University of São Paulo
Luiz de Queiroz College of Agriculture

Genetic diversity and susceptibility to Vip3Aa20 protein in Brazilian populations of *Helicoverpa armigera* and *Helicoverpa zea* (Lepidoptera: Noctuidae)

Natália Alves Leite

Thesis presented to obtain the degree of Doctor in Science. Area: Entomology

Piracicaba
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RESUMO

Diversidade genética e suscetibilidade à proteína Vip3Aa20 em populações brasileiras de *Helicoverpa armigera* e *Helicoverpa zea* (Lepidoptera: Noctuidae)

Helicoverpa armigera (Hübner) foi oficialmente reportada no Brasil em 2013. Esta espécie é estreitamente relacionada a *Helicoverpa zea* (Boddie) e tem causado danos significativos nas culturas no Brasil. O uso de plantas geneticamente modificadas, que expressam proteínas inseticidas de *Bacillus thuringiensis* (Berliner), tem sido uma das táticas de controle para o manejo dessas pragas. O milho geneticamente modificado que expressa Vip3Aa20 foi aprovado para comercialização no Brasil em 2009. O entendimento da diversidade genética e da suscetibilidade às proteínas de *B. thuringiensis* em populações de *H. armigera* e *H. zea* no Brasil são cruciais para o estabelecimento de programas de Manejo da Resistência de Insetos (MRI). Assim, os objetivos desse estudo foram: (a) inferir parâmetros demográficos e estrutura genética de *H. armigera* e *H. zea* no Brasil; (b) avaliar o fluxo gênico intra e interespecífico e a diversidade genética em *H. armigera* e *H. zea*; e (c) aferir a suscetibilidade de populações brasileiras de *H. armigera* e *H. zea* a proteína Vip3Aa20. Uma análise filogeográfica de populações de campo de *H. armigera* e *H. zea* foi realizada com o uso de sequências do gene *citocromo c oxidase I* (*COI*). Indivíduos de *H. armigera* foram mais prevalentes em dicotiledôneas e *H. zea* na cultura do milho. Ambas as espécies mostraram sinais de expansão demográfica e ausência de estrutura genética. Alta diversidade genética e ampla distribuição foram observadas em *H. armigera*. Análises conjuntas indicaram a presença de linhagens da China, Índia e Europa em populações brasileiras de *H. armigera*. A partir de um estudo de amplificação cruzada de microssatélites, sete locos amplificaram em ambas as espécies e evidenciaram a possibilidade de hibridização no campo. Estes mesmos locos foram usados para análises interespecíficas de *H. armigera* e *H. zea* do Brasil em comparação a *H. zea* dos EUA. Nas análises para cada espécie, 10 microssatélites foram usados para *H. armigera* e oito para *H. zea*. Alto fluxo gênico intraespecífico foi detectado em populações de *H. armigera* e *H. zea*. A diversidade genética foi similar em ambas as espécies. *H. armigera* foi mais similar a *H. zea* do Brasil que dos EUA e possíveis híbridos foram encontrados nas populações brasileiras. Houve um baixo fluxo gênico entre populações brasileiras e americanas de *H. zea*. A linha-básica de suscetibilidade a Vip3Aa20 resultou numa variação interpopulacional baixa em *H. zea* (3 vezes) e em *H. armigera* (5 vezes), baseada na CL_{50}. *H. armigera* foi mais tolerante a Vip3Aa20 que *H. zea* (≈ 40 to 75 vezes, baseado na CL_{50}). A concentração diagnóstica, baseada na CL_{99}, foi bastante alta (6.400 ng Vip3Aa20/cm^2) para *H. zea* e não validada para *H. armigera* devido à alta quantidade de proteína necessária para os bioensaios. A implementação de estratégias de MRI a Vip3Aa20 em *H. armigera* e *H. zea* serão um grande desafio no Brasil, principalmente devido à baixa suscetibilidade a Vip3Aa20 e alta diversidade genética e fluxo gênico em ambas as espécies, além da possibilidade de indivíduos híbridos entre *H. armigera* e *H. zea* nas condições de campo.

Palavras-chave: Manejo da resistência de insetos; *Helicoverpa* spp.; Genética de populações; Hibridização; Espécies invasoras; Milho transgênico

ABSTRACT

Genetic diversity and susceptibility to Vip3Aa20 protein in Brazilian populations of *Helicoverpa armigera* and *Helicoverpa zea* (Lepidoptera: Noctuidae)

Helicoverpa armigera (Hübner) was officially reported in Brazil in 2013. This species is closely related to *Helicoverpa zea* (Boddie) and has caused significant crop damage in Brazil. The use of genetically modified crops expressing insecticidal protein from *Bacillus thuringiensis* (Berliner) has been one of the control tactics for managing these pests. Genetically modified maize expressing Vip3Aa20 was approved to commercial use in Brazil in 2009. Understanding the genetic diversity and the susceptibility to *B. thuringiensis* proteins in *H. armigera* and *H. zea* populations in Brazil are crucial for establishing Insect Resistance Management (IRM) programs in Brazil. Therefore, the objectives of this study were: (a) to infer demographic parameters and genetic structure of *H. armigera* and *H. zea* Brazil; (b) to assess the intra and interspecific gene flow and genetic diversity of *H. armigera* and *H. zea*; and (c) to evaluate the susceptibility to Vip3Aa20 protein in *H. armigera* and *H. zea* populations of Brazil. A phylogeographic analysis of field *H. armigera* and *H. zea* populations was performed using a partial sequence data from the *cytochrome c oxidase I* (*COI*) gene. *H. armigera* individuals were most prevalent on dicotyledonous hosts and *H. zea* individuals were most prevalent on maize crops. Both species showed signs of demographic expansion and no genetic structure. High genetic diversity and wide distribution were observed for *H. armigera*. A joint analysis indicated the presence of Chinese, Indian, and European lineages within the Brazilian populations of *H. armigera*. In the cross-species amplification study, seven microsatellite loci were amplified; and showed a potential hybrid offspring in natural conditions. Interespecific analyses using the same microsatellite loci with Brazilian *H. armigera* and *H. zea* in compare to the USA *H. zea* were also conducted. When analyses were performed within each species, 10 microsatellites were used for *H. armigera*, and eight for *H. zea*. We detected high intraspecific gene flow in populations of *H. armigera* and *H. zea* from Brazil and *H. zea* from the USA. Genetic diversity was similar for both species. However, *H. armigera* was more similar to *H. zea* from Brazil than *H. zea* from the USA and some putative hybrid individuals were found in Brazilian populations.Tthere was low gene flow between Brazilian and USA *H. zea*. The baseline susceptibility to Vip3Aa20 resulted in low interpopulation variation for *H. zea* (3-fold) and for *H. armigera* (5-fold), based on LC_{50}. *H. armigera* was more tolerant to Vip3Aa20 than *H. zea* (≈ 40 to 75-fold, based on CL_{50}). The diagnostic concentration for susceptibility monitoring, based on CL_{99}, was fairly high (6,400 ng Vip3Aa20/cm^2) for *H. zea* and not validated for *H. armigera* due to the high amount of protein needed for bioassays. Implementing IRM strategies to Vip3Aa20 in *H. armigera* and *H. zea* will be of a great challenge in Brazil, mainly due to the low susceptibility to Vip3Aa20 and high genetic diversity and gene flow in both species, besides a potential of hybrid individuals between *H. armigera* and *H. zea* under field conditions.

Keywords: Insect resistance management; *Helicoverpa* spp.; Population genetics; Hybridization; Invasive species; Transgenic maize

1 INTRODUCTION

The two Heliothinae pests, *Helicoverpa armigera* (Hübner) and *Helicoverpa zea* (Boddie) (Lepidoptera: Noctuidae), are important pests due to their high polyphagy, high dispersal and rapid adaptation to various control tactics (FITT, 1989; HEAD et al., 2010; EDWARDS et al., 2013; YANG; LI; WU, 2013; RAZMJOU; NASERI; HEMATI, 2014; WALSH et al., 2014). *H. zea* is endemic in the Americas, while *H. armigera* were found only in the Old World until it was first detected in Brazil in 2013 (CZEPAK et al., 2013; SPECHT et al., 2013; TAY et al., 2013). The management of these pests around the world is based mainly on the use of insecticides and genetically modified (GM) crops that express *Bacillus thuringiensis* (Berliner) (*Bt*) proteins. These crops have been widely adopted throughout the world since 1996 (JAMES, 2014).

In Brazil, the adoption of *Bt* crops was on 32.5% of the total cultivated area with soybean, cotton and maize in 2014-2015 (CÉLERES, 2015). *Bt* maize was cultivated in an area of 12.5 million hectares, corresponding to 82.7% of the total area cultivated with this crop (CÉLERES, 2015). The introduction of *Bt* maize in Brazil occurred with hybrids expressing Cry1Ab protein in MON810 and Bt11 events, which were commercially available in 2008. In 2009, hybrids that express Vip3Aa20 protein, MIR162 event, were commercially released in Brazil.

Vip3Aa20 maize was released in Brazil to control: *Spodoptera frugiperda* (J. E. Smith) (Lepidoptera: Noctuidae), *Diatraea saccharalis* (Fabr.) (Lepidoptera: Crambidae) and *H. zea*. These species were considered the major target pests of *Bt* maize until the detection of *H. armigera*, another pest that also attacks maize (JALLOW; CUNNINGHAM; ZALUCKI, 2004). *H. zea* and *H. armigera* larvae feed on both vegetative and reproductive tissues of different crops, causing significant economic losses, even at low population densities (MITTER; POOLE; MATTHEWS, 1993). In 2012/13, *H. armigera* caused a loss of more than US$ 500 million, only in Bahia State, due to direct yield losses and resources spent on phytosanitary products in grains and fibers (MAPA, 2015).

The potential for resistant evolution in pest populations to *Bt* proteins is a major threat to the sustainable use of this technology (TABASHNIK, 1994; GOULD, 1998; ANDOW, 2008). Field-evolved resistance to *Bt* crops is a real concern because it has already been documented in some key pests exposed to Cry proteins,

such as in *Busseola fusca* (Fuller) (Lepidoptera: Noctuidae) to Cry1Ab maize in South Africa (VAN RENSBURG, 2007), in *S. frugiperda* to Cry1F maize in Puerto Rico (MATTEN; HEAD; QUEMADA, 2008), and in *Diabrotica virgifera virgifera* (Coleoptera:Chrysomelidae) to Cry3Bb1 maize in the USA (GASSMANN et al., 2011). To Cry1Ac cotton in *Pectinophora gossypiella* (Saunders) (Lepidoptera: Gelechiidae) in India (DHURUA; GUJAR, 2011), and in *H. armigera* in Pakistan (ALVI et al., 2012). Field-evolved resistance was also reported in *H. zea* to Cry1Ac cotton (TABASHNIK et al., 2008) and to Cry2Ab cotton in the USA (TABASHNIK; CARRIÈRE, 2010). Recently, field-evolved resistance was detected in *S. frugiperda* to Cry1F maize (FARIAS et al., 2014) and Cry1Ab maize (OMOTO et al., 2016) in Brazil. At this time, no field-evolved resistance was reported for Vip proteins. However, a high frequency of resistance alleles was reported in field populations of *H. armigera* (0.027) and *Helicoverpa punctigera* (Wallengren) (0.008) before the commercial release of *Bt* cotton expressing Vip3A in Australia (MAHON; DOWNES; JAMES, 2012). Vip3A protein has been an important tool for IRM programs, because there is no cross-resistance with Cry proteins (LEE et al., 2003).

The implementation of IRM strategies is dependent on the development of effective monitoring programs capable of detecting the resistance evolution at earlier stages (DENNEHY, 1987). Phenotypic or genotypic methods can be used for resistance monitoring (ANDOW; ALSTAD, 1998; ANDOW et al., 1998, ANDOW 2008). Among these methods, one of the most used is the phenotypic method of screening field-collected larvae (SIMS et al., 1996; WU; GU; HEAD, 2006; BERNARDI et al., 2014). In this method, neonates are exposed to a diagnostic concentration of the insecticidal protein incorporated in the diet or applied in its surface. The diagnostic concentration is a concentration of the protein that kills all or nearly all susceptible individuals but few or no resistant individuals (TABASHNIK et al., 2014). The initial step for the implementation of such programs is the establishment of a baseline susceptibility of the target pest of different geographical areas. With this information, changes in the susceptibility of populations in response to *Bt* selection pressure can be identified (FISCHHOFF, 1996).

The evolution of insect resistance to *Bt* proteins is a process governed by a number of factors that interact with each other and are related to transgenic plant characteristics, bioecology and genetics of the target pest, and crop and environment management. These factors are classified as genetic, bioecological and operational

(GEORGHIOU; TAYLOR, 1977a, 1977b; ROUSH; DALY, 1990). In order to delay resistance evolution, all these factors are taken into consideration in the development and implementation of management practices (HOKKANEN; WEARING, 1994).

The genetic diversity of the insects allows the adaptation to control tactics, and due to the high selection pressure carried out by the Brazilian cropping system, resistance selection can become an inevitable process. Additionally, gene flow may be able to maintain a uniformity of genetic diversity among populations as well as carry variation across populations (CAPRIO; TABASHNIK, 1992), enabling the alleles that confer resistance to spread among populations of *H. armigera* and *H. zea*. The intensive cropping system, the inadequate management (i.e. low adoption of refuge area), and the main tropical climate of Brazil create a unique complex scenario for pest populations (FARIAS et al., 2014).

The invasion of *H. armigera* into this complex agricultural scenario of Brazil brought questions about its population dynamics. A key aspect of understanding insect pest population dynamics in agricultural scenarios is the analysis of population genetic structure, i.e. the distribution of genetic variation within and among populations (RODERICK, 1996). Understanding population structures provides the most fundamental information for design of management strategies. Moreover, understanding the genetics of pest invasion may help to identify the number of introductions, the origin and the spread of the infestation of a pest in a specific region. Therefore, studies of population genetics of *H. armigera*, *H. zea* and other pests present in Brazil are extremely important to understand their population dynamics in order to acquire information for developing management strategies.

Studies of population genetics of *H. armigera* and *H. zea* using mitochondrial and nuclear markers revealed high genetic diversity and low structure in different regions of the world (SCOTT et al., 2005; BEHERE et al., 2007; ENDERSBY et al., 2007; LI et al., 2011; PERERA; BLANCO, 2011; BEHERE et al., 2013). For *H. zea*, 13 microsatellite loci were isolated and characterized (PERERA et al., 2007) and for *H. armigera* 20 loci were reported (TAN et al., 2001; JI et al., 2003; SCOTT et al., 2004; JI; WU; ZHANG, 2005). The transferability of microsatellite primers have already been tested for *H. armigera* to *H. zea* and other *Helicoverpa* species. Among the 14 loci tested, only four amplified for *H. zea* (GRASELA; MCINTOSH, 2005). The transferability of *H. zea* microsatellite primers to *H. armigera* has not been tested yet. The use of microsatellites that work on both species can be enlarged with the

transferability of *H. zea* primers to *H. armigera*. Therefore, population genetics studies relating both species can be performed.

H. armigera and *H. zea* are considered sibling species as they are phylogenetically close (MITTER; POOLE; MATTHEWS, 1993; CHO et al., 2008). Phylogeographic analysis of *H. armigera* and *H. zea* individuals suggest that *H. zea* has evolved from a small portion of *H. armigera* population or from a common ancestor that reached the Americas about 1.5 million years ago (MALLET et al., 1993; BEHERE et al., 2007). Therefore, they are able to mate and produce fertile offspring under laboratory conditions (LASTER; HARDEE, 1995; LASTER; SHENG, 1995). However, natural interspecific genetic flow between these two species has not been reported in the literature, since they did not occur in the same geographic region until the invasion of *H. armigera* in South America. This new scenario needs to be investigated, as there is the possibility that both species can mate in natural conditions and produce hybrids.

Hybridization and introgression are evolutionary processes that involve the creation of a new genome and are crucial at different times in the life history of a species (MALLET, 2007). In addition, hybridization and introgression events are described as important adaptation phenomena of invasive species into a new environment (ABBOTT, 1992; LEVIN; FRANCISCO-ORTEGA; JANSEN, 1996; RHYMER; SIMBERLOFF, 1996; SAKAI et al., 2001; ELLSTRAND; SCHIERENBECK, 2006). *H. armigera* rapidly dispersed across the American continent, with reports in all of South America in 2013/2014 (LEITE et al., 2014; MASTRANGELO et al., 2014; MURÚA et al., 2014; SENAVE, 2014), and more recently in Florida, USA, where *H. armigera* specimens were collected in pheromone traps in 2015 (APHIS, 2015; HAYDEN; BRAMBILA, 2015). The rapid and widespread success of an invasive species, such as *H. armigera*, in a new environment can be explained by the hybrid vigor event, which is an increase in a population fitness caused by the transference of genes directly linked to adaptive processes (BEEBEE; ROWE, 2004; FREELAND; PETERSEN; KIRK, 2011). Hybridization is extremely common in plants (ABBOTT, 1992; RIESEBERG et al., 2003; ELLSTRAND; SCHIERENBECK, 2006), but also occurs frequently in animals (ABERNETHY, 1994; ECHELLE; ECHELLE, 1997; PERRY; LODGE; FEDER, 2002; HASHIMOTO et al., 2012). In an IRM perspective, the occurrence of hybrids in nature is a new challenge,

as they may be better adapted to hosts, develop tolerance to climatic factors, pathogens, and control methods such insecticides and *Bt* plants.

In this context, the use of mitochondrial and nuclear markers can assist in the knowledge acquisition of the origin, demography, genetic diversity and intra and interspecific gene flow in *H. armigera* and *H. zea*. Furthermore, the use of native populations of *H. armigera* and/or *H. zea*, which had no contact with other species, gives more power to the identification of hybridization events between *Helicoverpa* species. This knowledge will serve as a base for understanding the adaptive processes involved in the success of *H. armigera* invasion in Brazil and other countries of the Western Hemisphere. In addition, the knowledge of the population dynamics of these insects will help in the development of management strategies. To implement an IRM program in Brazil, it is also important to perform a resistance risk analysis of *H. armigera* and *H. zea* to control tactics. To date, there is no information about the susceptibility of these pests in Brazil to the Vip3Aa20 protein. Therefore, our main objectives in this thesis were:

- To infer demographic parameters and genetic structure of *H. armigera* and *H. zea* populations of Brazil using cytochrome oxidase I (COI) marker;

- To assess the genetic diversity and gene flow of *H. armigera* and *H. zea* populations of Brazil using microsatellite markers;

- To identify the possible presence of hybrid specimens between *H. armigera* and *H. zea* in natural conditions;

- To evaluate the susceptibility to Vip3Aa20 protein in *H. armigera* and *H. zea* populations of Brazil.

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2 DEMOGRAPHICS AND GENETIC VARIABILITY OF THE NEW WORLD BOLLWORM (*Helicoverpa zea*) AND THE OLD WORLD BOLLWORM (*Helicoverpa armigera*) IN BRAZIL[1]

Abstract

Helicoverpa armigera is one of the primary agricultural pests in the Old World, whereas *Helicoverpa zea* is predominant in the New World. However, *H. armigera* was first documented in Brazil in 2013. Therefore, the geographical distribution, range of hosts, invasion source, and dispersal routes for *H. armigera* are poorly understood or unknown in Brazil. In this study, we used a phylogeographic analysis of natural *H. armigera* and *H. zea* populations to (1) assess the occurrence of *H. armigera* and *H. zea* on different hosts; (2) infer the demographic parameters and genetic structure of *H. armigera* and *H. zea* populations; (3) determine the potential invasion and dispersal routes for *H. armigera* within the Brazilian territory; and (4) infer the geographical origin of *H. armigera*. We analyzed partial sequence data from the cytochrome c oxidase subunit I (COI) gene. We determined that *H. armigera* individuals were most prevalent on dicotyledonous hosts and that *H. zea* were most prevalent on maize crops. The populations of both species showed signs of demographic expansion, and no genetic structure. The high genetic diversity and wide distribution of *H. armigera* in mid-2012 are consistent with an invasion period prior to the first reports of this species in the literature and/or multiple invasion events within the Brazilian territory. It was not possible to infer the invasion and dispersal routes of *H. armigera* with this dataset. However, joint analyses using sequences from the Old World indicated the presence of Chinese, Indian, and European lineages within the Brazilian populations of *H. armigera*. These results suggest that sustainable management plans for the control of *H. armigera* will be challenging considering the high genetic diversity, polyphagous feeding habits, and great potential mobility of this pest on numerous hosts, which favor the adaptation of this insect to diverse environments and control strategies.

Keywords: Molecular phylogeography; Cytochrome c oxidase I; *Helicoverpa*; Population genetics; Invasive species

2.1 Introduction

The Heliothinae (Lepidoptera: Noctuidae) subfamily has 381 described species, many of which are important agricultural pests from the *Helicoverpa* Hardwick and *Heliothis* Ochsenheimer genera (POGUE, 2013). The *Helicoverpa* genus contains two of the primary Heliothinae pest species: *Helicoverpa armigera* (Hübner) (Old World

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bollworm) and *Helicoverpa zea* (Boddie) (New World bollworm). Although the exact evolutionary relationship between *H. armigera* and *H. zea* remains uncertain, these insects are considered to be 'twin' or 'sibling' species, and they are able to copulate and produce fertile offspring under laboratory conditions (MITTER; POOLE; MATTHEWS, 1993; LASTER; HARDEE, 1995; LASTER; SHENG, 1995; CHO, S. et al., 2008). Some hypotheses propose that *H. zea* evolved from a small portion of the larger *H. armigera* population (i.e., a "founder effect") that reached the American continent approximately 1.5 million years ago, which is consistent with previous phylogeographic analyses of *H. armigera* and *H. zea* individuals (MALLET et al., 1993; BEHERE et al., 2007).

H. armigera is considered to be one of the most important agricultural pests in the world. This insect is widely distributed throughout Asia, Africa, Europe, and Australia, and it has been shown to attack more than 100 host species from 45 different plant families (FITT, 1989; POGUE, 2004; WU et al., 2008). In contrast, *H. zea* is restricted to the American continent and is of lesser economic importance; it is a secondary pest of cotton, tomato, and, most significantly, maize crops (DEGRANDE; OMOTO, 2013). However, the scenario in Brazil changed in 2013 when *H. armigera* individuals, which are considered to be A1 quarantine pests, were officially reported within the Brazilian territory (CZEPAK et al., 2013; SPECHT et al., 2013; TAY et al., 2013). This situation increased in severity due to the great dispersal ability of this insect as well as the steady reports from several regions of the world that described new *H. armigera* lineages showing tolerance/resistance to insecticides and genetically modified plants (MARTIN et al., 2005; YANG; LI; WU, 2013). It was estimated that *H. armigera* caused a loss of more than US$ 500 million to the 2012/13 Bahia State agriculture because of direct productivity losses and resources spent on phytosanitary products for grains and fibers (MAPA, 2015). Therefore, *H. armigera* is now one of the most important pest species with respect to agriculture in Brazil (MAPA, 2014).

High population densities of *Helicoverpa* spp. and the resulting economic damages to cultivated plants have been reported in different regions of Brazil, in particular in the Western state of Bahia (TAY et al., 2013). Therefore, these reports suggest the existence of an invasion period prior to the first official report of *H. armigera* in Brazil. This atypical and confusing scenario was likely caused by the significant morphological similarities between *H. zea* and *H. armigera* (POGUE,

2004; BEHERE et al., 2008) and by major changes in pest management programs over recent years. In addition, these population changes may have been related to the release and increased cultivation of crops that express *Bacillus thuringiensis* (*Bt*) genes in Brazil.

Aside from the identification of *H. armigera* individuals within the Brazilian territory, many basic pieces of information concerning this species, including its geographical distribution, the types of hosts it attacks, its invasion source, and its dispersal routes, remain poorly understood or completely unknown. Therefore, we attempted to address some of these outstanding questions using a phylogeographic approach by analyzing genetic sequence data from a portion of the cytochrome c oxidase subunit I (COI) gene of *Helicoverpa* spp. specimens isolated from different hosts and regions of Brazil. This study was performed with the following goals in mind: (1) to confirm and evaluate the occurrence of *H. armigera* and *H. zea* individuals from different hosts and regions of Brazil; (2) to assess the demographic parameters and genetic structure of *H. armigera* and *H. zea* populations within the Brazilian territory, with a focus on the region, season, and host; (3) to assess the potential invasion (single or multiple) and dispersal routes for *H. armigera* within the Brazilian territory; and (4) to determine the geographical origin of the *H. armigera* populations present in Brazil. This information will be essential for understanding the genetic diversity and population dynamics of these pests as well as for guiding both immediate control strategies (legal and/or phytosanitary) and subsequent long-term integrated management programs for the *Helicoverpa* spp. complex in Brazil.

2.2 Material and methods

2.2.1 Sampling procedures

Permit access to collect material used in our research at various crop sites was granted by respective growers. GPS coordinates of each location are listed in Table 2.1. Brazilian agriculture has shown successive and overlapping crops in space and time, and these crops can be largely separated into two harvest groups that are primarily characterized by their rainfall needs. In particular, winter crops are grown between May and September and require low rainfall, whereas summer crops are grown between October and April and require high rainfall. Our initial sampling

design was directed at understanding the *H. zea* population dynamics and primarily involved maize fields. However, attacks on soybean, cotton, bean, sorghum, and millet crops were also reported between May 2012 and April 2013 (Brazilian agricultural year). Therefore, we directed our sampling efforts towards a variety of crops and regions throughout Brazil. We also focused on the Western region of Bahia State, Brazil, which was the site of numerous *Helicoverpa* spp. attacks, to determine whether maize crops were the main source of *H*. *zea* in the Brazilian agricultural system. A total of 274 *Helicoverpa* larvae were collected at 19 sampling sites from six different crops (Table 2.1). In the absence of morphological characters or nuclear markers to reliably distinguish between *H. zea* and *H. armigera*, species identification was carried out using the sequence fragment of COI mitochondrial gene by comparing with *H. zea* and *H. armigera* species barcodes (BEHERE et al., 2007; ENCYCLOPEDIA OF LIFE, 2013; TAY et al., 2013) and determining homology with BlastN tool.

Table 2.1 - Sampling sites for *H. armigera* and *H. zea* in Brazil, including abbreviations, crops sampled, sample sizes for the mitochondrial gene (*COI*), geographic coordinates, dates sampled, and GenBank Accession.

(To be continue)

Site (City, State)	Abbreviation (Site, Crop)	Crop	Sample size		Lat. (S)	Lon. (W)	Date	GenBank Accession
			H. armigera	*H. zea*				
Winter cropping								
Barreiras, Bahia	BA1Co	Cotton	3	-	11°33′33”	46°19′47″	05.22.12	KM274936 – KM274938
Luís E. Magalhães, Bahia	BA2Co	Cotton	11	1	12°05′58″	45°47′54″	05.24.12	KM274939 – KM274950
Balsas, Maranhão	MA1Co	Cotton	10	-	07°31′59″	46°02′06″	06.23.12	KM274987 – KM274996
Luís E. Magalhães, Bahia	BA3Be	Bean	23	-	12°05′58″	45°47′54″	06.12.12	KM274979 – KM274986, KM275038 - KM275052
Luís E. Magalhães, Bahia	BA4Mi	Millet	6	3	12°05′58″	45°47′54″	05.10.12	KM274951 – KM274959
Luís E. Magalhães, Bahia	BA5Sr	Sorghum	16	-	12°05′58″	45°47′54″	05.10.12	KM274960 – KM274975
Capitólio, Minas Gerais	MG1Ma	Maize	-	14	20°36′17″	46°04′19″	06.08.12	KM274997 – KM275010
Luís E. Magalhães, Bahia	BA6Ma	Maize	-	13	12°05′58″	45°47′54″	06.12.12	KM274976 – KM274978, KM275053 - KM275062
Itapira, São Paulo	SP1Ma	Maize	-	7	22°26′11″	46°49′20″	06.12.12	KM275011 – KM275017
Assis, São Paulo	SP2Ma	Maize	-	7	22°39′40″	50°23′58″	06.15.12	KM275018 – KM275024

Table 2.1 - Sampling sites for *H. armigera* and *H. zea* in Brazil, including abbreviations, crops sampled, sample sizes for the mitochondrial gene (*COI*), geographic coordinates, dates sampled, and GenBank Accession.

(Continuation)

Sites (City, State)	Abbreviation (Site, Crop)	Crop	Sample size		Lat. (S)	Lon. (W)	Date	GenBank Accession
			H. armigera	*H. zea*				
Winter cropping								
São Gabriel do Oeste, Mato Grosso do Sul	MS1Ma	Maize	-	13	19°23′37″	54°33′49″	06.27.12	KM275025 – KM275038
Rondonópolis, Mato Grosso	MT1Ma	Maize	-	7	16°28′17″	54°38′14″	08.01.12	KM275063 – KM275069
Summer cropping								
Riachão das Neves, Bahia	BA7Sy	Soybean	8	-	12°08′54″	44°59′33″	10.21.12	KM275070 – KM275077
Luís E. Magalhães, Bahia	BA8Sy	Soybean	5	-	12°05′58″	45°47′54″	10.31.12	KM275078 – KM275082
Rondonópolis, Mato Grosso	MT2Sy	Soybean	13	-	16°28′17″	54°38′14″	11.08.12	KM275083 – KM275092, KM275156 - KM275158
Chapadão do Sul, Mato Grosso do Sul	MS2Sy	Soybean	6	-	18°46′44″	52°36′59″	11.29.12	KM275097 – KM275102
Balsas, Maranhão	MA2Sy	Soybean	10	-	07°31′59″	46°02′06″	01.06.13	KM275103 – KM275112
São Desidério, Bahia	BA9Sy	Soybean	10	-	12°21′08″	44°59′03″	01.15.13	KM275127 – KM275136
Limoeiro do Norte, Ceará	CE1Co	Cotton	-	4	05°08'56''	38°05'52''	10.08.12	KM275093 – KM275096

Table 2.1 - Sampling sites for *H. armigera* and *H. zea* in Brazil, including abbreviations, crops sampled, sample sizes for the mitochondrial gene (*COI*), geographic coordinates, dates sampled, and GenBank Accession.

