Anatomy of a pest control failure: Introgression of cytochrome P450 337B3 alleles from invasive old-world bollworm into native corn earworm.

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Abstract

The establishment of invasive species populations can threaten the ecological balance in naïve habitats and impact agricultural production practices. The old-world bollworm (OWBW), *Helicoverpa armigera,* and the corn earworm (CEW), *H. zea,* were geographically separated prior to the 2013 report of OWBW invasion into South America. Introgression of OWBW-specific cytochrome P450 337B3 (CYP337B3) gene into CEW was repeatedly detected across South America and the Caribbean. Two hybrids were documented among samples collected in Texas during 2019. In this study, screening insects collected in Olathe, Colorado, USA where a failure of pyrethroids to control CEW damage to conventional sweetcorn in 2023, detected 28.59% insects with the OWBW-specific CYP337B3 marker. Nucleotide sequencing of CYP337B3 gene identified 73.05 and 26.95% insects homozygous for CYP337B3v2 and CYP337B3v6 alleles, respectively. Based on prior data for distinct phylogeographic origins of CYP337B3v2 and v6 alleles, our results indicate Olathe samples were derived from two different introductions. Specifically, a novel origin based on restricted distribution of v6 allele to Ghana in West Arica, and possible South American or Caribbean origin of the v2 allele. One of the 1618 individuals screened also carried a ribosomal RNA internal transcribed spacer 1 (ITS1) derived from OWBW. Local selection pressures at the Olathe location imposed by repeated pyrethroid exposures are likely attributed to the prevalence of CYP337B3, where control practices hasten the accumulation of phenotypic resistance by adaptive introgression. Pyrethroid and other resistance factors carried by invasive OWBW may continue to impact CEW management tactics for across the Americas.

Keywords: *Helicoverpa armigera*, *Helicoverpa zea*, corn earworm, old world bollworm, Invasive pest, hybridization, introgression, pesticide resistance

Introduction

Invasive insect species can disrupt naïve ecosystems, impact the health of native species, and introduce challenges to agricultural production (Venette & Hutchison, 2021). An invasive species undergoes an initial establishment phase with varying levels of adaptation to new ecosystems and environmental conditions (Colautti et al., 2017). Ironically, invasive populations undergo a genetic bottleneck due to a small number of founders, where this reduced genetic diversity would reduce adaptive potential. Bottleneck effects can be alleviated through admixture following multiple introduction events from genetically distinct source populations, where increased genetic diversity in admixed populations can lead to higher fitness (Jaspers et al., 2021). Establishment can be facilitated by interspecific hybridization with native species which can transfer locally adapted alleles to invasive populations in a mechanism referred to as adaptive introgression (Arnold & Martin, 2009). Native populations may also acquire alleles with a selective advantage from an invasive species (Burgarella et al., 2019)

The sister species *Helicoverpa armigera* (Hübner,1808) and *H. zea* (Boddie, 1850) (Lepidoptera: Noctuidae) are morphologically similar but distinguishable via differences in male genitalia (Pouge, 2004). The endemic range of *H. zea* spans the Americas where it causes damage to multiple high-value crops (Reay-Jones, 2019). *Helicoverpa armigera* feeds on a broader range of host plants (Fitt, 1989) and is historically distributed across Africa, Asia, Europe, and Oceania (Kitching & Rawlins, 1998). Invasion and establishment of *H. armigera* into the Americas was first reported in Bahia, Goiás, and Mato Grosso states in Brazil in 2013 using morphological characters and the histology of male genitalia (Czepak et al., 2013). Speciation through analysis of mitochondrial DNA confirmed this invasion by *H. armigera* (Tay et al., 2013). Later, *H. armigera* was reported in other regions of Brazil and additional South American countries such as Argentina, Colombia, Paraguay, Uruguay, and Venezuela (Arnemann et al., 2016, Arnemann et al., 2019, Riaz et al., 2021, Specht et al., 2013). Evidence suggests these resulted from multiple independent introductions (Tembrock et al., 2019). Laboratory experiments document the capacity of *H. armigera* and *H. zea* to produce fertile progeny from reciprocal crosses and backcrosses (Laster & Sheng, 1995). Genomic analyses of *Helicoverpa* populations detected evidence of hybridization between field populations of *H. armigera* and *H. zea*, and the introgression of *H. armigera* alleles into *H. zea* (Valencia-Montoya et al., 2020). This involved

introgression of the *H. armigera* locus for a cytochrome P450, CYP338B3, associated with insecticide resistance (Walsh et al., 2018) into the *H. zea* genomic background.

High mobility of migratory *Helicoverpa* and its modeling predict a high probability of *H. armigera* spreading and establishing in North America (Kriticos et al., 2015). *Helicoverpa armigera* was initially detected outside South America in Puerto Rico and Florida, USA in 2014 and 2015, respectively (Smith, 2014, Hayden & Brambila, 2015, APHIS, 2024). Hybridization with *H. zea* was subsequently detected in Puerto Rico (Trujillo et al., 2023). The pest surveillance program of USDA Animal and Plant Health Inspection Service (USDA APHIS) later detected several *H. armigera* adults near the Chicago O'Hare International Airport between 2019 and 2022, however no individuals were detected in surrounding agricultural areas (APHIS, 2024). Introgression between these two species was shown by the detection of the invasive *H. armigera*-specific CYP337B3 allele in two *H. zea* collected from a Texas population in 2019 (North et al., 2023). Our current study focuses on a collection of *Helicoverpa* moths from Olathe, Colorado, where a failure to control damage to conventional sweetcorn was reported following repeated pyrethroid insecticide applications in 2023. Currently a limited number of genetic markers are available to screen invasive *H. armigera.* Regardless, our analyses of species-specific genetic markers at ITS1 (Perera et al., 2015) and CYP337B3 loci (Joußen et al., 2012) detected *H. armigera* alleles in the Olathe samples. Our work constitutes the second report of *H. zea* carrying genes introgressed from *H. armigera* within the USA. Pyrethroid resistance conferred by the movement of the CYP337B3 allele into the *H. zea* population has implications for the continued efficacy of insecticidal control tactics used to manage this crop pest.

