

# NON-CULTURE DEPENDENT SURVEY OF THE MICROBIOTA OF THE GLASSY-WINGED SHARPSHOOTER USING 454 PYROSEQUENCING

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**Reporting Period:** The results reported here are from work conducted March 2009 to December 2009.

**ABSTRACT**

The glassy-winged sharpshooter (GWSS; *Homalodisca vitripennis*) is an invasive pest that has spread across the southern and western United States. It is highly polyphagous, feeding on at least 100 species in 31 families (Hoddle et al., 2003; Turner and Pollard, 1959), and a voracious feeder, having been known to consume up to 100 times its weight in xylem fluid daily. This insect is a vector of the phytopathogen *Xylella fastidiosa* (*Xf*), which is the causative agent of Pierce's disease (PD) in grapevines. In order to evaluate the microbial flora associated with GWSS hemolymph, alimentary canal excretions and whole insect bodies were subjected to 16S pyrosequencing using the bTEFAP methodology and the resulting sequences (370-520 bp) were compared to a curated high quality 16S database derived from NCBI's GenBank. Species from the genera *Wolbachia*, *Delftia* (formerly *Pseudomonas*), *Pectobacterium*, *Moraxella*, *Serratia*, *Bacillus* and many others were detected and a comprehensive picture of the microbiome associated with GWSS was established. Some of the bacteria identified in this report are initial discoveries and having a breadth of knowledge as to the microbial flora of this insect pest can serve as a reservoir of information for developing biological control strategies. One method for biological control can be the genetic engineering of a particular bacterium to deliver certain molecules known to affect the life stage development of an insect. Another method, could be isolating a bacterium that competes with *Xf*, and re-delivering it to wild populations in excess of their natural bacterial load. Within this study, we have identified the types of bacteria which may be ubiquitous among GWSS providing us with targets to begin to investigate these future directions.

**LAYPERSON SUMMARY**

The glassy-winged sharpshooter is an insect pest that spreads the bacterium *Xylella fastidiosa*, the causal agent of Pierce's disease in the grapevine. Both wine and table grape production is affected by this disease and it has become a major financial burden on the industry. Bacterial DNA can be used to screen for such pathogens in insect populations and having knowledge of the bacterial pathogens of the insect can be used to develop a biological control strategy. Hemolymph, alimentary canal excretions and whole insect tissue were subjected to DNA sequencing and the resulting sequences were matched to groups of bacteria using NCBI's GenBank database. This study shows that bacteria such as *Wolbachia*, *Delftia*, *Pectobacterium*, *Moraxella*, *Serratia*, and *Bacillus* spp may be useful targets to develop biological control strategies.

**INTRODUCTION**

The glassy-winged sharpshooter (GWSS) is a highly mobile pest and transmits the xylem-limited bacterium *Xylella fastidiosa* (*Xf*). This bacterium can cause disease in many economically important plants including the grapevine, peach, and citrus and has become a major limiting factor in their mass production. The bacterium can also cause disease in ornamentals such as the oak, elm and sycamore. Once a plant has become infected with *Xf*, it becomes a reservoir for bacterium and can be easily spread from plant to plant by the near-continuous feeding of GWSS. Although pesticides are available for the control of the insect, resistance and harm to non-target insects is an issue of concern. Naturally occurring forms of control have been successful and should be further pursued as a complimentary strategy for insect and disease control.

Many insect taxa have obligate endosymbionts that supplement nutrition in exchange for vertical or horizontal transfer among individuals (Moran et al 2005, Buchner 1965). This mutualism has allowed insects to occupy or thrive in otherwise hostile niches. The GWSS, a xylem feeder, is known to host several bacterial species including *Baumannia cicadellinicola* and *Sulcia muelleri* (Wu et al 2006). The *B. cicadellinicola* genome is devoted to the biosynthesis of vitamins and cofactors but lacks most amino acid biosynthetic pathways, whereas *S. muelleri* apparently produces most of the amino acids needed for the host. DGGE has been used to find other symbiotic bacteria including *Wolbachia*, *Bacillus*, *Pseudomonas*, *Pedobacter*, *Methylobacterium*, and *Curtobacterium flaccumfaciens* which the authors suggest could be used as forms of symbiotic control (Lacava et al 2007). Curly et al (2007) identified bacteria closely related to *Stenotrophomonas* and *Acinetobacter* in hemolymph samples.

