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Short Communication

Detection and Identification of *Cymbidium mosaic virus* Infecting Vanilla (*Vanilla planifolia* Andrews) in India based on Coat Protein Gene Sequence Relationships

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Sixty five diseased vanilla (*Vanilla planifolia* Andrews) plants showing different kinds of symptoms were collected from different regions of Karnataka and Kerala. They were subjected to reverse transcription (RT) PCR using primers designed for coat protein (CP) gene of *Cymbidium mosaic virus* (CymMV). A ~650 bp RT-PCR amplified fragment was consistently seen in only those plants of vanilla showing mild chlorotic mottle or streaks on leaves. The amplicon obtained from one infected vanilla plant was cloned and sequenced. Sequenced region contained a single open reading frame of 672 nucleotides potentially coding for 223 amino acids. Sequence analyses confirmed the identity of the virus as a strain of CymMV. The CP gene of the virus showed the greatest identity with a CymMV isolate from Singapore (99.1%). An identity of 92.3 to 97.3% was seen with different CymMV isolates from India while with available partial CP sequences of CymMV isolates infecting vanilla identity ranged from 98.2 to 99.4%. Phylogenetic analyses confirmed the results of sequence alignment and showed no clear cut clustering of isolates either based on host they infect or geographical origin. This is the first report of occurrence of CymMV on *V. planifolia* from India.

Key words: *Cymbidium mosaic virus*, *Vanilla planifolia*, RT-PCR, detection, coat protein gene sequence, sequence analyses.

Cymbidium mosaic virus (CymMV) (genus: *Potexvirus*, family: *Flexiviridae*) is one of the most widespread plant viruses infecting many orchids worldwide (1). The virus induces floral and foliar necrosis in many orchids. The virus infection reduces plant vigour and lower flower quality (1). CymMV is known to infect *Vanilla planifolia* and *V. tahitensis* in French Polynesia and ReUnion Island (2). The infection of vanilla by CymMV is often asymptomatic or in certain cases, is accompanied by chlorotic flecks on leaves. There are no known vectors transmitting this virus. The virus is very stable and is spread mechanically by contaminated tools and pots (3). Transmission through planting material is also significant as vanilla is propagated through stem cuttings. CymMV has flexuous filamentous particle morphology with a single species of linear positive sense single stranded RNA. The virus nucleic acid is about 6 kb in length coding for five open reading frames with cap (M'Gppp) at the 5' end and poly (A) tail at 3' end (3).

In India occurrence of CymMV has been reported from a few orchids such as *Cymbidium* spp, *Cattleya* sp, *Phaius tankervilleae* etc. Vanilla (*Vanilla planifolia* Andrews), the intensive large scale cultivation of which started only recently in India is known to be affected by mosaic, necrosis and leaf curl diseases. Based on electron microscopy of leaf dip preparations, occurrence of three kinds of flexuous particles resembling to the members of genera *Potexvirus*, *Potyvirus* and *Closterovirus* and, an isometric particle have been reported (4). Of these, the isometric virus was identified as a strain of *Cucumber mosaic virus* belonging to subgroup IB (5). But exact identification of other causal viruses remained unaddressed. In this article, we report the occurrence of *Cymbidium mosaic virus* on the basis of coat protein gene sequence properties, a new record on *V. planifolia* in India.

Sixty-five vanilla plantations of Karnataka and Kerala states were surveyed and the plants showing viral like symptoms such as mosaic, mottling, chlorotic streaks parallel to venation, necrosis, leaf distortion and stunting were collected and maintained through vegetative propagation in an insect-proof glasshouse. Total RNA from 50 mg tissue was extracted using the protocol (6) except that extraction buffer contained 0.5% 2-mercaptoethanol (instead of 0.1%), and final RNA pellet was dissolved in 40

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Abbreviations: CymMV, *Cymbidium mosaic virus*; CP, coat protein.

