FINAL REPORT

For Public Release

Part 1 - Summary Details

CRDC ID: DAQ1601

Project Title: Surveillance and studies for endemic and exotic virus diseases of cotton

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Research Program: 2 Industry

Part 2 – Contact Details

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Signature of Research Provider Representative:

Date submitted: _____

Part 3 – Final Report

(The points below are to be used as a guideline when completing your final report)

Background

1. Outline the background to the project.

Viral diseases of cotton are of economic significance in many parts of the world. Only two virus diseases have been reported from Australian cotton crops, *Cotton bunchy top virus* (CBT) and *Tobacco streak virus* (TSV). However, many of the most economically damaging virus diseases of cotton remain serious biosecurity threats. These include Cotton leaf curl disease (CLCuD), *Cotton leaf roll dwarf virus* (CLRDV; causing Cotton blue disease) and *Cotton leaf crumple virus* (CLCrV). This is particularly relevant with the recent detection of *Cotton leafroll dwarf virus* (CLRDV – blue disease) from East Timor by projects DAQ1201 and DAQ1405 from samples collected by Northern Australian Quarantine Strategy (NAQS) staff conducting surveys for the International Plant Health Program (IPHP), Australian Department of Agriculture.

Results from CRDC project DAQ1201 indicate that Cotton blue disease is likely to be caused by different virus strains in Asia, East Timor and South America (little remains known about the causal agent in Africa). The recent detection of CLRDV from East Timor highlights the need for a better understanding of the likely host range of this virus, the potential for the East Timor strain to cause Blue disease in cotton and possible incursion pathways into Australia. CLRDV and other related viruses also infect pulse crops and little is known about the role these grain viruses may play in disease outbreaks in cotton in the same farming environment.

Results from project DAQ1201 identified many alternative hosts of Cotton bunchy top virus (CBTV) and also demonstrated that two distinct strains of CBTV, -A and –B, are commonly found in all cotton growing regions surveyed. It appears that CBTV-B is closely associated with disease but it remains unclear if CBTV-A plays a role in disease outbreaks.

Objectives

2. List the project objectives and the extent to which these have been achieved, with reference to the Milestones and Performance indicators.

Objective 1. Survey Australian cotton crops for endemic and exotic virus diseases to monitor for possible incursions and to determine the incidence, distribution and importance of endemic viruses.

This objective has been achieved. Virus disease surveys for endemic and exotic virus threats were conducted at least once during each growing season from 2012 to 2015 in central Queensland. Surveys were also conducted in southern Qld and northern NSW cotton crops in each season. Survey data has been collated and discussion of results presented to industry at least annually through project progress reports. Survey results were also presented at industry meetings as detailed further in Results section for Milestone 5.1.

Objective 2. Survey Gossypium species and other potential hosts in northern Australia for CBTV, CLRDV, other poleroviruses and exotic viruses. At least two virus surveys conducted during project.

This objective has been achieved. At least six surveillance trips were done in northern Australia between June 2016 and May 2019. Over 600 samples were collected and/ or tested by a range of assays including generic polerovirus tests and specific CLRDV PCRs. More than 30 plants of feral *Gossypium hirsutum* were collected and tested from Torres Strait Islands and Darwin. Three disease surveys were also done in commercial cotton production regions in Kununurra and northern QLD. Survey data has been collated and discussion of results presented to industry at least annually through project progress reports. Survey results were also presented at industry meetings as detailed further in Results section for Milestone 5.1.

Objective 3. Provide support and preparedness for viral biosecurity threats. Investigate if CLRDV is established in East Timor and if so, determine its possible host range and potential pathways into Australia. Make the most up to date diagnostic test for CLRDV and Contingency Plan available to industry.

This objective has been achieved as described in the associated Milestones and Performance Indicators. However, the results obtained did not provide conclusive evidence to determine if the CLRDV strains in Thailand or Timor-Leste could overcome resistance in a similar manner as observed for the "atypical" strains from South America.

Partial virus genome for CLRDV isolates from Thailand and Timor-Leste was determined for more than 12 samples from two regions of the virus genome. The analyses of these partial genome regions indicate that CLRDV isolates from both Thailand and Timor-Leste were most likely not the atypical strain from Brazil known to overcome CLRDV resistance. However, there was much greater diversity of CLRDV genome sequence from Timor-Leste and Thailand compared to CLRDV from South America.

Three surveys of Gossypium species and other potential hosts of CLRDV were done in Timor-Leste. Over 500 samples were collected from a wide range of host species from over 70 sites in eastern, central and western Timor-Leste. Over 100 Gossypium samples (37 *G. arboreum*, 40 *G. barbadense*, 25 *G. hirsutum*) were collected from 27 different sites and CLRDV was found to be relatively common in all three Gossypium species from 7 sites. Another 7 polerovirus species were detected from Timor-Leste for the first time. Results of surveys and CLRDV genome characterisation have been reported back to industry twice yearly through progress reports and via various extension material to industry.

The newly discovered diversity of CLRDV from Timor-Leste, Thailand and Uzbekistan has been used to update the CLRDV diagnostic test and the CLRDV Contingency Plan.

Objective 4. Determine relative importance of the two known strains of CBTV (-A and -B) in outbreaks of bunchy top disease.

This objective has been achieved. Almost complete genomes have been determined for the two *Cotton bunchy top virus* (CBTV) species from Australia and analysis has confirmed these are distinct polerovirus species, hereafter called CBTV-1 (previously strain –A) and CBTV-2 (previously strain –B). While there were limited significant outbreaks of CBTV

during this project, a total of 50 typical CBT and 122 non-symptomatic plants collected from 4 cotton blocks were tested for CBTV. No plants with typical bunchy top symptoms were found with only CBTV-1 and all symptomatic plants were infected with CBTV-2, either with or without CBTV-1. This data is in agreement with previous data from project DAQ1201 that CBTV-2 is always associated with disease symptoms in cotton while CBTV-1 is not.

Objective 5. Dissemination of disease management recommendations and provision of virology diagnostic services. Specifically, support CSIRO project CSP1401 by testing samples from CBTV host range experiments.

This objective has been achieved. At least 19 extension items (including 2 peer-reviewed journal articles) were prepared and published during this project via a range of extension opportunities including industry meetings such as FUSCOM and Spotlight magazine.

Sample extracts were tested in the CBTV multiplex PCR to support the host range studies of CSIRO project CSP1401.

Methods

3. **Detail the methodology and justify the methodology used. Include any discoveries in methods that may benefit other related research.**

Objective 1. Survey Australian cotton crops for endemic and exotic virus diseases to monitor for possible incursions and to determine the incidence, distribution and importance of endemic viruses.

The main aim of this objective was to ensure that the surveillance method used provided a high reliability of detecting endemic and exotic virus diseases in commercial cropping regions. Another aim was to determine the incidence of endemic virus diseases and this was done in situations where it appeared to be having an impact on the crop being inspected. To determine incidence, a randomised disease count method is preferred in which plants are inspected from a representative transect through the crop. In contrast, to maximise the probability of detecting any virus-affected plants, a more targeted surveillance method was adopted.

With a history of insect pest incursions in northern regions such as Silverleaf whitefly and solenopsis mealybug, and the proximity to possible pathways from northern Australia, surveillance in domestic crops was focused on central Queensland, southern QLD and emerging production regions in far north QLD and northern Western Australia.

Where possible, a standardised disease survey method was used for the purposes of maximising the chances of detecting virus diseases in crops. To maximise probability of detecting any virus, about 300-600 plants were inspected on the edge or corner of a block most likely to be exposed to possible virus sources such as weeds, ratoon or volunteer cotton, or bushland upwind. Additional plants were also inspected outside the standardised count area while moving through the crop so that typically about 1000 plants were sighted at each count location.

This surveillance method was adopted to provide approximately 95 % confidence that a virus-symptomatic plant would be detected in the search area if 1 % of plants were virus-

symptomatic. To provide this level of confidence, at least 300 plants need to be inspected (Cameron & Baldock, 1998; Sergeant, 2015). Total number of plants inspected was sometimes lower for the late season inspections due to the difficulty (and slower process) in checking larger plants.

Crops were inspected for any virus-like symptoms to check for endemic (*Cotton bunchy top virus* (CBTV) and *Tobacco streak virus* (TSV)) and exotic viruses (e.g. Cotton leaf curl disease (CLCuD) and other begomoviruses) and *Cotton leafroll dwarf virus* (CLRDV; causing Cotton blue disease). Representative samples of symptomatic plants were collected and tested either for TSV or CBTV depending on the observed symptoms. Occasional plants were also tested for exotic begomoviruses. TSV was tested for by Enzyme Linked Immuno-Sorbent Assay (ELISA) essentially as per the manufacturer's instructions (Reagent set SRA25500, Agdia, USA) to confirm that visual assessments were accurate. CBTV was tested for by reverse transcription PCR to distinguish between the two known species, CBTV-1 and CBTV-2 using the multiplex PCR developed as part of previous project DAQ1201.

Objective 2. Survey Gossypium species and other potential hosts in northern Australia for CBTV, CLRDV, other poleroviruses and exotic viruses. At least two virus surveys conducted during project.

At least six surveillance trips were done in northern Australia between June 2016 and May 2019. These included surveys in the Torres Strait Islands in June 2016, Darwin and surrounds in March 2017, April 2018, May 2018 and May 2019, Kununurra in May 2018 and May 2019, lower Cape York (Georgetown) in May 2017, and Weipa in June 2019. Two of these surveys were done in collaboration with the Northern Australian Quarantine Strategy teams (Torres Strait Islands and Weipa).

Over 600 samples from a wide range of potential hosts were collected and/ or tested by a range of assays including generic polerovirus tests and specific CLRDV PCRs. Hosts targeted for collection and testing were determined from previously determined host range of CBTV from project DAQ1201 and published host range of CLRDV as reported in the Contingency Plan prepared as part of project DAQ1201. During surveys, other potential hosts were also collected if they were found to be infested with aphid populations (the vector insect for poleroviruses). Potential hosts of begomoviruses were also collected.

The assays used for diagnostics generally included the use of Tissue Blot Immuno Assay (TBIA) using the monoclonal antibody 5G4 to *Bean leafroll dwarf virus* (BLRV; Katul, 1992). This antibody is known to cross react with a wide range of polerovirus species and has proved useful for the initial steps in detection and characterisation of new poleroviruses. TBIA is also a low tech assay that was used successfully during survey trips in northern Australia and Timor-Leste with all necessary equipment and consumables taken on these survey trips. Generally, samples were collected over a few days during a survey and if time permitted, a TBIA assay was done on a batch of samples (this assay takes about 1 day). Any positive TBIA results obtained during surveys could then inform targeting of further samples.

If a positive was obtained by TBIA, it was then tested by generic polerovirus PCRs (e.g. Pol3628F / Pol3982R, or Pol3870F / AS3) and sequenced. CBTV and CLRDV-specific PCRs were also used on selected samples.

Objective 3. Provide support and preparedness for viral biosecurity threats. Investigate if CLRDV is established in East Timor and if so, determine its possible host range and potential pathways into Australia. Make the most up to date diagnostic test for CLRDV and Contingency Plan available to industry.

I joined the International Plant Health Program (IPHP) team on three surveillance activities in Timor-Leste (East Timor) in March 2016, February 2017 and April 2018. The IPHP facilitates these surveillance activities which are done by NAQS staff and collaborating quarantine staff from Timor-Leste government. The program and location of the surveillance was determined by the IPHP and focused on the northern coastal region east of Dili. However, there was some focus on collection of Gossypium species after the recent detection of CLRDV in *G. barbadense*. The diagnostics used and the host range targeted during surveys was essentially the same as described for Objective 2.

The results from surveys and studies into diversity of CLRDV were used to improve the diagnostic assay for CLRDV and new information was used to update the CLRDV diagnostic test and the CLRDV Contingency Plan.

Objective 4. Determine relative importance of the two known strains of CBTV (-A and -B) in outbreaks of bunchy top disease.

CBTV-1 is thought to be non-symptomatic in cotton but to increase the chances of finding CBTV-1 infected plants, we targeted areas where there were spreading outbreaks of CBT plants and hence aphid activity. Symptomatic and non-symptomatic plants were collected close each other (often next to each other) to maximise the chances of aphids moving between plants with some of those aphids possibly only carrying CBTV-1 to cause non-symptomatic infection. Samples were tested by CBTV species-specific PCRs previously developed as part of project DAQ1201.

To further characterise the two species of CBTV, we selected a mixed infection of CBTV-1 and –2, originally isolated from cotton from Emerald. This was transmitted to chickpea via cotton aphids. The virus concentration in chickpea appears to be much higher than in cotton, so this was used as material for Ilumina next generation sequencing (NGS) done by the Australian Genome Research Centre.

Objective 5. Dissemination of disease management recommendations and provision of virology diagnostic services. Specifically, support CSIRO project CSP1401 by testing samples from CBTV host range experiments.

Extension material, based on research data obtained was prepared in collaboration with cotton extension and biosecurity staff and published to industry via a range of extension opportunities including industry meetings such as FUSCOM and Spotlight magazine. Some research data was also published in peer-reviewed journal articles. As a member of the Cotton Biosecurity Reference Group, I provided review and updates of virus threats for the Cotton Industry Biosecurity Plan on a yearly basis since about 2016.

Samples for virus diagnostics were received from collaborators (NAQS, CSIRO staff and NSW DPI pathologists) and agronomists. Generic polerovirus PCR and CBTV-specific PCRs were done as described for Objective 2.

Results

4. Detail and discuss the results for each objective including the statistical analysis of results.

Objective 1. Survey Qld and NSW cotton crops for endemic and exotic virus diseases.

Milestone 1.1. Annual viral disease surveys conducted in Australian cotton crops for exotic and endemic viruses.

Over 84,000 plants were inspected from within 158 standardised disease counts from 35 different farms across seasons in 2015/16 to 2018/19 from production areas in far north Queensland (Strathmore), central Queensland (Clermont, Emerald, Comet, Arcturus), Southern Queensland (Dalby, Cecil Plains, Macalister) and far north Western Australia (Kununurra) (Table 1). Additional plants were also inspected outside standardised counts while moving through the crop and this was occasionally effective for detecting trace levels of virus-infected plants or isolated patches within the crop. Hence, a combined surveillance approach is recommended which adopts both standardised counts on the crop edge where influx of virus is most likely and a more general observation further into the crop. The airborne habit of most virus insect vectors such as aphids and white flies means that infection loci are usually along a crop edge but can also be within the crop boundary.

