

# Reproductive isolation driven by the combined effects of ecological adaptation and reinforcement

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Recent years have seen a resurgence of interest in the process of speciation but few studies have elucidated the mechanisms either driving or constraining the evolution of reproductive isolation. In theory, the direct effects of reinforcing selection for increased mating discrimination where interbreeding produces hybrid offspring with low fitness and the indirect effects of adaptation to different environments can both promote speciation. Conversely, high levels of homogenizing gene flow can counteract the forces of selection. We demonstrate the opposing effects of reinforcing selection and gene flow in *Timema cristinae* walking-stick insects. The magnitude of female mating discrimination against males from other populations is greatest when migration rates between populations adapted to alternate host plants are high enough to allow the evolution of reinforcement, but low enough to prevent gene flow from eroding adaptive divergence in mate choice. Moreover, reproductive isolation is strongest under the combined effects of reinforcement and adaptation to alternate host plants. Our findings demonstrate the joint effects of reinforcement, ecological adaptation and gene flow on progress towards speciation in the wild.

**Keywords:** gene flow; hybridization; natural selection; speciation; *Timema*; walking-stick insects

## 1. INTRODUCTION

Speciation via natural selection can occur as an indirect by-product of adaptive divergence (Mayr 1963; Jiggins *et al.* 2001) and can also involve direct selection for reproductive isolation in geographical regions where hybridization is maladaptive (i.e. reinforcement) (Dobzhansky 1951; Howard 1993; Butlin 1995; Noor 1999). When speciation occurs as a by-product of adaptive divergence, ecologically divergent populations exhibit greater reproductive isolation than ecologically similar populations of similar age (reviewed in Funk 1998; Rundle *et al.* 2000; Schluter 2000; Funk *et al.* 2002). The key prediction of the reinforcement hypothesis is that non-allopatric (geographically contiguous or overlapping) populations will exhibit greater mating discrimination than allopatric (geographically separated) populations. Previous empirical studies of reinforcement have provided evidence for such a pattern (Noor 1995; Saetre *et al.* 1997; Rundle & Schluter 1998; Higgie *et al.* 2000) but there are few data on how ecological adaptation and reinforcement interact during the speciation process (Schluter 2000). Furthermore, although theoretical models predict that the outcome of reinforcement reflects a balance between the strength of reinforcing selection and the ability of homogenizing gene flow between populations to counteract selection (Sanderson 1989; Servedio & Kirkpatrick 1997; Cain *et al.* 1999; Servedio 2000), this prediction has never, to our knowledge, been tested using natural populations.

In this paper, we use *Timema* walking-stick insects to analyse the joint roles of ecological adaptation, reinforcement and gene flow in the evolution of reproductive isolation. *Timema* are wingless phytophagous insects

distributed throughout western North America (Crespi & Sandoval 2000). *Timema cristinae* exhibits two genetically determined colour-pattern morphs (Sandoval 1993), with an unstriped morph being more common on the host plant *Geonothus spinosus* and a striped morph being more common on *Adenostoma fasciculatum* (Sandoval 1994a). Predation on *T. cristinae* by birds and lizards is strong and each morph is most cryptic on the plant on which it is more common (Sandoval 1994a,b). Populations using different host plants have also diverged in a suite of other morphological and behavioural traits, including body size and shape, host preference and cryptic resting behaviour, where host preference refers to a preference to rest on a particular host, given the choice (Nosil *et al.* 2002; Nosil & Crespi 2003).

Levels of sexual isolation are higher between pairs of *T. cristinae* populations using different host plants ( $n = 15$  pairs) than between similar-aged pairs using the same host plant ( $n = 13$  pairs) (Nosil *et al.* 2002). Because insect colour morph (striped versus unstriped) does not influence between-population mate choice, the sexual isolation that has evolved between populations adapted to alternate hosts is independent of colour pattern (although colour pattern might influence within-population mate choice; Nosil *et al.* 2002). In this study, we expand previous work by considering the effects of reinforcing selection and gene flow on between-population mating preferences.

