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Diverse Array of New Viral Sequences Identified in Worldwide Populations of the Asian Citrus Psyllid (*Diaphorina citri*) Using Viral Metagenomics

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ABSTRACT

The Asian citrus psyllid, *Diaphorina citri*, is the natural vector of the causal agent of Huanglongbing (HLB), or citrus greening disease. Together; HLB and *D. citri* represent a major threat to world citrus production. As there is no cure for HLB, insect vector management is considered one strategy to help control the disease, and *D. citri* viruses might be useful. In this study, we used a metagenomic approach to analyze viral sequences associated with the global population of *D. citri*. By sequencing small RNAs and the transcriptome coupled with bioinformatics analysis, we showed that the virus-like sequences of *D. citri* are diverse. We identified novel viral sequences belonging to the picornavirus superfamily, the *Reoviridae*, *Parvoviridae*, and *Bunyaviridae* families, and an unclassified positive-sense single-stranded RNA virus. Moreover, a *Wolbachia* prophage-related sequence was identified. This is the first comprehensive survey to assess the viral community from worldwide populations of an agricultural insect pest. Our results provide valuable information on new putative viruses, some of which may have the potential to be used as biocontrol agents.

IMPORTANCE

Insects have the most species of all animals, and are hosts to, and vectors of, a great variety of known and unknown viruses. Some of these most likely have the potential to be important fundamental and/or practical resources. In this study, we used high-throughput next-generation sequencing (NGS) technology and bioinformatics analysis to identify putative viruses associated with *Diaphorina citri*, the Asian citrus psyllid. *D. citri* is the vector of the bacterium causing Huanglongbing (HLB), currently the most serious threat to citrus worldwide. Here, we report several novel viral sequences associated with *D. citri*.

Viruses are the most abundant microbes on our planet (1) and are found in all types of organisms. Insects are the largest and most diverse taxonomic class among animals, representing perhaps half of known animals, with more than one million species recognized worldwide (2). Insects are known to be hosts to viruses belonging to various viral taxa, including the *Baculoviridae*, *Parvoviridae*, *Flaviviridae*, *Ascoviridae*, *Togaviridae*, *Bunyaviridae*, and *Rhabdoviridae* (3). However, the number of currently described viral species infecting insects is relatively low compared to the number of viruses that have been discovered among prokaryotes, plants, and vertebrates. Furthermore, most of the insect viruses described to date have been discovered because of their pathogenic effects on their insect hosts or because they are pathogens of humans, other vertebrates, or economically important plants. Traditional viral detection methods that require prior knowledge of genome sequences may not be suitable for the discovery of new viruses and, in particular, viruses with a high level of genetic diversity. However, in the past decade, the development of high-throughput next-generation sequencing (NGS) technologies and bioinformatics applications has provided new opportunities for discovering viruses in many organisms, including humans (4–7), arthropods (8–29), and plants (30–45). In addition to transcriptome sequencing (RNA-seq) (15, 18, 28), deep sequencing of small RNAs (sRNAs) and the subsequent assembly of the sRNAs have been proven to be promising approaches for the discovery of both RNA and DNA viruses in plant and insect hosts (9, 41, 42, 46–50).

The Asian citrus psyllid, *Diaphorina citri* Kuwayama, is currently the most important insect associated with worldwide citrus production (51). *D. citri* is the vector of "*Candidatus* Liberibacter asiaticus," the causal agent of Huanglongbing (HLB). HLB, also known as citrus greening disease, is the most devastating disease of citrus trees and currently is a threat to world citrus production (52). HLB is native to Asia (53), and recently the American form, which is believed to have originated in China, was discovered and reported in South America, Mexico, and North America (United States) (54, 55). As there is no cure for HLB, disease control relies on a combination of approaches including insect vector management through chemical and biological control strategies.

In an attempt to discover putative viruses which may be associated with *D. citri*, we conducted a metagenomic study of populations of *D. citri* from various locations around the world. We used a multistep analysis pipeline consisting of high-throughput NGS of small RNAs and transcriptomes, *de novo* sequence assem-

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bly, *in silico* searches for sequence similarities to reference viruses, and confirmation of putative viral sequences via reverse transcription-PCR (RT-PCR) and PCR followed by Sanger sequencing. Here we describe the success of these methods to assess the diversity of viral sequences found in *D. citri*. Our findings demonstrate the presence of viral sequences from several distinct taxa, including the families *Reoviridae*, *Parvoviridae*, *Bunyaviridae*, the picornavirus superfamily, an unclassified positive-sense single-stranded RNA (ssRNA) virus, and the *Wolbachia* prophage WO.

MATERIALS AND METHODS

Sample collection and total RNA preparation. In 2013, RNA samples from *D. citri* collected from Florida were shipped to our laboratory at the University of California-Davis (UCD). We also received RNA samples from *D. citri* collected in China and Taiwan, the native geographic regions of *D. citri*, and from Brazil, where *D. citri* and HLB are listed as newly emerging. In 2014, we received RNA samples from other citrus-growing regions, including Hawaii, California, Texas, and Pakistan. RNA was extracted by homogenizing 50 to 60 whole wild-caught psyllids in TRIzol reagent according to the manufacturer's instructions (Life Science Research, Carlsbad, CA). *D. citri* RNAs were qualitatively and quantitatively evaluated on an Experion RNA analysis system using the Experion RNA SdtSens analysis kit (Bio-Rad, Hercules, CA) and a NanoDrop 1000 spectrophotometer (Thermo Scientific, Wilmington, DE), respectively. High-quality RNAs from the samples were selected for construction of small RNA and RNA-seq cDNA libraries for high-throughput NGS.

Small RNA and RNA-seq library construction and sequencing. Four sRNA libraries were generated from 2.0 μg of total RNA from Brazil, China, Taiwan, and Florida with RNA quality indicator (RQI) values of ≥7 using Illumina's TruSeq small RNA sample preparation kit according to the sample preparation instructions (Illumina, San Diego, CA). Briefly, 5′ and 3′ adapters designed based on the natural structure of small RNAs included in the kit were ligated to each end of the RNA molecules. cDNA was synthesized by reverse transcription (RT) followed by PCR amplification with common and indexed primers. The sRNA libraries were then gel size selected and purified, and the final cDNA libraries were validated on an Experion system using the Experion DNA 1K analysis kit (Bio-Rad). Final concentrations were adjusted to 10 nM, and 10 μl of each sample was shipped to the Beijing Genomics Institute (BGI), Hong Kong, for 50-bp single-read sequencing using the Illumina HiSeq 2000 platform in four lanes.

