ABSTRACT

Title of Thesis: TRANSMISSION OF *CYMBIDIUM MOSAIC VIRUS* IN *ONCIDIUM* ORCHIDS BY *PERIPLANETA AUSTRALASIAE*

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Cymbidium mosaic virus is the most common disease in orchids infecting a large number of cultivated orchids found in all phases of the industry and around the world. Its transmission occurs through contact by contaminated cutting tools, human hands, or water. Although insects known to transmit plant viruses have been exposed to orchid viruses, none have been found to successfully transmit *Cymbidium mosaic virus*. *Periplaneta australasiae*, the Australian cockroach, is a common greenhouse pest that is known to feed on orchid plants. In controlled conditions Australian cockroaches were given inoculation access through feeding activity on known CymMV positive orchid plants and then allowed to feed on virus free plants. The virus free plants were isolated from subsequent insect exposure and after a period of time samples from the feeding damage sites were analyzed for the presence of virus RNA through nested and hemi-nested PCR techniques. A statistically significant number of samples were positive

demonstrating that with high population numbers and long term exposure, virus transmission is possible.

TRANSMISSION OF *CYMBIDIUM MOSAIC VIRUS* IN *ONCIDIUM*

ORCHIDS BY *PERIPLANETA AUSTRALASIAE*

BY

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Chapter 1: Literature Review

Introduction

Worldwide, the orchid industry has enjoyed an unparalleled economic upswing in the past decade and a half. This economic boom has been marked in all phases of the industry: hobby, cut flower and the pot plant markets (Floriculture Crops 2011, Kiang Ho 2010). With this remarkable growth, a new awareness of orchid related pests and diseases has resulted in the need for improved standards and disease-prevention protocols. *Cymbidium mosaic virus* is the most prevalent orchid disease in all areas of the industry and in all countries where they are produced. CymMV is transmitted primarily by cutting tools, hands, and contaminated water sources (Wisler, personal correspondence August 12, 2009). Potex viruses are not normally known to be transmitted by insect vectors and lack a specific gene product for vector interactions (Hammond personal correspondence 2011). *Cymbidium mosaic virus* expression is observed in flower distortion, necrotic spotting and reduced plant vigor (Inouye 2008). The concept of a chewing insect route of transmission has been considered, but not pursued. *Periplaneta australasiae* is a common greenhouse and conservatory pest (Bell et al.1999) whose feeding damage has been suspected in the transmission of orchid virus disease.

History of Cymbidium Mosaic Virus

CymMV was first described in 1951 by Dilworth D. Jensen who observed black necrotic spotting on *Cymbidium spp* and named the virus Cymbidium Black Streak virus. Dr. Dilworth continued to discover other orchid virus diseases while at the College of Agriculture, University of California, Berkeley during the mid 1950's until the end of his life in 1973. His work with A. H. Gold successfully identified the *Cymbidium mosaic virus* particle via electron microscopy and described it as sinuous rods (about 18nm X 475 nm) (Gold and Jensen 1951) (Figure 1). He is credited as the pioneer of orchid virus research and was named a Fulbright Research Scholar in 1959-60 where he was assigned to the University of Utrecht in the Netherlands (Freitag, et al. 2011). He continued to research orchid virus transmission while in the Netherlands and found that both private and public collections and commercial producers there were observing similar symptoms as in the United States. He is credited with identifying 30 possible orchid viruses during his life. Although he is known for groundbreaking work in orchid virus disease, Jensen was an entomologist and doggedly looked for an insect vector for *Cymbidium mosaic virus*, but without success.

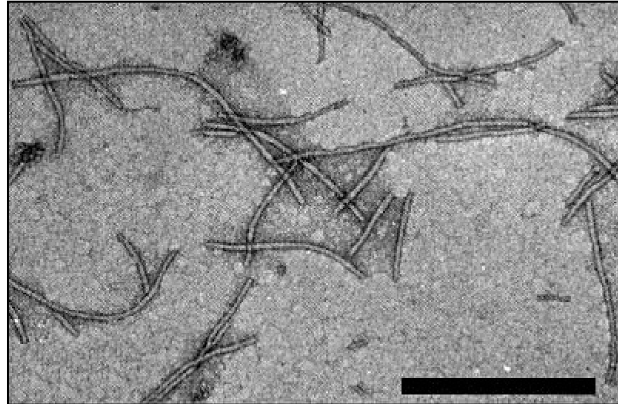


Fig. 1 CymMV Virus particles from purified preparation in uranyl acetate. Bar represents 500 nm.

Descriptions of Plant Viruses, http://www.dpvweb.net/dpv/index.php

Virus Symptoms

Virus disease in orchids can be expressed through a variety of abnormalities in both the leaves and the flowers. Reduced vigor is also attributed to the presence of virus infection, but that is harder to quantify and may be a function of culture and environmental conditions. Also, expression of virus symptoms may be latent in orchids that are well grown and under little stress (Inouye 2008). Early researchers had only the visual manifestation of virus disease as clues to the larger problem.

Cymbidium mosaic virus may be the most prevalent, but another virus disease, *Odontoglossum ringspot virus* is also a worldwide issue. Both virus diseases

typically have unique manifestations, but not consistently the same. Also an individual plant or group of plants may harbor both diseases simultaneously.

CymMV was described originally as Cymbidium Black Streak virus and that is an appropriate description of the leaf symptoms. Necrotic streaking or spotting is typical and these lesions can be found on both flowers and leaves (Figure 2).

Odontoglossum ringspot virus can be visualized as a mottling of the color of the leaf or as concentric necrotic rings (Figure 3).

Either virus can cause a condition known as 'color break' (Figure 4).

Fig. 2 Necrotic spotting on Phalaenopsis due to CymMV
Chin-An Chang

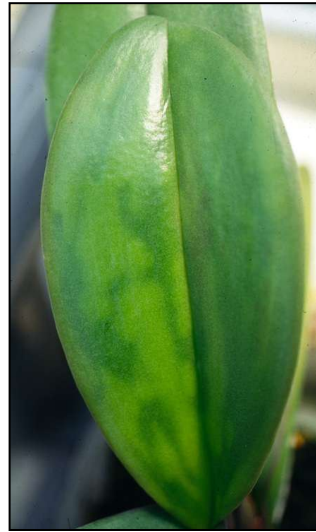


Fig. 3 Mottling due to infection by ORSV
Chin-An Chang

Fig 4 'Color break' abnormalities in flower coloration due to virus
Chin-An Chang

Determining CymMV Host Range

Researchers during the 1950's were working primarily with bioassay techniques; inoculating the sap from orchids with symptoms onto various plants, both orchid and non-orchid in order to establish the host range. In 1952, Dr. Jensen was successfully able to transmit this newly identified virus from *Laelia anceps* onto *Cymbidium sp*. and from *Cymbidium sp*. to *Cymbidium sp*. (Jensen 1952).

When inoculated with the sap from diseased orchids*, Datura stramonium* (White and Goodchild 1955), *Cassia occidentalis* (Corbett 1960), *Tropaeolum majus, Oryza sativa, Passiflora edulis*, and *Zinnia elegans* (Murakishi 1958) all proved to respond with the formation of leaf lesions. However, researchers were not able to distinguish between *Odontoglossum Ringspot virus* and *Cymbidium mosaic*

virus based on symptoms alone. Frequently both virus diseases were present in the same plant, adding to the confusion. Since each of these viruses has a different alternate host range, some of the early host range studies show contradiction among research groups.

Jensen seemed to have the best understanding of the two diseases and defined a diagnostic host range for CymMV that included only *Datura stramonium* and *Cassia occidentalis* (Jensen 1972). The hypersensitive response, leaf lesions or chlorosis, in a host plant enables it to be utilized as a bioassay or indicator plant in virus surveys. This localized reaction to CymMV puts *Chenopodium amaranticolor, C. quinoa, Tetragonia tetragonioides, Gomphrena globosa, Datura stramonium,* and *Cassia occidentalis* in that group of plants (Inouye 2008). The work of Inouye was further able to distinguish and separate the diagnostic host range of *Cymbidium mosaic virus* and *Odontoglossum ringspot virus* and determined that *Cassia occidentalis* was the most definitive test plant for visualizing CymMV lesions alone.

Detection

Bioassay – the inoculation of a host plant with a dilution of the sap from a test plant – is still widely employed by virologists as a quick and easy screening method. The development of Enzyme-linked immunosorbent assay (ELISA) was an advancement that made nursery wide screening easier and more accurate. ELISA testing uses immunology to detect a reaction of a specific antibody or antigen with the assistance of a color indicator.

However, ELISA testing requires laboratory equipment and is not available for most orchid growers. For on-the-spot screening, immunoassay test strips (ImmunoStrip, Agdia, Inc. 30380 County Road 6, Elkart, IN 46514) have been developed that do not require laboratory equipment. ImmunoStrips are sensitive for most CymMV isolates and are combined with ORSV detection. These are simple enough for greenhouse managers or curators to use for accurate and rapid on site testing.

RT-PCR (reverse transcription polymerase chain reaction) or one of the other tests that detect the presence of the cDNA can be more sensitive, especially for a low titer of virus particles. In RT-PCR, total RNA is extracted from the tissue being tested and is transcribed into cDNA by a reverse transcriptase enzyme. The viral cDNA is then amplified in the presence of template primers by DNA polymerase and can be visualized by electrophoresis on an agarose gel. If an exacting technique is employed in a clean laboratory, RT-PCR is a more reliable method of virus detection.

Unfortunately no detection technique is fool proof. Both false positives and false negatives are possible. Researchers have noted the time from virus inoculation to expression of symptoms can be from seven months, as in the case of *Potyvirus* in *Vanilla* in Tahiti (Wisler, personal communication 2009), to 30 months as reported in experiments with *Sophrolaeliocattleya* hybrids in Venezuela (Izaguirre-Mayoral et al., 1993). CymMV does associate with the vascular tissue and can move more rapidly than ORSV, which moves from cell to cell (Borth, et

al., 2006) but testing a newly acquired orchid can lead to false negatives if inoculation occurred at the time of division. The conscientious researcher or grower will employ more than one type of test to confirm the presence of virus infection and will repeat testing at regular intervals.

Worldwide Presence of Cymbidium Mosaic Virus

The relative incidence of CymMV in orchid crops has been studied by many researchers. A 1992 survey of approximately 3,600 orchid plants in Hawaii found that *Cymbidium mosaic virus* was found in 61% of the plants tested. ELISA testing was the protocol used. At that time most commercial *Dendrobium* hybrids (cut flower industry) were seed grown and the incidence of CymMV was 4% in plants less than three years old. However, cloned *Dendrobiums* showed an incidence of 45% (Hu 1993).

The Singapore Botanic Garden collection was tested between the years of 1988 to1991. 54.6% of the orchids tested were positive for *Cymbidium mosaic virus*. Most disturbing was that 50.5% of the in-house tissue cultured plants were infected with CymMV (Wong et al., 1994).

In 2003; bioassay, electron microscopy, ELISA, and RT-PCR testing was performed on a group of various orchid genera that appeared symptomatic in India This exhaustive protocol was employed as up to that time, it was believed that CymMV was not found in that country. The orchids proved to be positive for

virus and through the above testing protocols the particles were identified as *Cymbidium mosaic virus* (Sherpa et al., 2003).

The cut flower industry accounts for a significant segment of the Thai economy. For example, 2004 saw $56 million (US) in cut flower exports alone. ELISA testing was used to survey 280 vegetatively propagated *Dendrobium* plants and of those plants 64.5% were positive for CymMV. Similar to the Hawaii survey, in vitro cultured seedling plants showed no incidence of virus infection (Khentry et al., 2006).

In all of the above surveys the incidence of *Cymbidium mosaic virus* in cultivated orchids is significant. CymMV infection occurs worldwide in all genera, species and hybrids of orchids (Brunt, et al 1996). There are many countries where orchids are part of their world-based economy. The debilitating effects of the virus on an orchid crop reduce the potential for economic gain. Unfortunately in many cases where the orchid producer is growing for a throw-away pot plant market, as long as the plant is marketable, they are not deeply concerned about the presence of virus disease.

It is obvious that an industry wide renovation in attitude, cultivation techniques and parent plant virus screening is needed. With deeply ingrained protocols, minimum wage employees, and the expense of routine testing, it may be a long time before the incidence of CymMV will be reduced.

Description

Cymbidium mosaic virus is classified as a member of the family: Alphaflexiviridae, the genus: Potexvirus. It has a positive sense, single stranded RNA gnome. Positive sense RNA is similar enough to mRNA that it can be immediately translated by the host plant and is therefore immediately infective (Hull 1970). Virions are filamentous and not enveloped. The particle is flexuous, with a clear modal length of 480 nm X 13 nm wide. Axial canal is obscure. Basic helix is obvious with the pitch of basic helix 2.8 nm (Büchen-Osmond 2011) (Figure 5).

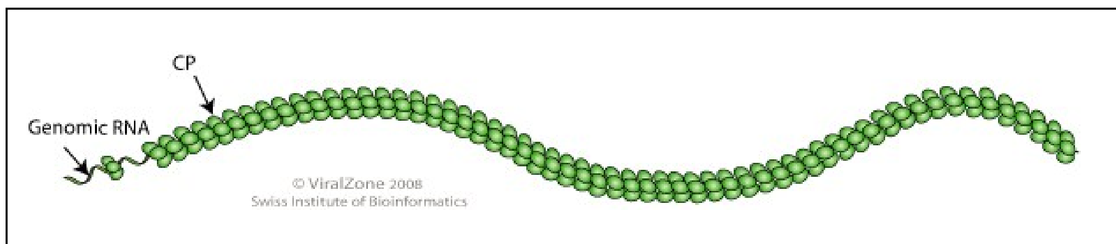


Fig. 5 Potex virus. Viral Zone

Genome

Cymbidium mosaic virus is approximately 6227 nucleotides in length not including the polyadenylated tail at the 3' end. The 5' end is capped. Like other potexviruses, it contains five open reading frames (ORF's): an RNA-dependent RNA polymerase (RdRp), three triple gene block (TGB1, TGB2, & TGB3), and a coat protein (Wong et al., 1997). Movement between cells and through the plant

host is facilitated cooperatively by the triple-gene-block proteins and the coat proteins (Lu et al., 2009). (Figure 6)

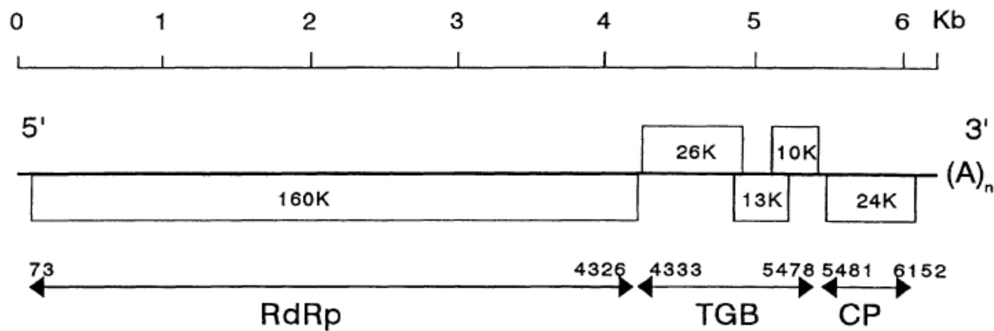


Fig 6 Schematic representation of the genome CymMV, genome organization with scale. Open boxes represent the coding regions for the RNA-dependent RNA polymerase (RdRp), 160 KDa, 26 KDa/13KDa/10 KDa triple gene block (TGB) and 24 KDa coat protein (CP). The 5' and 3' non-coding regions are represented as a single line. The $(A)_n$ represents the poly (A) tail. Numerals represent nucleotide positions. (Wong et al., 1997)

The virion RNA is infectious and serves as both the genome and viral messenger RNA. RNA-dependent RNA polymerase (RdRp) is translated directly from the genomic RNA. The other ORFS are transcribed presumably as monocistronic (translates only a single protein) subgenomic mRNAs (sgRNAs) (ViralZone 2011). Although there are a high number of isolates, the coat proteins and RdRp regions seem to be highly conserved (Moles et al., 2007).

Developing Resistance

CymMV has been observed worldwide in private and public collections, but with even greater economic significance in the cut flower industry as described previously. In traditional breeding programs flower count, size and color have been the ultimate goals. Breeding or screening for CymMV resistance in commercial orchid lines has not been a common research objective.

However, in 1988, Kuehnle, found that Dendrobiums that were susceptible to CymMV when bred to another susceptible Dendrobium produced susceptible offspring and resistant cultivars when crossed to another resistant cultivar produced resistant offspring. She determined that susceptibility was the dominant characteristic in cross breeding of types and that expression of floral necrosis was genetically controlled.

Researchers are actively working to develop CymMV resistant strains of *Dendrobium* varieties via genetic modification. In one case, a mutant movement protein gene, *mut11* was inserted via biolistics into two different *Dendrobium* hybrids and the plants were repeatedly challenged by inoculations of a 1:1000 dilution of CymMV. Though the sampling was small, with only 24 original plants, 9 of the transgenic plants remained CymMV free after 12 months (Obsuwan, et al 2009).

In Taiwan, another research group isolated CymMV and the cDNA of the CP gene was then synthesized and sequenced. Through particle bombardment, the

synthesized gene was transformed into very young *Dendrobium* plants. The presence of the gene was confirmed by PCR, Southern, Northern, and Western blot techniques. When these plants were challenged with CymMV they exhibited considerably milder symptoms (Chang et al., 2005).

Another research group in Taiwan worked with Phalaenopsis hybrids. Resistance in those plants was achieved by insertion of a CymMV coat protein and a nos terminator placed downstream of a maize ubiquitin promoter. Those plants exhibited improved resistance to CmyMV upon virus challenge (Liao, et al 2004).

***Periplaneta australasiae,* The Australian Cockroach**

Stejskal, et al. (2004) describe the Australian cockroach as a rapidly spreading pest moving from its native tropics into the temperate zone. It is thought to have originated in North Africa despite its common name. The pest species of cockroaches are believed to have dispersed with early human exploration (Kunkle 2007).

This cockroach infests not only food storage areas, but greenhouses and conservatories as well. Unlike some of its more well-known relatives, it can feed on tender plant material (Figure 7). Locally it can be found in damaging numbers at the Smithsonian complexes (Tom Mirenda, personal communication 2010) and the United States Botanic Garden. In greenhouses and conservatories its numbers can build rapidly especially when the targeted sprays of a strict program of integrated pest management are observed (Bell et al., 1999).

Fig 7 Australian cockroach damage to orchid roots (left) and a Cattleya flower (right)

Resembling the American cockroach, *Periplaneta australasiae* differs by the yellow band found encircling the thorax and a small yellow mark on its side near the wing base (Figure 8). Adults may reach twenty-seven to thirty-three mm in length (Cochran 1999)

Fig 8 Australian cockroach, *Periplaneta australasiae*

Biology and Life Cycle

Adult Australian cockroaches are believed to live for 6 – 8 months and maturation occurs at about five months of age. Their life cycle is one of gradual metamorphosis: egg, nymph, and adult. Nymphs undergo nine to twelve molting cycles before reaching maturity (Cochran 1999).

The females produce an egg case called an ootheca which can contain sixteen to twenty-four eggs. Hatch rate is influenced by temperature and humidity. Females may produce twenty to thirty egg cases in their life time. (Ramel 2001)
Australian cockroaches are in the order Blattodea (Cockroaches) and family, Blattidae.

Chewing Insects as Vectors

Researchers have reported some virus vectors in the orders Orthoptera (Grasshoppers) and Dermaptera (earwigs). More significant vectors are found in the order Coleoptera (Beetles), but no work has been reported on insects found in the order Blattaria (Cockroaches) as virus vectors in plants (Hull 1970)

Chapter 2: Materials and Methods

The cockroach colony and all experimental plants were housed in the University of Maryland research greenhouses. A southeast facing, 69.7 square meters (750 sq. ft.) section was chosen for its appropriate light levels for maximum orchid growth and health. Natural day length was allowed, supplemental lighting was used only to maintain a set point of 30 Klux during cloudy weather. Temperatures were set at 24°C day/18°C night.

Periplaneta australasiae, Australia Cockroach

The Australia cockroach colony was initiated with a purchase of 40 mature, male and female cockroaches (PNE, Inc., 169 Elsa Jane Lane, Pittsboro, NC 27312-5167). The cockroaches were housed in containers that were modified water proof document storage boxes. Water and dry dog chow were supplied ad libitum. The cockroaches were allowed to breed freely and were moved to other containers as the colony grew.

The cockroaches were subjected to a period of five weeks without food before their introduction to orchid plant tissue. *Periplaneta australasiae* will resist a change in diet up to the point of starvation (Barry Pawson, personal communication 2009). The period of deprivation enabled the cockroaches to accept the new food source rapidly.

Orchids

Three groups of orchids were used during the course of this experiment. Orchids of known virus infection made up the first group. These were plants of various genera that were donated from the United States Botanic Garden (United States Botanic Garden, 100 Maryland Avenue, SW, Washington, DC 20001). Testing to confirm virus infection was performed by a commercial laboratory (Agdia, Inc., 30380 County Road 6, Elkart, IN 46514) using enzyme linked immunosorbent assay. These plants all tested positive for *Cymbidium mosaic virus* and some were positive for *Odontoglossum ringspot virus* as well.

The second group of orchids were Oncidium cultivars and hybrids donated from private collections and through the generosity of a local commercial orchid grower (Orchid Enterprise, Inc., 6 Perch Place, Alexandria, VA 22309). Surveys were conducted via bioassay and ImmunoStrip (Agdia, Inc., 30380 County Road 6, Elkart, IN 46514) testing to ascertain virus infection.

