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A RAPD-PCR-based genetic diversity analysis of *Helicoverpa armigera* and *H. zea* populations in Brazil

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ABSTRACT. *Helicoverpa armigera* is the most significant pest of agriculture in Asia, Europe, Africa, and Australasia, causing damage to crops greater than US$2 billion annually and until 2013 it was not detected in Brazil. *Helicoverpa zea* is restricted to the American continent and is important to corn and a secondary pest of cotton and tomatoes. The wide range of crops exploited by *H. armigera* (mainly cotton, soybeans, chickpea, and corn), the possible mating between these species can promote population shifts, that could be assessed by RAPD-PCR technique. Therefore, the aim of this study was to determine the genetic diversity of *H. armigera* and *H. zea* populations by RAPD-PCR analysis. The most important result was the clustering of one *H. armigera* population in a group predominantly formed by *H. zea*. It could indicate a possible occurrence of an interspecific cross between these species. This is a concern to Brazilian agriculture due to the possibility of selection of hybrids well adapted to the American environment, which would be

inherited from *H. zea*. The other noxious fact is the possible development of new biotypes resistant to insectides or Bt toxins expressed in transgenic crops, came from *H. armigera* gene pool.

Key words: Old World bollworm; Corn earworm; Molecular markers; Population genetics; Genetic diversity

INTRODUCTION

The *Helicoverpa* and *Heliothis* are the two most important genera of agricultural pests within the Heliothinae subfamily (Lepidoptera: Noctuidae) (Pogue, 2013). According to Tay et al. (2013), *Helicoverpa armigera* is the most significant and impactful pest of agriculture in Asia, Europe, Africa, and Australasia, causing damage to crops estimated at greater than US$2 billion annually, excluding socio-economic and environmental costs associated with its control. In Brazil, regarding the agricultural years of 2012/2013, Spadotto et al. (2014) pointed out that decreasing yields and rise in phytosanitary costs in cotton, corn, beans, sorghum, and millet resulted in US$ 2 billion of loss. On the other hand, *Helicoverpa zea* is an important pest of corn and a secondary one for cotton and tomatoes and is restricted to American continent (King and Coleman, 1989). According to Bird and Downes (2014), chemical control and transgenic crops expressing Bt toxins are the main strategies to control *H. armigera* and *H. zea*.

In Brazil, *H. armigera* was considered as an A1 quarantine pest (not present, but of potential economic importance). However, the recent invasion of *H. armigera* populations into Brazilian crops has been independently confirmed by Czepack et al. (2013), Specht et al. (2013) and Tay et al. (2013). The introduction of *H. armigera* in the country is a major threat to Brazilian agriculture due to the following characteristics of this pest: a) long-distance dispersion (Farrow, 1984; Pedgley, 1985); b) wide range of host plants (Behere et al., 2013); c) high fecundity (Subramanian and Mohankumar, 2006); d) resistance to several insecticides (Bird and Downes, 2014); e) potential risk of resistance to *Bacillus thuringiensis* (*Bt*) proteins expressed in transgenic crops (Nair et al., 2013); f) possibility of interspecific crosses with *H. zea* populations and generation of fertile hybrid offspring (Laster and Sheng, 1995).

According to Behere et al. (2013), these characteristics alone or combined can alter the structure and population dynamics of this insect. Furthermore, evolutionary factors can imply in ecological specialization and adaptation to a particular host and these could shift the genetic flow between populations of this insect (Zalucki et al., 2012). In the specific case of *H. armigera*, Zhou et al. (2000) remarked that a high level of gene flow could allow spread of insecticide resistance genes in susceptible populations when this selective pressure is present, which is the case of several Brazilian agricultural systems. Regarding the interspecific cross between *H. armigera* and *H. zea*, the concern is the selection of hybrids well adapted to the new environment (American continent) and/or development of new biotypes resistant to insecticides or Bt toxins expressed in transgenic crops (cotton, maize, and soybeans) (Leite et al., 2014).

In this context, several molecular strategies have been used for genetic and evolutionary studies at population level (Guerra et al., 2010). One of these strategies is the PCR-based randomly amplified polymorphic DNA (RAPD), which is a type of polymerase chain reaction where the fragments of DNA are randomly amplified, using short synthetic oligonucleotides of random sequence (Williams et al., 1990). The RAPD marker system has many advantages, including the low cost, little technical demands, and no prior knowledge about DNA sequence

(Bhau et al., 2014). Besides, according to Anbalagan et al. (2012), RAPD marker system is suitable for studies of population structure because it allows for the detection of polymorphisms in coding and non-coding regions of both nuclear and mitochondrial genomes.

