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# Genetic diversity and population structure of *Helicoverpa gelotopoeon* populations from Argentina inferred by mitochondrial DNA COI and CytB gene sequences

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## Abstract

The South American bollworm, *Helicoverpa gelotopoeon* (Dyar), is a member of the Heliothinae complex in Northwestern Argentina and one of the most important pests affecting soybean in the country. Currently, management of Heliothinae pest species is based on the use of insecticides and transgenic *Bt* crops. However, many species in the Heliothinae complex have developed resistance to these control tactics. In this regard, studies of population structure and genetic diversity studies in these agricultural insect pests are of great importance for the effective implementation of management strategies. Mitochondrial DNA is one of the types of molecular markers available for insect studies successfully employed for population genetic studies in Lepidoptera. The aim of this study was to evaluate the genetic diversity and population structure of five *H. gelotopoeon* populations collected from different regions and crops in Argentina using analysis of three mitochondrial DNA regions. Results of genetic variability and gene flow analyses among *H. gelotopoeon* populations indicated, in general, a certain genetic structure in studied populations. Possible causes for this genetic structure include differences among regions, host plants and year of sampling. Additional studies contemplating the impact of biological and ecological features are necessary in understanding the source of genetic structure in these *H. gelotopoeon* populations.

**Key words:** South American bollworm, Heliothinae, insect resistance management, population genetics.

## Introduction

The South American bollworm, *Helicoverpa gelotopoeon* (Dyar) (Lepidoptera Noctuidae), is one of the pest species in the Heliothinae complex in Northwestern Argentina, also including *Helicoverpa zea* (Boddie), *Helicoverpa armigera* (Hubner), and *Chloridea virescens* (F.) (Murúa *et al*., 2016). Larvae of *H. gelotopoeon* is one of the most important soybean pests in Argentina, although they have been recorded feeding on 10 plant families, including chickpea *Cicer arietinum* L. (Fabales Fabaceae), cotton *Gossypium hirsutum* L. (Malvales Malvaceae), sunflower *Helianthus annuus* L. (Asterales Asteraceae), flax *Linum usitatissimum* L. (Malpighiales Linaceae), and maize *Zea mays* L. (Poales Poaceae) (Pastrana, 2004). Larvae are typically seed consuming pests, since they prefer soybean and chickpea pods, linen and cotton capsules, and sunflower flowers and seeds (Parisi and Iannone, 1978).

Currently, management of species in the Heliothinae complex is based on the use of insecticides and transgenic crops producing insecticidal proteins (*Bt* crops). However, many species in this complex have developed resistance to these control tactics (Forrester *et al.*, 1993; Gould *et al*., 1995; Armes *et al*., 1996; Hardee *et al*., 2001; Li *et al*., 2007; Mahon *et al*., 2007; Pietrantonio *et al*., 2007; Gao *et al*., 2009; Liu *et al*., 2010; Bird and Downes, 2014; Brévault *et al*., 2015; Reisig and Reay-Jones, 2015; Tay *et al*., 2015). Population structure and genetic diversity studies are of great importance for the effective implementation of management strategies of agricultural insect pests. Assessing the nature and extent of molecular diversity is essential for understanding the behaviour, response to selection pressure, structure and dynamics of different insect populations, and susceptibility to natural enemies and control tactics (Joyce *et al*., 2014). Thus, population structure and genetic diversity define the level of susceptibility of a population and its adaptive capacity to environmental changes (Kremer *et al*., 2012; Sridhar *et al*., 2016). In this regard, a better understanding of the genetic differences among populations of a polyphagous pest like *H. gelotopoeon* can be very useful to understand population dynamics and improve management tactics.

Among the different types of molecular markers available for insect studies, mitochondrial DNA (mtDNA) has been successfully employed in population genetic studies of several lepidopteran species (Behere *et al*., 2007; Albernaz *et al*., 2012; Asokan *et al*., 2012; Leite *et al*., 2014; Sridhar *et al*., 2016; Men *et al*., 2017; Tay *et al*., 2017). Due to their relatively small size, high rate of evolutionary change and maternal inheritance, mitochondrial markers are especially suitable for the analysis of populations and the history and evolution of closely related

taxa (Simon *et al*., 1994; Caterino *et al*., 2000; Albernaz *et al*., 2012).

Among mitochondrial genes, the evolutionary rates of cytochrome oxidase I (COI) and cytochrome B (CytB) are suitable for the detection of population level differences, and have been widely used to assess the genetic variation and phylogeny of several insect groups (Zhou *et al*., 2016; Men *et al*., 2017; Chen *et al*., 2018; Meng *et al*., 2018). However, there is scarcity of data on genetic diversity and population structure among populations of *H. gelotopoeon* using mitochondrial markers. The only available mitochondrial genetic studies on this species involved phylogeny estimates based on the regions of mitochondrial COI (Cho *et al*., 2008). Arneodo *et al*. (2015) compared COI sequences from *H. gelotopoeon* specimens from Argentina and observed low intraspecific genetic variability, Walsh *et al.* (2019) reported on the complete mitochondrial genome of two *H. gelotopoeon* individuals from Argentina. However, the number of individuals analysed in these studies was very low. Nevertheless, populations of other related species were assessed using these markers (Albernaz *et al*., 2012; Asokan *et al*., 2012; Leite *et al*., 2014; Mastrangelo *et al*., 2014; Anderson *et al*., 2016; Arnemann *et al*., 2019; Gonçalves *et al*., 2019; Tembrock *et al*., 2019).

The aim of this study was to evaluate the genetic diversity and population structure of five populations collected from different regions and hosts of Argentina by using three mtDNA regions.