No symptoms typical of exotic viruses were seen during the surveys of domestic crops. Generally, there was very low incidence of virus-like symptoms for endemic viruses (CBTV and TSV) in cotton crops inspected with the exception of a few sporadic disease outbreaks. CBTV was seen in spreading patches in a few crops such as paddocks WPms778-1 in Nov-2015, WPms17-198b in Nov-2017 and most strikingly in several paddocks around Macalister in early 2018 (Table 1).

One paddock at Farm 24, paddock WPms057, near MacAlister was inspected in more detail to determine the spread of CBT across the paddock (Fig. 1). There was about 30% CBT incidence close to the eastern edge of the crop along a margin of about 700 m. The incidence dropped to about 5% at 200 m (downwind) into the crop. There were no obvious infected ratoons along the edge of the crop but there was a fallow paddock with many CBT-symptomatic volunteers, with colonies of cotton aphids on a neighbouring farm about 150 m upwind, which had recently been sprayed out with herbicide. It appears the aphids had moved off the fallow paddock into the cotton crop. While we cannot be certain of the source of infection, this situation illustrates the need for area-wide control of ratoon and volunteer cotton to minimise the risk of CBT spread within and between farms. It also raises the issue of collective responsibility of industry members to maintain farm hygiene which would be particularly important if there was an incursion of an insect vectored pathogen.

In agreement with survey results from previous projects (DAQ0002 and DAQ1201), Tobacco streak virus was seen in crops from central Queensland but not from other regions. TSV was generally at low incidence and very localised to areas adjacent to infestations of parthenium or crownbeard weeds, the major alternative hosts for TSV. TSV was seen in many CQ crops during late 2016 but was generally only localised symptoms on one or more leaves and with no obvious impact on plant growth. As

observed previously, TSV sometimes occurred in mixed infections with CBTV (e.g. paddock WPms17-198b in Nov-2017) in which it appeared the systemic infection of CBTV enabled TSV to also systemically infect the plants resulting in more severe symptoms that CBTV alone.

CBTV-infected plants were seen in all growing regions surveyed except far north QLD (Strathmore) and far north WA (Kununurra) (Table 1). The surveillance strategy was effective at detecting diseased plants but it was important to also check surrounding volunteers and ratoons as they were often infected with CBTV but no infected plants were observed within the nearby crop. One example of this is included in Table 1 for paddock WPms18-183 in Nov-2018 where 65% of old volunteer cotton in an irrigation channel had CBTV but no CBTV was seen in the cotton paddocks in close proximity suggesting that only the lack of aphid vectors was stopping the movement of virus.

Table 1. Details of virus survey sites and results of surveillance for endemic viruses CBTV and TSV.

Month-year	Farm #	Nearest locality	Paddock Ref#	Total plants inspected in disease count	CBTV plants	TSV plants
Nov-2015	1	Emerald-east	WPms308	419	0	**1**
Nov-2015	1	Emerald-east	WPms443	637	**1**	**0**
Nov-2015	1	Emerald-east	WPms668	691	0	0
Nov-2015	2	Emerald-east	WPms775	610	0	0
Nov-2015	3	Emerald-east	WPms776	611	0	**1**
Nov-2015	3	Emerald-east	WPms777	350	0	**1**
Nov-2015	3	Emerald-east	WPms778-1	453	**43**	**1**
Nov-2015	3	Emerald-east	WPms778-2	300	0	0
Nov-2015	4	Comet	WPms558	663	0	0
Nov-2015	4	Comet	WPms681	600	0	0
Nov-2015	5	Arcturus	WPms246	604	0	0
Nov-2015	5	Arcturus	WPms780	600	0	0
Nov-2015	5	Arcturus	WPms330	600	0	**5**
Nov-2015	5	Arcturus	WPms329	560	0	0
Nov-2015	5	Arcturus	WPms781	350	0	0
Oct-2016	6	Emerald-west	Field4	606	0	**4**
Oct-2016	6	Emerald-west	WPms917	600	0	**3**
Oct-2016	7	Emerald-west	WPms918a	645	0	**13**
Oct-2016	7	Emerald-west	WPms918b	604	0	**40**
Oct-2016	7	Emerald-west	WPms919a	374	0	**7**
Oct-2016	7	Emerald-west	WPms919b	630	0	**17**
Oct-2016	2	Emerald-east	N-1	600	0	**7**
Oct-2016	4	Comet-west	WPms681a	600	0	**29**
Oct-2016	4	Comet-west	WPms558	600	0	**30**
Oct-2016	4	Comet-west	WPms437	602	0	**22**
Dec-2016	8	Frankfield	WPms922	308	0	**14**
Dec-2016	8	Frankfield	WPms923	306	0	**6**
Dec-2016	8	Frankfield	WPms924	400	0	**17**
Dec-2016	8	Frankfield	WPms926	126	0	**32**
Dec-2016	9	Pasha	WPms927	300	0	**15**
Dec-2016	9	Pasha	WPms928	311	**5**	**9**

Dec-2016	9	Pasha	WPms929a	337	0	**35**
Dec-2016	9	Pasha	WPms929b	312	0	**1**
Dec-2016	10	Arcturus	WPms932	343	0	**2**
Dec-2016	10	Arcturus	WPms933	319	0	0
Dec-2016	10	Arcturus	WPms934	335	0	0
Dec-2016	10	Arcturus	WPms935	341	0	0
Dec-2016	11	Arcturus	WPms936	338	0	**2**
Dec-2016	11	Arcturus	WPms937a	322	0	**1**
Dec-2016	11	Arcturus	WPms937b	306	0	0
Dec-2016	5	Arcturus	WPms140	352	0	**7**
Dec-2016	5	Arcturus	WPms938	331	0	**2**
Dec-2016	5	Arcturus	WPms939	300	0	**2**
Dec-2016	5	Arcturus	WPms781	319	0	**6**
Dec-2016	4	Comet	WPms437	300	0	**2**
Dec-2016	4	Comet	WPms437	300	0	**5**
Dec-2016	7	Emerald	WPms940	310	0	0
Feb-2017	12	Cecil Plains	WPms943	300	0	0
Feb-2017	13	Cecil Plains	WPms944	300	0	0
Feb-2017	14	Cecil Plains	WPms945	300	0	0
May-2017	15	Strathmore	WPms987	300	0	0
May-2017	15	Strathmore	WPms988	300	0	0
Nov-2017	3	Emerald-east	WPms17-190	600	**2**	0
Nov-2017	3	Emerald-east	WPms17-193	600	**3**	0
Nov-2017	3	Emerald-east	WPms17-198a	564	0	0
Nov-2017	3	Emerald-east	WPms17-198b	240	**45** [A]	**4**
Nov-2017	16	Emerald-east	Block 23	300	0	0
Nov-2017	16	Emerald-east	Block 5	640	0	0
Nov-2017	16	Emerald-east	Block 4	300	0	**3**
Nov-2017	16	Emerald-east	Block 8	300	0	**2**
Nov-2017	16	Emerald-east	Block 9	300	0	0
Nov-2017	16	Emerald-east	Block 24	300	0	0
Nov-2017	16	Emerald-east	Block 25	300	0	0
Nov-2017	17	Emerald-east	Block 3	300	0	0
Nov-2017	18	Emerald-west	Field T3	300	0	**2**
Nov-2017	18	Emerald-west	Field T2	600	**2**	0
Nov-2017	18	Emerald-west	Field T4	300	0	**2**
Nov-2017	4	Comet	WPms558a	600	0	0
Nov-2017	4	Comet	WPms558b	300	0	**1**
Nov-2017	4	Comet	WPms435	370	**1** [B]	0
Nov-2017	4	Comet	WPms681a	450	0	**1**
Nov-2017	4	Comet	WPms681b	340	0	0
Jan-2018	19	Dalby-east	WPms682-1	600	**3**	0
Jan-2018	19	Dalby-east	WPms682-2	2000	**9**	0
Jan-2018	19	Dalby-east	WPms682-3	2000	**2**	0
Jan-2018	20	Nandi-east	WPms030-1	2000	**3**	0
Jan-2018	20	Nandi-east	WPms030-2	2000	0	0
Jan-2018	21	Nandi	WPms687-1	1000	**1**	0
Jan-2018	22	Condamine Plains	WPms344	778	0	0
Jan-2018	22	Condamine Plains	Block W4A	300	0	0
Jan-2018	22	Condamine Plains	Block G6	712	0	0
Jan-2018	22	Condamine Plains	Block G5	773	0	0

Jan-2018	23	Branchview	WPms031	733	0	0
Jan-2018	23	Branchview	WPms833	640	0	0
Jan-2018	24	MacAlister	WPms051-1	417	**21**	0
Jan-2018	24	MacAlister	WPms051-2	343	**13**	0
Jan-2018	24	MacAlister	WPms057	188	**63** [C]	0
Feb-2018	25	MacAlister-SW	WPms18-010-1	700	**1**	0
Feb-2018	25	MacAlister-SW	WPms18-010-2	600	**36**	0
May-2018	26	Kununurra	WPms18-100	300	0	0
May-2018	27	Kununurra	WPms18-106	1000	0	0
May-2018	27	Kununurra	WPms18-110	600	0	0
May-2018	27	Kununurra	WPms18-111	600	0	0
May-2018	27	Kununurra	WPms18-112	600	0	0
May-2018	27	Kununurra	WPms18-113	300	0	0
May-2018	27	Kununurra	WPms18-114	600	0	0
May-2018	4	Comet	WPms18-169	600	0	**154**
Nov-2018	4	Comet	WPms18-171	600	0	**4**
Nov-2018	4	Comet	WPms18-172	600	0	**1**
Nov-2018	4	Comet	WPms18-173	600	0	**1**
Nov-2018	4	Comet	WPms18-174	600	0	0
Nov-2018	4	Comet	WPms18-175	600	0	0
Nov-2018	5	Arcturus	WPms18-177	600	0	0
Nov-2018	5	Arcturus	WPms18-178	600	0	**1**
Nov-2018	5	Arcturus	WPms18-179	600	0	0
Nov-2018	5	Arcturus	WPms18-180	1000	0	**1**
Nov-2018	5	Arcturus	WPms18-181	600	0	0
Nov-2018	5	Arcturus	WPms18-182	600	0	0
Nov-2018	5	Arcturus	WPms18-183	29	**19** [D]	0
Nov-2018	5	Arcturus	WPms18-184	300	0	0
Nov-2018	10	Arcturus	WPms18-186	600	0	0
Nov-2018	10	Arcturus	WPms18-187	600	0	0
Nov-2018	10	Arcturus	WPms18-188	600	0	0
Nov-2018	10	Arcturus	WPms18-189	600	0	0
Nov-2018	10	Arcturus	WPms18-190	600	0	0
Nov-2018	3	Emerald-east	WPms18-192	600	0	0
Nov-2018	3	Emerald-east	WPms18-193	600	0	0
Nov-2018	1	Emerald-east	WPms18-194	600	0	0
Nov-2018	1	Emerald-east	WPms18-195	300	0	0
Nov-2018	1	Emerald-east	WPms18-196	600	**2**	0
Nov-2018	1	Emerald-east	WPms18-197	300	**6**	0
Nov-2018	3	Emerald-east	WPms18-198	600	**1**	0
Nov-2018	3	Emerald-east	WPms18-199	300	0	0
Nov-2018	18	Emerald-east	WPms18-200	1000	**1**	0
Nov-2018	18	Emerald-east	WPms18-201	600	0	0
Nov-2018	18	Emerald-east	WPms18-202	600	0	0
Nov-2018	7	Emerald-west	WPms18-203	300	0	0
Nov-2018	7	Emerald-west	WPms18-204	600	0	0
Feb-2019	28	Cecil Plains	WPms19-009	500	0	0
Feb-2019	28	Cecil Plains	WPms19-010	500	0	0
Feb-2019	29	Cecil Plains	WPms19-011	600	0	0
Feb-2019	29	Cecil Plains	WPms19-012	600	0	0
Feb-2019	29	Cecil Plains	WPms19-013	600	0	0

Feb-2019	30	Condamine Plains	WPms19-014	600	0	0
Feb-2019	30	Condamine Plains	WPms19-015	600	0	0
Feb-2019	30	Condamine Plains	WPms19-016	600	0	0
Feb-2019	22	Cecil Plains	WPms19-018	600	0	0
Feb-2019	22	Cecil Plains	WPms19-019	600	0	0
Feb-2019	22	Cecil Plains	WPms19-020	600	0	0
Feb-2019	31	Brookstead	WPms19-021	600	0	0
Feb-2019	24	Macalister	WPms19-022	600	**1**	0
Feb-2019	24	Macalister	WPms19-023	300	0	0
Feb-2019	24	Macalister	WPms19-024	600	0	0
Feb-2019	32	Macalister	WPms19-026	600	**1**	0
Feb-2019	32	Macalister	WPms19-027	600	**3**	0
April-2019	33	Duaringa	WPms19-053	500	0	0
April-2019	33	Duaringa	WPms19-061 [E]	500	0	0
April-2019	1	Emerald-east	WPms19-072	600	0	0
April-2019	7	Emerald-west	WPms19-076	600	0	0
May-2019	34	Kununurra	WPms19-082	600	0	0
May-2019	35	Kununurra	WPms19-084	300	0	0
May-2019	35	Kununurra	WPms19-085	300	0	0
May-2019	27	Kununurra	WPms19-086	300	0	0
May-2019	27	Kununurra	WPms19-088	300	0	0
May-2019	26	Kununurra	WPms19-090	600	0	0
May-2019	35	Kununurra	WPms19-093	600	0	0
May-2019	35	Kununurra	WPms19-094	600	0	0
May-2019	35	Kununurra	WPms19-095	600	0	0

[A] Several plants seen with systemic-like symptoms of TSV as mixed infections with CBT.
[B] Patch of old (>1yr) volunteers in waterway about 50m from crop with 8/16 plants with CBT.
[C] This block had a large area of CBT disease outbreak. Numerous counts were done across the paddock as shown below in Fig. 1.
[D] This count was of old volunteer cotton plant in irrigation channel, next to cotton blocks which had no CBTV.
[E] Tall, abnormal plants seen in this crop at about 1% (5/500) with reduced boll set and size, deformed bolls and elongated growth above the main canopy.

