A Multiplex Real-Time Polymerase Chain Reaction Assay to Diagnose *Epiphyas postvittana* (Lepidoptera: Tortricidae)

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ABSTRACT A molecular assay for diagnosis of light brown apple moth, *Epiphyas postvittana* (Walker) (Lepidoptera: Tortricidae), in North America is reported. The assay multiplexes two TaqMan real-time polymerase chain reaction (RT-PCR) probe systems that are designed to target DNA segments of the internal transcribed spacer region 2 (ITS2) and 18S rRNA gene. The RT-PCR probe designed for the 18S target recognizes a DNA sequence conserved in all of the moths included in the study and functions as a control in the assay. The second probe recognizes a segment of the ITS2 specifically found in *E. postvittana* and not found in the other moths included in the study, i.e., this segment is not conserved. Inclusion of the two markers in a single multiplex reaction did not affect assay performance. The assay was tested against 637 moths representing >90 taxa in 15 tribes in all three subfamilies in the Tortricidae. The assay generated no false negatives based on analysis of 355 *E. postvittana* collected from California, Hawaii, England, New Zealand, and Australia. Analysis of a data set including 282 moths representing 41 genera generated no false positives. Only three inconclusive results were generated from the 637 samples. Spike experiments demonstrated that DNA contamination in the assay can affect samples differently. Contaminated samples analyzed with the ITS2 RT-PCR assay and DNA barcode methodology by using the *cytochrome oxidase* I gene can generate contradictory diagnoses.

KEY WORDS Tortricidae, barcodes, internal transcribed spacer, real-time polymerase chain reaction

The light brown apple moth, *Epiphyas postvittana* (Walker) (Lepidoptera: Tortricidae), is an invasive species that has recently been discovered in California (Brown et al. 2010). It is an important pest of fruit crops in Australia and New Zealand and is highly polyphagous, having been recorded feeding on >500 species of plants (Brown et al. 2010; Suckling and Brockerhoff 2010). The presence of this pest can have a negative economic impact on agriculture. For example, in addition to direct damage caused by larvae feeding on fruit and foliage, there are costs associated with the identification of suspect moths in all life stages, the quarantine and treatment of crops and nursery stock in infested areas, and the implementation of trade restrictions for infested areas leading to potential market loss (Venette et al. 2003, Barr et al. 2009, Suckling and Brockerhoff 2010).

Accurate identification of *E. postvittana* samples in California is not a trivial task. *E. postvittana* is one of over 40 described species in the Australian genus *Epiphyas* Turner (Baixeras et al. 2010). Although no other *Epiphyas* species have been detected in California (or North America), individuals of *E. postvittana* seem morphologically similar to many native North American tortricids (Gilligan and Epstein 2009). Adult *E. postvittana* can be positively identified by a combination of morphological characters, whereas all other life stages require DNA analysis for confirmation (Gilligan and Epstein 2009; Barr et al. 2009). Within California, it is important to quickly diagnose all *E. postvittana* life stages associated with nursery stock to determine whether costly quarantine or treatment is required (Barr et al. 2009). In these situations, DNA barcoding has been successfully implemented by the California Department of Food and Agriculture (CDFA) to identify immature *E. postvittana;* however, the time required to process DNA barcodes is often lengthy, with a turnaround time of several days from sample acquisition to DNA sequence. To address this problem, a conventional polymerase chain reaction (PCR) assay of the internal transcribed spacer region 2 (ITS2) locus was developed to reduce the diagnosis time for suspect moths (Barr et al. 2009). The ITS2 locus was determined to provide reliable identifications of *E. postvittana* in all

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Table 1. *E. postvittana* samples

Collection location	*N*	Source
USA: California	275	Barr et al. (2009)
USA: California: Los Angeles Co.	10	This study
USA: California: Sonoma Co.	4	This study
USA: California: Napa Co.	1	This study
USA: California: Yolo Co.	1	This study
USA: California: San Benito Co.	1	This study
USA: California: Santa Cruz Co.	1	This study
USA: Hawaii	8	Barr et al. (2009)
England	14	Barr et al. (2009)
New Zealand	19	Barr et al. (2009)
Australia	21	Barr et al. (2009)

life stages, and the ITS2 PCR assay required less labor, cost, and time than the DNA barcode assay. However, the conventional PCR method still required an electrophoresis step to visualize the PCR products.

Real-time (RT)-PCR is an important technology for the detection and diagnosis of biological organisms. Although RT-PCR has been more widely used in agriculture for plant pathogen detection (López et al. 2006, Nakhla et al. 2010), it also has been applied to taxonomic diagnosis of invertebrates (Yu et al. 2004, Barcenas et al. 2005, Walsh et al. 2005; Yu et al. 2005, Jones et al. 2006). The benefits of using RT-PCR versus conventional PCR include 1) reduced assay time, 2) elimination of post-PCR electrophoresis (and the concomitant reduction of amplified products exposure to the testing laboratory), 3) potential for scale up for high throughput testing, and 4) increased sensitivity and specificity when a quenched dye system (such as TaqMan) is incorporated (Logan and Edwards 2004).

Here, we develop a multiplex RT-PCR assay for *E. postvittana* diagnosis in North America (including California) that targets the ITS2 locus as a diagnostic marker and includes the 18S rRNA locus as an internal control. We report on the sensitivity and specificity of the assay and provide rules for interpreting assay results.

Materials and Methods

Collections of Light Brown Apple Moth. Three hundred and fifty-five light brown apple moth specimens were included in the study. These represent 337 trapped adult moths from California, Australia, New Zealand, Hawaii, and England previously analyzed by Barr et al. (2009), and another 18 adult moths from additional counties in California (Table 1). Barr et al. (2009) describe the collection information for the initial 337 specimens. The samples from New Zealand and Australia were provided as DNA isolates from the laboratory of R. Newcomb (Tooman et al. 2011).

Collections of Nontarget Tortricids. Two hundred and eighty-two specimens in the family Tortricidae representing > 90 taxa, 15 tribes, and all three subfamilies were included in the study (Table 2). The list of nontargets (i.e., not *E. postvittana*) includes taxa used in the Barr et al. (2009) study that represent North American genera containing species that are morphologically similar to *E. postvittana: Acleris* Hübner, *Archips* Hübner, *Argyrotaenia* Stephens, *Choristoneura* Lederer, *Clepsis* Guenée, and *Platynota* Clemens.

The moth included in the Barr et al. (2009) study as "tortricid species X" has since been tentatively diagnosed as *Clepsis fucana* (Walsingham) by using DNA barcodes of the *cytochrome oxidase* subunit I gene (JF505288). Additional DNA sequences from ITS2 (JF495725-JF495752) and *elongation factor* 1-*alpha* (JF495761–JF495766) support this identification. The abdomen of the specimen is saved as a voucher in the collection of CDFA (M.E.E.).

The tested specimens include 62 taxa collected from the United States and four species of *Epiphyas* from Australia. Although the number of specimens per taxon varied, the sample sizes of several species commonly found in California in conjunction with populations of *E. postvittana* were greater than four: *Archips argyrospila* (Walker) ($N = 8$), *Argyrotaenia franciscana* (Walsingham) ($N = 45$), *Choristoneura rosaceana* (Harris) ($N = 11$), *C. fucana* ($N = 17$), *Clepsis peritana* (Clemens) ($N = 22$), *Clepsis periscana* (Fitch) ($N = 5$), and *Platynota idaeusalis* (Walker) ($N = 5$).