To minimize the possibility of using test plants that had already been exposed to orchid viruses, newly de-flasked Odontocidium Catatante 'Pacific Sunspots', AM/AOS were purchased for the project (Carmela Orchids, P.O. Box 277. Hakalau, HI 96710) and comprise the third group of orchids. These plants were subsequently tested and found negative for virus infection by ImmunoStrip and polymerase chain reaction assay.

Experimental Units

The experimental unit was a clear fronted, screened enclosure (Rearing and Observation Cage, BioQuip Products, 2321 Gladwick Street, Rancho Dominguez, CA 90220) that successfully provided both insect containment and sufficient light for plant health and maintenance.

Thirty experimental units were set up on two greenhouse benches. The cages were protected from extreme light by draping with pieces of standard greenhouse shade cloth (60%) to keep the Australian cockroaches in a more comfortable environment. The cages were numbered and randomized on the benches.

In each enclosure elevated pierced flooring was provided by the insertion of a 12” X 12” piece of plastic egg crating. This elevation would prevent water contamination between plants. A 4 ½” standard, square plastic pot was used as an insect hide and water for the cockroaches was provided by a plastic petri dish fitted with an acrylic sponge soaked with water.

The cockroaches were distributed in groups of either ten or twenty individuals per experimental unit, ranging in size from 1.2 cm nymphs to mature adults. Gender ratio was not considered significant.

The Treatment

After a period of food deprivation of approximately five weeks duration, pieces of orchid leaf (approximately 2.5 cm X 2.5 cm) or an orchid flower were inserted into a slit in the sponge of the cage water source. Twenty five of the randomized

cages were supplied with leaf tissue from known virus infected orchids. To act as controls, five of the randomized cages were supplied with tissue from plants that had been repeatedly tested as virus free. The tissue samples were changed out after consumption or one week's time. To accustom the cockroaches to feeding on orchids, they were fed for a period of three weeks on orchid leaf tissue before orchid seedling test plants were placed in the cages.

Introduction of Seedling Test Plants

Five orchid seedlings were placed in each cage. The plants were numbered by cage and sequenced, 1 – 5. The cockroaches were allowed free access to feed. The orchid seedlings were examined for feeding damage several times per week and were removed as soon as damage occurred. A second period of deprivation was initiated in late March due to a lag in feeding activity. All food was removed for a period of approximately one month after which exposure to both infected plants and test plants was resumed. The inoculated seedlings were placed on a greenhouse bench to allow possible virus replication.

Time Interval for Virus Replication

Tissue samples were harvested a varying intervals to allow for virus replication within the inoculated tissue. This time period (Time B) was initially set at greater than 21 days. A longer period of time would have the advantage of a higher number of virus particles able to be detected. Samples were harvested at 56, 62, 65, 69, 71, 89, and 90 days post inoculation.

Analysis Protocol for Virus Detection

The sample tissue from inoculated and control plants was ground in a mesh extraction bag (Agdia, Inc. 30380 County Road 6, Elkart, IN 46514) containing 1.5 ml RLT, an RNeasy lysis buffer (QIAGEN Inc., 27220 Turnberry Lane, Valencia, CA 91355). RNA extraction was then performed by standard procedure using an RNeasy Plant Mini Kit (QIAGEN Inc.). For RNA extraction from ImmunoStrip, the preserved strips were soaked for 5 minutes in an 11:2 solution of RLT buffer then soaked for an additional 5 minutes in ethyl alcohol. The solutions were combined and 700 µl were then placed in the pink columns from the RNeasy Plant Mini Kit. Standard procedure was then followed.

Conversion from RNA to cDNA was performed on 5µl of RNA extract with the addition of 15 µl of a master mix containing: 1 µl Moloney Murine Leukemia Virus Reverse Transcriptase, 4 µl M-MLV Reverse Transcriptase 5X Reaction Buffer, 4 µl Deoxynucleotide Triphosphates 2.5mM, 5 µl, Primer NSNC-odT (5′ ATCCATGGCATGCATCGATTTTTTTTTTTTTTTV 3′, where V = A, G, or C), and 1 µl RNAsin (all reagents except NSNC-odt: Promega, 2800 Woods Hollow Road, Madison, WI 53711-5399. NSNC-odT designed by John Hammond and Michael Reinsel (USDA-ARS, USNA, FNPRU) and produced by Invitrogen, Life Technologies Corp., 3175 Staley Road, Grand Island, NY 14072). The samples were processed in a thermo cycler (Applied Biosystems, GeneAmp, PCR System 2700, Life Technologies Corporation, 5791 Van Allen Way, Carlsbad, CA 92008) using the following program: 42°C for 60 minutes, 95°C 5 minutes and 4°C to

hold until the cDNA was either sampled for the PCR step, or stored frozen for later use.

Initial testing of CymMV-infected and control plants was performed by PCR using one of several combinations of primers designed by Michael Reinsel (USDA-ARS, USNA, FNPRU) based on an alignment of multiple CymMV sequences available in GenBank, or on the ‘tag’ portion of cDNA primer NSNC-odT. These primers were: CymTGB2 (‘Forward’, 5′ TGCAATACATATCACCACCCCTGA 3′); CymCoatF (‘Forward’, 5′ TGGCGAGGGTTAAGTTACCA 3′); CymCoatR (‘Reverse’, 5′ TGCCAGTAGTGGAAACAAACTT 3′); and BNSNC (‘Reverse’, 5’ TTTATCGGATCCATGGCATGCATCG 3′) (Fig. 9). Each of these primer combinations yielded a CymMV-specific product of sizes (CymCoatF/CymCoatR, 763 bp; CymCoatF/BNSNC, 829 bp; CymTGB2/CymCoatR, 881 bp; CymTGB2/BNSNC, 947 bp), with minor yields of non-specific products. Although obvious CymMV-specific products were obtained from systemically-infected positive control orchids, no products were obtained in initial tests of plants exposed to cockroach feeding. Because the *Cymbidium mosaic virus* was suspected to be in very small amounts in the sample tissue, a hemi-nested PCR (Mullis and Faloona 1987) assay was then developed and used as the protocol of choice to increase the sensitivity of detection.

Primer pairs were selected by running a temperature gradient PCR (Appendix B) using samples of known positive, known negative as well as a plasmid positive control. Calculated annealing temperatures were determined for all primer pair combinations:

CymTGB2 (forward)/CymCoatR (reverse)	56° C
CymTGB2 (forward)/BNSNC (reverse)	63° C
CymCoatF (forward)/BNSNC (reverse)	58° C
CymCoatF (forward)/CymCoatR (reverse)	56° C

After analysis of the resulting cDNA product by gel electrophoresis, the primer pair CymCoatF (forward)/BNSNC (reverse) was determined to be the most advantageous for the initial PCR and CymCoatF (forward)/CymCoatR (reverse) for the hemi-nest. Amplification was further maximized by increasing the cycles from 35 to 40.

In the initial assay, sample plant tissue cDNA was subjected to PCR with amplification targeted at the virus coat protein and 3′ non-coding region using primers CymCoat F and BNSNC. That PCR product was then subjected to the hemi-nested reaction with amplification targeted to a narrower area of virus coat protein using primers CymCoat F and CymCoat R. The master mix used for all PCR assays was as follows: 0.2 µl GoTaq, 4 µl 5X Green GoTaq Reaction Buffer, 2 µl Deoxynucleotide Triphosphates 2.5mM (all reagents supplied by Promega), 1 µl forward primer, 1 µl reverse primer, and 10.8 µl dH_2O per sample. To 19 µl of

the master mix, 1 µl of sample cDNA or diluted (1:100) first PCR product was added. The samples were processed in a thermo cycler using the following protocol: 1 cycle 94° C for 3 minutes, 40 cycles: 94° C 30 seconds, 63° C 30 seconds, 72° C 90 seconds and then 1 cycle 72° C for 7 minutes.

The PCR products from both steps of the hemi-nested protocol were separately examined by Agarose gel electrophoresis. A standard 1% Agarose gel was formed by the formula: 0.6g Agarose (Separation > 500bp, Genetic Performance Certified, USB Corp, Cleveland, OH) dissolved in 60 ml tris-borate-EDTA buffer 0.5X. A 1 kb DNA Ladder (Promega) was used as a standard for the electrophoresis product. All PCR assays included known positive and negative control samples.

Since the amount of virus present in a sample could also affect the presence of the final PCR product, a dilution gradient was run. Dilutions of a known positive sample, a known negative sample, and a positive plasmid control were made at the ratios of 1:5, 1:25, 1:125, 1:625, and 1:3125. Serial dilutions were made from an initial 1:100 dilution of the first PCR product. The above hemi-nested protocol was performed and the PCR products were visualized on gel electrophoresis. Clear CymMV product bands were observed for all dilutions. (Appendix C)

A third set of primers were designed to be employed in future work with *Cymbidium mosaic virus*. These primers were: one forward primer, CymF23

(‘Forward’, 5′ GTGGTGTGGAATCTGATGCTGGC 3′) and two reverse primers, CymCP-R2 (‘Reverse’, 5′ GCAATGTTGGTGATGAGGTTGCCGG 3′) and CymR25 (‘Reverse’, 5′ CTTGGTGACCTCGGCAATGTTGG 3′). (Figure 9). An annealing temperature gradient was run on various combinations of existing and new forward and reverse primers. (Appendix D). Selected cDNA samples were run to test two of the new primer combinations.

A chi square test was applied to feeding interval data. (Appendix F)

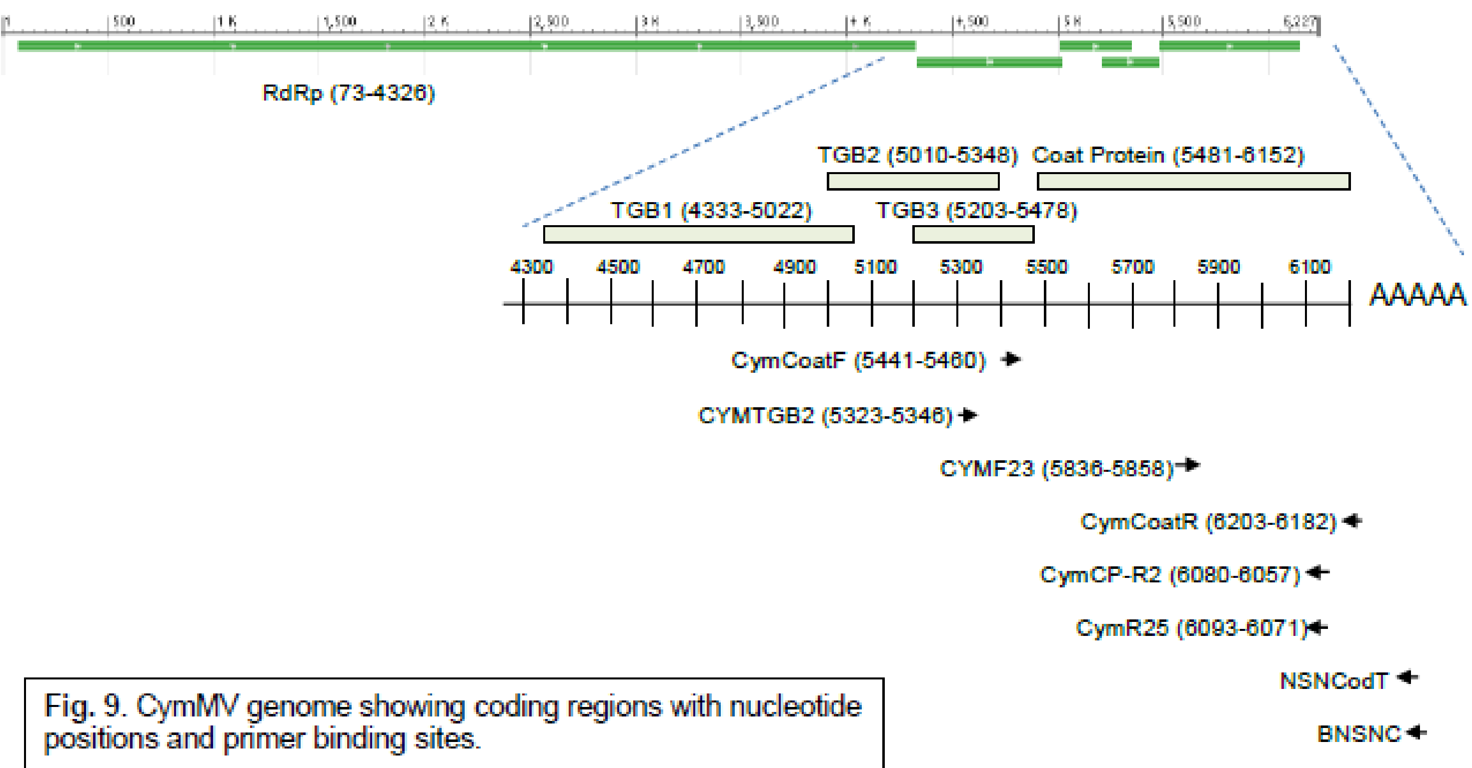


Fig. 9. CymMV genome showing coding regions with nucleotide positions and primer binding sites.

Chapter 3: Results

Australian Cockroach

Feeding damage was observed within ten days after the introduction of orchid leaf pieces. The damage resembled that of beetles (Fulton, et al. 1987) (Figure 10)

Fig 10 Feeding damage on leaf piece (left) and on test plant (right)

Damaged plants were removed as soon as observed and placed on the greenhouse bench. Groups of samples were taken for RNA extraction at 56, 62, 65, 69, 71, 89, and 90 days post inoculation. Most samples consisted of the chewing damage site and the surrounding leaf tissue; however there were two cases of pseudobulb damage and that tissue was tested as well.

The Test Plants

Two different time intervals are noted. The time between the exposure of the cockroaches to the infected plant material and the time of their access to feed on test plants is one critical period, labeled Time A (Appendix E). The other critical period is the time the virus has to replicate in the damaged test plant tissue, labeled Time B. For Time B, plants were grouped by number of days post feeding damage and labeled A through G. Damaged plant tissue was harvested according to this second time period and polymerase chain reaction analysis was performed.

Figure 11 shows the comparison between plants positive for *Cymbidium mosaic virus* and the time interval between exposure of the cockroaches to infected material and test plant feeding damage (Time A).

	Number of Plants	
Interval (days)	**Positive**	**Negative**
6-10	14	19
11-15	0	28
16-20	0	9
21-25	0	12

Fig 11. Incidence of positive and negative plants compared to time interval between cockroach exposure to infected material and feeding damage

Nineteen plants in the interval between 6 and 10 days were found to be negative for presence of CymMV. Fourteen plants were found positive for presence of

CymMV after feeding damage by Australian Cockroaches in the same interval. The results of the treatments are significantly significant at $p < .0001$. Forty-nine plants from longer time intervals between exposure to infected leaf and observed feeding damage were found to be negative for the presence to *Cymbidium mosaic virus*.

Polymerase Chain Reaction Analysis

Test Groups A, B & C

Samples 1 – 19 (Test Group A, B, & C) were run via PCR analysis as described above using CymTGB2 (forward) and CymCoatR (reverse). These samples were taken from plants 62, 69 and 71 days post inoculation. Three samples were from negative control cages.

Included in the initial polymerase reaction and the subsequent hemi- nested procedure were the following samples:

Sample number	Plant number	dpi (days post inoculation)
1	1-2	62
2	1-3	62
3	1-5	71
4	5-3	62
5	8-1	69

6	8-4	62	
7	8-5	69	
8	11-4	69	
9	12-3	62	
10	13-3	62	
11	16-3	62	
12	16-4	62	
13	17-2	69	
14	18-1	69	Negative control
15	18-2	69	Negative control
16	18-3	69	Negative control
17	21-5	69	
18	29-2	71	
19	29-5	71	

RNA extractions were made on 5/17/12, reverse transcription on 5/21/12, and the initial PCR was run on 6/19/12 with the primers CymCoatF/BNSNC as described.

Two DNA ladders (100 kb and 1 kb) were loaded on this gel for comparison of product size. In subsequent reactions only the 1 kb ladder was used as the anticipated product for CymMV would be found at 763 kb. A plasmid CymMV was used as the template for the positive control.

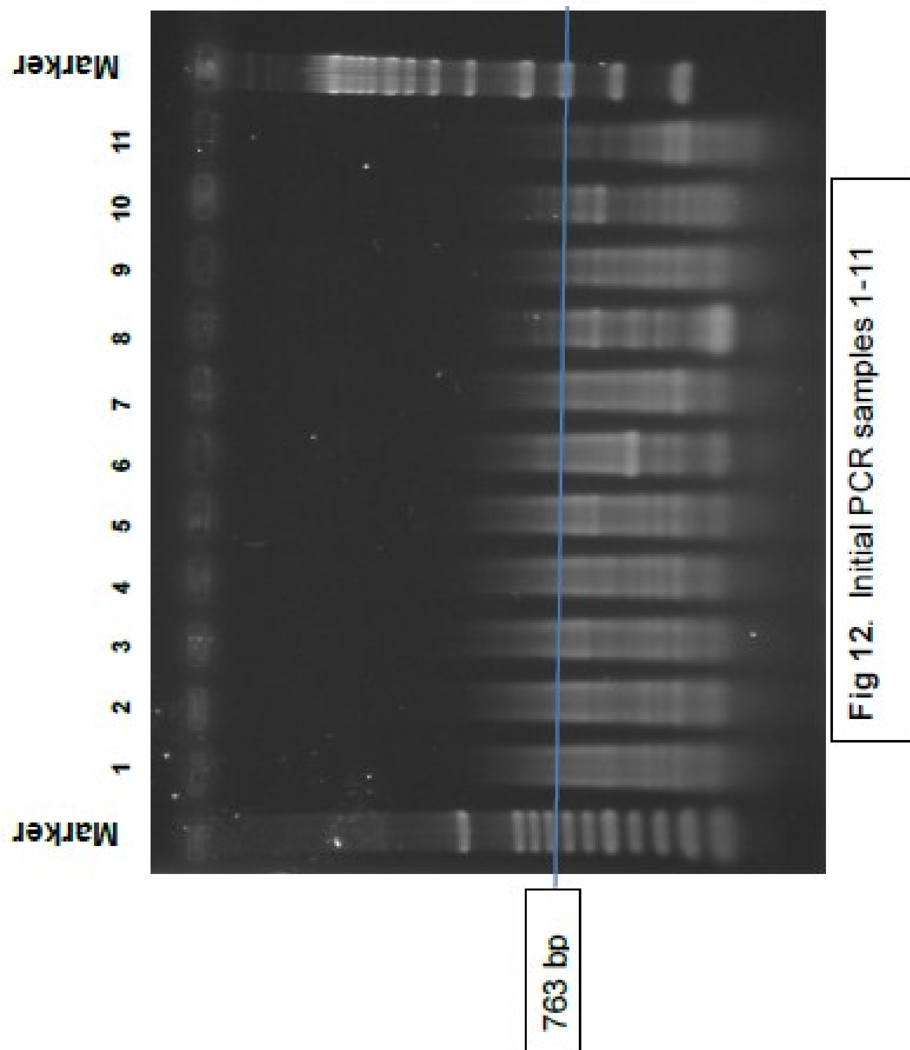


Fig 12. Initial PCR samples 1-11

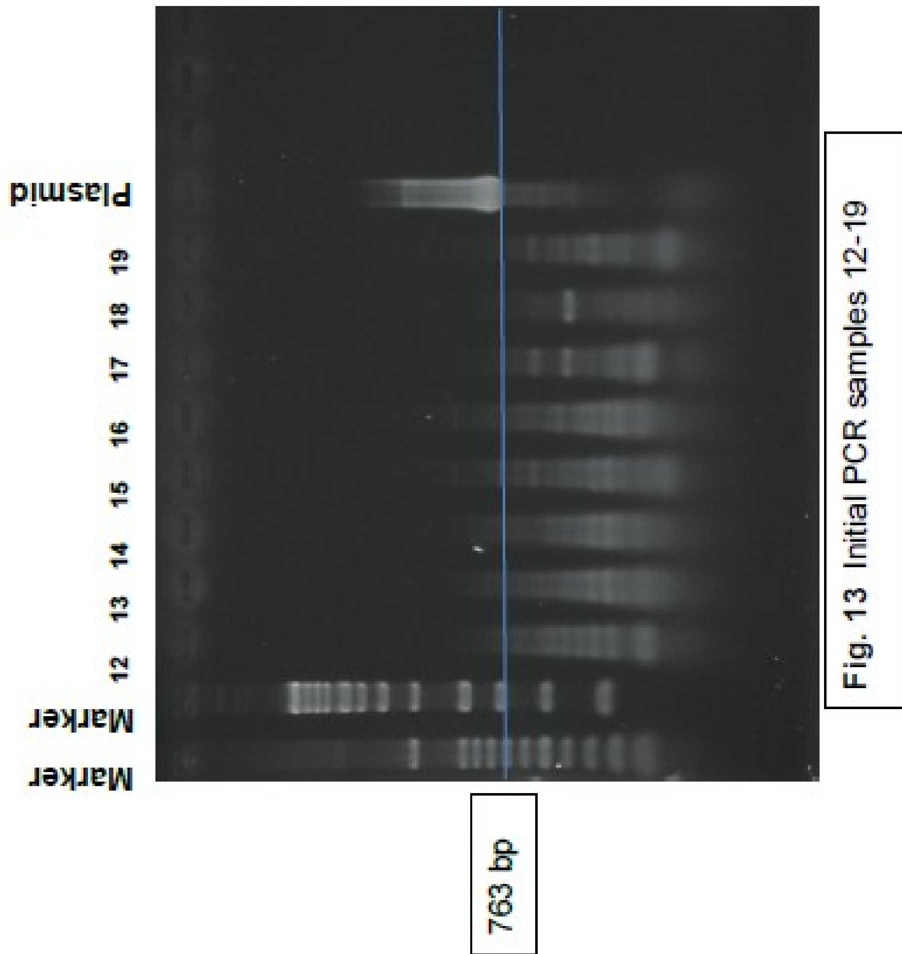


Fig. 13 Initial PCR samples 12-19

The initial PCR products were then diluted 1:100 with distilled water and a hemi-nested PCR analysis was run on 6/21/12. A dilution of the initial PCR products was made in an attempt to reduce non-specific product. The hemi-nested PCR was run with primers CymCoatF/CymCoatR.

