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A Global Comparison of *Bactericera cockerelli* (Hemiptera: Triozidae) Microbial Communities

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ABSTRACT The potato psyllid (*Bactericera cockerelli* Sulc) is an economically important insect pest of solanaceous crops such as potato, tomato, pepper, and tobacco. Historically, the potato psyllid's range included central United States, Mexico, and California; more recently, populations of this insect have been reported in Central America, the Pacific Northwest, and New Zealand. Like most phytophagous insects, potato psyllids require symbiotic bacteria to compensate for nutritional deficiencies in their diet. Potato psyllids harbor the primary symbiont, *Candidatus* Carsonella ruddii, and may also harbor many secondary symbionts such as *Wolbachia sp.*, *Sodalis sp.*, *Pseudomonas sp.*, and others. These secondary symbionts can have an effect on reproduction, nutrition, immune response, and resistances to heat or pesticides. To identify regional differences in potato psyllid bacterial symbionts, 454 pyrosequencing was performed using generic 16S rRNA gene primers. Analysis was performed using the Qiime 1.6.0 software suite, ARB Silva, and R. Operational taxonomic units were then grouped at 97% identity. Representative sequences were classified to genus using the ARB SILVA database. Potato psyllids collected in California contained a less diverse microbial community than those collected in the central United States and Central America. The crop variety, collection year, and haplotype did not seem to affect the microbial community in potato psyllids. The primary difference between psyllids in different regions was the presence and overall bacterial community composition of *Candidatus* Carsonella ruddii and *Wolbachia*.

KEY WORDS potato psyllid, *Bactericera cockerelli*, microbiome, Wolbachia, *Candidatus* Carsonella ruddii

The range of the phytophagous potato and tomato psyllid, *Bactericera cockerelli* (Sulc) (Hemiptera:Triozidae), changes seasonally following optimal breeding temperatures and extends from eastern Mexico south to Nicaragua, throughout the central United States, California, the Pacific Northwest, and New Zealand (Pletsch 1947; Munyaneza et al. 2007a,b, 2010; Secor et al. 2009; Teulon et al. 2009; Munyaneza 2012). Potato psyllids feed primarily on solanaceous plants, which include crops such as: potato, tomato, peppers, and tobacco (Knowlton 1934, Wallis 1955, Munyaneza 2012, Butler and Trumble 2012, Munyaneza et al. 2013). As well as damage caused by feeding, the potato psyllid is known to transmit the bacteria "*Candidatus* Liberibacter solanacearum" (Lso; aka *Candidatus* Liberibacter psyllaurous), the putative causal agent of Zebra Chip disease of potato (Secor et al. 2009). Zebra Chip causes physiological changes in potato tubers rendering them unsellable to the chip industry, which results in millions of dollars in crop losses annually (Munyaneza 2012).

The recent range expansions and extensive crop damage caused by potato psyllids have led to research into population dynamics of the potato psyllids. Molecular markers in the CO1 and ITS genes and ISSR have identified three unique haplotypes of potato psyllid; one located on the west coast of the United States and in New Zealand (western haplotype), one isolated in the Pacific Northwest (northwestern haplotype), and another found in the Central United States, Mexico, and Central America (central haplotype; Liu et al. 2006; Chapman et al. 2012; Swisher et al. 2012, 2013a).

Potato psyllids rely on bacterial symbionts in their gut to provide them with essential amino acids that are absent in their diet (Thao et al. 2000). This is common and many insects are host to a wide variety of mutu-

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alistic bacterial symbionts that can provide the insects with enhanced resistance to pesticides, enhanced immune response, nutrition, and reproductive advantages (Werren et al. 2008, Feldhaar and Gross 2009, Kikuchi et al. 2012). The primary symbiont of potato psyllids, *Candidatus* Carsonella ruddii, is encased in the bacteriocyte and coevolved with psyllids (Thao et al. 2000). *C. ruddii* likely provides the psyllids with essential amino acids lacking in their diet and the psyllids cannot synthesize on their own (Buchner 1965, Douglas 1998). There are also many secondary endosymbionts such as Lso, *Acinetobacter*, *Methylibium*, and *Wolbachia sp.* that can be acquired by psyllids through feeding or passed to offspring transovarially (Nachappa et al. 2011, Hail et al. 2012). The effects of these secondary symbionts on potato psyllids are unknown, but it can be assumed that some may provide the psyllids with physiological alterations.

