Incidence, Distribution, and Diversity of *Citrus tristeza virus* in the Hawaiian Islands

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ABSTRACT. *Citrus tristeza virus* (CTV), the causal agent of tristeza quick decline and stem pitting in citrus, and its most effective vector, the brown citrus aphid (BrCA) (*Toxoptera citricida* Kirk.), have been present together in Hawaii for over 50 yr. In this study the incidence, diversity, and population structure of Hawaiian CTV strains were examined. Citrus tissue samples collected on Kauai (8 sites, 91 trees), Oahu (13, 86), Molokai (4, 12), Maui (10, 87), and Hawaii (15, 129) were subjected to RT-PCR for the CTV coat protein gene as well as tissue blot immunoassays (TBIAs) using two CTV antibodies. Samples testing positive for either RT-PCR or TBIA were subjected to further RT-PCR using primers specific to CTV genotypes T3, T30, T36, and VT. The incidence of CTV on Kauai, Oahu, Molokai, Maui, and Hawaii was 59, 87, 58, 63, and 83% respectively, with an overall incidence of 74% (298/405). Strains with similarity to T3, T30, T36, and VT genotypes were identified in these samples, although their distribution was not uniform throughout the islands. Most infections, however, were composed of CTV strains that did not resemble any of these genotypes. The coat protein (CP) sequence was determined for over 100 Hawaiian CTV strains. Phylogenetic analysis using these sequences as well as CP sequences of CTV strains from other parts of the world indicate that Hawaii has previously uncharacterized strains of the virus.

Stem pitting, an economically important disease of citrus caused by *Citrus tristeza virus* (CTV) was first observed in Hawaii in 1952, although the disease was already widespread at the time (4). With the most effective vector, the brown citrus aphid (*Toxoptera citricida* Kirk.), abundant in the islands (9), it is likely that CTV was rapidly disseminated after its arrival. This disease has reduced Hawaii's citrus industry to its present day status of small farms and backyard plantings of mostly CTV-tolerant citrus.

As citrus is being revisited as a crop to vitalize Hawaii's agriculture industry, control of CTV must be undertaken. In order to develop a sound management strategy for CTV in Hawaii, we undertook a comprehensive study using molecular and serological techniques to determine the incidence, distribution, and diversity of CTV in the Hawaiian Islands.

MATERIALS AND METHODS

Citrus samples. Four hundred and five citrus trees were sampled from 50 sites on the islands of Kauai, Oahu, Molokai, Maui, and Hawaii (Fig. 1). These sites ranged from groves with >500 trees to single backyard plants. Young stem segments were collected from both vigorous and unthrifty trees at each site when possible. These samples were kept on ice in the field and were then stored at -20°C until analyzed.

Serological analysis. Tissue-blot immunological assays (TBIAs) based on the method described by Hu et al. (7), were used to detect CTV in the citrus samples. Two anti-CTV rabbit polyclonal antisera were used in these assays. The first (1212) was raised against purified virions of an Australian isolate of CTV. The second (kindly provided by the late D. J. Gumpf), designated CCPP, was raised against an *in vitro*-expressed