Extraction of DNA. Extractions were performed using either a leg, thoracic tissue plus legs, or the entire moth. A series voucher was saved for each collection, a specimen voucher was maintained using the nonextracted insect tissues, or both. Vouchers are stored in the CDFA California State Collection of Arthropods insect collection.

DNA was isolated from most samples using the animal tissue protocol of the DNeasy Blood and Tissue kit (QIAGEN, Valencia, CA). Samples extracted at the USDA laboratory in Texas were incubated overnight at 56°C and eluted in 100 μl of AE buffer (QIAGEN). Control of possible contamination at the DNA isolation step was accomplished by strict sanitation of materials and reagents that came in contact with the individual specimens, isolation and maintenance of specimen integrity throughout all extraction steps, and use of filter tips for all handling of liquid extracts and reagents. In addition, a no tissue extraction control was included with each extraction batch whenever possible.

PCR, Cloning, and Sequencing of DNA. All conventional PCR experiments were performed on GeneAmp 9700 thermal cyclers (aluminum plate, Applied Biosystems, Foster City, CA) using Ex *Taq* polymerase (Takara Mirus Bio, Kyoto, Japan) in total volumes of 25 μl. The reactions were performed using the manufacturer's recommended buffer (1×), primers at 0.2–0.4 μM concentrations, 0.75 U of *Taq* polymerase, and 1 μl of DNA extract (at unknown concentration). The controls included a blank DNA extraction sample (negative control of DNA isolation and PCR steps) and water (negative control of PCR step).

All primers were synthesized as salt-free oligos by Operon Biotechnologies (Huntsville, AL). Amplification of the ITS2 locus was performed using the ITSF and ITSR primer pair and the 18S locus was performed using the NS3-F and NS4-R primer pair (Table 3). For

Table 2. Various taxa included as representative moths for testing

Taxon	Present			*N*
	North America	USA	California	
Tortricinae: Phricanthini				1
Phricanthes asperana Meyrick	No	No	No	1
Scolioplecta comptana (Walker)	No	No	No	1
Tortricinae: Tortricini				
Acleris albicomana (Clemens)	Yes	Yes	No	2
Acleris cervinana (Fernald)	Yes	Yes	Yes	1
Acleris chalybeana (Fernald)	Yes	Yes	No	1
Acleris comariana (Lienig & Zeller)	Yes	Yes	No	1
Acleris hastiana (L.)	Yes	Yes	Yes	1
Acleris maculidorsana (Clemens)	Yes	No	No	2
Tortrix viridana L.	No	No	No	2
Tortricinae: Schoenotenini				
Diactenis tryphera Common	No	No	No	1
Tortricinae: Cochylini				
Eupoecilia ambiguella (Hubner)	No	No	No	1
Henricus umbrabasana (Kearfott)	Yes	Yes	Yes	4
Tortricinae: Cnephasiini				
Cnephasia longana (Haworth)	Yes	Yes	Yes	3
Decodes basiplagana (Walsingham)	Yes	Yes	Yes	1
Decodes fragariana (Busck)	Yes	Yes	Yes	2
Tortricinae: Epitymbiini				
Epitymbia eudrosa (Turner)	No	No	No	1
Tortricinae: Archipini				
Acropolitis sp.	No	No	No	1
Adoxophyes orana (Fischer Von Roslerstamm)	No	No	No	2
Adoxophyes sp. (Australia)	No	No	No	1
Archips argyrospila (Walker)	Yes	Yes	Yes	8
Archips cerasivorana (Fitch)	Yes	Yes	Yes	1
Archips georgiana (Walker)	Yes	Yes	No	2
Archips grisea (Robinson)	Yes	Yes	No	2
Archips infumatana (Zeller)	Yes	Yes	No	2
Archips podana (Scopoli)	Yes	Yes	No	3
Archips purpurana (Clemens)	Yes	Yes	No	1
Archips rileyana (Grote)	Yes	Yes	No	2
Archips semiferana (Walker)	Yes	Yes	No	2
Archips xylosteana (L.)	Yes	No	No	2
Argyrotaenia alisellana (Robinson)	Yes	Yes	No	1
Argyrotaenia floridana Obraztsov	Yes	Yes	No	2
Argyrotaenia franciscana (Walsingham)	Yes	Yes	Yes	45
Argyrotaenia juglandana (Fernald)	Yes	Yes	No	2
Argyrotaenia mariana (Fernald)	Yes	Yes	No	2
Argyrotaenia pinatubana (Kearfott)	Yes	Yes	No	1
Argyrotaenia quercifoliana (Fitch)	Yes	Yes	No	4
Argyrotaenia velutinana (Walker)	Yes	Yes	No	4
Choristoneura fractivittana (Clemens)	Yes	Yes	No	2
Choristoneura fumiferana (Clemens)	Yes	Yes	No	2
Chroistoneura retiniana (Walsingham)	Yes	Yes	Yes	5
Choristoneura rosaceana (Harris)	Yes	Yes	Yes	11
Clepsis clemensiana (Fernald)	Yes	Yes	Yes	1
Clepsis fucana (Walsingham)	Yes	Yes	Yes	17
Clepsis melaleucana (Walker)	Yes	Yes	No	2
Clepsis peritana (Clemens)	Yes	Yes	Yes	22
Clepsis persicana (Fitch)	Yes	Yes	Yes	5
Clepsis virescana (Clemens)	Yes	Yes	Yes	2
Diedra intermontana Rubinoff & Powell	Yes	Yes	Yes	3
Epiphyas ashworthana (Newman)	No	No	No	2
Epiphyas caryotis (Meyrick)	No	No	No	2
Epiphyas fabricata (Meyrick)	No	No	No	2
Epiphyas postvittana (Walker)	Yes	Yes	Yes	355
Epiphyas xylodes (Meyrick)	No	No	No	2
Homona mermerodes Meyrick	No	No	No	1
Homona spargotis Meyrick	No	No	No	1
Isotenes miserana (Walker)	No	No	No	1
Neocalyptis molesta (Meyrick)	No	No	No	1
Pandemis cerasana (Hubner)	Yes	No	No	3
Pandemis heparana [Denis & Schiffermller]	Yes	No	No	2
Pandemis lamprosana (Robinson)	Yes	Yes	No	2
Pandemis limitata (Robinson)	Yes	Yes	No	4

