Molecular Identification of the Light Brown Apple Moth (Lepidoptera: Tortricidae) in California Using a Polymerase Chain Reaction Assay of the Internal Transcribed Spacer 2 Locus

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ABSTRACT A molecular protocol using a hemi-nested polymerase chain reaction (PCR) of the internal transcribed spacer region 2 (ITS2) is reported for the diagnosis of light brown apple moth, *Epiphyas postvittana* (Walker) (Lepidoptera: Tortricidae), in California. This protocol distinguishes the light brown apple moth from other moths in California based on size differences of PCR amplicons that are visualized on agarose gels. The molecular diagnostic tool generated no false negatives based on analysis of 337 light brown apple moths collected from California, Hawaii, England, New Zealand, and Australia. Analysis of a data set including 424 moths representing other tortricid species generated correct identification for >95% of the samples and only two false positives. Of the 761 moths tested only fourteen produced no PCR amplicons and five generated inconclusive data.

KEY WORDS Tortricidae, barcodes, internal transcribed spacer, nested polymerase chain reaction

Native to Australia, the light brown apple moth, *Epiphyas postvittana* (Walker) (Lepidoptera: Tortricidae), is an invasive species that has spread to New Zealand, the British Isles, Hawaii, and most recently northern California (Meyrick 1937, Bradley et al. 1973, Zimmerman 1978, Fowler et al. 2007). Larvae of this pest are highly polyphagous and have been recorded feeding on >250 plant species in 50 families and 120 genera (Danthanarayana 1975, Wearing et al. 1991, Suckling and Brockerhoff 2010).

The presence and establishment of light brown apple moth in agricultural producing regions such as California can have a negative economic impact through lost trade; damage to commodities; and the cost of implementing eradication, quarantine, or other control strategies (Suckling and Brockerhoff 2010). For example, the presence of light brown apple moth in California has already affected commercial nurseries because the detection of a single light brown apple mot can trigger quarantine and treatment actions of the nursery stock. There are costs associated with quarantining a nursery either in response to detection of light brown apple moth or during the diagnosis process to confirm the identity of a suspected moth.

Although light brown apple moth is the only *Epiphyas* species reported from North America, the adult is similar to several taxa in the family Tortricidae (Gilligan and Epstein 2009). Therefore, the reliable and rapid identification of light brown apple moth in nonnative regions such as California is difficult. A morphology-based Lucid key for light brown apple moth identification in California that incorporates larval and adult life stages was recently published (Gilligan and Epstein 2009); however, currently morphological identification of light brown apple moth immature stages (i.e., eggs, larvae, pupae) remains especially problematic. As a result, The United States Department of Agriculture–Animal and Plant Health Inspection Service (USDA–APHIS) and The California Department of Food and Agriculture (CDFA) have applied DNA barcoding techniques to identify query moths trapped/detected within California by using *cytochrome oxidase* subunit I gene (COI) sequences. Each sequence generated from suspect light brown apple moths is compared with expert-identified reference sequences for a final molecular diagnosis.

Although the current tools based on morphology and DNA barcodes are useful, when large numbers of query specimens are submitted to a diagnostics facility the response time can increase because of limited resources (i.e., number of trained staff and/or available, onsite instruments and equipment). Consequently, to complement the existing identification tools, we have developed another DNA-based tool for identification of light brown apple moth within California. This tool uses the polymerase chain reaction (PCR) coupled with agarose gel electrophoresis to

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Table 1. LBAM collections used to develop/assess the ITS2 marker

LBAM collection	*N*	Amp.	Inconc.	Pos.	Neg.
USA: CA: Alameda County–2007	10	10	0	10	0
USA: CA: Alameda County–2008	19	19	0	19	0
USA: CA: Contra Costa County–2008	14	14	0	14	0
USA: CA: Los Angeles–2007	1	1	0	1	0
USA: CA: Marin County–2007	2	2	0	2	0
USA: CA: Marin County–2008	16	16	0	16	0
USA: CA: Monterey County–2007	5	5	0	5	0
USA: CA: Monterey County–2008	45	45	0	45	0
USA: CA: Napa County–2008	1	1	0	1	0
USA: CA: San Benito County–2008	2	2	0	2	0
USA: CA: San Francisco County–2007	7	7	0	7	0
USA: CA: San Francisco County–2008	21	21	1	20	0
USA: CA: San Mateo County–2008	21	21	0	21	0
USA: CA: Santa Barbara County–2008	1	1	0	1	0
USA: CA: Santa Clara County–2008	4	4	0	4	0
USA: CA: Santa Cruz County–2007	26	26	0	26	0
USA: CA: Santa Cruz County–2008	75	75	0	75	0
USA: CA: Solano County–2008	3	3	0	3	0
USA: CA: Sonoma County–2008	2	2	0	2	0
USA: HI: Hawai'i, Volcano–2008	8	8	0	8	0
Australia: Western–2008	11	11	0	11	0
Australia: New South Wales–2008	10	10	0	10	0
New Zealand: South Island–2008	10	10	0	10	0
New Zealand: North Island–2008	9	9	0	9	0
United Kingdom: England–2008	14	14	0	14	0
Total	337	337	1	336	0

N is the number of specimens tested; Amp. is the number of specimens that amplified for the ITS2 marker; Inconc. is the number of specimens that produced an inconclusive result because of banding pattern; Pos. is the number of true positives; and Neg. is the number of false negatives.

analyze a molecular marker developed from the internal transcribed spacer (ITS) region 2 locus. Application of the PCR method only requires instruments common to basic molecular biology labs and should facilitate future diagnosis of light brown apple moth.

Materials and Methods

Collections of Light Brown Apple Moth. Two hundred and seventy-five adult light brown apple moth specimens were trapped using Jackson pheromone traps set in 14 California counties (Table 1) during 2007–2008. The species-level identification of the 275 moths was verified using adult morphology. All specimens were stored dry and a voucher for each specimen is stored at the CDFA's California State Collection of Arthropods (CSCA). For the 2008 collections, a leg was removed from each moth for genetic analysis, and the remaining specimen was saved as a voucher. For the 2007 collections, only the abdomen was saved as a voucher and the remaining moth used for genetic analysis. Specimens from the 2007 collections were used to generate DNA sequences for primer design and to perform pilot studies to test the ITS2 markers.

