A Diagnostic Loop-Mediated Isothermal Amplification Method to Distinguish *Helicoverpa armigera* (Lepidoptera: Noctuidae) From Other Related Species in the New World

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Abstract

*Helicoverpa armigera* (Hübner) is a notorious agricultural pest native to the Old World. Recently, its invasion into South and Central America has become a serious problem in the New World. The rapid detection of invasive pests is essential to eradicate them and prevent establishment. However, an extremely similar species, *H. zeae* (Boddie) distributed in the New World makes identification difficult. *Helicoverpa armigera* and *H. zeae* have only minor differences in male genitalia to separate them morphologically. Both species are attracted to the same pheromone lure, and it takes considerable time and effort to identify them from bulk samples obtained during trap monitoring. Although several molecular approaches based on PCR have been reported, these methods require expensive equipment and are unsuitable for onsite diagnostics. Here, we developed a rapid and convenient diagnostic method based on the loop-mediated isothermal amplification to distinguish *H. armigera* from related species: *H. zeae*, *H. assulta* (Guenée), *H. punctigera* (Wallengren), and Chloridea virescens (Fabricius). The diagnostic method makes it possible to detect *H. armigera* within 90 min only using simple equipment. The method also worked with mixed DNA templates containing excess DNA from *H. zeae* at the ratio of 1:999 (*H. armigera*: *H. zeae*). This method can be an effective tool for onsite diagnostics during monitoring surveys for invasive *H. armigera*.

**Key words:** loop-mediated isothermal amplification, molecular identification, *Helicoverpa*

*Helicoverpa armigera* (Hübner) is widely distributed in the Old World and is considered as a highly important agricultural pest. *Helicoverpa armigera* feeds on more than 60 plant families including major crops such as corn, cotton, and soybean (Firr 1989, Cunningham and Zalucki 2014). In 2013, *H. armigera* was first reported in the New World in Brazil (Czepak et al. 2013), and it caused serious damage to soybean, corn, and cotton harvests (Bueno and Sosa-Gómez 2014). Recently, *H. armigera* has become established in Brazil and extended its geographical range rapidly into South and Central America, including Argentina, Bolivia, Paraguay, Peru, Uruguay, and Puerto Rico (SENAVE 2013, Tay et al. 2013, Murúa et al. 2014, NAPPO 2014, Gilligan et al. 2015, Castiglioni et al. 2016). The rapid spread of *H. armigera* in South and Central America suggests that North America is confronted with a serious risk of its invasion (Kriticos et al. 2015).

Rapid detection and accurate identification are essential for monitoring invasive pests. A sex pheromone lure containing Z11-16Al, Z9-16Al, and butylated hydroxytoluene has been used for monitoring surveys of *H. armigera* (USDA-APHIS-PPQ 2014). However, this pheromone combination also attracts *Helicoverpa zeae* (Boddie), which is widely distributed in the New World, and these two species are extremely similar and have only minor morphological differences in male genitalia (Pogue 2004). Dissection of specimens is time consuming even for a specialist and needs professional expertise.

Alternative molecular approaches have been reported to distinguish between these two species, including PCR restriction fragment length polymorphism (PCR-RFLP) analysis (Behere et al. 2008), DNA barcoding (Folmer et al. 1994, Mastrangelo et al. 2014), species-specific conventional PCR, high resolution melt analysis by real-time PCR (Perera et al. 2015), multiplex real-time PCR (Gilligan et al. 2015), and droplet digital PCR (ddPCR; Zink et al. 2017). Zink et al. (2017) focused on rapid detecting from bulk samples expected during trapping surveys, and their results suggested that ddPCR is capable of detecting a single leg of *H. armigera* in a background of up to 999 legs of *H. zeae*. Although ddPCR is an innovative technique, the method takes several hours to complete the analysis and requires highly expensive dedicated equipment.

Loop-mediated isothermal amplification (LAMP) is a rapid, simple, and accurate method to amplify a target gene (Notomi et al. 2000). Recently, several diagnostic assays using LAMP have been developed to detect pest insects and nematodes (e.g., Kikuchi et al. 2009;
Fekrat et al. 2015; Przybyska et al. 2015; Ide et al. 2016a,b; Peng et al. 2017; Blaser et al. 2018). The main feature of LAMP is its use of an isothermal reaction to amplify a target gene. Moreover, a positive LAMP reaction can be detected using the naked eye as turbidity or fluorescence, making gel electrophoresis and spectrophotometry unnecessary (Notomi et al. 2015). These features can make it possible to carry out onsite DNA diagnostics in a local small laboratory without expensive specialized devices.

