

Research Paper

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Phthorimaea absoluta (Meyrick) (Lepidoptera: Gelechiidae) draft mitogenomes and insecticide resistance gene characterisation support multiple maternal lineages in invasive African, Asian, and European populations

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Abstract

The tomato leafminer, *Phthorimaea absoluta* (synonym *Tuta absoluta* Meyrick, 1917), is a trans-boundary plant pest that poses a serious threat to global tomato cultivation and production, with significant negative social and environmental impact from increased insecticide usage for its management. We present three *P. absoluta* draft mitochondrial genomes (mitogenomes) from Malawi and South Africa, thereby increasing the mitogenome resources for this invasive agricultural pest. Comparative analysis with Spain, China, and Kenya samples revealed at least seven maternal lineages across its current invasive ranges, supporting multiple introductions as a major factor for the spread of invasive populations. Mitogenome results therefore identified unexpected diversity as compared to the use of the partial mitochondrial cytochrome oxidase subunit I (mtCOI/cox1) gene marker for the inference of *P. absoluta* invasion biology. The whole-genome sequencing approach further identified alternative mitochondrial DNA (mtDNA) gene regions necessary to improve diversity estimates, and enables concurrent characterisation of insecticide resistance genes. Characterisation of the VSSG (Para) and AChE-1/ace-1 gene profiles that underpin pyrethroid and organophosphate (OP) resistances, respectively, confirmed co-introductions of pyrethroid and OP resistance genes into Malawian and South African populations. Our study highlights the need for additional *P. absoluta* mitogenome resources, especially from native populations that is needed for more accurate interpretations of introduction pathways and the development of future sustainable management strategies.

Introduction

Over the past decades, the world has seen an increasing frequency in the introduction of invasive alien species (IAS) that significantly impacted plant health and agricultural productivity (e.g. Faulkner *et al.*, 2016; Mastrangelo *et al.*, 2014; Pozebon *et al.*, 2020; Sandvik *et al.*, 2022; Turbelin *et al.*, 2022). Examples of these included the silverleaf whitefly *Bemisia tabaci* species complex (De Barro *et al.*, 2011), *Thrips tabaci* (Balan *et al.*, 2018), *Frankliniella occidentalis* (Reitz *et al.*, 2020), *Helicoverpa armigera* (Czapak *et al.*, 2013; Jones *et al.*, 2019; Tay *et al.*, 2013), *Spodoptera frugiperda* (Goergen *et al.*, 2016; Tay *et al.*, 2022a), and the tomato leafminer *Phthorimaea absoluta* (synonym *Tuta absoluta*; Chang and Metz, 2021), highlighting the need to better understand, through genomics, mechanisms associated with the introduction and adaptation of these transboundary plant pests to improve global agricultural biosecurity preparedness and developing appropriate management solutions.

The dispersal of propagules from geographically distant locations is a major factor underpinning success of IAS introductions, enabling establishment potentials through higher levels of genetic diversity as compared with a single founder introduction event. These dispersal mechanisms and pathways, in turn, may manifest quantitatively (i.e. the number of individuals introduced to a location where it is not native) and qualitatively (i.e. different parental lineages), many of which are intrinsically linked to human-mediated dispersal routes, such as commercial pathways. The determination of genetic variability associated with species introduction is strongly influenced by the intensity and diversity of source populations. Considering these factors, variations in insect behaviour can result in distinct patterns of genetic diversity

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(Wilson *et al.*, 2009), although the choice of genetic markers used will also impact on detection of genetic patterns. Consequently, recently introduced invasive species such as *H. armigera* in Brazil (Czepak *et al.*, 2013; Tay *et al.*, 2013) where females randomly lay individual eggs, have been shown to exhibit higher genetic diversity in Brazil and its neighbouring countries (Anderson *et al.*, 2016; Arnemann *et al.*, 2016, 2019; Tay *et al.*, 2013) as compared to those that follow natural dispersal pathways, whose expansion is induced by climatic factors (Battisti *et al.*, 2017; Robinet *et al.*, 2015).

Various AIS (e.g. *P. absoluta*, Czepak *et al.*, 2018; *S. frugiperda*, Tay *et al.*, 2023) have been of particular global concern due to their economic impact (Adelino *et al.*, 2021; IPBES, 2023) and their increasing introduction frequencies (Mastrangelo *et al.*, 2014; Pozebon *et al.*, 2022). The economic cost of these invasive species, including *P. absoluta*, that has global footprints, remains poorly understood. For example, the combined economic losses due to *P. absoluta* for small-scale farmers in four African countries (Ethiopia, Kenya, Tanzania, and Uganda), range between US\$70 and US\$80 million annually, and contributed to a total of US\$1.1 billion when considering also other major invasive species, representing approximately 2.2% of the agricultural Gross Domestic Product (GDP) of the region (Pratt *et al.*, 2017).

The larvae of *P. absoluta* are polyphagous pests of solanaceous plants (e.g. egg plants *Solanum melongena*; potato *S. tuberosum*) (Smith *et al.*, 2018; EPPO, 2010), with *S. lycopersicum* (tomato) being its main host crop. The widespread detection of *P. absoluta* therefore raises significant concerns on tomato production (Hogea, 2020). Management of *P. absoluta* is challenging due to reported insecticide resistance (Haddi *et al.*, 2017; Silva *et al.*, 2021), and to its behaviour and high reproductive capacity, which involves overlapping generations and high-field infestation rates, facilitated by its flight dispersal ability, and the propensity to be accidentally introduced via anthropogenic-related activities, causing tomato productivity losses that can reach up to 100% (Biondi *et al.*, 2018). *Phthorimaea absoluta* females lay individual eggs randomly, preferentially on leaves, new shoots, and sepals (less on fruits). Adults can mate up to four times with implications to establishment success of bridgehead invasive populations (Guillemaud *et al.*, 2011). Multiply-mated female bridgehead founders undertaking range expansion have the potential to increase propagule genetic diversity even if no males were yet present in the new invasive range.

Native to South America and representing one of the main threats to tomato crops (Guedes and Picanço, 2012), *P. absoluta* was identified in 1917 and recorded as widely distributed in Peru (Rojas, 1981; USDA-APHIS, 2023). In 1980, it was reported in southern Brazil (Muszinski *et al.*, 1982) and brought with it concerns that included higher insecticide application frequencies with associated control costs, as well as issues relating to environmental contaminations with human health implications (Kim *et al.*, 2017; Tudi *et al.*, 2022). In 2006, *P. absoluta* was detected in Spain (Urbaneja *et al.*, 2007), followed by reports from other European countries (e.g. France, Italy, Switzerland, Malta, Portugal, Greece, the Netherlands), and the United Kingdom (Potting *et al.*, 2009). First reports of *P. absoluta* in northern Africa (e.g., Morocco, Algeria, Tunisia, Libya, Egypt) were between 2007 and 2008 (Mansour *et al.*, 2018), and in East and South African countries (e.g., Kenya, Malawi, South Africa) between 2014 and 2016 (e.g. IPPC, 2016; Visser *et al.*, 2017). In Asia, it was detected in Turkey in 2009 (Kilic, 2010) with an assumed rapid dissemination to reach central and southwestern parts of the continent (Biondi *et al.*, 2018). *P. absoluta* was reported in China (e.g. Yili, Xinjinag,

Yunnan) from 2017 onward (Li *et al.*, 2022; Zhang *et al.*, 2020), and confirmed in South Korea in 2024 (Lee *et al.*, 2024).