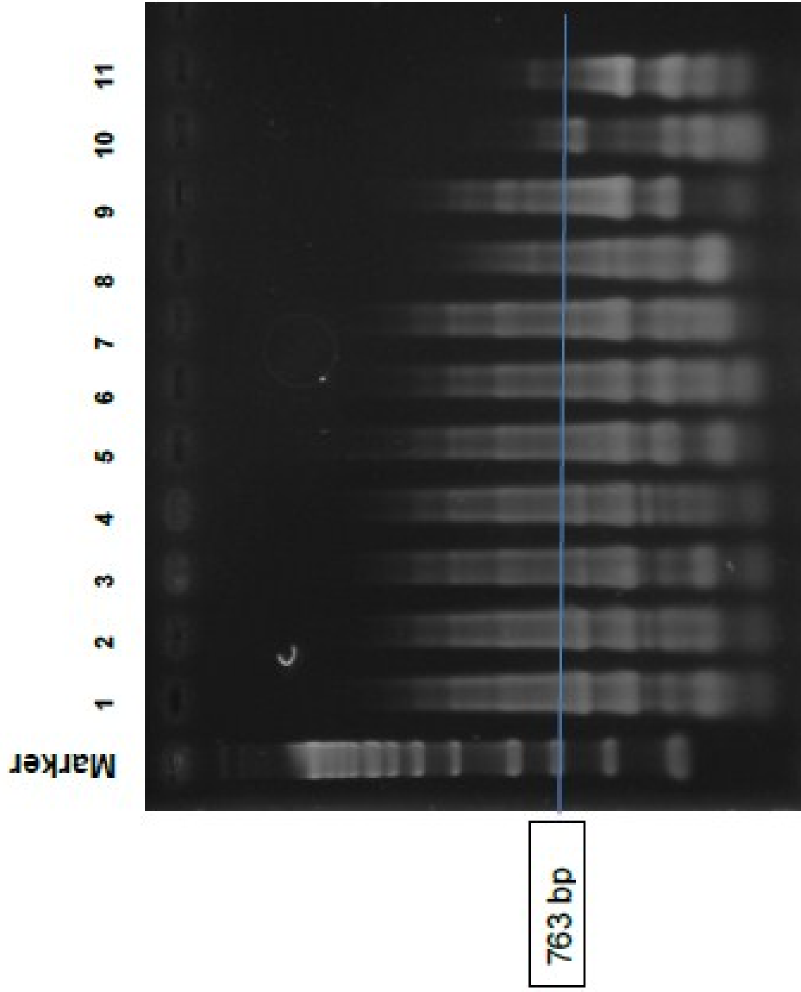


Fig. 14 Hemi-nested PCR samples 1-11

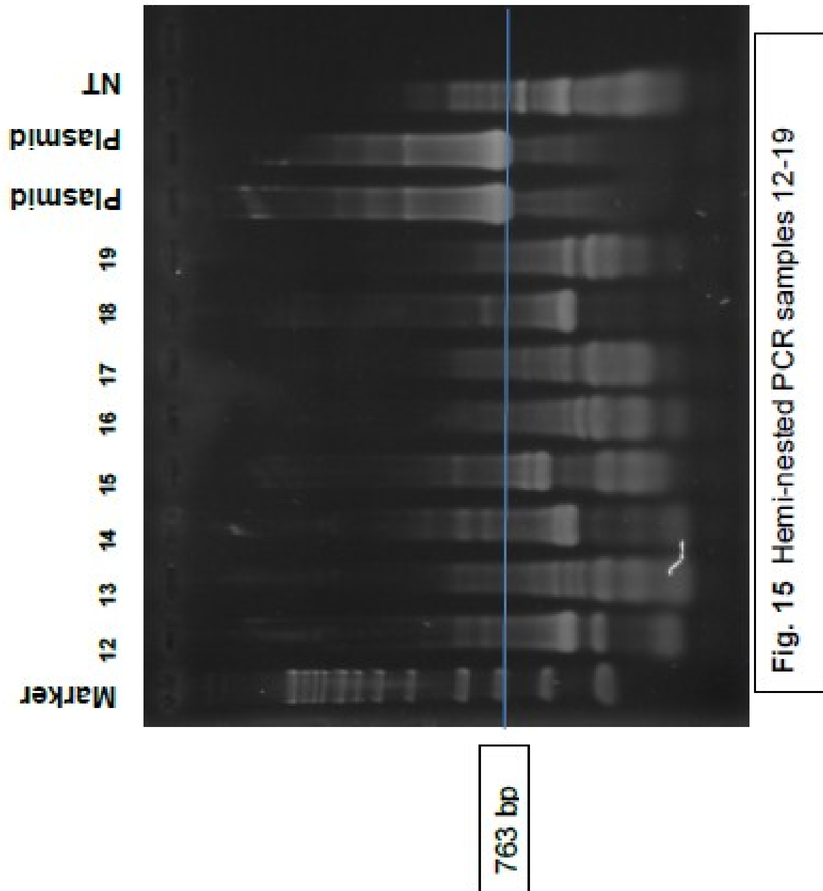


Fig. 15 Hemi-nested PCR samples 12-19

Although there were some non-specific products present and contamination in the 'No Template' lane, all cDNA samples were considered negative for *Cymbidium mosaic virus*. Markers and plasmid CymMV control were clearly visible.

Test Group D

Samples 20 – 42 (Test Group D) were run using the above described initial then hemi-nested protocol. These samples included chewing damage sites harvested 65 and 67 dpi. Also in this group were non-cockroach-exposed negative controls (negative control A & B). In addition, there were three samples that had been mechanically inoculated (Onc A, B, & C) and harvested 96 dpi. Four samples were extractions from previous testing with ImmunoStrips and two samples were from negative control cages.

Included in the initial polymerase reaction and hemi- nest procedures were the following samples:

Sample number	Plant number	dpi (Days Post Inoculation)	
20	1-4	65	
21	5-1	65	
22	8-2	65	
23	16-1	65	
24	18-4	65	Negative control
25	23-4	67	Negative control
26	25-2	65	

27	25-3	65
28	25-5	67
29	26-4	65
30	28-5	67
31	29-4	65
32	30-4	67
33	Onc A	96
34	Onc B	96
35	Onc C	96
36	Negative Control A	
37	Negative Control B	
38	3-1	from ImmunoStrip
39	14-5	from ImmunoStrip
40	19-3	from ImmunoStrip
41	20-4	from ImmunoStrip
42	27-1	from ImmunoStrip

RNA extraction was performed on 5/22/12 with reverse transcription run on 6/14/12. The initial PCR was run on 7/3/12 with the primers CymCoatF/BNSNC-R as described. Known infected plant number 871 was used as a positive control and 'no template' was used as the negative control.

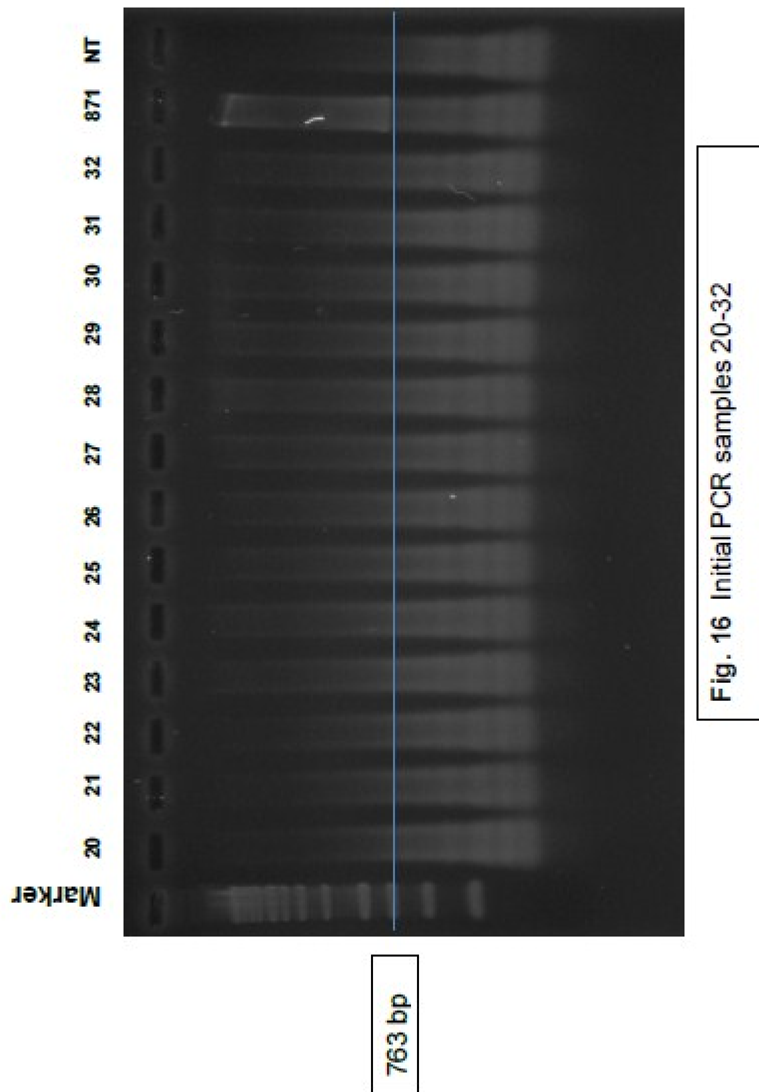


Fig. 16 Initial PCR samples 20-32

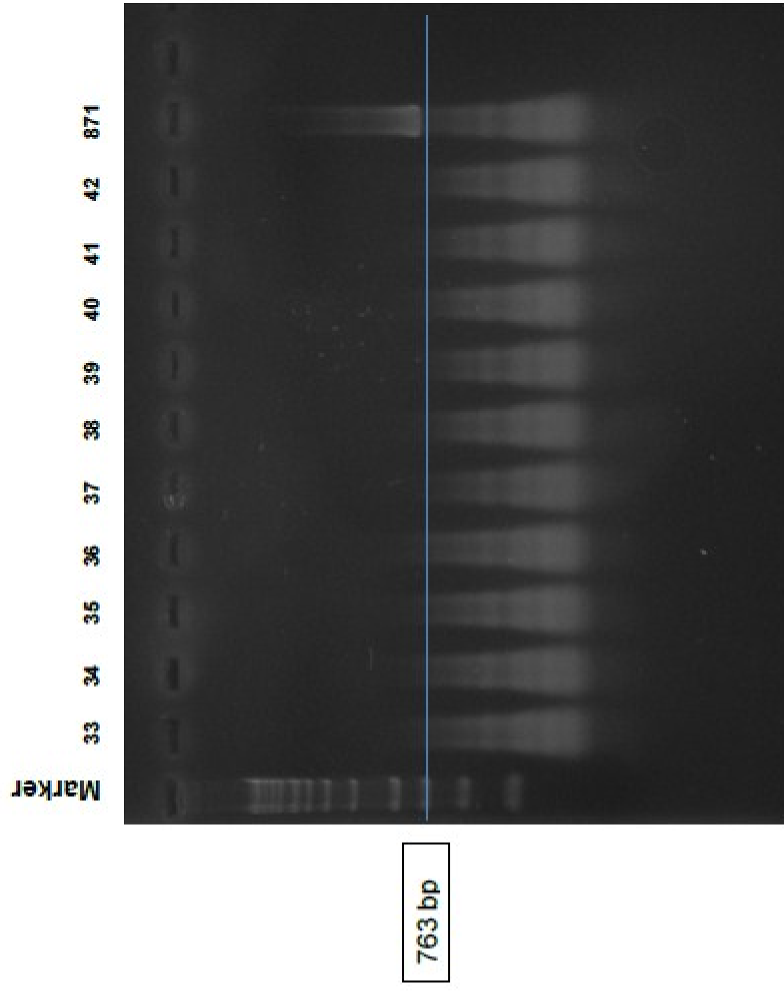


Fig. 17 Initial PCR samples 33-42

To better amplify the anticipated product, a hemi-nested PCR analysis was run using primers CymCoatF/CymCoatR.

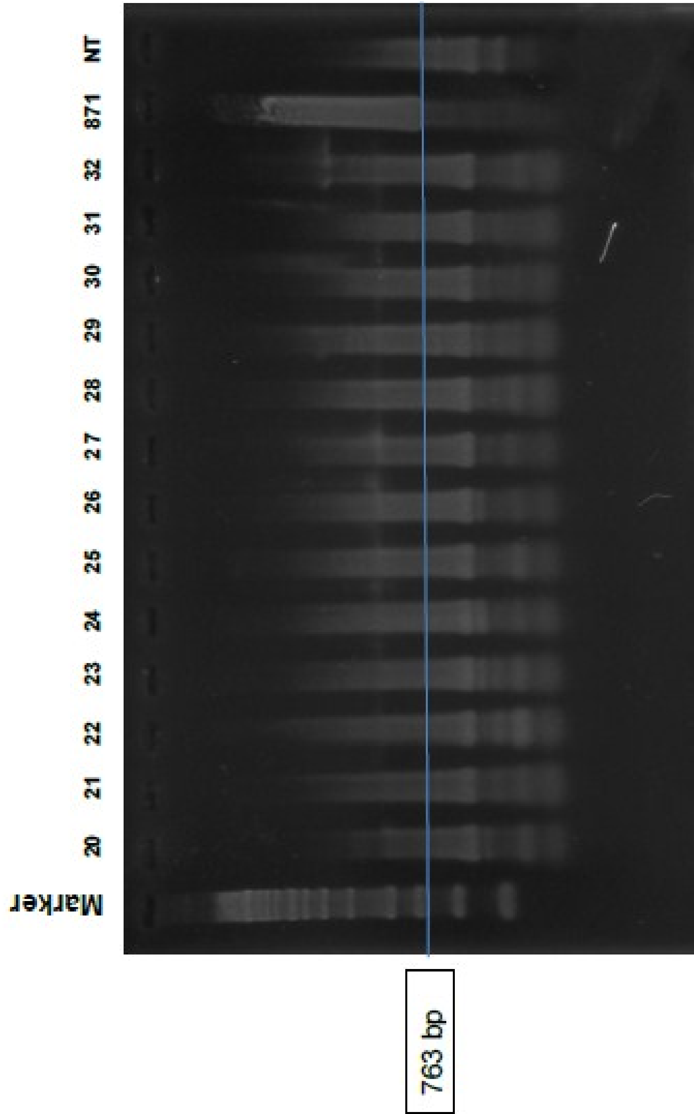


Fig. 18 Hemi-nested PCR samples 20-32

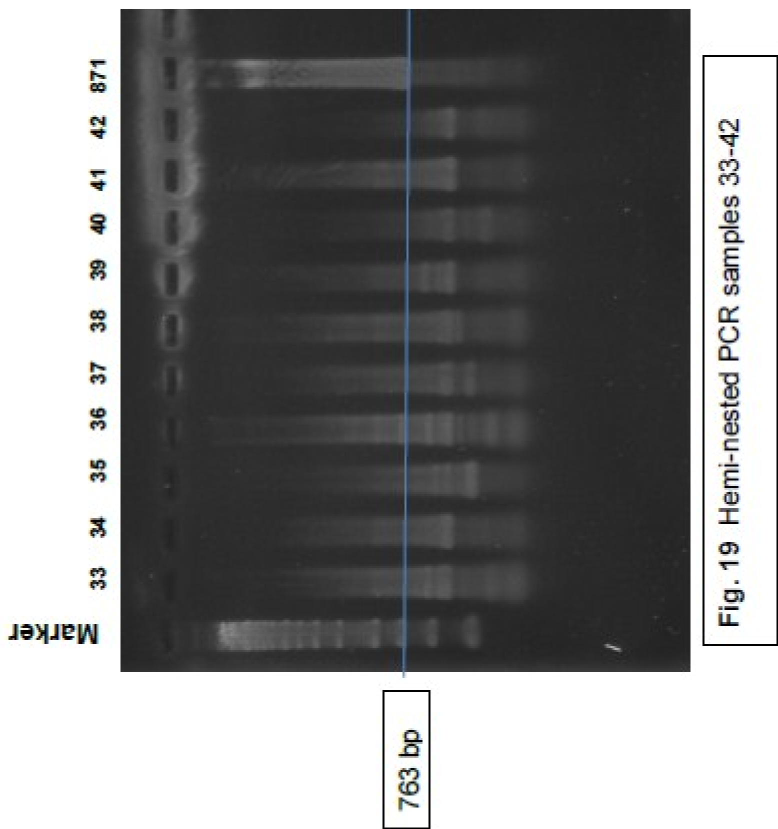


Fig. 19 Hemi-nested PCR samples 33-42

Although there were some non-specific products present and some contamination in the ‘No Template’ lane, all cDNA samples were considered negative for *Cymbidium mosaic virus*. Markers and positive control are clearly visible.

Test Group E

Samples 43 – 58 (Test Group E) were run using the above described initial PCR analysis followed by a hemi-nested PCR. These samples were harvested 89 dpi. Three of the samples were from negative control cages. Included in the initial polymerase reaction and hemi- nest procedures were the following samples:

Sample number	Plant number	dpi (Days Post Inoculation)	
43	4-2	89	
44	5-5	89	
45	7-3	89	
46	9-4	89	Negative control
47	9-5	89	Negative control
48	10-3	89	
49	11-1	89	
50	12-4	89	
51	15-5	89	
52	21-3	89	
53	21-1	89	
54	24-3	89	

55	25-4	89	
56	26-2	89	
57	27-5	89	Negative control
58	28-3	89	

RNA extraction was made on 6/26/12 with conversion to cDNA made on 6/28/12. Initial PCR analysis was performed on 7/10/12 and the hemi nested procedure performed on 7/12/12. The primers used in the initial PCR were CymCoatF/BNSNC and the primers used for the hemi-nested procedure were CymCoatF/CymCoatR. Known infected plant number 871 was used as a positive control and 'no template' was used as the negative control.