μl of sterile RNase free water. RT and PCR were performed in the same tube without any buffer changes in between as described previously (7). The primers designed for the CP gene sequences of CymMV (based on multiple sequence alignments of CP nucleotide sequences available in GenBank) were used to prime the amplification. Genome sense primer 5' ATGGGAGAGCCCACTCC 3' was derived from the beginning of the first 17 bases of the coding region while antisense primer, 5' TTATTCAGTAGGGGGTGC 3' represented last 18 bases of the coding region of the CP gene. The PCR reaction (50 μl) contained 100 ng each of the primers, 10 U Ribonuclease inhibitor, 5 U AMV reverse transcriptase, 1.50 U *Taq* Polymerase, 1x PCR buffer, 10 mM Dithiothreitol and 10 μM each of the dNTPs. PCR mix (13.5μl) containing the above components was added to the tubes containing the template RNA (5 μl). The final reaction volume was made up to 50 μl by adding RNase free water. Amplification was performed in an automated thermal cycler (Eppendorf Master Cycler Gradient) and the programme consisted of one cycle at 42 °C for 45 min for cDNA synthesis followed by 40 cycle reaction profile involving 30 sec of denaturation at 94 °C, 1 min of annealing at 50 °C, and 1 min of extension at 72 °C and a single cycle of final extension at 72 °C for 10 min. The reaction products (20 μl) were analyzed on 1% agarose gel along with 500 bp DNA ladder. The DNA bands were visualized and photographed using a UV transilluminator and a gel documentation apparatus (Alpha Innotech Corporation, CA, USA).

The amplified PCR product was purified after electrophoresis using Qiax II gel purification kit (Qiagen Inc, Chatsworth, CA, USA). Purified PCR fragments were ligated into a TA vector (Genei, Bangalore, India) and competent *Escherichia coli* strain DH5α were transformed by following standard molecular biology procedures (8). Recombinant clones were identified by PCR as well as restriction endonuclease digestion and the selected clones were sequenced at the automated DNA sequencing facility available at the Avestha GenGraine Technologies Pvt Ltd., Bangalore, India.

Sequence data were compiled using Seqaid Version 3.6 (9). Multiple sequence alignments were made using Clustal X (1.81). Per cent sequence identities were determined using Bio-Edit program version 5.0.9. Sequence phylogram was constructed by Neighborhood Joining Bootstrap method (Bootstrap analysis with 1000 replicates) in Clustal X (1.81) and rooted trees were generated using TREEVIEW software (Win 32) Version 1.6.6 (10). The CP nucleotide and amino acid sequences of other CymMV isolates used for comparison (Table 1) were obtained from GenBank. The BLAST programme was used to identify related sequences available from the GenBank database.

When total RNA extracted from vanilla plants showing different kinds of symptoms were used in RT-PCR, only plants showing mild chlorotic mottle and chlorotic streaks (Fig.1) resulted in successful amplification giving a band of expected size (Fig.2). No such band was seen in healthy plants. Of the 65 samples tested, 27 gave positive results for CymMV infection. The PCR amplified DNA fragment from one positive sample was cloned, sequenced and sequence data deposited at GenBank with accession number DQ 208422. The sequenced region contained a single open reading frame, which comprised of 672 nucleotides potentially coding for 223 amino acids. It was compared with CP gene sequences of all available CymMV isolates from India as well as a few representative isolates from other parts of the world. Similarly partial CP gene sequences of CymMV isolates infecting vanilla from French Polynesia and ReUnion Island were also used for comparison (Table 1). Nucleotide and deduced amino acid sequence of CP gene of CymMV infecting vanilla in India (IND-Vp) showed highest identity (98.8 and 99.1%) with a CymMV isolate from Singapore (SGR). Identity with other CymMV isolates ranged from 88.3 to 97.7% at nucleotide level. While at amino acid level, except for the two Korean isolates (KRA-Dd and KRA-orc), identity ranged from 92.3

Fig. 1. *Cymbidium mosaic virus* (CymMV) infected *Vanilla planifolia* leaf showing mild chlorotic mottle and streaks.

