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Detection of orchid viruses and molecular characterization of odontoglossum ringspot virus (ORSV) isolates

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ABSTRACT

During a survey (2018), 44 orchid samples collected from Sikkim and Darjeeling Hills of West Bengal. The samples were examined under electron microscope (EM) followed by DAC-ELISA and RT-PCR assays were performed. Electron microscopy showed the presence of rigid rods (300 × 18 nm), flexuous rods (475 nm × 13 nm and 800 nm × 12 nm), bacilliform or bullets shaped particles (40 × 100-140 nm) and enveloped quasi-spherical particles (80-110 nm). Based on the particle morphology, DAC-ELISA was performed using the antibodies specific to odontoglossum ringspot virus (ORSV), cymbidium mosaic virus (CymMV), calanthe mild mosaic virus (CalMMV) and groundnut bud necrosis virus (GBNV), which revealed the presence of four viruses namely CymMV, ORSV, GBNV and CalMMV. RT-PCR assays also confirmed the presence of four viruses using their specific primers. The cloning, sequencing and sequence analysis of the coat protein gene sequences of the four ORSV isolates was performed. The sequence analyses revealed up to 100% sequence identity among the ORSV isolates of this study and more than 99% identity with the different ORSV isolates available at NCBI GenBank database. The higher level of sequence homology among ORSV population suggests that they are under least evolutionary pressure. The occurrence of the multiple viruses in different orchids also reflects that the orchid cultivation is under major threat.

Keywords: Cloning, DAC–ELISA, Detection, Electron microscopy, Orchid viruses, Phylogenetic relationship , RT-PCR

Orchids are known for their beautiful flowers and play an important role in global floriculture trade (Lawson and Hsu 1995). There are about 28000 species of orchids in the world under 800 genera (Christenhusz and Byng 2016). India is a home of about 1256 species of orchids belonging to 155 genera, of which, about 714 species are reported from North-eastern region alone (Singh *et al*. 2019). The major constraints for the orchid cultivation in India are improper cultivation methods, lack of proper knowledge about pests and diseases and non-availability of elite germplasm to counter these biotic stresses. Orchids are infected by about 58 plant viruses (Zettler *et al*. 1990, Lawson and Hsu 1995, Manjunath 2020). In India, at least 10 viruses have been reported (Sherpa *et al.* 2003, 2004, Bhat *et al*. 2004, Sharma *et al.* 2005, Pant 2008, Pant *et al.* 2010, 2017, 2019). Of these, cymbidium mosaic virus (CymMV; genus *Potexvirus*, family *Flexiviridae*) and odontoglossum ringspot virus (ORSV; genus *Tobamovirus* family *Virgaviridae*) cause serious damage to orchid industry worldwide, including India (Zettler *et al*. 1990, Pant *et al*. 2010). The commercial cultivation of orchids in India was started only recently and screening of orchids viruses revealed that CymMV and ORSV are widespread in Indian orchids (Sharma *et al*. 2005, Bhat *et al*. 2006, Sherpa *et al*. 2006, Pant *et al*. 2010). Besides, these two viruses, some new viruses like orchid fleck virus (OFV; genus *Dichorhavirus*, family *Rhabdoviridae*), calanthe mild mosaic virus (CalMMV; genus *Potyvirus*, family *Potyviridae*), groundnut bud necrosis virus (GBNV; genus *Orthotospovirus*, family *Tospoviridae*) were also found infecting different orchid species/hybrids, thereby, posing a serious threat to orchid cultivation in India. Mixed infection of three viruses from *Phalaenopsis* orchid has also been reported from India recently (Pant *et al*. 2021). Present investigation reports the associations of four viruses and molecular characterization of the coat protein (CP) gene of four ORSV isolates collected from Sikkim and Darjeeling hills.