(Conclusion)

Sites (City, State)	Abbreviation (Site, Crop)	Crop	Sample size		Lat. (S)	Lon. (W)	Date	GenBank Accession
			H. armigera	*H. zea*				
Summer cropping								
São Desidério, Bahia	BA10Co	Cotton	14	-	12°21′08″	44°59′03″	01.15.13	KM275147 – KM275155, KM275202 - KM275206
Cândido Mota, São Paulo	SP3Ma	Maize	-	7	22°44′46″	50°23′15″	01.14.13	KM275113 – KM275119
Jardinópolis, São Paulo	SP4Ma	Maize	-	7	21°03′47″	47°45′05″	03.04.13	KM275120 – KM275126
Barreiras, Bahia	BA11Ma	Maize	4	-	11°33′33″	46°19′47″	02.21.13	KM275137 – KM275140
Luís E. Magalhães, Bahia	BA12Ma	Maize	-	9	12°05′58″	45°47′54″	03.28.13	KM275141 – KM275146, KM275207 - KM275209
Rolândia, Paraná	PR1Ma	Maize	-	12	23°19'13”	51°29'01”	01.24.13	KM275159 – KM275170
Passo Fundo, Rio Grande do Sul	RS1Ma	Maize	-	10	28°16'08”	52°37'15”	01.30.13	KM275171 – KM275180
Montividiu, Goiás	GO1Ma	Maize	-	10	17°19'19”	51°14'51”	02.05.13	KM275181 – KM275190
Capitólio, Minas Gerais	MG2Ma	Maize	-	11	20°36′17″	46°04′19″	03.10.13	KM275191 – KM275201
Total			**139**	**135**				

2.2.2 DNA extraction, PCR amplification, and gene sequencing

Genomic DNA was isolated from the thorax of each adult using an Invisorb Spin Tissue Kit (STRATEC Molecular, Berlin, Germany), according to the manufacturer's protocol. A fragment of the COI mitochondrial gene was amplified by polymerase chain reaction (PCR) with the primers LCO(F) (5´ - GGT CAA CAA ATC ATA AAG ATA TTG G - 3´) and HCO(R) (5´ - TAA ACT TCA GGG TGA CCA AAA AAT CA - 3´) (FOLMER et al., 1994). Amplification reactions were performed using 10 ng genomic DNA, 2.5 mM $MgCl_2$, 1.5 µL of BSA (2.5 mg/mL), 2.5 mM dNTPs, 5 pmol of each primer, 1 U Taq DNA Polymerase (Life Technologies, Carlsbad, CA, USA), and 10% 10× Taq Buffer in a final volume of 25 µL. The PCR program consisted of an initial denaturation step at 94°C for 3 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 45°C for 30 s, and polymerization at 72°C for 1.5 min, with a final extension step at 72°C for 10 min. Following amplification, the aliquots were visually inspected using agarose gel (1.5% w/v) electrophoresis. The amplicons were purified by ethanol precipitation, and a second round of amplification was performed using the Big Dye Terminator v3.1 Cycle Sequencing system (Applied Biosystems, Foster City, CA, USA), which was followed by further purification. DNA sequencing was performed using the ABI3500xl automated genetic analyzer (Applied Biosystems, Foster City, CA, USA) at the State University of Campinas (Universidade Estadual de Campinas, Campinas, São Paulo, Brazil).

2.2.3 Dataset assembly, haplotypes, and demographic analysis

All sequences were manually edited using the Chromas Lite version 2.01 (TECHNELYSIUM PTY LTD.) software program and were aligned using the ClustalW tool from the BioEdit version 7.0 (HALL, 1999) software program. After editing and aligning the COI sequences, we determined the 658 bp consensus sequence, which was then posteriorly compared with the *H. zea* and *H. armigera* species barcodes (ENCYCLOPEDIA OF LIFE, 2013) to determine homology using the BlastN tool, which is available online at NCBI (MADDEN, 2002).

The MEGA version 4 (TAMURA et al., 2007) software program was used to inspect the COI sequences from each species individually for the presence of numts (LOPEZ et al., 1994). In particular, we searched for the following numt signatures: (i)

insertions/deletions (*indels*); (ii) stop codons leading to premature protein termination; and (iii) increased rates of non-synonymous mutations. The presence of signatures (i) and (ii) was considered sufficient to regard a sequence as a COI numt. In the presence of signatures (i) or (ii), signature (iii) was used to confirm the sequence as a numt. The presence of signature (iii) alone was not considered sufficient to define a sequence as a numt.

Haplotype and nucleotide diversity parameters for each species were estimated using the DnaSP version 5 (LIBRADO; ROZAS, 2009) software program. Neutrality tests using Tajima's D (TAJIMA, 1989) and Fu's Fs (FU, 1997) were performed using the Arlequin version 3.1 (EXCOFFIER; LAVAL; SCHNEIDER, 2005) software program, and significance was determined using 1,000 random samples in coalescent simulations. Based on the recommendations in the Arlequin manual, we activated the "Infer from distance matrix" option under "Haplotype definition", and the Fu's Fs statistical values were considered to be significant at a level of 5% only when the *P*-value was below 0.02. The diversity estimates and neutrality tests were performed using all sampled individuals from each species, which were divided into winter-crop and summer-crop groups. A Mismatch Distribution Analysis using a spatial expansion model (ROGERS; HARPENDING, 1992) was also performed using the Arlequin version 3.1 software program, and significance was determined using 1,000 bootstrap replicates. We used the goodness-of-fit of the observed mismatch distribution to the expected distribution from the spatial expansion model and the sum of square deviations (SSD) and Raggedness as statistical tests (*p*-value support).

2.2.4 Population structure analysis

Using Arlequin 3.1, we also performed an AMOVA at the two- and three-hierarchy levels (EXCOFFIER; SMOUSE; QUATTRO, 1992). For the three-hierarchy AMOVA, we first separated the samples depending on whether they were collected on winter or summer crops and then further divided them by host plant (monocotyledonae or dicotyledonae).

2.2.5 Network analysis and Bayesian phylogenies

Genetic differences and connections between the *Helicoverpa* spp. haplotypes were determined by constructing a maximum parsimony network (TEMPLETON; CRANDALL; SING, 1992) using the TCS 1.21 software program (CLEMENT; POSADA; CRANDALL, 2000). To resolve ambiguities present in the haplotype network, we used the criteria of coalescence theory and population geography proposed by CRANDALL e TEMPLETON (1993).

We used the distance matrix option in the PAUP *4.0 software program to calculate the inter- and intra-species genetic distances, which were inferred using the nucleotide substitution model and the Akaike Information Criteria (AKAIKE, 1974) selected by MODELTEST 2 (NYLANDER, 2004). The MrBayes v3.2 software program (RONQUIST et al., 2012) was used to estimate Bayesian phylogenies. In particular, the Bayesian analysis was performed with 10 million generations using one cold and three heated chains. *Helicoverpa assulta* (Guenée) (GenBank Accession number: EU768937), *Helicoverpa gelotopoeon* Dyar (EU768938), and *Chloridea virescens* (Fabricius) (IN799050) sequences were included as outgroups for the Bayesian analysis. We obtained a 50%-majority-rule consensus tree with posterior probabilities that were equal to the bipartition frequencies.

2.2.6 Network analysis: Brazil vs. Old World

Seventy-two sequences from a variety of Old World sites that were present in GenBank were included with the 139 *H. armigera* sequences we collected in Brazil. In particular, 72 sequences were obtained from specimens collected in China (N = 35) [GenBank Accession numbers GQ892840 - GQ892855, GQ995232 - GQ995244 (LI et al., 2011), HQ132369 (Yang, 2010), JX392415, and JX392497 (not published)], Australia (1) [(EU768936) (CHO, SOOWON et al., 2008)], Pakistan (2) [(JN988529 and JN988530) (not published)], Europe (28) [(FN907979, FN907980, FN907988, FN907989, FN907996 - FN907999, FN908000 - FN908003, FN908005, FN908006, FN908011, FN908013 - FN908018, FN908023, FN908026, GU654969, GU686757, GU686955, and JF415782) (not published)] and India (6) [(HM854928-HM854932 and JX32104) (not published)] (Appendix C). This new data set was edited and aligned as follows. The sequences were different lengths; thus, the editing and

alignment processes generated a total of 212 sequences with 590 bp in length. The sequences from individuals collected in Brazil, which were previously analyzed using a fragment length of 658 bp, as entered into GenBank (see Table 2.1), were edited by removing the first 36 bp and the last 32 bp. Using the TCS 1.21 software program (CLEMENT; POSADA; CRANDALL, 2000), we subjected this data set to haplotype network analysis using a maximum parsimony network (TEMPLETON; CRANDALL; SING, 1992) to investigate the genetic connections between haplotypes from Brazil and the Old World as well as to infer the origins of maternal lineages within *H. armigera* populations in Brazil.

2.3 Results

2.3.1 Identification of *Helicoverpa* spp., hosts, and geographic locations

One hundred thirty-nine individuals from the 274 *Helicoverpa* spp. specimens initially sampled were identified as *H. armigera* (98-100% homology) and 135 individuals were identified as *H. zea* (98-100% homology) (GenBank Accession numbers KM274936- KM275209 are listed in Table 2.1). *H. armigera* was primarily found on soybean, bean, and cotton crops, and these insects were distributed throughout the Midwest and Northeast of Brazil during both crop periods (winter and summer) (Figure 2.1). *H. armigera* was also found on sorghum, millet, and maize crops. However, for maize, *H. armigera* individuals were only found at one site during the summer growing season in Northeastern Brazil (state of Bahia). *H. armigera* was not found on maize crops in the Midwest, Southeast, or South of Brazil. *H. zea* was primarily found on maize crops and was present in all sampled regions during both the winter and summer growing seasons. Of the winter crops, millet and cotton were exceptional in that they could simultaneously support *H. zea* and *H. armigera* (Figure 2.1). We found no correlations between specific *H. armigera* mitochondrial lineages (haplotypes) and specific hosts (Figure 2.1).

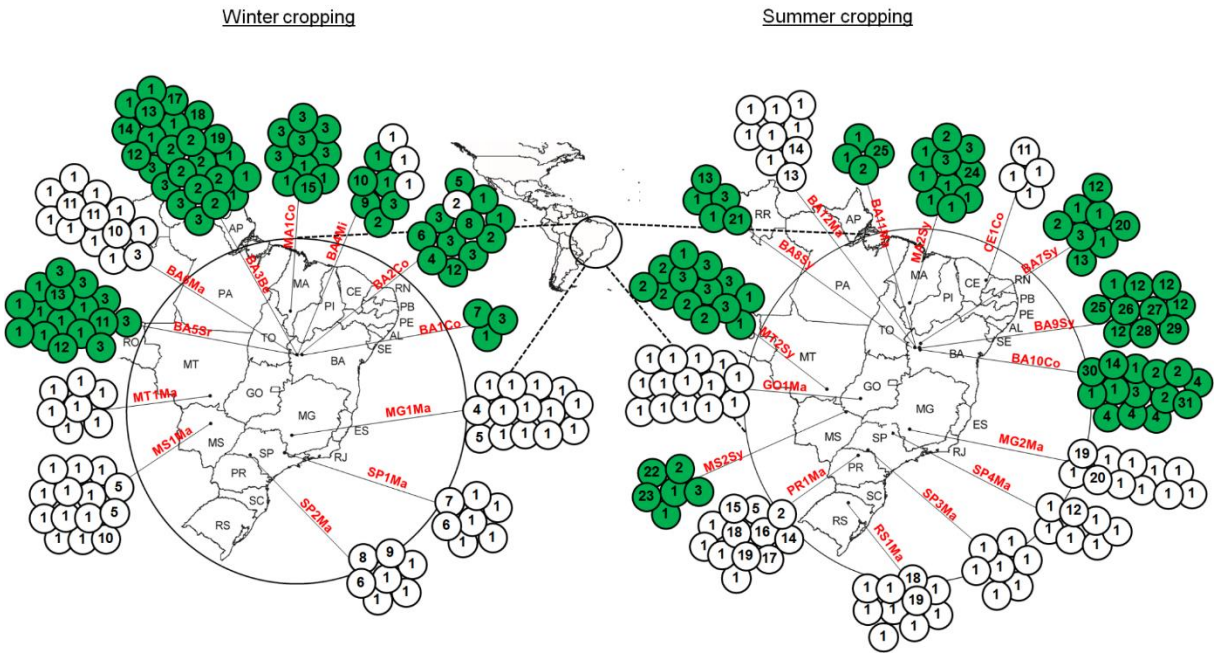

Figure 2.1 - Geographic distributions of COI haplotypes of *H. armigera* and *H. zea*. One hundred and thirty nine and 135 COI haplotypes were analyzed for these species, respectively. The samples were separated into two temporal groups (winter crops and summer crops). Each circle represents the haplotypes identified in a given population; a number within a circle denotes the COI haplotypes identified in that population. Colored circles refer to *H. armigera* specimens, and white circles refer to *H. zea* specimens. The abbreviations refer to the sampled locations and crops (Table 2.1).

2.3.2 Dataset assembly, haplotypes, and demographic analysis

Following alignment and editing, we were unable to identify indels or stop codons in the sequences from either species. However, using the most common haplotype for each species as a reference, eight non-synonymous substitutions were observed in 17 *H. armigera* individuals, and four non-synonymous substitutions were observed in eight *H. zea* individuals. However, considering the relatively high mutation rate reported for the *COI* gene in the *Helicoverpa* genus (LI et al., 2011), as well as the absence of indels and stop codons, it is unlikely that these sequences represent numts (nuclear mitochondrial DNA).

Twenty-six polymorphic sites were found among the 139 *H. armigera* individuals sampled, which yielded 31 haplotypes with a haplotype diversity (Hd) of 0.821 and a nucleotide diversity (Pi) of 0.0028. Sequence analysis of the 135 sampled *H. zea* individuals identified 19 polymorphic sites, which yielded 20 haplotypes with an Hd of 0.420 and a Pi of 0.0011 (Table 2.2). No significant

differences in Hd or Pi were found for either species when the individuals were separated by growing season according to the sampled crops (Table 2.2). The results from Tajima's D test were only not significant for *H. armigera* individuals (p = 0.07) sampled on summer crops; however, Fu's Fs test was significant (p < 0.01). The Tajima's D and Fu's Fs test results for both *H. armigera* and *H. zea* were negative and significant when the individuals were tested as a single group and when the individuals were split into groups based on the crop on which they were sampled (summer or winter; temporally). These results indicate an excess of low frequency polymorphisms and are consistent with either population expansion or purifying selection (Table 2.2). In addition, the model of sudden expansion (ROGERS; HARPENDING, 1992) did not reject the hypothesis of expansion demographics for *H. armigera* (SSD = 0.0012, p = 0.48; Raggedness = 0.0433, p = 0.61) or *H. zea* (SSD = 0.0002, p = 0.90; Raggedness = 0.1492, p = 0.72).

2.3.3 Statistical analysis of population structure

The results of the analysis of molecular variance (AMOVA) with two hierarchical levels showed that the greatest amount of total variation was accounted for by differences among individuals within populations: 92.89% for *H. armigera* (Φ_{ST} = 0.071) and 94.22% for *H. zea* (Φ_{ST} = 0.058) (Appendix A). For the AMOVA with three hierarchical levels for *H. armigera*, the largest percentage of variation occurred within populations, separating individuals into groups by time (winter and summer crops; 93.17%, Φ_{CT} = 0.006; Φ_{SC} = 0.074; Φ_{ST} = 0.068), host group (mono- and dicotyledonous; 99.24%, Φ_{CT} = -0.01; Φ_{SC} = 0.018; Φ_{ST} = 0.007), and each host type (crop; 93.19%, Φ_{CT} = -0.042; Φ_{SC} = 0.105; Φ_{ST} = 0.068) (Appendix A). The group separation for *H. armigera* was not significant for any of the three tested groups (p > 0.10). The AMOVA with three hierarchical levels divided the *H. zea* individuals into groups by time (winter and summer crops), which showed a larger variation within populations (93.76%, Φ_{CT} = 0.010; Φ_{SC} = 0.052; Φ_{ST} = 0.062); the group division was not significant (p > 0.10) (Appendix A).

Table 2.2 - Number of individuals, haplotype designation, and genetic diversity for the sampled populations grouped according to geographical origin.

Group	N. Individuals (samples)	N. haplotypes	Distribution of Haplotypes (n)	Haplotype Diversity (Hd)	Nucleotide diversity (Pi)	Tajima's D test (*p* value)	Fu's Fs test (*p* value)
			H. armigera				
Pooled	**139 (14)**	**31**	**-**	**0.821**	**0.0028**	**-1.729 (<0.01)**	**-26.361 (<0.01)**
Winter cropping	69 (6)	19	H1(22); H2(9); H3(21); H4(1); H5(1); H6(1); H7(1); H8(1); H9(1); H10(1); H11(1); H12(2); H13(2); H14(1); H15(1); H16(1); H17(1); H18(1); H19(1).	0.805	0.0028	-1.608 (=0.03)	-11.891 (<0.01)
Summer cropping	70 (8)	19	H1(22); H2(13); H3(11); H4(4); H12(4); H13(2); H14(1); H20(1); H21(1); H22(1); H23(1); H24(1); H25(2); H26(1); H27(1); H28(1); H29(1); H30(1); H31(1).	0.835	0.0028	-1.353 (=0.07)	-11.254 (<0.01)
			H. zea				
Pooled	**135 (16)**	**20**	**-**	**0.420**	**0.0011**	**-2.190 (<0.01)**	**-22.912 (<0.01)**
Winter cropping	65 (8)	11	H1(50); H2(1); H3(1); H4(1); H5(3); H6(2); H7(1); H8(1); H9(1); H10(2); H11(2).	0.408	0.0009	-2.156 (<0.01)	-9.735 (<0.01)
Summer cropping	70 (8)	13	H1(53); H2(1); H5(1); H11(1); H12(1); H13(1); H14(2); H15(1); H16(1); H17(1); H18(2); H19(3); H20(2).	0.427	0.0012	-1.967 (<0.01)	-10.411 (<0.01)

2.3.4 Network analysis and Bayesian phylogeny

Analysis of the genetic connections between the *Helicoverpa* spp. represented in the haplotype network revealed a close genetic relation between *H. armigera* and *H. zea*, which were separated by only 13 mutational steps (Figure 2.2). By separately analyzing the connections between the genetic haplotypes of each species, we inferred the existence of two predominant maternal lineages for *H. armigera*: H1 (31.65%) and H3 (23.02%), which were located at the center of the haplotype network. The other haplotypes of *H. armigera,* with the exception of haplotype H2 (15.83%), all had frequencies below 5%. Haplotypes H19, H18, H16, H12, H21, and H25 formed an outer cluster within the haplotype network of *H. armigera* (Figure 2.2). The haplotype network for *H. zea* revealed a genetic haplotype relationship with a single central high-frequency lineage (H1 = 76.30%) surrounded by low-frequency haplotypes (< 5%) (Figure 2.2).

The optimal nucleotide substitution model identified by the MODELTEST 2.3 software program was the GTR+I+G model (Generalized time reversible + Proportion of invariable sites + Gamma distribution model). The estimated model parameters were based on empirical base frequencies (A = 0.3092, C = 0.1463, G = 0.1312, and T = 0.4133), with the proportion of invariable sites (I) set to 0.7393 and the gamma distribution shape parameter set to 0.5778. The consensus tree generated by the Bayesian analysis divided the *Helicoverpa* spp. specimens sampled in Brazil into two monophyletic clades (*H. armigera* and *H. zea*) with an associated probability of 99% (Figure 2.3; Appendix B). The probabilities separating the *H. zea* individuals into groups within this species were not significant. A single *H. armigera* individual (MS2Sy6) was separated from the other individuals with an associated probability of 98%. Finally, *H. gelotopoeon* showed a closer phylogenetic relationship to *H. armigera* and *H. zea* compared with *H. assulta* (Figure 2.3; Appendix B).

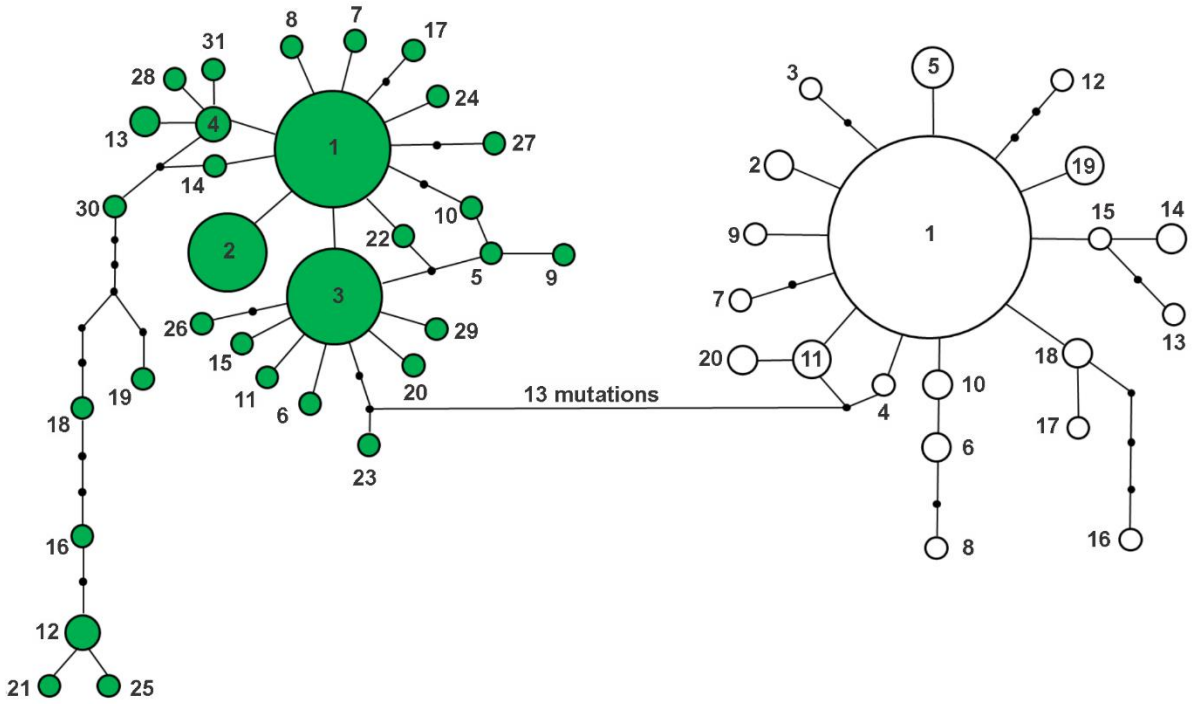

Figure 2.2 - Haplotype network based COI sequences from *H. armigera* and *H. zea* samples collected in Brazil. Partial mtDNA COI (658 bp) sequences from *H. armigera* (colored circles) and *H. zea* (white circles) were analyzed from samples collected in Brazil. Each haplotype is represented by a circle and is identified by a number from 1-31. The *H. armigera* and *H. zea* COI haplotypes are shown as described in Table 2.2. The numbers of nucleotide substitutions between the haplotypes are indicated by black circles. The total number of nucleotide substitutions separating the *H. armigera* specimens from the *H. zea* specimens is shown.

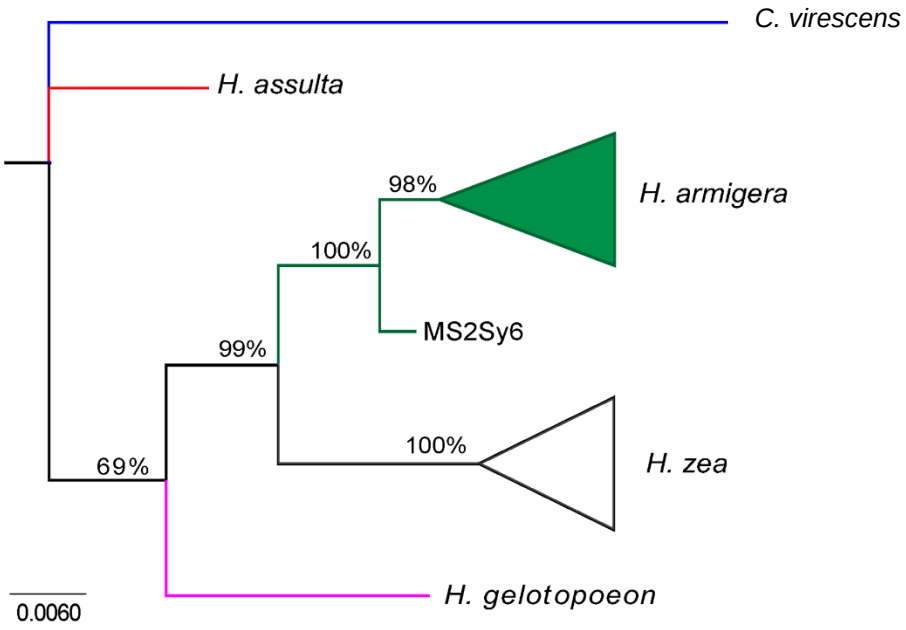

Figure 2.3 - Bayesian phylogenetic tree of *H. armigera* and *H. zea* individuals sampled in Brazil. This phylogenetic tree is based on partial COI haplotype sequences and includes *H. assulta* and *H. gelotopoeon* sequences. Numbers near the interior branches indicate posterior probability values. The outgroup used was *C. virescens*. *H. armigera* COI haplotypes and Genbank Accession numbers can be found in Appendix C.

2.3.5 Network analysis: Brazilian vs. Old World *Helicoverpa armigera*

The haplotype network constructed using the edited sequences collected in Brazil, along with numerous Old World sequences, identified 38 distinct haplotypes (Figure 2.4). H1 (28%) and H2 (24%), which are widely distributed throughout Brazil, Europe, and China, were the most frequent haplotypes and occupied the central region of the haplotype network. All other haplotypes, with the exception of H3 and H10, showed frequencies below 5%. Finally, the majority of haplotypes with low frequencies represented by singletons were located at the network extremities (Figure 2.4).

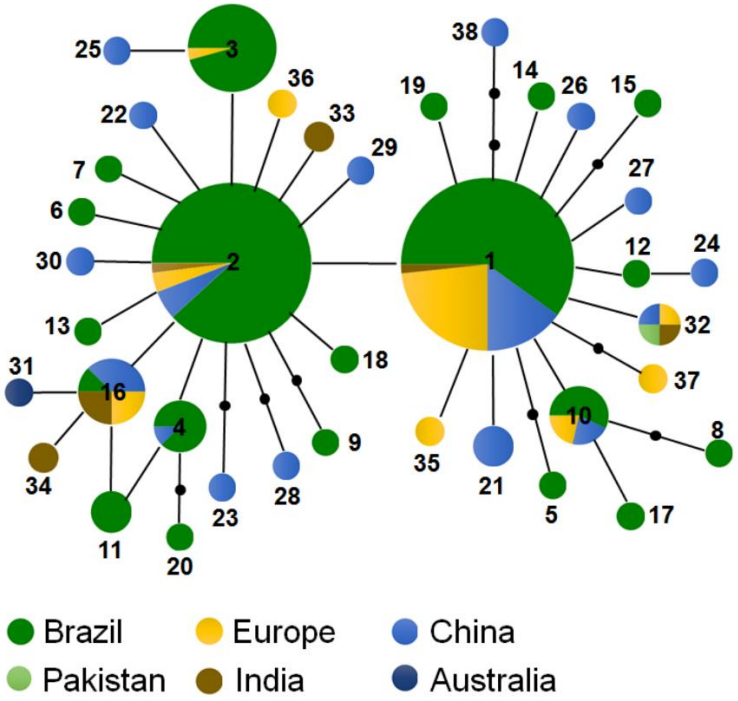

Figure 2.4 - Haplotype network based COI sequences from *H. armigera* samples from Brazil and Old World specimens. Partial mtDNA COI (590 bp) sequences from this species were analyzed. Thirty-eight haplotypes were identified from 211 individuals sampled from China (n = 35), Australia (n = 1), Pakistan (n = 2), Europe (n = 28), India (n = 6), and Brazil (n = 139). *H. armigera* COI haplotypes are shown as described in Appendix C. Each circle represents a haplotype and its number. The colors represent the frequency of each haplotype in the country/continent, with dark green (Brazil), light green (Pakistan), yellow (Europe), brown (India), light blue (China), and dark blue (Australia).

2.4 Discussion

Our results indicate a widespread distribution for *H. armigera* throughout the Midwest and Northeast of Brazil on a variety of crops, particularly dicotyledonous, beans, soybeans, and cotton as well as, to a lesser extent, millet, sorghum, and maize. This pest was not found on maize crops in the Midwest, Southeast, or South of Brazil, despite the fact that these crops were initially identified as sources of *H. armigera* in this system. *H. armigera* individuals associated with maize crops were only found at a single sampling site in the Northeast (state of Bahia) during February 2013. In contrast, *H. zea* individuals were essentially found only on maize crops, with the exception of a few individuals collected from millet and cotton crops, where *H. zea* individuals were found alongside *H. armigera* individuals. Before the documentation of *H. armigera* in Brazil in 2013, we had hypothesized that major

source of *Helicoverpa* spp. attacking different host plant was maize crops. However, our findings showed that targeting the control of *H. armigera* on maize crops may not be effective because *H. zea* was the predominant species in this host plant. The possibility of the formation of hybrid individuals between these two species, which has been reported under laboratory conditions (LASTER; HARDEE, 1995; LASTER; SHENG, 1995), needs to be investigated under field conditions to improve our pest management programs.

