GENOMIC SEQUENCING, DISCOVERY, AND CHARACTERIZATION OF VIRAL PATHOGENS IN THE GLASSY-WINGED SHARPSHOOTER

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ABSTRACT
A new viral pathogen of the glassy-winged sharpshooter, GWSS, (*Homalodisca vitripennis*, aka *H. coagulata*) was discovered and characterized. Few pathogens of leafhoppers have been discovered which have potential for use as a biological control agent. To identify new pathogens of GWSS we used a genomic approach to isolate, sequence, and identify expressed sequence tags, ESTs, produced from field collected GWSS populations. Viral sequences were identified out of the initial 9,620 ESTs generated from single-pass 5' end sequencing of the GWSS expression library. Processing produced 8,795 ESTs which had lengths greater than 100 nucleotides post quality and vector trimming. The ESTs had an average read length of 689 bp, and an average inset size of 899 bp. Sequences shorter than 200 contiguous bases were removed from analyses. After assembly there were 3,008 sequences, 799 contigs with an average length of 1,113 bp, and 2,209 singlets. Using these sequences to get a start, it was possible to completely sequence the full virus genome, and the virus was labeled, HoCV-1. Further analyses and characterization of HoCV-1 demonstrated that it infected and crossed the midgut barrier of GWSS. The virus was classified as a member of the family *Dicistroviridae,* which are single-stranded RNA viruses which do not have a DNA stage. Two other viruses were also identified which are currently being characterized and which were taxonomically unrelated to HoCV-1. These leafhopper viral pathogens appear to induce increased mortality, 40% or more, during the nymphal stages of leafhopper development and may have further applications in the management of leafhopper pests to reduce the spread of Pierce's disease of grapes.

INTRODUCTION
Leafhoppers are the second most serious agricultural pest, after aphids, both of which transmit plant diseases. Few leafhopper pathogens are known and efforts to discover pathogens can be costly. Where it occurs the glassy-winged sharpshooter, GWSS, *Homalodisca vitripennis* Germar 1821 (Hemiptera: Cicadellidae) (Takiya et al., 2006) is the primary vector in the spread of Pierce's disease, PD, of grapes. Pierce's disease is caused by strains of the plant-infecting, *Xylella* bacteria, which cause severe economic losses to viticulture and other tree crops in the USA. The GWSS readily flies long distances, thus spreading PD throughout and across grapes growing regions. To maximize our efforts we chose an approach which would advance our understanding of the genetic basis of leafhopper biology, while optimizing efforts to discover leafhopper pathogens. Therefore, we chose to create a large-scale 5′ end sequencing project of cDNA clones produced from adult GWSS. The use of expressed sequence tags (EST) have proven to be a rapid method to generate important genomic information which permits researchers to address difficult questions concerning insect biology, pathology and disease transmission.

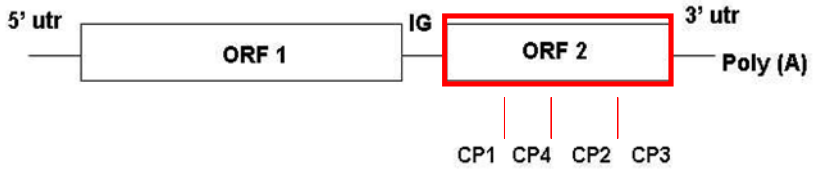
OBJECTIVES
Search for viral pathogens of sharpshooters using the molecular approach of cDNA libraries which provide a rapid, cost effective method that advances our understanding of an organism, plus identifies the invisible, unknown, internal/external organisms associated with the target species, the GWSS, or other leafhopper. Viral pathogens are tools for leafhopper management and open new avenues to reduce PD.

