Journal of Economic Entomology Advance Access published August 28, 2015

Rapid Communication

Molecular Identification of *Helicoverpa armigera* (Lepidoptera: Noctuidae: Heliothinae) in Argentina and Development of a Novel PCR-RFLP Method for its Rapid Differentiation From *H. zea* and *H. gelotopoeon*

JOEL D. ARNEODO,[1,2,3] EMILIA I. BALBI,[4] FERNANDO M. FLORES,[4] and ALICIA SCIOCCO-CAP[2]

J. Econ. Entomol. 1–6 (2015); DOI: 10.1093/jee/tov254

ABSTRACT *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae: Heliothinae) is among the most voracious global pests of agriculture. Adults of this species were identified recently in northern Argentina by dissection of male genitalia. In this work, a rapid and simple molecular tool was designed to distinguish *H. armigera* from the morphologically similar indigenous bollworms *Helicoverpa zea* (Boddie) and *Helicoverpa gelotopoeon* (Dyar), regardless of the life stage. Amplification of partial *COI* gene with a new primer pair, and subsequent digestion with endonuclease HinfI, yielded different RFLP profiles for the three main *Helicoverpa* pests currently present in South America. The method was validated in *Helicoverpa* specimens collected across Argentina, whose identity was further corroborated by *COI* sequencing and phylogenetic analysis. The data reported here constitute the first molecular confirmation of this pest in the country. The survey revealed the occurrence of *H. armigera* in northern and central Argentina, including the main soybean- and maize-producing area.

KEY WORDS old world bollworm, *Helicoverpa*, South America, biological invasion, barcode

The *Helicoverpa–Heliothis* complex (Lepidoptera: Noctuidae: Heliothinae) includes economically important agricultural pests worldwide. In southern South America, the polyphagous neotropical bollworms *Helicoverpa zea* (Boddie) and *Helicoverpa gelotopoeon* (Dyar) have been traditionally considered the principal members of the complex (Cork and Lobos 2003, Specht et al. 2013, Murúa et al. 2014). During the last years, the detection of the Old World bollworm *Helicoverpa armigera* (Hübner) in cotton, maize, and soybean fields in Brazil (Czepak et al. 2013, Specht et al. 2013, Tay et al. 2013) has raised the alarm about the spread of this exotic, invasive species in the region. *H. armigera* is one of the most destructive pests of agriculture in Eurasia, Africa, and Oceania (Tay et al. 2013). Its devastating impact is enhanced by the insect's outstanding capacity to develop resistance to chemical insecticides and to transgenic *Bacillus thuringiensis* (Bt) crop varieties (Yang et al. 2013).

Recently, *H. armigera* adults were identified based on male genitalia in NW Argentina (Murúa et al. 2014). Since Argentina is one of the top maize and soybean producers in the world, this finding has pointed out the need of establishing an extensive national monitoring program. The early detection of *H. armigera* in different agro-ecosystems is crucial to implement control measures. The observation of morphological characteristics alone (e.g., wing pattern design) may not be adequate to discriminate *Helicoverpa* species, especially when the integrity of pheromone-trapped specimens cannot be assured. This is particularly true for *H. armigera* and *H. zea*, which, besides their similar morphology and overlapping host range, are attracted by the same volatile compounds (Pogue 2004). At the larval stage, or in the case of frayed adult specimens, differentiation from *H. gelotopoeon* poses an additional complication. Microscopic analysis of the male genitalia (the traditional way of classification) is time-consuming and requires the examination of adult moths, retarding the diagnostic of larval infestations in the field. Direct sequencing of mitochondrial *cytochrome oxidase I* (*COI*) gene has proven its reliability for the correct identification of Heliothinae (Cho et al. 2008, Li et al. 2011). However, this technique is expensive and demands high-cost equipment not always available, especially in low complexity laboratories located in rural areas.

