Orchid fleck virus :molecular detection and *Brevipalpus*mite transmission

by

Raymond Ali

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School of Agricultural Science University of Tasmania

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Declaration

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Abstract

Orchids represent an important part of the global trade in cut flowers and potted plants. *Orchid fleck virus* (OFV) causes substantial negative impacts on quality of blooms and vigour of plants. A multiplex reverse transcription-polymerase chain reaction (mRT-PCR) assay was developed through a series of parameter optimisationsfor the simultaneous detection and differentiation of three widely prevailing viruses infecting orchids :*Cymbidium mosaic virus* (CymMV), *Odontoglossum ringspot virus* (ORSV), and *Orchid fleck virus* (OFV). Unique primers targeting conserved regions of the coat protein gene of the respective viruses amplified fragments of 845bp (ORSV), 505bp (CymMV) and 160bp (OFV) in the assay. The specificity of the mRT-PCR assay to detect CymMV, ORSV and OFV was determined by testing 29 samples from private collections in Tasmania, Victoria and New South Wales and comparing the results with Transmission Electron Microscopy (TEM). The mRT-PCR assay could detect more mixed infection samples than TEM. This is the first study to report the detection of these three viruses by multiplex RT-PCR and will have application in cost-effective and reliable screening of large number of plants as well as detecting infection in asymptomatic plants with low viral titre.

*Brevipalpus*mites constitute an economically important group of flat mites capable of vectoring one or more plant viruses in crop and ornamental plants. OFV has been shown to be transmitted in a persistent manner by *B. californicus*. However, little is known about the capacity for other *Brevipalpus*species to acquire and transmit OFV, particularly those that are pests on orchids. Studies on *Brevipalpus*mites are complicated by both the lack of reliable and informative morphological traits and the paucity of molecular resources available for phylogeny reconstruction. Ongoing work on the three most economically significant *Brevipalpus*mites (*B. californicus, B. obovatus*and *B. phoenicis*) seeks to resolve these taxonomic challenges, particularly through sequencing of the mitochondrial cytochrome oxidase c subunit I (COI) gene.

During the course of this study, a pest population of *Brevipalpus*mites was detected on *Brassiaverrucosa*(spider orchid) in Hobart, Tasmania. An integrative taxonomic approach using revised diagnostic morphological characters, in combination with COI sequencing, was unable to identify the mites to species level. Ongoing work is required, particularly with low temperature scanning electron microscopy, to resolve the identity of these mites. A biological transmission assay was performed to determine whether these mites could acquire OFV from infected cymbidium leaves and then transmit OFV to healthy cymbidiums using SYBR Green I quantitative reverse transcriptase polymerase chain reaction (RT-PCR) with OFV coat protein gene-specific primers. Primer-dimer formation confused the interpretation of results, however subsequent melt curve analysis tentatively suggests that nymphs and adult females may be able to vector OFV, albeit with poor efficiency, in a persistent manner. Future efforts to confirm vector competence in these mites should repeat quantitative RT-PCR with a carefully designed TaqManoligoprobe.

Literature Review

1. Introduction

The orchid family (Orchidaceae) is one of the most species-rich and widespread flowering plant families in the world (Hodgson, 1991). Orchids have great horticultural and ornamental importance, however viral infection in orchids is a major threat to industry, causing reduced vigour, poor quality blooms and increased susceptibility to secondary infection by fungal and bacterial pathogens (Matsui, 2010, Gibbs et al., 2000). There are at least 50 viral species from 8 different genera that infect cultivated orchids (Gibbs et al., 2000, Kubo et al., 2009a).

Past research has focused on the two most common viruses : the tobamovirus, *Odontoglossum ringspot virus* (ORSV), and the potexvirus, *Cymbidium mosaic virus* (CymMV), both spread by mechanical transmission (Gibbs et al., 2000). However, *Orchid fleck virus* (OFV), a negative sense, single stranded RNA virus with unenveloped bullet-shaped or bacilliform particles is of equal global importance due to its potential to infect a wide range of commercially important orchid genera (Peng et al., 2013, Kondo et al., 2006). OFV is transmitted in a persistent and propagative manner by the flat mite, *Brevipalpuscalifornicus*Banks. (Acari : Tenuipalpidae) (Kondo et al., 2003).

This literature review will overview the epidemiology, pathology and management of *Orchid fleck virus*, with particular emphasis on current detection methods. Some important aspects of temperature effects on viral disease and host resistance will be discussed. Following this, an overview of the biology of *Brevipalpus*mites will be given, concluding with a summary of persistent transmission of plant viruses.

2. Orchid Fleck Virus

2.1. Geographical distribution

OFV was first described in cymbidium leaves displaying necrotic fleck symptoms in Japan (Doi et al., 1977 in Kondo et al., 2003). Since then, the virions causing orchid fleck disease have been detected in orchids from Australia, Brazil, China, Columbia, Costa Rica, Denmark, Germany, Japan, Korea, South Africa and the United States (Kitajima et al., 2001, Kubo et al., 2009a, Kubo et al., 2009b, Peng et al., 2013). In other words, the distribution of OFV spans the five continents. Other *Brevipalpus*-transmitted viruses (eg.*Citrus leprosis virus-C, Coffee ringspot virus, Passion fruit green spot virus*) occur only in Western hemisphere nations (Kitajima et al., 2003). The worldwide occurrence of OFV is most likely a result of the global trade in orchids, making it challenging to determine its centre of origin(Peng et al., 2013).

2.2. Host range

Currently, there are 75 known plant species, from 48 genera of 12 different families, that have been confirmed susceptible hosts for OFV (Peng et al., 2013). Natural infections only occur in members of the Orchidaceae. There have been 50 orchid species from 31 genera that have been diagnosed with OFV infection (Peng et al., 2013, Blanchfield et al., 2001, Gibbs et al., 2000). Commercially important orchids that can be infected with OFV include *Cymbidium, Dendrobium, Phalaenopsis* and *Calanthe*species (Peng et al*.,* 2013). Experimentally, 25 further species from 11 non-orchid families are able to be infected either by mechanical or mite transmission (Peng et al., 2013, Kitajima et al., 2010). Global trading may result in an increase in the host range of the *Brevipalpus* vector, potentially increasing the host range of OFV. Expansion in host range by this manner has been shown many times before in viruses that are transmitted by insect vectors (Power, 2000).

2.3. Mode of Transmission

2.3.1 Vector Transmission

Transmission assays using two mite species, *Tetranychusurticae* and *Brevipalpuscalifornicus,*reared on OFV-infected cymbidiums, demonstrated that only *B. californicus*was able to transmit OFV into healthy leaves of native spinach (*Tetragoniatetragonoides*)and bean (*Phaseolus vulgaris*) (Kondo et al., 2003). Adults and nymphs, but not larvae, were able to transmit OFV (Kondo et al., 2003).Following moulting, adults and nymphs remained viruliferous for three weeks whilst maintained on OFV-immune *Camellia sinensis* plants (Kondo et al., 2003). The conclusion by Kondo et al. (2003) was that OFV transmission by *B. californicus*was persistent. Previous transmission assays have shown that several Hemipteran species from multiple suborders are unable to vector OFV (Doi et al., 1977 and Maeda et al., 1998 in Kondo et al., 2003). OFV has similar particle morphology to *Citrus leprosis virus-C, Coffee ringspot virus, Clerodendrum chlorotic spot virus, Hibiscus chlorotic spot virus* and *Viola ringspot virus*, all of which are transmitted by *Brevipalpusphoenicis*(Geijskes) (Kitajima et al., 2003). However, there is no experimental evidence to suggest that *B. phoenicis*is capable of transmitting OFV (Kitajima et al., 2001, Kubo et al., 2011). Furthermore, no transmission tests have been performed to assess the capacity of other *Brevipalpus*species to acquire or transmit OFV.

2.3.2. Mechanical Transmission

The role of mechanical transmission in natural OFV infections is thought to be minimal, since OFV particles are unstable outside of the host cell environment (Wilson, 1999). Successful infections by sap inoculation have been demonstrated experimentally in many herbaceous non-orchid species, including members of *Azociaceae, Chenopodiaceae and Solanaceae*(Wilson, 1999). For example, in *Chenopodiumamaranticolor, C. foliosum, C.alba*and *Nicotianaglutinosa,*local chlorotic mosaic symptoms developed 2-3 weeks after leaves were inoculated with infective sap (Wilson, 1999). Systemic symptoms were only observed in

*Chenopodiummurale*and *C.quinoa* and this occurred four weeks after inoculation (Wilson, 1999). Interestingly, cymbidium back bulbs were not able to be mechanically inoculated (Wilson, 1999). Homogenate in sap transmission studies has been shown to remain infective for 7-8 minutes following homogenization in summer, and approximately 15 minutes in winter (Inouye et al., 1996). Transmission rates have been observed to be higher at temperatures of 30^0C, while transmission rates tend to be lower at temperatures less than 20^0C, and when days shorten (Inouye et al., 1996, Wilson, 1999). However, these temperature effects on mechanical transmission have yet to be verified experimentally.

2.4. Pathology

2.4.1. Symptoms

In both orchid and non-orchid hosts, symptoms of infection can vary depending on host species, age of host, virus strain and environmental conditions (Gibbs et al., 2000). Typically, leaves are affected by chlorotic or necrotic flecks, spots or ring spots, although chlorotic mosaics and mottles have also been reported (Gibbs et al., 2000) (figure 1).

Figure 1. Orchid Fleck Virus symptoms.From left to right : *Cymbidium* leaf showing chlorotic and necrotic mosaics; *Cymbidium* leaf shows necrotic flecks; *Dendrobium speciosum* leaf with chlorotic ring spots; *Cymbidium* leaf with chlorotic flecks. Source : R. Ali (2013)

2.4.2 Cytopathology

Ultrathin sections of OFV-infected tissue viewed under electron microscopy reveal electron-lucent viroplasm in host cell nuclei, almost identical to inclusions caused by nucleorhabdoviruses, and multiple virion particles in the nuclei and cytoplasm (Kondo et al., 2003). Virion particles are bacilliform and appear to lack a lipid envelope, despite budding from the nuclear membrane (Kim et al., 2010, Jackson et al., 2005). In contrast, other plant rhabdoviruseshave been shown to acquire a lipid bilayer from host cell internal or surface membranes (Jackson et al., 2005). Characteristic 'spoke wheel' arrangements of virions are also observable (Kondo et al., 2003). Kubo et al*.,* (2009) demonstrated electron-dense viroplasm, similar to cytoplasmic-type of citrus leprosis virus (CiLV-C), in plant tissue infected with alternate isolates of OFV, suggesting the existence of a distinct cytoplasmic form of OFV. Furthermore, phylogenetic analysis based on viral N protein shows that OFV isolates can be divided into two distinct lineages (Blanchfield et al., 2001, Kubo et al., 2009a). Currently it is not certain whether they represent strains of the same species or in fact two different viral species (Peng *et al.,* 2013). Since OFV has unenvelopedvirion particles and a bipartite negative sense RNA genome, it has been proposed that it be the type species of a novel genus, *Dicorhabdovirus*(Kondo *et al.,* 2006).

2.5 Diagnosis

Currently, there is no cure for viral infection of orchids. Meristem tissue-culture has been reported to eliminate viral infection from high value plants, but is laborious, expensive and has variable success rates (Batchman, 2008). Therefore, viral detection and subsequent rogueing of an infected plant from a collection remains the cornerstone of managing the spread of disease (Gibbs et al., 2000). Serological tests, such as enzyme-linked immunosorbent assay (ELISA),is a commonly used diagnostic test for orchid viruses in commercial laboratories (Batchman, 2008). However, OFV virionscan not be purified due to their extreme fragility and host plant contaminants

complicate antibody production (Wilson, 1999). Alternatively, diagnosis of OFV by transmission electron microscopy (TEM) is routinely used to detect the presence of rod-shaped or bacilliform particles in negatively stained sap preparations (Peng et al., 2013). This technique can be time-consuming and relies on an experienced diagnostician to detect virus particles, since OFV titre is usually low in infected plants (Wilson,1999). A nucleic acid-based assay, such as reverse-transcriptase polymerase chain reaction (qRT-PCR), has the potential to offer greater sensitivity and specificity, particular in the early stages of infection, or in cases of co-infection with multiple viruses.

3. Temperature Effects on Viral Infection

The effects of temperature on viral infection vary widely with different hosts(Colhoun, 1973). This section will review experimental evidence regarding temperature effects on symptom expression, systemic infection and the equilibrium between virus multiplication and degradation within an infected host.

3.1 Hypersensitivity Response

Temperature is an important environmental factor that influences necrotic reactions in hypersensitivity response (HR)-mediated defence. High temperatures can induce a transition from localized necrotic lesions in resistant hosts to systemic symptoms (Li et al., 2009; and citations herein). In some of the earliest work, Samuel (1931) showed that *Nicotianaglutinosa*plants inoculated with *Tobacco mosaic virus*(TMV) did not display HR-type resistance when grown at temperatures over 28^0C. Following this, Kassanis (1952) provided convincing evidence that the threshold temperature for transition from necrotic local lesions to systemic symptoms was 36^0C in *N. glutinosa*inoculated with TMV. Likewise, necrosis-prone tobacco cultivars, reacting hyper-sensitively to tobacco ringspot virusat 24^0C, were shown to develop systemic symptoms at 35^0C (Hendrix, 1972). Both Roggero et al. (1996) and Moury et al. (1998)analysed the stability of HR-mediated defence in *Capsicum chinense*against tomato spotted wilt virus(TSWV). At constant high temperatures of 33^0C for 20 days,

systemic infection occurred in plants that all displayed necrotic local lesions at 18-24^{0}C (Roggero et al., 1996). In the same way, HR-mediated defence was broken after nine days at 32^{0}C in plants normally completely resistant at 22^{0}C (Moury et al., 1998). However, this effect was only seen in younger plants (1-2 expanded leaves) and not in older plants (4-5 expanded leaves) (Moury et al., 1998). Li et al. (2009) studied the more complex interaction of temperature on different strains of *Soybean mosaic virus* (SMV)in various allelic combinations of resistance genes in soybeans. They found that disruption of HR resistance at 30^{0}C was strain-specific, gene dose-dependent and influenced by host genetic background.

Recent investigation into the mechanisms of HR suppression has revealed that high temperatures may alter the production of reactive oxygen species in resistant plants (Kiraly et al., 2008). In their study, Kiraly et al. (2008) observed that TMV-infected tobacco plants grown at 30^{0}C had higher viral multiplication than infected plants grown at 20^0. Furthermore, at the higher temperature, tobacco plants became systemically infected. They attributed this to significantly lower levels of superoxide (O_2^{2-}) and NADPH oxidase in infected plants grown at 30^{0}C. Kiraly et al. (2008) speculated that at high temperatures, O_2^{2-}declined because of increased reactivity with other biomolecules and because of reduced expression of NADPH oxidase (known to play a role in O_2^{2-}production) (Kiraly et al., 2008).

Other mechanisms for HR suppression include interference with gene-for-gene recognition events in the early stages of HR-mediated defence at high temperatures(Moury et al., 1998). For example, Culver et al. (1991) suggests that subunits of TMV coat protein, the elicitor of N-gene mediated resistance in tobacco, may interact differently under high temperatures. In other interactions, it may be that at elevated temperatures, virus replication increases to such an extent that host resistance is overwhelmed (Celebi-Toprak et al., 2003). Alternatively, expression of gene products that normally resist viral movement at entry or exit sites of host cells (eg. bundle sheath cells, companion cells or sieve elements) may decrease at high temperatures (Celebi-Toprak et al., 2003). There may be evolutionary advantages to inhibition of disease resistance at high temperatures. For example, Wang et al. (2009) provide evidence for decreased virulence of pathogens at higher temperatures and

suggest that host plants balance the metabolically costly needs of defence with the potential virulence of the pathogen. However, there may be evolutionary limitations as well. At the molecular level, for example, certain proteins involved in HR-mediated defence, such as heat-shock proteins, may be co-opted for other more essential processes at elevated temperatures, limiting their availability in the HR pathway and thereby increasing the potential for systemic infection (Wang et al., 2009).

3.2 Viral Induced Gene Silencing

Under conditions favouring active and aggressive viral replication gene silencing has been shown to be crucial for survival in host plants (Qu et al., 2005, Zhang et al., 2012). For example, mutant *Arabidopsisthaliana* plants lacking critical gene silencing proteins (DCL-2 or AGO2) all died when infected with *Turnip crinkle virus* (TCV) at 26^0C whereas all infected wild type plants survived to set seed at these temperatures (Zhang et al., 2012). Additionally, wild type plants initially had more severe symptoms at 26^0C than wild type plants infected at 18^0C, but recovered to produce younger, less symptomatic leaves. Zhang et al., (2012) also detected higher concentration of both viral gRNA[1] and siRNA[2] in wild type plants grown at 26^0C than those grown at 18^0C. The higher levels of siRNA were attributed to upregulation of DCL-2 at the elevated temperature. Furthermore, the relative increase in siRNA was much greater than the increase in viral gRNA. This suggests that under high temperature conditions encouraging rigorous viral replication, the gene silencing pathway can confer a moderate competitive advantage to host plants (Zhang et al., 2012).

Often high temperatures can weaken viral symptoms (“heat masking”) and plants may recover rapidly from disease (Samuel, 1931, Kassanis, 1952, Colhoun, 1973). This may be because of increased viral degradation, mediated by temperature-dependent gene silencing pathways (Szittya et al., 2003, Sos-Hegedus

[1] gRNA = genomic RNA
[2] siRNA = small interfering RNA; double stranded RNA, 20-25 bp in length. One strand serves as the complementary template that is used to hunt for invading viral RNA.

et al., 2005). Conversely, low temperatures have been shown to compromise the activity of key gene silencing proteins. Szittya et al. (2003) established that *Nicotianabenthamiana* plants infected with *Cymbidium ringspot virus*(CymRSV) had undetectable levels of siRNAs at 15^0C. Furthermore, the levels of siRNAs increased as the plant growth temperature increased to 27^0C. A mutant CymRSV construct lacking viral suppressor function failed to infect plants at 27^0C, while at 15^0C there were strong viral symptoms. Based on these findings, Szittya et al. (2003) concluded that at low temperatures, gene silencing is ineffectual at protecting plants, even with viruses that lack silencing suppression. However, subsequent work in potato has revealed that alternative viral silencing pathways exist at these lower temperatures and that proteins involved in miRNA [3] pathway regulating gene expression are probably involved (Sos-Hegedus et al., 2005).

4. Brevipalpus species (Acari :Tenuipalpidae)

The subclass Acari, which comprises mites, is an important component of class Arachnida (which also includes spiders and scorpions). In this section, important biological aspects that influence the capacity of *Brevipalpus*mites to vector plant viruses will be reviewed.

4.1 Taxonomy and Morphology

The Tenuipalpidaeis a family of phytophagous mites and certain members are regarded as serious economic pests (Childers and Derrick, 2003, Childers and Rodrigues, 2011). *Brevipalpus*is the most important genus in the family, because of the potential for some species to transmit one or more plant viruses (Childers et al., 2003a). Of the 300 *Brevipalpus*species, three are vectors of viruses infecting tropical crops, subtropical crops and ornamentals : *B. phoenicis*(Geijskes), *B. obovatus*Donnadieu and *B. californicus*(Banks), (Kitajima et al., 2003, Childers and Rodrigues, 2011). *Brevipalpus*species are often difficult to detect. They are minute

[3]miRNA = micro RNA; another class of RNA thought to be primarily involved in post-translational gene silencing rather than viral induced gene silencing.

(measuring 0.25-0.4mm), slow-moving and dorso-ventrally flattened (Childers and Rodrigues, 2011, Welbourn et al., 2003). The taxonomy of *Brevipalpus*has not been fully resolved, with many species likely to represent cryptic or sister species complexes (Rodrigues and Childers, 2013). Indeed, taxonomic conclusions based on morphological traits have proven to be discordant with evidence derived from genetic data (Rodrigues and Childers, 2013). For example, Groot and Breeuwer (2006) demonstrated that mites appearing to be *B. phoenicis*morphologically were paraphyletic following mitochondrial *COI* sequence analysis. Current identification of *Brevipalpus*species relies on visual examination of slide-mounted specimens to assess key diagnostic features. These include number of dorsal setae, number of solenidia on the second tarsus and dorsal cuticular patterning (Welbourn et al., 2003). Factorscomplicating identification include difficulties in recognising diagnostic features, artefacts commonly arising from the slide mounting process, and intraspecific variation in cuticular patterning associated with age or feeding history(Welbourn et al., 2003). Other expensive investigative tools, such as low-temperature scanning electron microscopy (LTSEM), may offer more reliable character traits for diagnosis (Beard et al., 2012a).

4.2 Life Cycle

The *Brevipalpus*life cycle includes egg and four active stages - larva, protonymph, deutonymph and adult - with quiescent (chrysalis) developmental stages between each active stage (Childers et al., 2003a). Eggs are elliptical, shiny and reddish-orange, either lain singly or in clusters by the same female, often in cracks or areas of previous damage that offer shelter (Pritchard and Baker, 1953). An unknown adhesive agent, produced at the time of oviposition, enables eggs to be glued strongly to leaf surfaces making them difficult to remove (Childers et al., 2003a). Chrysalids also adhere strongly to leaf surfaces and evidence from LTSEM suggests that moulting flat mites can fasten to the leaf surface by insertion of the stylet into the plant tissue (Beard et al., 2012a). Newly-emerged larvae have three pairs of legs, while all other active stages have four pairs of legs and additional dorsal setae (Pritchard and Baker, 1953).Protonymphs are usually quite similar in morphological

appearance to deutonymphs, while adults are usually larger than both nymphal stages and tend to develop distinctive temperature-dependent dorsal pigmentation (Pritchard and Baker, 1953).