Since assembly of sRNA is difficult, and obtaining full-genome coverage is challenging due to the short lengths of the generated contiguous sequences (contigs), we also sequenced *D. citri* total RNA (RNA-seq) using an Illumina HiSeq 2000 platform. rRNA-depleted RNAs from the China, Brazil, Hawaii, and Florida populations were used to construct RNA-seq libraries with the ScriptSeq v2 RNA-Seq library preparation kit by following the manufacturer's instructions (epicenter/Illumina, San Diego, CA). Briefly, following DNase I digestion, rRNA was removed from total RNA using the epicenter/Illumina Ribo-Zero magnetic gold kit. The RNA was then fragmented and further processed according to the manufacturer's protocol. The libraries were multiplexed via PCR with ScriptSeq index PCR primers. Final indexed libraries were validated on the Experion system using the Experion DNA 1K analysis kit (Bio-Rad) and sequenced on the HiSeq 2000 platform (100-bp pair-end sequencing) at the Vincent J. Coates Genomics Sequencing Laboratory at University of California-Berkeley.

Small RNA and RNA-seq analysis and virus genome identification. Bioinformatics analysis of sRNA and RNA-seq data was performed using the CLC Genomic Workbench software package (CLC Bio-Qiagen, Boston, MA). Briefly, low-quality reads (<0.05) and adapter sequences were first removed from the raw sRNA data set. Trimmed sRNA sequences shorter than 15 nucleotides (nt) were discarded, and the remaining reads were mapped to the available recently assembled *D. citri* genome (GCF_000475195.1) to remove the host-related reads. Reads were then *de novo* assembled using two assemblers: the CLC Assembly Cell and Velvet (56), with word size/k-mer values ranging between 15 and 19. We used two assemblers because the length of contigs can vary based on the assembly programs and the parameter setting used for each program are specific, and using two assemblers also can give support of newly identified virus sequences (57). The resulting contigs were compared against the nonredundant viral protein database available in NCBI using BLASTx and tBLASTx at an E value of $<10^{-3}$ (58). BLAST results were then inspected manually to screen for potential viral sequences.

For the reads derived from RNA-seq, trimming and mapping were performed under the same conditions as described for sRNA. Reads were then assembled with word size/k-mer values ranging from 45 to 65. BLAST searches were conducted using contigs of >200 nt against the nonredundant viral protein database using BLASTx and tBLASTx at an E value of $<10^{-3}$ (58).

Viral genome sequence validation. To confirm the presence of the viral sequences identified in different populations of *D. citri*, RT-PCR and PCR assays were developed using specific primers designed based on *de novo*-assembled contigs with similarities to viral sequences. The (RT-)PCR products were sequenced by Sanger sequencing. We used RNA extracted from our *D. citri* colony maintained in the Contained Research Facility (CRF) at UC Davis, which was negative for identified viral sequences here based on (RT-)PCR results as the negative control in all (RT-)PCRs.

Phylogenetic analysis. Reference amino acid sequences of the respective viral RNA-dependent RNA polymerase (RdRp) proteins, and nonstructural (NS) proteins in the case of DNA viruses, were downloaded from GenBank. Multiple amino acid sequence alignments were performed with MUSCLE in MEGA version 6 with the default settings (59). Phylogenetic trees were constructed using the neighbor-joining (NJ) and maximum-likelihood (ML) methods in MEGA6 using the appropriate models for each group of viral sequences with 1,000 bootstraps. GenBank accession numbers of the reference sequences used in the phylogenetic analysis are shown in Table S3 in the supplemental material.

Accession numbers. All raw reads produced and used in this study were submitted to the National Center for Biotechnology Information (NCBI)'s Sequence Read Archive (SRA) under bioproject accession PRJNA293863. Sequences described in this paper were deposited in GenBank under the accession numbers KT698823 (DcBV L segment), KT698824 (DcBV M segment), KT698825 (DcBV S segment), KT698826 (DcACV RNA1), KT698827 (DcACV RNA2), KT698828 (DcDNV NS2), KT698829 (DcDNV VP1), KT698830 (DcRV segment 1), KT698831 (DcRV segment 8), KT698832 (DcRV segment 2), KT698833 (DcRV segment 10), KT698834 (DcRV segment 3), KT698835 (DcRV segment 4), KT698836 (DcRV segment 7), and KT698837 (DcPLV).

RESULTS

Small RNA profiles and RNA-seq analysis of *D. citri*. Because insects use RNA interference (RNAi), which results in the generation of abundant 21- to 30-nt virus-derived sRNAs, as a primary antiviral defense, NGS sequencing of sRNA libraries is a valid approach to identify insect-infecting viruses (9, 30, 60, 61). High-throughput NGS of the sRNA libraries generated 150 million to 200 million usable reads per library, with a length range of 15 to 30 nt. Both CLC Assembly Cell and Velvet assemblers generated more and longer contigs with a word window/k-mer value of 19 after subtracting the host reads. However, as we expected, the majority of the assembled contigs (70%) were ≤400 bp in length. Moreover, the number of reads that mapped to some detected virus-like sequences was low (see Table S2 in the supplemental material), but the mapping nevertheless suggested the presence of viral sequences in our libraries. Therefore, in order to generate longer contigs, four RNA-seq libraries were constructed and sequenced in multiplex. Approximately 50 million to 100 million paired-end raw reads of length 100 were produced from each li-

TABLE 1 Viral sequences identified in *D. citri*[a]

