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

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Abstract

The fall armyworm (FAW) *Spodoptera frugiperda* is present in over 70 countries in Africa, Asia, and Oceania. Its rapid dispersal since 2016 when it was 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. As an urgent first response we undertook bioassays on two Australian populations, one each from these initial points of establishment. To assist with preliminary sensitivity assessment, two endemic noctuid pest species, *Helicoverpa armigera* and *Spodoptera litura*, were concurrently screened to obtain larval LC50 estimates against various insecticides. We characterised known resistance alleles from the VGSC, ACE-1, RyR, and ABCC2 genes to compare 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 that reported from 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. The preliminary bioassay results and resistance allele patterns from invasive *S. frugiperda* populations suggest multiple introductions have contributed to the pest's spread 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), *S. frugiperda* 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 *S. frugiperda* populations from various African nations, India, China, Southeast Asia, and Australia also suggested that a high proportion of *S. frugiperda* 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 *S. frugiperda* 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 *S. frugiperda* damaging maize fields in Strathmore (Qld; *ca*. 1,000 km from the initial Bamaga detection site), followed by successive southward detections of *S. frugiperda* 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, *S. frugiperda* is expected to undertake seasonal migrations and may reach as far south as Victoria and Tasmania (du Plessis et al. 2018). Indeed, *S. frugiperda* was reported in Victoria in December 2020 and in Tasmania in April, 2021. Concurrently, and from the Western Australia Kununurra detection, *S. frugiperda* 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 *S. frugiperda* 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 *S. frugiperda* 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 based on 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); a similar conclusion reached also from analysis of multiple *S. frugiperda* populations from across China based on microsatellite DNA markers (Jiang et al. 2022). Recently, population genomic analysis of *S. frugiperda* populations from SEA (i.e., Philippines, Vietnam, Laos, Malaysia, Myanmar), from East Asia (i.e., South Korea), and Pacific/Oceania (i.e., Papua New Guinea; Australia) identified significant genetic diversity in these invasive *S. frugiperda* populations, and distinct population signatures between populations in close proximity (e.g., Kedah and Penang populations from Malaysia, and between Qld, WA and NT populations in Australia (Rane et al. 2022); between populations within e.g., Yunnan province (Tay et al. 2022), and populations from Anhui and Jiangsu (Jiang et al. 2022)). Such distinct population genomic structure contradicted the expected gene flow signatures of a single introduction and a west-to-east spread of this pest. Instead, they suggested relatively limited and localised spread of populations while also identified the likely multiple independent introduction pathways of *S. frugiperda* into the region including in SEA, Asia and Australia (Rane et al. 2022).

Distinguishing among single vs. multiple introductions within Australia (and elsewhere), and understanding the resistance profiles of existing and any new incursions, is therefore 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 the first bioassay experiments and resistance gene characterisation that aimed to understand how the initial 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 horticultural, grains and cotton industries. Since populations were presumed collected before selection occurred in Australia, these responses served as the first indication of selection against insecticides and Bt toxins used elsewhere (i.e., in the species' native range and in its recent invasive ranges from Southeast Asia, Asia, and/or Africa (Schlum et al. 2021; Tay et al. 2022; Jiang et al. 2022; Rane et al. 2022)) prior to arrival. While sampling from multiple Australian regions would be ideal (but impossible at the time as our laboratory had only been provided with these two lines during the pandemic travel

111 restriction), comparing the response differences between these two *S. frugiperda* populations from different
112 northern Australia regions nevertheless could demonstrate the use of bioassay data to distinguish signatures
113 of possible multiple points of entry compared with a single entry and spread event as generally assumed (e.g.,
114 Jing et al. 2021, Qi et al. 2021). To provide a first insight into the efficacy of key compounds and toxins we
115 compared the responses of *S. frugiperda* with two related major endemic crop pests in Australia, *Helicoverpa*
116 *armigera* (subspecies *conferta;* Hardwick 1965, Anderson et al. 2016, Pearce et al. 2017b, a, Zhang et al. 2022)
117 and *S. litura,* which are currently managed through adaptive resistance management plans. Finally, we
118 provided a critical review of resistance profiles in selected invasive populations of *S. frugiperda* globally to
119 better understand its invasion biology, and consider our work alongside a subsequent more comprehensive
120 examination in Australia of insecticide resistance profiles (Bird et al. 2022). Our study is the only available
121 information for Australian populations on the vulnerability of new arrived *S. frugiperda* to relevant Bt toxins.

122 **Material and Methods**

123 **Live insects**

124 Live FAW populations were sourced from Queensland (Qld) and Western Australia (WA). This population
125 (CSIRO code: 'Sf20-1' from Qld; see Table 2) consisted of 30 field-collected pupae (of which 29 pupated and
126 emerged as adults) for setting up the laboratory colony, was first noticed around 19th Feb, 2020, was collected
127 on 3rd March, 2020, and represented the first reported FAW detected attacking maize in Australia's agricultural
128 landscape. The sampling site for this population was from the University of Queensland (UQ) field station (Rex
129 Road, Walkamin Qld 4872) Strathmore Station, (Lat/Lon: -17.17892, 145.43359, Elevation 685m).

