

OVERVIEW

The roles and interactions of reproductive isolation mechanisms in fall armyworm (Lepidoptera: Noctuidae) host strains

ASTRID T. GROOT, MELANIE MARR, DAVID G. HECKEL

and GERHARD SCHÖFL¹ Department of Entomology, Max Planck Institute for Chemical Ecology, Jena, Germany

Abstract. 1. The moth *Spodoptera frugiperda* presents an interesting opportunity to study the evolution of reproductive isolation, because it consists of two host races that may be in the process of speciation.

2. The two races exhibit habitat isolation through host-plant preference, and two types of behavioural isolation, i.e. differences in sex pheromone composition and timing of mating activity at night.

3. In this paper, we review the selection pressures acting upon these three barriers as well as their genetic bases, to address the question of how divergence of the two strains may have evolved.

4. We also address possible interactions between the three barriers, whether and how they may have evolved in concert, and we view the evolution of these three prezygotic isolation barriers in the light of postzygotic isolation.

Key words. Behavioural isolation, fall armyworm, habitat isolation, postzygotic isolation, reproductive isolation, *Spodoptera frugiperda*.

Introduction

The generation of biological diversity by speciation is one of the central themes of evolutionary biology (Dobzhansky, 1937; Mayr, 1963; Howard & Berlocher, 1998; Coyne & Orr, 2004). To learn about the generalities of this process we will need to identify the factors causing divergence between natural populations in many case studies. Generally, the main factors likely to be important in the evolution of reproductive isolation between populations include a gradual accumulation of genetic incompatibilities by drift, adaptive divergence in response to environmental variation, and rapid genetic changes associated with founder events (Tregenza, 2002). Barriers to reproduction can be divided into prezygotic versus postzygotic isolation (e.g. Coyne & Orr, 2004). Comparisons between closely related *Drosophilid* species suggested that their relative importance may depend on whether populations diverge in geographic isolation or in sympatry (Coyne & Orr, 1989, 1997). However,

interspecific comparisons have the disadvantage that we cannot distinguish between divergence contributing to speciation and divergence occurring afterwards. Important insights in speciation are likely provided by examining contemporary patterns of reproductive isolation among partially isolated populations (e.g. Berlocher & Feder, 2002; Drès & Mallet, 2002; Via, 2002; Via & West, 2008). Understanding the process of speciation is important for explaining the diversity of life in general and, from a more applied perspective, crucial for conservation biology as well as pest management.

In this review we discuss the contributions of ecology, behavioural mechanisms, and genetic incompatibilities to reproductive isolation between fall armyworm host strains. We aim to assess whether and how these isolating barriers may act in concert to minimise gene flow between the two strains in sympatry and review the current state of knowledge on the underlying genetics of these traits.

Fall armyworm host strains

In the noctuid moth *Spodoptera frugiperda* J. E. Smith (fall armyworm; Lepidoptera: Noctuidae) two sympatrically occurring, major genetic groups have been recognised that show host-plant associated genetic variation (Pashley *et al.*,

Correspondence: Astrid T. Groot, Department of Entomology, Max Planck Institute for Chemical Ecology, Hans Knoell Strasse 8, 07745 Jena, Germany. E-mail: agroot@ice.mpg.de

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1985; Pashley, 1986). Larvae collected from maize, sorghum, and cotton typically represent the corn strain, whereas larvae collected from rice and various pasture grasses represent the rice strain (Pashley, 1986, 1989). Originally, a diagnostic esterase allozyme marker and three other significantly strain-biased protein variants were discovered by an electrophoretic survey of allozyme variation in samples from corn fields and rice paddies (Pashley *et al.*, 1985; Pashley, 1986). Subsequently, several additional strain-biased or strain-diagnostic molecular markers have been identified: the two strains differ in several DNA sequence variants in the mitochondrial cytochrome oxidase I and ND1 genes (Pashley, 1989; Pashley & Ke, 1992; Lu & Adang, 1996; Meagher & Gallo-Meagher, 2003; Nagoshi *et al.*, 2006a), strain-biased and strain-specific amplified fragment length polymorphism (AFLP) markers (McMichael & Prowell, 1999; Busato *et al.*, 2004; Prowell *et al.*, 2004), and the FR repetitive nuclear DNA sequence extensively present in the rice strain and mostly absent from the corn strain (Lu *et al.*, 1994; Nagoshi & Meagher, 2003b; Nagoshi *et al.*, 2008). To date, no diagnostic morphological features have been described that distinguish these two strains, but they differ in a number of physiological, developmental, and behavioural features (Pashley, 1988b; Pashley *et al.*, 1992, 1995; Veenstra *et al.*, 1995; Groot *et al.*, 2008; Lima & McNeil, 2009; G. Schöfl, A. Dill, D. G. Heckel, A. T. Groot, unpubl. res.). It has been suggested that these two genetically differentiated groups may actually be reproductively isolated cryptic sibling species (Pashley, 1986; Drès & Mallet, 2002). More recently, however, the detection of individuals non-concordant for two or more strain-specific markers (e.g. the esterase genotype of one strain and the mtDNA of the other) suggests hybridisation rates in the field of up to 16% (Prowell *et al.*, 2004) or even more (Nagoshi & Meagher, 2003a; Nagoshi *et al.*, 2006b; Machado *et al.*, 2008). Such high hybridisation rates argue for incipient, rather than completed speciation.

Despite these relatively high hybridisation rates in at least some of the areas of occurrence, the two strains have been found throughout South and North America (Meagher & Gallo-Meagher, 2003; Busato *et al.*, 2004; Prowell *et al.*, 2004; Nagoshi *et al.*, 2007; Machado *et al.*, 2008; Vélez-Arango *et al.*, 2008) and can be identified using the same strain-specific mitochondrial markers (Nagoshi *et al.*, 2007). Apparently, there is an array of reproductive isolating barriers active throughout the Western Hemisphere that prevents these strains from merging into one panmictic population. Apart from habitat isolation, two behavioural isolation mechanisms may contribute to reproductive isolation in *S. frugiperda*: differences in the female pheromone composition (Groot *et al.*, 2008; Lima & McNeil, 2009) and differential timing of reproductive activity at night (Pashley *et al.*, 1992; Schöfl *et al.*, 2009b G. Schöfl, A. Dill, D. G. Heckel, A. T. Groot, unpubl. res.). No intrinsic hybrid inviability has been found for the two host strains (G. Schöfl, unpubl. res.), but extrinsic ecological or behavioural sterility may affect overall reproductive isolation.