## Materials and methods

### Sample collection

Specimens of *H. gelotopoeon* were collected either as adults (using light traps) or larvae (through vertical sheets or direct sampling) from different hosts at five locations between 2013 and 2014 (table 1, figure 1). Species identification was performed by genitalia observation according Velasco de Estacul *et al*. (1969). Each sampling location was treated as a population. Moths collected using a light trap (Cha.Cot) were stored at –20 °C (table 1). To avoid DNA contamination with endoparasitoids, larvae were reared until adult stage, according to Herrero *et al.* (2017) and Murúa *et al*. (2021), and then stored at –20 °C until DNA extraction.

### DNA extraction

Genomic DNA was extracted from 73 *H. gelotopoeon* adults and seven *H. zea* (to be used as an outgroup) specimens collected from corn at Tucumán province. Insect tissues (legs and head) were homogenized in 480 µL of DNA extraction buffer (200 mM Tris-HCl, pH 8.0, 70 mM EDTA, pH 8.0, NaCl 5M, 1% β-mercaptoethanol). Homogenates were mixed with 120 µl of 10% CTAB and incubated at 65 °C for 5 minutes and sequentially digested with 6 µl of proteinase K (10 mg $ml^{-1}$). After incubation for 1 hours at 65 °C, microtubes were centrifuged at 16,000 g for 20 minutes. Nucleic acids were extracted in an equal volume (500 µl) of chloroform: isoamyl alcohol (24:1). After centrifugation for 20 minutes, the aqueous layer (≅ 400 µl) was collected and DNA was precipitated with an equal volume of cold isopropanol with 45% of ammonium acetate (180 µl). After gentle mixture, microtubes were incubated at 20 °C for 2 hours or at –4 °C overnight, and then centrifuged (16,000 g) for 20 minutes. The pellet was resuspended and washed in cold 70% ethanol, air-dried, and resuspended in TE buffer containing 0.8 µl of RNAse solution (40 µg $ml^{-1}$). DNA was then incubated at 27 ± 1 °C for 30 minutes to 2 hours for RNA degradation. Integrity of purified DNA

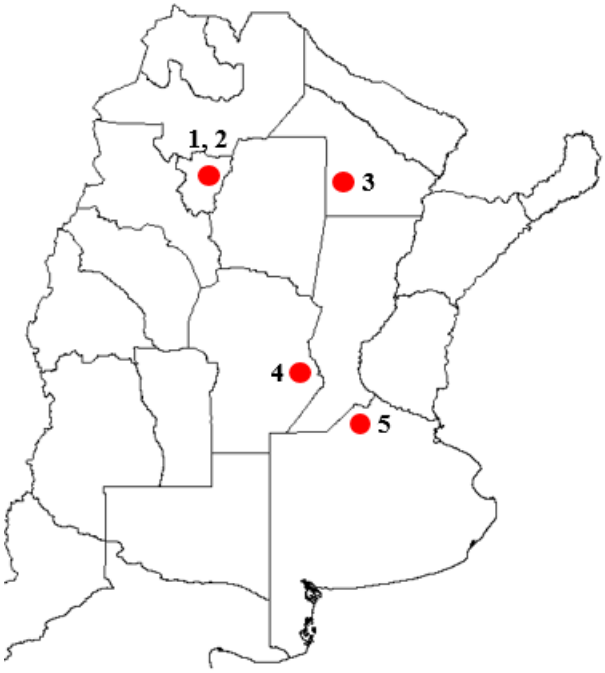


**Figure 1.** Geographic location of *H. gelotopoeon* sampling sites. 1: Cruz Alta, Tucumán (Chickpea); 2: Cruz Alta, Tucumán (Soybean); 3: Las Breñas, Chaco (Cotton); 4: Marcos Juarez, Córdoba (Chickpea); 5: Rojas, Buenos Aires (Soybean).

**Table 1.** Collection details of populations of *H. gelotopoeon* from Argentina.

| Geographic region | Province | County | GPS | Collection date | Host plant | n | Code |
|---|---|---|---|---|---|---|---|
| Northwest | Tucumán | Cruz Alta | 26°50'21"S 64°51'32"W | Jan 2013 | Soybean | 17 | Tuc.Soy |
| Northwest | Tucumán | Cruz Alta | 26°50'21"S 64°51'32"W | Sept 2014 | Chickpea | 16 | Tuc.Chic |
| Northeast | Chaco | Las Breñas | 27°05'20"S 61°06' 20"W | Oct 2013 | Light trap* | 13 | Cha.Cot |
| Pampas region | Buenos Aires | Rojas | 34°11'02"S 60°44'14"W | Feb 2014 | Soybean | 11 | BsAs.Soy |
| Pampas region | Córdoba | Marcos Juárez | 32°43'14.25"S 62°07'00.30"W | Oct 2014 | Chickpea | 16 | Cba.Chic |

*Individuals were collected from a light trap located in a cotton field.

was assessed using 1% agarose electrophoresis in 1 × TBE buffer at 120 V $cm^{-1}$ for 1 h and staining with 10 mg $ml^{-1}$ of ethidium bromide. DNA was visualized using a trans-illuminator and DNA concentration was determined using a NanoDrop 8000 Spectrophotometer v.2.0 (Thermo Fisher Scientific, Wilmington, DE, USA). The purified DNA samples were diluted to a final concentration of 5 ng $\mu l^{-1}$.