Analyzing insect bacterial symbionts has proven difficult in the past because many of these bacteria are unculturable; thus, a complete picture of the insect microbial communities was unavailable (Hugenholtz et al. 1998). With the advent of next-generation sequencing techniques, such as pyrosequencing, full identification of the complete insect symbiont composition is possible. Pyrosequencing allows for the simultaneous sequencing of millions of different 16S rDNA sequences per sample allowing for the identification of the entire microbial community without the need to culture the bacteria or create clone libraries (Tamaki et al. 2011). Pyrosequencing is also beneficial in that it provides a semiquantitative representation of the microbial community, not only presence and absence data provided by earlier cloning techniques.

Previous surveys of potato psyllid microbial communities by pyrosequencing have been performed using pooled insects of various life stages collected at the same time, location, and only from potato plants (Nachappa et al. 2011, Hail et al. 2012). This survey used similar techniques as the previous studies, but used single insect DNA extractions from many locations, years, host plants, and haplotypes. Molecular markers have been used to investigate potato psyllid migration patterns and recent range expansions, but these have not been able to identify population differences below haplotype. Microbial communities can differ between populations of insects and could potentially provide insight into migration patterns and also the range expansions into the Pacific Northwest and Central America. The potato psyllid microbial communities were also compared based on haplotype, year, and the crop the sample was collected from, to identify potential biotic influences on the microbial community. If proposed resident populations of potato psyllids and migration patterns do occur, regional differences in microbial community should be apparent. Understanding regional differences in microbial community could also lead to a better understanding of speculated pesticide resistance in some populations of potato psyllids because bacterial symbionts have been seen to impart pesticide resistance in other insects.

Materials and Methods

Sample Collection and Extraction. Psyllid samples were collected from both agricultural fields and from laboratory colonies. Potato psyllids were collected in 2008–2011 from fields in Texas, Kansas, Nebraska, Colorado, California, Oregon, Washington, Nicaragua, and New Zealand. Carrot psyllids (*Trioza apicalis* Forster) collected in Finland and three of the New Zealand potato psyllid samples were from laboratory-raised colonies. Agricultural samples were obtained with vacuum devices and not with sticky traps. After collection, samples were stored in 95% ethanol at −20°C until extraction.

Both Qiagen DNeasy Blood and Tissue kits (Qiagen, Valencia, CA) and an optimized CTAB protocol (Crosslin et al. 2011) were used to extract total DNA. Extracted DNA was stored at −20°C until pyrosequencing was performed.

Haplotype Identification. Psyllid haplotype was assessed using real-time melt analysis by the methods outlined by Chapman (2012). All samples were run with 2 μl of psyllid genomic DNA with concentrations of 2–20 ng/μl. The primers used were CO1 F1 5′ GGA TTC ATT GTT TGA GCA CAT C 3′ and CO1 R1 5′ TGA AAT AGG CAC GAG AAT CAA 3′, which amplify a 78-bp section of the psyllid mitochondrial CO1 gene. The PCR was run with 25 μl reactions using 12.5 μl of Qiagen Quanitfast PCR Master Mix (Qiagen), 2.5 μl of both primer, 2 μl of template DNA, and 7.5 μl of nanopure water. The PCR was run using the following thermal profile: 15 min hold at 95°C followed by 40 cycles of 95°C for 10 s and 53°C for 15 s. Following PCR and a 90-s premelt cycle, the product underwent a melt cycle from 65 to 80°C raising the temperature 0.25°C per step holding at each step for 5 s. The resulting differential melt curves were used to identify haplotype, with the central haplotype melting at ≈1°C lower than the western haplotype.