Habitat isolation

In phytophagous insects, habitat isolation may arise if genetically-based biases in habitat or host use decrease the probability of heterospecific encounters (Funk *et al.*, 2002; Coyne & Orr, 2004; Rundle & Nosil, 2005). Biases in host use may be caused either by differential performance on their respective host plants, by specific ovipositional preferences in adult females, or by a combination of both. There is also evidence in some species for 'natal habitat preference induction', in which early experience in a particular habitat increases the probability that habitat is chosen after adult dispersal (Davis & Stamps, 2004). Once a bias in host use is established, it may contribute to reproductive isolation, either directly through spatial or temporal segregation of the preferred host plants or pleiotropically by, for example, host-plant-mediated mate attraction or post-mating isolation if hybrid offspring show decreased viability because of a reduced capacity to use either parental host (Coyne & Orr, 2004; Rundle & Nosil, 2005). (Potential pleiotropic effects are discussed below.)

Analysing populations from Louisiana, Florida, Puerto Rico, Guadeloupe, and French Guiana, Prowell *et al.* (2004) found relatively high frequencies of rice-strain individuals (19%) on corn plants, and lower frequencies of corn-strain individuals (5%) on pasture grasses or rice plants. However, host use varied from region to region as well as from year to year (Prowell *et al.*, 2004). In Brazil (Rio Grande do Sul), Machado *et al.* (2008) found that all larvae collected from rice carried the rice-strain haplotype, while 83% of the larvae collected from corn carried the corn-strain haplotype. In Colombia, similar results were obtained; the corn strain was found exclusively on corn or cotton, while the rice strain was found mainly on rice and in low proportions on corn and cotton (Vélez-Arango *et al.*, 2008). Finally, similar patterns of asymmetric host use of the two strains were reported for a number of populations from Florida, Texas, and Brazil (Meagher & Nagoshi, 2004; Nagoshi *et al.*, 2006b; Nagoshi *et al.*, 2007). Thus, these studies indicate a similar differentiation in host use of the two strains throughout their range. The corn strain is largely restricted to corn, cotton, or sorghum. The rice strain predominates on pasture grasses and rice but may also utilise typical corn-strain habitats to variable degrees. However, the proximate causes for the differential host use between the host-associated strains in fall armyworm remain elusive.

Proximate causes of differential host use

Even if larvae of the two strains are able to utilise the same host plants, differences in their competitive abilities may lead to selection against 'immigrants' and thus promote host differentiation (Nosil *et al.*, 2005). *Spodoptera frugiperda* is polyphagous and can feed on many different plant species (Pashley, 1988a). For the fall armyworm host strains, a number of reports have described differential effects of plant hosts on viability and development of the two strains, but often their results are not consistent (Pashley, 1988b; Whitford *et al.*, 1988; Pashley *et al.*, 1995; Meagher *et al.*, 2004; Stuhl *et al.*, 2008). For example, concerning the rice strain, Pashley (1988b)

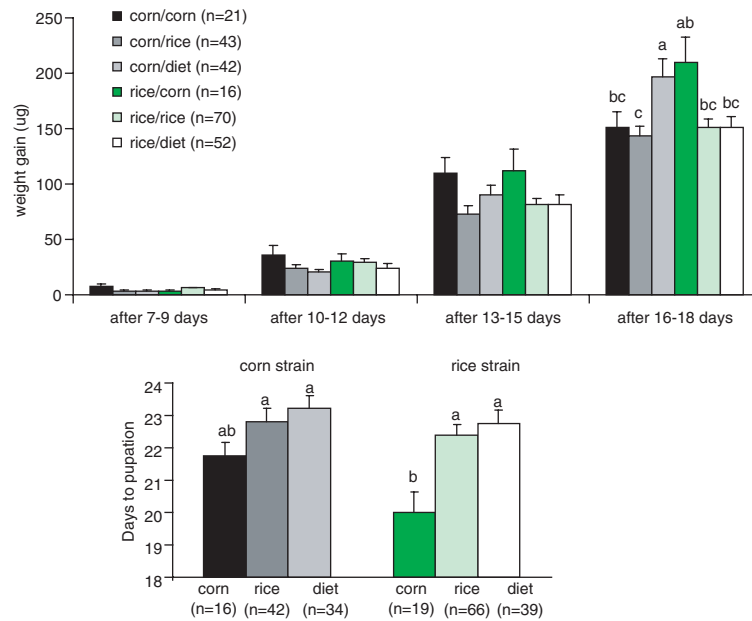


Fig. 1. Mean weight gain (\pm SEM) of *Spodoptera frugiperda* corn- and rice-strain larvae on different diets (corn leaves, rice leaves, artificial pinto bean diet). The eggs of nine fertile JS3C and 16 fertile OnaR families were transferred onto Petri dishes containing artificial pinto bean diet. The hatched larvae lived on diet for 4–6 days, after which they were transferred to plastic cups comprising fresh plant material or diet. Corn (*Zea mays*) and rice (*Oryza sativa*) plants were reared in the greenhouse and their leaves were cut into pieces and fed to the larvae. We started with 90 rice-strain and 60 corn-strain individuals per treatment (diet, corn leaves, rice leaves), so that a total of 270 OnaR rice-strain and 180 JS3C corn-strain individuals were used for the experiment. At the beginning of the experiment the plant material was changed every 3 days. After 10 days of the experiment the plant material was changed every day. The artificial pinto bean diet was cut into cubes [2 cm (h) \times 2 cm (l) \times 2 cm (d)] and exchanged every 9–12 days. Each larva was weighed every third day of the experiment until it pupated. The date of pupation, the date of eclosion, the sex of the eclosed individual, and the mortality rate up to pupation and eclosion was also examined.

and Pashley *et al.* (1992) found a negative effect on larval weight and larval developmental time when larvae were reared on corn plants as compared to rice plants, while pupal weight was significantly higher on corn, and larval survivorship was similar on both host plants. Whitford (1988) also reported a negative effect on larval weight and larval developmental time, but on pupal weight as well, when rice-strain larvae were reared on corn or sorghum, and found no effect of host plant on larval survival either. Concerning the corn strain, developmental differences with respect to host plants were less pronounced and more variable across these studies. Overall, both strains performed equally well on rice, but corn strain tended to outperform rice strain on corn plants (Pashley, 1988b; Whitford *et al.*, 1988; Pashley *et al.*, 1995). In contrast, Meagher *et al.* (2004) found that rice-strain larvae were significantly larger and developed faster on corn and sorghum–sudangrass hybrids (both corn-strain hosts) than corn-strain larvae, although typical rice-strain host plants were not tested.