130 A second FAW population (CSIRO code: 'Sf20-4' from WA; see Table 2) was collected as larvae from a maize
131 crop from the Northern Australian Crop Research Alliance Pty Ltd, Kununurra, Western Australia. These larvae
132 were collected on 17-Aug, 2020 from the Kununurra Airport field trail block (off Victoria Highway; -15.10090,
133 128.81342) on V4-6 growth stage maize, prior to applications of insecticides. A total of 30 larvae of different
134 instar stages arrived (= F_0), and 10 (six females and four males) survived to pupate and emerge as adult moths
135 to produce >200 F_1 individuals and to generate sufficient larvae to commence bioassays from F_4.

136 Two related endemic noctuid species, *S. litura* and *Helicoverpa armigera conferta* (hereafter '*H. armigera*'),
137 that are pests of a range of broadacre cropping and horticulture industries (and especially grains and cotton)
138 were also included in the bioassay experiments to assist with interpretating results such as base line
139 susceptibility and tolerance levels to the chemicals and toxins tested. Approximately 30 *S. litura* larvae with
140 unknown resistance profiles (from Mareeba and Toowoomba, Qld) pupated and were used to start the
141 laboratory colony. The *H. armigera* colony (CSIRO general rearing 'GR' strain) is a laboratory strain housed at
142 the Black Mountain Laboratory in the ACT that was established during the mid-1980's with individuals
143 originally collected as eggs from cotton fields around Narrabri NSW. Initially the GR colony was periodically
144 replenished to sustain population genetic diversity and minimise inbreeding but no new material has been
145 introduced for around two decades and the colony is susceptible to many chemical insecticides, including
146 organochlorines, organophosphates, carbamates and pyrethroids, as well as to the Cry1Ab, Cry1Ac, and

147 Cry2Ab Bt toxins (Pearce et al. 2017a, Pearce et al. 2017b). It therefore has tolerance levels that are not
148 dissimilar to those of a progenitor population created in 2011 that was deliberately maintained as susceptible
149 through screening (see Bird et al. 2021).The *H. armigera* GR population was confirmed by bioassays as having
150 no Cry1Ac/Cry2Ab/VIP3A resistance alleles. The field collected *S. frugiperda* strains were tested within 4
151 generations of establishment in the laboratory.

152 **Colony maintenance**

153 *Helicoverpa armigera* and *Spodoptera* species (*S. frugiperda* Sf20-1 and Sf20-4; *S. litura*) were reared by the
154 method of Teakle and Jensen (1985) at 25 °C, 50 ± 10% relative humidity, and with a light/dark cycle of 14:10
155 h. Artificial diet for *H. armigera* consisted of 81 g soya flour, 37.5 g wheat germ, 33 g brewer's yeast, 2 g
156 ascorbic acid, 2 g nipagen, 3 mL vegetable oil, and 13.5 g agar, which was processed in a microwave oven and
157 made up with water to 1 litre. Diet was poured into rearing cups or bioassay plates, allowed to cool and then
158 stored in the refrigerator (4-8°C) for no longer than 3 days. Diet for *S. frugiperda* was made to 1200mL with
159 water, and contained 100g navy bean flour, 25g soy flour, 60g wheat germ, 30g Brewer's yeast, 15g casein, 3g
160 Nipagen, 1.5g sorbic acid, 10g vitamin mixture, 4mL vegetable oil, and 18g agar. The mixture was processed in
161 a microwave oven prior to being poured into rearing cups/bioassay plates and allowed to cool as described
162 above for the *H. armigera* diet.

163

164 **Bioassays**

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

171 [Table 1]

172 All bioassays included a negative control for background mortality (i.e., to enable natural mortality rates to be
173 adjusted accordingly) which was treated with the diluent of the individual pesticide. An initial experiment
174 using 10x serial dilutions was performed to narrow the range for the detailed bioassay using 8-16 individuals
175 per dose replicated once. The full bioassay involved 2x serial dilutions at 6-7 doses with 16-24 individuals per
176 dose replicated 3-5 times. The bioassays involved diet incorporation, surface overlay, or topical application of
177 the insecticidal compounds or Bt toxins (Table 1). The selection among these exposure methods for an
178 insecticide was determined by its mode of entry (contact or ingestion) and the experience of similar bioassays
179 in related noctuid pests (i.e., *H. armigera* and *H. punctigera*; Bird 2015; Bird & Walker 2019).

180 The surface overlay of Bt toxin assays followed the approach outlined in Mahon et al. (2007b), Mahon et al.
181 (2008), Mahon et al. (2010), Mahon et al. (2012) and Walsh et al. (2014). They were conducted in 96-well trays

182 where each well contained approximately 300µl of rearing diet with a surface area of around 0.567 cm^2 per
183 well. When the diet cooled, 20µl solution containing an appropriate concentration of toxin was added and
184 allowed to air dry. One neonate was added to each well and the tray was heat sealed with a perforated lidding
185 material.

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

191 Topical bioassays of known concentrations were conducted for alpha-cypermethrin (group 3A) and methomyl
192 (group 1) and involved applying drops of pesticide as described in Bird (2018). Specifically, 1 µl of
193 acetone/insecticide solution was applied to the dorsal thorax of 3rd to 4th instar larvae (30-40g) using a 50 µl
194 micro-syringe. Bioassay trays were incubated at 25 ˚C, 45-55% RH, and a photoperiod 14:10 (L:D) h for 6 days
195 (e.g., see Bird and Akhurst 2007, Downes et al. 2009). The numbers of dead (moribund; incapable of
196 movement when prodded) and alive larvae (capable of coordinated movement when prodded) were counted
197 and the instars of surviving larvae recorded. The LC_{50} for each toxin was calculated from pooled raw data by
198 probit analysis using the POLO-PC program (LeOra Sorftware 1987).

