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Population genetics reveal multiple independent invasions of *Spodoptera frugiperda* (Lepidoptera: Noctuidae) in China

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Research Paper

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Abstract

The fall armyworm (*Spodoptera frugiperda*), a destructive pest that originated in South and North America, spread to China in early 2019. Controlling this invasive pest requires an understanding of its population structure and migration patterns, yet the invasion genetics of Chinese *S. frugiperda* is not clear. Here, using the mitochondrial cytochrome oxidase subunit I (*COI*) gene, triose phosphate isomerase (*Tpi*) gene and eight microsatellite loci, we investigated genetic structure and genetic diversity of 16 *S. frugiperda* populations in China. The *Tpi* locus identified most *S. frugiperda* populations as the corn-strains, and a few were heterozygous strains. The microsatellite loci revealed that the genetic diversity of this pest in China was lower than that in South America. Furthermore, we found moderate differentiation among the populations, distinct genetic structures between adjacent populations and abundant genetic resources in the *S. frugiperda* populations from China sampled across 2 years. The survival rate of *S. frugiperda* was significantly higher when it was fed on corn leaves than on rice leaves, and the larval stage mortality rate was the highest under both treatments. Our results showed that *S. frugiperda* probably invaded China via multiple independent introductions and careful pesticide control, continuous monitoring and further studies will be needed to minimize its potential future outbreak.

Introduction

The migration of many insects will advocate escaping a deteriorating environment, finding new resources, and avoiding competition and natural enemies (Dingle, 1972; Westbrook *et al.*, 2016; Smith *et al.*, 2020). Among the features that determine whether the invasion is successful, genetic characteristics are among the most important (Lee, 2002; Zhang *et al.*, 2019). For instance, reduced genetic diversity is the general trend in a single long-distance invasive event, while not in multiple invasions from different sources (Wilson *et al.*, 2009). The mechanisms underlying this phenomenon, which in turn may facilitate adaption, include admixing, the emergence of cryptic genetic variation and hybridization (Yang *et al.*, 2012; Parepa *et al.*, 2014; Exposito-Alonso *et al.*, 2018). Therefore, understanding the impact of evolutionary features on the population structure and diversification of an invading pest can improve our ability to predict its population dynamics changes (Sakai *et al.*, 2001; Porretta *et al.*, 2007). Such information is also critical to preventing further introductions of invasive species, as well as to control or eradicate them (Wei *et al.*, 2013; Arias *et al.*, 2019).

The fall armyworm, *Spodoptera frugiperda* (J. E. Smith) (Lepidoptera: Noctuidae), is a native crop pest in South and North America (Bentivenha *et al.*, 2017). *Spodoptera frugiperda* adults have the capacity for long-distance migration (Early *et al.*, 2018; Westbrook *et al.*, 2019). The detection time-line suggested that this pest invaded Africa in 2016 (Goergen *et al.*, 2016; Otim *et al.*, 2018) and had gradually invaded many Asian countries from Africa in 2018 (CABI, 2019). Since then, fall armyworm was assumed to have invaded Pu'er, Yunnan Province of China in January 2019 from Myanmar (Wu *et al.*, 2019; Zhang *et al.*, 2019) and soon was reported from 25 other provinces of China with outbreaks of varying degrees in just a few months. Crop cultivation has provided large amounts of food sources for humans, while *S. frugiperda* caused major harm to a large number of crops, especially corn (Sena *et al.*, 2003; Casmuz *et al.*, 2010). In China, this invasive pest has spread to more than 2.84 million acres, and the actual damage area was 0.41 million acres just in 2019 (MARA, 2019).

Two kinds of strains, the corn-strain and the rice-strain of *S. frugiperda*, have been identified (Pashley *et al.*, 1985). The corn-strain prefers corn and sorghum leaves, while the rice-strain prefers to eat rice and turfgrass (Pashley and Martin, 1987; Meagher and Nagoshi, 2004; Nagoshi and Meagher, 2004). Interestingly, these two types cannot be effectively distinguished by morphology and host plants (Nagoshi *et al.*, 2012), while molecular methods, including the analysis of genetic polymorphisms and mitochondrial haplotypes, can reliably distinguish them (Lu *et al.*, 1992).

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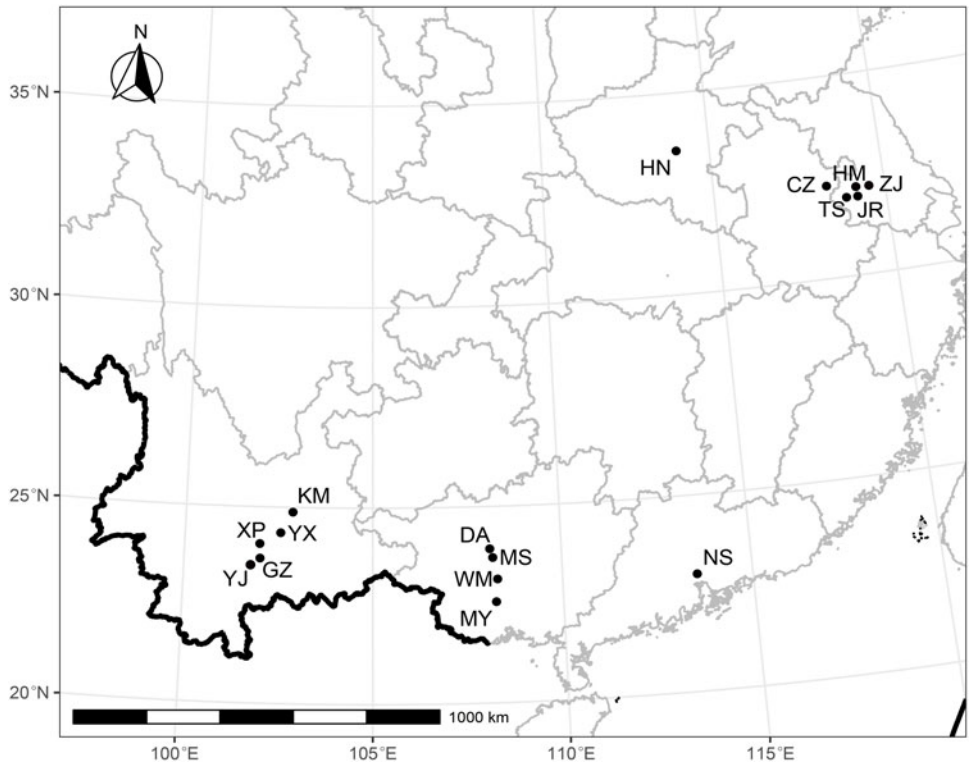

Figure 1. Map of sampling sites of *S. frugiperda* in China.