We observed a similar pattern as Meagher *et al.* (2004) in a feeding assay that assessed larval weight gain and developmental time of both strains on corn and rice plants, compared to a control using artificial diet. We reared both strains from neonates in individual cups on leaves of either plant, which were refreshed every 2–3 days. We did not find any difference in larval weight of either strain on the three diets until day 16–18 (Fig. 1). Rice-strain individuals were heavier when reared on corn leaves (although not significantly, likely due to

sample size) than on rice leaves or artificial diet, while corn-strain larvae were heaviest when reared on artificial diet. Rice-strain individuals developed significantly faster when reared on corn plants compared to the other food sources as well as compared to the corn strain (Fig. 1).

The variability across studies in the physiological responses of larvae of the two strains to different host plants may reflect differences in the geographic origin of the samples used, or they may reflect genetic and phenotypic variation in wild populations (Stuhl *et al.*, 2008). No consistent effects of host plant on survival have been found, and larval performance does not translate into differential fecundity (Pashley *et al.*, 1995). It thus seems unlikely that the consistent host differentiation found in field samples from a wide range of populations can be explained by differential host-plant adaptations of the larvae.

Adult oviposition preference can also contribute to host-plant specificity. The only study to date that examined oviposition preference of corn- and rice-strain females (Whitford *et al.*, 1988) showed a preference of corn-strain females to oviposit on corn and sorghum in two replicate trials, while rice-strain females preferred to oviposit on bermudagrass in only one of the two trials. While this finding is in line with the overall distribution of the strains in nature, more oviposition preference studies are needed, preferably with populations of several geographic origins. The possibility that post-natal experience could influence adult habitat choice should also

be investigated, as evidence for this phenomenon exists in *Spodoptera littoralis* (Anderson *et al.*, 1995).

Host differentiation and reproductive isolation

How much the bias in host use directly contributes to reproductive isolation between fall armyworm host strains is unknown. The fall armyworm is known to migrate long distances annually (Luginbill, 1928; Nagoshi & Meagher, 2008), which makes it unlikely that micro- or even macroscale segregation of suitable habitats alone can restrict encounters between the two strains. Many herbivore species mate on or near their host plants and oviposition sites, a tendency that may restrict encounters between heterospecifics (Funk *et al.*, 2002; Rundle & Nosil, 2005). We know of no reports that indicate whether fall armyworm females preferentially call or mate in proximity to their host plants or whether they seek out oviposition sites after having mated.

Two studies suggest a strong seasonal component to host use. In samples from Southern and Central Florida from corn (Nagoshi & Meagher, 2004) and from the Mato Grosso (Brazil) from sorghum (Nagoshi *et al.*, 2007), the corn strain dominated in spring collections while the rice strain dominated in autumn collections. This is likely due to seasonal differences in the availability of host plants, which may affect the abundance of the two strains and thus cause some seasonal temporal isolation (Pashley *et al.*, 1992).

Behavioural isolation

Behavioural isolation through sexual communication

The most common type of behavioural reproductive isolation in moths is through sexual communication. Females attract males from a distance by emitting a species-specific sex pheromone, usually consisting of two or more volatile compounds, that is released from the sex pheromone gland in the scotophase (e.g. Cardé & Haynes, 2004). So far the sex pheromones of about 1600 moth species have been identified (El-Sayed, 2008). The sex pheromone of *S. frugiperda* was identified in 1986 (Tumlinson *et al.*, 1986) and field experiments have been conducted in several regions (Mitchell *et al.*, 1985; Tumlinson *et al.*, 1986; Andrade *et al.*, 2000; Malo *et al.*, 2001; Batista-Pereira *et al.*, 2006). However, none of these studies mentioned whether corn or rice-strain females

were analysed or whether corn or rice-strain males were attracted to the different blends, even though both strains have been recognised in North America (Pashley *et al.*, 1985) as well as in Brazil (Busato *et al.*, 2004). One field experiment assessed strain-specific attraction: Pashley *et al.* (1992) used 1–2 day old live virgin females of each strain as bait in pheromone traps, one female per trap, in fields that contained host plants of both strains in Louisiana in two consecutive years. In this study a slight but significant strain-specific attraction was found, although corn-strain females also attracted a large number of rice males. This may not be so surprising, because a total of only 77 corn-strain males were caught against a total of 727 rice-strain males, indicating a much larger abundance of a rice-strain population at the time of the experiment.

To assess whether corn and rice-strain females differ in their sex pheromone composition, we analysed the sex pheromone from both strains (Groot *et al.*, 2008). The corn strain (JS3C) was obtained from corn plants near Homestead, Florida, in 2004, while the rice-strain (OnaR) colony originated from pasture grasses in Ona, Florida, in 2003. Glands were extracted from 2–3-day-old virgin corn and rice-strain females, as well as from hybrid female offspring (see Groot *et al.*, 2008 for a detailed description). Our analysis included the four compounds that have been found to be attractive for *S. frugiperda* males: the major component Z9-14:Ac; the crucial secondary pheromone component Z7-12:Ac without which *S. frugiperda* males are not attracted (Mitchell *et al.*, 1985; Tumlinson *et al.*, 1986; Descoins *et al.*, 1988; Andrade *et al.*, 2000; Malo *et al.*, 2001; Fleischer *et al.*, 2005; Batista-Pereira *et al.*, 2006); Z11-16:Ac, the addition of which attracted significantly more males in Pennsylvania (Fleischer *et al.*, 2005), Mexico and Costa Rica (Andrade *et al.*, 2000; Malo *et al.*, 2001), but not in Florida (Tumlinson *et al.*, 1986); and Z9-12:Ac, which also increased attraction of *S. frugiperda* males, at least in Pennsylvania (Fleischer *et al.*, 2005).

Considering these four components, we consistently found that Z11-16:Ac was significantly higher, while Z7-12:Ac and Z9-12:Ac were significantly lower in corn than in rice female glands (Fig. 2). The major component Z9-14:Ac was present in similar amounts in both females; however significantly lower amounts were found in corn females when they were injected with pheromone biosynthesis activating neuropeptide (PBAN). Hybrid females contained similar amounts of Z9-14:Ac and Z11-16:Ac as found in their mothers, indicating a maternal inheritance or maternal effect, while the other two components showed a corn-dominant inheritance (Groot *et al.*, 2008).

