Mitochondrial DNA genomes of five major *Helicoverpa* pest species from the Old and New Worlds (Lepidoptera: Noctuidae)

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**Funding information**
USDA-ARS; Fundação de Amparo à Pesquisa do Estado de Goiás, Grant/Award Number: Helicoverpa/20130102670001419; Commonwealth Scientific and Industrial Research Organisation, Grant/Award Number: R-03255-01, R-08681-01 and R-90204-01

**Abstract**
Five species of noctuid moths, *Helicoverpa armigera*, *H. punctigera*, *H. assulta*, *H. zea*, and *H. gelotopoeon*, are major agricultural pests inhabiting various and often overlapping global distributions. Visual identification of these species requires a great deal of expertise and misidentification can have repercussions for pest management and agricultural biosecurity. Here, we report on the complete mitochondrial genomes of *H. assulta assulta* and *H. assulta afra*, *H. gelotopoeon*, *H. punctigera*, *H. zea*, and *H. armigera armigera* and *H. armigera conferta* assembled from high-throughput sequencing data. This study significantly increases the mitogenome resources for these five agricultural pests with sequences assembled from across different continents, including an *H. armigera* individual collected from an invasive population in Brazil. We infer the phylogenetic relationships of these five *Helicoverpa* species based on the 13 mitochondrial DNA protein-coding genes (PCG’s) and show that two publicly available mitogenomes of *H. assulta* (KP015198 and KR149448) have been misidentified or incorrectly assembled. We further consolidate existing PCR-RFLP methods to cover all five *Helicoverpa* pest species, providing an updated method that will contribute to species differentiation and to future monitoring efforts of *Helicoverpa* pest species across different continents. We discuss the value of *Helicoverpa* mitogenomes to assist with species identification in view of the context of the rapid spread of *H. armigera* in the New World. With this work, we provide the molecular resources necessary for future studies of the evolutionary history and ecology of these species.

**KEYWORDS**
biosecurity, COI, lepidopteran pests, mitogenomes
1 | INTRODUCTION

Accurate species identification is the foundation for all biological research; however, the scientific community is often distracted by polarized support either for traditional morphological or for molecular identification of species (e.g., Hebert, Penton, Burns, Janzen, & Hallwachs, 2004; Rubinoff, 2006). However, it is also becoming increasingly clear that both methods contribute value and should be better integrated to provide stronger support for defining species status (e.g., Desalle, 2006). Confusion in the scientific literature, especially relating to visually similar organisms, can lead to substantial difficulty in formulating and developing management, trade, and economic policies. Furthermore, the availability of high-throughput sequencing data is revealing that hybridization between so-called species, is perhaps more common than was previously thought (Anderson et al., 2018; Anderson, Tay, McGaughran, Gordon, & Walsh, 2016; Elfekih et al., 2018).

Examples of this conflict between molecular and morphological identification include the stored grain beetle, Cryptolestes spp., where despite recent studies combining molecular data and morphology, confusion remains (Tay, Beckett, & De Barro, 2016; Wang et al., 2014). In contrast, a successful example of integrating DNA data, with morphological and phenotypic characters to differentiate species, is the differentiation Asian and European honeybee mite species, Varroa jacobsoni and V. destructor, respectively (Anderson & Trueman, 2000).

Confident and unambiguous identification of invasive organisms especially those with agricultural and economic significance is becoming increasingly important in a highly mobile world. This can be seen with the recent incursion of the Old World cotton bollworm, Helicoverpa armigera, into the New World (e.g., Czepak, Albernaz, Vivan, Guimarães, & Carvalhais, 2013; Tay et al., 2013), and the detection of both sister species of the fall army worm (FAW), Spodoptera frugiperda, in Africa (Cock, Beseh, Buddle, Cafa, & Crozier, 2017; Goergen, Kumar, Sankung, Togola, & Tamò, 2016; Nagoshi et al., 2017; Otim et al., 2018). Although the timing of S. frugiperda’s arrival to the African continent is as yet unknown, the arrival of H. armigera in Brazil occurred sometime before the cropping season of 2012/13 when it was first identified from historical sampling efforts (Sosa-Gómez et al., 2015). The morphological similarity between H. armigera and the New World H. zea was likely an important factor for the delay in detection. Various studies (Anderson et al., 2018, 2016; Arnemann, 2015; Arnemann et al., 2016; Arneodo, Balbi, Flores, & Sciocco-Cap, 2015; Mastrangelo et al., 2014; Tay et al., 2013) have shown that H. armigera populations in Brazil and neighboring countries had wide potential geographic origins from Asia, Africa, and Europe, with their introductions having a strong association with global agricultural and horticultural trade movements into South America (Tay, Walsh et al., 2017).

