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BACTERICERA COCKERELLI: RANGE EXPANSION, CANDIDATUS LIBERIBACTER SOLANACAERUM DETECTION, AND MICROBIAL COMMUNITY ANALYSIS

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by

ALEX ARP

A thesis submitted in partial fulfillment of the requirements for the degree of Master's of Science

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This is to certify that the Master's Thesis of

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Abstract

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The potato psyllid (Bactericera cockerelli) is an emerging pest of solanacious crops. Traditionally their range expanded from Guatemala north through the central United States and into southern California. Recent expansions have led to potato psyllids residing as far south as Nicaragua and as for north as Washington State. Potato psyllids were also accidentally imported into New Zealand. These expansions are alarming because potato psyllids transmit *Candidatus* Liberibacter solanacaerum (Lso), the bacteria associated with Zebra Chip disease in potato, which results in millions of dollars in damages annually. In this study, potato psyllid and Zebra Chip range expansion into Central America was explored; the current method of detecting Lso was compared to pyrosequencing to identify irregularities in infections of psyllids in fields and research labs; and the microbial communities of potato psyllids were

used to assess differences between populations and to provide evidence of migration patterns. The results of this research indicate that potato psyllids and Zebra Chip disease are spreading throughout Central America and infecting new plants such as tobacco, which could have severe economic impacts on the region. Current methods of detecting Lso are not sensitive enough to detect the pathogen at low levels of infection. Surveys of potato psyllid microbial communities provided insight into the population dynamics of potato psyllids. Potato psyllids in different regions contain drastically different microbial communities, mainly influenced by the proportions of *Wolbachia* and *Candidatus* Carsonella rudii. Haplotype, year, and host plant unexpectedly did not have an impact on potato psyllid microbial community.

Literature Review

Potato Psyllids

Description

The potato (tomato) psyllid (*Bactericera cockerelli* (Sulc)) is an economically important crop pest. Potato psyllids are insects, which lie in the order Hemiptera, sub-order Sternorrhyncha, and the family Triozidae. The sub-order Sternorrhyncha also includes other economically important sap-sucking insects such as aphids (Hemiptera: Aphididae) and white flies (Hemiptera:

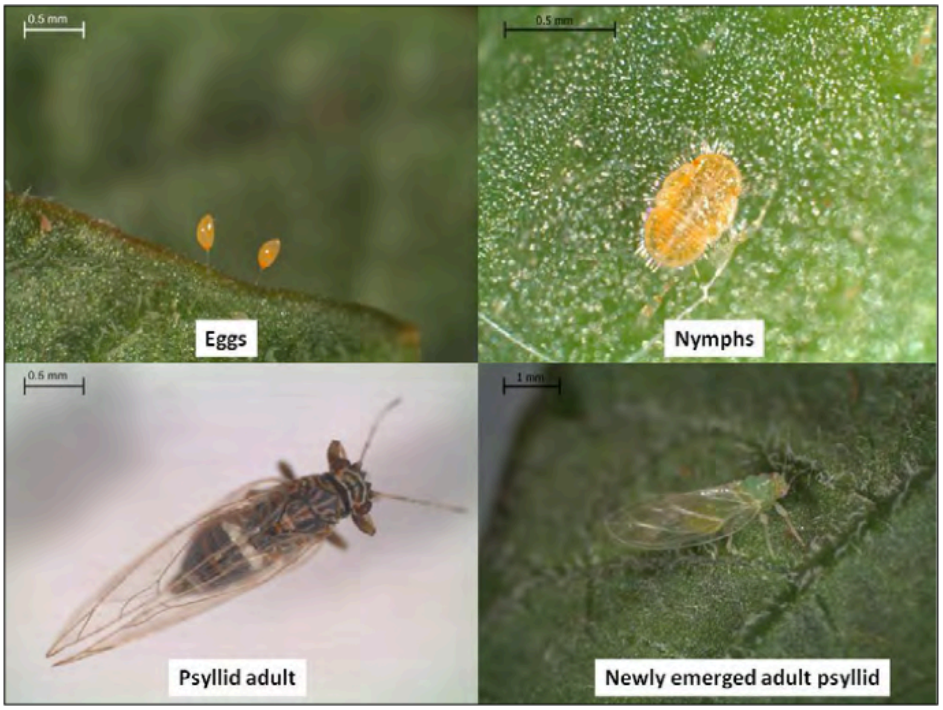

Figure 1.1: Life stages of *Bactericera cockerelli* (Rondon 2012)

Aleyrodidae). Like all hemipteran insects, potato psyllids are hemimetabolous,

and undergo five nymphal instars. Adult psyllids are black to brown with stripes of varying color along their abdomen. Both sexes have clear wings. They can be between 1.3 and and 1.9 mm in length (Lehman 1930, Liu and Trumble 2007). Early instar nymphs are white to yellow and later instars darken to green (Essig 1917).

Lifespan and development are heavily dependent on temperature but can also be influenced by host plant, sex, and regional differences in haplotype (Yang et al. 2010). Optimal temperature for potato psyllids has been determined to be 26.7°C, 32.2°C begins to be detrimental, and 38.8 is lethal to eggs, nymphs, and stops egg laying (List 1939). Psyllids lay their eggs on the top and bottom surfaces of leaves where they attach by a thin stalk (Knowlton and Janes 1931, Pletsch 1947). Eggs are yellow and oblong, 0.1mm long by 0.3mm wide (Compere 1915, Pletsch 1947). Eggs hatch after 3-15 days (Knowlton and Janes 1931, Yang and Liu 2009). Nymphal development takes between 12 and 44 days with an average of 15.4 days (Knowlton and Janes 1931, Yang et al. 2010). Nymph mortality is responsible for a majority of natural deaths (Abdullah 2008). Adult psyllids lifespan can range from 16-97 days . Females can begin laying eggs three days post-emergence but this can be delayed up to 25 days (Knowlton and Janes 1931, Abdullah 2008). Once mating has occurred a female will continue to lay eggs for up to 27.8 days, laying between 5-50 eggs every 24 hours (Knowlton and Janes 1931, Yang and Liu 2009). There is a 1:1 sex ratio of offspring (Knowlton and Janes 1931).

Potato psyllids are phytophagous, and predominantly feed on phloem. Adult psyllids generally feed on the underside of leaves but will also feed on the tops of leaves, stems, and petioles depending on the plant host and stage. Nymphs also generally feed on the undersides of leaves and rarely move (Lehman 1930). Potato psyllids have been observed feeding on over 20 families of plant but have only been seen to complete development on 40 species (Knowlton and Thomas 1934, Wallis 1951). Although psyllids seem to be a fairly generalist species, they show a preference for plants in the family Solanaceae (Wallis 1955), hence their common names of tomato or potato psyllid. Wintering psyllids have been found on evergreens in California and early spring breeding populations in Texas and Arizona have been found on *Lycuim sp*. (Romney 1939).

Range

Potato psyllids undergo a seasonal range variation or migration following optimal temperatures. They have been collected in aerial insect surveys at an elevation of 1200m and have been found high in the Sierra Nevada Mountains suggesting that psyllids migrate using air currents (Glick 1939, Papp and Johnson 1979). It is not currently known if their migration is dead-end or bi-directional although evidence suggests the psyllids are moving in a bidirectional pattern. Breeding populations are found in Southern Texas and Arizona in winter and early spring (Romney 1939). As temperatures rise, psyllid populations in this area disappear and begin to arrive farther north. Psyllids are regularly found in

Oklahoma, Kansas, Nebraska, North Dakota, South Dakota, Nevada, New Mexico, Montana, Minnesota, Idaho, Colorado, Utah, and Wyoming (Pletsch 1947, Munyaneza, Goolsby, et al. 2007, Munyaneza, Crosslin, et al. 2007, Secor et al. 2009, Munyaneza 2010). The upper reaches of the migration end in Canada where they have been observed in Alberta, Saskatchewan, British Columbia, and Ontario (Pletsch 1947, Wallis 1955, Ferguson et al. 2002). Year-round populations of potato psyllid occur in southern California but some of these psyllids seem to migrate north into Oregon and Washington (Munyaneza et al. 2009). It has been proposed that there is an isolated population in Washington and Oregon. Potato psyllid range also extends south through Mexico and Central America, where they have been observed in Guatemala, El Salvador, Honduras, and Nicaragua (Espinoza 2010, Munyaneza 2012, Powell et al. 2012, Bextine et al. 2013). Potato psyllids also have year-round breeding populations in New Zealand after an accidental immigration in 2005 (Teulon et al. 2009).

History

Karen Sulc described the potato psyllid in 1909 (Sulc 1909). They were not considered a potential pest until 1915 when an outbreak occurred on False Jerusalem Cherry in California that required the use of pesticides (Compere 1915). The first major outbreak took place in 1927, spreading from Utah and to other Rocky Mountain states. During this outbreak the first evidence of psyllid yellows was seen and thought to be caused by toxins in the psyllid's saliva (Linford 1928, Richards 1928). The earliest large-scale psyllid outbreaks

occurred in 1938 (Wallis 1946, Hill 1947). In 2001 a large outbreak of potato psyllids spread across the central and western United States and has since become an annual event. As well as becoming an annual pest across North and Central America, potato psyllids invaded New Zealand in 2005 and have become an established and costly agricultural pest (Teulon et al. 2009).

Psyllid Yellows

There are several diseases spread by potato psyllids that are of agricultural importance including psyllid yellows (PY) and Zebra Chip. Psyllid yellows, first seen during the 1927 outbreak, is characterized by reduction in growth and stem rigidity, chlorosis of the leaves, basal cupping of leaves, aerial tubers, and premature death (Richards et al. 1927, Richards 1928, Blood et al. 1933, Pletsch 1947). Potato psyllid nymph feeding is the direct cause of the disease in potatoes; though PY can be caused by adult psyllids in tomato (Blood et al. 1933, Daniels 1954). A bacterium or virus is not the cause of the disease, but the exact cause of the disease is currently unknown (Binkley and Metzger 1929, Daniels 1954, Abernathy 1991). Tubers from plants displaying the symptoms of psyllid yellows can be replanted and produce normal, though spindly, plants; thus, the symptoms are a direct result of psyllid feeding (Binkley and Metzger 1929). Diseased plants contain starch deposits in the stems and necrosis of the phloem (Schaal 1938, Carter 1939). Tubers of infected potatoes have lower starch content, thicker skins, and are reduced in size (Linford 1928,

Carter 1939). In both tomatoes and potatoes there is significant crop loss from PY (Liu and Trumble 2007).

(move to next page) Zebra Chip

In 1994 a new disease, commonly called Zebra Chip (ZC), arose in potato fields in Saltillo, Mexico. Potato psyllids were thought to be associated with the disease but the causal agent was not yet identified (Munyaneza et al. 2007). Later, the disease was found to be associated with the bacteria *Candidatus* Liberibacter solanacearum (Lso) which is transmitted by potato psyllids (Secor and Rivera-Varas 2004, Secor et al. 2009, Haas et al. 2011, Nachappa et al. 2011). ZC has now been reported as far north as Washington and Idaho and also into Central America as far as Nicaragua (Figure 1.2) (Munyaneza et al. 2009, Crosslin et al. 2012, Bextine et al. 2013).

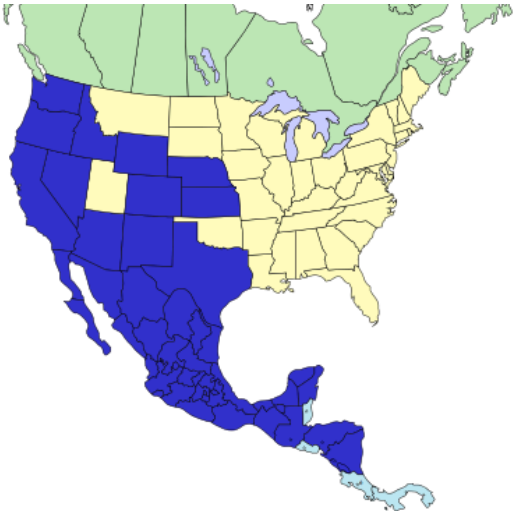

Figure 1.2: Map of ZC presence in North and Central America (Munyaneza et al. 2012)

ZC has foliar symptoms similar to psyllid yellows, but was identified and named based on the physiological changes in the tubers that are not caused by psyllid yellows (Arslan et al. 1985, Sengoda et al. 2009). Lso causes infected potato tuber medullary tissues to necrose, converting the starches to simple sugars, resulting in potatoes that turn dark and striped when fried rendering them unusable for the chipping industry and other uses. Lso is transmitted by infected potato psyllids feeding on the phloem of an infected solanaceous plant. It has been shown that one infected psyllid can infect a potato plant with Lso within six hours of feeding on that plant and only a short time is required for a psyllid feeding on an infected plant to acquire Lso (Buchman, Heilman, et al. 2011, Buchman, Sengoda, et al. 2011). Though some nymphs are infected through transovarial transmission, most psyllids acquire Lso by feeding on an infected plant as nymphs (Wu et al. 2006, Nachappa et al. 2011, Pitman et al. 2011). When these infected nymphs reach adulthood they then travel carrying Lso with them infecting fields farther north. When infected with Lso, that bacteria spreads throughout the plant and can infect sister tubers. The transmission to sister tubers results in the need to destroy an entire crop where ZC occurs to prevent the future spread of the disease (Buchner 1965, Shigenobu et al. 2000, Pitman et al. 2011).

Biotype and Haplotype

Subtle differences between different populations of an insect species, such as feeding preference, resistances, or mating behavior, can isolate

populations creating a unique biotype (Diehl and Bush 1984). Though these traits were traditionally all that was required to classify a biotype, current methods also require a genetic backing to determine biotype (Claridge and Hollander 1983). For example the whitefly, *Bemisia tabaci*, a worldwide agricultural pest, has multiple biotypes, biotype B which feeds on non-traditional host plants, while biotype Q has increased pesticide resistance (Jiang et al. 1999, Horowitz et al. 2003). In recent years different haplotypes of potato psyllid have been described by stable genomic variations in the CO1 gene, with ISSR, and the *Wolbachia wsp* gene. These stable genetic variations are regional with one primarily in the western United States, another in the central United States and Central America, and the newest in the Pacific Northwest and Idaho. (Shigenobu et al. 2000, Liu et al. 2006, Munyaneza, Crosslin, et al. 2007, Jackson et al. 2009, Swisher et al. 2012). The ability to detect these haplotypes has been beneficial in understanding the sources of new outbreaks in New Zealand and Central America. Physiological differences between these haplotypes are currently unknown, but if the haplotypes are observed to have different feeding behaviors, bacterial symbionts, or salivary toxins, they could be confirmed as a unique biotype. Identifying biotypes and populations most likely to cause agricultural damage could have economic advantages, especially if one biotype is more capable of causing agricultural damages or carrying Lso.

Insect-Bacterial Symbiosis

Symbiotic Bacteria in Insects

Many insects have specialized diets lacking in balanced nutrients. To account for insects' restrictive diet, many have developed symbiotic relationships with bacteria that aid them by providing necessary nutrients not found in their food source. Other bacteria may also form symbiotic relations with insects that give them other reproductive or physiological advantages. These bacteria that are required for the insects to live are called primary-symbionts; whereas, other facultative bacteria which may have more recently developed symbioses with the insect are called secondary-symbionts. Primary-symbionts are maternally transmitted, have co-evolved with their host insects. They reside in specialized cells called bacteriocytes or mycetocytes that can be part of larger structures such as the bateriome or mycetome (Buchner 1965, Moran and Mira 2001, McLean et al. 2010). The isolation and protection of primary-symbionts has resulted in their genomes being degraded and losing genes required for independent life (Thao et al. 2000, Moran and Mira 2001, Oliver et al. 2003, McLean et al. 2010)

Primary-symbionts in insects primarily provide the host with nutritional supplements that the insect cannot synthesis independently and is also lacking from the food source. Some examples of primary-symbionts are: aphids which rely on *Buchnera aphidicola* for amino acids (Chen et al. 1999, Shigenobu et al. 2000, Moran and Mira 2001); Tsetse flies, which rely on *Wigglesworthia*

glossinidia for vitamins and cofactors (Chen et al. 1999, Oliver et al. 2003, Wu et al. 2006); and sharpshooters, such as *Homolodisca vitrupenis*, which have dual primary-symbionts and rely on both *Baumannia cicadellinicola* and *Sulcia muelleri* for vitamins and amino acids (Buchner 1965, Montllor et al. 2002, Wu et al. 2006).

Secondary-symbionts can be maternally transferred or be obtained from the environment. Secondary-symbionts are not necessary for an insect's survival but they can provide beneficial physiological properties for the insect. It has been seen in aphids that their secondary-symbionts in the family *Enterobacteriaceae* can provide predator resistance (Oliver et al. 2003, Nakabachi et al. 2006), heat resistance (Clark et al. 2001, Montllor et al. 2002, Ferrari et al. 2007, McLean et al. 2010), and although proposed, does not allow for more diversity in host plants but greater fecundity (Thao et al. 2000, Clark et al. 2001, Wu et al. 2006, Ferrari et al. 2007, McLean et al. 2010).

Carsonella ruddii

All psyllid species harbor the *Carsonella ruddii* as their primary symbiont (Thao et al. 2000). *C. ruddii* is an endosymbiotic bacteria of psyllids that resides in specialized cells in the body cavity called bacteriocytes and is maternally transmitted to offspring (Buchner 1965, Chang and Musgrave 1969, Fukatsu and Nikoh 1998). It is proposed that *C. ruddii* provides the psyllids with essential amino acids lacking in their diet and the psyllids cannot synthesize on their own (Buchner 1965, Douglas 1998).

