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Chapter 4

Genetic basis of strain-specific female sex pheromone differences in *Spodoptera frugiperda* (Lepidoptera: Noctuidae)

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Abstract. The fall armyworm, *Spodoptera frugiperda*, consists of two distinct strains, the corn- and the rice-strain, which exhibit different prezygotic isolation mechanisms and seem to be in the process of ecological speciation in sympatry. Recent studies found that females of both strains showed significant differences in their sex pheromone composition and strain-specific sexual communication is considered as possible prezygotic isolation barrier between both strains. In this study, we investigated the inheritance of strain-specific sex pheromone blends by comparing the sex pheromone of pure strain, hybrid and backcross females. Furthermore, we conducted a QTL analysis and mapped different candidate genes to QTLs involved in the strain-specific production of (Z)-9-tetradecenyl acetate (Z9-14:OAc), (Z)-11-hexadecenyl acetate (Z11-16:OAc), (Z)-7-dodecenyl acetate (Z7-12:OAc), and (Z)-9-dodecenyl acetate (Z9-12:OAc). We found that all four pheromone compounds were differentially inherited and multiple factors influenced their inheritance. The QTL analysis showed that multiple genomic regions on 9 different chromosomes determined the strain-specific female sex pheromone. For three pheromone compounds, i.e. Z9-14:OAc, Z7-12:OAc and Z9-12:OAc, we found the involvement of one minor QTL each, whereas a total of seven different QTLs were significantly correlated with differential relative amounts of Z11-16:OAc. A delta-11-desaturase (*SfLPAQ*) mapped to one chromosome that explained a significant proportion of the variance in Z9-14:OAc and Z11-16:OAc, and showed the opposite-to-expected phenotypic pattern for both components. Interestingly, the circadian clock gene *vriille* (*SfVRI*), which appears to be responsible for strain-specific differences in the onset time of mating in the night, mapped to another chromosome that was involved in the production of the critical secondary sex pheromone component Z7-12:OAc. Our results suggest that two different prezygotic mating barriers in *S. frugiperda*, i.e. sexual communication and allochronic separation, may be genetically linked. If there is

genetic coupling of differential reproductive traits, evolution of prezygotic isolation in *S. frugiperda* could be facilitated.

Key Words. Fall armyworm, Corn- and rice-strain, QTL analysis, Pheromone extractions, Delta-11-desaturase, Delta-9-desaturase, *Vrille*, Prezygotic isolation, Timing of reproduction.

Introduction

Sex pheromones are commonly used amongst animals as premating signal to attract conspecific individuals for mating (Cardé and Minks, 1997; Rasmussen et al., 1997; Achiraman and Archunan, 2002, 2005; Rajanarayanan and Archunan, 2011; Buda et al., 2012). Within the order Lepidoptera, female moths usually produce species-specific sex pheromones in a pheromone gland to attract males over long distances (Cardé and Baker, 1984; Tamaki, 1985; Cardé and Minks, 1997; Cardé and Haynes, 2004). Because most adult moths are short-lived, a reliable sexual communication system between females and males is essential for the mating success and fitness of a species (Löfstedt, 1993; Cardé and Haynes, 2004). When the female pheromone signal changes due to mutations, the response of males needs to adapt to those changes or the mating partners will not find each other and reproduce. Due to this dependence of sender (female) and receiver (male), pheromone premating signals are expected to be under stabilizing selection (Löfstedt, 1993; Linn and Roelofs, 1995; Phelan, 1997). However, evolution solely under stabilizing selection processes is not able to explain the great diversification of moth species (Mitchell et al., 2000; Kristensen et al., 2007) and sex pheromones (El-Sayed, 2012). Other evolutionary forces like sexual selection (Coltman et al., 2002; Wade and Shuster, 2004; Andersson and Simmons, 2006; Irestedt et al., 2009; Sullivan-Beckers and Coccoft, 2010), genetic drift (Masel, 2011; Mendez et

al., 2011; Keller et al., 2012; Velo-Anton et al., 2012), or selection due to specific environmental factors like predators (Stowe et al., 1987; Haynes et al., 2002; Anton et al., 2011) or host adaptation (Nosil, 2007; Smadja and Butlin, 2009; Felix et al., 2011) may be able to act against stabilizing selection and generate diversification of moth pheromones. To understand the variability and the evolution of sexual communication systems in moths, intra- and inter-specific behavioral and genetic studies of sexual communication systems (premating signals and responses) are required.

An ideal model organism to study the evolution of sexual communication is the fall armyworm *Spodoptera frugiperda* (Lepidoptera: Noctuidae), which consists of a so-called corn-strain and rice-strain (Pashley, 1986). Both strains occur in sympatry in North and South America and hybridization rate between strains in the field of up to 16% (Prowell et al., 2004). Corn- and rice-strain populations seem to be in the process of sympatric speciation and exhibit several genetic and behavioral differences (Groot et al., 2010). Besides differential host plant preferences (Pashley, 1986; Meagher and Gallo-Meagher, 2003; Nagoshi et al., 2006; Nagoshi et al., 2007; Machado et al., 2008), both strains differ in their timing of mating in the night (Pashley et al., 1992; Schöfl et al., 2009), as well as in their female sex pheromone composition (Groot et al., 2008; Lima and McNeil, 2009; Unbehend et al., 2013).

