CHASING SYMPATRIC SPECIATION

THE RELATIVE IMPORTANCE AND GENETIC BASIS OF PREZYGOTIC ISOLATION BARRIERS IN DIVERGING POPULATIONS OF *SPODOPTERA FRUGIPERDA*

SABINE HÄNNIGER

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'Every scientific statement is provisional. […]. How can anyone trust scientists? If new evidence comes along, they change their minds.'
Terry Pratchett et al., *The Science of Discworld: Judgement Day*, 2005

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'I love deadlines. I love the whooshing noise they make as they go by.'
Douglas Adams, *The Salmon of Doubt*, 2002

SUMMARY

Speciation occurs when gene flow between groups of individuals of one species is reduced to such an extent that the genetic differences that have accumulated over time cause reproductive isolation. The most obvious cause of gene flow reduction occurs in allopatry, i.e. when populations become geographically isolated, but gene flow can also be reduced in sympatry, for example when individuals within a population start feeding on different types of food. Differentiation in feeding habits has been found in some herbivorous insects that develop on different host plants, and in cichlid fishes that feed on different types of prey. However, it is unlikely that one factor can cause a strong enough reduction in gene flow to cause reproductive isolation. More likely a number of factors, such as food choice and mate choice, interact to cause reproductive isolation. Determining which factors contribute to sympatric speciation, and the level and extent of their interaction, is the current challenge to understand under which circumstances and how sympatric speciation can occur.

This thesis investigates the mechanisms underlying three prezygotic isolation barriers between the corn- and the rice-strains of the noctuid moth *Spodoptera frugiperda* to determine possible interactions between these isolation barriers and their relative importance for sympatric speciation.

First, we investigated the role of *host plant differentiation* as isolation barrier between the two strains. **Chapter 2** summarizes studies addressing strain differences in host utilization, i.e. oviposition preference, larval host acceptance, larval choice, larval performance, pupal weight and total fecundity. Only oviposition preference showed some consistent differences between the two strains in the different experiments that were conducted. **Chapter 3** describes a population-genetic analysis with *S. frugiperda* populations collected from corn fields, rice fields and wild grasses at eight locations in Argentina, Brazil and Paraguay. This analysis shows that, although there is some host-associated genetic structure, host plant differentiation alone cannot explain the total genetic variance and thus other, additional factors must maintain the genetic differentiation. Consequently, we suggest calling these strains 'host forms' instead of 'host strains'.

Second, I focused on *differentiation in sexual communication* between the two strains. **Chapter 4** investigates the pheromonal divergence patterns of *S. frugiperda* strains in Florida, specifically whether the strain-specific pheromone blends cause strain-specific male responses. Males do not show strain-specific attraction in Florida. However, males did show a strain-specific difference in the attraction to the critical pheromone component Z7-12:OAc. Also, the different habitats influenced the male response, suggesting an interaction between host plant and sex pheromone volatiles. In **Chapter 5** we disentangled strain-specific variation from geographic

variation in male attraction of *S. frugiperda* by comparing male attraction in Canada, North Carolina, Florida, Puerto Rico, Peru and Argentina. We found that the response of corn-strain males varies between geographic regions, whereas rice-strain males did not show geographic variation in their response. Additionally, we found habitat to have an effect on the attraction of males to strain-specific lures, suggesting an interaction of host plant volatiles and sex-pheromones in the attraction of males in *S. frugiperda*.

The third and most consistent prezygotic isolation barrier between the two strains is *allochronic differentiation*, i.e. the strains differ in the daily timing of their sexual activities. In **Chapter 6**, we determined the genetic basis of this barrier, in combination with the pheromonal divergence, through quantitative trait locus (QTL) analysis. We found one major QTL for the circadian differentiation, explaining 19% of the variance between the strains. The circadian clock gene *vrille* maps to this locus, which is thus the major candidate gene underlying this circadian differentiation. Interestingly, the differentiation of the critical sex pheromone component (Z7-12:OAc) maps to the genomic locus, suggesting a genetic linkage between pheromonal divergence and allochronic differentiation. **Chapter 7** describes the annotation of nine genes in the corn-strain variant of the *S. frugiperda* genome project, which are involved in circadian rhythm. We successfully elucidated the exon-intron structure of these clock genes. Five of the nine genes are located on more than one scaffold, so that the annotation of these genes significantly contributed to the *S. frugiperda* genome assembly.

Finally, the *relative importance and possible interactions* between the three prezygotic isolation barriers between the two strains in *S. frugiperda* are discussed. The prezygotic timing of reproductive activity and the postzygotic hybrid sterility found in daughters of matings between rice-strain mothers and corn-strain fathers most likely make the strongest contribution to reproductive isolation. In addition, we propose that the corn-strain is the derived strain and the rice-strain the ancestral strain, because the rice-strain is genetically more diverse, shows a broader response to pheromone blends and is more likely to be found on host plants different from the 'typical' small grasses. In **Chapter 9**, I discuss the evolutionary potential of the different isolation barriers between the two strains of *S. frugiperda* and I propose an interaction between all prezygotic mating barriers which facilitate the divergence of the two strains, with allochronic differentiation being the driving force in these interactions. In conclusion, by investigating the different prezygotic isolation barriers between two sympatrically occurring strains of *S. frugiperda* in detail, I have determined their strengths and possible interactions, which has given important insights in the mechanisms underlying and facilitating the process of sympatric speciation.

SAMENVATTING

Soortvorming vindt plaats als genetische uitwisseling tussen groepen van individuen van een soort zodanig gereduceerd wordt dat de genetische verschillen die in de loop van de tijd geaccumuleerd zijn leiden tot reproductieve isolatie. De duidelijkste oorzaak van een vermindering van genetische uitwisseling is als populaties door geografische barrières worden gescheiden, dus allopatrische soortvorming. Echter, genetische uitwisseling tussen individuen kan ook verminderen zonder geografische barrière, in sympatrie, bijvoorbeeld als individuen binnen een populatie van verschillende voedselbronnen gaan eten. Differentiatie in voedingsgedrag is bijvoorbeeld gevonden in herbivore insecten, die zich op verschillende waardplanten kunnen ontwikkelen, en in cichlide vissen die verschillende prooien eten. Het is niet erg waarschijnlijk dat één specifieke factor een zodanige reductie in genetische uitwisseling kan veroorzaken dat reproductieve isolatie ontstaat. Waarschijnlijk zorgen meerdere factoren, zoals voedingsgedrag en paringsgedrag, samen en in interactie met elkaar voor een zodanige reductie in genetische uitwisseling dat reproductieve isolatie ontstaat. Bepalen welke factoren tot sympatrische soortvorming kunnen leiden, en hoe deze factoren interacteren, is de huidige uitdaging om te begrijpen wanneer en onder welke omstandigheden sympatrische soortvorming kan plaatsvinden.

Dit proefschrift beschrijft de mechanismen van drie prezygotische isolatiebarrières in het rijst- en mais-ras van de nachtvlinder *Spodoptera frugiperda* om hun onderlinge interacties en bijdrage aan sympatrische soortvorming te bepalen. Allereerst wordt de rol van waardplantdifferentiatie als isolatiebarrière tussen de twee rassen onderzocht. **Hoofdstuk 2** geeft een samenvatting van de verschillen tussen de twee rasssen in het gebruik van voedselplanten, met name ovipositievoorkeur van de volwassen vrouwtjes, de acceptatie van verschillende planten door de rupsen, de keuze van rupsen voor bepaalde planten en de vruchtbaarheid van de twee rassen op de verschillende planten. Alleen ovipositievoorkeur van de vrouwtjes bleek consistent te verschillen tussen de twee rassen in de verschillende experimentten die werden uitgevoerd. **Hoofdstuk 3** beschrijft een populatie-genetisch onderzoek aan *S. frugiperda* populaties verzameld van maisvelden, rijstvelden en wilde grassen van acht locaties in Argentinie, Brazilie en Paraguay. Deze analyse laat zien dat waardplantdifferentiatie alleen niet de genetische variatiepatronen tussen de twee rassen kan verklaren en dat derhalve andere factoren de genetische differentiatie handhaven. Daarom stellen wij voor om deze rassen niet (gastheer)rassen te noemen, zoals tot nog toe gebruikelijk was, maar (gastheer)vormen.

In het tweede deel van dit proefschrift analyseer ik de differentiatie in seksuele communicatie tussen de twee rassen. **Hoofdstuk 4** onderzoekt of de seksferomoonverschillen tussen de twee rassen in Florida tot ras-specifieke responsen van de mannetjes leiden. Dit blijkt niet het geval te zijn, hoewel we wel ras-specifieke verschillen in aantrekking tot een belangrijke feromooncomponent (Z7-12:OAc)

vonden. Habitat bleek ook de mannelijke respons te beïnvloeden, wat een interactie tussen planten- en seksferomoonstoffen suggereert. In **hoofdstuk 5** ontrafelen we ras-specifieke van geografische variatie in de seksuele aantrekking van mannetjes door dezelfde experimenten te doen in Canada, North Carolina, Florida, Puerto Rico, Peru en Argentinië. Uit deze studie blijkt dat de respons van maisras-mannetjes verschilt tussen gebieden, terwijl de rijstras-mannetjes geen geografische variatie vertonen. Ook hier vonden we dat de habitat een effect heeft op de aantrekking van mannetjes tot de ras-specifieke lokstoffen; dit suggereert een interactie tussen planten- en seksferomoonstoffen in de aantrekking van mannetjes in *S. frugiperda*.

De derde en meest consistente prezygotische isolatiebarrière tussen de twee rassen is allochrone differentiatie, d.w.z. de rassen verschillen in de dagelijkse timing van hun seksuele activiteit. **Hoofdstuk 6** beschrijft de genetische analyse van deze isolatiebarrière, in combinatie met de feromoondifferentiatie, door quantitative trait locus (QTL) analyse. De voornaamste QTL verklaarde een significant deel (19%) van de variantie in circadiane verschuivingen tussen de rassen. Het circadiane klokgen *vrille* bevindt zich op dit locus, wat daarmee het belangrijkste kandidaatgen is dat aan de circadiane rasverschillen ten grondslag ligt. Interessant genoeg vonden we dat het rasverschil in een van de kritieke seksferomooncomponenten zich ook op deze genomische locatie bevindt, wat suggereert dat feromoondifferentiatie en allochrone differentiatie genetisch gekoppeld zijn. **Hoofdstuk 7** beschrijft de annotatie van negen genen in het maisras van *S. frugiperda*, die betrokken zijn bij het circadiane ritme. We hebben de exon-intronstructuur van deze genen opgehelderd. Vijf van de negen genen zijn over meerdere scaffolds verspreid, waardoor hun annotatie belangrijk heeft bijgedragen tot de assemblage van het *S. frugiperda* genoom.

In het laatste deel van dit proefschrift worden de relatieve bijdrage en mogelijke interacties tussen de drie prezygotische reproductieve isolatiebarrières behandeld. De prezygotische timing van seksuele activiteiten en de postzygotische hybridde steriliteit in dochters van paringen tussen rijstvrouwtjes en maismannetjes zijn waarschijnlijk de belangrijkste bijdragen aan reproductieve isolatie tussen de rassen van *S. frugiperda*. Bovendien is het rijstras waarschijnlijk het oorspronkelijke ras en het maisras het afgeleide ras, omdat het rijstras genetisch diverser is, een bredere feromoonrespons laat zien en vaker voorkomt op andere waardplanten dan de 'typische' kleine grassen. In **hoofdstuk 9** bespreek ik de evolutionaire potentie van de verschillende isolatiebarrières tussen de twee rassen van *S. frugiperda* en stel ik een interactiemodel voor tussen de verschillende prezygotische barrières die de divergentie van de twee rassen faciliteren, waarin allochrone differentiatie de drijvende kracht is in deze interacties. Samenvattend heeft dit promotie-onderzoek verschillende prezygotische isolatiebarrières tussen twee sympatrisch voorkomende rassen van *S. frugiperda* in detail bestudeerd, hun mogelijke interacties in kaart gebracht, en daarmee belangrijke inzichten verschaft in de mechanismen die sympatrische soortvorming mogelijk maken.

1

GENERAL INTRODUCTION

SPECIATION IN SYMPATRY

'...whilst this planet has gone cycling on [...], from so simple a beginning endless forms most beautiful and most wonderful have been, and are being, evolved.'
Charles Darwin, *On the Origin of Species*, 1859

When Darwin introduced the idea of speciation in his famous book *On the origin of species* (1859), his theory included the evolution of species in sympatry as well as in allopatry. Sympatric speciation is the evolution of new species from an ancestral species without geographic isolation, whereas allopatric speciation is facilitated by geographical boundaries separating populations. Today it is widely accepted that also without spatial separation of populations gene flow may become limited between groups of individuals and allow reproductive isolation to evolve (recent studies include e.g. Nanda and Singh 2012; Nosil and Feder 2012; Strasburg et al. 2012; Boomsma and Nash 2014; Castiglia 2014; Scordato et al. 2014; Aboagye-Antwi et al. 2015). However, for over a century sympatric speciation was widely rejected and has been referred to as the 'ugly duckling' of evolutionary theory, which only in the past few decades grew up to be a 'swan' (Via 2001). Why has this theory been so controversial for so long? Ernst Mayr's decade-long wholehearted battle for the acceptance of allopatry as universal mechanism of speciation, and his influential opinion was instrumental in the rejection of sympatric speciation (Berlocher and Feder 2002). Ernst Mayr was convinced that gene flow is unavoidable between populations that are not spatially separated, and populations cannot genetically differentiate in the face of gene flow (Mayr 1947). Additionally, empiric evidence for speciation in sympatry was rare. One prominent example had already been discovered in the 1860s: Benjamin Walsh had observed that in the apple maggot fly (*Rhagoletis pomonella*) host-races had formed by shifting from native hawthorn (*Crataegus* spp.) to introduced domesticated apple (*Malus pumila*). Based on this observation, he proposed that phytophagous insect species may evolve in sympatry by shifting and adapting to new host plants (Walsh 1864, 1867). In the debate initiated by Mayr almost a century later, Mayr's former student Guy L. Bush picked up Walsh's studies from the 1860s and re-introduced *Rhagoletis pomonella* as potential case of sympatric speciation (Bush 1969). In 1988, Bush's concept of sympatric speciation in *Rhagoletis* was substantiated by the confirmation of genetic differentiation between the two host-races (Feder et al. 1988; McPheron et al. 1988). The host-shift of *Rhagoletis pomonella* has since become a role model for speciation in sympatry in phytophagous insects and subject to extensive research into the mechanisms involved in the shift (e.g. Feder et al. 1994; Nojima et al. 2003; Olsson et al. 2006). Today, many additional examples of diverging sympatric populations are known and investigated, for example the pea aphid (*Acyrthosiphon*

pisum) (Via 1999; Via et al. 2000), the Goldenrod ball-gall fly (*Eurosta solidaginis*) (Abrahamson and Weis 1997) and cichlid fishes (Cichlidae) (Schliewen et al. 1994; Danley et al. 2000; Wilson et al. 2000).

The extensive debate of the past decades forged a better picture of the circumstances favorable for sympatric speciation, especially when mediated by a host-shift. Among these circumstances are a broad sympatric overlap of host patches, limitation of gene flow by differing habitat choice and a genetic basis for habitat choice (Via 2001). Additionally, multiple selective forces rather than one single force seem to be necessary to drive speciation in sympatry (Rice and Hostert 1993; Via 2001).

Despite a better understanding of possible mechanisms that may cause sympatric speciation that was accumulated over the past decades (Smadja and Butlin 2011), quite a few questions still remain: Does sympatric speciation depend on specific starting criteria and can these criteria be generalized? Can the sequence in which different reproductive isolation barriers evolve in sympatry be predicted and is this comparable to the sequence of events in allopatric speciation? How rapidly must reproductive isolation evolve for speciation in sympatry to occur (Via 2001)? To answer these questions it is important to identify the causes and consequences of reduced gene flow in species where reproductive isolation has not yet been completed, i.e. in diverging races with different modes of reproductive isolation, which are partially isolated in the present rather than investigating fully isolated species (Berlocher and Feder 2002; Drés and Mallet 2002; Via 2002; Via and West 2008).

The fall armyworm *Spodoptera frugiperda* is one of the invaluable case studies with two diverging strains in sympatry. The two strains are hypothesized to currently be in an incipient stage of sympatric speciation and exhibit three major potential isolation barriers: differential host plant usage, differential sexual communication and differentiation in daily rhythms (Groot et al. 2010). Thus, these strains constitute an ideal model system to study a) incipient sympatric speciation and b) the contributions and interactions of different isolation barriers to speciation in sympatry.

THE MODEL SYSTEM AND AIM OF THIS THESIS

This thesis aims to investigate the relative importance of the different potential prezygotic isolation barriers for the divergence of the two strains of *S. frugiperda* in sympatry, by identifying the molecular differences underlying these isolation barriers, so that possible interactions between the isolation barriers can be determined. Identifying the mechanisms underlying prezygotic isolation barriers in two sympatrically occurring strains of a species will give important insight in the first steps of sympatric speciation.

Biology of Spodoptera frugiperda

Spodoptera frugiperda (Lepidoptera: Noctuidae) is a noctuid moth inhabiting in North- and South America (Sparks 1979). As a generalist, its larvae feed on a huge variety of plants. Exactly how many different plants are fed upon by *S. frugiperda* larvae is unknown, but plants of 80 different species in 23 plant families have been described so far (Luginbill 1928; Pashley 1988). Among the host plants are many important crop plants, such as rice (*Oryza sativa*), corn (*Zea mays*), sorghum (*Sorghum bicolor*) (all Poaceae) and cotton (*Gossypium hirsutum*, Malvaceae).

Life cycle

Dependent on the climate, *S. frugiperda* populations may occur the whole year in warm climate, overwinter as pupae in mild winters (Sparks 1979) or die in the cold season and repopulate an area in the next warm season through migration (Nagoshi et al. 2008b, 2012). One life cycle is completed in 4-13 weeks, depending on the temperature (Sparks 1979; Andrews 1988) and can be summarized as follows. Eggs are usually laid in clutches of up to hundreds of eggs which are multilayered and can be covered with scales (Meagher et al. 2011) (Figure 1A). In fertilized eggs, the head capsule of the developing larva becomes visible within 2-4 days after oviposition and the eggs appear black (Figure 1B). Neonate larvae hatch from the eggs approximately 1 day later (Figure 1C). Larvae develop on their host plants during six instars, growing continuously and shedding their cuticle between instars (Figure 1D, E). Sixth instars dig 1-3 inches deep into the soil, where they pupate (Figure 1F). The adults eclose underground after 1-5 weeks and then leave the soil to unfold their wings (Figure 1G). The adult moths are usually not reproductively active before the second night of their adulthood. Then, females sit as high as possible on a host plant, extrude their pheromone glands and emit a sex pheromone attractive to males (female calling, Figure 1H1). Males are attracted from a long range and in close range show a specific male calling behavior: They extrude hair pencils from their abdomen, perform wing fanning and attempt to mate with the female by bending the abdomen towards her (Figure 1H2). Several males can approach one female, and both females and males mate with one partner per night. With whom the female mates is probably mediated by close-range communication via a male pheromone (Birch et al. 1990). Once mated, the copulation can extend over several hours (Sparks 1979; Schöfl et al. 2009) (Figure 1I). Females can mate with different partners in consecutive nights, and eggs may be fertilized by sperm of several males (Meagher and Nagoshi 2010). The female oviposits for the first time in the night following the first successful copulation (thus at the earliest in the 3rd night of adulthood) and eggs are placed on host plants as well as on non-host plants and even man-made objects like car tires or window panes (R.L. Meagher, pers. comm.). This wide distribution of eggs may be important for larval dispersal, but could also be a strategy of predator and parasitoid avoidance (Meagher et al. 2011).

FIGURE 1. Life cycle of *Spodoptera frugiperda*. (A) Fresh egg clutch. (B) Mature egg clutch with head capsules of larvae visible. (C) Hatching neonate larvae. (D) Early instar larvae. (E) Late instar larva. (F) Pupa. (G) Adult moths, male left and female right. (H1) Female calling. (H2) Abdomen of calling male, arrow points to extruded hair pencils. (I) Mating couple. (J) Female oviposition.

Strain differentiation

Spodoptera frugiperda occurs as two morphologically indistinguishable, but genetically differentiated strains. These two strains were originally identified by allozyme analysis of *S. frugiperda* specimens sampled from corn plants in Louisiana and Puerto Rico, and rice plants in Puerto Rico and Bermuda grass plants in Louisiana (Pashley et al. 1985; Pashley 1986). Pashley found host-plant specific differentiation at five loci and proposed the existence of at least two sibling species that thus were reproductively isolated (Pashley 1986). Numerous studies followed

these findings and identified a variety of additional molecular markers that show strain-specific differentiation (summarized in Table 1).

TABLE 1. Summary of studies that identified strain-specific molecular markers or using these markers to further elucidate the strain-differentiation.

Genomic DNA: esterase allozymes	Pashley 1986
Mitochondrial DNA: cytochrome oxidase 1 (CO1) and NADH dehydrogenase 1 (ND1)	Pashley 1989, Pashley and Ke 1992, Lu and Adang 1996, Levy et al. 2002, Meagher and Gallo-Meagher 2003, Nagoshi et al. 2006, Lewter et al. 2006, Lewter et al. 2007, Machado et al. 2008, Juárez et al. 2012, Dumas et al. 2015a
Genomic DNA: FR tandem repeat sequence (present in rice-strain, absent in corn-strain)	Lu et al. 1994, Nagoshi and Meagher 2003, Nagoshi et al. 2008a
Genomic DNA: Amplified fragment length polymorphisms (AFLP)	McMichael and Prowell 1999, Busato et al. 2004, Prowell et al. 2004, Clark et al. 2007, Martinelli et al 2007, Juárez et al. 2014
Genomic DNA: Triose phosphate isomerase (*tpi*)	Nagoshi 2010, Juárez et al. 2014
Genomic DNA: Microsatellite markers	Dumas et al. 2015b

Currently, differences in the mitochondrial cytochrome oxidase 1 (CO1) gene are mainly used to discriminate the two strains, as this is a comparably fast and inexpensive method: a 600 bp amplicon of the gene is digested with *Sac*I and *Msp*I restriction enzymes. The corn-strain amplicon has only the *Msp*I restriction site, whereas *Sac*1 digests only the rice-strain amplicon. As mitochondria are maternally transmitted, hybrids can only be identified by additionally using a diagnostic nuclear marker, e.g. the triose phosphate isomerase gene (*tpi*) (Nagoshi 2010). With a combined analysis of mitochondrial and nuclear markers, up to 16% inter-strain hybrids have been detected in field populations (Nagoshi and Meagher 2003a; Prowell et al. 2004; Nagoshi et al. 2006b; Machado et al. 2008). While these high rates of hybridization suggest incipient rather than completed speciation, recent studies based on microsatellite markers and CO1 sequences indicate that the two strains are actually 'good' species (Dumas et al. 2015a,b). However, for the sake of consistency with the majority of fall armyworm publications, I will use strains throughout my thesis.

Besides molecular differences, the strains show differentiation in their host utilization (Pashley et al. 1985; Pashley 1986, 1988, 1989; Meagher and Gallo-Meagher 2003; Prowell et al. 2004; Nagoshi et al. 2006a; Machado et al. 2008), composition of female sex pheromone (Groot et al. 2008; Lima and McNeil 2009; Unbehend et al. 2013) and timing of reproductive activity (Pashley et al. 1992; Schöfl et al. 2009). These three prezygotic isolation differences will be introduced

in detail in part 3 of this introduction. In addition to the prezygotic isolation barriers, the strains show some postzygotic isolation. In laboratory experiments, RC hybrid (offspring of rice-strain ♀ and corn-strain ♂) females showed reduced fertility (Pashley and Martin 1987; Whitford et al. 1988; Groot et al. 2010). Interestingly, the majority of hybrids found in the field are RC hybrids (Nagoshi and Meagher 2003a; Prowell et al. 2004; Nagoshi et al. 2006b), which may thus partly explain the reduced gene flow between the strains. Recently, Kost et al. (2015) found that the reduced fertility of RC hybrid females is due to such females being sexually abstinent, i.e. they do not mate with any mating partner (R, C, RC or CR males). This unidirectional postzygotic isolation barrier, in combination with the prezygotic isolation barriers, probably make up the reproductive isolation syndrome in the *S. frugiperda* strains (Groot et al. 2010; Kost et al. 2015).

Prezygotic isolation between the two* S. frugiperda *strains

Spodoptera frugiperda larvae cause annual crop losses of up to millions of US dollars (Wiseman et al. 1983), but fall armyworm research extends beyond developing monitoring or pest management strategies (Mitchell et al. 1985; Sparks 1986; Andrews 1988; Hruska and Gladstone 1988; Pitre 1988; Wiseman and Isenhour 1988a,b; Andrade et al. 2000; Malo et al. 2001; Vergara and Pitre 2001; Molina-Ochoa et al. 2003a,b; Hoballah et al. 2004; Bueno et al. 2008). The fact that the species occurs as two genetically differentiated strains in sympatry has initiated a broad array of studies from different perspectives, which address the central question: 'What keeps the strains apart?'

Three main prezygotic isolation barriers have been identified in *S. frugiperda*: Habitat isolation, behavioral isolation through strain-specific sexual pheromone communication and behavioral isolation through strain-specific timing of reproduction.

Habitat isolation through strain-specific host utilization

The best investigated isolation barrier that drives divergence between sympatric herbivorous insects is habitat isolation. When two insect populations mainly utilize different host plant species, this reduces the probability of these populations of mating with each other, resulting in reduced gene flow. Genetic differences between the populations can thus accumulate in the populations, differentiation into host race formation, eventually enabling reproductive isolation and the appearance of new species (Schluter 2001; Drés and Mallet 2002; Funk et al. 2002, 2006; Coyne and Orr 2004; Rundle and Nosil 2005; Feder et al. 2012). Host races constitute an intermediate step in ecological speciation in sympatry, which is a continuous process from polymorphisms between populations of the same species to fully distinguished species (Berlocher and Feder 2002; Drés and Mallet 2002).

Research addressing ecological speciation in sympatry is mainly focused on specialist herbivorous insects, like the apple maggot fly *Rhagoletis pomonella*

(Bush 1969; Feder et al. 1994), the Goldenrod ball-gall fly *Eurosta solidaginis* (Craig et al. 1993; Craig and Itami 2011) and the treehopper *Enchenopa binotata* (Wood 1980; Guttman et al. 1981). However, there are also examples of generalist herbivorous insects that show host use differences between populations: in the European corn borer, *Ostrinia nubilalis*, the pheromone Z strain feeds on maize, whereas the E strain primarily feeds on mugwort, at least in France (Thomas et al. 2003; Bethenod et al. 2005); in the larch budmoth *Zeiraphera diniana* one biotype prefers larch (*Larix* spec.), whereas another biotype prefers pine (*Pinus* spec.) (Emelianov et al. 1995, 2003, 2004), and in the tobacco budworm *Heliothis virescens* two populations have been recently recognized that perform differently on chickpea (*Cicer arietinum*) and cotton (*Gossypium hirsutum*) (Blanco et al. 2008; Karpinski et al. 2014). If these differences have a genetic basis that can be selected for (Emelianov et al. 2003; Thomas et al. 2003; Karpinski et al. 2014), differential host plant choice could start the process of ecological speciation in sympatry.

Spodoptera frugiperda may be one more example of a generalist herbivorous insect undergoing sympatric speciation through habitat isolation. After Pashley's first identification (Pashley 1986), larvae collected from tall grasses like corn and sorghum (*Sorghum bicolor*) and from cotton were up to 80% corn-strain individuals, whereas larvae collected from smaller grasses, like rice or pasture (e.g. bermudagrass) were up to 95% rice-strain larvae (Pashley 1986, 1988, 1989; Meagher and Gallo-Meagher 2003; Prowell et al. 2004; Nagoshi et al. 2006a; Machado et al. 2008). In this thesis I further explore the level and extent of host plant differentiation of the two strains. An overview of the physiological and behavioural studies and their potential to explain the differential distribution in the field is given in **Chapter 2** (Hänniger et al. 2015a). In **Chapter 3** (Juárez et al. 2014), we determined the strains of specimens collected from different host plants in three South American countries, and found only a weak host-association for this large data set. In **Chapter 8** (Groot et al. 2015) we give an overview of the strain identities of various field collections reported in literature as well as our own collections. Main findings are that field observations, oviposition studies and larval performance studies overall show inconsistent results in terms of host preference and performance of the two strains, which leads to the question of how strong the host association of the two strains actually is and to what extent this contributes to reproductive isolation between the strains (discussed in **Chapter 9)**.

Behavioral isolation through strain-specific sexual communication

Besides host plant differentiation, the two strains of *S. frugiperda* exhibit differences in their sexual communication (Groot et al. 2008; Lima and McNeil 2009; Unbehend et al. 2013). In moths, the female uses species-specific pheromone signals to attract males over long distances (Tamaki 1985; Löfstedt and Kozlov 1997). When the female extrudes the pheromone gland from her abdomen,

pheromone is emitted (Tamaki 1985; Percy-Cunningham and MacDonald 1987). Males detect the female sex pheromone with their very sensitive antennae and in response start flying towards the female, following the pheromone signal (Baker et al. 1985; Mafraneto and Cardé 1994). At close range, males exhibit a typical male courtship behavior by extruding hairpencils from their abdomen and wing fanning (Tamaki 1985; Birch et al. 1990; Lassance and Löfstedt 2009). If both partners are attracted to each other, copulation will ensue.

The female sex pheromone is species-specific and thus usually only attracts conspecific mating partners (Tamaki 1985; Löfstedt and Kozlov 1997). This specificity is realized by the combination of specific pheromone components as well as a species-specific ratio of these components (Tamaki 1985; Jurenka 2004). A component of a sex pheromone in one blend of one species can be used in a lower concentration by a different species, e.g. the major compound in *S. frugiperda* pheromone, (Z)-9-tetradecenyl acetate (Tumlinson et al. 1986) is present in lower amounts in the pheromone of *S. litura* (Sun et al. 2002) without causing cross attraction. Some components may also repel closely related species to avoid attraction of co-occurring heterospecific males (Vickers and Baker 1997; Groot et al. 2006; Eizaguirre et al. 2007). As moths usually have a very short reproductive phase (generally < 2 weeks), it is essential to have a reliable sexual communication system that ensures mating success. If changes occur in either the female sender of a pheromone signal or the male receiver, this may initiate reproductive isolation (Löfstedt 1993; Cardé and Haynes 2004).

The most prominent example of behavioral isolation through differences in sexual communication in Lepidoptera is the European corn borer, *O. nubilalis*, with two pheromone strains, E and Z (Smadja and Butlin 2009; Wicker-Thomas 2011; Lassance et al. 2013). In the Z-strain, females produce a 3:97 ratio of (E)-11-tetradecenyl acetate to (Z)-11-tetradecenyl acetate, whereas E-strain females produce a pheromone ratio of 99/1 (E):(Z)-11-tetradecenyl acetate (Klun et al. 1973; Kochansky et al. 1975). The opposite pheromone ratio found in E- and Z-strain females is caused by the fatty acyl reductase *pg*FAR gene product (Lassance et al. 2010). As for male response, the Z-strain males have a narrow response-range, flying only towards a blend with a 3:97 ratio, whereas some E-strain males also respond to intermediate E/Z ratios and can even be attracted to a Z-strain female (Roelofs et al. 1987; Glover et al. 1990). Consequently, mainly hybrids between E-males and Z-females are found in the field (Liebherr and Roelofs 1975). Hybrid males respond to a broad range of E/Z-ratios, and rarely to the E-strain females (Roelofs et al. 1987; Glover et al. 1990). For the male response, a *resp* locus on the sex chromosome has been identified (Roelofs et al. 1987; Dopman et al. 2004), as well autosomal and sex-linked loci affecting the antennal response (Roelofs et al. 1987; Olsson et al. 2010). Thus, sexual communication constitutes a strong isolation barrier in *O. nubilalis*, which appears to undergo sympatric speciation through

sexual communication differentiation, and may already be sibling species (Cardé et al. 1978; Malausa et al. 2007; Lassance et al. 2010).

In *S. frugiperda*, at least two behaviorally active components constitute the female sex pheromone: the major sex pheromone component (Z)-9-tetradecenyl acetate (Z9-14:OAc) and the critical secondary sex pheromone component (Z)-7-dodecenyl acetate (Z7-12:OAc) that makes up only a few percent of the pheromone (Tumlinson et al. 1986). Interestingly, corn-strain females consistently exhibited lower relative amounts of Z7-12:OAc than rice-strain females in laboratory as well as field populations (Groot et al. 2008; Lima and McNeil 2009). We determined whether the different pheromone blends of the corn-strain and rice-strain females are differentially attractive to males from the same strain, in wind tunnel assays as well as in male trapping experiments (**Chapter 4,** Unbehend et al. 2013). We also determined whether sex pheromone differences as well as differences in the male response differ between geographic regions (**Chapter 5**, Unbehend et al. 2014). In addition, we conducted quantitative trait locus (QTL) analysis to determine the genetic basis of the pheromonal differences between the two strain (**Chapter 6**, Hänniger et al. 2015b).

Behavioral isolation through strain-specific timing of reproduction

The most pronounced difference between the two strains of *S. frugiperda* is their timing of reproductive activity at night (Pashley et al. 1992; Schöfl et al. 2009). Pashley et al. (1992) observed 16 pure strain matings and found the corn-strain to mate in the first six hours of the scotophase, whereas the rice-strain started to mate after the sixth hour into the scotophase. Repeating the experiment with a much larger sample size (320-400 matings), Schöfl et al. (2009) confirmed that the corn-strain mates significantly earlier than the rice-strain. If populations of the same species are (reproductively) active in different time-windows at night, this could constitute reproductive isolation and thus may drive speciation in sympatry. Allochronic speciation in insects has been suggested for crickets (Alexander and Bigelow 1960; Danley et al. 2007; Fergus et al. 2011; Fergus and Shaw 2013) as well as fruit flies (Tauber et al. 2003; Prabhakaran and Sheeba 2012) and mosquitoes (Rund et al. 2012). Such temporal differences may be seasonal (e.g. *Laupala*) or within a day (e.g. *Anopheles gambiae*), both narrow the possible time windows for mating between individuals with different time windows. Surprisingly little is known about the genetic changes underlying these timing differences (reviewed in Groot 2014). Candidate genes that could underlie changes in daily rhythms are genes involved in the circadian clock. The circadian clock is a complex network of genes and their products, which enhance and suppress each other in a rhythmic manner, and which are entrained by environmental cues, such as light, temperature and/or tides. These molecular networks and their evolution have been subject of extensive research since the 1960s (Aschoff 1960; Pittendrigh 1960,

1961, 1993) and we are now beginning to understand how these molecules interact and have evolved to form biological clocks in the different kingdoms of life (e.g. Hardin 2005, 2011; Zhan et al. 2011; Hermann et al. 2013).

Insect circadian clocks have been extensively studied in *Drosophila melanogaster* and also in the Monarch butterfly, *Danaus plexippus* (Zhan et al. 2011). In *D. plexippus*, as in *Drosophila* (Hardin 2005), proteins (written in upper case letters, e.g. CLOCK) and genes (in italicized lower case letters, e.g. *cycle*) form two interlocked feedback loops, connected by their mutual usage of CLOCK (CLK) and CYCLE (CYC). In the main feedback loop, the transcription of *period* (*per*), *timeless* (*tim*) and *cryptochrome 2* (*cry2*) is promoted by a heterodimer of CLK and CYC binding to E-box elements in the promoter of these genes. PER, TIM and CRY2 proteins co-locate and enter the nucleus together, where CRY2 inhibits the CLK:CYC mediated transcription, including its own transcription. Light-entrainment of this feedback loop is facilitated by the blue-light receptor CRYPTOCHROME 1 (CRY1) promoting TIM degradation. SUPERNUMERARY LIMBS (SLIMB) and JETLAG (JET) signal the degradation of PER and TIM, proteins involved in CRY2 degradation have not yet been identified. Kinases (e.g. CASEIN KINASE II (CKII) and DOUBLE-TIME (DBT)) and phosphatases (e.g. PROTEIN PHOSPHATASE 2A (PP2A)) are involved in the post-translational modification of PER and TIM (Zhan et al. 2011). In *Drosophila*, PER instead of CRY2 inhibits the CLK:CYC promoted transcription and only one cryptochrome, homologous to CRY1, is present (Hardin 2005). When in *Drosophila* PER is degraded with the onset of the photophase, CLK:CYC dimers are freed to promote the transcription of *per*, *tim* and *cry2* and restart the main feedback loop. Additionally, the CLK:CYC dimers promote the transcription of *vrille* (*vri*) and *PAR-domain protein 1* (*pdp1*), starting the second feedback loop (Hardin 2005). In *D. plexippus*, like in *Drosophila*, *vri* inhibits *clk* transcription by binding to a V/P-Box in the clock promoter. *Pdp1* counteracts *vri* by promoting *clk* transcription (Cyran et al. 2003; Hardin 2005; Zhan et al. 2011).

Spodoptera frugiperda is an ideal model organism to investigate a) the genetic basis of allochronic differentiation and b) the influence of allochronic differentiation as isolation barrier between the two strains. In **Chapter 6** we present the results of a QTL analysis addressing the strain-specific timing of reproductive activity. We also show strain-specific expression differences and sequence polymorphisms of the major candidate gene, *vrille*. In **Chapter 7** we summarize the results of the annotation of clock genes in the genome of the corn-strain variant of *S. frugiperda*.

In **Chapter 9**, I discuss the main findings of this thesis. First, I discuss the potential of the different isolation barriers to facilitate reproductive isolation between the two strains of *S. frugiperda*. Secondly, I discuss possible interactions of the isolation barriers and their potential to drive the reproductive isolation between

the strains. I propose an interaction of all prezygotic mating barriers to facilitate the divergence of the two strains, with allochronic differentiation being the strongest force in these interactions.

In conclusion, by determining the level and extent of the different prezygotic isolation barriers that exist between the two strains of *Spodoptera frugiperda*, I have furthered our understanding of the mechanisms underlying the divergence between the strains. This example of incipient sympatric speciation can contribute to a better understanding of speciation in the face of gene flow.

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SECTION 1

STRAIN-SPECIFIC HOST PLANT DIFFERENTIATION (OR NOT?)

'Negative results are just what I want.
They're just as valuable to me as positive results.
I can never find the thing that does the job best
until I find the ones that don't.'
Thomas A. Edison

2

NO STRAIN-SPECIFIC DIFFERENCES IN PREFERENCE AND PERFORMANCE OF *SPODOPTERA FRUGIPERDA* ON TYPICAL HOST PLANTS

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Abstract

Spodoptera frugiperda is a generalist moth species occurring as two separate strains. The strains are generally referred to as host strains, as they were originally identified on different host plants, i.e. the corn strain on tall grasses and the rice-strain on small grasses. Yet field observations, oviposition studies and larval performance studies overall show inconsistent results, which induces the question of how strong the host association of the two strains is. This study investigated oviposition preference and larval preference and performance in various bioassays and aims to determine within-strain host differentiation and between-strain host differentiation in our results and previous studies. The results are very variable between different published studies and show no consistent pattern. We conclude that the currently available data suggests only a weak involvement of host-differentiation in the divergence of the two *S. frugiperda* strains.

INTRODUCTION

For herbivorous insects, their host plant is not only a food source, but also provides protection from predators and pathogens, as well as mating sites. Therefore, a differentiation in host plant usage between populations of one species does not only affect nutrition and traits connected to it. Habitat isolation can reduce gene flow between populations when it reduces the probability to mate between populations. With reduced gene flow, genetic differences can accumulate, which can facilitate the formation of host races and reproductive isolation, and can thus lead to the differentiation into new species (Schluter 2001; Drés and Mallet 2002; Funk et al. 2002, 2006; Coyne and Orr 2004; Rundle and Nosil 2005; Feder et al. 2012). This ecological speciation in sympatry is hypothesized as a continuous process from polymorphisms between populations to species, and the existence of host races as intermediate state supports this hypothesis (Berlocher and Feder 2002; Drés and Mallet 2002).

Specialist herbivorous insects with a narrow range of host plants, like e.g. the apple maggot fly *Rhagoletis pomonella* (Bush 1969; Feder et al. 1994), the Goldenrod ball-gall fly *Eurosta solidaginis* (Craig et al. 1993; Craig and Itami 2011) and the treehopper *Enchenopa binotata* (Wood 1980; Guttman et al. 1981) are the main focus of research addressing ecological speciation in sympatry. However, many examples also show host usage differences between populations of generalists. For example, the European corn borer *Ostrinia nubilalis* is a generalist that consists of two pheromone strains, the E-strain and the Z-strain, but in Northern France the E-strain is feeding primarily on mugwort whereas the Z-strain is feeding on maize (Thomas et al. 2003; Bethenod et al. 2005). Also the two biotypes of the larch budmoth *Zeiraphera diniana* show differences in pheromones as well as host preference, with one biotype preferring larch (*Larix* spp.) and the other preferring pine (*Pinus* spp.) (Emelianov et al. 1995, 2003, 2004). Recently, host usage differences have also been shown for two laboratory strains of the generalist tobacco

budworm *Heliothis virescens* (Blanco et al. 2008; Karpinski et al. 2014), and a QTL analysis revealed a genetic basis of the difference in larval performance on different host plants. Such differences could be the start of host specialization.

The noctuid moth *S. frugiperda* occurs as two different host strains and is potentially an ideal model organism to study the mechanisms underlying habitat isolation and ecological speciation in sympatry. The two morphologically indistinguishable strains, the so-called corn-strain and the so-called rice-strain, are found in sympatry in the Americas. The strains can be discriminated by a number of molecular markers in the nuclear and mitochondrial DNA (Pashley 1989; Lu et al. 1992, 1994; Lu and Adang 1996; McMichael and Prowell 1999; Levy et al. 2002; Nagoshi and Meagher 2003a,b; Busato et al. 2004; Nagoshi et al. 2006; Clark et al. 2007; Martinelli et al. 2007; Belay et al. 2012). Besides genetic differences, the two strains show phenotypic differentiation, and the focus of this study is the host plant associated variation between the two strains (Pashley et al. 1985; Pashley 1986). Typically, larval collections from tall grasses like corn (*Zea mays*) and sorghum (*Sorghum bicolor*) and from cotton (*Gossypium hirsutum*) consist of up to 80% corn-strain individuals and only 20% rice-strain individuals, whereas up to 95% of larvae collected from smaller grasses, like rice (*Oryza sativa*) or pasture (e.g. bermudagrass, *Cynodon dactylon*) are rice-strain larvae (Pashley 1986, 1988a, 1989; Meagher and Gallo-Meagher 2003; Prowell et al. 2004; Nagoshi et al. 2006; Machado et al. 2008; Juárez et al. 2014). However, some field collections show different patterns. For example, males caught in a cotton field in Mississippi were mostly identified as rice-strain, whereas males and larvae collected from sorghum fields in Texas and Florida revealed more rice-strain individuals than corn-strain individuals (Nagoshi et al. 2006). Also in Argentina, Paraguay, and Brazil, larvae collected from rice plants mostly consisted of corn-strain individuals, whereas larvae collected from sorghum plants consisted exclusively of rice-strain individuals (Juárez et al. 2014). Thus, host plant adaptation of the two strains may not be as strict as previously thought.

Different mechanisms could underlie the distributional differences of the two strains observed in the field. Generally, host use biases may be due to different time points in insect/plant-interaction, i.e. by a) preference for oviposition sites in adult females, b) larvae that accept or do not accept the host they emerge on, c) differences in larval development and viability on different hosts, d) larval preference for different hosts, or through a combination of these preferences and performances. For the two *S. frugiperda* strains, behavioral differences in host use remains unclear, even though many assays have been conducted to investigate oviposition preferences of *S. frugiperda*, as well as the influence of host plants on development and viability of larvae (see Table 1). For example, Whitford et al. (1988) found that the corn-strain preferred to oviposit on corn and sorghum compared to bermudagrass, whereas the rice-strain preferred bermudagrass over

corn or sorghum in one of two conducted assays. Similarly, Meagher et al. (2011) found a preference of the rice-strain for pasture grass (*Cynodon nlemfuensis*) over corn in two oviposition assay, but the corn-strain did not show a preference for either plant. Many more studies have addressed larval performance differences on different host plants, but have found inconsistent results (e.g. Pashley 1988b; Meagher et al. 2004; Groot et al. 2010). Together, the inconsistent findings in field observations, oviposition studies and larval performance studies induce the question of how strong the host association of the two strains is.

In this study, we aimed to assess oviposition preference and larval preference and performance of the two strains of *S. frugiperda*. We conducted a number of bioassays, following the sequence of insect/plant-interaction events in nature: after females choose a plant as oviposition site (i. oviposition preference), larvae hatch and accept or do not accept the host plant (ii. larval host acceptance). When accepting and feeding on the host, larval development and viability may be influenced by the host plant (iii. larval performance). At different life stages, the larva can choose to move to a different plant (iv. larval preference). The ultimate aim of these assays was to verify or falsify the following two hypotheses: I. There is host-plant differentiation *within* the two strains, and II. There is a difference in performance and/or preference *between* the two strains. If both hypotheses are correct, host plant differentiation may underlie the strain differentiation in *S. frugiperda*.

MATERIALS AND METHODS

Insects and rearing

The bioassays in this study were conducted using three laboratory corn- and rice-strain populations. The oldest population originated from larval collections in Florida in 2003 and 2004. For the corn-strain, >100 corn-strain larvae were collected from sweet corn fields in Miami-Dade County (25°38’42”, 80°27’18”) in 2004. This population is referred to as JS3C. More than 200 rice-strain larvae were collected from different *Cynodon* pasture grasses at the Range Cattle REC near Ona (27°23’50”, 81°56’40”) in 2003 to establish the rice-strain population, referred to as OnaR. These populations were reared for 10 (corn-strain) and 21 (rice-strain) generations in mass culture at the USDA-ARS in Gainesville before shipment to the Max Planck Institute for Chemical Ecology in Jena, Germany (MPICE) in 2007. Larvae of these populations were used after 37 and 48 generations at MPICE, respectively.

The second population originated from larval collections of >120 individuals by Carlos A. Blanco in Mississippi in August 2008. Corn-strain larvae were collected from a corn field in Stoneville (+33°15’8.59”, -90°31’59.765”) and will be referred to as MSC. Rice-strain larvae were collected from a grass field in Raymond (+32°9’51.883”, -90°13’29.406”) and are referred to as MSR. After

collection, larvae were shipped to MPICE and reared on artificial diet. Larvae of these populations were used after 8 and 7 generations at MPICE, respectively.

The youngest population originated from field collections of 300 specimens in April 2010. Corn-strain larvae were collected in a corn field in the Everglades Research and Education Centre in Belle Glade, Florida (+26°40'7.20", −80°37'57.63") and are referred to as FLC. Rice-strain larvae were collected in a pasture field at the Graham Dairy Farm in Moore Haven, Florida (+26°53'3.04", −81°7'21.17") and are referred to as FLR. All larvae were shipped to MPICE and reared on artificial diet in the laboratory since then. Larvae of these populations were used after two generations at MPICE. Upon arrival at the MPICE, all individuals were screened for strain-specific COI markers (Nagoshi et al. 2006), and separated accordingly into strain-specific colonies. All populations were reared in incubators 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. Larvae were fed on artificial diet based on pinto beans (PBD).

