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A potyvirus in *Cymbidium* spp. in northern India

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Abstract. A potyvirus was isolated from diseased *Cymbidium pendulum* and *C. tigrinum* epiphytic orchids collected from Sikkim in northern India. ELISA, RT-PCR and Northern blot analysis revealed the association of potyvirus infection with disease symptoms. The sequencing of a RT-PCR-amplified amplicon using potyvirus group-specific primers showed that this virus is distinct but most closely related to *Calanthe mild mosaic virus*. This paper reports the occurrence of potyvirus in wild orchids especially in *Cymbidium* spp.

Potyviridae are one of the largest and most economically important families of plant viruses. Orchids are the largest family of flowering plants with ~30 000–35 000 species and they form a large part of the floriculture trade. *Odontoglossum ringspot tobamovirus* and *Cymbidium mosaic potexvirus* are the most frequently reported viruses in cultivated orchids, perhaps because both are highly contagious and spread by wounding when plants are pruned or divided for repotting. However, at least a dozen viruses, mostly potyviruses, have been recorded in orchids, including vanilla (Brunt *et al*. 1996). Most viruses are a serious threat to the orchid industry as they reduce the vigour of infected plants and decrease flower quality. The potyviruses previously isolated from orchids include *Dendrobium mosaic* (DeMV), *Habenaria mosaic*, *Pecteilis mosaic*, *Vanilla mosaic* and *Vanilla necrosis potyviruses* (Brunt *et al*. 1996). The polymerase chain reaction (PCR) has been shown to be very useful for detecting and characterising plant pathogens and is more sensitive than direct probing or serological techniques (Hadidi *et al*. 1995). There are now many published reports of the use of reverse transcription–PCR (RT-PCR) with degenerate primers, which produce specific DNA fragments from all species of a group.

A survey was undertaken for screening of major viruses infecting orchids, which revealed widespread occurrence of *Cymbidium mosaic virus* (CymMV) and *Odontoglossum ringspot virus* (ORSV) in Indian orchids (Sherpa *et al*. 2006*a*, 2006*b*). However, some plants of *Cymbidium pendulum* and *C. tigrinum* with symptoms of mild mosaic and stunting gave negative results for CymMV and ORSV. These epiphytic orchids grow as wild plants in Sikkim, in the north-eastern region of India. These plants were further tested using *Cucumber mosaic virus*, *Cymbidium ringspot virus* and potyvirus group-specific antibodies. ELISA tests using potyvirus group-specific antibodies indicated the association of a potyvirus with diseased orchid samples. To further elucidate and confirm the identity of the virus, total RNA was extracted from the diseased leaves of *C. pendulum* and *C. tigrinum* using the RNAqueous^{TM} Kit (Ambion, USA). RT-PCR was performed as described by Van der Vlugt *et al*. (1999) using the primer pair 'cp9502' (5′GCGGATCCTTTTTTTTTTTTTTT3′), specific for 3′ end of potyvirus genomes, and 'cpup' (5′TGAGGATCCT GGTGYATHGARAAYGG-3′, where Y = C or T, H = A, T or C, R = A or G), which hybridises with the coat protein region of potyvirus genomes. The cDNA was reverse transcribed in a 25-μL reaction mixture using 7 μL (1–2 μg) RNA, 0.2 μg reverse primer (cp9502), 2 μL of 40 mM dNTP mix, 10 units of human placental RNase inhibitor, 5 μL of 5× RT buffer and 200 units of M-MLV reverse transcriptase enzyme. The reaction mixture was incubated at 42°C for 75 min and after that it was incubated at 70°C for 5 min. PCR amplification was done in a GeneAmp PCR 9700 system (Applied Biosystems, USA) with a 50-μL reaction mixture containing 7 μL cDNA, 0.2 μg forward primer, 0.2 μg downstream primer, 5 μL of 10× PCR buffer, 3 μL of 10 mM dNTP mix and 1.5 units of *Taq* DNA polymerase. The DNA was amplified using 35 cycles of denaturation at 94°C for 1 min, annealing at 54°C for 2 min and elongation at 72°C for 1 min, and a final elongation at 72°C for 10 min. The amplified PCR-product was fractionated in a 1% agarose gel and then stained with ethidium bromide.

The amplified fragment was cloned into a pGEM-T Easy vector (Promega, USA) and was then sequenced and digested with *Eco*RI and used for probe preparation. DNA was mixed properly with a random hexamer mixture (100 ng), 10× Klenow buffer, 10 mM dNTPs (-dATP), alpha ^{32}P dATP and Klenow enzyme (5U/μL) in a reaction mixture of 30 μL. The mixture was centrifuged briefly and kept at 37°C for 1 h followed by incubation on ice for 5 min.

Northern slot blot hybridisation (Fig. 1) was carried out with slight modification of the methods described in ExpressHyb Hybridization Kit (BD Bio Sciences, USA). ExpressHyb solution was warmed at 68°C and stirred well to completely dissolve any precipitate. Membranes (10 × 10 cm) were prehybridised in a minimum total volume of 5–10 mL of ExpressHyb solution with continuous shaking at 68°C for 30 min. An equal volume of buffer A (500 mM Tris-HCl, 500 mM NaCl, 5 mM EDTA, 0.5% SDS) was added to the