The multi-component sex pheromone blend of *S. frugiperda* was first described by Tumlinson et al. (1986), consisting of the major pheromone component (Z)-9-tetradecenyl acetate (Z9-14:OAc), the critical secondary pheromone component (Z)-7-dodecenyl acetate (Z7-12:OAc), as well as other minor compounds like (Z)-11-hexadecenyl acetate (Z11-16:OAc). More than two decades later, Groot et al. (2008) reported the presence of strain-specific pheromone differences between

corn- and rice-strain females of laboratory populations. Corn-strain females had significantly higher relative amounts of Z11-16:OAc than rice-strain females, while rice-strain females exhibited higher relative amounts of Z7-12:OAc and (Z)-9-dodecenyl acetate (Z9-12:OAc) than corn-strain females (Groot et al., 2008). Similarly, pheromone extractions of *S. frugiperda* field populations confirmed the presence of corn- and rice-strain specific female pheromone blends (Unbehend et al., 2013). Based on a proposed pheromone biosynthesis pathway of *S. frugiperda* (Groot et al., 2008), different candidate genes like delta-9- or delta-11-desaturases could explain the pheromone differences between corn- and rice-strain females. Determining the genetic basis of strain-specific pheromone differences may help to understand how variability in a premating signal can arise.

A quantitative trait locus (QTL) analysis is a frequently used tool to determine the genetic basis of specific phenotypic traits in plants and animals (Gleason et al., 2005; Sheck et al., 2006; Groot et al., 2009; Manceau et al., 2011; Beecher et al., 2012; Merah et al., 2012). This genetic method has a wide area of applications ranging from the determination of genes responsible for favorable agricultural traits (Santos et al., 2012; Tsukazaki et al., 2012; Gao and Lin, 2013), up to the investigation of genes related to the evolution of species (Gleason et al., 2009; Groot et al., 2009; Gould et al., 2010; Lassance et al., 2010; Limousin et al., 2012). The advantage of working with a Lepidopteran model organism like *S. frugiperda* is the absence of crossing over in females (Heckel, 1993), which facilitates the genetic analysis of premating signals like sex pheromones because backcross females can be generated in which all markers on one chromosome co-segregate as one unit.

A recent QTL analysis of one prezygotic isolation barrier between both *S. frugiperda* strains, i.e. differential timing of reproduction in the night, showed that

the circadian clock protein *vriille* appears to be involved in the shifted onset time of mating between both strains in both sexes (Hänniger et al., 2013). Similar to the study conducted by Hänniger et al. (2013), the aim of this study was to investigate the genetic basis another potential prezygotic mating barrier, i.e. the strain-specific differences in pheromone composition of *S. frugiperda* corn- and rice-strain females. Therefore, we a) examined the inheritance of pheromone blends by comparing the pheromone phenotype of pure strain females (C, R), hybrid females (CR, RC) and backcross females (CR-R, CR-C, RC-R, RC-C); b) conducted a QTL analysis; and c) mapped different candidate genes to the QTLs involved in the differential production of four pheromone components (Z9-14:OAc, Z11-16:OAc, Z7-12:OAc, Z9-12:OAc).

Methods and Materials

Spodoptera frugiperda populations

To determine the genetic basis of strain-specific pheromone differences, we conducted experiments with a laboratory corn-strain and rice-strain population. Our laboratory corn-strain population originated from over 300 *S. frugiperda* larvae, which were collected in April 2010 from two corn fields in Santa Isabel in Puerto Rico (corn field1: +17° 59' 0.93", -66° 23' 29.88"; corn field 2: +17°57' 30.65", -66° 23' 32.43"). Our laboratory rice-strain population descended from around 300 larval specimens collected in May 2010 from a grass field at the Graham Farm in Moore Haven, Glades County in Florida (+26° 53' 3.04", -81° 7' 21.17"). All larvae were shipped to the MPICE and reared until adulthood on artificial pinto bean diet. To establish strain-specific colonies, the adults were screened for strain-specific COI markers (Nagoshi et al., 2006). Both populations were reared in climate chambers with reversed light:dark (L:D) cycle and 14:10 L:D photoperiod at 26°C

and 70% RH. Adults were fed with a 10% honey-water solution and random single-pair-matings were performed to maintain both populations.

Generation of backcrosses

We generated female-informative backcrosses to determine a) the pheromone composition of pure strain, hybrid and virgin females, as well as b) the genetic basis of strain-specific pheromone differences. Single pair matings between pure strain individuals were performed to generate F₁ hybrid females, which were then backcrossed to pure corn- and rice-strain males to produce different backcross families (Table 1). In the case of F₁ hybrid females, we crossed laboratory corn-strain females (6th generation) with rice-strain males (5th generation) to obtain CR hybrid females (first letter thus always referring to the female, second letter to the male), and rice-strain females (5th generation) were mated with corn-strain males (6th generation) to produce RC hybrid females (Table 1). One generation later, adult CR hybrid females were mated with either corn-strain males or rice-strain males to produce CR-C and CR-R backcross females (the first two letters of the backcross females refer to the mother, the last letter to the father of the female). Furthermore, RC hybrid females were backcrossed to corn-strain males and rice-strain males to generate RC-C and RC-R backcross females (Table 1). In total, we obtained five fertile CR-C backcross families, five CR-R families, one RC-C family and one RC-R family (Table 1). All individuals of two CR-C families (CR-C 2, CR-C 3), two CR-R families (CR-R 5, CR-R 19), the one RC-C family (RC-C 34) and the one RC-R family (RC-R 26) were reared until adulthood and used for pheromone extractions (see below). One CR-R backcross family (CR-R 19) was used for further genetic analysis.

