Resistance bioassays and allele characterisation inform *Spodoptera frugiperda* (Lepidoptera: Noctuidae) management and introduction pathways in Asia and Australia

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Abstract

The fall armyworm *Spodoptera frugiperda* is present in over 70 countries in Africa, Asia, and Oceania. Its rapid dispersal since 2016 since first reported in western Africa, and associated devastation to agricultural productivity, highlight the challenges posed by this pest. Currently, its management largely relies on insecticide sprays and transgenic *Bacillus thuringiensis* toxins, therefore understanding their responses to these agents and characteristics of any resistance genes enables adaptive strategies. In Australia, *S. frugiperda* was reported at the end of January 2020 in northern Queensland and by March 2020, also in northern Western Australia. We undertook bioassays on two Australian populations, one each from these initial points of establishment. To assist with resistance response interpretation, two related endemic noctuid pest species, *Helicoverpa armigera* and *Spodoptera litura,* were concurrently screened to obtained LC50 estimates for larvae against a range of insecticides. We characterised known resistance alleles from the VGSC, ACE-1, RyR, and ABCC2 genes for five populations to compared with published allele frequencies and bioassay responses from native and invasive *S. frugiperda* populations. An approximately 10x LC50 difference for indoxacarb was detected between Australian populations, which was approximately 28x higher than an Indian population. Characterisation of ACE-1 and VGSC alleles provided further evidence of multiple introductions in Asia, and multiple pathways involving genetically distinct individuals into Australia. Bioassay results and resistance allele patterns from invasive *S. frugiperda* populations therefore suggest multiple introduction events have contributed to the pest's spread in the Old World, and challenge the axiom of its rapid 'west-to-east' spread.

Keywords

FAW, whole genome sequencing, agricultural biosecurity, population genomics, invasion biology

Introduction

The fall armyworm (FAW) *Spodoptera frugiperda* (J. E. Smith), is a noctuid moth pest made up of two morphologically indistinguishable strains (C- and R-strains) that is native to tropical and subtropical regions of the Americas. It is highly polyphagous and feeds on host species from at least 76 plant families, principally Poaceae (106 spp.), Asteraceae (31) and Fabaceae (31) (Montezano et al. 2018). In the Americas, it causes significant economic damage to maize, rice, sorghum, millet, soya bean, wheat, alfalfa, cotton, turf, and fodder crops (Nagoshi et al. 2019a). Recognised globally as one of the top 20 arthropod pests (Willis 2017), the FAW was first officially confirmed outside of its native range in Western Africa in early 2016 (Goergen et al. 2016) and then detected across virtually all of Sub-Saharan Africa by February 2018 (Nagoshi et al. 2018, Otim et al. 2018, Botha et al. 2019). In July 2018, it was confirmed in Yemen and India, and by December 2018 in Bangladesh, Sri Lanka, Myanmar and Thailand, followed closely by China in January 2019. Nepal confirmed its presence in May 2019, and in July 2019 it was also reported in Egypt, Indonesia, Laos, Cambodia, Malaysia, Vietnam, Taiwan, The Republic of Korea, and Japan (FAO 2021). The moth's strong flight ability (Rose et al. 1975, Sparks 1979, Jones et al. 2019), its potential to contaminate certain commodities, and ability to act as a 'hitchhiker' in trade (Early et al. 2018) contribute to its rapid spread. Whole genome analyses of invasive FAW populations from various African nations, India, China, Southeast Asia, and Australia also suggested that a high proportion of FAW individuals were hybrids of the C- and R-strains (Gui et al. 2020, Tay et al. 2020, Zhang et al. 2020, Schlum et al. 2021, Tay et al. 2021, Rane et al. 2022).

Much of South and Southeast Asia, and much of Northern Australia, are highly suitable climatically for the FAW year-round (du Plessis et al. 2018, Early et al. 2018). It was confirmed in Queensland (Qld; Bamaga Northern Peninsula Area), Australia following clearance of lure traps on 31 January 2020, followed by reports also in north-Western Australia (WA; Kununurra) and in the Northern Territory (NT) in March 2020. Within two weeks of the Bamaga detection, there were reports of FAW damaging maize fields in Strathmore (Qld; *ca*. 1,000 km from the initial Bamaga detection site), followed by successive southward detections of FAW in Qld, marking the FAW's south-eastward antipodean expansion into New South Wales (NSW) in September 2020. Through CLIMEX simulation analysis based on irrigation patterns and rainfall records, the FAW is expected to undertake seasonal migrations and may reach as far south as Victoria and Tasmania (du Plessis et al. 2018). Indeed, the FAW was reported in Victoria in December 2020 and in Tasmania in April, 2021. Concurrently, and from the Western Australia Kununurra detection, the FAW followed the CLIMEX simulated southward expansion patterns along the WA's western coast, with larval populations detected in the Gingin area (< 80 Km north of the city of Perth) by February 2021.

The incursion of FAW into Australia is believed to have occurred through natural dispersal across the Torres Strait/Timor Sea into northern Australia via a single-entry point (Jing et al. 2021, Qi et al. 2021). However, incursion pathways to Australia could also involve multiple entry points as reported for the biting midges *Culicoides bravitarsis* (Tay et al. 2016), other anthropogenic routes such as infested commodities in trade, as well as through seasonal or on-going new arrivals of individuals from populations that have successfully established in Asia and Southeast Asia (SEA). The eastward spread of FAW across sub-Saharan Africa, the

Middle East, the Indian sub-continent, SEA, China, and the Far East (South Korea, Japan) mostly followed chronological detection and confirmation which led to a widely accepted belief that an invasive population established in west Africa (Goergen et al. 2016) through a single founder, similar to the 'invasive bridgehead effect' (Guillemaud et al. 2011), was the starting point for its subsequent global expansion. This hypothesis is supported by an overwhelmingly homogeneous genetic signature from single gene markers (e.g., Cock et al. 2017, Nagoshi et al. 2018). Whole genome analysis and genome-wide single nucleotide polymorphic marker analysis of invasive populations from Africa (Benin, Uganda, Malawi, Tanzania) and Asia (India, China) instead suggest multiple founding events (Tay et al. 2020, Zhang et al. 2020, Schlum et al. 2021).

Distinguishing among single vs. multiple introductions within Australia (and elsewhere), and understanding the resistance profiles of existing and any new incursions, is critical for informing the future management of this pest. For example, this information will assist with forecasting likely resistance profiles of invasive populations selected outside of Australia, and could prioritise efforts to prevent the introgression (potentially of multiple separate introductions) of known insecticide resistance genes and alleles from endemic populations (e.g., Carvalho et al. 2013, Banerjee et al. 2017, Boaventura et al. 2020a, Guan et al. 2020) into invasive populations, or through reciprocal introgression of newly selected/developed resistance traits from invasive populations to native populations.

In this study, we report on bioassay experiments and resistance gene characterisation that aimed to understand how the first invasive populations of *S. frugiperda* in Australia responded to selected approved insecticide compounds and *Bacillus thuringiensis* (Bt) toxins in commercial transgenic plant varieties and available as sprays for the grains and cotton industries. Since populations were presumed collected before selection occurred in Australia these responses indicate selection against insecticides used elsewhere prior to arrival. Comparing the response differences between two FAW populations from different northern Australia regions indicated the possibility of multiple points of entry *cf.* a single entry and spread event as generally assumed (e.g., Jing et al. 2021, Qi et al. 2021). To provide a first insight into the efficacy of key compounds and toxins we compared the responses of FAW with two related major endemic crop pests in Australia, *Helicoverpa armigera* (subspecies *conferta;* Hardwick 1965, Anderson et al. 2016, Pearce et al. 2017b, a, Zhang et al. 2022) and *S. litura*, which are currently managed through adaptive resistance management plans. Finally, we provided a critical review of resistance profiles in selected invasive populations to better understand the invasion biology of FAW.

