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Short Communication

Identification and Characterization of a *Badnavirus* Infecting Betel Vine and Indian Long Pepper

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***Badnavirus* infecting betel vine (*Piper betle* L) and Indian long pepper (*P. longum* L) were detected with primers designed from the open reading frame (ORF III) region of the virus using Polymerase Chain Reaction (PCR). The amplicons obtained from these infected hosts were cloned and sequenced. The sequenced region of ORF III contained 597 nucleotides in both the isolates. Sequence analysis with recognized badnaviruses revealed that *Badnavirus* infecting *P. betle* and *P. longum* had highest sequence identity (> 89.1% at nucleotide level and >93.4% at amino acid level) with an Indian isolate of *Piper yellow mottle virus* (PYMoV) infecting black pepper. Based on the sequence identity and phylogenetic relationship studies, it was concluded that *Badnavirus* infecting *P. betle* and *P. longum* in India is a strain of PYMoV. To our knowledge, this is the first report on the identification and characterization of PYMoV infecting *P. betle* in India and PYMoV infecting *P. longum* in India and elsewhere.**

***Key words*:** betel vine, Indian long pepper, *Badnavirus, Piper yellow mottle virus,* open reading frame, sequence analysis.

Betel vine (*Piper betle*) and Indian long pepper (*Piper longum*) are economically important species of the genus *Piper* after black pepper (*P. nigrum*). The betel vine is a spice cultivated for its leaves used for mastication. The aqueous extract of betel leaves is used for the treatment of throat inflammation, alleviation of cough and indigestion. Betel leaf is also used in certain indigenous medicinal preparations. Indian long pepper is a slender creeping plant cultivated for fruits and roots which possess diverse pharmacological applications. It is widely used as a medicinal ingredient in Ayurveda, Siddha and Unani systems of medicines particularly for the treatment of diseases of respiratory tract (1).

Incidence of viral diseases has been reported in various *Piper* species from South-East Asia. Association of a *Badnavirus* (*Piper yellow mottle virus*, PYMoV) infecting black pepper (*P. nigrum*) has been identified and characterized from Brazil, India, Indonesia, Malaysia, Philippines, Sri Lanka and Thailand (2-4). PYMoV infecting *P. betle* has also been reported from Thailand (2). Badnaviruses are plant pararetroviruses with non-enveloped bacilliform particles (30 x 130-150 nm) containing a circular double stranded DNA genome of 7.1 – 7.6 kb molecules (5). The genomes of distinct members have been sequenced which are similar to the genome of type member, *Commelina yellow mottle virus* (ComYMV) . The genomes of majority of badnaviruses encode three open reading frames (ORF I, ORF II, ORF III) except *Cacao swollen shoot virus* (CSSV), *Citrus yellow mosaic virus* (CYMV), *Taro bacilliform virus* (TaBV) and *Dracaena mottle virus* (DrMV). ORFs I and II encode putative proteins of unknown function, although the C terminus of the ORF II product of CSSV has nucleic acid-binding activities for both dsDNA and ssRNA. ORF III encodes a polyprotein that is cleaved post-translationally by the viral aspartic protease to four functional products - a virus movement protein, coat protein, aspartic protease and a replicase comprising reverse transcriptase and ribonuclease H (6). Here we report the identification and characterization of *Badnavirus* infecting betel vine and Indian long pepper in India based on sequence analysis and phylogenetic relationship of ORF III region of the virus.

Piper betle exhibiting mosaic, mottling and reduction in leaf size and *P. longum* showing mosaic, blisters, dark green patches and leaf distortion were collected from Calicut and Kasargod districts of Kerala, India. These isolates were maintained in insect proof glass house conditions.

Total DNA was isolated from the leaves of infected and healthy plants using DNeasy Plant Mini Kit (Qiagen)

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Abbreviations: PYMoV-Piper yellow mottle virus; ORF-open reading frame; PCR- Polymerase Chain Reaction.