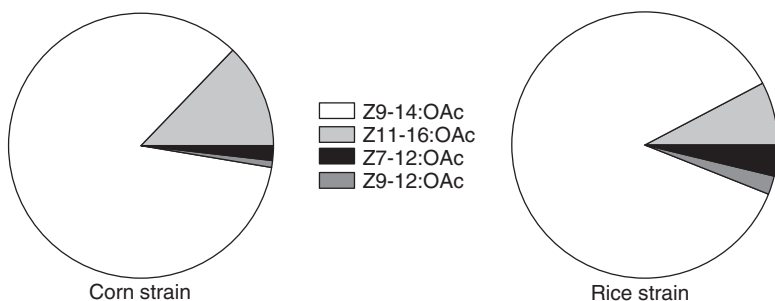


Fig. 2. Sex pheromone composition of the corn and rice strain when considering the four components that have been shown to be important in the attraction of males. The major pheromone component Z9-14:Ac is present in similar amounts in both strains, however corn-strain females contain significantly more Z11-16:Ac and significantly less Z9-12:Ac and Z7-12:Ac than rice-strain females. See Groot *et al.* (2008) for further details.

Lima and McNeil (2009) assessed strain-specific sex pheromone differences in three of the above-mentioned components (Z9-14:OAc, Z11-16:OAc, and Z7-12:OAc) in strains originating from Louisiana. They found that corn-strain females contained a higher amount of the major component Z9-14:Ac and lower amounts of Z11-16:OAc and Z7-12:OAc than rice-strain females. Thus, strains from Louisiana gave contrasting results to strains from Florida. Perhaps strain-specific differences in pheromone composition vary across geographic regions, as has been found in many other moth species (Klun, 1975; Cardé *et al.*, 1977; Guerin *et al.*, 1984; McElfresh & Millar, 1999, 2001; Gemeno *et al.*, 2000; Gries *et al.*, 2001; El-Sayed *et al.*, 2003; Groot *et al.*, 2009b). Interestingly, the major component Z9-14:OAc showed maternal inheritance in Louisiana hybrids as well as in Florida hybrids. To determine the biological significance of these sex pheromone differences, it will be important to evaluate whether different pheromone blends are differentially attractive to corn and rice-strain males in the field across the geographic regions.

In addition to the long-range sex pheromones of females, male moths may also emit short-range pheromones by extruding abdominal hairpencil scales (Birch *et al.*, 1990; Cardé & Haynes, 2004). Although these hairpencils are usually displayed extensively during courtship, their effects can range from no detectable influence on female behaviour (Gothilf & Shorey, 1976) to being a key stimulus in determining mate acceptance and mate choice (Conner *et al.*, 1981; Löfstedt *et al.*, 1989; Hillier & Vickers, 2004). In a number of noctuid species benzaldehyde has been identified as a component of the volatile male hairpencil secretions (Birch, 1974; Weatherston & Percy, 1977; Fitzpatrick *et al.*, 1985). Other chemicals commonly found in plants (e.g. linalool, cresol) have been identified in noctuid male hairpencils as well (Birch & Hefetz, 1987). Birch and Hefetz (1987) suggest that females may already have receptors for plant-derived compounds since they show behavioural responses to them (Birch & Hefetz, 1987). However, it is not known whether these compounds emitted from the male hairpencils are sequestered from plant hosts (as in e.g. Arctiids; Eisner & Meinwald, 1995) or synthesised *de novo*. In *S. frugiperda*, male pheromones have not yet been identified. Even so, males do possess abdominal hair pencils and display them overtly before mating (G. Schöfl, A. Dill, D. G. Heckel, A. T. Groot, unpubl. res.). Mate choice experiments in the laboratory indicate that non-random mating between the two host strains depends not only on the allochronic separation of reproduction during the night (see next section), but also involves a time-independent component of female choice, possibly influenced by male pheromones emitted during courtship (Schöfl *et al.*, 2009a).

Behavioural isolation through differential timing of reproduction

Spodoptera frugiperda offers a rare example of host-strain isolation by differential timing of reproductive activity at night. The two strains are allochronically separated in their timing of female calling (extrusion of the pheromone gland) and mating at night: the corn strain is active early at night, while

the rice strain is active late at night (Pashley *et al.*, 1992; G. Schöfl, A. Dill, D. G. Heckel, A. T. Groot, unpubl. res.). The only other example in lepidopterans where differences in the timing of reproductive activity at night have been described for two host strains is the rice stem borer *Chilo suppressalis* (Konno *et al.*, 1996; Samudra *et al.*, 2002). Differences in the timing of female calling have been found between closely related species, such as *Spodoptera latifascia* and *S. descoinsi* (Monti *et al.*, 1997), four sympatric limacodid moth species (Sasaerila *et al.*, 2000), two species of plume moths (Haynes & Birch, 1986), and the two closely related species *Heliothis virescens* and *H. subflexa* (Heath *et al.*, 1991). Recently, a study on a large number of co-occurring species of Neotropical skippers (Hesperiidae), demonstrated significant temporal partitioning of diurnal flight activity among subfamilies, genera, and species (Devries *et al.*, 2008). This suggests that temporal displacement of activity may be an important but hitherto largely neglected factor in reproductive isolation.

When the temporal displacement of reproductive activity between the two strains of *S. frugiperda* was first described (Pashley *et al.*, 1992), hardly any overlap in the timing of copulations at night was observed. Under similar conditions, but using populations of different geographic origin and a much larger sample size, we found that both strains were significantly differentiated for female calling and copulation times as well, but also showed a considerable overlap in the onset times of mating activity (G. Schöfl, A. Dill, D. G. Heckel, A. T. Groot, unpubl. res.). Specifically, onset of female calling and copulation in the rice strain was restricted to the last third of scotophase, while the corn strain was more flexible and would start calling and mating throughout the night. Interactions between the sexes in heterogamic pairings implied that rice-strain females are in general more restricted in the timing of copulation than rice-strain males (G. Schöfl, A. Dill, D. G. Heckel, A. T. Groot, unpubl. res.). This suggests that the allochronic reproductive isolation between the strains may be asymmetric between strains and sexes, and less pronounced than previously thought.

Possible interactions between the prezygotic isolation barriers

Viewed in isolation, each of the above-mentioned prezygotic isolation barriers seems unlikely to be sufficiently strong to prevent the two strains of *S. frugiperda* from merging into one panmictic population. However, the total reproductive isolation achieved in nature may potentially be much higher than the individual evaluations of habitat isolation, differential attraction to sex pheromones, and allochronic separation of reproductive activity suggest. Barriers may act simultaneously and could therefore contribute multiplicatively rather than cumulatively to reproductive isolation, or barriers may enhance each other through pleiotropic interactions (Fig. 3).

