
Mechanisms of reinforcement in natural and simulated polymorphic populations

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Reinforcement speciation is the process whereby selection against hybrids drives the evolution of enhanced pre-mating reproductive isolation. Work has focused on divergent mating preferences (assortative mating) but pre-mating isolation can also arise via various migration modification behaviours, such as divergent habitat preferences. The relative importance of these two different mechanisms of reinforcement remains unclear. A recent theoretical model (Yukilevich–True model) found that relative fixation probabilities between these mechanisms can vary. Additionally, natural populations of *Timema cristinae* walking-sticks exhibit variation (polymorphism) in both mechanisms, generating questions about the patterns expected for allele frequencies prior to fixation, during the early stages of the speciation process. In the present study, we report: (1) new analyses examining the correlation between fixation probabilities for assortative mating and migration modification in the Yukilevich–True model; (2) novel simulations examining allele frequencies in polymorphic populations; and (3) empirical patterns of reinforcement in *T. cristinae* in the context of theoretical predictions. Simulations of both types yielded congruent results, revealing that the outcome of reinforcement was dependent on the strength of selection. Under weak selection, reinforcement by either mechanism is unlikely. Under intermediate selection, the conditions favoring the rise and fixation of one mechanism favored the rise and fixation of the other. However, assortative mating evolved somewhat more readily than migration modification. Populations of *T. cristinae*, which experience such intermediate selection, supported these predictions. Under strong selection, the evolution of migration modification generally interfered with the evolution of assortative mating by decreasing migration between populations, thereby reducing selection for assortative mating. Congruence of the results for allele frequencies versus fixation probabilities suggests that similar patterns of reinforcement are expected during different stages of the speciation process. © 2008 The Linnean Society of London, *Biological Journal of the Linnean Society*, 2008, **95**, 305–319.

ADDITIONAL KEYWORDS: assortative mating – migration modification – reproductive isolation – simulation model – speciation – *Timema* walking-stick.

INTRODUCTION

Speciation has become a major research program in evolutionary biology, culminating in two recent synthetic books on the subject, one that emphasizes mostly (but not exclusively) empirical studies (Coyne & Orr, 2004) and the other focusing on theoretical models (Gavrilets, 2004). This interest in speciation from both approaches indicates that it is time to start bridging the gap between theory and speciation in

nature (Kirkpatrick & Ravigné, 2002). For example, simulation models of the contentious process of sympatric speciation have recently been coupled with empirical examples of this process in crater-lake cichlids and oceanic palms (Barluenga *et al.*, 2006; Savolainen *et al.*, 2006; Gavrilets & Vose, 2007; Gavrilets *et al.*, 2007). Another promising area to integrate theory and data is reinforcement, the process whereby natural selection against hybrids results in the evolution of pre-mating isolation (Dobzhansky, 1937; Servedio & Noor, 2003). Reinforcement is also a contentious mode of speciation,

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as many of the assumptions in theoretical models remain untested (Servedio & Kirkpatrick, 1997; Cain, Andreasen & Howard, 1999; Servedio, 2000, 2001, 2004; Lemmon, Smadja & Kirkpatrick, 2004) and because empirical studies often cannot exclude alternative interpretations (Servedio & Noor, 2003; Coyne & Orr, 2004 for review). Thus, it is critical to examine candidate natural systems of reinforcement (Noor, 1995; Rundle & Schluter, 1998; Higgie, Chenoweth & Blows, 2000; Pfennig, 2003; Hoskin *et al.*, 2005; Lukhtanov *et al.*, 2005) in light of theoretical models.

Traditionally, reinforcement is considered the evolution of divergent mate preferences in response to selection against hybrids. However, selection might favor the evolution of pre-mating isolation via other mechanisms, such as various migration modification behaviors (Fisher, 1930; Mayr, 1942, 1963; Balkau & Feldman, 1973; Yukilevich & True, 2006). An example of such a behavior is habitat preference, which reduces movement and thus gene flow between habitats (Coyne & Orr, 2004). Like mate preference, migration modification can evolve to avoid selection against hybrids. Furthermore, in addition to selection against hybrids, selection might often act on immigrant parental genotypes that migrate to an alternative habitat (Hendry, 2004; Nosil, Vines & Funk, 2005). Whether scenarios in which selection acts against immigrants should be considered examples of reinforcement in the traditional sense is debatable (Coyne & Orr, 2004). However, selection against hybrids and immigrants can clearly drive the evolution of both types of pre-mating isolation. Thus, we refer to both the evolution of divergent mate preferences and migration modification [hereafter designated assortative mating (AM) and migration modification (MM)], via either type of selection, as mechanisms of reinforcement in the broad sense *sensu* Servedio & Noor (2003).

Little is known about the conditions favoring the different mechanisms of reinforcement, and how they interact, particularly at different stages of the speciation process. This prompted Yukilevich & True (2006) to develop models [Yukilevich–True (YT) models] exploring the fixation probabilities of these reinforcement mechanisms, both when they evolve in isolation and when they evolve simultaneously. Two types of post-mating isolation were considered, an intrinsic postmating isolation model in which heterotypic matings suffer reduced fitness independent of habitat, and a niche-based model in which both heterotypic matings and immigrant homotypic matings have lower fitness in each habitat relative to resident homotypic matings (i.e. niche-based divergent selection). The results of the YT models revealed that AM generally fixed faster and under a broader range of biological conditions than MM. However, fixation of

MM over AM occurred when populations experienced strong, niche-based divergent selection.

Given that the two mechanisms differ from one another in fixation probabilities and can affect each others evolution, it is unclear what patterns are expected for allele frequencies within polymorphic populations (i.e. where AM or MM have not yet fixed). Theory aside, empirical data on the different mechanisms of reinforcement are almost entirely lacking (Servedio & Noor, 2003). These points motivated the present study, which aimed to: (1) expand the YT fixation model to polymorphic populations in the early stages of reinforcement; (2) compare theoretical patterns for fixed versus polymorphic populations, thereby generating predictions for different stages of the speciation process; and (3) test the role of the different isolating barriers in reinforcement in a natural system.

Empirically, we consider the *Timema cristinae* walking-stick system because there is evidence for reinforcement, and many of the parameters used in reinforcement models have been measured (Nosil, Crespi & Sandoval, 2003; Table 1). For example, the strength of selection against immigrants and hybrids is of major importance in explaining variable outcomes among different simulations, and this parameter has been estimated in *T. cristinae* (i.e. the simulation results applying to the empirical system are known). However, the genetic details of reinforcement in *T. cristinae* are unknown, and a different form of migration modification is considered relative to our simulations (habitat preference versus the tendency to not migrate, respectively). Thus, we stress that our aim is to generate general theoretical predictions and to outline empirical patterns in a well-parameterized system, rather than to build specific models for *Timema per se*.

