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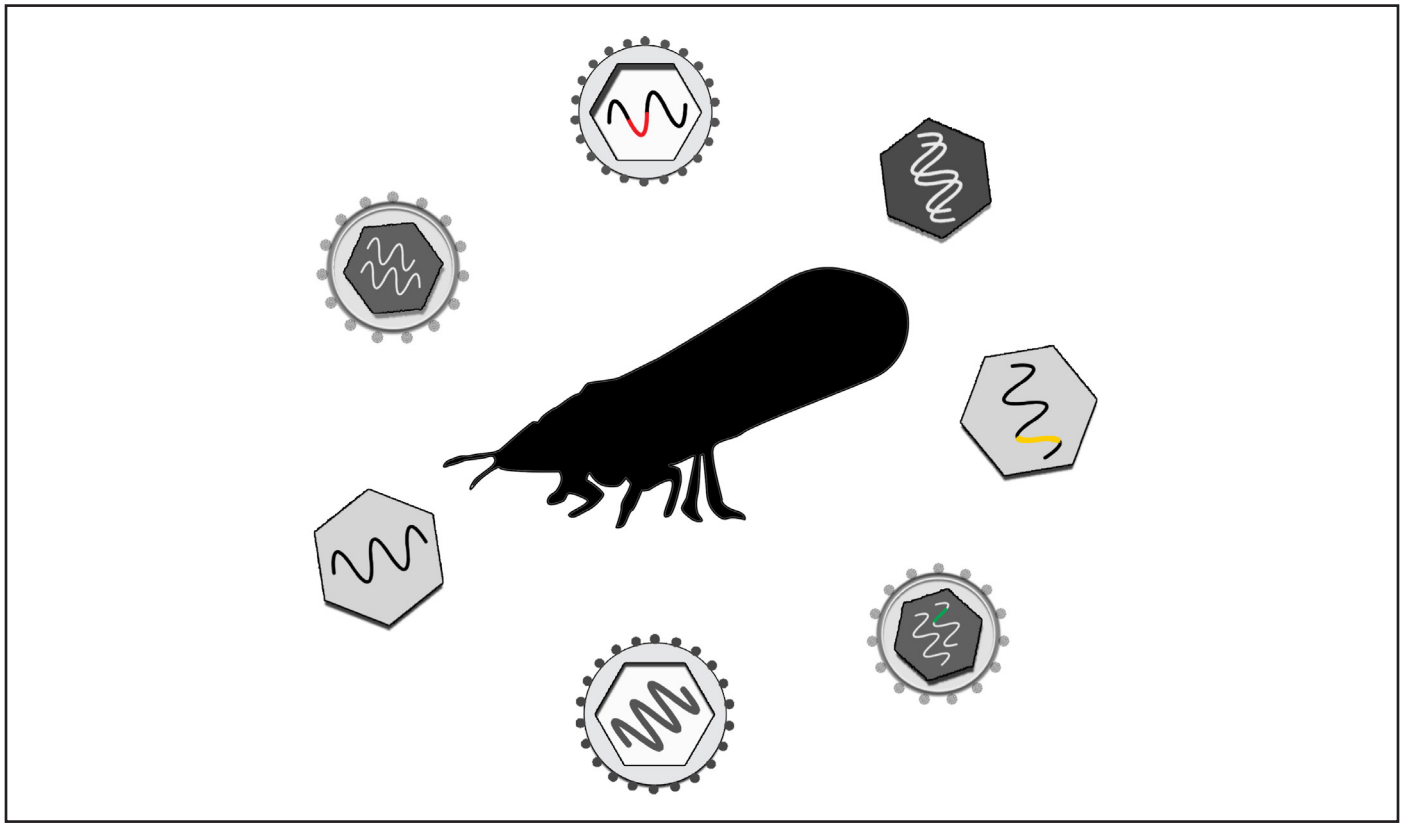

Illustration depicting the different types of viruses (black and white virus particles) found in worldwide populations of* Diaphorina citri *during this study. The two virus particles with colored sections of the viral genome represent potential recombinant viruses that could be developed to target* D. citri *and produce desired phenotypes.

ACP Are Full of Viruses. Can We Use Them Against HLB?