Habitat isolation and differential mate attraction via sex pheromones may interact pleiotropically if females preferentially call on or near their oviposition sites and if males tend to alight on their larval host plants. This effect may be further

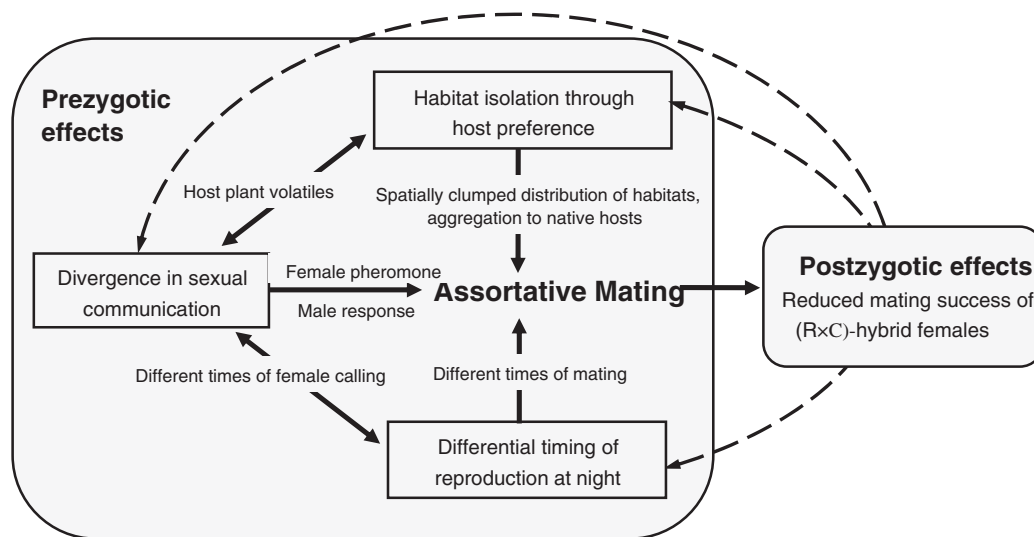


Fig. 3. Possible interactions between the different reproductive isolation barriers. Double arrows, potential reciprocal interaction; single arrows, one-way effect; dashed lines, potential extrinsic postzygotic effects, i.e. hybrids may be less effective in their sexual communication, not able to find their host plants, or timing reproduction differently than the parental strains.

enhanced by a spatially clumped distribution of the hosts. For instance, in two host races of the larch budmoth, assortative mate attraction is strongly affected by the tree species used as a calling substrate by the females and by the host neighbourhood structure (Emelianov *et al.*, 2001). This has been shown to be due to a genetically-based preference of adults of both sexes to alight on their on native host trees (Emelianov *et al.*, 2003). Adult attraction to native host plants has also been demonstrated in tephritid flies (Feder *et al.*, 1994) and aphids (Via, 1999). An association of females with host plants can be caused if, for example, female pheromone production is dependent on females perceiving the odour of a host plant (McNeil & Delisle, 1989; Raina *et al.*, 1992, 1997) or if pheromone release is affected by plant odours (Hendrikse & Vos Bunemeyer, 1987; McNeil & Delisle, 1989; Landolt & Phillips, 1997). In females, the coordination of pheromone production or release with the availability of a suitable host for oviposition is considered adaptive (Raina *et al.*, 1992).

Male attraction to host plant for the fall armyworm is more difficult to explain. In the cases of apple maggot flies and aphids, males are thought to rely on host cues to find mates (Feder *et al.*, 1994; Via, 1999). In the larch budmoth, mate attraction is through host-independent sex pheromones. Nevertheless, males show heritable alighting preferences for their respective host plants independent of potential mating partners (Emelianov *et al.*, 2003), which may also be the case in the fall armyworm. For this species, we do not know (yet) whether mating occurs mostly on or near host plants, and/or whether males are attracted solely to the female sex pheromone or to a combination of host-plant volatiles and strain-specific sex pheromone.

Host-plant chemistry might also directly affect pheromone production and/or the male response to female pheromones (reviewed by McNeil & Delisle, 1989; Landolt & Phillips, 1997). In Noctuidae, female pheromone is produced *de novo*

every night and the components do not have host-plant precursors (Bjostad *et al.*, 1987; Tillman *et al.*, 1999; Jurenka, 2004). However, male courtship pheromones that are active at close range have been found to be affected by the host plant on which the males were raised (reviewed by Birch *et al.*, 1990). At the receiver side, males may be more attracted to a combination of pheromone and plant volatiles than to pheromone alone (Dickens *et al.*, 1990, 1993; Landolt *et al.*, 1992; Hardie *et al.*, 1994). One study has been conducted to assess a possible synergistic effect between the commercial Trécé sex pheromone lure of *S. frugiperda* and phenylacetaldehyde (Meagher & Mitchell, 2001). This compound has been isolated from many flowering plants, including corn (Cantelo & Jacobson, 1979). However, when comparing traps baited with pheromone alone to traps baited with pheromone plus this flower compound in a corn field in Florida, *S. frugiperda*, males were more attracted to traps with pheromone alone than to traps where phenylacetaldehyde was added (Meagher, 2001).

Under field conditions, differential mate attraction via sex pheromones and temporal displacement of reproduction are also expected to reinforce each other as a barrier to reproduction. Corn-strain females will tend to attract males early in the night while rice-strain females attract males late at night. The only study so far to test for cross attraction between the two strains in the field, using live females as a lure (Pashley *et al.*, 1992), did not record the time of night at which potential mating partners homed in on the calling females.

Genetic basis of the prezygotic isolation barriers

Habitat isolation

The study of the genetic basis of host-plant preference is still in its infancy (Berlocher & Feder, 2002; Feder *et al.*,

2005) and nothing is known about the genetic architecture of either larval performance differences or oviposition preferences in *S. frugiperda*. One quantitative trait locus (QTL) analysis has been described for *Drosophila sechellia* where two QTL were identified to be involved in oviposition site preference (Jones, 1998). In F₁ hybrids, intermediate as well as dominant preferences for one of the parental plants have been found (e.g. Sheck & Gould, 1995; Sezer & Butlin, 1998; Via *et al.*, 2000; Emelianov *et al.*, 2003). Some studies found sex linkage of oviposition preference (Thompson, 1988; Scriber *et al.*, 1991), while others did not (Sheck & Gould, 1995). All studies so far indicate that host-plant utilisation is a trait governed by multiple, mostly autosomal loci (Hagen, 1990; Thompson *et al.*, 1990; Sheck & Gould, 1995). Recently, two genes were identified that affect the oviposition site preference in *Drosophila sechellia* (Matsuo *et al.*, 2007): odorant binding protein Obp57d and Obp57e. This finding makes the class of odorant binding proteins possible candidate genes that may be involved in oviposition preference in other species as well.