Materials and Methods

Insects were collected using *Helicoverpa zea* pheromone lures in bucket traps (Trece, Adair, OK) at 53 locations near Olathe, CO, USA, during the summer of 2023 (Table S1). A sweetcorn grower near these traps reported that repeated applications of pyrethroid insecticide failed to control insect damage to the crop, and local scouting provided visual identification of surviving larval *H. zea*. Initial screening of insects was carried out by pooling a single leg from each adult male from each trap collection (or all the detached legs in a collection bag) and extracting DNA using a modified squish buffer containing 10 mM Tris-HCl, 0.25 mM EDTA, and 0.25 mM NaCl (Perera et al., 2015). Two 3 mm stainless steel beads were added to the tubes containing insect

legs and homogenized using a bead beater (Biospec Products, Bartlesville, OK) for 1 min at the highest speed. Homogenates were centrifuged briefly and heated for 5 min in a heating block set to 100 °C, followed by centrifugation at 12,500 x g for 10 min. DNA was also extracted from *H. zea* collected in 2006 from 10 counties in Texas (*n* = 200) and 5 counties in Mississippi (*n* = 100), and included in this study. For these, a small piece of thoracic tissue from five insects was pooled into one tube to extract DNA using 150 µl of squish buffer as described above.

The supernatant from each extract was transferred to a new tube and used in the melt curve analysis (Perera et al., 2015). Briefly, 1 µl of each DNA pool was used in a 20 µl reaction containing final concentrations of 1X Fast SYBR Master mix (Life Technologies Corporation, Carlsbad, CA) 0.4 µM common forward primer 3373Ha_Hz_ITS1F for internal transcribed spacer 1 (ITS1) of ribosomal RNA gene, *H. armigera*-specific reverse primer 3374Ha_ITS1R, and 0.2 µM of *H. zea*-specific reverse primer 3377Hz_ITS1R (Table 1). DNA amplification and melt curve analysis were performed in an ABI7500 Fast real-time PCR instrument (Applied Biosystems, Foster City) with 95°C initial denaturation for 30 seconds followed by 40 cycles of denaturation at 95°C for 5 seconds and annealing and extension at 60°C for 30 seconds. A melt curve step was performed after the amplification cycles to determine the presence of species-specific amplicons. Agarose gel electrophoresis was performed to visualize the amplicons as a verification step.

Table 1. Primers used in PCR amplification and nucleotide sequencing of ribosomal RNA internal transcribed spacer 1 and cytochrome P450 337B, CYP337B3, from *Helicoverpa* samples collected from Olathe, Colorado, USA.

Primer Name	Sequence (5' to 3	Gene	Reference
3373Ha_Hz_ITS1F	GAGGAAGTAAAAGTCGTAACAAGGTTTCC	ITS1 (rRNA)	(Perera et al., 2015)
3374Ha_ITS1R	CGTTCGACTCTGTGTCCTCTAGTGG	ITS1 (rRNA)	(Perera et al., 2015)
3377Hz_ITS1R	TTGATTGTTAACGAACGCGCCG	ITS1 (rRNA)	(Perera et al., 2015)
310-18S-rDNA-2F	ATCATTTAGAGGAAGTAAAAGTCGTAACAAGGT	ITS1 (rRNA)	(Perera et al., 2015)
312-5.8S-rDNA-R	GAARTGTCGATGTTCAAATGTGTCCTGC	ITS1 (rRNA)	(Perera et al., 2015)
3713CYP337B3F	AATAATAACCAGTCCAAACGATGTGT	CYP337B3	(Joußen et al., 2012)
3714CYP337B3R	TGCAATAACAACTATTACATTTTGAATAAA	CYP337B3	(Joußen et al., 2012)
4268CYP337B3F	ATGTTGTTCGTRATATTACTCACGA	CYP337B3	This study
4269CYP337B1_B3R	TTATATGTCTCTTAACTTTAATTCATAACGAG	CYP337B3	This study

139 Full-length ITS1 was amplified from individual DNA using the primers 310-18S-rDNA-2F and
140 312-5.8S-rDNA-R (Table 1) in reactions containing 1X ThermoPol buffer (12.5mM tricine,
141 42.5mM KCl, 1.5mM MgCl2, 6% dextran; New England BioLabs, Ipswich, MA), 0.32 mM
142 dNTP mix, 0.4µM of each primer, 1 unit of *Taq* polymerase, and 10–100 ng of genomic DNA in
143 a 25 ml reaction. Amplification was performed in a PTC-200 thermal cycler (MJ Research,
144 BioRad, Hercules, CA) with a 30 s initial denaturation step at 95°C, 35 cycles of 15 s
145 denaturation at 95°C, 10 s annealing at 52°C followed by a final extension of 5 min at 72°C
146 (Perera et al., 2015). Amplicons were purified using AmPure XP magnetic beads according to
147 manufacturer instructions (Beckman Coulter, Brea, CA, USA). Approximately 2 ng of amplicon
148 from 82 separate pools was used as input for the Oxford Nanopore library construction reagent
149 set SQK-LSK114 and the native DNA barcode kit SQK-NDB-96. The resulting 82 indexed
150 libraries were sequenced in a FLO-MIN114.001 flow cell following the manufacturer's
151 instructions (Oxford Nanopore Technologies, New York, NY). Purified ITS1 DNA from sample
152 61e was also cloned into TA cloning vector (Invitrogen, Carlsbad, CA) and transformed into
153 DH5α competent cells. Plasmid DNA prepared from 24 colonies were screened for the presence
154 of *H. armigera*- and *H. zea*-specific amplicons as described previously. Nucleotide sequences of
155 clones positive for ITS1 specific for each species were obtained by Sanger sequencing. DNA
156 Sequences were analyzed using CLC Genome WorkBench v22.02 (Qiagen, Redwood City, CA).

