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Special Collection: World-Scale Ecology and Management of Fall Armyworm (*Spodoptera frugiperda*)

Resistance Bioassays and Allele Characterization Inform Analysis of *Spodoptera frugiperda* (Lepidoptera: Noctuidae) Introduction Pathways in Asia and Australia

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Abstract

The fall armyworm (FAW) *Spodoptera frugiperda* (Smith; Lepidoptera: Noctuidae) 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* (Hübner; Lepidoptera, Noctuidae) and *Spodoptera litura* (Fabricius; Lepidoptera, Noctuidae), were concurrently screened to obtain larval LC50 estimates against various insecticides. We characterized 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 10× LC50 difference for indoxacarb was detected between Australian populations, which was approximately 28× higher than that reported from an Indian population. Characterization of ACE-1 and VGSC alleles provided further evidence of multiple introductions in Asia, and multiple pathways involving genetically distinct individuals in 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.

Key words: FAW, whole genome sequencing, agricultural biosecurity, population genomics, invasion biology

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). Recognized 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, Xiao et al. 2020) its potential to contaminate certain commodities, and the 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; Zhang et al. 2020; Schlum et al. 2021; Tay et al. 2021, 2022a; 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 pest'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 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 subcontinent, 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 partial 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 (Zhang et al. 2020; Schlum et al. 2021; Tay et al. 2021, 2022a); 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. 2022a), 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 localized 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 versus 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 prioritize 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. 2021) 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 characterization 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, Jiang et al. 2022, Rane et al. 2022, Tay et al. 2022a)] before 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 restriction), comparing the response differences between these two *S. frugiperda* populations from different northern Australia regions nevertheless could demonstrate the use of bioassay data to distinguish signatures of possible multiple points of entry compared with 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 *S. frugiperda* with two related major endemic crop pests in Australia, *Helicoverpa armigera* (subspecies *conferta;* Hardwick 1965; Anderson et al. 2016; Pearce et al. 2017a,b; 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 of *S. frugiperda* globally to better understand its invasion biology, and consider our work alongside a subsequent more comprehensive examination in Australia of insecticide resistance profiles (Bird et al. 2022). Our study is the only available information for Australian populations on the vulnerability of new arrived *S. frugiperda* to relevant Bt toxins.

Material and Methods

Live Insects

Live *S. frugiperda* populations were sourced from Queensland (Qld) and Western Australia (WA). This population (CSIRO code:

'Sf20-1' from Qld; see Table 2) consisted of 30 field-collected pupae (of which 29 pupated and emerged as adults) for setting up the laboratory colony, was first noticed around 19th February 2020, was collected on 3rd March, 2020, and represented the first reported *S. frugiperda* detected attacking maize in Australia's agricultural landscape. The sampling site for this population was from the University of Queensland (UQ) field station (Rex Road, Walkamin Qld 4872) Strathmore Station, (Lat/Lon: –17.17892, 145.43359, Elevation 685 m).

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

Two related endemic noctuid species, *S. litura* and *Helicoverpa armigera conferta* (hereafter '*H. armigera*'), that are pests of a range of broadacre cropping and horticulture industries (and especially grains and cotton) were also included in the bioassay experiments to assist with interpreting results such as base line susceptibility and tolerance levels to the chemicals and toxins tested. Approximately 30 *S. litura* larvae with unknown resistance profiles (from Mareeba and Toowoomba, Qld) pupated and were used to start the laboratory colony. The *H. armigera* colony (CSIRO general rearing 'GR' strain) is a laboratory strain housed at the Black Mountain Laboratory in the ACT that was established during the mid-1980's with individuals originally collected as eggs from cotton fields around Narrabri NSW. Initially, the GR colony was periodically replenished to sustain population genetic diversity and minimize inbreeding but no new material has been introduced for around two decades and the colony is susceptible to many chemical insecticides, including organochlorines, organophosphates, carbamates, and pyrethroids, as well as to the Cry1Ab, Cry1Ac, and Cry2Ab Bt toxins (Pearce et al. 2017a,b). It, therefore, has tolerance levels that are not dissimilar to those of a progenitor population created in 2011 that was deliberately maintained as susceptible through screening (see Bird et al. 2022). The *H. armigera* GR population was confirmed by bioassays as having no Cry1Ac/Cry2Ab/VIP3A resistance alleles. The field collected *S. frugiperda* strains were tested within 4 generations of establishment in the laboratory.

Colony Maintenance

Helicoverpa armigera and *Spodoptera* species (*S. frugiperda* Sf20-1 and Sf20-4; *S. litura*) were reared by the method of Teakle and Jensen (1985) at 25°C, 50 ± 10% relative humidity, and with a light/dark cycle of 14:10 hr. Artificial diet for *H. armigera* consisted of 81 g soya flour, 37.5 g wheat germ, 33 g brewer's yeast, 2 g ascorbic acid, 2 g nipagen, 3 ml vegetable oil, and 13.5 g agar, which was processed in a microwave oven and made up with water to 1 litre. Diet was poured into rearing cups or bioassay plates, allowed to cool, and then stored in the refrigerator (4-8°C) for no longer than 3 d. Diet for *S. frugiperda* was made to 1,200 ml with water, and contained 100 g navy bean flour, 25 g soy flour, 60 g wheat germ, 30 g Brewer's yeast, 15 g casein, 3 g Nipagen, 1.5 g sorbic acid, 10 g vitamin mixture, 4 ml vegetable oil, and 18 g agar. The mixture was processed in a microwave oven before being poured into rearing cups/bioassay plates and allowed to cool as described above for the *H. armigera* diet.