Sexual communication

Most studies on moth pheromone genetics have concentrated on the genetic architecture of differences in sexual communication that can be explained by one gene (e.g. Jurenka *et al.*, 1994; Roelofs & Rooney, 2003; Dopman *et al.*, 2004). Analyses of the genetic basis of sex pheromone differences between two closely related noctuid moths, *Heliothis virescens* and *H. subflexa*, shows variation that cannot be explained by a single genetic change (Groot *et al.*, 2004, 2009a; Sheck *et al.*, 2006). These analyses revealed that five chromosomes and thus at least five autosomal loci are involved in the different pheromone blends between the two species. Since the sex pheromone of *S. frugiperda* is also a multi-component blend and differences between the two strains are complex (Groot *et al.*, 2008), these differences are likely caused by multiple genes as well. In addition to the multiple genes that probably affect the pheromone composition multiple component blends, other studies have found that male response genes are located in different genomic regions than female pheromone genes, at least in Lepidoptera (Butlin & Ritchie, 1989; Butlin & Trickett, 1997; Smadja & Butlin, 2009). Genetic correlations that have been found in Lepidoptera between long distance mate-signalling traits in one sex and response to those signals in the opposite sex appeared to be due to linkage disequilibrium and not pleiotropy (e.g. Linn *et al.*, 1997; Zhu *et al.*, 1997; Gray & Cade, 1999). Rare genotypes with a novel signal or response phenotype have been shown to be less attractive (e.g. Linn *et al.*, 1997; Zhu *et al.*, 1997). Thus, at least two independent mutations, one in sexual signal and one in response, are needed for a possible fitness advantage to arise, so that sexual communication signals can be considered complex traits.

Genes that are likely involved in the pheromone differences between moth races and species can be deduced from the biosynthetic pathways of sex pheromone production (Gould *et al.*, 2009; Groot *et al.*, 2008, 2009a). These biosynthetic pathways have been elucidated in a number of moth species (Tillman *et al.*, 1999; Jurenka, 2004) and typically share

common characteristics: most moth sex pheromones consist of multi-component blends, synthesised *de novo* through the fatty acid biosynthetic pathways, followed by a variety of modifications within the pheromone gland. However, so far genes encoding the enzymes involved in the biosynthetic pathway have not been identified, except for a family of desaturases in several moths (Knipple *et al.*, 2002; Roelofs & Rooney, 2003; Serra *et al.*, 2007) and a reductase in *Bombyx mori* (Moto *et al.*, 2003). Linking the pheromone differences that we found between the two strains of *S. frugiperda* to the hypothetical biosynthetic pathway of these compounds, we suggest that a $\Delta 11$ -desaturase and $\Delta 9$ -desaturases, and possibly a $\Delta 7$ -desaturase (not yet identified in insects) are candidate genes that may be differentially active between the two strains (Groot *et al.*, 2008). In the genus *Spodoptera*, $\Delta 9$ - and $\Delta 11$ -desaturases have been characterised in *S. exigua*, *S. littura* (Knipple *et al.*, 2002), and *S. littoralis* (Rodriguez *et al.*, 2004). These identifications will facilitate the assessment of whether and which of these genes vary between the two strains.

Differential timing of reproduction

A genetic analysis of temporal separation of sexual activity between the two closely related *Spodoptera* species, *S. descoinsi* and *S. latifascia*, suggested a polygenic control with mainly additive autosomal gene effects for onset time of female calling (Monti *et al.*, 1997). For the *S. frugiperda* host strains, different components of time-shifted reproductive behaviour (female calling, male courtship, copulation, and oviposition) have been found to show differing modes of inheritance: the timing of female and male calling was controlled mainly by maternal effects, the timing of copulation was controlled by a combination of maternal effects and corn strain dominant autosomal factors, and the timing of oviposition was inherited in a purely corn strain dominant fashion (G. Schöfl, A. Dill, D. G. Heckel, A. T. Groot, unpubl. res.). This suggests that the differential timing of reproductive behaviours between the two host strains is under complex genetic control.

In *S. frugiperda*, a temporal profile of general activity patterns (feeding and locomotion) parallels the temporal profile of reproductive behaviours between the two strains. Corn-strain individuals were active at relatively high levels from the beginning of scotophase until shortly before the end of scotophase. Rice-strain individuals were less active than corn-strain individuals during the first half of the night, but increased activity during the latter part of the night until shortly before photophase (G. Schöfl, A. Dill, D. G. Heckel, A. T. Groot, unpubl. res.). Such a co-ordinated time shift between reproductive traits and locomotor activity may suggest an involvement of genes associated with the central circadian system (Miyatake *et al.*, 2002; Tauber *et al.*, 2003). For example, it has been demonstrated that mating behaviour is gated by a circadian clock in *Drosophila* (Sakai & Ishida, 2001) and a cockroach (Rymer *et al.*, 2007). Differences in mating time between populations of melon flies and two sibling species of tephritid fruit fly have been correlated to differences in circadian fluctuations of two clock genes (*period* and *cryptochrome1*) (Miyatake *et al.*, 2002; An *et al.*, 2004). In *S. frugiperda*, three genes of the core

circadian clock (*period*, *timeless*, and *cryptochrome 2*) have been found to exhibit significant strain-specific differences in their daily transcription profiles (G. Schöfl, unpublished). Since central circadian clocks are driven by negative transcriptional feedback loops, the actual molecular difference(s) between the two strains may be located in any of the genes involved in the circadian clockwork.

Postzygotic incompatibilities

Postzygotic barriers to gene flow come into play only when prezygotic barriers are incomplete or absent. Prezygotic barriers are often thought to evolve rapidly in response to divergent selection in different habitats (Schluter, 1998), while intrinsic hybrid inviability and/or hybrid sterility commonly arise from sets of loci that interact epistatically and potentially accumulate much slower (Coyne & Orr, 1989, 1997; Orr, 1995). If one takes an ecological view on speciation and focuses on the order in which barriers to gene flow tend to evolve, intrinsic postzygotic effects may be of relatively little relevance to the early stages of the speciation process (Mallet *et al.*, 1998; Via, 1999; Funk *et al.*, 2002). On the other hand, for a large fraction ($\approx 2/3$) of sympatrically occurring Lepidopteran species that still hybridise in nature, intrinsic hybrid incompatibilities have been reported (Presgraves, 2002). This suggests that intrinsic postzygotic isolation may contribute to the maintenance of genetic integrity in many partially reproductively isolated species.

