Final report

Project title:

Improving surveillance strategies for tospoviruses and thrips to enhance the biosecurity of the nursery industry

Project leader:

Dr Andrew D. W. Geering

Delivery partner:

The University of Queensland

Report author/s:

Andrew Geering

Project code:

NY19007

Date:

29 November 2024

Disclaimer:

Funding statement:

Levy funds – R&D projects

This project has been funded by Hort Innovation, using the Greenlife Industry research and development levy and contributions from the Australian Government. Hort Innovation is the grower-owned, not-for-profit research and development corporation for Australian horticulture.

Publishing details:

Published and distributed by: Horticulture Innovation Australia Limited
ABN 71 602100149

Level 7
141 Walker Street
North Sydney NSW 2060

Telephone: (02) 8295 2300

www.horticulture.com.au

Contents

Public summary

Orthotospoviruses *(Bunyaviridae*) are among the most economically important plant viruses worldwide, which in part reflects the very large number of plant species that are susceptible to infection, numbering in the thousands. Ornamental plant species are important hosts of this group of viruses, and trade in plant propagules is an important movement pathway. Orthotospoviruses are spread by thrips, and the major virus vectors such as *Frankliniella occidentalis* and *Thrips palmi* are already present in Australia, hence the establishment potential for exotic tospoviruses is very high.

The overall aim of this project was to develop diagnostic and surveillance tools for thrips and orthotospoviruses for the greenlife industry. The project was undertaken by a PhD student at The University of Queensland, Mr Hsu-Yao Chao, who presented four major research chapters in his thesis. In the first research chapter, a new orthotospovirus species for Australia and the world was described, which was named Pterostylis blotch virus (PtBV). It was concluded that PtBV is indigenous to Australia, and unlikely a threat to the greenlife industry as its host range appears restricted to terrestrial greenhood orchids. In the second research chapter, the potential of using spider lilies (*Hymenocallis* spp.) as sentinel plants for orthotospoviruses was investigated. Spider lilies are very popular landscaping plants, are susceptible to a diverse range of orthotospoviruses, and display very prominent ringspot symptoms when they become infected. Strong geographical structuring of the populations of orthotospoviruses infecting spider lilies along the Queensland coast was observed, suggesting that infections in these plants were reflective of the overall abundance of orthotospoviruses in the local environment. A new vector for tomato spotted wilt virus was identified, namely *Taeniothrips eucharii*. This thrips species breeds on various species of Liliaceae and Amaryllidaceae, including *Crinum*, *Eucharis*, *Hymenocallis*, *Liriope*, *Lycoris*, *Narcissus* and *Zephyranthes*. In the third research chapter, new DNA barcoding assays were developed for both thrips and orthotospoviruses. These assays have potential for use in metabarcoding studies, in which environmental samples are collected to test for the presence of target organisms. The final research chapter was a compilation of two disease notes, one describing the first record of citrus leprosis C2 in Australia, and the second demonstrating tuber-borne transmission of PtBV.

Keywords

Thrips; orthotospovirus; *Bunyaviridae*; capsicum chlorosis virus; Pterostylis blotch virus; tomato spotted wilt virus; *Hymenocallis*; metabarcoding; high throughput sequencing; surveillance; biosecurity.

Introduction

Orthotospoviruses (genus *Orthotospovirus*, family *Tospoviridae*) are among the most economically important plant viruses worldwide. Currently, 26 species of orthotospovirus are officially recognized by the International Committee on Taxonomy of Viruses (https://talk.ictvonline.org). While orthotospoviruses can be found in both temperate and tropical environments of all arable continents, the major centres of diversity are Southeast Asia, the Indian subcontinent and Central and South America (Oliver and Whitfield 2016). Three species are commonly found in Australia, namely tomato spotted wilt virus (TSWV), iris yellow spot virus (IYSV) and capsicum chlorosis virus (CaCV) (Persley et al., 2006). All species are regarded as introduced, even though TSWV was first discovered in Melbourne more than 100 years ago. At the commencement of this project, no truly indigenous tospovirus was known, although a potentially novel species had been discovered infecting the native terrestrial orchid *Pterostylis nutans* (AJ Gibbs and ADW Geering, unpublished data) in a bushland reserve bordering the Canberra suburbs.

Two important features of orthotospovirus biology are the very wide host ranges of the member species, and the role of thrips as vectors. TSWV has the widest host range of any plant virus, standing at more than 1000 plant species in over 85 plant families, including both monocots and dicots (Sherwood et al. 2003). This very wide host range, including a multitude of crop, ornamental and weedy plant species, makes control through the elimination of sources of inoculum very difficult. All orthotospoviruses are transmitted in the persistent and propagative manner by thrips. Only larvae are able to acquire the viruses but adults are the principal transmitters. Worldwide, 15 species of thrips have been reported as vectors of orthotospoviruses but the vector status of some species is confounded by cryptic speciation (Rotenberg et al. 2015). Cryptic species is a term used to describe populations that are morphologically indistinguishable but genetically discrete using DNA sequence-based analytical techniques. For example, at least two cryptic species of *Frankliniella occidentalis*, and three cryptic species each of *Thrips palmi*, *Thrips tabaci* and *Frankliniella schultzei* have been reported.

Epidemics of orthotopoviruses are sporadic in occurrence, which is primarily related to vector activity. Different species of thrips transmit at different efficiencies and intraspecific variation has been observed. During the 1920s, major epidemics of TSWV occurred in tomato crops throughout Australia, and the main vector species was *F. schultzei* (Best, 1960). For reasons that are unclear, TSWV declined in importance post-WWII until the early 1990s, when the incidence of TSWV again began to rapidly climb across Australia. This sudden re-emergence of TSWV in the 1990s in Australia was attributed to the introduction of *F. occidentalis*, a very efficient vector of the virus (Persley et al. 2006).

For field crops in Australia, the two pillars of orthotospovirus control have been the deployment of virus resistance genes and strategic applications of systemic and residual insecticides such as imidacloprid. Within protected cropping systems, the use of biological control agents to reduce thrips populations has been beneficial, as well as the control of alternative virus host species near the glasshouses. The application of insecticides needs to be very judicious as some thrips species are prone to develop insecticide resistance. An active thrips monitoring system is required.

The aims of this project were to:

1) Improve knowledge on the status of endemic orthospoviruses in Australia.
2) Develop rapid diagnostic assays for orthotospoviruses and their thrips vectors.
3) Develop new surveillance tools for orthotospoviruses and their thrips vectors to provide early alert of the arrival of exotic species.

Methodology

Detailed descriptions of the methodology used in this project are provided in the accompanying PhD thesis. Brief descriptions of the experimental approaches are provided in Results and Discussion.

Results and discussion

The research in this project was divided into three discrete themes, each constituting an individual chapter in the PhD thesis of Mr Hsu-Yao (Chester) Chao. One of these chapters has already been published (Chao et al. 2022), a second has been accepted for publication (Chao et al. in press), and the third is currently under review. Two pieces of supplementary research have also been written for publication as disease notes and constitute a fourth chapter of the PhD thesis. Summaries of these thesis chapters are provided below.

1. Characterisation of Pterostylis blotch virus

In the first research chapter, a new orthotospovirus infecting ground orchids (*Pterostylis nutans* and *P. curta*) in the ACT and NSW was characterized. This virus had been described in the popular literature in 2000 (Gibbs et al. 2000) but very few details were provided to allow classification or determine whether the virus was a previously described virus or new to science. It was hypothesized by us that this virus, called Pterostylis blotch virus (PtBV), had been introduced into Australia and may have spread from garden plants in Canberra to the nearby bush. This seemed the most likely scenario, as every orthotospovirus that was known at the beginning of the project originated from either Eurasia or the Americas, and all known thrips vectors were also non-native.

To characterize PtBV, diseased plants of *P. nutans* were collected by Dr Mark Clements, orchid taxonomist at the Australian National Herbarium, from the same site on Black Mountain where the virus was originally found about 25 years ago (Fig. 1). Surveys were also done at several other locations in the ACT and in NSW, and entire plants with suspect symptoms dug out of the ground, rinsed of soil, and couriered to the Ecosciences Precinct, Dutton Park. The plants were then transplanted into a pot containing a sand:peat mix, and grown in a glasshouse until processed. Permission to collect the plants was provided by NSW Scientific Licence SL100750 and ACT License No. PL2017136.

