Detection and Genetic Diversity of a Heliothine Invader (Lepidoptera: Noctuidae) From North and Northeast of Brazil

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ABSTRACT The cotton bollworm, *Helicoverpa armigera* (Hübner), was recently introduced in Brazil. During the 2012–2013 harvest, producers reported reduced yields up to 35% on major crops. The economic losses reached US$ 1 billion only in western Bahia, triggering a phytosanitary crisis. The deficiencies in existing taxonomic keys to deal with the morphologically indistinct larvae of *H. armigera* and the native *Helicoverpa zea* (Boddie) constrained the detection of new incursions of this heliothine invader. This study explored the identity of heliothine larvae that were found infesting soybean- and corn-growing areas from Roraima state, northern Brazil, through sequences of the mitochondrial *cytochrome c oxidase* subunit I gene. The inter- and intraspecies sequence variations of DNA barcodes in *H. armigera* and *H. zea* were analyzed. The genetic diversity and population structure of the specimens from Roraima and two populations from Piauí and Bahia states, northeastern Brazil, were assessed by adding the *cytochrome c oxidase* subunit II gene to the analysis. Owing to the lack of studies on genetic introgression for the two species, the suitability of using three different nuclear genes to distinguish the two species was also investigated. The results showed strong evidence that the heliothine larvae from north and northeast of Brazil are conspecific with *H. armigera*, suggesting that this invasive moth has already crossed the Amazon basin. Surveys in the north of South America should start as soon as possible to monitor the entry or spread of this moth in the Caribbean, Central America, and the United States.

KEY WORDS *Helicoverpa armigera*, *mt*DNA, elongation factor-1α, isocitrate dehydrogenase, ribosomal protein S5

The cotton bollworm, *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae), is a polyphagous species, described as feeding on 181 plant species from >60 families (Venette et al. 2003, Pogue 2004, Srivastava et al. 2005). Each female moth can lay singly up to 1,500 eggs on several parts of the host preferably at night (European and Mediterranean Plant Protection Organization [EPPO] 1981). Larvae develop through six instars and feed on both vegetative and reproductive structures of the host (Wang and Li 1984). Large larvae (longer than 2.4 cm) are the most damaging stage, as they consume ≈80% of their overall diet in the fifth and sixth instars (Srivastava et al. 2005). Once larvae are fully grown, they pupate in the soil and can go into diapause to overwinter (Karim 2000). *H. armigera* is a polyvoltine species and, depending on the weather conditions, the entire life cycle can be completed in 4–6 wk (Fitt 1989).

Owing to its dispersal capability and omnivorous behavior, *H. armigera* is the widest distributed species in the genus *Helicoverpa* (Venette et al. 2003) and poses a constant threat in international trade of many horticultural crops (Lammers and MacLeod 2007). Venette et al. (2003) reported 4,431 interceptions of *Helicoverpa* species in the United States since 1985 on fruits, vegetables, and ornamentals. From 2000–2004, *Helicoverpa* spp. was intercepted >1,400 times at United States ports, and most samples were identified to two species only, *H. armigera* and *Helicoverpa assulta* (Guenee) (Passoa 2004).

Until recently, *H. armigera* was listed as an A1 quarantine pest (not present but of potential economic importance) in Brazil (Czepak et al. 2013, Embrapa 2013). However, disturbing reports started coming from a two million hectares agricultural area in the cerrado biome that encompasses parts of the Brazilian states of Maranhão, Tocantins, Piauí, and Bahia (also known as the MATOPIBA region) in early 2013. Producers, especially from western Bahia, observed population levels never seen before of larvae morphologically similar to *Helicoverpa zea* (Boddie), and the 2012–2013 harvest was severely affected. Infestations of larvae reduced yields up to 35% on soybean, cotton, corn, beans, sorghum, and millet. Most producers in-

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creased the number of pesticide applications by >15%, and the costs in cotton fields, for example, jumped from US$ 400 to US$ 800 per hectare. The extensive economic losses reached US$ 1 billion by July 2013, generating a phytosanitary crisis at the MATOPIBA region (Embrapa 2013). Research institutes identified the new pest as *H. armigera* based mainly on the morphology of male genitalia (Hardwick 1965, Pogue 2004) and also confirmed its presence in other two states from central Brazil (Goiás and Mato Grosso; Czepak et al. 2013, Tay et al. 2013). In 1992, *H. armigera* provoked a similar crisis in North China, with estimated losses of US$ 1.3 billion to agricultural crops in the Yellow River cotton region (Sheng 1993).

Infestations in legumes and other horticultural crops are being reported in other Brazilian states, but producers and entomologists are having trouble identifying the pest. The taxonomy based on morphological characters of *Helicoverpa* spp. is complicated (Pogue 2004) and presents three problems. First, as the available morphological keys are effective only for a particular life stage or gender, a large number of specimens collected from the field are discarded because they cannot be identified. Second, the use of these keys demands a high level of expertise, which increases the frequency of incorrect identifications. Finally, as *H. armigera* and *H. zea* are capable of interbreeding to produce fertile offspring (Laster and Hardee 1995), the nonmapped phenotypic variability in the characters of the hybrids could lead to misdiagnosis of the two species.

H. armigera and *H. zea* are difficult to distinguish especially in the larval stage. Currently, only genitalia and wing characteristics of male adults are being used to discriminate the two species (Pogue 2004, Czepak et al. 2013). The lack in Brazil of sufficient number of trained taxonomic experts in Lepidoptera and the deficiencies in existing morphology-based taxonomic keys to deal with the morphologically indistinct larvae of the two species prompted the need for a new identification approach.