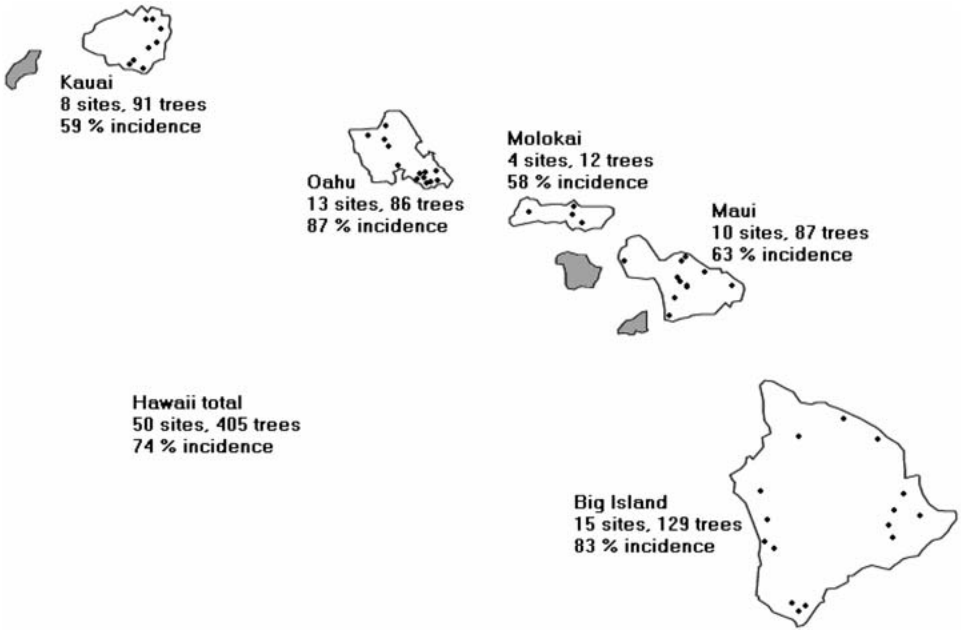

Fig. 1. Map of the major Hawaiian Islands. Noted are the sampling site locations (●), the number of trees sampled, and the percentage of samples testing positive for *Citrus tristeza virus*. The shaded islands of Niihau, Lanai, and Kahoolawe (from left to right) were not included in the survey.

CTV CP and is used by the California Citrus Clonal Protection Program for CTV detection. In order to screen each sample with multiple CTV antibodies, each stem segment was recut 0.5-1.0 mm above the previous cut and pressed successively onto separate membranes.

Molecular analysis. Approximately 100 mg of bark tissue was ground in liquid nitrogen and total nucleic acids (TNAs) were extracted as described by Stewart and Via (11), except that DIECA was omitted from the extraction buffer. First-strand cDNA synthesis was performed using 2 µl of TNAs, random hexamers, and MMLV-RT (Promega, Madison, WI) following the manufacturer's instructions. A PCR assay using the T36CP primer set was then used to detect CTV in these samples (6). Samples testing positive by either TBIA or the T36CP primer set were further characterized with 10 additional primer sets that amplified the 5'-UTR, K17, and POL markers of CTV genotypes T30, T36, and VT, and the K17 marker of the T3 genotype as previously described (5, 6).

For all PCR assays, 1 µl of the cDNA reaction was used as template in a 20 µl reaction containing 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 10 pmol of each CTV primer, 200 µM of each dNTP, 1 U of *Taq* DNA polymerase, overlaid with mineral oil. The amplification protocol consisted of one 5 min cycle at 94°C; 40 cycles at 94°C for 60 s, 56°C for 60 s, and 72°C for 60 s; ending with one 7 min cycle at 72°C. Amplification products were resolved in a 1% (w/v) agarose gel stained with ethidium bromide and viewed under UV light.

Sequence variation in Hawaiian CTV. Sequence variation in the Hawaiian CTV population was assessed for each island using the coat protein gene (CP) of CTV isolates. A CTV-positive sample from each site on an island was selected,

and l µl of the cDNA reaction (from above) for each of these samples was pooled. For example, on the island of Kauai we visited 8 sites and therefore the pooled Kauai cDNAs came from 8 CTV-positive samples. The pooled cDNA reactions were then used as template in PCR using the T36CP primer set as described above with only 30 amplification cycles. The resulting PCR product was ligated into pGEM-T Easy (Promega). Between 21 and 24 individual clones from each of the islands of Kauai, Oahu, Maui, and Hawaii, and 10 individual clones from Molokai were sequenced on an ABI377 cycle sequencer (PE Applied Biosystems, Foster City, CA). Plasmids were sequenced on one or both strands, depending on the signal strength of the electrophoretogram. In an exceptional case where sequencing of both strands could not resolve a base, the base was assigned the most common nucleotide at that position as determined by comparison to the other sequences.

Polymorphic positions in these 'Hawaiian' CTV CP sequences were compared with those of 'exotic' CTV CP sequences from California (strain SY568, GenBank accession number AF001623), Florida (T30, AF260651; T36, U1603), Israel (VT, U56902), Japan (NUagA, AB046398), Mexico (BC15-1, AF342894; QR2753-1, AF342893), Portugal (13C, AF184113; 19-21, AF184114), South Korea (SK1, AF339088; SK2, AF249279), and Spain (B35, L12175; T385, Y18420) using the Pretty function of SeqWeb 1.2 (GCG, University of Wisconsin, WI). Primer sequences were excluded from the comparison. Nucleotide sequence alignments of the CTV CP genes were generated by neighbor joining using ClustalX (1.81) and visualized by TreeView (1.6.6).

