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Molecular Cloning and Characterization of Coat Protein Gene of *Banana bract mosaic virus* Affecting Banana cv. Mysore Poovan (Aab)

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A B S T R A C T

Banana bract mosaic disease (BBrMD) is one of the most important viral diseases of banana which leads to a yield reduction. We have identified *banana bract mosaic virus* (BBrMV) in banana plants growing in the regions of southern India based on symptomatology, sequence homology, and Serodiagnostic assays. The viral sequence encoding the coat protein was specifically amplified by RT-PCR (Reverse Transcriptase – Polymerase chain reaction) using specific primers bordering the Coat Protein (CP) gene. The unique amplified product thus obtained was cloned into the pGEM-T vector and the authenticity of the cloned gene verified by colony PCR. The nucleotide sequences and the deduced amino acid sequences were compared with the other BBrMV isolates and found to be identical at both the nucleotide and amino acid sequence level of other isolates with 99 to 96 per cent and 95 to 83 per cent respectively. The phylogenetic analysis by the alignment of CP gene sequences of selected 22 isolates also revealed that the present isolate was more similar to KER2 (Kasaragod) isolate. The recombinant clones developed in the present study could be applied in serodiagnosis and genetic engineering. This could be also used as disease diagnostic probes for more sensitive molecular diagnostic techniques like Nucleic acid spot hybridization.

Introduction

Banana (*Musa* spp.), identified as a 'tropical treasure' is the most remunerative fruit crop which plays a pivotal role in the income security of farmers. The crop is adaptable to diverse environmental condition, could be cultivated throughout the year and suited for homesteads as well as an inter-crop. Banana is vulnerable to a number of pests and diseases which limit its production and productivity (Singh *et al.*, 2011). Among various diseases, the viral diseases caused by *Banana bunchy top virus* (BBTV), *Cucumber*

mosaic virus (CMV) *Banana streak virus* (BSV) and *Banana bract mosaic virus* (BBrMV) causes major significant yield loss in banana (Kumar *et al.,* 2015). The disease caused by *Banana bract mosaic virus* (BBrMV) is important one leads to average yield loss of 30 per cent (Selvarajan and Jeyabaskaran, 2006). This disease was first reported from Kerala as 'Kokkan' disease with unknown aetiology (Samraj *et al.,* 1996). Later, it was authentically reported that the kokkan disease in Nendran banana is caused by BBrMV (Rodini *et al.,* 1997). Now the disease has been recorded from many growing states *viz.,* Karnataka, Tamil Nadu, Andra Pradesh and Kerala (Balasubramanian *et al.,* 2012; Rodini *et al.,* 1997) and identified as one of the diseases of national importance in India. Mysore Poovan is an important commercial cultivar grown throughout the country with location specific ecotypes like palayankodan in Kerala, Poovan in Tamil Nadu, Karpura Chakkarakeli in Andhra Pradesh and Alpan in North Eastern Region. It is commonly cultivated as a perennial crop. Tamil Nadu is the leading producer of Poovan cultivar owing to its climatic and marginal soil condition. Poovan is also commercially cultivated for leaf industry throughout Tamil Nadu and in certain parts of Kerala. Fruit is slightly acidic, firm and has typical sour-sweet aroma. Fruits turn to attractive golden yellow on ripening. Medium sized bunch, closely packed fruits, good keeping quality and resistant to fruit cracking is its plus points. In addition, the recent studies revealed that presence of phenols, flavonoids in major amounts in the peals of Mysore Poovan and also shown Anti-Psoriatic activity (Durga and Kumar, 2015). But the variety is highly susceptible to Banana Bract Mosaic Viral (BBrMV) disease and Banana Streak Virus, (BSV), which cause considerable reduction in yield. BBrMV is a distinct member of the genus *Potyvirus* and family *Potyviridae,* has flexuous filamentous particles (660-760 x 12 nm) with single stranded positive sense RNA genome (Thomas *et al.,* 1997) of length 9711 bp long (Ha *et al.,* 2008) coding for 3,125 amino acids with yielding of ten functional protein. BBrMV is transmitted in a non-persistent manner by several aphid including *Rhopalosiphum maidis*, *Aphis gossypii* (Magnaye and Espino, 1990) and *Pentalonia nigronervosa Cocq* (Bateson and Dale, 1995). This virus spreads through vegetative planting materials such as suckers and tissue cultured plantlets but not soil-borne (Thomas and Magnaye, 1996). In case of any viral disease, early diagnosis is very important since symptomless hosts carry the viral inoculum. Development of molecular clones of viral genome has immense application in the field of disease diagnostics and management. Hence, we developed the molecular clones of coat protein (*CP*) gene of BBrMV and characterized in Mysore Poovan. These clones could use as diagnostic probes in Nucleic acid spot hybridization (NASH) and knowledge by characterisation would help in development of disease resistant banana lines through coat protein mediated resistant using transgenic technology.