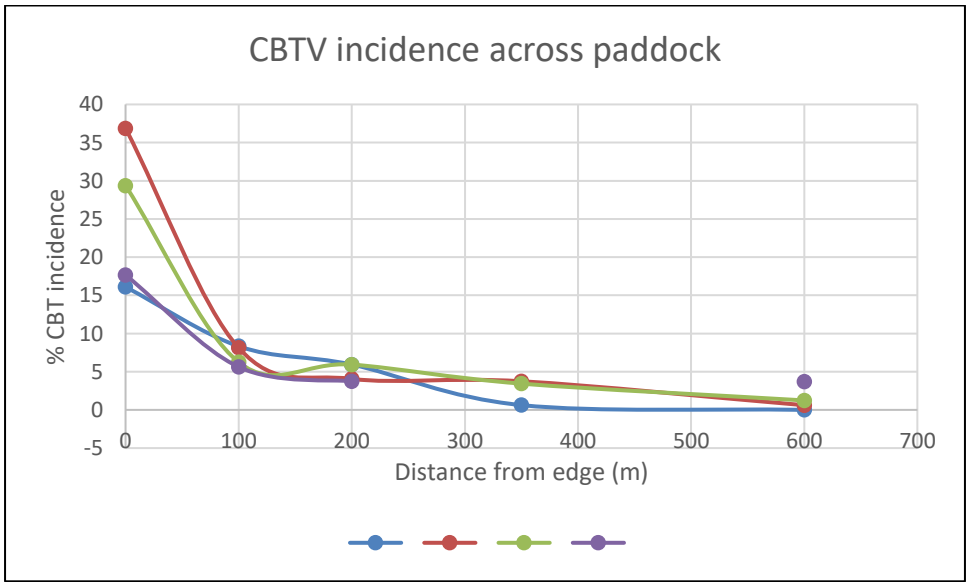

Fig. 1. Cotton bunchy top (CBT) disease incidence from the eastern edge of the crop in paddock "WPms057" from Jan-2018 (Table 1). Disease counts were done at the edge, 100 m, 200 m 350 m and greater than 600 m downwind into the crop. Four transects were surveyed (different coloured lines), each 200m apart.

Objective 2. Survey Gossypium species and other potential hosts in northern Australia for CBTV, CLRDV, other poleroviruses and exotic viruses. At least two virus surveys conducted during project.

Milestone 2.1. Survey Gossypium species and other potential hosts in northern Australia for CBTV, CLRDV, other poleroviruses and exotic viruses.

At least six surveillance trips were done in northern Australia between June 2016 and May 2019. These included surveys in the Torres Strait Islands in June 2016, Darwin and surrounds in March 2017, April 2018, May 2018 and May 2019, Kununurra in May 2018 and May 2019, lower Cape York (Georgetown) in May 2017, and Weipa in June 2019. Over 600 samples were collected and/ or tested by a range of assays including generic polerovirus tests and specific CLRDV PCRs. More than 30 plants of feral *G. hirsutum* were collected and tested from Torres Strait Islands and Darwin, none had virus symptoms and all were negative for polerovirus. No virus-like symptoms were seen in any commercial cotton surveyed in Kununurra in 2018 or 2019 (see Table 1 for cotton survey details). A range of other potential host species were tested for polerovirus and positives were found in several species but none contained poleroviruses known to affect cotton. A summary of the poleroviruses detected during these surveys is shown in Table 2.

Table 2. Polerovirus species detected and confirmed by partial coat protein sequencing during surveys in northern Australia in this study.

Virus species [A]	Host	Collection locations
CBTV-1	Ornamental hibiscus	Brisbane
CBTV-like virus (new)	Ornamental hibiscus	Kununurra, Darwin, Brisbane, N-NSW
SLPV (new)	Siratro	N-QLD, CQ, S-QLD
PVYV	Asthma plant	Thursday Island
CABYV-like virus (new)	Asthma plant	Torres Strait Islands
Cowpea polerovirus-2	Siratro	Kununurra
CuABYV	*Synedrella nodiflora*	Torres Strait Islands

[A] Virus abbreviations. Cotton bunchy top virus-1 (CBTV-1); Siratro latent polerovirus (SLPV); Pepper vein yellows virus (PVYV); Cucurbit aphid-borne yellows virus (CABYV); Cucumber aphid-borne yellows virus (CuABYV).

CLRDV or other exotic viruses known to affect cotton were not detected from the Torres Strait Islands. However, two poleroviruses, not previously reported from Australia were detected in samples from the Torres Strait Islands. One virus, Cucumber aphid borne yellows virus (CuABYV) was detected in *Synedrella nodiflora* (Cinderella weed) from three different islands separated by more than 200 km. The other virus, most similar to Cucurbit aphid borne yellows virus (CABYV) was detected in *Chamaesyce hirta* (asthma plant) from Dauan Island, about 10 km from the Papua New Guinea coast. These

diagnoses were based on relatively small parts of the virus genome sequence and further characterisation is still underway. Both of these have not been reported to affect cotton and are unlikely to do so but may pose unknown risks to horticultural industries.

Over 80 samples from about 12 plant species were collected from around Darwin and the Cox Peninsula in March 2017. A further 72 samples from 13 sites across the Darwin region (Fig. 2) were collected in April 2018, including 46 ornamental hibiscus (*Hibiscus rosa-sinensis*) from 9 sites and 12 feral cotton (*Gossyium hirsutum*) from 2 sites (Lee Point). Another 14 samples of potential polerovirus hosts were also collected. No virus-like symptoms were seen on the feral cotton. From these, 115 samples have been tested using Tissue Blot Immuno Assay (TBIA) with generic polerovirus antibodies and about 50 were positive. Of these 44 out of 51 (86%) of the ornamental hibiscus and 5 out of 6 chilli were positive. PCR and sequencing has been done on nine of these TBIA-positive samples and two polerovirues have been detected, a new CBTV-like species from ornamental hibiscus and Pepper vein yellows virus (PVYV) from chilli.

The new polerovirus from ornamental hibiscus has now been collected from numerous locations and the partial coat protein gene has been sequenced to show that they all share about 95% nt identity with each other. This virus species has now been found in ornamental hibiscus from Darwin, Kununurra, Brisbane and N-NSW (Fig 3). Along with the detection of CBTV-1 (Brisbane), also from ornamental hibiscus, this indicates ornamental hibiscus may be an important host for multiple poleroviruses, including those infecting cotton.

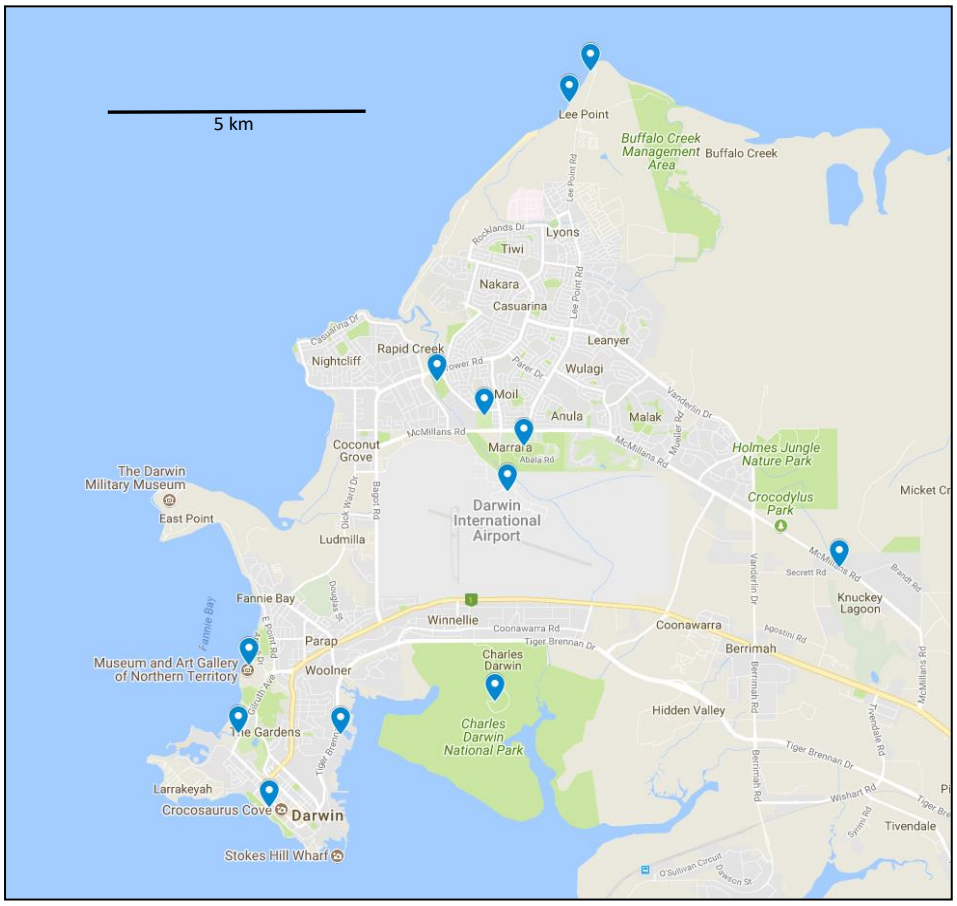

Fig. 2. Collection sites (shown by blue pins) for cotton and alternative hosts in the Darwin region. Survey was done 21-23rd April 2018. Ornamental hibiscus (*Hibiscus rosa-sinensis*) was collected from 9 sites. *Gossypium hirsutum* was collected from the two northern sites at Lee Point.

The genetic relationship of detected poleroviruses are shown in Figure 4. At least a couple of these detections have been from samples supplied by NAQS staff, illustrating the effectiveness of these collaborative links. It is also worth noting the potential importance of asthma plant as a host of poleroviruses and cotton aphids. At least three polerovirus species have been detected in asthma plant from locations including Emerald, Torres Strait Islands and Timor-Leste and from all locations, cotton aphid is commonly seen on this plant species.

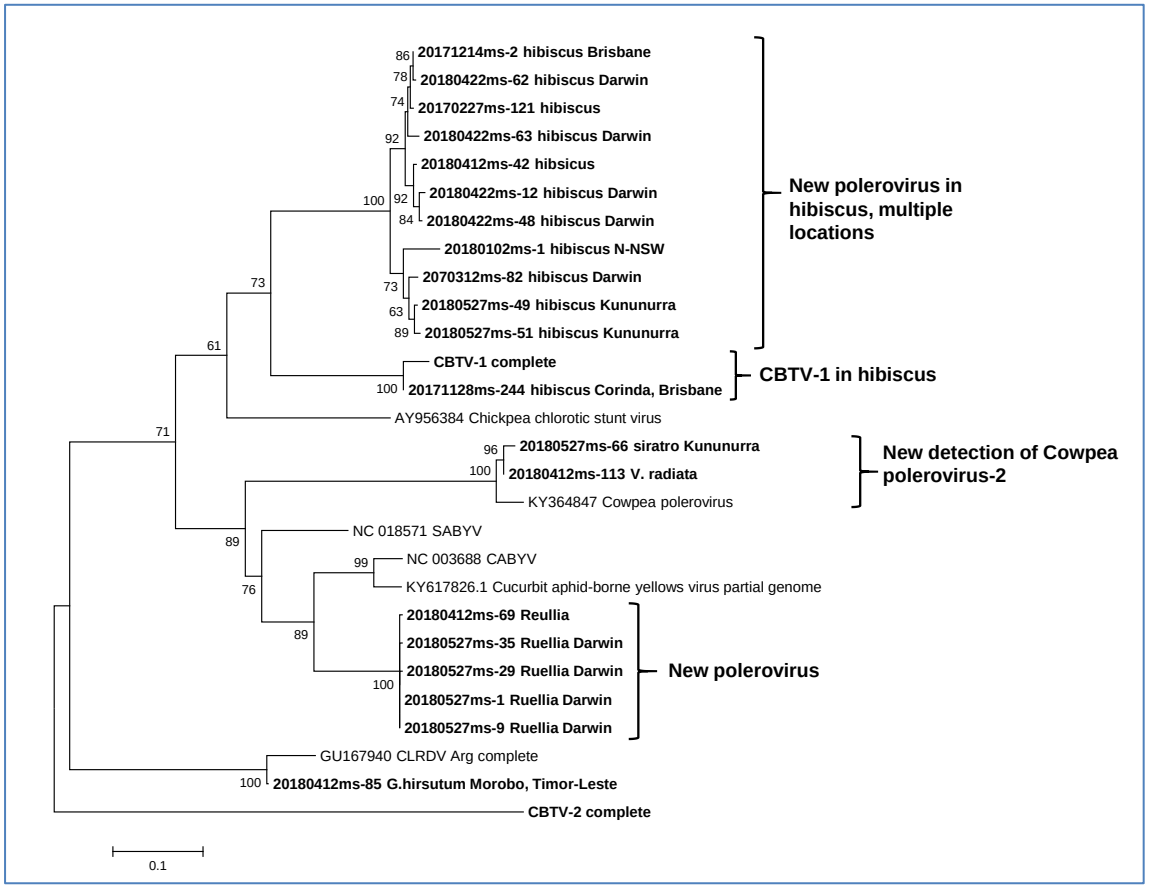

Fig. 3. Phylogram obtained from a maximum likelihood analysis for the partial coat protein gene of a selection of samples collected during this study (in bold) and previously published. The scale bar represents the number of nucleotide substitutions per site. Maximum likelihood support values (> 50%) from shown at the nodes as the upper values. Genebank accession numbers are shown for published sequences. Virus acronyms shown include CBTV (Cotton bunchy top viruses -1 and -2), CpCSV (Chickpea chlorotic stunt virus), CABYV (Cucurbit aphid-borne yellows virus), and CLRDV (Cotton leafroll dwarf virus).

