FINAL PROJECT REPORT

Project Title: Develop a PCR diagnostic for all life stages of *Drosophila suzukii*

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Percentage time per crop: Apple: 0% Pear: 0% Cherry: 60% Stone Fruit: 40%

Other funding sources: None

Total Project Funding: $5,500

Budget History:

Item	**Year 1:**
Salaries	$2,800
Benefits	$84
Wages	-
Benefits	-
Equipment	-
Supplies	$2,516
Travel	-
Plot Fees	$100
Miscellaneous	-
Total	$5,500

Footnotes: Salaries and Benefits are for one Undergraduate research assistant
Supplies include DNA oligos, reagents for DNA extraction, PCR, agarose gel electrophoresis and lab consumables
Miscellaneous item represent shipping costs for flies from different geographical locations

JUSTIFICATION

Drosophila suzukii Matsumura (Spotted Wing Drosophila [SWD]) is a recent invader and has become a serious economic pest of a wide variety of fruit crops in the United States, Canada, and Europe, leading to substantial yearly crop losses that are in the tens of millions of dollars (Hauser 2011, Lee et al. 2011, Walsh et al. 2011, Calabria et al. 2012). Unlike other cosmopolitan *Drosophilids* that oviposit in overripe and blemished fruits with no commercial value, SWD female has evolved a serrated ovipositor that enables this pest species to oviposit in ripe or ripening berry fruits, e.g. caneberry, soft-skinned fruits, e.g. cherry, and stone fruits, thus allowing the larvae to develop within the fruit. Although adult SWD flies have morphological characters, i.e. serrated ovipositors in females and spotted wings in males, that facilitate identification and differentiation from other common, non-pest *Drosophilids* that co-inhabit the same geographical regions, identification of SWD from larval stages or from poorly preserved specimens is difficult. Identification of SWD larvae requires researchers or fruit inspectors to rear the larvae to adulthood. This may require a week or more and such delays can result in substantial economic losses for exporters of these perishable fruits as well as delays to research on this important pest. Australia and New Zealand currently have a quarantine safeguarding against entry of SWD-infested fruits and such quarantines may be erected by other countries. Shipment delays due to identification can reduce fruit quality and increase handling costs. A method to identify the immature stage of *Drosophila* species, particularly to specifically identify *D. suzukii* is therefore warranted. We therefore set out to develop an efficient PCR-based molecular diagnostic test that can be used to quickly (i.e. 1 to 2 hours) identify SWD of all life stages and differentiate it from other common *Drosophilids* using minimal molecular biology equipments.

Since this project has been funded and initiated, there have been two published diagnostic designed for SWD (Dhami and Kumarasinghe 2014; Kim et al. 2014). However, the PCR-RFLP diagnostic designed by Kim et al. (2014) cannot differentiate between SWD and the closely-related *D. subpulchrella*, which also has a serrated ovipositor and has the potential to become a pest (Atallah et al. 2014). Whereas the diagnostic designed by Dhami and Kumarasinghe (2014) can differentiate between SWD and *D. subpulchrella*, it is based on a method (high-resolution melt analysis) that requires more sophisticated and expensive instrumentation, e.g. quantitative real-time PCR, which is not available in most common laboratories and export sorting facilities.

ORIGINAL OBJECTIVE OF PROJECT:

Objective 1: **Develop a reliable PCR-based molecular diagnostic test to identify SWD at all life stages**

Using the *Drosophila suzukii* (SWD) genome we recently published (G3: Genes, Genomes, Genetics; Chiu et al., December 2013), we will design SWD specific primers to differentiate SWD from other common *Drosophilids*. This one-step PCR molecular diagnostic can be completed within 2 hours and can be performed from limited starting materials, e.g. a single larva, and the most basic molecular biology equipments.

