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Research Article

Confirmation of *Spodoptera frugiperda* (Lepidoptera: Noctuidae) in Papua New Guinea by molecular diagnostics of mitochondrial DNA COI gene

Wee Tek Tay[1,*], Lastus Kuniata[2], William James[1] and Thomas Walsh[1]

[1]*CSIRO Black Mountain Laboratories, Clunies Ross Street, ACT 2601, Australia*
[2]*Ramu Agri Industries Ltd., Gusap Downs, PO Box 2183, Lae, Morobe Province, Papua New Guinea*
**Corresponding author*
E-mail: *weetek.tay@csiro.au*

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Abstract

Native to the Americas and the Caribbean, the highly invasive agricultural pest *Spodoptera frugiperda* (fall armyworm, FAW) gained global prominence in 2016 when its presence was confirmed in Nigeria and São Tomé and Principe. Since then, it has been reported from over 70 countries in the African and Asian continents including the Near East, South East Asia, and Oceania. In this report, we provide confirmation of the presence of the pest in the northeast province of Papua New Guinea (PNG) via molecular analysis (i.e., DNA barcoding) of the partial mitochondrial DNA cytochrome *c* oxidase subunit I (mtDNA COI) gene. Our analyses identified the suspected insect species as *S. frugiperda* with a gene sequence characteristic of the R-strain. We discussed the biosecurity implication of detecting FAW in this PNG region that neighbours the Bismarck Sea and the New Britain Province as well as the Solomon Islands. We further highlighted the need to survey for beneficial insects of FAW to assist with developing integrated pest management strategies, and to apply whole genome sequencing approach to characterize insecticide resistance gene in FAW populations from this region.

Key words: Fall armyworm, Madang Province, introduction pathways, biosecurity preparedness

Introduction

The fall armyworm (FAW) *Spodoptera frugiperda* (J.E. Smith) is a highly destructive agricultural insect pest that is native to the North, Central and South Americas as well as the Caribbean. This species is known for its migratory ability (e.g., Jones et al. 2019; Sparks 1979) and high level of polyphagy with reports of over 350 host plants (Montezano et al. 2018). *S. frugiperda* has been classified as either the corn- or rice-preferred fall armyworm that have been regarded both as host strains and as closely related sister species (Dumas et al. 2015a, b; Otim et al. 2018) with pre- and post-zygotic reproductive barriers (reviewed by Groot et al. 2010), while there is a general push to replace host-preferences with the use of C- and R-strains (i.e., *Sfc*, *Sfr*), respectively (Tay et al. 2023). Since 2016, FAW have been causing widespread agricultural damage especially in maize crops

across Africa, Asia (Silver 2019; Stokstad 2017; Sun et al. 2021), South East Asia (Hang et al. 2020; Zhang et al. 2019), and Oceania (FAO 2020a, b).

The spread of the fall armyworm across the Old World appears to have followed a west to east direction based on reported detections; from western Africa (Goergen et al. 2016) to Central/Southern Africa (Nagoshi et al. 2017) and East Africa (Otim et al. 2018), followed by Middle East (FAO 2019a), India (Ganiger et al. 2018; Sharanabasappa et al. 2018), Myanmar, Thailand (EPPO 2019) and China (FAO 2019b; Tay and Gordon 2019; Zhang et al. 2019) prior to spreading across other South East Asian regions, including Malaysia, Indonesia, Philippines (FAO 2019c) in mid-2019, and the pest's detection in the Torres Strait and northern Australia (FAO 2020a, b). Its presence in Mari and Daru, Papua New Guinea (PNG) has also been detected from trapping of two individuals (Mr David Tenakanai, NAQIA, *pers. comm.* 21 July, 2020). Genome-wide analyses of invasive populations of FAW in China suggested hybrids of C-strain and R-strain FAW were widespread (Zhang et al. 2020). Analyses of native (North, Central, South Americas; the Caribbean) and invasive (Africa, India and China) FAW populations by genome-wide single nucleotide polymorphic (SNP) markers confirmed the hybrid status of FAW from the invasive range, and provided evidence of multiple introductions involving FAW populations from different native geographical locations, as well as evidence that suggested the spread of FAW occurred, also, from Asia into Africa (Tay et al. 2022a; Yainna et al. 2020).