Materials and Methods

Naturally infected suckers of variety Mysore poovan (Grown in Kerala and Karnataka) showing the symptoms were collected from the fields of Banana Research Station, Kannara (Kerala). The collected suckers were maintained under insect proof net house in the Department of Plant Pathology, College of Horticulture, Thrissur, Kerala (India). Healthy tissue cultured plants were also maintained separately. These were used for further study.

Symptomatology

The types of symptoms expressed on different parts of the plant viz., leaves, pseudostem,

male-bud (bract) and bunches associated with Banana bract mosaic virus (BBrMV) infection were monitored and documented under natural field conditions.

Virus isolate

The infected suckers showing typical symptoms of BBrMD were collected from Banana Research Station (BRS), Kannara and maintained in the insect proof net house. Leave sample were collected and stored at -80^0 C.

Virus detection by serodiagnosis

Direct Antigen Coating-Enzyme linked immunosorbent assay (DAC-ELISA)

Titre for monoclonal antibody (Agdia Inc.) was determined using dilutions of 1:100, 1:200, 1:300 and 1:500 using procedure described by the Clark and Adams (1997). Determined the best one among four different dilutions based on the highest absorbance value. The result of the absorbance measured at 405 nm VERSAMAX ELISA reader. Using this titre, DAC- ELISA was performed and absorbance values of test sample and healthy sample were compared, if the absorbance value of test sample is more than twice that of healthy sample then the sample were considered as positive for virus infection.

DIBA (Direct antigen binding assay)

DIBA was done using procedure described by Banttari and Goodwin (1985) with slight modification. A desired size of Nitrocellulose membrane was cut and one cm^2 drawn on it. The membrane was washed with distilled water and air dried. 2 µl of sample containing crude antigen was spotted on appropriate square and air dried it for 15 min. After drying, membrane was immersed in blocking solution with gently shaking for one hour. Then it was washed three times with PBS-T for 3 min interval each. Primary antibody solution (Monoclonal antibody from Agdia Inc.; with 1: 200 dilution) was added on blot, incubated for 2 h at room temperature and followed by washing with PBS- T buffer thrice at 3 min interval each. Secondary antibody (Agdia Inc.) conjugated with alkaline phosphatase was added on the blot, incubated for 1 h followed by three times (5 min each) wash with PBS-T buffer. Finally, the membrane was rinsed in substrate solution and incubated under dark condition for 15-20 min. Then the membrane was washed with distilled water, air dried and observed for color development.

Primer designing

Virus specific primers were designed using coat protein sequences (Table 1) obtained from NCBI genbank were aligned by the program clustal omega (http://www.ebi.ac.uk/Tools/msa/clustalo/) and based on the homology, conserved boxes of 18 to 24 bases were selected throughout the sequence and the forward and reverse primers were selected from those conserved boxes based on ideal primer parameters (Faruk, 2013). Selected primer set was validated using *in-silico* tool OligoAnalyzer 3.1 (Integrated DNA technologies; http://eu. idtdna.com/site), named as BCF1 (Forward: 5' GATGATGACCCAAGCCGC 3') and BCR1 (Reverse 5' GCAGAGAG GCATATCAC 3')

Preparation of total RNA and cDNA synthesis

100 mg of leaf sample of infected plants were frozen in liquid N_2 and ground to a fine finder. Total RNA was isolated using the AmbionPureLink® Plant RNA reagent as per manufacturer's protocol (Thermo Scientific) and the complementary DNA (cDNA) was synthesized using RevertAid H Minus First

Strand cDNA synthesis kit as described by manufacturer (Thermo Scientific).First strand cDNA was confirmed by amplification with actin gene specific primers.