RNA was extracted from the leaves of the orchids and high throughput sequencing (HTS) done through the Australian Genome Research Facility. Standard methods were used to process the raw sequence reads, and the contigs obtained after *de novo* sequence assembly were annotated by doing BLASTX searches of the NCBI non-redundant protein database. Sequence contigs matching the L, M, and S RNA segments of a novel orthotospovirus species were identified for the type isolate of PtBV from Black Mountain (isolate 13365). Following 5' and 3' RACE, the complete genome segment sequences were 8636 nt for the L segment, 4697 nt for the M segment and 3159 nt for the S segment and these have been deposited in GenBank with accession numbers OL471332 to OL471334, respectively. The genome organization of PtBV was consistent with that of orthotospoviruses in general, confirming this classification.

Sequence contigs matching PtBV were also found in greenhood orchids from Gibraltar Falls and Hanging Rock in the Brindabella Ranges to the southwest of Canberra, and at Warrumbungle National Park in northern NSW. All infected plants had similar yellow blotch symptoms. Pairwise sequence comparisons showed that all the recovered genomes of PtBV had less than 40% aa sequence identity in the N protein to any other recognised or tentative orthotospovirus species but greater than 99% aa sequence identity to each other. To provide a genome-wide estimation of phylogeny, an analysis was carried out using a concatenated codon sequence alignment from five of the orthotospovirus genes (*RdRp*, *NSm*, *GP*, *NSs* and *N*). PtBV from Australia and Barleria chlorosis-associated virus (BCaV) from South Africa were shown to be sister taxa with 100% bootstrap support for the branch node and genealogical concordance with all but the NSs gene (Fig. 2). Both viruses were on long branches of the phylogenetic tree and fell outside of the currently recognized Eurasian and American clades, suggesting that they are indigenous to the continents on which they were originally found.

HTS is an agnostic sequencing approach, which does not rely upon prior knowledge of the genome of a virus to allow detection. This well-substantiated fact was again demonstrated in this study, with the discovery of many other viruses that were new to science in the orchid samples. Of greatest relevance to agriculture, a new polerovirus was discovered in greenhood orchids from Black Mountain. In a phylogenetic analysis, the Pterostylis polerovirus (PtPV) was shown to be most closely related to cereal yellow dwarf virus-RPS and cereal yellow dwarf virus-RPV. Given that PtPV grouped in a clade of introduced poleroviruses, it also is concluded to be non-native. Furthermore, all poleroviruses are transmitted in a persistent manner by aphids, and Australia has very few native aphids.

Figure 1. Nodding greenhood orchids (*Pterostylis nutans*) in natural habitat in Black Mountain Nature Park, ACT. The plant in the yellow frame is infected with Pterostylis blotch virus, and the plant in the red frame is uninfected. Photograph credit: Anne McKenzie, August 2020.

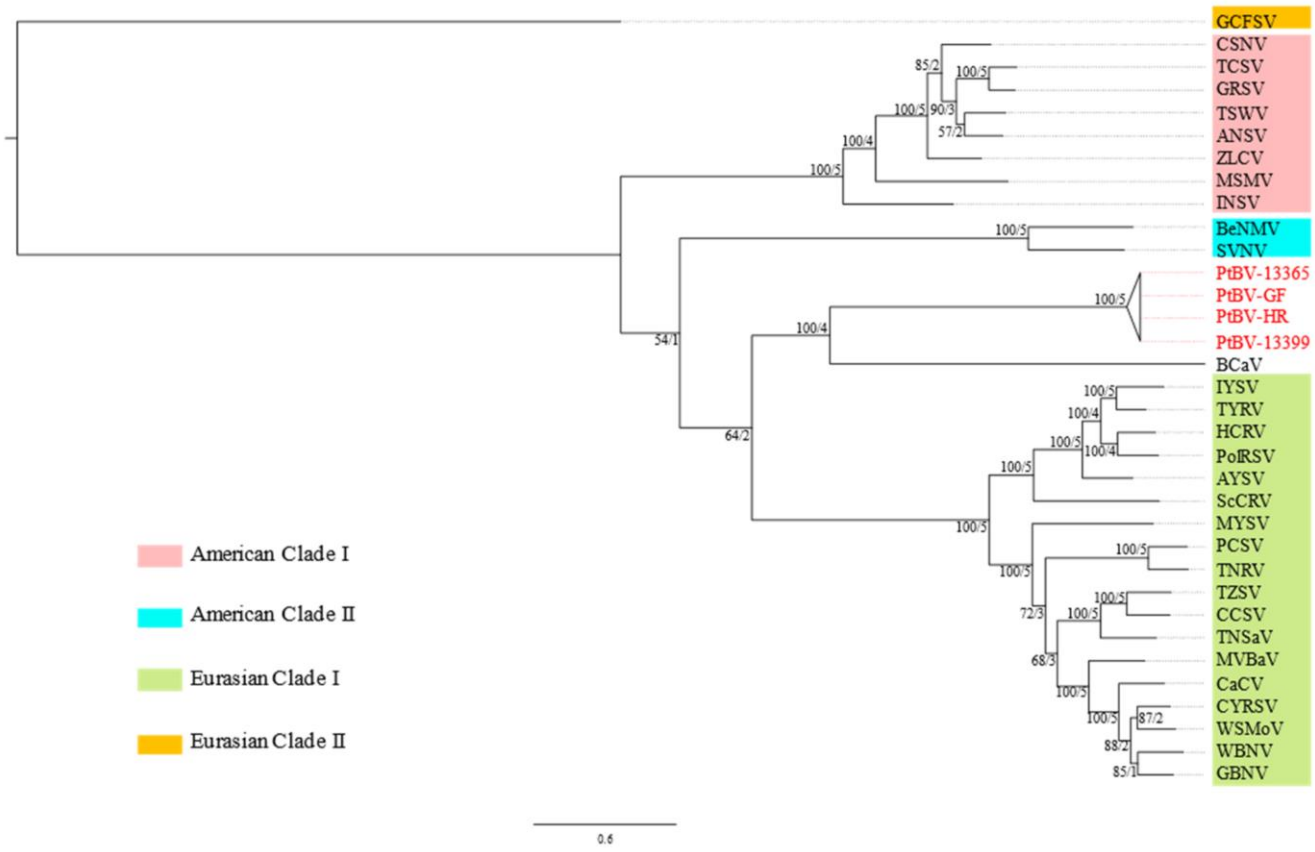

Figure 2. Maximum likelihood reconstruction of the phylogeny of orthotospoviruses based on concatenated sequence alignments of five genes (RNA-dependent RNA polymerase, non-structural protein M segment, glycoprotein precursor, non-structural protein S segment and nucleocapsid protein). The acronyms in red font correspond to the viruses discovered in this study. The numbers to the left of the slash next to the branches are non-parametric bootstrap values based on 1000 replicates; the numbers to the right of the slash are the number of decisive gene trees (5 at maximum) supporting the branch.

2. Evaluation of Spider Lilies (*Hymenocallis* spp.) as Sentinel Plants for Orthotospoviruses

Sentinels are highly susceptible plants or animals that are monitored for the purpose of early detection of a pathogen. The use of sentinel plantings allows surveillance for plant pathogens to be much more focused in area, which reduces the time and costs associated with the activity and may result in earlier detection of the pathogen. Furthermore, the use of sentinel plantings is a low technology solution for pathogen surveillance, and therefore is more likely to be adopted by the nursery industry.