Genetic comparisons of *C. ruddii* in different psyllid species suggest that this infection was passed down from a single infection of the common ancestor of all psyllids, yet *C. ruddii* shows unusually high genetic variation between host species of psyllids (Hugenholtz et al. 1998, Thao et al. 2000, Nakabachi et al. 2006). *C. ruddii* has the smallest genome of any known bacteria at 160kb, only 182 open reading frames, a low GC content of 16.5%, and 97.3% of the genome coding for proteins and RNA (Wolstenholme 1992, Clark et al. 2001, Nakabachi et al. 2006, Carlos et al. 2012). The reduced genome size and low CG content result in proteins that are ~9% smaller than orthologous proteins in other bacteria. There is also an absence of some ribosomal binding sites suggesting that translational coupling occurs similar to mitochondria (Wolstenholme 1992, Clark et al. 2001, Wu et al. 2006, Tedersoo et al. 2010). *C. ruddii* is missing or has highly degraded genes that code for proteins necessary for DNA replication and maintenance; thus, it is contested as to whether it can be considered an independent living organism or if it has become an organelle. *C. ruddii* is also missing genes that code for histidine, phenylalanine and tryptophan, which makes it unsuitable as the only primary symbiont in psyllids, which require these amino acids (Hugenholtz et al. 1998, Wu et al. 2006, Lee et al. 2009). For comparison, *Buchnera aphidicola*, the obligate symbiont in aphids, has a 450kb genome, with 564 ORFs, and with all necessary genes to function without aid from the host insect.

Wolbachia

Hertig and Wolbach, first described the genus *Wolbachia*, and α-proteobacter in the class Rickettsia, in 1936, where it was observed in the ovaries of *Culex pipiens* (Hertig and Wolbach 1924, Hertig 1936). No further research into *Wolbachia* was conducted until 1971 when Yen and Barr discovered that infected males' sperm could not fertilize non-infected female eggs (Yen and Barr 1971). Reproductive incompatibility has also been observed in many other insects such as *Drosophila* and wasps of the genus *Nasonia* (Breeuwer et al. 1992, Min and Benzer 1997). *Wolbachia* has also been observed in terrestrial isopods, mites, and nematodes. *Wolbachia* has been seen to affect the reproduction of insects in many ways, such as feminization of males, parthenogenesis, male killing, cytoplasmic incompatibility, increased fecundity, and pathenogenicity (Stouthamer et al. 1990, Rousset et al. 1992, Girin and Bouletreau 1995, Min and Benzer 1997, Fialho et al. 2000). Cytoplasmic incompatibility does not only occur between infected and non-infected individuals, in some cases different strains of *Wobachia* will cause reproductive incompatibility (Breeuwer and Werren 1990). It is thought that these modifications have played a part in the evolution of some insects and eusociality (Hurst 1997). Wolbachia has also been used in biological control where a strain that causes cytoplasmic incompatibility is added to mosquitoes that carry a desired gene, which are then released into wild populations and subsequently replace them (Bourtzis 2008).

Wolbachia is vertically transferred, but genetic comparisons of *Wolbachia* in different species has led to the conclusion that horizontal transfer is common. Horizontal transfer of *Wolbachia* between different species has been observed from Drosophila to a parasitoid wasp (Heath et al. 1999). Parasitoid transfer of *Wolbachia* is possibly common because of the abundance of parasitoid wasps and the diversity of their hosts (Godfray 1993).

In potato psyllids *Wolbachia* has been identified in eggs, nymphs, and adults (Hail et al. 2012). Currently no reproductive modifications or other behavioral differences have been associated with *Wolbachia* in psyllids but its presence in all life stages indicates that it is vertically transferred and likely has some effect on psyllids.

Analysis of microbial communities

Old School: Culturing and Cloning

Analysis of microbial communities prior to PCR and Sanger sequencing consisted of culturing and isolating bacteria on different nutrient media. Culturing bacteria limited microbial community analysis because it allowed for the visual identification of known and culturable bacteria. Post PCR, microbial communities could be identified and quantified by cloning 16s rRNA genes in libraries and then utilizing Sanger sequencing on the now isolated and amplified 16s rRNA genes. Sequencing bacterial 16s genes allowed for the identification and classification of unculturable bacteria, which account for 99% of all bacteria in most environmental samples. Results from sequencing 16s genes has led to the

identification of 40 new divisions of eubacteria, 13 of which are *Candidatus,* or not represented by a classified culturable organism (Hugenholtz et al. 1998, Lee et al. 2009, Amend et al. 2010, Carlos et al. 2012). Though this method allowed for a much better understanding of environmental bacterial samples, it was very labor intensive, expensive, and because it relied on isolated cultures for each sequence, was limited to very few isolates per sample (Schloss et al. 2009, Amend et al. 2010, Tedersoo et al. 2010, Caporaso et al. 2011, Huson et al. 2011, Carlos et al. 2012).

New School: Pyrosequencing

Pyrosequencing has eliminated the need for traditional methods of bacterial community analysis by reducing cost and time, greatly increasing the number of sequences per sample, and allowed for the processing of multiple samples at the same time.

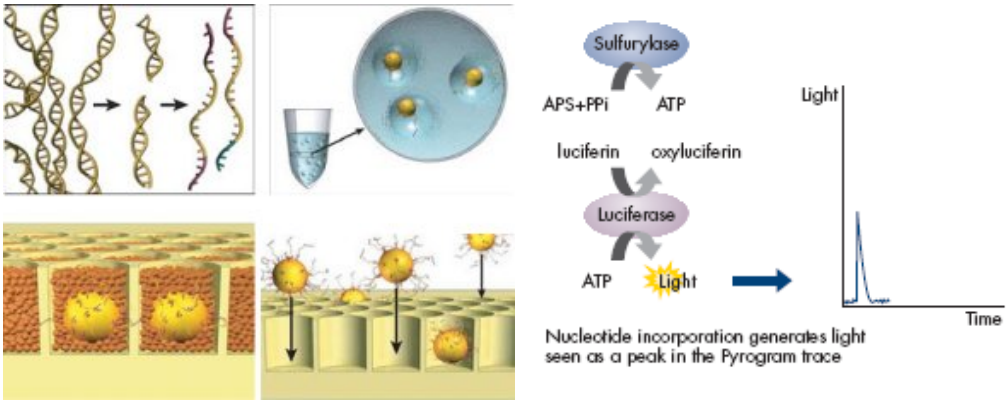

Figure 1.3: Diagrams detailing the steps of pyrosequencing. In the left images the process of attaching the DNA to beads and the beads being secured in the plate. The right image shows the enzymes responsible involved in the light reaction that signifies a nucleotide has been added and the resulting pyrogram. (www.pyrosequencing.com, www.454.com)

In pyrosequencing individual strands are given nucleotide barcodes then hybridized to beads. Once on these beads, the DNA is replicated millions of times so each bead contains millions of identical copies of single-stranded DNA. These beads are then added to a plate containing millions of wells, each well receives one bead. During the sequencing reaction, free nucleotides are washed over the plate in a sequential order. If the free nucleotide corresponds to the template sequence polymerase will add it to the strand and a phosphate is released. Released phosphates are used by sulfurylase to create ATP, which is then used by the enzyme luciferase to create light. This light is detected and because the nucleotides are added in sequential order, will tell the order of nucleotides in the sequence. The light readings are output on a graph called a pyrogram. Higher peaks on the pyrogram signify identical sequential nucleotides. If it is the improper nucleotide, the enzyme apyrase degrades the free nucleotides (Ronaghi 2001).

Although pyrosequencing has proven to be an accurate method of identifying environmental microbial communities, there are some biological and technical problems that need to be addressed to not create bias in the data or make false assumptions. Pyrosequencing results can be affected by primer selection, PCR, and extraction methods. To avoid impacts to diversity and species richness, protocols need to be established that will avoid biased data (Maidak et al. 1997, Rooney and Ward 2005, Meyer et al. 2008, Lee et al. 2009, Tedersoo et al. 2010, Sun et al. 2011, Markowitz et al. 2012). Another problem

that is apparent with pyrosequencing of 16s rRNA genes is the inaccurate quantification based on sequence abundance caused by gene copy number which is variable between and within species of fungus and bacteria (Rooney and Ward 2005, Lee et al. 2009, Amend et al. 2010, Haas et al. 2011). Although the actual count of sequences can be disproportionate to actual organisms in a sample, the overall percentage of abundance, or semi-quantification, in a sample is a fair way of describing presence in a sample (Schloss et al. 2009, Amend et al. 2010, Caporaso et al. 2011, Huson et al. 2011).

Analysis of 16s rRNA gene pyrosequencing data can be accomplished through many software packages such as QIIME, Mothur, and MEGAN (Liu et al. 2006, Schloss et al. 2009, Caporaso et al. 2011, Huson et al. 2011). There is also a growing number of online services like MG-RAST, the RDP, the CAMERA portal, and JGI IMG (Maidak et al. 1997, Munyaneza et al. 2007, Meyer et al. 2008, Sun et al. 2011, Markowitz et al. 2012).

Prior to analysis artifact reads that are too short (<200), abnormally long (>1000), chimeric, or low quality need to be removed from the dataset. These artifacts are the result of sequencing errors or recombination of sequences that are caused by the massively parallel nature of pyrosequencing. There are many programs that can filter bad reads or by sequence length, removal of chimeric sequences can be done with ChimeraSlayer (Haas et al. 2011, Nachappa et al. 2011).

Pyrosequencing datasets contain a large number of sequences that need to be grouped to aid in lowering the computational requirements for analysis. The sequences are grouped into OTUs, (operational taxonomic units) based on their similarity and length. OTU similarity is chosen by the user dependent on how precise of a classification is desired. In bacterial analysis OTU sequence identity of 97% is considered species level similarity and 94% similarity to genus. OTUs are then classified using a representative sequence from each OTU. There are many classification databases: Green Genes, SILVA, NCBI, RDP, and others. Depending on the dataset, each can provide classifications to genus or species. As more projects seek to classify environmental microbial communities these databases are becoming larger and are better suited to classifying samples but are still unable to classify many sequences.

Unidentifiable sequences are still important and can provide insight into microbial community diversity. To allow for these unidentified sequences to be included in the data analysis with the identified sequences, all representative sequences are aligned to a reference sequence and then a phylogenetic tree is created based on the alignment. Downstream, the complete phylogenetic tree will be used to assess similarities in microbial communities based on how related each sample's individual tree is to the other samples. OTUs closer on the tree will have less impact on diversity than OTUs closer on the tree.

Comparing Microbial Community Diversity

An important aspect of microbial community analysis is a comparison of how diverse these communities are. These comparisons can be qualitative, counting presence or absence only, or quantitative, accounting for the number of sequences in each OTU. When comparing alpha-diversity, the diversity within a sample, commonly used tests are choa1, Shannon-weaver, and rarefaction. When comparing beta-diversity, the diversity comparison between samples, commonly used indices are UniFrac, Sorensen index, and Jaccard index. Another way of looking at diversity is to look at the relatedness of bacteria present, called divergence-based diversity. These methods, such as UniFrac, use the phylogenetic tree of all OTUs and compare how each sample's OTU phylogeny compares within the OTU phylogeny of all samples combined. The advantage of the UniFrac method is that the similarity of the 16s rRNA gene sequence can be an indicator of metabolic or environmental similarity. If samples have different bacterial communities, but the bacteria perform the same function, the trees will be more similar than another sample with different metabolic needs (Ley et al. 2005, Lozupone and Knight 2008, Teulon et al. 2009).

Purpose Statement

The potato psyllid has become an economically important pest of solanacious crops and has seen a rapid range expansion into Central America, the Pacific Northwest, and New Zealand. Potato psyllids are associated with Zebra Chip disease in potatoes; which results in millions of dollars in damages to

potato crops annually. Previous studies have identified three haplotypes of psyllid with unique genetic markers in an attempt to better understand psyllid movements. Understanding dynamics that may be affecting potato psyllid range expansion could be beneficial to their control in the future. In this study, recent potato psyllid range expansions and *Candidatus* Liberibacter solanacaerum infections in solanaceous crops and potato psyllids in Central America were confirmed with molecular techniques, current molecular methods used to identify *Candidatus* Liberibacter solanacaerum infection were assessed to understand unusual patterns in infection levels of field and lab potato psyllids, and the global diversity in potato psyllid microbial community was assessed.

Literature Cited

Abdullah, N. M. M. 2008. Life history of the potato psyllid *Bactericera cockerelli* (Homoptera: Psyllidae) in controlled environment agriculture in Arizona. Afr J Agric Res. 3: 60–67.

Abernathy, R. L. 1991. Investigations into the nature of the potato psyllid toxin. M.S. thesis, Colorado State University, Fort Collins, CO.

Amend, A. S., K. A. Seifert, and T. D. Bruns. 2010. Quantifying microbial communities with 454 pyrosequencing: does read abundance count? Molecular Ecology. 19: 5555–5565.

Arslan, A., P. M. Bessey, K. Matsuda, and N. F. Oebker. 1985. Physiological effects of psyllid (*Paratrioza cockerelli*) on potato. Am. J. Pot Res. 62: 9–22.

Bextine, B. R., A. Arp, E. Flores, E. Y. Aguilar, L. Schindler, S. G. Freddy, C. Powell, and A. A. Rueda. 2013. First report of Zebra Chip and "*Candidatus* Liberibacter solanacearum" on potatoes in Nicaragua. Plant Dis.

Binkley, A. M., and C. H. Metzger. 1929. Psyllid Yellows (cause undetermined). US Dept. Agr. Bur. Plant Indus. Plant Disease Rptr. 68: 29.

Blood, H. L., B. L. Richards, and F. B. Wann. 1933. Studies of psyllid yellows of tomato. Phytopathology. 23: 930.

Bourtzis, K. 2008. *Wolbachia*-based technologies for insect pest population control. Adv Exp Med Biol. 104–113.

Breeuwer, J. A., and J. H. Werren. 1990. Microorganisms associated with chromosome destruction and reproductive isolation between two insect species. Nature. 346: 558–560.

Breeuwer, J. A., R. Stouthamer, S. M. Barns, D. A. Pelletier, W. G. Weisburg, and J. H. Werren. 1992. Phylogeny of cytoplasmic incompatibility micro-organisms in the parasitoid wasp genus *Nasonia* (Hymenoptera: Pteromalidae) based on 16S ribosomal DNA sequences. Insect Mol. Biol. 1: 25–36.

Buchman, J. L., B. E. Heilman, and J. E. Munyaneza. 2011. Effects of Liberibacter-Infective *Bactericera cockerelli* (Hemiptera: Triozidae) density on Zebra Chip Potato Disease incidence, potato yield, and tuber processing quality. Journal of Economic Entomology. 104: 1783–1792.

Buchman, J. L., V. G. Sengoda, and J. E. Munyaneza. 2011. Vector transmission efficiency of Liberibacter by *Bactericera cockerelli (*Hemiptera: Triozidae) in Zebra Chip Potato Disease: Effects of psyllid life stage and inoculation access period. Journal of Economic Entomology. 104: 1486–1495.

Buchner, P. 1965. Endosymbiosis of animals with plant microorganisms. Interscience Publishers.

Caporaso, J. G., C. L. Lauber, W. A. Walters, D. Berg-Lyons, C. A. Lozupone, P. J. Turnbaugh, N. Fierer, and R. Knight. 2011. Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. Proceedings of the National Academy of Sciences. 108: 4516–4522.

Carlos, N., Y.-W. Tang, and Z. Pei. 2012. Pearls and pitfalls of genomics-based microbiome analysis. Emerg Microbes Infect. 1: e45.

Carter, W. 1939. Injuries to plants caused by insect toxins. The Botanical Review. 5: 273–326.

Chang, K. P., and A. J. Musgrave. 1969. Histochemistry and ultrastructure of the mycetome and its “symbiotes” in the pear psylla, *Psylla pyricola* Foerster (Homoptera). Tissue and Cell. 1: 597–606.

Chen, X., S. Li, and S. Aksoy. 1999. Concordant evolution of a symbiont with its host insect species: molecular phylogeny of genus *Glossina* and its bacteriome-associated endosymbiont, *Wigglesworthia glossinidia*. J. Mol. Evol. 48: 49–58.

Claridge, M. F., and J. Den Hollander. 1983. The biotype concept and its application to insect pests of agriculture. Crop Protection. 2: 85–95.

Clark, M. A., L. Baumann, M. L. Thao, N. A. Moran, and P. Baumann. 2001. Degenerative minimalism in the genome of a psyllid endosymbiont. J. Bacteriol. 183: 1853–1861.

Compere, H. 1915. *Paratrioza cockerelli* (Sulc). Monthly Bulletin of California State Commission of Horticulture. 4: 574.

Crosslin, J. M., N. Olsen, and P. Nolte. 2012. First report of Zebra Chip Disease and "*Candidatus* Liberibacter solanacearum" on potatoes in Idaho. Plant Disease 96:3, 453-453.

Daniels, L. B. 1954. The nature of the toxicogenic condition resulting from the feeding of the tomato psyllid *Paratrioza Cockerelli* (Sulc). Ph.D. dissertation, University of Minnesota, St. Paul, MN.

Diehl, S. R., and G. L. Bush. 1984. An evolutionary and applied perspective of insect biotypes. Annu. Rev. Entomol. 29: 471–504.

Douglas, A. E. 1998. Nutritional interactions in insect-microbial symbioses: aphids and their symbiotic bacteria Buchnera. Annu. Rev. Entomol. 43: 17–37.

Espinoza, H. R. 2010. Facing the *Bactericera cockerelli*/*Candidatus* Liberibacter Complex in Honduras. 2010 Zebra Chip Reporting Session. 47.

Essig, E. O. 1917. The tomato and laurel psyllids. Journal of Economic entomology. 10: 433–444.

Ferguson, G., E. Banks, and H. Fraser. 2002. Potato psyllid–a new pest in greenhouse tomatoes and peppers. Ontario Ministry of Food and Agriculture.

Ferrari, J., C. L. Scarborough, and H. C. J. Godfray. 2007. Genetic variation in the effect of a facultative symbiont on host-plant use by pea aphids. Oecologia. 153: 323–329.

Fialho, R. F., L. Stevens, R. F. Fialho, and L. Stevens. 2000. Male-killing *Wolbachia* in a flour beetle. Proceedings of the Royal Society of London. Series B: Biological Sciences. 267: 1469–1473.

Fukatsu, T., and N. Nikoh. 1998. Two intracellular symbiotic bacteria from the mulberry psyllid *Anomoneura mori* (Insecta, Homoptera). Applied and Environmental Microbiology. 64: 3599–3606.

Girin, C., and M. Bouletreau. 1995. Microorganism-associated variation in host infestation efficiency in a parasitoid wasp, *Trichogramma bourarachae* (Hymenoptera: Trichogrammatidae). Cellular and Molecular Life Sciences. 51: 398–401.

Glick, P. A. 1939. The distribution of insects, spiders, and mites in the air. US Department of Agriculture.

