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Geographic patterns in the bacterial microbiome of the glassy-winged sharpshooter, *Homalodisca vitripennis* (Hemiptera: Cicadellidae)

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Abstract Numerous environmental and physiological factors influence the composition of the bacterial microbiome inhabiting an insect. Pyrosequencing of bacterial 16S rRNA sequences was used to identify bacterial taxa inhabiting 50 *Homalodisca vitripennis* collected from nine Texas vineyards collected during the summer of 2007. After quantifying distances between insects based on differences in bacterial community composition, it was clear that the geographical locations of the insects collected had a substantial influence on their internal bacterial microbiota composition. There was a clear tendency for insects collected from the same vineyard to cluster in principal component analyses using said distances. A single bacterial species, Candidatus *Baumannia cicadellinicola*, a primary endosymbiont, represented the majority of bacteria sampled. Many of the more common bacterial genera contain a majority of species known previously to inhabit soil and plants exclusively, suggesting that the insect's environment is a major source of abundant species of microflora. Many of the bacterial genera sampled had significant positive correlations with other bacteria, and many of these correlations were between genera with the same order.

Keywords Glassy-winged sharpshooter · *Homalodisca vitripennis* · *Xylella fastidiosa* · Microbiome

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1 Introduction

The glassy-winged sharpshooter, *Homalodisca vitripennis* Germar (Hemiptera: Cicadellidae), is a vector of multiple species of bacterial phytopathogens known to cause disease in a wide variety of plants from fruit and nut crops, as well as woody ornamentals, and alfalfa (Davis et al. 1978). This insect has been identified as the most efficient vector of *Xylella fastidiosa* Wells (Xanthomonadales: Xanthomonadaceae) *ssp. fastidiosa* the causal agent of Pierce's Disease in grapevine (*Vitis* sp.) (Davis et al. 1978). This species resides within the foregut of the insect, along with a number of other bacterial species. Bacteria may also persist within the cibarial region of the insect along with other regions of the alimentary canal and the bacteriome, a specialized structure for the housing of endosymbiotic bacteria, and the hemolymph of *H. vitripennis*. Thus far, only a few studies have investigated the diversity and possible roles of these bacteria (Table 1).

The bacterial species inhabiting insect organs are numerous and varied across and within insect species, and a number of insect environmental and physiological factors affect these internal bacterial communities or microbiomes. Insect bacterial endosymbionts can be divided into three major categories: primary or obligate endosymbionts, facultative endosymbionts, and environmentally associated symbionts. Primary endosymbiotic bacteria are mutualists and not only usually grow exclusively in the internal organs or cells of the insect but are also required by the insect for survival These endosymbionts are exemplified by the inability to grow outside of the host organism and require the host for transmission (Moran et al. 2008). Examples include *Buchnera aphidicola* that help synthesize essential amino acids for aphid species (Moran et al. 2008). Candidatus *Baumannia Cicadellinicola* and Candidatus *Sulcia muelleri* are the only two primary bacterial endosymbionts that have been identified within *H. vitripennis*. Both of these species live in the bacteriome of *H. vitripennis*, a specific organ adjacent to the gonads that

Table 1 Names of genera identified in *Homalodisca vitripennis* analyzed in the current study that have been also been found by previous similar studies with location of bacterium within the insect

Genus	Location
Exiguobacterium	Alimentary canal[a]
Ramlibacter	Alimentary canal[a]
Pelomonas	Alimentary canal[a]
Finegoldia	Alimentary canal[a]
Eubacterium	Alimentary canal[a]
Finegoldia	Alimentary canal[a]
Eubacterium	Alimentary canal[a]
Pantoea	Alimentary canal[a]
Chryseobacterium	Alimentary canal[a]
Lactobacillus	Alimentary canal[a]
Rhodoplanes	Alimentary canal[a]
Klebsiella	Alimentary canal[a]
Paenibacillus	Alimentary canal[a], head[b]
Bacillus	Alimentary canal[a], head[b,c]
Staphylococcus	Alimentary canal[a], head[b]
Baumannia	Bacteriome[e]
Sulcia	Bacteriome[f]
Ralstonia	Cibarial region[g], foregut[g], midgut[g]
Alcaligenes	cibarial region[g], foregut[g]
Nocardia	Head[b]
Brevibacillus	Head[b]
Brevundimonas	Head[b]
Methylobacterium	Head[b,d]
Rhodococcus	Head[b]
Sphingomonas	Head[b]
Clostridium	Hemolymph[a]
Raoultella	Hemolymph[a]
Bradyrhizobium	Hemolymph[a]
Acinetobacter	Hemolymph[a], alimentary canal[c]
Serratia	Hemolymph[a], alimentary canal[a], whole insect[a]
Stenotrophomonas	Hemolymph[c]
Pseudomonas	Hemolymph[c], alimentary canal[a], head[d]
Faecalibacterium	Whole insect[a]
Enterobacter	Whole insect[a]
Rahnella	Whole insect[a]

[a] Hail et al. 2011
[b] Gai et al. 2011
[c] Curley et al. 2007
[d] Lacava et al. 2007
[e] Wu et al. 2006
[f] Moran et al. 2008
[g] Bextine et al. 2004

functions exclusively to house primary symbionts, inside of the specialized cells known as bacteriocytes. Both of these symbionts are vertically transmitted, as are all other primary symbionts, from mother to offspring via the oocytes (McCutcheon et al. 2009).

Facultative symbionts are much more diverse in terms of where they may reside in the host, whether or not they provide a function, what that function may be, and how there are transmitted (Moran et al. 2008). Facultative symbionts are epitomized by their ability to utilize their host insect to support and increase their own persistence and transmission. This may be accomplished by the bacteria providing an advantageous phenotypic change to the host in return for host residence and enhanced persistence of the host. One of the most widely distributed of facultative symbionts in insects, and arthropods as a whole, is *Wolbachia pipientis.* This species has also been confirmed to be widespread among *H. vitripennis*. Facultative bacterial symbionts may be transmitted horizontally or vertically through oocytes depending on their location and role inside the host, and some, such as *Wolbachia sp*., may be transmitted by either mode.

Environmentally associated bacteria, appear to provide no discernible advantage or disadvantage to the insect and vary based on the environment and/or diet of the host insect. At this point, most of the bacterial symbionts identified within the glassy-winged sharpshooter can, at best, be lumped into this category. Further investigation of the internal microbiota of this insect is needed to understand the possible roles of their internal symbionts.