Plants

Seeds of sweetcorn hybrid SWEET G 90 (*Zea mays*) were obtained from Syngenta Seeds, Inc. (Boise, Idaho) and seeds of bermudagrass (*Cynodon dactylon*) were obtained from B&T World Seeds (Paguignan, France). Both plant species were cultivated in the greenhouse under L:D 14:10, 19 °C (night) – 24 °C (day) and 50-60% RH in 1-l pots. One corn plant was planted per pot, whereas for the bermudagrass enough grass plants were planted to gain a dense coverage of grass foliage in the pot (~50 plants per pot).

Bioassays

i) Oviposition preference

Female preference for oviposition sites was investigated in three different bioassays, presenting 1) plant leaf parts, 2) whole plants and 3) whole plants with and without gauze to mated females as potential oviposition sites.

Experiment 1: To determine oviposition preference in many females simultaneously, we first used leaf parts of corn and grass plants. 40 Single pair matings per strain were set up in plastic boxes (28 × 20 cm, Savelock). For the corn-strain matings, individuals from the JS3C population were used, whereas for the rice-strain matings the OnaR population was used. All matings were set up simultaneously in a walk-in climate chamber (L:D 14:10, 26 °C, 65% RH). Insects were provided with a 10% honey solution on a cotton ball. Boxes were covered with gauze and leaf parts of freshly cut corn and grass were placed in randomly chosen opposite corners on the gauze and covered with a moist paper towel. Leaf parts were renewed daily. Males and females were kept in the boxes and egg masses were counted daily for 4 days.

Experiment 2: To test oviposition preference using whole plants, 8 single pair matings per strain (FLC and FLR) were set up in mesh wire cages (60 × 60 × 60 cm) and provided with 10% honey solution. Additionally, one corn plant in a one liter pot and a one liter pot with grass plants were placed randomly in opposite corners of the cage. The set-ups were placed in a walk-in climate chamber (L:D 14:10, 24 °C, 55% RH). Egg masses were counted every day for 4 days in total.

Experiments 3: To investigate whether volatile cues of the plant are more important than tactile cues for stimulating oviposition, we repeated the above experiment, but covered the plants with gauze in 6 cages per strain and left the plants uncovered in additional 6 cages per strain. The gauze was sturdy, so it did not bend the plants, and the holes in the mesh were 1 mm^2 to allow insects to perceive plant volatiles. The experiment was performed in the same way as above, however this time with individuals from the FLC and FLR populations in their 4^{th} laboratory generation.

All statistical analyses were conducted using R Studio (RStudio 2012). The oviposition assays were analyzed individually and as a combination of experiment 1-3, using GLM with quasibinominal error structure.

ii) Larval host acceptance

As in nature, larvae are rarely in choice situations between plants, but can choose to start or not start feeding on the plant that they emerged on, we conducted two types of host acceptance assays: 4) larvae could move to and feed on leaf parts in a no-choice situation and 5) larvae were placed directly on the leaf part and feeding commencement was observed (Figure 1b.).

Experiment 4, where larvae needed to move to a leaf part, was conducted in small arenas (Petri dishes (Ø 9 cm, GBO, Frickenhausen, Germany)) or large arenas (plastic boxes (28 × 20 cm, Savelock)). Moist filter paper or paper towel were placed in the arenas and renewed prior to every assay for every new larva. Plants were always cut fresh for every assay and every new larva, and immediately placed in the arena. The tested corn-strain larvae originated from the MSC population and the rice-strain larvae from the MSR population and were of comparable size, 2^{nd} to early 3^{rd} instar. Larvae that were about to molt were excluded from the experiment. Such larvae can be recognized by typically having a darker skin and showing less or no movement until molting. A corn leaf part or some grass leaf parts, representing the approximate biomass of the corn leaf, on one side of each arena. The larva was placed in the center of the arena, i.e. 3.5 cm away from the plant in small arenas or 13 cm from the plant in large arenas. In the larger arenas, a directed movement towards the plant was more easily observable. Larvae were observed continuously for 30 minutes. In small arenas, four larvae were observed simultaneously by one observer. In the large arenas, two observers continuously observed 10 larvae

simultaneously. Since early instar larvae are rather small and several insects were observed at a time, a green gut content that was visible in the translucent larvae was also used as an indication for larval feeding on a plant in the larger arenas.

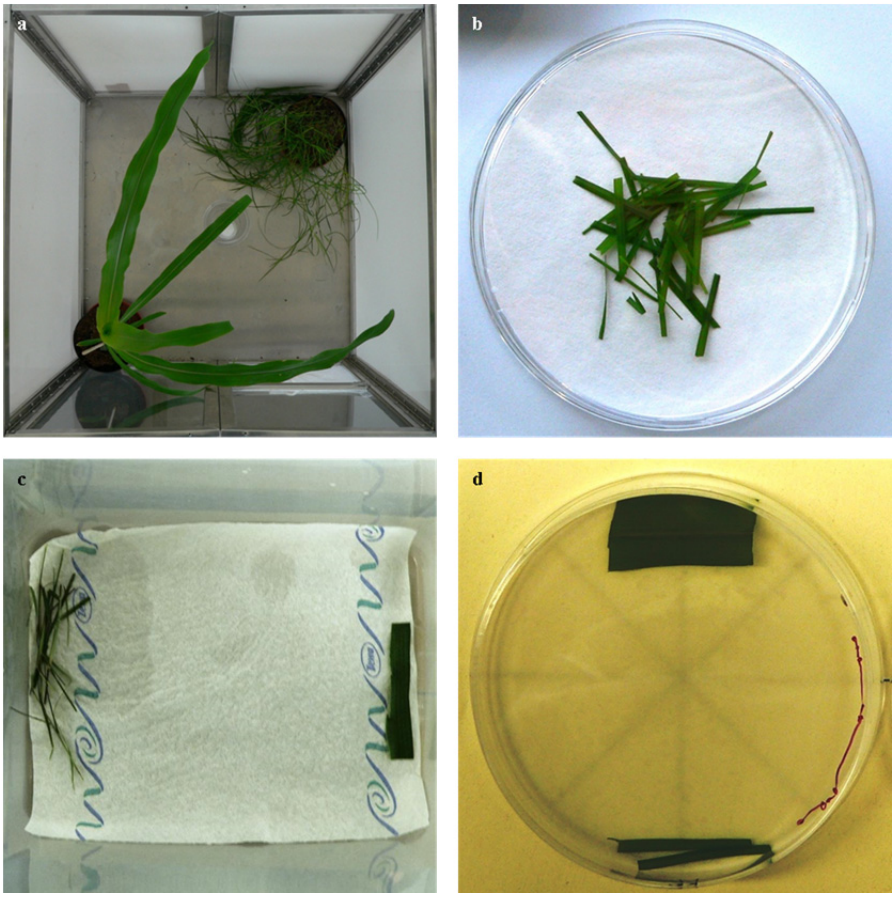

FIGURE 1. Setup of different bioassays. **a**. Oviposition preference with whole potted plants without gauze. **b**. Larval host acceptance. **c**. Larval host preference in large arena. **d**. Larval host preference in small arena.

Experiment 5, where larvae were placed directly on the plant, was carried out in small arenas. Fresh cut leaf material of corn or grass plants was placed in the middle of the arena on moist filter paper, 2nd to early 3rd instar were placed on the leaf parts and the time taken until feeding commencement was noted. Four larvae in individual arenas were observed simultaneously.

The variation within the strains was analyzed with Chi-squared tests. The between-strain variation was analyzed using a binominal comparison of proportions. The variation of the time taken to start feeding was analyzed using ANOVA on log-transformed data.

iii) Larval performance

Experiment 6: To determine whether different host plants have a different effect on the viability and the development of *S. frugiperda* larvae, we investigated larval performance of both strains on different plant-based diets. These diets were used, because plant parts dry out very quickly and need to be renewed every day, which causes a high larval death rate, and whole plants need a large amount of space, especially when testing >100 larvae per strain on each plant species.

The plant-based diets were based on lyophilized plant material. For these diets, corn plants and bermudagrass plants were grown in the greenhouse (L:D 14:10, 19 °C (night) – 24 °C (day), 50-60% RH), freshly harvested without roots and directly cut into pieces of ~10 cm length, immediately frozen at -80 °C in a chest freezer and lyophilized (Gefriertrocknungsanlage ALPHA 2-4 LD, CHRIST®, Germany). The lyophilized and dry plant material was powdered, after which 168 g plant powder, 2,200 ml water, 35 g agar, vitamins, tetracycline, sorbic acid and methyl paraben were mixed to produce the plant-tissue based diets (Blanco et al. 2008). The Pinto bean diet (PBD) that we generally use for our rearing was used as the control. All diets were irradiated for 1 h with UV light to kill microorganisms. Cubes of each diet, measuring ~2 cm^3, were placed in small (5 oz.) plastic cups. Eggs from both the FLC and FLR populations were collected and larvae were reared on Pinto bean diet until 2nd instar. 315 larvae of each strain were weighed and evenly distributed among the different diets, one larva per cup. Larvae were weighed every 3rd day until pupation, and pupae were weighed within a day after pupation. The date of eclosion was recorded, and adults were weighed within a day after eclosion. Also, larval and pupal death was documented. The growth rate between day 1 and day 4 of the experiment, i.e. after the larvae were placed on the plant-based diet, was calculated:

$$GR = (log10(weight\ day4) - log10(weight\ day1))/(3\ (days)\ .$$

This period was chosen, as some individuals had already pupated when the weight was measured at day 7. The differences between growth rates were analyzed using ANOVA. The survival rate was analyzed using Cox's proportional hazard and a parametric model.

iv) Larval host preference

Experiment 7: To investigate whether larvae prefer the host plant that they perform on best, we conducted choice assays in small or large arenas, described above for

the acceptance assays with movement (experiment 4). To examine a host preference, the larvae were presented with a choice between a corn leaf part and grass leaf parts, resembling the biomass of the corn leaf part. The different leaf parts were placed on opposite sides of each arena, i.e. ~7 cm apart, 3.5 cm away from the larva in small arenas or ~26 cm apart, 13 cm from the larva in large arenas. The tested larvae originated from the MSC and MSR population and were of comparable size, 2nd to early 3rd instar. As in the acceptance assays, larvae that were about to molt were excluded. Larvae were observed continuously for 30 minutes, as described in the acceptance assays above.

RESULTS

i) Oviposition preference

When females were offered leaf parts on gauze for oviposition (experiment 1, Figure 2a), only 9 of 40 (22.5%) of the tested corn-strain females laid eggs, and only 15 of 40 (37.5%) of the rice-strain females laid eggs. The corn-strain females oviposited on average 1.11 (± 0.48 SEM) egg clutches per female under the gauze under the corn leaf parts, compared to only 0.11 (± 0.11 SEM) egg clutches under the gauze under the grass leaf parts, whereas 2.56 (± 0.77 SEM) eggs were laid on the cage surfaces. Comparably, the rice-strain females only laid 0.53 (± 0.21 SEM) egg clutches under corn leaf parts, 0.67 (± 0.29 SEM) under grass leaf parts and 5.8 (± 1.04 SEM) on other surfaces of the cage. Thus, the majority of females of both strains did not lay eggs on gauze under the leaves. There was no significant difference between the corn- and rice-strain females in their oviposition behaviour in this first experiment.

When females were offered whole plants in planting pots for oviposition (experiment 2, Figure 2b), all females of both strains laid eggs, and all but one corn-strain female laid at least part of her egg masses on plants. On average, corn-strain females laid 1.5 (± 0.65 SEM) egg masses on corn plants, compared to 5 (± 1.78 SEM) egg masses on grass, and 1.75 (±0.49 SEM) egg clutches on the cage surfaces. Similarly, rice-strain females laid an average of 1.25 (± 0.56 SEM) egg masses on corn plants, 3.38 (± 0.71 SEM) egg clutches on grass plants, and 0.5 (± 0.38 SEM) egg clutches on the cage surfaces (Figure 2b). Thus, both strains laid most eggs on the grass plants. However, there were no significant differences between the strains or between plants.

When the same populations were tested one generation later in the same setup (experiment 3, Figure 2c), only 50% of the corn-strain females laid eggs, whereas 83% of the rice-strain females laid eggs. The corn-strain females laid on average 3.67 (± 3.67 SEM) egg clutches on corn plants, 1.33 (± 0.88 SEM) egg clutches on grass plants and none on the cage. In contrast, the rice-strain oviposited only 0.40 (± 0.40 SEM) egg clutches on corn, but 6.40 (± 2.50 SEM) egg clutches on grass and 1.6 (± 1.17 SEM) egg clutches on the cage surfaces. Thus, the corn-strain laid most

of the eggs on corn, whereas the rice-strain laid most eggs on grass. However, none of the differences were significant, probably due to low sample size.

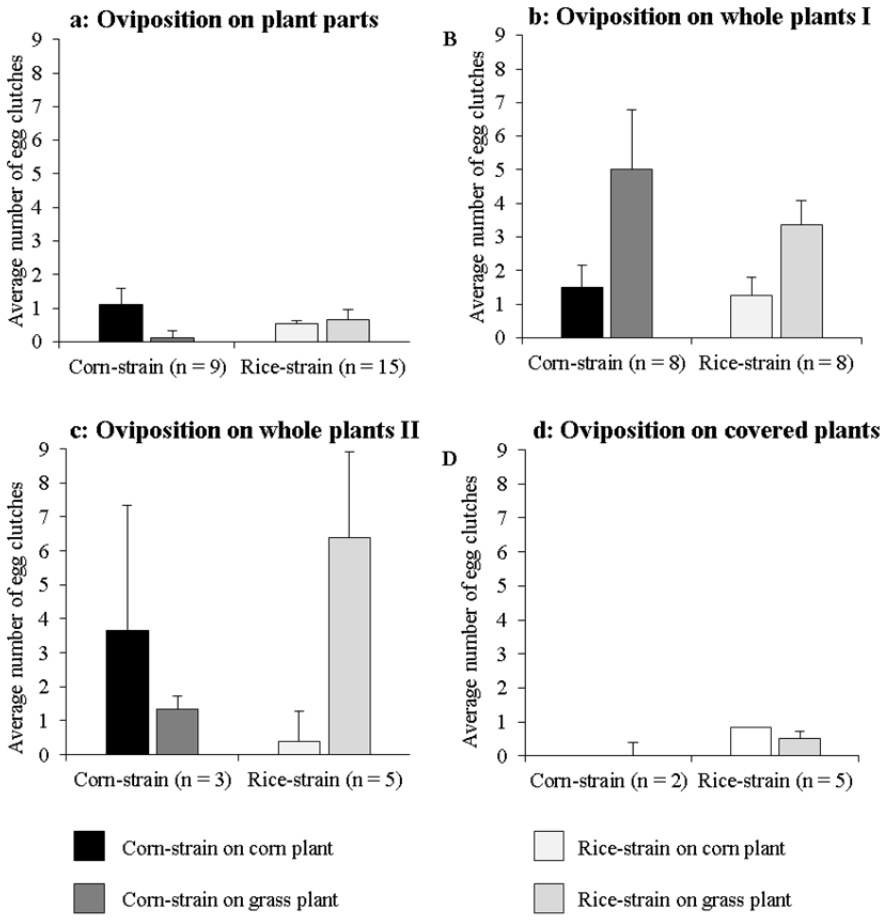

FIGURE 2. Oviposition choice (mean + SEM) of corn-strain and rice-strain female adults on **a** corn and grass plant parts, **b+c** whole corn plants and grass plants and **d** corn and grass plants covered in gauze. Figures show average number of egg masses laid per female on different plants per strain. n = number of females laying eggs. GLM with binomial error structure.

When plants were covered with gauze to determine the importance of tactile cues (experiment 3, Figure 2d), only 30% percent of the corn-strain females oviposited, whereas 100% of the rice-strain females laid eggs. The corn-strain females that did oviposit, laid an average of 1.50 (± 0.5 SEM) egg clutches on the cage walls and no eggs on either plant. In contrast, the rice-strain females laid on

average 0.83 (± 0.40 SEM) egg clutches on the corn plant, 0.5 (± 0.22 SEM) egg clutches on the grass plant and 1.17 (± 0.60 SEM) on the cage surfaces. Thus, oviposition was generally lower in cages with covered plants compared to the cages with uncovered plants (Figure 2d). Also, whereas both strains laid more eggs on their typical host plant in cages without gauze, both strains laid few egg masses, preferably on the cage, when the plants were covered with gauze.

A combined GLM analysis of oviposition experiments 1-3 (with leaf parts and with whole plants without gauze) shows that the strains chose different oviposition sites in each experiment and that there was no clear strain-specific choice for either plant.

ii) Larval host acceptance

In the acceptance assays where larvae needed to move towards a leaf part in small arenas, no significant differences were observed between the two strains and between the plant parts tested (Figure 3a, b): 50% of the corn-strain larvae fed on corn whereas 30% of the rice-strain larvae fed on corn, within 30 min. When offered grass leaf parts, 30% of the tested corn-strain larvae started feeding, compared to 50% of the rice-strain larvae. In the large arenas, we found no difference of host plant acceptance between the two strains either (Figure 3c, d): 35% of the corn-strain larvae and 40% of the rice-strain larvae fed on corn leaf parts within 30 minutes. On the grass plant parts, 40% of the corn-strain larvae and 35% of the rice-strain larvae started feeding within 30 min (Figure 3b). When larvae were directly placed on the leaf parts, more corn-strain than rice-strain larvae started feeding on either plant leaf parts (Figure 3e, f): On the corn plant, 64% of the corn-strain larvae started feeding compared to 47% of the rice-strain larvae. On the grass plant, 75% of the corn-strain larvae and 50% of the rice-strain larvae started to feed. Thus, the two strains did not differ significantly from each other in this host acceptance assay.

Larvae that did start feeding within 5 minutes, did so significantly later on the corn-plant than on the rice plant in both strains (Figure 4) ($P < 0.001$), with no difference between the two strains.

iii) Larval performance

Larvae of both strains showed significantly different growth rates when placed on the three different diets (Figure 5): on artificial pinto bean diet both larval strains grew fastest and on the lyophilized corn diet both larval strains grew slowest (Figure 5, $P < 0.001$). The corn-strain grew faster than the rice-strain on each diet, including the control diet ($P < 0.001$). There was no significant interaction effect between strain and diet, thus the differences between the strains on the different diets were due to the corn-strain generally performing better in this experiment.

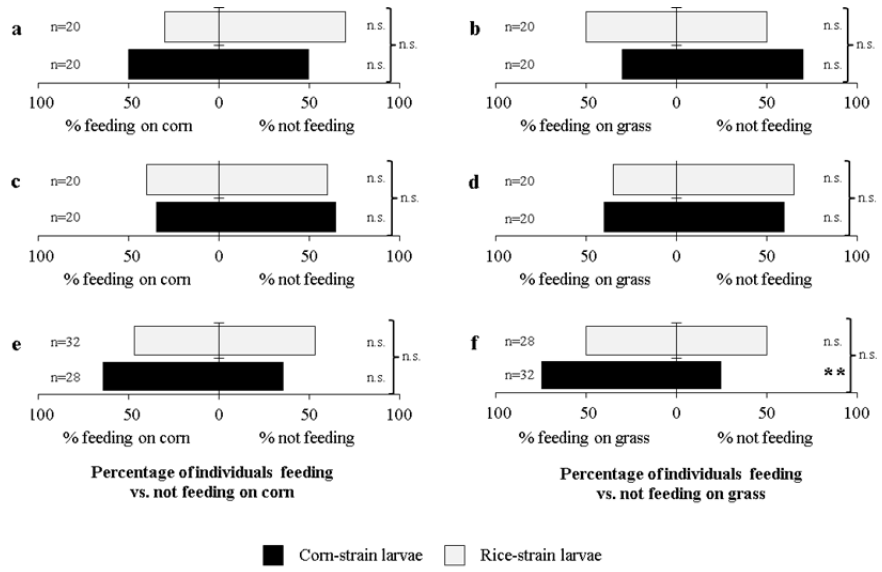

FIGURE 3. Larval host acceptance of corn-strain and rice-strain larvae. a-d No-choice assays with plant parts in small arenas (a+b; a corn plants, b grass plants) and big arenas (c+d; c corn plants, d grass plants). Figures show percentage of individuals moving to and feeding on the plant within 30 min vs. percentage of non- feeders. Within-strain variance: Chi-squared test. n.s. >0.05. Between-strain-variance: binominal comparison of proportions. n=individuals tested. e+f Food acceptance assays (e corn-plant, f grass plant). Figures show percentage of individuals starting to feed within 5 min vs. percentage of non-feeders. Within-strain variance: Chi-squared test, * P<0.05. Between-strain-variance: binominal comparison of proportions. n=number of individuals tested.

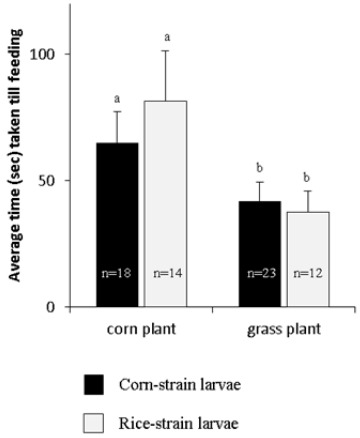

FIGURE 4. Food acceptance (mean + SEM) of corn-strain and rice-strain larvae on corn plant parts and grass plant parts. Figures show seconds taken till feeding starts. n= individuals feeding. Different letters indicate above the bars indicate significant differences (P<0.01).

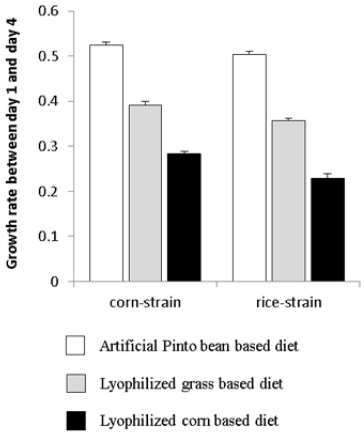

FIGURE 5. Larval growth rate of both strains on artificial pinto bean based diet (white bars), grass based diet (grey bars) and corn based diet (black bars. Error bars represent SEM.

Both strains showed the highest survival to adulthood on the diet based on lyophilized grass compared to lyophilized corn (Figure 6). Both strain and diet had a significant influence on the survival rate (both $P < 0.001$). i.e. the corn-strain had a significantly better survival rate than the rice strain and overall survival was best on grass based diet and poorest on corn-based diet.

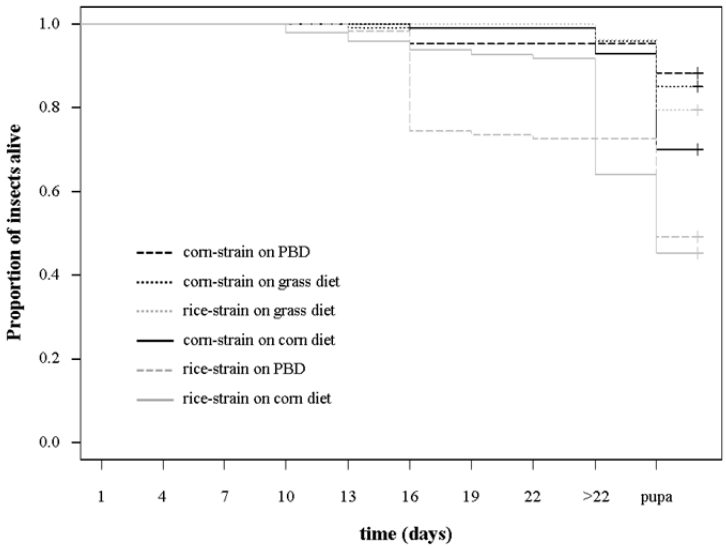

Figure 6. Kaplan-Meier survival plot of corn-strain (black line) and rice-strain (grey lines) larvae on three different diets based on lyophilized corn leaves (solid lines), lyophilized grass leaves (dotted lines) and on pinto beans (dashed lines).

iv) Larval preference

When larvae were given a choice between corn and grass leaf parts in small arenas, more individuals of both strains chose the corn leaf part (Figure 7a). All corn-strain larvae responded and 67% chose corn leaf parts, whereas 33% chose grass leaf parts. Of the rice-strain larvae, 87% responded, of which 69% chose corn leaf parts and 41% chose grass leaf parts. As these differences were not significant, the strains did thus not differ in their preference. In large arenas, 58% of the corn-strain larvae responded, of which 30% chose corn and 70% chose grass, whereas 50% of the rice-strain larvae responded, of which 25% chose corn and 74% chose grass (Figure 7b). Thus, the strains did not differ significantly in their preference in this assay either.

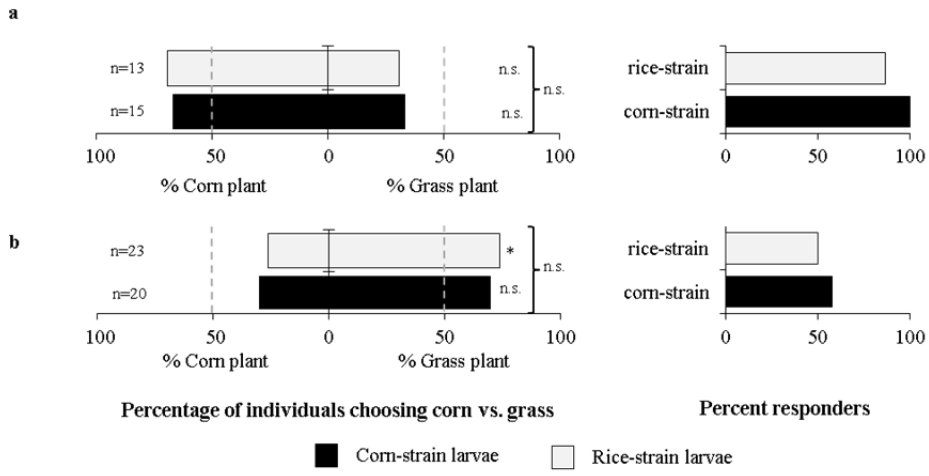

FIGURE 7. Larval preference (left) and response rate (right) of corn-strain and rice-strain larvae. Choice assays with plant parts in **A** small and **B** large arenas. Within-strain variation: Chi-squared test, significance levels: *P < 0.05. Between-strain variance: binominal comparison of proportions. n= individuals responding.

DISCUSSION

The main objective of this study was to identify the level and extent of host plant differentiation in the two strains of *S. frugiperda* to test the following two hypotheses: I. There is host-plant differentiation within the two strains, and II. There is host-plant differentiation between the two strains.

As we neither found an oviposition preference, nor a difference in larval performance or preference and results of other studies are not consistent either (see Table 1), it seems that host plant differentiation is not strongly developed in these two strains.

i) Oviposition preference

If *S. frugiperda* females of the two strains would choose different oviposition sites, this could cause a differential distribution of the two strains in the field, even if larvae would not perform differently on the different hosts. However, we found no significant differences in oviposition preference within or between the two strains. Even though our sample sizes were small, the same populations in the same setup showed variable results between experiments, so that a larger sample size would probably not have yielded different results. Whitford et al. (1988) also found different results for different experiments in the same study. In one experiment, corn-strain females preferred corn and sorghum as oviposition sites, whereas rice-strain females preferred bermudagrass, and the strains differed significantly from each other. However, in the second experiment, the two strains did not exhibit a difference (Whitford et al. 1988). Meagher et al. (2011) did consistently find that the rice-strain chose pasture grass over corn plants, whereas the corn-strain did not show a preference. However, if only rice-strain females show an oviposition preference, whereas corn-strain females do not, oviposition preference seems to play only a minor role, if any, in the host association of the two strains of *S. frugiperda*. In conclusion, only one of two experiments in the study of Whitford et al. (1988) verifies both hypothesis I (within-strain differentiation) and hypothesis II (between-strain differentiation), thus host plant differentiation between the two strains due to oviposition preference cannot be concluded from the available data.

Tactile cues do seem to play an important role as oviposition cues in *S. frugiperda*, because we found a generally much lower oviposition when we had covered the plants with gauze, to eliminate tactile cues, compared to when plants were uncovered. Similarly, Rojas et al. (2003) found that *S. frugiperda* females showed a strong preference for grooved or pitted surfaces over smooth surfaces, although the females did not prefer surfaces with host plant leaf extracts over control surfaces and were even repelled by high doses of extract. Thus, tactile cues rather than volatile cues seem to be involved in oviposition choice, at least at short distances.

ii) Larval host acceptance

If oviposition does not play a role in determining the distribution of the two strains in the field, the host acceptance of the larvae may contribute to host differentiation. In our experiments 4 and 5, corn-strain larvae showed a higher acceptance of the grass plant, i.e. the untypical host, and also accepted the grass plant faster than the corn plant, but only when directly placed on the leaf parts. When the larvae had to move to leaf parts in no-choice situations, the corn-strain did not show a differential acceptance of either plant. The rice-strain larvae accepted the grass plant significantly faster than the corn plant when directly placed on the leaf parts, but did not show a higher acceptance of either host in the other assays. Thus, one of three

assays addressing larval acceptance showed a difference within both strains (but the corn-strain favoured the 'wrong' host), verifying hypothesis I (within-strain differentiation). However, none of the larval host acceptance assays revealed a significant difference *between* the strains, so hypothesis II cannot be verified. Thus, we cannot conclude that larval host acceptance underlies the differential distribution of the two strains in the field. We are not aware of other studies testing *S. frugiperda* host acceptance.

iii) Larval performance

Since eggs of both strains are laid on the same plants and larvae do not differ in their host acceptance, the differential distribution of the two strains may be caused by a difference of larval development or viability on the different hosts. Both strains were heavier when reared on the grass plant diet and there was no difference between the strains beyond an overall higher weight of the corn-strain on all different diets. Both strains also performed better on a typical rice-strain host plant, and there was no difference between the strains. Thus, it cannot be concluded from our results that a differential larval performance on different plants underlies the observed host association in the field. Other studies have found contradictory results (see Table 1): some did find a difference between the strains (Pashley 1988b; Whitford et al. 1988; Pashley et al. 1995; Meagher et al. 2004), whereas others did not (Groot et al. 2010; Meagher and Nagoshi 2012). Larval developmental time, pupal weight and survival rates also differ widely between studies. Some studies show within-strain variation favouring the 'typical' host of the strains, i.e. corn or sorghum plants for the corn-strain and rice or different pasture grasses for the rice-strain (Pashley 1988b; Whitford et al. 1988; Pashley et al. 1995; Meagher and Nagoshi 2012), whereas other studies show between-strain variation in these developmental traits (Pashley 1988b; Whitford et al. 1988; Pashley et al. 1995; Veenstra et al. 1995; Meagher et al. 2004; Groot et al. 2010; Meagher and Nagoshi 2012). In summary, none of the studies, including our own, verifies both hypothesis I and II for larval performance, so that larval performance differences between the two strains of *S. frugiperda* strains cannot be concluded.

iv) Larval host preference

If neither oviposition preference, nor larval host acceptance or larval performance are likely to underlie a host association of *S. frugiperda*, the differential distribution of the strains in the field could still be caused by strain-specific larval choice for a particular host. In this study, larvae were tested in choice situations between leaf parts of corn plants and bermudagrass in two different arenas. In the larger arena, rice-strain larvae showed a preference for bermudagrass, their typical host, whereas the corn-strain did not prefer either plant. Though not significant, also more corn-strain larvae chose to feed on the bermudagrass. Thus, there was no difference

between the strains. In the smaller arenas, more larvae of both strains chose the corn leaf part, but the difference was not significant. There was also no difference between the strains. Our experiments cannot verify Hypothesis I (within-strain variation) or Hypothesis II (between-strain variation). Thus, a larval preference is also unlikely to be involved in the host association of *S. frugiperda*. We are not aware of other studies testing larval preference between typical corn-strain host plants and typical rice-strain host plants.

The existence of two distinct *S. frugiperda* strains is unquestioned, as proven by many studies identifying two distinct strains based on molecular markers (e.g. Nagoshi et al. 2006, 2008, 2012a,b; Nagoshi 2010; Kergoat et al. 2012; Juárez et al. 2014). The question thus remains what separates the two strains, if not their host association, and prevents them from forming one panmictic population. There are two additional potential isolation barriers: differences in the composition of the female sex pheromone (Groot et al. 2008; Unbehend et al. 2013a,b; Hänniger et al. 2015) and allochronic separation of mating activity at night (Pashley et al. 1992; Schöfl et al. 2009, 2011; Hänniger et al. 2015). Host plants could act together with pheromonal divergence and/or allochronic separation to form isolation mechanisms that are not addressed in our bioassays or in the referenced studies. For example, it is possible that different host plant volatiles enhance the attraction of males to the pheromone composition of one or both strains, as described for example for *Grapholita molesta* (Varela et al. 2011). If corn plant volatiles would enhance the attraction of males to corn-strain females, and females would prefer corn plants as oviposition sites, this would facilitate a bias of mated corn-strain females in corn fields and could possibly lead to oviposition bias without an oviposition site preference. Another possibility of how host plants could interact with an isolation barrier is the synchrony of rhythms between different plants and the different strains. The volatile emission of flowers as well as leaves shows circadian rhythms in plants (e.g. Loughrin et al. 1991, 1994; Staudt et al. 1997). A possible scenario would be that corn plants emit certain attractive volatiles earlier in the night than pasture grasses and are thus attracting the early active corn-strain, whereas the grasses attract the later active rice-strain. This may not be the case when the plants are in close vicinity to each other in bioassays, as their volatiles may be mixed too much. Also, tactile cues may be more important at close range than volatile cues, as our experiment (with gauze covered plants) and Rojas et al. (2003) suggest.

Summary of studies on host plant preference and performance of the two strains of S. frugiperda

Table 1 summarizes all published studies that address host plant use of the two *S. frugiperda* strains to verify or falsify our two main hypotheses: I. There is host-plant differentiation *within* the two strains, and II. There is a host-plant differentia-

TABLE 1: Verification or falsification of the two main hypotheses by the different experiments of this and other studies.

Experiment	H1: Differentiation within strains		H2: Differentiation between strains	H1+H2
	Corn-strain	Rice-strain	Corn-strain vs. rice-strain	Can underlie host differentiation?
Oviposition preference on plant parts	-	-	-	no
Oviposition preference on whole plants I	-	-	-	no
Oviposition preference on whole plants II	-	-	-	no
Oviposition preference on whole plants with gauze	-	-	-	no
Oviposition preference I (Whitford et al. 1988)	C	R	+	yes
Oviposition preference II (Whitford et al. 1988)	C	C	-	no
Oviposition preference cage (Meagher et al. 2011)	-	R	+	no
Oviposition preference pool (Meagher et al. 2011)	-	R	+	no
Larval host acceptance small arena	-	-	-	no
Larval host acceptance large arena	-	-	-	no
Larval host acceptance placed on plant	R	-	-	no
Larval host acceptance timing	R	R	-	no
Larval developmental time (Groot et al. 2010)	-	C	+	no
Larval developmental time (Meagher et al. 2004)	n.a.	n.a.	+	n.a
Larval developmental time (Meagher and Nagoshi 2012)	C	C	+	no
Larval developmental time (Pashley 1988)	-	R	-	no
Larval developmental time (Pashley et al. 1995)	R	R	+	no
Larval developmental time (Veenstra et al. 1995)	n.a.	n.a.	-	n.a
Larval developmental time (Whitford et al. 1988)	-	C	+	no
Larval survival	R	R	+	no
Larval survival (Meagher et al. 2004)	n.a.	n.a.	+	n.a
Larval survival (Pashley 1988)	-	R	+	no
Larval survival (Pashley et al. 1995)	-	-	-	no
Larval survival (Veenstra et al. 1995)	n.a.	n.a.	-	n.a
Larval survival (Whitford et al. 1988)	-	-	+	no
Larval weight	R	R	-	no
Larval weight (Groot et al. 2010)	-	-	-	no
Larval weight (Meagher et al. 2004)	n.a.	n.a.	+	n.a
Larval weight (Meagher and Nagoshi 2012)	C	C	-	no
Larval weight (Pashley 1988)	R	R	+	no
Larval weight (Pashley et al. 1995)	R	R	+	no
Larval weight (Whitford et al. 1988)	-	-	+	no
Larval weight day 8 (Veenstra et al. 1995)	n.a	n.a.	+	n.a
Larval weight II(Veenstra et al. 1995)	-	-	-	no
Larval weight last molt (Veenstra et al. 1995)	n.a.	n.a.	-	n.a
Pupal weight (Meagher et al. 2004)	n.a.	n.a.	+	n.a
Pupal weight (Meagher and Nagoshi 2012)	C	-	-	no
Pupal weight (Pashley 1988)	C	C	-	no
Pupal weight (Pashley et al. 1995)	C	C	+	no
Pupal weight (Veenstra et al. 1995)	n.a.	n.a.	+	n.a
Pupal weight (Whitford et al. 1988)	-	R	-	no
Larval choice small arena	-	-	-	no
Larval choice large arena	-	R	-	no
Total fecundity (Pashley et al. 1995)	-	-	-	no

tion *between* the two strains. Grey rows summarize experiments of this study. 'C' indicates preference for or better performance on host plants typical for the corn-strain (e.g. corn, sorghum), 'R' indicates preference for or better performance for host plants typical for rice-strain (e.g. rice, pasture grasses), '+' indicates verification, '–' indicates falsification, n.a. = not available due to experimental design or missing statistical analysis. Only the oviposition preference study by Whitford et al. (1988) shows both levels of host plant differentiation.

In conclusion, the inconclusive results of this and other studies suggest that host plants only have a minor influence, probably in interaction with other isolation barriers, on the divergence of the two *S. frugiperda* strains. Possibly, as already indicated by Juárez et al. (2014), the strains should be called host forms instead of host strains.

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3

POPULATION STRUCTURE OF *SPODOPTERA FRUGIPERDA* MAIZE AND RICE HOST FORMS IN SOUTH AMERICA: ARE THEY HOST STRAINS?

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Abstract

Determining which factors contribute to the formation and maintenance of genetic divergence and to evaluate their relative importance as a cause of biological differentiation is among the major challenges in evolutionary biology. In *Spodoptera frugiperda* (Smith) (Lepidoptera: Noctuidae) two host strains have been recognized in the 1980s: the corn-strain prefers maize, sorghum, and cotton, whereas the rice-strain prefers rice and wild grasses. However, it is not clear to what extent these so-called 'strains', which have also been called 'host races' or even 'sibling species', are really associated with host plants. Due to the indeterminate evolutionary status, we will use the term 'host forms' (sensu Funk). Here, we characterized populations collected from maize, rice, and wild grasses from three countries in South America. Using two mitochondrial cytochrome oxidase I (mtCOI) markers and 10 polymorphisms in the triose phosphate isomerase (*Tpi*) gene, we found various patterns of host association. Two hundred twenty-seven nuclear amplified fragment length polymorphisms (AFLPs) markers revealed significant genetic differentiation among populations, which was generally correlated to the host from which the larvae were collected. Using a multivariate discriminant analysis and a Bayesian clustering approach, we found that individuals could be grouped into 2-5 genetically distinct clusters, depending on the method. Together, our results indicate that although host-associated differentiation is present in this species, it does not account for all observable genetic variation and other factors must be maintaining genetic differentiation between these forms. Therefore, the term 'host strains' should be abandoned and 'host forms' should be used instead for *S. frugiperda*.

INTRODUCTION

Phytophagous insect species often show a population-specific preference for only a few host plant species. This choice and adaptation to a reduced number of host plants may cause reproductive isolation in which the final outcome can be the formation of new species (Walsh 1867; Bush 1969; Schluter 2001; Funk et al. 2006; Feder et al. 2012). One intermediate stage between polymorphic populations and full species along the speciation process is host races. According to Drés and Mallet (2002), host races can be defined as genetically differentiated sympatric populations that are incompletely reproductively isolated with an appreciable rate of gene flow; they exhibit host fidelity by the use of different host taxa in the wild, display a correlation between host choice and mate choice, and have higher fitness on natal than alternative hosts (Drés and Mallet 2002). After this definition, several papers have been published reporting the existence of host races in many insect species with few clear examples in which their existence has been recognized; e.g., the apple maggot fly *Rhagoletis pomonella* (Walsh) (Walsh 1864; Bush 1969; Feder et al. 1994, 2012), the larch budmoth *Zeiraphera diniana* (Guenée) (Emelianov et al. 1995; Drés and Mallet 2002), and the leaf beetle *Neochlamisus bebbianae* (Brown) (Funk 1998, 2012). Recently, Funk (2012) stated that this number is relatively low

because the evidence in many biological systems is still inconclusive given the extensive amount of work required to determine whether a certain organism meets the defined criteria. In this sense, Funk emphasizes that it is necessary to introduce terms aimed at describing different kinds of biological variation in entities in which the existence of host-associated differentiation has been proven but its evolutionary status has not yet been determined. One such term is 'host form' which consists of 'a group of individuals or populations exhibiting host-associated biological variation in which the kind of variation has not yet been diagnosed' (Funk 2012). Determining which factors contribute to the formation and maintenance of genetic divergence to evaluate their relative importance as a cause of biological differentiation is among the major challenges in evolutionary biology (Feder et al. 1988; Berlocher and Feder 2002; Egan et al. 2008).

The noctuid moth *Spodoptera frugiperda* (Smith) (Lepidoptera: Noctuidae) exemplifies this problem. This species seems to be under a process of ecological divergence in sympatry due to host-associated differentiation, as two so-called 'host strains' have been recognized in the 1980s, which are morphologically indistinguishable but show some genetic differentiation in association with different host plants (Pashley et al. 1985; Pashley 1986). Larvae of the so-called corn-strain (C) infest maize (*Zea mays* L.), sorghum [*Sorghum bicolor* (L.) Moench subsp. *bicolor*], and cotton (*Gossypium hirsutum* L.) and have been associated to large grasses, whereas larvae of the so-called rice-strain (R) are found mostly on small grasses as rice (*Oryza sativa* L.) and wild grasses, such as Johnson grass [*Sorghum halepense* (L.) Pers.] and Bermuda grass [*Cynodon dactylon* (L.)] (Pashley 1986, 1988a). Although the term 'strain' or even 'race' and 'sibling species' have been widely used in the literature on this species (Pashley 1986, 1988a; Pashley and Martin 1987; Whitford et al. 1988; Pashley et al. 1995; Drés and Mallet 2002; Prowell et al. 2004; Schöfl et al. 2009, 2011; Meagher et al. 2011), here we will follow Funk (2012) due to the yet indeterminate evolutionary status and use the term 'host form' instead of 'host strain'. These two host forms exhibit some degree of reproductive isolation, including (1) ecological isolation caused by differential use of host plants (Pashley 1986, 1988a; Prowell et al. 2004), larval performance differences (Pencoe and Martin 1981; Pashley 1988b; Whitford et al. 1988; Pashley et al. 1995), and oviposition preference (Whitford et al. 1988; Meagher et al. 2011); (2) temporal isolation caused by temporal partitioning of nocturnal mating activities (Pashley et al. 1992; Schöfl et al. 2009); (3) female-mediated differential mating preferences (Schöfl et al. 2011); and (4) potential sexual isolation caused by differences in the composition of female sex pheromones (Groot et al. 2008; Lima and McNeil 2009) and directionally biased incompatibility and low viability in hybrids (Pashley and Martin 1987; Whitford et al. 1988; Groot et al. 2010).

The two host forms of *S. frugiperda* can be identified by a number of genetic markers. These markers include differences in mtDNA sequences identified in the

cytochrome oxidase I (COI), and NADH dehydrogenase (ND1) genes (Pashley 1989; Lu and Adang 1996; Levy et al. 2002; Nagoshi et al. 2006a), as well as nuclear DNA differences, including restriction length fragment polymorphisms (RFLPs) (Lu et al. 1992), amplified fragment length polymorphisms (AFLPs) (McMichael and Prowell 1999; Busato et al. 2004; Clark et al. 2007; Martinelli et al. 2007; Belay et al. 2012), polymorphisms in tandem-repeat sequences (FRs) (Lu et al. 1994; Nagoshi and Meagher 2003a,b), and 10 polymorphisms in the sex-linked triose phosphate isomerase gene (*Tpi*) (Nagoshi 2010). Although restriction site polymorphisms in COI have been widely accepted to be the most suitable to characterize populations and assess host association with their respective plants, recently 10 SNPs in the *Tpi* gene have been proposed to be more consistent than COI for these purposes (Nagoshi 2012).

Irrespective of the markers, the studies described above on this species show that host association is not always absolute, ca. 80% of individuals collected from maize habitats belong to the corn-form, whereas ca. 85-90% of larvae collected from rice habitats belong to the rice-form. In South American populations there seem to be some differences, as we recently found no consistent pattern of host association between the two forms and their respective host plants when using two restriction site polymorphisms in COI (Juárez et al. 2012). The combined use of mitochondrial and nuclear markers which have different inheritance mechanisms allows inferring the rates and directionality of hybridization. In using this combination, about 16% of field-collected samples from Louisiana, Florida (both USA), Puerto Rico, Guadeloupe, and French Guiana (Prowell et al. 2004) were found to be potential hybrids due to discordance for at least one marker (mtDNA, esterase, and AFLP), with both types of hybrids (RC and CR; first letter always referring to the female) equally frequent, mostly in maize habitats. Similar findings were found with Colombian populations using COI gene and FR-sequence (Saldamando and Velez-Arango 2010). Others found mainly RC-hybrids (Nagoshi and Meagher 2003a; Nagoshi et al. 2006b; Nagoshi 2012). Most of the work published using molecular markers has been used to identify both forms and assess their host specificity, but provide little information about the genetic diversity and population structure of *S. frugiperda*, with a few exceptions (McMichael and Prowell 1999; Busato et al. 2004; Clark et al. 2007; Belay et al. 2012).

Thus, even though the two host forms of the fall armyworm have been considered as host races or even as sibling species, it is not clear whether these forms are associated with specific host plants along the entire range of their distribution or whether there is a constant level of genetic differentiation between populations from different host plants. Therefore, in this study we characterized populations of *S. frugiperda* in the southern limit of its distribution obtained from different hosts, to determine whether these host forms can be considered host races. To do so, we analyzed the combination of two genetic markers that are generally

used to distinguish the two strains (mtCOI and *Tpi* genes) and we studied the genetic structure among the various populations using 227 AFLP markers.

MATERIALS AND METHODS

Insect collection

Fall armyworm larvae were collected from three hosts at six localities from Argentina, one from Brazil, and two from Paraguay (Figure 1). The sampling design aimed at sampling two regions: the eastern region comprising Northeast Argentina, Paraguay, and Southern Brazil, and the western region comprising Northwest Argentina. Within these two regions, one of the characteristic hosts of each form was chosen. In the eastern region, larvae were collected from maize and rice. These two crops are widely cultivated next to each other in extensive areas. In the western region, rice is not cultivated and for this reason the alternative hosts sampled for the rice-form were Bermuda grass and Guinea grass [*Panicum maximum* (Jacq)], that grow spontaneously in the surroundings of maize plantations. Collections took place during November (spring) to February (summer) from 2007 to 2010. Each population was assigned a code denoting the host plant of each form (i.e., C for maize and R for rice or wild grasses), the year of collection, and the region, as detailed in Table 1.