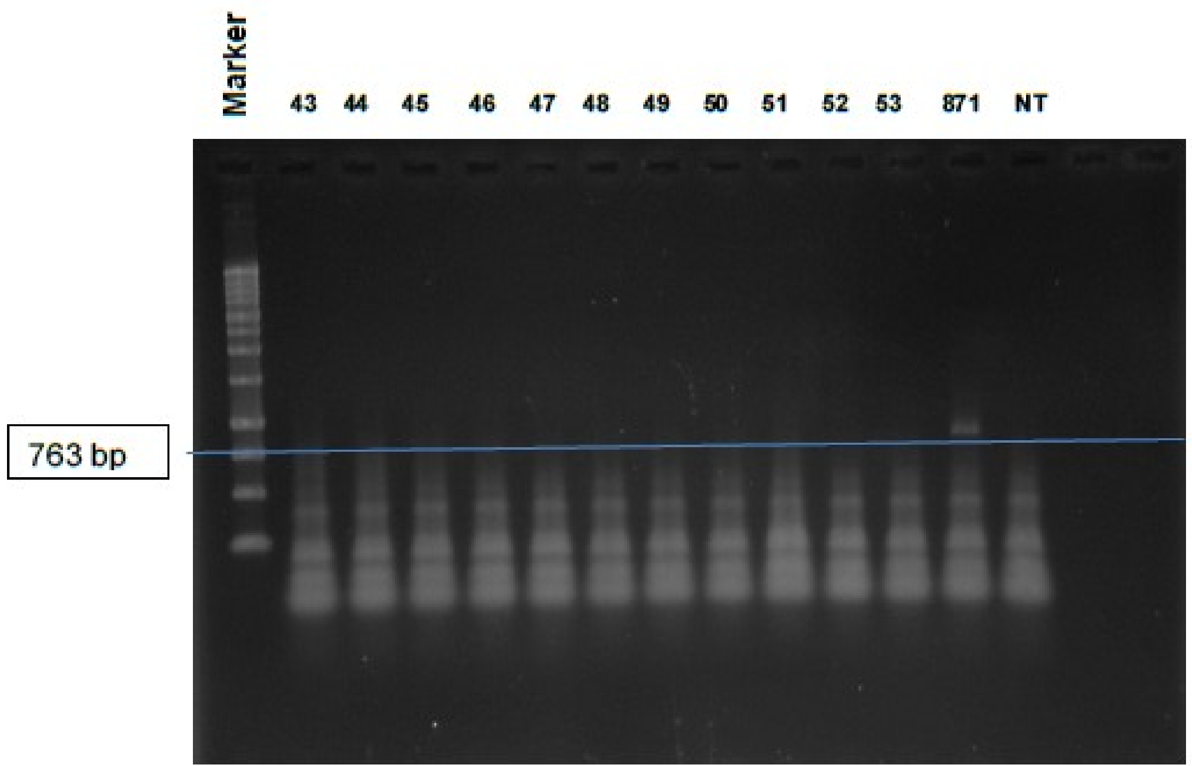


Fig. 20. Initial PCR samples 43-53

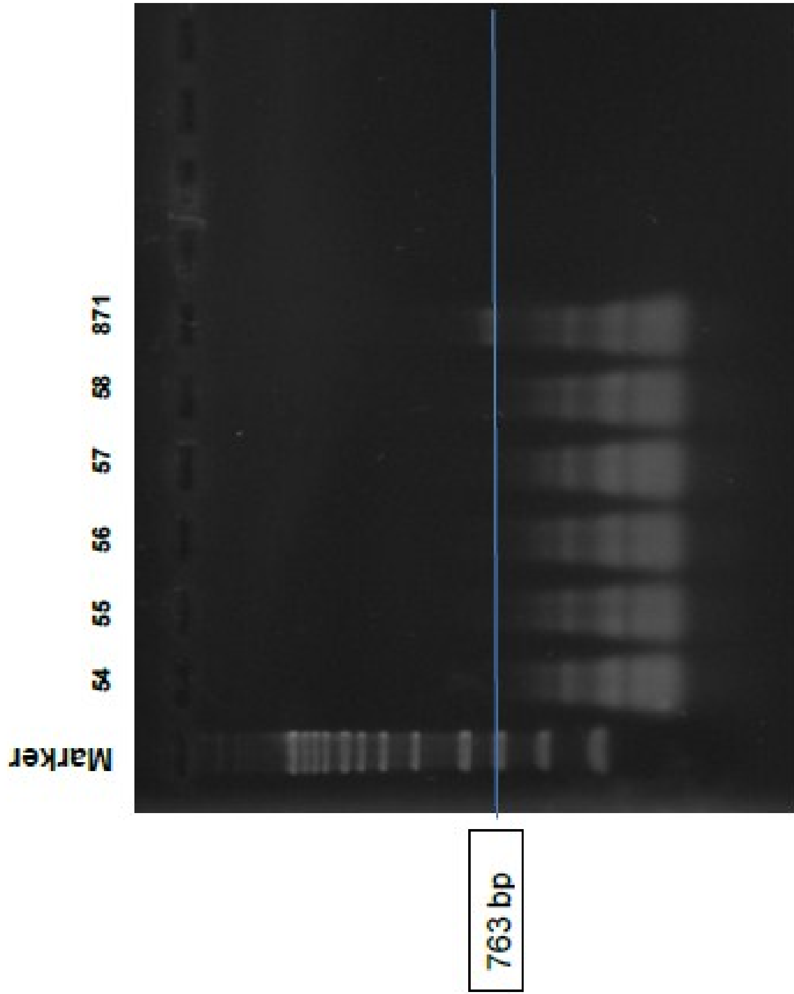


Fig. 21. Initial PCR samples 54-58

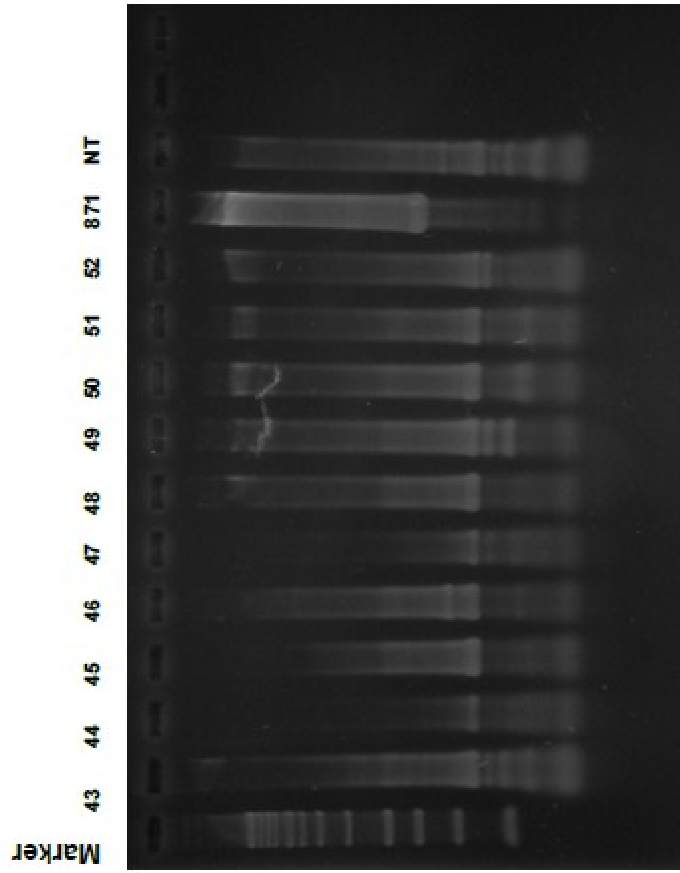


Fig. 22. Hemi-nested PCR samples 43-52

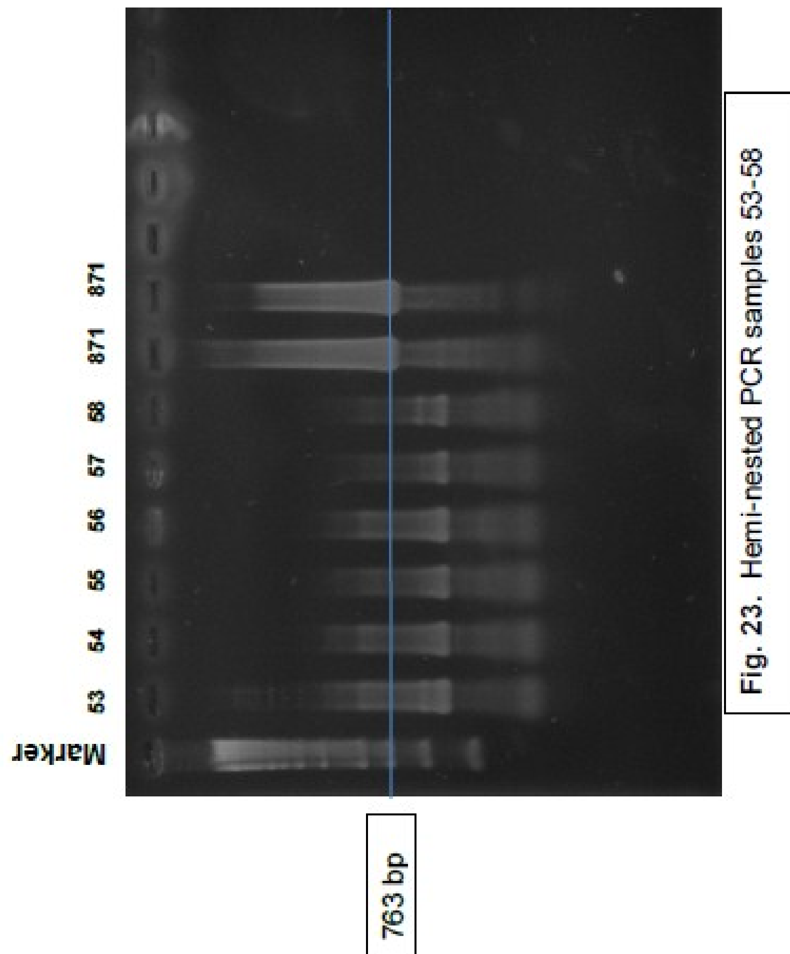


Fig. 23. Hemi-nested PCR samples 53-58

All samples were negative for CymMV-specific product after gel electrophoresis, though there was nonspecific product present and some contamination in the ‘No Template’ lane. Ladder and positive control are visible.

Test Group F

Samples 59-73 (Test Group F) were run using the above described protocol. These samples were 90 dpi. Four samples originated from negative control cages. The leaves from the manual inoculation were tested again. Included in the initial polymerase reaction and hemi- nest procedures were the following samples:

Sample number	Plant number	dpi (Days Post Inoculation)	
59	4-3	90	
60	5-4	90	
61	9-1	90	Negative Control
62	9-2	90	Negative Control
63	10-1	90	
64	10-2	90	
65	13-4	90	
66	13-5	90	
67	14-1	90	Negative Control
68	20-4	90	
69	26-1	90	
70	27-2	90	Negative Control

71	Onc A	145
72	Onc B	145
73	Onc C	145

RNA extraction was performed on 7/11/12 with reverse transcriptase to cDNA on 7/12/12. The initial PCR using primers CymCoatF/BNSNC was performed on 7/23/12. The hemi-nest PCR with primers CymCoatF/CymCoatR was run on the same day. The positive control was cDNA from a known infected plant, number 871 and 'no template' was used as a negative control.

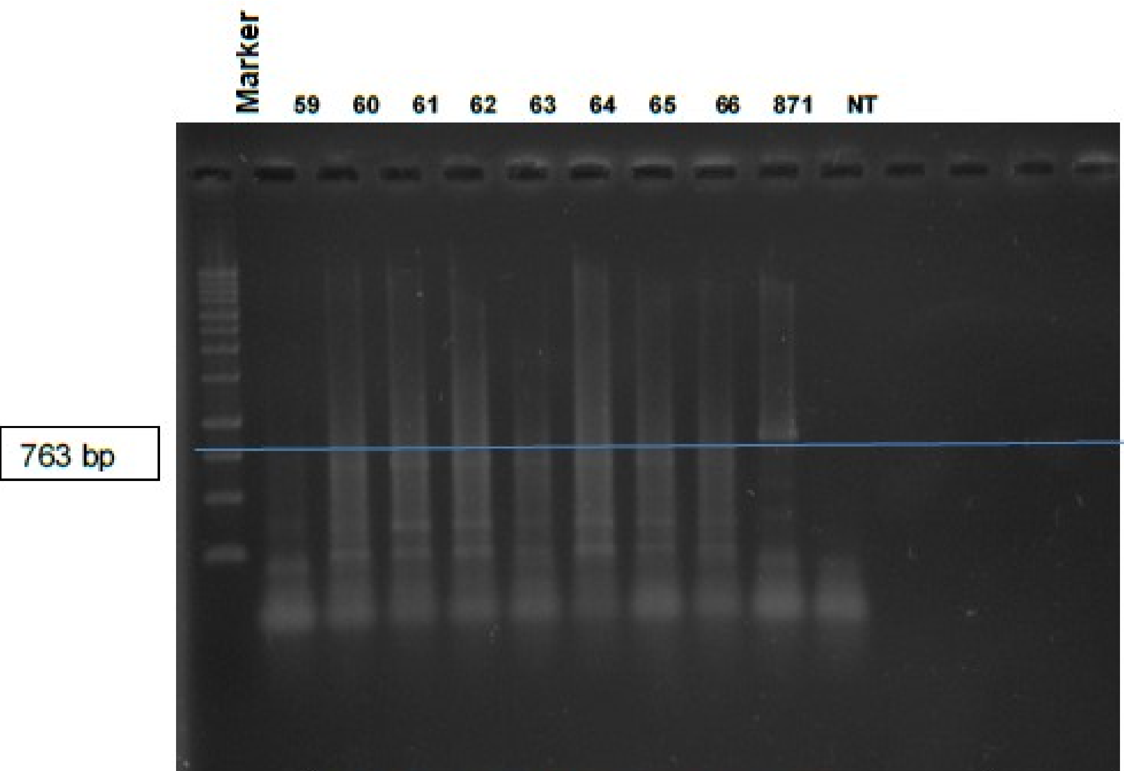


Fig. 24. Initial PCR samples 59-66

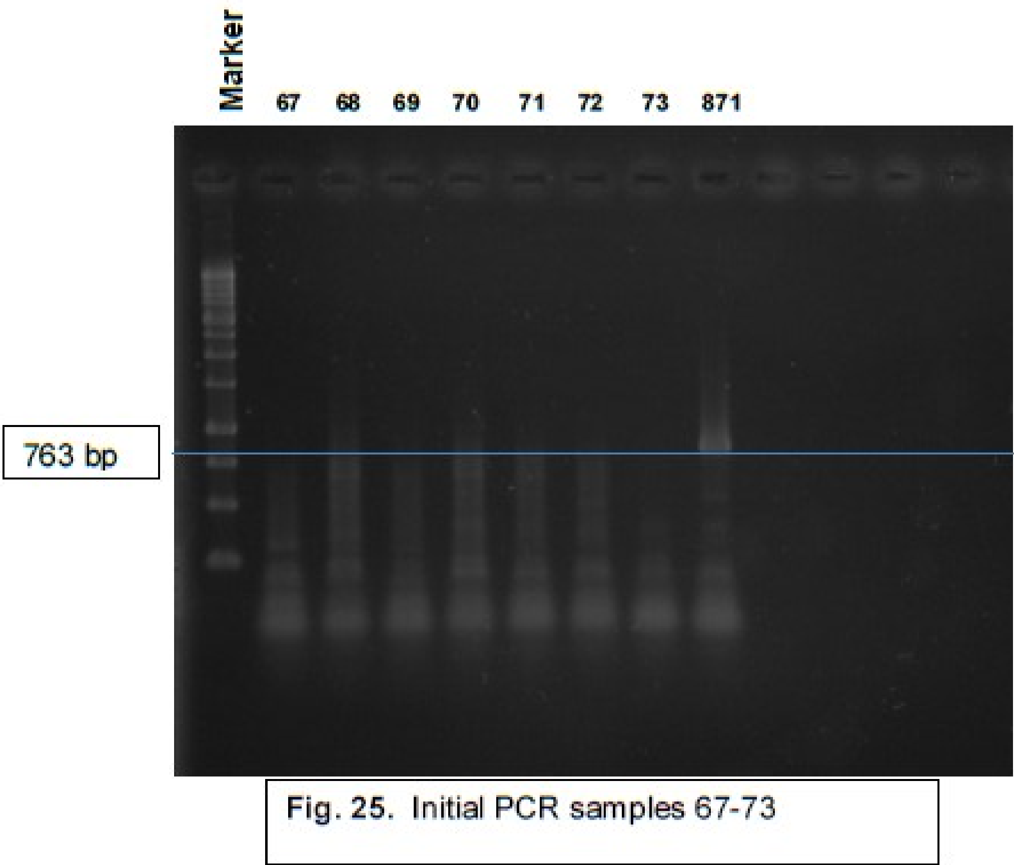


Fig. 25. Initial PCR samples 67-73

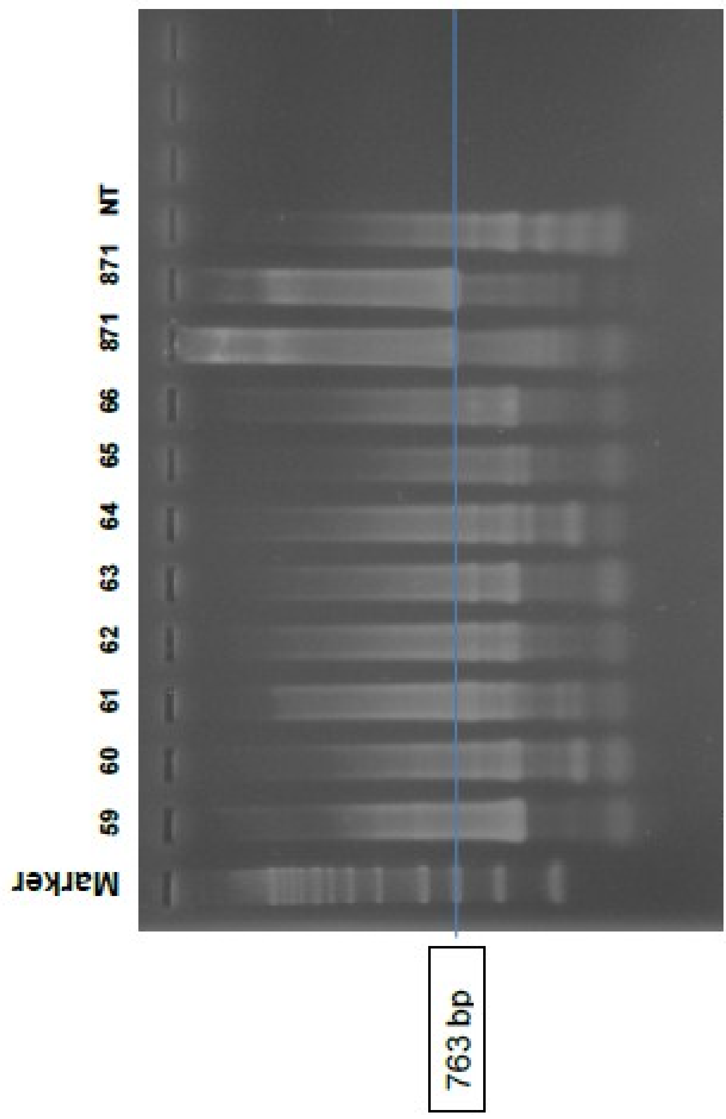


Fig. 26. Hemi-nest PCR samples 59-66

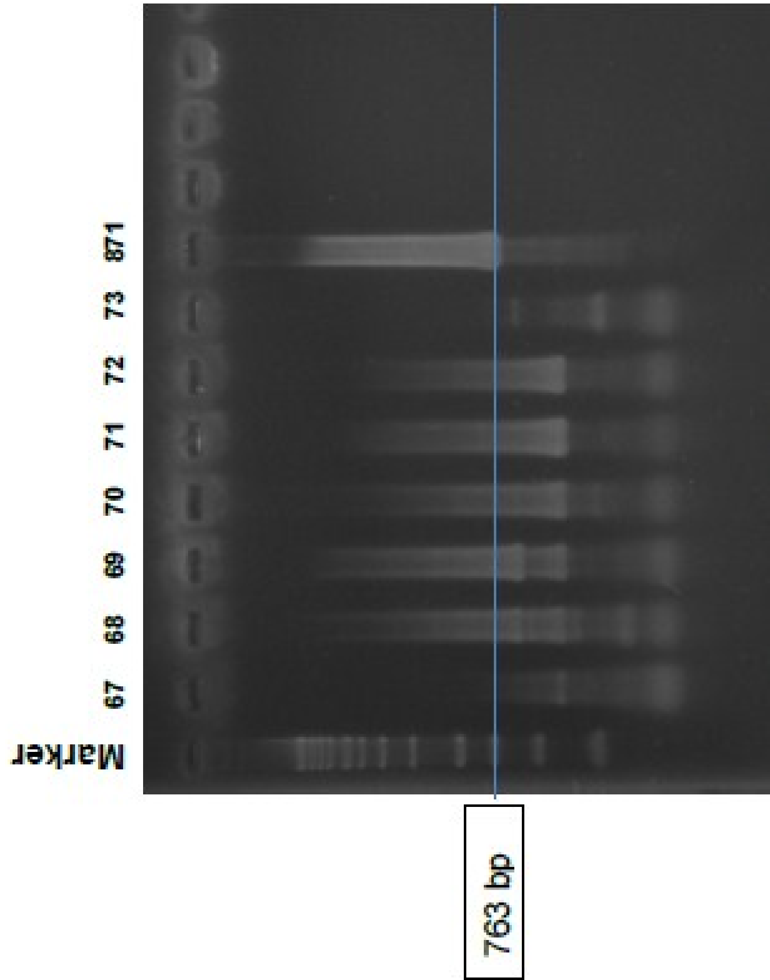


Fig. 27. Hemi-nest PCR samples 67-73

All samples were negative for CymMV-specific product after gel electrophoresis, though there was nonspecific product present and some contamination in the 'No Template' lane. Ladder and positive control are visible

Test Group G

Samples 74-93 (Test Group G) were run using the above described hemi-nested protocol. These samples were 56 dpi. There were two samples from negative control cages. Included in the initial polymerase reaction and hemi- nest procedures were the following samples:

Sample number	Plant number	dpi (Days Post Inoculation)
74	1-1	56
75	2-4	56
76	4-1	56
77	4-5	56
78	7-1	56
79	7-5	56
80	8-3	56
81	11-2	56
82	13-1	56

83	14-5	56	Negative Control
84	15-1	56	
85	15-4	56	
86	21-2	56	
87	22-5	56	
88	24-2	56	
89	24-4	56	
90	27-1	56	Negative Control
91	28-1	56	
92	28-2	56	
93	30-5	56	

The RNA extraction was performed on 7/25/12 with conversion to cDNA on 7/26/12. The initial PCR was made on 7/26/12 using primers CymCoatF/BNSNC. A 1:100 dilution of the initial PCR product was made with distilled water. The hemi-nested PC was run on 7/27/12 using primers CymCoatF/CymCoatR.

