

## RESEARCH

# Rapid Identification of *Helicoverpa armigera* and *Helicoverpa zea* (Lepidoptera: Noctuidae) Using Ribosomal RNA Internal Transcribed Spacer 1

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**ABSTRACT.** Rapid identification of invasive species is crucial for deploying management strategies to prevent establishment. Recent *Helicoverpa armigera* (Hübner) invasions and subsequent establishment in South America has increased the risk of this species invading North America. Morphological similarities make differentiation of *H. armigera* from the native *Helicoverpa zea* (Boddie) difficult. Characteristics of adult male genitalia and nucleotide sequence differences in mitochondrial DNA are two of the currently available methods to differentiate these two species. However, current methods are likely too slow to be employed as rapid detection methods. In this study, conserved differences in the internal transcribed spacer 1 (ITS1) of the ribosomal RNA genes were used to develop species-specific oligonucleotide primers that amplified ITS1 fragments of 147 and 334 bp from *H. armigera* and *H. zea*, respectively. An amplicon (83 bp) from a conserved region of 18S ribosomal RNA subunit served as a positive control. Melting temperature differences in ITS1 amplicons yielded species-specific dissociation curves that could be used in high resolution melt analysis to differentiate the two *Helicoverpa* species. In addition, a rapid and inexpensive procedure for obtaining amplifiable genomic DNA from a small amount of tissue was identified. Under optimal conditions, the process was able to detect DNA from one *H. armigera* leg in a pool of 25 legs. The high resolution melt analysis combined with rapid DNA extraction could be used as an inexpensive method to genetically differentiate large numbers of *H. armigera* and *H. zea* using readily available reagents.

**Key Words:** *Helicoverpa*, bollworm, early detection, invasive species, melt curve

Rapid detection of invasive species is of paramount importance for land management and conservation practices. Invasive species are estimated to cost the United States more than \$120 billion in damages annually (Pimentel et al. 2005) and result in a major loss of biodiversity (Sala et al. 2000). Accurate identification of invasive species is crucial for early preventive strategies. Alternative eradication strategies are more expensive and generally have limited success once established (Lodge et al. 2006).

The bollworms, *Helicoverpa armigera* (Hübner) and *Helicoverpa zea* (Boddie) are two of the most damaging pests of agricultural crops around the world. Until recently, *H. armigera* was not established in North or South America. *H. zea* is a native and serious agricultural pest in the Americas (Metcalf and Flint 1962, Hardwick 1965) and *H. armigera* recently established reproducing populations in Brazil (Czepak et al. 2013, Tay et al. 2013), Argentina, and Paraguay (Murúa et al. 2014), placing North America at risk of invasion by *H. armigera*. The Animal and Plant Health Inspection Service of the United States Department of Agriculture (USDA-APHIS) reported detection of *H. armigera* in Puerto Rico in 2014 (North American Plant Protection Organization [NAPPO] 2014), and a single *H. armigera* moth was captured in June 2015 in a pheromone trap in Bradenton, Florida (Anonymous 2015). Continued surveillance of this invasive pest is important for agricultural production in the United States.

Differentiation of *H. armigera* and *H. zea* is difficult, since larvae and adults are morphologically similar. Identification by morphological traits requires examination of adult male genitalia (Siverly 1947, Lödl 2001, Pogue 2004). Alternative molecular approaches include mitochondrial cytochrome oxidase 1 barcode region sequencing and restriction fragment length polymorphism (RFLP) analysis of polymerase chain reaction (PCR)-amplified mitochondrial DNA (mtDNA) (Folmer et al. 1994, Behere et al. 2008, Mastrangelo et al. 2014). There are several other

potential methods based on enzyme-linked immunosorbent assays (ELISA), which assess antigen variation for differentiation of other closely related lepidopteran pest species. ELISA has been used to distinguish *H. armigera* and *Helicoverpa punctigera* (Wallengren) (Trowell et al. 2000), eggs of *Heliothis virescens* (F.) and *H. zea* (Greenstone 1995, Zeng et al. 1998), and *Helicoverpa* species and *Helio. virescens* (Goodman et al. 1997). Near-infrared spectroscopy has been used to distinguish *H. zea* and *Helio. virescens* (Jia et al. 2007). However, these methods have not been evaluated for their ability to distinguish *H. armigera* from *H. zea*. Additionally, mtDNA sequence-based methods have been developed for identification of several *Helicoverpa* species (Behere et al. 2008, Mastrangelo et al. 2014). While these molecular methods are capable of differentiating *H. armigera* and *H. zea*, they require long process times, and it is difficult to scale up detection to analyze large number of samples. This makes barcode sequencing and RFLP-based methods inefficient for rapid detection. Alternatively, high resolution melt (HRM) analysis is capable of differentiating small differences in melting temperature ( $T_m$ ) of PCR products. HRM is a fast method for detecting small molecular differences that could be scaled up for species identification (Winder et al. 2011).

The aim of this study was to identify molecular methods to screen and differentiate large numbers of *Helicoverpa* moth samples that could rapidly detect *H. armigera* in North America. Repetitive DNA sequences with conserved nucleotide sequences within a species should provide both the sensitivity and species specificity required for molecular assays. Ribosomal RNA (rRNA) in most eukaryotes exists as clusters of tandemly arrayed multicopy genes (Arnheim et al. 1980, Coen et al. 1982). rRNA genes in animals also undergo concerted evolution to preserve homogeneity of rRNA repeat units within a cluster (Mullins and Fultz 1989, Schlötterer and Tautz 1994). In animals, rRNA gene copy numbers range from 39 to 19,300 (Prokopowich et al. 2003).

Therefore, conserved differences in rRNA genes between different species are suitable targets for developing molecular species identification tools. Intergenic spacer and internal transcribed spacer (ITS) regions of rRNA genes have been used to develop species identification methods for mosquitoes (Collins and Paskewitz 1996, Perera et al. 1998). In this study, a molecular method based on conserved differences within ITS (ITS1) of rRNA genes of *H. armigera* and *H. zea* were used to develop species-specific primers to positively identify each species. HRM analysis was then used to rapidly measure  $T_m$  differences in ITS1 amplicons for semiautomated species identification. In addition, a process for rapidly obtaining genomic DNA for PCR from a small amount of tissue (e.g., a single neonate or a single leg from an adult) was optimized to further expedite the detection process. These tools could be scaled up to screen large numbers of samples within a short time to expedite *H. armigera* detection programs.

