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Brief Report

Citrus tristeza virus strains present in New Zealand and the South Pacific.

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Abstract

Citrus are an important subsistence and commercial crop across the South and Central Pacific. Unfortunately, the spread of plant material has contributed to the spread of citrus pathogens, such as *Citrus tristeza virus* (CTV). In this study, we examined the incidence and diversity of CTV strains present in New Zealand and select island nations of the South and Central Pacific. We found that all currently described strains are present, and exist as complex mixtures of strains. Phylogenetic analysis showed little difference in strain diversity between locations, suggesting extensive movement of infected planting material occurred in the past.

Keywords: *Citrus tristeza virus*, Pacific, strain survey

Introduction

From early European settlement of the Pacific, citrus has been an important subsistence crop, and is grown across Melanesia, Micronesia, Polynesia, and Oceania. Citrus planting material in the South Pacific, and in particular New Zealand, has come from a diverse range of sources including North and South America, Asia, and South Africa, often via Australia (Mooney et al. 2000). This movement of citrus material, particularly prior to the establishment of quarantine restrictions on the importation of plant material, has resulted in the inadvertent introduction of many citrus diseases (Pearson and Grisoni 2002).

One of the most widespread and potentially devastating pathogens of citrus in the South Pacific is *Citrus tristeza virus* (CTV) (Pearson and Grisoni 2002; Harper et al. 2009; Harper et al. 2010). Unlike most viruses, CTV causes a range of diseases, including quick decline, stem pitting, and seedling yellows (Moreno et al. 2008). Also, unlike most viruses, CTV exists as a complex of strains that are often found as a population in the same host (Scott et al. 2013). The most recent classification of CTV strains (Harper 2013) identified 6 strains, defined by separation of their complete genome phylogenies and distance between groups, named T36, VT, T3, RB, T68, and T30, with a putative seventh strain, HA16-5, also being reported (Melzer et al. 2010). The link between individual strain and symptoms produced is unclear, for example multiple strains can produce stem-pitting, and mixtures of the same strains can produce a gradient of different symptoms (Harper, unpublished).

Despite knowledge of the presence of a wide range of CTV isolates in New Zealand, revealed through indexing studies (Dawson and Mooney 2000; Mooney et al. 2000), comparatively little is known about the molecular composition of the isolates present. Even less is known about CTV in the Pacific Islands. Such information is necessary to understand disease etiology, and also for the development of control measures based on strain homology, such as mild strain cross protection (Folimonova et al. 2010). In this study we examined the incidence of the 6 described CTV strains in *Citrus*, *Poncirus*, and *Fortunella* species from the North Island of New Zealand and from the Pacific Island nations of Samoa, Tonga, Tahiti, Wallis and Futuna, and the Marianas, using a multiple molecular marker (Hilf et al. 2005), and sequence based approach.

Materials and Methods

To examine CTV strain diversity present in New Zealand, a total of 96 trees, representing 23 species from the genera *Citrus*, *Fortunella*, and *Poncirus*, as well as hybrids thereof, were sampled from the New Zealand citrus budwood collection (courtesy of Plant and Food Research, Kerikeri, New Zealand) in the winter of 2007. An additional 14 samples from Samoa, Tahiti, the Marianas, and Wallis and Futuna were retrieved from dried tissue held in the Pacific Island Plant Virus

Collection at the University of Auckland (courtesy of R Davis, Australian Quarantine and Inspection Service). Total RNA was extracted from 100 mg of tissue and extracted using an RNeasy Plant Mini kit (Qiagen Inc., Valencia, CA) as per the manufacturer's instructions.