Mitochondrial DNA (mtDNA) sequencing as a genetic tool can reveal the origins of invasive populations, genotypes and paths of migration (Behura, 2006; Nagoshi *et al.*, 2007a; Valade *et al.*, 2009). The mitochondrial cytochrome oxidase subunit I (*COI*) gene was one of the most commonly used molecular markers in mtDNA. Simple sequence repeats (*SSR*) markers (Arias *et al.*, 2011; Pavinato *et al.*, 2013), have also been frequently used as a marker method for agricultural pests to study genetic diversity and genetic structure. *SSR* markers have the advantage of detecting high levels of polymorphism even for closely related individuals (Chen and Dorn, 2010). For instance, studies in South America have distinguished haplotypes and studied the hybridization, structure and gene flow pattern of *S. frugiperda* through the *COI* gene and *SSR* markers (Pair *et al.*, 1986; Nagoshi *et al.*, 2007b; Arias *et al.*, 2019). Furthermore, polymorphisms in triose phosphate isomerase (*Tpi*) gene (Nagoshi, 2010) can identify specific host strains of *S. frugiperda* (Nagoshi, 2012; Nagoshi and Meagher, 2016). Therefore, it is first necessary to know its genetic diversity, genetic structure and diffusion pathways in controlling *S. frugiperda* of China.

In this study, we investigated the genetic structure and genetic diversity of 16 *S. frugiperda* populations in China by analyzing the *COI* and the *Tpi* gene, and by inference using microsatellite loci. To better understand the feeding behaviors of *S. frugiperda*, this pest was fed on both rice and corn leaves. Our goals were to determine (1) the genetic differentiation and diversity, (2) possible invasive patterns and (3) feeding behaviors of this pest in China.

Materials and methods

Sample collection and DNA extraction

All 322 *S. frugiperda* samples were sampled in Yunnan Province (population numbers $n = 5$), Guangxi Province ($n = 4$), Jiangsu Province ($n = 4$), Anhui Province ($n = 1$), Henan Province ($n = 1$) and Guangdong Province ($n = 1$) (fig. 1 and Table S1). These samples were collected on corn except for two sites (Yuxi, Xinping) where larvae were collected on sorghum. Samples were collected between May 2019 and October 2020 (Table S1), when *S. frugiperda* migrated rapidly across the Chinese mainland. All *S. frugiperda* larvae were put in 1.5 ml tubes containing 95% ethanol and stored at −20°C. DNA was extracted with the Wizard SV Genomic DNA purification System (Promega) according to the manufacturer's instructions.

Identification of S. frugiperda *strains through* COI *and* Tpi *genes*

A mtDNA *COI* gene fragment was amplified in all *S. frugiperda* individuals ($n = 322$) and sequences searched against National Center for Biotechnology Information (*NCBI*; https://www.ncbi.nlm.nih.gov/genbank/) database for species identification (Table S2) (Levy *et al.*, 2002; Nagoshi *et al.*, 2007b). The Polymerase Chain Reactions (PCR) were conducted as described by Nagoshi *et al.* (2007b) The PCR mixture contained 12.5 µl 2 × Taq PCR Master Mix (+ Dye) (TSINGKE Biotechnology Co., Ltd. China), 0.5 µl of *COI*-893 sense, 0.5 µl *COI*-1303 primer antisense, 1 µl of DNA made up to 25 µl with ddH_2O. The PCR amplification conditions were set as follows: 94°C(1 min), 33 cycles at 92°C(45 s), 60°C(45 s), 72°C(1 min), and 72°Cfor 3 min as a final step. The PCR products were sequenced to obtain 410 bp fragments and SnapGene v.1.1.3 software (https://www.snapgene.com/) was used to detect the presence of EcoRV which only present in the rice-strain (Fig. S1). In this study, we compared the above 322 *COI* sequences with 55 *COI* sequences from Brazil (populations numbers $n = 2$) and Paraguay ($n = 4$) in South America from *NCBI* (accession numbers ranged from

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Table 1. The genetic diversity index of 16 *S. frugiperda* populations

Pop code	*N*	N_A	A_R	H_O	*He*	*F*	*I*	$\chi 2$
MS	22	7.750	5.625	0.318	0.690	0.504	1.782	90.619***
DA	18	8.125	5.788	0.292	0.775	0.625	1.565	122.755***
MY	10	6.000	6.000	0.313	0.704	0.538	1.729	109.546***
WM	22	8.750	6.000	0.335	0.741	0.560	1.483	105.756***
YX	15	7.125	5.875	0.283	0.739	0.583	1.260	114.278***
GZ	32	9.125	5.750	0.301	0.730	0.593	1.782	110.185***
YJ	32	8.750	5.500	0.316	0.695	0.516	1.565	122.198***
XP	22	8.375	6.000	0.284	0.741	0.629	1.729	128.953***
KM	24	6.500	5.000	0.271	0.674	0.584	1.483	112.34***
ZJ	11	5.000	4.891	0.239	0.639	0.635	1.260	115.239***
JR	20	6.250	4.875	0.294	0.688	0.544	1.641	106.027***
TS	24	4.875	3.625	0.224	0.626	0.632	1.693	82.031***
HM	12	5.375	4.875	0.333	0.678	0.493	1.594	107.137***
CZ	18	5.250	4.500	0.257	0.677	0.627	1.683	108.557***
HN	20	8.000	4.875	0.406	0.702	0.442	1.437	35.547**
NS	18	4.125	3.625	0.625	0.571	−0.080	1.214	79.611***
Average	20	6.836	5.175	0.318	0.692	0.527	1.354	

N, Sample number; N_A, Allele number; A_R, mean allelic richness over eight microsatellites (based on $n = 10$); H_O, Observed heterozygosity; *He*, Expected heterozygosity; *F*, Fixation Index; *I*, Shannon Index; *** : Significant deviation from Hardy-Weinberg equilibrium ($P < 0.001$).