Populations of FAW in East Africa and China have been shown to carry resistance alleles to organophosphate insecticides (Guan et al. 2020) but lacking resistance to pyrethroids, while Zhang et al. (2020) and Boavantura et al. (2020) reported resistance to pyrethroids in China and Indonesian FAW populations, respectively. Insecticide resistances of the invasive FAW detected to-date likely represented introduction events of genes of biosecurity importance from native American populations rather than as recently selected phenomena in their invasive range (Tay and Gordon 2019; Rane et al. 2023; Tay et al. 2022b). Insecticide resistance status to different classes of conventional insecticidal chemistries as well as to various *Bacillus thuringiensis* (Bt) toxins will also need to be determined in the pest's invasive range to assist with development of resistance management strategies for this pest, and to establish baseline allele frequencies of various resistance genes. Confirmation of the presence of the pest represents the first step to its long-term future management.

Differentiating between the invasive FAW and native related noctuid species (e.g., *S. litura* from Asia/Oceania; *Helicoverpa* species including *H. armigera* and *H. assulta from* Asia/Oceania; *H. punctigera from* Oceania) especially relating to larval identification, is often difficult via morphological characterisation, but can be readily achieved using the maternally inherited mitochondrial DNA cytochrome *c* oxidase subunit 1 (mtDNA COI) gene,

including identification using LAMP (loop-mediated isothermal amplification) assay as reported by e.g., Kim et al. (2021) and Agawal et al. (2022). Furthermore, differentiating between *Sf*C and *Sf*R has also been traditionally done using the mtDNA COI gene (Levy et al. 2002), although the nuclear gene Triose Phosphate Isomerase (*TPI*) on the Z chromosome has also been used in recent times (Nagoshi 2010). While both *Sf*C and *Sf*R (by mitochondrial marker) have been detected across Africa (Goergen et al. 2016; Otim et al. 2018), India (e.g., Tay et al. 2022a), China (Tay et al. 2022a; Zhang et al. 2020), and Australia (B. Thistleton DPIR, N.T., *pers. comm.* 7 May, 2020; Piggott et al. 2021). In our understanding, gaps relating to the pest's potential to both utilise different crop hosts and adaptation to their new environments remain, and characterisation of the pest into either *Sf*C or *Sf*R should be continued.

In this report we applied the mtDNA COI gene marker to rapidly and confidently confirm the presence of FAW in the Madang province of PNG, to demonstrate the use of the mtDNA COI gene as marker to differentiate FAW from *H. armigera*, and to provide characterization of their predicted host plant preference and discussed the need for biosecurity preparedness strategies and management options for PNG and surrounding regions in the South Pacific.

Materials and methods

Samples collection

Suspected FAW specimens were collected from Ramu Sugar Estate (5°58.154S; 145°53.252E) located at the Madang Province, PNG (Figure 1), from 3–4 weeks maize/corn plants. These maize crops were grown for the purpose of producing seeds for planting commercial crops/silage. The plot was less than 0.5 ha and was surrounded by commercial sugarcane crops. Collection of suspected specimens were done between May 15 and June 7, 2020. Samples were collected as multi-instar larvae (n = 17) but also included one adult moth and stored in 95% ethanol, prior to shipping to the CSIRO Black Mountain Laboratories in the ACT, Australia, for molecular diagnostics.