Bioassays

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

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

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

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

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

Spodoptera frugiperda Specimens for Genomic Analyses

Populations of *S. frugiperda* from South Korea (SK), Papua New Guinea (PNG), and Peru were included for whole genome sequencing (Table 2). The methods for SK and PNG specimen preservation involved collection from fields and placing larvae directly into high concentrations of ethanol (95–99.9%) to transport to the laboratory where they were then stored at –20°C and replaced with fresh

Table 1. List of insecticides and Bt toxins used in the bioassay experiments

Insecticides/Bt toxins	Commercial name	Date	Approach	Larval stage	Species tested S. frugiperda	H. armigera	S. litura
Chlorantraniliprole (200 g/liter)	Coragen	Manufactured: 28 June 2019	DI	2nd/early 3rd	Yes	Yes	No
Indoxacarb (150 g/liter)	Steward 150	Manufactured: 30 Mar. 2019	DI	2nd/early 3rd	Yes	Yes	No
Emamectin (17 g/liter)	Affirm 017 EC	Unspecified[a]	DI	2nd/early 3rd	Yes	Yes	No
Spinetoram (120 g/liter)	Dow AgroSciences Success Neo	Manufactured: Feb 2018	DI	2nd/early 3rd	Yes	Yes	No
Alpha cypermethrin (100 g/liter)	Dominex Duo	Manufactured: 30 April 2019	TA	Late 3rd/early 4th	Yes	Yes	No
Methomyl		Expiry date: Feb 2022	TA	Late 3rd/early 4th	Yes	Yes	No
Advertised as: Cry2A, Cry2B, Cry1Ab, Cry1Aa, Cry1Ac	Yates Nature's Way DIPEL	Expiry date: 27 May 2022	ST	Neonates	Yes	Yes	Yes
Advertised as: Cry1D, Cry1C, Cry1Ab, Cry1Aa	XenTari WG Bta	Manufactured: 05 Feb 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

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: (1) diet incorporation (DI); (2) Topical application (TA); or (3) 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 30 mg. Species tested were: *Spodoptera frugiperda*, *Helicoverpa armigera*, and *Spodoptera litura*. Commercial names of insecticides/Bt toxins, their manufactured or expiry dates are provided where available. N/A: not available. These Bt toxins were produced and purified directly for the bioassay purpose. N/A: not available. These Bt toxins were produced and purified from *B. thuringiensis* cultures (Cry1Ac and Cry2Ab) or cloned into vectors in *Escherichia coli* (Cry1F and Vip3A) and grown at the CSIRO Black Mountain Laboratories.

[a]Likely manufactured recently as this was requested from Syngenta Australia Pty Ltd and provided directly for the bioassay purpose.

Table 2. *Spodoptera frugiperda* samples from Australia, Papua New Guinea, South Korea, and Peru used in whole genome sequencing for resistance gene characterization

Country	State/Province	Population	N	Date	Note	R: C
Australia	WA	Kununurra	9	16 Aug. 2020	'Sf20-4', represent the original WA field-collected individuals	9: 0
	Qld	Strathmore Station, Walkamin	29	03 Mar. 2020	'Sf20-1', represent the original Qld field-collected individuals	29: 0
South Korea	Milyang	MF	7	Feb. 2020	Milyang-si, Gyeongsangnam-do, G_4 lab; GPS: 35°29.29 N, 128°44.31 E	7: 0
	Goryong	GR	1	Sept. 2019	Goryeong-gun, Gyeongsangbuk-do, Corn-field; GPS: 35° 64.04 N, 128°39.08 E	1: 0
	Haenam	MN	1	Aug. 2019	Haenam-gun, Jeollanam-do, Corn field; GPS: 34°24.38 N, 126°37.55 E	1: 0
	Milyang	MY	2	Sept. 2019	Milyang-si, Gyeongsangnam-do, Corn field; GPS: 35°29.29 N, 128°44.31 E	0: 2
	Muan	MA	1	Aug. 2019	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 June 2020	5°58.154 S, 145°53.252 E, Maize host	16: 0
	Central Province	Yule Island Junction	1	5 June 2020	corn host	1: 0
Peru	n/a	Pre-border intercepted specimens on imported agricultural commodities	16	2016–2019	Samples from Tay et al. (2022a) (Supp Table 1 [online only], individuals PE01–PE16)	0: 16

Australian *S. frugiperda* populations were Sf20-1 from Qld and Sf20-4 from WA. Number of specimens from each location is indicated by 'N'. Where available, GPS coordinates and elevation are provided. The number of individuals belonging to the *S. frugiperda* R-strain or the C-strain mitochondrial DNA COI haplotypes is also provided.

ethanol after 24–48 hr. Peru specimens were samples intercepted from Australian pre-border inspections of imported agricultural/horticultural commodities between 2016 and 2019 (see Tay et al. 2021, 2022a). Australian samples (Sf20-1 from Qld, Sf20-4 from WA) were F_0 individuals and represented individuals obtained directly from fields. Samples from SK, PNG, and Peru were sent to CSIRO and stored at –20°C until DNA extraction.

DNA Extraction and Genome Library Preparation

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

Processing of Genome Sequences

Genome sequencing data for individuals were trimmed to remove adapter sequences using trim_galore (v 0.6.6; https://www.bioin-formatics.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 was completed using SAMtools (v1.9) (Li et al. 2009).