The three characteristics that would make a good sentinel plant for orthotospoviruses are: i) they are susceptible to infection by a broad range of orthotospoviruses; ii) they display distinctive and easily recognisable disease symptoms; and iii) they are popular in cultivation and are perennial in growth habit. Spider lilies (*Hymenocallis* spp.) appear to have all the features that would make them a good sentinel plant for orthotospoviruses. Worldwide, spider lilies are susceptible to infection by at least five species of orthotospovirus, including calla lily chlorotic spot virus, hippeastrum chlorotic ringspot virus (HCRV), capsicum chlorosis virus (CaCV), impatiens necrotic spot virus (INSV) and tomato spotted wilt virus (TSWV) (Dietzgen et al. 2018; Liu et al. 2012; Xu et al. 2013; Liu et al. 2010; Huang et al. 2017). Spider lilies have a lot of ornamental appeal and are very commonly used for landscaping throughout frost-free, coastal areas of Australia. Finally, large ringspot symptoms are produced when the plants become infected with any of the aforementioned viruses.

Disease surveys of spider lilies (*Hymenocallis littoralis* and *H. speciosa*) were conducted in three distinct regions of Queensland: southeast Queensland (Qld) (Coolangatta to Noosa Heads), central Qld (Sarina to Gumlu), and far north Qld (Innisfail to Chillagoe) (Fig. 3). These regions are geographically distinct, being about 500–1500 km apart, and are separated by drier livestock grazing country. A variety of disease symptoms were observed, with ringspots being the most

common, followed by streaks or irregular blotches. The symptomatic areas were either chlorotic, strongly pigmented red or necrotic, sometimes a mixture of all three.

Symptomatic plants were tested for infection, initially using a near-universal RT-PCR assay followed by direct sequencing of the amplicon. A trend soon emerged that all plants were infected with either TSWV or CaCV, and therefore the diagnostic protocol changed to using species-specific RT-PCR assays only. Different profiles of orthotospoviruses were observed in each region (Fig. 3). In south-east Qld, symptomatic plants were common across the entire region, and nearly all were infected with TSWV, with a small minority (two of 65 plants) being infected with CaCV. However, in central Qld the composition of viral infections markedly changed, with all 21 sampled plants testing positive for CaCV, and TSWV was absent. Furthermore, symptomatic plants were less common than in southeast Qld, with most virus records coming from Bowen and Gumlu, which are major vegetable growing areas. No infections were identified in Mackay, and only a small number from the Pioneer River Valley where sugarcane is the major crop. The composition of viral infections in far north Qld resembled central Qld, although TSWV was detected in two plants from Ravenshoe, the highest town on the Atherton Tableland. Symptomatic plants were very rare along the coast of far north Qld, where sugarcane and banana are the two major crops. Most virus records came from the Atherton Tableland, an area of mixed horticulture.

During the virus surveys, symptoms were observed on several other plant species, and these also were placed through the diagnostic pipeline. TSWV-infected ornamental plants included bridal bouquet (*Plumeria pudica* Jacq.) from Cleveland, Dahlia 'Café au Lait' from Anstead, cast iron plants (*Aspidistra elatior* Blume) from Dutton Park, petunia (*Petunia × atkinsiana* (Sweet) W.H.Baxter) from Toowong, agapanthus (*Agapanthus praecox*) from Moorooka and Toowong, and swamp lily (*Crinum pedunculatum* R.Br) from Surfers Paradise. CaCV-infected ornamental plant species included two native waxplants (*Hoya australis* R.Br. and *H. macgillivrayi* F.M.Bailey) and a hippeastrum (*Hippeastrum* sp.) in Babinda. Some of these plants were found next to clumps of infected spider lilies, suggesting cross-infection between the species.

The N gene of all orthotospoviral isolates was sequenced and a phylogenetic analysis done to investigate whether there was finer geographic structuring of the viral populations. Conceptually translated protein sequences were clustered using the online server of CLUSS1 version 2.0. This algorithm was used as it conducts motif matching and is more sensitive to subtle differences among similar protein sequences compared to conventional phylogenetic tree reconstruction methods (e.g., maximum likelihood and distance-based methods like neighbour-joining), which treat each position in an alignment independently. CLUSS effectively groups sequences with similar patterns of sequence conservation.

The sequence clustering analysis based on complete nucleocapsid (N) protein sequences showed that most of the TSWV samples collected in southeast Queensland formed a single clade that was sister to other TSWV isolates reported from capsicum plants in Bowen and Gatton, Queensland (Figure). However, one of the southeast Queensland TSWV isolates collected from a spider lily plant in Boonah and the bridal bouquet sample from Redland City (TSWV sample number 3-1 from Cleveland) formed part of the same clade as TSWV from capsicums in central (Bowen) and southeast (Gatton) Queensland. The TSWV isolates from Ravenshoe in far north Queensland were more closely related to TSWV isolates recorded in Western Australia and South Australia than those found in spider lily plants in southeast Queensland. Together these results suggest TSWV infections in spider lily plants cannot simply be explained by multiplication of a single source of infected germplasm through vegetative propagation practices, but instead, multiple, independent infection events. Similarly, the population of CaCV isolates in spider lilies in Queensland was also not clonal (Fig. 5).

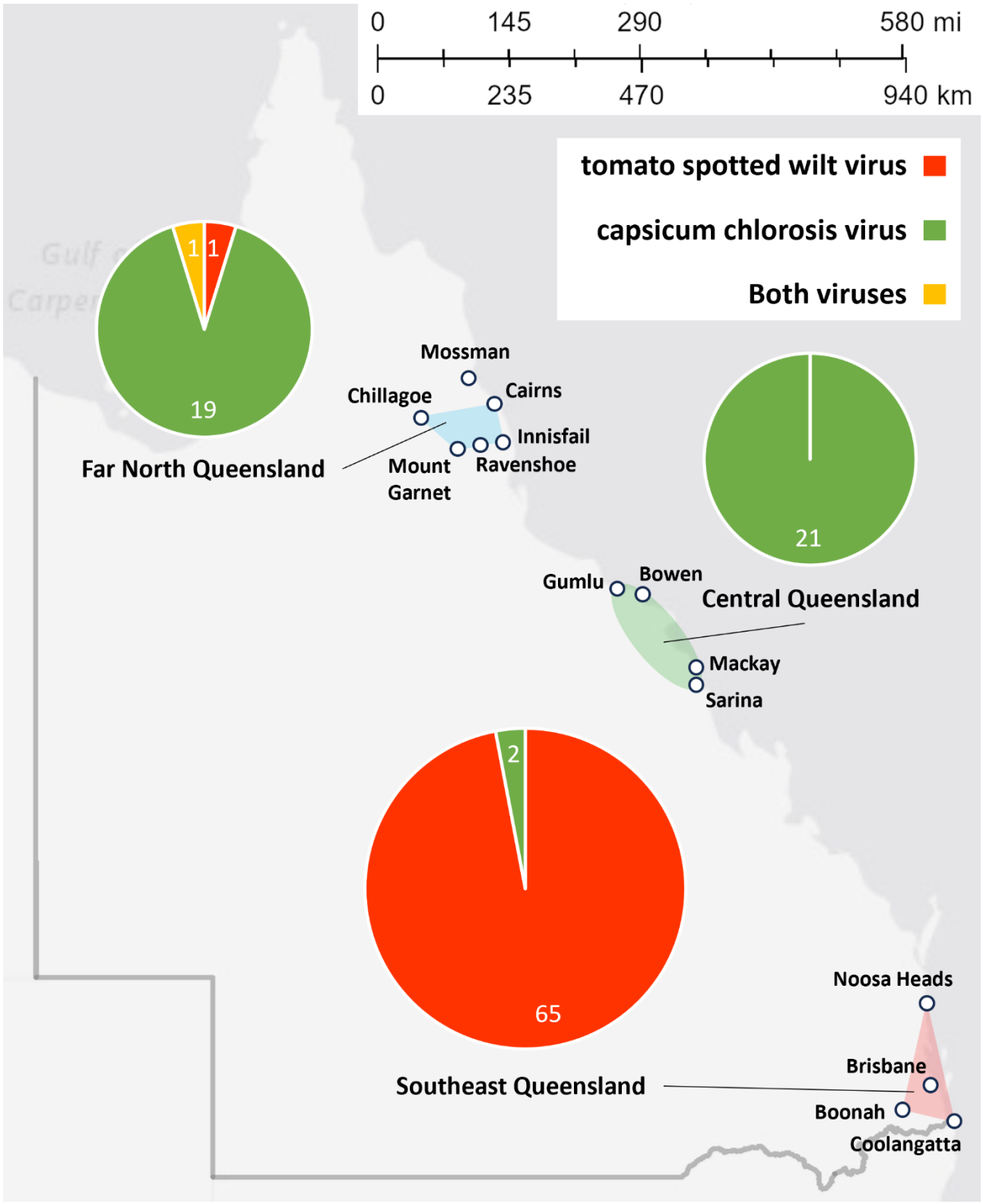

Figure 3. Regions surveyed and numbers of locations where orthotospovirus-infected spider lilies were found in this study**.**