Reverse transcription PCR

The reverse transcription (RT)-PCR was carried out in a reaction mixture containing dNTP mix, BCPF1/R1 primer and 10× PCR buffer with $MgCl_2$ and *Taq*DNA Polymerase (Thermo Fisher Scientific, USA) to obtain amplified product of *CP* gene of BBrMV. The PCR was carried out in Agilent Technologies (USA) with PCR programme set with an initial denaturation at 94°C for 3 min, followed by 30 cycles of denaturation at 94°C for 45 s, annealing at 58.2°C (Optimized by gradient PCR) for one min. and extension at 72°C for one min. The final extension was carried out at 72°C for 10 min.

Cloning

The synthesized RT-PCR products were resolved in 1.5 % agarose gel electrophoresis, and the fragments were eluted using GenElute Gel Extraction Kit (Sigma, USA), ligated into pGEM-T Vector (Promega, USA), and transformed into competent *E. coli* DH5α cells as per manufacturer's instructions. Transformed colonies (white colonies) were selected based on blue / white selection were resuspended in 20 µl of distilled water, heated at 98° C for 3 min. followed by centrifugation at 12000 rpm for 2 min. The supernatant was taken in fresh PCR tube and was used as template DNA for colony PCR reaction to confirm presence of insert in recombinant plasmid. The colony PCR was carried out in a reaction mixture containing dNTP mix, plasmid primers (T7 and SP6), 10× PCR buffer with $MgCl_2$ and *Taq* DNA Polymerase (Thermo Fisher Scientific, USA) to obtain amplified product of cloned *CP* gene. The thermo-cycling conditions were as follows: 2 min at 94 °C (1 cycle), 94 °C for 45 s, 55 °C for 1 min. and 72 °C for 1 min (30 cycles), and a final extension at 72 °C for 10 min. Amplified PCR product from colony PCR were Sequenced at the SciGenome Pvt. Ltd, Kochi.

Sequencing analysis

The trimmed sequence was compared with sequence available in the National Centre for Biological Information (NCBI) database using BLASTn tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi; Altshul *et al.,* 1997) to find best aligned sequence. The *CP* gene sequences of BBrMV isolates generated in this study was aligned with 22 *CP* gene sequences of BBrMV isolates of banana and cardamom from India and Southeast Asia were retrieved from NCBI for analysis (Table 2). Alignments of total 23 nucleotide (nt) sequences were done using Clustal Omega (http://www.ebi.ac.uk/Tools/ msa/clustalo) and the phylogenetic relationship was inferred using the Neighbor-Joining method (Saitou and Nei, 1987) among BBrMV isolates from different geographical region and conducted using MEGA 7.0 software by constructing phylogenetic tree.

Results and Discussion

Symptomatology is very essential for early detection of disease. The banana plants infected with BBrMV showed different kinds of symptoms on different parts of the plant viz., leaves, pseudostem, male-bud, and bunches. The primary symptoms of BBrMV infected plants exhibiting longitudinal irregular reddish streaks of varying sizes on the psuedostem of Mysore Poovan (Fig. 1). The orientation of infected leaves became fan shaped which resembled travelers palm and such symptoms were noticed (Fig. 2A). Infected leaves showed spindle shaped lesions running parallel to the veins (Fig. 2B) and

mosaic pattern were visible on the lower side of petiole which extended throughout the midrib (Fig. 2C).

The symptoms seen on the bract were the main indication of disease in almost all the cultivars. The disease infected bracts showed a distinct reddish streaks and mosaic pattern (Fig. 3A). The infected bunches were under sized with malformed fingers (Plate 3C) and mosaic pattern observed on peduncle (Fig. 3B).