MK372747 to MK372820). We used the Mega 7.0 (Kumar *et al.*, 2016) to align and trim all the mitochondrial sequences into 367 bp fragments based on the base quality and overlap *COI* region of Chinese and South American populations. The haplotype data file of the 367 bp fragments was generated using DnaSP v.6.0 software (Librado and Rozas, 2009). Finally, the haplotyping data results were imported into POPART software (Leigh and Bryant, 2015) to obtain the network of *COI* haplotypes. Besides, the *Tpi* $e4_{183}$ on the Z (sex) chromosome was selected for analysis (Fig. S2) and *Tpi* $e4_{183}$ SNP is C for corn strain (*Tpi-C*) or T for rice strain (*Tpi-R*) in females. For males, there are more possibilities of overlap in the PCR process, which possess an overlapping C and T at $e4_{183}$ (Nagoshi, 2010; Nagoshi *et al.*, 2017). In order to overcome this problem, two pairs of *Tpi* primers (Table S2) were used for PCR amplifications, including *Tpi*-282F, *Tpi*-412F and *Tpi*-850R (Nagoshi, 2010; Nagoshi and Meagher, 2016). PCR amplifications for all segments were performed in a 25 µl reaction (the same as above) in the follow conditions: initial denaturation at 94°C (1 min), followed by 33 cycles of 92°C (30 s), 56°C (45 s), 72°C (45 s), and a final segment of 72°C for 3 min.

Microsatellites markers and genotyping

Sixteen populations of *S. frugiperda* were amplified using 8 microsatellite loci (Table S3), four of which were described in Pavinato *et al.* (2013) and the other four loci were used in Arias *et al.* (2011) The Hardy-Weinberg and linkage disequilibrium were estimated using Fstat v.2.9.3 (Goudet, 2002). Null alleles were tested using Micro-Checker 2.2.3 (Van-Oosterhout *et al.*, 2004). Microsatellite fluorescently labeled primers were customed for 8 microsatellite loci: FAM for Spf01 and Spf06, HEX for Spf05 and Spf343, ROX for Spf09 and Spf670, TAM for Spf918 and Spf1502. Microsatellites loci were amplified in PCR reaction and the specific reaction system is as follows: 12.5 µl 2xTaq PCR Master Mix (+ Dye), 0.5 µl of primer sense, 0.5 µl primer antisense, 1 µl of DNA and made up to 20 µl with ddH_2O. PCR amplification procedure was the same as above (*COI* sequence) and the PCR products were sent to TSINGKE Company for polymorphism evaluation performed on an ABI PRISM 3730xl DNA Analyzer (Applied Biosystems®). Subsequent data processing and genetic polymorphism analysis were performed using GenAlEx 6.503 software (Peakall and Smouse, 2012) and FSTAT v.2.9.3 (Goudet, 2002). A Mantel test was performed between genetic distance and geographic distance to detect an Isolation-by-Distance (IBD) effect, with over 10^4 permutations of significance tests.

Population genetics and diversity analysis

Based on the eight microsatellite loci, 320 individuals from the 16 populations were selected for analysis (table 1). We calculated allele number (N_A), observed heterozygosity (H_O), expected heterozygosity (H_e), fixation index (F), SHANNON Index (I), effective number of migrants successfully entering a population per generation (Nm) among 8 loci and 16 populations using GenAlEx 6.503. Allele richness (A_R) of 16 populations were calculated by Fstat v.2.9.3. Tests of Hardy-Weinberg equilibrium were applied in Genepop version 4.7.5 (Rousset, 2008).

Analysis of Molecular Variance (AMOVA) and Principal Coordinates Analysis (PCoA) in 16 populations were obtained using GenAlEx 6.503. The Unweighted Pair-group Method with Arithmetic Means (UPGMA) cluster analysis of these populations was analyzed by MEAG7.0. Population differentiation including genetic differentiation coefficient (F_{ST}) and gene flow as estimated

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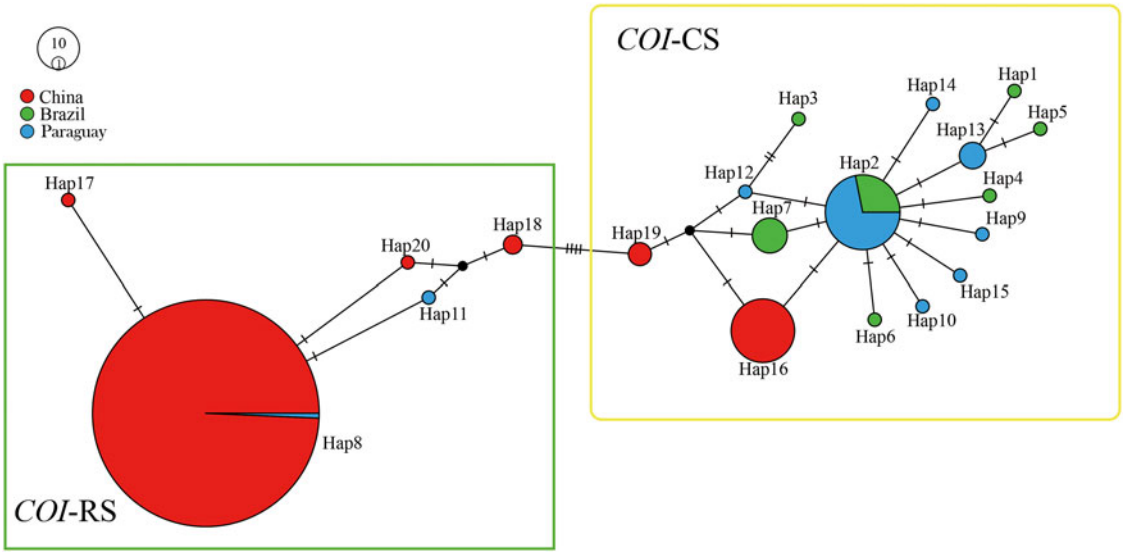

Figure 2. Haplotype network diagram based on *COI* fragments. Circles represent haplotypes and the sizes represent the frequencies. (China: red, Brazil: green, Paraguay: blue). COI-RS: rice-strains; COI-CS: corn-strains.

based on effective number of migrants (*Nm*) were obtained through *SSR* data (Pritchard *et al.*, 2000; Falush *et al.*, 2003, 2007). The ancestral population proportions of the invasive *S. frugiperda* were inferred using the STRUCTURE software with Length of Burin Period = 10^5 and Number of MCMC Reps length after Burin = 10^6. The K (Evanno *et al.*, 2005) values were set between 1 and 10 and repeated 10 times to obtain the possible number of populations. We performed Structure Harvester (Earl and vonHoldt, 2012) to identify the best *K* value and ran CLUMPP (Jakobsson and Rosenberg, 2007) to get *Q*-matrix files of groups and individuals, and then used Distruct 1.1 (Rosenberg, 2004) to draw the final figure.