Material and Methods

Live insects

Live FAW populations were sourced from Queensland (Qld) and Western Australia (WA). This population (CSIRO code: 'Sf20-1') consisted of 30 field-collected pupae for setting up the laboratory colony, was first noticed around 19th Feb, 2020, was collected on 3rd March, 2020, and represented the first reported FAW detected attacking maize in Australia's agricultural landscape. The sampling site for this population was from

107 the University of Queensland (UQ) field station (Rex Road, Walkamin Qld 4872) from Strathmore Station,
108 (Lat/Lon: -17.17892, 145.43359, Elevation 685m).

109 A second FAW population (CSIRO code: 'Sf20-4') was collected as larvae from a maize crop from the Northern
110 Australian Crop Research Alliance Pty Ltd, Kununurra, Western Australia. These larvae were collected on 17-
111 Aug, 2020 from the Kununurra Airport field trail block (off Victoria Highway; -15.10090, 128.81342) on V4-6
112 growth stage maize, and were all collected prior to applications of insecticides. A total of 30 larvae of different
113 instar stages arrived (= F_0), and 10 (six females and four males) survived to pupation to produce >200 F_1
114 individuals and to generate sufficient F_2 larvae to commence bioassays.

115 Two related endemic noctuid species, *S. litura* and *Helicoverpa armigera conferta* (hereafter as '*H. armigera*'),
116 that are pests of a range of broadacre cropping and horticulture industries (and especially grains and cotton)
117 were also included in the bioassay experiments to assist with interpretating results such as base line
118 susceptibility and tolerance levels to the chemicals and toxins tested. Approximately 30 *S. litura* larvae (from
119 Mareeba and Toowoomba, Qld) with unknown resistance profiles were used to start the laboratory colony.
120 The *H. armigera* colony (CSIRO general rearing 'GR' strain) was a long-established laboratory strain housed at
121 the Black Mountain Laboratory in the ACT, with individuals originally collected as eggs from cotton fields in
122 and around Narrabri NSW and were periodically replenished to sustain population genetic diversity and
123 minimise inbreeding. The *H. armigera* GR population was confirmed by bioassays as having no
124 Cry1Ac/Cry2Ab/VIP3A resistance alleles.

125 **Colony maintenance**

126 *Helicoverpa armigera* and *Spodoptera* species (*S. frugiperda* Sf20-1 and Sf20-4; *S. litura*) were reared by the
127 method of Teakle and Jensen (1985) at 25 °C, 50 ± 10% relative humidity, and with a light/dark cycle of 14:10
128 h. Cooked artificial diet consisted of 81 g soya flour, 37.5 g wheat germ, 33 g brewer's yeast, 2 g ascorbic acid,
129 2 g nipagen, 3 mL vegetable oil, and 13.5 g agar, and was made up with water to 1 litre. Diet was poured into
130 rearing cups or bioassay plates, allowed to cool and then stored in the refrigerator (4-8°C) for no longer than 3
131 days.

132 **Bioassays**

133 The bioassay experiments involved commercially available insecticidal compounds and Bt toxins (available in
134 transgenic varieties of cotton and as commercial sprays), as well as Bt toxins that were produced and purified
135 by CSIRO (Table 1). For all insecticides and Bt toxins, Sf20-1 and Sf20-4 FAW populations were tested. The *H.*
136 *armigera* 'GR' line was tested alongside the FAW against all chemicals and Bt toxins except Cry1F because it is
137 known to be insensitive to it. We included *S. litura* in all Bt toxin bioassays as it is regarded as a pest of interest
138 by the Australian cotton industry who depend on Bt varieties.

139 [Table 1]

140 All bioassays included a negative control for background mortality which was treated with the diluent of the
141 individual pesticide. An initial experiment using 10x serial dilutions was performed to narrow the range for the

142 detailed bioassay using 8-16 individuals per dose replicated once. The full bioassay involved 2x serial dilutions
143 at 6-7 doses with 16-24 individuals per dose replicated 3-5 times. The bioassays involved diet incorporation,
144 surface overlay, or topical application of the insecticidal compounds or Bt toxins (Table 1). The selection
145 among these exposure methods for an insecticide was determined by its mode of action (contact or ingestion)
146 and the experience of similar bioassays in related noctuid pests (i.e., *H. armigera* and *H. punctigera*; Bird
147 2015).

148 The surface overlay of Bt toxin assays followed the approach outlined in Mahon et al. (2007b), Mahon et al.
149 (2008), Mahon et al. (2010), Mahon et al. (2012) and Walsh et al. (2014). They were conducted in 96-well trays
150 where each well contained approximately 300µl of rearing diet with a surface area of around 0.567 cm^2 per
151 well. When the diet cooled, 20µl solution containing an appropriate concentration of toxin was added and
152 allowed to air dry. One neonate was added to each well and the tray was heat sealed with a perforated lidding
153 material.

154 For diet incorporation, commercial grade insecticides were diluted to the appropriate concentration with
155 water, added to 150ml of artificial diet and mixed well to produce a homogenous solution. Insecticide-
156 incorporated diet was then dispensed into 45-well bioassay trays, each with approximately 1.5 ml of diet-
157 insecticide mix. One late-second or early-third instar larva was added to each well and the tray was heat
158 sealed with a perforated lidding material (as per Bird 2015).

159 Topical bioassays of known concentrations were conducted for alpha-cypermethrin (group 3A) and methomyl
160 (group 1) and involved applying drops of pesticide as described in Bird (2018). Specifically, 1 µl of
161 acetone/insecticide solution was applied to the dorsal thorax of 3rd to 4th instar larvae using a 50 µl micro-
162 syringe. Bioassay trays were incubated at 25 ˚C, 45-55% RH, and a photoperiod 14:10 (L:D) h for 6 days (e.g.,
163 see Bird and Akhurst 2007, Downes et al. 2009). The numbers of dead (moribund; incapable of movement
164 when prodded) and alive larvae (capable of coordinated movement when prodded) were counted and the
165 instars of surviving larvae recorded. The LC_{50} for each toxin was calculated from pooled raw data by probit
166 analysis using the POLO-PC program (LeOra Sorftware 1987).
167

168 **FAW specimens for genomic analyses**

169 Populations of *S. frugiperda* from South Korea (SK), Papua New Guinea (PNG), and Peru were included for
170 whole genome sequencing (Table 2). The methods for SK and PNG specimen preservation involved collection
171 from fields and placing larvae directly into high concentrations of ethanol (95-99.9%) to transport to the
172 laboratory where they were then stored at -20˚C and replaced with fresh ethanol after 24-48 hours. Peru
173 specimens were samples intercepted from Australian pre-border inspections of imported
174 agricultural/horticultural commodities between 2016 and 2019 (see Tay et al. 2020). Australian samples (Sf20-
175 1 from Qld, Sf20-4 from WA) were F_0 individuals and represented individuals obtained directly from fields.
176 Samples from SK, PNG, and Peru were sent to CSIRO and stored at -20˚C until DNA extraction.

177 **DNA extraction and genome library preparation**

178 DNA of individual FAW samples was extracted using the Qiagen DNA extraction kit and eluted in 200µL elution
179 buffer following protocols as provided by the manufacturer (Qiagen, Hilden Germany). Genomic DNA libraries
180 for individual samples were prepared, quantified and sent for commercial sequencing by the Australian
181 Genome Research Facility (AGRF) in Melbourne, Victoria, Australia.

182 **Processing of genome sequences**

183 Genome sequencing data for individuals were trimmed to remove adapter sequences using trim_galore (v
184 0.6.6; https://www.bioinformatics.babraham.ac.uk/projects/trim_galore/) and aligned to the *S. frugiperda* rice
185 genome (v1.0) (Gouin et al. 2017) using bwa_mem2 (v2) (Vasimuddin et al. 2019). Duplicate alignments were
186 removed using SAMBLASTER (v 0.1.26) (Faust and Hall 2014) and sorting completed using SAMtools (v1.9) (Li
187 et al. 2009).