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Table 2. Continued

Taxon	Present			*N*
	North America	USA	California	
Pandemis pyrusana Kearfott	Yes	Yes	Yes	5
Planostocha cumulata (Meyrick)	No	No	No	1
"Cacoecia" mnemosynana Meyrick	No	No	No	1
Syndemis afflictana (Walker)	Yes	Yes	Yes	1
"Technitis" sp.	No	No	No	1
Tortricinae: Sparganothini				
Amorbia cuneana (Walsingham)	Yes	Yes	Yes	1
Amorbia humerosana Clemens	Yes	Yes	Yes	1
Platynota exasperatana (Zeller)	Yes	Yes	No	2
Platynota flavedana Clemens	Yes	Yes	No	5
Platynota idaeusalis (Walker)	Yes	Yes	No	5
Platynota nigrocervina Walsingham	Yes	Yes	No	1
Platynota rostrana (Walker)	Yes	Yes	No	2
Platynota stultana Walsingham	Yes	Yes	Yes	4
Sparganothis diluticostana (Walsingham)	Yes	Yes	No	2
Sparganothis directana (Walker)	Yes	Yes	Yes	2
Sparganothis lamberti Franclemont	Yes	Yes	No	1
Sparganothis niveana (Walsingham)	Yes	Yes	No	1
Sparganothis pettitana (Robinson)	Yes	Yes	No	1
Sparganothis reticulatana (Clemens)	Yes	Yes	No	2
Sparganothis saracana (Kearfott)	Yes	Yes	No	2
Sparganothis senecionana (Walsingham)	Yes	Yes	Yes	1
Sparganothis sulfureana (Clemens)	Yes	Yes	No	2
Sparganothis tunicana (Walsingham)	Yes	Yes	Yes	1
Sparganothoides lentiginosana (Walsingham)	Yes	Yes	No	2
Tortricinae: Euliini				
Eulia ministrana (L.)	Yes	Yes	No	3
Olethreutinae: Microcorsini				
Cryptaspasma bipenicilla Brown & Brown	Yes	Yes	No	1
Cryptaspasma lugubris (Felder & Rogenhofer)	Yes	No	No	1
Cryptaspasma n. sp.	Yes	No	No	7
Olethreutinae: Olethreutini				
Dactylioglypha sp.	No	No	No	1
Lobesia botrana [Denis & Schiffermller]	Yes	Yes	Yes	3
Metrioglypha sp.	No	No	No	1
Olethreutinae: Enarmoniini				
Enarmonia formosana (Scopoli)	Yes	Yes	No	1
Fibuloides sp.	No	No	No	1
Olethreutinae: Eucosmini				
Rhopobota sp. (Australia)	No	No	No	2
Olethreutinae: Grapholitini				
Cydia splendana (Hubner)	No	No	No	2
Grapholita funebrana (Treitschke)	No	No	No	1
Chlidanotinae: Chlidanotini				
Trymalitis sp.	No	No	No	1

Species are arranged by subfamily and tribe and ordered alphabetically by genus. Presence of each taxon within North America, United States, and California is indicated along with sample size (*N*) for each taxon included in the study.

most reactions, PCR conditions (for both loci) included an initial step of 94°C (3min), 30 cycles of 94°C (20 s)/60°C (20 s)/72°C (30 s), and an extension step of 72°C (5 min). The number of cycles was increased to 40 and the annealing time was increased to 30 s, for problematic reactions.

The TOPO TA cloning kit (Invitrogen, Carlsbad, CA) was used to clone amplicons of the ITS2 and 18S loci. Transformed colonies were grown and selected on Luria-Bertani-agar plates with kanamycin and ampicillin. Multiple transformed colonies were selected for each individual (i.e., cloned PCR product) to test for intra-individual variation. The plasmid DNA was purified using a miniprep kit (QIAGEN) and sequenced on an ABI 3730 sequencer using universal M13 forward and reverse primers at the Davis Sequencing facility (Davis Sequencing Inc., Davis, CA).

ITS2 sequences reported by Barr et al. (2009) for *E. postvittana* (EPOS 1.1; GQ281283) and *C. fucana* (CFUC 1.1; GQ281284; Tortricid sp. X) were downloaded from GenBank. Additional sequences of the ITS2 locus were generated from cloned PCR products of *Epiphyas ashworthana* (Newman) (three clones, EASH 1.1–1.3; JF495714–JF495716); *Epiphyas caryotis* (Meyrick) (three clones, ECAR 1.1–1.3; JF495711–JF495713); *Epiphyas fabricata* (Meyrick) (four clones, EFAB 1.1–1.4; JF495721–JF495724); *Epiphyas xylodes* (Meyrick) (four clones, EXYL 1.1–1.4; JF495717–JF495720); two *E. postvittana* specimens: one from California (same individual used for EPOS 1.1; three clones, EPOS 1.2–1.4; JF495704–JF495706) and one from Western Australia (four clones, EPOS 2.1–2.4; JF495707–JF495710); and *Clepsis fucana:* four from the "species X" individual (CFUC 1.2–1.5; JF495725–

Table 3. Primers and probes used in this study

Name	Locus (description)	Sequence	Tm (°C)	Source
NS3-F	18S	5-GCAAGTCTGGTGCCAGCAGCC	68.4	White et al. (1990)
NS4-R	18S	5-CTTCCGTCAATTCCTTTAAG	56.3	White et al. (1990)
RT-18S-F2	18S (primer 1 for RT)	5-ACCGCCCTAGTTCTAACCGTAAA	62.8	This study
RT-18S-R2	18S (primer 2 for RT)	5-CCGCCGAGCCATTGTAGTAA	62.4	This study
RT-18S-P2	18S (probe for RT)	5-CAL Fluor Red 610-TGTCATCTAGCGATCCGCCGA-BHQ-2	66.08	This study
ITSF	5.8-ITS2–28S	5-TTGAACATCGACATTTCGAACGCAC	62.9	Barr et al. (2009); Hillis and Dixon (1991)
ITSR	5.8-ITS2–28S	5-TCCTCCGCTTATTGATATGC	58.4	Barr et al. (2009); White et al. (1990)
RT-LBAM-ITS2-F5	ITS2 (primer 1 for RT)	5-TTGTCGTTGTCACATTGCTCTCT	61	This study
RT-LBAM-ITS2-R5	ITS2 (primer 2 for RT)	5-CCGCCACCAGCTCGTACT	64.5	This study
RT-BAM-TS2-P5	ITS2 (probe for RT)	5-FAM-CTCGCCCTCTCTCGCCGAGATAGG-BHQ-1	71.43	This study

JF495728) and eight from two additional specimens (CFUC 2.1–2.4 and 3.1–3.4; JF495729–JF495736).

The 18S locus was directly sequenced using PCR product from *E. postvittana* (JF495699-JF495701), *P. idaeusalis* (JF495697), and *Choristoneura retiniana* (Walshingham) (JF495698). Additional sequences of *E. postvittana* (JF495702–JF495703) were generated from cloned PCR product and used for dilution experiments. Sequences were edited using Sequncher 4.8 (Gene Codes, Ann Arbor, MI) and aligned using either the Megalign program in DNA Star (Madison, WI) or the alignment editor function in MEGA 4 (Kumar et al. 2008).

Design of TaqMan Probes and Primers. The ITS2 locus was selected as a possible diagnostic marker for *E. postvittana* based on previous work by Barr et al. (2009). The 18S locus was selected as a control to ensure that failure to amplify the ITS2 target was because the specimen was derived from a nontarget taxon. Failure to amplify the 18S control indicated that the DNA used in the analysis was either of insufficient quality or quantity for amplification or included inhibitory compounds. The 18S locus was selected as the control because it represents a conserved locus within the rDNA array that includes the ITS2 locus; therefore, the two loci should be present in equal copy numbers within each specimen (Hillis and Dixon 1991).

Primers and probes for the ITS2 and 18S loci were designed from an *E. postvittana* sequence by using Primer Express 2 (Applied Biosystems). The sequence GQ281283 was used as the RT-PCR template for the 5.8-ITS2–28S locus; and the sequence JF495699 was used for the 18S template. Primers and probes selected using Primer Express (for the ITS2 system) were then compared with locations in an ITS2 alignment (Barr et al. 2009) to select combinations that would maximize discrimination between *E. postvittana* and other species. Likewise, primers and probes selected using Primer Express for 18S were then compared with locations in an 18S alignment (this study) to select combinations that should recognize all tortricids.

These putative markers were then tested using the web-based Netprimer (Premier Biosoft, Palo Alto, CA) for secondary structure. The successful oligonucleotides were ordered as primers (Operon Biotechnologies) and tested using SYBR Premix Ex *Taq* (Perfect Real Time, 2×; Takara Mirus Bio) to confirm amplification. The TaqMan probes were synthesized by Biosearch Technologies (Novato, CA) and stored in 1× TE buffer (10 mM Tris-HC1, 1 mM EDTA-Na2, pH 8.0) at −20°C. The ITS2 probe was labeled with FAM and the 18S probe with CAL RED 610 (i.e., Texas Red). The ITS2 and 18S primers generate amplicons of 83 and 68 bp, respectively. Primer and probe information is reported in Table 3. All primers were stored as 10 μM working solutions.

