Reassortment of Genome Segments Creates Stable Lineages Among Strains of Orchid Fleck Virus Infecting Citrus in Mexico

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ABSTRACT

The genus *Dichorhavirus* contains viruses with bipartite, negative-sense, single-stranded RNA genomes that are transmitted by flat mites to hosts that include orchids, coffee, the genus *Clerodendrum*, and citrus. A dichorhavirus infecting citrus in Mexico is classified as a citrus strain of orchid fleck virus (OFV-Cit). We previously used RNA sequencing technologies on OFV-Cit samples from Mexico to develop an OFV-Cit-specific reverse transcription PCR (RT-PCR) assay. During assay validation, OFV-Cit-specific RT-PCR failed to produce an amplicon from some samples with clear symptoms of OFV-Cit. Characterization of this virus revealed that dichorhavirus-like particles were found in the nucleus. High-throughput sequencing of small RNAs from these citrus plants revealed a novel citrus strain of OFV, OFV-Cit2. Sequence comparisons with known orchid and citrus strains of OFV showed variation in the protein products encoded by genome segment 1 (RNA1). Strains of OFV

clustered together based on host of origin, whether orchid or citrus, and were clearly separated from other dichorhaviruses described from infected citrus in Brazil. The variation in RNA1 between the original (now OFV-Cit1) and the new (OFV-Cit2) strain was not observed with genome segment 2 (RNA2), but instead, a common RNA2 molecule was shared among strains of OFV-Cit1 and -Cit2, a situation strikingly similar to OFV infecting orchids. We also collected mites at the affected groves, identified them as *Brevipalpus californicus* sensu stricto, and confirmed that they were infected by OFV-Cit1 or with both OFV-Cit1 and -Cit2. OFV-Cit1 and -Cit2 have coexisted at the same site in Toluca, Queretaro, Mexico since 2012. OFV strain-specific diagnostic tests were developed.

Keywords: Brevipalpus, negative sense RNA virus, Rhabdovirus, virus evolution

Leprosis was perhaps the first disease of citrus with viral etiology to have been described, although at the time of its first report in Florida, it was incorrectly attributed to infection by a fungus and called nail-head rust. The disease was first described on sweet orange (*Citrus sinensis* L.) trees in Tampa, Florida but spread statewide. Symptoms began with chlorotic lemon-colored spots on green branches or fruit that developed a necrotic border. Infestations led to severe dieback. Grapefruit (*Citrus paradisi* Macf) was not affected, but losses to sweet orange production reached 60% in groves that were not treated with biocides to control the disease (Fawcett 1911).

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Curiously, the leprosis disease disappeared in Florida in the mid-1960s. This has been attributed to the introduction of a new miticide and a severe freeze that caused the removal of diseased trees (Childers et al. 2003). The similarity of the symptoms to those caused by ringspot viruses led to the proposal that the disease was of viral rather than fungal etiology (Bitancourt 1955). Researchers in Argentina and later, Florida associated mites in the genus *Brevipalpus* with the disease (Knorr 1950, 1968).

Viruses that cause the disease originally described from Florida replicate and form particles in the nucleus of infected cells and belong to the dichorhavirus group. The members of the genus *Dichorhavirus* (family Rhabdoviridae; order Mononegavirales) have a bipartite, single-stranded, and negative-sense RNA genome (Dietzgen et al. 2014, 2017), and the type member of the genus is orchid fleck dichorhavirus (Amarasinghe et al. 2019; Kondo et al. 2006). Genome segment 1 (RNA1; 6,413 nucleotides [nt]) consists of five open reading frames (ORFs) that are thought to encode the capsid protein (N), a phosphoprotein, a movement protein (MP), a matrix protein, and a glycoprotein (G). Genome segment 2 (RNA2; 6,001 nucleotides) has only one ORF that encodes the large protein (L), an RNA-dependent RNA polymerase (Kondo et al. 2006). The genomic structure of dichorhaviruses is very similar to that of the genus *Nucleorhabdovirus* in the family Rhabdoviridae, with substantial sequence homology between ORF1 (N), ORF5 (G), and ORF6 (L). The function of the putative protein encoded by ORF3 is similar to the cell to cell movement protein identified in the unsegmented nucleo- and cytorhabdoviruses infecting plants (Huang et al. 2005; Mann et al. 2016). The dichorhavirus that infects citrus is remarkably similar to orchid fleck virus (OFV) in

gene order, content, and sequence, and it is considered to be a citrus strain of orchid fleck virus (OFV-Cit1) (Amarasinghe et al. 2019; Roy et al. 2015b).

Although dichorhavirus infecting citrus was first reported from Brazil in 1972 (Kitajima et al. 1972), subsequent reports of dichorhavirus from this country have been rare (Bastianel et al. 2010). New and distinct dichorhavirus infecting citrus, orchids, coffee, and clerodendrum have been described in Brazil, including citrus chlorotic spot virus (CiCSV), citrus leprosis virus N (CiLV-N), coffee ringspot virus (CoRSV), and *Clerodendrum* chlorotic spot virus (Amarasinghe et al. 2019; Blanchfield et al. 2001; Chabi-Jesus et al. 2018; Ramos-González et al. 2017). Dichorhavirus that infect citrus also have been reported from Panama (de Dominguez et al. 2001), Mexico (Roy et al. 2015b), Colombia (Roy et al. 2014a), and in 2019, South Africa (Cook et al. 2019). The dichorhavirus strains that are associated with citrus are generally found in groves at relatively high elevation and low humidity (Freitas-Astúa et al. 2018; Roy et al. 2015a). Within the genus, CiLV-N is a virus species present in Brazil, and it is transmitted by *Brevipalpus phoenicis* sensu stricto (Ramos-González et al. 2017). CiLV-N is quite distinct from the dichorhavirus species found in citrus in Mexico and Colombia, which are citrus strains of OFV (Roy et al. 2013c, 2014a, 2015b). OFVs that infect citrus in South Africa are more closely related to subgroup I of the orchid strains of OFV (Cook et al. 2019). Both citrus and orchid strains of OFV are transmitted by *Brevipalpus californicus* (García-Escamilla et al. 2017; Kondo et al. 2003).

The availability of nucleotide sequence data (Roy et al. 2013c) and advances in mite taxonomy (Beard et al. 2015; Sánchez-Velázquez et al. 2015) have revealed a previously unappreciated diversity of virus, vector, and host interactions in dichorhavirus (Bastianel et al. 2010; Roy et al. 2015a). Genome sequence data from a herbarium sample of the original strain of CiLV-N collected in 1948 in Florida revealed that it was a distinct virus. This virus was named CiLV-N0, because it was the first of the nuclear-type leprosis viruses to be reported (Hartung et al. 2015). CiLV-N0 is recognized as a distantly related strain of OFV. Nucleotide sequence data have also been obtained from several citrus strains of dichorhavirus from Mexico (Roy et al. 2014b) as well as from other dichorhavirus that cause distinct diseases in Brazil reported as CiLV-N and CiCSV (Chabi-Jesus et al. 2018). Sequence diversity in orchid strains of dichorhavirus has also been used to recognize two subgroups of strains of OFV designated as orchid fleck virus subgroup I (OFV-Orc1) and orchid fleck virus subgroup II (OFV-Orc2) (Kondo et al. 2017).

In this report, we describe a novel dichorhavirus from citrus in Mexico (OFV-Cit2) in which the genome sequence of RNA1 is related to but clearly distinct from the previously described strain of OFV from citrus (OFV-Cit1) (Roy et al. 2015b) and other dichorhavirus reported from citrus (Chabi-Jesus et al. 2018; Ramos-González et al. 2017). However, the RNA2 genome segments of OFV-Cit1 and -Cit2 are nearly identical, and they are apparently derived by genomic reassortment from other strains of OFV. Analysis of the available dichorhavirus sequence data suggested that sensitive reverse transcription PCR (RT-PCR) and its downstream assays would be useful to distinguish these viruses, and we report the development of these assays.

