Chasing sympatric speciation: The relative importance and genetic basis of prezygotic isolation barriers in diverging populations of Spodoptera frugiperda

Hänniger, S.

SECTION 3

STRAIN-SPECIFIC ALLOCHRONIC DIFFERENTIATION

'An experiment is a question which Science poses to Nature, and a measurement is the recording of Nature's answer.'
Max Planck

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GENETIC BASIS OF PREZYGOTIC ISOLATION IN THE FALL ARMYWORM

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Abstract

The two host strains of the fall armyworm *Spodoptera frugiperda* (Noctuidae) seem to be in the process of ecological speciation in sympatry. The strains exhibit allochronic differentiation in their mating time and also differ in female sex pheromone composition, which together seem to act as isolation barriers driving divergence between the strains. We conducted two QTL analyses addressing these two isolation barriers. We identified one major QTL for the allochronic divergence of mating, which to our knowledge is the first time that a genomic location is identified that underlies differentiation in circadian timing of mating activity in two strains in the process of speciation. We identified the homologous chromosome in *Bombyx mori*, on which the clock gene *vrille* is located, which thus became our major candidate gene. In *S. frugiperda*, *vrille* showed strain-specific polymorphisms and circadian expression differences corresponding to the phenotypic differences in mating time. Interestingly, another QTL that affects the production of the critical sex pheromone component Z7-12:OAc, maps to the same chromosome as the timing QTL. Our results suggest that allochronic differentiation and sex pheromonal divergence are genetically linked, which could facilitate the evolution of prezygotic isolation in *S. frugiperda*.

INTRODUCTION

In the past 150 years, evolutionary biologists have attempted to understand how new species can evolve and how this process creates the rich biodiversity found on earth. The formation of new species requires some sort of limitation of gene flow between populations, finally resulting in reproductive isolation (Coyne and Orr 2004). Physical barriers separating populations may lead to allopatric speciation, while ecological isolation in terms of habitat or behavioral isolation can cause speciation in sympatry (Coyne and Orr 2004; Smadja and Butlin 2011; Boughman 2013; Butlin et al. 2014). Studying the genetic basis of strong, but incomplete, reproductive isolation barriers between closely related species, or even better within species between differentiating populations, can help to reveal the initial steps causing speciation and to identify genes driving divergence.

An ideal model organism to study speciation and the evolution of reproductive isolation is the noctuid moth *Spodoptera frugiperda* (Lepidoptera: Noctuidae), as it consists of two morphologically identical, but behaviorally and genetically different strains (Pashley 1986). These so-called corn- and rice-strains seem to be in the process of ecological speciation in sympatry (Groot et al. 2010). Although the hybridization rate is up to 16% in the field (Prowell et al. 2004), the two strains do not merge into one panmictic population, which is probably prevented by different isolation barriers (Pashley et al. 1992; Groot et al. 2010). So far, three possible prezygotic mating barriers have been described in this species: a) differential host plant choice (Pashley 1986; Meagher and Gallo-Meagher 2003; Nagoshi et al. 2006, 2007; Machado et al. 2008), b) strain-specific timing of mating in the night (Pashley et al. 1992; Schöfl et al. 2009), and c) female sex pheromone differences (Groot et al. 2008; Lima and McNeil 2009; Unbehend et al. 2013). Recent studies showed that

host preference in the field is not as specific as previously thought (Juárez et al. 2012, 2014; Groot et al. 2015). Also, studies on inter-strain differences in host plant utilization are not consistent in their results (Pashley 1988; Whitford et al. 1988; Pashley et al. 1995; Meagher et al. 2004). For example, rice-strain larvae have been found to develop faster and grow significantly larger when fed on corn than do corn-strain larvae (Meagher et al. 2004), while other studies show that the corn-strain outperforms the rice-strain on corn plants (Pashley 1988; Whitford et al. 1988; Pashley et al. 1995). Additionally, numerous bioassays in our lab, including larval performance and choice assays and oviposition choice assays, failed to show any difference between the strains (Hänniger, unpubl.). Therefore, habitat isolation seems to be a weak prezygotic mating barrier. Allochronic divergence and sex pheromone differentiation are each incomplete mating barriers, but may interact to form a behavioral isolation barrier that promotes strain separation (Groot et al. 2010, 2015). Here, we test this hypothesis by assessing the genetic basis of both traits.

Allochronic divergence seems to be a major barrier separating the two *S. frugiperda* strains, as both strains consistently differ in their timing of reproductive activity at night (Pashley et al. 1992; Schöfl et al. 2009). The corn-strain calls, mates and oviposits approximately three hours earlier than the rice-strain, with only a small overlapping time-window between the two (Pashley et al. 1992; Schöfl et al. 2009). Allochronic speciation has been suggested for several insect species, e.g. crickets (Danley et al. 2007; Fergus and Shaw 2013), fruit flies (Tauber et al. 2003; Prabhakaran and Sheeba 2012) and mosquitoes (Rund et al. 2012). However, surprisingly little research has been conducted on the importance and exact genetic changes underlying temporal speciation (reviewed in Groot 2014). It is likely that changes in biological clocks are involved in temporal differentiation between closely related species, and in timing of reproductive activity in *S. frugiperda*. Biological clocks can be described as a network of genes and gene products that enhance and suppress each other in a rhythmic manner, entrained by environmental factors like light, temperature or tides (Hardin 2005; Kaiser et al. 2011). Within insects, the clock gene network is best described in the fruit fly *Drosophila melanogaster*, where the network consists of two interlocked feedback loops (Cyran et al. 2003; Hardin 2005): one feedback loop involving the genes *vrille* (*vri*), *PAR-domain protein 1* (*PDP1*), *clock* (*clk*) and *cycle* (*cyc*)*;* a second feedback loop involving *period* (*per*), *timeless* (*tim*), *clk* and *cyc* (Figure 1). In addition, kinases phosphorylate clock proteins (e.g. phosphorylation of PER by DOUBLETIME (DBT) and CASEIN KINASE 2 α (CK2α)) and facilitate their accumulation (Hardin 2005), while *cryptochrome 1* (*cry1*) functions as circadian photoreceptor. Most of these genes are also present in Lepidoptera (Zhu et al. 2005; Trang et al. 2006; Yuan et al. 2007; Zhu et al. 2009), and are thus good candidate genes that may underlie the timing differences between the corn- and the rice-strain. Additionally, a second cryptochrome, *cryptochrome 2* (*cry2*), is present in Lepidoptera and is able to repress CLK:CYC mediated transcription (Zhu et al. 2005; Yuan et al. 2007).

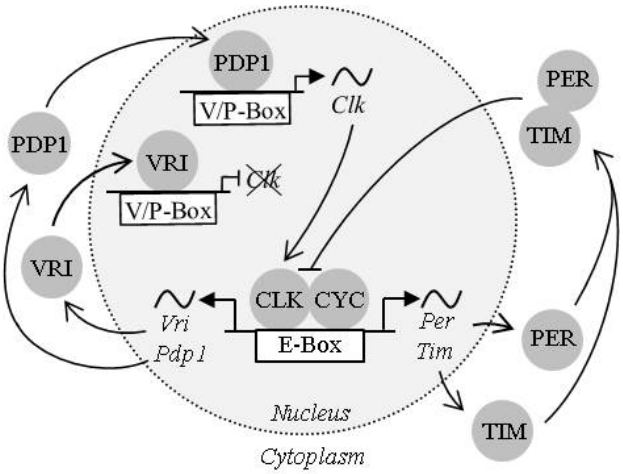

FIGURE 1. Two feedback loops that define the circadian rhythm in *Drosophila*. Adapted from Cyran et al. (2003), Hardin (2005).

In addition to allochronic differentiation, differences in sexual communication have been found between the two strains of *S. frugiperda* (Groot et al. 2008; Lima and McNeil 2009; Unbehend et al. 2013). Behavioral isolation via sexual communication differences is known from several insect orders, such as Diptera (e.g. *Drosophila*) and Lepidoptera (e.g. *Ostrinia*) (Smadja and Butlin 2009; Wicker-Thomas 2011). A, a reliable sexual communication system between females and males is essential for the mating success and fitness of a species. Therefore, changes in the sender (female) and receiver (male) of a pheromone signal can have tremendous fitness effects and drive prezygotic isolation (Löfstedt 1993; Cardé and Haynes 2004).

The sex pheromone of *S. frugiperda* consists of at least two behaviorally active components, the major pheromone component (Z)-9-tetradecenyl acetate (Z9-14:OAc), and the critical minor component (Z)-7-dodecenyl acetate (Z7-12:OAc) (Tumlinson et al. 1986). Other pheromone compounds have also been identified from the female gland, e.g. (Z)-11-hexadecenyl acetate (Z11-16:OAc) and (Z)-9-dodecenyl acetate (Z9-12:OAc), but their behavioral function is unclear (Unbehend et al. 2013). Pheromone extractions of laboratory and field populations showed that corn-strain females consistently exhibited lower relative amounts of Z7-12:OAc than rice-strain females (Groot et al. 2008; Lima and McNeil 2009; Unbehend et al. 2013). Interestingly, field studies in Florida suggest that males have adapted to the strain-specific amount of Z7-12:OAc in females, which could promote isolation between both strains (Unbehend et al. 2013). Based on a proposed pheromone biosynthesis pathway of *S. frugiperda* (Groot et al. 2008), different candidate genes, e.g. delta-9- or delta-11-desaturases, could influence the pheromone differences between corn- and rice-strain females.

In this study, we determined the genetic basis of the two most promising potential prezygotic mating barriers, i.e. allochronic differentiation and sexual communication variation, in the two strains of *S. frugiperda*. We conducted quantitative trait locus (QTL) analyses for both isolation mechanisms, we mapped different candidate genes to the QTLs involved in the differential timing of reproduction and in the production of different pheromone components, and we assessed strain-specific differences in the structure and expression of the candidate gene *vri*, key to the allochronic differentiation between the two strains.