In this context, the objective of this study was to assess the genetic divergence among *H. armigera* and *H. zea* populations using information generated by the RAPD marker system.

MATERIAL AND METHODS

Specimens

H. armigera and *H. zea* laboratory-reared caterpillars collected in Midwest, South and Southeast regions from Brazil were used in the present study. The identification of the *Helicoverpa* populations at species level was done via sequencing of a taxonomically informative segment of the mitochondrial DNA (mtDNA) cytochrome oxidase I (COI) gene and comparing the obtained sequences with the available *H. armigera* and *H. zea* barcode (Behere et al., 2008; Tay et al., 2013) sequences using the BLASTn tool. In total, five *H. armigera* and eight *H. zea* populations were included in the present study. All samples were preserved in 75% ethanol and stored at -20°C prior to DNA extraction. The population codes, sampling dates, plant hosts, geographic coordinates and sampling sites are described in Table 1.

Table 1. Abbreviation, number of individuals/sample, sampling date, host plant, geographic coordinates, and sampling sites of 12 *Helicoverpa armigera* (HA) and *Helicoverpa zea* (HZ) populations collected in Brazil.

Abbreviation*	N	Sampling date	Host plant	Geographic coordinates	Sites[1] (Cities-States)	Climate
HA-BA	8	10/24/2014	Cotton	11°19'40,1"S 46°11'16,8"W	Formosa do Rio Preto-BA	Tropical (Aw)
HA-DF1	15	01/22/2014	Soybean	15°43'30,0"S 47°36'39,0"W	Brasília-DF	Tropical (Aw)
HA-DF2	10	12/17/2013	Soybean	15°43'11,9"S 47°35'51,8"W	Brasília-DF	Tropical (Aw)
HA-SP	20	11/04/2014	Soybean	22°45'40,0"S 47°09'15,0"W	Paulínea-SP	Subtropical (Cfa)
HZ-DF1	20	11/11/2014	Soybean	15°52'07,0"S 47°24'3,50"W	Brasília-DF	Tropical (Aw)
HZ-DF2	20	11/13/2014	Sweetcorn	15°57'2,0"S 47°56'2,0"W	Brasília-DF	Tropical (Aw)
HZ-DF3	20	01/09/2014	Corn	15°43'30,0"S 47°36'39,0"W	Brasília-DF	Tropical (Aw)
HZ-GO1	10	07/25/2014	Corn	18°20'25,9"S 49°11'11,03"W	Itumbiara-GO	Tropical (Aw)
HZ-GO2	10	10/01/2014	Corn	18°20'27,9"S 49°11'11,0"W	Itumbiara-GO	Tropical (Aw)
HZ-GO3	12	10/15/2014	Corn	17°43'52,0"S 49°05'58,0"W	Morrinhos-GO	Tropical (Aw)
HZ-PR	10	01/06/2015	Corn	24°42'45,0"S 53°44'35,0"W	Toledo-PR	Subtropical (Cfa)
HZ-SC	10	01/19/2015	Corn	26°45'39,5"S 53°10'21,0"W	Maravilha-SC	Subtropical (Cfa)

[1]Abbreviations of the collection sites: BA = Bahia State; DF = The Federal District; GO = Goiás State; SP = São Paulo State; PR = Paraná State, and SC = Santa Catarina State.

DNA extraction

Genomic DNA was extracted from the last three abdominal segments of the larvae (Behere et al., 2013) from eight individual caterpillars in each population, following a

modified cetylmethylammonium bromide (CTAB) protocol (Subramanian and Mohankumar, 2006). Larvae were ground with 1.0 mL 2% cetyl trimethyl ammonium bromide buffer (CTAB), 100 mM Tris-HCI (pH 8.0), 1.4 M sodium chloride, 20 mM EDTA, and 0.1% 2-mercaptoethanol. This mixture was incubated at 65°C for 2 h and then equal volume of chloroform: isoamylalcohol (24:1) was added. The suspension was centrifuged at 800 *g* for 15 min at 4°C. Then, the DNA was precipitated by adding equal volume of ice-cold isopropanol, centrifuged at 8000 *g* and the pellet was washed with 70% ethanol and dissolved in 100 µL of TE + RNase (10 mM Tris-HCl, 1 mM EDTA, 8 µL RNase, pH 8.0) and short term stored at 4ºC. The DNA from each sample was quantified by spectrophotometry (NanoDrop 2000 spectrophotometer, Thermo Fischer Scientific, USA) and the concentrations were adjusted with TE + RNase to get a working solution of 20 ng/µL.

