FINAL PROJECT REPORT

Project Title: Molecular gut content analysis to pinpoint where psylla overwinter

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Other funding sources: None

Total Project Funding: $29,000

Budget History:

Item	Year 1: 2016	Year 2: 2017
WTFRC expenses		
Salaries	$7500	
Benefits	$2500	
Wages		
Benefits		
Equipment		
Supplies	$17,500	
Travel		
Plot Fees	$1500	
Miscellaneous		
Total	$29,000	No-cost extension

OBJECTIVES

1. Design PCR primers to detect shelter plant DNA.
2. Determine the number of sequences required to identify previous shelter hosts.
3. Determine how long the plant DNA signal persists in winterform psylla.
4. Develop and test flight interception traps that (a) effectively trap returning psylla before they have entered the orchard and fed extensively on pear, but (b) do not compromise the plant (DNA) signal within the guts of those returning insects.

SIGNIFICANT FINDINGS

1. Verification that pear psylla do indeed feed on multiple species of shelter plants
2. Successful development of methods for direct sequencing
3. Evidence that specimens from sticky traps may degrade too extensively to provide consistent sequencing results; tests with a preservative-filled trap (developed for citrus psyllid) show promise in collecting dispersing winterforms.
4. Several hundred specimens of winterform psylla from multiple habitats, with and without known dietary history, collected and stored for assay.

RESULTS AND DISCUSSION

Many winterform pear psylla disperse from pear orchards beginning in early- to mid-September following leaf fall in pear, and colonize a wide-variety of shelter plants including conifer and deciduous windbreaks and other fruit tree orchards such as peach, apple, or cherry (Kaloostian 1970, Fye 1982, Horton et al. 1994a). Psylla adults begin returning to pear orchards in late February and March. Although dispersal of winterform psylla from pear orchards is well documented, it is not known what shelter habitats are preferred by dispersing psylla, or what proportion of the winterform population remains in pear. Since habitats surrounding orchards can vary by location, better knowledge of winterform dispersal and use of shelter plants could improve predictions of which orchards or regions within orchards are most at risk of colonization by overwintered pear psylla.

Technology to investigate landscape-level movements of pear psylla are not currently available. We previously developed a PCR-based method to identify dietary history of the potato psyllid, *Bactericera cockerelli* (Cooper and Horton 2016). This method mimics aspects of molecular gut content analyses of insect predators (Harwood and Obrycki 2005). Although psyllids primarily feed on phloem contents that presumably lack plant DNA, nearly 40% of the time spent stylet-probing involves contact with non-vascular tissues including DNA-containing parenchyma cells (Civolani et al. 2011, Sandanayaka et al. 2014). Potato psyllid apparently acquires plant DNA during these stylet penetrations within parenchyma tissues.

Feeding behavior of winterform pear psylla is poorly understood, but published and preliminary results indicate that winterform pear psylla likely obtain water from shelter hosts. Horton et al. (1994b) reported that winterform psylla caged on shelter plants during the winter survived, but psylla confined to dead pear limbs died confirming the need for a moisture source. Also, dispersing winterform pear psylla are known vectors of the pathogen that causes peach yellow leaf roll disease in peach (Purcell and Suslow 1985, Blomquist and Kirkpatrick 2002). This disease is caused by a phloem-limited bacterium that is transmitted to peach when the insect feeds and salivates. It seems possible that winterform psylla may acquire shelter plant DNA during the stylet-probing activities. Acquisition of shelter plant DNA would allow us to identify which shelter plants pear psylla had previously visited and fed upon.

Our goal was to adapt methods that we developed for analyzing gut contents of potato psyllid (Cooper et al. 2016) to identify plant species that are fed upon by wintering pear psylla. The technology would allow us basically to look back in time at the winter diet of psylla that are captured and assayed weeks later as they return to the orchard. While the basic premise of this technique is simple – amplify plant DNA from psylla using PCR, clone PCR products into bacteria vectors, sequence PCR products to identify plants that had been visited by the insect (Figure 1) – many

challenges remained in the development of this technology: (1) designing PCR primers that efficiently amplify short but variable regions of chloroplast DNA from a wide-variety of possible shelter plants, (2) establishing the minimum number of sequenced clones required to identify the most recent shelter plants visited by any given insect, (3) determining how long chloroplast DNA persists in living pear psylla, and (4) developing flight interception traps that capture returning psylla but that will not complicate DNA extraction or the detection of the plant signal in captured insects