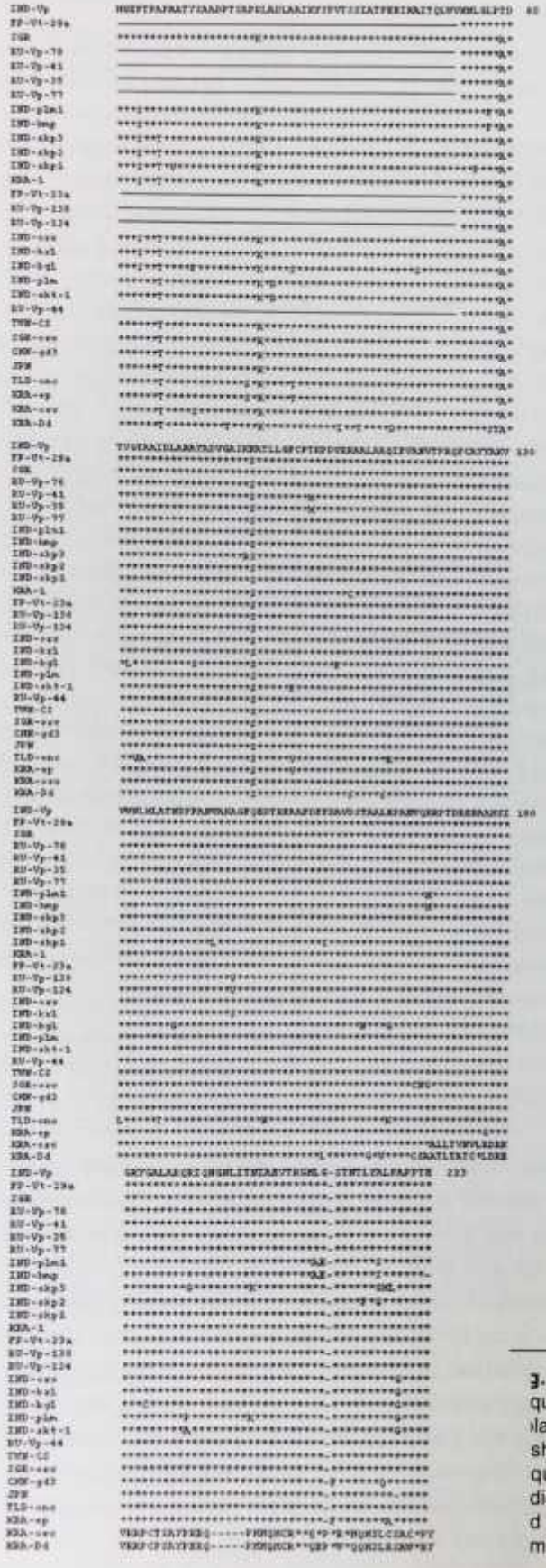
Fig. 2. Agarose gel showing RT- PCR amplification of CP gene of *Cymbidium mosaic virus* (CymMV). lane M: 500 bp DNA size ladder and numbers on the left indicate their size in kb; lane 1: healthy vanilla (negative control); lane 2: a known CymMV infected vanilla (positive control); lanes 3-10: CymMV infected vanilla isolates.

Table 1. Source of *Cymbidium mosaic virus* coat protein gene sequence used in the study

Host	Country	Designation	GenBank Accession No.
Vanilla planifolia	India	IND-Vp	DQ208422 (This study)
	ReUnion Island	RU-Vp-138	AJ301850
	ReUnion Island	RU-Vp-124	AJ301849
	ReUnion Island	RU-Vp-78	AJ301848
	ReUnion Island	RU-Vp-77	AJ301847
	ReUnion Island	RU-Vp-44	AJ301846
	ReUnion Island	RU-Vp-41	AJ301845
	ReUnion Island	RU-Vp-35	AJ301844
V. tahitensis	French Polynesia	FP-Vt-29a	AJ311914
	French Polynesia	FP-Vt-23a	AJ311908
Cattleya sp	India (Kerala)	IND-krl	AJ698947
	India (Sikkim)	IND-skt1	AJ581997
Phaius tankervilleae	India (Palampur)	IND-plm1	AM055720
	India (Himachal Pradesh)	IND-hmp	AJ564562
Orchids	India (Orissa)	IND-ors	AJ871374
	India (Bangalore)	IND-bgl	AJ620244
	India (Sikkim)	IND-skp3	AJ585204
	India (Sikkim)	IND-skp2	AJ585203
	India (Sikkim)	IND-skp1	AJ585202
	India (Himachal Pradesh)	IND-plm	AJ581998
	Korea	KRA-orc	AF206274
	Singapore	SGR-orc	X62665
	Singapore	SGR	NC001812
Cymbidium spp	Japan	JPN	AB197937
	Korea	KRA-1	AF016915
	Taiwan	TWN-CS	AY429021
	China	CHN-gd3	AY360410
Dendrobium sp	Korea	KRA-Dd	X81051
Epidendrum sp	Korea	KRA-ep	AY050650
Oncidium sp	Thailand	TLD-onc	AY376393
Papaya	-	PaMV	AY017187

to 98.2%. Korean isolates showed an identity of 69.4% (KRA-Dd) and 75.6% (KRA-orc) with the present isolate. When all CymMV isolates available from India were compared, identity ranged from 94.3 to 96.4 % and 92.3 to 97.3% at nucleotide and amino acid levels respectively. Similarly IND-Vp showed high levels of identities (95.5 to 97.0% and 98.2 to 99.4%) with partial CP sequence of CymMV isolates infecting vanilla from French Polynesia and ReUnion Island at nucleotide and amino acid levels.

