Mitochondrial DNA COI characterization of *Helicoverpa armigera* (Lepidoptera: Noctuidae) from Paraguay and Uruguay

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ABSTRACT. Since its detection in Brazil in 2013, the Old World cotton bollworm *Helicoverpa armigera* has been reported in Argentina, Paraguay, and Bolivia. Here we present evidence extending the South American range of *H. armigera* to Uruguay, using polymerase chain reaction and sequencing of the partial mitochondrial DNA (mtDNA) *cytochrome oxidase I* region. Molecular characterization of this gene region from individuals from Paraguay also supports previous morphological identification of *H. armigera* in Paraguay. Shared mtDNA haplotypes in *H. armigera* from Brazil, Uruguay, and Paraguay were identified. Additional surveying of populations in this region will be imperative to better monitor and understand factors that are underpinning its presence and successful adaptation in

these South American regions. We discuss our findings with respect to the development of resistance pest management strategies of this invasive insect pest in a predominantly monoculture soybean crop landscape in the Southern Cone region.

Key words: Parana River; Sea port; Southern Cone; Molecular detection; South America

INTRODUCTION

The incursion of the highly polyphagous Old World cotton bollworm *Helicoverpa armigera* into Brazil was reported by Czepak et al. (2013), based on morphological characters, and confirmed by Tay et al. (2013), based on mtDNA markers. Since then, *H. armigera* has been reported from countries neighbouring Uruguay; including Paraguay (Senave, 2013), Argentina (Murúa et al., 2014; Arneodo et al., 2015), and Bolivia (Kriticos et al., 2015). Kriticos et al. (2015) modeled the anticipated spread of *H. armigera* across the southern and northern American continents. The pest has since been detected in Puerto Rico and Florida in the United States of America (Animal and Plant Health Inspection Service, 2015). To date, Brazil has been the only country where extensive surveys of population genetic diversity of *H. armigera* have been carried out (Leite et al., 2014; Mastrangelo et al., 2014), with attempts to better understand host plant usages in different seasons combined with pest genetic diversity across populations from northern and central Brazilian states. Sequence data (i.e., three mtDNA *cytochrome oxidase I* (COI) haplotypes; KP984522, KP984523, and KP984524) for *H. armigera* from Argentina was also recently reported by Arneodo et al. (2015). In Paraguay, the presence of *H. armigera* was reported, but to date, no genetic diversity data and no official record of *H. armigera* in Uruguay exists.

The landscape of the South America is highly connected and there are substantial soybean areas available in Uruguay (1.3 million hectares in 2014; USDA, 2015a) and neighbouring countries, including Brazil (31.5 million ha in 2015, CONAB, 2015), Argentina (20.3 million ha in 2015; USDA, 2015a), and Paraguay (3.6 million ha in 2014; USDA, 2015a). In addition, there are ongoing trade activities between countries of the Southern Cone (Cone Sul) region (i.e., Brazil, Uruguay, Paraguay, and Argentina). It is reasonable to expect that *H. armigera*, a pest species with high flight ability (Fitt, 1989), is present in Uruguay. A southern range expansion of invasive pests from Brazil to neighbouring countries has been suggested for the neotropical invasive brown stink bug *Euschistus heros* (Saluso et al., 2011). The likely presence of *H. armigera* in Uruguay was previously reported based on morphological characters (Castiglioni E, Perini CR, Chiaravalle W, Arnemann JA, et al., unpublished results). Based on high similarity with previously published mtDNA COI haplotypes, we now provide molecular confirmation that *H. armigera* is indeed present in Uruguay. This represents the southern most region in South America to record this highly invasive pest species to date. This study is also the first to report the mtDNA COI haplotype diversity of *H. armigera* in Paraguay, since its detection and identification based on morphological characters (Senave, 2013).

MATERIAL AND METHODS

Suspected *H. armigera* adult moths were collected using delta traps baited with the female sexual pheromone ISCAlure armigera® (ISCA Tecnologias LTDA, Ijuí, RS, Brazil) installed in soybean fields in Uruguay (Rocha, -34.556354; -54.409917) and Paraguay (Alto Paraná, -24.997165;

