FINAL PROJECT REPORT
WTFRC Project Number: TR-15-102A

Project Title: Genetic analysis of Western Cherry Fruit Fly to facilitate species ID

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Percentage time per crop: Cherry: 70% Apple: 30%

Other funding sources: None

WTFRC Collaborative expenses: None

Budget 1
Organization Name: UC Davis **Contract Administrator:** Guyla Yoak
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Item	2015	2016
Salaries	$9,198	$9,565.92
Benefits	$3,716	$3,864.63
Wages	-	-
Benefits	-	-
Equipment	-	-
Supplies	$5,000	$5,000.00
Travel	-	-
Miscellaneous	$3,600	$1,000.00
Plot Fees	-	-
Total	$21,514	$19,430.55

Footnotes: Salary is to support a technician at 25% effort in both years 1 and 2 of the project. Benefits are calculated at UC Davis specified rate of 40.4%. Supplies include reagents for DNA/RNA extraction, Illumina sequencing library preparation, quality control of sequencing libraries, PCR enzymes, standard laboratory consumables and chemicals for molecular biology (PCR and agarose gel electrophoresis). Miscellaneous costs include transcriptome sequencing costs, which will be performed at BGI@UCD in Sacramento, CA, in Year 1 of the project. Miscellaneous costs for year 2 will be for publications and reporting costs to facilitate implementation of the diagnostic assay.

Budget 2

Organization Name: OSU-MCAREC **Contract Administrator:** L. J. Koong
Telephone: 541-737-4066 **Email address:** l.j.koong@oregonstate.edu

Item	2015	2016
Salaries	-	-
Benefits	-	-
Wages	-	-
Benefits	-	-
Equipment	-	-
Supplies	-	-
Travel	-	-
Plot Fees	-	-
Miscellaneous	$750	$750
Total	$750	$750

Footnotes: Miscellaneous costs include costs for collecting and shipping insect samples from Oregon to California for transcriptome sequencing (Year 1) and testing and validating of the molecular diagnostic (Year 2).

JUSTIFICATION

The Western Cherry Fruit Fly (WCFF), *Rhagoletis indifferens*, is a serious pest of cultivated cherries in the Western U.S. and British Columbia, Canada (Yee et al. 2011; Yee et al. 2014a; Yee 2014b; Kumar et al. 2014). It damages the crop directly, and more importantly, becomes a quarantine and quality issue if found in fruit by domestic or oversea inspectors. Once fly larvae that remotely resemble WCFF are found by inspectors at packing houses or export facilities, fruit shipments are halted until a positive or negative species ID is determined. Distinguishing larvae from other insect species that infest cherries can be difficult, and rearing to adulthood for more reliable ID is not practical if marketability of the shipment were to be maintained. When even one suspect larva is found, an entire load of fruit can be rejected, and all subsequent fruit shipments will undergo intensive inspection to uphold the zero tolerance policy.

In order to (1) speed up species ID and (2) ensure reliability of ID to prevent false positives, which can lead to unnecessary quarantine measures and intensive inspection, both leading to increased economic burden to the Cherry industries, we propose to develop a molecular diagnostic test that can be used to rapidly (less than 2 hours) identify WCFF and differentiate it from other insect larvae, including the apple maggot (AMF), *Rhagoletis pomonella* (Green et al. 2013), which is an occasional pest in cherries, as well as the Spotted Wing Drosophila (SWD; *Drosophila suzukii*) (Beers et al. 2011; Walsh et al. 2011). There are a number of commonly used PCR-based molecular diagnostics that have been used for species ID, but these approaches often vary in cost and duration to obtain the results (Behura 2006, Gariepy et al. 2007, Hebert et al. 2003, Williams et al. 1990, Wyman and White 1980). So far, only microsatellite markers have been designed for the molecular identification of WCFF (Maxwell et al. 2009; St. Jean et al. 2013), but results generated using microsatellites are generally difficult to interpret, even for trained scientists. The molecular diagnostic we propose to develop will be a simple, easy-to-interpret, one-step PCR amplification using WCFF-specific primers that is not dependent on sequencing or restriction enzyme digestion, procedures that add both cost and processing time. LAMP PCR approach, which will not require a thermocycler, will also be tested, and can potentially further reduce processing time. Our goal is to develop a molecular diagnostic that is easy to interpret, accurate, and require minimum processing time and equipment.