MATRIALS AND METHODS

Insects

We conducted two QTL analyses with two laboratory corn- and rice-strain populations. Details about the populations are given in the supplementary materials and summarized below. Individuals used for the timing QTL analysis descended from Florida and were also used by Schöfl et al. (2009). We refer to these populations as CL1 and RL1 (Table S1). Since these two populations died after six years of laboratory rearing, we established new laboratory populations for the pheromone QTL analysis, originating from Florida (rice-strain) and Puerto Rico (corn-strain) We refer to these populations as CL2 and RL2 (Table S1). We confirmed genetic similarity between the corn-strain populations from Florida (CL1) and Puerto Rico (CL2) by determining the mitochondrial haplotype profile of 47 Florida and 43 Puerto Rico individuals (Table S2; Nagoshi et al. 2007). All 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 set up to maintain the populations and minimize inbreeding.

Generation of backcrosses

For the two QTL analyses, we generated female-informative backcrosses (Table S3). Single pair matings between pure corn- and rice-strain individuals were performed to obtain F_1 hybrid females, which were then backcrossed to pure rice-strain males to produce different backcross families (Table S3). Two backcross families (BCs) were used for the timing QTL analysis (BC_A: RCxR, BC_B: CRxR), while one BC (BC_C: CRxR) was used for the pheromone QTL analysis (the first two letters of a backcross refer to the mother, the last letter to the father). The two rice-strain fathers used to generate both backcrosses for the timing QTL were kin.

Phenotyping of backcrosses

To determine the phenotype for the timing QTL analysis, we observed the mating behavior of a) pure strain individuals in intra-strain (CxC, RxR) and inter-strain matings (CxR, RxC), b) hybrid females backcrossed to pure strain males (CRxC, CRxR, RCxC, RCxR), and c) female backcross offspring crossed to pure strain

males (CR-RxC, CR-RxR, RC-RxC, RC-RxR). The observations of mating behavior, i.e. copulation, were performed as described by (Schöfl et al. 2009) and are summarized here. One to four day old virgin females and males were set up in single pairs in clear plastic cups (16 oz.) and provided with 10% honey solution. All matings were set up simultaneously and placed in a walk-in climate chamber (26 °C, 70% RH, L:D 14:10) two hours before scotophase. In total, 320 to 400 couples were observed throughout the scotophase and one hour into photophase (in total 11 hours), with a 30 min interval, i.e. each couple was observed once every 30 min. All pairs were observed for three consecutive nights starting at the first day of the mating. The onset time of the first mating, on whichever night it occurred, was the phenotype for the timing QTL analysis. After observation, all individuals were frozen at -80 °C for further genetic analysis.

For the pheromone QTL analysis, pheromone glands were extracted from 2-3 day-old virgin pure strain females (C, R), hybrids (CR, RC), and backcross females (CR-R), as described in detail in Unbehend et al. (2013) and in the supplementary data and summarized here. The pheromone glands were extracted in the scotophase at the strain-specific peaks of calling times, i.e. 4-5 h into scotophase for corn-strain females, 6-7 h into scotophase for the rice-strain females. For the hybrid females, we used the approximate times of the mothers, i.e. 3.5-5.5 h into scotophase for the CR hybrids, 5-6 h into scotophase for the RC hybrids, and 4-7 h into scotophase for the CR-R backcross females. Gas chromatography analysis was performed using a HP7890 gas chromatograph with a polar capillary column (DB-WAXetr (extended temperature range); 30 m × 0.25 mm × 0.5 μm) and a flame-ionization detector. Female pheromone compounds were identified by comparing retention times with synthetic standards of Z9-14:OAc, Z7-12:OAc, Z11-16:OAc, and Z9-12:OAc (Pherobank, Wageningen, the Netherlands). After pheromone extraction, all females were stored at -20 °C for further analysis.

DNA extraction and AFLP marker analysis

All DNA extractions were performed as described in Unbehend et al. (2013), using Cetyltrimethylammonium bromide and isopropanol for DNA precipitation. For the timing QTL analysis, DNA of 90 randomly chosen backcross females (44x RC-R, 46x CR-R) covering the full range of the timing phenotype (i.e. early maters to late maters) as well as of their parents and grandparents were used to generate AFLP markers. For the pheromone QTL analysis, we selected 88 females covering the full range of relative amount of Z7-12:OAc (lowest to highest amount) in the female glands, as this is significantly different between the two strains (Unbehend et al. 2013): We chose 36 females with low amounts of Z7-12:OAc (1-2%), 16 females with medium amounts (~2.5%), and 36 females with high amounts of Z7-12:OAc (> 3.5%), as well as their parents and grandparents.

After DNA extraction, AFLP markers were generated and analyzed as described in Groot et al. (2009), detailed in the supplementary material and summarized here:

200 ng DNA of each sample was digested with *EcoR*I and *Mse*I (New England Biolabs, Ipswich, MA, USA), and *EcoR*I- and *Mse*I-adapters were ligated to the fragments which were then preamplified (Wilding et al. 2001). The preamplified DNA was selectively amplified with different *EcoR*I- and *Mse*I-primer combinations (Table S4). The generated AFLP fragments were analyzed scored with AFLP-Quantar Pro 1.0 (KeyGene, Wageningen, the Netherlands).

Genetic map construction and QTL analyses

After scoring of at least 300 AFLP markers per backcross (Table S4), we constructed a linkage map for each QTL analysis with MapMaker 3.0 (www.broadinstitute.org/ftp/distribution/software/mapmaker3/). Markers were clustered into linkage groups (LG) using a LOD of 4.5 (timing QTL analysis) and a LOD of 6.5 (pheromone QTL analysis). In each QTL analysis, 30 LGs were identified that refer to the 30 autosomes in a backcross family, as there is no crossing over in female Lepidoptera (Heckel 1993). The chromosome names (chromosome 1 to 30) were chosen arbitrarily for each QTL analysis, so that the same numbers in the different linkage maps are not necessarily homologous. For the timing QTL analysis, markers present in both backcrosses (Table S4) were used to homologize the chromosomes of these backcrosses. To identify candidate QTL, each chromosome was tested for a significant difference in the phenotype (timing QTL: onset time of first mating, pheromone QTL: female pheromone composition) between the homo- and heterozygous backcross females. The two backcrosses for the timing QTL were combined for analysis, to increase the sample size and thus the possibility to detect QTL. For this combination, markers of both backcrosses were set such that all 'present' markers originated from the corn-strain grandparent. We also show the results of QTL analyses for each BC individually (Table S8), but focus on the results of the combined analysis. Statistical analysis was performed with R 2.5.0 (R Development Core Team, 2007) and SAS (SAS institute, Cary, NC, USA, 2002-2008). We conducted a two-sided t-test and a GLM to assess how much of the variance is explained by the different QTLs (R^2 value). The female pheromone data were log transformed to stabilize the variance. Chromosomes with a significant correlation ($P < 0.05$) were considered a QTL. For the timing QTL we additionally used a t-test based marker regression as implemented in R/qtl (Broman et al. 2003). A LOD score derived from the t-statistics provides evidence for a QTL (Broman et al. 2003). Permutation tests using 10,000 permutations empirically established significance thresholds for LOD scores (Figure S1). For the pheromone QTL, the same analysis did not yield any QTL chromosomes above the significance level. We performed power simulations for the timing QTL with 10,000 simulation replicates for backcross sizes of 50, 85 and 100 progeny to estimate the power to detect QTL with the observed effect sizes (Figure S2). The probability to detect our major timing QTL was 0.58 with the given setup, thus it could easily have been missed. Therefore we also describe the pheromone QTL, even though they were not above the significance thresholds established by permutation tests.

Homologizing linkage maps to* Bombyx mori *chromosomes

To identify candidate genes in the QTL regions, the linkage map of the timing QTL analysis was homologized to the reference genome of *B. mori*, using restriction site associated DNA (RAD) analysis (see Baxter et al (2011) and Groot et al. (2013). DNA of parents, female grandparents and 11 backcross individuals per backcross family was barcoded, pooled, sheared and amplified, following the procedure described in Groot et al. (2013). The pool was paired-end sequenced by FASTERIS (Geneva, Switzerland) with a HiSeq Illumina sequencer, resulting in 76 million reads. The reads were separated by barcodes into pools per individual and filtered for quality (q10=99%). On average, there were 5-10 different paired-end reads per forward read (Table S5). Forward sequences were transformed to binary segregation patterns obtained with the AFLP markers in the backcross individuals using RAD tools (Baxter et al. 2011). All sequences matching an AFLP segregation pattern were pooled across the individuals, after which the paired-end sequences were retrieved, resulting in 30 FASTA files (one file per chromosome). Each group was assembled using CLC Genomics Workbench (CLC bio version 5.0.1; www.clcbio.com). Sequences were trimmed for length and quality with standard settings (nucleotide mismatch cost = 2; in/del cost = 2; length fraction = 0.35; similarity = 0.9; when bases conflicted, the base with highest frequency was chosen) and assembled *de novo.*

Resulting contigs from the paired-end RAD sequences were blasted per chromosome in SilkDB (www.silkdb.org) and KAIKObase (http://sgp.dna.affrc.go.jp/ KAIKObase) using BLASTX and TBLASTX. Homology between *S. frugiperda* chromosomes and *B. mori* chromosomes was confirmed when (in hierarchical order) a) contigs from both BC_A and BC_B produced significant blast hits to the same *Bm* chromosome and/or b) different contigs of the same *Sf* chromosome produced significant blast hits to the same *Bm* chromosome and/or c) in cases where multiple *Bm* chromosomes hit contigs of one *Sf* chromosome, the hit with the lowest e-value was chosen. Hence, if contigs from both backcrosses produced significant hits from one *Sf* chromosome to the same *Bm* chromosome, this was evaluated as most powerful, while one contig from one backcross producing a highly significant hit to one *Bm* chromosome, but also significant hits to other chromosomes, this was evaluated as least powerful. Table S8 summarizes the results of this homologizing procedure and shows the e-value of the best blast result of all contigs of one *Sf* chromosome to one *Bm* chromosome at the intersection of these chromosomes. For the pheromone QTL, we only constructed a genetic map using AFLP markers, after which we mapped the candidate gene *vri*, located on the major timing QTL, as well as both candidate genes that could explain the pheromone variation, delta-9-desaturase and delta-11-desaturase.