## Materials and Methods

**Insects.** Cloning and sequencing of the 18S rRNA subunit sequence and ITS1 were initially conducted using insects obtained from a *H. armigera* colony maintained at the Department of Genetics, University of Valencia, Valencia, Spain ( $n = 7$ ). In addition, insects from laboratory colonies of *H. zea* ( $n = 5$ ), and *Helio. virescens* ( $n = 2$ ) maintained at the USDA-ARS Southern Insect Management Research Unit and *Heliothis subflexa* (Guenée) ( $n = 1$ ) obtained from North Carolina State University were used to obtain ITS1 nucleotide sequences. Subsequent development and validation of the species-specific primers for ITS1 were carried out using preserved *H. armigera* specimens collected from Australia ( $n = 45$ ), China ( $n = 48$ ), India ( $n = 36$ ), New Zealand ( $n = 15$ ), and Kenya ( $n = 12$ ), one  $F_1$  hybrid male (a cross between a female *H. zea* and a male *H. armigera*), and two *Helicoverpa assulta* (Guenée) preserved as pinned specimens in the USDA-ARS Southern Research Quarantine Facility in Stoneville, MS (Laster and Sheng 1995). Exact geographical coordinates were not available for pinned *H. armigera* specimens preserved from 1983 to 1993. *H. zea* ( $n = 384$ ) were collected across the Mississippi Delta from field locations previously described (Ali et al. 2006, Perera and Blanco 2011).

**DNA Extractions.** DNA extractions from thorax tissue were performed using MasterPure DNA extraction reagents (Epicenter Technologies, Madison, WI) following manufacturer's instructions. Briefly, tissue samples were homogenized in 188  $\mu$ l of lysis buffer supplemented with 2  $\mu$ l of 20 mg per ml proteinase K (Life Technologies, Carlsbad, CA) and incubated at 65°C for 1 h. At the end of incubation period, the tissue homogenates were equilibrated to 37°C and 10  $\mu$ l of 2 mg per ml RNase A solution was added, mixed gently by inverting tubes several times, and incubated at 37°C for 30 min. At the end of RNase digestion, 120  $\mu$ l of protein precipitation solution was added to each tube and incubated on ice for 15 min prior to centrifugation at 12,000  $\times g$  for 10 min at 4°C to pellet protein and other contaminants. The supernatant from each tube was carefully

aspirated and transferred to a new tube, and 200  $\mu$ l of 100% isopropanol (Sigma-Aldrich, St. Louis, MO) was added and mixed by inverting the tubes 25 times. The tubes were incubated at room temperature for 10 min before centrifuging at 16,000  $\times g$  for 15 min at 4°C to pellet DNA. Supernatant was discarded, and the DNA pellets were washed twice with 70% ethanol (Sigma-Aldrich, St. Louis, MO). After the second wash, the DNA pellets were air-dried at room temperature and resuspended in 35  $\mu$ l of 10 mM Tris-HCl, pH 7.4. All DNA preparations were quantified using a NanoDrop 2000C instrument (NanoDrop, Wilmington, DE) and stored at -20°C until used in experiments. Genomic DNA extracted from the legs of the pinned specimen of the  $F_1$  hybrid male (Laster and Sheng 1995) and *H. assulta* were repaired with preCR DNA repair reagents following manufacturer's instructions (New England BioLabs, Ipswich, MA) prior to PCR amplification of ITS1.

**ITS1 and 18S rRNA Subunit Sequencing.** To amplify and sequence 18S rRNA subunit and ITS1, primers for *Helicoverpa* 18S and 5.8S rRNA subunits (flanking regions of the ITS1) were developed by aligning previously cataloged ITS1 sequences of *H. armigera* (Ji et al. 2003 and accessions AB620127.1 and AF401740.1), *Attacus ricini* (Wang et al. 2003, accession AF463459.1), *H. assulta* (accession EU057177.1), *Papilio xuthus* (Futahashi et al. 2012, accession AB674749.1), and *Spodoptera exigua* (Wu et al. 2012, accession JN863293.1), obtained from public databases (Table 1). These primers were then used to amplify 18S and ITS1 regions of each species using Crimson LongAmp *Taq* polymerase (New England BioLabs, Ipswich, MA). The reactions contained 1X Crimson Long Amp buffer (12.5 mM tricine, 42.5 mM KCl, 1.5 mM MgCl<sub>2</sub>, 6% dextran, and acid red), 0.32  $\mu$ M dNTP mix, 0.4  $\mu$ M primer mix for each amplicon, 1 unit of Crimson LongAmp *Taq*, and 10–100 ng of genomic DNA in a 25  $\mu$ l reaction. Amplification reactions were performed in a PTC-100 thermal cycler (MJ Research, BioRad, Hercules, CA) with a 45 s initial denaturation step at 95°C, 35 cycles of 15 s denaturation at 95°C, 10 s annealing at 52°C, and 4 min extension at 72°C followed by final extension of 5 min at 72°C. Amplicons were resolved in 1.2% agarose in 1X Tris-Acetate-EDTA buffer (40 mM Tris-acetate, 0.1 mM EDTA, pH 7.4). Amplicons of products were cloned into pCR2.1 T-A cloning vector (Life Technologies, Carlsbad, CA) following manufacturer's instructions. At least eight independent clones from each amplicon were sequenced using universal M13 forward and reverse primers followed by gene-specific primers designed to conserve regions of 18S, 5.8S rRNA subunits (Table 1). Nucleotide sequencing was performed at the USDA-ARS Genomics and Bioinformatics Research Unit, Stoneville, MS. Vector NTI Suite v11.5 was used for nucleotide sequence assembly, alignment, manual curation, annotation, and primer design. All primers used in this study were purchased from Integrated DNA Technologies (Coralville, IA).

**Species-Specific Primer Development and Melt Curve Analysis.** An alignment of ITS1 of *H. armigera*, *H. assulta*, *H. zea*, and *Helio. virescens* was used to search for species-specific nucleotide sequences

**Table 1. Nucleotide sequences of primers used for PCR amplification, 18S rRNA, and ITS1 sequencing, and qPCR assays**

Primer name	Sequence	Target	Purpose
310-18S-rRNA-F	ATCATTAGAGGAAGTAAAAGTCGTAACAAGGT	3'-end of 18S	ITS1 PCR and sequencing
312-5.8S-rDNA-R	GAARTGTGCATGTTCAAATGTGTCCTGC	5'-end of 5.8S	ITS1 PCR and sequencing
412_18S_1893R	TTCMCCTACGGAAACCTTGTTACGACTT	3'-end of 18S	18S rRNA PCR per sequencing
3355_18S_80F	AAGCGGATACCCGAATGGCT	18S	18S rRNA PCR per sequencing
3356_18S_196R	CACTGTGCAGATTTCTGATTGCA	18S	18S rRNA sequencing
3357_18S_800F	GTGCTCTCGGTGAGTGTGAGG	18S	18S rRNA sequencing
3358_18S_858R	CAGCATTTTGAGCCCGCTTG	18S	18S rRNA sequencing
3359_18S_1418F	TAACGAACGAGACTCTAGCCTGC	18S	18S rRNA sequencing
3373Ha_Hz ITS1-F	GAGGAAGTAAAAGTCGTAACAAGTTTCC	ITS1	Common forward
3374Ha ITS1-R	CGTTGCACTGTGTCCTCTAGTGG	ITS1	<i>H. armigera</i> -specific reverse
3377Hz ITS1-R	TTGATTGTTAACGAACGCGCCG	ITS1	<i>H. zea</i> -specific reverse
3695_18S_1150F	GCAGCTCCGGGAAACCAA	18S	18S Control amplicon
3696_18S_1232R	GCCCTTCGTCAATTCCTTAAGT	18S	8S Control amplicon