RESULTS

Incidence of CTV in Hawaii. Samples testing positive in either TBIA or RT-PCR assays were considered positive. The results of the two assays, however, were not in full agreement. Of the 298 samples considered positive, 226 were positive with both assays, 236 (226+10) with the RT-PCR assay, and 288 (226+62) with the TBIA. The incidence of CTV for the islands of Kauai, Oahu, Molokai, Maui, and Hawaii was found to be 59, 87, 58, 63, and 83%, respectively, with an overall incidence in the islands of approximately 74% (298/405) (Fig. 1).

Characterization of Hawaiian CTV isolates. A 411 bp fragment was amplified from many samples using the T36POL primer set. As the expected marker size is 732 bp, this product was at first assumed to be non-specific. Cloning and sequencing of this amplicon, however, identified it as a partial CTV p33 gene. Sequence comparisons showed this fragment was most homologous to the corresponding sequence from the CTV p33 of strains T385 and SY568 (92% identical at the nucleotide level). This amplification product was designated as marker 'p33'.

For the 298 CTV-positive samples, 143 different marker patterns were identified (Table 1). None of the samples were positive with all 12 RT-PCR markers; the T36POL marker was conspicuously absent in all but one of the samples tested (Table 1, Column B, Row 61).

The most common marker pattern, found for 29 samples, had only the p33 marker amplified (Table 1, Column B, Row 70). The second most common pattern, found for 11 samples, had the T36CP and p33 markers amplified (Table 1, Column B, Row 59). The third most common pattern was found in 10 samples that were positive for TBIA, but negative for all 12 markers (Table 1, Column B, Row 71). Most of the marker patterns, however, were represented by only a single sample (Table 1).

The distribution of these markers was not uniform throughout the islands. The T3 genotype, which has

TABLE 1
RT-PCR MARKER PATTERNS OF HAWAIIAN *CITRUS TRISTEZA VIRUS* GENOTYPES. MARKERS SPECIFIC TO CTV GENOTYPES T3, T30, T36, AND VT, AS WELL AS A P33 MARKER (SEE TEXT) WERE USED TO CHARACTERIZE HAWAIIAN CTV ISOLATES. THESE MARKERS INCLUDED THE COAT PROTEIN (CP), 5'-UTR (5), K17 (K), AND POL (P) REGIONS (5, 6). THE T36 CP MARKER IS NOT SPECIFIC TO T36 GENOTYPES AND WAS USED IN THIS STUDY FOR GENERAL DETECTION OF CTV IN CITRUS SAMPLES. A ● INDICATES THE MARKER WAS PRESENT. THE NUMBERS TO THE RIGHT OF EACH COLUMN INDICATE THE NUMBER OF SAMPLES FOR EACH ISLAND THAT HAD THE MARKER PATTERN TO THE LEFT. ABBREVIATIONS ARE KA (KAUAI); OA (OAHU); MO (MOLOKAI), MA (MAUI); HA (HAWAII)