MATERIALS AND METHODS

Collection of plant and mite samples. The OFV-Cit-affected citrus grove and residential citrus trees were identified in Toluimán, Querétaro, Mexico located at 21°03'08"N, 99°49'069"W and 20°53'35.9"N, 99°54'59.5"W at altitudes of 1,369 and 1,607 m above sea level, respectively. Both plant and flat mite samples were collected from these locations (Table 1 and Supplementary Table S1). Brevipalpid mites from the leprosis-infected citrus orchard were sent to Beltsville for taxonomic identification and molecular

identification of possible viruses. The identification of mites was made using differential interference contrast microscopy with a Zeiss AxioScope and low-temperature scanning electron microscopy (Bolton et al. 2014).

Transmission electron microscopy of OFV-Cit2 suspect leaf samples. Leaf tissue was cut from the yellow halo surrounding chlorotic and necrotic leprosis leaf lesions and from healthy leaf samples. Tissues were fixed in 3% glutaraldehyde in Sorenson's 0.066 M phosphate buffer, pH 6.8, for 24 h at 4°C following the protocol described by Roy et al. (2015b). The sections were placed on 200-mesh Formvar-coated copper grids and stained with 2% aqueous uranyl acetate followed by Reynold's lead citrate. Observations and micrographs were done on a Morgagni 268 transmission electron microscope (FEI Company).

RT-PCR assay for detection of citrus strains of OFV from leaf tissue and mites. Healthy and leprosis symptomatic mandarin (*Citrus reticulata*), grapefruit (*Citrus × paradisi*), lime (*Citrus aurantiifolia*), sweet or navel lime (*Citrus limetta*), Persian lime (*Citrus × latifolia*), Mexican and royal lemon (*Citrus × limon*), sour orange (*Citrus aurantium*), and Valencia sweet orange (*Citrus × sinensis*) leaves were collected in Toluimán, Querétaro, Mexico in 2012, 2015, and 2019. OFV-Cit1 was detected in samples from multiple citrus species in 2012. These samples were archived and included in this study. Additional symptomatic lesions were excised using stainless steel razor blades, ground in liquid nitrogen, and preserved at -80°C for downstream applications. Fifty-four OFV/CiLV-N suspect leaf samples were selected for RNA extraction with the RNeasy Plant Mini Kit (Qiagen). All samples were analyzed for the presence of known citrus-infecting cilevirus, higrevirus, and dichorhavirus following previously described RT-PCR protocols (Roy et al. 2013a, 2015b). Sets of generic primers for citrus and orchid strains of OFV were designed after aligning the RNA2 segment of all known OFVs and utilized to detect strains of OFV infecting citrus in Mexico (R2-Dicho-GF: ¹⁰²⁶ATC TGT GTM GGT GAG ATA ATC AAC AGG¹⁰⁵² and R2-Dicho-GR: ¹⁴²¹GAC CTG CTT GAT GTA ATT GCT TCC TTC¹³⁹⁵) using the previously published RT-PCR protocol for detection of CiLV-N (Roy et al. 2015b).

A minimum of 10 mites collected from leprosis-symptomatic citrus in the Toluimán region of Querétaro state in 2015 and 2019 were frozen in liquid nitrogen and crushed, and total RNA was purified using the RNeasy fibrous tissue mini kit (Qiagen) following the manufacturer's protocol. RNA was eluted in 15 µl of elution buffer, and RNA yield varied from 5 to 20 ng/µl depending on the number of mites included in the extract process. The RNA was quantified with a NanoDrop ND-1000 spectrophotometer and stored at -20°C. OFV generic and strain-specific primers were used in an RT-PCR assay to detect OFV in the mites (Roy et al. 2015b).

Determination of the complete viral genome sequence using high-throughput sequencing of small RNAs, overlapping, and rapid amplification of complementary DNA ends RT-PCR. Suspected OFV-Cit1 symptomatic sour orange (S9), sweet lime (QR020), and sweet orange (QR017 and QR024) leaf samples that were negative (S9, QR017, and QR024) and positive (QR020) in OFV-Cit1-specific RT-PCR assays were selected (Table 1). Total RNA was isolated from these four isolates using the RNeasy Mini Kit (Qiagen). RNA yield and quality were determined using the Qubit 3.0 Fluorometer (Life Technologies/Invitrogen) and the Tape Station system (Agilent). RNA integrity numbers (RINs) were calculated. Fifty microliters of RNA having RINs between five and eight was sent to Genewiz for high-throughput sequencing using an Illumina HiSeq platform. The four libraries contained 15- to 45-base pair fastq reads and were analyzed using a subtractive approach (Roy et al. 2013b) with the alignment tool Bowtie (Langmead et al. 2009). The Velvet/Oases programs (Zerbino and Birney 2008) were used to assemble the sequences into longer contigs with the default k-mer of 15 (Roy et al. 2015b).

BlastX (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) was used to compare the assembled contigs with the nonredundant protein database at the NCBI GenBank.

To amplify nucleotides in sequence gaps between adjacent contigs, OFV-Cit2-specific primers were designed, and overlapping amplicons were produced by RT-PCR following a previously described protocol (Roy and Brilansky 2010). The production of amplicons approximately 1.1 to 1.6 kilobases in length with an overlap of 153 to 1,051 nucleotides (Supplementary Table S2) between adjacent contigs eliminated the assembly of mosaic sequences of different OFV strains.

Sequence-specific rapid amplification of complementary DNA ends (RACE) primers were designed to amplify the 5'- and 3'-UTR sequences of OFV-Cit2-RNA1 (Supplementary Table S2). Published RACE primers were used to determine the untranslated regions (UTR) sequences of OFV-Cit2 RNA2 (Roy et al. 2015b). The RNA ligase-mediated RACE kit (Thermo Fisher Scientific) was used, and 5'- and 3'-UTR-encoding amplicons were obtained. Inner UTR RACE amplicons were cloned in the TOPO TA cloning vector (Thermo Fisher Scientific), and 8 to 10 clones for each terminal amplicon were bidirectionally sequenced. Primers for RT-PCR were designed to completely amplify genome segment RNA1 of OFV-Cit2 and are listed in Supplementary Table S2. Genome segment RNA2 was amplified using primers previously developed for OFV-Cit1 (Roy et al. 2015b).

Phylogenetic and statistical analyses. To determine the phylogenetic relationships among dichorhavirus, an evolutionary analysis was conducted using the maximum likelihood method and Tamura-Nei model with the MEGA X program (Kumar et al. 2018; Tamura and Nei 1993). The calculated phylogeny was tested with the maximum composite likelihood (MCL) approach using the nearest neighbor interchange algorithm parameter and 1,000 bootstrap replications. The best tree topology was selected based

on a superior log likelihood value. The trees were drawn to scale, with branch lengths measured as the number of substitutions per site and the percentage of trees in which the associated taxa clustered together shown at the branch points. The net evolutionary divergence among four groups of OFV sequences was estimated with MEGA X using the MCL model (Tamura et al. 2004). All results were based on pairwise analysis of eight sequences after removing all ambiguous positions for each sequence pair. The net average divergence among four groups of OFV sequences was estimated utilizing the number of base substitutions per site.

Tajima's test of neutrality was conducted in MEGA X to determine if the DNA sequences evolved by random (neutral) or purposive (directional or balancing) selection (Tajima 1989). The population genetics parameter for the number of segregating sites and the nucleotide diversity within the population of OFV was also estimated using Tajima's test of neutrality. In general, when the number of samples included in the analysis is small, the correlation between these two estimates will be large and will decrease slowly as the sample size increases. A similar approach using Tajima's test statistic (*D*) analysis protocol was followed previously to determine the genetic variation among citrus tristeza virus genotypes (Roy and Brilansky 2010). A negative *D* value among the different coding regions signifies an excess of low-frequency polymorphism, whereas a positive *D* value signifies low levels of both low- and high-frequency polymorphisms.