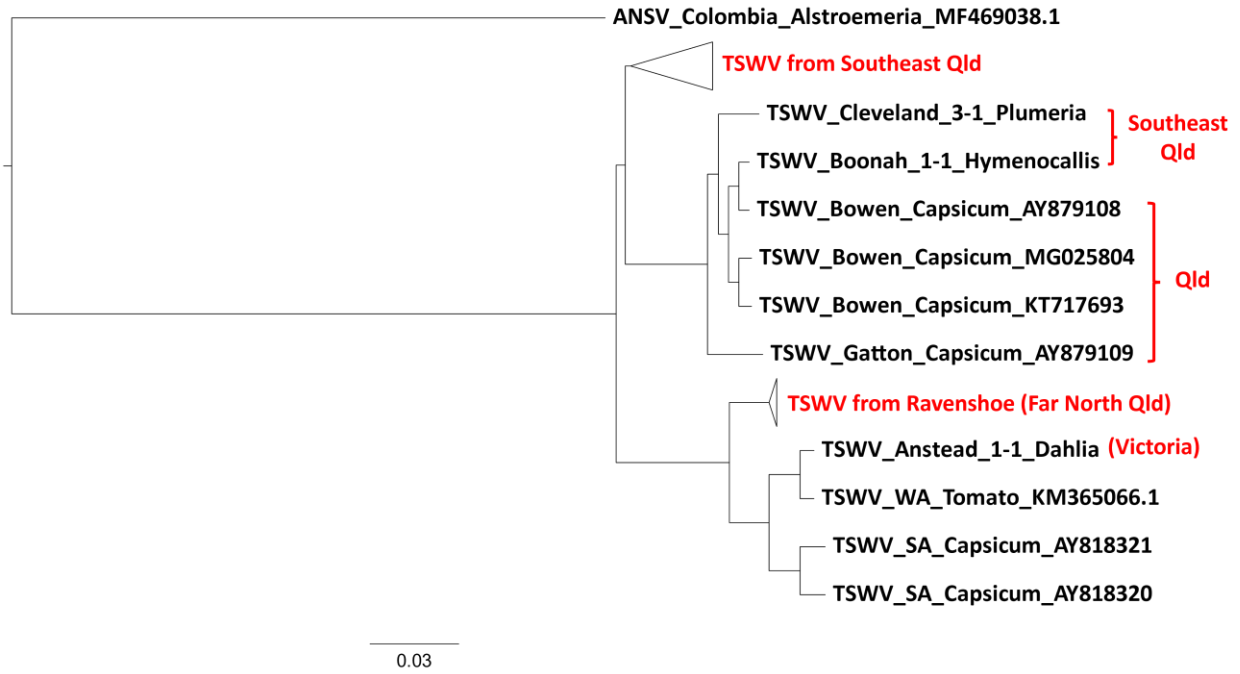

Figure 4. Dendrogram representing the sequence clustering results for tomato spotted wilt virus (TSWV) nucleocapsid protein sequences. Sequences from this study are labelled in the format of TSWV_suburb/town_sample number_host genus, while sequences with a GenBank accession number are from other studies. The dahlia sample was collected in Anstead, Queensland, but the plant was known to be from Victoria. All sequences from this study (including those in collapsed clades) are described in Additional file 1: Table S1. ANSV: Alstroemeria necrotic streak virus. Qld: Queensland. WA: Western Australia. SA: South Australia.

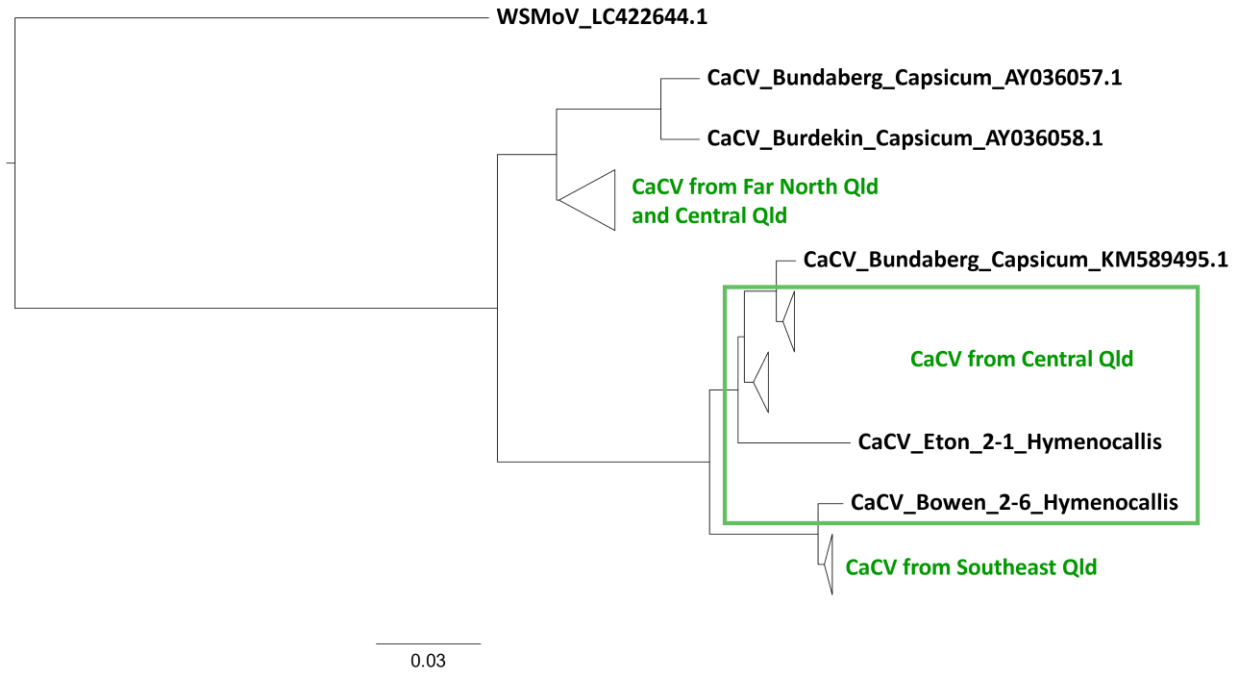

Figure 5. Dendrogram representing the sequence clustering results for capsicum chlorosis virus (CaCV) nucleocapsid protein sequences. Sequences from this study are labelled in the format of CaCV_suburb/town_sample number_host genus, while sequences with a GenBank accession number are from other studies. All sequences from this study (including those in collapsed clades) are described in Additional file 1: Table S1. WSMoV: watermelon silver mottle virus. Qld: Queensland.

During the surveys it was noted that many spider lily plants had discrete ringspots on an otherwise green leaf lamina. It was hypothesised that each ringspot represented a local lesion that developed at the point of inoculation by thrips. To test this hypothesis, TSWV-infected spider lily leaves showing ringspot symptoms were collected, with the leaf lamina sampled at multiple spots from the leaf and tested for the presence of virus as illustrated in Fig. 6. The test results suggested that TSWV was restricted to the symptomatic areas. Such experiments were repeated with ten more leaves collected from naturally infected spider lily plants, including both *H. littoralis* and *H. speciosa*, and the same pattern of infection was observed.