The recent migration rates among the sixteen populations were inferred by BayesAss 3.0.4 software (Wilson and Rannala, 2003). Then we combined 10 long-running trace files to calculate mean migration with a burn-in of 5×10^7 according to Tracer 1.6 (Rambaut *et al.*, 2018), and plotted the heatmap of the migration rates matrix using Origin.2018 (https//www.originlab.com).

Bioassays

Additional *S. frugiperda* first instar larvae collected from Tangshan population (TS; see Table S1) in Jiangsu province were fed on corn leaves or rice leaves to examine their feeding characteristics. These first instar larvae originally fed on corn were individually put into test tubes to prevent them from attacking each other (Outer diameter × length: 18 mm × 180 mm) and then we randomly selected 40 developmentally synchronous larvae to feed on corn leaf disks (10 cm in length) and rice leaves (10 cm in length) respectively. Sufficient leaves from both rice and corn plant hosts were provided every other day to ensure the growth of larvae. We treated these larvae as the F1 generation and it took almost one month from hatching to laying eggs, so we obtained the F5 generation about five months later. The larvae were fed at 25 ± 0.5°C, and 60 ± 5% relative humidity and under a 14:10 (light:dark) condition (Busato *et al.*, 2005). The bioassays including survival and mortality were carried out with 40 samples from the egg stage to eclosion for each generation. To obtain egg production and hatchability, we put single female and male adults together under dark condition for 7 days (24 h × 7 days). Three replications were conducted for each treatment. All significant differences between groups and treatments were analyzed with the SPSS 21.0 software (IBM Inc., Chicago, IL, USA) by two-way ANOVA of variance and followed by the LSD test for multiple comparisons ($P < 0.05$). These experimental data mainly included egg production, hatchability and mortality of different generations under two feeding treatments and survival rate at different periods under two feeding treatments.

Results

COI *fragment haplotype network and molecular identification*

The haplotype network of 377 samples (322 individuals from China and 55 individuals from Brazil and Paraguay) based on *COI* sequences showed that the rice- and corn-strains were separated into two distinct groups (fig. 2). A total of 20 different haplotypes were found based on partial sequences of *COI* genes (*Hd* of *COI*-RS = 0.453, *Hd* of *COI*-CS = 0.639), with six haplotypes belonging to China, of which four (i.e., Hap8, Hap17, Hap18, Hap20) were rice (i.e., COI-RS; Fig. 2) and two (i.e., COI-CS; Fig. 2). The COI-RS Hap8 was the main haplotype detected in China of which also shared with Paraguay, which differed from the Paraguay Hap11 by a single substitutional step. In the COI-CS haplotype, Hap16 was the most common haplotype detected in China, and differed from Hap2 group consisted of native Brazil and Paraguay FAW also by a single base substitutional step.

We further used the *Tpi* gene for molecular identification, which is another method for typing *S. frugiperda*. *Tpi* $e4_{183}$ SNP is C for corn strain (*Tpi-C*) or T for rice strain (*Tpi-R*) and an overlapping C and T at $e4_{183}$ for heterozygous strain (*Tpi*-h). DNA from 50 larvae were randomly selected for PCR amplification followed by sequencing. Based on polymorphisms at the *Tpi* $e4_{183}$ SNP, 43 of 50 larval samples belonged to the corn strain (*Tpi-C*), six samples showed heterozygous polymorphisms (*Tpi*-h), and the remaining one was *Tpi-R*. The haplotype of more than 80% of the larvae was *Tpi-C* (fig. 3), which was consistent with samples collected from corn and sorghum fields.

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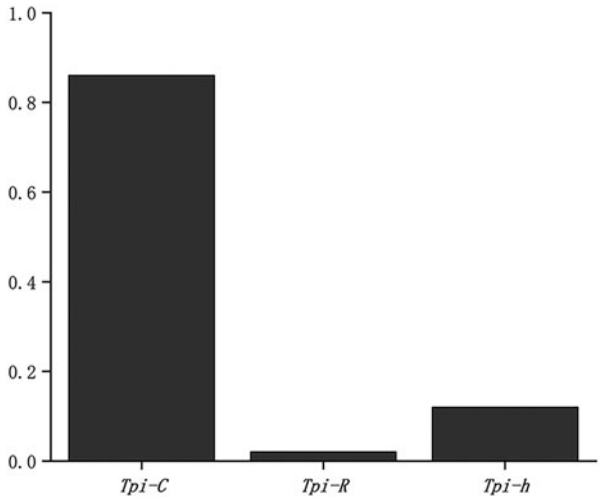

Figure 3. Frequencies (*y*-axis) of *Tpi* gene and haplotypes composition (*x*-axis).

Genetic diversity in different S. frugiperda *populations*

The genetic diversity of 16 *S. frugiperda* populations were analyzed based on microsatellite data. For these 320 individuals, the average number of effective alleles (N_A) of *S. frugiperda* among the 16 geographic populations was 6.836, ranging from 4.125 to 9.125 (table 1). The allele richness (A_R) per population ranged from 3.625 to 6.000 (table 1), indicating the alleles in the collection are abundant. Effective number of migrants (*Nm*) were > 1 (Table S4), which revealed sufficient gene flow to negate the effects of genetic drift (Wright, 1931). Observed heterozygosity (*Ho*) was lower than expected heterozygosity (*He*) in all populations except for NS (table 1). The apparent heterozygosity differed from the expected heterozygosity in most of the groups, revealed diminished genetic diversity and departures from the Hardy-Weinberg equilibrium which were verified by global tests in Genepop (table 1). The GZ population from Yunnan Province had more alleles and higher genetic diversity. Generally, gene flow and F_{ST} value are inversely proportional. F_{ST} ranged from 0.018 to 0.153 whereas the lowest gene flow was between ZJ and NS, and the highest between CZ and JR (Table S5).