RT-PCR Multiplex Protocol. All RT-PCR experiments were performed using Premix Ex *Taq* (Perfect Real Time) [2×] (Takara Mirus Bio) and a SmartCycler II instrument (Cepheid, Sunnyvale, CA). The ITS2 (FAM) and 18S (CAL RED) probe systems were optimized as a multiplex assay. Conditions were optimized on a subset of samples to ensure 1) that the two probe systems generated relatively similar cycle/crossing threshold (Ct) values (i.e., detection levels for the two targets were within three Cts of each other) and 2) that the 18S control probe (CAL RED) was less sensitive than the diagnostic ITS2 probe (to limit the probability of false negatives; Barr et al. 2009). The Ct measures how many PCR cycles are required to detect DNA template in the reaction. The Ct value should be higher when fewer copies of the target DNA are present in the PCR template. When the DNA target is not present in the template, the Ct value should be zero.

The optimized multiplex, RT-PCR assay was performed in a 25-μl reaction, by using 12.5 μl of Takara Premix Ex *Taq* [2×], 0.5 μl (0.2 μM) of each of the three ITS2 oligonucleotides (see primers and probe in Table 3), 1.25 μl (0.5 μM) of each of the two 18S primers (Table 1), 1 μl (0.4 μM) of the 18S probe (Table 1), 6.5 μl of sterile water (Sigma-Aldrich, St. Louis, MO), and 1 μl of DNA template or water (negative control). The real-time cycling parameters were as follows: 95°C (30 s) to denature the template followed by 45 cycles of 95°C (5 s)/60°C (15 s) with a threshold level set at 30.

The multiplex assay was tested on all DNA isolates, and the Ct values for the two probes were recorded.

The default settings in the SmartCycler were used for calculating the Ct values. Values of zero indicated amplification was not detected within the tested 45 cycles. The Ct values were rounded to two decimal places for data analysis.

Sensitivity Assays of RT-PCR Protocols. Additional tests were performed on serial dilutions of *E. postvittana* DNA by using plasmids with the ITS2 insert, plasmids with the 18S insert, and total nucleic acid extractions of adult individuals to determine the performance of the multiplex PCR at different template concentrations. All dilutions were performed in water. The concentrations of DNA samples were estimated using an ND-1000 spectrophotometer (NanoDrop, Wilmington, DE). For each sample, a PCR assay was performed that included 1 μl of template at the original sample concentration and then a 10-fold dilution series. The Ct values for each dilution were plotted against DNA concentration using a logarithmic scale. Sensitivity curves for the cloned inserts were plotted using the mean Ct value from three independent plasmids.

The serial dilution experiment was repeated using the insect DNA sample (i.e., the total nucleic extraction) and two modified RT-PCR protocols that were not multiplexed. In the first modified RT-PCR protocol, only the ITS2 probe system was tested by substituting the 18S reagents (primers and probe in the multiplex protocol) with water. The second RT-PCR protocol was designed to test the 18S probe by substituting water for the ITS2 reagents. The Ct values were plotted against DNA concentration and graphed against the multiplex Ct values to observe possible effects of multiplexing the two probe systems.

Spike Experiments. To test the effect of *E. postvittana* contamination on RT-PCR results of nontargets, DNA isolated from *A. franciscana* was tested in the multiplex RT-PCR assay after being spiked with 1 μl of *E. postvittana* DNA. The experiment was performed on samples including $\approx$1.5 ng of *A. franciscana* DNA as a genetic background and variable amounts of "spiked" *E. postvittana* DNA. In total, eight spiked samples were tested: 5 ng of *E. postvittana* DNA and a series of 10-fold dilutions ranging from 1 ng to 1×10^{-4} ng. Two additional samples including either *E. postvittana* DNA (5 ng) or *A. franciscana* DNA (1.5 ng) were included as controls. The experiment was repeated using *P. idaeusalis* DNA ($\approx$7.9 ng) as the genetic background.

The series of spiked samples tested in the RT-PCR assay also were analyzed using a DNA barcode of the *cytochrome oxidase* subunit I (COI) gene, to compare contamination effects on different molecular methods. The spiked samples were used as template in a PCR to amplify the COI barcode gene. The Folmer et al. (1994) primers [LCO-1490 (5′-GGTCAACAAATCATAAAGATATTGG) and HCO-2198 (5′-TAAACTTCAGGGTGACCAAAAAATCA)] were used to amplify the barcode region. The reactions were performed using Ex-*Taq* polymerase (Takara Mirus Bio) in 25-μl reaction volumes. The PCR thermal cycling conditions for the COI barcode, performed on an ABI 9700 thermal cycler, were 94°C (3 min) to denature the template followed by 39 cycles of 94°C (20 s)/50°C (20 s)/72°C (30 s) and an extension step at 72°C (5 min).

The PCR product was purified using QIAquick columns (QIAGEN) and sequenced at the Davis Sequencing facility by using both of the Folmer et al. (1994) primers. The trace files were assembled using Sequencher (Gene Codes). The spiked-sample barcodes were compared with the expected barcodes for the two species.

Evidence of heterozygous sites (i.e., double peaks characteristic of a mixture of DNA templates) was observed by visual inspection of the trace files. The aligned bidirectional trace sequences were compared for evidence of multiple peaks at all nucleotide sites. The samples were classified as consistent with the barcode of the contaminant (*E. postvittana*), consistent with the nontarget background moth (*A. franciscana* or *P. idaeusalis*), or consistent with evidence of a mixture of genetic templates (MIX).

Results

DNA Sequences and Probe Development. As expected, moth DNA sequences of the highly conserved 18S gene fragment were nearly identical in our study. There were only three base changes across all individuals in the $\approx$600-bp fragment. Using this information, primers and a probe were developed in a 68-bp section of the 18S fragment that showed 100% identity.

The *E. postvittana* and *C. fucana* sequences reported in Barr et al. (2009) were used to design the primers and probe for the RT-PCR assay of the ITS2 locus. Based on primer and probe searches and a comparison of similarity values, the regions selected as targets are located in an 83-bp section of the *E. postvittana* DNA sequence. This section was previously used to develop the conventional ITS2 marker (Barr et al. 2009). The targeted DNA sequences from *E. postvittana* are aligned with *C. fucana* and four additional *Epiphyas* species in Fig. 1 to demonstrate similarity in binding sites.

Evidence of intra-genomic (i.e., intraindividual polymorphism) variation in ITS2 was observed for most specimens (Fig. 1). Analysis of four transformed colonies (plasmids) generated from the Californian *E. postvittana* specimen resulted in three forms (1.1, 1.2, and 1.4). Analysis of four colonies from the Australian *E. postvittana* specimen also resulted in three forms (2.1, 2.2, and 2.3). All eight sequences have the expected primer binding sites (F5 and R5; Fig. 1), but not all forms had the recognition site for the probe (P5; Fig. 1). Both *E. postvittana* specimens had at least one copy of the form with the necessary P5 recognition sites for the RT-PCR assay.

Intragenomic variation was observed for most of the other *Epiphyas* specimens (Fig. 1). The only exception was the individual of *E. ashworthana.* Intragenomic variation for the ITS2 locus has been reported previously for other insect taxa (Onyabe and Conn 1999, Harris and Crandall 2000, Leo and Barker 2002).

