE AND THE REVERSE PRIMER OF THE SEQUENCE GGC GTC ATT AGC ATA GGC AAT WERE USED TO AMPLIFY THE COAT PROTEIN REGION OF MYMV. THE REACTION MIXTURE WAS PREPARED BY ADDING 25µL OF MASTER MIX, 2 µL OF FORWARD PRIMER,2 µL OF REVERSE PRIMER AND 2 µL OF DILUTED TEMPLATE DNA. THE FINAL VOLUME IS ADJUSTED TO 50 µL USING NUCLEASE FREE WATER. THE POLYMERASE CHAIN REACTION WAS CARRIED OUT IN MASTERCYCLER® NEXUS GRADIENT THERMAL CYCLER USING A TEMPERATURE PROFILE OF INITIAL DENATURATION AT 94°C FOR 2 MINUTES FOLLOWED BY 35 CYCLES OF DENATURATION AT 95°C FOR 1 MINUTE, ANNEALING AT 54°C FOR 2 MINUTES AND ELONGATION AT 72°C FOR 3 MINUTES, THE FINAL EXTENSION STEP WAS CARRIED OUT AT 72°C FOR 10 MINUTES. THE AMPLIFIED PCR PRODUCTS WERE ANALYZED AND THE SIZE OF AMPLICONS WAS INTERPRETED UTILIZING ELECTROPHORESIS ON 1 PER CENT AGAROSE GEL STAINED WITH ETHIDIUM BROMIDE USING 1X TAE BUFFER. IN A UV-GEL DOCUMENTATION UNIT (ALPHAIMAGER, USA), THE GEL WAS OBSERVED, AND THE FINDINGS WERE RECORDED.

TABLE 1. DETAILS OF PRIMERS USED AND ANTICIPATED AMPLICON SIZES

S.NO	NAME OF THE PRIMER	SEQUENCE 5' TO 3'	ANNEALING TEMPERATURE (°C)	ANTICIPATED AMPLICON SIZE	REFERENCE
1	PALR772	5'GGNAARATHTGGGATGGA3'	55	~1.5KB	[15]
2	PALC1960	5'ACNGGNAARACNATGTGGGC3'			
3	MYMV-CP-F	ATGGG(T/G)TCCGTTGTATGCTTG	54	1000BP	[7]
4	MYMV-CP-R	GGCGTCATTAGCATAGGCAAT			

2.5 SEQUENCING, SEQUENCE ANALYSIS AND CONSTRUCTION OF PHYLOGENETIC TREE THE SAMPLES AMPLIFIED FOR COAT PROTEIN REGION OF MYMV USING GENE SPECIFIC PRIMERS WERE SEQUENCED BY SANGER SEQUENCING. THE SEQUENCE OBTAINED WAS SEARCHED IN NCBI- BLAST TO KNOW THE SEQUENCE SIMILARITY WITH OTHER BEGOMOVIRUSES. SEQUENCE IDENTITY MATRIX TOOL, BIOEDIT SOFTWARE WAS USED TO ASSEMBLE AND ANALYZE THE DATA. MULTIPLE ALIGNMENTS WERE MADE AND AMINO ACID SEQUENCE WAS OBTAINED BY CLUSTRAWLX PROGRAMME. THE SEQUENCE WAS DEPOSITED IN NCBI BLAST AND THE ACCESSION NUMBERS WERE OBTAINED (TABLE). THE PHYLOGENETIC TREE WAS CONSTRUCTED BASED WITH THE AVAILABLE SEQUENCES USING CLUSTRALW PROGRAMME

TABLE 2. LIST OF ACCESSION NUMBERS PROVIDED BY NCBI TO THE MYMV ISOLATE AND COAT PROTEIN OF THIS STUDY

S. NO	LOCATION OF SAMPLE COLLECTION	ISOLATE NAME	PRIMERS USED	ACCESSION NUMBER
1	NATIONAL PULSE RESEARCH STATION, VAMBAN	MUNGBEAN YELLOW MOSAIC VIRUS TAMIL NADU ISOLATE IN BLACKGRAM	PALR772 AND PALC1960	OP121618
2	DEPARTMENT OF PULSES, TNAU	COAT PROTEIN GENE OF MUNGBEAN YELLOW MOSAIC VIRUS TAMIL NADU ISOLATE	MYMV-CP-F AND MYMV-CP-R	OP121617