Candidate genes

As for the timing QTL, all QTL chromosomes were homologized to the *B. mori* chromosomes, we assessed the location of candidate genes involved in the circadian

rhythm (Figure 1), using KAIKObase (http://sgp.dna.affrc.go.jp/KAIKObase). The position of *vri* on the timing QTL chromosome *Sf*_C25 (*Bm*_C27) was verified by mapping it via single nucleotide polymorphisms (SNPs) to the generated QTL map. Initially, eight backcross individuals were used and the pattern of SNPs in the coding sequence, that were present in both backcrosses, were compared to the pattern of the AFLP markers. This was sufficient, as no linkage group other than *Sf*_C25 had the same AFLP segregation pattern in these 8 individuals. The position of *vri* was later also verified when sequencing the full CDS of *vri* in 17 BC individuals and comparing SNPs in these individuals to AFLP segregation patterns.

In the pheromone QTL analysis, the two candidate genes delta-11-desaturase (*Sf*LPAQ) and delta-9-desaturase (*Sf*KPSE), as well as *vri*, were mapped onto the genetic map using SNPs as well, for which 24 backcross individuals were used. Based on insect ESTs and genomic sequences (*vri*: gb|AY526608.1, gb|AY576272.1, gb|AADK01019845.1; *Sf*KPSE: gb|DY793393.1, gb|DV079258.1; *Sf*LPAQ: gb|FP368185.1, gb|FP366982.1), primers were designed for mapping (Table S6). To identify segregating SNPs in the candidate genes, PCR amplifications were conducted (Table S7) with the grandparents, parents, and 8 to 24 backcross females of all three backcross families. The generated amplification products were mixed with 3µl loading dye and ran on a 1.5% agarose gel at 120 V for 2 h. The obtained bands were cut from 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 in Vogel et al. (2011), and analyzed with Sequencher 4.10.1 (Gene Codes Corporation, Ann Arbor, MI, USA).

Structure analysis of vri

To assess strain-specific structure differences in *vri*, the sequence of the gene was established stepwise, using degenerate primers and DNA Walking SpeedUp™ Kit II (SEEGENE, Eschborn, Germany). This elucidated the coding sequence and ~1 kbp of the 5' untranslated region and the promoter region. Subsequently, thanks to the Whole Genome Sequencing project supported by the Fall Armyworm International Public Consortium (The FAW-IPC, in prep.), the *S. frugiperda* genome for both strains (http://www6.inra.fr/lepidodb/SfruDB) became available as well as an in-house RNAseq database of larval guts. With these tools (as detailed in the supplementary material) the full length of *vri* could be obtained, including a large intron with 11 Ebox elements in the 5' UTR. The regions surrounding the Ebox elements was then amplified and sequenced in 12 corn-strain and 12 rice-strain individuals from the CL1 and RL1 populations, respectively.

Expression analysis

To determine strain-specific expression differences in the candidate gene *vri* that mapped onto the major QTL (*Bm*_C27), we conducted reverse transcription-

quantitative real-time PCR (RT-qPCR) experiments with mRNA from heads of female and male *S. frugiperda* of both strains (CL1 and RL1; Table S1). For 24 h, every two hours 10 females of both strains were transferred from the rearing cups to a 10-ml Falcon tube, immediately frozen in liquid nitrogen and kept at -80 °C. RNA was isolated from two pools of five heads, providing two biological replicates per strain per time point. RNA extraction, cDNA synthesis and qRT-PCR reaction were conducted, as described in Groot et al. (2013) and summarized here. Heads were ground with mortar and pistil in liquid nitrogen, RNA was isolated using TRIsure (Bioline, Luckenwalde, Germany) and the RNA pellet was dissolved in 90 µl nuclease free water (Ambion, LIFE TECHNOLOGIES, Darmstadt, Germany). DNase was digested by adding 10 µl 10x Turbo DNase buffer and 1 µl Turbo DNase (Ambion, LIFE TECHNOLOGIES, Darmstadt, Germany) to the 90 µl sample and incubating for 30 min at 37˚C. The RNA samples were contaminated with dark pigments from the eyes. We found the 10x Turbo DNase buffer to be capable of precipitating these pigments. Thus, an additional precipitation step was conducted as followed: 10 µl 10x Turbo DNase buffer was added to the 101 µl samples and samples were vortexed until pigments were dissolved and again incubated for 30 min at 37˚C. Then samples were centrifuged for 20 min at high speed (16000 g) and the supernatant was transferred to a new tube. This step was repeated followed by a cleanup with RNeasy MinElute Cleanup-Kit (QIAGEN, Hilden, Germany). cDNA was synthesized from 1000 ng RNA using Verso cDNA synthesis kit (Thermo Fisher Scientific, Schwerte, Germany). RT-qPCRs were conducted with 5 ng cDNA per reaction, 3 technical replicates on each plate, using ABsolute Blue QPCR SYBR Green Mix (Thermo Fisher Scientific, Schwerte, Germany) and a Bio-Rad CFX machine (Bio-Rad Laboratories GmbH, München, Germany). The reaction ran 15 min at 95 °C, 40 cycles of 15 s at 95 °C, 30 s at 58 °C, 30 s at 72 °C followed by 10 s at 95 °C and a melt curve 55-95 °C with an increment of 0.5 °C. Three potential reference genes, eukaryotic translation initiation factor 1α (eIF1α), eukaryotic translation initiation factor 4α (eIF4α) and ribosomal protein subunit 18 (RPS18), were tested on a subset of 10 samples pooled over both biological replicates (both strains; time points 1, 3, 5, 7 and 9 hours into scotophase) to identify the gene that is most stable over time. eIF1α was the gene with the least variation between the samples and was thus chosen as the reference gene for this study and amplified for all samples (see Table S6 for all primers and Table S7 for detailed protocol). Relative expression levels were calculated as copy numbers per 1000 copies eIF1α.

RESULTS

QTL analyses

In the timing QTL analysis, a total of 465 (in BC family A) and 514 (in BC family B) informative AFLP markers were used to identify the 30 *S. frugiperda* autosomes, while 303 markers (BC_C) were scored to construct a genetic map for the pheromone QTL analysis (Table S4). In BC_B only 29 chromosomes could be

identified, even though more markers were scored to find the additional chromosome. The higher amount of scored markers in BC_A and BC_B was also needed to homologize the two linkage maps to each other. Each chromosome consisted of at least two AFLP markers from different primer combinations up to a maximum of 26 markers. Some markers did not map to any linkage group (17 markers in each BC in the timing QTL analysis, seven markers in the pheromone QTL analysis). Three chromosomes could not be homologized between the two timing linkage maps, i.e. three chromosomes in BC_A and only 2 chromosomes in BC_B, as there is one linkage group missing in BC_B.

QTLs were identified by testing each linkage group for a significant association with the relevant phenotypic trait. Because of the absence of crossing-over in females, each identified QTL corresponds to an individual chromosome, on average 1/31 of the genome. For the timing, we found one QTL (*Sf*_C25, P<0.0001, R^2=0.19) that explained most of the variance in the strain-specific timing of mating, which was consistent in both backcrosses (Table S8). This QTL is homologous to *Bombyx mori* chromosome 27 (*Bm*_C27) (Table S8) and explained 19% of the variance of the onset time of first mating (Figure 2). *Bm*_C27 is 14.5 Mb in size (52.8 cM) and represents 3.3% of the total *B. mori* genome (Xia et al. 2008; Shimomura et al. 2009). Additionally we detected three minor QTL: *Sf*_C28 (*Bm*_C2, P=0.014, R^2=0.08), *Sf*_C30 (*Bm*_C6, P=0.0104, R^2= 0.08) and *Sf*_C20 (*Bm*_C12, P=0.234, R^2=0.06).

For the pheromone variation, we found one minor QTL *Sf*_C28 (*P*=0.028, R^2 = 0.05), that explained the strain-specific differences in the relative amount of Z7-12:OAc, the critical sex pheromone component that is essential for male attraction (Figure 2). Heterozygous as well as homozygous backcross individuals exhibit a higher relative percentage of Z7-12:OAc/pheromone gland compared to the pure strain individuals, which is due to a lower abundance of the major component Z9-14:OAc. We found several other genomic regions that explained some of the variation in the pheromone blend, i.e. *Sf*_C02 (*P*=0.050, R^2=0.04) for the major sex pheromone component Z9-14:OAc, *Sf*_C11 (*P*=0.033, R^2=0.05) for Z9-12:OAc, and a total of seven minor QTLs for Z11-16:OAc, i.e. *Sf*_C01 (*P*=0.022, R^2=0.06), *Sf*_C02 (*P*=0.014, R^2=0.07), *Sf*_C03 (*P*=0.003, R^2=0.10), *Sf*_C17 (*P*=0.040, R^2=0.05), *Sf*_C22 (*P*=0.023, R^2=0.06), *Sf*_C25 (*P*=0.004, R^2=0.09) and *Sf*_C30 (*P*=0.042, R^2= 0.05, Figure S3).

Homologizing linkage map to* Bombyx mori *chromosomes

Of the 30 autosomes of our linkage map for the timing QTL, we homologized 16 to *B. mori* chromosomes. All four QTL chromosomes were among the homologized ones (Table S8). Two chromosomes, which had not been homologized between the timing linkage maps, could be homologized in addition (BC_A:11 to BC_B:19 and BC_A:32 to BC_B:4), because the RAD sequences mapped to the same *Bm* chromosome.

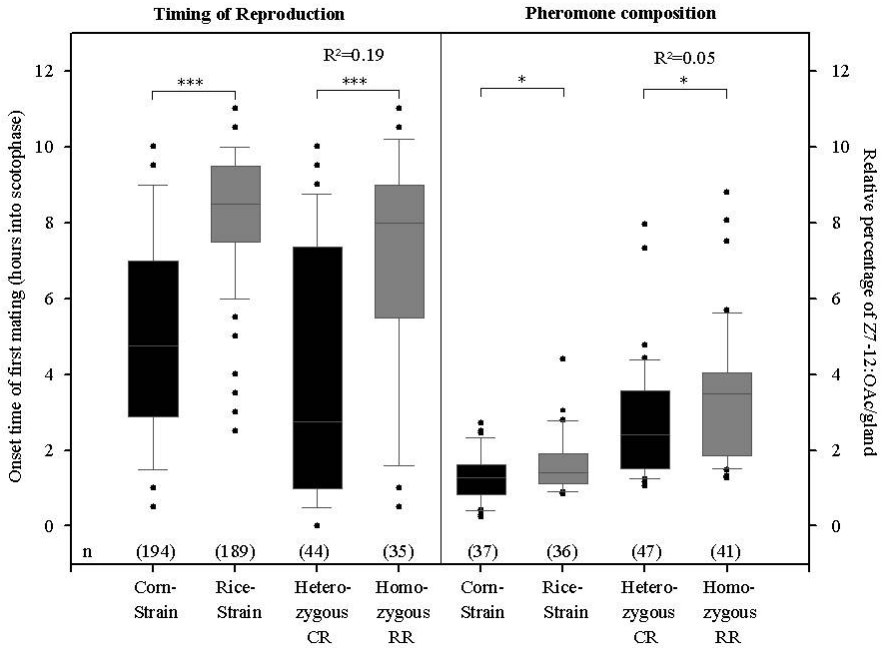

FIGURE 2. Phenotypes for major QTL chromosome *Sf*_C25 (=*Bm*_C27) of pure strain CC and RR individuals vs. heterozygous CR and homozygous RR backcross individuals. For the timing QTL: Onset time of first mating (hours into scotophase). For the pheromone QTL: Relative percentage of Z7-12:OAc per gland.