We start with theory, first reviewing the relevant aspects of the published YT fixation model, and then present new simulations examining allele frequencies in polymorphic populations that are in the process of evolving AM and MM. We conclude these sections by outlining parallels and contrasts between the results from the two types of simulations. We then turn to empirical data, beginning with a description of the general features of the *T. cristinae* system, and then present novel analyses of previously published data on MM and AM. The final step is to integrate the theoretical and empirical results.

THEORETICAL MODEL

SUMMARY OF THE YT MODEL

Here, we summarize the YT fixation model (Yukilevich & True, 2006), which considered two popula-

Table 1. Summary of reinforcement in *Timema cristinae*

Parameter	Summary	Details	Reference
Intrinsic selection against hybrids	None known	F_1 hybrid hatching success from between-population crosses is not reduced relative to within-population crosses	Nosil <i>et al.</i> (2007)
Niche-based selection against immigrants and hybrids	Intermediate in strength	Upon secondary contact: $s = 0.53$ in parapatry; s ranges from 0.02 to 0.30, mean = 0.15	Nosil (2004); Nosil <i>et al.</i> (2007)
Evidence for ongoing gene flow	Yes	Lower molecular and morphometric divergence between parapatric versus allopatric pairs of populations: F_{ST} in mtDNA: mean, maximum F_{ST} in parapatry = 0.07, 0.25 F_{ST} in allopatry = 0.31, 0.79	Sandoval (1994a); Nosil <i>et al.</i> (2003); Nosil & Crespi (2004); Nosil <i>et al.</i> (2008)
Estimated migration rates	Intermediate	Range of $m = 0.001$ – 0.232 Mean $m = 0.043$	Nosil <i>et al.</i> (2003)
Segregation of AM and MM upon secondary contact	Both segregate	Allopatric populations show variation in both forms of pre-mating isolation (i.e. neither mechanism is fixed): mean isolation = 0.20 and 0.13 for MM, AM respectively; Direct evidence for genetic variation within populations in MM exists	Nosil <i>et al.</i> (2002, 2006a,b)

AM, assortative mating; MM, migration modification.

tions that diverged historically in allopatry, and are now in secondary contact. Because *T. cristinae* fits a niche-based model with simultaneous evolution of both reinforcement mechanisms (see below), we consider only such models here. The niche-based model examined three biallelic loci in haploid organisms that undergo a transient diploid stage during reproduction. The first locus is a fitness/phenotypic marker locus conferring adaptation to different environments. In this model, heterotypic matings and immigrant homotypic matings had equally lower fitness in each habitat relative to resident homotypic matings (and this strength of selection against immigrants and hybrids was denoted 's'). The second locus examines the mating behavior of females, depending on whether the female has a random-mating or positive assortative mating allele. The third locus determines migratory behavior, depending on whether individuals have an allele with a tendency to migrate or an allele with a tendency to be sedentary. Thus, MM involves reduced movement rather than habitat choice *per se*. The actual probability of migration for individuals with the migratory allele was dependent on the initial migration rate between populations (m), which is assumed to be symmetric. The simultaneous evolution YT model examined fixation probabilities at

AM and MM loci during the joint evolution of both mechanisms of reinforcement. A one-allele scenario was considered for both AM and MM (Felsenstein, 1981), with no costs to mate choice (i.e. all females mate). These conditions are favourable for reinforcement (Gavrilets, 2004) but are a reasonable starting point that allows the mechanisms to be examined in an 'all-else-equal scenario'.

Monte Carlo simulations were performed in BASIC. In all the results reported here, including the new simulations described below, AM and MM each segregated at one percent at the initiation of the simulations. The order of events as specified by the simulation program is as follows. First, 1000 starting individuals of each sex are sampled from the initial haplotype frequencies in each population. Second, each female chooses a male, depending on her mating behavior locus and her phenotype at the marker locus. This creates 1000 mating pairs in each population. Third, each pair produces progeny with a maximum clutch size (fecundity) of ten offspring with a fitness of 1. The production of progeny haplotypes depended on the haplotypes of both parents and the recombination rates between different loci. Selection acted on less fit matings by decreasing the fecundity of those matings by a certain fraction, as determined

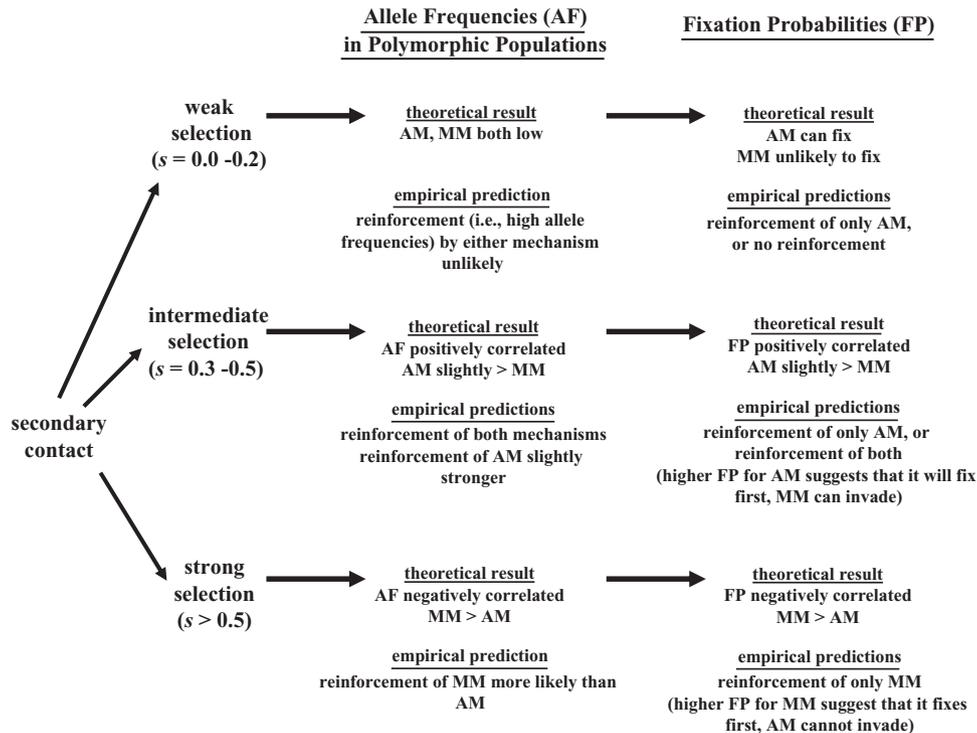


Figure 1. Summary of theoretical results for allele frequencies and fixation probabilities of assortative mating (AM) versus migration modification (MM) in the simultaneous evolution YT fixation model. In general, the outcome of reinforcement was highly dependent on selection strength but congruent results were seen for allele frequencies and fixation probabilities. The latter observation indicates that similar patterns of reinforcement are expected at different stages of the speciation process. The results shown consider a scenario where both mechanisms segregate at 1% upon secondary contact (i.e. at the initiation of the simulations). Some other scenarios were explored in the fixation model. For example, if MM was set as fixed at the onset of the simulation and AM was set at 1%, AM did not evolve and reinforcement of MM alone is observed. By contrast, if AM was set as fixed at the onset of the simulations, MM could invade (in fact the evolution of MM was facilitated) such that reinforcement of both mechanisms was likely.

by s . The progeny count was then updated in each population and each offspring then either migrates or remains within the local habitat, depending on its allele at the migration locus and the probability of migration (m). Finally, the post-selection, post-migration haplotype frequencies were updated in both populations, and the program looped back to the beginning of the reproductive cycle to initiate the next generation.