Developmental times for *Brevipalpus*species vary with temperature, relative humidity and host plant species fed upon (Childers et al., 2003a, Childers et al., 2003b). Increased population density has been shown to shorten developmental times in *B. phoenicis*and promote paedogenesis, a phenomenon referred to as “phase variation” (Kennedy, 1995). Mean developmental times for *B.californicus*at temperatures of 21 - 30^0C were 8.6 days for larvae, 6.2 days for protonymphs, 7.0 days for deutonymphs and 3.6 days for quiescent stages (Manglitz and Cory, 1953). Pre-oviposition period for adult females following final moult was 3.8 days, after which time mothers laid one egg daily over 25 days or more (Manglitz and Cory, 1953). Similarly, high rates of fertility and longevity have been seen in *B. phoenicis* and *B. obovatus*(Childers et al., 2003a).

4.3 Host Range

Brevipalpusphoenicis, *B. obovatus* and *B. californicus*are extremely polyphagous, making them formidable peststo agriculture in many countries across the world (Childers et al., 2003b). *Brevipalpus*mites are capable of aerial dispersal by lifting the anterior half of their bodies off leaf surfaces and moving to neighbouring hosts via wind currents (Childers and Rodrigues, 2011). Childers et al. (2003b) found 316 plant species, including several orchid genera, recorded as suitable hosts for *B. californicus.* In addition,928 plants species, from 513 different genera within 139 different families, were recorded as hosts for all three species (*B. phoenicis*, *B. obovatus* and *B. californicus).* Childers et al. (2003b) argue that given the diversity of plant genera reported, and the low host plant species to genus ratio recorded, it is likely that they have identified only a fraction of the actual host plant range of the three *Brevipalpus*species mentioned. Based on this assumption, Childers et al. (2003b) recommend countries that are free of *Brevipalpus*-transmitted viruses consider stricter biosecurity controls on the exchange of host plants originating from countries that have known records of these viruses.

4.4 Feeding Biology

All Tenuipalpids possess a pair of elongate stylets that fuse together to form a hollow feeding tube through which plant cell fluid contents are sucked up by vacuum forces (Childers and Rodrigues, 2011, Beard et al., 2012a). The stylets are adapted to pierce between cells or directly into cells and are often furnished with ornamentation that assists with shredding host tissue (Beard et al., 2012a). Toxic saliva injected into feeding sites is thought to cause numerous symptoms, such as galling, malformation, stunting, necrosis or scab-like lesions (Childers et al., 2003a). However, the extent to which saliva aids in pre-oral digestion is not known, especially in those Tenuipalpids that seem incapable of generating a vacuum force to suck plant cell contents (Beard et al., 2012a). Recently, the feeding biology of Tenuipalpid mites has been called into question. Beard et al. (2012a) used LTSEM to show that *Raoiellaindica*Hirst., a Tenuipalpid pestiferous on palm crops, feeds via stomata and not by piercing surface epidermal cells as previously thought. This feeding behaviour may be an adaptation to bypass plant structural defences, (eg. waxy cuticles and thick-walled epidermal and hypodermal cells), prominent in many monocots, including orchids (Beard et al., 2012a). Cell walls within stomatal chambers are frequently thinner and therefore more susceptible to attack (Esau, 1977). Furthermore, many monocot species have isobilateral leaves with abundant stomata arranged in parallel longitudinal confirmations (Esau, 1977). Stomata appear to be a clear target for flat mites that are otherwise incapable of penetrating structural leaf defences. Therefore, the analysis of stomatal morphology and stomatal response in host plants will help to understand why a particular host is fed upon, as well as how feeding damage and virus transmission are affected by abiotic stress, such as drought or heat waves. No studies found in this review have been able to definitively describe the feeding behaviour of other *Brevipalpus*pests, in particular *B. californicus,* on host plants.

4.5 Reproductive Biology

Many *Brevipalpus*mites, including *B. phoenicis, B. obovatus*and *B. californicus* are capable of thelytokous parthenogenesis, whereby daughters develop from unfertilized haploid eggs (Weeks et al., 2001, Groot, 2006). This haploid state in females is exceptionally rare in metazoans and theoretically places considerable limitations on the success and evolutionary longevity of such asexual species (Weeks et al., 2001). However, Groot et al. (2005) proposed a "Frozen Niche" model to explain the relative success and widespread distribution of thelytokous*Brevipalpus*species. Assuming the presence of intraspecific specialist populations adapted to unique components of the geographical range, this model proposes that specialist populations do not easily change from one host plant to another, because of their unique adaptations. This was shown experimentally for *Brevipalpusphoenicis*(Groot et al., 2005). If the Frozen Niche model holds true for other thelytokous populations, the threat of alternative host plants serving as reservoirs for pestiferous or viruliferous mites may not warrant such strict biosecurity measures recommended by Childers et al. (2003b).

The phenomenon of haploid thelytoky in *Brevipalpusphoenicis, B. obovatus*and *B. californicus*can be attributed to infection by endosymbiont bacteria in the genus *Cardinium.* This bacteriahas the ability to feminize unfertilized eggs, although the mechanism isnot certain (Weeks et al., 2001, Groot and Breeuwer, 2006). The effects of *Cardinium*was first discovered by Weeks et al. (2001), who applied tetracycline antibiotic to female *B. phoenicis*mites, promoting significantly more male offspring. The presence of *Cardinium*has been successfully detected in select populations of *B. phoenicis, B. obovatus*and *B. californicus* by PCR-based techniques and direct visualization of the endosymbiont has been possible with transmission electron microscopy (Kitajima et al., 2007). *Cardinium*bacteria is present in almost every part of the mite body, including eggs, eye complexes, epidermal cells, epithelial cells of the digestive tract and nerve cells surrounding nerve ganglion (Kitajima et al., 2007). This suggest that the endosymbiont may have additional effects on mite biology (Kitajima et al., 2007). For example, *Cardinium*infection could affect fitness of mites or influence the ability for mites to vector plant viruses. So far, only one study has

attempted to address this possibility in *B. californicus,* with no significant effects of *Cardinium*infection observed on fitness or transmission efficiency of OFV (Chigira and Miura, 2005). Interestingly, sexual reproduction may play a more important role in thelytokous*Brevipalpus*species than previously thought. For example, Rodrigues and Childers (2013) recently published evidence of elaborate guarding behaviour in *B. phoenicis*males over female quiescent stages, with subsequent mating on emergence of the adult female.

The many unknown aspects of reproductive biology in *Brevipalpus*mites continue to confound attempts at managing these pests.With regards to OFV, it is very likely that multiple biotypes of *B. californicus*are capable of acquiring and transmitting virus and that each biotype has its unique host range, feeding behaviour, reproductive strategies and other local adaptations. Furthermore, taxonomic ambiguities and lack of knowledge regarding other aspects of biology, such as feeding behaviour, means that it is quite conceivable that other *Brevipalpus*species, especially those found to be pests on Orchidaceae hosts, are capable of vectoring OFV.

4.6. Arthropods as Vectors of Plant Virus

In contrast to animal viruses that rely on the mobility of their host for dissemination, the vast majority of plant viruses rely on an arthropod vector for dissemination (Hogenhout et al., 2008). For many plant viruses, there is a high degree of vector specificity, with transmission occurring via one or a few related species (Ammar el et al., 2009). The International Committee on Taxonomy of Viruses recognizes 697 different plant virus species, over half of which are spread by Hemipteran vectors (predominantly aphids and whiteflies, but also leafhoppers and planthoppers) (Hogenhout et al., 2008). Only a small fraction of these plant viruses are transmitted by mites (approximately 1%) (Hogenhout et al., 2008). In order to understand the specific events involved in vector-borne plant virus transmission in nature, it is necessary to first identify the anatomical and physiological barriers that viruses must overcome in order to move and replicate within the vector. Most of our understanding of these barriers comes from research on virus interactions with insect

vectors, however fundamental principles revealed from this research may still apply to acarine vectors.

4.6.1. Persistent transmission

Persistent viruses are classified as either circulative (virus does not replicate within vector) or propagative (viral replication occurs within vector as well as host plant) (table 1). Following acquisition, the general model for spread of a persistent virus within a vector involves : (1) movement into the columnar epithelium of the midgut, followed by (2) transfer across the basement membrane into longitudinal and circular muscle cell layers surrounding the midgut, then (3) escape and dissemination into the haemolymph, allowing the virus access to the salivary glands by (4) crossing the basement membrane of the salivary ducts (Hogenhout et al., 2008, Ammar el et al., 2009, Whitfield et al., 2005). In the case of propagative persistent transmission, the virus may replicate in columnar epithelial cells of the midgut or muscle cells surrounding the midgut (Hogenhout et al., 2088). Transmission finally occurs by excretion of viral particles at the time of release of saliva during re-feeding (Hogenhout et al., 2008). Each stage in this model of propagative virus transmission constitutes a barrier involving highly specific molecular interactions between virus and host cells (Hogenhout et al., 2008). For example, tospovirus transmission in thrips (Thysanoptera) requires virus particles to cross up to six different membranes in order to get from the alimentary canal to the salivary glands of the vector (Whitfield et al., 2005).

Table 1. Comparison between circulative and propagative plant viruses (Hogenhout*et al.*, 2008)

	Circulative	**Propagative**
Acquisition access period	hours, days *	hours, days*
Latent period	hours, days	days, weeks
Retention in vector	days, weeks	lifetime
Present in haemolymph?	yes	yes
Replication in vector?	no	yes
Transovarial transmission?	no	frequently

** depends on virus location in plant (eg. phloem limited viruses take longer to acquire than viruses in epidermal or mesophyll cells)*

4.6.2. Mechanisms of Persistent Transmission

For most insects, the midgut has a lining of columnar epithelial with microvilli (referred to as a brush border) with a multilayered covering of extracellular chitin and proteins (peritrophic membrane) that prevents invasion by microorganisms (Chapman, 2003). The fact that immature stages have thinner midgut linings may explain why, in some virus-vector complexes, nymphs are more efficient at transmission than adults (Ammar el et al., 2009). Other reasons why certain viruses are not able to overcome the midgut barrier include enzymatic hydrolysis within the foregut or midgut lumen, innate immune response by the vector, or lack of recognition of virus due to absence of receptor sites for attachment and movement across the microvillar membrane (Ammar el et al., 2009). Very little has been published on the gut anatomy of Tenuipalpidmites, although based on ultrastructural findings in other Acarids, the midgut is likely to comprise a ventriculus, caecum and colon with a lining analogous to the peritrophic membrane of insects (Sobotnik et al., 2008).

Within the haemolymph of some insect vectors, circulative viruses have been shown to interact with proteins (GroEL homologues[4]) produced by endosymbiotic bacteria (Akad et al., 2007, Hogenhout et al., 2008, Ammar el et al., 2009). The interaction is thought to prevent destruction of the virus within the vector haemolymph(Ammar el et al., 2009). Early examples of this phenomenon have been detected in *Myzuspersicae*(green peach aphid) infected with a luteovirus and in *Bemisiatabaci*(whitefly) infected with a begomovirus, suggesting a conserved function for these endosymbiont proteins (probably transportation of macromolecules, including viral particles, through the haemolymph)(van den Heuvel et al., 1994, Morin et al., 1999). Interestingly, Akad et al., (2007) demonstrated that transgenic tomatoes expressing whitefly GroEL homologues in phloem cells were resistant to infection with the begomovirus, *Tomato yellow leaf curl virus*. Further investigation is required to determine what role, if any, endosymbionts play in the movement of persistent plant viruses in *Brevipalpus*mites.

[4]GroEL = chaperonin family of proteins involved in post-translational folding and subunit assembly of other proteins (Akad et al., 2007)

Differences in the morphology and physiology of the salivary gland apparatus may also inhibit vector transmission of propagative viruses, even if virus particles are able to overcome all other barriers. Alternative routes to the salivary glands may be from the nerves, ganglia or brain tissue (referred to as the neurotropic pathway) or via visceral muscles and the tracheal system (Ammar et al., 2009). In thrips, movement of tospovirus from the midgut into the primary salivary glands only occurs when there is direct contact between the midgut muscle cell and salivary gland cell membranes (Moritz et al., 2004). In the case of *Frankliniellaoccidentalis,* this direct contact is only present in early second instars, whereas older thrips develop a spatial separation of these tissues, preventing entry of the virus into salivary glands (Moritz et al., 2004) These ontogenetic changes explain why viral acquisition must occur during larval stages for successful tospovirus transmission by thrips(Whitfield et al., 2005). Furthermore, thrips with detectable levels of tospovirus may not necessarily transmit these viruses to susceptible host plants (Nagata et al., 2004). For example, *Thripspalmi*infected with *Chrysanthemum stem necrosisvirus* at early larval stages were unable to transmit the virus, indicating that though the virus was able to enter and replicate within the insect, the salivary gland entry barrier was not overcome (Nagata et al., 2004).

Other studies in insects have revealed that developmental stage, gender and biotype can all influence the interaction between vector and plant viruses (Hogenhout et al., 2008). Therefore, investigations into the ability for other *Brevipalpus*species to acquire and/or transmit OFV should take into account these factors.

5. Conclusion

Orchid fleck virus (OFV) infects commercially important orchids from multiple genera and is transmitted in a persistent manner by the flat mite, *Brevipalpuscalifornicus* (Banks). Currently there are two main groups of OFV isolates and it is uncertain whether they represent strains of the same species or in fact two different viral species. It is likely that mechanical transmission plays a minor role in natural infections, however elevated temperatures may increase risk. There is some evidence that higher rates of OFV transmission by sap inoculation occur at temperatures over 30^0C, but this requires experimental verification. Plant growth temperatures are known to influence viral replication and movement through the host by modulating hypersensitivity response and gene silencing. Understanding this interaction will assist in predicting disease outbreaks. Current diagnostic tools for OFV infection are limited to electron microscopy, therefore a sensitive and specific high throughput test, such as RT-PCR, will assist growers to detect asymptomatic plants and early infection. With regards to the vector of OFV, it is likely that several biotypes of *B. Californicus*exist. Until the taxonomy of the species can be resolved, unique host preferences or biological properties of these biotypes will be difficult to examine. Furthermore, little is known about the capacity of congenerics to acquire and/or transmit OFV, especially those that are pests on orchids.

Development of a multiplex reverse transcription-polymerase chain reaction for detection of three major RNA viruses infecting orchids

Introduction

Orchids represent an imporant part of the global floriculture trade in cut flowers and potted plants. *Orchid fleck virus* (OFV), *Cymbidium mosaic virus* (CymMV) and *Odontoglossum ringspot virus* (ORSV) are the three most important and widespread viruses affecting orchids (Wilson et al., 1999). All three viruses cause substantial negative impacts on marketable yield in commercial industries and decrease value and productivity of hobby collections (Wisler, 1983, Zettler et al., 1990, Wilson et al,.1999, Yamane et al., 2008). Mixed infections are common and may cause more severe symptoms compared to single infections (Wisler, 1983). Symptoms of viral infection in orchids are diverse and may be extremely varied between genera and even between individual plants of the same species (Gibbs et al., 2000). Furthermore, infected plants may be asymptomatic and difficult to distinguish from healthy plants based on visual symptoms alone (Gibbs et al., 2000). The ease of transmission of OFV, CymMV and ORSV by common propagation methods and horticultural tools means that all three viruses may rapidly spread through nursery and private collections (Wilson et al., 1999). Diagnosis is critical for effective control so that infected plants may be destroyed and propagation material sourced from virus-free germplasm (Wisler, 1983). Common detection methods for orchid viruses have included serological assays and electron microscopy (Gibbs et al., 2000). However, commercial sources of antisera for OFV is not available due to difficulties in purification of its virion, and electron microscopy is an inefficient detection method for multiple sample testing and may not be sufficiently sensitive to detect virus present at low titre in leaf extracts (Wilson et al., 1999).

Multiplex polymerase chain reaction (multiplex PCR) is increasingly becoming the preferred method of detection for plant pathogens worldwide (Kuwabara et al., 2010, Varanda et al., 2010, Lin et al., 2012, Liu et al, 2012, Zhang et al., 2013). Multiplex PCR can be used for the simultaneous detection of multiple viruses and is potentially more economical and sensitive than other methods (Mumford et al., 2006). For RNA viruses, complementary DNA (cDNA) synthesised by reverse transcription (RT) is used as a template in multiplex PCR

assay. In this study, a multiplex RT-PCR assay was optimised and established for simultaneous detection and differentiation of OFV, CymMV and ORSV in orchids. In addition, the sensitivity and specificity of the assay was evaluated. This is the first study to report the detection of these three virus species using multiplex RT-PCR.

Materials and Methods

Plant Materials

Cymbidium leaf samples with single infections of OFV, CymMVand ORSV were kindly provided by Mr. Peter Cross, Plant Biosecurity and Diagnostics Branch, Department of Primary Industries, Parks, Water and Environment, Tasmania. Virus identity was previously verified by Transmission Electron Microscopy (TEM). Healthy,virus-free cymbidium plants were kindly provided by Mr. Graham Morris, Cymbidium Orchid Club of south Australia. Leaves from healthy and virus-infected cymbidiums were stored at - 80^0C for later use.

Isolation of Total Nucleic Acid and Reverse Transcription

Total RNA was extracted from 50mg of cymbidium leaves using PowerPlant® RNA Isolation Kit (MO-BIO, Carlsbad, California) according to the manufacturers instructions and eluted into a final volume of 100µL. First-strand cDNA was synthesized in a reaction mixture using 2µL of total RNA extraction, 1µL of random hexamers, 1µL of oligo dT(18) primer, 4µL of 5 x Moloney murine leukemia virus (MMLV) reverse transcriptase buffer, 1µL of dNTP mixture (each dNTP 10mmol/L), 1µL of RNase inhibitor (10U/µL), 1µL of MMLV reverse transcriptase (200U/µL, Bioline) and DEPC-treated water to a final volume of 20µL. The reaction solution was incubated at 25^0C for 10 minutes, then 45^0C for 30 minutes, heated to 85^0C for 5 minutes and chilled on ice before being stored at -20^0C.

Design of Virus-specific Primers

Species-specific primer pairs were designed based on published nucleotide sequences of conserved regions within the coat protein gene, available on the GenBank database provided by the National Centre for Biotechnology Information (NCBI). Coat protein sequences for each of the three viruses were aligned using a ClustalW multiple alignment (Thompson et al., 1994). For OFV, degenerate primer pairs were designed to anneal to conserved regions of the coat protein gene from three distinct strains previously identified byWilson (1999).Primer pair sequences were chosen based on comparable annealing temperatures, and absence of predicted secondary structure and primer dimer formation, using the PrimerSelect module of DNASTAR 5.01 (DNASTAR, Inc., Madison, Wisconsin). It was essential that expected amplicon sizes could be easily differentiated on gel electrophoresis for simultaneous detection of all three viruses in a single reaction system. The uniqueness of primer sequences was confirmed by using the NCBI Basic Local Alignment Search Tool (BLAST). Primer sequences, expected RT-PCR product size, sequence location within the viral genome and NCBI accession numbers for isolates are given in Table 2.

Table 2.Primer sequences and location within the viral genome, annealing temperature, expected PCR amplification product size and target gene.

Target	Primer name	Sequence (5' – 3')[a]	Location[b]	Tm (^{0}C)	Product size (bp)	Amplified gene
OFV	OFV-ALL1F	GRCTKGCWGCGGAGGCWGAC	nt 1339 – 1358 (RNA 1)[c]	63.8	160	Coat protein
	OFV-ALL1R	CTGGCGGAWGGKGGTGTGAACAG	nt 1476 – 1498 (RNA 1)			
CymMV	CYM-505F	ACCCCACTTCTGCACCAAAA	nt 5527 - 5546	57.4	505	Coat protein
	CYM-505R	CCGTACTTCCCGATCGAGTG	nt 6012 - 6031			
ORSV	ORSV-845F	ATTTAAGCTCGGCTTGGGCT	nt 5758 – 5777	57.2	845	Coat protein
	ORSV-845R	CTACCCGAGGTAAGGGGGAA	nt 6583 - 6602			

[a] redundancy code : R = A/G; K = G/T; W = A/T

[b] The reference accession numbers (NCBI) for determining primer location are as follows : NC009608, AF343871, AF343873, AF343874, AF343875 and AF321775 for OFV; NC001812, AY360408, DQ208422, AB693982, AB541572, AB541571 for CymMV; U34586, DQ915440, AB693988, JN584484, AB541516, AB693991, EU653020, FJ372909, AF406777 for ORSV.

[c] RNA 1 refers to the 6413nt strand of the OFV bipartite genome.

Single and Multiplex RT-PCR Amplification of OFV, CymMV and ORSV

Each of the three primer pairs was tested by single PCR containing positive cDNA template. For single PCR reactions, 1µL of cDNA template was added to 19µL of PCR mixture, which consisted of 10µL of HotstarTaq*Plus* MasterMix (Qiagen, Doncaster, Victoria), 1µL of forward and reverse primers (10µM each) and 7µL of RNase-free water. Primer pairs were then tested by multiplex PCR containing mixtures of cDNA template. For multiplex PCR, individual cDNA templates were used in addition to equimolar mixtures of cDNA templates to detect all possible combinations of the three viruses (ie. OFV + CymMV; OFV + ORSV; CymMV + ORSV; OFV + CymMV + ORSV). The multiplex PCR mixture contained 1µL of cDNA (single or pooled templates) added to 19µL of PCR mixture, which consisted of 10µL of HotstarTaq*Plus* MasterMix (Qiagen), 1µL of each forward primer (3µL in total of forward primer), 1µL of each reverse primer (3µL in total of reverse primer) and 3µL of RNase-free water. A series of optimization steps were performed to determine the most appropriate annealing temperature, cycle number and proportion of each of the three virus-specific primer pairs in the multiplex reaction mixture. For multiplex PCR, the optimal concentration for OFV primers was 0.4µM, while for both CymMV and ORSV primer pairs was 10µM. Amplifications were performed in an Eppendorf Mastercycler Gradient 5331. Annealing temperature in the multiplex assay was determined by gradient PCR using single incremental temperature units from 50.0^0C to 70.5^0C. Cycling conditions for both single and multiplex PCR consisted of initial heat activation at 95^0C for 5 minutes; followed by 35 cycles of denaturation at 95^0C for 1 minute, primer annealing at 60^0C for 1 minute, and primer extension at 72^0C for 1 minute; terminating in final extension at 72^0C for 10 minutes. Reaction products (5µL) were analysed on 1.5% agarose gel electrophoresis buffered in 1mM sodium borate solution, run at 180V for 25 minutes. Reaction products were then visualized under UV transillumination after staining with 0.1µL/1mL SYBR® Safe DNA Gel stain (Life Technologies, Mulgrave, Australia) using a 50bp ladder to determine size of products. To confirm the lack of nonspecific amplification, every RT-PCR run included water as a non-template control and cDNA from a healthy plant sample as negative control.