Response to challenges of rapid and widespread invasive agricultural pest population establishments often involves insecticide applications that can be both excessive, inappropriate, and inaccurate (Desneux *et al.*, 2011; Guenaoui *et al.*, 2013; Palumbo *et al.*, 2016; Potting, 2013; Wan and Yang, 2016), thereby contributing to the evolution of insecticide resistance and secondary pest outbreaks (Aynalem, 2022; Biondi *et al.*, 2018; Khan *et al.*, 2020). The spread and successful population establishment of invasive pests may also be facilitated by the presence of resistance alleles that contributes to reduced insecticide effectiveness, as already reported in *P. absoluta* in South America (Silva *et al.*, 2016a, 2016b, 2016c), Europe (Roditakis *et al.*, 2015), Asia (Zhu *et al.*, 2024; Zibae *et al.*, 2018), and Africa (Bala *et al.*, 2019; Ong'onge *et al.*, 2023). Therefore, identifying resistant genotypes underpinning resistance phenotypes in *P. absoluta* populations will be crucial for understanding its propagation processes and for the designing of management strategies.

Low levels of nucleotide variation in the partial mitochondrial DNA cytochrome oxidase subunit I (mtCOI/cox1) gene were previously reported in native (e.g. South America; Cifuentes *et al.*, 2011) and invasive *P. absoluta* populations from the Mediterranean Basin (Cifuentes *et al.*, 2011), India, and Nepal (Shashank *et al.*, 2018). Similar findings were reported also in other significant transboundary pest species such as *S. frugiperda* (Cock *et al.*, 2017; Goergen *et al.*, 2016) and *Oryctes rhinoceros* (Tay *et al.*, 2024), emphasizing the general similar nucleotide characteristics for the mtCOI barcoding partial gene region in insects/arthropods.

An over reliance of the partial mtCOI gene region to infer population founding histories is known to result in conflicting population genetic signatures, especially when compared with full mitochondrial genome-derived population signatures (e.g. Elfekih *et al.*, 2018; Tay *et al.*, 2022c, 2024). The circular arthropod mitochondrial genomes are typically high in A–T compositions (e.g. Pozebon *et al.*, 2023; De Souza *et al.*, 2016; Kunz *et al.*, 2019a), and due to its overall non-recombinant nature (i.e. maternally inherited; Crozier, 1990) and relatively small size (i.e. typically ranged between 15kb and 28kp; Behere *et al.*, 2019; Piper *et al.*, 2016; Tay *et al.*, 2022b; Morgan *et al.*, 2022), enabled ease of genome assembly that also afforded significant advantages for more comprehensive inference of population establishment history. Kunz *et al.* (2019b) and Vyskocilová *et al.* (2018) also showed that at both inter- and intra-specific levels, amino acid conservation enabled detections of pseudogenes and therefore elimination of poorly annotated mtCOI barcode gene to significantly improve on species identification, while quality control of assembled mitogenomes enabled assembly errors to be identified through comparative genome analyses (e.g. see Walsh *et al.*, 2019).

Based on simple sequence repeat markers (SSR) analysis, Guillemaud *et al.* (2015) concluded that the invasive Mediterranean *P. absoluta* populations likely originated from central Chile, while the shared mtCOI genetic similarity linked the species' invasion patterns from southern and central Europe to the Indian sub-continent and to northern and sub-Saharan Africa (Cifuentes *et al.*, 2011; Fiaboe *et al.*, 2021; see also review by Santana *et al.*, 2019). To-date, very few *P. absoluta* mitogenome resources are available, including one from Spain (Zhang *et al.*, 2019), two from China (i.e. one each from Xinjiang and Yunnan; Li *et al.*, 2022), and one from Kenya (Ajene *et al.*, 2024). Li *et al.* (2022) concluded that multiple mtDNA markers would be needed to increase population genetic diversity estimate accuracy of

P. absoluta. This is especially apposite to better understand and assess the scenarios on the African continent that accounts for approximately 18% of the world's human population, is highly vulnerable to climate change, and where severe food insecurity may result due to invasive pests.

Here, we report three new mitochondrial genome resources for *P. absoluta* from Malawi and South Africa, and demonstrate the benefit of utilising a whole-genome sequencing (WGS) approach to concurrently characterise known resistance genes and alleles, using the organophosphate (OP) and pyrethroid resistance/DDT resistance genes, i.e. Acetylcholinesterase (AChE-1) and Para-type voltage-gated sodium channel (VGSC), respectively, as examples. We further compare *P. absoluta* mitogenome gene variability of Spain, Kenya, and China, for re-assessments of mitogenome assembly quality. We also review *P. absoluta* published partial mtCOI gene sequences to assess the marker's suitability for understanding both *P. absoluta* and general arthropods' invasion biology.

Material and methods

Samples for WGS

Two *P. absoluta* individuals were collected from Malawi in East Africa (sample code: MAL19, MAL20) and one individual from South Africa (sample code: ZA1) (Table 1). The Malawian *P. absoluta* individuals were collected from tomato plant hosts from Thyolo District (−16.054, 35.062) in Malawi (elevation 1,045 m) on 12 June 2018. The South African *P. absoluta* individual was from tomato near Baltimore (Limpopo province, South Africa) (−23.217337, 28.304087). Samples were collected as early instar larvae and were stored in 100% ethanol until used in DNA extraction. DNA of each individual was extracted using the QIAGEN DNeasy Blood & Tissue DNA extraction Kit (Dusseldorf, Germany) following manufacturer's recommended protocol. Extracted genomic DNA was quantified using the Qubit 2.0 Fluorometer (Life Technologies). Illumina DNA libraries were prepared as per the manufacturer's protocols at the Biomolecular Resource Facility at the John Curtin School of Medical Research, Australian National University, Canberra, Australia. WGS of these libraries was performed on an Illumina NextSeq 2000 sequencer, P1 flowcell, (2 x 300bp, PE).

Mitogenome assembly and annotation

Mitogenome assembly was performed in Geneious Prime (v11.1.5 from Biomatters Ltd, Auckland, NZ), using the Geneious Mapper program with default settings (Algorithm: Auto; Scoring matrix: 200PAM/K = 2; Gap open penalty: 1.53; Offset value: 0.123). Initial assembly of a complete mitochondrial genome was undertaken for sample MAL19, commencing by mapping the MAL19 whole-genome sequence data against a reference partial mtCOI gene (GenBank MT897989). We build the full mitogenome of MAL19 by genome assembly walking out from the initial assembled partial cox1 gene. Assembled partial mitogenome contig versions were improved iteratively until a draft assembled circularised mitogenome for MAL19 was obtained. Ambiguous nucleotides and sub-optimally assembled regions were checked manually to ensure resolutions. Annotation of the assembled mitogenome was performed using Mitos (Bernt *et al.*, 2013) selecting genetic code 5 (invertebrate mitochondrial genome). Mitos annotated bedfile (.bed) was imported into Geneious to assist with fine tuning of the MAL19 final version of mitogenome (Pa-MAL19; Magalhaes

et al., 2024). Fine tuning of mitogenomes involved identifying a 'Methionine' (Met) start codon and a termination codon for each of the 13 protein-coding gene (PCG). To infer the stop codon for the 13 PCGs, gene length was extended until the first stop codon was identified, irrespective of whether the predicted PCG over-lapped a transfer RNA (tRNA) gene or not. This approach differed from those used to predict stop codons of the 13 PCGs of the Chinese (Yunnan, Xinjiang) and Spanish *T. absoluta* mitogenomes (Li *et al.*, 2022; Zhang *et al.*, 2019), where the stop codon ended with single T in the cox1, cox2, nad5, and nad1 genes.