The YT fixation model ended each run when variation was lost at any locus. Possible outcomes included: a single allele at the marker/fitness locus is fixed in both populations, the AM allele is lost in both populations, the MM allele is lost in both populations, the AM allele is fixed in both populations, or the MM allele is fixed in both populations (fixation of AM or MM was designated full speciation). If none of these outcomes was realized in 1000 generations the simulation was stopped, but this scenario was very rare. For each of ten selection regimes ranging from $s = 0.10$ to 0.95, Yukilevich & True (2006) simulated

15 different migration regimes ranging from 0.02 to 0.30 in intervals of 0.02. Values of s smaller than 0.10 were not explored because reinforcement is unlikely under such weak selection (Servedio & Noor, 2003). The fixation probability for each migration-selection regime is the proportion of the 25 independent model runs under each regime in which AM or MM fixed. Here, we summarize the findings of the fixation models, focusing on previously unreported correlations between fixation probabilities for MM versus AM.

THEORETICAL RESULTS: FIXATION PROBABILITIES

The outcome when both mechanisms initially segregate upon secondary contact was highly dependent on the strength of selection (Figs 1, 2). Under weak selection ($s < 0.30$), AM fixed frequently (probability of fixation was 0.0–0.5), whereas MM almost never fixed. Thus, under weak selection, fixation of AM alone is expected (Fig. 2A). When selection strength

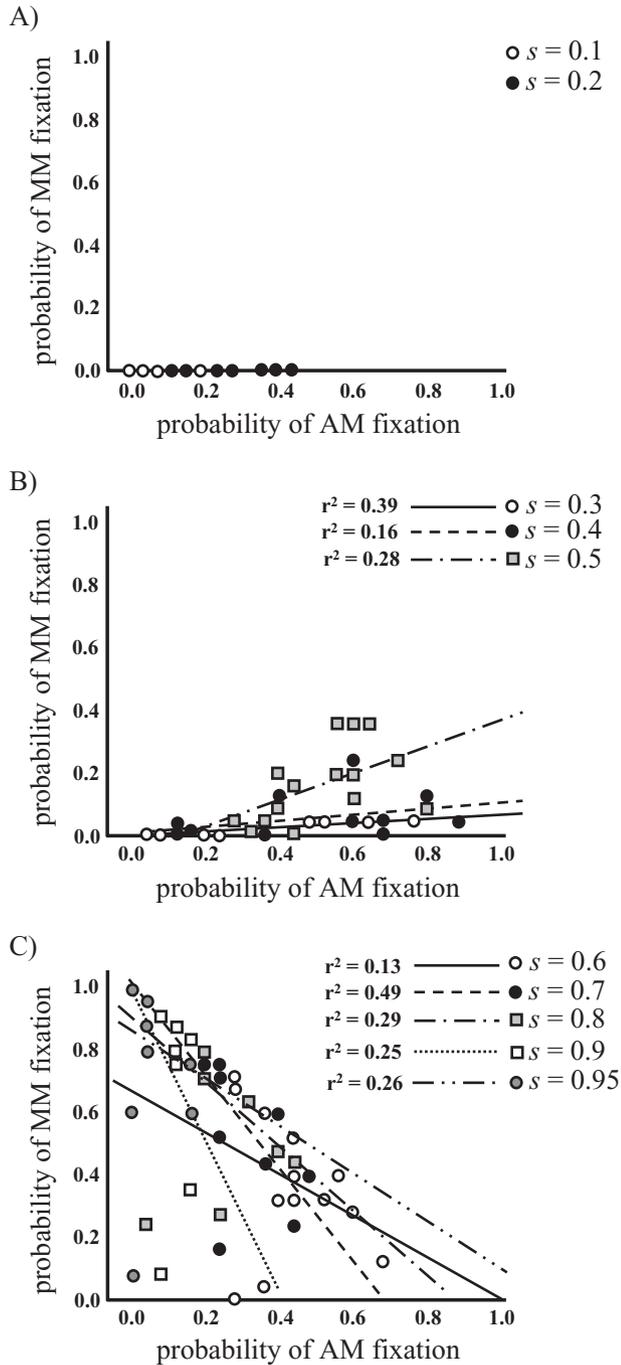


Figure 2. Correlation between the probability of fixation for assortative mating (AM) versus migration modification (MM) during simultaneous evolution under the niche-based isolation model. The data are from Yukilevich & True (2006), although these authors did not report these correlations. Penetrance of alleles is 100% and the two secondary contact populations are symmetrical in size. The 's' in the graphs is short-hand for selection strength against immigrant and hybrid matings in both niches. A, weak selection ($s < 0.3$). B, intermediate selection ($s = 0.3\text{--}0.5$). C, strong selection ($s > 0.5$).

was intermediate ($s = 0.3\text{--}0.50$), both mechanisms readily evolved and a positive correlation between their fixation probabilities was observed (Fig. 2B). However, absolute fixation probabilities are higher for AM, suggesting that it will fix first. By exploring cases in which AM fixed first and then seeing what happens to MM when it initially segregates at 1%, the YT models found that AM actually facilitated the evolution of MM. These results generate the prediction that both mechanisms might often be fixed under intermediate selection (Fig. 1). Under strong selection ($s > 0.5$), a negative correlation was observed between AM and MM fixation probabilities (Fig. 2C). The absolute fixation probabilities were much higher for MM, suggesting that it will evolve first. By seeing what happens to AM (segregating at 1%) when MM is fixed first, Yukilevich & True (2006) found that MM generally prevented the evolution of AM, by reducing migration and thus the opportunity for selection for AM. The predicted outcome under strong selection is thus the fixation of only MM (Fig. 1). These results are complex but illustrate that, when it comes to fixation probabilities, the order of evolution and the strength of selection are important for the final outcome of reinforcement.