### DNA amplification and sequencing

Two fragments of the *H. gelotopoeon* mitochondrial COI gene (Cox1fin and Cox1in) and one fragment of the mitochondrial CytB gene were amplified and sequenced using the following primers: COI-F02 (5'-CTCAAATTAATTACTCCCCATC-'3) and COI-R02 (5'-GGAGGTAAGTTTTGGTATCATT-3') for Cox1fin; COI LCO1490-J-1514 (5'-GGTCAACAAATCATAAAGATATTGG-3') and COI HCO2198-N-2175 (5'-TAAACTTCAGGGTGACCAAAAAATCA-3') for Cox1in; CytBF02 (5'-GAATCCTTTAATTTAAAATATAC-3') and CytBR02 (5'-AAATATGGGTTAGTTAAAGTTAA-3') for CytB (Behere *et al*., 2008; Specht *et al*., 2013).

The total reaction volumes of 25 µl contained 6 µl of total DNA (5 ng/µl), 2.5 µl of PCR Buffer 10×, 1.2 µl of MgCl2 50 mM, 2 µl of dNTPs 2.5 mM, 2 µl of DMSO 5%, 1 µl of each primer (5 µM), 0.2 µl of Taq polimerase and 9.1 µl of Milli-Q water to reach the final volume. Amplification was performed using an initial denaturation step at 94 °C for 4 minutes followed by 35 cycles at 91 °C for 30 seconds, 50 °C for 30 seconds, 72 °C for 1 minute and a final extension at 72 °C for 10 minutes. The amplified products were resolved on 1.3% agarose gel and then submitted to electrophoresis in an automatic sequencer.

Obtained sequences were edited with the programme Sequencher® version 5.4.6 and aligned with ClustalW program available in BioEdit v.7.0.5.3 software (Hall, 1999).

### Data analysis

Sequences of the COI gene (Cox1fin and Cox1in) were concatenated to yield a total length of 1086 bp, and the CytB gene sequence was analysed separately. The BsAs.Soy population was excluded from CytB analyses due to lack of amplification for this region, but this population was included in COI analyses.

Estimates of the number of haplotypes (H), haplotype diversity (HD), nucleotide diversity ($\pi$) and average number of nucleotide differences (k) were carried out using DnaSP v.5 software (Librado and Rozas, 2009).

To depict relationships among haplotypes, a network was constructed with the TCS v. 1.21 software (Clement *et al*., 2000) using a 95% connection limit, which uses the statistical probability based on the parsimony criterion to estimate genealogies of haplotypes (Templeton *et al*., 1992).

The level of genetic differentiation between pairs of populations was estimated using the index of pairwise population differentiation ($F_{ST}$) (Weir and Cockerham, 1984) computed after 10,000 permutations in Arlequin 3.5 (Excoffier *et al*., 2010).

Analysis of molecular variance (AMOVA) was also calculated in Arlequin 3.5 to assess the genetic structure among and within populations of *H. gelotopoeon*. Four analyses were conducted to quantify the distribution of the molecular variation attributed to the presence of genetic structure: (i) among individuals from all populations (non-hierarchic), (ii) among individuals sampled from different hosts, (iii) among individuals sampled from different regions, and (iv) among individuals from different years of sampling. The significance of the variance component estimates for the different hierarchical subdivisions was determined after 1000 non-parametric permutations.

Additionally, selective neutrality tests based on Tajima's parameter *D* (Tajima, 1989), and Fu's parameter $F_s$ (Fu, 1997) were also estimated. These analyses were also developed on Arlequin 3.5.

Genetic distances were calculated based on pairwise matrix of sequence divergences using Kimura's two parameter methods implemented in MEGA 6.0 software. The maximum likelihood method was used for phylogeny reconstruction of haplotype sequences. The reliability of each branch was determined by using the non-parametric boot-strapping procedure with 1000 replicates (Felsenstein, 1985).

## Results

### Genetic diversity

Among the two sequenced regions, COI presented the highest number of haplotypes (47), haplotype diversity (0.967) and nucleotide diversity (0.03712). This marker also showed the highest percentage of variable sites (18.42%) and average number of nucleotide differences (40.31). In contrast, the CytB marker presented lower values for these parameters (H = 10; HD = 0.407 ± 0.086; $\pi$ = 0.00402 ± 0.00245; 8.25%; k = 1.56) (table 2).

The Tajima *D* and Fu $F_s$ statistics when considering all the populations were significant and negative, which would indicate an ancestral population expansion (table 2).

For the COI region, the Tuc.Soy, Cha.Cot and BsAs.Soy populations presented the highest haplotype diversity (HD = 1.00 ± 0.030; 1.00 ± 0.006 and 1.00 ± 0.039, respectively) (table 3). These populations presented 13, 7 and 11 haplotypes, respectively, all of them exclusive as they were not shared with any other population. In contrast, the Cba.Chic population presented the lowest haplotype diversity (HD = 0.850 ± 0.077).

For the CytB region, the Cba.Chic population showed the highest haplotype diversity (HD = 0.591 ± 0.108), with three haplotypes (two of them of exclusive occurrence) (table 4). The population that showed the lowest haplotype diversity was Tuc.Chic (HD = 0.222 + 0.166) (table 4).