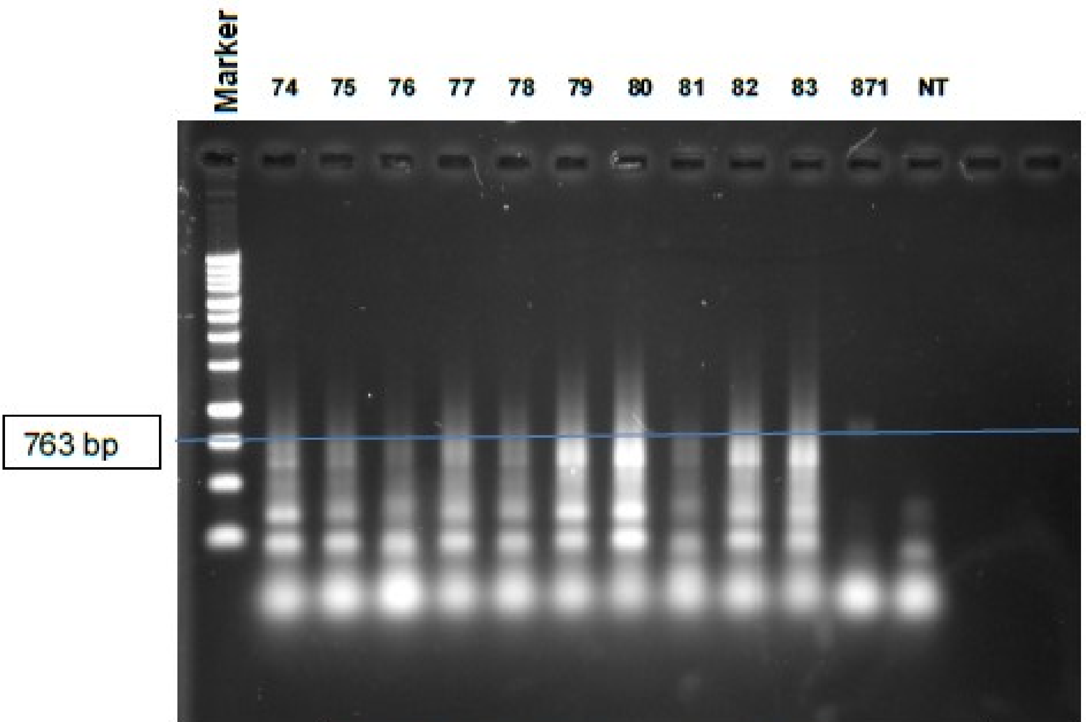


Fig. 28. Initial PCR samples 74-83

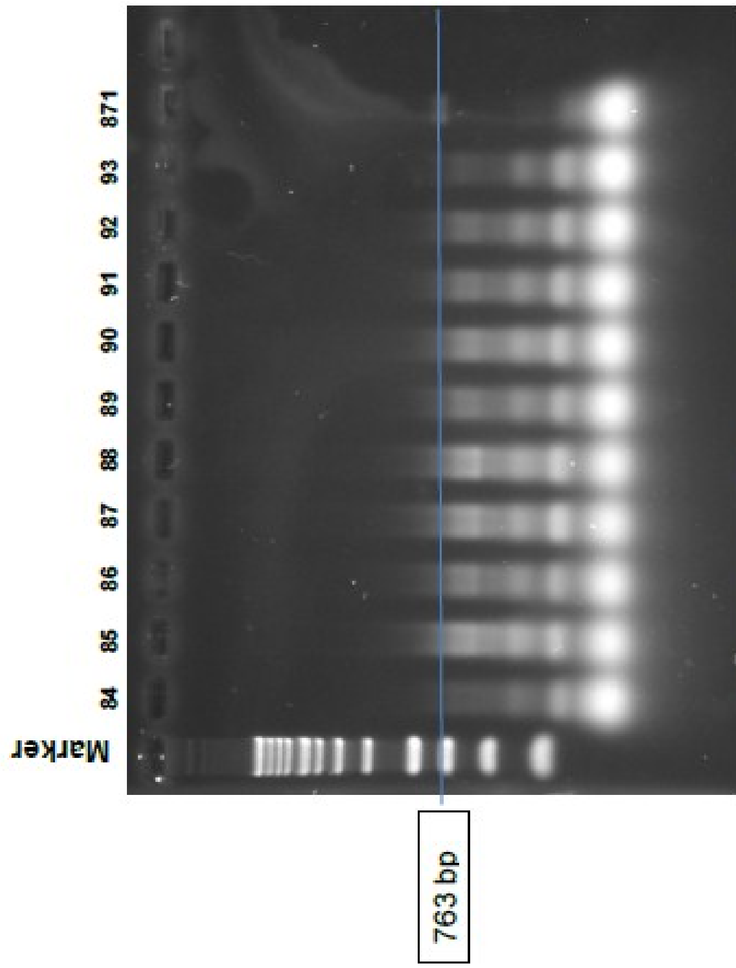


Fig. 29. Initial PCR samples 84-93

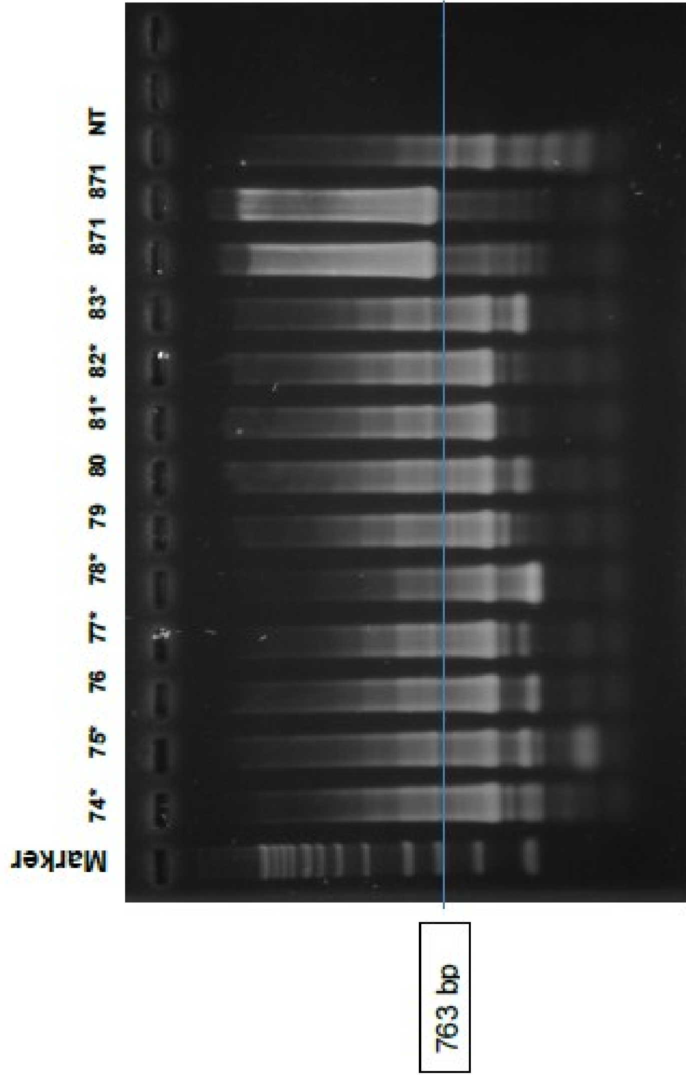


Fig. 30. Hemi-nested PCR samples 74-83.
* indicates positive sample

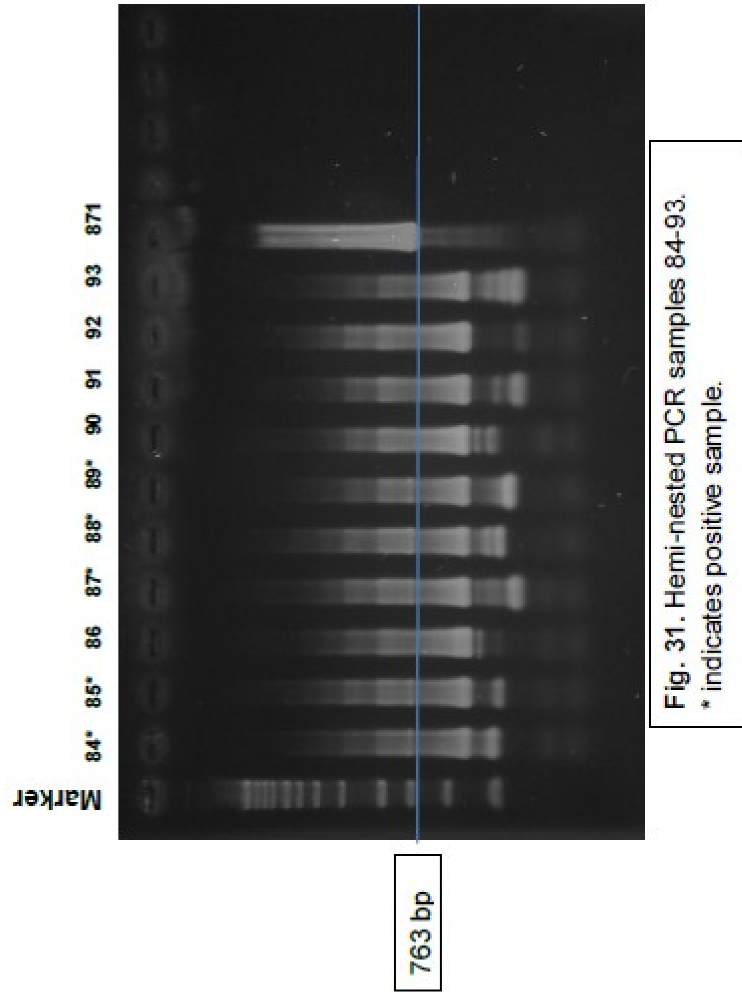


Fig. 31. Hemi-nested PCR samples 84-93.
* indicates positive sample.

The initial PCR was unremarkable showing no bands indicating the presence of *Cymbidium mosaic virus* in any of the samples. However the gels from the hemi-

nested PCR demonstrate bands at approximately the 763 kb position. Lanes 74, 75, 77, 78, 81, 82, 83, 84, 85, 87, 88, and 89 indicate the potential for CymMV product. Unfortunately, the ‘No Template’ lane indicates contamination and that contamination may have effect on the bands at the 763 kb position.

The PCR analysis was re-run on 8/6/12 from the cDNA with a different set of initial primers, CYMTGB2/CymCoatR and then the hemi-nest with CymCoatF/CymCoatR. A fresh dilution of the CymCoatF primer eliminated the persistent contamination issue. Controls were run with cDNA from a plant known to be free of CymMV, number 062 and a known positive, number 871 and were run with the primers CYMTGB2/CymCoatR

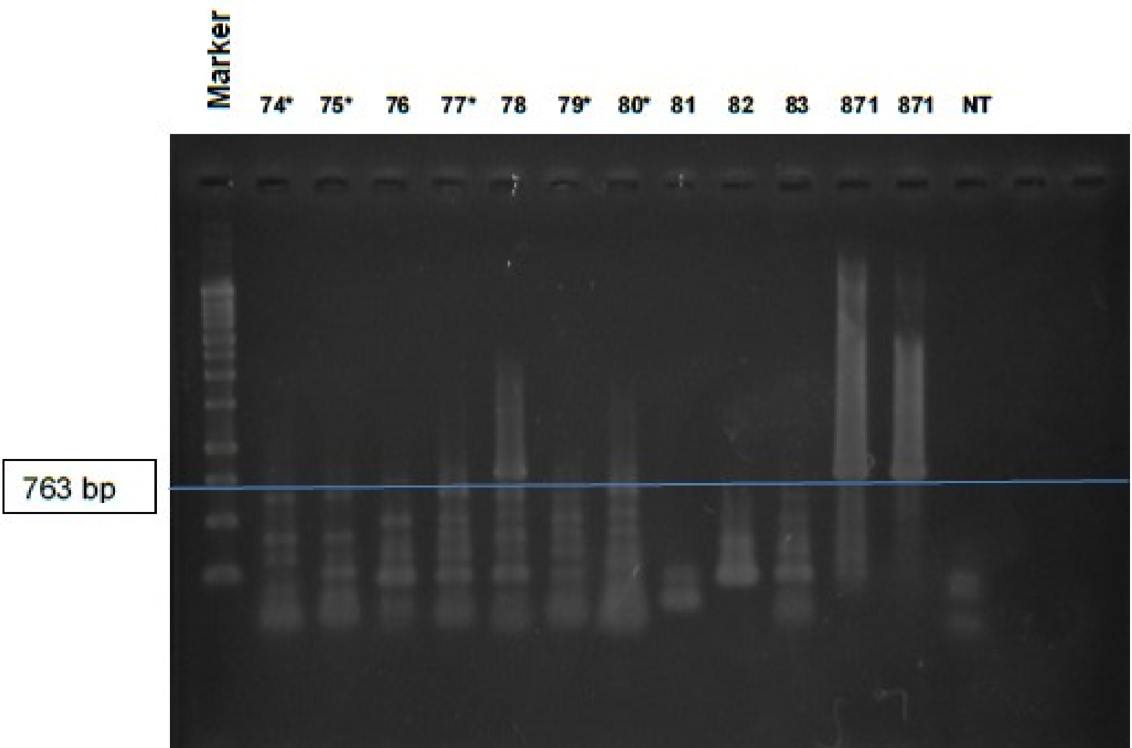
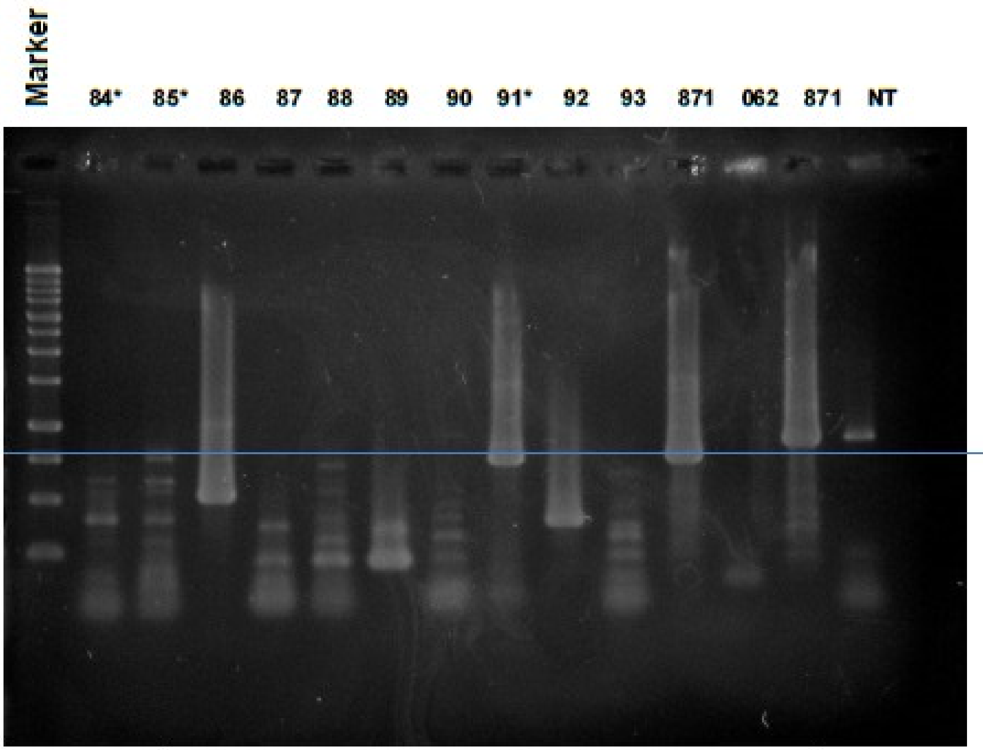


Fig. 32. Hemi-nested PCR samples 74-83 with initial primers of CYMTGB2/CymCoatR.
* indicates positive sample.



763 bp

Fig. 33. Hemi-nested PCR samples 84-93 with initial primers of CYMTGB2/CymCoatR. * indicates positive sample.

This change in primer pairs resulted in a number of possible CymMV bands. Noted were samples number 74, 75, 77, 79, 80, 84, 85, and 91. There was some bleeding of the positive control into the 'No Template' lane when the gel was loaded and some primer-dimers are evident as non-specific product.

Selected Samples

Three new primers were designed and tested on a selection of previously tested cDNA samples listed below:

Sample number	plant number	tested & date
75	2-4	POS 7/27 & 8/6
76	4-1	NEG 7/27 & 8/6
78	7-1	POS 7/27 & NEG 8/6
82	13-1	POS 7/27 & NEG 8/6
83	14-5	POS 7/27 & NEG 8/6
85	15-4	POS 7/27 & 8/6
86	21-2	NEG 7/27 & 8/6
88	24-2	POS 7/27 & NEG 8/6
90	27-1	NEG 7/27 & 8/6
91	28-1	NEG 7/27 & POS 8/6

The 1:100 diluted products originated from the PCR analyzed on 8/2 and used primers CYMTGB2/CymCoatR. The sample #871 was used as a positive control and 'No Template' was used as the negative control.

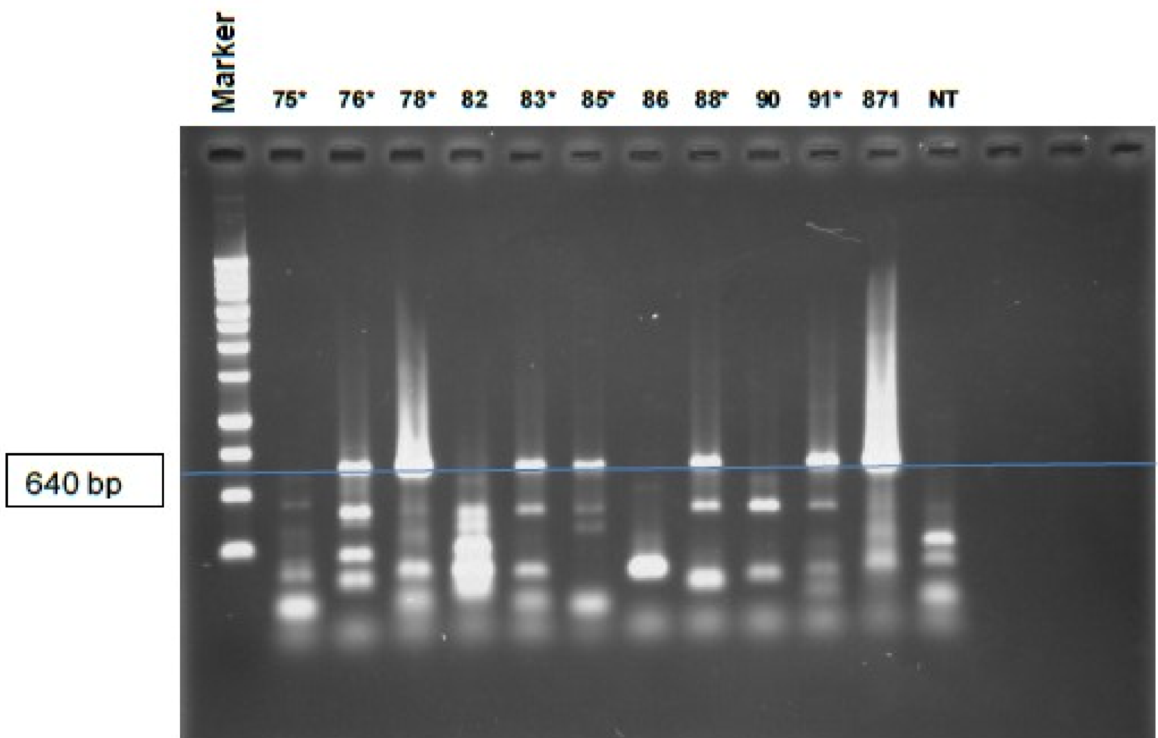


Fig. 34. Nested PCR of selected samples with primers CYMCoatF/CymCP-R2. * indicates positive sample.

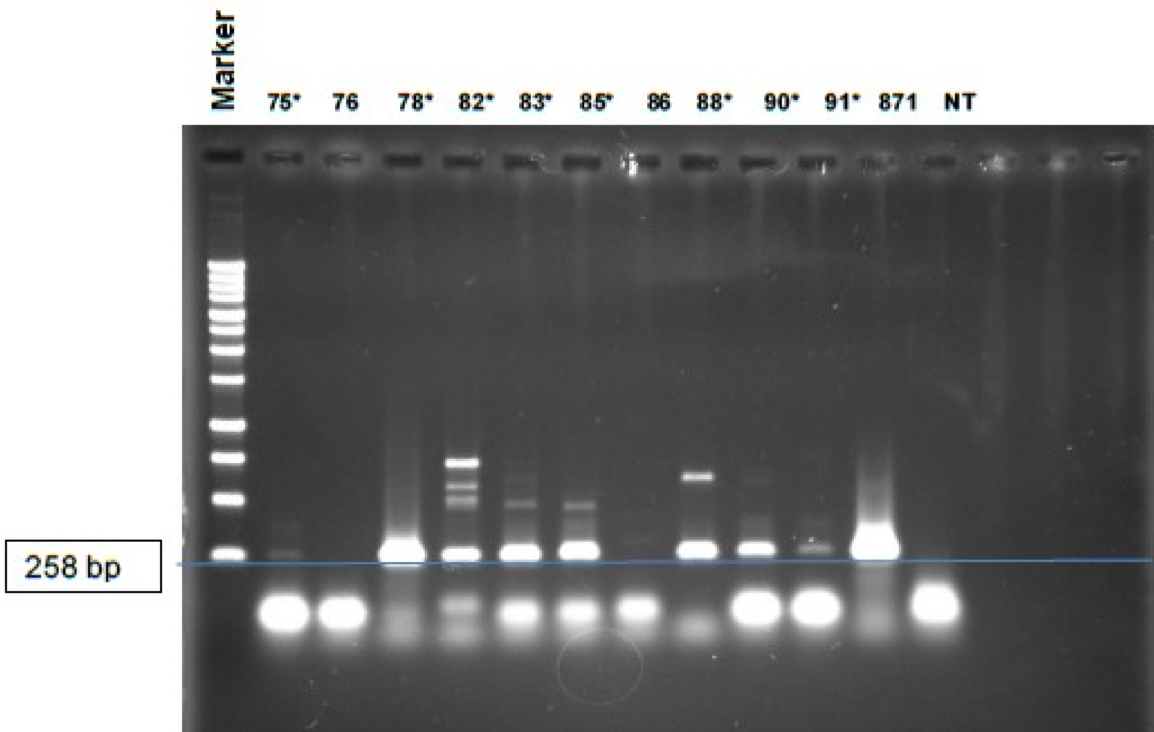


Fig. 35. Nested PCR of selected samples with primers CymF23/CymR25. * indicates positive sample.