454 Pyrosequencing. Bacterial tag-encoded FLX amplicon pyrosequencing (bTEFAP) was performed by Research and Testing Laboratory (Lubbock, TX). All DNA sample concentrations were adjusted to 100 ng/μl. A 1-μl aliquot was used from each sample in 50-μl PCR reactions. Primers used for pyrosequencing were Gray28F 5′ TTT GAT CNT GGC TCA G 3′ and Gray519R 5′ GTN TTA CNG CGG CKG CTG 3′ (Dowd et al. 2008; Hail et al. 2010, 2011). Initial generation of the sequencing library used a one-step PCR with a total of 30 cycles using HotStar *Taq* Plus Master Mix (Qiagen) and the following thermal protocol: 94°C for 3 min followed by 30 cycles of 94°C for 30 s; 60°C for 40 s and 72°C for 1 min; and a final elongation step at 72°C for 5 min. A two-step PCR was performed for 454 amplicon sequencing using the same conditions using fusion primers with different tag sequences as described previously (Dowd et al. 2008). A second PCR was performed to prevent amplification biases, which are caused by the tags and linkers in the first

PCR. Next, amplicon products were mixed in equal volumes, and purified with Agencourt Ampule beads (Agencourt Bioscience Corporation, MA).

The double-stranded DNA was combined with DNA capture beads, then amplified using emulsion PCR. After bead recovery and bead enrichment, the bead-attached DNA strands were denatured with NaOH, and sequencing primers were annealed. A two-region 454 sequencing run was performed on a PicoTiterPlate (PTP) using the Genome Sequencer FLX System (Roche, Nutley, NJ), and all FLX procedures were performed according to the manufacturer's instructions. Tag-encoded FLX amplicon pyrosequencing analyses used Roche 454 FLX instrument with Titanium reagents (Roche, Nutly, NJ).

Data Analysis. Sequence analysis was performed using the Qiime 1.6.0 metagenomic analysis package (Caporaso et al. 2010). Samples with <1,000 sequences were removed to filter samples with low sequencing depth. OTU (operational taxonomic unit) picking was performed using the USEARCH pipeline scripts with a sequence identity of 97% (Edgar 2010). All OTUs with a length <200bp were removed before classification. Representative sequences for each OTU were classified against the ARB SILVA database to genus using the SILVA Incremental Aligner (SINA; Pruesse et al. 2012, Quast et al. 2012). Total counts for each classification were calculated and used for downstream analysis. Genus classification results as opposed to OTU counts were used because of inconsistencies in sequencing results from multiple pyrosequencing runs causing artificial sample separations based on run.

To compare similarities between bacterial communities, classical multidimensional analysis (CMDS) with a Bray–Curtis distribution were performed using R with the vegan package (Dixon 2003). Psyllid bacterial communities were compared based on their locations, the year they were collected, the host plant they were collected from, and their haplotype.

Results

Microbial Community Analysis. Pyrosequencing of 117 individual potato psyllids and 10 carrot psyllids resulted in >788,000 quality sequences, ≈6,200 per psyllid (min 1,013; max 32,552). Four potato psyllids returned <1,000 sequences and were removed before analysis. Clustering the sequences into OTUs at 97% sequence identity resulted in 1,623 OTUs. Classification of these OTUs reported the presence of 14 Phyla, 28 classes, 63 orders, 114 families, and 105 genera of bacteria. No OTUs that were identified as eukaryotes were used in the analysis.

The bacterial sequences returned from pyrosequencing were similar in composition to the previous findings of Hail et al. (2012) and Nachappa et al. (2011). Returned sequences consisted of 53% *Wolbachia sp.*, 19% *Candidatus* Carsonella ruddii, 4% *Acintobacter sp.*, 4% *Pseudomnas sp.*, and 2% *Candidatus* Liberibacter (Fig. 1). The remaining 18% consisted of sequences that were either uncommon in the entire dataset or could not be classified to the genus level. When looking at individual samples, other bacteria such as *Pantodea sp.*, *Porphyromonas sp.*, *Prevotella sp.*, *Sodalis sp.*, *Streptococcus sp., and Veilonella sp.*, were common. Bacteria in the families Burkholderiales and Rhizobiales were also common.

