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Population Genetics of the Potato Psyllid, Bactericera Cockerelli

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POPULATION GENETICS OF THE POTATO PSYLLID, *BACTERICERA COCKERELLI*

By

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Table of Contents

List of Tables

List of Figures

Abstract

POPULATION GENETICS OF THE POTATO PSYLLID, *BACTERICERA COCKERELLI*

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The potato psyllid (*Bactericera cockerelli*) is native to North America and occurs from Honduras to Canada. A new disease of potatoes, Zebra Chip, has recently been associated with potato psyllid occurrence. Potato psyllids transmit a gram-negative α-proteobacter, *Candidatus* Liberibacter solanacearum, the putative causal agent of Zebra Chip in potatoes. Symptoms of Zebra Chip first appeared in potato plants grown outside of Saltillo, Mexico in 1994, and by 2000, this invasive disease complex was found in potatoes in south Texas. Since then, Zebra Chip has spread northward, throughout the central plains of the US and has also been reported on the west coast, including the Pacific Northwest. Potato psyllid population management is further complicated by the presence of two biotypes, one of which is more likely to be a vector of *Candidatus* Liberibacter solanacearum. Improved detection of *Candidatus* Liberibacter solanacearum is needed to maintain supply for potatoes in the United States. In this study, potato psyllid biotypes were delineated by melt temperature analysis following Sybr Green qrt-PCR, and then were grouped further into populations using ISSR-PCR. Potato psyllid populations were mapped, and showed a northward migration throughout the 2009 growing season. The putative causal agent of Zebra Chip, *Ca.* Liberibacter solanacearum, was detected using pyrosequencing at low levels, including a sample with only one cell. These molecular tools will increase efficiency in the management of potato crops in zones of high probability of interaction with inoculative populations of potato psyllids.

Chapter One

Literature Review

Potatoes and Potato Psyllids

Potatoes are the world's fourth most produced food crop, after wheat, rice, and maize (Vandermeiren et al., 2005). According to the International Potato Center in Lima, Peru, the annual global production of potatoes exceeds 320 million tons. Potato is a popular food source, especially for developing countries, because it is more efficient with water usage than grains, and one hectare of potatoes can be two to four times more productive than grain crops. In the United States, 41.3 billion pounds of potatoes were produced in 2008, according to the United States Potato Board.

Potato crops are affected by a number of pests, including *Phytophthora infestans*, the protist responsible for late blight, *Corynebacterium sepedonicum*, the bacterium responsible for brown rot, and *Corynebacterium sepedonicum*, fungi that produce powdery scab (Hooker 1981). Phytoplasma specific diseases of potato include potato purple top, potato hair sprouts, and putatively, zebra chip complex. Phytoplasmas are unculturable bacterium lacking cell walls (Crosslin et al. 2006). Phytoplasma diseases of plants are phloem limited and obligatorily passed from one plant to another by a vector (Weintraub and Jones 2010). Common vectors of phytoplasma diseases include insects of the suborder Homoptera, and the families Cicadillidae,Cixiidae, Cercopidae, Delphacidae, Derbidae, Flatidae, Menoplidae, and Psyllidae (Rohas-Martínez).

The potato psyllid, *Bactericera cockerelli* (Sulc) (order: Hemiptera, suborder: Homoptera, family: Psyllidae), also known as the tomato psyllid, is a native crop pest of the Americas, which prefers to feed on popular agricultural, solanaceous plants such as tomatoes, eggplant, peppers, and potatoes (Liu et al. 2006, Pletsch 1947). The potato psyllid feeds from the phloem of the plants, and is subsequently a vector for phytoplasms (Liu et al. 2006, Rohas-Martínez). Psyllids are quite small and difficult to identify to the species level using morphology. They are often misidentified by producers and scouts as other insecta including whiteflies (family Aleyrodidae) and aphids (family Aphididae).

Potato psyllids have been traditionally associated with crop diseases such as "psyllid yellows" in solanaceous plants, characterized by rolling and yellowing of leaves, aerial tubers, and small tubers underground (Richards 1927; Eyer and Crawford 1933). Psyllid yellows was first identified in Utah in 1927 (Richards and Wann 1928), and subsequent outbreaks were observed in Nebraska, Colorado, Wyoming, Montana, and New Mexico in 1938 that caused complete crop destruction (Wallis 1946). Outbreaks of *B. cockerelli* and psyllid yellows have been noted in California since the 1930's, but these outbreaks were always sporadic and did not continue annually (Pletsch 1947). In the United States, the potato psyllid was often identified in the interior of the country, including the states of Utah, Colorado, Wyoming, and Texas (Pletsch 1947, Liu and Trumble, 2007). Populations were noted to arrive in Colorado, Wyoming, Utah, and Nebraska in June (Cranshaw 2001, Munyaneza 2009). More recently, populations of potato psyllids have been observed in south-central Washington from July to September, and nymphs have also been observed, signifying reproductive populations (Munyaneza et

al. 2009). In recent years, potato psyllids have also caused increased damage to crops including tomato, pepper, and eggplant in the Lower Rio Grande Valley of Texas, an area where potatoes are a main host plant, but other crops are in close proximity to potatoes, allowing for efficient potato psyllid dispersal (Yang and Liu 2009). Previously, potato psyllids were observed during warm months, with little crop damage. Populations of potato psyllids likely overwintered in Mexico and south Texas, and moved north each year as monsoon winds blew inland from the Gulf of Mexico (Abdullah, 2008). The migration coincides with the growing season, starting in February in the Lower Rio Grande Valley of Texas, and moves northward throughout the year. Potato psyllids have a relatively narrow temperature requirement, so as temperatures heat up in New Mexico, Arizona, and Texas, or as temperatures drop in the later part of the year, populations would disappear (Liu and Trumble, 2007). The preferred temperature for the potato psyllid is 26^0 C (80^0 F); temperatures greater than 30^0 C (90^0 F) will reduce or stop egg laying, egg hatching, and the survival of the nymphs (Abdullah, 2008). Sustained temperatures of 38^0 C (100^0 F) for a period of 2 hours causes death of the eggs and nymphs (Abdullah, 2008).

In 1994, a new disease was observed in potato fields near Saltillo, Mexico (Secor and Rivara-Varas 2004). This disease re-immerged in the Rio Grande Valley of Texas, USA, in 2000-2001, and has become a reoccurring issue every year since. The disease was named zebra chip, because the pattern of tissue necrosis in the medullary tissue of the tubers created radial striations, which were especially prevalent when the tubers were cut and fried for chips (Munyaneza et al. 2007). Zebra chip incidence have been identified in other states including New Mexico, Arizona, Colorado, Nevada, Kansas, and

Nebraska, and was also identified in Honduras in 2002, and New Zealand in 2008 (Munyaneza et al. 2007, Espinoza 2010, Liefting et al. 2008). However, zebra chip incidence was absent among potato fields in Washington state (Munyaneza et al. 2009). In 1999-2000, invasive populations of potato psyllids were identified in Baja, Mexico and California. The populations caused significant crop losses of 80% in Baja, and 50% in California (Liu and Trumble 2007).

Potato psyllids were the common denominator in potato fields exhibiting zebra chip, which tend to host unusually high loads of the insects. However, in some cases, potato psyllid colonies did not cause zebra chip in potato fields (Munyaneza et al. 2008). Zebra chip symptom severity may vary depending on the host plant species, for example, potato psyllids reared on bell pepper or eggplant tend to cause more severe symptoms than potato psyllids reared on tomato or potato (Gao et al. 2009, Liu and Trumble 2006). This variation may be attributed to a yet unknown physiological phenomenon (Gao et al. 2009, Liu and Trumble 2006). The severity of the zebra chip symptoms also varied with the age of the potato plant, with more severe symptoms occurring in younger plants (Gao et al. 2009). When tissue from potato plants with zebra chip symptoms was grafted onto healthy potato plants, the majority of the previously healthy individuals displayed symptoms of zebra chip, which supported the hypothesis that zebra chip is caused by a pathogen (Crosslin and Munyaneza 2009, Secor et al. 2009).

One factor that may link potato psyllids to the incidence of zebra chip is the α-proteobacter "*Candidatus* Liberobacter solanacearum". Zebra chip has been associated with *Candidatus* Liberibacter solanacearum, an unculturable class of bacteria which have been identified as putative plant pathogens. Citrus greening disease, or Huanglongbing,

has three *Candidatus* Liberibacter species associated with it; *Ca.* L. asiatucus (Jagoueix et al. 1994; Jagoueix et al. 1996), *Ca.* L. africanus (Jagoueix et al. 1994; Planet et al. 1995; Jagoueix et al. 1996; Garnier et al. 2000), and *Ca.* L. americanus (Teixeira et al. 2005). When compared to *Candidatus* Liberibacter asiatucus, africanus, and americanus in a Neighbor-Joining tree using 16S rRNA sequences, *Candidatus* Liberibacter solanacearum fell in between *Candidatus* Liberibacter afticanus and *Candidatus* Liberibacter americanus (Lin et al. 2009). In comparison to the other *Candidatus* Liberibacter sequences in GenBank, *Ca.* L. solanacearum was 94-97% similar (Secor et al. 2009). In the summer of 2005-06 potato psyllid outbreaks occurred in New Zealand, and by 2009 potato psyllids became a widespread pest (Teulon et al. 2009). In 2009 greenhouse-grown plants were found infected with a new strain of α-proteobacter, *Candidatus* Liberobacter *solanacearum* (*Candidatus* Liberibacter psyllaurous) caused by the outbreak of *B. cockerelli* (Liefting 2009), which was concurrently associated with zebra chip in the United States (Hansen et al. 2008).

High Resolution Melt Temperature Analysis

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) is a method that increases the number of target sequences exponentially, then, with the use of fluorescent molecules, the temperature at which the hydrogen bonds break between the strands of DNA can be observed. High resolution melt curve analysis takes advantage of the differences between nucleotide sequences of DNA (Meuzelaar et al. 2007). An intercalating dye, such as SYBR Green I (Molecular Probes, Inc.) binds to the major groove of the double helix. When temperatures increase, the hydrogen bonds between the helices pull apart, releasing the intercalating molecule. The molecule fluoresces, and

a machine such as the iCycler5 (Bio-Rad, Hercules, CA), records the amount of light emitted in the reaction tube.

RT-PCR is a fast, efficient, cost-effective way to distinguish biotypes. Ninety-six samples can be analyzed simultaneously, and the program takes approximately 1.5 hours to run. Single nucleotide polymorphisms (SNPs) are found within certain genetic sequences which differentiate the biotypes. One gene in particular, cytochrome oxidase 1 (CO1), is often used in arthropod phylogenetics to differentiate at the species level. Typically, the Biological Species Concept is used to define species. It states that a species is "groups of actually or potentially interbreeding natural populations, which are reproductively isolated from other such groups" (Mayr 1942, 1963). However, there are some issues with this concept. At times reproductive barriers do not cut off reproduction, therefore some gene flow occurs, and speciation can occur within an interbreeding population (Hausdorf, 2011). Some species, such as the whitefly (*Bemisia tabaci*), have high phenotypic plasticity, which results in multiple biotypes within a species (DeBarro and Driver 1997). The whitefly biotypes vary with location and host plant (DeBarro and Driver 1997).