Previous works employed DNA-based techniques to discriminate among Heliothinae species. PCR-RFLP methods have been conceived to distinguish *Heliothis virescens* (F.) from *H. zea* (Roehrdanz, 1997) and *H. armigera* from *Helicoverpa assulta* (Guenée) (Orui et al. 2000, Kranthi et al. 2005). Instead, Ming and

[1] Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Av. Rivadavia 1917, (1033) Buenos Aires, Argentina.
[2] Instituto de Microbiología y Zoología Agrícola (IMyZA) – Instituto Nacional de Tecnología Agropecuaria (INTA), Nicolás Repetto y de los Reseros, (1686) Hurlingham, Argentina.
[3] Corresponding author, e-mail: arneodo.joel@inta.gob.ar.
[4] EEA- INTA Marcos Juárez, Ruta 12 km 3, (2580) Marcos Juárez, Argentina.

Wang (2006) used AFLP markers to discriminate *H. armigera* and *H. assulta*. In a more comprehensive research, Behere et al. (2008) developed a molecular method to differentiate *H. armigera*, *H. assulta*, *Helicoverpa punctigera* (Wallengren), and *H. zea* through amplification of two regions of the mitochondrial DNA and digestion with two endonucleases. The present study provides a new molecular tool to discriminate *H. armigera* from *H. zea,* taking also into account a previously disregarded, though economically important species: *H. gelotopoeon*. Identification is based on the restriction patterns of a partial sequence in the *COI* gene amplified with a new primer pair and digested with a single endonuclease. Consequently, a sensitive reduction in time and costs can be achieved by diminishing the number of reactions needed. The method was assessed in silico and further validated by processing *Helicoverpa* specimens sampled over a wide geographic range. This paper reports the first molecular evidence on the occurrence of *H. armigera* in Argentina, and expands the current knowledge about the distribution of the pest in diverse agricultural regions of the country. A brief molecular phylogeny of the *COI* sequences obtained to confirm the identity of the insects is also reported.

Materials and Methods

Helicoverpa specimens were collected from different production areas of central and northern Argentina. Adults were captured with light traps, while larvae were caught directly from the affected crops (in all cases, between October 2014 and January 2015). Sampling sites and host plants are detailed in Fig. 1. Eighty-five specimens were chosen for PCR-RFLP analysis. *H. armigera*, *H. zea*, and *H. gelotopoeon* controls consisted of adult males identified by macroscopic features and microscopic examination of their genitalia.

DNA was extracted from single specimens using a CTAB-based protocol (Doyle and Doyle 1987). A new set of primers was designed to amplify an 812-bp region, based on Heliothinae *COI* sequences from GenBank: H3Fw (5′-CGAGCAGAATTAGGTAAYCC-3′) and H3Rv (5′-GCTGATGTRAAATAAGCTCGAG-3′). PCR conditions were as follows: 95°C for 5 min, 40 cycles of 95°C for 30 s, 50°C for 30 s, and 72°C for 1 min, and final extension at 72°C for 2 min. In silico digestions were performed to identify an endonuclease that would produce a distinctive band pattern for *H. armigera COI* sequences, compared to the two other *Helicoverpa* spp. commonly found in South America and to *Heliothis virescens* (a cotton pest of northern Argentina). Although not reported in the New World, solanaceous specialist *Helicoverpa assulta* was included in the bioinformatic screening because, as for *H. zea*, this species is quite difficult to distinguish from *H. armigera*, particularly at the larvae stage (Li et al. 2011). The polyphagous Australian pest *H. punctigera*, also absent from the Americas, was considered as well. The endonuclease HinfI (Promega, Madison, USA) was selected using Vector NTi and AlignX software (InfoMax, Bethesda, USA), after an exhaustive restriction analysis of published *COI* sequences of all the above-mentioned species (Cho et al. 2008, Li et al. 2011, Albernaz et al. 2012, Leite et al. 2014, Walsh 2014, Zahiri et al. 2014). PCR products, before and following digestion, were resolved in 1% agarose gels.

