Synthesis
Divergent Host Plant Adaptation and Reproductive Isolation between Ecotypes of *Timema cristinae* Walking Sticks

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Submitted August 6, 2006; Accepted September 22, 2006; Electronically published January 2, 2007

Online enhancements: appendix, color version of figure 2.

**Abstract:** Theoretical and empirical studies have demonstrated that divergent natural selection can promote the evolution of reproductive isolation. Three unresolved questions concern the types of reproductive barriers involved, the role of geography, and the factors determining the extent of progress toward complete speciation. Here I synthesize studies of *Timema cristinae* host plant ecotypes to address these issues. The approach is to compare the magnitude of multiple reproductive barriers among different ecological and geographic scenarios, where pairs of populations within each scenario are the unit of replication. Application of this approach to *T. cristinae* revealed that divergent host adaptation can promote the evolution of diverse reproductive barriers, including those that are not inherently ecological. Gene flow in parapatry tended to constrain divergence, with the notable exception of the reinforcement of sexual isolation. Thus, geography affected progress toward speciation but did not influence all reproductive barriers in the same way. Studies of any single pair of taxa often capture only certain stages of the speciation process. For example, reproductive isolation between *T. cristinae* ecotypes is incomplete, and so only the stages before the completion of speciation have been examined. Studies of more divergent taxa within the genus are required to determine the factors that complete speciation.

**Keywords:** ecological divergence, ecological speciation, divergent natural selection, *Timema* walking sticks, phytophagous insects.

Divergent natural selection can promote speciation by causing populations in distinct ecological environments to diverge in ecologically important traits (Funk 1998; Schluter 2000, 2001; Coyne and Orr 2004; Gavrilets 2004). If these ecological traits, or those that are genetically correlated with them, incidentally cause reproductive isolation, then speciation occurs as a by-product of ecological divergence (Muller 1942; Mayr 1963). This process of speciation via divergent natural selection has been termed “ecological speciation,” and there is convincing evidence for its occurrence in nature (Schluter 2000; Funk et al. 2002; Rundle and Nosil 2005). Whether divergent natural selection can promote speciation is no longer in question, but many details of the process remain unknown. Some unresolved questions concern the types of reproductive barriers involved, the role of geography, and the factors determining the extent of progress toward complete speciation (Coyne and Orr 2004). Here I synthesize studies of *Timema cristinae* host plant ecotypes to address these issues. This article has three main goals: to outline a general framework for isolating the role of ecology and geography in the evolution of diverse reproductive barriers, to review studies of *T. cristinae* within this standardized framework, and to point out new and integrated insights that emerge from the synthesis of multiple studies but were not apparent from the individual studies on these insects.

**Comparing Reproductive Barriers and Geographic Scenarios**

Over recent years, a general comparative framework for analyzing ecological speciation has emerged (Schluter and Nagel 1995; Funk 1996, 1998; Rundle et al. 2000; Schluter 2001; Funk et al. 2002, 2006; Vines and Schluter 2006). The main prediction underlying this framework is that ecologically divergent pairs of populations exhibit greater levels of reproductive isolation than ecologically similar pairs of populations (fig. 1). This prediction assumes an "all else equal" situation with respect to the time and levels of gene flow separating population pairs. Ecologically similar pairs of populations can diverge via processes such as genetic drift, sexual conflict, and some forms of sexual...
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Figure 1: “Different-host/same-host” framework for studying reproductive isolation between populations of herbivorous insects. In each plot, three scenarios are compared. Scenario 1 examines reproductive isolation between geographically separated (allopatric) pairs of populations on the same host. Scenario 2 examines reproductive isolation between geographically separated pairs of populations on different hosts. Scenario 3 examines reproductive isolation between geographically contiguous (parapatric) pairs of populations on different hosts. Environment-independent processes can cause divergence under any scenario, although gene flow may negate a strong role for genetic drift in scenario 3. The likelihood that other processes can contribute to the evolution of reproductive isolation is depicted below the X-axis.

A) Reproductive isolation

- 'lots' separation
- 'none' separation

Genetic Drift: YES YES NO
Adaptation: NO YES YES
Reinforcement: NO NO YES
Gene Flow: NO NO YES

B) Reproductive isolation

- 'lots' contact
- 'none' contact

Timema cristinae Host Plant Ecotypes

Timema walking sticks are wingless insects inhabiting southwestern North America (Vickery 1993; Crespi and Sandoval 2000). Individuals feed and mate on the host plants on which they rest. Timema cristinae uses two host species that differ strikingly in foliage and general morphology (Ceanothus spinosus: Rhamnaceae and Adenostoma fasciculatum: Rosaceae; fig. 2). Ceanothus is relatively large, treelike, and broad leaved. Adenostoma is small and bushlike and exhibits thin, needlelike leaves. Timema cristinae ecotypes are defined by the host species they are found on: individuals from populations using Ceanothus are considered the “Ceanothus ecotype,” and individuals from populations using Adenostoma are considered the “Adenostoma ecotype.” Thus, multiple populations associated with each host make up the T. cristinae ecotypes. For convenience, I use the term “hybridization” to refer to interbreeding between ecotypes without claiming species status.