Table 1 Generation of female informative *Spodoptera frugiperda* backcrosses

Female strain	Male strain	Generated offspring		No. paired	No. fertile	% fertile
Corn	Corn	C	Pure strains	97	54	56
Rice	Rice	R		75	48	64
Corn	Rice	CR	F ₁ hybrids	30	5	17
Rice	Corn	RC		16	5	31
CR hybrid	Corn	CR-C	Backcrosses	19	9	47
CR hybrid	Rice	CR-R		19	8	42
RC hybrid	Corn	RC-C		49	1	2
RC hybrid	Rice	RC-R		34	1	3

Pheromone extractions

To determine the pheromone phenotype of intrastain- and interstrain-specific crosses, pheromone extractions of virgin pure strain females (C and R), hybrid females (CR and RC) and backcross females (CR-R, CR-C, RC-C, RC-R) were performed. Pheromone glands of 3 days old corn-strain (6th generation) and rice-strain (5th generation) females were extracted during the scotophase according to strain-specific female calling times (corn-strain: 4 h, rice-strain: 6-7 h). CR hybrid females were extracted 3.5-5.5 h into scotophase at the age of 3 days and pheromone glands of 3 days old RC hybrid females were extracted 5.5 h into scotophase. Pheromone extractions of 2-3 days old backcross females were performed 4-7 hours into scotophase. Pheromone glands were excised from the female abdomen and singly placed into a glass vial containing 50 µl hexane and 125 ng pentadecane as internal standard. After an extraction time of 30 min, the gland was removed from the vial and the extract was stored at -20°C until gas chromatography analysis (see below).

Chemical analysis

Gas chromatography (GC) analysis was performed according to methods used by Unbehend et al. (2013), using a HP7890 gas chromatograph with a polar capillary column (DB-WAXetr; 30 m × 0.25 mm × 0.5 μm) and a flame-ionization detector (FID). Female pheromone extracts were reduced from 50 μl to 2 μl under a gentle stream of nitrogen. Together with 2 μl octane, the reduced pheromone extracts were singly transferred into a glass vial and injected into the gas chromatograph. Female pheromone compounds were identified by comparing retention times with synthetic standards of Z9-14:OAc, Z11-16:OAc, Z7-12:OAc and Z9-12:OAc, which were bought from Pherobank (Wageningen, the Netherlands).

DNA extraction and AFLP marker analysis

Genetic analysis was performed with one backcross family (CR-R 19), which was most fertile amongst all backcross matings and produced 159 offspring females. Out of all CR-R 19 backcross females, we selected 88 females for further AFLP analysis based on the amount of Z7-12:OAc found in the female glands. We chose 36 females which exhibited low amounts of Z7-12:OAc (1-2%), 16 females with medium amounts of Z7-12:OAc (~ 2.5%), and 36 females with high amounts of Z7-12:OAc (> 3.5%). We extracted DNA of the 88 selected CR-R females, their parents (CR hybrid female and rice-strain male) and their maternal grandparents (corn-strain female and rice-strain male). DNA extractions were performed as described by Unbehend et al. (2013), and 200 ng DNA of each sample was digested with EcoRI and MseI (New England Biolabs, Ipswich, MA, USA) at 37°C for 2 h, according to Wilding et al. (2001). After the restriction digest, EcoRI- and MseI-adapters were ligated to the EcoRI and MseI restriction sites and all DNA fragments that contained an adapter were preamplified (Wilding et al., 2001).

Table 2 Number of informative AFLP-makers scored per primer combination

MseI primer	EcoRI primer	No. markers
AAG	AAG	16
	ACG	18
	ACC	12
	ACT	10
ACA	AAG	14
	ACG	13
	ACC	13
	ACT	14
	CGA	10
	CGC	13
ACG	AAG	17
	ACG	13
	ACC	9
	ACT	9
AGG	AGG	13
	ACG	14
	ACT	15
CAT	AAG	12
	ACG	7
	CGA	11
	CGC	4
CGA	AAG	11
	ACG	10
	ACC	8
	ACT	5
CTG	ACG	12
Total markers		303

The preamplified DNA was diluted with ddH₂O (1:50) and selectively amplified with 26 specific EcoRI- and MseI-primer combinations, consisting of a core sequence (EcoRI-primer: 5'-GACTGCGTACCAATTC; MseI-primer: 5'-GATGAGTCCT-GAGTAA) plus three selective bases at the end of each primer (Table 2). The generated AFLP fragments were analyzed on a 6.5% polyacrylamide gel using a LI-COR 4300 DNA analyzer (LI-COR Biosciences,

Lincoln, NE, USA) according to methods by Groot et al. (2009). AFLP gels were scored with AFLP-Quantar Pro 1.0 (KeyGene, Wageningen, the Netherlands). To identify corn-strain specific markers, we searched for AFLP markers that were present in the grandmother (C female), the mother (CR hybrid female) and half of the offspring females (heterozygote CR-R females), but absent in the grandfather (R male), the father (R male) and the homozygote CR-R females. For identification of rice-strain specific markers, we scored markers present in the grandfather (R male), the mother (CR female) and half of the offspring females (homozygote CR-R females), but absent in the grandmother (C female) the father (R male) and the heterozygote CR-R females.