After PCR-RFLP analysis, partial sequencing of *COI* gene was performed on three individuals per *Helicoverpa* species collected across the country. H3Fw/H3Rv-primed products were purified with ADN Puri-Prep-GP kit (INBIO, Tandil, Argentina) and directly sequenced (both strands) in an ABI PRISM 3500 XL genetic analyzer (Applied Biosystems, Foster City, USA) at CICVyA-INTA (Hurlingham, Argentina). Hence, the taxonomy of the specimens was confirmed through phylogenetic analysis by the maximum likelihood method (1,000 bootstrap replicates) using MEGA version 5.2 (Tamura et al. 2011). Reference sequences from GenBank were incorporated in the phylogenetic tree and *Spodoptera exigua* (a non-Heliothinae noctuid) served as outgroup.

Results and Discussion

Amplification of partial *COI* sequences with primer pair H3Fw/H3Rv yielded fragments of the expected size from all *Helicoverpa* specimens tested (not shown). When digesting PCR products with HinfI (which targets the 5′-G▾ANT C-3′ sequence), distinctive restriction patterns were observed for each of the three key *Helicoverpa* pests in southern South America. No digestion occurred in *H. zea* samples ($n = 30$), whereas a restriction site was present in the partial *COI* sequences of *H. gelotopoeon* ($n = 30$) and *H. armigera* ($n = 25$). In the case of *H. gelotopoeon*, the 812 bp PCR product was cleaved into two fragments of 638 and 174 bp, while the digestion of *H. armigera* amplicons resulted in two fragments of 462 and 350 bp, as predicted from in silico analysis. These differences could be clearly visualized through conventional agarose-gel electrophoresis (Fig. 2). HinfI restriction sites found in the obtained partial *COI* sequences reported later (5′-G▾AAT C-3′ for *H. armigera* and 5′-G▾ATT C-3′ for *H. gelotopoeon*) were in accordance with the observed fragment sizes. Since mitochondrial DNA can be extracted at any time throughout the insect's life cycle, this method constitutes an easy and low-cost alternative for the rapid identification of *H. armigera* without the necessity of examining adult males. Even if individuals of *Heliothis virescens* and the exotic pests *Helicoverpa assulta* and *H. punctigera* were not empirically tested, bioinformatic analysis evidenced the absence of HinfI restriction sites in the equivalent *COI* fragments. The lesser studied, but regionally significant, pest *H. gelotopoeon* also showed a distinctive PCR-RFLP pattern (Fig. 2). Despite causing considerable losses, and possibly due to its restricted geographical distribution, this species was not included in previous works involving the use of molecular markers. This novel PCR-RFLP method proved to be quick and simple, as only one *COI* sequence and a single restriction enzyme are sufficient to discriminate the three major *Helicoverpa* pests currently known in South America.