Virus category	Tentative name for putative virus	Closest relative virus (BLASTx)	Tentative virus family or superfamily	Tentative virus genus	Largest sequence length (bp) obtained	Source of representative *D. citri* populations
dsRNA	DcRV	*Nilaparvata lugens reovirus*	*Reoviridae*	*Fijivirus*	Seg. 1: 4,454 Seg. 2: 3,530 Seg. 3: 3,814 Seg. 4: 3,445 Seg. 7: 2,129 Seg. 8: 1,737 Seg. 10: 1,216	CH, TW, FL, HW, TX
ssRNA	DcPLV	*Deformed wing virus*	Picorna-like virus	Unclassified	9,580	BR, CH, TW
ssDNA	DcDNV	*Cherax quadricarinatus densovirus*	*Parvoviridae*	Unclassified	NS1: 1,299 NS2: 1,344 VP1: 1,767 VP2: 618	CH, TW, Pak
ssRNA	DcBV	*Wuchang cockroach virus 1*	*Bunyaviridae*	Unclassified	L-seg.: 1,911 M-seg.: 1,852 S-seg.: 438	CH, TW
ssRNA	DcACV	*Pea enation mosaic virus-2* *Chronic bee paralysis virus*	Unclassified Unclassified	*Umbravirus* Unclassified	RNA1: 1,111 RNA2: 1,764	CH, CA, TX, FL
dsDNA	WO Prophage	*WO prophage*	Unclassified phages	Unclassified	8,615	All

[a] DcPLV, *Diaphorina citri picorna-like virus; DcRV, Diaphorina citri reovirus*; DcDNV, *Diaphorina citri densovirus*; DcBV, *Diaphorina citri bunyavirus*; DcACV, *Diaphorina citri associated C virus*; Seg., segment; CH, China; TW, Taiwan; FL, Florida; BR, Brazil; HW, Hawaii; TX, Texas; CA, California; Pak, Pakistan.

brary. Raw reads were cleaned and assembled *de novo* as described for the sRNA libraries.

BLASTx searches with all the generated contigs from both sRNA and RNA-seq libraries suggested the presence of viral sequences from several distinct taxa. Viral sequences similar to those within the *Reoviridae* family and the picornavirus superfamily comprised the majority of viral sequences identified in *D. citri* (see Table S1). A smaller number of reads and contigs were observed to have similarity with viruses of the families *Bunyaviridae* and *Parvoviridae* and with *Chronic bee paralysis virus*, an unclassified positive-sense single-stranded RNA virus (see Table S1). Furthermore, a bacteriophage-like contig was identified in all *D. citri* populations. Most of the identified viral sequences shared less than 50% amino acid identity to known viral sequences, suggesting that they represent novel viral sequences. Table S2 in the supplemental material provides a list of all viruses in the viral database that showed significant BLASTx hits to contigs produced in this study.

Contigs related to picorna-like viruses. Contigs that showed similarity to picorna-like viruses (Iflaviruses) were assembled from sRNA and RNA-seq libraries from the *D. citri* populations from Brazil, China, and Taiwan (Table 1; see also Table S2). Picorna-like viruses, which belong to the picornavirus superfamily, a major division of eukaryotic positive-strand RNA viruses (62), are a large group of positive-sense, single-stranded RNA viruses which includes important pathogens of humans, plants, and insects. The genomes of viruses in the picornavirus superfamily are characterized by an RdRp, a chymotrypsin-like 3C protease, a putative helicase, and a genome-linked protein (VPg) (62–65). The picornavirus superfamily currently has 14 divergent families of viruses and several unclassified genera and species (62). Five of these families, *Picornaviridae*, *Iflaviridae*, *Dicistroviridae*, *Marnaviridae*, and *Secoviridae*, are further classified in the order *Picornavirales*, which includes extremely diverse viruses and virus-like elements (64). Iflaviruses are members of a relatively newly recognized family called *Iflaviridae*, members of which all belong to the genus *Iflavirus* and possess a monopartite, single-stranded, positive-sense RNA genome ranging from 8.5 to 10 kb in length (66). The genome encodes a single polyprotein of ~3,000 amino acids that is processed to produce a helicase, a protease, an RdRp, and four structural proteins (VP1 to VP4). All known iflaviruses are insect-infecting viruses with a wide range of hosts belonging to the orders *Lepidoptera*, *Hemiptera* and *Hymenoptera* and also bee parasitic mites (11, 12, 14–16, 20, 21, 26, 29, 67). However, a plant-infecting iflavirus-like virus, *Tomato matilda virus*, was recently reported from tomato (*Solanum lycopersicum*) (68). There are currently seven definitive groups within the genus *Iflavirus* recognized in the *Ninth Report of the International Committee on Taxonomy of Viruses* (69). However, several tentative viruses have been identified that show sequence similarity to the members of the genus *Iflavirus* yet are classified as unassigned viruses at this time (12, 16, 21, 70, 71). Previous phylogenetic analysis suggested that iflaviruses have evolved from different origins since the viruses infecting insects from the same order do not form a single clade (3, 17).

Through bioinformatics analysis of both sRNA and RNA-seq data, we were able to assemble more than 80% of the predicted genome sequence of a putative picorna-like virus tentatively named *Diaphorina citri picorna-like virus* (DcPLV). The presence of this virus in the RNA samples was subsequently confirmed by RT-PCR and Sanger sequencing using specific primers. Using a primer walking strategy to fill in the gaps, 9,580 nucleotides of the genome of DcPLV were determined (Table 1). Bioinformatics analysis predicted one possible open reading frame (ORF) of 8,496 nt. Sequence analysis showed that the DcPLVs found in the *D. citri* populations from China, Taiwan, and Brazil shared 97 to 99% nucleotide identity (data not shown), suggesting that they are members of the same species. BLASTx and tBLASTx searches showed that the DcPLVs shared low sequence identity (less than 40%) with members of *Iflaviridae* at the amino acid level (Table 2). However, DcPLV has a distinctly different genome organization from iflaviruses, which have a single large ORF encoding a

TABLE 2 Query coverage and maximum amino acid identity (BLASTx) between the proteins encoded by the putative viruses found here and the most closely related species or genus[a]