Column 1

	Marker Pattern												# of Samples with Marker Pattern Island						
	T36	T3	T30			T36			VT			p33							Tot.
	CP	K	5	K	P	5	K	P	5	K	P		Ka	Oa	Mc	Ma	Ha		
1	●	●	●	●	●	●	●		●	●	●	●	2	2					4
2	●	●	●	●	●	●	●		●	●	●	●		5			3		8
3	●	●	●	●	●	●			●	●	●	●		4					4
4	●	●	●	●		●	●		●	●	●	●	1						1
5	●	●	●		●	●	●		●	●	●	●		2					2
6	●		●	●	●	●	●		●	●	●	●	2						2
7	●	●	●	●	●	●			●	●	●			1			1		2
8	●	●	●	●	●	●			●	●	●	●		1					1
9	●	●	●	●			●		●	●	●	●		1					1
10	●	●	●		●	●			●	●	●	●		1					1
11	●	●		●	●	●			●	●	●	●					1		1
12	●		●	●	●	●	●		●	●	●		1	1					2
13	●		●	●	●	●			●	●	●	●		1			1		2
14	●		●	●	●		●		●	●	●	●	1			1			2
15	●		●	●		●	●		●	●	●	●	3			1			4
16	●			●	●	●	●		●	●	●	●	1						1
17	●	●	●	●		●			●	●	●			1					1
18	●	●	●	●		●			●	●	●	●					1		1
19	●	●	●	●			●		●	●	●		1						1
20	●	●			●	●	●		●	●	●						1		1
21	●	●	●		●	●			●	●	●			1					1
22	●	●	●			●	●		●	●	●			3					3
23	●	●	●			●			●	●	●	●					1		1
24	●	●	●				●		●	●	●	●				3			3
25	●	●		●	●	●			●	●	●	●		1					1
26	●	●		●		●			●	●	●	●		1					1
27	●	●			●	●	●		●	●	●	●		2					2
28	●	●			●		●		●	●	●	●					1		1
29	●	●				●	●		●	●	●	●						1	1
30	●		●	●	●	●			●	●	●	●		1			1		2
31	●		●	●	●	●			●	●	●	●		2			1		3
32	●		●	●		●	●		●	●	●	●				1			1
33	●		●	●			●		●	●	●	●		1					1
34	●		●		●	●	●		●	●	●	●		1					1
35	●		●		●		●		●	●	●	●	1			1			2
36	●			●		●	●		●	●	●	●	3						3
37	●	●			●	●			●	●	●			1					1
38	●	●	●			●			●	●	●	●		1					1
39	●	●	●				●		●	●	●	●				1			1
40	●	●	●				●		●	●	●	●					1		1
41	●	●		●			●		●	●	●	●					1		1
42	●	●			●	●	●		●	●	●	●		1					1
43	●	●			●		●		●	●	●	●		1					1
44	●	●				●	●		●	●	●	●				1			1
45	●	●				●	●		●	●	●	●				1			1
46	●	●				●			●	●	●	●		1			2		3
47	●		●	●	●		●		●	●	●	●				1			1
48	●		●		●	●	●		●	●	●			1					1
49	●		●			●			●	●	●	●		1					1
50	●		●				●		●	●	●	●				2			2
51	●			●	●	●	●		●	●	●			1					1
52	●			●			●		●	●	●	●	3				1		4
53	●				●	●	●		●	●	●	●					1		1
54	●				●		●		●	●	●	●				1			1
55	●					●	●		●	●	●	●	1						1
56	●	●	●				●		●	●	●	●	1						1
57	●	●				●	●		●	●	●					1			1
58	●	●				●	●		●	●	●	●				2			2
59	●	●				●			●	●	●						3		3
60	●	●				●			●	●	●	●		3			2		5
61	●	●					●		●	●	●	●					1		1
62	●	●					●		●	●	●	●				2			2
63	●		●	●					●	●	●	●		1		1			2
64	●		●			●			●	●	●	●		1					1
65	●		●			●			●	●	●	●				1			1
66	●		●				●		●	●	●	●		1					1
67	●			●	●		●		●	●	●		1						1
68	●			●		●	●		●	●	●						1		1
69	●			●		●	●		●	●	●	●					1		1
70	●			●			●		●	●	●						1		1
71	●			●			●		●	●	●	●		1		2	1		4
72	●				●	●	●				●	●	1						1