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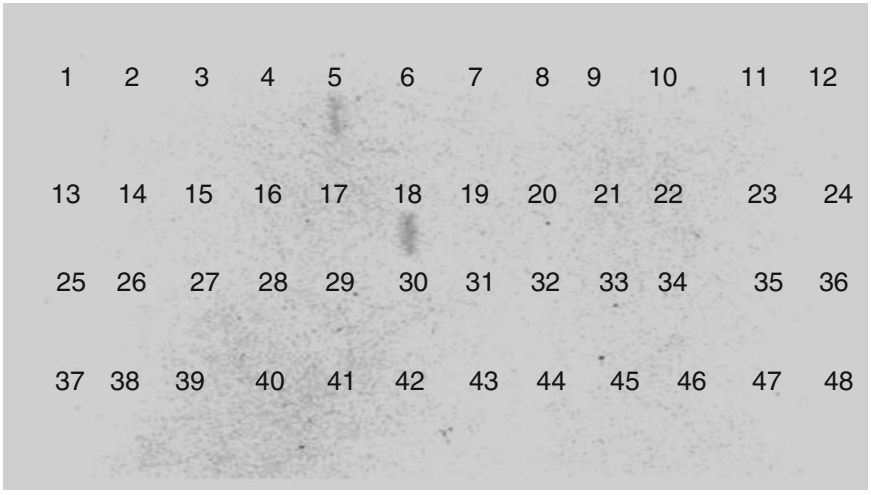

Fig. 1. Detection of potyvirus by Northern slot blot hybridisation. Lane 5 and lane 18 show a positive reaction for the probe prepared from the Cymbidium potyvirus partial coat protein gene cloned in pGEM-T Easy vector and digested with *Eco*RI.

radioactively labelled DNA probe and denatured at 95–100°C for 2–5 min. Then it was chilled quickly on ice and added to 5–10 mL of fresh ExpressHyb hybridisation solution. ExpressHyb hybridisation solution was replaced with the fresh solution containing the radiolabelled DNA probe. All air bubbles were removed from the container and hybridisation solution was evenly distributed over the entire blot. It was incubated with continuous shaking at 68°C for 1 h and the blot was rinsed with wash solution I at room temperature for 30–40 min with continuous agitation with four changes of fresh wash solution I. The blot was washed in wash solution II with continuous shaking at 50°C for 40 min with one change of fresh solution. The blot was removed with forceps and excess wash solution was drained off. Immediately, the blot was put on Whatman paper and wrapped with plastic wrap. The X-ray film was exposed to the blot at −70°C with two intensifying screens and developed after overnight incubation.

These tests showed the presence of a potyvirus in the diseased orchids. In this survey, fifty orchid species and hybrids representing 10 genera, including *Aerides multiflora*, *Coelogyne cristata*, *Cymbidium aloifolium*, *C. iridiodes*, *C. pendulum*, *C. tigrinum*, *Cymbidium* hybrid (Great flower), *Dendrobium nobile*, *Epidendrum* sp., *Phaius tankervillae*, *Pholiodota* sp., *Rhynchostyly retusa*, *Strauropris undulata* and *Vanda* spp., were collected from different nurseries and local habitats in Sikkim and were tested for the presence of potyviruses. Their presence was detected by ELISA, RT-PCR and Northern blot in *C. pendulum* and *C. tigrinum*. These are epiphytic orchids found growing naturally in the Sikkim region. In nature, these wild virus-infected plants exhibit only mild mosaic along with growth retarded symptoms. In RT-PCR, a fragment of ~650 bp (Fig. 2) was amplified, including a sequence of 453 bp that was homologous to the 3′ coat protein gene and 3′ non-coding region of potyviruses. The 3′ non-coding region is 173 nucleotides long and is followed by a polyA tail. The sequence of the PCR-amplified fragment from *C. pendulum* was submitted to EMBL GenBank as Accession Code No: AJ871476. It showed

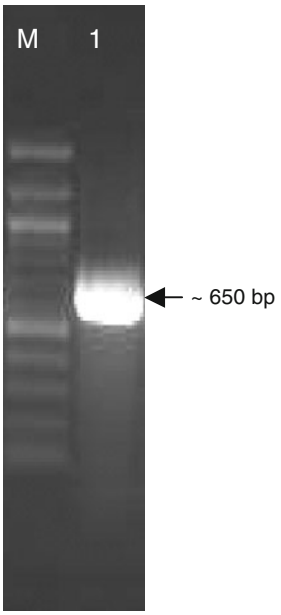

Fig. 2. Amplification of Cymbidium potyvirus partial coat protein gene by RT-PCR. Lane M: 100 bp DNA marker, lane 1: PCR amplified product of ~650 bp.

88% identity with *Calanthe mild mosaic virus* (AB011404), 50% with *Bean yellow mosaic virus* (NC_003492), 48% with *Vanilla mosaic virus* (AJ616720), 47% with *Dasheen mosaic virus* (NC_003537), *Turnip mosaic virus* (NC_002509) and *Dendrobium mosaic virus* (DMU23564), 46% with *Watermelon mosaic virus 2* (L22907) and 44% with *Cypripedium mosaic virus* (AF185955) at the nucleotide level (Table 1) and it was tentatively named Cymbidium potyvirus. This is the first report of a new potyvirus infecting *C. pendulum* and *C. tigrinum* orchids from natural habitats of Sikkim, and is only the second report of a virus in a wild orchid as all

Table 1. Relationship of Cymbidium potyvirus (identity of coat protein gene) with previously reported orchid infecting potyviruses

Virus (accession number)	Identity (%)
Calanthe mild mosaic virus (AB011404)	88
Bean yellow mosaic virus (NC_003492)	50
Vanilla mosaic virus (AJ616720)	48
Dasheen mosaic virus (NC_003537)	47
Turnip mosaic virus (NC_002509)	47
Dendrobium mosaic virus (DMU23564)	47
Watermelon mosaic virus 2 (L22907)	46
Cypripedium mosaic virus (AF185955)	44

previous records are of virus infection in cultivated orchids (Gibbs *et al*. 2000).

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