A few studies have concentrated on identifying bacterial endosymbionts within *H. vitripennis*, mostly in effort to identify the level of how widespread specific symbionts are across insect individuals and the locations of these symbionts inside the insects in order the identify candidates for use in symbiont therapy. Most of these studies have utilized partial 16S rRNA gene sequencing to identify the bacteria living within whole glassy-winged sharsphooter and specific parts of the insect. For example, Lacava et al. (2007) identified the major bacteria living inside the heads of *H. vitripennis* as *Bacillus*, *Pseudomonas*, *Pedobacter*, *Methylobacterium*, and *Curtobacterium flaccumfaciens* and identified that the major factors affecting the composition of bacteria in *H. vitripennis* heads as host plant and developmental stage of the insect. Another study, looking specifically at sharpshooter vectors of *X. fastidiosa spp. pauca* (not including *H. vitripennis*), found the most abundant genera in sharpshooter heads to be, much like the previous paper *Methylobacterium* and *Curtobacterium* (Gai et al. 2011). Additionally, a study by Curley et al. in 2007 identified *Pseudomonas*, *Stenotrophomonas*, and *Acinetobacter* in the hemolymph of *H. vitripennis* but were unable to confirm the source of the bacteria. Finally, Hail et al. (2011) found that bacteria from the genera *Cardiobacterium*, *Pectobacterium*, *Serratia*, and *Wolbachia* to be the most prevalent in 20 laboratory reared glassy-winged sharpshooters.

The purpose of this study was to survey the microbiomes of *H. vitripennis* in Texas. A total of 50 *H. vitripennis* were collected from nine different Texas vineyards, and 16S rRNA pyrosequencing was utilized to identify and catalogue specific bacterial genera founding living with the insects sampled. Based on these results, inferences were made regarding

the major factors (i.e. sampling location) contributing to differences and similarities in microbiome community structure between these insects, and correlations between prevalence and quantities of bacterial genera were identified across multiple sampling sites.

2 Materials and methods

2.1 Trapping of *Homalodisca vitripennis*

A total of 207 adult *H. vitripennis* were collected on 23 × 14 cm double-sided bright yellow sticky traps (Seabright Laboratories www.seabrightlabs.com) coated with Stikem Special® glue (www.seabrightlabs.com) from nine vineyards in Texas from early August to early September 2007. After collection, sticky traps were placed into plastic zip bags stored at −20 °C.

2.2 *H. vitripennis* genomic DNA extractions

Sharpshooters were removed from the sticky traps using orange oil (Citrus King, www.citrusdepot.net) and sterile tweezers, immediately surface sterilized by submerging in 10 % bleach followed by 95 % ethanol, then washed with sterile DI water (Marshall et al. 2010). The abdomen and thorax of each insect were immediately removed from the insect and stored together at −20 °C in a clean, sterile 1.5 µl microcentrifuge tube. Each sharpshooter abdomen and thorax was homogenized individually in sterile PBS using a sterile, disposable microtube pestle and total DNA was extracted using a Qiagen DNeasy blood and tissue kit according to the manufacturer's protocol. Out of all of the sharpshooters collected, 3–8 from each vineyard were selected randomly for use in the current study, totaling 50.

2.3 *X. fastidiosa* detection PCR

The presence of *X. fastidiosa* in all collected *H. vitripennis* were determined by real time PCR and melt curve analysis of genomic DNA extracted from *X. fastidiosa* extracted from collected *H. vitripennis*. For all samples of extracted DNA from glass-winged sharpshooter foregut content samples, real time PCR was used to amplify a region of 198 base pairs of the gyrase B gene of *X. fastidiosa*. Analyses using the Rotor Gene Q RT-PCR machine and the Rotor Gene SYBR green PCR kit (Qiagen, www.qiagen.com), with the set-6 gyrase B primers (Bextine and Child 2007). Melting curves were measured of each reaction mix following amplification. Samples were only considered positive for *X. fastidiosa* if they showed fluorescence above the no template controls and a sharp peak within the expected region of the specific product to be amplified on the negative derivative curve of the melt curve. For the purpose of analysis, each insect was classified as positive or negative for *X. fastidiosa*.

2.4 454 pyrosequencing of bacterial 16S sequences

Total genomic DNA was used for 454 pyrosequencing where a specific region of the 16S-coding sequence of bacterial rRNA from individual insects are sequenced using the Roche 454 FLX pyrosequencing platform in two runs of the machine, with 25 different insects analyzed for each run, totaling 50 insects across all sampling locations.

2.5 Insect age determination

Wings were collected from all adult *H. vitripennis* samples, and the forewings were used to age the insects according to the methods described in Timmons et al. (2011). Briefly, the relative age value for each insect was calculated by measuring the red pixilation values of specific points on the forewings of each insect. To determine the pixilation values, wings were photographed through a dissecting microscope (Nikon SMZ-1) with a 7.1 M pixel Canon (www.usa.canon.com) digital camera, and red pixilation values were determined using the Paint Shop Pro 7.02 image analysis software. The sites that were measured on each forewing were the most proximal junction of subcosta vein and subcosta-radial cross vein, the junction of first branch of the subcosta vein, and the junction of the first branch of the cubitus vein. This totaled 3–6 red pixilation values for each insect (for 1 or 2 wings for each insect). To maintain consistency between data, red pixilation values were recorded across at least 50 pixels at each junction. The average of all readings for each insect was used for the relative age of each insect. For the purpose of analysis, relative ages were measured for all 207 insects, and three age groups of equal range quantity were designated. Each of the 50 insects analyzed was placed in one of the three qualitative groups for the purpose of analysis.

2.6 Analysis of 16S rRNA sequences

All of the analysis described were completed separately for pyrosequencing runs 1 and 2 because of sequence variation between runs was greater that seen within runs leading to false clustering based on distance measures. Using the QIIME (Quantitative Insights into Microbial Ecology) software package (Caporaso et al. 2010), the raw 454 16S sequence data were clustered according to 97 % percent identity (This level of identity is thought to cluster short (~400 bp) 16S rRNA sequences at approximately species level). Consensus sequences or operational taxonomic units (OTUs) were generated for each cluster, and bacterial community composition profiles were then be generated for each sample based on the percent of sequences belonging to each OTU. These profiles

were then used for direct comparison between samples. The taxonomic classification of each OTU was determined to the lowest level of classification possible (down to an 80 % confidence level) using the ARB SILVA SINA online 16S rRNA sequence aligner and classifier (Pruesse et al. 2012) based on the Green Genes 16S rRNA gene database (DeSantis et al. 2006). Alpha diversity curves were then generated through calculation of phylogenetic diversity (PD) using QIIME. Beta diversity was also calculated using weighted and unweighted Unifrac (Lozupone and Knight 2005) phylogenetically aware distances were calculated for each possible sample pair. Principle component analysis using weighted and unweighted Unifrac distances were then used to cluster samples according to community composition profiles and the phylogenetic distances between OTUs included in the profiles. Contribution to the clustering of insect samples of a specific set of variables was determined for the following variables relating to the *Homalodisca vitripennis* samples used for the analysis: source vineyard location, date of collection, insect age, and insect *Xylella fastidiosa* presence/absence.