On the gel for the reaction using primers CymCoatF/CymCP-R2 bands are seen at the 640 bp region as expected. Samples 75, 76, 78, 83, 85, 88 and 91 appear to be positive for CymMV. The positive control #871 and the maker are clearly visible. The 'No Template' lane shows left over primers. There are considerable non-specific products present in some other lanes.

On the gel using primers CymF23/CymR25 bands are seen at the 258 bp region. The PCR product was anticipated to be visible in this area. Samples 75, 78, 82, 83, 85, 88, 90, and 91 appear to be positive for presence of *Cymbidium mosaic virus*. There is considerable primer material that was not used and there are bands in several lanes above the virus product.

Sample	Plant	Primers A	Primers B	Primers C	Primers D
75	2-4	Pos	Pos	Pos	Pos
76	4-1	Neg	Neg	Pos	Neg
78	7-1	Pos	Neg	Pos	Pos
82	13-1	Pos	Neg	Neg	Pos
83	14-5	Pos	Neg	Pos	Pos
85	15-4	Pos	Pos	Pos	Pos
86	21-2	Neg	Neg	Neg	Neg
88	24-2	Pos	Neg	Pos	Pos
90	27-1	Neg	Neg	Neg	Pos
91	28-1	Neg	Pos	Pos	Pos

A - CymCoatF/BNSNC, CymCoatF/CymCoatR

B - CYMTGB2/CymCoatR, CymCoatF/CymCoatR

C - CYMTGB2/CymCoatR,CymCoaF/CymCP-R2

D - CYMTGB2/CymCoatR, CymF23/CymR25

Fig. 36. Comparison of selected samples and four different primer sets

Chapter 4: Conclusion

Research frequently does not go as expected and this study is certainly an example of that. What started as a relatively simple plant science experiment has morphed into a study of molecular biology techniques. It has moved so far from its original intent, that I am hard pressed to state that the hypothesis is strongly supported. Can Australian cockroach transfer *Cymbidium mosaic virus*? Yes, it seems that they can, but at such a low level that it is hard to prove unequivocally.

Many factors conspire to negate that proof. First is the previously established slow rate of movement of CymMV in orchids (Appendix A, conclusion). Any study that seeks to clearly detect CymMV infection in orchid test subjects will have to be of several years duration just to allow the virus to move into new growth tissue.

Second is the seemingly very small amount of virus particles that the cockroaches move as they feed from infected plant to uninfected plant. Both of these factors conspire to make detection difficult. Hence the transformation of this plant science study into one of molecular biology.

There are some other aspects of the possible transfer of virus particles from infect plant to uninfected plants by cockroach feeding that can be addressed as well.

First, we know that the phenomenon of Australian Cockroach feeding damage on orchids and other plants in conservatories and greenhouses has been well documented (Bell et al.1999). Virus transfer could be hindered by the cockroaches having been taken out of their natural or adapted habitat and placed in confined and rather sterile cages. However, as demonstrated in this study, with only a little manipulation they readily took to the foods that were offered and adapted well enough to breed freely. The test plant chewing damage observed under these controlled circumstances appeared to be equivalent to the damage that has been seen in conservatories.

The low number of positive transmissions may also be a result of the type of feeding damage. Successful virus transmission depends on wounded, but living cells that will allow the movement and replication of the virus particles. The cockroach damage in many instances appears as a shredding of the leaf area, resulting in a wide band of dead plant cells. This band of dead cells could be limiting virus transmission.

Cockroach feeding behavior may have reduced virus transmission as well. It is suspected that they engage in a fast and gorge type of behavior. If a cockroach satisfies its hunger on an infected plant and then goes for longer than seven days before feeding again, there could be a reduction in virus particle viability by the time it eats another plant and potentially transmits the virus. As stated in ICTVBdB Index of Viruses (http://ictvdb.bio-mirror.cn/Ictv/index.htm), CymMV

virions are capable of infection only within a period of seven days at room temperature. That stated period of time was supported by the observations made during this study. The number of positive transmissions of CymMV occurred within 6 – 10 days of exposure. Overall efficiency of transmission would be affected by the short period of virion viability.

Other aspects of cockroach behavior could affect efficiency of virus transmission as well. Mutual grooming behavior has been observed in cockroaches. This could work to either spread the virus particles from one cockroach to another or to effectively clean plant material residues and virus particles from the cockroaches' mandibles. Follow-the-leader type of behavior when confronted with a new food source would also affect virus spread through feeding. Possible interaction between the virus particles and the insect saliva should also be considered. Further study of cockroach behavior would shed light on the efficiency of transmission from feeding damage.

The orchid plant has its own defenses to prevent virus transmission. The orchid collection that was maintained for this study was well supported and well grown. Though the plants were donated and were not in good health when received, they quickly put on vigorous new growth and bloomed frequently. In most instances it was impossible to determine by sight that a plant was virus infected. A healthy orchid leaf has a very thick, protective cuticle and that cuticle could also decrease

the efficiency of virus transmission. Orchids plants that were manually inoculated, even though they were pre-treated with a detergent, failed to be infected. The cuticle barrier would also deter transmission through casual wounding by insects.

The resistance pathway in orchids to CymMV has not been established. Neither has innate resistance been cataloged in either species, intraspecies or intergeneric hybrids. As *Cymbidium mosaic virus* is not found in wild populations of orchids, natural resistance needs to be considered. Orchid growers have noted that a well grown orchid plant may be positive for virus infection and yet not express symptoms. Is that lack of symptom expression part of the natural resistance of the orchid to virus infection? Research on the resistance pathways would potentially be able to answer that question.

One factor that favors cockroach virus transmission is the high number of the insects present in a typical conservatory and their frequently unlimited access to susceptible plant material. That scenario can be compared to aphid transmission of *Lettuce mosaic virus* (Broadbent et al., 1951) in field grown lettuce crops. This was a case of a very small percent of seed born virus resulting in wide spread crop loss when the crop was fed on by aphids. Researchers found that the most effective method of virus control was reduction of virus infected seeds. The incidence of LMV in lettuce seed was brought down to 0 in 30,000 seeds and only then could a crop be protected. A large population of virus vectors, in the above

case, aphids, when applying constant feeding pressure had resulted in significant transmission.

This study reinforced the concept that the period of time between cockroach exposure to infected material and timely access to uninfected plants was a significant factor. The other aspect that was considered was the time allowed for the virus to replicate in the orchid tissue. This time period did not seem to influence the number of infected plants. This aspect needs to be further studied and a longer period of time allowed between cockroach feeding and sample harvest. Perhaps less sensitive means of detection would then be effective if a greater time period and therefore a higher titer of virus particles present had been allowed.

The success of this study revolved around the sensitivity of detection and a very large effort was made in improving those methods. The standard polymerase chain reaction protocol was further enhanced with the use of a hemi-nest and nested technique. Although at the initiation of this study, three specific primers were available, when greater sensitivity was required, three more primers were designed.

The most sensitive primer pair, CymF23/CymR25 has the optimum characteristics of equivalent length, Tm and GC content. This primer pair yielded the greatest number of positive samples and enhanced very low virus titer. Faint bands on a

gel can be the visual indication of a low virus titer. By comparing the gel products of less sensitive primer pairs to the more sensitive pair it can be seen that the additional amplification by the more sensitive primers resulted in stronger bands of greater width. A good example of that more efficient amplification is the comparison of samples #82 and #90 in the gel from the primer combination of CymCoatF/Cym CP-R2 and the gel from primer pair CymF23/CymR25. The improved efficiency of primer pairs by complimentary chemistry and cycling conditions is demonstrated by the work of Arif, et al. 2012.

Effective limitation of the spread of *Cymbidium mosaic virus* depends on controlling the Australian cockroach. Allowing high numbers of Australian cockroaches has serious impact on conservatory collections or greenhouse crops. Though the trend towards an integrated pest management system with targeted pesticide applications is a laudable effort, pest control personnel need to be also monitoring and controlling what up until now was considered only a nuisance pest.

As public awareness grows concerning the large number of *Cymbidium mosaic virus* infected orchids entering the market place, improved and reliable testing methods need to be developed and used by orchid growers and breeders. *Cymbidium mosaic virus* has been established as a world-wide occurring pathogen that is capable of great economic impact in the orchid industry. Its control will

need a multidisciplinary approach involving consumer awareness, improved asepsis in plant handling, grower compliance, improved methods of testing at the greenhouse level, and the introduction of resistant plants.

Appendix A

Transmission of Virus in Orchids Through the Feeding Damage of Australian Cockroach, *Periplaneta australasiae*

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Keywords: Australian cockroach, *Cymbidium* mosaic virus, CymMV, *Odontoglossum* ring spot virus, ORSV

Abstract

The project goal was to demonstrate the possibility of orchid virus transmission by a chewing insect, the Australian cockroach (*Periplaneta australasiae*) under controlled conditions. The experiments were housed in aluminum frame screen cages to contain the cockroaches. Two plants were placed in each cage: an orchid that tested positive for orchid virus and a young clone of *Oncidium* Sweet Sugar 'Kalender' that tested as virus free. Australian cockroaches were introduced into four of the cages. Two cages were used as controls containing the above plant material, but no cockroaches. Approximately one third of the Australian cockroaches used were "wild" caught in a nearby conservatory and the rest were purchased from a commercial supplier. The cockroaches were communally housed for a period of one week. We assumed that any of the wild-caught cockroaches that carried an orchid virus would distribute the virus particles by mutual grooming. The Australian cockroaches were housed with the plant material until sufficient feeding damage was observed. At that time, the orchid virus testing was repeated on the *Oncidium* Sweet Sugar 'Kalender'. Samples of new growth tissue were initially tested at a commercial laboratory and were subjected to an orchid virus screen that identifies nine viral agents known specifically to orchids. Test results were negative for presence of virus. Four weeks after feeding damage was observed, testing was repeated. Tissue from the feeding sites was tested for presence of CymMV and ORSV with Agdia's immunoStrip kits. Two sites were faintly positive for CymMV. Testing was repeated 18 weeks later with Agdia's immunoStrip kits and the same sites showed strong response for both CymMV and ORSV.

INTRODUCTION

The Australian cockroach (*Periplaneta australasiae*) is a persistent pest in many North American conservatories and botanic gardens. The tender shoots and root tips of orchids (Fig. 1) are some of its preferred foods. The possibility of virus transfer is a common topic for debate among curators and conservatory gardeners. To date, viral transmission from cockroaches to orchids has not been demonstrated under controlled conditions.

MATERIALS AND METHODS

Housing and Environment

Two controlled environment chambers were employed with a temperature of 24 ^{o}C night and 29 ^{o}C day, with relative humidity levels between 65 and 85%. One chamber was used to house the virus-infected plants until exposed to the Australian cockroaches. The second and larger unit was used for the experiment cages and the unexposed plants.

Aluminum frame, screen cages (46 cm × 46 cm × 76 cm) were used to contain the cockroaches with the plants. Each cage contained: a virus-infected orchid and a virus-free *Oncidium* Sweet Sugar 'Kalender'. An average of 10 cockroaches was introduced into four of the cages and two cages were used as controls. Care was taken so that the plants did not touch, and strict asepsis was observed in handling the plant material during the experiment to prevent casual contamination.

Plant Material

The following mature orchid plants were obtained from a local conservatory: *Bifrenaria harrisoniae* 'Ruth' AM/AOS 01-0893, *Calanthe* Baron Schroder 02-0206A, *Laeliocattleya* Irene Finney 98-2954C, *Oncidium* unknown, *Vuylstekeara* Linda Isler 'Red' 04-0356C, *Oncidium* unknown BG 21794, *Brassia* Starex 02-0507, *Calanthe* William Murray 01-0916A, *Miltassia* Charles M. Fitch 'Izumi' AM/AOS, and *Odontocidium* Big Mac 020171A. These plants were suspected of possible virus infection because of their age and provenance. Samples were sent to a commercial laboratory for testing of nine possible orchid virus pathogens: cucumber mosaic virus, *Cymbidium* mosaic virus, *Cymbidium* ringspot virus, *Impatiens* necrotic spot virus, *Odontoglossum* ringspot virus (ORSV), *Potyvirus* group, tobacco mosaic virus, tomato ringspot virus, and tomato spotted wilt virus. All plants were positive for either *Cymbidium* mosaic virus (CymMV), *Odontoglossum* ringspot virus, or both.

Twelve young plants of the clone *Oncidium* Sweet Sugar 'Kalendar' were purchased from a commercial supplier in Hawaii. Samples of each were sent to a commercial laboratory and also subjected to virus screening. All results were negative.

Cockroaches

Australian cockroaches were obtained from two sources. Approximately 18 adult and late instar juveniles were captured from a nearby conservatory, and 40 adult cockroaches were purchased commercially. The cockroaches were allowed one week to socialize in a common cage. We assumed that if any of the "wild caught" insects were contaminated with virus particles; the cockroach's behavior of mutual grooming would distribute the pathogen.

RESULTS

Five weeks after the Australian cockroaches were introduced into the experimental cages, evidence of feeding on roots and flowers was observed. New growth samples of the *Oncidium* Sweet Sugar 'Kalander' were taken one month after feeding damage was observed and sent to a commercial laboratory for virus testing. All samples returned negative. Samples were taken again four weeks later, but this time from the actual feeding sites. Agdia ImmunoStrip test kits were used in-house for CymMV and ORSV. Of seven samples, two tested positive for presence of CymMV (sample No. 2 and 3) (Fig. 2).

The plants were tested again 18 weeks later and one site (sample 2a) showed a strong response to both CymMV and ORSV (Fig. 3). The site that had previously tested positive for CymMV (sample No. 3) had been consumed by the cockroaches.

DISCUSSION

The initial results indicate possible virus pathogen transmission by Australian cockroach through feeding damage. Periodic sampling will track the distribution of virus infection through the test plants of *Oncidium* Sweet Sugar 'Kalander' over time. There are limitations to conducting a one-year test as orchid virus movement through the plant can take a considerable amount of time. CymMV associates with the vascular tissue and can move more rapidly than ORSV, which moves from cell to cell (Borth, et al., 2006). This could account for the initial detection of CymMV at the two-month interval and not ORSV. We postulate that future sampling of new growth will not only test positive for CymMV, but also ORSV and necrotic spotting will be observed.

CONCLUSION

Trace-back to the source of virus infection in a large orchid collection can be a daunting, if not impossible task. Sources of virus transmission can be a poorly disinfected tool, water dripping from an overhead contaminated orchid, or a worker's fingertips. (Wisler 2009) The time from virus inoculation to expression of symptoms can be from seven months, as in the case of *Potyvirus* in *Vanilla* in Tahiti (Wisler, 2009), to 30 months as reported in experiments with *Sophrolaeliocattleya* hybrids in Venezuela (Izaguirre-Mayoral, 1993). This time lag easily allows for multiple source inoculation and inability to pin-point the exact contaminating source. Large, conservatory-sized plants can be 50 years old or more and multiple owners, conservatories, and opportunities for virus infection are possible.

The most common orchid viruses, CymMV and ORSV, are found in a high number of plants in older collections. (Wisler, 2009) The potential for virus transmission by Australian cockroach emphasizes the need for the systematic testing and removal of virus-infected plants if a virus-free collection is to be maintained.

ACKNOWLEDGMENTS

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Literature Cited

Borth, W.B., Barry, K., Obsuwan, K., Xu, M.Q., Liu, R.W., Kuehnle, A.R. and Hu, J.S. 2006. Movement of cymbidium mosaic virus and transgenic resistance in Dendrobium orchids. Acta Hort. 722:137-146. http://www.actahort.org/books/722/722_17.htm

Izaguirre-Mayoral, M.L., de Uzcategui, R.C. and Carballo, O. 1993. Crassulacean acid metabolism in two species of orchids infected by tobacco mosaic virus-orchid strain and/or Cymbidium mosaic virus. J. Phytopathology 137:272-282.

Lamborn, J.S.. 2009. Account Manager, Testing Services and Ornamental Diagnostics. Agdia, Inc. Elkhart, IN. Personal correspondence. August 19, 2009.

Wisler, G.C. 2009. National Program Leader, Horticulture and Sugar. Agriculture Research Service. Beltsville, MD. Personal correspondence. August 12, 2009.

Figures

Fig. 1.

Australian cockroach damage on orchid roots (top) feeding damage on bromeliad leaves (middle) and feeding damage on *Cattleya* flower (bottom).

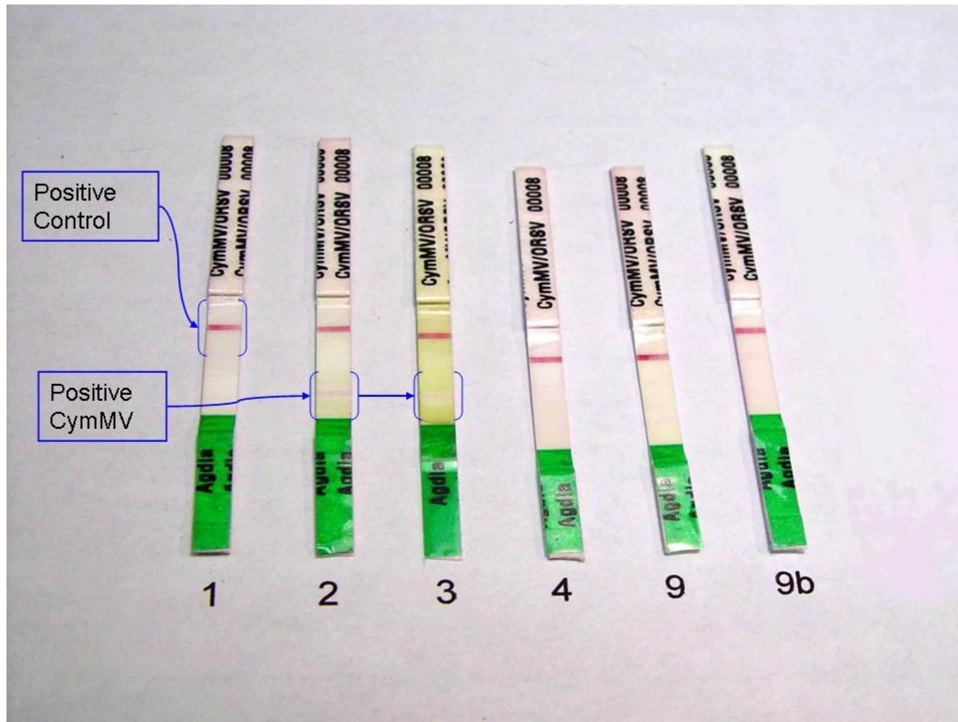


Fig. 2. Agdia ImmunoStrip tests. Upper line indicates a positive control; lower line indicates a positive response to CymMV. Note the faint lower line on test strips 2 and 3.

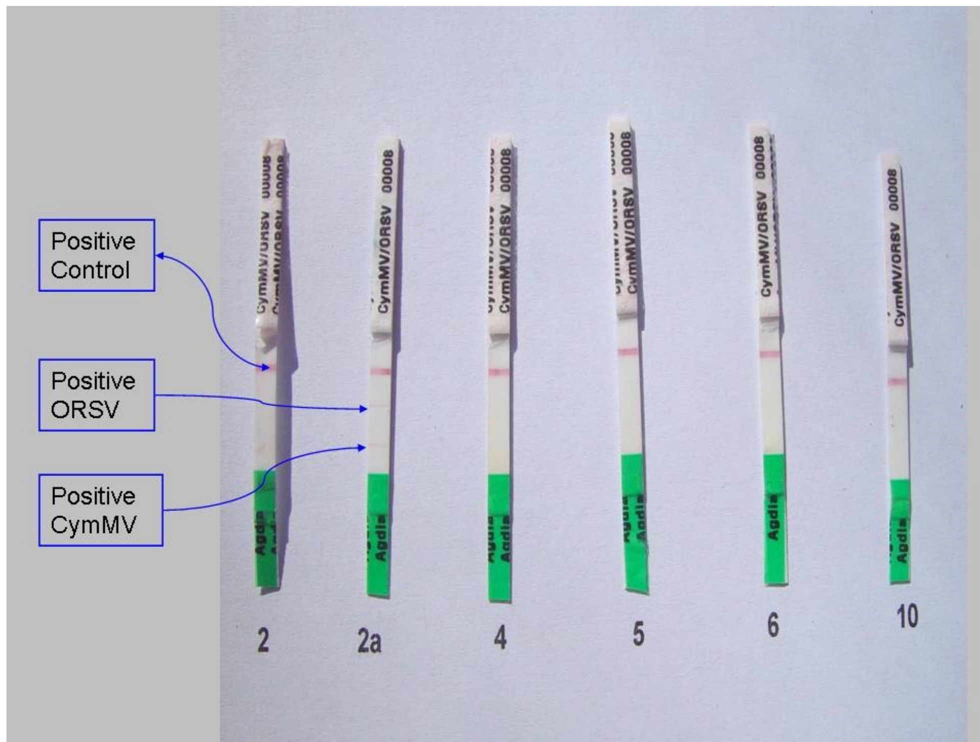
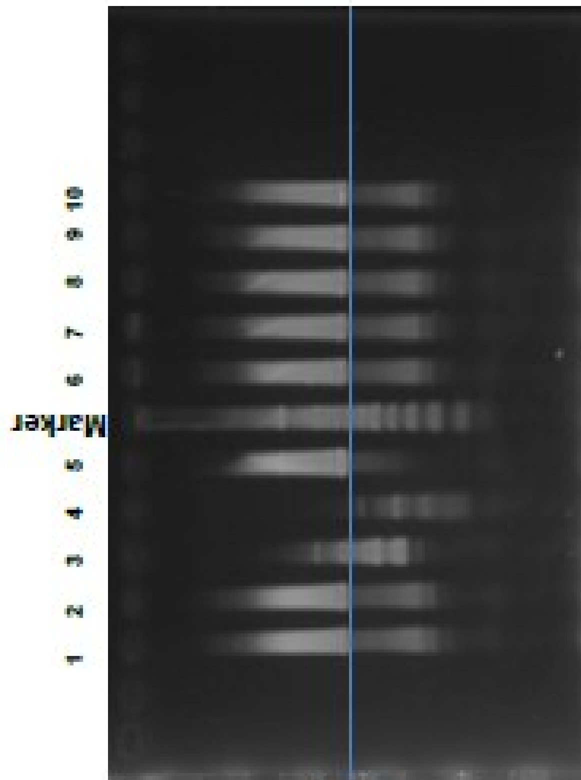


Fig. 3. Agdia ImmunoStrip tests. Test was taken 22 weeks after feeding damage. Upper line indicates a positive control; lower lines indicate a positive response to ORSV and CymMV. Note the faint lower lines on test strip 2a.