DNA extraction, PCR and sequencing, sequence analysis and molecular diagnostics

Total genomic DNA from all 18 suspect specimens were individually extracted using the Qiagen Blood and Tissue DNA extraction kit following manufacturer's instructions. Extracted DNA from each sample was eluted in 100 µL elution buffer EB and stored in −18 °C until readied for PCR. We followed the methods as described in Arnemann et al. (2019) for PCR profiles using the diagnostic markers Noc-COI-F (5′-GCGAAAATGACTT TATTCAAC-3′) and Noc-COI-R (5′- CCAAAAAATCAAAATAAATGTTG-3′) that amplifies the 5' terminal region (708 base pair) of the mtDNA COI gene

Figure 1. Sampling site in Ramu (Madang Province), Papua New Guinea. The map also shows Mari Village and Daru (Western Province, PNG) where two individuals of *Spodoptera frugiperda* were detected in February 2020. The Torres Strait islands of Saibai and Erub which are within Australia's Torres Strait Protected Zone, were also the first Australian locations where *S. frugiperda* were detected (late January, 2020) (FAO 2020a). Bamaga at northern Queensland, was the first mainland location in Australia where *S. frugiperda* was trapped in early February 2020 (FAO 2020b).

of noctuid moths, including for the molecular diagnostics of FAW, although this gene region in FAW generally lacks sequence variation to enable inference of intra-species genetic diversity (Tay et al. 2022a).

The PCR amplicon were visualized by gel electrophoresis on a 1.5% TAE agarose gel stained with GelRed® Nucleic Acid Gel Stain (Biotium, San Francisco) and viewed under UV-transilluminator. Amplicons were purified using Qiaquick PCR purification kit prior to setting up of Sanger sequencing reaction as described in Behere et al. (2007, 2008). Sanger sequencing was carried out at the Australian National University (Canberra, ACT) Biological Resource Facility at the John Curtin School of Medical Research. Trace files were analysed using the Pregap4 and Gap4 programs within the Staden sequence analysis package 2.0.0b10 (Staden et al. 2000) to assemble DNA contigs, editing of trace files, and to assess for sequencing accuracy. Assembled DNA contigs were checked for premature stop codons prior to identity search against GenBank DNA "nr" (non-redundant) dataset using the Blastn search program.

Mitochondrial DNA COI phylogenetic inferences

To provide statistical confidence (i.e., via high bootstrap support values and/or high nucleotide identity values) of species identity via the molecular diagnostic approach, we aligned the partial mtCOI gene (694 bp; matching

nucleotide positions 1,473 to 2,166 of MT897450) from the suspected PNG FAW specimens against 15 *Sfr* and 16 *Sfc* individuals from native and invasive ranges (Tay et al. 2022a), and compared against related invasive non-native (i.e., *S. orchrea* KJ6344308; *S. eridania* JQ605405; *S. cosmioides* HQ571028; and *S. littoralis* MT816470) and native (e.g., *S. litura* KF701043) *Spodoptera* species to further confirm the suspect species to be FAW *cf.* other related *Spodoptera* species. Based on the partial mtCOI sequences, one of the 18 samples showed low nucleotide similarity with *Spodoptera* species and was therefore suspected to likely be that of the closely related *Helicoverpa armigera* species known also to attack corn host plants. For this suspected PNG *H. armigera* specimen, we aligned 636 bp of partial mtCOI gene (matching nt 11 to 704 of MG437193) against mitochondrial genome sequences of related species (Walsh et al. 2016; Walsh 2019; Yin et al. 2010), selected published partial mtCOI sequences (Gilligan et al. 2015) and with the top 20 best-matched publicly available sequences from GenBank (see Supplementary material Table S1) that showed 100% nucleotide identity across the 636 bp partial mtCOI gene (Cho et al. 2008; Juen et al. 2012) through BLAST (Basic Local Alignment Search Tool) searches against the standard non-redundant (nr) database.

All sequences used in phylogenetic analyses with the suspected PNG *S. frugiperda* and *H. armigera* specimens were downloaded from GenBank. Sequences of PNG specimens are accessible from CSIRO public data repository as detailed in Rane et al. (2023). Multiple sequence alignments were carried out using MAFFT v7.450 (Katoh and Standley 2013) and trimmed to the desired lengths within the bioinformatics software Geneious v11.1.5 (Biomatters, Auckland NZ). We used the default option settings for MAFFT alignments (Algorithm: Auto; Scoring matrix: 200PAM / K = 2; Gap open penalty: 1.53; Offset value: 0.123), with the aligned sequences exported in fasta format to IQ-Tree 1.6.12 (Trifinopoulos et al. 2016) for Maximum Likelihood phylogenetic inferences using 1,000 UltraFast bootstrap (UFBoot) approximations (Minh et al. 2013). Visualisation of phylogenies was by Dendroscope 3 (Huson and Scornavacca 2012).

