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:** Jessica Kaur
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Item	2015	2016
Salaries	$9,198	$9,565.92
Benefits	$3,716	$3,864.63
Supplies	$5,000	$5,000.00
Miscellaneous	$3,600	$1,000.00
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
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 is 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.

We aim to (1) speed up species ID and (2) ensure reliability of ID to prevent false positives. False positives can lead to unnecessary quarantine measures and intensive inspection, both leading to increased economic burden to the Cherry industries. Therefore, we propose to develop a molecular diagnostic test that can be used to rapidly (in 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, both which add cost and 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.

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. The assay will be validated using WCFF and closely related insect specimens.

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 western cherry fruit fly (WCFF), apple maggot fly (AMF), olive fruit fly (OFF), blueberry maggot fly (BMF), and spotted wing Drosophila (SWD).
- We have successfully developed a molecular diagnostic to differentiate WCFF from AMF, SWD, OFF, and BMF. We ensured that internal control primers worked for all five species and that the

divergent primers worked strictly for WCFF. The protocol is designed to be fast, simple, and reliable.

- We designed an alternative assay (iPLEX MassArray) that uses single nucleotide polymorphisms (SNPs) to differentiate between WCFF, AMF, and SWD. This method is fast, even more reliable than the PCR and gel-based diagnostic, and allows users to process up to 95 samples at one time. This assay relies on the interrogation of 15 SNPs simultaneously in one single assay.

TO BE COMPLETED:

- A manuscript describing the PCR diagnostic and the iPLEX SNP genotyping assay for species identification is in preparation.

RESULTS AND DISCUSSION:

Objective 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 reduced the 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 spotted wing Drosophila, *Drosophila suzukii* (Chiu et al. 2013), to design molecular diagnostic markers that can be used to differentiate these species.

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 bases, 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, blueberry maggot fly, and spotted wing Drosophila have been collected in preparation 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: 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. In addition, we will develop a high-quality diagnostic that can be used by export and government quarantine facilities. Upon completion of the project, we will work with stakeholders and scientists to implement our species diagnostic into their monitoring or research programs.

Multiplex PCR primer design to identify WCFF

The multiplex PCR diagnostic test for SWD 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, SWD, OFF and BMF to confirm the presence of good quality DNA in the reaction and to verify the success of the PCR reaction. These two internal control primer pairs were designed from the coding region of the Dark gene that is highly conserved among the three species we used for 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). 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') (Figure 2).

Genomic DNA extraction and PCR reactions

To test the specificity of our multiplex PCR, we extracted genomic DNA from pupae or adult WCFF, AMF, SWD, OFF, and BMF using a conventional CTAB protocol, that 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 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 wide geographical range as genetic variations exist between populations from different collection sites.

Alternative Method for Species Identification: iPLEX SNP genotyping Assay

In addition to the Multiplex PCR diagnostic, we have also designed an iPLEX SNP genotyping assay (Gabriel et. al 2009) that can distinguish between WCFF, AMF, and SWD. The design of this high-performance assay is made possible by the large amount of sequence data obtained from our WCFF transcriptome analysis. By performing comparative bioinformatic analysis of the WCFF transcriptomes with previously sequenced SWD genome (Chiu et al. 2013), and transcriptomes of Apple Maggot fly (Schwarz et al. 2009), we identified hundreds of single nucleotide polymorphisms (SNPs) between these species, and selected a panel of 15 SNP markers to design a robust assay to distinguish these three species. Each specimen can be interrogated for all 15 SNP markers simultaneously on the MassARRAY system using iPLEX chemistry for SNP genotyping (Agena Bioscience, San Diego, CA), and the results are highly accurate. This assay takes about two hours of processing time after gDNA extraction and is more accurate than the method described above because of the ability to look at multiple DNA markers simultaneously. The iPLEX assay workflow consists of a multiplex PCR reaction to amplify all 15 gene regions containing the various SNPs. The primers we designed for this first multiplex PCR step can be found in Table 2. The reactions are then treated with a chemical to neutralize any free nucleotides after the first PCR. The samples then undergo one more multiplex PCR reaction consisting of chain-terminating nucleotides and extensions primers. The extension primers for this step can be found in Table 3. For each SNP marker, the amplicons from different species will give variable spectra readings when run in a mass spectrophotometer because of the sequence difference at the SNP marker site (Gabriel et. al 2009). We validated this assay using 27-29 specimens for each species (WCFF, AMF, and SWD), and the

accuracy for all SNP markers is a perfect 100%. The life stages of the specimens used for validation are indicated in Table 4, and the validation results for the iPLEX SNP genotyping assay are shown in Table 5. Finally, the entire process for this species ID assay is performed on one machine and is expected to decrease risk of contamination during sample processing.