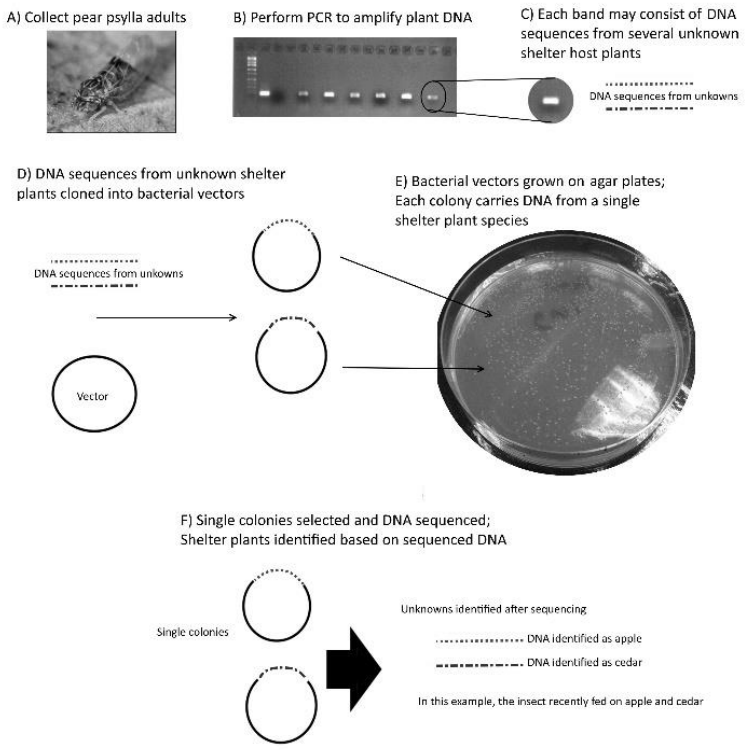

Figure 1. Basic process for identifying dietary history of psyllids using Sanger-based sequencing.

Objective 1: Design PCR primers to detect shelter plant DNA

Our previously published primers for chloroplast DNA (Cooper et al. 2016) amplify sequences from plants within the Solanaceae with high efficiency, but do not adequately amplify sequences from other plant Families. Several other universal primer sets were tested, but most did not consistently amplify plant DNA from psylla. We identified primers which consistently amplify 400-500 bp regions of the host chloroplast genes, *trnL* and *trnF*, from pear psylla. Both regions of chloroplast are highly variable and are suitable for identifying host plants to Family, and in many cases to genus or species. These primers were used for PacBio sequencing (Obj. 2).

Objective 2: Determine the number of sequences required to identify previous shelter plants

Studies in Year 1 indicated that a psylla visited a large number of shelter hosts or feeding hosts. The large number of sequences required to fully assess the dietary history of winterform pear psylla would be cost prohibitive using our previously described methods involving cloning and sequencing PCR products (Figure 1). We requested a no-cost extension for 2017 to explore methods that would reduce costs and improve our ability to detect plant DNA signals.

Following discussions with other researchers and managers of University CORE facilities, we concluded that direct sequencing of PCR products using a PacBio system at the WSU CORE facility in Pullman, WA would be cost-effect and provide a large sequence database to identify dietary history of psylla. This process includes DNA extraction from psylla and PCR using barcoded primers to amplify plant DNA in the guts of psylla. The primer barcodes allow us to identify which samples the sequences belong to after sequencing. Products from all samples are pooled and shipped to the CORE facility at WSU where they are processed for direct sequencing. The resulting dataset is far more extensive that could be achieved by cloning and sequences (Figure 1).

During winter and spring of 2016/2017, we collected a large number of winterform psylla having known and unknown dietary histories (Table 1). An initial experiment was designed using PacBio direct sequencing to 1) determine whether direct sequencing was suitable for gut content analysis of pear psylla, 2) examine to what extent psylla feed on non-pear plants, and 3) determine whether capture of psylla on sticky traps leads to unacceptable levels of DNA degradation (Objective 4).