Demographic analyses using neutrality tests and a Mismatch Distribution Analysis indicated an expansion of the *H. armigera* and *H. zea* populations within the Brazilian territory. Population expansions were also consistent with the Haplotype network structure, which was characteristic of species undergoing processes of demographic expansion (EXCOFFIER; HOFER; FOLL, 2009). Brazilian *H. armigera* individuals showed two primary maternal lineages, whereas *H. zea* showed a single primary lineage, all of which were surrounded by numerous lower-frequency haplotypes. Therefore, these central high-frequency haplotypes represent the ancestral haplotypes, with the low-frequency haplotypes more recently derived (CRANDALL; TEMPLETON, 1993). Furthermore, signs of the *H. armigera* population expansion are likely because of the introduction of this pest into Brazil. Following the founder event, during which a portion of the overall genetic diversity of the species was introduced to Brazil, the *H. armigera* population further propagated. According to Nibouche et al. (1998), *H. armigera* can migrate as far as 2,000 km, which likely facilitated the colonization of a variety of crops. The migration and colonization of crop areas by a small group of individuals can cause bottleneck effects, which, combined with pest population-suppression strategies (e.g., insecticide use that kills all but a small portion of the population), can lead to the types of demographic expansions observed for *H. zea* and *H. armigera* in Brazil (ENDERSBY et al., 2007; ALBERNAZ et al., 2012; DOMINGUES et al., 2012). In addition, the expansion of maize, soybean, and cotton crops into the North and Northeast of Brazil over the previous decade may also be responsible, in part, for the demographic expansion of these species, specifically *H. zea.* Additionally, assuming that not all COI variation is neutral, *Helicoverpa* spp. populations could be suffering selection, especially considering that populations have colonized new environments. However, further studies using a larger number of molecular markers from nuclear and mitochondrial genome regions would answer these questions. The *H. armigera* and *H. zea*

population genetics were not structured according to space, time (winter and summer crops), or host (crops). Unstructured genetic networks have been reported for other populations of these two pest species in other parts of the world, which were based on several molecular markers, including mtDNA, allozymes, and microsatellites (NIBOUCHE et al., 1998; ZHOU et al., 2000; HAN; CAPRIO, 2002; BEHERE et al., 2007; ENDERSBY et al., 2007). Both species showed wide spatial haplotype distributions, and no genetic relationships were identified using a haplotype network analysis or an AMOVA. This scenario may be because these populations have a polyphagous feeding habit and migratory characteristics.

The unstructured population of *H. armigera* and the wide distribution of the two ancestral maternal lineages within the Brazilian territory did not allow us to infer any hypothetical invasion or dispersal routes for this species within the region. However, we noted that the haplotype and nucleotide diversities found for *H. armigera* in Brazil are similar to or greater than those reported for natural *H. armigera* populations in the Old World (BEHERE et al., 2007; LI et al., 2011). For example, one outer branch of the *H. armigera* haplotype network, formed by haplotypes H19, H18, H16, H12, H21, and H25, is noteworthy for having the greatest genetic distance from the central haplotypes (H1 and H3), and these haplotypes have yet to be identified in Old World populations (BEHERE et al., 2007; LI et al., 2011). In addition, joint analysis of the haplotypes from Brazil and the Old World yielded an overall structure that was similar to the haplotype network obtained only from the Brazilian individuals. In particular, the two most frequent haplotypes were identified throughout Brazil, Europe, China, and India, whereas the majority of the singletons were from Brazil and China. The cited literature, along with our results that showed a wide geographic distribution for *H. armigera* during the first half of 2012, support the hypothesis of an invasion period prior to the first reports of this species in Brazil. Alternatively, these findings are also consistent with a invasion that involved a large gene pool, multiple invasion events, or some combination of these events.

The low genetic divergence observed between *H. armigera* and *H. zea* in the haplotype network analysis and the Bayesian phylogeny confirms the close genetic relatedness of these two species. Therefore, the reported co-occurrence of these species in time and space, as well as on the same hosts (as described here), could allow for the formation of hybrid individuals, which has been reported under laboratory conditions (LASTER; HARDEE, 1995; LASTER; SHENG, 1995). Although

the existence of hybrids in the wild remains unconfirmed, this scenario is of significant concern. In particular, recombination or introgression phenomena between *H. armigera*, which is reportedly resistant to control methods, and *H. zea*, which has adapted to the environmental conditions of the American continent, may enable gene transfer and fixation in some individuals. Therefore, hybridization may enable the selection of breeds with enhanced hybrid vigor and the ability to rapidly adapt to current management and suppression methods.

The population studies described in this study indicate a demographic expansion and a high mitochondrial genetic diversity for *H. armigera* and *H. zea* in Brazil. Therefore, the sustainable management of *H. armigera* will likely become a significant challenge for Brazilian entomology in the coming years, especially considering the polyphagous feeding habit, the great dispersal ability, and the numerous reports of resistance to insecticides and *Bt* crops for this insect (FITT, 1989; NIBOUCHE et al., 1998; MARTIN et al., 2000; GUNNING et al., 2005; ZHANG et al., 2009; ACHALEKE; BRÉVAULT, 2010; NAIR et al., 2013). This scenario requires immediate attention, as there is an imminent risk of *H. armigera* expanding throughout the American territory and perhaps reaching agricultural areas in Central and North America. However, it was not possible to trace the invasion and dispersal routes of *H. armigera* in the Brazilian territory. Nevertheless, the hypotheses of an invasion period prior to the first reports in the literature and/or an invasion that involved a diverse gene pool are both consistent with the observed high incidence and rapid adaptation of *H. armigera* in the Brazilian territory. Our confirmation that the predominant maternal lineages in the Brazilian territory are the same compared with those in Europe and Asia may represent a starting point to guide *H. armigera* management programs. Indeed, control strategies have a greater chance of success when reliable information is gathered in the regions where the pests, their hosts, and their natural enemies have co-evolved over a significant period of time.

2.5 Conclusions

- *H. armigera* individuals are most prevalent on dicotyledonous hosts and *H. zea* individuals are most prevalent on maize crops.

- *H. armigera* and *H. zea* are on demographic expansion, and do not show genetic structure.

- The high genetic diversity and wide distribution of *H. armigera* in mid-2012 are consistent with an invasion period prior to the first reports of this species in the literature and/or multiple invasion events within the Brazilian territory.

- There are Chinese, Indian, and European lineages within the Brazilian populations of *H. armigera*.

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APPENDIXES

Appendix A - Hierarchical analysis of molecular variance (AMOVA), for population genetics structure of *H. armigera* and *H. zea* with a mithocondrial (COI) region marker.

(To be continue)

Helicoverpa armigera

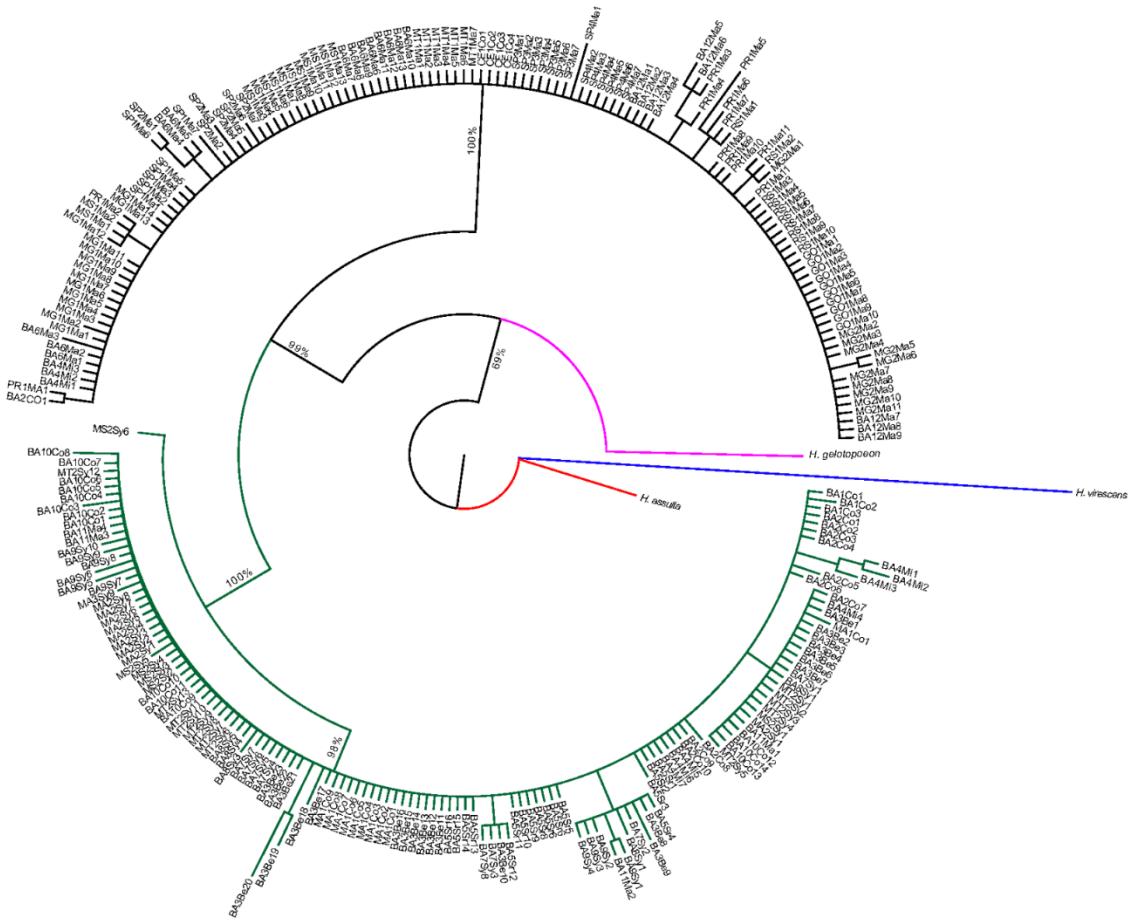
Hierarchical levels	d.f.	Sum of Squares	Variance components	Variance (%)	Fixation Indices	P value
Two-hierarchical-levels						
Among populations	15	21.897	0.0669Va	7.11	Φ_{ST}=0.071	<0.01
Within populations	127	111.019	0.8741Vb	92.89		
Total	142	132.916	0.9411			
Three-hierarchical-levels (winter x summer cropping)						
Among groups	1	1.276	-0.0062Va	-0.66	Φ_{CT}=0.006	=0.64
Among populations within groups	14	20.621	0.0703Vb	7.49	Φ_{SC}=0.074	<0.01
Within populations	127	111.019	0.8741Vc	93.17	Φ_{ST}=0.068	<0.01
Total	142	132.916	0.9382			
Three-hierarchical-levels (di x mono)						
Among groups	1	0.807	-0.01056Va	-1.11	Φ_{CT}=-0.011	=0.79
Among populations within groups	4	5.304	0.01783Vb	1.87	Φ_{SC}=0.018	=0.05
Within populations	133	125.522	0.94378Vc	99.24	Φ_{ST}=0.007	=0.12
Total	138	131.633	0.95104			
Three-hierarchical-levels (six crops)						
Among groups	5	6.111	-0.04007Va	-4.21	Φ_{CT}=-0.042	=0.74
Among populations within groups	9	15.503	0.10492Vb	11.02	Φ_{SC}=0.105	<0.01
Within populations	124	110.019	0.88725Vc	93.19	Φ_{ST}=0.068	<0.01
Total	138	131.633	0.95210			

Appendix A - Hierarchical analysis of molecular variance (AMOVA), for population genetics structure of *H. armigera* and *H. zea* with a mithocondrial (COI) region marker.

(Conclusion)

Helicoverpa zea

Hierarchical levels	d.f.	Sum of Squares	Variance components	Variance (%)	Fixation Indices	P value
Two-hierarchical-levels						
Among populations	13	7.081	0.02111Va	5.78	Φ_{ST}=0.057	<0.025
Within populations	121	41.637	0.34411Vb	94.22		
Total	134	48.719	0.36522			
Three-hierarchical-levels (winter x summer cropping)						
Among groups	1	0.826	0.00391Va	1.07	Φ_{CT}=0.010	=0.127
Among populations within groups	12	6.255	0.01897Vb	5.17	Φ_{SC}=0.052	<0.025
Within populations	121	41.637	0.34411Vc	93.76	Φ_{ST}=0.062	<0.023
Total	134	48.719	0.36700			



Appendix B - Bayesian phylogenetic tree of *H. armigera* and *H. zea* individuals sampled in Brazil. This phylogenetic tree is based on partial COI haplotype sequences and includes *H. assulta* and *H. gelotopoeon* sequences. Numbers near the interior branches indicate the posterior probability values. The outgroup used was *C. virescens*. *H. armigera* COI haplotypes and Genbank Accession numbers can be found in Appendix C.

Appendix C - Global *H. armigera* including the Brazilian haplotypes, and relevant GenBank Accession numbers. Numbers of individuals sequenced from each locality are indicated in parentheses.

(To be continue)

Country or Continent	Locations	COI Haplotypes	GenBank Accession numbers
China	Kunming (8)	H1, H2, H3, H16, H21, H26, H30	GQ892840, GQ892842, GQ892854, GQ995232, GQ995234, GQ995235, GQ995244, GQ995239
	Tibet (2)	H2, H10	JX392497, JX392415
	Miaofengshan (4)	H1, H10, H32	JX509766, JX509765, JX509764, JX509739
	…(1)	H38	HQ132369
	Dali (15)	H1, H2, H4, H16, H21, H22, H23, H24, H25, H27, H28, H29	GQ892846, GQ892847, GQ892848, GQ892849, GQ892850, GQ892851, GQ892852, GQ892853, GQ995233, GQ995236, GQ995237, GQ995240, GQ995241, GQ995242, GQ995243
	Henan (2)	H1, H16	GQ995238, GQ892855
	Lijiang (1)	H21	GQ892845
	Yuxi (2)	H1, H21	GQ892843, GQ892844
Europe	...(24)	H1, H2, H3, H10, H16, H32, H35, H36, H37	FN907979, FN907980, FN907988, FN907989, FN907995, FN907996, FN907997, FN907998, FN907999, FN908000, FN908001, FN908002, FN908003, FN908005, FN908006, FN908011, FN908013, FN908014, FN908015, FN908016, FN908017, FN908018, FN908023, FN908026
	Germany (4)	H1, H3, H16	GU654969, GU686757, GU686955, JF415782
Australia	Toowoomba (1)	H1	EU768936
India	...(6)	H1, H16, H32, H33, H34	HM854928, HM854929, HM854930, HM854931, HM854932, JX532104
Pakistan	...(2)	H29, H32	JN988529, JN988530
Brazil	Barreiras, Bahia (7)	H1, H2, H3, H6, H17	KM274936 - KM274938, KM275137 - KM275140

Appendix C - Global *H. armigera* including the Brazilian haplotypes, and relevant GenBank Accession numbers. Numbers of individuals sequenced from each locality are indicated in parentheses.

(Conclusion)

Country or Continent	Locations	COI Haplotypes	GenBank Accession numbers
Brazil	Luís Eduardo Magalhães, Bahia (61)	H1, H2, H3, H4, H5, H7, H8, H9, H10, H11, H13	KM274939 - KM274941, KM274943 - KM274950, KM274951 - KM274953, KM274957 - KM274975, KM274979 - KM274986, KM275038 - KM275052, KM275078 - KM275082
	Riachão das Neves, Bahia (8)	H1, H2, H3, H10, H11, H14	KM275070 - KM275077
	São Desidério, Bahia (24)	H1, H2, H3, H4, H10, H17, H18, H19, H20	KM275127 - KM275136
	Balsas, Maranhão (20)	H1, H2, H3, H12, H16	KM274987 - KM274996, KM275103 - KM275112
	Rondonópolis, Mato Grosso (13)	H1, H2, H3	KM275083 - KM275092, KM275156 - KM275158
	Chapadão do Sul, Mato Grosso do Sul (6)	H1, H2, H3, H15	KM275097 - KM275102

3 CROSS-SPECIES AMPLIFICATION AND POLYMORPHISM OF MICROSATELLITE LOCI IN *Helicoverpa armigera* AND *Helicoverpa zea* (LEPIDOPTERA: NOCTUIDAE) IN BRAZILIAN CROPPING SYSTEMS[1]

Abstract

The Old World bollworm *Helicoverpa armigera* (Hübner) was recently discovered in Brazil. This species is closely related to the New World bollworm *H. zea* (Boddie), and mating between these species has already been reported under laboratory conditions. Here, we tested the cross-species amplification of 20 microsatellite (SSR) loci in field populations of *H. armigera* and *H. zea* collected from Brazilian cropping systems. Seven SSR loci were successfully amplified and polymorphic in both species except for the locus HaC14, which was monomorphic for *H. zea*. All SSR loci were in linkage equilibrium, and deviations from Hardy-Weinberg equilibrium were only observed for the locus HarSSR1 in the HaRS-2 population, where null alleles were present. A moderate level of polymorphism was detected in *H. armigera* and *H. zea* populations with a mean allele number of 4.14, and 2.24, respectively. Interestingly, most of the populations of the invader *H. armigera* showed higher genetic diversity and inbreeding coefficients than *H. zea* populations. The genetic identity of each species was recovered using a STRUCTURE analysis, where the populations formed two clusters (K = 2) according to their species. STRUCTURE also suggested the occurrence of potential hybrid offspring between *H. armigera* and *H. zea* individuals in natural conditions. These SSR loci will be valuable in characterizing population differentiation, invasion routes, adaptation, reproductive behavior, and intra- and interspecific gene flow in *H. armigera* and *H. zea* populations in Brazil, the USA, and other areas where these two pests occur.

Keywords: *Helicoverpa*; Microsatellite; Hybridization; Old World bollworm; Corn earworm; Invasive species

3.1 Introduction

A native of Oceania, the Old World bollworm, *Helicoverpa armigera* (Hübner), is one of the most severe agricultural pests in the world (POGUE, 2013). It has invaded several other continents, including Asia, Europe, and Africa, and was first reported in Brazil in 2013 (CZEPAK et al., 2013; SPECHT et al., 2013; TAY et al., 2013). This invasive pest rapidly spread throughout Brazil (LEITE et al., 2014; MASTRANGELO et al., 2014) and was also reported in other South American

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countries, such as Argentina (MURÚA et al., 2014), Paraguay and Uruguay (ARNEMANN et al., 2016). In 2015, it was found in Florida, USA (APHIS, 2015; HAYDEN; BRAMBILA, 2015), and now threatens crop production throughout the Americas. Historically, the New World bollworm, *Helicoverpa zea* (Boddie), is the most important species of the *Helicoverpa* genus in the Americas, including Brazil (DEGRANDE; OMOTO, 2013). Although the exact evolutionary relationship between *H. armigera* and *H. zea* is uncertain, they are considered “twin” or “sibling” species, and are able to mate and produce fertile offspring under laboratory conditions (MITTER; POOLE; MATTHEWS, 1993; LASTER; HARDEE, 1995; LASTER; SHENG, 1995; CHO et al., 2008).

Both *H. armigera* and *H. zea* are polyphagous and have high reproductive and dispersal capacities that favor their rapid adaptation to various control tactics (e.g., insecticides and genetically modified plants) (FITT, 1989; HEAD et al., 2010; EDWARDS et al., 2013; YANG; LI; WU, 2013; RAZMJOU; NASERI; HEMATI, 2014; WALSH et al., 2014). Due to the invasion of *H. armigera*, both species now coexist in Brazilian landscapes. Severe economic damage to different crops, such as cotton, millet, bean, sorghum, and soybean, has been reported in different regions of Brazil (LEITE et al., 2014), which confirms the significant adaptation of *H. armigera* in Brazilian landscapes.

Population genetic studies of these two pests have been performed worldwide. Studies with mitochondrial and nuclear markers revealed high genetic diversity and low genetic structure (spatial, temporal, and host) (SCOTT et al., 2005; BEHERE et al., 2007; ENDERSBY et al., 2007; LI et al., 2011; PERERA; BLANCO, 2011; BEHERE et al., 2013). Similar results have been observed in Brazil (LEITE et al., 2014; MASTRANGELO et al., 2014). However, in Brazil and other countries where both species occur, analyses with markers that evolve rapidly in the genome, such as microsatellites, are important in order to acquire information about intra- and interspecific gene flow between *H. armigera* and *H. zea* populations. This information is important to better understand the reproductive behavior, population structure, and potential hybridization events between *H. armigera* and *H. zea*.

Microsatellites or SSRs (simple sequence repeats) are polymorphic DNA loci that, in general, consist of one to six nucleotide sequences repeated in tandem. These are widespread and randomly dispersed in the genomes of all eukaryotic organisms (LITT; LUTY, 1989; TAUTZ, 1989). SSR markers have been used in

population genetic studies on *Helicoverpa*, owing to their higher informational content when compared to other types of molecular markers (SUBRAMANIAN; MOHANKUMAR, 2006; PERERA; BLANCO, 2011). Furthermore, SSR primers described for one species could be used to detect polymorphisms in other, closely related species, becoming useful tools in the detection of hybridization zones. Testing cross-species amplification of SSR is important to generate data more rapidly than by developing new SSR primers, and cheaper than using other new technologies (i.e., next generation sequencing).

Grasela and Mcintosh (2005) tested cross-species amplification of SSR markers developed for *H. armigera* on *H. zea* populations from the USA, generating only four loci that lead to amplification in both species. SSR markers developed for *H. zea* (PERERA et al., 2007) were never tested in *H. armigera*. Thus, our main objectives were (i) to test the cross amplification of 13 microsatellite loci previously characterized from *H. zea* in *H. armigera*, (ii) to retest for the cross-amplification of seven microsatellite loci from *H. armigera* (JI et al., 2003; SCOTT et al., 2004; JI; WU; ZHANG, 2005) in *H. zea*, in order to determine whether they also work in Brazilian populations, and (iii) to characterize the polymorphism of these microsatellites and develop a set of polymorphic markers available to researchers for investigations of genetic diversity and mating systems of these species in Brazil. Ultimately, our aim in this paper was a preliminary evaluation of the usefulness of these markers on the detection of potential hybrids in a few Brazilian populations of *H. armigera* and *H. zea.*

3.2 Material and methods

3.2.1 Sampling and DNA extraction

Sixty-four *H. armigera* larvae and 72 *H. zea* larvae were collected at six sampling sites (Table 3.1 and Figure 3.1). *Helicoverpa* spp. larvae were maintained on an artificial diet modified from that used by Greene; Lepla and Dickerson (1976), and under controlled laboratory conditions, at 25 ± 1°C, 60 ± 10% relative humidity, and 14:10 h (L:D) photoperiod. After adult emergence, genomic DNA was isolated from the thorax of each individual using an Invisorb Spin Tissue Kit (STRATEC Molecular, Berlin, Germany), according to the manufacturer protocol. Species

identification was confirmed using a method based on interspecific polymorphisms in the cytochrome c oxidase subunit I (COI) gene as described by Behere et al. (2008).

Table 3.1 - Identification code, location, collection site (crop), date of collection of *H. armigera* and *H. zea* populations, and number of individuals (N) used in the cross-amplification test with the 20 microsatellite loci previously published.

Population Code	City, state	Crop	Latitude	Longitude	Date	N
H. armigera						
HaGO-2	Mineiros, GO	Soybean	17°34'10" S	52°33'04" W	Jan. 2014	24
HaBA-44	Luís Eduardo Magalhães, BA	Cotton	12°05'58" S	45°47'54" W	Feb. 2014	23
HaRS-2	Itaara, RS	Soybean	29°36'35" S	53°45'53" W	Mar. 2014	17
H.zea						
HzBA-32	Luís Eduardo Magalhães,BA	Maize	12°05'58" S	45°47'54"W	Jun. 2013	24
HzMG-4	Capitólio,MG	Maize	20°36′17″ S	46°04′19"W	Feb. 2014	24
HzSP-13	Cândido Mota,SP	Maize	22°44′46″ S	50°23′15"W	Mar. 2014	24

Figure 3.1 - Distribution of *Helicoverpa armigera* (HaBA-44, HaGO-2, and HaRS-2) and *H. zea* (HzMG-4, HzSP-13, and HzBA-32) populations sampled in Brazil.

3.2.2 Microsatellite cross-species amplification

Thirteen pairs of microsatellite primers described for *H. zea* were tested in *H. armigera*, and seven pairs of microsatellite primers described for *H. armigera* were tested in *H. zea* (Table 3.2). The forward primers were modified with the addition of the M13 forward sequence (5′-CACGACGTTGTAAAACGAC-3′) at the 5′ end. PCR

amplification was performed in a 10-μL reaction mixture containing 0.5 μM each primer, 2.5 mM dNTP, 3.75 mM $MgCl_2$, 0.5 μL BSA (2.5 mg/mL), 0.25 pmol M13 forward primer (modified with IRDye 700 or IRDye 800 fluorescence), 10% 10x Taq Buffer, 1 U Taq DNA polymerase (Life Technologies, Carlsbad, CA, USA), and 50 ng genomic DNA. PCR amplifications proceeded according to the following protocol: 95°C for 5 min followed by 30 cycles at 95°C for 45 s; different annealing temperatures (°C) for each locus/species for 45 s; and 72°C for 45 s. For the M13 reactions, the 30 cycles were immediately followed by eight cycles at 94°C for 45 s, 53°C for 45 s, and 72°C for 45 s, with a final extension at 72°C for 10 min. Amplified fragments were visualized on 2% (w/v) agarose gels with a 1-kb DNA ladder. After PCR optimization, the loci that showed clear and robust band amplification on the agarose gels were selected for polymorphism analysis. The amplification products were separated under denaturing conditions on 5% (v/v) polyacrylamide gels containing 8 M urea and 1x TBE (0.045 M Tris-borate and 1 mM EDTA) in a semi-automated DNA sequencer (LI-COR 4300S DNA Analysis System; LI-COR Biosciences, Lincoln, Nebraska, USA) for approximately 2 h at 70 W. The loci were genotyped using Saga software (LI-COR Biosciences).

Table 3.2 - Characteristics of 20 microsatellite loci previously published for *Helicoverpa zea* and *H. armigera*. Locus name, forward (F) and reverse (R) primer sequences, repeat motif, expected size and size range of observed alleles, and reference.

(To be continue)

Locus	Primer sequence (5′-3′)	Repeat motif	Allele size and size range (bp)	Reference
HzMS1-4	F:CAAGTGATAAAAGACGCCG AAGAT R:TTGATCGTCAAGGAAGTG GCTAT	$(TGA)_6$	118 (111-144)	PERERA et al. (2007)[z]
HzMS1-6	F:GTTTTGTCATTTGTCAAGC CGAA R:AGCTCCCATACAACAAACG TGATT	$(TGA)_7$	237 (208-245)	PERERA et al. (2007)
HzMS3-1	F:CAGTAGTTCCTGAGATTAG CGCGT R:ATCACGTTCTCGAAAAACA TTGCT	$(CAAA)_4$	113 (106-110)	PERERA et al. (2007)
HzMS3-4	F:GGTCAAGATTCGTGCCGAT AACTA R:TTTTCGGTTCAGTGGCTTG TAGTAG	$(TCTG)_4$	118 (115-117)	PERERA et al. (2007)
HzMS3-11	F:ACTTCAAAGTTCGATTCTT GGGAT R:GCTCAAAGAGGACTACGT AGCTGA	$(AGCT)_4$	106 (93-106)	PERERA et al. (2007)
HzMS3-41	F:AAATTTCAACCAAATCGGT CTAGC R:TGGCCGAACTATAATATCT TACTTCCTA	$(ACAT)_4$	121 (121-135)	PERERA et al. (2007)
HzMS3-48	F:GGTGAAATGGAAATTGTTA TCTATCCC R:TCAGTCCAGTGGTTTAGAC GTGAA	$(TCTG)_4$	101 (94-102)	PERERA et al. (2007)
HzMS3-86	F:GGGGAAAAGAGGAAACAA ATCATC R:GAAACACGTTTGAGGAGG TCAGAT	$(CAT)_4$	140 (136-151)	PERERA et al. (2007)
HzMS4-3	F:ACTTTCCGCATCCGATTAA AATGT R:CAAATCGGACCAGTAGTTC CTGAG	$(GTTT)_4$	122 (122-126)	PERERA et al. (2007)
HzMS4-10	F:CTAGAACGGGCTTCATGGT GAG R:AAAAATAAAATGTATTCCG GGCGT	$(ATT)_4$	113 (110-113)	PERERA et al. (2007)
HzMS4-14	F:CAACATACAACATTCAGCC TGTCC R:TCAGTCGTCAGTTTTTGTC TTTGC	$(AC)_7$	132 (110-135)	PERERA et al. (2007)
HzMS4-16	F:AGTGTATACGGAGCAAGAA TTGGA R:TTTTGCAAATCAAACTATT GAAAAGTAA	$(ACAT)_6$	147 (134-149)	PERERA et al. (2007)

Table 3.2 - Characteristics of 20 microsatellite loci previously published for *Helicoverpa zea* and *H. armigera*. Locus name, forward (F) and reverse (R) primer sequences, repeat motif, expected size and size range of observed alleles, and reference.