Results

Species diagnostics

Trace files edited using the Gap4 program within the Staden sequence analysis package (Staden et al. 2000) enabled the assembly of partial mtDNA COI DNA contigs for all 18 lepidopteran samples ranging between 680–700 bp. These DNA contigs were grouped into two separate contigs based on shared sequence similarity, with one group of contigs consisted of 17 sequences that shared 100% sequence identity. The second unique contig consisted of one sample. Blast search of the contig representing 17 of the suspect specimens (i.e., 16 larvae and one adult moth) identified these to

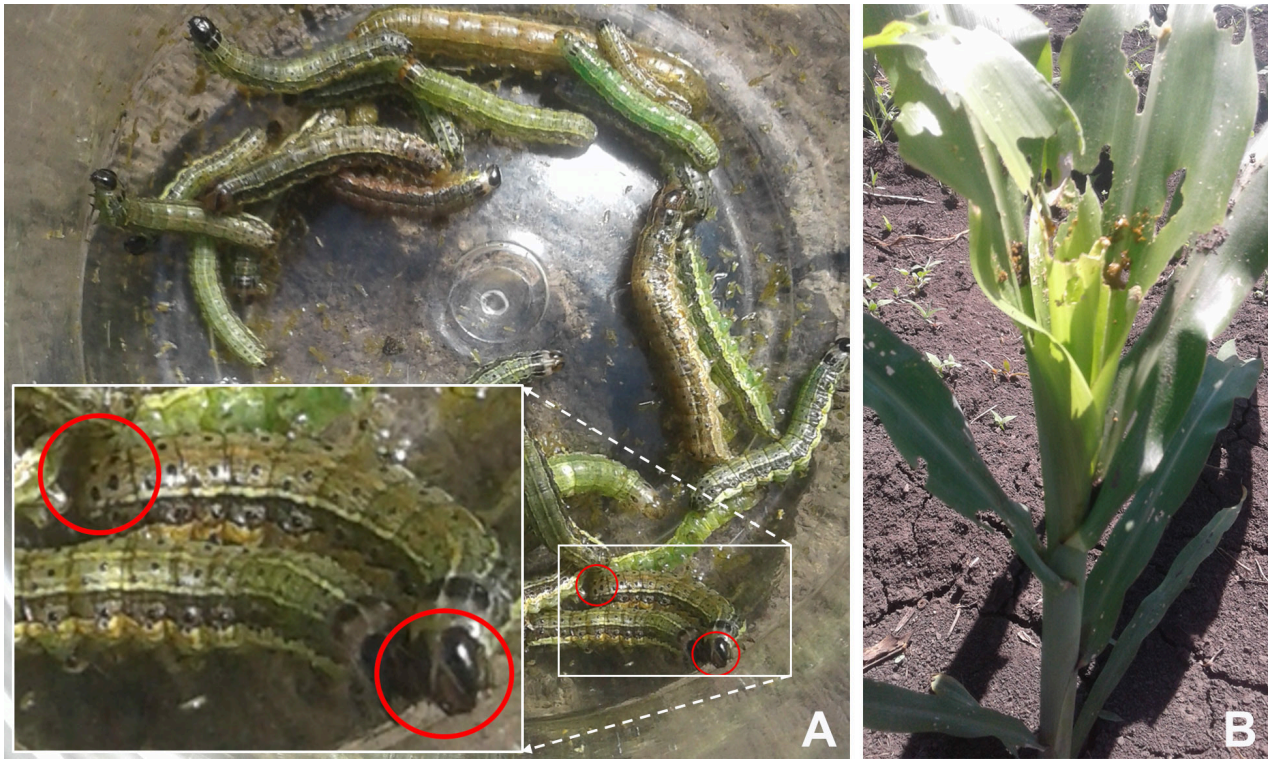

Figure 2. (a) A photograph of the sampled *Spodoptera frugiperda* individuals. Red circles on the caterpillar showed the characteristic morphological features of the species (inverted "Y" marking on the head and the four "square dots" at the end segments of the abdomen; see also enlarged insert), and (b) characteristic leaf-damage signs with frass on maize crop. Photos taken from the Ramu Sugar sampling site, Madang Province.

