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Asymmetrical patterns of speciation uniquely support reinforcement in *Drosophila*

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Abstract: Understanding how often natural selection directly favors speciation, a process known as reinforcement, has remained an outstanding problem for over 70 years. While reinforcement has been strongly criticized in the past, it is once again seen as more realistic due to the seminal discovery of enhanced prezygotic isolation among sympatric species and to a handful of well-studied examples. Nevertheless, the pattern of enhanced isolation in sympatry has alternative explanations, highlighting the need to uncover unique signatures of reinforcement to determine its overall frequency in nature. Using a novel dataset on asymmetrical prezygotic and postzygotic isolation among *Drosophila* species, I uncover new patterns explicitly predicted by reinforcement. Broadly, I found that almost all sympatric species had concordant isolation asymmetries, where the more costly reciprocal mating has greater prezygotic isolation relative to the less costly mating. No such patterns exist in allopatry. Using simulations, I ruled out alternative explanations and showed that concordant isolation asymmetries in sympatry are likely unique signatures of reinforcement. These results allowed me to estimate that reinforcement may impact 60% to 83% of all sympatric *Drosophila* and enhance premating isolation by 18% to 26%. These findings suggest that reinforcement plays a key role in *Drosophila* speciation.

Among all of the mechanisms of speciation, reinforcement has been one of the most widely discussed topics in evolutionary biology. While speciation is typically assumed a byproduct of other evolutionary processes, reinforcement uniquely posits that natural selection directly favors the speciation process (Servedio and Noor 2003). Reinforcement has had a turbulent history from early enthusiasm by its founders Th. Dobzhansky (1940) and R. A. Fisher (1930), to strong criticism in the 1980's (Paterson 1978; Felsenstein 1981; Templeton 1981; Butlin 1987; Butlin 1995). The theory is once again seen as realistic, due in large part to the discovery of enhanced sexual isolation in sympatric *Drosophila* species relative to allopatric species of similar genetic age, first theorized and studied by Dobzhansky et al. (1968) and later generalized by Coyne and Orr (1989, 1997; also see Noor 1997). This pattern stimulated others to study the theoretical plausibility of reinforcement (e.g. Liou and Price 1994; Kelly and Noor 1996; Servedio and Kirkpatrick 1997; Servedio 2000; Yukilevich and True 2006), to identify the pattern of enhanced isolation in other organisms (Howard 1993; Lukhtanov et al. 2005, but see Moyle et al. 2004), and to characterize several empirical examples (Noor 1995; Saetre et al. 1997; Pfennig and Simovich 2002; Nosil et al. 2003; Hoskin et al. 2005; Jaenike et al. 2006).

Despite this progress, the overall frequency of reinforcement remains uncertain. First, many have favored an alternative explanation for the enhancement of sexual isolation in sympatry, known as the Templeton effect (see Paterson 1978; Templeton 1981; Powell 1997; Coyne and Orr 2004). It argues that enhanced isolation in sympatry arises because strongly prezygotic isolated allopatric pairs survive better in secondary contact than weakly isolated allopatric pairs, which either go extinct or fuse back into each other, hence biasing what we observe in sympatry. Because Coyne and Orr (1989, 1997) studied a limited sample of allopatric taxa, it is unknown whether more sampling would uncover these young, but strongly prezygotic isolated taxa in allopatry and hence support the Templeton effect (Noor 1997). Second, while several important studies in *Drosophila* (Noor 1995; Jaenike et al. 2006), walking-stick insects (Nosil et al. 2003), birds (Saetre et al. 1997) and amphibians (Pfennig and Simovich 2002; Hoskin et al. 2005) have found support for reinforcement, it is still unclear how exceptional these cases are in nature and whether studies that fail to support reinforcement go unpublished and thus bias our perception of reinforcement's overall impact in nature (Howard 1993; Noor 1997; Coyne and Orr 2004). Thus in their recent book on speciation, Coyne and Orr (2004; pg. 381)

argue that until additional predictions of reinforcement are identified and tested, we will not be able to determine the overall impact of this process in nature.

Interestingly, reinforcement theory makes another set of key predictions that so far have remained largely unexplored. Broadly, reinforcement predicts that selection pressure to evolve prezygotic isolation should be a function of the hybridization cost both within and across species pairs (e.g. Liou and Price 1994; Kelly and Noor 1996; Servedio and Kirkpatrick 1997; Servedio 2000; Yukilevich and True 2006). Cost in this context is defined as the loss of fitness from mating with the foreign species individuals and failing to produce fully viable, fertile or ecologically and behaviorally functional hybrids.