### Haplotype networks

The network analysis for the COI region resulted in two major sub-networks, two minor sub-networks consisting of four and three individuals, and eight independent haplotypes (figure 2A). The first and largest sub-network consisted of individuals belonging to all populations. The

**Table 2.** Statistical summary of mitochondrial sequences of COI and CytB markers.

| | COI | CytB |
|---|---|---|
| Number of sequences | 63 | 51 |
| Total base pairs (bp) | 1086 | 388 |
| Variable sites (% variation) | 200 (18.42%) | 32 (8.25%) |
| Number of haplotypes (H) | 47 | 9 |
| Haplotype diversity (HD) | 0.967 ± 0.014 | 0.407 ± 0.086 |
| Nucleotide diversity ($\pi$) | 0.03712 ± 0.00567 | 0.00402 ± 0.00245 |
| Average number of nucleotide differences (k) | 40.31 | 1.56 |
| Tajima's *D* test (*P* value) | −0.80 ($P > 0.10$) | −2.59 ($P < 0.001$) |
| Fu's $F_s$ test (*P* value) | −4.546 ($P < 0.10$) | −2.98 ($P < 0.02$) |

**Table 3.** Haplotype distribution and genetic diversity indices of *H. gelotopoeon* populations obtained from COI region.

| Population | N[1] | NH[2] | H (Number I)[3] | HD ± SD[4] | $\pi$ ± SD[5] |
|---|---|---|---|---|---|
| Cha.Cot | 7 | 7 | H1(1), H2(1), H3(1), H4(1), H5(1), H6(1), H7(1) | 1.000 ± 0.006 | 0.01871 ± 0.00254 |
| Tuc.Soy | 13 | 13 | H8(1), H9(1), H10(1), H11(1), H12(1), H13(1), H14(1), H15(1), H16(1), H17(1), H18(1), H19(1), H20(1) | 1.000 ± 0.030 | 0.02431 ± 0.00286 |
| Tuc.Chic | 16 | 10 | H21(6), H22(1), H23(1), H24(2), H25(1), H26(1), H27(1), H28(1), H29(1), H30(1) | 0.867 ± 0.079 | 0.00163 ± 0.00028 |
| Cba.Chic | 16 | 9 | H21(3), H24(6), H31(1), H32(1), H33(1), H34(1), H35(1), H36(1), H37(1) | 0.850 ± 0.077 | 0.02670 ± 0.02670 |
| BsAs.Soy | 11 | 11 | H26(1), H38(1), H39(1), H40(1), H41(1), H42(1), H43(1), H44(1), H45(1), H46(1), H47(1) | 1.000 ± 0.039 | 0.06072 ± 0.00881 |

[1] N = Number of individuals analysed; [2] NH = Number of haplotypes found in each population; [3] H (Number I) = Haplotype (Number of individuals that presented that haplotype); [4] HD ± SD = Haplotype diversity ± standard deviation; [5] $\pi$ ± SD = Nucleotide diversity ± standard deviation.

**Table 4.** Haplotype distribution and genetic diversity indices of *H. gelotopoeon* populations obtained from CytB region.

| Population | N[1] | NH[2] | H (Number I)[3] | HD ± SD[4] | $\pi$ ± SD[5] |
|---|---|---|---|---|---|
| Cha.Cot | 13 | 2 | H1(12), H6(1) | 0.275 ± 0.148 | 0.00988 ± 0.00790 |
| Tuc.Soy | 17 | 5 | H1(13), H2(1), H3(1), H4(1), H5(1) | 0.426 ± 0.147 | 0.00182 ± 0.00085 |
| Tuc.Chic | 9 | 2 | H1(8), H7(1) | 0.222 ± 0.166 | 0.00057 ± 0.00043 |
| Cba.Chic | 12 | 3 | H1(7), H9(8), H9(1) | 0.591 ± 0.108 | 0.00254 ± 0.00103 |

[1] N = Number of individuals analysed; [2] NH = Number of haplotypes found in each population; [3] H (Number I) = Haplotype (Number of individuals that presented that haplotype); [4] HD ± SD = Haplotype diversity ± standard deviation; [5] $\pi$ ± SD = Nucleotide diversity ± standard deviation.

second sub-network comprised individuals from Tuc.Soy, Cba.Chic and Cha.Cot. Minor sub-networks comprised individuals only from the BsAs.Soy population. Independent haplotypes corresponded to individuals from all populations except for Tuc.Chic.

The CytB region showed a single network that comprised individuals from all populations, and one independent haplotype corresponding to the Cha.Cot population. In this network, H1 was the most common haplotype, and was present in all populations (figure 2B).

## Genetic structure

For the COI region, results from AMOVA analysis revealed that the highest source of genetic variation existed within populations (61.54%), whereas the remaining (38.46%) came from variation among populations (table 5). However, AMOVA showed significant F-statistics ($F_{ST}$ = 0.38) when all populations were compared. No significant values were found when populations were partitioned according to host plant, region and year of sampling (table 5).

For the CytB region, AMOVA showed low genetic differentiation among populations ($F_{ST}$ = 0.01877), with 98.12% of variation occurring within populations. No significant values were found when populations were partitioned according to host plant, region and year of sampling (table 6).

Genetic differentiation among populations was also evaluated using $F_{ST}$ pairwise population comparisons, where $F_{ST}$ values near one indicate higher differentiation and values near zero indicate more homogeneous populations (Hartl and Clark, 1997). The highest genetic differentiation for the COI region was found between BsAs.Soy and Tuc.Chic populations (table 7). In the case of CytB regions, significant values were only detected between Cba.Chic and Tuc.Soy populations (table 8).