Recombination analyses. Full-genome sequences of 15 dichorhavirus were included for recombination/reassortment analyses using the Recombination Detection Program v.4.97 (RDP4) with default parameter values (Martin et al. 2015). The bipartite or concatenated genomes of the dichorhavirus studied were aligned with MAFFT version 7 with default parameters (Katoh and Stanley 2013). Aligned genomes were subjected to

TABLE 1. Summary of collection sites, hosts, isolates, and infection types of samples used in this study^a

Year and collection site	Isolate	Host	OFV-Cit1 RT-PCR		Viruses detected ^b	Type of infection	Confirmative assays
			RNA1	RNA2			
2012							
Barrio del Molino, Toliman, Querétaro	S9	Sour orange	Negative	Positive	OFV-Cit2	Single	RT-PCR, RFLP, Sanger sequencing, and HTS
Barrio del Molino, Toliman, Querétaro	S13	Sweet orange	Negative	Positive	OFV-Cit2	Single	RT-PCR, RFLP, and Sanger sequencing
Toliman, Querétaro	M6	Sour orange	Positive	Positive	OFV-Cit1 and -Cit2	Mixed	RT-PCR, RFLP, and Sanger sequencing
Toliman, Querétaro	N9	Sweet orange	Negative	Positive	OFV-Cit2	Single	RT-PCR, RFLP, and Sanger sequencing
Toliman, Querétaro	N10	Sweet orange	Negative	Positive	OFV-Cit2	Single	RT-PCR, RFLP, and Sanger sequencing
2015							
Colon, Querétaro	QR007	Grapefruit	Positive	Positive	OFV-Cit1 and -Cit2	Mixed	RT-PCR, RFLP, and Sanger sequencing
San Pablo, Toliman, Querétaro	QR008	Lime	Positive	Positive	OFV-Cit1 and -Cit2	Mixed	RT-PCR, RFLP, and Sanger sequencing
San Pablo, Toliman, Querétaro	QR009	Sour orange	Positive	Positive	OFV-Cit1 and -Cit2	Mixed	RT-PCR, RFLP, and Sanger sequencing
Colon, Querétaro	QR010	Royal lemon	Positive	Positive	OFV-Cit1 and -Cit2	Mixed	RT-PCR, RFLP, and Sanger sequencing
Barrio del Molino, Toliman, Querétaro	QR017	Sweet orange	Negative	Positive	OFV-Cit2	Single	RT-PCR, RFLP, Sanger sequencing, and HTS
Horno de Cal, Toliman, Querétaro	QR020	Sweet lime	Positive	Positive	OFV-Cit1 and -Cit2	Mixed	RT-PCR, RFLP, Sanger sequencing, and HTS
Horno de Cal, Toliman, Querétaro	QR022	Sweet lime	Positive	Positive	OFV-Cit1 and -Cit2	Mixed	RT-PCR, RFLP, and Sanger sequencing
2019							
Barrio del Molino, Toliman, Querétaro	QR024	Sour orange	Negative	Positive	OFV-Cit2	Single	RT-PCR, RFLP, Sanger sequencing, and HTS
Barrio del Molino, Toliman, Querétaro	QR025	Sweet orange	Negative	Positive	OFV-Cit2	Single	RT-PCR, RFLP, and Sanger sequencing
Rancho del Molino, Toliman, Querétaro	QR026	Sour orange	Negative	Positive	OFV-Cit2	Mixed	RT-PCR, RFLP, and Sanger sequencing

^a HTS, high-throughput sequencing; OFV-Cit, citrus strain of orchid fleck virus; RFLP, restriction fragment-length polymorphism; RNA1, genome segment 1; RNA2, genome segment 2.

^b All samples were tested by reverse transcription PCR (RT-PCR) for cileviruses CiLV-C and CiLV-C2 (Roy et al. 2013a, b, c). Cilevirus sequences were not detected in any sample.

putative recombination/reassortment analyses using specific MAS and PIR file formats and used as input for Gblocks Version 0.91b to eliminate poorly aligned positions and divergent regions of aligned DNA sequence. Sequences were trimmed with Gblocks and used for analysis with the RDP4 package of programs. To identify the most probable unique recombination events among dichorhavirus, all sequences studied were analyzed with several recombination analysis programs, including RDP (Martin et al. 2015), GENECONV (Sawyer 1999), Bootscan (Salminen et al. 1995), MaxChi (Smith 1992), Chimera (Posada and Crandal 2001), SiScan (Gibbs et al. 2000), and 3SEQ (Boni et al. 2007). Lineages of recombinant genomes were identified with RDP4 when the two most closely related parental sequence regions were amalgamated to form a recombinant (daughter) sequence. If the

same recombination event was detected by at least three of the above programs, the event was considered as positive evidence for recombination (Kondo et al. 2017; Roy and Brlansky 2010).

RT-PCR/restriction fragment-length polymorphism. Sets of generic forward (OFV-NP-GF: ²²⁸ATGGCTAACCCAAGT GAGATMGATTAC²⁵³) and specific reverse primers (Cym07-OFV-Gr1-NP-R: ¹⁵⁵⁴TTATTCATTGTTTCATGGCATCCAGTGC¹⁵⁸⁰; So-OFV-Gr2-NP-R: ¹⁵⁵⁸TCAGTCACTGTTTCATGGCATCCA¹⁵⁸⁰; M2345-OFV-Gr3-NP-R: ¹⁵⁵⁵TCATTGCTGTTCATAGCATC TAGGG¹⁵⁸⁰; and S9-OFV-Gr4-NP-R: ¹⁵⁵⁷TCACTCGCTGTT CATAGCATCCAG¹⁵⁸⁰) for strains of OFV from orchids and citrus were designed using the nucleocapsid (N) gene region. These were used in standard RT-PCR assays for detection and differentiation of OFV-Cit2 from OFV-Cit1 and other citrus-associated

Fig. 1. Citrus leprosis disease symptoms in the state of Querétaro in Mexico are associated with single infection of **A**, orchid fleck virus citrus strain 1 (OFV-Cit1) mandarin, **B**, OFV-Cit2 sweet orange (isolate QR025) and **C**, sour orange (QR024), and mixed infection of **D**, OFV-Cit1 and -Cit2 sour orange (QR026), **E**, grapefruit (QR007), **F**, royal lemon (QR010), **G**, lime (QR008), and **H**, sweet lime (QR020).

dichorhavirus in single and mixed infections (Roy et al. 2013a, 2015b). To confirm the identity of the virus(es) associated with the leprosis symptoms, the amplicons produced by RT-PCR were sequenced, blasted, and aligned with other dichorhavirus sequences available in GenBank. Unique restriction sites that could be used to differentiate OFV strains were identified using all complete N gene sequences from OFV strains available from GenBank. Gene sequences from all four strains of OFV (two from citrus and two from orchids) were mapped in silico using Webcutter 2.0 to create restriction maps. A 1,353-nt amplicon obtained from OFV-Orc1, OFV-Orc2, OFV-Cit1, and OFV-Cit2 strains was amplified as described (Roy et al. 2015b). The amplicons were purified using a PCR purification kit (Qiagen). To confirm the presence of restriction sites identified by in silico analysis, restriction digests were performed in two separate tubes with *Bam*HI and *Pst*II

Fig. 2. Virions in the nucleus of symptomatic leaf samples. **A**, Low magnification showing the nucleus (N) and nucleolus (Nu) with numerous virus particles (vp). **B**, Higher magnification of the boxed area of the same nucleus.

endonucleases and subjected to electrophoresis through 2% agarose gels. Fragment sizes were estimated by comparison with 1Kbp plus MW markers (Life Technologies). The remaining RT-PCR products were purified and sent for DNA sequencing.