First, at the within-species pair level, the cost of hybridization, and hence selection pressure to avoid it, may differ between the two reciprocal matings (Noor 1995; Pfennig and Simovich 2002; Jaenike et al. 2006; Cooley 2007). These asymmetries in postzygotic isolation are widespread in nature and have been documented since Darwin (recently referred to as “Darwin’s Corollary”; see Turelli and Moyle 2007). Thus, whenever postzygotic isolation is asymmetrical, reinforcement predicts that prezygotic isolation in sympatry should evolve to be asymmetrical in the same direction. This agreement between the directions of prezygotic and postzygotic isolation asymmetries is henceforth referred to as “*concordant isolation asymmetries*”. In contrast, “*discordant isolation asymmetries*” can be defined as when prezygotic and postzygotic isolation asymmetries are in the opposite direction between reciprocal matings. Since in allopatry, prezygotic isolation is expected to evolve independently of postzygotic isolation, *concordant and discordant isolation asymmetries* should be equally common. Moreover, at the level of many species pairs, we also expect that prezygotic isolation will be related to the cost of hybridization in sympatry. No one has yet tested these theoretical predictions in any group of organisms.

Second, sexual selection theory broadly predicts that costs of hybridization should be greater for females than for males due to females’ greater investment in each reproductive event (e.g. Darwin 1859, 1871; Williams 1966; Andersson 1994: e.g. pgs.3-5, 146-148; Partridge and Parker 1999; Coyne and Orr 2004). Further, females from a rarer or smaller population size species are expected to experience greater effective cost of hybridization than females from the more common species because the rarer females should encounter more heterotypic mating attempts by foreign males. As a result of these two assumptions, reinforcement predicts that the

selection pressure to evolve prezygotic isolation should be stronger for females of the species with the smaller range or population size even when postzygotic isolation is symmetrical across reciprocal matings (Noor 1995; Bordenstein et al. 2000; Hoskin et al. 2005). This second prediction of reinforcement will be referred to as the “*rarer-female effect*”. While the *rarer-female effect* has been found in the few cases of reinforcement above, there has never been a general test of this prediction across many taxa to see if this is a common pattern in nature.

The present work accumulates data from the literature on over 630 *Drosophila* species pairs for both prezygotic (exclusively premating because prezygotic postmating isolation data is largely lacking in *Drosophila*) and postzygotic isolation indexes, their asymmetries across the two reciprocal matings, and their geographical range sizes and overlap (extending the meta-analysis of Coyne and Orr 1989, 1997). The *Drosophila* genus still remains unique in having the most detailed information about isolation indexes across many species. In this paper, I first determine if more intensive sampling of allopatric and sympatric species pairs would support the Templeton effect hypothesis, then proceed to test the two new predictions of asymmetrical reinforcement: 1) by first asking if *concordant isolation asymmetries* are enriched in sympatry relative to allopatry and 2) then determining if data supports the *rarer-female effect* in sympatry. I then develop a computer model of how these asymmetrical patterns can result from reinforcement and study whether alternative processes, such as the Templeton effect, can also explain these asymmetries in sympatry. Finally, I combine these asymmetrical patterns of speciation with the other signature of reinforcement (i.e. enhanced premating isolation in sympatry) to provide a new estimate of the impact of reinforcement on sympatric *Drosophila* species.

Methods

THE DATA

All of the data on: 1) premating isolation, 2) postzygotic isolation, 3) genetic distances using Nei's D, 4) geographical range sizes, and 5) geographical range overlap of species pairs was accumulated directly from primary literature on over 630 species pairs in the genus of *Drosophila*. Effort was made to check the consistency of all data with previous meta-analyses of Coyne and Orr (1989, 1997) and of Bock (1984), the latter study focusing exclusively on

postzygotic isolation across the genus of *Drosophila*. In those cases where there was some discrepancy in the values between the present analysis and previous work, the data were identified as such (see data spreadsheets below). The raw and the phylogenetically corrected data on all species pairs are available online at: <http://www.Drosophila-speciation-patterns.com>. Geographical and ecological information not directly used in the present paper is also available. All references used to accumulate data are linked with particular species pairs. The goal of this public dataset is to maintain and increase the knowledge of these basic parameters across the *Drosophila* genus. Experts of particular species groups with new knowledge of their biology are encouraged to email: yukilevr@union.edu. Their names, affiliation, and email address will be associated with this update if the information has not yet been published.