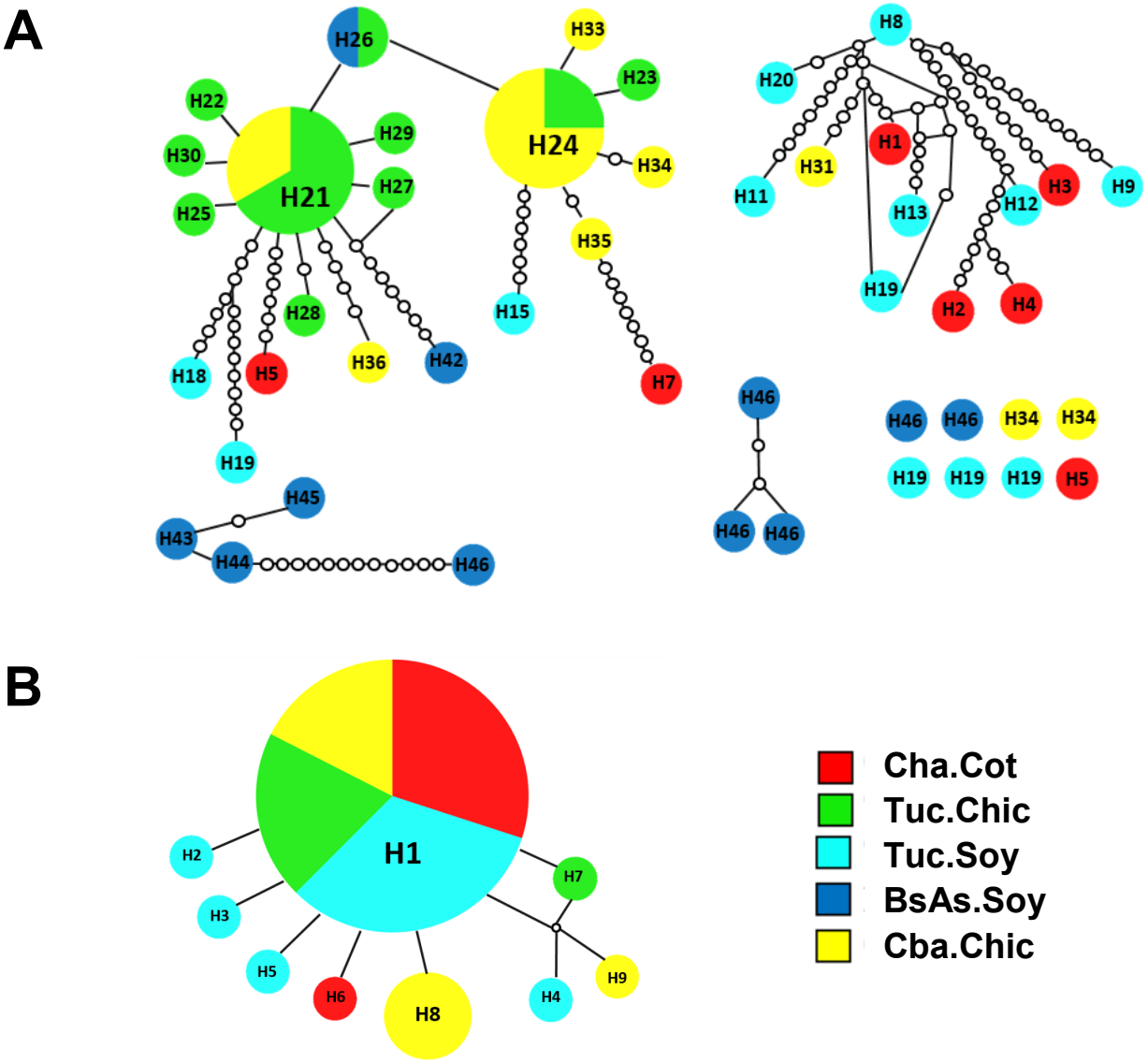


**Figure 2.** Haplotype networks of *H. gelotopoeon* for (**A**) COI and (**B**) CytB regions. Each circle represents a haplotype. Circle size is proportional to haplotype frequency, and the smallest circle represents one haplotype.

**Table 5.** Analysis of molecular variance of *H. gelotopoeon* populations categorized by populations, host plants, regions and year of sampling using COI region.

| Model | Source of variation | d.f. | Fixation indices | Percentage of variation |
|---|---|---|---|---|
| A. Populations | Among populations | 4 | $F_{ST}$: 0.38456 | 38.46 |
| | Within populations | 58 | | 61.54 |
| B. Host plants | Among host plants | 2 | $F_{CT}$: −0.02954 | −2.95 |
| | Among populations within host plants | 2 | $F_{SC}$: 0.39770 | 40.94 |
| | Within populations | 58 | $F_{ST}$: 0.37990 | 62.01 |
| C. Regions | Among regions | 3 | $F_{CT}$: 0.14274 | 14.27 |
| | Among populations within regions | 1 | $F_{SC}$: 0.29575 | 25.35 |
| | Within populations | 58 | $F_{ST}$: 0.39628 | 60.37 |
| D. Year of sampling | Among year of sampling | 1 | $F_{CT}$: 0.08957 | 8.96 |
| | Among populations within year of sampling | 3 | $F_{SC}$: 0.35115 | 31.97 |
| | Within populations | 58 | $F_{ST}$: 0.40927 | 59.07 |

$F_{CT}$ = fixation index within groups; $F_{SC}$ = fixation index among populations within groups.