All samples were first tested for the presence of CTV using coat protein specific primers (CP-F: 5'-AAAGAAGGCGACGATGTTGT-3' and CP-R: 5'-AGCTCCGGTCAAGAAATCTG-3') with the Invitrogen Superscript™ III One-Step RT-PCR System with Platinum *Taq* (Invitrogen, Carlsbad, CA) kit. Following this, CTV-positive samples were strain-typed by one-step RT-PCR as above using the multiple-PCR marker system described by Hilf et al. (2005). Additional primer pairs targeting the ORF1a 'K17' region were designed for the discrimination of 2 more recently described strains, RB (RB-K17F: 5'-GTTTTCACGTCTGAAACGAAAG-3' and RB-K17R: 5'-CCAACACATCAAAAATAGCCTG-'3), the trifoliate resistance-breaking strain (Harper et al. 2010), and a local subtype of strain T68, isolates B18/B165 (B18-K17F: 5'- GTTGTCGCGCTTAAAGTT CGGT-3' and B18-K17R: 5'- GTACGACGTCAAAAA TAGCTGA-3') (Harper et al. 2009; Harper 2013; Roy et al. 2010). All reactions were performed in a 20 µl final volume containing 400 nM of forward and reverse primers, 1.5 mM of $MgSO_4$, and 200 ng of total RNA extract. Reaction conditions were as follows: 50 °C for 30 min, 94 °C for 2 min, then 35 cycles of 94 °C for 10 s, 56 °C for 30 s, and 68 °C for 45 s. Strains were assigned on the basis of amplification of both the appropriate ORF1a-K17 and ORF1b-p33 POL markers (Table 1).

Table 1
PCR marker pairings used to discriminate CTV strains in this study. Due to markers in common between strains, both the appropriate POL and K17 markers were required to make an identification. PCR markers are described in Hilf et al. (2005) and this study.

Marker	Strain					
	T36	T30	RB	VT	T3	T68
T36-POL	+					
T36-K17	+					
T30-POL		+	+			
T30-K17		+				
RB-K17			+			
VT-POL				+	+	+
VT-K17				+		
T3-K17					+	
B18-K17						+

Due to the potential presence of defective RNAs in field samples, which are often derived from ORF1a (Mawassi et al. 1995), amplification of the K17 marker alone was not considered definitive to assign a CTV strain. Strain identification was confirmed by direct sequencing of amplicons (Macrogen, Seoul, Korea). Sequences were aligned against representative isolates from each strain (Harper 2013), and neighbor-joining phylogenetic trees were constructed using the Tamura-Nei model, with a 10^3 replicate bootstrap test for support. Sequences generated during this study were deposited in the NCBI GenBank database under accession numbers FJ529147 through FJ529166 and GU594045 through GU594055. Statistical analyses to test for associations between CTV strains or mixtures, and species were performed using a chi-squared test for association.

Results

CTV was found to be present in 104 of the 110 samples examined, of which 96 were found to have one or more CTV strains present; the remaining 8 produced non-standard profiles to which no strain could be assigned. Of the 96 plants from both New Zealand and the Pacific Islands that were able to have strains assigned, 64 (71.1%) were positive for the RB strain. The related VT, T3, and T68 strains were found in 32 (35.6%), 36 (40.0%), and 32 (35.6%) plants respectively. The T30 strain was found in 28 (31.1%) plants, while the T36 strain was rare with only 7 (7.8%) plants positive (Table 2). Taking into account differences in sample size, there were no significant differences in strain presence in New Zealand versus the island nations.

Table 2
CTV strain frequencies in samples from New Zealand and other Pacific Island nations, as identified by multiple-molecular PCR marker assays.

Country	No. CTV Positive Samples	No. Plants Infected with CTV Strains					
		RB	VT	T3	T68	T30	T36
New Zealand	82	55	27	26	23	22	5
Samoa	5	4	2	3	3	4	0
Tahiti	3	3	3	3	3	2	1
Wallis & Futuna	3	1	0	3	1	0	1
Marianas	3	1	0	1	2	0	0

Mixtures of CTV strains were found to be extremely prevalent in *C. sinensis* (76.2%), *C. unshiu* (60%), *C. reticulata* (60%), and *C. limon* (69.2%) samples, whereas they are comparatively rare in *P. trifoliata* and hybrid rootstock species (11.1%). Infection by a solitary strain was found in 26 of 90 (28.9%) CTV-positive samples. The RB strain was most frequently found alone (12 of 26 plants) although this is biased by the inclusion of *P. trifoliata* and trifoliate hybrid species that only RB can readily infect (Harper et al. 2010). Two strains were found in 31 samples, 3 strains in 21 samples, 4 strains in 7 samples, and a mixture of 5 strains in 4 plants; no samples were found with all 6 CTV strains tested for. The RB strain was again prevalent in these mixtures, being found in 41 of the 61 plants containing 2 or 3 strains, and all plants containing mixtures in which 4 or 5strains were present. Moreover, the majority (82.5%) of mixtures found contained RB with VT, T3, or T68, suggesting that

the RB strain does not exclude these more aggressive strains. A smaller percentage of samples (43.1%) contained both RB and T30, although this is proportionate to the overall incidence of T30, and suggests that T30 is rarely found in isolation in New Zealand and the Pacific.