157 Phylogenetic analysis was performed using Molecular Evolutionary Genetic Analysis (MEGA)
158 11 (Tamura et al., 2021) based on a multiple sequence alignment of the ITS1 region from *H.*
159 *armigera*, *H. zea* and *H. assulta* (KT342378.1, KT343380.1, and KT343379.1, respectively), and
160 our Sanger read data for Olathe, CO individual 61e. Relationships were estimated using the
161 Maximum Likelihood (ML) method (Nei & Kumar, 2000, Beerli & Felsenstein, 2001) applying
162 the Kimura-2-Parameter model of sequence evolution (Kimura, 1980), that implemented a
163 discrete Gamma shape parameter, and node support inferred from 10,000 bootstrap pseudo
164 replications (Felsenstein, 1985) of the aligned data. The tree was rooted by the *Heliothis*
165 *virescens* ITS1 (GenBank accession KT342279.1)

166 A short fragment of the cytochrome P450 337B3 (CYP337B3) gene was amplified from Olathe
167 Colorado, Texas and Mississippi samples using gene-specific primers 3713CYP337B3F and
168 3714CYP337B3R (Table 1) following the protocol described in Joußen et al. (2012). Amplicons

169 were resolved by 1.0% agarose gel electrophoresis to identify insects positive for the CYP337B3
170 gene. For each insect identified as positive for CYP337B3, the full-length CYP337B3 gene was
171 then amplified with primers 4268CYP337B3F and 4269CYP337B1_B3R. PCR amplification
172 reaction and thermal cycling parameters were the same as those used for amplification of the
173 short fragment of CYP337B3, except for an annealing temperature of 50°C and extension time
174 increased to 2.75 min. Amplicons were purified using AmPure XP beads (Beckman Coulter)
175 prior to use in sequencing reactions. Barcoded Oxford Nanopore libraries were constructed and
176 sequenced as described above for full length CYP337B3 gene sequences from 143 insects.
177 CYP337B3 allele versions v1 through v8 (JQ959292.1, KJ636466.1, KM675665.1,
178 KM675666.1, KX958388.1, KX958389.1, KX958390.1, and KX958391.1, respectively),
179 CYP337B1v1, and CYP337B2v2 (JQ995291) were used as references for subsequent alignment
180 of Oxford Nanopore amplicon sequences. Evolutionary relationships of the CYP337B3 alleles
181 from Olathe, CO population and previously published CYP337B genes was inferred in a ML tree
182 constructed using MEGA 11 as described above.

183 **Results**

184 Initial screening of 1618 insects collected from Olathe, CO in 2023 occurred within 82 pools by
185 PCR with *H. armigera*-specific ITS1 primers, where an amplicon was generated from a single
186 pool, pool 61. This indicated at least one *H. armigera* or a hybrid insect in pool 61 carried an *H.*
187 *armigera* ITS1 region sequence whereas none were detected in the Texas or Mississippi samples
188 collected in 2006. Analysis of Oxford Nanopore sequence data from pool 61 also identified ITS1
189 sequences closely resembling *H. armigera* ITS1. Screening of DNA from individual insects from
190 pool 61 identified one insect (61e) from which both *H. armigera*- and *H. zea*-specific ITS1
191 amplicons were produced. Subsequent alignment found that the ribosomal RNA (rRNA) gene
192 sequence of insect 61e contained both *H. zea* rRNA repeats, and a variant of *H. armigera* rRNA
193 repeats. Specifically, this was shown from Oxford Nanopore sequencing and Sanger sequencing
194 of cloned full length ITS1 PCR products from 61e (Fig. 1) where insertion/deletion (indel)
195 mutations were shown in the *H. armigera* compared to *H. zea* ITS1 references sequences at
196 positions 138-167, 401-403, and 431-433. Sequence data from sample 61e showed two variants,
197 with indels in each identical to respective *H. armigera*- and H. zea ITS1 reference sequences.
198 However, there were 23 single nucleotide polymorphisms in the *H. armigera*-like ITS1 sequence

from sample 61e that were identical to the *H. zea* ITS1 reference. Phylogenetic reconstruction of ITS1 region showed clustering of the two ITS region variants from Olathe, CO individual 61e with corresponding *H. armigera* (KT342378.1) and *H. zea* references (KT343380.1; Fig. 2). ITS1 nucleotide sequences from the Olathe, CO sample 61e were deposited in the GenBank database under accession numbers PP456864.1 (Olathe_61e_Ha_ITS1), and PP456865.1 (Olathe_61e_Hz_ITS1).