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Project Summary

We examined worldwide populations of Diaphorina citri, *also known as the Asian citrus psyllid (ACP), for viruses that might be useful in developing new strategies against* D. citri *and its ability to transmit '*Candidatus *Liberibacter asiaticus' (CLas) to citrus plants. In this survey, we discovered six previously undescribed viruses. Because these viruses are newly described, nothing is known of their biology; so for five of these viruses, we have established separate* D. citri *colonies infected with one of the viruses within the University of California, Davis (UC Davis) Biosafety Level-3 (BSL-3) Contained Research Facility (CRF). We also have established colonies using ACP collected from four geographically distinct locations. None of these viruses induce obvious negative effects in* D. citri, *but we are attempting to manipulate them to interfere with* D. citri *and/or its ability to transmit CLas. For this approach, we also are using two well-characterized insect viruses, Flock house virus and Cricket paralysis virus. We hope that by gaining a greater fundamental understanding of* D. citri *and virus interactions, we can use virus(es) for novel approaches against* D. citri *and huanglongbing (HLB).*

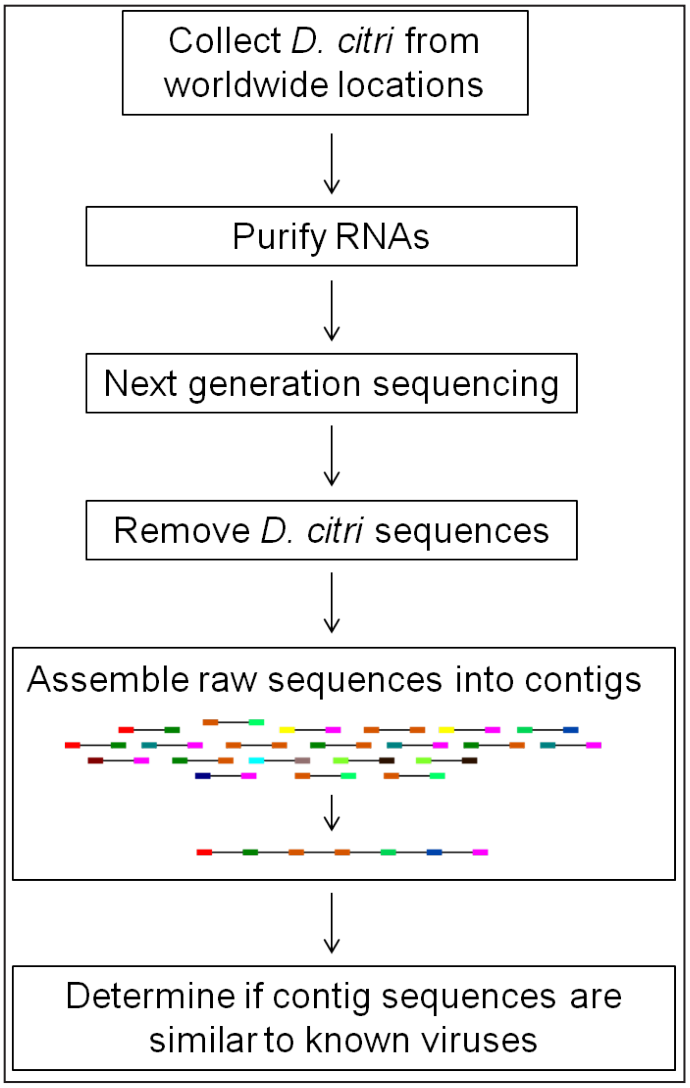

Figure 1. General outline of procedures used to identify new viruses from worldwide* Diaphorina citri *populations.

Viruses are the most abundant microbes on the planet. With so many viruses floating around in our environment, can we put them to use in the fight against HLB?

Before 2015, there was only a single report of a possible virus from *D. citri*. The ACP was collected in Florida and was based on limited sequence data that suggested a possible virus could be found in some Florida *D. citri* (Marutani-Hert et al. 2009). We felt that there must be more, so we contacted colleagues throughout the world's citrus growing areas and asked them to collect samples from wild *D. citri* populations and then ship dead *D. citri* to us. We planned to use these samples for next generation sequencing[1] (NGS) and bioinformatics[2] analysis to identify genomic sequences[3] corresponding to possible *D. citri*-infecting viruses (**Figure 1**).