Sensitivity Testing of Individual and Multiplex PCR assays

To determine the limits of detection for both single and multiplex PCR assays, a tenfold serial dilution of cDNA virus templates was performed (ie. from 10^{0} to 10^{-4}). Single and multiplex PCR reactions using the cDNA template dilutional series were carried out simultaneously in order to directly compare assay sensitivities. Amplification products were analysed as previously reported.

Field Validation of Multiplex PCR assay and Comparison with TransmissionElectron Microscopy

Following optimization, the multiplex RT-PCR assay was applied to orchid leaf samples provided by Mr. Shane Hossel (TASAG ELISA and Pathogen Testing Service). Orchid leaf samples came from private collections in New South Wales and Victoria and displayed symptoms strongly suggestive of virus infection. In addition, a local Hobart collection was surveyed and further orchid leaf samples were taken to detect the presence of virus infections using the multiplex PCR assay. A total of 29 leaf samples from orchids comprising five different genera (*Cymbidium, Phalaenopsis, Masdevallia, Oncidium, Dendrobium*) were tested. Multiplex PCR test results were compared to diagnoses made by TEM on the same leaf samples.

For examination of samples by TEM, a 2-3 mm square section of symptomatic plant tissue was macerated and plant tissue sap extracted in a drop of ammonium molybdate stain on a glass microscope slide. Sap extracted in the negative stain was then transferred to a carbon-coated parlodion film grid by placing the grid copper-side down in the extraction mixture. Copper grids were allowed to air dry for 10 minutes before being examined with a Phillips 201 Transmission Electron Microscope (20000x). Results from TEM were re-confirmed by either Mr. Peter Cross or Mr. Shane Hossel (TASAG ELISA and Pathogen Testing Service).

Results

Specificity and Compatibility of Primer Sets

A series of single reverse transcriptase-polymerase chain reaction (RT-PCR) assays were performed in a gradient thermocycler to determine optimal annealing temperatures. For OFV and ORSV primers, visible amplicon occurred over a wide range of annealing temperatures in single RT-PCR : 50.0^0C – 70.5^0C and 50.0^0C – 68.1^0C respectively (Fig. 2a, 2b). In contrast, detection of CymMV amplicon in single RT-PCR assays occurred over a narrow range of annealing temperatures from 50.0^0C – 63.5^0C (Fig. 2c)

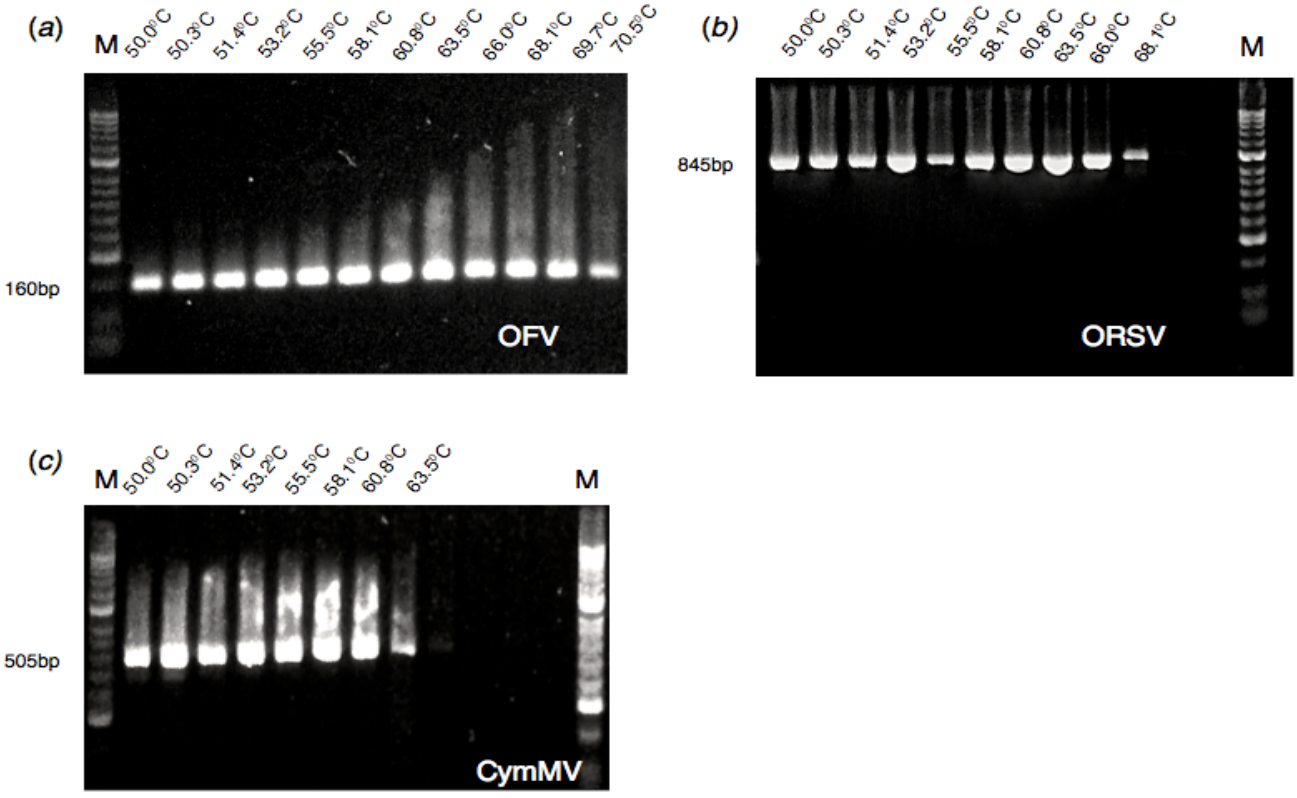


Figure 2. Optimisation of annealing temperature using orchid fleck virus (OFV), odontoglossum ringspot virus (ORSV) and cymbidium mosaic virus (CymMV) coat protein gene-specific primers to amplify single target virus cDNA in multiplex reverse transcription-polymerase chain reaction (mRT-PCR). Analysis of amplification products (5µL) was on 1.5% agarose gel electrophoresis. Details on primers in Table 2. Lanes M = 50bp DNA size marker. Lanes from 50.0^0C – 70.5^0C indicate incremental annealing temperatures in Gradient PCR. ***(a)***mRT-PCR to amplify OFV in cymbidium. ***(b)***mRT-PCR to amplify ORSV in cymbidium. ***(c)***mRT-PCR to amplify CymMV in cymbidium.

In multiplex RT-PCR, equimolar concentrations of all primer pairs (10μM) resulted in suppression of CymMV at annealing temperature below 55.5^{0}C with no amplification above 66.0^{0}C (Fig. 3). Furthermore, with all primers at 10μM the OFV amplicons were considerably more intense than those for CymMV and ORSV (Fig. 3). In order to optimise primer concentrations, a series of multiplex reactions with a mixture of all three viral cDNA template and increasing dilutions of OFV primer from 10 to 0.1μM was performed. In these reactions concentrations of CymMV and ORSV primer was constant at 10μM. The dilutional series revealed that the most favourable OFV primer concentration for multiplex assay was 0.4μM (Fig. 4). Under these conditions, amplification of all three viral cDNAs was relatively uniform (Fig. 4). Therefore, subsequent multiplex reactions were performed with 0.4μM of OFV primers and 10μM of CymMV and ORSV primers.

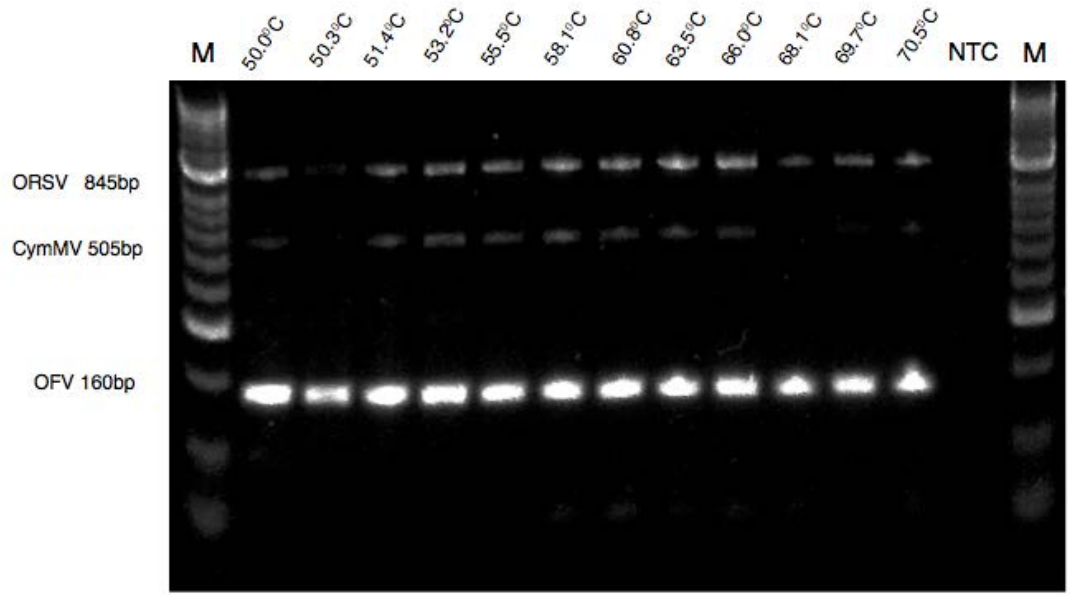


Figure 3 Optimisation of annealing temperature using orchid fleck virus (OFV), odontoglossum ringspot virus (ORSV) and cymbidium mosaic virus (CymMV) coat protein gene-specific primers for simultaneous amplification of all three target virus cDNAs in multiplex reverse transcription-polymerase chain reaction (mRT-PCR). Analysis of amplification products (5μL) was on 1.5% agarose gel electrophoresis. Details on primers in Table 2. Lanes M = 50bp DNA size marker. Lanes from 50.0^{0}C – 70.5^{0}C indicate incremental annealing temperatures in Gradient PCR.

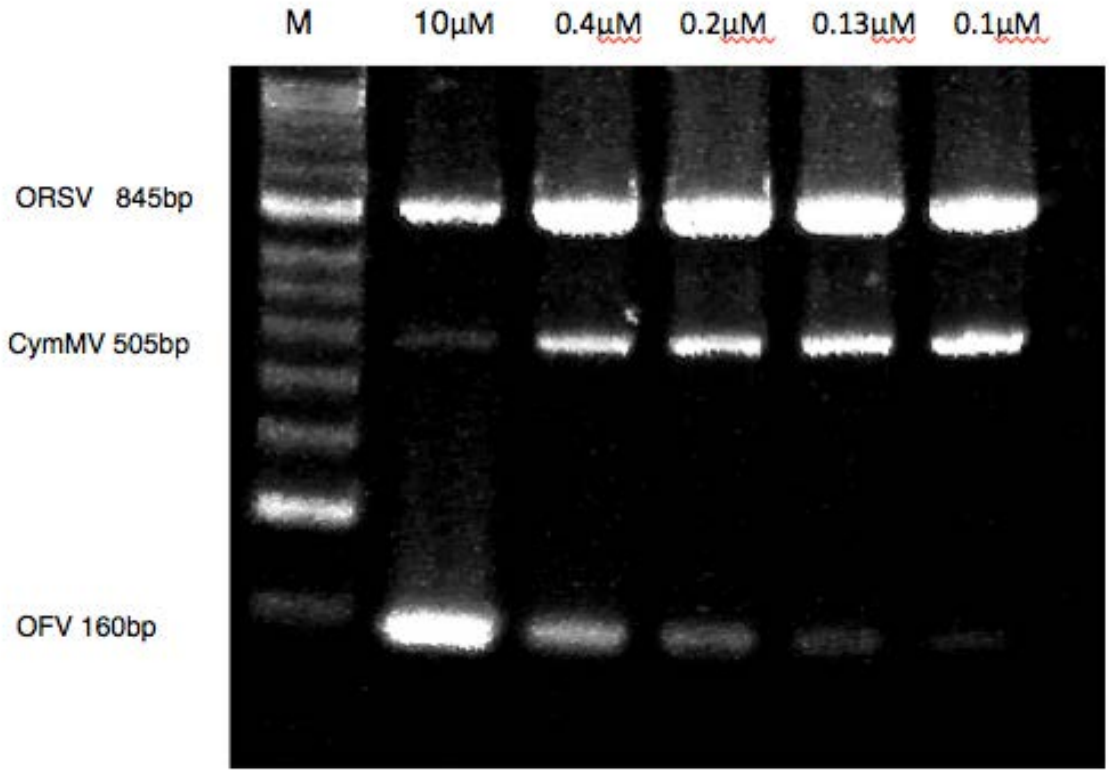


Figure 4. Optimisation of primer concentration of orchid fleck virus (OFV) coat protein gene-specific primer in a multiplex reverse transcription-polymerase chain reaction (mRT-PCR) with fixed concentrations (10µM) of odontoglossum ringspot virus (ORSV) and cymbidium mosaic virus (CymMV) coat protein gene-specific primers. Each multiplex RT-PCR mixture contained an equimolar concentration of OFV, ORSV and CymMVcDNAs. Annealing temperature for all reactions was 60^0C. Analysis of amplification products (5µL loaded in each lane) was on 1.5% agarose gel electrophoresis. Details on primers in Table 2. Lane M = 50bp DNA size marker; Lanes from 10µM to 0.1µM refer to concentration of OFV-specific primer in mRT-PCR.

Detection of OFV, CymMV and ORSV by multiplex RT-PCR was deemed optimal at an annealing temperature of 60^0C for 1 minute (OFV primers 0.4µM; CymMV and ORSV primers 10µM) using 35 amplification cycles. Three different sizes of amplified product specific to the targeted viruses were observed on 1.5% agarose gels : 160bp for OFV, 505bp forCyMV and 845bp for ORSV (Fig. 5, lanes 2-4). Furthermore, all possible combinations of viral cDNA mixture in multiplex reactions produced virus-specific amplicon with no evidence of primer dimer formation or non-specific primer annealing (Fig. 5, lanes 5-9). Therefore, all primer pairs were deemed compatible with each other and without significant interference when used in the same reaction.

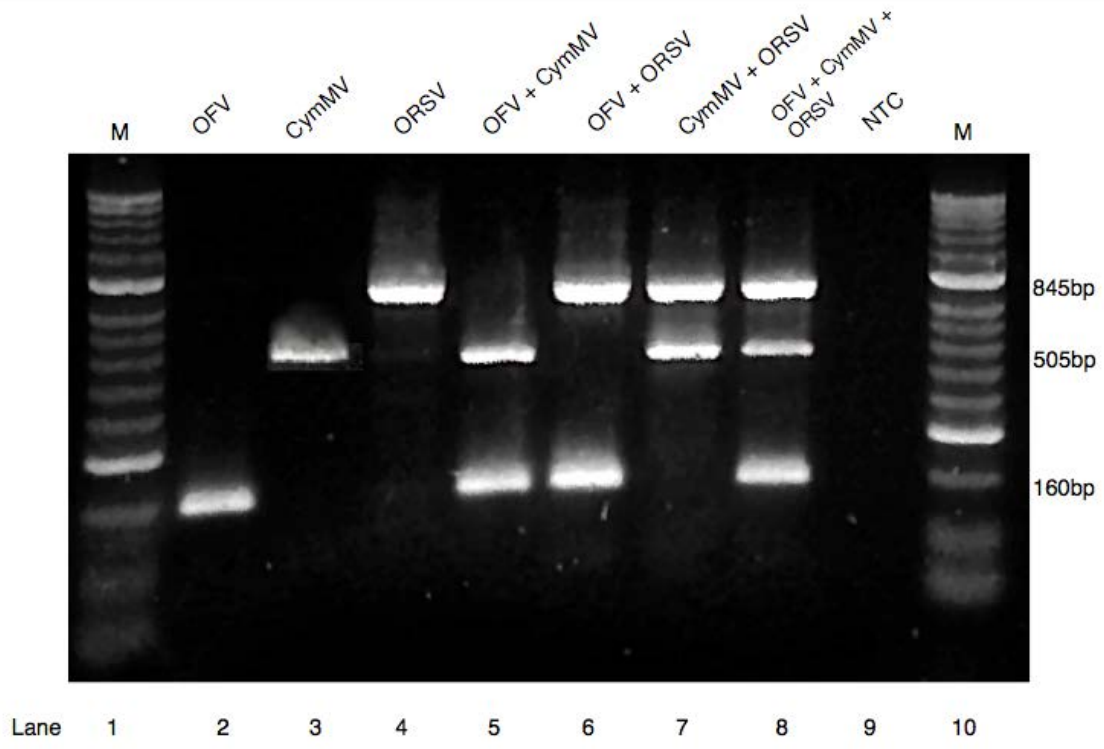


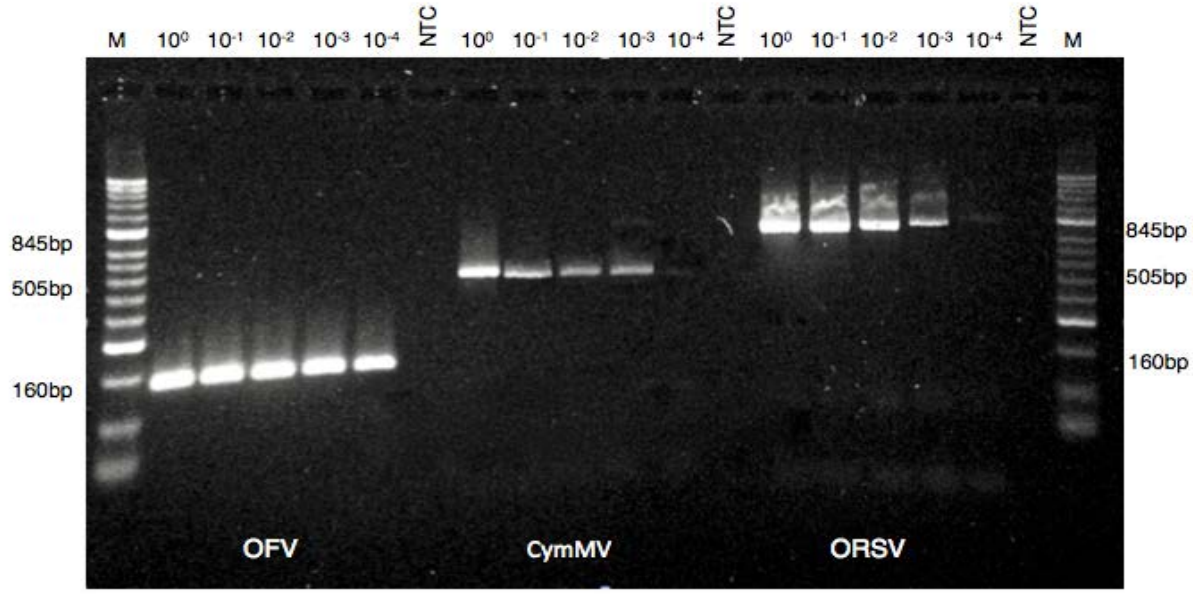
Figure 5. Multiplex reverse transcriptase-polymerase chain reaction (mRT-PCR) assay for detection of orchid fleck virus (OFV), cymbidium mosaic virus (CymMV)and odontoglossum ringspot virus (ORSV) in cymbidiums with coat protein-specific primers. Analysis of amplification products (5µL loaded in each lane) was on 1.5% agarose gel electrophoresis. Details on primers in Table 2. Lane 1 and 10 = 50bp DNA size marker; lane 2, 3 and 4 = single cDNA templates of OFV, CymMV and ORSV respectively. Lane 5 – 9 contain mixtures of cDNA templates as follows : lane 5 = OFV + CymMV; lane 6 = OFV + ORSV; lane 7 = CymMV + ORSV; lane 8 = OFV + ORSV + CymMV. Lane 9 = healthy cymbidium control.

Single and Multiplex RT-PCR Sensitivity

In order to compare the relative sensitivity of single and multiplex RT-PCR assays, serial tenfold dilutions of cDNA template from positive controls of OFV, CymMV and ORSV were used. Detection limits of single RT-PCR assays using virus-specific primer pairs varied. Virus-specific bands for OFV were easily observed after cDNA template had been diluted to an endpoint of 10^{-4} (Fig. 6a). However, detection limits for CymMV and ORSV occurred at an endpoint of 10^{-3} (Fig. 6a). In the multiplex RT-PCR assay, cDNA mixtures of all three viruses could be detected after mixed template had been diluted up to 10^{-3} (Fig. 6b).