199

200 ***Spodoptera frugiperda* specimens for genomic analyses**

201 Populations of *S. frugiperda* from South Korea (SK), Papua New Guinea (PNG), and Peru were included for
202 whole genome sequencing (Table 2). The methods for SK and PNG specimen preservation involved collection
203 from fields and placing larvae directly into high concentrations of ethanol (95-99.9%) to transport to the
204 laboratory where they were then stored at -20˚C and replaced with fresh ethanol after 24-48 hours. Peru
205 specimens were samples intercepted from Australian pre-border inspections of imported
206 agricultural/horticultural commodities between 2016 and 2019 (see Tay et al. 2020). Australian samples (Sf20-
207 1 from Qld, Sf20-4 from WA) were F_0 individuals and represented individuals obtained directly from fields.
208 Samples from SK, PNG, and Peru were sent to CSIRO and stored at -20˚C until DNA extraction.

209 **DNA extraction and genome library preparation**

210 DNA of individual *S. frugiperda* samples was extracted using the Qiagen DNA extraction kit and eluted in
211 200µL elution buffer following protocols as provided by the manufacturer (Qiagen, Hilden Germany). Genomic
212 DNA libraries for individual samples were prepared, quantified and sent for commercial sequencing by the
213 Australian Genome Research Facility (AGRF) in Melbourne, Victoria, Australia.

214 **Processing of genome sequences**

215 Genome sequencing data for individuals were trimmed to remove adapter sequences using trim_galore (v
216 0.6.6; https://www.bioinformatics.babraham.ac.uk/projects/trim_galore/) and aligned to the *S. frugiperda* rice

genome (v1.0) (Gouin et al. 2017) using bwa_mem2 (v2) (Vasimuddin et al. 2019). Duplicate alignments were removed using SAMBLASTER (v 0.1.26) (Faust and Hall 2014) and sorting completed using SAMtools (v1.9) (Li et al. 2009).

[Table 2]

Strain identification and resistance alleles characterisation by whole genome sequencing

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

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

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

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

Results

Bioassays of Individual Bt proteins

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

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

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

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

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

<u>Foliar Bt</u>

The performance against FAW of the sprayable products containing multiple Bt toxins (XenTari and DIPEL) showed both products to differ in efficacies against *H. armigera, S. frugiperda,* and *S. litura*. XenTari with the mixture of Cry1 toxins including Cry1C was formulated to provide *Spodoptera* control (valentbiosciences, last accessed 13-Jan, 2022) but also for cabbage looper and the diamondback moth (DBM), while DIPEL was formulated for broad spectrum caterpillar control (valentbiosciences, last accessed 13-Jan, 2022). Both DIPEL and XenTari are reported to contain different toxin complements but the relative amounts are unclear. DIPEL contains Cry1Ac and Cry2-type toxins and was less effective against both tested FAW populations than XenTari which contains Cry1Aa, Cry1Ab, Cry1C and Cry1D. Relative to *H. armigera, S. frugiperda* Sf20-1 was 24x less sensitive, *S. frugiperda* Sf20-4 was 18x less sensitive and *S. litura* was 17x less sensitive to Dipel. XenTari

287 toxicity ratios relative to *H. armigera* ranged 2 to 4x for *S. frugiperda* Sf20-1, *S. frugiperda* Sf20-4 and *S. litura*
288 (Figs. S5 and S6; Table 3).

289 Conventional pesticides

290 Cypermethrin is a pyrethroid pesticide and the bioassay results suggest that *S. frugiperda* (i.e., the Sf20-1 and
291 Sf20-4 laboratory cultures) is less sensitive to it than the laboratory strain of *H. armigera* (56x and 145x
292 respectively: Fig. S7; Table 4).

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

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

300 The dose response and LC_{50} for chlorantraniliprole show similar sensitivity for *H. armigera* and *S. frugiperda*
301 Sf20-1, with the latter showing a 3x difference in the LC_{50}. Interestingly, the *S. frugiperda* Sf20-4 exhibited a
302 15x reduction in sensitivity compared to *H. armigera* (Fig. S10; Table 5), and a 5x response variation to
303 chlorantraniliprole when compared to the Sf20-1 line.

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

307 [Table 3, Table 4, Table 5]

308 **Resistance alleles by whole genome sequencing**

309 The loci examined for potential resistance alleles were present in the data set with the appropriate level of
310 coverage to accurately call the genotype. Resistance alleles to carbamate/organophosphates (ACE-1) were the
311 only resistance alleles identified in the Kununnura, Strathmore, South Korea, Papua New Guinea, and Peru
312 populations. No resistance alleles associated with target site mutations were detected for pyrethroid (VGSC) or
313 for diamide insecticides (RyR), although there are likely other genes (e.g., detoxification genes) that confer
314 resistance to synthetic pyrethroid in these laboratory lines of *S. frugiperda* (Bird et al. 2022). Resistance allele
315 profile differences between the two Australian populations were evident between the A201S and the F290V
316 amino acid substitutions. Similar ACE-1 resistance allele profiles were detected between Strathmore and South
317 Korean populations, while the Papua New Guinea population shared ACE-1 allele profiles with Strathmore
318 population (A201S) and with Kununnura (F290V). The Peruvian FAW population was the only population that
319 have heterozygous individuals with resistance allele for G227A amino acid substitution (Table 6).