2.7 Co-occurrence and concordance analysis

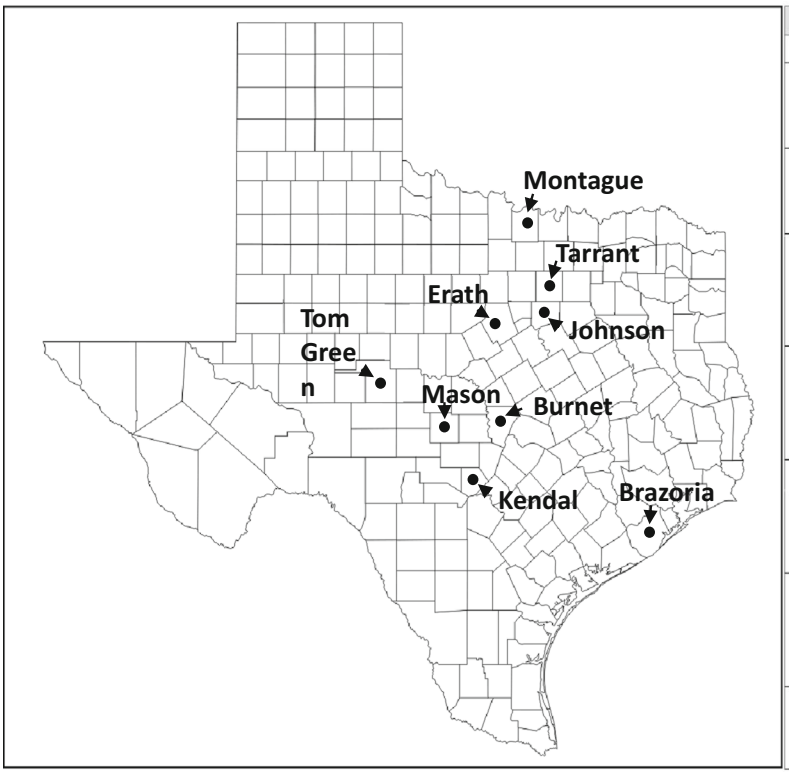
Analysis of co-occurrence and concordance relationships between bacterial genera found across *H. vitripennis* samples was analyzed using two software packages. The CoOccurence (Ulrich 2007) software package was used to determine overall patterns of non-random community assemblage for each pyrosequencing run by calculation of global co-occurrence statistics such as C-scores with all statistics calculated for presence-absence and abundance genera matrices. Significance was assessed with a two-tailed probability test based on 100 randomizations. Additionally, Kendall's global coefficient (W) of concordance was calculated as a measure of global concordance between genera to test for positive non-random community assemblage. W was calculated using a Hellinger- transformed genera abundance matrix for each pyrosequencing run data separately using Vegan package in R (Oksanen et al. 2013). Significance of this test was assessed using a two-tailed probability test based on 999 permutations. To measure non-random assemblage patterns (co-occurrence/concordance) between specific genera the Spearman rank-order correlation coefficients (r) between all sets of genera present in at least five *H. vitripennis* samples were calculated for each pyrosequencing run separately. These correlations were calculated based relative abundance data for all genera using R (R Core Team 2013). Correlation coefficients were considered significant if $r > 0.6$ or < -0.6 and $p < 0.01$. The correlations of *Xylella fastidiosa* with all other genera was calculated by representing its abundance data as that determined by quantitative PCR (Fig. 1).

3 Results

More than 300 unique bacterial taxa were identified across all samples. Most had not been previously identified within *Homalodisca vitipennis* and are of unknown origin and role within this species. At the level of phylum, the microbiome community composition of all insect samples was similar for the two sequencing runs. For example, Proteobacteria represented, by far, the most abundant phylum for both runs. Firmicutes and Actinobacteria are among the next three most abundant taxa for both runs as well. Run 2 contained a relatively high percentage of Cyanobacteria, a group composed exclusively of photosynthetic bacteria. For both runs, the most abundant bacteria below the level of family was the *H. vitripennis*-specific primary endosymbiont, Ca. *B. cicadellinicola.* (Fig. 2) This also happened to be the species that was found in the most insects tested, specifically 48 out of the total 50 (Fig. 3). The other genera that appeared in highest relative abundance were *Wolbachia*, *Pseudomonas*, *Pantoea*, *Ralstonia*, and the only other known primary endosymbiont known to inhabit *H. vitripennis*, Ca. *Sulcia* (Figs. 2 and 3).

General differences in alpha diversity between samples were measured. The major contributing factor for differences in alpha diversity is most likely linked to the relative abundance of Ca. *B. cicadellinicola* in that sample and/or the relative abundance of the most abundant taxa as Ca. *B. cicadellinicola* relative abundance is significantly and negatively correlated with phylogenetic diverdity for samples in runs 1 and 2 ($r = 0.434$; $p = 0.00407$; alpha diversity values were averaged for all ten iterations for a sampling depth of 1750 sequences; data not shown). Sampling depth was enough so that this should have had minimal influence on the number of taxa sampled for each sharpshooter, but the relatively high abundance of specific taxa (most often *Baumannia*) had strong influence on beta diversity measures. Alpha diversity is not significantly correlated with vineyard, age, or *X. fastidiosa* foregut load (data not shown), and no other known variables seem to contribute to the differences seen.

Measures of beta diversity were calculated in order to view overall patterns relating sharpshooter internal bacterial communities using the Unifrac metric (Lozupone and Knight 2005). Principal component analysis (PCA) plots were generated using weighted and unweighted Unifrac metric distances between individual insect bacterial communities to assess whether clustering patterns across the samples sequenced in runs 1 and 2 correlated to the following variables: insect age, *X. fastidiosa* infectivity and foregut load, and vineyard location. For runs 1 and 2, no clustering was visible for PCAs using unweighted Unifrac distance for all of the variables listed above (data not shown). For PCAs using weighted Unifrac distance, clustering was visible only based on



Run 1		Run 2	
vineyard/county	Sample ID	vineyard/county	Sample ID
Burnet	32A	Burnet	35A
	33A	Johnson	8B
	34A		40E
Montague	23A		51B
	24A		52K
	23B	Mason	25A
Johnson	10D		25B
	43D		25E
	12E	Kendall	29B
	40I		30C
Kendall	29A		30A
	31A	Brazoria	54I
	26B		54J
	30B		57D
Brazoria	53A		57S
	57G	Tarrant	2E
	57J		3A
	57V		4D
Erath	17A		2F
	18A		4B
	22A	Erath	19A
	21B		20A
Tom Green	27C		21A
	27E		22B
	27K	Tom Green	27B

Fig. 1 The location and ID for all samples analyzed in runs 1 and 2

vineyard location (Fig. 4). Therefore, neither the ages nor states of *X. fastidiosa* infectivity of *H. vitripennis* adults correlate with the structures of their overall microbiome communities in the abdomen and thorax. However, the geographic locations of the insects do correlate with their microbiome structure but only when the relative abundances of the bacterial taxa present are accounted for (Fig. 4). Although the classifications of bacterial endosymbionts varied between individual insects, no variables accounted for in this study correlated with these differences when the abundances of bacterial taxa was not considered.