RAPD-PCR

The initial screening for molecular polymorphisms among the pest total DNA samples was performed with 120 oligonucleotide primers (Operon Technologies, USA). Sixteen primers were chosen based upon the production of scorable amplicons (bands) across the *Helicoverpa* populations (Table 2). Each RAPD-PCR was performed in a total volume of 25 µL containing 40 ng DNA template, 2.5 µL 10X TaqDNA polymerase buffer, 1.5 mM $MgCl_2$, 0.5 U *Taq* DNA polymerase (Invitrogen, Brazil), 0.2 mM of each dNTP, 20 pmol primer and sufficient Milli Q sterile water to complete the final volume. PCR was performed in a GeneAmp® PCR System 9700 thermal cycler (Applied Biosystems, USA) programmed with one cycle of initial denaturation at 94°C for 1 min; 35 cycles each of 94°C for 30 s, annealing at 36°C for 1 min, extension at 72°C for 1 min and 30 s, and final extension at 72°C for 7 min. The PCR products were separated on a 1.5% agarose gel stained with ethidium bromide (10 mg/mL) in 1X TBE buffer, using 1 kb plus DNA ladder (Invitrogen, USA) as a molecular weight standard. Gels were visualized and photographed under UV light using the Quantity One® (BioRad, USA) software. This procedure was repeated using the same equipment, supplies, and DNA template in order to confirm the band patterns.

Table 2. Sixteen genetically informative RAPD primers and their respective number of polymorphic amplicons observed after analyzing total genomic DNA of 12 *Helicoverpa armigera* and *H. zea* populations collected across four geographic Brazilian regions.

Primer code	Primer sequence (5'-3')
OPA-02	TGCCGAGCTG
OPA-07	GAAACGGGTG
OPA-12	TCGGCGATAG
OPA-14	TCTGTGCTGG
OPA-16	AGCCAGCGAA
OPB-01	GTTTCGCTCC
OPC-01	TTCGAGCCAG
OPC-02	GTGAGGCGTC
OPC-04	CCGCATCTAC
OPC-05	GATGACCGCC
OPC-06	GAACGGACTC
OPC-11	AAAGCTGCGG
OPC-13	AAGCCTCGTC
OPC-20	ACTTCGCCAC
OPF-02	GAGGATCCCT
OPF-06	GGGAATTCGG

Statistical analysis

The presence '1' or absence '0' of bands in each population for each selected primer was visually scored on the gel and entered into a binary matrix. Loci were selected for which the most common allele in each population occurred at a frequency greater than or equal to 75%, as suggested by McMichael and Prowell (1999). The data matrix was used to calculate the arithmetic complement of Jaccard index (Sneath and Sokal, 1973). Based on this information, a dendrogram was obtained by using the unweighted pair group method with arithmetic average (UPGMA) with bootstrapping (500 replicates). Moreover, the dissimilarity values were used in other two cluster analysis, one based on the Tocher method (Rao, 1952) and the other using a principal component analysis (PCA) bidimensional scatter plot. UPGMA and principal component analysis were performed by the NTSYS software version 2.02 (Rohlf, 2000) and Tocher's cluster analysis was done using the GENES software package (Cruz, 2013).

RESULTS

One-hundred seventeen (117) polymorphic amplicons were obtained with a set of 16 RAPD primers selected in preliminary screening assays (Figure 1). The number of informative bands for each primer in each population ranged from 4 to 12 with a mean of 7.5. The amplicon sizes ranged from 200 to 3000 bp. Based on Jaccard index the highest genetic distance was found between HA-DF2 and HA-BA (0.86). On the other hand, the lowest distances were observed between HZ-GO2 and HZ-GO3 (0.06), and HZ-PR and HZ-SC (0.06) (Table 3). Genetic relationships between *Helicoverpa* populations can also be observed in Table 3, where the average genetic distance was 0.68 for *H. armigera* and 0.37 for *H. zea*.

