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Cross-species amplification and polymorphism of microsatellite loci in *Helicoverpa armigera* and *Helicoverpa zea* (Lepidoptera: Noctuidae) in Brazilian cropping systems

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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 recent 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.

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

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). This invasive pest rapidly spread throughout Brazil (Leite et al., 2014; Mastrangelo et al., 2014) and was also reported in other South American countries, such as Argentina (Múrua et al., 2014), Paraguay (Senave, 2014), Bolivia, and Uruguay (Czepak C, unpublished results). In 2015, it was found in Florida, USA (Hayden and Brambila, 2015), and now threatens crop production throughout the Americas.

Historically, the New World bollworm, *H. zea* (Boddie), is the most important species of the *Helicoverpa* genus in the Americas, including Brazil (Kogan et al., 1989). 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 (Laster and Hardee, 1995; Laster and Sheng, 1995; Cho et al., 2008, Pogue, 2013).

Both *H. armigera* and *H. zea* are highly 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; Yang et al., 2013; Razmjou et al., 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) (Endersby et al., 2007; Li et al., 2011; Perera and Blanco, 2011; Behere

et al., 2007, 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 and 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 and Mohankumar, 2006; Perera and 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 to i) test the cross-amplification of 13 microsatellite loci previously characterized from *H. zea* in *H. armigera*, ii) retest for the cross-amplification of seven microsatellite loci from *H. armigera* (Scott et al., 2004; Ji et al., 2003, 2005) in *H. zea*, in order to determine whether they also work in Brazilian populations, and iii) 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*.

MATERIAL AND METHODS

Sampling and DNA extraction

Sixty-four *H. armigera* larvae and 72 *H. zea* larvae were collected at six sampling sites (Table 1 and Figure 1). *Helicoverpa* spp larvae were maintained on an artificial diet modified from that used by Greene et al. (1976), in which white beans were used under controlled laboratory conditions, at 25° ± 1°C, 60 ± 10% relative humidity, and 14:10-h light/dark 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).

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 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 mL 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, NE, USA) for approximately 2 h at 70 W. The loci were genotyped using the Saga software (LI-COR Biosciences).

Table 1. Identification code, location, collection site (crop), date of collection of *Helicoverpa 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 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.

Table 2. Characteristics of 20 microsatellite loci previously published for *Helicoverpa zea* and *H. armigera*.