320 [Table 6]

321 Resistance allele characterisation by sequencing approaches from this study and published studies for the ACE-
322 1 gene is summarised in (Fig. 1, Table S2 and references therein). The most common resistance allele detected
323 in the invasive and native population (456 individuals examined in total) was the F290V mutation with 66
324 homozygous and 222 heterozygous resistant genotypes detected. This mutation is a T to G single nucleotide
325 polymorphism (SNP) that changes the codon encoding the amino acid from TTT to GTT leading to a
326 phenylalanine (F) to valine (V) change in the protein sequence encoded by the ACE-1 gene. This mutation was
327 present at all locations and evenly distributed between the invasive and native populations. In Australia, of the
328 sequenced individuals (n = 146), 24.7% (n = 36) were heterozygous, and 8% (n = 12) were homozygous for the
329 resistance allele. Heterozygous and homozygous individuals were found in populations from all four states
330 (i.e., WA, NT, Queensland, and NSW) as well as from the single individual from Erub Island, suggesting it is
331 common across Australia.

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

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

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

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

354 None of the known and validated Cry1 resistance ABCC2 mutations were identified in the individuals
355 sequenced for this work however, some variation from the reference was observed (Fig. S13). Numerous
356 synonymous mutations and a total of 31 non-synonymous mutations were observed in the coding sequence of

357 the ABCC2 gene in all sequenced individuals. Several of these non-synonymous mutations were associated
358 with a common deletion and insertion motif in the first exon where an 11bp deletion and a 2 bp insertion
359 maintain the reading frame but replace and change several amino acids. Most of the other mutations are the
360 result of one (i.e., single nucleotide variant; SNV) or two (i.e., multiple nucleotide variant; MNV) nucleotide
361 changes. Several of these mutations are found in other assemblies of *S. frugiperda* and likely reflect natural
362 variation. An alternative explanation for the variation is that they are associated with the c-strain which is
363 thought to make up at least some of the genome of the invasive populations. Only one mutation, a 2 bp
364 deletion in one individual collected from the Burdekin in Queensland was identified that might cause a frame
365 shift mutation (see Supplemental Figures S14 and S15) as has been identified in Bt resistant individuals in
366 other studies. It was present as a heterozygote in the individual and has not been experimentally validated.

367 [Figure 1]

368 **Discussion**

369 In this study, we showed insecticide and Bt response differences in two of the first reported *S. frugiperda*
370 populations in Queensland (i.e., SF20-1) and Western Australia (Sf20-4) in Australia, with the Queensland
371 (Sf20-1) population being less tolerant to various insecticides such as methomyl, chlorantraniliprole, and
372 indoxacarb compared to the Western Australian (Sf20-4) population. On the other hand, the Queensland
373 population exhibited at least a two-fold higher tolerance to Cry1Ac, Cry2Ab, Cry1F and Vip3A Bt toxins than
374 the WA population. Characterisation of the ABCC2 resistance gene identified one *S. frugiperda* individual from
375 Queensland as potentially being heterozygous with a 2 bp deletion that could underpin Cry1F resistance,
376 although confirmation of the detected mutation and of the resistance phenotype is required. This could be
377 accomplished via PCR and Sanger sequencing (e.g., Guan et al. 2020) as well as via the CRISPR/Cas9 gene
378 editing approach (e.g., see Wang et al. 2017). The response differences to chemical insecticides and Bt toxins
379 of the two studied invasive *S. frugiperda* populations in Australia and their ACE-1 resistance allele profile
380 differences (Table 6) may reflect the diverse genetic composition across the pest's recent expanding range
381 (Tay et al. 2020, Schlum et al. 2021, Rane et al. 2022), and suggest that separate pathways were involved in
382 establishment of these Queensland and Western Australian populations. This is contrary to the current
383 postulation of a single introduction pathway for the arrival of *S. frugiperda* to Australia based on an
384 assumption (Jing et al. 2021) or reverse trajectory simulation (Qi et al. 2021) and highlights the importance of
385 harmonising simulation studies with genomic and phenotypic evidence.

386 With significant economic impacts on agriculture from *S. frugiperda* reported in over 80 countries (excluding
387 the New World native range) from Africa, Middle East, Asia, Southeast Asia, and Oceania, its response to
388 different insecticidal and Bt control agents is increasingly investigated at recently impacted localities (e.g.,
389 Worku and Ebabuye 2019, Deshmukh et al. 2020, Zhang et al. 2020, Kulye et al. 2021, Lv et al. 2021). However,
390 the diverse bioassay methods (approach, larval stage, scoring criteria) used in these studies complicate
391 meaningfully comparison of findings. Taking emamectin benzoate and indoxacarb as examples, Zhang et al.
392 (2022) used a topical application bioassay on 3rd instar larvae, Deshmukh et al. (2020) used leaf-dip bioassays
393 on 2nd instar larvae, while Hardke et al. (2011) used a diet-incorporation approach on 3rd instar larvae.