according to the manufacturer's instructions. Total DNA isolated from healthy plant served as the negative control, while DNA isolated from infected black pepper plant served as the positive control. Degenerate primers pairs (forward – 5′ CTNTAYGAATGGYTWGTDATGCC -3′ and reverse -5′ CAYTTRCAKAYKSCHCCCC – 3′) designed on the basis of multiple sequence alignments of ORF III region of several badnaviruses were used for amplification. PCR was performed in a 50 µl reaction volume containing 1x PCR reaction buffer (Genei, Bangalore, India), 2.5 mM $MgCl_2$, 250 µM each of dNTPs (Merck, India), 1.5 U *Taq* DNA polymerase (Genei, Bangalore, India), 15 pmole each of the primers and 5.0 µl template DNA. Samples were amplified in a thermocycler (Eppendorf) using a reaction profile programmed for an initial DNA denaturation at 94°C (3 min), followed by 35 cycles of denaturation at 94°C (30 sec), primer annealing at 58°C (1 min), primer extension at 72°C (1 min) and a final extension at 72°C (10 min).

The amplified products were analyzed on 1% agarose gel containing ethidium bromide. DNA was visualized and photographed using an UV transilluminator and a gel documentation apparatus (Alpha Innotech Corporation, CA, USA). The amplified fragments were eluted from the gel using Perfect prep gel cleanup kit (Eppendorf, Germany). The purified products were then cloned into TA cloning vector (Genei, Bangalore, India). Competent *E. coli* strain DH5α was transformed with this recombinant vector by following standard molecular biology procedures. Selected recombinant clones identified by restriction digestion and PCR were sequenced using automated sequencing facility at Avestha GenGraine Technologies Pvt Ltd, Bangalore, India.

Multiple nucleotide and amino acid sequence alignments were made using Clustal X and the aligned sequences were compared using BioEdit Sequence Alignment Editor (7). Phylogenetic relationships among badnaviruses were estimated using Neighborhood Joining Bootstrap Method (Bootstrap analysis with 1000 replicates) in Clustal X (1.81). Rooted Trees were generated using TREEVIEW software (Win 32) (8). Nucleotide and amino acid sequences corresponding to the ORF III region of other *Badnavirus* isolates used for confirming the robustness of grouping are listed in Table 1. The BLAST programme was used to identify related sequences from GenBank.

Table 1. Viruses with accession numbers from GenBank database used for sequence analysis

GenBank Acc no.	Abbreviations used	Virus isolates
DQ451009	BSV-GD	*Banana streak virus* isolate GD
DQ092436	BSV-Yun	*Banana streak virus* Acuminata Yunnan
AY805074	BSV-Mys	*Banana streak Mysore virus*
AJ534983	CSSV-TN	*Cacao swollen shoot virus*
AJ609020	CSSV-N1A	*Cacao swollen shoot virus,* isolate N1A
AJ609019	CSSV-PEKI	*Cacao swollen shoot virus,* isolate Peki
AF347695	CYMV	*Citrus yellow mosaic virus*
X52938	ComYMV	*Commelina yellow mottle virus*
DQ473478	DrMV	*Dracaena mottle virus*
AJ277091	SCBV-IM	*Sugarcane bacilliform IM virus* isolate Ireng Maleng
M89923	SCBV-Mor	*Sugarcane bacilliform Mor virus*
AF357836	TaBV	*Taro bacilliform virus*
X94576	DABV- L85-20	*Dioscorea alata bacilliform virus* (clone L85-20)
X94575	DABV- L85-36	*Dioscorea alata bacilliform virus* (clone L85-36)
DQ103759	BSCVBV	*Bougainvillea spectabilis chlorotic vein-banding virus*
DQ822074	DBV	*Dioscorea bacilliform virus* clone B39-6
DQ836227	PYMoV-CLT	*Piper yellow mottle virus*(Calicut, India isolate)
DQ836235 (Present study)	PYMoV-PB	*Piper yellow mottle virus* from *Piper betle*
DQ836237 (Present study)	PYMoV-PL	*Piper yellow mottle virus* from *Piper longum*
OUTGROUP		
AF220561	RTBV	*Rice tungro bacilliform virus* Chainat isolate

A PCR product of approximately 600 bp was successfully amplified from the ORF III region of the *Badnavirus* infecting these symptomatic hosts (Fig. 1). The amplicons obtained from both hosts were cloned and sequenced from both the directions. The sequenced region of both isolates contained 597 nucleotides potentially coding for 199 amino acids and the sequences were deposited in GenBank (Accession nos. DQ836235, DQ836237). Sequence analysis with other recognized *Badnavirus* species in the GenBank revealed that *P. betle* and *P. longum* isolates showed 97.4% and 89.1% homology at the nucleotide level and 97.4% and 93.4% homology at the amino acid level, respectively with a PYMoV black pepper isolate (E value = 0). Pair-wise comparison revealed an identity of 88.7% and 93.9% between *P. betle* and *P. longum* isolates at nucleotide and amino acid levels, respectively (Table 2). In contrast, identity of <67.3% and < 72.8% was seen with other distinct *Badnavirus* species at nucleotide and amino acid levels.