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 a 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 Supp Table 1 [online only]), 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 'nonvariant' 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*: (1) A201S, (2) G227A, and (3) 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) and invasive populations (Benin, Uganda, Kenya, Tanzania, Zambia, Malawi; India, Indonesia, China, Australia) (see Supp Table 2 [online only] 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. 2021, 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 Supp Table 2 [online only]).

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 (Supp Fig. 1 [online only]; Table 3). Based on the LC_{50}, and relative to *H. armigera*, *S. frugiperda* Sf20-1 was 174× less sensitive, *S. frugiperda* Sf20-4 was 99× less sensitive, and *S. litura* was 120× 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 (Supp Fig. 2 [online only]; Table 3); *S. frugiperda* Sf20-1 was 13× less sensitive, *S. frugiperda* Sf20-4 was 4× less sensitive and *S. litura* was 10× 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 (Supp Fig. 3 [online only]; 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 2× less sensitive to Cry1F. In terms of field control there is unlikely to be any distinguishable difference in Cry1F efficacy against *S. frugiperda* versus *S. litura*.

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

Based on published studies which show resistance ratios of >2,500 fold in *H. armigera* and *S. frugiperda* 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.

Foliar Bt

The performance against *S. frugiperda* 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 (valent biosciences, last accessed 13 January 2022) but also for cabbage looper and the diamondback moth (DBM), while DIPEL was formulated for broad spectrum caterpillar control (valent biosciences, last accessed 13 January 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 *S. frugiperda* populations than XenTari which contains Cry1Aa, Cry1Ab, Cry1C, and Cry1D. Relative to *H. armigera*, *S. frugiperda* Sf20-1 was 24× less sensitive, *S. frugiperda* Sf20-4 was 18× less sensitive and *S. litura* was 17× less sensitive to Dipel. XenTari toxicity ratios relative to *H. armigera* ranged 2–4× for *S. frugiperda* Sf20-1, *S. frugiperda* Sf20-4, and *S. litura* (Supp Figs. 5 and 6 [online only]; Table 3).

Conventional Pesticides

Cypermethrin is a pyrethroid pesticide and the bioassay results suggest that S. *frugiperda* (i.e., the Sf20-1 and Sf20-4 laboratory cultures) is less sensitive to it than the laboratory strain of *H. armigera* (56× and 145× respectively: Supp Fig. 7 [online only]; Table 4).

Bioassay results for the carbamate pesticide methomyl are not as repeatable as other toxins with considerable variability between replicates (Supp Fig. 8 [online only]; Table 4). However, overall *S. frugiperda* Sf20-4 shows 52× less sensitivity to methomyl than *H. armigera* while *S. frugiperda* Sf20-1 shows a 4× lower sensitivity. This difference is consistent with some level of heterogeneity between the first invasive *S. frugiperda* populations in Australia.

The dose response and LC_{50} for indoxacarb (Supp Fig. 9 [online only]; Table 5) show that *S. frugiperda* Sf20-1 and *S. frugiperda* Sf20-4 are less sensitive to this chemistry than *H. armigera* (22× and 208× respectively). This difference is consistent with some level of heterogeneity between the first invasive *S. frugiperda* populations in Australia.

The dose response and LC_{50} for chlorantraniliprole show similar sensitivity for *H. armigera* and *S. frugiperda* Sf20-1, with the latter showing a 3× difference in the LC_{50}. Interestingly, the *S. frugiperda* Sf20-4 exhibited a 15× reduction in sensitivity compared to *H. armigera* (Supp Fig. 10 [online only]; Table 5), and a 5× response variation to chlorantraniliprole when compared to the Sf20-1 line.

The dose response and LC_{50} data for emamectin benzoate and spinetoram (Supp Figs. 11 and 12 [online only]; Table 5) suggest that *S. frugiperda* Sf20-1 and Sf20-4 are not significantly different in their level of sensitivity compared to *H. armigera*.

Resistance Alleles by Whole Genome Sequencing

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

Table 3. Summary bioassay data on *Spodoptera frugiperda* populations from Queensland and Western Australia, *S. litura*, and *Helicoverpa armigera* involving surface treatment of the diet with Bt toxins and products