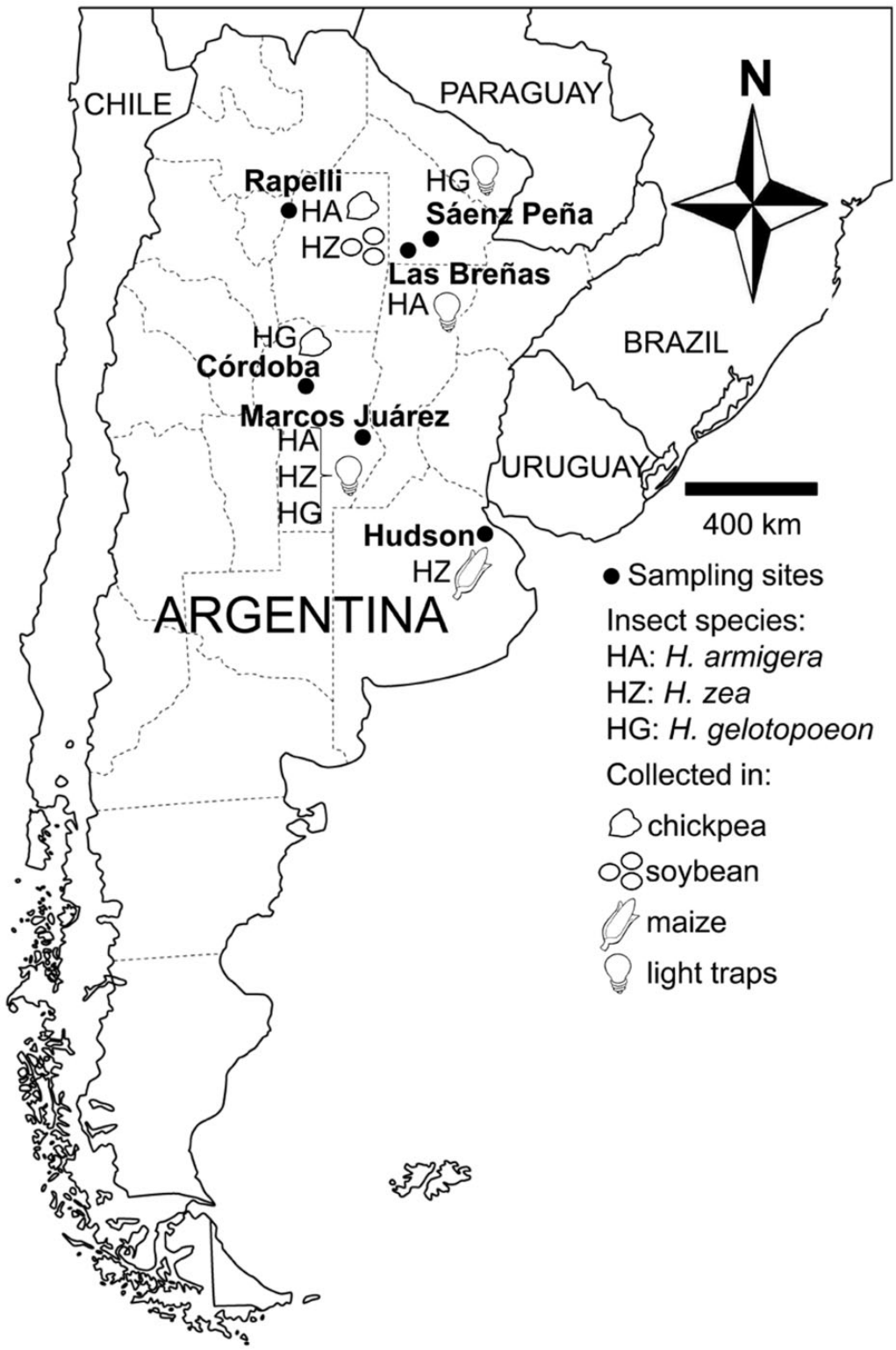

Fig. 1. Origin of the *Helicoverpa* specimens analyzed in this study.

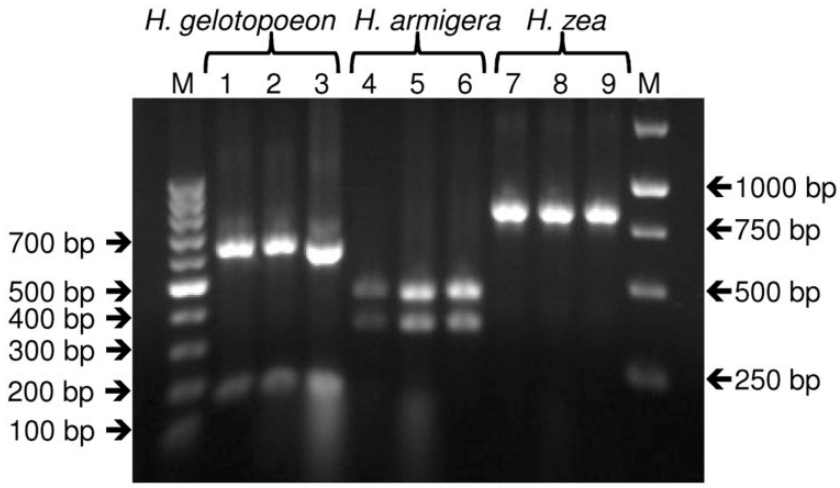

Fig. 2. Agarose gel electrophoresis of PCR products digested with HinfI restriction enzyme, showing different RFLP patterns for the *Helicoverpa* spp. analyzed. M: molecular weight markers. Lanes 1–3: *H. gelotopoeon* specimens from Marcos Juárez, Córdoba, and Sáenz Peña, respectively; lanes 4–6: *H. armigera* specimens from Marcos Juárez, Rapelli, and Las Breñas, respectively; lanes 7–9: *H. zea* specimens from Marcos Juárez, Rapelli, and Hudson, respectively.