RESULTS AND DISCUSSION

We had previously described the first complete genome sequence of dichorhavirus OFV-Cit1 (isolate M2345) from Toliman, Querétaro state in Mexico (Roy et al. 2013c). Samples were collected in 2019 from the same locations sampled previously in 2012 and 2015 and archived to further validate previously published OFV-Cit1-specific and newly developed generic OFV RT-PCR assays (Table 1). Symptomatic foliage was collected from eight species of citrus in Toliman, Querétaro. We noted that, although the symptoms of leprosis were clear on all citrus species, the symptoms observed in 2012 (relatively narrow chlorotic halos surrounding relatively larger necrotic lesions) (Fig. 1A) (Roy et al. 2015b) differed from the symptoms in 2015 and 2019 (Fig. 1B to H). Symptoms on green stems and fruit were not observed.

Symptomatic samples S9 and QR024 from sour orange (*C. aurantium* L) and S13 from sweet orange (*C. sinensis*) did not produce amplification products after RT-PCR as expected for OFV-Cit1 (data not shown). Because these samples failed to produce amplification products with OFV-Cit1-specific primers, samples were prepared for transmission electron microscopy. Transmission electron micrographs revealed the presence of numerous bullet-shaped virions in the nuclei of cells examined from lesions from samples S9 and S13 (Fig. 2). The particles ranged from 29 to 44 nm in width to 110 to 120 nm in length. No virions were present in the cytoplasm and endoplasmic reticulum as previously reported with cilevirus (Roy et al. 2013a).

Fig. 3. Schematic diagram of the genomic organization of orchid strains of orchid fleck virus (OFV; OFV-Orc1 and -Orc2) and citrus strains of OFV (OFV-Cit1 and -Cit2). Black denotes intergenic regions and blue denotes 5' and 3' UTRs.

To explore the possibility of variation among OFV-Cit1 isolates, RT-PCR assays were performed to amplify the OFV-Cit1 genome segments RNA1 and RNA2. Interestingly, although primers for OFV-Cit1 genome segment RNA2 produced expected amplicons,

primers for genome segment RNA1 failed to produce amplicons from symptomatic leaf samples S9, S13, and QR024. New generic OFV-RNA1 primers were developed and produced amplicons that were sequenced. Specific primers were then designed to fill the gaps

A

RNA1:	Reverse complementary negative strand sequence					Complementary RNA strand sequence					Region
		Elements: I	II	III		Elements: I	II	III			
OFV-Cit2:	1415' TACACG	ATATAAACAAAA	CAAC	AAG	T3' 166	AUGUGC	UAUUAUUUGUUU	GUUG	UUC	A	3' -/ORF1
OFV-Cit1:	1415' TACATG	ATGTAACAAAA	CAAC	AAG	T3' 166	AUGUAC	UACAUUUUGUUU	GUUG	UUC	A	3' -/ORF1
OFV-Orc1:	1415' TACACG	ATGTAACAAAA	CAAC	AAG	T3' 166	AUGUGC	UACAUUUUGUUU	GUUG	UUC	A	3' -/ORF1
OFV-Orc2:	1415' TACATG	ATGTAACAAAA	CAAC	AAG	T3' 166	AUGUAC	UACAUUUUGUUU	GUUG	UUC	A	3' -/ORF1
OFV-Cit2:	16455' GTGTGC	ATTTAAATAAAA	CATC	AAC	A3' 1668	CACACG	UAAAUUUUUUUU	GUAG	UUG	U	ORF1/ORF2
OFV-Cit1:	16355' GTGTGC	ATTTAAATAAAA	CAAC	AAC	A3' 1660	CACACG	UAAAUUUUUUUU	GUUG	UUG	U	ORF1/ORF2
OFV-Orc1:	16375' ATATGC	ATTTAAATAAAA	CATC	AAC	A3' 1662	UAUACG	UAAAUUUUUUUU	GUAG	UUG	U	ORF1/ORF2
OFV-Orc2:	16345' GCGTGC	ATTTAAATAAAA	CAAC	AAC	A3' 1659	CGCACG	UAAAUUUUUUUU	GUUG	UUG	U	ORF1/ORF2
OFV-Cit2:	25845' ACATGC	ATTTAAATAAAA	CCTC	AAC	C3' 2608	UGUACG	UAAAUUUUUUUU	GGAG	UUG	G	ORF3
OFV-Cit1:	25805' ACATGC	ATTTAAATAAAA	CATC	AAC	T3' 2605	UGUACG	UAAAUUUUUUUU	GUAG	UUG	A	ORF3
OFV-Orc1:	25775' ACATGC	ATTTAAATAAAA	CATC	AAT	A3' 2608	UGUACG	UAAAUUUUUUUU	GUAG	UUA	U	ORF3
OFV-Orc2:	25755' TCATGC	ATTTAAATAAAA	CATC	AAC	T3' 2600	AGUACG	UAAAUUUUUUUU	GUAG	UUG	A	ORF3
OFV-Cit2:	37655' ATCGTA	ATTTAAATAAAA	CAAC	AAT	C3' 3790	UAGCAU	UAAAUUUUUUUU	GUUG	UUA	G	ORF3/ORF4
OFV-Cit1:	37605' ACCATA	ATTTAAATAAAA	CAAC	AAT	C3' 3785	UGGUAU	UAAAUUUUUUUU	GUUG	UUA	G	ORF3/ORF4
OFV-Orc1:	37575' ATCGTG	ATTTAAATAAAA	CAAC	AAC	C3' 3782	UAGCAC	UAAAUUUUUUUU	GUUG	UUG	G	ORF3/ORF4
OFV-Orc2:	37555' ACCGTA	ATTTAAATAAAA	CAAC	AAT	C3' 3780	UGGCAU	UAAAUUUUUUUU	GUUG	UUA	G	ORF3/ORF4
OFV-Cit2:	45475' CCCCGG	ATTTAAATAAAA	CATC	AAT	G3' 4572	GGGGCC	UAAAUUUUUUUU	GUAG	UUA	C	ORF4/ORF5
OFV-Cit1:	45325' ACCCTT	ATTTAAATAAAA	CAAT	AAT	G3' 4557	UGGGAA	UAAAUUUUUUUU	GUUA	UUA	C	ORF4/ORF5
OFV-Orc1:	45455' GTTTGG	ATTTAAATAAAA	CAAC	AAT	G3' 4570	CAAAAC	UAAAUUUUUUUU	GUUG	UUA	C	ORF4/ORF5
OFV-Orc2:	45325' AATTGG	ATTTAAATAAAA	CAAT	AAT	G3' 4552	UUAUAC	UAAAUUUUUUUU	GUUA	UUA	C	ORF4/ORF5
OFV-Cit2:	62695' AATAAA	ATTTAAATAAAA	AAAA	AAA	A3' 6294	UUAUUU	UAAAUUUUUUUU	UUUU	UUU	U	ORF5/-5'
OFV-Cit1:	62535' TAATAA	ATTTAAATAAAA	AAAA	AAA	A3' 6278	AUUUAU	UAAAUUUUUUUU	UUUU	UUU	U	ORF5/-5'
OFV-Orc1:	62665' CAATAA	ATTTAAATAAAA	CCAC	CAT	C3' 6291	GUUAUU	UAAAUUUUUUUU	GGUG	GUA	G	ORF5/-5'
OFV-Orc2:	62485' TGATAA	ATTTAAATAAAA	CTAT	CTC	T3' 6273	ACUAUU	UAAAUUUUUUUU	GAUA	GAG	A	ORF5/-5'