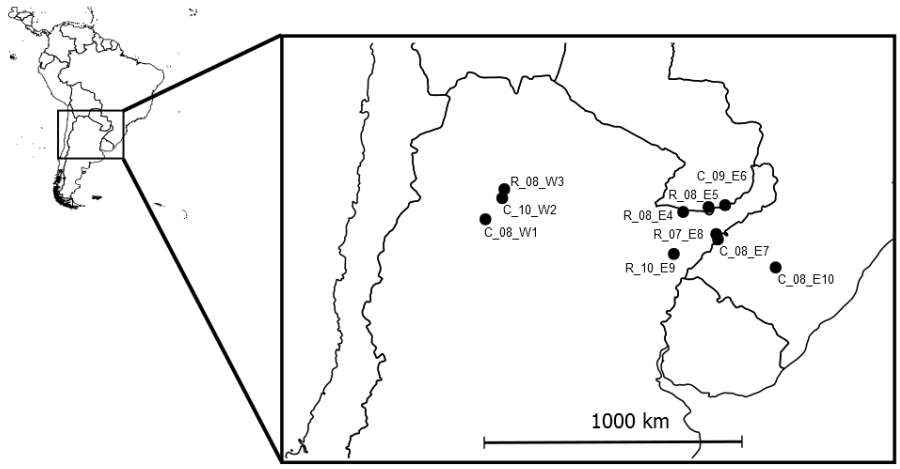

FIGURE 1. *Spodoptera frugiperda* sampling sites in Argentina, Brazil, and Paraguay.

In a given field, approximately 30 sites with 10 plants each were sampled randomly. To avoid any homogenization effect, at least 250 larvae were collected (one per plant) and placed individually in glass tubes (12 cm high, 1.5 cm diameter)

with leaves of the host plant. Larvae were taken to the laboratory and reared in chambers at 27 ± 2 °C, 70-75% r.h., and L14:D10 photoperiod until adult emergence. Late instars and adults were examined to confirm that all individuals were fall armyworm based on diagnostic taxonomic characters. Populations from each sampled host in each locality were maintained separately and 200 adults were used from each population to establish laboratory colonies. In separate mating cages (30 cm high, 10-cm-diameter cylindrical polyethylene-terephthalate cages with nylon mesh cloth) 4-5 females of <24 h old and 4-5 males were introduced. We had in total about 20 mating cages per population. The cages contained pieces of paper that allowed females to rest and to lay eggs. Food was provided via a cotton plug saturated with a 1:1 (vol:vol) mixture of honey and water, which was renewed every day. Cages were checked daily for oviposition and adult mortality. To minimize loss of genetic variability, once females started to lay egg masses, approximately 15 egg masses from each cage were collected and deposited in glass tubes (12 cm high,

TABLE 1. Collection sites, years, and host plants of *Spodoptera frugiperda*

Region	Country	Site	Longi-tude	Lati-tude	Host plant	Year	Population code
West	Argentina	La Cocha	65°34'47.04"W	27°46'25.38"S	Maize	2008	C_08_W1
	Argentina	Los Pereyra	64°53'36.9"W	26°55'09.0"S	Maize	2010	C_10_W2
	Argentina	Benjamín Aráoz	64°48'26.79"W	26°33'28.64"S	Grass	2008	R_08_W3
East	Argentina	Berón de Astrada	57°29'53.90"W	27°28'35.01"S	Rice	2008	R_08_E4
	Paraguay	San Cosme y Damián	56°27'46.1"W	27°16'43.8"S	Rice	2008	R_08_E5
	Paraguay	Capitán Miranda	55°47'12.4"W	27°12'02.5"S	Maize	2009	C_09_E6
	Argentina	Santo Tomé	56°04'26.8"W	28°34'58.3"S	Maize	2008	C_08_E7
	Argentina	Santo Tomé	56°08'47.1"W	28°22'12.9"S	Rice	2007	R_07_E8
	Argentina	Mercedes	57º52'42.4"W	29º10'23.1"S	Rice	2010	R_10_E9
	Brazil	Santa María	53°43'05.2"W	29°43'10.4"S	Maize	2008	C_08_E10

Population code: first letter denotes the host plant the larvae were collected from (C for maize, R for rice and wild grasses); number refers to year of collection; second letter denotes the geographic location of population (eastern or western); the number behind it is the population number. Bermuda grass and Guinea grass are referred to as grass.

1.5 cm diameter). Once emerged, 15 neonate larvae from each of the egg masses were placed individually in glass tubes with artificial diet (Osores et al. 1982) which was renewed every 2-3 days. As larvae pupated, they were placed in cylindrical cages until adult emergence. On average, 200 adults were used again to initiate a new generation. After establishing a colony from each population and host, larvae from the second generation were stored at -20 °C until DNA extraction.

DNA extraction and identification by COI markers

Total DNA was extracted using a modification of Black & DuTeau (1997) CTAB (hexadecyltri- methylammonium bromide) method. Buffer and running conditions were performed according to Sambrook et al. (1989). All samples were characterized using two mtCOI markers by amplifying a 600-bp fragment and digesting separately with *Msp*I (producing 510- and 90-bp fragments in the corn-form) and *Sac*I (producing 450- and 150-bp fragments in the rice-form) (Juárez et al. 2012). Populations were considered to belong to one of the two host forms if the frequency of the corresponding haplotype was above 80%. If the frequency was between 0.8 and 0.2, we characterized the population as a mixture of haplotypes (Juárez et al. 2012).

Characterization of fall armyworm host form by Tpi polymorphisms

Identification of two haplotypes of the fall armyworm was performed using the 10 polymorphic nucleotide sites (SNPs) located in the Z-linked *Tpi* gene as described by Nagoshi (2010). Primers *Tpi*-282F (5-GGTGAAATCTCCCCTGCTATG-3) and *Tpi*-850gR (5-AATTTTATTACCTGCTGTGG-3) (Nagoshi 2010) were synthesized by Metabion (Martinsried, Germany). PCR amplicons from genomic DNA, generated using these primers, were sequenced from both ends using the same primers in separate reactions. Sequencing was performed at the Entomology Department of the Max Planck Institute for Chemical Ecology (Jena, Germany). The DNA sequences were aligned and compared using the program Geneious Pro 5.4.3 (Biomatters, Auckland, New Zealand) (Drummond et al. 2011).

As previously described, each of the 10 sites has a specific nucleotide associated with each host form, making it possible to obtain a consensus sequence for the corn-form (*Tpi*-C) and the rice-form (*Tpi*-R). We followed the criterion proposed by Nagoshi (2010), in which at least seven of the 10 sites must match to the consensus *Tpi*-C or *Tpi*-R sequence in order to identify an individual as corn- or rice-form, respectively. Nagoshi (2010) defined all other configurations of the 10 sites as intermediate, *Tpi*-int. Due to sex-linkage, all females carry only one *Tpi* allele and can be classified in this way. However, males carry two *Tpi* alleles, and therefore can be homozygous or heterozygous. Nagoshi (2010) classified homozygous males in the same manner as females, but only classified heterozygous males if both of their *Tpi* alleles were of the same strain category, e.g., *Tpi*-C or *Tpi*-

R with three or fewer double peaks indicating heterozygous SNPs. *Tpi*-C/*Tpi*-R heterozygotes were not distinguished from *Tpi*-C/*Tpi*-int, *Tpi*-R/*Tpi*-int, or *Tpi*-int/*Tpi*-int heterozygotes, and none of these heterozygotes were included in the analysis of Nagoshi (2010). Here, we scored all 10 of the polymorphic SNPs, because we sequenced all amplicons from both ends, and so we distinguished among genotypic classes in the following way. C, R, and IHo refer to individuals hemizygous (females) or homozygous (males) for a *Tpi*-C, *Tpi*-R, or *Tpi*-int sequence, respectively; i.e., with no double peaks in the sequencing chromatogram. CHe and RHe refer to individuals heterozygous for two different *Tpi*-C or *Tpi*-R sequences, respectively; i.e., showing double peaks at one, two, or three sites, but matching the consensus *Tpi*-C or *Tpi*-R sequences at the other sites. IHe (heterozygous intermediates) include all other heterozygous classes. Our analysis included all these classes.

To consider the possibility of hybridization, we denote the COI and *Tpi* types of individuals by a configuration code in which the first letter represents the COI haplotype (C or R) and the rest represents the *Tpi* type as defined above. For example, the configuration C/RHo has a COI haplotype of C, and is hemizygous or homozygous for *Tpi*-R.

Genome-wide random nuclear markers

AFLP markers were developed following Vos et al. (1995) with some modifications. Genomic DNA (200 ng) was digested with restriction enzymes, *Eco*RI (5U) and *Mse*I (3U) in a 12.5-µl reaction mix. *Eco*RI adapter (5 pmol μl^{-1}) and *Mse*I adapter (50 pmol μl^{-1}) were ligated to generate template DNA for the amplification of DNA fragments by PCR. The adapters had the following sequences: *Eco*RI adapter: 5'-CTCGTAGACTGCGTACC, 5'-AATTGGTACGCA GTCTAC, and *Mse*I adapter: 5'-GACGATGAGTCCTGAG, 5'-TACTCAGGACT CAT) (Metabion). After the pre-amplification step, the selective amplifications were conducted using 11 primer combinations (Table 2).

TABLE 2. AFLP primer combinations used to find 227 genetic markers *in Spodoptera*

Primer combinations			
Mse	*Eco*R	*Mse*	*Eco*R
AAG	AAG700/ACG800	CGA	ACC700/ACT800
AAG	ACC700/ACT800	ACA	TAC700/GTA800
ACA	AAG700/ACG800	CAT	AAG700/ACG800
ACA	ACC700/ACT800	CAT	ACC700/ACT800
ACG	ACC700/ACT800	CTT	ACC700/ACT800
CGA	AAG700/ACG800		

Two 96-well gels were used for each primer combination, where the samples of one population were equally divided among the two gels, as well as within the gels. Twelve individuals were represented on both plates. In this way, we included a total of 177 individuals in the analysis. For visualization in the polyacrylamide gel, all *Eco*RI primers were labeled with an infrared dye (IRD) of 700 or 800 nm. AFLP fragments were separated based on size with a Li-Cor 4300 DNA analyzer that simultaneously detects infrared DNA fragments of 700 and 800 nm. The samples were run on a 6.5% polyacrylamide gel and loaded into 96 wells with a Hamilton syringe (Hamilton, Reno, NV, USA). A labeled standard (Li-Cor STR marker, 50-700 bp) was loaded in the first and last well of each gel (1-100). We scored the gels using image analysis software AFLP-Quantar Pro 1.0 (KeyGene Products, Wageningen, The Netherlands). AFLP markers were identified by scoring the presence (1, indicating the dominant homozygote or the heterozygote) or absence (0, indicating the recessive homozygote) of the bands for every selective primer combination in each gel. The repeated 12 individuals were used to indicate the same markers on both gels. Only those markers that were scored consistently on both gels were used for subsequent analysis.

Genetic diversity and genetic structure

To assess whether the available loci allow for an acceptable precision for genetic analyses, the software BOOTSIE (https://code.google.com/p/bootsie/) was used to calculate the coefficient of variation for genetic distances across 100 bootstrap samples for a decreasing number of loci. Population genetic parameters were estimated based on the AFLP markers using the program AFLPsurv 1.0 (Vekemans 2002). We used two criteria to define populations and assign individuals to each population: (1) 10 populations were defined depending on their origin (i.e., based on sampling site, year of collection, and host plant from which the larvae were collected), and (2) 21 populations were defined depending on their origin (as above) and the combination of mitochondrial (COI) and nuclear (*Tpi* genes) genotypes obtained with both markers. To estimate allele frequencies, a Bayesian method with non-uniform prior distribution (Zhivotovsky 1999) was used. The parameters of genetic diversity and population genetic structure estimated were: total gene diversity (Ht), average gene diversity within populations (Hw), average gene diversity among populations (Hb), and Wright's F_{ST}. Parameters were estimated using the approach of Lynch & Milligan (1994) and assuming Hardy-Weinberg equilibrium. Pairwise Wright's fixation indices (F_{ST}) and pairwise Nei's distances were used to estimate the genetic differentiation and distance between populations. To test the significance level of genetic differentiation among populations, a permutation test using 2,000 replications was performed. Based on pairwise F_{ST} values, a phenogram representing genetic differentiation between populations was reconstructed using the *bionj* neighbor-joining algorithm (Gascuel 1997) and

visualized using the R package APE (Paradis et al. 2004). To infer bootstrap confidence on tree nodes, Neighbor and Consense procedures from the PHYLIP software ver. 3.6 were used (Felsenstein 2005). Consensus was obtained using the 'Majority rule' option, from 1,000 matrices of pairwise F_{ST} generated by AFLPsurv. Nodes are considered well supported if they occur in at least 500 (50%) bootstrap tree reconstructions.

The significance of the correlation between geographic distance and genetic distance matrices was estimated using the Mantel test implemented in the R package ADE4 (Chessel et al. 2004). The P-value for the Mantel coefficient *r* was obtained after performing 2,000 permutations.

To determine the presence of outlier-F_{ST} loci, we performed two sets of analyses using the software MCHEZA (Antao and Beaumont 2011) with 50,000 simulations. The first set of analyses was made to detect outliers that contribute to geographic and host differentiation among all populations. For that, first we considered the 10 populations defined by their origin and then we considered nine populations excluding the population from grasses. The second set of analyses was made to detect outliers that contribute to differentiation between hosts only (i.e., excluding geographic differentiation). For that, first we pooled all 10 populations from the same host (maize or rice/grass) and then we pooled the nine populations without the grass population (i.e., maize or rice). Once all outlier loci were identified, we estimated the population genetic parameters from only neutral loci using the program AFLP-SURV 1.0 and performed the same two hierarchical analysis of variance (ANOVA) components (see above).

Hierarchical analysis of population structure

We performed two hierarchical ANOVA components (Wright 1978) using the HIERFSTAT package (Goudet 2006) from the statistical software R (R Development Core Team, 2012). In the first analysis, the estimation of hierarchical variance components considered four levels: populations (defined by their origin as shown in Table 1), regions (western and eastern), populations within each region, and individuals within each population. In the second analysis, the hierarchical components considered populations (defined by their origin as shown in Table 1), host plant species (maize, rice, and wild grasses), populations within each host plant species, and individuals within each population. Thus, in the first case the highest hierarchical level tested was the geographic distance, whereas in the second case this level was represented by the host plant from which individuals were collected.

Probabilistic analysis of population structure

To identify the level of clustering of genetically related individuals we applied two approaches: an exploratory multivariate method and a model-based Bayesian method.

Discriminant analysis of principal components (DAPC)

DAPC (Jombart et al. 2010) combines the multivariate principal component analysis (PCA) with a discriminant analysis (DA) and makes no assumptions about Hardy-Weinberg equilibrium or linkage disequilibrium. The analysis was performed using the R package ADEGENET (Jombart 2008). The clustering of individuals was determined without prior information on population groupings using the function 'find.clusters', which runs successive K-means clustering with increasing number of clusters (k) to achieve the optimal number of groups (Jombart et al. 2010). The optimal number was based on the minimum value of the Bayesian information criterion (BIC). The association of individuals in clusters and the correspondence between clusters and their original populations was shown by means of a scatter plot of individuals on the first two components of the DAPC, where the grouping factor was defined by the clusters recognized by 'find.clusters'. Each individual was identified by a color key of the sampling population. This scatter plot was obtained with the function 's.class' of the package ADE4 of R (Dray and Dufour 2007). The reliability of the results was corroborated by comparing the a priori assignment with the a posteriori assignment of each individual.

Bayesian clustering analysis

Bayesian approaches to genotypic clustering of individuals typically use explicit population genetic models to sort individuals into clusters such that deviations from equilibrium within clusters are minimized. We estimated the number of clusters and the assignment of individuals into clusters without prior information on population groupings using the methods implemented in the programs STRUCTURE 2.3.1 (Pritchard et al. 2000; Falush et al. 2003) and STRUCTURAMA (Huelsenbeck and Andolfatto 2007).

Using STRUCTURE, the most likely number of clusters is estimated by determining the change in the marginal likelihood of the data Pr (X|K) when the number of clusters (K) is fixed to different values (K = 1, 2,..., 10). We used an ancestry model that allowed for admixture and correlated allele frequencies between populations. Under this model, individuals are fractionally assigned to clusters using a membership coefficient. We ran eight replicate Markov chains with a burn-in period of 200,000 iterations followed by a sampling period of 800,000 iterations for each K. We also used the ΔK method of Evanno et al. (2005) to detect the amount of structuring beyond which a further subdivision does not substantially improve the fit of the admixture model.

An individual was assigned to the cluster for which it had the highest average membership coefficient across runs, after 'label switching' heterogeneity had been accounted for using the software CLUMPP (v. 1.1.1) (Jakobsson and Rosenberg 2007).

Using STRUCTURAMA, the number of clusters and the assignment of individuals to clusters were estimated simultaneously by applying a Dirichlet

process prior, which treats both the assignment of individuals to populations and the number of populations as random variables. STRUCTURAMA implements the basic no-admixture model of STRUCTURE and additionally allows setting the concentration parameter α of the Dirichlet process prior (which shapes the prior probability of the number of clusters) by specifying the prior mean of the number of clusters. We performed eight analyses, varying the prior mean of the number of clusters from two to nine. Each analysis consisted of a single Markov chain run for 2,000,000 cycles. Samples were drawn from the chain every 100th cycle. The first 10,000 of the resulting 20,000 samples were removed as burn-in prior to analysis. The posterior probabilities of the number of populations given the data Pr(K|X) were averaged across runs. At each step in the MCMC chain each individual was assigned to a cluster. To summarize the results of this partitioning of individuals, the partition that minimizes the squared distance to all sampled partitions during an MCMC-run was calculated and reported (mean partition). The distance measure is the number of individuals that must be deleted between two partitions to make them the same (Huelsenbeck and Andolfatto 2007).

RESULTS

Coefficient of variation of AFLP markers

The relationship between the coefficient of variation and the number of AFLP markers allows determining the robustness of genetic variability estimates. The coefficient of variation calculated for all 227 markers was 4.8%, indicating that this number of markers was sufficient to perform unbiased analyses of genetic structure and diversity (Figure S1).

Identification of the corn and rice genotypes with the Z-linked nuclear marker Tpi

The association between *Tpi* genotypes and the host plant from which larvae were collected was more pronounced for populations from maize and grasses than for those collected from rice. The analysis identified homozygous and heterozygous individuals for *Tpi*-C (C and CHe), *Tpi*-R (R and RHe), and intermediate (IHo and IHe) types (Tables 3, S1-S11; Figures 2, S2). In three of the five populations collected from maize fields, more than 80% of the individuals could be classified as *Tpi*-C; of the two remaining populations, one also showed a high percentage of *Tpi*-C individuals (75%) whereas the other population showed a broader distribution of *Tpi* types with 52, 4, and 44% of the individuals belonging to *Tpi*-C, *Tpi*-R, and intermediate, respectively. In the population collected from grasses, 86% of the individuals were *Tpi*-R, consistent with the preference of this type for this habitat. In contrast, none of the four populations collected from rice were characterized as *Tpi*-R. In two of these populations, the *Tpi*-C type was predominant whereas in the other two, intermediate types prevailed.

TABLE 3. Number and percentage (in parenthesis) of *Tpi* genotypes in populations of *Spodoptera frugiperda* collected from Argentina, Brazil, and Paraguay.

Population	n	*Tpi*-C	*Tpi*-CHe	*Tpi*-R	*Tpi*-RHe	*Tpi*-IHe	*Tpi*-IHo
C_08_W1	25	7 (28)	6 (24)	0	1 (4)	10 (40)	1 (4)
C_10_W2	15	9 (60)	6 (40)	0	0	0	0
R_08_W3	14	0	0	7 (50)	5 (36)	2 (14)	0
R_08_E4	21	0	1 (5)	4 (20)	2 (9)	12 (57)	2 (9)
R_08_E5	19	2 (10)	0	3 (16)	1 (5)	13 (70)	0
C_09_E6	19	4 (21)	15 (79)	0	0	0	0
C_08_E7	20	6 (30)	13 (65)	0	0	1 (5)	0
R_07_E8	15	13 (86)	1 (7)	1 (7)	0	0	0
R_10_E9	5	1 (20)	3 (60)	0	0	1 (20)	0
C_08_E10	16	2 (12)	10 (63)	0	0	4 (25)	0

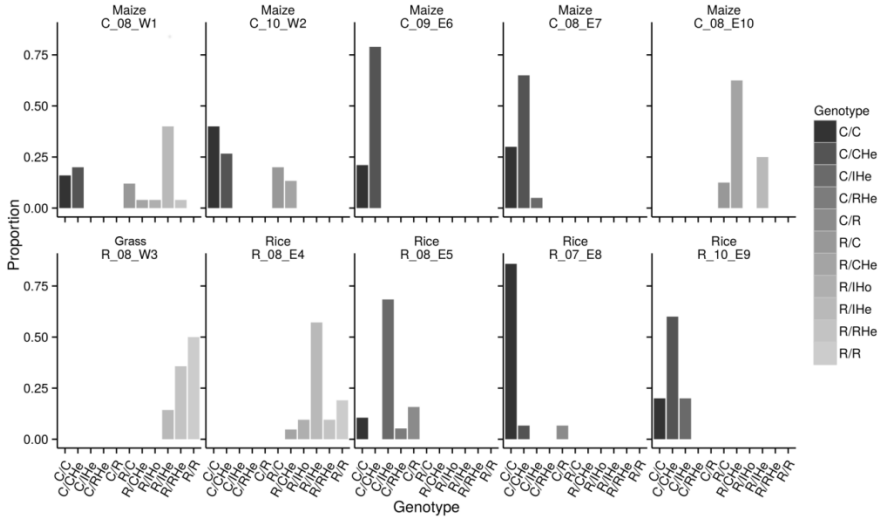

FIGURE 2. Proportion of different configurations of COI and Tpi types in the populations of Spodoptera frugiperda collected from Argentina, Brazil, and Paraguay.

Correlation between Tpi and COI markers and their association with host plants

The combined analysis using both molecular markers showed the following patterns (Figures 2, S2; Tables S1-S11):

Concordance between Tpi and COI markers with association to the host plant
In 3 of the 10 populations, the *Tpi* types were largely consistent with both the previously characterized mitochondrial haplotypes and the host plants from which larvae were obtained. Two of these populations were collected from maize (C_09_E6 and C_08_E7) with 100 and 95% individuals characterized as corn-form (C/C and C/CHe) by both markers, and one population was collected from grasses (R_08_W3) where 86% of the individuals were characterized as rice-form (R/R and R/RHe) by both markers.

Concordance between Tpi and COI markers without association to the host plant
In two of the 10 populations, the *Tpi* types were largely consistent with the mitochondrial haplotypes, but not with the host plants. These populations were collected from rice (R_07_E8, R_10_E9), with 93 and 80% of individuals identified as corn-form (C/C and C/CHe), respectively.

Discordance between Tpi and COI markers
In the remaining five populations, the *Tpi* types were not consistent with the mitochondrial haplotypes and indicated different configurations. In C_08_W1, there were 36% COI-C and 64% COI-R types, and 44 and 16% of individuals had an R/I (R/IHo and R/IHe) and R/C (R/C and R/CHe) configuration, respectively (Figure 2). Similar results were found for C_10_W2 from maize, with 67% COI-C and 33% COI-R types, and 33% of individuals having the RC (R/C and R/CHe) configuration. In C_08_E10 from maize, 100% of larvae analyzed carried COI-R, whereas 75% of them had the *Tpi*-C (C and CHe) and 25% had the intermediate (IHe) type. The remaining two populations were collected from rice. In R_08_E4, 100% of larvae analyzed were COI-R, with 28% having *Tpi*-R (R/R and R/RHe), 67% as intermediate (R/IHe and R/IHo), and 5% with *Tpi*-C (R/CHe). Population R_08_E5 showed the opposite pattern, with 100% of larvae as COI-C and only 11% *Tpi*-C (C/C), 68% intermediate (C/IHe), and 21% *Tpi*-R (C/R and C/RHe) (Figure 2).

Taken together, of the 169 individuals analyzed from the 10 populations, 46% were determined as corn-form with both markers (C/C and C/CHe), 11% as rice-form with both markers (R/R), whereas the remaining 43% showed discordance between the mitochondrial and nuclear markers, C/R (3%), C/I (9%), R/C (13%), and R/I (18%).

Genetic diversity and population structure
We scored a total of 227 genomic AFLP markers, 215 (94%) of which were polymorphic and were thus used for the population genetic analysis.

Populations defined according to their origin
The overall gene diversity (Ht) was 0.28, with the highest component represented by the within-population diversity (Hw = 0.20) and a relatively low among-

populations diversity (Hb = 0.084). The overall genetic differentiation among populations was highly significant (F_{ST} = 0.31, P<0.0001; Table 5). The most differentiated population compared to all others was the population obtained from wild grasses (R_08_W3). When this population was excluded from the analysis, F_{ST} and Hb decreased to 0.18 and 0.045, respectively. Thus, roughly half of the total genetic differentiation among populations was due to the differentiation between the samples from wild grasses and all other populations. The differentiation between populations still remained significant after the samples from wild grasses were removed (P<0.0001; Table 5).

The lowest genetic distance between populations as estimated by Nei's distance and F_{ST} coefficients (Table S12) was observed between C_08_E7 and C_09_E6 (both from maize). The largest genetic distance was observed between R_08_W3 and R_07_E8 (from wild grasses and rice, respectively). Correlation analysis using a Mantel test showed an absence of significant isolation by distance between *S. frugiperda* populations (r = 0.148, P = 0.31) (Figure S3).

A neighbor-joining tree derived from F_{ST}-values showed no support for clustering of populations by geographic region (Figure 3A). It did, however, indicate a separation of populations by host plant. Samples collected from rice or maize plants were clearly separated from the wild grass population, which had the highest bootstrap support.

Populations defined according to their origin and the combination of mitochondrial and nuclear types

The overall gene diversity (Ht = 0.29), was mainly represented by the within-population diversity (Hw = 0.20), with relatively low between-population diversity (Hb = 0.09). The overall genetic differentiation among populations was highly significant (F_{ST} = 0.31, P<0.0001). After excluding the diverged samples collected from wild grass, F_{ST} and Hb decreased to 0.21 and 0.06, respectively, but F_{ST} remained significant. The genetic distances between populations as estimated by Nei's distance and F_{ST} coefficients are shown in Table S13. The lowest genetic distance was observed between C_08_W1 (C/C) and C_08_W1 (C/CHe), R_08_W3 (R/R) and R_08_W3 (R/RHe), C_10_W2 (C/CHe) and C_10_W2 (C/C), C_08_E7 (C/C) and C_08_E7 (C/CHe), C_09_E6 (C/CHe) and C_09_E6 (C/C), C_08_W1 (R/C) and C_08_W1 (R/IHe), all these from the same collection site. The largest genetic distance was observed between R_08_W3 (R/RHe) and R_07_E8 (C/C) (Nei's distance), and R_08_W3 (R/R) and C_08_W1 (C/C) (F_{ST}), respectively.

A neighbor-joining tree derived from F_{ST}-values (Figure 3B) showed that most populations grouped together on the basis of their geographic origin and not on the basis of their genotype composition. The only exception was the individuals from C_08_W1, which formed two clearly separated groups based on their COI haplotype. Individuals carrying the COI-C haplotype clustered with the rest of the

samples derived from maize fields, whereas individuals carrying the COI-R haplotype formed a cluster relatively basal in the tree indicating a very distant position from all other groups. The sample C_08_W1 thus seems to be comprised of individuals from two genetically diverged populations. This may explain why C_08_W1 is not clustered with the remaining populations collected from maize in Figure 3A. In addition, high bootstrap support was found between the populations R_08_W3 (R/RHe) and R_08_W3 (R/R), R_08_E4 (R/R) and R_08_E4 (R/IHe), C_08_W1 (R/C) and C_08_W1 (R/IHe), and between C_08_W1 (C/Che) and C_08_W1 (C/C) populations.

Analysis to detect the presence of outlier-F_{ST} loci that contribute to geographic and host differentiation among populations (first set of analyses) showed that, considering the 10 populations together, no outliers were found but when we considered the nine populations, six of the 227 loci were identified as outliers and thus candidates to be under positive selection (Figure S4AB). In this case, the overall genetic differentiation among populations was 0.19 ($P<0.0001$). The hierarchical analysis revealed significant differences among regions and hosts, whereas the differentiation among populations within each region and host was highly significant (Tables S14-S16). No outlier-F_{ST} loci were found when all populations from the same host were considered, including or not the wild grass population (second analysis) (Figure S5AB).

Hierarchical analysis of population structure

A hierarchical ANOVA component considering populations, regions, populations within regions, and individuals within populations revealed no significant differences between regions; in contrast, the differentiation among populations and populations within each region was highly significant (Table 4). The hierarchical analysis considering populations, host plant species, populations within each host plant species, and individuals within populations revealed borderline significant differences among host species and highly significant differences among populations and among populations within host species (Table 5).

Probabilistic analysis of population structure

Discriminant analysis of principal components (DAPC) by find.clusters function

Applying the Bayesian information criterion (BIC), the total sample was best divided into 10 groups (Figure 4). Mostly, these groups were composed of individuals from the same locality and sampling year. Major exceptions were groups A and K, which were composed of individuals from C_08_W1; group A was composed of individuals carrying the C/C and C/CHe configurations, whereas group K was composed of individuals with R/RHe, R/C, R/CHe, R/Iho, and R/IHe configurations. Group I was composed of all individuals from C_08_E7 and R_10_E9, which carried the C/C, C/CHe, and C/IHe configuration, and by some

individuals from C_09_E6 and R_08_E5 with C/C, C/CHe, C/R, and CIHe configurations. In addition, DAPC indicated five clusters of groups (gray ellipses in Figure 4). The largest cluster was composed of five groups: G (R_07_E8), D (C_10_W2), H (most individuals from R_08_E5), I, and F (most individuals from C_09_E6). All these groups were represented by individuals collected from maize and rice and carrying the C/C, C/CHe, and C/IHe configurations. The cluster represented by group J (R_08_E4) was very close to the former cluster and included individuals collected from rice and carrying mostly the R/IHe, R/R, and R/RHe configurations. A third cluster was formed by group A (C_08_W1), composed of individuals carrying the C/C and C/CHe configurations, and group B (C_08_E10), composed of individuals carrying the R/C, R/CHe, and R/IHe configurations; both were collected from maize and exhibited a slight overlap. The more isolated clusters are group L, consisting of R_08_W3, including individuals from wild grasses carrying R/R, R/RHe, and R/IHe configurations, and group K (C_08_W1), that includes individuals from maize carrying the R/C, R/CHe, R/RHe, R/IHo, and R/IHe configurations. The distribution of the groups represented here seems to constitute a hierarchical islands model (Jombart et al. 2010). The agreement comparing the prior and posterior assignments was 84%.

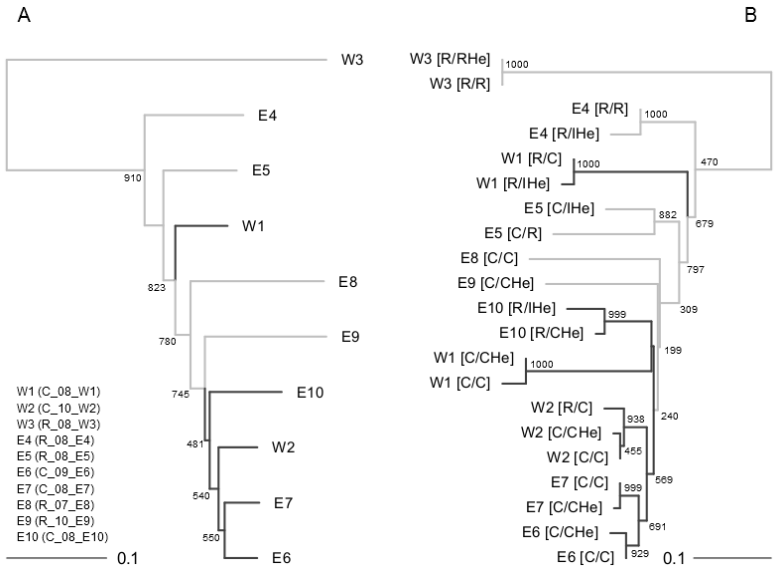

Figure 3. Neighbor-joining trees, (A) based on F_{ST} values calculated from AFLP markers, from *Spodoptera frugiperda* populations defined according to their origin: sampling site, year of collection and host plant, and (B) with populations defined by their origin and combination of mitochondrial and nuclear haplotypes. Populations collected from maize are indicated by black branches, populations collected from rice and grasses by gray branches.

TABLE 4. Hierarchical analysis of *Spodoptera frugiperda* populations among populations, among regions, among populations within regions, and among individuals within each population.

	Variance	F	95% confidence interval	P
Populations – total	33.707	0.416	0.387-0.445	<0.0005
Regions – total	1.040	0.013	-0.005-0.031	0.46
Populations – regions	32.667	0.408	0.381-0.437	<0.0005
Individuals – populations	47.384			

'Regions' denotes eastern (incl. R_08_E4, R_08_E5, C_09_E6, C_08_E7, R_07_E8, R_10_E9, C_08_E10) or western (incl. C_08_W1, C_10_W2, R_08_W3) geographic location of populations (see also Table 1).

TABLE 5. Hierarchical analysis of *Spodoptera frugiperda* populations among populations, among host plants, among populations within host plant, and among individuals within each population.

	Variance	F	95% confidence interval	P
Populations – total	39.663	0.456	0.426-0.487	<0.0005
Hosts – total	1.808.641	0.208	0.172-0.244	0.041
Populations – hosts	21.577	0.313	0.287-0.344	0.001
Individuals – populations	47.384			

'Hosts' denotes the plant species from which populations were sampled: maize (incl. C_08_W1, C_10_W2, C_09_E6, C_08_E7, C_08_E10), rice (incl. R_08_E4, R_08_E5, R_07_E8, R_10_E9), and grasses (incl. R_08_W3) (see also Table 1).

Bayesian analysis

The Bayesian inference of structural patterns among the individuals gave no consistent results. The minimal number of genetic clusters necessary to explain the data as suggested by Evanno's ΔK was 2 (Figure S6). The posterior probability of the number of clusters derived from STRUCTURAMA was highest at K = 4 (Figure S6). Average log-likelihoods across 10 replicate STRUCTURE runs showed no marked plateau before K = 8 [Figure S6; empirical evidence suggests that a biologically meaningful number of K may be indicated by a declining rate of increase in Pr(X|K) as K increases, rather than by the absolute maximum likelihood (Pritchard et al. 2000; Evanno et al. 2005). Figure 5 presents the assignments of individuals to different clusters by both programs for K≤4. With K≥4, assignments became increasingly inconsistent across replicate runs and hence difficult to interpret and summarize. At K = 2, R_08_W3 (from wild grasses) consistently formed one cluster, whereas all other samples were joined in a second cluster (Figure 5). At K = 3, a third cluster was split off, comprising R_08_E4, R_08_E5

(both from rice) and most individuals from C_08_W1 (from maize). At K = 4, four alternative clusters were observed across 10 runs, all of which introduced a split among populations R_08_E4, R_08_E5, R_07_E8, and C_08_W1 (Figure 5). These are most populations collected from rice and the genotypically mixed population C_08_W1. When K was treated as a random variable (using STRUCTURAMA), the overall patterns were largely similar.

However, as noted previously (Groot et al. 2011), STRUCTURAMA tends to introduce additional populations comprised of only very few individuals, which often lack a biologically meaningful interpretation. Thus, despite STRUCTURAMA detecting between four and six clusters as the most likely number of K across multiple runs (compare Figure 5), the vast majority of individuals were assigned to either two or three clusters (Figure 5). As with STRUCTURE, if two major clusters were inferred, one cluster contained all individuals from R_08_W3, whereas most other individuals were placed in the second cluster. If three major clusters were inferred, the third comprised R_08_E4, R_08_E5, R_07_E8, and C_08_W1 in varying combinations (Figure 5).

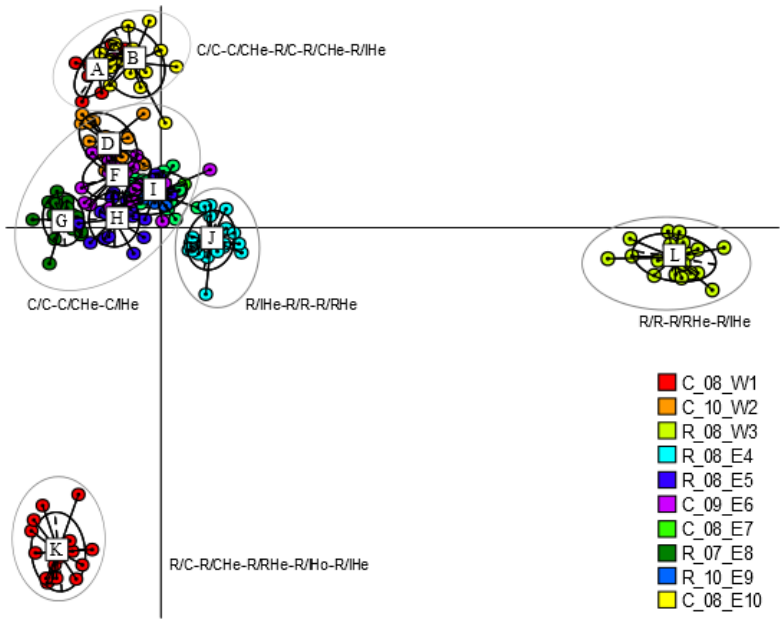

FIGURE 4. Population analysis by discriminant analysis of principal components (DAPC). Groups of individuals were identified by the *find.clusters* function without prior information on population groupings: A (C_08_W1), J (R_08_E4), L (R_08_W3), G (R_07_E8), B (C_08_E10), K (C_08_W1), D (C_10_W2), H (R_08_E5), I (including all individuals from C_08_E7, all individuals from R_10_E9, and some individuals from R_08_E5 and C_09_E6), and F (C_09_E6).

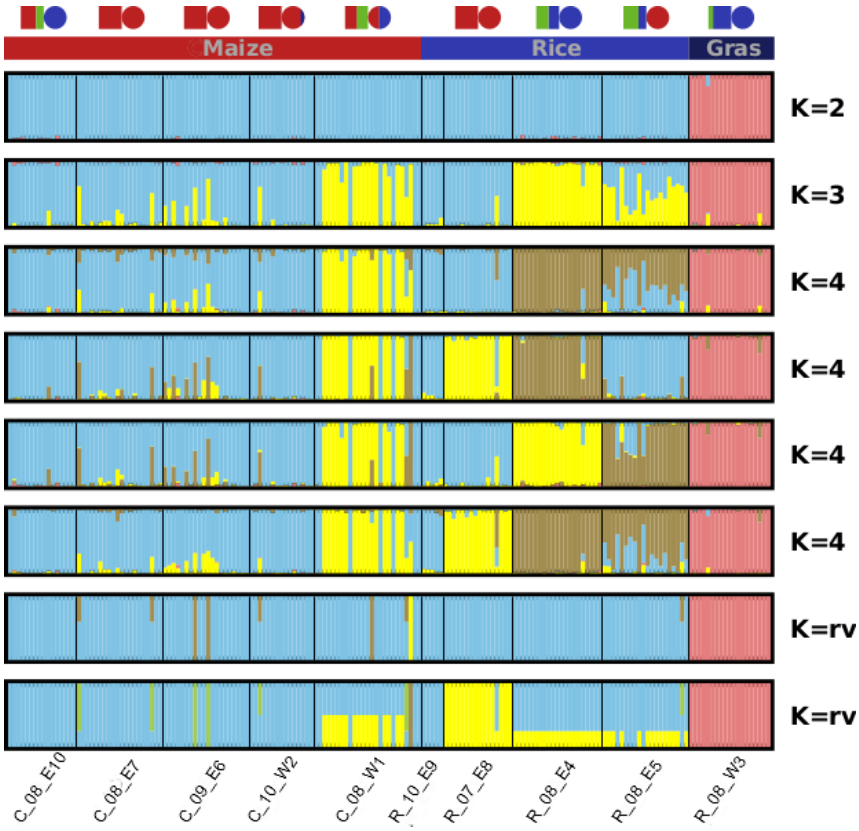

FIGURE 5. Estimated population structure of *Spodoptera frugiperda* populations. The analysis with STRUCTURE was performed under an admixture model with the number of clusters (K) fixed to different values (K = 2, 3, and 4). The analysis with STRUCTURAMA was performed under a no-admixture model where the assignment of individuals to populations and the number of populations as random variables (rv).

DISCUSSION

The present study aimed to determine whether *S. frugiperda* is under a process of speciation through host associated differentiation, to support or reject the existence of host races. We characterized different South-American populations with the Z-linked nuclear marker *Tpi* and 227 nuclear AFLP markers to complement a study by Juárez et al. (2012) that used two restriction site polymorphisms in the mtDNA COI. In this study, we found a different pattern of association between the host species from which the populations were sampled and the two molecular markers, COI and *Tpi*, and highly significant genetic variability, with strong genetic differentiation of some populations.

Population characterization and the association with host plants

Populations collected from maize and wild grasses mostly showed the expected *Tpi* genotypes. This result is in agreement with Nagoshi (2012), who found that this marker is an accurate indicator and therefore should be considered as the most appropriate marker developed so far to assign host form identity. However, the combined analysis of *Tpi* and COI revealed that only two out of the five populations collected from maize were characterized as belonging to the corn-form (i.e., *Tpi* and COI correlated). The other three showed various combinations of the corn-, rice-, or intermediate forms of the markers, indicating some sort of hybridization. Because the individuals we sequenced come from the second generation of laboratory-maintained colonies founded by field-collected individuals, we cannot determine how much of this hybridization occurred in the field, and how much is due to crossing in the laboratory of different, non-hybrid forms collected from the same field. However, the large difference in COI haplotype frequencies and *Tpi* allele frequencies in three of the populations indicates that some hybridization must be occurring in the field, because these frequency differences cannot have arisen within two generations. In any event, this means that *Tpi* marker alone is not sufficient to characterize the populations as it is unable by itself to provide information on possible mixed genomes. Populations collected from rice revealed an unexpected situation. None of the populations were characterized by both markers as rice-form, and two populations (R_07_E8 and R_10_E9) were genetically identified as corn-form (both by COI and *Tpi*).

The high frequency of corn-form individuals in rice plants is unexpected; this result is unaffected by the fact that the analyzed individuals come from the second generation of a field collection. Previous studies found that corn-form individuals seemed restricted to their host plant (Lu and Adang 1996; Nagoshi and Meagher 2003a, 2004; Velez-Arango et al. 2008) and this could be attributed to the low levels of toxic cyanogenic compounds found in this plant compared to wild grasses (Hay-Roe et al. 2011). It could be argued that rice is also less toxic than wild grasses and hence this host can be exploited by corn-form individuals which lack the capacity to cope with high levels of these toxic compounds (Hay-Roe et al. 2011). The remaining samples derived from two populations collected from rice were composed mostly of individuals bearing intermediate *Tpi* types. In one of these populations the haplotype COI-R was predominant, whereas in the other the haplotype COI-C prevailed. This revealed a more complex pattern and the presence of individuals with mixed genomes. In the populations we sampled overall, rice occurs as a host in which the pure rice-form of *S. frugiperda* was almost absent and in which pure corn-form individuals and individuals with mixed genomes can develop.

Genetic variability and population structure

We found a high genetic diversity between the sampled populations, which could be clustered into 2-5 genetically distinct groupings. This poses the question of what maintains this genetic differentiation: geographical distance, host fidelity, or strain identity, and how they are related. The highest amount of diversity was found among individuals and the correlation analysis confirmed no genetic isolation by geographic distance between *S. frugiperda* populations, which is in agreement with Martinelli et al. (2007), Clark et al. (2007), and Belay et al. (2012). The ANOVA components revealed no differences between eastern and western regions and showed a marginally significant differentiation between host plants. In the neighbor-joining trees based on F_{ST}-values, populations grouped together mainly based on their geographic origin and not on their haplotype composition or region (Figure 3B). To a lesser extent, there was also an association based on the host. There were, however, two main exceptions. One was R_08_W3, collected from wild grass, which never grouped with any population collected from rice and appeared in a separate branch showing that the least amount of gene flow is between this population and the rest of populations. The other was C_08_W1, where individuals formed two clearly separated groups; one included those individuals bearing the COI-R haplotype and the other those bearing the COI-C haplotype irrespective of their *Tpi*. The same pattern was observed with the DAPC and Bayesian clustering methods. It can be assumed that COI-R individuals from the C_08_W1 population were recent immigrants. In this region (Western Argentina) rice is not cultivated and hence, the migrant individuals possibly have derived from the surrounding grasses (C_08_W1 and R_08_W3 are only 160 km apart). However, the neighbor-joining analysis and the DAPC failed to merge these individuals, revealing the need of more sampling to determine whether wild grasses act as a reservoir of pure rice individuals or even of other genetically isolated populations. DAPC grouped the individuals mostly based on the site and year of sampling. Additionally, the groups with C-mitochondrial haplotype tended to cluster, whereas most of the groups with the R-mitochondrial haplotype were more isolated. STRUCTURE and STRUCTURAMA also showed that the populations collected from maize clustered and were homogeneous. Similar to the DAPC analysis, populations collected from rice formed three distinct groups, showing indicating a much higher level of heterogeneity. The outlier analysis showed that they did not contribute significantly to the geographical and host differences and their removal did not alter the conclusions compared to the analysis that included the total number of loci.

A possible cause of bias in our study was the introduction of the populations into the laboratory, which might have caused changes in the distribution of allele frequencies. To minimize this problem, we maximized the number of adults from which we started the colonies and sampled an equal number of larvae from egg

masses and mating cages to reduce any skew in reproductive success. In addition, if we consider that the mtCOI is maternally transmitted and does not recombine, a significant change in allele frequencies is not expected within two generations of laboratory rearing. The *Tpi* marker is sex-linked and may recombine only in males, but, due to the close linkage between SNPs, a significant number of intermediate patterns by recombination in only one or two generations is highly unlikely. Therefore, we assume that the *Tpi* frequencies have also not changed significantly during two generations in the laboratory. The other aspect of sampling second-generation laboratory populations is a loss of information about the naturally-occurring frequency of hybrids. From samples with large discrepancies in frequencies of mitochondrial and nuclear markers, we can infer that some hybridization is occurring in the field, but we cannot estimate its frequency. Our study and the work done by Nagoshi (2012) infer hybridization values greater than 40% with individuals from the laboratory, whereas the hybridization rates found by Prowell et al. (2004) are near 16%. As the study of Prowell et al. (2004) is based on field-collected samples, hybridization rates of 16% are likely to be an accurate estimation.

Evolutionary and ecological implications

Our results provide additional information for understanding the population structure and the host-associated differentiation in the two host forms of *S. frugiperda*. In this study, the utility of the host as indicator of population identity was variable as shown by the different molecular markers. The genotypes identified by *Tpi* revealed high frequency of populations matching their respective hosts, and the AFLP analysis showed that populations collected from the same host tended to be more associated, but this was not confirmed by the mtCOI markers. In addition, the majority of individuals from populations collected from rice were more heterogeneous than individuals collected from maize.