The candidate genes from the circadian rhythm are located on the following chromosomes (Table S8): *per*, *clk*, *cyc* and *PdP1* on the sex chromosome (*Bm*_C01), *jetlag* on *Bm*_C3 (*Sf* undetermined), *tim* on *Bm*_C4 (*Sf*_undetermined), *CK2α* on *Bm*_C5 (*Sf*_C05), *cry2* on *Bm*_C15 (*Sf*_C23), *CK2β* and *cry1* on *Bm*_C15 (*Sf*_C23), *dbt* on *Bm*_C17 (*Sf*_C17), *shaggy* on *Bm*_C18 (*Sf*_C13), *clockwork orange* on *Bm*_C22 (*Sf*_undetermined), *slimb* on *Bm*_C24 (*Sf*_C12, 32), *vri* on *Bm*_C27 (*Sf*_C25) and *CK1α* on *Bm*_scaf256 (not integrated in *B. mori* chromosomes, cannot be homologized). Thus, of all candidate genes, only *vri* mapped to the major QTL chromosome, *Bm*_C27 (*Sf*_C25).

The candidate gene for the pheromone variation, delta-11-desaturase (*Sf*LPAQ), mapped to *Sf*_C02, which explained a small but significant portion of the variance of Z9-14:OAc (P=0.050, R^2=0.04) and Z11-16:OAc (P=0.014, R^2=0.07). However, this QTL showed an opposite-to-expected phenotypic pattern for both compounds (Figure S3). A similar delta-11-desaturase can be found on *Bm*_C23 (gi|162809332|ref|NP_001037017.2), indicating that *Sf*_C02 of this backcross is homologous to *Bm*_C23. The candidate gene delta-9-desaturase (*Sf*KPSE) mapped to *Sf*_C05, which was not associated with strain-specific differences in any of the four pheromone compounds (Figure S3). This chromosome is probably homologous

to *Bm*_C12, because *Bm*_C12 contains a similar delta-9-desaturase to the one we found (gi|34538645|gb|AAQ74257.1). Interestingly, *vri* mapped to *Sf*_C28 in this backcross family, i.e. *Bm*_C27, which is the most significant QTL for the critical sex pheromone component Z7-12:OAc (Figures 2 and S1). Thus, the strain-specific variance in two potential prezygotic mating barriers of *S. frugiperda* mapped to the same chromosome.

Structure and expression analysis of vri

Vrille is a short gene without introns in the protein coding region, coding for a 367 aa protein, followed by a 1234 bp 3' UTR. The 5' UTR is divided into a 45 bp segment and a 375 bp segment by an intron containing regulatory elements, namely 11 Ebox elements (Ebox A-K) with the core sequence CACGTG (Figure 3). Near Eboxes E, F and I, 5 polymorphisms between the investigated corn-strain and rice-strain populations and the maternal grandmothers of BC_A and BC_B were identified (Table 1).

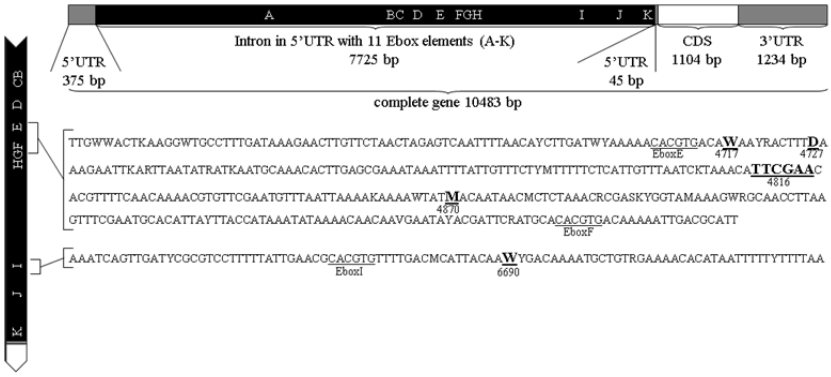

FIGURE 3. Structure of *vri* in the corn- and rice-strain of *S. frugiperda* and strain-specific polymorphisms in the intron in the 5' UTR.

TABLE 1. Variation in the regulatory intron in the 5' UTR of *vri*. Single nucleotide polymorphisms (SNPs) and insertions/deletion (IN/DEL) between 12 individuals from a corn-strain population and 12 individuals from a rice-strain population as well as in the maternal grandmothers (mgm) BC_A and B (originating from these populations. Mgm = maternal grandmother; Sample name followed by C (= corn-strain) or R (= rice-strain); n.a. not available due to sequencing restrictions. Dark grey cells indicate corn-strain alleles and light grey cells indicate rice-strain alleles.

Position	Close to	Type	Population		Individual	
			CL_1 C	**RL_1 R**	**mgmA R**	**mgmB C**
4717	EboxE	SNP	T	A	A	T
4727	EboxE	SNP	T	A	A	T
4816	EboxE	IN/DEL	_ _ _ _ _	TTCGAA	TTCGAA	_ _ _ _ _
4870	EboxF	SNP	A	C	C	A
6690	EboxI	SNP	T	A	A	n.a.

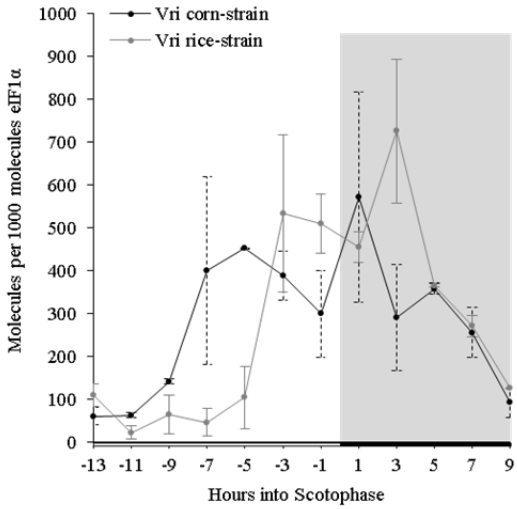

FIGURE 4. Expression of *vri* over time in heads of *S. frugiperda* corn- and rice-strain females. Five individuals were used per biological replicate, two biological replicates per time point. Grey field indicates scotophase. Expression is shown as molecules per 1000 molecules eIF1α.

When analyzing the strain-specific differences in *vri* expression by RT-qPCR, both strains showed two peaks of *vri* expression. The corn-strain females had the highest *vri* expression five hours before and one hour into scotophase, while rice-strain females exhibited one peak three hours before scotophase and one peak three hours into the scotophase (Figure 3). Both peaks thus showed a time-shift between the strains by two hours.

DISCUSSION

In this study, we tested the hypothesis that two prezygotic mating barriers are genetically linked in the two strains of *S. frugiperda*, i.e. allochronic differentiation and sexual communication variation. We found one consistent QTL for the differences in the onset time of mating in the two strains, Sf_C25, which is homologous to Bm_C27. Interestingly, this is also the one QTL that we found for the strain-specific variation in the critical sex pheromone component Z7-12:OAc, which suggests that both traits may indeed be genetically linked. Since the majority of lepidopteran species have 31 chromosomes, the chance of finding the same chromosome in two separate QTL studies is 1/31 or 0.03. The timing of behavior is a complex trait, as it is dependent on the circadian clock, which is a complex network of genes and their products that participate in interlocked feedback loops of transcription and translation (see Figure 1). Thus, it is remarkable to only find one major consistent QTL in two backcross families. Our QTL is one autosome and the homologous autosome in *B. mori* is 14.5 Mb (52.8 cM), which is in the range of

other QTL studies (Gleason and Ritchie 2004; Moehring and Mackay 2004; Shaw et al. 2007; Gleason et al. 2009). Within this region, multiple clock related genes could be located. However, in *B. mori* only one candidate clock gene is known to be located on this chromosome, namely *vri*. All other known clock genes map to different chromosomes in *B. mori* (see also Table S8).

A limitation of our indirect mapping approach is the different number of autosomes in *B. mori* (28) and *S. frugiperda* (30). As we could not homologize all chromosomes between the species, we were not able to determine which chromosomes are fused in *B. mori* compared to *S. frugiperda.* We would expect two *Bm* chromosomes to be fused such that two *Sf* chromosomes would be homologous to one fused *Bm* chromosome. However, as the position of *vrille* is confirmed on our major *Sf* QTL chromosome, a second *Sf* chromosome homologous to Bm_C27 would not affect this result. Also, all minor QTL chromosomes have a confident homologue in *B. mori* and none of these contain known clock genes. If a second *Sf* chromosome would map to the same *Bm* chromosome, this would not lead to a clock gene on the QTL chromosome but would rather mean one more *Sf* chromosome without a known clock gene. Nevertheless, translocations of genes between the two species cannot be ruled out completely, but the synteny between *B. mori* and *S. frugiperda* is highly conserved, which makes *B. mori* an ideal reference genome for *S. frugiperda* (d'Alencon et al. 2010). The high synteny thus also supports our conclusion that *vrille* is the only clock gene located on a QTL chromosome in *S. frugiperda*.