MATERIALS AND METHODS

Sample collection: Total 44 samples were collected (6 samples from the Cymbidium Development Centre Rumtek, Sikkim; 22 samples from the National Research Centre for Orchids, Pakyong, Sikkim; 6 samples from the

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Table 1 Primers used for RT-PCR assays for the detection of plant viruses associated wth the orchid samples

Virus	Primer pair	Sequence (5' to 3')	Tm (°C)	Amplicon size in bp
ORSV	RKJ42F	GGATCCATGTCTTACACTATTACAGA	49	477
	RKJ43R	TCTAGATTAGGAAGAGGTCCAAGT		
CymMV	RKJ38F	TTGGATCCATGGGAGAGCCCACT	49	672
	RKJ39R	TTTCTAGATTATTCAGTAGGGGG		
GBNV	Gb-F	GTTGAAAAGAGCAAGAATGATGC	52	271
	CaGbWb-R	CYTTRCAMACCTGTTCATARGTAGA		
OFV	Orchid flex (F)	ATGGCTAACCCAAGTGAGATT	50	380
	Orchid flex (R)	TCAGTCACTGTTCATGGCATC		
CalMMV	Cal-forward	GGGGACAAGAGTGAGTTGGAT	62	800
	Cal-reverse	CATATAGCGGGCACCATTGAG		

National Research Centre for Orchids, Darjeeling Campus; and 10 samples from the Everest Nursery, Kalimpong). Samples were cleaned and stored in deep freezer (-80°C) for further analysis.

Electron microscopy: Samples were initially subjected to leaf dip electron microscopy (Milne 1984) and examined under JEOL-1011 electron microscope (EM) (Tokyo, Japan) attached with Megaview G2 CCD camera (Olympus, SIS, Munster, Germany) and operated at 80 KV.

Serological assays: Based on the type of virus particles visualized under EM, the direct antigen coating-enzyme linked immunosorbent assays (DAC-ELISA) were performed using the polyclonal antibodies (PAbs) of CymMV, ORSV, CalMMV and GBNV to analyse the presence of specific virus species in the orchid samples (Clark and Adams 1977; Mandal and Jain 2010). All the antibodies were available in the Division of Plant Pathology, Indian Agricultural Research Institute, New Delhi.

Nucleic acid based assays: Total RNA was extracted from a 0.1 g symptomatic leaves of each of the EM and DAC-ELISA positive samples using Total RNA extraction kit (RNeasy Plant Mini Kit, Qiagen, USA). Total RNA were used as the template for the complementary DNA (cDNA) synthesis by using reverse transcriptase enzyme (Improm II, Promega), followed by the RT-PCR assays were carried out by using the specific primers designed to amplify the CP gene of CalMMV, CymMV, GBNV, OFV and ORSV (Table 1).

Molecular characterization and phylogenetic analysis of ORSV isolates: The gel purified PCR products of ORSV CP gene resulted from four different orchid species, viz. *P. tankervilleae* (NRCO Sikkim); the cymbidium hybrids (Baltic Glacier Mint Ice and SHOL-2, Rumtek, Sikkim; and *Epidendrum* sp. (Everest Nursery Kalimpong, West Bengal) were cloned in the pGEM-T Easy cloning vector (Promega). The recombinant clones were screened by colony PCR and restriction digestion analysis, and outsourced for sequencing (Sanger Sequencing at Delhi University, South Campus, New Delhi). The resultant sequences of cloned products were subjected to NCBI BLAST analysis and assembled the virus specific reads using BioEdit Software (Hall 1999). For the comparative analysis, the ORSV CP sequences already available in the NCBI GenBank database were retrieved by using CLUSTAL W (Thompson *et al*. 2013). The phylogenetic tree was constructed using MEGA 7.0 sequence analyses tool through the Maximum Likelihood method employing the default settings with 1000 bootstrap replications (Tamura *et al.* 2013).