Putative virus	Encoded protein	Query % coverage	Maximum % identity	E value	Closely related species or genus	GenBank accession no.
DcRV	Seg. 1_RdRP	97	36	0.0	*Nilaparvata lugens reovirus*	NP_619776
	Seg. 2_136.6KD	33	28	2e−18		NP_619777
	Seg .3_major core capsid protein	87	29	6e−43		NP_619778
	Seg. 4_130KD	99	26	5e−81		NP_619779
	Seg. 7_73.5KD	96	24	3e−17		NP_619782
	Seg. 8_major outer capsid protein	87	26	5e−37		NP_619775
	Seg. 10_polypeptide	82	24	1e−18		NP_619774
DcPLV	Polyprotein	70	33	2e−112	*Deformed wing virus*	ADK55526
DcDNV	NS1	37	33	1e−09	Uncharacterized protein in *Diaphorina citri*	XP_008482940
	NS2	96	35	5e−69	*Cherax quadricarinatus densovirus*	YP_009134732
	VP1	37	31	6e−15	Densovirus SC1065	AFH02754
	VP2	43	42	2e−14	*Periplaneta fuliginosa densovirus*	NP_051016
DcBV	RdRp	85	31	6e−62	*Wuchang cockroach virus 1*	AJG39258
	Glycoprotein precursor	80	32	1e−25		AJG39291
	Nucleocapsid	90	36	2e−20		AJG39319
DcACV	RdRp	70	33	8e−25	*Pea enation mosaic virus-2*	AAU20330
	Hypothetical protein_s2gp2	8	39	0.001	*Chronic bee paralysis virus*	YP_001911140

[a] DcPLV, *Diaphorina citri Picorna-like virus; DcRV, Diaphorina citri reovirus*; DcDNV, *Diaphorina citri densovirus*; DcBV, *Diaphorina citri bunyavirus*; DcACV, *Diaphorina citri associated C virus.*

polyprotein that is cleaved into both structural and nonstructural proteins with the structural proteins (capsid proteins) located N terminal to the nonstructural proteins (Fig. 1A). In contrast, the DcPLV structural proteins are located C terminal to the nonstructural proteins in the predicted DcPLV polyprotein (Fig. 1B). The organization of the DcPLV genome appears to be most like that of *Heterosigma akashiwo RNA virus* (HaRNAV), which belongs to the family *Marnaviridae* (72). The family *Marnaviridae* consists of a single genus, *Marnavirus*, with HaRNAV as the type species (73). Interestingly, DcPLV and HaRNAV are phylogenetically distant, sharing only limited (20%) sequence identity at the amino acid level (Fig. 2A). Therefore, we believe that DcPLV is not an iflavirus, a marnavirus, or a dicistrovirus (Fig. 1C) but is a new, unclassified picorna-like virus. A phylogenetic tree generated based on

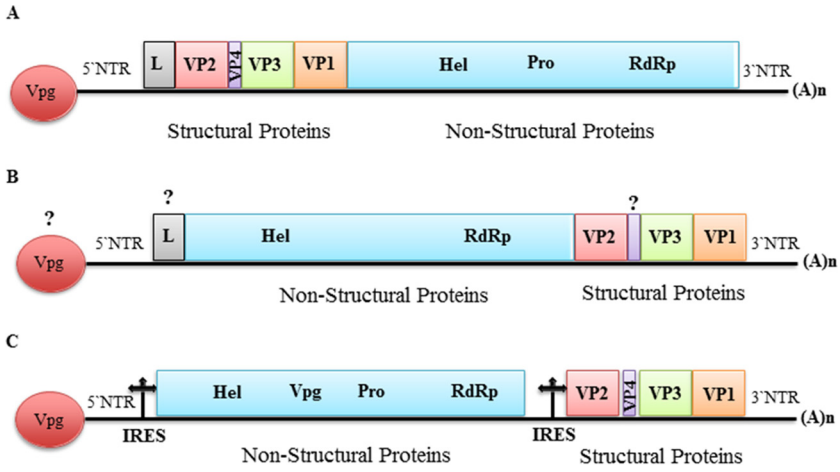

FIG 1 Schematic illustration of the predicted genome organization of DcPLV. (A) A typical *Iflavirus* genome showing a single ORF and the encoded structural proteins in the N-terminal region, while nonstructural proteins are located in the C-terminal region; (B) DcPLV predicted genome showing the unique position of the viral proteins in the polyprotein indicated; (C) a typical bipartite *Dicistrovirus* genome showing two ORFs. The nonstructural proteins are encoded by ORF1, and structural proteins are encoded by ORF2. L, leader protein; VP, virion protein; Hel, superfamily 3 helicase; Vpg, genome-linked protein; Pro, chymotrypsin-like cysteine protease; RdRp, RNA-dependent RNA polymerase; IRES, internal ribosome entry site.