-55.164035), in the 2014/15 season. Adult moths collected were preserved in 99.9% ethanol for molecular characterization purposes. Total gDNA from individual moths was extracted, using the Qiagen Blood and Tissue DNA extraction kit (Cat# 69506) in 50 µL AE buffer, and stored at -20°C until further analysis. A 707-bp fragment of the mtDNA COI gene was amplified by polymerase chain reaction (PCR), using the primers NOC-COI-F (5' GCGAAAATGACTTTATTCAAC 3') and COI-R (5' GCGAAAATGACTTTATTCAAC 3'). The PCR was sun under the following conditions: denaturing at 95°C for 5 min, 34 cycles of alternating denaturing, annealing, and extension steps each for 30 s at 95, 61, and 72°C, respectively, followed by a final extension cycle of 72°C for 5 min. The PCR amplification of individual DNA samples was carried out in a total reaction volume of 25 µL, and contained 25 ng genomic DNA, 0.5 µM both forward and reverse primers, 0.2 mM dNTPs, 1X Phusion HF Buffer (NEB, Ipswich, Massachusetts, USA), and 1.25 U Phusion DNA polymerase (NEB, Ipswich, Massachusetts, USA).Amplicons were purified using the QIAquick® PCR purification Kit (Qiagen), prior to Sanger sequencing. The sequencing reaction (ABI BigDye® dideoxy chain termination sequencing system V3.1; Applied Biosystems, Carlsbad, California, USA) and post sequencing reaction clean-up were performed as required by the sequencing facility (Australian National University Biomolecular Resource Facility). The programs Pregap and Gap4 within the Staden package (Staden et al., 2000) were used for editing and analysis of the DNA sequences, as well as to generate sequence contigs (i.e., haplotypes). Assembled partial mtDNA COI contigs were checked for premature stop codons that may be indicative of pseudogenes. The evolutionary divergence between haplotypes was estimated using the maximum composite likelihood model (Tamura et al., 2004), and included all (i.e., 1^{st} + 2^{nd} + 3^{rd}) codon positions using MEGA6 (Tamura et al., 2013). Estimates of haplotype diversity (h ± SE) and nucleotide diversity (π ± SE) were carried out using the molecular evolution software package DNA sequence polymorphism (DnaSP) v. 5.10.01 (Librado and Rozas, 2009).

The maximum-likelihood phylogenetic software PhyML 3.0 (Guindon et al., 2010) was used, to infer phylogeny based on 541 bp partial mtCOI genes by selecting the “automatic model selection” option for optimizing the substitution model and 1000 bootstrap replications for estimating node confidence. We included sequences of four *H. zea* (KM275162, KM274998, KM274954, and EU768942), four *H. punctigera* (HQ951242, HQ951243, KF977797, and EU768941), the two most common haplotypes of *H. armigera*, identified in Brazil by Mastrangelo et al. (2014; KF624850) and Leite et al. (2014; KM274936), three sequences from Uruguay (five individuals, three haplotypes, KU255535, KU255536, and KU255537), and six sequences from Paraguay (10 individuals, six haplotypes, KU255538, KU255539, KU255540, KU255541, KU255542, and KU255543). The *Chloridea subflexa* “C” sequence (KT598689) was used as the out-group (de Souza et al., 2015).

RESULTS

All 10 specimens collected from Paraguay and five of the 11 collected from Uruguay were successfully sequenced for the mtDNA COI fragment, using the NOC-COI-F/R primer pairs. Note that KU255537 from Uruguay and KU255542 from Paraguay are identical to the previously published sequences KM275131 (Leite et al., 2014) and KF624850 (Mastrangelo et al., 2014), respectively. A sequence identity search of the NCBI GenBank database confirmed that all suspected moths significantly matched (i.e., 99-100% nucleotide identity) published *H. armigera* sequences. The evolutionary divergence over sequence pairs against the *H. armigera* sequences (using sequences from Paraguay, Uruguay, and the two most common haplotypes from Brazil), *C. subflexa*, *H. zea*, and *H. punctigera* sequences were estimated. The average evolutionary

divergence within *H. armigera* was 0.004 (± 0.002 SE) and ranged from 0 to 0.009 (± 0.002-0.003 SE). As previously reported, the most similar species to *H. armigera* was *H. zea* (0.032 ± 0.006 SE), and the most divergent was *H. punctigera* (0.06 ± 0.01 SE) (Behere et al., 2007) and *C. subflexa* (0.066 ± 0.010 SE) (Table 1).

Table 1. Estimates of evolutionary divergence over sequence pairs between *Helicoverpa armigera* sequences.

	H. armigera	*C. subflexa*	*H. zea*	*H. punctigera*
H. armigera	-	**0.010**	**0.006**	**0.010**
C. subflexa	0.066	-	**0.010**	**0.010**
H. zea	0.032	0.070	-	**0.009**
H. punctigera	0.060	0.066	0.055	-

Using sequences from Paraguay, Uruguay, and the two most common haplotypes from Brazil, *Chloridea subflexa*, *H. zea*, and *H. pucntigera*. SE estimates from 500 bootstrap replications are shown in the upper right triangle (in bold).