RESULTS AND DISCUSSION

Symptomatology: The collected samples showed variable symptoms. Cymbidium hybrids exhibited the symptoms like mosaic, brown and black spots, and necrotic streak symptoms; necrotic spots on leaves; necrotic flecks and black ringed lesions on leaves. *Cymbidium* spp. and hybrids exhibited necrotic rings, flecks and chlorotic lesions on the leaves. *P. tankervilleae* plants exhibited mild mosaic and chlorotic streak symptoms; *Cattleya* hybrids exhibited the typical color-breaking symptoms on flower petals; Leaves of *Phalaenopsis* plants exhibited mild chlorotic depressed spots/lesions.

Electron microscopy: EM observation of cymbidium hybrids exhibiting mosaic, necrosis and black spot symptoms revealed the association of straight and rigid rods of 300 nm × 18 nm indicated the presence of ORSV (Fig 1a). Samples showing mosaic and necrosis showed flexuous filamentous rods of 475 nm × 13 nm indicative of CymMV (Fig 1b). The leaves of *P. tankervilleae* with mild mosaic symptoms showed the presence of long flexuous particles measuring 800 nm × 12 nm resembling (potyvirus) CalMMV (Fig 1c). The *Phalaenopsis* plants showed the presence of enveloped quasi-spherical particles measuring 80-110 nm in diameter resembling the tospovirus (Fig 1d). Sample showing severe necrosis and sunken pits showed mixed infection of ORSV and CymMV (Fig 1e). The bacilliform or bullet-shaped particles measuring 40 × 100-140 nm (OFV) were also observed on samples showing necrotic flecks and black spots (Fig 1f).

Serological diagnosis: In the current investigation, out of 44 orchid samples examined by DAC-ELISA, 13 were failed to react with the PAbs to CymMV, ORSV, CalMMV, GBNV and OFV in DAC-ELISA, whereas, remaining