Although less extensively studied, extrinsic postzygotic isolation (i.e. reduced hybrid fitness due to ecological disadvantages of hybrid phenotypes in the parental environments) can be a direct result of adaptive evolution in different habitats and therefore more commonly observed during early stages of speciation (Coyne & Orr, 1998, 2004; Schluter, 1998). Another form of postzygotic barrier arises when hybrids are unable to secure mates (Coyne & Orr, 2004). This form of post-mating isolation may be intrinsic if, for example, behavioural dysfunctions render hybrids incapable of courtship, or extrinsic if, for example, intermediate courtship phenotypes render hybrids unattractive to members of the parental populations (Servedio & Noor, 2003; Coyne & Orr, 2004).

In the fall armyworm, Pashley and Martin (1987) found that in no-choice matings between corn females and rice males ($C \times R$) no spermatophores were transferred, while the reciprocal cross ($R \times C$) produced viable offspring. However, a second study could not replicate these results (Whitford *et al.*, 1988), since the strains crossed successfully in both directions at similar rates. Pashley (1988a) suggested that the discrepancy between the two studies was due to the age of the laboratory colonies used. Pashley and Martin (1987) had used colonies that had been reared for one to five generations in the laboratory, while Whitford *et al.*'s (1988) colonies were at least 3 years (~ 30 generations) old. This objection was tested using colonies of different age ranging from two generations to 25 years in the laboratory (Quisenberry, 1991). Again, no reproductive incompatibilities between the strains were found. Independent of colony age, both cross directions proved fertile to a similar extent (Quisenberry, 1991). Nevertheless, it is

unlikely that these differences in results are due to chance. Possibly there is geographic variation in the success rate between the two reciprocal cross directions; Pashley and Martin (1987) used individuals collected either in Puerto Rico or Louisiana, while Whitford *et al.* (1988) used fall armyworm collected in Louisiana and Mississippi.

When considering backcrosses, results between different studies are more consistent. Pashley and Martin (1987) found that RC-hybrid females mated moderately successfully only with their hybrid brothers, but not with males of either parental strain (RC-hybrid males mated successfully with females of either parental strain). Whitford *et al.* (1988) found that interhybrid crosses between either CR- or RC-hybrids did produce fertile clutches, although $RC \times RC$ pairs were significantly less successful than $CR \times CR$ pairs (Fisher's exact test, $P < 0.001$, reanalysis of data from Whitford *et al.*, 1988). Similarly, when we performed between-strain crosses between rice-strain and corn-strain individuals originating from Florida, we found no reproductive isolation in either cross direction (Table 1), but we did consistently find that backcrossing RC-hybrid females to males of either parental strain mostly failed, while all other backcross combinations, including RC-hybrid males, yielded fertile clutches (Table 1). RC-hybrid females did mate successfully with their RC-hybrid brothers, although at significantly reduced rates (Table 1), similar to what was reported previously (Pashley & Martin, 1987; Whitford *et al.*, 1988).

Since RC-hybrid females hardly ever mate successfully with males of either parental strain but may mate successfully with RC-hybrid males, this behavioural sterility could be extrinsic in nature. On the other hand, the significantly lower rate of RC interhybrid matings as compared to CR interhybrid matings suggests some intrinsic component to behavioural sterility. In either case, the behavioural sterility of RC-hybrid females in the laboratory predicts that gene flow across strains boundaries

Table 1. Proportion of pairs that laid fertile clutches within 7–9 days after being placed together in a mating cup.

Female strain	Male strain	No. paired	No. fertile	% fertile
Corn	Corn	181	109	0.60 ^a
Corn	Rice	70	38	0.54 ^a
Rice	Corn	60	28	0.47 ^a
Rice	Rice	124	75	0.60 ^a
($C\text{♀} \times R\text{♂}$) hybrid	Corn	35	28	0.80 ^a
($C\text{♀} \times R\text{♂}$) hybrid	Rice	27	18	0.67 ^a
($R\text{♀} \times C\text{♂}$) hybrid	Corn	31	5	0.16 ^b
($R\text{♀} \times C\text{♂}$) hybrid	Rice	31	1	0.03 ^b
Corn	($C\text{♀} \times R\text{♂}$) hybrid	30	28	0.93 ^a
Rice	($C\text{♀} \times R\text{♂}$) hybrid	23	15	0.65 ^a
Corn	($R\text{♀} \times C\text{♂}$) hybrid	29	23	0.79 ^a
Rice	($R\text{♀} \times C\text{♂}$) hybrid	22	14	0.64 ^a
($C\text{♀} \times R\text{♂}$) hybrid	($C\text{♀} \times R\text{♂}$) hybrid	73	66	0.90 ^a
($C\text{♀} \times R\text{♂}$) hybrid	($R\text{♀} \times C\text{♂}$) hybrid	22	12	0.55 ^b
($R\text{♀} \times C\text{♂}$) hybrid	($C\text{♀} \times R\text{♂}$) hybrid	22	5	0.23 ^b
($R\text{♀} \times C\text{♂}$) hybrid	($R\text{♀} \times C\text{♂}$) hybrid	37	13	0.35 ^b

Different letters indicate significant differences (unplanned *G*-tests of independence, $\alpha = 0.05$).

will be sex-biased with reduced female-mediated gene flow relative to male-mediated gene flow.

For the fall armyworm, assessments of rates and directionality of hybridisation in the field are based on discordancies between strain-specific mitochondrial haplotypes and nuclear markers (esterase genotypes, AFLPs or FR-repeat). Prowell *et al.* (2004) estimated that 16% of 162 field-collected individuals were potential hybrids with the majority being second or later generation. The observed discordancies between mitotype on the one hand and esterase or AFLPs on the other hand suggest that roughly equal proportions of hybrids derive from $C \times R$ and $R \times C$ matings. However, in a series of recent publications Nagoshi and co-workers (Nagoshi & Meagher, 2003a; Nagoshi *et al.*, 2006b; Machado *et al.*, 2008) used discordancies between mitotypes and the sex-linked tandem-repeat sequence (FR-repeat) to infer directional interstrain mating biases. The FR-repeat is abundantly present in rice-strain genomes (FR⁺) but absent or present only in low copy numbers in the corn strain (FR⁰) (Lu *et al.*, 1994; Nagoshi & Meagher, 2003b) and can be diagnosed by PCR amplification patterns. Surprisingly, in samples from Florida, Texas, and Brazil, substantial proportions of mt^RFR⁰ genotypes were discovered, which can only be accounted for by RC hybrid females backcrossing to corn males. In Florida and Texas, FR⁰ was found in the majority of all individuals that carried a rice-strain mitochondrial haplotype, while the reciprocal configuration, mt^CFR⁺, was rarer (Nagoshi & Meagher, 2003a; Nagoshi *et al.*, 2006b; Machado *et al.*, 2008).