Co-occurring with H. armigera across the Old World is the Solanaceae specialist H. assulta, while H. punctigera, a major agricultural pest in itself, is endemic to Australia (for a review see Hardwick, 1965). Helicoverpa armigera, H. punctigera, and H. zea are morphologically similar and identifying them has traditionally relied on dissecting the adult male and female genitalia (e.g., Hardwick, 1965; Pogue, 2004), which is both time consuming and technically challenging. Studies by Behere, Tay, Russell, and Batterham (2008) and Fang et al. (1997) have previously assessed mtDNA and nuclear DNA genes to distinguish between the major Helicoverpa pest species. Behere et al. (2008) developed a PCR-RFLP method of identifying H. armigera, H. zea, H. punctigera, and H. assulta based on partial mtDNA COI and cytochrome b (Cytb) genes, Arneodo et al. (2015) applied the concept of Behere et al. (2008) and developed a RFLP method to assist with the rapid differentiation between New World H. zea and H. gelotopoeon and H. armigera. However, both Behere et al. (2008) and Arneodo et al. (2015) used different mtDNA COI gene regions, and identification by PCR-RFLP between these five Helicoverpa pest species would therefore require different PCR amplicons.

Recent studies relating to the molecular characterization of complete mitochondrial DNA genomes (mitogenomes) have used high-throughput sequencing technology that enables rapid mitogenome assembly of a wide range of insect species. High-throughput sequencing platforms with improved bioinformatic pipelines for assembling mitogenomes have also been shown to be an ideal option for studying historical specimens, in vertebrates (e.g., Anmarkrud & Lifjeld, 2017) as well as insects (e.g., Tay, Elfekih et al., 2017), where genomic DNA is typically fragmented due to the age of samples, and/or poor preservation conditions. These factors represent a significant challenge to the Sanger method (Sanger & Coulson, 1975) of sequencing PCR amplicons. Furthermore, applying high-throughput sequencing methods also bypasses potential primer annealing issues, gDNA template limitations, and reduces the chances and impact of contamination.

Currently, there are published mitogenomes of H. armigera (Yin, Hong, Wang, Cao, & Wei, 2010) from China, H. punctigera (Walsh, 2016) from Australia, H. zea (Perera, Walsh, & Luttrell, 2016) from the United States of America, two mitogenomes of H. assulta from China, but no mitogenomes for H. gelotopoeon. For the H. assulta mitogenomes, one has been published (Li, Zhang, Luo, Cui, & Dong, 2016; GenBank KP015198), while the second is unpublished but publicly available (GenBank KR149448). In this study, we report on the molecular characterization of an additional 15 mitogenomes that include the New World H. gelotopoeon from Argentina and a Brazilian H. zea, the Old World H. assulta (subspecies assulta, present in Asia, Australasia, Europe) and subspecies atra (present in Africa, south of the Sahara Desert); (Hardwick, 1965), H. armigera conferta, (present in Australia) and H. armigera armigera (present in Asia, Europe, Africa (Hardwick, 1965) and South America (Anderson et al., 2018, 2016), and increase the mitogenome resources of the Australian endemic H. punctigera (Table 1). We show that the available H. assulta mitogenomes are affected by misidentification (KP015198) and sequencing errors (KR149448). We also consolidate the current PCR-RFLP methods for species identification to include all five Helicoverpa pest species. Furthermore, we discuss the biosecurity implications of our study with respect to pest species identification and the importance
of accurately characterized mitogenomes, while providing the molecular resources necessary for future studies of the evolutionary history and ecology of these Helicoverpa pest species.

2 | MATERIAL AND METHODS

2.1 | Helicoverpa species DNA library construction and sequencing

Fifteen mitogenomes were sequenced in this work: H. assulta assulta (n = 2), H. assulta apha (n = 1), H. gelotopoeon (n = 2), H. zea (n = 1), H. punctigera (n = 2), H. armigera conferta (n = 2), H. armigera armigera (n = 5), and H. zeze (n = 1) draft mitogenomes (mtGenome) lengths, collection dates of individuals, country of origins, and GenBank accession numbers assembled for this study.