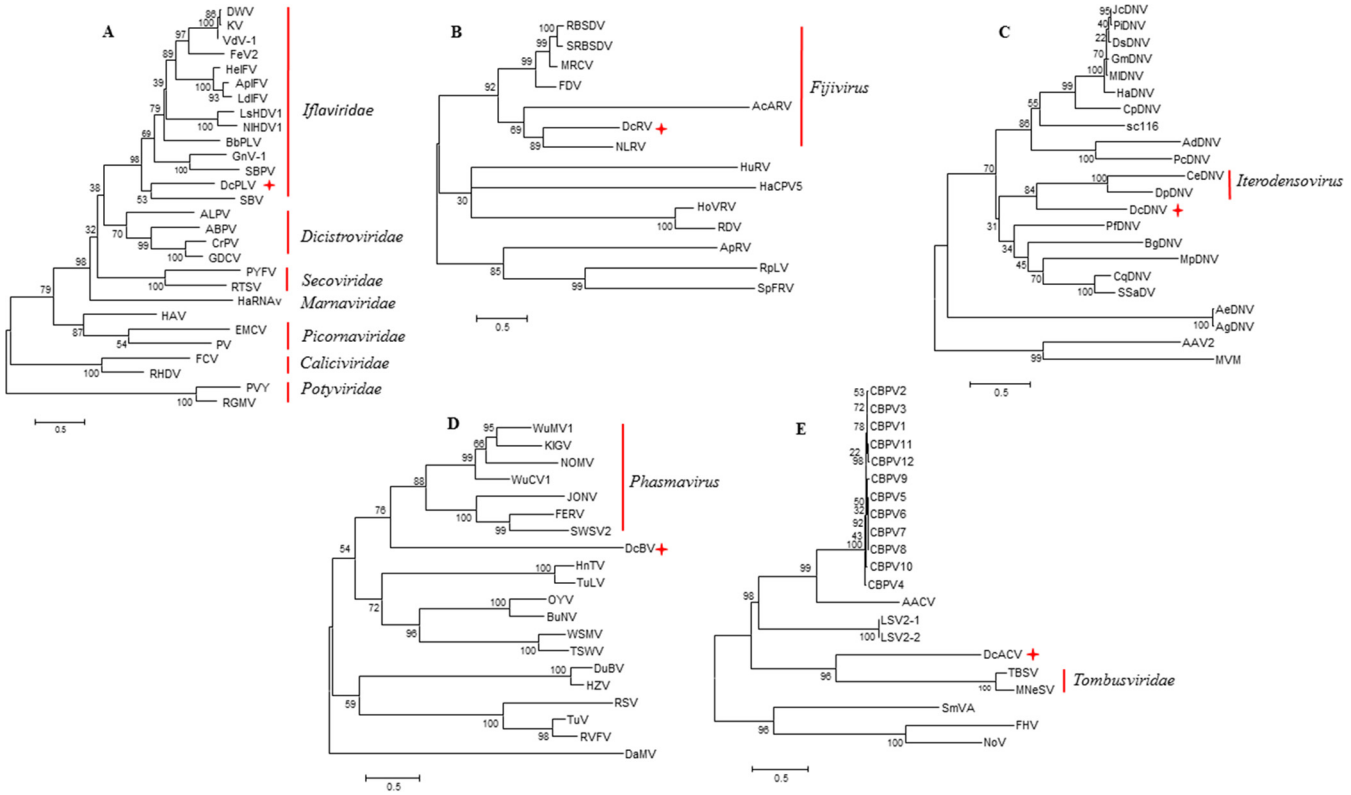

FIG 2 Maximum-likelihood trees of RdRp/nonstructural (NS2) protein amino acid sequences from representative viruses from picorna-like virus (A), *Reoviridae* (B), *Parvoviridae* (C), *Bunyaviridae* (D), and unclassified viruses, *Tombusviridae*, and *Nodiviridae* (E). Phylogenetic trees were constructed using the MEGA 6.0 program, with the LG+G+I evolutionary model for picorna-like virus and unclassified-*Tombusviridae-Nodiviridae* trees and the WAG+G+F model for *Reoviridae*, *Parvoviridae*, and *Bunyaviridae* trees with 1,000 bootstrap replications. The topology of the NJ trees was similar to that of the ML trees. Table S3 in the supplemental material shows the accession numbers of the reference sequences. DcPLV, *Diaphorina citri picorna-like virus*; DcRV, *Diaphorina citri reovirus*; DcDNV, *Diaphorina citri densovirus*; DcBV, *Diaphorina citri bunyavirus*; DcACV, *Diaphorina citri-associated C virus*. Pluses indicate novel viruses discovered in the current study.

the RdRp placed DcPLV close to the *Iflaviridae* and related to other members of the order *Picornavirales* (Fig. 2A). To assess the presence of DcPLV in other *D. citri* populations, we examined RNA from different populations using RT-PCR with specific primers. The results showed the presence of DcPLV in all of the populations from China and Brazil but not in the populations from the United States, Pakistan, or Taiwan, except one (see Table S4 in the supplemental material).

Reovirus-like sequences. Reovirus-like sequences were identified in the *D. citri* samples from China, Taiwan, Florida, and Hawaii from both sRNA and RNA-seq libraries. These sequences displayed similarities with viruses belonging to the family *Reoviridae*, genus *Fijivirus* (Table 1; see also Table S2). RT-PCR and Sanger sequencing confirmed the presence of the identified viral sequences in the above-mentioned samples and also in one sample from Texas (see Table S4). *Reoviridae* is a family of double-stranded RNA (dsRNA) viruses with members having 10 to 12 genome segments (69). In general, different reoviruses infect a wide range of different hosts, including mammals, birds, reptiles, fish, arthropods, fungi, protists, and plants. Reoviruses infecting plant-feeding hemipteran insects are classified into three genera: *Phytoreovirus* (74, 75), *Fijivirus* (76, 77), and *Oryzavirus* (78). Fijiviruses have 10 linear dsRNA segments encoding 12 proteins (69). Segment lengths can range from 1.4 to 4.5 kb, and the total genome size is about 27 to 30 kb.

In this study, nearly complete nucleotide sequences of seven putative reovirus segments ranging from 1,216 to 4,454 nt in length were identified in *D. citri*. RT-PCR further confirmed the presence of these sequences in our *D. citri* RNA extracts, and these sequences were not amplified by PCR, thus suggesting they were derived from dsRNAs. In 2009, a putative reovirus called *Diaphorina citri reovirus* (DcRV) was reported as a new species from the genus *Fijivirus*, family *Reoviridae*, naturally infecting ca. 55% of wild *D. citri* in Florida (79). However, only partial nucleotide sequences (a range of 400 to 800 nt) of six individual genome segments (predicted segments 1, 2, 4, 7, 8, and 10) of that virus were determined by Sanger sequencing of the cDNA libraries (79). The reovirus-like sequences obtained in the current study showed the highest amino acid similarity to the previously reported DcRV from Florida. However, due to the limited amount of sequence data available for the previously described DcRV, query coverage for our sequences was very low (<30%) for BLAST hits to the previously described DcRV, but in each case amino acid identity was high (≥93%). The second highest score of amino acid similarity belonged to *Nilaparvata lugens reovirus* (NLRV) (80) and compared to the case with DcRV from Florida, query coverage was much greater with NLRV, likely because a complete genome sequence is available for this virus (Table 2). Consistent with the previous report, our phylogenetic analysis based on segment 1, which encodes the RdRp, suggests that DcRV is a new putative