In this study, we developed a rapid and convenient diagnostic method based on the LAMP reaction to distinguish *H. armigera* from related species, including *H. zea*. We also considered detection from bulk samples obtained during pheromone-trapping monitoring surveys for *H. armigera*.

### Materials and Methods

#### Insects

Pinned dry specimens were obtained from the collection of the Japanese plant protection station intercepted during plant inspections in egg and larval stages and reared to identify species in adult between 2015 and 2019 (Table 1). Male *H. armigera* (*n* = 109) were used as the positive target species and male *H. zea* (*n* = 59) as the negative target. In addition to *H. zea*, *H. assulta* (Guenée) (*n* = 2), *H. punctigera* (Wallengren) (*n* = 2), and *Chloridea virescens* (Fabricius) (*n* = 5) were used as the negative target species.

#### DNA Extraction

Total DNA was extracted from a femur of single specimens. To confirm a suitable extraction method, two different DNA extraction methods were used to compare five pairs of middle femora from *H. armigera*. The methods were as follows.

**Rapid Method**

PrepMan Ultra Sample Preparation Reagent (Applied Biosystems, Inc., Foster City, CA) was used as the rapid method. A femur removed from each pinned specimen was macerated with a pestle in 50 μl of PrepMan reagent and treated at 99.9°C for 15 min.

**Spin Column Method**

A DNeasy Blood and Tissue Kit (QIAGEN, Valencia, CA) was used for the spin column method. The femora opposite those used in the rapid method was used. One hour of incubation time was employed in this study, and all other procedures were conducted according to the manufacturer’s instructions.

Time requirements, and the quantity and purity of DNA samples from two methods were compared. The extracted DNA concentration was measured by using a NanoDrop Lite spectrophotometer (Thermo Fisher Scientific, Waltham, MA) and the purity of DNA was evaluated by the optical density ratio (OD$_{260}$/OD$_{280}$).

#### PCR Assays

In order to confirm the extraction of genomic DNA, part of the internal transcribed spacer 1 of the ribosomal gene (ITS1) was amplified using a universal primer set for *Helicoverpa* spp. (Perera et al. 2015) with partial modification of the reverse primer (Table 2).

PCR was performed in a total of 20 μl reaction volume and contained 2 μl of 10× Ex Taq buffer, 200 μl dNTP Mixture, 0.5 U Takara Ex Taq HS (Takara Bio Inc., Shiga, Japan), 0.5 μM each primer, and 1 μl of the template DNA. DNA extracts diluted 100-fold were used as the template DNA. The PCR protocol had following condition: 1 cycle at 94°C for 4 min; 30 or 35 cycles at 94, 65, and 72°C for 30 s each; and at 72°C for 5 min as a final extension. The PCR products were separated by 2% agarose gel electrophoresis containing 0.01% GelRed (Wako Pure Chemical Industries, Ltd., Osaka, Japan), and a 100-bp DNA ladder (Takara Bio Inc.) was used for molecular size estimating.

#### Primer Design and Development of the Diagnostic LAMP Method

In accordance with Perera et al. (2015), the ITS1 region was selected as a potential diagnostic marker for the LAMP method. We designed a *H. armigera*-specific LAMP primer set (Table 2) using PrimerExplorer V5 (https://primerexplorer.jp/lampv5/index.html) in accordance with the following GenBank sequences: five *H. armigera* (KT343377, KT343378, AJ577253, AB620127, and AF401740) and two from *H. zea* (KT343380 and KT343381).

The LAMP reaction was performed in a total of 12.5 μl reaction volume and contained 6.25 μl 2× reaction mix (Eiken Chemical, Tokyo, Japan), 1.6 μM each forward inner primer and backward inner primer, 0.2 μM each F3 and B3 primer, 0.8 μM loop primer B, 0.5 μL Fluorescent Detection Reagent (Eiken Chemical), 0.5 μl Bst DNA Polymerase (Eiken Chemical), and 1 μl DNA template. DNA extracts diluted 100-fold were used as a template. The LAMP protocol was performed at 68°C for 30, 60, or 90 min and then terminated at 95°C for 2 min. Amplicons of the LAMP reaction were confirmed by fluorescence observation or electrophoresis. Fluorescence was observed under UV light. Electrophoresis was performed with 2% agarose gels containing 0.01% GelRed. The LAMP assays were tested in two independent runs for all samples.