**OBJECTIVES**

1. Identify major groups of bacteria in the hemolymph, alimentary canal and whole insect.
2. Identify species of bacteria for possible transgenesis and biological control.

## RESULTS AND DISCUSSION

Using 16S pyrosequencing based upon the bTEFAP methodology (Dowd et al., 2008a; Dowd et al., 2008b) optimized for the Titanium pyrosequencing platform (Roche, Indianapolis, IN), we were able to identify 17 orders (**Figures 1-3**), 28 families and at least 38 genera (**Figures 4-6**) of bacteria. Sequences were taken from separately prepared extracts of hemolymph, alimentary canal excretions and macerated whole insects suspended in 1X PBS. The sequences were approximately 500 bp (370-520 bp) and were compared to NCBI's basic local alignment search tool (BLAST) for homologies. Some of the shorter sequences aligned to multiple genera and were placed in a separate category called "Other" because it was not clear which identification was appropriate.

The hemolymph extracts (**Figures 1 and 4**) contained over 1000 sequences aligning with the order Enterobacteriales although no *Enterobacter* were found at the genus level. Up to 27 sequences from Burkholderiales were found in the hemolymph but no *Burkholderia*, *Bordetella* or *Oxalobacter* related sequences were found at the genus level. A single sequence aligning with Rhizobiales and two sequences aligning with *Clostridium* were also found in the hemolymph.

The alimentary canal excretions (**Figures 2 and 5**) contained Enterobacteriales related sequences. This coupled with the Enterobacteriales found in the hemolymph may be a sign of cross-contamination with respect to preparation of samples. The hemolymph vessels and the alimentary canal lie close to one another in the body of the insect and may have been punctured in some trials. Thirteen sequences from *Bacillus*, 52 *Moraxella* (an opportunistic cattle and human pathogen) and 72 *Serratia* (a human pathogen and lab-colony limiting agent) were recovered as well.

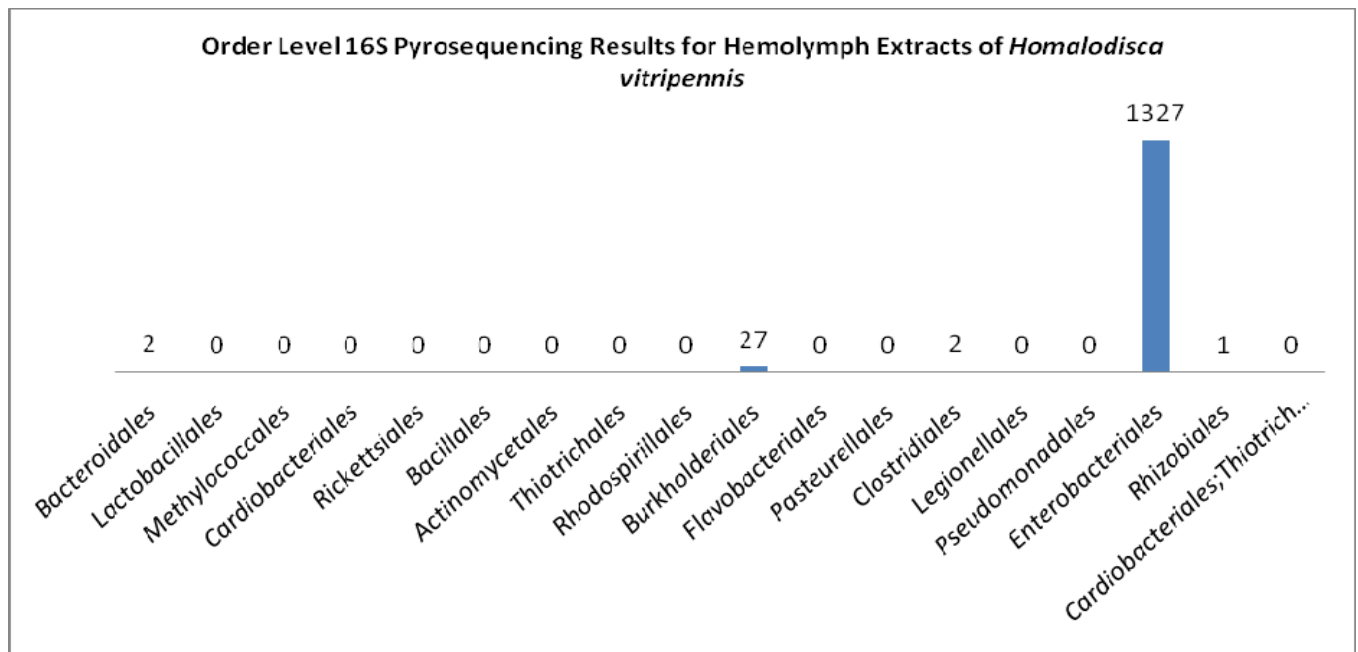
Whole insect macerations (**Figures 3 and 6**) contained 74 sequences related to *Wolbachia*, a well-known intracellular insect pathogen that has been characterized in previous studies. This symbiont has been shown to be obligate in many arthropods and nematode species (Mavingui et al 2005) and may be a target for limiting populations of GWSS. Sequences related to *Cardiobacterium* spp. were also recovered in large numbers from all but hemolymph samples. It is not clear why this particular bacterium is present.