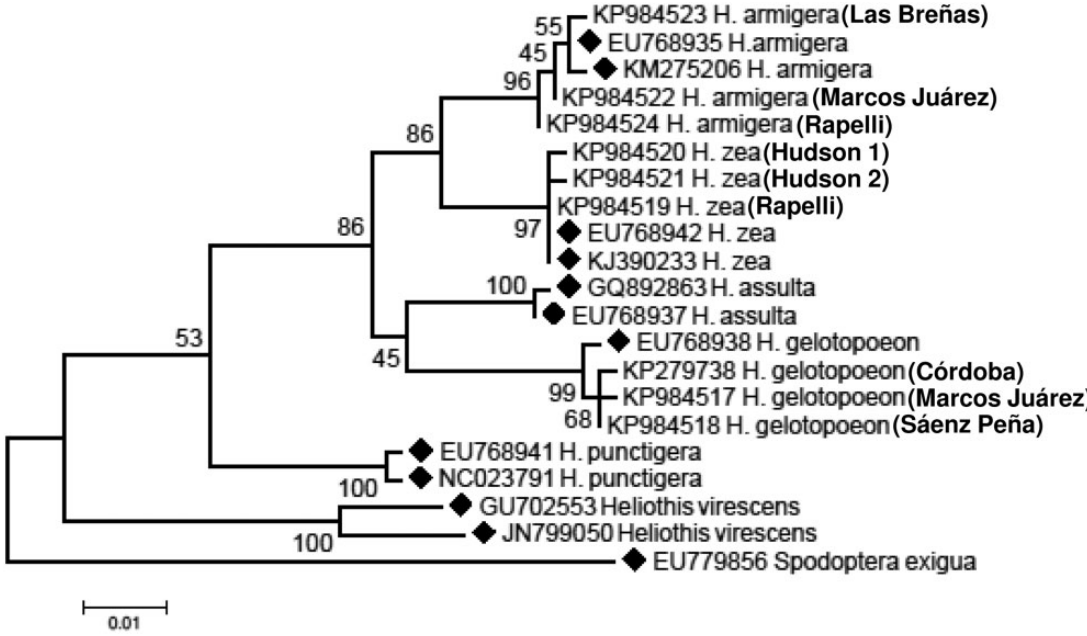

Fig. 3. Molecular phylogeny of partial *Helicoverpa* spp. *COI* sequences obtained in this study (insect collection sites in parentheses). Reference sequences of *Helicoverpa* spp. and other Heliothinae are included in the tree. *S. exigua* served as outgroup. Black diamond indicates previously reported sequences. GenBank accession numbers are indicated at the branch tips. Branch lengths are measured in number of substitutions per site. Bootstrap percentages are shown at nodes.

In contrast, in order to accurately distinguish *H. zea* from other Heliothinae spp. (native pests or potential invaders), the use of additional enzymes and/or genomic regions is to be envisaged. In this sense, earlier research carried out by Behere et al. (2008) on *H. assulta* and *H. punctigera* provides valuable information.

Partial *COI* gene sequences were obtained from specimens of geographically distant Argentinean populations of *Helicoverpa* spp. After removal of poor-quality reads, sequences ranging from 704 to 812 bp were deposited in GenBank under accession numbers KP984522, KP984523, KP984524 (*H. armigera*), KP984519, KP984520, KP984521 (*H. zea*), and KP279738, KP984517, KP984518 (*H. gelotopoeon*). Sequences were trimmed to a common length of 539 bp and compared to those available at public

databases. The resulting phylogenetic tree (Fig. 3) confirmed the morphological and PCR-RFLP identifications of the field-sampled insects, which grouped into three well-defined clades (96–100% bootstrap support). Low intraspecific genetic variability was observed among obtained *COI* sequences, and also when contrasted with those previously reported (>99% identity). The utility of *COI* gene as a barcode for noctuid species is well known (Behere et al. 2008, Cho et al. 2008, Li et al. 2011, Zahiri et al. 2014).