3. RESULTS AND DISCUSSION

THE ISOLATED DNA FROM DISEASED PLANTS HAS SOWN A CLEAR NON SMEARED BAND ON 0.8% AGAROSE GEL. THE QUANTITY OF DNA USING NANODROP REVEALED THAT ALL THE DNAS ARE AT GOOD QUANTITY OF 1000-2000NG/µL.

TABLE 3 THE NANODROP READINGS OF ISOLATED PLANT GENOMIC DNA REPRESENTING IT'S CONCENTRATION

S.No	A260/230	A260/280	Conc. (ng/µl)
1	1.539	1.516	2175.4
2	1.306	1.344	2119.2
3	0.946	1.096	2156.0
4	0.926	1.073	2225.6
5	1.097	1.300	2043.3
6	1.515	1.513	2238.3
7	1.295	1.337	2272.0
8	1.573	1.634	2156.9
9	1.853	1.790	2070.8
10	1.629	1.789	2041.0

THE DILUTED DNA WERE SUBJECTED FOR AMPLIFICATION USING ROJA'S PRIMER, THEY SHOWED AN AMPLICON AT ~1.5KB WHICH IS THE EXPECTED SIZE (Fig. 2). THEN THE SAMPLES AMPLIFIED USING GENE SPECIFIC PRIMERS THAT WERE DNA A (COAT PROTEIN) SHOWED THE AMPLIFICATION AT 1000BP(Fig. 3). FOR FURTHER CONFIRMATION THE SAMPLES OF DNA A AMPLIFICATION WERE GIVEN FOR SEQUENCING. THE NUCLEOTIDE SEQUENCE HAS BLASTED IN NCBI AND SHOWED SIMILARITY OF 98% QUERY COVERAGE WITH COAT PROTEIN GENES OF MYMV EXISTING IN THE DATABASE. THE SEQUENCE WAS DEPOSITED IN NCBI DATABASE AND ACCESSION NUMBER WAS OBTAINED. THE ACCESSIONS NUMBERS OBTAINED WERE OP121618 FOR MUNGBEAN YELLOW MOSAIC VIRUS TAMIL NADU ISOLATE IN BLACKGRAM AND OP121617 FOR COAT PROTEIN GENE OF MUNGBEAN YELLOW MOSAIC VIRUS TAMIL NADU ISOLATE.PHYLOGENETIC TREE WAS CONSTRUCTED USING MEGA 11 SOFTWARE USING BOOTSTRAP SAMPLING WITH 1000 REPLICATIONS IN WHICH OUR ISOLATE HAS SHOWED SEQUENCE SIMILARITY WITH OTHER DEPOSITED MYMV DNA A SEGMENT SEQUENCES (Fig. 4). OUR ISOLATE HAS SHOWED CLOSE SIMILARITY WITH *MUNGBEAN YELLOW MOSAIC VIRUS* BANGLORE COAT PROTEIN GENE (ACCESSION NO: MK409376).

Fig.2 Agarose gel electrophoresis image of infected plant genomic DNA amplified with Universal primers