(Conclusion)

Locus	Primer sequence (5′-3′)	Repeat motif	Allele size and size range (bp)	Reference
HzMS4-23	F:GTTCAGCGGTTTAGATGTGAAAGG R:TAAGGGTTCGTGTAGAAGTTCCCA	$(GACA)_4$	135 (130-139)	PERERA et al. (2007)
HaB60	F:CACCACCTGACATAACGC R:AAGGAGCAGCAATTGCAAGC	$(CTG)_2(TTG)_3(CTG)_5(TTG)_2$	162[1]	SCOTT et al. (2004)[a]
HaC87	F:ACGCGAGCACCAACTGTAA R:GAGACCAATAGCAGTAGTTC	$(TC)_5$	118[1]	SCOTT et al. (2004)
HaC14	F:TCCACACAGTTTGCATTATGA R:CGCCATAATCCTATTGATTC	$(ATTT)_5$	161[1]	SCOTT et al. (2004)
HarSSR1	F:TAGGTGATTGTGGCTCAGTTTT R:CAAACCCATCAGCAAATGCAAC	$(TGC)_2GAT(TGY)_4GAT(TGY)_{35}(TGA)_2AGC(TGY)_8$	240 (228-288)	JI et al. (2003)[a]
HarSSR6	F:TGTTGTTGCAGAGCTGCC R:TTCAGCAACACAACCGTACA	$(GHT)_{43}$	(292-340)[1]	JI; WU and ZHANG (2005)[a]
HarSSR7	F:AAGCAATAATTACCAGAAACAG R:GTTTATTCGTGTATTCATTAAATAG	$(GAT)_4$	(80-176)[1]	JI; WU and ZHANG (2005)
HarSSR9	F:AGCTCCACAACTCTTAACTAC R:GCAAACGATCACTGATATTAAC	$(CA)_{15}$	(189-261)[1]	JI; WU and ZHANG (2005)

[1]No further information provided
[z]*Helicoverpa zea*
[a]*Helicoverpa armigera*

3.2.3 Population genetic statistics

To estimate polymorphism, allele frequencies, species-specific alleles (private alleles), expected and observed heterozygosities, and the inbreeding coefficient (*f*), we used the GDA software (LEWIS; ZAYKIN, 2001). Hardy-Weinberg equilibrium (HWE) and linkage disequilibrium were tested using FSTAT software (GOUDET, 2002). The significance of each test was assessed based on 20,000 permutations, and a Bonferroni correction was used to correct for multiple testing (WEIR, 1996). The null-allele frequency was estimated using the program FreeNA (CHAPUIS; ESTOUP, 2007).

To detect the possible presence of hybrids within *Helicoverpa* spp. populations in Brazil, Bayesian assignment tests were performed using STRUCTURE v. 2.3.3 (PRITCHARD; STEPHENS; DONNELY, 2000). This software uses a Bayesian approach based on a Markov chain Monte Carlo (MCMC) algorithm, which divides individuals within "*K*" clusters (i.e., populations) in which the HWE is maximized and the linkage disequilibrium is minimized. Ten independent runs were performed with a 100,000 burn-in period followed by 500,000 MCMC steps. The *K* number for simulations ranged from 1 to 9, as suggested by Evanno; Regnaut and Goudet (2005). The consensus values for *K* were obtained with CLUMPP v. 1.1.2 (JAKOBSSON; ROSENBERG, 2007). The best *K* was recognized according to the ΔK method of Evanno; Regnaut and Goudet (2005), as calculated with the web application Structure Harvester (EARL, 2012).

3.3 Results

3.3.1 Microsatellite cross-species amplification

Among the 13 SSR loci previously published for *H. zea*, three loci were successfully amplified and evaluated in *H. armigera* (Table 3.3). Primers HzMS1-6, HzMS3-1, and HzMS4-3 failed to consistently amplify in all *H. armigera* samples, even at various annealing temperatures. Primers HzMS3-48, HzMS3-86, HzMS4-10, HzMS4-14, HzMS4-16, and HzMS4-23 amplified, but were non-specific, showing multiple bands during electrophoresis. For *H. zea*, the loci HaB60, HaC87, HaC14, and HarSSR1 were successfully amplified. The other three loci, HarSSR6, HarSSR7, and HarSSR9, did not amplify. All cross-amplified loci were polymorphic within both species with the exception of locus HaC14, which was monomorphic for *H. zea*. The most polymorphic locus was HasSSR1, which showed 12 alleles in *H. armigera* and five alleles in *H. zea*. Overall, the number of alleles varied from three (HzMS3-41 and HaC87 loci) to 12 for *H. armigera* with a mean number of 4.142 and from one (HaC14 locus) to five in *H. zea* with a mean number of 2.238 (Tables 3.3 and 3.4). *H. armigera* showed 17 species-specific alleles, while *H. zea* showed two species-specific alleles (Table 3.2). The allele sizes were similar to those reported from the authors that developed the primers, matching the expected repeat sizes (Tables 3.2 and3.3).

Table 3.3 - Locus name, annealing temperature (T_A), number of alleles, allele size and amplitude, and species-specific (private) alleles for both species of the seven microsatellite loci in *H. armigera* and *H. zea* populations.

Locus	T_A (°C)	*H. armigera* (N = 64)[1]		*H. zea* (N = 72)[1]		Allele size and size range (bp)
		Number of alleles	Alleles[2]	Number of alleles	Alleles	
HzMS1-4	60	4	**113**, 116, 119, 122	3	116, 119, 122	116 (113-122)
HzMS3-11	60	4	98, **102**, 106, 110	3	98, 106, 110	110 (98-110)
HzMS3-41	60	3	121, 125, 129	3	121, 125, 129	121 (121-129)
HaB60	55	4	**164**, 167, 170, 173	4	**161**, 167, 170, 173	167 (161-173)
HaC87	50	3	110, **114**, 118	2	110, 118	110 (110-118)
HaC14	55	6	**142**, **150**, **154**, 158, **162**, **166**	1	158	158 (142-166)
HarSSR1	58	12	242, 248, **251**, 254, 257, **260**, **263**, **266**, **269**, **275**, **284**, **287**	5	242, **245**, 248, 254, 257	242 (242-287)

[1]Number of individuals evaluated.
[2]Alleles highlighted in bold are the species-specific.

Table 3.4 - Genetic diversity estimates for each populations of *H. armigera* and *H. zea* based on seven microsatellite loci.

(To be continue)

Population	Diversity indices	HzMS1-4	HzMS3-11	HzMS3-41	HaB60	HaC87	HaC14	HarSSR1	Mean
HaBA-44	*A*	3.000	2.000	3.000	3.000	3.000	3.000	11.000	4.000
	He	0.298	0.359	0.554	0.236	0.152	0.573	0.879	0.436
	Ho	0.238	0.363	0.857	0.260	0.052	0.350	0.739	0.408
	f	0.206	-0.012	-0.568	-0.104	0.660	0.395	0.162	0.064
	a	0.067	0.000	0.000	0.000	**0.137**	**0.141**	0.055	
HaGO-2	*A*	4.000	3.000	3.000	4.000	2.000	5.000	12.000	4.714
	He	0.354	0.507	0.219	0.373	0.047	0.730	0.871	0.443
	Ho	0.409	0.388	0.238	0.347	0.047	0.500	0.695	0.375
	f	-0.159	0.239	-0.086	0.071	0.000	0.320	0.205	0.157
	a	0.000	0.084	0.000	0.024	0.000	**0.137**	0.091	
HaRS-2	*A*	3.000	4.000	2.000	4.000	2.000	4.000	7.000	3.714
	He	0.301	0.333	0.370	0.477	0.239	0.777	0.847	0.478
	Ho	0.333	0.272	0.470	0.625	0.000	0.444	0.416*	0.366
	f	-0.111	0.189	-0.280	-0.321	1.000	0.443	0.519	0.239
	a	0.000	0.000	0.000	0.000	**0.230**	**0.171**	**0.227**	
H. armigera mean	A	3.333	3.000	2.666	3.666	2.333	4.000	10.000	4.142
	He	0.317	0.411	0.406	0.359	0.138	0.689	0.876	0.452
	Ho	0.327	0.352	0.525	0.387	0.036	0.431	0.655	0.383
	f	-0.033	0.143	-0.296	-0.078	0.739	0.376	0.254	0.155

Table 3.4 - Genetic diversity estimates for each populations of *H. armigera* and *H. zea* based on seven microsatellite loci.

(Conclusion)

Population	Diversity indices	HzMS1-4	HzMS3-11	HzMS3-41	HaB60	HaC87	HaC14	HarSSR1	Mean
HzMG-4	*A*	3.000	2.000	2.000	2.000	1.000	1.000	4.000	2.143
	He	0.327	0.500	0.130	0.130	0.000	0.000	0.233	0.188
	Ho	0.291	0.421	0.136	0.136	0.000	0.000	0.250	0.176
	f	0.110	0.162	-0.050	-0.050	0.000	0.000	-0.073	0.066
	a	0.036	0.045	0.000	0.000	0.001	0.001	0.000	
HzSP-13	*A*	3.000	2.000	2.000	2.000	1.000	1.000	4.000	2.143
	He	0.121	0.493	0.241	0.043	0.000	0.000	0.335	0.176
	Ho	0.125	0.523	0.272	0.043	0.000	0.000	0.291	0.179
	f	-0.029	-0.064	-0.135	0.000	0.000	0.000	0.132	-0.018
	a	0.000	0.000	0.000	0.000	0.001	0.001	0.060	
HzBA-32	*A*	2.000	3.000	3.000	3.000	2.000	1.000	3.000	2.429
	He	0.042	0.565	0.449	0.138	0.085	0.000	0.510	0.256
	Ho	0.042	0.500	0.590	0.142	0.000	0.000	0.608	0.269
	f	0.000	0.118	-0.325	-0.034	1.000	0.000	-0.198	-0.053
	a	0.000	0.093	0.000	0.000	**0.139**	0.001	0.000	
H. zea mean	A	2.666	2.666	2.333	2.333	1.333	1.000	3.666	2.238
	He	0.163	0.519	0.273	0.103	0.028	0.000	0.359	0.207
	Ho	0.153	0.481	0.333	0.107	0.000	0.000	0.383	0.208
	f	0.066	0.075	-0.225	-0.036	1.000	0.000	-0.068	-0.007

A, Number of alleles; *He*, expected heterozygosity; *Ho*, observed heterozygosity; *f*, inbreeding coefficient; *a*, null alleles. Significant values are highlighted in bold. Deviation from HWE: * $p \leq 0.0024$.

3.3.2 Population genetic statistics

There was no linkage disequilibrium for any loci combination, and all populations showed *p*-values higher than 0.00238 (FSTAT corrected *p*-value). Null alleles were detected in all *H. armigera* populations at the HaC14 locus, in two populations (HaBA-44 and HaRS-2) at the HaC87 locus, and in the HaRS-2 population at the HarSSR1 locus (Table 3.4). Within *H. zea*, null alleles were only detected for the population HzBA-32 at the HaC87 locus. Despite the presence of null alleles, the tests of HWE showed that all populations and all loci were in equilibrium, except for population HaRS-2 with the locus HarSSR1 ($p \leq 0.00238$) (Table 3.4).

H. armigera showed an average heterozygosity (expected/observed) of 0.452/0.383, varying from 0.436/0.408 in population HaBA-44, to 0.478/0.366 in population HaRS-2. The average *f* was 0.155, varying from 0.064 (population HaBA-44) to 0.239 (population HaRS-2) (Table 3.4). For *H. zea*, the average heterozygosity was 0.207/0.208, varying from 0.176/0.179 in population HzSP-13, to 0.256/0.269 in population HzBA-32. The *f* varied from -0.053 (population HzBA-32) to 0.066 (population HzMG-4) with an average of -0.007 (Table 3.3). Furthermore, most of the loci in the *H. armigera* populations showed higher values of observed heterozygosity than in the *H. zea* populations, except for the HzMS3-11 locus.

The STRUCTURE analysis indicated that the best *K* when *H. armigera* and *H. zea* individuals were analyzed jointly was $K = 2$ (Figure 3.2). The individuals were divided in two genetic clusters according to their respective species (Figure 3.3). In the *H. armigera* cluster there were nine individuals with greater similarity (> 0.50 assignment) to *H. zea* individuals; the same occurred with one individual of the *H. zea* cluster (Figure 3.3).

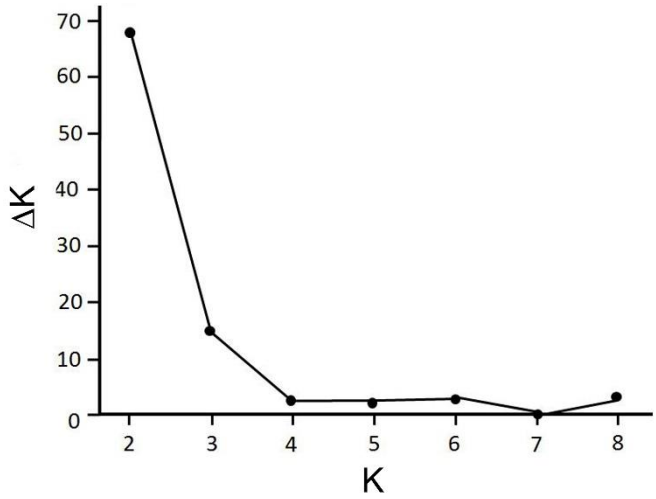

Figure 3.2 - Graphical plot of ΔK for *H. armigera* and *H. zea*; the maximum value of ΔK was considered to be the value of *K* (genetic groups or clusters) that best fit the data; *K* = 2.

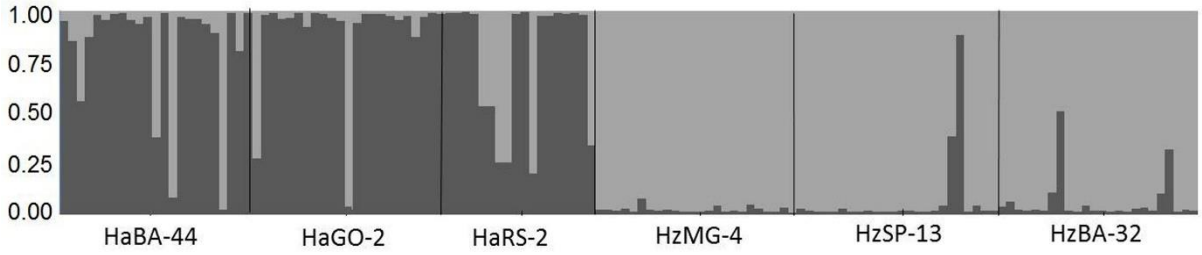

Figure 3.3 - Bar plot for *K* = 2 clusters for *H. armigera* (HaBA-44, HaGO-2, and HaRS-2) and *H. zea* (HzMG-4, HzSP-13, and HzBA-32) individuals assigned by STRUCTURE, based on seven microsatellite loci. Each color represents a different cluster.

3.4 Discussion

Cross-species amplification tests of SSR loci is an important and rapid method to generate informative markers, even for species for which microsatellites have not yet been characterized. The alternative is the development of species-specific SSR markers, which may be costly and take a long time to develop. For invasive species, using previously developed microsatellites from related species can generate data quickly to establish patterns of migration and gene flow as well as detect potential hybridization. Seven loci were successfully cross-amplified in *H. armigera* and *H. zea* and can be useful to further understand the invasion routes, gene flow, population structure, and mating systems of both species. Furthermore, the detection of cross-species SSR markers is extremely important for population studies in areas where *H. armigera* and *H. zea* individuals occur in sympatry, which can result in interspecific

gene flow (by hybridization) between natural populations of these species (LASTER; HARDEE, 1995; LASTER; SHENG, 1995).

The cross-species amplification success of SSR primers between *H. armigera* and *H. zea* was low. Only three of the 13 primers previously described for *H. zea* were successfully cross-amplified in *H. armigera*. Among the primers that we re-tested, the same four of the seven primers described for *H. armigera* were successfully cross-amplified in *H. zea.* This is consistent with the results of previous cross-amplification tests of 14 SSR primers described for *H. armigera* in *H. zea* (GRASELA; MCINTOSH, 2005), where the same four loci were amplified. This confirms the low cross-amplification success of SSR primers between the two species. These results suggest the rapid evolution of some primer biding sites in both species' genomes. This genetic divergence between the species is a paradox because they are able to produce fertile hybrid offspring in the laboratory (LASTER; HARDEE, 1995; LASTER; SHENG, 1995; CHO et al., 2008; POGUE, 2013).

The SSR analyses confirmed the utility of these markers in population studies of *H. armigera* and *H. zea* in Brazil, and presumably in South America. This is suggested by the fact that the loci did not show linkage disequilibrium, indicating that they can be used as independent genetic markers. Deviation from HWE was detected for locus HarSSR1 in the HaRS-2 population; for the same locus and population, null alleles were detected. In the other loci and populations where null alleles were detected, there were no deviations from HWE. The presence of null alleles at microsatellite loci is a major cause of deviations in HWE proportions because it confounds genotyping and leads to an accounting of more homozygotes (CHAKRABORTY et al., 1992). Null alleles are quite common among Lepidoptera (MEGLECZ et al., 2004), but the lack of a high null allele frequency and few HWE deviations suggest that they do not pose a significant problem for *Helicoverpa*. This may be due to the conservation of primer sites with cross-amplified loci; primers that work in both species would tend not to carry mutations that would prevent amplification and present null alleles. In general, the SSR loci evaluated showed a moderate level of polymorphism, and can be useful for simultaneous population genetic studies in *H. armigera* and *H. zea*.

H. armigera is an invasive pest recently reported in Brazil, which may account for the higher inbreeding coefficient in relation to *H. zea* populations. However, *H. armigera* populations showed consistently higher levels of genetic diversity, as

demonstrated by the higher observed heterozygosity values than those of *H. zea* populations. Leite et al. (2014) reported similar results in *H. armigera* and *H. zea* Brazilian populations using the mitochondrial COI gene sequence. Three hypotheses can explain these results: first, there could be higher intrinsic genetic diversity in *H. armigera* species relative to *H. zea*, since it is hypothesized that *H. zea* populations were established via a founder event from *H. armigera* individuals on the American continent (BEHERE et al., 2007). Second, *H. armigera* populations may have resulted from multiple independent introductions, with subsequent gene flow among the populations increasing their genetic diversity. Third, since more *H. armigera* loci work in *H. zea*, these may evolve or mutate slower because they are more conserved. If we had more *H. zea*-specific loci, they might actually be more polymorphic in *H. zea*. Lastly, the increased genetic diversity is related to potential hybridization and introgression events between *H. armigera* and other *Helicoverpa* species (LASTER; HARDEE, 1995; LASTER; SHENG, 1995).

The genetic identity of each individual detected with the STRUCTURE analysis showed that the individuals were mostly separated into two distinct clusters (K = 2) according to the *H. armigera* and *H. zea* species as identified by mtDNA. However, nine *H. armigera* individuals and one *H. zea* individual, previously identified by their mitochondrial COI gene sequence, showed greater than 0.50 (50%) genetic similarity to each other. This result suggests natural hybridization among individuals of different species, with an individual carrying a cytoplasmic genome from one species or population and a partial nuclear genome from the other species (FREELAND; PETERSEN; KIRK, 2011). However, the SSR loci were not useful to characterize a large number of species-specific alleles, mainly for *H. zea* (see Table 3.3), which complicates the specific identification of hybrid individuals from interspecific crosses between *H. armigera* and *H. zea*. Further analyses and population studies using this set of microsatellites should be done on *H. armigera* and *H. zea* populations collected prior to *H. armigera* introduction in Brazil and/or in the regions where these two species occur separately, to enable accurate and unambiguous differentiation between both species alleles.

We hope that the cross-species amplification and validation of the seven SRR loci from *H. armigera* and *H. zea* populations will contribute to a better understanding of the genetic structure, reproductive behavior, intra- and interspecific gene flow, and

adaptation processes of these two important agricultural pests. There is a scarcity of such information, especially for populations in the Americas, where *H. armigera* and *H. zea* populations occur simultaneously in different crops and landscapes throughout the year.

3.5 Conclusions

- Seven SSR loci successfully cross-amplify and are polymorphic in *H. armigera* and *H. zea* species except for the locus HaC14, which is monomorphic for *H. zea*.

- Apparently, *H. armigera* has higher genetic diversity than *H. zea*, based on microsatellites.

- There is potential hybrid offspring between *H. armigera* and *H. zea* individuals in natural conditions.

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4 GENETIC DIVERSITY AND INTRA AND INTERSPECIFIC GENE FLOW IN *Helicoverpa armigera* AND *Helicoverpa zea* (LEPIDOPTERA: NOCTUIDAE)

Abstract

The invasion of *Helicoverpa armigera* into the New World allowed the co-occurrence with its sibling species *H. zea* in the field, thus resulting in the possibility of natural hybridization. *H. armigera* rapidly spread in South America, and was also reported in Florida, USA. The genetic diversity, and intra and interspecific gene flow in *H. armigera* and *H. zea* in the Western Hemisphere is poorly understood. Therefore, our goals in this study were to (a) investigate the genetic diversity and gene flow in *H. armigera* populations from Brazil; (b) investigate the genetic diversity and gene flow between *H. zea* populations from Brazil and the USA; and, (c) identify the possible presence of hybrid specimens of *H. armigera* and *H. zea* in different hosts and regions from Brazil. We analyzed seven microsatellites that amplified in both species, for interspecific analyses. Ten microsatellites were used for Brazilian *H. armigera*, and eight were used for Brazilian and USA *H. zea* when intraspecific analyses were performed. Data analyses were performed with 17 populations of *H. armigera*, and 12 of *H. zea* collected in Brazil in 2012, 2013, and 2014; and five populations of *H. zea* collected in the USA in 2015. We detected high intraspecific gene flow (i.e. no genetic structure) in *H. armigera* and *H. zea* from Brazil and the USA. Genetic diversity was higher in *H. armigera*. Pairwise F_{st} and private alleles showed that *H. armigera* is more similar to *H. zea* from Brazil than *H. zea* from the USA. STRUCTURE analysis showed that there is low gene flow between Brazilian and USA *H. zea*. STRUCTURE analysis also strongly suggested the presence of hybrid individuals in *H. armigera* and *H. zea* populations of Brazil, mainly in Bahia state, which could have favored the rapid expansion of *H. armigera* in the Western Hemisphere. Our results are very important for the implementation of management of *H. armigera* and *H. zea* in Brazil and the USA because populations of both species need to be considered as one panmictic unit within each country. The detection of possible natural hybridization between these species poses a new challenge for Insect Resistance Management.

Keywords: Microsatellite; Hybridization; Old World bollworm; Corn earworm; Invasive species; Genetic structure

4.1 Introduction

Helicoverpa armigera (Hübner) (Lepidoptera: Noctuidae) is an Old World destructive invasive pest in the American continent. It was first reported in Brazil in 2013, causing damage to maize, soybean and cotton crops with losses of 35% and increased insecticide applications that have resulted in an estimated loss of US$ 1 billion per year (CZEPACK et al., 2013; MAPA, 2015; SPECHT et al., 2013; TAY et al., 2013). *H. armigera* rapidly dispersed across the American continent, with reports

in almost all South America in 2013/2014 (ARNEMANN et al 2016; LEITE et al., 2014; MASTRANGELO et al., 2014; MURÚA et al., 2014), and more recently in Florida, USA, where *H. armigera* specimens were collected in pheromone traps in 2015 (APHIS, 2015; HAYDEN; BRAMBILA, 2015).

Helicoverpa zea (Boddie) is endemic in the Americas, and it was the main pest of the genus *Helicoverpa* before the invasion of *H. armigera* (MALLET et al., 1993). *H. zea* is an important pest of cotton and maize crops in Brazil and the USA (LUTTRELL; JACKSON, 2012; DEGRANDE; OMOTO, 2013). In the USA, this species is also a soybean pest, while in Brazil *H. zea* has not been found in this crop (SWENSON; PRISCHMANN-VOLDSETH; MUSSER, 2013; LEITE et al., 2014). *H. zea* is polyphagous but seems to have a smaller host range and voracity than *H. armigera* (FITT, 1989; CUNNINGHAM; ZALUCKI, 2014; LEITE et al., 2014). The exact evolutionary relationship between *H. armigera* and *H. zea* remains unclear but is hypothesized that *H. zea* populations were established in the American continent via a founder event (approximately 1.5 million years ago) from a common ancestor of *H. armigera* (BEHERE et al., 2007). This hypothesis is supported by high morphological and genetic similarity between them and their ability to produce fertile offspring under laboratory conditions (MITTER; POOLE; MATTHEWS, 1993; LASTER; HARDEE, 1995; LASTER; SHENG, 1995).

Recent demographic studies with mitochondrial (mtDNA) markers revealed high genetic diversity, and a wide distribution of both species in Brazil with *H. armigera* preferring dicotyledonous hosts and *H. zea* preferring maize (LEITE et al., 2014; MASTRANGELO et al., 2014). The dispersion and adaptation of *H. armigera* in the American continent can be justified by its wide polyphagy, high reproductive rate and dispersal ability (FITT, 1989; RAZMJOU; NASERI; HEMATI, 2014). In addition, the tropical climate and new agronomic technology in South America facilitate an intense cropping system where many crop varieties are continuously cultivated across the landscape in large acreage (FARIAS et al., 2014). In these areas, polyphagous pests with high dispersal ability can easily move and have up to nine generations per year. Furthermore, the continuous maize, soybean and cotton cultivation during almost the entire year in South America could select for host-feeding preferences strains of polyphagous insect specimens based on limited inter-mating (DRÈS; MALLET, 2002; SCHOONHOVEN; VAN LOON; DICKE, 2005; MACHADO et al., 2008).

An additional, non-mutually exclusive hypothesis for rapid *H. armigera* adaptation and dispersal in South America is hybridization with *H. zea* that could introduce previously selected genes to *H. armigera* and its invasive genome (ABBOTT, 1992; LEVIN; FRANCISCO-ORTEGA; JANSEN, 1996; RHYMER; SIMBERLOFF, 1996; SAKAI et al., 2001; ELLSTRAND; SCHIERENBECK, 2006). Hybrids can have a higher potential for adaptability (SEEHAUSEN, 2004; MALLET, 2007), which may facilitate colonization and establishment in novel niches within the invaded range not typically occupied by any of its ancestors (YODER et al., 2010; WILLIAMS et al., 2014). For Insect Resistance Management (IRM), hybridization could result in novel genotypes that could be less susceptible to control tactics, due to the transfer and combination of beneficial alleles (SNOW; ANDERSEN; JØRGENSEN, 1999; WHITNEY; RANDELL; RIESEBERG, 2006; RIESEBERG et al., 2007). Native populations of *H. armigera* and *H. zea* have high genetic diversity and intraspecific gene flow (SCOTT; LAWRENCE; et al., 2005; BEHERE et al., 2007; ENDERSBY et al., 2007; LI et al., 2011; PERERA; BLANCO, 2011; BEHERE et al., 2013). Where they co-occur, like in Brazil, there is no report of inter and intraspecific gene flow based on nuclear molecular markers.

In this context, nuclear molecular markers that evolve faster than mtDNA are useful tools to help us understand the population dynamics and gene flow of *H. armigera* and *H. zea*. Therefore, a comparative study using microsatellite markers among *H. armigera* from Brazil, and *H. zea* from Brazil and the USA can help determine population structure and genetic diversity of these two important pests in the American continent. Moreover, nuclear markers can confirm the presence of field hybrid individuals, and identify possible hybridization zones between *H. armigera* and *H. zea* populations in Brazilian territory. Thus, our specific objectives were: (a) investigate the genetic diversity and gene flow in *H. armigera* populations from Brazil; (b) investigate the genetic diversity and gene flow in and between *H. zea* populations from Brazil and the USA; and, (c) identify the possible presence of hybrid specimens of *H. armigera* and *H. zea* in different hosts and regions from Brazil.

4.2 Material and methods

4.2.1 Sampling and DNA extraction

Larvae of *H. armigera* (n = 316) were collected from 17 Brazilian localities on six crops in 2012, 2013 and 2014 (Appendix A, Figure 4.1a). Larvae of *H. zea* (n = 255) were collected on 12 localities from Brazil on maize in 2012, 2013, and 2014 (Appendix A, Figure 4.1a). In addition, four populations of *H. zea* (n = 118) were collected in the USA in 2015 with pheromone traps (adults) on maize, with an additional collection of larvae in North Carolina (NC) on soybean (Appendix A, Figure 4.1b).

The maintenance of the larvae, DNA isolation, and species identification of insects collected in Brazil were done as described in Chapter 3. The insects from the USA were immediately frozen after collection. Genomic DNA was isolated from the thorax of each adult individual, and from the larvae using the ‘DNeasy Blood and Tissue kit’ (Qiagen), according to the manufacturer’s protocol.

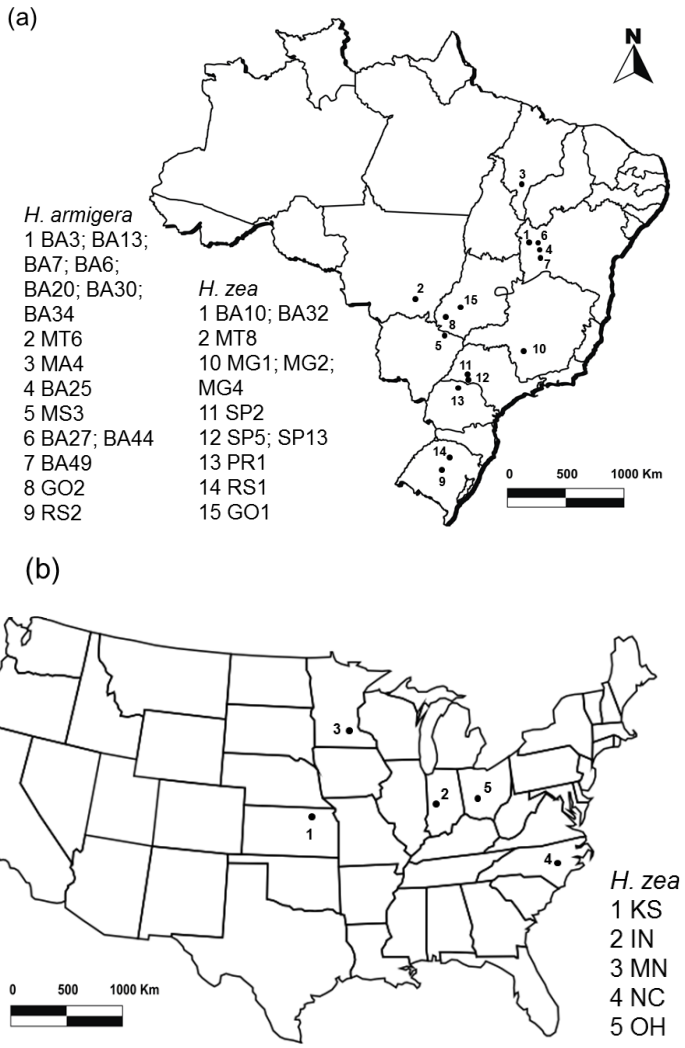

Figure 4.1 – Sampling sites for *H. armigera* and *H. zea* populations. (a) Populations of *H. armigera* and *H. zea* collected in Brazil. (b) Populations of *H. zea* collected in the USA.