Genetic map construction and QTL analysis

We scored in total 303 AFLP-markers (Table 2), and constructed a linkage map with MapMaker 3.0 (<http://www.broadinstitute.org/ftp/distribution/software/mapmaker3/>). Using a LOD score of 10, 30 linkage groups were identified which refer to the 30 autosomes in the CR-R19 backcross family, because there is no crossing over in Lepidoptera females (Heckel, 1993). The chromosome names (chromosome 1 to 30) were chosen arbitrarily. To identify candidate QTL, we tested for each chromosome whether the homozygote CR-R backcross females had significantly different relative amounts of Z9-14:OAc, Z11-16:OAc, Z7-12:OAc and Z9-12:OAc than the heterozygote CR-R backcross females (see statistical analysis).

Mapping of candidate genes

To determine whether candidate genes that are likely involved in the pheromone biosynthetic pathway may be responsible for strain-specific pheromone differences, we mapped a delta-11-desaturase and a delta-9-desaturase to our

generated QTL map. Furthermore, we mapped the circadian clock protein *vriIle* to our map, which appears to be involved in the strain-specific differences of timing of reproduction in the night (Hänniger et al., 2013). Based on *Spodoptera* sequences (ESTs) published at NCBI (<http://www.ncbi.nlm.nih.gov/>), we designed primers for a delta-11-desaturase (*SfLPAQ*) and a delta-9-desaturase (*SfKPSE*). Based on *S. frugiperda vriIle* sequences obtained from SH (Hänniger et al., 2013), we designed primers for *vriIle* (*SfVRI*) (Table 3).

To identify SNPs in the candidate genes that could be used to map them onto our genetic map, PCR amplification of the different genes were conducted with the grandparents (C female, R male), the parents (CR hybrid female, R male) and 12 to 24 backcross females of the backcross family CR-R 19. PCR amplifications were performed using 1 µl DNA, 11.92 µl dH₂O, 2 µl 10x Taq buffer, 2 µl 2 mM dNTPs, 3 µl 10 mM primer mix (Table 3) and 0.08 µl Taq polymerase (Metabion, Martinsried, Germany). The thermo cycler program started with 2 min incubation time at 94°C, followed by 35 cycles of 45 s at 94°C, 45 s at the primer-specific annealing temperature T_a (Table 3), 60 s at 72°C and a final elongation at 72°C for 10 min. The generated amplification products were mixed with 3 µl loading dye and ran on a 1.5% agarose gel at 120 V for 2 h.

The obtained products were cut out of the gel and extracted with a QIAGEN gel extraction kit (QIAGEN, Hilden, Germany). After gel extraction, all products were sequenced using Sanger-sequencing, according to methods described by Vogel et al. (2011), and analyzed with Sequencher 4.10.1 (Gene Codes Corporation, Ann Arbor, MI, USA).

Table 3 Primer combinations and annealing temperatures (T_a) of candidate genes

Gene		Primer	T_a
Delta-11- desaturase	SfLPAQ	Forward: 5'AACATTTGGGGAAGGTTTCC	53°C
		Reverse: 5'CAAATGCAACATTATAAACTTCA	
Delta-9- desaturase	SfKPSE	Forward: 5'TCATTATGCCACGGTGATT	53°C
		Reverse: 5'ATGACAGTGAAAAGGAAGACAT	
Vrille	SVRI	Forward: 5'GAGGCGCTTCAATGACATGG	60°C
		Reverse: 5'GGCTCCTGCTTTATGTGCTGAA	

Statistical analysis

Statistical analysis was performed with R 2.5.0 (R-Development-Core-Team, 2007). Data of the female pheromone extractions were log transformed to stabilize the variance and analyzed using a generalized linear model (GLM). To identify candidate QTL, we conducted a 2-sided t-test, and we used a GLM to assess how much of the variance can be explained by the different QTL (R^2 value).

Results

Strain-specific pheromone differences

Corn- and rice-strain females exhibited significant differences in the relative amount of Z9-14:OAc, Z11-16:OAc, Z7-12:OAc and Z9-12:OAc (Fig. 1). While corn-strain females produced significantly higher relative amounts of Z11-16:OAc than rice-strain females, rice-strain females had larger relative amounts of Z9-14:OAc, Z7-12:OAc and Z9-12:OAc than corn-strain females (Fig. 1).

Inheritance of pheromone compounds

We found differential modes of inheritance for all four pheromone compounds of *S. frugiperda*, i.e. Z9-14:OAc, Z11-16:OAc, Z7-12:OAc and Z9-12:OAc (Fig. 1).