ORIGINAL OBJECTIVES OF PROJECT:

Objective 1: Sequence the transcriptomes (all expressed genes) of WCFF and perform bioinformatic and comparative sequence analysis with other insect pests of cherries (common and occasional) in the Pacific Northwest as well as closely related species to identify appropriate species-specific molecular diagnostic markers. Genetic analysis will also pave the way for future molecular analysis of WCFF to improve management strategies, e.g. evaluation of response to insecticide treatments and development of RNAi biopesticide (Year 1).

Objective 2: Develop an accurate PCR-based molecular diagnostic test to identify WCFF at all life stages from limited starting materials, e.g. a single larva. Both conventional and LAMP PCR will be tested to design a user-friendly and economical diagnostic assay. The assay will be validated using WCFF and closely related insect specimens (Year 2).

SIGNIFICANT FINDINGS:

- We obtained WCFF larvae, pupae, and adult samples and sequenced their transcriptomes.
- We completed the bioinformatic analysis to assemble the first transcriptome for WCFF.
- We collected closely related insect species as well as species that co-inhibit cherry hosts for validation of our molecular diagnostic (Table 1). Insect specimens include WCFF, apple maggot fly, olive fruit fly, walnut husk fly, blueberry maggot fly, and spotted wing Drosophila.
- We have successfully developed a molecular diagnostic to differentiate WCFF from apple maggot fly and spotted wing Drosophila. These three species co-inhabit the pacific northwest,

and more importantly, at the larval stage, they share extensive similarity in morphology, which makes them difficult to differentiate and make a positive ID. We ensured that internal control primers (positive control for the PCR reaction) worked for all three species and that the divergent primers amplify only DNA from WCFF (diagnostic PCR). The protocol has been designed to be fast, simple, and reliable.

IN PROGRESS OR TO BE COMPLETED:

- A manuscript describing the transcriptome sequencing of WCFF and Apple Maggot fly, as well as development of the PCR diagnostic is in preparation.
- Currently, we are optimizing the protocol so that DNA extraction will no longer be necessary. We aim to finish the optimization and develop this direct tissue PCR diagnostic by April 2017, before the end of the project period.
- Three other closely related but non-target species, walnut husk fruit fly, olive fruit fly, and blueberry maggot fly, will be validated for the WCFF diagnostic. Based on sequence analysis of the diagnostic primers and internal control primers, we expect that the WCFF diagnostic primers will not amplify DNA from these three species.
- We will make a video protocol to illustrate the procedure of the diagnostic test, and it will be available to stakeholders and other interested parties.

RESULTS AND DISCUSSION:

Objective 1 (Year 1): Sequencing and assembly of WCFF transcriptome

Overview: In order to design an accurate and efficient one-step PCR diagnostic that can differentiate WCFF from other common and occasional cherry pest species, we first needed to obtain substantial sequence information of WCFF, which was not available. We therefore sequenced the transcriptomes of WCFF using different life stages as starting material. Sequencing the transcriptomes instead of full genomes will reduce cost of the project by at least 50%. Bioinformatic analysis was then performed to compare WCFF sequences with sequence data of other pest species that infest cherries, e.g. the apple maggot *Rhagoletis pomonella,* (Schwarz et al. 2009) and *Drosophila suzukii* (SWD) (Chiu et al. 2013) to design molecular diagnostic markers that can be used to differentiate these species (Figure 1).

RNA extraction, Transcriptome Sequencing, and assembly

Total RNA was extracted from individual specimens collected from the Pacific Northwest using Tri-reagent (Sigma). We collected WCFF larvae, pupae, and adults and generated three RNA sequencing libraries (Illumina) for each of the WCFF life stages. We then performed paired end sequencing on an Illumina HiSeq 3000 platform, and obtained a total of 778,742,672 100-bp reads.

Trimmomatic v0.35 was used to trim adaptor sequences and low quality ends for quality control. 99.07% of nucleotide bases were retained after trimming indicating high quality sequence data, and subsequently passed on to Trinity 2.1.1 (Grabherr et al. 2011) for transcriptome assembly. To reduce runtime and computing resource requirements, *in silico* read normalization was performed as part of the Trinity assembly process. A total of 230,770 transcript sequences and 204,659,650 bases were assembled. The transcript contig N50 is 1,943, demonstrating good quality sequence assembly. The GC content is 38.81%. Paired reads were mapped back to the assembly using STAR v2.5.0c and passed to Corset v1.04 for clustering into genes. Corset generated 96,628 clusters, representing possible number of expressed genes in WCFF. We anticipated that the number of expressed genes to be lower. Future genome sequencing can likely further improve WCFF transcriptome assembly. Nevertheless, the transcriptome data we generated was sufficient for the development of molecular diagnostic for WCFF and apple maggot fly species ID.