When we compared the citrus species to the frequency and type of strains identified (Table 3), we found only one significant association, that of the RB strain in *P. trifoliata* and citrange rootstock samples (X^2 = 74.58, $p<0.05$, df=54). This is unsurprising, for as mentioned above, RB is the only strain that can readily infect these hosts. While scion species show a much greater CTV incidence and strain variability than rootstocks, there was no statistically significant association between any particular strain and scion species (X^2 = 36.49, $p<0.05$, df=42). There were general trends however, with a high incidence of T3 and T68-like strains being observed in *C. limon* and *C. sinensis* cultivars, while *C. paradisi* and hybrids possessed a greater than average incidence of T36 isolates, and an absence of T3 isolates; these likely reflect the ability of different strains to infect specific species, as reported by Harper et al. (2014). Sample numbers of other species were too low to form an accurate picture of strain-host frequencies.

Phylogenetic analysis of polymerase region fragments amplified in this study (Fig. 1) suggested that there is no obvious geographic segregation of CTV strains, for New Zealand and Pacific Island samples group with each other, and with sequences of isolates from most major citrus growing regions of the world. Furthermore, these data indicate that the RB strain, first described in New Zealand (Harper et al. 2010), is present in several island nations across the Pacific and has not noticeably diverged; average nucleotide identity for the New Zealand and Pacific Island RB isolates was 97.4%, which is comparable to that between extant RB genomes (Harper et al. 2010). There was also no obvious association observed between phylogenetic position and host species (data not shown), and no evidence of recombination was observed within the sequenced region for any of the New Zealand or Pacific Island isolates.

Table 3
The frequencies of CTV strains in citrus from New Zealand and the Pacific Islands, as identified by multiple-molecular PCR marker assays, and divided by host species.

Species	No. Tested	CTV Absent / No strains assigned	CTV Strain					
			RB	VT	T3	T68	T30	T36
Citrus aurantifolia (Christm.) Swing.	1				1			
Citrus aurantium L.	2				2	2		
Citrus clementina hort. ex Tanaka	1	1						
Citrus deliciosa Ten.	1		1			1	1	
Citrus hystrix DCitrus	1						1	
Citrus junos Siebold ex Tanaka	1		1		1			
Citrus latifolia (Yu. Tanaka) Tanaka	2			1	2		1	
Citrus limon (L.) Burm. f	13	1	8	7	10	6	5	2
Citrus madurensis Loureiro	1		1	1			1	
Citrus maxima (Burm.) Merr.	4	1	2	1	2			
Citrus medica L.	2			1	2			
Citrus meyeri Yu. Tanaka	2		2	2		1	1	
Citrus myrtifolia Raf.	1			1	1			
Citrus paradisi 'Duncan' x *Citrus tangerina*	2	1		1				
Citrus paradisi Macfad.	7	2	3	3		1		2
Citrus paradisi x *Citrus reticulata*	6	1	4	2	2	3		1
Citrus reticulata Blanco	10		7	3	3	5	2	1
Citrus sinensis (L.) Osbeck	21	2	16	7	7	11	9	1
Citrus unshiu Marcow.	10	1	8	2	2	2	4	
Citrus unshiu x *Citrus sinensis*	1	1						
Fortunella spp.	3	1	1	1	1		1	
Poncirus trifoliata L. Raf.	9	3	6				1	
Poncirus trifoliata x *C. sinensis*	9	5	4				1	