(a)

(b)


M
10⁰
10⁻¹
10⁻²
10⁻³
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NTC
10⁰
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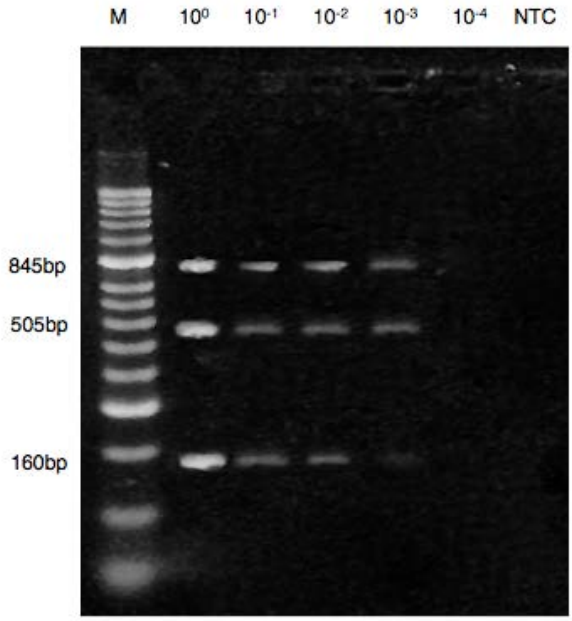


Figure 6. Comparison of the sensitivity of single and multiplex reverse transcriptase-polymerase chain reaction (RT-PCR) assays. Analysis of amplification products (5µL loaded in each lane) was on 1.5% agarose gel electrophoresis. Details on primers in Table 2.**Panel a** : Limits of detection in each single RT-PCR. Lane M = 50bp DNA size marker; Lanes 10^0 to 10^{-4} indicate 10-fold serial dilutions of orchid fleck virus (OFV), cymbidium mosaic virus (CymMV) and odontoglossum ringspot virus (ORSV) cDNA templates; Lane NTC = negative control with healthy cymbidium cDNA. **Panel b** : Limits of detection in multiplex RT-PCR. . Lane M = 50bp DNA size marker; Lanes 10^0 to 10^{-4} indicate 10-fold serial dilutions of OFV, CymMV and ORSV cDNA templates. Lane NTC = negative control with healthy cymbidium cDNA.

Field Sample Detection by multiplex RT-PCR and TEM

The multiplex RT-PCR assay was applied to orchid leaf samples displaying symptoms suggestive of viral infection but without any previous confirmed diagnoses. A total of 29 orchid leaf samples were gathered from New South Wales, Victoria and a single private collection in Hobart for testing. The multiplex RT-PCR assay was successful in detecting infection in all genera of orchids tested (Fig. 8). All 29 of the sample plants gathered tested positive for single or mixed infections of OFV, CymMV and ORSV (Fig. 8). Transmission Electron Microscopy (TEM) was used to verify all diagnoses in the same leaf samples, with no false negatives noted. However, TEM failed to detect co-infection with OFV in three samples diagnosed as single infection with CymMV or ORSV, despite a repeat analysis (Fig. 8).

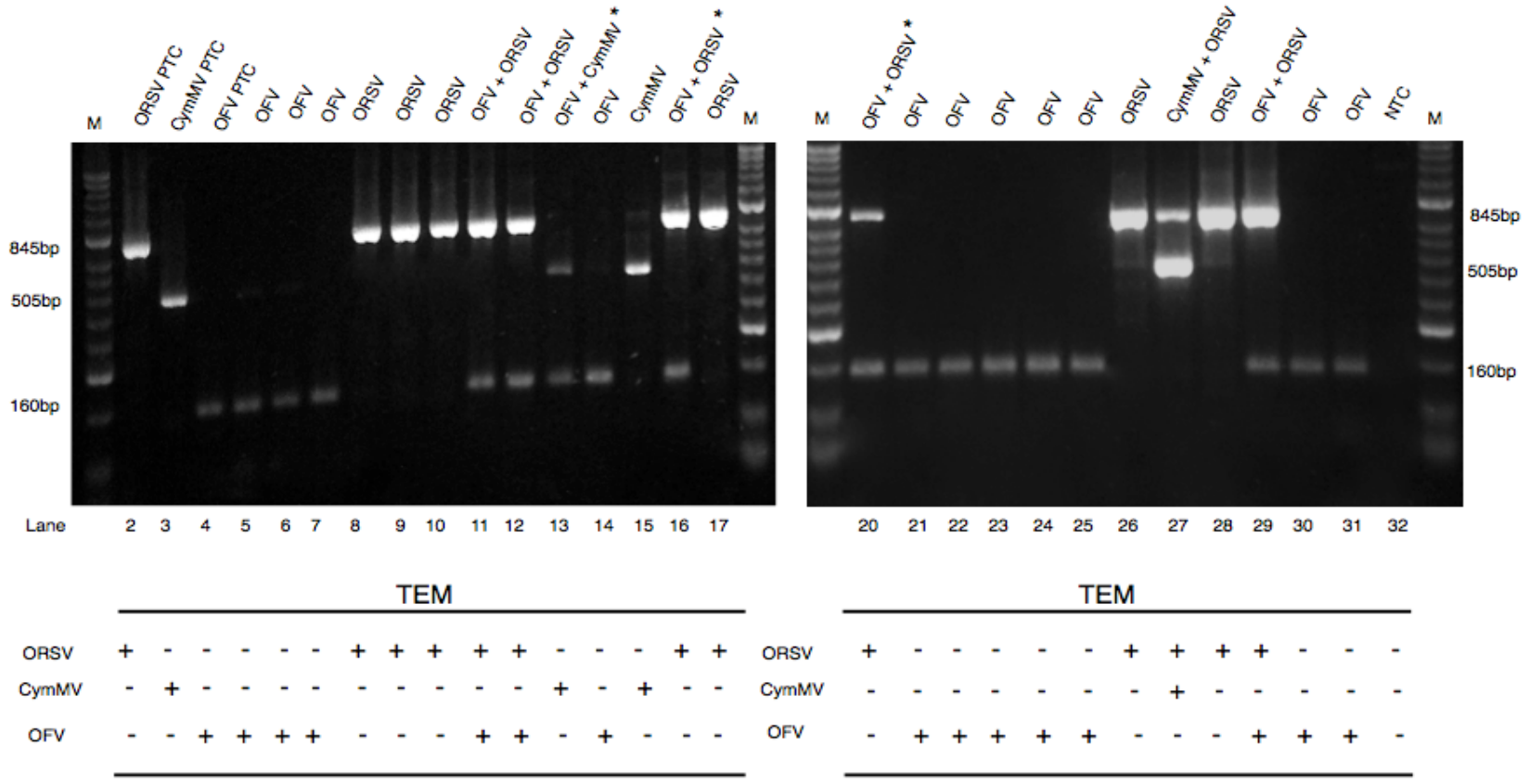


Figure 8. Orchid leaf samples (n=29) originating from private collections around Australia were tested by multiplex reverse transcriptase-polymerase chain reaction (mRT-PCR) and Transmission Electron Microscopy (TEM) for orchid fleck virus (OFV), cymbidium mosaic virus (CymMV)and odontoglossum ringspot virus (ORSV). For mRT-PCR, analysis of amplification products (5µL loaded in each lane) was on 1.5% agarose gel electrophoresis. Details on primers in Table 2. Lane 1, 18, 19, 33 = 50bp DNA size marker (M); lane 2, 3 and 4 = positive cDNA template controls (PTC) for ORSV, CymMV and OFV respectively. Lanes 5 – 17 and lanes 20 – 31 contain field samples. Lane 32 = healthy cymbidium control. Infected and uninfected samples, based on TEM results are indicated by + and –, respectively. * Denotes co-infection with OFV missed on Transmission Electron Microscopy (TEM).

Discussion

This study is the first to describe the use of a multiplex reverse transcriptase-polymerase chain reaction (multiplex RT-PCR) assay for simultaneous detection and differentiation of the three most common viruses infecting commercial orchids worldwide ie. cymbidium mosaic virus (CymMV), odontoglossum ringspot virus (ORSV) and orchid fleck virus (OFV). Following several optimisations, the multiplex RT-PCR assay developed in this study was able to detect all possible combinations of the three RNA viruses and was successfully applied in diagnosis of viral infection in orchids from multiple genera. Over the last two decades, there have been significant advances in molecular phytodiagnostic approaches enabling detection of viruses at extremely low concentrations (Mumford et al., 2006, James et al., 2006). Multiplex RT-PCR has enabled rapid detection of multiple viruses in one reaction, doing away with the need for numerous, resource-intensive single RT-PCR assays (Sanchez-Navarro et al., 2005, Gambino and Gribaudo, 2006). Multiplex RT-PCR has been used extensively for virus detection in cereals, fruit trees, berries, vegetables and ornamentals (Tao et al., 2012, Ma et al., 2008, Chang et al., 2007, Quintero-Vasquez et al., 2013, Hu et al., 2009). The development of a multiplex RT-PCR assay requires a systematic approach, with repeated tests to determine optimal reaction conditions (Henegariu et al., 1997, Wei et al., 2008). Primer selection plays a fundamental role in the specificity of the assay (Henegariu et al., 1997). By designing primers that target conserved regions of the viral genome, the multiplex RT-PCR assay may detect multiple strains or isolates despite natural intraspecific sequence variability (Henegariu et al., 1997). In this study, redundant nucleotides at multiple positions in OFV primer sequences allowed for natural isolate variation in the coat protein gene, thereby increasing chances of detecting most, if not all variants of OFV. Further primer analysis with computer software can predict incompatible primer interactions in a multiplex assay, such as primer self-annealing, self-looping and annealing between forward and reverse primers (Wei et al., 2009). However, empirical testing is still necessary to

manipulate efficiency of primer amplification of target product in the multiplex reaction (Tao et al., 2012). In particular, inappropriate ratios of primers may weaken amplification or cause non-specific amplification of target templates (Tao et al., 2012). In this study, the coat protein genes of OFV, CymMV and ORSV were shown to be suitable genomic regions for reliable detection of these viruses using multiplex RT-PCR. All primers in this study were designed correctly, with no evidence of incompatible interactions, and were able to be used at concentration of 10μM in simplex RT-PCR assay. However, successful detection of all three viruses in multiplex RT-PCR required adjustment of concentration ratios of primers. Using equimolar concentrations of all three primer pairs resulted in preferential amplification of the shortest fragment, OFV (160bp), and suppression of longer fragments, CymMV (505bp) and ORSV (845bp). Previous studies have reported the tendency for shorter fragments to amplify with greater efficiency than longer fragments (Garson et al., 1998, Hu et al., 2009, Du et al., 2006, Wei et al., 2009). However, lowering the concentration of the OFV primers to 0.4μM, while maintaining the ORSV and CymMV primers at 10μM improved detection of all three viruses, producing sufficiently intense bands that could be easily differentiated on agarose gel electrophoresis. Therefore, this study, alongside other previous studies (Roy et al., 2010), has demonstrated that amplicon sizes of 800-950bp can be successfully used in multiplex PCR provided primer concentration ratios are optimised. The other parameter that required optimisation was annealing temperature of primer pairs. By using gradient PCR, an annealing temperature of 60^0C was determined as ideal in both simplex and multiplex RT-PCR assays.

In this study, the sensitivity of multiplex RT-PCR was shown to be lower than simplex RT-PCR for detection of OFV, consistent with findings comparing simplex with multiplex RT-PCR for other plant viruses (Tao et al., 2012, Gambino and Gribaudo, 2006, Ge et al., 2013). This is to be expected, since a combination of several primer pairs in a multiplex PCR assay necessitates the simultaneous consumption of polymerase enzyme, buffer and dNTPs by more than one amplicon (Tao et al., 2012). Furthermore, in a multiplex reaction, a mixture of

primer pairs, instead of one pair, are competing for cDNA templates, sometimes in an antagonistic fashion (Gambino and Gribaudo, 2006). However, the level of sensitivity of multiplex RT-PCR reported in this study has been deemed acceptable for the routine testing of plant samples by other authors (Tao et al., 2012, Gambino and Gribaudo, 2006, Ge et al., 2013). In this study, specific bands produced in multiplex RT-PCR could be detected for OFV, CymMV and ORSV on agarose gel electrophoresis at positive cDNA template dilutions of up to 1:1000. This is equivalent to 0.05mg of infected leaf tissue in a total leaf sample of 50mg using commercial spin column kitsfor nucleic acid extraction. Therefore, the multiplex RT-PCR developed in this study will benefit growers who require mass-sampling from large collections, since detection of a single positive sample in a bulk extract is possible.

In general, diagnosis of viral infection in orchids has relied upon either direct visualization of particle morphology by transmission electron microscopy (TEM) or immunological techniques, such as enzyme-linked immunosorbent assay (ELISA) (Ryu et al., 1995, Wilson, 1999). Although serological diagnosis is readily available for CymMV and ORSV, this is not the case for OFV because of virion instability thwarting attempts to purify virus particles (Wilson, 1999). Laboratory testing services still continue to diagnosis OFV by direct examination of the leaf sap with TEM (Wilson, 1999, Batchman, 2008, Gibbs, 2000). In contrast to TEM and serological techniques such as ELISA, multiplex RT-PCR can be promptly applied in independent laboratories once primer sequences and reaction protocols have been confirmed and made accessible (Peiman and Xie, 2006, Viswanathan et al., 2010). Furthermore, TEM is potentially unreliable for the diagnosis of OFV since the virus often occurs at very low concentrations (Wilson, 1999). Importantly, orchid plants infected with CymMV or ORSV may have co-infection with OFV, as this study has demonstrated. Multiplex RT-PCR has greater sensitivity than TEM for detecting viral infection at low concentrations, in addition to being able to detect more than one virus simultaneously (Viswanathan et al., 2010). In this study, application of multiplex RT-PCR to orchid samples from various private collections demonstrated that the

molecular assay was more sensitive than TEM in detecting co-infection with OFV. In three plants infected with CymMV or ORSV, multiplex RT-PCR detected co-infection with OFV where TEM did not. Both CymMV and ORSV typically occur at higher concentrations in symptomatic plants than OFV (Wilson, 1999). Consequently, abundant filamentous and rod-shaped particles of CymMV and ORSV may reduce the chance of visualizing sporadic bullet-shaped particles of OFV using TEM (analogous to looking for a needle in a haystack).

Although not observed in this study, false negatives remain an important limitation of RT-PCR assays for virus detection (Vincelli and Tisserat, 2008, James et al., 2006). False negatives in RT-PCR assays may arise from RNA degradation or from plant extract contaminants (eg. acidic polysaccharides or polyphenolic compounds) that contain inhibitors of reverse transcriptase or Taq polymerase (Ge et al., 2013, Ma et al., 2008). Increasingly, multiplex RT-PCR assays for virus detection incorporate internal controls to take into account the possibility of false negatives (Ge et al., 2013, Ma et al., 2008, Gambino and Gribaudo, 2006, Hu et al., 2010). Suitable internal controls amplify fragments of highly conserved endogenous regulatory genes whose expression is unaffected by seasonal, ontogenic or pathophysiological factors (Mumford et al., 2006). Furthermore, these regulatory genes may be co-extracted from samples along with target virus nucleic acid if present. In multiplex RT-PCR assays for RNA viruses, inclusion of primers carefully designed to target spliced regulatory gene sequences can confirm both the success of total RNA extraction as well as the RT-PCR process (Menzel et al., 2002, Mumford et al., 2006). Therefore, further refinement of the multiplex RT-PCR assay developed in this study could be achieved by addition of compatible primers for co-amplification of an orchid mRNA internal control. Possible internal controls include the 18s ribosomal RNA (rRNA) used in potato (Du et al., 2006) or the mitochondrial *nad5* gene (NADH dehydrogenase subunit 5) used successfully in pome fruit, grapes and ornamentals including orchids (Ma et al., 2008, Gambino and Gribaudo, 2006, Hu et al., 2010, Lee and Chang, 2006). However, use of the *nad5* gene as an internal control in this study may be limited given that the amplicon size reported

by Lee and Chang (2006) in *Phalaenopsis* orchid extracts (185bp) may be poorly differentiated from OFV amplicon (160bp). Inclusion of an internal control to the multiplex RT-PCR assay in this study will likely require a new series of optimization reactions, since amplification efficiency of internal controls may vary between plant species and has been shown to interfere with virus amplification (Hu et al., 2010).

In conclusion, a new multiplex RT-PCR assay was established to detect OFV, CymMV and ORSV simultaneously in infected orchids from multiple genera. This assay provides a highly specific and sensitive test for detection of these serious viral pathogens in single and mixed infections. Since mixed infections with OFV, CymMV and ORSV do occur in orchids, this assay will be useful for future epidemiological studies as well as ongoing analysis of genetic variation between isolates. Control of viral infection in orchids depends on roguing of infected plants and propagation from virus-free germplasm (Zettler et al., 1990, Wilson et al., 1999). This multiplex assay is well suited to screening large numbers of plants or for screening nursery stock intended to be used as propagative material in tissue culture or as flasked seedlings. In addition, this assay can be used to identify asymptomatic plants with low titre infections to stop the spread of virus in private collections and commercial nurseries. Given that OFV, CymMV and ORSV all have a global distribution and are the three most common viruses infecting orchids, this multiplex RT-PCR assay has high practical use.

Capacity of a potential novel *Brevipalpus*species (Acari : Tenuipalpidae) to acquire and transmit *Orchid fleck virus*

Introduction

Flat mites (family Tenuipalpidae) are closely related to spider mites (Tetranychidae), with all members possessing a characteristic pair of enlongated stylet-like mouthparts used to pierce and feed on plant cell contents (Childers and Rodrigues, 2011, Beard et al., 2012, Rodrigues and Childers, 2013). Multiple species in the genus *Brevipalpus*are considered economic pests of crop or ornamentalplants (Childers et al., 2003b). In addition to feeding injury to leaves, *Brevipalpus*mites are known to inject toxic saliva into plant tissue, causing diffuse chlorotic blotching on leaves, corky scabs on fruit and galling in stems of many host plants (Childers et al., 2003a, Prabheena and Ramani, 2013). The most significant impact of *Brevipalpus*mites in agricultural and horticultural production systems is in the spread of one or more plant viruses, including *Citrus leprosis virus C* (CiLV-C), *Passion fruit green spot virus* (PFGSV), *Coffee ringspot virus* (CoRSV) and *Orchid fleck virus* (OFV) (Bastianel et al., 2010, Kitajima et al., 2003, Chagas et al., 2003, Kondo et al., 2003). OFV was shown to be spread in a persistent manner by *B. californicus*using immunoelectron microscopy and negative staining electron microscopy(Kondo et al., 2003). OFV has also been detected in *B. californicus*mites by reverse transcriptase-polymerase chain reaction (RT-PCR) with OFV coat protein gene-specific primers (Kubo et al., 2011). However, mites previously referred to as *B. californicus*are now believed to constitute a species complex representing at least three distinct lineages (Beard et al., 2012, Navia et al., 2013). Attempts to resolve the challenging systematics of *Brevipalpus*species still continues, through DNA sequencing and detailed morphological analysis using light microscopy and low temperature scanning electron microscopy (Beard et al., 2012). Besides the presence of cryptic species complexes within the genus, little is known about the competence of other *Brevipalpus*species to vector OFV, particularly those that are pests on orchids. Recently, a population of *Brevipalpus*mites was detected in Hobart, Tasmania. Using this population, the aims of this study were to (i) identify these *Brevipalpus*mites to species level; and (ii) determine whether or not these mites

could acquire and transmit OFV to healthy cymbidiums using quantitative RT-PCR.

Materials and Methods

Source populations and rearing techniques

A population of *Brevipalpus*mites was detected on a collection of spider orchids (*Brassiaverrucosa*Bateman ex Lindl.) in a single location in Hobart, Tasmania. Mites were detected by direct inspection using a hand lens (20x) and stereomicroscope (40x). Infested leaves of *B. verrucosa*host plants were excised and stored in sealed plastic bags for transport back to the laboratoryon 18/02/2013, 15/03/2013 and 22/04/2014. Stock colonies were initiated from adult female and male specimens present on excised leaves using a single paint-brush hair to transfer mites individually. Stock colonies were maintained on virus-free cymbidium plants kindly provided by Mr. Graham Morris, South Australian Cymbidium Growers Association. Stock colonies were kept in an isolated rearing room in which transmission assays described below were also conducted.

Morphological analysis

In order to study diagnostic morphological features, adult female specimens were cleared in Kono's solution for 48-72 hours,then mounted in the dorso-ventral position with Hoyer's medium on microscopic slides. Mounted specimens were protected with a cover slip sealed with a ring of waterproof paint after being oven dried for 2 days at 40^0C. Slide mounted specimens were examined by phase contrast and differential interference contrast microscopy under 40x and 100x objectives using a Nikon Eclipse 80i microscope. Extended depth of field images of certain morphological structures were obtained from several partially focused images (taken with a 12.6 megapixel digital camera connected to a computer) that were blended using CombineZP free software.

Morphological identification of specimens to species level was attempted using an online interactive key designed with Lucid3 software (Beard et al., 2012).

Molecular Analysis : DNA extraction, amplification and sequencing

Genomic DNA was extracted from six samples each containing 10 adult female mites using the DNeasy Tissue Kit (Qiagen, Doncaster, Victoria) following the protocol for animal cultured cells. Fresh mites were placed in each of the microcentrifuge sample tubes and crushed using a sterile glass pestle. All steps of the Qiagen protocol were followed except that all volumes of reagents were halved and genomic DNA was recovered by eluting with 50µL of DEPC-treated water. Amplification of a fragment of the mitochondrial cytochrome *c* oxidase subunit I gene (COI) by PCR was achieved using the primers DNF 5' TAC AGC TCC TAT AGA TAA AAC 3' and DNR 5' TGA TTT TTT GGT CAC CCA GAA G 3' (Navia et al., 2013). For the PCR, 1µL of mite cDNA template was added to 19µL of reaction mixture which consisted of 10µL of HotstarTaq*Plus*MasterMix (Qiagen), 1µL of forward primer,1µL of reverse primer and 7µL of RNase-free water. Cycling conditions for the PCR consisted of initial heat activation at 95^0C for 5 minutes; followed by 35 cycles of denaturation at 95^0C for 1 minute, primer annealing at 52^0C for 1 minute, and primer extension at 72^0C for 1.5 minutes; terminating in final extension at 72^0C for 10 minutes. PCR products (5µL) were checked by visualizing on a 1.5% agarose gel stained with 0.1µL/1mL SYBR® Safe DNA Gel stain (Life Technologies). Target amplicon of sufficient concentration was then purified using a QIAquick PCR Purification kit (Qiagen). Purified amplified fragments were sent to the Australian Cancer Research Foundation Biomolecular Resource Facility (Canberra, ACT) for Sanger sequencing, yielding a total of five COI fragment sequences (433bp) with 100% sequence homology.