In the past decade, Hebert et al. (2003) proposed the first half of the mitochondrial *cytochrome c oxidase* subunit I gene (COI) as the core ("DNA barcode") of a global identification system for metazoans and showed a model COI profile that was 100% successful in correctly identifying 200 specimens from closely allied species of lepidopterans. Sequences of COI and other genes from mitochondrial DNA (*mt*DNA) have been successfully used for population genetic studies of Lepidoptera groups (Caterino et al. 2000, Behere et al. 2007, Albernaz et al. 2012). Armstrong and Ball (2005) also demonstrated the suitability of DNA barcodes in providing a practical, cost-effective, and flexible framework for the accurate diagnostic of morphologically indistinct intercepted specimens. Increasingly, molecular diagnostic tests are accepted as an essential component of detection and identification systems of exotic invasive species (Jenkins et al. 2012).

The exact distribution and extension of economic damages of the recently introduced *H. armigera* in Brazil is still not known. In July 2013, heliothine larvae were found infesting soybean- and corn-growing areas from Roraima state, northern Brazil. The major goal of this study was, therefore, to identify these larvae from the north by using sequences of mitochondrial genes. The interspecific and intraspecific sequence variation of the COI gene fragment ("DNA barcode") in the two morphologically alike species, *H. armigera* and *H. zea*, was also analyzed. The genetic diversity and population structure among the specimens from Roraima and two populations from Piauí and Bahia states, northeastern Brazil, were assessed by adding a second mitochondrial gene, *cytochrome c oxidase* subunit II gene (COII), to the analysis.

Many aspects of heliothine phylogeny remain unclear by either morphology or some molecular markers (Cho et al. 2008), and the genetic boundaries between distinct lineages of *H. armigera* and *H. zea* were not established yet owing to the lack of studies on mitochondrial introgression for the two species. In view of this, the suitability of using three different nuclear genes, which are used in phylogenomic studies with lepidopterans owing to their stability and considerable nucleotide substitution rates (Wahlberg and Wheat 2008), was investigated for the populations analyzed.

Materials and Methods

Specimens and DNA Extraction. In total, 65 heliothine larvae suspected to be *H. armigera* were collected from infested farms of eight geographic sites from the states of Roraima ($n = 14$), Piauí ($n = 39$), and Bahia ($n = 12$). The specimens from Bahia came from populations from which adult moths had been previously identified as *H. armigera* based on morphological characters (Czepak et al. 2013). Larvae were collected directly from host plants and immediately preserved in 100% ethanol at −20°C until DNA extraction.

Total DNA was extracted from the last three abdominal segments of the larvae (Behere et al. 2013) using the phenol: chloroform method, adapted for microcentrifuge tubes (Lyra et al. 2009), resuspended in 100 μl of TE buffer and stored at −20°C. The DNA from six *H. zea* larvae reared at laboratory for four generations (Callahan 1962) at the Piauí State University (UESPI) and two wild *Spodoptera frugiperda* (Smith) (Lepidoptera: Noctuidae) larvae (collected from corn in Bonfim, Roraima) was also extracted and used as control for some of the molecular analyses. Absence of cross-contamination during the extractions was confirmed by inclusion of a blank extraction among each extraction batch.

PCR Amplification and Sequencing. The partial sequences of the COI gene were amplified by PCR using the primers COI*F* (5′-ATTCAACCAATCATAAA GATATTGG-3′) and COI*R* (5′-TAAACTTCTGGAT GTCCAAAAAATCA-3′; Li et al. 2011). The primers A-*t*LEU (5′-ATGGCAGATTAGTGCAATGG-3′) and B-*t*LYS (5′-GTTTAAGAGACCAGTACTTG-3′; Liu and Beckenbach 1992) were used to amplify part of

the COII gene. Partial sequences of three protein-encoding nuclear genes, namely elongation factor-1α (EF-1α), isocitrate dehydrogenase (IDH), and ribosomal protein S5 (RpS5), were amplified by the pairs of primers HibEfrcm4 (5′-ATTAACCCTCACTAAAGACAGCV ACKGTYTGYCTCATRTC-3′) and HibAlf (5′-TAAT ACGACTCACTATAGGGGAGGAAATYAARAARG AAG-3′), IDH*deg*27*f* (5′-TAATACGACTCACTATA GGGGGWGAYGARATGACNAGRATHATHTGG-3′) and IDH*deg*R (5′-ATTAACCCTCACTAAAGTTY TTRCAIGCCCANACRAANCCNCC-3′), HibRPS5F_F (5′-TAATACGACTCACTATAGGGATGGCNGARGA RAAYTGGAAYGA-3′) and HibRPS5F_R (5′-ATTAA CCCTCACTAAAGCGGTTRGAYTTRGCAACACG-3′; Wahlberg and Wheat 2008), respectively.

The PCR reactions were conducted separately with ≈25 ng of total DNA, 2.5 mM $MgCl_2$, 0.25 mg/ml of BSA, 100 μM dNTPs, 0.5 mM of each primer, 1.5 U of *Taq*DNA polymerase (Fermentas International Inc., Burlington, Canada), and 10× TaqBuffer for a final reaction volume of 25 μl. Amplification was carried out on a GeneAmpPCR System 9700 thermal cycler (Applied Biosystems, Foster City, CA), with the following conditions: an initial denaturation at 94°C for 3 min, followed by 35 cycles of 94°C for 30 s, 55°C for 30 s (45°C for COI and 48°C for COII), 70°C for 1:30 s (72°C for COII), and ended with a 7 min final extension at 70°C (72°C for COII). After amplification, 2-μl aliquots were analyzed by 1% agarose gel electrophoresis in 1× TAE (40 mM Tris-acetate, 1 mM EDTA).

Amplicons were purified with the IllustraGFX kit (GE Healthcare, Bucks, UK) and sequenced bidirectionally, to ensure correct basecalling, by the ABI3730xl DNA Analyzer sequencer (Applied Biosystems, Foster City, CA), with the same primers used for the PCR reactions.