Population structure of S. frugiperda

Based on STRUCTURE analysis, 3 genetic clusters ($K = 3$) can best explain the observed allelic frequencies of 10 populations collected in 2019 (fig. 4a and Fig. S3a). An admixture analysis of *S. frugiperda* genotypes with STRUCTURE revealed a strong genetic structure of 16 populations collected between 2019 and 2020 (fig. 4b). Delta K reached its maximum value at $K = 6$ (Fig. S3b). The composition of six different colors indicates that all samples can be divided into 6 groups, including the genetic characteristics of individuals from different geographical groups.

As YN was the first province in which *S. frugiperda* was found, it showed a high degree of admixture among five populations (fig. 4). GX and YN were the provinces that were initially invaded by *S. frugiperda*. The main clusters in these two provinces were different, which suggests that GX and YN were invaded by different sources. The population structures at the ZJ and HM sites in JS province were similar to the population structure of YN, which may suggest that JS and YN samples shared a common origin (fig. 4b). The TS population in JS province and the NS population in GD province consisted of almost a single color composition, while other populations were admixed. As we avoided sampling siblings by collecting one larva per plant (each plant was at least 1 m apart from the others), the single population composition maybe the result of additional single founder events derived from other independent invasions. The genetic structures of the four populations from different hosts (YX and XP on sorghum, ZJ and KM on corn) were basically similar.

Based on the results of BayesAss, most populations lacked strong recent gene flow (the mean value of m ranged from 0.0077 to 0.1889), except for a few with asymmetrical strong gene flow. For example, the migration rates were high from GZ to MY, from YJ to MS, from ZJ to MY, from TS to WM and from HN to YX (fig. 5), which was consistent with the STRUCTURE that the recipient populations exhibited mixed ancestral population proportions. The most mixed population proportions of XP, KM and GZ populations, without any signal of recent Chinese population immigration (fig. 5), may be the results of long-term gene flow before invading China. The recent migration rate detected in distantly separated populations (from HN to YN and JS to GX) might suggest recent gene flow in last several ancestors.

Principal coordinate and genetic distance analyses

A UPGMA cluster analysis using the *Nei's* standard genetic distance showed that the ZJ population in Jiangsu has the farthest genetic distance from the remaining 15 populations (fig. 6a and Table S6), while the shortest genetic distance existed in GZ and YJ, CZ and JR populations. In addition, no evidence of a correlation between genetic distance and geographic distance was found by the Mantel test based on an Isolation-by-Distance analysis in 2019 ($R^2 = 0.5718$, $P = 0.8973$) (Fig. S4a). Similarly, there was no correlation between genetic distance and geographic distance combining 2 years ($R^2 = 0.2467$, $P = 0.7517$) (Fig. S4b). According to a PCoA analysis (fig. 6b), the first two principal coordinates, PC1 and PC2, accounted for 19.83 and 17.44% of the molecular variation, respectively. ZJ population was far from other populations, suggesting it has a unique origin. CZ from Anhui was similar to JR and TS from Jiangsu because of the close distance and frequent gene flow between them.

Population differentiation

AMOVA results revealed that 51.9% ($P < 0.001$) of molecular variance among populations and 48.1% ($P < 0.001$) within populations (table 2). F_{ST} among the 16 populations revealed that 81.67% of *S. frugiperda* groups were moderately differentiated (Table S7).

Bioassay data analysis

For all five generations, egg production by adults that were fed on corn leaves (fig. 7a) or rice leaves (fig. 7b) was not significantly different by both the error bars and two-way ANOVA (LSD test: $F_{1,20} = 1.61$, $P = 0.219$). For each generation except F1, the hatchability rate was significantly higher for females fed on corn leaves than on rice leaves (LSD test: $F_{1,15} = 133.02$, $P < 0.05$; Fig. 7c). For each generation, the mortality of *S. frugiperda* fed on rice leaves was significantly higher than on corn leaves (LSD test: $F_{1,20} = 80.36$, $P < 0.05$; Fig. 7d). Throughout the developmental stages, the survival rate of *S. frugiperda* fed with

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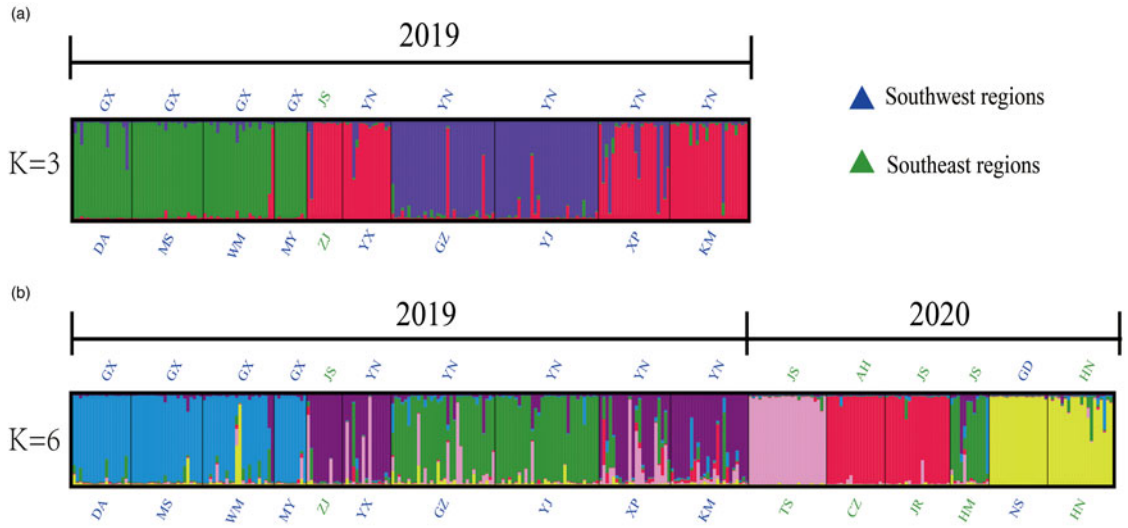

Figure 4. Genetic structure of *S. frugiperda* populations based on eight microsatellite markers. (a) The genetic structure of 10 *S. frugiperda* populations collected in 2019. (b) The genetic structure of 16 *S. frugiperda* populations collected in 2019 and 2020. The colored bars represent the composition of the inferred populations. red, P1; purple, P2; green, P3; blue, P4; pink, P5; yellow, P6. Labels above the colored bars represent provinces and labels below the colored bars represent population codes. Province: GX, Guangxi; JS, Jiangsu; YN, Yunnan; AH, Anhui; GD, Guangdong; HN, Henan. Population codes: DA, Duan; MS, Mashan; WM, Wuming; MY, Mingyang; ZJ, Zhenjiang; YX, Yuxi; GZ, Ganzhuang; YJ, Yuanjiang; XP, Xinping; KM, Kunming; TS, Tangshan; CZ, Chuzhou; JR, Jurong; HM, Huangmei; NS, Nansha; HN, HeNan (Luohe).