The near absence of pure rice-form populations on rice is interesting, given that previous reports indicate that this form is predominant in ca. 85-90% of the collections from rice or wild grasses and only in ca. 20% of the collections from maize (Lu and Adang 1996; McMichael and Prowell 1999; Nagoshi and Meagher 2003a,; Machado et al. 2008; Velez-Arango et al. 2008). Most studies on the rice-form have focused on its association with wild grasses. These findings, together with the physiological and biological evidence from laboratory studies showing the capacity of both strains to develop equally well on maize and rice plants (Pashley et al. 1995; Meagher et al. 2004; Groot et al. 2010), suggest that host plants do not exert the same selective pressure towards differential host use. It also suggests that other factors are relevant in the process of host shift. For example, Pashley et al. (1995) reported that over 2 years of sampling, *S. frugiperda* larval mortality caused by parasites and pathogens was higher in pasture than in maize fields, suggesting

that the maize habitat may constitute a more protected environment compared to rice habitats. The unexpected high frequency of corn individuals in rice raises doubt whether host-driven selection can still create population divergence even when host fidelity is weak.

The high genetic variability that we found within and among populations may have arisen from new genotypic combinations, providing a high capacity to adapt to changes in agricultural environments with an evolutionary potential (Domingues 2011). Another possible explanation is that divergent genotypes are not of recent origin and they existed and still exist on native grasses (grass-form) and some simply expanded their host ranges into maize and rice with the domestication and introduction of these crops in America. In some areas of the distribution of *S. frugiperda*, this has resulted in *Tpi* and COI haplotype patterns that correlate with host plant, but in other places these markers do not show a consistent pattern. The greater homogeneity found in the populations collected from maize, and the greater heterogeneity found in the populations collected from rice, suggests that the rice-form is the ancestral type.

Conclusions

Reiterating the definition of host races (Drés and Mallet 2002), our study sheds light on whether the two forms of *S. frugiperda* can be considered host races. The two forms use different host taxa in the wild, at least to some extent; the two forms consist of a number of individuals that exhibit host fidelity; the two forms coexist in sympatry in at least part of their distribution range; and they are genetically differentiated at more than one locus. However, we found that the two forms are not always more genetically differentiated from populations on another host in sympatry than from some geographically distant populations on the same host. Our study did not examine whether the two forms display a correlation between host choice and mate choice, but we did find that they undergo gene flow at an appreciable rate. Previous studies have shown the two forms do not have higher fitness on natal than on alternative hosts, or more accurately, inconsistent results were found across studies (Pashley 1988b; Whitford et al. 1988; Pashley et al. 1995; Meagher et al. 2004; Stuhl et al. 2008; Groot et al. 2010). However, hybrid incompatibility has been shown for RC hybrid females, which mate at much lower rates and few matings result in fertile egg clutches (Pashley and Martin 1987; Whitford et al. 1988; Groot et al. 2010). Overall, we have insufficient information to conclude that the two forms are true host races, and thus 'host form' (Funk 2012) is the appropriate terminology at this stage of our knowledge. Our results indicate that although host-associated differentiation is confirmed as one of the diverging mechanisms, this species is composed of genetically distinct entities that are most likely diverging due to (additional) factors other than host specialization.

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SUPPLEMENTARY MATERIAL

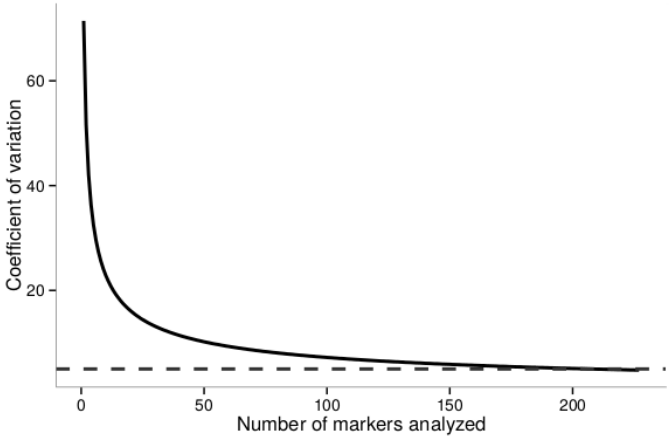

FIGURE S1. The coefficient of variation of *Spodoptera frugiperda* AFLP markers with 100 bootstrap replicates. The horizontal dashed line indicates a coefficient of variation of 5%.

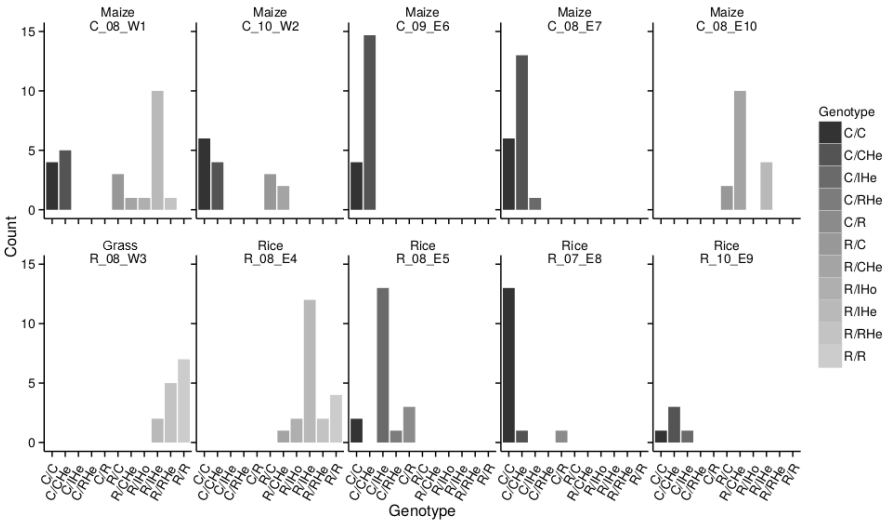

FIGURE S2. Count of different configurations of COI and *Tpi* types in populations of *Spodoptera frugiperda* collected from maize, rice, and grasses in Argentina, Brazil, and Paraguay.

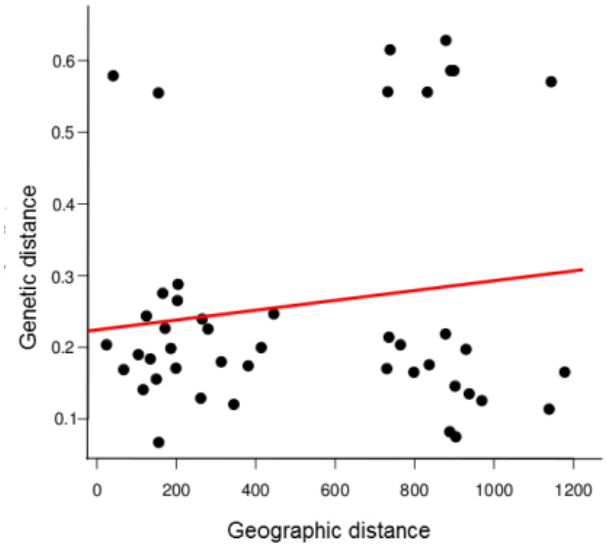

FIGURE S3. Isolation by distance for *Spodoptera frugiperda* populations collected from Argentina, Brazil, and Paraguay.

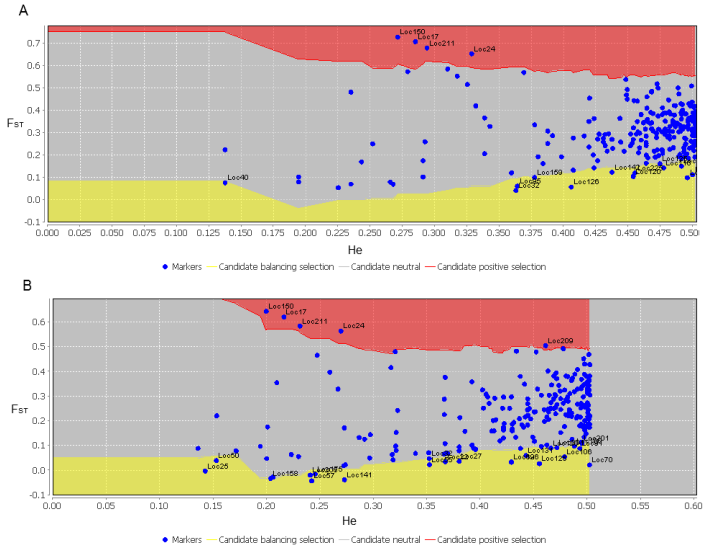

FIGURE S4. (A) F_{ST} / H_E plot showing neutral loci, loci candidate for balancing selection, and loci candidate for positive selection derived from 10 populations defined by their origin. (B) F_{ST} / H_E plot showing neutral loci, loci candidate for balancing selection, and loci candidate for positive selection derived from nine populations defined by their origin and (excluding the population from grasses).

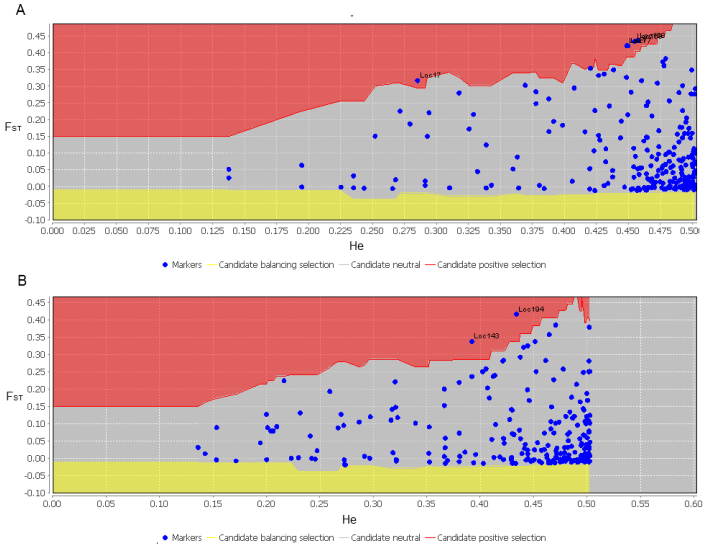

FIGURE S5. (A) F_{ST} / H_E plot showing neutral loci, loci candidate for balancing selection, and loci candidate for positive selection derived from all populations from the same host (maize or rice/grass). (B) F_{ST} / H_E plot showing neutral loci, loci candidate for balancing selection, and loci candidate for positive selection derived from nine populations from the same host without the grass population (maize or rice).

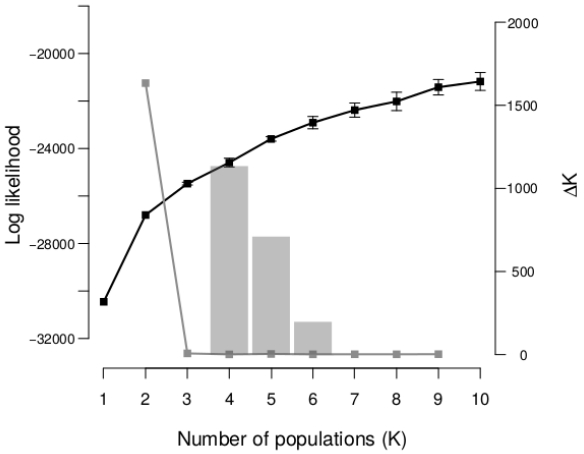

FIGURE S6. Estimates of the most likely number *of Spodoptera frugiperda* populations. Black squares show the marginal log likelihoods of the data Pr (X|K) when the number of clusters (K) is fixed to different values. The grey squares denote ΔK, an ad hoc indicator of the uppermost hierarchical level of structure detected, based on the rate of change in Pr (X|K) between successive K values. The grey bar denotes the posterior probability distributions Pr (K|X) for the number of populations where K is treated as a random variable.

TABLE S1. Proportion of different configurations of COI haplotypes and *Tpi* genotypes in populations of *Spodoptera frugiperda* collected from Argentina, Brazil, and Paraguay.

Population	N	COI-C Tpi-C	COI-C Tpi-CHe	COI-C Tpi-R	COI-C Tpi-RHe	COI-C Tpi-IHe	COI-R Tpi-R	COI-R Tpi-RHe	COI-R Tpi-C	COI-R Tpi-CHe	COI-R Tpi-IHe	COI-R Tpi-IHo
C_08_W1	25	4	5	0	0	0	0	1	3	1	10	1
C_10_W2	15	6	4	0	0	0	0	0	3	2	0	0
R_08_W3	14	0	0	0	0	0	7	5	0	0	2	0
R_08_E4	21	0	0	0	0	0	4	2	0	1	12	2
R_08_E5	19	2	0	3	1	13	0	0	0	0	0	0
C_09_E6	19	4	15	0	0	0	0	0	0	0	0	0
C_08_E7	20	6	13	0	0	1	0	0	0	0	0	0
R_07_E8	15	13	1	1	0	0	0	0	0	0	0	0
R_10_E9	5	1	3	0	0	1	0	0	0	0	0	0
C_08_E10	16	0	0	0	0	0	0	0	2	10	4	0

TABLE S2-10. The orange color denotes the nucleotide corresponding to the corn genotype sequence; the blue color denotes the nucleotide corresponding to the rice genotype sequence; the green color indicates the nucleotide for the intermediate genotype. The purple color denotes a different nucleotide for the corn- and rice-form sequences. The intermediate genotype represents individuals where the SNPs from the two genotypes (corn and rice) were in similar proportions or individuals where the same SNPs presented two alternative nucleotides. In addition, it shows information on mitochondrial haplotype (COI) and host plants from which the individuals were collected. C, R, and IHo refer to individuals hemizygous (females) or homozygous (males) for a *Tpi*-C, *Tpi*-R, or *Tpi*-int sequence respectively. CHe and RHe refer to individuals heterozygous for two different *Tpi*-C or *Tpi*-R sequences respectively. IHe (heterozygous intermediates) include all other heterozygous classes.

TABLE S2. Polymorphic nucleotide sites (SNPs) located in the *Tpi* gene region present in population C_08_W1.

Samples / SNP	74	95	173	174	184	185	253	352	355	377	COI	TPI	Host
AA10	T	T	G(A)	A	C	T	T	C	T	C	corn	CHe	corn
AA1	T	T	A	T	C	T	T	C	T	C	corn	corn	corn
AA2	T	T	A	T	C	T	T	C	T	C	corn	corn	corn
AA3	T	T	A	T	C	T	T	C	T	C	corn	corn	corn
AA4	T	T	A	T	C	T	T	C	T	C	corn	corn	corn
AA5	T	T	G/A	A/T	C(T)	T	T	C	T	C	corn	CHe	corn
AA6	T	T	G/A	A/G	C/T	T	T	C/G	T	C/A	corn	CHe	corn
AA7	T	T	G(A)	A	C	T	T	C	T	C	corn	CHe	corn
AA9	T	T	G/A	A/G	C/T	T/C(A)	T	C	T	C	corn	CHe	corn
AA110	C	C	C	A	C/T	T	T	T	C	T	rice	IHe	corn
AA114	C	C	G/A	A	T	A(T)	C	T	C	T	rice	IHe	corn
AA19	C/T	C/T	C/A	A(T)	C(T)	T(C)	T(G)	T	C/T	T	rice	IHe	corn
AA23	T	T	A	T	C	T	T	C	T	C	rice	corn	corn
AA26	C/T	C/T	C/A	A/T	C/T	T	T(A)	T/C	C/T	T/C	rice	IHe	corn
AA28	T	T	A	T	C	T	T	C	T	C	rice	corn	corn
AA116	C	C	C	A	C/T	T	T	T	C	T	rice	IHe	corn
AA117	C(T)	C	G/A	A(T)	T(C)	A(T)	C	T	C	T	rice	RHe	corn
AA18	T	T	A	T	C	T	T	C	T	C	rice	corn	corn
AA30	C/T	C/T	A/C	A/T	C(T)	T	T	C/T	C/T	C/T	rice	IHe	corn
AA32	T/C	T	A	T	C(T)	T	T/G	C/G	T	C	rice	CHe	corn
AA44	C	C	C	A	C/T	T	T	T	C	T	rice	IHe	corn
AA47	C	C	C	A	C(T)	T(C)	T	T	C	T	rice	IHo	corn
AA20	C/T	C/T	C/A	A/T	C/T	T(A)	T(A)	T(C)	C(T)	T(C)	rice	IHe	corn
AA22	C/T	T/C	A/C	A/T	C(T)	T(C)	T	C/T	T/C	C/T	rice	IHe	corn
AA21	T/C	C/T	A(G)	T	C/T	T	T(G)	C	T	C	rice	IHe	corn

TABLE S3. Polymorphic nucleotide sites (SNPs) located in the *Tpi* gene region present in population C_10_W2.

Samples / SNP	74	95	173	174	184	185	253	352	355	377	COI	TPI	Host
III11	T	T	A	T	C	T	T	C	T	C	corn	corn	corn
III14	T	T	A	T	C	T	T/C	C	T	C	corn	CHe	corn
III16	T	T	A	T	C	T	T	C	T	C	corn	corn	corn
III18	T	T	A	T	C	T	T	C	T	C	corn	corn	corn
III25	T	T	A/G	A/T	C	T(A)	T	C	T	C	corn	CHe	corn
III3	T	T(G)	A	T(A)	C/T	T	T(A)	C	T	C	corn	CHe	corn
III4	T	T	A	T	C	T	T	C	T	C	corn	corn	corn
III6	T	T	A	T	C	T	T	C	T	C	corn	corn	corn
III7	T	T	A/G	A/T	C	T	T	C	T/G	C	corn	CHe	corn
III21	T	T	A	T	C(T)	T	T	C	T	C	corn	corn	corn
III10	T	T	A	T	C	T	T	C	T	C	rice	corn	corn
III17	T	T	A/G	A/T	C	T	T(G)	C	T(G)	C	rice	CHe	corn
III19	T	T	A	T	C	T	T	C	T	C	rice	corn	corn
III27	T	T	A/G	A/T	C	T	T	C	T	C	rice	CHe	corn
III9	T	T	A	T	C	T	T	C	T	C	rice	corn	corn

TABLE S4. Polymorphic nucleotide sites (SNPs) located in the *Tpi* gene region present in population R_08_W3.

Samples / SNP	74	95	173	174	184	185	253	352	355	377	COI	TPI	Host
T12	C	C	G	A	T	C	C	T	C	T	rice	rice	grasses
T15	C	C	G	A	T	C	C	T	C	T	rice	rice	grasses
T17	C	C	A/G	A	T	C/T	C(G)	T	C	T	rice	RHe	grasses
T18	C	C	G	A	T	C	C	T	C	T	rice	rice	grasses
T19	C	C	G	A	T	C	C	T	C	T	rice	rice	grasses
T21	C	C	G	A	T	C	C	T	C	T	rice	rice	grasses
T22	C(T)	C	G/A	A	T	C/T	C(G)	T	C	T	rice	RHe	grasses
T23	C(T)	C(G)	G(A)	A	T	C/T	C	T	C	T	rice	RHe	grasses
T25	C	C	G/A	A	T	C	C	T	C	T	rice	RHe	grasses
T4	C	C	G	A	T	C	C	T	C	T	rice	rice	grasses
T6	A	-	G/A	A(T)	C	T	T	T	C	T	rice	IHe	grasses
T7	C	C	G(A)	A	T	C(T)	C	T	C	T	rice	rice	grasses
T8	C	T	G/A	A(T)	C	T	T	T	C	T	rice	IHe	grasses
T9	C	C(G)	G/A	A	T	C/T	C	T	C(T)	T	rice	RHe	grasses

TABLE S5. Polymorphic nucleotide sites (SNPs) located in the *Tpi* gene region present in population R_08_E4.

Samples / SNP	74	95	173	174	184	185	253	352	355	377	COI	TPI	Host
MM10	C	C(A)	G(A)	A(G)	T	C(T)	C	T/C	C	T/C	rice	RHe	rice
MM112	C	C	G	A	T	A	C	C	T	T	rice	rice	rice
MM16	C/T	T	G/A	A(T)	T/C	C/T	T	C	C(T)	T(C)	rice	IHe	rice
MM17	C(T)	T	G(A)	A(T)	T(C)	C/T	T(A)	C	C(T)	T(C)	rice	IHe	rice
MM1	C	T	G/A	A(G)	T(C)	C(T)	T(G)	C	C	T(C)	rice	IHe	rice
MM4	T	T	A	T(A)	C/T	T	T	C	T	C	rice	CHe	rice
MM9	C	T(C,A)	G/A	A(G)	T(C)	C	T(C,G)	C/G	C	T	rice	IHe	rice
MM8	C	C	G	A	T	C	C	T	C	T	rice	rice	rice
MM95	C	T	G	A	T	C	T	C	C	-	rice	rice	rice
MM31	C(T)	T	G/A	A	T(C)	C/T	T(A)	C	T	C	rice	IHe	rice
MM40	C(T)	C(T)	G/A	A(T)	T	A(T)	C	C	T	T	rice	IHe	rice
MM46	C(T)	T	G/A	A(T)	T(C)	C/T	T(A)	C	C	T/C	rice	IHe	rice
MM38	C/T	T	G/A	A/T	T/C	C/T	T(G)	C	C(T)	T/C	rice	IHe	rice
MM3	C(T)	T(C,A)	A/G	A/T	T/C	C/T	T(C,G)	C	C	T(C)	rice	IHe	rice
MM20	C/T	T	A/G	A/T	T/C	T(C)	T/G	C	T/C	T/C	rice	IHe	rice
MM27	T	T	A	A	T	C	T	T	C	T	rice	IHo	rice
MM30	T	T	A	A	T	C	T	T	C	T	rice	IHo	rice
MM2	C/T	T	G/A	A/T	T/C	C/T	T	C	C/T	T/C	rice	IHe	rice
MM15	T/C	T/A	A/G	A	T	C	T/A	T	C	T	rice	RHe	rice
MM18	C(T)	T	G/A	A	T(C)	C/T	T(A)	C	C	T/C	rice	IHe	rice
MM22	T	T	A	A	T(C,G)	C	T	T	C	T	rice	rice	rice

TABLE S6. Polymorphic nucleotide sites (SNPs) located in the *Tpi* gene region present in population R_08_E5.

Samples / SNP	74	95	173	174	184	185	253	352	355	377	COI	TPI	Host
PA10	T/C	T/C	A(G)	T/A	C/T	C/T	T/C	C(G)	T	C/T	corn	IHe	rice
PA11	T/C	T/C	A(G)	T/A	C/T	T/C	T/C	C	T	C(T)	corn	IHe	rice
PA16	T	T	A	T	C	T	T	C	T	C	corn	corn	rice
PA115	C	C	G	A	T	C	C	T	C	T	corn	riCe	rice
PA127	C	C	G	A	T	C	C	T	C	T	corn	riCe	rice
PA136	T	T	A	T	C	T	T	C	T	C	corn	corn	rice
PA137	T(C)	T	G	A(G)	T	C	C	T	C	T	corn	riCe	rice
PA6	T/C	T/C	A(G)	T/A	C/T	C/T	T/C	C	T	C(T)	corn	IHe	rice
PA7	T/C	T/C	A	T/A	C/T	T/C	T(C)	C	T	C	corn	IHe	rice
PA9	C	C	G	A	C/T	T(C)	C	T	C	T	corn	RHe	rice
PA17	T/C	T/C	A	T/A	C/T	T/C	T(C)	C	T	C(T)	corn	IHe	rice
PA19	T/C	T/C	A(G)	T/A	C/T	T(C,A)	T(C)	C	T	C	corn	IHe	rice
PA12	C/T	T/C	A/G	T(A)	T/C	C/T	C/T	C(G)	T/C	C/T	corn	IHe	rice
PA143	T	T(C)	A/G	G/A	T	C/T	C(A,T)	T	C	T	corn	IHe	rice
PA14	C/T	C/T	G/A	A(T)	T(C)	C(T)	C(T)	G	C	T(C)	corn	IHe	rice
PA57	C/T	C/T	A/G	A/T	T/C	C/T	C/T	C/G	C	C/T	corn	IHe	rice
PA1	C/T	T/C	-	-	T(C)	C/T	T/C	T(C)	T	C	corn	IHe	rice
PA3	C/T	T/C/A	G(A)	A/G	T(C)	C/T	C (T,A)	T	C	T	corn	IHe	rice
PA4	T/C	T/C	A/G	A/T	C/T	C/T	T/C	C(G)	T(C)	C/T	corn	IHe	rice

TABLE S7. Polymorphic nucleotide sites (SNPs) located in the *Tpi* gene region present in population C_09_E6.

Samples / SNP	74	95	173	174	184	185	253	352	355	377	COI	TPI	Host
PM116	T	T(G)	A	T	C/T	T	T	C	T	C	corn	CHe:	corn
PM11	T	T	A/G	A/T	C	T	T	C	T	C	corn	CHe:	corn
PM12	T	T	A	T	C	T	T	C	T	C	corn	corn	corn
PM14	T	T	A	T	C(T)	T	T/C	C	T	C	corn	CHe:	corn
PM15	T	T	A	T	C	T	T	C	T	C	corn	corn	corn
PM16	T	T	A	T	C	T	T/C	C	T	C	corn	CHe:	corn
PM17	T	T	G/A	A/T	C(T)	T	T	C	T	C	corn	CHe:	corn
PM181	T	T	A(G)	T/A	C	T	T	C	T	C	corn	CHe:	corn
PM224	T	T	A	T	C	T	T	C	T	C	corn	corn	corn
PM20	T	T	A/G	A/T	C	T	T	C	T	C	corn	CHe:	corn
PM216	T	T	G/A	A/T	C	T	T	C(G)	T	C	corn	CHe:	corn
PM21	T	T	A/G	A/T	C/T	T	T	C	T	C	corn	CHe:	corn
PM25	T	T	A/G	A/T	C	T	C/T	C	T	C	corn	CHe:	corn
PM27	T	T	A	T	C	T	T	C	T	C	corn	corn	corn
PM29	T	T	G/A	A/T	C(T)	T	T	C	T	C	corn	CHe:	corn
PM6	T	T	G/A	A/T	C/T	T	T	C	T	C	corn	CHe:	corn
PM7	T	T	A/G	A/T	C(T)	T	C/T	C	T	C	corn	CHe:	corn
PM9	T	T	A	T	C(T)	T	T/C	C	T	C	corn	CHe:	corn
PM26	T	T	G/A	A(T)	C(T)	T	T	C	T	C	corn	CHe:	corn

TABLE S8. Polymorphic nucleotide sites (SNPs) located in the *Tpi* gene region present in population C_08_E7.

Samples / SNP	74	95	173	174	184	185	253	352	355	377	COI	TPI	Host
JJ103	T	T	A	T	C	T	T	C	T	C	corn	corn	corn
JJ10	T	T	A/G	T/A	C	T	T	C	T	C	corn	CHe:	corn
JJ12	T	T	A	T	C	T	T	C	T	C	corn	corn	corn
JJ13	T	T(C)	A/G	A/T	C/T	T(C)	T	C	T	C	corn	CHe:	corn
JJ14	T	T	G(A)	A	C	T	T	C	T	C	corn	CHe:	corn
JJ15	T	T	A	T	C	T	T	C	T	C	corn	corn	corn
JJ17	T	T	G/A	A(T)	C	T	T	C	T	C	corn	CHe:	corn
JJ20	T	T	A	T	C	T	T	C	T	C	corn	corn	corn
JJ1	T	T	A	T	C	T	T	C	T	C	corn	corn	corn
JJ22	T	T	A/G	A/T	C(T)	T	T	C	T	C	corn	CHe:	corn
JJ26	T	T	A/G	T/A	C	T	T	C	T	C	corn	CHe:	corn
JJ2	T	T	G(A)	A	C	T	T	C	T	C	corn	CHe:	corn
JJ33	T	T	A/G	T/A	C(T)	T	T	C	T	C	corn	CHe:	corn
JJ3	T	T	A/G	A/T	C	T	T	C	T	C	corn	CHe:	corn
JJ4	T	T	A/G	A/T	C	T	T	C	T	C	corn	CHe:	corn
JJ39	T	T	G/A	A(T)	T/C	T	T	C	T/G	C	corn	IHe	corn
JJ44	T	T	A/G	A/T	C	T	T	C	T	C	corn	CHe:	corn
JJ5	T	T	G(A)	A	C	T	T	C	T	C	corn	CHe:	corn
JJ7	T	T	A	T	C	T	T	C	T	C	corn	corn	corn
JJ8	T	T	A/G	A/T	C(T)	T	T	C	T	C	corn	CHe:	corn

TABLE S9. Polymorphic nucleotide sites (SNPs) located in the *Tpi* gene region present in population R_07_E8.

Samples / SNP	74	95	173	174	184	185	253	352	355	377	COI	TPI	Host
C11	T	T	A	T	C	T	T	C	T	C	corn	corn	rice
C12	T	T	A	T	C	T	T	C	T	C	corn	corn	rice
C13	T	T	A	T	C	T	T	C	T	C	corn	corn	rice
C15	T	T	A	T	C	T	T	C	T	C	corn	corn	rice
C17	T	T	A	T	C	T	T	C	T	C	corn	corn	rice
C20	T	T	A	T	C	T	T	C	T	C	corn	corn	rice
C1	T	T	A	T	C	T	T	C	T	C	corn	corn	rice
C21	T	T	A(T,G)	T(A)	C/T	T(C,G)	T	C	T	C	corn	CHe:	rice
C22	T	T	A	T	C	T	T	C	T	C	corn	corn	rice
C23	T	T	A	T	C	T	T	C	T	C	corn	corn	rice
C24	T	T	A	T	C	T	T	C	T	C	corn	corn	rice
C2	T	T	A	T	C	T	T	C	T	C	corn	corn	rice
C3	C	C	G	A	C	T	C	T	C	T	corn	rice	rice
C5	T	T	A	T	C	T	T	C	T	C	corn	corn	rice
C6	T	T	A	T	C	T	T	C	T	C	corn	corn	rice

TABLE S10. Polymorphic nucleotide sites (SNPs) located in the *Tpi* gene region present in population R_10_E9.

Samples / SNP	74	95	173	174	184	185	253	352	355	377	COI	TPI	Host
ZZ24	T	T	A	T	C	T	T	C	T	C	corn	corn	rice
ZZ25	T	T	G/A	A/T	C/T	T	T	C	T	C	corn	CHe:	rice
ZZ33	T(C)	T	G/A	T/G	C/T	T	T	C	T	C	corn	CHe:	rice
ZZ37	T/C	T	A/G	A(G,T)	C/T	T(C)	T	C	T	C	corn	IHe	rice
ZZ28	C/T	T(C)	A	A/T	C/T	T(C)	T	C	T	C	corn	CHe:	rice

TABLE S11. Polymorphic nucleotide sites (SNPs) located in the *Tpi* gene region present in population C_08_E10.

Samples / SNP	74	95	173	174	184	185	253	352	355	377	COI	TPI	Host
BM11	T	T	A	T(A)	C/T	T(A)	T	C	T	C	rice	CHe:	corn
BM13	T	T/C	A/T	G/T	C/T	T	G/T	C/G	G/T	C/G	rice	IHe	corn
BM14	T	T	A	G(A)	C/T	T	T	C	T	C	rice	CHe:	corn
BM17	T	T	A/G	A/T	T(A)	C(T)	G(A)	C	T	C	rice	IHe	corn
BM20	T	T	A	T(A)	C/T	T	T	C	T	C	rice	CHe:	corn
BM21	T	T	A/G	A/T	C	T	T	C	T	C	rice	CHe:	corn
BM2	C/T	C/T	A/G	G/T	T	C/T	G/C	C	G	C	rice	IHe	corn
BM3	T	T(G)	A/G	G/T	C/T	T	T	C	T	C	rice	CHe:	corn
BM4	T/C	T/C	A/G	A,G,T	C/T	T/A	G/T	C/G	T(G)	C	rice	IHe	corn
BM6	T	T(C)	A/G	A/T	C	T(A)	T(G)	C/G	T	C	rice	CHe:	corn
BM8	T	T	A	T	C	T	T	C	T	C	rice	corn	corn
BM9	T/C	T	A	T(G,A)	C/T	T	T	C	T	C	rice	CHe:	corn
BM32	T	T	A/G	A/T	C	T	T	C	T	C	rice	CHe:	corn
BM33	T	T	A/G	A(T)	C	T	T	C	T	C	rice	CHe:	corn
BM27	T	T	A/G	A(T)	C	T	T	C	T	C	rice	CHe:	corn
BM1	T	T	A	T	C	T	T	C	T	C	rice	corn	corn

The orange color denotes the nucleotide corresponding to the corn haplotype sequence; the blue color denotes the nucleotide corresponding to the rice haplotype sequence; the green color indicates the nucleotide for the intermediate haplotype. The purple color denotes a different nucleotide for the corn and rice strains sequences. The intermediate haplotype represents individuals where the SNPs from the two haplotypes (corn and rice) were in similar proportions or individuals where the same SNPs presented two alternative nucleotides. In addition, it shows information on mitochondrial haplotype (COI) and host plants from which the individuals were collected. CHe, corn heterozygote. RHe, rice heterozygote. IHe, intermediate heterozygote. IHo, intermediate homozygote.

TABLE S12. Matrix of genetic distance between populations of *Spodoptera frugiperda* defined according the sampling site, year of collection, and host plant estimated by pairwise Wright's indices F_{ST} (upper diagonal) and pairwise Nei's genetic distance (below diagonal).

Populations	C_08_W1	C_10_W2	R_08_W3	R_07_E8	C_08_E7	R_08_E4	R_10_E9	R_08_E5	C_09_E6	C_08_E10
C_08_W1	------	0.1408	0.5551	0.1971	0.135	0.1651	0.2036	0.1459	0.1256	0.1653
C_10_W2	0.0468	-------	0.5789	0.2185	0.0822	0.2139	0.1703	0.1756	0.0751	0.1137
R_08_W3	0.3303	0.325	------	0.6284	0.5861	0.5567	0.6152	0.5561	0.586	0.5707
R_07_E8	0.0691	0.0716	0.4022	-------	0.2036	0.2755	0.2653	0.2437	0.1836	0.2254
C_08_E7	0.0435	0.0225	0.3266	0.0638	-------	0.1984	0.1706	0.1556	0.0673	0.1287
R_08_E4	0.0559	0.0704	0.2861	0.097	0.0624	-------	0.2879	0.1897	0.2265	0.2467
R_10_E9	0.0699	0.0505	0.3605	0.0881	0.0495	0.1003	------	0.2395	0.1797	0.1995
R_08_E5	0.0522	0.0594	0.3171	0.0887	0.0499	0.0643	0.084	-------	0.1689	0.1741
C_09_E6	0.0406	0.0207	0.3333	0.0568	0.018	0.0755	0.0536	0.0561	-------	0.1202
C_08_E10	0.0568	0.0331	0.3114	0.0745	0.0373	0.0852	0.0615	0.0586	0.035	-------

TABLE S13. Matrix of genetic distance between populations of *Spodoptera frugiperda* defined by site and year of sampling and combination of nuclear and mitochondrial haplotype (COI/TPI) estimated by pairwise Wright's indices F_{ST} (upper diagonal) and pairwise Nei's genetic distance (below diagonal).

Population	C_W1 C/C	C_W1 C/CHe	C_W1 R/IHe	C_W1 R/C	R_W3 R/R	R_W3 R/RHe	C_W2 R/C	C_W2 C/CHe	C_W2 C/C	R_E8 C/C	C_E7 C/C	C_E7 C/CHe	R_E4 R/R	R_E4 R/IHe	R_E9 C/CHe	R_E5 C/IHe	R_E5 C/R	C_E6 C/CHe	C_E6 C/C	C_E10 R/CHe	C_E10 R/IHe
C_W1_C/C	-----	0.0000	0.4088	0.3720	0.6482	0.6396	0.2633	0.2445	0.2455	0.3582	0.2506	0.2717	0.3202	0.3850	0.3311	0.3031	0.3688	0.2502	0.2415	0.2676	0.2755
C_W1_C/CHe	0.0000	-----	0.3570	0.3240	0.6250	0.6196	0.1856	0.1666	0.1665	0.3189	0.1702	0.1986	0.2489	0.3076	0.2828	0.2535	0.3269	0.1765	0.1636	0.2047	0.2438
C_W1_R/IHe	0.1794	0.1430	-----	0.0005	0.6020	0.5993	0.2990	0.2532	0.2587	0.3335	0.2416	0.2681	0.2038	0.2633	0.3269	0.2663	0.3454	0.2779	0.2124	0.2915	0.3348
C_W1_R/C	0.1673	0.1344	0.0000	-----	0.5741	0.5724	0.2616	0.2253	0.2304	0.2800	0.2077	0.2320	0.1897	0.2576	0.3083	0.2329	0.3083	0.2469	0.1803	0.2638	0.2990
R_W3_R/R	0.4201	0.3769	0.3826	0.3750	-----	0.0000	0.5749	0.5437	0.5468	0.6450	0.5500	0.5724	0.4909	0.5536	0.6208	0.5572	0.5976	0.5815	0.5409	0.5495	0.5776
R_W3_R/RHe	0.4082	0.3728	0.3834	0.3777	0.0000	-----	0.5768	0.5414	0.5465	0.6444	0.5563	0.5766	0.4923	0.5565	0.6210	0.5568	0.6028	0.5802	0.5410	0.5487	0.5794
C_W2_R/C	0.0876	0.0555	0.1182	0.1065	0.3291	0.3376	-----	0.0404	0.0197	0.2468	0.0677	0.0816	0.1901	0.2444	0.2262	0.2169	0.2696	0.0972	0.0581	0.1248	0.1490
C_W2_C/CHe	0.0863	0.0528	0.1014	0.0945	0.3172	0.3185	0.0118	------	0.0000	0.2412	0.0896	0.0891	0.1435	0.1957	0.1739	0.1740	0.2532	0.1002	0.0682	0.1152	0.1626
C_W2_C/C	0.0838	0.0511	0.1010	0.0941	0.3080	0.3123	0.0054	0.0000	------	0.2282	0.0477	0.0610	0.1374	0.1967	0.1855	0.1758	0.2370	0.0602	0.0175	0.1083	0.1472
R_E8_C/C	0.1297	0.1089	0.1299	0.1092	0.4277	0.4337	0.0821	0.0869	0.0779	------	0.2164	0.2303	0.2729	0.3289	0.3150	0.2959	0.3767	0.2230	0.1576	0.2566	0.2708
C_E7_C/C	0.0833	0.0508	0.0886	0.0791	0.2994	0.3133	0.0193	0.0285	0.0140	0.0701	------	0.0000	0.1058	0.1681	0.1910	0.1472	0.2102	0.0489	0.0077	0.1069	0.1500
C_E7_C/CHe	0.0908	0.0600	0.0997	0.0893	0.3216	0.3336	0.0231	0.0276	0.0177	0.0741	0.0000	------	0.1385	0.2017	0.1941	0.1682	0.2386	0.0767	0.0279	0.1222	0.1511
R_E4_R/R	0.1313	0.0913	0.0771	0.0768	0.2542	0.2596	0.0696	0.0536	0.0492	0.1057	0.0350	0.0466	------	0.0146	0.2359	0.1517	0.2563	0.1717	0.0996	0.1790	0.2298
R_E4_R/IHe	0.1534	0.1076	0.0948	0.1008	0.2851	0.2938	0.0844	0.0685	0.0667	0.1212	0.0529	0.0648	0.0037	-----	0.3187	0.2002	0.3099	0.2476	0.1612	0.2457	0.2876
R_E9_C/CHe	0.1142	0.0909	0.1258	0.1262	0.3760	0.3829	0.0729	0.0565	0.0594	0.1086	0.0596	0.0592	0.0860	0.1153	-----	0.2733	0.3395	0.2150	0.1720	0.2295	0.2554
R_E5_C/IHe	0.1147	0.0893	0.1060	0.0962	0.3269	0.3313	0.0789	0.0649	0.0635	0.1135	0.0494	0.0565	0.0559	0.0688	0.1009	-----	0.1927	0.1973	0.1497	0.1798	0.2473
R_E5_C/R	0.1349	0.1121	0.1361	0.1250	0.3305	0.3449	0.0921	0.0921	0.0811	0.1438	0.0667	0.0769	0.0956	0.1094	0.1209	0.0621	------	0.2521	0.2186	0.2462	0.2883
C_E6_C/CHe	0.0804	0.0514	0.1045	0.0968	0.3341	0.3372	0.0279	0.0313	0.0173	0.0706	0.0135	0.0213	0.0603	0.0848	0.0672	0.0688	0.0826	------	0.0000	0.1232	0.1711
C_E6_C/C	0.0840	0.0511	0.0790	0.0700	0.3094	0.3142	0.0173	0.0225	0.0053	0.0496	0.0021	0.0079	0.0348	0.0532	0.0553	0.0535	0.0746	0.0000	------	0.1008	0.1346
C_E10_R/CHe	0.0901	0.0633	0.1144	0.1083	0.2934	0.2969	0.0378	0.0374	0.0338	0.0871	0.0322	0.0365	0.0648	0.0855	0.0747	0.0623	0.0815	0.0367	0.0318	------	0.0597
C_E10_R/IHe	0.0953	0.0811	0.1438	0.1322	0.3417	0.3503	0.0472	0.0572	0.0490	0.0954	0.0485	0.0476	0.0910	0.1086	0.0878	0.0962	0.1036	0.0551	0.0451	0.0169	------

TABLE S14. Analysis of genetic structure of nine *Spodoptera frugiperda* populations excluding the population from grasses and outlier-F_{ST} loci.

Population genetic structure				
N	Ht	Hw	Hb	F_{ST}
9	0.250	0.2041	0.0468	0.1870

TABLE S15. Hierarchical analysis of nine *Spodoptera frugiperda* populations among regions, among populations within regions, and among individuals within each population, excluding the population from grasses and outlier-FST loci.

	Variance	F	Conf. int. (95%)	P
Regions – Total	3.277	0.044	0.025-0.061	0.024
Population – Regions	21.859	0.307	0.284-0.330	<0.0005
Individuals – Population	49.299			

TABLE S16. Hierarchical analysis of nine *Spodoptera frugiperda* populations among host plants, among populations within host plant, and among individuals within each population,excluding the population from grasses and outlier-F_{ST} loci.

	Variance	F	Conf. int. (95%)	P
Hosts – Total	5.318	0.070	0.055-0.086	0.05
Population – Hosts	20.359	0.292	0.272-0.312	<0.0005
Individuals – Population	49.299			

SECTION 2

STRAIN-SPECIFIC DIFFERENTIATION OF SEXUAL COMMUNICATION

'The entire universe has been neatly divided into things to
(a) mate with, (b) eat, (c) run away from,
and (d) rocks.'
Terry Pratchett, *Equal Rites*

4

PHEROMONAL DIVERGENCE BETWEEN TWO STRAINS OF *SPODOPTERA FRUGIPERDA*

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Abstract

Spodoptera frugiperda consists of two genetically and behaviorally different strains, the corn- and the rice-strain, which seem to be in the process of sympatric speciation. We investigated the role of strain-specific sexual communication as a prezygotic mating barrier between both strains by analyzing strain-specific variation in female pheromone composition of laboratory and field strains, and also male attraction in wind tunnel and field experiments. Laboratory-reared and field-collected females from Florida exhibited strain-specific differences in their relative amount of (Z)-7-dodecenyl acetate (Z7-12:OAc) and (Z)-9-dodecenyl acetate (Z9-12:OAc). In wind tunnel assays, we did not find strain-specific attraction of males to females. However, in field experiments in Florida, we observed some differential attraction to synthetic pheromone blends. In a corn field, the corn-strain blend attracted more males of both strains than the rice-strain blend, but both blends were equally attractive in a grass field. Thus, habitat-specific volatiles seemed to influence male attraction to pheromones. In dose-response experiments, corn-strain males were more attracted to 2% Z7-12:OAc than other doses tested, whereas rice-strain males were attracted to a broader range of Z7-12:OAc (2–10%). The attraction of corn-strain males to the lowest dose of Z7-12:OAc corresponds to the production of this compound by females; corn-strain females produced significantly smaller amounts of Z7-12:OAc than rice-strain females. Although corn-strain individuals are more restricted in their production of and response to pheromones than rice-strain individuals, it seems that differences in sexual communication between corn- and rice-strain individuals are not strong enough to cause assortative mating.

INTRODUCTION

Many insect species produce sex pheromones that mediate sexual communication between males and females (Tamaki 1985; Löfstedt and Kozlov 1997). In Lepidoptera, females usually produce species-specific sex pheromones that exclusively attract conspecific males over long distances (Cardé and Baker 1984; Tamaki 1985; Cardé and Haynes 2004). To find a suitable mating partner, males need to respond to the specific chemical signal that is emitted by a conspecific female (Löfstedt 1993; Cardé and Haynes 2004). Thus, changes in the pheromone signal of a female may result in reproductive isolation, which in turn can lead to speciation (Roelofs and Cardé 1974; Phelan 1992; Baker 2002; Smadja and Butlin 2009). A model species to study the evolution of sexual communication is the European corn borer, *Ostrinia nubilalis* (Lepidoptera: Crambidae), which consists of two strains, the Z- and the E-strain, that differ in their female-produced pheromone production and male response to pheromones (Klun 1975; Smadja and Butlin 2009; Lassance et al. 2010; Lassance 2010; Wicker-Thomas 2011). While Z-strain females produce 97:3 (*Z*)/(*E*)-11-tetradecenyl acetate (Z/E11-14:OAc) (Klun et al. 1973), E-strain females emit 1:99 Z/E11-14: OAc (Kochansky et al. 1975). The production of different ratios Z/E11-14:OAc is based on a strain-specific allelic

variation in a fatty-acyl reductase gene (*pg*FAR), which causes different substrate specificities of the enzyme and, thus, different female pheromones (Lassance et al. 2010). Males of both strains are specifically attracted to females of their own strain, although E-strain males have a broader response to pheromones than Z-strain males (Lassance 2010). The two *O. nubilalis* strains seem to be sibling species, which mate assortatively, and exhibit low hybridization rates in the field due to strain-specific sexual communication (Lassance 2010).

Similar to the European corn borer, the fall armyworm, *Spodoptera frugiperda* (J.E. Smith) (Lepidoptera: Noctuidae), is an ideal model organism to study speciation because it also consists of two distinct strains, the corn- and the rice-strains (Pashley 1986). Corn-strain individuals mainly occur in habitats that contain large grasses like corn and sorghum, whereas the rice-strain inhabits areas consist of small grasses like rice, bermuda grass, or turf grass (Pashley 1986, 1989; Lu and Adang 1996; Levy et al. 2002; Nagoshi et al. 2006, 2007; Machado et al. 2008). However, in most fields, both kinds of strains can be found in different proportions, and habitats containing only one strain are rare (Pashley 1989; Meagher and Gallo-Meagher 2003; Nagoshi et al. 2006, 2007). Although both strains are morphologically indistinguishable from each other, they exhibit several genetic differences in the mitochondrial cytochrome oxidase I (COI) and NADH dehydrogenase (ND1) genes (Pashley 1989; Pashley and Ke 1992; Lu and Adang 1996; Levy et al. 2002; Meagher and Gallo-Meagher 2003; Prowell et al. 2004; Nagoshi et al. 2006; Machado et al. 2008), esterase allozyme loci (Pashley 1986), amplified fragment length polymorphisms (AFLP) loci (McMichael and Prowell 1999; Busato et al. 2004; Prowell et al. 2004; Clark et al. 2007; Martinelli et al. 2007; Juárez et al. 2012), the copy number and organization of the fall armyworm rice-strain sequence (FR) (Lu et al. 1994; Nagoshi and Meagher 2003), and in their triose phosphate isomerase (TPI) gene (Nagoshi 2010). Furthermore, both strains differ in their timing of mating in the scotophase; corn-strain individuals call, mate, and oviposit approximately 3 hr earlier than rice-strain individuals (Pashley et al. 1992; Schöfl et al. 2009).