suitable for PCR primers. Nucleotide sequence differences sufficient to design primers to amplify species-specific amplicons were identified in ITS1. Two different primer mixes for ITS1, each containing one primer common to both species and one primer specific to *H. armigera* or *H. zea* were designed and tested. Initial tests of species-specific fragment amplification were carried out using Crimson LongAmp *Taq* polymerase with a 45 s initial denaturation step at 95°C followed by 35 cycles of 15 s denaturation at 95°C, 10 s annealing at 60°C, and 30 s extension at 72°C. Upon verification of amplicon sizes expected from each species by agarose gel electrophoresis and nucleotide sequencing, the primer mixes were tested in an ABI7500-Fast instrument to evaluate the feasibility of using melt curve analysis to distinguish *H. armigera* from *H. zea*. First, approximately 25 ng of genomic DNA extracted from each species was used in a 20 µl amplification reaction containing 1X TaqMan Fast master mix (Life Technologies, Carlsbad, CA), 5 µM SYTO-9 dye (Life Technologies, Carlsbad, CA), and 0.4 µM primer mix. After initial denaturation for 1 min, 40 cycles of amplification with a 5 s denaturation step at 95°C and 30 s anneal and extension step at 60°C were performed. Amplification of unique products was verified by dissociation curve analysis and gel electrophoresis of amplification products. Dissociation (or melt) curve analysis is performed by gradually increasing the temperature of the reaction while continually recording the fluorescence produced by dye molecules (fluorophores) intercalated to double-stranded DNA (dsDNA). As the rising temperature denatures dsDNA, fluorophores dissociate from dsDNA leading to a gradual loss of fluorescence. The rate of fluorescence loss increases when the temperature approaches the  $T_m$  of a dsDNA. The rate of dsDNA melting (inferred using the rate of fluorescence loss) is determined by plotting the negative first regression of relative fluorescence against the change of temperature. This plot (dissociation curve) yields a peak that represents the  $T_m$  of the dsDNA molecules present in the reaction. If multiple dsDNA molecules with sufficiently different  $T_m$  are present in a reaction, multiple peaks representing  $T_m$  of each dsDNA molecule may be observed in a dissociation curve. The dissociation curve analysis of amplicons consisted of an initial denaturation step of 15 s at 95°C, annealing step of 1 min at 60°C followed by ramping of temperature to 95°C at a rate of 0.1°C per second. The HRM analysis was performed by importing dissociation curve data into the HRM v 2.0 software (Life Technologies, Carlsbad, CA).

After optimization of the species-specific primer combination, a primer pair (Table 1) designed to a conserved nucleotide sequence region of 18S rRNA subunit was used to amplify a positive control amplicon that would be amplified from all heliothine species listed previously. In HRM analysis, this positive control amplicon would generate a peak that is distinct from peaks generated by species-specific amplicons of *H. armigera* and *H. zea*. This amplicon would be absent in failed amplification reactions and would be the only amplicon present in a reaction containing DNA from a heliothine species other than *H. armigera* or *H. zea*. Concentrations of 18S and ITS1 primers in amplification reactions were optimized by testing final concentrations of 0.025, 0.05, 0.1, and 0.2 µM of 18S primer pair with 0.2, 0.3, and 0.4 µM of species-specific ITS1 primers 3374 and 3377. Common ITS1 primer 3373 was kept constant at 0.4 µM in all reactions.

Limits of detection was examined using genomic DNA from *H. zea* and *H. armigera* quantified using Qbit DNA quantification system (Life Technologies, Carlsbad, CA). Five serial dilutions ranging from approximately 1 ng per µl to 0.1 pg per µl were prepared using the genomic DNA of each species. Triplicate amplifications of standards and genomic DNA dilutions were used in quantitative PCR (qPCR) analysis on an ABI7500-Fast instrument using the thermal cycling profile previously described for HRM analysis. Experimental design was identical to that of HRM analysis described above.

**Evaluation of the Best Methods for Obtaining Amplifiable Genomic DNA.** Screening large numbers of samples to identify invasive insects is greatly facilitated by cost-effective and rapid DNA isolation methods. Quantitative real-time PCR generally requires very small

amounts of DNA, but polymerases could be inhibited by impurities carried over from crude extractions. To identify DNA isolation methods suitable for species-specific qPCR, two different rapid DNA isolation methods were evaluated. The reagents evaluated were DNAzol Direct (Molecular Research Center, Inc., Cincinnati, OH), a commercial product marketed for direct PCR amplification of lysates, and a “squish” buffer modified from Gloor et al. (1993). To prevent inhibition of PCR amplification, the squish buffer formulation was modified by eliminating proteinase K and reducing EDTA and NaCl concentrations by 50% to obtain final concentrations of 10 mM Tris-HCl, 0.5 mM EDTA, 12.5 mM NaCl, and pH 8.2. One or two legs of adult moths or one neonate of *H. zea* was used with each reagent. Each sample was placed in a single well of a 96-well PCR plate. Two stainless steel ball bearings (2 mm) were placed in each well followed by addition of 25 µl of squish buffer or DNAzol Direct to each well. Ten replicates of *H. zea* DNA isolations were prepared per sample (neonate or legs) per reagent type. The PCR plate was covered with an adhesive film and the samples were homogenized for 5 min using a Mini-BeadBeater-96 (BioSpec Products, Inc., Bartlesville, OK) and centrifuged briefly at 3,700 × *g* in an Eppendorf 5810R centrifuge (Eppendorf AG, Hamburg, Germany) to bring the liquid to the bottom of wells. It was then incubated at 80°C for 10 min on a thermal cycler block. After heating, the samples were centrifuged at 3,700 × *g* for 5 min to pellet tissue debris. The lysates were tested first by PCR amplification using the species-specific primer cocktail, and the amplicons were resolved on a 1.2% agarose gel with Tris-Acetate-EDTA buffer system (40 mM Tris-Acetate, 0.1 mM EDTA). After verification of amplification, dissociation curve analysis was performed using 1 µl of lysate and real-time PCR conditions, described earlier.