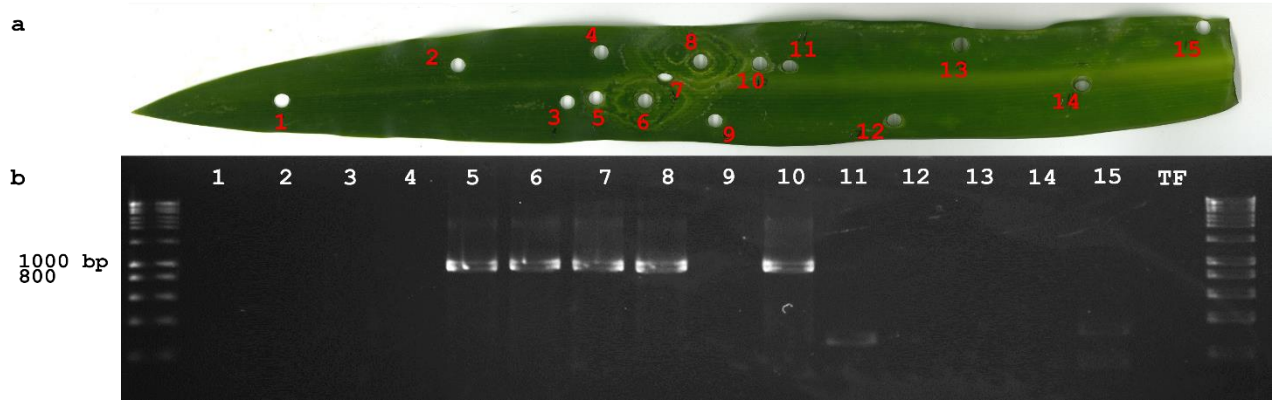

Figure 6. Detection of tomato spotted wilt virus (TSWV) in a *Hymenocallis speciosa* leaf with discrete ringspots. **a** Image of the leaf showing the location of the tissue sampling sites (numbered 1 to 15) relative to the ringspot symptoms. **b** Detection of TSWV by reverse trancriptase polymerase chain reaction. The lane number corresponds to the sampling site in **a**. The target amplicon is about 900 bp, and TF is the template-free control. Photograph credits: Hsu-Yao Chao.

To examine whether TSWV can systemically infect spider lilies, five plants (two *H. littoralis* and three *H. speciosa*) with discrete ringspot symptoms were dug up from gardens and maintained in the glasshouse. After approximately 4 months and the emergence of about nine uninfected and asymptomatic leaves, one *H. speciosa* plant became obviously symptomatic. Later, one *H. littoralis* plant also developed systemic infection. TSWV was detectable in the offsets of the two systemically infected plants, further confirming the systemic nature of the infection. The other three spider lily plants remained asymptomatic and uninfected over a total of 6 months of observation and testing.

The final research question that was addressed in this body of work was which thrips species may be responsible for spread of TSWV between spider lily plants. Blue sticky traps were set up close to TSWV-infected spider lilies at five different locations in Brisbane. Only 33 thrips from these locations tested positive for TSWV, and each was identified as *Taeniothrips eucharii* by DNA barcoding (>99% pairwise nt sequence identity to *T. eucharii*, GenBank Accession KT946651.1).

To confirm that *T. eucharii* could transmit TSWV, a colony was established on a systemically infected spider lily plant in an insect cage. Individual adults from this colony were then transferred to either detached leaves or entire seedlings of *Emilia sonchifolia* and allowed an inoculation access period of c. 16 h. The detached leaves were kept alive by floating on water. Eight out of 15 detached leaves of *Emilia sonchifolia* (L.) DC. showed disease symptoms (chlorotic and necrotic lesions) 7 days after inoculation with TSWV-viruliferous thrips, and these leaves tested positive for the virus by RT-PCR. Additionally, three out of four *E. sonchifolia* plants became systemically infected after viruliferous thrips fed on the plants.

The main conclusions of this body of work were that spider lilies are good indicator plants of the orthotospoviruses circulating in the local environment, and the number of ringspot lesions on a leaf may be used to provide an estimate of the infection pressure. Spider lilies could provide a useful low technology surveillance tool for orthotospoviruses. *Taeniothrips eucharii* was also shown for the first time anywhere in the world to be a vector of TSWV, and it likely plays an important role in the epidemiology of this virus.

3. Improving Molecular Diagnostics and Surveillance for Orthotospoviruses and Their Vectors

Orthotospoviruses and thrips each pose challenges for identification. All orthotospoviruses produce similar symptoms in plants, and they can only accurately be distinguished using molecular diagnostic methods. Thrips can be identified using morphological features, but this requires great technical expertise to firstly clear and properly mount the specimens on microscope slides, and then to be able interpret the discriminating features. Furthermore, cryptic speciation is observed with some thrips, meaning that closely related species are indistinguishable using morphological features.

The cytochrome c oxidase subunit I (COI) gene in the mitochondrial genome has achieved status around the world as the universal DNA barcode for insect taxonomy. Sequences of this gene have broad taxonomic coverage in the international nucleotide sequences databases, and it is one of the most used genetic loci for phylogenetic analyses and species demarcation. However, use of the COI gene for DNA barcoding has problems, one being that there are not strictly conserved DNA sequences in the COI gene that can be used for universal PCR primer design to cover all insect taxa. It is often necessary to develop taxon-specific DNA barcoding PCR primers. Universal primers such as LCO-1490/HCO-2198 (Folmer et al. 1994) or C1-J-1751/C1-N-2191 (Simon et al. 1994; Lin et al. 2004) are still commonly used for thrips DNA barcoding, but these were designed up to 30 years ago using sequences from organisms that are completely unrelated to thrips (Folmer et al. 1994).

The concept of DNA barcoding also extends to viruses, although there is not a single orthologous gene common to all viruses. For viruses, the term universal usually only applies to a virus genus. Universal orthotospoviral PCR primers have been designed, but because of recent discoveries of new orthotospoviruses such as PtBV, there is a need to update the primers to account for new sequence variation that has been found.

The aims of this research chapter were to develop new universal PCR primers for both orthotospoviruses and thrips. To achieve these aims, a new software pipeline was written in Python to identify conserved regions for designing universal PCR primers. Input sequence data were collected from National Center for Biotechnology Information (NCBI) databases. The selection of candidate primer sequences took into consideration sequence conservation at both the amino acid and the nucleotide/codon levels, aiming for fewer variants, ideally just one.

In total 322 orthotospovirus RdRp protein/coding sequences were collected. Based on the protein sequence alignment, two conserved motifs in the RNA dependent RNA polymerase protein were chosen for primer design. Three forward (F1, F2 and F3) and three reverse (R1, R2 and R3) PCR primers were designed, which together accounted for all observed sequence variation observed across *c*. 30 officially recognized or tentative orthotospovirus species (Table 1). The observed primer motif patterns consisted of six combinations: F1R1, F1R2, F1R3, F2R2, F2R3 and F3R3. The F1R1 pattern spanned several clades and was observed in most orthotospoviruses in an American clade (American I), some Eurasian orthotospoviruses (Eurasian II) and Pterostylis blotch virus (PtBV) (Fig. 7). The F1R2 pattern was observed in most Eurasian orthotospoviruses (Eurasian I) and some American orthotospoviruses (CSNV and zucchini lethal chlorosis virus, ZLCV). The F1R3 pattern was only found in BCaV, while the F2R2 pattern was only seen in soybean vein necrosis orthotospovirus (SVNV). The F2R3 pattern occurred in bean necrotic mosaic virus (BeNMV) and soybean thrips-associated orthotospovirus 1 (STaTV1). Lastly, only the RdRp of lisianthus necrotic ringspot virus (LNRV) contained the F3R3 pattern and was the only target of primer F3.

To achieve universal orthotospovirus detection, the aforementioned primers were “multiplexed”, i.e., all primers were included in one reaction. The amount of each primer per reaction was proportional to the degree of primer degeneracy. The orthotospovirus RdRp assay was independently tested on six different targets, each containing one of the observed primer binding motif combinations in orthotospoviruses (F1/R1, F1/R2, F1/R3, F2/R2, F2/R3, and F3/R3). When tested on dsDNA templates, the best performance of the assay was visible target amplification from 10^{-7} fmol or 10^{-8} fmol of the template in a 10-µL PCR. Ten different orthotospoviruses were also detected in a range of plant hosts and thrips vectors (Fig. 8).