The pheromone composition of *S. frugiperda* females has been studied several times at different geographic regions (Tumlinson et al. 1986; Descoins et al. 1988; Batista-Pereira et al. 2006; Groot et al. 2008; Lima and McNeil 2009). However, most studies have focused on the general composition of the female sex pheromone, irrespective of the female strain. The first pheromone component identified in *S. frugiperda* females was the major component, (*Z*)-9-tetradecenyl acetate (Z9-14:OAc) (Sekul and Sparks 1967). Analyses of female pheromone glands and volatiles have shown that females from Florida emit ratios of 4.9:3.1:1.7:3.5:86.9 of dodecyl acetate (12:OAc), (*Z*)-7- dodecenyl acetate (Z7-12:OAc), 11-dodecenyl acetate (11-12:OAc), (*Z*)-11-hexadecenyl acetate (Z11-16:OAc), and Z9-14:OAc (Tumlinson et al. 1986). In addition to the major pheromone component, Z9-

14:OAc, the critical secondary sex pheromone component, Z7-12:OAc, is important to attract *S. frugiperda* males in North and South America (Tumlinson et al. 1986; Andrade et al. 2000; Batista-Pereira et al. 2006). Because Z9-14:OAc and Z7-12:OAc are biologically active for male attraction they will be referred to as 'pheromone components', according to the definition of Tamaki (1985). The importance of Z11-16:OAc, Z9-12:OAc and other minor compounds in attraction of males is not yet understood (Jones and Sparks 1979; Tumlinson et al. 1986; Andrade et al. 2000; Fleischer et al. 2005; Batista-Pereira et al. 2006), so these will be referred to as 'pheromone compounds'.

Two independent studies have investigated strain-specific differences in the pheromone composition of females (Groot et al. 2008; Lima and McNeil 2009). Each study found that females of both strains produce strain-specific relative amounts of different pheromone compounds (Groot et al. 2008; Lima and McNeil 2009). However, the strain-specific pheromone variation differed between the two studies. We found that corn-strain females from Florida exhibited significantly higher relative amounts of Z11-16:OAc, and lower relative amounts of Z7-12:OAc and (*Z*)-9-dodecenyl acetate (Z9-12:OAc) than rice-strain females (Groot et al. 2008). In contrast, Lima and McNeil (2009) found that corn-strain females from Louisiana produced significantly larger relative amounts of Z9-14:OAc and lower relative amounts of Z7-12:OAc and Z11-16:OAc compared to rice-strain females. The differing results of these studies suggest that geographic variation might influence the strain-specific pheromone composition of *S. frugiperda* females.

Considering all genetic, as well as behavioral (e.g., host plant choice, timing of reproduction, female pheromone) strain-specific differences, it seems that the two strains of *S. frugiperda* are in the process of sympatric speciation (Groot et al. 2010). Sympatric speciation requires the evolution of reproductive isolation mechanisms to reduce recombination between groups of individuals, as well as the coexistence of newly formed groups within the same area (Coyne and Orr 2004). Strain-specific pheromone differences of corn- and rice-strain females could act as a reproductive isolation barrier if males show differential attraction to the different pheromone blends. The aim of our study was to examine the importance of sexual communication as a prezygotic mating barrier between the two strains of *S. frugiperda*. We determined a) whether lab- and field-collected corn- and rice-strain females differed in their pheromone composition, and b) the biological relevance of strain-specific female sex pheromone differences on male mate choice in wind tunnel assays and pheromone attraction experiments in the field.

MATERIALS AND METHODS

Spodoptera frugiperda *populations*

We conducted experiments with two different populations of each host-strain from Florida. The so-called laboratory populations, i.e. corn-strain (JSC3) and rice-strain

(OnaR), originated from 100 to 200 larvae collected by RLM in Florida. JSC3 individuals were collected from corn plants near Homestead, Miami-Dade County in 2004, and OnaR larvae were collected from pasture grasses at the Range Cattle Research and Education Center, Ona, Hardee County in 2003. Populations were reared on artificial pinto bean diet for 2–3 yr in a mass culture at USDA, Gainesville, Florida, after which specimens of both strains were sent to the MPICE in 2006 to establish a colony. All individuals were screened for strain-specific cytochrome oxidase subunit I (COI) markers to confirm strain-identity, and reared for another 3 yr on artificial pinto bean diet. Since these two populations have been reared up to 6 yr under laboratory conditions, they will be referred to as the laboratory populations in all experiments.

The so-called field population of both strains descended from around 300 larval specimens collected in 2010 in Florida. Corn-strain larvae (FLC) were collected from a corn field at the Everglades Research and Education Center, Belle Glade, Palm Beach County (+26°40'7.20", −80°37'57.63"), and rice-strain individuals (FLR) from a grass field at the Graham Farm in Moore Haven, Glades County (+26°53'3.04", −81°7'21.17"). All larvae were shipped to the MPICE, and reared until adulthood on artificial pinto bean diet. Adults were screened for strain-specific COI markers to establish strain-specific colonies. Experiments with these populations were conducted after the colony was established (2nd laboratory generation); these populations will be referred to as field populations in all experiments.

All insects were reared in climate chambers on a reversed light:dark (L:D) cycle, and a 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 avoid inbreeding and maintain both populations. Although we collected all insects from different locations in Florida, we do not assume genetic differences between populations because *S. frugiperda* is a highly migratory species (Sparks 1979), that overwinters in Florida (Luginbill 1928), suggesting high gene flow among populations. Furthermore, genetic analyses of corn-strain haplotypes in different habitats indicated a genetically homogenous corn-strain population in Florida (Nagoshi and Meagher 2008).

Pheromone extractions

To determine strain-specific differences in the pheromone composition, and consistency of these differences between laboratory and field populations, pheromone extractions of the field population were compared to the pheromone extractions done previously and reported in Groot et al. (2008). Pheromone extractions of the field populations were performed in summer 2010 with newly collected corn-strain (FLC, 2nd generation) and rice-strain (FLR, 2nd generation) field populations from Florida. Pheromone glands of 2–4 d-old corn-strain and rice-

strain virgin females were extracted during the scotophase according to strain-specific female calling times (corn-strain: 2-4 h, rice-strain: 5-7 h). Pheromone glands were excised from the female abdomen and placed singly into a glass vial containing 50 µl hexane and 125 ng pentadecane as internal standard. After extraction for 30 min, the gland was removed from the vial and the extract was stored at −20 °C until gas chromatographic analysis (see below).

Chemical analysis

Gas chromatography (GC) was performed using an HP7890 GC with a 7683 automatic injector, which injected 2–4 µl of each sample into a splitless inlet attached to a high resolution polar capillary column (DB-WAXetr (extended temperature range); 30 m × 0.25 mm × 0.5 µm), using a flame-ionization detector (FID) at 250 °C. The GC was programmed from 60 °C with a 2 min hold to 180 °C at 30 °C/min, 230 °C at 5 °C/min, and finally, to 245 °C at 20 °C/min with a 15 min hold. Pheromone extracts of females were reduced from 50 µl to 2 µl under a gentle stream of nitrogen. The reduced 2 µl extract and 2 µl octane were transferred into a 50 µl vial within a crimp capped glass vial and injected into the GC. An internal standard containing four pheromone compounds of *S. frugiperda* (Z9-14:OAc, Z11-16:OAc, Z7-12:OAc, Z9-12:OAc) was injected into the GC each day before the first samples were analyzed to confirm retention times.

Preparation of lures

Synthetic pheromone lures were prepared to test attraction of *S. frugiperda* males. The four pheromone compounds identified from *S. frugiperda* females (Z9-14:OAc, Z11-16:OAc, Z7-12:OAc, Z9-12:OAc) were purchased from Pherobank (Wageningen, the Netherlands) to prepare lures (Table 1). Each pheromone lure consisted of a red rubber septum (Thomas Scientific, Swedesboro, NJ, USA) that was loaded with 100 µl of hexane containing 300 µg of the major component Z9-14:OAc (100%) plus different amounts of minor compounds (0–18%) relative to 300 µg Z9-14:OAc (Table 1). Before use, rubber septa were soaked in hexane over night and air dried for 1 d. Pheromone solutions for the four different experiments were prepared according to Table 1.

To test the quality and quantity of the synthetic pheromone blends, 2 µl of each solution were analyzed by GC; the relative percentages of all lure compounds were confirmed by peak area integration, and lures were stored at −20 °C until use. Heath et al. (1986) showed that release rates of C12-C14 acetates (Z9-14:OAc, Z7-12:OAc, Z9-12:OAc) are similar to loading percentages of these compounds on rubber septa; however, the results of Tumlinson et al. (1990) suggest that the release rates of Z11-16:OAc from our lures might have been lower than the loaded percentages.

TABLE 1. Field experiments to test the attraction of *Spodoptera frugiperda* males.

Experiment	A) Strain-specific blends[1]	B) Z7-12:OAc dose-response[1]	C) Z11-16:OAc dose-response[2]	D) Z9-12:OAc dose-response[2]
Pheromone blends	Corn-strain blend: 2% Z7-12:OAc 13% Z11-16:OAc 1% Z9-12:OAc	0% Z7-12:OAc	0% Z11-16:OAc	0% Z9-12:OAc
	Rice-strain blend: 4% Z7-12:OAc 8% Z11-16:OAc 2% Z9-12:OAc	2% Z7-12:OAc	8% Z11-16:OAc	1% Z9-12:OAc
	Hexane	4% Z7-12:OAc 10% Z7-12:OAc	13% Z11-16:OAc 18% Z11-16:OAc	2% Z9-12:OAc 4% Z9-12:OAc
Field	Corn field, Belle Glade, FL Grass field, Moore Haven, FL		Corn field, Hague, FL	Peanut/grass field, Williston, FL

[1]All septa contained 300 µg Z9-14:OAc, which was set to 100%.
[2]All septa contained 300 µg (100%) Z9-14:OAc and 6 µg (2%) Z7-12:OAc
Other pheromone concentrations were as follows: 18% = 54 µg, 13% = 39 µg, 10% = 30 µg, 8% = 24 µg, 4% = 12 µg, 1% = 3 µg.

Wind tunnel experiments

To assess strain-specific attraction of *S. frugiperda* males in the wind tunnel, experiments were performed in November 2009 in the laboratory of Prof. Manfred Ayasse at the Institute of Experimental Ecology, University of Ulm, Germany. Strain-specific attraction of *S. frugiperda* males was tested in a wind tunnel (200×75×75 cm) at 23 °C, 30 cm/s airflow, and 23% RH. To adapt males to the low humidity, we placed all males, which were located in round plastic tubes covered with gauze, for about 1 h in the wind tunnel before the experiments started. Attraction of males was tested with choice experiments because in nature both kinds of strains can occur within one habitat and, thus, females might be located close to each other during calling. Choice experiments were conducted with the laboratory corn-strain (JS3C, 38th generation) and rice-strain (OnaR, 49th generation). Single 2-5-d-old, virgin males and females were placed in round plastic tubes (9.5 cm, 3.5 cm diam) that were closed with gauze at both ends. One plastic tube containing a male was mounted on a stand 30 cm high, and placed downwind in the middle of the wind tunnel. After the gauze was removed, each male was able to fly upwind, and given a choice between corn-strain and rice-strain females; three females of each strain were housed separately in round plastic tubes (9.5 cm, 3.5 cm diam) on stands above each other at 30 cm, 45 cm, and 60 cm height. We used 3 females to increase the chance that at least 1 of the females would call. The stands holding females of each strain were positioned upwind 26 cm apart.

We examined the response of males on 5 consecutive nights; 18 males per night (9 corn-strain males and 9 rice-strain males) were tested repeatedly, so that every male was tested up to five times per night. After testing males one night, they were excluded from the experiment, and another subset of 18 males was tested in the following night. All males that were completely inactive for 4 min were excluded from a trial. Each active male was allowed 5 min to start upwind flight before exclusion from a trial. Active males that started upwind flight were observed until they contacted the source (i.e., one of the female tubes), and displayed courtship behavior. Various male behaviors (e.g., activity status, presence/absence zigzag flight, source contact, courtship) were recorded for 2–9 h within scotophase.

Field experiments

To assess strain-specific attraction of *S. frugiperda* males in the field, four different male trapping experiments were performed using: (A) strain-specific blends, (B) a range of Z7-12:OAc dosages, (C) a range of Z11-16:OAc dosages, and (D) a range of Z9-12:OAc dosages (Table 1). Plastic green-yellow-white Unitraps (Pherobank, Wageningen) were baited with synthetic pheromone lures, and attached to a bamboo stick 1–2 m above the ground; traps were at least 15 m apart, as well as from field borders. All traps contained a Vaportape II insecticide strip (Hercon Environmental, Emigsville, PA, USA) to kill males after they were trapped. These males were stored at -20 °C for strain-identification at a later stage (see below). All experiments were conducted using a complete randomized block design with 3 biological replicates per field. Traps were rotated and emptied every 2–3 d. Field experiments were conducted at the following sites in Florida: 1) a corn field at the Everglades Research and Education Center in Belle Glade (+26°40’7.20”, −80°37’57.63”) (experiments A and B); 2) a grass field at the Graham Farm in Moore Haven (+26°53’3.04”, −81°7’21.17”) (experiments A, B and C); 3) a corn field in Hague (+29°47’7.40”, −82°25’3.66”) (experiments C and D); and 4) in a peanut/pasture field in Williston (+29°20’ 28.72”, −82°34’18.88”) (experiment D).

DNA extractions

To determine the strain identity of all trapped males, one third of the thorax of each trapped male was homogenized in 500 µl TES buffer (100 mM tris(hydroxymethyl)aminomethane hydrochloride pH 8, 10 M ethylene-diamine-tetraacetic acid, 2% sodiumdodecylsulfate), and 2.5 µl proteinase K and incubated at 55 °C overnight. Cetyltrimethyl-ammonium bromide (80 µl, 10% CTAB) and 170 µl 5 M sodium chloride were added to each sample followed by an incubation time of 10 min at 65 °C. After addition of 750 µl chloroform-isoamyl alcohol (24:1) and 30 min incubation on ice, the sample was centrifuged for 10 min at 10,000 rpm at 4 °C. Approximately 650 µl of the upper phase, together with 650 µl 100% isopropanol, were transferred into a new tube, and incubated on ice for 1 h. The

mixture was centrifuged for 45 min at 13,000 rpm at 4 °C, and the resulting DNA pellet was washed with 500 µl 70% ethanol, and centrifuged for 10 min at 13,000 rpm at 4 °C. The extracted DNA was dissolved in 50 µl TE buffer, and stored at 4 °C until PCR amplification. All chemicals and buffers used for DNA extractions were purchased from Carl Roth GmbH & Co. (Karlsruhe, Germany).

Strain identification

To determine the strain-identity of each individual, strain-specific polymorphisms at the mitochondrial cytochrome oxidase I (COI) gene, as described by Nagoshi et al. (2006), were used. PCR amplifications were conducted using 1 µl DNA, 11.92 µl dH_2O, 2 µl 10x Taq buffer, 3 µl 10 mM primer mix, 2 µl 2 mM dNTPs, and 0.08 µl Taq polymerase (Metabion, Martinsried, Germany). CO1-58 (5'-GGAATTTGAGC AGGAATAG-TAGG-3') was used as forward primer, and JM77 (5'-ATCACCTC CWCCTGCAGGATC-3') as reverse primer (Nagoshi et al. 2006). The thermo cycler was programmed for 2 min incubation time at 94 °C, followed by 35 cycles of 45 s at 94 °C, 45 s at 56 °C, 60 s at 72 °C, and a final elongation at 72 °C for 10 min. The generated amplification products were further digested for 2 h at 37 °C with MspI and SacI (New England Biolabs, Ipswich, MA, USA). For this digestion, 4 µl PCR product were mixed with 0.6 µl NEB buffer 4, 1 µl H_2O and 0.4 µl MspI (MspI digest) as well as 0.6 µl NEB buffer 1, 0.06 µl 100x BSA, 0.94 µl H_2O, and 0.4 µl SacI (SacI digest). Each digest was mixed with 3 µl loading dye, and 4.5 µl of this mix were loaded on a 1% agarose gel, and run at 110 V for 45 min. MspI digestion proved corn-strain identity, whereas SacI digestion detected rice-strain individuals.

Statistical and graphical analysis

Statistical analysis was performed with R 2.11.1 (R Development Core Team 2007). Female pheromone data were log transformed to stabilize the variance, and analyzed using a multivariate analysis of variance (MANOVA) and a generalized linear model (GLM). A graphical illustration of the female pheromone production was generated with SigmaPlot 8.0 (Figure 1). The attraction experiments in the wind tunnel were analyzed using a Pearson's Chi-square test and a GLM. The attraction experiments in the field were analyzed with a GLM using a Poisson distribution. If a treatment caught no moths, it was removed from the analysis. The quasi-Poisson distribution was used whenever the residual deviance of the data was larger than the residual degrees of freedom (over-dispersion). Graphical illustrations of the wind tunnel and field experiments were made with Microsoft Office Excel 2007.

RESULTS

Strain-specific variation in the pheromone blend

In this study, we compared the pheromone composition of a field population with previous data from our laboratory population (Groot et al. 2008). Corn- and rice-

strain females of the laboratory and field populations from Florida showed consistent strain-specific differences in their amount of Z7-12:OAc and Z9-12:OAc (Figure 1). Rice-strain females of both the laboratory, and field populations produced significantly higher relative amounts of Z7-12:OAc and Z9-12:OAc compared to corn-strain females of both populations (Figure 1). As for Z11-16:OAc, laboratory corn-strain females exhibited significantly higher relative amounts of Z11-16:OAc compared to laboratory rice-strain females (Figure 1). Such a difference was not found in the field populations (Figure 1). The relative amount of the major sex pheromone component Z9-14:OAc was not significantly different between corn- and rice-strain females in either population (P=0.918, Figure 1).

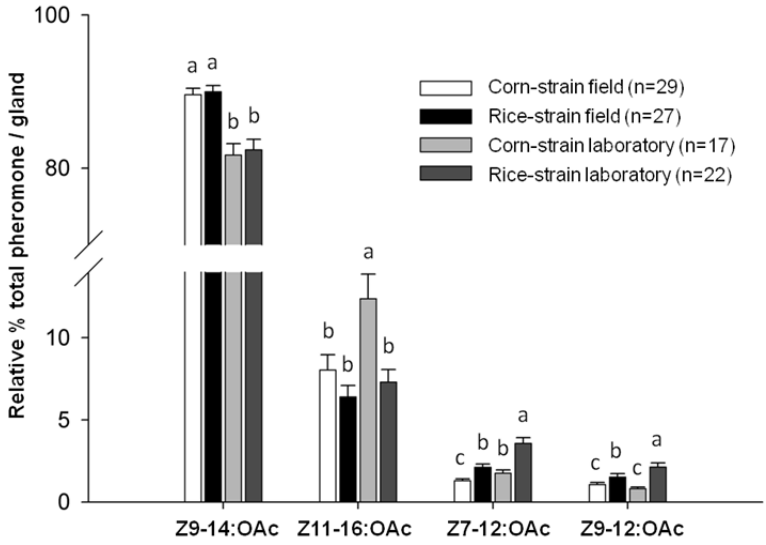

FIGURE 1. Pheromone composition of *Spodoptera frugiperda* corn-strain and rice-strain virgin females from laboratory and field populations originated from Florida. The sum of all components adds to 100%. Different letters above the bars indicate significant differences. Pheromone data of laboratory populations refer to Groot et al., 2008. n= sample size.

Population-specific variation in the pheromone blend

In addition to strain-specific pheromone differences, we also found differences between laboratory and field corn-strain females, as well as between laboratory and field rice-strain females for all four pheromone components (P<0.001 for Z9-14:OAc and Z7-12:OAc, P=0.009 for Z11-16:OAc, P=0.043 for Z9-12:OAc, Figure 1). Corn-strain females of the field population produced lower relative amounts of Z7-12:OAc than corn-strain females of the laboratory population (Figure 1).

Similarly, field rice-strain females produced lower relative amounts of Z7-12:OAc than laboratory rice-strain females (Figure 1). Corn-strain females of field and laboratory populations exhibited similar relative amounts of Z9-12:OAc, whereas rice-strain field females had significantly less Z9-12:OAc than laboratory rice-strain females (Figure 1). Field and laboratory rice-strain females produced similar relative amounts of Z11-16:OAc, whereas corn-strain laboratory females contained higher relative Z11-16:OAc amounts than corn-strain field females (Figure 1). The relative amount of the major component Z9-14:OAc was significantly lower in laboratory corn- and rice-strain females than in field corn-and rice-strain females (Figure 1).

Male attraction to females in the wind tunnel

Corn- as well as rice-strain males showed no strain-specific attraction in wind tunnel choice assays (Chi-square test: P=0.421, Figure 2a). No significant strain-, choice- or strain × choice-effect was observed (Figure 2a). Although males were flown multiple times per night, we could not detect any effect of flight experience on the male choice. Of all tested corn- and rice-strain males, 29–44% were inactive and did not respond to any of the six presented females upwind (Figure 2a). Most of the inactive males showed no response during the whole night (i.e., in all trials of one experiment), and generally males did not become inactive when tested multiple times per experiment. All active males that reached a source did fly within the odor plume, and did not reach a female just by chance. None of the tested males showed only zigzag flight behavior without afterwards contacting a tube containing females. Active males of both strains were attracted to strain-specific females (18–22%), to females of the other strain (16–27%), as well as females of both strains (22%, Figure 2a). We observed that most males responded to every female that was currently calling, irrespective of the female strain.

Around 80% of the active males of both strains were attracted to calling corn-strain females at the beginning of the scotophase, and to calling rice-strain females at the end of the scotophase (Figure 2b).

Male attraction to synthetic lures in the field

In the field, the strain-specific blends tested in experiment (A) revealed differential attraction to the corn- and rice-strain blend between habitats, but equal attraction to both blends within habitats (Figure 3). Males of both strains were significantly more attracted towards the synthetic corn-strain lure than to the synthetic rice-strain lure in the corn field (Figure 3a). However, both strains were equally attracted towards corn- and rice-strain lures within the grass field (Figure 3b).

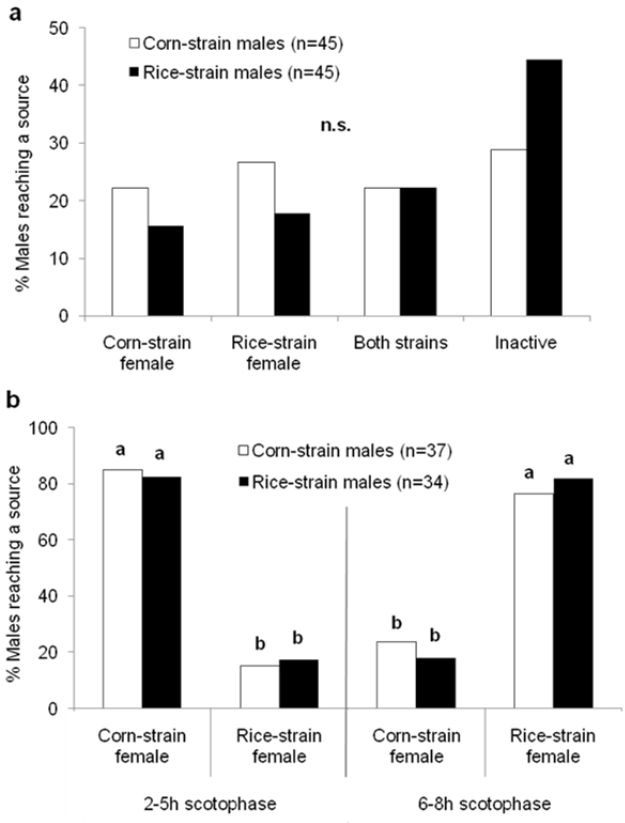

FIGURE 2. Attraction of *Spodoptera frugiperda* corn-strain and rice-strain males to calling females in a wind tunnel. **a** Data represent the sum of all males that showed source contact, and male calling within five experiments performed on five consecutive nights. n.s. not significant. **b** Data represent the sum of all active males that showed source contact and male calling within five experiments in the early scotophase (2–5 h), and four experiments at the end of the night (6–8 h). Different letters above the bars indicate significant differences.

Experiment (B), the dose–response experiment to Z7-12:OAc, evidenced strain-specific responses of corn- and rice-strain males within both kinds of habitats (Figure 4). Corn-strain males in corn and grass habitats were significantly more attracted to 2% Z7-12:OAc than to traps baited with 4 or 10% Z7-12:OAc (Figure 4). Rice-strain males were attracted equally to traps with lures containing 2 and 4% Z7-12:OAc and were even attracted to 10% of this component within both fields (Figure 4). Males of both strains were attracted only towards lures containing Z9-14:OAc when Z7-12:OAc was added (Figure 4).

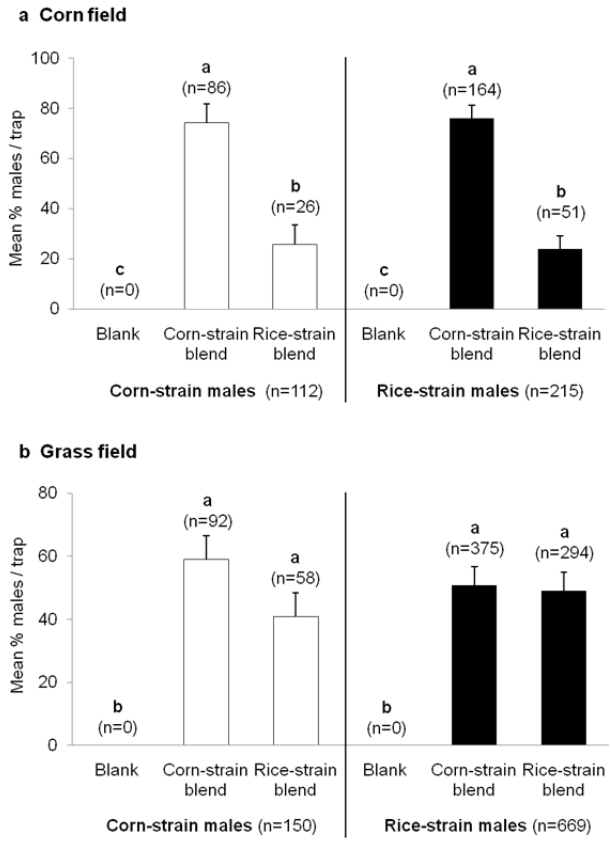

FIGURE 3. Attraction of *Spodoptera frugiperda* corn-strain and rice-strain males to strain-specific synthetic pheromone lures in a corn field (a) and in a grass field (b) in Florida. Different letters above the bars indicate significant differences. n = sample size.

The male response towards different doses of Z11-16:OAc (experiment C) was equal between both strains and both corn and grass habitats. Corn-strain males were similarly attracted to binary blends (100% Z9-14:OAc and 2% Z7-12:OAc; n_{corn} field=153, n_{grass} field=28) as to three-component blends containing 8% Z11-16:OAc (n_{corn} field=220, n_{grass} field=51), 13% Z11-16:OAc (n_{corn} field=188, n_{grass} field=40), or 18% Z11-16:OAc (n_{corn} field=162, n_{grass} field=30).

Although not statistically significant, corn-strain males seemed to be more attracted to the three-component blend containing 8% Z11-16:OAc in both fields. Similar to the response of corn-strain males, rice-strain males did not differentiate between binary blends (n_{corn} field=202, n_{grass} field=276) and three-component blends containing different doses of Z11-16:OAc (8%: n_{corn} field=194, n_{grass} field=331; 13%: n_{corn} field=198, n_{grass} field=360; or 18%: n_{corn} field=161, n_{grass} field=252).

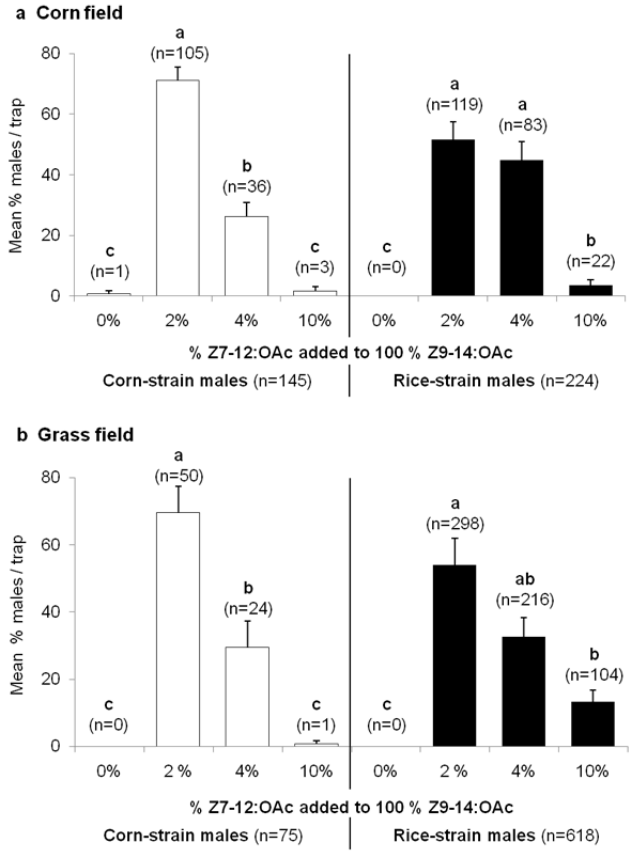

FIGURE 4. Attraction of *Spodoptera frugiperda* corn-strain and rice-strain males towards different doses (0%, 2%, 4%, 10%) of Z7-12:OAc added to 100% Z9-14:OAc in a corn field (**a**), and in a grass field (**b**), in Florida. Different letters above the bars indicate significant differences. n=sample size

In both corn and grass habitats, the addition of 1, 2, or 4% of Z9-12:OAc to the binary blend (experiment D) did not significantly increase trap catches compared to the binary blend. Corn-strain males responded similarly to binary blends (n_{corn} field=96, n_{grass} field=54) as to three-component blends containing either 1% Z9-12:OAc (n_{corn} field=139, n_{grass} field=60), 2% Z9-12:OAc (n_{corn} field=111, n_{grass} field=59) or 4% Z9-12:OAc (n_{corn} field=111, n_{grass} field=70). Like the corn-strain, rice-strain males were equally attracted to binary blends (n_{corn} field=84, n_{grass} field=207) as to three-component blends containing different doses of Z9-12:OAc (1%: n_{corn} field=116, n_{grass} field=196; 2%: n_{corn} field=73, n_{grass} field=187; or 4%: n_{corn} field=72, n_{grass} field=243). Within the corn field, males of both strains showed a slight, but not significant, increase in attraction when 1% Z9-12:OAc was added to the binary blend.

DISCUSSION

In this study, we assessed the importance of sex pheromone differences between the two strains of *S. frugiperda* for differential male attraction, in order to estimate the role of sexual communication as a prezygotic mating barrier between both strains. We found: a) consistent pheromone variation between corn- and rice-strain females; but also b) significant pheromone variation between laboratory and field populations within the strains; c) no differential attraction of males in wind tunnel experiments; and d) some differential attraction of males to synthetic lures in the field. Although experiments were conducted with insect colonies that have been reared many years under laboratory conditions, we do not assume that laboratory breeding influenced their reproductive behavior because the same colonies were used by Schöfl et al. (2009) who found similar strain-specific timing differences in the reproduction as found previously (Pashley and Ke 1992).

a) Consistent pheromone variation between strains

Our finding that rice-strain females collected from the field contain higher relative amounts of Z7-12:OAc and Z9-12:OAc than corn-strain females from the field confirms our previous results when we analyzed laboratory populations also originating from Florida (Groot et al. 2008). Since our field collections were from 2009, and the laboratory populations originated from field-collected larvae in 2003, this indicates that the strain-specific pheromone differences of *S. frugiperda* females are not an artifact that may have developed during laboratory rearing. Nevertheless, our findings contrast with those of Lima and McNeil (2009), who found that corn-strain females exhibited larger relative amounts of Z9-14:Ac, as well as lower relative amounts of Z7-12:OAc and Z11-16:OAc, compared to rice-strain females (Lima and McNeil 2009). Most likely, the different findings are due to the fact that females from different geographic regions were used. We extracted laboratory and field females originating from Florida, whereas Lima and McNeil (2009) used females from Louisiana.

The pheromone differences of females from Florida and Louisiana could be related to different corn-strain specific mitochondrial COI haplotype profiles existing in the Florida and Louisiana populations (Nagoshi et al. 2008). The different haplotype profiles reflect the migration of corn-strain individuals through North America in two migration routes: an Eastern route from Florida northwards to Georgia and along the Atlantic coast, and a Western route from Texas northeastwards to Louisiana, Mississippi, Alabama, and into the Ohio Valley to the northeast (Nagoshi and Meagher 2008; Nagoshi et al. 2008). If haplotype profile and migration differences influence female pheromone composition, then pheromones of females from regions of the Eastern migration route should be similar to each other but different from pheromones of females from the Western migration route and vice versa. To disentangle geographic from strain-specific

variation, further pheromone studies from different geographic regions will be necessary.

The critical secondary sex pheromone component, Z7-12:OAc, showed similar strain-specific variation between corn- and rice-strain females from Florida and Louisiana (Groot et al. 2008; Lima and McNeil 2009). In different geographic regions, *S. frugiperda* males are attracted to the binary blends containing Z7-12:OAc and Z9-14:OAc (Andrade et al. 2000; Fleischer et al. 2005; Batista-Pereira et al. 2006). This consistent attraction of males, together with the geographically independent strain-specific variation in Z7-12:OAc in females, indicates that the critical component Z7-12:OAc is under stabilizing selection. In contrast, variation in Z11-16:OAc and Z9-12:OAc, both in the female glands (Groot et al., 2008; Lima and McNeil, 2009) and in the male response (see part d), indicates that Z11-16:OAc and Z9-12:OAc are under much less stabilizing selection.

b) Pheromone variation within strains

The fact that we found population-specific pheromone differences between our laboratory and field females implies that either differences were present before individuals were bred in laboratory, or that differences developed in the course of laboratory rearing. We do not assume population-specific pheromone differences per se based on the same origin of both populations in Florida. Laboratory rearing may influence the pheromone composition of *S. frugiperda* females to some degree, although strain-specific pheromone variation is preserved. The higher probability of inbreeding, genetic drift, founder effects, and bottlenecks, as well as the loss of long-range mate search and inter-specific interactions, could result in a reduction of selection pressures acting on laboratory bred populations, which could cause an alteration of the pheromone composition of laboratory-bred females (Miller and Roelofs 1980; Haynes and Hunt 1990). Examples of such changes have been found in *Argyrotaenia velutinana* (Miller and Roelofs 1980), *Agrotis segetum* (Löfstedt et al. 1985), and *Trichoplusia ni* (Haynes and Hunt 1990). Long lasting laboratory pure-strain matings of *S. frugiperda* likewise could have changed the pheromone composition in the females in our case, resulting in an increase of all minor components in at least one of both laboratory strains compared to field females (see Figure 1). Based on a proposed pheromone biosynthesis pathway of *S. frugiperda*, a single-gene mutation in a fatty acyl reductase (FAR) would be sufficient to reduce the amount of the major pheromone component Z9-14:OAc, which would in turn lead to an increase in the amount of the minor compounds Z11-16:OAc, Z7-12:OAc, and Z9-12:OAc (Groot et al., 2008).

c) No differential attraction of males in wind tunnel experiments

Wind tunnel experiments showed that males of both strains were attracted mainly to corn-strain females at the beginning of the night and to rice-strain females at the end

of the night, which corresponds to the strain-specific female calling times of *S. frugiperda* (Pashley et al. 1992; Schöfl et al. 2009). Although we found no strain-specific male attraction to females of their own strain in wind tunnel choice assays, Lima and McNeil (2009) reported an experiment that showed that *S. frugiperda* males of both strains exhibited 'different responses to an array of concentrations and blends in the wind tunnel' (McNeil et al. unpublished data). These data imply that *S. frugiperda* males are able to show differential responses to pheromone blends in the wind tunnel and, thus, other factors might have influenced the male response in our experiments. It is known that lepidopteran males show differential attraction behavior in the wind tunnel depending on host plant volatiles (Landolt et al. 1994; Deng et al. 2004; Yang et al. 2004), their age (Rojas 1999), the pheromone dosage of the source and the ambient temperature (Charlton et al. 1993), chemical noise and wind turbulences (Liu and Haynes 1993), as well as the wind speed, flight altitude, and ground pattern (Foster and Howard 1999). We observed that *S. frugiperda* males were highly sensitive to slight changes in the wind tunnel parameters (e.g., temperature, wind speed), and stopped their response to any stimulus when environmental conditions were inadequate. Due to the construction of the wind tunnel, it was not possible to obtain higher percentages than 23% relative humidity in the wind tunnel, which might explain why more than one third of all males were inactive and did not respond to any of the presented females. Most likely, males may have shown differential attraction in the wind tunnel if we had optimal environmental conditions and shifted the calling times of the females, so that corn- and rice-strain females would have called simultaneously.

d) Some differential attraction of males in the field

In a corn field, we found that corn- and rice-strain males preferred the synthetic corn-strain blend over the synthetic rice-strain blend. Such a preference was not found when the same blends were tested in a grass field. The presence of response to strain-specific lures could be explained by synergistic effects of specific corn field volatiles. For many lepidopteran species, it has been shown that the presence of host plant volatiles can synergize the male orientation towards female sex pheromones (Landolt et al. 1994; Landolt and Phillips 1997; Ochieng et al. 2002; Deng et al. 2004; Reddy and Guerrero 2004; Yang et al. 2004). Although synergistic plant volatile effects have not been described for *S. frugiperda*, adult moths can perceive at least 16 different host plant volatiles, and males show greater EAG responses to plant odors than females (Malo et al. 2004). Thus, host plant volatiles may enhance the attraction of both strains towards the corn-strain blend in a corn field.

Plant semiochemicals and non-host green leaf volatiles also can have an inhibitory effect on insect behavior by repelling them from certain hosts, thus providing proper host-selection (Reddy and Guerrero 2004). In *Spodoptera*

littoralis, a closely related species, plant terpenes can antagonize the pheromone signal in a reversible way and are able to reduce the firing response of pheromone receptor neurons that respond to the major pheromone component (*Z*)-9-(*E*)-11-tetradecadienyl acetate (Party et al. 2009). If plant volatiles are able to modulate pheromone perception in *S. frugiperda*, grass volatiles could reduce the ability of males to quantify doses of pheromone and differentiate between blends, which could explain why both strains did not differentiate between the synthetic corn- and rice-strain blend in a grass field.

The fact that we found no strain-specific attraction to the four-component blends suggests either that males of both strains have a similar response range and are not differentiated in this respect, or that the blends that we tested were not strain-specific enough. Even though we and Lima and McNeil (2009) found that Z7-12:OAc is present in significantly lower amounts in corn-strain females than in rice-strain females, variation in the other two compounds Z9-12:OAc and Z11-16:OAc is not consistent between the strains and is variable within the strains. This may have confounded a possible strain-specificity of our so-called corn-strain and rice-strain pheromone blend. That such a confounding factor may have occurred seems to be confirmed by our dose-response experiment, varying the dose of the critical pheromone component Z7-12:OAc.

Corn-strain males were significantly more attracted when 2% of Z7-12:OAc was added to the major component compared to 4 and 10%, whereas rice-strain males showed a much wider response range, from 2 to 10% of Z7-12:OAc. These differences in response are in accordance with the strain-specific female pheromone production, as corn-strain females produce smaller relative amounts of Z7-12:OAc than rice-strain females. These results suggest that *S. frugiperda* males from Florida are adapted to the strain-specific Z7-12:OAc differences in the females. The fact that corn-strain males differentiated between 2 and 4% Z7-12:OAc in our dose–response experiments shows that males were able to detect minor differences of 2% between the tested synthetic lures. This in turn suggests that males similarly detected the differences between our strain-specific corn- and rice-strain blends that also differed in their amount of Z7-12:OAc by 2%. Furthermore, we found that no males of either strain were attracted when Z7-12:OAc was absent (0%), which confirmed previous findings of Tumlinson et al. (1986) that this secondary component is necessary for male attraction to the major pheromone component, Z9-14:OAc.

Similar to previous field experiments conducted in Florida and Brazil (Tumlinson et al. 1986) Batista-Pereira et al., 2006), our Z11-16:OAc dose–response experiments showed that the addition of Z11-16:OAc to binary blends containing Z9-14:OAc and Z7-12:OAc did not increase capture rates compared to binary blends. This male response is in accordance with the female pheromone production, because Floridian field females of both strains do not differ in their

relative amount of Z11-16:OAc. Nevertheless, the amount of Z11-16:OAc in female pheromone glands (Groot et al. 2008; Lima and McNeil 2009), as well as male attraction to this compound, differs between different geographic regions (Tumlinson et al. 1986; Andrade et al. 2000; Fleischer et al. 2005; Batista-Pereira et al. 2006). Field trapping experiments in Costa Rica showed that addition of Z11-16:OAc did marginally increase capture rates of binary blends (Andrade et al. 2000), and even doubled the attraction of males in Pennsylvania when Z11-16:OAc, together with Z9-12:OAc, was added to the binary blend (Fleischer et al. 2005). In contrast, EAG studies of laboratory *S. frugiperda* males from Mexico showed that males respond electrophysiologically to Z9-14:OAc, Z7-12:OAc, and Z9-12:OAc, but not to Z11-16:OAc (Malo et al. 2004).

We found no strain-specific attraction of males towards different doses of Z9-12:OAc, which is in contrast to the strain-specific Z9-12:OAc differences that we found between Floridian corn- and rice-strain females. However, males of both strains showed similar attraction in both fields to binary blends (Z9-14:OAc, Z7-12:OAc), but differential attraction between fields to four-component blends containing Z9-12:OAc. This differential attraction may be due to the addition of Z9-12:OAc. Furthermore, field experiments in Florida and Costa Rica showed that traps baited only with Z9-12:OAc were attractive for *S. frugiperda* males (Jones and Sparks 1979; Andrade et al. 2000), and addition of Z9-12:OAc and Z11-16:OAc to binary blends doubled the attraction of males in Pennsylvania (Fleischer et al., 2005). If the amount/presence of Z9-12:OAc and Z11-16:OAc is unimportant for male attraction, results of the test of strain-specific blends should be similar to results of our Z7-12:OAc dose–response experiment where we tested 2 and 4% Z7-12:OAc, because strain-specific blends differed in their amount of Z7-12:OAc (corn-strain blend: 2%, rice-strain blend: 4%), Z9-12:OAc and Z11-16:OAc. However, when testing the strain-specific blend we found differences between habitats, whereas the Z7-12:OAc dose response experiment showed similar results between habitats. Thus, we cannot exclude the biological relevance of Z9-12:OAc or Z11-16:OAc for male attraction and/or synergistic effects of these compounds in combination with other pheromone components or plant volatiles, which could influence male attraction in the field.

In summary, overall, we found some consistent strain-specific differences in the sexual communication system of *S. frugiperda*. Laboratory and field females showed strain-specific pheromone differences in their relative amount of Z7-12:OAc and Z9-12:OAc. Although males were not attracted to females of their own strain in wind tunnel assays, which was most likely due to differential calling times of the females, we observed some differential attraction of males in the field. In a corn field, both corn- and rice-strain males were more attracted to our synthetic corn-strain blend than our synthetic rice-strain blend, whereas these blends were similarly attractive in a grass field. Furthermore, males of both strains showed

strain-specific responses towards the critical component Z7-12:OAc. While corn-strain males were mainly attracted to 2% Z7-12:OAc, rice-strain males were attracted to 2% up to 10% of this component. Together, these data suggest that strain-specific differences in sexual communication alone are marginal and probably not sufficient to cause assortative attraction.

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5

GEOGRAPHIC VARIATION IN SEXUAL ATTRACTION OF *SPODOPTERA FRUGIPERDA* CORN- AND RICE-STRAIN MALES TO PHEROMONE LURES

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Abstract

The corn- and rice-strains of *Spodoptera frugiperda* exhibit several genetic and behavioral differences and appear to be undergoing ecological speciation in sympatry. Previous studies reported conflicting results when investigating male attraction to pheromone lures in different regions, but this could have been due to inter-strain and/or geographic differences. Therefore, we investigated whether corn- and rice-strain males differed in their response to different synthetic pheromone blends in different regions in North America, the Caribbean and South America. All trapped males were strain-typed by two strain-specific mitochondrial DNA markers. In the first experiment, we found a nearly similar response of corn- and rice-strain males to two different 4-component blends, resembling the corn- and rice-strain female blend we previously described from females in Florida. This response showed some geographic variation in fields in Canada, North Carolina, Florida, Puerto Rico, and South America (Peru, Argentina). In dose-response experiments with the critical secondary sex pheromone component (Z)-7-dodecenyl acetate (Z7-12:OAc), we found some strain-specific differences in male attraction. While the response to Z7-12:OAc varied geographically in the corn-strain, rice-strain males showed almost no variation. We also found that the minor compound (Z)-11-hexadecenyl acetate (Z11-16:OAc) did not increase attraction of both strains in Florida and of corn-strain males in Peru. In a fourth experiment, where we added the stereo-isomer of the critical sex pheromone component, (E)-7-dodecenyl acetate, to the major pheromone component (Z)-9-tetradecenyl acetate (Z9-14:OAc), we found that this compound was attractive to males in North Carolina, but not to males in Peru. Overall, our results suggest that both strains show rather geographic than strain-specific differences in their response to pheromone lures, and that regional sexual communication differences might cause geographic differentiation between populations.

INTRODUCTION

Geographic variation in the sexual communication signals of animals is a widespread phenomenon, being reported in frogs (Ryan et al. 1996; Bernal et al. 2005; Pröhl et al. 2006), birds (Slabbekoorn and Smith 2002; Tack et al. 2005; Podos and Warren 2007), fish (Gonzalez-Zuarth et al. 2011) and insects (Ackerman 1989; Miller et al. 1997; Zhu et al. 2009). This variation can be the result of isolation by distance, with a positive correlation between genetic dissimilarity and geographic distance (Balaban 1988; MacDougall-Shackleton and MacDougall-Shackleton 2001; Lampert et al. 2003), but this is not always the case (Seppä and Laurila 1999; Leblois et al. 2000; Kaefer et al. 2012). Furthermore, mating signals can be influenced by environmental factors such as temperature (Delisle and Royer 1994; Olvido et al. 2010; Roeser-Mueller et al. 2010; Green et al. 2012), humidity (Kumar and Saxena 1986; Royer and McNeil 1991, 1993), photoperiod length (Delisle and McNeil 1987; Gemeno and Haynes 2001), host plant volatiles (Landolt and Phillips 1997; Reddy and Guerrero 2004) or interspecific olfactory cues (Groot et al. 2010b) that vary geographically.

