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ORIGINAL ARTICLE

Whole-genome sequencing to detect mutations associated with resistance to insecticides and Bt proteins in *Spodoptera frugiperda*

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Abstract The fall armyworm (FAW), *Spodoptera frugiperda*, is a major pest native to the Americas that has recently invaded the Old World. Point mutations in the target-site proteins acetylcholinesterase-1 (ace-1), voltage-gated sodium channel (VGSC) and ryanodine receptor (RyR) have been identified in *S. frugiperda* as major resistance mechanisms to organophosphate, pyrethroid and diamide insecticides respectively. Mutations in the adenosine triphosphate-binding cassette transporter C2 gene (*ABCC2*) have also been identified to confer resistance to Cry1F protein. In this study, we applied a whole-genome sequencing (WGS) approach to identify point mutations in the target-site genes in 150 FAW individuals collected from China, Malawi, Uganda and Brazil. This approach revealed three amino acid substitutions (A201S, G227A and F290V) of *S. frugiperda ace-1*, which are known to be associated with organophosphate resistance. The Brazilian population had all three *ace-1* point mutations and the 227A allele (mean frequency = 0.54) was the most common. Populations from China, Malawi and Uganda harbored two of the three *ace-1* point mutations (A201S and F290V) with the 290V allele (0.47–0.58) as the dominant allele. Point mutations in VGSC (T929I, L932F and L1014F) and RyR (I4790M and G4946E) were not detected in any of the 150 individuals. A novel 12-bp insertion mutation in exon 15 of the *ABCC2* gene was identified in some of the Brazilian individuals but absent in the invasive populations. Our results not only demonstrate robustness of the WGS-based genomic approach for detection of resistance mutations, but also provide insights for improvement of resistance management tactics in *S. frugiperda*.

Key words Bt resistance; insecticide resistance; mutation detection; *Spodoptera frugiperda*; whole-genome sequencing

Introduction

The fall armyworm (FAW) *Spodoptera frugiperda* (J.E. Smith) is endemic to the tropical and subtropical regions of the American continent. This highly polyphagous pest feeds on as many as 353 host plants (Montezano *et al*., 2018), including economically important crops such as corn, rice, sorghum, sugarcane, cotton, and soybean (Hardke *et al*., 2015; Early *et al*., 2018; Assefa & Ayelew, 2019). In its native and invasive ranges, two major different lineages of the FAW have been identified, named the rice-preferring and corn-preferring FAW according to their preferred host plants (Nagoshi, 2010; Dumas *et al*., 2015; Goergen *et al*., 2016; Nagoshi *et al*.,

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2017, 2019; Otim *et al*., 2018; Zhang *et al*., 2019). The mitochondrial DNA *cytochrome oxidase subunit* I (mt-COI) (Dumas *et al*., 2015; Otim *et al*., 2018) and the *triose phosphate isomerase* (*Tpi*) exon4 nt370 gene locus (Nagoshi, 2010) are the two molecular diagnostic marker systems currently used in identification of FAW species status and to distinguish the two plant host-preferring lineages respectively.

FAW detection was first officially confirmed in West Africa in 2016 (Goergen *et al*., 2016), followed by confirmations also in central (Cock *et al*., 2017) and eastern (Otim *et al*., 2018) Africa and by February 2018 in all sub-Saharan African nations. India, Yemen, Sri Lanka, Thailand and Myanmar confirmed the detection of this pest in 2018 (CABI, 2018; FAO, 2019), followed by official confirmation in Yunnan China in January 2019 (Tay & Gordon, 2019; Jing *et al*., 2020). In 2020, the FAW was detected in the Torres Strait Islands, part of Australia (IPPC, 2020) and on mainland Australia (Queensland Government Department of Agriculture and Fisheries, 2020). The FAW is now confirmed in at least 64 nations from Africa, the Middle East, South-East Asia, Asia and Australia (Czepak *et al*., 2019; IPPC, 2020). Since the successful invasive establishments of FAW, global maize production has been severely impacted, with productivity declines of as much as 50% reported from Africa and southern Asia since 2016 (Assefa & Ayelew, 2019; Silver, 2019).

Control of these FAW populations currently depends on beneficial insects (Shylesha *et al*., 2018), biopesticides (Behle & Popham, 2012), spraying of chemical insecticides and planting of transgenic crops expressing *Bacillus thuringiensis* (Bt) proteins (Burtet *et al*., 2017). In Africa and Asia, the use of insecticides is by far the most common control approach; however, FAW in the Americas is controlled using transgenic Bt crops, and biological control agents, in addition to insecticides (Hruska, 2019). The evolution of resistance to both synthetic insecticides and Bt corn further increases the dependence on spraying chemical insecticides to control this pest (Gutiérrez-Moreno *et al*., 2019). This often leads to more frequent and high-dose sprays, which increase costs and could cause harm to users, consumers and the environment. In Africa and Asia, recommendations for insecticide sprays are not based on an understanding of the resistance status of the invasive FAW populations.