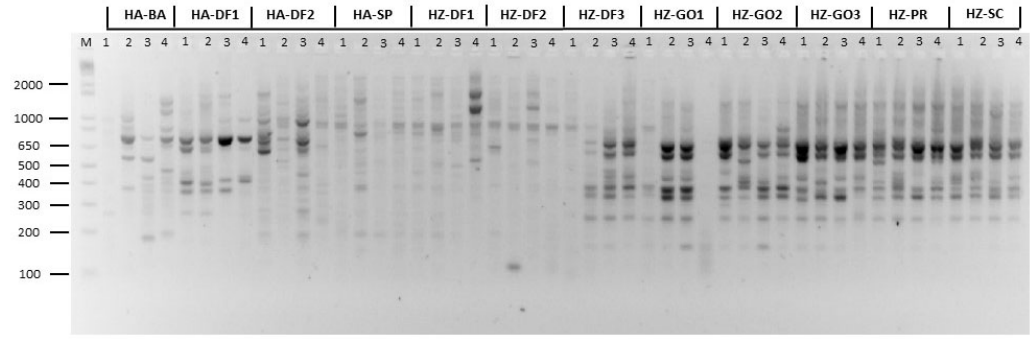

Figure 1. DNA samples from four caterpillars of twelve different populations of *Helicoverpa* spp amplified with RAPD primer OPC13. Population acronyms details are present in Table 1. *Lane M* = size markers (1 kb plus DNA ladder from Invitrogen).

The dendrogram, based on UPGMA, separated *Helicoverpa* populations in three distinct groups (Figure 2). The group 1 was composed by all *H. zea* populations, except HZ-GO1. The group 2 was formed by two populations of *H. armigera* (HA-BA and HA-SP) and the third cluster (group 3) was composed by two populations of *H. armigera* (HA-DF1 and HA-DF2) and one of *H. zea* (HZ-GO1).

Table 3. Pairwise dissimilarity matrix of 12 populations of *Helicoverpa armigera* and *H. zea* according to Jaccard index using genetic information derived from 117 polymorphic RAPD-PCR bands obtained in the amplification profiles of 16 RAPD primers.

	HA-BA	HA-DF1	HA-DF2	HA-SP	HZ-DF1	HZ-DF2	HZ-DF3	HZ-GO1	HZ-GO2	HZ-GO3	HZ-PR	HZ-SC
HA-BA	0.00											
HA-DF1	0.85	0.00										
HA-DF2	0.86	0.33	0.00									
HA-SP	0.58	0.68	0.70	0.00								
HZ-DF1	0.61	0.80	0.81	0.60	0.00							
HZ-DF2	0.62	0.71	0.73	0.56	0.28	0.00						
HZ-DF3	0.66	0.82	0.83	0.59	0.30	0.27	0.00					
HZ-GO1	0.83	0.30	0.35	0.64	0.79	0.35	0.79	0.00				
HZ-GO2	0.62	0.80	0.82	0.59	0.21	0.21	0.17	0.80	0.00			
HZ-GO3	0.62	0.82	0.84	0.61	0.26	0.21	0.20	0.84	0.06	0.00		
HZ-PR	0.62	0.78	0.80	0.61	0.23	0.25	0.24	0.79	0.15	0.16	0.00	
HZ-SC	0.62	0.79	0.81	0.59	0.20	0.20	0.19	0.78	0.10	0.10	0.06	0.00

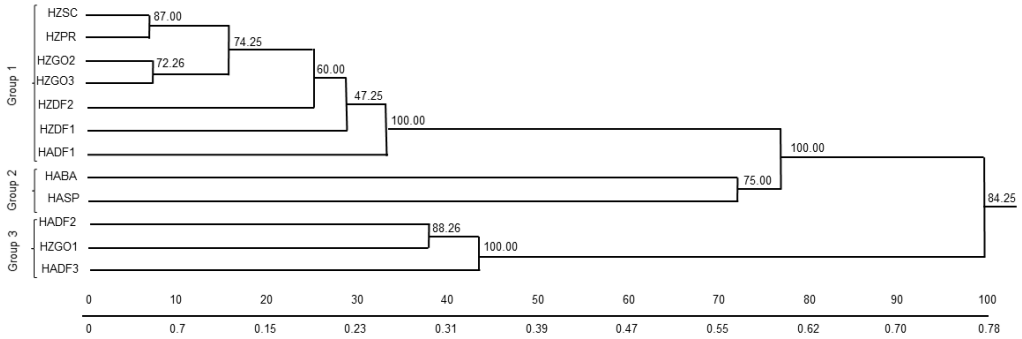

Figure 2. UPGMA dendrogram of 12 populations of *Helicoverpa* spp based on Jaccard index. Bootstrap support (percent of 400 replicates) is indicated for each branch.