RNA2:	Reverse complementary negative strand sequence					Complementary RNA strand sequence					Region
		Elements: I	II	III		Elements: I	II	III			
OFV-Cit2:	1165' TACACG	GATCATGCAAAA	CAACC	AAA3' 141	AUGUGC	CUAGUACGUUUU	GUUGG	UUU		3' -/ORF6	
OFV-Cit1:	1165' TACACG	GATCATGCAAAA	CAACC	AAA3' 141	AUGUGC	CUAGUACGUUUU	GUUGG	UUU		3' -/ORF6	
OFV-Orc1:	1165' TTCACG	GATCATGCAAAA	CAACC	AAC3' 141	AAUGUC	CUAGUACGUUUU	GUUGG	UUG		3' -/ORF6	
OFV-Orc2:	1165' TTCACG	GATCATAAAAA	CAACC	AAC3' 141	AAUGUC	CUAGUAUUUUU	GUUGG	UUG		3' -/ORF6	
OFV-Cit2:	58345' ATATTC	ATTTAAGGAAAA	AAAAA	AAA3' 5859	UAUAAG	UAAAUUCCUUUU	UUUUU	UUU		ORF6/-5'	
OFV-Cit1:	58345' ATATTC	ATTTAAGGAAAA	AAAAA	AAA3' 5859	UAUAAG	UAAAUUCCUUUU	UUUUU	UUU		ORF6/-5'	
OFV-Orc1:	58345' ATATAC	ATTTAAGGAAAA	CCGGG	TAT3' 5859	UAUAUG	UAAAUUCCUUUU	GGCCC	AUA		ORF6/-5'	
OFV-Orc2:	58345' ATATAC	ATTTAAGGAAAA	CCGGG	TAT3' 5859	UAUAUG	UAAAUUCCUUUU	GGCCC	AUA		ORF6/-5'	

B

Virus		Consensus Sequence		
		Elements: -I	-II	-III
OFV-Cit2	RNA1:	UAAAUUUUUUUU	GU (A/U) G	UUN
	RNA2:	UAAAUUCCUUUU	GUUGG	UUU
OFV-Cit1	RNA1:	UAAAUUUUUUUU	GUUG	UUN
	RNA2:	UAAAUUCCUUUU	GUUGG	UUU
OFV-Orc1	RNA1:	UAAAUUUUUUUU	GU (U/A) G	UU (A/G)
	RNA2:	UAAAUUCCUUUU	GUUGG	(U/A) U (G/A)
OFV-Orc2	RNA1:	UAAAUUUUUUUU	GUU (G/A)	UU (G/A)
	RNA2:	UAAAUUCCUUUU	GUUGG	(U/A) U (G/A)
CiLV-N	RNA1:	UAAAUUUUUUUU	GU (A/U) G	UU (A/G) (C/U)
	RNA2:	UAAAUUUUUUUU	GAUU	UUUG
CiCSV	RNA1:	UAAAUUUUUUUU	G (U/G) AG	UUCU
	RNA2:	UAAAUUAGUUU	GUAC	ND

Fig. 4. A, Alignment and comparison of the gene junction sequences among the orchid strains of orchid fleck virus (OFV; OFV-Orc1 and -Orc2) and citrus strains of OFV (OFV-Cit1 and -Cit2) genome segment 1 (RNA1) and genome segment 2 (RNA2). Conserved nucleotide sequences of elements I (transcription termination polyadenylation signal), II (intergenic region), and III (transcription start site) are highlighted. Start codon AUG is indicated with bold underlined letters. **B**, Comparison of the consensus sequences of OFV-Cit2 with other dichorhavirus infecting citrus and orchid. Consensus sequences included citrus leprosis virus N (CiLV-N), OFV-Orc and OFV-Cit strains, and citrus chlorotic spot virus (CiCSV). Nucleotide positions of the reference sequences are based on the following accession numbers: LC222629 (OFV-Orc1), AB244417 (OFV-Orc2), KF209275 (OFV-Cit1), and MK578000 (OFV-Cit2). ND, not determined; ORF, open reading frame.

and determine the complete sequence of the ORFs of the OFV-Cit2 genome segment RNA1. The 5'- and 3'-UTR sequences were determined using RACE RT-PCR. To demonstrate that leprosis samples maintained the same genome segment RNA2 sequences over time, small RNA sequencing was conducted using sample S9 collected in 2012, samples QR017 and QR020 collected in 2015, and sample QR024 collected in 2019 (Table 1).

The nucleotide sequences of genome segments RNA1 and RNA2 of OFV-Cit1 and -Cit2 were compared with those of orchid strains of OFV (OFV-Orc1 and -Orc2). The lengths of the 5'-UTR and ORF1, ORF2, and ORF4 of OFV-Cit2 RNA1 sequences were identical to all known OFVs, whereas ORF3 (MP) was 6 nucleotides shorter and ORF5 (G) was 78 to 108 nucleotides longer than corresponding ORFs of OFV-Cit1 and OFV-Orc strains. The length of the OFV-Cit2 genome segment RNA2 was identical to that of OFV-Cit1 and 141 nucleotides shorter than the corresponding OFV-Orc sequences. The differences in the overall lengths of genome segments RNA1 and RNA2 between OFV-Cit1 and -Cit2 were accounted for by variations in the gene junction regions between the ORFs and 12 nucleotides at the 3'-UTR (Fig. 3). Each gene junction consisted of three elements (I, II, and III) that denote the polyadenylation signal, the intergenic spacer region, and the putative gene transcription start site (Dietzgen

et al. 2018). Comparison of the consensus sequences of all three elements among strains of OFVs and other dichorhavirus infecting citrus is shown (Fig. 4). Elements of segment RNA2 are more conserved than the elements of segment RNA1. The genome sequences of the novel citrus strain, designated OFV-Cit2 from Querétaro state, Mexico, were submitted to the NCBI GenBank under accession numbers MK578000 (RNA1) and MK578001 (RNA2).

The relationship of OFV-Cit2 to other dichorhavirus from citrus was estimated using the nucleotide sequence data for genome segments RNA1 and RNA2 (Table 2). The nucleotide sequence identity of the five ORFs of RNA1 of OFV-Cit2 with OFV-Cit1 and the orchid viruses OFV-Orc1 and -Orc2 varied from 74 to 97%. The entire molecule of OFV-Cit2 RNA1 shared 80% nucleotide identity with RNA1 of OFV-Orc1 (isolate Cym07), 81 to 82% with OFV-Orc2 (isolate So), and 78 to 81% with OFV-Cit1 (isolate M2345). The amino acid sequences encoded by the ORFs of genome segment RNA1 were more conserved than the nucleotide sequences. Nucleotide sequence variation has been shown in the RNA1 genome segment of strains OFV-Orc1 and -Orc2, but the nucleotide sequence of genome segment RNA2 of OFV-Orc1 and -Orc2 was 99% identical (Kondo et al. 2017). Highly conserved nucleotide sequences were also observed between the RNA2

TABLE 2. Percentages of nucleotide and amino acid identities of citrus strain 2 of orchid fleck virus (OFV-Cit2) genome segment 1 (RNA1) and genome segment 2 (RNA2) with other dichorhavirus infecting orchid and citrus

	Nucleotide position	OFV-Cit1 ^a	OFV-Orc1	OFV-Orc2	CiLV-N	CiCSV
OFV-Cit2 RNA1 ^b						
ORF-1/N	228–1580	83/96–97	83/96	86/97	55/49–50	54/50
ORF-2/P	1737–2450	86/96	86/96	87/95	46–47/35	44/34
ORF-3/MP	2691–3698	77/83	85/92	87/95	59/60	59/54
ORF-4/M	3840–4391	80–81/92–94	83–84/94–95	86/95–96	46–47/35–37	46/40
ORF-5/G	4492–6228	74–75/83	74/82	76/82–83	38–40/31–32	41/29
All RNA1	1–6296	78–81/–	80/–	81–82/–	46–47/–	45/–
OFV-Cit2 RNA2						
ORF-6/L	183–5816	96–98/97/99	89–90/97	89/97	59/57	59/58
All RNA2	1–5860	93–99/–	87–88/–	87/–	56/–	57/–

^a Subgroups and isolates used in the analysis: orchid strain 1 of orchid fleck virus (OFV-Orc1) includes citrus isolate CL and orchid isolates Cym07 and Br. OFV-Orc2 includes orchid isolates So and NHHS1. OFV-Cit1 includes citrus isolate M2345, and OFV-Cit2 includes citrus isolate S9. Citrus leprosis virus N (CiLV-N) includes isolates MAS1 (KY751404 and KY751405), Ibi (KX982176 and KX982179), SRq (KX982177 and KX982180), and SBS (KX982178 and KX982181). Citrus chlorotic spot virus (CiCSV) includes isolate Trs1 (KY700685 and KY700686).