Sequence alignment and phylogenetic analysis

A data set was compiled using the COI fragment sequence from this study and a further 46 *Brevipalpus*COI fragment sequences obtained from GenBank (NCBI) that had been published in peer-reviewed journals. For the outgroup, a COI sequence of *Cenopalpuspulcher* (Canestrini&Fanzago; Tenuipalpidae) was used based on previous phylogenetic analysis by (Navia et al., 2013). Details of the GenBank accession numbers of sequences used, assigned species names, geographical location, host plant association and references are presented in Table 3. Alignment of the longest reading frame of each COI sequences was performed using the ClustalW multiple alignment procedure (Thompson et al., 1994) in Molecular Evolutionary Genetics Analysis version 5.2. (MEGA5) (Tamura et al., 2011). No manual adjustments to the alignment were required. Genetic distances were determined using Kimura's 2-parameter model (Kimura, 1980) for nucleotide sequences to build a phylogenetic tree using the Neighbour-Joining (NJ) algorithm in MEGA5. Estimates of standard error were performed with a bootstrap procedure (1000 replications).

Table 3.Overview for *Brevipalpus*COI fragment sequence data set, including *Cenopalpus*outgroup used in phylogenetic analysis.

Host plant species, family	Country	GenBank accession	Identification	Reference
Brassiaverrucosa, Orchidaceae	Hobart, Tasmania	N/A	*B.* n. sp.	this study
unknown	The Netherlands	DQ450480	*B.* n. sp.	Groot (2006)
unknown	The Netherlands	DQ450481	*B.* n. sp.	Groot (2006)
*Ligustrum*sp., Oleaceae	Brazil	KC291366	*B. phoenicis*type 2	Navia et al. (2013)
*Ligustrum*sp., Oleaceae	Brazil	KC291367	*B. phoenicis*type 2	Navia et al. (2013)
Malpighiaglabra, Malpighiaceae	Brazil	KC291368	*B. phoenicis*type 2	Navia et al. (2013)
Citrus sp., Rutaceae	Brazil	KC291370	*B. phoenicis*type 2	Navia et al. (2013)
Hibiscus sp., Malvaceae	Brazil	KC291371	*B. phoenicis*type 2	Navia et al. (2013)
Coffea Arabica, Rubiaceae	Brazil	KC291372	*B. phoenicis*type 2	Navia et al. (2013)
Malpighiaglabra, Malpighiaceae	Brazil	KC291373	*B. phoenicis*type 2	Navia et al. (2013)
Citrus sp., Rutaceae	Brazil	KC291374	*B. phoenicis*type 2	Navia et al. (2013)
Hibiscus sp., Malvaceae	Brazil	KC291375	*B. phoenicis*type 2	Navia et al. (2013)
Citrus sp., Rutaceae	Brazil	KC291376	*B. phoenicis*type 2	Navia et al. (2013)
Hibiscus sp., Malvaceae	Brazil	KC291377	*B. phoenicis*type 2	Navia et al. (2013)
Spondiaspurpurea, Anacardiaceae	Brazil	KC291378	*B. phoenicis*type 2	Navia et al. (2013)
Citrus sp., Rutaceae	Brazil	KC291379	*B. phoenicis*type 2	Navia et al. (2013)
Malvaviscusarboreus, Malvaceae	Brazil	KC291380	*B. phoenicis*type 2	Navia et al. (2013)
Malvaviscusarboreus, Malvaceae	Brazil	KC291381	*B. phoenicis*type 2	Navia et al. (2013)
Hibiscus sp., Malvaceae	Florida, USA	AY320014	*B. phoenicis*type 2	Rodrigues et al. (2004)
Citrus sinensis, Rutaceae	Florida, USA	KC291382	*B. phoenicis*type 2	Navia et al. (2013)
Citrus sp., Rutaceae	Brazil	DQ789582	*B. phoenicis*type 2	Groot and Breeuwer (2006)
Hibiscus sp., Malvaceae	Brazil	KC291369	*B. phoenicis*?	Navia et al. (2013)
Cocos nucifera, Arecaceae	Brazil	KC291390	*B.* n. sp.	Navia et al. (2013)
Unknown	Florida, USA	KC291402	*B. californicus*	Navia et al. (2013)
Rhododendron sp., Ericaceae	Brazil	DQ789591	*B. californicus*	Groot and Breeuwer (2006)
Thevetiaperuviana, Apocynaceae	The Netherlands	DQ789594	*B. californicus*	Groot and Breeuwer (2006)
*Ligustrum*sp., Oleaceae	Brazil	KC291387	*B. phoenicis*type 1	Navia et al. (2013)
Alnussubcordata, Betulaceae	Brazil	KC291388	*B. phoenicis*type 1	Navia et al. (2013)
Alnussubcordata, Betulaceae	Brazil	KC291389	*B. phoenicis*type 1	Navia et al. (2013)
Malpighiaglabra, Malpighiaceae	Brazil	DQ789586	*B. phoenicis*type 1	Groot and Breeuwer (2006)
Ocimumbasilicum, Lamiaceae	Brazil	KC291383	*B. obovatus*	Navia et al. (2013)
Ocimumbasilicum, Lamiaceae	Brazil	KC291384	*B. obovatus*	Navia et al. (2013)
Ocimumbasilicum, Lamiaceae	Brazil	KC291385	*B. obovatus*	Navia et al. (2013)
Cestrum nocturnum, Solanaceae	Brazil	KC291386	*B. obovatus*	Navia et al. (2013)
Camellia sinensis	Charleston, USA	AY320028	*B. obovatus*	Rodrigues et al. (2004)
Hibiscus rosa-sinensis, Malvaceae	Brazil	DQ789590	*B. obovatus*	Groot and Breeuwer (2006)
Ligustrumsinense, Oleaceae	Chile	KC191391	*B. chilensis*	Navia et al. (2013)
Juglansregia, Juglandaceae	Chile	KC291392	*B. chilensis*	Navia et al. (2013)
Ligustrumsinense, Oleaceae	Chile	KC291393	*B. chilensis*	Navia et al. (2013)
Citrus sinensis, Rutaceae	Chile	KC291394	*B. chilensis*	Navia et al. (2013)
Cestrum parqui, Solanaceae	Chile	KC291395	*B. chilensis*	Navia et al. (2013)
*Crataegus*sp., Rosaceae	Chile	KC291396	*B. chilensis*	Navia et al. (2013)
Ligustrumsinense, Oleaceae	Chile	KC291397	*B. chilensis*	Navia et al. (2013)
Vitisvinifera, Vitaceae	Chile	KC291398	*B. chilensis*	Navia et al. (2013)
Ligustrumsinense, Oleaceae	Chile	KC291399	*B. chilensis*	Navia et al. (2013)
Ligustrumsinense, Oleaceae	Chile	KC291400	*B. chilensis*	Navia et al. (2013)
Ligustrumsinense, Oleaceae	Chile	KC291401	*B. chilensis*	Navia et al. (2013)
Unknown	Unknown	AY320029	*Cenopalpuspulcher*	Rodrigues et al. (2004)

Transmission Assays

In order to determine the capacity for *Brevipalpus*mites to acquire and transmit OFV, biological transmission assays were carried out using groups of larvae, nymphs (proto- and deuto-nymphs), adult females and adult males. Juvenile stages were not separated on the basis of gender due to the lack of reliable diagnostic features visible under a stereomicroscope. An Australian OFV isolate, maintained in a cymbidium plant displaying chlorotic fleck symptoms, was used as the source plant. Mite larvae, nymphs and adults were reared separately on excised OFV infected leaves and allowed to feed for acquisition access periods of one hour, one day and one week during early autumn in an enclosed room. Virus-infected leaves were embedded in an agar medium held in a vertical position. After each acquisition access period, a proportion of mites from each group was tested for OFV by quantitative RT-PCR. The remaining proportion of mites was used to inoculate healthy virus-free cymbidiums. For each acquisition period (one hour, one day, one week), five healthy cymbidiums were inoculated with 15-20 mites. Due to the limited number of healthy cymbidiums accessible for this experiment, a total of 15 healthy cymbidiums were inoculated with mixed batches of mites (almost equal numbers of larvae, nymphs and adults).Inoculations occurred in early autumn and cymbidiums were grown in a glasshouse environment and observed for symptom expression for six months.

Isolation of total RNA, cDNA synthesis and SYBR Green I real-time PCR

Extraction of nucleic acids from *Brevipalpus*mites was performed with PowerPlant® RNA Isolation Kit (MO-BIO, Carlsbad, California) according to the manufacturers instructions. Samples of 3-5 fresh mites (larvae, nymphs, adult females and adult males) that had fed on OFV-infected leaves were placed in microcentrifuge sample tubes and crushed with a sterile glass pestle. All steps of the MO-BIO protocol were followed except that all reagent volumes were halved and total RNA was recovered by eluting with 50µL of DEPC-treated water. First-

strand cDNA was synthesized in a reaction mixture using random hexamers, oligo dT(18) primer and M-MLV enzyme (Bioline). Total RNA was extracted from cymbidium leaf tissue six months post-inoculation using PowerPlant® RNA Isolation Kit (MO-BIO) and eluted into a final volume of 100µL. First-strand cDNA was synthesized in a reaction mixture and under conditions as previously described in section 3.2.2.

Detection of OFV in mite and cymbidium samples was carried out using iQ^{TM} SYBR® Green ISupermix (Bio-Rad, Gladesville, New South Wales). The primer pair used was OFV-ALL1F (5' GRC TKG CWG CGG AGG CWG AC) and OFV-ALL1R (5' CTG GCG GAW GGK GGT GTG AAC AG). The dye-based quantitative PCR cocktail contained 1µL of cDNA template in 9µL reaction mixture, which consisted of 5µL iQ SYBR Green Supermix (2x), 1µL of forward and reverse primer (100µM), and 1µL of DEPC-treated water. For analysis with SYBR Green I, PCR cycling consisted of an initial polymerase heat activation and DNA denaturation step of 95^{0}C for 3 minutes, followed by 40 cycles of denaturation at 95^{0}C for 15 seconds and annealing/extension at 60^{0}C for 60 seconds. Amplifications were performed in a two channel Corbett Rotor-Gene 6000 72-well real time PCR cycler with high resolution melt analyzer (Qiagen, Doncaster, Victoria). Post-amplification melt curve analysis was performed to check for primer-dimer artefact and reaction specificity. Melting temperatures of double-stranded DNA products are affected by length, percentage of GC content, base mismatches and other factors (Mackay et al., 2002, Raymaekers et al., 2009). Therefore, melt curve analysis can differentiate different PCR products based on their melting characteristics. Melt curve analysis was conducted from 60^{0}C to 95^{0}C at 0.5^{0}C increments per second with a smooth curve setting averaging 1 point. Rotor-Gene Q Series Software version 1.7 (Qiagen) was used to visualise melting curve peaks from plots of the first derivative against melting temperature. The highest curve peaks on these plots therefore represented the melting temperature (T_m) for amplicon present in the reaction.

Results

Mite Biology

Mites were successfully maintained on cymbidium plants for the duration of the study. In contrast, attempts to maintain mites on *Chenopodiummurale, C. amaranticolor, C. alba, Nicotianaglutinosa, Phaseolus vulgaris, Tetragoniaexpansa, Capsicum annuum*and *Citrus x limon*host plants failed. All four active stages typical for the genus *Brevipalpus*(ie. larva, protonymph, deutonymph and adult) and intervening dormant chrysalid stages were observed in the stock colonies collected from *Brassiaverrucosa*leaves (fig.9). The average length of adult females was 0.25 ± 0.007 mm (n=10). Females were noted to lay one egg per day over a period of over 30 days. Eggs were bright orange-red, elliptical and typically laid in clusters in leaf tissue cracks, mid-ribs and at the folded base of *Brassia*leaf sheathes (fig. 9). Adult males occurred in low frequency in stock colonies on cymbidium plants. Adult males demonstrated stereotypic sexual behaviour by guarding immature female teleiochrysalids, followed by copulation with the fully developed female on emergence (fig. 10). Rudimentary observations on the average duration of the developmental stages of mites (n=10) maintained on excised cymbidium leaves between 18-24^{0}C were : 11.4 ± 0.6 days for larvae; 9.3 ± 0.4 days for protonymphs; 10.0 ± 0.6 days for deutonymphs; and quiescent stages 5.6 ± 1.7 days each. Mites dispersed along the entire length of *Brassia*leaves, on both adaxial and abaxial surfaces, but tended to show a preference for settling along the midrib. Feeding injury on *Brassia*leaves resulted in sunken, rusty-brown lesions which were often widespread in large infestations (fig. 11).

Figure 9.*Brevipalpus*n. sp. developmental stages.Counter clockwise, starting from bottom left : clusters of eggs adhere tightly to leaf surfaces; larva (bottom) with 3 pairs of legs and protonymph (top) with 4 pairs of legs; quiescent chrysalis stage fixed to leaf surface; deutonymph; adult male; adult female.

Figure 10. Stereotypical sexual behaviour in *Brevipalpus*n. sp. Adult males expend energy guarding immature female quiescent chrysalids and attempt copulation with female after emergence.

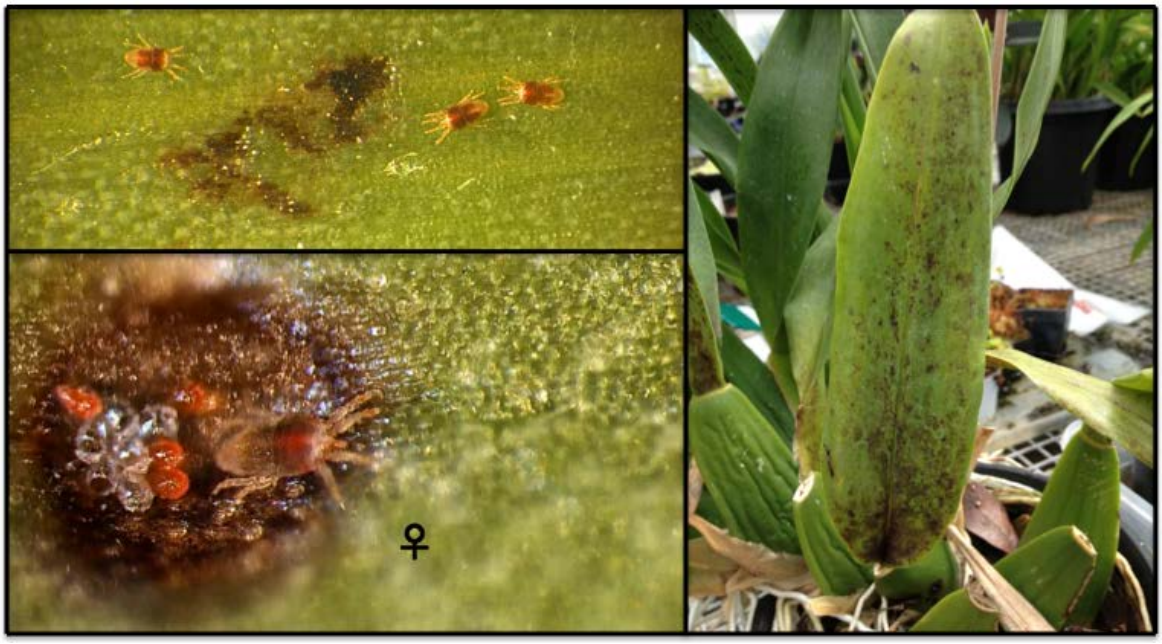


Figure 11. Feeding damage on *Brassiaverrucosa*(spider orchid) by *Brevipalpus*n. sp. mites. Adult females often lay clusters of eggs in these sunken, brown necrotic lesions.

Morphological Analysis

Morphological analysis, using informative characters of adult females described by Beard et al. (2012), confirmed the mites in this study belonging to the genus *Brevipalpus.* Adult female specimens were observed to have all four diagnostic features of *Brevipalpus*mites : six pairs of lateral opisthosomal setae, four palp segments, a fully exposed gnathosoma and a broad extension over the first three coxae and gnathosoma (fig. 12). However, identification of mites to species level was not possible using currently defined morphological criteria. Important morpholgical observations in adult females were as follows :*f2* dorsal opisthosomal setae present; tarsus II with one solenidion; ventral plate with distinct margins; and ventral plate with small to medium sized rounded cells (fig. 13 and 14). Based on these findings, the diagnosis of *Brevipalpuscalifornicus, B. phoenicis, B. obovatus, B. lewisi*or *B. chilensis*(ie. currently known economically important species) was excluded.

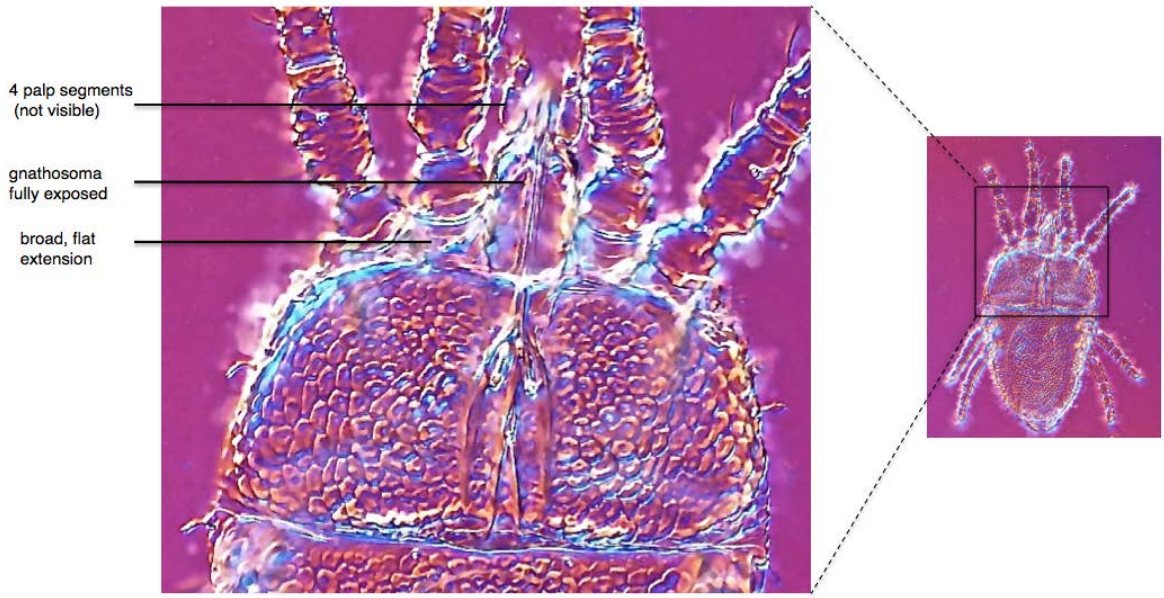


Figure 12. Diagnostic morphological features for the genus *Brevipalpus*as described by Beard et al. (2012b). **Adult female mite under differential interference contrast microscopy (100x).**

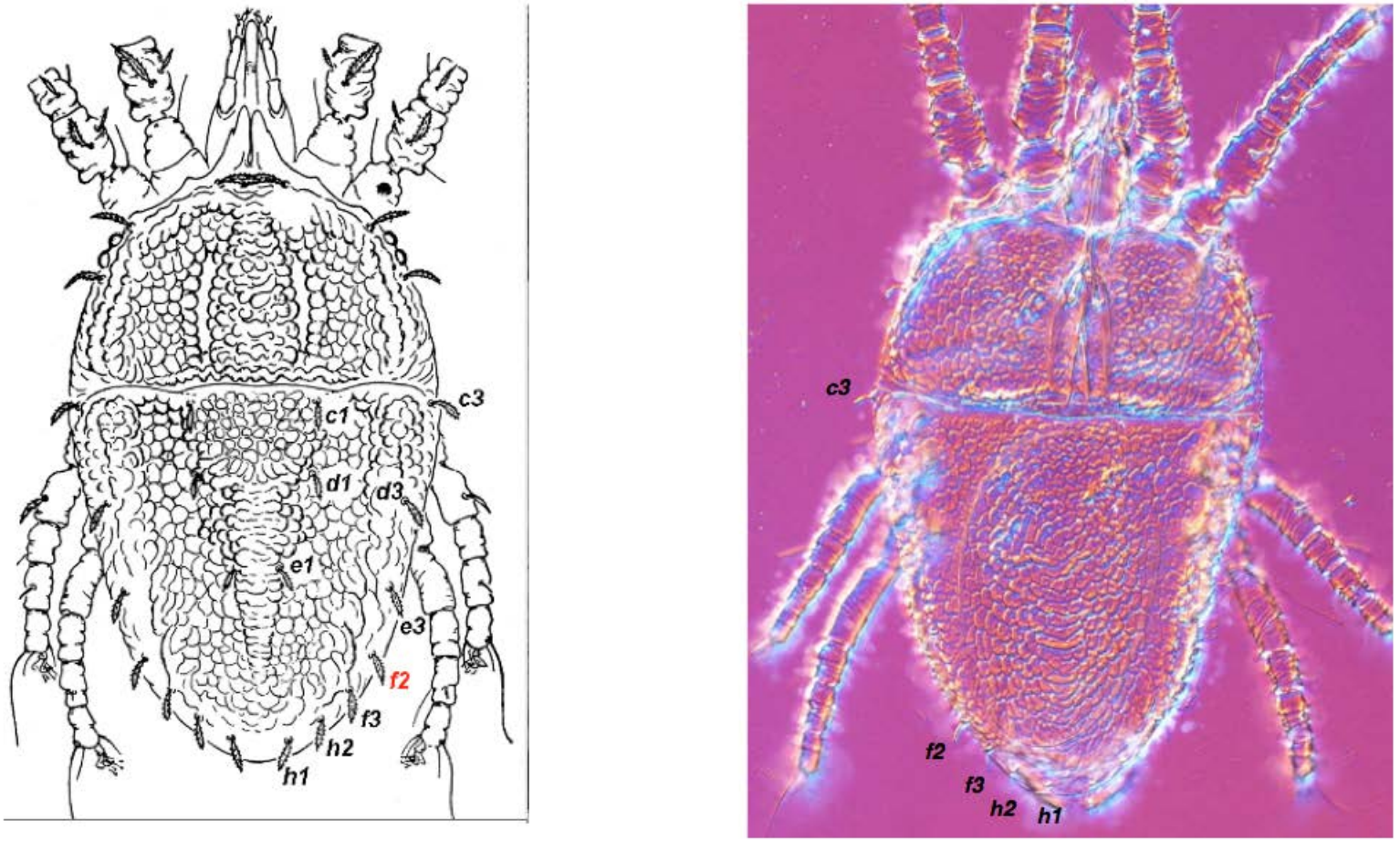


Figure 13. Diagnostic features for *Brevipalpus*species identification. Left : Dorsal opisthosomal setae with diagnostic *f2* highlighted in red (courtesy of Beard et al., 2012b); Right : Adult female mite in this study under differential interference contrast microscopy (100x) demonstrating presence of *f2* seta.