Godfray, H. C. J. 1993. Parasitoids: behavioral and evolutionary ecology. Princeton University Press.

Haas, B. J., D. Gevers, A. M. Earl, M. Feldgarden, D. V. Ward, G. Giannoukos, D. Ciulla, D. Tabbaa, S. K. Highlander, E. Sodergren, B. Methe, T. Z. DeSantis, The Human Microbiome Consortium, J. F. Petrosino, R. Knight, and B. W. Birren. 2011. Chimeric 16S rRNA sequence formation and detection in Sanger and 454-pyrosequenced PCR amplicons. Genome Research. 21: 494–504.

Hail, D., S. E. Dowd, and B. Bextine. 2012. Identification and location of symbionts associated with potato psyllid (*Bactericera cockerelli*) lifestages. Environmental Entomology. 41: 98–107.

Heath, B. D., R. D. Butcher, W. G. Whitfield, and S. F. Hubbard. 1999. Horizontal transfer of *Wolbachia* between phylogenetically distant insect species by a naturally occurring mechanism. Curr. Biol. 9: 313–316.

Hertig, M. 1936. The rickettsia, *Wolbachia pipientis* and associated inclusions of the mosquito, *Culex pipiens*. Parasitology. 28: 453–486.

Hertig, M., and S. B. Wolbach. 1924. Studies on rickettsia-like micro-organisms in insects. The Journal of medical research. 44: 329.

Hill, R. E. 1947. An unusual weather sequence accompanying the severe potato psyllid outbreak of 1938 in Nebraska. Journal of the Kansas Entomological Society. 20: 88–92.

Horowitz, A. R., K. Gorman, G. Ross, and I. Denholm. 2003. Inheritance of pyriproxyfen resistance in the whitefly, *Bemisia tabaci* (Q biotype). Arch. Insect Biochem. Physiol. 54: 177–186.

Hugenholtz, P., B. M. Goebel, and N. R. Pace. 1998. Impact of culture-independent studies on the emerging phylogenetic view of bacterial diversity. J. Bacteriol. 180: 4765–4774.

Hurst, G. D. 1997. *Wolbachia*, Cytoplasmic incompatibility, and the evolution of eusociality. Journal of theoretical biology. 184: 99–100.

Huson, D. H., S. Mitra, H. J. Ruscheweyh, N. Weber, and S. C. Schuster. 2011. Integrative analysis of environmental sequences using MEGAN4. Genome Research. 21: 1552–1560.

Jackson, B. C., J. Goolsby, A. Wyzykowski, N. Vitovsky, and B. Bextine. 2009. Analysis of genetic relationships between potato psyllid (*Bactericera cockerelli*) populations in the United States, Mexico and Guatemala using ITS2 and Inter Simple Sequence Repeat (ISSR) data. Subtropical Plant Science. 61: 1–5.

Jiang, Y. X., H. Lei, J. L. Collar, B. Martin, M. Muniz, and A. Fereres. 1999. Probing and feeding behavior of two distinct biotypes of *Bemisia tabaci* (Homoptera: Aleyrodidae) on tomato plants. Journal of Economic entomology. 92: 357–366.

Knowlton, G. F., and M. J. Janes. 1931. Studies on the biology of *Paratrioza cockerelli* (Sulc). Annals of the Entomological Society of America. 24: 283–292.

Knowlton, G. F., and W. L. Thomas. 1934. Host plants of the potato psyllid. Journal of Economic entomology. 27: 547.

Lee, Z. M. P., C. Bussema, and T. M. Schmidt. 2009. rrnDB: documenting the number of rRNA and tRNA genes in bacteria and archaea. Nucleic Acids Research. 37: 489–493.

Lehman, R. S. 1930. Some observations on the life history of the tomato psyllid (*Paratrioza Cockerelli* Sulc.) (Homoptera). Journal of the New York Entomological Society. 38: 307–312.

Ley, R. E., F. Bäckhed, P. Turnbaugh, C. A. Lozupone, R. D. Knight, and J. I. Gordon. 2005. Obesity alters gut microbial ecology. Proceedings of the National Academy of Sciences. 102: 11070–11075.

Linford, M. B. 1928. Psyllid yellows (cause undetermined). Plant Dis. Rep. 59: 95–99.

List, G. M. 1939. The effect of temperature upon egg deposition, egg hatch and nymphal development of *Paratrioza cockerelli* (Sulc). Journal of Economic entomology. 32: 30–36.

Liu, D., and J. T. Trumble. 2007. Comparative fitness of invasive and native populations of the potato psyllid (*Bactericera cockerelli*). Entomol Exp Appl. 123: 35–42.

Liu, D., J. T. Trumble, and R. Stouthamer. 2006. Genetic differentiation between eastern populations and recent introductions of potato psyllid (*Bactericera cockerelli*) into western North America. Entomol Exp Appl. 118: 177–183.

Lozupone, C. A., and R. Knight. 2008. Species divergence and the measurement of microbial diversity. FEMS Microbiology Reviews. 32: 557–578.

Maidak, B. L., G. J. Olsen, N. Larsen, R. Overbeek, M. J. McCaughey, and C. R. Woese. 1997. The RDP (Ribosomal Database Project). Nucleic Acids Research. 25: 109–111.

Markowitz, V. M., I. M. A. Chen, K. Palaniappan, K. Chu, E. Szeto, Y. Grechkin, A. Ratner, B. Jacob, J. Huang, P. Williams, M. Huntemann, I. Anderson, K. Mavromatis, N. N. Ivanova, and N. C. Kyrpides. 2012. IMG: the integrated microbial genomes database and comparative analysis system. Nucleic Acids Research. 40: 115–122.

McLean, A. H. C., M. van Asch, J. Ferrari, and H. C. J. Godfray. 2010. Effects of bacterial secondary symbionts on host plant use in pea aphids. Proceedings of the Royal Society B: Biological Sciences. 278: 760–766.

Meyer, F., D. Paarmann, M. D'Souza, R. Olson, E. M. Glass, M. Kubal, T. Paczian, A. Rodriguez, R. Stevens, A. Wilke, J. Wilkening, and R. A. Edwards. 2008. The metagenomics RAST server – a public resource for the automatic phylogenetic and functional analysis of metagenomes. BMC Bioinformatics. 9: 386.

Min, K. T., and S. Benzer. 1997. *Wolbachia*, normally a symbiont of Drosophila, can be virulent, causing degeneration and early death. Proc. Natl. Acad. Sci. U.S.A. 94: 10792–10796.

Montllor, C. B., A. Maxmen, and A. H. Purcell. 2002. Facultative bacterial endosymbionts benefit pea aphids *Acyrthosiphon pisum* under heat stress. Ecological Entomology. 27: 189–195.

Moran, N. A., and A. Mira. 2001. The process of genome shrinkage in the obligate symbiont *Buchnera aphidicola*. Genome Biol. 2: 1–0054.12.

Munyaneza, J. E. 2010. Psyllids as vectors of emerging bacterial diseases of annual crops. Southwestern Entomologist. 35: 471–477.

Munyaneza, J. E. 2012. Zebra Chip Disease of potato: Biology, epidemiology, and management. Am. J. Pot Res. 89: 329–350.

Munyaneza, J. E., J. A. Goolsby, J. M. Crosslin, and J. E. Upton. 2007. Further evidence that zebra chip potato disease in the lower Rio Grande Valley of Texas is associated with *Bactericera cockerelli.* Subtropical Plant Science. 59: 30-37.

Munyaneza, J. E., J. M. Crosslin, and J. E. Upton. 2007. Association of *Bactericera cockerelli* (Homoptera : Psyllidae) with “zebra chip,” a new potato disease in southwestern United States and Mexico. Journal of Economic entomology. 100: 656–663.

Munyaneza, J. E., J. M. Crosslin, and J. L. Buchman. 2009. Seasonal occurrence and abundance of the potato psyllid, *Bactericera cockerelli*, in south central Washington. Am. J. Pot Res. 86: 513–518.

Munyaneza, J. E. 2012. Zebra Chip Disease of potato: Biology, epidemiology, and management. Am. J. Pot Res. 89: 329–350.

Nachappa, P., J. Levy, E. Pierson, and C. Tamborindeguy. 2011. Diversity of endosymbionts in the potato psyllid, *Bactericera cockerelli* (Hemiptera: Triozidae), vector of Zebra Chip Disease of potato. Curr Microbiol. 62: 1510–1520.

Nakabachi, A., A. Yamashita, H. Toh, H. Ishikawa, H. E. Dunbar, N. A. Moran, and M. Hattori. 2006. The 160-Kilobase genome of the bacterial endosymbiont Carsonella. Science. 314: 267–267.

Oliver, K. M., J. A. Russell, N. A. Moran, and M. S. Hunter. 2003. Facultative bacterial symbionts in aphids confer resistance to parasitic wasps. Proc. Natl. Acad. Sci. U.S.A. 100: 1803–1807.

Papp, R. P., and J. B. Johnson. 1979. Origins of psyllid fallout in the central Sierra Nevada of California (Homoptera). Pan-Pacific Entomologist. 55: 95–98.

Pitman, A. R., G. M. Drayton, S. J. Kraberger, R. A. Genet, and I. A. W. Scott. 2011. Tuber transmission of "*Candidatus* Liberibacter solanacearum" and its association with zebra chip on potato in New Zealand. Eur J Plant Pathol. 129: 389–398.

Pletsch, D. J. 1947. The potato psyllid, *Paratrioza cockerelli* (Sulc): its biology and control. Montana State College, Agricultural Experiment Station.

Powell, C. M., J. King, and B. R. Bextine. 2012. Cytochrome B sequences of potato psyllids, *Bactericera cockerelli* (Sulc) 1, from North and Central America. Southwestern Entomologist. 37: 521–524.

Richards, B. L. 1928. A new and destructive disease of the potato in Utah and its relation to the potato psyllid. Phytopathology. 18: 140–141.

Richards, L. B., H. L. Blood, and M. B. Linford. 1927. Destructive outbreak of unknown potato disease in Utah. Plant Disease Report. 11: 93–94.

Romney, V. E. 1939. Breeding areas of the tomato psyllid, *Paratrioza cockerelli* (Sulc). Journal of Economic entomology. 32: 150–151.

Ronaghi, M. 2001. Pyrosequencing sheds light on DNA sequencing. Genome Research. 11: 3–11.

Rondon, S., A. Schreiber, A. Jensen, P. Hamm, J. Munyaneza, P. Nolte, N. Olsen, E. Wenninger, D. Henne, C. Wohleb, and A. T. Waters. 2012. Potato psyllid vector of Zebra Chip Disease in the Pacific Northwest. 1–8.

Rooney, A. P., and T. J. Ward. 2005. Evolution of a large ribosomal RNA multigene family in filamentous fungi: birth and death of a concerted evolution paradigm. Proceedings of the National Academy of Sciences. 102: 5084–5089.

Rousset, F., D. Bouchon, B. Pintureau, P. Juchault, and M. Solignac. 1992. *Wolbachia* endosymbionts responsible for various alterations of sexuality in arthropods. Proceedings of the Royal Society of London. Series B: Biological Sciences. 250: 91–98.

Schaal, L. A. 1938. Some factors affecting the symptoms of the psyllid yellows disease of potatoes. Am. J. Pot Res. 15: 193–206.

Schloss, P. D., S. L. Westcott, T. Ryabin, J. R. Hall, M. Hartmann, E. B. Hollister, R. A. Lesniewski, B. B. Oakley, D. H. Parks, C. J. Robinson, J. W. Sahl, B. Stres, G. G. Thallinger, D. J. Van Horn, and C. F. Weber. 2009. Introducing mothur: Open-source, platform-independent, community-supported software for describing and comparing microbial communities. Applied and Environmental Microbiology. 75: 7537–7541.

Secor, G. A., and V. V. Rivera-Varas. 2004. Emerging diseases of cultivated potato and their impact on Latin America. Rev. Latinoam. de la Papa (Suppl.). 1: 1–8.

Secor, G. A., V. V. Rivera, J. A. Abad, I. M. Lee, G. Clover, L. W. Liefting, X. Li, and S. H. De Boer. 2009. Association of "*Candidatus* Liberibacter solanacearum" with Zebra Chip Disease of potato established by graft and psyllid transmission, electron microscopy, and PCR. Plant Dis. 93: 574–583.

Sengoda, V. G., J. E. Munyaneza, J. M. Crosslin, J. L. Buchman, and H. R. Pappu. 2009. Phenotypic and etiological differences between psyllid yellows and Zebra Chip Diseases of potato. Am. J. Pot Res. 87: 41–49.

Shigenobu, S., H. Watanabe, M. Hattori, Y. Sakaki, and H. Ishikawa. 2000. Genome sequence of the endocellular bacterial symbiont of aphids *Buchnera sp.* APS. Nature. 407: 81–86.

Stouthamer, R., R. F. Luck, and W. D. Hamilton. 1990. Antibiotics cause parthenogenetic *Trichogramma* (Hymenoptera/Trichogrammatidae) to revert to sex. Proc. Natl. Acad. Sci. U.S.A. 87: 2424–2427.

Sulc, K. 1909. Trioza cockerelli n. sp., a novelty from North America, being also of economic importance. Acta Societatis Entomologicae Bohemiae. 6: 102–108.

Sun, S., J. Chen, W. Li, I. Altintas, A. Lin, S. Peltier, K. Stocks, E. E. Allen, M. Ellisman, J. Grethe, and J. Wooley. 2011. Community cyberinfrastructure for Advanced Microbial Ecology Research and Analysis: the CAMERA resource. Nucleic Acids Research. 39: D546–D551.

Swisher, K. D., J. E. Munyaneza, and J. M. Crosslin. 2012. High resolution melting analysis of the Cytochrome Oxidase I gene identifies three haplotypes of the potato psyllid in the United States. Environmental Entomology. 41: 1019–1028.

Tedersoo, L., R. H. Nilsson, K. Abarenkov, T. Jairus, A. Sadam, I. Saar, M. Bahram, E. Bechem, G. Chuyong, and U. Kõljalg. 2010. 454 Pyrosequencing and Sanger sequencing of tropical mycorrhizal fungi provide similar results but reveal substantial methodological biases. New Phytologist. 188: 291–301.

Teulon, D., P. J. Workman, K. L. Thomas, and M.-C. Nielson. 2009. *Bactericera cockerelli*: incursion, dispersal and current distribution on vegetable crops in New Zealand. New Zealand Plant Protection Society. 62: 136–144.

Thao, M. L., N. A. Moran, P. Abbot, E. B. Brennan, D. H. Burckhardt, and P. Baumann. 2000. Cospeciation of psyllids and their primary prokaryotic endosymbionts. Applied and Environmental Microbiology. 66: 2898–2905.

Wallis, R. L. 1946. Seasonal occurrence of the potato psyllid in the North Platte Valley. Journal of Economic entomology. 39: 689–694.

Wallis, R. L. 1951. Potato psyllid selection of host plants. Journal of Economic entomology. 44: 815–817.

Wallis, R. L. 1955. Ecological studies on the potato psyllid as a pest of potatoes. US Dept. of Agriculture.

Wolstenholme, D. R. 1992. Animal mitochondrial DNA: structure and evolution. Int Rev Cytol. 141: 173–216.

Wu, D., S. C. Daugherty, S. E. Van Aken, G. H. Pai, K. L. Watkins, H. Khouri, L. J. Tallon, J. M. Zaborsky, H. E. Dunbar, and P. L. Tran. 2006. Metabolic complementarity and genomics of the dual bacterial symbiosis of sharpshooters. Plos Biol. 4: e188.

Yang, X.-B., and T.-X. Liu. 2009. Life history and life tables of *Bactericera cockerelli* (Homoptera: Psyllidae) on eggplant and bell pepper. Environmental Entomology. 38: 1661–1667.

Yang, X.-B., Y.-M. Zhang, L. Hua, and T.-X. Liu. 2010. Life history and life tables of *Bactericera cockerelli* (Hemiptera: Psyllidae) on potato under laboratory and field conditions in the Lower Rio Grande Valley of Texas. Journal of Economic entomology. 103: 1729–1734.

Yen, J. H., and A. R. Barr. 1971. New hypothesis of the cause of cytoplasmic incompatibility in *Culex pipiens* L.. Nature 232: 657 – 658.

Range Expansion of *Bactericera cockerelli* and New Reservoirs for *Candidatus* Liberibacter solanacaearum in Central America

Abstract

The potato psyllid (*Bactericera cockerelli*) is an established agricultural pest of solanacious crops in the United States, Canada, and Mexico, that has recently seen range expansions in Central America, the Pacific Northwest, and New Zealand. These expansions are alarming because potato psyllids transmit *Candidatus* Liberibacter solanacaerum (Lso), the bacteria associated with Zebra Chip disease in potato that results in millions of dollars in damages annually. Potato psyllids have been observed on tomato, potato, peppers, and eggplant, but recent reports have shown potato psyllid feeding on tobacco resulting in symptoms similar to Zebra Chip. In this survey, plants and psyllids from Honduras, Nicaragua, and El Salvador, were tested for the presence of Lso to confirm visual indications of infection. The haplotype of the potato psyllids was also identified to identify possible sources of import. Plants, including tobacco, from all countries tested positive for Lso, but no potato psyllids tested positive. This is the first report of Lso in Nicaragua and El Salvador. The potato psyllids were all central haplotype indication of a natural range expansion into new areas of Central America.

Introduction

The potato psyllid (*Bactericera cockerelli*) is an established agricultural pest of solanacious crops in the United States, Canada, and Mexico, that has recently expanded its range south into Honduras, into the Pacific Northwest, and an accidental import to New Zealand has resulted in an established presence there (Pletsch 1947, Secor and Rivera-Varas 2004, Munyaneza, Goolsby, et al. 2007, Munyaneza, Crosslin, et al. 2009, Espinoza 2010, Munyaneza et al. 2010, Crosslin et al. 2012). Until the early 2000's potato psyllids were not a hugely damaging or regular pest of great importance. The cause of their recent range expansions and regular annual outbreaks is yet unknown but could be the result of climate changes or the psyllids themselves gaining new environmental resistances or adaptations allowing them to breed more prolifically.