188 [Table 2]

189 For identification of FAW R- or C-strain, the used the partial mtCOI gene sequences for the R-strain (GenBank
190 MF197867) and the C-strain (GenBank MF197868) of Otim et al. (2018) as reference sequence for mapping
191 against the whole genome sequence data for each individual FAW from Sf20-1, Sf20-4, SK, PNG, and Peru.
192 Mapping of partial mtCOI gene was carried out within Geneious v11.1.5 (Biomatters Ltd, Auckland, NZ) using
193 the Geneious Mapper program with assembly parameters specified to 'Low Sensitivity / Fastest', with no
194 trimming before mapping, and the 'Fine Tuning' option set to 'Iterate 2 times' to map reads to the consensus
195 from the previous iteration. Full mitochondrial DNA genomes of all individuals were also assembled following
196 the procedures as outlined in (Otim et al. 2018) and have been reported by Rane et al. (2022). For all
197 resistance alleles of interest (ABCC2, ACE-1, RyR, VGSC; see Table S1), base-sequence at the genomic location
198 for individuals listed in Table 1 was extracted from the alignment file using BCFTOOLS MPILEUP followed by
199 CALL, since BBMAP does not report 'non-variant' sites. Results were tabulated for inference.

200

201 **Comparisons of published ACE-1 and VGSC resistance allele frequencies and published indoxacarb and**
202 **chlorantraniliprole LC_{50} values**

203 Three resistance loci for the organophosphate/carbamate ACE-1 gene have been reported to-date in FAW: (i)
204 A201S, (ii) G227A, and (iii) F290V. To understand the frequencies of these alleles, we surveyed specimens from
205 invasive (i.e., Australia, PNG, South Korea) and native (Peru) FAW populations, and combined this information
206 with reported allele frequencies for these three loci from other native (Brazil, French Guiana, Mexico
207 Guadeloupe, Puerto Rico, USA) and invasive populations (Benin, Uganda, Kenya, Tanzania, Zambia, Malawi;
208 India, Myanmar, Laos, Vietnam, Malaysia, Indonesia, China, Australia) (see Table S2 and references therein).
209 For the pyrethroid *para* sodium channel resistance gene VGSC, three loci have been identified to date in FAW:
210 (i) L1014F, (ii) L932F, and (iii) T929I (e.g., Carvalho et al. 2013, Guan et al. 2020, Yainna et al. 2021). Resistance
211 and susceptible allele frequencies in these three VGSC loci were also surveyed from published studies from
212 native and invasive FAW populations (see Table S2).

213 For comparisons of bioassay LC_{50} data for indoxacarb and chlorantraniliprole between published data and our
214 study, we considered only those that reported broadly similar methodologies (see Table 1) such as clearly
215 stated route of delivery (i.e., diet incorporation/ingestion) for the insecticidal compounds, and using
216 comparable developmental stages of larvae (i.e., 2nd/early 3rd instar) and scoring criteria.

217

218 **Results**

219 **Bioassays of Individual Bt proteins**

220 Cry1Ac was not highly effective against the *Spodoptera* species tested in this study (Figure S1; Table 3). Based
221 on the LC_{50}, and relative to *H. armigera,* FAW Sf20-1 was 174x less sensitive, FAW Sf20-4 was 99x less
222 sensitive, and *S. litura* was 120x less sensitive. This suggests that in *S. frugiperda,* Cry1Ac would give similar
223 control in Australia to that found in *S. litura* but far less than for *H. armigera*.

224 The *Spodoptera* species are less sensitive to Cry2Ab than *H. armigera* but the differences are not as striking as
225 for Cry1Ac (Figure S2; Table 3); FAW Sf20-1 was 13x less sensitive, FAW Sf20-4 was 4x less sensitive and *S.*
226 *litura* was 10x less sensitive. This suggests that in *S. frugiperda,* Cry2Ab would give similar control in Australia
227 to that found in *S. litura* which is unlikely to be different from *H. armigera* in terms of field control.

228 Cry1F is a Bt protein that has been deployed in certain genetically modified plants to target *Spodoptera.*
229 Though not effective against *H. armigera sensu lata* even at a relatively high dose, Cry1F was effective against
230 *S. frugiperda* at a much lower dose (Figure S3; Table 3), indicating that this species is much more sensitive to
231 this Bt protein than *H. armigera*. Relative to *S. litura,* the LC_{50} data shows that FAW Sf20-1 and FAW Sf20-4 are
232 2x less sensitive to Cry1F. In terms of field control there is unlikely to be any distinguishable difference in Cry1F
233 efficacy against FAW vs. *S. litura.*

234 FAW Sf20-1 and FAW Sf20-4 showed a similar tolerance to Vip3A as *S. litura* (~2.34x and 1.2x higher,
235 respectively) and all three populations were more sensitive to Vip3A than *H. armigera* (0.15x, 0.08x, and 0.07,
236 respectively). In terms of field control there is unlikely to be any distinguishable difference in efficacy of VIP3A
237 against FAW relative to *H. armigera* and *S. litura* (Figure S4; Table 3).

238 Based on published studies which show resistance ratios of >2,500 fold in *H. armigera* and FAW that are
239 homozygous for recessive resistance alleles against Bt toxins (e.g., Mahon et al. (2007a) - Cry2Ab, *H. armigera*;
240 Horikoshi et al. (2016), Cry1F, Cry1A, Vip3A, FAW) it is unlikely that either of the founding FAW populations in
241 Australia carried resistance alleles to Cry1Ac, Cry2Ab or Vip3A in homozygous states.

242 Sprayable Bt

243 The performance against FAW of the sprayable products containing multiple Bt toxins (XenTari and DIPEL)
244 showed both products to differ in efficacies against *H. armigera,* FAW, and *S. litura*. XenTari with the mixture
245 of Cry1 toxins including Cry1C was formulated to provide *Spodoptera* control (valentbiosciences, last accessed
246 13-Jan, 2022) but also for cabbage looper and the diamondback moth (DBM), while DIPEL was formulated for

247 broad spectrum caterpillar control (valentbiosciences, last accessed 13-Jan, 2022). Both DIPEL and XenTari are
248 reported to contain different toxin complements but the relative amounts are unclear. DIPEL contains Cry1Ac
249 and Cry2-type toxins and was less effective against both tested FAW populations than XenTari which contains
250 Cry1Aa, Cry1Ab, Cry1C and Cry1D. Relative to *H. armigera*, FAW Sf20-1 was 24x less sensitive, FAW Sf20-4 was
251 18x less sensitive and *S. litura* was 17x less sensitive to Dipel. XenTari toxicity ratios relative to *H. armigera*
252 ranged 2 to 4x for FAW Sf20-1, FAW Sf20-4 and *S. litura* (Figure S5 and Figure S6; Table 3).

253 Conventional pesticides

254 Cypermethrin is a pyrethroid pesticide and the bioassay results suggest that FAW Sf20-1 and FAW Sf20-4 are
255 much less sensitive to it than *H. armigera* (56x and 145x respectively: Figure S7; Table 4). Broadly speaking,
256 given that pyrethroid resistance is common in the field in *H. armigera* (Walsh et al. 2018), this result suggests
257 that it may be difficult to control *S. frugiperda* with pyrethroids. It also indicates some heterogeneity in the
258 response to pyrethroids in the first invasive FAW populations in Australia.

259 Bioassay results for the carbamate pesticide methomyl are not as repeatable as other toxins with considerable
260 variability between replicates (Figure S8; Table 4). However, overall FAW Sf20-4 shows 52x less sensitivity to
261 methomyl than *H. armigera* while FAW Sf20-1 shows a 4x lower sensitivity. This difference is consistent with
262 some level of heterogeneity between the first invasive FAW populations in Australia.

263 The dose response and LC_{50} for indoxacarb (Figure S9; Table 5) show that FAW Sf20-1 and FAW Sf20-4 are less
264 sensitive to this chemistry than *H. armigera* (22x and 208x respectively). This difference is consistent with
265 some level of heterogeneity between the first invasive FAW populations in Australia.

266 The dose response and LC_{50} for chlorantraniliprole show similar sensitivity for *H. armigera* and FAW Sf20-1,
267 with the latter showing a 3x difference in the LC_{50}. FAW Sf20-4 demonstrated a 15x reduction in sensitivity
268 compared to *H. armigera* (Figure S10; Table 5).