Eight adult moths were collected from Volcano, Hawai'i Volcano Experiment Station, Hawai'i, in July 2008. The specimens were stored in 95% ethanol and sent to M. Epstein for identification. A leg from each moth was sent to the Mission Lab for molecular analysis and the remaining moth was saved as a voucher (CDFA–CSCA).

Fourteen adult moths were collected from Southsea, Hampshire, England, in August 2008. The specimens were stored dry and sent to T. Gilligan for identification. A leg from each moth was sent to the Mission Lab for molecular analysis and the remaining moth specimen was saved as a voucher (CDFA–CSCA).

Aliquots of 40 DNA extractions generated using the DNeasy method (QIAGEN, Valencia, CA) at the laboratory of R. Newcomb (HortResearch, NZ) were sent to the Mission Lab for molecular analysis. Nineteen of the extractions were from New Zealand collections: nine were from a collection made in Hort, Palmerston North, on the North Island in 2008 and 10 were from a collection made in Paroa on the west cost of the South Island in 2008. Twenty-one of the extractions were from Australian collections: 11 were from a collection made in Applecross, Dalkeith, and Kensington, Western Australia, Australia, in 2008 and 10 were from a collection made in Merungle Hill, Leeton, New South Wales, Australia, in 2008.

Collections of Nontarget Tortricids. *Reference Collection.* Nine adult moths representing the genera *Acleris* Hübner, *Archips* Hübner, *Argyrotaenia* Stephens, *Choristoneura* Lederer, *Clepsis* Guenée, and *Platynota* Clemens were analyzed at the Mission Lab (Table 2). These moths represent species that can be misidentified as light brown apple moth in California. Extractions were performed using a leg, thoracic tissue, or entire moths. A series voucher were saved for each collection, and/or a specimen voucher was maintained using the nonextracted insect tissues. Vouchers are stored in the CDFA–CSCA insect collection. In addition, a 10th adult tortricid specimen of uncertain taxonomy (called tortricid sp. X in this study) was included in this reference collection because based on DNA barcodes by using COI, it is more similar to light brown apple moth (≈93% identity) than the other

Table 2. Collections of non-LBAM moths used to assess the ITS2 marker

Nontarget moth sample[a]	*N*	Amp.	Inconc.	Pos.	Neg.
Reference collection					
A. cerviana	1	1	0	0	1
Archips argyrospila (Walker)	2	2	0	0	2
A. franciscana	1	1	0	0	1
C. retiniana	1	1	0	0	1
C. peritana	1	1	0	0	1
Platynota stultana	3	3	0	0	3
Tortricid sp. X	1	1	0	0	1
CDFA (barcoded) collection	414	400	4	2	394
Total	424	410	4	2	407

N is the number of specimens tested; Amp. is the number of specimens that amplified for the ITS2 marker; Incon. is the number of specimens that produced an inconclusive result because of banding pattern; Pos. is the number of false positives; and Neg. is the number of true negatives.

[a] The *A. cerviana* sample was collected in Mississippi, but all other nontarget moths were collected in California.

nine identified moth species. Based on a search of the BOLD data base (Ratnasingham and Hebert 2007) on 23 March 2009, this specimen is most similar to the Australasian genus *Merophyas* (95.3%).

CDFA Database. To test the utility of the ITS2 diagnostic on nontargets and estimate the rate of false positives, a data set of 414 query specimens identified by CDFA as not light brown apple moth (based on DNA barcoding using COI sequences) was analyzed. These DNA extractions were from immature and adult query moths submitted to CDFA for DNA barcode analysis from 20 June 2007 to 26 November 2008 because they were morphologically similar to light brown apple moth. All DNA extracts were inconsistent with the COI genetic profile of light brown apple moth by uncorrected genetic distances of at least 9%.

Extraction of DNA. Samples were extracted using the DNeasy extraction method for animal tissue (Qiagen, Valencia, CA). Extractions were performed using overnight incubation of samples at 56°C and eluted in 100 μl of buffer. Whenever possible a no tissue extraction control was included with each extraction batch to control for possible contamination at the DNA isolation step. The optional RNase treatment (QIAGEN) was not performed for the Mission Lab samples.

PCR, Sequencing, and Cloning of ITS2. All PCR experiments were performed on Applied Biosystem GeneAmp 9700 thermal cyclers (aluminum plate; Applied Biosystems, Foster City, CA) by using Ex *Taq* polymerase (Takara Mirus Bio, Otsu, Shiga, Japan) in total volumes of 25 μl. The reactions were performed using the manufacturer's recommended buffer (1×) and primer (0.2–0.4 μM) concentrations, 0.75 U of *Taq* polymerase per reaction, and 1 μl of DNA template, blank DNA extraction (negative control of Extraction and PCR steps), or 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 (5-TTGAACATCGACATTTCGAACGCAC, T_m = 62.9, forward; 5.8d, Hillis and Dixon 1991) and ITSR (5-TCCTCCGCTTATTGATATGC, T_m = 58.4, reverse; ITS4, White et al. 1990) primer pair. Several PCR file conditions were tested in the experiments to test effects of annealing temperature, annealing time, and cycle number on the markers. In general the programs included an initial step of 94°C × 3 min, 30–40 cycles of (94°C × 20 s, 60–64°C × 20–30 s, 72°C × 30 s), and an extension step of 72°C × 5–7 min.

The TA cloning kit (Invitrogen, Carlsbad, CA) was used to clone amplicons of light brown apple moth the tortricid sp. X sample, and *Platynota stultana* Walsingham. Transformed colonies were grown and selected on Luria broth (LB) and LB-agar plates with antibiotic. The plasmid DNA was isolated and purified using the miniprep kit (QIAGEN). Miniprep DNA was sequenced using the universal M13 forward and reverse sequencing primers at the Davis Sequencing facility (Davis, CA). Additional DNA sequences were generated at the Davis facility by direct sequencing using the ITS2 PCR primers as sequencing primers.