Sequences of *Pectobacterium* were recovered from all extracts of the glassy-winged sharpshooter. This bacterium is known to cause soft rot and black leg in potato plants through its arsenal of extracellular pectinases (Chan et al 2009). This identification is believed to be the first report of this phytopathogen in GWSS.

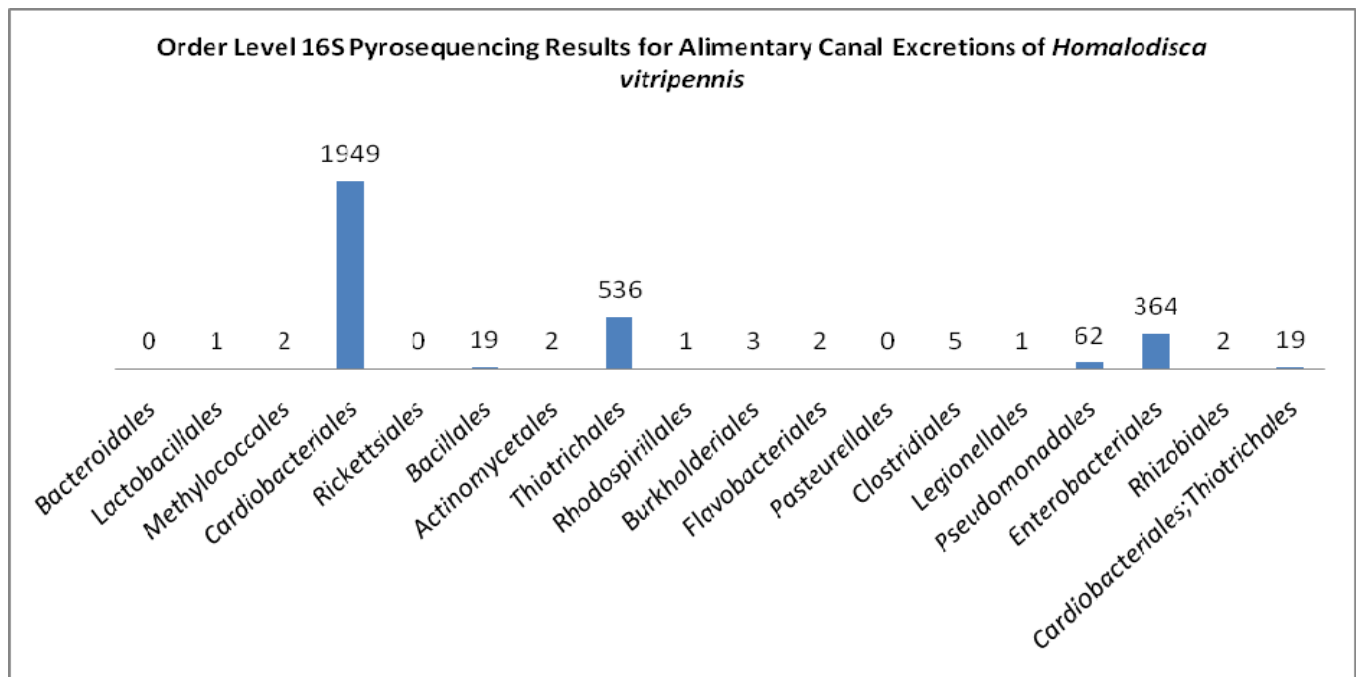
Although not clearly understood at this moment, sequences relating to *Delftia* sp were only recovered from the hemolymph extracts. *Delftia* sp are ubiquitous, rod-shaped, gram-negative bacteria (Hai et al 2007) that are able to degrade di-n-butylphthalate (DBP), an industrial pollutant and phthalate derivative, as a sole source of carbon and energy (Neelakanteshwar et al 2006).

