Fruit Fly ID Australia

Fruit fly molecular diagnostics

REAL-TIME PCR HIGH RESOLUTION MELTING (HRM) ASSAY FOR THE DETECTION OF THE SPOTTED WING DROSOPHILA, DROSOPHILA SUZUKII (DIPTERA: DROSOPHILIDAE)

INTRODUCTION

D. suzukii is an economically pest because females are able to lay eggs in healthy, unwounded fruits (Walsh et al. 2011), with a wide host range (Lee et al. 2011, Kacsoh and Schlenke 2012). Estimates of the economic burden associated with this pest due to crop loss and control efforts are dramatic in both Europe and North America (Bolda et al. 2010, Goodhue et al. 2011, Cini et al. 2012), therefore it is important to have effective tools to detect and managing this invasive pest.

Several countries where *D. suzukii* is not present, such as New Zealand and Australia, impose strict border controls to reduce the risk of invasion (Berry 2012, DAFF 2013). A large proportion of suspected drosophilids detected in imported produce or discovered in orchard surveys are in the larval stages, as their feeding leads to observable damage to the produce (Berry 2012). Unfortunately, like for most insects, it is impossible to identify the larvae, pupae and eggs accurately, unless they are reared to adults. Therefore, it is necessary to develop a molecular-based assay to accurately identify the species in the early development stages.

DNA barcoding based on the cytochrome oxidase subunit I (COI) gene has been developed for *D. suzukii* and is currently used for its accurate identification (Hauser 2011). A number of COI sequences of *D. suzukii* are available in GenBank (Benson et al. 2005) and BOLD (Ratnasingham and Hebert 2007) databases. COI DNA sequences could therefore be used to develop a highly specific and sensitive real-time PCR assay to differentiate this species from other closely related species of this subgroup. We selected the DNA-binding SYBR dye-based chemistry which is simple (Maeda et al. 2003), and requires non-fluorescent oligos that reduce the cost. This real-time PCR approach is coupled with High Resolution Melt (HRM) analysis, which can be used to differentiate between the target and the non-target DNA (Reed et al. 2007).

AIM

This assay aims to provide a rapid method for the identification of the *Drosophila suzukii*, from any life stage, using the real-time PCR HRM method.

TARGET

Drosophila suzukii

PROCEDURE

DNA extraction protocols

See DNA extraction page on Fruit Fly Identification website.

Real-time PCR assay for *Drosophila suzukii*

All the real-time PCR setup and analysis are the same as for *B. tryoni* complex (see Real time PCR page of the Fruit Fly Identification Australia website) except the following reagents and steps listed below.

Equipment and material

- Primers (Dhami and Kumarasinghe 2014)
    - Forward primer: Dsuz1F, 5'- AATTGTTACCGCACATGC -3'
    - Reverse primer: Dsuz6R, 5'- GGAATGCTATATCTGGGTCC -3'
- Real-time PCR mastermix
    - SsoFast™ EvaGreen® Supermix (Cat#: 172-5200 or 172-520, BioRad), 2x qPCR mix, containing dNTPs, Eva Green Dye, Sso7d-fusion polymerase, $MgCl_2$ and stabilisers.

Note: * other SYBR Master Mix can be used, suggest validating the assay before applying in diagnostics.

- Positive control to monitor the performance of the real-time PCR
    - DNA samples of *Drosophila suzukii*
    - Plasmid DNA of COI insert of *Drosophila suzukii* (available from MPI PHEL, NZ on request)

Method

Real-time PCR compositions

Table: Real-time HRM PCR for *D. suzukii* protocol using SsoFast EvaGreen Supermix (BioRad)

Reagents	1 x reaction (µl)[a]	10x reactions (µl) [b]
Sterile distilled H_2O	4.8	48
2 x EvaGreen Supermix	10.0	100
5 µM Dsuz1F (300 nM)	1.2	12
5 µM Dsuz6R (300 nM)	1.2	12
BSA (10mg/ml)	0.8	16
DNA template	2.0	

