

Molecular identification of *Helicoverpa armigera* (Noctuidae: Heliothinae) in Amambay Department, Paraguay

Identificación molecular de *Helicoverpa armigera* (Noctuidae: Heliothinae) en el departamento de Amambay, Paraguay

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Conflict of interest:
The authors declare no conflict of interest.

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History:
Received: 06/02/18;
Accepted: 28/06/18

ABSTRACT

The *Helicoverpa armigera* (Noctuidae: Heliothinae) is a polyphagous and cosmopolitan pest distributed throughout South America. The objective of this research was to identify by PCR-RFLP molecular analysis, the presence of *H. armigera* in the Amambay region in Paraguay. Delta traps with sexual pheromone were deployed in the 2016/2017 season soybean crop distributed at the following locations: Pedro Juan Caballero, Zanja Pytã, Capitán Bado, Karapãĩ and Bella Vista Norte. Sampling cloth was used for collecting caterpillars. The collected specimens were sorted by external morphological characters, individualized in labeled jars containing pure alcohol. 24 samples were sent to the Arthropods Molecular Ecology Laboratory ESALQ/USP for identification by PCR-RFLP molecular analysis. Ten specimens of *H. armigera* belonging to Fortuna and Zanja Pytã were identified. Ten other specimens were identified as *H. zea* belonging to Cerro Cora'i, Captain Bado, Cerro Kuatiã and Karapãĩ. The remaining four samples did not amplify by PCR-RFLP. The analysis revealed the occurrence of *H. armigera* in soybean and maize crops in the northern region of Paraguay.

Key words: *H. armigera*, pheromone, delta trap, molecular identification.

RESUMEN

Helicoverpa armigera (Noctuidae: Heliothinae) es una plaga polífaga y cosmopolita que se distribuye por América del Sur. El objetivo de esta investigación fue identificar mediante técnica molecular PCR-RFLP la presencia de la *H. armigera* en la región del Amambay, Paraguay. Se instalaron trampas tipo delta con feromona sexual distribuidas en las localidades de Pedro Juan Caballero, Zanja Pytã, Capitán Bado, Karapãĩ y Bella Vista Norte en cultivos de soja zafra 2016/2017. Se utilizó paño de muestreo para colecta de orugas. Los especímenes colectados fueron seleccionados mediante caracteres morfológicos externos, individualizados en frascos, etiquetados con alcohol puro. Para identificación por análisis molecular PCR-RFLP se remitieron 24 muestras al laboratorio de Ecología Molecular de Artrópodos, ESALQ/USP. Se identificaron diez especímenes de *H. armigera* provenientes de las localidades de Fortuna y Zanja Pytã. Otros diez especímenes se identificaron como *H. zea* provenientes de Cerro Cora'i, Capitán Bado, Cerro Kuatiã y Karapãĩ. Las cuatro muestras restantes no amplificaron por la técnica PCR-RFLP. El análisis reveló la ocurrencia de la *H. armigera* en cultivos de soja y maíz de la región norte del Paraguay.

Palabras clave: *H. armigera*, feromona, trampa tipo delta, identificación molecular.

INTRODUCTION

The Old World cotton bollworm *Helicoverpa armigera* is one of the most important agricultural pests in the world. Distributed in Asia, Africa, Europe, Australia and reported in 180 cultivated agricultural crops (Tay 2013). Recently it invaded South and Central America, and it appears to be spreading rapidly (Kriticos et al. 2015). Its voracity is worrisome, in its larval stage can feed on leaves, stems, shoots, inflorescences, fruits and pods (Acevedo Reyes 2015).

In the American continent, its occurrence was reported in several Brazilian regions, which resulted in significant economic losses in soybean (*Glycine max* (L.) Merrill), cotton (*Gossypium hirsutum* L.), maize (*Zea mays* L.) and other crops. In Brazil, the species shows a high adaptation capability, strong population growth, widespread distribution within different regions, and the ability to feed and develop on different host species. Considered a quarantine pest until mid-2013, its presence was officially confirmed by morphological characteristics of the genitalia and by molecular PCR technique in soybean crops in Bahia and cotton in Mato Grosso State (Czepak et al. 2013; Embrapa 2013; Specht et al. 2013; Leite et al. 2014; Sosa-Gómez et al. 2016).