SIGNIFICANT FINDINGS:

- We have successfully developed a molecular diagnostic to differentiate SWD from 8 other species of common Drosophilids, including *D. biarmipes*, *D. subpulchrella*, and *D. takahashii*, which are closely related to SWD and belong to the *suzukii-takahashii* subgroup.
- The molecular diagnostic has been validated using 9 species of *Drosophila* species (SWD and non-SWD) and 19 populations of SWD collected from different geographical regions in the US and around the world (CA, OR, WA, MD, Hawaii, Japan, South Korea, Italy).
- The SWD molecular diagnostic protocol has been optimized to be fast, simple, and reliable (1 to 2 hours to completion). It does not require the procedure of DNA extraction, and requires minimal starting material (e.g. one single larva).

- A manuscript describing this PCR diagnostic is in revision in the Bulletin of Entomological Research. It was also presented in the annual Entomological Society of America meeting (Portland, OR) in November 2014 as part of a symposium talk.

RESULTS & DISCUSSION:

Comparative genomics analysis facilities primer design

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 any Drosophilid to confirm the presence of good quality DNA in the reaction and to verify the success of the PCR reaction. This internal control primer pair was designed from the coding region of a gene (*D. suzukii* ortholog [SpottedWingFlybase ID: DS10_00001395] of *sec61alpha* in *D. melanogaster* [FlyBase ID: FBgn0086357 and FBpp0078896]) that is highly conserved among the fifteen *Drosophila* species we used for our comparative genomic analysis to yield a 1,248 base pair (bp) product (forward primer: 5'-ATCCCTTCTACTGGATCCGTG-3' and reverse primer: 5'- ACAGCAGCGTGCCCATG-3') (Figure 1A).

The second set of primers was designed from a gene (*D. suzukii* ortholog [SpottedWingFlybase ID: DS10_00004458] of *D. melanogaster* FBgn0035268 and FBpp0072657) that is more divergent. Since there is currently no publicly available genome or transcriptome data available for the closely related species *D. subpulchrella,* for which females also have serrated ovipositors (Atallah *et al.*, 2014), we sequenced a gene region within the *D. subpulchrella* ortholog of *D. suzukii* DS10_00004458 (*D. melanogaster* FBgn0035268) to locate primer sequences that are sufficiently diverged between *D. suzukii* and *D. subpulchrella* to differentiate these two species in addition to the other *Drosophila* species included in our comparative genomics analysis. To obtain the sequence for the *D. subpulchrella* ortholog of *D. suzukii* DS10_00004458, PCR was performed using *D. subpulchrella* genomic DNA as a template with forward primer 5'-AGTTTTGCGTCAGCGGATCC -3' and reverse primer 5'-TCGTCGTCGGAGCTGTTG -3'. These primer sequences were designed based on *D. suzukii* sequences, but were sufficiently conserved to amplify *D. subpulchrella* genomic DNA. Sanger sequencing of the amplified gene region was performed by the UC Davis sequencing facility. Alignment of the resulting *D. subpulchrella* gene region with its orthologs from other *Drosophila* species were performed to identify primer sequences with sufficient sequence polymorphisms to yield a species-specific primer set (Figure 1B). We chose a primer pair that amplifies a 263 bp product. The forward primer (5'-ACTTGTGTCTTGTCCCTCACATAC -3') is located within an intron and the reverse primer (5'-TCCAGATCTTTACGTCATGCTCC -3') is located within the coding region.

Comparative genomic analysis provides a more robust framework for primer design and the theoretical foundation for the reliability of our PCR test. More importantly, since fly populations are continuously evolving, our comprehensive genomics analysis provides the basis for the design of additional diagnostic markers in the case that DNA sequence changes render our diagnostic marker ineffective for specific fly populations.

Multiplex PCR diagnostic differentiates *Drosophila suzukii* from other *Drosophila* species

The internal control primer pair amplifies a 1248 bp product that is conserved in all Drosophilids for quality control purposes. The presence of the control band in non-SWD samples signals that DNA quality and amplification condition are acceptable, and the lack of amplification for the species-specific primers is not due to suboptimal PCR conditions. The SWD species-specific primer pair amplifies a 263 bp product. Thus, a positive test should result in the amplification of two DNA fragments, one at 263 bp, and another at 1248 bp, visible upon DNA gel electrophoresis.