Design of Diagnostic Marker. The ITS2 sequences from light brown apple moth (GQ281283), tortricid sp. X (GQ281284), and *P. stultana* (GQ281285) were aligned by hand in MEGA4 (Kumar et al. 2008) by using the alignment editor function. The 5.8S and 28S sequences were identified by comparison with GenBank sequences of other moth species: *Maruca vitrata* (F.) (FJ041109) and *Perina nuda* (F.) (FJ041110). This alignment was used to search for diagnostic primers.

The nucleotide composition of each sequence was investigated in MEGA by using overall estimates of each nucleotide and a nonoverlapping sliding window analysis of 20-bp steps for each nucleotide and dinucleotide combination (e.g., C+G and C+T).

Diagnostic primers were designed using sections of the light brown apple moth sequence that 1) were not conserved with the nontarget sequences (i.e., based on the alignment) and 2) exhibited either unbiased nucleotide composition or bias toward base compositions not common to other non–light brown apple moth sequences (e.g., exclude C+G-rich DNA regions of the light brown apple moth sequence for primer design if the nontarget sequences also exhibit regions of C+G-rich DNA).

The primers were tested using Primer3 (Rozen and Skaletsky 2000) for PCR success by using the light brown apple moth sequence as template and either the ITSF or ITSR primer as the second primer. To search for possible nonspecific binding sites in nontarget sequences, each diagnostic primer sequence (and its complement) was aligned using ClustalW (pairwise alignment, gap penalty set at 100; MEGA4) to each nontarget and light brown apple moth sequence.

Additional ITS2 sequences of *Acleris cervinana* (Fernald) (GQ281286), *Argyrotaenia franciscana* (Walsingham) (GQ281287–88), *Choristoneura retiniana* (Walsingham) (GQ281289), and *Clepsis peritana* (Clemens) (GQ281290) were generated through direct DNA sequencing for comparison to the original

alignment and to search for potential binding sites of the diagnostic primers.

Pilot Study and Protocol Development. The putative diagnostic primers were tested using subsets (5–10) of the light brown apple moth DNA extractions from California moths collected in 2007 (Table 1) and the nontarget reference samples. DNA extractions were first tested using equal ratios of the three primers (using final primer concentrations of 0.2 μM) and a program file with an annealing temperature of 60°C (see above). The primer ratios, total primer concentration, and PCR program variables (e.g., annealing temperature and cycle number) were then adjusted to minimize background amplification and enhance the intensity of the expected band patterns. These optimization experiments were performed using 1 μl of template DNA taken directly from the QIAGEN extraction tube (i.e., without dilution).

Primer ratios that consistently generated relatively high amounts of the ITSF-ITSR amplicon with respect to the light brown apple moth diagnostic amplicon were not used in the ITS2 protocol. The ITSF and ITSR primers amplify DNA from all moths and are expected to generate an amplicon for all reactions. Therefore, the ITSF-ITSR amplicon serves as an internal control of the PCR assay because its presence distinguishes between profiles consistent with nontarget moths (i.e., presence of the ITSF-ITSR amplicon) and PCR failure (i.e., no amplification). However, if the ITSF-ITSR primer pair performs better (i.e., is more sensitive) than the light brown apple moth-specific primer pair during PCR, then the rate of false negatives increases. To develop a conservative diagnostic test, it is better to have the internal control (e.g., ITSF-ITSR) less sensitive than the diagnostic marker because then PCR failure for the internal control band results in an inconclusive result.

Additional tests were performed on serial dilutions of the purified light brown apple moth plasmid and an light brown apple moth total nucleic acid extraction to determine the performance of the multiplex PCR at different template concentrations. The concentrations of DNA samples were estimated using an ND-1000 spectrophotometer (NanoDrop, Willmington, DE). For each sample a PCR assay was performed that included template at the original sample concentration, a dilution at 100 ng/μl, a dilution at 50 ng/μl, and then 10-fold serial dilutions ranging from 1×10^{1} to 1×10^{-5}. A second PCR assay using a set of two-fold dilutions starting at 1×10^{-2} ng/μl for the plasmid sample was performed to provide a greater level of resolution. All dilutions were performed in water.

PCR Analysis of Specimens With ITS2 Multiplex Marker. The ITS2 marker was amplified from DNA samples in a total volume of 25 μl including 1 μl of template, 0.5 μl of primer F1 (at 10 μM), 0.5 μl of primer ITSF (at 10 μM), 1 μl of primer ITSR (at 10 μM), 2.5 μl of 10× buffer (Takara Mirus Bio), 2 μl of dNTP mix (Takara Mirus Bio), and 0.15 μl of Ex *Taq* (5 U/μl; Takara Mirus Bio). The PCR was performed under the following conditions: 94°C × 3 min; 35 cycles of 94°C × 20 s, 60°C × 30 s, 72°C × 30 s; 72°C × 5 min; and hold at 4°C. Positive and negative controls were included in all PCR runs.

Amplicons were run on 1.2% Tris-acetic acid-disodium EDTA (Bio-Rad Laboratories, Hercules, CA) gels using general purpose agarose (Sigma-Aldrich, St. Louis, MO). An additional 2% agarose gel was run for a subset of samples to compare resolution with the 1.2% gel (see Fig. 4). All gels were prestained with ethidium bromide (0.5 μg/ml final concentration; Sigma-Aldrich) and visualized on a UV light source (Fluor-S MultiImager, Bio-Rad Laboratories, or GeneFlash imaging system, SynGene Bio Imaging, Frederick, MD). Samples for electrophoresis consisted of 25 μl of PCR amplicon and 5 μl of Blue/Orange 6× loading dye (Promega). Amplicon size was estimated based on comparison with 10 μl of 100-bp TriDye ladder and/or 50-bp ladder (New England Biolabs, Ipswich, MA).