Appendix B

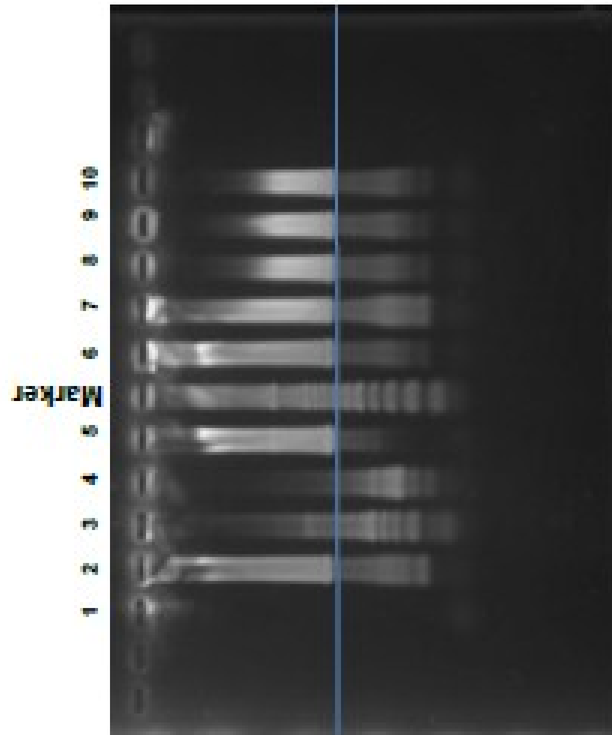
Dilution Gradient: Initial PCR – CymTGB2F/CymCoatR, Hemi-nest – CymCoatF/CymCoatR using 1:100 dilution of initial PCR product.



763 bp

Lane 1- #871, 50° C
Lane 2 - #871, 65° C
Lane 3 - #082, 50° C
Lane 4 - #082, 65° C
Lane 5- Plasmid positive control
Lane 6 – 1:5
Lane 7 – 1:25
Lane 8 – 1:125
Lane 9 – 1:625
Lane 10 – 1:3125

Dilution Gradient: Initial PCR – CymCoatF/BNSNC, Hemi-nest – CymCoatF/CymCoatR using 1:100 dilution of initial PCR product.



763 bp

Lane 1- #871, 50° C
Lane 2 - #871, 65° C
Lane 3 - #062, 50° C
Lane 4 - #062, 65° C
Lane 5- Plasmid positive control
Lane 6 – 1:5
Lane 7 – 1:25
Lane 8 – 1:125
Lane 9 – 1:625
Lane 10 – 1:3125

Appendix C

Annealing Temperature Gradient

A calculated annealing temperature gradient was run on all combinations of four primers, BNSNC, CymTGB2-F, CymCoat-F, CymCoat-R.

Primer TM at 50mM NaCl

BNSNC 25nt	72.09^{o} C	48 % GC
CymTGB2 24nt	65.46^{o} C	45.8% GC
CymCoat-F 20nt	60.40^{o} C	50% GC
CymCoat-R 22nt	58.81^{o} C	40.9% GC

Primer	TM degrees C	Calculated Annealing Temperature
CymTGB2-F	65.46	56° C
CymCoat-R	58.81	
CymTGB2	65.46	63° C
BNSNC	72.09	
CymCoat-F	60.49	58° C
BNSNC	72.09	
CymCoat-F	60.49	56° C
CymCoat-R	58.81	

Figure 1. Calculated annealing temperatures for primer pair combinations

The four primer pairs above were analyzed by PCR at eight annealing temperatures using a known negative sample, #062 and a known positive sample, #871. A plasmid CymMV was used as a positive control and 'No Template' was used as a negative control.

Lane	Primer pair	sample	temperature degrees C
1	CymTGB2/BNSNC	Plasmid	50
2		871	65
3		871	63.9
4		871	62.1
5		871	59.4
6		871	55.9
7		871	53.4
8		871	51.4
9		871	50
10		Marker	
11		062	65
12		062	63.9
13		062	62.1
14		062	59.4
18		062	55.9
19		062	53.4
20		062	51.4
21		062	50
22		Marker	50
23	CymTGB2/CymCoatR	871	65
24		871	63.9
25		871	62.1
26		871	59.4
27		871	55.9
28		871	53.4
29		871	51.4
30		871	50
31		Plasmid	50

Figure 2. Legend for gel #1

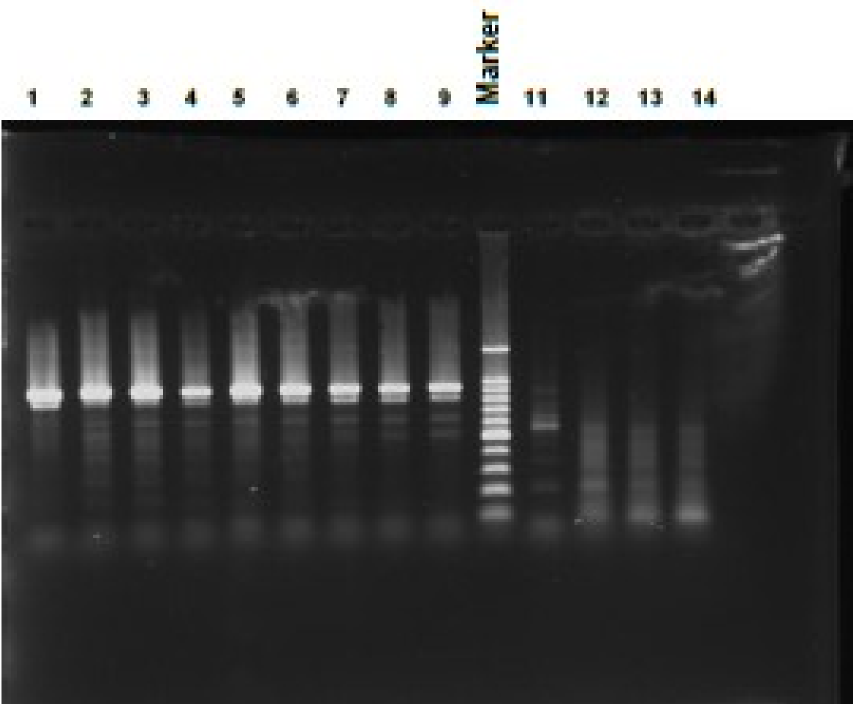


Figure 3. Temperature gradient lanes 1 – 14
Gel #1

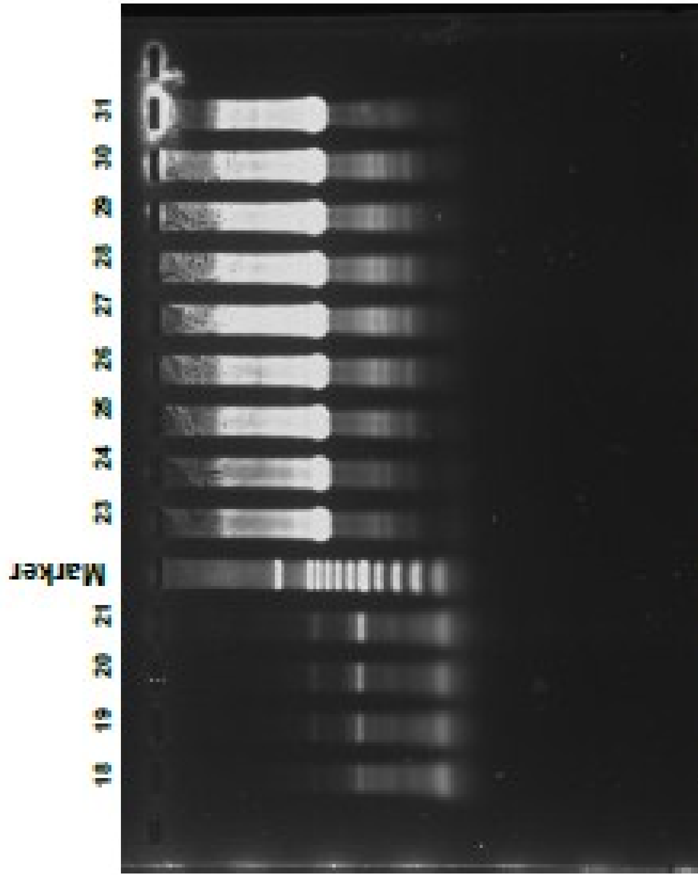


Figure 4. Temperature gradient lanes 18 – 31
Gel #1

Lane	Primer pair	sample	temperature degres C
1	CymTGB2/CymCoatR	062	65
2		062	63.9
3		062	62.1
4		062	59.4
5		062	55.9
6		062	53.4
7		062	51.4
8		062	50
9		Marker	50
10	CymCoatF/BNSNC	871	65
11		871	63.9
12		871	62.1
13		871	59.4
14		Plasmid	50
18		871	55.9
19		871	53.4
20		871	51.4
21		871	50
22		Marker	50
23		062	65
24		062	63.9
25		062	62.1
26		062	59.4
27		062	55.9
28		062	53.4
29		062	51.4
30		062	50

Figure 3. Legend for gel #2

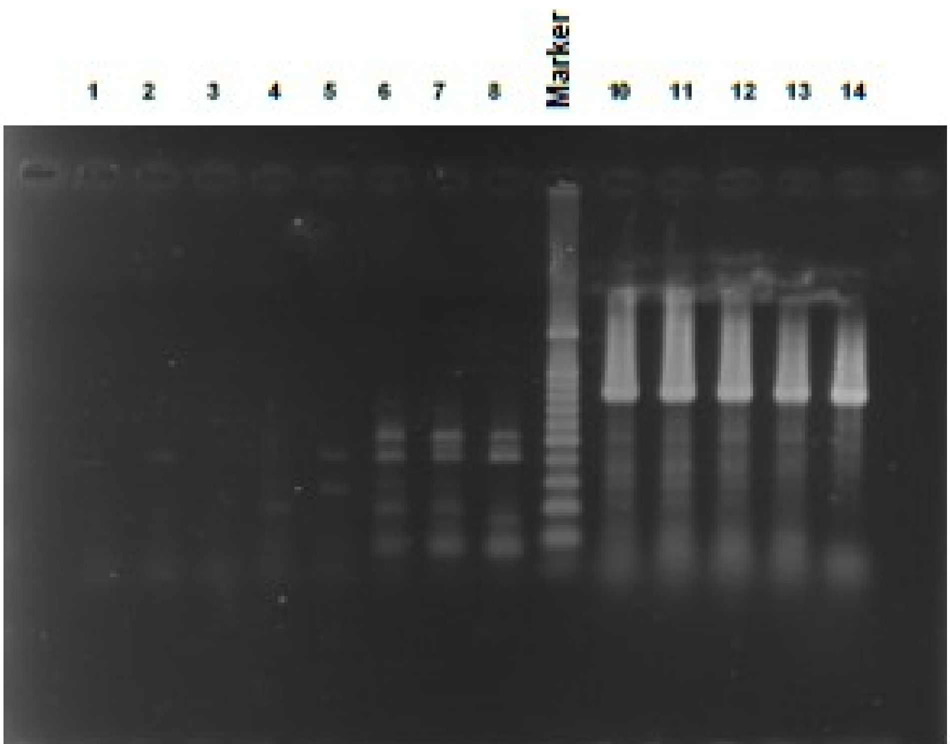


Figure 5. Temperature gradient lanes 1 – 14
Gel #2

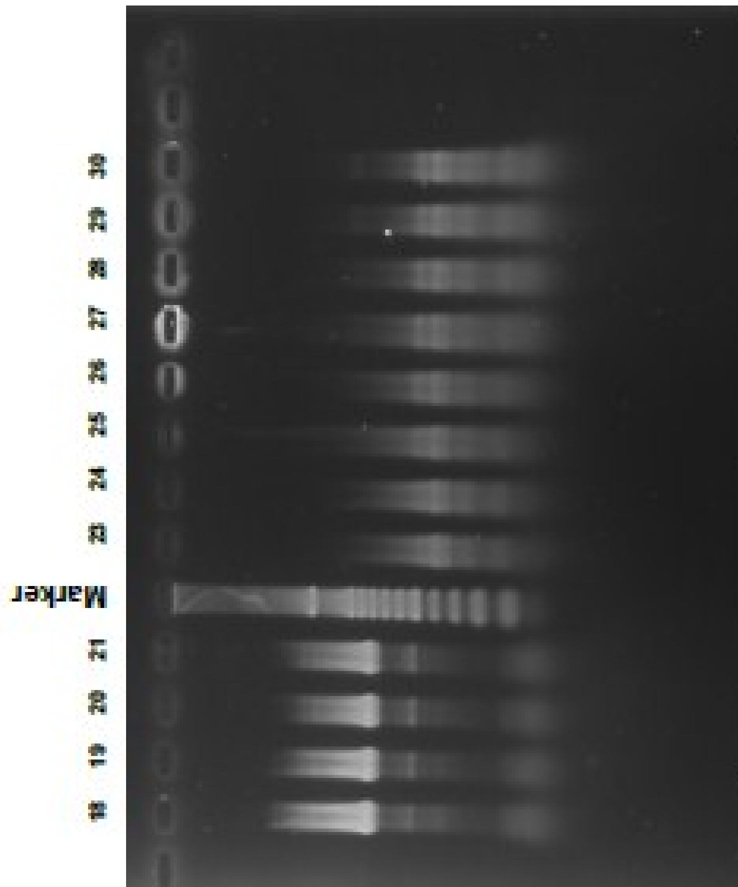


Figure 6. Temperature gradient lanes 18 – 30 Gel #2

Lane	Primer pair	sample	temperature degres C
4	CymCoatF/CymCoatR	Plasmid	50
5		871	65
6		871	63.9
7		871	62.1
8		81	59.4
9		871	55.9
10		871	53.4
11		871	51.4
12		871	50
13		Marker	50
21		062	65
22		062	63.9
23		062	62.1
24		062	59.4
25		062	55.9
26		062	53.4
27		062	51.4
28		062	50
29		Marker	50

Figure 4. Legend for gel #3

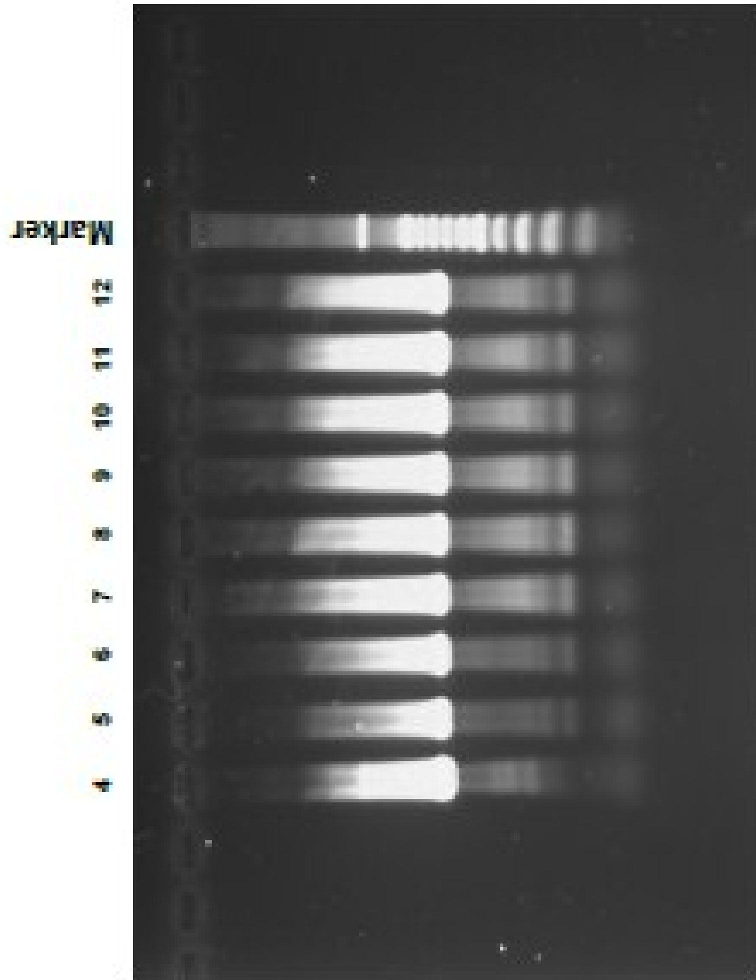


Figure 7. Temperature gradient lanes 4-13 Gel #3

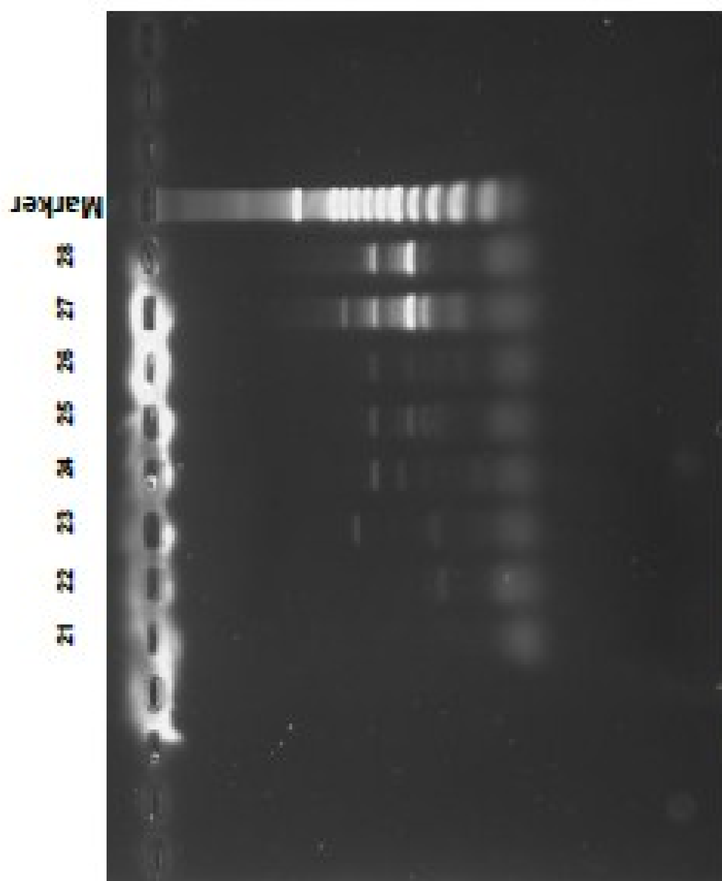


Figure 8. Temperature gradient lanes 21 – 29
Gel #3

Appendix D

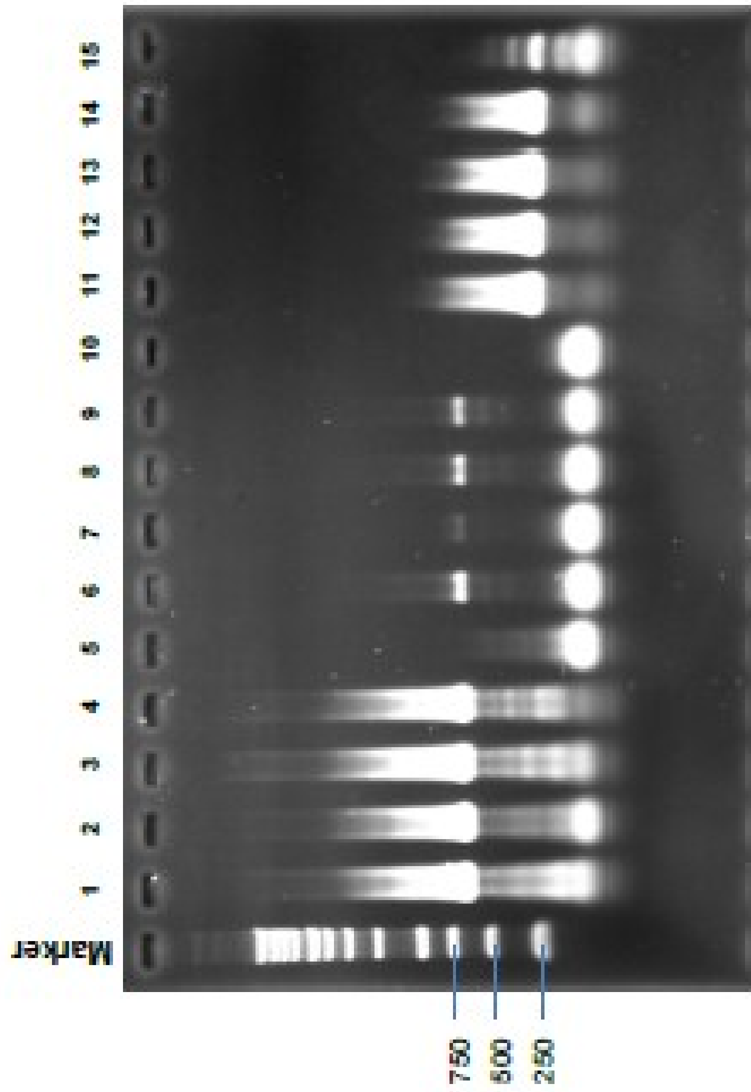
New Primer Annealing Temperature Gradient