Pooling DNA from two or more insects could be used to increase the sample screening throughput. In the event a *H. armigera* was pooled with one or more *H. zea*, the peak profile generated in HRM analysis would be similar to that of a hybrid. The ability to detect *H. armigera* in a pool of two or more insects per reaction was tested by mixing *H. armigera* and *H. zea* DNA in 1:1, 1:4, 1:9, 1:14, 1:19, and 1:24 ratios. Lysates of adult legs were produced using 25 µl of modified squish buffer per leg. Lysates were clarified by centrifugation and *H. armigera* and *H. zea* leg lysates were mixed in separate tubes in the ratios given above. In addition, adult *H. armigera* legs (freeze dried and shipped from Australia at ambient temperature) and legs of *H. zea* males captured in pheromone traps (stored at ambient temperature for 60 days) were pooled 1:19 and 1:24 ratios in a 1 ml deep-well plate and homogenized in 500 and 625 µl, respectively, of modified squish buffer. Legs were homogenized in a mini bead-beater using 4 mm steel balls and the lysates were processed as described previously to obtain clarified homogenates. Ten technical replicates were used to test each DNA ratio using 1 µl of the lysate pool and the primer combination containing optimal concentrations of the primers for 18S rRNA control and the species-specific amplicons. All amplifications with pooled tissue lysates were optimized using KAPA SYBR Fast 2X qPCR Master Mix (KAPA Biosystems, Boston, MA, Item no. KK4604) and LongAmp *Taq* DNA polymerase and buffer (New England BioLabs, Ipswich, MA, Item no. M0323S).

## Results

**ITS1 Sequencing and HRM-Based Species Identification.** Complete 18S rRNA and ITS1 sequences of *H. armigera*, *H. zea*, and *Helio. virescens*, and ITS1 sequences of *H. assulta*, the F<sub>1</sub> hybrid male (*H. zea* ♀ × *H. armigera* ♂), and *Helio. subflexa* were deposited in GenBank (accession numbers KT343375.1 through KT343382.1, and KT762150.1). Nucleotide sequences of ITS1 amplicons from the F<sub>1</sub> hybrid male identified two ITS1 sequences, one matching *H. zea* (accession no. KT343375.1) and the other matching *H. armigera* (accession no. KT343376.1). Nucleotide sequences of ITS1 of *H. armigera* from Australia, China, India, and Kenya were 97, 98, 98, and 98%



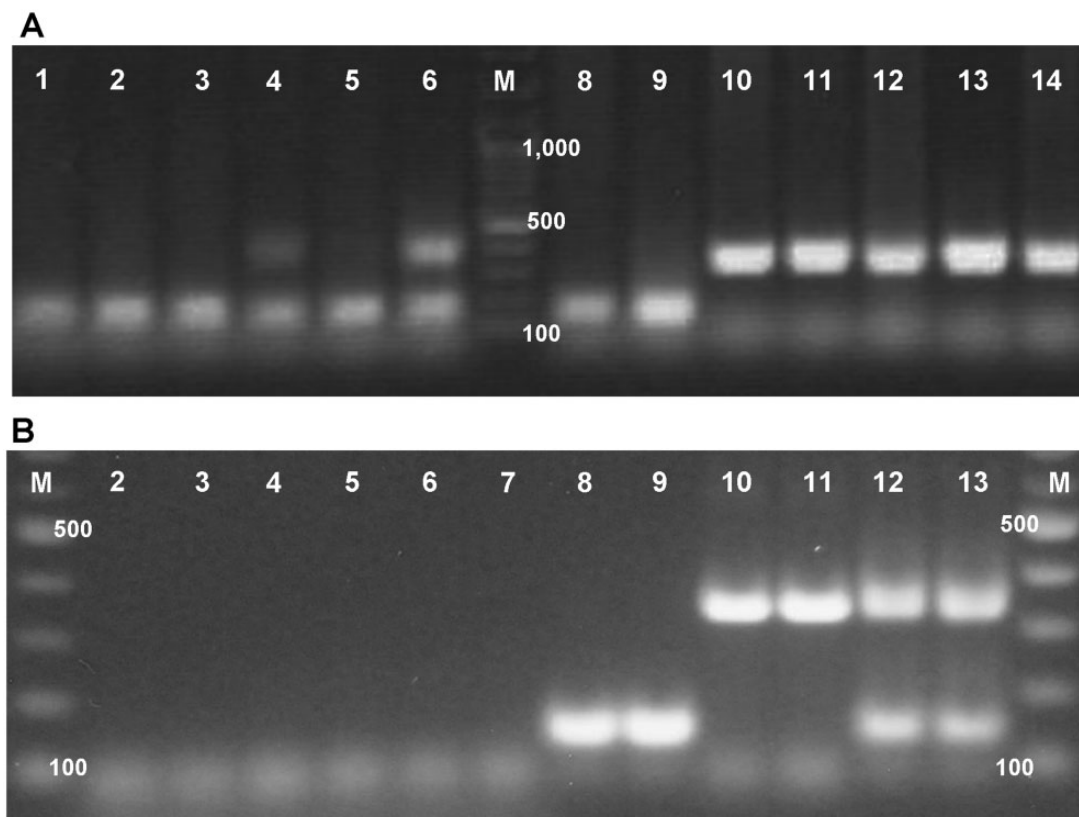
efficiency of the 314 bp *H. zea* ITS1 fragment from the F1 hybrid was low compared with the artificial hybrid. DNA degradation in the preserved F1 hybrid was the most likely reason for inefficient amplification of the larger ITS1 fragment of *H. zea* compared with the smaller (147 bp) ITS1 fragment from *H. armigera*.

During the second phase of assay development, amplifications were also performed using the species-specific primer set (3373, 3374, and 3377) combined with the primer pair for 18S rRNA subunit (3695 and 3696) designed to generate positive control amplicon. Optimization experiments identified that final concentrations of 0.1  $\mu$ M of 18S primer pair (3695 and 3696), 0.4  $\mu$ M of common forward primer (3373) and *H. armigera*-specific primer (3374), and 0.2  $\mu$ M *H. zea*-specific primer (3377) provided the best results when DNA was pooled (Fig. 2b). Only the positive control 18S rRNA amplicon (83 bp) was amplified from the nontarget species *H. assulta*, *Helio. subflexa*, and *Helio. virescens* (Fig. 2b, Lanes 2–3, 4–5, and 6–7, respectively). DNA from target species *H. armigera* and *H. zea* produced species-specific amplicons and the positive control amplicon (Fig. 2b, Lanes 8–9 and 10–11, respectively). The artificial hybrids produced amplicons specific to both parent species and the control amplicon (Fig. 2b, Lanes 12 and 13)