Results and Discussion

Sequence Data and Properties of the Marker. The light brown apple moth sequence generated with the ITSF and ITSR primer pair is 534 bp and includes 76 bp of the 5.8 locus and 69 bp of the 28S locus (using the *Perina* sequence as the guide, Fig. 1). The ITS2 locus is 389 bp and has an overall base composition biased toward C+G (61%) content. Additional ITS2 DNA sequences generated through direct sequencing of light brown apple moth extractions (GQ332410-12) produced no differences with the cloned sample.

The total length of the ITS2 region varies for the other tested taxa: tortricid sp. X (391 bp), *P. stultana* (493 bp), *C. peritana* (405 bp), *A. cervinana* (379 bp), *C. retiniana* (424 bp), and *A. franciscana* (410 bp). The expected ITSF-ITSR amplicons for these species range from 524 bp to 638 bp. Consequently, the internal control band (i.e., ITSF-ITSR amplicon) varies in size for the moth species included in the study.

The overall nucleotide composition was similar among the nontarget ITS2 sequences (T, 23–27%; C, 26–29%; A, 19–21%; and G, 27–29%). Based on a sliding window analysis using 20-bp windows, all sequences had regions with relatively elevated C+G bias (80–90%) and A+T bias (70–95%). However, only the light brown apple moth sequence exhibited a C+T content of 95% within a 20-bp window. The other sequences had maximum C+T contents between 60 and 70% for the sliding window analysis. This section of light brown apple moth was used to develop a putative diagnostic forward primer called F1 (5-GCTCTCTCTCTCTCCCTCTCG, T_m = 66.5; Fig. 1). Alternative diagnostic primers were designed but found to not perform as well as the F1 primer (N.B.B., unpublished).

The F1-ITSR primer pair generates an expected diagnostic light brown apple moth band of 420 bp. The F1 primer did not share >71% identity to regions of the seven nontarget sequences, based on complement or reverse complement comparisons. Although these data do not preclude nonspecific binding, they suggest that the primer sequence is unique to the light brown apple moth target DNA sequence.

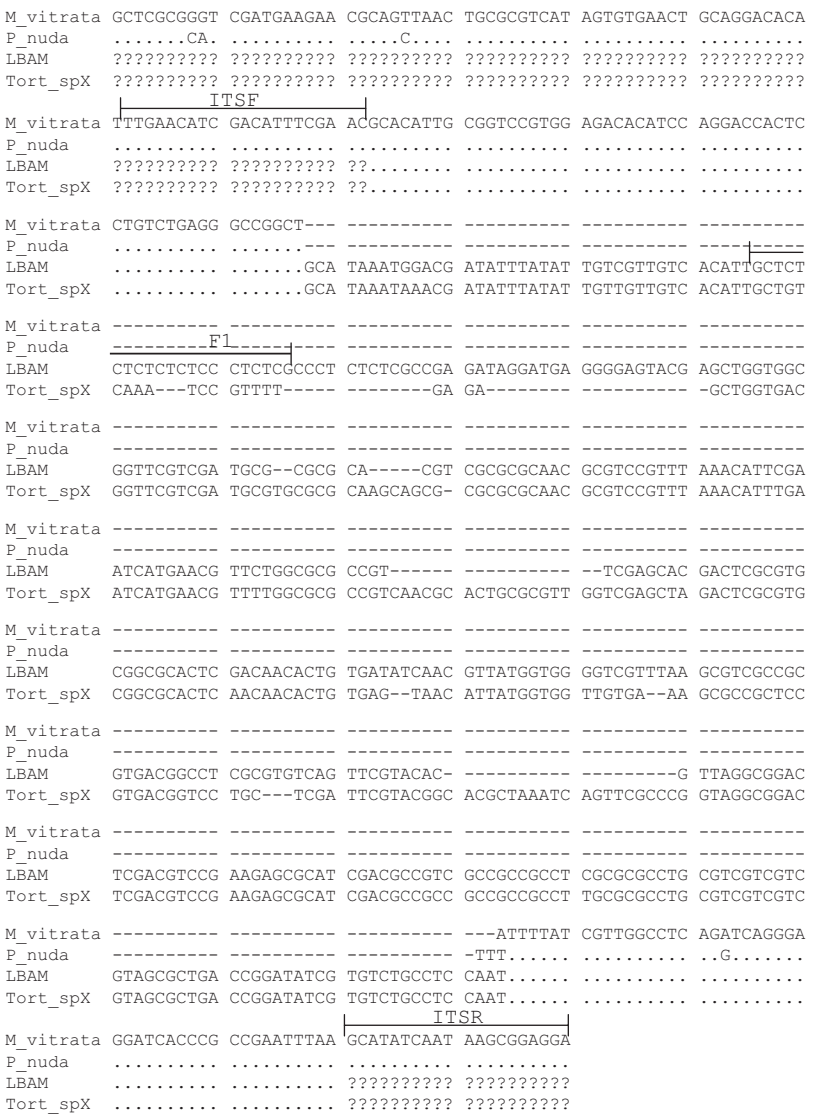

Fig. 1. Alignment of 5.8-ITS2–28S DNA sequences of light brown apple moth and the tortricid sp. X plus the 5.8 and 28S sequences from two additional lepidopterans (*M. vitrata* and *P. nuda*) to indicate the location of these loci. The primers reported in the ITS2 study are indicated in boxes.

Pilot Studies to Test Amplification of the Light Brown Apple Moth Clone and Moth Samples. Initial tests of light brown apple moth DNA by using a heminested, multiplex PCR including ITSF, ITSR, and F1 primers produced the expected ITSF-ITSR band (534 bp) and F1-ITSR band (420 bp). The F1 primer generated only the expected two bands for the DNA extracts included in the pilot study; additional, nonspecific bands do appear when higher DNA concentrations are analyzed with F1 (see below). As expected, the 13 nontarget tortricid DNA extracts generated single bands (i.e., the ITSF-ITSR amplicon), by using the multiplex PCR, that are distinct from the diagnostic band generated by the F1-ITSR pair (e.g., Fig. 4, lanes 11 and 12).