$\textbf{Table 1}$ | Samples and sequences used in this work. Helicoverpa gelotopoeon (n = 2), H. assulta assulta (n = 1), H. assulta apha (n = 1), H. zea (n = 1), H. punctigera (n = 2), H. armigera conferta (n = 2), H. armigera armigera (n = 5), and H. zeze (n = 1) draft mitogenomes (mtGenome) lengths, collection dates of individuals, country of origins, and GenBank accession numbers assembled for this study.

| Helicoverpa sp. | Draft mitogenome length (bp) | Collection date | Country | GenBank |
|----------------|-------------------------------|-----------------|---------|---------|
| gelotopoeon    | 15,226                        | 2013            | Argentina | MG437199 |
| gelotopoeon    | 15,230                        | 2013            | Argentina | MG437189 |
| assulta assulta| 15,184                        | 2013            | Australia | MG437197 |
| assulta assulta| 15,400                        | 1986            | Thailand | KT626655 |
| assulta assulta| 15,403                        | 1981            | Tanzania | MG437198 |
| punctigera     | 15,382                        | 2013            | Australia | KF977797 |
| punctigera     | 15,382                        | 2013            | Australia | MG437200 |
| punctigera     | 15,374                        | 2013            | Australia | MG437201 |
| armigera armigera | 15,347                     | NA              | China    | GU188273 |
| armigera conferta | 15,347                     | 2013            | Australia | MG437194 |
| assulta apha   | 15,351                        | NA              | China    | KP015198 |
| armigera conferta | 15,311                     | 2013            | Australia | MG437193 |
| armigera armigera | 15,344                     | 2013            | Brazil   | MG437190 |
| armigera armigera | 15,234                     | 2013            | Spain    | MG437191 |
| armigera armigera | 15,249                     | 2005            | Uganda   | MG437196 |
| armigera armigera | 15,322                     | 2004            | India    | MG437192 |
| armigera armigera | 15,373                     | 2006            | Madagascar | MG437195 |
| zeze            | 15,343                        | 2014            | USA      | KJ930516 |
| zeze            | 15,352                        | 2012            | Brazil   | MG437202 |

This $H. assulta$ individual (KP015198) is highly likely to be a misidentified $H. armigera$ individual from China based on nucleotide sequence identity and phylogenetic analysis as presented in this current study.

With the exception of the pinned historical $H. assulta$ from Tanzania and $H. assulta$ from Thailand, all Helicoverpa specimens were stored in ≥95% ethanol. DNA was purified using the Blood and Tissue DNA extraction kit (Qiagen), prior to quantification using Qubit (Life Technologies). Sequencing libraries were constructed as reported in Walsh (2016). DNA extraction from $H. assulta$ (KT626655) was as reported in Perera et al. (2015), and the DNA library was constructed as described in Perera et al. (2015). DNA library sequencing was performed at the Australian National University Biomolecular Resource Facility (Canberra, Australia) and the USDA-ARS Genomics and Bioinformatics Research Unit, (Stoneville, MS, USA).

Initial identification of adult $H. gelotopoeon$ specimens from $H. zeze/H. armigera$ as described by Hardwick (1965) using the adult morphology and subsequently confirmed by partial mtCOI
sequence identity prior to complete mtDNA genome characterization. For the differentiation between subspecies of *H. assulta*, we followed the guidelines outlined by (for a review see Hardwick, 1965); that is, *H. a. assulta* present in Asia, Australasia, Europe; *H. a. afra* present in Africa, south of the Sahara Desert.

2.2 Mitogenome assembly

For the assembly of the *Helicoverpa* mitogenomes (Table 1), we used two separate methods involving different assembly programs of either the genomic software Geneious R8 version 8.1.9 (Biomatters Pty Ltd., Auckland, NZ) or the CLC Genomic Workbench v8.5 (Qiagen). The mitogenome of *H. assulta* from Thailand was de novo assembled using CLC Genome Workbench, and the remainder of the mitogenomes was assembled using mitogenomes of *H. punctigera* (KF977797), *H. armigera* (GU188273), and *H. zea* (KJ930516) as reference sequences. After the initial sequence assembly based on the appropriate reference mitogenome, we reassembled these mitogenomes against their first version mitogenome templates. With each subsequent reassembly, we fine-tuned and removed all ambiguity by manually checking for potential misassembled regions. This procedure was repeated between three to eight times until a complete draft mitogenome was obtained. Draft mitogenomes from this study are available in GenBank (MG437189-MG437202, KT626655; Table 1).