Organophosphate insecticides (OPs) and pyrethroids have been commonly and intensively used for FAW control in the Americas, and as a result, resistance to these insecticides has been reported in several countries from South, Central and North America (Yu, 1991; Carvalho *et al*., 2013; Gutiérrez-Moreno *et al*., 2019). Biochemical and molecular studies have shown that insensitivity of the target sites is an important resistance mechanism to both OPs and pyrethroids in *S. frugiperda*, although a metabolic mechanism is also involved (Yu *et al*., 2003; Carvalho *et al*., 2013). Three amino acid substitutions (A201S, G227A and F290V, numbered as *Torpedo californica* acetylcholinesterase, PDB ID: 1EA5) in the OP target protein (acetylcholinesterase-1, AChE-1), and three point mutations (T929I, L932F and L1014F, numbered as *Musca domestica* sodium channel, GenBank X96668) in the pyrethroid target protein (voltage-gated sodium channel, VGSC) have been identified to be associated with resistance to OPs and pyrethroids respectively in Brazilian and Mexican field-derived populations of *S. frugiperda* (Carvalho *et al*., 2013; Herrera-Mayorga *et al*., 2018). More recently, the I4734M mutation in *S. frugiperda* ryanodine receptor (SfRyR) (equivalent to I4790M in *Plutella xylostella* RyR) was associated with 225-fold and >5 400-fold resistance to chlorantraniliprole and flubendiamide respectively in a laboratory-selected population of FAW from Brazil (Boaventura *et al*., 2020).

Transgenic corn and cotton expressing Bt proteins have been planted since 1996 to control some major lepidopteran pests including FAW. However, the FAW evolved resistance to Bt crops expressing Bt Cry1F and Cry1A toxins in the United States, Brazil and Argentina (Storer *et al*., 2010; Bernardi *et al*., 2014; Huang *et al*., 2014; Chandrasena *et al*., 2018). Resistance to the Cry1F Bt toxin in FAW has been reported and attributed to various disruptions of the *ABCC2* gene (Banerjee *et al*., 2017; Flagel *et al*., 2018; Boaventura *et al*., 2019).

Molecular genetics tools are of major importance to confirm the arrival of suspect incursions and monitor invasive dynamics (Jones *et al*., 2018; Walsh *et al*., 2018; Tay & Gordon, 2019). They also provide insights into the presence, frequency and spread of insecticide resistance alleles. Recently, the whole-genome sequencing (WGS) technique was used to detect resistance allele frequencies to pyrethroids in the redlegged earth mite, *Halotydeus destructor* (Edwards *et al*., 2017), and the amplicon sequencing approach was employed to monitor resistance allele frequencies to Bt protein Cry1Ac in the cotton bollworm, *Helicoverpa armigera* (Jin *et al*., 2018).

The determination of resistance allele frequencies in the FAW populations of both its native and invasive ranges is required to design effective management strategies for this pest. To-date, efforts to characterize resistance genes have been slow for the Old World, especially Africa which is the first continent to have reported incursions by this pest. Here we used a WGS approach to ascertain species identity and estimate frequencies of