### Specificity Comparison between the LAMP Method and the Specific PCR with Mixed DNA Samples

Specificity of the LAMP method and the specific PCR method for *H. armigera* with pooled DNA samples was compared using mixed DNA templates with 1:1, 1:9, 1:99, 1:999, 1:0 (as a positive control), and 0:1 (as a negative control) ratios (*H. armigera*: *H. zea*). Each mixed DNA template was prepared to include 50 ng of DNA in 1-μl template. The specific PCR was carried out according to the protocol mentioned above using the species-specific primer set (Table 2).
described by Perera et al. (2015). The PCR was done for 35 cycles. Each ratio was replicated 10 times per method. In addition, the time required for each method was compared.

**Results**

The result of comparison between two different DNA extraction methods is shown in Table 3. Both the yield and purity of the extracted DNA were significantly higher by the rapid method than those by the spin column method. Time consumption of rapid method was much shorter (18 min) than spin column method (90 min).

PCR amplification with the universal primer set yielded a single PCR product of ≈550 bp for the five species (Fig. 1) from all 177 individuals. The size of the amplicons corresponded with the estimated length from sequence data.

The LAMP reaction was first performed with one specimen each of *H. armigera* and *H. zea* to determine the appropriate incubation time. Amplification was observed as bright signals of fluorescence (Fig. 2) and unique ladder-like LAMP bands on the gel (Fig. 3) in the mixtures with *H. armigera* incubated for 60 and 90 min. In contrast, amplification was not observed in the mixtures with *H. zea* for all incubation times. Thus, a 60 min of incubation time was employed in the following LAMP assays.

LAMP assays were performed with the remaining 175 individuals using *H. armigera*, *H. zea*, *H. assulta*, *H. punctigera*, and *C. virescens*. Fluorescence was only observed in mixtures with *H. armigera* (Fig. 4) in all cases. Moreover, amplification was also observed under room light as a color change from yellow to lime green in all mixtures with *H. armigera* (Fig. 4). In contrast, no amplification was observed in mixtures with any of the other species (Fig. 4).

The results of the LAMP assay and the specific PCR assay with mixed templates are shown in Figs. 5 and 6, respectively. *Helicoverpa armigera* was detected as bright signals of fluorescence in the LAMP assay (Fig. 5) and as single specific bands of ≈150 bp in the PCR assay (Fig. 6). In terms of specificity, the LAMP method and the specific PCR method produced consistent results for all of the mixing ratios in all 10 replications (100%; Table 4). In addition, the time required for the LAMP method was less than half that for the specific PCR (Table 4).

**Discussion**

The rapid DNA extraction method by PrepMan Ultra Sample Preparation Reagent using only one femur from adult specimen yielded enough quantity and quality of DNA template for the LAMP and PCR assays. According to Huang et al. (2009), the Chelex extraction method is superior to the other methods in terms of cost and time. However, in view of onsite application, the rapid extraction method was adopted in this study because that method does not need any centrifugation steps.

Several diagnostic methods based on molecular biological techniques to distinguish *H. armigera* from other related species have been already reported (e.g., Behere et al. 2008, Gilligan et al. 2015, Perera et al. 2015, Zink et al. 2017). All these methods are reliable, and each has its own characteristics. PCR-RFLP and multiplex PCR enable us to distinguish several target species with only conventional PCR equipment. Conventional PCR is convenient and amenable for small laboratories. However, these methods are not suitable for large numbers of samples because using electrophoresis to detect the amplification products can be a rate-determining step. Real-time PCR and ddPCR allows the rapid analysis of larger numbers of samples simultaneously, but it requires expensive equipment. In contrast, neither electrophoresis nor real-time monitoring was needed in LAMP method.

In this study, *H. armigera* was distinguished from related species *H. zea*, *H. assulta*, *H. punctigera*, and *C. virescens* using the diagnostic LAMP method. This method takes less than 90 min including the DNA extraction procedure. It is faster than conventional PCR.