As we aim to develop an efficient assay that can be performed using limited starting materials, we tested our PCR diagnostic using crude extract isolated from either single larva or preserved adult specimen (when larva is not available) without any prior genomic extraction steps. To ensure reproducibility, at least five biological replicates were performed for each SWD and non-SWD population. A total of eight non-SWD *Drosophila* species were tested (Figure 2A and Table 1). There

were no false positives in that all non-SWD larvae showed robust amplification only for the control 1248 bp product. The size of the internal control band in the different species showed slight variation indicating possible in/del within the regions spanned by the control primer set. As sequence polymorphisms might have accumulated over time as *D. suzukii* populations spread, we tested the utility of our PCR diagnostic using larvae or preserved adult specimens from nineteen *D. suzukii* populations (Figure 2B and 2C, Table 1). Freshly sacrificed larvae were used to assay *D. suzukii* populations from the continental U.S.A. including California, Oregon, Washington and Maryland, as well as populations from Hawaii and Japan, as live cultures of these populations were available. On the other hand, preserved adult specimens were used to assay *D. suzukii* populations from Italy and South Korea because we only have access to RNAlater-preserved specimens for these sites. All of the *D. suzukii* larvae originated from different geographical regions in the U.S.A. as well as from Hawaii and Japan tested positive and showed amplification at the 263 bp (SWD-specific band) (Figure 2B and 2C). Moreover, adult *D. suzukii* specimens collected in South Korea and Italy and preserved in RNA*later* also tested positive (Figure 2C). This represents a 100% success rate.

As our diagnostic relies on multiplex PCR, there is competition between the primer sets for reaction components such as ATP and deoxynucleotide triphosphates (dNTPs). When one product is favored, it can outcompete the other reaction resulting in uneven amplification of the two products. Short amplicons, e.g. the 263 bp SWD-specific products, are often amplified with higher efficiency than long amplicons, e.g. the 1248 bp control product, because the polymerase is more likely to fully extend a larger percentage of the short products as the reaction proceeds. Thus, we designed the control primers to produce a longer amplicon than the SWD-specific primers so that when *D. suzukii* DNA is provided as the template, the SWD-specific product will be favored. This design increases the sensitivity of our assay, yet retain the advantage of having an internal control to illustrate the difference between robust vs. weak non-specific amplification. As shown in Figure 2B, the SWD-specific 263 bp product is clearly more robust than the 1248 bp control amplified fragment in all SWD samples. In a few of our test samples, the SWD-specific primers even outcompete the internal control primers, resulting in a single band at 263 bp (e.g. Figure 2B, lane 12; Figure 2C, lane 1). When the 263bp SWD-specific product is present and 1248bp control product is absent, a single band at 263bp can be interpreted as a positive test result.

This assay was also performed using crude extract from ethanol preserved larvae, pupae, and adult samples because this is a common collection and storage condition and produced amplification results and conclusions identical to those in reactions using live samples (data not shown). This direct larval tissue PCR method is economical and practical for rapid identification of single larva because the PCR reaction can be assembled in minutes, while the use of extracted genomic DNA typically requires at least an hour as well as additional equipment and reagents. Finally, we verified that our multiplex PCR diagnostic assay also performs well with genomic DNA extracted from all nine *Drosophila* species and observed that the SWD-specific primers only amplified *D. suzukii* DNA (data not shown).