Pilot Studies to Optimize Primer Ratio for Amplification of the ITS2 Products. Adjustment of the primer ratio to 5:3:10 (F1:ITSF:ITSR) generated both of the expected bands, reduced background noise in light brown apple moth and nontarget moths and generated an F1-ITSR band more intense than the ITSF-ITSR band for the majority (>85%) of samples. The only exceptions (i.e., where the ITSF-ITSR band was more intense) were samples of high DNA concentration and these generated intense bands of both amplicon sizes. This trend for a relatively more intense ITSF-ITSR band at higher DNA concentrations (>50 ng/μl) and a relatively more intense F1-ITSR band at lower DNA concentrations was also observed using dilutions of plasmid DNA (Fig. 3, see below for a

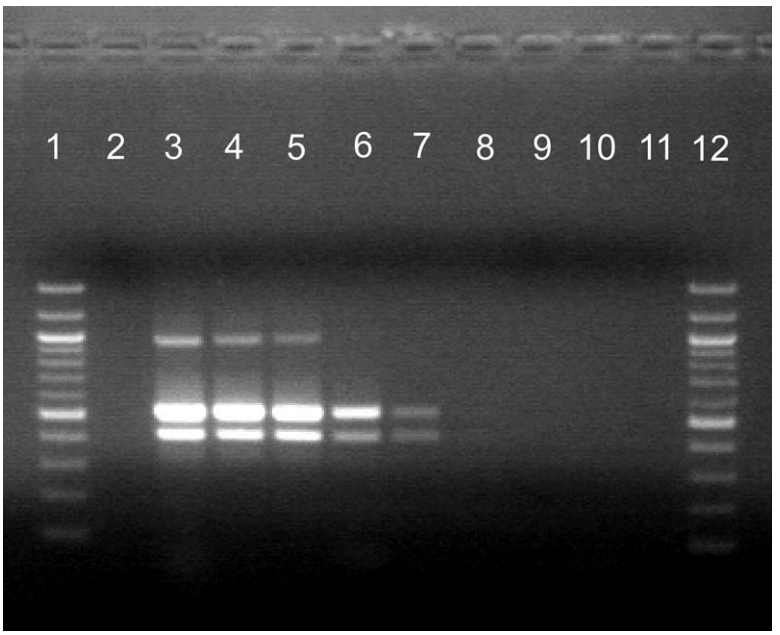

Fig. 2. PCR results of the ITS2 marker using dilutions of an light brown apple moth total nucleic acid extraction: lanes 1 and 12, 100-bp TriDye; lane 2, negative control; lane 3, 915 ng/μl; lane 4, 100 ng/μl; lane 5, 50 ng/μl; lane 6, 1×10^{1} ng/μl; lane 7, 1×10^{0} ng/μl; lane 8, 1×10^{-1} ng/μl; lane 9, 1×10^{-2} ng/μl; lane 10, 1×10^{-3} ng/μl; and lane 11, 10^{-4} ng/μl.

description). This trend, however, is not a rule because at least one moth sample generated a more intense ITSF-ITSR band (Fig. 2, lane 6) by using 10 ng/μl of template DNA. None of the tested ratios and final primer concentrations produced the two light brown apple moth amplicons in equal amounts for all of the samples tested. If the two bands are unequal in intensity, then it is preferable to have the diagnostic band as the more intense of the two (see Material and Methods).

DNA Template Concentration Effects on the ITS2 PCR Assay. A light brown apple moth DNA extract that had high concentrations of isolated DNA (i.e., 915 ng/μl) was selected to study the effect of DNA concentration on the hemi-nested PCR. Amplicon patterns generated using dilutions of the extract are reported in Fig. 2. The protocol was capable of detecting the marker when the nucleic acid concentration was $\geq$1 ng/μl. When the sample concentration was $\geq$50 ng/μl an additional, large ($\approx$1-kb) band was produced. Template concentrations $\geq$50 ng/μl for DNA extracts in our study were not common (<10%) for the light brown apple moth samples that were collected in traps and generated using a single leg. However, DNA concentration will depend on the quality of the tissue and efficiency of extraction protocol used for DNA isolation.

The large (1-kb) band was gel excised and sequenced using the three primers (i.e., ITSF, ITSR, and F1) to determine its origin. Despite being $\approx$1 kb, the sequencing reactions of the amplicon only generated useful data for $\approx$550 bp; the quality of the downstream base calls is poor because of multiple peaks and reduced signal. Based on the good quality data, the sequencing reactions confirmed that the band was ITS2 from light brown apple moth (GQ332416). One possible explanation for this PCR artifact is that in the presence of high DNA template concentrations the newly synthesized DNA strands can bind at the ends to produce an amplicon consisting of two ITS2 copies in tandem. Consequently, during the sequencing reaction each primer binds at two locations within the amplicon template and generates identical base calls for these two copies. The regions immediately flanking the two copies are not identical (a result of connecting them in tandem) so the two reactions (the result of the same primer binding at two different locations in the template) generates heterogeneous data.

For comparison, this dilution experiment was repeated using an light brown apple moth sample that was extracted using the DNeasy method with RNase treatment (gel not shown). This sample generated profiles similar to the other extraction: the detection limit is at 1 ng/μl and at concentrations $\geq$50 ng/μl the 1-kb band is produced.

The dilution assay using the miniprep of the plasmid generated a pattern similar to the total DNA extraction results (Fig. 3). At plasmid DNA concentrations above 1×10^{-3} ng/μl, a large ($\approx$1-kb) amplicon was generated. Sequencing of this amplicon generated the light brown apple moth ITS2 sequence (GQ332417) supporting the previous concept of PCR-generated tandem copies of ITS2. This artifact may be restricted to particular DNA sequences because it is not generated in serial dilutions of a plasmid with an insert of ITS2 derived from *P. stultana* (data not shown).