We received *D. citri* samples from Florida, Hawaii, Texas, Pakistan, China, Taiwan, Brazil, Puerto Rico and Uruguay. We extracted and purified the complement of RNAs from these *D. citri* samples and sent them to commercial companies for NGS sequencing. Bioinformatics analysis showed that most of the sequences corresponded to *D. citri* RNAs. The *D. citri* sequences were removed and the remaining sequences were assembled into contigs[4] of larger sequences. These contigs then were compared to a publicly available database of all known genetic sequences to see if any of the sequences found in *D. citri* were similar to previously described viruses (**Figure 1**). We found that some of the putative viral sequences from *D. citri* were similar, but not identical, to sequences corresponding to known viruses, suggesting the presence of previously unidentified viruses (Nouri et al. 2015). Using additional bioinformatic tools, we confirmed the existence of six previously undescribed insect viruses and one bacterial virus in *D. citri* (**Table 1**). Not all viruses were present in all *D. citri* populations. Six of the seven viruses were found in *D. citri* collected from China, five of the seven were found in Florida *D. citri* and only two in California *D. citri*. This could be a reflection of our narrow and small sample sizes or it may indicate differences in the susceptibility of different *D. citri* populations to infection with these viruses.

UC Davis has a BSL-3 CRF to research exotic plant pests and pathogens. No live insects, plants or infectious materials can be removed from the CRF. We obtained necessary federal, state and university permits that allowed us to recover infectious viruses from *D. citri* from other countries. In some cases, we were able to import live *D. citri* infected with specific viruses (Taiwan, Uruguay and Hawaii) for research in the CRF.

As all of the viruses identified by NGS and bioinformatics were newly discovered, we did not know anything of their biological or molecular properties. Since these are important considerations for determining which, if any, viruses may be useful for our long-term goals, we currently are focusing efforts on five of the six insect viruses we identified in *D. citri* - *Diaphorina citri* reovirus (DcRV), *Diaphorina citri* flavi-like virus (DcFLV), *Diaphorina citri* picorna-like virus (DcPLV), *Diaphorina citri* densovirus (DcDV) and *Diaphorina citri*-associated C virus (DcACV) (**Table 1**).

We have studied several aspects of DcRV, which is a typical "reovirus." Reoviruses are widespread, common viruses found among many different types of hosts, including plants, animals and humans. One thing that interested us initially about DcRV is that it infects almost 100 percent of the *D. citri* we tested from Hawaii. While Hawaii has had high *D. citri* populations for several years, there have been no observed cases of HLB in the state. Could DcRV have adverse effects on *D. citri*, or the HLB-causing bacterium, CLas, and thus have a role in the lack of HLB in Hawaii? We identified DcRV virus particles using electron microscopy. The virus particles are extremely abundant within all tissues of DcRV-infected *D. citri* (**Figure 2**), yet DcRV-infected *D. citri* show no obvious symptoms of infection. We found that DcRV is passed from infected female *D. citri* to 100 percent of the progeny and can be passed from DcRV-infected male *D. citri* to a low percentage of females during mating (Chen et al. 2019). However, DcRV was not readily spread from infected to

Table 1. Viruses identified from worldwide populations of *Diaphorina citri*.

Closest Family/Order	Virus Name	Closest Virus Species	*D. citri* population*	Maintained in the UC Davis CRF
Reoviridae	*Diaphorina citri* reovirus	Nilaparvata lugens reovirus	CH, TW, FL, TX, HI	Yes
Flaviviridae	*Diaphorina citri* flavi-like virus	Gentian Kobu-sho associated virus	CH, FL, HI	Yes
Iflaviridae	*Diaphorina citri* picorna-like virus	Deformed wing virus	BR, CH, TW, UY	Yes
Parvoviridae	*Diaphorina citri* densovirus	Mythimna loreyi densovirus	PK, BR, UY	Yes
Bunyavirales	Not yet named	Wuchang cockroach virus 1	CH, FL, TW	No
Unclassified	*Diaphorina citri* associated C virus	Chronic bee paralysis virus	CH, CA, TX, FL	Yes
Unclassified	Not yet named	*Wolbachia* prophage	BR, CH, HI, FL, CA, TX	No

CH=China; TW=Taiwan; BR=Brazil; PK=Pakistan; UY=Uruguay; FL=Florida; TX=Texas; CA=California; HI=Hawaii.

healthy *D. citri* (often referred to as horizontal transmission) in our studies, thus we do not know how efficiently it may spread among an existing *D. citri* population if DcRV-infected insects were introduced into a healthy population. We currently are conducting experiments to see if DcRV affects CLas, or vice versa in *D. citri*.