^b Nucleotide/amino acid homologies for open reading frame 1 (ORF1) to -6 encoding the nucleoprotein (N), phosphoprotein (P), movement protein (MP), matrix protein (M), glycoprotein (G), and large protein (L), respectively.

Fig. 5. Phylogenetic relationships among dichorhavirus infecting citrus, orchid, the genus *Clerodendrum*, and coffee based on the nucleotide sequences of **A**, genome segment 1 (RNA1) and **B**, genome segment 2 (RNA2). CiCSV, citrus chlorotic spot virus; CiLV-N, citrus leprosis virus N; CICSV, *Clerodendrum* chlorotic spot virus; CoRSV, coffee ringspot virus; OFV-Cit, citrus strain of orchid fleck virus; OFV-Orc, orchid strain of orchid fleck virus.

molecules of OFV-Cit2 and -Cit1. Therefore, for all strains of OFV tested from citrus and orchids, the nucleotide sequence of genome segment RNA1 was variable (78 to 82% identity). However, the nucleotide sequence of genome segment RNA2 is divergent between the citrus and orchid strains of OFV at only 87 to 88% nucleotide sequence identity (Table 2). The CiLV-N and CiCSV dichorhavirus from citrus in Brazil were very well separated from the viruses in the OFV group, with only 45 to 47% sequence identity

for segment RNA1 and 56 to 57% sequence identity for segment RNA2.

Citrus strains of dichorhavirus from Brazil, designated CiLV-N (Ramos-González et al. 2017), were included in a separate analysis based on nucleotide sequence data of genome segments RNA1 and RNA2. All members of the OFV group clustered together, whether isolated from citrus or orchids, and were clearly separated from the newly described CiLV-N isolates from Brazil regardless of whether

Fig. 6. Phylogenetic relationships among orchid strains of orchid fleck virus (OFV; OFV-Orc) and citrus strains of OFV (OFV-Cit) based on the nucleotide sequences of the nucleocapsid protein.

the analysis was based on RNA1 or RNA2 (Fig. 5). CiCSV and CoRSV, also reported from Brazil, were well phylogenetically distinct from other members of the dichorhavirus group. The results were the same when nucleotide sequence data from the nucleoprotein, glycoprotein, or L protein were analyzed separately (Supplementary Fig. S1). Phylogenetic analyses were performed with all isolates of OFV available in GenBank based on partial nucleocapsid sequence data (ORF1/RNA1). Four distinct clades or subgroups were apparent in the phylogenetic tree representing the orchid and citrus strains of OFV (Fig. 6).

Genetic variation within orchid and citrus strains of OFV. The net evolutionary divergence among the four subgroups of OFV was estimated with the bootstrap approach and MCL analysis. To do this, the average nucleotide distances among sequences from the six genomic regions between the orchid and citrus strains of OFV were analyzed pairwise (Table 3). The average nucleotide distance for RNA1 between OFV-Cit2 subgroup IV and OFV-Orc2 subgroup II (0.2036) was the most distant relationship among the subgroups. Among the five ORFs of OFV-Cit2 RNA1 (subgroup IV), the average nucleotide distance of ORF5 sequences encoding glycoprotein was considerably greater than for other ORFs regardless of the subgroups of OFV compared (Table 3). The lowest average distance among the subgroups of OFV RNA1 was observed for ORF2 encoding phosphoprotein. The average intra-subgroup distances for genome segment RNA2 among orchid strains (subgroups I and II) and citrus strains (subgroups III and IV) were 0.0004 and 0.0048, respectively, which are very close to zero. The inter-subgroup average nucleotide distances for the RNA2 genome segment of OFV infecting orchids and citrus was greater (between 0.0904 and 0.1051), at least 21-fold greater than between other subgroups (I versus IV, II versus IV, I versus III, and II versus III) (Table 3).

Eight complete genome sequences were included for phylogenetic analyses of OFVs infecting orchids and citrus (Table 4). The total number of segregating sites and the average number of mutations between nucleotide pairs in the sample were compared utilizing Tajima's neutrality test. The nucleotide diversity (π) values among the eight complete OFV RNA1 and RNA2 genome segments were

0.1232 and 0.0591, respectively. Among the five ORFs of RNA1, ORF5 showed the highest proportion of segregating sites (p_s ; 0.3156), highest Watterson's estimator value (Θ ; 0.1217), and highest nucleotide diversity (π ; 0.1474) compared with ORF1 to ORF4, consistent with the MCL analysis (Table 4). ORF6 of the RNA2 genome segment showed the lowest proportion of segregating sites (p_s ; 0.1431) with least nucleotide diversity (π ; 0.0629) (Table 4). Tajima's D value for genome segment RNA1 was 1.196245, and for genome segment RNA2, it was 0.719226 when the eight complete OFV genomes were analyzed. The consistently positive value of Tajima's D for the six ORFs was significant at the 95% confidence level, indicating low levels of polymorphism. The mean evolutionary diversity of the entire population was estimated as 0.16.

Reassortment of genome segments among strains of OFV. The possibility of recombination events in the RNA1 and RNA2 genome segments of citrus strains (OFV-Cit1 and -Cit2) as observed previously for OFV orchid strains (OFV-Orc1 and -Orc2) (Kondo et al. 2017) was investigated using RDP4 (Martin et al. 2015). Concatenated sequences of genome segments RNA1 and RNA2 of dichorhavirus isolates that infect orchids, citrus, coffee, and the genus *Clerodendrum* were included for reassortment analysis. No recombination events were detected within either RNA1 or RNA2 of OFV-Cit2 and other OFV isolates that infect citrus and orchids. Two significant recombination events were detected among the isolates of CiLV-N (Fig. 7 and Table 5). The region close to the RNA1–RNA2 junctions in the concatenated sequences was anticipated for possible reassortment breakpoints. A similar location was identified for the reassortment phenomenon between the OFV-Orc1 and -Orc2 (Kondo et al. 2017). The whole-genome reassortment analysis of the dichorhavirus isolates associated with citrus showed that isolates of OFV-Cit1 and -Cit2 were formed by conserved lineages of RNA2 paired with variable lineages of RNA1, such as was the case with OFV-Orc1 and -Orc2 (Fig. 7).

RT-PCR/restriction fragment-length polymorphism assay to differentiate strains of OFV infecting orchid and citrus. Nucleotide sequence data suggested that RT-PCR tests would be useful to distinguish among these strains. The N-gene

TABLE 3. Intergroup net evolutionary divergence among four subgroups of orchid fleck virus based on six open reading frame (ORF) sequences from genome segment 1 (RNA1) and genome segment 2 (RNA2)

Genome segment/ORF	IV versus III	IV versus II ^a	IV versus I	III versus II	III versus I	II versus I
RNA1	0.1894	0.2036	0.1757	0.0863	0.1866	0.1897
ORF1	0.1742	0.1856	0.1489	0.0820	0.1547	0.1635
ORF2	0.0698	0.0760	0.0682	0.0276	0.0754	0.0768
ORF3	0.1539	0.1689	0.1382	0.0785	0.1590	0.1756
ORF4	0.2542	0.2070	0.1712	0.0952	0.2217	0.2105
ORF5	0.2243	0.2407	0.2262	0.0997	0.2240	0.2227
RNA2	0.0046	0.1025	0.1040	0.0877	0.0888	0.0004
ORF6	0.0048	0.1037	0.1051	0.0904	0.0913	0.0004

^a The number of base substitutions per site between groups of sequences is based on the analyses of eight orchid fleck virus (OFV) sequences conducted in MEGA X using the maximum composite likelihood method. Subgroup I: OFV-Orc1-CL (citrus), OFV-Orc1-Cym07, and OFV-Orc1-Br (orchid). Subgroup II: OFV-Orc2-So and OFV-Orc2-NHHS1 (orchid). Subgroup III: OFV-Cit1-M2345 (citrus). Subgroup IV: OFV-Cit2-S9 (citrus).