NEW SIMULATIONS REGARDING ALLELIC FREQUENCIES IN A NICHE-BASED MODEL

With the goal of generating predictions for polymorphic populations, we examined allele frequencies for MM and AM prior to fixation (again using Monte Carlo simulation and the code from Yukilevich & True, 2006). The fixation model stopped runs when variation was lost at any of the loci. The key difference in the present simulations is that we now stop the simulation run before either reinforcement allele reaches fixation in either population. This is done to study the correlation between segregating AM and MM alleles.

To determine which generation to stop the run, we used one generation less than the generation number corresponding to the fastest time for fixation of either reinforcement allele for each migration-selection regime; average rates are in Yukilevich & True (2006). For example, if the fastest time for allelic fixation for $s = 0.3$ and $m = 0.1$ was 240 generations, we used 239 as the generation number to stop the run. This approach is necessary because the speed of fixation varies across different migration-selection regimes. For each of ten selection regimes (ranging from $s = 0.10$ to 0.95), we simulated 15 different migration regimes (ranging from 0.02 to 0.30 in intervals of 0.02).

Finally, we repeated these analyses using random stopping points, rather than the criteria noted above

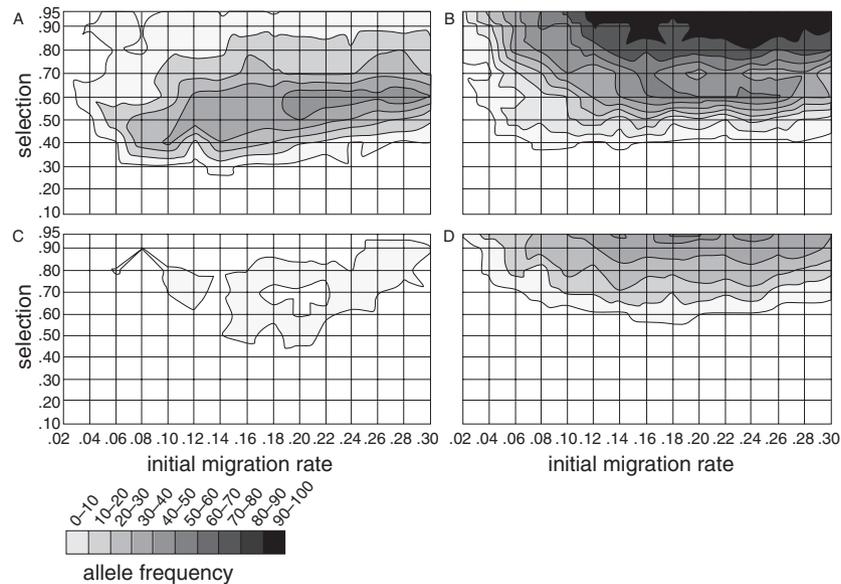


Figure 3. Results of the model examining allele frequencies for assortative mating and migration modification in polymorphic populations. Shown is the allele frequency under each selection and migration regime (averaged across different runs within each selection/migration regime). A, assortative mating, maximal stopping procedure. B, migration modification, maximal stopping procedure. C, assortative mating, random stopping procedure. D, migration modification, random stopping procedure.

(hereafter referred to as ‘maximal’ and ‘random’ stopping procedures, respectively). In the latter approach, runs were stopped randomly within predetermined generational ranges, where the range varied from the first generation to the fastest generation time for fixation (within each selection and migration regime). The random stopping procedure stops evolution in earlier generations than the maximal procedure, and thus the results stemming from it represent the earliest stages of reinforcement. Each procedure involved a total of 3750 runs, and congruent results were obtained from the different procedures (see below), providing confidence in our results. Although analytical theory to compliment the simulations would clearly be desirable, reinforcement theory is complex enough that simulations are generally required even when only a single mechanism is examined (Servedio & Kirkpatrick, 1997; Cain *et al.*, 1999; Servedio, 2000, 2001, 2004; Lemmon *et al.*, 2004). Thus, our simulations allow an initial exploration of the evolution of AM versus MM. Complete tables of allelic frequencies are available upon request.

THEORETICAL RESULTS: ALLELE FREQUENCIES

The patterns emerging from the new simulations are depicted in Figure 3, and predictions stemming from them are outlined in Figure 1. The highest allele frequencies for both AM and MM generally occurred under intermediate migration rates. The evolution of

both AM and MM was also highly dependent on selection strength, with different patterns observed for the different mechanisms of reinforcement. For MM, allele frequencies increased with increasing strength of selection, reaching maximal values under very strong selection ($s > 0.70$). For AM, low allele frequencies were observed under both weak ($s = 0.10$ – 0.30) and strong selection ($s > 0.70$), and allele frequencies reached maximal values under intermediate selection strengths. These results can be explained as follows: under weak selection, neither mechanism can reach high frequency; under intermediate selection, both mechanisms evolve to intermediate frequencies (with both mechanisms perhaps on their way to fixation); and, under strong selection, MM inhibits the evolution of AM.

We used two additional analyses to illustrate the inhibiting effect of MM on AM under strong selection. In both analyses, we averaged the mean allele frequencies from population 1 and 2. First, using the 25 runs within each selection and migration regime, we calculated the correlation between allele frequencies for AM versus MM. Averaged across migration regimes, this correlation tended to be positive for intermediate selection strengths but negative for strong selection (Table 2). Second, we examined differences in absolute allele frequencies by calculating the following value for each of the 3750 simulation runs: AM allele frequency minus MM allele frequency. Thus positive values indicate greater AM

Table 2. Statistical analyses suggesting that migration modification (MM) tends to inhibit the evolution of assortative mating (AM) under strong selection

Selection strength	Correlation between allele frequencies for AM vs. MM		Difference in allele frequencies: AM frequency minus MM frequency	
	Mean r	SD	Mean difference	SD
Maximum stop				
0.10	-0.49	0.13	0.005	0.017
0.20	-0.03	0.42	0.014	0.049
0.30	0.27	0.45	0.046	0.113
0.40	0.50	0.43	0.120	0.228
0.50	0.50	0.24	0.108	0.312
0.60	0.23	0.37	-0.093	0.419
0.70	0.02	0.34	-0.241	0.358
0.80	-0.10	0.40	-0.502	0.369
0.90	-0.07	0.43	-0.626	0.361
0.95	-0.10	0.44	-0.710	0.339
Random stop				
0.10	-0.17	0.21	0.002	0.002
0.20	-0.22	0.16	0.006	0.017
0.30	-0.14	0.37	0.012	0.039
0.40	0.06	0.32	0.020	0.051
0.50	0.38	0.34	0.030	0.103
0.60	0.55	0.20	0.031	0.111
0.70	0.66	0.16	-0.091	0.196
0.80	0.57	0.14	-0.178	0.257
0.90	0.53	0.20	-0.295	0.335
0.95	0.46	0.16	-0.367	0.359

Correlations are shown between allele frequencies for MM and AM, and differences in allele frequency between MM and AM (for details, see text). To highlight shifting trends, positive mean values are in bold. Sample sizes within each selection strength are 15 for correlations between allele frequencies (one correlation for each of 15 migration regimes within each selection regime) and 375 for differences in allele frequency.

than MM, values near zero indicate similar allele frequencies for AM and MM, and negative values indicate greater MM than AM. These values tended to be near zero or slightly positive for weak and intermediate selection because either neither mechanism was readily evolving or because both evolved to some extent. Conversely, under strong selection, these values tended to be highly negative, indicating much higher MM than AM (Table 2). Thus, the absolute value of the difference in allele frequency between AM and MM increased with increasing strength of selection ($r = 0.86$).