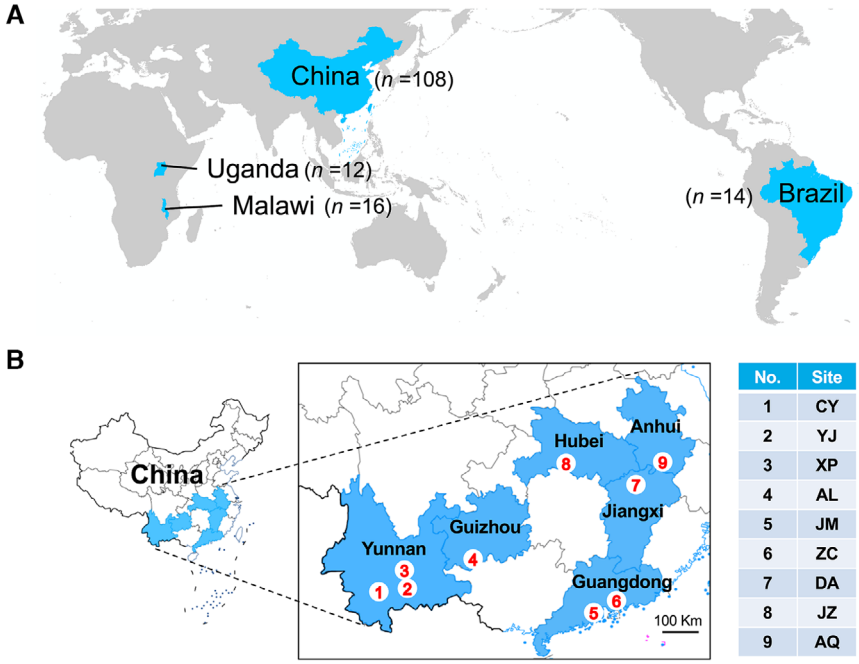

Fig. 1 Maps showing sampling sites of *Spodoptera frugiperda*. (A) Four sampling countries (Brazil, Uganda, Malawi and China). (B) Sampling sites in the six provinces of China: Cangyuan (CY), Yuanjiang (YJ), Xinping (XP), Anlong (AL), Jiangmen (JM), Zengcheng (ZC), Dean (DA), Jingzhou (JZ), and Anqing (AQ). [GS(2020)1349].

target-site resistance alleles in field FAW populations derived from China, Brazil, Uganda and Malawi. We focused on the detection of point mutations on three different target-site genes of *S. frugiperda* (*ace-1*, *VGSC* and *RyR*) involved in resistance to conventional chemical insecticides, and we characterized the *ABCC2* gene known to be responsible for resistance to Bt toxin Cry1F. Resistance evolved in the native range and carried by the invasive populations will compromise control measures not only in the local areas but also across Africa, Asia and Oceania. Thus, our results provide insights and baseline data necessary for developing insecticide resistance management tactics for *S. frugiperda* in both its native and recently invaded areas.

Materials and methods

FAW collection

The FAW samples were collected from 28 sites in four countries [19 sites from Brazil, Malawi and Uganda, nine sites from China (Fig. 1)]. The nine Chinese populations were collected between April and July of 2019 from five provinces that included Yunnan (Cangyuan, Yuanjiang, Xinping), Guizhou (Anlong), Guangdong (Jiangmen, Zengcheng), Jiangxi (Dean), Hubei (Jingzhou) and Anhui (Anqing). All Chinese FAW specimens were collected from maize host crops. Brazilian FAW samples were collected from maize hosts from the State of Goiás in 2014 and 2019 for establishing of laboratory colonies (sequenced individuals from the 44th and 2nd generations, respectively). The Malawi samples were collected from nine districts: Blantyre, Chiradzulu, Machinga, Mulanje, Thyolo and Zomba (southern region); Salima (central region), and Karonga, Mzimba and Nkhata Bay (northern region) in 2018. Ugandan samples were collected from eight districts: Amolatar (northern region); Katakwi, Kumi, Ngora, Pallisa and Soroti (eastern region); Mbarara (western) and Wakiso (central region) from maize plants in 2017. All samples were stored in 100% ethanol. Information for all samples is detailed in Table S1.

DNA extraction

Insect larva or moth was homogenized by ball mill (MM400, Retsch, Germany), then DNA was extracted using the phenol/chloroform method described in previous research (Jin *et al*., 2018).

Whole-genome sequencing (WGS)

For the Chinese FAW samples, DNA library preparation and Illumina sequencing were completed by Novogene Co. Ltd (Beijing, China). The DNA degradation and purity were checked on 1% agarose gels and NanoPhotometer spectrophotometer (IMPLEN, CA, USA). The DNA concentration was quantified using a Qubit 2.0 Flurometer (Life Technologies, Carlsbad, CA, USA) using Qubit DNA Assay Kit. DNA was fragmented by sonication to 350 bp fragments and used for library preparation using the NEBNext Ultra DNA Library Prep Kit (New England Biolabs, Ipswich, MA, USA). The constructed libraries were sequenced by Illumina NovaSeq6000 generating 150 bp PE data. For samples from Brazil, Uganda and Malawi, DNA was extracted using the Qiagen Blood and Tissue kit following the manufacturer's protocol. The DNA quality and quantity were assessed by gel electrophoresis (2% agarose) and Qubit 2.0 Fluorometer (Life Technologies, USA), respectively. Libraries were prepared using the Nextera Flex DNA Library Prep Kit following the manufacturers' instructions and sequenced by Illumina NovaSeq6000 S4 300 sequencing system at the Australian Genome Research Facility (AGRF).

Quality control and mapping

The raw data were quality trimmed using Trimmomatic 0.36 with the following parameters: "HEADCROP:5 LEADING:10 TRAILING:3 SLIDINGWINDOW:4:20 MINLEN:75" (Bolger *et al*., 2014). The clean data were mapped to FAW genome (corn strain) using the MEM module in Burrows-Wheeler Aligner (BWA) software (Li, 2013; Gouin *et al*., 2017). The sequence alignment/map (SAM) files were transferred to the binary files BAM and sorted using SAMtools (Li *et al*., 2009). Then we marked the polymerase chain reaction (PCR) duplicates in sorted BAM files using Picard module in GATK version 4.1.2.0 (GATK4) and indexed the BAM files using SAMtools (Li *et al*., 2009; McKenna *et al*., 2010).