Locus	Primer sequence (5'-3')	Repeat motif	Allele size and size range (bp)	Reference
HzMS1-4	F: CAAGTGATAAAAGACGCCGAAGAT R: TTGATCGTCAAGGAAGTGGCTAT	$(TGA)_6$	118 (111-144)	Perera et al. (2007)[z]
HzMS1-6	F: GTTTTGTCATTTGTCAAGCCGAA R: AGCTCCCATACAACAAACGTGATT	$(TGA)_7$	237 (208-245)	Perera et al. (2007)
HzMS3-1	F: CAGTAGTTCCTGAGATTAGCGCGT R: ATCACGTTCTCGAAAAACATTGCT	$(CAAA)_4$	113 (106-110)	Perera et al. (2007)
HzMS3-4	F: GGTCAAGATTCGTGCCGATAACTA R: TTTTCGGTTCAGTGGCTTGTAGTAG	$(TCTG)_4$	118 (115-117)	Perera et al. (2007)
HzMS3-11	F: ACTTCAAAGTTCGATTCTTGGGAT R: GCTCAAAGAGGACTACGTAGCTGA	$(AGCT)_4$	106 (93-106)	Perera et al. (2007)
HzMS3-41	F: AAATTTCAACCAAATCGGTCTAGC R: TGGCCGAACTATAATATCTTACTTCCTA	$(ACAT)_4$	121 (121-135)	Perera et al. (2007)
HzMS3-48	F: GGTGAAATGGAAATTGTTATCTATCCC R: TCAGTCCAGTGGTTTAGACGTGAA	$(TCTG)_4$	101 (94-102)	Perera et al. (2007)
HzMS3-86	F: GGGGAAAAGAGGAAACAAATCATC R: GAAACACGTTTGAGGAGGTCAGAT	$(CAT)_4$	140 (136-151)	Perera et al. (2007)
HzMS4-3	F: ACTTTCCGCATCCGATTAAAATGT R: CAAATCGGACCAGTAGTTCCTGAG	$(GTTT)_4$	122 (122-126)	Perera et al. (2007)
HzMS4-10	F: CTAGAACGGGCTTCATGGTGAG R: AAAAATAAAATGTATTCCGGGCGT	$(ATT)_4$	113 (110-113)	Perera et al. (2007)
HzMS4-14	F: CAACATACAACATTCAGCCTGTCC R: TCAGTCGTCAGTTTTTGTCTTTGC	$(AC)_7$	132 (110-135)	Perera et al. (2007)
HzMS4-16	F: AGTGTATACGGAGCAAGAATTGGA R: TTTTGCAAATCAAACTATTGAAAAGTAA	$(ACAT)_6$	147 (134-149)	Perera et al. (2007)
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	$(TGC)_2GAT(TGY)_4GAT$ $(TGY)_{35}(TGA)_2$ $AGC(TGY)_8$	240 (228-288)	Ji et al. (2003)[a]
HarSSR6	F: TGTTGTTGCAGAGCTGCC R: TTCAGCAACACAACCGTACA	$(GHT)_{43}$	(292-340)[1]	Ji et al. (2005)[a]
HarSSR7	F: AAGCAATAATTACCAGAAACAG R: GTTTATTCGTGTATTCATTAAATAG	$(GAT)_4$	(80-176)[1]	Ji et al. (2005)
HarSSR9	F: AGCTCCACAACTCTTAACTAC R: GCAAACGATCACTGATATTAAC	$(CA)_{15}$	(189-261)[1]	Ji et al. (2005)

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

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 and Zaykin, 2001). Hardy-Weinberg equilibrium (HWE) and linkage disequilibrium were tested using the 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 and 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 et al., 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 et al. (2005). The consensus values for K were obtained with CLUMPP v. 1.1.2 (Jakobsson and Rosenberg, 2007). The best K was recognized according to the ΔK method of Evanno et al. (2005), as calculated with the web application Structure Harvester (Earl and vonHoldt, 2012).

RESULTS

Microsatellite cross-species amplification

Among the 13 SSR loci previously published for *H. zea*, the loci HzMS1-4, HzMS3-11 and HzMS3-41 were successfully amplified and evaluated in *H. armigera* (Table 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 and 4). *H. armigera* showed 16 species-specific alleles, while *H. zea* showed two species-specific alleles (Table 2). The allele sizes were similar to those reported from the authors that developed the primers, matching the expected repeat sizes (Tables 2 and 3).

Table 3. Locus name, annealing temperature (Ta), number of alleles, allele size and amplitude, and private alleles for both species of the seven microsatellite loci in *Helicoverpa armigera* and *H. zea* populations.

Locus	Ta (°C)	*H. armigera* (N = 64)[1]		*H. zea* (N = 72)[1]		Allele size and size range (bp)
		No. of alleles	Alleles	No. 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. Highlighted in bold are the species-specific alleles.

Population genetic statistics

There was no linkage disequilibrium for any locus 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 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 4).

Table 4. Genetic diversity estimates for each populations of *Helicoverpa armigera* and *H. zea* based on seven microsatellite loci.