Currently, two biotypes of *B. cockerelli* are accepted. Morphologically, the biotypes look identical; however, there are genetic differences. The biotypes are differentiated using a SNP in the CO1 mitochondrial gene (Liu et al. 2006). The mid-continental biotype moves throughout the middle of the United States during the growing season, and is identified by the presence of an adenine - thymine nucleotide pair. The west coast biotype migrates northward along the west coast of the United States during the growing season, and is identified by a cytosine - guanine nucleotide pair in place of

an adenine - thymine pair. The CO1 gene is highly conserved, and it is passed to offspring maternally, resulting in an important molecular tool for population differentiation.

Inter-Simple Sequence Repeats

Inter-simple sequence repeat polymerase chain reaction (ISSR-PCR) is a genetic fingerprinting technique in which multiple loci of the nuclear DNA are amplified using a non-anchored primer, with a repeating sequence such as 'CACACACACACACACA' (Zietkiewicz et al., 1994). ISSR-PCR uses longer primers compared to other techniques used for DNA fingerprinting, which allows for a higher annealing temperature, making this technique highly specific and reproducible (Fang et al. 1997, Liu et al. 2010). Genetic markers do not need to be known for ISSR-PCR to work since primers are designed using nucleotide repeats, and the sequences between the repeats are amplified. This gives ISSR-PCR an advantage over other techniques used to identify polymorphisms in a genome, such as simple sequence repeats (SSR), also known as microsatellites, because prior sequence knowledge is required to develop SSR primers (McGregor et al. 2000). ISSR-PCR is also relatively fast and inexpensive, unlike the amplified fragment length polymorphism technique, which is accurate, but takes more time because of extra steps such as restriction enzyme digestion and amplification of selected fragments (McGregor et al. 2000). Once the inter-simple sequences are amplified, the resulting PCR product is separated by molecular size using gel electrophoresis. The molecular bands resulting from the gel electrophoresis visibly show polymorphisms between individual DNA samples. Each band on the gel represents a dominant trait, which is assumed to represent a single locus (Williams et al. 1990, Liu et

al. 2010). ISSR-PCR allows for a visualization of many polymorphisms using gel electrophoresis, which can be important when working with populations located within a small geographical space (Soliani et al., 2010).

ISSR PCR is often used in plant genomics to determine hybridization or to delineate subspecies (Liu et al., 2006, Wolfe et al., 1998). However, the use of ISSR has been broadened to include arthropods such as *Folsomia candida*, a soil dweller (Sullivan et al., 2009), the mosquito, *Aedes aegypti* (Soliani et al., 2010), bird sexing, population analysis and migration (Wink et al. 1998, Wink et al. 2002, Wink 2006). ISSR-PCR analysis of potato psyllid populations from Guatemala, Mexico, and McAllen, Texas determined that McAllen psyllid populations were more related to populations from Mexico than populations from Guatemala, and furthermore, populations from McAllen and Mexico were more closely related to each other than they were related to Guatemala populations (Jackson et al. 2009).

Chapter Two

Rapid Biotype Differentiation of the Potato Psyllid (*Bactericera cockerelli)* Using Quantitative Real-Time PCR Melt Curve Analysis of Partial CO1 Sequence

Abstract

The potato psyllid (*Bactericera cockerelli*) is native to North America and occurs from Honduras to Canada. A new disease of potatoes, Zebra Chip, has recently been associated with *B. cockerelli* occurrence. *B. cockerelli* transmit a gram-negative α-proteobacter, *Candidatus* Liberibacter solanacearum, the putative causal agent of Zebra Chip in potatoes. Not only do *B. cockerelli* feed on potatoes (*Solanum tuberosum*), but also other solanaceous crops including tomato (*Lycopersicon esculentum*), peppers (*Capsicum annuum*), and eggplant (*Solanum melongena*). *B. cockerelli* population management is further complicated by the presence of two biotypes, one of which is more likely to be a vector of *Candidatus* Liberibacter solanacearum. The two biotypes were previously described using traditional PCR (Liu et al. 2007). In this study, two *B. cockerelli* biotypes were delineated by melt temperature analysis following Sybr Green quantitative real-time PCR. This rapid identification tool was used to characterize *B. cockerelli* from populations in Colorado and North Dakota, USA. This tool will increase efficiency in the management of potato crops in zones of high probability of interaction with inoculative populations of *B. cockerelli*.

Introduction

The potato psyllid, *Bactericera cockerelli,* is a native agricultural pest in North America (Pletsch 1947; Liu and Trumble 2006). *B. cockerelli* are phyllophagous insects that feed primarily on solanaceous crops such as tomatoes *(Lycopersicon esculentum),* peppers *(Capsicum annuum),* eggplant *(Solanum melongena)* and potatoes *(Solanum tuberosum).* Psyllids are quite small and difficult to identify to the species level using morphology. They are often misidentified by producers and scouts as other Insecta including whiteflies (family Aleyrodidae) and aphids (family Aphididae). Potato psyllids have been traditionally associated with crop diseases such as "psyllid yellows" in solanaceous plants, characterized by rolling and yellowing of leaves, aerial tubers, and small tubers underground (Richards 1927; Eyer and Crawford 1933). Psyllid yellows was first identified in Utah in 1927 (Richards and Wann 1928), and subsequent outbreaks were observed in Nebraska, Colorado, Wyoming, Montana, and New Mexico in 1938 that caused complete crop destruction (Wallis 1946). Outbreaks of *B. cockerelli* and psyllid yellows have been noted in California since the 1930's, but these outbreaks were always sporadic and did not continue annually (Pletsch 1947).

In 1994, a new disease was observed in potato fields near Saltillo, Mexico (Secor and Rivara-Varas 2004). This disease re-immerged in the Rio Grande Valley of Texas, USA, in 2000-2001, and has become a reoccurring issue every year since. The disease was named Zebra Chip, because the pattern of tissue necrosis in the tubers created radial striations, which were especially prevalent when the tubers were cut and fried for chips (Munyaneza et al. 2007). Zebra Chip incidence has been identified in other states

including New Mexico, Arizona, Colorado, Nevada, Kansas, and Nebraska (Munyaneza et al. 2007b).

In North America, *B. cockerelli* migrate from southern wintering locations in Mexico and south Texas northward throughout the crop growing seasons on monsoon winds from the Gulf of Mexico (Pleasch 1947; Wallis 1946; Al-Jabar 1999). Migration patterns are thought to be the result of a narrow temperature tolerance of *B. cockerelli* (Liu and Trumble 2007). Temperatures outside of the 15.5^0 C – 32.2^0 C (60.0^0 - 90.0^0 F) inhibit reproduction and survival (List 1939a; 1939b). Population similarities were explored with inter-simple sequence repeat (ISSR) polymerase chain reaction (PCR) using populations of *B. cockerelli* s from Orange and Ventura counties in southern California, Baja, Mexico, Colorado, Nebraska, and Coahuila, Mexico (Liu et al. 2006). *B. cockerelli* in southern California were more closely related to potato psyllids in Baja, Mexico, and potato psyllids found in the states of Colorado and Nebraska were more closely related to potato psyllids in Coahuila, Mexico. ISSR-PCR was again used to compare populations from Guatemala, Mexico, and Texas (Jackson et al. 2009). The *B. cockerelli* in Texas were more related to *B. cockerelli* in Mexico than in Guatemala. Genetic structure of *B. cockerelli* populations may help explain the variable transmission of *Candidatus* Liberibacter solanacearum to potato plants.

Zebra chip has been associated with a bacteria-like organism, *Candidatus* Liberibacter, an unculturable class of bacteria which have been identified as putative plant pathogens. Citrus greening disease, or Huanglongbing, has three *Ca.* L. species associated with it; *Ca.* L. asiatucus (Jagoueix et al. 1994; Jagoueix et al. 1996), *Ca.* L. africanus (Jagoueix et al. 1994; Planet et al. 1995; Jagoueix et al. 1996; Garnier et al.

2000), and *Ca.* L. americanus (Teixeira et al. 2005). In the summer of 2005-06 *B. cockerelli* outbreaks occurred in New Zealand, and by 2009 the psyllids became a widespread pest (Teulon et al. 2009). In 2009 greenhouse-grown plants were found infected with a new strain of α-proteobacter, *Candidatus* Liberobacter solanacearum (*Candidatus* Liberibacter psyllaurous) caused by the outbreak of *B. cockerelli* (Liefting 2009), which was concurrently associated with Zebra Chip in the United States (Hansen et al. 2008).

Zebra Chip is the term used to describe the symptoms caused by *Candidatus* Liberibacter solanacearum, as when tubers from the plants are sliced and fried, a darkened radial design is evident on the chip, caused by a starch build up in necrotic tissue of the tuber (Munyaneza et al. 2007b). Potato crop losses to Zebra Chip are significant, causing millions of dollars in losses and abandonment of potato fields (Secor and Rivera-Varas 2004; Munyaneza et al. 2007a; Liefting et al. 2008). Incidence of Zebra Chip has been prevalent in Texas, New Mexico, Arizona, Colorado, Nevada, Kansas, Nebraska, Mexico and Central America (Munyaneza et al. 2007b). However, Zebra Chip has not occurred in all areas where *B. cockerelli* are present, as reports of severe crop losses have not been reported from west coast states such as California, Oregon, and Washington, or from northern states such as Idaho, Montana, North or South Dakota. Genetic variability among populations has revealed separate biotypes within *B. cockerelli* which concur with geographic separation of potato psyllid populations and the incidence of Zebra Chip (Liu et al. 2006). Insect biotypes reflect some variation between populations of the same species which can include morphological variation, the ability to survive or reproduce or cause disease in different plants than their counterparts (Shufran

and Payton 2009). Biotypes were identified using a single nucleotide polymorphism (SNP), from guanine to adenine, in the cytochrome oxidase I (COI) gene of the mitochondria (Liu et al. 2006). Separating biotypes of *B. cockerelli* can direct attention to the biotype involved with Zebra Chip transmission, saving valuable time and resources.

Differentiated sequences of DNA can be separated using melt temperature analysis. Quantitative real time PCR (qrt-PCR) uses fluorescence from an intercalating dye, such as SYBR Green I (Qiagen, Valencia, CA) to detect the denaturation of amplified DNA strands. The variation in nucleotide content of the DNA strands results in a predictable melting temperature (*T*m). The amplicons resulting from the qrt-PCR are slowly heated to determine the precise *T*m. This technique has become a convenient method for applications including identifying virulent strains of bacteria, such as *Mycobacterium tuberculosis* (Ramirez et al. 2010), cancer-related gene mutations (Tricario et al. 2011), and characterization of wildlife populations (Smith et al. 2009).