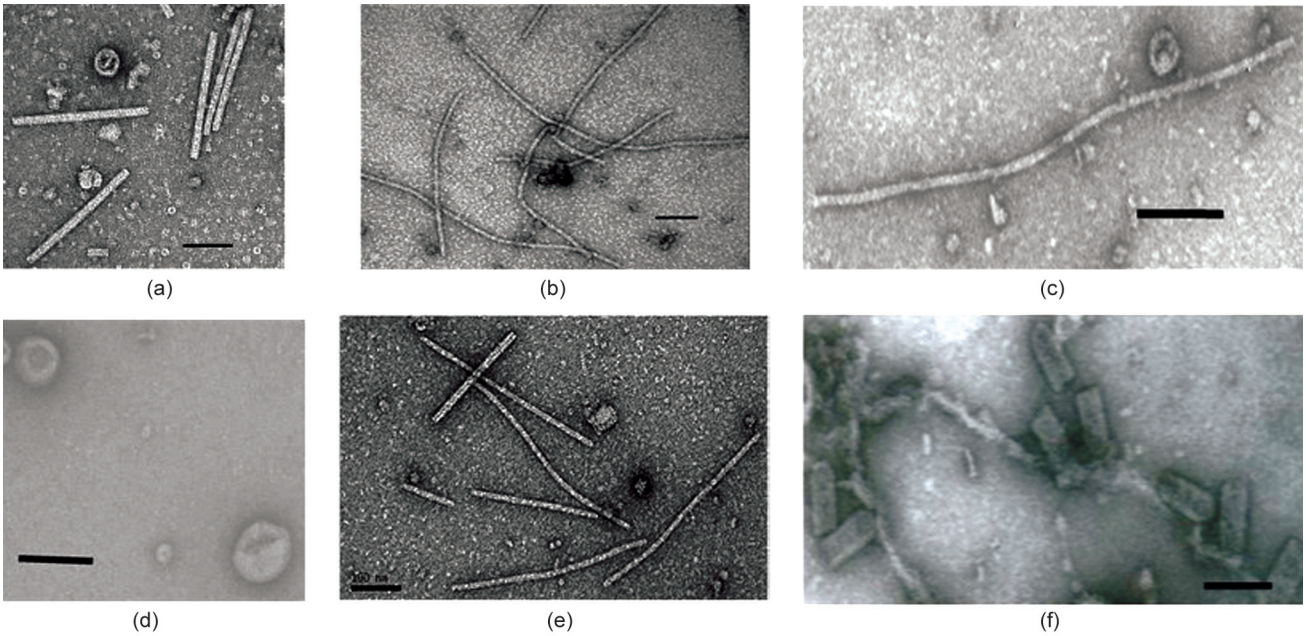

Fig 1 Electron micrographs showing the association of straight/rigid rods measuring ~300 ×18nm (a); short flexuous rods ~450 nm (b); Long flexuous rods of 800 × 12 nm (c); enveloped quasi-spherical particles, ~80-110 nm in diameter (d); mixed infection of rigid rods ~300 × 18nm and flexuous rods ~450 nm × 12 nm (e); bacilliform or bullet-shaped particles measuring 40 × 100-140 nm (f). Bar = 100nm.

31were reacted with the PAbs to CymMV, ORSV CalMMV and GBNV. However, 20 samples reacted with the PAbs to either CymMV alone or both CymMV and ORSV. The positive signals for the association of CalMMV and GBNV were found with one sample each of *Phaius tankervilleae* and *Phalaenopsis* sp. respectively. These finding suggested that CymMV and ORSV are the most prevalent viruses of orchids in India. Similar results were also observed by many workers (Beetham and Moran 1988, Zettler *et al*. 1990, Wong *et al.* 1994, Pant *et al*. 2010, 2021).

Reverse transcription – polymerase chain reaction: RT-PCR analysis of orchid samples of four different viruses, viz. ORSV, CymMV, CalMMV and GBNV achieved the amplicons of ~477 bp, ~672 bp, ~800 bp, and ~271 bp lengths respectively in the agarose gel electrophoresis. In the RT-PCR assay, a total of 27 samples found positive for ORSV, 25 for CymMV, 2 samples each for GBNV and CalMMV. The number of samples turned positive in the RT-PCR was higher than that observed in DAC-ELISA results (Table 2). This increase in number of positive samples in RT-PCR over DAC-ELISA could be due to a higher sensitivity of the technique. These results thus confirmed the presence of CymMV, ORSV, CalMMV and GBNV on collected samples and also the mixed infections of CymMV and ORSV on orchids grown in Sikkim and Darjeeling Hills.

In some of the samples, the bacilliform/bullet-shaped particles were observed under EM (Pant 2008), but their association could not be established by RT-PCR, which needs further investigation. RT-PCR based detection has been widely used for indexing the orchid viruses in India (Sherpa *et al*. 2003, 2006, Bhat *et al*. 2004, 2006).

Molecular characterization of ORSV isolates: The CP gene fragments that were cloned and sequenced were resulted in the nucleic acid sequences of 477 bp length which encoded 159 amino acids. When the sequences were submitted to the NCBI GenBank database, they got assigned with the accession numbers as MNO27917, MNO27918, MNO27919 and MNO27920 for *P. tankervilleae*, cymbidium hybrid, *Epidendrum* sp. and SHOL-2 isolates respectively. The pair wise sequence comparison and genetic homology analysis of 477 bp sequences of all the four ORSV isolates of this study revealed that they shared 100% identity among themselves and 99.79 percent with the ORSV isolates of Taiwan (AY571290.1), USA (U89894), China B (AM398154) and China (AM398154) isolates; up to 99.58% with the isolates of Singapore (AF455273) and Brazil (AF515606); 99.37%, with the isolate of Germany (AJ429092); and 99.16% with the isolate of Thailand (AY376394).

Phylogenetic analysis: Phylogenetic analysis of the CP sequences of the four ORSV isolates of this study with those sequences of the other isolates available in the NCBI GenBank database revealed their close phylogenetic relationship by forming single major sub-cluster comprising all the ORSV isolates of the world. However, a separate sub-cluster was formed at the base of the tree comprising only two ORSV isolates originated one each from Korea (Q84122.3) and USA (NP056812.1) (Fig 2). These results showed clearly that the oldest known isolate of ORSV originated from USA (Jenson and Gold 1952) was forming the base of the tree with a very high level of sequence identity (>99%) among all the ORSV isolates of the world. Similar observations were also reported earlier from India

Table 2 Details of the DAC-ELISA and RT-PCR results revealing the association of different plant viruses with the orchid samples collected from Sikkim and Darjeeling hills.