Population	Diversity indices	HzMS1-4	HzMS3-11	HzMS3-41	HaB60	HaC87	HaC14	HarSSR1	Mean
HaBA-44	N_A	3.000	2.000	3.000	3.000	3.000	3.000	11.000	4.000
	H_E	0.298	0.359	0.554	0.236	0.152	0.573	0.879	0.436
	H_O	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	N_A	4.000	3.000	3.000	4.000	2.000	5.000	12.000	4.714
	H_E	0.354	0.507	0.219	0.373	0.047	0.730	0.871	0.443
	H_O	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	N_A	3.000	4.000	2.000	4.000	2.000	4.000	7.000	3.714
	H_E	0.301	0.333	0.370	0.477	0.239	0.777	0.847	0.478
	H_O	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	N_A	3.333	3.000	2.666	3.666	2.333	4.000	10.000	4.142
	H_E	0.317	0.411	0.406	0.359	0.138	0.689	0.876	0.452
	H_O	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
HzMG-4	N_A	3.000	2.000	2.000	2.000	1.000	1.000	4.000	2.143
	H_E	0.327	0.500	0.130	0.130	0.000	0.000	0.233	0.188
	H_O	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	N_A	3.000	2.000	2.000	2.000	1.000	1.000	4.000	2.143
	H_E	0.121	0.493	0.241	0.043	0.000	0.000	0.335	0.176
	H_O	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	N_A	2.000	3.000	3.000	3.000	2.000	1.000	3.000	2.429
	H_E	0.042	0.565	0.449	0.138	0.085	0.000	0.510	0.256
	H_O	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	N_A	2.666	2.666	2.333	2.333	1.333	1.000	3.666	2.238
	H_E	0.163	0.519	0.273	0.103	0.028	0.000	0.359	0.207
	H_O	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

N_A, number of alleles; H_E, expected heterozygosity; H_O, observed heterozygosity; *f*, inbreeding coefficient; *a*, null alleles. Significant values are highlighted in bold. Deviation from HWE: *P ≤ 0.0024.

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 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 4). 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 2). The individuals were divided into two genetic clusters according to their respective species (Figure 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).

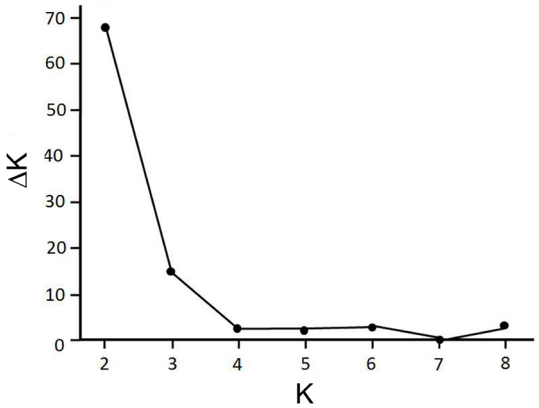

Figure 2. Graphical plot of ∆*K* for *Helicoverpa armigera* and *H. zea*; the maximum value of ∆*K* was considered to be the value of *K* (genetic groups or clusters) that best fit the data; *K* = 2. Each color (dark and light gray) represents a different cluster.

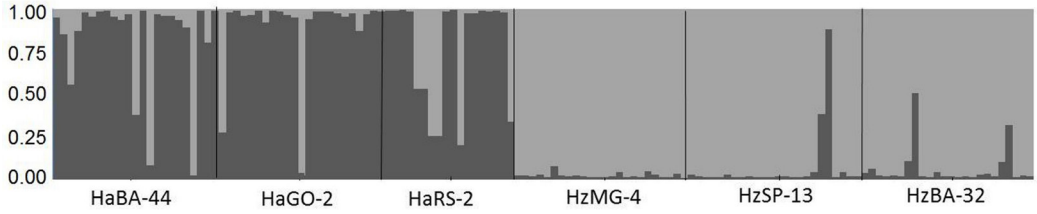

Figure 3. Bar plot for *K* = 2 clusters for *Helicoverpa armigera* (HaBA-44, HaGO-2, and HaRS-2) and *H. zea* (HzMG-4, HzSP-13, and HzBA-32) individuals assigned by STRUCTURE, based on seven microsatellite loci. Each color represents a different cluster.

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 and Hardee, 1995; Laster and 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 retested, 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 and 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 and Hardee, 1995; Laster and Sheng, 1995).

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 and Hardee, 1995; Laster and 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 et al., 2011). However, the SSR loci were not useful to characterize a large number of species-specific alleles, mainly for *H. zea* (see Table 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.

Conflicts of interest

The authors declare no conflict of interest.

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