Pesticide	Populations/ species	N	Slope	LC_{50}	95% CI	LC_{99}	95% CI	χ^2 (Degrees of Freedom)	P	Toxicity ratio (*H. armigera*= 1)
Cry1Ac (µg/cm²)	*H. armigera*	741	1.837 ± 0.126	0.025	0.021–0.029	0.465	0.316–0.773	31.66 (29)	0.334	-
	Sf20-1	504	0.827 ± 0.102	4.34	1.81–8.15	80.6	36.85–249.17	18.38 (19)	0.497	174
	Sf20-4	575	1.449 ± 0.149	2.48	1.79–3.29	85.34	53.32–159.68	17.77 (22)	0.720	99
	S. litura	501	1.626 ± 0.135	2.99	2.06–4.20	80.7	38.60–284.54	49.14 (19)	<0.001	120
Cry2Ab (µg/cm²)	*H. armigera*	476	1.501 ± 0.143	0.049	0.034–0.068	1.74	0.86–6.57	31.834 (18)	0.023	–
	Sf20-1	575	1.649 ± 0.136	0.655	0.435–0.951	16.88	7.24–86.01	86.222 (22)	<0.001	13
	Sf20-4	551	2.201 ± 0.246	0.178	0.138–0.221	2.03	1.33–4.94	39.076 21	0.010	4
	S. litura	859	1.197 ± 0.081	0.511	0.335–0.734	44.9	20.179–154.39	91.946 33	<0.001	10
Cry1F (µl/cm²)	Sf20-1	576	1.531 ± 0.115	0.025	0.015–0.038	0.838	0.365–3.949	87.309 (22)	<0.001	–
	Sf20-4	575	1.809 ± 0.149	0.021	0.014–0.029	0.394	0.211–1.138	19.947 (22)	0.586	–
	S. litura	574	1.524 ± 0.121	0.0088	0.0069–0.011	0.294	0.187–0.535	58.968 (22)	<0.001	–
VIP3a (µl/cm²)	*H. armigera*	763	1.486 ± 0.093	0.0062	0.0051–0.0075	0.230	0.140–0.400	33.738 (30)	0.291	–
	Sf20-1	619	1.881 ± 0.225	0.0021	0.0010–0.0031	0.049	0.021–0.257	68.846 (24)	<0.001	0.152
	Sf20-4	599	2.398 ± 0.212	0.0019	0.0016–0.0023	0.018	0.013–0.028	20.934 (23)	0.585	0.078
	S. litura	575	1.693 ± 0.129	0.00065	0.0005–0.0008	0.015	0.088–0.035	39.108 (22)	0.013	0.065
Dipel (IU/cm²)	*H. armigera*	526	1.832 ± 0.154	2.11	1.59–2.72	39.2	22.43–92.76	33.878 (20)	0.007	–
	Sf20-1	644	1.832 ± 0.154	52.02	36.55–70.05	2612.6	1379.89–6752.99	29.258 (25)	0.253	24
	Sf20-4	574	1.703 ± 0.153	38.93	23.97–56.35	904.1	469.80–2779.63	53.767 (22)	0.002	18
	S. litura	788	1.426 ± 0.098	35.96	28.23–44.99	1540.8	916.66–3110.88	40.916 (31)	0.110	17
XenTari (DBM/cm²)	*H. armigera*	620	1.530 ± 0.136	5.95	4.69–7.40	197.2	118.7–398.3	23.051 (24)	0.516	–
	Sf20-1	647	2.375 ± 0.289	11.81	8.33–15.17	112.7	69.8–270.1	39.18 (25)	0.035	2
	Sf20-4	549	1.333 ± 0.180	19.01	9.27–29.8	1058.9	379.5–10739.7	43.25 (21)	0.003	3
	S. litura	741	2.375 ± 0.289	24.86	17.08–31.14	206.9	126.2–597.5	47.92 (29)	<0.001	4

S. frugiperda populations were from Walkamin, Queensland (Sf20-1) and Kununurra, Western Australia (Sf20-4); *S. litura* was from Mareeba Queensland, and *Helicoverpa armigera* was a laboratory 'GR' line. The concentration of each pesticide required to kill 50% and 99% of the test subjects (*H. armigera*, *S. frugiperda*, *S. litura*) are given as LC_{50} (50% lethal concentration) and LC_{99} (99% lethal concentration), respectively. 95% confidence intervals (95% CI) for both LC_{50} and LC_{99} are also provided. Sample sizes (*N*) of *H. armigera*, *S. litura*, and the two *S. frugiperda* laboratory culture lines used in the bioassay tests are indicated. *P*-values (*P*) associated with the χ^2 tests are also provided.

Table 4. Summary bioassay data on *Spodoptera frugiperda* populations and *Helicoverpa armigera* (GR laboratory line) involving topical application to the insect with contact insecticides

Toxin	Population/Species	N	Slope	95% C.I.	LC_{50}	95% C.I.	LC_{99}	95%CI	χ^2 (Degrees of Freedom)	P	Toxicity ratio (*H. armigera* = 1)
Alpha cypermethrin µg/larvae	*H. armigera*	1328	2.849 ± 0.140	0.0032–0.0041	0.0036	0.018–0.032	0.023	0.018–0.032	109.84 (57)	<0.001	–
	Sf20-1	675	2.399 ± 0.154	0.171–0.239	0.201	1.31–3.06	1.88	1.31–3.06	43.903 (28)	0.028	56
	Sf20-4	766	2.186 ± 0.132	0.427–0.641	0.523	3.99–10.85	6.06	3.99–10.85	67.208 (32)	<0.001	145
Methomyl µg/larvae	*H. armigera*	858	0.809 ± 0.059	0.031–0.097	0.057	10.27–53.93	43.12	10.27–53.93	135.89 (36)	<0.001	–
	Sf20-1	765	1.064 ± 0.076	0.177–0.356	0.254	15.81–156.30	39.2	15.81–156.30	72.309 (32)	<0.001	4
	Sf20-4	631	0.874 ± 0.07	1.87–4.66	2.96	380.11–10950.47	1363.7	380.11–10950.47	57.160 (26)	<0.001	52

S. frugiperda from Walkamin Queensland and from Kununurra Western Australia are coded Sf20-1 and Sf20-4, respectively. *Helicoverpa armigera* laboratory line is coded as 'GR'. The concentration of each pesticide required to kill 50% and 99% of the test subjects (*H. armigera*, *S. frugiperda*) are given as LC_{50} (50% lethal concentration) and LC_{99} (99% lethal concentration), respectively. 95% confidence intervals (95% CI) for both LC_{50} and LC_{99} are also provided. Sample sizes (N) of *H. armigera* and *S. frugiperda* laboratory culture lines used in the bioassay tests are indicated. *P*-values (*P*) associated with the χ^2 tests are also provided.

Kununurra (F290V). The Peruvian *S. frugiperda* population was the only population that had heterozygous individuals with resistance allele for G227A amino acid substitution (Table 6).