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A

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Dsuz  ATCCCTTCTACTGGATCCGTG ---------- CATGGGCACGCTGCTGT
Dsec  ATCCCTTCTACTGGATCCGTG ---------- CATGGGCACGCTGCTGT
Dtak  ATCCCTTCTACTGGATCCGTG ---------- CATGGGCACGCTGCTGT
Dgri  ATCCCTTCTACTGGATACGTG ---------- CATGGGCACGCTGCTGT
Dana  ATCCCTTCTACTGGATCCGTG ---------- TATGGGCACGCTGCTGT
Dvir  ATCCCTTCTACTGGATCCGTG ---------- CATGGGCACGCTGCTGT
Dsim  ATCCCTTCTACTGGATCCGTG ---------- CATGGGCACGCTGCTGT
Dmoj  ATCCCTTCTACTGGATACGTG ---------- CATGGGCACGCTGCTGT
Dyak  ATCCCTTCTACTGGATCCGTG ---------- CATGGGCACGCTGCTGT
Dwil  ATCCCTTCTACTGGATTCGTG ---------- CATGGGCACACTGCTGT
Dper  ATCCCTTCTACTGGATTCGTG ---------- CATGGGCACGCTGCTGT
Dmel  ATCCCTTCTACTGGATCCGTG ---------- CATGGGCACGCTGCTGT
Dbia  ATCCCTTCTACTGGATCCGTG ---------- CATGGGCACGCTGCTGT
Dere  ATCCCTTCTACTGGATCCGTG ---------- CATGGGCACGCTGCTGT
Dpse  ATCCTTTCTACTGGATTCGTG ---------- CATGGGCACGCTGCTGT
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B

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Dsuz  TCGCAACACCAAGGATGGCGAGTACTTGCATTGCTACGAGGGACACAT|GTAAGTCCACGT
Dmel  TCGGAACTACAACAGAGGCGACTATTTGCACTGCTATAAGGGTCACAT|GTAAGTAAGCCC
Dsim  TCAGAACTACAACAGAGGCGACTACTTGCACTGCTATAAGGGTCACAT|GTAAGTTCACCA
Dsec  TCAGAACTACAACAGAGGCGACTACTTGCACTGCTATAAGGGTCACAT|GTAAGTTCACCA
Dyak  TCGGAACAACAAGGACGGCGAGTACTTGCATTGCTACCAGGGTCACAT|GTTAGTACTGCC
Dere  TCGGAACAACCAGCCCGGCGAGTACTTGCACTGCTACCAGGGTCACAT|GTAAGTACTGCC
Dbia  TCGCAGCACCAAGGATGGCGAGTACTTGCATTGCTACGAGGGACACAT|GTAAGTCCACGT
Dsub  TCGCAACACCAAGGATGGCGAGTACTTGCATTGCTACGAGGGACACAT|GTAAGTCCATGT
Dtak  TCGGAATACCAAAGACGGCGAGTATCTGCACTGCTACGAGGGGCACAT|GTAGGTTTATAG
Dana  CCGCAACTACACGGATGGCGAGACCCTGCACAGCTACAGGGGACATGT|GTAAG------G
Dwil  TCGCAATTGTA---ATGGCGGCATTCTGCGTACCTATCGAGGACACTT|GTAAGTTGTTAA

Dsuz  TGCATCACTTG--TGTCTTGTCCCT----------CACATACCCCT-CCT-GTCGCCCA
Dmel  TGTTTCCCCCA-ATGTTCTGACACTGA---------CAAATATCATCTCCT-CTTTCCCA
Dsim  TGCCTCCCCCA-ATGTTCTGATATT-----------TATCATCTCCT-CTTTTCCA
Dsec  TGCCTCCCCCA-ATGTTCTGATATT-----------TATCATCTCCTCTTTTTCCA
Dyak  TGCAGCCCAGA-GTGTTCTGGCACT----------CAAATATCATCTCCT-GTTACCCA
Dere  TGCAGCACCGA-GTGTTCTGGCACT----------CACATATCATCTCCT-GTCCCTCA
Dbia  TGCAGCGCTAC--TGTGCTAGCAGT----------CACATACCCCTGCTT-GTCGCCCA
Dsub  TGCATCGCGTG--TGTCTTGGCACT----------CACATACCCCT-CTT-GTCGCCCA
Dtak  AGCATCACTAGTATGTCCTGGTACT----------CACATTTCTCC-----TTCTACCT
Dana  TGCAAGTAAAA-ATGTTCAGCCTTC----------TACTAACTTAGAACTTTGGTCATT
Dwil  TCCCACCAAAG-ATATTGGCCTAGTTAAATCTATCGCACACACACAC------ACACACA