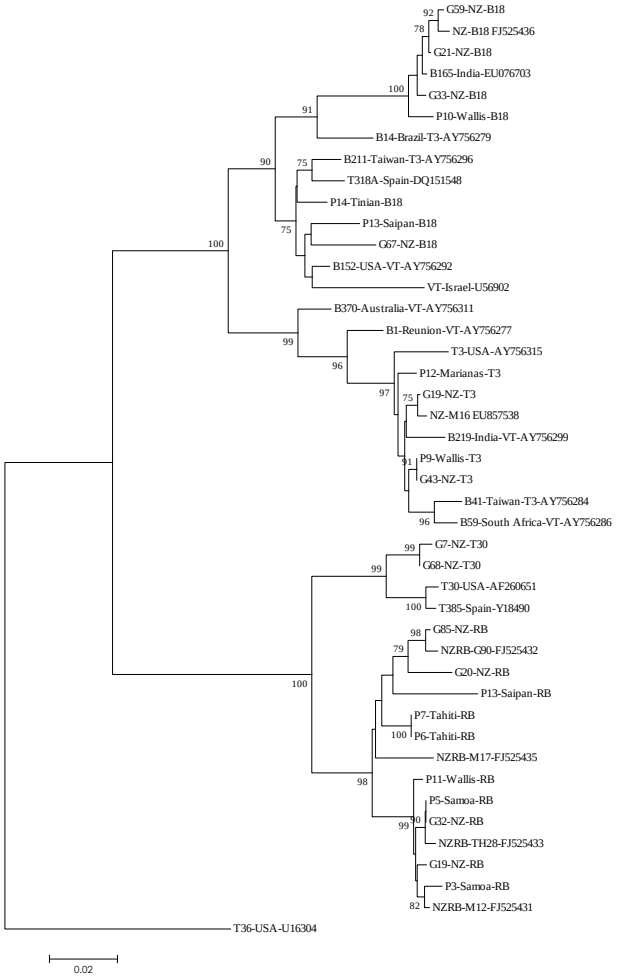

Fig 1. Neighbor-joining nucleotide-based phylogeny of the ORF1b-p33 POL marker of selected CTV samples from New Zealand and the Pacific islands, coded by sample number-country-strain amplified, compared to representative sequences from Hilf et al. (2005). Bootstrap values from 10^3 replicates.

Discussion

In this study, we found that all 6 of the major CTV strains, T36, T3, T30, T68, VT, and RB, were present in both New Zealand and across the South Pacific. CTV incidence in New Zealand across all citrus species is approximately 88%; similar levels of infection are observed in Argentina and Florida where, as in New Zealand, the brown citrus aphid *Toxoptera citricida* is well established (Iglesias et al. 2008; Powell et al. 2005). In addition, New Zealand has no certified virus-free budwood scheme and employs no centralized disease control or tree removal program, unlike California, South Africa, and Spain (Rocha-Pena et al. 1995), whilst virus vector control is inconsistently applied.

It has been suggested that high CTV diversity may be correlated with growing practices and the range of citrus varieties grown (Moreno et al. 2008; Rocha-Pena et al. 1995). The diversity of CTV strains within the large number of varieties tested in New Zealand in this study would appear to support this. These samples were taken from a cultivar collection that had been assembled from around the country over several decades, and had been maintained in its present form, under aphid pressure, in 1 of the 3 major citrus production regions of the country. This permitted the movement of CTV strains into a diverse range of host cultivars and likely contributed to the range of mixtures observed. However, in the Pacific Islands we see the same high level of diversity and range of mixtures, yet the citrus plants are generally produced as a subsistence crop and cultivar diversity is low. Where then, did the isolates in New Zealand and the South Pacific originate?

Phylogenetic analysis suggests that there is little divergence, and no obvious segregation, between the isolates of New Zealand and the Pacific Islands. This may suggest that either the Pacific Islands acquired their planting material from New Zealand or vice versa, which is unlikely as little material moves between New Zealand and the Pacific Islands due to quarantine restrictions, or that all locations acquired their CTV stock from one or more third parties. Given the apparent stability of CTV sequences (Albiach-Marti et al. 2000), this is the most likely situation, for it is known that much of the citrus material in New Zealand has been acquired from South-East Asia, South Africa, and the Americas, via Australia.

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