Resistance allele characterization by sequencing approaches from this study and published studies for the ACE-1 gene is summarized in (Fig. 1, Supp Table 2 [online only] and references therein). The most common resistance allele detected in the invasive and native population (456 individuals examined in total) was the F290V mutation with 66 homozygous and 222 heterozygous resistant genotypes detected. This mutation is a T to G single nucleotide polymorphism (SNP) that changes the codon encoding the amino acid from TTT to GTT leading to a phenylalanine (F) to valine (V) change in the protein sequence encoded by the ACE-1 gene. This mutation was present at all locations and evenly distributed between the invasive and native populations. In Australia, of the sequenced individuals (*N* = 146), 24.7% (*N* = 36) were heterozygous, and 8% (*N* = 12) were homozygous for the resistance allele. Heterozygous and homozygous individuals were found in populations from all four states (i.e., WA, NT, Queensland, and NSW) as well as from a single individual from Erub Island, suggesting it is common across Australia.

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

The G227A mutation was the least common (from 456 individuals) with 20 heterozygous individuals and 2 homozygous individuals detected. This mutation is a G to a C SNP which alters the codon from GGA to CGA encoding a glycine (G) to alanine (A) amino acid change. Interestingly G227A was only present in individuals from the native range (Brazil, USA, Puerto Rico, Peru) but absent in individuals from across the invasive range that was surveyed in this study and from the related studies of Tay et al. (2021, 2022a). When compiling allele frequencies for both the VGSC and ACE-1 resistant genes from this study and from published whole genome sequencing and targeted PCR/Sanger sequencing data (Fig. 1; see also Supp Table 1 [online only]), the rare G227A resistance allele was present only in the Indonesian (Boaventura et al. 2020a) and Hubei populations (Guo et al. 2020) but absent in African, Australian, and other Asian (e.g., Indian, South Korea) populations, including populations from six other Chinese provinces.

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

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,

Table 5. Summary bioassay data on *Spodoptera frugiperda* and *Helicoverpa armigera* involving diet incorporation of insecticides

Pesticide	Population/Species	N	Slope	LC$_{50}$	95% CI	LC$_{99}$	95%CI	χ^2 (Degrees of Freedom)	P	Toxicity ratio (*H. armigera* = 1)
Chlorantraniliprole (µg/ml diet)	*H. armigera*	540	3.199 ± 0.234	0.011	0.009–0.013	0.056	0.038–0.109	72.27 (22)	<0.001	-
	Sf20-1	613	2.484 ± 0.191	0.032	0.024–0.043	0.28	0.162–0.777	99.976 (25)	<0.001	3
	Sf20-4	897	2.065 ± 0.152	0.163	0.132–0.201	2.19	1.40–4.10	99.895 (38)	<0.001	15
Indoxacarb (µg/ml diet)	*H. armigera*	653	1.684 ± 0.122	0.054	0.028–0.089	1.29	0.49–12.59	226.42 (27)	<0.001	–
	Sf20-1	697	2.359 ± 0.159	1.203	1.031–1.398	11.66	8.50–17.62	34.789 (29)	0.212	22
	Sf20-4	541	1.817 ± 0.130	11.206	9.254–13.654	213.85	136.20–391.41	24.511 (22)	0.321	208
Emamectin (µg/ml diet)	*H. armigera*	810	2.465 ± 0.164	0.0107	0.0087–0.0131	0.0945	0.065–1.607	75.940 (34)	<0.001	–
	Sf20-1	631	2.606 ± 0.175	0.0158	0.013–0.019	0.124	0.082–2.292	67.73 (26)	<0.001	1
	Sf20-4	720	3.234 ± 0.226	0.0179	0.016–0.020	0.094	0.723–1.331	40.00 (30)	0.105	2
Spinetoram (µg/ml diet)	*H. armigera*	472	1.711 ± 0.173	0.086	0.023–0.157	1.977	0.686–8.409	154.67 (19)	<0.001	–
	Sf20-1	831	3.220 ± 0.198	0.118	0.101–0.137	0.623	0.457–0.990	94.168 (35)	<0.001	1
	Sf20-4	542	4.921 ± 0.489	0.102	0.092–0.112	0.301	0.247–0.400	13.119 (22)	0.930	1

S. frugiperda populations are Sf20-1 from Walkamin Queensland and Sf20-4 from Kununurra Western Australia, *H. armigera* 'GR' belongs to a laboratory line The concentration of each pesticide required to kill 50% and 99% of the test subjects (*H. armigera*, *S. frugiperda*) are given as LC$_{50}$ (50% lethal concentration) and LC$_{99}$ (99% lethal concentration), respectively. 95% confidence intervals (95% CI) for both LC$_{50}$ and LC$_{99}$ are also provided. Sample sizes (N) of *H. armigera*, and the two *S. frugiperda* laboratory culture lines used in the bioassay tests are indicated. *P*-values (*P*) associated with the χ^2 tests are also provided.

some variation from the reference was observed (Supp Fig. 13 [online only]). Numerous synonymous mutations and a total of 31 nonsynonymous mutations were observed in the coding sequence of the ABCC2 gene in all sequenced individuals. Several of these nonsynonymous mutations were associated with a common deletion and insertion motif in the first exon where an 11 bp 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 c-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 (see Supp Figs. 14 and 15 [online only]) 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.