Fifty two (52) sequences associated with *Moraxella* spp. were recovered from the alimentary canal excretions. This bacterium is a polymorphous gram-negative opportunistic pathogen of both humans and cattle and is known to cause conjunctivitis in both animals. It also causes ear, nose and throat infections and is known to be transmitted by flies (Ala'Aldeen 2007).

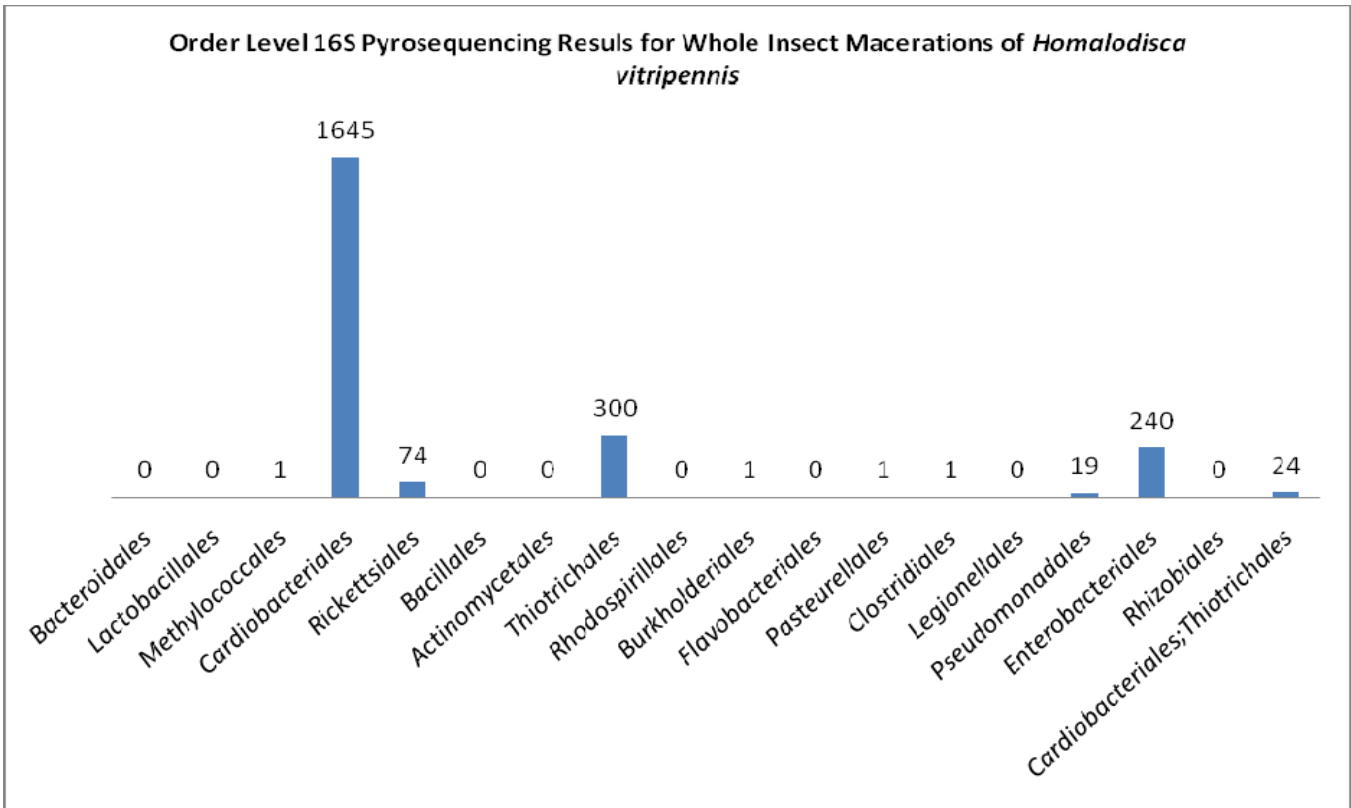
Although many different types of bacterial taxa were recovered from the extracts of GWSS it is important to recall that these sequences are small relative to even the whole 16S gene. While these 500 bp sequences are sufficient to identify bacteria to the order and perhaps even family level, their predictive ability can be less absolute at the genus and species level. However, this data can be used to design primers to "walk" down the gene and may be more adept at resolving the species level identifications. In addition, because it is estimated that less than 10% of all bacteria have ever been fully identified this study has shown that many novel genera may be associated with the GWSS microbiome. Further study of the microflora of GWSS will be needed to identify possible targets of paratransgenesis or obligate symbiont knockdown.