4.2.2 Microsatellite amplification

4.2.2.1 Population genetics

Data were collected from 10 microsatellites for *H. armigera*. Seven of these amplify for both *H. armigera* and *H. zea* (HzMS1-4, HzMS3-11, HzMS3-41, HaB60, HaC87, HaC14, and HarSSR1) (Chapter 3), and other three are species-specific to *H. armigera* (HarSSR2, HarSSR3, and HarSSR9) (JI et al., 2003; JI; WU; ZHANG, 2005). For *H. zea* from Brazil and the USA, data were collected from eight microsatellites. Five (HzMS1-4, HzMS3-11, HzMS3-41, HaB60, and HarSSR1) described in Chapter 3, and the other three were species-specific (HzMS4-16,

HzMS3-48, and HzMS4-23) (PERERA et al., 2007). Loci HaC87 and HaC14 were not used for *H. zea* because they had low polymorphism for Brazilian *H. zea* and were monomorphic for USA *H. zea*. However, when interspecific analyses were performed, the seven common microsatellites between both species were used.

PCR conditions, loci amplification, and genotyping of *H. armigera* and *H. zea* from Brazil were described in Chapter 3. For USA *H. zea*, the following protocol was used: the forward primers were modified with the addition of the M13 forward sequence (5′-CACGACGTTGTAAAACGAC-3′) at the 5′ end (SCHUELKE, 2000). PCR amplification was performed in a 20-µL reaction mixture containing PCR Master Mix, 2x (Promega), 1 U of *Taq* polymerase, 0.5 µM of each primer, 0.4 µM of Dye, and ≈ 10 ng of DNA. PCR amplifications proceeded according to Chapter 3. The different annealing temperatures (°C) for the species-specific loci are given in Ji et al. (2003), Ji; Wu and Zhang (2005), and Perera et al. (2007). PCR products were diluted, pooled when possible, and genotyped using Beckman-Coulter CEQ8800KL (Fullerton, CA). Allele sizes were determined by visual inspection using CEQ Fragment Analysis Software (version 9.0.25). A population of Brazil (MG4, n = 24) was used as a control to determine the allele sizes among different genotyping platforms.

4.2.3 Statistics

4.2.3.1 Intraspecific population genetics

To estimate the general descriptive statistics [mean sample size of insects (*N*) over all loci, mean number of alleles per locus (*A*), allelic richness (*AR*), expected heterozygosity (*He*), and observed heterozygosity (*Ho*)] we used the GDA software for each population (LEWIS; ZAYKIN, 2001).

To verify if the allele frequencies are in agreement with those expected under random mating, we estimated Hardy-Weinberg equilibrium (HWE) using FSTAT 2.9.3.2 (GOUDET, 2002). FSTAT 2.9.3.2 was also used to calculate linkage disequilibrium, the inbreeding coefficient (*f*), and genetic differentiation (pairwise F_{st}). Significance across multiple tests was determined based on Bonferroni-corrected *p* values after 20,000 random permutations (WEIR, 1996). The null-allele frequency was estimated through the maximum likelihood method using FreeNA (CHAPUIS;

ESTOUP, 2007). Molecular analysis of variance (AMOVA) was also used to estimate population differences using Arlequin v.3.1 (EXCOFFIER; LAVAL; SCHNEIDER, 2005).

Bayesian assignment tests were performed using STRUCTURE v. 2.3.3 (PRITCHARD; STEPHENS; DONNELY, 2000) for *H. armigera*. This software uses a Bayesian approach based on a Markov chain Monte Carlo (MCMC) algorithm, which divides individuals within "*K*" clusters (i.e., populations) in which the HWE is maximized and the linkage disequilibrium is minimized. We performed the analyses with the Loc Prior model, which uses prior weight on clustering outcomes that are correlated with sampling locations. The Loc Prior method performs better for data sets where there are too few loci or individuals, or not enough divergence (HUBISZ et al., 2009). Ten independent runs were performed with a 250,000 burn-in period followed by 1,000,000 MCMC steps under the admixture model. The *K* number for simulations ranged from 1 to *K* stabilization. Consensus values for *K* were obtained with CLUMPP v. 1.1.2 (JAKOBSSON; ROSENBERG, 2007). The best *K* was recognized according to the ΔK method of EVANNO; REGNAUT and GOUDET (2005), as calculated with the web application Structure Harvester (EARL, 2012).

To search for evidence of bottleneck during *H. armigera* invasion, and/or a bottleneck caused by management on this species we used BOTTLENECK version 1.2.0.2 (CORNUET; LUIKART, 1996; PIRY; LUIKART; CORNUET, 1999). This analysis is based on the assumption that bottlenecked populations will show an excess of heterozygotes relative to allelic diversity. BOTTLENECK was run under three mutation models: the infinite alleles (IAM), two-phased (TPM) and stepwise mutation (SMM). The TPM was set at 95% stepwise mutation model and 5% multi-step mutations, as recommended by PIRY; LUIKART and CORNUET (1999). To identify heterozygosity excess the sign test and 2-Tail Wilcoxon signed-rank test were performed with 10,000 iterations (PIRY; LUIKART; CORNUET, 1999). In addition, the Garza-Williamson index (G-W) (GARZA; WILLIAMSON, 2001), an indicator of historical bottlenecks, was computed using Arlequin v.3.1 (EXCOFFIER; LAVAL; SCHNEIDER, 2005). For comparison purposes, we also performed bottleneck tests for *H. zea* in Brazil and the USA.

4.2.3.2 Comparative population genetics

To compare allelic diversity among *H. armigera*, *H. zea* from Brazil, and from the USA, descriptive statistics were gathered with the seven loci in common. Allelic richness (*AR*), observed heterozygosity (H_o), and gene diversity (H_s) was estimated using the comparison among groups of samples of FSTAT 2.9.3.2 (GOUDET, 2002), and 20,000 random permutations. For the other analyses, each species belonging to one location was considered as one population. FSTAT 2.9.3.2 was also used to calculate the genetic differentiation (pairwise F_{st}). The significance of this test was determined based on Bonferroni-corrected *p* values after 20,000 random permutations (WEIR, 1996).The number of private alleles (A_{priv}) was calculated using GDA (LEWIS; ZAYKIN, 2001).

Bayesian assignment tests were also performed to investigate gene flow among *H. zea* populations from Brazil and the USA using STRUCTURE v. 2.3.3 as described above. The same eight loci genotyped were used for this analysis, and each of the 17 populations were considered separately.

To search for hybrids, Bayesian assignment tests were performed among *H. armigera*, *H. zea* from Brazil and *H. zea* from the USA, with the data of the seven loci genotyped in common using STRUCTURE v. 2.3.3 as described above. Also, in the comparative analysis between Brazilian and USA *H. zea* and in the search of hybrids, consensus values for *K* were obtained with CLUMPP v. 1.1.2 (JAKOBSSON; ROSENBERG, 2007). The best *K* was recognized according to the ΔK method of EVANNO; REGNAUT and GOUDET (2005), as calculated with the web application Structure Harvester (EARL, 2012).

4.3 Results

4.3.1 Intraspecific population genetics

4.3.1.1 *H. armigera*

No linkage disequilibrium was observed for any pair of loci after Bonferroni correction. Therefore, further analyses were performed on multi-locus data from all 10 microsatellites for *H. armigera*. Significant deviation from HWE was observed for

H. armigera in only two situations: the BA7 population with locus HarSSR9, and BA30 with locus HaC14 ($p < 0.0003$) (Appendix B). The presence of null alleles may have contributed to these departures from HWE as significant evidence for null alleles was detected in these populations (Appendix B). The general descriptive statistics are presented in Table 4.1. The mean number of alleles per locus was 3.91, varying from 2.80 (MT6) to 4.60 (BA49, MS3, BA20). The mean expected heterozygosity (H_e) was 0.40, varying from 0.28 (MT6) to 0.50 (BA25). The mean observed heterozygosity (H_o) was 0.35, varying from 0.18 (MT6) to 0.45 (RS2). The mean inbreeding coefficient (*f*) was 0.141, but was only significant (does not differ from zero) for populations BA25, MA4, BA7, BA6, and MT6. We considered that populations went through a bottleneck if heterozygosity excess matched in at least the three tests (sign rank, Wilcoxon, and Garza-Williamson), and in one model. Several populations of *H. armigera* showed a genetic signal of a bottleneck (Table 4.2) including GO2, BA49, MA4, BA7, BA3, BA20, BA6 and MT6 ($p < 0.05$).

Pairwise F_{st} showed little to moderate differentiation (most of them not significant), and no pattern among populations (Appendix E). Although AMOVA showed that the higher proportion of variation was within populations (94.74%, $\Phi_{ST}=0.053$, $p < 0.001$), we observed a significant variation among populations (5.26%) (Table 4.3). STRUCTURE analysis indicated the best *K* as 2, separating the populations into two groups, orange and gray (Figure 4.2). However, the groups were not consistent with year of collection, geographic distance or host.

4.3.1.2 *H. zea* from Brazil

No linkage disequilibrium was observed for any pair of loci after Bonferroni correction. Therefore, further analyses were performed on multi-locus data from all eight microsatellites for *H. zea*. No deviation from HWE was found in Brazilian *H. zea* populations (Appendix C). The mean number of alleles per locus was 2.97, varying from 2.50 (SP2) to 3.63 (MG1) (Table 4.1). The mean expected heterozygosity (H_e) was 0.43, varying from 0.37 (SP13) to 0.47 (RS1). The mean observed heterozygosity (H_o) was 0.49, varying from 0.40 (MG2 and BA10) to 0.57 (RS1). The mean inbreeding coefficient (*f*) was -0.145, but no *f* was significant for any population. No population showed a genetic signal of a bottleneck (Table 4.2).

Pairwise F_{st} showed little to moderate differentiation (most of them not significant), and no pattern among populations (Appendix F). AMOVA showed that a higher proportion of variation existed within populations of *H. zea* from Brazil (98.95%, Φ_{ST}=0.010, $p < 0.001$) (Table 4.3).

4.3.1.3 *H. zea* from the USA

No linkage disequilibrium was observed for any pair of loci after Bonferroni correction. Therefore, further analyses were performed on multi-locus data from all eight microsatellites for *H. zea*. Significant deviation from HWE was observed for USA *H. zea* populations IN, NC, and OH, in HzMS3-11 locus, and MN, NC, OH, and KS in HzMS4-16 locus ($p < 0.001$) (Appendix D). Null alleles may have contributed to these departures from HWE (Appendix D). The mean number of alleles per locus was 3.05, varying from 3.00 (IN) to 3.38 (MN and OH) (Table 4.1). The mean expected heterozygosity (H_e) was 0.38, varying from 0.33 (OH) to 0.42 (IN). The mean observed heterozygosity (H_o) was 0.29, varying from 0.22 (OH) to 0.34 (MN and IN). The mean inbreeding coefficient (*f*) was 0.237, and *f* was significant for KS, NC, and OH. No population showed a genetic signal of a bottleneck (Table 4.2). Pairwise F_{st} showed little to moderate differentiation (most of them not significant), and no pattern among populations (Appendix G). AMOVA showed that a higher proportion of variation existed within populations of *H. zea* from the USA (98.23%, Φ_{ST}= 0.087, $p < 0.001$) (Table 4.3).

Table 4.1 - Summary of genetic diversity of *H. armigera* with 10 microsatellite loci at 17 sampled locations; *H. zea* from Brazil with eight microsatellite loci at 12 sampled locations, and *H. zea* from the USA with eight microsatellite loci at five sampled locations. Mean sample size (*n*) over all loci, mean number of alleles per locus (*A*), allelic richness (*AR*), expected heterozygosity (*He*), observed heterozygosity (*Ho*), and inbreeding coefficient (*f*). See Appendixes B, C, and D for raw data.

Population	***N***	***A***	***AR***	H_E	H_O	***f***
Helicoverpa armigera	**17.59**	**3.91**	**3.16**	**0.40**	**0.35**	**0.141**
Barreiras, BA (BA44)	19.50	3.90	2.86	0.42	0.38	0.084
Mineiros, GO (GO2)	19.70	4.50	3.13	0.45	0.39	0.137
Itaará, RS (RS2)	9.50	3.80	3.23	0.48	0.45	0.066
São Desidério, BA (BA25)	8.20	3.80	3.43	0.50	0.36	0.302*
Luís E. Magalhães, BA (BA34)	23.20	4.50	3.02	0.43	0.39	0.097
Correntina, BA (BA49)	22.60	4.60	3.06	0.42	0.40	0.050
Balsas, MA (MA4)	7.90	3.50	3.08	0.37	0.26	0.320*
Barreiras, BA (BA27)	13.50	2.90	2.32	0.32	0.33	-0.026
Luís E. Magalhães, BA (BA30)	23.00	3.40	2.64	0.42	0.41	0.031
Luís E. Magalhães, BA (BA33)	18.50	3.30	2.49	0.34	0.34	-0.001
Chapadão do Sul, MS (MS3)	12.90	4.60	3.40	0.44	0.42	0.046
Luís E. Magalhães, BA (BA7)	21.30	4.20	3.00	0.41	0.28	0.313*
Luís E. Magalhães, BA (BA3)	23.70	4.20	2.76	0.37	0.31	0.172
Luís E. Magalhães, BA (BA13)	26.40	4.40	2.74	0.36	0.30	0.176
Luís E. Magalhães, BA (BA20)	19.10	4.60	3.10	0.44	0.40	0.105
Luís E. Magalhães, BA (BA6)	17.80	3.50	2.68	0.34	0.27	0.213*
Rondonópolis, MT (MT6)	12.30	2.80	2.40	0.28	0.18	0.368*
***Helicoverpa zea* from Brazil**	**20.20**	**2.97**	**2.90**	**0.43**	**0.49**	**-0.145**
Capitólio, MG (MG4)	23.75	2.88	2.58	0.38	0.42	-0.102
Cândido Mota, SP (SP13)	21.75	2.75	2.49	0.37	0.41	-0.114
Luís Eduardo Magalhães, BA (BA32)	22.88	3.13	2.77	0.45	0.55	-0.237
Montividiu, GO (GO1)	15.38	2.75	2.62	0.44	0.56	-0.288
Rolândia, PR (PR1)	20.25	3.13	2.82	0.43	0.50	-0.158
Passo Fundo, RS (RS1)	12.50	2.75	2.71	0.47	0.57	-0.232
Capitólio, MG (MG1)	22.88	3.63	2.97	0.45	0.51	-0.141
Capitólio, MG (MG2)	21.13	2.88	2.48	0.40	0.40	-0.023
Rondonópolis, MT (MT8)	22.50	3.00	2.56	0.42	0.50	-0.186
Cândido Mota, SP (SP5)	23.88	3.00	2.62	0.44	0.56	-0.267
Luís E. Magalhães, BA (BA10)	21.13	3.25	2.96	0.46	0.40	0.144
Assis, SP (SP2)	14.38	2.50	2.45	0.40	0.45	-0.126
***Helicoverpa zea* from the USA**	**23.08**	**3.05**	**3.22**	**0.38**	**0.29**	**0.237**
Palmer, KS (KS)	23.25	3.25	3.18	0.37	0.27	0.288*
Lafayette, IN (IN)	23.00	3.00	2.97	0.42	0.34	0.189
Rosemount, MN (MN)	22.75	3.38	3.31	0.40	0.34	0.163
Wayne County, NC (NC)	23.00	3.25	3.21	0.40	0.31	0.227*
Springfield (WARS), OH (OH)	23.38	3.38	2.34	0.33	0.22	0.340*

*Imbreeding coefficient (*f*) significant at $p < 0.05$.

Table 4.2 – Bottleneck detection for *H. armigera* and *H. zea* populations based on G-W index, and Sign and 2-Tail Wilcoxon Tests under three models of microsatellite mutation: infinite allele model (IAM), two-phase model (TPM) and stepwise mutation model (SMM).

Population	G-W index (sd)	Sign Test (*p*-value)			2-Tail Wilcoxon Test (*p*-value)		
		IAM	TPM	SMM	IAM	TPM	SMM
H. armigera							
BA44	0.317 (0.091)	0.427	0.327	0.315	0.652	0.164	0.164
GO2	0.301 (0.067)	0.250	***0.016***[(1)]	***0.002***	0.625	***0.010***	***0.005***
RS2	0.341 (0.081)	0.372	0.131	0.124	0.496	0.164	0.164
BA25	0.312 (0.099)	0.605	0.148	0.134	0.820	0.250	0.164
BA34	0.335 (0.065)	0.250	0.067	0.067	0.492	***0.014***	***0.014***
BA49	0.328 (0.123)	0.346	***0.025***	***0.026***	0.426	***0.020***	***0.014***
MA4	0.303 (0.110)	0.354	***0.003***	***0.003***	0.375	***0.008***	***0.008***
BA27	0.293 (0.072)	0.246	0.639	0.632	0.688	0.688	0.688
BA30	0.344 (0.096)	0.160	0.264	0.259	0.105	0.770	0.557
BA33	0.316 (0.091)	0.280	0.065	0.073	0.641	0.074	0.074
MS3	0.290 (0.088)	0.098	0.074	0.083	0.492	0.084	0.084
BA7	0.326 (0.084)	0.383	0.119	***0.029***	0.652	***0.049***	***0.027***
BA3	0.313 (0.075)	0.385	***0.005***	***0.000***	0.301	***0.004***	***0.002***
BA13	0.350 (0.106)	0.275	0.069	0.064	0.275	0.019	0.019
BA20	0.332 (0.102)	0.094	***0.002***	***0.002***	0.160	***0.010***	***0.005***
BA6	0.347 (0.111)	***0.041***	***0.023***	***0.004***	0.131	***0.005***	***0.003***
MT6	0.322 (0.157)	***0.024***	***0.010***	***0.009***	***0.020***	***0.008***	***0.008***
***H. zea* from Brazil**							
MG4	0.346 (0.096)	0.635	0.258	0.267	0.641	0.844	0.844
SP13	0.294 (0.067)	0.610	0.542	0.531	0.547	0.742	0.742
BA32	0.311 (0.084)	0.181	0.441	0.439	0.383	0.945	0.844
GO1	0.310 (0.093)	0.396	0.515	0.519	0.250	0.844	0.945
PR1	0.308 (0.110)	0.164	0.220	0.219	0.383	0.547	0.461
RS1	0.336 (0.103)	0.164	0.513	0.511	0.055	0.844	0.945
MG1	0.306 (0.114)	0.182	0.066	0.071	0.383	0.074	0.074
MG2	0.311 (0.114)	0.138	0.451	0.532	0.383	0.945	1.000
MT8	0.323 (0.120)	0.353	0.275	0.258	0.383	0.844	0.742
SP5	0.319 (0.112)	0.137	0.553	0.539	***0.039***	0.742	0.945
BA10	0.314 (0.108)	0.168	0.216	0.221	0.195	0.641	0.547
SP2	0.331 (0.102)	0.209	0.326	0.328	0.078	0.938	0.938
***H. zea* from the USA**							
KS	0.312 (0.123)	0.322	0.224	0.220	1.000	0.195	0.195
IN	0.328 (0.108)	0.374	0.490	0.479	0.313	0.742	0.742
MN	0.357 (0.115)	0.622	0.272	0.288	0.945	0.641	0.547
NC	0.307 (0.090)	0.585	0.110	0.111	0.688	0.297	0.078
OH	0.359 (0.100)	0.290	0.616	0.609	0.461	0.945	0.945

[(1)]Numbers in bold and italic: significant at $p < 0.05$.

Table 4.3 - Analysis of molecular variance (AMOVA), for population genetics structure of *H. armigera* with 10 microsatellite markers, and *H. zea* from Brazil and the USA with eight microsatellite markers.

Source of variation	d.f.	Sum of square	Variance components	Fixation Indices	Variation (%)
H. armigera					
Among populations	16	87.114	0.09906 Va	Φ_{ST}=0.053*	5.26
Within populations	615	1097.035	1.78380 Vb		94.74
Total	631	1184.149	1.88286		
***H. zea* from Brazil**					
Among populations	11	24.893	0.01658 Va	Φ_{ST}=0.010*	1.05
Within populations	498	776.824	1.55989 Vb		98.95
Total	509	801.718	1.57647		
***H. zea* from the USA**					
Among populations	4	10.896	0.02651 Va	Φ_{ST}= 0.087*	1.77
Within populations	231	340.193	1.47270 Vb		98.23
Total	235	351.089	1.49921		

* Significant to $p < 0.05$

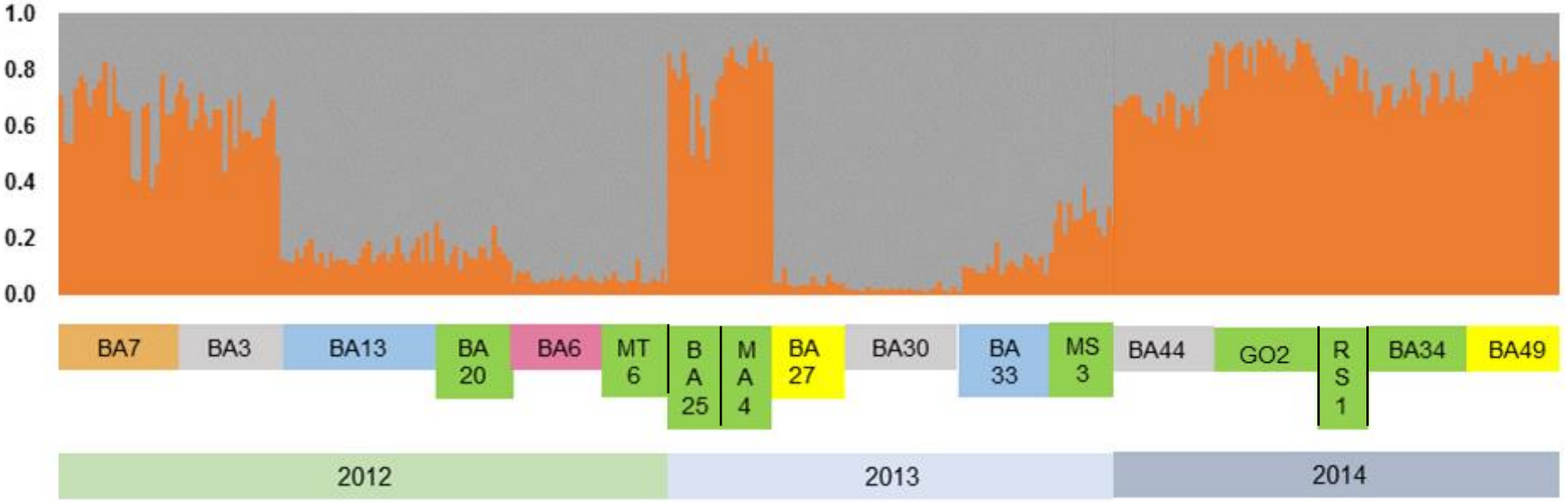

Figure 4.2 - Bar plot for *K* = 2 clusters for *H. armigera* individuals assigned by STRUCTURE (Loc Prior Model), based on ten microsatellite loci. Colors assigned to populations mean the crop where populations were collected (gray = cotton; green = soybean; yellow = maize; blue = bean; orange = millet; pink = sorghum).

4.3.2 Comparative population genetics

4.3.2.1 Comparative analyses

Descriptive statistics using the seven loci indicated that the genetic diversity of *H. armigera* is higher than *H. zea* as demonstrated by allelic richness (*AR*), observed heterozygosity (H_o), and gene diversity (H_s) ($p < 0.05$). The mean number of *AR*, which standardizes populations to a uniform sample size, was 3.05 for *H. armigera*, 1.85 for *H. zea* from Brazil, and 1.76 for *H. zea* from the USA. H_o was equal to 0.39 for *H. armigera*, 0.26 for Brazilian *H. zea*, and 0.15 for USA *H. zea*. H_s was equal to 0.42 for *H. armigera*, 0.24 for Brazilian *H. zea*, and 0.19 for USA *H. zea*. Pairwise F_{st} was 0.166 between *H. armigera* and *H. zea* from Brazil, 0.218 between *H. armigera* and *H. zea* from the USA, and 0.028 between Brazilian and USA *H. zea* (all F_{st} values were significant, $p < 0.05$). In addition, we observed six private alleles in *H. zea* from the USA, when compared to Brazilian *H. zea* and *H. armigera* (Table 4.4). On the other hand, *H. zea* from Brazil had one private allele (161 allele in HaB60 locus) in comparison with *H. armigera*. *H. armigera* showed the highest number of private alleles (n = 17).

STRUCTURE analysis between *H. zea* from Brazil and the USA showed that the populations from the two continents are substantially different, i.e. gene flow between *H. zea* from the two Americas might be very low (Figure 4.3). The best *K* was 5, however, it showed two distinct clusters corresponding to *H. zea* from Brazil and the USA. No clustering within countries was apparent. This result was corroborated by AMOVA, described above.

4.3.2.2 Detection of Putative Hybrids

STRUCTURE analysis with the entire data set clearly showed that some individuals in Brazil shared genomic relatedness with both *H. armigera* and *H. zea* (Figure 4.4). The best *K* (for the entire data set) was 2, mostly distinguishing *H. armigera* from *H. zea*. Within *H. armigera* populations, 34 individuals were more similar to the *H. zea* cluster (≥ 0.5 of similarity); alternatively in Brazilian *H. zea* populations, nine individuals were more similar to the *H. armigera* cluster. In the USA, no individuals had a high similarity with *H. armigera* individuals; the highest

similarity was 0.3. The presence of putative hybrids were higher in populations collected in 2012 and 2013, and in the state of Bahia (Appendix A) where large hectares of shared hosts (soybean, maize and cotton) are present. In terms of number of hybrids, the populations of *H. armigera* BA27, BA13 and BA7 showed the highest proportion (40%, 32%, and 18%, respectively). Considering *H. zea* populations, BA10 showed the highest proportion (18%) of hybrids among the insects collected.

Table 4.4 **-** Locus name, number of alleles, allele size and amplitude, and private alleles of the seven microsatellite loci in *H. armigera* and *H. zea* populations from Brazil and the USA.

Locus	*H. armigera* (*n* = 316)[1]		Brazilian *H. zea* (*n* = 255)[1]		USA *H. zea* (*n* = 118)[1]	
	Number of Alleles	Alleles	Number of Alleles	Alleles	Number of Alleles	Alleles
HzMS1-4	5	**110**[2],113, 116, 119, 122	3	113, 116, 119, 122	4	116, 119, 122, **125**
HzMS3-11	4	98, **102**, 106, 110	3	98, 106, 110	4	**94**, 98, 110, **114**
HzMS3-41	3	121, 125, 129	3	121, 125, 129	3	**113**, 121, 125,
HaB60	4	164, 167, 170, 173	5	161[3], 164, 167, 170, 173	3	161, 167, 170
HaC87	7	**106**, 110, **114**, 116, 118, **120**, **122**	3	110, 116, 118	2	**104**, 118
HaC14	8	**142**, **146**, 150, 154, 158, 162, **166**, **170**	4	150, 154, 158, 162	2	150, 158
HarSSR1	17	233, 242, 245, 248, 251, 254, 257, 260, **263**, 266, **269**, **272**, **275**, **278**, **281**, **284**, 287	8	242, 245, 248, 251, 254, 257, 266, 287	8	**227**, 233, 242, 245, 248, 251, 254, 260

[1]*n*, number of individuals evaluated.
[2]Numbers in bold are private alleles comparing the three samples.
[3]Numbers subscribed are private alleles to only *H. zea*.

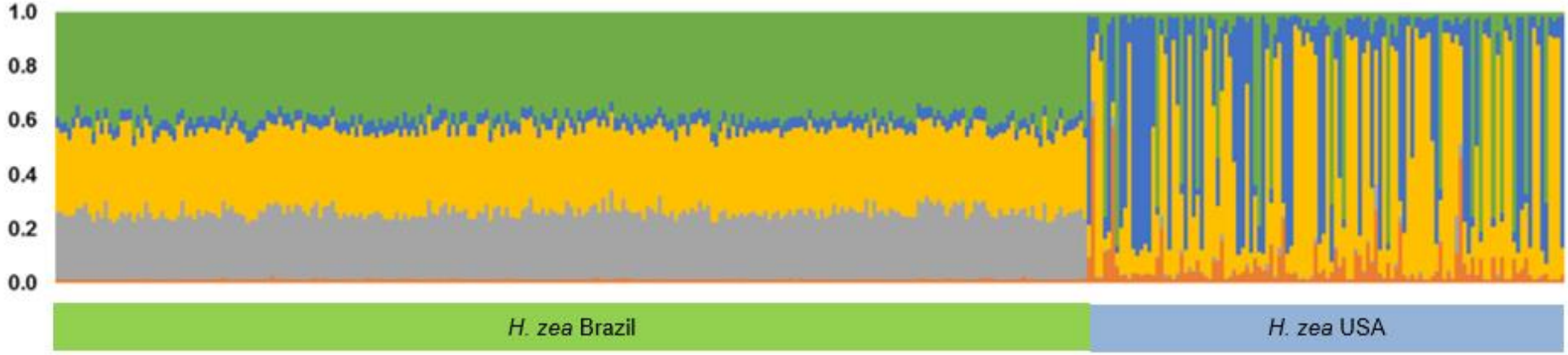

Figure 4.3 - Bar plot for *K* = 5 clusters for *H. zea* (Brazil and the USA) individuals assigned by STRUCTURE (Loc Prior Model), based on eight microsatellite loci.