Within the network of the circadian clock genes in insects, *vri* is a powerful player (e.g. in fire ants (Ingram et al. 2012), pea aphids (Cortes et al. 2010) and bean bugs (Ikeno et al. 2008)) and best described in *Drosophila* (Blau and Young 1999; Cyran et al. 2003; Glossop et al. 2003; Hardin 2005). VRI inhibits *clk* transcription, and since a dimer of CLK and CYC promotes many E-Box promoted genes, *clk* inhibition represses transcription of the core clock genes. Consequently, *vri* mutants have altered behavioral rhythms (Blau and Young 1999). Hence, in *S. frugiperda* a strain-specific difference in *vri* expression may cause a strain-specific expression difference in other clock genes, leading to a timing difference in behavior. Our qPCR results indicate that *vri* expression is indeed time-shifted between the strains in females, correlating with the behavioral time shift: expression in the corn-strain is two hours earlier than in the rice strain. The differences between the peaks of behavioral activity, i.e. onset time of mating, are approximately three hours (Schöfl et al. 2009). Since we extracted RNA every two hours, it is not possible to determine whether the qPCR peaks differed by three hours as well. Also, since the variation between the replicates was high, these experiments need to be confirmed to verify our findings. However, together with *vri*'s location on the major QTL chromosome, the expression difference strongly suggests its involvement in the allochronic differentiation in the two strains of *S. frugiperda*.

In a search for sequence differences in *vri* that might account for the timing difference, we have obtained the full sequence consisting of the coding region (1101

bp), the 3' UTR (1234 bp), a split 5' UTR (45 and 375 bp) and an intron in the 5' UTR (7725 bp), including 11 Ebox elements. Within the regulatory intron in close vicinity to Ebox elements, we identified 5 polymorphisms between a corn-strain and a rice-strain population from Florida, five of which were also found in the parental generation of the backcross families for the timing QTL. Since the binding specificity of basic helix-loop-helix transcription factors like CLK and CYC is influenced by the genomic region surrounding the Ebox binding site (Gordan et al. 2013), a less efficient binding of a transcription factor to the active *vri* Ebox element(s) in e.g. the rice-strain could facilitate a later expression of *vri*. Alternatively, a cis-regulatory element regulating this gene could be situated on the same chromosome in a more distant region that we did not yet sequence. Mutations in cis-regulatory elements generally cause expression differences (Wittkopp et al. 2008b, a) and are hypothesized to be key elements of evolutionary changes (Wray 2007). A more distant cis-acting regulatory element could also influence genes involved in the production of the critical pheromone component Z7-12:OAc.

All other known clock genes did not map to any QTL (see Table S8) in the two timing backcross families. The involvement of the sex chromosome in the timing differentiation between the two strains can be excluded based on the fact that the reciprocal F1 hybrids (CR and RC) did not differ in their onset time of mating (Figure S4; Schöfl et al. 2009), which thus excludes *per*, *clk*, *cyc* and *PdP1* that are located on the sex chromosome.

In the strain-specific pheromone differences, we found several genomic regions on 9 different chromosomes to explain at least some of the variance between the two strains (Figure S3). 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 suggests that Z11-16:OAc is not under strong stabilizing selection, compared to Z9-14:OAc, Z7-12:OAc and Z9-12:OAc, which is confirmed by dose-response experiments showing that this compound is not required for male attraction (Unbehend et al. 2013).

Mapping the candidate desaturases to our generated *S. frugiperda* map, we found that the delta-11-desaturase *Sf*LPAQ mapped to QTL chromosome 2 (*Sf*_C02, homologous to *Bm*_C23), involved in the production of Z9-14:OAc and Z11-16:OAc. Thus, strain-specific differences in this desaturase (*Sf*LPAQ) could at least partly explain that corn-strain females produce higher relative amounts of Z11-16:OAc and lower percentages of Z9-14:OAc than rice-strain females. Because Z11-16:OAc and Z9-14:OAc are biosynthetically linked, overproduction of one component consequently leads to the reduction of the other component (Groot et al. 2008). In contrast to delta-11-desaturase *Sf*LPAQ, the delta-9-desaturase (*Sf*KPSE) did not map to a QTL, i.e. to chromosome 5 (*Sf*_C05, homologous to *Bm*_C12). Thus, sequence variation within the delta-9-desaturase *Sf*KPSE can be ruled out to be involved in strain-specific differences in any of the four pheromone components.

Our most interesting finding is that *vri* mapped to pheromone QTL chromosome 28 (*Sf*_C28, homologous to *Bm*_C27), affecting the production of the critical minor component Z7-12:OAc, which is essential for male attraction (Tumlinson et al. 1986; Unbehend et al. 2013). Thus, genes involved in strain-specific Z7-12:OAc production and in strain-specific timing of mating in the night are located on the same chromosome. This suggests that these two prezygotic mating barriers may be genetically linked and/or influenced by the same set of genes or regulatory elements. If strain-specific differences in a cis-regulatory element do exist and influence *vri* expression, it is possible that the same regulatory element also influences another gene, responsible for differential production of Z7-12:OAc in females. A number of 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). Fine-scale mapping and further genetic analysis will be necessary to evaluate which genes are responsible for the strain-specific production of Z7-12:OAc and whether and how this is related to strain-specific timing of mating activity.

In summary, we identified one major QTL chromosome for the timing difference in mating between the two *S. frugiperda* strains. The clock gene *vrille* (*vri*) is located on this QTL chromosome and thus the major candidate for the strain-specific timing differences. Strain-specific expression differences of *vri*, resembling the phenotypic timing differences, as well as strain-specific polymorphisms in the regulatory region of *vri* support the hypothesis that *vri* plays a role in the timing differentiation of these two strains. Interestingly, we found the same QTL involved in the differential pheromone composition of corn- and rice-strain females, namely the production of the critical secondary sex pheromone component Z7-12:OAc. Together, our results indicate that the two prezygotic mating barriers, i.e. allochronic separation and sexual communication, may be genetically linked, which could facilitate the evolution of prezygotic isolation in *S. frugiperda*.

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SUPPORTING INFORMATION

Detailed methods

Insects

Individuals used for the timing QTL analysis descended from > 200 rice-strain larvae and > 100 corn-strain larvae, collected from different fields in Florida in 2003 and 2004, respectively (Table S1). These populations were reared for 10 (corn-strain) and 21 (rice-strain) generations in mass culture at the USDA-ARS in Gainesville, FL, before shipment to MPICE in 2007. These populations were also used by (Schöfl et al. 2009). We refer to these populations as CL1 and RL1 (Table S1). Unfortunately, these two populations died after six years of laboratory rearing. Therefore, we established new laboratory populations for the pheromone QTL analysis, starting with ~300 larvae collected in Florida (rice-strain) and Puerto Rico (corn-strain) in 2010 (Table S1), which were shipped directly to MPICE, where all adults were screened for strain-specific COI markers (Nagoshi et al. 2006), and separated accordingly into strain-specific colonies. We refer to these populations as CL2 and RL2 (Table S1). We confirmed genetic similarity between the corn-strain populations from Florida (CL1) and Puerto Rico (CL2) by determining the mitochondrial haplotype profile of 47 Florida and 43 Puerto Rico individuals (Table S2, Nagoshi et al. (2007)). All 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 set up to maintain the populations and minimize inbreeding.

Phenotyping of pheromone QTL backcrosses

For the pheromone QTL analysis, pheromone glands were extracted from 2-3 day-old virgin pure strain females (C, R), hybrids (CR, RC), and backcross females (CR-R), as described in detail in Unbehend et al. (2013). The pheromone glands were extracted in the scotophase at the strain-specific peaks of calling times, i.e. 4-5 h into scotophase for corn-strain females, 6-7 h into scotophase for the rice-strain females. For the hybrid females, we used the approximate times of the mothers, i.e. 3.5-5.5 h into scotophase for the CR hybrids, 5-6 h into scotophase for the RC hybrids, and 4-7 h into scotophase for the CR-R backcross females. 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. Gas chromatography analysis was performed according to methods and using equipment used in (Unbehend et al. 2013), using a HP7890 gas chromatograph with a polar capillary column (DB-WAXetr (extended temperature range); 30 m × 0.25 mm × 0.5 µm) and a flame-ionization detector. Female pheromone extracts were reduced from 50 µl to 2 µl (with a nitrogen stream), and the reduced extracts were injected singly into the gas chromatograph. Female pheromone compounds were identified by comparing retention times with synthetic standards of Z9-14:OAc, Z7-12:OAc, Z11-16:OAc, and Z9-12:OAc (Pherobank, Wageningen, the Netherlands). After pheromone extraction, all females were stored at -20 °C for further analysis.

DNA extraction and AFLP marker analysis

After DNA extraction, AFLP markers were generated as described in Groot et al. (2009): 200 ng DNA of each sample was digested with *Eco*RI and *Mse*I (New England Biolabs, Ipswich, MA, USA), and *Eco*RI- and *Mse*I-adapters were ligated to the fragments which were then preamplified (Wilding et al. 2001). The preamplified DNA was selectively amplified with different *Eco*RI- and *Mse*I-primer combinations (Table S4). 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). AFLP gels were scored with AFLP-Quantar Pro 1.0 (KeyGene, Wageningen, the Netherlands). To identify corn-strain specific markers, we scored markers that were present in the corn-strain grandparent (C grandmother or grandfather), the hybrid mother (RC or CR), and half of the offspring females (heterozygote females), but absent in the rice-strain grandparent (R), the backcross male (R), and the homozygote backcross (CR-R and RC-R) females. For identification of rice-strain specific markers, we scored markers present in the rice-strain grandparent, the hybrid mother and the homozygote offspring females, but absent in the corn-strain grandparent, the father and the heterozygote backcross females. All markers were converted to the same phase by inverting the absence/presence patterns of all rice-strain specific markers.

Structure analysis of vrille

First degenerate primers based on insect ESTs and genomic sequences (gb|AY526608.1, gb|AY576272.1, gb|AADK01019845.1) were used to obtain partial sequences. After obtaining the sequences, primers were designed to sequence further. The DNA Walking SpeedUp™ Kit II (SEEGENE, Eschborn, Germany) was used to obtain the sequence upstream of the coding sequence (see Table S6 for all primers used). To determine exon/intron structure, the coding region was sequenced from cDNA. Subsequently, parts of the gene were sequenced in 88 different samples (including backcross individuals and corn- and rice-strain individuals from different regions; Table S1), using Sanger-sequencing and Sequencher 4.10.1 for analysis. All obtained sequences from the coding region and ~1kbp upstream are available on GenBank (accession numbers KM675483-658).

TABLE S1. Collection information of *Spodoptera frugiperda* populations used for the different experiments.