Although absolute allele frequencies were generally lower when runs were stopped randomly instead

of maximally, similar relative allele frequencies were observed between the two types of simulations (Fig. 3). Using random stopping, the correlation between allele frequencies for AM versus MM was strongest under intermediate selection strengths, and weakened under strong selection (although it did not become negative; Table 2). As observed for the maximal stopping procedure above, differences in the allele frequency between AM and MM were slightly positive under weak to intermediate selection and negative under strong selection, with the absolute value of the difference again highly correlated with selection strength ($r = 0.82$; and differences were correlated between the two stopping procedures, $r = 0.81$).

SUMMARY OF THEORETICAL PREDICTIONS

The results from the new simulations examining allele frequencies were highly congruent with the past work on fixation probabilities. In general, when selection and migration regimes are unfavorable for reinforcement, both mechanisms will be prevented from evolving to either high frequency or to fixation. When selection strength is intermediate, then both mechanisms are likely to increase in frequency, and to fix, although AM is expected to be somewhat stronger than MM. Under strong selection, we observed a negative correlation for the probability of fixation of the different mechanisms, and for their allele frequencies, because MM can evolve to high frequency thereby decreasing selection favoring AM.

EMPIRICAL STUDY SYSTEM

GEOGRAPHIC SCENARIOS

Timema walking-sticks are wingless insects inhabiting the chaparral of Southwestern North America (Crespi & Sandoval, 2000). *Timema cristinae* exhibits populations feeding on one of two host-plant species (*Ceanothus* versus *Adenostoma*). A 'population' of walking-sticks is defined as all of the insects collected within a homogenous patch of a single host-plant species (as in Nosil *et al.*, 2002, 2003). Thus 'parapatric' insect populations are in contact with an adjacent population of insects adapted to the alternative host. Conversely, 'allopatric' populations are separated from all other populations adapted to the alternative host by distances more than 50 times the 12 m per-generation gene flow distance (Sandoval, 1993, 2000). The regions between allopatric populations are occupied by unsuitable hosts. Under this scheme, the geographic categorization of populations is determined by the distribution of the host plants, rather than by characteristics of the insects themselves. This allows us to focus on the conditions favoring isolating

barriers, rather than their consequences for population designation.

MIGRATION MODIFICATION AND ASSORTATIVE MATING

In *T. cristinae*, there is experimental evidence for host-specific selection against immigrants and hybrids (Sandoval, 1994a,b; Nosil, 2004; Nosil & Crespi, 2006) but intrinsic F_1 hybrid egg inviability is absent (Nosil *et al.*, 2007). Thus, individuals moving between hosts suffer a fitness cost, and this species best fits a niche-based isolation model. Previous work has shown that populations on different hosts exhibit genetically-based pre-mating isolation caused by divergent mate and host preferences, and that selection against immigrants and hybrids contributes to the evolution of both forms of pre-mating isolation (Nosil *et al.*, 2002, 2003; Nosil, 2004; Nosil *et al.*, 2005, 2006a,b). Additionally, reproductive character displacement occurs in some cases, there is molecular and morphological evidence for ongoing interbreeding between populations ('hybridization'), and alternatives to reinforcement were tested but unsupported (Nosil, *et al.*, 2003; Nosil, Egan & Funk, 2008). Thus, divergent mate and host preferences in *T. cristinae* can be considered AM and MM respectively, with reinforcement contributing to their evolution.

We present data from the 12 populations studied for AM in Nosil *et al.* (2003; eight parapatric and four allopatric populations) because all these were also examined by Nosil *et al.* (2006a) for MM. The magnitude of reproductive isolation between allopatric populations resulting from each mechanism is comparable (0.20 versus 0.13 respectively, where 0 = random mating and 1 = complete isolation; Nosil, 2007). Thus, we assume the mechanisms segregate at equal levels and evolve simultaneously upon secondary contact. Because allopatric population pairs of *T. cristinae* exhibit both partial MM (Nosil *et al.*, 2006a) and partial AM (Nosil *et al.*, 2002), it is probable that genetic variation for both mechanisms of reinforcement segregates upon secondary contact (i.e. the system is polymorphic for both mechanisms). However, partial reproductive isolation cannot distinguish between genetic variation versus fixation of alleles with weak effects or incomplete penetrance. There is direct quantitative genetic evidence for genetic variation in host preference within populations (Nosil *et al.*, 2006b). Although comparable data do not exist for mate preference, the available data suggest that polymorphism for MM and AM is the most likely scenario. Even if this were not the case, any match of empirical data to the simulation results is still informative because we observed qualitatively similar patterns for fixation probabilities versus allele frequencies.

REINFORCEMENT VERSUS REPRODUCTIVE CHARACTER DISPLACEMENT

The classic signature of the process of reinforcement is the pattern of reproductive character displacement (RCD): greater pre-mating isolation in regions where maladaptive hybridization occurs (sympatry/parapatry) relative to where it does not (allopatry). However, the process of reinforcement can occur even if the pattern of RCD is not produced (Servedio & Noor, 2003; Lemmon *et al.*, 2004). Divergent selection on mate or host preference between allopatric populations (e.g. due to ecological differences between populations) can cause strong divergence between allopatric populations, obscuring RCD (Day, 2000; Servedio, 2001, 2004; Kirkpatrick & Ravigné, 2002). Additionally, gene flow counteracts reinforcing selection such that the process of reinforcement may be occurring in parapatry but without generating stronger pre-mating isolation than observed in allopatry. Reinforcement could be 'weakly successful', counteracting the effects of gene flow only to the extent that similar levels of divergence are seen in allopatry versus parapatry.

Thus, reinforcement can be studied independent of RCD if the criteria for reinforcement are met and if the evolution of pre-mating isolation in allopatric populations is understood. Both these conditions are satisfied in *T. cristinae*. The evidence for reinforcement is outlined above and pre-mating isolation among allopatric populations evolves as a by-product of adaptation to different hosts (Nosil *et al.*, 2002, 2006a; Nosil, 2007). Thus, we can study reinforcement in *T. cristinae* by examining the strength of pre-mating isolation: (1) among parapatric populations that exhibit variable effects of reinforcement and (2) for parapatric versus allopatric populations (even if RCD is lacking).