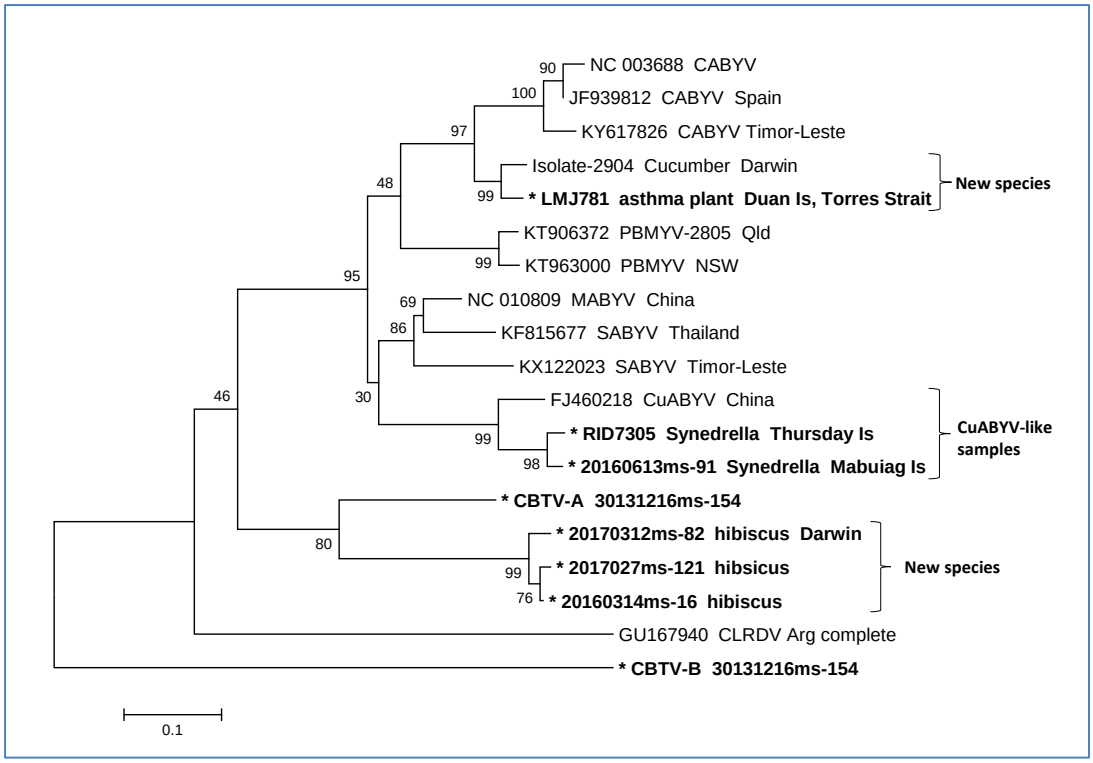

Fig. 4. Phylogram obtained from a maximum likelihood analysis for the partial coat protein gene (290 nt positions in common) of various poleroviruses from this study (**in bold and marked with ***) and previously published. Genebank accession numbers are shown for published sequences. The scale bar represents the number of nucleotide substitutions per site. Maximum likelihood support values shown at the nodes. Other Luteoviridae members shown are, *Cucurbit aphid borne yellows virus* (CABYV), *Phasey bean mild yellows virus* (PBMYV), *Melon aphid-borne yellows virus* (MABYV), *Suakwa aphid-borne yellows virus* (SABYV), *Cucumber aphid-borne yellows virus* (CuABYV), *Cotton bunchy top virus* (CBTV-A and –B), and *Cotton leafroll dwarf virus* (CLRDV).

A virus survey was done at Strathmore Station in north Qld in early May 2017. About 120 samples from various polerovirus hosts were also collected between Cairns and Strathmore for testing. A selection of these have been tested by either TBIA and/or PCR. Polerovirus was confirmed in siratro (*Macroptilium atropurpureum*) from 13 of 71 tested (from 3 of 4 locations collected) by TBIA and several of these were confirmed by PCR as a recently described polerovirus species, *Siratro latent polerovirus* (SLPV). There is no evidence SLPV infects cotton or other Malvaceae species. Five samples of upland cotton, 5 native cotton, 11 native hibiscus and 17 asthma plant were tested by generic polerovirus PCR and none were positive. The cotton, native cotton and hibiscus samples also tested negative for begomovirus by PCR (Avcore / Accore).

In addition to virus testing, Solenopsis mealybug was confirmed (ID by Mark Schutze, QDAF) on a Malvaceae species west of Mount Garnet and from native hibiscus at Strathmore station. Cotton aphids were also confirmed on native hibiscus from Strathmore and from road side asthma plant from east of Mount Garnet. Asthma plant has been seen as a host of cotton aphids in many locations in Qld, Torres Strait and Timor-Leste and is also a host of at least three poleroviruses. It may play an important role in the persistence of aphids and poleroviruses such as CBTV in some locations.

Nineteen samples from PNG, Torres Strait, N-QLD, NT and Kununurra from 11 hosts (almost all were known polerovirus hosts), were submitted for virus testing from NAQS

staff, Lynne Jones and Richard Davis. All were tested by generic polerovirus PCR and one sample of asthma plant from Thursday Island had a weak positive and sequence identified *Pepper vein yellows virus*.

Following the detection of a new polerovirus species in *Ruellia tuberosa* from the April 2018 survey, I collected samples of this species from three locations across Darwin in May 2018 to check if the same virus may be present in Australia. From the 36 plants collected, 14 were positive in a generic polerovirus TBIA assay and 4 of these were selected for PCR and sequencing of the partial coat protein gene. All four samples from Darwin share almost 100% nt identity (Fig. 3). Aphids were collected from plants in Darwin for identification but they appeared to be cotton aphids.

Siratro weed was collected and tested from Darwin and Kununurra. No polerovirus was detected from 8 samples from Darwin, but 2 of the 11 samples from Kununurra were positive by TBIA. One of these was confirmed by PCR and sequencing and was found to be a new detection for Australia for *Cowpea polerovirus-2*. The Kununurra and sample share about 95% nt identity to the only other published sequence from Africa.

The very high nt identity of two newly found poleroviruses in Darwin and Timor-Leste and also Kununurra and Timor-Leste may indicate an active link between northern Australia and Timor-Leste. This example of potential virus movement is concerning with the expansion of cotton production in the Kununurra region because we know *Cotton leafroll dwarf virus* is present in Timor-Leste.

From a survey done in Weipa in June 2019, 84 samples were collected and/ or tested for polerovirus, consisting of ornamental hibiscus, siratro, synedrella weed and phasey bean. Positive samples were found in 3 of the 11 ornamental hibiscus and 1 of the 4 phasey bean tested. Further analysis is needed for these samples to confirm which polerovirus is present.

A large number of asthma plant samples have been collected from various sites in northern Australia (often with cotton aphids present). These have been stored but there was not enough time in this project to test most of these by PCR for polerovirus. I hope to be able to use these stored samples as a resource for further testing as part of a new diagnostics project focusing on northern Australia.

Objective 3. Provide support and preparedness for viral biosecurity threats. Investigate if CLRDV is established in East Timor and if so, determine its possible host range and potential pathways into Australia. Make the most up to date diagnostic test for CLRDV and Contingency Plan available to industry.

Milestone 3.1. Determine genetic relatedness of Thailand and East Timor CLRDV to the "atypical" strains from Brazil known to overcome CRLDV-resistance.

Partial genome sequence was determined for two CLRDV isolates from Thailand (Q3513 and Q3514). PCR products were amplified from the Thailand samples using primer pairs Pol3167F/AS3, Pol3870F/AS3 and Pol3870F / Pol4333R, and sequenced (Table 3). The resulting partial genome fragments were 1113 nucleotides (nt) in length, consisting of partial RNA-Dependent-RNA-Polymerase gene, the intergenic region, and the complete coat protein gene. Samples Q3513 and Q3514 shared greater than 99.5 % nt identity over the 1113 nt length with only a single nt difference. As such, further analysis was done for sample Q3513 only. Over a 1113 bp region, sample Q3513 had a 92 % nt identity with CLRDV from Argentina (GU167940). Sample Q3513 also had a 96 % amino acid identity

with the Argentinian CLRDV for the putative coat protein. This new sequence derived for the Thailand CLRDV samples indicates they are closest to the standard strain of CLRDV from Brazil and Argentina and are not the "atypical" strain.

A similar approach was used to obtain further partial genome sequence for the original CLDRV isolate from Timor-Leste (isolate Q6232), collected during a NAQS survey in 2013. It appears the irradiation treatment required by the quarantine import permit, may have affected amplification of large viral genome fragments from isolate Q6232 but a few overlapping PCR products were sequenced. The resulting consensus sequence was 1005 nt (GenBank accession KT223789) and the closest match was 97 % nt identity to *Chickpea stunt disease associated virus* (CpSDaV; accession Y11530) but this Genbank accession for partial CpSDaV genome is only 577 nt. Over the 1005 nt sequence of the Timor Leste sample the closest match was 94 % with a standard CLRDV isolate from Argentina (accession GU167940). A similar level of nt identity was also found when compared to other CLRDV sequences from Thailand, Brazil and India (accessions KP176644, HQ827780 and JN033875). Figure 5 shows the phylogenetic relationship of the partial genome of Timor-Leste and Thailand CLRDV samples characterised in this project compared to other published CLRDV.

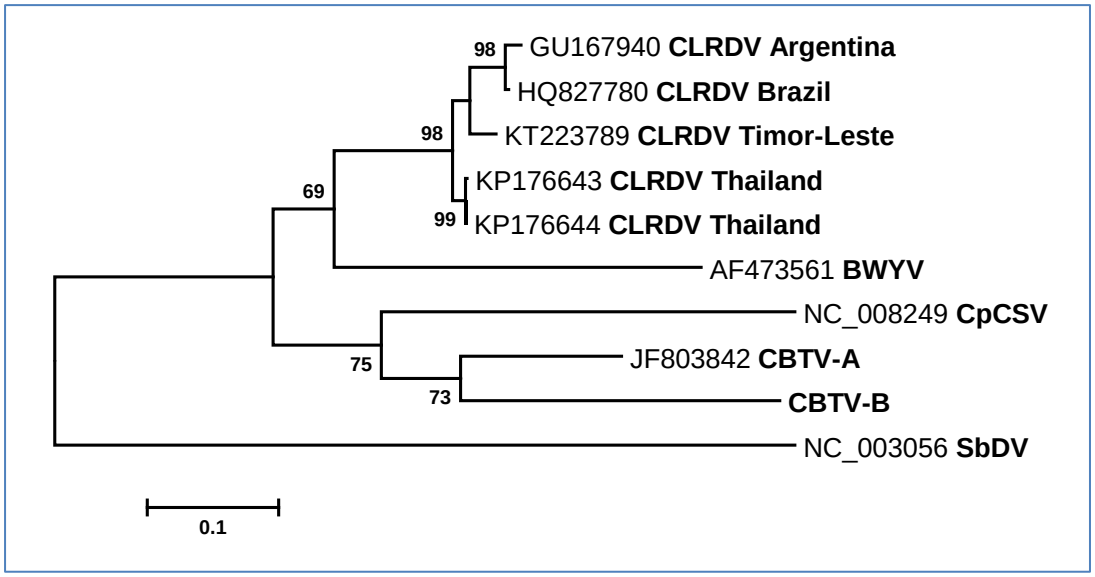

Fig. 5 Phylogram obtained from a maximum likelihood analysis for the partial genome fragment (922 nt positions in common) consisting of partial 3' end of RdRp gene, intergenic region, and almost complete coat protein gene of CLRDV samples from Timor-Leste, Thailand, Brazil and Argentina, and other previously published Luteoviridae members. The scale bar represents the number of nucleotide substitutions per site. Maximum likelihood support values (> 50%) from shown at the nodes as the upper values. Genebank accession numbers are shown on phylogram. Other related Luteoviridae members shown are Cotton bunchy top virus strains-A and –B (CBTV), *Beet western yellows virus* (BWYV), *Chickpea chlorotic stunt virus* (CpCSV) and *Soybean dwarf virus* (SbDV).

The almost-complete coat protein gene sequence from Timor-Leste isolate Q6232 was analysed to determine phylogenetic relationships to published sequences. Over a 197 amino acid (aa) region, the partial putative coat protein sequence of the Timor-Leste sample was closely related to CLRDV from Thailand (KP176643), India (JN033875), Brazil (HQ827780) and Argentina (GU167940), and CpSDaV from India (Y11530) with between 98 % to 96 % aa identity. The high levels of identity determined indicate the Timor-Leste sample is closely related to CLRDV and CpSDaV which are likely to be

synonyms for the one species. It appears that CpSDaV reported by Naidu et al. (1997), may be the same virus as CLRDV and until further evidence indicates otherwise, CpSDaV should be considered synonymous with CLRDV.

These analyses of coat protein gene sequences indicated that CLRDV isolates from both Thailand and Timor-Leste were most likely not the atypical strain from Brazil known to overcome CLRDV resistance. However, research from groups in Brazil and Argentina (Agrofoglio et al., 2017; Cascardo et al., 2015; da Silva et al., 2015) have important implications for this milestone. The complete genomes of two CLRDV isolates known to overcome CLRDV-resistance have recently been characterised. These CLRDV strains cause "atypical" cotton blue disease in resistant cotton lines. When compared to the complete genomes of typical CLRDV, these atypical-CLRDV isolates had a high degree of similarity across most of the genome except for open reading frame 0 (ORF-0) which is reported to control RNA-silencing suppression. More simply, ORF-0 encodes from the P0 protein which is reported to control the ability of the virus to overcome plant resistance and thus is likely to determine if the virus will be a resistance breaking strain. In this region of the genome, these atypical CLRDV isolates were markedly different to typical isolates, indicating the function of ORF-0 is critical for enabling atypical CLRDV to overcome plant resistance.

In order to determine if we can make any prediction about the likely resistance-breaking status of the Thailand or Timor-Leste CLRDV strains, I developed a nested PCR which enables the amplification of the partial PO gene. This is described in more detail below for Milestone 3.3. In brief, I designed primers to amplify the almost complete ORF-0 gene based on available published sequences for CLRDV (Table 3). This has been used to get almost complete ORF-0 gene sequence from 20 CLRDV isolates from six locations in Timor-Leste (16 samples), Thailand (2) and Uzbekistan (2).

Figure 6 shows the CLRDV ORF-0 sequences from this study compared with published sequences from standard and resistance-breaking strains from Brazil and Argentina. The standard (typical) and resistance-breaking strains from Brazil and Argentina form their own clades which share about 90-91% nt identity between the clades. By contrast, the ORF-0 gene sequences from Uzbekistan, Thailand and Timor-Leste show far greater diversity.

From within Timor-Leste, there is some obvious clustering of diversity based on locality (e.g. Dili, Luro and Lospalos, Fig. 6) and does not appear to be related to Gossypium species. The diversity between CLRDV samples from two sites in the eastern area of Timor-Leste separated by only 21 km (Lospalos and Luro), share about 86 % nt identity. From the Bobanaro region of western Timor-Leste, a number of CLRDV samples collected shared less than 90 % nt identity in the ORF-0 gene, even when collected from sites separated by less than 1 km. The landscape in this region of Bobanaro is rugged and diverse and may be related to the highly diverse CLRDV strains. This level of genetic diversity in the ORF-0 gene of CLRDV isolates from Timor-Leste represents greater diversity than that between the reported standard and resistance-breaking CLRDV samples from across vast distances and the two countries of Brazil and Argentina.

Further diversity in the ORF-0 gene is also present in the CLRDV samples from Thailand and Uzbekistan which both form their own clades (Fig. 6). This new data shows a wide diversity of PO gene sequences across Uzbekistan, Thailand and Timor-Leste and may suggest that Asia is a more likely centre of origin (and diversity) for CLRDV than South America. It may also suggest that CLRDV has either been present in Timor-Leste for a long

time and has diversified across the landscape, or there have been multiple arrivals and establishment of different strains of CLRDV into Timor-Leste.