Experiment	Strain	Population Origin		Field	Date	Name
Timing QTL	Corn	Florida	Homestead	Corn	2004	CL1
	Rice	Florida	Ona	Grass	2003	RL1
Pheromone QTL	Corn	Puerto Rico	Santa Isabel	Corn	2010	CL2
	Rice	Florida	Moore Haven	Grass	2010	RL2
Haplotyping	Corn	Florida	Belle Glade	Corn	2010	CL3
Structure analysis of Vrille	Corn	Argentina	Los Pereyra	Corn	2010	CF1
		Argentina	Santo Tomé	Corn	2008	CF2
		Florida	Homestead	Corn	2004	CL1
		Puerto Rico	Santa Isabel	Corn	2010	CL2
	Rice	Argentina	Benjamín Aráoz	Grass	2008	RF1
		Argentina	Berón de Astrada	Rice	2008	RF2
		Paraguay	San Cosme y Damián	Rice	2008	RF3
		Texas	College Station	Corn	2010	CF3
		Florida	Ona	Grass	2003	RL1
		Florida	Moore Haven	Grass	2010	RL2
	Corn & Rice	Florida	Hague	Corn	2011	Pheromone trapping
		Argentina	La Cocha	Corn	2008	CF4
Expression analysis	Corn	Florida	Homestead	Corn	2004	CL1
	Rice	Florida	Ona	Grass	2003	RL1

With the full length mRNA acquired from the RNAseq database and blasted against the genome, the full sequence of *vri* was obtained, including a large regulatory intron in the 5' UTR. The corn-strain genome was not complete in this region, thus two BAC clones (AUA0AAA25YL06FM1, AUA0AAA20YH15RM1) spanning the region were obtained from the Centre National de Ressources Génomiques Végétales (CNRGV, Toulouse, France) and shotgun sequenced using Sanger sequencing and Sequencher for analysis. Based on an alignment of the rice-strain genome from SfruDB and the BAC clone sequences, additional parts of the regulatory intron were sequenced in 12 corn-strain and 12 rice-strain individuals from the CL_1 and RL_1 populations as well as the parental and F_1 generations of the timing QTL backcross families.

TABLE S2. Mitochondrial haplotype profiles of *Spodoptera frugiperda* corn-strain individuals from Florida and Puerto Rico.

Population Origin	**Tested Individuals**	**Sample Size**	**Nucleotide**[1]		**Haplotype subgroup**[2]
			Site 1164	**Site 1287**	
Florida[3]	Males	26	Guanosine	Guanosine	CS-h4
	Females	21	Guanosine	Guanosine	CS-h4
Puerto Rico[4]	Males	19	Guanosine	Guanosine	CS-h4
	Females	24	Guanosine	Guanosine	CS-h4

[1]Determination of the mitochondrial Cytochrome oxidase I (COI) haplotype profiles was conducted as described by Nagoshi et al. (2007). After PCR amplification, a part of the COI gene was sequenced at the MPICE (Vogel et al., 2011), and screened for corn-strain specific polymorphisms at the sites 1164 and 1287 (Nagoshi et al., 2007).
[2]The corn-strain haplotype subgroup 4 (CS-h4) is typical for populations from Florida and Puerto Rico (Nagoshi et al., 2007; Nagoshi et al., 2010).
[3]Laboratory population CL3 (Table S1)
[4]Laboratory population CL2 (Table S1)

TABLE S3. Generation of female-informative backcross families for QTL analyzes.

Analysis	**Backcross Family**	**Female Strain**[*]	**Male Strain**[*]	**Generated Offspring**
Timing QTL	A	Rice (33)	Corn (22)	F_1 hybrid (RC)
		RC hybrid (1)	Rice (34)	Backcross (**RC-R**)
	B	Corn (22)	Rice (33)	F_1 hybrid (CR)
		CR hybrid (1)	Rice (34)	Backcross (**CR-R**)
Pheromone QTL	C	Corn (6)	Rice (5)	F_1 hybrids (CR)
		CR hybrid (1)	Rice (6)	Backcross (**CR-R**)

[*]Number in brackets show the generation time of the laboratory populations (Table S1) used for the crosses.

TABLE S4. AFLP markers (Number of informative AFLP-makers scored per primer combination in the three different backcross families (BC) A-C.)

Primer[1]		Number of AFLP-markers		
MseI	EcoRI	Timing QTL BC A	Timing QTL BC B	Pheromone QTL BC C
AAG	AAG	21	18	16
	ACC	9	5	12
	ACG	14	11	18
	ACT	6	8	10
	CGA	6	7	-
	CGC	4	5	-
ACA	AAG	19	17	14
	ACC	8	11	13
	ACG	7	8	13
	ACT	14	16	14
	CGA	11	11	10
	CGC	11	13	13
ACC	AAC	7	10	-
	ACA	6	9	-
ACG	AAG	6	9	17
	ACC	5	3	9
	ACG	5	2	13
	ACT	4	5	9
	CGA	7	7	-
	CGC	4	3	-
ACT	AGA	7	20	-
	AGC	6	8	-
AGG	AAG	15	10	-
	ACC	10	11	-
	ACG	3	2	14
	ACT	7	6	15
	AGG	-	-	13
	CGA	5	5	-
	CGC	4	2	-
CAA	AGG	2	4	-
	ATG	5	9	-
CAC	CAT	7	15	-
	TAC	6	9	-
CAG	GTA	3	3	-
	TCT	3	3	-
CAT	AAG	6	10	12
	ACC	9	11	-
	ACG	5	6	7
	ACT	6	3	-
	CGA	9	12	11
	CGC	6	3	4
CCA	ACA	5	11	-
	TTA	-	3	-
CCC	GTA	6	7	-
	TTA	5	5	-
CCG	AGC	7	6	-

	TAC	3	8	-
CCT	AGA	4	4	-
	AGG	2	2	-
CGA	AAG	13	8	11
	ACC	1	8	8
	ACG	9	6	10
	ACT	11	9	5
	CGA	11	5	-
	CGC	3	3	-
CTC	AAC	12	12	-
	CAT	6	10	-
CTG	AAG	7	9	-
	ACC	13	15	-
	ACG	6	7	12
	ACT	3	2	-
	CGA	4	5	-
	CGC	1	3	-
CTT	AGG	13	15	-
	ATG	22	21	-
Total markers		465[2]	514[2]	303

[1]All primers have a core sequence (MseI- primer: 5'-GATGAGTCCTGAGTAA; EcoRI-primer: 5'-GACTGCGTACCAATTC) plus three selective bases at the end (according to the table).
[2]Of all markers scored in the timing QTL analysis, 294 markers were present in both backcross families A and B.

TABLE S5. Coverage of RAD sequences (Distribution of RAD sequences per individual sample.

Individual	Average FW read Stackhight	Standard-deviation	Standard-error	Max FW read Stackhight	Min FW read Stackhight	Median FW read Stackhight	Number of different FW reads	Average Copy of PE reads	Standard-deviation	Standard-error	Max PE reads	Min PE reads	Median PE reads	Average Different PE read per FW read	Median Different PE read per FW read
mgmA	426.6307	1018.028	15.83725	32219	2	243	4132	90.00413	111.4997	1.734577	979	1	56.25287	7.683204	3
mgfA	458.7566	2385.873	36.01754	107630	2	225	4388	86.1971	106.1036	1.601758	829	1	56.5	7.999544	3
moA	431.8876	1204.144	18.66273	40573	2	241	4163	85.79489	103.4392	1.603179	884	1	58	7.895268	3
faA	415.4024	1197.019	17.18288	41120	2	224	4853	87.56805	107.9381	1.549422	882	1	55.5	7.377911	3
bcA02	387.7372	1056.735	15.08236	38044	2	210	4909	88.96191	107.6816	1.536898	864	1	59.75	7.242819	3
bcA08	413.1341	1047.458	15.58173	38200	2	228	4519	88.63931	110.1302	1.638269	898	1	57	7.6289	3
bcA18	409.1944	974.1887	13.85632	35374	2	230	4943	87.96394	108.8721	1.548536	855	1	58.5	7.60085	3
bcA24	364.366	1146.624	15.83548	45840	2	189	5243	83.94609	104.3151	1.440646	847	1	54.8	6.664124	2
bcA25	393.1814	1075.532	15.52882	35691	2	217	4797	83.85207	105.0766	1.517124	849	1	53.5	8.147801	3
bcA26	421.4899	1251.074	16.42882	40588	2	195	5799	73.1337	95.20368	1.250193	817	1	43.57143	8.608036	3
bcA39	436.8314	1237.587	16.08613	56709	2	205	5919	79.68049	102.0066	1.32588	1170	1	51.36364	7.618855	3
bcA40	387.1765	929.5574	13.85856	29871	2	212	4499	85.72081	103.8102	1.547682	824	1	58.5	7.287842	3
bcA57	426.8583	1106.231	15.43144	42410	2	223	5139	87.98172	114.7766	1.601084	905	1	51	8.52014	3
bcA60	450.5923	1951.403	25.8856	92590	2	201	5683	82.85137	108.4086	1.438054	951	1	46.4	8.675875	3
bcA71	411.6647	1056.592	14.80832	45594	2	221	5091	92.14743	116.1186	1.627422	900	1	57.25	7.097623	3
mgmB	439.8348	1749.672	25.00552	65713	2	190	4896	84.30461	108.096	1.54486	881	1	51	8.24857	3
mgfB	374.7253	1247.66	19.53048	49200	2	204	4081	87.46333	109.3928	1.712401	789	1	54.8	6.598873	2
moB	415.3913	1221.099	16.44736	43634	2	214	5512	82.76193	107.7499	1.451316	881	1	50.5	8.097242	3
faB	319.5755	529.0829	14.25277	8100	2	222	1378	110.9249	133.7814	3.603886	962	1	73.83333	5.197388	2
bcB62	420.5553	1406.466	17.20455	54590	2	177	6683	72.77307	98.2771	1.202172	860	1	37	8.438276	3
bcB49	420.7763	1953.068	25.45483	109348	2	167	5887	74.83013	94.73558	1.234713	853	1	46.5	7.651266	3
bcB47	457.8441	2020.614	25.69915	106689	2	196	6182	80.17825	105.2327	1.338401	972	1	46.21324	8.549822	3
bcB43	427.5193	1501.815	19.71979	69494	2	205	5800	79.48143	100.2758	1.316685	1093	1	51.0625	8.03931	3
bcB32	460.2086	2572.537	33.6229	136510	2	191	5854	74.8997	98.23262	1.283895	826	1	41	8.406218	3
bcB26	513.6885	2049.415	22.44393	98066	2	115	8338	63.37613	89.00555	0.974734	884	1	17.84659	10.40621	3
bcB25	408.9109	1531.764	20.82732	66322	2	189	5409	80.31784	101.9278	1.385907	780	1	48.6875	8.137549	3
bcB20	468.9908	1946.305	23.00301	99304	2	173	7159	71.97056	96.79316	1.14398	941	1	37.375	8.692136	3
bcB09	430.441	1186.454	16.18009	43249	2	224	5377	84.28062	107.3992	1.464641	998	1	51	8.310954	3
bcB08	393.7708	1149.8	14.46433	38380	2	154	6319	76.13915	105.1656	1.322969	843	1	38	7.464947	2
bcB05	403.9736	2023.704	26.74837	110009	2	190	5724	81.34371	99.26521	1.312041	810	1	52.59839	6.885744	3

TABLE S6. Primer combinations and annealing temperatures (T_a) of candidate genes.