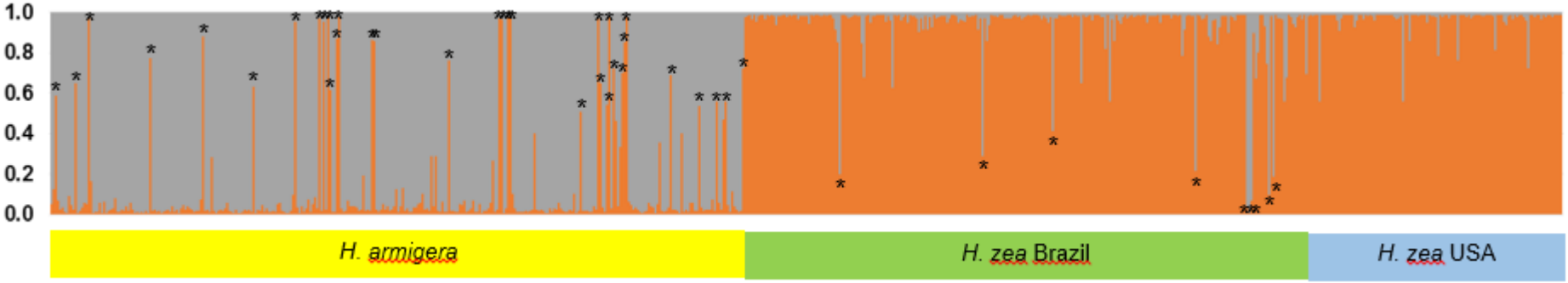

Figure 4.4 - Bar plot for *K* = 2 clusters for *H. armigera*, and *H. zea* (Brazil and the USA) individuals assigned by STRUCTURE (Loc Prior Model), based on seven microsatellite loci. *Putative hybrid individuals.

4.4 Discussion

Our aims in this study were to investigate the genetic diversity and gene flow in and among Brazilian *H. armigera* and *H. zea*, and USA *H. zea*. Since *H. armigera* is an invasive pest in the New World, we hypothesized that its adaptation was due to its high genetic diversity and hybridization with *H. zea*. We also believed that these species would show no genetic structure (i.e. high gene flow) due to their high mobility. However, we expected a difference between *H. zea* from Brazil and from the USA, since Brazilian *H. zea* almost feeds only on maize (LEITE et al. 2014), and USA *H. zea* feeds on soybean and cotton too (LUTTRELL; JACKSON, 2012; SWENSON; PRISCHMANN-VOLDSETH; MUSSER, 2013).

Our intraspecific results showed an incipient structure (5.3% variation among populations) for Brazilian *H. armigera* and virtually no structure for Brazilian *H. zea* (1.0%), and USA *H. zea* (1.7%). Both species can migrate over than 1,000 Km (PEDGLEY, 1985; FARROW; DALY, 1987) and the results suggest that the gene flow in these species is sufficient to homogenize allele frequencies in populations of different regions. This study supports previous findings of little population subdivision in Australian *H. armigera* and USA *H. zea*. Allozyme studies with 12 populations of Australian *H. armigera* (DALY; GREGG, 1985), and 39 USA *H. zea* (HAN; CAPRIO, 2002), suggested limited population structure for these species, F_{st} = 0.01 and 0.007, respectively. Similarly, analysis of mitochondrial sequences reveals minimal differentiation among global (BEHERE et al., 2007) and Brazilian samples (LEITE et al., 2014; MASTRANGELO et al., 2014) with most of the variation distributed throughout the species range. A survey of Australian *H. armigera* with five variable microsatellite loci, revealed that reduced migration ratio between cropping regions resulted in significant genetic structure (SCOTT; WILKINSON; et al., 2005). However, this isolation by distance was most pronounced in years with limited migration. These authors, attributed these results to different seasonal migration patterns, as *H. armigera* has voluntary migration (FARROW; DALY, 1987). Thereafter, Scott et al. (2006) detected that maize acted as a major sink for immigrants from cotton and from outside the region of Murrumbidgee Valley, Australia. We collected *H. armigera* in six crops in Brazil, however we did not find any pattern of structure related to crops. Also, our collections were made in three years (2012, 2013, and 2014), similar to Endersby et al. (2007), that sampled in Victoria, Australia in 1999, 2001, and 2004.

As well as these authors, we did not find any structure over time. *H. zea* populations were collected in the same three years in Brazil, but only on maize, and in the USA in 2015 on pheromone traps on maize and sorghum. We did not find genetic structure in *H. zea* populations in these countries, by year or crop. Similar results were found when *H. zea* populations were collected in the USA on four hosts, in 2005 (PERERA; BLANCO, 2011).

Exon-primed-intron-crossing (EPIC) markers were developed by Tay et al. (2008). These primers bind to conserved exon sequences, reducing the frequency of null alleles and allowing the characterization of the more variable intronic sequences. Behere et al. (2013) used these markers to study the genetic structure of *H. armigera* in India, and were able to detect evidence of substructure by host, time and space, without, however, a clear biological reason for these structures being evident. We also found evidence of substructure in *H. armigera* for Brazil, however, STRUCTURE groups showed no pattern. We suspect that these small differences among populations were related to random sampling and different management practices as insecticides application and genetically modified crops that express *Bacillus thuringiensis* (*Bt*) proteins

In Brazil, studies with other lepidopteran pests showed no evidence of genetic structure. Ten populations of *Spodoptera frugiperda* (J. E. Smith) (Lepidoptera: Noctuidae) were analyzed using polymorphic DNA (RAPD) – polymerase chain reaction (PCR) (MARTINELLI et al., 2006), and seven populations using AFLP (MARTINELLI et al., 2007) to investigate their association with maize and cotton, however no genetic structure was observed. *Chloridea virescens* (Fabricius) (Lepidoptera: Noctuidae) showed no structure regardless of geographical scale, time, and host, with most genetic variation occurring within populations, using mtDNA markers (ALBERNAZ et al., 2012). This is consistent with other studies performed in other countries with *S. frugiperda* (CLARK et al., 2007), when not considering rice and maize strains (PASHLEY, 1986), and *C. virescens* (HAN; CAPRIO, 2004; GROOT et al., 2011). Therefore, almost all studies with lepidopteran pests reported no genetic structure. These results mean that panmixia within countries should be considered for resistance management of *H. armigera* and *H. zea*.

Genetic analysis of Lepidoptera populations may be affected by the occurrence of null alleles. HWE deviation caused by the excess of homozygotes was found in HarSSR9 locus in BA7 population and in HaC14 locus in BA30 population of

H. armigera. HWE deviation was also observed for three populations of USA *H. zea* in HzMS3-11 locus, and four populations in HzMS4-16 locus. The possibility of inbreeding should be discarded because heterozygous deficiency in a locus indicates the presence of null alleles, whereas inbreeding or other population processes are generally reflected in all loci. Other explanation could be the Wahlund effect, which is the occurrence of within populations subdivisions (WEIR, 1996). However, the lack of genetic structure and the fact that Endersby et al. (2007) also have found null alleles in HaC14 (frequence = 7.8%) indicate that this is the main cause of deviations in HWE observed in our study. These authors demonstrated that markers with low or moderate level of null alleles were powerful to enable a correct interpretation of gene flow patterns, a consistent conclusion with population genetics simulation studies (CHAPUIS; ESTOUP, 2007; CARLSSON, 2008). Chapuis and Estoup (2007) also concluded that F_{st} estimates were more accurate when markers with null alleles were not excluded and were less biased in populations with unrestricted gene flow.

Bottleneck was only observed in *H. armigera* populations. The Garza-Williamson index was low for both species (less than 0.4) (Table 4.2), indicating a recent reduction in population size. However, considering all the three tests (G-W, Sign and Wilcoxon), eight populations of *H. armigera* showed signs of genetic bottleneck. Leite et al. (2014) using mtDNA showed higher genetic diversity for *H. armigera* in Brazil, than *H. zea*, and also a population expansion for these species. These results suggest that initial *H. armigera* populations were established by a large number of individuals, were derived from introductions from different sources and/or were submitted to a bridgehead effect (GUILLEMAUD et al., 2011). In a bridgehead effect an invasive population acts as the source of the colonists that invade another place, with some admixture in the source population (LOMBAERT et al., 2010). This scenario is evolutionarily more parsimonious, because it considers only one evolutionary shift. However, these hypotheses are not mutually exclusive. Therefore, the bottleneck observed in *H. armigera* could be due to the management practices applied to the populations. When *H. armigera* started causing problems in 2012, there was a massive use of insecticides to control it, which continued and increased in 2013 and 2014. This could have reduced some populations of this pest that might have been detected as recent bottlenecks. On the other hand, a bottleneck signal in *H. zea* might not have been detected due to its presence in the Americas for a long time (MALLET et al., 1993; BEHERE et al., 2007).

Surprisingly, microsatellite data of seven interspecific loci showed that genetic diversity is higher in *H. armigera* than *H. zea*, despite the recent invasion and evidence for a bottleneck in *H. armigera*. This result is corroborated by studies with allozyme electrophoresis that found significantly less heterozygosity in *H. zea* populations than other related heliothine species (SLUSS et al., 1978). In addition, mitochondrial DNA showed more haplotypes in *H. armigera* in Brazil than *H. zea* (LEITE et al., 2014). The higher genetic diversity of *H. armigera* could explain its greater adaptability potential when compared to *H. zea*.

Private alleles and pairwise F_{st} showed clear divergence between *H. armigera* and *H. zea* species revealing that the genetic identity is preserved between them. Also, STRUCTURE analysis showed low gene flow between Brazilian and USA *H. zea*. Interesting, pyrethroid tolerance is also different from *H. zea* populations from Brazil and the USA. Brazilian *H. zea* populations are susceptible to pyrethroids, while there are evidences for susceptibility decrease in USA *H. zea* (BROWN et al., 1998; HUTCHISON et al., 2007). Therefore, gene flow between populations of the two countries may not be sufficient to spread alleles that confer resistance to pyrethroids, which means that gene flow is low. It would be interesting to do a comparative study using populations from Central America. *H. zea* populations from Central America could be intermediate similarly with Brazilian and USA *H. zea*. In the USA, *H. zea* migrates northward, however, there is no evidence of its return to south (SANDSTROM; CHANGNON; FLOOD, 2007). Therefore, the migration pattern of *H. zea* could be from south (Brazil) to north (USA), using Central America as bridge.

Our study strongly suggested that *H. zea* and *H. armigera* in Brazil are mating in the field, producing hybrid offspring. The individuals that were seen to be similar to the other species (> 50%) with microsatellite, were identified as being from the original species with the PCR-RFLP technique proposed by Behere et al. (2008), based on cytochrome oxidase I (COI) gene. USA *H. zea* was used as a “pure” strain to identify the hybrids, since *H. armigera* has not yet established in the USA. USA *H. zea* populations showed a maximum of 30% of similarity to *H. armigera*. This can be considered a threshold for alleles identical in size due to convergent mutation (i.e. size homoplasy) (ESTOUP; JARNE; CORNUET, 2002). Populations from Bahia showed a higher number of hybrids in 2012 and 2013 (Appendix A). *H. armigera* was first reported in Brazil in 2013, although a recent study showed that this pest has been present in Brazil since 2008 (SOSA-GÓMEZ et al., 2015). The field problem started earlier in 2012, mainly in the state of Bahia on cotton and soybean crops. The damage led to economic losses estimated at more than 2 billion dollars. After that, this pest was detected all over Brazil. Its expansion and niches occupation was extremely fast, causing damage in several crops (BUENO et al., 2014; LEITE et al., 2014). Thus, an explanation for this rapid occupation of this pest in Brazil and in South America may be due to generation of hybrids in the field with *H. zea*.

Hybridization is increasingly recognized as potentially important in the invasion process after introduction (ABBOTT, 1992; LEVIN; FRANCISCO-ORTEGA; JANSEN, 1996; RHYMER; SIMBERLOFF, 1996; SAKAI et al., 2001; ELLSTRAND; SCHIERENBECK, 2006). This event is extremely common in plants (see ABBOTT, 1992; RIESEBERG et al., 2003; ELLSTRAND; SCHIERENBECK, 2006), but also occurs frequently in animals (ECHELLE; ECHELLE, 1997; PERRY; LODGE; FEDER, 2002; HASHIMOTO et al., 2012; LEVIN, 1974). Invasive species can negatively impact native ones, especially in relation to competition (FACON et al., 2005). On the other hand, it may eventually create new species (ABBOTT, 1992; BAUMEL; AINOUCHE; LEVASSEUR, 2001). In an IRM perspective, the creation of new species or the occurrence of hybrids is a new challenge. Hybrids could have higher fitness than their parents, a phenomenon known as heterosis (or hybrid vigour). Heterosis is expected when there is some overdominance and/or cooperative epistasis between alleles inherited from the parental taxa (KELLER; WALLER, 2002). Therefore, hybrids may be better adapted to hosts, develop tolerance to climatic factors, pathogens, and control methods such insecticides and *Bt* plants.

The current study provides important information on population genetics of *H. armigera* and *H. zea* in Brazil and the USA for designing and implementing sustainable pest management strategies. These species are not structured in Brazil and the USA, which highlights the importance of maintaining regional coordinated IRM strategies, due to their large territorial areas. Regarding to *Bt* crops, if resistance emerges in populations of one crop (i.e. cotton), the high movement onto other crops might spread the alleles for resistance. This is more important to *H. armigera* and USA *H. zea*, which attack different hosts. On the other hand, for Brazilian *H. zea*, maize populations should be constantly monitored. In the USA, there is still time to implement management plans to prevent the wide dispersion of *H. armigera* in this territory. Also, our findings support the formation of hybrids between these species. This may have contributed to the rapid expansion of *H. armigera* in South America. Further studies on the bioecology of these insects, as well as susceptibility to different control methods should be performed for a better understanding of their population dynamics.

4.5 Conclusions

- Apparently, genetic diversity is higher in *H. armigera* than *H. zea*.

- There is high intraspecific gene flow in Brazilian *H. armigera* and *H. zea*, and USA *H. zea*.

- There is low gene flow between Brazilian and USA *H. zea*.

- There is putative hybrid offspring between *H. armigera* and *H. zea* in natural conditions, which may have favored the rapid expansion of *H. armigera* in South America.

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APPENDIXES

Appendix A - Sampling sites for *H. armigera* and *H. zea* in Brazil, and *H. zea* in the USA, including abbreviations, crops sampled, sample sizes for the microsatellites loci, geographic coordinates, and sampling dates.

(To be continue)

Site (City, State, Abbreviation)	Crop	Sample size		Hybrid individuals (total number)	Latitude	Longitude	Date
		H. armigera	*H. zea*				
Brazilian populations							
2012 cropping							
Luís E. Magalhães, Bahia (BA3)	Cotton	25	-	-	12°05′58″ S	45°47′54″ W	05.24.12
Luís E. Magalhães, Bahia (BA13)	Bean	28	-	7;15;16;19;20; 22;26;27;28 (9)	12°05′58″ S	45°47′54″ W	06.12.12
Luís E. Magalhães, Bahia (BA7)	Millet	22	-	17;18;21;22 (4)	12°05′58″ S	45°47′54″ W	05.10.12
Luís E. Magalhães, Bahia (BA6)	Sorghum	19	-	13 (1)	12°05′58″ S	45°47′54″ W	05.10.12
Luís E. Magalhães, Bahia (BA20)	Soybean	20	-	20 (1)	12°05′58″ S	45°47′54″ W	10.31.12
Rondonópolis, Mato Grosso (MT6)	Soybean	14	-	2;6;14 (3)	16°28′17″ S	54°38′14″ W	11.08.12
Capitólio, Minas Gerais (MG1)	Maize	-	23	16 (1)	20°36′17″ S	46°04′19″ W	06.08.12
Luís E. Magalhães, Bahia (BA10)	Maize	-	22	12;13;14;22 (4)	12°05′58″ S	45°47′54″ W	06.12.12
Assis, São Paulo (SP2)	Maize	-	15	2 (1)	22°39′40″ S	50°23′58″ W	06.15.12
2013 cropping							
Balsas, Maranhão (MA4)	Soybean	9	-	4 (1)	07°31′59″ S	46°02′06″ W	01.06.13
São Desidério, Bahia (BA25)	Soybean	9	-	-	12°05′58″ S	45°47′54″ W	01.15.13
Chapadão do Sul, Mato Grosso do Sul (MS3)	Soybean	13	-	7 (1)	18°46′44″ S	52°36′59″ W	05.23.13
Luís E. Magalhães, Bahia (BA30)	Cotton	24	-	15;16 (2)	12°05′58″ S	45°47′54″ W	06.17.13
Luís E. Magalhães, Bahia (BA33)	Bean	19	-	-	12°05′58″ S	45°47′54″ W	09.20.13
Barreiras, Bahia (BA27)	Maize	15	-	6;8;10;11;14;15 (7)	11°33′33″ S	46°19′47″ W	02.21.13
Cândido Mota, São Paulo (SP5)	Maize	-	25	13 (1)	22°44′46″ S	50°23′15″ W	01.14.13
Luís E. Magalhães, Bahia (BA32)	Maize	-	24	-	12°05′58″ S	45°47′54″ W	03.28.13
Rondonópolis, Mato Grosso (MT8)	Maize	-	23	-	16°28′17″ S	54°38′14″ W	04.22.13
Rolândia, Paraná (PR1)	Maize	-	22	20 (1)	23°19'13” S	51°29'01” W	01.24.13

Appendix A - Sampling sites for *H. armigera* and *H. zea* in Brazil, and *H. zea* in the USA, including abbreviations, crops sampled, sample sizes for the microsatellites loci, geographic coordinates, and sampling dates.

(Conclusion)

Site (City, State, Abbreviation)	Crop	Sample size		Hybrid individuals (total number)	Latitude	Longitude	Date
		H. armigera	*H. zea*				
Brazilian populations							
2013 cropping							
Passo Fundo, Rio Grande do Sul (RS1)	Maize	-	14	-	28°16'08" S	52°37'15" W	01.30.13
Montividiu, Goiás (GO1)	Maize	-	18	-	17°19'19" S	51°14'51" W	02.05.13
Capitólio, Minas Gerais (MG2)	Maize	-	22	-	20°36′17″ S	46°04′19″ W	03.10.13
2014 cropping							
Correntina, Bahia (BA49)	Maize	24	-	9 (1)	13°20′36″ S	44°38'12″ W	04.23.14
Barreiras, Bahia (BA44)	Cotton	20	-	3;12;18 (3)	11°33′33″ S	46°19'47″ W	02.22.14
Luís E. Magalhães, Bahia (BA34)	Soybean	24	-	10 (1)	12°05′58″ S	45°47′54″ W	01.31.14
Mineiros, Goiás (GO2)	Soybean	21	-	-	17°34′10″ S	52°33′04″ W	01.03.14
Itaará, Rio Grande do Sul (RS2)	Soybean	10	-	5 (1)	29°36′35″ S	53°45′53″ W	03.10.14
Capitólio, Minas Gerais (MG4)	Maize	-	24	-	20°36′17″ S	46°04′19″ W	02.17.14
Candido Mota, São Paulo (SP13)	Maize	-	23	20 (1)	22°44′46″ S	50°23′15″ W	03.17.14
Total Brazil		316	255	43			
USA populations							
Palmer, Kansas (KS)	Maize	-	24	-	39°38′21″ N	97°03′45″ W	08.09.15
Lafayette, Indiana (IN)	Maize	-	24	-	40°23′57″ N	86°51′41″ W	08.26.15
Rosemount, Minnesota (MN)	Maize	-	23	-	44°42′20″ N	93°05′09″ W	08.11.15
Wayne County, North Carolina (NC)	Soybean	-	23	-	35°07′51″ N	78°07′32″ W	08.13.15
Springfield (WARS), Ohio (OH)	Maize	-	24	-	39°51'47" N	83°40′21″ W	09.17.15
Total USA		-	118	-			
Total Brazil and USA		316	373	-			

Appendix B - Summary of genetic variation of 10 microsatellite loci at 17 locations for *H. armigera* populations of Brazil.

(To be continue)

Population		HzMS1-4	HzMS3-11	HzMS3-41	HaB60	HaC87	HaC14	HarSSR1	HarSSR9	HarSSR2	HarSSR3	Average
	A	3.000	2.000	3.000	3.000	2.000	3.000	11.000	6.000	1.000	5.000	3.900
	AR	2.489	1.985	2.691	2.076	1.333	2.929	7.033	3.606	1.000	3.459	2.860
BA44	H_0	0.263	0.400	0.894	0.250	0.056	0.368	0.750	0.400	0.000	0.450	0.383
	H_E	0.325	0.385	0.568	0.229	0.056	0.590	0.888	0.567	0.000	0.564	0.417
	f	0.196	-0.041	-0.602	-0.092	0.000	0.382	0.159	0.300	0.000	0.206	0.084
	NA	0.066	0.000	0.000	0.000	0.000	0.137	0.052	0.132	0.001	0.091	
	A	3.000	3.000	3.000	4.000	2.000	5.000	10.000	6.000	4.000	5.000	4.500
	AR	2.366	2.811	2.076	2.819	1.316	4.257	6.279	3.988	1.900	3.498	3.131
GO2	H_0	0.350	0.353	0.250	0.381	0.053	0.524	0.714	0.714	0.150	0.411	0.390
	H_E	0.308	0.501	0.229	0.403	0.053	0.744	0.859	0.711	0.146	0.547	0.450
	f	-0.137	0.302	-0.092	0.056	0.000	0.302	0.172	-0.005	-0.027	0.235	0.137
	NA	0.000	0.100	0.000	0.018	0.000	0.132	0.064	0.013	0.000	0.030	
	A	3.000	4.000	2.000	4.000	2.000	4.000	6.000	8.000	1.000	4.000	3.800
	AR	2.705	3.235	2.000	3.333	1.853	3.97	5.34	5.804	1.000	3.053	3.229
RS2	H_0	0.400	0.333	0.800	0.889	0.000	0.444	0.500	0.800	0.000	0.300	0.447
	H_E	0.358	0.399	0.505	0.608	0.189	0.778	0.821	0.742	0.000	0.363	0.476
	f	-0.125	0.172	-0.636	-0.505	1.000	0.443	0.404	-0.083	0.000	0.182	0.066
	NA	0.000	0.000	0.000	0.000	0.204	0.171	0.166	0.000	0.001	0.000	
	A	3.000	2.000	2.000	3.000	4.000	4.000	9.000	6.000	1.000	4.000	3.800
	AR	2.642	1.993	1.999	2.500	3.799	3.857	7.600	5.436	1.000	3.493	3.432
BA25	H_0	0.444	0.375	0.555	0.250	0.000	0.428	0.875	0.375	0.000	0.250	0.355
	H_E	0.385	0.325	0.424	0.242	0.653	0.736	0.891	0.816	0.000	0.516	0.499
	f	-0.164	-0.167	-0.333	-0.037	1.000	0.438	0.020	0.558	0.000	0.533	0.302

Appendix B - Summary of genetic variation of 10 microsatellite loci at 17 locations for *H. armigera* populations of Brazil.
(Continuation)

Population		HzMS1-4	HzMS3-11	HzMS3-41	HaB60	HaC87	HaC14	HarSSR1	HarSSR9	HarSSR2	HarSSR3	Average
BA25	***NA***	0.000	0.000	0.000	0.000	0.387	0.149	0.000	0.209	0.001	0.146	
	A	5.000	4.000	3.000	3.000	5.000	4.000	9.000	7.000	3.000	2.000	4.500
	AR	3.017	2.653	2.030	2.008	2.438	3.447	6.421	4.422	1.719	1.995	3.015
BA34	H_0	0.333	0.500	0.250	0.227	0.086	0.416	0.739	0.708	0.130	0.541	0.393
	H_E	0.461	0.431	0.227	0.210	0.244	0.618	0.870	0.718	0.126	0.438	0.435
	f	0.282	-0.162	-0.099	-0.082	0.649	0.331	0.154	0.014	-0.031	-0.241	0.097
	NA	0.076	0.000	0.000	0.000	0.172	0.116	0.052	0.034	0.000	0.000	
	A	5.000	3.000	3.000	3.000	3.000	5.000	11.000	7.000	1.000	5.000	4.600
	AR	3.372	2.367	2.561	2.724	1.951	3.754	6.824	3.469	1.000	2.561	3.058
BA49	H_0	0.347	0.291	0.583	0.727	0.000	0.478	0.916	0.521	0.000	0.142	0.401
	H_E	0.494	0.296	0.451	0.524	0.173	0.595	0.882	0.535	0.000	0.265	0.422
	f	0.302	0.018	-0.301	-0.400	1.000	0.200	-0.040	0.026	0.000	0.469	0.051
	NA	0.083	0.000	0.000	0.000	0.202	0.059	0.000	0.000	0.001	0.115	
	A	5.000	2.000	1.000	2.000	1.000	3.000	9.000	7.000	1.000	4.000	3.500
	AR	3.902	1.950	1.000	1.750	1.000	3.000	7.443	5.872	1.000	3.846	3.076
MA4	H_0	0.333	0.000	0.000	0.125	0.000	0.166	0.875	0.666	0.000	0.428	0.259
	H_E	0.483	0.233	0.000	0.125	0.000	0.530	0.883	0.836	0.000	0.648	0.374
	f	0.324	1.000	0.000	0.000	0.000	0.706	0.010	0.213	0.000	0.357	0.320
	NA	0.116	0.224	0.001	0.000	0.001	0.202	0.000	0.074	0.001	0.094	
	A	4.000	3.000	2.000	2.000	1.000	3.000	10.000	2.000	1.000	1.000	2.900
	AR	2.799	2.400	1.993	1.429	1.000	2.994	7.090	1.462	1.000	1.000	2.317
BA27	H_0	0.400	1.000	0.533	0.071	0.000	0.333	0.857	0.076	0.000	0.000	0.327
	H_E	0.544	0.549	0.404	0.071	0.000	0.660	0.894	0.076	0.000	0.000	0.320

Appendix B - Summary of genetic variation of 10 microsatellite loci at 17 locations for *H. armigera* populations of Brazil.
(Continuation)

Population		HzMS1-4	HzMS3-11	HzMS3-41	HaB60	HaC87	HaC14	HarSSR1	HarSSR9	HarSSR2	HarSSR3	Average
BA27	***f***	0.273	-0.875	-0.333	0.000	0.000	0.510	0.043	0.000	0.000	0.000	-0.026
	NA	0.078	0.000	0.000	0.000	0.001	0.187	0.030	0.000	0.001	0.001	
BA30	***A***	2.000	3.000	3.000	2.000	2.000	3.000	11.000	3.000	2.000	3.000	3.400
	AR	1.250	2.441	2.773	1.776	1.988	2.728	6.492	2.213	1.993	2.762	2.642
	H_0	0.041	1.000	0.583	0.200	0.291	0.041	0.954	0.136	0.318	0.541	0.411
	H_E	0.041	0.549	0.465	0.184	0.403	*0.440**	0.864	0.341	0.426	0.520	0.424
	f	0.000	-0.852	-0.260	-0.086	0.281	0.907	-0.107	0.606	0.258	-0.042	0.031
	NA	0.000	0.000	0.000	0.000	0.083	0.294	0.000	0.173	0.076	0.000	
BA33	***A***	3.000	2.000	1.000	3.000	2.000	5.000	8.000	3.000	1.000	5.000	3.300
	AR	2.232	2.000	1.000	1.992	1.316	4.053	5.727	2.284	1.000	3.343	2.495
	H_0	0.368	1.000	0.000	0.187	0.052	0.388	0.833	0.210	0.000	0.315	0.336
	H_E	0.317	0.513	0.000	0.179	0.052	0.628	0.831	0.382	0.000	0.448	0.335
	f	-0.167	-1.000	0.000	-0.047	0.000	0.388	-0.002	0.457	0.000	0.301	-0.001
	NA	0.000	0.000	0.001	0.000	0.000	0.120	0.013	0.120	0.001	0.124	
MS3	***A***	5.000	2.000	2.000	3.000	2.000	6.000	14.000	4.000	3.000	5.000	4.600
	AR	3.576	2.000	1.860	2.182	1.720	4.626	9.326	2.902	1.923	3.913	3.403
	H_0	0.461	1.000	0.230	0.230	0.000	0.461	1.000	0.153	0.153	0.500	0.419
	H_E	0.507	0.520	0.212	0.218	0.147	0.778	0.956	0.347	0.150	0.547	0.439
	f	0.094	-1.000	-0.091	-0.059	1.000	0.417	-0.047	0.568	-0.021	0.090	0.046
	NA	0.000	0.000	0.000	0.000	0.182	0.154	0.000	0.150	0.000	0.000	
BA7	***A***	3.000	3.000	3.000	2.000	5.000	4.000	11.000	5.000	1.000	5.000	4.200
	AR	2.008	2.388	2.141	1.925	2.999	3.159	7.354	3.816	1.000	3.243	3.003
	H_0	0.227	0.363	0.238	0.238	0.227	0.400	0.545	0.190	0.000	0.400	0.283

Appendix B - Summary of genetic variation of 10 microsatellite loci at 17 locations for *H. armigera* populations of Brazil.
(Continuation)

Population		HzMS1-4	HzMS3-11	HzMS3-41	HaB60	HaC87	HaC14	HarSSR1	HarSSR9	HarSSR2	HarSSR3	Average
	H_E	0.210	0.347	0.221	0.284	0.363	0.532	0.901	*0.695**	0.000	0.533	0.409
BA7	***f***	-0.082	-0.047	-0.075	0.167	0.381	0.253	0.401	0.731	0.000	0.255	0.313
	NA	0.000	0.000	0.000	0.044	0.135	0.104	0.179	0.294	0.001	0.104	
	A	3.000	2.000	3.000	4.000	3.000	5.000	12.000	5.000	1.000	4.000	4.200
	AR	2.003	1.679	2.139	1.750	2.106	3.965	6.773	3.712	1.000	2.499	2.763
BA3	H_0	0.240	0.160	0.250	0.125	0.080	0.650	0.760	0.450	0.000	0.375	0.309
	H_E	0.219	0.150	0.230	0.122	0.222	0.696	0.873	0.675	0.000	0.529	0.372
	f	-0.095	-0.067	-0.087	-0.022	0.644	0.068	0.132	0.340	0.000	0.296	0.172
	NA	0.000	0.000	0.000	0.000	0.160	0.000	0.060	0.126	0.001	0.085	
	A	2.000	3.000	3.000	3.000	3.000	5.000	12.000	3.000	4.000	6.000	4.400
	AR	1.988	2.177	2.261	1.643	1.919	3.062	7.355	2.230	2.019	2.744	2.739
BA13	H_0	0.291	0.464	0.285	0.115	0.200	0.400	0.777	0.192	0.037	0.250	0.301
	H_E	0.403	0.370	0.259	0.112	0.186	0.410	0.899	0.506	0.176	0.320	0.365
	f	0.281	-0.258	-0.102	-0.027	-0.071	0.026	0.137	0.624	0.794	0.224	0.176
	NA	0.083	0.000	0.000	0.000	0.000	0.013	0.035	0.204	0.173	0.000	
	A	3.000	2.000	3.000	3.000	4.000	6.000	11.000	6.000	4.000	4.000	4.600
	AR	2.366	2.000	2.337	1.600	2.870	4.436	6.481	3.704	2.331	2.834	3.095
BA20	H_0	0.350	0.625	0.300	0.100	0.300	0.526	0.823	0.600	0.050	0.315	0.400
	H_E	0.308	0.508	0.273	0.098	0.419	0.684	0.857	0.576	0.234	0.485	0.445
	f	-0.137	-0.240	-0.101	-0.013	0.290	0.236	0.041	-0.041	0.791	0.355	0.105
	NA	0.000	0.000	0.000	0.000	0.098	0.109	0.000	0.000	0.195	0.087	
	A	4.000	2.000	3.000	2.000	4.000	4.000	9.000	3.000	2.000	2.000	3.500
	AR	2.992	1.617	2.595	1.797	2.545	3.084	6.510	2.176	1.538	1.940	2.679
BA6	H_0	0.421	0.125	0.421	0.105	0.210	0.333	0.647	0.235	0.105	0.066	0.267

Appendix B - Summary of genetic variation of 10 microsatellite loci at 17 locations for *H. armigera* populations of Brazil.