Dsuz  CAG|CAATAGCCGCACCATTAAGGGAGTG-------- GGAGCATGACGTAAAGATCTGGA
Dmel  CAG|CAACAGCCGCACCATAAAGGGTGTG-------- AGAGCACGACGTCAAGATCTGGA
Dsim  TAG|CAACAGCCGCACCATCAAGGGTGTG-------- AGAGCACGACGTCAAGATCTGGA
Dsec  TAG|CAACAGCCGCACCATCAAGGGTGTG-------- AGAGCACGACGTCAAGATCTGGA
Dyak  CAG|CAACAGCCGCACCATCAAGGGAGTG-------- GGAGCACGATGTCAAGATCTGGA
Dere  CAG|CAACAGCCGCACCATCAAGGGAGTG-------- TGAGCACGACGTCAAGATCTGGA
Dbia  CAG|CAATAGCCGCACCATCAAGGGAGTG-------- GGAGCACGACGTTAAGATCTGGA
Dsub  CAG|CAATAGCCGCACCATCAAGGGAGTG-------- GGAGCACGACGTTAAAATCTGGA
Dtak  TAG|CAATAGCCGCACCATCAAGGGAGTG-------- GGAGCATGACGTCAAGATCTGGA
Dana  TAG|TAACAGCCGCACCATCAAGGGTGTG-------- GGAACACGACGTTAAAATTTGGG
Dwil  CAG|GAATAGTCGTACCATCAAGGGTGTT-------- GGAGCATGACGTTAAATTATGGG
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Figure 1. Alignments for the gene regions in which the conserved and diverged primer sets are located. (A) Alignment of *D. suzukii sec61alpha* (DS10_00001395; annotation of SpottedWingFlybase (Chiu *et al.*, 2013)) to orthologs from 14 other *Drosophila* species spanning multiple groups in the subgenus *Sophophora* (*D. melanogaster* (FBgn0086357), *D. simulans* (FBgn0193973), *D. sechellia* (FBgn0172841), *D. yakuba* (FBgn0235854), *D. erecta* (FBgn0115759), *D. ananassae* (FBgn0092730), *D. pseudoobscura* (FBgn0081850), *D. persimilis* (FBgn0163685), *D. willistoni* (FBgn0220690), *D. takahashii (*KB461656.1), *D. biarmipes (*KB462833.1)) and subgenus *Drosophila* (*D. virillis* (FBgn0205065), *D. mojavensis* (FBgn0140032), *D. grimshawi* (FBgn0120726)). Only the gene sequences corresponding to the location of the forward and reverse primers were shown, and sequence polymorphisms as compared to *D. suzukii sec61alpha* were indicated in grey. *D. suzukii* primer sequences are shaded in grey. (B) Alignment of *D. suzukii* DS10_00004458 (annotation in SpottedWingFlybase (Chiu *et al.*, 2013)) to orthologs from 10 other *Drosophila* species: *D. melanogaster* (FBgn0035268), *D. simulans* (FBgn0185353), *D. sechellia* (FBgn0169366), *D.ananassae* (FBgn0101826), *D. yakuba* (FBgn0238459), *D. erecta* (FBgn0107076), *D. takahashii (*KB461143.1), *D. biarmipes (*KB462838.1), *D. willistoni* (FBgn0218640), and *D. subpulchrella* (KM208658). *Drosophila* species including *D. grimshawi*, *D. virillis*, *D. mojavensis*, and *D. persimilis* have a larger and more diverged intron and were excluded from this figure. 66-bp are shown upstream of the forward primer and 52-bp are shown downstream to anchor the alignment and indicate the polymorphic nature of the intronic region used for the forward primer. Vertical lines mark exon-intron boundaries. The 8 dashes immediately before the reverse primer sequence indicate the portion of the sequence that is not shown in the alignment. Grey color highlights polymorphic base pairs or deletions in the other *Drosophila* sequences as compared to *D. suzukii* primer sequences, which are shaded in grey.