CALCULATING PREMATING AND POSTZYGOTIC ISOLATION AND ASYMMETRIES

Premating isolation between each species pair was based on the percentage of successful copulations for each mating type during the period of the experiment (henceforth referred to as *mating %*). No-choice, single-choice and multiple-choice designs were considered. No-choice experiments involved placing individual females with either one or certain number of males into separate vials of each mating type (e.g. homotypic, heterotypic) and then counting the percentage of females that were inseminated (determined by evidence of sperm in female reproductive tract upon dissection). Single-choice and multiple-choice tests involved placing one or both sexes of each species with both conspecific and heterospecific individuals of the opposite sex and then counting the percentage of inseminations of each mating type by dissection or observation. At least two studies instead counted the number of females producing viable zygotes (one in *quinaria* group and one in *buzzati* complex; see spreadsheet online). This design confounds pre mating and postzygotic isolation. However, their inclusion did not affect the results of the paper. Results were averaged whenever more than one experiment was performed for a given species pair. The overall pre mating isolation index is equal to $1 - (\text{sum of heterotypic matings \%}) / (\text{sum of homotypic matings \%})$. Each reciprocal heterotypic mating isolation index is equal to $(\text{reciprocal heterotypic mating \%}) / (\text{average mating \% between both homotypic matings})$. Premating isolation asymmetry is equal to reciprocal mating #1 isolation index – reciprocal mating #2 isolation index.

Postzygotic isolation between each species pair was based on the percentage of F1 hybrid male and female sterility and/or inviability for each reciprocal mating. All hybrid sterility and inviability effects were considered. For each reciprocal mating, postzygotic isolation can range from 0, where both hybrid females and males are fertile and viable, to 0.5, where either hybrid females or males are sterile or inviable, to 1, where both hybrid sexes are either sterile or inviable. Partial sterility and inviability of each hybrid sex was also accounted whenever available by multiplying the above indexes by percentage of hybrid sterility or inviability. The overall postzygotic isolation index is the average of the reciprocal mating indexes. The postzygotic isolation asymmetry is calculated the same way as for premating isolation above.

PHYLOGENETIC CORRECTIONS

Phylogenies of each species group were either based on allozyme or molecular datasets from the literature. I considered all phylogenies whenever more than one phylogenetic analysis was performed on a particular species group. In the few cases where different phylogenetic analyses did not agree, I considered each one separately in the overall analysis. I used the same phylogenetic correction as in Coyne and Orr (1989, 1997) and Fitzpatrick and Turelli (2006) by averaging non-independent values. Star phylogenies were averaged over all unresolved species pairs.

ESTIMATING RELATIVE RANGE SIZES OF SPECIES AND THEIR PERCENT OF GEOGRAPHICAL OVERLAP (% SYMPATRY)

I used known range size maps from the literature and determined the absolute area for each species in square kilometers using Google Map Area Calculator: <http://www.daftlogic.com/projects-google-maps-area-calculator-tool.htm>. The relative range size of each species is equal to absolute range of species / sum of absolute species ranges of both species.

Using the same approach, I then determined the absolute range size overlap between each species pair in square kilometers. The percent of overlap for each species is equal to absolute range size overlap between the two species / absolute range size of that species. I then calculated the average percent of geographical overlap between these species by averaging these values. This calculation differs from that of Fitzpatrick and Turelli (2006) because in their analysis,

whenever one species range was completely contained within another range, they assumed 100% overlap, whereas in the present case, I calculated the overlap for each species and then averaged them. Thus in my analysis, the species with the larger range encounters the species with the smaller range by some percentage less than 100%.

ESTIMATING AVERAGE COST OF HYBRIDIZATION

The average cost of hybridization was calculated as the product of the average postzygotic isolation between species and the average percent of geographical overlap between species (see above for details).