*Timema cristinae* satisfies all of the preconditions for reinforcement to contribute to the reproductive isolation observed between populations. First, interbreeding and gene flow between adjacent populations using different host plants (i.e. 'hybridization' between the host-adapted forms) is strongly suggested by the observations that individuals from adjacent populations on different hosts are well within one per-generation dispersal distance of each other (Sandoval 1993, 1994a) and that between-morph

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matings are common in nature where populations using different hosts are in contact (Nosil *et al.* 2002). Moreover, geographical variation in morphology is indicative of a balance between host-specific selection and gene flow between adjacent patches (Sandoval 1994a; Nosil & Crespi 2003), and the populations are conspecific. In this study, we use mitochondrial DNA (mtDNA) sequence variation to provide further evidence of interbreeding and gene flow between adjacent populations of *T. cristinae*.

Second, the evolution of reinforcement traditionally requires reduced hybrid fitness. In *T. cristinae*, between-host migrants are more likely to be of the locally non-matching colour morph than are residents (Sandoval 1994a) and the non-matching morph is at a large selective disadvantage owing to differential predation (the relative fitness of the less-cryptic morph is 0.30; Sandoval 1994a,b). Thus offspring derived from between-host mating (i.e. 'hybridization') will tend to exhibit reduced fitness relative to offspring derived from within-population mating because females who mate with locally less-cryptic males produce a higher frequency of offspring that are the locally non-matching colour morph or intermediate in colour pattern (i.e. bear a faint stripe) than females that mate with cryptic males (experimental genetic crosses show that only 5% of within-morph crosses produce  $F_1$  offspring of the alternate morph or intermediates, whereas 80% of between-morph crosses produce both morphs or intermediates; Sandoval 1993). Although previous work demonstrates that females producing offspring of the non-matching colour-pattern morph will be at a selective disadvantage (Sandoval 1994a,b), the cost of producing offspring that are intermediate in colour pattern is less well known. In the current study, we test whether selection against the intermediate colour-pattern morph also imposes reinforcing selection, predicting that if the intermediate morph exhibits low fitness then its frequency will decline through time (i.e. between sequential time periods and age classes; Endler 1986). Because populations using different hosts have diverged in a number of traits other than colour pattern (Nosil *et al.* 2002; Nosil & Crespi 2003), selection against 'hybrids' may extend beyond that based upon colour pattern alone.

Finally, we note that recent theoretical work indicates that direct selection against hybrid mating can promote reinforcement, even without reduced hybrid fitness (Servedio 2001). In *T. cristinae*, the male rides on the back of the female during the mating period and thus females that mate with locally less-cryptic males are likely to suffer reduced individual survival during mating, favouring mating discrimination against migrants from the alternate host.

Given that the preconditions for reinforcement are met, we tested for its presence by assessing levels of female mating discrimination against foreign males (males that were collected from a different population from the females) for walking-stick insects collected from eight parapatric populations (reinforcement possible) and from four allopatric populations (no opportunity for reinforcement). Mating trials were conducted for all 66 possible pairwise combinations of the 12 study populations (figure 1). Most patches of these two host-plant species are distributed in parapatric patches of varying size, forming a mosaic at the scale of a mountain slope. However, some host patches

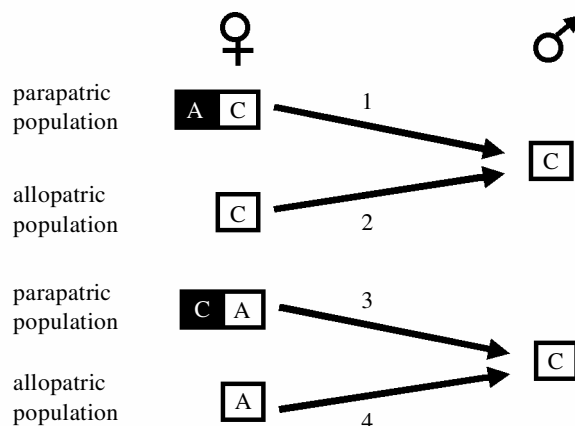


Figure 1. Representative examples of the four 'types' of between-population crosses (C, *Ceonothus* population; A, *Adenostoma* population). Each square represents a homogeneous patch of a single host plant, which may or may not have a neighbouring population using the alternative host plant (parapatric and allopatric populations, respectively). 'Study' populations used in mating trials are represented by unfilled boxes, and black boxes represent populations that are adjacent to a study population but were not used in mating trials. In the figure, males from allopatric *Ceonothus* populations are used as an illustrative example; in the mating trials males from each of the 12 study populations were used (i.e. males from allopatric and parapatric populations on each host). 1, female parapatric, male from the same host; 2, female allopatric, male from the same host; 3, female parapatric, male from the alternative host; 4, female allopatric, male from the alternative host.

are geographically separated from all other host patches by regions lacking suitable hosts. We define a 'population' of walking-stick insects as all the insects collected within a homogeneous patch of a single host plant. 'Parapatric' insect populations are in contact with a population of insects adapted to the alternate host, while 'allopatric' populations are separated from all populations adapted to the alternative host by distances of more than 50 times the 12 m per-generation gene-flow distance (Sandoval 1993). Reinforcement would be supported by higher between-population copulation frequencies in mating trials involving allopatric females than in trials involving parapatric females.