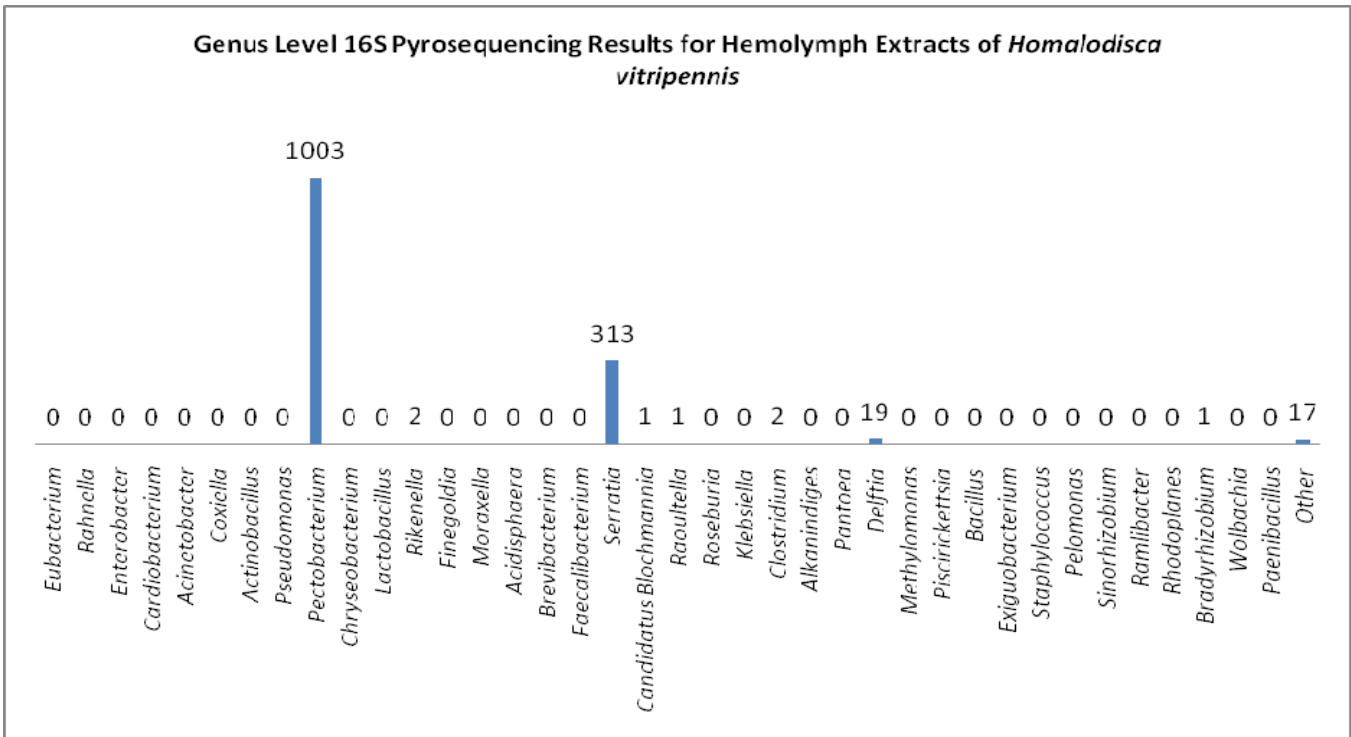
**Figure 1.** Order level sequencing results for the hemolymph of the GWSS. Larger numbers of sequences related to Enterobacteriales were recovered as well as Bacteroidales and Burkholderiales.