(Conclusion)

Population		HzMS1-4	HzMS3-11	HzMS3-41	HaB60	HaC87	HaC14	HarSSR1	HarSSR9	HarSSR2	HarSSR3	Average
	H_E	0.438	0.120	0.364	0.193	0.288	0.490	0.868	0.221	0.102	0.287	0.337
BA6	***f***	0.040	-0.034	-0.161	0.462	0.276	0.327	0.261	-0.067	-0.029	0.774	0.213
	NA	0.011	0.000	0.000	0.107	0.097	0.106	0.109	0.000	0.000	0.198	
	A	3.000	2.000	2.000	1.000	3.000	4.000	6.000	4.000	2.000	1.000	2.800
	AR	2.395	1.922	1.429	1.000	2.638	3.283	6.000	2.540	1.683	1.000	2.389
MT6	***H_0***	0.230	0.272	0.071	0.000	0.071	0.416	0.500	0.214	0.000	0.000	0.178
	H_E	0.335	0.246	0.071	0.000	0.415	0.434	0.848	0.267	0.137	0.000	0.276
	f	0.321	-0.111	0.000	0.000	0.833	0.043	0.434	0.204	1.000	0.000	0.368
	NA	0.090	0.000	0.000	0.001	0.260	0.000	0.157	0.000	0.176	0.001	

A, total number of alleles; *AR*, allelic richness, H_O, observed heterozygosity; H_E, expected heterozygosity; *f*, inbreeding coefficient; *NA*, frequency of null alleles. *Italic: deviation from HWE, $p < 0.05$.

Appendix C - Summary of genetic variation of eight microsatellite loci at 12 locations for *H. zea* populations of Brazil.

(To be continue)

Population		HzMS1-4	HzMS3-11	HzMS3-41	HaB60	HarSSR1	HzMS4-16	HzMS3-48	HzMS4-23	Average
MG4	***A***	3.000	2.000	2.000	2.000	4.000	4.000	3.000	3.000	2.888
	AR	2.780	2.000	1.895	1.895	2.892	3.343	2.943	2.895	2.580
	H_0	0.292	0.348	0.167	0.167	0.250	0.565	0.958	0.625	0.421
	H_E	0.327	0.464	0.156	0.156	0.233	0.554	0.598	0.577	0.383
	f	0.110	0.254	-0.070	-0.070	-0.074	-0.021	-0.623	-0.085	-0.102
	NA	0.036	0.076	0.000	0.000	0.000	0.000	0.000	0.000	
SP13	***A***	2.000	2.000	2.000	2.000	4.000	4.000	3.000	3.000	2.750
	AR	1.686	2.000	1.969	1.435	3.486	3.638	2.708	2.988	2.489
	H_0	0.087	0.524	0.238	0.043	0.304	0.565	1.000	0.555	0.415
	H_E	0.085	0.494	0.215	0.043	0.348	0.621	0.554	0.627	0.373
	f	-0.023	-0.062	-0.111	0.000	0.127	0.092	-0.840	0.117	-0.114
	NA	0.000	0.000	0.000	0.000	0.059	0.000	0.000	0.012	
BA32	***A***	2.000	3.000	3.000	3.000	3.000	5.000	3.000	3.000	3.125
	AR	1.417	2.922	2.708	2.208	2.908	4.024	2.952	2.999	2.767
	H_0	0.042	0.500	0.591	0.143	0.609	0.750	0.870	0.917	0.552
	H_E	0.042	0.566	0.449	0.138	0.510	0.652	0.598	0.638	0.449
	f	0.000	0.118	-0.345	-0.034	-0.198	-0.153	-0.469	-0.500	-0.237
	NA	0.000	0.093	0.000	0.000	0.000	0.000	0.000	0.000	
GO1	***A***	2.000	2.000	3.000	2.000	3.000	4.000	3.000	3.000	2.750
	AR	1.983	2.000	2.889	1.909	2.810	3.955	2.897	2.556	2.625
	H_0	0.214	0.688	0.400	0.091	0.889	0.625	1.000	0.611	0.564
	H_E	0.198	0.514	0.349	0.091	0.552	0.716	0.577	0.541	0.442
	f	-0.083	-0.352	-0.151	0.000	-0.639	0.130	-0.780	-0.133	-0.288

Appendix C - Summary of genetic variation of eight microsatellite loci at 12 locations for *H. zea* populations of Brazil.

(Continuation)

Population		HzMS1-4	HzMS3-11	HzMS3-41	HaB60	HarSSR1	HzMS4-16	HzMS3-48	HzMS4-23	Average
GO1	***NA***	0.000	0.000	0.000	0.000	0.000	0.004	0.000	0.000	
	A	3.000	2.000	3.000	2.000	3.000	5.000	3.000	4.000	3.125
	AR	2.527	2.000	2.920	1.500	2.903	4.397	2.959	3.376	2.823
PR1	H_0	0.176	0.364	0.476	0.050	0.526	0.727	0.895	0.773	0.498
	H_E	0.220	0.444	0.400	0.050	0.525	0.668	0.599	0.553	0.433
	f	0.200	0.184	-0.198	0.000	-0.003	-0.091	-0.515	-0.411	-0.158
	NA	0.000	0.053	0.000	0.000	0.000	0.000	0.000	0.000	
	A	3.000	2.000	2.000	2.000	3.000	3.000	3.000	4.000	2.750
	AR	2.976	2.000	2.000	2.000	2.996	2.992	2.983	3.714	2.708
RS1	H_0	0.417	0.333	0.714	0.100	0.545	0.538	0.929	1.000	0.572
	H_E	0.366	0.464	0.476	0.100	0.450	0.612	0.611	0.672	0.469
	f	-0.146	0.290	-0.529	0.000	-0.224	0.125	-0.551	-0.517	-0.232
	NA	0.000	0.082	0.000	0.000	0.000	0.005	0.000	0.000	
	A	3.000	2.000	3.000	2.000	4.000	7.000	3.000	5.000	3.625
	AR	2.661	2.000	2.683	1.829	3.263	5.041	2.435	3.831	2.968
MG1	H_0	0.348	0.695	0.478	0.130	0.435	0.696	0.609	0.682	0.509
	H_E	0.305	0.464	0.389	0.125	0.495	0.695	0.503	0.606	0.448
	f	-0.143	-0.517	-0.234	-0.048	0.124	-0.001	-0.215	-0.129	-0.140
	NA	0.000	0.000	0.000	0.000	0.021	0.000	0.000	0.006	
	A	4.000	2.000	2.000	2.000	2.000	4.000	3.000	4.000	2.875
	AR	2.684	2.000	2.000	1.847	1.998	3.701	2.455	3.163	2.481
MG2	H_0	0.143	0.272	0.545	0.136	0.235	0.762	0.454	0.682	0.404
	H_E	0.182	0.444	0.406	0.130	0.299	0.617	0.527	0.555	0.395

Appendix C - Summary of genetic variation of eight microsatellite loci at 12 locations for *H. zea* populations of Brazil.

(Continuation)

Population		HzMS1-4	HzMS3-11	HzMS3-41	HaB60	HarSSR1	HzMS4-16	HzMS3-48	HzMS4-23	Average
MG2	***f***	0.221	0.391	-0.355	-0.050	0.220	-0.243	0.141	-0.235	-0.023
	NA	0.000	0.119	0.000	0.000	0.058	0.000	0.035	0.000	
MT8	***A***	3.000	2.000	2.000	2.000	4.000	5.000	2.000	4.000	3.000
	AR	2.781	2.000	2.000	1.793	2.000	3.728	2.645	4.034	2.558
	H_0	0.261	0.818	0.545	0.130	0.435	0.727	0.545	0.522	0.498
	H_E	0.308	0.507	0.406	0.125	0.397	0.604	0.474	0.554	0.422
	f	0.157	-0.636	-0.355	-0.048	-0.097	-0.211	-0.156	0.059	-0.186
	NA	0.068	0.000	0.000	0.000	0.000	0.000	0.000	0.000	
SP5	***A***	3.000	2.000	2.000	2.000	2.000	5.000	3.000	5.000	3.000
	AR	2.753	2.000	2.000	1.793	2.000	3.728	2.645	4.034	2.619
	H_0	0.167	0.760	0.542	0.120	0.421	0.875	0.680	0.920	0.561
	H_E	0.297	0.480	0.403	0.115	0.444	0.614	0.547	0.657	0.445
	f	0.444	-0.600	-0.353	-0.043	0.053	-0.438	-0.248	-0.412	-0.267
	NA	0.134	0.000	0.000	0.000	0.008	0.000	0.000	0.000	
BA10	***A***	3.000	2.000	2.000	3.000	6.000	4.000	3.000	3.000	3.250
	AR	2.835	2.000	1.981	2.411	4.914	3.695	2.866	2.994	2.962
	H_0	0.381	0.454	0.272	0.142	0.473	0.500	0.524	0.428	0.397
	H_E	0.333	0.495	0.241	0.219	0.666	0.576	0.577	0.591	0.462
	f	-0.147	0.083	-0.135	0.355	0.294	0.135	0.095	0.280	0.144
	NA	0.000	0.019	0.000	0.092	0.090	0.006	0.067	0.108	

Appendix C - Summary of genetic variation of eight microsatellite loci at 12 locations for *H. zea* populations of Brazil.

(Conclusion)

Population		HzMS1-4	HzMS3-11	HzMS3-41	HaB60	HarSSR1	HzMS4-16	HzMS3-48	HzMS4-23	Average
SP2	***A***	3.000	2.000	2.000	1.000	5.000	3.000	2.000	2.000	2.500
	AR	2.867	2.000	1.998	1.000	4.818	2.897	2.000	2.000	2.448
	H_0	0.333	0.533	0.333	0.000	0.642	0.533	0.571	0.667	0.452
	H_E	0.301	0.515	0.287	0.000	0.574	0.579	0.508	0.460	0.403
	f	-0.111	-0.037	-0.167	0.000	-0.125	0.082	-0.130	-0.475	-0.126
	NA	0.000	0.000	0.000	0.001	0.000	0.001	0.000	0.000	

A, total number of alleles; *AR*, allelic richness, H_O, observed heterozygosity; H_E, expected heterozygosity; *f*, inbreeding coefficient; *NA*, frequency of null alleles. *Deviation from HWE, $p < 0.05$.

Appendix D - Summary of genetic variation of eight microsatellite loci at 12 locations for *H. zea* populations of the USA.

(To be continue)

Population		HzMS1-4	HzMS3-11	HzMS3-41	HaB60	HarSSR1	HzMS4-16	HzMS3-48	HzMS4-23	Average
KS	***A***	4.000	3.000	2.000	3.000	3.000	6.000	3.000	2.000	3.250
	AR	3.874	2.994	1.913	2.986	3.000	5.826	2.875	2.000	3.184
	H_0	0.250	0.087	0.043	0.083	0.286	0.261	0.667	0.458	0.267
	H_E	0.301	0.363	0.043	0.196	0.261	*0.784**	0.531	0.552	0.373
	f	0.171	0.765	0.000	0.580	-0.096	0.672	-0.262	0.090	0.288
	NA	0.060	0.225	0.000	0.116	0.000	0.284	0.000	0.023	
IN	***A***	3.000	2.000	2.000	2.000	4.000	5.000	3.000	3.000	3.000
	AR	3.000	2.000	2.000	1.875	4.000	4.875	2.913	3.000	2.957
	H_0	0.348	0.000	0.522	0.042	0.333	0.458	0.522	0.478	0.338
	H_E	0.308	*0.348**	0.433	0.042	0.368	0.739	0.530	0.552	0.415
	f	-0.132	1.000	-0.211	0.000	0.097	0.385	0.017	0.136	0.189
	NA	0.000	0.277	0.000	0.000	0.029	0.145	0.000	0.029	
MN	***A***	4.000	2.000	2.000	2.000	7.000	4.000	4.000	2.000	3.375
	AR	3.826	2.000	2.000	1.913	6.817	3.994	3.913	2.000	3.308
	H_0	0.217	0.000	0.783	0.043	0.409	0.261	0.521	0.478	0.339
	H_E	0.241	0.169	0.487	0.043	0.485	*0.702**	0.590	0.511	0.404
	f	0.098	1.000	-0.630	0.000	0.160	0.633	0.119	0.066	0.163
	NA	0.000	0.195	0.000	0.000	0.081	0.257	0.014	0.014	

Appendix D - Summary of genetic variation of eight microsatellite loci at 12 locations for *H. zea* populations of the USA.

(Conclusion)

Population		HzMS1-4	HzMS3-11	HzMS3-41	HaB60	HarSSR1	HzMS4-16	HzMS3-48	HzMS4-23	Average
NC	***A***	3.000	3.000	3.000	1.000	5.000	5.000	2.000	4.000	3.250
	AR	2.994	2.994	2.913	1.000	4.994	4.994	2.000	3.826	3.214
	H_0	0.174	0.000	0.522	0.000	0.522	0.391	0.304	0.565	0.310
	H_E	0.204	*0.240**	0.405	0.000	0.550	*0.733**	0.507	0.550	0.399
	f	0.150	1.000	-0.297	0.000	0.052	0.472	0.405	-0.029	0.227
	NA	0.000	0.237	0.000	0.001	0.000	0.194	0.129	0.000	
OH	***A***	3.000	2.000	2.000	2.000	2.000	4.000	2.000	2.000	2.375
	AR	2.826	2.000	2.000	1.875	2.000	4.000	2.000	2.000	2.338
	H_0	0.087	0.000	0.043	0.042	0.182	0.250	0.542	0.583	0.216
	H_E	0.086	*0.433**	0.125	0.042	0.241	*0.719**	0.503	0.454	0.325
	f	-0.011	1.000	0.656	0.000	0.250	0.657	-0.079	-0.293	0.340
	NA	0.000	0.308	0.121	0.000	0.065	0.271	0.000	0.000	

A, total number of alleles; *AR*, allelic richness, H_O, observed heterozygosity; H_E, expected heterozygosity; *f*, inbreeding coefficient; *NA*, frequency of null alleles. *Deviation from HWE, $p < 0.05$.

Appendix E - Matrix of F_{st} values for each pairwise combination of 17 *H. armigera* populations based on 10 microsatellite loci.[a]

Population	BA44	GO2	RS2	BA25	BA34	BA49	MA4	BA27	BA30	BA33	MS3	BA7	BA3	BA13	BA20	BA6
GO2	0.028															
RS2	0.033	0.038														
BA25	0.037	0.022	0.021													
BA34	0.016	0.006	0.046	0.022												
BA49	0.021	**0.036**	0.003	0.046	0.027											
MA4	0.050	0.000	0.069	0.000	0.009	0.052										
BA27	0.072	0.120	0.110	0.144	0.091	0.078	0.182									
BA30	**0.075**	**0.107**	**0.114**	0.116	**0.076**	**0.101**	0.140	0.104								
BA33	**0.064**	**0.063**	**0.107**	0.118	**0.039**	**0.074**	0.098	0.056	**0.055**							
MS3	0.057	0.052	**0.077**	0.087	**0.053**	**0.074**	0.100	0.027	**0.070**	0.028						
BA7	0.017	0.006	**0.056**	0.019	0.002	**0.034**	-0.002	0.120	**0.089**	**0.059**	**0.076**					
BA3	0.032	0.019	**0.070**	0.042	0.007	**0.055**	0.016	0.142	**0.105**	**0.072**	0.077	0.009				
BA13	**0.051**	**0.058**	**0.086**	0.087	0.028	**0.049**	0.048	0.069	**0.080**	0.044	**0.074**	0.031	0.041			
BA20	**0.042**	**0.050**	**0.068**	0.049	**0.030**	**0.061**	0.053	0.072	**0.041**	0.018	0.033	**0.039**	**0.056**	0.040		
BA6	**0.054**	**0.100**	**0.084**	0.098	**0.056**	**0.041**	0.098	0.085	**0.090**	**0.079**	**0.087**	**0.056**	**0.077**	0.040	0.041	
MT6	0.086	0.103	0.128	0.115	0.053	0.064	0.116	0.101	0.107	0.067	0.100	0.064	0.092	0.043	0.043	0.009

[a]Significant values are indicated in bold ($p < 0.001$).

Appendix F - Matrix of F_{st} values for each pairwise combination of 12 *H. zea* populations from Brazil based on eight microsatellite loci.[a]

Population	MG4	SP13	BA32	GO1	PR1	RS1	MG1	MG2	MT8	SP5	BA10
SP13	-0.004										
BA32	**0.031**	0.014									
GO1	0.039	0.027	0.023								
PR1	0.013	0.013	-0.001	0.007							
RS1	0.022	0.016	-0.004	0.032	0.005						
MG1	**0.023**	0.016	**0.025**	**0.045**	0.020	0.008					
MG2	0.004	0.003	**0.021**	**0.042**	**0.012**	0.008	0.004				
MT8	0.021	0.013	**0.037**	**0.044**	**0.036**	0.015	-0.004	0.006			
SP5	**0.026**	**0.019**	**0.022**	**0.054**	**0.025**	0.009	-0.005	-0.003	0.006		
BA10	0.019	0.018	**0.033**	0.035	**0.018**	**0.030**	0.005	0.010	0.019	0.003	
SP2	0.026	0.013	**0.033**	0.043	**0.027**	0.021	0.000	0.017	0.000	0.013	0.007

[a]Significant values are indicated in bold ($p < 0.001$).

Appendix G - Matrix of F_{st} values for each pairwise combination of five *H. zea* populations from the USA based on eight microsatellite loci.[a]

Population	KS	IN	MN	NC
IN	0.0157			
MN	0.0442	-0.0134		
NC	**0.0179**	-0.0039	-0.0029	
OH	0.0008	0.0268	0.0555	0.0156

[a]Significant values are indicated in bold ($p < 0.001$).

5 SUSCEPTIBILITY TO Vip3Aa20 IN BRAZILIAN POPULATIONS OF *Helicoverpa armigera* AND *Helicoverpa zea* (LEPIDOPTERA: NOCTUIDAE)

Abstract

The cultivation of maize events that express the Vip3Aa20 insecticidal protein is increasing in Brazil. To evaluate the susceptibility of *Helicoverpa armigera* (Hübner) and *Helicoverpa zea* (Boddie) to Vip3Aa20, as a part of an Insect Resistance Management (IRM) program, we characterized the baseline susceptibility and validated a diagnostic concentration for resistance monitoring. Diet-overlay bioassays were conducted with neonates exposed to Vip3Aa20 for seven days. The baseline susceptibility data was obtained for seven field populations of *H. armigera* and six of *H. zea* collected from major soybean-, cotton-, and maize-producing areas in Brazil. To validate the diagnostic concentration, 11 field populations of *H. zea* were tested from 2014 to 2015. The LC_{50} for *H. armigera* populations ranged from 2.97 to 8.41 µg Vip3Aa20/cm^2 (3-fold variation), and for *H. zea* populations from 0.04 to 0.21 µg Vip3Aa20/cm^2 (5-fold variation). The EC_{50} for *H. armigera* ranged from 0.099 to 0.455 µg Vip3Aa20/cm^2 (5-fold variation), and for *H. zea* from 0.004 to 0.020 µg Vip3Aa20/cm^2 (5-fold variation). *H. armigera* was more tolerant to Vip3Aa20 protein than *H. zea* (≈ 40 to 75-fold, based on LC_{50}). Based on the LC_{99} value, the concentration of 6.4 µg Vip3Aa20/cm^2 was defined as a diagnostic concentration for susceptibility monitoring in *H. zea*. A diagnostic concentration was not validated for *H. armigera* because of the high amount of protein needed for bioassays. Our baseline susceptibility data to Vip3Aa20 in *H. armigera* and *H. zea* populations will be important in IRM programs in Brazil.

Keywords: Old world bollworm; Corn earworm; Vip3A; *Bt* maize; Resistance management

5.1 Introduction

Vip3Aa20 maize was released in Brazil to control three pests: *Spodoptera frugiperda* (J. E. Smith) (Lepidoptera: Noctuidae), *Diatraea saccharalis* (Fabr.) (Lepidoptera: Crambidae) and *Helicoverpa zea* (Boddie) (Lepidoptera: Noctuidae). These species were considered the major target pests of genetically modified maize expressing *Bacillus thuringiensis* (Berliner) (*Bt*) genes until the detection of *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae) in 2013 (CZEPACK et al., 2013; SPECHT et al., 2013), another pest that also attacks maize (JALLOW; CUNNINGHAM; ZALUCKI, 2004). However, *H. zea* predominates in maize and *H. armigera* in dicotyledonous hosts in Brazil (LEITE et al., 2014). In addition, their larvae feed on both vegetative and reproductive tissues of different crops, causing significant economic losses, even at low population densities (MITTER; POOLE;

MATTHEWS, 1993). In 2012/13, *H. armigera* caused a loss of more than US$1 billion to the Brazilian agriculture due to direct yield losses and resources spent on phytosanitary products in grains and fibers (MAPA, 2015). Hence, this pest is now one of the most important pest species to the Brazilian agriculture (MAPA, 2014), and is also a new Heliothinae target to Vip3Aa20 maize. Therefore, the risk of resistance evolution in *H. zea* and *H. armigera* populations to Vip3Aa20 protein in Brazil should be investigated.

Field-evolved resistance to genetically modified crops expressing proteins from *Bacillus thuringiensis* (Berliner) (*Bt*) has been widely reported. In *Busseola fusca* (Fuller) (Lepidoptera: Noctuidae) to Cry1Ab maize in South Africa (VAN RENSBURG, 2007), in *S. frugiperda* to Cry1F maize in Puerto Rico (STORER et al., 2010), in *Pectinophora gossypiella* (Saunders) (Lepidoptera: Gelechiidae) to Cry1Ac cotton in India (DHURUA; GUJAR, 2011), and in *Diabrotica virgifera virgifera* (Coleoptera: Chrysomelidae) to Cry3Bb1 maize in USA (GASSMANN et al., 2011). Also in *H. armigera* to Cry1Ac cotton in Pakistan (ALVI et al., 2012), and in *H. zea* to Cry1Ac and Cry2Ab in the USA (TABASHNIK et al., 2008; TABASHNIK; CARRIÈRE, 2010). However, there are discussions about field-evolved resistance in *H. zea*, due to the fact that these reports were based on resistance monitoring data, and not on control failures in the field.

In the Brazilian cropping systems, resistance can evolve very quickly. In less than five years, *S. frugiperda* evolved resistance in the field to Cry1F maize (FARIAS et al., 2014), and to Cry1Ab maize in Brazil (OMOTO et al., 2016). The adoption of *Bt* crops in this country, such as maize, cotton and soybean was on 32.5% of the total area (13.8 million hectare) in 2014-2015 (CÉLERES, 2015). Considering only maize, 82.7% of the area (12.5 million hectare) was cultivated with *Bt* technology (CÉLERES, 2015). This high adoption of *Bt* crops in an intense crop production system increases selection pressure on pest populations, and consequently the risk of resistance evolution. All reported field evolved resistance cases were to Cry proteins. No field-evolved resistance was reported to the Vip3A protein group.

The presence of Vip3A resistance alleles has been reported under laboratory studies. A laboratory resistant strain of *Chloridea virescens* (F.) (Lepidoptera: Noctuidae) has already been selected with Vip3A (> 1,000 fold) (GULZAR et al., 2012). In Australia, a high frequency (0.027) of resistance alleles in field populations of *H. armigera* was reported before the commercial release of Vip3A cotton (MAHON;

DOWNES; JAMES, 2012). In this context, the knowledge of the susceptibility of *H. armigera* and *H. zea* populations to Vip3Aa20 protein is necessary to support IRM programs in order to delay resistance evolution in Brazil.

Vip3Aa20 maize hybrids (MIR162 event) were first commercialized in Brazil in 2009. In 2010, maize hybrids that express Vip3Aa20 and Cry1Ab (Bt11×MIR162×mEPSPS) were released. Recently, in 2015, pyramided maize that express Vip3Aa20, Cry1F (TC1507) and Cry1Ab (MON810, Bt11) were launched (CTNBIO, 2014). Vip3A insecticidal proteins are an alternative to Cry proteins for IRM. They are produced by *B. thuringiensis* in the vegetative stage of growth, while Cry proteins are produced in the sporulation stage, and the mode of action of VIP3A proteins is different from the Cry proteins (SELVAPANDIYAN et al., 2001; LEE et al., 2003). Hence, there is no cross-resistance between these insecticidal proteins in insects (JACKSON et al., 2007; SENA; HERNANDEZ-RODRIGUEZ; FERRÃO, 2009; KURTZ, 2010; GULZAR et al., 2012). In Brazil, the adoption of Vip3Aa20 maize is still low (BERNARDI et al., 2015). However, its cultivation will tend to increase in next few years with the introgression of this event in other maize hybrids. Therefore, in this study, we established the baseline susceptibility of Brazilian populations of *H. armigera* and *H. zea* to Vip3Aa20 insecticidal protein, and validated the diagnostic concentration for resistance monitoring.

5.2 Materials and methods

5.2.1 Populations

To establish baseline susceptibility data in field populations of *H. armigera* and *H. zea*, larvae were collected in distinct geographic regions of Brazil (> 85 larvae/location) (Tables 5.1 and 5.2, and Figure 5.1a and 5.1b). *H. armigera* were collected in different hosts; while *H. zea* were sampled only on maize. After the collections, *H. armigera* and *H. zea* larvae were transported to the laboratory and were individually placed in 50 mL plastic cups with 20 mL of artificial diet proposed by Greene; Lepla and Dickerson (1976). However, in this study pinto beans were substituted for white beans. The plastic cups were sealed with an acrylic sheet, and the larvae remained in the cups until pupation. Pupae were placed in cylindrical PVC

cages (40 cm diameter × 60 cm), ~50 pupae/cage, lined with newsprint and closed at the top and bottom with tulle fabric. Adult food was a solution of 10% honey in a plastic cup (50 mL) which was plugged with water absorbent cotton. Eggs were collected every two days and stored in plastic containers (500 mL) with filter paper moistened with distilled water. The neonate larvae (< 24 h) were inoculated in plastic cups (100 mL) with 20 mL of artificial diet, and at third instar larvae were placed individually in plastic cups (50 mL) with 20 mL of artificial diet and sealed with an acrylic sheet, and remained in the cups until pupation. Procedures were repeated each generation. Insects were reared at 25 ± 1°C, 60 ± 10% relative humidity, and 14:10 h (L:D) photoperiod.