Geographical Arrangement of Populations

I focus on divergence between populations, where a “population” of walking sticks is defined as all of the insects
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Figure 2: Host plant ecotypes of *Timema cristinae* depicted above their respective hosts. The most conspicuous difference is in the frequency of individuals with the presence of a dorsal stripe. Populations rarely show fixed differences, but the unstriped morph is more common in *Ceanothus* (mean frequency of 81%), and the striped morph is more common on *Adenostoma* (mean frequency of 72%). Thus most, but not all, individuals of the *Adenostoma* ecotype are striped, and the ecotypes also differ in many other traits. Individuals from the *Ceanothus* ecotype tend to exhibit bright bodies, lack a dorsal stripe, and have relatively narrow heads and long legs. In contrast, individuals from the *Adenostoma* ecotype tend to have smaller and duller bodies, large and bright dorsal stripe patterns, and relatively wide heads and short legs. A color version of this figure is available in the online edition of the *American Naturalist*.

collected within a homogenous patch of a single host plant species (Nosil et al. 2002, 2003). Thus, “parapatric” populations are those in contact with a population of stick insects using the alternative host, and parapatric pairs consist of two parapatric populations that are adjacent to each other. Conversely, “allopatric” populations are geographically separated from all populations using the alternative host by distances ≥50 times the per generation gene flow distance. The appendix in the online edition of the *American Naturalist* provides a schematic diagram and further details concerning these geographic scenarios (see also Sandoval 2000).

Evidence for Divergent Adaptation

The ecotypes differ in a suite of traits including color, color pattern, body size, and body shape (Sandoval 1994a, 1994b; Nosil et al. 2002; Nosil and Crespi 2004, 2006b). Figure 2 depicts representative specimens of each ecotype, and figure 3 illustrates the nature of the differences between ecotypes. The most conspicuous morphological difference between ecotypes is in the frequency of individuals with or without a dorsal stripe (striped or unstriped color pattern morphs, respectively; Sandoval 1994b). The striped morph is more common in the *Adenostoma* ecotype, and the unstriped morph is more common in the *Ceanothus* ecotype. However, the striped pattern is usually not fixed on either host, and the ecotypes differ in many other traits. Thus, the striped morph should not, for example, be equated with the *Adenostoma* ecotype. Covariance among different traits within ecotypes depends on genetic independence among traits, on whether migration between divergent populations generates linkage disequilibrium between loci affecting different traits, and on genetic variation within populations (Nosil et al. 2006b).

The morphological differences between ecotypes are adaptive and genetically based (Sandoval 1994a, 1994b; Nosil and Crespi 2004, 2006b; Nosil et al. 2007). For example, figure 3A depicts how the direction and magnitude of host-specific morphological divergence in 11 traits are strongly related to the direction and magnitude of host-specific selection when selection is estimated in the presence of visual predation. This association between selection and trait divergence is not detected in the absence of predation (fig. 3B). Thus, there are strong fitness trade-offs in survival due to visual predation (Nosil 2004; Nosil and Crespi 2006b). In contrast, both ecotypes show higher fecundity in the absence of predation on *Ceanothus*, indicating a lack of physiological trade-offs in host use (Sandoval and Nosil 2005). Thus, as in other herbivores (Via et al. 2000; Funk et al. 2002), host-associated selection plays a critical role in divergent host adaptation. However, rather than the typical situation of physiological trade-offs, divergent adaptation in *T. cristinae* appears to be driven by divergent selection from predators for host-specific crypsis.
Ecological Divergence and Reproductive Isolation

In this section, seven diverse reproductive barriers are reviewed. I first compare reproductive isolation between allopatric different-host population pairs and allopatric same-host pairs. I then turn my attention to the role of geography by considering only different-host pairs and comparing parapatric to allopatric pairs. For the first five reproductive barriers, there is sufficient replication of population pairs that these barriers are analyzed under a common statistical framework and simultaneously plotted in figure 4 (indirect evidence is discussed for the other two). When estimates of reproductive isolation were obtained for all pairwise combinations between populations, Mantel \( t \)-tests (with 10,000 randomizations) were used to test for differences between scenarios (Manly 1997). When each population was used in only one pairwise comparison, standard \( t \)-tests were applied. The results of these statistical tests are presented in table 1.