In Argentina, this pest was detected at many locations, confirming the presence of *H. armigera* for the first time in the Tucuman province (Murúa et al. 2014). Previously, Chiarelli de Gahan & Touron (1954) reported the occurrence of *H. armigera* in Argentina but clearly this was a misidentification as stated by Hardwick (1965). In 2015, *H. armigera* larvae was collected from chickpea in Rapelli (Santiago del Estero province), and adults were captured with light traps near leguminous and cotton fields in Las Breñas (Chaco province) North of the country. Also, the propagation of *H. armigera* to the south to the Argentine belt of corn and soybeans was demonstrated. In fact, 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 (Arneodo et al. 2015).

Specimens of *H. armigera* from Uruguay were recently identified by molecular characterization at the following locations: Flores, Lavalleja, Cerro Largo and Rocha Department (Castiglioni et al. 2016). In Paraguay, the National Plant and Seed

Quality and Health Service, SENAVE, in 2013, reported its presence in Cantina kué, Alto Paraná Department in soybean crops. However, DNA analysis to confirm the species has not been performed and its distribution in the country is unknown. Thus, the objective of this research was to demonstrate the occurrence of *H. armigera*, identified by molecular analysis, in the Amambay Department, Paraguay.

To obtain the *H. armigera* specimens, delta traps were installed with sex pheromone (Isalure armigera) attached to fixed bases according to the manufacturer's instructions. The traps were distributed in soybean crops located in the municipalities of Pedro Juan Caballero, Zanja Pytã, Capitán Bado, Karapã'i and Bella Vista. In addition, sampling cloth was used for collecting caterpillars. All the specimens collected were pre-selected using description techniques and morphological characteristics for caterpillars and adults corresponding to the genus *Helicoverpa* (Azambuja 2016). Once individualized in jars containing pure alcohol, they were sent to the Laboratory of Molecular Ecology of Arthropods of the Department of Entomology of the Escola Superior de Agricultura "Luiz de Queiroz", ESALQ/USP - Piracicaba/SP/Brazil, where DNA extraction, amplification and identification by PCR-RFLP were carried out.

For molecular identification, DNA extraction of the thoracic tissues of both caterpillars and adults were carried out following the Doyle and Doyle protocol (1990). Head, abdomen, wings, and legs of insects were separated using a scalpel. The thorax was individualized in 1.5 ml microtubes under extractor hood and were added 650 µL of extraction buffer (10 ml CTAB 2% + 20 µg of proteinase K 20 µg / ml + 20 µl β-mercaptoethanol) and crushed with previously autoclaved pistils. Once crushed, the samples were subjected to a water bath at 65°C for one hour.

Subsequently, 650 µl of CIA 24: 1 (24 chloroform and 1 isoamyl alcohol) were removed and the sample stirred and centrifuged at 12,000 rpm at 4°C for 5 minutes. The supernatant fluid was then extracted with a micropipette and transferred to another 1.5 ml microtube. Again, 650 µl of the CTAB 2% extraction buffer (without proteinase K and β-mercaptoethanol) and 200 µl of CIA 24: 1 were added, being centrifuged as described above. This cleaning process was repeated until the sample was

in condition. Once this process was completed, the supernatant fluid was separated into another 1.5 ml microtube by adding 200 μ l of isopropanol and kept in a freezer at -20°C for two hours. It was then centrifuged at 12000 rpm at 4°C for 10 minutes, then the isopropanol was discarded and the resulting pellet was washed twice with 70% alcohol for 10 minutes. Afterwards, the alcohol was discarded and the pellet was left to dry in the overnight vacuum, finally, 49 μ l of purified double-distilled water and 1 μ l of RNase were added, and kept in a freezer at -20°C , to be observed in agarose gel electrophoresis and in this way, evaluated the quality and quantity of extracted DNA, used for amplification in PCR and identification of *H. armigera*.