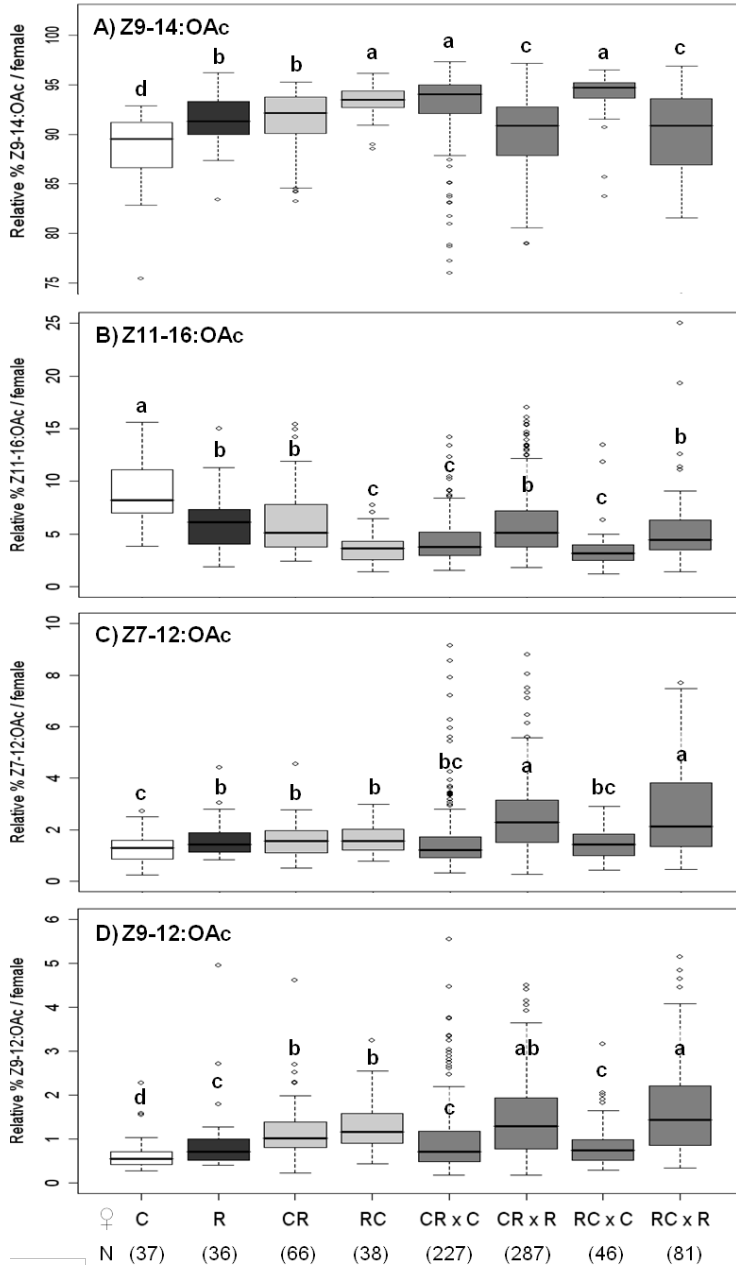


Figure 1. Relative percentage of A) Z9-14:OAc, B) Z11-16:OAc, C) Z7-12:OAc, and D) Z9-12:OAc in *Spodoptera frugiperda* pure strain (C/R), hybrid (CR/RC)

and backcross (CR-C/ CR-R/RC-C/RC-R) females. Each box plot shows the median (black line), the smallest/largest observations (dashed lines) as well as outliers (circles). Different letters above the box plots indicate significant differences. N= sample size. Information about the crossing schemes used to generate the different females can be found in Table 1.

The major sex pheromone component Z9-14:OAc was present in similar relative amounts in rice-strain and hybrid CR ($C_{\text{♀}} \times R_{\text{♂}}$) females, suggesting a paternal inheritance (Fig. 1A). Hybrid RC ($R_{\text{♀}} \times C_{\text{♂}}$) females and hybrids that were backcrossed to corn-strain males, i.e. CR-C and RC-C backcross females, produced significantly higher relative amounts of Z9-14:OAc than all other female crosses, while corn-strain females produced overall the lowest amount of Z9-14:OAc (Fig. 1A). In contrast, CR-R and RC-R females, i.e. backcrosses of hybrid females with rice-strain males, produced lower relative amounts of Z9-14:OAc than rice-strain or CR ($C_{\text{♀}} \times R_{\text{♂}}$) hybrid females (Fig. 1A).

The relative amount of Z11-16:OAc was similar in rice-strain females, hybrid CR ($C_{\text{♀}} \times R_{\text{♂}}$) and CR-R/RC-R backcross females, suggesting rice-strain specific paternal inheritance (Fig. 1B). In contrast, hybrid RC ($R_{\text{♀}} \times C_{\text{♂}}$) and CR-C/RC-C backcross females, i.e. crosses to corn-strain males, resulted in the overall lowest amount of Z11-16:OAc in all crosses (Fig. 1B).

The critical minor component Z7-12:OAc was inherited as a dominant trait from the rice-strain in hybrid CR and RC females (Fig. 1C). Backcross CR-R and RC-R females, i.e. crosses between hybrids and rice-strain males, exhibited significantly higher relative amounts of Z7-12:OAc than all other crosses (Fig. 1C). On the contrary, crosses between hybrids and corn-strain males, i.e. CR-C and RC-C backcross females, produced relative amounts of Z7-12:OAc which were intermediate between those of corn- and rice-strain females (Fig. 1C).

The relative amount of the minor compound Z9-12:OAc was significantly higher in CR/RC hybrids and backcrosses to rice-strain males (CR-R, RC-R) than in pure strain females of both strains (Fig. 1D). Whenever a hybrid female was backcrossed to a corn-strain male, backcross CR-C/RC-C females had similar relative amounts of Z9-12:OAc than rice-strain females (Fig. 1D).