Each reproductive barrier likely has a strong genetic basis and is largely unaffected by host rearing environment.

Figure 3: Divergent, host-associated selection due to predation. “C minus A” refers to Ceanothus value minus Adenostoma value (X-axis: trait values standardized to mean 0 and variance 1; Y-axis: standardized directional selection differentials). A, Predation present. The direction and magnitude of divergent selection in a field experiment was positively correlated with the direction and magnitude of trait divergence observed between ecotypes in nature. B, Predation absent. There was no association between selection and trait divergence. Moreover, absolute divergent selection was stronger in the presence than in absence of predation for 10 of 11 traits (paired \( t \)-test, \( p < 0.01 \)). Reprinted with permission from the National Academy of Sciences of the USA (Nosil and Crespi 2006b).

Figure 4: Effects of ecological divergence and geography on reproductive isolation. Bars represent mean reproductive isolation, and each black dot depicts results from an individual population pair (table 1 for statistics). A, Role of ecological divergence, where all comparisons are between allopatric population pairs. In general, different-host population pairs exhibit significantly greater reproductive isolation than do same-host pairs, across reproductive barriers. B, Role of geography, where all comparisons are between different-host pairs. In general, allopatric pairs exhibit greater levels of reproductive isolation than do parapatric pairs. The notable exception is sexual isolation, which is reinforced in parapatry. Two outliers below \(-0.3\) for parapatric pairs are not depicted for cryptic isolation and hybrid inviability but were used in statistical analyses.
The genetic details underlying each reproductive barrier are unknown (e.g., number of genes, same genes in different populations, mode of inheritance, physical location on chromosomes, role of pleiotropy), and unraveling these details offers a promising avenue of further research. The existing information on genetic bases is presented in table 1, along with a summary of the results for each reproductive barrier and original references that can be consulted for further detail.

Habitat Isolation

Habitat isolation occurs when divergent habitat preferences result in a reduced number of encounters, and thus reduced mating, between individuals from different populations (Bush 1969; Feder et al. 1994; Coyne and Orr 2004). Habitat isolation was inferred from divergence between populations in host preferences (the difference between a population pair in the proportion of individuals choosing Ceanothus in laboratory choice experiments). Nosil et al. (2006a) examined 27 populations. Ignoring geography and considering all possible pairwise comparisons, 176 different-host population pairs exhibited significantly greater differences in host preference (mean = 0.27) than did 175 same-host population pairs (mean = 0.15). The 21 pairwise comparisons from the seven allopatric populations (of the total 27) yielded the same pattern.

Geography affected evolution in such a way that allopatric population pairs exhibited more divergent host preferences than did parapatric pairs. For example, comparing preference divergence (percentage of individuals from Ceanothus preferring Ceanothus minus percentage of individuals from Adenostoma preferring Ceanothus) between allopatric and parapatric different-host pairs revealed a tendency for allopatric pairs to show greater divergence (experiment 1 [depicted in fig. 4B] and experiment 3 mean difference = −7% and 22%, respectively; combined $P < 0.05$). Preference divergence sometimes occurs between parapatric populations but tends to be stronger between allopatric pairs. Thus, gene flow appears to have a homogenizing rather than a reinforcing effect on habitat isolation in Timema cristinae.

Immigrant Inviability

Reproductive isolation can arise when there is natural selection against immigrants from divergent habitats. Although not typically considered a form of reproductive isolation, such “immigrant inviability” can decrease the rate of heterotypic mating encounters and thus reduce interbreeding between populations (see Nosil et al. 2005 for a review). Because immigrant inviability is a consequence of divergent natural selection, it is interesting to elucidate not only its existence during ecological speciation but also the agents of selection. In T. cristinae, integration of two types of data, morph-specific survival probabilities and the actual distribution of different morphs within populations, suggests that visual predation generates selection against between-host migrants (i.e., immigrant inviability; Nosil 2004).