There are three known haplotypes of potato psyllid; one found primarily in the central United States and Mexico, another along the west coast of the United States and New Zealand, and another recently identified haplotype that is found in Washington, Oregon, and Idaho (Liu et al. 2006, Thomas et al. 2011, Chapman et al. 2012, Swisher et al. 2012). These three haplotypes do not have any morphological differences and have been categorized through molecular means. Single nucleotide polymorphisms (SNPs) in the CO1 mitochondrial gene have been used successfully as well as ISSR, and the *Wolbachia wsp* gene to identify haplotype (Liu et al. 2006, Chapman et al. 2012, Swisher et al. 2012). The Cytochrome B gene was proposed as another gene for detecting haplotype

to create more robust protocol for haplotype identification, but there was not enough genetic variation between haplotypes (Powell et al. 2012). There are no known physiological or behavioral differences between these haplotypes, though it is thought that the western haplotype has an increased resistance to pesticides. Though psyllids have unknown differences between haplotypes, in other insects different biotypes have been seen to have differing feeding preferences, breeding behaviors, pesticide resistances, and other economically important differences (Claridge and Hollander 1983).

Potato psyllids have been observed feeding on over 20 families of plants but have only been seen to complete development on 40 species (Knowlton and Thomas 1934, Wallis 1951), though they show a preference for plants in the family Solanaceae (Wallis 1955). Many important food crops are within the family Solanaceae such as tomato, potato, peppers, and eggplant. These plants have all been observed displaying symptoms of psyllid yellows, a disease caused by potato psyllid nymphs feeding on the plant (Blood et al. 1933, Eyer 1937). Psyllid yellows are characterized by a reduction in growth and stem rigidity, chlorosis of the leaves, basal cupping of leaves, aerial tubers, and premature death (Richards et al. 1927, Richards 1928, Blood et al. 1933, Pletsch 1947).

Damages from psyllid yellows alone are reason to monitor and control potato psyllids, but they also transmit *Candidatus* Liberibacter solanacaerum (Lso), the putative causal agent of Zebra Chip (ZC) disease in potatoes (Munyaneza, Crosslin, et al. 2007). Lso has been found in many crops such as

carrot, tomato, peppers, tamarillo, and potato (Liefting et al. 2008, Crosslin and Bester 2009, McKenzie and Shatters 2009, Munyaneza, Sengoda, et al. 2009, Munyaneza et al. 2010). ZC displays similar foliar symptoms as psyllid yellows, but in potatoes also results in a necrosis of medullary tissues in the tuber that causes potatoes to stripe when fried (Munyaneza, Crosslin, et al. 2007). These symptoms make the potatoes unmarketable and because the Lso is retained in the tubers they cannot be used as seeds for future plantings.

Honduras, El Salvador, and Nicaragua have seen recent outbreaks of potato psyllids and ZC has been observed in many solanaceous crops. Monitoring the spread of potato psyllids and ZC disease into new countries and crops is necessary to properly control future outbreaks and understand if the spread is natural or caused by negligent agricultural practices. In this study tomato plants from El Salvador, potato and pepper plants from Nicaragua, and tomato, eggplant and tobacco from Honduras were tested for Lso infection by PCR and sequencing. Potato psyllids from Nicaragua were tested with real-time melt analysis to identify their haplotype and were also tested for infection with Lso.

Materials and Methods

Sample Collection and DNA Extraction

DNA from four tomato plants from El Salvador; five potato plants, one pepper plant, and three potato psyllids from Nicaragua; two eggplant, four tomato, and twelve tobacco plants from Honduras. All DNA samples from Central

America were extracted using the CTAB protocol described by Crosslin et al. (2011) and stored at -20°C before and after shipping.

Haplotype

Psyllid haplotype was assessed using real-time melt analysis by the methods outlined by Chapman et al. (2012). All samples were run with 2μl of psyllid genomic DNA with concentrations of 2-20ng/μl. The primers used were CO1 F1 5'GGA TTC ATT GTT TGA GCA CAT C and CO1 R1 5' TGA AAT AGG CAC GAG AAT CAA, which amplify a 78bp section of the psyllid mitochondrial CO1 gene. The PCR was run with 25μl reactions using 12.5μl of Qiagen Quanitfast PCR Master Mix (Qiagen, Valencia, CA), 2.5μl of both primer, 2 μl of template DNA, and 5.5μl of nanopure water. The PCR was run using the following thermal profile: 15 minute hold at 95°C followed by 40 cycles of 95°C for 10 seconds and 53°C for 15 seconds. Following PCR and a 90 second pre-melt cycle, the product underwent a melt cycle from 65°C to 80°C raising the temperature 0.25°C per step holding at each step for 5 seconds. The resulting differential melt curves were used to identify haplotype, with the central haplotype melting at approximately 1°C lower than the western haplotype.

Diagnostics to determine Lso infection in insects and plants.

Extracted potato psyllid DNA was analyzed for the presence of the 16s rRNA gene of *C*Ls using conventional PCR. Each 25 μl reaction included 12.5 μL Ampli Taq Gold 360 master mix (Applied Biosystems, Foster City, CA), 1μL GC enhancer (Applied Biosystems, Foster City, CA), 1 μL of each primer OA2

and OI2c (Liefting et al. 2008, Crosslin et al. 2011), 7.5 μL water, and 2 μL extracted DNA suspended in buffer AE (DNeasy Blood and Tissue DNA extraction Kit, Qiagen, Valencia, CA). The thermal profile was run as follows: cycle 1, 94.0^0 C for 2 min (1x), cycle 2, 94.0^{0}C for 30s, 65.0^{0}C for 30s, 72.0^{0}C for 60s (x40), and cycle 3, 72.0^{0}C for 5 min (x1). Gel electrophoresis was run using 1% agarose gels stained with ethidium bromide.

Sequencing

PCR products from nine samples positive for Lso were purified using a Qiagen Gel Extraction Kit (Qiagen, Valencia, CA) according to manufacturer protocol. Purified product less than 20ng/μL was condensed with a CentriVap DNA Vacuum Concentrator (Labconco, Kansas City, MO) or rerun following the PCR protocol seen above and purified again by gel extraction. Sequencing was done at the Yale Sequencing Core on an Applied Biosystems 3730*xl* DNA Genetic Analyzer with 96-capillaries (Life Technologies, Grand Island, NY). Each sample was repeated with four forward and four reverse primers. Returned sequences were aligned using Geneius and consensus sequences were compared with BLAST.

Results & Discussion

Biotype

Three psyllids from Nicaragua and 11 from Honduras were subjected to real-time PCR and Melt analysis of a 78bp section of their CO1 mitochondrial

gene containing a SNP that differentiates western and central haplotypes of potato psyllid (Chapman et al. 2012). All potato psyllids collected in Honduras and Nicaragua displayed the same haplotype real-time melt curve as potato psyllids from the central United States. One potato psyllid from Honduras was excluded from because of low DNA concentrations and a late amplification that indicated poor DNA quality possibly caused by improper extraction or shipping. Swisher et al. (In Press) confirmed these results using the Honduran samples and others from Nicaragua and El Salvador also using the CO1 gene but a different primer par that will also differentiate the Pacific Northwest haplotype (Swisher et al. 2012). These results indicate that the spread of the potato psyllid into this region was not caused by accidental import from California or the Pacific Northwest, but a natural range expansion south from Mexico. Weather patterns and winds in Central America provide a north-south corridor that the psyllids could use, similar to the winds they use to migrate in the United States. The southward expansion of potato psyllids is alarming as they transmit pathogens and cause extensive damages to crops. Agricultural practices in Central America are also a concern when considering the spread of potato psyllids to this area. Crop rotations of solanaceous crops do not remove food and breeding locations for psyllids and thus over-use of pesticides are being used to control them, which could result in the development of resistance (Observations by Blake Bextine and Joe Munyaneza). To alleviate problems with growing techniques in this region

there needs to be more education outreach to promote more environmentally conscious agriculture.

Insects Infected

None of the psyllids collected from Nicaragua tested positive for *Candidatus* Liberibacter solanacaerum with traditional PCR methods. These results are not evidence that potato psyllids are not transmitting Lso in this region. In previous studies, only 2% of field-collected insects tested positive for Lso using traditional detection methods because of low Lso titers below the minimum detection threshold (Arp et al. Submitted). Further testing using more sensitive techniques, such as pyrosequencing, need to be utilized to identify the presence of Lso in these insects. Pyrosequencing will also be useful in detection of potential microbial community differences with other populations of potato psyllid that may be present from dietary changes from the available solanaceous plants in the region such as tobacco and other native flora.

Plants Infected

DNA extracted from tomato plants from El Salvador; potatoes and pepper plants from Nicaragua; and eggplant, tomato, and tobacco plants from Honduras were tested for Lso. All samples tested positive for Lso, with exception of the pepper plant sample from El Salvador, with traditional PCR using the OI2c and OA2 primer pair. Gel extracted PCR product from three tomato samples from El Salvador, three potatoes from Nicaragua, and two eggplants and one tobacco from Honduras were sequenced to confirm positive results. The resulting

sequences were checked with BLAST and returned >99% identity with *Candidatus* Liberibacter solanacaerum.

Table 2.2: Plant samples that tested positive for Lso by traditional PCR with the OI2c/OA2 primer pair and sequencing

	Tomato		Potato		Pepper		Eggplant	
Country	PCR	Sequencing	PCR	Sequencing	PCR	Sequencing	PCR	Sequencing
El Salvador	4/4	3/3	n/a	n/a	n/a	n/a	n/a	n/a
Nicaragua	n/a	n/a	5/5	3/3	0/1	n/a	n/a	n/a
Honduras	4/4	n/a	n/a	n/a	n/a	n/a	2/2	2/2

These results are the first reported cases of Lso in Nicaragua and El Salvador. The rapid expansion of areas affected with Lso is alarming and a greater effort to monitor and control the spread is needed. Lso was most likely spread to these countries by the potato psyllids whose range is also expanding into new areas of Central America. Better control methods for potato, and regulations on transporting crops that can act as reservoirs of Lso need to be put in place to stop the spread.

Figure 2.1: Leaf curl and chlorosis caused by Lso infection in tobacco plants found in Honduras

Tobacco plants had previously not been identified as a reservoir Lso. Like tomatoes and peppers, which display symptoms of infection from Lso similar to psyllid yellows, tobacco plants show symptoms of chlorosis and leaf curl. Tobacco production in Central America provides many jobs and creates a large export. In 2012 tobacco production in Nicaragua in created $95 million in export revenue and provided 22,000 jobs. Widespread infection of tobacco plants by Lso in Central America could be highly detrimental to these countries and adds to the need for better psyllid controls and a better understanding of their range expansion.

Literature Cited

Arp, A., R. I. Chapman, J. M. Crosslin, and B. R. Bextine. Low-level detection of *Candidatus* Liberibacter solanacearum in extracted *Bactericera cockerelli* (Hemiptera: Triozidae) DNA by 454 Pyrosequencing. Submitted Nov 2012.

Blood, H. L., B. L. Richards, and F. B. Wann. 1933. Studies of psyllid yellows of tomato. Phytopathology. 23: 930.

Chapman, R. I., J. F. Macias-Velasco, A. P. Arp, and B. R. Bextine. 2012. Using quantitative real time PCR melt curve analysis of partial CO1 sequence for rapid biotype differentiation of *Bactericera cockerelli* (Hemiptera: Triozidae). Southwestern Entomologist. 37: 475–484.

Claridge, M. F., and J. Den Hollander. 1983. The biotype concept and its application to insect pests of agriculture. Crop Protection. 2: 85–95.

Crosslin, J. M., and G. Bester. 2009. First report of “Candidatus Liberibacter psyllaurous” in Zebra Chip symptomatic potatoes from California. Plant Dis. 93: 551–551.

Crosslin, J. M., H. Lin, and J. E. Munyaneza. 2011. Detection of “Candidatus Liberibacter Solanacearum” in the potato psyllid, *Bactericera cockerelli* (Sulc)1, by conventional and Real-Time PCR. Southwestern Entomologist. 36: 125–135.

Crosslin, J., J. Munyaneza, J. Brown, and L. Liefting. 2012. A history in the making: potato Zebra Chip Disease associated with a new psyllid-borne bacterium. A Tale of Striped Potatoes. APSnet Features.

Crosslin, J. M., N. Olsen, and P. Nolte. 2012. First report of Zebra Chip Disease and "Candidatus Liberibacter solanacearum" on potatoes in Idaho. Plant Disease. 96: 453-453.

Espinoza, H. R. 2010. Facing the *Bactericera cockerelli*/*Candidatus* Liberibacter complex in Honduras. 2010 Zebra Chip Reporting Session. 47.

Eyer, J. R. 1937. Physiology of psyllid yellows of potatoes. Journal of Economic entomology. 30: 891–898.

Knowlton, G. F., and W. L. Thomas. 1934. Host plants of the potato psyllid. Journal of Economic entomology. 27: 547.

Liefting, L. W., L. I. Ward, J. B. Shiller, and G. R. G. Clover. 2008. A New "Candidatus Liberibacter" species in Solanum betaceum (Tamarillo) and Physalis peruviana (Cape Gooseberry) in New Zealand. Plant Dis. 92: 1588–1588.

Liu, D., J. T. Trumble, and R. Stouthamer. 2006. Genetic differentiation between eastern populations and recent introductions of potato psyllid (*Bactericera cockerelli*) into western North America. Entomol Exp Appl. 118: 177–183.

McKenzie, C. L., and R. G. Shatters. 2009. First Report of “Candidatus Liberibacter psyllaurous” associated with Psyllid Yellows of tomato in Colorado. Plant Dis. 93: 1074–1074.

Munyaneza, J. E., J. A. Goolsby, J. M. Crosslin, and J. E. Upton. 2007. Further evidence that Zebra Chip potato disease in the lower Rio Grande Valley of Texas is associated with *Bactericera cockerelli*. Subtropical Plant Science. 59: 30-37.

Munyaneza, J. E., J. M. Crosslin, and J. E. Upton. 2007. Association of *Bactericera cockerelli* (Homoptera : Psyllidae) with “Zebra Chip,” a new potato disease in southwestern United States and Mexico. Journal of Economic entomology. 100: 656–663.

Munyaneza, J. E., J. M. Crosslin, and J. L. Buchman. 2009. Seasonal occurrence and abundance of the potato psyllid, *Bactericera cockerelli*, in south central Washington. Am. J. Pot Res. 86: 513–518.

Munyaneza, J. E., T. W. Fisher, V. G. Sengoda, S. F. Garczynski, A. Nissinen, and A. Lemmetty. 2010. First report of “Candidatus Liberibacter solanacearum” associated with asyllid-affected carrots in Europe. Plant Dis. 94: 639–639.

Munyaneza, J. E., V. G. Sengoda, J. M. Crosslin, J. A. Garzón-Tiznado, and O. G. Cardenas-Valenzuela. 2009. First report of “Candidatus Liberibacter solanacearum” in pepper plants in México. Plant Dis. 93: 1076–1076.

Pletsch, D. J. 1947. The potato psyllid, Paratrioza cockerelli (Sulc): its biology and control. Montana State College, Agricultural Experiment Station.

Powell, C. M., J. King, and B. R. Bextine. 2012. Cytochrome B sequences of potato psyllids, *Bactericera cockerelli* (Sulc) 1, from North and Central America. Southwestern Entomologist. 37: 521–524.

Richards, B. L. 1928. A new and destructive disease of the potato in Utah and its relation to the potato psyllid. Phytopathology. 18: 140–141.

Richards, L. B., H. L. Blood, and M. B. Linford. 1927. Destructive outbreak of unknown potato disease in Utah. Plant Disease Report. 11: 93–94.

Secor, G. A., and V. V. Rivera-Varas. 2004. Emerging diseases of cultivated potato and their impact on Latin America. Rev. Latinoam. de la Papa (Suppl.). 1: 1–8.

Swisher, K. D., A. Arp, B. R. Bextine, E. Y. Aguilar, J. M. Crosslin, and J. E. Munyaneza. n.d. Haplotyping studies of the potato psyllid, *Bactericera cockerelli*, in Mexico and Central America. Submitted Feb 2013.

Swisher, K. D., J. E. Munyaneza, and J. M. Crosslin. 2012. High resolution melting analysis of the Cytochrome Oxidase I gene identifies three haplotypes of the potato psyllid in the United States. Environmental Entomology. 41: 1019–1028.

Thomas, K. L., D. C. Jones, L. B. Kumarasinghe, J. E. Richmond, G. Gill, and M. S. Bullians. 2011. Investigation into the entry pathway for tomato potato psyllid *Bactericera cockerelli*. New Zealand Plant Protection. 64: 259–268.

Wallis, R. L. 1951. Potato psyllid selection of host plants. Journal of Economic entomology. 44: 815–817.

Wallis, R. L. 1955. Ecological studies on the potato psyllid as a pest of potatoes. US Dept. of Agriculture.

“Pure growth industry”.http://www.laprensa.com.ni/2012/05/30/activos/103113-industria-puro-crecimiento. Accessed Mar 9, 2013

Low-level Detection of *Candidatus* Liberibacter solanacearum in Extracted *Bactericera cockerelli* (Hemiptera: Triozidae) DNA by 454 Pyrosequencing

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Abstract

Accurate detection and quantification of *Candidatus* Liberibacter solanacearum, the putative causal agent of zebra chip disease of potato (*Solanum tuberosum),* in the potato psyllid, *Bactericera cockerelli,* has become necessary to better understand the biology of the disease cycle. Studies on the transmission efficiency of potato psyllids have shown inconsistencies with field surveys. There have also been reports of laboratory colonies inexplicably losing and regaining *Candidatus* Liberibacter solanacearum infection. Until now, DNA primers were used to detect *Candidatus* Liberibacter solanacearum in potato psyllid tissue using conventional polymerase chain reaction and gel

electrophoresis or by real-time quantitative PCR. In this study, *Candidatus* Liberibacter solanacearum was detected using bacterial tag-encoded FLX amplicon pyrosequencing (bTEFAP) at levels identifiable by PCR, and low levels, including samples with only one cell of *Candidatus* Liberibacter solanacearum. Potato psyllids with <300 pyrosequencing reads did not show positive using conventional PCR. These results explain why it is possible for laboratory colonies to seemingly lose and regain *Candidatus* Liberibacter solanacearum infection and possibly why some populations of potato psyllids that do not test positive for *Candidatus* Liberibacter solanacearum still occur in potato fields with symptoms of zebra chip.