Genetic mapping of strain-specific pheromone differences

A total of 303 informative markers were used to identify 30 linkage groups (chromosomes) of *S. frugiperda*. Each linkage group consisted of at least two markers (from different primer combinations) up to a maximum of 22 markers. On average, each linkage group contained around 10 different markers. A total of 7 markers did not map to any linkage group. QTL analysis of strain-specific pheromone differences showed that multiple genomic regions were involved in the production of strain-specific pheromone blends (Fig. 2).

In total, one minor QTL was found which explained the strain-specific differences in the relative amount of the major sex pheromone component Z9-14:OAc (Fig. 2A), a different minor QTL was found for the critical sex pheromone component Z7-12:OAc (Fig. 2B), yet another minor QTL was found for Z9-12:OAc (Fig. 2C), and a total of seven minor QTL were found for the variance in Z11-16:OAc, i.e. C01, C02, C03, C17, C22, C25, C30, (Fig. 2D). Chromosome 2 (C02) explained 4% and 7% of the variance of Z9-14:OAc and Z11-16:OAc, respectively (Fig. 2A, D). C28 was associated with higher percentages of Z7-12:OAc in homozygous (RR) females than in heterozygous (CR) females (Fig. 2B), and C11 affected the production of Z9-12:OAc, which was found in higher proportions in homozygous (RR) females than in heterozygous (CR) females (Fig. 2C).

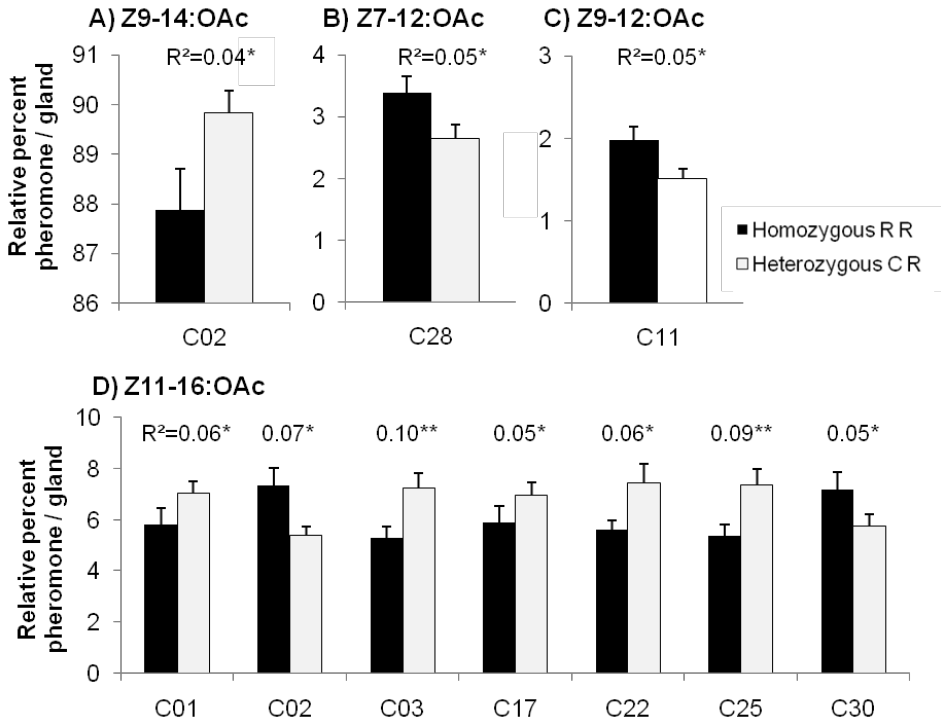


Figure 2. QTL found in *Spodoptera frugiperda* backcross family CR-R 19, showing the effect of different chromosomes on the relative amount of A) Z9-14:OAc, B) Z7-12:OAc, C) Z9-12:OAc and D) Z11-16:OAc in homozygous RR (black) and heterozygous CR (white) backcross females. Homozygous females had significantly different pheromone amounts than heterozygous females for all shown chromosomes ($P < 0.05^*$, $P < 0.01^{**}$). Bars show the mean percentage of pheromone per gland (+SE). The R^2 value for each chromosome is given above the bars, showing how much of the variation is explained by each chromosome.

Seven out of the ten above-described QTL affected the pheromone production in the expected direction, i.e. heterozygous (CR) females produced smaller amounts of Z7-12:OAc and Z9-12:OAc as well as higher amounts of Z11-16:OAc than homozygous (RR) females (Fig. 2). Contrary to expectation, C02 and C30 showed

the opposite-to-expected pattern for Z9-14:OAc (C02) and Z11-16:OAc (C02, C30), i.e. heterozygous (CR) females had higher proportions of Z9-14:OAc and lower amounts of Z11-16:OAc than homozygous (RR) females (Fig. 3A, D), while these compounds are present in lower and higher amounts in C females than in R females, respectively (Fig.1).