Column 2

	Marker Pattern												# of Samples with Marker Pattern Island						
	T36	T3	T30			T36			VT			p33							Tot.
	CP	K	5	K	P	5	K	P	5	K	P		Ka	Oa	Mc	Ma	Ha		
1	●				●				●	●	●	●	1						1
2	●					●	●		●	●	●	●					1		1
3	●					●			●	●	●	●						2	2
4	●						●		●	●	●	●	1				1		2
5	●		●	●					●			●						1	1
6	●		●	●					●	●	●							1	1
7	●					●			●	●				1					1
8	●					●			●	●								2	2
9	●					●			●			●		2				1	3
10	●						●		●	●			1				2		3
11	●								●	●	●							1	1
12	●								●	●		●	1				2		3
13	●		●	●						●							1		1
14	●		●	●					●			●		1					1
15	●								●								2		2
16	●		●	●								●					1	1	2
17	●		●	●					●	●				1					1
18	●		●	●					●	●	●			1					1
19	●		●	●					●	●	●		1						1
20	●								●			●	2					1	3
21	●								●	●		●		1					1
22	●								●	●		●					1		1
23	●								●	●	●							1	1
24	●								●	●	●	●						1	1
25	●								●			●	1				1		2
26	●								●	●	●	●	4				1	4	9
27	●								●			●						1	1
28	●								●			●					1		1
29	●								●			●						1	1
30	●								●			●		1					1
31	●								●			●						1	1
32	●								●			●		1					1
33	●								●			●	1						1
34	●								●			●	1						1
35	●								●			●	1				2		3
36	●								●			●	1				2		3
37	●								●	●		●				1		2	3
38	●								●	●		●	1					1	2
39	●								●	●		●	1					3	4
40	●								●									1	1
41	●								●								1		1
42	●								●			●						1	1
43	●											●						1	1
44	●								●	●						1		1	2
45	●								●	●		●				1			1
46	●								●	●						1		3	4
47	●								●			●					2	1	3
48	●								●			●				1		1	2
49	●								●	●		●						2	2
50	●								●	●		●				1	1	1	3
51	●								●	●		●						1	1
52	●								●	●		●						1	1
53	●								●	●		●				1		1	2
54	●								●								1		1
55	●											●				2			2
56	●								●									1	1
57	●								●										1
58	●											●				1			1
59	●											●	1	4	1	4	1		11
60	●											●					1		1
61	●								●								1		1
62	●								●								1		1
63	●								●			●					1	1	2
64	●								●	●								1	1
65	●											●						6	6
66	●															1		3	4
67	●																	1	1
68	●																2	1	4
69	●											●						2	2
70	●																		29
71	●																		10
72	●																		1
													9	3	3	2	12		
													1	2		1	6		
												Total	54	75	7	55	107		298

only the K17 marker available, was found in 50% of the samples from Oahu, 13% of the samples from Kauai, and none of the samples from Molokai. In total, it was found in 32% of the samples (Fig. 2A).

Genotypes T30, T36, and VT, each have three markers that amplify the 5'-UTR, K17, and POL regions of their genomes. A large proportion of the samples tested positive with only one or two of these primer sets; 32, 58, and 34% for strains T30, T36, and VT, respectively. This suggests that CTV genotypes infecting these plants are distinct from the type strain genotype. To simplify the data for the purpose of examining the distribution of these markers in the islands, the results for each group of three markers were designated as 0/3 (type strain genotype not present), 1/3 (partial similarity to type strain genotype), 2/3 (moderate similarity), and 3/3 (high similarity). Samples containing genotypes with some degree of similarity to strain T30 were common on the northwestern islands of Kauai and Oahu, less common on the southeastern islands of Maui and Hawaii, and absent on Molokai. Samples from Oahu had the highest incidence of CTV with genotypes similar to T30, including 24% that had high similarity to T30 (Fig. 2B).

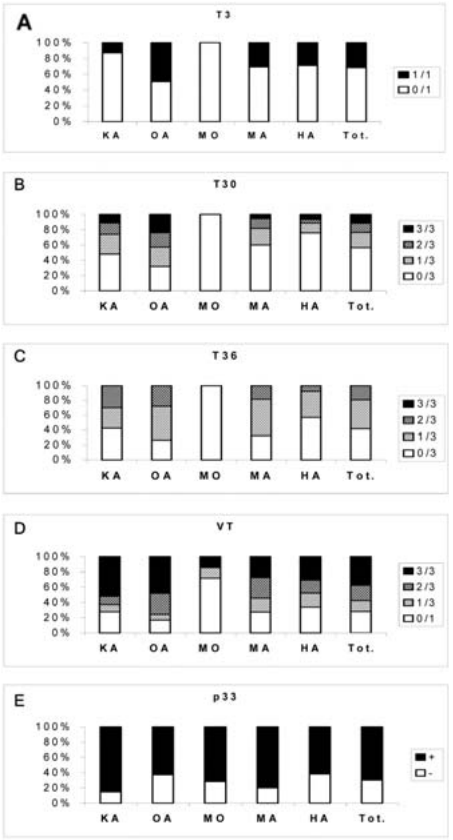

Fig. 2. Distribution of *Citrus tristeza virus* genotypes in the Hawaiian Islands. Stack graphs indicate the percentage of samples from each island that had the given number of markers amplified for each genotype. For genotype T3 (A) one marker was used (0/1, T3 genotype absent; 1/1, T3 genotype present). For genotypes T30 (B), T36 (C), and VT (D) three markers were used (0/3, genotype absent; 1/3, partial similarity to genotype; 2/3, moderate similarity; and 3/3, high similarity). E, the percentage of samples in which the p33 marker was amplified. Abbreviations are KA (Kauai); OA (Oahu); MO (Molokai); MA (Maui); HA (Hawaii), Tot (total for all islands).