share 100% sequence identity with individuals from the R-strain FAW (i.e., *Sf*R) thus far identified across the invasive range including Uganda (GenBank accession MF197867), South Korea (GenBank accession MN599982), China (GenBank accession MN068212), Nepal (GenBank accession MT103345), and India (MT791633). A picture of FAW larvae from Ramu sampling site and a picture of damaged symptoms on maize are presented in Figure 2a and 2b, respectively. Phylogenetic analysis involving individuals representing global populations (Tay et al. 2022a) of FAW provided further confirmation that the Madang Province population of FAW belonged to the R-strain (*Sfr*) based on the 694 bp partial mtCOI gene sequence, where all *Sfr* individuals from the invasive range (e.g., China, India, Uganda, Tanzania, Malawi Benin, and PNG) and selelcted individuals from the native range (e.g., Mississippi MT897394; French Guiana MT897428; Brazil MT897450; USA Florida MT897374; and Puerto Rico MT897402) all shared 100% sequence identity (Figure 3).

A Blast search of the remaining one specimen's partial mtDNA COI gene showed that it shared 100% nucleotide sequence identity with four previously reported *Helicoverpa armigera conferta* partial mtDNA COI sequences from Australia (GenBank accessions MG437193, KT946077, HQ951239, and EU768936), but also a *H. armigera* sequence (JQ240198) that was misidentified as *H. punctigera* (Juen et al. 2012), as confirmed by phylogenetic analysis (Figure 4). Our *H. armigera* sequence represents the