Ca. *B. cicadellinicola* was the most abundant species in most of the insects sampled, and in those samples for which this was not true, another genus of bacteria represented the majority of the taxa sampled. The nature of the weighted Unifrac metric infers that the most abundant taxa will contribute more, quantitatively, to the calculated distances. This fact is depicted for this data set in Fig. 3. The samples in run 1 can be divided qualitatively into five groups based on the taxa that appear in highest relative abundance within that group. As would be expected, these groups correspond roughly with UPGMA clustering based on weighted Unifrac distances between insect microbiomes (Fig. 3). Four out of five of the sharpshooters from Tarrant County sequenced during run 2 clustered into group 1, based primarily on abundance of *Ochrobactrum* in these samples. For run 1, group 1 contains the highest abundance of *Paenibacillus* whereas groups 2, 3, 4, and 5 contained, on average higher abundances of *Ralstonia*, Ca. *Baumannia*, *Pseudomonas*, and *Pantoea* respectively (Fig. 3a). All of these genera have been identified previously within the gut of glassy-winged sharpshooter (Hail et al. 2011).

Multiple measures of global non-random community assembly patterns were calculated; all of these measures detected positive correlations between genera found within the sharpshooters (Table 2). For example, for runs 1 and 2 the C-score indices for presence-absence and abundance data for all genera were significantly higher than simulated indices (Table 2). Additionally, Kendall's global coefficient of concordance was positive for both runs 1 and 2 (Table 2). Figure 5 is depictions of networks of significant positive correlations between bacterial genera found in samples sequenced by runs 1 and 2, respectively. No significant negative correlations were found between any genera for runs 1 or 2. Out of those interactions, only those between *Escherichia* and *Enterobacter*, *Bradyrhizobium* and *Mesorhizobium*, *Acidovorax* and *Ralstonia*, *Klebsiella* and *Leclercia*, and Ca. *Sulcia* and Ca. *Baumannia* were present within runs 1 and 2 (Fig. 6). Except for that between Ca. *Baumannia* and Ca. *Sulcia*, all of these correlations were between genera from the same order. Between all of those pairs of genera, only

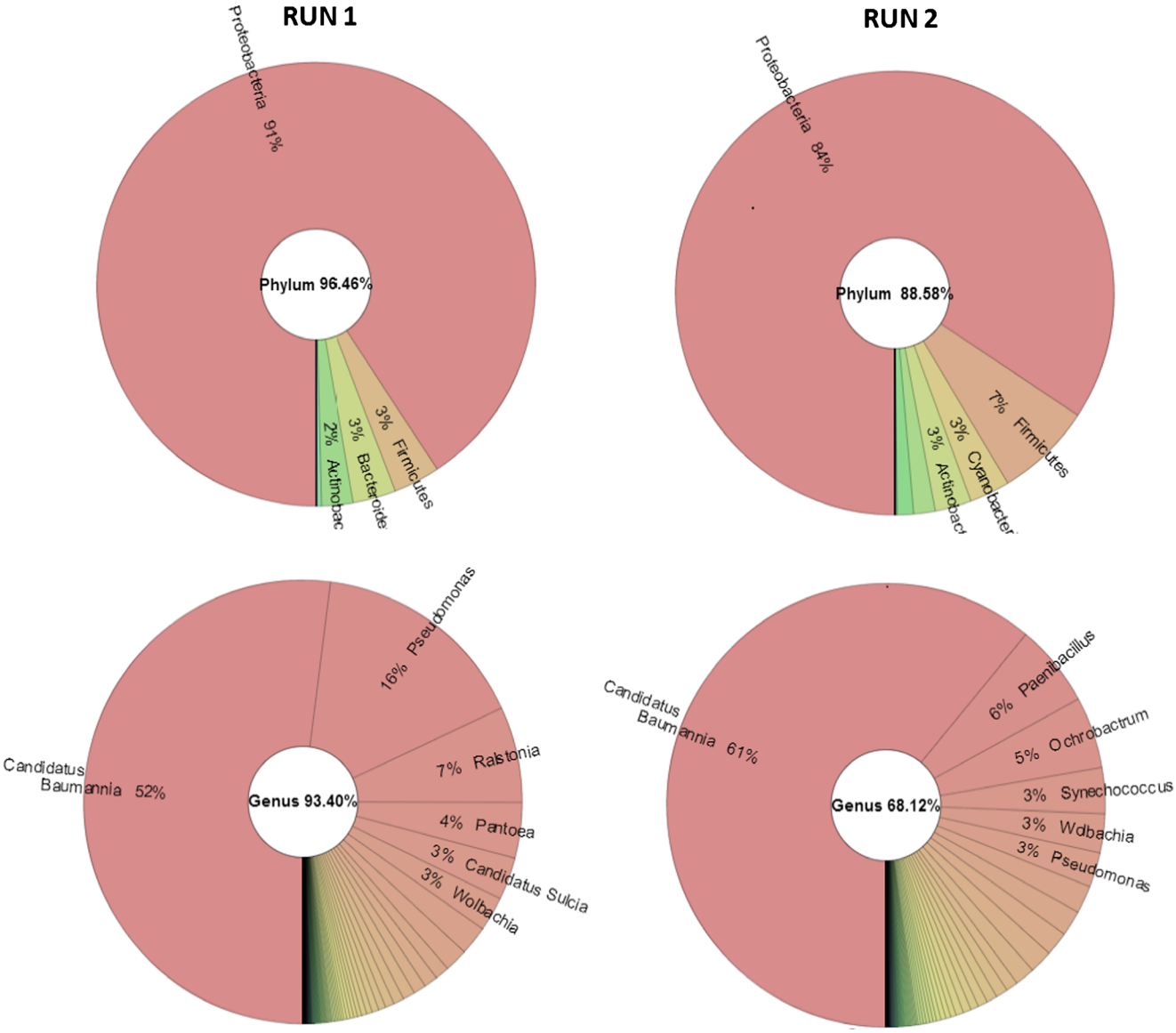


Fig. 2 The percentages of the most abundant taxa identified across all *H. vitripennis* samples at the levels of phylum and genus

sequences from *Escherichia* and *Enterobacter* were more genetically similar to one another than any other sequences. This suggests that misclassification of sequences, which could have placed groups of sequences belonging to one genus into more than one, played little if any role in influencing the analysis aimed at identifying these correlations between genera.