A mark-recapture field experiment revealed that in the absence of predation, striped and unstriped color pattern morphs exhibit equal fitness on both hosts. In contrast, in the presence of predation, the unstriped morph had higher survival on Ceanothus than did the striped morph and vice versa on Adenostoma. This information alone does not estimate selection against between-host migrants, because both morphs occur on both hosts. What must also be determined is the degree to which adaptive divergence between populations in morph frequency results in residents being, on average, more likely than immigrants to be the locally cryptic morph. Greater divergence implies stronger selection against immigrants. To this end, color pattern was scored on 5,233 randomly sampled individuals from 15 population pairs (see the appendix for raw data). Insects captured within a focal population were considered “residents,” and insects captured within a different study population were considered “immigrants.” Each individual was assigned the survival value exhibited by its color pattern phenotype in the field experiment on the focal population’s host and in the presence of predation. Immigrant inviability was estimated as $1 - (\text{immigrant survival/resident survival})$, treating each population in turn as focal and averaging across a population pair. This approach is indirect and estimates immigrant inviability based on a single character (presence vs. absence of the stripe), but it allows estimation in numerous population pairs.

Same-host population pairs exhibit similarity in morph frequencies and the selective environment such that selection against immigrants does not occur ($s = 0.00$). Allopatric different-host pairs are strongly differentiated in morph frequency and under a scenario of secondary contact are predicted to exhibit strong immigrant inviability ($s = 0.53$). Among 12 parapatric population pairs (mean $s = 0.15$), asymmetry in gene flow homogenized color pattern morph frequencies in such a way that immigrant inviability weakened as asymmetry in population size increased.

Sexual Isolation

Sexual isolation occurs when divergent mate preferences and traits result in reduced heterotypic mating relative to homotypic mating. The magnitude of sexual isolation between T. cristinae populations was estimated using the $I_{ps}$
Table 1: Summary of the reproductive isolating barriers between host-associated populations of *Timema cristinae*

<table>
<thead>
<tr>
<th>Barrier</th>
<th>Ecological Divergence</th>
<th>Geography</th>
<th>Heritability</th>
<th>Notes</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Habitat isolation</td>
<td>Habitat isolation</td>
<td>3 A &gt; 6 P (t = 1.49, df = 7, P = .18)</td>
<td>Persists using genetic crosses; no effects of host rearing environment in reciprocal-rearing experiment; F1 hybrids have intermediate preferences</td>
<td>Migration generates genetic covariance between host preference and cryptic color pattern</td>
<td>Nosil et al. 2006a, 2006b</td>
</tr>
<tr>
<td>Immigrant inviability</td>
<td>DH &gt; SH</td>
<td>A &gt; P</td>
<td>Heritable population divergence in traits subject to divergent selection</td>
<td>Caused by visual predation, not physiological trade-offs in host plant use</td>
<td>Nosil 2004; Sandoval and Nosil 2005</td>
</tr>
<tr>
<td>Natural selection against hybrids</td>
<td>DH &gt; SH likely</td>
<td>A &gt; P likely</td>
<td>Heritable population divergence in traits likely to reduce hybrid fitness</td>
<td>Never directly measured, but likely given less cryptic hybrid color patterns</td>
<td>Nosil 2004; Nosil et al. 2007</td>
</tr>
<tr>
<td>Sexual isolation</td>
<td>Sexual isolation</td>
<td>3 A &lt; 15 P (t = 2.49, df = 18, P &lt; .05); 3 A &lt; 3 P (t = 2.46, df = 4, P = .07)</td>
<td>Levels of sexual isolation independent of host rearing environment in a reciprocal-rearing experiment</td>
<td>Intermediate migration rates promote reinforcement; involves pheromone and olfactory communication</td>
<td>Nosil et al. 2002, 2003, 2007</td>
</tr>
<tr>
<td>Cryptic isolation</td>
<td>Cryptic isolation</td>
<td>3 A = 8 P (t = 1.49, df = 9, P = .17)</td>
<td>Persists in common garden and is independent of whether female is reared on native host</td>
<td>Observed for fecundity and oviposition rate but not for female longevity</td>
<td>Nosil and Crespi 2006a</td>
</tr>
<tr>
<td>Intrinsic hybrid incompatibility</td>
<td>Intrinsic hybrid incompatibility</td>
<td>3 A = 8 P (t = .34, df = 9, P = .75)</td>
<td>Not available because of lack of reproductive isolation</td>
<td>Only F1 hatching success was examined</td>
<td>Nosil et al. 2007</td>
</tr>
<tr>
<td>Natural selection against hybrids</td>
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<td>A &gt; P likely</td>
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</tr>
</tbody>
</table>

Note: The role of ecological divergence is examined by comparing reproductive isolation between different-host (DH) and same-host (SH) population pairs. The role of geography is examined by comparing reproductive isolation between allopatric (A) and parapatric (P) population pairs. Numbers preceding each type of population pair represent the number of population pairs studied. Where applicable, statistical comparisons between the different types of population pairs are presented. The heritability column summarizes evidence that reproductive isolation has a genetic basis. Further notes and original references are also provided.