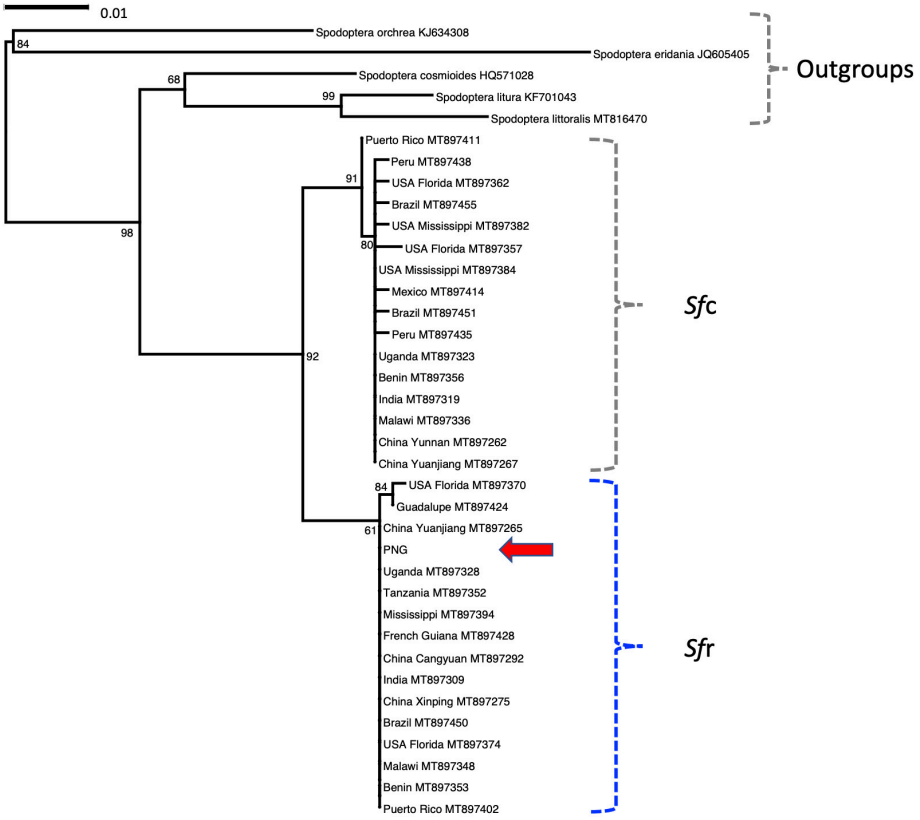

Figure 3. Maximum Likelihood (ML) phylogenetic analysis based on partial (694 bp) mtCOI gene of the suspected Papua New Guinea (PNG) *Spodoptera frugiperda* (red arrow) against specimens from invasive ranges (Benin, Uganda, Malawi, Tanzania, India, China) and from native ranges (Florida, Mississippi, Puerto Rico, Mexico, French Guiana, Guadalupe, Peru, Brazil). R-strain and C-strain are *Sf*r and *Sf*c, respectively. Outgroups are *S. litura* (Li et al. 2015), *S. littoralis, S. eridania, S. ochrea* (Van de Vossenberg and Straten 2014), and *S. cosmioides*. GenBank accession numbers and branch node support with > 60% confidence values are shown.

first partial mtDNA COI gene characterisation of the species from PNG. *Helicoverpa armigera* represents a complex of three sub-species based on morphological characterisation (Hardwick 1965), consisting of *H. armigera armigera*, *H. a. conferta*, and *H. a. commoni*. Differentiation between subspecies of *H. armigera* is not achievable using the mtDNA COI gene (Behere et al. 2007). Based on morphological characters, *H. armigera* from Australia, New Guinea, Fiji, and New Zealand were considered as belonged to the *H. a. conferta* sub-species (Hardwick 1965). Molecular characterisation based on whole genome sequencing (Pearce et al. 2017) and on genome-wide single nucleotide polymorphic marker analyses (e.g., Anderson et al. 2016, 2018; Valencia-Montoya et al. 2020; Zhang et al. 2022) have been shown to differentiate between the sub-species of *H. a. armigera* and *H. a. conferta,* as well as a candidate new sub-species of *H. armigera* from Xinjiang Province, China (Zhang et al. 2022), and such approaches will be needed to further confirm the sub-species and/or hybrid (i.e., between *H. a. armigera*/ *H. a. conferta*) status of the specimen from this study.