**Table 6.** Analysis of molecular variance of *H. gelotopoeon* populations categorized by population, host plants, regions and year of sampling using CytB region.

| Model | Source of variation | d.f. | Fixation indices | Percentage of variation |
|---|---|---|---|---|
| A. Populations | Among populations | 3 | $F_{ST}$: 0.01877 | 1.88 |
| | Within populations | 47 | | 98.12 |
| B. Host plants | Among host plants | 2 | $F_{CT}$: 0.03782 | 3.78 |
| | Among populations within host plants | 1 | $F_{SC}$: −0.01562 | −1.50 |
| | Within populations | 47 | $F_{ST}$:0.02279 | 97.72 |
| C. Regions | Among regions | 2 | $F_{CT}$: 0.09431 | 9.43 |
| | Among populations within regions | 1 | $F_{SC}$: −0.06772 | −6.13 |
| | Within populations | 47 | $F_{ST}$: 0.03297 | 96.70 |
| D. Year of sampling | Among year of sampling | 1 | $F_{CT}$: 0.00765 | 0.76 |
| | Among populations within year of sampling | 2 | $F_{SC}$: 0.01384 | 1.37 |
| | Within populations | 47 | $F_{ST}$: 0.02138 | 97.86 |

$F_{CT}$ = fixation index within groups; $F_{SC}$ = fixation index among populations within groups.

**Table 7.** Pairwise population estimates of $F_{ST}$ for *H. gelotopoeon* populations using COI region.

| | Tuc.Soy | BsAs.Soy | Tuc.Chic | Cba.Chic | Cha.Cot |
|---|---|---|---|---|---|
| Tuc.Soy | 0.0000 | | | | |
| BsAs.Soy | 0.47020* | 0.0000 | | | |
| Tuc.Chic | 0.48708* | 0.59083* | 0.0000 | | |
| Cba.Chic | 0.26327* | 0.35788* | 0.07219* | 0.0000 | |
| Cha.Cot | 0.03533 | 0.42662* | 0.53162* | 0.17440 | 0.0000 |

* Significance test using 1000 permutations ($P < 0.01$).

### Phylogenetic analysis

The maximum likelihood analysis did not show significant clustering due to geographical origin, year of sampling or host plant for none of the three regions. However, each mitochondrial region showed a similar trend to the haplotype networks obtained.

The COI region tree showed three principal clades. One clade included individuals from the outgroup (*H. zea*), and the other mostly involved individuals from the BsAs.Soy population. The third clade subdivided in three principal subclades, one of them formed mainly by Cba.Chic and Tuc.Chic populations and the other by individuals from the remaining populations (Cha.Cot, Cba.Chic, BsAs.Soy and Tuc.Chic) (figure 3).

The CytB region tree showed two clades, one composed of individuals from the outgroup, and the other by all *H. gelotopoeon* analysed individuals, with no clear subclade differentiation (figure 4).

**Table 8.** Pairwise population estimates of $F_{ST}$ for *H. gelotopoeon* populations using CytB region.

| | Tuc.Soy | Cha.Cot | Tuc.Chic | Cba.Chic |
|---|---|---|---|---|
| Tuc.Soy | 0.0000 | | | |
| Cha.Cot | 0.00687 | 0.0000 | | |
| Tuc.Chic | −0.02478 | −0.02677 | 0.0000 | |
| Cba.Chic | 0.10334* | 0.02333 | 0.08447 | 0.0000 |

* Significance test using 1000 permutations ($P < 0.01$).

## Discussion

Results of genetic variability and gene flow analysis among *H. gelotopoeon* populations evaluated through mitochondrial markers indicated certain genetic structure in studied populations. This represents the first estimation of *H. gelotopoeon* genetic structure using mitochondrial markers. The $F_{ST}$ value observed in this study for the analysed COI region (0.38456) was substantially higher than those found for other species of *Helicoverpa* using mitochondrial markers (ranging from $7.10^{-5}$ to 0.071) (Leite *et al*., 2014; Mastrangelo *et al*., 2014; Wang *et al*., 2018). However, the CytB region showed similar values (0.01877) to those reported in these previous studies.

The HD estimation for COI was 0.967 ± 0.014, which can be considered high and similar to those observed for *H. armigera* populations from Paraguay (Arnemann *et al*., 2016). However, π estimations for *H. gelotopoeon* populations showed higher values compared to those observed in Paraguay for *H. armigera*. The CytB region showed a lower HD value (0.407 ± 0.086) for *H. gelotopoeon* populations, which is similar to values for *H. armigera* populations from Uruguay and *C. virescens* from Brazil (Albernaz *et al*., 2012; Arnemann *et al*., 2016).

Haplotype networks and phylogenetic trees showed a similar clustering trending, yet no clear influence of host plant, region or year of sampling was observed.