Discussion

In this study, we showed insecticide and Bt response differences in two of the first reported *S. frugiperda* populations in Queensland (i.e., SF20-1) and Western Australia (Sf20-4) 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. Characterization of the ABCC2 resistance gene identified one *S. frugiperda* 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. 2021) 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 *S. frugiperda* 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 (Schlum et al. 2021, Rane et al. 2022, Tay et al. 2022a), and suggest that separate pathways were involved in the establishment of these Queensland and Western Australian populations. This is contrary to the current postulation of a single introduction pathway for the arrival of *S. frugiperda* to Australia based on an assumption (Jing et al. 2021) or reverse trajectory simulation (Qi et al. 2021) and highlights the importance of harmonizing simulation studies with genomic and phenotypic evidence.

With significant economic impacts on agriculture from *S. frugiperda* 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 the 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.

Table 6. ACE-1 locus of *Spodoptera frugiperda* individuals from Australia, South Korea, Papua New Guinea, and Peru characterized via whole genome

Population	N	ACE-1 (A201S)			ACE-1 (G227A)			ACE-1 (F290V)		
		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

Number of *Spodoptera frugiperda* individuals (*N*) from populations from Australia (Kununnura 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 characterized via whole genome sequence data of the ACE-1 locus involving the A2001S, G227A, and F290V amino acid substitutions.

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.

It is important to stress the challenge and difficulty to compare bioassay results between studies due to the different genetic background of the test samples (e.g., due to different number of individuals used to establish test populations), methods and approaches between research groups, and the general different rearing conditions of laboratory cultures that could contribute to varied response outcomes. A further challenge for invasive *S. frugiperda* 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) versus 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 *S. frugiperda* 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 *S. frugiperda* populations with diet incorporation assays (on 3rd instar larvae), Yu (1991) also included a native susceptible *S, frugiperda* 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 *S. frugiperda* 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 *S. frugiperda* 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 *S. frugiperda* 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, resistance ratios 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 were 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 *S. frugiperda* populations from Florida USA (Yu 1991) and with the South African invasive population of *S. frugiperda* (Eriksson 2019) showed that the Queensland population as similar in its response as the susceptible Florida *S. frugiperda*, while the Western Australia population was around 5 times more tolerant. This contrasted with the South African population which was 110 times more tolerant than the Florida susceptible *S. frugiperda* strain, and around 8 times more tolerant than the resistant *S. frugiperda* strain from Florida (Table 8). Taken as a whole, similar bioassay results between native (i.e., Brazil, USA) and various invasive populations suggested potential significant genetic diversity in introduced populations. This concurs with population genomic and genetic analyses (Zhang et al. 2020, Schlum et al. 2021, Jiang et al. 2022, Rane et al. 2022, Tay et al. 2022a) that suggested multiple origins for the invasive African, Asian (Indian, Chinese), and Southeast Asian (e.g., Malaysia) *S. frugiperda* populations.