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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 (WCFF)	*Rhagoletis indifferens*	Yakima, WA	USDA ARS Research Station	A. Abrams
Apple Maggot Fly (AMF)	*Rhagoletis pomonella*	Hood River, OR	OSU	P. Shearer
Olive Fruit Fly (OFF)	*Bactrocera oleae*	Davis, CA	Plant Pathology Field Station, UC Davis	N. Nicola
Blueberry Maggot Fly (BMF)	*Rhagoletis mendax*	Benton Harbor, MI	Southwest Michigan Research and Extension Center	R. Isaacs
Spotted Wing Drosophila (SWD)	*Drosophila suzukii*	Hood River, OR	OSU	P. Shearer

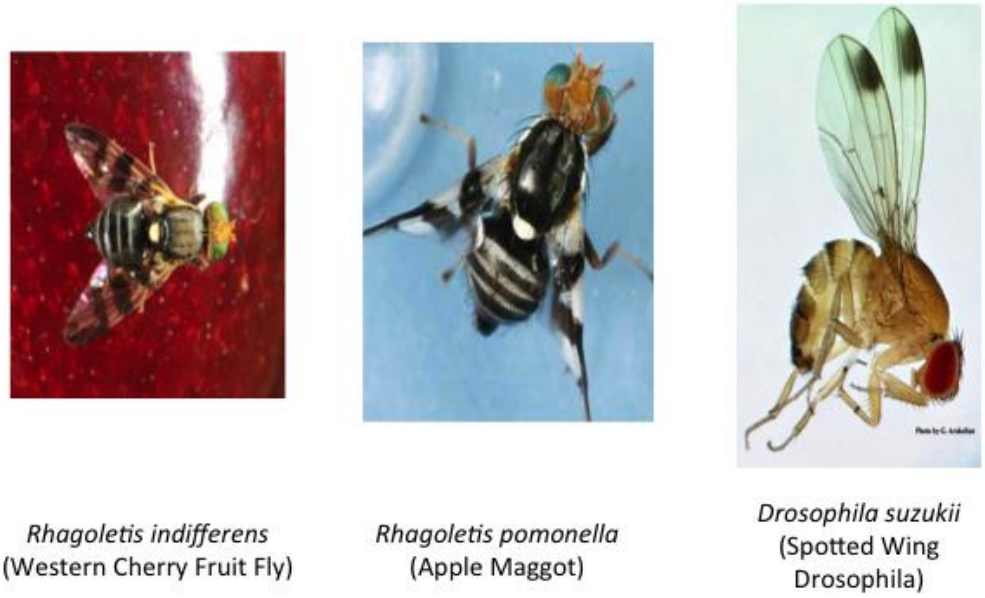

Figure 1: Western Cherry Fruit Fly is difficult to differentiate phenotypically from closely related species. At the larval stage, it is difficult to identify WCFF from closely related species. More so, it does not vary much phenotypically from Apple Maggot Fly (AMF) in the adult stage.