Name of sample	OD value at 405 nm[a]				RT-PCR				
	CymMV	ORSV	CalMMV	GBNV	CymMV	ORSV	CalMMV	GBNV	OFV
Acanthephippium sylhetense	0.45	0.04	0.06	0.24	+	+	-	-	-
Aranda hybrid	0.03	0.02	0.04	0.02	-	-	-	-	-
Cattleya hybrid	0.49	0.05	0.15	0.05	+	-	-	-	-
Cattleya hybrid	0.46	0.54	0.10	0.04	+	-	-	-	-
Cattleya hybrid	0.07	0.49	0.02	0.05	+	-	-	-	-
Cleisocentron pallens	0.02	0.04	0.09	0.05	-	-	-	-	-
Coelogyne elata	0.03	0.03	0.01	0.03	+	+	-	-	-
Cymbidium Baltic glacier mint ice	0.02	0.57	0.22	0.03	+	+	-	-	-
Cymbidium cherry pepper hot stock	1.53	0.04	0.01	0.06	+	+	-	-	-
Cymbidium hybrid	0.07	0.05	0.01	0.26	+	+	-	-	-
Cymbidium hybrid	0.40	0.03	0.01	0.25	+	+	-	-	-
Cymbidium hybrid	0.68	0.59	0.01	0.34	+	+	-	-	-
Cymbidium hybrid	1.28	0.48	0.12	0.23	+	+	-	-	-
Cymbidium hybrid	0.01	0.55	0.11	0.34	+	+	-	-	-
Cymbidium hybrid SHOL-2	0.05	0.17	0.10	0.24	+	+	-	-	-
Cymbidium hybrid SHOL-7	0.02	0.65	0.10	0.25	+	+	-	-	-
Cymbidium pendulum	0.02	0.03	0.04	0.06	-	-	-	-	-
Dendrobium madam pink	0.04	0.04	0.09	0.08	-	-	-	-	-
Dendrobium erica	0.04	0.49	0.11	0.03	-	+	-	-	-
Dendrobium hybrid	0.07	0.49	0.01	0.05	+	+	-	-	-
Dendrobium hybrid	0.59	0.49	0.07	0.03	+	+	-	-	-
Dendrobium moschata	0.05	0.04	0.08	0.03	+	+	-	-	-
Epidendrum sp.	0.09	0.05	0.12	0.02	-	-	-	-	-
Epidendrum sp.	0.44	0.64	0.12	0.03	+	+	-	-	-
Eria javanica sp.	0.02	0.02	0.01	0.05	-	-	-	-	-
Gloriosa russeliana	0.09	0.05	0.01	0.36	-	-	-	-	-
Mokara hybrid	0.62	0.18	0.11	0.03	+	+	-	-	-
Mokara hybrid	0.62	0.03	0.01	0.06	+	+	-	-	-
Mokara hybrid	0.44	0.02	0.03	0.05	+	-	-	-	-
Mokara hybrid happy beauty	1.05	0.04	0.01	0.03	-	-	-	-	-
Oncidium sp	0.02	0.08	0.01	0.05	+	+	-	-	-
Oncidium sp.	0.50	0.20	0.10	0.04	+	+	-	-	-
Oncidium spp.	0.55	0.12	0.12	0.05	-	+	-	-	-
Paphiopedilum sp.	0.12	0.01	0.01	0.04	-	+	-	-	-
Paphiopedilum sp.	0.11	0.04	0.04	0.06	-	+	-	-	-
Paphiopedilum sp.	0.05	0.59	0.10	0.04	-	+	-	-	-
Paphiopedilum sp.	0.03	0.59	0.03	0.02	-	+	-	-	-
Phaius tankervilleae	0.02	0.04	0.42	0.07	-	-	-	+	-
Phaius tankervilleae	0.13	0.54	0.12	0.04	+	+	-	+	-
Phalaenopsis sp.	0.58	0.40	0.01	0.45	-	-	+	-	-
Phalaenopsis sp.	0.48	0.52	0.01	0.12	-	-	+	-	-
Rhynchostylis retusa	0.78	0.03	0.03	0.06	-	-	-	-	-
Rhynchostylis retusa	0.44	0.19	0.10	0.04	+	+	-	-	-
Vanda sp.	0.626	0.08	0.06	0.06	-	-	-	-	-
Healthy control	0.18	0.11	0.10	0.14					
Buffer control	0.17	0.13	0.13	0.18					
Positive control	2.03	2.15	2.08	2.07					