The fully assembled and annotated MAL19 mitogenome (Pa-MAL19; Magalhaes *et al.*, 2024) was then used as the reference genome for the assembly of MAL20 (Pa-MAL20; Magalhaes *et al.*, 2024) and ZA1 (Pa-ZA1; Magalhaes *et al.*, 2024) draft mitogenomes. The published mitogenomes of *P. absoluta* individuals from Spain (GenBank accession number MK654754), China (Xinjiang, GenBank accession number MT897989; Yunnan, GenBank accession number MT897990), and Kenya (SRR22312267; Ajene *et al.*, 2024) were downloaded from GenBank for comparison against our African individuals. The *Helicoverpa* species' mitogenome resources reported in Walsh *et al.* (2019) were downloaded to enable comparative genome analysis assessments of *P. absoluta* mitogenomes assembly quality via amino acid residue compositions and consideration of their expected biochemical properties.

Assembly and identification of OP and pyrethroid resistance genes

To assess and characterise the resistance genes Acetylcholinesterase 1 (AChE-1/ace-1) responsible for OP and the Para-type VGSC gene responsible for pyrethroid and DDT resistance, we downloaded the *Tuta absoluta* strain TA1 ace-1 mRNA partial coding sequence KU985167 from GenBank for use as reference sequence to assist with assembly of the AChE-1 gene in our Malawi and South African individuals. Similarly, we downloaded the *T. absoluta* partial coding sequence KY767010 from GenBank to assist with assembly of the pyrethroid/DDT resistance gene 'VGSC' of our African *P. absoluta* individuals. In both AChE-1 and VGSC, resistance to OP and to pyrethroid, respectively, have been identified in various lepidopteran pest species (e.g., *S. frugiperda*; *H. armigera*; *P. absoluta*) as due to nucleotide base substitution resulting in amino acid changes (Table S1). For *P. absoluta*, the known nucleotide substitutions for KU985167 and KY767010 are compared and as provided in Carvalho *et al.* (2013) (Table S1).

Molecular diagnostics

To provide confidence that the Malawian and South African larvae sequenced were *P. absoluta* and to better understand the implications between utilisation of partial mtCOI gene versus full mitogenome PCGs in understanding the species' invasion biology, we undertook molecular diagnostics and pairwise nucleotide comparison of published and our assembled mtCOI partial gene sequences. We selected representative sequences encompassing various worldwide locations from GenBank (Table 1) for alignment using the MAFFT Align tool (Katoh and Standley, 2013) in the Geneious software and trimmed all to 570 bp to ensure no missing data in all sequences prior to downstream analyses.

Table 1. Samples of *Phthorimaea absoluta* analysed in this study for their partial mtCOI gene. The draft mitogenomes of the African samples (indicated by ‘†’) can be downloaded from CSIRO’s data access portal (Magalhaes *et al.*, 2024), the partial gene region used in this analysis ranged between nucleotide positions 1,572-2,142 of the MAL19 mitogenome (i.e., 570 bp) (see also Figure 1)

Continent	Country	GenBank Accession number	References
Africa	Malawi	Pa-MAL19†, Pa-MAL20†	This study
	South Africa	Pa-ZA1†	This study
	Algeria	MK848296	Ndiaye <i>et al.</i> , 2021
	Benin	MH882438	Karlsson <i>et al.</i> , 2018
	Burkina Faso	MK117028	Ndiaye <i>et al.</i> , 2021
	Congo	MG693224	Mukwa <i>et al.</i> , 2021
		MK000732	Singh <i>et al.</i> , 2023
	Kenya	KU565715	Kinyanjui <i>et al.</i> , 2019
		KP324752	Tonnang <i>et al.</i> , 2015
	Niger	MK848304	Ndiaye <i>et al.</i> , 2021
	Nigeria	MK189155	Ndiaye <i>et al.</i> , 2021
	Senegal	MK116937	Ndiaye <i>et al.</i> , 2021
	Togo	MN759250	Fiaboe <i>et al.</i> , 2021
	Tunisia	JQ749676	Bettaïbi <i>et al.</i> , 2012
Asia	China	MT897989, MT897990	Li <i>et al.</i> , 2022
	India	KY619685	Shashank <i>et al.</i> , 2018
	Iran	MT328192	Mehrkhou <i>et al.</i> , 2021
	Nepal	KY619677	Shashank <i>et al.</i> , 2018
	Oman	KT452897	Patankar and Al-Wahaibi, 2015
	Saudi Arabia	JN255972	Hudaib, 2013
	Turkey	MT328202	Mehrkhou <i>et al.</i> , 2021
Europe	Bosnia Herzegovina	KC852871	Đurić <i>et al.</i> , 2014
	France	MK117032	Ndiaye <i>et al.</i> , 2021
	Greece	MK848321	Ndiaye <i>et al.</i> , 2021
	Italy	MK848322	Ndiaye <i>et al.</i> , 2021
	Montenegro	KC852872	Đurić <i>et al.</i> , 2014
	Serbia	JN417242	Toševski <i>et al.</i> , 2011
	Spain	MK654754	Zhang <i>et al.</i> , 2019
South America	Argentina	MK848320	Ndiaye <i>et al.</i> , 2021
	Chile	HQ873077	Cifuentes <i>et al.</i> , 2011
	Colombia	MK848319	Ndiaye <i>et al.</i> , 2021
	Ecuador	HQ873080	Cifuentes <i>et al.</i> , 2011
	Peru	KX443108, KX443110, KX443111	Sint <i>et al.</i> , 2016
	Uruguay	HQ873075	Cifuentes <i>et al.</i> , 2011

Quality control of partial mtCOI (*cox1*) gene sequences

The Staden sequence assembly package’s Pregap and Gap4 programs (Staden *et al.*, 2000) were employed for contig assembly and quality checking of the selected published mtCOI partial sequences (Table 1). Identified base substitutions in clean sequences were translated into amino acids and the likelihood

of possible incorrect base-calling in these published sequences were assessed through amino acid residue biochemical properties (Kunz *et al.*, 2019b). Presence of premature stop codons that may indicate candidate nuclear mitochondrial (NuMt) sequence was also assessed through amino acid translation. Sequence alignment also enable possible insertion/deletion (INDEL) mutations to be identified.

