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

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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 sympathy (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**

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. frugiperta 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. frugiperta, or host plant response to S. frugiperta eggs, has been published.

Another aspect of egg-plant-interaction that has not been explored in S. frugi-perda 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. frugiperta 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 (R² ~ 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 ni*) 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 indicated, 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).
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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CHAPTER 9