Reinforcing selection is predicted to be frequency dependent, with increasing rarity of a population (relative to the population with which it co-occurs) raising the probability of individuals from the co-occurring population mating with individuals of the wrong population and thus the opportunity for reinforcing selection (Howard 1993; Noor 1995). However, increased opportunity for between-population matings also increases the potential for gene flow between populations, which can retard or prevent reinforcement. Thus, the actual magnitude of female mating discrimination that evolves is expected to reflect a balance between the opposing forces of reinforcing selection and gene flow, with the effects of reinforcement being greatest when population sizes are similar (Sanderson 1989; Servedio & Kirkpatrick 1997; Cain *et al.* 1999; Servedio 2000). Because we quantified female mating discrimination in multiple populations of

differing sizes, we were able to test these key predictions of the reinforcement hypothesis.

Collectively, we provide an assessment of the joint effects of host adaptation, reinforcing selection and gene flow on the evolution of reproductive isolation, predicting that: (i) reinforcing selection strengthens premating isolation; (ii) high levels of gene flow counteract the effects of reinforcing selection; and (iii) the strongest reproductive isolation is found under the combined influence of divergent host-plant adaptation and reinforcement.

## 2. MATERIAL AND METHODS

### (a) No-choice mating experiment

*Timema cristinae* were collected from multiple study sites in the Santa Ynez Mountains, California, USA, in February 2001 and 2002 using sweep nets. Study sites were chosen such that a wide range of geographical arrangements of populations was represented. Other species of *Timema* do not occur in sympatry with populations from these sites. Animals were captured in the first instar and reared to maturity in the laboratory on the foliage of either their native or the alternative host.

Protocols for the no-choice mating trials used in this study have been previously published (Nosil *et al.* 2002) ( $n = 3320$  trials; 1024 of these from Nosil *et al.* (2002); median number of mating trials per pairwise comparison = 275, range of 75–497). One male and one female were placed in a 10 cm Petri dish and at the end of 1 h we scored whether the male and the female were paired (male on female without genital contact) or not, and copulating or not. Individuals were selected randomly from each population, such that mating trials were conducted using natural morph frequencies. In mating trials conducted for the current study, each individual was tested only once.

Copulation frequencies (proportion of male–female pairs that were copulating at the end of 1 h) were analysed using logistic regression, assessing significance using likelihood ratio tests (LR). We tested for reinforcement in a model that examined the dependence of copulation with males from each of the 12 study populations on male host, female host, male population, female population, allopatry (female from an allopatric or parapatric population) and all possible interaction terms. Host-specific sexual isolation is indicated by a significant interaction between male host and female host. Reinforcement is indicated by a significant allopatry term. We do not report the significances of the other terms in the model, as they are peripheral to the topic of the study. All results are from a reduced regression model derived using backward elimination (the model initially included all factors and interactions but then removed all terms for which the significance of  $-2 \log LR$  was more than 0.10). Including rearing environment (insect raised on its native or the alternative host) in the logistic regression models yielded no significant interactions (all main effects and interactions:  $p > 0.25$ ), indicating that differences in mate preference probably have a genetic basis. Analogous analyses conducted using males (male from parapatric or allopatric populations) did not yield evidence of reinforcement (all main effects and interactions with allopatry:  $p > 0.25$ ).

#### (i) Population rarity and the opportunity for reinforcement

In *T. cristinae*, the area of the host-plant patch of the study population relative to that of its parapatric neighbour serves as a measure of the study population's rarity: the larger the patch inhabited by neighbouring population and the smaller the patch

inhabited by study population, the 'rarer' the individuals of the study population become relative to individuals of the neighbouring population.