RESULTS & DISCUSSION
GWSS Genomics: Adult GWSS were collected from a citrus grove near Riverside, CA. Of the initial 9,620 ESTs generated from single-pass 5' end sequencing of the GWSS expression library, 8,795 ESTs had lengths greater than 100 nucleotides post quality and vector trimming. The ESTs had an average read length of 689 bp, and an average inset size of 899 bp. Approximately 500 of these sequences were identified as having significant homology to a virus. After assembly of the dataset there were 3,008 sequences, 799 contigs with an average length of 1,113 bp, and 2,209 singlets, average length of 681 bp. Of the total assembled 3,008 sequences, 1,574 or 52.3% corresponded with putative matches in GenBank at an E-value of <-10, while 1,434 sequences, or 47.7%, had 'no significant homology' to sequences currently listed in GenBank,nr database by *in silico* analyses (BLASTX, TBLASTX, BLASTN). The sequence data described in this paper have been submitted GenBank Accession Numbers CF194966 through CF195393. National Center for Biotechnology Information, NCBI. The capsid protein sequence of HoCV-1 was submitted into GenBank (accession number: DQ308403). The genomic architecture was determined (Figure 1) and phylogenetic analyses performed as in Hunnicutt et al., 2006, (Figure 2), (Table 1).

Genomic characterization of the virus: demonstrated that HoCV-1 was a close member of the Family: *Dicistroviridae,* which are single-stranded, RNA viruses, with no DNA stage. The complete nucleotide sequence of HoCV-1 infecting GWSS was determined and further *in silico* analysis revealed a genome containing 9,321-polyadenylated nucleotides encoding two large open reading frames (ORF1 and ORF2) separated by a 182-nt intergenic region (IGR) (Figure 1). The deduced amino acid sequence of the 5′-proximal ORF (ORF1, nt 420–5807) exhibited conserved core motifs characteristic of the helicases, cysteine proteases, and RNA-dependent RNA polymerases of other insect-infecting picorna-like viruses. These viruses are often reported to be in association with increased mortality of their infected host, as with fire ants, and honey bees. Virus analysis and detection from salivary gland (Sg) and midgut (Mg) tissues of *Homalodisca vitripennis* adults tested for presence of HoCV-1, using rtPCR. Both types of tissues from individual insects were dissected and analyzed in a pairwise fashion. Only midgut tissues were shown to test positively for virus presence. Tissues were then prepared for examination by transmission electron microscopy (Figure 3.). Virions were observed to be present in high numbers within midgut tissues of GWSS which tested positive for HoCV-1, and absent when GWSS tested negatively. The virions appeared to be taken up by the microvilli of the midgut and to propagate within the basal laminae (Fig 3).

Geographic Distribution: A host range for HoCV-1 was conducted. Adult leafhoppers were collected over a two year period throughout various geographic locations, including Florida, Georgia, South Carolina, North Carolina, California, and Hawaii. Other leafhopper species were also evaluated. The presence of HoCV-1 was detected in both sexes and all developmental stages of GWSS, including eggs (Hunnicutt et al., 2007). Although no mode(s) of transmission could be conclusively accepted/rejected, these results suggest that infection may be maintained through both transovarial and transstadial transmission. In Florida viruliferous samples were detected only in Gadsden and Suwannee counties, two of the three localities in which GWSS were most abundant. This account is similar with a distribution study conducted by Hoddle et al. (2003) which found that significantly more GWSS inhabit north Florida than central and south Florida. Infected GWSS were found in Georgia, South Carolina, and North Carolina with the incidence of infection ranging from 8–100% (Hunnicutt et al., 2007). Conversely, our assay failed to detect *HoCV-1* in any of the GWSS collected from the island of Oahu, HI. Virus infection was distributed among populations regardless of the host plant from which the insect was harvested. Adults of two additional sharpshooter vector species, *H. insolita* and *O. nigricans*, collected in north Florida were also demonstrated to be natural hosts for *HoCV-1* However, neither *D. minerva* nor *G. atropunctata* tested positive for the virus. These findings suggest that while *HoCV-1* is not limited to *H. vitripennis*, infection was not ubiquitous to all sharpshooter genera evaluated.

Figure 1. Genomic organization of HoCV-1, Capsid proteins are encoded at the 3′ end. Organization follows that of other Dicistroviruses. The complete nucleotide sequence of HoCV-1 infecting GWSS *in silico* analysis revealed a genome containing 9,321-polyadenylated nucleotides encoding two large open reading frames (ORF1 and ORF2) separated by a 182-nt intergenic region (IG).