Bioinformatic analysis to design species-specific diagnostic PCR primers

Bioinformatic analysis was performed to compare the WCFF transcriptome and sequence data from apple maggot fly, spotted wing Drosophila, and other closely related species which allowed us to design species-specific diagnostic PCR. Specimens of WCFF, apple maggot fly, olive fruit fly, walnut husk fly, blueberry maggot fly, spotted wing Drosophila have been collected in year 1 of the project to pepare for testing and validation of the molecular diagnostic. Although not within the scope of this project, comparative transcriptome analysis from different stages of WCFF will also advance our understanding of WCFF biology at different life stages.

Objective 2 (Year 2): Design a species-specific molecular diagnostic for WCFF

Overview: Our goal is to develop a PCR diagnostic that is easy to interpret, accurate, and requires minimum processing time and equipment. Upon completion of the project, we will work with stakeholders and scientists who are interested in implementing our species diagnostic into their monitoring or research programs.

Multiplex PCR primer design to identify WCFF

The multiplex PCR diagnostic test for WCFF relies on the use of two primer sets in a multiplex PCR reaction. The first set of primers was designed to amplify a product from WCFF, AMF, and SWD to confirm the presence of good quality DNA in the reaction and to verify the success of the PCR reaction. This represents our built-in quality control. Two separate internal control primer pairs were designed from the coding region of the "*Dark"* gene that is highly conserved among the three species based on our comparative genomic analysis to yield 427 and 462 base pair (bp) products (forward primer: 5'-TCAAATAAACACGAAGGCGC-3' and reverse primers: 5'- GTGGCACAAAATCGTATAATGC-3' and 5'-CATCAGATCGATCTGTGACGG-3' respectively) (Figure 2).

The second set of primers was designed from the Trehalose-6-phosphate synthase gene that is more divergent in sequence between the species of interest such that only the addition of WCFF DNA in the PCR reaction results in successful PCR amplification. We chose a primer pair that amplifies a 249 bp product (forward primer: 5'- GCGTTATGGATTATTCGCAG -3' and reverse primer: 5'- GGTATGTTGGAGGCTGAAATC -3').

Genomic DNA extraction and PCR reactions

To test the specificity of our multiplex PCR, we extracted genomic DNA from larvae, pupae or adult WCFF, AMF, and SWD, using a conventional CTAB protocol, which is routine in our lab, for use as template for PCR reactions. PCR was performed using Taq DNA polymerase (Life Technologies, Grand Island, NY) in a Mastercycler Pro PCR machine (Eppendorf, Hauppauge, NY). The amplified DNA products were resolved by agarose gel electrophoresis and visualized under UV light to assess (i) presence/absence of the two types of DNA bands, and (ii) size of DNA bands as compared to a size standard. To ensure utility of our WCFF diagnostic, we will validate the assay using specimens collected from a multiple geographical locations as genetic variations exist between populations from different collection sites.

Direct larval tissue PCR

Since PCR amplification using extracted genomic DNA has been successful, we are now proceeding to test our molecular diagnostic using crude larval extract as starting material. Our goal is to optimize our diagnostic to enable use of crude extract isolated from as little as one WCFF larva. The use of crude extract as starting material for the PCR will also greatly reduce necessary chemical reagents as well as processing time to allow for SWD identification in less than 2 hours. Currently, DNA extraction adds about one hour to the entire procedure (total of 3 hours). Individual larvae will be cut in half with a sterile razor blade and incubated in PCR-grade water. The samples will be vortexed briefly and a small aliquot of the crude extract will be used directly as input to our PCR diagnostic. PCR amplification and

DNA visualization will be the same as described above. We have previous success optimizing our species diagnostic for SWD (Murphy et al. 2015) to be performed using crude larval extract with DNA extraction.

EXECUTIVE SUMMARY:

We have designed an accurate PCR diagnostic that can unambiguously differentiate Western Cherry Fruit Fly from at least two other fly species that co-inhabit the Pacific Northwest in cherry crops, the Apple Maggot Fly and Spotted Wing Drosophila. These flies are difficult to ID through morphological examination, especially at the immature stages. Our diagnostic method relies on a multiplex PCR workflow that does not require sequencing or restriction digestion. We sequenced the transcriptome of the Western Cherry Fruit Fly and used a comparative genomic approach to facilitate the discovery of the diagnostic marker we presented here. In order to increase the utility of this PCR diagnostic, we are actively working to optimize this diagnostic method and further decrease the time of the workflow from 3 hours to 2 hours by eliminating the DNA extraction step. We anticipate the optimization to be completed by April 2017.