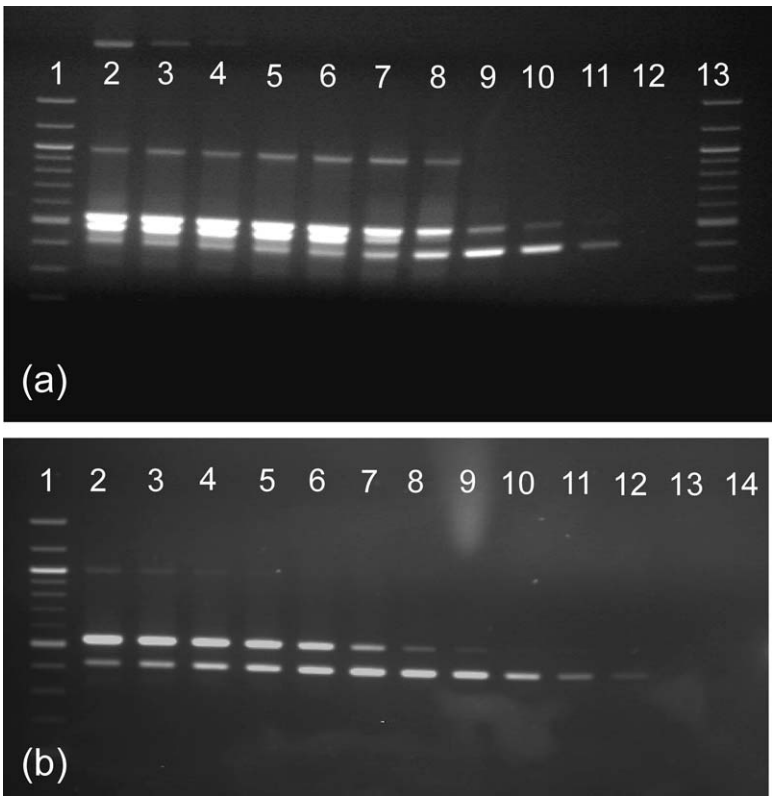

Fig. 3. PCR results of the ITS2 marker by using a serial dilution of a plasmid with the light brown apple moth ITS2 insert. The top gel (a) represents a test using a wider range of concentrations and 10-fold dilutions: lanes 1 and 13, 100-bp TriDye; lane 2, 250 ng/μl; lane 3, 100 ng/μl; lane 4, 50 ng/μl; lane 5, 1×10^{1} ng/μl; lane 6, 1×10^{0} ng/μl; lane 7, 1×10^{-1} ng/μl; lane 8, 1×10^{-2} ng/μl; lane 9, 1×10^{-3} ng/μl; lane 10, 1×10^{-4} ng/μl; lane 11; 1×10^{-5} ng/μl; and lane 12, negative control. The bottom gel (b) represents a second test using a more narrow range of concentrations and two-fold dilutions: lane 1, 100-bp TriDye; lane 2, 1×10^{-2} ng/μl; lane 3, 5×10^{-3} ng/μl; lane 4, 2.5×10^{-3} ng/μl; lane 5, 1.25×10^{-3} ng/μl; lane 6, 6.25×10^{-4} ng/μl; lane 7, 3.125×10^{-4} ng/μl; lane 8, 1.56×10^{-4} ng/μl; lane 9, 7.81×10^{-5} ng/μl; lane 10, 3.9×10^{-5} ng/μl; lane 11, 1.95×10^{-5} ng/μl; lane 12, 9.7×10^{-6} ng/μl; lane 13, 4.8×10^{-6} ng/μl; and lane 14, negative control.

At plasmid DNA concentrations above 1×10^{-2} ng/μl, a band near 500 bp is generated (Fig. 3a). This artifact could be the result of a nonspecific binding site within the ITS2 locus. As the copy number of the 534-bp ITS2 sequence increases during PCR, it is possible that suboptimal binding becomes more common. This, however, does not explain why the 420-bp (F1-ITSR) band decreases in intensity as the amount of template increases (Fig. 3). The opposite effect is observed for the ITSF-ITSR band (Fig. 3). Perhaps the F1 primer binds more efficiently than the ITSF primer to genomic DNA but F1 does not bind as well to amplicons. This could cause a competition between primers based on the amount and source (i.e., genomic versus amplicon) of the template.

Based on these results, the assay is sufficiently sensitive to detect the light brown apple moth bands from general DNA extracts (with or without RNase treatment) and a plasmid concentration between 1.25×10^{-3} and 6.25×10^{-4} ng/μl (Fig. 3b, lanes 5 and 6) should work well as a positive control of the ITS2 tool.

Amplicon Profiles of Light Brown Apple Moth Samples. The ITS2 diagnostic marker successfully amplified DNA from all 337 light brown apple moth extracts included in the analysis (Table 1). Of these samples, 336 generated the two expected light brown apple moth bands. One extraction generated the smaller diagnostic (F1-ITSR) band but not the larger (ITSF-ITSR) band. Several extractions also produced additional nontarget amplicons that were much larger (e.g., Fig. 4a, lane 14) or smaller (e.g., Fig. 4a, lane 9) than the expected band sizes. Additional bands generated in these reactions are likely caused by variable amounts of DNA template in the reaction (i.e., the larger band occurs when greater DNA concentrations are used in the serial dilution test) and/or differences in the genetic background of the particular moth. These are regarded as PCR artifacts and do not interfere with amplification of the target bands.