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                          1                                                        60
  Hz_ITS1_KT343380   (1)  ATCATTTAGAGGAAGTAAAAGTCGTAACAAGGTTTCCGTAGGGGAACCTGCGGAAGGATC
Olathe_61e_Hz_ITS1   (1)  ATCATTTAGAGGAAGTAAAAGTCGTAACAAGGTTTCCGTAGGGGAACCTGCGGAAGGATC
  Ha_ITS1_KT343378   (1)  ATCATTTAGAGGAAGTAAAAGTCGTAACAAGGTTTCCGTAGGGGAACCTGCGGAAGGATC
Olathe_61e_Ha_ITS1   (1)  ATCATTTAGAGGAAGTAAAAGTCGTAACAAGGTTTCCGTAGGGGAACCTGCGGAAGGATC
                          61                                                      120
  Hz_ITS1_KT343380  (61)  ATTAACGTGTAAACGCAAGACGCGACTGGCTCGCGACGCGCGTGTTATAACGTGAACAAT
Olathe_61e_Hz_ITS1  (61)  ATTAACGTGTAAACGCAAGACGCGACTGGCTCGCGACGCGCGTGTTATAACGTGAACAAT
  Ha_ITS1_KT343378  (61)  ATTAACGTGTAAATGCAAGACGCGACTGGCTCGCGACGCGCGTGTTATAACGTAAACAAT
Olathe_61e_Ha_ITS1  (61)  ATTAACGTGTAAACGCAAGACGCGACTGGCTCGCGACGCGCGTGTTATAACGTGAACAAT
                          121                                                     180
  Hz_ITS1_KT343380 (121)  AATCCACACCACTATGCGCATGCATATATTGCATCGCGCATATCCAGAGGACACAGAGTC
Olathe_61e_Hz_ITS1 (121)  AATCCACACCACTATGCGCATGCATATATTGCATCGCGCATATCCAGAGGACACAGAGTC
  Ha_ITS1_KT343378 (121)  AATCCACACACCA------------------------------CTAGAGGACACAGAGTC
Olathe_61e_Ha_ITS1 (121)  AATCCACACCACT------------------------------CCAGAGGACACAGAGTC
                          181                                                     240
  Hz_ITS1_KT343380 (181)  GT--GGACGCTGTGCGTTGAAT---AGCCGATGATTCGGTGTTGTTCGTCGCGCTCGTCC
Olathe_61e_Hz_ITS1 (181)  GT--GGACGCTGTGCGTTGAAT---AGCCGATGATTCGGTGTTGTTCGTCGCGCTCGTCC
  Ha_ITS1_KT343378 (151)  GAACGGACGCTGTGCGTTGAATGATAGCCGATGATTCGGTGTTGTTCGTCGCGCTCGTCC
Olathe_61e_Ha_ITS1 (151)  GT--GGACGCTGTGCGTTGAAT---AGCCGATGATTCGGTGTTGTTCGTCGCGCTCGTCC
                          241                                                     300
  Hz_ITS1_KT343380 (236)  GTCGTTATGTGTTCTCGCGTCGCGTCATGCGCACGACACGCAGAAGGGTACAGAT--CAC
Olathe_61e_Hz_ITS1 (236)  GTCGTTATGTGTTCTCGCGTCGCGTCATGCGCACGACACGCAGAAGGGTACAGAT--CAC
  Ha_ITS1_KT343378 (211)  GTCGTTATGTGTTCTCGCGTCGCGTCATGCGCACGACACGCAGAAGGGTACACAAAACAC
Olathe_61e_Ha_ITS1 (206)  GTCGTTATGTGTTCTCGCGTCGCGTCATGCGCACGACACGCAGAAGGGTACAGAT--CAC
                          301                                                     360
  Hz_ITS1_KT343380 (294)  AAAAATCCGCGTCCCCGAGTGTCCGCACGG-CGCGTTCGTTAACAATCAAACACATGTCC
Olathe_61e_Hz_ITS1 (294)  AAAAATCCGCGTCCCCGAGTGTCCGCACGG-CGCGTTCGTTAACAATCAAACACATGTCC
  Ha_ITS1_KT343378 (271)  AAAAATCCGCGTCCCCGAGTGTCTGCATGCACGCGTTCGTTAACAATCAAACACAAGTCC
Olathe_61e_Ha_ITS1 (264)  AAAAATCCGCGTCCCCGAGTGTCCGCACGG-CGCGTTCGTTAACAATCAAACACATGTCC
                          361                                                     420
  Hz_ITS1_KT343380 (353)  CTTGCAACGCGGTGGGATAATATCATTTTCTTTTTT---ATACATACACACATGTATATA
Olathe_61e_Hz_ITS1 (353)  CTTGCAACGCGGTGGGATAATATCATTTTCTTTTTT---ATACATACACACATGTATATA
  Ha_ITS1_KT343378 (331)  CTTGCAACGCGGTGGGATGATATCATTTTATTTTTATACATACAAACACGCGTGTATATA
Olathe_61e_Ha_ITS1 (323)  CTTGCAACGCGGTGGGATAATATCATTTTCTTTTTATACATACATACACACATGTATATA
                          421                                                     480
  Hz_ITS1_KT343380 (410)  AAATACAACACACACACAAGTCTATATCATGTAACACTAGATTACGCGAACAGCGGTAAT
Olathe_61e_Hz_ITS1 (410)  AAATACAACACACACACAAGTCTATATCATGTAACACTAGATTACGCGAACAGCGGTAAT
  Ha_ITS1_KT343378 (391)  GAATACA---CACTCACAAGTCAATTTAATGTAACACTAGATTACGCAAACAGCGGTAAT
Olathe_61e_Ha_ITS1 (383)  AAATACA---CACACACAAGTCTATATCATGTAACACTAGATTACGCGAACAGCGGTAAT
                          481                                                     540
  Hz_ITS1_KT343380 (470)  TTAAAACTATTACCCTGGACGGTGGATCACTTGGCTCGCGGGTCGATGAAGAACGCAGTT
Olathe_61e_Hz_ITS1 (470)  TTAAAACTATTACCCTGGACGGTGGATCACTTGGCTCGCGGGTCGATGAAGAACGCAGTT
  Ha_ITS1_KT343378 (448)  TTAAAACTATTACCCTGGACGGTGGATCACTTGGCTCGCGGGTCGATGAAGAACGCAGTT
Olathe_61e_Ha_ITS1 (440)  TTAAAACTATTACCCTGGACGGTGGATCACTTGGCTCGCGGGTCGATGAAGAACGCAGTT
                          541                                           591
  Hz_ITS1_KT343380 (530)  AACTGCGCGTCATAGTGTGAACTGCAGGACACATTTGAACATCGACATTTC
Olathe_61e_Hz_ITS1 (530)  AACTGCGCGTCATAGTGTGAACTGCAGGACACATTTGAACATCGACATTTC
  Ha_ITS1_KT343378 (508)  AACTGCGCGTCATAGTGTGAACTGCAGGACACATTTGAACATCGACATTTC
Olathe_61e_Ha_ITS1 (500)  AACTGCGCGTCATAGTGTGAACTGCAGGACACATTTGAACATCGACATTTC
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Figure 1. Alignment of reference ITS1 sequences of *Helicoverpa armigera* and *H. zea* with ITS1 sequences obtained from Olathe, CO sample 61e. Nucleotides identical in both *H. armigera* and *H. zea* ITS1 sequences are shown in black. The ITS1 sequence Olathe_61e_Hz_ITS1 was identical to the ITS1 sequence of *H. zea*. Olathe_61e_Ha_ITS1 sequence was highly similar to *H. zea* ITS1 sequence except for the *H. armigera*-specific ITS1 sequences (text with gray background). Differences between *H. armigera* ITS1 sequence and Olathe_61e_Ha_ITS1 sequence are shown by white text with black background.