2.3 Molecular characterization of *Helicoverpa* draft mitogenomes

To characterize the assembled draft mitogenomes of all *Helicoverpa* species, we used the program MITOS (Bernt et al., 2013), specifying the invertebrate genetic code (code #5) for identifying all tRNAs, rRNAs, and the start of protein-coding genes (PCGs). The origin of replication in our assembled mitogenomes was putatively identified, and due to its low complexity nature, we inserted a string of 5 N’s to indicate potential assembly difficulty across this region. The characterized mitogenomes were manually adjusted for stop codons to indicate the end of the PCGs using the published *H. zea* mitogenome PCGs as reference (Perera et al., 2016, KJ930516), although we note that to identify the most likely stop codon would require sequencing of RNA reverse transcribed cDNA (Gissi & Pesole, 2003).

2.4 Confirmation of species identity

We examined all mitogenome PCGs using BlastN (Altschul et al., 1997) to confirm species identity. To confirm our putative *H. assulta assulta* individuals (MG437197; KT626655), we compared our *H. assulta assulta* PCGs against both the published (Li et al., 2016; KP015198 and NC_026199) and unpublished *H. assulta* mitogenome (GenBank KR149448). The published mitogenome (KP015198) was reported from an individual collected from a cotton host in Anyang (Henan Province, China). We aligned our newly assembled *H. assulta* mitogenomes and those available in GenBank using the nucleotide alignment program MAFFT v7.017 (Katoh, Misawa, Kuma, & Miyata, 2002), implementing default settings (i.e., automatic algorithm option, Scoring Matrix: 200PAM/K2, Gap open penalty 1.53; Offset value: 0.123) within Geneious v8.1.9. Due to the significant differences detected between our two *H. assulta* mitogenomes (MG437197, KT626655.1) and the previously reported mitogenomes (GenBank KR149448, NC_026199/KP015198) from both MAFFT alignment and BlastN searches, we realigned these four *H. assulta* mitogenomes against the published *H. armigera* mitogenome available in GenBank (GU188273; Yin et al., 2010).

2.5 Phylogenetic analysis

We performed a phylogenetic analysis using all 13 protein-coding genes (PCGs) found in the publicly available mitogenomes of selected noctuid species: (a) *Agrotis segetum* (KC894725, Wu, Cui, Du, Gu, & Wei, 2014), (b) *A. ipsilon* (KF163965, Wu, Cui, & Wei, 2015), (c) *Spodoptera frugiperda* (KM362176, (Liu, Chai et al., 2016), (d) *S. litura* (JQ647918, Fan, Kim, & Kim, 2013) (KF543065, Liu, Zhu et al., 2016), (e) *Chloridea (Heliolithis) subflexa* (KT598688, de Souza, Tay, Czekaj, Elafikh, & Walsh, 2016), selected available *Helicoverpa* species mitogenomes that included (f) *H. punctigera* (KF977797, Walsh, 2016), *H. assulta assulta* (KP015198, Li et al., 2016) KR149448, (unpublished), *H. zea* (KJ930516, Perera et al., 2016), and *H. armigera armigera* (GU188273, Yin et al., 2010). All complete mitogenome sequences were first aligned using the MAFFT alignment program with default parameters as detailed above. Next, all PCG’s were re-adjusted to include a stop codon, while the start codon was as determined by MITOS (Bernt et al., 2013), prior to extraction to Geneious R8 for fine-scale alignment and sequence trimming where necessary (Supporting Information Data S1: Aligned PCGs). Phylogenetic analysis of the concatenated PCGs from all noctuid species was carried out using the PhyML web-based program (Guindon et al., 2010) with 1,000 bootstrap replications and automatic model selection. Visualization of the inferred phylogeny was conducted within the program Dendroscope v3.2.10 (Huson & Scornavacca, 2012).