While no samples tested positive for all three markers, samples containing genotypes with low or moderate similarity to strain T36 were common throughout the islands, with the exception of Molokai (Fig. 2C). All of these samples were positive for only the 5' UTR and/or K17 markers, except for one sample from the island of Hawaii which was positive for the K17 and POL markers, but was negative for the 5' UTR marker (Table 1, Column B, Row 61). To determine whether the T36 primers were in fact amplifying T36-like markers and not non-specific sequences, the 409 bp T36 K17 marker amplified from an Oahu sample was cloned and sequenced. This clone was found to have 92, 81, 80, 78, and 77% amino acid similarity to strains T36, T30, T385, VT, and SY568, respectively, confirming that the amplification products were T36-like.

Samples that contained VT-like genotypes were common on all the islands except Molokai. Like the samples containing T30-like genotypes,

the VT-like genotypes were most prevalent in samples from the northwestern islands of Kauai and Oahu. On these islands, genotypes with high similarity to VT were found in over half of the samples (Fig. 2D).

The p33 marker was often found in samples that were negative for all other markers, including the T36CP marker that was used for general CTV detection. This marker was common on all islands, but most prevalent on Kauai where it was found in 85% of the samples. Its overall incidence in the samples tested was about 70% (Fig. 2E).

Sequence variation in Hawaiian CTV. Between 21 and 24 CP sequences for each island of Kauai, Oahu, Maui, and Hawaii, and 10 sequences from Molokai were initially analyzed for sequence variation. These were designated as 'Hawaiian' sequences. Also included in the analysis were 14 CTV CP sequences from locations outside Hawaii, which were designated as 'foreign' sequences. Surprisingly, only a few of the Hawaiian sequences were identical to each other, and none were identical to any foreign sequences. Of the 102 sequences obtained, we found 85 different variations of the CP sequence.

Of the 626 bases analyzed for the Hawaiian and foreign sequences, 217 were polymorphic: 92 of these polymorphic bases were found in both Hawaiian and foreign sequences, 25 were unique to the foreign sequences, and 100 were unique to the Hawaiian sequences. The lowest sequence homology between two Hawaiian sequences, two foreign sequences, and a Hawaiian and a foreign sequence was found to be 91.7, 91.5, and 91.4%, respectively.

DISCUSSION

CTV and the brown citrus aphid (BrCA) have been present in the Hawaiian Islands for over 50 yr, during which time no major control attempts have been made. This relationship has led to the high incidence of CTV in Hawaii that in this study was found to be 74%. This is somewhat lower than the value of 91% found by Garnsey et al. (3). The fact that several of the farms visited in this study were recently established with certified CTV-free trees may account for this lower incidence as most of these farms had not been established at the time of the previous survey. Our study's 10-fold increase in sample size over the previous work may have provided a more realistic reflection of CTV incidence in the islands. At 49 of the 50 sites visited, at least one tree tested positive for CTV. The only CTV-free site was an isolated tree growing near a sugarcane field on Kauai. Many of these sites were established with certified CTV-free trees within the last 5 yr, indicating how rapidly CTV is disseminated in Hawaii.

The TBIA and RT-PCR detection assays were not in full agreement, indicating that a single assay with either the T36CP primers or the two antibodies used in this study is not adequate for CTV detection in Hawaii. The TBIA assay was most reliable, as only 10 samples that were positive with the T36CP marker RT-PCR assay tested negative with TBIA. It is unclear whether these 10 negative samples were not recognized by any of the antibodies, or were results of low virus titer (2), or false positive results due to PCR contamination. Conversely, 58 samples in which the T36CP marker was not amplified by RT-PCR were positive with the TBIA. It is clear from this study, that alternative or additional primers should be used if RT-PCR is to be used as the sole CTV detection assay.