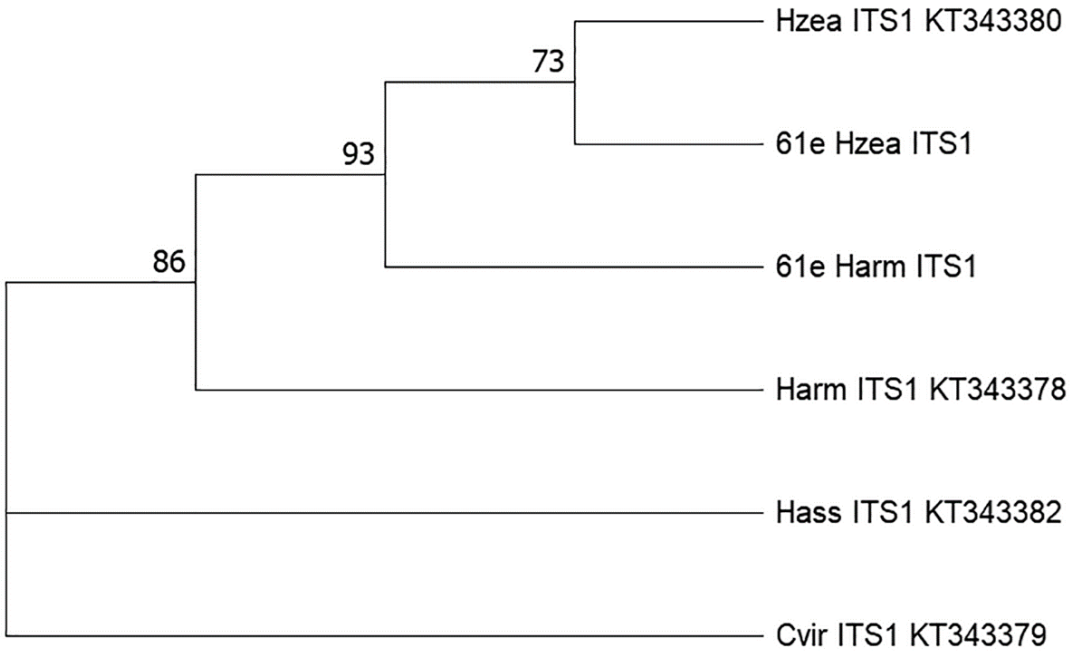

Figure 2. The evolutionary history was inferred among internal transcribed spacer 1 (ITS1) regions using Maximum Likelihood based on the Kimura 2-parameter model of sequence evolution (Kimura, 1980). The phylogeny among ITS1 regions among *Helicoverpa armigera* (Harm), *H. zea* (Hzea) and *H. assulta* (Hass) was rooted by the *Chloridea* (*Heliothis*) *virescens* (Cvir) ITS1 (GenBank accession provided at branch tips), based on a 591 nucleotide-long consensus alignment. The bootstrap consensus at each node was inferred from 10,000 replicates (Felsenstein, 1985) and partitions with less than 50% bootstrap support are collapsed. Neighbor-Join and BioNJ algorithms were applied to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach and the topology with superior log likelihood value was selected to obtain the initial tree(s). A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+*G*, parameter = 0.2548)). Evolutionary analyses were conducted in MEGA11 (Tamura et al. 2021).

Ribosomal RNA units in eukaryotes are arranged as head-to-tail tandem repeats at one or more loci (Nei & Rooney, 2005, Naidoo et al., 2013). One study indicated that the rRNA gene copy number in animals is highly variable, ranging from 39 in the mosquito *Sabethes cyaneus* (Fabricius) (Diptera: Culcidae) to 19,300 in the salamander *Amphiuma means* Garden in Smith 1821 (Urodela: Amphiumidae) (Prokopowich et al., 2003). Although precise copy number estimates are not available for noctuid moths, the silk moth *Bombyx mori* (L.) (Lepidoptera: Bombicidae) has approximately 240 copies of rRNA genes (Prokopowich et al., 2003). In the reference *H. zea* genome assembly, ilHelZeax1.1 (RefSeq assembly GCF_022581195.2; (Stahlke et al., 2023), BioProject: PRJNA818699), one complete set of rRNA repeats and one 18S rRNA subunit are annotated at one end of the chromosome 30 (Accession: NC_061481.1; XR_006986104.1, XR_006986099.1, and XR_006986099.1). Similarly, one rRNA repeat unit is annotated at one end of chromosome 30 in *H. armigera* (Accession: OZ005796.1; BioProject: PRJEB72184). Collapse of identical rRNA repeat units during the assembly of the genome may have reduced the number of repeats in the final assembly.