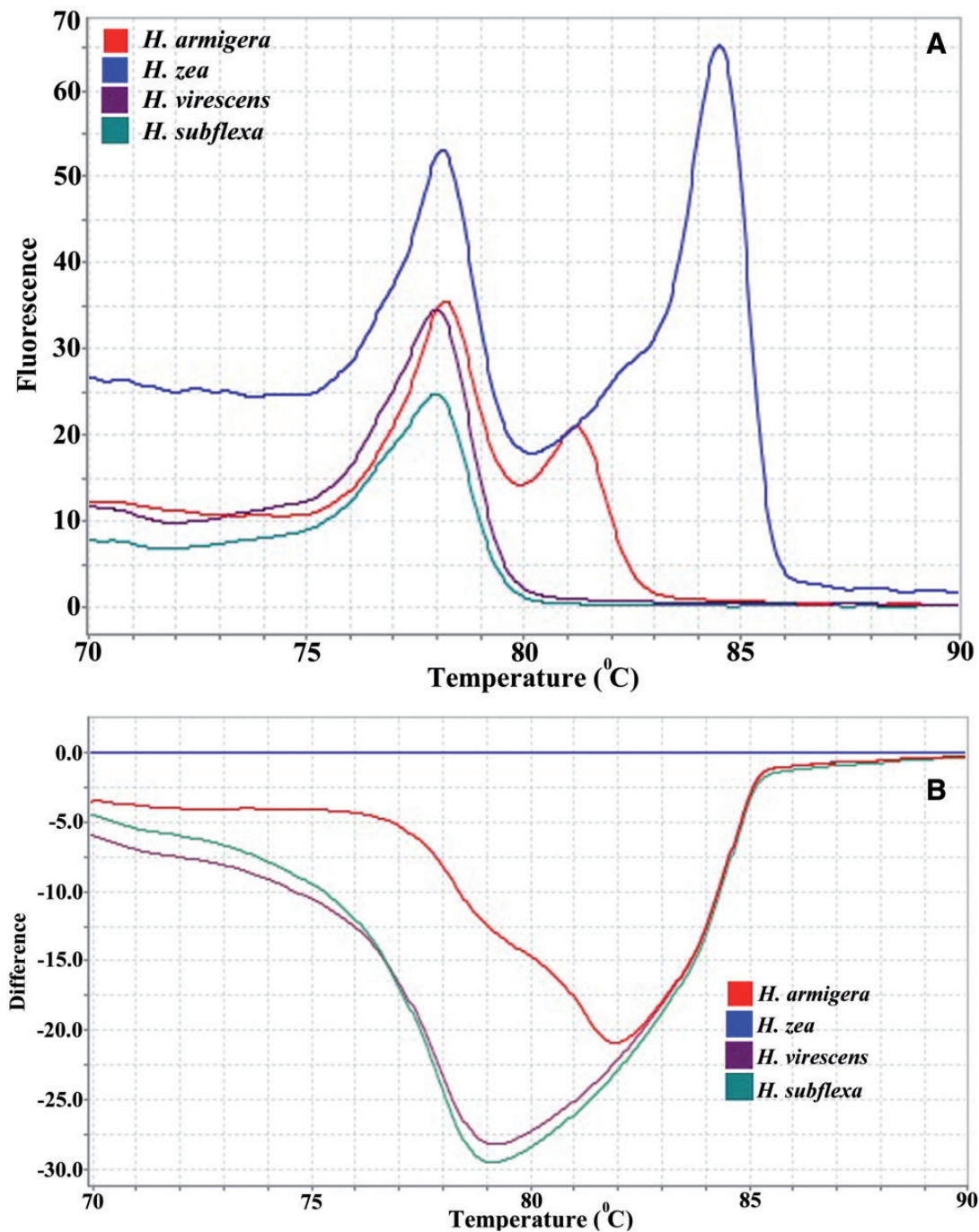
Dissociation curve analysis indicated ITS1 amplicon melting temperatures ( $T_m$ ) of 81.2°C for *H. armigera* and 84.6°C for *H. zea* (Fig. 3a). The empirical  $T_m$  value for *H. armigera* was similar to the software predicted  $T_m$  value of 81.5°C. The 18S rRNA control amplicon had predicted and empirical  $T_m$  values of 78.6 and 78.3, respectively. However, the  $T_m$  value of 90.2°C predicted for *H. zea*-specific ITS1 amplicon was 5.6°C higher than the actual  $T_m$ . This discrepancy is most likely a result of using prediction algorithms designed for calculating

$T_m$  of short oligonucleotide sequences for calculating the  $T_m$  of a larger DNA molecule.

When HRM analysis was performed on the DNA amplified with optimal concentrations identified for 18S rRNA control primers and the species-specific ITS1 primers, only the peak representing the control amplicon (18S rRNA subunit) was produced in the reactions with DNA from *Helio. subflexa* and *Helio. virescens* (Fig. 3a). DNA from *H. armigera* and *H. zea* yielded peaks representing both the control amplicon and the amplicon specific to each species (Fig. 3a). The difference plot produced by HRM v2.0 software with *H. zea* as reference (Fig. 3b) produced distinct plots for *H. armigera* and *H. zea* that distinguished them from nontarget species *Helio. subflexa* and *Helio. virescens* (Fig. 3b). In addition to the species-specific dissociation curves produced by *H. armigera* and *H. zea*, DNA from artificial hybrids (mixture of DNA from both species) produced dissociation curves containing three peaks (Fig. 4a). These three peaks represented melting points of the 18S rRNA control amplicon and the species-specific amplicons of *H. armigera* and *H. zea*. The difference plots generated using the dissociation curves of each species and the hybrids were also readily distinguishable from each other (Fig. 4b). It was noted that the height of the dissociation curve peaks were directly correlated to the length of the amplicon and the quantity of amplified product. This effect is due to proportional variation in the strength of fluorescence signal from SYTO-9 dye intercalated to DNA molecules as observed with a low peak height of the 147 bp *H. armigera*-specific ITS1 amplicon compared with the 334 bp *H. zea*-specific amplicon (Figs. 3a and 4a). When dissociation curves were analyzed using HRM software, distinct difference plots were generated for *H. armigera*, *H. zea*, and the F<sub>1</sub> hybrid male (Figs.



**Fig. 2.** Gel images of ITS1 amplicons from a representative set of DNA amplified using the oligonucleotide primer mix developed for species detection. (A) Amplicons produced with common (3373) primer, and primers specific to *H. armigera* (3374) and *H. zea* (3377) and 0.4  $\mu$ M final concentration. Lanes 1–3, 5, 8, and 9: 147 bp amplicon of *H. armigera*; Lanes 10–14: 314 bp amplicon of *H. zea*; Lane 4: F1 male hybrid of *H. zea* and *H. armigera*; Lane 6: a mixture of *H. zea* and *H. armigera* DNA. (B) Amplicons produced with optimal concentrations of primers for species-specific amplicons and the 83 bp 18S rRNA subunit control amplicon. Lanes 2 and 3: *H. assulta*; Lanes 4 and 5: *Helio. subflexa*; Lanes 6 and 7: *Helio. virescens*; Lanes 8 and 9: *H. armigera*; Lanes 10 and 11: *H. zea*; Lanes 12 and 13: mixed *H. armigera* and *H. zea* DNA. M: 2- $\log$  DNA ladder (New England Biolabs, Ipswich, MA) with major DNA band sizes shown in base pairs.