As regarded in cluster analysis based on the Tocher method (Table 4), all *Helicoverpa* populations were subdivided into three groups. The group 1 was formed by all evaluated populations of *H. zea* except HZ-GO1. The group 2 involved two populations of *H. armigera* and one of *H. zea* and group 3 was composed by the remaining populations of *H. armigera* (HA-BA and HA-SP). Besides, considering the average distance within and between groups, it could be inferred that the most divergent groups were 1 and 2 (16.67) and the least variable was the group 3 (0.58).

Table 4. Average distance within and among clusters of 12 *Helicoverpa* populations based on Tocher's methodology using genetic information derived from 117 polymorphic RAPD-PCR bands obtained in the amplification profiles of 16 RAPD primers.

Cluster	1 (HZ-SC, HZ-PR, HZ-GO2, HZ-GO3, HZ-DF1, HZ-DF2, and HZ-DF3)	2 (HA-DF1, HZ-GO1 and HA-DF2)	3 (HA-BA and HA-SP)
1 (HZ-SC, HZ-PR, HZ-GO2, HZ-GO3, HZ-DF1, HZ-DF2, and HZ-DF3)	4.04	16.67	8.51
2 (HA-DF1, HZ-GO1 and HA-DF2)	-	0.98	4.55
3 (HA-BA and HA-SP)	-	-	0.58

The accumulated variance of the first two eigenvalues, generated by PCA was almost 70%. As postulated by Sparks et al. (1999), in this situation, it is reasonable to study the divergence of the *Helicoverpa* populations in a bidimensional space, with negligible distortion degree (Figure 3). The same trend observed in UPGMA dendogram (Figure 1) and using the Tocher method (Table 4) was observed in PCA biplot, which also revealed three distinct clusters.

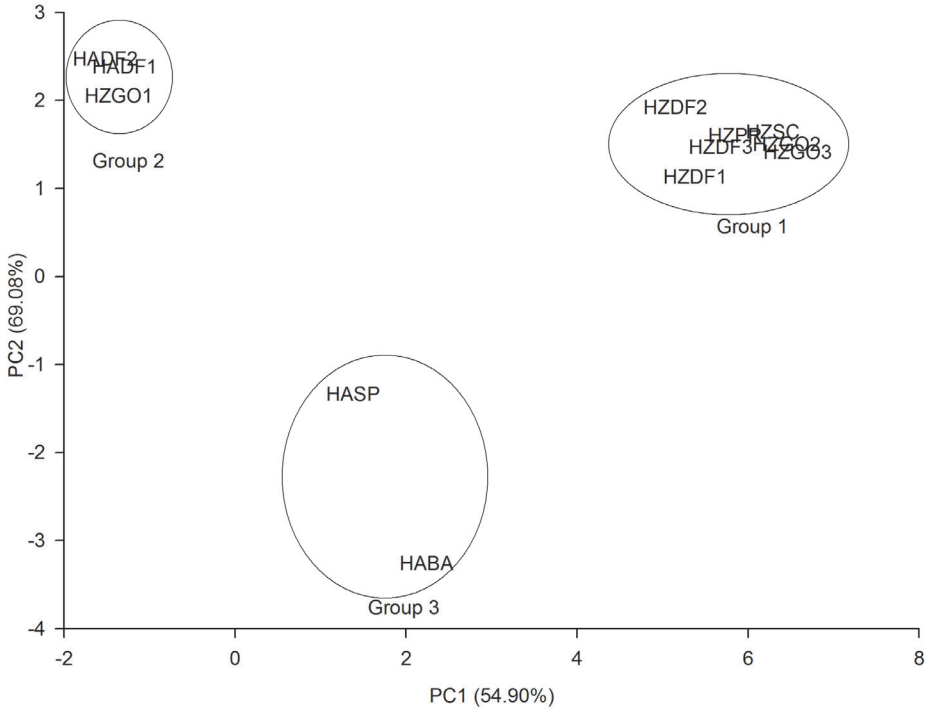

Figure 3. Scatter plot of principal component analysis of 117 polymorphic RAPD-PCR loci. Numbers in the parenthesis, located in the X- and Y-axes, indicate cumulative percentages of the eigenvalues.