TABLE 4. Evolutionary analyses for all orchid fleck virus sequences were conducted in MEGA X using Tajima's neutrality test (all ambiguous positions were removed for each sequence pair using the pairwise deletion option)

Genome segment/ORF ^a	m^b	n	S	p_s	Θ	π	D
RNA1	8	6975	1830	0.2624	0.1012	0.1232	1.1962
ORF1	8	1372	341	0.2485	0.0958	0.1176	1.2495
ORF2	8	1004	157	0.1563	0.0603	0.0751	1.3460
ORF3	8	1113	252	0.2265	0.0873	0.1060	1.1755
ORF4	8	574	152	0.2648	0.1021	0.1236	1.1536
ORF5	8	1749	552	0.3156	0.1217	0.1474	1.1595
RNA2	8	6211	842	0.1356	0.0523	0.0591	0.7192
ORF6	8	5658	810	0.1431	0.0552	0.0629	0.7718

^a ORF, open reading frame; RNA1, genome segment 1; RNA2, genome segment 2.

^b m , number of sequences; n = total number of sites; S = number of segregating sites; $p_s = S/n$; $\Theta = p_s/a_1$; π = nucleotide diversity; D = the Tajima's test statistic.

sequence was chosen for this, because the N gene is relatively conserved in dichorhavirus, yet the sequence data showed that the sequence diversity was sufficient to develop tests that would differentiate strains. RT-PCR/restriction fragment-length polymorphism (RFLP) analysis was first performed in silico, and it identified *Bam*HI and *Pst*I as restriction enzymes with differential target sites in the nucleocapsid sequence of dichorhavirus that infect citrus and orchids. The lack of restriction sites for either enzyme in the predicted amplicon produced from the newly described CiLV-N strains from Brazil meant that these strains would be readily distinguished from both OFV-Cit1 and -Cit2. However, the nucleocapsid sequences of OFV-Orc1 (subgroup I) and

OFV-Cit2 (subgroup IV) contain differential *Pst*I sites. The nucleocapsid sequences of OFV-Cit2 (subgroup IV) contained a single *Pst*I and two *Bam*HI restriction sites that distinguish it from other known citrus and orchid strains of OFV. CiLV-N0, the extinct OFV citrus strain (Florida 1948) had two sites for each enzyme. The in silico predictions (Supplementary Table S3) were confirmed by laboratory experimentation. Generic forward and strain/subgroup-specific reverse primers were designed to amplify the complete ORF1 nucleocapsid gene by RT-PCR. *Bam*HI and *Pst*I endonuclease-digested nucleocapsid amplicon sizes were used to distinguish orchid and citrus strains of the OFV as predicted by the in silico analysis (Fig. 8 and Supplementary Table S3).

Fig. 7. Recombination or reassortment analysis of dichorhavirus genomes with the Recombination Detection Program v.4.97 (RDP4) software. Unique recombination events within dichorhavirus genomes are illustrated with different colors and concatenated sequence alignments of genome segment 1 (RNA1) and genome segment 2 (RNA2). Unique recombination events are demarcated by the boxes below the genome of each of the dichorhavirus. CiCSV, citrus chlorotic spot virus; CiLV-N, citrus leprosis virus N; CiCSV, *Clerodendrum* chlorotic spot virus; CoRSV, coffee ringspot virus; OFV-Cit, citrus strain of orchid fleck virus; OFV-Orc, orchid strain of orchid fleck virus.

The RT-PCR/RFLP assay was applied to study the infestation of citrus by citrus leprosis disease in Querétaro, Mexico (Roy et al. 2015b). Single infections of OFV-Cit2 were documented in archived samples collected in 2012 and 2015 and samples in 2019 of either or both sweet and sour orange plants. Double infections of both OFV-Cit1 and -Cit2 were also documented in samples 2012-M6, 2015 QR007-010, QR020-022, and QR026. Mixed infections of OFV-Cit1 and -Cit2 were observed and characterized from six citrus varieties grown in Querétaro state, Mexico (Table 1). Because these samples were obtained in 2012, 2015, and January 2019, the pathosystem of host, vector, and mite has been stable at the location sampled in Querétaro, Mexico over a period of 8 years.

Taxonomic identification of vector *Brevipalpus* sp. associated with the possible transmission of OFV-Cit2. Mites from symptomatic Mexican lime, grapefruit, lime, Persian lime, sweet orange, and sour orange trees were collected from the area of infestation in Querétaro in 2015 and 2019 and sent to Beltsville, Maryland for taxonomic identification. The genus *Brevipalpus* is one of the largest groups of mites in terms of the number of species (Mesa et al. 2009). The genus is defined by the presence of a three- to four-segment palpus, strongly developed ventral and genital plates, and hysterosoma with six or seven dorsolateral setae (Beard et al. 2013, 2015). The genus *Brevipalpus* was divided into six groups based on the number of dorsal setae, number of solenidia on leg II, palp segmentation, and number of setae on the terminal segment of the palpus (Baker and Tuttle 1987).

Of these morphological groups, two are important. The *B. californicus* group has seven dorsolateral setae (c3, d3, e3, f2, f3, h2, and h1), and the *B. phoenicis* group has six dorsolateral setae (without f2). Members of both groups are often confused and misidentified. The species identified with OFV-Cit1 dichorhavirus in this study is *B. californicus* (Banks) and has round and strong spermatheca, two solenidia on tarsus II, and a V pattern between setae d1 and e1 (Beard et al. 2012). *B. californicus* has been previously associated with transmission of OFV-Cit1 (García-Escamilla et al. 2017). Many different dichorhavirus have been shown to infect citrus, and they are often in association with *Brevipalpus* mite: in Florida (extinct) (Hartung et al. 2015), Brazil (Chabi-Jesus et al. 2018; Ramos-González et al. 2017), Mexico (Cruz-Jaramillo et al. 2014, Roy et al. 2014b), Panama (de Dominguez et al. 2001), Colombia (Roy et al. 2014a), and South Africa (Cook et al. 2019). In this study, mites collected from Querétaro in 2015 and 2019 were identified as *B. californicus* sensu stricto (Banks) (Fig. 9 and Supplementary

Table S1). Results of RT-PCR assays using total RNA obtained from mites collected from the six different citrus species showing leprosis-like symptoms (Fig. 1B to E and G) demonstrated that the *B. californicus* sensu stricto was the carrier of both citrus strains of OFV. In a previous study, *B. californicus* sensu stricto was identified as vector of the OFV-Cit1 (García-Escamilla et al. 2017). We hypothesize that the same mite species is the vector of OFV-Cit2. We are currently conducting transmission experiments following the optimized parameters of the OFV-Cit1 transmission study (García-Escamilla et al. 2017).

There are parallels between the population structure of strains of OFV that infect orchids and citrus. Both are composed of two distinct but related subgroups of strains: OFV-Orc1 and -Orc2 and OFV-Cit1 and -Cit2. The nucleotide sequences of the five ORFs encoded by RNA1 vary within and between the citrus and orchid subgroups of OFV. The nucleotide sequences of RNA2 between the subgroups of OFV-citrus and -orchid are also conserved but with only 90% identity. The nucleotide sequence of RNA2 was 99% identical within both orchid subgroups I and II (isolates Cym07 and So) and citrus subgroups III and IV (isolates M2345 and S9). Analysis of the orchid and citrus isolates of OFV showed that recombination events within genome segment RNA1 had not occurred and could not account for the sequence diversity observed within RNA1. The genetic data from these strains of OFV have been used to establish that OFV-Orc1 and OFV-Cit1 originated as a result of a reassortment event (Kondo et al. 2017). OFV-Cit1 is a possible major parent for both orchid strains of OFV, whereas OFV-Orc1 (subgroup I) is the major parent of OFV-Cit2. RNA2 from the orchid strain OFV-Orc2 has paired with RNA1 of OFV-Orc1 or vice versa. In this work, we show that yet another RNA1 genome segment from a citrus lineage has been paired with an RNA2 genome segment from OFV-Cit1 to create OFV-Cit2.