4 Discussion

The microbiome of *Homalodisca vitripennis* was elucidated between nine geographic locations in Texas. More than 300 bacterial taxa were indentified within the insects sampled. Most of which were not previously identified within this species. The two bacteria that are most often associated with the glassy-winged sharpshooter are the only known primary endosymbionts associated with the insect Ca. *B. cicadellinicola*, and Ca. Sulcia. Both bacteria live exclusively within the bacteriome of the glassy-winged sharpshooter (B. *cicadellinicola*) or several species of sharpshooters (*Sulcia*) are vertically transmitted through generations, and provide essential metabolic functions (McCutcheon et al. 2009). Theoretically, both genera should have appeared in all of the samples. However, Ca. *B. cicadellinicola* was not represented in two out of the 50 insects sampled, and Ca. Sulcia was represented in 29 samples. The lack of these genera in certain samples was possibly due to sampling error because of low sequencing depth and/or high

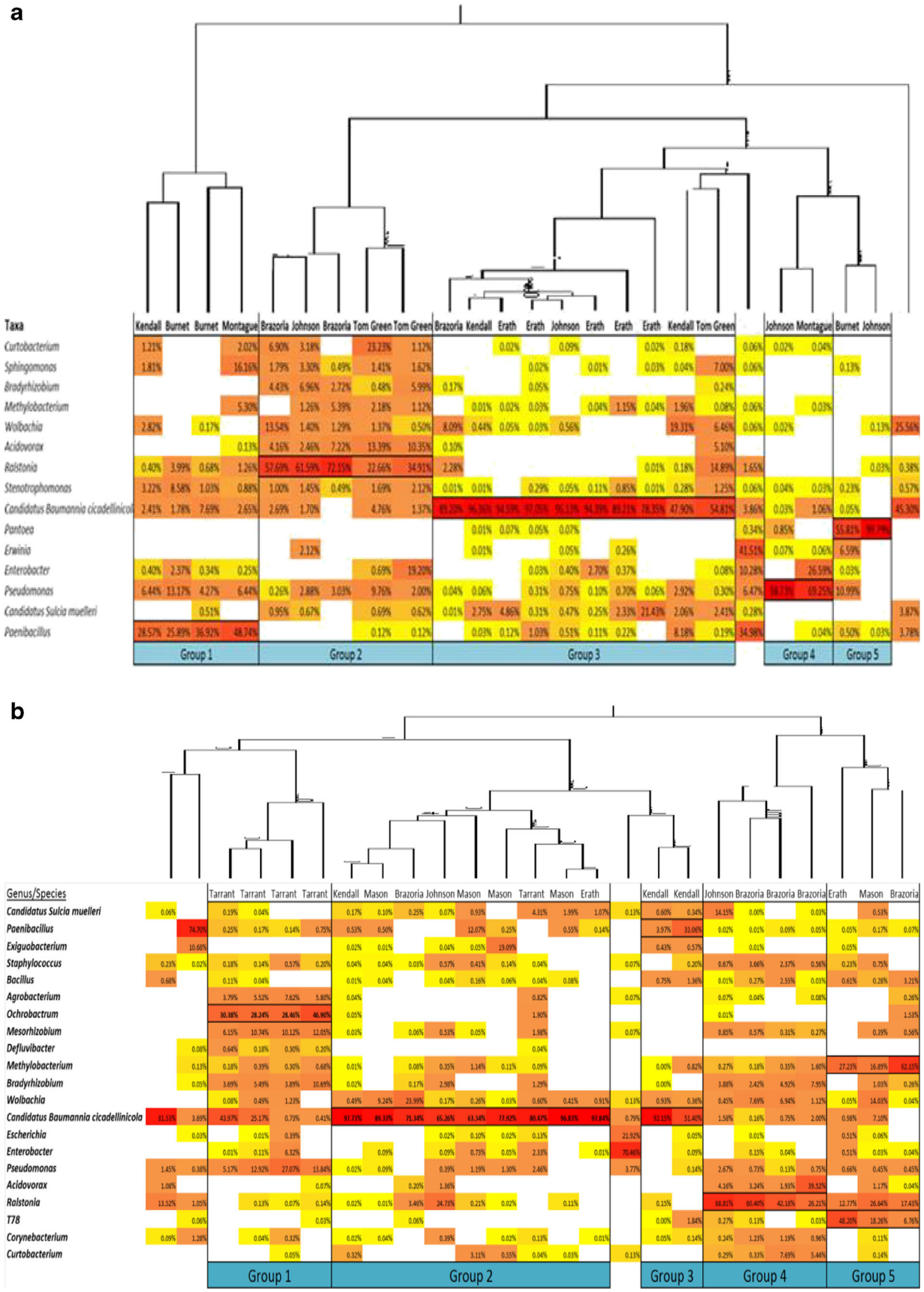


Fig. 3 Relatedness between *H. vitripennis* samples based on weighted Unifrac metric showing clustering groups for **a** run 1 and **b** run 2

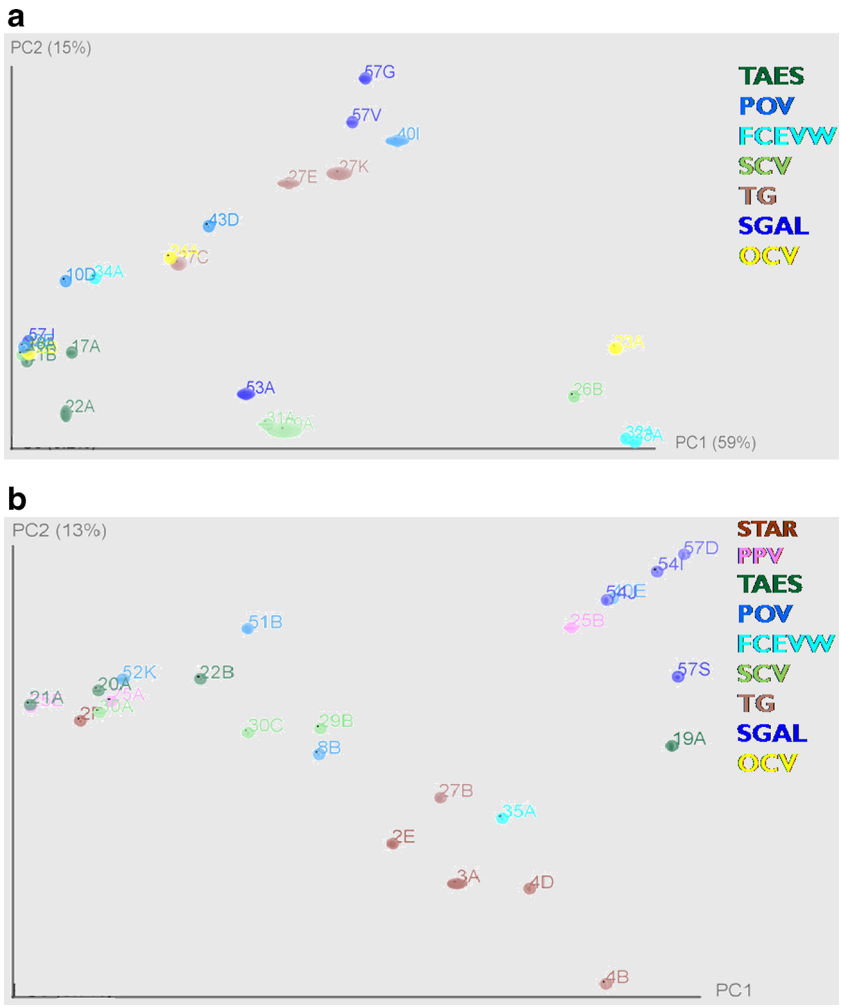


Fig. 4 Jackknifed PCA based on weighted Unifrac metric distance for pyrosequencing shows clustering of *H. vitripennis* samples based on vineyard location for pyrosequencing **a** run 1 and **b** run 2. *Numbers* and *letters* represent specific samples

relative abundance of other genera (*Ralstonia* and *Pantoea*) in the samples in which it was lacking (Fig. 2). Ca. *Sulcia* probably was excluded from the sampling more often because of its lower relative abundance and was therefore more likely to be underrepresented. The common facultative arthropod endosymbiont *Wolbachia* was also, not surprisingly, prevalent among the samples.