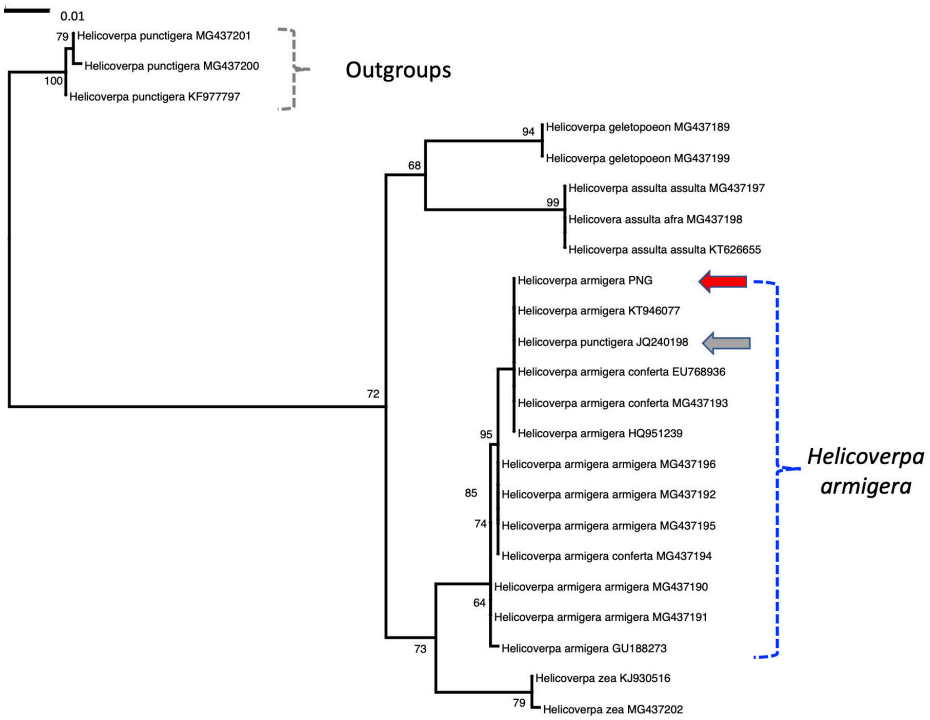

Figure 4. Maximum Likelihood (ML) phylogeny based on 636 bp of partial mtCOI gene, providing support that the PNG *Helicoverpa* sample to be *H. armigera* (red arrow). Grey arrow indicates likely misidentification of a *H. armigera* sequence from Australia (JQ2440198) as an Australian endemic related *H. punctigera* by Juen et al. (2012). All sequences from Walsh et al. (2019) except the following: KF977797 (Walsh 2016); KT946077 (Gilligan et al. 2015), JQ240198 (Juen et al. 2012); EU768936 (Cho et al. 2008), and GU188273 (Yin et al. 2010). KT626655 and HQ951239 are as GenBank released but unpublished sequences. Branch node support from 1,000 ultrafast bootstrap approximations of ≥ 60% are shown.

Discussion

Based on a molecular diagnostic approach via DNA characterization of the partial mtDNA COI gene, 17 of the 18 suspect lepidopteran specimens from the Ramu Sugar, Madang Province were identified as *Spodoptera frugiperda* and as R-strain FAW. Previously, two suspect lepidopteran individuals were collected in February 2020, from Mari and Daru, Western Province of PNG and confirmed as *S. frugiperda* by the Department of Agriculture, Water and the Environment (DAWE), Australia (Mr David Tenakanai, NAQIA, *pers. comm*. 21 July, 2020), representing the earliest report of FAW in PNG, although whether these were mtDNA COI C-strain and/or R-strain FAW was not known. The strain status based on the *TPI* gene marker for PNG FAW specimens confirmed in this study was not undertaken and was therefore not known. Nevertheless, presence of FAW in PNG is now confirmed for two provinces (i.e., Western, Madang; Figure 1) that are located at opposite ends of the country and separated by the highlands region in the middle, with the Madang province populations being further confirmed as having the R-strain (*Sfr*) mtCOI signature by phylogenetic analysis (Figure 3). FAW is a strong flyer, and with favourable wind current conditions is able to travel long distances of at least 100 miles (*ca*. 160 km) (Jones et al. 2019; Rose et al. 1975; Sparks 1979). Large scale

corn farming is being carried out in the Ramu (Madang Province) and Markham (Morobe Province) valleys, PNG and in 2020 the FAW seriously affected the crops (Sim Sar *pers comm.*). Small-holder corn plots were also affected. With the proximity of the Morobe Province to the West New Britain Province, monitoring of the spread of FAW via either the "continent-island" (i.e., assumes that gene flow occurs from a donor population (continent) to a recipient (island) population in a unidirectional spread) (Wright 1931) and/or the "stepping stone migration" models (i.e., each population is connected by other neighbouring populations with constant migration rates between populations occurring in each generation; Kimura and Weiss 1964) across the Bismarck Sea and the Solomon Sea should commence. They would assist with prevention and preparedness of potential natural spread to PNG's Island Regions and the Solomon Islands. Rapid characterisation of the mtCOI gene to differentiate between *Sfr* and *Sfc* could also inform of population change between years, especially if baseline *Sfr*/*Sfc* ratios were known for target populations, thereby further emphasising the benefit of DNA barcoding approach of population surveys. With the species capable of covering long distances (e.g., > 1,600 km over 30 hours; Rose et al. 1975) under favourable conditions, monitoring for its spread to other parts of Papua New Guinea and across the South Pacific regions, and exploration of integrated pest management utilising either endemic parasitoids, or widely introduced (e.g., see Otim et al. 2021) and highly effective parasitoid species (e.g., *Telenomus remus* Nixon) (Hymenoptera: Platygastridae) should commence.