Multivariate Analysis of Influences on Potato Psyllid Microbial Community. The proportions of *Wolbachia sp.* and *Candidatus* Carsonella ruddii influenced the psyllids groupings (Fig. 2). Carrot psyllids were included as an outlier and their isolation from most of the potato psyllids validates the model data. Carrot psyllids did not harbor *Wolbachia sp.* but did have a very high proportion of their microbial community represented by *Candidatus* Liberibacter sp. (≈24%). The high proportion of *Candidatus* Liberibacter sp. also present in the colony potato psyllids from New Zealand could explain their proximity in the CMDS plot.

Within the United States, there was a great diversity within the microbial community of psyllids (Fig. 3). Although the patterns are not precise, there are some groupings that imply certain regions have unique microbial communities. Of the 27 potato psyllids collected in California, all but two grouped closely and show little microbial diversity with *Wolbachia* sp. comprising a majority of their microbial community (>80%). The two outlying samples from California, collected in Riverside in 2009, had a microbial community composed of much higher percentages of *Candidatus* Carsonella ruddii (≈25%), *Sodalis sp.*(≈15%), and lower percentage of *Wolbachia* sp.(≈1%) than other samples collected in California. Potato psyllids collected from Texas, Nebraska, Kansas, Colorado, and Nicaragua possessed much more microbial diversity than samples collected in California. The samples from Washington grouped together with their microbial community being primarily composed of *Candidatus* Carsonella ruddii (>80%) and a *Pantodea sp.* Samples collected in Oregon did not group exclusively with samples from Washington. These samples portrayed different groupings with similarities to Washington, California, and Texas samples.

Potato psyllid haplotype was assessed using a single SNP in the CO1 mitochondrial gene using the real-time melt analysis protocol developed by Chapman et al. (2012). Seventy potato psyllids from Texas, Kansas, Nebraska, Colorado, Washington, Oregon, California, Nicaragua, and Auckland, New Zealand, were tested for haplotype. Using the haplotype as the signifier, the bacterial communities were plotted with CMDS. There were no apparent patterns or clustering of bacterial community in relation to haplotype (Fig. 4).

Potato psyllids of the central haplotype from Texas, Kansas, and Nebraska, from 2008, 2009, and 2010, were also separately compared using CMDS based on their microbial communities (Fig. 5). These samples were chosen because of their wide diversity in microbial communities and the likelihood of identifying patterns related strictly to annual changes as opposed to environmental or physiological differences. Although there is an unusual separation in the samples from