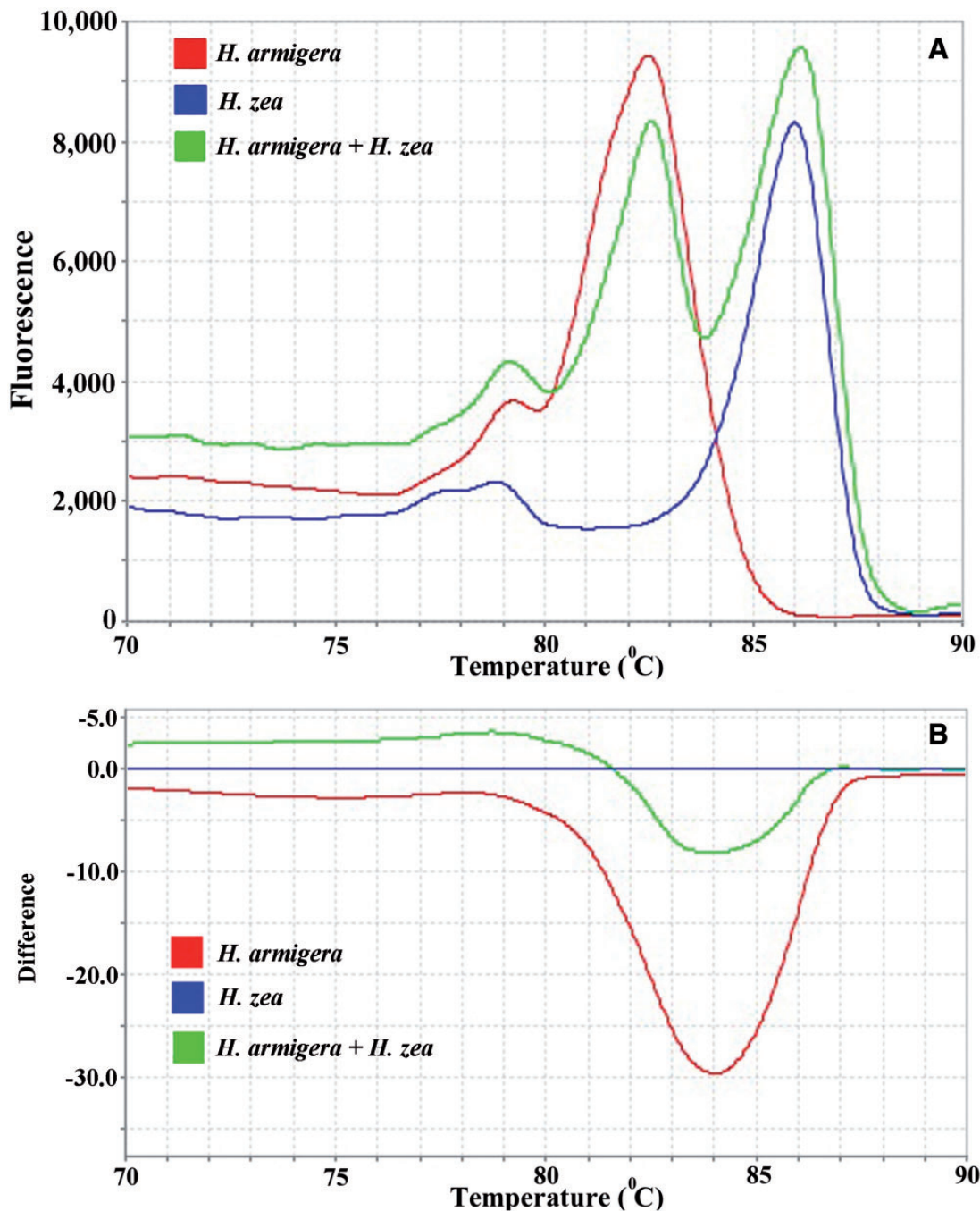


**Fig. 3.** Derivative dissociation curves and difference plots produced by the control and species-specific amplicons of *H. armigera*, *H. zea*, *Helio. subflexa*, and *Helio. virescens*. (A) Derivative dissociation curves of *H. armigera* (red) and *H. zea* (blue), *Helio. subflexa* (green), and *Helio. virescens* (purple). The peaks produced by 18S rRNA subunit amplicon (~78°C) is present in all species. Dissociation curves of *H. armigera* and *H. zea* also contains peaks specific to each species. (B) Difference plot of dissociation curves generated by HRM v2.0 software using *H. zea* as the standard.

3b and 4b), facilitating reliable, semiautomated identification of *H. armigera*, *H. zea*, and F<sub>1</sub> hybrids.

**Evaluation of Detection Limits and DNA Isolation Methods.** Genomic DNA serially diluted from 1 ng to 0.1 pg resulted in adequate amplification of species-specific amplicons from both *H. armigera* and *H. zea* although DNA concentrations above 10 pg produced best amplification results with  $C_T$  values greater than  $28.16 \pm 0.15$  (mean  $\pm$  SD). Lysates prepared from neonates using DNAzol Direct and the squish buffer produced amplicons in quantities that could be easily visualized by agarose gel electrophoresis. In qPCR assay, lysates of neonates

prepared with DNAzol Direct and squish buffer produced  $C_T$  values  $23.81 \pm 1.19$  and  $18.05 \pm 0.56$ , respectively. All lysates of single-leg samples prepared with squish buffer amplified in both PCR and qPCR amplifications. One of the single-leg lysates prepared with DNAzol Direct did not amplify by PCR during initial testing (data not shown) but did amplify when used in qPCR. Amplifications of DNAzol Direct and squish buffer lysates from single legs produced  $C_T$  values  $25.23 \pm 3.08$  and  $20.48 \pm 3.37$ , respectively. Both DNAzol Direct and squish buffer lysates prepared with two legs amplified the target without any failures and yielded  $C_T$  values  $23.88 \pm 1.89$  and  $17.33 \pm 0.51$ , respectively.



**Fig. 4.** Derivative dissociation curves and difference plots produced by the control and species-specific amplicons produced using optimized primer combination and squish buffer lysates from legs of *H. armigera*, *H. zea*, and 1:24 ratio of *H. armigera*: *H. zea* legs. KAPA YBR Fast Master Mix was used for DNA amplification. (A) Dissociation curves of *H. armigera* (red) and *H. zea* (blue) have the peaks from control amplicon and the species-specific amplicon. Mixed DNA (green) has all three peaks. (B) Difference plot of dissociation curves generated by HRM v2.0 software using *H. zea* as the standard.