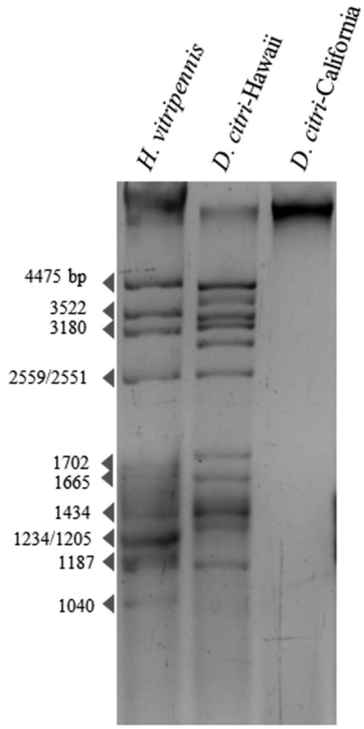

FIG 3 Double-stranded RNAs recovered from the Hawaiian *D. citri* population. CF11-cellulose-purified dsRNAs were electrophoresed in a 1.5% agarose gel. dsRNAs of *Homalodisca vitripennis reovirus* (HoVRV; 4,475 to 1040 bp) were used as the size standards. Californian *D. citri* was used as a negative control.

species in the genus *Fijivirus*, most closely related to NLRV (Fig. 2B). Interestingly, we were not able to identify three predicted genome segments (segments 5, 6 and 9) through our computational analysis. However, by examining dsRNAs extracted from the Hawaiian *D. citri* population according to the previously described method (81), we observed at least 10 dsRNAs. No dsRNAs were seen in our Californian *D. citri* (used as a negative control), and the resulting dsRNA pattern closely resembled that of another insect-infecting reovirus, *Homalosisca vitripennis reovirus* (81), suggesting that predicted segments 5, 6, and 9 were present but could not be identified by our NGS and bioinformatics analysis (Fig. 3). This can be explained if the missing segments are too divergent from known sequences in databases and were thus not recognized by our BLAST searches.

Densovirus-like sequences. High-throughput NGS analysis of sRNA and RNA-seq libraries from the China and Taiwan populations revealed densovirus (DNV)-like sequences (Table 1; see also Table S2 in the supplemental material). Similar sequences were also detected in the population from Pakistan and confirmed in the populations from China and Taiwan using PCR (see Table S4). DNVs, which belong to the subfamily *Densovirinae* in the family *Parvoviridae*, are characterized by small, nonenveloped virions which contain a linear, single-stranded DNA genome 4 to 6 kb in length (69, 82). DNVs are classified into five genera, *Ambidensovirus*, *Brevidensovirus*, *Hepandensovirus*, *Iteradensovirus*, and *Penstyldensovirus*, on the basis of genome characteristics, gene expression strategy, and structure of the terminal hairpins (69, 82, 83). Our analysis generated contigs that displayed significant BLASTx hits against nonstructural (NS) and structural (VP) genomic regions of DNVs. However, amino acid identity between reference sequences and these contigs was low (<40%). In order to obtain more genome coverage, a primer-walking approach was performed using primers based on the contigs generated in our analysis. This approach yielded a product of 4,836 nt, and computational analysis predicted four ORFs, including two overlapping NS genes on the same strand, tentatively called NS1 (1,299 nt) and NS2 (1,344 nt), and two nonoverlapping VP genes on the opposite strand, tentatively called VP1 (1,767 nt) and VP2 (618 nt) (Fig. 4). This putative novel virus is tentatively named *Diaphorina citri densovirus* (DcDNV). A BLASTx search using the ~4.8-kb sequence indicated the highest similarity with an ambidensovirus called *Cherax quadricarinatus densovirus* (GenBank accession no. YP_009134732) (Table 1). Table 2 shows the maximum amino acid identity of each DcDNV-encoded protein to homologous reference proteins. Members of the *Ambidensovirus* genus have an ambisense genome organization wherein both complementary strands have the capacity to encode functional proteins, a feature that is present among members of the family *Parvoviridae* (69). All members of *Ambidensovirus* are pathogenic to their insect hosts, and members of this genus are known to infect insects belonging to at least five orders (*Lepidoptera*, *Diptera*, *Orthoptera*, *Odonata*,

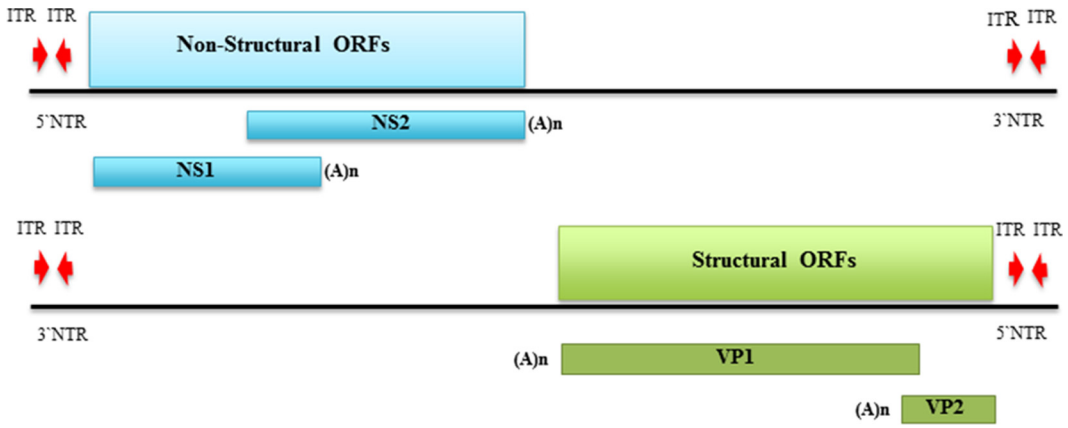

FIG 4 Schematic illustration of the predicted genome organization of DcDNV. The 4,836-bp ambisense genome contains four open reading frames (NS1, NS2, VP1, and VP2) which are flanked by inverted terminal repeats (ITR). NS, nonstructural protein; VP, virion protein; NTR, nontranslated region.

and *Hemiptera*) (83). A mutualistic association between a novel densovirus, *Helicoverpa armigera densovirus-1* (HaDNV-1), and its host, *Helicoverpa armigera*, has recently been reported (84). However, a phylogenetic tree constructed based on NS2 amino acid sequences placed DcDNV closer to the viruses from the genus *Iteradensovirus* (Fig. 2C) which possess a monosense genome (69). Interestingly, the NS1 sequence displayed the highest similarity with an uncharacterized insect protein (Table 2).