269 The dose response and LC_{50} data for emamectin benzoate and spinetoram (Figure S11, Figure S12; Table 5)
270 suggest that FAW Sf20-1 and FAW Sf20-4 are not significantly different in their level of sensitivity compared to
271 *H. armigera*.

272 [Table 3, Table 4, Table 5]

273 **Resistance alleles by whole genome sequencing**

274 The loci examined for potential resistance alleles were present in the data set with the appropriate level of
275 coverage to accurately call the genotype. Resistance alleles to carbamate/organophosphates (ACE-1) were the
276 only resistance alleles identified in the Kununnura, Strathmore, South Korea, Papua New Guinea, and Peru
277 populations. No resistance alleles were detected for pyrethroid (VGSC) or for diamide insecticides (RyR).
278 Resistance allele profile differences between the two Australian populations were evident between the A201S
279 and the F290V amino acid substitutions. Similar ACE-1 resistance allele profiles were detected between
280 Strathmore and South Korean populations, while the Papua New Guinea population shared ACE-1 allele
281 profiles with Strathmore population (A201S) and with Kununnura (F290V). The Peruvian FAW population was

282 the only population that have heterozygous individuals with resistance allele for G227A amino acid
283 substitution (Table 6).

284 [Table 6]

285 Resistance allele characterisation by sequencing approaches from this study and published studies for the ACE-
286 1 gene is summarised in (Figure 1, Table S2 and references therein). The most common resistance allele
287 detected in the invasive and native population (456 individuals examined in total) was the F290V mutation
288 with 66 homozygous and 222 heterozygous resistant genotypes detected. This mutation is a T to G single
289 nucleotide polymorphism (SNP) that changes the codon encoding the amino acid from TTT to GTT leading to a
290 phenylalanine (F) to valine (V) change in the protein sequence encoded by the ACE-1 gene. This mutation was
291 present at all locations and evenly distributed between the invasive and native populations. In Australia, of the
292 sequenced individuals (n = 146), 24.7% (n = 36) were heterozygous, and 8% (n = 12) were homozygous for the
293 resistance allele. Heterozygous and homozygous individuals were found in populations from all four states
294 (i.e., WA, NT, Queensland, and NSW) as well as from the single individual from Erub Island, suggesting it is
295 common across Australia.

296 The second most common allele detected was A201S (456 individuals examined) with 90 heterozygous
297 individuals detected. This mutation is a C to A SNP which changes the codon from GCG to TCG leading to an
298 alanine (A) to serine (S) amino acid change. While less common, this mutation also appears to be in both the
299 invasive and native range with no obvious pattern. In Australia, of the sequenced individuals (n = 146), 15.8%
300 (n = 23) were heterozygous, and 0 were homozygous for the resistance allele. Heterozygous individuals were
301 found in WA and Queensland, also suggesting it is common across Australia.

302 The G227A mutation was the least common (from 456 individuals) with 20 heterozygous individuals and 2
303 homozygous individuals detected. This mutation is a G to a C SNP which alters the codon from GGA to CGA
304 encoding a glycine (G) to alanine (A) amino acid change. Interestingly G227A was only present in individuals
305 from the native range (Brazil, USA, Puerto Rico, Peru) but absent in individuals from across the invasive range
306 that were surveyed in this study and from the related studies of Tay et al. (2020) and Tay et al. (2021). When
307 compiling allele frequencies for both the VGSC and ACE-1 resistant genes from this study and from published
308 whole genome sequencing and targeted PCR/Sanger sequencing data (Figure 1; see also Table S1), the rare
309 G227A resistance allele was present only in the Indonesian (Boaventura et al. 2020a) and Hubei populations
310 (Guo et al. 2020) but absent in African, Australian, and other Asian (e.g., Indian, Myanmar, etc.) populations,
311 including populations from six other Chinese provinces.

312 No alleles predicted to cause resistance to pyrethroids or the group 28 pesticides were identified in this work.
313 However, while the previously identified resistance alleles (Bolzan et al. 2019, Boaventura et al. 2020b) were
314 not detected, considerable variation was present in the RyR gene at the potential resistance loci. This should
315 be further investigated in conjunction with bioassays to establish whether any of the variants could contribute
316 to resistance.

317 **ABCC2 resistance alleles in Australia, Papua New Guinea, South Korea, and Peru populations**

None of the known and validated Cry1 resistance ABCC2 mutations were identified in the individuals sequenced for this work however, some variation from the reference was observed (Figure S13). Numerous synonymous mutations and a total of 31 non-synonymous mutations were observed in the coding sequence of the ABCC2 gene in all sequenced individuals. Several of these non-synonymous mutations were associated with a common deletion and insertion motif in the first exon where an 11bp deletion and a 2 bp insertion maintain the reading frame but replace and change several amino acids. Most of the other mutations are the result of one (i.e., single nucleotide variant; SNV) or two (i.e., multiple nucleotide variant; MNV) nucleotide changes. Several of these mutations are found in other assemblies of *S. frugiperda* and likely reflect natural variation. An alternative explanation for the variation is that they are associated with the corn strain which is thought to make up at least some of the genome of the invasive populations. Only one mutation, a 2 bp deletion in one individual collected from the Burdekin in Queensland was identified that might cause a frame shift mutation as has been identified in Bt resistant individuals in other studies. It was present as a heterozygote in the individual and has not been experimentally validated.

[Figure 1]

Discussion

In this study, we showed insecticide and Bt response differences in two of the first established FAW populations in Australia, with the Queensland (Sf20-1) population being less tolerant to various insecticides such as methomyl, chlorantraniliprole, and indoxacarb compared to the Western Australian (Sf20-4) population. On the other hand, the Queensland population exhibited at least a two-fold higher tolerance to Cry1Ac, Cry2Ab, Cry1F and Vip3A Bt toxins than the WA population. Characterisation of the ABCC2 resistance gene identified one FAW individual from Queensland as potentially being heterozygous with a 2 bp deletion that could underpin Cry1F resistance, although confirmation of the detected mutation and of the resistance phenotype is required. This could be accomplished via PCR and Sanger sequencing (e.g., Guan et al. 2020) as well as via the CRISPR/Cas9 gene editing approach (e.g., see Wang et al. 2017). The response differences to chemical insecticides and Bt toxins of the two studied invasive FAW populations in Australia and their ACE-1 resistance allele profile differences (Table 6) may reflect the diverse genetic composition across the pest's recent expanding range (Tay et al. 2020, Schlum et al. 2021, Rane et al. 2022), and suggest that separate pathways were involved in establishment of these Queensland and Western Australian populations. This is contrary to the current postulation of a single introduction pathway for the arrival of FAW to Australia based on an assumption (Jing et al. 2021) or reverse trajectory simulation (Qi et al. 2021) and highlights the importance of harmonising simulation studies with genomic and phenotypic evidence.

With significant economic impacts on agriculture from FAW reported in over 80 countries (excluding the New World native range) from Africa, Middle East, Asia, Southeast Asia, and Oceania, its response to different insecticidal and Bt control agents is increasingly investigated at recently impacted localities (e.g., Worku and Ebabuye 2019, Deshmukh et al. 2020, Zhang et al. 2020, Kulye et al. 2021, Lv et al. 2021). However, the diverse bioassay methods (approach, larval stage, scoring criteria) used in these studies complicate meaningfully comparison of findings. Taking emamectin benzoate and indoxacarb as examples, Zhang et al. (2022) used a

topical application bioassay on 3rd instar larvae, Deshmukh et al. (2020) used leaf-dip bioassays on 2nd instar larvae, while Hardke et al. (2011) used a diet-incorporation approach on 3rd instar larvae. Responses have been expressed as LD_{50} (e.g., Deshmukh et al. 2020) or as EC_{50} (e.g., Kulye et al. 2021), further making it difficult to rigorously compare outcomes.