2.6 PCR-RFLP analysis of all five *Helicoverpa* pest species

Two previous studies have reported methods for distinguishing between *Helicoverpa* species through interrogation of mitochondrial markers, though each was limited in scope. Behere et al. (2008) utilized two restriction enzymes to differentiate *H. punctigera*, *H. armigera*, *H. assulta*, and *H. zea* based on RFLPs from the 3’ region of the mtDNA COI gene (511 bp) and a partial Cyt b gene region (434 bp), but the study lacked the South American *H. gelotopoeon* species that is also a polyphagous pest. The second study, that of Arneodo et al. (2015), used a single restriction enzyme (Hinfl) on the 5’ end of the partial COI gene (812 bp) to generate specific RFLP patterns for *H. armigera*, *H. zea*, and *H. gelotopoeon*. Mitogenome resources from this study enabled surveys of the relevant gene regions of...
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TABLE 2  Helicoverpa armigera (GU188273), suspected misidentified H. assulta (KP015198), and two chimeric H. assulta/H. armigera (KR149448) and (KR149448) mtDNA gene regions (i.e., 3’ region of COI genes of H. gelotopoeon, trimmed to 511 bp; 5’ region of Cyt b gene, trimmed to 411 bp). To update the method of Arneodo et al. (2015), we analyzed the 5’ COI gene regions of H. punctigera and H. assulta. Sequences were identified for restriction enzyme(s) and predicted RFLP patterns using the CLC Sequence Viewer 7 program. Relevant partial COI and Cyt b gene regions previously reported (Arneemann et al., 2016; Arneodo et al., 2015; Behere et al., 2008; Leite et al., 2016; Tay, Walsh et al., 2017) were also included in the current study (Supporting Information Data S2 and S3—GenBank accession numbers and sequences used for RFLP).

3 | RESULTS

3.1 | Molecular characterization of the mitogenomes of Helicoverpa (sub)species

The assembled mitogenomes of the five Helicoverpa species were estimated to be between 15,226 bp and 15,403 bp in length (Table 1). We identified all 13 PCG’s, two rRNA genes, and 22 tRNA genes for all assembled mitogenomes. Perfect synteny was observed between mitogenomes of H. gelotopoeon, both subspecies of H. assulta, and previously published Helicoverpa species mitogenomes (i.e., Perera et al., 2016; Walsh, 2016; Yin et al., 2010). Intraspecific nucleotide sequence identities were generally low ranging between 93.03% (between H. punctigera KF977797 and MG437197) and 94.83% (between H. armigera GU188273 and H. assulta KT626655), while between the two most recently diverged species H. armigera and H. zea this ranged between 96.90% and 97.40% (average = 97.24%). The average intraspecific nucleotide sequence identities were high at the mitogenome level, (i.e., 99.83%, H. punctigera; 99.67%, H. gelotopoeon; 98.88%, H. assulta (excluding chimeric and Sanger sequencing error individuals, see below); 99.46%, H. armigera; and 99.51%, H. zea).

Comparing the H. assulta mitogenomes from this study (MG437197, MG437198, KT626655) to the published (KP015198, Li et al., 2016) and the unpublished (KR149448) H. assulta mitogenomes highlighted some issues (Table 2). While both H. assulta mitogenomes assembled in this study were >99% identical, they shared 98% sequence identity with the unpublished H. assulta mitogenome (KR149448), and only 95% sequence identity with the published H. assulta mitogenome (KP015198, Li et al., 2016). Similarly, comparison across the 13 PCG’s and the two rRNA genes (Table 2) showed that the H. assulta (KP015198) and the H. armigera (GU188273) mitogenomes were highly similar, sharing >99%–100% nucleotide identity. For the unpublished H. assulta mitogenome (KR149448), the lower level of nucleotide sequence identity (98%) with our H. assulta mitogenomes was predominantly due to low identity from