Bunyavirus-like sequences. Sequences similar to the L, M, and S segments of a typical member of the family *Bunyaviridae* were assembled from the sRNA and RNA-seq libraries from China and Taiwan (Table 1; see also Table S2 in the supplemental material), and the presence of these sequences in *D. citri* was confirmed by one-step RT-PCR. Bunyaviruses, members of the family *Bunyaviridae*, have segmented, single-stranded, negative-sense RNA genomes comprised of three RNAs designated L (large), M (medium), and S (small), which together total 11 to 19 kb. The L, M, and S genome segments encode the RdRp, envelope glycoproteins (Gn and Gc), and nucleocapsid protein (N), respectively (69). The family *Bunyaviridae* includes the genera *Orthobunyavirus*, *Nairovirus*, *Phlebovirus*, *Hantavirus*, and *Tospovirus* as well as a recently proposed genus tentatively named Phasmavirus (85, 86). We identified several contigs with amino acid similarity to members of the putative genus *Phasmavirus* (85). Significant hits included the L, M, and S segments of *Wuhan mosquito virus 1* (AJG39267), *Wuchang cockroach virus 1* (AJG39258), and *Kigluaik phantom virus* (AIA24559) (see Table S2). Table 2 shows the query coverage and the maximum amino acid similarities for individual protein sequences of the putative bunyavirus identified in the current study, which is tentatively named *Diaphorina citri bunyavirus* (DcBV). DcBV was phylogenetically most closely related to the phasmaviruses based on the analysis of the L segment (Fig. 2D).

Unclassified CBPV-like sequences. We identified a 1.7-kb contig in the *D. citri* populations from China and Florida from RNA-seq libraries that displayed 39% amino acid identity to RNA 2 of *Chronic bee paralysis virus* (CBPV) (Table 2). RT-PCR and Sanger sequencing confirmed and also revealed similar sequences in the populations from California and Texas (see Table S4 in the supplemental material). CBPV is an unclassified single-stranded RNA virus with no close relatives among known sequenced viruses. It infects adult honey bees, resulting in paralysis and death (87). The CBPV genome is comprised of two RNA molecules of 3,674 and 2,305 bases. Both RNA molecules have a 5′ cap structure and are not polyadenylated on the 3′ end (88). A previous phylogenetic study based on the RdRp domains of CBPV suggested an intermediate phylogenetic position for the virus between *Nodaviridae* and *Tombusviridae* (87). Although our analysis was unable to identify any sequence related to RNA 1 of CBPV, we detected a contig of 1.1 kb with 33% amino acid sequence identity to the RdRp of tombusviruses (Table 2). Furthermore, the presence of both fragments in the same RNA samples was confirmed by RT-PCR. Therefore, we believe that these two segments belong to a putative novel virus tentatively named *Diaphorina citri-associated C virus* (DcACV). Phylogenetic analysis based on the RdRp amino acid sequences revealed that DcACV was most closely related to tombusviruses (Fig. 2E).

Bacteriophage-like sequences. Our approach also generated contigs from both sRNA and RNA-seq libraries prepared from the *D. citri* samples from China, Taiwan, Brazil, Florida, and Hawaii that displayed 100% identity to nucleotide sequences from *Wolbachia* phage WO (Table 1; see also Table S2 in the supplemental material). The prokaryotic endosymbiont *Wolbachia* is present in 66% of all arthropod species (89), and prophage WO is the most widespread bacteriophage, infecting many *Wolbachia* species (90). To assess the presence of the prophage WO-like sequences in different populations of *D. citri*, PCR amplifications with primers specific for the minor capsid gene (*orf7*) (91) were performed, and specific 1-kb amplicons were produced in all populations (data not shown).

DISCUSSION

HLB and its associated natural insect vector, *D. citri*, are the major threats to the world's citrus industry (52). The goal of the current study was to examine populations of *D. citri* from both native areas (China and Taiwan) and those where *D. citri* has more recently emerged (United States and Brazil) through high-throughput NGS of small RNAs and transcriptomes in an attempt to discover putative viruses associated with *D. citri*. Such metagenomic approaches with similar goals have been successfully implemented to discover highly diverse and novel viruses from field-collected mosquito, bat, and *Drosophila* fly samples (25, 47, 60, 92–94). A desirable translational outcome of this work would be to identify *D. citri*-infecting viruses which might have the potential to be used as biological agents to control *D. citri* and slow the spread of HLB. In this study, we were able to identify and assemble nearly complete genome sequences of several putative novel viruses associated with *D. citri*, including a picorna-like virus, a reovirus, a densovirus, a bunyavirus, and an unclassified (+) ssRNA virus. We also detected sequences similar to that of *Wolbachia* phage WO. To trace the identified virus-like sequences in geographically distant *D. citri* populations, specific primers were designed based on the fragments obtained from bioinformatics analysis and used to screen additional *D. citri* populations which were not analyzed by NGS, including populations from Brazil, China, Taiwan, Pakistan, and the United States. To the best of our knowledge, this is the first comprehensive high-throughput NGS-based survey of the viral sequences associated with the global population of an agricultural insect pest, and our work demonstrates the success of this approach for field-collected insects.

NGS and bioinformatics analyses alone are very useful for virus identification but yield only viral sequences, some of which may not be representative of viruses actually infecting the host sampled. Therefore, we took several approaches in attempts to assess if the viral sequences identified in this study represented viruses of *D. citri*. We intentionally chose sRNA NGS because this has proven to be a powerful means to identify active RNA and DNA viruses of both plants and insects (42, 49, 95). Both insects and plants use RNAi as a primary defense against virus infections. RNAi activity results in distinct populations of overlapping sRNAs derived from the RNAi-targeted viral genomes, and in insects, 21-nt sRNAs represent the primary size class (9, 60, 61, 96–98). In our study, the size distribution of sRNAs mapping to the putative viruses had a prominent peak at 21 nt (data not shown), suggesting that these putative viruses may originate from viral infections of *D. citri* and may be processed by the antiviral RNAi machinery. In a recent study, the presence of virus-derived 21-nt small RNAs was used to support the majority of identified putative viruses in *Drosophila melanogaster* as bona fide *Drosophila* infections (60). As a second approach, we used RT-PCR, PCR, and Sanger sequencing to further confirm the origin of our sequences and their