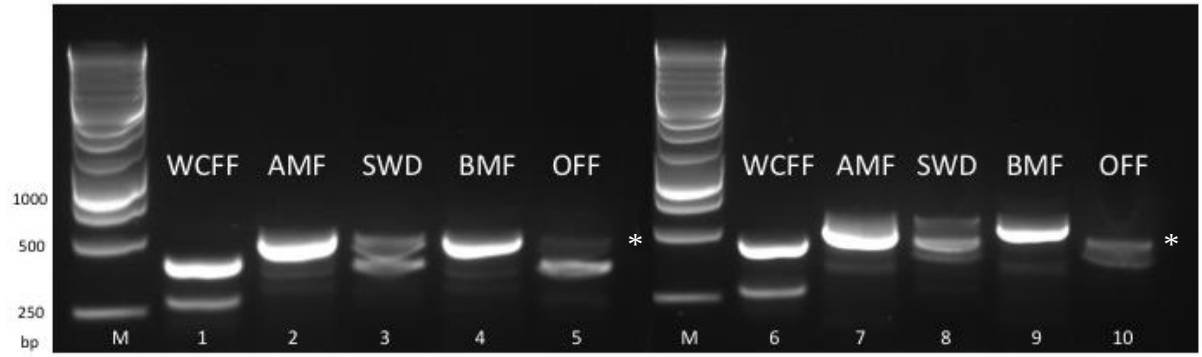

Figure 2: DNA agarose gel electrophoresis showing results of multiplex PCR. Multiplex PCR was performed using WCFF, AMF, SWD, OFF, and BMF. The top band (~427 bp for lanes 1-5 and ~462 bp for lanes 6-10) confirms the presence of high quality DNA. The lower band (~249 bp) confirms the presence of WCFF. Therefore, two bands indicate that the organism is WCFF. The upper band present in lanes 2-5 and 7-10 (denoted by the asterisk) is likely a splice variant amplified by the convergent primers which explains its larger size.

Table 2: Primer sequences used in the first PCR step of the iPLEX assay to amplify regions containing the SNPs between WCFF, AMF, and SWD.

SNP Marker Name		5'- Sequence- 3'
A00472:13742	1st	ACGTTGGATGTAACGTATCCTCCTCTTCGG
A00472:13742	2nd	ACGTTGGATGGTTATCGACATAATCGCCATC
A00725:00046	1st	ACGTTGGATGACTCAATTAACCACCAAGAC
A00725:00046	2nd	ACGTTGGATGGTCGTCCTGTAAGGACAAAC
A83745:00333	1st	ACGTTGGATGATGATAAGAGACAGTCCGAG
A83745:00333	2nd	ACGTTGGATGGGTCGTTCGGATGTAGAATC
A84595:00185	1st	ACGTTGGATGTCCATAGGTGCAATGTGCTG
A84595:00185	2nd	ACGTTGGATGCCTCAGGACGATTTGCTTTG
A89356:00611	1st	ACGTTGGATGTAATACGTCAGCTGAAACGG
A89356:00611	2nd	ACGTTGGATGACCACATGAAAAGTGCCACG
A89440:00936	1st	ACGTTGGATGGAGCCTGGATTTATTTCAG
A89440:00936	2nd	ACGTTGGATGTCGGCGCTTGGAATTTTGAC
A91696:00017	1st	ACGTTGGATGGTGCATTGTAGCTACGTTTG
A91696:00017	2nd	ACGTTGGATGTACAGCGAAGCAGAGTTTCC
A92071:01834	1st	ACGTTGGATGCGCCTTTTCCAAGTATCAAC
A92071:01834	2nd	ACGTTGGATGGCCCACAGAATCAGCTAATG
A92394:00563	1st	ACGTTGGATGGCAACTCATCCGAATAGCAC
A92394:00563	2nd	ACGTTGGATGAATGTACAGCCATGGTGGAG
A92493:03064	1st	ACGTTGGATGCGGCTTCAAAATAGAAGAGG
A92493:03064	2nd	ACGTTGGATGATGCGGTCCGACTTTGCTTG
A92728:00898	1st	ACGTTGGATGTATCTGCTGGGCAAACTCTC
A92728:00898	2nd	ACGTTGGATGTGTTACGCAACTGTTCCAGC
A92743:00078	1st	ACGTTGGATGTTTGCATTCTAGTCTTCACC
A92743:00078	2nd	ACGTTGGATGCCTTTTCGGAGGTATTGCAG
A92752:02475	1st	ACGTTGGATGGCAGCAAATCAATAATAGGAC
A92752:02475	2nd	ACGTTGGATGGTTCTGTTCGGAGGAATGTC
A92822:00048	1st	ACGTTGGATGCAATCCAAGTCCAAGCCATC
A92822:00048	2nd	ACGTTGGATGGATCCCGAACGTAGACAAAG
A92844:00736	1st	ACGTTGGATGACATGGGTAAGCTTAGTTCC
A92844:00736	2nd	ACGTTGGATGCCCACTACCACTGATTTTTC
A92875:01375	1st	ACGTTGGATGGCGGCAGAAGTGCTAAGTAG
A92875:01375	2nd	ACGTTGGATGATTACTGCTGGCAACCGTG

Table 3: Extension primers used with chain-terminating nucleotides to identify SNPs between WCFF, AMF, and SWD.