A further challenge for invasive FAW management is the lack of base-line values representing susceptible responses in newly populated areas which makes it difficult to monitor changes through time in insecticide efficacy due to resistance. Knowledge of whether the introduction occurred once (e.g., single 'invasive bridgehead effect'; Guillemaud et al. 2011) vs. multiple times (e.g., multiple 'mass dispersal'; Wilson et al. 2009) and patterns of gene flow also impact the long-term monitoring of insecticide resistance evolution. Using native FAW colonies established in 2005 from cotton fields in Louisiana, USA, Wilson et al. (2009) undertook diet-incorporation bioassays (on 3rd instar larvae), using insecticides including chlorantraniliprole, indoxacarb, and spinetoram (see Table 7). Yu (1991) undertook topical application bioassays against methomyl (on 4th instar larvae) which included comparison with a susceptible population free from insecticide exposure since 1975, and a resistant population collected from a maize field in Gainesville, Florida (see Table 8). In comparing insecticide responses in Indian invasive FAW populations with diet incorporation assays (on 3rd instar larvae), Yu (1991) also included a native susceptible FAW population from Brazil to assist with interpreting changes to insecticide responses (including chlorantraniliprole and spinetoram) at spatial and temporal scales.

In the absence of native FAW populations in Australia, and in addition to the information collected herein for *H. armigera* and *S. litura*, we used the information from these global studies on native and newly invaded populations to assist interpretation of the early data collected in this study on invasive FAW populations in Australia. These examples used broadly similar approaches, instars and scoring criteria to our study for bioassays with specific insecticides. Relative to the indoxacarb results from Hardke et al. (2011) on native FAW populations from the USA, our bioassay findings for the invasive Western Australian and Queensland populations suggest a 28- and 3-folds difference, respectively (Table 7). For spinetoram, the responses of the two Australia populations (which were like each other) is around 10 and 1.5 times higher than for the native populations from Brazil and the USA respectively. In contrast the ratios for the invasive populations from India was 1.3 and 0.3 relative to the Brazil and USA native populations. The differences are even more pronounced for chlorantraniliprole where the Sf20-4 WA population exhibited ratios that were 32 and 2.4 times higher than both the native Brazilian and American populations, which are at least around 3-fold higher than for the invasive populations in South Africa and India (Table 7) and also for the invasive Indian population studied by Deshmukh et al. (2020) using a leaf dip assay (and hence not reported in Table 7).

Methomyl resistance alleles have been reported in invasive populations from China, Indonesia, Africa, and in this study. Comparisons between susceptible and resistant strains of native FAW populations from Florida USA (Yu 1991) and with the South African invasive population of FAW (Eriksson 2019) showed that the Queensland population was similar in its response as the susceptible Florida FAW, while the Western Australia population was around 5 times more tolerant. This contrasted the South African population which was 110 times more

392 tolerant than the Florida susceptible FAW strain, and around 8 times more tolerant than the resistant FAW
393 strain from Florida (Table 8). Taken as a whole, similar bioassay results between native (i.e., Brazil, USA) and
394 various invasive populations suggested potential significant genetic diversity in introduced populations. This
395 concurs with population genomic analyses (Tay et al. 2020, Zhang et al. 2020, Schlum et al. 2021, Rane et al.
396 2022) that suggested multiple origins for the invasive African, Asian (Indian, Chinese), and Southeast Asian
397 (e.g., Malaysia) FAW populations.

398 It is possible that the differential response between the two Australian FAW populations to insecticides and Bt
399 toxins represents natural variation in the national population which has emerged from a single founding
400 incursion. It is unlikely that the differential responses within Australian populations represents local selection
401 pressures because the period between populations establishing and being collected for this study was
402 presumed to be too short to enable this opportunity. It could be that separate incursions involving different
403 source populations occurred in WA compared to the eastern states of Australia. Different selection pressures
404 on the global population which recently originated from multiple Asian, Southeast Asian, and African
405 incursions (Tay et al. 2020, Zhang et al. 2020, Schlum et al. 2021, Rane et al. 2022) may have driven different
406 phenotypes which entered the expanding ranges tested in our study and others reported herein. However,
407 Kulye et al. (2021) demonstrated in FAW populations collected in India during 2018, 2019, and 2020 that large
408 response changes such as those observed in South African (e.g., methomyl; Eriksson 2019) and Western
409 Australian (chlorantraniliprole, indoxacarb; this study) populations were unlikely considering the short time
410 frame since the very recent arrival of FAW especially assuming a west-to-east spread (Goergen et al. 2016,
411 Cock et al. 2017, Nagoshi et al. 2018, Nagoshi et al. 2019b). Another explanation for the differential responses
412 of the two Australian populations that we studied is that they have different fitness because of variation in
413 genetic diversity which reflects the number of individuals founding the populations. However, if this was the
414 case one might expect sensitivity levels to be consistently higher or lower to all of the agents that we tested
415 but this was not the case.

416 [Table 7, Table 8]

417 Alien invasive agricultural pests are increasingly being shown to carry novel insecticide resistance genes (e.g.,
418 Anderson et al. 2018, Walsh et al. 2018, Tay and Gordon 2019). This includes the FAW in which invasive
419 populations have been confirmed via whole genome sequence analyses (e.g., Guan et al. 2020, Zhang et al.
420 2020, Yainna et al. 2021) and molecular characterisation to harbour selected resistance genes (Boaventura et
421 al. 2020b, Boaventura et al. 2020a, Zhao et al. 2020). Our review of reported ACE-1 and VGSC resistance allele
422 frequency differences (Figure 1; Table S2 and references therein) suggests that the invasive FAW populations
423 within China (Guo et al. 2020), Indonesia (Boaventura et al. 2020a), Malaysia and Queensland, Australia (Tay et
424 al. 2021, Rane et al. 2022), and South Korea (this study) were genetically diverse and likely originated from
425 different native populations (Figure 1). This further supports the perceived rapid spread of FAW across Africa,
426 Asia, and Oceania as likely to also involve multiple independent introduction events.

427 Given the insecticide resistance allele frequency differences in FAW (e.g., Boaventura et al. 2020a, Guan et al.
428 2020, Lv et al. 2021, Yainna et al. 2021) and variation between the two populations sampled herein in bioassay

429 responses to some of the approved insecticides for broadacre cropping, effort in Australia and indeed, for
430 other invasive regions where possible, should now be directed to establishing baseline susceptibilities against
431 key chemistries for multiple populations across geographies. For instance, our comparisons with *H. armigera*
432 and *S. litura* suggest that pyrethroids like cypermethrin are unlikely to provide good control in Australia against
433 FAW but the large discrepancies between WA and Qld populations for methomyl and indoxacarb require study
434 of further populations before drawing firm conclusions. This information will be critical for on-going
435 monitoring of resistance allele frequencies to key chemistries and determining their field application
436 efficiencies, among geographies which is required to inform resistance management plans. It will also be
437 important to understand gene flow patterns between different populations. Early detection of potential future
438 introductions into Australia of novel resistance genes/alleles should also be a priority; for example, the VGSC
439 L1014 resistance allele and the ACE G227A resistance allele from Southeast Asia (Boaventura et al. 2020a) and
440 China (Guo et al. 2020), ryanodine receptor (RyR) resistance alleles from Brazil and China (Bolzan et al. 2019,
441 Boaventura et al. 2020b, Lv et al. 2021) and ABCC2 resistance alleles from the Americas (Banerjee et al. 2017,
442 Flagel et al. 2018, Guan et al. 2020, Yainna et al. 2021).