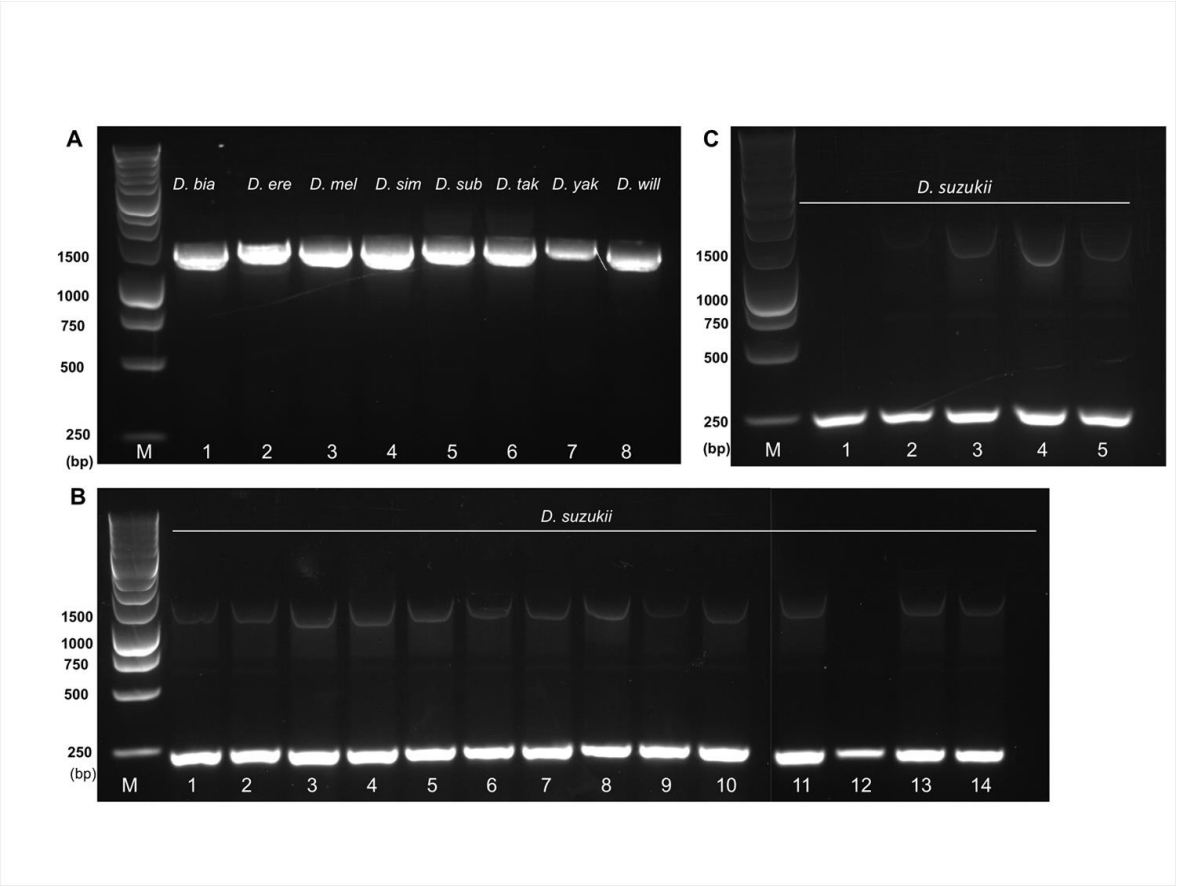

Figure 2: **DNA agarose gel electrophoresis showing results of multiplex PCR.** Multiplex PCR was performed using (A) multiple *Drosophila* species (see Table 1 for strain information) including *D. suzukii* (genome strain WT3 F10) and (B) *D. suzukii* collected from different regions in the Western United States (Lanes 1-14: genome strain WT3 F10, lime, grape, Mark Bolda WAT, Wolfskill IFL WO-2 [from CA], HR3 F4, TD3 F5, PD3 F5, ARS, OS1 [from OR], colony #8, colony #9, colony #10, and colony #11 [from WA]). PCR amplification for (A) and (B) was performed using crude larval extract isolated from a single larva that was freshly sacrificed. All crude DNA extractions and corresponding PCR reactions for (A) and (B) were repeated at least five times using biological replicate samples. Representative results are shown here. (C) Multiplex PCR was performed using crude extract from *D. suzukii* specimens collected from regions outside of the western United States. Adult flies were collected in South Korea and Italy (Table 1) and preserved in RNA*later* for storage (Lane 1: South Korea, Lane 2: Italy). Freshly sacrificed larva from strains collected in Japan, Hawaii, and Maryland, U.S.A. (Lane 1: MTY3, Japan, Lane 2: Oahu, Hawaii, Lane 5: Maryland).