As with DcRV, DcFLV, DcPLV, DcACV and DcDV induced no obvious negative effects on infected *D. citri*. We also infected California *D. citri* with DcRV, DcFLV and DcACV via injection, but none of these viruses so far readily spread by horizontal transmission among *D. citri*. We are attempting to engineer some of these viruses to use in *D. citri*.

We also have used two model insect viruses with *D. citri* to help provide proof of concept for our approach. These are Cricket paralysis virus (CrPV) and Flock house virus (FHV). Both are well-studied viruses that we can manipulate in the lab using recombinant DNA technologies. CrPV and FHV have relatively wide host ranges among insects, and both infect *D. citri*. FHV does not induce any obvious symptoms in FHV-infected *D. citri*, but CrPV is lethal when injected into *D. citri*. Therefore, we have further modified CrPV to make it less virulent in *D. citri*. We are genetically engineering both viruses to induce specific RNA interference (RNAi) effects to modulate gene expression in *D. citri*. RNAi approaches are useful for targeting insect pests and plant pathogens (Rosa et al. 2018), and some successes obtained by several groups are very encouraging. But when RNAi inducers are delivered to insects by feeding, the resulting RNAi effects are most prominent in the insect gut, and not necessarily in distal tissues such as salivary glands. However, many viruses spread throughout the insect body, including to the salivary glands, during infection (*see our results with DcRV*). Therefore, using recombinant viruses[5] that induce RNAi effects could be a way to give desired RNAi effects in many different *D. citri* tissues. We now are attempting to use recombinant FHV and CrPV to target specific *D. citri* RNAs in various tissues. Our longer-term goal is to build on information gained from our work with CrPV and FHV by using our *D. citri* viruses to interfere with *D. citri* and/or its ability to transmit CLas.