443 Being alert to new FAW incursions carrying novel resistance genes is relevant also for the Bt toxins. Our
444 bioassay findings suggest Australia's FAW populations likely do not carry Cry1F resistance alleles in
445 homozygous state known to exist in native range FAW populations (e.g., Banerjee et al. 2017, Flagel et al.
446 2018, Guan et al. 2020, Yainna et al. 2021), however the 2 bp deletion identified via whole genome sequencing
447 in a single heterozygous individual from Burdekin (Queensland) will require further confirmation. Field-
448 selected lines of VIP3A20 resistant FAW have been reported in Brazil and the USA where they occur as a
449 recessive trait (Yang et al. 2013, Bernardi et al. 2015, Bernardi et al. 2016, Yang et al. 2018, Yang et al. 2019).
450 The candidate resistance gene(s) and associated mutation(s) underpinning this resistance are yet to be
451 identified, therefore phenotypic bioassays are required to detect resistance to VIP3A. Using what was
452 essentially an F_0 screen of material we did not detect resistance to VIP3A in our field-derived laboratory-
453 maintained FAW colonies, however bioassays involving F_2-crosses (Andow and Alstad 1998) would be needed
454 to detect individuals that are heterozygous for a recessive form of VIP3A resistance allele. VIP3A remained
455 highly effective when tested against both populations, and should be a valuable tool in protecting the
456 Australian cotton industry against FAW due to the > 90% up-take of Bollgard III cotton containing Cry1Ac,
457 Cry2Ab and Vip3A proteins. To protect agriculture in Australia (and elsewhere) from an accidental introduction
458 of VIP3A resistant populations from the Americas (e.g., from Brazil, Bernardi et al. 2016) into global invasive
459 populations, national (e.g., pre-border) and industry biosecurity preparedness strategies must be co-ordinated
460 to prevent and to increase the chances of early detection of such novel introductions of new traits.

461 Finally, to enable meaningful comparisons of bioassay findings from across the FAW invasive range, there is a
462 need to globally standardise approaches for testing of insecticides and Bt toxins. Localised novel resistance
463 traits in populations of FAW would likely serve as a new resistance management challenge to other
464 neighbouring regions (Kalyebi 2020), and movements of FAW in the new invasive ranges could lead to as yet

465 unknown and complex gene flow patterns that could significantly hinder the development of suitable pest and
466 resistance management strategies for this global pest complex.

467

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722 **Table 1:** List of insecticides and Bt toxins used in the bioassay experiments. All insecticides and Bt toxins were
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724 Western Australia (Sf20-4, from Kununurra). Commercial names of insecticides/Bt toxins, their manufactured
725 or expiry dates are provided where available. Bioassays involved either: (i) diet incorporation (DI); (ii) Topical
726 application (TA); or (iii) Surface treatment (ST) of diet to be consumed. The larval stages used in the bioassays
727 are specified. For topical application we used larvae from late 3rd to early 4th instar with each larva weighing
728 approximately 30mg. Species tested were: *Helicoverpa armigera*, *Spodoptera frugiperda* (FAW), and
729 *Spodoptera litura*.

Insecticides/Bt toxins	Commercial name	Date	Approach	Larval stage	Species tested		
					FAW	*H. armigera*	*S. litura*
Chlorantraniliprole (200g/L)	Coragen	Manufactured : 28/06/2019	DI	2nd/early 3rd	Yes	Yes	No
Indoxacarb (150g/L)	Steward 150	Manufactured : 30/03/2019	DI	2nd/early 3rd	Yes	Yes	No
Emamectin (17g/L)	Affirm 017 EC	Unspecified†	DI	2nd/early 3rd	Yes	Yes	No
Spinetoram (120g/L)	Dow AgroSciences Success® Neo	Manufactured : 02/2018	DI	2nd/early 3rd	Yes	Yes	No
Alpha cypermethrin (100g/L)	Dominex Duo	Manufactured : 30/04/2019	TA	Late 3rd/ early 4th	Yes	Yes	No
Methomyl		Expiry date: 02/2022	TA	Late 3rd/ early 4th	Yes	Yes	No
Advertised as: Cry2A, Cry2B, Cry1Ab, Cry1Aa, Cry1Ac	Yates Nature's Way DIPEL	Expiry date: 27/05/2022	ST	Neonates	Yes	Yes	Yes
Advertised as: Cry1D, Cry1C, Cry1Ab, Cry1Aa	XenTari WG Bta	Manufactured : 05/02/2019	ST	Neonates	Yes	Yes	Yes
Cry1Ac	CSIRO; from HD73 strain	NA	ST	Neonates	Yes	Yes	Yes
Cry2Ab	CSIRO; from Bt expressing Cry2Ab	NA	ST	Neonates	Yes	Yes	Yes
Cry1F	CSIRO; from *E. coli* expressing Cry1F	NA	ST	Neonates	Yes	Yes	Yes
VIP3A	CSIRO; from *E. coli* expressing Vip3A	NA	ST	Neonates	Yes	Yes	Yes

730 **Note:** † likely manufactured recently as this was requested from Syngenta Australia Pty Ltd and provided
731 directly for the bioassay purpose. N/A: not available. These Bt toxins were produced and purified from *B.*
732 *thuringiensis* cultures (Cry1Ac and Cry2Ab) or cloned into vectors in *Escherichia coli* (Cry1F and Vip3A) and
733 grown at the CSIRO Black Mountain Laboratories.

734 **Table 2:** *Spodoptera frugiperda* samples from Australian populations (Sf20-1, Qld; Sf20-4, WA), Papua New Guinea, South Korea, and Peru used in whole genome
735 sequencing for resistance gene characterisation. Number of specimens from each location is indicated by 'N'. Where available, GPS co-ordinates and elevation are
736 provided. The number of individuals belonging to the *S. frugiperda* R-strain or the C-strain mitochondrial DNA COI haplotypes is also provided.

Country	State/Province	Population	N	Date	Note	R : C
Australia	WA	Kununurra	9	16-Aug-20	'Sf20-4', represent the original WA field-collected individuals	9: 0
	Qld	Strathmore Station, Walkamin	29	03-Mar-20	'Sf20-1', represent the original Qld field-collected individuals	29 : 0
South Korea	Milyang	MF	7	Feb-20	Milyang-si, Gyeongsangnam-do, G_4 lab, GPS: 35˚29.29 N, 128˚44.31 E	7 : 0
	Goryong	GR	1	Sep-19	Goryeong-gun, Gyeongsangbuk-do, Corn-field; GPS: 35˚ 64.04 N, 128˚39.08 E	1 : 0
	Haenam	MN	1	Aug-19	Haenam-gun, Jeollanam-do, Corn field; GPS: 34˚24.38 N, 126˚37.55 E	1 : 0
	Milyang	MY	2	Sep-19	Milyang-si, Gyeongsangnam-do, Corn field; GPS: 35˚29.29 N, 128˚44.31 E	0 : 2
	Muan	MA	1	Aug-19	Muan-gun, Jeollanam-do, Corn field; GPS: 34˚52.21 N, 126˚31.11 E	1 : 0
Papua New Guinea	Madang Province	Ramu Sugar Estate	16	15-17 Jun-2020	5°58.154 S, 145°53.252 E, Maize host	16 : 0
	Central Province	Yule Island Junction	1	5-Jun-20	corn host	1 : 0
Peru	n/a	Pre-border intercepted specimens on imported agricultural commodities	16	2016 - 2019	Samples from Tay et al. (2020) (Suppl. Table 1, individuals PE01 – PE16)	0 : 16

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739 **Table 3:** Summary bioassay data on *Spodoptera frugiperda* populations from Walkamin, Queensland (Sf20-1) and Kununurra, Western Australia (Sf20-4), *S. litura* (Mareeba)
740 and *Helicoverpa armigera* (GR laboratory line) involving surface treatment of the diet with Bt toxins and products.