Pear psylla were collected in November of 2016 from a pear orchard located at the USDA research farm near Moxee, WA (Figure 2A). Sequencing results indicated that psylla within these collections included specimens that had fed upon one or more of the following: pear, apple, Juniper, *Salix*, *Solanum*, pine, or plants within the Asteraceae. The detection of pear sequences was expected because psylla were collected directly from the canopies of pear trees. Windbreaks composed of Juniper and *Salix* are located to the west of the orchard, and an apple orchard is located to the east of the orchard (Figure 2A). Potato was planted to the north of the orchard between the pear orchard and the Juniper windbreak (Figure 2A). Pine trees are also located on the farm. Asteraceae is a large plant family that includes many weed species located on the orchard floor. The results indicate that some winterform psylla present in pear orchards in November had at one time left the orchard and fed upon trees and plants outside of the orchard, and then subsequently returned to the orchard where they were collected (in November). This behavior by winterform psylla has not previously been documented. The presence of Asteraceae sequences in winterform psylla suggests that the insects also fed upon annual weeds located on the orchard floor. These feeding events may have occurred as psylla were displaced from trees by autumn leaf-fall, suggesting that psylla which have dropped from the canopy (either associated with leaf fall or voluntarily), will 'drink' from herbaceous weeds before returning to the pear canopy.

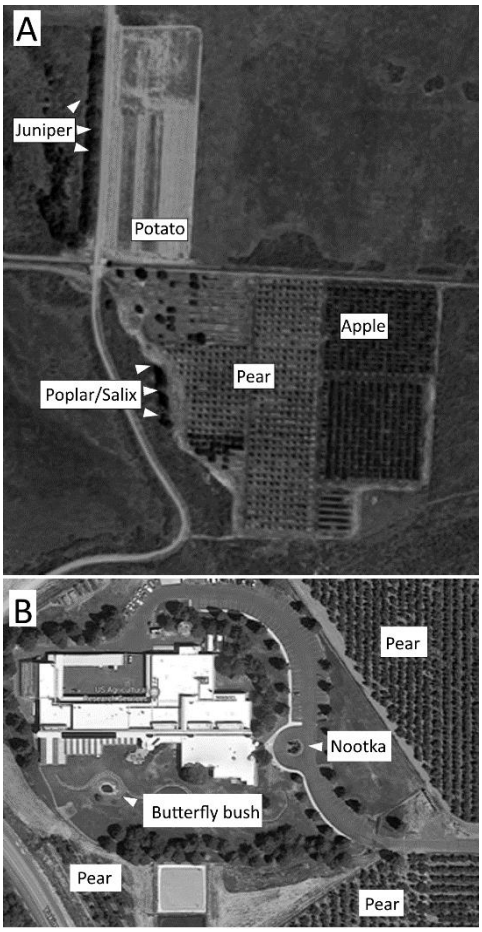

Figure 2. Winterform pear psylla were collected in November 2016 from a pear orchard located at the USDA experimental farm near Moxee, WA (A) and from weeping Nootka located near the ARS laboratory in Wapato, WA (B).

Pear psylla were also collected from Weeping Nootka (an ornamental conifer) located near the ARS laboratory in Wapato, WA in November of 2016 (Figure 2B). Sequences identified from these psylla included Juniper and butterfly bush, which are both planted on the grounds of the Wapato lab. Although the presence of butterfly bush was not known to Cooper before identification of sequences, Horton has seen exceptionally large populations of winterform pear psylla accumulating on this large bush during leaf-fall in pear, followed by disappearance from the bush as the ornamental in turn drops its leaves in late autumn. Our sequence results indicate that many psylla migrated from pear to butterfly bush, then migrated to weeping Nootka from where they were collected for gut content analysis.

Our results confirm that winterform psylla feed from non-host shelter plants, and that direct sequencing using the PacBio platform provides a cost- and labor-effective method for gut content analysis of pear psylla. Results of our pilot study also reveal compelling patterns in autumn migrations of diapausing winterform pear psylla. We have collected and stored a large number of pear psylla with known and unknown dietary histories, and will continue to add to this collection (Table

1). These psylla will be used during the spring of 2018 for a more comprehensive study of shelter plant use by winterform pear psylla using methods developed from this industry-funded pilot study.