Primer Name		5'- Sequence- 3'
A00472:13742	U1	TCGACATAATCGCCATCATTCCGTA
A00725:00046	U1	GAGGACAAACCTCTATGGTTTAGCTC
A83745:00333	U1	TTTCGGATGTAGAATCGGTAGATT
A84595:00185	U1	CGGTGGTTCGATTATTTC
A89356:00611	U1	CGGTCGTTGTAAGATTTC
A89440:00936	U1	GGTTGACGTAGTCTAACACCGGAAGG
A91696:00017	U1	GCGGTGGGTATGATAAT
A92071:01834	U1	TCTCAGCTAATGTTCCAACTGC
A92394:00563	U1	CGGCGCCTGCAAGAGACCAAAAC
A92493:03064	U1	GCGGTCCGACTTTGCTTGGAATTTA
A92728:00898	U1	ATGGCTCCAAATTCCTG
A92743:00078	U1	CGGCATGGGTGCCACCTGCATTGT
A92752:02271	U1	CTCGAATTGCACTACAC
A92822:00048	U1	CCGTAGACAAAGATTTGTGCG
A92844:00736	U1	AACATGATGTTTGGCTTAGG
A92875:01375	U1	CCATATTCCGTTCGTATCC

Table 4: Life stages of specimens used for iPLEX species ID validation.

	AMF	SWD	WCFF
Larva	20	10	19
Pupa	8	10	8
Adult	0	9	0
Total	28	29	27

Table 5: Validation of the iPLEX MassArray assay to distinguish WCFF, AMF, and SWD.

Marker Name	SNP Identified	Species	n =	% accuracy
A00472:13742	A	AMF/WCFF	28/27	**100/100**
	G	SWD	29	**100**
A00725:00046	A	AMF/WCFF	28/27	**100/100**
	G	SWD	29	**100**
A83745:00333	C	WCFF	27	**100**
	T	AMF	28	**100**
A89356:00611	C	WCFF	27	**100**
	T	AMF	28	**100**
A89440:00936	T	WCFF	27	**100**
	C	AMF	28	**100**
A91696:00017	A	WCFF	27	**100**
	T	AMF	28	**100**
A92071:01834	T	WCFF	27	**100**
	C	AMF	28	**100**
A92394:00563	G	WCFF	27	**100**
	C	AMF	28	**100**
A92493:03064	G	WCFF	27	**100**
	A	AMF	28	**100**
A92728:00898	G	WCFF	27	**100**
	A	AMF	28	**100**
A92743:00078	A	WCFF	27	**100**
	G	AMF	28	**100**
A92752:02271	C	WCFF	27	**100**
	T	AMF	28	**100**
A92822:00048	A	WCFF	27	**100**
	T	AMF	28	**100**
A92844:00736	C	WCFF	27	**100**
	T	AMF	28	**100**
A92875:01375	G	WCFF	27	**100**
	A	AMF	28	**100**

EXECUTIVE SUMMARY:

We have sequenced the transcriptomes of adult, larval, and pupal stages of Western Cherry Fruit Fly (WCFF). By performing comparative genomic analysis of the WCFF transcriptomes with available sequences for Spotted Wing Drosophila (Chiu et al. 2013) and Apple maggot fly (Schwarz et al. 2009), we have designed two accurate diagnostic approaches that can unambiguously distinguish any life stages of Western Cherry Fruit Fly from the Apple Maggot fly and Spotted Wing Drosophila. The DNA source can be extracted from a small amount of materials, e.g. a single larva. The first assay is a simple PCR gel-based diagnostic that relies on a set of diagnostics primers and an internal control primer set to confirm the quality of the DNA and the success of the PCR reaction. The second assay is a high-throughput high-performance assay that utilizes 15 single nucleotide polymorphic (SNP) markers simultaneously on a iPLEX SNP genotyping MassARRAY platform. Both assays are expected to take around 2-3 hours for species identification and validation results show 100% accuracy. To increase the utility of these species diagnostic, we will work with any researchers (e.g. in USDA), quarantine facilities, and other interested parties to adopt these assays for species ID.