COMPUTER SIMULATIONS

The life cycle. The code (written in Basic and is available upon request) describes an individual-based population genetic model with diploid individuals, each genotype having four genetic loci. The simulation begins with two distinct “island” populations that have accumulated some low level of premating isolation and some level of intrinsic postzygotic isolation in allopatry and may also experience divergent natural selection between the two inhabiting environments. In each population, individuals are initially generated from the initial allelic frequencies at each locus. The model then divides each population into females and males and lets each female choose a mating partner based on her mating preference locus and his sexual mating cue locus (see details below). Females and males then undergo meiosis to generate the respective gametes that can undergo recombination (which can be zero in males to reflect recombination in *Drosophila*). Each mating pair then produces a number of zygote progeny determined by a fecundity parameter. The offspring of each mating pair then experience viability selection which can be asymmetrical between the two reciprocal matings (see details below). The surviving juveniles can then experience divergent ecological selection against migrant homozygote genotypes at the ecological trait locus and against hybrids at that locus (see details below). Surviving adults are then randomly assigned to remain in the local population or to migrate to the other population based on the migration rate parameter. The next generation begins with sampling new mating partners from post-selection, post-migration pools in each population.

Genetic architecture and parameters. The model is composed of four diploid loci: The first two are fitness loci **A** and **B** that generate the intrinsic fitness cost of hybridization. Upon

secondary contact, population 1 is fixed for alleles $A1$ and $B1$ and population 2 is alternatively fixed for alleles $A2$ and $B2$. I assumed that $A1$ has never seen $B2$, and $A2$ has never seen $B1$ (i.e. the ancestral state is $A0B0$ such that both local populations have evolved unique alleles at both loci). As a result, this creates Dobzhansky-Muller incompatibility in hybrids that have either: $A1-B2$ or $A2-B1$ alleles between their chromosomes (e.g. $A1A2B1B2$ F1 hybrid). This results in intrinsic postzygotic isolation between these populations. To model the *asymmetry* in hybridization cost, I assumed that the Dobzhansky-Muller incompatibilities can be asymmetrical between the two reciprocal matings. Specifically, the incompatibility between the $A-B$ alleles from the two populations is assumed to be strongly affected by the maternal cytoplasm (see Turelli and Moyle 2007 for additional mechanisms). Thus, the $A1-B2$ or $A2-B1$ incompatibility in hybrids is stronger when the $A1$ or $B1$ alleles come from the *mother* and are weaker when $A1$ or $B1$ alleles come from the *father*. This generates asymmetrical selection (s) against offspring with the above allelic combinations between reciprocal matings: $s1$ = selection against offspring from $\text{♀}A1B1 \times \text{♂}A2B2$ matings is greater than $s2$ = selection against offspring from $\text{♀}A2B2 \times \text{♂}A1B1$ matings. Offspring coming from backcrosses between hybrids and parental genotypes or between F1 hybrids experience the same rule of selection as those coming from parental crosses (see above).

The third locus C represents a “magic trait” which is both a male sexual cue and ecological trait experiencing divergent ecological selection across the two populations (see Servedio et al. 2011). My results do not require this trait to be involved in both functions and hold equally well when this locus is exclusively a neutral male sexual cue trait (data not shown). I assumed that the two populations are initially alternatively fixed for the two alleles at locus C , with population 1 fixed for $C1$ and population 2 fixed for $C2$. Homozygote genotypes at C locus can experience divergent ecological selection ($s3$) by migrating to the wrong environment; $C2C2$ migrants in population 1 and $C1C1$ migrants in population 2. Also, $C1C2$ hybrids can “fall-in-between ecological niches” of the two populations and thus can experience their ecological selection $s4$.

The fourth locus D is a female mating preference locus, determining which male the female will mate with based on the male sexual cue trait at locus C . It can have five different alleles, depending on assumptions. First, $D1D1$ female homozygotes prefer to mate with $C1C1$ homozygote males, regardless of what the female is at the C locus. Similarly, $D2D2$ female

homozygotes prefer to mate with *C2C2* homozygote males. This is an example of a “two-allele” mating preference model sensu Felsenstein (1981). If the above females do not find their preferred male after a given number of tries, they then mate randomly with the next male they encounter. Females carrying a *D3* allele mate randomly with the first male they encounter. Thus I assumed that mating preference alleles are recessive to *D3* (for empirical evidence, see Coyne and Orr 2004, pg. 227). I also assumed that the two preference alleles are recessive in hybrids such that the *D1D2* hybrid females show *no* preference (i.e. mate randomly; for empirical evidence, see Coyne and Orr 2004, pg. 227). The preference penetrance of the *D1D1* and *D2D2* females is controlled by an independent parameter (range: 0-100% genetic penetrance). I assumed that mating preference alleles *D1* and *D2* are initially introduced into each population by mutation and have drifted or have been selected in allopatry to some low frequency (e.g. $p = 0.05-0.10$).