In this study, melt curve analysis of DNA was performed using qrtPCR and SYBR Green I with melt curve analysis to differentiate between two *B. cockerelli* biotypes. The SNP region of the mitochondrial COI gene provided a focal point for primer design. Melt curve analysis allowed for the detection of the SNP without gel electrophoresis.

Materials and Methods

Sample Collection and DNA Extraction

Adult *B. cockerelli* were collected from potato fields in Alamosa (late July, 2010) and Saguache (August 8, 2010) counties, Colorado, and Lisbon (1 sample collected late July, 2010), Linton (2 samples collected late July, 2010), and Inkster (1 sample collected August 16, 2010), North Dakota, using sweep nets, and placed in 95% ethanol until processed. The DNA was extracted by crushing individual insects in PBS buffer, followed by DNA extraction using a DNeasy blood and tissue kit (Qiagen, Valencia, CA) per provided protocol. DNA concentrations were measured using 2uL of DNA in a Nanodrop 1000 Spectrophotometer (Thermo-Scientific, Waltham, MA, USA).

Primer Design.

Species-specific PCR primers were designed for the *B. cockerelli* COI gene region using CLUSTALX (Thompson et al., 1997) sequence alignment. Primers were designed to amplify 150-500 base pair fragments of the COI gene using PRIMER3 online oligonucleotide design software (http://frodo.wi.mit.edu/primer3/). Theoretical melt temperature (*T*m) values for sequences were entered into the Poland program (www.biophys.uni-dusseldorf.dc/local/POLAND/poland.html) (Steger, 1994). The program parameters were: temperature range of 72–92 1^0C, 0.5 1^0C step size, with no mismatched positions or strand concentration. The thermodynamic parameters were set at DNA (100mM NaCl) with a dissociation constant (b) selected for a double strand at the default (b = 1.0 E*1*3/M). The program generated a *T*m curve for each of the entered sequences. Peak *T*m values for each biotype were calculated using 4 primer sets. Poland algorithm data were analyzed in three ways to determine the feasibility of genotype

identification. The four primer sets were tested for validity (Bioneer, Alameda, CA USA) (Table 1). Reverse primers BB BC melt COI R3, R4, and R5 were not used for this study because the melting curves gave either ambiguous results, or resulted in a non-uniform curve which suggested incomplete denaturation of the target sequence.

Quantitative Real-Time Polymerase Chain Reaction

Quantitative real-time polymerase chain reaction was performed with Rotor-gene SYBR® Green I PCR kit (Qiagen, Valencia, CA) and forward and reverse primers in 25µL reactions as follows; 12.5uL Rotor-gene SYBR® Green I, 2.5uL forward primer, 2.5uL reverse primer, 5uL RNase free water, and 2uL DNA. Melt curve analysis was performed in duplicate. The following PCR protocol was a two-cycle PCR with an optical fluorescence reading for melt temperature following the PCR. The reaction was performed in a Bio-Rad iCycler (Hercules, CA) using the following thermal profile: Cycle one, 95°C for 5m, Cycle two, 95°C for 5s and 67°C for 10s for 40 cycles, Cycle 3 (fluorescence reading), 55°C for 20s, 70°C for 20s increased by half degree increments until 90^0C.

Species Separation

The primer set BB Bc melt COI F and BB Bc melt COI R was checked for species specificity by running a qrt-PCR with multiple species of insect including Asian citrus psyllid (*Diaphorina citri*), American palm cixiid (*Haplaxius crudus*), glassy-wing sharpshooter (*Homalodisca vitripennis*), and invasive fire ant (*Solenopsis invicta*) using the aforementioned two-step PCR protocol, including melt temperature analysis. Gel electrophoresis was used to verify qrt-PCR amplification results on a 1% agarose gel at

100v for 30 minutes with a 1000kbp ladder. The gel was viewed and recorded with a UVP BioDock-It Imaging System (UVP, LCC, Upland, CA, USA).

Biotype Separation

Biotypes of *B. cockerelli* were separated by the melt temperature of each individual sample. The SNP in the COI gene sequence caused the hydrogen bonds between the double helices to melt at a difference from each other of two degrees Celsius. Typically, the mid-continental *B. cockerelli* had a melt temperature at 73.0^0 C, and the west coast *B. cockerelli* had a melt temperature at 75.0^0 C. Any small deviation from these melt temperatures could be explained by the DNA extraction method used or DNA sequence variation.

Sequence Conformation.

To confirm the results of the melt temperatures, DNA from ten samples were sequenced. Five samples from Alamosa County, CO and five samples from Saguache County, CO were selected for sequencing because they represented each melting temperature. Sequencing was completed by the DNA Analysis Facility at Yale (New Haven, CT, USA). The resulting sequences were aligned in FASTA files (Figure 1).

Results

Primers.

Primers were designed using Poland's algorithm at the Institut für Physikalische at Heinrich-Heine-Universität Düsseldorf website: http://www.biophys.uni-duesseldorf.de/local/POLAND/. Of the four primer sets ordered, BB Bc melt COI F1 and BB Bc melt COI R1 were the most successful at producing clear, unambiguous melt peaks, and were therefore used to analyze the samples (Table 1). The other primer sets,

including BB Bc melt COI F1 and BB Bc melt COI R3, BB Bc melt COI F1 and BB Bc melt COI R4, and BB Bc melt COI F1 and BB Bc melt COI R5 resulted in melt curves with multiple peaks, which is a sign of incomplete denaturation of the target DNA strands, or nonspecific binding of PCR products during melt temperature analysis.

Species Separation

Primers were checked using multiple species to confirm specificity of the BB Bc melt F1 and BB Bc melt R1 primers with the potato psyllid. Species included; imported fire ant (*Solenopsis invicta*), glassy-wing sharpshooter (*Homalodisca vitripennis*), American palm cixiid (*Haplaxius crudus*), Asian citrus psyllid (*Diaphorina citri*), and potato psyllid (*Bactericera cockerelli*). The melt curve analysis resulted in two distinct melt curve peaks for the potato psyllid samples at 73.0^0 C and 75.0^0 C (Figure 2), which represented the separate *B. cockerelli* biotypes.

Biotype Separation

The *B. cockerelli* DNA samples from North Dakota melted at 73°C, and the Colorado samples split, with samples at 73^0 C and 75^0 C (Figure 3). Since the North Dakota samples were the same biotype, they were excluded from DNA sequencing. Five Colorado samples from each melt temperature were sequenced. There was a single nucleotide polymorphism between the samples, which had a consensus sequence of 421 base pairs, a base pair change from guanine to adenine at nucleotide 297, with a 99.8% confidence interval (Figure 1).

Discussion

Separating species and biotypes of a species can be an important factor in pest management, as one biotype may be relatively benign, and the other more destructive (De Barro and Driver 1997). There are two known biotypes of *Bactericera cockerelli* when separated by the percentage of polymorphic loci (Liu et al. 2006). The SNP in the COI gene of *B. cockerelli* was accurately identified using melt temperature analysis. The biotype that dominantly occurred in the Central United States was separated from the invasive biotype along the West Coast of the United States at 73.0^0 C and 75.0^0 C, respectively (Figure 3). It must also be noted that both biotypes of *B. cockerelli* were found to occur in Colorado, which seems to be one location where populations intermingle. The native biotype is primarily found in the central portion of the United States and Mexico, and the invasive biotype is primarily found along the west coast of the United States, and Baja, Mexico. As temperatures warm throughout the year, *B. cockerelli* migrate northward on warm winds to more favorable breeding areas.

It is speculated that the invasive west coast *B. cockerelli* biotype carries *Candidatus* Liberibacter solanacearum, and therefore, is the invasive *B. cockerelli* biotype is the primary vector of Liberibacter solanacearum in potatoes. The ability to quickly and inexpensively differentiate populations of each biotype will be crucial for potato producers. Melt temperature analysis is a tool that can identify pests with the ability to devastate crops to the species level or lower, which makes it indispensable for population control in an agricultural setting. Melt temperature analysis is accurate, fast, and inexpensive which makes it a smart choice for future management of *B. cockerelli* populations and useful for a variety of other applications.

The use of the COI gene in biotype identification adds to other useful identification sequences within the *B. cockerelli* genome including ITS2 (Liu et al. 2006 & Jackson et al. 2009), *wsp* Bac1, *wsp* Bac2 (Liu et al. 2006). Results from melt temperature analysis of the COI gene give another locus to determine *B. cockerelli* biotype.

For the analysis of the *Bactericera cockerelli* COI gene, melt temperatures were consistent throughout all samples processed. Melt temperatures were inexpensive and fast, and similar to a standard PCR reaction in comparison to other methods that would require more supplies and time.

Chapter Three

Using ISSR-PCR to Track Potato Psyllid (*Bactericera cockerelli*) Migratory Movements through the Central United States

Abstract

Potatoes are a popular global food crop, which has recently been affected by a new disease in Mexico and the United States, termed Zebra Chip. The likely vector of Zebra Chip is the potato psyllid (*Bactericera cockerelli*), which is native to North America. In this study, potato psyllid DNA from Texas, Kansas, and Nebraska was separated into genetic biotypes, then grouped further, into populations using inter-simple sequence repeat polymerase chain reaction (ISSR-PCR). Populations were mapped over time and space, and potato psyllid populations showed a northward migration throughout the 2009 growing season. Potato psyllid migration information from this study will be helpful for potato pest management and Zebra Chip control.

Introduction

Potatoes are one of the most popular food crops in the world, along with wheat, rice, and maize (Vandermeiren et al. 2005). According to the International Potato Center in Lima, Peru, the annual global production of potatoes exceeds 320 million tons. Potato is a popular food source, especially for developing countries, because it is more efficient with water usage than grains, and one hectare of potatoes can be two to four times more productive than grain crops. In the United States, 41.3 billion pounds of potatoes were produced in 2008, according to the United States Potato Board. In Mexico, 3.5 billion pounds of potatoes were produced in 2007, according to the World Potato Atlas. Recently, a new disease of potatoes (*Solanum tuberosum*), Zebra Chip, has appeared in Mexico and the United States. The likely vector for zebra chip is the potato psyllid (*Bactericera cockerelli*). This disease is caused by the bacterium, *Candidatus* Liberibacter solanacearum, and is transmitted to new plants by the potato psyllid (*Bactericera cockerelli)*.