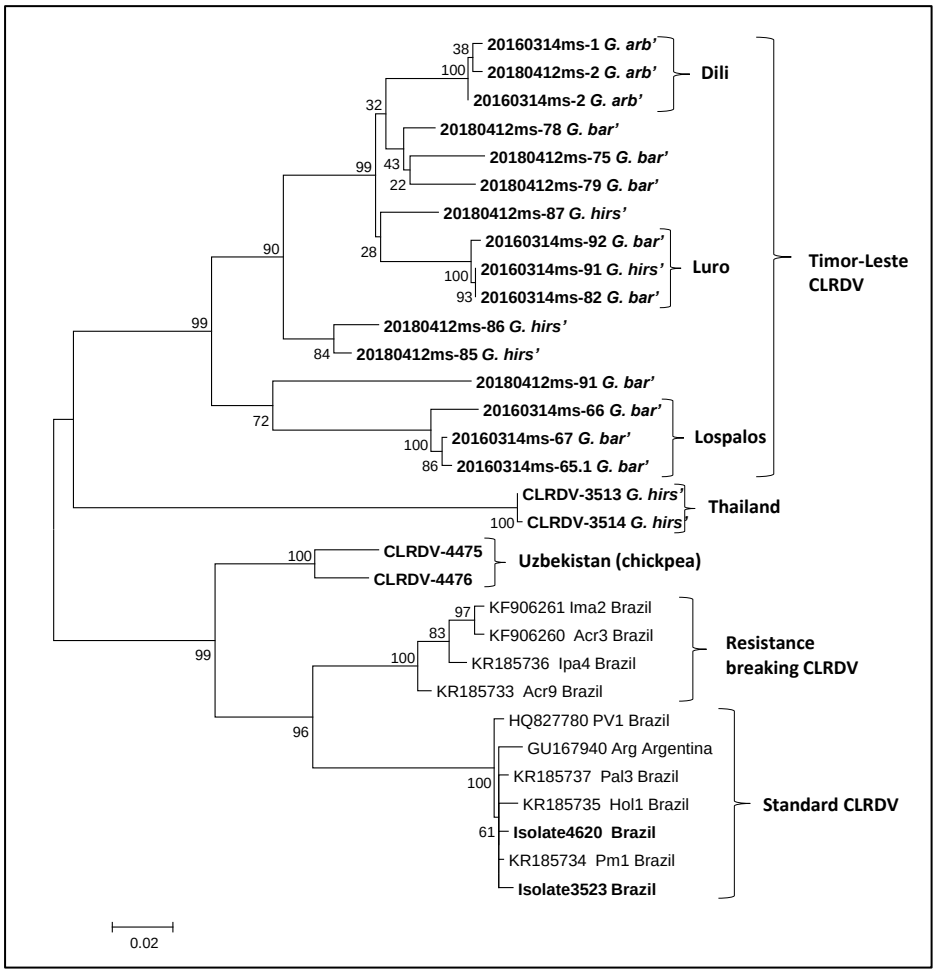

Fig. 6. Phylogram obtained from a maximum likelihood analysis for the partial ORF-0 gene (~ 667 nt positions in common) of various CLRDV samples from this study and previous CRDC projects (in bold) and previously published. The scale bar represents the number of nucleotide substitutions per site. Genebank accession numbers are shown for published sequences. Clusters of standard and resistance-breaking CLRDV are shown for samples from Brazil and Argentina. Collection locations (e.g. Lospalos) are shown for CLRDV samples from Timor-Leste. All samples not labelled with a location from Timor-Leste were from within 6 km of each other from the Bobanaro region. Abbreviations of Gossypium species are shown for Timor-Leste samples for *G. arboreum*, *G. barbadense* and *G. hirsutum.*

None of the new ORF-0 gene sequences from Thailand or Timor-Leste CLRDV fall within the clade for known resistance-breaking strains of CLRDV from Brazil and Argentina. Hence, we probably still cannot predict if the Thailand or Timor-Leste CLRDV strains may overcome resistance. However, the greater diversity of CLRDV strains in the SE Asia region may suggest that a strain capable of breaking resistance could be present and selected for in the presence of a plant resistance gene.

Based on this data I think it is most likely that CLRDV was a relatively recent incursion into South America. This genetic bottle neck means there is little diversity between CLRDV samples across Brazil and Argentina but enough diversity to select a resistance-breaking strain in the presence of cotton containing a CLRDV resistance gene.

A Next Generation Sequencing (NGS) attempt using an irradiated CLRDV sample collected from Timor-Leste in 2016 was unsuccessful and this is likely due to the fragmentation of the RNA genome caused by irradiation which was a required condition for the import permit. However, using different import permit condition (no irradiation), 59 cotton samples were imported from Timor-Leste from the 2018 survey. A non-irradiated CLRDV-infected sample has now been used successfully in a recent NGS analysis (August 2019). Sequence analysis is continuing but it appears the almost complete CLRDV genome from the Timor-Leste sample has been obtained.

Table 3. Diagnostic PCR primers developed for the detection of CLRDV and other poleroviruses, and for barcoding Gossypium species.

PCR primer name	Sequence (5' – 3')	Target [A]	Reference
CLRDV3675F	CCACGTAGRCGCAACAGGCGT	All known strains of CLRDV (not CBTV)	Ray et al (2016)
CLRDV3659F	CAATGGAAGAAGACGACCACGTAGRCGCAACAGGCGT	All known strains of CLRDV (not CBTV)	This project
CLRDV4001R	GGAACCGGAGGATGTTGAAGMGGCYTCGGWGAYGAACT	All known strains of CLRDV	This project
Pol3167F	YTVGGTTTYAAAGTCGAGG	CLRDV, CBTV, CABYV, BWYV, PLRV	Sharman et al (2015)
AS3	CACGCGTCIACCTATTTIGGRTTITG	Polerovirus generic	Abraham et al. (2008)
Pol3870F	ATCACBTTCGGGCCGWSTYTWTCAGA	CLRDV, CpCSV, CABYV, PLRV, BWYV, TuYV, BLRV, CBTV, SbDV	Sharman et al 2015
Pol4333R	GGRTTKCCYTCATAACCCCA	CLRDV, CABYV	Sharman et al 2015
CLRDV_ORF0F	GTCTCGTGTATGTTGAATTTGATCAT	P0 gene of all known strains of CLRDV	This project
CLRDV_ORF0R	CTCAACTGCTYTCTCCTTCAC	P0 gene of all known strains of CLRDV	This project
CLRDV90F	GCAGARTYTCTTCCGCAGCTCT	P0 gene of all known strains of CLRDV	This project
CLRDV794R	CGCCTTCATCGTCAAAATGGTA	P0 gene of all known strains of CLRDV	This project
Pol4021R	GGRTCMAVYTCRTAAGMGATSGA	Polerovirus generic	Project DAQ1201
Pol3982R	CGAGGCCTCGGAGATGAACT	Polerovirus generic	Sharman et al 2015
matK472Ra	CCCATCCATCTRGAAATCTTRGTTC	Maturase K gene for barcoding Gossypium species	Yu et al (2011)
matK1248Ra	CCACTRTRATAATGAGAAAGATTTCTG	Maturase K gene for barcoding Gossypium species	Yu et al (2011)

[A] Virus abbreviations: Cotton leafroll dwarf virus (CLRDV), Cotton bunchy top virus (CBTV), Cucurbit aphid-borne yellows virus (CABYV), Beet western yellows virus (BWYV), Potato leaf roll virus (PLRV), Chickpea chlorotic stunt virus (CpCSV), Bean leaf roll virus (BLRV), Soybean dwarf virus (SbDV).

Milestone 3.2. Survey Gossypium species and other potential hosts for CLRDV in East Timor.

I joined the International Plant Health Program (IPHP) team on three surveillance activities in Timor-Leste (East Timor) in March 2016, February 2017 and April 2018. The IPHP surveillance activities were done in collaboration with the Timor-Leste Ministry of Agriculture and Fisheries under a memorandum of understanding. As such, all records of new pathogens can only be published with the approval of both the IPHP administrators and the Timor-Leste Ministry of Agriculture and Fisheries. Specific details of new virus species or hosts from Timor-Leste have been omitted from this public release version of the Final Report.

Over 500 samples were collected from a wide range of host species from over 70 sites in eastern, central and western Timor-Leste (Fig 7, Table 4). Over 100 Gossypium samples (37 *G. arboreum*, 40 *G. barbadense*, 25 *G. hirsutum*) were collected from 27 different sites (Fig 7, Table 5). It appears fairly common for one to several cotton plants to be grown by some families in each village. The Timorese have a tradition of weaving ceremonial fabrics and probably grew more cotton in the past but now mostly import spun thread. It appears that the occasional cotton plant is still grown as a culturally important plant even if it yields virtually no lint. Villagers who did not have electricity also said they grew cotton to mix the fibre with candle nut oil to burn as candles for lighting. As electricity becomes more widespread, it is likely less cotton will be grown for this purpose.

Fig. 7. Map of Timor-Leste showing sample sites. Sites that had Gossypium species present are shown with a black star and sites with other potential alternative hosts are shown with a pin. See Tables 4 and 5 for summary of samples collected and sites from which CLRDV was detected.

CLRDV was confirmed in one or more Gossypium plants from 7 of the 27 collection sites. In total, 56 of the collected Gossypium samples have been tested by CLRDV-specific PCR

and CLRDV was confirmed by specific PCR (and some also by partial genome sequencing) from 7/13 *G. arboreum*, 10/30 *G. barbadense*, and 4/18 *G. hirsutum*. These results indicate that CLRDV is established and relatively common in many locations in Timor-Leste.

Table 4. Summary of samples collected during survey trips to Timor-Leste, the number of sites collected from, the number of different species collected, samples tested by TBIA for polerovirus and number positive, the number of Gossypium samples collected and sites collected from.

Year	Samples collected (sites)	Number of species	Samples tested by TBIA (positive)	Samples imported	Gossypium samples (sites)
2016	173 (24)	25	173 (22)	123	30 (11)
2017	190 (23)	20	100 (21)	150	10 (7)
2018	148 (30)	26	102 (18)	116	59 (11)

Table 5. Summary of Gossypium samples collected from each survey year and number of samples positive for CLRDV from total number of plants tested to date.

	2016		2017		2018	
	plants collected	positive/ total tested	plants collected	positive/ total tested	plants collected	positive/ total tested
G. arboreum	10	2/4	2	2/2	25	3/7
G. barbadense	12	6/12	10	0/9	18	4/9
G. hirsutum	9	1/9	1	0/0	15	3/9

Virus-like symptoms were seen in many of the *G. barbadense* and *G. arboreum* plants including mild chlorotic mosaic around the leaf margins of occasional leaves and some bunchy terminal growth on *G. arboreum*. Mild virus-like symptoms were seen occasionally on *G. hirsutum*, particularly from site WPms18-063 (Table 6) and included diffuse chlorosis on leaf margins and some down cupping of leaves. However, no severe blue disease symptoms were seen on any Gossypium plants and virtually no virus-like symptoms were seen on *G. hirsutum* plants.

Even though some Gossypium plants from Timor-Leste appeared to have virus-like symptoms, the testing for CLRDV did not indicate a close association between the mild symptoms seen and infection. There was only one sample of CLRDV-infected *G. hirsutum* collected from the Bobanaro region (site WPms18-063) that had some stunting and cupping of leaves more typical of blue disease. It may be possible that the strain/s of CLRDV present in Timor-Leste do not produce pronounced symptoms (much like the apparently non-symptomatic *Cotton bunchy top virus-1* (CBTV-1) in Australian cotton) or the varieties of cotton grown in Timor-Leste have some tolerance to CLRDV. It remains unclear if CLRDV from Timor-Leste would cause a severe disease in Australian cotton varieties. As also discussed in results for Milestone 3.1, we did find wide genetic diversity of CLRDV in Timor-Leste which is of concern as it may indicate disease-inducing or resistance breaking strains may be present.

While a number of Gossypium plants collected in 2016 and 2018 were found to be CLRDV-infected, none of the 10 *G. barbadense* collected from 6 locations in the 2017 survey were positive (Table 5). There were CLRDV-positive *G. arboreum* samples collected in 2017 but these were from the same site at Dili as previously tested (site WPms16-791, Table 6). All other sample sites in the 2017 survey were at higher altitudes

than those surveyed in 2016 with most sites at about 700-1000 m in elevation and it appears that Gossypium was less common in the highlands compared to lower areas sampled in 2016 and 2018.

A wide range of other potential host species were also collected and most of these were tested during the surveys in Timor-Leste using a low-tech generic polerovirus Tissue blot immune assay (TBIA). A number of positives were obtained but the TBIA does not distinguish between polerovirus species so further polerovirus PCR and sequencing was done after treatment and import of samples to Australia under Biosecurity Permit conditions. TBIA testing for polerovirus was an effective way to quickly screen many samples in-country but was not very accurate for identification of all CLRDV-positive Gosssypium samples due to the low titre of virus. This is very similar to TBIA results for cotton bunchy top virus in Australia. At least 12 plant species were found to host 8 different polerovirus species. These results demonstrate the surveillance strategy and diagnostic assays used were effective for detection of CLRDV and other poleroviruses from a wide range of hosts and locations.

Another notable result from testing of survey samples from Timor-Leste was the detection of viruses from weed species that are common and widespread in both Timor-Leste and northern Australia, suggesting that these weed species may be worth monitoring for symptoms in Australia as indicator species of incursion pathways.

From the 3 survey trips, over 370 samples from Timor-Leste have been tested by at least polerovirus TBIA. However, a large number of samples were not suitable for TBIA and at least 100 samples remain untested or could be tested for other viruses. These remain stored in the QDAF collection and could be tested for priority targets at a later time.

Table 6. Sample site details for Gossypium species in Timor-Leste from surveys in 2016, 2017 and 2018, and presence of CLRDV detected.