Objective 3: Determine how long the plant DNA signal persists in winterform psylla

Pear psylla were collected from Juniper and confined to pear shoots using sleeve cages every two weeks from 20-January 2017 to 3-March 2017. Psylla were also collected directly from Juniper when psylla were retrieved from the sleeve cages on 17-March. Dietary history of psylla was assessed using methods described in Figure 1. The Juniper DNA signal was detected in psylla that were moved from Juniper to pear on 3-March indicating that the Juniper signal persisted in psylla confined to pear for at least 2 weeks. DNA other than pear, including maple and several perennial weed species, were detected in psylla moved from Juniper to pear in February and January. These same weed species were also detected in psylla collected directly from Juniper on 17-March. These results confirm our previous finding with potato psyllid that the plant DNA signal persists in psyllids for an extended period of time. We anticipate the length of time in which the plant DNA signal persists in psyllids to be dependent upon temperature, and to therefore be substantially shorter in psylla collected later in the year when temperatures rise.

Objective 4: Develop and test flight interception traps that (a) effectively trap returning psylla before they have entered the orchard and fed extensively on pear, but (b) do not compromise the plant (DNA) signal within the guts of those returning insects.

Psylla were collected by cooperator Louis Nottingham from yellow sticky traps placed on the perimeter of pear orchards near Wenatchee WA during the spring re-entry period. Traps were hung for a week, and most of the collected insects were highly desiccated and coated in TangleTrap. We were unable to detect plant DNA from these insects suggesting that the DNA was too highly degraded for gut content analysis.

Efficiency of several alternative interception traps for capture of winterform adults were compared in spring of 2016. Interception traps with low-tack tape were not effective at capturing psylla, and will not be suitable for capturing psylla for gut content analysis. Mesh traps treated with horticultural oil were very effective at capturing psylla, but were messy to work with. Brown and olive green traps developed for citrus psyllid (Figure 3) successfully captured winterform psylla. Because these traps capture psylla directly into preservative, there is no need to remove horticultural oil or sticky trap residue from psylla before DNA extraction. We will continue work this winter and spring with mesh traps and 3D traps, and determine whether trapping methods compromise the plant DNA signal.

Figure 3. Prototype 3D-printed traps that capture winterform pear psylla directly into a preservative.

Conclusions. Our results provide the strongest evidence to date that winterform pear psylla indeed do feed extensively upon multiple species of non-developmental shelter plants, and that PacBio sequencing of plant barcoding genes can be used to identify the sometimes highly complex dietary history of dispersing pear psylla. Our long-term objective is to use molecular gut content analysis along with other landscape-ecology approaches to study the landscape-level movements of winterform psylla. To this end we have collected a large number of winterform pear psylla with known and unknown dietary histories from various locations. These specimens along with psylla collected by collaborators in other pear growing regions of the

Pacific Northwest will allow a more comprehensive investigation of shelter plant use by winterform pear psylla. A better understanding of winterform dispersal and overwintering habitats could then enable growers to predict which orchards or areas within orchards are most at-risk of being colonized by overwintered psylla. This information will also enable researchers to develop and test landscape-level approaches of managing the overwintering population of pear psylla.

Table 1. Winterform psylla were collected mid-November to early-December 2015, 2016 and 2017 from miscellaneous orchard and shelter plants at four locations. Collections (1)-(4): specimens were collected directly from shelter plants and are being used to confirm the utility of our molecular methods for psyllids having a partially known dietary history. Collection (5): dispersing winterforms were collected in mid-November from the side of a house in West Yakima, located some 2 miles from the nearest pear orchard; these specimens will allow us to examine our methods for psylla having an unknown dietary history.

	Numbers of winterforms collected and now in storage (-80 °C)
(1) Known plant sources (Moxee farm; winter 2015-2017) Pear orchard, apple orchard, rabbitbrush, sagebrush coniferous windbreak	200+
(2) Known plant sources (West Yakima; Nov-Dec 2016) *Juniperus* windbreak Mixed creekside vegetation (*Rosa, Populus, Salix, Cornus*) Ponderosa pine (*Pinus ponderosa*) Weeping Nootka false cypress (*Chamaecyparis nootkatensis*) Unidentified coniferous Golden currant (*Ribes* sp.) Unknown ornamental fir (*Abies* sp.) Lilac bush (*Syringa vulgaris*) Gold Cone Cedar (*Cedrus deodara*)	 22 6 5 7 3 2 9 3 28
(3) Known plant sources (YARL-Wapato; Nov-Dec 2017) Butterfly bush (*Buddleja* sp.) Unknown ornamental fir (*Abies* sp.) Western Cedar (*Thuja plicata*) Weeping Nootka false cypress (*Chamaecyparis nootkatensis*) Ponderosa pine (*Pinus ponderosa*) Oregon grape (*Mahonia aquifolium*) Rosa	 41 35 42 64 30 39 3
(4) Known plant sources (Naches region; Nov-Dec 2016) Ponderosa pine (*Pinus ponderosa*) Douglas fir (*Pseudotsuga menziesii*) Western cedar (*Thuja plicata*) Mugo pine (*Pinus mugo*)	 1 4 14 9
(5) Unknown dietary history (West Yakima Nov-Dec 2017) Unknown dietary history (preservative-filled traps to be placed on perimeter of orchards)	300+