| Species | COI (%) | COII (%) | COIII (%) | ATP6 (%) | ATP8 (%) | COB (%) | CO9L (%) | CO10L (%) | CO11L (%) | CO12L (%) | ND1 (%) | ND2 (%) | ND3 (%) | ND4 (%) | ND4L (%) | ND5 (%) | ND6 (%) |
|---------|---------|----------|-----------|----------|----------|---------|----------|-----------|-----------|-----------|---------|---------|---------|---------|---------|---------|---------|
| GU188273 | 99.74 | 99.75 | 99.94 | 99.79 | 99.62 | 99.12 | 98.13 | 99.62 | 99.13 | 98.13 | 99.45 | 99.68 | 99.62 | 99.13 | 98.13 | 99.45 | 99.68 |
| KP015198 | 99.79 | 99.80 | 99.81 | 99.64 | 99.19 | 99.64 | 99.64 | 99.19 | 99.64 | 99.64 | 99.19 | 99.64 | 99.64 | 99.19 | 99.64 | 99.64 | 99.19 |
| KR149448 | 99.80 | 99.81 | 99.82 | 99.64 | 99.19 | 99.64 | 99.64 | 99.19 | 99.64 | 99.64 | 99.19 | 99.64 | 99.64 | 99.19 | 99.64 | 99.64 | 99.19 |
| MG437197 | 99.80 | 99.81 | 99.82 | 99.64 | 99.19 | 99.64 | 99.64 | 99.19 | 99.64 | 99.64 | 99.19 | 99.64 | 99.64 | 99.19 | 99.64 | 99.64 | 99.19 |
| KT626655 | 99.80 | 99.81 | 99.82 | 99.64 | 99.19 | 99.64 | 99.64 | 99.19 | 99.64 | 99.64 | 99.19 | 99.64 | 99.64 | 99.19 | 99.64 | 99.64 | 99.19 |

Note: Shaded regions indicate higher than expected nucleotide sequence identity at the interspecific level between H. armigera (GU188273) and the presumed H. assulta (KP015198; KR149448).
**TABLE 3**  SNP alignment at the mtDNA Cyt b gene between *Helicoverpa armigera* (GU188273), two putative *H. assulta* individuals (KP015198, KR149448), and two *H. assulta* (MG437197; KT626655.1) as reported in this study

|        | GU188273 | KP015198 | KR149448 | MG437197 | KT626655 |
|--------|----------|----------|----------|----------|----------|
| SNP    | C C A T C T C T A T C T C G A T T T T C A C T C A C T C T T T T C C |
| 1     | 1 1 1 1 1 1 2 2 2 2 2 2 2 2 3 3 3 3 4 4 4 4 4 5 5 5 5 5 5 5 6 |
| 3     | 1 3 3 1 3 3 5 7 8 9 1 2 3 4 5 6 7 7 7 7 7 7 0 3 5 6 5 6 9 9 0 3 7 7 9 9 1 |
|       | 3 8 7 8 2 5 6 6 2 9 5 6 3 4 6 8 7 1 3 7 9 2 5 9 0 2 5 8 7 4 6 9 1 4 2 |

Note. Nucleotide positions of detected SNPs are indicated (the start of the Cyt b CDS marks position 1). SNPs are compared to the GU188273 *H. armigera* and identical polymorphisms are indicated by ‘’. The orange shaded cell at nucleotide position 355 marks the first detectable change over of the *H. assulta* SNP pattern for KR149448 to one showing high homology with *H. armigera*. 
TABLE 4

| TAXON          | mtCOI1   | mtCOI2   | mtCOI3   | mtCOI4   |
|----------------|----------|----------|----------|----------|
| H. armigera    |          |          |          |          |
| H. assulta     |          |          |          |          |

Note: Gaps inserted during alignment are indicated by ‘-’ and ‘.’ indicate nucleotides identical to those in the H. armigera rrnS gene. The cell highlighted in orange at nucleotide position 425 represents the last identical nucleotide position between KR149448 and H. assulta. The sequence reverts back to H. assulta rrnS sequence between positions 426 and 485 predominantly sharing SNPs with H. assulta -343 and H. assulta OP.