Collection year	Waypoint #	Latitude, Longitude	Gossypium species present	Abundance	CLRDV detected
2016	WPms16-791	S8.55046° E125.52420°	*G. arboreum*	Multiple plants	yes
2016	WPms16-798	S8.46241° E126.42845°	*G. barbadense*	Single plant	no
2016	WPms16-801	S8.49654° E126.99327°	*G. barbadense*	Single plant	no
2016	WPms16-806	S8.52451° E127.00917°	*G. barbadense*	Multiple plants	yes
2016	WPms16-807	S8.47906° E126.81552°	*G. arboreum, G. hirsutum*	Few plants	no
2016	WPms16-810	S8.52121° E126.84039°	*G. barbadense, G. hirsutum*	Few plants	yes
2016	WPms16-814	S8.47062° E126.45450°	*G. barbadense*	Single plant	no
2016	WPms16-815	S8.47239° E126.45404°	*G. barbadense*	Single plant	yes
2016	WPms16-818	S8.53340° E125.73813°	*G. hirsutum*	Few plants	no
2016	WPms16-819	S8.53351° E125.75074°	*G. hirsutum*	Few plants	no
2016	WPms16-820	S8.55011° E125.52560°	*G. arboreum*	Multiple plants	no
2017	WPms17-950	S8.71225° E125.43855°	*G. barbadense*	Few plants	no
2017	WPms17-952	S8.71896° E125.43610°	*G. barbadense*	Few plants	no
2017	WPms17-957	S8.70407° E125.56128°	*G. barbadense*	Single plant	no
2017	WPms17-961	S8.70107° E125.52540°	*G. barbadense*	Single plant	no
2017	WPms17-964	S8.55184° E125.52719°	*G. barbadense*	Single plant	no
2017	WPms17-968	S8.84206° E125.61126°	*G. barbadense*	Single plant	no
2017	WPms16-791	S8.55046° E125.52420°	*G. arboreum*	Multiple plants	yes
2018	WPms16-791	S8.55046° E125.52420°	*G. arboreum*	Multiple plants	yes
2018	WPms18-047	S9.33100° E125.24657°	*G. arboreum*	Multiple plants	no
2018	WPms18-050	S9.27903° E125.21571°	*G. hirsutum*	Single plant	no
2018	WPms18-054	S9.34266° E125.20245°	*G. hirsutum*	Multiple plants	no
2018	WPms18-057	S9.40530° E125.14240°	*G. barbadense, G. arboreum*	Few plants	no
2018	WPms18-058	S9.39078° E125.15078°	*G. hirsutum*	Few plants	no
2018	WPms18-061	S9.02736° E125.37345°	*G. barbadense*	Single plant	yes
2018	WPms18-063	S8.99254° E125.32702°	*G. barbadense, G. hirsutum*	Multiple plants	yes
2018	WPms18-064	S8.98904° E125.33456°	*G. barbadense, G. arboreum, G. hirsutum*	Multiple plants	yes
2018	WPms18-065	S8.98647° E125.33875°	*G. barbadense*	Multiple plants	no
2018	WPms18-073	S8.96718° E125.05564°	*G. barbadense*	Single plant	n/t

Milestone 3.3. Modify CLRDV diagnostic test and Contingency Plan as required to incorporate relevant new information.

The CLRDV PCR developed as part of project DAQ1201 worked well on cotton but had significant problems with non-specific PCR amplifications from Malvaceae hosts other than cotton. In an attempt to overcome this issue, new PCR primers, CLRDV3659F and CLRDV4001R (Table 3) were designed and three different PCR options were compared. The options include a standard PCR using primers CLRDV3675F / Pol3892R (Fig. 8a), a nested PCR firstly with Pol3628F / Pol4021R with the second round PCR primers also being CLRDV3675F / Pol3892R (Fig. 8b), or a PCR with primers CLRDV3659F / CLRDV4001R with stringent parameters to improve specificity (Fig. 8c). This comparison indicates that the nested PCR (Fig. 8b) has the highest sensitivity and specificity for detection of CLRDV. This is illustrated by the false-positive in sample 3 (Fig. 8a) which is clearly negative in other PCRs (Fig. 8b and c). Further optimisation may be possible with the PCR shown in Fig. 3c which currently has comparable specificity but less sensitivity than the nested PCR (Fig. 8b).

The nested PCR has been used successfully on a range of CLRDV samples from Timor-Leste, Brazil and Uzbekistan. The details of the CLRDV nested PCR are shown in Appendix 1.

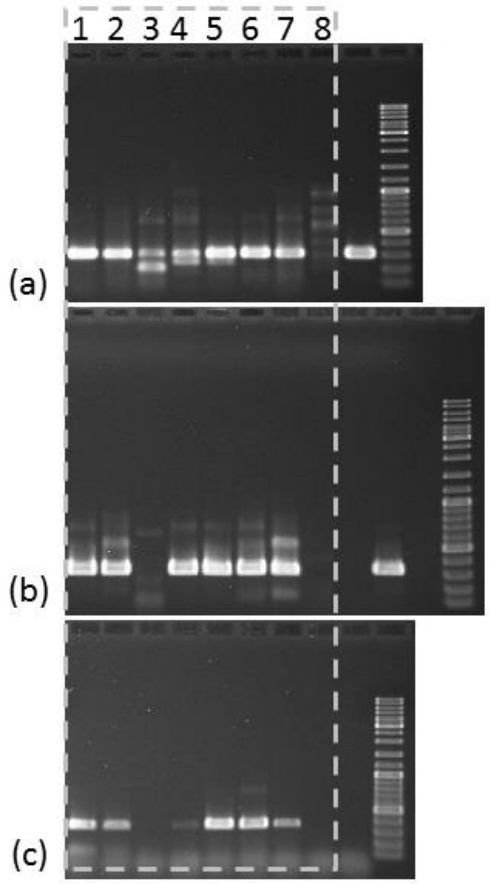

Fig. 8. Comparison of CLRDV-specific PCRs for detection of CLRDV samples from Timor-Leste. Samples within the boxed outline are the same between PCR options and are: tree cotton (samples 1-2 and 8), hibiscus (3) and sea island cotton (4-7). Lanes outside the boxed outline are different controls for each PCR and the marker lane. PCRs are: CLRDV3675 / Pol3982R (Fig. 8a), nested PCR of Pol3628F/Pol4021R followed by CLRDV3675F / Pol3982R (Fig. 8b), and CLRDV3659F / CLRDV4001R (Fig. 8c).

The currently used nested PCR for detection of CLRDV worked well on all known positive samples and its high sensitivity has enabled the detection of CLRDV from poor quality

samples from Timor-Leste. However, during testing of Torres Strait samples, a false positive was obtained from *Synedrella nodiflora* infected with *Cucumber aphid borne yellows virus* (CuABYV) (Table 2). This was the first detection of CuABYV from Australia and it appears that the CLRDV PCR primers cross react with this polerovirus species. I have designed a PCR assay to distinguish between CLRDV and CuABYV which can be used for any suspect samples. However, this known cross reaction is unlikely to cause issues for CLRDV testing because there is no indication that CuABYV can infect cotton so any positives from cotton should be assumed to be CLRDV. Any CLRDV-positive sample that may represent a new host should also be sequenced to confirm the PCR results.

The CLRDV national diagnostic assay and Contingency Plan have been updated to include the use of the nested PCR for CLRDV and the known cross reaction with CuABYV.

As also discussed in results for Objective 1, a nested PCR was developed for another region of the CLRDV genome, the open reading frame 0 gene (ORF-0). The first round PCR is done using CLRDV_ORF0F / CLRDV_ORF0R primers, then second round PCR (nested) is done using CLRDV90F / CLRDV794R primers (Table 3, Appendix 2). These worked well on CLRDV strains from Thailand, Brazil, Timor-Leste and Uzbekistan. The fact that the nested P0-gene PCR worked on genetically diverse strains of CLRDV means it could be a useful assay for detection of CLRDV if the coat protein gene PCR (CLRDV3675F / Pol3982R) does not detect variant strains. It is also useful to characterise the P0-gene to try to determine the likely resistance-breaking status of a CLRDV strain or its likely geographical origin.

An updated National Diagnostic Protocol for Cotton leafroll dwarf virus (Version 1.1) has been lodged with the Sub-committee on Plant Health Diagnostics (SPHD). This is also included in this report as an addition document. The main areas of change in the new version is the inclusion of new references and details for nested PCRs for the coat protein and P0 genes. The nested PCR for the coat protein gene improves sensitivity and specificity which has been an issue screening irradiated samples imported from overseas. It also enables detection of the greater diversity of CLRDV found in Timor-Leste. The nested PCR for the P0 gene provides a secondary confirmation of CLRDV infection and the sequence information from this region is likely to be important for determining if the CLRDV strain is similar to the reported resistance-breaking strains from South America.

An updated version of the draft CLRDV Contingency Plan (Version 1.1) includes reference to new literature and unpublished data about host and geographic range and an updated risk assessment section based on the new knowledge about host range.

Objective 4. Determine relative importance of the two known strains of CBTV (-A and -B) in outbreaks of bunchy top disease.

Milestone 4.1. Characterise the CBTV strains associated with symptomatic and non-symptomatic infections during field outbreaks of bunchy top disease.

Previous data from project DAQ1201 indicate that there were two distinct strains of CBTV (A and B) which were genetically distinct. Further data from this project now confirms that these strains are distinct polerovirus species. Given CBTV-A was the first virus reported and in keeping with accepted virus naming convention, these two species are hereafter called CBTV-1 (previously CBTV-A) and CBTV-2 (previously CBTV-B). The distinction between these two species will be discussed further below.

CBTV-1 is thought to be non-symptomatic in cotton but to increase the chances of finding CBTV-1 infected plants, we targeted areas where there were spreading outbreaks of CBT plants and hence aphid activity. Symptomatic and non-symptomatic plants were collected close each other (often next to each other) to maximise the chances of aphids

moving between plants with some of those aphids possibly only carrying CBTV-1 to cause non-symptomatic infection.

During this project a low number of CBTV disease outbreaks were found during field surveys (Table 1). In November 2015, 20 CBT typical symptomatic and 30 non-symptomatic samples were collected from eastern Emerald. In December 2016, 50 plants with typical CBT symptoms and 80 plants with no obvious symptoms were collected from small outbreaks of cotton bunchy top from eastern Emerald. In early 2018, 50 plants with typical CBT symptoms and 57 plants with no obvious virus symptoms were collected from within outbreak patches from around Macalister.

A total of 50 typical CBT and 122 non-symptomatic plants collected from 4 cotton blocks from CQ and 3 from the Downs have been tested by CBTV species-specific PCR (Table 8). From the CBT plants, 92% had CBTV-2-only infections and only 1 out of the 7 cotton blocks studied had CBT plants with mixed infections of CBTV-1 and CBTV-2. No typical CBT plants had only CBTV-1. From the 122 non-sympomtatic plants, 8 were positive for CBTV-2. This confounds our previous results that indicated CBTV-2 was always associated with typical CBT symptoms. There are some possible reasons for these new results. CBTV-1 was actually pretty rarely found last season (i.e. only found from 4 plants as mixed infections from one cotton block) so the chances of finding it in non-symptomatic plants was low. The non-symptomatic plants collected were taken from within areas of active CBT disease spread and development, so it is quite likely that we may be able to detect CBTV-2 by PCR in recently infected plants during the latency period, before symptoms have developed. It would be more thorough to have sampled and marked the non-symptomatic plants, returned to check the same plants again in 3 weeks and if they were still non-symptomatic, then test the originally collected sample. The extra travel and time required was not feasible within this project for the crops from CQ and the Downs.

Table 8. Summary of the number of plants tested by PCR for CBTV-1 and CBTV-2 from symptomatic and non-symptomatic cotton plants collected from areas of CBT outbreak in crops.

		Viruses detected by PCR			
		None	CBTV-1	CBTV-2	CBTV-1 & -2
Symptoms	typical CBT	0	0	46	4
	none	114	0	8	0

During this project we characterised about 98 % of the complete genomes of CBTV-1 (syn. CBTV-A) and CBTV-2 (syn. CBTV-B). A mixed infection of CBTV-1 and –2, originally isolated from cotton from Emerald was transmitted to chickpea via cotton aphids. The virus concentration in chickpea appears to be much higher than in cotton, so this was used as material for Ilumina next generation sequencing (NGS). The resulting consensus sequences were approximately 5,900 nt in length and covered all six coding regions (open reading frames) of the genomes.

Across the almost complete genomes, the CBTV species share only 77 % nucleotide (nt) identity. They are more closely related to each other than to other poleroviruses (Fig. 9) but this is a similar level of identity shared between other distinct polerovirus species such as CABYV and MABYV or TuYV and BWYV (see Fig. 9 for details of abbreviations).

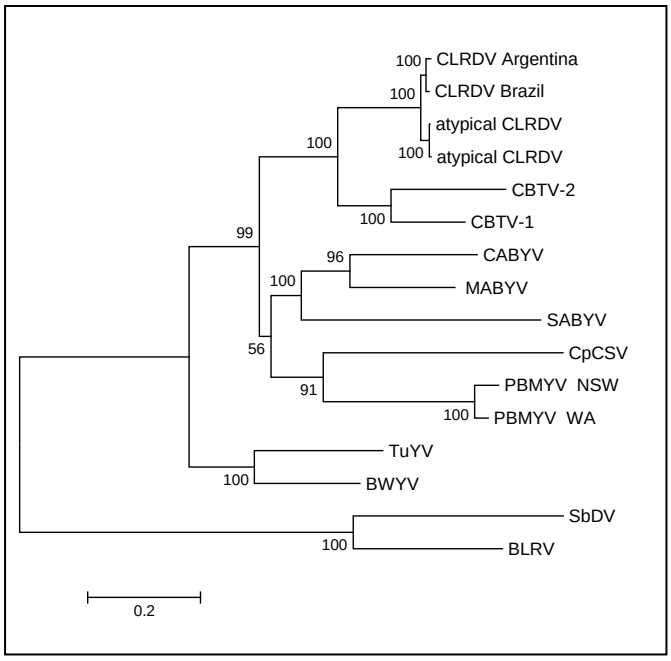

Fig. 9. Phylogram obtained from a maximum likelihood analysis for the complete genome (3693 nt positions in common) of CBTV-A and CBTV-B, CLRDV and other previously published Luteoviridae members. The scale bar represents the number of nucleotide substitutions per site. Maximum likelihood support values (> 50%) from shown at the nodes as the upper values. Genebank accession numbers have been removed but are available. Other Luteoviridae members shown are, *Cucurbit aphid borne yellows virus* (CABYV), *Melon aphid-borne yellows virus* (MABYV), *Suakwa aphid-borne yellows virus* (SABYV), *Chickpea chlorotic stunt virus* (CpCSV), *Phasey bean mild yellows virus* (PBMYV), *Turnip yellows virus* (TuYV), *Beet western yellows virus* (BWYV), *Soybean dwarf virus* (SbDV) and *Bean leafroll virus* (BLRV).

For the complete coat protein amino acid sequence, the CBTV strains share only 70 % amino acid (aa) sequence identity and CBTV-1 has a higher sequence identity to *Chickpea chlorotic stunt virus* (Fig. 10). For the complete P0 protein, the CBTV strains share only 50 % aa sequence identity (Table 9, Fig. 11). The International Committee for the Taxonomy of Viruses lists the criteria used to demarcate species to include: differences in amino acid sequence identity of any gene product of greater than 10%, differences in breadth and specificity of host range, and failure of cross-protection. CBTV-1 and CBTV-2 differ by greater than 10% amino acid identity for all predicted gene products (Table 9), they can co-infect the same plant and hence fail to cross-protect, and our data from project DAQ1201 suggests they also have some differences in host range. This new genome sequence data for the CBTV species, in combination with the differences in disease expression, provides strong evidence that they are distinct polerovirus species.