Sequence Analyses. Sequences were assembled into a contig for each specimen by using the Geneious 6.0.6 Software (Biomatters Ltd., Auckland, New Zealand), considering *Phred* values (Ewing et al. 1998). All sequences were aligned for each of the five genes separately using the multiple sequences alignment algorithm implemented in Clustal Ω (Sievers et al. 2011). Protein coding sequences were checked for the presence of open reading frames in MEGA 5.1 Software (Tamura et al. 2011).

Phylogenetic Analyses and Sequence Divergence. Only COI sequences were used for phylogenetic analyses owing to the lack of available sequences in public databases for the species and genetic regions investigated in this study. The 65 COI fragments from the heliothine specimens from Roraima (RR01–RR14), Piauí (PI01–PI39), and Bahia (BA01–BA12) were used to perform the phylogenetic analyses along with sequences of three *S. frugiperda* (two from Roraima ("*S. frugiperda* (01)–(02)") and a voucher under the GenBank GU090724 ("*S. frugiperda* (03)")), one *Heliothis virescens* F. (Lepidoptera: Noctuidae) (GU087832), one *Helicoverpa punctigera* (Wallengren) (EU768941), one *Helicoverpa gelotopoeon* (EU768938), one *Helicoverpa hawaiiensis* (EU768939), three *H. assulta* (GQ892856, GQ892857, GQ892859), five *H. zea* ("*H. zea* 01–03" were from UESPI laboratory, "*H. zea* 04" was a laboratory strain from Mississippi (EU768942), and "*H. zea* 05" was a Brazilian voucher (HQ571107)), and the 16 *H. armigera* haplotypes (*arm*01–*arm*16; EU768935, EU768936, GQ892840, GQ892845, GQ892846, GQ892848, GQ892849, GQ892850, GQ892853, GQ892854, GQ995238–995244) reported by Li et al. (2011).

Behere et al. (2007) investigated the genetic diversity of *H. armigera* from Asia and Africa, but the COI sequences of this study could not be compared with theirs because they used only a 511 bp fragment located in the region 980–1490 of the COI gene that was not overlapped with the region 39–696 analyzed in this study and by Li et al. (2011).

Neighbor-joining (NJ) distance analysis (Saitou and Nei 1987) and sequence divergences were calculated in MEGA 5.1 software (Tamura et al. 2011) with Kimura two-parameters (K2P; Kimura 1980) and uncorrected sequence divergences (*p*-distances) models. Node supports were measured by 5,000 replicates of bootstrap (BS).

The Bayesian inference (BI) and maximum-likelihood (ML) methods were also used for the phylogenetic analyses. The best fitted substitution model selection for the dataset was carried out using MrAIC 1.4.4 (Nylander 2004) software. The favored model for COI was the GTR+I (General Time Reversible; I = invariable sites). BI analysis was conducted using mrBayes v3.1.2 (Ronquist and Huelsenbeck 2003) with the favored substitution model selected previously. Two independent analyses were run for 10,000,000 generations (sample frequency = 1,000), with 25% of burn-in after checking for convergence. Node supports were analyzed by their posterior probabilities in the 70% majority rule consensus tree. The ML analysis was conducted using PhyML (Guindon and Gascuel 2003) with SPR tree topology search operation, 10 random starting trees as parameters and the MrAIC 1.4.4-favored substitution model. Node supports were accessed with 1,000 replicates of BS.

The DnaSP.V5 software (Librado and Rozas 2009) was used to investigate polymorphisms in COI sequences and to identify possible diagnostic characters for species discrimination between *H. armigera* and *H. zea.*

A haplotype network was constructed based on the 96 COI sequences used in the phylogenetic analyses by TCS version 1.21 (Clement et al. 2000), following the guidelines proposed by Bandelt et al. (2000). The connection between haplotypes was limited by a probability of parsimony for DNA pairwise differences lower than 95%.

Structure and Genetic Diversity of the Populations Sampled. The partial sequences of the COI and COII genes from the 65 heliothine specimens from Roraima, Piauí, and Bahia were concatenated for the genetic diversity analyses. Individual sequences were collapsed in haplotypes and, in the absence of detailed knowledge of gene flow and for the purpose of this study, each one of the three Brazilian states sampled was regarded as a different "population" in the anal-

yses. Haplotype frequencies, haplotype diversity ($\hat{H}$), and nucleotide diversity (π), as defined by Nei (1987), were estimated using Arlequin v.3.5 (Excoffier and Lischer 2010).

Analysis of molecular variance (AMOVA) was performed to access the genetic structure among and within the three populations as implemented in Arlequin v.3.5 (Excoffier and Lischer 2010). Genetic differences among populations were determined through pairwise F_{ST} statistics (Reynolds et al. 1983, Slatkin 1995). Statistical significance was accessed by 10,000 permutations, the computed distance matrix used pairwise difference, and the gamma α value was considered zero for the analysis.

Sequence Divergence Using Nuclear Genes. The partial sequences of the nuclear genes EF-1α, IDH, and RpS5 for 6 *H. zea* from laboratory rearing and 22 heliothine larvae from Roraima, Piauí, and Bahia (one representative from each one of the 22 different haplotypes resulting from the concatenation of the COI and COII fragments) were used for the estimation of inter- and intraspecific genetic distances between *H. zea* and *H. armigera.* Sequence divergence with K2P (Kimura 1980) and uncorrected sequence divergence (*p*-distances) models were calculated by using MEGA 5.1 software (Tamura et al. 2011).