Originally detected in Tucumán province (Murúa et al. 2014), this sampling revealed the occurrence of *H. armigera* in further agricultural areas of Argentina (Fig. 1). *H. armigera* larvae collected from chickpea in Rapelli (Santiago del Estero province), and adults captured with light traps near leguminous and cotton fields in Las Breñas (Chaco province), suggest that this new pest has already settled over a wide area in the North of the country. Moreover, this preliminary survey demonstrated the spread of *H. armigera* southern to the Argentine corn and soybean belt. Indeed, adults were caught by light traps adjacent to maize and soybean fields in Marcos Juárez (Córdoba province), at the core of the country's main agricultural lands (Fig. 1). A model estimating the potential distribution of the pest can be found in Kriticos et al. (2015). From where and when *H. armigera* entered Argentina is uncertain. The analysis of *COI* alone does not allow tracing the origin(s) of this biological invasion. Supplementary research, including additional genomic regions, is needed to elucidate the gene flow of this species at a global and local scale.

The results presented here constitute the first molecular evidence on the presence of *H. armigera* in Argentina, and provide a useful tool for its differentiation from the main South American Heliothinae. A broader sampling is mandatory to monitor the spread of the pest across the country, in order to adopt integrated management strategies for minimizing its impact on agriculture.

Acknowledgments

We are indebted to Eleonora Da Riva (AgIdea, Pergamino, Argentina), Mariela Folgar (EEA-INTA Sáenz Peña, Argentina), and Patricia Fichetti (Universidad Nacional de Córdoba, Córdoba, Argentina) for providing some of the insects used in this study. The work was funded by INTA and CONICET (Argentina).

References Cited

Albernaz, K. C., K. L. Silva-Brandao, P. Fresia, F. L. Consoli, and C. Omoto. 2012. Genetic variability and demographic history of *Heliothis virescens* (Lepidoptera: Noctuidae) populations from Brazil inferred by mtDNA sequences. Bull. Entomol. Res. 102: 333–343.

Behere, G. T., W. T. Tay, D. A. Russell, and P. Batterham. 2008. Molecular markers to discriminate among four pest species of *Helicoverpa* (Lepidoptera: Noctuidae). Bull. Entomol. Res. 98: 599–603.

Cho, S., A. Mitchell, C. Mitter, J. Regier, M. Matthews, and R. Robertson. 2008. Molecular phylogenetics of heliothine moths (Lepidoptera: Noctuidae: Heliothinae), with comments on the evolution of host range and pest status. Syst. Entomol. 33: 581–594.

Cork, A., and E. A. Lobos. 2003. Female sex pheromone components of *Helicoverpa gelotopoeon*: first heliothine pheromone without (Z)-11-hexadecenal. Entomol. Exp. Appl. 107: 201–206.

Czepak, C., K. C. Albernaz, L. M. Vivan, H. O. Guimarães, and T. Carvalhais. 2013. Primeiro registro de ocorrência de *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae) no Brasil. Pesq. Agropec. Trop. 43: 110–113.

Doyle, J. J., and J. L. Doyle. 1987. Isolation of DNA from fresh plant tissue. Focus 12: 13–15.

Kranthi, S., K. R. Kranthi, A. A. Bharose, and S. N. Syed. 2005. A PCR-RFLP tool for differentiating *Helicoverpa armigera* and H. assulta (Lepidoptera: Noctuidae). Curr. Sci. 89: 1322–1323.

Kriticos, D. J., N. Ota, W. D. Hutchison, J. Beddow, T. Walsh, W. T. Tay, D. M. Borchert, S. V. Paula-Moreas, C. Czepak, and M. P. Zalucki. 2015. The potential distribution of invading *Helicoverpa armigera* in North-America: is it just a matter of time? PLoS ONE 10: e0119618. (doi:10.1371/journal.pone.0119618).