BBrMV best detected in 1:200 primary antibody dilution along with 1:500 secondary antibody (alkaly-phosphate conjugated) dilution and can gave clear difference between healthy and infected sample by DAC-ELISA. DIBA also gave positive reaction for infected leaf sample and this could be detected by the purple coloured spots on nitrocellulose membrane (Fig. 4). The species specific primer pair was designed to amplify of the coat protein gene of virus based on the most favorable combination of conserved regions in the multiple aligned nucleotide sequences and named as BCPFl and BCPRl respectively. RT-PCR analysis with this primer yielded an amplicon of ~850 bp in infected samples (Fig. 5). The positive samples were gel eluted and cloned into *E-coli* DH 5 cells. Colony PCR using plasmid specific primer (T7 and SP6) which yielded amplicons of expected band size of 1150 bp (Fig. 6). BLAST analysis of *CP* coding region of the virus has maximum homology of 99 percent to KER2 isolate (Kasargod, Kerala; accession KF385491). The sequence exhibited significant nucleotide identity (99 to 96 percent) and amino acid identity (95 to 83 percent) with other BBrMV nucleotide and protein sequence of BBrMV in the database.

The Phylogenetic analysis (Fig. 7) by the alignment of *CP* gene sequences of 23 isolates also revealed that the present isolate was more similar to KER2 isolate which *is* clustering with TN13 (KF385477; Tanjore, Tamil Nadu) and the Indian isolates did not show any relationship based on geographical origin.

Table.1 Details of selected sequences from NCBI which were for primer designing

Sl.No.	Accession Number	Isolate name	Source	Size (bp)	Reference
1	EU009210	Trichy	Tamil Nadu	914	Selvarajan and Balasubramanian, 2007
2	KF385480	AP7	Andra Pradesh	900	
3	KF385483	AS2	Assam	900	Balasubramanian and Selvarajan, 2014
4	KF385491	KER 2	Kerala	900	
5	KF385490	KAR 3	Karnataka	900	
6	KF385473	TN9	Tamil Nadu	900	

Table.2 Details CP sequence used for phylogenetic analysis

SI.NO	Accession No	Isolate name	Source	Reference
1	HQ709165	Card1	Madikere, Karnataka	Siljo *et al.*, 2012
2	HQ709166	Card2	Mudigere, Karnataka	Siljo *et al.*, 2012
3	HQ709164	Card3	Sirsi, Karnataka	Siljo *et al.*, 2012
4	HQ709163	Card5	Idukki, Kerala	Siljo *et al.*, 2012
5	HQ709162	Card6	Wynadu, Kerala	Siljo *et al.*, 2012
6	EU009210	TN4	Trichy, Tamilnadu	Selvarajan, R and Balasubramanian, V., unpublished data, 2014; unreferenced
7	KF385470	TN6	Pudukottai, Tamilnadu	Balasubramanian and Selvarajan, 2014
8	KF385472	TN8	Theni, Tamilnadu	Balasubramanian and Selvarajan, 2014
9	KF385474	TN10	Karur, Tamilnadu	Balasubramanian and Selvarajan, 2014
10	KF385476	TN12	Cuddalore, Tamilnadu	Balasubramanian and Selvarajan, 2014
11	KF385477	TN13	Tanjore, Tamilnadu	Balasubramanian and Selvarajan, 2014
12	KF385480	AP7	West godhavari, AP	Balasubramanian and Selvarajan, 2014
13	KF385481	KAR2	Bangolore, KA	Balasubramanian and Selvarajan, 2014
14	KF385488	KER3	Kayankulam, KER	Balasubramanian and Selvarajan, 2014
15	KF385490	KAR3	Arabhavi,KAR	Balasubramanian and Selvarajan, 2014
16	KF385491	KER2	Kasargod, KER	Balasubramanian and Selvarajan, 2014
17	AY953427	AP1	Kovur, AP	Ramesh, B., Sreenivasulu, P. and Krishna prasadji, J. unpuplished data, 2005; unreferenced
18	EU414267	P5	Philippines	Iskra Caruana, M.L., Bringaud, C. and Bousalem, M. unpuplished data, 2008; unreferenced
19	AF071585	P2	Philippines	Rodoni *et al.*,1999
20	AF071587	WS1	Western Samoa	Rodoni *et al.*,1999
21	AF071588	VT1	Vietnam	Rodoni *et al.*,1999
22	AF071589	TH1	Thialand	Rodoni *et al.*,1999