PCR screening of insects from the Olathe, CO, collection from 2023 with primers 3713CYP337B3F and 3714CYP337B3R identified 243 of 850 (28.6%) PCR positive and carrying at least one copy of a CYP337B3 gene. None of the samples from Texas or Mississippi collected in 2006 generated amplicons for the *H. armigera* CYP337B3 gene. Oxford Nanopore sequencing and mapping of individual reads from 113 full-length amplicons and 28 short amplicons from 141 CYP337B3 positive insects from Olathe, CO, predicted 100 and 99.33% nucleotide identity to the reference genomic sequences of CYP337B3 alleles v2 (KJ636466.1) and v6 (KX 958389.1), respectively. The subsequent consensus built from mapped reads (coverage > 200-fold) indicated that CYP337B3 alleles v2 (accession#: PP461124) and v6 (accession# PP461125) were present among individuals from Olathe, CO. Approximately 26.95% of the analyzed samples (38 out of 141) had CYP337B3v6 while the remaining were from the CYP337B3v2 allele. Four of the samples identified with the CYP337B3v2 allele also contained 5.3 to 7.6% Oxford Nanopore sequence reads that mapped to the CYP337B3v6 allele. The percentages of CYP337B3v6 reads identified in these four samples were significantly lower than the 50% reads expected from a heterozygous genotype with v2 and v6 alleles. These reads may have resulted from cross-contamination of samples during collection in traps and shipping in bags as bulk samples, or PCR bias is unknown primer binding site mutations exist. Therefore,

our data likely shows that the 141 individuals sequenced in this study were homozygous for either CYP337B3 v2 or v6 allele. Alignment of the mRNA and genomic sequences of CYP337B1v1, CYP337B2v2, and CYP337B3 alleles v1-v8, including two consensus CYP337B3v2 and CYP337B3v6 alleles sequences found in the Olathe, CO, showed Olathe CYP337B3v2 genomic and mRNA nucleotide sequences were nearly 100% identical to the reference CYP337B3v2 (KJ636466.1) sequence (Figs. S1 and S2). Specifically, there was one synonymous T to C substitution in the coding regions between the reference CYP337B3v2 allele (KJ636466.1) and the CYP337B3v2 gene variant present in the Olathe, CO population (Fig. S3). Alignments also showed that CYP337B3v6 genomic DNA and mRNA sequences were 99.4% and 98.9% identical to the reference CYP337B3v6 (KX 958389.1) allele, respectively. There were 16 SNPs between the reference CYP337B3v6 allele from Uganda (KX958389.1) and the v6 allele variant found in Olathe, CO (Fig S4), but only a single mutation, an A to T transversion at the nucleotide position 232, resulted in a conservative isoleucine to leucine substitution at the amino acid position 78 (I78L). Although conserved amino acid substitutions generally do not affect the function of a protein, alteration of function have been observed in some isoleucine to leucine substitutions (Brown et al., 2002, Christoffers et al., 2002, Sitbon et al., 1991). Therefore, implications of this I78L substitution on resistance to pyrethroids remain unknown and are in need if future empirical assessments. The consensus ML tree indicated that predicted CYP337B3v2 and v6 alleles from the Olathe, CO population clustered with corresponding reference alleles with node support from ≥ 99% of the bootstrap iterations (Fig 3).

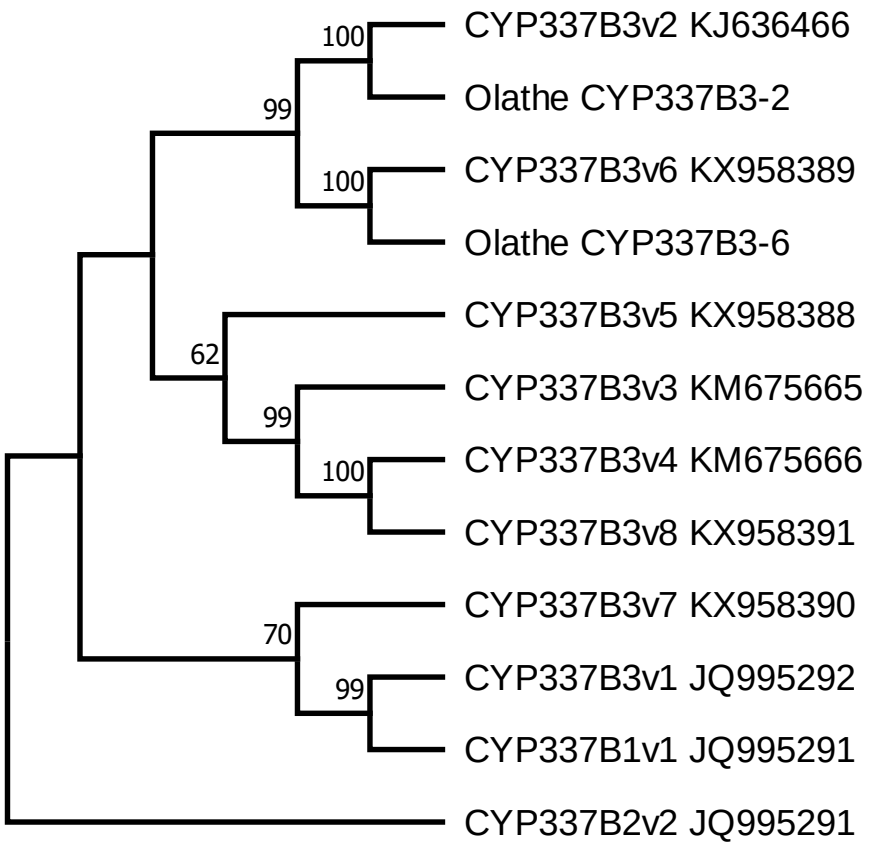

Figure 3. The evolutionary history was inferred among cytochrome P450 337B orthologs, CYP3B1-3, by using Maximum Likelihood and based on the Kimura 2-parameter model of sequence evolution (Kimura, 1980) with 10,000 bootstrap replicates (Felsenstein, 1985) as implemented in Mega 11 (Tamura et al. 2021). Relationships of alleles from the Olathe, CO population are shown with respect to reference sequences for CYP337B1, CYP337B2 and CYP337B3 paralogs (GenBank accessions include at respective branch tips) and based on 3605 positions in a consensus sequence alignment. Neighbor-Join and BioNJ algorithms were applied to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach and the topology with superior log likelihood value was selected to obtain the initial tree(s). A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+*G*, parameter = 0.3794) and the tree with the highest log likelihood (-9917.03) is shown.