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Table 1: Specimens for validation of WCFF and Apple Maggot Fly molecular diagnostics

Common Name	Species Name	Source	Institution	Collector
Western Cherry Fruit Fly	*Rhagoletis indifferens*	Yakima, WA	USDA ARS Research Station	A. Abrams
Apple Maggot Fly	*Rhagoletis pomonella*	Hood River, OR	OSU	P. Shearer
Olive Fruit Fly	*Bactrocera oleae*	Davis, CA	Plant Pathology Field Station, UC Davis	N. Nicola
Walnut Husk Fly	*Rhagoletis completa*	CA	UC Berkeley	B. van Steenwyk
Blueberry Maggot Fly	*Rhagoletis mendax*	Benton Harbor, MI	Southwest Michigan Research and Extension Center	R. Isaacs
Spotted Wing Drosophila	*Drosophila suzukii*	Hood River, OR	OSU	P. Shearer

Rhagoletis indifferens
(Western Cherry Fruit Fly)

Rhagoletis pomonella
(Apple Maggot Fly)

Drosophila suzukii
(Spotted Wing
Drosophila)

Figure 1: Western Cherry Fruit Fly is difficult to differentiate phenotypically from closely related species, especially at immature stages. At the larval stage, it is difficult to identify WCFF from closely related species. More so, it does not vary much phenotypically from Apple Maggot Fly in the adult stage. All species co-inhabits the Pacific Northwestern of the U.S.

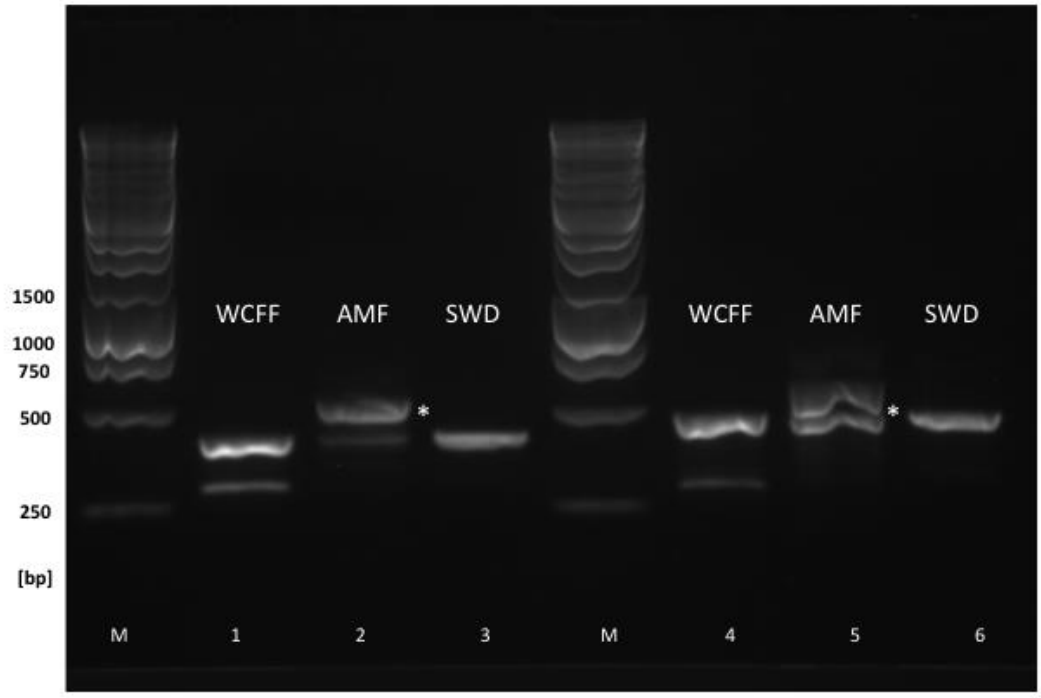

Figure 2: DNA agarose gel electrophoresis showing results of multiplex PCR. Multiplex PCR was performed using WCFF (Western Cherry Fruit Fly), AMF (Apple Maggot Fly), and SWD (Spotted Wing Drosophila). The top band (~427 bp for lanes 1-3 and ~462 bp for lanes 4-6) confirms the presence of high quality DNA in the PCR reaction. The lower band (~249 bp) confirms the identification of WCFF, e.g. in lanes 1 and 4. Therefore, two bands indicate that the organism is WCFF. The upper band present in lanes 2 and 5 (denoted by the asterisk) is a size variant of the gene amplified by the conserved internal control primers, and can be used to confirm the ID of AMF.