Since an mt^RFR⁰ hybrid combination can only arise as a result of RC-hybrid female \times C-male backcrosses or RC-hybrid female \times RC-hybrid male matings, these results disagree with the findings that under laboratory conditions this type of backcross happens only at a very low frequency, if at all (Pashley & Martin 1987; Whitford *et al.*, 1988, Table 1).

Nagoshi and Meagher (2008) suggest that this high-frequency genotype class might represent an additional hybrid subpopulation, besides the two recognised host strains, which could show different patterns of host use, behaviours, or susceptibilities to pesticides. While this is an intriguing possibility, an alternative explanation could be that the mt^RFR⁰ samples are not of hybrid origin after all. Z chromosomes of the rice strain harbour an about 100-fold lower copy number of the FR-repeat than the W chromosome (Lu *et al.*, 1994; Nagoshi & Meagher, 2003a) and may additionally be naturally polymorphic with respect to copy number. Thus, rice-strain variants of the Z chromosome could exist that give rise to an amplification pattern similar to what is usually classified as the corn-strain variant. To clarify whether the observed mt^RFR⁰ genotypes indeed represent hybrids between the rice and the corn strain, it needs to be tested whether these individuals also exhibit discordant patterns with respect to the other known diagnostic markers, specifically the Z-linked *Est3* allozyme and the strain-specific AFLP patterns.

Permeability of the host strain genomes

Overall, surprisingly few fixed nuclear genetic differences have been found between the two strains. Sequencing the

nuclear ITS1 gene (Prowell, 1998) and an intron at the *para* sodium channel (Adamczyk *et al.*, 1996) revealed no strain-specific or strain-biased variation. Studies using nuclear AFLP markers to identify strain-specific markers found that only a small fraction of the AFLP loci exhibited diagnostic allelic differences (McMichael & Prowell, 1999; Busato *et al.*, 2004; Prowell *et al.*, 2004). For example, McMichael and Prowell (1999) investigated more than 1000 loci to find 10 strain-specific AFLP loci; Busato *et al.* (2004) examined more than 200 loci and detected two strain-specific loci. This suggests that less than 1% of the nuclear genome may be fixed for different alleles between the two strains. Loci that show such an excessive level of genetic differentiation between populations may identify parts of the genome that resist the homogenising effects of between-strain gene flow, perhaps indicating regions that are under divergent selection between the two strains (Beaumont, 2005). Such a pattern is generally expected under divergence-with-gene-flow models of speciation (e.g. Emelianov *et al.*, 2004; Via & West, 2008), and emphasises that probably just a few key traits act to reduce interstrain mating and reproduction between fall armyworm host races.

By contrast, large and consistent differences exist in the mitochondrial genome which forms two distinct strain-specific clusters of haplotypes (Lewter *et al.*, 2006). When comparing two corn-strain populations from Florida (Homestead, Miami-Dade Co.; Hague, Alachua Co.) and two rice-strain populations from Florida (Ona, Hardee Co.), and Mississippi (Washington Co.), we found 11 fixed differences between rice and corn mitotypes in the 16S-ND1 mitochondrial region, (Tables 2 and 3), which corresponds to a mean pairwise sequence divergence (uncorrected) of 2.1% between the two strains. Combining this estimate with a previous estimate of observed divergence between strains for the mitochondrial cytochrome oxidase I and II genes (0.66%, Lewter *et al.*, 2006), the average estimate of sequence divergence for a 1.1 kb portion of the mitochondrial genome is 1.3%, whereas within-strain

Table 2. Table of polymorphic sites among fall armyworm haplotypes at a 516 bp fragment of the 16S-ND1 mitochondrial region.

```
position 0000111111 1111111111 1112222244 5
position 6677000000 0000111112 7883578977 1
position 3838012345 6789012346 6589898114 3
```

```
C1      A*TT***** *****T TTTTAAACGA T
C2      .....T ATATATATAC AGAA..... .
C3      ..... ..TATATA. .... .
C4      ..... ..TATA. .... .
R1      GTA*ATATAT ATATATATA. ....GGGTAG A
R2      GTA*..... ..TATA. ....GGGTAG A
R3      GTA*..... ..TATA. ....GGGTAG A
          ▲▲▲▲                               ▲▲▲▲▲▲ ▲
```

Sequences were obtained from 83 individuals from two corn-strain and two rice-strain populations (see text). Triangles identify fixed differences between corn-strain and rice-strain populations. Differences between mitochondrial haplotypes within strains are almost exclusively due to variation at a microsatellite locus.

Table 3. Frequency of fall armyworm corn- and rice-strain haplotypes at a 516 bp fragment of the 16S-ND1 mitochondrial region in two corn-strain and two rice-strain populations.

Location	Host	C1	C2	C3	C4	R1	R2	R3
Homestead, Miami-Dade Co., FL	Corn	–	–	13	2	–	3	–
Hague, Alachua Co., FL	Corn	8	12	2	–	–	1	–
Ona, Hardee Co., FL	Pasture	–	–	–	–	–	14	5
Washington Co., MS	Pasture	–	–	–	–	23	–	–

mitochondrial haplotypes differed almost exclusively due to variation at a fast-evolving microsatellite locus. A preliminary estimate of divergence time between the corn- and the rice-strain can be based on a molecular clock rate estimate of 2.3% pairwise mtDNA sequence divergence per million years for various arthropod taxa (Brower, 1994). By assuming tentatively that the *S. frugiperda* mtDNA is evolving neutrally, the maximum uncorrected pairwise interstrain sequence divergence corresponds to an age of separation of the two strains of approximately 600 000 years.

The difference in permeability between the nuclear genome and the mitochondrial genome supports the notion that female-mediated gene flow between the two strains may be more reduced than male-mediated gene flow.

Conclusions

Even though *S. frugiperda* has been the subject of numerous studies since the discovery of the two host strains in the 1980s, there are still many unresolved issues in how the two strains maintain their identity. Now that different reproductive isolation barriers have been recognised, it is important to assess their relative contribution to the reproductive isolation between the strains. The main questions that need to be resolved are the following. Is the occurrence on different host plants due to differential oviposition preference? What is the relative importance of the female sex pheromone differences versus the differential timing of reproduction in the assortative attraction of males in the field? Is there geographic variation in host preference and/or sexual communication and/or differential timing of reproductive activity and/or postzygotic incompatibility? What is the genetic basis of the reproductive isolation barriers? And finally, how do the different reproductive isolation barriers interact? Elucidating these questions will give insight into the speciation process that seems to be ongoing in this intriguing species.

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