Candidate genes

A delta-11-desaturase (*SfLPAQ*) mapped to the QTL chromosome 2, which explained a significant portion of the variance of Z9-14:OAc and Z11-16:OAc, and showed an opposite-to-expected phenotypic pattern for both components (Fig. 2). A similar delta-11-desaturase can be found on chromosome 23 in *Bombyx mori* (ID: BMgn011563 on KAIKObase, <http://sgp.dna.affrc.go.jp/KAIKObase/>), which suggests that our *Sf* C02 could be homologous to *Bm* C23. A delta-9-desaturase (*SfKPSE*) mapped to chromosome 5, which was not associated with strain-specific differences in any of the four pheromone components (Fig. 2).

Interestingly, the circadian clock protein *vriille* (*SfVRI*) mapped to chromosome 28, which affected the production of the critical secondary sex pheromone component Z7-12:OAc (Fig. 2). *Vriille* is located on chromosome 27 in *B. mori* and our *Sf* C28 is homologous to *Bm* C27 (Hänniger et al., 2013).

Discussion

In this study, we investigated the inheritance and genetic basis of strain-specific pheromone differences between both *S. frugiperda* strains and found a) differential inheritance of all pheromone compounds, b) the involvement of multiple genomic regions determining the female pheromone composition c) the potential contribution of a delta-11-desaturase (*SfLPAQ*) in strain-specific pheromone

production of Z9-14:OAc and Z11-16:OAc, and d) a possible genetic linkage between two prezygotic mating barriers of *S. frugiperda*, i.e. strain-specific production of the critical secondary sex pheromone component Z7-12:OAc and the onset time of mating in the night, through the circadian clock gene *vriIle*.

a) Differential inheritance of pheromone compounds

To assess the inheritance of each pheromone compound of *S. frugiperda*, we performed pheromone extractions of pure strain, hybrid and backcross females and found that all four pheromone compounds, i.e. Z9-14:OAc, Z11-16:OAc, Z7-12:OAc and Z9-12:OAc, were differentially inherited. The inheritance of the major pheromone component Z9-14:OAc seemed to be influenced by strain-specific paternal genes, which caused either overproduction of Z9-14:OAc (in RC, CR-C and RC-C females), or reduced amounts of Z9-14:OAc (in CR-R and RC-R females). Similarly, the amount of Z11-16:OAc in hybrid and backcross females was influenced by corn- and rice-strain male-specific factors. The critical secondary sex pheromone component Z7-12:OAc was rice-strain dominant in hybrid females, and paternal factors seemed to influence the amount of this component in backcross females. In contrast, the inheritance of Z9-12:OAc appeared to be influenced by different factors and a higher production in heterozygotes was found in hybrid and most backcross females.

The results of this study are significantly different compared to results of our previous study on the inheritance of strain-specific sex pheromone differences (Groot et al., 2008). In our previous study, where we examined the pheromone composition of pure strain and hybrid females, we found that Z9-14:OAc and Z11-16:OAc were both maternally inherited, while Z7-12:OAc was suppressed in hybrid females and Z9-12:OAc showed a corn-strain dominant inheritance (Groot et al., 2008). In contrast, this study showed that Z9-14:OAc was overexpressed in

CR hybrid females ($C_{\text{♀}} \times R_{\text{♂}}$) and paternally inherited in RC ($R_{\text{♀}} \times C_{\text{♂}}$) hybrid females, while Z11-16:OAc was suppressed in both hybrids, Z7-12:OAc was rice-strain dominant in CR and RC females, and Z9-12:OAc was overexpressed in both hybrids. These differential results could be explained by differing experimental procedures and/or by different *S. frugiperda* populations used in both studies. In our previous study, pheromone gland extractions were performed using females that were previously injected with pheromone biosynthesis activating neuropeptide (PBAN), to induce pheromone production and perform pheromone extractions in the photophase (Groot et al., 2008). In this study, we extracted only untreated females within the scotophase, i.e. their natural time of pheromone production, because PBAN was shown to influence the pheromone composition of *S. frugiperda* females (Groot et al., 2008). Concerning the populations used, the first study used laboratory corn- and rice-strain populations from Florida (Groot et al., 2008), while experiments in this study were conducted with a corn-strain population from Puerto Rico and a different rice-strain population from Florida. Although both studies showed different results, they also confirmed that multiple genomic regions and differential modes of inheritance are involved in the heredity of strain-specific sex pheromone differences in corn- and rice-strain females.

b) Multiple genomic regions influence the female pheromone composition

We investigated the genetic basis of strain-specific pheromone differences in both *S. frugiperda* strains and found that multiple genomic regions on 9 different chromosomes were involved in the production of corn- and rice-strain specific female pheromone blends. A delta-9-desaturase (*Sf*KPSE), a delta-11-desaturase (*Sf*LPAQ) and the circadian clock gene *vriille* were mapped to a generated *S. frugiperda* map, whereupon the last two genes were associated with strain-specific pheromone differences. Interestingly, for three pheromone compounds, i.e. Z9-14:OAc, Z7-12:OAc and Z9-12:OAc, we found the involvement of one QTL each,

whereas a total of seven different QTLs were significantly correlated with the amount of Z11-16:OAc. This result suggest that Z11-16:OAc might not be under strong stabilizing selection, compared to Z9-14:OAc, Z7-12:OAc and Z9-12:OAc.

c) Involvement of desaturases in strain-specific pheromone production

To identify candidate genes which may be responsible for the strain-specific pheromone differences in *S. frugiperda*, we mapped two desaturases to our generated *S. frugiperda* map and found that the delta-11-desaturase *SfLPAQ* mapped to chromosome 2, which was involved in the production of Z9-14:OAc and Z11-16:OAc. Thus, strain-specific differences in this desaturase (*SfLPAQ*) could, at least partly, explain why corn-strain females produce higher relative amounts of Z11-16:OAc and lower percentages of Z9-14:OAc than rice-strain females.