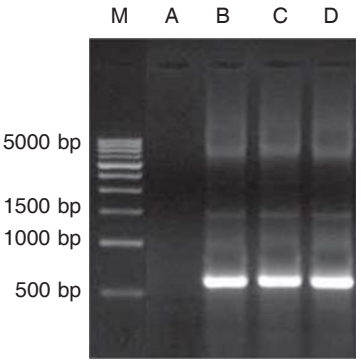

Fig. 1. PCR products obtained from the primers of ORF III region of *Badnavirus* infecting betel vine and Indian long pepper. Lane M: 500 bp DNA size ladder, lane A: Healthy (Negative control), lane B: Infected black pepper (Positive control), lane C: *Piper betle*, and *lane* D: *P. longum*.

The results of multiple sequence alignments of ORF III regions of different *Badnavirus* species were used to generate phylograms. In the phylogram generated on the basis of amino acid sequences of ORF III, isolates of *P. betle and P. longum* were clustered together with PYMoV isolates. Further, among the isolates, *P. betle* isolate was closer to PYMoV black pepper isolate than to *P. longum* isolate (Fig. 2). Similar results were also obtained in the phylogram generated on the basis of multiple alignment based on the nucleotide sequences (data not shown). Thus, based on the results of sequence identity and phylogenetic relationship studies, it can be concluded that *Badnavirus* infecting *P. betle* and *P. longum* in India is a strain of PYMoV.

Table 2. Per cent nucleotide sequence (above diagonal line) and amino acid sequence (below diagonal line) identities of 597 bp region of ORF III of *Badnavirus* infecting *Piper betle* and *P. longum* with other selected *Badnavirus* isolates. Designations given to the isolates are listed in Table 1

Seq->	BSV-GD	BSV-Yun	BSV-Mys	PYMoV-PB	PYMoV-CLT	PYMoV-PL	SCBV-IM	SCBV-Mor	ComYMV	CSSV-PEKI	CSSV-N1A	CSSV-TN	CYMV	DABV-L85-36	DABV-L85-20
BSV-GD		71.5	70	67.3	67.1	64.1	61.3	61.3	65.2	65.4	65.1	64.3	66.4	52	52.1
BSV-Yun	78.3		68.5	65.4	65.4	65.3	60.3	60.8	62.7	64.9	64.4	64.3	64.3	49.8	50.8
BSV-Mys	77.8	77.3		67.1	67.3	66.3	60.8	61.5	62.3	61.4	60.8	61.8	64.1	50.8	51.6
PYMoV-PB	69.8	66.3	71.8		97.4	88.7	62.2	60.2	62.2	62.8	62.8	63.6	64.3	47.1	50.1
PYMoV-CLT	69.8	66.3	71.8	97.4		89.1	62.3	60.5	62.7	63.6	63.6	64.3	64.4	46.6	49.4
PYMoV-PL	69.8	66.3	72.8	93.9	93.4		62.8	61.5	62.7	64.9	64.9	65.4	64.9	46.3	49.3
SCBV-IM	61.8	60.3	63.8	61.3	61.3	60.8		79	63.8	58.6	59	58.1	59.6	43.6	44.6
SCBV-Mor	65.3	63.3	64.8	60.8	60.8	60.3	90.9		62.1	60.3	60	60.5	59.8	45.9	45.9
ComYMV	66.3	62.8	67.3	63.8	63.8	64.3	63.3	64.3		60	59.5	60.3	60.7	46.1	47.1
CSSV-PEKI	65.3	65.8	64.3	63.8	63.3	64.3	59.2	60.8	59.2		98.3	89.7	66.1	45.1	46.4
CSSV-N1A	65.3	65.8	64.3	63.8	63.3	64.3	59.2	60.8	59.2	99.4		89.1	66.3	44.4	46.9
CSSV-TN	62.8	64.8	62.3	63.8	63.3	64.3	58.2	59.7	57.7	94.4	93.9		66.3	44.4	46.1
CYMV	67.8	65.3	70.3	68.3	68.3	66.3	59.7	60.3	63.8	69.8	69.8	69.3		48.1	47.8
DABV-L85-36	40.6	41.5	42.5	38.7	39.2	38.7	34.1	35	34.5	37.8	37.8	38.3	37.8		88.6
DABV-L85-20	41.1	42.5	43.4	39.7	40.1	39.7	34.1	35	35.9	37.3	37.3	37.3	38.3	96.5	