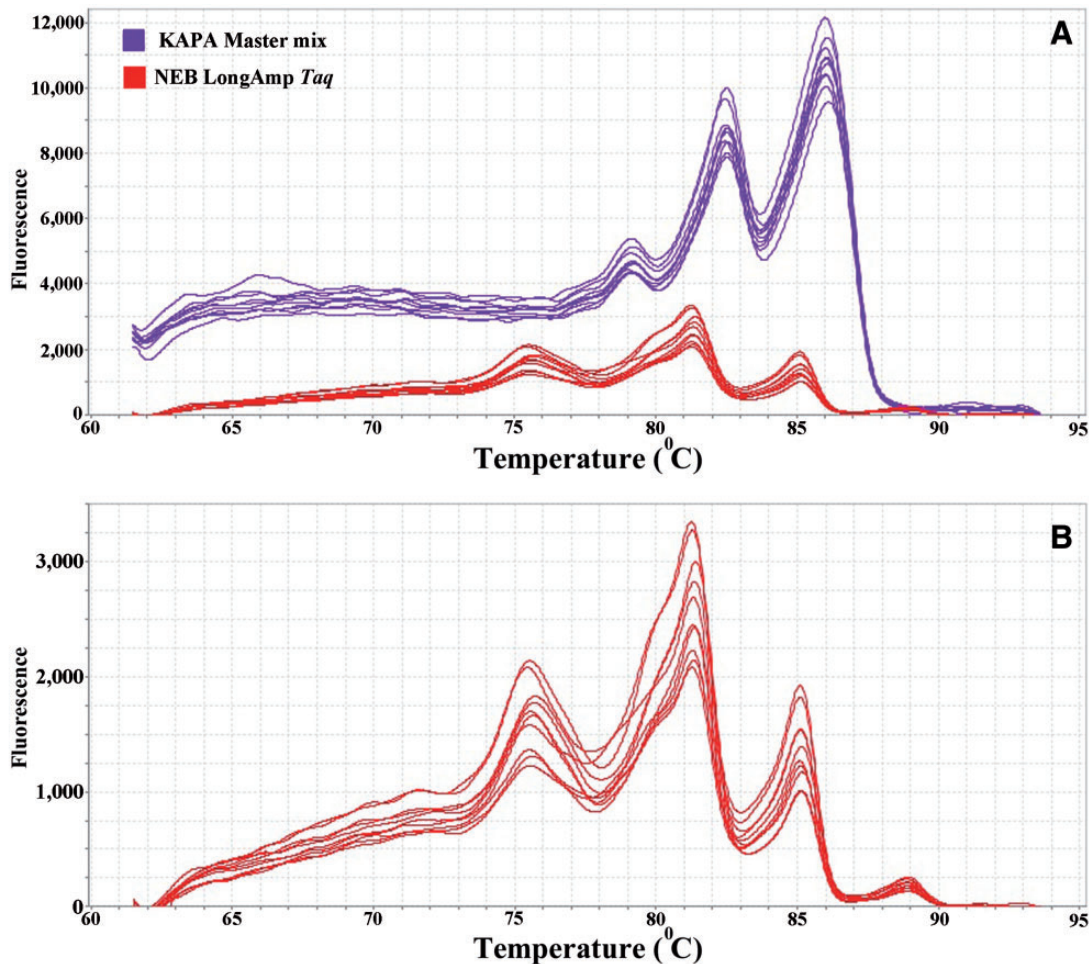
Compared with DNAzol Direct lysates, squish buffer lysates had lower  $C_T$  values in all three experiments, and Student's *t*-test indicated that the differences were significant ( $P \leq 0.005$ ) (Table 2). The difference in  $C_T$  values ( $\Delta C_T$ ) between two DNA preparation methods represents an amplification difference equivalent to  $2^{\Delta C_T}$ . Therefore, the DNA samples prepared with squish buffer amplified with at least 4-fold higher efficiency ( $\Delta C_T > 2$ ) compared with the samples prepared with DNAzol Direct. It was also noted that relative to the melting temperatures of the amplicons produced with purified DNA (dissolved in 2.5 mM Tris-HCl), amplicons produced by squish buffer lysates were slightly higher at 83.7 and 86.4°C for *H. armigera*- and *H. zea*-specific amplicons, respectively.

Conversely, melting temperatures of amplicons from both species in reactions with DNAzol Direct lysates were shifted about 1.5°C lower. This effect was similar to the  $T_m$  shifts observed with different reaction buffers (Fig. 5) and with different  $Mg^{++}$  ion concentrations (Supp Fig. S1 [online only]). However, these  $T_m$  shifts did not affect the melting temperature difference between species-specific amplicons or the ability to distinguish two *Helicoverpa* species. As with dissociation curve analysis, HRM analysis produced discrete melt curves that were characteristic of the species-specific amplicons of ITS1 from *H. armigera* and *H. zea*. Slight variations between some samples were observed due to high sensitivity of HRM analysis to nucleotide polymorphisms within amplicons.

**Table 2. Threshold cycle ( $C_T$ ) values obtained in qPCR amplification of neonate and leg extracts obtained using either modified squish buffer or DNAzol Direct reagent**

Tissue (per reaction)	No. of replicates	Reagent	Threshold cycle ( $C_T$ ) $\pm$ SD	Confidence interval (95%)	P value (Student's <i>t</i> -test)
One neonate	10	Squish buffer	18.05 $\pm$ 0.56	17.43–18.67	$P < 0.0001$
	10	DNAzol Direct	23.81 $\pm$ 1.19	23.19–24.43	
One leg	10	Squish buffer	20.48 $\pm$ 3.37	18.33–22.62	$P = 0.0041$
	10	DNAzol Direct	25.23 $\pm$ 3.08	23.08–27.37	
Two legs	10	Squish buffer	17.33 $\pm$ 0.51	16.41–18.25	$P < 0.0001$
	10	DNAzol Direct	23.88 $\pm$ 1.89	22.97–24.80	

Student's *t*-test was performed on the  $C_T$  values for DNA extraction reagent pairs used for each tissue. Degrees of freedom for all tests were 18.



**Fig. 5.** Derivative dissociation plots produced by different reagents using one microliter of lysate produced by homogenizing one *H. armigera* leg with 24 *H. zea* legs in 625  $\mu$ l of modified squish buffer. (A) Amplification reactions made with KAPA SYBR Fast Master Mix (purple) and NEB LongAmp Taq polymerase and buffer with 2.5 mM MgCl<sub>2</sub> (Red). Peaks representing 18S rRNA control amplicon and species-specific amplicons of *H. armigera* and *H. zea* are present in all reactions, but melting temperatures were different in two reagents. (B) An enlarged view of the derivative dissociation plot produced by amplification reactions with NEB LongAmp Taq polymerase and buffer. Although variations in dissociation plots between samples were observed, the 3-peak dissociation plot was clearly identifiable.

Nevertheless, melt curves produced by the amplicons specific to each species were distinct and sufficient to separate the two species.