The potato psyllid, *Bactericera cockerelli* (Sulc) (Order: Hemiptera), also known as the tomato psyllid, is a native crop pest of the Americas, which prefer to feed on the phloem of popular agricultural, solanaceous plants such as tomato (*Lycopersicon esculentum*), eggplant (*Solanum melongena*), pepper (*Capsicum annuum*), and potato (*Solanum tuberosum)* (Liu, 2006). In the United States, potato psyllid populations were often identified in the interior of the country, including the states of Utah, Colorado, Wyoming, and Texas (Liu & Trumble 2007). The insects were observed during warm months, with little crop damage. Populations of potato psyllids likely overwintered in Mexico and south Texas (Abdullah 2008), and moved north each year as monsoon winds

blew inland from the Gulf of Mexico. The migration coincided with the growing season, starting in February in the Lower Rio Grande Valley of Texas, and moved northward throughout the year. Potato psyllids have a relatively narrow temperature requirement, so as temperatures heated up in New Mexico, Arizona, and Texas, or as temperatures dropped in the later part of the year, populations would disappear (Liu & Trumble 2007). According to Abdullah (2008), the preferred temperature for the potato psyllid is 26^0 C (80^0 F), and temperatures greater than 30^0 C (90^0 F) will reduce or stop egg laying, egg hatching, and the survival of the nymphs, and at 38^0 C (100^0 F), for a period of 2 hours, causes death of the eggs and nymphs (Abdullah 2008).

In 1999-2000, an invasive population of potato psyllids was identified in Baja, Mexico and California. This population caused a crop loss of 80% in Baja, and 50% in California (Liu et al. 2007). The biotypes cannot be distinguished using morphological characterizations; they are identified using changes of nucleotides in DNA sequences, such as a single nucleotide polymorphism in the mitochondrial cytochrome oxidase I (COI) gene (Liu et al. 2006). The biotypes can be separated using real-time melt temperature analysis, in which short strands of COI DNA are replicated using conventional PCR, and then the hydrogen bonds of the DNA strands are "melted". The temperature at which the strands pull apart is recorded using a fluorescent signaling molecule, and native potato psyllid COI DNA melt at 73.0^0C, and invasive potato psyllid DNA melts at 75.0^0C (Chapman et al. submitted).

Although potato psyllids cause a variety of problems with their host plants, Zebra Chip is considered a new potato disease characterized by symptoms including leaf yellowing and curling, reduced plant size, reduced tuber size, and death; however, the

distinguishing characteristic of Zebra Chip is most apparent after a tuber has been sliced and fried. The potato chips have a dark, radial pattern, caused by starch build-up in the tuber which likely occurs when vascular tissues of the plant are constricted by *Candidatus* Liberibacter solanacearum (Gau et al. 2009).

Potato psyllids were the common denominator in potato fields exhibiting Zebra Chip, which tended to host unusually high loads of the insects. One factor that may link potato psyllids to the incidence of Zebra Chip is the α-proteobacter "*Candidatus* Liberibacter solanacearum". *Candidatus* Liberibacter solanacearum is likely carried in the invasive populations of potato psyllids, so identifying populations with the ability to transmit Zebra Chip would provide an important pest management tool.

Inter-simple sequence repeat -polymerase chain reaction (ISSR-PCR) is a genetic fingerprinting technique in which multiple loci of the nuclear DNA are amplified using a non-anchored primer, with a repeating sequence such as 'CACACACACACACACA' (Zietkiewicz et al. 1994). One advantage of this technique is the genetic sequence does not need to be known, since all genomes contain repeating sequences. ISSR-PCR is also relatively fast and inexpensive. Once the sequences are amplified using PCR, the resulting PCR product is separated by molecular size using gel electrophoresis. The molecular bands resulting from the gel electrophoresis visibly show polymorphisms between individual DNA samples. Genetic markers do not need to be known for ISSR-PCR to work since primers are designed using nucleotide repeats, and the sequences between the repeats are amplified. ISSR-PCR allows for a visualization of many polymorphisms using gel electrophoresis, which can be important when working with populations located within a small geographical space (Soliani et al. 2010)

ISSR-PCR is often used in plant genomics to determine hybridization or to delineate subspecies (Liu et al. 2006, Wolfe et al. 1998). However, the use of ISSR has been broadened to include arthropods such as *Folsomia candida*, a soil arthropod (Sullivan et al. 2009), and the mosquito, *Aedes aegypti* (Soliani et al. 2010).

In this study, ISSR markers were used to further characterize potato psyllid populations collected in the United States, and it was hypothesized that populations of potato psyllids move northward through the central United States throughout the potato growing season. Potato psyllids were collected from locations in North Dakota, Colorado, Nebraska, Kansas, and Texas in an attempt to track population movements within the mid-continental United States.

Materials and Methods

Potato psyllid samples were collected from the Lower Rio Grande Valley of Texas (near the southernmost border in Texas), from February to April, 2009, Pearsall, TX (Southwest of San Antonio) from March to September, 2009, Dalhart, TX (Northwest of Amarillo) from May to October, 2009, Kansas from June to August, 2009, and Nebraska from July to September, 2009. Individual potato psyllid samples were placed in 95% ethanol until processing. DNA was extracted from the samples using the CTAB method (Daire et al. 1997). The samples were tested for *Candidatus* Liberibacter solanacearum using conventional PCR and primers OA2/o12c (Munyaneza et al. 2009).

The 102 samples were tested for potato psyllid biotype using quantitative real-time polymerase chain reaction (QRT-PCR). Species specific primers and QRT-PCR protocol were developed for this procedure; BB Bc melt COI F (5'-GGA TTC ATT GTT TGA GCA CAT C) and BB Bc melt COI R (5'-TGA AAT AGG AAT CAA), and the

reaction was performed in a Bio-Rad iCycler (Hercules, CA) using the following thermal profile: Cycle one, 95°C for 5m, Cycle two, 95°C for 5s and 67°C for 10s for 40 cycles, Cycle 3 (fluorescence reading), 55°C for 20s, 70°C for 20s increased by half degree increments until 90^0C (Chapman et al. submitted).

Potato psyllid biotypes were separated for two reasons; first, to confirm that the samples were in fact potato psyllids, and second to group the samples into biotypes prior to further separation into populations. For population separation the samples were amplified by PCR using ISSR primer 847 (Liu et al. 2006). Reaction volumes were 50µL per sample and contained 25µL AmpliTaq Gold (Applied Biosystems, Foster City, CA), 2µL GC enhancer (Applied Biosystems, Foster City, CA), 10µL ISSR primer 847 (5'-CACACACACACACACARC) (Liu et al., 2006) (Bioneer, Alameda, CA), 11µL water, and 2µL DNA.

ISSR-PCR was performed in duplicate for each individual DNA sample, and then samples were subjected to gel electrophoresis. Samples were run out on 1% agarose gels at 80 volts for approximately 90 minutes. This allowed for sufficient separation of DNA bands for measurement against the molecular ladder.

Multivariate Statistical Package (MVSP) v. 3.2 (Exeter Software, Setauket, New York) was used to perform a cluster analysis on the presence or absence of bands at specific lengths obtained by PCR amplification of ISSR sequences. A dendogram was produced using Unweighted Pair Group Method with Arithmetic Means (UPGMA) following the distance matrix constructed using Simpson's coefficient. The dendogram is a visual representation of the similarity between groups that are clustered within the same node. Similarity between groups can be expressed using the Simpson's coefficient such

that more similar groups have coefficients approaching one. The dendogram split the DNA samples into five related groups, or clades (Figure 4). Each clade represented separate populations. The sample locations and dates for each clade were plotted on maps, so that there was one map for each population.

Results

Electrophoresis gel results ranged from little variation between samples, with only one allele, to lots of variation between samples, with eight alleles. The average number of alleles for all of the samples was 3.46, and five samples were not included as they exhibited no alleles. Each gel lane was scored with bands as present or absent, resulting in 25 loci and 52 different patterns of heterozygosity, with 13 samples of the most prevalent pattern, a band at 250 base pairs (Figure 5). All loci were present in Texas samples, with the exception of one sample that included a band at 575 base pairs (bp). Five bands were specific only to Texas samples, including bands at 1300bp, from Dalhart, 1200bp, from the Lower Rio Grande Valley, 675bp, from the Lower Rio Grande Valley, 425bp, from the Lower Rio Grande Valley, Pearsall, and Dalhart, and 175bp, from Dalhart. All of these included multiple samples, except the haplotype including the band at 1300bp from Dalhart, and 675bp from the Lower Rio Grande Valley (Figure 5). Samples from Texas had the highest number of banding patterns, 40, followed by Kansas, 11, then Nebraska, six.

The resulting dendogram from the UPGMA cluster analysis separated the populations of potato psyllids into five clades (Figure 4). The arms of the dendogram are relative in size to the relatedness of the individual DNA samples, for example, shorter lines between samples infer a closer relationship, and longer lines between samples show

less relatedness between those two samples. Individual samples were plotted on maps with all of the samples represented within that clade (Figure 6). Time was represented on the maps with color coded dots, which are darker colored for the early months of 2009, and gradually become lighter later in the year (Figure 6). In the map representing clade one, related individuals were collected from potato fields in Pearsall, Texas, and later in the year related individuals were collected in Dalhart, Texas, and Nebraska (Figure 6). In clade two, related individuals were collected from the Lower Rio Grande Valley in Texas early in the year in February and March, and late in the growing season, in nearby Pearsall, Texas in September and October (Figure 6). Related individuals were collected during the summer months of the potato growing season in Dalhart, Texas and Kansas (Figure 6). In clade three, related individuals were collected in February and March in the Lower Rio Grande Valley of Texas, in Pearsall, Texas in April and September, Dalhart, Texas in May and June, and Kansas in June, July, and August, showing a clear northward movement (Figure 6). In the fourth clade, individual samples were collected early in the year in the Lower Rio Grande Valley and Pearsall, Texas, and again, later in the potato growing season in Dalhart, Texas, Kansas, and Nebraska (Figure 6). In clade five, a similar northward migration pattern appeared with individuals collected early in the year in the Lower Rio Grande Valley, and Pearsall, Texas, and related individuals collected later in the growing season in Dalhart, Texas, Kansas, and Nebraska (Figure 6). Clade five contained the only *Candidatus* Liberibacter solanacearum positive DNA sample.

Discussion

The ISSR-PCR gel electrophoresis showed high amounts of heterozygosity throughout the DNA samples, especially in southern Texas. The variation is evidence that the populations of potato psyllids in the central United States have occurred in that region for a long time as native populations, as opposed to the invasive populations on the West Coast of the United States (Liu et al., 2006, personal observation of unpublished ISSR data). As expected, there were a greater number of haplotypes present in samples from Texas, especially in the Lower Rio Grande Valley region, and fewer haplotypes noted from south to north. Unfortunately, the data likely does not form a complete picture, as there are more samples from southerly locations, and less samples northward, for example, there are 36 samples from the Lower Rio Grande Valley, and eight samples from Nebraska. It should also be noted that although there was some sample number bias in location, compartmentalizing the data according to state may not be the best way to view the data, as Texas is spread over such a large area. The distances between collection sites are approximately 200 miles between the Lower Rio Grande Valley and Pearsall, Texas, 500 miles between Pearsall and Dalhart, Texas, only 100 miles between Dalhart, Texas and collection sites in Kansas, and approximately 200 miles between collection sites in Kansas and Nebraska.