Based on a proposed pheromone biosynthesis pathway of *S. frugiperda*, pheromone production starts with one product of the fatty acid synthesis, i.e. 16-carbon acyl-CoA (16:CoA), which can be modified by a delta-11-desaturase to produce Z11-16:CoA, that can further be reduced and acetylated to produce Z11-16:OAc (Groot et al., 2008). However, the precursor Z11-16:CoA may also be modified by chain-shortening enzymes to produce Z9-14:CoA, the precursor of Z9-14:OAc (Groot et al., 2008). Because Z11-16:OAc and Z9-14:OAc are linked via their biosynthetic pathway, overproduction of one component leads to the reduction of the other component (Groot et al., 2008). If corn-strain, but not rice-strain females, would posses a higher activity of a delta-11-desaturase that could convert 16:CoA to Z11-16:CoA, e.g. *SfLPAQ* on *SfC02*, it could explain why corn-strain females exhibited higher amounts of Z11-16:OAc and lower percentages of Z9-14:OAc than rice-strain females.

However, chromosome *SfC02* showed the opposite-to-expected pattern for both components, i.e. heterozygous (CR) females had higher proportions of Z9-14:OAc and lower amounts of Z11-16:OAc than homozygous (RR) females. This suggests that *SfLPAQ*, if it exhibits strain-specific sequence or expression differences, may act together with another enzyme to produce strain-specific differences in the relative amount of Z9-14:OAc and Z11-16:OAc. We found that seven different chromosomes were significantly correlated with the relative amount of Z11-16:OAc and *SfC02* accounted for only 7% of the variance, while all seven chromosomes explained together 48% of the variance of Z11-16:OAc. The involvement of multiple genomic regions in the strain-specific production of Z11-16:OAc suggests that, either different enzymes, and /or different activating or inhibiting regulatory elements act together to produce strain-specific Z11-16:OAc and Z9-14:OAc differences.

In addition to the delta-11-desaturase *SfLPAQ*, we mapped another desaturase, i.e. a delta-9-desaturase (*SfKPSE*) to *SfC05*, which was which was not associated with strain-specific pheromone differences. Thus, the delta-9-desaturase *SfKPSE* is probably not responsible for strain-specific differences in any of the four pheromone components. To verify whether the delta-11-desaturase *SfLPAQ* is responsible for strain-specific differences in the amount of Z9-14:OAc and Z11-16:OAc, further experiments will be necessary. Structure elucidation will help to determine strain-specific sequence differences and heterologous expression studies can prove whether *SfLPAQ* is able to strain-specifically convert 16:CoA to Z11-16:CoA.

d) Genetic linkage between pheromone production and timing of reproduction

To examine whether strain-specific differences in two prezygotic mating barriers of *S. frugiperda*, i.e. sex pheromone composition and differential timing of reproduction, may be determined by the same genomic regions, we mapped the circadian clock protein *vriille* to our generated *Sf* map, which was recently found to be responsible for strain-specific differences in timing of reproduction (Hänniger et al., 2013). Interestingly, we found that *vriille* (*SfVRI*) mapped to chromosome 28 (*Sf* C28, homologous to *Bm* C27), which affected the production of the critical minor component Z7-12:OAc, that is known to be essential for male attraction to the major sex pheromone component Z9-14:OAc (Tumlinson et al., 1986; Unbehend et al., 2013). Thus, genes involved in strain-specific Z7-12:OAc production and timing of mating in the night are located on the same chromosome. This suggests that these two prezygotic mating barriers might be genetically linked and influenced by the same set of genes and/or regulatory elements.

Genetic analysis of strain-specific differences in timing of reproduction showed that one *S. frugiperda* chromosome, which is homologous to *Bm* C27, explained around 30% of the variance in the differential onset time of mating between both strains (Hänniger et al., 2013). Hänniger et al. (2013) found that the circadian clock gene *vriille* mapped to this chromosome and although *vriille* showed no strain-specific sequence differences, its relative expression levels cycled in a *clock*-dependent manner and *vriille* expression peaks were shifted between both strains in both sexes. Hänniger et al. (2013) suggested that differential *vriille* expression levels might be caused by strain-specific differences in a cis-regulatory enhancer element, which still needs to be investigated.

If strain-specific differences in a cis-regulatory element do exist and influence *vriI* expression, it could be possible that the same regulatory element also influences another gene, responsible for differential production of Z7-12:OAc in females. Due to the fact that Z7-12:OAc consists only of 12 carbons and pheromone production starts with 16:CoA, many different enzymes could be responsible for the production of Z7-12:OAc, i.e. desaturases, chain-shortening enzymes, reductases and acetyl transferases (Groot et al., 2008). If a cis-regulatory enhancer element strain-specifically affects the expression and/or substrate specificity of one of these genes, it could explain why corn-strain females produced lower relative amounts of Z7-12:OAc than rice-strain females. Further genetic analysis will be necessary to evaluate which genes are responsible for the strain-specific production of Z7-12:OAc.

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