394 Responses have been expressed as LD_{50} (e.g., Deshmukh et al. 2020) or as EC_{50} (e.g., Kulye et al. 2021), further
395 making it difficult to rigorously compare outcomes.

396 It is important to stress the challenge and difficulty to compare bioassay results between studies due to the
397 different genetic background of the test samples (e.g., due to different number of individuals used to establish
398 test populations), methods and approaches between research groups, and the general different rearing
399 conditions of laboratory cultures that could contribute to varied response outcomes. A further challenge for
400 invasive *S. frugiperda* management is the lack of base-line values representing susceptible responses in newly
401 populated areas which makes it difficult to monitor changes through time in insecticide efficacy due to
402 resistance. Knowledge of whether the introduction occurred once (e.g., single 'invasive bridgehead effect';
403 Guillemaud et al. 2011) vs. multiple times (e.g., multiple 'mass dispersal'; Wilson et al. 2009) and patterns of
404 gene flow also impact the long-term monitoring of insecticide resistance evolution. Using native *S. frugiperda*
405 colonies established in 2005 from cotton fields in Louisiana, USA, Wilson et al. (2009) undertook diet-
406 incorporation bioassays (on 3rd instar larvae), using insecticides including chlorantraniliprole, indoxacarb, and
407 spinetoram (see Table 7). Yu (1991) undertook topical application bioassays against methomyl (on 4th instar
408 larvae) which included comparison with a susceptible population free from insecticide exposure since 1975,
409 and a resistant population collected from a maize field in Gainesville, Florida (see Table 8). In comparing
410 insecticide responses in Indian invasive *S. frugiperda* populations with diet incorporation assays (on 3rd instar
411 larvae), Yu (1991) also included a native susceptible *S, frugiperda* population from Brazil to assist with
412 interpreting changes to insecticide responses (including chlorantraniliprole and spinetoram) at spatial and
413 temporal scales.

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

428 Methomyl resistance alleles have been reported in invasive populations from China, Indonesia, Africa, and in
429 this study. Comparisons between susceptible and resistant strains of native *S. frugiperda* populations from
430 Florida USA (Yu 1991) and with the South African invasive population of *S. frugiperda* (Eriksson 2019) showed

431 that the Queensland population was similar in its response as the susceptible Florida *S. frugiperda*, while the
432 Western Australia population was around 5 times more tolerant. This contrasted the South African population
433 which was 110 times more tolerant than the Florida susceptible *S. frugiperda* strain, and around 8 times more
434 tolerant than the resistant *S. frugiperda* strain from Florida (Table 8). Taken as a whole, similar bioassay results
435 between native (i.e., Brazil, USA) and various invasive populations suggested potential significant genetic
436 diversity in introduced populations. This concurs with population genomic and genetic analyses (Tay et al.
437 2020, Zhang et al. 2020, Schlum et al. 2021, Rane et al. 2022, Jiang et al. 2022) that suggested multiple origins
438 for the invasive African, Asian (Indian, Chinese), and Southeast Asian (e.g., Malaysia) FAW populations.

439 It is possible that the differential response between the two Australian *S. frugiperda* populations to
440 insecticides and Bt toxins represents natural variation in the national population which has emerged from a
441 single founding incursion. However, it is unlikely that the differential responses within Australian populations
442 represents local selection pressures because the period between populations establishing and being collected
443 for this study was presumed to be too short to enable this opportunity. It could be that separate incursions
444 involving different source populations occurred in WA compared to the eastern states of Australia, as
445 supported by genome-wide SNP marker population genomic studies (Rane et al. 2022). Different selection
446 pressures on the global population which recently originated from multiple Asian, Southeast Asian, and African
447 incursions (Tay et al. 2020, Zhang et al. 2020, Schlum et al. 2021, Rane et al. 2022) may have driven different
448 phenotypes which entered the expanding ranges tested in our study and others reported herein. However,
449 Kulye et al. (2021) demonstrated in *S. frugiperda* populations collected in India during 2018, 2019, and 2020
450 that large response changes such as those observed in South African (e.g., methomyl; Eriksson 2019) and
451 Western Australian (chlorantraniliprole, indoxacarb; this study) populations were unlikely considering the
452 short time frame since the very recent arrival of *S. frugiperda* especially assuming a west-to-east spread
453 (Goergen et al. 2016, Cock et al. 2017, Nagoshi et al. 2018, Nagoshi et al. 2019b). Another explanation for the
454 differential responses of the two Australian populations that we studied is that they have different fitness
455 because of variation in genetic diversity which reflects the number of individuals founding the populations.
456 However, if this was the case one might expect sensitivity levels to be consistently higher or lower to all of the
457 agents that we tested but this was not the case.