Experiment	Candidate Gene	Primer	T_a (°C)
QTL 1	Vrille	PC19 Forward: 5' CGACCCAAATGACTACTCTCCT PC19 Reverse: 5' CGTCAGCTTACTCCTCTTGGTT	58
		PC34 Forward: 5' ACCGGCTCATAATTGATCGTT PC34 Reverse: 5' GTCGGTTGCAAAAACTGAATGTC	58
		PC38 Forward: 5' CGG GGC AAC CGA CAA AAA AAT PC38 Reverse: 5' GCGTTCAATAAAAAGGACGCGGATCA	58
		EBOX1 Forward: 5' GATCCGCGTCCTTTTTATTGAAC EBOX1 Reverse: 5' CGAAAGCATCACTCAACACAATG	58
QTL 2	Delta-11-desaturase (*Sf*LPAQ)	Forward: 5' AACATTTGGGGAAGGTTTCC Reverse: 5' CAAATGCAACATTATAAAACTTCA	53
	Delta-9-desaturase (*Sf*KPSE)	Forward: 5' TCATTATGCCCACGGTGATT Reverse: 5' ATGACAGTGAAAAGGAAGACAT	53
	Vrille	Forward: 5' GAGGCGCTTCAATGACATGG Reverse: 5' GGCTCCTGCTTTATGTGCTGAA	60
Structure analysis of *Vrille*	Vrille	PC8 Forward: 5' GTCCGCCGAAACATGGTYGCMG PC8 Reverse: 5' GDACTGAACCGGGDGGTTCG	61-50
		PC19 Forward: 5' CGACCCAAATGACTACTCTCCT PC19 Reverse: 5' CGTCAGCTTACTCCTCTTGGTT	58
		PC21 Forward: 5' CCCTACCAGGAGAGGCTACC PC21 Forward: 5' TCAGTGCTCGWGCSMGCGSG	58
		PC34 Forward: 5' ACCGGCTCATAATTGATCGTT PC34 Reverse: 5' GTCGGTTGCAAAAACTGAATGTC	58
		PC38 Forward: 5' CGG GGC AAC CGA CAA AAA AAT PC38 Reverse: 5' GCGTTCAATAAAAAGGACGCGGATCA	58
		CDS+5' Forward: 5' TGTCACGTGTTTCAAGCATGGTA CDS+5' Reverse: 5' TGTTCTGGTGCATCATGTTCTTC	58
		EBOXBCD Forward: 5' ATTTCACGTTCTTCGATCAC EBOXBCD Reverse: 5' TAAATGCAAATGCACAGAAC	55
		EBOXDE Forward: 5' CTAATCGCGGTTCTAATGAC EBOXDE Reverse: 5' CATTCGAAACTTAAGGTTGC	55
		EBOXFGH Forward: 5' GCAACCTTAAGTTTCGAATG EBOXFGH Reverse: 5' ACGGTGACGACACTCTAAAT	55
		EBOXI Forward: 5' GATCCGCGTCCTTTTTATTGAAC EBOXI Reverse: 5' CGAAAGCATCACTCAACACAATG	58
		EBOXJ Forward: 5' ACCGGCTCATAATTGATCGTT EBOXJ Reverse: 5' GCGGATTTCTTCCGTTACAA	58
		EBOXK Forward: 5' TGTCACGTGTTTCAAGCATGGTA EBOXK Reverse: 5' GTCGGTTGCAAAAACTGAATGTC	58
		DNAwalk1 TSP1: 5' GCGTCAGCTTACTCCTCTTGGTT DNAwalk1 TSP2: 5' GCTGTGCTTTGAGTACGTGGTTC DNAwalk1 TSP3: 5' GCGCTGTCCAAAGAACTCCTTGC	65 65 66
		DNAwalk2 TSP1: 5' TGAGCCGGTAATACAGGAAGTGTAA DNAwalk2 TSP2: 5' TGCCTATTGTGGCGACTTAGTTTGAT DNAwalk2 TSP3: 5' GCGTTCAATAAAAAGGACGCGGATCA	64.1 64.4 66.2
Expression analysis	Vrille	Forward: 5' CTGTGCTTTGAGTACGTGGTTC Reverse: 5' GCAAACAGAGGGAGTTCATACC	58
	eIF1α	Forward: 5' AGGAGTTGCGTCGTGGTTAC Reverse: 5' CTTTGATTTCGGCGAACTTG	58

TABLE S7. PCR conditions of different experiments.

Experiment	Candidate Gene	PCR components[1]		PCR program	
QTL 1	Vrille	1 µl	DNA	2 min	94 °C
		11.92 µl	dH_2O	45 s	94 °C
		2 µl	10x Taq buffer	35x 45 s	T_a[2]
		2 µl	2 mM dNTPs	60 s	72 °C
		3 µl	10 mM primer mix[2]	10 min	72 °C
		0.08 µl	Taq polymerase		
QTL 2	Delta-11-desaturase Delta-9-desaturase Vrille	1 µl	DNA	2 min	94 °C
		11.92 µl	dH_2O	45 s	94 °C
		2 µl	10x Taq buffer	35x 45 s	T_a[2]
		2 µl	2 mM dNTPs	60 s	72 °C
		3 µl	10 mM primer mix[2]	10 min	72 °C
		0.08 µl	Taq polymerase		
Structure analysis of *Vrille*	Vrille	1 µl	DNA	2 min	94 °C
		11.92 µl	dH_2O	45 s	94 °C
		2 µl	10x Taq buffer	35x 45 s	T_a[2]
		2 µl	2 mM dNTPs	90 s	72 °C
		3 µl	10 mM primer mix[2]	10 min	72 °C
		0.08 µl	Taq polymerase		
	Vrille (touchdown PCR for degenerate primers)	1 µl	DNA	3 min	94 °C
		11.92 µl	dH_2O	30 s	94 °C
		2 µl	10x Taq buffer	28x 30 s	T_a[2] *
		2 µl	2 mM dNTPs	60 s	(decrease by
		3 µl	10 mM primer mix[2]	30 s	0.7)
		0.08 µl	Taq polymerase	23x 30 s	72 °C
				60 s	94 °C
					Lowest T_a[2]
					72 °C
Expression analysis	Vrille eIF1α	1 µl	cDNA	10 min	90 °C
		10 µl	dH_2O	30 s	95 °C
		1 µl	10 mM primer fw	40x 60 s	58 °C
		1 µl	10 mM primer rv	60 s	72 °C
		12 µl	SYBR Mix[3]	60 s	95 °C
				30s	58 °C
				30s	95 °C

[1]Taq polymerase, dNTPs, buffer and primers were purchased from Metabion, Martinsried, Germany

[2]Primers and corresponding annealing temperatures (T_a) can be found in Table S6

[3] ABsolute Blue QPCR SYBR Green Mix from Thermo Fisher Scientific, Schwerte, Germany

TABLE S8. Overview homologized chromosomes, QTL values and mapping genes of timing QTL (E-values of blast hits are given in intersection of *Bm* and *Sf* chromosomes. Dark fields indicate successfully homologized chromosomes based on e-values in dark fields.)

Mapping GOI		BC	Confident homologue in *B. mori*																	
clk, cyc, per, PdP1	Bm_C1	A																		
		B	2.E-02																	
	Bm_C2	A																		5.E-09
		B																		5.E-09
CK2α	Bm_C5	A		4.E-11															4.E-62	
		B																	1.E-25	
	Bm_C6	A																		
		B															1.E-18	1.E-25	
	Bm_C10	A														1.E-15			
		B	1.E-20						1.E-02								2.E-19			
	Bm_C11	A		3.E-10					7.E-17							2.E-02				
		B	3.E-18						8.E-02							6.E-14				
	Bm_C12	A													1.E-12					
		B	5.E-28		3.E-02									4.E-02	2.E-10					
	Bm_C14	A								2.E-05				4.E-02	4.E-03					
		B								8.E-38		4.E-02		1.E-13						
cry2, CK2β	Bm_C15	A						6.E-02					2.E-16							
		B							1.E-02				5.E-14						2.E-02	
cry1, dbt	Bm_C17	A										2.E-17								
		B										5.E-11								
sgg	Bm_C18	A									7.E-38	3.E-09				4.E-18				
		B									4.E-11								2.E-02	
	Bm_C19	A		6.E-04					1.E-03	8.E-03					1.E-32					
		B								1.E-17										
	Bm_C21	A							6.E-77						2.E-03					
		B	2.E-20						1.E-25										2.E-02	
	Bm_C25	A						2.E-21		1.E-02						8.E-19				
		B						2.E-15												
vri	Bm_C27	A					4.E-19													
		B					5.E-08	8.E-02		4.E-19										
	Bm_C28	A				4E-16												3.E-04		
		B			1.E-08															
slimb	Bm_C24	A		2.E-14											6.E-04					
		B	4.E-36							4.E-11										
lark, jetlag	Bm_C3	A																		
		B													2.E-05				3.E-02	
tim	Bm_C4	A															1.E-08			
		B	2.E-42																	
	Bm_C7	A							8.E-14					2.E-13	3.E-07			3.E-03		
		B						1.E-14												
	Bm_C8	A											1.E-14	2.E-13	3.E-07				2.E-04	
		B						1.E-14					1.E-02							
	Bm_C9	A																		
		B																	1.E-25	
	Bm_C13	A							6.E-02											
		B	3.E-41																	
	Bm_C16	A							8.E-02						1.E-19					
		B	5.E-12																1.E-25	
	Bm_C20	A																		
		B	1.E-18																3.E-09	
cwo	Bm_C22	A							4.E-02											
		B																	2.E-11	
	Bm_C23	A																		
		B	2.E-19																	
	Bm_C26	A													2.E-03					
		B					3.E-02													
	unknown	A											2.E-40				3.E-24			
		B																		
		A		Sf C32		Sf C11	Sf C25	Sf C2	Sf C26	Sf C8	Sf C13	Sf C17	Sf C23	Sf C3	Sf C20	Sf C21	Sf C14	Sf C30	Sf C5	Sf C28
		B	C4		C19		C27	C17	C12	C8	C14	C20	C7	C18	C21	C22	C16	C10	C5	C29
QTL results	BCA+B	P					1E-04								0.023			0.01		0.014
		R²					0.191								0.065			0.082		0.077
QTL results	BCB	P					7E-04													0.033
		R²					0.279													0.12
QTL results	BCA	P					0.021											0.008		
		R²					0.129											0.165		