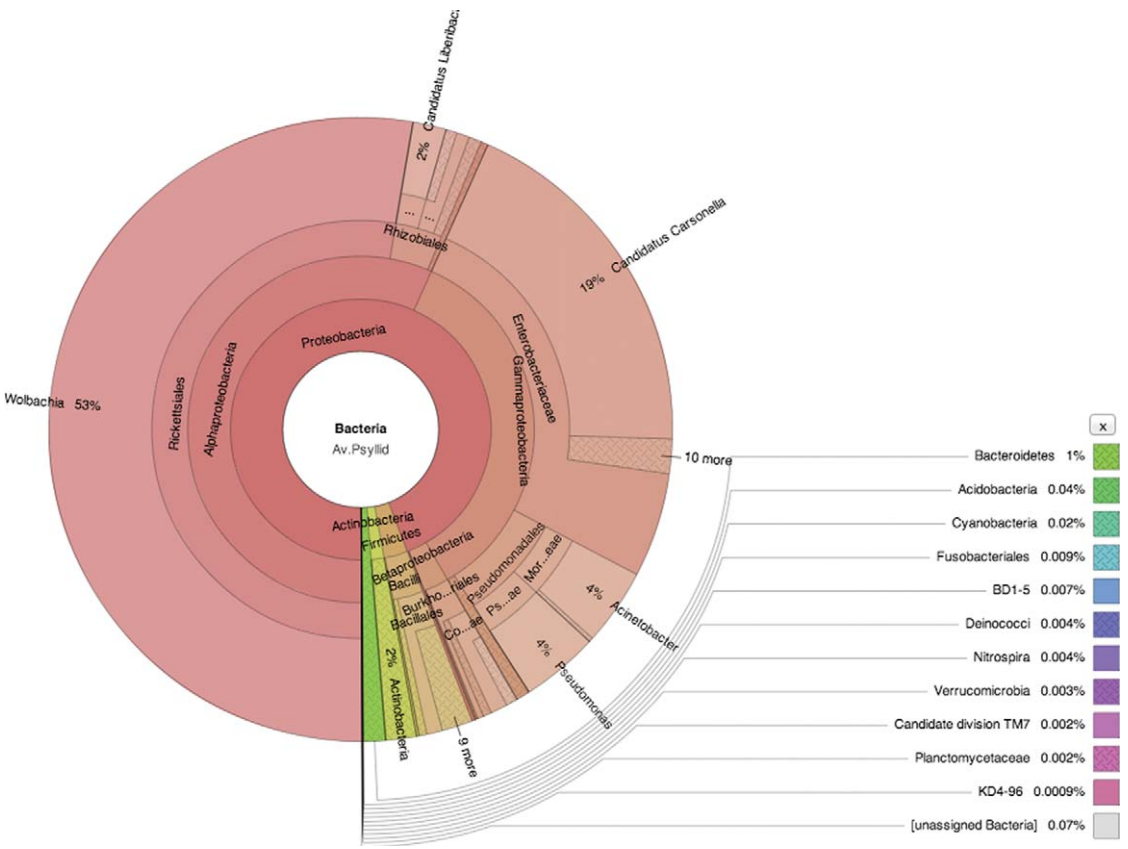

Fig. 1. Representation of all sequences returned from 454 pyrosequencing. (Online figure in color.)

2009, a majority of the samples from all 3 yr overlapped and did not separate in any meaningful way.

Potato psyllids collected from three counties and three host plants in southern California were also compared by their bacterial communities to assess whether host plant has an effect on the psyllid microbial community (Fig. 6). The psyllids were collected from Orange, and Ventura Counties from potato, pepper, and tomato plants; while the potato psyllids collected in Riverside County were only collected from potato plants. With the proximity of the sites and the assumption of their haplotype being the same, external influence other than plant host variety on the groupings was limited. When plotted there were no observed differences based on host pant or county. Overlap of counties was expected because of their proximity.

Discussion

Microbial Community Analysis. The overall results returned from pyrosequencing matched previous studies into potato psyllid microbial communities, though between individual samples there was a great amount of diversity not yet observed in psyllid microbial community surveys. Previous surveys by Hail et al. (2012) and Nachappa et al. (2011) used potato psyllids collected from potato fields in central Texas, or from colonies that were originally collected from potato fields in Texas. As well as using similar locations, these previous studies used DNA extracted from pooled insects, which could have washed out unique diversity in the individuals of the pooled samples. Performing the survey with individual insects over a much wider area has provided a substantially deeper insight to the regional diversity present in the microbial communities of potato psyllids.

The majority of difference between samples was the levels of *Wolbahia* sp. and *Candidatus* Carsonella ruddii, which appear to be competing for dominance (Figs. 2 and 7). Further studies with cell culture and insect culture should be done to investigate this relationship. The absence of *Candidatus* Carsonella ruddii in some samples was unexpected because it is considered the primary symbiont to psyllids and is required to provide the psyllids with amino acids that they cannot synthesize, yet some samples did not contain any or a very low percentage of sequences that matched to *Candidatus* Carsonella ruddii. After the whole genome of *Candidatus* Carsonella ruddii was sequenced, it was found that it did not contain genes for production of all three amino acids lacking from the psyllid and it would be necessary for potato psyllids to have a second primary symbiont (Tamames et al. 2007). It is possible that *Wolbachia* or another