- All the populations from experiment 1 in Nosil et al. 2006a.
- Only the allopatric populations from experiment 1 in Nosil et al. 2006a.
- Experiment 1 from Nosil et al. 2006a.
- Experiment 3 from Nosil et al. 2006a (insufficient data from experiment 2).
- Both populations in a pair allopatric versus at least one population parapatric.
- Both populations allopatric or both parapatric.
- Fitness component is fecundity.
- Fitness component is oviposition rate.
index (0 = random mating, +1 = complete sexual isolation; Rolán-Alvarez and Caballero 2000). Nosil et al. (2002) conducted 1,024 no-choice mating trials between 28 pairs of populations that were geographically separated from one another. The magnitude of sexual isolation detected between 15 different-host population pairs was significantly greater than the magnitude detected between 13 same-host pairs (fig. 4A). This result suggests that some sexual isolation evolved as a by-product of adaptation to different hosts.

In T. cristinae, the preconditions for reinforcement are met. For example, there is evidence for ongoing hybridization and for selection against hybrids (Nosil et al. 2003, 2007). Examination of sexual isolation revealed evidence for reinforcement of female mating preferences (n = 3,320 mating trials; Nosil et al. 2003). For example, the 15 different-host pairs where at least one population in the pair was parapatric exhibited significantly stronger sexual isolation than did the three different-host pairs where both populations were allopatric (fig. 4B). Similar results were observed when only unique population pairs were considered so that no population was used in more than one pairwise comparison (mean \( I_{PSI} \) for three allopatric and three parapatric pairs is 0.31 and 0.49, respectively). Moreover, congruent patterns occurred when considering single focal populations rather than pairs: females from seven of eight parapatric populations exhibited between-population copulation frequencies that were lower than the average observed for females from allopatric populations (Wilcoxon signed-ranks test: \( Z = 2.24, P < .05 \)).

For sexual isolation, it is particularly interesting to consider the effects of geography in more depth. From an empirical standpoint, there is abundant variation in levels of sexual isolation among parapatric T. cristinae populations, ranging from levels typical of allopatric pairs to twice that level. Theoretical models offer a potential explanation: high gene flow between populations can erode the effects of reinforcing selection (Sanderson 1989; Servedio and Kirkpatrick 1997; Cain et al. 1999; Servedio and Noor 2003). However, gene flow also generates the opportunities for selection against hybrids that reinforcement requires (Howard 1993; Noor 1995). As noted by Coyne and Orr (2004, p. 371), “reinforcement requires some gene flow, but not too much” and might be most likely when gene flow is intermediate (Kirkpatrick 2000). Nosil et al. (2003) provide indirect evidence supporting this hypothesis in T. cristinae. That study showed that as the relative size of the population adjacent to a focal population increased, the mitochondrial DNA gene flow rate into the focal population increased. Results for sexual isolation were highly consistent with the dual influence of gene flow: sexual isolation was greatest when the estimated sizes of adjacent populations were similar, and levels of gene flow were intermediate. A number of questions remain, particularly, which traits are divergent mate preferences based on? Nosil et al. (2007) review these factors in detail, including evidence that sexual isolation involves pheromones and olfactory cues.

Cryptic Postmating Isolation

Postmating barriers that do not involve the fitness of hybrids are sometimes referred to as “cryptic” reproductive isolation (Price et al. 2001; Coyne and Orr 2004). For example, reduction in female fitness after heterotypic mating lowers the reproductive output of these matings, thereby acting as a form of reproductive isolation (i.e., few hybrids produced). For T. cristinae, cryptic isolation was estimated using data on female fitness after between-population or within-population mating. No effects were found for female survival. Two forms of cryptic postmating isolation were detected, measured as 1 − (mean lifetime fecundity of females used in between-population mating/mean lifetime fecundity of females used in within-population mating) and 1 − (mean oviposition rate of females used in between-population mating/mean oviposition rate of females used in within-population mating).

Both within-population and between-population crosses were conducted for three allopatric different-host population pairs (n = 130 crosses) and three same-host pairs (n = 182 crosses). Reductions in female fecundity following between-population mating, relative to within-population mating, tended to be greater for crosses between different-host pairs than for crosses between same-host pairs (fig. 4A). Using oviposition rate as the component of fitness yielded the same pattern. Moreover, the reduction in female fitness for between-population versus that for within-population crosses was greater for all three different-host pairs than for any of the three same-host pairs (for both fecundity and oviposition rate). The probability of this pattern arising by chance is \( (3!)(3!)/(6!) = 0.05 \) for each component of fitness. Thus, statistical support exists for a positive association between ecological divergence and cryptic isolation.