Introduction

The potato psyllid, *Bactericera cockerelli* (Sulc), is an emerging agricultural pest of solanaceous crops (e.g. potato, tomato, and pepper). Potato psyllids are endemic to the central and western United States, Canada, Mexico, and Central America (Pletsch 1947, Munyaneza et al. 2009, 2010, Ferguson et al. 2002). Historically, the potato psyllid was an agricultural pest of little consequence with infrequent outbreaks causing damages to solanaceous crops such as tomato, potato, and pepper throughout the western United States (Richards et al. 1927, Wallis 1955, Hill 1947). In 2000 a large outbreak of potato psyllids spread across the central and western United States and has since become an annual event (Secor et al. 2006). As well as becoming an annual pest across North and Central America, potato psyllids invaded New Zealand in 2005 and have become an established and costly agricultural pest (Teulon et al. 2009).

When plants are fed on by potato psyllids, they often present symptoms referred to as “psyllid yellows”, that include the yellowing and curling of leaves, reduced tuber growth in potatoes, and premature plant death (Arslan et al. 1985). The specific cause of “psyllid yellows” has not been identified (Abernathy 1991). In 1994 a new disease arose in potato fields in Saltillo, Mexico commonly called Zebra Chip (Munyaneza et al. 2007). Zebra Chip has foliar symptoms similar to psyllid yellows, but was identified and named based on the physiological changes in the tubers that are not present in “psyllid yellows” (Arslan et al. 1985, Sengoda et al. 2009). Zebra Chip is identified by necrosis of the medullary

tissues resulting from conversion of the starches to simple sugars, and increases in phenolic content and polyphenol oxidase, that result in potatoes that turn dark and striped when fried rendering them unusable for the chipping industry and other uses (Alvarado et al. 2012). The causal agent of Zebra Chip was determined to be transmitted by potato psyllids, and has been identified as the bacteria *Candidatus* Liberibacter solanacearum (*C*Ls), though because this organism cannot cultured, traditional Koch's postulate has not been completed (Secor et al. 2009, Hansen et al. 2008, Liefting et al. 2009). Therefore, CLs remains the putative causal agent despite mounting circumstantial evidence of pathogenicity. *C*Ls is transmitted by a *C*Ls infected psyllid nymph or adult feeding on the phloem of a solanaceous plant. One infected psyllid can infect a potato plant with *C*Ls within six hours of feeding and only a short time is required for a non-infected psyllid feeding on an infected plant to acquire *C*Ls (Buchman et al. 2011a, 2011b). Though some nymphs can be infected with *C*Ls through transovarial transmission, most psyllids acquire *C*Ls by feeding on infected plants as nymphs (Nachappa et al. 2011). When infected with *C*Ls, the bacteria spread throughout the plant and can infect daughter tubers. Because of this, to prevent the spread the disease, it is necessary to destroy an entire crop where zebra chip is found (Pitman et al. 2010).

Many xylem & phloem-feeding insects have symbiotic relationships with bacteria to aid in metabolism of amino acids not present in their diet that have been well described (Baumann 2005). The potato psyllid is a phytophagous

insect and relies on an obligate symbiont bacterium, *Candidatus* Carsonella rudii, to provide for the lack of essential amino acids in the phloem that the psyllids cannot synthesize (Thao et al. 2000). Potato psyllids also harbor *Wolbachia pipientis*, a bacterium that is known to have an effect on reproduction in insects, but it is not known what, if any, effect *Wolbachia* has on psyllids (Nachappa et al. 2011, Liu et al. 2006). Along with these obligate symbionts, potato psyllids harbor many other bacteria such as *Rhizobium, Gordonia, Mycobacterium, Xanthomonas, Staphylococcus,* and others that can be obtained through vertical or horizontal transfer (Hail 2010). The effect these bacteria have on the psyllid is unknown. The relationship between psyllids and *C*Ls is currently unknown, but has been observed to shorten lifespan, increase nymph mortality, and lower fecundity (Nachappa et al. 2012).

Next generation sequencing gives the opportunity to look at these microbial communities in more depth, teasing out sequences that are at such low levels that traditional sequencing missed (Kumar et al. 2011). These methods also allow for the detection and relative abundance of non-culturable prokaryotes (Nonnenmann et al. 2010). Ongoing studies are currently using this method to analyze global soil microbial communities, the human microbiome, and marine plankton surveys (Delmont et al. 2011, Human Microbiome Project Consorium 2012, Delong 2009). More targeted research has used pyrosequencing to survey for West Nile Virus in mosquitos, identify cattle gut bacteria through their feces, and to identify potential causal agents of colony collapse disorder in the honey

bee (*Apis mellifera*) (Bishop-Lilly et al. 2010, Dowd et al. 2008a, Cox-Foster et al. 2009). Using pyrosequencing as a more specific and quantifiable tool is becoming more feasible as costs have begun to decrease to levels similar to qRT-PCR.

Detection of *C*Ls is most commonly done with traditional PCR and gel electrophoresis methods or by quantitative PCR. These results often show inconsistencies of infection levels, such as infected lab colonies of psyllids seemingly losing their *CLs* infection only to regain infection in future generations, or low numbers of *C*Ls positive psyllids collected from fields with Zebra Chip. To better understand the *C*Ls infection levels of potato psyllids in lab colonies, in wild populations, and *C*Ls transmission rates, 110 psyllids collected from 2007 – 2011 from the United States, New Zealand, and Nicaragua were subjected to testing for *C*Ls with PCR and bacterial tag-encoded FLX amplicon pyrosequencing (bTEFAP).

Materials and Methods

Sample collection and DNA extraction.

Adult potato psyllids were collected from 2009-2011 growing seasons from potato fields in Texas, Kansas, Nebraska, southern California, Washington, Oregon, Auckland, New Zealand; and from colonies kept at Plant and Food Research (Auckland, New Zealand) from the initial import of potato psyllids to New Zealand in 2005. The samples were placed in 95% ethanol and stored at -20^0 C until processed. Individual potato psyllid nucleic acid extractions were

done using the CTAB (cetyltriethylammonium bromide) buffer method (Zhang et al. 1998).

Traditional diagnostics to determine Liberibacter-infected insects

Extracted potato psyllid DNA was analyzed for the presence of the 16s rRNA gene of *C*Ls using conventional PCR. Each 25 μl reaction included 12.5 μL Ampli Taq Gold 360 master mix (Applied Biosystems, Foster City, CA), 1μL GC enhancer (Applied Biosystems, Foster City, CA), 1 μL of each primer OA2 and OI2c (Liefting et al. 2008, Crosslin et al. 2011), 7.5 μL water, and 2 μL extracted DNA suspended in buffer AE (DNeasy Blood and Tissue DNA extraction Kit, Qiagen, Valencia, CA). The thermal profile was run as follows: cycle 1, 94.0^0 C for 2 min (1x), cycle 2, 94.0^{0}C for 30s, 65.0^{0}C for 30s, 72.0^{0}C for 60s (x40), and cycle 3, 72.0^{0}C for 5 min (x1). Gel electrophoresis was run using 1% agarose gels stained with ethidium bromide, and visualized using a UVP Bio-Dock It Imaging System (Upland, CA).

Massively parallel bTEFAP

Bacterial tag-encoded FLX amplicon pyrosequencing (bTEFAP) was performed by Research and Testing Laboratory (Lubbock, TX). All DNA sample concentrations were adjusted to 100ng/μl. A 1μl aliquot was used from each sample in 50μl PCR reactions. Primers used for pyrosequencing were Gray28F 5'TTTGATCNTGGCTCAG and Gray519r 5'GTNTTACNGCGGCKGCTG (Dowd et al. 2008a, Dowd et al. 2008b, Hail et al. 2010, Hail et al. 2011). Initial generation of the sequencing library utilized a one-step PCR with a total of 30

cycles using HotStar Taq Plus Master Mix (Qiagen, Valencia, CA) and the following thermal protocol: 94°C for 3 minutes followed by 30 cycles of 94°C for 30 seconds; 60°C for 40 seconds and 72°C for 1 minute; and a final elongation step at 72°C for 5 minutes. 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. 2008a, 2008b). 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, USA).

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 utilized Roche 454 FLX instrument with Titanium reagents (Roche, Nutly, NJ).

Bacterial Diversity and Data Analysis

After sequencing, failed sequence reads and low quality sequence ends, tags and primers were removed. Black Box Chimera Check (B2C2) (Research and Testing, Lubbock, TX) was used for the depletion of chimeras from the

bacterial 16s data set. A distributed BLASTn.NET algorithm was used to remove base substitutions and PCR chimeras, assemble the sequences into clusters, and query the sequences against a database of high quality 16s bacterial sequences from NCBI. High quality sequences were characterized similar to the Ribosomal Database project, version nine (Cole et al 2009). The resulting BLASTn outputs were compiled using a .NET and C# analysis pipeline to validate taxonomic distance methods and data reduction analysis.

Bacterial Identification

The bacteria were classified at appropriate taxonomic levels based on the BLASTn outputs. The following criteria were met for each level of taxonomy; sequence identity scores compared to known or well characterized 16s sequences with a greater than 97% identity (less than 3% divergence) were resolved at the species level, sequences between 95% and 97% were resolved at the genus level, sequences between 90% and 95% were resolved at the family level, sequences between 85% and 90% were resolved at the order level, sequences 80% to 85% were resolved at the class level, and 77% to 80% were resolved at the phyla level. The percentage of sequence likeness was analyzed for each sample, as long as there was ample information among individual samples, based on the number of reads within each sample. Taxonomic evaluations were matched to the identification of the closest relative.

Results

454 Pyrosequencing Results

Pyrosequencing of the 110samples resulted in 750,075 unique 16s bacterial sequences. 28,959 (3.86%) of these sequences were identified as *C*Ls. *Wolbachia sp.* and *Candidatus* Carsonella rudii were the most abundant bacteria present. *Candidatus* Caronella rudii was identified in 99 psyllids (90.0%) accounting for 343,298 (45.77%) of the total reads. *Wolbachia sp.* was identified in 103 psyllids (93.63%) accounting for 140,470 (18.73%) reads. *Rhizobium* (561 reads)*, Gordonia* (43 reads)*, Mycobacterium* (74 reads)*, Xanthomonas* (44 reads)*, Staphylococcus*(828 reads) were present in many samples though at far lower amounts than the obligate symbiont bacteria.

Comparison of detection methods between PCR and pyrosequencing

Of 110 samples sent for bacterial tag-encoded FLX amplicon pyrosequencing (bTEFAP), six samples previously tested positive for *C*Ls using PCR and gel electrophoresis. On return of the bTEFAP results, fifteen additional samples showed positive results at low levels (Table X). The samples that tested positive for *C*Ls with PCR all had cell counts greater than 300 when pyrosequenced. Psyllids reared in the laboratory had an average *C*Ls cell count of 7847 accounting for an average of 73% of overall reads. *C*Ls infected field-collected psyllids had *C*Ls cell counts from 1 to 4901 accounting for .01% to 81.78% of their overall reads, though all but one of these psyllids had a cell count below 400 and *C*Ls accounting for less than 1% of their overall bacterial reads.

Table 3.1. Comparison of detection of *C*Ls by pyrosequencing and traditional PCR.

Location	Date Collected	No. Reads	Percent of total reads	PCR
New Zealand Colony	Jan. 2012	12611	94%	+
New Zealand Colony	Jan. 2012	6796	69%	+
New Zealand Colony	Jan. 2012	4135	57%	+
LRGV, TX	Mar. 2008	377	0.01%	+
Kingsville, TX	Mar. 2010	55	1.90%	-
Auckland, New Zealand	Jan. 2012	41	0.12%	-
Bridgeport, NE	Sept. 2009	11	0.17%	-
Auckland, New Zealand	Jan. 2012	2	0.02%	-
Auckland, New Zealand	Jan. 2012	1	0.01%	-
Lower Rio Grande Valley, TX	Feb. 2009	4901	81.78%	+
Riverside County, CA	May 2009	1	0.01%	-
Dalhart, TX	June 2009	1	0.02%	-
Ventura County, CA	Sept. 2009	9	0.09%	-
Dalhart, TX	Oct. 2009	2	0.04%	-
Ventura County, CA	Oct. 2009	2	0.02%	-
Orange County, CA	June 2010	2	0.02%	-
Orange County, CA	Aug. 2010	1	0.01%	-
Ventura County, CA	Aug. 2010	2	0.03%	-
Ventura County, CA	Sept. 2010	1	0.01%	-
Ventura County, CA	Oct. 2010	2	0.02%	-
Ventura County, CA	Oct. 2010	2	0.03%	-
Ventura County, CA	Oct. 2010	2	0.03%	-
Orange County, CA	Nov. 2010	1	0.01%	-
Ventura County, CA	Nov. 2010	1	0.01%	-

Discussion

In a traditional monitoring program, yellow sticky cards are used to monitor populations of psyllids. Potato fields are often treated with insecticides in response to the first influx of these pest insects. With these collections of insect samples, presence or absence of *C*Ls can be determined, allowing estimations of pathogen risk to be determined. This data is only useful if the detection of the pathogen is correct. Current methods may only be detecting pathogen levels that are above a threshold that is inadequate for determining the risk. In our study, 107 wild caught psyllids were tested for *C*Ls, 21 (19.63%) of the samples had positive results when tested using 454 pyrosequencing; 3 (2.80%) when tested with traditional PCR methods. In a study where potato psyllids were collected from symptomatic potato fields in Texas, approximately 25% *C*Ls infection using conventional PCR was reported (Secor et al. 2009). Recently, in laboratory colonies of potato psyllid positive for *C*Ls, only 95% of individuals tested using conventional PCR result in positive results (Crosslin et al. 2011). In our survey, all *C*Ls positive laboratory colony psyllids were positive for *C*Ls by PCR and pyrosequencing. It is likely that a higher percent of *C*Ls positive laboratory colony psyllids will test positive with pyrosequencing versus traditional PCR methods.

In research studies involving colonies of potato psyllids that are being maintained in the laboratory, a phenomenon of insect colonies testing negative for *C*Ls, then becoming positive, has been observed by several research teams. In these studies plants grown from seeds and in isolation from the psyllids were

used throughout the life of the colony, and the likelihood of contamination (infected individuals being accidentally introduced) was low; and an explanation has yet to be derived. This study provides circumstantial evidence that individuals in these colonies probably had levels of *C*Ls that were below the detection threshold; thus the insects exhibited false negatives.

The extremely high bacterial load of *C*Ls in the laboratory reared potato psyllids in comparison with field-collected psyllids is an important factor that future research into *C*Ls transmission and the effects of *C*Ls on psyllids need to address. The disproportionately high bacterial load of *C*Ls is possibly caused by keeping laboratory colonies on too few plants with multiple generations of psyllid feeding on the same infected plants compounding *C*Ls exposure. In a recent study, Nachappa (2012) found that laboratory psyllids infected with *C*Ls had lower fecundity and a shorter lifespan than non-infected psyllids. Using 454 pyrosequencing analysis could help to explain if these results were caused by an unnatural overabundance of *C*Ls interfering with the potato psyllid's natural microbial biota and thus affecting reproduction or nutrition. In studies by Buchman et al (2011 a, 2011b), the transmission efficiency of *C*Ls by potato psyllids at different life stages was observed. This study also used laboratory colonies of *C*Ls infected psyllids. The psyllids were tested for *C*Ls with the OA2/OI2c primer pair for presence of *C*Ls only. Our findings of laboratory psyllids' high levels of infection possibly indicate that the results from this study could be skewed by the inflated *C*Ls presence of laboratory psyllids.

Even though bTEFAP can be a valuable tool in the management of *C*Ls, the advantages and disadvantages of this method must be weighed. Traditional PCR cannot compare to the diagnostic clarity that bTEFAP gives, as bTEFAP can detect the presence of one cell, and PCR requires hundreds of cells for detection. Unfortunately, bTEFAP is much more costly than PCR. PCR is more readily available, as equipment is relatively inexpensive and can be done in-house in most labs. Pyrosequencing requires expensive equipment, so the work needs to be contracted out in most cases. However, price is becoming more reasonable with the next-next generation of sequencers making their way to the commercial market.

This study was used to give a baseline of *C*Ls data using the pyrosequencing platform. The extra cost of 454 pyrosequencing has advantages other than low level detection of *C*Ls, it also provides additional data, which can be mined to identify potential interactions between *C*Ls and other microbes. In the end, this is a great method that may be too expensive for high volume sampling, but it can be used to determine how effective other methods of detection are.

Literature Cited

Abernathy, R.L. 1991. Investigations into the nature of the potato psyllid toxin. MS thesis. Colorado State University, Fort Collins, CO. 54 p

Alvarado, V. Y., D. Odokonyero, O. Duncan, T. E. Mirkov, and H. B. Scholthof. 2012. Molecular and physiological properties associated with zebra complex disease in potatoes and its relation with *Candidatus* Liberibacter contents in psyllid vectors. PLoS One. 7: e37345.

Arslan, A., P. M. Bessey, K. Matsuda, and N. F. Oebker. 1985. Physiological effects of psyllid (*Paratrioza cockerelli*) on potato. Am J Potato Res. 62: 9–22.

Baumann, P. 2005. Biology bacteriocyte-associated endosymbionts of plant sap-sucking insects. Annu Rev Microbiol. 59: 155–189.

Bishop-Lilly, K. A., M. J. Turell, K. M. Willner, A. Butani, N. M. E. Nolan, S. M. Lentz, A. Akmal, A. Mateczun, T. N. Brahmbhatt, S. Sozhamannan, C. A. Whitehouse, and T. D. Read. 2010. Arbovirus Detection in insect vectors by rapid, high-throughput pyrosequencing. PLOS Neglect Trop D. 4: e878.

Buchman, J. L., B. E. Heilman, and J. E. Munyaneza. 2011a. Effects of Liberibacter-infective *Bactericera cockerelli* (Hemiptera: Triozidae) density on zebra chip potato disease incidence, potato yield, and tuber processing quality. J Econ Entomol. 104: 1783–1792.

Buchman, J. L., V. G. Sengoda, and J. E. Munyaneza. 2011b. Vector transmission efficiency of Liberibacter by *Bactericera cockerelli* (Hemiptera: Triozidae) in Zebra Chip Potato disease: effects of psyllid life stage and inoculation access period. J Econ Entomol. 104: 1486–1495.