SELECTION: MIGRATION REGIMES IN *T. CRISTINAE*

Timema cristinae fits a niche-based model with AM and MM segregating upon secondary contact. However, at least two additional factors are needed for interpreting reinforcement. The first is the strength of selection against immigrants and hybrids. In *T. cristinae*, Nosil (2004) estimated the mean strength of selection against immigrants upon secondary contact between allopatric populations at $s = 0.53$. The strength of selection against immigrants between 12 parapatric population pairs ranged from $s = 0.02$ to 0.30 (mean = 0.15), depending on migration regime. In addition to varying between geographic scenarios, selection estimates also vary slightly among populations, experiments, and immigrants versus hybrids (Nosil *et al.*, 2003, 2007). Thus, a range of selection estimates from $s = 0.10$ to 0.50 applies well to the system (Table 1). The second factor is migration rates,

which in *T. cristinae* were inferred with coalescent-based analyses applied to mitochondrial DNA sequence variation (Nosil *et al.*, 2003; Nosil & Crespi, 2004). The migration rates into parapatric populations from adjacent populations are comparable to the range of 0.02 to 0.30 explored in the YT model (*T. cristinae*, range of $m = 0.001$ – 0.232 , mean $m = 0.043$).

EMPIRICAL METHODS

MIGRATION REGIMES, ASSORTATIVE MATING AND MIGRATION MODIFICATION

Our simulations examined the conditions favoring the evolution of the different mechanisms of reinforcement. Determining their levels in *T. cristinae* populations under different migration regimes provides an empirical evaluation of such conditions. Therefore, we examined the association between an index of migration and measures of each isolating barrier. The index of migration reflects the size of a focal population relative to its adjacent population on the alternative host. Population size is estimated from host-plant patch size, such that the index value for each focal population is the proportion of the total area (focal population plus adjacent population) occupied by the alternative host. Thus, allopatric populations are assigned a value of zero and a parapatric population equal in size to its adjacent population would be assigned a value of 0.5. This index has been validated as a meaningful measure of the migration rate into a focal population by field (Sandoval, 1994a) and molecular studies (Nosil *et al.*, 2003).

For AM, we estimated the mean level of between-population mating discrimination that females from each of the populations examined in Nosil *et al.* (2003) exhibit against males from all other study populations using the alternate host plant (calculated for each of the twelve study populations as mean copulation frequency of females with males from their own population subtracted from the mean copulation frequency of females with foreign males from other populations). This averaging among populations provides a general measure of the degree to which a focal population is sexually-isolated from other populations and should not bias the results, although it decreases precision. This approach is required to minimize non-independence among population pairs. Finally, averaging among populations is warranted because parapatric *T. cristinae* populations exhibit similar levels of pre-mating isolation against all other populations (be they adjacent to them or not). This ‘universal effect’ of reinforcement indicates that mean data from single populations are valid measures of overall mating discrimination (Zouros & d’Entremont, 1980; Hoskin *et al.*, 2005). For MM, we estimated the

proportion of individuals picking their population’s native host in choice tests (Nosil *et al.*, 2006a).

CORRELATION BETWEEN ASSORTATIVE MATING AND MIGRATION MODIFICATION

We also tested whether the effects of reinforcement on levels of AM are correlated with the effect of reinforcement on MM. We quantify the effect of reinforcement for each parapatric population using the difference between the pre-mating isolation of the focal parapatric population (AM or MM as estimated above) and the mean pre-mating isolation of allopatric populations. This difference measures the level of pre-mating isolation a parapatric population exhibits relative to the average of allopatric populations (which do not undergo reinforcement). For AM, the allopatric mean was attained by calculating the individual AM values for each of the four allopatric populations, and then averaging them. For MM, the allopatric mean was calculated by averaging the individual values for allopatric populations that use the same host as the parapatric population. We tested for the expected positive association (Figs 1, 2) between these measures of the effects of reinforcement using one-tailed correlation analysis.

EMPIRICAL RESULTS

CONDITIONS FAVORING ASSORTATIVE MATING AND MIGRATION MODIFICATION

Strong effects of reinforcement occurred under a wider range of migration rates for AM than for MM. RCD was only observed for AM (Fig. 4A versus 4B). Thus, the association between preference for the native host and our index of migration was linear and negative for populations on both hosts ($r = -0.83$, -0.56 , $P = 0.021$, 0.123 for *Ceanothus* and *Adenostoma*, respectively, combined $P < 0.0025$; Fig. 4B). Conversely, the association between AM and our index of migration was ‘humped’, with the strongest AM occurring under intermediate migration rates (quadratic regression coefficient = -0.79 , $P = 0.042$; Fig. 4A; for further support for this trend, see Nosil *et al.*, 2003). These results are qualitatively consistent with the YT fixation model, which found that, under these (weak to intermediate) selection regimes and (low to intermediate) migration rates, AM fixed under a much broader range of migration conditions relative to MM (Fig. 1). Furthermore, the YT models also exhibited a ‘humped’ AM evolution pattern, with intermediate migration rates giving rise to highest probability of AM fixation (Yukilevich & True, 2006; Fig. 3). Presumably, this occurs because reinforcement requires some gene flow to generate the hybrids that are selected against, but too much gene flow