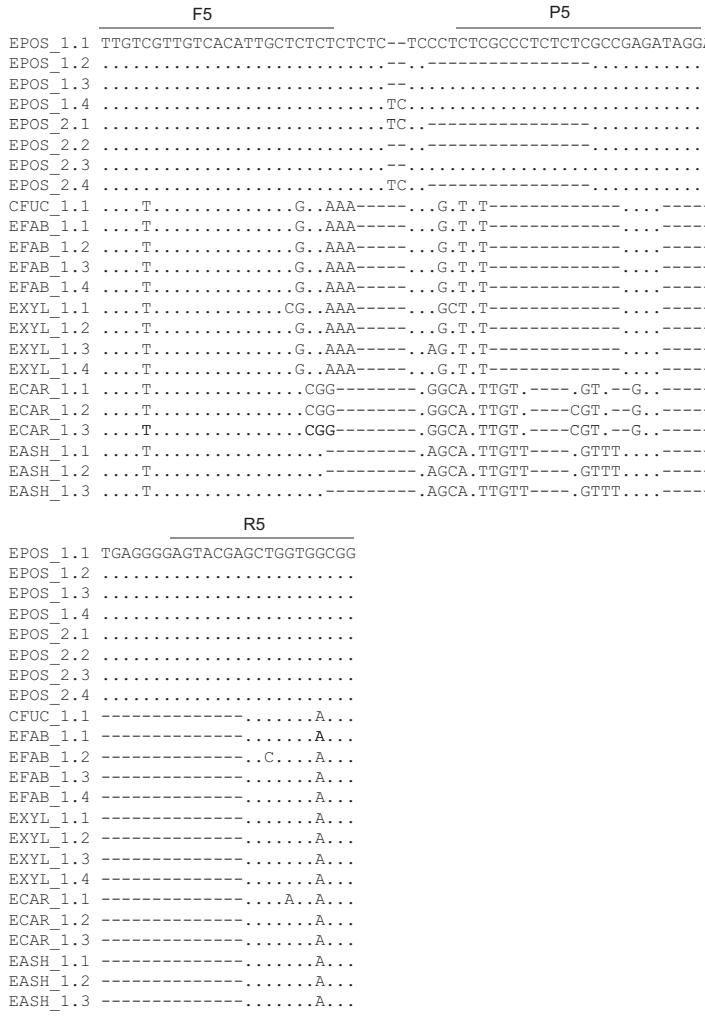

Fig. 1. An alignment of the 83-bp target region including primers (F5 and R5) and probe (P5) sequences used in the ITS2 TaqMan RT-PCR assay. Sequences are generated from cloned PCR products of *E. postvittana* (EPOS), *E. fabricata* (EFAB), *E. xylodes* (EXYL), *E. caryotis* (ECAR), *E. ashworthana* (EASH), and *C. fucana* (CFUC). Each sequence represents a distinct plasmid insertion event. Clone EPOS 1.1 is GQ281283 and CFUC 1.1 is GQ281284 (Barr et al. 2009), with intra individual variation from EPOS 1 (California), EPOS2 (Western Australia), EFAB, EXYL, and ECAR shown.

The three cloned *E. postvittana* 18S inserts were not identical because of a single base substitution (that was outside of the primer and probe recognition sites). This provides further evidence of intragenomic variation in the rDNA array of the species. Cloning error or PCR could have introduced mutations, thereby generating additional genetic forms. The large number of genetic forms observed for the ITS locus, however, would require an unexpectedly high error rate for these two routine molecular procedures. Additional experiments are required to estimate the contribution of PCR and cloning error rates for the loci.

Performance of Multiplex RT-PCR Assay on *E. postvittana*. All of the *E. postvittana* samples tested with the multiplex RT-PCR assay generated Ct values for the ITS2 (FAM) and 18S (CAL RED) markers. The Ct values of the reaction decrease as DNA template concentrations increase, as expected (Figs. 2 and 3). Increasing DNA concentration above 1 ng by using a plasmid template does not continue that trend because the values asymptote (data not shown).

Using DNA isolated from an adult leg, the ITS2 (FAM) probe system generates the same Ct value for the specimen regardless if it is multiplexed or not multiplexed with 18S in the assay (Fig. 3). The same result was observed for the 18S (CAL RED) probe indicating that multiplexing the two probe systems does not negatively affect the assay.

The Ct values of the regressions also indicate that the 18S (CAL RED) probe generates a higher Ct value (i.e., is less sensitive) than the ITS2 (FAM) probe. This experiment was repeated using seven additional specimens and supports the aforementioned observations: no effect of multiplexing the two probes and a trend

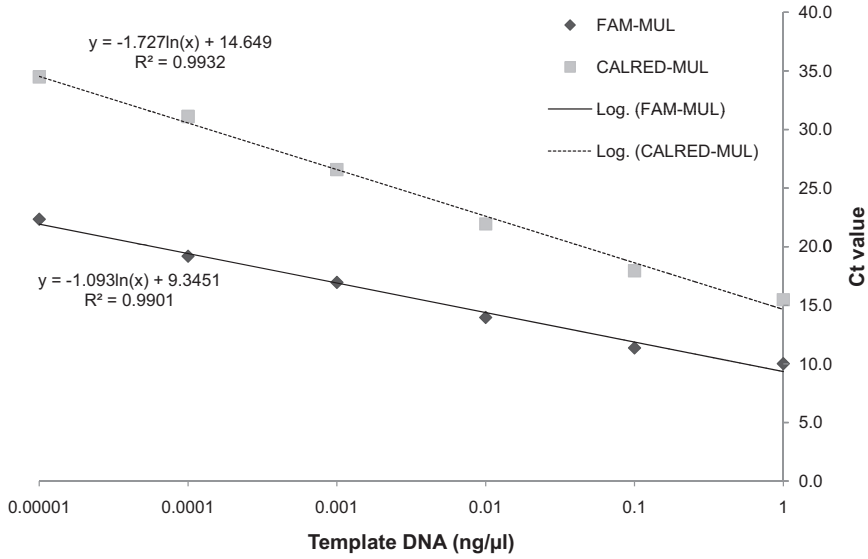

Fig. 2. Standard curve of RT-PCR Ct values for serial dilutions of plasmids with *E. postvittana* ITS2 insert (FAM) or the *E. postvittana* 18S insert (CAL RED) by using the multiplex protocol. Curves are established using mean Ct values for template-cloned plasmids in triplicate (independent plasmids).

for greater FAM sensitivity relative to CAL RED (data not shown). Based on these eight *E. postvittana* DNA isolates, DNA concentrations ≥0.1 ng/µl are adequate for generating Ct values below 40.

A pairwise comparison of *E. postvittana* 18S and ITS2 Ct values for each sample demonstrates a strong trend for higher 18S Ct values (i.e., lower sensitivity) relative to the ITS2 Ct values (Fig. 4). With the exception of one sample, the ITS2 (FAM) probe generated Ct values 1 to 5 cycles lower than the 18S (CAL RED) Ct value. The single exception generated a Ct for ITS2 (FAM) that was 2.24 cycles higher than the 18S (CAL RED) Ct value. The differences between 18S and ITS2 Ct values for the other 354 specimens were no greater than 5 (Fig. 4), with a mean of 3.37 and standard deviation of 0.78.