I also explored two additional scenarios: First, the two populations can have different random mating alleles to begin with instead of one (*D3D3* in population 1 and *D4D4* in population 2). Thus, in this scenario, the two preference alleles *D1* and *D2* evolve under different genetic backgrounds. Second, instead of the two mating preference alleles, I assumed there was a single “assortative mating allele” *D5* evolving against the random mating allele *D3* in both populations (sensu Felsenstein 1981).

For all simulations, the linkage map of the loci is as follows: A-B-C-D, with neighboring loci allowed to recombine according to the rules of meiosis (maximum recombination range: 0-0.5 and 0-0.25 for *Drosophila*). The population sizes and carrying capacity were finite and were typically assumed to be 3,000 in each local population (but see tests of the *rarer-female effect* below). Genetic drift was clearly evident, but selection gave consistent results. Migration rate was allowed to range from 0-0.5 and was symmetrical between populations.

Results

MORE INTENSIVE SAMPLING DISFAVORS THE TEMPLETON EFFECT

First, I asked whether more intensive sampling would uncover the previously-missing young, but strongly premating isolated taxa in allopatry and thus support the Templeton effect. Fig. 1 shows premating isolation between all 129 allopatric species pairs, and for comparison, the 125

sympatric pairs as a function of their genetic distance using Nei's D (phylogenetically corrected data shows qualitatively same results: Fig. S1).

Remarkably, even after this extensive search, no single case of strong premating isolation is found during incipient speciation in allopatry (e.g. index > 0.6 for $D < 0.25$; Fig. 1A). This finding is unlikely to be a result of inadequate sampling, since the present study did uncover previously-unobserved cases of weak premating isolation in sympatry (Fig. 1B). These results are completely consistent with Coyne and Orr (1989, 1997) and show that unlike in sympatry, there appears to be a fundamental and definable limit to how fast premating isolation can evolve in allopatry, presently best characterized by an exponential equation: $0.12 + 2.32x + 3.82$, $R^2 = 0.987$, $P < 0.0001$ (see Fig. 1A insert).

Given that the Templeton effect hypothesis relies on the existence of young and strongly premating isolated taxa in allopatry, these results strongly disfavor it as a viable explanation for the observed pattern of enhanced isolation in sympatry. While these findings are more consistent with reinforcement, other alternatives, such as parapatric or sympatric speciation, are possible for at least 25% of these young sympatric taxa that lack intrinsic postzygotic isolation under laboratory conditions (Coyne and Orr 1989, 1997). Below I test two novel predictions of reinforcement for those cases where postzygotic isolation is evident; in other words, where the theory predicts reinforcement should most likely occur (e.g. Liou and Price 1994; Servedio and Noor 2003; Yukilevich and True 2006).

TESTING THE “*CONCORDANT ISOLATION ASYMMETRIES*” PREDICTION OF REINFORCEMENT

Below I asked if premating and postzygotic isolation asymmetries are correlated within species pairs. Out of the 630 species pairs, 89 allopatric and 74 sympatric pairs contained complete information for both isolation indexes for both reciprocal matings (Table S1). A total of 28 allopatric and 29 sympatric pairs contained asymmetries in postzygotic isolation between reciprocal matings (Table S1). Premating isolation asymmetries were assumed to be meaningful if the reciprocal mating indexes differed by 0.05 or greater. This resulted in 20 allopatric and 16 sympatric species pairs that contained asymmetries in both postzygotic and premating isolation indexes between matings. More stringent cutoffs for premating isolation asymmetry (i.e. indexes differences > 0.1 or 0.2) gave the same results (see below).

I determined the direction of asymmetries for pre-mating and postzygotic isolation for each species pair and asked if the direction was *concordant* or *discordant* (Table 1 and Table S2 for details). For 20 allopatric pairs, the percentage of concordant and discordant isolation asymmetries was equal at 50% (Binomial: one-tailed sign test: $P = 0.59$; Table 1). Because each asymmetry is a within-species pair phenomenon, correcting these results for phylogenetic non-independence is unnecessary (Coyne and Orr 1989; Coyne and Orr 1997), but was also performed to be conservative (see Table S3). This resulted in 13 corrected points that showed similar percentages of concordant versus discordant isolation asymmetries (42% or 40% concordant versus 58% or 60% discordant, depending on the combination of non-independent data; Table S3). Neither case was significantly different from random (Binomial: one-tailed sign test: $P = 0.61$ or 0.62). There was also no relationship between pre-mating isolation and postzygotic isolation across these matings ($n = 40$, Spearman's Rho = 0.168, $P = 0.30$; Fig. 2A). These results suggest that pre-mating isolation evolves independently of postzygotic isolation in allopatry.