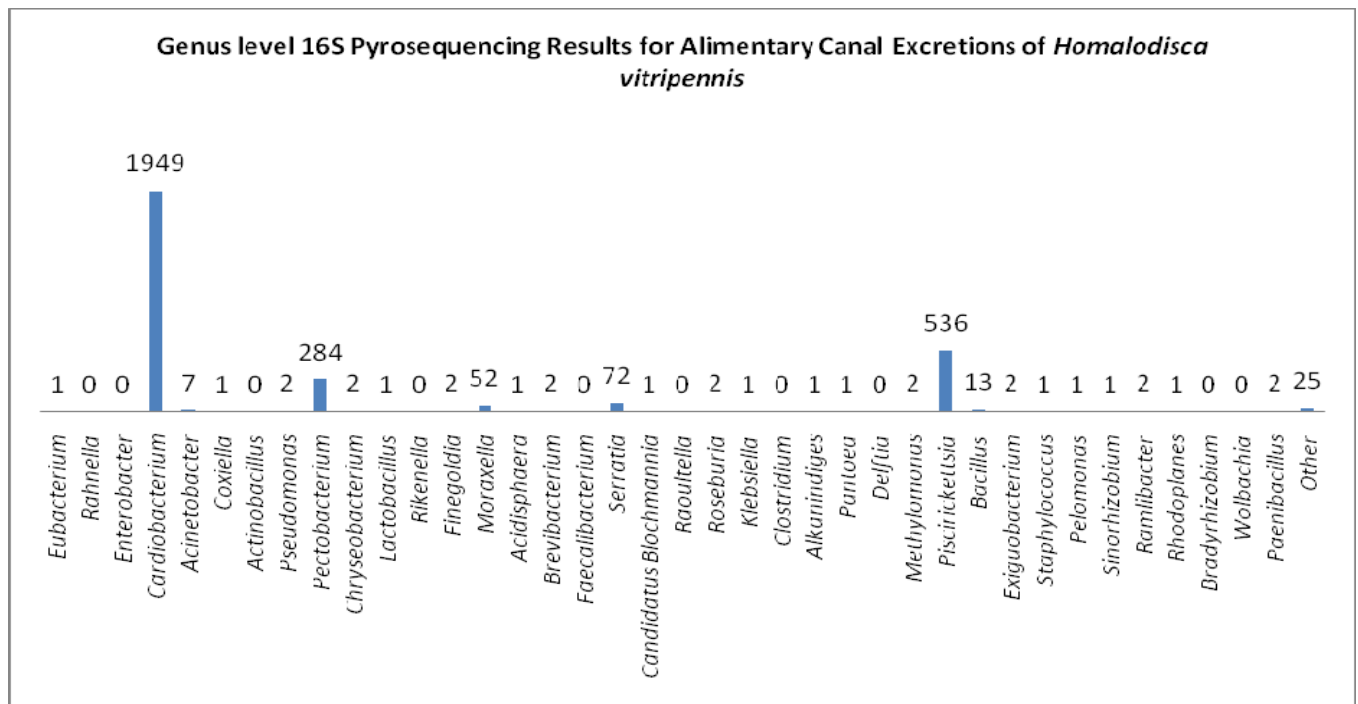
**Figure 2.** Order level sequencing results for the alimentary canal excretions of the GWSS. Sequences related to Cardiobacteriales, Thiotrichales, Enterobacteriales and others were recovered.