3.2 | Phylogenetic analysis

PhyML identified the optimal nucleotide evolutionary model as GTR+G+I+F (lnL -33794.41; AIC 71706.82; BIC 72137.99). Phylogenetic analysis using the GTR substitution model, 0.616 proportion of invariable sites, four substitution rate categories and an estimated 1.140 gamma-shape parameter with 1,000 bootstraps was performed. The phylogeny (Figure 1) is based on the trimmed 13 PCG’s and includes as outgroups A. segetis, S. frugiperda, S. littera, Chloridea subflexa, resulting in H. punctigera being sister to all other Helicoverpa species, namely H. gelotopeon, H. assulta, and with H. zeay and H. armigera as the most recently diverged sister clades. As expected, the H. assulta mitogenome with partial H. armigera contamination (i.e., KR149448) was sister to the two subspecies of H. assulta. Similarly, the published H. assulta (KP015198) clustered within the global representation of H. armigera (i.e., H. armigera armigera and H. armigera conferta). Subspecies status (i.e., within H. assulta, and within H. armigera) could not be differentiated based on mitochondrial DNA genomes. All nodes had high (99.8%-100%) support values, with 100% bootstrap values obtained for each of the Helicoverpa species. The phylogenetic relationships between the pest Helicoverpa species were well-resolved using the complete mitogenome protein-coding gene sequences of 10,310 bp (Supporting Information Data S1), with the same tree topology as previously reported by (Anderson et al., 2016). The phylogenetic relationship between H. zeay and H. armigera suggested these two species shared a most recent common ancestor that diverged approximately 1.5-2 million years ago (Behere et al., 2007; Pearce et al., 2017a, 2017b). The monophyly of each of the subspecies of H. assulta (i.e., H. assulta assulta, H. assulta afra) and of H. armigera (i.e., H. armigera armigera, H. armigera conferta) was not well-resolved, with low bootstrap values at internal nodes (Figure 1). However, Anderson et al. (2016) was able to differentiate Australian H. armigera conferta from Old World H. armigera armigera based on genome-wide SNP data. A similar genome-wide SNP approach might be used to differentiate subspecies of H. assulta.
3.3 | RFLP analysis

The PCR-RFLP method of Behere et al. (2008) and Arneodo et al. (2015) that used 511 bp of the c-terminal COI gene, and 698 bp (trimmed from 732 bp) from the N-terminal COI gene, respectively, were revised such that either the N-terminal or C-terminal region of partial COI gene could be used to differentiate all five pest Helicoverpa species through a combination of four restriction enzymes (Table 5). The restriction enzymes selected represented a more comprehensive in silico analysis of publicly available partial COI sequences and avoided the need to include the partial Cyt b gene RFLP analysis originally designed by Behere et al. (2008). The revised RFLP method has not included the restriction enzymes identified by both Arneodo et al. (2015) and Behere et al. (2008) because of novel mtCOI haplotypes identified from global populations of *H. armigera* including from South America (e.g., Arnemann, 2015; Arnemann et al., 2016; Leite et al., 2016; Tay, Walsh et al., 2017).

4 | DISCUSSION

In this study, we increased the mitogenome resources for the five globally significant agricultural Helicoverpa species including subspecies for *H. assulta* and *H. armigera*, as well the South American endemic *H. gelotopoeon*. Furthermore, we report on species identification and mitogenome assembly issues with existing data for the Solanaceae specialist *H. assulta*. These mitogenome resources include *H. armigera armigera* subspecies from its Old World range as well as its New World invasive range. We also report the complete draft mitogenome of *H. zea* from Brazil, where previously only data from the North American continent were available (Perera et al., 2016). Finally, we have revised existing PCR-RFLP methods to allow for identification between these Helicoverpa pest species, taking into consideration the increase in partial mtDNA sequences resources in public DNA database. In addition, this method removes the need to survey the Cyt b gene region for confirmation of species, thereby eliminating possible detection failures due to PCR amplification failures for the Cyt b gene.
### Table 5 Predicted PCR-RFLP patterns on 698 bp (5' region) and 511 bp (3' region) partial COI gene regions of the five *Helicoverpa* species

| *Helicoverpa* species | COI (698 bp) | COI (511 bp) |
|-----------------------|--------------|--------------|
|                       | BseI         | BsaI         | BsaBI         | BspEI         | BstBI         | BspEI         | AquVI         | BseRI         | EcoRI         |
| punctigera            | × 1.0.698 (698 bp) | ✓ 1.0.494 (494 bp) | 494.0.698 (204 bp) | ✓ 1.0.698 (698 bp) | 1.0.511 (511 bp) | × 1.0.511 (511 bp) | ✓ 1.0.468 (468 bp) | 469.0.511 (43 bp) | × 1.0.511 (511 bp) |
| armigera              | × 1.0.698 (698 bp) | ✓ 1.0.494 (494 bp) | 494.0.698 (204 bp) | ✓ 1.0.698 (698 bp) | 1.0.511 (511 bp) | × 1.0.511 (511 bp) | ✓ 1.0.468 (468 bp) | 469.0.511 (43 bp) | × 1.0.511 (511 bp) |
| assulta               | ✓ 1.0.461 (461 bp) | 461.0.698 (237 bp) | ✓ 1.0.461 (461 bp) | 461.0.698 (237 bp) | 1.0.511 (511 bp) | ✓ 1.0.267 (267 bp) | 268.0.511 (244 bp) |
| zea                   | ✓ 1.0.461 (461 bp) | 461.0.698 (237 bp) | ✓ 1.0.461 (461 bp) | 461.0.698 (237 bp) | 1.0.511 (511 bp) | ✓ 1.0.468 (468 bp) | 469.0.511 (43 bp) |
| gelotopoeon           | ✓ 1.0.461 (461 bp) | 461.0.698 (237 bp) | ✓ 1.0.461 (461 bp) | 461.0.698 (237 bp) | 1.0.511 (511 bp) | ✓ 1.0.468 (468 bp) | 469.0.511 (43 bp) |