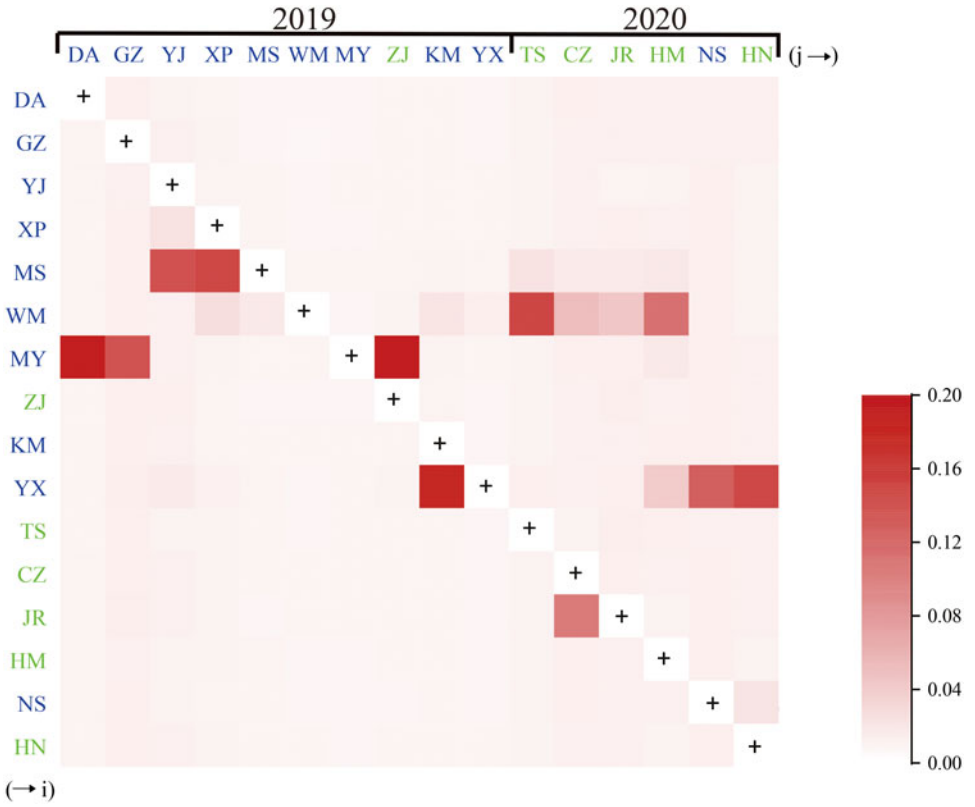

Figure 5. Heatmap of migration rate among 16 populations of *S. frugiperda* across China. Note that '*j* --> *i*' is the fraction of individuals in population *i* that are migrants derived from population *j* per generation. Dark color indicates that the gene flow level of the (*j*) – (*i*) population is high, while the light color is the opposite. The scale represents the range of migration rate among 16 populations.

corn leaves was significantly higher than that of *S. frugiperda* fed with rice leaves (LSD test: $F_{1,12} = 2569.82$, $P < 0.001$, fig. 7e). Whether feeding on rice leaves or corn leaves, there was an interaction between developmental stage and survival rate (LSD test: corn: $F_{2,12} = 38.17$, $P < 0.001$; rice: $F_{2,12} = 93.29$, $P < 0.001$, fig. 7e). The percentage of survival from egg stage to larval

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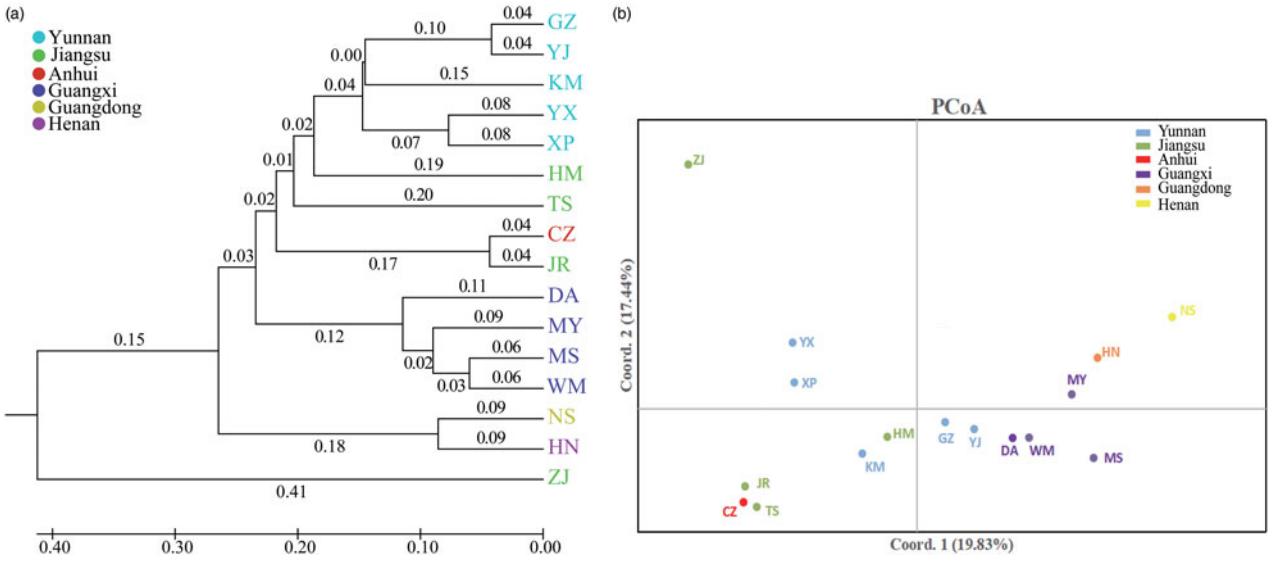

Figure 6. UPGMA and PCoA analysis of 16 *S. frugiperda* populations in China. (a) UPGMA cluster analysis using the Nei's standard genetic distance indices for *S. frugiperda*. (b) PCoA analysis in different sites of China.