5.2.2 Baseline susceptibility

To characterize the baseline susceptibility of Brazilian populations of *H. armigera* and *H. zea* to Vip3Aa20 protein, bioassays were conducted with seven populations of *H. armigera* and six populations of *H. zea* (Table 5.1 and Figure 5.1a). The Vip3Aa20 insecticidal protein was provided by Syngenta Seeds Ltd. (Uberlândia, Brazil) with 86.5% purity and stored in a freezer at - 80°C. For the diet-overlay bioassays, we used the artificial diet proposed by Greene; Lepla and Dickerson (1976), but with white beans. After preparation, the diet was poured on the bioassay trays (BIO-BA-128, CD International Inc., Pitman, NJ), containing 128-wells (1 mL per well). Afterwards, Vip3Aa20 protein was diluted in distilled water to prepare the different concentrations to be tested. Triton X-100 at 0.1% was added to obtain a uniform spread of the solution over the diet surface. The control treatment was composed of distilled water + surfactant. For each population of *H. armigera* and *H. zea,* seven to 14 concentrations of Vip3Aa20 were tested, which were applied on the diet surface with a replication pipette (30 µl per well) (four to eight replicates of 16 larvae/concentration). The diet surface area in each well was 1.5 cm^2. Vip3Aa20 concentrations ranged from 36 to 36,000 ng/cm^2 for *H. armigera*, and from 3.6 to 3,600 ng/cm^2 for *H. zea* to enable mortality from 10 to >90%. After a drying period, one neonate larvae (0 - 24 h old) of *H. armigera* or *H. zea* was added to each well using a fine brush. The trays were sealed with self-adhesive plastic sheets (BIO-CV-16, CD International Inc.) that allow for gas exchange with the external environment and then placed in a climatic chamber (temperature 27 ± 1°C, 60 ± 10% RH, and a

photoperiod of 14:10 h [L:D]). The biological activity of the Vip3Aa20 insecticidal protein was assessed after seven days. The mortality (with larvae that did not go beyond the first instar also being considered dead) and weight of the surviving larvae was used as response criteria.

Table 5.1 – Field populations of *H. armigera* and *H. zea* used to establish the baseline susceptibility data to Vip3Aa20 insecticidal protein.

Population Code	Crop	n[a]	City, State	Latitude	Longitude	Date
H. armigera						
BA16	Bean	118	Luís Eduardo Magalhães,BA	12°05'58" S	45°47'54" W	Sept. 2012
BA25	Soybean	104	São Desidério,BA	12°21'08" S	44°59'03" W	Jan. 2013
BA45	Cotton	97	São Desidério,BA	12°21'08" S	44°59'03" W	Feb. 2014
MS5	Cotton	285	Costa Rica,MS	18°32'38" S	53°07'45" W	Oct. 2013
GO2	Soybean	600	Mineiros,GO	17°34'10" S	52°33'04" W	Jan. 2014
MT9	Maize	200	Itiquira,MT	17°07'35" S	54°31'02" W	May 2014
BA49	Maize	200	Correntina,BA	13°45'03" S	46º16'07" W	Apr. 2014
H. zea						
GO1	Maize	85	Montividiu,GO	17°19'19" S	51°14'51" W	May 2013
RS1	Maize	152	Passo Fundo,RS	28°16'08" S	52°37'15" W	Jan. 2013
PR1	Maize	98	Rolândia,PR	23°19'13" S	51°29'01" W	Jan. 2013
SP5	Maize	87	Cândido Mota,SP	22°44′46″ S	50°23'15" W	Jan. 2013
MG2	Maize	102	Capitólio,MG	20°36′17″ S	46°04'19" W	Mar. 2013
BA32	Maize	113	Luís Eduardo Magalhães,BA	12°05'58" S	45°47'54" W	Jun. 2013

[a] n = Number of insects collected

5.2.3 Validation of diagnostic concentration of Vip3Aa20 to *H. zea*

To validate a diagnostic concentration for monitoring the susceptibility of *H. zea* populations to Vip3Aa20 protein, we used 11 populations collected in maize fields, in 2014 and 2015 (Table 5.2 and Figure 5.1b). The bioassay procedure for resistance monitoring program was identical to the previously described. The diagnostic concentration of the Vip3Aa20 insecticidal protein was defined from the joint analysis of the baseline susceptibility data. In the bioassays we used 1,024

neonates per population (64 replications of 16 neonates in the diagnostic concentration treatment and four replications of 16 neonates in the control treatment). The mortality and larval weight was assessed after seven days, using the same criteria described above.

Table 5.2 – Identification, site, and date of collection of *H. zea* populations used to validate a diagnostic concentration of the Vip3Aa20 insecticidal protein for resistance monitoring.

Population code	n[a]	City, State	Latitude	Longitude	Date
2014 year					
SP10	250	Cândido Mota,SP	22°44'46"S	50º23'15"W	Jan. 2014
SP12	200	Jaboticabal,SP	21°15'17"S	48°19'20"W	Jan. 2014
PR3	220	Londrina,PR	23°18'37"S	51°09'46"W	Jan. 2014
MG4	200	Capitólio,MG	20°36'17"S	46°04'19"W	Feb. 2014
SP14	200	Casa Branca,SP	21°42'17"S	46°59'09"W	May 2014
PR4	200	Castro,PR	24°47'34"S	49°53'58"W	May 2014
2015 year					
SP16	463	Eng. Coelho,SP	22°29'18"S	47°12'57"W	Mar. 2015
RS3	880	Carazinho,RS	28°16'42"S	52°45'59"W	Apr. 2015
SP17	330	Eng. Coelho,SP	22°29'18"S	47°12'57"W	May 2015
MT14	1000	Campo Verde,MT	15°32'44"S	55°09'59"W	May 2015
MG5	126	Capitólio,MG	20°36'17"S	46°04'19"W	Jun. 2015

[a]n = Number of insects collected

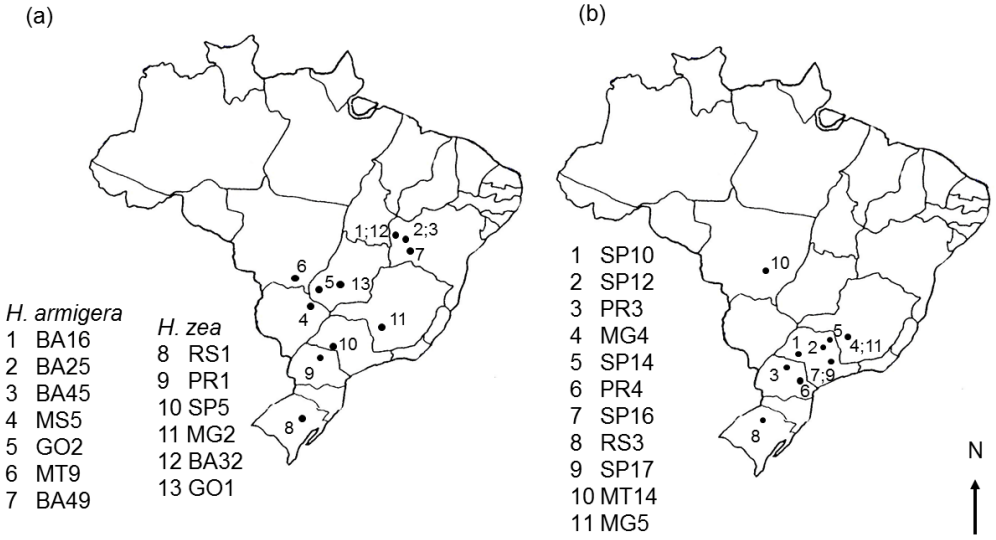

Figure 5.1 - Field populations of *H. armigera* and *H. zea*; (a) collected to establish the baseline susceptibility data to Vip3Aa20 insecticidal protein. (b) Field populations of *H. zea* used to validate a diagnostic concentration to monitor *H. zea* susceptibility to Vip3Aa20.

5.2.4 Statistical analysis

To estimate the LC_{50} (lethal concentration that kills 50% of the insects) and the respective CI's (confidence intervals), the concentration-mortality data of each population were submitted to Probit analysis (PROC PROBIT, SAS Institute 2002). Weight from all surviving insects was analyzed with nonlinear regression analysis to estimate EC_{50} (effective concentration required to cause 50% growth inhibition) and to estimate the mean EC_{90} (effective concentration required to cause 90% growth inhibition) of all populations, JMP SAS® (SAS Institute 2012). The nonlinear logistic models used for the computation of EC_{50} (Sims et al. 1996) and EC_{90} (adapted from Sims et al. 1996) were, respectively:

$$\text{Weight} = W_0/[1+ (\text{concentration}/EC_{50})^B]$$

$$\text{Weight} = W_0 / [1 + (\text{concentration} / EC_{50})]^{[\log 9/(\log (EC50 / EC90))]}$$

Where W_0 is the expected control weight, concentration is the amount of Vip3Aa20 protein/cm^2 of diet, and B is the logistic function slope parameter (SIMS et al., 1996).

To estimate the diagnostic concentration, the concentration-mortality data of all populations were analyzed jointly, according to the method proposed by SIMS et al. (1996). In the joint analysis, mortality data were fitted with a binomial model using the log-log complement connection function (gompit) in SAS 9.1 (PROC PROBIT, SAS INSTITUTE INC., 2004) to obtain LC_{99}. The diagnostic concentration for the resistance monitoring of *H. zea* to Vip3Aa20 insecticidal protein were designated based on ~2 times the estimate LC_{99} value. The monitoring data were analyzed by estimating the 95% CI's on the probability of success of survival in a binomial distribution analysis. This analysis was performed using the function *binom.probit* from the package *binom* (DORAI-RAJ, 2009) in R 2.15.1 (R DEVELOPMENT CORE TEAM, 2012). Survival data at diagnostic concentration were considered significantly different when their 95% CI_s did not overlap. The weight of surviving larvae was used to calculate the growth inhibition. Growth inhibition (GW) was calculated with the equation adapted from Abbott (1925):

$$GW\ (\%) = 100*(X - Y)/X$$

Where X = weight of the larvae in the untreated treatment, and Y = weight of the larvae of the treated treatment. The growth inhibition data were submitted to the Kruskal-Wallis test (BRESLOW, 1970), and means were compared with the multiple comparison test of Kruskal-Wallis in R 2.15.1 (R DEVELOPMENT CORE TEAM, 2012).

5.3 Results

5.3.1 Baseline susceptibility

H. armigera populations were more tolerant to Vip3Aa20 protein than *H. zea* populations (≈ 40 to 75-fold, based on LC_{50}) (Table 5.3). Considering the populations of both species collected in the same states and crop (maize) the variation was 21-fold in the LC_{50} between BA49 (*H. armigera*) and BA32 (*H. zea*). For the populations sampled in Goiás state, GO2 (*H. armigera*) and GO1 (*H. zea*), collected on soybean and maize, respectively this difference were higher 41-fold. The interpopulation variation in the LC_{50} was low for both species. For the seven populations of *H. armigera* the variation was ~3-fold, ranging from 2.9 (population MT9) to 8.4 (population GO2) µg Vip3Aa20/cm^2. For the six populations of *H. zea* the variation was ~5-fold, ranging from 0.040 (population MG2) to 0.207 (population GO1) µg Vip3Aa20/cm^2. Estimated EC_{50} values ranged from 0.099 (population BA25) to 0.455 (population BA49) µg Vip3Aa20/cm^2 in *H. armigera*, and from 0.004 (population BA25) to 0.017 (population BA49) ng Vip3Aa20/cm^2 in *H. zea*. Susceptibility variation among populations in each species was ~ 5-fold as indicated by the EC_{50}.

By the joint analysis of *H. zea* populations, the LC_{99} was estimated to be 2.7 [CI 95% (2.1 – 3.5)] µg Vip3Aa20/cm^2 (*n* = 2,956; Slope (±SE) = 1.38 (±0.06); χ^2 = 7.44; df = 7), and the EC_{90} was estimated to be 0.6 [CI 95% (0.3 – 1.4)] µg Vip3Aa20/cm^2 (*n* = 1,532). From the LC_{99}, the candidate diagnostic concentration of 6.4 µg Vip3Aa20/cm^2 (~2 times the LC_{99}) was designated for the resistance monitoring of *H. zea* to Vip3Aa20 insecticidal protein. The joint analysis of *H. armigera* populations estimated a high concentration for the LC_{99} of 44 [CI 95% (34 –

60)] µg Vip3Aa20/cm^2 (*n* = 3,758; Slope (±SE) = 1.93 (±0.11); χ^2 = 15.91; df = 7) and for the EC_{90} of 3.0 [CI 95% (1.0 – 10)] µg Vip3Aa20/cm^2 (*n* = 2,209).

Table 5.3 – Lethal concentration (LC_{50}; ng/cm^2) and effective concentration (EC_{50}; µg/cm^2) of Vip3Aa20 insecticidal protein to *H. armigera* and *H. zea* neonates.

Pop. Code	Generation	*n*	Slope ± SE	LC_{50} (95% CI)[a]	χ^2 (df)[b]	EC_{50} (95% CI)[a]
***H. armigera* populations**						
GO2	F_2	445	2.32 ± 0.45	8.41 (6.13 - 10.45)	3.34 (4)	0.200 (0.099 - 0.364)
MS5	F_4	505	1.17 ± 0.15	7.64 (5.55 - 11.43)	4.93 (5)	0.262 (0.169 – 0.373)
BA16	$F_{1;2}$	890	1.69 ± 0.21	4.21 (3.23 - 5.19)	3.55 (6)	0.222 (0.149 – 0.332)
BA45	F_3	575	1.86 ± 0.25	4.05 (2.63 - 5.46)	6.73 (6)	0.114 (0.044 – 0.199)
BA25	F_3	568	2.38 ± 0.24	3.74 (3.15 - 4.39)	8.23 (6)	0.099 (0.041 – 0.159)
BA49	F_3	447	2.12 ± 0.21	3.57 (2.91 - 4.28)	3.74 (4)	0.455 (0.374 – 0.531)
MT9	F_3	448	2.03 ± 0.25	2.97 (2.17 – 3.78)	0.44 (4)	0.195 (0.112 – 0.289)
***H. zea* populations**						
GO1	F_1	531	1.55 ± 0.13	0.21 (0.16 - 0.26)	7.57 (6)	0.020 (0.001 - 0.002)
RS1	F_1	631	1.25 ± 0.15	0.18 (0.11 - 0.25)	6.44 (7)	0.008 (0.005 - 0.011)
BA32	F_2	633	2.22 ± 0.36	0.17 (0.11 - 0.21)	8.02 (7)	0.009 (0.002 - 0.018)
PR1	F_1	497	1.55 ± 0.33	0.14 (0.04 - 0.26)	10.97 (5)	0.014 (0.002 – 0.026)
SP5	F_1	609	1.26 ± 0.15	0.05 (0.03 - 0.07)	10.78 (7)	0.004 (0.003 – 0.005)
MG2	F_1	496	2.32 ± 0.31	0.04 (0.03 - 0.05)	2.59 (5)	0.009 (0.007 – 0.012)

[a] LC_{50}: concentration of Vip3Aa20 (ng/cm^2) required to kill 50% of larvae in the observation period of seven days. Similarly, EC_{50} is the effective concentration of Vip3Aa20 (µg/cm^2) required to cause reduce 50% growth inhibition in the observation period of seven days.
[b] $p > 0.05$ in the goodness-of-fit test (degrees of freedom).

5.3.2 Diagnostic concentration for resistance monitoring of *H. zea*

There was a low variation in the susceptibility of the 11 field populations of *H. zea* to the diagnostic concentration of 6.4 µg/cm^2 of Vip3Aa20 (Table 5.4). Throughout the years of 2014 and 2015, mortality and growth inhibition was similar among populations. Six populations (SP10, PR3, MG4, RS3, SP17, and MG5)

showed mortality ranging from 93.4% to 97.9% differing from the populations SP14 and SP16, which showed complete mortality. The mortality of other three (SP12, PR4, MT14) populations did not differ significantly from the mortality of SP14 and SP16. Despite these differences, all 11 populations showed high mortality (> 90%) to Vip3Aa20 protein.

Furthermore, the growth inhibition was high for all populations, above 94%. This differ significantly (χ^2 = 53.9 ; df = 7; p < 0.0001) among populations, ranging from 94.3% (PR4) to 99.5% (MT14) (Table 5.4). Larval growth inhibition was not reported for the population SP10, since no larvae were weighed. In addition, no survival larvae reached the third instar in any population fed with the diet treated with Vip3Aa20 protein.

Table 5.4 – Mortality and growth inhibition of *H. zea* larvae exposed to the diagnostic concentration of 6.4 µg/cm^2 of Vip3Aa20 protein in diet-overlay bioassay.

Pop. Code	Generation	Mortality (%) (IC 95%)	Growth inhibition (%) (± SE)[b]
2014 year			
SP14	F_2	100.0 (99.6 - 100.0)	NE[c]
SP12	F_1	99.7 (99.1 - 99.9)	97.1 ± 0.6a
PR4	F_2	99.4 (98.7 - 99.7)	94.3 ± 0.6b
PR3	F_1	94.6 (93.1 - 95.9)[a]	96.8 ± 1.4b
MG4	F_1	95.4 (94.0 - 96.6)[a]	95.7 ± 1.3b
SP10	F_2	95.1 (93.6 - 96.3)[a]	NE
2015 year			
SP16	F_1	100.0 (99.6 - 100.0)	NE
MT14	F_1	99.5 (98.9 - 99.8)	99.5 ± 0.6a
SP17	F_1	97.9 (97.0 - 98.6)[a]	98.9 ± 1.3a
RS3	F_1	95.9 (94.6 - 96.9)[a]	98.2 ± 1.2a
MG5	F_1	93.4 (91.7 - 94.8)[a]	95.8 ± 1.5b

[a] Populations that differ from each other's in the susceptibility to Vip3Aa20 protein due to no overlap of the 95 CIs.
[b] Means followed by the same letter do not differ significantly by Kruskal-Wallis multiple comparison test, p < 0,05.
[c] NE = not evaluated.

5.4 Discussion

In our study, *H. armigera* was more tolerant to Vip3Aa20 insecticidal protein than *H. zea*. Because we did not have susceptible reference populations of *H. zea* and *H. armigera* in our study, we considered the most susceptible field populations as a reference for the susceptibility for both species. For *H. armigera* populations, the

variation in the susceptibility to Vip3Aa20 protein considering the LC_{50} was from 2.9 to 8.4 µg/cm^2, a 3-fold variation. The variation in the susceptibility of *H. armigera* to *Bt* proteins in terms of LC_{50} is demonstrated in several studies around the world. Similar low interpopulation variation was found in Australian populations for Cry1Ac from 0.023 to 0.108 µg/cm^2 (4.6-fold) (BIRD; AKHURST, 2007), and for Cry2Ab protein, from 0.065 to 0.420 µg/cm^2 (6.6-fold) (BIRD; AKHURST, 2007). Also for Cry1Ac, the variation was low among populations of India from 0.002 to 0.014 µg/cm^2 (7-fold) (JALALI et al., 2004), and for Cry2Ab the variation was from 0.102 to 1.0 µg/cm^2 (10-fold) in populations from four countries of the West Africa (BREVAULT et al., 2009). Otherwise, other researchers found a higher variation for Cry1Ac in Indian populations from 0.0002 to 0.013 µg/cm^2 (67-fold) (KRANTHI; KRANTHI; WANJARI, 2001), and among populations of the West Africa from 0.008 to 0.334 µg/cm^2 (44-fold) (BREVAULT et al., 2009). In Brazil, interpopulation variation in the susceptibility to Vip3Aa20 was low, approximately 6-fold, for *S. frugiperda* (0.092 to 0.612 µg/cm^2) and *D. saccharalis* (61 to 368.0 ng/cm^2) populations (BERNARDI et al., 2014).

Furthermore, the LC_{50} to Vip3Aa in a colony of *H. armigera* was also high, 1.7 µg/cm^2 (CI 95% 1.0 – 2.5) in a study that compared the activity of different Vip proteins against lepidopteran pests (DE ESCUDERO et al., 2014). In addition, high resistance allele frequency (0.027) to Vip3A protein was found in field populations of *H. armigera* in Australia before the commercial release of a Vip-cotton event (MAHON; DOWNES; JAMES, 2012). These authors used in their discriminating assays 10 µg Vip3A/cm^2. After resistance selection, individuals from two colonies survived at the maximum concentration of 220 µg Vip3A/cm^2, with only 2.4% of mortality. However, Vip3A cotton plants provided high efficacy against *H. armigera* in field conditions in the Eastern Australia (LLEWELLYN; MARES; FITT, 2007). This could be explained by differences in the Vip3A protein used in the laboratory (MAHON; DOWNES; JAMES, 2012) than that expressed by the cotton plant (LLEWELLYN; MARES; FITT, 2007). In our study, the LC_{50} of the least susceptible *H. armigera* population (GO2) was 8.4 µg Vip3A/cm^2, closed to the value of discriminating concentration used in Australia, which could indicate a resistant population. However, in the lack of a susceptible population from Brazil, we could not calculate the resistance ratio of resistance.

For *H. zea* the interpopulation variation in the baseline susceptibility was from 0.040 to 0.207 µg Vip3Aa20/cm^2, a 5-fold difference. Our results showed a lower variation for *H. zea* to Vip3A protein in terms of LC_{50} when compared to USA populations, which varied from 0.020 to 1.5 µg/cm^2 (75-fold variation) (ALI; LUTTRELL, 2011). This variation was also high for Cry1Ac and Cry2Ab2 proteins. For field populations from USA for Cry1Ac, LC_{50} varied from 0.047 to 2.6 µg/cm^2 (53-fold) (ALI; LUTTRELL; III, 2006); and for Cry2Ab2 from 0.088 to 4.1 µg/cm^2 (47-fold) (ALI; LUTTRELL, 2007). However, a lower variation was observed for *H. zea* to Cry1Ab protein from 0.096 to 0.221 µg/cm^2 (2-fold), in USA (SIEGFRIED; SPENCER; NEARMAN, 2000). Also in USA, efficacy bioassays showed that both Vip3A and Cry1Ab cotton lines provided similar moderate mortality (~ 60%) against *H. zea* (ADAMCZYK JR; MAHAFFEY, 2008). Consistently, in another study, the survivorship of *H. zea* ranged from 4 to 28% on Vip3A cotton plant structures (BOMMIREDDY; LEONARD, 2008). In contrast, Vip3A maize was high efficient in controlling *H. zea*, more than 99% of control (BURKNESS et al., 2010). These differences in field control could be explained by the expression of Vip3A on cotton and maize. Analyses performed by Syngenta® showed that Vip3A levels on cotton leaves, where higher levels were found at all sampling stages, varied from 5 to 118 µg/g dry weight (ARTIM, 2003), while on maize kernels varied from 123.8 to 140.1 µg/g dry weight (EPA, 2009).

Although interpopulation variation in susceptibility to Vip3Aa20 was observed, the magnitude of the differences was small in *H. armigera* and *H. zea* populations of Brazil. This variation may not be due to selection pressure by Vip3Aa20 crops. Maize hybrids expressing Vip3Aa20 protein (MIR162 and Bt11×MIR162×GA21) have been commercialized in Brazil since 2009 (CTNBIO, 2014). However, the cultivated area with these *Bt* maize events is small, less than 10% of the total maize area (BERNARDI et al., 2015). Many factors may contribute to variation in baseline susceptibility reported in Brazil and in different laboratories around the world. These factors can be associated with bioassay methods, insect generation, mortality criteria, the source of the insecticidal protein, and the general vigor tolerance of different populations (ROSSITER; YENDOL; DUBOIS, 1990; LUTTRELL; WAN; KNIGHTEN, 1999; ALI; LUTTRELL; III, 2006; WAQUIL et al., 2004). General vigor can impact on an insect's ability to withstand the stress imposed by *Bt* proteins, it varies widely among *Helicoverpa* strains and is usually poor when strains originate

from a narrow genetic basis (ROSSITER; YENDOL; DUBOIS, 1990; BIRD; AKHURST, 2007). According to these authors, we just tested populations that were originated with a minimum of 50 individuals to minimize the possibility of inbreeding depression in our test populations. The small variation found in our study is more likely to represent the natural variation of the susceptibility of *H. armigera* and *H. zea*, and is an indication of the ability of these insects to adapt to the insecticidal protein. Also, in an IRM perspective, our previous studies demonstrated that there are intraspecific gene flow among populations of Brazil with cytochrome oxidase I (COI) gene (LEITE et al., 2014), and microsatellite markers (Chapter 4). This provides chances to repeated colonization of *Bt* fields and opportunities for rapid local adaptation (CARRIERE; CROWDER; TABASHNIK, 2010).

In Chapter 4, an interspecific analysis with Brazilian *H. armigera* and *H. zea*, and USA *H. zea* showed the presence of hybrid individuals within *H. armigera* and *H. zea* populations of Brazil. Some of these populations were tested in this study for their susceptibility to the Vip3Aa20 protein. The few populations that presented one hybrid individual tested here were: BA49 (*H. armigera*), PR1 and SP5 (*H. zea*). We did not detected hybrids within another populations evaluated in the present study. Therefore, we did not find any correlation with the presence of hybrids and higher or lower susceptibility to Vip3Aa20 protein. Still, we do not know how far is the interference of these individuals on the susceptibility of the populations, their fitness, and if they are capable of breeding in the field. Hybridization can increase genetic variation, therefore augmenting evolutionary potential of species (STEBBINS, 1959; ABBOTT, 1992). Studies with populations of hybrids should be done to understand their population dynamics and response to control methods.

Our monitoring data for *H. zea* showed that the populations' responses were similar around Brazil, with a high mortality (> 90%) to the concentration of 6.4 µg Vip3Aa20/cm^2. For *S. frugiperda* and *D. saccharalis*, also in Brazil, two diagnostic concentrations of 2.0 and 3.6 µg Vip3Aa20/cm^2 were defined for monitoring resistance, these caused up to 90% mortality (BERNARDI et al., 2014). Therefore, *H. zea* can be considered less susceptible than *S. frugiperda* and *D. saccharalis* to Vip3Aa20 protein in Brazil. The differences between LC_{50} and EC_{50} values ranged between 4- and 12-fold for *H. zea*, and between 15- and 42-fold for *H. armigera*. Consequently, lower concentrations than those causing mortality affected growth and

development, and this difference is more expressive for *H. armigera*. *Bt* proteins can affect growth inhibition, preventing insects from reaching adulthood, and thus completing their life cycle (DULMAGE; MARTINEZ, 1973). The EC_{90} could be validated and used for resistance monitoring of *H. zea* in order to decrease the amount of protein for the bioassays. However, we recommend the use of mortality data for large-scale monitoring, because collecting mortality data are easier and faster, although the higher amount of protein needed.

We did not monitor *H. armigera* populations due to their less susceptibility to Vip3Aa20. As a result, the designation of a diagnostic concentration equivalent to LC_{99} was difficult to achieve because of the high amount of protein required for bioassays. Other option would be to use EC_{90} to designate a diagnostic concentration, however our CI's were very high for this estimate (data not shown). Also, this species occurs sporadically in maize in Brazil (LEITE et al., 2014), which is currently the only cultivated crop that express Vip3Aa20 protein in this country. Thus, the chances of detecting changes in susceptibility in a pest that is not under selection pressure are low. It will be extremely important to monitor this pest in the future, when other crops such as cotton and soybean expressing Vip3Aa20 protein might be commercially released in Brazil.

In conclusion, our data show that *H. armigera* is less susceptible to Vip3Aa20 protein than *H. zea*. The knowledge of the natural variation in response to this protein among these species populations, before widespread commercial use of *Bt* maize, is necessary to avoid unwarranted concerns about resistance to Vip3Aa20 in field surveys of *H. armigera* and *H. zea* populations. However, some questions need to be evaluated such as the frequency of resistant alleles in both species and the effective dominance of resistance to predict resistance evolution. In this context, future efforts should be concentrated on a comprehensive monitoring program for evaluating the susceptibility of *H. armigera* and *H. zea* populations to Vip3Aa20, which will be useful for assessing the efficacy of current resistance management strategies.

5.5 Conclusions

- *H. armigera* is less susceptible to Vip3Aa20 insecticidal protein than *H. zea*.

- There is a low interpopulation variation in the susceptibility to Vip3Aa20 in populations of *H. armigera* and *H. zea* from Brazil.

- The concentration of 6,400 ng/cm^2 in a diet-overlay bioassay is appropriated for the resistance monitoring of *H. zea* populations to Vip3Aa20 protein in Brazil.

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6 FINAL CONSIDERATIONS

The current study provides important information on population structure of *H. armigera* and *H. zea* specimens in Brazil and in the USA for designing and implementing sustainable strategies of pest management. Also, it provides information on the tolerance of these pests to the Vip3Aa20 protein in Brazil. These species have a wide distribution in Brazil and the USA (only *H. zea*), which highlights the importance of maintaining regional coordinated Integrated Pest Management (IPM) and IRM strategies. If resistance to *Bt* or insecticides emerges in one area or crop, the high movement might spread the resistance alleles quickly. This is more important to polyphagous pests such as *H. armigera* and USA *H. zea*, which attack different hosts. On the other hand, for Brazilian *H. zea*, maize populations must be constantly monitored to pesticides and *Bt* maize crop resistance. Regarding to the Vip3Aa tolerance, *H. armigera* showed to be less tolerant than *H. zea*. Therefore, this protein is more recommended to manage *H. zea*.

We believe that *H. armigera* invasion is eminent in the USA due the bioecology of this pest and migration potencial of other Noctuidae pests as *H. zea* (here reported) and *S. frugiperda*. Thus, information about biology, ecology, biological control agents, insecticides and *Bt* crops susceptibility of *H. armigera* population will be valuable to the success of IRM plans in Brazil and USA territories.