The possibility of distinguishing *H. armigera* in a pool of DNA containing increasing amounts of *H. zea* DNA was evaluated by either mixing lysates of adult legs from the two species in various proportions or homogenizing one *H. armigera* leg with 19 or 24 *H. zea* legs. HRM analysis of different DNA ratios of *H. armigera* and *H. zea* indicated that *H. armigera*:*H. zea* DNA ratios up to 1:24 produced the three-peak dissociation curve profile characteristic of the hybrids (Fig. 5a). Amplification reactions containing the lowest ratios of *H. armigera* (i.e., 1:19 and 1:24 *H. armigera*:*H. zea*) produced the best results with

the ready-to use 2X master mix (e.g., KAPA Biosystems). LongAmp Taq polymerase and reaction buffer with 2.5 mM MgCl<sub>2</sub> (New England Biolabs) produced dissociation curves with lower intensity than ready-to-use master mixes but sufficient to detect *H. armigera* in 1:24 ratio of *H. armigera*:*H. zea* tissue lysate (Figs. 5a and b). Therefore, pooling a small amount of tissue (e.g., legs) to produce a lysate of up to 25 insects per reaction could be used to screen up to 2,400 (96-well plate) or 9,600 (384-well plate) samples per run in approximately 2 h (starting with squish buffer lysate preparation to HRM analysis, excluding insect tissue collection time). Although fluorescent signal was much lower (most likely due to low amplification efficiency) in assays with



standard *Taq* polymerase, the DNA concentration could be increased to produce better results either by using less squish buffer volume when homogenizing the samples or increasing the volume of lysate added to the reaction. In this study, all lysates were prepared using 25  $\mu$ l per adult leg. Although 625  $\mu$ l of squish buffer was used for preparing lysates from 25 legs, the volume could be reduced to 200  $\mu$ l to increase the DNA concentration in lysates to facilitate better amplification with standard *Taq* polymerases. In addition, other inexpensive PCR reagents may produce equal or superior results compared with proprietary master mixes, and the users may have to optimize the protocol to adapt to specific reagents or instruments. A step-by-step protocol that includes reagents and processes used in this study for analyzing pools of insects is given in the [Supp Data S3](#) (online only).

## Discussion

Differentiation of morphologically similar species using standard taxonomic characteristics is challenging, time consuming, and requires expertise. Morphological features that could reliably distinguish *H. armigera* and *H. zea* are limited to male genitalia (Lödl 2001, Pogue 2004), and identification of adult females and immature stages requires alternative techniques. RFLP analysis of mtDNA is a reliable method for identification of *Helicoverpa* species but involves PCR amplification, restriction enzyme digests of PCR products, and agarose gel electrophoresis. Mitochondrial cytochrome oxidase subunit 1 barcode analysis involves PCR amplification, nucleotide sequencing, and bioinformatics analysis to identify species. Therefore, aforementioned types of analyses are difficult to scale up for rapid screening of large numbers of samples for detecting a few individuals of an invasive species but are well suited for confirmative analysis of small numbers of samples after initial detection. ELISA is another reliable technique for distinguishing morphologically similar species and assays to identify different combinations of heliothine species are available (Greenstone 1995, Goodman et al. 1997, Zeng et al. 1998, Trowell et al. 2000). However, none of these assays have been evaluated for their ability to distinguish *H. armigera* from *H. zea*. In addition, developing antibodies that distinguish all life stages of both species requires distinct antigens that are expressed in all life stages. Given the remarkable nucleotide identity observed in expressed genes of both species (O.P.P., unpublished data), identification of suitable targets may be difficult. On the other hand, genomic DNA remains the same throughout all life stages, and conserved differences between two species could be exploited to develop assays to identify species. Detection of invasive *H. armigera* requires screening of large numbers of samples, and therefore, the DNA-based assays should be simple and scalable. In this study, conserved differences in ITS1 sequences of *H. armigera* and *H. zea* were used to develop and validate a PCR-based method for reliable species identification. This method used a three-primer cocktail that amplified ITS1 fragments specific to each species. Amplicons specific to each species could be identified either by agarose gel electrophoresis using the size difference or by dissociation curve analysis using a real-time PCR instrument and measuring the difference in melting temperature. A control amplicon produced from a conserved nucleotide sequence region of 18S rRNA served as a positive control to confirm amplification (i.e., to distinguish PCR amplification failures from reactions containing DNA from nontarget species). This method is simple yet efficient, cost effective, and can be readily performed in most laboratories. Conventional agarose gel electrophoresis is time consuming and not suitable for screening large numbers of insects for detecting invasive *H. armigera*, but gel instruments that could resolve up to 96 samples and 8 marker lanes are now available for analysis of large numbers of samples. Dissociation curve (and HRM analysis) requires real-time PCR instruments, but at least 96 samples can be analyzed simultaneously, and with appropriate instruments, 384 samples could be analyzed at a time. In addition to increasing the throughput per run by 4-fold, reagent cost

could be reduced by at least 50% since 10  $\mu$ l reactions could be set up with 96- or 384-well plates ([Supp Fig. 2](#) [online only]). Pooling of samples could be used to further expedite the analysis. For example, if tissues from two or more insects were pooled in DNA extractions, the number of samples analyzed simultaneously could be increased proportionately. However, if at least one *H. armigera* was present in a lysate pool, the assay results would be similar to that of a hybrid of *H. armigera* and *H. zea*. A second round of assays would be needed to identify each sample in the pool, followed by analysis with other confirmatory molecular and morphological methods. The squish buffer extraction method modified from Gloor et al. (1993) significantly improved the efficiency of sample processing. A single moth leg (fresh weight  $688.9 \pm 177.9 \mu$ g) or a neonate (fresh weight  $46.8 \pm 3.4$  ng) could be used to obtain DNA sufficient for multiple PCR amplifications in about 30 min, and from 96 (single leg per well) to 2,400 samples (pools of 25 legs per well) could be processed simultaneously. Furthermore, this method could use only a fraction of an appendage if archiving of specimens is necessary. Dissociation curve analysis developed in this study does not require proprietary reagents or costly fluorescent labeled primers or probes and it provides robust results for pools of up to 25 samples per reaction. The analysis could be performed using either a commercially available master mixes or an inexpensive *Taq* polymerase and a DNA intercalating fluorescent dye (e.g., SYBR green or SYTO-9). The cost of 5 ml of proprietary 2X master mixes (e.g., KAPA SYBR Fast) is close to \$350 and 1,000 reactions could be prepared at 10  $\mu$ l reaction volume (i.e., 5  $\mu$ l of 2X master mix per reaction). Therefore, the reagent cost per reaction is approximately \$0.35. If 25 insects were pooled per reaction, the per insect cost is approximately 1.5 cents. Cost per sample could be further reduced by using an inexpensive *Taq* polymerase and a buffer combination. When combined with home-made squish buffer for DNA preparation, time and reagent cost per sample would be substantially lower than other detection methods that depend on fluorescent tagged primers or probes.

## Supplementary Data

Supplementary data are available at *Journal of Insect Science* online.

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