To design COI DNA barcoding primers for thrips, 190 thrips COX1 sequences were assembled, consisting of 25 species in the suborder Terebrantia and 10 species in the suborder Tubulifera. As most existing COI DNA barcodes for thrips were generated using either the LCO1490/HCO2198 or C1-J-1751/C1-N-2191 primer pairs, the *COX1* primers designed in this study (

Table) all flanked this common region. Two equally conserved sites were identified upstream of the target region.

Interestingly, the sequences of both sites were conserved within but not between the Terebrantia and Tubulifera (Table 1), making them potentially useful for differentiating these two suborders of thrips. Three equally conserved sites were identified downstream of the target region. As all known orthotospovirus vectors belong to the family Thripidae in the suborder Terebrantia, only COX1 primers compatible with Terebrantia sequences were subjected to further examination. The two Terebrantia-specific forward primers and three reverse primers constitute six different primer pairs generating amplicons ranging from approximately 450 bp (Trb-F2/R3) to 900 bp (Trb-F1/R1).

Primer Name	Primer Sequence	Codon Sequence	Amino Acid Sequence
Orthotospovirus RNA-dependent RNA polymerase			
R1	CARAGRACIAARACIGAYAGRGA	CARAGRACNAARACNGAYAGRGAR	QRTKTDRE
R2	CARAGRACIAARATGGAYAGRGA	CARAGRACNAARATGGAYAGRGAR	QRTKMDRE
R3	CARAGRACIAARGTIGAYAGRGA	CARAGRACNAARGTNGAYAGRGAR	QRTKVDRE
F1	CCAYTTIGAYTGRTCIGCIGA	TCNGCNGAYCARTCNAARTGG	SADQSKW
F2	CCAYTTIGAYTGRTCIGAIGA	TCNTCNGAYCARTCNAARTGG	SSDQSKW
F3	CCAYTTIGACATRTCIGCIGA	TCNGCNGAYATGTCNAARTGG	SADMSKW
Insect translation elongation factor 1-alpha			
F	TTYAARTAYGCITGGGTIYTIGAYAA	TTYAARTAYGCNTGGGTNYTNGAYAAR	FKYAWVLDK
R	CCICCIATYTTRTAIACRTCYTG	CARGAYGTNTAYAARATHGGNGGN	QDVYKIGG
Thrips cytochrome c oxidase subunit I			
Trb-F1	ATYGGIGGITTYGGIAAYTG	ATYGGNGGNTTYGGNAAYTGR	IGGFGNW
Tbl-F1	ATRGGIGGITTYGGIAAYTG	ATRGGNGGNTTYGGNAAYTGR	MGGFGNW
Trb-F2	CCIGAYATRGCITTYCCICG	CCNGAYATRGCNTTYCCNCGN	PDMAFPR
Tbl-F2	CGIGGRTARCAYATRTCIGG	CCNGAYATRTGYTAYCCNCGN	PDMCYPR
R1	ACRTARTGRAARTGIGCIACIAC	GTNGTNGCNCAYTTYCAYTAYGTN	VVAHFHYV
R2	TGIGCYCAIACRATRAAICC	GGNTTYATYGTNTGRGCNCAY	GFIVWAH
R3	TCIGGRTGICCRAARAAYCA	TGRTTYTTYGGNCAYCCNGAR	WFFGHPE

Table 1. Primers designed in this study and their corresponding codon and amino acid sequences. “F” in the primer names indicates forward primers, while “R” in the primer names indicates reverse primers. Primers with the prefix “Trb-“ in their names are specific to the suborder Terebrantia, and those with the prefix “Tbl-“ specific to the suborder Tubulifera. The underscored letters highlight the differences in the codon or amino acid residue.

PCR amplicons of the expected size were obtained using all six COX1 primer pairs designed in this study (Table 1) as well as the primer pair C1-J-1751/C1-N-2191 for all four thrips species tested in this study, namely *F. occidentalis*, *F. schultzei* (Trybom), *Thrips tabaci* and *Taeniothrips eucharii* (Whetzel). To assess the quantitative bias of different *COX1* primer pairs in reflecting relative abundance of the different thrips species, lyophilised specimens of *F. schultzei*, *T. eucharii* and *F. occidentalis* were mixed in a dry-mass ratio of 1 mg: 2 mg: 3 mg to create a mock community. Nanopore sequencing results of the amplicons showed that only the *COX1* primer pair Trb-F1/R2 consistently yielded numbers of reads that highly correlated with the dry masses of thrips, albeit not necessarily reflecting the same ratio of 1:2:3. This indicates the impracticability of pursuing a metabarcoding assay that will directly yield quantitative results in every scenario.

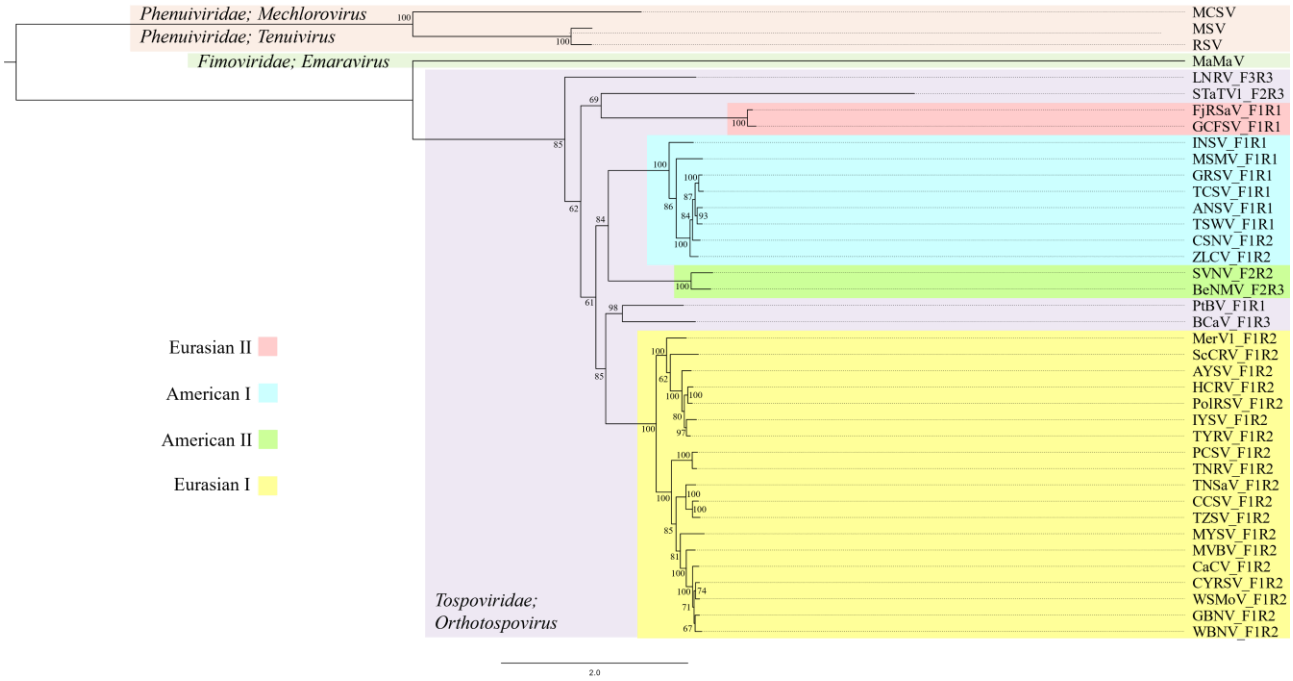

Figure 7. Maximum likelihood tree reconstructed from the protein sequence alignment of virus RNA-dependent RNA polymerase. The tree was rooted to Phenuiviridae. The substitution model used for tree reconstruction was LG+F+I+G4. The "FXRY" following the acronyms of orthotospoviruses indicates the binding site sequence pattern combination of the primers in

Table 1. The numbers at each branch/node represent the the bootstrap value.