Geographic variation in sexual communication systems has been reported in several lepidopteran species (Toth et al. 1992; McElfresh and Millar 1999; Wu et al. 1999; Gemeno et al. 2000; Kawazu et al. 2005; Groot et al. 2009) and is of interest because changes in the sex pheromone signal and/or response to sex pheromones could result in reproductive isolation and subsequently may lead to speciation (Roelofs and Cardé 1974; Phelan 1992; Baker 2002; Smadja and Butlin 2009). Furthermore, geographically varying sexual communication is of interest for pest management, as many lepidopteran insects are pest species which are commonly monitored, disrupted or killed via pheromone-mediated methods (Cardé and Minks 1995; El-Sayed et al. 2009; Witzgall et al. 2010).

The fall armyworm, *Spodoptera frugiperda* (J.E. Smith) (Lepidoptera: Noctuidae), consists of two genetically and behaviorally distinct strains, the corn- and rice-strain, occurring sympatrically throughout North- and South America (Pashley 1986). Both strains appear to be undergoing ecological speciation in sympatry and reveal several possible prezygotic isolation barriers (Groot et al. 2010c). These include differential host plant choice (Pashley 1986, 1989; Lu and Adang 1996; Nagoshi et al. 2006a; Machado et al. 2008), strain-specific mating times in the scotophase (Pashley et al. 1992; Schöfl et al. 2009), as well as differences in the female sex pheromone composition (Groot et al. 2008; Lima and McNeil 2009; Unbehend et al. 2013). Among these prezygotic mating barriers, the strain-specific timing of reproduction seems to be the most important one that differentiates both strains, as host plant preference is not as clear cut as previously thought (Nagoshi et al. 2006a, 2007; Groot et al. 2010c; Juárez et al. 2012), and no strain-specific mating based on sex pheromone differences could be shown so far (Pashley et al. 1992; Meagher and Nagoshi 2013; Unbehend et al. 2013). Additionally, a postzygotic mating barrier, i.e. reduced fertility of RC (rice-strain ♀ × corn-strain ♂) hybrid females, contributes to the divergence of the two strains and separates them in nature (Groot et al. 2010c). Besides being an excellent model to study the evolution of reproductive isolation (Groot et al. 2010c), *S. frugiperda* is a serious pest species that feeds on a large variety of agricultural crops (Pashley 1988), and can cause annual damages of up to ~300 million dollars in the United States (Sparks 1986).

The sex pheromone of *S. frugiperda* was identified by Tumlinson et al. 1986) to consists of (Z)-9-tetradecenyl acetate (Z9-14:OAc) as the major sex pheromone component, and (Z)-7-dodecenyl acetate (Z7-12:OAc) as critical secondary sex pheromone component. A number of other minor compounds like (Z)-11-hexadecenyl acetate (Z11-16:OAc) and (Z)-9-dodecenyl acetate (Z9-12:OAc) have also been identified from the gland (Tumlinson et al. 1986; Groot et al. 2008; Lima and McNeil 2009), but with unclear behavioral function so far (Unbehend et al. 2013). Analysis of sex pheromone gland extracts from females collected in Florida showed that corn-strain females contained significantly lower relative amounts of

Z7-12:OAc and Z9-12:OAc than rice-strain females (Groot et al. 2008; Unbehend et al. 2013). However, different male trapping experiments conducted in Louisiana and Florida showed no consistent attraction of males to females of their own strain (Pashley et al. 1992; Meagher and Nagoshi 2013; Unbehend et al. 2013), which suggests that differences in the female pheromone are not sufficient to cause assortative mating in the field.

There is evidence that there are geographic differences in this species in the female sex pheromone blend (Tumlinson et al. 1986; Batista-Pereira et al. 2006; Groot et al. 2008; Lima and McNeil 2009), as well as in the male response (Jones and Sparks 1979; Mitchell et al. 1985; Tumlinson et al. 1986; Andrade et al. 2000; Fleischer et al. 2005; Batista-Pereira et al. 2006; Unbehend et al. 2013). For example, while females from Brazil (Batista-Pereira et al. 2006) produce (E)-7-dodecenyl acetate (E7-12:OAc), those from Florida, Louisiana or French Guyana do not (Descoins et al. 1988; Groot et al. 2008; Lima and McNeil 2009). In addition, studies on females originating from Florida and Louisiana provide evidence of geographic variation in the production of sex pheromone by females of both strains (Groot et al. 2008; Lima and McNeil 2009; Unbehend et al. 2013). Numerous studies have shown that the number of males caught varies with the pheromone blend used. For example, while the minor compound Z11-16:OAc did not affect male attraction in Florida and Brazil (Tumlinson et al. 1986; Batista-Pereira et al. 2006; Unbehend et al. 2013), it did marginally increase capture rates in Costa Rica (Andrade et al. 2000), and addition of Z11-16:OAc and Z9-12:OAc to binary blends (of Z9-14:OAc and Z7-12:OAc) almost doubled the attraction of males in Pennsylvania (Fleischer et al. 2005). However, most of these studies did not determine the strain identity of the males captured. Consequently, the variation in male attraction observed in these studies could either be due to strain-specific and/or due to geographic differences.

To disentangle strain-specific variation from geographic variation in male response, we investigated the response of corn- and rice-strain males to different synthetic pheromone blends in six different countries in North America, the Caribbean and South America. We tested (A) two synthetic 4-component blends (Blend 1 and 2) in different fields in Canada, North Carolina, Florida, Puerto Rico, Peru and Argentina; (B) different doses of Z7-12:OAc in Florida, Puerto Rico and Peru; (C) different doses of Z11-16:OAc in Florida and Peru; and (D) different doses of E7-12:OAc and Z7-12:OAc in Peru and North Carolina. We found that corn- and rice-strain males showed some variation in their response to different synthetic pheromone blends in different geographic regions. Overall, our results suggest that there is less strain-specific than geographic variation in male response and that regional sexual communication differences might cause geographic differentiation between populations.

MATERIALS AND METHODS

Ethics statement

The examined species is neither endangered nor protected. Trapping experiments in Canada, North Carolina and Peru were conducted at experimental research stations of the University of Western Ontario, the North Carolina State University, and the Universidad Nacional Agraria La Molina, with permission of the local university members/experimenters (Table 1). Trapping experiments in Florida were conducted at a) the Everglades Research and Education Center of the University of Florida in Belle Glade with permission of Gregg S. Nuessly, b) a private field in Hague with permission obtained by RLM, and c) private fields at the Graham Farm in Moore Haven with permission of the farm manager Tommy Toms (Table 1). Field experiments in Puerto Rico were conducted at field sites of the seed companies Monsanto and 3rd Millenium Genetics with permission of the company employees Wilson Rivera González and Jose Santiago, respectively. In Argentina, experiments were conducted in a private field with permission obtained by MLJ (Table 1).

Male trapping experiments

To test whether a certain synthetic pheromone blend is equally attractive for corn- and rice-strain males in different geographic regions, four different trapping experiments were conducted in six different regions in North America, the Caribbean and South America (Table 1). For experiment (A), we prepared two synthetic 4-component blends (Blend 1 and 2) based on strain-specific pheromone differences found in laboratory females from Florida by Groot et al. (2008). Both blends consisted of 100% Z9-14:OAc, but with different percentages of Z11-16:OAc, Z7-12:OAc and Z9-12:OAc (Table 2), as described by Unbehend et al. (Unbehend et al. 2013). Both blends were tested in Canada, the United States (North Carolina and Florida), Puerto Rico, Peru and Argentina (Table 1).

To evaluate the relative importance of Z7-12:OAc for male attraction in Florida, Puerto Rico and Peru (experiment B), different percentages of Z7-12:OAc (0, 2, 4, 10%) were added to the major sex pheromone component Z9-14:OAc alone (Table 2). The percentages used were chosen to examine whether Z7-12:OAc is necessary for male attraction in all regions and fields (0%, lures baited only with Z9-14:OAc), to test whether males can distinguish between 2 and 4% Z7-12:OAc, which is the difference that we found between corn- and rice-strain females (Groot et al. 2008), and to investigate a possible repellent effect of high dosages of Z7-12:OAc (10%).

To assess whether Z11-16:OAc would affect male attraction, we conducted experiment (C), in which different amounts of Z11-16:OAc (0, 8, 13, 18%) were added to a ‘minimal blend’, consisting of 100% Z9-14:OAc and 2% Z7-12:OAc (Table 2). The ‘minimal blend’ (0% Z11-16:OAc) was used as control, while 8 and 13% Z11-16:OAc reflect the percentages found in rice- and corn-strain females

from Florida, respectively (Groot et al. 2008). To test possible repellent effects, 18% was used as the highest concentration. Experiment C was conducted in Florida and Peru.

TABLE 1. *Spodoptera frugiperda* trapping experiments conducted in North America, the Caribbean and South America.

Country	Location & Coordinates		Field	Experiment[1]	Experimenter	Date
Canada	Ontario	+43°4'26.08", -81°20'21.81"	Corn	A	JMN	Sept. 2011
North Carolina	Plymouth	+35°50'46.19", -76°39'46.24"	Soybean	A, D	DR	Sept. 2011
		+35°51'01.93", -76°39'11.28"	Cotton; Grass			
		+35°51'48.80", -76°39'33.61"	Soybean; Corn			
Florida[2]	Belle Glade	+26°40'7.20", -80°37'57.63"	Corn A	A, B	MU, SH	April-May 2010
	Hague	+29°47'7.40", -82°25'3.66"	Corn B	C	RLM	Sept. 2011
	Moore Haven	+26°53'3.04", -81°7'21.17"	Grass	A, B, C	MU, SH	April-May 2010
Puerto Rico	Santa Isabel	+17°59'0.93", -66°23'29.88" +17°57'30.65", -66°23'32.43"	Corn A	A	MU,SH, ATG,DAJ	April 2010
			Corn B	A, B		
Peru	Lima	-12°4'51.56", -76°57'9.14"	Corn	A, B, C, D	GV	May-July 2011
Argentina	El Molino	-27°20'11.1", -65°41'25.8"	Corn	A	MLJ	Dec. 2010-Jan. 2011

[1]Experiments: A) Test of strain-specific blends, B) Z7-12:OAc dose-response, C) Z11-16:OAc dose-response, D) Importance of E7-12:OAc.
[2]Data adapted from Unbehend et al. 2013.

To test the importance of the isomers Z7-12:OAc and E7-12:OAc in North and South America (experiment D), we added different doses of E7-12:OAc and Z7-12:OAc (0, 1, 2%) to 100% Z9-14:OAc (Table 2). The 'minimal blend' (2% Z7-12:OAc + 100% Z9-14:OAc) was used as control, and as an equivalent we prepared an E-blend with 2% E7-12:OAc and 100% Z9-14:OAc. To investigate a possible interaction effect of both isomers together, 1% as well as 2% of E- and Z7-12:OAc were added to 100% Z9-14:OAc. The fourth experiment (Exp. D) was carried out in North Carolina and Peru.

We were not able to conduct all four experiments in all countries, due to technical limitations (i.e. limited time availability of collaborators, limited access to infested field sites and variability of moth population densities). All data from trapping experiments in Florida were published previously (Unbehend et al. 2013) and were included in this study for comparison. In all experiments, the synthetic pheromone lures were placed in plastic green-yellow-white Unitraps (Pherobank, Wageningen, the Netherlands), which contained a Vaportape II insecticide strip (Hercon Environmental, Emigsville, PA, USA) to kill the males captured. At each site, traps were hung just above the crop canopy (1-2 m above the ground depending on crop phenology), spaced 15 m apart and at least 15 m from the edge of the field using a complete randomized block design. There were three replicates per treatment per field (n=3), except for experiments conducted in North Carolina, where each replicate was conducted in a different field (Table 1). Traps were rotated and emptied three or four times, depending on the number of treatments (Exp. A: n=3; Exp. B-D: n=4), and traps were rotated every 1-6 days, depending on the population density in the field. The males captured were stored at -20 °C until strain-identification in the laboratory (see below).

Preparation of pheromone lures

All pheromone compounds used to prepare lures were bought from Pherobank (Wageningen, the Netherlands), and had a purity of $\geq$99%. Red rubber septa (Thomas Scientific, Swedesboro, NJ, USA) were soaked in hexane for 24 hours and air dried before they were loaded with 100 µl hexane containing 300 µg of the major pheromone component, Z9-14:OAc, plus different amounts (relative to 300 µg Z9-14:OAc) of the minor compounds Z11-16:OAc, Z7-12:OAc, Z9-12:OAc, and E7-12:OAc (Table 2). To avoid variable loading of our multi-component lures, all components of one specific lure were mixed together in hexane, formulated into one blend, and checked on the gas chromatograph before they were loaded onto a septum. All lures within one experiment were prepared using one master solution to avoid variation between replicates. The loaded relative amounts of each minor pheromone component reflected the relative amounts present in the female pheromone gland (Groot et al. 2008). To avoid variation in attraction due to differential amounts of the major component, the amount of Z9-14:OAc was set to

100% in all blends (Table 2), similar to studies conducted by Batista-Pereira et al. (2006), and Groot et al. (2007, 2010a). Therefore, the sum of all components in our multi-component blends was always larger than 100%, and the total amount of pheromone per lure ranged from 300 up to 360 µg (Table 2). All prepared lures were stored in glass vials at -20 °C until used 1-3 months later in the field. Each lure was only used once within one experiment (for ~1-3 weeks), and we did not observe a consistent decrease in lure-effectiveness at the end of an experiment (Figure S1). However, we cannot completely exclude a decrease in emission rates over time.

Chemical analysis

The purity and composition of the prepared pheromone solutions were verified by gas chromatography (GC) analysis, using a HP7890 gas chromatograph with a 7683 automatic injector. A 2 µl aliquot of each pheromone solution used for the preparation of the pheromone lures (Table 2) was injected into a splitless inlet attached to a polar capillary column (DB-WAXetr; 30 m × 0.25 mm × 0.5 µm) and a flame-ionization detector (FID). The GC program ran from 60 °C, with a 2 min hold, to 180 °C at 30 °C/min, 230 °C at 5 °C/min and to 245 °C at 20 °C/min, followed by a 15 min hold at 245 °C to clean the column for the next sample. The FID detector was held at 250 °C.

TABLE 2. Composition of pheromone lures to test the attraction of *Spodoptera frugiperda* males in the field.

Experiment and Lures[1]			Z9-14:OAc	Z11-16:OAc	Z7-12:OAc	E7-12:OAc	Z9-12:OAc
A	Blend 1		100%	13%	2%	-	1%
	Blend 2			8%	4%	-	2%
	Blank (Hexane)		-	-	-	-	-
B	0%	Z7-12:OAc	100%	-	-	-	-
	2%			-	2%	-	-
	4%			-	4%	-	-
	10%			-	10%	-	-
C	0%	Z11-16:OAc	100%	-	-	-	-
	8%			8%	-	-	-
	13%			13%	-	-	-
	18%			18%	-	-	-
D	2%	Z7-12:OAc	100%	-	2%	-	-
	2%	E7-12:OAc		-	-	2%	-
	1+1%	Z/E7-12:OAc		-	1%	1%	-
	2+2%	Z/E7-12:OAc		-	2%	2%	-

[1]Compound concentrations were as follows: 100% = 300 µg, 18% = 54 µg, 13% = 39 µg, 10% = 30 µg, 8% = 24 µg, 4% = 12 µg, 2% = 6µg, 1% = 3 µg.

Strain identification

The strain identity of all trapped males was determined via two strain-specific markers, i.e. MspI- and SacI-digest of the mitochondrial COI gene, which are known to be diagnostic for strain-identification of the two fall armyworm strains in North and South America (Meagher and Gallo-Meagher 2003; Nagoshi et al. 2006a, 2007). DNA of all males captured was extracted as described by Unbehend et al. (Unbehend et al. 2013) using CTAB (Cetyltrimethylammonium bromide) and isopropanol for DNA precipitation. The extracted DNA was tested at MPICE for strain-specific polymorphisms at the mitochondrial COI gene by amplification and strain-specific digestion (Nagoshi et al. 2006a; Unbehend et al. 2013). The amplified part of the COI gene was digested with MspI as well as SacI and analyzed electrophoretically on a 1% agarose gel (Unbehend et al. 2013). *Msp*I digestion detected corn-strain individuals, whereas *Sac*I digestion proved rice-strain identity (Nagoshi et al. 2006a).

Statistical analysis

Data of each field site of one experiment (Exp. A-D) were analyzed separately, using a generalized linear model (GLM) with a Poisson distribution, or a quasi-Poisson distribution if the residual deviance of the data was larger than the residual degrees of freedom (over-dispersion), using the R software 2.11.1 (R Development Core Team 2007). To assess whether there was any effect of geographic location, field crop, and/or any strain-specific effect that influenced male attraction, data of experiment A and B were additionally analyzed with a multivariate analysis of variance (MANOVA) and a Wilks Lambda test. Untransformed data were used for all GLM analyses, while data were square root transformed for the MANOVA. Treatments that did not catch any moths within any rotation in any of the three biological replicates per field were excluded from the statistical analysis (e.g. blanks in Experiment A). Whenever a certain blend attracted one or more males in at least one trap within one biological replicate, possible zero catches of the other biological replicates were included in the analysis. In all graphs, we averaged the number of males of all rotations of one treatment, calculated one percentage value for each of the 3 biological replicates, and plotted the mean percentage of males per trap (i.e. the average of the 3 biological replicates). In the statistical analysis, only raw data (no means or percentages) were used.

RESULTS

Overall, the field tests showed that *S. frugiperda* males of both strains exhibited some geographic variation in their attraction to two different synthetic 4-component-blends (Blends 1 and 2) in North America, the Caribbean and South America (Figure 1). We found a significant effect of geographic region, field crop, and an interaction effect between geographic region × strain ($P<0.001$, Table 3).

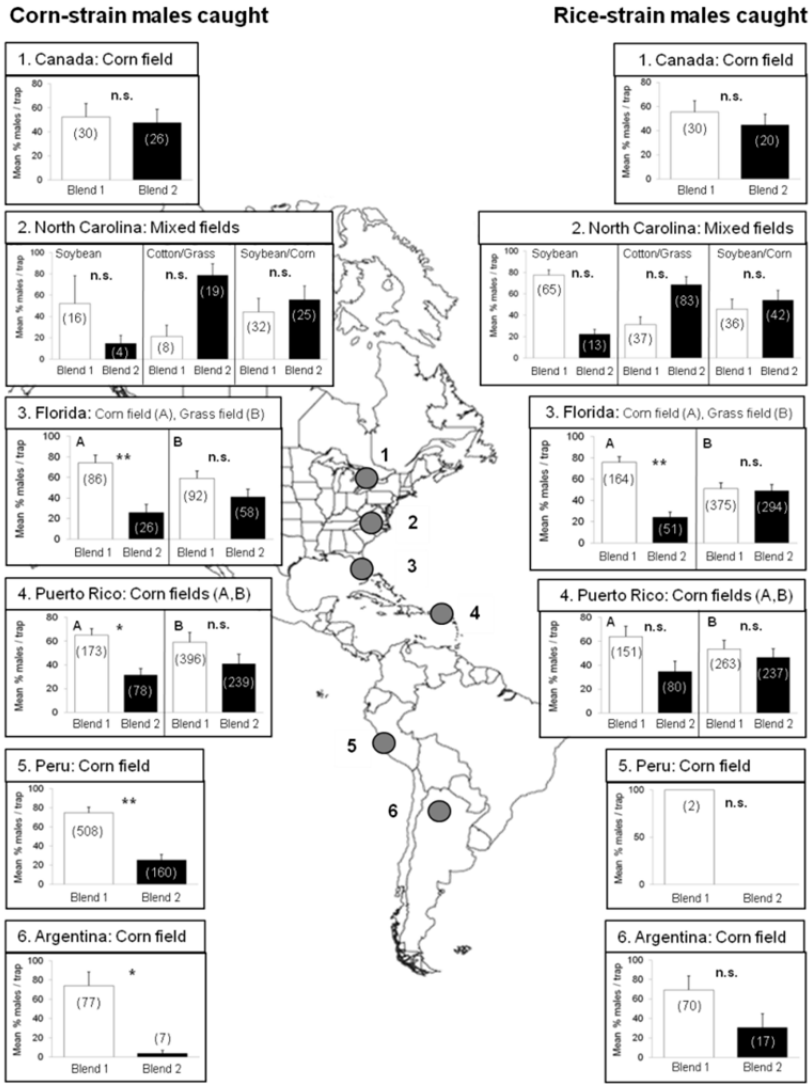

FIGURE 1. Attraction of *Spodoptera frugiperda* corn- and rice-strain males to two 4-component blends in different regions. Bars show the mean percentage of males caught per trap (Blend 1: 100% Z9-14:OAc + 13% Z11-16:OAc + 2% Z7-12:OAc + 1% Z9-12:OAc; Blend 2: 100% Z9-14:OAc + 8% Z11-16:OAc + 4% Z7-12:OAc + 2% Z9-12:OAc) and per biological replicate. There were three biological replicates per field (n=3), except for all fields in North Carolina (n=1) and for rice-strain males in Peru (n=1), where only one replicate caught males. The standard errors in all fields in North Carolina show the variation between rotations in one replicate (n=3), while all other error bars show the variation between biological replicates (n=3). Numbers in brackets/bars represent the total number of males caught, *= $P<0.05$, **= $P< 0.01$, n.s.=not significant. Data from Florida are adapted from Unbehend et al. (2013).

TABLE 3. Test statistics on the *Spodoptera frugiperda* male trap catches of different experiments.

	EXPERIMENT A			**EXPERIMENT B**		
	Wilks	F	P	Wilks	F	P
Country	0.448	12.152	**< 0.001**	0.196	26.711	**< 0.001**
Strain	0.975	1.551	0.216	0.697	9.245	**< 0.001**
Field	0.619	8.345	**< 0.001**	0.907	2.181	0.078
Country:Strain	0.471	11.245	**< 0.001**	0.425	11.333	**< 0.001**
Strain:Field	0.899	1.680	0.104	0.787	5.743	**< 0.001**

Experiments A (Test of two 4-component blends: Blend 1 and Blend 2) and B (Z7-12:OAc dose-response experiment) were analyzed individually using square root transformed data in a MANOVA and a Wilks' Lambda test. Bold P-values show a significant effect of geographic region, strain-identity of males, and/or the field crop, influencing the attraction of fall armyworm males to synthetic pheromone blends. Mean values and standard errors are shown in Figure 1 (Exp. A) and Figure 2 (Exp. B).

Corn-strain males showed a significantly higher attraction to Blend 1 than to Blend 2 in corn fields in Florida, Puerto Rico (field A), Peru and Argentina, but did not show a preference for any of the two blends in corn fields in Canada and Puerto Rico (field B), the mixed habitats in North Carolina or a grass field in Florida (Figure 1). Rice-strain males were equally attracted to Blend 1 and Blend 2 in all cases, with only one exception in a corn field in Florida, where Blend 1 was more attractive than Blend 2. Both strains only differentiated between the two blends when the experiment was conducted in a corn field, where males of both strains were more attracted to Blend 1 than to Blend 2 (Figure 1). Control traps baited with hexane were usually empty in all fields (data not shown), but did trap some males in Argentina (n=2) and in Puerto Rico (n=19 in field A, n=2 in field B). Interestingly, 18 out of the 19 males found in control traps in corn field A in Puerto Rico were caught during the first trap rotation at a time where male density was extremely high (over 50% of all males caught in this experiment were caught at the date of the first rotation, Figure S1).

In experiment B, the Z7-12:OAc dose-response experiment, we found a significant effect of geographic region and strain, as well as an interaction effect between geographic region × strain, and field crop × strain (P<0.001; Table 3). Interestingly, corn-strain males exhibited a greater differentiation in their response to Z7-12:OAc than rice-strain males (Figure 2), i.e. the highest number of corn-strain males was captured with lures containing 2% Z7-12:OAc, which was significantly different from all other ratios at three field sites in Florida and Puerto Rico. In Peru, lures with 2 and 4% Z7-12:OAc captured equal numbers of corn-strain males (Figure 2A). In Puerto Rico, lures containing no Z7-12:OAc, which is

the critical secondary sex pheromone component of this species, attracted 37 corn-strain males. In contrast to corn-strain males, rice-strain males showed a similar response to different concentrations of Z7-12:OAc in all regions tested, being equally attracted to blends containing 2 or 4% Z7-12:OAc, and showing some level of response to lures with 10% Z7-12:OAc (Figure 2B). No data of rice-strain males could be gathered in Peru, as only corn-strain males were found in this field (Figure 2A).

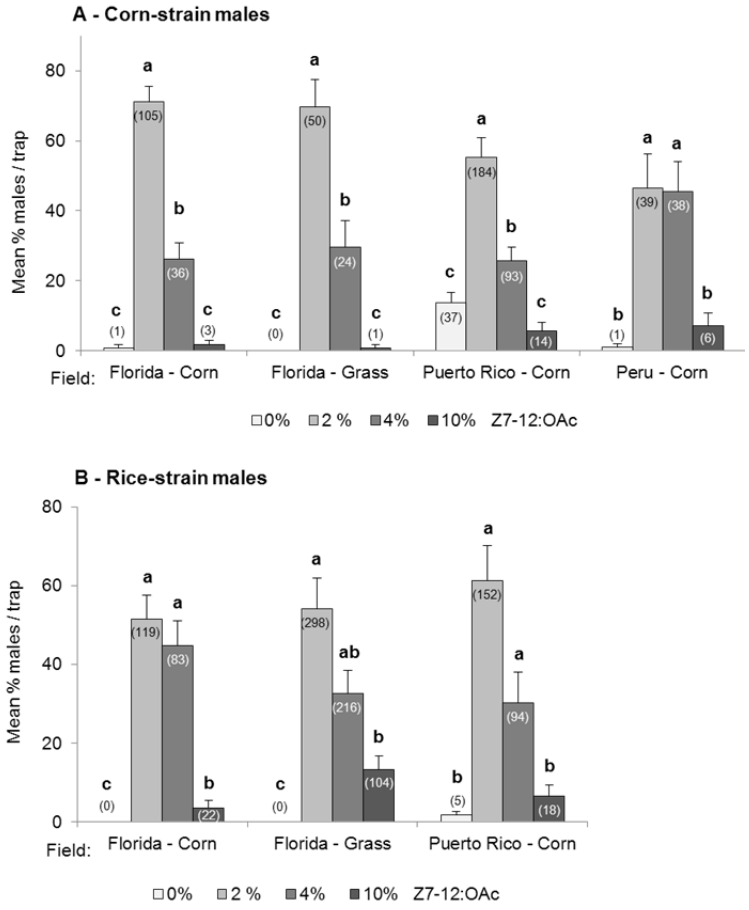

FIGURE 2. Attraction of corn-strain (A) and rice-strain (B) males to different doses of Z7-12:OAc. Bars represent the mean percentage of males caught per trap (0%, 2%, 4%, or 10% Z7-12:OAc + 100% Z9-14:OAc) and per biological replicate (n=3). Different letters above the bars indicate significant differences. Error bars show the variation between biological replicates (n=3). Numbers in brackets/bars represent the total number of males caught. Data from Florida are adapted from (Unbehend et al. 2013).

Testing different doses of Z11-16:OAc (experiment C) added to the minimal blend (i.e. 100% Z9-14:OAc and 2% Z7-12:OAc), revealed that corn-strain males were similarly attracted to binary blends as to three-component blends containing 8, 13 or 18% Z11-16:OAc (Figure 3A). This was true in Peru and Florida. Similarly, addition of Z11-16:OAc did not influence the attraction of rice-strain males in Florida (Figure 3B). In Peru, no rice-strain males were caught in the corn field.

In experiment D, when testing different doses (0, 1, 2%) of E7-12:OAc and Z7-12:OAc added to 100% Z9-14:OAc, we found that corn-strain males from Peru were not attracted to traps baited only with 2% E7-12:OAc added to Z9-14:OAc, but were equally attracted to all other blends tested (Figure 4). In North Carolina, *S. frugiperda* males were equally attracted to synthetic blends to which E7-12:OAc, Z7-12:OAc or E- and Z-7-12:OAc was added (Figure 4). Unfortunately, we were not able to identify the strain-type of any of the trapped males in North Carolina because of DNA degradation of the samples.

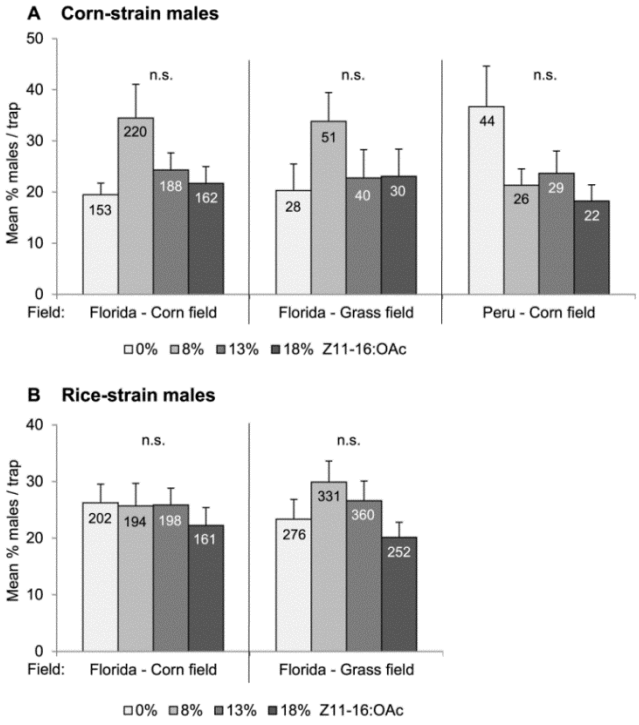

FIGURE 3. Attraction of corn-strain (A) and rice-strain (B) males to different doses of Z11-16:OAc. Bars represent the mean percentage of males caught per trap (0%, 8%, 13%, or 18% Z11-16:OAc + 100% Z9-14:OAc + 2% Z7-12:OAc) and per biological replicate (n=3). Error bars show the variation between biological replicates (n=3). Numbers in the bars represent the total number of males caught. n.s.=not significant. Data from Florida are adapted from (Unbehend et al. 2013).

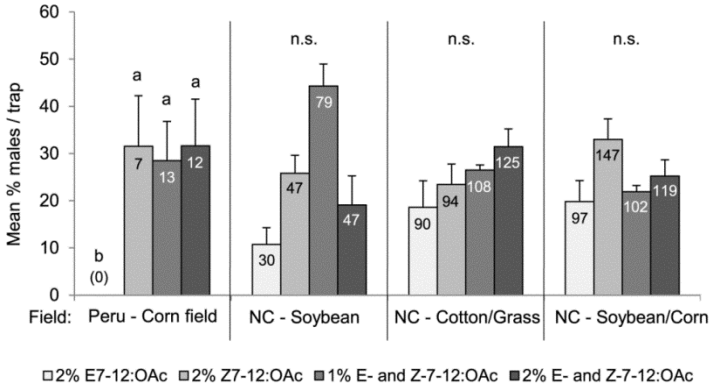

FIGURE 4. Attraction of *Spodoptera frugiperda* males to different doses of E7-12:OAc and Z7-12:OAc in different fields. Bars show the mean percentage of males caught per trap (100% Z9-14:OAc + a: 2% E7-12:OAc, b: 2% Z7-12:OAc, c: 1% E- and Z-7-12:OAc, d: 2% E- and Z-7-12:OAc) and per biological replicate. The standard errors in all fields in North Carolina (NC) show the variation between rotations in one replicate (n=3), while error bars in Peru show the variation between biological replicates (n=3). Males from Peru are corn-strain males, while males from NC could not be strain-typed but probably belonged to both strains. Numbers in brackets/bars represent the total number of males caught. Different letters above the bars indicate significant differences. n.s.=not significant.

DISCUSSION

We investigated the variation in attraction of *S. frugiperda* corn- and rice-strain males in North America, the Caribbean and South America, and found a) geographic variation in corn-strain male attraction to synthetic 4-component blends and to different doses of Z7-12:OAc, b) some geographic variation in rice-strain male attraction to different synthetic blends, c) no variation in male attraction to the minor compound Z11-16:OAc, and d) some evidence of geographic variation in response to E7-12:OAc, the compound identified in females from Brazil. Taken together our results indicate that both strains exhibit rather geographic than strain-specific variation in their response to synthetic pheromone lures.

We realize that our field data should be interpreted with some degree of caution for several reasons. Firstly, the composition of the lures of the first experiment (Blend 1 vs. Blend 2) was based on the relative amount of compounds observed in pheromone gland extracts (Groot et al. 2008), and not from airborne collections, which are somewhat different in composition (Tumlinson et al. 1986). We decided to load rubber septa with compound ratios and concentrations that occur in the female gland, assuming a similar emission of volatiles from the pheromone septum as from the female gland surface. Secondly, abiotic conditions may affect the rates at which the pheromone components are released from the lures, as well as the responses of males to a pheromone source (McNeil 1991). As the experiments were

carried out over a large geographic range, it is inevitable that there were intra- and inter-site variability in climatic conditions during the trapping periods. Thirdly, due to the fact that we used mitochondrial markers for strain diagnosis, which are maternally inherited, we were not able to differentiate between homozygote and heterozygote corn- and rice-strain males in our experiments. Therefore, it is possible that also hybrid males were attracted to our lures. Hybridization in the field has been found to be around 16% (Prowell et al. 2004), and several studies have found a bias of inter-strain matings between rice-strain females and corn-strain males, while corn-strain females hybridize less frequently (Nagoshi and Meagher 2003; Prowell et al. 2004; Nagoshi et al. 2006b; Nagoshi 2010). Consequently, the majority of hybrids occurring in nature have a rice-strain mitochondrial COI gene. If our lures attracted hybrid males, they were thus probably typed as rice-strain males. However, despite these limitations we believe our findings support the idea of geographic variability in the sexual communication system of the fall armyworm.

Geographic variation in corn-strain male responses

Testing two different 4-component blends revealed that corn-strain males were equally attracted to both blends in Canada and North Carolina, but preferred Blend 1 over Blend 2 in South America, i.e. in Argentina and Peru. Interestingly, Blend 1 mimics the pheromone composition previously reported for corn-strain females in Florida (Groot et al. 2008). However, corn-strain males in Florida and Puerto Rico showed a preference for Blend 1 in one of the two fields tested at each site, but were equally attracted to Blend 1 and 2 in the other field (Figure 1). This differential male attraction between fields could be caused by habitat-specific volatile differences. For example, the two corn fields in Puerto Rico, which were only 4 km apart, were planted with different corn varieties, were in different phenological states during the trapping period, and were treated with different insecticides. This could result in different background odor profiles, which in turn may have influenced the attraction of corn-strain males to the two different 4-component blends used in the first experiment. Previously, it was shown that corn-strain males varied in their attraction to sex pheromone blends in different fields with different host plants in north and south Florida (Meagher and Nagoshi 2013; Unbehend et al. 2013). Furthermore, both strains have some host plant preferences (Pashley 1986; Nagoshi et al. 2006a; Machado et al. 2008), and *S. frugiperda* males show EAG responses to at least 16 different plant volatiles (Malo et al. 2004). Thus, it seems likely that corn-strain males exhibit different responses to female sex pheromones in different habitats, emitting host or non-host volatiles.

The differential attraction of corn-strain males could also be explained by genetic differences between *S. frugiperda* populations from North America, the Caribbean and South America. Population genetic analyses of *S. frugiperda* samples collected throughout the Western Hemisphere generally found no isolation by

distance between populations from different regions (Clark et al. 2007; Martinelli et al. 2007; Belay et al. 2012), which indicates no geographically restricted gene flow probably due to the high migratory ability of *S. frugiperda* (Luginbill 1928; Sparks 1979). However, these analyses did not take into account strain-specific differences and in several cases the strain-type of captured individuals was unknown (Clark et al. 2007; Belay et al. 2012). Genetic studies on populations from Arkansas and Florida showed significant genetic variation among populations, both within and between the two strains (Lewter et al. 2006). Furthermore, corn-strain individuals exhibit different mitochondrial haplotype profiles between populations from a) Florida, Puerto Rico, Georgia and b) Texas, Brazil, Mississippi, Alabama, Louisiana (Nagoshi et al. 2007, 2008a,b, 2010). Thus, genetic differences could play a role in differential attraction of corn-strain males to synthetic blends in different regions.

Besides different host plant volatiles or genetic differences, the responses of corn-strain males could have been influenced by geographically varying environmental factors like temperature or humidity, which are known to influence sexual communication in insects (McNeil 1991). In a previous wind tunnel study, we observed that males of both strains were highly sensitive to changes in temperature or humidity and stopped their response to calling females whenever humidity or temperature was low (Unbehend et al. 2013). Thus, it is possible that males may respond differently to pheromone blends in regions with dry and cold climate compared to (sub) tropical climate zones.

In addition to variation in response to two synthetic 4-component blends, corn-strain males also exhibited significant geographic differences in their attraction to different doses of Z7-12:OAc. In Florida and Puerto Rico, corn-strain males were more attracted to the 2% dose than to other doses tested, but were equally attracted to 2 and 4% Z7-12:OAc in Peru (Figure 2). Furthermore, some corn-strain males from Puerto Rico were attracted to Z9-14:OAc alone, even though Z7-12:OAc has been considered an essential secondary sex pheromone component, without which males are not attracted (Tumlinson et al. 1986; Unbehend et al. 2013). The fact that the response of corn-strain males to Z7-12:OAc varied between regions suggests that females may also vary in their relative amount of Z7-12:OAc across different regions. Although previous data indicated that the production of Z7-12:OAc is under strong stabilizing selection (Unbehend et al. 2013), in light of the data presented here, selection pressures may be different in different regions.

Geographic variation in rice-strain male responses

In general, rice-strain males were equally attracted to Blend 1 and Blend 2 in different fields in North America, the Caribbean and South America (Figure 1). Although the response of rice-strain males was significantly different to the one of corn-strain males in a corn field in Puerto Rico and Argentina, the attraction of both

strains looks relatively similar in the first experiment. The results of our statistical analysis should be handled with care, as factors like time-dependent density changes within a population, differences between replicates, as well as seasonal/regional differences caused a considerable variation in trap catches (Figure S1). Overall, the results of our first experiment show that both strains respond similarly to the two different 4-components blends that we tested, but exhibit some geographic variation in their response.

Interestingly, rice-strain males from Florida and Puerto Rico showed a broader response spectrum to different doses of Z7-12:OAc compared to corn-strain males (Figure 2). This small but significant strain-specific difference is likely to be important, because Z7-12:OAc is the critical secondary sex pheromone component which is usually crucial for male attraction (Tumlinson et al. 1986; Unbehend et al. 2013). However, it is possible that the broader response spectrum of rice-strain males is due to attraction of hybrid males, if hybrids prefer more extreme blend ratios than the parental pure strains. This should be tested further, although we do not assume that the response of rice-strain males is masked by the presence of hybrid males, because hybridization frequency in nature is relatively low [70]. In conclusion, the results of our experiments suggest that both strains exhibit rather geographic than strain-specific differences in their response, although the response of rice-strain males seems to be broader than that of corn-strain males.

Male attraction to the minor compound Z11-16:OAc

Testing the importance of Z11-16:OAc for male attraction showed that corn-strain males from Peru were equally attracted to blends with and without different doses of Z11-16:OAc, similar to the response of corn- and rice-strain males in Florida (Unbehend et al. 2013). In general, addition of Z11-16:OAc to Z9-14:OAc and Z7-12:OAc did also not decrease male attraction in Florida, Peru, Costa Rica and Pennsylvania (Tumlinson et al. 1986; Andrade et al. 2000; Fleischer et al. 2005; Unbehend et al. 2013). These data suggest that Z11-16:OAc is not an essential component for *S. frugiperda* male attraction, which is supported by the observation that *S. frugiperda* males from Mexico did not respond electrophysiologically to Z11-16:OAc (Malo et al. 2004).

Geographic variation in male attraction to E7-12:OAc

So far, the E-isomer of the critical secondary sex pheromone component Z7-12:OAc has only been found in *S. frugiperda* females from Brazil, and males from this region responded electrophysiologically to E7-12:OAc and exhibited a higher attraction to binary blends (Z9-14:OAc and Z7-12:OAc) when E7-12:OAc was added (Batista-Pereira et al. 2006). In our trapping experiments, we found that corn-strain males from Peru were not attracted to traps baited only with E7-12:OAc and Z9-14:OAc, but were similarly attracted to all other blends that contained Z7-

12:OAc (Figure 4). Thus, corn-strain males from Peru appear to distinguish between both isomers and need Z7-12:OAc, but not E7-12:OAc, for attraction. This result contrasts our findings in North Carolina, where males did not differentiate between these two isomers. However, while in Peru males captured were corn-strain individuals, males caught in North Carolina could not be strain-typed due to DNA degradation, but probably belonged to both strains. Hence, we currently cannot exclude the possibility that corn- and rice-strain males show differential strain-specific attraction to E- and Z-7-12:OAc. Different isomers of a pheromone component are usually critical for attraction of males and can even lead to speciation, as shown in the two pheromone strains of *Ostrinia nubilalis* (Hübner) (Lassance 2010). Taken together, geographic variation in response to E7-12:OAc seems to exist, but additional experiments are required to evaluate the importance of E7-12:OAc for both strains in different regions.

Implication for pest management

As the fall armyworm is a serious agricultural pest, an efficient monitoring system for both strains in different habitats and regions would be helpful to detect infestations early and start pest control. To avoid high costs, monitoring via pheromone baited traps requires an effective 'minimal' synthetic lure that equally attracts both strains in all habitats and regions. The results of our study showed that only Z9-14:OAc and Z7-12:OAc are usually required to attract *S. frugiperda* in the field, and although both strain exhibit some strain-specific responses towards different doses of Z7-12:OAc, their response ranges also overlap (Figure 2). More precisely, both strains responded equally well to 2% Z7-12:OAc and therefore, we recommend a monitoring blend consisting of 100% Z9-14:OAc (300 µg) and 2% Z7-12:OAc (6 µg).

Conclusions

Corn- and rice-strain males exhibited some similarities in their attraction to the different blends that we tested, although corn-strain males showed more differentiation in their response than rice-strain males. We found some geographic variation in attraction of corn- and rice-strain males to two synthetic 4- component blends. In contrast, rice-strain males, but not corn-strain males, showed almost no geographic variation in their attraction to different doses of Z7-12:OAc in different regions. One aspect that merits further attention is the possibility that habitat-specific volatiles influence the male response to pheromone blends in different fields. Furthermore, the minor compound Z11-16:OAc does not seem to affect attraction of *S. frugiperda* males, while region-specific differences in the attraction seem to occur to the compound that has only been identified from female glands in Brazil, E7-12:OAc. Overall, the data show some geographic variation in the response of *S. frugiperda* males to pheromone blends. If this variation coincides

with geographic variation in the female pheromone composition, then geographic differentiation between populations could occur.

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SUPPLEMENTARY MATERIAL

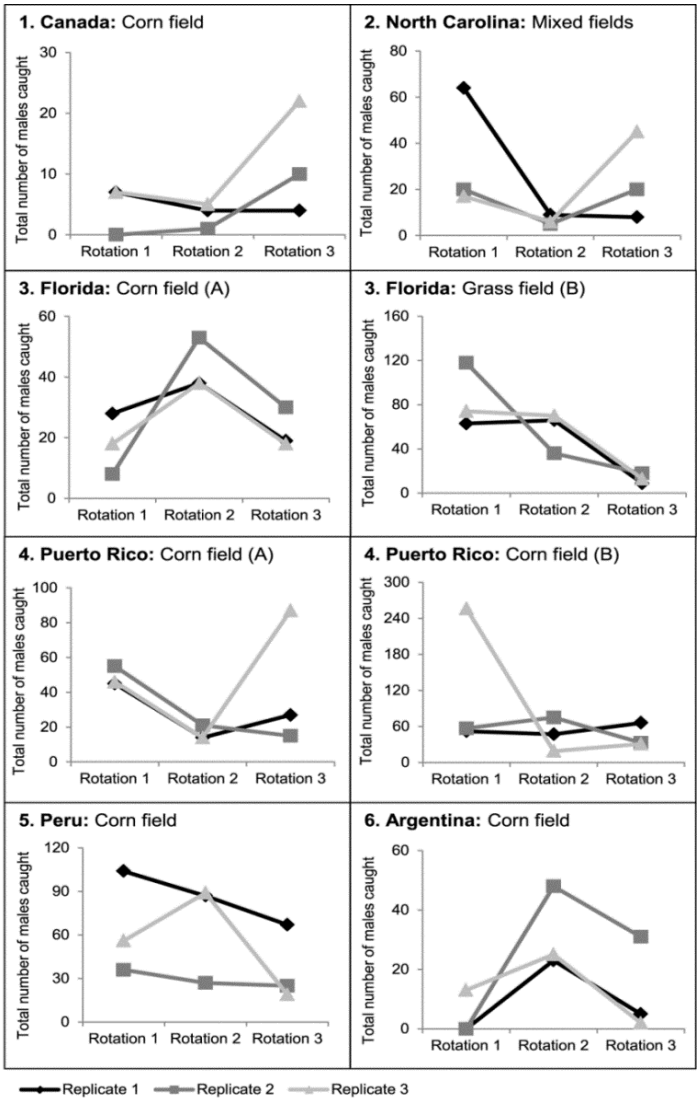

FIGURE S1. Variation in trap catches of *Spodoptera frugiperda* males in different fields and regions. Graphs show the total number of corn- and rice-strain males that were caught with Blend 1 (100% Z9-14:OAc + 13% Z11-16:OAc + 2% Z7-12:OAc + 1% Z9-12:OAc) in different regions in the first experiment (Figure 1). All traps were rotated three times and all replicates were conducted in the same field, except for North Carolina, where each replicate was conducted in a different field (replicate 1: soybean field, replicate 2: cotton and grass field, replicate 3: soybean and corn field).

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

6

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).

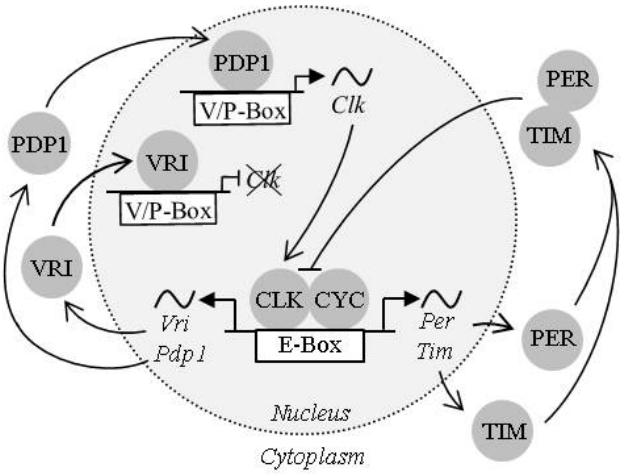

FIGURE 1. Two feedback loops that define the circadian rhythm in *Drosophila*. Adapted from Cyran et al. (2003), Hardin (2005).

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.