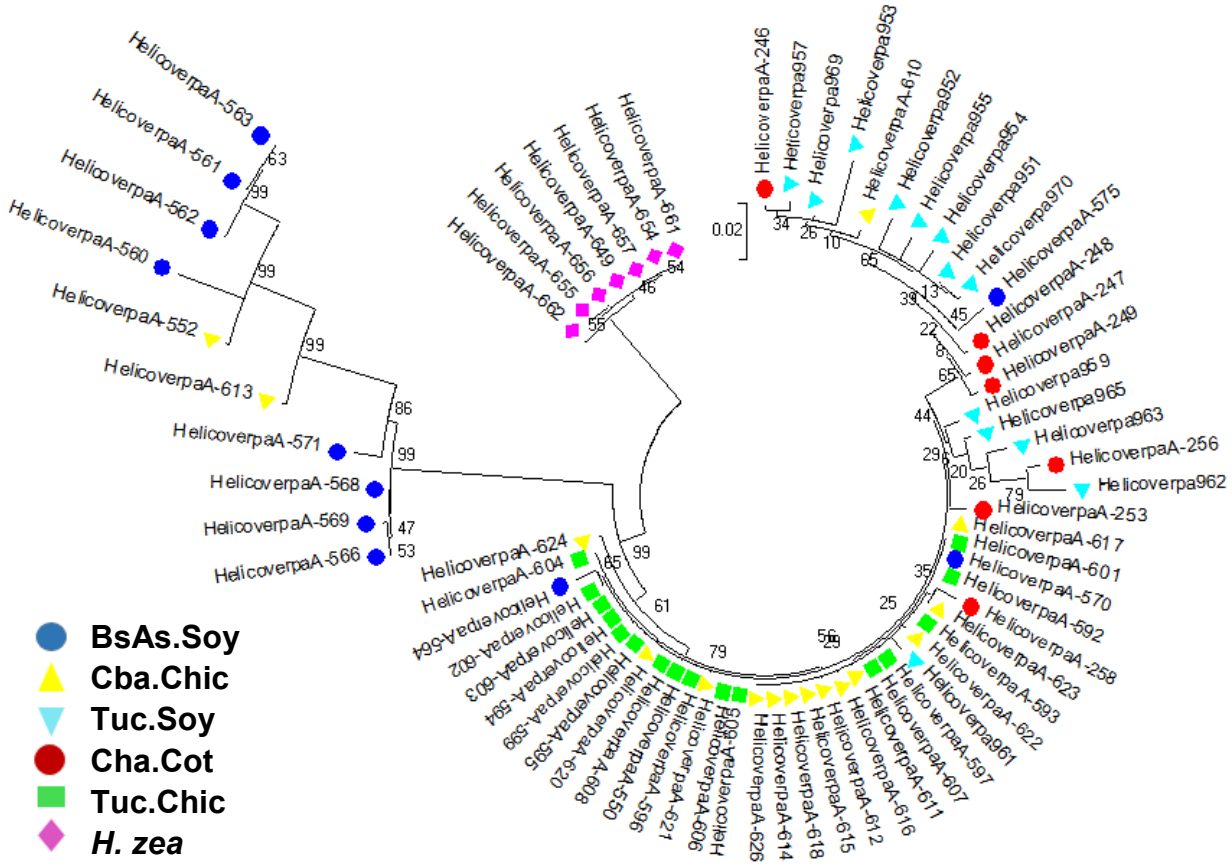


**Figure 3.** Maximum likelihood tree of *H. gelotopoeon* populations estimated under Kimura 2-parameters model for COI region.

Possible causes that could explain the genetic structure observed for *H. gelotopoeon*, include differences among regions and climatic influences. In this respect, the phylogenetic tree of COI region shows BsAs.Soy and Cba.Chic populations (both belonging to the same region) grouped in a widely differentiated cluster. Another possible cause for genetic structure of *H. gelotopoeon* populations is the host plant. This was reflected by the haplotype distribution among populations, since Cba.Chic and Tuc.Chic populations (although distant) were the populations that shared the highest number of haplotypes for both mitochondrial regions. This association was not as clear for Tuc.Soy and BsAs.Soy populations. However, these last populations were collected in different years, in contrast to Tuc.Chic and Cba.Chic, that were collected in the same growing season. Thus, it is also necessary to consider that year of sampling may have had certain influence in population structure. This was also reflected by the haplotype network structure of the COI region, in which Tuc.Soy and Cha.Cot populations, collected during the same year, formed a unique network separated from the rest. Regarding this observation, Scott *et al*. (2005) demonstrated that high, moderate and low gene flow occur among different *H. armigera* Australian populations, with gene flow between populations from distant growing regions, varying depending on the year, evidencing that gene flow is highly variable among years. This is probably due to moth movement differs from season to season, highlighting the importance of extending this kind of studies over consecutive years. Therefore, short-term sampling may be misleading when population dynamics and migration change so significantly.

It is also interesting to consider that even though *H. gelotopoeon* is present in all the regions considered in this study, the relative importance of this pest is not the same. This is especially relevant because it will influence what kind of management will be applied in each county, resulting in different degrees of selective pressure upon these populations. In the case of Rojas County, this species does not cause major problems in soybean and it is generally found in low frequency, compared to NOA and NEA populations. This is reflected in haplotype distribution of BsAs.Soy: the COI network shows two sub-networks constituted exclusively by BsAs.Soy individuals. In addition, a clade constituted almost exclusively by BsAs.Soy individuals was observed in COI phylogenetic tree.

Results of this study show many exclusive alleles, and in some cases independent, that could not be related with any net. Studies conducted by Albernaz *et al*. (2012) in *C. virescens* using mtDNA also revealed a high percentage of unique occurrence haplotypes. These authors proposed the hypothesis that rare haplotypes might arise by new mutational events and that alternatively, these low frequency haplotypes might come from individuals living on wild host plants found nearby crop fields every