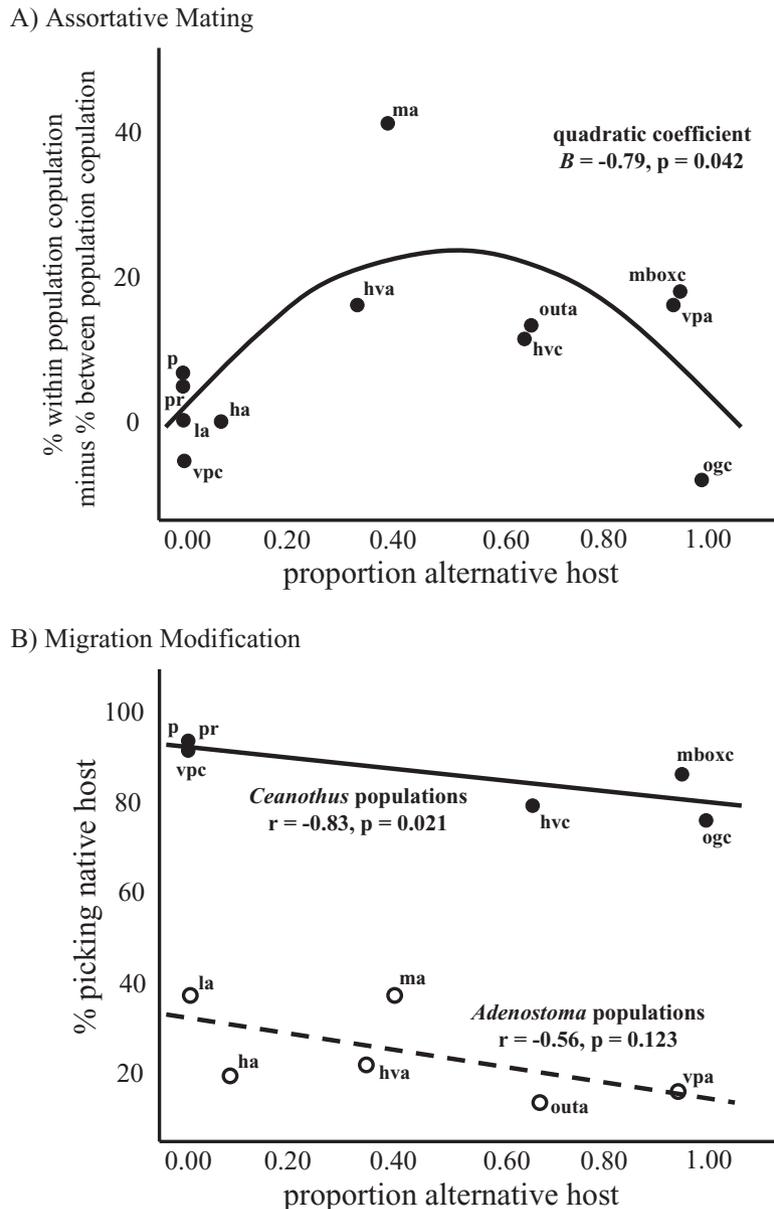


Figure 4. Reinforcement in *Timema cristinae* in relation to migration regimes. The y-axis represents the magnitude of pre-mating isolation. The x-axis represents the proportion of the total area (area of the study population plus the area of the adjacent population using the alternate host) occupied by the alternative host. As the value on the x-axis increases, migration into the study population, from its adjacent population, increases (Nosil *et al.*, 2003) A, assortative mating, line fit using quadratic regression. B, migration modification, line fit using linear regression. Closed circles, *Ceanothus* populations; open circles, *Adenostoma* populations.

swamps the effects of reinforcing selection (Kirkpatrick, 2000; Servedio & Noor, 2003; Coyne & Orr, 2004; Gavrillets, 2004).

CORRELATION BETWEEN ASSORTATIVE MATING AND MIGRATION MODIFICATION

We detected a positive correlation between the effect of reinforcement on AM and the effect on MM

($r = 0.67, P = 0.034$). This correlation was greatly influenced by one population with very high values for both forms of pre-mating isolation ('ma'; Fig. 5). Thus, we also examined the correlation using a jack-knife approach. For six of the seven regressions with 'ma' included, the correlation remained strong and significant (all $r > 0.65$, all $P < 0.05$). In the seventh case, the result approached significance ($r = 0.66, P = 0.055$). However, the regression with 'ma'

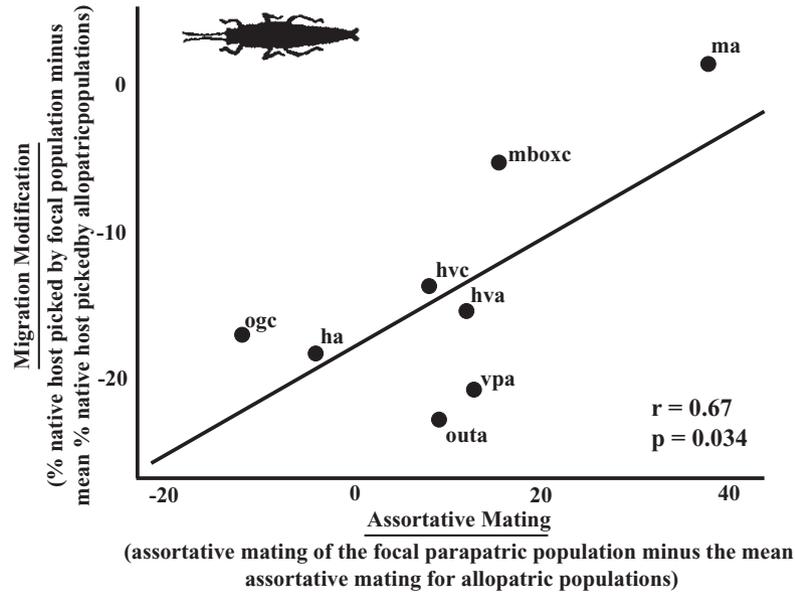


Figure 5. Among eight parapatric populations, the effects of reinforcement on levels of assortative mating were positively correlated with the effects of reinforcement on migration modification.

excluded was not significant ($r = 0.23$, $P = 0.31$) and a nonparametric rank correlation with all eight populations was not significant ($\rho = 0.48$, $P = 0.12$). Thus, the relationship between AM and MM is positive, but strongly influenced by one extreme (yet accurate) data point. This correlation was not reported in past studies, and was unexpected given the disparate patterns of RCD reported above. These results are consistent with the past YT model and with our new simulations which, under these selection-migration conditions, produce a positive correlation between AM and MM (Figs 1, 2).

DISCUSSION

REINFORCEMENT IN *T. CRISTINAE* AND YT MODELS

In *T. cristinae*, the effects of reinforcement appear stronger for AM than for MM, such that RCD occurs only for AM. This pattern holds even if the correlation between AM and MM in Figure 5 does not, and is thus our most robust empirical finding. If selection on pre-mating isolation in allopatry is strong (e.g. via ecological selection), it could result in strong allopatric divergence, thereby precluding a pattern of RCD (Servedio, 2001, 2004). For host preference specifically, search and efficiency costs in allopatric populations can favor increased preference for the single host in the environment because individuals without strong preferences accrue lower fitness for reasons other than switching to an alternate host (Jaenike, 1990; Bernays & Wcislo, 1994; Janz & Nylin, 1997; Carriere, 1998; Bernays & Funk, 1999). For example,

such individuals might take longer to locate or to decide whether to feed on the utilized host, thereby wasting time and energy and increasing predation risk. In the case of *T. cristinae*, stronger selection on host versus mate preference in allopatric population could result in RCD being less likely for MM. Or perhaps mating decisions occur more commonly than host choice decisions because insects can move whereas plants cannot, such that the opportunity for reinforcement is greater for AM (Nosil *et al.*, 2006a). More generally, the results of the current study highlight the utility of considering reinforcement as a process, one which is predicted to generate RCD, but may not always do so, depending on selection in allopatric populations, and rates of migration (Servedio, 2001, 2004). Focusing exclusively on RCD would not have permitted the analyses in the present study.