The rarity of each study population was calculated as (size of neighbouring patch)/(size of study patch + size of neighbouring patch). The areas of each population and its neighbour (in the case of parapatric patches) were calculated using aerial photographs. Patch size has been shown to be strongly and positively correlated with population size ( $r^2 = 0.63$  and  $0.53$  for *Ceonothus* and *Adenostoma* patches, respectively;  $n = 13$  patches of each host; Sandoval 1994a). The strength of female mating discrimination against foreign males was calculated for each of the 12 study populations as the absolute value of the mean copulation frequency of females with foreign males subtracted from the mean copulation frequency of females with males from their own population.

#### (ii) DNA sequencing and estimates of gene flow

A total of 107 mtDNA (cytochrome oxidase 1) sequences, 467 bp in length, were collected from the 12 study populations and from each of the populations that neighbour the parapatric study populations (mean number of individuals per population = 6.3, range of 3–11; protocols and 40 sequences from Nosil *et al.* (2002)). Haplotypes from the 67 sequences acquired in this study have been deposited in GenBank under accession numbers AY311411–AY311477.

We then estimated levels of gene flow between adjacent patches to test whether gene flow into a parapatric population, from its neighbouring population of the alternate host, increases with increasing relative size of its neighbouring population. Gene flow was estimated using the coalescent-based methods of Beerli & Felsenstein (2001). First, we used default settings in the program MIGRATE to obtain estimates of the number of migrants per generation ( $N_m$ ) into each of the parapatric study populations, from their neighbouring population of the alternate host. We then estimated migration rates using two independent approaches. First, we estimated  $m$  (the proportion of the population consisting of migrants, migration rate) from  $N_m$  by calculating total population size (using previously published regression equations for patch size versus population size; Sandoval 1994a), dividing this number by two to obtain female population size (mtDNA is maternally inherited) and then multiplying by 0.10 to obtain effective population sizes (Frankham 1995) (changing this final scaling value alters only the absolute estimates of  $m$ , whereas results of our analyses and their interpretation depend only on variation in relative migration rates). Second, we also report the migration parameter  $M$  ( $M = m/\text{mutation rate}$ ), obtained from MIGRATE. We note that our analyses of the relationship between gene flow and neighbouring-population size depend on relative migration rates and thus estimating  $m$  from  $M$  using different mutation rates will not affect our results.

### (b) Selection against the intermediate pattern morph

The intermediate morph is rare in most populations of *T. cristinae* (less than 2%). However, one site (Refugio) has relatively high frequencies of the intermediate morph, providing an opportunity to test for selection against the intermediate morph within a natural population. This site contained both host plants, each of which was sampled during March and April in 1996 and 1997. Captured individuals were scored for colour pattern (unstriped, striped, intermediate) and age class (juvenile,

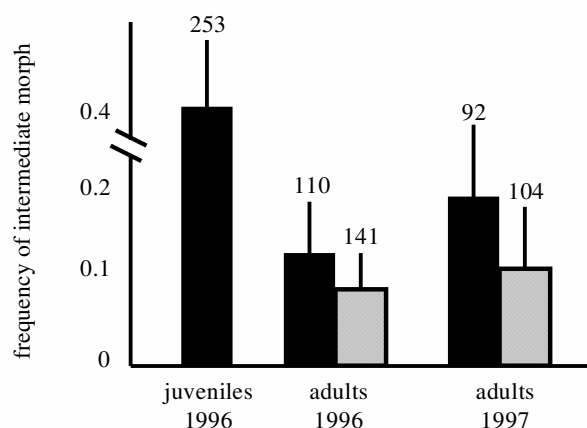


Figure 2. The frequency of the intermediate colour-pattern morph declined in successive age classes within a sample period and in successive sample months (black bars, March; grey bars, April). Numbers above each bar refer to the sample size.

adult; we found juveniles only during March 1996) and released where captured. All specimens were scored by one individual (C.P.S.).

We first assessed whether the frequency of the intermediate morph was dependent on age class by testing for an association between morph (intermediate or other) and age class (using the March 1996 sample) in a three-way log-linear contingency analysis that included morph, age class and host as factors. Second, we tested whether the frequency of the intermediate morph within adults was dependent upon sample month using a four-way log-linear analysis that also included sample year as a factor. For both analyses, we examined the effects of the interactions in question independent from the effects of other factors by using partial  $\chi^2$ -values and by assessing the significance of higher-order interactions (Norusis 1993).