Table 2 Global co-occurrence and concordance statistics for genera identified within *H. vitripennis* samples

	C-score index	Simulated index	95 % Confidence interval
Run 1:			
Presence-absence analysis:	4.23	4.72	4.63–4.77
Abundance analysis:	4.19	5.08	4.38–5.79
Run 2:			
Presence-absence analysis:	4.6	6.25	6.15–6.34
Abundance analysis:	4.6	2.64	2.10–3.14
	W	*p*-value	
Run 1:	0.0369	0.001	
Run 2:	0.0872	0.001	

For a large percentage of the classifications found, their presence could be explained through environmental acquisition, possibly through feeding. For example, the abundance of proteobateria across all samples is not surprising considering that some of the most abundant genera for both runs, Ca. *B. cicadellinicola*, *Psuedomonas*, *Ralstonia*, *Pantoea*, *Ochrobactrum*, and *Wolbachia* belong to Proteobacteria. However, many of the other genera of proteobacteria are known pathogens and soil bacteria. The same is true for Actinobacteria. The presence of *Pseudomonas* could have been expected, as it has been previously isolated from grapevine xylem sap, as well as the hemolymph (Curley et al. 2007), alimentary canal (Hail et al. 2011), and head (Gai et al. 2011) of *H. vitripennis*. *Pseudomonas* exists commonly as a soil or plant-associated bacteria. At this