346 **Discussion**

347 The chimeric CYP337B3 gene associated with and the functional cause of pyrethroid resistance
348 in *H. armigera* evolved from an unequal crossover between paralogs CYP337B1 and CYP337B2
349 and is not present in susceptible *H. armigera* (Joußen et al., 2012, Rasool et al., 2014).
350 CYP337B3 gene has not been reported in non-hybrid *H. zea*. The *H. armigera* CYP337B3 gene
351 confers resistance to the pyrethroid class of insecticides, including cypermethrin, deltamethrin,
352 and fenvalerate (Durigan et al., 2017, Rasool et al., 2014). Evidence of hybridization between *H.*
353 *armigera* and *H. zea* was previously reported in South America based on the introgression of the
354 CYP337B3 gene into sympatric populations (Valencia-Montoya et al., 2020, Tay et al., 2013,
355 Anderson et al., 2018, Ivey & Hillier, 2023). The initial report of hybridization in North America
356 was based on the detection of CYP337B3 in two *H. zea* collected in Texas during 2019 (North et
357 al., 2023). In this study we found that approximately 28.6% of the *H. zea* collected in sweetcorn
358 fields in Olathe, CO, where severe control failure occurred during the 2023 growing season,
359 carried one of two different alleles, CYP337B3v2 or CYP337B3v6, at the CYP337B3 locus.
360 Reference *H. zea* populations from Texas and Mississippi collected in 2006 prior to the
361 documented *H. armigera* invasion of South America or North America (USDA-APHIS 2023) did
362 not contain the CYP337B3 gene. Our results thus suggest that the *H. armigera* invasion into the
363 United States occurred after 2006 or was previously undetectable due to low frequency during its
364 initial establishment. Regardless, a more comprehensive spatial and temporal screening of
365 archived samples would be needed to approximate the time(s) and the location(s) of introduction.
366 Previous population genetic studies postulated that the CYP337B3 locus may be under strong
367 selection in Brazil due to the high frequency of this gene being associated with field failures of
368 pyrethroid insecticides to control damage (Valencia-Montoya et al., 2020, Durigan et al., 2017).
369 Pyrethroid resistance due to knock-down resistance (*kdr*) mutations in the voltage-gated sodium
370 channel and significantly increased expression of cytochrome P450 6B (CYP6B) genes was
371 previously reported in the *H. zea* populations in the USA (Hopkins & Pietrantonio, 2010,
372 Pietrantonio et al., 2007, Hopkins et al., 2011). In contrast, *kdr* mutations were not reported
373 among *H. armigera* populations in China, whereas the CYP337B3 gene was fixed among all
374 samples tested (Ni et al., 2023).

375 The above lines of evidence, along with introgression of genome regions flanking CYP337B
376 (Valencia-Montoya et al., 2020, Durigan et al., 2017; North et al., 2023), support the premise that
377 the chimeric CYP337B3 detected in the Americas originated from invasive *H. armigera*. Olathe
378 is in western Colorado, where a discontinuous cropping landscape exists punctuated by expanses
379 of non-arable land and high mountain elevations. Thus, the detection of the *H. armigera*
380 CYP337B3 in *H. zea* from such remote and isolated fields suggests that the effects of
381 introgression might be widespread across North America and potentially even surprising given
382 known mobility (Westbrook & López, 2010, Jones et al., 2019) and high gene flow of *H. zea*
383 within the continent (Perera et al., 2020, Seymour et al., 2016). The fact that two *H. zea* was
384 found in samples collected in 2019 from Texas (North et al., 2023) and CYP337B3 allele
385 frequencies ranging from 0.67 to 21.50% detected in samples collected in New York, Texas, and
386 Virginia from 2016 to 2023 (Perera, unpublished) most likely indicate that hybrid *H. zea* with
387 genes introgressed from *H. armigera* is omnipresent within the continent. Therefore, in our
388 opinion, quarantine, area-wide management programs, or regulatory action to limit the spread of
389 hybrid *H. zea* may not be successful. Regardless, continuous, and repeated use of pyrethroid
390 insecticides in the United States, especially in commercial sweetcorn production, likely provide
391 high selection pressures as previously hypothesized in South America (Valencia-Montoya et al.,
392 2020, Durigan et al., 2017). Thus, in contrast to adaptive introgression resulting in the transfer of
393 locally-adapted alleles from indigenous populations of genetically-compatible relative species to
394 invasive species (Edelman & Mallet, 2021), our results and prior evidence suggests the reverse
395 scenario exists in *Helicoverpa* due to the influence of selection to pyrethroid insecticide.
396 Evaluating phenotypic levels of pyrethroid resistance conferred by *H. zea* kdr and CYP6B
397 mechanisms compared to those by *H. armigera* CYP337B3 may provide an estimate of selective
398 advantages attained by introgression.