identities. When we performed PCR with DNA extracted from *D. citri* for all of the RNA virus-like sequences identified in this study, no products were amplified. In contrast, RT-PCR did give the expected products, confirming that the sequences represented nonintegrated RNA virus sequences. We did amplify products for DcDNV by both RT-PCR and PCR, but Southern blot hybridization analysis using DNA extracted from a Taiwanese *D. citri* population and a probe based on our DcDNV sequence showed a single DNA molecule of ~5 kb, suggesting that the DcDNV sequences were not integrated into the genome of *D. citri* (data not shown). Finally, our phylogenetic analyses also strongly support the idea that all of the putative viruses reported here are closely related to known insect-specific viruses. Taken together, our cumulative data indicate that all of the putative viruses identified in this study are in fact episomal viruses and do not represent genomic integration events or contamination. Moreover, the fact that similar viral sequences were found in geographically distant *D. citri* populations collected at different times and from different plants suggests that the viral sequences identified in this study are likely not derived from environmental factors, such as ingested plant material. Finally, the goal of the present study was to utilize metagenomic approaches to identify novel putative viruses associated with *D. citri*, and in that we were successful. While it is very likely that many viruses of *D. citri* remain to be discovered, here we have identified four putative RNA viruses and one putative DNA virus that are good candidates for further study.

All of the virus sequences identified here represent new putative viruses. We detected DcPLV in RNA samples from China, Taiwan, and Brazil. The genome organization of DcPLV suggests that it is a new unclassified picorna-like virus which is phylogenetically close to *Iflaviridae*, a relatively newly recognized insect virus family. The reo-like virus DcRV has been previously described to occur in a subset of the natural *D. citri* population in Florida, and here we provide additional genome sequence information and also evidence for its incidence in *D. citri* from Hawaii, China, and Taiwan.

Identification of a densovirus from RNA libraries is not surprising. Detection of DNA viruses via small-RNA and total-RNA sequencing has been previously reported (42, 47, 99). In the case of transcriptome sequencing, viral mRNA transcribed from DNA genomes and even small amounts of DNA have been implicated as explanations for the detection of a DNA virus via RNA sequencing (99). The underlying mechanism for detection of DNA viruses by sequencing of small RNA libraries has been provided by evidence that antisense transcripts with the potential to form dsRNA by base pairing with sense transcripts are produced in different families of DNA viruses, and these could be targeted by RNAi activity (95, 100–102). It is worth mentioning that densoviruses have some features that make them attractive for use as biological control agents (103). In fact, developing genetically modified densoviruses to express genes of interest and their successful application *in vitro* has been discussed as an alternative approach to control mosquito populations (103–105).

The putative bunyalike virus identified in this study displayed the highest amino acid similarities with newly discovered bunyavirids (18, 106). In 2014, Ballinger et al. discovered the most divergent group of bunyavirids, informally referred to as phasmaviruses, including *Kigluaik phantom virus* (KIGV) and *Nome phantom virus* (NOMV) in phantom midges (106). The phasmaviruses shared only 30% amino acid identity with the RdRps of other bunyaviruses (106). One year later, another group (18) sequenced RNAs extracted from 70 arthropod species in China, and they discovered sequences in the mosquito (*Wuhan mosquito virus 1*), cockroach (*Wuchang cockraoch virus 1*), and water strider (*Gerridae*) (*Sanxia water strider virus 2*) that displayed high amino acid similarities to phasmaviruses. During preparation of the manuscript, two other novel bunyavirids isolated from mosquito cell lines, *Jonchet virus* (JONV) and *Ferak virus* (FERV), were described (85). JONV and FERV phylogenetically branch from an old common ancestor in a similar manner to unclassified Phasmaviruses (85). The discovery of this new group of bunyaviruses in distantly related arthropods highlights the importance of these viruses as potential emerging agents.

Two CBPV-related viruses, *Anopheline-associated C virus* (AACV) and Dansoman virus, have recently been reported from mosquito field populations and *Drosophila melanogaster*, respectively (25, 60). These two viruses displayed protein sequence identities of <30% to the reference CBPV RNA 2, which is consistent with our observation in this study for DcACV. Thus, these recent discoveries show that the incidence of CBPV-related viruses is not restricted to only honeybees and that this group of viruses probably has a wide distribution.

Metagenomic approaches for virus discovery have been used for a range of organisms, mostly including those of potential medical importance. Compared with the viral communities found among mosquitoes and bats by approaches similar to those used in this study (13, 18, 25, 107, 108), the diversity of viruses found in this agricultural pest insect was much lower. Obviously, host range of the target sampled can play an important role in the diversity of viral sequences detected. Mosquitoes are blood-feeding arthropods and are known to feed on a wide range of sources, including humans, nonhuman primates, other mammals, birds, and even plant nectar (13, 25). As the second most diverse group of mammals, bats are natural reservoirs of many emerging viruses and feed on a diverse array of biota (107, 109, 110). Thus, for bats and mosquitoes, the viral sequences detected through metagenomics likely reflect viruses infecting the host sampled as well as biota on which these hosts feed. It is only recently that metagenomic approaches have been applied to viral discovery in insect pests of plants. For example, a vector-enabled metagenomics study of *Bemisia tabaci* from a single site in Florida resulted in the detection of mostly plant viruses and some insect virus-like sequences (111). Unlike the animal-feeding targets mentioned above (bats and mosquitoes), *D. citri* feeds almost exclusively on citrus. Thus, our analyses supporting the idea that the putative viruses described here are active insect viruses suggests that *D. citri*, rather some other unknown insect, is their likely host.

In summary, by pairing Illumina high-throughput NGS technology with a dedicated bioinformatics workflow, we obtained a snapshot of the viral sequences associated with *D. citri* populations from various world regions. Additionally, this initial characterization sheds light on the diversity of putative viruses in non-blood-feeding insects and will aid further studies in identifying and finding biotechnological applications for insect viruses. However, the data presented here likely do not depict the total diversity of viral sequences in *D. citri*. Sample collection was a limitation in this study. Samples were collected in different geographic locations and at different times and probably reflect different ages of *D. citri*. We also do not know enough about the genetic diversity among the *D. citri* populations studied here, and it has been demonstrated

that the viromes of mosquitoes and bats vary by species, age, space, time, and sample type (13, 107).

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