Mapping GOI		BC		No confident homologue in *B. mori*													
clk, cyc, per, PdP1	Bm_C1	A		5.E-06													
		B															
	Bm_C2	A													2.E-01		
		B															
CK2α	Bm_C5	A							7.E-02								
		B												4.E-62			
	Bm_C6	A		1.E-05													
		B											1.E-11				
	Bm_C10	A		1.E-05		7.E-02											
		B															
	Bm_C11	A															
		B															
	Bm_C12	A															
		B								3.E-01							1.E-05
	Bm_C14	A															
		B															
cry2, CK2β	Bm_C15	A						1.E-01						8.E-02			
		B															3.E-12
cry1, dbt	Bm_C17	A															3.E-09
		B									6.E-11					1.E-06	
sgg	Bm_C18	A															
		B									5.E-03						
	Bm_C19	A				2.E-33											
		B															
	Bm_C21	A		5.E-11													8.E-26
		B															
	Bm_C25	A															
		B															2.E-27
vri	Bm_C27	A									8.E-03						
		B												3.E-20			
	Bm_C28	A				9.E-07											
		B											6.E-59				
slimb	Bm_C24	A			1.E-05							3.E-14					
		B													0,37		5.E-12
lark, jetlag	Bm_C3	A		1.E-10													
		B								3.E-01			2.E-57				7.E-10
tim	Bm_C4	A			2.E-13		1.E-06										
		B								4.E-01							
	Bm_C7	A		5.E-11													
		B						7.E-24		6.E-14			4.E-09	4.E-05			
	Bm_C8	A		5.E-11									0,13				
		B								6.E-14	6.E-14			4.E-05			
	Bm_C9	A								3.E-22							
		B								0,37				8.E-58			
	Bm_C13	A		1.E-10													
		B															
	Bm_C16	A								0,3					1.E-01	6.E-12	
		B									2.E-02					2.E-10	
	Bm_C20	A															
		B															
cwo	Bm_C22	A				7.E-02											
		B								9.E-11				7.E-19	2.E-22		
	Bm_C23	A										2.E-02				7.E-20	
		B															
	Bm_C26	A															
		B															
	unknown	A									8.E-14	5.E-46					2.E-07
		B				6.E-19						5.E-91					
		A		Sf C22	Sf C31	Sf C29	Sf C24	Sf C19	Sf C18	Sf C16	Sf C15	Sf C12	Sf C10	Sf C9	Sf C6	Sf C4	Sf C1
		B			C15	C28	C24	C25	C30	C3	C1	C13	C11	C9	C6	C2	C23
QTL results	BCA+B	P															
		R²															
QTL results	BCB	P							0.0076		0.0014						
		R²							0.1819		0.2517						
QTL results	BCA	P		0.0352										0.044			
		R²		0.1088										0.9999			

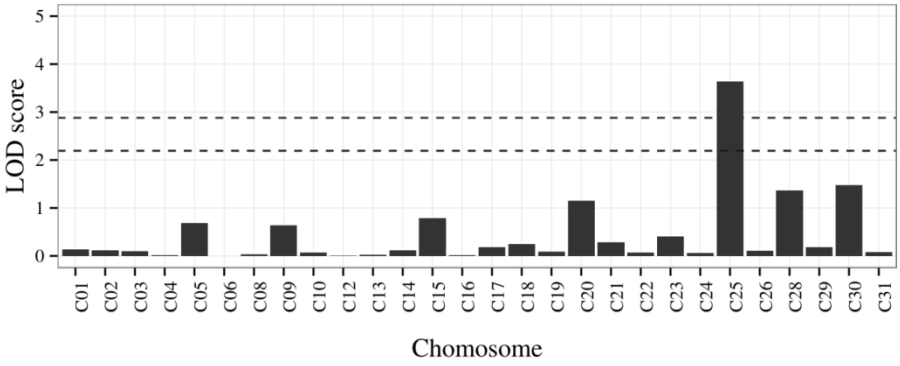

FIGURE S1. LOD scores for all linkage groups in the combined analysis of the two timing backcross families, empirically determined by 10,000 permutations. 0.05 and 0.10 significance thresholds are represented by dashed lines.

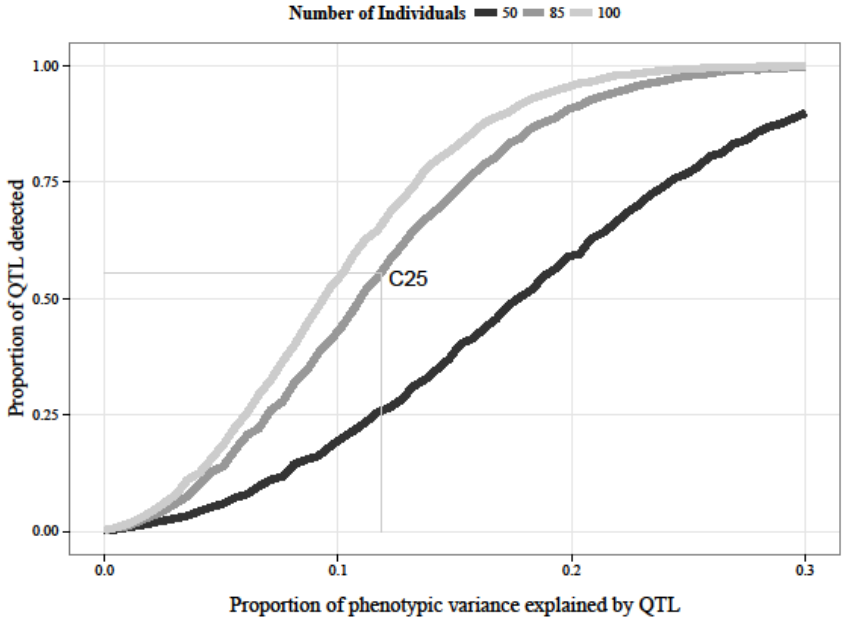

FIGURE S2. Power analysis for backcross families with 50 (black line), 85 (timing QTL, dark grey line) and 100 (black line) progeny, respectively. The probability of detecting a QTL is plotted as a function of the fraction of phenotypic variance explained by the QTL.

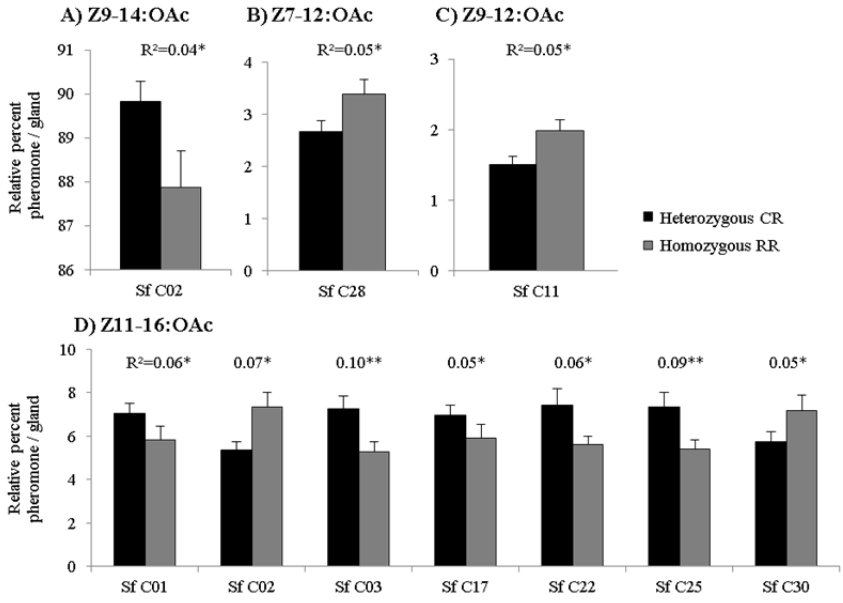

FIGURE S3. All QTL found in pheromone QTL analysis (The effect of different chromosomes on the relative amount of **A)** Z9-14:OAc, **B)** Z7-12:OAc, **C)** Z9-12:OAc and **D)** Z11-16:OAc in pheromone glands of heterozygous (CR, black bars) and homozygous (RR, grey bars) *S. frugiperda* backcross individuals (BC C).)

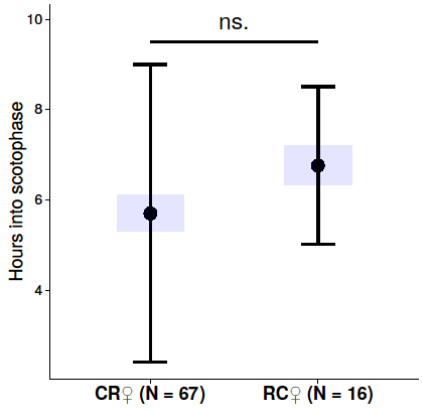

FIGURE S4. Mating time in *S. frugiperda* hybrids. Onset time of first mating in *S. frugiperda* hybrid females. The reciprocal crosses (CR= corn-strain mother, rice-strain father; RC= rice-strain mother, corn-strain father) do not show differences in mating time. This excludes the involvement of the sex chromosome in the timing differentiation.