Fig.1 Symptoms on pseudostem showing linear red lesions on Mysore Poovan

Fig.2 Symptoms on leaf (A). Fan like arrangement of leaves; (B). Spindle shaped lesions running parallel to the veins on the leaf lamina; (C). Mosaic on leaf petiole

Fig.3 Symptoms on Bract and fruits: (A). Reddish streaks and mosaic pattern on bract; (B). Mosaic on peduncle

Fig.4 Detection of virus by dot immuno binding assay (DIBA). H- Healthy sample; B- Buffer control; D- Infected sample

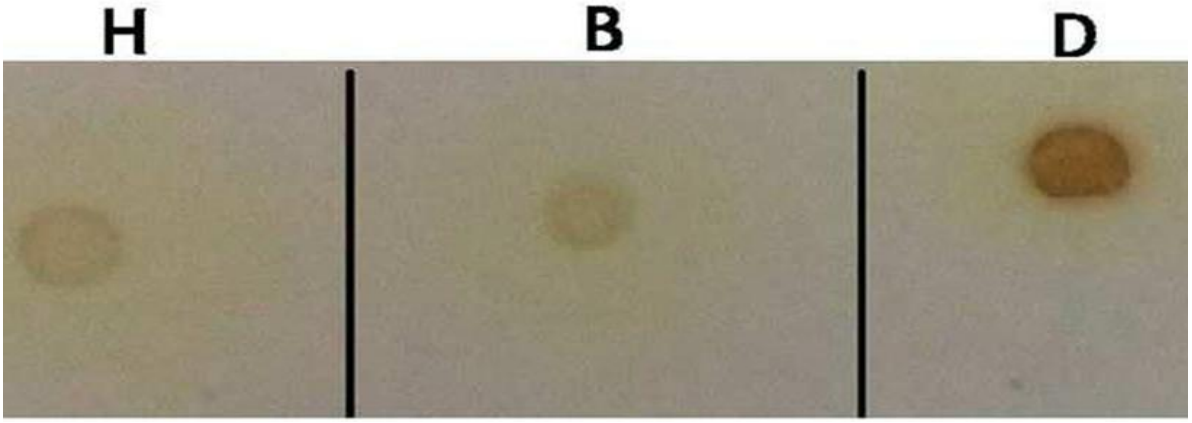


Fig.5 Amplification of CP region by BC F1/R1: M- Marker (1kb, Genei); Lane 1 - Healthy control; Lane 2 and 3- Infected samples

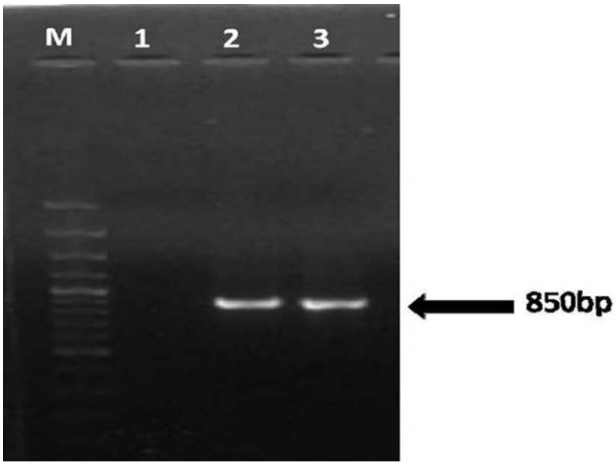


Fig.6 Analysis of recombinant clones using colony PCR: M- Marker (1kb, Genei); Lane 1 to 6-Recombinant plasmid; Lane7-Positive control (DNA insert); Lane 8-Negative control