Results

All specimens yielded high-quality DNA and were successfully sequenced for the fragments of COI (658 bp), COII (554 bp), EF-1α (779 bp), IDH (560 bp), and RpS5 (593 bp) genes. The COI sequences obtained for the specimens from Roraima, Piauí, and Bahia were blasted against GenBank database and checked in BOLD Identification System (BOLD 2007), and all the 65 heliothine larvae matched 99–100% with *H. armigera* sequences. Chromatograms were reliable for all the gene fragments and, after terminal cutoff owing to sequences ambiguities, the absence of indels made the alignment straightforward. The *mt*DNA and nuclear sequences generated in this study have been deposited in GenBank (KF624811–KF625029).

Phylogenetic Analyses and Sequence Divergence. Four different phylogenetic analyses (NJ under *p*-distance and K2P models, BI, and ML under MrAIC 1.4.4-favored substitution model) conducted for the COI sequences gave nearly identical tree topologies (Fig. 1). NJ and BI proved to be reliable measures of species delimitation, as all identifiable species with more than one sequence (*S. frugiperda*, *H. assulta*, and *H. zea*) had all individuals joined by >98% BS and 1.0 posterior probability (PP). The 65 heliothine specimens from Roraima, Piauí, and Bahia joined by >95% BS and 0.9 PP values formed a monophyletic group with the 16 *H. armigera* sequences reported by Li et al. (2011). The *H. armigera* group formed a robustly supported monophyletic clade with *H. zea* (80%/77% NJ BS, 0.91 BI PP, and 79% ML BS), as expected for these species (Cho et al. 2008).

The genetic distance (Table 1) between the investigated heliothine specimens from Roraima, Piauí, and Bahia and the 16 *H. armigera* COI sequences reported by Li et al. (2011) revealed very low sequence divergence (0–0.8%), suggesting conspecificity among the specimens (Avise 2000). For instance, the sequence divergence between the 65 heliothine specimens and the *H. zea* group exceed that divergence, ranging approximately from 2 to 3%, likely the range observed between the *H. armigera* group and *H.zea* (Table 1). For both distance models used (K2P and *p*-distance), there was substantial sequence divergence between the 65 heliothine specimens and the four other species of the genus *Helicoverpa* (≈2.6–6.3%), *He. virescens* (≈7–8%), and *S. frugiperda* (≈10–12%; Table 1).

The sequences from the 65 heliothine specimens (RR01–RR14; PI01–PI39; and BA01–BA12) and the 16 *H. armigera* (*arm*1–*arm*16; Li et al. 2011) were collapsed into 21 different haplotypes, which were linked in a unique parsimony network (Fig. 2). The general topology of the network showed one clade containing all *H. armigera* haplotypes, separated by >13 mutational steps from *H. zea* and 11 steps from the other heliothine species. The two most common *H. armigera* COI haplotypes, designated by Li et al. (2011) as "*arm*1" (found in Thailand and the Chinese province of Yunnan) and "*arm*2" (found in Yunnan and Henan provinces, China), are most likely to be ancestral (Castelloe and Templeton 1994). Five specimens from Roraima, 18 from Piauí, and 8 from Bahia were identical to "*arm*1," and 6, 11, and 2 specimens from Roraima, Piauí, and Bahia, respectively, were identical to "*arm*2" (Fig. 2). Most of the other *H. armigera* haplotypes were rare (single individuals) and their frequency was comparatively high (15 out of 21). Haplotypes of *H. armigera* differed from the center of the network by no more than three mutational steps. No haplotype was shared among the six *Helicoverpa* species, and *He. virescens* differed by 62 mutational steps from the *H. armigera* group (Fig. 2).

The DNA barcodes generated for *H. armigera* and *H. zea* (Table 2) based on combinations of diagnostic nucleotides allowed the observation of 30 nucleotide substitutions between the two species, with 14 unique diagnostic characters that could be used to discriminate the species.

Structure and Genetic Diversity of the Populations Sampled. The COI and COII fragments from the 65 specimens from Roraima, Piauí, and Bahia were concatenated, generating a final 1212-bp sequence for each individual. In total, 22 different haplotypes were defined (Fig. 3). Haplotypes were shared among samples from the three Brazilian states, especially the haplotype one, the most frequent, suggesting a significant gene flow among these regions. In general, high haplotype diversity ($\hat{H}$-mean > 0.85) and low nucleotide diversity (π-mean < 0.003) were observed for the locations analyzed (Fig. 3). AMOVA detected no genetic structure ($F_{ST} = 7.10^{-5}$; $P = 0.44$), with 99.9% of variation accounted for at the within population level and with only 0.01% variation observed among