+ = Positive; - = Negative; a = the absorbance value (mean of three triplicates) at A_{405nm} after 1 h of adding the substrate. Samples with absorbance values more than two times of healthy control were considered as ELISA positive.

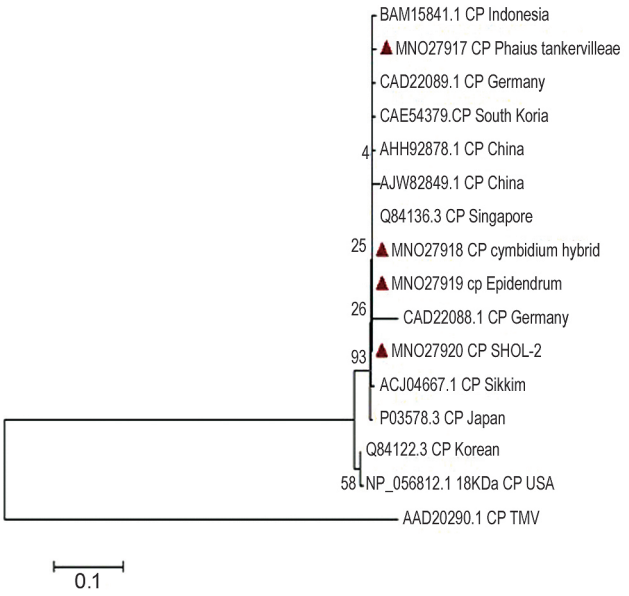

Fig 2 Phylogenetic analysis based on multiple alignments of the coat protein nucleic acid sequence of ORSV isolates. The isolates characterized in the present study are highlighted with the "filled triangle" symbol. CP gene sequence of TMV (AAD20290.1CP TMV; Yunnan) was used as the out-group).

and Indonesia (Rhu and Park 1995, Sherpa *et al*. 2004, Lakani *et al.* 2010).

Present investigation revealed that CymMV and ORSV are the most predominant viruses on Indian orchids and the results were convincing with the earlier reports (Zettler *et al*. 1990, Wong *et al.* 1994, Ajjikuttira *et al.* 2002). GBNV is a well-known tospovirus species predominant in several crops in the Asian sub-continent has also been found to infect orchids, which indicated a possible serious threat to the orchid industry in India. At present GBNV and CalMMV is confined only to *Phalaenopsis* and *P. tankervilleae* respectively in Sikkim and Darjeeling hills. However, the possible widespread occurrence of these viruses cannot be ruled out, and hence, this needs further investigation. The presence of virus diseases in the commercial nurseries suggested the inadvertent spread of viruses due to the poor knowledge about the virus diseases.

Present study also indicated that the ORSV isolates of India and the world were highly conserved with over 99% sequence identities and an oldest known isolate of ORSV originated from USA (Jenson and Gold 1952) was forming the base of the phylogenetic tree. This indicated that the ORSV isolates reported elsewhere from the world could have been moved from the USA through the planting material. These observations further necessitate tightening the quarantine regulations to avoid any further entry of the other viruses hitherto not reported in the country.

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