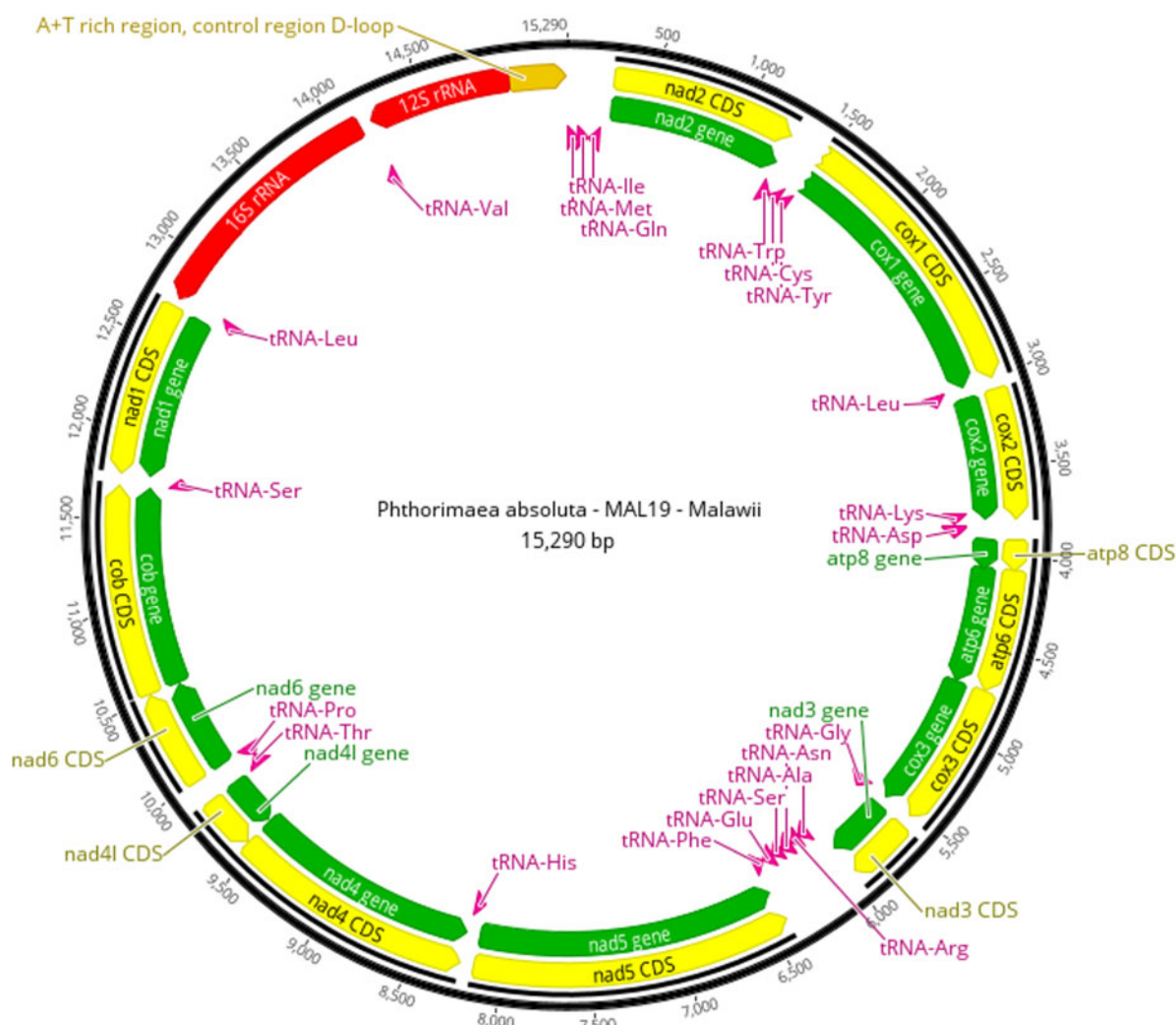


Figure 1. Circular mitochondrial genome (mitogenome) of the *phthorimaea absoluta* individual MAL19 (Pa-MAL19; Magalhaes *et al.*, 2024) from Malawi, showing 13 protein-coding genes (green), 22 tRNA genes (pink), 2 rRNA genes (red), and an A + T rich control region (Orange).

Basic nucleotide statistics and haplotype network construction

Pairwise nucleotide analysis was conducted to compare all 13 PCGs of the published mitogenomes and mitogenomes of the three African *P. absoluta* samples reported in this study. Each full length PCG was extracted with the MAL19 PCG gene lengths used as standards for pairwise gene alignment using MAFFT with default parameters (Auto algorithm, scoring matrix: 200PAM/k = 2, Gap open penalty: 1.53, Offset value: 0.123). Once aligned, the genetic variability from the 13 PCGs were recorded as a base substitution table (Table 2) to enable a mitogenome haplotype network to be constructed (Fig. 2). For the haplotype network analysis, SNP data were converted to the Phylip format prior to being analysed using the TCS v1.23 program (Clement *et al.*, 2000), with the connection limit for the haplotype network set at 15 steps. This approach allowed for the visualization and analysis of the samples' network structure, highlighting evolutionary patterns and genetic relationships among them (Fig. 2).

Results

The mitogenome organisation and diversity of *Phthorimaea absoluta*

A total of 107 manual mitogenome assembly iterations was needed to generate our final draft mitogenome for MAL19. All three African *P. absoluta* mitochondrial genomes (MAL19, MAL20, ZA1) exhibited the expected circular genome structure, as shown for the MAL19 mitogenome (Fig. 1), with draft mitogenome lengths of 15,290 bp for both MAL19 and MAL20, and 15,292 bp for ZA1. The draft mitogenomes of MAL19, MAL20, and ZA1 all contained 13 PCGs, 2 ribosomal RNA (rRNA) genes, 22 tRNA genes, and a control region. Among the 37 genes, 23 were encoded on the heavy strand (H-), while four PCGs (nad5, nad4, nad4l, and nad1), eight tRNAs (trnC-tgc, trnY-tac, trnF-ttc, trnH-cac, trnP-cca, trnL1-cta, trnV-gta, and trnQ-caa), two rRNAs were encoded on the light strand (L-), and an approximately 320 bp non-coding region that is also rich in A + T nucleotide composition (91.9%).

Table 2. Summary of base substitutions of the Malawian (MAL19, MAL20) and South African (ZA1) *P. absoluta* samples and including published mitochondrial genomes of Spain (MK654754) and China (MT897989, MT897990) *P. absoluta* samples. Base substitutions were identified in eight mtDNA PCGs and for the 16S rRNA gene

Gene	cox2		atp8	atp6	cox3		nad5		nad4		cob	nad1		16S-rRNA	
Position	3490	3574	4002	4147	5359	5367	7203	7278	8360	9160	10659	12317	12513	12903	13464
MAL19	T	A	G	G	T	T	A	A	T	C	T	C	G	A	A
MAL20	C	A	A	A	T	T	G	A	C	T	T	C	G	A	T
ZA1	T	A	A	G	T	T	A	A	T	T	T	C	G	G	A
MT897989	C	A	A	A	T	T	A	A	T	T	T	C	G	A	A
MT897990	C	A	A	G	T	T	A	A	T	T	T	C	G	A	A
MK654754	C	G	A	A	C	G	A	G	T	T	C	T	A	A	A

Note: No base substitutions were present in the full mtCOI gene of these six full mitogenomes compared. An INDEL was detected in the 16S-rRNA gene of ZA1 at alignment position 13,930 (ZA1 has a 'T' which was not detected in the other five mitogenomes).