Identification of host strain status of S. frugiperda samples

The mitochondrial DNA *cytochrome oxidase subunit I* (mtCOI) (Dumas *et al*., 2015; Otim *et al*., 2018) and the *Tpi* gene (Nagoshi, 2010) are the two molecular diagnostic marker genes widely used to identify species status and distinguish plant host preferences, respectively. First, we performed *de novo* assembly of the partial mtCOI sequences using MITObim v1.9.1 with "quick" flag (Hahn *et al*., 2013). Then we performed BLASTN search of the assembled partial mtCOI genes to the non-redundant (nr) DNA database. If multiple records returned, we used the most similar record to indicate the closest host strain. We characterized the partial mtCOI gene region to ascertain the maternal lineages of whether the FAW was a corn-preferred or rice-preferred *S. frugiperda*. We also characterized the *Tpi* locus (Nagoshi, 2010; Nagoshi *et al*., 2017) to ascertain the associated nucleotide compositions (i.e., "nuclear corn" as a "C" at nucleotide position exon4 nt370; "nuclear rice" as a "T", or as heterozygotes with C/T; see Nagoshi., 2010 Fig. 3; Nagoshi *et al*., 2017).

Calling variants on mutation sites of resistance genes

The reference sequences harboring resistance mutations were downloaded from GenBank (*ace-1*: KC435023; *VGSC*: KC435025; *RyR*: MK226188; *ABCC2*: KY489760) and then mapped to the *S. frugiperda* genome using BLASTN (Camacho *et al*., 2009). Then we marked the coding sites of resistance mutation in the genome. The variants were called using HaplotypeCaller module in GATK4 (McKenna *et al*., 2010) with "-L" parameter for target region in the genome. Variants were selected for analysis and filtered using the SelectVariants and VariantFiltration modules in GATK4 (McKenna *et al*., 2010). We filtered the variants with the following parameter: "QD $<$ 2.0 $\|$ MQ $<$ 40.0 $\|$ FS $>$ 60.0 $\|$ SOR $>$ 3.0 $\|$ MQRankSum $<$ $-$12.5 $\|$ ReadPosRankSum $<$ –8.0" for SNPs (single nucleotide polymorphisms) and "QD $<$ 2.0 $\|$ FS $>$ 200.0 $\|$ SOR $>$ 10.0 $\|$ MQRankSum $<$ $-$12.5 $\|$ ReadPosRankSum $<$ $-$8.0" for INDELs (insertions or deletions). The sequencing depth in the target region was calculated according to the Genomic Variant Cell Format temp files. The samples with regional minimal depth of $<$4 were discarded. Finally, the preliminary positive INDELs called by GATK were checked by observing alignment files using the TVIEW module in SAMtools (Li *et al*., 2009).

Verification of the positive mutations in the ace-1 gene

We confirmed the mutations in *ace-1* gene revealed by WGS using Sanger sequencing. Primers were designed (ace1-F: 5'-ATGCTGTGGGTCTTTGG-3'; ace1-R: 5'-CCTACTTATCCCTACATTCTC-3') to amplify the location of the three mutations A201S, G227A and F290V. The 25 μL PCR reactions consisted of 12.5 μL of 2 $\times$ Taq PCR Master Mix, 1 μL of each primer, 1 μL of gDNA and 9.5 μL distilled water. Temperature cycling

conditions were 94 °C for 3 min followed by 35 cycles of 94 °C for 30 s, 50 °C for 30 s and 72 °C for 40 s, followed by a final extension of 72 °C for 10 min. The amplified fragments of the expected size (711 bp) were visualized on 1% agarose gels, purified and directly sequenced by Sanger sequencing using the reverse primer (ace1-R) by TSINGKE company (Beijing, China).

Verification of the INDEL detected in Brazilian FAW ABCC2 gene

The *SfABCC2* coding sequences (GenBank accession number KY489760) were mapped to the genome of *S. frugiperda* (Gouin *et al*., 2017) and used as reference to assemble the *ABCC2* genes from native South American and invasive African and Chinese FAW populations. Assembly of the *ABCC2* gene used the same pipeline as described above for the *ace-1* gene assembly. The putative disruption to the ABCC2 exon was verified by PCR using the primers SfABCC2-exon15-F (AGATCCTGARTTGRACACTCAAGT) and SfABCC2-exon15-R (CACTATTGATTTGCACTTACCCGATG) using the PCR profiles 95 °C for 3 min; 35 cycles consisted of 95 °C for 30 s/50 °C for 30 s/72 °C for 30 s per cycle; 72 °C for 10 min, and incubation at 10 °C post-PCR amplification. Amplicons were visualized on 1.25% TBE agarose gel prior to Sanger sequencing following the protocol previously described (Tay *et al*., 2012). Sanger sequence trace files were analyzed and contigs assembled using the Pre-Gap4 and Gap4 programs within the Staden sequence analysis package (Staden *et al*., 2000). Confirmation of sequenced amplicons as the target ABCC2 gene region was by BLASTN search (Camacho *et al*., 2009) against the non-redundant (nr) GenBank DNA database in the National Center for Biotechnology Information.