Pesticide	Populations/species	LC_{50}	95% CI	LC_{99}	Toxicity ratio (*H. armigera* = 1)	Slope
Cry1Ac	*H. armigera*	0.025	0.021 - 0.029	0.465	-	1.837 ±0.126
(µg/cm^2)	Sf20-1	4.34	1.81 - 8.15	2813.9	174	0.827 ±0.102
	Sf20-4	2.48	1.79 - 3.29	99.9	99	1.449 ±0.149
	S. litura	2.99	2.06 - 4.20	80.7	120	1.626 ±0.135
Cry2Ab	*H. armigera*	0.049	0.034 -0.068	1.74	-	1.501 ±0.143
(µg/cm^2)	Sf20-1	0.655	0.435 - 0.951	16.88	13	1.649 ±0.136
	Sf20-4	0.178	0.138 - 0.221	2.03	4	2.201 ±0.246
	S. litura	0.511	0.335 - 0.734	44.9	10	1.197 ±0.081
Cry1F	Sf20-1	0.025	0.015 - 0.038	0.838	-	1.531 ±0.115
(µl/cm^2)	Sf20-4	0.021	0.014 - 0.029	0.394	-	1.809 ±0.149
	S. litura	0.0088	0.0069 - 0.011	0.294	-	1.524 ±0.121
VIP3a	*H. armigera*	0.0062	0.0051 - 0.0075	0.23	-	1.486 ±0.093
(µl/cm^2)	Sf20-1	0.0021	0.0010 - 0.0031	0.035	0.152	1.881 ±0.225
	Sf20-4	0.0019	0.0016 - 0.0023	0.018	0.078	2.398 ±0.212
	S. litura	0.00065	0.0005 - 0.0008	0.015	0.065	1.693 ±0.129
Dipel	*H. armigera*	2.11	1.59 - 2.72	39.2	-	1.832 ±0.154
(IU/cm^2)	Sf20-1	52.02	36.55 - 70.05	2612.6	24	1.832 ±0.154
	Sf20-4	38.93	23.97 - 56.35	904.1	18	1.703 ±0.153
	S. litura	35.96	28.23 - 44.99	1540.8	17	1.426 ±0.098
XenTari	*H. armigera*	5.95	4.69 - 7.40	197.2	-	1.530 ±0.136
(DBM/cm^2)	Sf20-1	11.81	8.33 - 15.17	112.7	2	2.375 ±0.289
	Sf20-4	19.01	9.27 - 29.8	1058.9	3	1.333 ±0.180
	S. litura	24.86	17.08 - 31.14	206.9	4	2.375 ±0.289

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743 **Table 4:** Summary bioassay data on *Spodoptera frugiperda* populations and *Helicoverpa armigera* (GR laboratory line) involving topical application to the insect with
744 contact insecticides. Sf20-1 = *S. frugiperda* from Walkamin Queensland; Sf20-4 = *S. frugiperda* from Kununurra Western Australia.

Toxin	Population/Species	LC_{50}	95% C.I.	LC_{99}	Toxicity ratio (*H. armigera* = 1)	Slope
Alpha cypermethrin	*H. armigera*	0.0036	0.0032 - 0.0041	0.023	-	2.849 ±0.140
µg/larvae	Sf20-1	0.201	0.171 - 0.239	1.88	56	2.399 ±0.154
	Sf20-4	0.523	0.427 - 0.641	6.06	145	2.186 ±0.132
Methomyl	*H. armigera*	0.057	0.031 - 0.097	43.12	-	0.809 ±0.059
µg/larvae	Sf20-1	0.254	0.177 - 0.356	39.2	4	1.064 ±0.076
	Sf20-4	2.96	1.87 - 4.66	1363.7	52	0.874 ±0.07

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746 **Table 5:** Summary bioassay data on *Spodoptera frugiperda* populations (Sf20-1 from Walkamin Queensland; Sf20-4 from Kununurra Western Australia) and *Helicoverpa*
747 *armigera* (GR laboratory line) involving diet incorporation of insecticides.

Pesticide	Population/Species	LC_{50}	95% CI	LC_{99}	Toxicity ratio (*H. armigera* = 1)	Slope
Chlorantraniliprole	*H. armigera*	0.011	0.009 -0.013	0.056	-	3.199 ±0.234
(µg/ml diet)	Sf20-1	0.032	0.024 - 0.043	0.28	3	2.484 ±0.191
	Sf20-4	0.163	0.132 - 0.201	2.19	15	2.065 ±0.152
Indoxacarb	*H. armigera*	0.054	0.028 - 0.089	1.29	-	1.684 ±0.122
(µg/ml diet)	Sf20-1	1.203	1.031 - 1.398	11.66	22	2.359 ±0.159
	Sf20-4	11.206	9.254 - 13.654	213.85	208	1.817 ±0.130
Emamectin	*H. armigera*	0.0107	0.0087 - 0.0131	0.0945	-	2.465 ±0.164
(µg/ml diet)	Sf20-1	0.0158	0.013 - 0.019	0.124	1	2.606 ±0.175
	Sf20-4	0.0179	0.016 - 0.020	0.094	2	3.234 ±0.226
Spinetoram	*H. armigera*	0.086	0.023 - 0.157	1.977	-	1.711 ±0.173
(µg/ml diet)	Sf20-1	0.118	0.101 - 0.137	0.623	1	3.220 ±0.198
	Sf20-4	0.102	0.092 - 0.112	0.301	1	4.921 ±0.489

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750 **Table 6:** Number of *Spodoptera frugiperda* individuals (N) from populations from Australia (Kununurra SF20-4; Strathmore SF20-1), South Korea, Papua New Guinea, and
751 Peru with homozygous susceptible (S/S), heterozygous (S/R) and homozygous resistance (R/R) profiles characterised via whole genome sequence data of the ACE-1 locus
752 involving the A2001S, G227A, and F290V amino acid substitutions.

		ACE-1 (A201S)			**ACE-1 (G227A)**			**ACE-1 (F290V)**		
Population	**N**	S/S	S/R	R/R	S/S	S/R	R/R	S/S	S/R	R/R
Kununnura	9	9	0	0	9	0	0	2	4	3
Strathmore	30	26	4	0	30	0	0	0	0	30
South Korea	12	11	1	0	12	0	0	0	0	12
Papua New Guinea	17	15	2	0	17	0	0	1	12	4
Peru	16	4	12	0	10	6	0	2	14	0

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755 **Table 7:** Comparisons of bioassay results for selected insecticides fed to laboratory populations of *Spodoptera*
756 *frugiperda* via diet incorporation approaches. *S. frugiperda* populations were from the native ranges (USA,
757 Louisiana State University (LSU) culture; see Hardke et al. 2011); Brazil SUS-2005, (Kulye et al. 2021) and
758 introduced ranges from the original 2018 populations from Kanartaka, India (population I-2018; Kulye et al.
759 2021); a population from Groblersdal, Mpumalanga province, South Africa (see Discussion above, Eriksson
760 2019), and from our Sf20-1 and Sf20-4 laboratory populations from Queensland and Western Australia,
761 Australia, respectively. Potency ratios of the invasive populations were measured against the native Brazilian
762 (SUS-2005) population as reported by Kulye et al. (2021). Diet incorporation approach was for the Brazil, USA
763 (LSU), India I-2018, and Australia (Sf20-1; Sf20-4) populations.

Insecticides	**Populations**	**Range**	**LC_{50} (ppm) / EC_{50} (µg/mL)**	**Fiducial limits† (95%)**	**Slope ± SE**	**Toxicity ratios**
Spinetoram	Brazil (SUS-2005)#	native	0.010	0.009 - 0.012	2.35 ± 0.37	-
	USA (LSU)	native	0.066	0.053 - 0.081	2.54 ± 0.36	6.6
	India (I-2018)#	invasive	0.013	0.012 - 0.014	2.25 ± 0.13	1.3
	Sf20-1 (Qld)	invasive	0.118	0.101 - 0.137	3.220 ±0.198	11. 8
	Sf20-4 (WA)	invasive	0.102	0.092 - 0.112	4.921 ±0.489	10.2
Chlorantraniliprole	Brazil#	native	0.005	0.005 - 0.006	4.54 ± 0.76	-
	USA (LSU)	native	0.068	0.317 - 0.481	2.55 ± 0.23	13.6
	India (I-2018)#	invasive	0.009	0.008 - 0.009	2.39 ± 0.11	1.8
	South Africa	invasive	0.06	0.01 - 0.16	1.01 ± 0.42	12
	Sf20-1 (Qld)	invasive	0.032	0.024 - 0.043	2.484 ± 0.191	6.4
	Sf20-4 (WA)	invasive	0.163	0.132 - 0.201	2.065 ±0.152	32.6
Indoxacarb	USA (LSU)	native	0.392	0.317 - 0.481	2.35 ± 0.25	-
	Sf20-1 (Qld)	invasive	1.203	1.031 - 1.398	2.359 ±0.159	3.07
	Sf20-4 (WA)	invasive	11.206	9.254 - 13.654	1.817 ±0.130	28.6

764 **Note:** †Fiducial limits are similar to confidence limits but are based on logistic growth or S-shaped curve
765 distribution. #EC_{50} reported instead of LD_{50} by Kulye et al. (2021). In this comparison we assumed both EC_{50}
766 and LD_{50} estimates to be broadly similar.