A total of 24 *Helicoverpa* specimens were analyzed using the PCR-RFLP marker of a fragment of the mitochondrial gene Cytochrome Oxidase Subunit I (COI), previously used by (Specht et al. 2013, Leite et al. 2014). The extracted DNA was amplified by PCR in final volume of 10 μ l of reaction containing 1 μ l of buffer Taq DNA polymerase (10X), 1.5 μ l 25 mM MgCl_2 , 1 μ l of 10 mM dNTP, 1 μ l forward primer LCO (F) (59 - GGT CAA CAAATC ATA AAG ATA TTG G - 39), and 1 μ l of reverse primer HCO (R) (59 - TAA ACTTCA GGG TGA CCA AAA AAT CA - 39), 0.10 μ l of taq DNA polymerase, 3.4 μ l of purified H_2O and 1 μ l of diluted DNA (2 μ l of DNA extracted in 48 μ l

of purified water). For the PCR reaction a cycle was performed at 94°C for 3 minutes, followed by 35 cycles of 94°C for 40 seconds, 55°C for 40 seconds, 72°C for 70 seconds and a final extension cycle at 72°C for 10 minutes. Next, digestion with the restriction enzyme was performed in another microtube of 5 μ l of the PCR product, 2 μ l of enzyme buffer, 0.5 μ l of restriction enzyme and finally completed with 7.8 μ l of purified water giving a final volume of 15 μ l and incubated at 37°C for 6 hours or overnight. Finally, the samples were analyzed in 1.5% agarose gel with SYBER SAFE (TAE 1X) and observed in UV ultraviolet transilluminator (Figure 1).

Of twenty-four samples subjected to analysis and characterization by molecular techniques, ten individuals were identified as *H. armigera*, Zanja Pytã district (a location of Zanja Pytã (n = 1), Fortuna (n = 7) and Pedro Juan Caballero (Mafussi location n = 2). Other ten individuals were identified as *H. zea*, four specimens from Pedro Juan Caballero district, Cerro Kora'i, three from Karapa'i district, and two from Zanja Pytã district, Cerro Kuatiã location (Figure 1, Figure 2). However, four samples did not amplify (Table 1). These results reveal the occurrence of *H. armigera* in soybean and maize crop 2016/2017 in the Amambay Department, Paraguay.

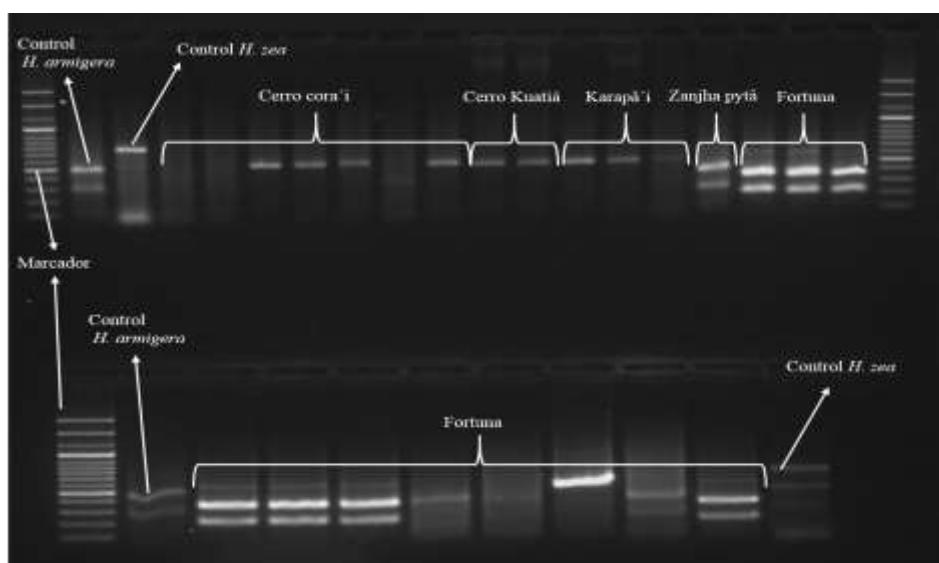


Figure 1. PCR-RFLP Agarose gel (1,5%) for amplification that characterizes the bands of the specimens after cutting with the restriction enzyme (One band: *Helicoverpa zea*; Two bands: *Helicoverpa armigera*).