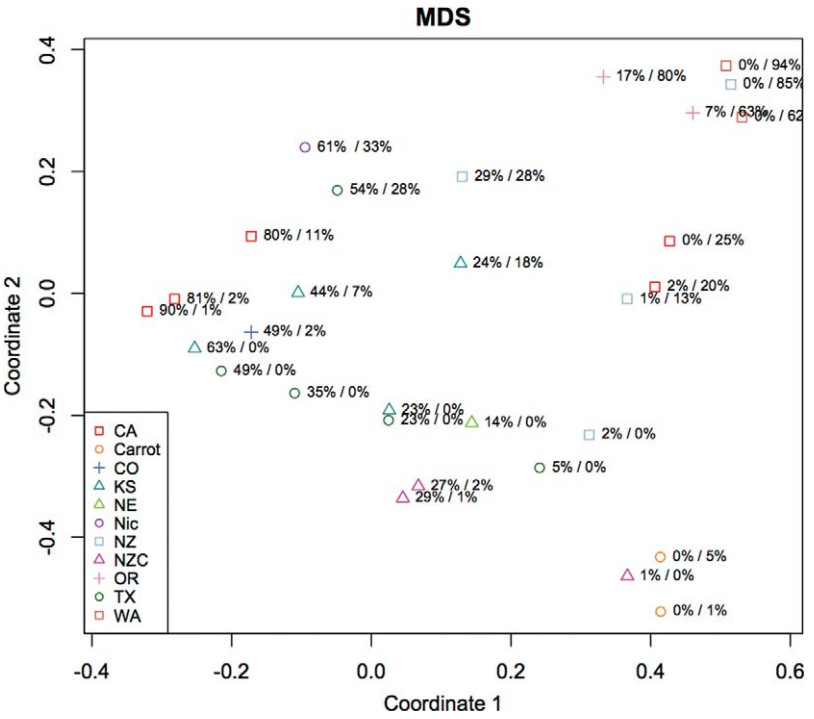

Fig. 2. Classic multidimensional dimensional scaling of individual potato psyllids compared by their bacterial community composition. Samples are labeled by their percent bacterial community composition of *%Wolbachia*/*% Candidatus* Carsonella ruddii. (Online figure in color.)

secondary symbiont could be providing these amino acids to psyllids, but whole genome sequencing of the *Wolbachia* species found in potato psyllids would be needed to provide the evidence of this relation.

Multivariate Analysis of Influences on Potato Psyllid Microbial Community. Regional differences in potato psyllid microbial community have provided an interesting insight into questions about potato psyllid

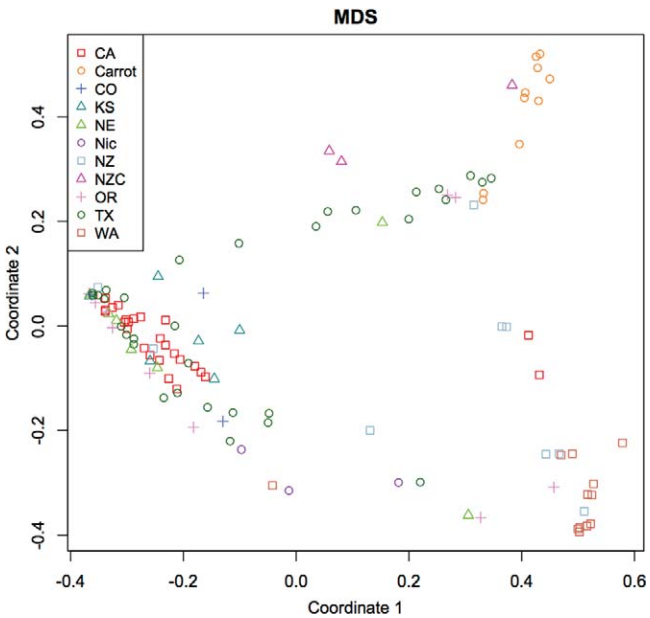

Fig. 3. Classic multidimensional dimensional scaling of individual potato psyllids compared by their bacterial community composition. (Online figure in color.)