### 3. RESULTS

#### (a) *Gene flow and hybridization in the wild*

Adjacent pairs of populations using different host plants are weakly or not differentiated at mtDNA (mean  $F_{st} = 0.07$ , range of 0.00–0.25,  $n = 7$  pairs) while geographically separated populations are strongly differentiated (mean  $F_{st} = 0.31$ , range of 0.00–0.79,  $n = 129$  pairs; Mantel's  $t = 2.33$ ,  $p < 0.01$ ). These data suggest that substantial gene flow between neighbouring populations occurs in the wild (see also coalescent-based estimates of gene flow § 3d). Thus, although incomplete lineage sorting (between neighbouring populations only) could produce similar patterns, the requirement for reinforcement of recent hybridization appears to be fulfilled.

#### (b) *Selection against intermediate colour-pattern morphs*

The frequency of the intermediate morph was higher in juveniles than in adults sampled during the same time period (partial  $\chi = 53.40$ , d.f. = 1,  $p < 0.001$ ; higher-order interaction:  $p > 0.10$ ; figure 2). In addition, the frequency of the intermediate morph within the adult age class decreased between March and April. This reduction occurred in both sample years, but the overall results were marginally non-significant (partial  $\chi = 2.96$ , d.f. = 1,

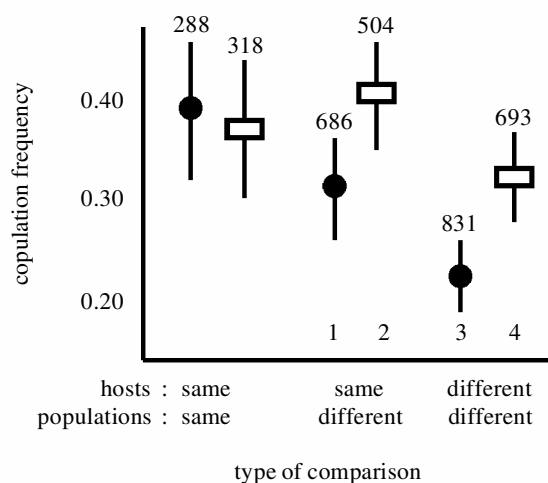


Figure 3. Copulation frequencies for male–female pairs of *Timema cristinae* walking-stick insects. Consistent with reinforcement, between-population copulation frequencies were higher for mating trials involving females from allopatric populations (rectangles) than for trials involving females from parapatric populations (circles) ( $p < 0.001$ ). The increased mating discrimination of parapatric females was independent of host-specific sexual isolation (male host  $\times$  female host, LR = 11.70, d.f. = 1,  $p < 0.001$ ; all other interactions,  $p > 0.15$ ). Thus reproductive isolation has evolved both via reinforcement and as a by-product of adaptation to different habitats. Numbers of mating trials for each comparison are shown above each 95% confidence interval. The numbers above the x-axis refer to the type of between-population mating trial as outlined in figure 1. All combinations of mating trials were conducted, such that some females from both parapatric and allopatric populations were tested with males from their own population, some with males from different populations using the same host and some with males from different populations using the alternate host.

$p = 0.08$ ; all higher-order interactions:  $p > 0.10$ ). These results demonstrate selection against the intermediate morph during the juvenile stages, possibly continuing into adult life. Ongoing interbreeding between the parental forms (i.e. striped and unstriped morphs) could account for the persistence of the intermediate morph at the study site, despite selection against it.

#### (c) *Reinforcement of premating isolation*

Female mating discrimination against foreign males is significantly stronger when the females are from populations where the two host-adapted forms hybridize than when the females are from geographically isolated populations (figure 3; mean between-population copulation frequency in mating trials involving females from parapatric populations = 28%, s.d. = 0.45; in trials using females from allopatric populations = 35%, s.d. = 0.47; allopatry term: LR = 11.93, d.f. = 1,  $p < 0.001$ ). Notably, the copulation frequencies of females with males from their own population were similar for females from parapatric and allopatric populations (allopatry term in a model including within-population mating trials only: LR = 1.40, d.f. = 1,  $p = 0.24$ ), indicating that reinforcement has strengthened female mating discrimination against foreign males without reducing mating frequencies with local males. Moreover, between-population copulation frequencies were