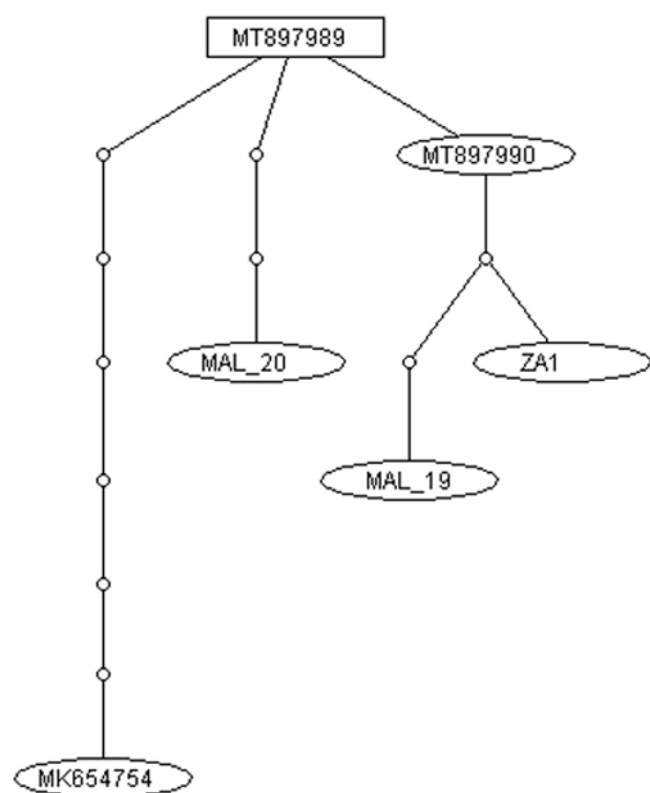


Figure 2. *Phthorimaea absoluta* draft mitogenome haplotype network. Number of base changes between haplotypes are indicated by open circles.

No nucleotide base substitutions were detected for six of the mitochondrial genes (i.e. nad2, cox1, atp6, nad3, nad4l, nad6), and one to two base substitutions observed among the cox2, atp8, atp6, nad5, nad4, and 16S rRNA genes (Table 2) between the published and our African *P. absoluta* individuals.

The nucleotide composition of the draft *P. absoluta* mitogenome based on MAL19 was as follows: 39.3% A, 40.6% T, 8.1% G, and 12.0% C, respectively, and exhibiting an overall high A + T bias (79.8%). Of the 13 coding genes, one started with the CGA codon (cox1), six with the ATG codon (cox2, atp6, nad4, nad4l, cob, cox3), four with the ATT codon (atp8, nad5, nad3, nad2), and two with the ATA codon (nad6, nad1), and all 13 PCGs terminate

with the TAA codon (Table 3). The mitogenomes between the African, European, and Asian *P. absoluta* showed high degree of similarity (i.e. between 99.90 and 99.98%; Table 4), while 17 intergenic sequences and four overlapping sequences were also identified (Table 3).

Through comparisons of gene lengths within individuals from Africa, from the mitogenomes, it was observed that the genes exhibited similar lengths, except for the 16S-rRNA (Rnl) region, where the ZA1 individual showed a slightly longer length (1,367 bp) as compared with MAL19 and MAL20 (1,366 bp). Previously, Li *et al.* (2022) reported differences only in the lengths of intergenic spacers between two Chinese individuals and a Spanish individual. However, in the current study, discrepancies in gene lengths were identified between our African individuals and the others due to the different approaches for determining start and stop codon positions for the PCGs. These differences were particularly notable in the cox1, cox2, nad5, nad1, and 16S-rRNA (Rnl) regions, where African individuals exhibited longer lengths (Table 3).

Assessments of mitogenome assembly quality prior to calculating intraspecific nucleotide variability between the reported *P. absoluta* mitogenomes and our African *P. absoluta* mitogenomes was undertaken (Table S2). Across the seven mitogenomes now being reported, we identified 44 base substitutions that resulted in 27 synonymous and 17 non-synonymous amino acid changes in the 13 PCGs. Of the 17 non-synonymous amino acid changes, one occurred in MAL19 at nucleotide positions (nt) 8358-8360 (within the nad4 gene) and involved a change from Glutamic Acid (E; negatively charged side chain) in all *P. absoluta* to Glycine (G; 'special case') in MAL19. Two non-synonymous amino acid residue changes were detected in the Spain specimen (MK654754, Zhang *et al.*, 2019), and involved a change from Valine (V, hydrophobic side chain) to Glycine (cox3, nt5366-5368), and from Serine (S, polar uncharged side chain) to Phenylalanine (F, hydrophobic side chain) at nt12511-12513 (nad1 gene). The remaining unexpected 14 non-synonymous amino acid changes at the intraspecific level were all detected in the Kenyan *P. absoluta* mitogenome (SRR22312267; Ajene *et al.*, 2024).

Nucleotide base and amino acid residue substitutions at positions corresponding to those of *P. absoluta* were also compared at both intra- and interspecific levels in five different *Helicoverpa* species that included also four known subspecies (*H. armigera armigera* (Haa), *H. armigera conferta* (Hac), *H. assulta assulta* (Haas), *H. assulta afra* (Haaf); Hardwick, 1965; Walsh *et al.*, 2019). At the interspecific level, in the five genetically distinct *Helicoverpa*

Table 3. Annotation of *P. absoluta* mitochondrial genomes between MAL19 from Malawi (Pa-MAL19; Magalhaes et al., 2024) and published mitogenomes from China (Xinjiang, GenBank MT897989; Yunnan, GenBank MT897990) by Li et al. (2022) and Spain (GenBank MK654754) by Zhang et al. (2019)

Name	Strand	Malawi	Xinjiang	Yunnan	Spain	Gene length (bp)	Start/anti codon	Stop codon	Intergenic nucleotide
tRNA-Met	+	1–69	1–69	1–69	1–69	69	TTA		
tRNA-Ile	+	70–134	70–134	70–134	70–134	65	AAT		1
tRNA-Gln	-	204–136	204–136	204–136	204–136	69	TAT		56
nad2	+	261–1274	261–1274	261–1274	261–1274	1014	ATT	TAA	
tRNA-Trp	+	1273–1340	1273–1340	1273–1340	1273–1340	68	AAG		-2
tRNA-Cys	-	1398–1333	1398–1333	1398–1333	1398–1333	66	AGT		4
tRNA-Tyr	-	1468–1403	1468–1403	1468–1403	1468–1403	66	AAT		3
cox1	+	1472–3007	1472–3002	1472–3002	1472–3002	1536	CGA	TAA	
tRNA-Leu1	+	3003–3070	3003–3070	3003–3070	3003–3070	68	TCT		
cox2	+	3071–3787	3071–3752	3071–3752	3071–3752	717	ATG	TAA	-35
tRNA-Lys	+	3753–3823	3753–3823	3753–3823	3753–3823	71	CAT		8
tRNA-Asp	+	3832–3899	3832–3899	3832–3899	3832–3899	68	AAA		
atp8	+	3900–4070	3900–4070	3900–4070	3900–4070	171	ATT	TAA	-7
atp6	+	4064–4741	4064–4741	4064–4741	4064–4741	678	ATG	TAA	
cox3	+	4742–5530	4742–5530	4742–5530	4742–5530	789	ATG	TAA	2
tRNA-Gly	+	5533–5599	5533–5599	5533–5599	5533–5599	67	ATT		
nad3	+	5600–5953	5600–5953	5600–5953	5600–5953	354	ATT	TAA	69
tRNA-Ala	+	6023–6088	6031–6096	6029–6094	6023–6088	66	AGG		
tRNA-Arg	+	6089–6152	6097–6160	6095–6158	6089–6152	64	AAA		2
tRNA-Asn	+	6155–6220	6163–6228	6161–6226	6155–6220	66	TTA		2
tRNA-Ser1	+	6223–6288	6231–6296	6229–6294	6223–6288	66	GAA		
tRNA-Glu	+	6289–6354	6297–6362	6295–6360	6289–6354	66	ATT		-2

(Continued)

Table 3. (Continued.)