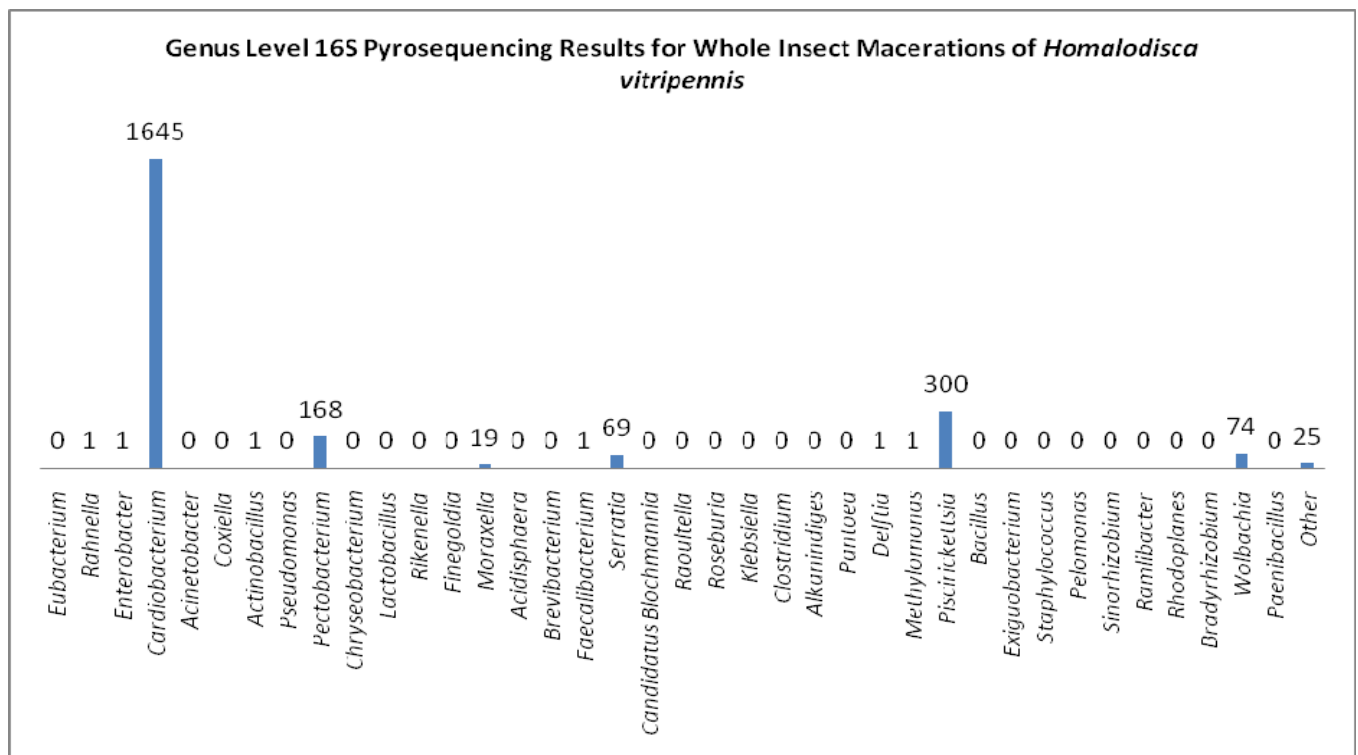
**Figure 3.** Order level sequencing results for the whole insect macerations of the GWSS. Sequences related to Cardiobacteriales, Thiotrichales, Enterobacteriales and others were recovered.



**Figure 4.** Genus level sequencing results for the hemolymph of the GWSS. Large numbers of *Pectobacterium* and *Serratia* were recovered. Some *Delftia* (formerly *Pseudomonas*) and other non-specific identifications were made. Note that no sequences from *Wolbachia*, an intracellular symbiont, were recovered.



**Figure 5.** Genus level sequencing results for alimentary canal excretions of the GWSS. Many sequences of *Cardiobacterium*, *Pectobacterium*, *Piscirickettsia* and *Serratia* were recovered. Other non-specific identifications were made. Note that no sequences from *Wolbachia*, an intracellular symbiont, were recovered.



**Figure 6.** Genus level sequencing results for whole insect macerations of the GWSS. Many sequences of *Cardiobacterium*, *Pectobacterium*, *Piscirickettsia* and *Serratia* were recovered. Other non-specific identifications were made. Note that sequences from *Wolbachia*, an intracellular symbiont, were only recovered from whole tissue extracts.

## CONCLUSIONS

Studies on the tsetse fly (*Glossina* spp.) have shown that transinfection by a genetically transformed strain of endosymbiont and expression of foreign gene products can be used to block transmission of a disease causing agent (Aksoy et al 2008). Paratransgenesis in the glassy-winged sharpshooter has not yet been extensively studied but one trial (Bextine et al 2004) did succeed in delivering *Alcaligenes xylosoxidans denitrificans*, expressing a red fluorescent protein to GWSS using a novel

feeding strategy. The bacterium was able to occupy the same area in the foregut normally associated with *Xf* infection. Using this system as a template and the results from the 16S pyrosequencing, we can now pursue multiple avenues of paratransgenesis.

The presence of *Delftia* sp exclusively in the hemolymph of GWSS provides an exciting possibility for this bacterium as a potential tool. A study by Wang et al (2008) showed that a new formulation of media was successful in culturing *Delftia tsuruhatensis* to a level 4.7 times higher than with un-optimized media. Other culture media are also available.

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#### FUNDING AGENCIES

Funding for this project was provided by the Texas Pierce's Disease Research and Education Program, and the USDA Animal and Plant Health Inspection Service.