A clear northward migration of related potato psyllid individuals was plotted on maps, which represented movement of populations through time and space (Figure 6). All of the populations were migratory; however, not all populations had a continuous northward migration throughout the growing season. Some populations stayed in the southern half of Texas through the year. The individuals that were collected in Kansas

and Nebraska were genetically related to populations in Texas, no samples were unrelated. An interesting observation was that clade five included the only sample positive for *Candidatus* Liberibacter solanacearum, and samples from clade five were distributed across the sample range, suggesting this genetic profile can carry *Candidatus* Liberibacter solanacearum, and they are highly mobile.

It was important to biotype the samples in this study prior to processing with ISSR. The melt temperature analysis primers were species-specific, to ensure only potato psyllid DNA was used (Chapman et al. submitted). Once genetic biotypes were delineated, the ISSR analysis was used to group biotypes into populations. If ISSR analysis was completed without first grouping samples into biotypes, the data would not group in a logical, useful way.

Insect populations that were found in northern locations such as Dalhart, TX, Kansas, and Nebraska were unlikely to be endemic as evidence of the temperature thresholds for development of the potato psyllid (Liu & Trumble 2007, Abdullah 2008). While our data does not directly show that the insects found in these locations migrated from southern locations, it does show genomic homogeneity in between insects collected from these geographic extremes. This evidence, combined with known wind patterns that blow from south to north during the summer, and the predictable times of year that the insects "arrive" in each location is an indication that migration occurs. This supports the hypothesis that potato psyllids migrate northward throughout the growing season (Pletsch 1947, Cranshaw 2001, Liu et al. 2006, Liu & Trumble 2007, Abdullah 2008, Jackson et al. 2009, Munyaneza 2009). Further studies to prove this are expensive, difficult to complete, and outside the scope of this work. Mass mark and recapture would be the best

way to support the migration hypothesis; however, the species and area of this study may cause difficulties. Typical methods for marking individuals for these studies include paint, dye, fluorescent dust, radioactive labels, genetic markers, physical mutilation, rubidium markers, or Immunoglobulin markers (Paulson & Akre 1991, Prasifka et al. 2001, Blackner et al. 2004). For a species as small as a potato psyllid, external physical markers are not practical. Internal markers may be a viable option, however, some markers such as rubidium break down over time, and, like monarch butterflies (*Danaus plexippus*), multiple generations of potato psyllids are likely needed to make a mass migration into the northern United States, rendering most internal labeling methods useless (Prasifka et al. 2001, Batalden et al. 2007). In general, mass mark recapture studies tend to be expensive because they require frequent sampling and additional time and resources to mark individuals, there is a possibility of causing injury or death to the individual while marking, and behaviors may be altered, also altering the likelihood of recapture (Haddad et al. 2008).

In the future, this project should be expanded to include more locations such as California, the Four Corners region (including New Mexico, Arizona, Utah, and Colorado), the Pacific Northwest, and the northern Great Plains region. Sample collection should be more uniform than it was for this study, as a greater number of samples were collected from southern Texas than northern Texas, Kansas, and Nebraska. Also, it would be interesting to see how populations are changing over multiple years, and how that compares to biotype frequency and presence of *Candidatus* Liberibacter solanacearum.

Chapter Four

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

Abstract

Improved detection of *Candidatus* Liberibacter solanacearum (the putative causal agent of Zebra Chip of potato (*Solanum tuberosum*) in the potato psyllid, *Bactericera cockerelli*, is needed to maintain demand for potatoes in the US. Symptoms of Zebra Chip first appeared in potato plants grown outside of Saltillo, Mexico in 1994. By 2000, this invasive disease complex was found in potatoes grown in south Texas. Since then, Zebra Chip has spread northward, throughout the central plains of the US and has been reported on the west coast of the US. In 2011, Zebra Chip was reported and confirmed in potatoes grown in the Pacific Northwest, the largest contributor to the potato crop in the U.S.A. Until now, DNA primers were used to detect *Candidatus* Liberibacter solanacearum in potato psyllid tissue using conventional polymerase chain reaction and gel electrophoresis. In this study, we detected *Candidatus* Liberibacter solanacearum using bacterial tag-encoded FLX amplicon pyrosequencing (bTEFAP) at levels identifiable by PCR, and low levels, including a sample with only one cell of *Candidatus* Liberibacter solanacearum. The low level presence of *Candidatus* Liberibacter solanacearum may help explain 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

Sequencing Technology

Pyrosequencing emerged in 1998 as way to read DNA sequences while they were being synthesized, as opposed to the more traditional Sanger sequencing, which reads nucleotides of already synthesized strands of DNA (Ronaghi et al. 1998). In 2004, the Roche GS-FLX 454 Genome Sequencer became commercially available (Zhang et al. 2011). The advantages pyrosequencing offered over Sanger sequencing included increased number of strands of DNA synthesized at one time, by millions, increased sensitivity to low levels of DNA, and reduced master mix components such as sample volume, solvents, and reagents used, from milliliters to nanoliters or picoliters (Franca et al. 2002, Trietsch et al. 2011). Furthermore, massively parallel sequencing allows researchers to run millions of experiments at the same time, which lowers the chance of variability in the experiments (Trietsch et al. 2011).

Currently, there are five next-generation platforms used commercially, the Roche GS-FLX 454 Pyrosequencer, the Illumina/Solara Genome Analyzer, the SOLiD 5500xd system from Applied Biosystems, the the Polonator G.007 by Dover, and the Heliscope by Helicos.

The Roche GS-FLX 454 pyrosequencing method uses emulsion PCR to encapsulate PCR reactants in aqueous micelles (Margulies et al. 2005, Zhang et al. 2011). Sequences are created by synthesis, and inorganic phosphate is released while building the strand, and is converted into light in a series of enzymatic reactions as DNA polymerase synthesizes the strand (Margulies et al. 2005, Zhang et al. 2011). Limited amounts of single deoxyribonucleotides (dNTPs) are added to the reaction, and when

incorporated to a strand, the DNA polymerase extends the primer, and pauses until another complimentary dNTP is added to the strand (Margulies et al. 2005, Zhang et al. 2011). The current Roche GS-FLX 454 Genome Sequencer can read up to 600 base pairs, which is still much less than the Sanger method which can read up to 1200 base pairs, however, the massively parallel format of pyrosequencing makes the lack of base pair length a moot issue. It should be noted that the raw data accuracy of the Roche GS-FLX 454 is greater than 99%, however there tend to be some errors with insertions and deletions (Zhang et al. 2011).

Another popular Next-Generation sequencing platform is the Illumina/Solara Genome Analyzer. The Illumina/Solara platform became commercially available in July of 2007, and uses sequencing by ligation rather than pyrosequencing (von Bubnoff 2008, Zhang et al. 2011). For this method, the DNA is fractionated into short strands, and adaptor sequences are ligated onto the ends of the short strands. The strands are separated by size using gel electrophoresis, and DNA strands of appropriate length, 200-300 base pairs, are removed from the gel for sequencing. Oligonucleotides that will bind to the flow cell are ligated to the ends of the DNA strands, and then the strands are washed over the flow cell, where the oligonucleotides attach at random. As the strands are elongated, the DNA creates a bridge to the flow cell, where the oligonucleotides of the new strand attach, and the cycle continues, leaving the area covered with identical sequences. Nucleotide bases are added to the template strands across the flow cell one at a time and fluorescence is captured after the addition of each base. The raw base pair accuracy for this method is greater than 99.5% (Zhang et al. 2011).

Another platform available that sequences by ligation is the SOLiD 5500xd system from Applied Biosystems. Prior to sequencing the DNA is fragmented into sizes of known length, either a fragment library with single DNA fragments, or a mate-paired library with two DNA fragments ligated together with a known distance between the two fragments. DNA ligation is used to link oligonucleotides to the target strands, and the fourth and fifth bases of the oligonucleotides are encoded with a specific fluorescence. Like the Roche GS-FLX 454 system, SOLiD uses emulsion PCR to replicate sequences of interest attached to glass beads, however, this system uses DNA read lengths of 50-100 base pairs, unlike Roche's 400-600 base pairs (Zhang et al. 2011). The beads are then covalently attached to a glass slide. A nice feature of the SOLiD system is the open slide format, and duel slide run option. The open slide format allows the glass beads to be packed in a relatively dense format, and the duel slides can either be used in tandem, or staggered so two experiments can be run simultaneously. Universal primers are used for sequencing, and each base position is sequenced twice during each cycle, which gives greater than 99.94% accuracy, according to Applied Biosystems.

Another system that uses emulsion PCR and sequencing by ligation is the Polonator G.007, by Dover. Sequence reads from this platform are 26 base pairs, with 92% mappable reads. The focus of the Polonator G.007 platform is affordability, as program software and protocols are freely downloadable, taking some of the sting out of working with the resulting gigabytes of data.

Lastly, the Heliscope by Helicos offers the first commercially available single molecule sequencer (Zhang et al. 2011). This system cleaves DNA into sections 100-200 base pairs in length, and then attaches a poly-A section with a fluorescent marker to the

3’ end of the strands. Stands are washed over a glass plate with Poly-T strands already attached, and the poly-A sections attach randomly to the poly-T strands. A laser illuminates the flow cell, and a CCD camera takes detailed images of the flow cell in sections, so the location of each of the DNA strands is recorded. The fluorescent markers are washed away after the flow cell is imaged. Fluorescently labeled nucleotides are then introduced along with DNA polymerase. When incorporated, the nucleotide fluoresces and the camera records images of the flow cell. This process is repeated, and read lengths are 30-35 base pairs, with a raw accuracy of greater than 99% (Zhang et al. 2011).

The increased volume of sequencing data, the reduced cost of sequencing, and the increased reliability of sequencing has made the sequencing of genomes, transcriptomes, and metagenomes much more feasible, as these feats require the entirety of an organism’s DNA or RNA. Next generation sequencing gives the opportunity to look at microbial communities in more depth, teasing out sequences that are at such low levels that Sanger sequencing has missed detecting them (Kumar et al. 2011). These methods also allow for the detection of non-culturable prokaryotes as well as an estimate of their relative abundance (Nonnenmann et al. 2010). In addition to the host’s eukaryotic genome and transcriptome, pyrosequencing provides an opportunity to analyze the metagenome, the total of all genomes found in a habitat (Proal et al. 2009). For example, humans, *Homo sapiens,* are hosts to millions of organisms living internally within the gut and externally on the skin. The discipline of metagenomics attempts to understand the interaction of the eukaryotic host with microbial communities with which it shares both time and space (Proal et al. 2009).

An important sub discipline within genomics, transcriptomics, and metagenomics is agriculture and agriculture related pests. For agriculture, the ability to understand the important variations between individuals has much value in increased production and hardiness (Green et al. 2007). Furthermore, the study of metagenomics in humans, especially in relation to diseases such as inflammatory bowel disease, obesity, and antibiotic associated diarrhea, have ignited the study of metagenomics in other species such as bovines, swine, soybean, rice, and honeybees (Dowd et al. 2008a, Dowd et al. 2008b, Durso et al. 2011, Frank 2011, Ikeda et al. 2010, Cox-Foster et al. 2007). Genome, transcriptome, and metagenome knowledge of agricultural pests allows us to explore genes important for pest management (Hail et al. 2010).