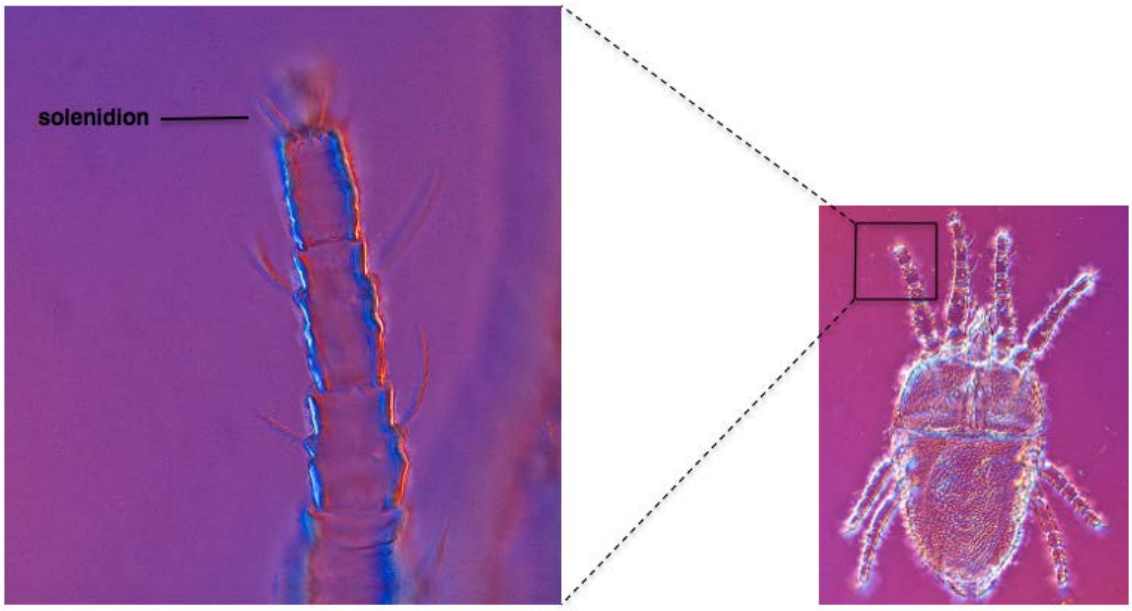


Figure 14. Diagnostic features for *Brevipalpus* species identification. Adult female mite under differential interference contrast microscopy (100x) demonstrating single solenidion on tarsus II.

Molecular Analysis

The DNA extraction technique used on bulks of 10 mites enabled six out of nine samples to amplify a product of 400-500bp in PCR using COI gene-specific primers (fig. 15). Amplified product was of sufficient concentration in five out of these six positive samples for Sanger sequencing. All amplified fragments were sequenced successfully with high quality chromatograms demonstrating uniformly spaced peaks and minimal background noise in both strands (fig. 16). A consensus sequence was produced in MEGA5 and required no manual adjustment.

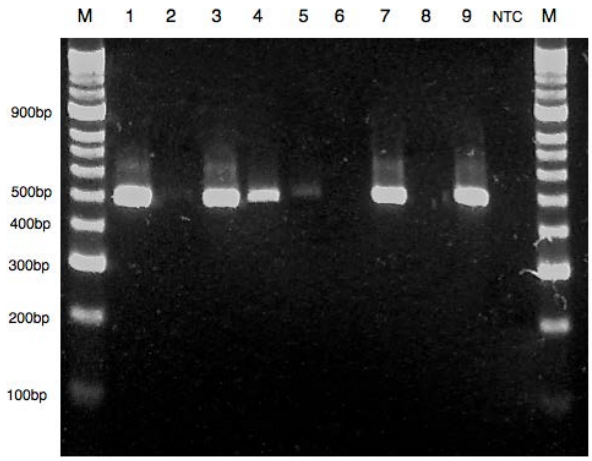


Figure 15. PCR amplification with mitochondrial COI-specific primers on DNA extracts of *Brevipalpus*n. sp.Analysis of amplification products (5µL loaded in each lane) was on 1.5% agarose gel electrophoresis. Lane M = 50bp DNA size marker; Lane 1-9 = mite DNA templates; lane NTC = negative template control.

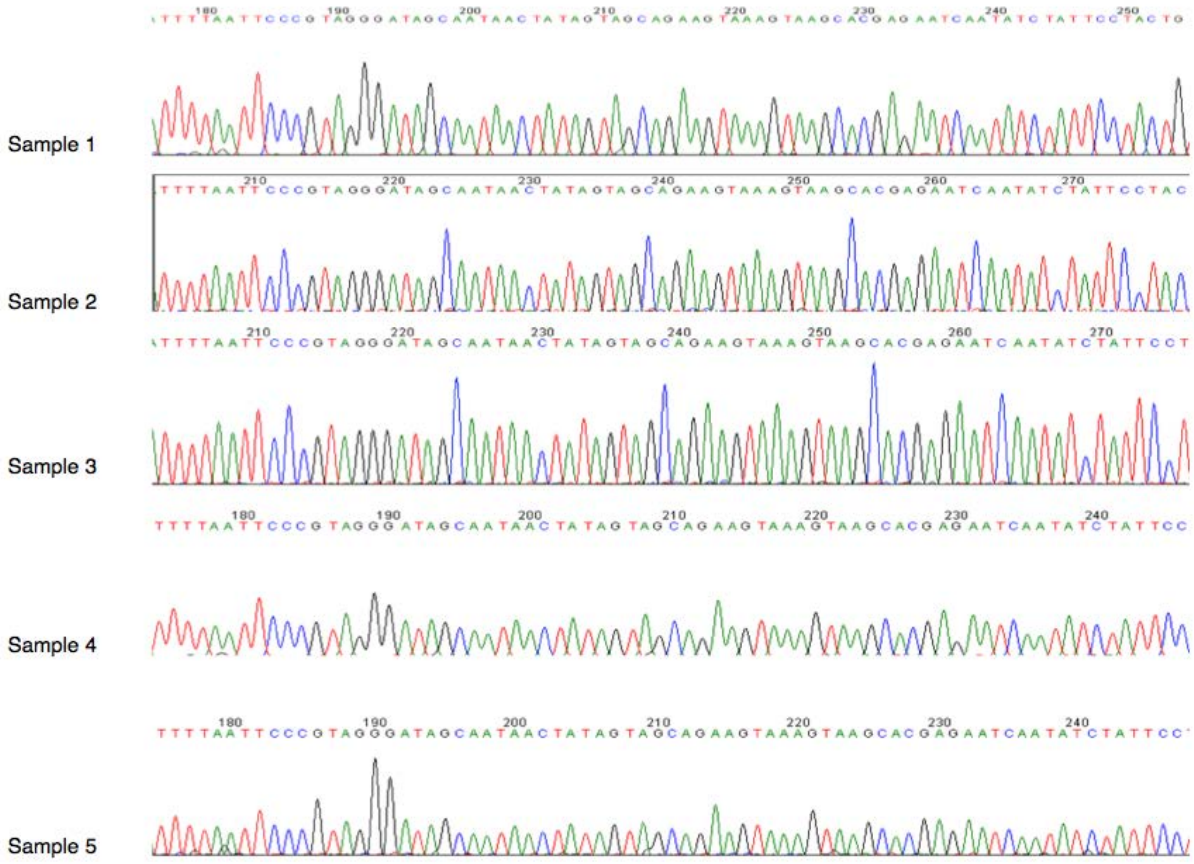


Figure 16. Chromatograms provided by automatic sequencing for determining the nucleic acid sequence of PCR amplicon using mitochondrial COI-specific primers on DNA extracts from five bulk samples of *Brevipalpus*n. sp.

The data set for phylogenetic analysis comprised 47 COI sequences and one outgroup. The alignments required no insertions or deletions. Conserved domains matching all *Brevipalpus*sequences gave suitable alignments from data retrieved from GenBank. Based on the COI nucelotide sequences used, the general topology of the phylogenetic tree inferred by neighbour-joining method (NJ) was similar to other published phylogenies (Navia et al., 2013). *Brevipalpus*samples, including the one from this study, formed a monophyletic group (fig. 17). However, the *Brevipalpus* sample from this study clustered into a distinct clade with two other unidentified *Brevipalpus*samples from Groot (2006) that was separate from clades formed by remaining samples (fig. 17). Topological support for most lineages was variable, with bootstrap values ranging from 72 to 99%; however, there was low support for basal branches (bootstrap values as low as 34%), except for the unidentified *Brevipalpus*clade containing the sample from this study (87%) (fig. 17). Although species identification of the *Brevipalpus*population in this study was not determined, an integrative approach using both molecular and morphological analysis excluded *B.californicus, B. phoenicis, B. obovatus*and *B. chilensis.*

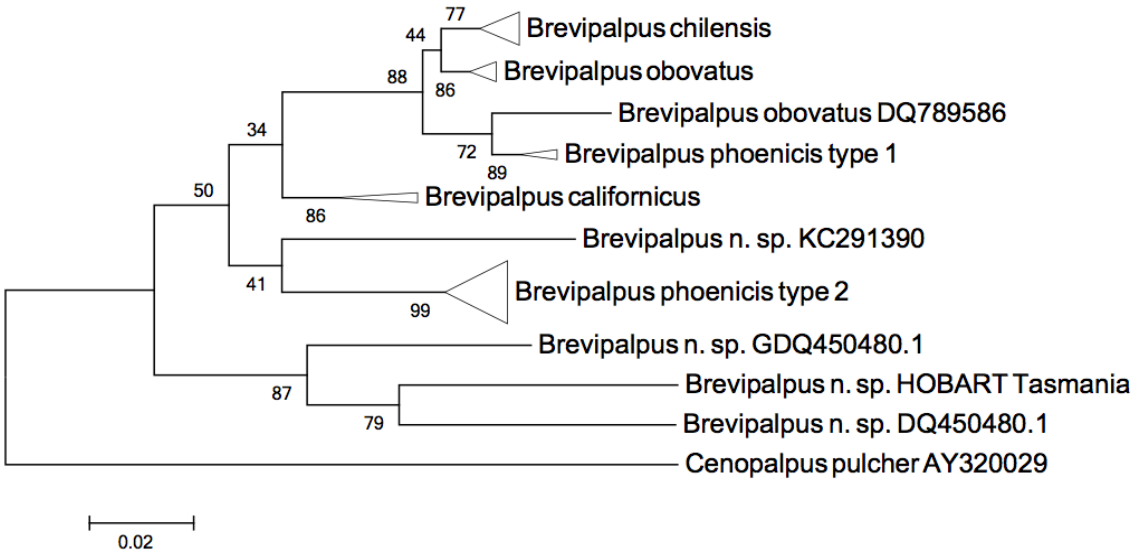


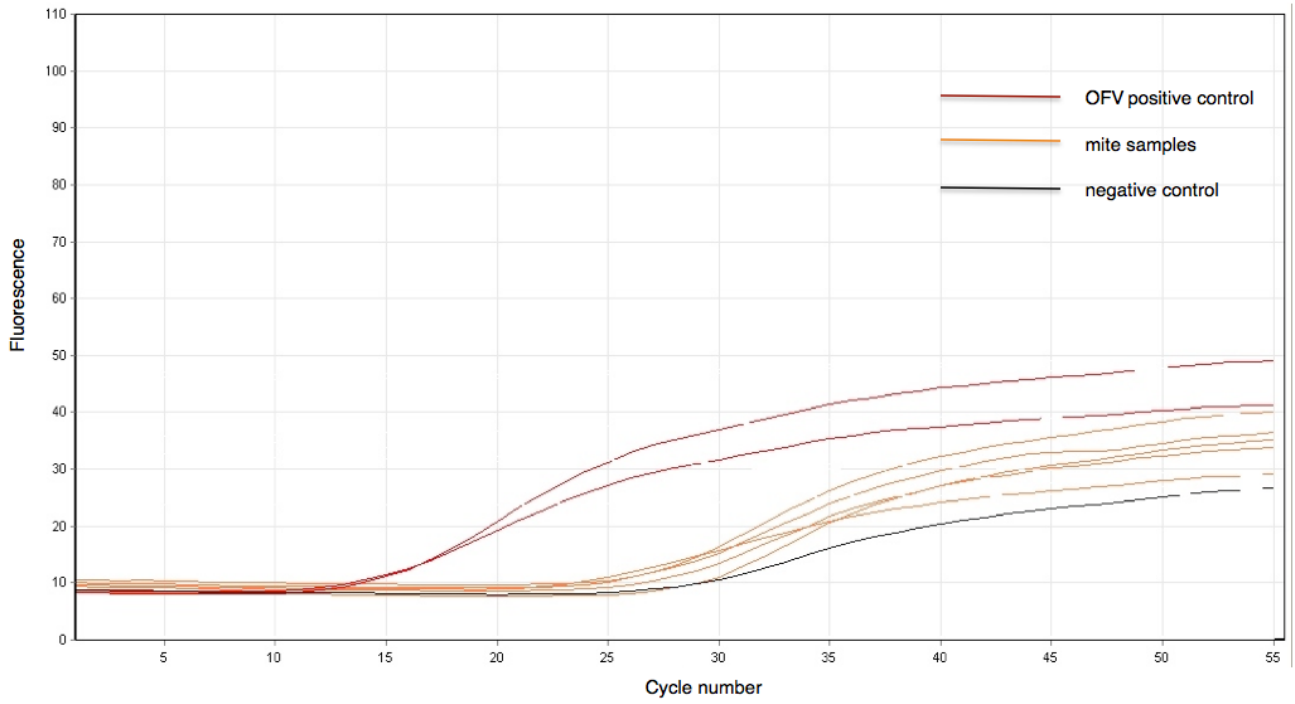
Figure 17.NJ phylogeny tree inferred from 358bp mitochondrial COI sequences of *Brevipalpus*mites obtained from GenBank and this study (*Brevipalpus*n. sp. HOBART Tasmania). Details of GenBank accessions are given in table 3. NJ bootstrap values (1000 replications) are shown above branches.

Transmission Assay

The ability for *Brevipalpus*mites to acquire OFV was tested by SYBR Green I quantitative RT-PCR, using OFV coat protein gene-specific primers (table 2) on extracted cDNA of batches of larvae, nymphs, adult males and adult females that had been allowed to feed on infected leaves for one hour, one day or one week. An OFV cDNA positive control was used in the assay in addition to DEPC-treated water as a negative control. In contrast to conventional RT-PCR, which involves end-point analysis, quantitative RT-PCR detects and measures the accumulation of amplicon as the reaction progresses (Mackay et al., 2002, Wilhem and Pingoud, 2003). The DNA-binding flurophore SYBR Green I emits a detectable fluorescent signal when associated with dsDNA (Mackay et al., 2002). A detectable increase in fluorescence above background suggests amplification of target product, with the amount of fluorescence being proportional to the amount of amplification product produced in the reaction (Mackay et al., 2002, Wilhem and Pingoud, 2003).

Of the twelve mite samples analysed by quantitative RT-PCR, five samples produced an observable increase in fluorescence (fig. 18a). These samples were from cDNA synthesised from extracts of adult females that had been given an acquisition access period of one hour, one day or one week; and nymphs that had an acquisition period of one week and one hour. Increase in fluorescence in these mite samples occurred later in the reaction than positive controls (ie. higher cycling threshold, C_t), suggesting that viral template, if present, occurred in much lower concentrations (fig. 18a). However, an increase in fluorescence late in the reaction was noted in the negative control, complicating the intepretation of results (fig. 18a). Subsequent melt curve analysis revealed similar melting points (T_m) for products arising from positive template controls (84.1^0C) and mite samples (83.5^0C), suggesting that the amplicon may have been the same (ie. OFV coat protein cDNA fragment) (fig. 18b). Furthermore, T_m for product in the negative control was lower, indicating a smaller amplicon, most likely primer-dimer (fig. 18b).

(a)



(b)

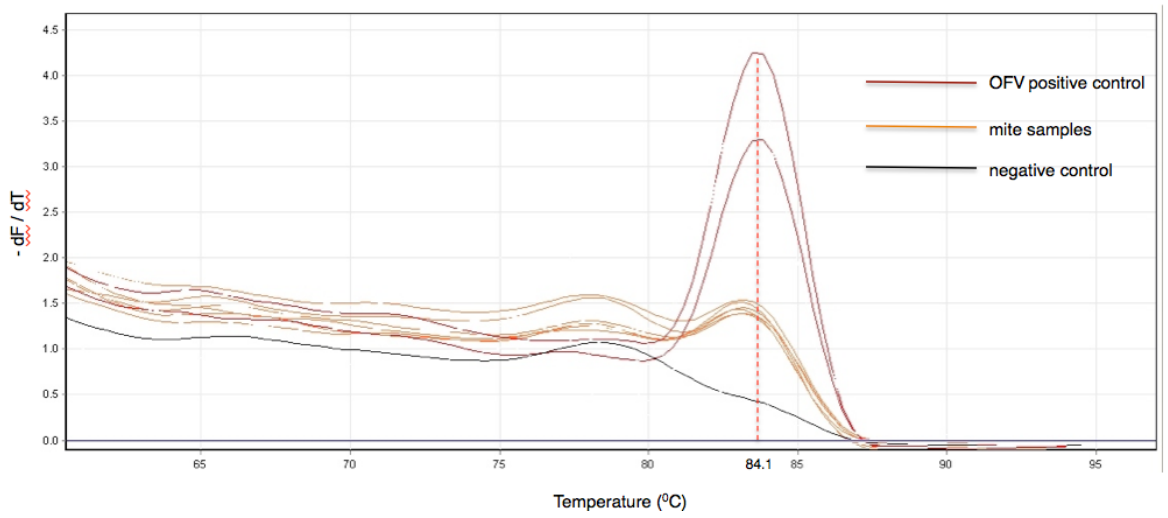
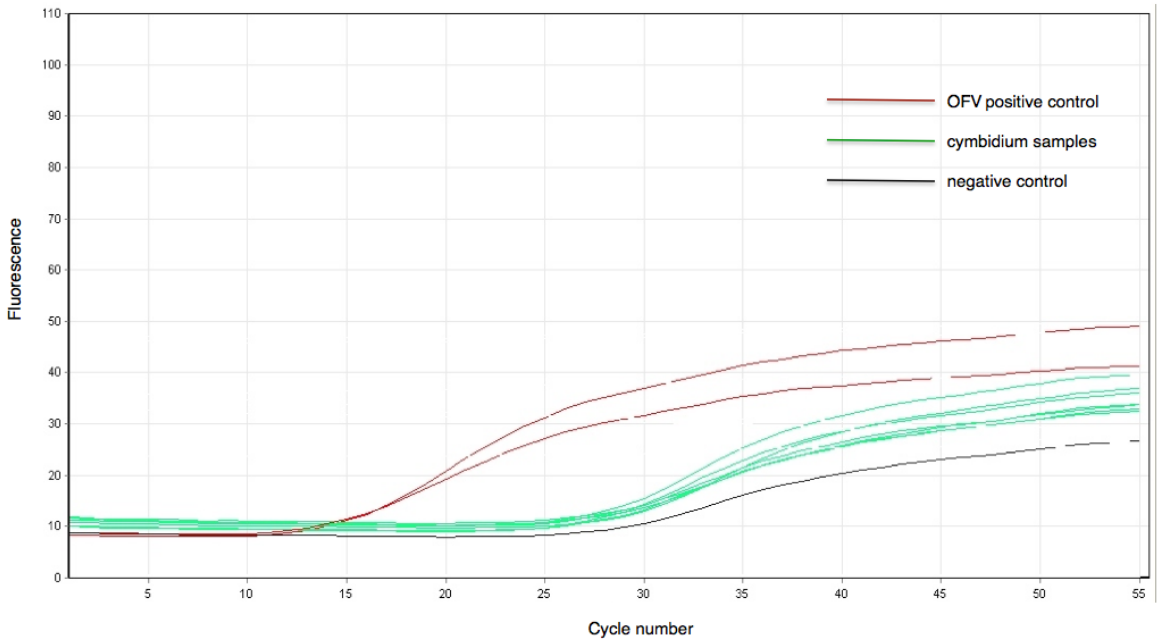


Figure 18. Analysis of SYBR Green quantitative RT-PCR with OFV coat protein gene-specific primers on extracts of mite cDNA samples :(a) Amplification plot showing early amplification of positive controls and late amplification in mite cDNA samples and negative control; (b) PCR products were distinguished by melt curve analysis. Melting temperatures (T_m) for positive controls (84.1^0C) and mite cDNA samples (83.5^0C) were distinct from negative control.