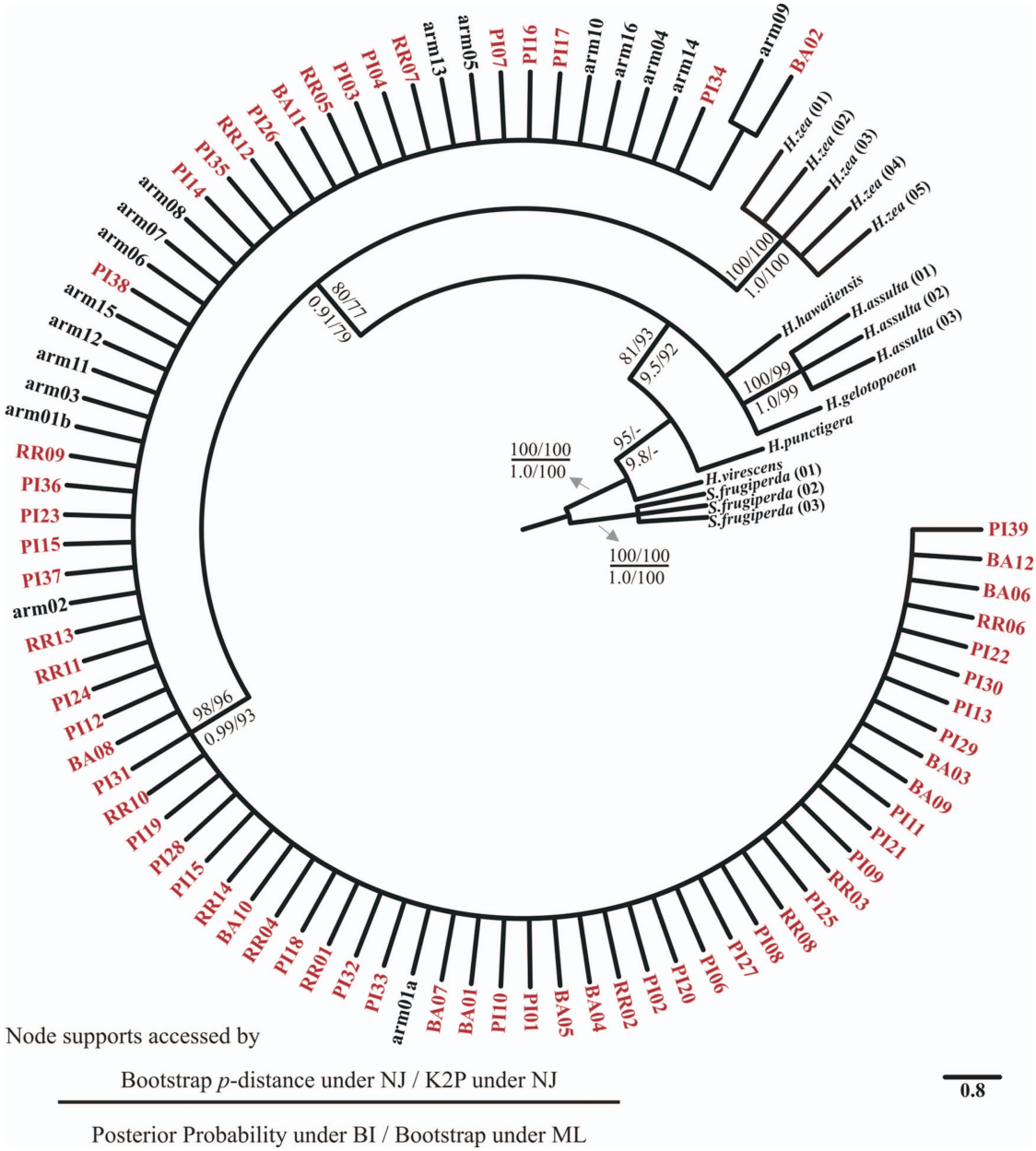

Fig. 1. NJ strict consensus tree (topology under *p*-distance model) inferred using COI complete dataset for Heliothine species. The 65 specimens from Roraima (RR01–RR14), Piauí (PI01–PI39), and Bahia (BA01–BA12) are in red. Sequences of *S. frugiperda*, *He. virescens*, and *H. punctigera* were used as outgroups. Numbers above branches refer to NJ BS proportions among 5,000 replicates, while numbers below branches refers to BI PP and ML BS proportions among 1,000 replicates. The four analyses gave nearly identical tree topologies, and node supports values below 70% (BS), 0.7 (PP), or both, were not recorded in the tree (−).

populations. Pairwise F_{ST} values (considering the individual three Brazilian states as separate populations) were low and not significant ($F_{ST} < 0.03$; $P > 0.05$) in the studied specimens (Table 3).

Sequence Divergence Using Nuclear Genes. The genetic distances between 22 heliothine specimens from Roraima, Piauí, and Bahia and 6 *H. zea* from laboratory rearing estimated by three different nuclear genes were low (K2P and uncorrected *p*-distance <3%; Table 4). The highest number of variable sites (2.7%) was observed for the fragments of the RpS5 gene, but the highest sequence divergence (2.2%) was given by the EF-1α gene. As the intraspecific range of differences from both *H. armigera* and *H. zea* overlapped the interspecific ranges (Table 4), the three nuclear gene fragments investigated failed to

Table 1. Mitochondrial DNA COI pairwise genetic distances for the investigated heliothine specimens from Brazil and other Lepidoptera species

Species compared			Kimura two-parameters (K2P)	Uncorrected sequence divergences (*p*-distance)
			Range	Range
Investigated *Heliothine* specimens[a]	vs	*S. frugiperda*	0.114–0.120	0.099–0.103
		He. virescens	0.076–0.079	0.068–0.071
		H. punctigera	0.058–0.063	0.053–0.058
		H. gelotopoeon	0.035–0.040	0.033–0.038
		H. assulta	0.032–0.37	0.030–0.035
		H. hawaiiensis	0.027–0.032	0.026–0.030
		H. zea	0.023–0.027	0.024–0.029
		H. armigera	0.000–0.008	0.000–0.008
H. armigera[b]	vs	*H. zea*	0.026–0.031	0.024–0.029

[a] The investigated heliothine specimens comprised the 65 larvae sampled from Roraima, Piauí, and Bahia states.
[b] The *H. armigera* group encompassed only the 16 COI haplotypes described by Li et al.(2011).

robustly discriminate the two morphologically alike species.

Discussion

The *mt*DNA COI phylogeny strongly supported that the 65 heliothine larvae from Roraima, Piauí, and Bahia are conspecific with *H. armigera.* The four phylogenetic analyses grouped these specimens with 16 *H. armigera* COI sequences described by Li et al. (2011) as a single monophyletic group. The other defined species (*S. frugiperda*, *He. virescens*, and the other five *Helicoverpa* species) were recovered in distinct positions or clades on strongly supported branches (Fig. 1). The *H. zea* clade was monophyletic with the *H. armigera* clade, and this same phylogenetic pattern had been observed in previous studies (Fang et al. 1997, Cho et al. 2008). Behere et al. (2007) suggested that the American *H. zea* populations were established via founder event from *H. armigera* (or from their joint common ancestor; Mallet et al. 1993) no >1.5 million years ago, which is further supported by the high similar morphology and the possibility of mating compatibility between the two species (Laster et al. 1985, Laster and Hardee 1995, Laster and Sheng 1995).