Hemipteran symbionts

Prokaryotes are everywhere, many prokaryotic organisms have symbiotic relationships with eukaryotes, and the prokaryotes living in eukaryotic organisms have some influence over that organism (Buchner 1965, Margulis 1976). These relationships are categorized as obligate or facultative. Obligate relationships can be defined as a symbiotic relationship that is necessary for the host survival and/or reproduction, and facultative relationships are not necessary for host survival and/or reproduction, but may still influence host behaviors and/or phenotype (Hansen et al. 2007, Moya et al. 2008). In some cases, facultative endosymbionts occur along with obligate endosymbionts, which may or may not work collectively with facultative species, depending on the relationship (Tsuchida et al. 2002). Endosymbionts have been found residing in host tissues and cells, or specialized cells called bacteriocytes (Tsuchida et al. 2002, Moya et al. 2008). Hemipteran association with endosymbionts has allowed diet diversity and specification

by making up for nutrient deficiencies (Margulis 1976, Moya et al. 2008). Oftentimes, when symbiotic relationships have been occurring for a very long time, the prokaryotes involved lose portions of their genome unnecessary for living within the safe confines of their host, such as the thick cell wall of many gram negative bacteria, making the prokaryote unculturable in lab conditions (Moya et al. 2008). Next generation sequencing can overcome this obstacle, as the prokaryotes do not need to be cultured, while the metagenomics and symbiotic relationships can be studied, thereby allowing a closer look at the species involved in the prokaryotic communities. Insects and their endosymbionts are one of the largest studied symbiotic systems (Moya et al. 2008). In hemiptera, many of the species feed on the phloem of plants, which leads to deficiencies in amino acids, vitamins and cofactors, as well as fatty acids necessary for survival and reproduction (Moya et al. 2008). The endosymbiotic prokaryotes assist in the synthesis of the necessary amino acids, vitamins and cofactors, and fatty acids in which the species are deficient, and in return gain various metabolites from the host (Baumann and Moran 1997, Moya et al. 2008). An example of these symbiotic relationships is the pea aphid, *Acyrthosiphon pisum*, the obligate endosymbiont *Buchnera sp.*, and the facultative endosymbiont, *Hamiltonella defensa* (Buchner 1965, Tsuchida et al. 2002, Moya et al. 2008, Oliver et al. 2008). The obligate symbiont, *Buchnera sp.*, is a γ-proteobacteria that is essential for amino acid acquisition for the host, and when removed from the host using antibiotics or heat results in slowed growth, sterility, or death of the host (Tsuchidea et al. 2002). The facultative endosymbiont, *Hamiltonella defensa*, which is inherited, protects the host against parasitism by *Aphidius ervi*, a wasp species (Oliver et al. 2008).

Psyllid Symbionts

Psyllids harbor endosymbionts, some of which are unculturable, such as *Carsonella ruddii*, and *Candidatus* Liberibacter species. The complete genome of *Carsonella ruddii* has been sequenced, and the genome is greatly reduced as it has been associated with psyllids for a very long time (Nakabachi et al. 2006, Moya et al. 2008). The *Candidatus* class of bacteria has been identified as putative plant pathogens. The Asian citrus psyllid (*Diaphorina citri*) has three *Candidatus* Liberibacter species associated with it; *Ca.* L. asiatucus (Jagoueix et al. 1994, Jagoueix et al. 1996), *Ca.* L. africanus (Jagoueix et al. 1994, Planet et al. 1995, Jagoueix et al. 1996, Garnier et al. 2000), and *Ca.* L. americanus (Teixeira et al. 2005). Recently, the potato psyllid has been identified as the primary vector of an α-proteobacter, *Candidatus* Liberibacter solanacearum (*Candidatus* Liberibacter psyllaurous) the putative causal agent of Zebra Chip in potatoes (Liefting et al. 2008, Hansen et al. 2008). In the insect, this microbe appears to be a passive mutualist; however, descriptions of this relationship are in their infancy.

In 1994 a new disease of potatoes was recognized in Saltillo, Mexico (Munyaneza et al. 2007). It was characterized by leaf yellowing and rolling, reduced tuber size, and when cut, the tubers revealed a tissue necrosis that resembled zebra stripes and was more distinct when fried, hence the name, Zebra Chip (Munyaneza et al. 2007). Since 1994, Zebra Chip incidence has radiated out from Saltillo, Mexico, and has been recorded in Central America, Texas, New Mexico, Arizona, Colorado, Nevada, Kansas, and Nebraska (Munyaneza et al. 2007).

In potato psyllids, *Candidatus* Liberibacter solanacearum was identified using polymerase chain reaction to amplify sections of the 16S rRNA, then the results were run on an agarose gel to identify a sample as positive or negative for *Candidatus* Liberibacter solanacearum (Liefting et al. 2008, Secor et al. 2009). Traditional diagnostics have depended on PCR and quantitative real-time PCR methodologies to detect this pathogen in potential insect vectors. While this methodology allows for large numbers of insect samples to be analyzed, false negatives may be an inherent problem because of low microbe levels in the insects. Since analysis of symbiont populations by pyrosequencing can provide more sensitive diagnostics, which return quantitative results and sequencing data, this may be an optimal technique to determine how effective current diagnostic practices are. In this study, bacterial tag-encoded FLX amplicon pyrosequencing (bTEFAP) was used to identify levels of *Candidatus* Liberibacter solanacearum in DNA samples extracted from whole potato psyllids, organisms in which only one sample was previously identified as positive for *Ca.*Liberibacter solanacearum using conventional PCR.

Materials and Methods

Sample collection and DNA extraction

Adult potato psyllids were collected throughout the 2009 and 2010 growing season from potato fields in Texas, Kansas, Nebraska, and southern California. 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 (+) insects

Whole extracted potato psyllid DNA was analyzed for the presence of the 16s rRNA gene of *Candidatus* Liberibacter solanacearum 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, 94.0^0C for 30s, 65.0^0C for 30s, and 72.0^0C for 60s (x40), and cycle 3, 72.0^0C 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 (Lubbock, TX) (www.medicalbiofilm.org). 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. 2008). A second PCR was performed to prevent amplification biases, which are caused by the tags and linkers in the first PCR. Next, amplicon products were mixed in equal volumes, and purified with Agencourt Ampule beads (Agencourt Bioscience Corporation, MA).

The double-stranded DNA was combined with DNA capture beads and 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, 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

Comparison of detection methods between PCR and pyrosequencing

Of 49 samples sent for bacterial tag-encoded FLX amplicon pyrosequencing (bTEFAP), only one sample had previously tested positive for *Candidatus* Liberibacter solanacearum using PCR and gel electrophoresis (Figure 7). On return of the bTEFAP results, fourteen additional samples showed positive results at low levels (Table 2). The original positive sample was collected in the Lower Rio Grande Valley of Texas in February, 2009. The other positive samples were collected in Dalhart, Texas in June and October, 2009, Riverside county, California May, 2009, Ventura county, California,

September, 2009, Ventura county, California, October, 2009, Orange county, California, June, 2010, Orange and Ventura counties, August, 2010, Ventura county, September, 2010, Ventura county, October, 2010, and Orange and Ventura counties, November 2010. The sample from the Lower Rio Grande Valley of Texas from February of 2009 tested positive using conventional PCR and gel electrophoresis, and had a *Candidatus* Liberibacter solanacearum cell count of 4,901. The sample collected from Dalhart, Texas in June 2009, had a *Candidatus* Liberibacter solanacearum cell count of one, the sample collected in Dalhart in October, 2009 had a cell count of two, the sample from Riverside, CA, May 2009, had a cell count of one, the sample from Ventura, CA, September, 2009, had a cell count of nine, the sample from Ventura, CA, October, 2009, had a cell count of two, the sample from Orange, CA, June, 2010, had a cell count of two, the sample from Orange, CA, August, 2010 had a cell count of 10, the sample from Ventura, CA, August 2010 had a cell count of two, the sample from Ventura, CA, September, 2010, had a cell count of one, the samples from Ventura, CA, October, 2010, had cell counts of two for each of three samples, the sample from Orange, CA, November, 2010 had a cell count of one, and the sample from Ventura, CA, November, 2010, had a cell count of one.

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 *Candidatus* Liberibacter solanacearum 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 this study, 49 samples were tested for *Candidatus* Liberibacter solanacearum, 15, or 30.61% of the samples had positive results. In a study where potato psyllids were collected from symptomatic potato fields in Texas, there was approximately a 25% *Candidatus* Liberibacter solanacearum infection rate using conventional PCR (Secor et al.2009). Recently it was reported that in laboratory colonies positive for *Candidatus* Liberibacter solanacearum, only 95% of individuals tested using conventional PCR result in positive results (Crosslin et al. 2011), therefore 454 pyrosequencing would likely increase the rate of detection of *Candidatus* Liberibacter solanacearum in those colonies.

Standard PCR can be accomplished with a minimal investment of funds and time in-house, and typically samples need to be sent to another lab for pyrosequencing, as the machinery is more expensive, and not as readily available. Cost of pyrosequencing, along with the additional time it takes to ship samples off and wait for someone else to process the samples, makes pyrosequencing unsuitable for some experiments.

In studies involving colonies of laboratory potato psyllids a phenomena of insect colonies testing negative for *Candidatus* Liberibacter solanacearum, then becoming positive, has been observed by several research teams. In these studies "clean" plants were used throughout the life of the colony, 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 Candidatus Liberibacter solanacearum that was below the detection threshold; thus the insects exhibited false negatives.

Even though bTEFAP can be a valuable tool in the management of *Bactericera cockerelli*, 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 *Candidatus* Liberibacter solanacearum data using the pyrosequencing platform. The extra cost of 454 pyrosequencing has advantages other than low level detection of *Candidatus* Liberibacter solanacearum; it also provides additional data, which can be mined to identify potential interactions between *Candidatus* Liberibacter solanacearum 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 the effectiveness of detection of other methods.

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List of Tables

Table 1. Melt Temperature Primers. Primer sets were designed using the potato psyllid COI gene.

Primer Name	Primer Sequence
BB Bc melt COI F	5' - GGA TTC ATT GTT TGA GCA CAT C
BB Bc melt COI R	5' - TGA AAT AGG AAT CAA
BB Bc melt COI R3	5' - AGG CAC GAG AAT CAA CAT CT
BB Bc melt COI R4	5' - GAA ATA GGC ACG AGA ATC AAC A
BB Bc melt COI R5	5' - TGA AAT AGG CAC GAG AAT CAA

Table 2. *Candidatus* Liberibacter solanacearum-Positive Potato Psyllid DNA Samples. Samples are listed along with location, year, and month collected. The sample from the Lower Rio Grande Valley, TX was the only sample to test positive using conventional PCR techniques.