Table 2. AMOVA analysis of the 320 *S. frugiperda* individuals using the *SSR* alleles

Source of variation	d.f.	Sum of squares	Var	Percentage of variation (%)
Among populations	15	684298.44	2193.23	51.9
Within populations	304	618875.60	2035.78	48.1
Total	319	1303174.04	4229.01	100

Fixation Index F: 0.519 $P < 0.001$.

stage decreased significantly than the other two stages according to the results of pairwise comparisons ($P < 0.001$ for both feeding treatments, fig. 7e).

Discussion

China is one of the world's largest corn and rice-growing countries due to its many regions that have a warm and suitable climate. As a result, these regions are ripe for colonization by *S. frugiperda*. Thus, further research on the migration and harm of *S. frugiperda* infestation is needed. The results of molecular identification of *S. frugiperda* using the *COI* gene showed that most of them were rice-strains and a few were corn-strains, while the identification results of the *Tpi* gene showed that corn-strains accounted for the majority and a small proportion were rice-strains. The conclusions drawn by the *Tpi* gene tend to be consistent with the sampled host plants, which suggests that the *Tpi* gene can be useful for the rapid and accurate identification of Chinese *S. frugiperda*. These larvae were all collected from corn and sorghum, but the rice-strain was also found, so we should pay more attention to the potential damage of this pest in rice fields.

Microsatellites are widely used molecular markers in population genetics and can be used to infer the invasion routes of many invasive insects (Yang *et al.*, 2012). Microsatellite *SSR* markers were used to study the current gene flow and genetic structure of different geographical populations of *S. frugiperda* in China. In a short period of time after being reported in China, five microsatellite loci of *S. frugiperda* showed moderate differentiation, and three loci showed obvious differentiation (Table S4). Compared with the genetic diversity of *S. frugiperda* in South America (Arias *et al.*, 2019), the higher average value of inbreeding coefficient and the lower mean allelic richness of Chinese invasive populations reflected the evolutionary history of this recently introduced pest with limited founding populations. To obtain more accurate population genetics of *S. frugiperda*, it will be necessary to analyze larger numbers of samples from a larger number of outbreak areas, especially Southeast Asia and northern China. Most of the *S. frugiperda* populations had moderate population differentiation which was much higher than that of the migrant white-blacked planthopper in local China (Sun *et al.*, 2014), raising the possibility that the populations of this notorious pest in China were not established by a single panmictic introduction. The *Nm* for the populations were >1 but < 4, suggesting that there was sufficient genetic diversity to nullify the effects of genetic drift however they were nevertheless not large enough to be considered as a panmictic population (Wright, 1931). Based on the moderate population differentiation and low recent gene flow among most populations, a scenario that multiple populations of *S. frugiperda* underpinned the pest's introductions to China and then spread across mainland China multiple times was a more parsimonious explanation than an assumption of a single introduction.

This scenario was further supported by the strong genetic structure of the 16 populations. At the early stage of the 2019 invasion, the strong and distinct genetic structure of populations in Yunnan province where *S. frugiperda* was first found in China (Wu *et al.*, 2019; Zhang *et al.*, 2019) suggested at least two independent groups had established in YN before 2020. The additional genetic composition of GX populations further suggested multiple sources of this invasive pest. There was a high possibility that these different invasive populations of YN derived from

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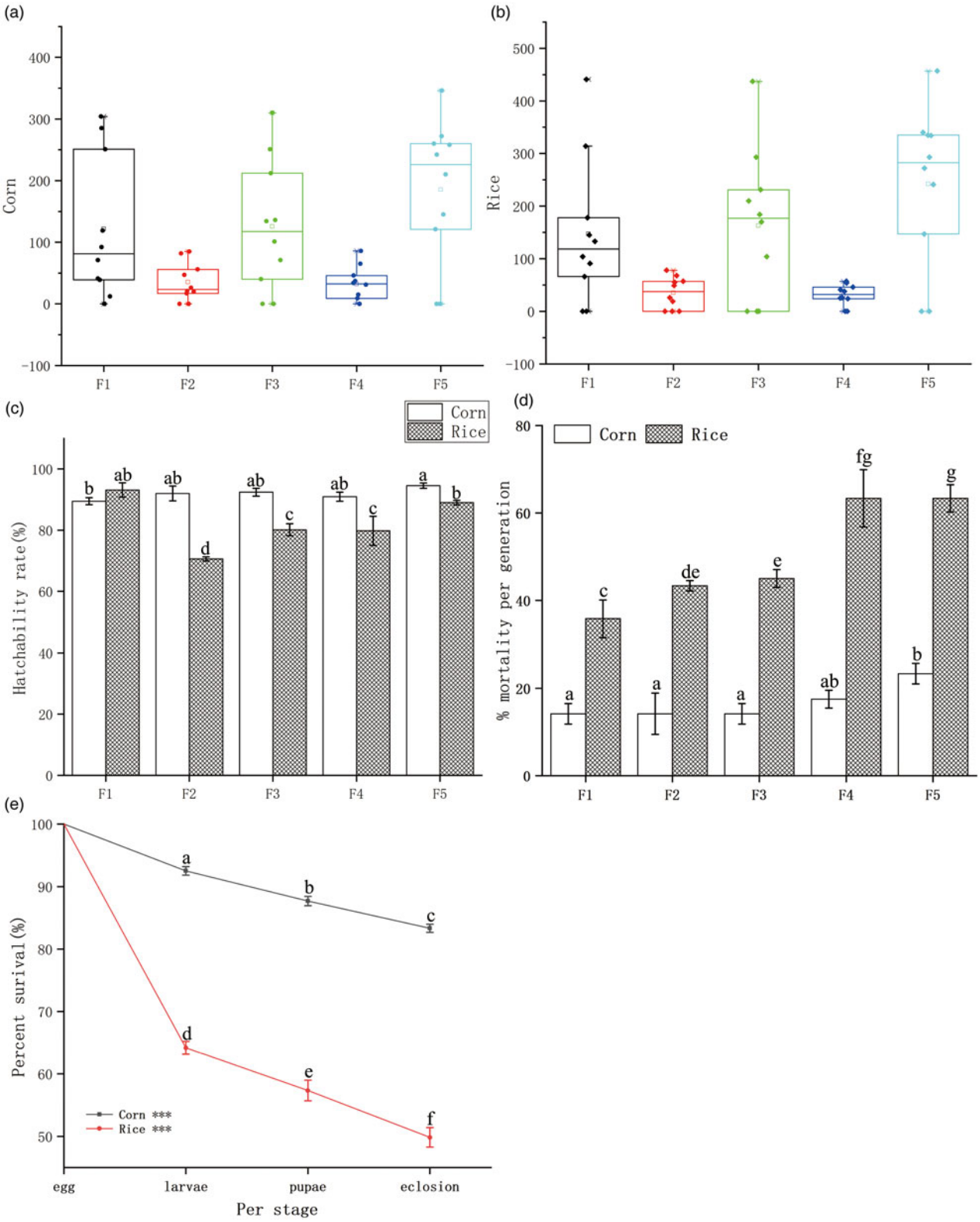