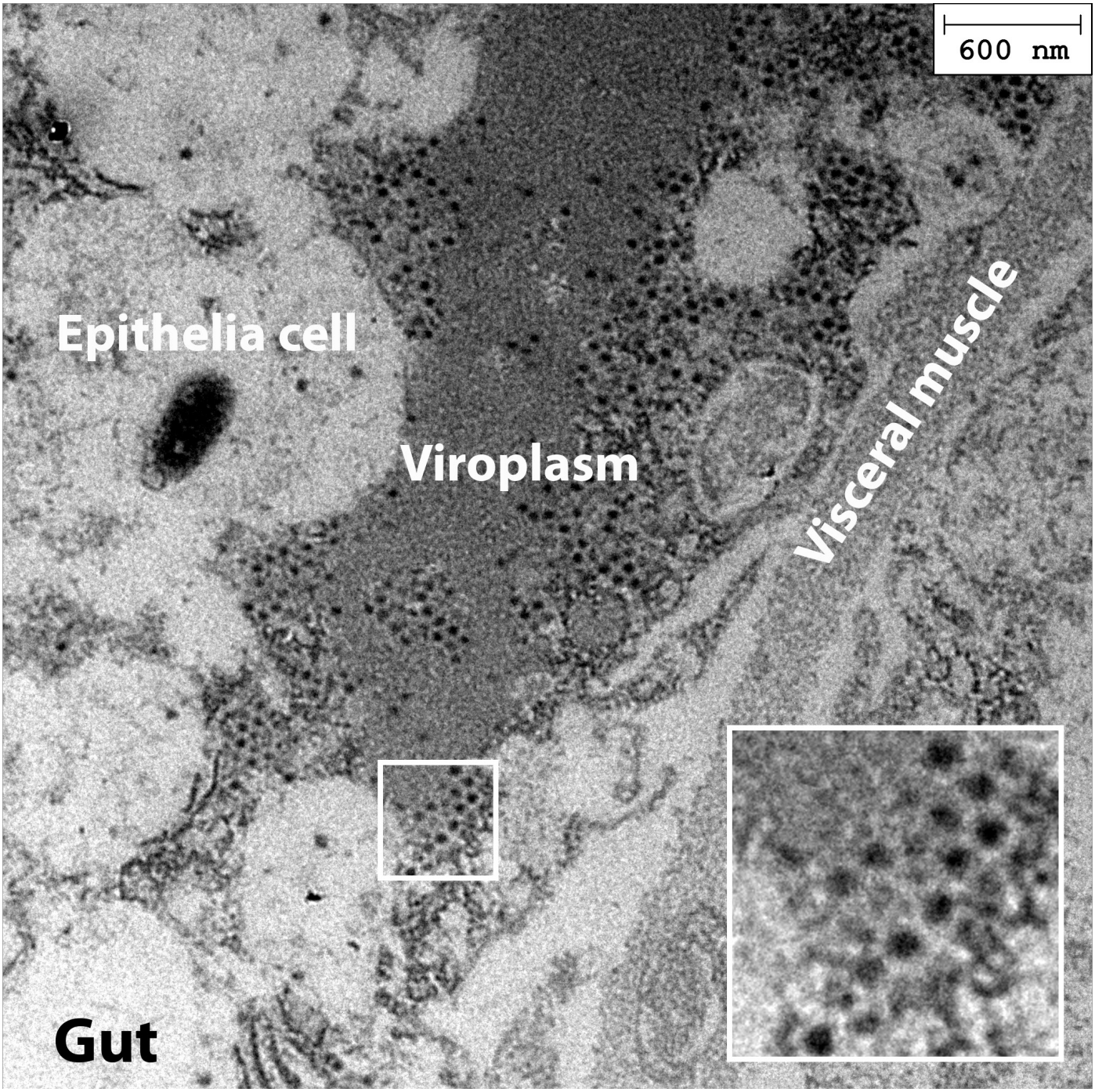

Figure 2. Transmission electron micrograph of* Diaphorina citri *reovirus (DcRV)-infected* D. citri *showing gut epithelial cells and abundant virus particles in a viroplasm. A viroplasm or inclusion body is a structure in a virus-infected cell where viral replication and assembly occurs. Similar features are seen in other* D. citri *tissues including salivary glands.

Using viruses as tools in biology raises many important questions. Are they safe? Will they spread to non-target hosts? Have viruses ever been used this way before? We already can answer some of these questions, and additional research is necessary to help answer others. First, recombinant viruses have been used for decades in the environment as very effective tools to combat rabies. Rabies is caused by the Rabies virus (RV)and is almost always lethal in humans without medical intervention. RV has a wide host range among many mammals and can be spread easily between different mammals by activities such as biting. That is why we vaccinate our dogs and cats against rabies, which provides excellent control in urban environments. However, what about some wild animals that are known to be very important reservoirs of RV? While injection-based immunization is impractical for wild animals, rabies control has been achieved in those populations using baits containing recombinant Vaccinia virus (VV) genetically engineered to contain one gene from RV. Baits containing recombinant VV are spread by a variety of means into wild

environments such as National Parks. The animals eat the bait and become infected with the recombinant VV, which causes a very mild infection, but they also become immunized against RV (Maki et al. 2017).

While our work above describes using *D. citri* viruses, there also are other ongoing efforts now to use a recombinant virus in citrus plants to help manage HLB. W. O. Dawson, Ph.D., and colleagues at the University of Florida have engineered mild forms of *Citrus tristeza virus* (CTV) to contain and express foreign genetic sequences encoding for anti-microbial peptides that may be able to target CLas and other CTVs to express sequences that may induce RNAi effects when *D. citri* feed on the infected plants (Dawson et al. 2015). Southern Gardens Citrus Nursery in Florida applied for a permit to test recombinant CTV expressing anti-microbial peptides in field grown citrus in Florida (*http://citrusindustry.net/2017/04/07/usda-announces-intent-prepare-eis-ge-citrus-tristeza-virus/*).

Thus, our efforts with *D. citri* viruses are not without precedent in terms of using viruses for beneficial accomplishments, even in citrus, but they are new for *D. citri*. It is important to remember that all of our current research is done in the CRF, not in the open environment. We are in the research/experimental phase and believe that such fundamental research is necessary and will lead to translational opportunities in the future.

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Glossary

[1]Next generation sequencing: NGS technologies are used to determine the nucleotide sequences of DNA and RNA molecules in a high throughput and robust manner. Millions of sequences are generated and used for bioinformatics analyses.

[2]Bioinformatics: Computer-based tools used to analyze sequences.

[3]Genomic sequences: Short segments of DNA or RNA.

[4]Contigs: Overlapping genomic DNA or RNA sequences used to assemble genomes.

[5]Recombinant virus: Virus containing specific genetic sequences derived from another source.

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