Despite the recent history of separation between the two species (Behere et al. 2007) and the low sequence divergences expected for the order Lepidoptera (Caterino et al. 2000, Hebert et al. 2003), the information content of the COI fragment selected in this study was sufficient to enable recognition of the divergence among the lepidopterans analyzed. The estimated genetic distances (K2P and *p*-distance) indicated divergence values between species >2%. Setting this value as a threshold for species diagnosis in this study allowed the discrimination of all heliothine species analyzed (Table 1). The low divergence (0–0.8%) between the 65 heliothine specimens from north and northeast of Brazil and the *H. armigera* sequences from Li et al. (2011) indicated conspecificity among these individuals, and both groups diverged similarly with *H. zea* (≈2–3% for K2P and *p*-distance;Table 1). Hebert et al. (2003) also designated a 2–3% *mt*DNA COI sequence divergence as a "threshold" to discriminate insect and mammal species. However, as rates of genetic change between taxa are dynamic processes (Rubinoff et al. 2006), thresholds used to define species may vary widely among studies with lepidopterans. Hebert et al. (2004) used a threshold as low as 0.32% for skipper butterflies, and Whinnett et al. (2005) observed divergences from 0.23 to 6.4% in Ithoninae butterflies, demonstrating that species thresholds can be extremely fluid.

The *mt*DNA COI haplotype network showed that the 65 heliothine samples from Roraima, Piauí, and Bahia formed a homogeneous group of *H. armigera* haplotypes (Fig. 2). The connections between haplotypes was similar to that reported by Li et al. (2011), with *H. armigera* haplotypes differing from the center of the network by no more than three mutational steps and no shared haplotypes among the different species. Two Chinese haplotypes, "*arm*1" (EU768935) and "*arm*2" (EU768936; Cho et al. 2008, Li et al. 2011), encompassed 47.7 and 29.2%, respectively, of the haplotypes found in the samples from Roraima, Piauí, and Bahia, indicating that "*arm*1" is a potential ancestral haplotype of *H. armigera. H. zea* appeared linked to the *H. armigera* group (by 15 mutational steps) through the haplotypes from samples PI-34, PI-3, PI-4, and RR-7 (Fig. 2). In the haplotype network reported by Behere et al. (2007), the *H. armigera* and *H. zea* groups were separated by ≈20 mutational steps.

This study observed shared haplotypes between the three Brazilian states, not significant F_{ST} values, and low nucleotide diversity between the sampled regions from north and northeast of Brazil (Table 3; Fig. 3). In general, high values of haplotype diversity and low nucleotide diversity, combined with a high number (15 out of 22) of low frequency haplotypes (Fig. 3), are characteristic of species that have undergone a process of recent population expansion (Excoffier et al. 2009).

Many authors, using different molecular methods, have verified similar patterns of genetic variation, which seems to be common in insect pests capable of rapid spatial expansion and long-range dispersion such as *H. armigera.* Daly and Gegg (1985) observed very little genetic variation (F_{ST} = 0.012) between Australian *H. armigera* populations from a 3,000-km study area using isozymes. Nibouche et al. (1998) verified