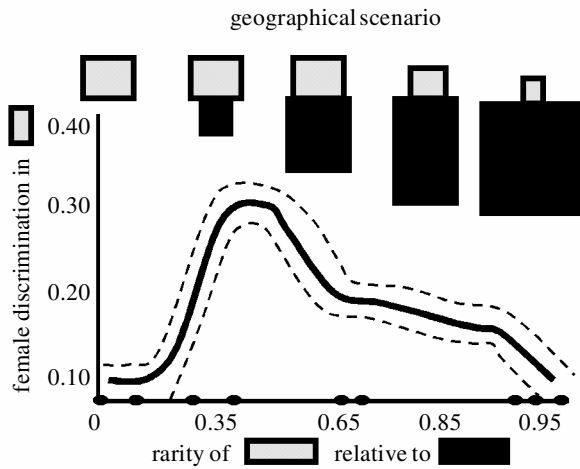


Figure 4. Female mating discrimination against males from other populations is strongest when the rarity of the study population is intermediate (males from the alternative and same host, respectively;  $t = -2.37, -2.25$ , both  $p < 0.05$ , quadratic term in regression model including both linear and quadratic terms;  $r^2$  change between a linear and quadratic model = 0.37, 0.33, both  $p < 0.05$ , partial  $F$ -test). Shown here is the relationship between the rarity of the study population (relative to its neighbouring population on the alternative host; values for each study population denoted by black circles on the  $x$ -axis) and female mating discrimination against foreign males that use the alternative host (absolute value of mean copulation frequency with foreign males subtracted from mean copulation frequency with resident males, for each of the 12 study populations). Boxes illustrate the different geographical scenarios, where the grey box denotes the study population and the black box denotes the neighbouring population. The curve was estimated using the non-parametric cubic spline (dashed lines show standard errors from 1000 bootstrap replicates) (Schluter 1988).

lower for females from the parapatric population than the average from the four allopatric populations in seven out of the eight population comparisons (Wilcoxon's signed ranks test (WSR):  $Z = 2.24$ ,  $p < 0.05$ ). These data provide strong evidence for reinforcement of premating isolation.

#### (d) Population size, gene flow and reinforcement

Coalescent-based analyses indicate that the opportunity for gene flow to erode the effects of reinforcing selection increases with increasing population rarity: levels of gene flow into the parapatric study populations, from their neighbouring populations, were highly variable (range of number of migrants ( $N_m$ ) = 0.68–14.7, mean = 3.13; range of migration rates estimated using population sizes ( $m$ ) = 0.001–0.232, mean = 0.043; range of migration parameter ( $M$ ) = 22.70–300.43, mean = 177.83) and increased with increasing population rarity ( $r = 0.86, 0.62, 0.92$ ,  $p < 0.01, 0.05, 0.01$ , respectively; Spearman rank correlations). Consistent with the balancing effects of frequency-dependent reinforcing selection and gene flow, mating discrimination is low when the study population is allopatric or large relative to its neighbour, it increases rapidly until the sizes of the study and neighbouring populations are similar, and it then decreases when the study population is relatively rare (figure 4).

#### (e) Combined effects of host-adaptation and reinforcement

Host-plant adaptation and reinforcement contributed independently and additively to the evolution of reproductive isolation (figure 3). Thus, copulation frequency was reduced when the sexes were from populations using alternate hosts (for both allopatric and parapatric females) and mating discrimination is highest when both adaptation and reinforcement occur.

#### (f) Alternative hypotheses

Numerous processes have been presented that could account for increased mating discrimination in parapatric or sympatric populations relative to allopatric populations (reviewed in Howard 1993; Butlin 1995; Noor 1999). Each of these hypotheses can be viewed as an alternative to reinforcement. As described below, each of these alternative hypotheses was unsupported.

First, when similar phenotypes from different populations compete most strongly for resources, frequency-dependent disruptive selection drives population divergence (Slatkin 1980). This phenomenon, called ecological character displacement, results in populations that are in geographical contact with one another exhibiting greater adaptive divergence than allopatric pairs of populations. As a consequence of this greater trait divergence, mating discrimination can be stronger between parapatric than between allopatric pairs of populations. Previous work has shown that ecological character displacement in morphology does not occur in *T. cristinae*: divergences in morph frequency, body size and body shape are greater between allopatric pairs of populations using different hosts than between parapatric pairs (Sandoval 1994a; Nosil & Crespi 2003). In this study, we tested for character displacement in resting behaviour and in a correlate of physiology (survival to maturity in the laboratory) by assessing the effects of host (*Ceonothus* versus *Adenostoma*), allopatry (allopatry versus parapatry) and an interaction term on variability among the 12 study populations. Resting behaviour refers to whether the insects were found resting where visible from the side (versus above or below) in 1073 host-preference trials (see Nosil *et al.* (2002) for details). For these binary variables, we assessed the effects of host, allopatry and the interaction term using logistic regression.