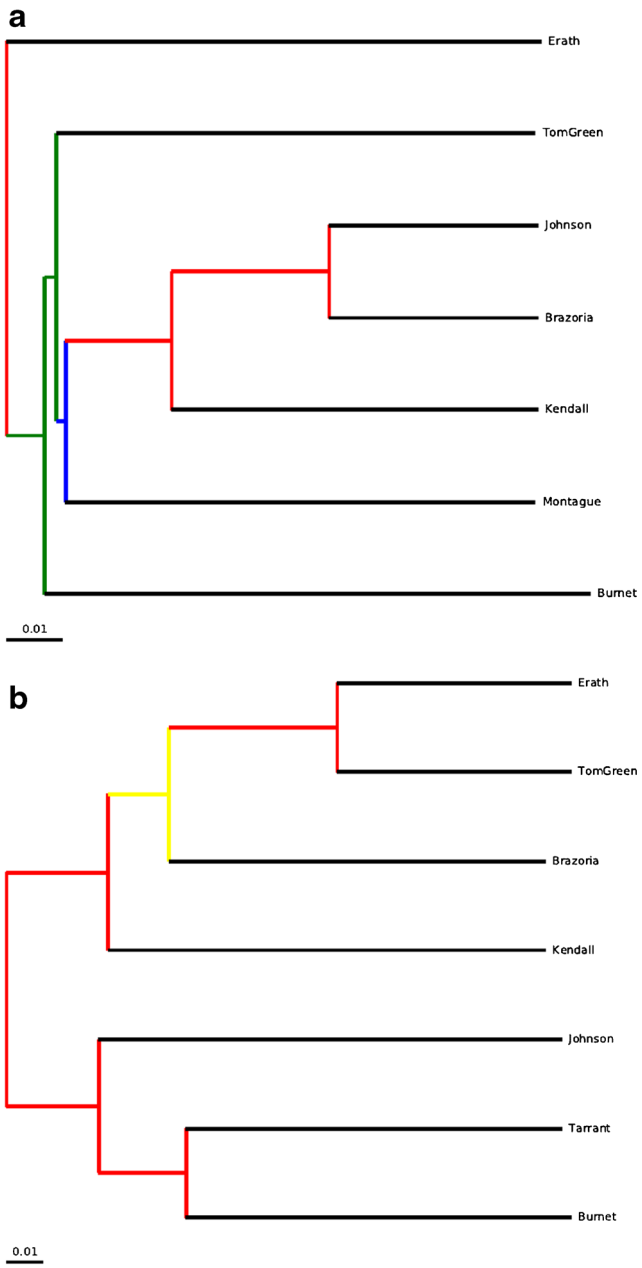
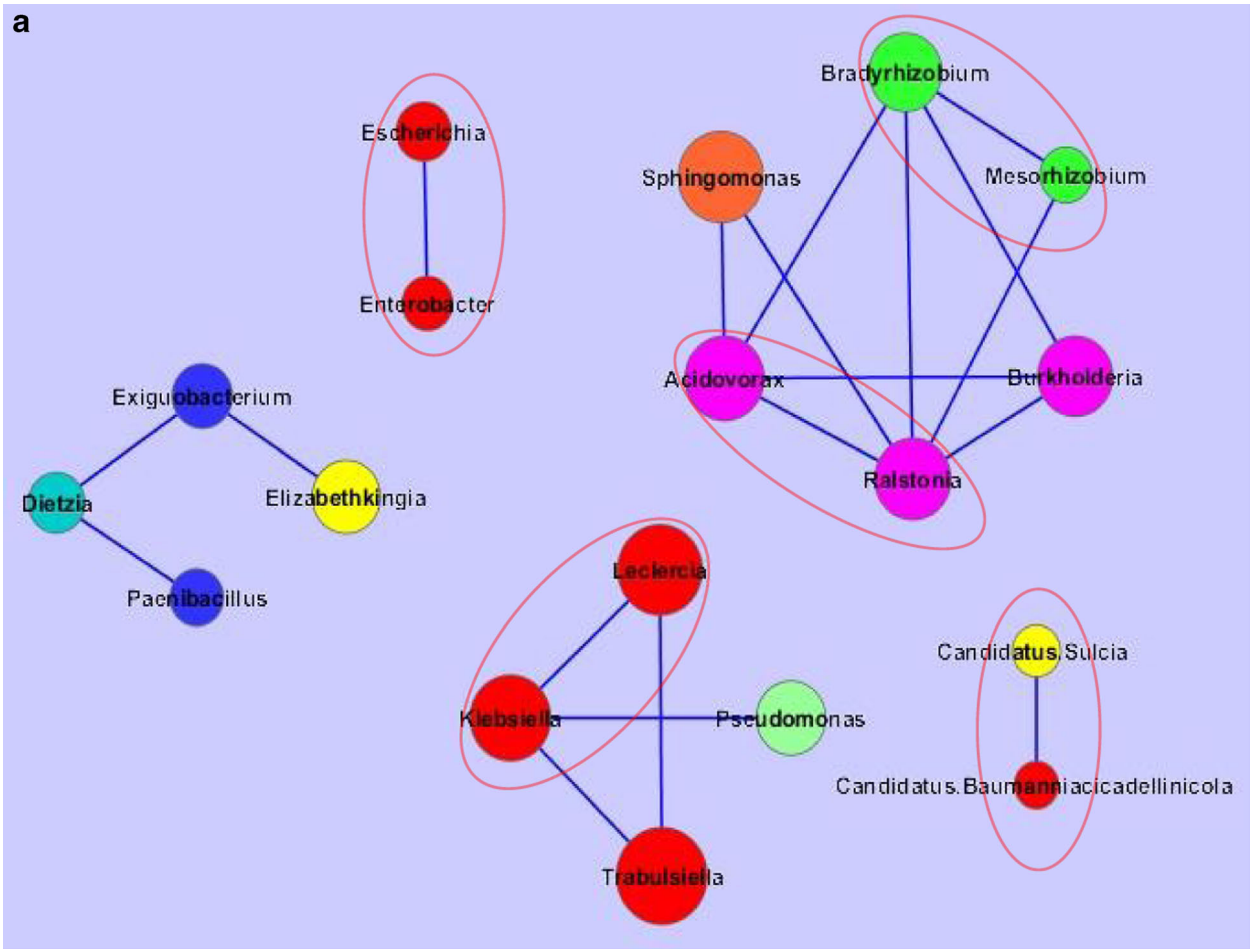
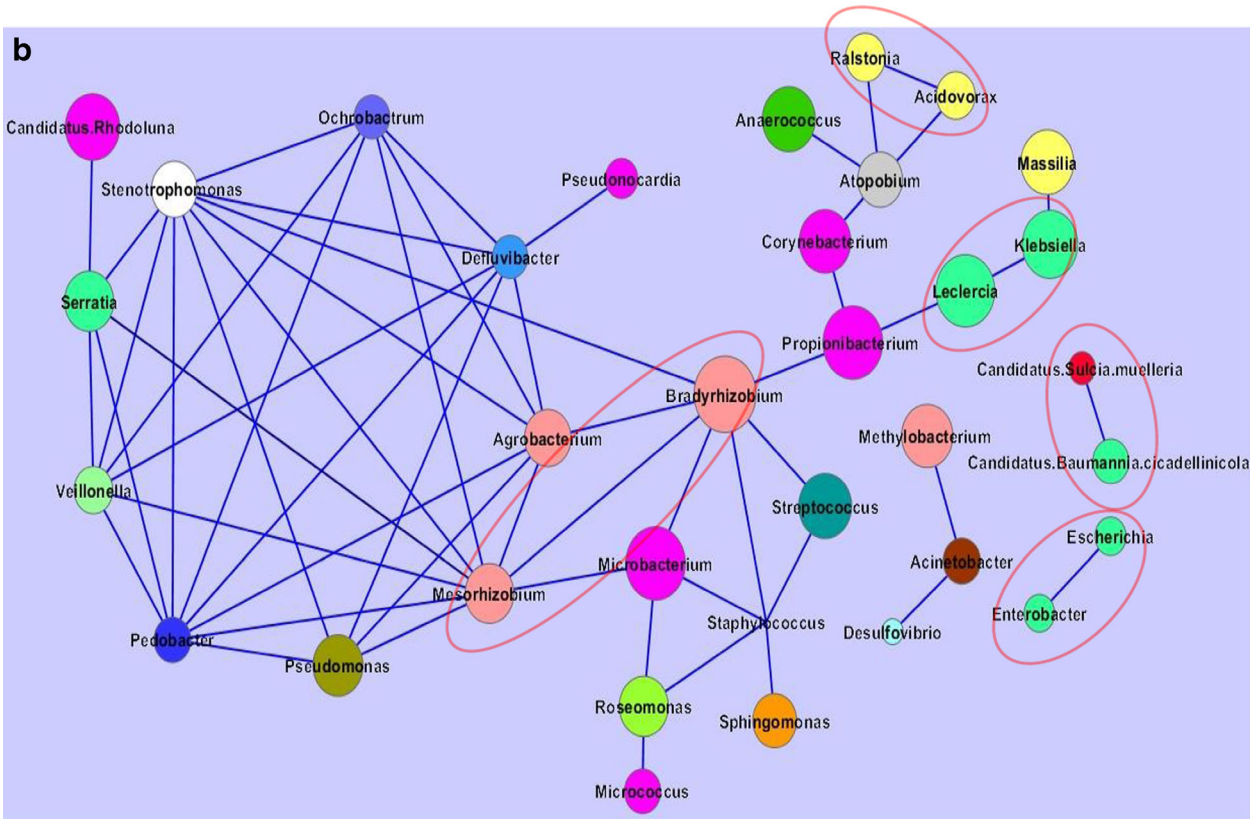


Fig. 5 UPGMA clustering tree showing relatedness between taxa found at different sampling locations based on weighted Unifrac metric showing clustering groups for **a** run 1 and **b** run 2

point, whether is it simply acquired by the insect from environment or it confers some advantage to the insect is unknown. *Pantoea*, a commonly plant-associated bacteria probably acquired through feeding, has been found in the alimentary canal of the glassy-winged sharpshooter (Hail et al. 2011). *Ralstonia*, a known grape endophyte commonly living within the vascular tissues of plants (Phloem-and xylem-restricted plant pathogenic bacteria), has also been identified within multiple regions of the gut of this sharpshooter and was also most likely acquired thought feeding (Bextine et al. 2004).