When geography was considered (n = 377 crosses using eight parapatric pairs), patterns were less clear. Very few of the individual parapatric pairs exhibited significant reductions in female fecundity after between-population mating, yet no statistical differences were found between allopatric and parapatric pairs. The trends for oviposition rate were more suggestive of cryptic isolation in parapatry; lower oviposition rates were observed in between-population crosses for seven of eight parapatric pairs, and a significant reduction was detected when individuals were pooled among pairs. Thus, whether cryptic isolation oc-
curs between parapatric populations requires further study.

Intrinsic Hybrid Incompatibilities
Intrinsic postmating isolation (hybrid inviability or sterility) can result from between-locus genetic incompatibilities (Dobzhansky 1937; Orr and Turelli 2001; Gavrilets 2004). Although these incompatibilities might be accentuated in harsher environments, they can reduce hybrid fitness without an ecological interaction between phenotype and environment (Rundle and Whitlock 2001). In *T. cristinae*, a component of intrinsic hybrid inviability was estimated by comparing hatching success in between-population and within-population crosses. Intrinsic F1 hybrid egg inviability was estimated as $1 - \left(\frac{\text{percentage of eggs hatched in broods derived from between-population crosses}}{\text{percentage of eggs hatched in broods derived from within-population crosses}}\right)$. Both within-population and between-population crosses were conducted for three allopatric different-host pairs ($n = 116$ crosses) and for three allopatric same-host pairs ($n = 159$ crosses). There was no evidence for intrinsic reductions in F1 hatching success in any case (Nosil et al. 2007 for details). Thus, different-host and same-host pairs do not differ in the magnitude of this reproductive barrier.

Similar patterns were detected when geography was considered, because again inviability was not observed. The three allopatric different-host pairs did not differ in F1 hybrid hatching success from the eight parapatric pairs (parapatric crosses). Intrinsic inviability might be more pronounced in F2 or backcross individuals, which were not examined. Furthermore, hybrid sterility was not examined, and it generally evolves before inviability (Coyne and Orr 2004). At the very least, F1 hybrids do not show intrinsic egg inviability.

Natural and Sexual Selection against Hybrids
There are no direct estimates of the ecological or sexual fitness of hybrids between the ecotypes. However, the possibility of ecological selection against hybrids is supported by estimates of selection on color pattern and inferences about the color pattern of hybrids (Nosil et al. 2007 for review). Populations on different hosts exhibit adaptive and genetically based divergence in color pattern morph frequencies. Thus, hybrids may suffer reduced fitness due to exhibiting maladaptive color patterns. Nosil et al. (2007) review this possibility in detail, including evidence for selection against intermediate color patterns. The divergent mate preferences of the parental ecotypes suggest that sexual selection against hybrids might also occur, with hybrids exhibiting intermediate mating traits and preferences that are discriminated against by the parental ecotypes. Again, direct data are lacking.

Total Reproductive Isolation
Figure 5 summarizes the four forms of reproductive isolation that were detected using multiple population pairs and also depicts total reproductive isolation (which is calculated by multiplying across the individual contributions of all barriers and can be interpreted as the magnitude of gene flow that is blocked by the combined effects of all barriers). Components of reproductive isolation were estimated using procedures outlined in Ramsey et al. (2003). In brief, the mean level of reproductive isolation caused by each barrier in isolation (i.e., its individual contribution, depicted by bars in fig. 4 for allopatric and parapatric pairs, respectively) was used to calculate different components of reproductive isolation. For all barriers, allopatric different-host population pairs exhibited greater reproductive isolation than same-host pairs. Thus, total reproductive isolation between these allopatric populations was estimated at 0.83 and 0.19, respectively. For all barriers except sexual isolation, divergence was weaker in parapatry. Thus, total reproductive isolation between parapatric pairs was 0.66, weaker than that observed between allopatric different-host pairs but much stronger than that between same-host pairs.
Reproductive Isolation Often Increases with Time, Where Time Is Estimated Using Genetic Distance at Neutral Loci (Coyne and Orr 2004). In *Timema cristinae*, time since divergence is unlikely to confound the observed association between ecological divergence and reproductive isolation. Three lines of evidence support this claim. First, geographically separated different-host population pairs are not more genetically divergent from one another than geographically separated same-host pairs, for either a mitochondrial (cytochrome oxidase I [COI]) or a nuclear gene (internal transcribed spacer 2 [ITS-2]). This similarity in genetic distance between ecological scenarios is depicted in the appendix, along with statistical details.