Results

Identification of FAW host strain status

To identify our sampled populations as either belonging to the corn-preferring or rice-preferring FAW, we assembled the relevant partial mtCOI gene region (635 bp) using the locus MF197868 previously reported by Otim *et al*. (2018). We observed four haplotypes within our 28 sampled populations in the partial mtCOI. Two of these haplotypes matched exactly to *S. frugiperda* records, that is, MF197868.1 (corn strain) and MF197867.1 (rice strain) respectively, which were also reported in Uganda (Otim *et al*., 2018), and another two haplotypes (MN820654, MN820655) were very similar to MF197868.1 (99.84%) and only detected from three Brazilian samples. Most of our samples (127/150) were identified as the rice strain based on the partial COI gene fragment we analyzed (Table S1).

The polymorphism at the exon4 nt370 of the *Tpi* gene has been proposed as a marker for the host strain (Nagoshi, 2010), where the corn strain haplotype is a "C" at this locus and rice strain "T". Based on this SNP at the *Tpi* exon4 nt370 locus, almost all individuals (146 out 150) would be classified as being the corn strain, one as the rice strain (CY19), and only three as hybrids (AL02, CY14 and CC46) (Table S1).

These results showed that all individuals detected are *S. frugiperda*, with the majority having the *Tpi* marker associated with the corn strain and the mitochondrial DNA genome of the rice strain. It is impossible to infer hybrid status of FAW based on the mtCOI gene marker alone and in this case there is a contrast between the mtCOI haplotype and the *Tpi* haplotype which could suggest hybridization. However, any identification of host strains and hybrids based on a single allele from a partial *Tpi* gene is likely to be inaccurate and therefore should be discouraged.

Detection of the target-site point mutations associated with insecticide resistance

In the present study, we examined eight mutations previously identified as involved in resistance to chemical insecticides: three mutations (A201S, G227A and F290V) in acetylcholinesterase-1 (*ace-1*), three mutations (T929I, L932F and L1014F) in the *VGSC* and two mutations (I4790M and G4946E) in the ryanodine receptor (*RyR*). The raw data from WGS were analyzed according to the pipeline described in the Materials and Methods section. In order to decrease the false positive rate, samples with minimal depth of <4 at the codon sites were discarded. A total of 150 samples were used for downstream analysis. The mean sequencing depth was 22 for Brazil (SD = 13), 52 for China (SD = 18), 17 for Malawi (SD = 8) and 21 for Uganda (SD = 11) (Table S2).

We identified all three candidate point mutations in the *ace-1* gene in our samples (Fig. 2, Table S2). The F290V mutation was detected in all populations including all nine Chinese populations (mean allele frequency = 0.58; range 0.46–0.68), the African populations (0.5 for Uganda and 0.47 for Malawi), and the Brazilian population (0.23). A201S mutation was present in seven of nine Chinese populations (mean allele frequency = 0.07, range 0–0.23), in populations from both African

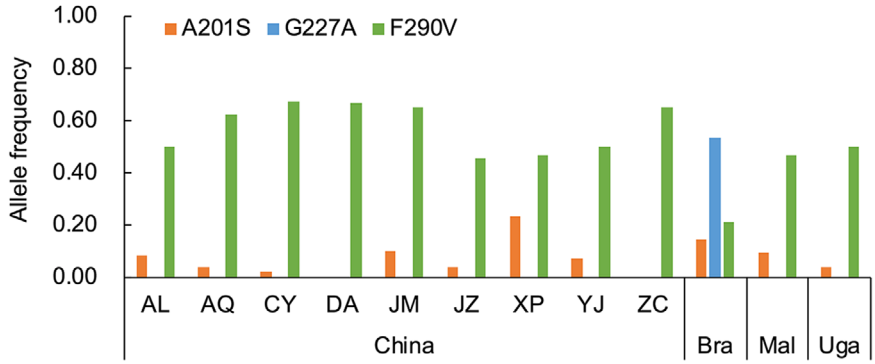

Fig. 2 Frequencies of the point mutations of the acetylcholinesterase-1 gene (*ace-1*) of *Spodoptera frugiperda* from China, Brazil (Bra), Uganda (Uga) and Malawi (Mal). Nine Chinese populations are: Cangyuan (CY), Yuanjiang (YJ) and Xinping (XP) from Yunnan, Anlong (AL) from Guizhou, Jiangmen (JM) and Zengcheng (ZC) from Guangdong, Dean (DA) from Guizhou, Jingzhou (JZ) from Hubei, and Anqing (AQ) from Anhui provinces.

nations (0.09 for Malawi and 0.04 for Uganda), and the Brazilian population (0.14). The G227A mutation was only detected in the Brazilian population with an allele frequency of 0.54.