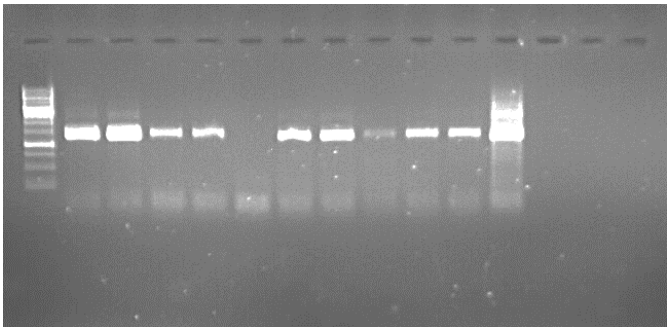


Fig.3 Agarose gel electrophoresis image of Gene specific amplification of MYMV

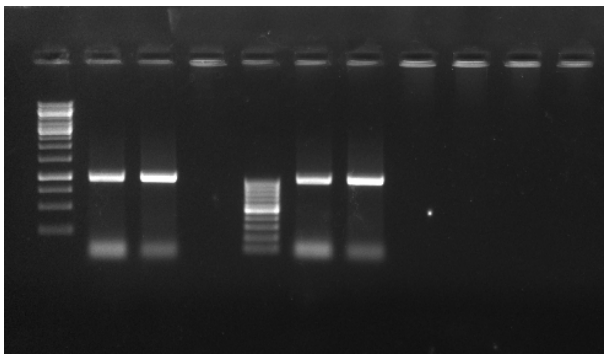
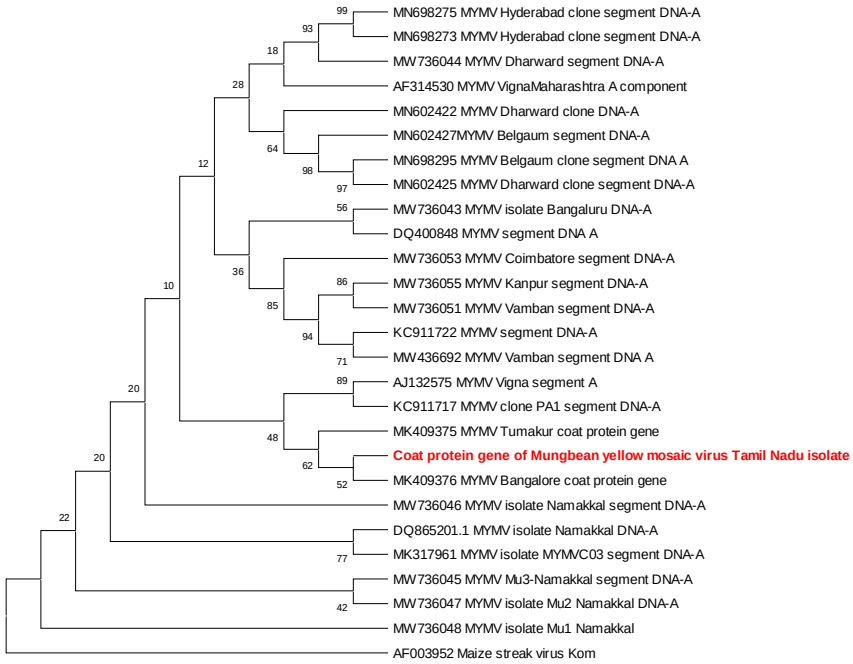


Fig.4 Phylogenetic tree of coat protein gene of Mungbean yellow mosaic virus Tamil Nadu isolate



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4. CONCLUSION

THE DISEASED SAMPLES COLLECTED FROM FIELDS OF DEPARTMENT OF PULSES HAS SHOWED THE TYPICAL SYMPTOMS OF MYMV. THE DNA ISOLATED FROM DISEASED SAMPLES USING GEM-CTAB METHOD IS OF GOOD QUALITY AS WELL AS THE QUANTITY. THE EXPECTED AMPLICON SIZE WAS SHOWED WITH ROJA'S PRIMER AND GENE SPECIFIC PRIMERS. THE COAT PROTEIN OF MYMV IS SEQUENCED AND SUBMITTED IN BLAST. THE PHYLOGENETIC TREE HAS SHOWN SIMILARITY WITH MUNGBEAN YELLOW MOSAIC VIRUS BANGLORE COAT PROTEIN GENE (ACCESSION NO: MK409376).

COMPETING INTERESTS

The authors have no conflicts of interest to declare. All co-authors have seen and agree with the contents of the manuscript and there is no financial interest to report. We certify that the submission is original work and is not under review at any other publication.

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