Second, adjacent populations using different hosts are weakly differentiated or undifferentiated at COI (mean $F_{st} = 0.07$, range $0.00–0.25$, $n = 7$ pairs), while geographically separated populations are strongly differentiated (mean $F_{st} = 0.31$, range $0.00–0.79$, $n = 129$ pairs; Mantel’s $r = 2.33$, $P < .01$). Although nuclear DNA data are lacking, morphological divergence is also weaker between parapatric populations (Sandoval 1994a, 1994b; Nosil and Crespi 2004). Despite weaker neutral differentiation, parapatric population pairs on different hosts show greater reproductive isolation than do allopatric pairs on the same host (fig. 5).

Third, for sexual isolation it is possible to explicitly quantify the association between reproductive isolation and genetic distance, because genetic data are available for all the population pairs examined. The results show that the degree of sexual isolation observed between populations is not correlated with the genetic distance between them (see the appendix for a depiction of these results and statistical details). Collectively, these analyses provide evidence that ecological divergence in host plant use, rather than neutral differentiation, predicts the magnitude of reproductive isolation. However, these results should be interpreted with some caution because data for all population pairs examined exist only for sexual isolation, and estimates from multiple, unlinked nuclear loci are lacking.

**Novel Insights into Reproductive Barriers from the Ecotype Studies**

From synthesis of multiple reproductive barriers and geographic scenarios, some new insights into speciation emerge. Most generally, divergent natural selection, rather than genetic drift, appears to drive the evolution of reproductive isolation in this system (fig. 6). A more novel insight is that divergent natural selection can promote the evolution of multiple and diverse reproductive barriers, including those that are not inherently ecological. This pattern could not be inferred from any individual study. Additionally, synthesis of different studies revealed that geography affects the evolution of multiple reproductive barriers but does not affect all barriers in the same way. For most barriers, gene flow eroded divergence in such a way that allopatry promoted speciation. The clear counterexample was sexual isolation, where moderate gene flow between parapatric populations facilitated reinforcement. Thus, roughly comparable levels of total reproductive isolation between allopatric and parapatric different-host pairs arose via different contributions of various reproductive barriers. Another finding that emerges from analysis of multiple barriers is that habitat isolation in *Timema cristinae*, unlike many other insect herbivores, is weak both in absolute terms and relative to other barriers. Thus, the early stages of speciation in this system proceed via multiple reproductive barriers and not only through divergence in host preference. These diverse barriers evolve through a combination of the incidental effects of divergent adaptation and reinforcement. Finally, the results suggest that comparative frameworks hold promise for analyzing speciation in other systems.

**Reproductive Isolation Is Incomplete**

The studies of *Timema cristinae* isolated a role of ecological divergence in the evolution of reproductive isolation. However, studies of a single pair of taxa, such as the *T. cristinae* ecotypes, often capture only a portion of the process of speciation. For example, reproductive isolation be-
between the *T. cristinae* ecotypes is incomplete in that complete speciation has not occurred. Experimental, molecular, and morphological data suggest ongoing gene flow in parapatry and thus incomplete reproductive isolation. Experimental data indicate that for even the most extreme cases of allopatric divergence, there is only an 80% barrier to gene flow (fig. 5). Total barriers to gene flow are weaker in parapatry. Even if unquantified barriers such as ecological selection against hybrids are considered, reproductive isolation is likely incomplete.

Molecular data support this claim of incomplete reproductive isolation: adjacent pairs of populations using different hosts exhibit consistently and significantly weaker levels of mitochondrial DNA differentiation than do geographically separated populations (Nosil et al. 2003). This pattern is indicative of ongoing gene flow between parapatric populations (Coyne and Orr 2004). Indeed, coalescent-based estimates of gene flow (*m*) into parapatric populations range among eight populations from 0.001 to 0.232, with a mean migration rate of 0.043 (Nosil et al. 2003). The pattern of weaker population divergence in parapatry is also seen for morphological traits (Sandoval 1994b; Nosil and Crespi 2004). For example, consider novel analyses of data from Nosil and Crespi (2006b), where 604 individuals were scored for 11 traits. For illustrative purposes, and because similar patterns are seen for most traits, I focus here on two traits: a general aspect of multivariate morphology (the first axis from a principal components analysis, PC1) and the univariate trait under the strongest divergent selection between hosts (body brightness). Whether divergence between individuals on different hosts differs between allopatric and parapatric populations can be analyzed using the host use × geography interaction in ANOVAs (host use and geography are factors with two levels, *Ceanothus* vs. *Adenostoma* and allopatry vs. parapatry, respectively). For both traits and sexes, this interaction is significant (all *P* < .05). In all cases, the magnitude of divergence between hosts is greater for allopatric populations than for parapatric populations. Figure 6 depicts the weaker divergence in parapatry for male PC1, and complete statistics are presented in the appendix. Gene flow between parapatric populations, indicative of incomplete reproductive isolation, constrains divergence.