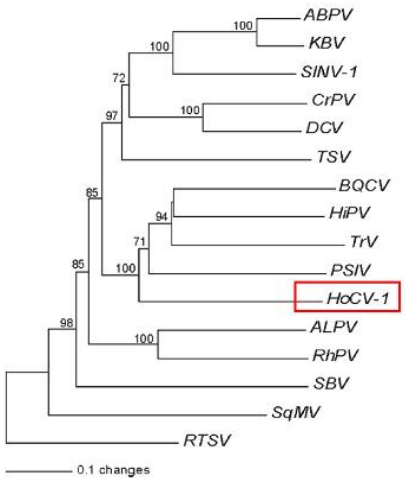


Figure 2. Phylogenetic analysis of HoCV-1 and other positive-sense ssRNA viruses based on amino acid sequence of the putative RNA-dependent RNA polymerase (RdRp). Neighbor-joining were produced using PAUP 4.0b software, 1000 bootstrap replicates. Outgroup was Sacbrood virus (SBV).

Table 1. Comparison of amino acid sequence of *Homalodisca vitripennis virus*, HoCV-1, capsid polyprotein to other viruses in *Dicistroviridae*. Percent identity and similarity from BLASTX.

Capsid Polyprotein		Identity %	Similarity %
HoCV-1	**905 aa**	**100**	**100***
PSIV	874 aa	25	44
TrV	915 aa	27	44
HiPV	889 aa	26	44
BQCV	885 aa	27	44
DCV	937 aa	24	40
CrPV	926 aa	23	39
ALPV	888 aa	26	40
RhPV	893 aa	26	41
ABPV	968 aa	22	38

Drosophila C virus (DCV) (Johnson and Christian 1998), *Cricket Paralysis virus* (CrPV) (Koonin and Gorbalenya 1992), *Aphid lethal paralysis virus* (ALPV) (Munster et al., 2002), *Rhopalosiphum pisum* virus (RhPV) (Moon et al., 1998), *Triatoma virus* (TrV) (Czibener et al., 2000), *Plautia stali intestine virus* (PSIV) (Sasaki et al., 1998), *Himetobi P virus* (HiPV) (Nakashima et al., 1999), *Black Queen Cell virus* (BQCV) (Leat et al., 2000), *Acute bee paralysis virus* (ABPV) (Govan et al., 2000). (From Hunter et al., 2006).

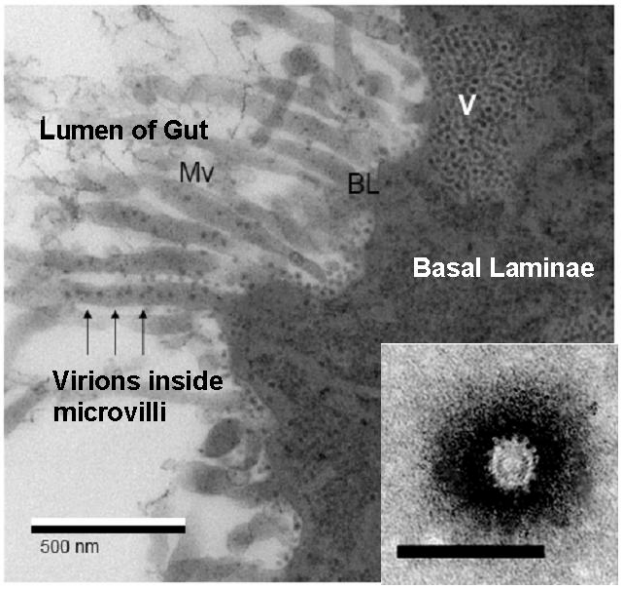


Figure 3. Electron micrograph of a single virion prepared from infected adult leafhoppers, *Homalodisca vitripennis*, glassy-winged sharpshooter. Scale bar = 100 nm.

CONCLUSIONS

The production of cDNA libraries provides rapid and cost effective methods that advance our understanding of an organism and the interactions of the invisible internal/external organisms associated with the target species, the GWSS. We have been using this method for the last four years to discover leafhopper pathogens, and to gather insights into the genetic basis of leafhopper biology, pathogen interactions. The first leafhopper virus characterized, HoCV-1, has demonstrated that it may have use to decrease GWSS population numbers. Insect viruses can cause indirect mortality by making the infected insects more susceptible to insecticide applications, and/or to parasitization and predation by reducing the activity of the leafhopper. Two other new leafhopper viruses which we have been discovered are currently being further characterized to determine if they too have application as biological control agents, and/or as gene delivery tools, to be used in the management of GWSS and other leafhopper pests, aimed as reducing the spread of Pierce's disease of grapes as well as other *Xylella* caused plant diseases.

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