The estimates of *h* and π for the Uruguay sequences were 0.4 (0.237 SD) and 0.00073 (0.00043 SD), respectively. For the Paraguay samples, *h* was 0.889 (0.075 SD) and π was 0.00537 (0.00057 SD). The *h* for the *H. armigera* samples from Uruguay can be considered low, similar to those observed in populations from China, Pakistan, and Burkina Faso (Behere et al., 2007). The low π observed for *H. armigera* is likely due to the small sample size. In Paraguay, the *h* was similar to that found for the Brazilian population by Leite et al. (2014), and higher than those found in populations from India, Pakistan, Burkina Faso, and Uganda (Behere et al., 2007). On the other hand, the π was considerably higher in Paraguay, when compared with *H. armigera* populations from Brazil, India, Pakistan, Burkina Faso, and Uganda.

Sequence analysis of our Paraguay and Uruguay *H. armigera* individuals using PhyML indicated that these individuals were clustered together with a strong bootstrap support value of 97.4%. This cluster also included the previously published *H. armigera* haplotypes (KM275131 and KF624850). The previously published *H. zea* and *H. punctigera* haplotypes, also included in this study, clustered separately with strong bootstrap support (98.4 and 91.3%, respectively) from all our *H. armigera* individuals (Figure 1).

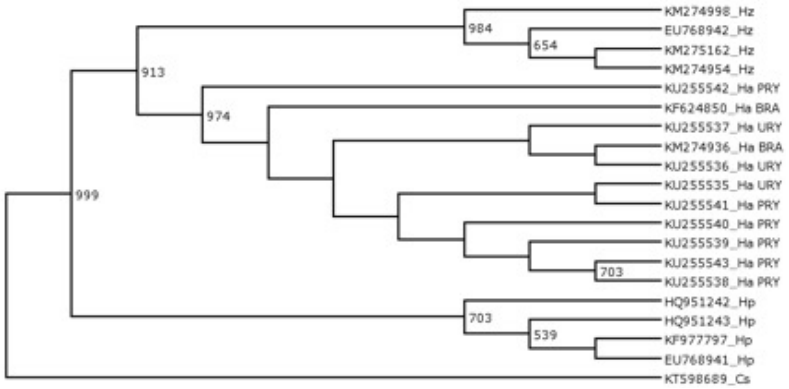

Figure 1. Maximum likelihood phylogeny analysis using PhyML (Guindon et al., 2010) of *Helicoverpa* (Hz = *H. zea*, Ha = *H. armigera*, and Hp = *H. punctigera*) and *Chloridea subflexa* (Cs = *C. subflexa*) species. The tree was created using substitution model: GTR+G6, AIC: 2274.45, Gamma shape parameter: 0.029, proportion of invariable sites: 0.0, and was based on 541 bp partial mtCOI gene. The out group is *C. subflexa* (KT598689) (de Souza et al., 2015). BRA = Brazil, PRY = Paraguay, and URY = Uruguay. Bootstrap values of >50% are shown. The iBoL ID or GenBank accession numbers for all samples used are provided.

DISCUSSION

Based on shared mtDNA COI partial gene identity, we have been able to confirm that *H. armigera* has extended its range in South America to Uruguay. The density and distribution of this pest insect within Uruguay remains to be studied in detail. Our data were limited to those obtained from five individuals caught using a pheromone trap. We were not able to amplify the DNA from the other six individuals. This failure may have been due to poor gDNA and/or the fact that they belonged to other lepidopteran species. In Paraguay, the presence of *H. armigera* has previously been reported based on morphological characterization (Senave, 2013). However, due to the difficulty of species identification among closely related congeners (e.g., *H. zea*, Hardwick, 1965; Pogue, 2004), confirmation using molecular characterization would also be desirable. The results presented here represent the first insight into genetic diversity of *H. armigera* in Paraguay.

Soybean is being planted to an increasing extent in the Cone Sul region, especially in Argentina, Paraguay, and Uruguay. The heterogeneous cropping patterns, distribution, and the substantial soybean cropping area in the Cone Sul region (i.e., over 56.8 million ha of combined planting areas from Brazil, Argentina, Uruguay, and Paraguay) results in availability of this host crop over many months to support *H. armigera*. Although the majority of the soybean fields have one cropping season (September to May) in Brazil, Argentina, and Uruguay, these regions too contain large areas with two cropping seasons. For example, some regions of Paraguay can have two soybeans crops per year. In Bolivia, soybean is planted in the winter (June-July) and harvested in mid spring/early summer (October-December). This is followed by another summer planting (November-December) and harvested in late summer/early autumn (March-April) (USDA, 2015b). This offers a continuous food source to support populations of agricultural pests. Furthermore, the highly polyphagous nature of *H. armigera* suggests that it is also able to utilize diverse, alternative agricultural and horticultural hosts (e.g., tomatoes, sunflower, and citrus) cultivated in these regions (e.g., in Uruguay and Paraguay), in addition to soybean.