Leite, N. A., A. A. Pereira, A. S. Correa, M. I. Zucchi, and C. Omoto. 2014. Demographics and Genetic variability of the new world bollworm (*Helicoverpa zea*) and the old world bollworm (*Helicoverpa armigera*) in Brazil. PLoS ONE 19: e113286. (doi:10.1371/journal.pone.0113286).

Li, Q. Q., D. Y. Li, H. Ye, X. F. Liu, W. Shi, N. Cao, and Y. Q. Duan. 2011. Using COI gene sequence to barcode two morphologically alike species: the cotton bollworm and the oriental tobacco budworm (Lepidoptera: Noctuidae). Mol. Biol. Rep. 38: 5107–5113.

Ming, Q. L., and C. Z. Wang. 2006. Genetic differentiation of *Helicoverpa armigera* (Hübner) and *H. assulta* (Guenée) (Lepidoptera: Noctuidae) based on AFLP markers. Insect Sci. 13: 437–444.

Murúa, M. G., F. S. Scalora, F. R. Navarro, L. E. Cazado, A. Casmuz, M. E. Villagrán, E. Lobos, and G. Gastaminza. 2014. First record of *Helicoverpa armigera* (Lepidoptera: Noctuidae) in Argentina. Fla. Entomol. 97: 854–856.

Orui, Y., H. Matsuzawa, Y. Koike, and S.-I. Yoshimatsu. 2000. Discrimination of *Helicoverpa armigera* (Hübner) and *H. assulta* (Guenée) (Lepidoptera: Noctuidae) by PCR-RFLP analysis, and application to surveying occurrence of *H. armigera* in tobacco fields of Japan. Jpn. J. Appl. Entomol. Z. 44: 73–79.

Pogue, M. G. 2004. A new synonym of *Helicoverpa zea* (Boddie) and differentiation of adult males of *H. zea* and *H. armigera* (Hübner) (Lepidoptera: Noctuidae: Heliothinae). Ann. Entomol. Soc. Am. 97: 1222–1226.

Roehrdanz, L. R. 1997. Identification of tobacco budworm and corn earworm (Lepidoptera: Noctuidae) during early developmental stages by polymerase chain reaction and restriction fragment length polymorphism. Ann. Entomol. Soc. Am. 90: 329–332.

Specht, A., D. R. Sosa-Gómez, S. V. de Paula-Moraes, and S.A.C. Yano. 2013. Identificação morfológica e molecular de *Helicoverpa armigera* (Lepidoptera: Noctuidae) e ampliação de seu registro de ocorrência no Brasil. Pesq. Agropec. Bras. 48: 689–692.

Tamura, K., D. Peterson, N. Peterson, G. Stecher, M. Nei, and S. Kumar. 2011. MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol. Biol. Evol. 28: 2731–2739.

Tay, W. T., M. F. Soria, T. Walsh, D. Thomazoni, P. Silvie, G. T. Behere, C. Anderson, and S. Downes. 2013. A

brave New World for an Old World pest: *Helicoverpa armigera* (Lepidoptera: Noctuidae) in Brazil. PLoS ONE 8: e80134. (doi:10.1371/journal.pone.0080134).

Walsh, T. K. 2014. Characterization of the complete mitochondrial genome of the Australian Heliothine moth, *Australothis rubrescens* (Lepidoptera: Noctuidae). Mitochondrial DNA 22: 1–2. (doi:10.3109/19401736.2013.878927).

Yang, Y., Y. Li, and Y. Wu. 2013. Current status of insecticide resistance in *Helicoverpa armigera* after 15 years of *Bt* cotton planting in China. J. Econ. Entomol. 106: 375–381.

Zahiri, R., J. D. Lafontaine, B. C. Schmidt, J. R. DeWaard, E. V. Zakharov, and P. D. N. Hebert. 2014. A transcontinental challenge – A test of DNA barcode performance for 1541 species of Canadian Noctuoidea (Lepidoptera). PLoS ONE 9: e92797. (doi: 10.1371/journal.pone.0092797)

Received 2 July 2015; accepted 6 August 2015.