The wide range of marker patterns found in this study indicates that a diverse population of CTV exists in Hawaii. Many samples tested positive for all markers from more than one genotype, suggesting mixed infections are common. The strongest evidence for this is the sam-

ples that tested positive with nearly all markers. Most of the samples had only one or two markers amplified from multiple genotypes. We suggest that these are either mixed infections of genotypes with various degrees of similarity to the characterized genotypes, or they represent recombinant genotypes, or both. Recombination in CTV genome is not novel (10), and has been proposed to explain the asymmetrical sequence variation in the T36 genome (6, 8), as well as regions of high sequence homology between the genomes of SY568, T30, and T385 (1).

Although the 5'-UTR and K17 markers for the T36 genotype were common in Hawaiian CTV samples, the POL marker for this genotype was conspicuously absent. This marker was found in only one sample from the Kainaliu Experiment Station on the island of Hawaii. This sample was from an 'Abhay Apury' finger lime (*Microcitrus australasica* (F. Muell) Swingle) imported from Australia. It has been proposed that the T36 genome was formed by a recombination event between the 3'-terminal region of a 'common' CTV genome and the 5'-terminal region of some distantly related CTV genome; the crossover point being between the helicase motif and the RNA-dependent RNA polymerase (8). It is intriguing to speculate that the T36-like genotypes commonly found in Hawaii, which are similar to T36 upstream of this crossover point (5'-UTR and K17 regions) but dissimilar to T36 downstream of this crossover point (POL region), might be this putative distantly related T36 parental strain.

Hilf and Garnsey (5) found heterologous amplification products when characterizing CTV isolates T3, T30, and VT with the strain-specific primer sets. For example, the VT 5' and K17 markers were amplified in samples thought to contain only the T30 genotype. Similarly, the VT 5' and POL markers were amplified in samples thought to contain only the T3 genotype, and the T30 K17 marker was amplified in samples thought to contain only the VT genotype. We also found these heterologous amplification products in some samples. Samples corresponding to Table 1, Column A, Row 47 and Column B, Row 19 show the marker patterns for T30 and VT, respectively, as described by Hilf and Garnsey (5) that include the heterologous amplification products. However, we also found samples that contained only the markers for the given strain, with no heterologous amplification products produced. Samples for Table 1, Column B, Row 37 did not have the T30 K17 marker amplified and may represent a different VT genotype than that described in the Hilf and Garnsey (5) study. Similarly, a sample with a T3 genotype was also found that lacked heterologous amplification products (Table 1, Column B, Row 54).

The wide range in genotype patterns identified in this study may be partially due to fundamental differences in the testing procedure. An immunocapture (IC) RT-PCR assay has been used in most CTV genotyping applications, whereas in this study RT-PCR was performed on TNA. The reasoning for using TNA was that the first polyclonal antibody tested (1212) was incapable of detecting CTV in large percentage of Hawaii's CTV-positive samples, making its use as a capture antibody undesirable (data not shown). We are currently performing IC-RT-PCR on select samples from this study using the polyclonal antibody designated CCPP.

The sequence variation found in the Hawaiian CTV CP sequences supports the level of diversity that was found with the RT-PCR markers. Despite the great deal of diversity found in Hawaiian CTV CP sequences, it is certainly an underestimation of the true diversity present. This is because the PCR-based strategy used to obtain these

sequences may be biased towards the amplification of some CP sequences and not others that may be even more divergent, as the T36CP primer set did not produce amplicons from several samples that were CTV-positive by TBIA.

The control of CTV is essential for citrus to be a successful industry in Hawaii. Cross-protecting CTV strains appear to be established in some citrus production areas in Hawaii; many productive trees in the survey were infected with CTV. Additionally, resistant or tolerant citrus varieties such as pummelo and some mandarins which are popular in local markets are grown productively. There appear to be no CTV-free areas within the islands where quarantine procedures for new plantings could be implemented. Removing infected plants to establish such CTV-free areas would be ineffective, as the BrCA would soon re-infect virus-free plantings. To allow the industry to further expand, however, we feel that additional control strategies must be adopted. The high incidence, distribution, and diversity of CTV in Hawaii documented in this study, however, will make this a challenge.

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