767

768 **Table 8:** Comparisons of topically applied methomyl bioassay results on late 3rd and/or early 4th instar larvae of
769 FAW between resistant or susceptible populations from Gainesville Florida, USA (FL) (Yu 1991; see Discussion
770 above), and invasive populations from South Africa (Eriksson 2019; see Discussion above) and Queensland or
771 Western Australia, Australia.

Populations	LC_{50} (ppm)	Fiducial limits (95%)	Slope ± SE	Toxicity ratios
USA (FL susceptible)	0.57	0.45 - 0.78	4.9	-
South Africa	62.98	17.22 – 52.12	11.14 ± 9.20	110.5
Sf20-1 (Qld)	0.254	0.177 - 0.356	1.064 ±0.076	0.45
Sf20-4 (WA)	2.96	1.87 - 4.66	0.874 ±0.07	5.2
USA (FL resistance)	8.23	4.86 - 11.6	2.9	-
South Africa	62.98	17.22 – 52.12	11.14 ± 9.20	7.65
Sf20-1 (Qld)	0.254	0.177 - 0.356	1.064 ±0.076	0.031
Sf20-4 (WA)	2.96	1.87 - 4.66	0.874 ±0.07	0.36

772

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775

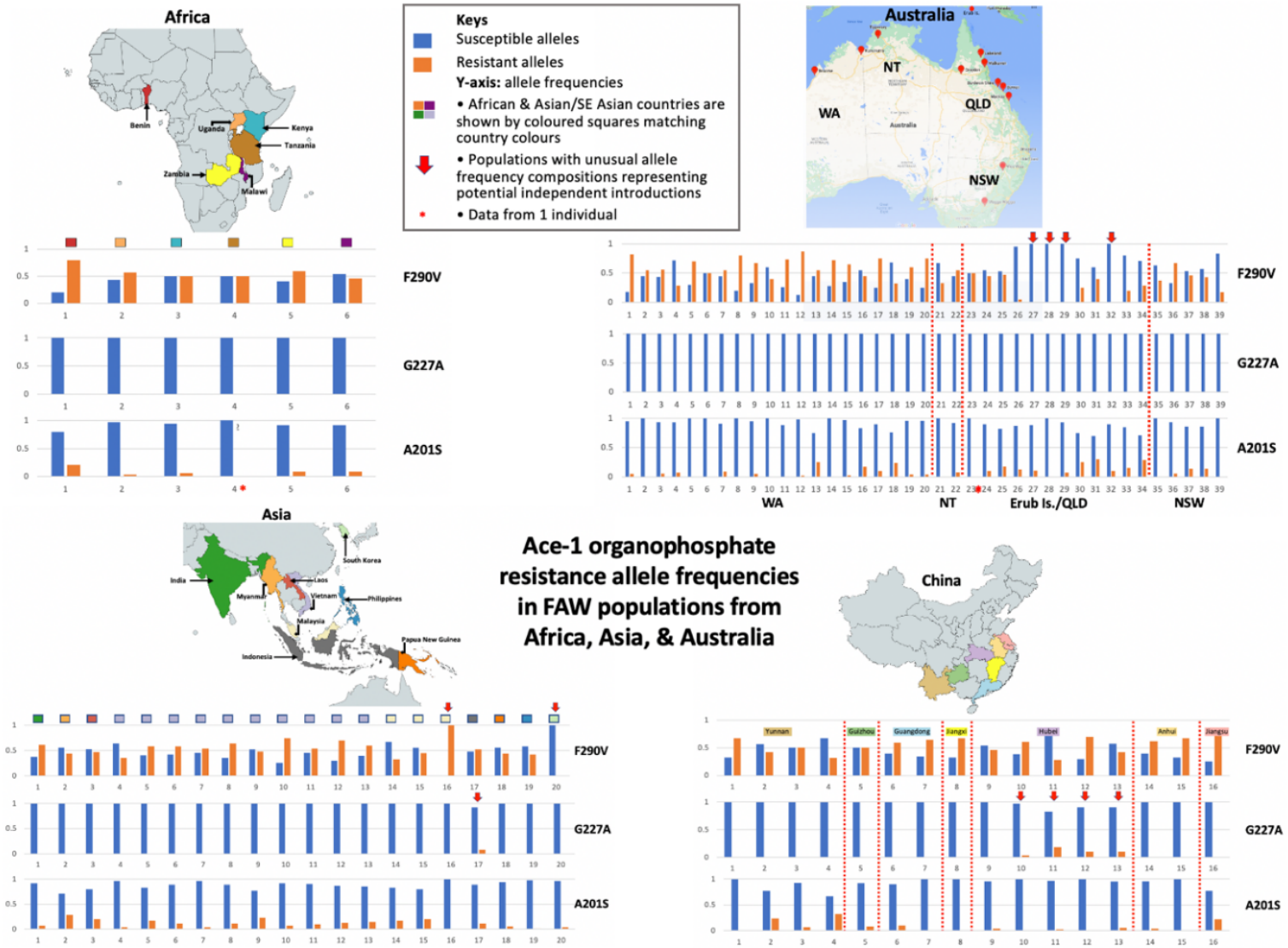
776 **Figure 1 caption**

777

778 **Figure 1:** Summary of Acytylcholinesterase (ACE-1) susceptible and resistance allele frequencies in *Spodoptera*
779 *frugiperda* populations from 17 countries (i.e., Benin, Uganda, Kenya, Tanzania, Zambia, Malawi, India,
780 Indonesia, Papua New Guinea (PNG), South Korea, China, Australia) across the invasive range. A total of 1,246
781 individuals representing 76 populations in the Table S2 were used to compile the data below. Population
782 identity is as provided in Table S2 that combined data from this study (Australia SF20-1 and SF20-4 (generation
783 0 representing field-collected individuals), PNG, South Korea) and from published studies (Boaventura et al.
784 2020a, Guan et al. 2020, Zhang et al. 2020, Zhao et al. 2020, Nguyen et al. 2021, Tay et al. 2021, Yainna et al.
785 2021, Rane et al. 2022). Susceptible and resistant alleles from the three previously reported loci (i.e., F290V,
786 G227A, A201S) from the ACE-1 gene provided evidence to support multiple independent introductions across
787 the invasive FAW populations, such as in Indonesia (#17; G227A), South Korea (#20; F290V), and China (Hubei
788 province (#10-13); G227A) as indicated by the red arrows. In Australia, newly established FAW populations
789 between Queensland (e.g., #27, #28 (Walkamin), #29 (Strathmore), #32 (Burdekin), and Western Australia (i.e.,
790 #2 (Kununurra)/Northern Territory (e.g., #21 (Bluey's Farm)) suggested this likely involved multiple
791 introductions from diverse populations from neighbouring countries and likely arrived via separate pathways
792 and entry points.

793

Figure 1


Africa
Benin
Uganda
Kenya
Tanzania
Zambia
Malawi
Keys
Susceptible alleles
Resistant alleles
Y-axis: allele frequencies
• African & Asian/SE Asian countries are shown by coloured squares matching country colours
• Populations with unusual allele frequency compositions representing potential independent introductions
• Data from 1 individual
Australia
NT
WA
QLD
NSW
F290V
G227A
A201S
WA
NT
Erub Is./QLD
NSW
Asia
India
South Korea
Laos
Myanmar
Vietnam
Philippines
Malaysia
Papua New Guinea
Indonesia
Ace-1 organophosphate resistance allele frequencies in FAW populations from Africa, Asia, & Australia
China
Yunnan
Guizhou
Guangdong
Jiangxi
Hubei
Anhui
Jiangsu