It is possible that the differential response between the two Australian *S. frugiperda* populations to insecticides and Bt toxins represents natural variation in the national population which has emerged from a single founding incursion. However, it is unlikely that the differential responses within Australian populations represents local selection pressures because the period between populations establishing and being collected for this study was presumed to be too short to enable this opportunity. It could be that separate incursions involving different source populations occurred in WA compared to the eastern states of Australia, as supported by genome-wide SNP marker population genomic studies (Rane et al. 2022). Different selection pressures on the global population which recently originated from multiple Asian, Southeast Asian, and African incursions (Zhang et al. 2020, Schlum et al. 2021, Rane et al. 2022, Tay et al. 2022a) may have driven different phenotypes which entered the expanding ranges tested in our study and others reported herein. However, Kulye et al. (2021) demonstrated in *S. frugiperda* populations collected in India during 2018, 2019, and 2020 that large response changes such as those observed in South African (e.g., methomyl; Eriksson 2019) and Western Australian (chlorantraniliprole, indoxacarb;
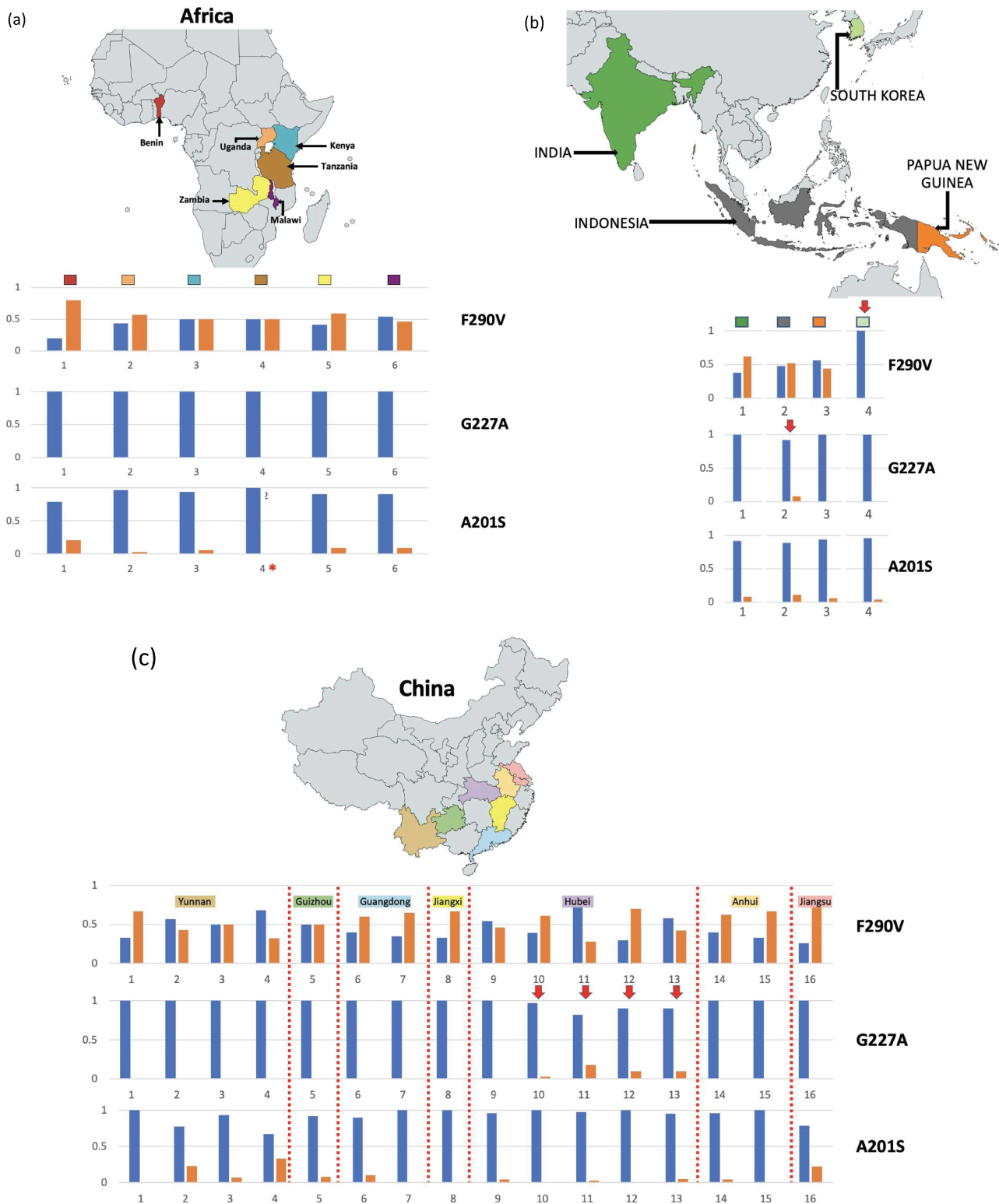
Fig. 1. Summary of Acytylcholinesterase (ACE-1) susceptible and resistance allele frequencies in invasive range *Spodoptera frugiperda* populations from: (a) six African countries (i.e., Benin (#1), Uganda (#2), Kenya (#3), Tanzania (#4), Zambia (#5), Malawi (#6)), (b) Asia (i.e., India, Indonesia, Papua New Guinea (PNG), South Korea), (c) China, and (d) Australia. A total of 1,177 individuals representing 75 populations in Supp Table 2 (online only) were used to compile the data below. Population identity is as provided in Supp Table 2 (online only) 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, Zhang et al. 2020, Zhao et al. 2020, Guan et al. 2021, 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 (b) Indonesia (#2; G227A) and South Korea (#4; F290V), and in (c) China (Hubei province (#10-13); G227A) as indicated by the arrows. In (d) 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 neighboring countries and likely arrived via separate pathways and entry points (see also Rane et al. 2022).
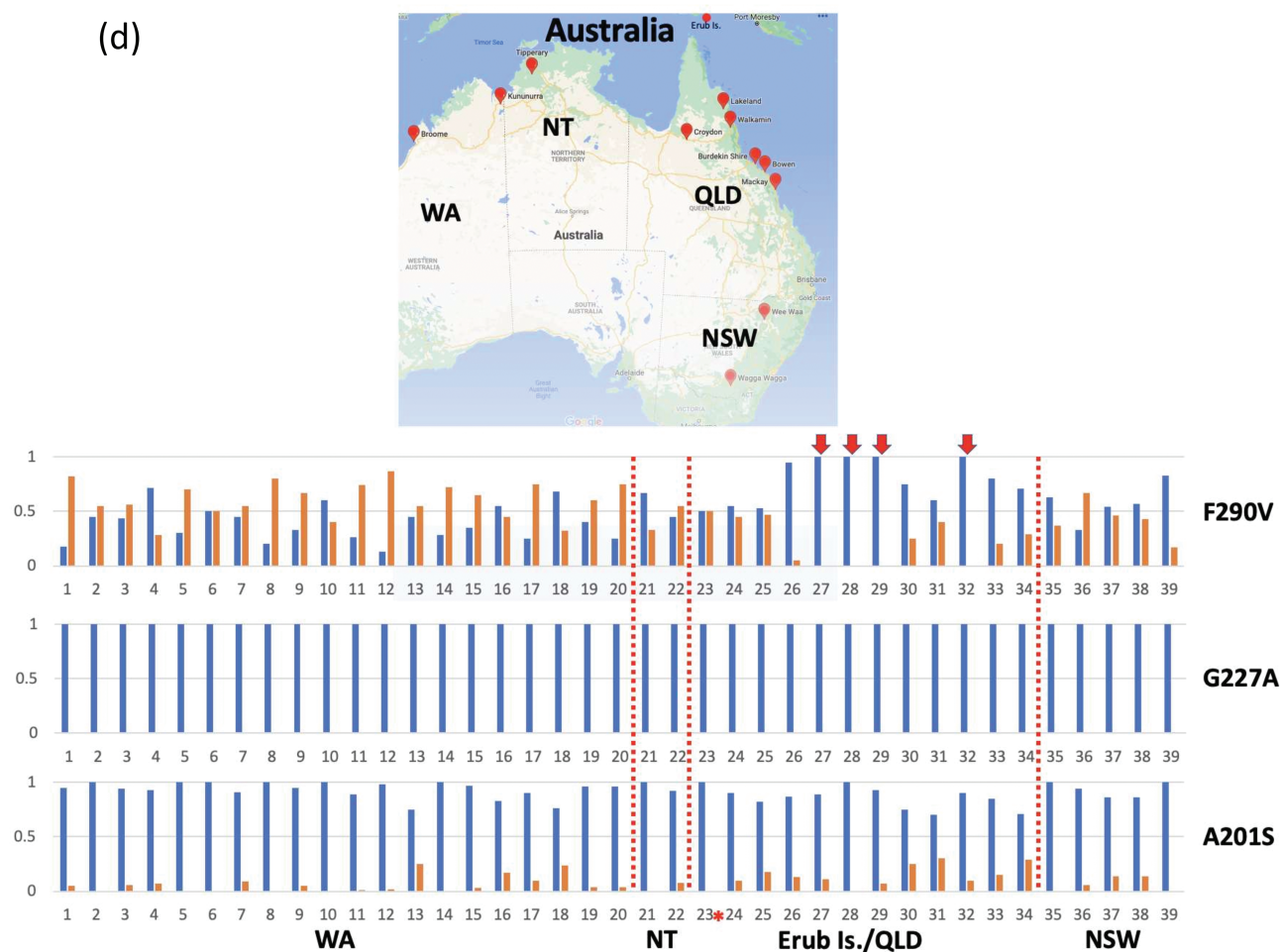
Fig. 1. Continued