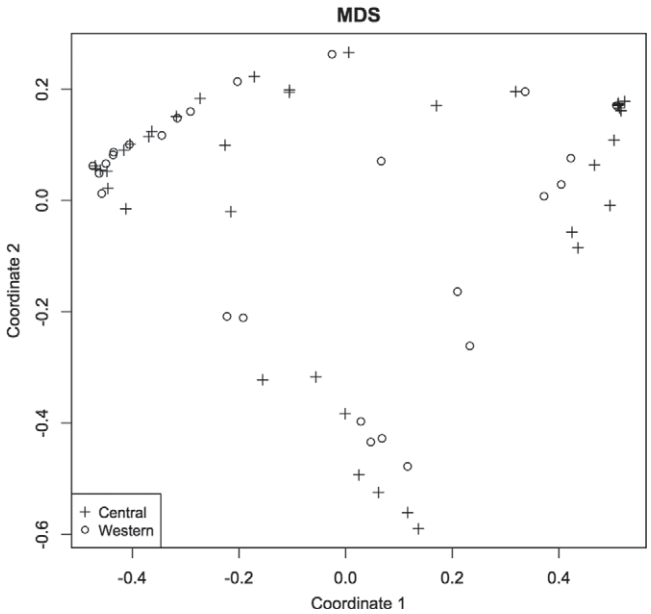

Fig. 4. Classic multidimensional dimensional scaling of bacterial community similarities of potato psyllids compared by the psyllids' haplotype.

range expansion, pesticide resistance, and new introductions. Samples collected in Washington and Oregon were suspected to share microbial communities similar to the potato psyllids collected in California because of the traditional northward range expansion seen in the central United States, but this pattern is not supported in the western United States. The reason for the dissimilarity between Washington and Oregon samples given their geographic proximity is unknown.

Potato psyllid haplotype was expected to be a primary influence on the microbial community, as many primary and secondary symbionts are vertically transferred and comprise a majority of the microbial community in most insects. The results of this survey did not reflect the expected separation of samples based on their haplotype. A possible explanation for part of these results could be caused by the methods used to identify the potato psyllid's haplotype. At the time of

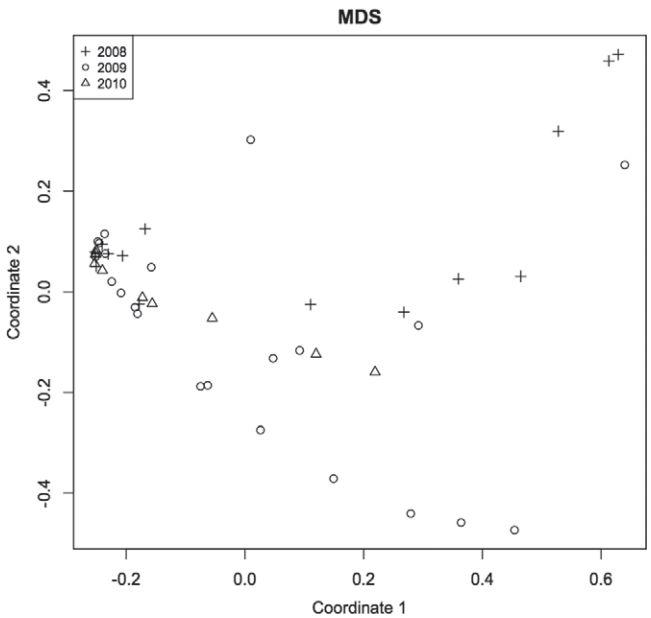

Fig. 5. Classic multidimensional dimensional scaling of bacterial community similarities of potato psyllids collected from TX, NE, and KS compared by the year in which the psyllids were collected.