Name	Strand	Malawi	Xinjiang	Yunnan	Spain	Gene length (bp)	Start/anti codon	Stop codon	Intergenic nucleotide
tRNA-Phe	-	6418 – 6353	6426 – 6361	6424 – 6359	6418 – 6353	66	ATT		-17
nad5	-	8153 – 6402	8161 – 6427	8159 – 6425	8153 – 6419	1752	ATT	TAA	
tRNA-His	-	8219 – 8154	8227 – 8162	8225 – 8160	8219 – 8154	66	ACT		7
nad4	-	9657 – 8227	9575 – 8235	9573 – 8233	9567 – 8227	1341	ATG	TAA	1
nad4l	-	9862 – 9569	9870 – 9577	9868 – 9575	9862 – 9569	294	ATG	TAA	2
tRNA-Thr	+	9865 – 9929	9873 – 9937	9871 – 9935	9865 – 9929	65	GTT		
tRNA-Pro	-	9994 – 9930	10,002 – 9938	10,000 – 9936	9994 – 9930	65	CAA		2
nad6	+	9997 – 10,520	10,005 – 10,535	10,003 – 10,533	9997 – 10,527	531	ATA	TAA	9
Cob	+	10,537 – 11,685	10,545 – 11,693	10,543 – 11,691	10,537 – 11,685	1149	ATG	TAA	7
tRNA-Ser2	+	11,693 – 11,759	11,701 – 11,767	11,699 – 11,765	11,693 – 11,759	67	AAT		-7
nad1	-	12,712 – 11,753	12,723 – 11,790	12,721 – 11,788	12,715 – 11,782	960	ATA	TAA	4
tRNA-Leu2	-	12,784 – 12,717	12,792 – 12,725	12,790 – 12,723	12,784 – 12,717	68	GCT		-24
16S-rRNA (Rml)	-	14,126 – 12,761	14,134 – 12,793	14,132 – 12,791	14,126 – 12,785	1366	GGG		
tRNA-Val	-	14,194 – 14,129	14,202 – 14,137	14,200 – 14,135	14,194 – 14,129	66	CAA		
12S-rRNA (Rms)	-	14,970 – 14,195	14,978 – 14,203	14,976 – 14,201	14,970 – 14 – 195	776	AAT		
Control region		14,971 – 15,290	14,979 – 15,298	14,977 – 15,296	14,971 – 15,290	320	TAT		

Note: Detailed annotation of *P. absoluta* mitochondrial genomes for MALL19, MAL20, and ZAI are also provided in Magalhaes *et al.* (2024). Predicted secondary structures of transfer RNAs (tRNAs) are provided in Fig. S1.

Table 4. Comparison of pairwise nucleotide distances (*p*-dist) among the 13 concatenated (11,286bp) PCGs from the mitogenomes of *Phthorimaea absoluta* individuals from Malawi (MAL19, MAL20), South Africa (ZA1), Spain (MK654754), and China (MT897989, MT897990)

GENOME	MAL19, %	MAL20, %	ZA1, %	MT897989, %	MT897990, %
MAL20	99.95				
ZA1	99.96	99.94			
MT897989	99.92	99.92	99.91		
MT897990	99.94	99.93	99.93	99.98	
MK654754	99.92	99.93	99.91	99.90	99.90

species (Hardwick 1965; Pearce *et al.*, 2016; Anderson *et al.*, 2016; 2018), only one non-synonymous amino acid change was observed (i.e. *H. armigera* (Haa, Hac), *H. zea* (Hz), *H. punctigera* (Hp) versus *H. assulta* (Haas, Haaf), *H. geletopoeon* (Hg)), and involved a change from Asparagine (N, uncharged side chain) to Glycine (G) at nt10650-10652 (cob gene) (Table S2). Excessive non-synonymous amino acid changes especially at the intraspecific level (e.g. Kenyan *P. absoluta* SRR22312267 versus all other *P. absoluta*; Table S2) are not expected for mitogenomes in arthropods (e.g. Kunz *et al.*, 2019b; Walsh *et al.*, 2019) and the assembly quality of the Kenyan mitogenome will need to be re-checked to ensure accuracies. Base substitutions specific to the Kenyan *P. absoluta* that did not result in amino acid residue changes were identified (e.g. in *cox2*, *atp6*, *cox3*, *nad5*, *nad4*, *cob*, *nad1*; Table S2), suggesting that this Kenyan *P. absoluta* likely belonged to yet another unique (i.e. 8th) maternal lineage. However, suspected mitogenome assembly issues nevertheless precluded calculations and analyses of haplotype network, nucleotide diversity, and intra-specific comparisons of mitogenome characterization involving this Kenyan individual.

Assembly and identification of OP and pyrethroid-resistance genes

We assembled the partial AChE-1 and the partial VGSC gene regions in our *P. absoluta* samples for characterisation of OP and pyrethroid-resistance alleles, respectively. We successfully

identified OP resistance alleles in all three *P. absoluta* specimens from Malawi and from South Africa. In MAL19 and ZA1, resistance to OP would be expected due to changes to the A201S and the F290V amino acid residues, while for MAL20, base substitution from GCT to TCT is expected to result in OP resistance due to the A201S amino acid change (Table 5). Pyrethroid resistance profiles obtained from the partially assembled VGSC gene identified super-kdr for MAL19 due to the presence of M918T + L1014 amino acid substitutions, and for the first time, amino acid substitution profiles of T929I + L1014F in ZA1 which corresponded to the 1B pyrethroid-resistance profile in *Musca domestica*, expected to be of greater resistance to pyrethroid insecticides than super-kdr (Roca-Acevedo *et al.*, 2023). For MAL20, poor coverage prevented characterisation of the L1014F amino acid residue, although the T929I amino acid residue change was detected (Table 5).

Mitogenome haplotype network

The network analysis showed that MAL19 and the Spain (MK654754) mitogenomes were most divergent from each other, separate by 11 base substitutions, followed by between MAL19 and MAL20 (7 base substitutions). South African (ZA1) and China *P. absoluta* shared close maternal evolutionary relationships, being separated by two (i.e. between ZA1 and MT897990) and three (i.e., between ZA1 and MT897989) base substitutions. The two

Table 5. Nucleotide substitutions and amino acid residue changes in the Acetylcholine esterase-1 (AChE-1/ace-1) and the Para-type VGSC genes, associated with OP and pyrethroid knockdown resistance (kdr), respectively, in *phthorimaea absoluta* individuals from Malawi (MAL19, MAL20) and South Africa (ZA1). Missing genome data coverage is indicated by '-'; indetermined amino acid residue is indicated by '?'. Single amino acid residue letter codes used: alanine (A), serine (S), glycine (G), phenylalanine (F), valine (V), methionine (M), threonine (T), isoleucine (I), leucine (L)

AChE-1	A201S	codon	G227A	codon	F290V	codon		
MAL19	A	GCT	G	GGA	F	TTT		
	S	TCT	G	GGA	V	GTT		
MAL20	A	GCT	G	GGA	F	TTT		
	S	TCT	G	GGA	F	TTT		
ZA1	A	GCT	G	GGA	F	TTT		
	S	TCT	G	GGA	V	GTT		
VGSC	M918T	Codon	T929I	codon	L932F	codon	L1014F	codon
MAL19 (super-kdr)	T	ACG	T	ACC	L	TTG	F	TTT
MAL20	M	ATG	I	ATC	L	TTG	?	-
ZA1 (1B)	M	ATG	I	ATC	L	TTG	F	TTT

Note: VGSC Kdr (L1014F); super-kdr (M918T + L1014); kdr-his (L1014H); 1B (T929I + L1014F); type N (D600N + M918T + L1014F). General level of protection conferred by these alleles against pyrethroids: kdr-his < kdr < Type N ≤ super-kdr ≤ 1B (see Roca-Acevedo *et al.*, 2023). Novel concomitant mutations L932F + I936V (Sugiura *et al.*, 2021) for VGSC were not detected in the three *P. absoluta* individuals.