Morphological differentiation can also be examined using a cluster-based concept, which generates more information concerning species status than is provided by simply comparing means (Mallet 1995). In general, stable and bimodal genotypic clusters in sympatry (i.e., bimodal distributions with few intermediates) suggest the existence of genetically independent and reproductively isolated species (Jiggins and Mallet 2001). Thus, a lack of strong bimodality, or the collapse of bimodality in the face of gene flow, is evidence for incomplete speciation/reproductive isolation. Here I pool individuals from different hosts into a single distribution and test for bimodality in PC1 and body brightness within allopatry and parapatry. Bimodality is examined by visually inspecting frequency distributions and by testing statistically whether each distribution is better represented by a single normal distribution or by a mixture of two normal distributions (Brewer 2003; Hendry et al. 2006; see the appendix for details).

The results concerning bimodality are summarized via three main points. First, strong statistical support for bimodality was common (see the appendix for details). Second, visual inspection of frequency distributions revealed that despite statistical support, bimodality was extremely weak and that many intermediates are present. Thus, there is much overlap between the two distributions, and certainly, distinct clusters are not evident. Representative results are depicted for male PC1 in figure 6, and similar patterns were seen in other cases. Third, there was some evidence for the collapse of bimodality in parapatry (i.e., in the face of gene flow). For example, there was strong support for a mixture of two normal distributions for PC1 in allopatric males. In contrast, there was strong support for only a single normal distribution for PC1 in parapatric males. Thus, weak and variable bimodality indicates that strong and stable genotypic clustering has not occurred even for divergently selected traits (e.g., body brightness). Comparable results from multilocus genotypes at neutral loci would provide additional support for a finding of incomplete speciation.

**Synthesis: Transitions across the Species Boundary**

Synthesizing the studies of *Timema cristinae* revealed that ecological divergence and geography can both affect progress toward speciation. Future research on these ecotypes could focus on the genetic details of divergence and the unknown mechanisms underlying some of the forms of reproductive isolation. However, synthesis of the individual studies also revealed that complete speciation has not occurred. The *T. cristinae* ecotypes represent either an ongoing speciation event or population divergence that has reached equilibrium. A new question emerges: why has complete speciation not occurred? Time since divergence (i.e., recent origin of the populations) could play a role but is unlikely to be the complete explanation, because allopatric population pairs exhibit substantial sequence divergence indicative of reasonably ancient divergence times (on average, 3%–4% and 1%–2% divergence at COI and ITS-2, respectively; Nosil et al. 2002, 2003). Thus, addressing questions concerning incomplete speciation will likely require studies of more divergent *Timema* taxa.

In essence, comparison of multiple pairs of closely related taxa that exhibit different and wide-ranging amounts
of divergence can be used to analyze different stages of speciation (Mallet et al. 1998; Dres and Mallet 2002). Preferably, both early and late stages would be examined. This approach might shed light onto the ecological, geographic, or genetic factors that drive transitions across the species boundary (from host race or ecotype to species) and thus complete speciation. Perhaps more extreme host shifts (i.e., stronger selection) or a longer history of allopatry and lower gene flow upon secondary contact are required. Under what conditions can selection counteract gene flow strongly enough to result in complete reproductive isolation? Perhaps the genetic details matter. Questions concerning progress toward speciation can also be thought of in terms of the structure of hybrid zones: what factors predict whether unimodal or bimodal hybrid zones will occur (Jiggins and Mallet 2001)? Clearly, for *T. cristinae* ecological selection is central to divergence, indicating that the population-genetic processes acting within contemporary populations can also influence the formation of new species (Charlesworth et al. 1982). Extending these studies across the species boundary, using other species of *Timema*, will likely lead to novel and powerful insights into the process of speciation and how it unfolds from beginning to end.

**Acknowledgments**

B. J. Crespi and C. P. Sandoval were instrumental to all the *Timema* research. I also thank S. P. Egan, D. J. Funk, A. Mooers, T. E. Reimchen, H. Rundle, D. Schluter, and two anonymous reviewers for discussions regarding speciation and comments on previous versions of the article. Funding was provided by the Natural Sciences and Engineering Research Council of Canada.

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