458 [Table 7, Table 8]

459 Alien invasive agricultural pests are increasingly being shown to carry novel insecticide resistance genes (e.g.,
460 Anderson et al. 2018, Walsh et al. 2018, Tay and Gordon 2019). This includes *S. frugiperda* in which invasive
461 populations have been confirmed via whole genome sequence analyses (e.g., Guan et al. 2020, Zhang et al.
462 2020, Yainna et al. 2021) and molecular characterisation to harbour selected resistance genes (Boaventura et
463 al. 2020b, Boaventura et al. 2020a, Zhao et al. 2020). Our review of reported ACE-1 and VGSC resistance allele
464 frequency differences (Fig, 1; Table S2 and references therein) suggests that the invasive *S. frugiperda*
465 populations within China (Guo et al. 2020), Indonesia (Boaventura et al. 2020a), Malaysia and Queensland,
466 Australia (Tay et al. 2021, Rane et al. 2022), and South Korea (this study) were genetically diverse and likely

originated from different native populations (Fig, 1). This further supports the perceived rapid spread of *S. frugiperda* across Africa, Asia, and Oceania as likely to also involve multiple independent introduction events.

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

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

be co-ordinated to prevent and to increase the chances of early detection of such novel introductions of new traits.

Novel introductions leading to unique population structure has been reported for western African *S. frugiperda* (Nagoshi et al. 2022), in populations from China (Jiang et al. 2022), in Africa (e.g., Benin vs. Malawi; Tay et al. 2022), and in, e.g., Australia, Malaysia, and Myanmar vs. China populations (Rane et al. 2022), suggesting that the widely anticipated long distance migration of *S. frugiperda* especially in the invasive range could likely be less and may be impacted by localised ecological and climatic determinants. Factors impacting the founding for the two Australian populations (e.g., founding number (i.e., involving one/few individuals vs. many); gene flow dynamics at spatial and temporal scales, and frequencies and impact from potential novel and on-going introductions) used in our bioassay studies is at present unknown. While bioassays testing of a larger population pooled from several sites or several individual populations from multiple sites would be especially relevant in a pest capable of high dispersal across the landscape, however the spread of *S. frugiperda* in the invasive range is increasingly being recognised as may not be as rapid and widespread as originally believed (e.g., Naghoshi et al. 2022; Rane et al. 2022; Tay et al. 2022; Jiang et al. 2022). As such, we advocate cautionary approaches to avoid premature assumptions that extensive population admixture via gene flow in Australia landscape would lead to homogenised populations and therefore the approach to combine multiple distantly sampled populations in bioassay studies (e.g., Bird et al. 2022).

Finally, to enable meaningful comparisons of bioassay findings from across the *S. frugiperda* invasive range, there is a need to globally standardise approaches for testing of insecticides and Bt toxins. Localised novel resistance traits in populations of *S. frugiperda* would likely serve as a new resistance management challenge to other neighbouring regions (Kalyebi 2020), and movements of *S. frugiperda* in the new invasive ranges could lead to as yet unknown and complex gene flow patterns that could significantly hinder the development of suitable pest and resistance management strategies for this global pest complex.

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Table 1: List of insecticides and Bt toxins used in the bioassay experiments. All insecticides and Bt toxins were tested on two populations of *Spodoptera frugiperda* from Queensland (Sf20-1, from Walkamin) and Western Australia (Sf20-4, from Kununurra). Bioassays involved either: (i) diet incorporation (DI); (ii) Topical application (TA); or (iii) Surface treatment (ST) of diet to be consumed. The larval stages used in the bioassays are specified. For topical application we used larvae from late 3rd to early 4th instar with each larva weighing approximately 30mg. Species tested were: *Spodoptera frugiperda, Helicoverpa armigera,* and *Spodoptera litura*.

Insecticides/Bt toxins	Commercial name	Date	Approach	Larval stage	Species tested		
					S. frugiperda	***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

Note: Commercial names of insecticides/Bt toxins, their manufactured or expiry dates are provided where available. † likely manufactured recently as this was requested from Syngenta Australia Pty Ltd and provided directly for the bioassay purpose. N/A: not available. These Bt toxins were produced and purified from *B.*

803 *thuringiensis* cultures (Cry1Ac and Cry2Ab) or cloned into vectors in *Escherichia coli* (Cry1F and Vip3A) and
804 grown at the CSIRO Black Mountain Laboratories.

805 **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
806 sequencing for resistance gene characterisation. Number of specimens from each location is indicated by 'N'. Where available, GPS co-ordinates and elevation are
807 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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810 **Table 3:** Summary bioassay data on *Spodoptera frugiperda* populations from Walkamin, Queensland (Sf20-1) and Kununurra, Western Australia (Sf20-4), *S. litura*
811 (Mareeba) and *Helicoverpa armigera* (GR laboratory line) involving surface treatment of the diet with Bt toxins and products.