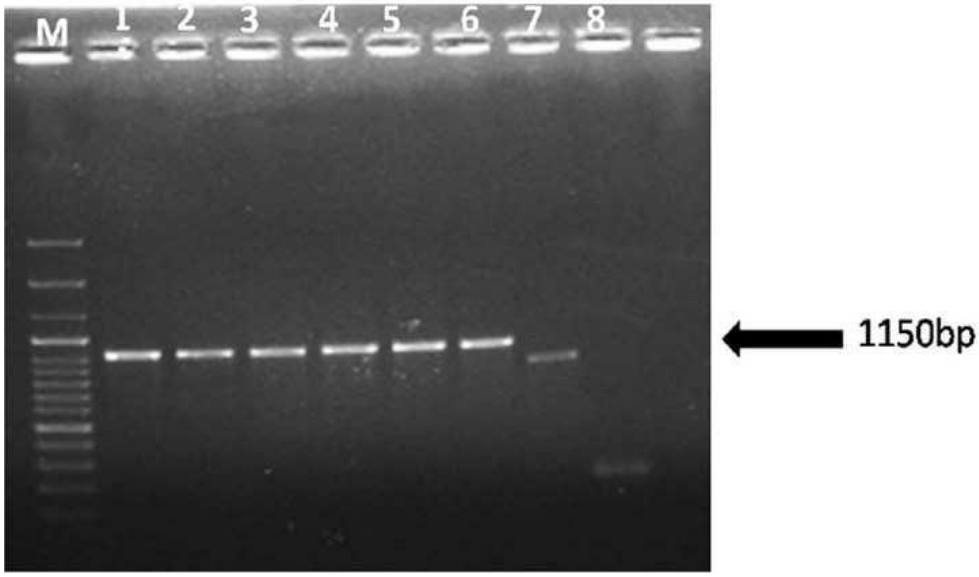
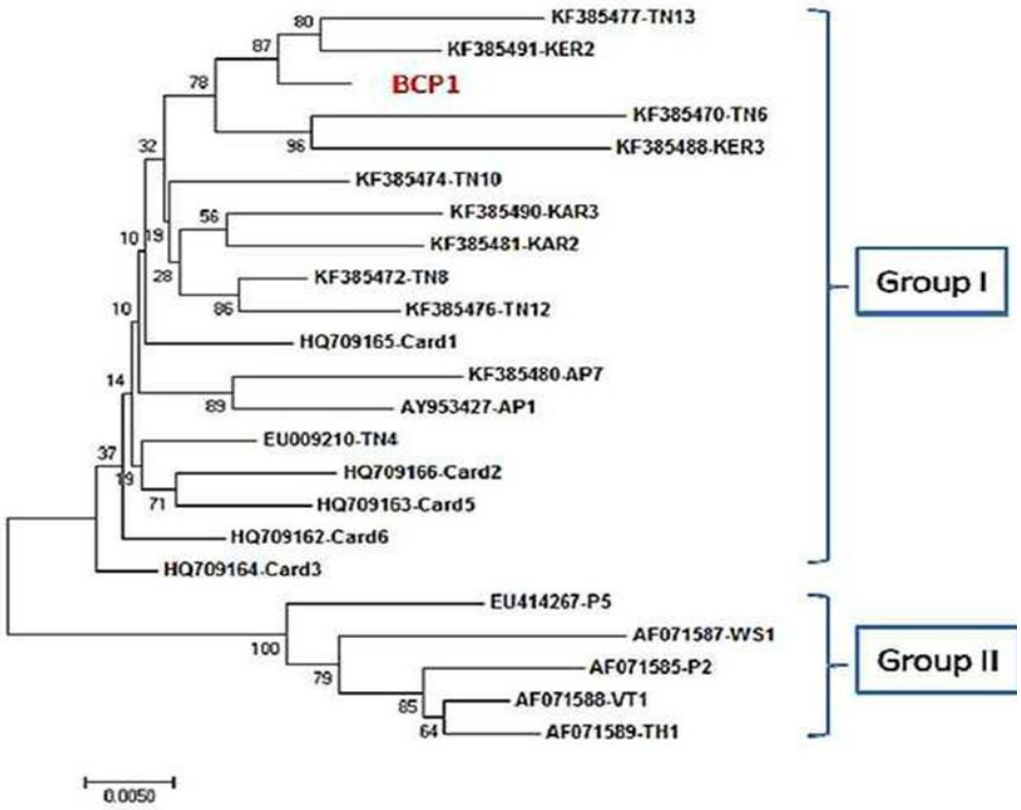


