Development of a Molecular Test (Diagnostic PCR) for the Identification of Lepidopteran pests in Almonds and Pistachios

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Introduction

The California almond and pistachio industries have a combined value of >$6.6 billion, and comprise an astounding 1.4 million acres of California crop land (CDFA, 2016). As acreage has increased insect pests of these crops have become increasingly problematic. A variety of lepidopterous pests in particular have been a consistant source of yield loss, with some pests damaging >20% of harvestable kernels if mismanaged. Key to making correct management decisions is the rapid and accurate identification of these moths at all lifestages. Common moths and later lifestages are often easily identified. However, each year orchard managers and scouts find less common moth pests attacking nut crops, including moth larvae in fresh or old nuts such as oriental fruit moth (*Grapholita molesta*), carob moth (*Ectomyelois ceratoniae*), filbert worm (*Cydia latiferreana*), Indian meal moth (*Plodia interpunctella*), and raisin moth (*Cadra figulilella*).

While these 'other' moth species are typically a non-issue for farm managers, outbreak populations can be found, and require quick identification to make proper management decisions. However, what is collected is typically an adult in a pheromone trap with few 'scales' remaining on the wings, which makes sight identification difficult. Further, early instar moth larvae are difficult to identify as they are nearly identical morphologically, and are often smashed in the nut or on a leaf during collection. Consequently, accurate identification requires rearing these immature larvae to a later larval instar or even adult stage for identification. Here we describe work being done by a graduate student in our lab developing molecular tools to identify both the common and less common moth pests in pistachio and almonds. These tools include a traditional diagnostic PCR as well as the newer approache of qPCR meltcurve analysis.

Methods

Diagnostic PCR

PCR, or polymerase chain reaction, is a technique used to amplify a single copy of a DNA sequence by several orders of magnitude, producing millions or billions of copies. In order to amplify a specific region of the organism's genome, researchers must design DNA primers (short strands of DNA artificially produced in a laboratory) that match the DNA sequences flanking the target region. Consequently, a PCR can be a diagnostic tool if the primers are designed to only match the DNA sequence of a single species of interest. Diagnostic PCR is a common technique used to identify morphologically similar insect species in both the public health and agriculture fields (Phuc *et al*, 2003; Daane *et al*, 2011)

Here we designed species specific primers targeting the Folmer region of the barcode gene Cytochrome Oxidase-1 (CoI) (Jalali, Ojha, Venkatesan, 2015) for six direct pests of tree nuts: navel orangeworm, peach twig borer, oriental fruit moth, filbert worm, indian meal moth, and raisin moth. Primers were designed with two traits, first they were build to be species specific, only undergoing a successful PCR in the presence of template DNA from a single moth species. Second, primers were also designed to produce different sized amplicons for each species, allowing us to rapidly on the species present, and were combined into two separate multiplexes.

qPCR Melt Curve Analysis

Quantitative PCR (qPCR) uses flouresent dyes and sensors to actively measure the amount of double stranded DNA produced during a PCR reaction. Inversely it can be used to measure how quickly double stranded DNA dissociates when exposed to high temperatures. Given that the rate of DNA dissociation is directly related to the amplicon's sequence, the rate and shape of melt curves produced on a qPCR machine can be diagnostic to the species level. Indeed melt curve analyses have been used to rapidly identify insects to species rapidly and without the need of species specific primers, albeit at a higher cost than traditional PCR (Winder *et al* 2011, Ajamma *et al* 2016).

Using this approach, we are currently working to develop diagnostic melt curves for each of the six moth species that we developed the diagnostic PCR test for. Much like the diagnostic PCR this test amplifies the Folmer region of the CoI gene, however in this case the entire gene was amplified using the universal insect primers LepF and LepR. After amplification the PCR product was subjected to a slow denaturation, increasing the sample temperature stepwise 0.2°C with a 20 second hold. For each species melt curve profiles were analyzed using the POLAND algorithm.

Results & Discussion

After two rounds of primer design, the diagnostic PCR has proven to be successful at differentiating the six almond/pistachio pest species we targetted (Figure 1, Table 1). Perhaps the most useful feature of this test was that it produces differently sized species specific DNA bands. This allows for rapid identification of the pest just by visual inspection of the agarose gel. The qPCR based approach continues to be developed, with no updates at the time of this writing.

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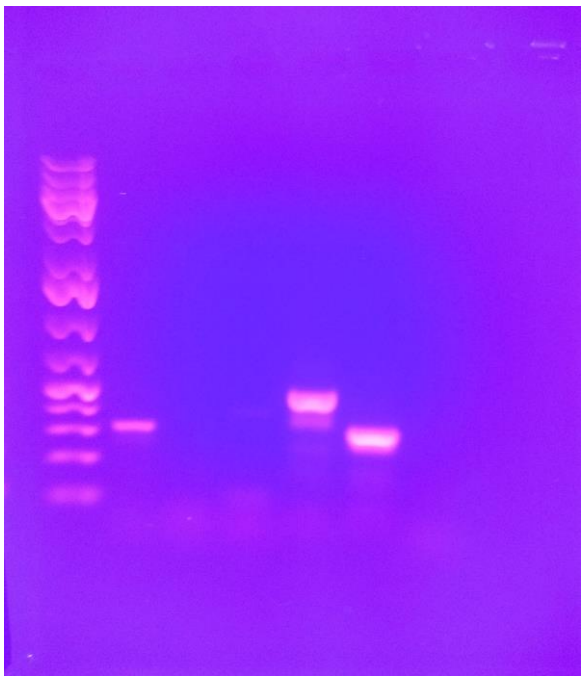

Figure 1: Image of diagnostic PCR. Wells 1) Ladder, 2) NOW, 3) OFM, 4) OBLR, 6) Indianmeal Moth

Table 1: Moth species included in diagnostic PCR with associated species amplicon size.

Moth Species	Amplicon Size (bp)
Filbertworm	56-76
Raisin Moth	202-222
Navel Orangeworm	263-283
Oblique Banded Leafroller	319-342
Indianmeal Moth	421-441
Oriental Fruit Moth	517-541

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