The resolution of differences and similarities in bacterial community structure between *H. vitripennis* across geographic space provides clues concerning the importance of microbial fauna in *H. vitripennis*. The composition of insect microbiomes clustered according to geographic sampling location. Therefore, microbiome composition could also cluster according to a number of other environmental variables such as most recent insect diet (although all can be presumed to have fed most recently on grapevine as they were collected between the rows of a vineyard), soil type, local climate, etc. Fig. 5 illustrates that robust differences between vineyards exist, but lack any pattern regarding the relationships between vineyards. This suggests that geographic location of the insect probably does have influence on the makeup of its microbiome, but there is no clear indication that closer geographic locations are likely to have insects with more similar microbiomes. This suggests that location climate probably plays a minimal role in shaping microbiome makeup. Additional controlled studies are needed to tease apart the fine scale conditions that influence what bacteria may exist within this species and their abundances. For each run, the insects were qualitatively clustered into groups based on the presence and abundance of the most prevalent genera sampled. As could be expected, this clustering method aligned well with the UPGMA clustering tree based on weighted UniFrac distances between samples. For run 1, group 1 contains the highest abundance of *Paenibacillus*, whereas groups 2, 3, 4, and 5 contained, on average, higher abundances of *Ralstonia*, Ca. *Baumannia*, *Pseudomonas*, and *Pantoea* respectively (Fig. 3a). All of these genera have been identified previously within the gut of glassy-winged sharpshooter (Hail et al. 2011). *Paenibacillus* and *Ralstonia* both contain species that exist as grape endophytes and *Pseudomonas* and *Pantoea*, as mentioned arlier, both contain species known to live within plants. The differences in abundances for these taxa could be explained by differences in insect feeding. For Ca. *B. cicadellinicola*, since it has such a strong metabolic role in these insects, any regional differences in its abundance may have a link to insect diet or metabolic differences within the insects themselves. For run 2, the genera that influenced clustering the most were Ca. *Baumannia*, *Ralstonia*, *Ochrobactrum*, and *Methylobacterium*. *Ochrobactrum* is known to live ein a multitude of environments; the basis behind its variation in this dataset is unknown (Fig. 3b). However, *Methylobacterium* has been identified by multiple studies as one of the primary bacteria living in the heads of glassy-winged sharpshooters and other sharpshooter species (Lacava et al. 2007; Gai et al. 2011) and has also formed symbiotic relationships with *Vitis sp.* and other plant species. For run 2, the genera that influenced clustering the most were Ca. *Baumannia*, *Ralstonia*, *Ochrobactrum*, and


a
Escherichia
Enterobacter
Bradyrhizobium
Sphingomonas
Mesorhizobium
Acidovorax
Burkholderia
Ralstonia
Exiguobacterium
Dietzia
Elizabethkingia
Paenibacillus
Leclercia
Klebsiella
Pseudomonas
Trabulsiella
Candidatus.Sulcia
Candidatus.Baumanniacicadellinicola


b
Ralstonia
Acidovorax
Anaerococcus
Atopobium
Massilia
Corynebacterium
Klebsiella
Leclercia
Propionibacterium
Candidatus.Sulcia.muelleria
Candidatus.Baumannia.cicadellinicola
Candidatus.Rhodoluna
Ochrobactrum
Stenotrophomonas
Pseudonocardia
Defluvibacter
Serratia
Bradyrhizobium
Agrobacterium
Methylobacterium
Veillonella
Streptococcus
Microbacterium
Acinetobacter
Escherichia
Mesorhizobium
Staphylococcus
Desulfovibrio
Enterobacter
Pedobacter
Pseudomonas
Roseomonas
Sphingomonas
Micrococcus

◀ **Fig. 6** Co-occurrence associations between genera identified in *H. vitripennis* samples analyzed by pyrosequencing **a** run 1 and **b** run 2. Nodes sizes are proportional to mean of all Spearman r's calculated for each genus. Colors are based on order. Networks were created with Cytoscape. Interactions circled by a red circle are present in both pyrosequencing runs

Methylobacterium. Additionally, group 1 (high % of *Ochrobactrum*) was composed only of 4 out of 5 of the Tarrant county insects, two of the Kendall county insects composed group 3, and three out of the four members of group 4 (high % of *Ralstonia*) were from Brazoria county.

Out of the taxa that were identified within these samples, many had been previously found within *H. vitripennis*. The roles of these bacteria within *H. vitripennis* have not been elucidated. However, it is possible that associations formed by these bacteria may give some clues to possible roles within this insect. Many of these bacteria have been found in the heads of GWSS or other sharpshooter *X. fastidiosa* vectors (Table 2). Several of these bacteria, specifically species belonging to *Pedobacter* and *Sphingomonas*, are often found closely associated with the soil (Kwon et al. 2011; Xie and Yokota 2006). Interestingly, *Pedobacter* was only identified within H. vitripennis from the Tarrant county vineyard. Tarrant County also showed a higher average *X. fastidiosa* foregut load than all other vineyards (data not shown). Since *Pedobacter* and *X. fastidiosa* are both closely associated with the head of the insect, there may be some link between these two findings. Others, specifically *Curtobacterium*, *Methylobacterium*, *Pseudomonas* and *Paenibacillus*, are often associated with plant tissue. Species of *Paenibacillus*, also found in the alimentary canal of *H. vitripennis*, included grape endophytes and obligate symbionts in insects. These were likely acquired from feeding. Other genera have been found in the alimentary canal (Table 1). Out of those, *Ralstonia* is a known grape endophyte (Curley et al. 2007). *Klebsiella* has been found to participate in nitrogen fixation with *Enterobacter* in the green stinkbug (found in whole insect but not gut) (Table 1). *Alcaligenes* (found in gut) was used previously in one study investigating its potential use as a control agent through occupation of the cibarium (Bextine et al. 2004). Others have been found in the hemolymph of sharpshooters (Table 1). Out of those, some have been found in other areas of the insect. For example, *Delftia* was identified within the hemolymph and whole insect (Table 1). *Serratia*, a genus containing common insect pathogens and symbionts, one species that acting as a primary symbiont in aphids to help with toleration of high temperatures (Moran et al. 2008), has been found in hemolymph, alimentary canal, and whole insect (Table 1). *Pseudomonas* has also been found living practically ubiquitously among *H. vitripennis* (Table 1). Found in the hemolymph, alimentary canal, and head of GWSS, *Pseudomonas* is a bacterium that has been found within grape sap (Curley et al. 2007). *Stenotrophomonas* is genus containing species that are most often found associated with plants was also found in hemolymph (Zhu et al. 2011). Other bacteria have been identified previously within the whole insect, meaning they were associated with no organs specifically (Table 1). Out of these, *Enterobacter* was the most common, appearing in 30 out of 50 samples, and contains species that have also found in the guts of *Drosophila* and mosquito species (Eappen et al. 2013; Christopher and Gilmore 2007). *Enterobacter* also belongs to *Enterbacteriaceae*, a family known to contain a multitude of animal symbionts (Moran et al. 2008). Out of taxa mentioned above, the most common among all 50 samples were *Pseudomonas* (43 samples), *Paenibacillus* (39), *Ralstonia* (36), *Methylobacterium* (35), and *Stenotrophomonas* (35).

Important aspects of bacterial communities are the interactions between the individuals and taxa living with those communities. Negative correlations between abundances of taxa across locations or communities could indicate competition or a preference for divergent environmental conditions, with positive correlations possibly suggesting mutualistic relationships between bacteria or host insects or a preference for similar environmental conditions. Multiple positive correlations between the genera were identified across the insects sampled, with multiple possible explanations for each correlations. For example, the association between Ca. *Baumannia* and Ca. *Sulcia* can be explained by their shared location in the bacteriome of the insect and their shared ubiquity among all *H. vitripennis*.

The fact that most of the positive correlations identified were between genera from the same order suggests that positive associations between bacterial genera inside *H. vitripennis* are more likely to occur between genera within the same higher levels of classification. This could be because that bacteria that are more phylogenetically similar are more likely to share a similar environment and, thus, are more likely to be acquired by the same set of insects.

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