DISCUSSION

H. armigera has been recorded as feeding on 181 different plant species and this pest can attack the most important agricultural crops, such as: cotton, sorghum, soybeans, maize, vegetables, and subsistence crops like chickpea and pigeon pea (Subramanian and Mohankumar, 2006). As stated by King and Coleman (1989), its major natural enemies are *Microplitis croceipes* and *Bracon brevicornis* (Hymenoptera: Braconidae), *Trichogramma* spp, *Eucelatoria bryani* and *Archytas marmoratus* (Diptera: Tachnidae), *Chrysopa* spp (Neuroptera: Chrysopidae), *Coleomegilla maculata* (Coleoptera: Coccinellidae), and *Geocoris* spp (Hemiptera: Geocoridae). Besides, *H armigera* larvae usually feed on flower buds and/or fruits and because of this, they become protected from predators and parasites as well as exposed to sub lethal doses of insecticides. These authors also remark that the insecticides are often used to suppress *Helicoverpa* populations because of their availability, portability, and potential for quick intervention and prevention of serious plant damage.

The greatest dissimilarity observed between the populations of *H. armigera* (HA-DF2 and HA-BA) cannot be explained only by the fact that they were collected in far apart geographic locations, because it is well known that this species can be dispersed at long distances (Farrow, 1984; Pedgley, 1985). According to Nibouche et al. (1998), this species can migrate as far as 2000 km, and this value is greater than the distance between these two geographic sites. Feng et al. (2005) studied the long-distance migration of this species in China and based on radar observations concluded that the moth flight could reach up to 2000 m in altitude and, using airflows, moths can overcome distances over than 2000 km, considering a three-day travel and a single night flight up to 700 km.

Moreover, as supported by Jallow et al. (2004), Subramanian and Mohankumar (2006) and Behere et al. (2013), the differences between these two populations could be due to their natural hosts, since HA-BA caterpillars were collected infesting cotton plants, whereas HA-DF3 caterpillars were collected in soybean. There are many differences on agricultural practices between these crops, considering the flowering periods, pesticides spray and exposure to allelochemicals. Acording to Behere et al. (2013), the different cropping systems, to which *H. armigera* is exposed, may also be important underlying factors that contributed to population substructure differences. Host crops with short flowering periods (e.g., soybeans) generally support no more than one or two *H. armigera* generation; while hosts such as cotton with prolonged flowering periods are capable of supporting three consecutive generations. Populations that feed on cotton are under tremendous selection pressure from insecticide applications, and from varying levels of the allelochemical gossypol associated with different life stages and specific cotton varieties

In general, all studied *H. zea* populations were grouped into a single cluster, except HZ-GO1, indicating low genetic variability of this species in our samples. According to Pogue (2004), *H. armigera* is widely distributed in the Old World and, from this region, it was spread to Oceania. First proposed by Mallet et al. (1993) and confirmed by Behere et al (2007) through allozyme and mtDNA COI sequences respectively, using phylogeographic analyses of *H. armigera* and *H. zea* individuals, these authors postulated that *H. zea* evolved from a small portion of the larger *H. armigera* population (i.e., the "founder effect") that reached the American continent approximately 1.5 million years ago.

The populations of *H. armigera* assessed in the present study were subdivided into two distinct clusters, and one of these was composed by two geographically distant populations (HA-BA and HA-SP) and the other displayed an opposite trend. As previously discussed, *H. armigera* adults have long distance dispersion and adaptation to multiple host plants, which allow a high level of gene flow and a decrease on genetic variation. Furthermore, as postulated by Tay et al. (2013) and Leite et al. (2014), the invasion of *H. armigera* into the New World evolved from at least two maternal lineages. This fact gives support to the assignment of our *H. armigera* populations in two distinct clusters.

An important result found in the present study was the presence of a *H. zea* population (HZ-GO1) in a cluster with predominance of *H. armigera* populations. This observation can indicate potential interspecific crosses between these species. As observed by Laster and Hardee (1995) and Laster and Sheng (1995), despite their period for speciation, these two species can mate and generate fertile offspring under laboratory conditions.

In summary, the results of this research are significant because they indicate a possible interspecific cross between *H. armigera* and *H. zea*. Based on Leite et al. (2014), this recombination or introgression phenomena could allow the transfer of insecticide resistance

genes from *H. armigera* to *H. zea*. On the other side, *H. zea* could donate genes for adaptation to environmental conditions of the American continent to *H. armigera*. Therefore, hybridization may enable the selection of breeds with enhanced hybrid vigor and the ability to rapidly adapt to current management and suppression methods used in Brazil.

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