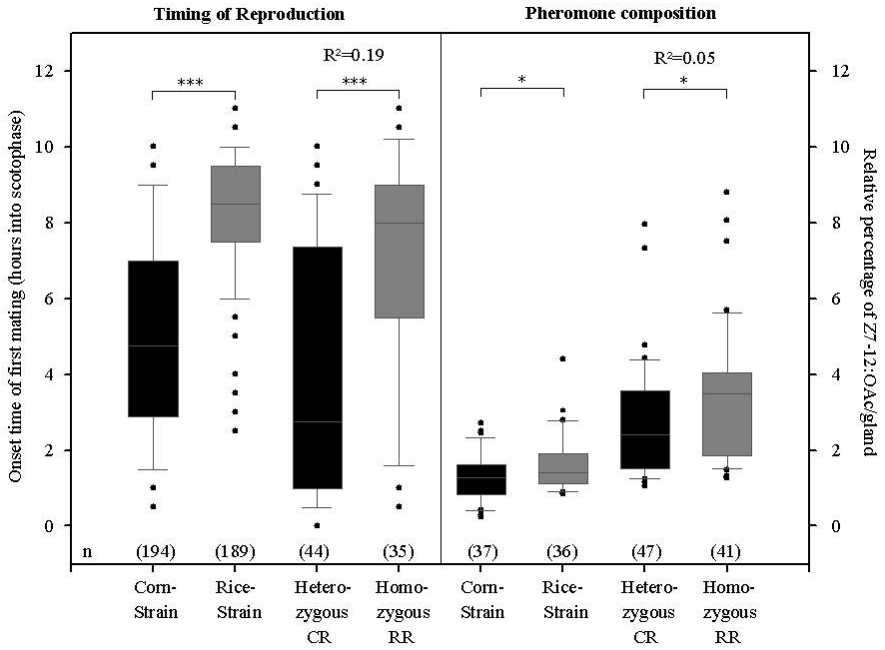

FIGURE 2. Phenotypes for major QTL chromosome *Sf*_C25 (=*Bm*_C27) of pure strain CC and RR individuals vs. heterozygous CR and homozygous RR backcross individuals. For the timing QTL: Onset time of first mating (hours into scotophase). For the pheromone QTL: Relative percentage of Z7-12:OAc per gland.

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).

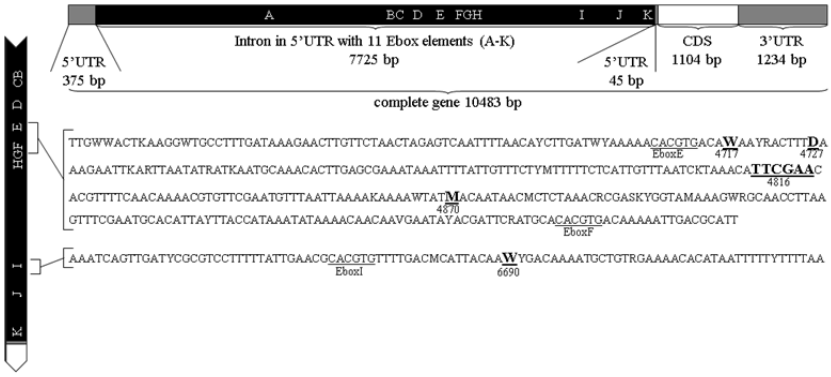

FIGURE 3. Structure of *vri* in the corn- and rice-strain of *S. frugiperda* and strain-specific polymorphisms in the intron in the 5' UTR.

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.

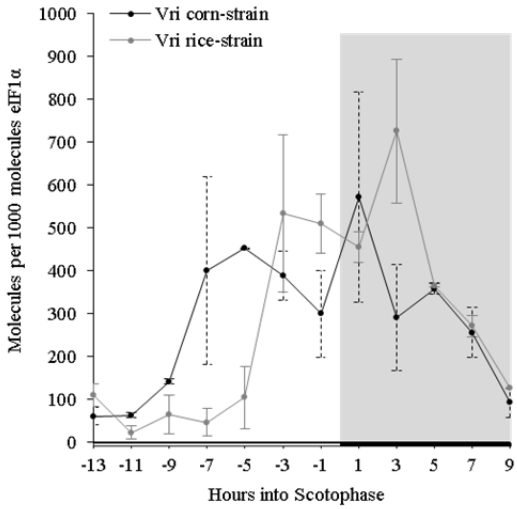

FIGURE 4. Expression of *vri* over time in heads of *S. frugiperda* corn- and rice-strain females. Five individuals were used per biological replicate, two biological replicates per time point. Grey field indicates scotophase. Expression is shown as molecules per 1000 molecules eIF1α.

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			

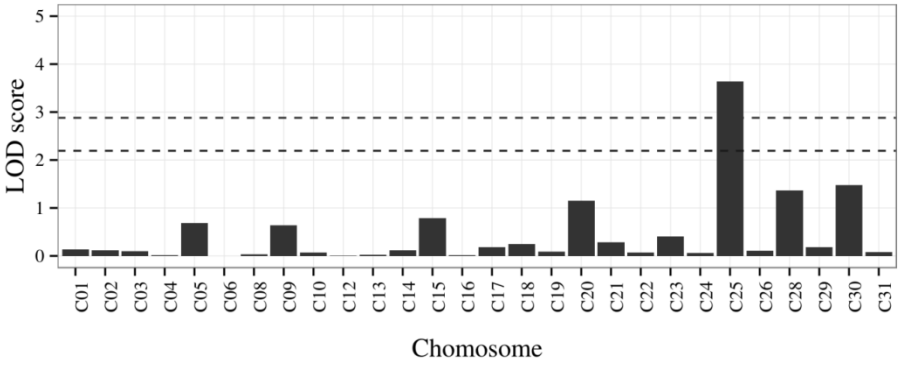

FIGURE S1. LOD scores for all linkage groups in the combined analysis of the two timing backcross families, empirically determined by 10,000 permutations. 0.05 and 0.10 significance thresholds are represented by dashed lines.

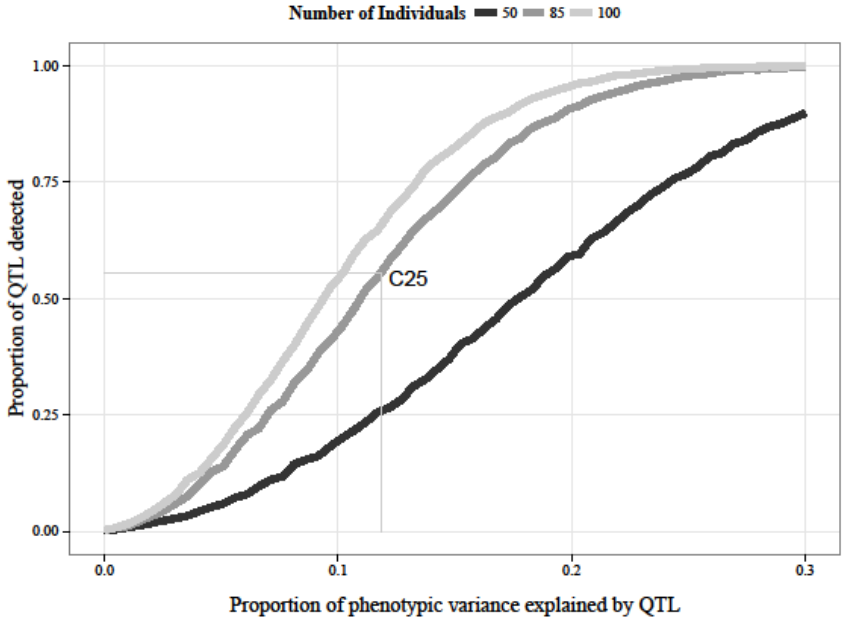

FIGURE S2. Power analysis for backcross families with 50 (black line), 85 (timing QTL, dark grey line) and 100 (black line) progeny, respectively. The probability of detecting a QTL is plotted as a function of the fraction of phenotypic variance explained by the QTL.

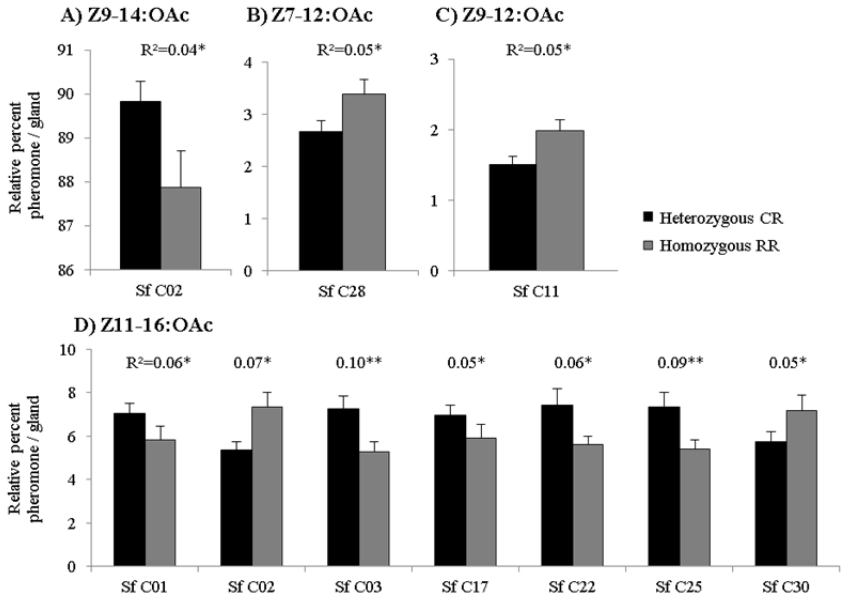

FIGURE S3. All QTL found in pheromone QTL analysis (The effect of different chromosomes on the relative amount of **A)** Z9-14:OAc, **B)** Z7-12:OAc, **C)** Z9-12:OAc and **D)** Z11-16:OAc in pheromone glands of heterozygous (CR, black bars) and homozygous (RR, grey bars) *S. frugiperda* backcross individuals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

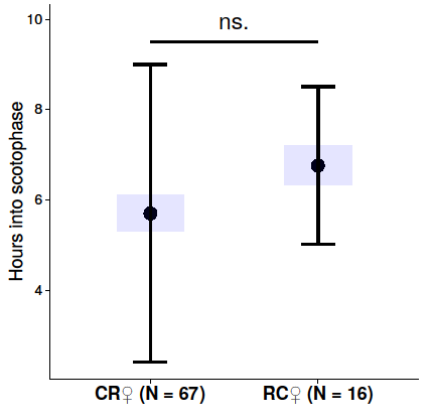

FIGURE S4. Mating time in *S. frugiperda* hybrids. Onset time of first mating in *S. frugiperda* hybrid females. The reciprocal crosses (CR= corn-strain mother, rice-strain father; RC= rice-strain mother, corn-strain father) do not show differences in mating time. This excludes the involvement of the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

7

ANNOTATION OF CIRCADIAN CLOCK GENES IN THE GENOME OF *SPODOPTERA FRUGIPERDA*

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Part of *The* Spodoptera frugiperda *Whole Genome Sequencing Project*.
The Fall armyworm International Public Consortium (FAW-IPC) (in prep.)
http://www6.inra.fr/lepidodb/SfruDB

INTRODUCTION

All life on earth is subject to rhythmical changes of light and temperature as day and night alternate in a rhythm of roughly 24 hours. Consequently, organisms have evolved reliable internal clocks that are entrained by changing external factors like light and temperature. These circadian clocks (from Latin circa = approximately, dia = day) enable them to predict these changes and 'schedule' physiological as well as behavioral processes in beneficial time windows, e.g. to avoid heat stress. Hence, circadian clocks are involved in almost all physiological and behavioral processes in animals such as mating, feeding and cell-division (Dunlap 1999; Wijnen and Young 2006). They are composed of genes and their protein products that form interlocked transcriptional/translational feedback loops, which repeat a feedback cycle every approximately 24 hours (Edery 2000). Key genes involved in the circadian clock are well characterized in a number of model organisms and are conserved within kingdoms and particularly strongly conserved within the animal kingdom (reviewed e.g. in Rear and Allada 2012). For example the important signal sensor region PAS-B of the protein CLOCK shows 80-88% sequence similarity between the silkmoth *Antheraea pernyi*, the fruitfly *Drosophila melanogaster* and the mouse *Mus musculus*. Similarly the basic helix-loop-helix (bHLH) domain of the CLK protein, facilitating its DNA binding, shows 59-76% sequence similarity between these species (Chang et al. 2003).

Homologues of most of the known *Drosophila* clock genes are found in Lepidoptera (Sandrelli et al. 2008; Zhan et al. 2011). In general, the lepidopteran clockwork (like the clockwork of *Drosophila*) is proposed to consist of two feedback loops (Hardin 2005; Zhan et al. 2011). They are interlocked by both involving the genes (always named in lower case italic letters) *clock* (*clk*) and *cycle* (*cyc*) and their protein products (always named in upper case letters) CLOCK (CLK) and CYCLE (CYC). In the core transcriptional/translational feedback loop CLK:CYC heterodimers drive *timeless* (*tim*), *period* (*per*) and *cryptochrome 2* (*cry2*) transcription. TIM, PER and CRY2 form a complex that enters the nucleus, where CRY2 inhibits the transcription mediated by CLK:CYC, including transcription of *tim*, *per* and *cry2*. The light-dependent CRYPTOCHROME 1 (CRY1) in involved in TIM degradation, facilitating light-entrainment of the clock. The degradation of TIM and PER is signaled by SUPERNUMERARY LIMBS (SLIMB) and JETLAG (JET) and kinases and phosphatases like CASEIN KINASE II (CKII), DOUBLETIME (DBT) and PROTEIN PHOSPHATASE 2A (PP2A) are involved in posttranslational modifications of PER and TIM. In the modulatory feedback loop, VRILLE (VRI) inhibits *clk* transcription and PAR DOMAIN PROTEIN 1 (PDP1) promotes *clk* transcription. Both *vri* and *pdp1* transcription are driven by the CLK:CYC heterodimer. The amplitude of the clock is modified by CLOCKWORKORANGE (CWO) (Hardin 2005; Zhan et al. 2011). From this list of circadian clock genes (Table 1), in total nine critical genes involved in the core

feedback loop (*clk*, *cyc*, *per*, *tim*, *cry2*), the modulatory feedback loop (*vri*, *pdp1*), the light entrainment (*cry1*) and in the posttranslational modification (*dbt*) were chosen for annotation in as a starting set.

The annotation of the circadian clock genes is part of the *Spodoptera frugiperda* whole genome sequencing project of The Fall Armyworm International Public Consortium (FAW-IPC) (in prep.). The project aims to sequence and assemble the whole genome of both the corn-strain and rice-strain of *S. frugiperda* and annotate the genomes by identifying genetic elements (genes and transposable elements) in the genome and adding relevant biological information (name and function) to these elements. The database SfruDB, which provides the genome sequences, transcriptional data and an annotation interface (WebApollo) to the consortium is hosted by the French National Institute for Agricultural Research (INRA) and can be found at http://www6.inra.fr/lepidodb/SfruDB.

Table 1. List of clock genes with abbreviations, their part in the clockwork and their annotation status.

Gene name	Abbrev.	Clockwork part	Annotation status
Clock	clk	core feedback loop	yes
Cycle	cyc	core feedback loop	yes
Timeless	tim	core feedback loop	yes
Period	per	core feedback loop	yes
Cryptochrome 2	cry2	core feedback loop	yes
Cryptochrome 1	cry1	photic entrainment	yes
Supernumerary limbs	slimb	TIM and PER degradation	not yet
Jetlag	jet	TIM and PER degradation	not yet
Casein kinase II	ckII	posttranslational modification	not yet
Doubletime	dbt	posttranslational modification	yes
Protein phosphatase 2A	pp2a	posttranslational modification	not yet
Vrille	vri	modulatory feedback loop	yes
PAR domain protein 1	pdp1	modulatory feedback loop	yes
Clockworkorange	cwo	amplitude modification	not yet

METHODS

To annotate the clock genes, we conducted the following steps. First, in the GenBank database of NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi), DBT, TIM, CRY1, CRY2, CLK and PER amino acid sequences were identified in the closely related species *Spodoptera exigua*. As for PDP1 and CYC, no sequence was available from *S. exigua*, but PDP1 was identified in *Drosophila melanogaster* and CYC in *Danaus plexippus*. For VRI, the *S. frugiperda* DNA sequence obtained in our laboratory was used.

Secondly, for CLK, PER, CYC, VRI and PDP1, homologs were BLAST searched in an RNAseq assembly from larval midguts of both strains using tblastn and the program SEQtools (Rasmussen 2002) and using the protein sequences obtained from NCBI. The used RNAseq assemblies are now available on SfruDB WebApollo: A1: corn strain midguts (larvae fed on maize), B1: corn strain midguts (larvae fed on pinto bean diet), C1: rice strain midguts (larvae fed on maize), D1: rice strain midguts (larvae fed on pinto bean diet). The cDNA sequences of *clk*, *per*, *cyc*, *vri* and *pdp1* that were obtained from the RNAseq assemblies were then used for the next step. For DBT, TIM, CRY1 and CRY2 we used the protein sequences of *S. exigua* that were obtained from the GenBank database of NCBI in the first step.

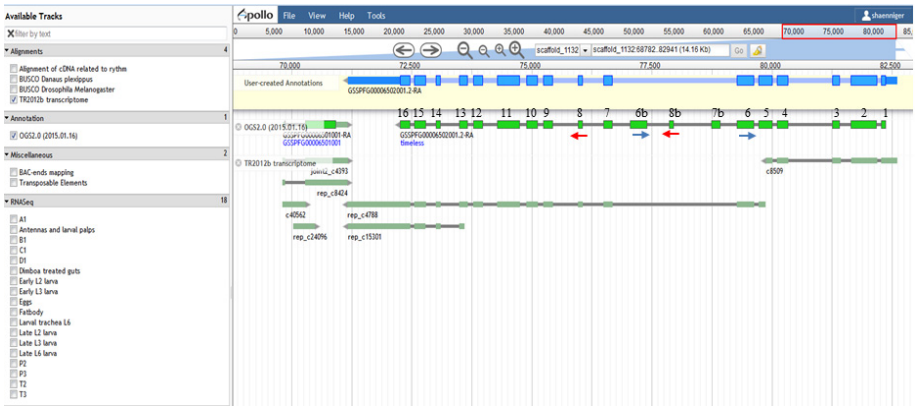

FIGURE 1. Screenshot of the annotation of timeless in the WebApollo annotation platform of the SfruDB. Available resources can be chosen on the left panel. The User-created Annotations (UCA) shows own annotations and that of other users. Below the UCA field appear the resources chosen on the left panel. They can be dragged and dropped onto the UCA field. In this example, a previous annotation (OGS2.0) was corrected (3 exons deleted) and the UTRs were added based on the TR2012b transcriptome (see results and discussion for details). Numbers over OGS2.0 correspond to exon numbers. Arrows under OGS2.0 indicate the approximate position of primers (see results and discussion for details).

In the third step, we BLAST searched *Spodoptera frugiperda* homologs of the nine chosen clock genes in the corn-strain variant of the genome in the SfruDB database using tblastn (for protein query sequences) or blastn (for cDNA query sequences). In the annotation platform WebApollo, we annotated the corresponding transcripts, if present, and corrected the exon-intron structure based on homology between the *S. frugiperda* and *S. exigua* sequences. We further corrected the gene structure, including 5' and 3' UTRs, based on transcriptome data available in WebApollo (RNAseq and TR2012b). Figure 1 shows an example for *timeless*.

In the fourth step, we carefully named the alleles and parts of all genes, if present.

As a final check, we retrieved the created protein sequences from the annotated genes in WebApollo and performed blastp against insects on the NCBI blast server to confirm homology in other lepidopteran insects.

RESULTS AND DISCUSSION

In the corn-strain variant of the *Spodoptera frugiperda* genome in SfruDB, we found the homologs of all nine chosen clock genes: c*lock*, *cycle*, *timeless*, *period*, *cryptochrome 2*, *cryptochrome 1*, *double-time*, *vrille* and *pdp1*. The genes have not been annotated in the rice-strain variant yet, as it only became available for annotation when this thesis was submitted.

The exon-intron structures of the annotated genes as well as the distribution on different scaffolds are summarized in Table 2 and shown in Figure 2 below.

Most of the annotated circadian clock genes, i.e. *clk*, *cyc*, *per*, *pdp1* and *cry1*, are located on several scaffolds (see Table 2). Since the genome assembly is still very fragmented, i.e. consists of some large and several thousand small scaffolds that are not connected, this information is useful to merge scaffolds or at least determine the right order and orientation of the scaffolds and will be used by the bioinformatics experts developing and improving the assembly in the near future.

For *clk* the 3' UTR and the first exon could not be annotated, because a homologue was missing in the genome assembly. The annotated parts of the gene spread over five scaffolds and consist of 12 exons, thus five scaffolds could be arranged in the right order or possibly even merged. For *cyc*, all parts could be annotated, thirteen exons were distributed over three scaffolds. The 25 exons and the 3' UTR of *per* were distributed over 7 scaffolds. Exon 12 is missing in the genome assembly and could thus not be annotated. Also the 5' UTR could not be annotated. All parts of *pdp1* could be annotated. The seven exons were distributed over four scaffolds. The twelve exons and the 3' UTR of *cry1* were annotated on three different scaffolds. The 5' UTR could not be annotated.

As evident from Table 2, many UTRs could only be annotated partially or not at all. This is because these UTRs are (partially) located on a separate scaffold and the Web Apollo interface does not allow the annotation of a sequence as UTR when it is not connected to a coding sequence. This extra information could be taken into account for merging or arranging the scaffolds, equivalent to the information from separated exons. In case of *vri* we can contribute one missing piece of sequence information to the genome assembly. The coding region of *vri*, consisting of only one exon, was annotated on one scaffold. However, the upstream part of the 5' UTR is located on a separate scaffold. The 5' UTR is split by a large (>7,000 bp) intron that is not fully present in the genome assembly. Because of this large gap the two scaffolds cannot be merged. We could obtain the sequence of this intron by

sequencing two BAC clones (AUA0AAA25YL06 and AUA0AAA20YH15) whose ends were mapping to the scaffolds containing *vri*. This sequence information will be useful to merge the two scaffolds and close a large gap in the genome assembly.

TABLE 2. Summary of annotated circadian clock genes. Numbers in brackets include the exons that could not be annotated and their unknown scaffolds.

Gene name	Symbol	Nr of exons	Nr of scaffolds	5' UTR annotated?	3' UTR annotated?
Clock	CLK	12 (13)	5 (6)	no	yes
Cycle	CYC	13	3	partially	yes
Timeless	TIM	16	1	yes	yes
Period	PER	24 (25)	7 (8)	partially	partially
Cryptochrome 2	CRY2	9	1	no	partially
Vrille	VRI	1	1	partially	yes
PAR domain protein 1	PDP1	7	3	yes	yes
Double-time	DBT	8	1	yes	no
Cryptochrome 1	CRY1	12	3	partially	yes

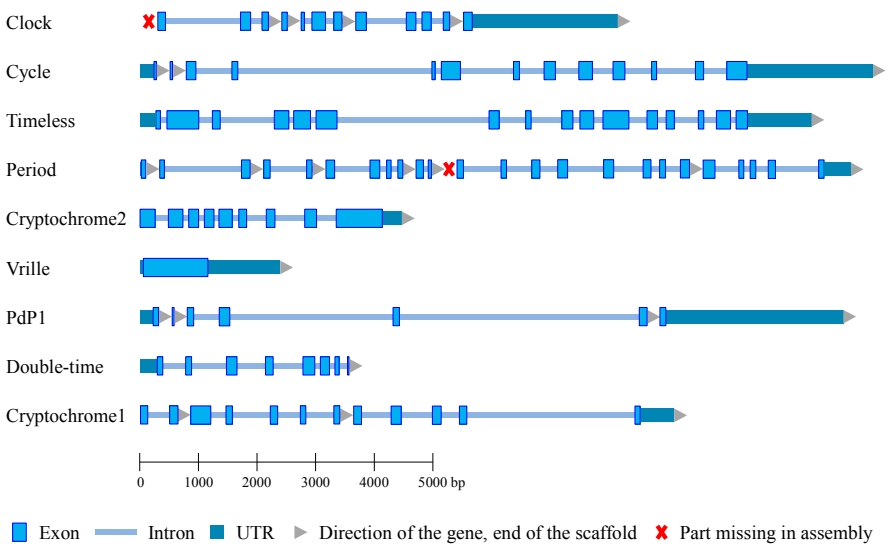

FIGURE 2. Exon-intron structure of the clock genes annotated in the whole genome assembly of *S. frugiperda*. The 3' end of a gene part that is located on one scaffold is indicated by a grey arrow head. Introns that are spanning scaffolds are not depicted. Exons that could not be annotated are indicated by a red cross.

A special case occurred when annotating *tim*. All 16 exons and the UTRs of the gene are located on one scaffold (see Figure 1). The sequences of exons 6, 7 and 8 are replicated in concert on this scaffold as follows: 6-7-8-6-7-8, resulting in 19 exons instead of 16 (see OGS2.0 in Figure 1, numbers above the OGS2.0 correspond to exon numbers, duplicated exons are named 6b, 7b and 8b). The sequences of these exons are only present once in e.g. the *S. exigua tim* gene. The transcriptomic and RNAseq data available in Web Apollo indicate that the replication is a mistake in the genome assembly rather than a genuine duplication of this part of the gene. This can be seen in the missing transcriptomic information in TR2012b for the exons 6b, 7b and 8b of OGS2.0 in Figure 1. To confirm a mistake in the genome assembly and rule out a duplication in the *tim* gene of *S. frugiperda*, we used a forward primer in exon 8 (red arrows in Figure 1) and a reverse primer in exon 6 (blue errors in Figure 1) for a PCR, which would only amplify a sequence if (a second) exon 6 (6b) would follow an exon 8 (8b). The PCR did not amplify a product. Thus, the exons 6b, 7b and 8b were removed from the annotation, leaving *tim* with the expected 16 exons and a sequence homologous to that of *S. exigua tim*.

In conclusion, the annotation of the nine clock genes in SfruDB has resulted in the elucidation of the exon-intron structure of these genes in the corn-strain of *S. frugiperda*. Through this annotation, three sequences of three scaffolds each can be arranged in the right order or could possibly be merged, as well as one sequence of five scaffolds and one sequence of seven scaffolds. The arrangement of the scaffolds, and with this the overall genome annotation of *S. frugiperda*, would benefit from the possibility to annotate UTRs that are located on separate scaffolds and not attached to a coding sequence of a gene.

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8

EVOLUTION OF REPRODUCTIVE ISOLATION OF *SPODOPTERA FRUGIPERDA*

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INTRODUCTION

Spodoptera frugiperda, the fall armyworm, is a noctuid moth occurring in North and South America with two host strains (a corn- and a rice-strain) identified in the 1980s (Pashley et al. 1985; Pashley 1986). These two strains were originally characterized by a polymorphism in an esterase allozyme marker and three other strain-biased protein variants in larvae collected from corn fields and rice paddies in Puerto Rico (Pashley 1986). Since then, several additional strain-biased or strain-diagnostic molecular markers have been identified: the two strains differ in mitochondrial DNA sequences in the cytochrome oxidase I (COI) and NADH dehydrogenase 1(ND1) genes (Pashley 1989; Pashley and Ke 1992; Lu and Adang 1996; Levy et al. 2002; Meagher and Gallo-Meagher 2003; Prowell et al. 2004; Nagoshi et al. 2006a; Machado et al. 2008). There are also strain-biased and strain-specific amplified fragment length polymorphisms (AFLP) (McMichael and Prowell 1999; Busato et al. 2004; Prowell et al. 2004; Clark et al. 2007; Martinelli et al. 2007; Juárez et al. 2012), restriction length fragment polymorphisms (RFLP) (Lu et al. 1992), a so-called Frugiperda Rice (FR) repetitive nuclear DNA sequence, present in high copy number in the rice-strain and mostly lower copy number in the corn-strain (Lu et al. 1994; Nagoshi and Meagher 2003b; Nagoshi et al. 2008) and nucleotide polymorphisms within the triose phosphate isomerase gene (Tpi, Nagoshi 2010).

Recently, sex pheromone differences have been found among populations of the two strains (Groot et al. 2008; Lima and McNeil 2009; Unbehend et al. 2013). However, these differences were not consistent among studies, suggesting that geographic variation may be confounded with strain-specific variation, or that pheromones may vary within strains as well. The relative importance of the pheromone differences between the two strains still needs to be established, i.e. are all pheromone compounds in the pheromone glands behaviorally important and/or are males of the two strains differentially attracted to the different pheromone blends? Since other physiological, developmental, and behavioral differences have been found among the strains (Pashley and Martin 1987; Pashley 1988b; Pashley et al. 1992, 1995; Veenstra et al. 1995; Meagher et al. 2004, 2011; Schöfl et al. 2009, 2011; Groot et al. 2010; Meagher and Nagoshi 2012), this overview integrates strain-specific variation in sexual communication (variation in the pheromone gland composition and variation in male response) with other possible pre- and postmating barriers that likely contribute to isolation of the two strains. First, we show that the naming of the two strains is somewhat misleading, as the host specificity of the two strains is not as clear-cut as the names suggest. Then we focus on the two types of prezygotic isolating mechanisms that have been demonstrated to differ between the two strains: a) the diel pattern of reproductive activity, and b) pheromone signal and response traits. In addition to the premating barriers, we also consider postmating barriers that may isolate the two strains. Finally, based on recent findings, we discuss a possible evolutionary scenario for the evolution of the two strains.

ARE THE TWO STRAINS REALLY HOST STRAINS?

Allozyme differences at five loci, including one apparently strain-specific esterase allele, provided the first evidence of partial genetic differentiation of populations collected from adjacent corn and rice fields in Puerto Rico and Louisiana (Pashley et al. 1985; Pashley 1986). Differences in mitochondrial DNA RFLP patterns were also found among these populations (Pashley 1988a). Subsequently, the same genetic differences were found in populations collected from other host plants and localities, and used to assign them to either the corn- or rice-strain. The so-called corn-strain was found to infest mainly corn (*Zea mays*), sorghum, (*Sorghum bicolor* subsp. *bicolor*), and cotton (*Gossypium hirsutum*), whereas the so-called rice-strain was found mostly in rice (*Oryza sativa*), sugar cane (*Saccharum officinarum*), and grasses such as Johnson grass (*Sorghum halepense*), and Bermuda grass (*Cynodon dactylon*). Genetic differentiation between these two strains has been confirmed in several regions in North and South America, using different molecular markers. The host associations of the two strains are summarized below.

Host associations based on mitochondrial COI polymorphism

Among the molecular markers available to distinguish the two strains, the most widely used target mitochondrial DNA. For example, the two strains show differences in their cytochrome oxidase I (COI) gene and can be identified by a polymorphism in the restriction sites for *Sac*I and *Aci*I (both present in rice-strain and absent in the corn-strain), and for *Hinf*I, *Bsm*I and *Msp*I (all present in the corn-strain and absent in the rice-strain). The polymorphisms in *Sac*I and *Msp*I are used in most studies (Lu and Adang 1996; Levy et al. 2002; Meagher and Gallo-Meagher 2003; Nagoshi et al. 2006a). Based on the restriction site polymorphisms mentioned above, especially in the double digestion with *Sac*I and *Msp*I, the identity of the strains has been evaluated for different habitats and it has been demonstrated that the association is not always absolute.

Approximately 80% of individuals collected from corn habitats were identified as corn-strain and the remaining 20% as rice-strain (Pashley 1989; Lu and Adang 1996; Levy et al. 2002; Nagoshi et al. 2006a, 2007b). However, exceptions from this percentage of distribution have been found as well: Prowell et al. (2004) identified samples collected from corn predominantly (i.e. 50% or more of the individuals) as rice-strain in French Guiana and in Louisiana. Nagoshi et al. (2006a) also found mostly rice-strain individuals in a sorghum field in Texas, which is considered a corn-strain habitat. In the case of larvae collected from rice fields, up to 95% of individuals have been identified as rice-strain (Nagoshi and Meagher 2003a, 2004; Machado et al. 2008; Velez-Arango et al. 2008). Recently, Juárez et al. (2012) did not find a consistent pattern between the two strains and their respective host plants (especially, in rice habitats), when using COI markers in South American populations.

Some of these shifts in strain distributions may be due to seasonal and temporal variation in the distributions of the two strains and in the distribution of available host plants or different migration patterns of the two strains (Nagoshi et al. 2007a). For example, Nagoshi et al. (2007c) showed that the corn-strain predominated in collections from sorghum in the fall (March-June) in Brazil, but was less common in spring collections (September-November), while in Florida rice-strain larvae predominated in collections made from sorghum in the fall (September-November), and corn-strain larvae were mostly present in the spring season in sorghum (February-April). In Louisiana, Pashley et al. (1992) found that corn-strain populations were detected in the corn fields in the spring, while rice-strain populations remained at low density on various grasses until late summer when they increased in number. Together, these findings suggest that the migration pattern of the two strains may not be the same (Nagoshi and Meagher 2004).

In Figure 1 we provide an overview of collections of the fall armyworm over a period of 27 years (from 1983 until 2010) from a number of different habitats. In 17 of 20 populations sampled from predominantly rice habitats (rice and pasture/Bermuda grass), most individuals were identified as rice-strain, whereas in 29 of 44 populations habitats (corn, cotton and sorghum), most individuals were identified as corn-strain. Although mitochondrial markers generally show a strong correlation between strain type and host plant, in many of the collections this association is lacking, especially in predominantly corn habitats (see Figure 1).

Host associations based on genome-wide AFLP markers

Although some studies have found a close association between the two strains and their host plants using AFLP markers (e.g., McMichael and Prowell 1999; Busato et al. 2004 in the USA and Brazil, respectively), others have not (e.g., Martinelli et al. (2007) in Mexico, Brazil, Argentina and the USA). Recently, we found that individuals from populations collected from corn plants from several locations in Argentina, Paraguay and Brazil tended to cluster together and showed a high degree of homogeneity in AFLP markers (Juárez et al. 2014). This finding thus contrasts the trend found in the COI marker, where 15 out of 44 populations collected from corn (see Figure 1) showed a significant portion of rice-strain individuals. Individuals from the populations collected from rice from several locations in Argentina and Paraguay formed three distinct groups and showed a much higher level of heterogeneity in their AFLP markers (Juárez et al. 2014). Overall, individuals collected from corn-strain habitats were clustered separately from individuals collected from rice-strain habitats, although there were some marked exceptions (Juárez et al. 2014).

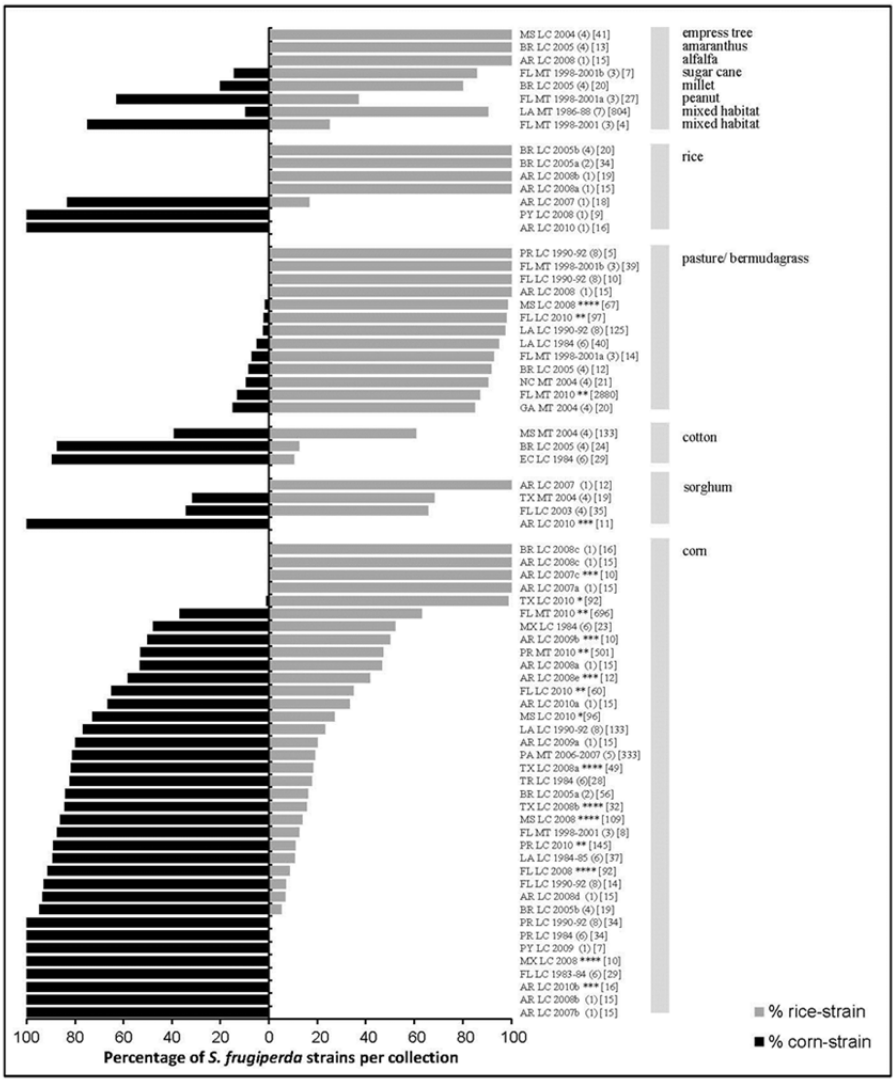

FIGURE 1. Distribution of *Spodoptera frugiperda* host strains in different habitats and geographic regions. Each bar shows the percentage of the identified strains per collection, based on mitochondrial markers. Habitats are indicated on the right. Numbers in [ ] indicate total n of collection. MS-Mississippi, BR-Brazil, AR-Argentina, LA-Louisiana, FL-Florida, PY-Paraguay, PR-Puerto Rico, NC-North Carolina, GA-Georgia, EC-Ecuador, TX-Texas, MX-Mexico, PA-Pennsylvania, TR-Trinidad; LC-larval collection, MT-male trapping experiments. Data from: * C. Blanco, ** S. Hänniger & M. Unbehend, *** M. L. Juárez, **** G. Schöfl, (1) Juárez et al. 2014, (2) Machado et al. 2008, (3) Meagher & Gallo-Meagher 2003, (4) Nagoshi et al. 2006a, (5) Nagoshi et al. 2009, (6) Pashley 1989, (7) Pashley et al. 1992, (8) Prowell et al. 2004.

Host association based on mitochondrial and nuclear markers

Combining mitochondrial and nuclear markers with their different modes of inheritance, the rate and directionality of hybridization between the strains in the field can be identified. Prowell et al. (2004) analyzed populations from Louisiana, Florida, Puerto Rico, Guadeloupe, and French Guiana with different molecular markers (mitochondrial haplotype, esterase genotypes, AFLPs) reported that 16% of the samples were potential hybrids due to discordance for at least one marker. The authors found evidence of crosses between the strains in both directions: when using mtDNA and esterase markers, 66% of the hybrids were inferred to be derived from rice-strain females mated with corn-strain males, i.e. RC hybrids, while in multilocus comparisons using the three markers, 54% of the hybrids were RC hybrids and 46% were from the reciprocal cross, i.e. CR hybrids. In addition, Prowell et al. (2004) found that these hybrids occurred mostly in the corn habitats. Similar results were found by Saldamando and Vélez-Arango (2010) with Colombian populations. In contrast, Nagoshi and Meagher (2003b) and Nagoshi et al. (2006b), using mitochondrial haplotypes and the nuclear FR tandem-repeat sequence, found 40-56% of all males collected in pheromone traps having the RC configuration, while only 3-3.5% were CR-hybrids, and hybrids occurred in both corn and rice habitats.

Recently, Nagoshi (2010) identified 10 polymorphic diagnostic sites in the Z-linked (sex-linked) triose phosphate isomerase (*Tpi*) gene that can be associated with the corn- or rice-strain of the fall armyworm (as in most Lepidoptera females are the heterogametic sex, ZW). With this marker, Nagoshi (2012) analyzed 12 populations (9 collected from corn and 3 from rice) with the COI marker, and then reanalyzed the same samples with the *Tpi* marker, and found that 60 and 7% of the COI-R typed individuals were *Tpi*-C in the corn and rice habitats, respectively (i.e. RC hybrids). The reverse constellation, COI-C and *Tpi*-R (i.e. CR hybrids), occurred in 8 and 22% of the COI-C typed individuals from corn and rice habitats, respectively. Like Nagoshi (2012), when we combined the COI marker with the Z-linked *Tpi* marker, we also found discordance between the mitochondrial and nuclear markers (43%) (Juárez et al. 2014). These configurations consisted of four different combinations: RC (30% of all hybrids), CR (7% of all hybrids), CI (20% of all hybrids), and RI (42% of all hybrids). The I stands for a Tpi-intermediate haplotype, i.e. individuals in which corn and rice SNPs were present in similar proportions or heterozygous individuals in which SNPs showed the two alternative nucleotides. The latter individuals must be hybrid males, as in Lepidoptera the females carry only one copy of the Z-linked Tpi gene. Nagoshi (2010) and Nagoshi et al. (2012) also found this intermediate configuration in a very low frequency and proposed that they may represent hybrid individuals as well.

In summary, both types of hybrids seem to occur in nature, although recent studies suggest that the RC-hybrids are more common. These hybrids are mostly

found in corn habitats, while other hybrids (CR, CI, RI) are mostly found in rice habitats. Overall, the two strains seem to be predominantly found in the habitats from where they were originally described, but significant exceptions have been found with all markers used. Therefore, our preliminary conclusion is that divergence between the strains is not likely due to host plant specialization, or at least not alone. We hypothesize that an interaction between ecological and behaveioral mechanisms has contributed to reproductive isolation between the two strains (Groot et al. 2010).

BEHAVIORAL ISOLATION MECHANISM 1: TIMING OF REPRODUCTIVE ACTIVITY

Differences in the diel pattern of mating activity between strains would create a powerful barrier to hybridization. Strain-specific differences in the timing of reproductive activity of the two strains have been consistently found, independent of the geographic origin of the strains (Pashley et al. 1992; Schöfl et al. 2009, 2011): the corn-strain is active early in the scotophase, while the rice-strain is active late in the scotophase. Schöfl et al. (2009) showed that different reproductive behaviors (calling, copulation and oviposition) are differentially inherited and thus under complex genetic control. The coordinated timing difference between the two strains in reproductive activity and general locomotor activity suggested the involvement of the circadian clock.

When testing whether allochronic separation causes assortative mating in the laboratory, Schöfl et al. (2011) found an interaction between strain-specific timing of mating and time-independent intrinsic preferences that influenced the mating choice of both strains. Furthermore, mate choice changed over time in consecutive nights and was influenced by the timing of introduction of the mating partners, i.e. when they were introduced at the onset of the scotophase or introduced 6 hours after the onset of scotophase, thus when the ricestrain is more active (Schöfl et al. 2011). In general, females were more restricted in their mate preference than males and approximately 30% of the isolation between both strains was generated by female mate preference, suggesting the involvement of a male-specific sex pheromone that mediates close-range courtship behavior (Schöfl et al. 2011). Also, this mate-choice experiment indicates that the level of assortative mating caused by allochronic separation alone is not strong enough to cause reproductive isolation between strains.

Although the importance of differential timing of reproduction is probably not as strong as suggested by Pashley et al. (1992), the consistent timing differences between the strains, independent of the geographic origin, suggests that this behavioral difference could have a stronger influence as prezygotic isolation barrier than host plant choice. Therefore, we are tempted to argue that both strains are ‘timing strains’ rather than ‘host strains’.

BEHAVIORAL ISOLATION MECHANISM 2: VARIATION IN SEXUAL COMMUNICATION

In the early 1990s, Pashley et al. (1992) found that males of both strains showed a slight preference for females of the same strain, 60-65% of corn- and rice-strain males being attracted to corn- and rice-strain females, respectively. These findings indicate that in addition to the differences in timing of reproduction, pheromone differences might be important for mate choice and cause assortative mating in the two strains, although Pashley et al. (1992) suggested that 'pheromone chemistry may play a small role (if any) in strain separation.' The sex pheromone composition of *S. frugiperda* females has been studied in different geographic regions (Mitchell et al. 1985; Tumlinson et al. 1986; Descoins et al. 1988; Batista-Pereira et al. 2006; Groot et al. 2008; Lima and McNeil 2009; Unbehend et al. 2013). While earlier studies mainly focused on the general composition of the female sex pheromone without distinguishing the two strains, later studies investigated strain-specific differences in the female pheromone composition (Groot et al. 2008; Lima and McNeil 2009; Unbehend et al. 2013). In general, the fall armyworm sex pheromone consists of the primary sex pheromone component Z9-14Ac and the critical secondary sex pheromone Z7-12Ac (Tumlinson et al. 1986; Batista-Pereira et al. 2006; Groot et al. 2008; Lima and McNeil 2009). The behavioral effect of other secondary compounds in the female gland remains unclear (Tumlinson et al. 1986; Andrade et al. 2000; Fleischer et al. 2005; Groot et al. 2008; Unbehend et al. 2013). However, twice as many males were caught when Z11-16Ac or Z9-12Ac were added to the binary blend (Fleischer et al. 2005), suggesting at least a synergistic effect of these compounds. It has been shown that corn- and rice-strain females exhibit strain-specific differences in their relative amount of Z7-12Ac (relative to the amounts of other gland compounds), as well as in the relative amount of Z9-14Ac, Z11-16Ac and Z9-12Ac, although the type of variation found seems to vary in different geographic regions (Groot et al. 2008; Lima and McNeil 2009; Unbehend et al. 2013).

Disentangling geographic from strain-specific variation

Extractions of the pheromone glands of females from a colony, that was initiated with larvae collected in Florida, revealed that rice-strain females produce significantly higher relative amounts of Z7-12Ac and Z9-12Ac, and lower relative amounts of Z11-16Ac, than corn-strain females (Groot et al. 2008). However, laboratory rice-strain females originating from Louisiana contained lower relative amounts of the major component Z9-14Ac, as well as larger relative amounts of Z7-12Ac and Z11-16Ac, compared to laboratory corn-strain females from Louisiana (Lima and McNeil 2009). Taken together, only Z7-12Ac showed consistent strain-specific variation in females from Florida and Louisiana (Groot et al. 2008; Lima and McNeil 2009; Unbehend et al. 2013). Apparently, the selection pressure on Z7-

12Ac is similar in both regions but different between the two strains. The inconsistent variation in the major sex pheromone component Z9-14Ac between the two regions suggests geographic rather than strain-specific variation. The importance of Z11-16Ac and Z9-12Ac in the attraction of fall armyworm males is not completely understood yet, but their variation suggests that these components are not under strong stabilizing selection.

Geographic variation in the strain-specific pheromone composition of females from Florida and Louisiana may be related to different haplotype profiles in Floridian and Louisianan corn-strain populations. There seem to be two main migration routes of the fall armyworm, based on haplotype patterns in the corn-strain (Nagoshi et al. 2008; Nagoshi et al. 2010). These patterns suggest an eastern migration route, i.e. populations originating from Puerto Rico and Florida move northwards to Georgia, and a western migration route, i.e. populations from Texas move northeastwards to Louisiana, Mississippi, Alabama and Pennsylvania (Nagoshi et al. 2008; Nagoshi et al. 2009). If no other geographic effects influence the female pheromone, then pheromone profiles of females from Texas, Louisiana, Mississippi, Alabama and Pennsylvania may be more similar to each other than to pheromone profiles of females from Florida, Puerto Rico and Georgia.