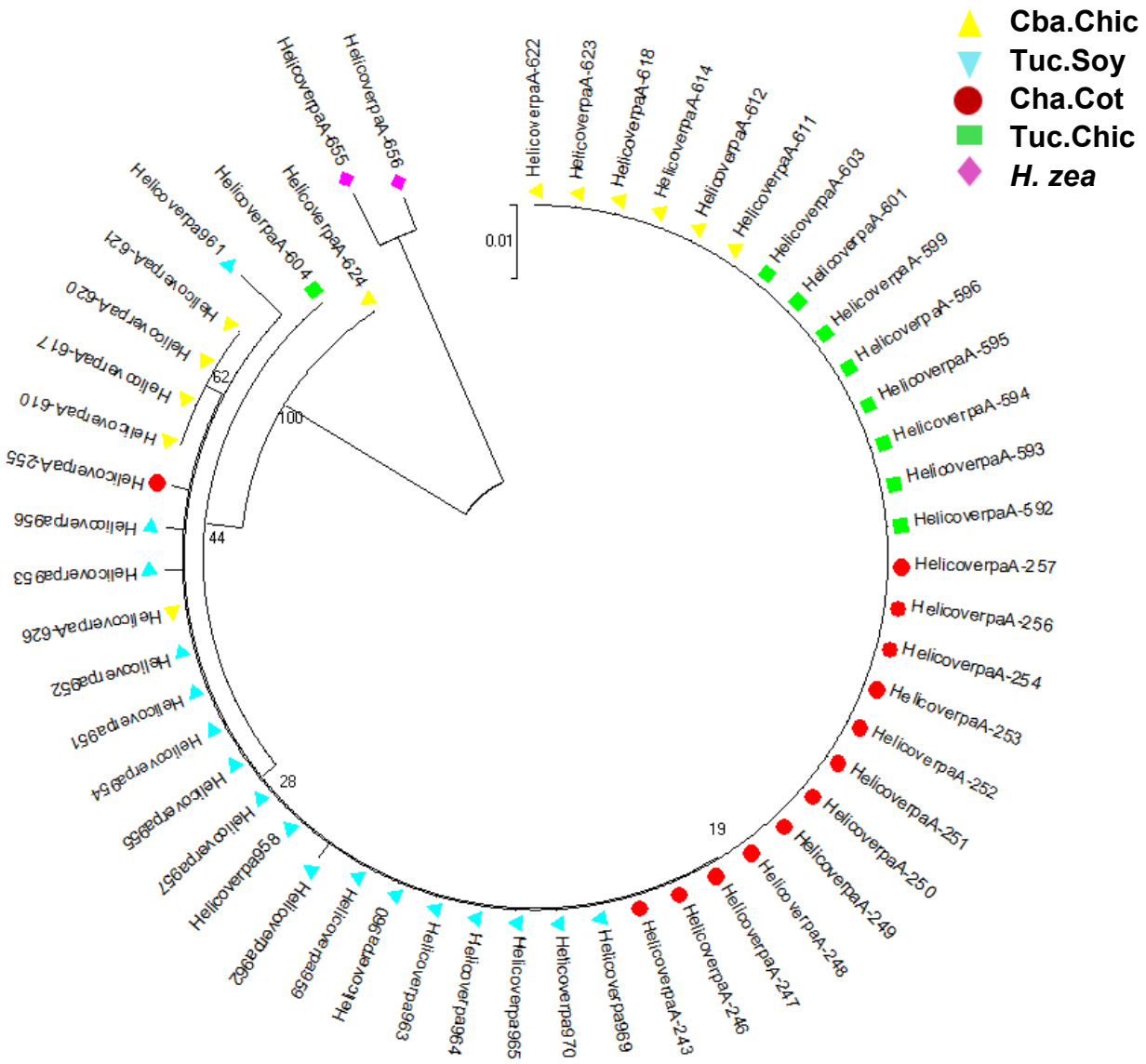


**Figure 4.** Maximum likelihood tree of *H. gelotopoeon* populations estimated under Kimura 2-parameters model for CytB region.

season. In addition, these rare haplotypes could be different for every sampled region, since each area presents different natural vegetation associated to Brazilian biomes. This hypothesis is further supported by *H. gelotopoeon* being a polyphagous pest and the fact that that sampled provinces belong to different agroecological regions with different natural vegetation associated.

Furthermore, the pattern of genetic variability observed in *H. gelotopoeon* populations, reflected in the high values of haplotype diversity and the high number of low frequency haplotypes, is characteristic of a species that has undergone a process of recent population expansion, which is supported by negative and significant values for Fu's *Fs* and Tajima's *D* neutrality test.

These different scenarios are not exclusive and all of them could have contributed to *H. gelotopoeon* population structure observed in this study. It would be necessary to develop additional studies to clarify what other factors may be intervening in genetic structure of this species.

Our results diverge from those of Herrero *et al*. (2021) for the same pest using microsatellite markers, since those authors found no evidence of genetic structuring among *H. gelotopoeon* populations. However, there are reports on inconsistent results between population analysis using mitochondrial and nuclear markers. Albernaz *et al*. (2012) observed low genetic structure in Brazilian populations of *C. virescens* using mtDNA sequences. Conversely, Domingues *et al*. (2012) showed moderate to high genetic structure and low levels of gene flow among Brazilian populations of *C. virescens* employing microsatellites.

Difference between both types of markers could be due to different kinds of inheritance. The mtDNA, being uniparentally inherited, is haploid and it behaves as a single inherited unit and therefore, as a single locus marker. Data from a single locus allow us to retrace the history of only a single genetic unit, which may or may not be concordant with the history of the species in question (Freeland *et al*., 2011). Because of their reduced effective population sizes

relative to nuclear DNA (25%), their haplotypes have a greater probability of going extinct. Therefore, if the size of a population is temporarily reduced, even if the population recovers quickly, it will have relatively few surviving mitochondrial haplotypes compared with nuclear genotypes. This could lead to infer and oversimplified population history or to underestimate levels of genetic diversity (Freeland *et al*., 2011).

This highlights the importance of using different molecular markers for population structure studies, since using only a single marker could not be representative of the genome as a whole. In this case, microsatellites and mtDNA provide complementary information, since each one emphasizes different aspects of genetic variability.

Therefore, despite the significant genetic structure found among *H. gelotopoeon* populations using mtDNA markers, more studies contemplating other biological and ecological features are necessary to understand the source of genetic structure in these populations and to apply this information in the management of this pest.

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