The stronger effects of reinforcement on AM in *T. cristinae* are consistent with the simulation results. For example, the original Yukilevich & True (2006) simulations predicted that AM evolves under a wider range of migration rates than MM when selection is low or intermediate. Yukilevich & True (2006) suggested that this pattern occurs because the MM allele can only reduce hybridization by not immigrating into other patches but it cannot prevent mating with incoming migratory individuals, and is thus less effective relative to AM which is actually involved in mating decisions. Furthermore, the MM allele, by its quality of being more sedentary, cannot spread between populations and thus often fails to rescue

itself from being lost by genetic drift when it is rare (e.g. when selection is not strong). Our new simulations in polymorphic populations also suggest that AM tends to be somewhat stronger than MM under weak to intermediate selection regimes (Table 2). Our new simulations further suggest that positive correlations between allele frequencies for the different reinforcement mechanisms are expected under intermediate selection strengths, again as observed in *T. cristinae* (assuming the correlation holds as shown in Fig. 5).

Thus, some general conclusions emerge from our collective results. Both the probabilities of fixation and allele frequencies for the different mechanisms of reinforcement vary according to selection and migration regimes. Under intermediate selection strengths, the different mechanisms are expected to be positively correlated but with AM stronger than MM, as supported by both types of simulations and the example from nature. The similarity of results from fixation probabilities and allele frequencies generates the prediction that similar patterns of reinforcement are expected within and among species in nature, and thus during different stages of the speciation process (but see also Jiggins, *et al.*, 2001; Jiggins, Estrada & Rodrigues, 2004).

THEORY FROM RELATED STUDIES

As noted by Yukilevich & True (2006), most reinforcement models tend to examine mate preference alone. It is worth pointing out that a different class of models, namely those dealing with sympatric speciation, have sometimes examined the joint evolution of AM and MM. For example, Johnson *et al.* (1996) examined the joint evolution of AM (their 'nonhabitat mating sympatric divergence') and MM (their habitat preference or 'habitat-mating sympatric divergence'), via selection on a third locus that affects fitness. This model examines sympatric speciation, rather than reinforcement, but these two types of models often share many common features (e.g. the evolution of AM via selection on a fitness character/locus; see also Kondrashov & Kondrashov, 1999). One main difference is that reinforcement, unlike sympatric speciation, is often facilitated by some degree of initial divergence in allopatry (Liou & Price, 1994; Kirkpatrick & Ravigné, 2002). Johnson *et al.* (1996) found that each type of pre-mating isolation facilitates the effectiveness of the other two types such that they operate together to promote speciation, as was observed in some instances in the YT model (e.g. when AM fixes first). A clear difference between the models is that the YT models examine fixation probabilities and allele frequencies at single loci, whereas Johnson *et al.* (1996) focused on the build up of

linkage disequilibrium among loci. Moreover, the Johnson *et al.* (1996) model examines a smaller range of selection coefficients, focusing on weak to intermediate selection, in which a positive correlation between AM and MM is also expected based upon the present study. Future modeling efforts examining the interactions between different types of reproductive barriers are clearly warranted.

RELATED EMPIRICAL STUDIES OF REINFORCEMENT

There are few empirical studies examining both AM and MM in the context of reinforcement (Servedio & Noor, 2003). Empirical studies have sometimes examined the evolution of these different reproductive barriers, but usually the role of reinforcement is unclear (e.g. allopatric populations were studied, hybrid fitness is unknown, etc.). Both forms of pre-mating isolation have been examined in sympatric ecotypes of *Littorina saxatilis*; AM was reasonably strong (Rolan-Alvarez *et al.*, 1999), but MM was weak (Cruz *et al.*, 2004). Nasil *et al.* (2005) examined numerous reproductive barriers in 20 taxa. In four of these taxa, both AM and MM were measured. In all four cases, $MM > AM$ and selection against immigrants was strong ($s > 0.6$), consistent with the YT model. Funk, Filchak & Feder (2002) reviewed pre-mating isolation in numerous herbivorous insects, detecting ten study systems in which both host preference (MM) and mate preference (AM) had been quantified. In seven cases, both AM and MM were observed, and in the other three only MM was observed. These patterns are consistent with two scenarios in the YT model: (1) there is strong selection such that only MM is observed and (2) AM fixes first, and MM invades such that both are observed. Although these patterns are consistent with the YT model, they are not from examples of reinforcement per se and focused empirical studies of the different mechanisms of reinforcement are needed.

CAVEATS, QUALIFIERS AND FUTURE DIRECTIONS

Further studies are required before generalities will emerge. In particular, empirical systems in which multiple populations can be studied are required to examine associations between AM and MM under different conditions. Because theoretical outcomes are dependent on basic parameters, such as the strength of selection, migration rates, and segregation of AM versus MM, systems in which such parameters can be measured will be particularly useful. Once such systems become available, we can continue to bridge the gap between data and theory. Furthermore, the flow of information should not be viewed as unidirectional and, as empirical data emerges, it will calibrate theoretical models.

Our results are most applicable to scenarios where populations use different habitats, a scenario that is likely common given that habitat heterogeneity is widespread. Finally, we note that, even in scenarios without distinct habitat differences, some forms of MM might evolve. For example, although habitat preference such as observed in *T. cristinae* will not evolve without habitat differences, reduced movement could evolve, with this latter form of MM promoting speciation (Yukilevich & True, 2006).

An obvious unknown factor in the present study, and one that is known to affect reinforcement in theory (Servedio & Noor, 2003), is the genetic architecture of reinforcement. Although an empirical example of the one-allele mechanism examined in our models recently emerged (Ortiz-Barrientos & Noor, 2005), *T. cristinae* may not match this mechanism. However, for this to confound our comparisons between data and theory requires that qualitatively different predictions emerge from modeling one-allele versus two-allele mechanisms of reinforcement. Although reinforcement is less likely in absolute terms under a two-allele mechanism (Felsenstein, 1981; Servedio & Noor, 2003), the effects of genetic architecture on the relative roles of the different mechanisms of reinforcement is unknown. This is clearly an area where further modeling efforts are required, and where empirical data on the genetics of reinforcement can help inform theory (Sezer & Butlin, 1998; Ortiz-Barrientos, Counterman & Noor, 2004; Ortiz-Barrientos & Noor, 2005).

Our most general theoretical conclusion is that similar patterns are expected for allele frequencies versus fixation probabilities during reinforcement. Identifying congruence between empirical and theoretical patterns indicates that the YT model might be quite general. However, it is premature to make strong conclusions based upon only a few models and empirical systems, and further work on multiple mechanisms of speciation is likely to be informative.

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