Analysis of the 355 adult specimens generated mean ± SD Ct values of 20.71 ± 2.43 for ITS2 (FAM) and 24.08 ± 2.91 for 18S (CAL RED). The ITS2 Ct values ranged from 13.96 to 33.61 for FAM, and plotting these on a histogram resulted in evidence of a bimodal distribution (S1). The 18S Ct values ranged from 15.14 to 38.62 and also approximated a bimodal pattern in the histogram.

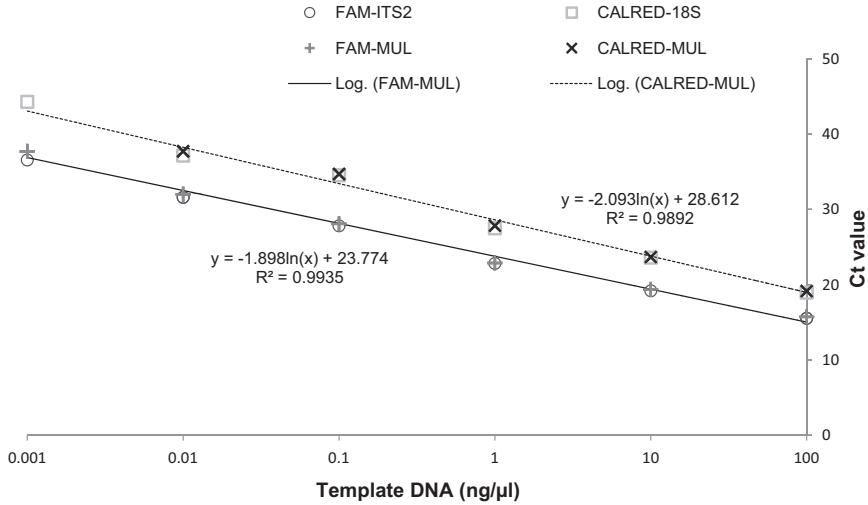

Fig. 3. Standard curve of RT-PCR Ct values for serial dilution of genomic *E. postvittana* DNA (isolated from adult individual) using the multiplex protocol (FAM and CAL RED probes together), the ITS2 protocol (FAM probe separately), and the 18S protocol (CAL RED probe separately).

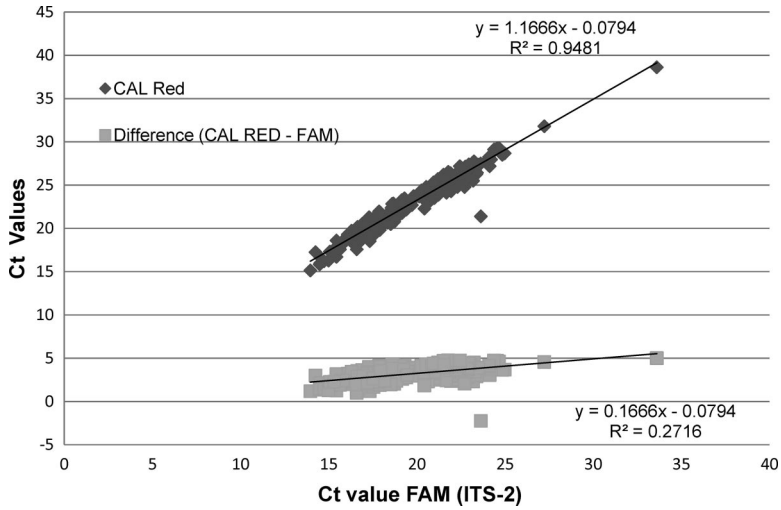

Fig. 4. Comparison of FAM and CAL RED Ct values for 355 *E. postvittana* samples. Regression of CAL RED against FAM Ct values and the difference in CAL RED and FAM Ct values against FAM values.

The bimodal pattern seems to be caused by variation in DNA isolation practices, DNA present in insect tissues, or both. The majority of DNA isolates generated from both thoraces and legs by using moths collected in 2007 from California and 2008 from Hawaii resulted in Ct values below 20. A subsample of 52 isolates from this group had DNA concentrations that ranged from 16.7 to 290.2 ng/μl (mean, 65.5 ng/μl). Likewise, the 40 DNA samples generated at the laboratory of Richard Newcomb (HortResearch, New Zealand; DNA estimates of 50 ng/μl) from Australian and New Zealand specimens generated Ct values below 20.

In comparison, the majority of DNA isolates generated in 2008–2010 by using only legs of Californian specimens resulted in Ct values >20. Based on a sample of 65 DNA extracts, the DNA concentration of these isolates ranged from 0.6 to 9.9 ng/μl (mean, 2.7 ng/μl).

Performance of Multiplex RT-PCR Assay on Nontarget Moths. All 282 nontarget samples tested in the multiplex assay generated Ct values for the 18S (CAL RED) probe; these Cts ranged between 16.67 and 35.71. Unlike the *E. postvittana* samples, a histogram of the nontarget Ct values for 18S (CAL RED) approximated a normal distribution (S2), with a mean ± SD of 26.75 ± 3.20.

Only one of the nontarget samples generated a Ct value with the ITS2 (FAM) probe. This sample [*Henricus umbrabasana* (Kearfott)] generated a relatively high Ct for FAM (37.38) in comparison to the range of values reported for *E. postvittana* samples. One explanation for the Ct value is that it represents a template with a low concentration of DNA. However, its 18S (CAL RED) Ct value (27.14) was within the expected range for a normal DNA concentration. These Ct values for the two probes are not consistent with a profile of *E. postvittana* because the difference between the values is over 10 cycles, and the FAM value is greater than the CAL RED value. Other assayed specimens representing this species did not generate Ct values for the ITS2 (FAM) probe. Although steps were taken to preserve specimen integrity during this study, the possibility that the sample was contaminated with a low level of *E. postvittana* DNA at some point either before or during the study cannot be ruled out.

Spike Experiment. All samples spiked with low levels of E. *postvittana* DNA using either the *A. franciscana* or *P. idaeusalis* genetic background generated Ct values for the 18S (CAL RED) probe (Figs. 5 and 6). These Ct values ranged from 23 to 28 for the tests.

For the *A. franciscana* test, the ITS2 (FAM) probe was detected only when samples contained ≥0.01 ng of *E. postvittana* DNA (Fig. 5). A regression of the Ct values against spiked DNA concentration suggests that at concentrations ≥0.01 ng the samples have relative 18S and ITS2 Ct values similar to the profile of a pure *E. postvittana* template (Fig. 5).

Analysis of spiked samples using the *P. idaesalis* genetic background generated similar but not identical RT-PCR results (Fig. 6). The ITS2 (FAM) probe was detected when samples contained ≥0.001 ng of *E. postvittana* DNA and the 18S/ITS2 Ct values were similar to pure *E. postvittana* samples at concentrations ≥0.01 ng (Fig. 6).