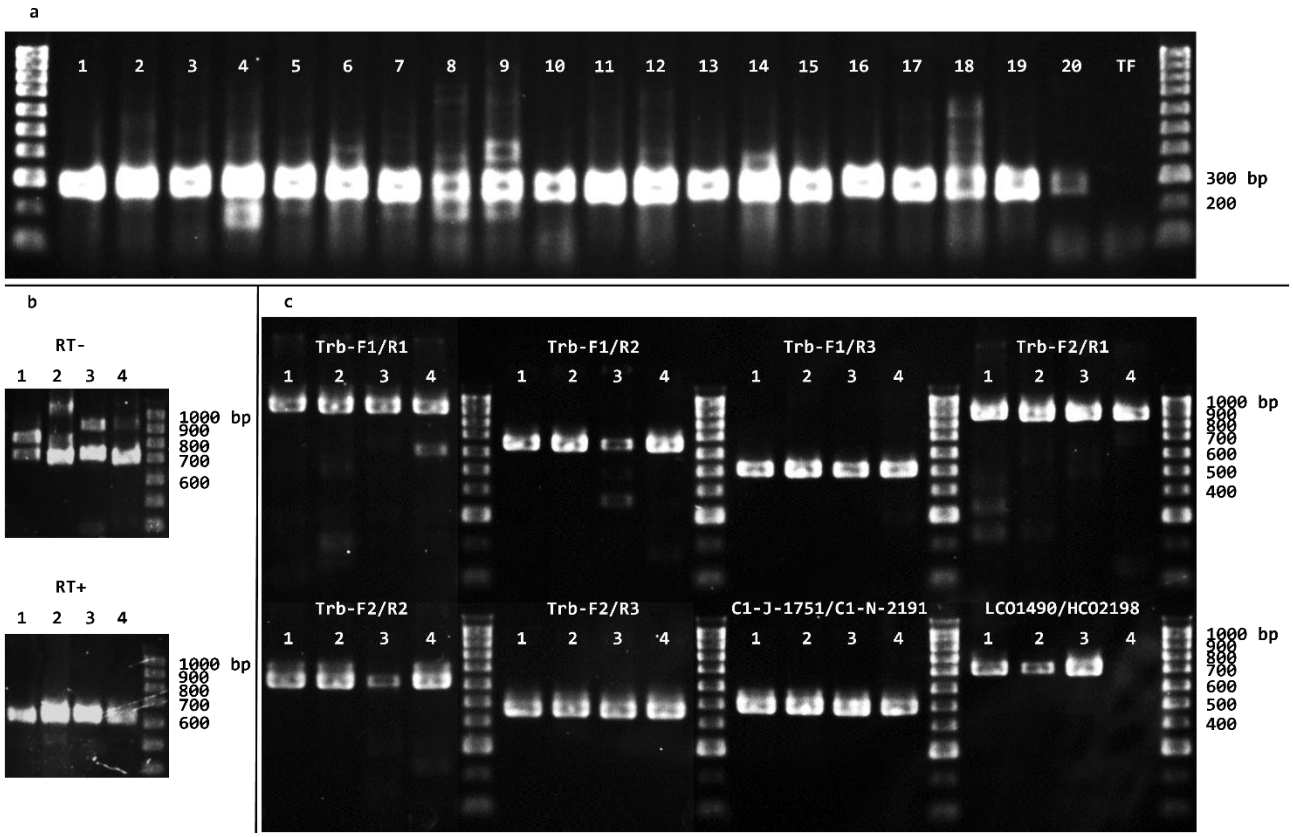

Figure 8. Test results of polymerase chain reaction (PCR)-based assays developed in this study on biological samples. **a** Orthotospoviruses detected in different hosts using the reverse transcription-PCR (RT-PCR) assay targeting the orthotospovirus RNA-dependent RNA polymerase gene. The target amplicons were approximately 250 bp. Samples 1 to 19 are different orthotospovirus species, while sample 20 was a *Taeniothrips eucharii* (Whetzel) specimen carrying tomato spotted wilt virus. TF: template-free control. **b** Amplification results of the RNA internal control RT-PCR assay targeting the translation elongation factor 1-alpha gene on four different thrips species using total nucleic acids without reverse transcription (RT-) and DNase I-treated RNA with reverse transcription (RT+). **c** Amplification results of PCR targeting the cytochrome c oxidase subunit I (COX1) gene in four different thrips species with different primer pairs. The thrips species tested in **b** and **c** were 1: *Frankliniella schultzei* (Trybom); 2: *Frankliniella occidentalis* (Pergande); 3: *Thrips tabaci* Lindeman; 4: *Taeniothrips eucharii*. The partial *COX1* sequences of each thrips species are available through GenBank accessions PQ368741–PQ368744.

4. Disease notes

The final research chapter of the PhD thesis is a compilation of two disease notes.

The first disease note dealt with the discovery of the hibiscus strain of citrus leprosis virus C2 (CLV-C2H) in a cultivated plant of *Hoya macgillivrayi* from the Brisbane suburb of Yeerongpilly. This discovery was serendipitous, as the virus was detected during efforts to sequence the genome of an isolate of capsicum chlorosis virus infecting the same plant. A near-complete genome of CLV-C2H was assembled with 98–99% nt identity to the genome of the reference isolate of the virus infecting a *Hibiscus rosa-sinensis* plant from Hawaii. This detection led to a biosecurity incursion response, as CLV-C2H had not previously been recorded in Australia and is a threat to Australia's citrus industry. Follow-up surveys were done in Brisbane, but no other examples of infection were identified.

The second disease note addressed the question, how does PtBV persist from one year to the next? The host range of PtBV appears to be very narrow, and its main plant host, *Pterostylis nutans*, dies back to a tuber during summer. To test whether PtBV is tuber-borne, entire infected plants were dug up during spring, transplanted into a pot, and allowed to naturally go into dormancy during summer within a screenhouse. A subset of plants was destructively sampled to examine the distribution of PtBV within the plant. PtBV was detectable in the root and tubers of infected plants by RT-

PCR, indicating systemic infection. The plants that regenerated the following year from tubers had symptoms in the very first leaves that emerged and tested positive for PtBV. It was concluded that PtBV was transmitted through the tubers. Insects were also collected from infected plants in the field, as part of preliminary investigations as to what vectors may be involved in horizontal transmission of the virus. The only thrips species that was collected was *Dichromothrips australiae*, and this possibly is the vector of PtBV. Additionally, about 50 morphologically similar aphids were collected and a subset identified as *Aulacorphum solani* by DNA barcoding. It is hypothesized that this aphid species is the vector of Pterostylis polerovirus.

Outputs

Table 2. Output summary

Output	Description	Detail
Newly trained early career scientist specializing in plant virology.	• Mr Chester (Hsu-Yao) Chao successfully completed his PhD	Mr Chester (Hsu-Yao) Chao submitted his PhD thesis in August 2024
Research publications.	• Each thesis chapter submitted for publication.	See Chao et al. (2022), Chao et al. (in press)
New knowledge on Pterostylis blotch virus (PtBV)	• Genome sequence for PtBV • New diagnostic assay for PtBV. • Distributional and host range data for PtBV	See PhD thesis
New diagnostic protocols for thrips and orthospoviruses	• New universal DNA barcoding PCR assays for both thrips and orthotospoviruses	See PhD thesis
New surveillance protocols for orthotospoviruses and thrips.	• New knowledge on effectiveness of spider lilies as sentinel plants for orthotospoviruses • Protocols for metabarcoding thrips catches using MinION.	See PhD thesis
New knowledge on orthotospovirus transmission	• Evidence of vertical transmission of PtBV through tubers. • Identification of *Taeniothips euchari* as a new vector species of tomato spotted wilt virus (TSWV)	See PhD thesis
New knowledge on citrus leprosis virus C2	• First record of virus in Australia • New host record of virus	See PhD thesis
Extension notes for production nursery managers and owners	Outputs of project described in two industry extension notes	https://yourlevyatwork.com.au/project-update-spider-lilies-can-be-used-to-warn-of-tospovirus-infections/ Thrips identification using a laptop computer and a pocket-sized DNA sequencer (in press). See Appendices 3 and 4.