The Brazilian population had all three *ace-1* point mutations and the G227A allele was the most common resistance allele. Seven of the nine populations from China, as well as populations from both Malawi and Uganda harbored two of the three *ace-1* point mutations (A201S and F290V) with the F290V allele being the most common. The low A201S allele frequencies across all sampled invasive populations suggested that the majority of these resistant FAW possessed only the F290V resistance allele.

However, none of the mutations in the other two resistance genes (*VGSC* and *RyR*) were found in our samples from the species' native range of Brazil, and the East African (i.e., Malawi, Uganda) and Asian (i.e., China) invasive ranges.

Verification of the mutations with Sanger sequencing

Using direct Sanger sequencing of PCR products, we genotyped all 108 individuals from China for the *ace-1* mutations. The A201S and F290V were detected by sequencing a PCR-amplified fragment harboring all three mutation sites (Fig. 3). The result of positive A201S and F290V mutations detected by WGS was consistent with that derived from the Sanger sequencing. No significant detection error was observed and suggested that the WGS-based detection was suitable and effective for detecting the multiple resistance mutations in the present study. The negative results for the other mutations in *VGSC* and *RyR* were also confirmed by Sanger sequencing.

Detection of a novel Cry1F resistance allele of ABCC2 from Brazil

From two (rCC5 and rCC25, Table S1) of the four Brazilian individuals collected in 2019, the WGS approach identified a 12 bp insertion at exon 15 of the *ABCC2* locus, leading to a premature stop codon (Fig. 4A). PCR amplification followed by Sanger sequencing confirmed the 12 bp insertion is heterozygous in both Brazilian individuals. Screening of the *ABCC2* locus in all invasive populations as well as all Brazilian FAW individuals except for rCC5 and rCC25 did not detect this novel *ABCC2* allele or the three other major resistance alleles in FAW.

Our novel INDEL mutation represents another mutation in the *ABCC2* gene in addition to those previously identified in FAW (Banerjee *et al*., 2017; Flagel *et al*., 2018; Boaventura *et al*., 2019) and which we designate as r4. The 12 bp insertion was detected at the intracellular loop between transmembrane domains (TMDs) VIII and IX, potentially resulting in the loss of TMDs IX, X, XI, and XII, as well as the adenosine triphosphate-binding cassette 2 and the carboxyl terminus (Fig. 4B).

Discussion

Previous studies (Liu *et al*., 2019; Zhang *et al*., 2019; Jing *et al*., 2020) showed that FAW carrying markers associated with both corn and rice host strains have been detected in China. Our results showed that, at least at the *Tpi* locus, the corn strain was most commonly identified,

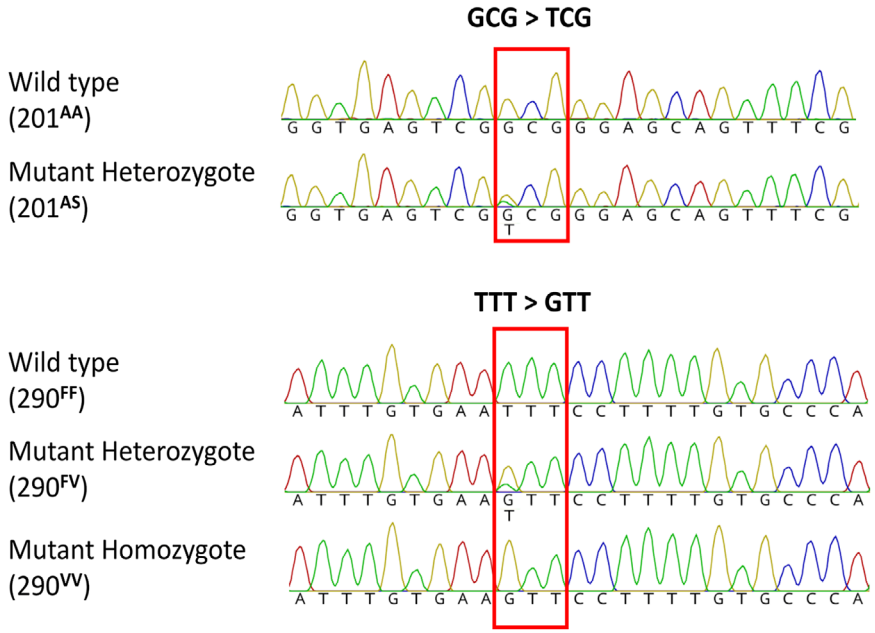

Fig. 3 Sequencing chromatograms of genomic DNA fragments, including the A201S and F290V mutation sites in the acetylcholinesterase-1 gene (*ace-1*) of *Spodoptera frugiperda* in China. The triple codons corresponding to the mutations are boxed.