Location	Year	Month	Copy Number
Lower Rio Grande Valley, TX	2009	February	4,901
Riverside County, CA	2009	May	1
Dalhart, TX	2009	June	1
Ventura County, CA	2009	September	9
Dalhart, TX	2009	October	2
Ventura County, CA	2009	October	2
Orange County, CA	2010	June	2
Orange County, CA	2010	August	10
Ventura County, CA	2010	August	2
Ventura County, CA	2010	September	1
Ventura County, CA	2010	October	2
Ventura County, CA	2010	October	2
Ventura County, CA	2010	October	2
Orange County, CA	2010	November	1
Ventura County, CA	2010	November	1

List of Figures

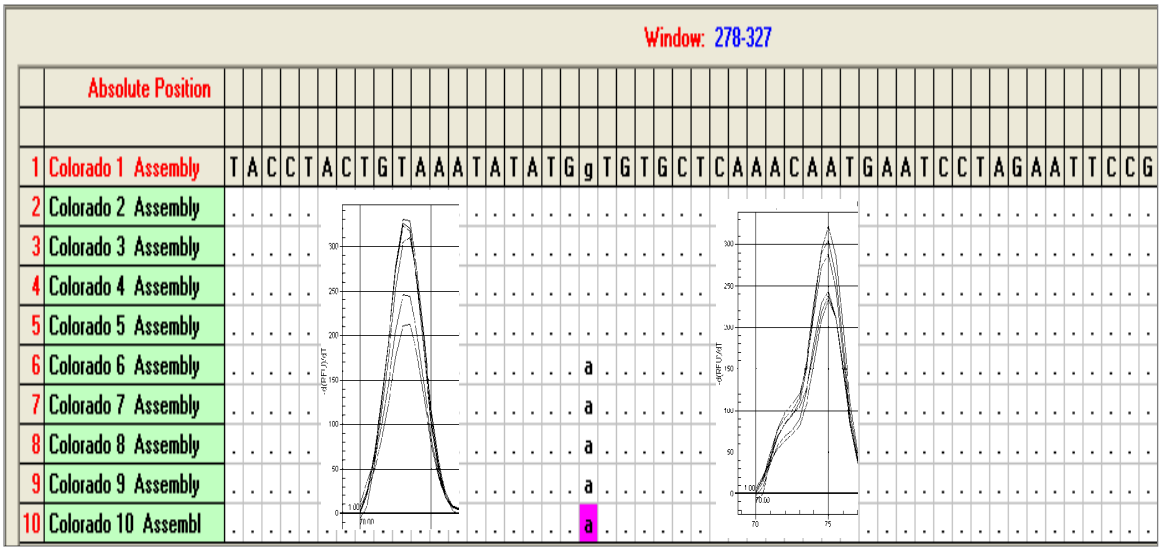

Figure 1. Alignment of Partial COI Sequences and Corresponding Melt Curves. Sequencing of the *Bactericera cockerelli* COI gene from ten samples with different biotypes revealed the G-A base pair change. The melt curve on the left corresponds with the adenine base, and the melt curve on the right corresponds with the guanine

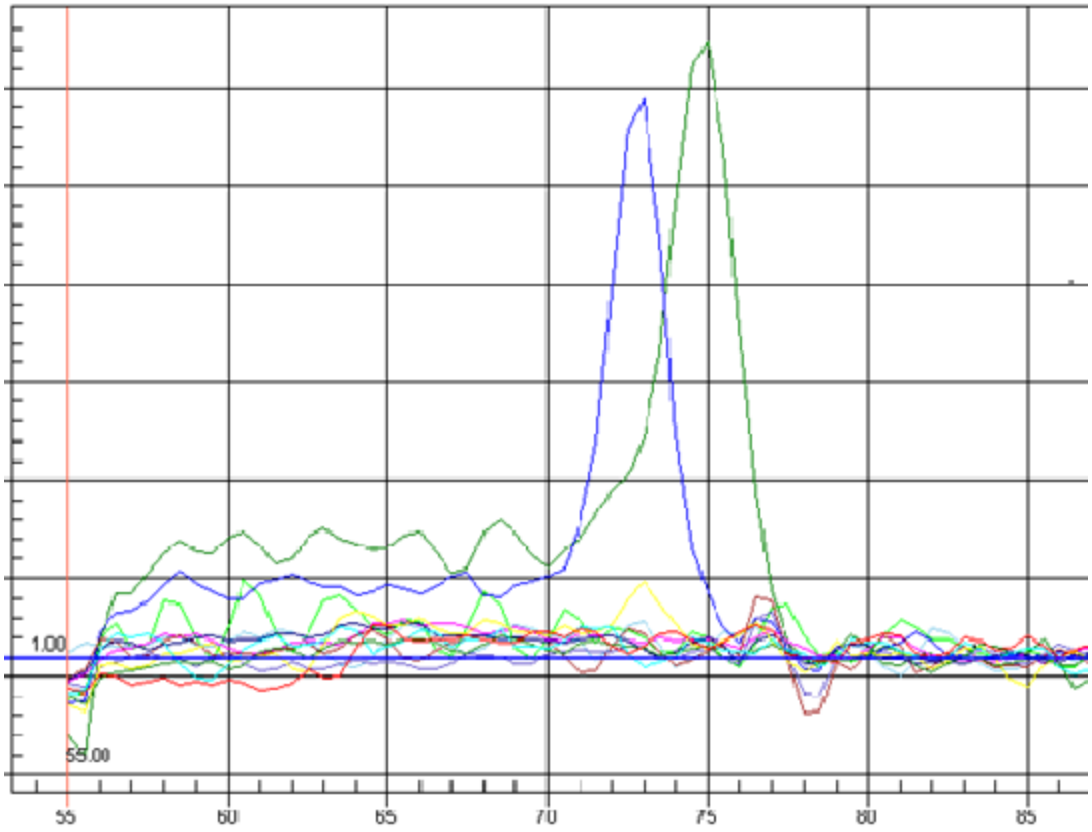

Figure 2. Potato Psyllid COI Species-Specific Primer Melt Curve. The melt temperatures were a result of the primer set BB Bc melt COI F1 and BB Bc melt COI R1. The melt curves show peaks at 73.0° and 75.0°C, which correspond with the two potato psyllid biotypes. The other DNA samples included imported fire ant, glassywing sharpshooter, American palm cixiid, and Asian citrus psyllid.

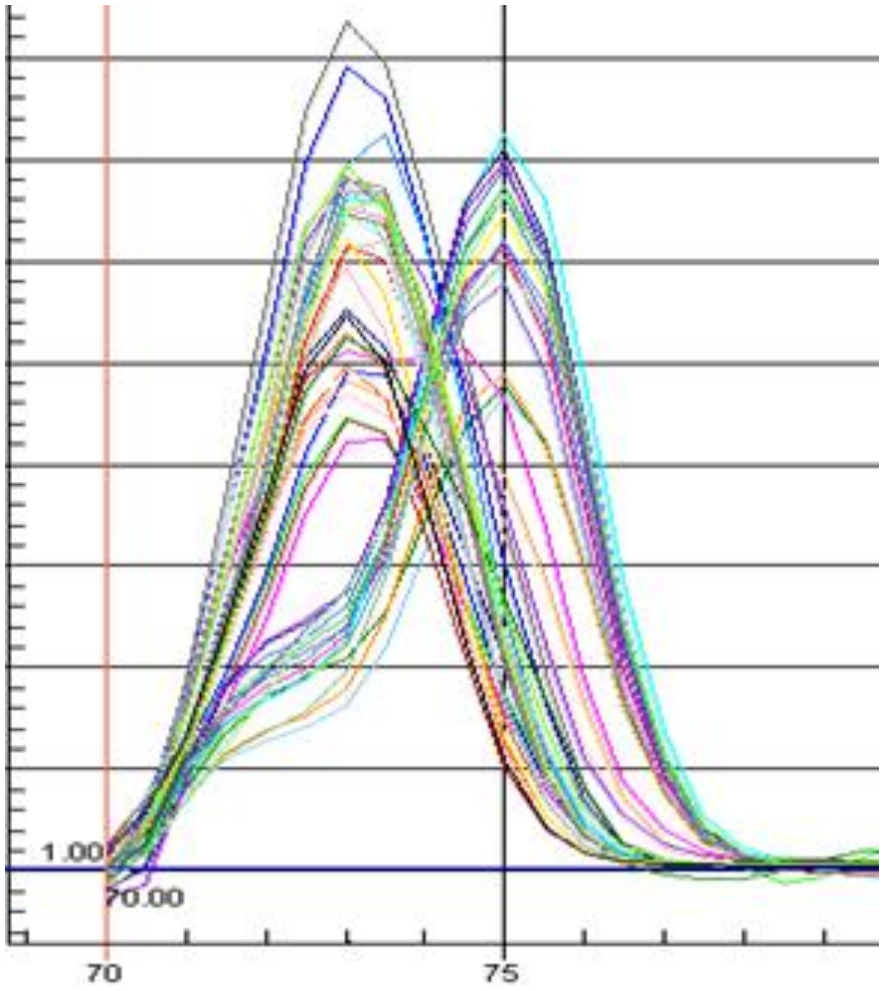

Figure 3. Potato Psyllid COI Biotype-Specific Melt Curve. Melt temperatures for samples from North Dakota and Colorado resulted in melt peaks at 73.0º and 75.0ºC.

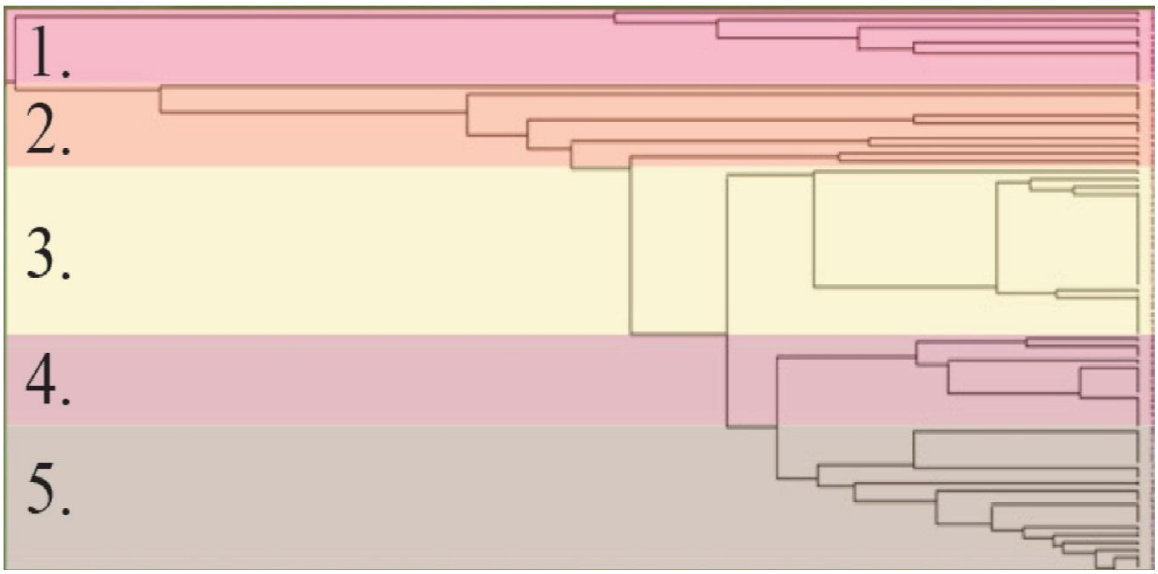

Figure 4. Potato Psyllid Clades Resulting from ISSR-PCR and UPGMA Analysis. This dendogram was the result of UPGMA analysis of ISSR loci. The dendogram split the loci into five distinct clades.