this study) populations were unlikely considering the short time frame since the very recent arrival of *S. frugiperda* especially assuming a west-to-east spread (Goergen et al. 2016; Cock et al. 2017; Nagoshi et al. 2018, 2019b). Another explanation for the differential responses of the two Australian populations that we studied is that they have different fitness because of variation in genetic diversity which reflects the number of individuals founding the populations. However, if this was the case one might expect sensitivity levels to be consistently higher or lower to all of the agents that we tested but this was not the case.

Alien invasive agricultural pests are increasingly being shown to carry novel insecticide resistance genes (e.g., Anderson et al. 2018, Walsh et al. 2018, Tay and Gordon 2019). This includes *S. frugiperda* in which invasive populations have been confirmed via whole genome sequence analyses (e.g., Zhang et al. 2020, Guan et al. 2021, Yainna et al. 2021) and molecular characterization to harbor selected resistance genes (Boaventura et al. 2020a,b; Zhao et al. 2020). Our review of reported ACE-1 and VGSC resistance allele frequency differences (Fig. 1; Supp Table 2 [online only] and references therein) suggests that the invasive *S. frugiperda* populations within China (Guo et al. 2020), Indonesia (Boaventura et al. 2020a), Queensland, 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 *S. frugiperda* (e.g., Boaventura et al. 2020a, Guan et al. 2021, 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 *S. frugiperda* 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 are 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. 2021, 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 *S. frugiperda* populations likely do not carry Cry1F resistance alleles in the homozygous state known to exist in native range *S. frugiperda* populations (e.g., Banerjee et al. 2017, Flagel et al. 2018, Guan et al. 2021, 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 VIP3Aa20 resistant *S. frugiperda* have been reported in Brazil and the USA where they occur as a recessive trait (Yang et al. 2013, 2018, 2019; Bernardi et al. 2015, 2016). 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 further confirm the status of VIP3A resistance allele in both populations. The F_2-crosses approach should be a valuable tool in protecting the Australian cotton industry against *S. frugiperda*, given 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 coordinated 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. 2022a), and in, e.g., Australia, Malaysia, and Myanmar versus 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 localized ecological and climatic determinants (Tay et al. 2022b). 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. 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 recognized as may not be as rapid and widespread as originally believed (e.g., Jiang et al. 2022, Nagoshi et al. 2022, Rane et al. 2022, Tay et al. 2022a). As such, we advocate cautionary approaches to avoid premature assumptions that extensive population admixture via gene flow in Australia landscape would lead to homogenized 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 standardize approaches for testing of insecticides and Bt toxins. Localized novel resistance traits in populations of *S. frugiperda* would likely serve as a new resistance management challenge to other neighboring 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

Table 7. Comparisons of bioassay results for selected insecticides fed to laboratory populations of *Spodoptera frugiperda* via diet incorporation approaches

Insecticides	Populations	Range	LC_{50} (ppm)/EC_{50} (µg/ml)	Fiducial limits[a] (95%)	Slope ± SE	Toxicity ratios
Spinetoram	Brazil (SUS-2005)[b]	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)[b]	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[b]	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)[b]	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

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 Brazil, USA (LSU), India I-2018, and Australia (Sf20-1; Sf20-4) populations. LC_{50}: 50% lethal concentration [in parts per million (ppm)]: The concentration of the pesticide required to kill 50% of the test subject, EC_{50}: 50% effective concentration (in µg/ml).
[a]Fiducial limits are similar to confidence limits but are based on logistic growth or S-shaped curve distribution.
[b]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 on *S. frugiperda* between resistant or susceptible native populations from USA (FL) and invasive populations from South and 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

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. LC$_{50}$ (50% lethal concentration): The concentration of each pesticide required to kill 50% of the test subject. Fiducial limits are similar to confidence limits but are based on logistic growth or S-shaped curve distribution.

development of suitable pest and resistance management strategies for this global pest complex.

Acknowledgments

Supplementary Data

Supplementary data are available at *Journal of Economic Entomology* online.

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