399 It was postulated that all allelic variants of CYP337B3 formed independently in different
400 geographic regions (Walsh et al., 2018). In Olathe, CO, population, the CYP337B3v2 allele was
401 present in 73.05% of the hybrid insects, while the remaining hybrid insects had the CYP337B3
402 v6 allele. The high-throughput sequencing of CYP337B3 amplicons interestingly did not indicate
403 the presence of heterozygotes among all 141 samples sequenced. Assuming the increase
404 metabolic capacity of homozygotes, their high prevalence may be anticipated at locations such as
405 Olathe, CO, where strong selection pressure likely resulted following repeated exposures to

406 pyrethroid applications. Regardless, mating between lineages each carrying different haplotypes
407 at the CYP337B3 locus would predict the formation of recombinants (heterozygotes) in the
408 population unless admixture is suppressed due to recency of contact or yet undescribed barriers
409 to gene flow exist. The CYP337B3v2 allele was first reported in Pakistan and subsequently in
410 Australia, China, and the African and European continents (Rasool et al., 2014, Han et al., 2015,
411 Walsh et al., 2018). In contrast, the CYP337v6 allele has only thus far been reported in Uganda
412 (Walsh et al., 2018). In a previous study of *Helicoverpa*, almost all *H. armigera* analyzed from
413 Brazil (n = 92) were homozygous for the CYP337B3v2 allele. At the same time, one insect was
414 heterozygous for CYP337B3v2/ CYP337B3v5 alleles and found one of 59 *H. zea* carried the
415 CYP337B3v2 allele (Walsh et al., 2018). Thus, the source population(s) for the introgressed
416 CYP337B3v2 allele in Olathe, CO may be the South American *H. armigera* or hybrid *H. zea-*
417 *armigera* populations that migrated or were transported northward, but an independent
418 introduction source of CYP337B3 into North America cannot be ruled out. Our study is the first
419 to report the African-derived CYP337B3v6 allele in the Americas, providing evidence that a
420 proportion of individuals in the Olathe population may be derived from an African source
421 population and thus is unique to the introduction into South America. It is possible that the *H.*
422 *armigera* population detected near O'Hare airport in Chicago, IL since 2019 could be a source of
423 one or both CYP337B3 alleles introgressed into *H. zea* in Olathe, CO, population. However, the
424 genetic composition of *H. armigera* from this interception from Chicago, IL remains unavailable,
425 leaving open the potential that CYP337B3 alleles we detected in Olathe, CO may be derived
426 from other point sources. Nevertheless, the two likely origins of CYP337B3 alleles detected
427 among *H. zea* from Olathe in this study indicate that at least two different hybridization events
428 occurred between *H. armigera* and *H. zea* in the Americas, and the CYP337B3v6 allele could
429 represent signature of one or more new *H. armigera* introductions into the Americas and the
430 USA.

431 The *H. armigera* rRNA ITS1 sequence identified in one insect (61e) in Olathe, CO population
432 further confirms introgression from *H. armigera* into *H. zea* through hybridization. Because
433 rRNA genes are not found on other chromosomes of either *Helicoverpa* species' genome
434 assembly, we inferred that orthologous rRNA genes are located only in chromosome 30. It is
435 conceivable that the crossover of orthologous chromosome regions in a hybrid progeny of *H. zea*
436 and *H. armigera* could result in the introgression of some of the rRNA repeats into the *H. zea*

437 genome. Regardless, several repetitive gene families in prokaryotes and eukaryotes are known to
438 undergo concerted evolution, which functions to limit variation between repeat units (Ganley &
439 Kobayashi, 2007, Peel et al., 2006, Rooney & Ward, 2005, Bettencourt & Feder, 2002, Liao,
440 2000, Benedict et al., 1996, Naidoo et al., 2013, Mullins & Fultz, 1989, Coen et al., 1982). This
441 insect 61e may have had rRNA gene repeats introgressed from *H. armigera* during a crossover
442 event between *H. zea* and *H. armigera* haplotypes and in the process of gene conversion and
443 repeated backcrossing to *H. zea.* However, it is not possible to determine the structure of the
444 rRNA repeats in this insect and the origin of the sequences resembling ITS1 of both *H. armigera*
445 and *H. zea* without haplotype phased sequence data from the segment of chromosome 30
446 containing the rRNA repeats. Concerted evolution would predict that the single nucleotide
447 polymorphisms (SNP) specific to *H. armigera* ITS1 or the *H. zea* ITS1 would be converted over
448 time, and unconverted variants may thus be indicative of how recent the introgression of the
449 rRNA genes occurred. It should be noted that sample 61e was not positive for the CYP337B3
450 gene, thus indicating it may have originated from a hybridization event independent of those that
451 led to the introgression of CYP337B1v2 and v6 alleles. Due to the lack of linkage between the
452 ITS region and CYP337B loci, a recombination in a backcross could also have disentangled
453 these two *H. armigera*-derived loci.

454 Invasive species are a threat to agricultural production in the United States and worldwide
455 (Coates et al., 2015). A scenario of adaptive introgression facilitated by the transfer of a novel
456 gene from an invasive to a native insect, as predicted for the *Helicoverpa* pest complex, provides
457 a unique opportunity to study evolution and selection in real-time. Specifically, our evidence
458 supports the admixture of previously geographically distinct introgressed CYP337B3v2 and
459 CYP337B3V6 alleles into a single population. Although any hybrid vigor or advantageous
460 phenotypic effects conferred by a unique combination of two pyrethroid resistance alleles in the
461 North American population remains to be determined and the prevalence of these alleles across
462 the continent remain unknown, high gene flow between North American *H. zea* populations
463 (Perera et al., 2020) predicts rapid spread of these genes across the continent. CYP337B3 gene is
464 only an indicator of the extent of gene transfer between these two species. We expect other genes
465 with selective advantage (e.g. resistance to *Bacillus thuringiensis* insecticidal proteins) might be
466 present in hybrid *H. zea* populations. The impacts of genes derived from invasive *H. armigera* on
467 grower production practices remain uncertain, but continued selection for pest adaptions to

practices that rely on insecticidal agents may be predicted to drive introgression of associated resistance alleles into *H. zea* .

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Author contributions

Conceptualization: MIN, OPP, and BSC. Methodology: MIN, OPP, CAP, DJ, and JG. Investigation: MIN, OPP, CAP, BSC, CAA, and DJ; Formal Analysis: MIN, OPP, BSC, CAA, and JG; Writing - Original Draft: MIN and OPP. Writing - Review & Editing: All authors. Resources: PON, MC, TT, and GVPR; Supervision: OPP.

Declaration of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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