Table 9. Comparison of amino acid identity for each predicted Open Reading Frame for CBTV-1 and CBTV-2.

Open Reading Frame #	**% amino acid identity between CBTV-1 and CBTV-2**
ORF-0	51 %
ORF-1	65 %
ORF-1+2	75 %
ORF-3	70 %
ORF-4	59 %
ORF-3+5	60 %

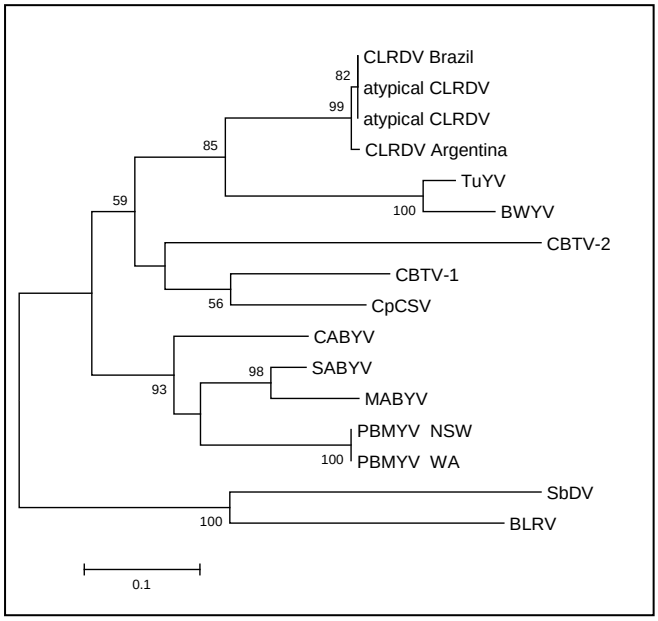

Fig. 10. Phylogram obtained from a maximum likelihood analysis for the complete coat protein (166 aa positions in common) of CBTV-A and CBTV-B, CLRDV and other previously published Luteoviridae members. The scale bar represents the number of amino acid substitutions per site. Maximum likelihood support values (> 50%) from shown at the nodes as the upper values. Genebank accession numbers have been removed but are available. See Fig. 9 for virus abbreviations.

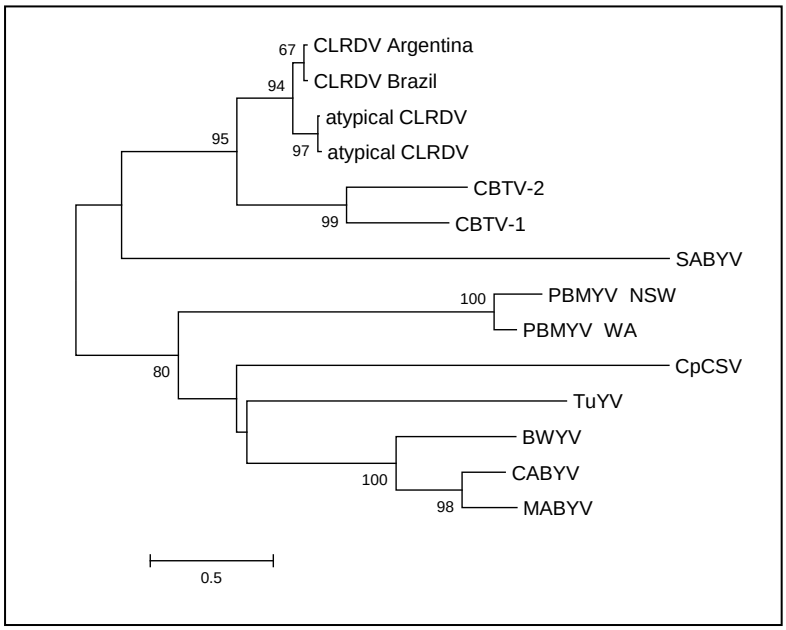

Fig. 11. Phylogram obtained from a maximum likelihood analysis for the complete P0 protein (209 aa positions in common) of CBTV-A and CBTV-B, CLRDV and other previously published Luteoviridae members. The scale bar represents the number of amino acid substitutions per site. Maximum likelihood support values (> 50%) from shown at the nodes as the upper values. Genebank accession numbers have been removed but are available. See Fig. 9 for virus abbreviations. SbDV and BLRV are in the Luteovirus genus that lack a PO protein and are not included in this phylogram.

Objective 5. Dissemination of disease management recommendations and provision of virology diagnostic services. Specifically, support CSIRO project CSP1401 by testing samples from CBTV host range experiments.

Milestone 5.1. Preparation of extension material.

During the course of this project, various extension material has been prepared and published via different media to industry and the scientific community.

The following peer-reviewed articles were published:

- Sharman M, Lapbanjob S, Sebunruang P, Belot JL, Galbieri R, Giband M, Suassuna N (2015) First report of Cotton leafroll dwarf virus in Thailand using a species-specific PCR validated with isolates from Brazil. Australasian Plant Disease Notes 10 (1):1-4. Published in July 2015 from research done in project DAQ1201.
- Ray JD, Sharman M, Quintao V, Rossel B, Westaway J, Gambley C, 2016. Cotton leafroll dwarf virus detected in Timor-Leste. Australasian Plant Disease Notes 11, 29.

Other extension material was prepared included:

- Sharman, M., Ray, J. D., Gambley, C. F., Lapbanjob, S., & Quintao, V. (2015). Cotton leafroll dwarf virus detected in Thailand and Timor Leste. Paper presented at the 2nd Australian Cotton Research Conference, Toowoomba, 8-10th September.
- Sharman M. (2015) Aphid-transmitted polerovirus threats to cotton and grain industries in Australia. Presented talk at APPS regional meeting, Darwin, 30th November.
- Sharman M. (2015) Aphid-transmitted virus threats to cotton and pulse crops. Presented talk at NAQS Scientific Discipline Meeting, Darwin, 1-3rd December.
- Australian CottonGrower magazine article about viruses and diagnostic services, Jan 2016.
- Sharman M, Ray J, Ximenes A, Soares G, Quintao V (2016). Cotton leafroll dwarf virus is common in Timor-Leste. Proceedings of the 18th Australian Cotton Conference, Gold Coast, 2-4th August. Oral presentation to about 200 people.
- Sharman M (2016). Update on Cotton bunchy top virus and other virus threats. Talk at the CottonInfo Crop Protection meeting in Emerald, 18 Oct 2016.
- Sharman M., Ray J., Gambley C. (2016) Cotton virus update. FUSCOM meeting, 12 April. Toowoomba.
- Sharman M. (2016) Cotton virus surveying in CQ. Central Queensland Cotton Update, Issue 4, December.
- Sharman M. (2016) Cotton virus surveying in CQ. Central Queensland Cotton Update, Issue 4, December.
- Sharman M. (2017) Presented summary of CRDC funded virus work to Plant Health Australia board, 21 Feb. Narrabri.
- Sharman M. (2017) Diversity of Cotton bunchy top virus in Australia and Cotton leafroll dwarf virus in Timor-Leste. FUSCOM, Goondiwindi, 8-9th August. Talk and abstract presented at meeting to about 40 researchers and key industry members.
- Sharman M. (2017) Diversity of Cotton leafroll dwarf virus in Thailand and Timor-Leste. 2nd Australian Cotton Research Conference, Canberra, 5-7th September. Talk and abstract presented to about 200 delegates.
- Sharman M. (2017) Update on biology and effects of phytoplasma in pigeon pea refuge and cotton. CSD field day and trial walk, Cecil Plains. Talk presented to about 40 growers and agronomists.
- Sharman, M. (2018) Project update (DAQ1601): Surveillance and studies for endemic and exotic virus diseases of cotton. FUSCOM meeting, Griffith, 28-29th August.
- Sharman M, Wilson L, et al. (2019). Endemic cotton-infecting viruses from Australia, and detection and studies of the exotic cotton leafroll dwarf virus. Talk

presented at cotton blue disease workshop as part of Beltwide cotton pathology conference, New Orleans, USA, 9th Jan 2019.
- Sharman M (2019). Preparing for exotic threats: focus on blue disease overseas. Article in Spotlight magazine, Winter 2019, p17-18.
- As a member of the Cotton Biosecurity Reference Group, I have provided review and updates of virus threats for the Cotton Industry Biosecurity Plan on a yearly basis since about 2016.

Milestone 5.2. Provision of virology diagnostic service as required by Industry members.

During this project approximately 84 cotton samples have been submitted for diagnostics by clients from about 20 locations from Giffith in southern NSW to Mareeba in north QLD. The majority of these were submitted for virus testing to check for endemic and/ or exotic viruses. No exotic viruses were detected and CBTV was found from several locations. Testing results were communicated back to clients.

Samples of tall, sterile (no or few fruit) cotton plants were submitted from Griffith in 2016 and several locations in NSW in 2019. I also collected similar samples from Duaringa in QLD in 2019. These samples were tested using generic polervirus PCRs but none were positive and it is not clear what the cause of the unusual growth was. Some of the affected plants were also tested with one set of general phytoplasma PCR primers and they were negative. To determine if a biotic agent may have been involved I grafted several branches from affected plants onto Sicot 71 plants to try to see if the symptoms are transmissible. No obvious symptoms were seen on the test plants. If these plants are seen again in the field and are of concern for growers, it would be advisable to try grafting again and perhaps consider an NGS analysis for unknown pathogens.

Eighteen samples (non-cotton hosts) were received from NAQS collaborators in 2016 for polerovirus testing. Two samples were positive in generic polerovirus PCRs and were sequenced to confirm one sample of Synedrella nodiflora (20160920-12) with a new polerovirus similar to Cucumber aphid-borne yellows virus in and one sample of asthma plant (20160920-15) with a virus similar to Cucurbit aphid-borne yellows virus.

Twelve extracts from various hosts were submitted for CBTV testing by Tanya Smith (CSIRO – ACRI) to support the host range studies of CSIRO project CSP1401. These were tested in the CBTV multiplex PCR and all were negative.

Outcomes

5. Describe how the project's outputs will contribute to the planned outcomes identified in the project application. Describe the planned outcomes achieved to date.

Results from this project have provided a better understanding of the distribution and host range of CBTV and other poleroviruses in northern Australia. At least six surveillance trips were done in northern Australia including areas around Torres Strait Islands, Darwin, Kununurra, lower Cape York (Georgetown) and Weipa. No cotton bunchy top virus was detected in cotton production areas of Kununurra and northern QLD. However, six different polerovirus species were detected in other hosts with at least one from all regions surveyed. At least 5 of these polerovirus species are either new records for Australia or new undescribed species. None of the newly detected poleroviruses are thought be able to infect cotton but it does indicate there is a diverse range of poleroviruses in northern Australia. The detection of three polerovirus species with high levels of genome identity from northern Australia and Timor-Leste could represent natural movement of virus-infected aphids in wind currents from Timor-Leste. This

example of potential virus movement is concerning with the expansion of cotton production in the Kununurra region because we know *Cotton leafroll dwarf virus* and other viruses are present in Timor-Leste.

Research and findings from this project has provided a significant increase in our understanding of the incidence, distribution and to a lesser extent the host range of *Cotton leafroll dwarf virus* (CLRDV) in Timor-Leste. While cotton is not produced on a commercial scale in Timor-Leste, CLRDV was found to be relatively common and widespread in three Gossypium species (*G. arboreum, G. barbadense* and *G. hirsutum*) from 7 of the 27 sample sites from which Gossypium was collected. We also found CLRDV in ornamental hibiscus in Timor-Leste and it appears that this host could be an important perennial host for at least a few different polerovirus species. We found significant diversity of CLRDV from Timor-Leste and Thailand, far greater than that found in south-America. This new knowledge about CLRDV and other poleroviruses in Timor-Leste and northern Australia has been used to improve preparedness for these biosecurity threats by improving the diagnostic assays and Contingency Plan.

While there were limited significant outbreaks of CBTV, the findings from this project are in agreement with previous results from project DAQ1201 and data still indicates that CBTV-2 is always associated with disease symptoms in cotton while CBTV-1 is not. No plants with typical bunchy top symptoms were found with only CBTV-1 and all symptomatic plants were infected with CBTV-2, either with or without CBTV-1. This will help to focus any future work on the control of CBTV-2 which is the causal agent for disease while it appears that CBTV-1 is most like non-symptomatic in cotton and of little concern.

This project has provided virus-specific surveillance in cotton production regions. In particular, surveillance in new emerging regions in Kununurra and far northern QLD has provided confidence that no virus diseases were found. This will provide a valuable baseline in disease surveillance data for future surveys in case there are changes in the viruses present or new incursions.

6. **Please describe any:-**
   a) **technical advances achieved (eg commercially significant developments, patents applied for or granted licenses, etc.);**
   b) **other information developed from research (eg discoveries in methodology, equipment design, etc.); and**

An improved nested-PCR diagnostic for CLRDV coat protein gene was developed in this project (see Appendix 1). A nested PCR was also developed for the PO gene region of CLRDV (see Appendix 2). Both of these diagnostics have been incorporated into an updated draft National Diagnostic Protocol for CLRDV.

   c) **required changes to the Intellectual Property register.**

An updated IP register (Schedule 2) has been included with this report. Some addition notes about IP related to survey samples collected from Timor-Leste and reporting of new disease records from those samples have been included.

Conclusion

7. **Provide an assessment of the likely impact of the results and conclusions of the research project for the cotton industry. What are the take home messages?**

At least six surveys targeting viruses in northern Australia were done over 4 years in areas that may be exposed to possible incursion pathways and also in emerging cotton production regions. No viruses were detected in cotton production areas of Kununurra

and northern QLD. However, six different polerovirus species were detected in other hosts with at least one from all regions surveyed. None of the newly detected poleroviruses are thought be able to infect cotton but it does indicate there is a diverse range of poleroviruses in northern Australia. The detection of three polerovirus species with high levels of genome identity from northern Australia and Timor-Leste could represent natural movement of virus-infected aphids in wind currents from Timor-Leste. This example of potential virus movement is of concern for new cotton production in the Kununurra region and continued surveillance in this and other emerging production regions is advisable.