Note: Differentiation between all five *Helicoverpa* pest species for the N-terminal (698 bp) COI gene region required restriction endonucleases of BseI, BsaI, BsaBI, and BspEI. For the PCR-RFLP of the 511 bp C-terminal region of partial COI gene, Bco5I, AquVI, BseRI, and EcoRI restriction endonucleases were identified. The presence and absence of restriction enzyme cut sites within amplicons are indicated by ✓ and X, respectively. Expected restriction fragment lengths from either the 698 bp or 511 bp partial COI gene regions are indicated accordingly.
for potential H. armigera-H. gelotopoeon, H. assulta-H. gelotopoeon, H. assulta-H. zeae, and H. armigera-H. assulta hybrids remained to be tested. Failure to monitor for these interspecific hybrids may lead to invasive genotypes such as enhanced resistance to insecticides being spread unchecked (Walsh et al., 2018). The identification of naturally occurring hybrids will be difficult and will require significant coordination efforts between governmental departments (e.g., quarantine services, molecular detection, and identification facilities), and the development and adoption of new biosecurity policies. However, most of these are not yet recognized by policy makers as novel, potential and/or imminent national biosecurity threats.

Regardless of the shortcomings of mitochondrial genes in assisting with species confirmation, it is nevertheless desirable to obtain well-characterized mtDNA genes to bolster biosecurity and pest management practices. The importance of rechecking the assembled mtDNA against public DNA databases (e.g., NCBI Genbank, BoLD) is often not emphasized and has on occasion, led to the misidentification of species and mitogenomes (Tay, Elfeikh, Court, Gordon, & Barro, 2016; Walsh, 2016). While providing the much needed mitogenome resources for the Helicoverpa pest species across the Old and New Worlds, our study is not aimed at criticizing mistakes and oversights, but is rather, a cautionary reminder of the need to check sequence data against that readily available public DNA databases.

ACKNOWLEDGMENTS

Calvin A Pierce, USDA-ARS Southern Insect Management Research Unit for technical assistance. The authors thank the late Prof. Fernando Navarro (Entomología - Investigador CONICET, Universidad Nacional de Tucumán, Argentina) for providing and identifying H. gelotopoeon adults. CSIRO Health and Biosecurity project “Genes of Biosecurity Significance” (R-8681-1) to WTT and TKW. CSIRO Land and Water for funding TKW (R-90204-01) CSIRO Office of the Chief Executive postdoctoral fellowship funding (R-03255-01) to CA. CC was funded by FAPEG (Fundação de amparo a pesquisa do estado de Goiás; Grant number: Helicoverpa/2013102670001419). USDA-ARS for funding research of OPP. TW, WTT and KHG would like to thank CMK for motivation and support.

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CONFLICT OF INTEREST

None declared.

AUTHOR CONTRIBUTIONS

WTT, TW, KG, and AM conceived and designed the study. WTT, TW, OP, CA, and AM contributed to the work. All the authors contributed material, data, analysis and to the writing of the manuscript.

DATA ACCESSIBILITY

All assembled mitogenomes are available in NCBI Genbank (Table 1). Raw data is available from the CSIRO data portal (https://doi.org/10.4225/08/5ab8fd3e72d3).

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

How to cite this article: Walsh TK, Perera O, Anderson C, et al. Mitochondrial DNA genomes of five major Helicoverpa pest species from the Old and New Worlds (Lepidoptera: Noctuidae). Ecol Evol. 2019;9:2933–2944. https://doi.org/10.1002/ece3.4971