EXECUTIVE SUMMARY

Preventing unacceptably high densities of pear psylla during the growing season requires effective management of the post-winter generation. A factor complicating these efforts is the tendency of winterform psylla to disperse from orchards in autumn and overwinter on non-pear shelter plants. In late winter prior to pear budbreak, psylla leave these shelter plants, return to pear orchards, and begin laying eggs destined to become the first summerform generation. We have a very poor understanding of what habitats are preferred by wintering psylla, other than that plants suitable for maintenance feeding by psylla apparently are necessary, and that many different types of plants can provide the needed resources. Better understanding of this part of psylla's life cycle would help us predict whether a given orchard is likely to receive a large post-winter influx of psylla (i.e., orchards near favorable overwintering habitat) versus a small influx (i.e., orchards surrounded by less-favorable habitat). The objective of our study was to develop methods for gut content analysis to identify the dietary history of winterform pear psylla as they return to the pear orchard in spring.

Summary of Findings. We previously demonstrated that plant DNA can be PCR-amplified from potato psyllid, and that the dietary history of the potato psyllid could be identified by cloning and sequencing the PCR products. Although this method was appropriate as a proof-of-concept, it is not cost- or labor-effective for wild psyllids that potentially feed upon numerous plant species. Our initial studies in 2016 demonstrated that overwintering pear psylla may feed upon a large number of shelter plant species. We therefore requested a no-cost extension in 2017 to examine whether direct sequencing using a PacBio platform would provide suitable and cost-effective data. Psylla were collected in November from a pear orchard near Moxee, WA, and from a coniferous ornamental (weeping Nootka) located on the grounds of the ARS laboratory in Wapato, WA. Sequences from pear, apple, *Salix*, and juniper were identified in psylla collected from Moxee. All plant species that were detected in psylla specimens occur somewhere on the farm-grounds and within dispersal distance from the source pear orchard. In addition, sequences from potato and from weeds within the Asteraceae were identified from these psylla. A potato field was located immediately below the pear orchard, suggesting that specimens of winterform psylla collected in our source pear orchard in November had at some time preceding the November collection date visited this stand of potatoes. This result was completely unexpected. Asteraceae is a large plant family that includes weeds that are common on the orchard floor, and we suggest that the Asteraceae signal in winterforms is evidence that psylla had visited the orchard floor and fed on weedy Asteraceae before being collected from the tree canopy in November. Sequences from butterfly bush and juniper were identified in psylla collected on the grounds of the Wapato laboratory. Large numbers of winterform psylla often can be found on this stand of butterfly bush during leaf drop in pear, but those psylla then disappear from this plant as it loses its leaves in late autumn. The butterfly bush signal was found in late-autumn in psyllids collected from a coniferous ornamental, indicating that the signal was detectable even following movement onto the coniferous shelter host. Collectively, our results demonstrate that winterform pear psylla feed upon and acquire DNA from non-pear shelter plants and that direct sequencing provides quality data useful for identifying dietary history of winterform psylla. Results also reveal insight into patterns of autumn dispersal by winterform pear psylla that would be impossible to demonstrate using other approaches. These results will be useful as we design more broadly ranging studies in the future.

Another goal of our study was to develop a trap that captures pear psylla but does not interfere with our ability to detect plant DNA. DNA isolated from psylla captured on yellow sticky cards was too degraded to amply plant sequences. We evaluated capture of psylla using 3D-printed traps originally designed for monitoring citrus psyllid and adapted for monitoring potato psyllid. Although the traps are not as efficient as yellow sticky cards, they are less messy and capture psylla directly into a preservative that prevents degradation of plant DNA.

Our longer term objectives are to use these methods to examine landscape-level movements and shelter plant use by winterform psylla from pear growing regions occupying any of a range of native habitats (coniferous forest [Wenatchee, Hood River] to native rangeland [Medford, Wapato]).