Cymbidium plants inoculated with mites (larvae, nymphs, adult males and adult females) that had fed on infected leaves showed no visible symptoms of OFV six months after initial inoculation. The ability for mites to transmit OFV to healthy plants was tested by SYBR Green I quantitative RT-PCR, using the same primers as above, on cDNA synthesised from cymbidium leaf extracts six months post-inoculation. Of the 15 cymbidium samples tested, seven samples produced an increase in fluorescence above background but all at a higher C_t value than positive control. The higher C_t value indicated that infection, if present, in the inoculated cymbidiums was at much lower titre than the postive controls (fig. 19a). Similar to the mite transmission experiment, an increase in fluorescence above background occurred in the negative control, suggesting primer-dimer formation or non-specific binding of primers in the reaction (fig. 19a). However, melt curve analysis again demonstrated that inoculated cymbidium samples contained amplification product of similar T_m (83.5^0C) as that of positive controls (84.1^0C), while the lower T_m associated with negative control was best explained by primer-dimer formation (fig. 19b). Moreover, amplicon T_m of mite samples and inoculated cymbidium samples using OFV coat protein-gene specific primers was identical (fig. 18b and 19b respectively).

Overall, the results of the transmission experiment tentatively suggest that the *Brevipalpus*mites may be able to acquire OFV after prolonged feeding times and that they may be able to subsequently transmit OFV to healthy cymbidiums at very low viral titres.

(a)



(b)

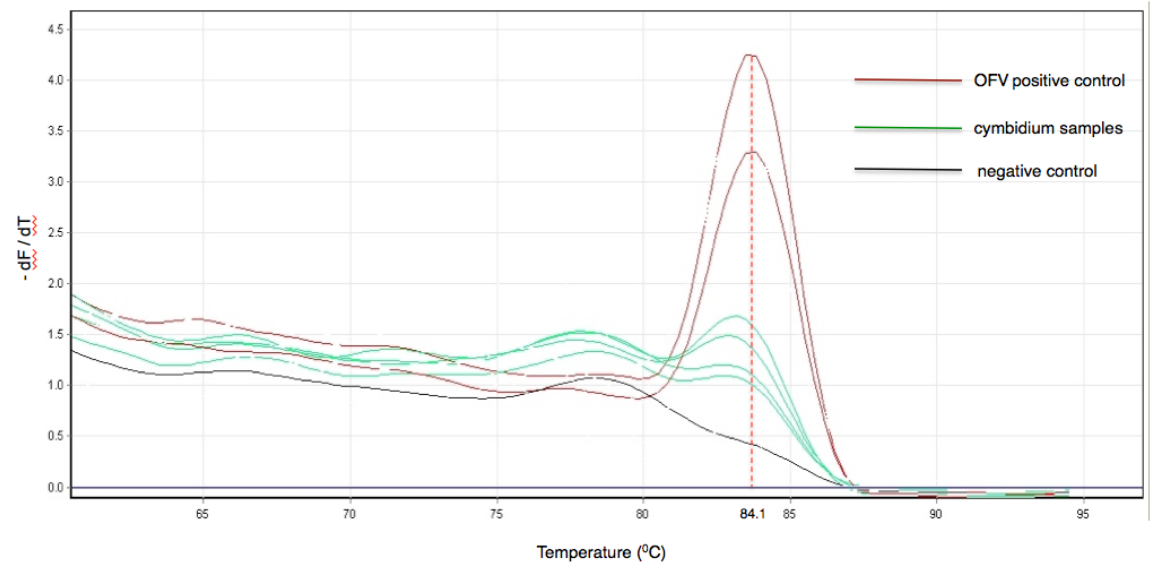


Figure 19.Analysis of SYBR Green quantitative RT-PCR with OFV coat protein gene-specific primers on extracts of inoculated cymbidium cDNA samples :(a) Amplification plot showing early amplification of positive controls and late amplification in inoculated cymbidium cDNA samples and negative control; (b) PCR products were distinguished by melt curve analysis. Melting temperatures (T_m) for positive controls (84.1^0C) and cymbidium cDNA samples (83.5^0C) were distinct from negative control.

Discussion

The taxonomy of flat mites in the genus *Brevipalpus*remains an ongoing challenge for acarologists(Rodrigues and Childers, 2013, Navia et al., 2013, Beard et al., 2012b). Many different classifications have been presented in the past (Pritchard and Baker, 1953, Meyer, 1979, Welbourn et al., 2003, Mesa et al., 2009) and valuable morphological characteristics have since been reprioritised (Beard et al., 2012b). However, the three most economically significant *Brevipalpus*species (*B. californicus, B.phoenicis*and *B.obovatus*)continue to be misidentified (Childers et al., 2003b; Beard et al., 2012b). Beard et al. (2012b) believe that the taxon names *B. californicus*and *B. phoenicis*represent cryptic species complexes. This has been supported by recent integrative taxonomic work by Navia et al., (2013). Importantly, it has been suggested that information in the literature pertaining to *B. californicus*and *B. phoenicis*is 'close to useless' until the correct identities of these two species complexes are resolved (Beard et al., 2012b). An integrative taxonomic approach combining molecular diagnostic methods is now accepted as the best approach to identify morphologically indiscriminable species (Armstrong and Ball, 2005, Navia et al., 2013).

In this study, sequence fragments of the mitochondrial cytochrome *c* oxidase subunit I (COI) gene were used for phylogeny reconstruction to provide additional diagnostic information relating to a *Brevipalpus*mite population discovered as a pest on *Brassiaverrucosa*(spider orchid) plants. Previous work has confirmed that identification systems based on analysis of the COI gene are highly effective for animal phyla (Lunt et al., 1996, Hebert et al., 2003, Armstrong and Ball, 2005). However, the morphological and molecular analysis in this study was unable to resolve the identity of *Brevipalpus*mites to species level. Given the current ambiguity surrounding *Brevipalpus*systematics this was not unexpected. Several ethanol preserved specimens of adult female mites from this study have since been sent to Dr. Jennifer Beard at the Department of Entomology, University of Maryland, USA, for more detailed morphological observations under low-temperature scanning electron microscopy (LTSEM). Conventional slide mounting techniques can alter the way in which details of the propodosoma and opisthosoma

appear under light microscopy, reducing their reliability as diagnostic markers (Welbourn et al., 2003, Navia et al., 2013). In contrast, LTSEM can provide remarkably detailed views of micromorphology (Beard et al., 2012a). At the time of writing, results from LTSEM analysis are still pending. Other genes with highly conserved and variable regions may be exploited in future DNA barcoding work to identify a wider range of *Brevipalpus*species : these include *cox1*, 16S, 18S and elongation factor-1α genes (Armstrong and Ball, 2005). In addition, several next generation sequencing technologies (Roche-454 pyrosequencing, ABI SOLiD, Illumina GA) enable far greater amounts of sequence data to be available to researchers than the current traditional Sanger sequencing method and offer exciting possibilities to broaden the scope of species identification on a molecular level (Harismendy et al., 2009). For example, Vera et al. (2008) were able to assemble the transcriptome of *Melitaeacinxia* (Lepidoptera: Nymphalidae), a butterfly with no previous genomic data available, by next generation sequencing. The authors were able to produce over 600,000 expressed sequence tags (ESTs) from a genetically varied pool of larvae, pupae and adults, and then form over 45,000 sets of overlapping fragments of DNA (contigs) by pyrosequencing (Vera et al., 2008). Over the next coming decade, many independent laboratories will be capable of using such an approach for phylogeny reconstruction of non-model species, including mites in the genus *Brevipalpus*(Harismendy et al., 2009).

The accurate identification of agricultural pests is imperative to implementing effective prevention and control strategies. Global trade, widespread international human movement, climage change and modified land use has meant ever increasing risks of introduction of exotic pest species into novel environments (Bradley et al., 2012). The continued growth in horticultural trade between continents of cultivated orchids has the potential to expose countries to introductions of polyphagous pest species, like *Brevipalpus*flat mites. Although Australia is currently free of many of the more serious *Brevipalpus*mite pests, reliable and accurate methods of species recognition must be in place. Furthermore, the extent to which different *Brevipalpus*species, particularly those that vector devestating viral diseases in crops and ornamentals, are able to

colonise and reproduce on commerical orchid species is largely unknown (Childers and Derrick, 2003, Childers and Rodrigues, 2011, Beard et al., 2012b).

Most research on vector transmission of persistent viruses has come from insects since the vast majority of recognised plant viruses are spread by hemipterans (aphids, planthoppers, leaf hoppers, whiteflies) (Hogenhout et al., 2008). Previous studies have demonstrated a complex sequence of events essential for the insect to acquire and later transmit a virus in a susceptible host plant (Moritz et al., 2004, Whitfield et al., 2005, Akad et al., 2007, Ammar el et al., 2009). In many pathosystems, viral coat proteins interact with insect cell membrane components to facilitate endocytosis and exocytosis of the virion across four potential internal insect barriers : midgut escape barrier; salivary gland infection barrier; salivary escape barrier; and transovarial transmission barrier (reviewed by Hogenhout et al., 2008). These intimate interactions studied in insects suggest that for most persistently transmitted viruses, a high degree of fidelity exists between pathogenic microbe and arthropod vectors, including *Brevipalpus*mites (Hogenhout et al., 2008, Ammar el et al., 2009). In this study, biological transmission assays were performed to determine the competence of a population of *Brevipalpus*mites to vector OFV in cymbidiums. Acquisition access periods selected in this study were based on previous findings by Kondo et al. (2003), who demonstrated that mites identified as *B. californicus*could acquire OFV after a wide range of feeding periods (between 30 minutes and 3 days) on OFV infected cymbidiums. In the Kondo et al. (2003) study, mites remained viruliferous for up to 3 weeks and vector transmission of OFV to healthy cymbidiums and *Phaseolus vulgaris* was demonstrated by electron microscopy with negative staining. In this study, quantitative RT-PCR was used to determine OFV vector competence in *Brevipalpus*mites.

Quantitative RT-PCR is an extremely sensitive method of detecting amplicon visually, even with extremely low concentrations of template (Mackay et al., 2002). Five major fluorescence chemistries are commonly used in quantitative

RT-PCR (Mackay et al., 2002). The DNA-binding fluorophore, SYBR Green I, was chosen in this study for ease of application and reduced cost compared to other chemistries. However, limitations of the chosen fluorescence chemistry should be noted, since the formation of either primer-dimer or non-specific amplicon in negative controls confused the interpretation of flurorescence data acquired from the mite and cymbidium assays. Analysis of the amplicon melting curves provided initial evidence that mites may be able to acquire and transmit OFV at very low efficiency. This is based on the theory that shorter primer-dimer may be discriminated from target amplicon by the lower temperature at which dsDNA of primer-dimer is denatured (Mackay et al., 2002). Interestingly, the findings by Kondo et al. (2003) that only adult *Brevipalpus*mites and nymphs, not larvae, could transmit OFV in a persistent manner are consistent with the preliminary results for the mites used in this study, although no definitive conclusions can be made at this point. Future work to resolve the vector competence of the *Brevipalpus*mites in this study should attempt to use an alternative fluorochemistry in quantitative RT-PCR. For example, the use of 5' nuclease or TaqManoligoprobe, designed to bind to OFV coat protein gene-specific sequences between current primers, has the potential to offer less ambiguity in the analysis of the biological transmission assays. Furthermore, quantitative RT-PCR using a carefully designed oligoprobe can be used for absolute quantification and can address the possibility of temperature-dependenteffects on virus acquisition by *Brevipalpus*mite, as well as transmission efficiency and subsequent virus replication in inoculated cymbidiums.

Given that the *Brevipalpus*mites in this study are pests on orchids, there are some very interesting questions that could be addressed in the future, particularly related to the effects of *Cardinium*endosymbionts on vector competence. One of the major discoveries in entomology and acarology of recent times is that a large number of species are host to bacterial endosymbionts that have profound and wide-ranging effects on the ecology, behaviour and reproduction of their arthropod host (Feldhaar, 2011). The occurrence in *Brevipalpus*mites of bacterial endosymbionts in the genus *Cardinium*has been known for over a decade (Weeks et al., 2001). The best characterised arthropod-microbe system is in pea aphids,

host to an obligate bacterial endosymbiont, *Buchneraaphidicola*, that provides the aphid host a ‘nutritional upgrade’ by producing essential amino acids such as tryptophan (Shigenobu and Wilson, 2011). Importantly, *Buchneraaphidicola*also produces a protein (GroEL) that binds to luteo- and polero-viruses that pass through the digestive tract of aphids, preventing the degradation of these viruses in the aphid haemocel and thereby facilitating virus transmission (Feldhaar, 2011). Homologues of GroEL from other endosymbionts have been discovered in other insect vectors of plant viruses, including the whitefly, *Bemisiatabaci*(Morin et al., 1999, Akad et al., 2007). Although past research has demonstrated the feminizing effects of natural infection with *Cardinium* in several *Brevipalpus*species (Groot, 2006, Kitajima et al., 2007, Novelli et al., 2007), little is known about the effects of the endosymbiont on persistent transmission of viruses such as OFV. Given the increased awareness of the important roles that endosymbionts play in virus-vector associations, it is important to continue studying the effects of *Cardinium*bacteria in multiple *Brevipalpus*populations, including the one described in this study using next-generation sequencing, transcriptomics and metabolomics. Novel methods of control of virus vectors through manipulation of endosymbionts have been achieved in recent times (McGraw and O'Neill, 2013). Furthermore, expansion of the molecular dataset for *Brevipalpus*species by DNA sequencing of *Cardinium*endosymbionts provides an additional independently evolving genomic region that can be used in phylogeny reconstruction.

In conclusion, a potential novel *Brevipalpus*species was detected as a pest on *Brassiaverrucosa*orchids. Although the majority of *Brevipalpus*species are believed to be predominantly thelytokous, males in the population described in this study were observed to be sexually functional. An integrative taxonomic approach, combining morphological observations with analysis of mitochondrial COI gene sequences, was not able to resolve the identity of the mites to species level. Efforts to obtain a species diagnosis are ongoing. The ability for these mites to acquire and transmit OFV to cymbidiums remains in doubt, although preliminary investigations using SYBR Green I quantitative RT-PCR suggest that adult females and nymphs may be able to transmit OFV with poor efficiency in a persistent

manner. Vector competence in these mites may best be addressed in the future using TaqMan quantitative RT-PCR.

Conclusion

This study is the first to report a multiplex RT-PCR for simultaneous detection and differentiation of the three most common viruses affecting orchids :*Cymbidium mosaic virus, Odontoglossum ringspot virus* and *Orchid fleck virus.* The multiplex RT-PCR is a highly specific and sensitive molecular tool aimed at improving detection of low viral titre infections and mixed infections. Primers were specifically designed to target conserved sequences of the coat protein gene of all three viruses. The multiplex RT-PCR is well suited to screening large numbers of plants, particularly nursery stock, and ensuring that propagative material for tissue culture or flasked seedlings is virus free. It is hoped that the application of the multiplex RT-PCR may assist growers in limiting the spread of these serious viruses in their private or commercial collections. The same OFV coat protein gene-specific primers were used in a quantitative RT-PCR experiment to determine the vector competence of a potentially novel *Brevipalpus*species found on *Brassiaverrucosa*orchids in Hobart, Tasmania. An integrative taxonomic approach was not able to resolve the identity of the mites to species level, however ongoing efforts will be made to determine whether micromorphological traits observed on low-temperature scanning electron microscopy may be informative. This study was able to generate important nucleotide sequence data from a fragment of the mitochondrial cytochrome oxidase c subunit I (COI) gene, which will serve as a contribution to global efforts to increase the molecular resources for DNA barcoding of the *Brevipalpus*genus. The ability for these mites to acquire and transmit OFV to cymbidiums remains in doubt but the use of TaqManoligoprobe in quantitative RT-PCR may provide a more definitive answer.

References

AKAD, F., EYBISHTZ, A., EDELBAUM, D., GOROVITS, R., DAR-ISSA, O., IRAKI, N. & CZOSNEK, H. 2007. Making a friend from a foe: expressing a GroEL gene from the whitefly Bemisia tabaci in the phloem of tomato plants confers resistance to tomato yellow leaf curl virus. *Archives of Virology,* 152**,** 1323-39.

AMMAR EL, D., TSAI, C. W., WHITFIELD, A. E., REDINBAUGH, M. G. & HOGENHOUT, S. A. 2009. Cellular and molecular aspects of rhabdovirus interactions with insect and plant hosts. *Annual Review of Entomology,* 54**,** 447-68.

BATCHMAN, L. 2008. Detecting Virus in Orchids. *Orchids***,** 342-343.

BEARD, J. J., OCHOA, R., BAUCHAN, G. R., WELBOURN, W. C., POOLEY, C. & DOWLING, A. P. 2012a. External mouthpart morphology in the Tenuipalpidae (Tetranychoidea): Raoiella a case study. *Experimental and Applied Acarology,* 57**,** 227-55.

BEARD, J. J., OCHOA, R., REDFORD, A. J., TRICE, M. D., WALTERS, T. W. & MITTER, C. 2012b. *Flat Mites of the World - Part I Raoiella and Brevipalpus. Identification Technology Program, CPHST, PPQ, QPHIS, USDA; Fort Collins, CO, USE.* [Online]. Available: http://idtools.org/id/mites/flatmites/ [Accessed 15th October 2013].

BLANCHFIELD, A., MACKENZIE, A., GIBBS, A., KONDO, H., TAMADA, T. & WILSON, C. 2001. Identification of Orchid fleck virus by reverse transcriptase-polymerase chain reaction and analysis of isolate relationships. *Journal of Phytopathology,* 149**,** 713-718.

BRADLEY, B. A., BLUMENTHAL, D. M., EARLY, R., GROSHOLZ, E. D., LAWLER, J. J., MILLER, L. P., SORTE, C. J. B., D'ANTONIO, C. M., DIEZ, J. M., DUKES, J. S., IBANEZ, I. & OLDEN, J. D. 2012. Global change, global trade, and the next wave of plant invasions. *Frontiers in Ecology and the Environment,* 10**,** 20-28.

CELEBI-TOPRAK, F., SLACK, S. A. & RUSSO, P. 2003. Potato Resistance to Cucumber Mosaic Virus is Temperature Sensitive and Virus-strain Specific. *Breeding Science,* 53**,** 69-75.

CHANG, L., ZHANG, Z., YANG, H., LI, H. & DAI, H. 2007. Detection of Strawberry RNA and DNA Viruses by RT-PCR Using Total Nucleic Acid as a Template. *Journal of Phytopathology,* 155**,** 431-436.

CHAPMAN, R. F. 2003. *The Insects : Structure and Function, fourth edition.* Cambridge University Press.

CHIGIRA, A. & MIURA, K. 2005. Detection of 'Candidatus Cardinium' bacteria from the haploid host Brevipalpus californicus (Acari: Tenuipalpidae) and effect on the host. *Experimental and Applied Acarology,* 37**,** 107-116.

CHILDERS, C. & DERRICK, K. S. 2003. Brevipalpus mites as vectors of unassigned rhabdoviruses in various crops. *Experimental and Applied Acarology,* 30**,** 1-3.

CHILDERS, C., FRENCH, J. V. & RODRIGUES, J. C. 2003a. Brevipalpus californicus, B. obovatus, B. phoenicis, and B. lewisi (Acari: Tenuipalpidae): a review of their biology, feeding injury and economic importance. *Experimental and Applied Acarology,* 30**,** 5-28.

CHILDERS, C., RODRIGUES, J. C. & WELBOURN, W. C. 2003b. Host plants of Brevipalpus californicus, B. obovatus, and B. phoenicis (Acari: Tenuipalpidae) and their potential involvement in the spread of viral diseases vectored by these mites. *Experimental and Applied Acarology***,** 29-105.

CHILDERS, C. & RODRIGUES, J. C. V. 2011. An overview of Brevipalpus mites (Acari: Tenuipalpidae) and the plant viruses they transmit*. *Zoosymposia,* 6**,** 180-192.

COLHOUN, J. 1973. Effect of Environmental Factors on Plant Disease. *Annual Review of Phytopathology,* 11**,** 343-364.

CULVER, J. N., LINDBECK, A. G. C. & DAWSON, W. O. 1991. Virus-host interactions: induction of chlorotic and necrotic responses in plants by tobamoviruses. *Annual Review of Phytopathology,* 29**,** 193-217.

DU, Z., CHEN, J. & HIRUKI, C. 2006. Optimization and application of a multiplex RT-PCR system for simultaneous detection of five potato virus using18S rRNA as an internal control. *Plant Disease,* 90**,** 185-189.

ESAU, K. 1977. Anatomy of seed plants, 2nd edition.

FELDHAAR, H. 2011. Bacterial symbionts as mediators of ecologically important traits of insect hosts. *Ecological Entomology,* 36**,** 533-543.

GAMBINO, G. & GRIBAUDO, I. 2006. Simultaneous detection of nine grapevine viruses by multiplex reverse transcription-polymerase chain reaction with coamplification of a plant RNA as internal control. *Phytopathology,* 96**,** 1223-9.

GARSON, J. A., RING, C. J. A. & TUKE, P. W. 1998. Improvement of HCV genome detection with 'short' PCR products. *Lancet,* 338**,** 1466-7.