Amplicon Profiles of Nontarget CDFA Query Moth Samples. Analysis of the CDFA nontarget data set produced a greater number of banding patterns than that expected based on the pilot study. Similar to the

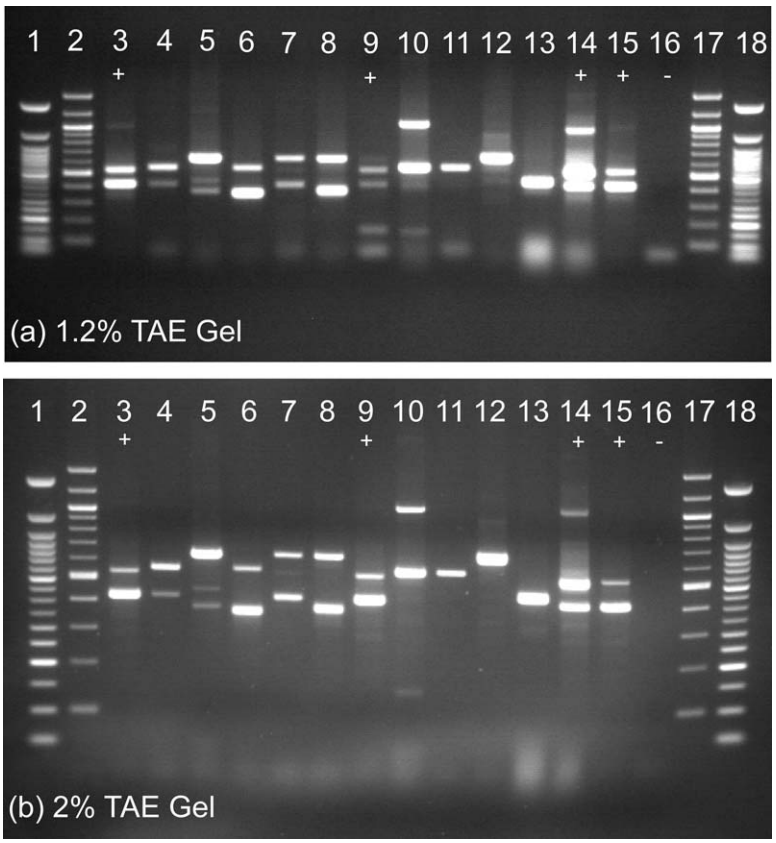

Fig. 4. Example profiles generated for the ITS2 marker when run on a 1.2% gel (a) and a 2% gel (b) by using general purpose agarose. Lanes 1 and 18, 50-bp DNA ladder (New England Biolabs); lanes 2 and 17, 100-bp TriDye ladder (New England Biolabs); lanes 3, 9, 14, and 15, light brown apple moth ; lane 4, nontarget (code DD); lane 5, nontarget; lane 6, nontarget (code KL); lane 7, nontarget (code F); lane 8, nontarget (code AL); lane 10, nontarget; lane 11, nontarget; lane 12, nontarget; lane 13, nontarget (single band near 420 bp—inconclusive result in ITS2 test); and lane 16, negative control.

pilot study result, 89.4% of the samples generated single bands that were distinct from the light brown apple moth-diagnostic band (e.g., Fig. 4a, lanes 11 and 12). ≈6% of the samples generated multiple bands that did not include both of the sizes expected for light brown apple moth , 3% of the samples failed to generate any PCR product, 0.5% produced two bands near the expected sizes for light brown apple moth , and 1% generated a single band close to the diagnostic (F1-ITSR) light brown apple moth band.

Four of the nontarget CDFA larval samples that generated amplicon patterns like the light brown apple moth profile were selected for further characterization by using PCR and DNA sequencing. To determine whether the light brown apple moth-like profiles were caused by the F1 primer, the four DNA extracts were amplified using the ITSF-ITSR primer pair and the F1-ITSR primer pair separately. These moths are referred to using the codes DD, KL, F, and AL (assigned during intake at the Mission Lab), and their respective ITS2 amplicon profiles are included in Fig. 4 (lanes 4, 6, 7, and 8). The DD sample generates two bands near the expected light brown apple moth bands under both 1.2% and 2% gel conditions. The KL, F, and AL samples generate profiles similar to the light brown apple moth profile under the 1.2% gel conditions. The results of the PCR and DNA sequence data are discussed below.

Light Brown Apple Moth-Like Amplicon Profiles of Nontarget Moth Samples Caused by Parasitoids. The KL and AL nontarget samples (Fig. 4a, lanes 6 and 8) generated the same light brown apple moth-like pattern when amplified using only the ITSF-ITSR primer pair. Amplification using the F1-ITSR primer pair generated no amplicons for these samples. These results suggest that the F1 primer was not causing the ≈400 bp amplicon for these two samples.

The larger and smaller bands (Fig. 4, lanes 6 and 8) of KL and AL generated using the ITSF-ITSR primers were gel excised and sequenced. The DNA sequence of the larger KL band (Fig. 4a, lane 6) matched best with the ITS2 sequence of *A. franciscana* (the only variation between the two sequences was a 2-bp gap). The DNA sequence of the larger AL band (Fig. 4a, lane 8) matched best with the ITS2 sequence of *P. stultana* (the only variation between the two sequences was a 3-bp gap).

The smaller amplicon for both of the samples generated sequences (GQ332418) consistent with Hymenoptera (Ichneumonoidea; based on BLAST searches of >90% similarity to 5.8S wasp sequences). Therefore, it is possible to amplify nontarget DNA from parasitoids or other animal contaminants using this protocol. Because a mixture of Lepidoptera and Hymenoptera ITS2 sequences can generate double band profiles similar to light brown apple moth , highly parasitized moth populations could be problematic for our ITS2 marker. Therefore, analysis using 2% gels is recommended to detect the difference in size between the diagnostic-light brown apple moth band and the hymenopteran ITS2 band (Fig. 4b, lane 6).

Light Brown Apple Moth-Like Amplicon Profiles of Nontarget Moth Samples Caused by Nonspecific Binding. The DD (Fig. 4a, lane 4) and F (Fig. 4a, lane 7) nontarget samples were amplified and sequenced using the ITSF and ITSR primers. Neither sample generated the light brown apple moth-like diagnostic amplicon (≈420 bp) by using the primer pair; but each sample did generate a single band of expected size for nontarget species. The ≈550-bp band from the DD sample matched best with the *A. franciscana* ITS2 sequence (differ by 2-bp gap; GQ332413) and the ≈640-bp band from the F sample was identical to the *P. stultana* ITS2 sequence (GQ332415).