It is important to consider that *H. armigera* samples were collected from locations that limit geographically with Mato Grosso do Sul state, Brazil (1 to 20 km) (Figure 2). Extensive areas of soybeans and corn from of Pedro Juan Caballero, Zanja Pytä and Capitan Bado districts form a green

bridge between Paraguay and Brazil, which facilitates the dispersal of *H. armigera* from Brazil to Paraguayan crops, assuming that the pest was detected for the first time in the America continent in Brazil (Czepak et al. 2013).

Table 1. Detection of *Helicoverpa armigera* and *Helicoverpa zea* in soybean and maize crops at Amambay districts, identified by molecular analysis.

Amambay Districts	Location	Coordinates UTM (XY)		Crop, Plant or Trap	Species (n)
Pedro Juan Caballero	Cerro Kora'í	641643.5	7445518.4	<i>Zea mays</i>	<i>Helicoverpa zea</i> (4)
	Mafussi	626987.3	7498338.7	<i>Glycine max</i> , <i>Z. mays</i> , Feromone	<i>H. armigera</i> (2)
Capitán Bado	Cerro kuatiá	641643.5	7445518.4	<i>G.max</i> , <i>Z. mays</i> , Feromone	<i>H. zea</i> (2)
Karapã'ĩ	Karapã'ĩ	630362.6	7410439.8	<i>Z. mays</i>	<i>H. zea</i> (3)
	Zanja Pytä	641036.2	7491406.8	<i>G. max</i> , Feromone	<i>H. armigera</i> (1)
Zanja Pytä	Fortuna	627540.8	7497567.6	<i>G. max</i> , <i>Sorghum alepense</i>	<i>H. armigera</i> (7)
				<i>Z. mays</i> , Feromone	<i>H. zea</i> (1)

* Samples from Cerro Kora'i (n = 3) and Fortuna (n = 1) did not amplify. Total = 24 samples

In Paraguay, the National Seeds and Plants Health Service (SENAVE), reported in 2013 the presence of *Helicoverpa* sp. in soybean in Cantina kué, a location in the Alto Paraná department, however, only external morphological characteristics were used as mean of identification without carrying out molecular DNA analysis to confirm the species presence In Brazil, morphological identification was based on the male genitalia and molecular analysis of mitochondrial genes sequences of cytochrome B and the *cox1-tRNA^{Leu}-cox2* region were amplified, later they were deposited at the GenBank (Embrapa 2013, Czepak et al. 2013, Avila et al. 2013). These *H. armigera* specimes were captured with light traps. *H. armigera* has spread to the northeast and the midwestern region of Brazil, causing great losses. Recently, its presence in the state of Minas Gerais, Viçosa, was confirmed by molecular analysis. The insects were collected from transgenic and non-transgenic soybean, cotton and corn crops (Pinto et al. 2017).

The Argentine National Pest Surveillance and Monitoring System (SINAVIMO) also recorded *H. armigera* in eight provinces and twenty counties in Argentina, in soybean, chickpea, sunflower and

cardus Carduus acanthoides crops. Being this the first report of *H. armigera* in sunflower and cardus in Argentina (Murúa et al. 2014). In the same year, specimens of *H. armigera* were also identified by molecular characterization in Uruguay at the following locations Flores, Lavalleja, Cerro Largo and the Rocha department (Castiglioni et al. 2016).

There is evidence that *H. armigera* has spread to Uruguay and Paraguay, polymerase chain reaction and sequencing of the partial mitochondrial DNA (mtDNA) cytochrome oxidase also supports these findings. Molecular characterization of this gene region from individuals from Paraguay also supports previous morphological identification of *H. armigera* in Paraguay (Arnemann et al. 2016).

Research about the potential invasion of *H. armigera* in North America shows that this is a matter of time, given that the species has already been detected in Argentina, Bolivia, Uruguay, Puerto Rico and now Paraguay. Furthermore, the extensive production of host crops, facilitates their abundance and propensity to disperse in wind systems, thus, they will continue to spread beyond South and Central America (Kriticos et al. 2015)



Figure 2. Detection of *H. armigera* and *H. zea* in soybean and maize crop 2016/2017 from districts of Amambay Department, Paraguay.

ACKNOWLEDGMENTS

To CONACYT, Consejo Nacional de Ciencia y Tecnología - Paraguay for financing the research.

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