The concentration of spiked *E. postvittana* DNA affected the DNA barcode analysis for the *A. franciscana* genetic background (Figs. 5 and 7). At the highest levels of contamination (≥1 ng of spiked DNA), there is clear evidence of two templates in the trace file (Fig. 7). At the 0.1-ng level for spiked DNA, there was very minor background peaks consistent with *E. postvittana* DNA; but under normal editing practices, this could be treated as noise rather than conflicting signal (Fig. 7). At lower levels (<0.1 ng) of spiked DNA, the resulting trace files lacked any evidence of the *E. postvittana* DNA sequence. Consequently, sam-

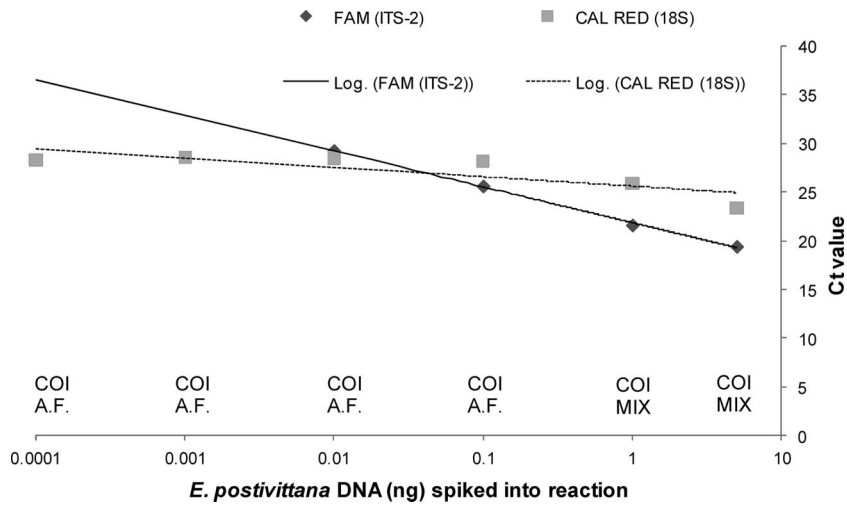

Fig. 5. Spike experiment using *A. franciscana* background (1.5 ng of DNA). Results of DNA barcoding spiked DNA samples are reported above the DNA spike concentration (along the x axis): A.F. indicates that the trace file showed evidence of the *A. franciscana* COI barcode; MIX indicates that the trace file showed evidence of both *A. franciscana* and *E. postvittana* COI barcodes.

ples spiked with <1 ng of DNA, generated the *A. franciscana* barcode sequence (Fig. 5).

The DNA barcode results of the *P. idaesalis* test were not consistent with those from the *A. franciscana* test. At spiked DNA concentrations ≤0.01 ng, the barcodes were identical to *P. idaeusalis.* At concentrations ≥0.01 ng, the barcodes were identical to *E. postvittana.* None of the tested spiked concentrations resulted in evidence of contamination or mixed signal (Fig. 6; trace files available from authors).

Development of Interpretation Rules. The detection of fluorescence in a reaction (i.e., any numerical Ct value) is important because it signifies the presence of target DNA or a PCR artifact (Barcenas et al. 2005). It does not, however, provide information on the source of the target DNA (e.g., contamination). Including the relative Ct values for 18S and ITS2 in the interpretation process provides additional information that can identify unusual results that should be treated without confidence (i.e., inconclusive). For example, when using 1 μl of template in the RT-PCR assay, 99.7% of the *E. postvittana* samples generate an 18S Ct value greater than the ITS2 Ct value. Therefore, samples that have values inconsistent with this pattern should be treated with suspicion in the diagnosis.

Despite the trend in the assay for a greater sensitivity to the ITS2 target, it is possible for an *E. postvittana* sample to generate a measurable Ct for 18S and

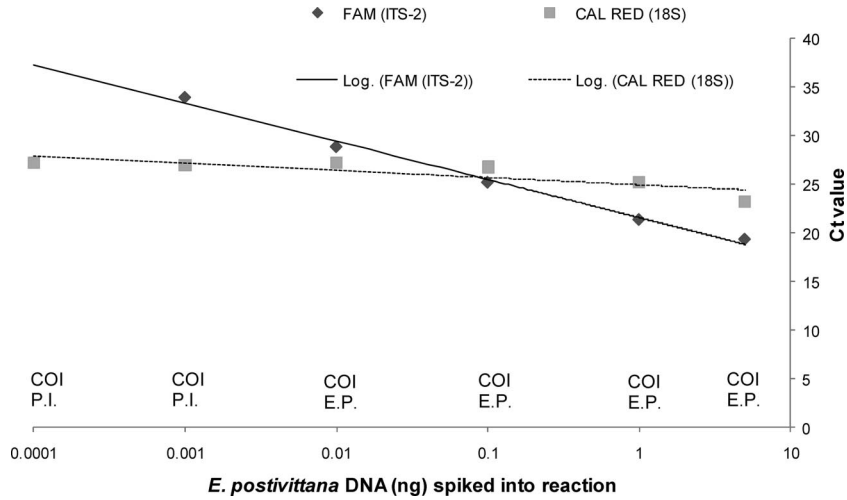

Fig. 6. Spike experiment using *P. idaeusalis* background (7.9 ng). Results of DNA barcoding spiked DNA samples are reported above the DNA spike concentration (along the x axis): P.I. indicates that the trace file showed evidence of the *P. idaeusalis* COI barcode; E.P. indicates that the trace file showed evidence of the *E. postvittana* COI barcode.

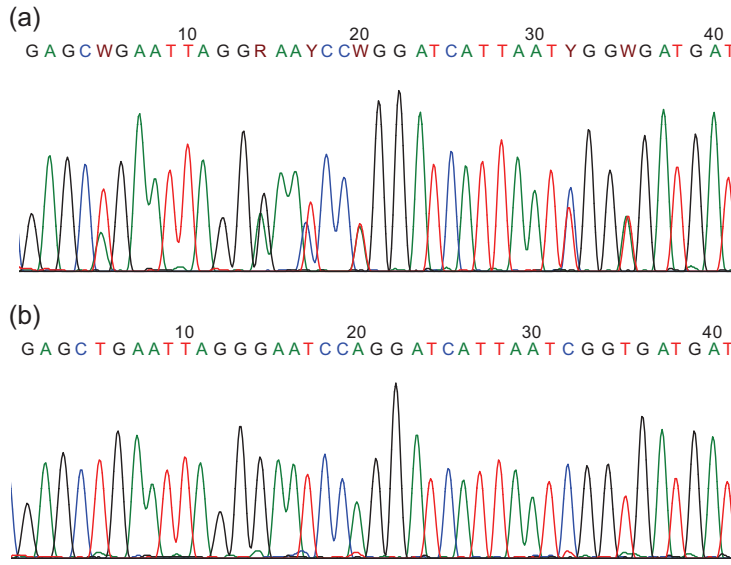

Fig. 7. Example of COI trace files for spike of 1 ng of *E. postvittana* (a) and 0.1 ng of *E. postvittana* (b). Both samples were spiked DNA against 1.5 ng of *A. franciscana* background. The 0.1-ng trace file has no evidence of double peaks (i.e., heterozygous sites). (Online figure in color.)

have no measurable fluorescence for the ITS2 probe (Fig. 4). This could occur if the 18S Ct values are close to the maximum number of cycles in RT-PCR assay (i.e., 40 cycles in the assay) and the ITS2 crosses the threshold soon after the final cycle. Simply increasing or decreasing the number of cycles on the RT-PCR run would not resolve this problem. Therefore, the selection of maximum Ct values for consideration in the diagnosis is important.

Based on the RT-PCR data sets, the following rules for interpreting Ct values were generated: 1) The 18S CAL RED Ct value must be >0 and ≤35 Ct to ensure that the sample has adequate DNA for analysis; 2) The ITS2 FAM Ct must be equal to 0 for the sample to be diagnosed as not *E. postvittana;* 3) The 18S CAL RED Ct value must be equal to or greater than the ITS2 FAM value for the sample to be diagnosed as *E. postvittana;* and 4) all other arrangements of Ct values result in inconclusive data. These rules are integrated into the data interpretation process that is reported in Fig. 8.