PCR of these samples using the F1-ITSR primer pair generated no bands for the DD sample and a very faint band for the F sample. It is possible that the ≈400-bp amplicon generated from the DD extract is an artifact of PCR using the two forward primers (i.e., ITSF-F1 pair). There is no evidence that this artifact is caused by alternate binding sites within ITS2 because at least one other CDFA sample that has an identical DNA sequence (GQ332414) does not generate nonspecific bands. For the F sample, the F1 primer might be binding nonspecifically when sufficient template is available. The accumulation of ITSF-ITSR amplicon can provide additional sites for this nonspecific binding. Reduced intensity of the c. 400 bp amplicon when the ITSF primer is excluded is consistent with this explanation.

Additional samples that produced single amplicons similar in size to the diagnostic F1-ITSR band (e.g., Fig. 4a, lane 13; Table 2, inconclusive results) were also sequenced to confirm that the amplicons were of moth origin and not from a parasitoid. These sequences (N.B.B., unpublished) were distinct from other ITS2 sequences generated in our study, but the 5.8 and 28S portions matched with lepidopteran sequences reported in our data set and in GenBank. They were not consistent with hymenopteran DNA sequences.

Development of Interpretation Rules. Based on the agarose gel electrophoresis banding patterns and DNA sequences of the light brown apple moth and nontarget species, a positive identification (i.e., a genetic profile consistent with the light brown apple moth profile) is restricted to multiplexed amplicons that include both the 534 bp (ITSF-ITSR) and 420 bp (F1-ITSR) bands. This rule is included in the tool because some moth DNA extracts can generate amplicons similar in size to the diagnostic band (e.g., parasitoids, nonspecific binding sites, and shorter moth copies of the ITS2 locus) and the larger band functions as a control in the assay. Consequently, amplification profiles that include a single PCR product similar to the diagnostic light brown apple moth band are treated as inconclusive.

Table 3. Rules for interpreting ITS2 banding profiles on agarose gels[a]

Rule	Observation	Conclusion
1	No amplification	Inconclusive
2	Two bands near 534 and 420 bp (ignoring additional bands)	Consistent with LBAM
3	A profile including two or more bands but not including both the 534- and 420-bp bands	Not consistent with LBAM
4	A profile of one band that is not equal to the diagnostic 420-bp band	Not consistent with LBAM
5	A profile including one band that is equal to the diagnostic 420-bp band	Inconclusive

[a] The gel conditions described in the study do not allow resolution of bands to the exact predicted sizes; consequently, bands should be scored as near 534 bp (i.e., between 500 and 550 bp) and 420 bp (i.e., between 400 and 450 bp).

It is also possible for the three primers (or PCR products) to bind nonspecifically in the genome and generate additional bands during the PCR. In Fig. 4 (lane 14) a third band of ≈1 kb is produced for the light brown apple moth profile. Although this band contains the light brown apple moth ITS2 sequence, it is probably an artifact of PCR that generates tandem copies of the locus (Figs. 2 and 3). Similarly, smaller bands (Fig. 4a, lane 9) are generated for some reactions. Consequently, these extra bands are regarded as artifacts of nonspecific binding (with the genome or primer sequences) and are treated as nondata in the diagnostic tool. The exclusion of such amplicons as data in the tool simplifies interpretation of true positives based on light brown apple moth data and does not affect the scoring of negatives.

PCR failure (i.e., generation of no amplicons) using this diagnostic assay is regarded as an inconclusive result. All templates are expected to generate a band using the highly conserved ITSF-ITSR primers. The absence of data has no taxonomic meaning in the assay because all moths should generate data.

Amplicon profiles that include a single band (that is not near 420 bp) are treated as not consistent with the light brown apple moth genetic profile (i.e., negative identifications). Similarly, PCR amplicon profiles that include multiple bands but do not include both of the expected bands (534 bp + 420 bp) for the light brown apple moth profile also are treated as not consistent with the light brown apple moth profile (i.e., negative identifications). Rules for interpretation of ITS2 amplicon profiles are given in Table 3.

Results of PCR Assay of Moth Extracts Using the ITS2 Marker. The protocol for PCR of the ITS2 marker successfully amplified from all 342 light brown apple moth extracts included in the analysis (Table 1). No false negatives were observed and only one moth

produced inconclusive results. The inconclusive score was the result of a profile without a visible ITSF-ITSR band. The F1-ITSR (diagnostic) band amplified successfully from all the light brown apple moth extractions. Using this method >99% of the samples produced positive results.

Of the 424 nontarget samples tested (414 CDFA + 10 reference samples, Table 2) only 3.3% failed to amplify using the marker. Excluding PCR failure, the success rate of correctly identifying the nontarget samples was 98.5%. The rates of false positives and inconclusive data were <0.5% and <1%, respectively.

Assessment and Application of the ITS2 Marker. In this study we have reported a new molecular protocol for the identification of light brown apple moth in California. Based on available samples, our protocol generates no false negatives and a low level of false positives under the conditions tested. This method is less sensitive than the currently applied DNA barcoding technique for light brown apple moth identification; 100% of the CDFA samples amplified using the COI barcode, but only 97% amplified using the ITS2 marker.

The DNA barcode method also provides a greater level of taxonomic information that can be used to identify nontarget moths. The ITS2 marker using PCR is based on amplicon patterns and is intended to assess whether a profile is consistent with light brown apple moth or not. Our protocol does not currently discriminate among the possible nontarget species (although there is the potential to distinguish some taxa using this approach).

It is also important to note that the tool was developed to analyze moths within California. Consequently it does not include genetic patterns for many moth species that might be encountered outside of California or at ports of entry. Application of the tool to samples from outside of California or the United States could increase the rate of false positives but should not affect the rate of false negatives.

The advantage of using the ITS2 marker is that it is less expensive to run than DNA barcoding in terms of labor, reagents, and equipment. This is important when large numbers of moths are to be analyzed and the results are used to determine a regulatory action with associated costs. Given the various strengths and weaknesses of the available ITS2, COI barcode, and morphological tools, it is possible to arrange light brown apple moth identification programs to integrate all three. For example, it is possible to use the ITS2 marker to screen large samples of immature moths and then use the COI DNA barcode to confirm positives or identify nontargets that generate novel ITS2 profiles.

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