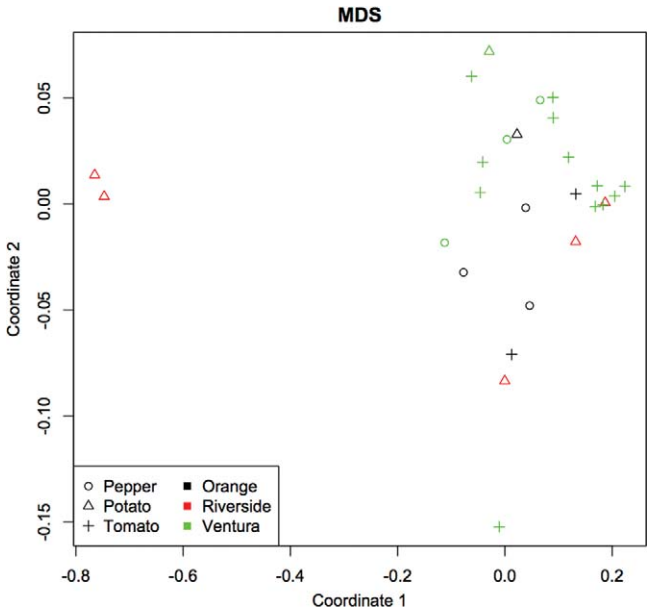

Fig. 6. Classic multidimensional dimensional scaling of bacterial community similarities of potato psyllids collected from three counties in California compared by the host plant the psyllids were on when collected. (Online figure in color.)

our survey, there was only one method of haplotype detection to differentiate between the central and western haplotypes, but a newer method has been developed that separated a new third haplotype present primarily in Washington and Oregon (Chapman et al. 2012; Swisher et al. 2012, 2013b). Using the two-haplotype method, this third haplotype shows similarity to the central haplotype. Using the three-haplotype detection could add detail necessary to explain some of these results, especially because Washington and some Oregon samples grouped far separate than samples from the central United States and California.

Similar to haplotype, no separation occurs based on the years the psyllid samples from the central United States were collected. This information is promising for planning IPM, as some symbiotic bacteria have been shown to provide resistance to pesticides and annual shifts in microbial community could lead to shifts in resistance (Kikuchi et al. 2012). Annual consistency in the diversity of microbiota in this region lends further evidence that a better understanding of potato psyllid populations and their migration patterns is needed.

The potato psyllid samples from southern California were collected from pepper, tomato, and potato

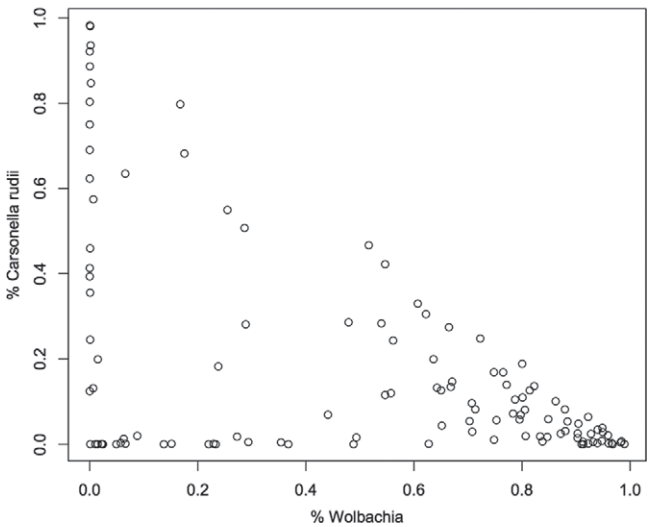

Fig. 7. Correlation of total percent of the total microbial community composition of *Wolbachia* to *Candidatus* Carsonella ruddii in potato psyllids ($P < 0.001$).

plants. It was thought that host plant would cause differences in the potato psyllids' microbial community because part of the microbial community in insects is obtained through feeding. The host plant the potato psyllids were collected from did not have an impact on the similarity of potato psyllid microbial communities. These results are interesting, but it is important to note that these psyllids were field collected and the diet of the nymphs and adults before collection were not known. Plant material from where the psyllids were collected was also not analyzed, so what bacteria the psyllids were possibly obtaining from the plants are also unknown.

The results from our survey indicate that potato psyllid microbial communities are primarily influenced by location, though these differences are not affected by host plant, haplotype, and do not shift annually. Understanding whether these differences have an effect on pesticide resistance or "*Candidatus* Liberibacter solanacearum" uptake or transmission is unknown. Future studies into how these microbial communities affect potato psyllids should be conducted, as these differences have potential for effective pest and disease control.

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