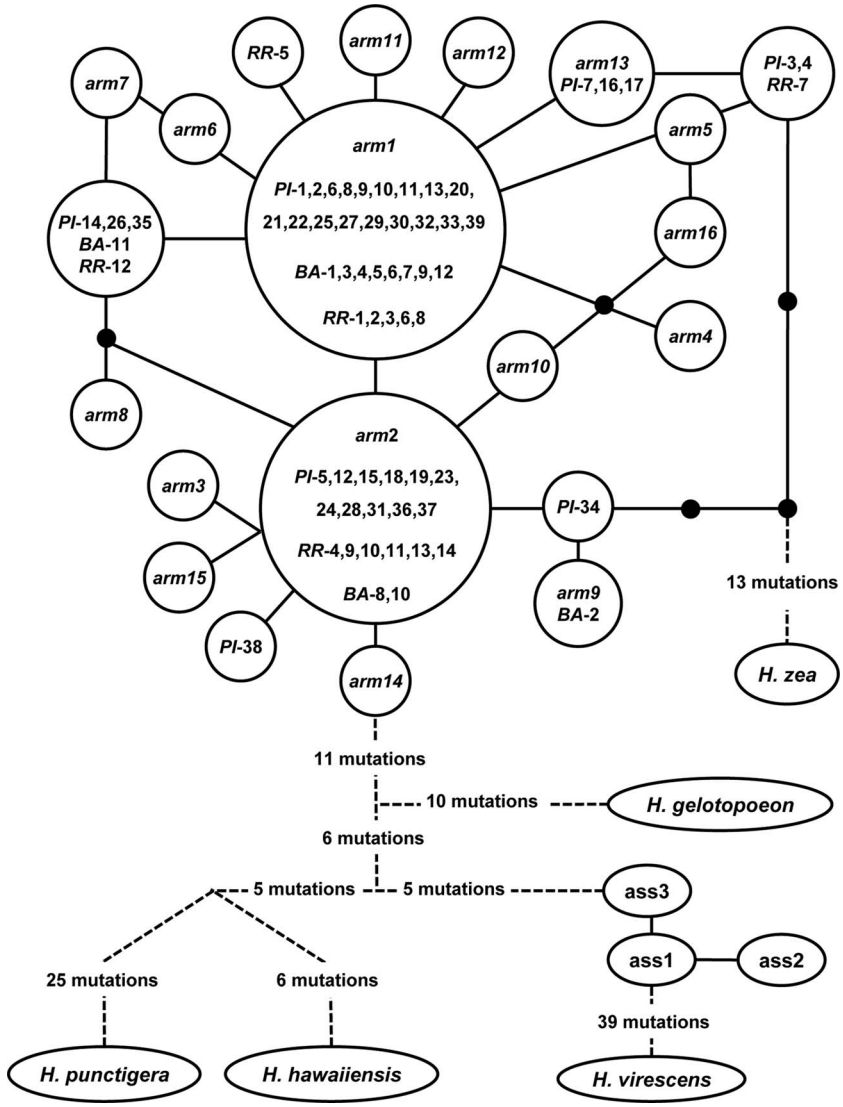

Fig. 2. Haplotype network based on partial *mt*DNA COI (658 bp) of heliothine species. Each haplotype is represented by a circle. The original names of the haplotypes reported by Li et al. (2011) (*arm*1–*arm*16) and the 65 specimens from Roraima (RR01–RR14), Piauí (PI01–PI39), and Bahia (BA01–BA12) were maintained. Full lines between haplotypes represent one mutational step, while the dots are presumptive intermediate haplotypes that were not observed.

that barriers such as the Sahara desert had not prevented long-distance migration in *H. armigera*, as no significant isozyme allele frequency differences were found between populations from either side of the desert. Zhou et al. (2000) reported very low genetic distances among Israeli and Turkish *H. armigera* populations using RAPD-PCR analysis. Using microsatellite loci, Endersby et al. (2007) found no evidence of genetic structure among samples from Australia and New Zealand collected at different times. Behere et al. (2007) suggested the occurrence of long-distance gene flow in *H. armigera*, based on low F_{ST} values and low among-group haplotype variance across Australia, China, India, Pakistan, Burkina Faso, and Uganda. No significant population substructure across India, irrespective of cropping seasons, were inferred from EPIC-PCR DNA markers (Behere et al. 2013).

Long-distance migration of this heliothine pest has also been suggested by capture of adult moths on Ascension Island (2,000 km from the African coast; Widmer and Schofield 1983) and Willis Island in the Coral Sea (450 km from the coast of Australia; Daly and Gregg 1985). Borne by wind, *H. armigera* can reach the United Kingdom from southern Europe (>1,000 km; Pedgley 1985). Mark–recapture experiments have shown that *H. armigera* moths could fly 200–300 km in a single night (Armes and Cooter 1991).

Correct and prompt detection of this highly mobile invasive pest is an essential step before initiating quarantine actions, proper control measures, and quick

Table 2. DNA barcodes for *H. armigera* and *H. zea* based on combinations of diagnostic nucleotides represented by the International Union of Pure and Applied Chemistry symbols

N.P[a]	*H. armigera*	*H. zea*	N.P	*H. armigera*	*H. zea*
40	R	A	340	***C***	T
85	Y	T	346	M	A
115	***T***[b]	C	368	R	G
127	***T***	C	385	Y	T
130	R	A	386	***C***	T
181	K	T	418	***C***	T
217	***A***	G	439	Y	T
238	***C***	T	477	***G***	A
262	R	A	478	***C***	T
268	***T***	C	496	R	A
274	Y	T	536	***T***	C
277	***T***	C	539	Y	T
278	***T***	C	548	***T***	C
286	R	A	595	Y	C
313	Y	T	616	Y	T

[a] N.P. indicates the nucleotide position which is relative to the beginning of the fragment investigated in this study.
[b] The 14 pure diagnostic characters are shaded in bold italics.

Table 3. Pairwise F_{ST} estimates among three investigated heliothine populations from Brazil based on combined genes COI and COII

Populations	Piauí	Bahia	Roraima
Piauí	0.0000		
Bahia	0.0255 (P=0.15)	0.0000	
Roraima	−0.0056 (P=0.48)	−0.041 (P=0.91)	0.0000

response to any incursion (Li et al. 2011). The current study demonstrated the effectiveness of DNA barcodes for distinguishing *H. armigera* from *H. zea* (Table 2). The use of DNA barcodes has also succeeded in species-level identification for >50,000 species of Lepidoptera so far (Hebert et al. 2004, Hajibabaei et al. 2006, Burns et al. 2008, Silva-Brandão et al. 2009, Jinbo et al. 2011).

Despite the high resolution to distinguish the six *Helicoverpa* species analyzed, DNA barcodes should not be used blindly in the context of biosecurity (Armstrong and Ball 2005, Darling and Blum 2007). There are some shortcomings of *mt*DNA for "barcoding" identification, which includes recombination, inconsistent mutation rate, heteroplasmy, maternal inheritance, and introgression (Rubinoff et al. 2006, Krishnamurthy and Francis 2012). Although the possibility of mating incompatibility via mechanical isolation between *H. armigera* and *H. zea* was reported (Hardwick 1965), mating compatibility between laboratory *H. zea* from the United States and wild *H. armigera* from Asia and Australia under controlled conditions (Laster et al. 1985, Laster and Hardee 1995, Laster and Sheng 1995) is also possible. These studies, allied to the inexistence of hybridization trials between *H. armigera* and *H. zea* populations from Central and South America, raise the question if the 65 heliothine larvae from north and northeast of Brazil are from a pure strain of the recently introduced *H. armigera* or a hybrid originated from the cross between a *H. zea* male and a *H. armigera* female (Caterino et al. 2000, Hebert et al. 2003).