Discussion

We have demonstrated that a real-time PCR assay using the ITS2 locus can provide accurate identifications of *E. postvittana* samples in California. Like the conventional PCR assay, the RT-PCR assay includes an internal control to reduce the number of reactions and labor required for making a conclusive determination for diagnostic purposes. Inclusion of this internal control does not interfere with sensitivity of the assay. This RT-PCR technique is quicker to perform than the conventional PCR assay of Barr et al. (2009). The RT-PCR assay has an analysis time of ≈45 min when analyzing isolated DNA. In comparison, the conventional PCR assay requires 4 h. The DNA barcode method would require >24 h to analyze the same sample. Therefore, the RT-PCR technique represents a significant savings in time and labor.

The RT-PCR assay is designed to minimize both false positives and false negatives. Six hundred and thirty-seven samples were assayed in the study, and 99.5% of these samples resulted in correct identifications. Inconclusive information was generated for just three samples (0.5%). No false positives were generated from any samples. Sensitivity curves demonstrate that the probes perform well when the DNA template contains ≥0.1 ng of DNA, and the assay is appropriate for diagnosing samples based on a standard DNA isolate volume. To minimize the cost and labor involved in estimating DNA concentrations during the diagnosis process, the protocol was developed to assay an objective template volume. Based on our data, this approach works on all of the *E. postvittana* adult samples collected within California. The assay also has been tested successfully on *E. postvittana* egg masses, larvae, and pupae reared from a laboratory colony in Albany, CA (data not shown).

Similar to conclusions reached in Barr et al. (2009), application of this tool to tortricid moths intercepted at a port of entry or collected in Australia or New Zealand is problematic. This is because the genus *Epiphyas* includes >40 species (Baixeras et al. 2010), and *E. postvittana* is part of a putative species complex (B. Hitchcock, personal communication). All members of this diverse genus are not included in the current study, so the taxonomic specificity of the assay remains uncertain. Furthermore, it is not possible to predict what *Epiphyas* species might be intercepted at a port in the future.

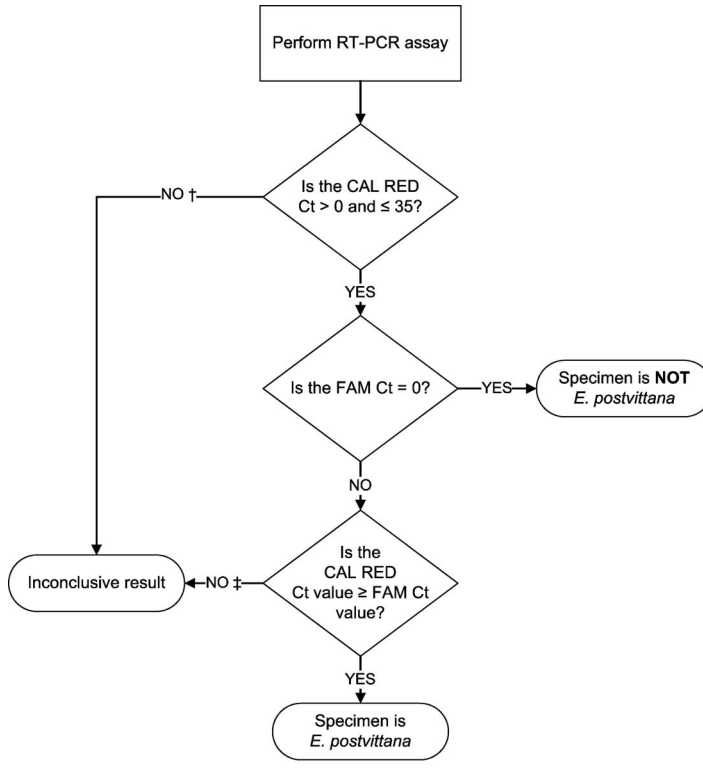

Fig. 8. Decision process for interpreting Ct values generated using the RT-PCR assay and tortricid samples collected in the United States.

Our study does, however, include four additional *Epiphyas* species not present in the Barr et al. (2009) study. These species do not cross-react with the ITS2 probe, indicating that more distantly related moths (i.e., those outside of the genus *Epiphyas*) also should not cross-react with the diagnostic probe. Currently, *E. postvittana* is the only *Epiphyas* species present in North America. As long as this geographic distribution holds true, the RT-PCR assay should be applicable to North America and other regions where *Epiphyas* taxonomic diversity is limited to *E. postvittana.*

The 18S locus was selected as an internal control in the assay because it is located in the rDNA array with ITS2 (Hillis and Dixon 1991). Although we expected the Ct value for the 18S and ITS2 probes to differ (because of difference in dye chemistries and kinetics), we expected the difference in Ct values between the two probes to be similar across DNA isolates because the number of targets in the template should be similar. This was not observed in our data.

One explanation for variation in differences in Ct values is that the number of ITS2 copies with its targeted binding site is not equal to the number of 18S copies with its targeted binding site. This is supported by DNA sequences of cloned ITS2 and 18S PCR products. Intragenomic variation for both loci in *E. postvittana* specimens and for ITS2 in four *Epiphyas* species is displayed in Fig. 1. Although similar results have been reported for other species (Onyabe and Conn 1999; Harris and Crandall 2000; Leo and Barker 2002), this is the first reporting of intra individual variation for the ITS2 locus within the genus *Epiphyas.*

An interesting result from our study is the potential effects of contamination on the assay. The risk of contamination can be high for laboratories that process large numbers of query moths (or other insects). We performed spiking experiments to simulate contamination using both the RT-PCR assay and a DNA barcode assay. These experiments were performed to estimate sensitivity of the markers for LBAM DNA against a DNA background from a nontarget species and compare sensitivity of the two assays.

For the ITS2 RT-PCR assay, contamination with *E. postvittana* DNA at concentrations ≥0.01 ng could result in false positives. This was true for both of the tested genetic backgrounds. Although relatively high concentrations of contamination can generate Ct profiles consistent with *E. postvittana*, these false positives

should be detected in the negative controls included during the DNA isolation and PCR steps.

Although the same quantity and quality of *E. postvittana* template was used to spike the two genetic backgrounds (*A. franciscana* and *P. idaeusalis*), the analytical sensitivity for the *E. postvittana* ITS2 target was not the same for these experiments. The difference in detection levels for the two moth species could be the result of differences in the quality, quantity, and genetic composition of the DNA isolates used as genetic background. Additional experiments are needed to adequately understand these factors, but two important conclusions can be drawn from the current experiments: 1) contamination can generate Ct values for the target ITS2 probe resulting in false positives and 2) sensitivity to contamination can vary for different DNA samples. Therefore, inclusion of proper controls is crucial for the RT-PCR assay.

The DNA barcode assay resulted in variable results for the two genetic backgrounds when spiked with identical *E. postvittana* DNA. Although contamination (evidence of mixed signal) was detectable against the *A. franciscana* genetic background, this was not true for the *P. idaeusalis* genetic background. At low levels of contamination, the *P. idaeusalis* samples were identified as *P. idaeusalis.* At higher levels, the samples were identified as *E. postvittana.* Although the RT-PCR and DNA barcode assays generated similar results for the *P. idaeusalis* background, these assays using the *A. franciscana* genetic background generated conflicting interpretations. These results demonstrate that it is possible for a contaminated sample to have Ct values in the RT-PCR assay that would result in a diagnosis as *E. postvittana* and still have a COI barcode DNA sequence that would result in a diagnosis as not *E. postvittana* (Fig. 5). Therefore, a diagnostician cannot assume that DNA barcodes will generate evidence of contamination in the analysis process. These experiments also demonstrate that variation in DNA quality and quantity can affect molecular identification methods (RT-PCR and DNA barcoding) in different ways.

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