Real-time PCR cycling parameters

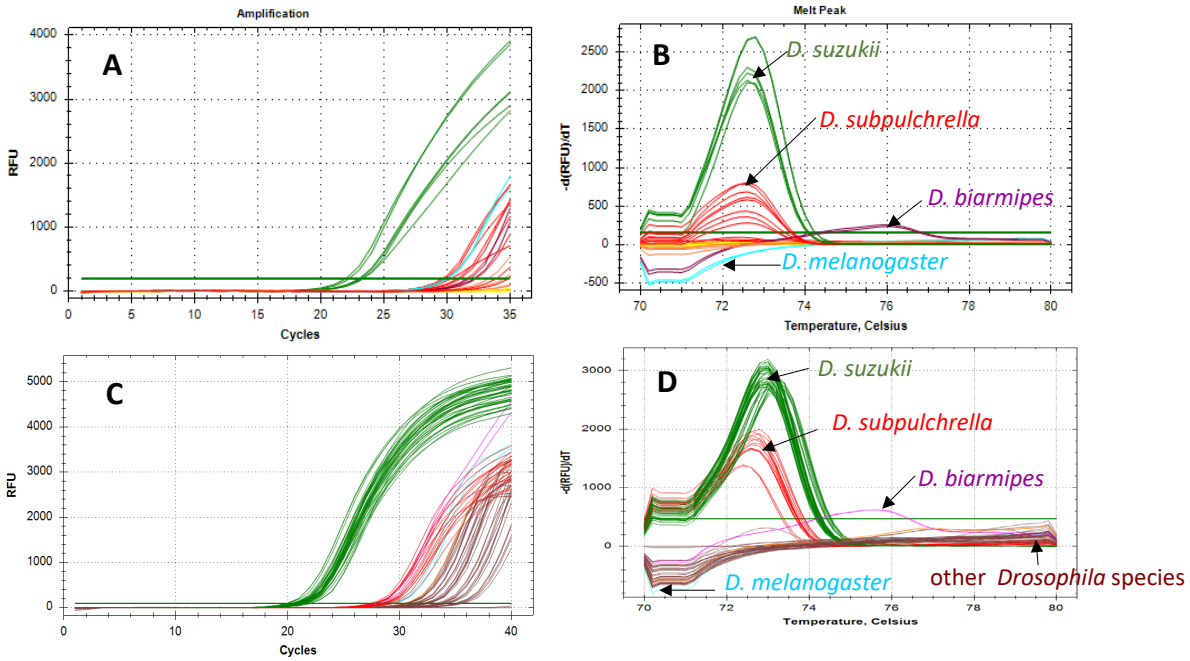
1 x cycle	95 °C, 2 min
35 x cycles	95 °C, 15 sec
	61 °C, 40 sec
	72 °C, 20 sec
	Plate read after each cycle
1x cycle	95 °C, 10 sec
1x cycle	70 °C, 10 sec
Melting	70°C – 80°C, ramp speed: 0.2°C for 2 sec
	Plate read

ANALYSIS OF REAL-TIME PCR HRM RESULTS

Since SYBR binds to any dsDNA, besides the target product, non-specific products and primer dimers can be detected. Therefore, the melting curve analysis is needed to determine whether the PCR products is the positive amplification, the non-specific products or the primer dimers.

1. Open BioRad CFX Manager 3.0
2. Click file, open the data file, and choose the real-time PCR data you have saved for the assay
3. Click on quantification tab, the amplification curve will appear on the screen, see Figure 16A for an example
4. Click on the melting curve tab, the melting peaks will appear on the screen, see Figure 16B for an example
5. Interpretation of the results – *D. suzukii* assay: The positive or negative of the samples need to be decided by the melting peak and the *Cq* values.
    a. If the melting peak is in the range of 72.60-73.40°C with *Cq* <25 cycles, the sample is positive for *D. suzukii*
    b. If the melting peak is 72.4-72.60°C with Cq > 30 cycles, the sample might be *D. subpulchrella*, further tests are needed to confirm its identity
        I. Run the real-time PCR assay for 40 cycles with the unknown samples, and several DNA samples from *D. suzukii* and *D. subpulchrella* to conduct precision melt analysis after the run
        II. DNA barcoding needs to be conducted to confirm the species
    a. If the melting peak is >73.40°C, the samples should be considered as negative for *D. suzukii,* and identification by another method is necessary
6. The real-time PCR run at 40 cycles gave better melting peaks for *D. subpulchrella* and *B. biarmipes* samples. Using the melting peaks (figures C and D below), we can clearly separate *D. biarmipes* from *D. subpulchrella* and *D. suzukii*. It showed that the melting peak for *D. biarmipes* is around 75.20°C, however, no further validation for *D. biarmipes* have been conducted, therefore it is not suggested to use this assay to identify *D. biarmipes*.
7. Late amplification for a number of *Drosophila* species were also observed (figure A below), but there were no melting peaks formed (figure D below). The *Drosophila* species tested, including: *D. buskii, D. eugracilis, D. hydei, D. lucipennis, D. lutescens, D. melanogaster, D. mimetica, D. pseudoobscura, D. replete, D. takahashii,* and *D. simulans.*

Figure: Real-time PCR assays for *D. suzukii* were run for 35 (A and B) and 40 (C and D) cycles, respectively. Amplification curves (A and C) and melting peaks (B and D) for various *Drosophila* species samples tested.



Green:	*D. suzukii,* target species
Red:	*D. subpulchrella, the* closely related species
Purple:	*D. biarmipes*, non-target species
Blue:	*D. melanogaster*, non-target species
Brown:	*D. takahashii, non-target species*
Yellow:	other species tested

References: see References page on the Fruit Fly Identification Australia website