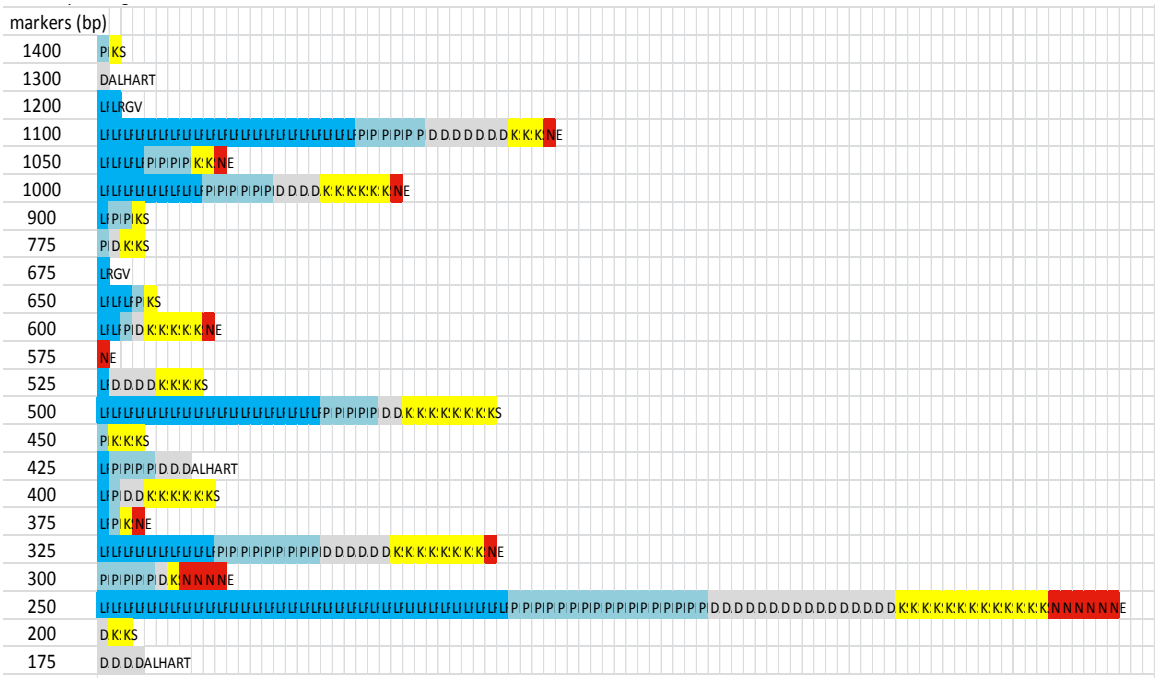

Figure 5. ISSR-PCR Loci Markers and Corresponding Locations. ISSR loci markers were associated with the locations of their corresponding samples. ISSR markers were not found uniformally throughout the collection sites. Blue/Grey-Texas, Yellow-Kansas, Red-Nebraska.

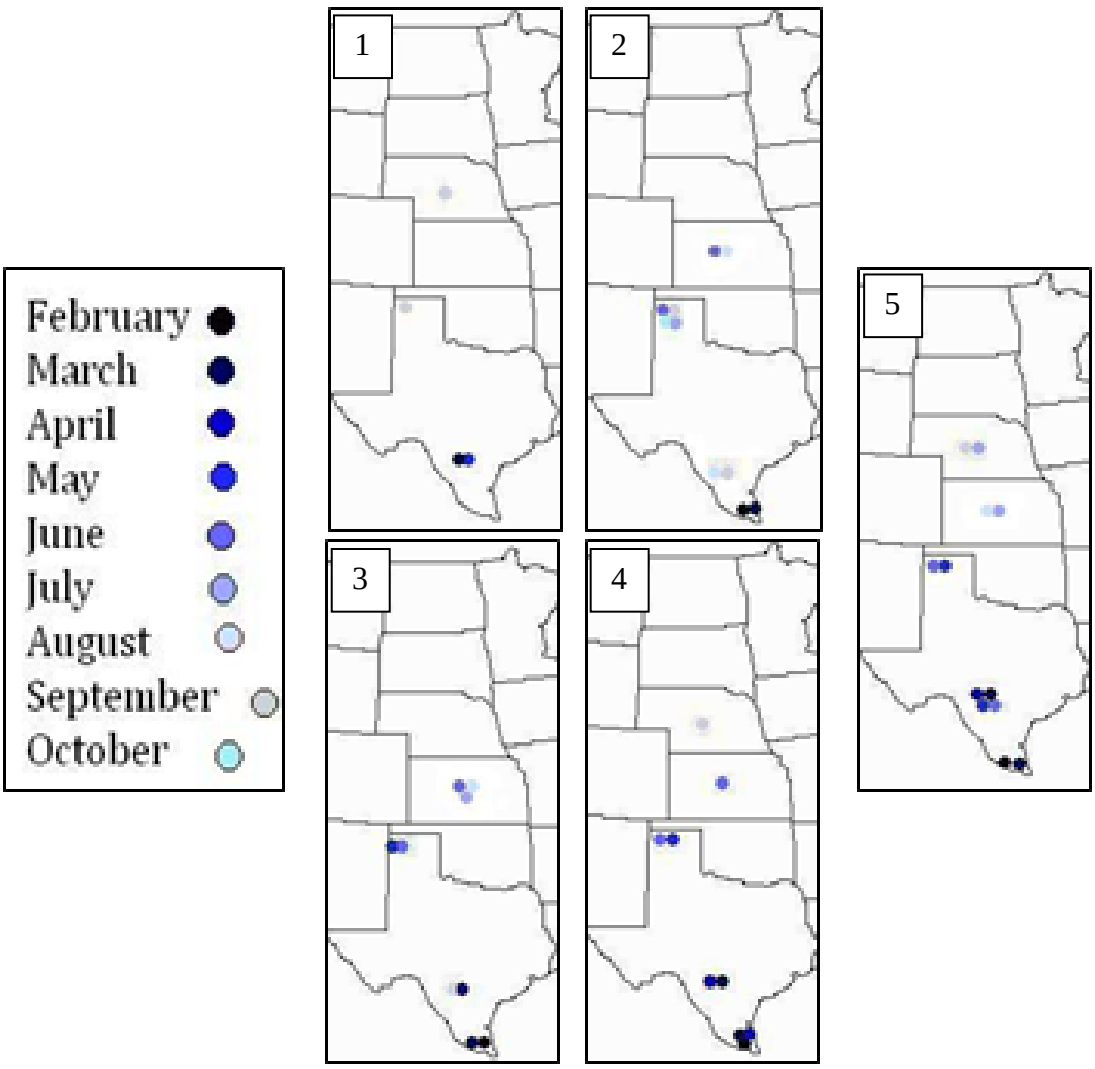

Figure 6. Potato Psyllid Clades. Sample collection locations, from south to north, included the Lower Rio Grande Valley, TX, Pearsall, TX, Dalhart, TX, Kansas and Nebraska. Clade 5 was the only clade to include a *Candidatus* Liberibacter solanacearum-positive sample.

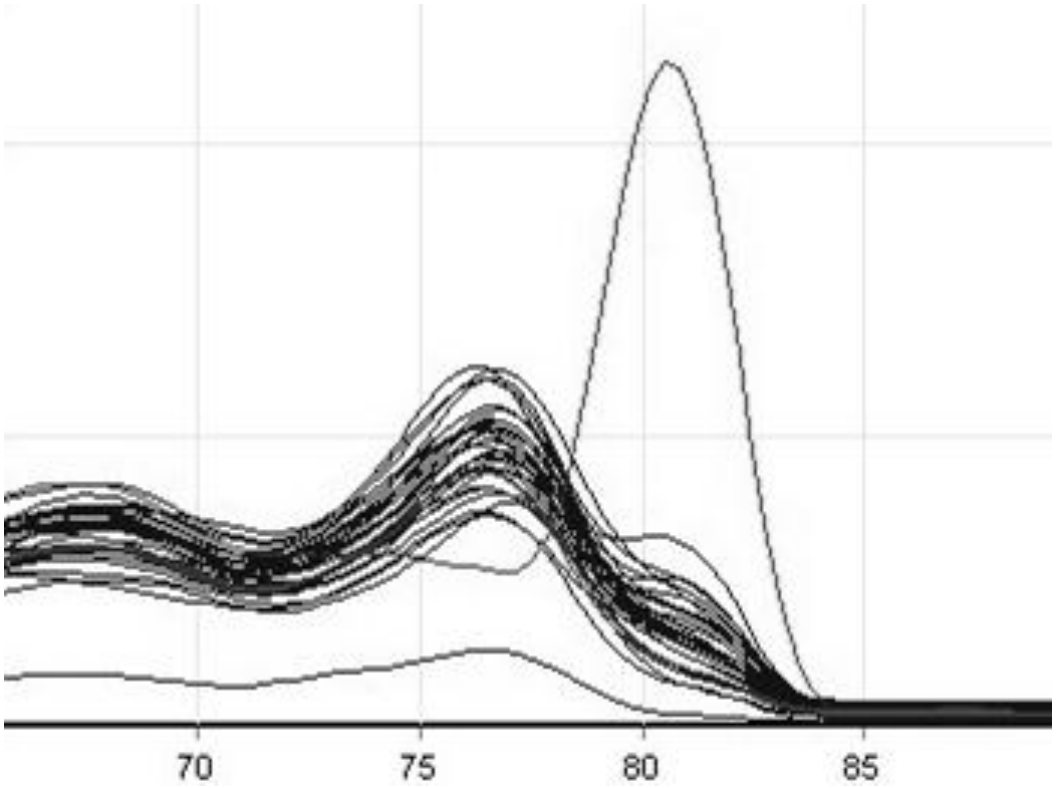

Figure 7. *Candidatus* Liberibacter solanacearum-Positive Potato Psyllid DNA High Resolution Melt Curve. This high resolution melt curve analysis shows one potato psyllid DNA sample that was positive for *Candidatus* Liberibacter solanacearum.