Cole J. R., Q. Wang, E. Cardenas, J. Fish, B. Chai, R. J. Farris, A. S. Kulam-Syed-Mohideen, D. M. McGarrell, T. Marsh, G. M. Garrity, and J. M. Tiedje. 2009. The ribosomal database project: improved alignments and new tools for rRNA analysis. Nucleic Acids Res. 37: 141-145.

Cox-Foster, D. L., S. Conlan, E. C. Holmes, G. Palacios, J. D. Evans, N. A. Moran, P-L Quan, T. Briese, M. Hornig, D. M. Geiser, V. Martinson, D. vanEngelsdorp, A. L. Kalkstein, A. Drysdale, J. Hui, J. Zhai, L. Cui, S. K. Hutchinson, J. F. Simons, M. Egholm, J. S. Pettis, and W. I. Lipkin. 2007. A metagenomic survey of microbes in honey bee colony collapse disorder. Science. 318: 283-287.

Crosslin, J. M., H. Lin, and J. E. Munyaneza. 2011. Detection of '*Candidatus* Liberibacter solanacearum' in the potato psyllid, *Bactericera cockerelli* Sulc, by conventional and real-time PCR. Southwestern Entomol. 36: 125-135.

Delmont, T. O., P. Robe, S. Cecillon, I. M. Clark, F. Constancias, P. Simonet, P. R. Hirsch, and T. M. Vogel. 2011. Accessing the soil metagenome for studies of microbial diversity. Appl Environ Microb. 77: 1315–1324.

DeLong, E. F. 2009. The microbial ocean from genomes to biomes. Nature. 459: 200–206.

Dowd, S. E., T. R. Callaway, R. D. Wolcott, Y. Sun, T. McKeehan, R. G. Hagevoort, and T. S. Edrington. 2008a. Evaluation of the bacterial diversity in the feces of cattle using 16S rDNA bacterial tag-encoded FLX amplicon pyrosequencing (bTEFAP). BMC Microbiol. 8:125.

Dowd, S. E., Y. Sun, R. D. Wolcott, A. Domingo, and J. A. Carroll. 2008b. Bacterial tag-encoded FLX amplicon pyrosequencing (bTEFAP) for microbiome studies: bacterial diversity in the ileum of newly weaned *Salmonella*-infected pigs. Foodborne Pathog and Dis. 5: 459-472.

Ferguson, G., E. Banks and H. Fraser. 2010. Potato psyllid - a new pest in greenhouse tomatoes and peppers. Ontario Ministry of Food and Agriculture, Canada, Ontario, Canada. http://www.omafra.gov.on.ca/english/crops/facts/potato_psyllid.htm

Hail, D., W. B. Hunter, S. E. Dowd, and B. R. Bextine. 2010. Expressed sequence tag (EST) survey of life stages of the potato psyllid, *Bactericera cockerelli*, using 454 pyrosequencing. Southwestern Entomol. 35: 463-466.

Hail, D., I. Lauzíere, S. E. Dowd, and B. Bextine. 2011. Culture independent survey of the microbiota of the glassy-winged sharpshooter (*Homolodisca vitripennis*) using 454 pyrosequencing. Environ Entomol. 40: 23-29.

Hansen, A. K., J. T. Trumble, R. Stouthamer, and T. D. Paine. 2008. A new huanglongbing species, "*Candidatus* Liberibacter psyllaurous," found to infect tomato and potato, is vectored by the psyllid *Bactericera cockerelli* (Sulc). Appl Environ Microb. 74: 5862–5865.

Hill, R. E. 1947. An unusual weather sequence accompanying the severe potato psyllid outbreak of 1938 in Nebraska. J Kansas Entomol Soc. 20:88-92.

Human Microbiome Project Consortium. 2013. Structure, function and diversity of the healthy human microbiome. Nature. 486: 207–214.

Kumar, P. S., M. R. Brooker, S. E. Dowd, and T. Camerlengo. 2011. Target region selection is a critical determinant of community fingerprints by 16S pyrosequencing. PLoS One, 6: e20956.

Liefting, L. W., Z. C. Perez-Egusquiza, G. R. G. Clover, and J. A. D. Anderson. 2008. A new 'Candidatus Liberbacter' species in *Solanum tuberosum* in New Zealand. Plant Dis. 92: 1474-1474.

Liefting, L. W., B. S. Weir, S. R. Pennycook, and G. R. G. Clover. 2009. "*Candidatus* Liberibacter solanacearum", associated with plants in the family *Solanaceae*. Int J Syst Evol Micr. 59: 2274–2276.

Munyaneza, J. E., J. M. Crosslin, and J. E. Upton. 2007. Association of *Bactericera cockerelli* (Homoptera: Psyllidae) with “zebra chip”, a new potato disease in southwestern United States and Mexico. J Econ Entomol. 100: 656-663.

Munyaneza, J. E., J. M. Crosslin, and J. L. Buchman. 2009. Seasonal occurrence and abundance of the potato psyllid, *Bactericera cockerelli*, in south central Washington. Am J Potato Res. 86: 513-518.

Munyaneza, J. E. 2010. Psyllids as vectors of emerging bacterial diseases of annual crops. Southwestern Entomol. 35:471-477.

Nachappa, P., J. Levy, E. Pierson, and C. Tamborindeguy. 2011. Diversity of endosymbionts in the potato psyllid, *Bactericera cockerelli* (Hemiptera: Triozidae), vector of zebra chip disease of potato. Curr Microbiol. 62: 1510–1520.

Nachappa, P., A. A. Shapiro, and C. Tamborindeguy. 2012. Effect of “Candidatus Liberibacter solanacearum” on fitness of its insect vector, *Bactericera cockerelli* (Hemiptera: Triozidae), on tomato. Phytopathology. 102: 41–46.

Nonnenmann, M. W., B. Bextine, S. E. Dowd, K. Gilmore, and J. L. Levin. 2010. Culture-independent characterization of bacteria and fungi in a poultry bioaerosol using pyrosequencing: a new approach. J Occup Environ Hyg. 7: 693-699.

Pitman, A. R., G. M. Drayton, S. J. Kraberger, R. A. Genet, and I. A. W. Scott. 2010. Tuber transmission of “Candidatus Liberibacter solanacearum” and its association with zebra chip on potato in New Zealand. Eur J Plant Pathol. 129: 389–398.

Pletsch, D. J. 1947. The potato psyllid *Paratrioza cockerelli* (Sulc), its biology and control. Montana Agricultural Experiment Station Bulletin 446: 95.

Secor, G. A., V. V. Rivera, J. A. Abad, I-M Lee, G. R. G. Clover, L. W. Liefting, X. Li, and S. H. De Boer. 2009. Association of ‘*Candidatus* Liberibacter solanacearum’ with zebra chip disease of potato established by graft and psyllid transmission, electron microscopy, and PCR. Plant Dis. 93: 574-583.

Sengoda, V. G., J. E. Munyaneza, J. M. Crosslin, J. L. Buchman, and H. R. Pappu. 2009. Phenotypic and etiological differences between psyllid yellows and zebra chip diseases of potato. Am J Potato Res. 87: 41–49.

Teulon, D., P. J. Workman, K. L. Thomas, and M.-C. Nielson. 2009. *Bactericera cockerelli*: incursion, dispersal and current distribution on vegetable crops in New Zealand. N Z Plant Protect-Se. 62: 136–144.

Thao, M. L., N. A. Moran, P. Abbot, E. B. Brennan, D. H. Burckhardt, and P. Baumann. 2000. Cospeciation of psyllids and their primary prokaryotic endosymbionts. Appl Environ Microb. 66: 2898–2905.

Wallis, R.L. 1955. Potato psyllid as a pest of potatoes. USDA Techinical Bulleting February 1955: 1–28.

Zhang, Y-P, J. K. Uyemoto, and C. Kirkpatrick. 1998. A small-scale procedure for extracting nucleic acids from woody plants infected with various phytopathogens for PCR assay. J Virol Methods. 71: 45-50.

A Global Comparison of *Bactericera cockerelli* Microbial Communities

Abstract

The potato psyllid (*Bactericera cockerelli*) is an emergent pest of solanacious crops such as potato, tomato, and tobacco. Historically the potato psyllid's range included central United States, Mexico, and California; more recently populations 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. The primary-symbiont of psyllids is Candidatus Carsonella ruddii; they 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 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. OTUs were then grouped at 95% 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.

Introduction

The potato psyllid, *Bactericera cockerelli* (Sulc), is a phytophagous hemipteran insect in the family Triozidae. Potato psyllids' range changes seasonally following optimal breeding temperatures that extends from eastern Mexico south to Nicaragua, throughout the central United States, California, the Pacific Northwest, and New Zealand (Pletsch 1947, Munyaneza, Goolsby, et al. 2007, Munyaneza, Crosslin, et al. 2007, Secor et al. 2009, Teulon et al. 2009, Munyaneza 2010). Potato psyllids feed primarily on solanaceous plants, which include crops such as: potato, tomato, peppers, and tobacco (Knowlton and Thomas 1934, Wallis 1951). The potato psyllid is known to transmit the bacteria *Candidatus* Liberibacter solanacaeurm (Lso), the putative causal agent of Zebra Chip disease in 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. The recent range expansions and extensive crop damages caused by potato psyllids have led to research looking at population dynamics of the potato psyllids. Using molecular markers in the CO1 gene and ISSR have identified three unique haplotypes of potato psyllid, one along the west coast of the United States and New Zealand, one isolated in the Pacific Northwest and Idaho, a haplotype in the Central United States, Mexico, and Central America (Liu et al. 2006, Chapman et al. 2012, Swisher et al. 2012).

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 mutualistic bacterial symbionts that can provide the insects with enhanced resistance to pesticides, enhanced immune response, nutrition, and reproductive advantages (Feldhaar and Gross 2009, Kikuchi et al. 2012).

These bacteria are grouped into two categories, primary and secondary symbionts. Primary-symbionts have co-evolved with their host and are necessary for their host's survival and are passed vertically, from parent to offspring. They are usually held in specialized cells called bacteriocytes, or mycetocytes, that may be clustered into specialized organs called bacteriomes (Buchner 1965, Moran and Mira 2001, McLean et al. 2010). Many sap-feeding insects, such as psyllids and hoppers, co-evolved with bacteria that provide essential amino acids not present in the plant fluids they ingest (Baumann 2005).

Secondary-symbionts can provide the host with advantages, are not necessary for their host's survival and do not share a long evolutionary history. Secondary symbionts can either be intracellular or reside in the hemolymph and are obtained through feeding or by physical contact (Buchner 1965). These secondary facultative symbionts can affect the reproduction, predator resistance, host feeding preference, pesticide resistance, or heat resistance (Stouthamer et al. 1999, Montllor et al. 2002, Oliver et al. 2003, Casteel et al. 2012).

The primary symbionts contained in potato psyllid bacteriocytes are *Candidatus Carsonella ruddi,* an endosymbiont that co-evolved with psyllids (Thao et al. 2000). There are also many secondary endosymbionts such as Lso, *Acinetobacter*, *Methylibium,* and *Wolbachia sp*. that are transmitted to the psyllids through feeding or passed to offspring from the mother (Nachappa et al. 2011, Hail et al. 2012).

Analyzing insect bacterial symbionts has proven difficult in the past. Many of these bacteria are unculturable, so 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, we are now able to fully identify the complete insect symbiont composition. 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). Pryosequecing is also beneficial in that it provides a quantitative representation of the microbial community, not only presence and absence data provided by earlier cloning techniques.

Previous pyrosequencing surveys of potato psyllid microbial communities have been performed on various life stages and using pooled insects collected at the same time and location (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 haplotype. 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 and three of the New Zealand samples were from lab-raised colonies. Agricultural samples were obtained with aspirators 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 and an optimized CTAB protocol were used to extract complete DNA. Extracted DNA was stored at -20°C.

Haplotype

Psyllid haplotype was assessed using real-time melt analysis by the methods outlined by Chapman (2013). All samples were run with 2μl of psyllid genomic DNA with concentrations of 2-20ng/μl. The primers used were CO1 F1 5'GGA TTC ATT GTT TGA GCA CAT C and CO1 R1 5' TGA AAT AGG CAC GAG AAT CAA, which amplify a 78bp section of the psyllid mitochondrial CO1 gene. The PCR was run with 25μl reactions using 12.5μl of Qiagen Quanitfast PCR Master Mix (Qiagen, Valencia, CA) , 2.5μl of both primer, 2 μl of template DNA, and 7.5μl of nanopure water. The PCR was run using the following thermal profile: 15 minute hold at 95°C followed by 40 cycles of 95°C for 10 seconds and 53°C for 15 seconds. Following PCR and a 90 second pre-melt cycle, the product underwent a melt cycle from 65°C to 80°C raising the temperature 0.25°C per step holding at each step for 5 seconds. The resulting differential melt curves were used to identify haplotype, with the central haplotype melting at approximately 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 100ng/μl. A 1μl aliquot was used from each

sample in 50μl PCR reactions. Primers used for pyrosequencing were Gray28F 5'TTTGATCNTGGCTCAG and Gray519r 5'GTNTTACNGCGGCKGCTG (Dowd et al. 2008a, Dowd et al. 2008b, Hail et al. 2010, Hail et al. 2011). Initial generation of the sequencing library utilized a one-step PCR with a total of 30 cycles using HotStar Taq Plus Master Mix (Qiagen, Valencia, CA) and the following thermal protocol: 94°C for 3 minutes followed by 30 cycles of 94°C for 30 seconds; 60°C for 40 seconds and 72°C for 1 minute; and a final elongation step at 72°C for 5 minutes. 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. 2008a, 2008b). 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, USA).

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 utilized 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. Samples with less than 1000 sequences returned 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%. All OTUs with a length <200bp were removed prior to classification. Samples with less than 1000 sequences returned were also removed to filter samples with low sequencing depth. Representative sequences for each OTU were classified against the ARB SILVA database to genus. 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 runs causing artificial sample separations based on run.

To compare similarities between bacterial communities, classical multidimensional analysis with a Bray-Curtis distribution were performed using R with the vegan package. 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 over 788,000 quality sequences, approximately 6,200 per psyllid (min

1013; max 32,552). Four potato psyllids returned less than 1000 sequences and were removed prior to 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.

The bacterial sequences returned from pyrosequencing were similar in composition to the previous findings of Hail (2012) and Nachappa (2011). Returned sequences consisted of 53% *Wolbachia sp.*, 19% *Candidatus* Carsonella rudii, 4% *Acintobacter sp.*, 4% *Pseudomnas sp.*, and 2% *Candidatus* Liberibacter (Figure 4.1). 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* Carsonsella rudii mostly influenced the psyllids groupings (Figure 4.4). Carrot psyllids (*Trioza apicalis*) were included as an outlier and their isolation from most of the potato psyllids validates the model data. It was found that carrot psyllids do not harbor *Wolbachia sp.* but do have a very high proportion of their microbial community represented by *Candidatus* Liberibacter sp.(~24%). The high proportion of

Candidatus Liberibater sp. also present in the colony potato psyllids from New Zealand could explain their close proximity in the MDS plot.

Within the United States there is a great diversity within the microbial community of psyllids (Figure 4.3). Although the patterns are not precise there are some groupings of note. Of the 27 potato psyllids collected in California all but two grouped very similar and show little microbial diversity with Wolbachia sp. comprising a majority of their microbial community. The two outlying samples from California, collected in Riverside in 2009, had a microbial community composed of much higher percentages of *Candidatus* Carsonsella rudii (~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 ruddi 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). 70 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 classical multidimensional scaling. There were no apparent patterns or clustering of bacterial community in relation to haplotype (Figure 4.5). It can be inferred from these results that the microbial community of psyllids is not influenced by haplotype and is most likely the result of vertical transfer at a population level or environmental interactions.

Potato psyllids of the central haplotype from Texas, Kansas, and Nebraska, from 2008, 2009, and 2010 were also separately compared using classical multidimensional scaling based on their microbial communities (Figure 4.6). 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 2009, a majority of the samples from all three years overlapped and did not separate in any meaningful way.