Chinese individuals (MT897989 and MT897990) showed the closest maternal lineage relationship, being separated by only one step. We were unable to confidently assess the Kenyan *P. absoluta* mitogenome haplotype due to the high frequencies of significant amino acid residue changes at the intraspecific level (Table S2). Raw WGS data should ideally be made available for independent confirmation, and if the mitogenome assembly quality was confirmed, this highly divergent mitogenome haplotype would further support current widespread detections of *P. absoluta* involved multiple origins of genetically diverse native populations.

Molecular diagnostics and re-assessment of *P. absoluta* partial mtCOI gene

Comparison of the mtCOI partial gene region (i.e. the 'DNA barcoding' gene region, Hebert *et al.*, 2003) typically used in molecular diagnostics supported the *P. absoluta* species identity for the three African individuals with 100% sequence identity versus partial mtCOI sequences available in GenBank for selected Africa ($N = 15$), Asia ($N = 8$), Europe ($N = 7$), and South America ($N = 8$) sequences. We further observed that, specifically in the *cox1* region, there is evidence of only two maternal lineages (i.e. KP324752 from Kenya versus all others) (Fig. 3). Taken

together, and assuming that this base substitution in KP324752 (i.e. a substitution between 'T' and 'G') to be a potential real SNP (amino acid for all other partial mtCOI gene with 'TGG' codon = (Gly), amino acid for KP324752 = 'GGG' (Gly), i.e. there is no change to the predicted glycine amino acid residues), there would appear at least seven separate maternal lineages identified in the *P. absoluta* invasive populations from Europe, Africa, and Asia.

Discussion

In this study, we presented draft mitochondrial genomes of two Malawian and one South African *P. absoluta*, representing the first mitogenome resources from these eastern and southern African countries. When compared with the individual from Spain and two individuals from Xinjiang and Yunnan, China (Tables 2, S2), and the partial mtCOI gene survey from a Kenyan *P. absoluta* population (Fig. 3), we demonstrated that at least seven different maternal lineages were present in African, European, and Asian invasive *P. absoluta* populations. We also characterised the AChE-1 and VGSC partial gene regions responsible for OP and pyrethroid resistance in these African *P. absoluta* individuals. Our results revealed surprising mitogenome haplotypes, as well as OP and pyrethroid resistance diversity in *P. absoluta*, and highlighted the limitation of

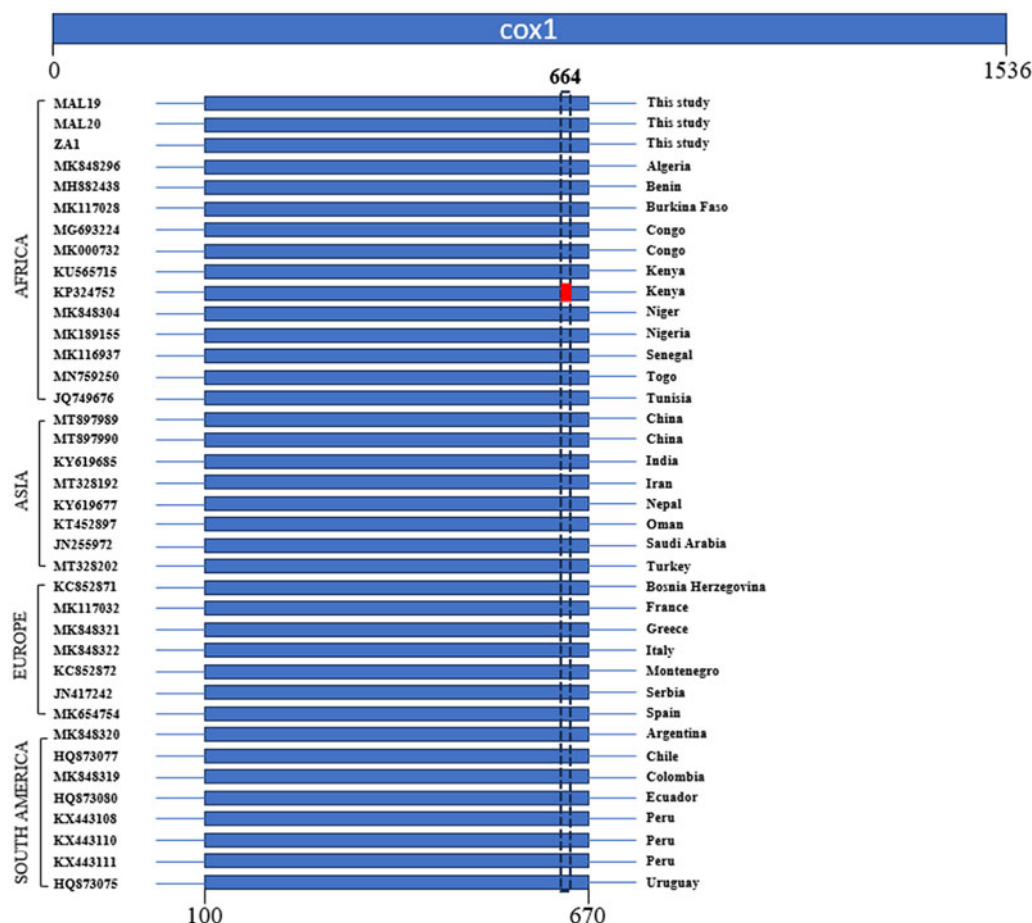


Figure 3. Graphical representation comparing the partial mtCOI gene of *Phthorimaea absoluta* samples from different parts of the world. A base substitution (G) in the Kenyan partial mtCOI gene (KP324752) that differed from all other partial mtCOI gene at nucleotide position 644 (T) is shown in red rectangle.

Note: The Congo sample MG693224 as reported in Mukwa *et al.* (2021) to be 84% identical to other *P. absoluta* partial mtCOI sequences appeared to have been an error. Recalculation of pairwise nucleotide identity showed 100% identity with all reported *P. absoluta* partial mtCOI sequences (except for the sample KP324752 as detailed above). Sample from South Africa (KY212128) was identical to sequences from Tunisia (JQ749676) and Peru (KX443108 and KX443111) (Visser *et al.*, 2017).

using partial mtCOI gene as DNA marker to infer invasion biology of often complex global introduction pathways and incursion frequencies of agriculturally significant AIS.