Dichorhavirus are distinct from other rhabdoviruses. Their particles are not enveloped and their genome is segmented, with the L protein, encoding RNA polymerase on its own genome segment. The two genome segments are packaged in separate virions and determine the length of the bullet-shaped nuclear rhabdovirus particles. Bipartite dichorhavirus are mostly transmitted by brevipalpid mites in a persistent manner consistent with replication in the mite vector, whereas monopartite nucleo- and cytorhabdoviruses are transmitted by aphids, planthoppers, or leafhoppers (Kondo et al. 2006). The packaging of genome segments RNA1 and RNA2 in a separate particles would facilitate genome reassortment as observed first in orchid strains of OFV (Kondo et al. 2017) and in citrus strains of OFV.

TABLE 5. Detection of recombination sites in genome segments 1 and 2 of leprosis-associated dichorhavirus using multiple recombination-detecting programs (RDPs)

Dichorhavirus group	Isolate/accession no.	Start and end break points ^a	Software ^b	<i>P</i> value ^c	Parent isolates (major × minor) ^d	Recombination between groups ^e
OFV-Orc1/I	Cym07/LC222629-30	5941-11902	RGBMCSi3s	7.713 × 10 ⁻¹¹⁸	OFV-Cit1 × OFV-Orc2	III versus II
OFV-Orc1/I	CL/MK522804-05					
OFV-Orc1/I	Br/MK522806-07					
OFV-Orc2/II	So/AB244417-18	5941-11902	RGBMCSi3s	1.601 × 10 ⁻¹¹⁴	OFV-Cit1 × OFV-Orc1	III versus I
OFV-Orc2/II	NHHS1/AB516441-42					
OFV-Cit2/IV	S9/MK578000-01	5930-11956	RGBMCSi3s	9.182 × 10 ⁻¹²³	OFV-Orc1 × OFV-Cit1	I versus III
CiLVN/V	Ibi1/KX982176 and KX982179	6328-11342	RMCSi3s	6.420 × 10 ⁻¹³	CiLVN-SRq1 × CiLVN-MAS1	V versus V
CiLV-N/V	MAS1/KY751404-05	5930-11922	RBSi3s	8.463 × 10 ⁻⁰⁹	CiLVN-SRq1 × CiLVN-SBS1	V versus V
CiLV-N/V		8691-11958	GM3s		CiLVN-SRq1 × CiLVN-SBS1	V versus V

^a Nucleotide positions are indicated based on actual genome sequence (accession number) of the type species of each group.

^b Recombinant isolates identified by the RDPs: B, Bootscan; C, Chimaera; G, GENECONV; M, MaxChi; R, RDP; Si, SiScan; and 3S, 3Seq programs in the RDP4 program.

^c The *P* value is for the program in bold type in column 4, and it is the most significant calculated *P* value among the isolates.

^d The identified parental sequences participating in the recombination events identified by RDP4.

^e Type of 'group': I (orchid strain 1 of orchid fleck virus [OFV-Orc1-like]), II (OFV-Orc2-like), III (citrus strain 1 of orchid fleck virus [OFV-Cit1] like) and IV (OFV-Cit2-like) and V (CiLVN-like). No significant recombination events were detected in OFV-Cit1 (KF209275-76), CiNSV (KF198064-65), CoRSV, CiCSV, CiCSV and SBS1 and SRq1 isolates of CiLVN.

Symptoms similar to those caused by what are known as dichorhavirus were observed in the first quarter of the 20th century in Argentina and Brazil, where the disease was referred to as “lepra explosiva” and later, “leprosis” (Bastianel et al. 2010). It has become clear over the years that two distinct viruses that shared hosts in the genus *Citrus*, local lesion symptoms, transmission by mites in the genus *Brevipalpus*, bipartite genomes, and bacilliform particles similar in size had become conflated, revealing an interesting example of convergent evolution. The virus that causes the disease originally described from South America as the cause of lepra explosiva differs from dichorhavirus in that it replicates and

forms particles in the cytoplasm, has a positive-sense single-stranded RNA genome, and is known as citrus leprosis virus-cytoplasmic type (*Cilevirus*) (Bastianel et al. 2010). It is tempting to speculate that replication in the mite vector is enabled by segmented genomes (Kondo et al. 2006). This is supported by the fact that cileviruses are also transmitted by brevipalpid mites and have segmented genomes (Roy et al. 2013a).

The citrus leprosis complex is clearly of emerging importance. It remains a mystery why these viruses have not appeared in Florida, Texas, or California, although flat mites, orchids, and citrus are present. This may be owing to good fortune, but the history of

Fig. 8. Reverse transcription PCR restriction fragment-length polymorphism-based detection of orchid strains of orchid fleck virus (OFV; OFV-Orc) and citrus strains of OFV (OFV-Cit). Lane M, Size markers. Lanes 2 and 3, OFV-Orc1 from orchids. Lane 4, OFV-Orc1 from citrus. Lane 5, OFV-Orc2 from orchids. Lane 6, OFV-Cit2. Lane 7, Healthy citrus control for reverse transcription (no amplicon). Lane 9, Citrus infected with OFV-Cit1 and Cit2. Lane 10, OFV-Cit1 from citrus. Restriction digestion of nucleocapsid amplicons with endonucleases **A**, *Bam*HI and **B**, *Pst*I. nt, nucleotide.

disease and pest incursions into the U.S. citrus industry in the past 100 years argues otherwise. The absence of the leprosis disease complex in the United States and the Mediterranean basin in spite of the presence of abundant hosts and flat mites (Jacobucci et al. 2018) suggests a degree of vector/virus/host specificity. OFV-Cit1 is widespread in Mexico and has a wide host range within citrus, but it is confined to higher elevations with a cooler and drier climate (Roy et al. 2015a). This is different from the CiLV-N described in Brazil

in 2017, which has a narrow host range in citrus (Ramos-González et al. 2017). Florida is low in elevation and very humid, and therefore, the environment there may not be conducive to the establishment of the dichorhavirus complex prevalent in Mexico. Growers of conventional and organic citrus in the current era of huanglongbing control are using significant amounts of systemic insecticide and other insect suppression methods that could be expected to secondarily suppress populations of flat mites as well.

Fig. 9. *Brevipalpus californicus* s.s. **A**, Female dorsal habitus. 1, Reticulated pattern of propodosoma midarea; 2, smooth to rugose wrinkled area; 3, opithosoma with wrinkled central area and posterior weak v-shaped folds; 4, seven lateral setae (f2 present). **B**, Female ventral habitus. 1, Smooth central region with soft striations. **C**, Female propodosoma. 1, Leg II, tarsus with two solenidia. **D**, Female opisthosoma. 1, Ventral plate; 2, genital plate.

These control measures may protect the industry from both dichorhavirus and cileviruses, but home owner trees would still be vulnerable. The report of OFV-Orc1 in *Phalaenopsis* hybrid orchids in the United States in 2015 (Bratsch et al. 2015) and its association with *Brevipalpus* species indicate that the virus is established in the United States. Orchids are distributed in states including Florida, Texas, and California. Furthermore, the 2019 report of natural infection of OFV-Orc1 in different species of citrus and orchids in South Africa (Cook et al. 2019) demonstrates the potential threat to citrus. For these reasons, the U.S. Department of Agriculture-APHIS includes the citrus leprosis disease complex in its biosecurity program for citrus, and the RT-PCR/RFLP assay described herein in addition to assays previously developed for cileviruses (Melzer et al. 2013; Roy et al. 2013a, 2018) enhance the biosecurity of citrus in the United States.

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