Pesticide	Populations/species	N	Slope	LC_{50}	95% CI	LC_{99}	95% CI	χ2	Toxicity ratio (*H. armigera* = 1)
Cry1Ac	*H. armigera*	741	1.837 ±0.126	0.025	0.021 - 0.029	0.465	0.316 - 0.773	31.66	-
(µg/cm^2)	Sf20-1	504	0.827 ±0.102	4.34	1.81 - 8.15	80.6	36.85 - 249.17	18.38	174
	Sf20-4	575	1.449 ±0.149	2.48	1.79 - 3.29	85.34	53.32 - 159.68	17.77	99
	S. litura	501	1.626 ±0.135	2.99	2.06 - 4.20	80.7	38.60 – 284.54	49.14	120
Cry2Ab	*H. armigera*	476	1.501 ±0.143	0.049	0.034 -0.068	1.74	0.86 - 6.57	31.834	-
(µg/cm^2)	Sf20-1	575	1.649 ±0.136	0.655	0.435 - 0.951	16.88	7.24.- 86.01	86.222	13
	Sf20-4	551	2.201 ±0.246	0.178	0.138 - 0.221	2.03	1.33 - 4.94	39.076	4
	S. litura	859	1.197 ±0.081	0.511	0.335 - 0.734	44.9	20.179 - 154.39	91.946	10
Cry1F	Sf20-1	576	1.531 ±0.115	0.025	0.015 - 0.038	0.838	0.365 - 3.949	19.947	-
(µl/cm ^{2})	Sf20-4	575	1.809 ±0.149	0.021	0.014 - 0.029	0.394	0.211 - 1.138	87.309	-
	S. litura	574	1.524 ±0.121	0.0088	0.0069 - 0.011	0.294	0.187 - 0.535	58.968	-
VIP3a	*H. armigera*	763	1.486 ±0.093	0.0062	0.0051 - 0.0075	0.230	0.140 - 0.400	33.738	-
(µl/cm ^{2})	Sf20-1	619	1.881 ±0.225	0.0021	0.0010 - 0.0031	0.049	0.021 - 0.257	68.846	0.152
	Sf20-4	599	2.398 ±0.212	0.0019	0.0016 - 0.0023	0.018	0.013 - 0.028	20.934	0.078

	S. litura	575	1.693 ±0.129	0.00065	0.0005 - 0.0008	0.015	0.088 - 0.035	39.108	0.065
Dipel	*H. armigera*	526	1.832 ±0.154	2.11	1.59 - 2.72	39.2	22.43 - 92.76	33.878	-
(IU/cm^2)	Sf20-1	644	1.832 ±0.154	52.02	36.55 - 70.05	2612.6	1379.89 - 6752.99	29.258	24
	Sf20-4	574	1.703 ±0.153	38.93	23.97 - 56.35	904.1	469.80 - 2779.63	53.767	18
	S. litura	788	1.426 ±0.098	35.96	28.23 - 44.99	1540.8	916.66 - 3110.88	40.916	17
XenTari	*H. armigera*	620	1.530 ±0.136	5.95	4.69 - 7.40	197.2	118.7 - 398.3	23.06	-
(DBM/cm^2)	Sf20-1	647	2.375 ±0.289	11.81	8.33 - 15.17	112.7	69.8 - 270.1	39.18	2
	Sf20-4	549	1.333 ±0.180	19.01	9.27 - 29.8	1058.9	379.5 - 10739.7	43.25	3
	S. litura	741	2.375 ±0.289	24.86	17.08 - 31.14	206.9	126.2 - 597.5	47.92	4

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

Toxin	**Population/Species**	**N**	**Slope**	**LC_{50}**	**95% C.I.**	**LC_{99}**	**95%CI**	**χ2**	**Toxicity ratio (*H. armigera* = 1)**
Alpha cypermethrin	*H. armigera*	1328	2.849 ±0.140	0.0036	0.0032 - 0.0041	0.023	0.018 - 0.032	109.84	-
µg/larvae	Sf20-1	675	2.399 ±0.154	0.201	0.171 - 0.239	1.88	1.31 - 3.06	43.903	56
	Sf20-4	766	2.186 ±0.132	0.523	0.427 - 0.641	6.06	3.99 - 10.85	67.208	145
Methomyl	*H. armigera*	858	0.809 ±0.059	0.057	0.031 - 0.097	43.12	10.27 - 53.93	135.89	-
µg/larvae	Sf20-1	765	1.064 ±0.076	0.254	0.177 - 0.356	39.2	15.81 - 156.30	72.309	4
	Sf20-4	631	0.874 ±0.07	2.96	1.87 - 4.66	1363.7	380.11 - 10950.47	57.160	52

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

Pesticide	Population/Species	N	Slope	LC_{50}	95% CI	LC_{99}	95%CI	χ2	Toxicity ratio (*H. armigera* = 1)
Chlorantraniliprole	*H. armigera*	540	3.199 ±0.234	0.011	0.009 -0.013	0.056	0.038 - 0.109	72.27	-
(µg/ml diet)	Sf20-1	613	2.484 ±0.191	0.032	0.024 - 0.043	0.28	0.162 - 0.777	99.976	3
	Sf20-4	897	2.065 ±0.152	0.163	0.132 - 0.201	2.19	1.40 - 4.10	99.895	15
Indoxacarb	*H. armigera*	653	1.684 ±0.122	0.054	0.028 - 0.089	1.29	0.49 - 12.59	226.42	-
(µg/ml diet)	Sf20-1	697	2.359 ±0.159	1.203	1.031 - 1.398	11.66	8.50 - 17.62	34.789	22
	Sf20-4	541	1.817 ±0.130	11.206	9.254 - 13.654	213.85	136.20 - 391.41	24.511	208
Emamectin	*H. armigera*	810	2.465 ±0.164	0.0107	0.0087 - 0.0131	0.0945	0.065 - 1.607	75.940	-
(µg/ml diet)	Sf20-1	631	2.606 ±0.175	0.0158	0.013 - 0.019	0.124	0.082 - 2.292	67.73	1
	Sf20-4	720	3.234 ±0.226	0.0179	0.016 - 0.020	0.094	0.723 - 1.331	40.00	2
Spinetoram	*H. armigera*	472	1.711 ±0.173	0.086	0.023 - 0.157	1.977	0.686 - 8.409	154.67	-
(µg/ml diet)	Sf20-1	831	3.220 ±0.198	0.118	0.101 - 0.137	0.623	0.457 - 0.990	94.168	1
	Sf20-4	542	4.921 ±0.489	0.102	0.092 - 0.112	0.301	0.247 - 0.400	13.119	1