and the rice strain was only present in a small fraction of individuals from China (1/108, 0.92%). The proportion of heterozygous individuals was also small (2/108, 1.83%), significantly lower than the resequencing data of Jing *et al*. (2020). Identifying hybrids in globally invasive insect pests is challenging and is ideally accomplished using genome-wide SNPs (e.g., *H. armigera*/*H. zea*, Anderson *et al*., 2016; Anderson *et al*., 2018; Valencia-Montoya *et al*., 2019; *Bemisia* cryptic whiteflies species, Elfekish *et al*., 2018, 2019) and interpretations based on a SNP such as is widely used for FAW based on the single *Tpi* exon4 nt370 marker should proceed with caution.

Mutations in the acetylcholinesterase gene conferring resistance to organophosphate insecticides have been documented in several insect species (Andrews *et al*., 2004; Baek *et al*., 2005; Cassanelli *et al*., 2006; Hsu *et al*., 2006; Lee *et al*., 2007; Wu *et al*., 2015). Three mutations in this gene (A201S, G227A and F290V) found in a Brazilian population of *S. frugiperda* were correlated with an 18.1-fold resistance to the organophosphate chlorpyrifos (Carvalho *et al*., 2013). Among the three mutant alleles, the G227A allele was the most common (67.5%), while the F290V (32.5%) and A201S (17.5%) alleles were at relatively lower frequencies (Carvalho *et al*., 2013). In the present study, a similar frequency distribution (54% for G227A, 21% for F290V, and 14% for A201S) was observed in the three mutant alleles of *ace-1* in our Brazilian laboratory-maintained FAW population. In contrast, only two alleles (A201S and F290V) were present in the populations from China, Uganda and Malawi and the F290V allele was at higher frequencies, suggesting a different distribution pattern for the resistance alleles from the Brazilian population we tested.

The mutation frequencies of A201S on the *ace-1* gene of rice stem borer, *Chilo suppressalis* were strongly correlated with levels of resistance to the organophosphate triazophos (Jiang *et al*., 2009). The A201S and G227A mutations in the *ace-1* gene of *P. xylostella* were identified to confer resistance to organophosphates (Baek *et al*., 2005; Lee *et al*., 2007; Sindhu *et al*., 2018). In the codling moth, *Cydia pomonella*, F290V in *ace-1* could confer a low level of resistance (6.7-fold) to organophosphate azinphos-methyl but a high level of resistance (130-fold) to carbamate carbaryl (Cassanelli *et al*., 2006). This evidence suggests that A201S, G227A or F290V could confer resistance alone or enhance resistance in combination. The ace-1 mutations detected in the Chinese and African populations are most likely carried from the New World native populations and the observed frequency is not dramatically different to the Brazilian population. However, future local selection could rapidly increase the frequencies of these resistance mutations, so organophosphate and carbamate insecticides should be used with caution if at all.

A range of previous studies have shown that the mutations in *VGSC* and *RyR* were involved in resistance