Figure 4.1: Representation of all sequences returned from 454 pyrosequencing

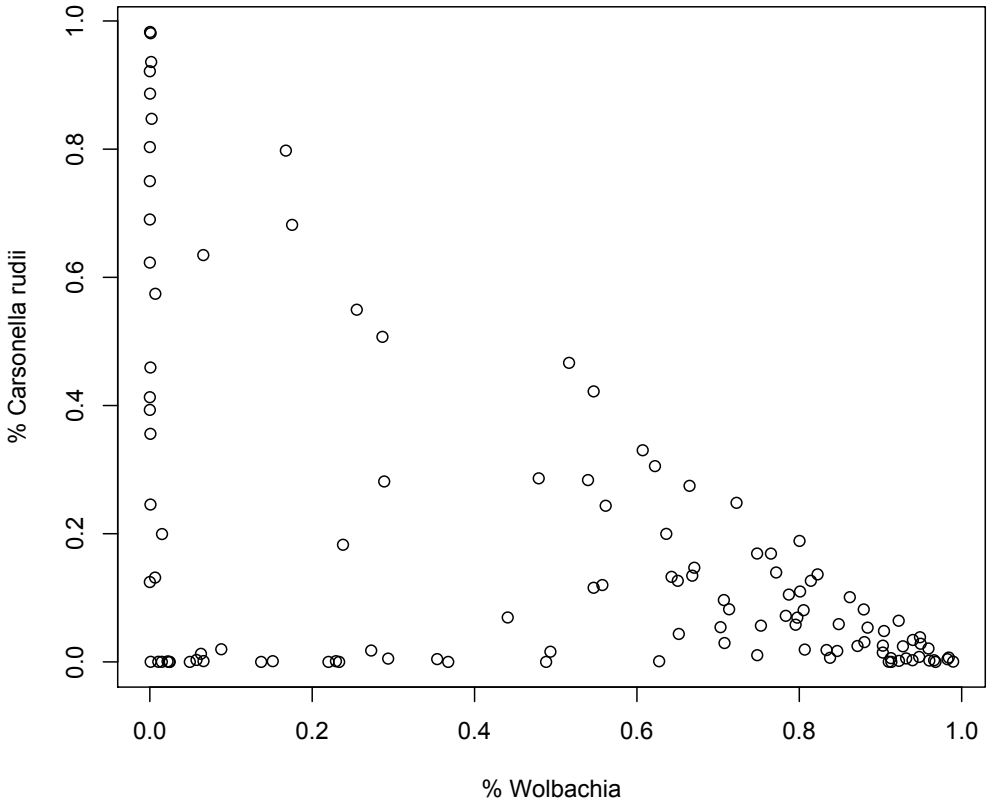

Figure 4.2: Correlation of % the total microbial community composition of Wolbachia to C. ruddii in potato psyllids ($p < 0.001$)

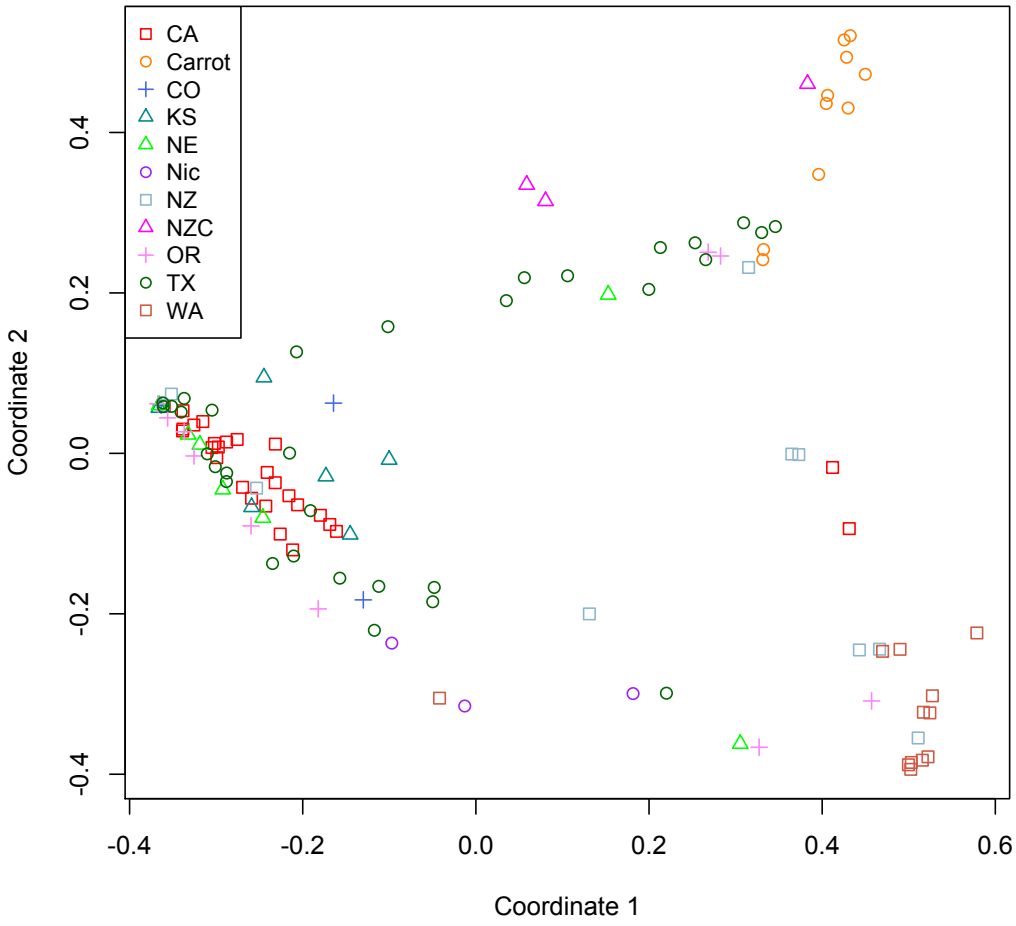

Figure 4.3: Individual potato psyllids compared by their bacterial community and labeled based on the state they were collected

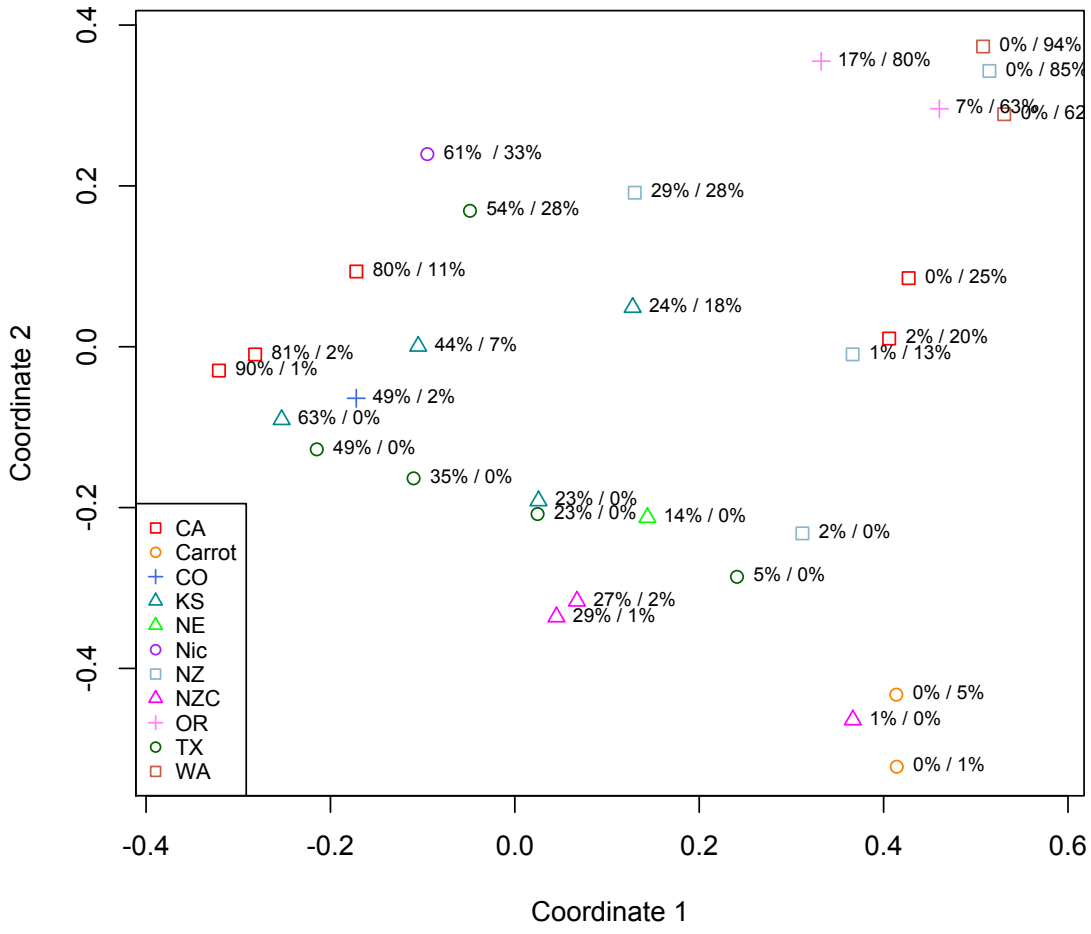

Figure 4.4: Individual potato psyllids compared by their bacterial community and labeled by their percent bacterial community composition of %Wolbachia / % C. rudii

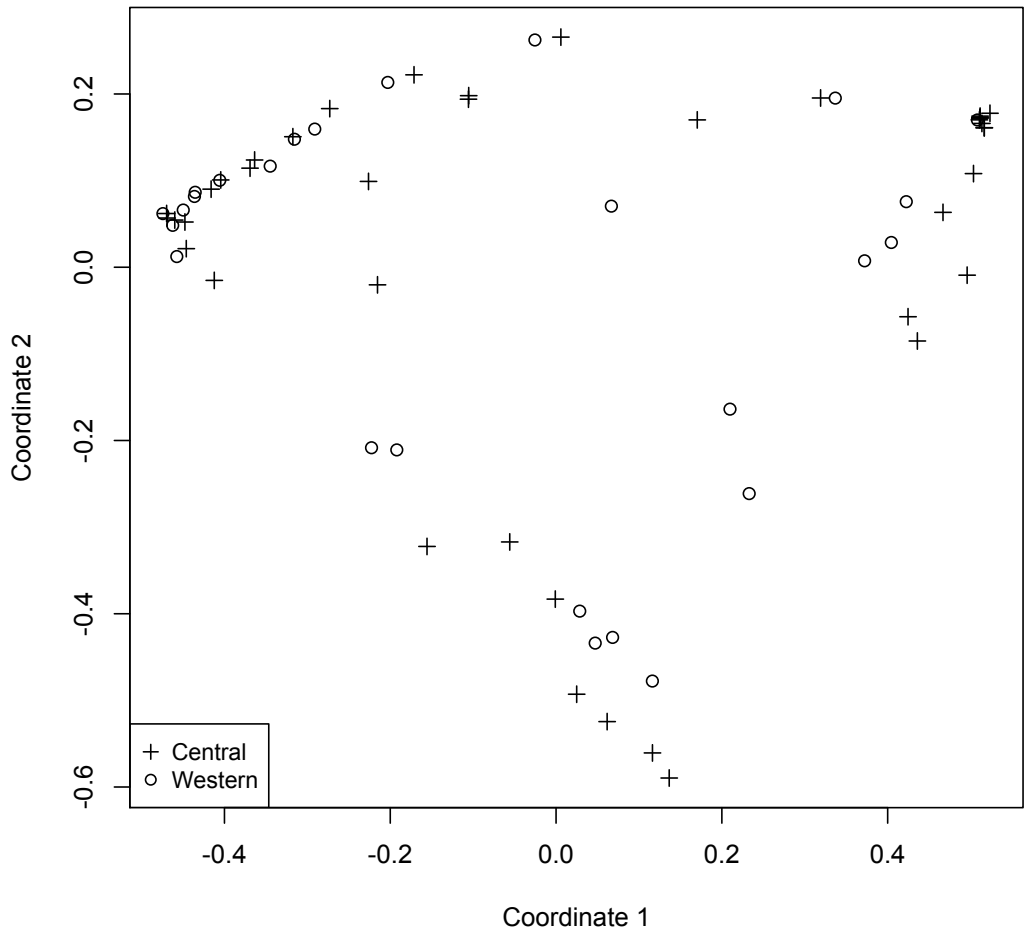

Figure 4.5: Bacterial community similarities of potato psyllids compared by the psyllids' haplotype

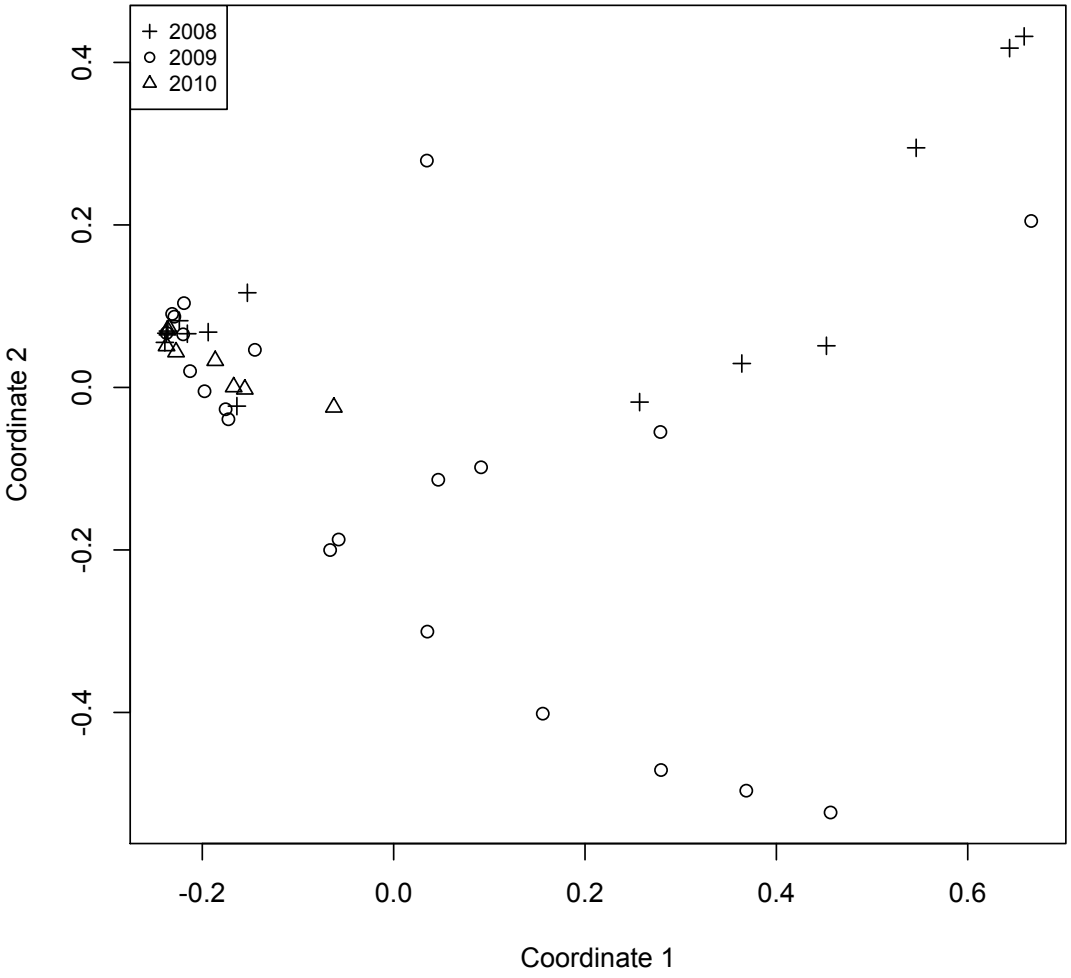

Figure 4.6: Bacterial community similarities of potato psyllids collected from TX, NE, and KS compared by the year in which the psyllids were collected

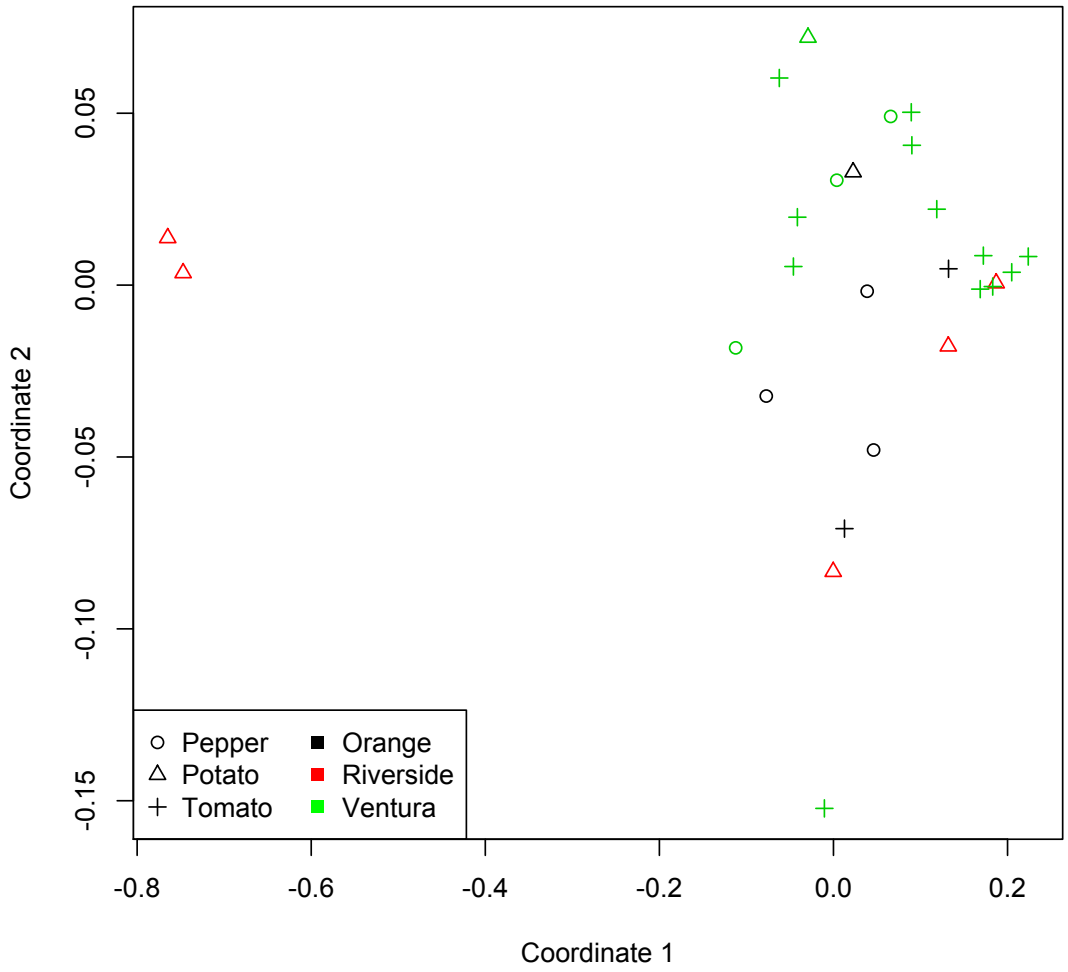

Figure 4.7: Bacterial community similarities of potato psyllids collected from three counties in California compared by the host plant the psyllids were on when collected

Potato psyllids collected from three counties in southern California and were collected from three different host plants, were also compared by their bacterial communities to assess whether host plant has an effect on the psyllid microbial community (Figure 4.7). The counties the psyllids were collected from were Riverside, Orange, and Ventura counties. Potato psyllids collected in Ventura and Orange County were collected from potato, pepper, and tomato

plants; while the potato psyllids collected in Riverside County were only collected from potato plants. It was assumed that the close proximity of the sites, and the assumption of their haplotype being the same, would limit the external influence other than plant host variety on the groupings. When plotted there were no differences based on host pant or county. Overlap of counties was expected because of their close proximity. The result of the MDS shows that the host plant the psyllids were collected from does not seem to have an effect on microbial community.