Table 1. Species and strains used for testing the *Drosophila suzukii* molecular diagnostic.

Species	**Strain**	**Location of collection**	**Collector/Source**
D. suzukii	colony #8	Bray's Landing, WA, USA N47.738425 W120.167644	Beers lab, WSU
D. suzukii	colony #9	Daroga Park, WA, USA N47.705933 W120.19128	Beers lab, WSU
D. suzukii	colony #10	Royal City, WA, USA N46.837533 W119.5099	Beers lab, WSU
D. suzukii	colony #11	Rock Island, WA, USA N47.2350 W120.0727	Beers lab, WSU
D. suzukii	lime	Davis, CA, USA N38.55 W121.78	Begun Lab, UCD
D. suzukii	grape	Davis, CA, USA N38.55 W121.78	Begun Lab, UCD
D. suzukii	genome strain, WT3 F10	Watsonville, CA, USA N36.94 W121.76	Begun Lab, UCD
D. suzukii	Mark Bolda, WAT	Watsonville, CA, USA N36.94 W121.76	Zalom Lab, UCD
D. suzukii	Wolfskill IFL WO-2	Winters, CA, USA N38.49 W121.98	Begun Lab, UCD
D. suzukii	HR3 F4	Hood River, OR, USA N45.410860 W121.321011	Shearer Lab, OSU
D. suzukii	TD3 F5	The Dalles, OR, USA N45.351738 W121.131167	Shearer Lab, OSU
D. suzukii	PD3 F5	Parkdale, OR, USA N45.310333 W121.351362	Shearer Lab, OSU
D. suzukii	ARS	Corvallis, OR, USA N45.010035 W122.564377	Shearer Lab, OSU
D. suzukii	OS1	Corvallis, OR, USA N45.010035 W122.564377	Shearer Lab, OSU
D. suzukii	wild population	South Korea	Betsy Miller, OSU
D. suzukii	wild population	Scurelle, Trentino, Italy	Claudio Loriatti, FEM-IASMA
D. suzukii	MTY	Ehime, Japan	Kopp Lab, UCD
D. suzukii	wild population	Oahu, Hawaii	Kopp Lab, UCD
D. suzukii	wild population	MD, USA	Hamby Lab, UM
D. biarmipes	genome strain, 361.0-isol e-11	Ari Ksatr, Cambodia	Kopp Lab, UCD
D. erecta	14021-0224.01	Tucson Stock Center	Begun Lab, UCD
D. melanogaster	Oregon R	Roseburg, Oregon	Zalom Lab, UCD
D. subpulchrella	NGN5	Nagano, Japan	Begun Lab, UCD
D. simulans	W501	Genome strain	Begun Lab, UCD
D takahashi	genome strain, 311.5-iso4	Yun Shui, Taiwan	Kopp Lab, UCD
D. willistoni	14030-0814-10	Guadeloupe Island, France	Begun Lab, UCD
D. yakuba	CY28	Cameroon, Africa	Begun Lab, UCD

EXECUTIVE SUMMARY:

We have designed an accurate PCR diagnostic that can unambiguously differentiate *D. suzukii* from other common *Drosophila* species using the crude homogenate of a single larva as the DNA source without the need for additional steps such as genomic DNA extraction, sequencing, or restriction digestion. The use of the recently sequenced *D. suzukii* genome (Chiu et al. 2013) and a comparative genomic approach facilitated the discovery of the diagnostic marker we presented here. In order to increase the utility of this PCR diagnostic, we are actively working with researchers (e.g. in USDA), growers, and other interested parties to optimize this diagnostic for use with SWD samples of various conditions, e.g. flies trapped and soaked in apple cider vinegar, and larvae embedded in fruit samples.