In fall armyworm females from Brazil, another minor sex pheromone component, E7-12Ac, was demonstrated to be attractive to Brazilian males in the field (Batista-Pereira et al. 2006). Addition of E7-12Ac to binary blends, containing Z9-14Ac and Z7-12Ac, significantly increased the number of males captured in Brazil, i.e. from an average of 70 males per trap to an average of 100 males per trap (Batista-Pereira et al. 2006). The fact that E7-12Ac has not been found in females from Florida, Louisiana or French Guyana (Descoins et al. 1988; Groot et al. 2008; Lima and McNeil 2009) suggests the existence of geographic variation in female pheromone production. In conclusion, the two *S. frugiperda* strains do differ in their female sex pheromone composition (Groot et al. 2008; Lima and McNeil 2009; Unbehend et al. 2013), but geographic variation seems to influence the strain-specific pheromone production. To disentangle geographic from strain-specific variation, additional strain-specific pheromone extractions of different populations from North and South America will be necessary.

Variation in pheromone composition within the strains

In addition to strain-specific and geographic variation in the pheromone composition, pheromone differences between females of the same strain have been observed between artificially reared and field-collected corn- and rice-strain females from Florida (Unbehend et al. 2013). Females of both laboratory strains produced significantly lower relative amounts of the major pheromone component Z9-14Ac and usually higher relative amounts of Z7-12Ac, Z11-16Ac and Z9-12Ac, compared to the field-collected females, although strain-specific pheromone variation was main-

tained (Unbehend et al. 2013). To estimate how much within-strain variation occurs in nature, we analysed the pheromone composition of females from seven different corn-strain families, originating from single pair matings of individuals that were collected one generation earlier from a corn field in Florida (Marr 2009). The females of these families exhibited significant differences in their pheromone composition compared to our laboratory populations (Marr 2009). The variation of Z9-14Ac, Z7-12Ac, Z11-16Ac and Z9-12Ac was strongly heritable and a broad-sense heritability analysis showed that the variation in gland compounds within the different families is determined mainly by genetic rather than environmental effects (Marr 2009). However, the within-strain variation found in laboratory- and field-females, in addition to the geographic variation, indicates that laboratory rearing and environmental factors influence the pheromone composition of females. The challenge is to determine the factors that cause variation in the pheromone composition and why. Understanding the cause of variation in the pheromone composition and its genetic control will be important to understand how variation in sexual communication influences reproductive isolation and how sexual communication systems may evolve (Baker and Cardé 1979; Löfstedt 1993; Butlin and Trickett 1997; Ritchie 2007).

Male response to strain-specific pheromone

The existence of strain-specific sex pheromone blends can only contribute to differentiation between the strains if this leads to differential attraction of fall armyworm males in the field. Although several trapping experiments of *S. frugiperda* males have been conducted in the field (Mitchell et al. 1985; Tumlinson et al. 1986; Meagher and Mitchell 1998; Andrade et al. 2000; Batista-Pereira et al. 2006), only one investigated strain-specific differences in the male attraction toward different pheromones (Pashley et al. 1992). In Louisiana fields containing both host plants, 60% of all rice-strain males trapped in pheromone traps were attracted to a virgin rice-strain female, while 65% of all trapped corn-strain males were caught in traps baited with virgin corn-strain females (Pashley et al. 1992). Thus, males of both strains exhibited only a slight bias toward females of their own strain in mixed habitats, suggesting that strain-specific sexual communication is a weak prezygotic isolation barrier (Pashley et al. 1992). Similarly, Lima and McNeil (2009) argued that it is quite unlikely that strain-specific sex pheromone differences alone 'would be sufficient to ensure reproductive isolation of the two strains.' To evaluate whether fall armyworm males exhibit strain-specific attraction towards females of their own strain, we conducted wind tunnel choice assays and male trapping experiments in Florida (Unbehend et al. 2013). Wind tunnel experiments without plant volatiles revealed that *S. frugiperda* males from laboratory populations show no strain-specific attraction to virgin females of their own strain. Interestingly, males of both strains were mainly influenced by the timing of female calling, and

did not discriminate among calling females (Unbehend et al. 2013). However, when testing pheromone lures mimicking the pheromone gland composition of Floridian corn-strain females (i.e. 100% Z9-14Ac, 13% Z11-16Ac, 2% Z7-12Ac, 1% Z9-12Ac), 74% of all trapped corn-strain males in a corn field were attracted to this corn-strain lure, and only 26% to the rice-strain lure, i.e. 100% Z9-14Ac, 8% Z11-16Ac, 4% Z7-12Ac, 2% Z9-12Ac (Figure 2).

In rice fields, such a similar strain-specific attraction was not observed, and only 59% of all trapped corn-strain males were attracted to the synthetic corn-strain lure, while 41% were attracted to the rice-strain lure (Figure 2). This result suggests that strain-specific attraction to different lures depends on the respective (volatile) environment, and hints to a synergistic effect of sex pheromones and host plant volatiles. However, similar to corn-strain males, rice-strain males were also mostly attracted to the synthetic corn-strain lure in the corn field with 76% of all trapped rice-strain males were caught in traps baited with the corn-strain lure (Figure 2). The pheromone traps that were baited with the so-called rice-strain lure (100% Z9-14Ac, 8% Z11-16Ac, 4% Z7-12Ac, 2% Z9-12Ac) did not specifically attract rice-strain males in a grass field and only 49% of all trapped rice-strain males were attracted to the rice-strain lure (Unbehend et al. 2013). Together, these results indicate that in Florida corn-strain lures are most attractive for both strains in a corn habitat, while there is no preference for a corn- or rice-strain lure in a rice habitat.

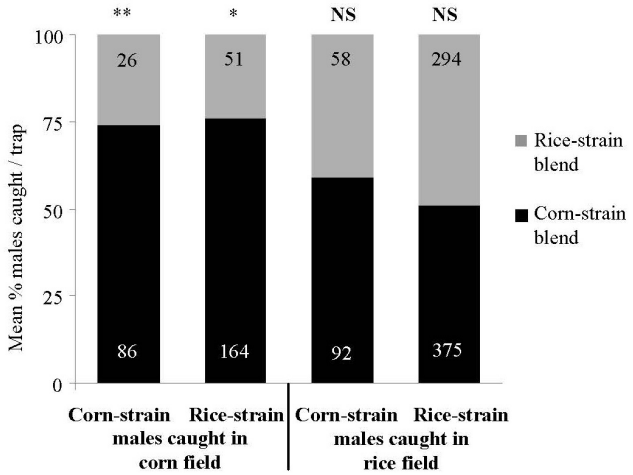

FIGURE 2. Mean percent of corn-strain and rice-strain males caught in sex pheromone traps baited with synthetic pheromone lures in a corn field and a grass field in Florida. The corn-strain blend consisted of 300 µg Z9-14Ac, which was considered 100%, 6 µg (2%) Z7-12Ac, 39 µg (13%) Z11-16Ac and 3 µg (1%) Z9-12Ac. The rice-strain blend was constructed in a similar way, only with 12 µg (4%) Z7-12Ac, 24 µg (8%) Z11-16Ac and 6 µg (2%) Z9-12Ac. Numbers in the bars indicate total number of males caught. See Unbehend et al. (2013) for more details. ** $P < 0.01$; * $P < 0.05$; NS: not significant.

Importance of different pheromone components for male attraction in the field

To assess strain-specific male response towards the different pheromone components, we also evaluated the importance of single pheromone components in the attraction of corn- and rice-strain males in a corn and a grass field in Florida (Unbehend et al. 2013). As mentioned earlier, fall armyworm males can vary in their attraction towards E7-12Ac, but show stable geographic-independent attraction towards binary blends containing Z9-14Ac and Z7-12Ac (Tumlinson et al. 1986; Andrade et al. 2000; Fleischer et al. 2005; Batista-Pereira et al. 2006; Unbehend et al. 2013). We tested different doses of the critical secondary component Z7-12Ac and found that corn-strain males had a much more pronounced optima centered at the 2% Z7-12Ac blend, while the rice-strain male optima was less pronounced with no discrimination between 2 and 4% (Unbehend et al. 2013; Figure 3). This strain-specific male response is consistent with the strain-specific female pheromone production in Florida, at least in the corn-strain, because corn- and rice-strain females produce around 2 and 4% Z7-12Ac, respectively (Groot et al. 2008; Unbehend et al. 2013). These results suggest that fall armyworm corn-strain males in Florida are adapted to the strain-specific female pheromone differences in the amount of Z7-12Ac, i.e. 2 vs. 4%.

The relative importance of Z11-16Ac is still unclear. In Costa Rica, the ternary blend of Z11-16Ac, Z9-14Ac and Z7-12Ac captured marginally more males than the binary blend of Z9-14Ac and Z7-12Ac in one test and marginally fewer in another, although neither effect was statistically significant (Andrade et al. 2000).

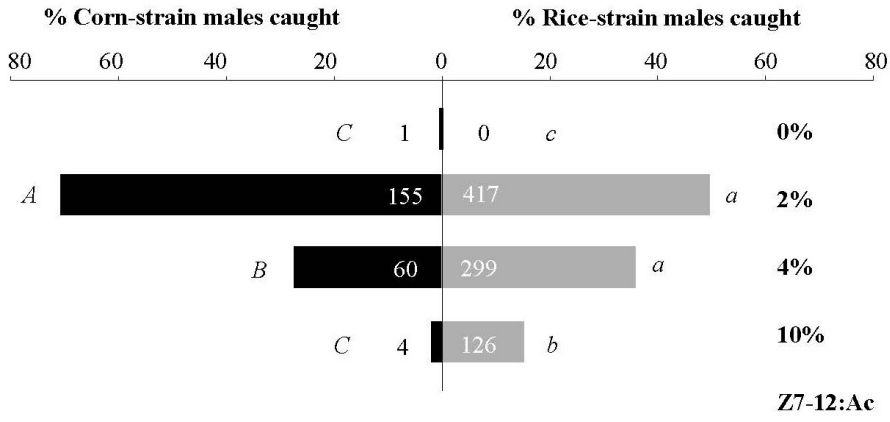

FIGURE 3. Strain-specific response of *S. frugiperda* males towards different doses of Z7-12Ac added to 300 µg Z9-14Ac in a corn and grass field in Florida. Different letters next to the bars indicate significant differences. Numbers in the bars indicate total number of males caught. See Unbehend et al. (2013) for more details.

Similarly, addition of Z11-16Ac to binary blends did not significantly increase trap catches in Brazil (Batista-Pereira et al. 2006) or Florida (Tumlinson et al. 1986; Unbehend et al. 2013). However, trapping experiments in Pennsylvania suggest that the addition of Z11-16Ac, together with Z9-12Ac, enhances male attraction to Z9-14Ac and Z7-12Ac (Fleischer et al. 2005). Also, in our field experiments in Florida we found that males were differentially attracted to the two-component blends without Z11-16Ac compared to the four-component blends with Z9-12Ac and Z11-16Ac between corn- and rice-strain habitats, suggesting a synergistic effect between the compounds (Unbehend et al. 2013)

The compound Z9-12Ac has been reported to occur in glands of females from North and South America (Descoins et al. 1988; Batista-Pereira et al. 2006; Groot et al. 2008). In Costa Rico and Florida, fall armyworm males were attracted to traps containing only Z9-12Ac (Jones and Sparks 1979; Andrade et al. 2000). When conducting experiments where we added different relative amounts of Z9-12Ac to the binary blend of Z9-14Ac and Z7-12Ac, we found that all ternary blends containing Z9-12Ac were similarly attractive as the binary blends without Z9-12Ac, both in corn- and rice-strain habitats in Florida (Unbehend et al. 2013). However, as pointed out above, a synergistic effect between Z9-12Ac and the other compounds cannot be excluded.

In summary, corn- and rice-strain males in Florida were mostly attracted to a corn-strain pheromone blend, at least in corn fields. Thus, there may be synergistic effects of host plant volatiles and sex pheromone components in corn fields. In grass fields, we did not find a preference for a corn- or a rice-strain pheromone blend in either strain. Strain-specific responses were found towards different doses of Z7-12Ac added to the major pheromone component Z9-14Ac, where corn-strain males were mostly attracted to 2% Z7-12Ac and rice-strain males were attracted to a wider range (2-10%). Together, these data suggest that strain-specific differences in the sexual communication of both strains do not cause assortative mating in Florida and thus are a weak prezygotic isolation barrier between the corn- and the rice-strain.

LEVEL AND DIRECTION OF HYBRIDIZATION BETWEEN THE TWO STRAINS

The fact that hybridization between the two strains can be observed in the field raises the question: are these strains in the process of divergence or convergence? RC-hybrid females have been found to be less likely to mate with any kind of male (C, R, RC or CR) and to produce a lower number of egg masses when they do mate (Pashley and Martin 1987; Whitford et al. 1988; Groot et al. 2010). Interestingly, RC-hybrid males did not show this deficiency and mated readily with all types of females (C, R and CR) (Groot et al. 2010). The fact that RC hybrid females are found to be mostly sterile in laboratory experiments seems to conflict with the field observation where mainly RC hybrids are found (see Section I). However, this contradiction makes the 'reproductive problem' of RC hybrid females a perfect postzygotic isolation barrier: if the most abundant individuals are at the same time

the least fertile ones, gene flow is maximally prevented at this stage. This thus indicates that these strains are in the process of divergence rather than convergence. Given the existence of RC hybrid females in the field, while in the laboratory these hybrid females are hardly able to reproduce, this hybrid incompatibility represents an essential contribution to the process of speciation between the two strains.

POSSIBLE EVOLUTIONARY SCENARIOS ON REPRODUCTIVE ISOLATION IN THE TWO STRAINS

Since the host association of the two strains does not seem to be as strict as early studies indicated, ecological specialization based on host plant choice does not seem the most likely cause of differentiation between the two strains in *S. frugiperda*. Other factors may have influenced a host association between the strains. One of these factors may be the presence of competitors or natural enemies on the ancestral host as has been suggested for other phytophagous insects (Berlocher and Feder 2002). Pashley et al. (1995) reported that over a two year period, fall armyworm larval mortality caused by parasites, predators and pathogens was higher in pastures than in corn fields. For this reason, the corn habitat may constitute a more protected environment than the rice habitat.

On the basis of the distribution of the two strains, particularly the distribution of the respective hybrids, and the behavioral differences between the two strains, we hypothesize that the rice-strain is the ancestral strain and corn-strain the derived strain (Juárez et al. 2014). Higher levels of genetic and behavioral homogeneity observed in the corn- than in the rice-strain suggests that the corn-strain went through a bottle-neck, i.e. that the corn-strain arose from a few individuals. Additionally, in corn fields a significant portion of rice-strain individuals as well as hybrids are found, specifically RC hybrids, while in rice fields the percent of corn-strain individuals or hybrids is generally much lower (Prowell et al. 2004; Saldamando and Velez-Arango 2010). The observation that males of both strains are mostly attracted to a corn-strain sex pheromone blend in corn fields, while this preference is not found in rice fields, is consistent with these results. Hybrid incompatibility is between R mothers and C fathers and not vice versa, i.e. RC hybrids are incompatible with any kind of male, whereas CR hybrids produce fertile and viable offspring. Together, these findings suggest that the rice-strain is the ancestral strain and the corn-strain is the derived strain.

CONCLUSION

In reviewing many studies on the host plant association of the two strains, host associations do not seem to be consistent when the mitochondrial COI marker is considered. In corn fields, more rice-strain individuals seem to be found than vice versa, and RC hybrids are also mostly found in corn habitats. Thus, habitat isolation alone does not seem to be a strong prezygotic isolation barrier between the corn- and the rice-strains. Similarly, strain-specific differences in the sexual communication system of

both strains alone do not appear strong enough to cause assortative mating within strains. However, differences in diel patterns of reproductive behaviors seem to be much more consistent than host-plant associations or differential sexual communication between the strains. Since a shift in timing can immediately inhibit gene flow, the strains may be 'timing strains' rather than 'host strains' or 'pheromone strains.' Furthermore, the postmating barrier of RC hybrid female sterility seems to be most likely a key element in the divergence of these two strains.

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9

GENERAL DISCUSSION

In the past 3 decades, ever since Dorothy Pashley discovered the two strains of *S. frugiperda* in 1985 (Pashley et al. 1985; Pashley 1986), the divergence of these strains and the underlying mechanisms have been subject to numerous molecular and behavioral studies. Even though the strains occur in sympatry and hybridize in the field, they do not form one panmictic population and are hypothesized to be in an incipient stage of ecological speciation in sympatry (Groot et al. 2010). Three prezygotic isolation barriers were identified that may influence the divergence to different extents: strain-specific host utilization, strain-specific sexual communication and strain-specific timing of reproductive activity during the night.

My thesis aimed to identify the relative importance of these isolation barriers for the divergence of the strains in three steps: (A) determining the strength and consistency of the phenotypic differentiation, (B) identifying the molecular basis of the isolation barriers and (C) evaluating the contribution of the isolation barriers to reproductive isolation between *S. frugiperda* strains.

Based on behavioral and molecular studies, I will first discuss the relative contributions of the prezygotic isolation barriers to the reproductive isolation between the two *S. frugiperda* strains for (1.1) host plant utilization, (1.2) sexual communication and (1.3) timing of reproductive activity. I will then discuss how these isolation barriers may effectively act together to minimize gene flow between the strains: (2.1) Synergistic effect of host plant volatiles and female sex pheromones, (2.2) adaptation of moth's circadian rhythm to host plant circadian rhythm, and (2.3) genetic linkage of female sex pheromone divergence and differential timing of reproductive activity.

IMPORTANCE OF THE THREE DIFFERENT ISOLATION BARRIERS

Strain-specific host plant differentiation

Strength and consistency of phenotypic differentiation

Even though the two strains were initially identified by detecting allozyme differences between specimens collected from different host plants (Pashley et al. 1985; Pashley 1986), recent studies as well as a critical comparison of results of previous studies indicate a weaker host association in the field than previously thought (see chapters 4 (Juárez et al. 2014) and 8 (Groot et al. 2015)), and results from bioassays in different laboratories differ widely (see chapters 3 (Hänniger et al. 2015a) and 8 (Groot et al. 2015)). However, oviposition preferences of female adult *S. frugiperda* do show some consistency between studies and some degree of host-association cannot be denied. So how is it possible that laboratory bioassays are not able to elucidate the behavior underlying this host association? Is it possible that laboratory studies have not yet addressed all possible mechanisms?

While many studies have addressed larval and reproductive stages of the fall armyworm, the eggs have so far been overlooked. The shape of egg clutches differs depending on the plant they are laid on (longer and thinner on bermudagrass com-

pared to corn) and some females prefer to lay many small egg clutches compared to the usual one or few big clutches (S. Hänniger, pers. obs.). The eggs could also differ in other features, possibly in a strain-specific manner. Within-species differences in egg adhesion, for example, are known from two populations of Melissa blue butterflies (*Lycaeides melissa*), one alpine and one from a lower elevation. While the eggs from the alpine population are only loosely attached and easily fall off, the population from the lower elevation fastens the eggs so strongly that they can overwinter on the host plants (Fordyce and Nice 2003). Eggs of the codling moth *Cydia pomonella* have also been shown to have a different adhesion to the leaves of different apple cultivars and are more easily blown off from some cultivars than others (Al Bitar et al. 2012). Maybe eggs of the two *S. frugiperda* strains also differ in adhesion. If, for example, eggs of the corn-strain show a weaker adhesion to the host plant, i.e. tall grasses such as corn and sorghum, the eggs would most likely fall into the whorl, where eggs are protected from predators and neonate larvae are in direct contact with their food. On small grasses, such as rice and bermudagrass, the eggs are more likely to fall to the ground so that emerging neonates have to locate and move to a food source. This scenario would lead to corn-strain larvae being predominantly found on corn or sorghum and a broader distribution of the rice-strain, which is the pattern reported in e.g. Pashley (1989) and Juárez et al (2014). When the eggs of both strains in e.g. corn fields are not subject to harsh weather conditions, both strains may develop, resulting in mixed populations, which are also observed (Pashley 1989; Juárez et al. 2014). This mechanism alone could possibly be sufficient to cause strain-specific distributions, even when neither larvae nor female adults show additional differences in preference or performance. However, as far as I know so far no study addressing egg adhesion in *S. frugiperda*, or host plant response to *S. frugiperda* eggs, has been published.

Another aspect of egg-plant-interaction that has not been explored in *S. frugiperda* is the broad spectrum of defensive plant responses that oviposition can induce, ranging from changes in the plant's volatile organic compounds to attract parasitoids or deter herbivores to direct defenses against the eggs, e.g. leaf necrosis or ovicidal substances (reviewed in Hilker and Fatouros 2015). For example, the eggs of the noctuid moth *Heliothis subflexa* elicit direct responses in *Physalis* plants. The plants form necrotic tissue and/or a bump of cells under the eggs of 64% of *H. subflexa* eggs, which leads to reduced hatching and increased removal of the egg from the plant (Petzold-Maxwell et al. 2011). A strain-specific plant response to the eggs of *S. frugiperda* could lead to a differential distribution of larvae in the field.

Molecular basis of host plant differentiation

Even though many studies have found molecular markers to show some extent of host association (Pashley 1989; Lu et al. 1992, 1994; Pashley and Ke 1992; Lu and Adang 1996; McMichael and Prowell 1999; Levy et al. 2002; Meagher and Gallo-

Meagher 2003; Nagoshi and Meagher 2003; Busato et al. 2004; Prowell et al. 2004; Nagoshi et al. 2006, 2008; Clark et al. 2007; Martinelli et al. 2007; Machado et al. 2008; Nagoshi 2010; Juárez et al. 2012), no study has specifically addressed the genetic basis of the host plant association. A QTL analysis could shed light on the genetic basis of host differentiation in *S. frugiperda*. However, such an analysis depends on a well-defined and reproducible strain-specific phenotype that can be measured in a large number of individuals in a short time. As behavioral and physiological studies have not yet yielded a bioassay that shows such a consistent phenotypical difference between the two strains, a QTL analysis cannot be conducted, so that the genetic basis of a possible host plant differentiation remains unclear.

Contribution of host plant differentiation to reproductive isolation

Field distributions do not exhibit a clear differential host association of the two strains and the currently available data are not sufficient to understand the mechanism(s) underlying the pattern of host association in *S. frugiperda*. Thus, host association is less pronounced than previously thought. Additionally, Kergoat et al. (2012) suggest a divergence of the strains more than 2 MY ago. This is long before the domestication or introduction of grasses like corn, sorghum, rice and sugarcane in the Americas, where the two strains of *S. frugiperda* occur (Munkacsi et al. 2007). Thus, a corn-strain preference for corn and sorghum and a rice-strain preference for rice and pasture grasses are not likely to be primarily responsible for the divergence between the strains (although other tall and small grasses were probably present 2 MY ago). It seems more likely that host plant differentiation between the strains interacts with the two other isolation barriers, sexual communication and allochronic differentiation, which together facilitate reproductive isolation between the strains.

Strain-specific differentiation of sexual communication

Strength and consistency of phenotypic differentiation

Female sex pheromone signal

Pheromone glands from rice-strain females from Florida showed consistently higher relative amounts of the critical secondary sex pheromone component Z7-12:OAc (and of Z9-12:OAc) than glands from corn-strain females, but there is also intra-strain variation in both strains. As M. Unbehend (née Marr) describes in her diploma thesis, the relative amounts of Z9-14:OAc, Z11-16:OAc, Z7-12:OAc and Z9-12:OA differ significantly between corn-strain females of different families (Marr 2009). It is not known whether rice-strain females exhibit a similar intra-strain variation in their pheromone composition, but this is likely.

Additionally, sex pheromone blends of corn-strain and rice-strain females vary between geographic locations (Tumlinson et al. 1986; Batista-Pereira et al. 2006;

Groot et al. 2008; Lima and McNeil 2009; Unbehend et al. 2013). For example, the relative amount of the major pheromone component Z9-14:OAc seems to vary between pheromone blends of corn-strain and rice-strain females in Louisiana (Lima and McNeil 2009) and Florida (Groot et al. 2008; Unbehend et al. 2013). Also, the E-isomer E7-12:OAc of the critical secondary component Z7-12:OAc shows a high variability between regions, as this is absent in the pheromone blends of females in Florida, Louisiana or French Guyana (Tumlinson et al. 1986; Descoins et al. 1988; Groot et al. 2008; Lima and McNeil 2009) but present in females from Brazil, where it is also behaviorally active in males (Batista-Pereira et al. 2006).

The laboratory environment may also affect the sex pheromone composition. Pheromonal changes induced by laboratory rearing are also known from other moth species, e.g. the redbanded leafroller moth, *Argyrotaenia velutinana* (Miller and Roelofs 1980), the turnip moth, *Agrotis segetum* (Löfstedt et al. 1985) and the cabbage looper moth, *Trichoplusia ni* (Haynes and Hunt 1990). Laboratory-reared corn- and rice-strain females from Florida exhibited a significant difference in their pheromone composition compared to field-collected females (Chapter 4 (Unbehend et al. 2013)): the relative amount of all three minor compounds (Z7-12:OAc, Z11-16:OAc and Z9-12:OAc) increased significantly in laboratory strains compared to field collected females, although the amount of the major sex pheromone component remained the same.

In summary, corn-strain as well as rice-strain females show within-strain variability in their sex pheromone, depending on different environmental factors. Despite this variability, the strains also exhibit a differentiation in the pheromone signal between strains that could enhance reproductive isolation, depending on the male response.

Male response to sex pheromone differences

Spodoptera frugiperda corn- and rice-strain males showed some inter-strain differences in their attraction to different pheromone blends. Corn-strain males were mostly attracted to pheromone lures with 2% Z7-12:OAc in Florida (Chapter 4 (Unbehend et al. 2013)), and corn-strain males showed some geographic variation in their response (Chapter 5 Unbehend et al. (2014)). In contrast, rice-strain males responded to a range of 2-10% Z7-12:OAc in the blend (Chapter 4 (Unbehend et al. 2013)), which was similar in all geographic regions (Chapter 5). These results suggest that the rice-strain males respond to a broader spectrum of pheromone blends than corn-strain males. However, in wind tunnel assays as well as field experiments, females or strain-specific pheromone blends did not attract significantly more males of their own strain (Chapter 4 and 5). Interestingly, trap catches depended on the field in which experiments were conducted (Chapter 5), suggesting an interaction effect between sexual communication and host plant volatiles (see also part 2 below).

Molecular basis of sexual communication

Our QTL analysis addressing strain-specific differences in the female sex pheromone composition revealed that multiple genomic regions are involved in the biosynthesis of the pheromone blend and its differentiation (Chapter 6 (Hänniger et al. 2015b)). However, all identified QTLs only explained a minor proportion of the variation between the strains ($R^2 \sim 4$-10%). Interestingly, one QTL for the critical secondary component Z7-12:OAc mapped to a region underlying a different isolation barrier, allochronic differentiation (Chapter 6), which will be discussed below (2.3. in this chapter). As for male response, the differences in abundance and sensitivity of component-specific or blend-specific odorant receptor neurons in the male antennae could underlie the different response ranges, like e.g. described for the European corn borer *Ostrinia nubilalis* (Anton et al. 1997), where Z-strain males have a very narrow response range compared to E-strain or hybrid males.

Contribution of sexual communication variation to reproductive isolation

Since the strain-specific blends as well as virgin females did not attract significantly more males of their own strain, it seems that sexual communication only constitutes a weak isolation barrier between the two strains of *S. frugiperda* (Chapters 4 and 5). This is consistent with findings of other studies from Florida (Meagher and Nagoshi 2013) and Louisiana (Pashley et al. 1992), where males of both strains were attracted to females of both strains. However, male trap catches differed depending on the field site (Chapter 5), and one genetic locus underlying female sex pheromone differences between the strain overlaps with the locus underlying the allochronic differentiation between the strains (Chapter 6). Thus, weak sexual communication differences may interact with other isolation barriers to facilitate reproductive isolation between the strains.

Strain-specific differentiation in daily rhythm

Strength and consistency of phenotypic differentiation

The strong strain-specific differentiation in daily rhythm is consistently found in different populations of *S. frugiperda* (Pashley et al. 1992; Schöfl et al. 2009, 2011; Hänniger et al. 2015b, unpubl.). Pashley et al. (1992) found almost no overlap between the mating time of the corn-strain early in the night (0–6 hours into scotophase) and the rice-strain late in the night (5–10 hours into scotophase), but these observations were based on 16 mating pairs. Using > 300 pairs, Schöfl et al. (2009) also found the corn-strain to mate significantly earlier than the rice-strain, but the time windows of the strains did overlap. This study revealed that not only was the mating time between the two strains shifted, but also all other activities including feeding and female and male calling (Schöfl et al. 2009). This time shift constitutes a strong phenotypic differentiation with the potential to interact with the other premating barriers, as discussed below.

Molecular basis of circadian differentiation

Only a few studies address the genetic basis of differentiation in timing behaviors in insects (reviewed in Groot 2014), all of them in flies (Tychsen and Fletcher 1971; Smith 1979; Ritchie and Kyriacou 1994; Sakai and Ishida 2001; Miyatake et al. 2002; Tauber et al. 2003). However, the timing of fly behavior is hardly comparable to that of moths, as flies can show activity throughout the day and also at night, while noctuid moths are truly night-active and even at night exhibit some hours of inactivity (Groot 2014). Investigating the molecular basis of the clear-cut timing differentiation in *S. frugiperda* thus bears a great potential to understand the molecular changes that underlie this phenotypic differentiation.

In our QTL analysis addressing the genetic basis of the strain-specific timing differences in *S. frugiperda*, we identified a major QTL that contained the candidate gene *vrille*, as part of the feedback loop of the circadian clock (Hänniger et al. 2015b, chapter 6). No other known clock gene was mapped to a QTL. We also identified strain-specific sequence polymorphisms in the vicinity of *vrille* promoter elements and *vrille* showed strain-specific expression differences.

As the circadian clock constitutes of interlocked transcriptional/translational feedback loops, a change in expression of one gene, e.g. *vrille*, should have an impact on the other gene products, e.g. *clock*, whose transcription is inhibited by VRILLE and which promotes the transcription of all other clock genes. The circadian clock is a pacemaker for physiological and behavioral processes and thus it seems possible that a change in the expression of one clock gene has an effect on the entire circadian clock and with this also on the timing of behavior.

Contribution of circadian differentiation to reproductive isolation

The most obvious mechanism of how circadian differentiation can contribute to the reproductive isolation between the two strains of *S. frugiperda* is that two partners with different time windows of activity, i.e. an early active corn-strain individual and a late active rice-strain individual, will rarely meet to mate (Schöfl et al. 2011). This constitutes a powerful reproductive isolation barrier on its own. Yet, there are some additional scenarios in which timing differentiation may interact with the other isolation barriers to contribute even more strongly to the divergence between the two strains of *S. frugiperda* (discussed below).

POSSIBLE INTERACTIONS BETWEEN ISOLATION BARRIERS

Synergistic effect of host plant volatiles and female sex pheromones

Spodoptera frugiperda males are able to perceive 16 different plant volatile organic compounds (VOCs) (Malo et al. 2004). Odor receptors in insect antennae are the first instance in odor perception and specific for the molecules they bind, i.e. VOCs from plant leaves (e.g. (*E*)-3-hexenol) do not bind to the receptors specific for pheromone components (e.g. (*Z*)-7-dodecenyl acetate) or for floral odorants (e.g.

phenylacetaldehyde) (reviewed in Hallem et al. 2006). Since males do not need to find plants as suitable oviposition sites, it is interesting that they express specific odor receptors for plant VOCs, which makes it likely that plant VOCs play an important role in the biology of the males.

Plant VOCs enhance the attraction of male moths to female sex pheromones (McNeil and Delisle 1989; Raina et al. 1992; Landolt and Phillips 1997; Reddy and Guerrero 2004; Yang et al. 2004). For example, in the codling moth (*Cydia pomonella*), a blend of the sex pheromone codlemone and different apple VOCs attracted significantly more males that codlemone (Yang et al. 2004). Also in *S. frugiperda*, host plant VOCs could play an important role in the attraction of male mating partners and could enhance strain-specific attraction to strain-specific pheromone blends. Male trapping experiments with pheromones in the field showed that male attraction is to some degree dependent on the host plant surroundings, suggesting a synergistic effect of host plant volatiles and female sex pheromones (Chapters 4, 5 and 8).

In addition to the possible interaction of host plant VOCs and female sex pheromones, plant compounds could influence the close-range male pheromone (Birch et al. 1990). While female Lepidoptera generally produce sex-pheromone *de novo* (Bjostad et al. 1987; Tillman et al. 1999; Jurenka 2003, 2004), this is not always true for males. For example, male queen butterflies (*Danaus gilippus*) have been shown to obtain pyrrolizidine alkaloids (PA) from their host plants and use them as precursors of the male pheromone emitted from their hair pencils (Eisner and Meinwald 2003). The male sex pheromone of *S. frugiperda* has not yet been identified, but the male sex pheromone may play a major role in sexual communication of *S. frugiperda*, as Schöfl et al. (2011) found evidence for female mating preference in both strains at close range. Thus, the male pheromone itself as well as potential host plant effects on the male pheromone biosynthesis could be a very interesting research field in *S. frugiperda*.

Adaptation of moth's circadian rhythm to host plant circadian rhythm

For herbivorous insects, it is generally important to time their own behavior according to the phenology of their host plants, e.g. to eclose from the eggs when the host is in high abundance (Berlocher and Feder 2002). When a timing difference in emergence period affects a difference in adult mating period (Wood and Keese 1990; Pratt 1994; Craig et al. 1997; Feder and Filchak 1999; Groman and Pellmyr 2000), allochronic isolation can occur (Berlocher and Feder 2002). Besides this well documented possibility of host plants influencing the seasonal timing of behavior, a daily circadian influence is possible too. *S. frugiperda* adults are frequently observed to mate in fields of suitable larval host plants (e.g. corn) (Luginbill 1928; Sparks 1979). They most likely use plant VOCs to locate host plants and mating sites, at least to some extent, as both sexes are able to perceive plant volatiles (Malo

et al. 2004). The emission of some VOCs of corn is under control of the circadian rhythm (Christensen et al. 2013) and thus follows a predictable pattern. Green leaf volatiles (GLVs) as well as mono-, homo- and sesquiterpenes are mainly emitted during the day, but larval feeding during the night induces the nightly emission of GLVs (Christensen et al. 2013). A comparative study of the emissions of VOCs of different grasses during the night has not been published, but different plants do emit volatiles at different times of the day or night, which is well studied for floral odors (e.g. Kolosova et al. 2001). It is possible that tall and small grasses exhibit different emission rates during the night, e.g. corn plants could emit VOCs earlier in the night than rice plants. Since the two strains of *S. frugiperda* are active in different time windows at night, they may encounter the emitted VOCs of different grasses and orientate towards them to find an oviposition and/or mating site. This may lead to a strain-specific distribution of eggs and thus larvae in the field.

Also the rhythmic activity of larvae may play a role in this context. When cabbage looper (*Trichoplusia n*i) larvae and *Arabidopsis thaliana* plants developed in the same photophase, the plant defended itself against herbivory very efficiently and suffered only minor damage, while larvae grew slowly. However, when larvae were time-shifted for 12 hours, they caused more damage and grew faster and more (Goodspeed et al. 2012; Jander 2012). Strain-specific timing of larval feeding has not been investigated in *S. frugiperda*. On artificial diet, larvae of both strains appear to be feeding continuously (S. Hänniger, personal observation). However, larvae on artificial diet do not need to deal with plant defense traits and larvae feeding on plants may thus exhibit a different behavioral pattern.

Genetic linkage of female sex pheromone divergence and differential timing of reproductive activity

When a female fall armyworm emits her pheromone to attract males, it is not only important *what* she emits, but also *when* she emits it. In wind tunnel assays, the males responded to the calling females, in their time window of activity, regardless of her pheromone composition (Unbehend et al. 2013) (Chapter 4). A female noctuid moth calling during the day will only rarely attract a mating partner, however attractive her pheromone composition may be. Thus, timing of pheromone emission and attractiveness of the pheromone are tightly linked. In two QTL analyses, one addressing the differential timing of mating activity and the other addressing the pheromonal divergence between the strains, we found one QTL that underlies both timing of mating and the relative amount of one of the critical sex pheromone components, suggesting a hitchhiking effect (Hänniger et al. 2015b) (Chapter 6). Via and West (2008) show that genomic regions that differ between two host races of the pea aphid (*Acyrthosiphon pisum pisum)* cluster around QTLs for traits driving ecological speciation. When inter-strain mating is reduced, the probability of recombination between the strains is reduced for the loci that defined

the strains and their vicinity. Via and West (2008) propose that this 'divergence hitchhiking' may greatly increase the possibility of speciation in sympatry.

THE RELATIVE IMPORTANCE OF PREZYGOTIC ISOLATION BARRIERS IN THE DIVERGENCE OF *SPODOPTERA FRUGIPERDA*

The two strains of *S. frugiperda* seem to have diverged to a point somewhere between 'host forms' (Juárez et al. 2014) and 'distinct species' (Dumas et al. 2015).

In Figure 1 I summarize my interpretation of the relative contributions of the different isolation barriers to the divergence of the strains, as well as possible interactions between the isolation barriers that could facilitate reproductive isolation. Host differentiation appears to be a weak isolation barrier, as field collections as well as behavioral assays addressing host association of the strains show inconsistent results (Juárez et al. 2014; Groot et al. 2015; Hänniger et al. 2015a) (Chapters 2,3 and 8). Also, pheromonal divergence seems to be a weak isolation barrier. While the female sex pheromone shows strain-specific differences, males do not seem to discriminate between the different blends (Unbehend et al. 2013, 2014) (Chapters 4 & 5).

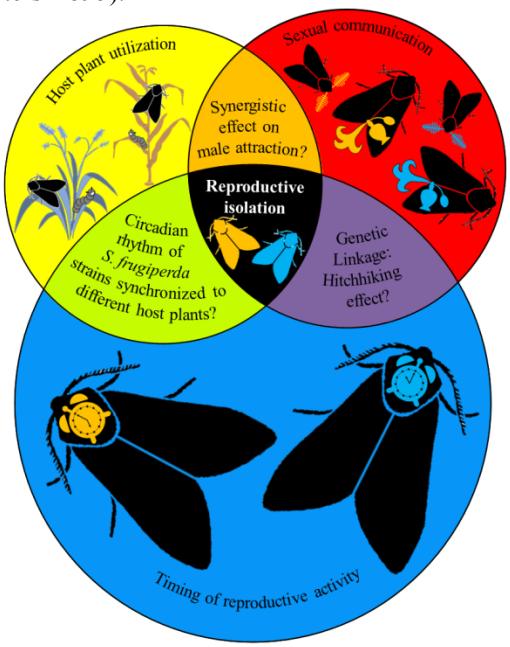

FIGURE 1. Suggested interactions and relative importance of the different prezygotic reproductive isolation barriers involved in the divergence of the corn- and rice-strain of *S. frugiperda*. The isolation barriers are represented in circles, the size of which corresponds to the relative importance. Possible interactions are indictaed, where circles overlap. The overlap of all 3 isolation barriers indicates that all three are necessary to drive the divergence of the two *S. frugiperda* strains.

The timing of reproductive activity seems to be the strongest of the isolation barriers, as it is consistently exhibited by the strains in different studies (Pashley et al. 1992; Schöfl et al. 2009, 2011; Hänniger et al. 2015b). Nevertheless, the time windows of reproductive activity do overlap between the strains (Schöfl et al. 2009), so that differential timing alone is most likely not sufficient to drive the divergence between the strains. It is likely that all three prezygotic isolation barriers interact and together facilitate reproductive isolation is *S. frugiperda.*

OUTLOOK

It is often said that in science a result raises more questions than it answers. Therefore, I would like to point out future research directions addressing the divergence between the two *S. frugiperda* strains that in my opinion ask the most interesting questions.

Experiments addressing egg-plant interactions

Despite the facts that the two strains of *S. frugiperda* are generally referred to as host-strains but solid evidence for a mechanism underlying a potential host differentiation has not yet been found, one important life stage, i.e. the egg stage and its interactions with the host plant, has been neglected so far. Also, more oviposition preference experiments are needed to determine the role of oviposition choice in the differential distribution of the fall armyworm strains in the field. Egg adhesion in both strains under different weather conditions should be analyzed, as well as direct or indirect anti-egg defense responses in plants.

Understanding the molecular clock

The two timing strains of *S. frugiperda* give a unique opportunity to investigate the molecular basis of timing differentiation of daily activity and its impact on speciation in sympatry. Considering *vrille* as the main candidate gene and protein, the effect of variation in the E-boxes of this gene may give new insights into the molecular basis of allochronic differentiation, and the functioning of the circadian clock in Lepidoptera in general. To elucidate interactions between the central and the peripheral clocks, it would also be interesting to investigate strain-specific differences in peripheral clocks of *S. frugiperda*, e.g. in the pheromone glands, and relate them to differences in the central clock in the brain.

The Gold Eye Mutation

Recently, a mutant eye color was discovered in a laboratory rice-strain population of *S. frugiperda* at the Max Planck Institute for Chemical Ecology, Jena (S. Hänniger, unpublished). The mutation causes the eye color of homozygous adult moths to be a bright yellow to orange, while wild type individuals have dark brown eyes (Figure 2D), hence the mutated strain is called Gold Eye Mutant (GEM).

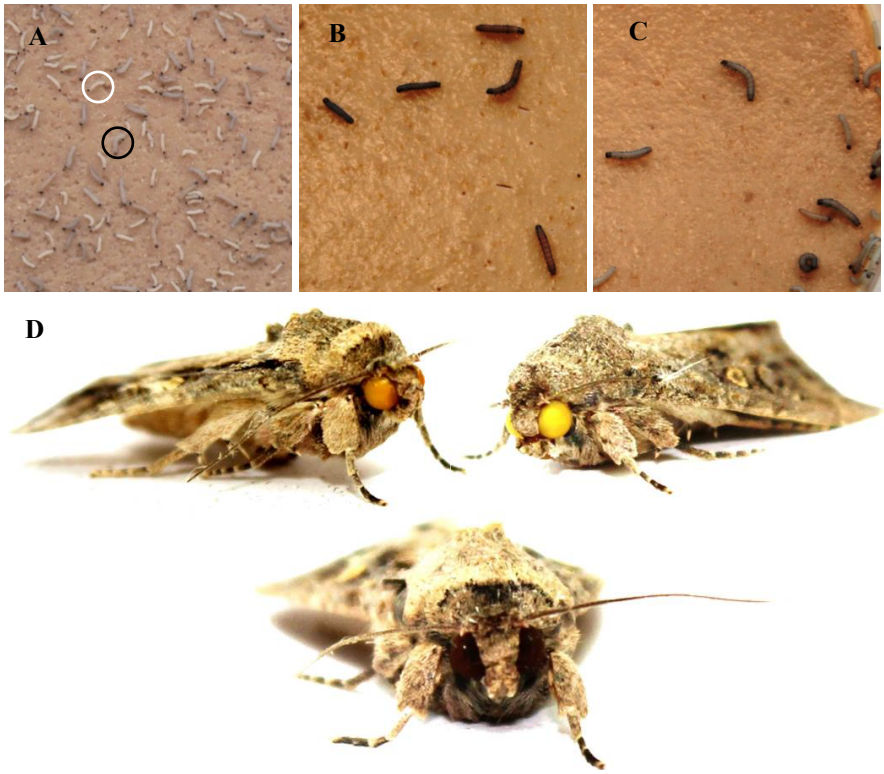

FIGURE 2. *S. frugiperda* GEM mutant and wild type larval skin color and adult eye color. (A) Second instar larvae with GEM mutation (e.g. white circle) and wild type (e.g. black circle). (B) Wild type larvae three days later. (C) Larvae with GEM mutation three days later. (D) Adult moths with GEM mutation (upper left: male, upper right: female) and wild type male (below).

Early instar larvae that are homozygous for the mutated allele appear much lighter than heterozygous or wild type larvae (Figure 2A-C), so the GEM mutants can be easily separated from the wild type in the early developmental phases. This mutation occurred only in the rice-strain. It may be useful as a visible marker in future crossing experiments or in transfection assays.

In summary, *Spodoptera frugiperda* is a fascinating species (complex) and still harbors many open questions. The answers to these questions can significantly advance the field of ecological sympatric speciation and should thus be explored diligently.

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2: No strain-specific differences in preference and performance of *Spodoptera frugiperda* larvae on typical host plants
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Revision of manuscript: M. Unbehend, S. Hänniger, R.L. Meagher, D.G. Heckel, A.T. Groot

5: Geographic variation in sexual attraction of *Spodoptera frugiperda* corn- and rice-strain males to pheromone lures
M. Unbehend, S. Hänniger, G.M. Vázquez, M.L. Juárez, D. Reisig, J.N. McNeil, R.L. Meagher, D.A. Jenkins, D.G. Heckel & A.T. Groot
Experimental design: M. Unbehend, S. Hänniger, D.G. Heckel, A.T. Groot
Conduct of experiments: M. Unbehend, S. Hänniger, G.M. Vázquez, M.L. Juárez, D. Reisig, J.N. McNeil, R.L. Meagher, D.A. Jenkins, A.T. Groot
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6: Genetic basis of prezygotic isolation in the fall armyworm
S. Hänniger, M. Unbehend, G. Schöfl, S. Gebauer-Jung, H. Vogel, D.G. Heckel & A.T. Groot
Conduct of experiments: G. Schöfl, S. Hänniger, M. Unbehend, A.T. Groot
Data analysis: S. Hänniger, M. Unbehend, G. Schöfl, S. Gebauer-Jung, H. Vogel, D.G. Heckel, A.T. Groot
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Revision of manuscript: S. Hänniger, M. Unbehend, G. Schöfl, S. Gebauer-Jung, H. Vogel, D.G. Heckel, A.T. Groot

7: Annotation of clock genes in the genome of *Spodoptera frugiperda*
S. Hänniger, P. Dumas, D.G. Heckel & A.T. Groot
Annotation: S. Hänniger, P. Dumas
Preparation of manuscript: S. Hänniger, P. Dumas
Revision of manuscript: D.G. Heckel, A.T. Groot

8: Evolution of reproductive isolation in *Spodoptera frugiperda*
A.T. Groot, M. Unbehend, S. Hänniger, M.L. Juárez, S. Kost & D.G. Heckel
Preparation of manuscript: A.T. Groot, M. Unbehend, S. Hänniger, M.L. Juárez, S. Kost
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CURRICULUM VITAE

Sabine Hänniger was born on July 9th, 1980 in Heiligenstadt, Germany, where she attended primary school and high school. Sabine started studying to obtain a 1st Staatsexamen (degree for high school teachers, equivalent to MA) in October 1999 at the Friedrich Schiller University in Jena, Germany. She took a detour via English and Politics, but then focused on Biology and Philosophy/Ethics. She completed her thesis in the field of Botany and Nutritional science entitled 'Untersuchungen zur infragenerischen Variabilität von Carotinoiden in der Gattung *Rosa*' ('Investigating infrageneric variability of carotenoids in the genus *Rosa*'). Sabine obtained her 1st Staatsexamen in Biology and Philosophy/Ethics in April 2009. She started to work on her PhD thesis at the Max Planck Institute for Chemical Ecology in Jena, Germany in May 2009, supervised by Astrid T. Groot, David G. Heckel and Steph B. J. Menken. During the time of her PhD student position she was on maternity leave for 11 months. In August 2015, Sabine accepted a teacher's trainee position in Jena, Germany.

'Getting an education was a bit like a communicable sexual disease.
It made you unsuitable for a lot of jobs and then
you had the urge to pass it on.'
Terry Pratchett, *Hogfather*