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Table 6: Number of *Spodoptera frugiperda* individuals (N) from populations from Australia (Kununurra SF20-4; Strathmore SF20-1), South Korea, Papua New Guinea, and 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 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

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

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

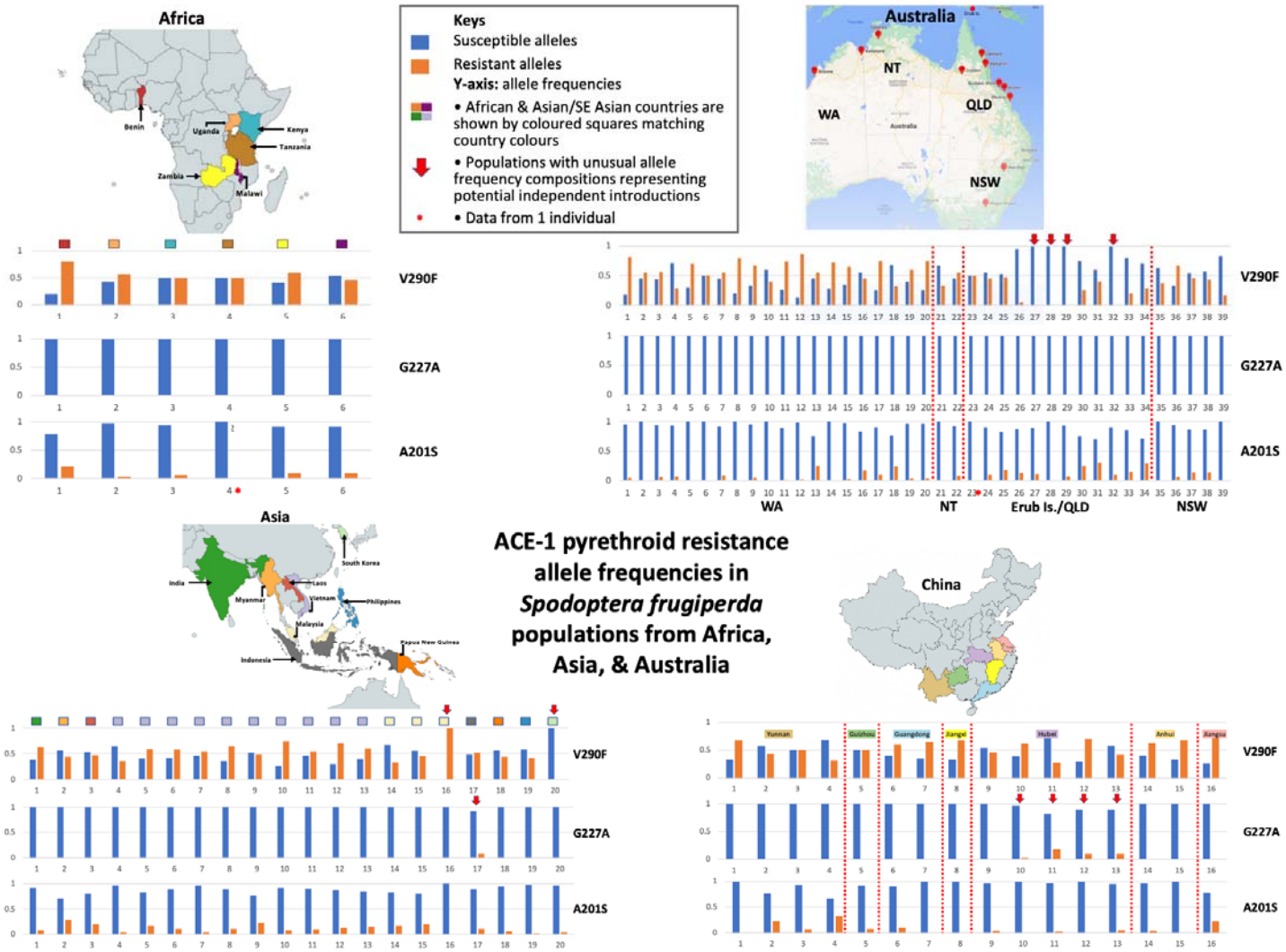
Table 8: Comparisons of topically applied methomyl bioassay results on late 3rd and/or early 4th instar larvae of *S. frugiperda* between resistant or susceptible populations from Gainesville Florida, USA (FL) (Yu 1991; see Discussion above), and invasive populations from South Africa (Eriksson 2019;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

Figure 1 Caption

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

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
V290F
G227A
A201S
WA
NT
Erub Is./QLD
NSW
Asia
India
South Korea
Laos
Myanmar
Vietnam
Philippines
Malaysia
Papua New Guinea
Indonesia
ACE-1 pyrethroid resistance allele frequencies in *Spodoptera frugiperda* populations from Africa, Asia, & Australia
China
Yunnan
Guizhou
Guangdong
Jiangxi
Hubei
Anhui
Jiangsu