The character-displacement hypothesis is untenable because individuals from parapatric populations using different hosts are less divergent from one another than are individuals from allopatric populations using different hosts (mean trait values for individuals from parapatric versus allopatric populations; resting behaviour, 8% and 36%, respectively; LR from interactions term = 28.03,  $p < 0.001$ ; survival in laboratory, 4% and 5%, respectively; LR = 0.25,  $p > 0.10$ ).

Second, we evaluated whether population ancestry (i.e. time since divergence) might contribute to levels of reproductive isolation by testing whether values of an index of reproductive isolation ( $I_{PSI}$ ) (Rolan-Alvarez & Caballero 2000) were correlated with neutral mtDNA differentiation (range of nucleotide divergence of 0–5%) in the 66 pairs of study populations, or with differentiation at a nuclear locus in eight out of the 12 populations used in this study ( $n = 28$  pairs of populations; data from Nosil *et al.* (2002)).

The population-ancestry hypothesis is unsupported because levels of reproductive isolation are uncorrelated with neutral differentiation (mtDNA,  $r = -0.11$ ,  $p > 0.50$ , Mantel test; nuclear DNA,  $r = 0.29$ ,  $p = 0.10$ , data from Nosil *et al.* (2002)). Moreover, levels of gene diversity, defined as the probability that two randomly chosen haplotypes from the sample are different, tend to be correlated with population age (Nei 1987) but do not differ between parapatric and allopatric populations (mean of 0.75, s.d. 0.14 versus mean of 0.82, s.d. 0.05, respectively;  $t$ -test:  $p > 0.25$ ).

Third, the biased-extinction hypothesis predicts that non-allopatric pairs of populations tend to exhibit greater mating discrimination because non-allopatric populations without strong reproductive isolation fuse upon secondary contact, or one population goes extinct (see Noor (1999) for a review). We tested the key prediction of this hypothesis, that some allopatric populations will exhibit levels of mating discrimination similar to those observed in parapatric populations.

The biased-extinction hypothesis is unsupported because all four allopatric populations used in this study exhibited lower discriminations than the average parapatric population (one-tailed WSR:  $Z = 1.83$ ,  $p < 0.05$ ). However, this hypothesis cannot be unequivocally rejected using this approach because only four allopatric populations were sampled (see Noor (1999) for a discussion). We do note that biased extinction does not apply as readily to cases with gene flow among conspecific populations (versus cases with little or no gene flow between distinct species), where populations are defined by the local geography of their host plant, and where populations are parapatric rather than sympatric (i.e. *T. cristinae*) (Noor 1995, 1999).

Fourth, we tested for male preference for allopatric females because such male preferences could result in higher copulation frequencies when males are paired with allopatric versus parapatric females. For a subset of the mating trials ( $n = 160$ ), we recorded the position of the male every 10 min, over a 4 h interval. For trials where pairing occurred, we assessed whether males paired more rapidly with females from three allopatric populations ( $n = 74$ ) than with females from a parapatric population ( $n = 22$ ). Male post-copulatory guarding behaviour towards allopatric versus parapatric females was examined by observing single male–female pairs for two weeks after the first copulation event and noting when the male first stopped guarding the female (pairs observed twice a day,  $n = 30$ , half of the trials conducted with females from allopatric populations, all pairs were from populations using different hosts).

The male-preference hypothesis is unsupported because males do not pair more rapidly with allopatric than with parapatric females (mean time until first pairing of 55 and 35 min respectively; Mann–Whitney  $U$ -test:  $Z = 0.67$ ,  $p > 0.50$ ) and males do not preferentially guard allopatric versus parapatric females (mean time until males dismount of 3.38 and 3.88 days, respectively; Mann–Whitney  $U$ -test:  $Z = 0.35$ ,  $p > 0.50$ ).