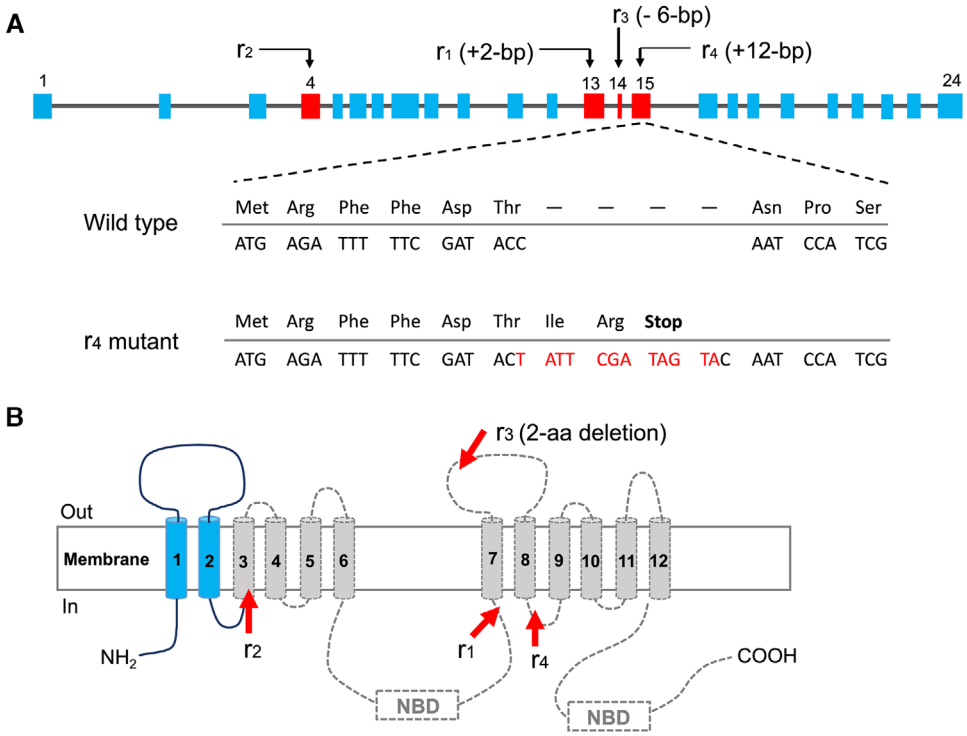

Fig. 4 Four mutant alleles (r_1–r_4) of *SfABCC2* associated with Cry1F resistance in *Spodoptera frugiperda*. (A) Genomic structure of *SfABCC2*. (B) Protein structure of SfABCC2. The r_1, r_2 and r_3 alleles were reported respectively by Banerjee *et al*. (2017), Flagel *et al*. (2018) and Boaventura *et al*. (2019). The r_4 allele was detected in the present study.

to pyrethroid and diamide insecticides respectively (Sattelle *et al*., 2008; Dong *et al*., 2014; Silver *et al*., 2014). T929I, L932F and L1014F of *VGSC* were detected at low allele frequencies (5% for each mutation) in the PYR strain of *S. frugiperda* from Brazil with ~30-fold resistance to lambda-cyhalothrin (Carvalho *et al*., 2013). A recent study documented that the I4734M substitution in *S. frugiperda RyR* confers high levels of resistance to diamides in a laboratory-selected *S. frugiperda* strain from Brazil (Boaventura *et al*., 2020). A G4946E mutation causing diamide resistance was validated with the reverse genetic approach in *Spodoptera exigua* (Zuo *et al*., 2017). However, neither of these resistance-conferring mutations in *VGSC* or *RyR* was detected in the populations of *S. frugiperda* screened in this study. Metabolic mechanisms cannot be excluded so it will be necessary to bioassay sensitivity to pyrethroids and diamides in these field populations and then decide to employ the insecticides with little or no resistance in the field.

The WGS-based detection method we used is time- and cost-effective and can identify the pest species, provide information on the spread and population genomics, as well as detecting multiple resistance mutations simultaneously. In the present study, consistent results between WGS-based and traditional PCR-based methods indicate the robustness of the WGS-based method we developed for detection of the single-base mutations in insecticide target sites in *S. frugiperda*. Furthermore, not only is it able to detect previously identified mutations, but this approach also allows the identification of new candidate resistancemutations such as the potential novel Cry1F resistance allele in the *ABCC2* gene. Field FAW populations likely have diverse resistance alleles in the *ABCC2* gene, so a WGS-based method is a valuable approach for the simultaneous detection of known and potential resistance alleles. Increasing evidence suggests that splice variation may be responsible for some types of Bt resistance (e.g., Fabrick *et al*., 2014; Mathew *et al*., 2018). WGS is limited in its ability to detect this type of resistance directly but does provide a dataset to return to for further analysis. Monitoring for changes in these resistance allele frequencies may indicate imminent failures/diminishing efficacies of the toxin in Bt crops (Downes *et al*., 2016).

Molecular characterization of resistance loci in the native and invasive populations can help in understanding the origins of invasive insect pests, as demonstrated by Walsh *et al*. (2018) using the *CYP337B3* locus for the cotton bollworm *H. armigera* in Brazil. We cannot use

the resistance alleles and allele frequencies to identify potential origins of the invasive FAW as we do not have sufficient data across the native range.

Partial mitochondrial DNA genes together with partial *Tpi* gene characterization of FAW populations from the New World and various sub-Saharan African countries have suggested limited introduction of FAW into Africa and point toward Florida and the Greater Antilles as a source population (Nagoshi *et al*., 2018, 2019). For the related noctuid *H. armigera*, the use of multiple partial genes as markers and/or single gene incorporating simulation have enabled inference of potential incursion pathways (e.g., trade-related, Tay *et al*., 2017; and introduction frequencies, Arnemann *et al*., 2019). Nevertheless, there is increasing use of WGS approaches, as exemplified by this study, for understanding invasive populations' resistance profiles and to identify novel resistance alleles. Such studies of the FAW and in other invasive agricultural pests will provide even greater confidence in the understanding of likely population origins, potential introduction pathways or natural dispersal (Anderson *et al*., 2016; Elfekih *et al*., 2018), and frequencies of the introductions that led to the successful establishment of this dominant and formidable lepidopteran pest in the Old World and Oceania.

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Disclosure

The authors declare they have no conflicts of interest.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1 Basic information of the *Spodoptera frugiperda* samples detected in this study.

Table S2 Substitution variations in the ace-1 of *Spodoptera frugiperda* samples called with GATK software.