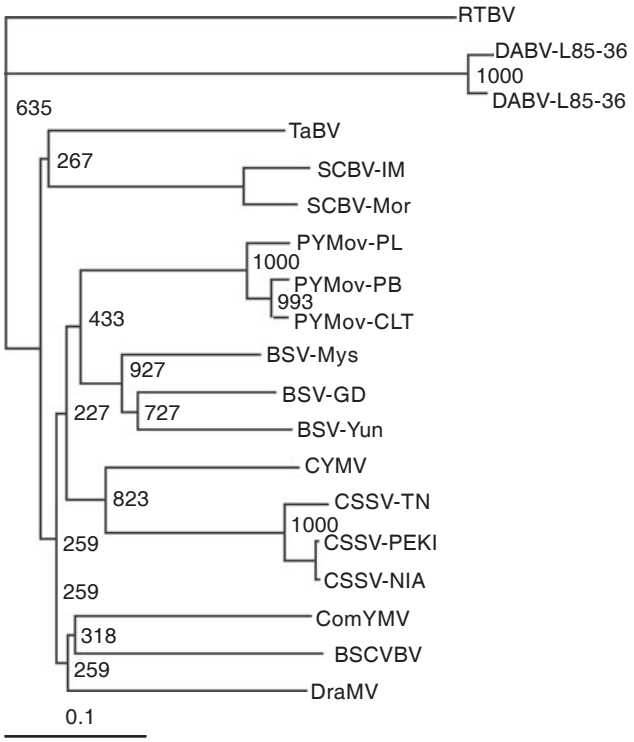

Fig 2. Phylogenetic tree depicting relationship of *Badnavirus* infecting *Piper betle* (PYMoV-PB) and *P. longum* (PYMoV-PL) with distinct *Badnavirus* species and their strains based on multiple sequence alignment of a part of ORF III amino acid sequences. Virus names and GenBank accession numbers are given in Table 1. The bootstrap values are shown at the individual node. RTBV was used as outgroup.

Further confirmation of the identity of the virus was done by cloning a portion of ORF I from both the infected hosts. The sequenced region of ORF I contained 469 and 471 nucleotides in *P. betle* and *P. longum* isolates, respectively and the sequences were deposited in the GenBank (Accession nos. DQ836234, DQ836236). Sequence comparison and phylogenetic relationship studies based on ORF I region with other *Badnavirus* species from databases also showed that the causal virus is a strain of PYMoV (data not shown). In *Badnavirus*, ORF III sequences are highly conserved within the species while ORF I sequences are known to be variable and diverse within a species (9, 10). Hence, ORF I sequence analyses were not taken into account in the present study.

Relatively low sequence identities of ORF III region between PYMoV infecting *P. betle* and *P. longum* suggest their discrete and independent origin. A large number of PYMoV isolates from different *Piper* species and geographical locations will have to be examined to look into any variation existing in different isolates. High nucleotide and amino acid sequence identity observed between PYMoV isolates infecting betel vine and black pepper needs to be investigated further to see whether the isolates are cross- infective to each other. To our knowledge, this is the first report on the identification of PYMoV infecting *P. betle* in India and PYMoV infecting *P. longum* from India and elsewhere in the world. Occurrence of disease symptoms on the cultivated Indian long pepper is reported from Calicut (Kerala) and Nellore (Andhra Pradesh) (11). Similarly occurrence of symptoms on betel vine is reported from Karnataka (12). Further, in certain cases plants showing such symptoms in betel vine and long pepper were also infected with *Cucumber mosaic virus* (CMV). Hence a detailed and systematic study is needed to look into the extent of occurrence of symptoms and associated viruses both in long pepper and betel vine in different regions. The PCR based method described in the present study can be used for the detection of PYMoV in different *Piper* species.

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