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 have only 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, previous studies used DNA extracted from pooled insects, which could have washed out unique diversity found within the pooled samples. Performing the survey with individual insects over a much wider area has provided much 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

(Figure 4.2 and Figure 4.4). 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 all three amino acids lacking from the psyllid and it would be necessary for potato psyllids to have a second primary symbiont. It is possible that *Wolbachia* 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 range expansion, pesticide resistance, and new introductions. In previous surveys of the potato psyllid microbial community only potato psyllids collected from Texas were used and these insects were pooled, removing any possibility of detecting unique differences between the psyllids (Nachappa et al. 2011, Hail et al. 2012). 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 US, but this pattern is

not supported in the western United States. The reason for the dissimilarity between Washington and Oregon samples given their close 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 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 (Swisher et al. 2012). 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 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 seen to provide resistances to pesticides and annual shifts in microbial community could lead to shifts in resistances (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 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 prior to 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.

Literature Cited

Baumann, P. 2005. Biology bacteriocyte-associated endosymbionts of plant sap-sucking insects. Annu. Rev. Microbiol. 59: 155–189.

Buchner, P. 1965. Endosymbiosis of animals with plant microorganisms. Interscience Publishers.

Casteel, C. L., A. K. Hansen, L. L. Walling, and T. D. Paine. 2012. Manipulation of plant defense responses by the tomato psyllid (*Bactericerca cockerelli*) and its associated endosymbiont *Candidatus* Liberibacter psyllaurous. PLoS ONE. 7: e35191.

Chapman, R. I., J. F. Macias-Velasco, A. P. Arp, and B. R. Bextine. 2012. Using quantitative real time PCR melt curve analysis of partial CO1 sequence for rapid biotype differentiation of *Bactericera cockerelli* (Hemiptera: Triozidae). Southwestern Entomologist. 37: 475–484.

Feldhaar, H., and R. Gross. 2009. Insects as hosts for mutualistic bacteria. International Journal of Medical Microbiology. 299: 1–8.

Hail, D., S. E. Dowd, and B. Bextine. 2012. Identification and location of symbionts associated with potato psyllid (*Bactericera cockerelli*) lifestages. Environmental Entomology. 41: 98–107.

Hugenholtz, P., B. M. Goebel, and N. R. Pace. 1998. Impact of culture-independent studies on the emerging phylogenetic view of bacterial diversity. J. Bacteriol. 180: 4765–4774.

Kikuchi, Y., M. Hayatsu, T. Hosokawa, A. Nagayama, K. Tago, and T. Fukatsu. 2012. Symbiont-mediated insecticide resistance. Proceedings of the National Academy of Sciences. 109: 8618–8622.

Knowlton, G. F., and W. L. Thomas. 1934. Host plants of the potato psyllid. Journal of Economic entomology. 27: 547.

Liu, D., J. T. Trumble, and R. Stouthamer. 2006. Genetic differentiation between eastern populations and recent introductions of potato psyllid (*Bactericera cockerelli*) into western North America. Entomol Exp Appl. 118: 177–183.

McLean, A. H. C., M. van Asch, J. Ferrari, and H. C. J. Godfray. 2010. Effects of bacterial secondary symbionts on host plant use in pea aphids. Proceedings of the Royal Society B: Biological Sciences. 278: 760–766.

Montllor, C. B., A. Maxmen, and A. H. Purcell. 2002. Facultative bacterial endosymbionts benefit pea aphids *Acyrthosiphon pisum* under heat stress. Ecological Entomology. 27: 189–195.

Moran, N. A., and A. Mira. 2001. The process of genome shrinkage in the obligate symbiont *Buchnera aphidicola*. Genome Biol. 2: 1–0054.12.

Munyaneza, J. E. 2010. Psyllids as vectors of emerging bacterial diseases of annual crops. Southwestern Entomologist. 35: 471–477.

Munyaneza, J. E., J. A. Goolsby, J. M. Crosslin, and J. E. Upton. 2007. Further evidence that zebra chip potato disease in the lower Rio Grande Valley of Texas is associated with *Bactericera cockerelli*. Subtropical Plant Science. 59: 30-37.

Munyaneza, J. E., J. M. Crosslin, and J. E. Upton. 2007. Association of *Bactericera cockerelli* (Homoptera : Psyllidae) with "Zebra Chip," a new potato disease in southwestern United States and Mexico. Journal of Economic entomology. 100: 656–663.

Nachappa, P., J. Levy, E. Pierson, and C. Tamborindeguy. 2011. Diversity of endosymbionts in the potato psyllid, *Bactericera cockerelli* (Hemiptera: Triozidae), vector of Zebra Chip disease of potato. Curr Microbiol. 62: 1510–1520.

Oliver, K. M., J. A. Russell, N. A. Moran, and M. S. Hunter. 2003. Facultative bacterial symbionts in aphids confer resistance to parasitic wasps. Proc. Natl. Acad. Sci. U.S.A. 100: 1803–1807.

Pletsch, D. J. 1947. The potato psyllid, Paratrioza cockerelli (Sulc): its biology and control. Montana State College, Agricultural Experiment Station.

Secor, G. A., V. V. Rivera, J. A. Abad, I. M. Lee, G. Clover, L. W. Liefting, X. Li, and S. H. De Boer. 2009. Association of "*Candidatus* Liberibacter solanacearum" with Zebra Chip Disease of potato established by graft and psyllid transmission, electron microscopy, and PCR. Plant Dis. 93: 574–583.

Stouthamer, R., J. A. Breeuwer, and G. D. Hurst. 1999. *Wolbachia pipientis*: microbial manipulator of arthropod reproduction. Annu. Rev. Microbiol. 53: 71–102.

Swisher, K. D., J. E. Munyaneza, and J. M. Crosslin. 2012. High resolution melting analysis of the Cytochrome Oxidase I gene identifies three haplotypes of the potato psyllid in the United States. Environmental Entomology. 41: 1019–1028.

Tamaki, H., C. L. Wright, X. Li, Q. Lin, C. Hwang, S. Wang, J. Thimmapuram, Y. Kamagata, and W.-T. Liu. 2011. Analysis of 16S rRNA amplicon sequencing options on the Roche/454 Next-Generation titanium sequencing platform. PLoS ONE. 6: e25263.

Teulon, D., P. J. Workman, K. L. Thomas, and M.-C. Nielson. 2009. *Bactericera cockerelli*: incursion, dispersal and current distribution on vegetable crops in New Zealand. New Zealand Plant Protection Society. 62: 136–144.

Thao, M. L., N. A. Moran, P. Abbot, E. B. Brennan, D. H. Burckhardt, and P. Baumann. 2000. Cospeciation of psyllids and their primary prokaryotic endosymbionts. Applied and Environmental Microbiology. 66: 2898–2905.

Wallis, R. L. 1951. Potato psyllid selection of host plants. Journal of Economic entomology. 44: 815–817.

Appendix A

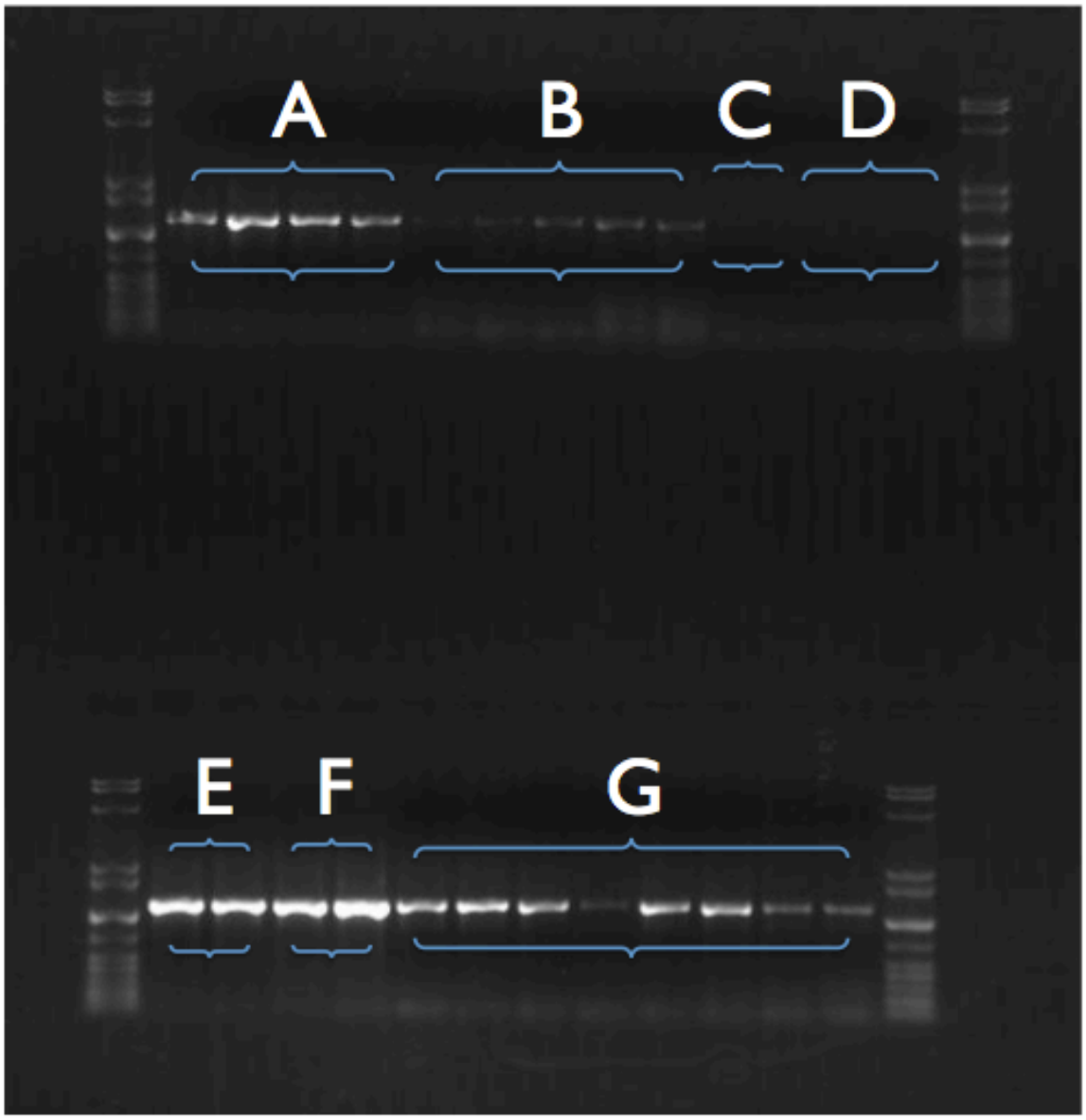

Figure 0.1: Gel result of Lso test of Central American samples. A: El Salvador Tomato; B: Nicaragua Potato; C: Nicaragua Pepper; D: Nicaragua Psyllid; E: Honduras Eggplant; F: Honduras Tomato; G: Honduras Tobacco

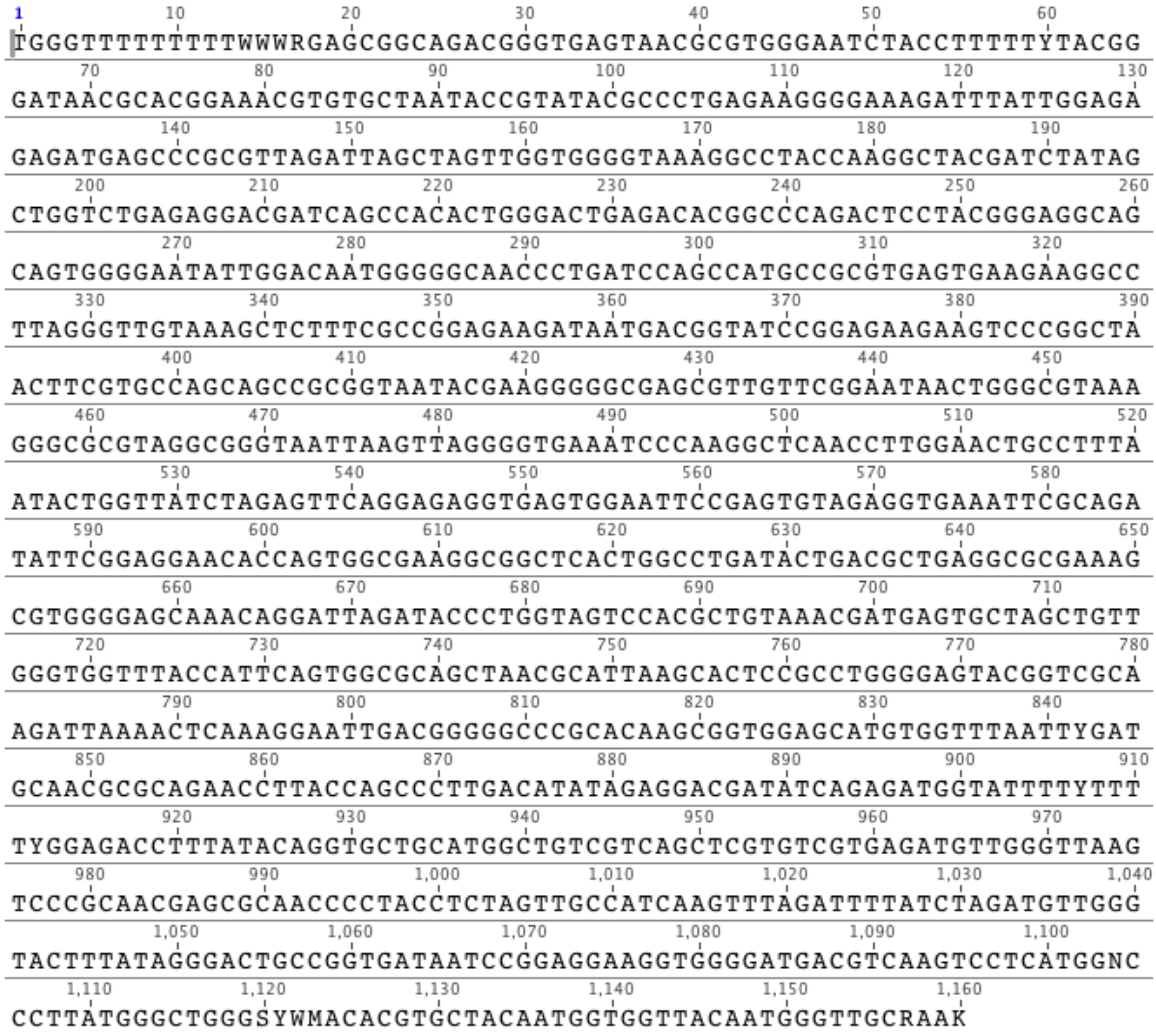

Figure 0.2: Consensus sequence from OI2c/OA2 primer pair targeting the 16s gene of Lso from Sample 31

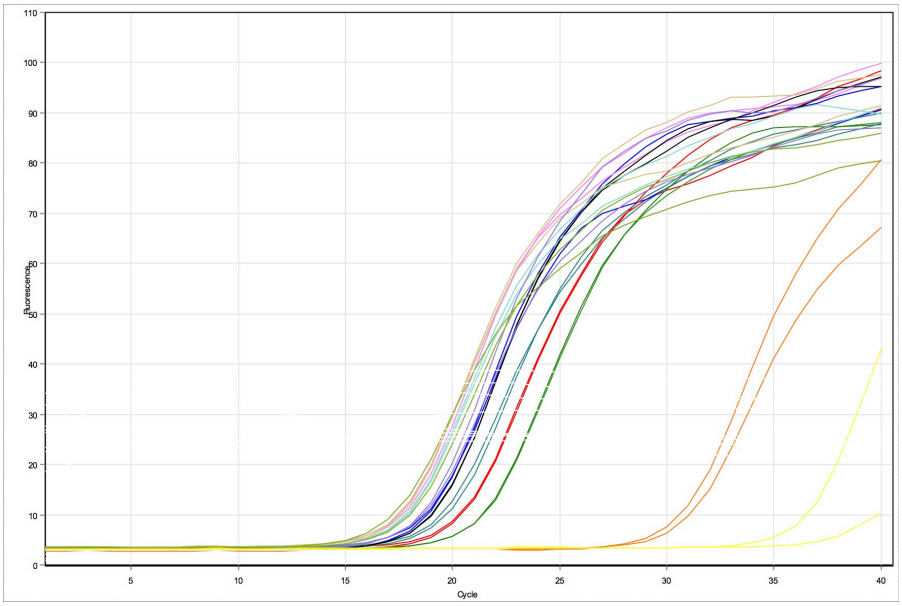

Figure 0.3: PCR amplification curve for haplotype detection of psyllids collected in Honduras. Samples amplifying later than cycle 20 were excluded.

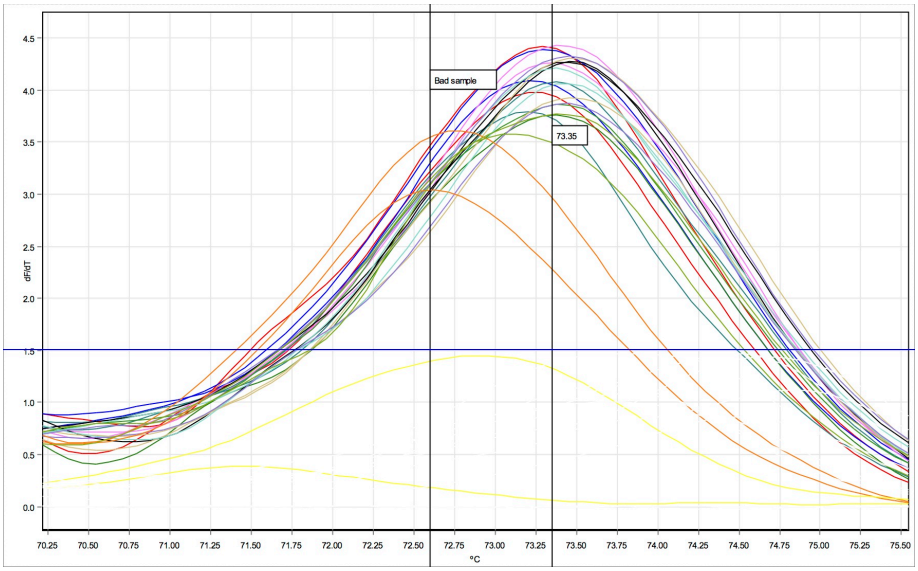

Figure 0.4: Melt analysis for haplotype detection of potato psyllids from Honduras. The curve matches that of the central haplotype

Figure 0.5: Results of Lso detection in samples that were positive for Lso by pyrosequencing. All samples were run in duplicate. The number above the bands indicates the number of returned sequences from pyrosequencing that were identified as Lso.