Multiple sequence alignment based on deduced CP amino acid sequences of CymMV isolates showed that IND-Vp and SGR differed only at two positions at the N-terminal region (Fig. 3). Analysis of CP identified one amino acid position (R22) as unique to IND-Vp. Similarly one amino acid position (N81) was found to be unique among IND-Vp and SGR isolates. When available partial CP sequence of vanilla isolates of CymMV were considered, IND-Vp and FP-Vt-29a differed only at one position (Fig.3). Among Indian isolates, CymMV isolate from Bangalore (IND-bgl) showed maximum variation. Multiple alignment showed that core region of CP were more conserved than N- and C-terminal regions. C-terminal region accounted for maximum variation especially in two isolates from Korea (KRA-orc and KRA-Dd).



Phylogram illustrating phylogenetic relationship among CymMV isolates generated based on CP amino acid sequences showed that IND-Vp was most closely related to a vanilla isolate of CymMV from French Polynesia (FP-Vt-29a) followed by a CymMV isolate from Singapore (SGR) (Fig.4). Except for two isolates from Korea (KRA-orc and KRA-Dd), results showed that sequences were highly conserved among different isolates of CymMV infecting different hosts and regions. No clear cut clustering either based on host infected or geographical distribution of the isolates was seen in the study.

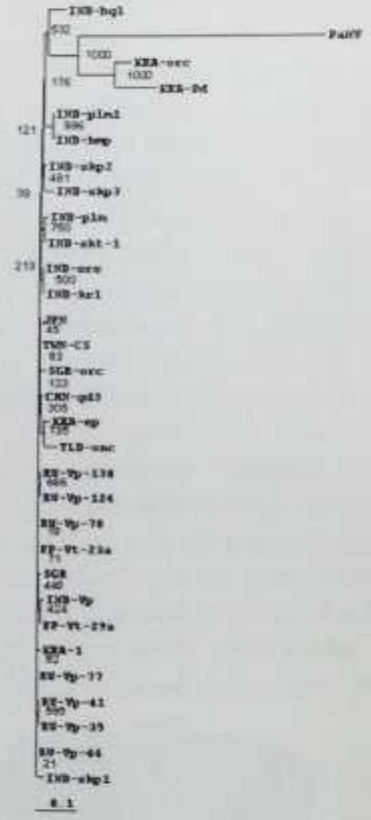


Fig. 4. Phylogram, drawn by Neighborhood Joining Bootstrap method in Clustal X (1.81), illustrating phylogenetic relationships based on the multiple alignments of the coat protein amino acid sequences of 29 distinct isolates of *Cymbidium mosaic virus* (CymMV) and vanilla isolate of CymMV (IND-Vp). *Papaya mosaic virus* (PaMV) was used as outgroup. Sequences for comparisons were obtained from GenBank and designation given to each of the isolates and their GenBank accession numbers are given in Table 1. The bootstrap values are shown at the individual nodes.

g. 3. Multiple sequence alignment of deduced coat protein amino acid quences of CymMV infecting vanilla in India (IND-Vp) with other CymMV olates using Clustal X. Asterisk indicates identity at a given position and a sh indicates deletion at a given position. The N-terminal 52 amino acid quences of nine vanilla isolates were not available for comparisons dicated with a line). Sequences for comparisons were obtained from GenBank d designation given to each of the isolates and their GenBank accession mbers are given in Table 1.

The results presented show the occurrence and identification of CymMV on vanilla on the basis of CP sequence similarities. This is the first report of identification of CymMV infecting vanilla in India. However, CymMV is known to infect many orchids in India. But the relatively low sequence identities observed with all Indian isolates of CymMV suggest that vanilla isolate of CymMV did not originate locally. Occurrence of CymMV on vanilla in French Polynesia and ReUnion Island is also known (1, 2). Infection of vanilla by CymMV is often symptomless or cause only mild symptoms. This coupled with easy mechanical transmission of the virus from plant to plant (through injuries caused during cultural operations such as looping of vines and manual pollination) could lead to widespread dissemination of the virus. Another factor that might contribute to high incidence of CymMV is the use of tissue culture for propagation using shoot tips from apparently healthy (but infected with virus) plants. Hence it is essential to index planting material for the presence of virus before they are being used. RT-PCR method described in the present studies can be efficiently used to identify CymMV free plants. This would help in identifying and certifying planting material to check spread of the virus.

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