We identified three *H. armigera* individuals from Paraguay that had one of the most common mtDNA COI haplotypes from Brazil, as reported by Mastrangelo et al. (2014; KF624850). Three individuals from Uruguay shared the other most common mtDNA COI haplotype from Brazil (Leite et al., 2014; KM274936). These may represent movements from Brazil, although we caution on inferences of pest movements based on the most common haplotypes especially at the global scale. We also identified new mtDNA COI haplotypes from the Paraguay (KU255538, KU255539, KU255540, KU255541, and KU255543) and Uruguay (KU255535 and KU255536) populations. The existing trade movements between countries in the Southern Cone region (e.g., southern Brazil (Parana state, PR; Santa Catarina, SC; and Rio Grande do Sul, RS), Uruguay, Paraguay, and Argentina), together with the high migration ability of this pest (Fitt, 1989) may constitute factors that contribute to the movements of *H. armigera* within this region. However, the Parana River port (Rosario) in Argentina, the sea port Montevideo in Uruguay, and movements of goods along the Parana River from Argentina to the Paraguay region, may also act as potential entry points for new incursions of agricultural pests, including the highly invasive *H. armigera*.

The eradication of *H. armigera* from Paraguay and Uruguay is unlikely due to its high dispersal capacity, the successful establishment of this pest in neighbouring countries such Argentina (Murúa et al., 2014; Arneodo et al., 2015), Bolivia (Kriticos et al., 2015) and Brazil (Czepak et al., 2013; Tay et al., 2013; Mastrangelo et al., 2014; Leite et al., 2014), and the availability of diverse plant hosts in the Cone Sul region. The anticipated costs associated with containment and eradication of this highly invasive pest in both Paraguay and Uruguay will likely be economically

unattractive. Furthermore, even with successful eradication, the likelihood of re-infestation from neighbouring countries that serve as source populations will be high.

To minimize potential costs associated with significant loss of agricultural crops and loss of insecticide chemical compounds for effective control of *H. armigera* in this region, a well-designed resistance pest management (RPM) strategy will need to be developed and implemented. *H armigera* has a history of rapidly evolving resistance to insecticides such as pyrethroids in India (Kranthi et al., 2001), China (Yang et al., 2004), Africa (Martin et al., 2002), and Australia (Gunning et al., 1991). The Brazilian *H. armigera* is suspected to have originated from Asia and Europe, although African and/or Australian origins cannot be ruled out, due to the limited availability of DNA sequence data from these two continents (Leite et al., 2014). Until the population origins are identified, an effective RPM for *H. armigera* in the South American region will need to consider resistance profiles of populations from across the Old World and Australasia.

Genetically modified (GM) crops are not features of the European agricultural landscape; however, GM cotton (*Gossypium* spp) is currently cultivated in Africa, Australia, and across Asia, expressing either the *Bacillus thuringiensis* (Bt) Cry1Ac toxin and/or Cry1Ac and Cry2Ab toxins. However, GM soybean, cotton, and maize (*Zea mays*) are present in the Cone Sul region (e.g., in Brazil, GM soybean expressing the Bt Cry1Ac toxin in the 2013/14 season was estimated to be ca. 6-7 million ha), including the Mato Grosso do Sul, PR, SC, and RS states neighbouring Paraguay, Argentina, and Uruguay. Resistances to Cry1Ac toxins in *H. armigera* are known to occur in China (Yang et al., 2007) and India (Kranthi et al., 2006), and it is important to note that the majority of the soybean crops planted in the Cone Sul region are GM plants expressing the Cry1Ac toxin. Bt corn expressing the Cry2Ab toxin is also cultivated in the Cone Sul region and monitoring of *H. armigera* will also need to consider resistance to Cry2Ab (Tay et al., 2015). When developing RPM strategies, the nature of the GM crops expressing particular classes of Bt toxins and the types of economic crops (e.g., soybean, cotton, or corn) cultivated in the Cone Sul region will need to be taken into consideration, and considerable efforts should be directed towards keeping the *H. armigera* populations at low levels in infested areas. This may be done through the use of pheromone traps or biological control agents, including parasitoids. In addition, strategies should examine the potential need for introducing refuge strategy. It may also be necessary to tighten the sowing time and harvesting season between regions as well as introduce crop rotation. Actions such as mitigating measures to inhibit the dispersal of *H. armigera* between these regions, including policies to prevent its dissemination through unchecked anthropogenic activities, and strict control of movement of host plant material from infested areas are also crucial, since a broad-scale application of chemicals in the Cone Sul region, as a strategy to reduce *H. armigera* dispersal, would be economically and environmentally costly.

Conflicts of interest

The authors declare no conflict of interest.

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