New gene regions have been successfully sequenced for Lepidoptera and primers for the genes

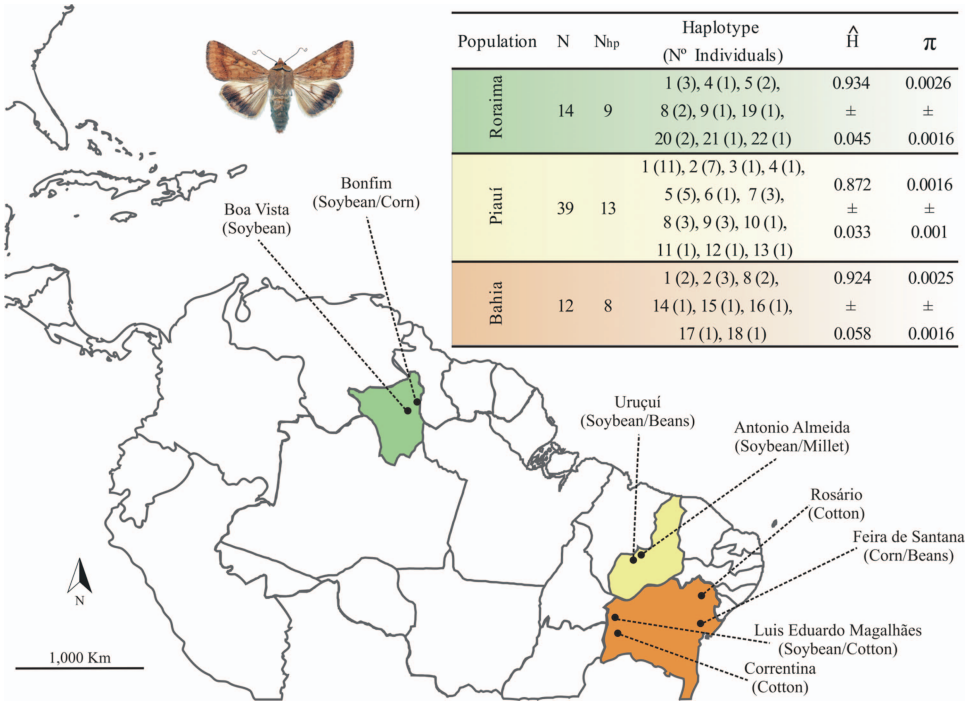

Fig. 3. Heliothine sampled sites, haplotype distribution, and genetic variability indices (±SE).

Table 4. Interspecies and intraspecies divergences given by three nuclear genes for the *H. armigera* and *H. zea* investigated

Gene	No. of specimens sequenced	Amplicon length	No. of variable sites	Genetic distances					
				Interspecific		Intra. *H.armigera*		Intra. *H. zea*	
				K2P	*p*-distance	K2P	*p*-distance	K2P	*p*-distance
EF-1α	28 (6 *H. zea* and 22 *H. armigera*	779bp	17 (2.1%)	0.004–0.022	0.004–0.022	0–0.008	0–0.006	0–0.022	0–0.022
IDH		560bp	7 (1.25%)	0.004–0.018	0.004–0.018	0–0.008	0–0.008	0	0
RpS5		593bp	16 (2.7%)	0–0.019	0–0.018	0–0.017	0–0.016	0	0

EF-1α, IDH, and RpS5 were suggested to be universal in Lepidoptera, giving robustness for most species (Wahlberg and Wheat 2008). Trying to overcome the unresolved hybridization issue, sequence divergence between *H. zea* and *H. armigera* was also explored using these three nuclear genes. Nonetheless, the variation of the fragments analyzed was not sufficient to robustly discriminate *H. armigera* from *H. zea* (Table 4). The highest sequence divergence obtained was 2.2%, but inter- and intraspecific divergences were overlapped. In general, nuclear DNA undergoes relatively slow mutation compared with *mt*DNA, which may provide for the first a smaller degree of taxonomic resolution at the species level (Waugh 2007). A monophyletic relationship between *H. zea* and *H. armigera* was inferred previously based on the EF-1α gene (Cho et al. 1995) and the Dopa Decarboxylase (DDC) gene (Fang et al. 1997). The insufficient variation in the EF-1α, IDH, and RpS5 genes to accurately reconstruct the recent divergence between the species may be a consequence of the common ancestry (Mallet et al. 1993, Behere et al. 2007).

Further studies to search for combining evidence from *mt*DNA and more rapidly evolving nuclear DNA markers with pure strains and hybrids from *H. armigera* and *H. zea* will be necessary to enhance the robustness of any key and assignment of identification for these two species. While comprehensive protocols with new molecular markers giving higher taxonomic resolution are not developed, *mt*DNA-based identification systems will undoubtedly continue to provide diagnostic and geographic origin information that exceeds that which can be achieved by using morphological studies alone (Armstrong and Ball 2005, Behere et al. 2007). The *mt*DNA-based systems can also help to speed the work of policy makers interested in the anticipatory detection of heliothine invasive species.

The strong evidence that the heliothine larvae from Roraima are conspecific with *H. armigera* under significant gene flow with northeastern populations suggest that this invasive moth is not restricted to crop fields from northeast and central Brazil and probably already has crossed the Amazon basin. The data of the current study, however, do not allow to precise the likely source of the invasions. Tay et al. (2013) were also unable to define the origin of the *H. armigera* introduced in Brazil, but suggested that the samples from Mato Grosso might have come from either multiple recent incursions or a single incursion from mixed populations of *H. armigera.*

A broader geographic sampling in northern Brazil and surveys in Venezuela and Colombia should start as soon as possible to monitor its entry or spread in the Caribbean, Central America, and the United States. The increasing rates of *H. armigera* infestations in Brazil were favored by many conditions (tropical climate, extensive agriculture, wide host availability, insecticide resistance, and drought in northeastern states; Behere et al. 2013, Czepak et al. 2013). Finding similar conditions in other regions of America, this heliothine invader could trigger more phytosanitary crisis.

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