**Table 2. Sequences of the PCR and LAMP primers used in this study**

| Primer   | Sequence (5′–3′)                     | Reference                  |
|----------|-------------------------------------|----------------------------|
| AM_F3    | TTCCGTAGGGGAACCTGC                  | This study                 |
| AM_B3    | GTTTTTGGTACCTTCTGTC                 | This study                 |
| AM_FIP   | CTTGCGCTCTCTAGTGGTGGTGGGATGTAATGC   | This study                 |
| AM_BIP   | GAACGGACGCTGTTCCGGTGACAGGAGACACATAAACGACG | This study     |
| AM_LB    | TAGCCGTAGATTCCGGTGTTGGT             | This study                 |
| 3373Ha_Hz_ITS1-F | GAGGAAGTAAAAGTCGTAACAAGGTGGTTCC | Perera et al. (2015) |
| Ha_Hz_ITS1-R    | GTCGATGTTCAAAATGTCGCTGC            | This study                 |
| 3374Ha_ITS1-R    | GTGCCACTCTGTCCTCTCTTAAGTGG         | This study                 |

AM_F3, AM_B3, AM_FIP, AM_BIP, and AM_LB are LAMP assay primers. 3373Ha_Hz_ITS1-F and Ha_Hz_ITS1-R are primers for the universal primer PCR. 3373Ha_Hz_ITS1-F and 3374Ha_ITS1-R are primers for the specific PCR.

**Table 3. Time required, quantity, and purity comparison between two different DNA extraction methods**

| Method               | Time (min) | DNA yield (µg/femur) | P value | Purity (OD260/OD280) | P value |
|----------------------|------------|----------------------|---------|----------------------|---------|
| Rapid method         | 18         | 13.4 ± 2.3           | <0.01   | 1.89 ± 0.19          | <0.05   |
| Spin column method   | 90         | 0.7 ± 0.1            |         | 1.62 ± 0.07          |         |

Student’s t-test was used to compare DNA yield and purity of two methods. Values are means ± SD of five replications.
methods and as fast as real-time PCR (Gilligan et al. 2015, Perera et al. 2015). The LAMP method just requires simple equipment such as a water bath or a block heater to amplify the target DNA, and amplification can be confirmed using the naked eye. Although *H. assulta* and *H. punctigera* are not known in the New World (Plantwise 2019a,b), this result also suggested LAMP potential usage in monitoring *H. armigera* in field.

We detected *H. armigera* from mixed DNA templates containing these two species at a ratio of 1:999 (*H. armigera*: *H. zea*) using the LAMP method and the specific PCR method. Although, the LAMP method worked as well as the specific PCR method in our specificity comparison test with mixed DNA templates, the PCR method is considered to be superior in specificity to the LAMP method in this moment because the specific PCR method is capable of detecting the target from actual tissue mixed samples (Perera et al. 2015). Moreover, the ddPCR method shows much higher specificity than
Fig. 5. The fluorescence observation of LAMP products obtained from mixed DNA templates. Each tube contains LAMP products amplified with DNA templates mixed at ratio of 1:1, 1:9, 1:99, 1:999, 1:0 (as a positive control), and 0:1 (as a negative control). Ratios of Helicoverpa armigera:Helicoverpa zea. Helicoverpa armigera was clearly detected as fluorescence at all ratios except in the negative control. Similar results were obtained in all 10 replications.

LAMP method and real-time PCR method (Zink et al. 2017). At present, we have not conducted batch tests with mixed leg samples because of a lack of samples. Although validation tests using batch leg samples should be performed before applying for trapping surveys, the LAMP method has advantages in terms of time required and convenience. This research demonstrates that the diagnostic LAMP method can be an efficacious tool in detecting H. armigera.

This is the first report of the application of a LAMP protocol to the study of detecting the invasive agricultural pest H. armigera. This method should help local laboratories in field concerned about potential danger from H. armigera to make rapid and simple onsite identifications.

Table 4. Comparison of time required and specificity between the LAMP method and the specific PCR method using mixed DNA templates containing Helicoverpa armigera and Helicoverpa zea.

| Diagnostic method | Timea | Template mixing ratios (H. armigera:H. zea)b |
|-------------------|-------|---------------------------------------------|
|                   |       | 1:1  | 1:9  | 1:99 | 1:999 | 0:1  |
| LAMP              | 70 min| 10/10| 10/10| 10/10| 10/10 | 10/10|
| Specific PCR      | 2.5 h | 10/10| 10/10| 10/10| 10/10 | 10/10|

aTime required excludes DNA extraction procedure.
bValues are positive detections/replications.

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