Fig.7 Phylogenetic analysis of coat protein gene of banana bract mosaic virus Isolates. Tree was constructed by Neighbor-joining method using mega 7



Among the solid phase serological detection methods such as DIBA and ELISA, DIBA was simple and convenient for field level application since it does not require sophisticated instruments such as ELISA-reader, PCR and could detect virus within 4 hrs. A field level diagnostic kit based on DIBA was developed for detection of *Cassava mosaic geminivirus* (CMG) and it was able to obtain the result within 4 h (Nair, 2012). Both ELISA and DIBA could be considered as efficient method for detection of BBrMV, although probability of non-specific reaction of antibody and components of plant sap is higher in DIBA than in multi well ELISA (Dhanya *et al.,* 2007). Molecular cloning of CP gene has been carried out and the main objective of cloning plant viruses has been the improvement of virus detection and diagnosis (Jelkmann *et al.,* 1989).

Many potyvirus RNAs had been partially or completely cloned (Nagel and Hiebert, 1985; Rosner *et al.,* 1986). The complete genome sequences of isolates BBrMV- PHI from Philippines (Ha *et al.,* 2008) and BBrMV-TRY from India (Balasubramanian and Selvarajan, 2012) were determined using cloning. In the family Potyviridae, species demarcation criteria are based on genetic information mainly based on *CP* gene (Berger *et al.,* 2005). Characterization of *CP* gene will be used to establish evolutionary relationships at both species and strain levels and used to develop pathogen derived resistance against potyvirus through coat protein mediated resistance by means of genetic engineering. Therefore, it could be a target of selection in the present study.

In the present study, *CP* gene specific primer was designed to amplify the product size of 850 bp and this could be utilized for detection of *BBrMV* in the samples for routine molecular detection purpose. Phylogenetic analysis was used to study genetic diversity *BBrMV* based on *CP* gene sequence information. As *CP* gene sequences are frequently used to develop pathogen derived resistance against *potyvirus* by means of genetic engineering, BBrMV diversity could help in predicting the risk of breakdown of resistance in the developed resistant transgenic banana lines. Hence the efficient long term management strategies could be achieved by preventing the loss-of resistance of *CP* mediated virus resistant due to the evolution of new variants. In this study, we compared *CP* gene sequence of BBrMV isolates with 22 previously reported isolates originating from different geographical regions. Phylogenetic tree was constructed from *CP* gene sequences showed two monophyletic clusters in the world population of BBrMV. However, the Indian isolates did not show any relationship according to geographical origins and the hosts from which they were isolated. This finding is consistence with result of Balasubramanian and Selvarajan (2014) who reported, using phylogenetic analysis based on *CP* coding region of 49 BBrMV isolate. A probable reason for the geographical distribution of BBrMV is that the virus has moved as a separate event. Perhaps through different infected cultivars of banana and BBrMV exist in India for a longer period of time (Rodoni *et al.,* 1999). BBrMV was first noticed in 1966 (Samraj *et al.,* 1996); because of prolonged presence, high divergence of BBrMV populations might have occurred. However, BBrMV was noticed first in southern parts of Kerala, has moved to three neighboring states *viz.,* Andra Pradesh, Tamil Nadu and Karnataka during the past five decades either through infected planting material or through aphid vector. There is no domestic quarantine enforced to restrict the movement of banana suckers between the states. This virus has recently been reported to infect small cardamom which is grown along with banana

in Western Ghats region of Kerala and Karnataka (Siljo *et al.*, 2012).

Statement of author contributions

The project was initiated by Dr. Anitha Cherian (Author 2) who is well known plant pathologist and the project was funded by Kerala Agricultural University, Thrissur. Author 1 (Darshangowda M.R) did the main work as a part of Master's research for two years. Author 3 (SaakreManjesh) did the gene sequence studies of coat protein and primer designing. Author 4 (Dr. Abida P. S) was the member of this committee helped in various ways such as cloning, standardization of PCR profile for cDNA amplification. Author 5 (Mr. Ashwathappa Reddy) is from is a background of both Agriculture and Plant pathology was contributed in this project in various ways.

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