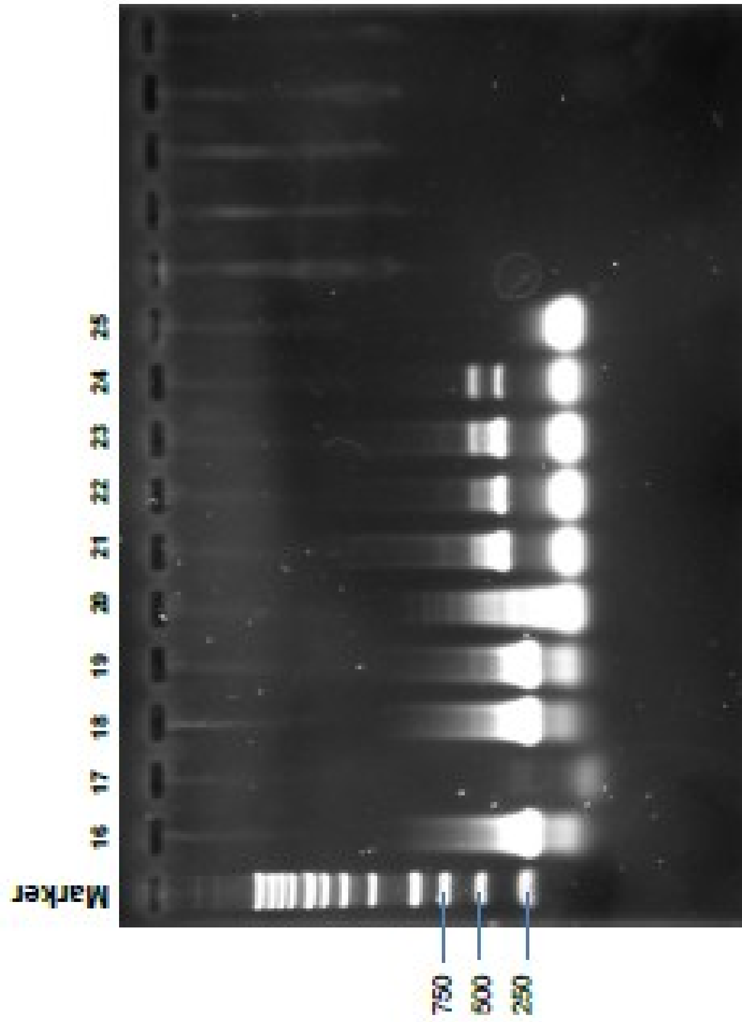
The primer combination CymCoatF/CymCP-R2 gave strong bands in the range of 640 bp and when compared to the other combinations, less non-specific product. Bands were strong at all annealing temperatures.

The primer combination CymF23/CymR25 gave strong bands in the range of 258 bp. In this trail there seemed to be little or no non-specific product. The bands were strong at all annealing temperatures. There was, however some product in the 'No Template' lane.

Group	Lane #	Template	Forward	Reverse	Annealing Temperature	Product length
A	1	cDNA #871, 1:100 dil	Cym Coat F	CymCP -R2	50 (50)	640 bp
	2	cDNA #871, 1:100 dil	Cym Coat F	CymCP -R2	55 (55.0)	
	3	cDNA #871, 1:100 dil	Cym Coat F	CymCP -R2	60 (60.6)	
	4	cDNA #871, 1:100 dil	Cym Coat F	CymCP -R2	65 (65.2)	
	5	NT	Cym Coat F	CymCP -R2	50 (50)	
B	6	cDNA #871, 1:100 dil	Cym Coat F	CymR2 5	50 (50)	653 bp
	7	cDNA #871, 1:100 dil	Cym Coat F	CymR2 5	55 (55.0)	
	8	cDNA #871, 1:100 dil	Cym Coat F	CymR2 5	60 (60.6)	
	9	cDNA #871, 1:100 dil	Cym Coat F	CymR2 5	65 (65.2)	
	10	NT	Cym Coat F	CymR2 5	50 (50)	
C	11	cDNA #871, 1:100 dil	Cym	CymR2	50 (50)	258

			F23	5		bp
	12	cDNA #871, 1:100 dil	Cym F23	CymR2 5	55 (55.0)	
	13	cDNA #871, 1:100 dil	Cym F23	CymR2 5	60 (60.6)	
	14	cDNA #871, 1:100 dil	Cym F23	CymR2 5	65 (65.2)	
	15	NT	Cym F23	CymR2 5	50 (50)	
D	16	cDNA #871, 1:100 dil	Cym F23	CymCP -R2	50 (50)	245 bp
	17	cDNA #871, 1:100 dil	Cym F23	CymCP -R2	55 (55.0)	
	18	cDNA #871, 1:100 dil	Cym F23	CymCP -R2	60 (60.6)	
	19	cDNA #871, 1:100 dil	Cym F23	CymCP -R2	65 (65.2)	
	20	NT	Cym F23	CymCP -R2	50 (50)	
E	21	cDNA #871, 1:100 dil	Cym F23	CymCo atR	50 (50)	368 bp
	22	cDNA #871, 1:100 dil	Cym F23	CymCo atR	55 (55.0)	
	23	cDNA #871, 1:100 dil	Cym F23	CymCo atR	60 (60.6)	
	24	cDNA #871, 1:100 dil	Cym F23	CymCo atR	65 (65.2)	
	25	NT	CymF2 3	CymCo atR	50 (50)	


Marker
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
750
500
250


Marker
16
17
18
19
20
21
22
23
24
25
750
500
250

Appendix E

Inoculation interval (Time A)

X indicates no sample

Plant No.	Inoculation date	interval	CymMV
1-1	5/20	10	POS
1-2	3/1	15	Neg
1-3	3/1	15	Neg
1-4	3/1	17	Neg
1-5	3/1	6	Neg
2-1	x	x	
2-2	x	x	
2-3	5/30	7	
2-4	5/20	10	POS
2-5	x	x	
3-1	3/1	6	Neg
3-2	5/30	7	
3-3	5/30	14	
3-4	5/30	14	
3-5	x	x	
4-1	5/20	10	POS
4-2	3/18	11	Neg
4-3	3/18	25	Neg
4-4	5/30	7	
4-5	5/20	10	POS
5-1	3/1	17	Neg
5-2	5/30	7	
5-3	3/1	15	Neg
5-4	3/18	25	Neg
5-5	3/18	11	Neg
6-1	5/30	14	
6-2	5/30	7	
6-3	x	x	
6-4	5/30	14	
6-5	x	x	
7-1	5/20	10	POS

7-2	x	x	
7-3	3/18	11	Neg
7-4	5/30	24	
7-5	5/30	10	Neg
8-1	3/1	8	Neg
8-2	3/1	17	Neg
8-3	5/20	10	POS
8-4	3/1	15	Neg
8-5	3/1	8	Neg
9-1	3/18	25	Neg
9-2	3/18	25	Neg
9-3	x	x	
9-4	3/18	11	Neg
9-5	3/18	11	Neg
10-1	3/18	25	Neg
10-2	3/18	25	Neg
10-3	3/18	11	Neg
10-4	x	x	
10-5	5/30	7	
11-1	3/18	11	Neg
11-2	5/20	10	POS
11-3	5/30	7	
11-4	3/1	8	Neg
11-5	5/30	14	
12-1	5/30	7	
12-2	5/30	7	
12-3	3/1	15	Neg
12-4	3/18	11	Neg
12-5	5/30	7	
13-1	5/20	10	POS
13-2	5/30	7	
13-3	3/1	15	Neg
13-4	3/18	25	Neg
13-5	3/18	25	Neg
14-1	3/18	25	Neg
14-2	x	x	
14-3	3/1	8	Neg
14-4	5/30	24	
14-5	5/20	10	POS
15-1	5/20	10	POS

15-2	5/30	24	
15-3	5/30	24	
15-4	5/20	10	POS
15-5	3/18	11	Neg
16-1	3/1	17	Neg
16-2	5/30	14	
16-3	3/1	15	Neg
16-4	3/1	15	Neg
16-5	5/30	24	
17-1	5/30	7	
17-2	3/1	8	Neg
17-3	5/30	24	
17-4	5/30	7	
17-5	5/30	14	
18-1	3/1	15	Neg
18-2	3/1	8	Neg
18-3	3/1	8	Neg
18-4	3/1	17	Neg
18-5	5/30	7	
19-1	x	x	
19-2	x	x	
19-3	x	x	
19-4	x	x	
19-5	5/30	7	
20-1	x	x	
20-2	x	x	
20-3	x	x	
20-4	3/18	25	Neg
20-5	5/30	7	
21-1	3/18	11	Neg
21-2	5/20	10	Neg
21-3	3/18	11	Neg
21-4	5/30	7	
21-5	3/1	8	Neg
22-1	5/30	14	
22-2	x	x	
22-3	x	x	
22-4	x	x	
22-5	5/20	10	POS
23-1	x	x	

23-2	5/30	7	
23-3	x	x	
23-4	3/1	15	Neg
23-5	5/30	24	
24-1	5/30	14	
24-2	5/20	10	POS
24-3	3/18	11	Neg
24-4	5/20	10	POS
24-5	x	x	
25-1	5/30	7	
25-2	3/1	17	Neg
25-3	3/1	17	Neg
25-4	3/18	11	Neg
25-5	3/1	6	Neg
26-1	3/18	25	Neg
26-2	3/18	11	Neg
26-3	5/30	14	
26-4	3/1	17	Neg
26-5	5/30	14	
27-1	5/20	10	Neg
27-2	3/18	25	Neg
27-3	x	x	
27-4	x	x	
27-5	3/18	11	Neg
28-1	5/20	10	Neg
28-2	5/20	10	Neg
28-3	3/18	11	N
28-4	5/30	7	
28-5	3/1	15	Neg
29-1	5/30	7	
29-2	3/1	6	Neg
29-3	5/30	7	
29-4	3/1	17	Neg
29-5	3/1	6	Neg
30-1	5/30	14	
30-2	5/30	7	
30-3	5/30	24	
30-4	3/1	15	Neg
30-5	5/20	10	Neg

Appendix F

Chi square statistical analysis

Interval	Status	A
A	P	14
A	N	19
B	P	0
B	N	28
C	P	0
C	N	9
D	P	0
D	N	12

```
proc print data=innocchi;
run;
proc freq data=innocchi;
tables interval*status/chisq;
weight A;
run;
```


The FREQ Procedure

Table of INTERVAL by STATUS

INTERVAL(INTERVAL) STATUS(STATUS)

Frequency Percent Row Pct Col Pct	N	P	Total
A	19 23.17 57.58 27.94	14 17.07 42.42 100.00	33 40.24
B	28 34.15 100.00 41.18	0 0.00 0.00 0.00	28 34.15
C	9 10.98 100.00 13.24	0 0.00 0.00 0.00	9 10.98
D	12 14.63 100.00 17.65	0 0.00 0.00 0.00	12 14.63
Total	68 82.93	14 17.07	82 100.00

Statistics for Table of INTERVAL by STATUS

Statistic	DF	Value	Prob
Chi-Square	3	25.0677	<.0001
Likelihood Ratio Chi-Square	3	29.9681	<.0001
Mantel-Haenszel Chi-Square	1	15.1941	<.0001
Phi Coefficient		0.5529	
Contingency Coefficient		0.4839	
Cramer's V		0.5529	

WARNING: 38% of the cells have expected counts less

than 5. Chi-Square may not be a valid test.

Sample Size = 82

Bibliography

Arif, M., Fletcher, J., Ochoa Corona, F. M. Application of primer and probe modifications in detection, biosecurity, and microbial forensics. Phytopathology 102, number 7 (Supplement 4) S4.6. July 2012

Bell, H. A., Cooke, D., Wildey, K.B., Baker, L.F., Mosson, J., and Short, J. Long-Term Management of a Population of Australian Cockroaches (Periplaneta australasiae) in a Tropical Plant House in the United Kingdom using the Juvenile Hormone Analogue (S)-Hydroprene. Proceedings of the 3rd International Conference on Urban Pests. 1999.

Borth, W.B., Barry, K., Obsuwan, K., Xu, M.Q., Liu, R.W., Kuehnle, A.R. and Hu, J.S. 2006. Movement of cymbidium mosaic virus and transgenic resistance in Dendrobium orchids. Acta Hort. 722:137-146. http://www.actahort.org/books/722/722_17.htm

Broadbent, L. Tinsley, T. W., Buddin, W., and Roberts, E. T. The spread of lettuce mosaic in the field. Annals of Applied Biology 1951 38, 689-706

Brunt, A.A., Crabtree, K., Dallwitz, M.J., Gibbs, A.J., Watson, L. and Zurcher, E.J. (eds.) (1996 onwards). ‘Plant Viruses Online: Descriptions and Lists from the VIDE Database. Version: 20th August 1996.' URL: http://biology.anu.edu.au/Groups/MES/vide/. Accessed May 19, 2011

Büchen-Osmond, Cornelia. *Cymbidium mosaic virus.* ICTVdB - The Universal Virus Database. http://www.ictvdb.rothamsted.ac.uk/ICTVdB/00.056.0.01.007.htm. Accessed May 19, 2011

Chang, Ching-An. Orchid virus diseases in Taiwan and their control strategies. International Commercial Orchid Growers Organization. http://www.icogo.org/images/Dr._Ching-An_Chang_-_Orchid_Viruses.pdf Accessed 5/21/11

Chang, C., Chen, Y. C., Hsu, Y. H., Wu, J. T., Hu. C. C., Chang, W. C., Lin, N. S., Transgenic resistance to *Cymbidium mosaic virus* in *Dendrobium* expressing the viral capsid protein gene. Transgenic Research 2005 Feb; 14 (1): 41-46.

Cochran, Donald G. Cockroaches, their biology, distribution, and control. World Health Organization, Communicable Diseases Prevention and Control, WHO Pesticide Evaluation Scheme (WHOPES). 1999

Corbett, M. K. Purification by density gradient centrifugation, electron microscopy, and properties of *Cymbidium mosaic virus*. Phytopathology 50: 346-351. 1960

Floriculture Crops 2011 Summary (May 2012) USDA, National Agricultural Statistics Service. http://usda01.library.cornell.edu/usda/current/FlorCrop/FlorCrop-05-31-2012.pdf. Accessed July 2012

Freitag, J. H., Frazier, N. W., and Sylvester, E. S. Dilworth D. Jensen. Essig Museum of Entomology Collections. 2011. http://content.cdlib.org/view?docId=hb9k4009c7&doc.view=content&chunk.id=div00027&toc.depth=1&&anchor.id=0. Accessed May 20, 2011.

Fulton, J. P., Gergerich, R. C., and Scott, H. A. Beetle transmission of plant viruses. Annual Review of Phytopathology. 25 (111-123) 1987

Gold, A. H. & Jensen, D. D. An electron microscope study of Cymbidium mosaic virus. American Journal of Botany 38: 577-578., 1951

Hu, J. S. Ferreira, S., Wang, M., Xu, M. Q. Detection of Cymbidium Mosaic Virus, Odontoglossum Ringspot virus, Tomato Spotted Wilt Virus, and Potyviruses Infecting Orchids in Hawaii., American Phytopathological Society DOI:10.1094/PD-77-0464. 1993

Hull, Roger. Matthew's Plant Virology, Fourth Edition. Academic Press, London, UK. 1970

Inouye, Narinobu. Viruses of Orchids, Symptoms, Diagnosis, Spread, and Control. Blue Bird Publishers, The Netherlands. 2008

Izaguirre-Mayoral, M.L., de Uzcategui, R.C. and Carballo, O. 1993. Crassulacean acid metabolism in two species of orchids infected by tobacco mosaic virus-orchid strain and/or Cymbidium mosaic virus. J. Phytopathology 137:272-282.

Jensen, D. D. Virus Diseases of Orchids. California Agriculture. 6: 7, 15, 16. January and February 1952.

Jensen, D. D. Virus diseases of orchids in The Netherlands., Netherlands Journal of Plant Pathology. 76 (1970) 135-139.

Khentry, Y., Paradornuwat, A., Tantiwiwat, S., Phansiri, S., Thaveechai, N. Incidence of *Cymbidium mosaic virus* and *Odontoglossum ringspot virus* in *Dendrobium spp*. in Thailand. Crop Protection 25 (2006) 926-932

Kiang Ho, Wong. Orchid Industry in Malaysia and Global Market. Commercial Orchid Grower Association of Malaysia. 2010. http://sdvi.fama.net.my/maha/seminar/upload_module/paper_MAHA2010/Sesi02_Kertas05.pdf. Accessed July 2012

Kim, J. D., Koo, Y. B., Chang, M. U. Genome characterization of a Korean isolate of Cymbidium mosaic virus. Molecules and Cells. 1997 8 (2) 181-188

Kuehnle, A. R., Breeding and biotechnology of orchids with special emphasis Dendrobiums. USDA Research, Education, & Economics Information System. http://www.reeis.usda.gov/web/crisprojectpages/0014694-breeding-and-biotechnology-of-orchids-with-special-emphasis-on-dendrobiums.html. Accessed 7/22/12

Kunkle, Joseph G. The Cockroach Homepage. Biology Department, University of Massachusetts. 2007. http://www.bio.umass.edu/biology/kunkel/cockroach.html. Accessed 8/9/12

Liao, L. J., Pan, I. C., Chan, Y. L, Hsu, Y. H., Chen, W. H., Chan, M. T., Transgene silencing in Phalaenopsis expressing the coat protein of Cymbidium mosaic virus is a manifestation of RNA-mediated resistance. Molecular Breeding 2004 13(3) 229-242

Lu, Hsiang-Chia, Chen, Cheng-En, Tsai, Meng-Hsiun, Wang, Hsiang-iu, Su, Hong-Ji, Yeh, Hsin-Hung. Cymbidium mosaic potexvirus isolate-dependent host movement systems reveal two movement control determinants and the coat protein is dominant. Virology 388 (2009) 147-159

Moles, M., Delatte, H., Farreyrol, K., Grisoni, M. Evidence that *Cymbidium mosaic virus* (CymMV) isolates divide into two subgroups based on nucleotide diversity of coat protein and replicase genes. Archives of Virology, 152:4 (705-715) 2007

Mullis, K. B. and Faloona, F. Specific synthesis of DNA in vitro via a polymerase-catalyzed chain reaction. Methods Enzymole, 155 (335-350) 1987

Murakishi, H. H., Host range, symptomalology, physical properties, and cross-protection studies of orchid virus isolates. Phytopathology 48: 132-137. 1958

Namba, R. and Ishii, M. Failure of Aphids to transmit the Odontoglossum Ringspot and Cymbidium mosaic viruses to orchid plantlets derived from meristem cultures. Phytopathology 61:582-583. 1971

Obsuwn, K., Hieber, D. A., Mudalige-Jayawickrama, R. G., Kuehnle, A. R. A dysfunctional CymMV movement protein confers resistance to CymMV in *Dendrobium* Orchid., Acta Horticulturae 2009 No. 836 pp. 149-154

Potexvirus. ViralZone. http://www.expasy.ch/viralzone/all_by_species/272.html. Accessed 5/20/11

Ramel, Gordon. The Blattodea or Cockroaches. 2001. http://www.earthlife.net/insects/blatodea.html. Accessed 8/9/12

Sherpa, A. R., Hallan, V., Pathak, P., Zaidi, A. A. Complete nucleotide sequence analysis of Cymbisium mosaic virus Indian isolate: further evidence for natural recombination among Potexviruses. Journal of Bioscience 32 (4) June 2007, 663-669

Sherpa, A. R., Hallan, V., Ram, R., Vij, S. P., Pathak, P., Garg, I. D., Zaidi, A. A. The first report of *Cymbidium mosaic virus* (CymMV) in orchids from India. BSPP Web, New Disease Reports (2003) 7, 10. http://www.ndrs.org.uk/article.php?id=007010. Accessed 5/20/11

Stejskal, V., Lukas, J., and Aulicky, R. Temperature-dependent development and mortality of Australian Cockroach, Periplaneta australasias (Fabricius) Plant Protection Science 40(1) 11-15. 2004

ViralZone www.expasy.org/viralzone, Swiss Institute of Bioinformatics. Accessed July 2012

White, N. H. and Goodchild, D. J. Mosiac or black streak disease of Cymbidium and other orchid hybrids. Journal Australian Institute of Agricultural Science 21: 36-38. 1955

Wong, S. M., Chng, C. G., Lee, Y. H., Tan, K., Zettler, F. W. Incidence of Cymbidium mosaic and Odontoglossum ringspot viruses and their significance in orchid cultivation in Singapore. Crop Protection, 13:235-239. 1994

Wong, S. M., Mahtani, P.H., Lee, K. C., Yu, H. H., Tan, Y., Neo, K. K., Chan, Y., Wu, M., and Cheng, C. G. Cymbidium mosaic potexvirus RNA: complete nucleotide sequence and phylogenetic analysis. Archives of Virology, 142: 383-391. 1997