GE, B., LI, Q., LIU, G., LU, M., LI, S. & WANG, H. 2013. Simultaneous detection and identification of four viruses infecting pepino by multiplex RT-PCR. *Archives of Virology,* 158**,** 1181-7.

GIBBS, A., MACKENZIE, A., BLANCHFIELD, A., CROSS, P., WILSON, C., KITAJIMA, E., NIGHTINGALE, M. & CLEMENTS, M. 2000. Viruses of Orchids in Australia; their identification, biology and control. *The Australian Orchid Review***,** 11-20.

GROOT, T. V. & BREEUWER, J. A. 2006. Cardinium symbionts induce haploid thelytoky in most clones of three closely related Brevipalpus species. *Experimental and Applied Acarology,* 39**,** 257-71.

GROOT, T. V. M. 2006. *The Effects of Symbiont Induced Haploid Thelytoky on the Evolution of Brevipalpus Mites.* PhD, University of Amsterdam.

GROOT, T. V. M., JANSSEN, A., PALLINI, A. & BREEUWER, J. A. J. 2005. Adaptation in the Asexual False Spider Mite Brevipalpus phoenicis: Evidence for Frozen Niche Variation. *Experimental and Applied Acarology,* 36**,** 165-176.

HARISMENDY, O., NG, P. C., STRAUSBERG, R. L., WANG, X., STOCKWELL, T. B., BEESON, K. Y., SCHORK, N. J., MURRAY, S. S., TOPOL, E. J., LEVY, S. & FRAZER, K. A. 2009. Evaluation of next generation sequencing platforms for population targeted sequencing studies. *Genome Biology,* 10**,** R32.

HEBERT, P. D., RATNASINGHAM, S. & DEWAARD, J. R. 2003. Barcoding animal life: cytochrome c oxidase subunit 1 divergences among closely related species. *Proceedings of Biological Science,* 270 Suppl 1**,** S96-9.

HENDRIX, J. W. 1972. Temperature-dependent resistance to tobacco ringspot virus in L8, a necrosis-prone tobacco cultivar. *Phytopathology,* 62**,** 1376-1381.

HENEGARIU, O., HEEREMA, N. A., DLOUHY, S. R., VANCE, G. H. & VOGT, P. H. 1997. Multiplex PCR : critical parameters and step-by-step protocol. *BioTechniques,* 23**,** 504-511.

HODGSON, M. 1991. *A guide to orchids of the world*, William Collins Publishers Ltd. .

HOGENHOUT, S. A., AMMAR EL, D., WHITFIELD, A. E. & REDINBAUGH, M. G. 2008. Insect vector interactions with persistently transmitted viruses. *Annual Review of Phytopathology,* 46**,** 327-59.

HU, W.-C., HUANG, C.-H., LEE, S.-C., WU, C.-I. & CHANG, Y.-C. 2009. Detection of four calla potyviruses by multiplex RT-PCR using nad5 mRNA as an internal control. *European Journal of Plant Pathology,* 126**,** 43-52.

INOUYE, N., MATSUMOTO, J., MAEDA, T., MITSUHATA, K., KONDO, H. & TAHARA, M. 1996. Orchid fleck virus, the causal agent of a yellowish fleck mosaic disease of Calanthe. *Bulletin of the Research Institute for Bioresources Okayama University,* 4**,** 119-135.

JACKSON, A., DIETZGEN, R., GOODIN, M., BRAGG, J. & DENG, M. 2005. Biology of Plant Rhabdoviruses. *Annual Review of Phytopathology,* 43**,** 623-660.

JAMES, D., VARGA, A., PALLAS, V. & CANDRESSE, T. 2006. Strategies for simultaneous detection of multiple plant viruses. *Canadian Journal of Plant Pathology,* 28**,** 16-29.

KASSANIS, B. 1952. Some Effects of High Temperature on the Susceptibility of Plants to Infection with Viruses. *The Annals of Applied Biology,* 39**,** 358-369.

KENNEDY, J. S. 1995. Phase variation—a possible adaptive character for the false spider mite, Brevipalpus phoenicis (Geijskes 1939). *Journal of Applied Entomology,* 119**,** 259-261.

KIM, S. R., YOON, J. Y., CHOI, G. S., CHANG, M. U., CHOI, J. K. & CHUNG, B. N. 2010. Molecular characterization and survey of the infection rate of orchid fleck virus in commercial orchids. *Plant Pathology Journal,* 26**,** 130-138.

KIMURA, M. 1980. A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. *Journal of Molecular Evolution,* 16**,** 111-120.

KIRALY, L., HAFEZ, Y. M., FODOR, J. & KIRALY, Z. 2008. Suppression of tobacco mosaic virus-induced hypersensitive-type necrotization in tobacco at high temperature is associated with downregulation of NADPH oxidase and superoxide and stimulation of dehydroascorbate reductase. *Journal of General Virology,* 89**,** 799-808.

KITAJIMA, E. W., CHAGAS, C. M. & RODRIGUES, J. C. V. 2003. Brevipalpus-transmitted plant virus and virus-like diseases : cytopathology and some recent cases. *Experimental and Applied Acarology,* 30**,** 135-160.

KITAJIMA, E. W., GROOT, T. V., NOVELLI, V. M., FREITAS-ASTUA, J., ALBERTI, G. & DE MORAES, G. J. 2007. In situ observation of the Cardinium symbionts of Brevipalpus (Acari: Tenuipalpidae) by electron microscopy. *Experimental and Applied Acarology,* 42**,** 263-71.

KITAJIMA, E. W., KONDO, H., MACKENZIE, A., REZENDE, J., GIORIA, R., GIBBS, A. & TAMADA, T. 2001. Comparative Cytopathology and Immunocytochemistry of Japanese, Australian and Brazilian Isolates of Orchid fleck virus. *Journal of General Plant Pathology,* 67**,** 231-237.

KITAJIMA, E. W., RODRIGUES, J. C. V. & FREITAS-ASTUA, J. 2010. An annotated list of ornamentals naturally found infected by Brevipalpus mite-transmitted viruses *Sci.Agric.(Piracicaba, Braz.),* 67**,** 348-371.

KONDO, H., MAEDA, T., SHIRAKO, Y. & TAMADA, T. 2006. Orchid fleck virus is a rhabdovirus with an unusual bipartite genome. *Journal of General Virology,* 87**,** 2413-21.

KONDO, H., MAEDA, T. & TAMADA, T. 2003. Orchid fleck virus : Brevipalpus californicus mite transmision, biological properties and genome structure. *Experimental and Applied Acarology* 30**,** 215-223.

KUBO, K. S., FREITAS-ASTÚA, J., MACHADO, M. A. & KITAJIMA, E. W. 2009a. Orchid fleck symptoms may be caused naturally by two different viruses transmitted by Brevipalpus. *Journal of General Plant Pathology,* 75**,** 250-255.

KUBO, K. S., NOVELLI, V. M., BASTIANEL, M., LOCALI-FABRIS, E. C., ANTONIOLI-LUIZON, R., MACHADO, M. A. & FREITAS-ASTUA, J. 2011. Detection of Brevipalpus-transmitted viruses in their mite vectors by RT-PCR. *Experimental and Applied Acarology,* 54**,** 33-9.

KUBO, K. S., STUART, R. M., FREITAS-ASTUA, J., ANTONIOLI-LUIZON, R., LOCALI-FABRIS, E. C., COLETTA-FILHO, H. D., MACHADO, M. A. & KITAJIMA, E. W. 2009b. Evaluation of the genetic variability of orchid fleck virus by single-strand conformational polymorphism analysis and nucleotide sequencing of a fragment from the nucleocapsid gene. *Archives of Virology,* 154**,** 1009-14.

KUWABARA, K., YOKOI, N., OHKI, T. & TSUDA, S. 2010. Improved multiplex reverse transcription-polymerase chain reaction to detect and identify five tospovirus species simultaneously. *Journal of General Plant Pathology,* 76**,** 273-277.

LEE, S. & CHANG, Y. 2006. Multiplex RT-PCR detection of two orchid viruses with an internal control of plant nad5 mRNA. *Plant Pathology Bulletin,* 15**,** 187-196.

LIN, L., LI, R., MOCK, R. & KINARD, G. 2012. One-step multiplex RT-PCR for simultaneous detection of four pome tree viroids. *European Journal of Plant Pathology,* 133**,** 765-772.

LIU, F., FENG, L., CHEN, X., HAN, Y., LI, W., XU, W., CAI, B. & LIN, M. 2012. Simultaneous Detection of Four Banana Viruses by Multiplex PCR. *Journal of Phytopathology,* 160**,** 622-627.

LI, D., CHEN, P., SHI, A., SHAKIBA, E., GERGERICH, R. & CHEN, Y. 2009. Temperature affects expression of symptoms induced by soybean mosaic virus in homozygous and heterozygous plants. *Journal of Heredity,* 100**,** 348-54.

LUNT, D. H., ZHANG, D., SZYMURA, J. M. & HEWITT, G. M. 1996. The insect cytochrome oxidase I gene : evolutionary patterns and conserved primers for phylogenetic studies. *Insect Molecular Biology,* 5**,** 153-165.

MA, B., NIU, J., MORLEY-BUNKER, M., PAN, L., ZHANG, H. & ZHANG, L. 2008. Detection of three pear viruses by multiplex RT-PCR assays with co-amplification of an internal control. *Australasian Plant Pathology,* 37**,** 117-122.

MCGRAW, E. A. & O'NEILL, S. L. 2013. Beyond insecticides : new thinking on an ancient problem. *Nature Reviews Microbiology,* 11**,** 181-193.

MACKAY, I. M., ARDEN, K. E. & NITSCHE, A. 2002. Real-time PCR in virology. *Nucleic Acid Research,* 30**,** 1292-1305.

MANGLITZ, G. R. & CORY, E. N. 1953. Biology and control of *Brevipalpus australis*. *Journal of Economic Entomology,* 46**,** 116-119.

MATSUI, A. 2010. The Strategy of one-quarter occupation marketing. *International Commercial Orchid Growers Association Bulletin.,* 4**,** 3-5.

MESA, N. C., OCHOA, R., WELBOURN, W. C., EVANS, G. A. & DE MORAES, G. J. 2009. A catalog of Tenuipalpidae Berlese (Acari: Prostigmata) of the World, with key to genera. *Zootaxa,* 2098.

MENZEL, W., JELKMANN, W. & MAISS, E. 2002. Detection of four apple viruses by multiplex RT-PCR assays with coamplification of plant mRNA as internal control. *Journal of Virological Methods,* 99**,** 81-92.

MEYER, M. K. P. 1979. The Tenuipalpidae (Acari) of Africa with keys to the world fauna. *Entomology Memoir.* Department of Agriculture Republic South Africa, Pretoria, .

MORIN, S., GHANIM, M., ZEIDAN, M., CZOSNEK, H., VERBEEK, M. & VAN DEN HEUVEL, J. F. 1999. A GroEL homologue from endosymbiotic bacteria of the whitefly Bemisia tabaci is implicated in the circulative transmission of tomato yellow leaf curl virus. *Virology,* 256**,** 75-84.

MORITZ, G., KUMM, S. & MOUND, L. 2004. Tospovirus transmission depends on thrips ontogeny. *Virus Research,* 100**,** 143-49.

MOURY, B., SELASSIE, K. G., MARCHOUX, G., DAUBEZE, A. & PALLOIX, A. 1998. High temperature effects on hypersensitive resistance to Tomato Spotted Wilt Tospovirus (TSWV) in pepper (Capsicum chinense Jacq.). *European Journal of Plant Pathology,* 104**,** 489-498.

MUMFORD, R., BOONHAM, N., TOMLINSON, J. & BARKER, I. 2006. Advances in molecular phytodiagnostics – new solutions for old problems. *European Journal of Plant Pathology,* 116**,** 1-19.

NAGATA, T., ALMEIDA, A. C. L., RESENDE, R. O. & DE AVILA, A. C. 2004. The competence of four thrips species to transmit and replicate four tospoviruses. *Plant Pathology,* 53**,** 136-140.

NAVIA, D., MENDONCA, R. S., FERRAGUT, F., MIRANDA, L. C., TRINCADO, R. C., MICHAUX, J. & NAVAJAS, M. 2013. Cryptic diversity in *Brevipalpus* mites (Tenuipalpidae). *Zoologica Scripta,* 42**,** 406-426.

NOVELLI, V. M., FREITAS-ASTUA, J., ARRIVABEM, F., LOCALI-FABRIS, E. C., HILF, M. E., GOTTWALD, T. R. & MACHADO, M. A. 2007. Effects of temperature, storage period and the number of individuals on the detection of the false spider mite Cardinium endosymbiont. *Experimental and Applied Acarology,* 42**,** 17-21.

PENG, D. W., ZHENG, G. H., ZHENG, Z. Z., TONG, Q. X. & MING, Y. L. 2013. Orchid fleck virus: an unclassified bipartite, negative-sense RNA plant virus. *Archives of Virology,* 158**,** 313-23.

PEIMAN, M. & XIE, C. 2006. Sensitive detection of potato viruses, PVX, PLRV and PVS, by RT-PCR in potato leaf and tuber. *Australasian Plant Disease Notes,* 1**,** 41-46.

POWER, A. G. 2000. Insect transmission of plant viruses: a constraint on virus variability. *Current Opinion in Plant Biology,* 3**,** 336-340.

PRITCHARD, A. E. & BAKER, E. W. 1953. *Pritchard-Baker : False Spider Mites*, University of California Publications in Entomology.

QU, F., YE, X., HOU, G., SATO, S., CLEMENTE, T. E. & MORRIS, T. J. 2005. RDR6 has a broad-spectrum but temperature-dependent antiviral defense role in Nicotiana benthamiana. *Journal of Virology,* 79**,** 15209-17.

QUINTERO-VASQUEZ, G. A., BAZAN-TEJEDA, M. L., MARTINEZ-PENAFIEL, E., KAMEYAMA-KAWABE, L. & BERMUDEZ-CRUZ, R. M. 2013. Multiplex PCR to detect four different tomato-infecting pathogens. *Folia Microbiol (Praha),* 58**,** 269-76.

RAYMAEKERS, M., SMETS, R., MAES, B. & CARTUYVELS, R. 2009. Checklist for optimization and validation of real-time PCR assays. *Journal of Clinical Laboratory Analysis,* 23**,** 145-51.

RODRIGUES, J. C. & CHILDERS, C. C. 2013. Brevipalpus mites (Acari: Tenuipalpidae): vectors of invasive, non-systemic cytoplasmic and nuclear viruses in plants. *Experimental and Applied Acarology,* 59**,** 165-75.

RODRIGUES, J. C., GALLO-MEAGHER, M., OCHOA, R., CHILDERS, C. C. & ADAMS, B. J. 2004. Mitochondrial DNA and RAPD polymorphisms in the haploid mite Brevipalpus phoenicis (Acari: Tenuipalpidae). *Experimental and Applied Acarology,* 34**,** 275-290.

ROGGERO, P., LISA, V., NERVO, G. & PENNAZIO, S. 1996. Continuous high temperature can break the hypersensitivity of Capsicum chinense 'PI 152225' to tomato spotted wilt tospovirus (TSWV). *Phytopathologia Mediterranea,* 35**,** 117-120.

ROY, A., ANANTHAKRISHNAN, G., HARTUNG, J. S. & BRLANSKY, R. H. 2010. Development and application of a multiplex reverse-transcription polymerase chain reaction assay for screening a global collection of Citrus tristeza virus isolates. *Phytopathology,* 100**,** 1077-88.

RYU, K. H., YOON, K. E. & PARK, W. M. 1995. Detection by RT-PCR of Cymbidium Mosaic Virus in Orchids. *Journal of Phytopathology,* 143**,** 643-646

SAMUEL, G. 1931. Some Experiments on Inoculating Methods with Plant Viruses, and on Local Lesions. *The Annals of Applied Biology,* 18**,** 494-507.

SANCHEZ-NAVARRO, J. A., APARICIO, F., HERRANZ, M. C., MINAFRA, A., MYRTA, A. & PALLAS, V. 2005. Simultaneous detection and identification of eight stone fruit viruses by one-step RT-PCR. *European Journal of Plant Pathology,* 111**,** 77-84.

SHIGENOBU, S. & WILSON, A. C. C. 2011. Genomic revelations of a mutualism: the pea aphid and its obligate bacterial symbiont. *Cellular and Molecular Life Sciences,* 68**,** 1297-1309.

SOBOTNIK, J., ALBERTI, G., WEYDA, F. & HUBERT, J. 2008. Ultrastructure of the digestive tract in Acarus siro (Acari: Acaridida). *Journal of Morphology,* 269**,** 54-71.

SOS-HEGEDUS, A., LOVAS, A., KONDRAK, M., KOVACS, G. & BANFALVI, Z. 2005. Active RNA silencing at low temperature indicates distinct pathways for antisense-mediated gene-silencing in potato. *Plant Molecular Biology,* 59**,** 595-602.

SZITTYA, G., SILVAHY, D., MOLNAR, A., HAVELDA, Z., LOVAS, A., LAKATOS, L., BANFALVI, Z. & BURGYAN, J. 2003. Low temperature inhibits RNA silencing-mediated defence by the control of siRNA generation. *The European Molecular Biology Organization Journal,* 22**,** 633-640.

TAMURA, K., PETERSON, D., PETERSON, N., STECHER, G., NEI, M. & KUMAR, S. 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Molecular Biology and Evolution,* 28**,** 2731-9.

TAO, Y., MAN, J. & WU, Y. 2012. Development of a multiplex polymerase chain reaction for simultaneous detection of wheat viruses and a phytoplasma in China. *Archives of Virology,* 157**,** 1261-7.

THOMPSON, J. D., HIGGINS, D. G. & GIBSON, T. J. 1994. Clustal-W - Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acid Research,* 22**,** 4673-80.

VAN DEN HEUVEL, J. F., VERBEEK, M. & VAN DER WILK, F. 1994. Endosymbiotic bacteria associated with circulative transmission of potato leafroll virus by Myzus persicae. *Journal of General Virology,* 75 2559-65.

VARANDA, C., CARDOSO, J. M. S., ROSÁRIO FÉLIX, M., OLIVEIRA, S. & CLARA, M. I. 2010. Multiplex RT-PCR for detection and identification of three necroviruses that infect olive trees. *European Journal of Plant Pathology,* 127**,** 161-164.

VERA, J. C., WHEAT, C. W., FESCEMYER, H. W., FRILANDER, M. J., CRAWFORD, D. L., HANSKI, I. & MARDEN, J. H. 2008. Rapid transcriptome characterization for a nonmodel organism using 454 pyrosequencing. *Molecular Ecology,* 17**,** 1636-47.

VINCELLI, P. & TISSERAT, N. 2008. Nucleic Acid–Based Pathogen Detection in Applied Plant Pathology. *Plant Disease,* 92**,** 660-669.

VISWANATHAN, R., KARUPPAIAH, R. & BALAMURALIKRISHNAN, M. 2010. Detection of three major RNA viruses infecting sugarcane by multiplex reverse transcription–polymerase chain reaction (multiplex-RT-PCR). *Australasian Plant Pathology,* 39**,** 79-84.

WANG, Y., BAO, Z., ZHU, Y. & HUA, J. 2009. Analysis of Temperature Modulation of Plant Defense Against Biotrophic Microbes. *Molecular Plant-Microbe Interactions,* 22**,** 498-506.

WEEKS, A. R., MAREC, F. & BREEUWER, J. A. J. 2001. A mite species that consists entirely of haploid females. *Science,* 292**,** 2479-2482.

WEI, T., LU, G. & CLOVER, G. 2008. Novel approaches to mitigate primer interaction and eliminate inhibitors in multiplex PCR, demonstrated using an assay for detection of three strawberry viruses. *Journal of Virological Methods,* 151**,** 132-139.

WEI, T., LU, G. & CLOVER, G. R. G. 2009. A multiplex RT-PCR for the detection of Potato yellow vein virus, Tobacco rattle virus and Tomato infectious chlorosis virus in potato with a plant internal amplification control. *Plant Pathology,* 58**,** 203-209.

WELBOURN, W. C., OCHOA, R., KANE, E. C. & ERBE, E. F. 2003. Morphological observations on Brevipalpus phoenicis (Acari: Tenuipalpidae) including comparisons with B. californicus and B. obovatus. *Experimental and Applied Acarology,* 30**,** 107-133.

WHITFIELD, A. E., ULLMAN, D. E. & GERMAN, T. L. 2005. Tospovirus-Thrips Interactions. *Annual Review of Phytopathology,* 43**,** 459-89.

WILSON, C. 1999. Detection and characterisation of orchid fleck virus. Gordon, NSW: Horticultural Research & Development Corporation.

WISLER, G. C. 1989. *How to control orchid viruses : the complete guidebook*, Maupin House Publishers.

YAMANE, K., OYAMA, K., IUCHI, E., OGAWA, H., SUZUKI, T. & NATSUAKI, T. 2008. RT-PCR Detection of Odontoglossum ringspot virus, Cymbidium mosaic virus and Tospoviruses and Association of Infections with Leaf-Yellowing Symptoms in Phalaenopsis. *Journal of Phytopathology,* 156**,** 268-273.

ZETTLER, F. W., KO, N. J., WISLER, G. C., ELIOTT, M. S. & WONG, S. M. 1990. Viruses of orchids and their control. *Plant Disease,* 74**,** 621-626.

ZHANG, J., WANG, R., SONG, J., LUO, Z., YANG, J. & LIN, F. 2013. One-step Multiplex RT-PCR for Simultaneous Detection of Four Viruses in Tobacco. *Journal of Phytopathology,* 161**,** 92-97.

ZHANG, X., ZHANG, X., SINGH, J., LI, D. & QU, F. 2012. Temperature-dependent survival of Turnip crinkle virus-infected arabidopsis plants relies on an RNA silencing-based defense that requires dcl2, AGO2, and HEN1. *Journal of Virology,* 86**,** 6847-54.