Figure 7. Ecological effects of *S. frugiperda* under rice and corn leaves feeding treatments. (a) and (b) Box diagram of egg production per generation under rice and corn leaves feeding treatments. (c) Hatchability rate per generation of *S. frugiperda.* (d) Mortality per generation of *S. frugiperda.* (e) The percent survival per stage of *S. frugiperda.* The letters above the error bars represent whether there is a significant difference between groups and treatments. Different letters represent there is a significant difference and containing the same letter indicates no significant difference between groups and treatments.

Myanmar based on the three-dimensional trajectory analytical approach (Chen *et al.*, 2020b). However, this conclusion needed more genetic evidence covering populations from the Southeast Asia. While adding 6 populations (TS, CZ, JR, HM, NS and HN) collected in 2020, 3 more genetic compositions were suggested by STRUCTURE (best $K = 3$ vs. $K = 6$, fig. 4b). As new

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population structure formation typically required many generations under limited gene flow, the new genetic structure of TS, CZ, JR, NS and HN populations indicated complex population evolutionary history and insufficient field sampling of *S. frugiperda* in 2019. The consistent genetic competent between HM population in 2020 and YN populations in 2019 suggested that the HM population potentially originated from Yunnan province. The obvious differences in the population genetic structures in Guangxi and Yunnan possibly corresponded to the previously two predicted migratory routes in China originated in Myanmar and Indochina respectively (Chen *et al*., 2020a; Li *et al*., 2020), but these invasive populations couldn't be the descendants of a single introduction linked to Africa, further suggesting likely independent introduction of *S. frugiperda* in Southeast Asia. Even if there was no gene flow between the above-mentioned different sources of *S. frugiperda,* the new composition of populations in 2020 could not have been formed in such a short time. Hence, there were at least three independent introductions of *S. frugiperda* into China after our sampling in 2019. Moreover, distantly separated populations (HN, NS; ZJ, YX) appeared to share the same hypothetical ancestral genetic clusters, which may mean that these populations have similar origins. Four populations separated by short distances (TS, JR; HM, ZJ) exhibited distinct genetic background, which further indicated the complexity of the genetics of the Chinese *S. frugiperda* populations, and suggested this region of the country could represent a biosecurity hotspot for the accidental introductions of this and potentially other alien species. Despite of invading one year earlier, the HM and ZJ populations that shared the same ancestors with Yunnan did not become the major *S. frugiperda* in Southeast of China, which could be due to potentially high levels of genetic variability of this pest in Asia, assuming the establishment of Chinese populations involved other Asian populations (Wu *et al*., 2021), that there were other possible origins beyond Myanmar and Indochina, as well as agricultural trades with other countries including direct trade between China and *S. frugiperda*'s native range countries. All these hypotheses need a broader population genetic studies and if possible, using the whole genome SNPs. The genetic distance between different populations was demonstrated by UPGMA and PCoA results (fig. 6). Both UPGMA and PCoA analyses revealed a close genetic relationship in adjacent populations as well as some geographically distant populations, such as NS and HN, which was likely caused by long-distance migration or separate introductions from the same source populations of *S. frugiperda.* All the populations in the southeast of China, except for HM, exhibited large genetic distance (fig. 6) and distinct population structure with southwest, indicating the invading of *S. frugiperda* from new sources had continued in 2020.

Associating with Asian monsoon climate, *S. frugiperda* relied on the southeast wind to damage the domestic crops along two paths northward in summer and with the effect of the northwest wind. Meanwhile, this pest does not survive prolonged freezing, which explains why it had not gone beyond Yunnan and Guangxi provinces which are the northern boundary of overwintering *S. frugiperda* in China. Because *S. frugiperda* is severely destructive and does not undergo diapause, it is vital to do a long-term study on *S. frugiperda* of these two provinces in the future. The invasion of *S. frugiperda* in China caused serious damage to different domestic crops in just one year. In early 2020, *S. frugiperda* broke out again in Yunnan and Guangxi when control measures were insufficient (Cui *et al*., 2020). Besides, this pest in Guangxi, Yunnan, Jiangsu, Anhui, Guangdong and Henan had six different regional structures (fig. 4b), which was different from another migratory pest *Plutella xylostella* which is distributed in different areas of China (Wei *et al*., 2013). Due to the abundant genetic resources of the invasive *S. frugiperda* populations, insecticides should be carefully applied in pest control to avoid the rapid development of resistance. For well-established populations in Guangxi and Yunnan, comprehensive measures combining prevention and pesticide control should be adopted. In other crop-growing areas in China, especially cornfields, monitoring and prevention throughout the year will be extremely important.

Combining genetics and ecology, we can understand this pest from multiple perspectives and provide guidance for the follow-up pesticide control. In the host leaf-feeding bioassay experiment, the survival rate of *S. frugiperda* was higher when fed on corn leaves than on rice leaves ($P < 0.001$, fig. 7d and e) suggesting that this pest had a higher fitness on corn leaves than on rice leaves. This could explain why the rice-strain showed a preference to feed on corn leaves. Moreover, the larval stage had the highest mortality rate under both treatments, which indicated the importance of this stage for the prevention and elimination of this pest. Therefore, future biological indicators researches (e.g., weight of larvae at different stages) and the development of pesticides should target the larval stages that could lead to more effective control of *S. frugiperda*.

In conclusion, this population genetic analysis revealed distinct population structures in field populations of *S. frugiperda* to support multiple introductions of this pest in China, and revealed potential biosecurity weakness at regional levels underpinned the spread of this pest. Further studies with more samples and loci from the Americas, Asia, and Africa will be necessary to better understand its outbreak process, and to help with the development of effective and sustainable management strategies for this pest.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S0007485322000190

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