Outcomes

Table 3. Outcome summary

Outcome	Alignment to fund outcome, strategy and KPI	Description	Evidence
More effective and cost-efficient surveillance tools for tospoviruses and thrips	Improve industry biosecurity preparedness and resilience, including prevention, protection and recovery from exotic and endemic plant pest incursions and responses	New diagnostic and surveillance tools for thrips and orthotospoviruses	New diagnostic and surveillance protocols have been submitted for publication in open access journals, making them freely available to all biosecurity agencies in Australia
Better market access for Australian horticultural produce	Improve access to interstate and international nursery products through the national adoption of a creditable biosecurity system	Data on presence or absence of orthotospoviruses in Australia	Data in the process of being published in open access journals. Virus isolates deposited in Queensland DPI plant virology collection.
Better IPM strategies for thrips	Improve industry biosecurity preparedness and resilience, including prevention, protection and recovery from exotic and endemic plant pest incursions and responses	More accurate and rapid identification of thrips, allowing tailoring of control methods	New diagnostic and surveillance protocols have been submitted for publication in open access journals, making them freely available to all biosecurity agencies in Australia

Monitoring and evaluation

Table 4. Key Evaluation Questions

Key Evaluation Question	Project performance	Continuous improvement opportunities
To what extent has the project achieved its expected outcomes?	There was a good mix of applied and academic outcomes from this project, as to be expected with a PhD project. Importantly, the student submitted his PhD thesis on time.	Some flexibility needs to be provided for PhD projects, as it is a learning experience and consideration needs to be given to the interests and talents of the student.
How relevant was the project to the needs of intended beneficiaries?	The project was very relevant to the greenlife industry as a whole, less so to individual nurseries. Both high and low technology solutions for orthospovirus surveillance were provided.	Practice change needs be advocated within the biosecurity agencies
How well have intended beneficiaries been engaged in the project?	Not as well as hoped. At the beginning of the project, there were field visits to Pohlman's Nursery, but experimental work at this site discontinued due to changes in the experimental direction.	In a project such as this, engagements with industry partners are on a needs basis
To what extent were engagement processes appropriate to the target audience/s of the project?	Extension notes on project progress were published at the end of years 2 and 3. Scientific findings were presented at the Australasian Plant Virology Workshop and Biennial Conference of Australasian Plant Pathology Society	Communication activities were appropriate but could be more extensive, such as webinars
What efforts did the project make to improve efficiency?	Fortnightly meetings were held between the student and supervisory team to fine tune the project goals and set minor milestones.	None

Recommendations

The recommendations of this project are:

1) More research is required to understand the epidemiological factors leading to the discrete geographical distributions of orthotospoviruses in Australia. Data was provided that CaCV is the dominant orthotospovirus in the tropics, whereas TSWV is much more abundant in the subtropics of Queensland. It is hypothesized that differences in the populations of thrips vectors in the various regions is the principal reason for the differences in the prevalence of orthotospoviruses. However, very little is known about virus-vector relationships, for example the comparative vectoring capabilities of the brown and yellow morphs of *Frankliniella schultzei* for each of the orthotospovirus species.

2) Domestic quarantine regulations are justified to prevent the introduction of impatiens necrotic spot virus (INSV) into Queensland. Evidence was provided that this orthotospovirus is still absent in Queensland, despite having been introduced into NSW and Victoria.

3) More research is required to identify the variety of plant viruses carried by ornamental plant species in Australia. It was again demonstrated in this project that ornamental plant species do represent movement pathways for economically important pathogens of food and fibre crops. Vegetatively propagated plants carry the greatest risk of spreading plant viruses, as infections are perpetuated in propagules such as cuttings or tissue culture plants.

4) There is a need to create a DNA barcode library for the thrips of Australia, particularly as traditional taxonomic expertise for this group of insects has almost disappeared. DNA barcoding provides a much easier method of identification of thrips, and there is scope to utilize the sequence data to develop rapid diagnostic tools such as LAMP assays.

5) Metabarcoding of thrips catches should be incorporated into future surveillance activities by the biosecurity agencies for both thrips and orthotospoviruses.

6) Engaging two PhD students on this project, one in Victoria, and the second in Queensland, has proven to be a very cost effective and comprehensive research approach, especially as the environments are so different between these states. It is impossible to generalize results produced in Queensland across all of Australia.

Refereed scientific publications

Journal article

Chao, H.-S., Clements, M.A., Mackenzie, A.M., Dietzgen, R.G., Thomas, J.E., Geering, A.D.W., 2022. Viruses infecting greenhood orchids (Pterostylidinae) in eastern Australia. Viruses 14:365

Chao, H.-S., Dietzgen, R.G., Thomas, J.E., Geering, A.D.W., in press. New molecular diagnostic and surveillance tools for orthotospoviruses and their thrips vectors. Phytopathological Research

Chapter in a book or paper in conference proceedings

Chao, H.-S., Clements, M.A., Mackenzie, A.M., Dietzgen, R.G., Thomas, J.E., Geering, A.D.W., 2022. Viruses infecting greenhood orchids in Eastern Australia, Australasian Plant Virology Workshop. Melbourne, VIC, Dec 2022.

Chao, H., Geering, A., Thomas, J., Dietzgen, R. (2023). Development of highly sensitive RT-PCR assays for universal detection of orthotospoviruses and internal control in thrips testing. 24th Biennial Conference of the Australasian Plant Pathology Society, Adelaide, SA Australia, 20-24 November 2023.

References

Chao, H.-Y., Clements, M.A., Mackenzie, A.M., Dietzgen, R.G., Thomas, J.E., Geering, A.D.W., 2022. Viruses infecting greenhood orchids (Pterostylidinae) in eastern Australia. Viruses 14 (2):365

Chao, Y.-S., Dietzgen, R.G., Thomas, J.E., Geering, A.D.W., in press. New molecular diagnostic and surveillance tools for orthotospoviruses and their thrips vectors. Phytopathological Research

Dietzgen, R.G., Freitas-Astúa, J., Salaroli, R.B., Kitajima, E.W., 2018. Tomato spotted wilt virus infects spider lily plants in Australia. Australas Plant Dis Notes 13 (1):26.

Folmer, O., Black, M., Hoeh, W., Lutz, R., Vrijenhoek, R., 1994. DNA primers for amplification of mitochondrial cytochrome c oxidase subunit I from diverse metazoan invertebrates. Molecular Marine Biology and Biotechnology 3 (5):294-299

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:11-21

Huang, Y., Hong, H., Zhao, X.H., Li, J., Tao, X.R., 2017. Complete genome sequence of a Capsicum chlorosis virus in China and the structural variation and evolutionary origin of its S RNA intergenic region. Arch Virol 162 (10):3229-3232.

Lin, C.-P., Danforth, B.N., Wood, T.K., 2004. Molecular phylogenetics and evolution of maternal care in Membracine treehoppers. Syst Biol 53 (3):400-421.

Liu YT, Lu XP, Zhi L, Zheng YX, Chen XJ, Xu Y, Wu FJ, Li YZ (2012) Calla lily chlorotic spot virus from Spider Lily (Hymenocallis litteralis) and Tobacco (*Nicotiana tabacum*) in the South-west of China. Journal of Phytopathology 160 (4):201-205.

Liu YT, Zheng YX, Li YZ, Li ZY (2010) First Report of Impatiens necrotic spot virus on Spiderlily in China. Plant Dis 94 (4):484-484.

Persley, D.M., Thomas, J.E., Sharman, M., 2006. Tospoviruses—an Australian perspective. Australasian Plant Pathology 35 (2):161-180.

Simon C, Frati F, Beckenbach A, Crespi B, Liu H, Flook P (1994) Evolution, Weighting, and Phylogenetic Utility of Mitochondrial Gene Sequences and a Compilation of Conserved Polymerase Chain Reaction Primers. Ann Entomol Soc Am 87 (6):651-701.

Xu, Y., Lou, S.G., Li, XL, Zheng, Y.X., Wang, W.C., Liu, Y.T., 2013. The complete S RNA and M RNA nucleotide sequences of a hippeastrum chlorotic ringspot virus (HCRV) isolate from Hymenocallis littoralis (Jacq.) Salisb in China. Arch Virol 158 (12):2597-2601.

Intellectual property

No project IP or commercialisation to report.