Cotton leafroll dwarf virus (CLRDV) in Timor-Leste was found to be relatively common and widespread in three Gossypium species (G. arboreum, G. barbadense and *G. hirsutum*). CLRDV was also found in ornamental hibiscus in Timor-Leste and it appears that this host could be an important perennial host for at least a few different polerovirus species. We found significant diversity of CLRDV from Timor-Leste and Thailand, far greater than that found in south-America. While we did not find evidence that the CRLDV strains in Timor-Leste are similar to the "atypical" strain from south America that are resistance breaking, the high diversity of CLRDV from Timor-Leste and Asia more broadly is a concern as there may be strains capable of breaking resistance.

Improved diagnostic PCRs for CLRDV have been developed which have been used to detect diverse strains of CLRDV from Timor-Leste, Thailand and Uzbekistan. These diagnostic assays contribute to improved preparedness for any possible incursions of CLRDV into Australia.

This project has confirmed that CBTV-1 and CBTV-2 are distinct polerovirus species and that that CBTV-2 is always associated with disease symptoms in cotton while CBTV-1 is not. No plants with typical bunchy top symptoms were found with only CBTV-1 and all symptomatic plants were infected with CBTV-2, either with or without CBTV-1. This will help to focus any future work on the control of CBTV-2 which is the causal agent for disease while it appears that CBTV-1 is most likely non-symptomatic in cotton and of little concern.

While there were only a few sporadic outbreaks of CBTV found during virus surveys in commercial crops during this project, it appears that old volunteer or ratoon cotton is the major source of virus moving into crops. As such it is recommended to maintain effective crop hygiene and to break the infection cycle and reduce the risk of virus disease outbreaks.

Extension Opportunities

8. Detail a plan for the activities or other steps that may be taken:

(a) to further develop or to exploit the project technology.

(b) for the future presentation and dissemination of the project outcomes.

- A peer reviewed journal article co-authored with several collaborators is planned (has been for a long time) to report the host range, diagnostic assays, geographic distribution and genetic diversity studies for CBTV in Australia (currently in draft).
- A peer reviewed journal article to report the improved diagnostics for CLRDV, the new knowledge of host range and distribution in Timor-Leste and the significant diversity of CLRDV from Timor-Leste, Thailand and Uzbekistan.
- First report of CLRDV from chickpea in Uzbekistan (currently in draft).

(c) for future research.

- Further improvement and validation is planned for diagnostic assays for CLRDV. It is hoped to develop more rapid diagnostic assay that could be used in the event of delimiting surveys for CLRDV to quickly and accurately distinguish it from the endemic CBTV.
- It remains unclear if the CLRDV strains found in Timor-Leste or Thailand may overcome resistance genes being used in cotton. It would be useful to conduct CLRDV transmission tests in Timor-Leste onto cotton lines containing the resistance gene.

9. A. List the publications arising from the research project and/or a publication plan. (NB: Where possible, please provide a copy of any publication/s)

See Milestone 5.1, Objective 5 above for more complete list of extension material prepared during this project. Below is a list of more significant publications.

- Sharman M, Lapbanjob S, Sebunruang P, Belot JL, Galbieri R, Giband M, Suassuna N (2015) First report of Cotton leafroll dwarf virus in Thailand using a species-specific PCR validated with isolates from Brazil. Australasian Plant Disease Notes 10 (1):1-4. Published in July 2015 from research done in project DAQ1201.
- Ray JD, Sharman M, Quintao V, Rossel B, Westaway J, Gambley C, (2016). Cotton leafroll dwarf virus detected in Timor-Leste. Australasian Plant Disease Notes 11, 29.
- Sharman, M., Ray, J. D., Gambley, C. F., Lapbanjob, S., & Quintao, V. (2015). Cotton leafroll dwarf virus detected in Thailand and Timor Leste. Paper presented at the 2nd Australian Cotton Research Conference, Toowoomba, 8-10th September.
- Australian CottonGrower magazine article about viruses and diagnostic services, Jan 2016.
- Sharman M, Ray J, Ximenes A, Soares G, Quintao V (2016). Cotton leafroll dwarf virus is common in Timor-Leste. Proceedings of the 18th Australian Cotton Conference, Gold Coast, 2-4th August.
- Sharman M. (2017) Diversity of Cotton leafroll dwarf virus in Thailand and Timor-Leste. 2nd Australian Cotton Research Conference, Canberra, 5-7th September.
- Sharman M, Wilson L, et al. (2019). Endemic cotton-infecting viruses from Australia, and detection and studies of the exotic cotton leafroll dwarf virus. Talk presented at cotton blue disease workshop as part of Beltwide cotton pathology conference, New Orleans, USA, 9th Jan 2019.
- Sharman M (2019). Preparing for exotic threats: focus on blue disease overseas. Article in Spotlight magazine, Winter 2019, p17-18.

B. Have you developed any online resources and what is the website address?

No.

Part 4 – Final Report Executive Summary

Provide a one-page summary of your research that is not commercial in confidence, and that can be published on the internet. Explain the main outcomes of the research and provide contact details for more information. It is important that the Executive Summary highlights concisely the key outputs from the project and, when they are adopted, what this will mean to the cotton industry.

This project aimed to enhance and support the sustainability of the Australian cotton industry by: providing continued capacity in plant virology expertise and diagnostics, building industry awareness of viral disease threats, and developing preparedness for viral diseases that pose serious biosecurity threats to the Australian cotton industry.

Disease surveys targeting viruses in northern Australia were done over 4 years in areas that may be exposed to possible incursion pathways for biosecurity threats and also in emerging cotton production regions in far northern Western Australia and Queensland. No viruses were detected in cotton production areas of Kununurra and northern QLD. However, six different polerovirus species were detected in other hosts. None of the newly detected poleroviruses are likely to affect cotton but it does indicate there is a diverse range of poleroviruses in northern Australia and emerging cotton production regions may be exposed to new virus threats.

Disease surveys were also done in commercial cotton in Queensland. From 158 disease counts from 35 farms, no symptoms typical of exotic viruses were seen. Generally, there was very low incidence of virus-like symptoms for endemic viruses (*Cotton bunchy top virus* – CBTV and *Tobacco streak virus* - TSV) in cotton crops inspected with the exception of a few sporadic disease outbreaks of CBTV. Old volunteer or ratoon cotton appears to be the major source of infection for CBTV moving into crops and as such it is recommended to maintain effective crop hygiene to break the infection cycle and reduce the risk of virus disease outbreaks.

This project has confirmed that two distinct polerovirus species infect cotton in Australia, CBTV-1 and CBTV-2. We found that CBTV-2 is always associated with disease symptoms in cotton while CBTV-1 is not. All symptomatic plants were infected with CBTV-2, either with or without CBTV-1. This will help to focus any future work on the control of CBTV-2 which is likely the only causal agent for disease while it appears that CBTV-1 is most likely non-symptomatic in cotton and of little concern.

Three virus surveys were done in Timor-Leste (East Timor) with the primary focus to establish how common *Cotton leafroll dwarf virus* (CLRDV) is and what potential threat there may be for incursion into Australia. Gossypium samples were collected from across much of the country and CLRDV was detected from more than 30% of plants tested from several sites. Hence, CLRDV in Timor-Leste was found to be relatively common and widespread in three Gossypium species (*G. arboreum, G. barbadense and G. hirsutum*). Improved diagnostics developed for CLRDV strains from Timor-Leste, Thailand and other countries will support preparedness for this biosecurity threat.

Other new poleroviruses were also found in Timor-Leste and northern Australia, suggesting there may be natural movement of virus-infected aphids in wind currents from Timor-Leste. This example of potential virus movement may be of concern for the expansion of cotton production in northern Australia.

Appendix 1

CLRDV-specific nested RT-PCR (Pol3628F / Pol4021R, then CLRDV3675F / Pol.cp.3982R) for coat protein gene.
Version 20150609

This nested RT-PCR should detect partial coat protein gene from both the typical and atypical strains of Cotton blue disease from Brazil, Timor-Leste, Thailand and Uzbekistan. This nested PCR does not detect the two known Cotton bunchy top virus species from Australia. However, the first PCR is not CLRDV-specific and may detect other poleroviruses and must be used with the second PCR for specific detection of CLRDV.

Reverse Transcription (cDNA synthesis)

- To the tubes add:

	Vol per tube (1x)	Master mix **X**	**Check added**
Primer: Pol.4021R (10 uM)	1.0 µl		
ddH2O	3.5 µl		
Total nucleic acid extract (CTAB method)	1.5 µl		

- Heat to 80°C for 10min, chill on ice and spin down.
- From a master mix, add to tubes:

	Vol per tube (1x)	Master mix **X**	**Check added**
5×1st strand buffer	2.0 µl		
0.1M DTT	1.0 µl		
10mM dNTPs	0.5 µl		
SuperScript III 200U/µl (Invitrogen)	0.25 µl		
0.6ug/µl BSA	0.25 µl		

- Incubate at 55°C for 45min then 70°C for 10min, chill and spin down. Store on ice or at -20°C.

1st PCR

PCR Reaction Mix	**Vol per tube (1x)**	Master mix **X**	**Check added**
10 x PCR buffer (Invitrogen, Cat # 18038-067)	2.5 µl		
$MgCl_2$ (50 mM) (Invitrogen, Cat # 18038-067)	0.875 µl		
dNTPs (10 mM) (Invitrogen, Cat # 10297-018)	0.5 µl		
Primer: **Pol3628F** (10µM)	0.5 µl		
Primer: **Pol4021R** (10µM)	0.5 µl		
Taq (5U/µl) (Invitrogen, Cat # 18038-067)	0.2 µl		
H_2O	18.925 µl		
Template (cDNA)	1.0 µl		

2nd PCR

This RT-PCR should detect both the typical and atypical strains of Cotton blue disease from Brazil, Thailand and East Timor. This PCR does not detect the two known species of Cotton bunchy top virus from Australia. Amplifies about half the 5' end of coat protein gene; expected size product **~307 bp**.

PCR Reaction Mix	Vol per tube (1x)	Master mix X	Check added
10 x PCR buffer (Invitrogen, Cat # 18038-067)	2.5 µl		
$MgCl_2$ (50 mM) (Invitrogen, Cat # 18038-067)	0.875 µl		
dNTPs (10 mM) (Invitrogen, Cat # 10297-018)	0.5 µl		
Primer: **CLRDV3675F** (10µM)	0.5 µl		
Primer: **Pol3982R** (10µM)	0.5 µl		
Taq (5U/µl) (Invitrogen, Cat # 18038-067)	0.2 µl		
H_2O	18.925 µl		
Template (1:10 of 1st PCR product)	1.0 µl		
Total:	**25.0 µl**		

Primers: **Pol3628F** (TAATGAATACGGYCGYGGSTAG)
Pol4021R (GGRTCMAVYTCRTAAGMGATSGA) – cDNA primer
CLRDV3675F (CCACGTAGRCGCAACAGGCGT)
Pol3982R (CGAGGCCTCGGAGATGAACT) – cDNA primer

Cycling parameters: 95ºC for 1:00 min, then 35 cycles of (95ºC for 15 sec, 62ºC for 20 sec, 56°C for 10 sec, 72ºC for 20 sec), then 1 cycle of 72ºC for 3:00 min.

Appendix 2

CLRDV-specific RT-PCR nested for P0 gene, ORF-0 (CLRDV_ORF0F / CLRDV_ORF0R, then CLRDV90F / CLRDV794R)
Verion 20161221

This RT-PCR has been shown to detect the partial P0 gene (ORF0) of both the typical and atypical strains of Cotton blue disease from Brazil, Timor-Leste, Thailand and Uzbekistan. This PCR does not detect the two known species of Cotton bunchy top virus from Australia.

Reverse Transcription (cDNA synthesis)

- To the tubes add:

	Vol per tube (1x)	Master mix **X**	**Check added**
Primer: CLRDV_ORF0R (10 uM)	2.0 µl		
ddH2O	3.5 µl		
Total nucleic acid extract (CTAB method)	1.5 µl		

- Heat to 80°C for 10min, chill on ice and spin down.
- From a master mix, add to tubes:

	Vol per tube (1x)	Master mix **X**	**Check added**
5×1st strand buffer	2.0 µl		
0.1M DTT	1.0 µl		
10mM dNTPs	0.5 µl		
SuperScript III 200U/µl (Invitrogen)	0.25 µl		
0.6ug/µl BSA	0.25 µl		

- Incubate at 55°C for 45min then 70°C for 10min, chill and spin down. Store on ice or at -20°C.

1st PCR
Amplifies all (?) of P0 gene; expected size product **~790 bp**.

PCR Reaction Mix	**Vol per tube (1x)**	Master mix **X**	**Check added**
10 x PCR buffer (Invitrogen, Cat # 18038-067)	2.5 µl		
$MgCl_2$ (50 mM) (Invitrogen, Cat # 18038-067)	0.875 µl		
dNTPs (10 mM) (Invitrogen, Cat # 10297-018)	0.5 µl		
Primer: **CLRDV_ORF0F** (10µM)	0.5 µl		
Primer: **CLRDV_ORF0R** (10µM)	0.5 µl		
Taq (5U/µl) (Invitrogen, Cat # 18038-067)	0.2 µl		
H_2O	18.925 µl		
Template (cDNA)	1.0 µl		

Cycling parameters: 95ºC for 1:00 min, then 35 cycles of (95ºC for 15 sec, 62ºC for 20 sec, 56°C for 10 sec, 72ºC for 30 sec), then 1 cycle of 72ºC for 3:00 min.

2nd PCR
Amplifies about 85 % of the partial P0 gene; expected size product **~705 bp**.

PCR Reaction Mix	**Vol per tube (1x)**	Master mix **X**	**Check added**

10 x PCR buffer (Invitrogen, Cat # 18038-067)	2.5 µl		
$MgCl_2$ (50 mM) (Invitrogen, Cat # 18038-067)	0.875 µl		
dNTPs (10 mM) (Invitrogen, Cat # 10297-018)	0.5 µl		
Primer: **CLRDV90F** (10µM)	0.5 µl		
Primer: **CLRDV794R** (10µM)	0.5 µl		
Taq (5U/µl) (Invitrogen, Cat # 18038-067)	0.2 µl		
H_2O	18.925 µl		
Template (1:10 of 1st PCR product)	1.0 µl		
Total:	**25.0 µl**		

Primers: **CLRDV_ORF0F** (GTCTCGTGTATGTTGAATTTGATCAT)
CLRDV_ORF0R (CTCAACTGCTYTCTCCTTCAC) – cDNA primer
CLRDV90F (GCAGARTYTCTTCCGCAGCTCT)
CLRDV794R (CGCCTTCATCGTCAAAATGGTA) – cDNA primer

Cycling parameters: 95ºC for 1:00 min, then 35 cycles of (95ºC for 15 sec, 62ºC for 20 sec, 50°C for 10 sec, 72ºC for 25 sec), then 1 cycle of 72ºC for 3:00 min.

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