Until now, genetic analyses of *P. absoluta* populations have largely relied on the partial mtCOI gene widely used in species diagnostics (Hebert *et al.*, 2003; but see also, e.g., Guillemaud *et al.* (2015); Cifuentes *et al.* (2011)), and reported high genetic homogeneity among populations (e.g., Flores *et al.*, 2003; Cifuentes *et al.*, 2011; Shashank *et al.*, 2018). A survey of the partial mtCOI gene of multiple native and invasive *P. absoluta* populations demonstrated this gene region to be lacking in nucleotide polymorphisms (Fig. 3). Here, we identified eight mitochondrial genes where base substitutions were detected that could facilitate the development of alternative DNA markers for both molecular diagnostics and investigation of population diversity of this important Solanaceae pest. Specifically, by combining the *cox2*, *atp6*, *atp8*, and the 16S-rRNA partial genes could enable differentiation of all six maternal lineages. Li *et al.* (2022) analysed multiple partial mtDNA genes (i.e. *cox1*, *cox2*, *cox3*, *atp6*, *cob*, *nad1*, *nad5*) to demonstrate that populations between Xinjian and Yunnan were genetically different at the *cox2*, *atp6*, *nad1*, and *nad5* partial gene regions. Going forward, there is a need to standardise the set of multiple partial *P. absoluta* mtDNA gene markers for use in population genetic studies, although adoption of the WGS approach for the characterisation of the mitogenome would be more economic and efficient. Finally, these new *P. absoluta* mitogenomes will also further contribute to the in-depth understanding of lepidopteran systematics especially for the Gelechiidae family, and between the Gelechioidea superfamily (i.e. for which *P. absoluta* belonged) and other lepidopteran species as shown by Zhang *et al.* (2019) and Chen *et al.* (2022).

Assessment of the Kenyan *P. absoluta* individual identified exceptionally high amino acid composition changes in this individual as compared with mitogenomes of other *P. absoluta* individuals (Table S2). Across many of these mitogenome polymorphic sites, amino acid composition conservation was detected not only between *P. absoluta* individuals (i.e. intraspecific comparison), but also between lepidopteran species (i.e. *Helicoverpa* species; Table S2) from evolutionary diverged lineages. High amino acid conservation patterns in the partial mtCOI gene region have been shown in the cryptic *Bemisia tabaci* species complex (Kunz *et al.*, 2019b; Vyskocilová *et al.*, 2018), and the detection of the high number of intraspecific amino acid residue changes between the Kenyan and other African, Asia, and Europe *P. absoluta* individuals was therefore unexpected and suggested potential assembly inaccuracies.

With rapidly decreasing WGS cost, investigation of the invasive biology for *P. absoluta* would benefit from analysis of genome-wide SNP loci and full mitogenomes, as already demonstrated for other invasive Lepidoptera (e.g. *S. frugiperda*, Tay *et al.*, 2022d; Rane *et al.*, 2023; and *H. armigera*, Anderson *et al.*, 2016; Zhang *et al.*, 2022) that could also enable characterisation of known resistance genes (e.g. Guan *et al.*, 2021; Walsh *et al.*, 2018). There is as yet no *P. absoluta* population genomic studies based on genome-wide loci although Guillemaud *et al.* (2015) inferred population genetics of *P. absoluta* using low numbers of SSR loci, and concluded that there was an overall absence of genetic sub-structure across the Mediterranean Basin populations that very probably corresponded to a single introduction in Africa or Spain, followed by a rapid expansion and little noticeable demographic bottleneck effect, but acknowledging also the recognised difficulties of scoring SSR loci in the Lepidoptera due to high null allele frequencies.

The presence of null alleles and other related factors such as allele drop out and loci not in Hardy Weinberg equilibrium, could be due to association with transposable elements (e.g. Gordon *et al.*, 2009; Tay *et al.*, 2010) and/or due to Wahlund effect and could contribute to poor lepidopteran population genetic result interpretations. A closer examination of the *P. absoluta* structure analysis (Guillemaud *et al.*, 2015) identified potential genetic diversity that could have been missed especially in the Gre_lef (Greece), Fra_bal/Fra_ber (France), and the Cyp_emp (Cyprus) populations which differed from, e.g., Isr_wga (Israel), Mar_lar (Morocco), and Spa_alm (Spain) populations. Increasing the number of SSR and/or adopting the EPIC-PCR loci approach (e.g. Behere *et al.*, 2013; Tay *et al.*, 2008), and avoiding potential marker-association with mobile elements, including using of multiple mtDNA gene markers (e.g. Li *et al.*, 2022; Otim *et al.*, 2018), or by adopting genome-wide SNP loci population genomic approaches (e.g. Elfekih *et al.*, 2022), could increase sensitivity to detect potential signatures of population substructure in *P. absoluta* invasive populations.

Analyses of draft mitogenomes in this study therefore demonstrated that *P. absoluta* populations in the invasive ranges are genetically diverse and complex, although more mitogenomes from invasive populations are needed. There is also currently no draft mitogenome resources for *P. absoluta* from native range populations or from South American invasive range such as from Brazil. These diverse maternal lineages increased genetic variability in the invasive populations and underpinned the likelihood of better adaptation to new environments, to better finding suitable colonisation sites, as well as increasing the potential of introducing insecticide resistance alleles (Suinaga *et al.*, 2004) that will enable the pest to better survive within agricultural environments (Lockwood *et al.*, 2005). Zhu *et al.* (2024) showed that pyrethroids, organophosphates, and carbamates are likely to be ineffective in controlling *P. absoluta* and the related *P. operculella* due to the widespread detection of resistance alleles in invasive populations studied. In this study, we demonstrated that WGS data simultaneously enabled characterisation of both mitogenome and known OP and pyrethroid resistance genes in our limited African samples. The diversity of insecticide resistance gene profiles between *P. absoluta* populations from the many invaded countries remained to be adequately characterised, although targeted PCR tests have been developed and carried out for both AChE-1 and *kdr* alleles, e.g. in Kenyan populations (Haddi *et al.*, 2012; Ong'onge *et al.*, 2023).

Our characterisation of the pyrethroid resistance gene VGSC identified the '1B' (T929I + L1014F) *kdr* resistance allele first described in *Musca domestica*, and which was shown to have higher resistance to permethrin + piperonyl butoxide (PBO) and deltamethrin + PBO (Kasai *et al.*, 2017; Roca-Acevedo *et al.*, 2023). Co-occurrence of the Isoleucine I and the Phenylalanine amino acids in T929I and in L101 F, respectively, in *P. absoluta* is likely in various populations due to the L101F resistance amino acid being fixed in survey populations, and the T929I amino acid change detected in approximately 50% of the tested individuals (Haddi *et al.*, 2021). The detection of the *M. domestica* '1B' *kdr* phenotype in South Africa is nevertheless demonstrated for the first time, although the level of resistance to pyrethroids in this 1B mutational alleles as compared with other *kdr* mutation alleles remained to be investigated. A review of currently known insecticide resistances in *P. absoluta* is provided (Table S3).

Diversity in AChE-1 OP resistance alleles in the invasive lepidopteran pest *S. frugiperda* has been linked to multiple

independent introductions across the world (e.g. see Boaventura *et al.*, 2020; Tay *et al.*, 2022c) and different selective pressures on these pest populations. With the intensity and variety of source populations playing a major role in determining the genetic diversity of the invasive populations (Wilson *et al.*, 2009), it is therefore unsurprising that evidence from both mitogenomes and insecticide resistance gene diversity (e.g. this study; Ong'onge *et al.*, 2023; Zhu *et al.*, 2024) supported complex global introduction history of *P. abosluta*. A whole genome sequencing approach incorporating well-designed and well-coordinated insecticide/biopesticide bioassay experiments is now the logical next step to enable development of environmentally responsible future management solutions for *P. abosluta* at a global scale.

Supplementary material. The supplementary material for this article can be found at <https://doi.org/10.1017/S0007485325000252>.

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