Across the species' invasive range (i.e., Africa, India, China), whole genome sequencing analyses have confirmed the invasive FAW populations were predominantly from admixed *Sfc* and *Sfr* (e.g., Tay et al. 2022a; Yainna et al. 2020; Zhang et al. 2020). Specific difference between Sfc/Sfr ratios have been reported between populations in Southeast Asia (e.g, Vietnam, Myanmar, Laos, Cambodia; Rane et al. 2023), and China (e.g., Zhang et al. 2020), and Australia (e.g., Piggott et al. 2021; Rane et al. 2023). While this current Papua New Guinea population has been shown to be of hybrid signature (Rane et al. 2023), whether other Papua New Guinea populations were also hybrids of R- and C-strains FAW will require whole genome sequence analysis, since signatures of multiple FAW introductions into Asia and Africa have been detected (Tay et al. 2022a; Yainna et al. 2020), and the genetic compositions of PNG's FAW will need on-going monitoring to help identify their likely invasive and/or native population origins, and to determine if multiple introductions of FAW also occurred in PNG, which will have on-going pest management implications including insecticide resistance management to Australia's primary crop producers.

While FAW populations have established on mainland Australia since early February 2020, Australia could reasonably expect on-going migration of this pest from neighbouring countries including Indonesia and PNG to

arrive (Rane et al. 2023). Regular monitoring of resistance status of FAW populations in PNG and neighbouring countries (e.g., New Caledonia; FAW detected in specific areas of Ouenghi in Boulouparis on 16 Dec., 2020; FAO-IPPC 2021) would be needed to provide early warning of potential introduction of novel resistance genes representing genes of biosecurity importance, thereby enabling relevant agricultural sectors in countries along the insect's natural migration and spread pathways to modify, adopt, and undertake timely up-dated management strategies to this pest, including via whole genome sequencing to detect arrival of novel insecticide and *Bacillus thuringiensis* (Bt) resistance alleles (Guan et al. 2020).

Similarly for the cotton pest *H. armigera* which is also highly polyphagous and has a global distribution including the recently expended invasive South and Central America ranges (e.g., Czepak et al. 2013; Tay et al. 2013; Arnemann et al. 2016), its evolutionary genetics remained unknown for populations in Papua New Guinea. In the Oceania (i.e., Australia, New Zealand), Hardwick (1965) identified the presence of the subspecies *H. a. conferta*, while in Southeast Asia and Asia this was replaced by the subspecies *H. a. armigera*. These subspecies are known to have different insecticide gene profiles (e.g., Walsh et al. 2018) including resistance to Bt toxins such as Cry2Ab (e.g., Tay et al. 2015; Wang et al. 2017). The contact zone between *H. a. armigera* and *H. a. conferta* is poorly understood, and there is a need to undertake population genomic analysis for Papua New Guinea *H. armigera* as contact between closely related *Helicoverpa* species (e.g., between *H. armigera* and *H. zea*) have been shown to lead to introgression of resistance gene (Anderson et al. 2018; Valencia-Montoya et al. 2020) which could have significant insecticide resistance management implication to the Asia/Pacific region.

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Author's contribution

WTT, TKW, LK contributed to study design. LK collected and provided suspect material, and provided the photo of *Spodoptera frugiperda* larva (Figure 2). WJ carried out the laboratory experiments. WTT and TKW wrote the manuscript. WTT analysed the sequence data and interpreted the results. WTT, TKW, LK, and WJ contributed to editing the manuscript.

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Supplementary material

The following supplementary material is available for this article:

Table S1. Top 20 best matches to the Papua New Guinea (PNG) candidate *Helicoverpa armigera*.

This material is available as part of online article from:
http://www.reabic.net/journals/bir/2023/Supplements/BIR_2023_Tay_etal_SupplementaryMaterial.pdf