#### 4. DISCUSSION

We detected strong evidence for reinforcement in *T. cristinae*: the assumptions of the reinforcement hypothesis

were met, and as predicted females from populations where the two host-adapted forms interbreed exhibit greater mating discrimination against foreign males than do females from geographically isolated populations. Moreover, each of the alternative explanations for the increased mating discrimination of parapatric females was unsupported (Noor 1999). By contrast, two additional key predictions of the reinforcement hypothesis were supported (Sanderson 1989; Servedio & Kirkpatrick 1997; Cain *et al.* 1999; Servedio 2000). Specifically, migration between divergent populations acted as both a homogenizing and a diversifying force, such that reinforcement was most likely when migration was high enough to facilitate reinforcement but low enough to prevent gene flow from eroding adaptive divergence in mate choice. These are, to our knowledge, the first empirical data to demonstrate the roles of relative population sizes and levels of gene flow in the evolution of reinforcement. We note that, although maternal effects on mating tendencies have not been explicitly ruled out, such maternal effects are unlikely to produce patterns of mating discrimination that are consistent with a balance between selection and gene flow.

Reinforcement requires a cost to hybridization. In *T. cristinae*, the costs of between-host mating are twofold. First, colour pattern in *T. cristinae* is genetically determined (Sandoval 1993) and immigrant males from the alternate host plant tend to exhibit the locally less-cryptic colour pattern (Sandoval 1994a,b). Thus females that mate with males from the alternate host plant tend to produce a higher frequency of less-cryptic offspring (i.e. the locally non-matching morph or a morph that is intermediate in colour pattern) than do females that mate with resident males. Second, females pairing with males from the alternative host might themselves suffer increased predation rates, favouring increased female mating discrimination. Under both these scenarios, natural selection favours mating discrimination against foreign males (see Servedio (2001) for a discussion). Although we are not able to disentangle these two costs of hybrid mating completely, we note that direct benefits would probably be obtained at the level of male–female pairing, rather than willingness to copulate in a confined area. Mating trials were conducted in small Petri dishes, allowing us to assess whether females discriminate against males once the opportunity for direct benefits is reduced (i.e. males can easily pair with the female but cannot force copulations; the frequency of pairing is 65%), rather than testing whether females discriminate against males prior to pairing (i.e. by actively fleeing).

Previous work has demonstrated that indirect effects of adaptation to alternative host plants also increase reproductive isolation between *T. cristinae* populations (Nosil *et al.* 2002). Our augmented dataset affirms these findings, and demonstrates that host-plant adaptation and reinforcement contribute independently and additively to the evolution of reproductive isolation (i.e. mating discrimination is highest when both processes occur). The indirect effects of such host-plant adaptation may provide the initial degree of divergence in mate preference that has been predicted to make reinforcement evolve more readily (Liou & Price 1994; Kelly & Noor 1996).

Consistent with the independence of the effects of reinforcement and host adaptation, reinforcing selection

exerted a 'universal' effect on mating preferences. Thus, although females are selected to be more discriminating against males from an adjacent population, this selection has indirectly resulted in increased mating discrimination against foreign males from multiple other populations that use either host (trials involving females from parapatric populations exhibited lower copulation frequencies than those involving allopatric females for males from 10 out of the 12 populations, one-tailed WSR:  $Z = 1.83$ ,  $p < 0.05$ ; significant differences detected only in this direction and in six comparisons, LR: all  $p < 0.05$ ). Such 'universal' effects of reinforcement may be caused by females recognizing and preferring males from their own population based on a 'population-specific' trait (rather than a 'host-specific' trait) (Kelly & Noor 1996; Higgin et al. 2000). Similar findings have been reported in *Drosophila mojavensis*, where females from mainland populations discriminate against conspecific males from other regions, and evidence is presented that this discrimination is a by-product of selection for sexual isolation between mainland *D. mojavensis* and its sympatric sibling species *D. arizonensis* (Zouros & D'Entremont 1980). If such 'universal' effects of reinforcement are common, then reinforcement will contribute to speciation between both ecologically similar and ecologically divergent pairs of species and between conspecific populations.

Our findings have broad implications for the study of speciation. First, our results indicate that the outcome of reinforcement depends on the spatial distribution of populations and on relative population sizes. These results concord with recent theoretical developments indicating that ecological interactions between populations are likely to drive speciation (Doebeli & Dieckmann 2003). Second, our results indicate that, even within a single species, natural selection can favour the evolution of reproductive isolation in two distinct ways, via indirect pleiotropic effects of ecological adaptation and via direct selection for increased premating isolation. The greatest progress towards speciation occurs when both processes operate.

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