Sympatric species showed very different results. First, 13 out of the total 29 sympatric pairs with postzygotic isolation asymmetries lacked pre-mating isolation asymmetries at the above cutoff level. Interestingly, with the exception of two species pairs (one of which had only 2.5% sympatric overlap, these pairs showed nearly complete pre-mating isolation (avg. index = 0.978, st.dev. = 0.029; see Table S4). Thus the main reason for their lack of pre-mating isolation asymmetries appears to be because these species already nearly attained complete speciation. The remaining 16 sympatric pairs with pre-mating isolation asymmetries had significantly lower pre-mating isolation, representing speciation in action (avg. index = 0.768, st.dev. = 0.213; Wilcoxon-Kruskal-Wallis test: Z -value = 3.83, $P < 0.0001$). Strikingly, all but one of these species pairs (94%) showed concordant isolation asymmetries (Binomial: one-tailed sign test: $P < 0.0003$; Table 1). The only exception is a weak discordant isolation asymmetry between *ananassae* and *pallidosa-like* (*Wau*). To correct the above data for phylogenetic non-independence, two alternative competing phylogenies available for the *ananassae* complex had to be considered. Using the first phylogeny led to the conclusion that all points above are phylogenetically independent. The second phylogeny led to the conclusion that there are 14 independent points with 100% concordant isolation asymmetries (see Table S3).

Further, premating isolation and postzygotic isolation in sympatry are also highly correlated across these matings ($n = 32$, Spearman's $Rho = 0.55$, $P = 0.0011$; Fig. 2B). On average, premating isolation is dramatically different between reciprocal matings with zero hybridization cost versus maximum cost (avg. $index_{zero\ cost} = 0.576$, avg. $index_{max.\ cost} = 0.963$; $n = 17$, Chi-square value = 6.30, $P < 0.012$; Fig. 2B). Because these differences occur within the same species pairs, this relationship is independent of genetic distance between species. Similarly, at the broad taxonomic level, there is a significant positive relationship between premating isolation and average cost of hybridization across species pairs (Spearman's $Rho = 0.264$, $P < 0.034$ for 67 corrected data points, Fig. S2A, and Spearman's $Rho = 0.283$, $P < 0.014$ for 75 uncorrected points, Fig. S2B; see *Methods* for definition of cost). However, at this level, the significant relationship is not independent of genetic distance between species (partial correlation between prem. isol. and hybrid. cost = 0.219, $P = 0.138$) and thus is only consistent with, but not indicative of reinforcement.

Since none of the above pairs have attained complete intrinsic postzygotic isolation, this pattern cannot be driven by “reproductive character displacement” after speciation (Butlin 1987; Powell 1997). Instead, the overwhelming enrichment of concordant isolation asymmetries in sympatry is directly predicted by reinforcement. After identifying this pattern in *Drosophila*, I briefly searched the literature for sympatric cases with asymmetrical postzygotic isolation in other taxa. Three cases were identified (in *Nasomia* wasps (Bordenstein et al. 2000); in *Spea* frogs (Pfennig and Simovich 2002), in *Litoria* frogs (Hoskin et al. 2005)). All showed similar concordant isolation asymmetries, implying that this pattern may extend to other taxa.

TESTING THE “RARER-FEMALE EFFECT” PREDICTION OF REINFORCEMENT

The above test of the relationship between premating and postzygotic isolation is independent of whether females or males drive the observed mating asymmetries. This is in contrast to the hypotheses of Kaneshiro (1980) and Watanabe and Kawanishi (1979) that assume that mating isolation asymmetries exclusively arise from female mating preference differences. While this assumption appears too restrictive, some have argued that the costs of hybridization are still likely to be greater for females than for males (e.g. Partridge and Parker 1999; Coyne and Orr 2004: pg. 380). If we assume the latter, then reinforcement makes a second key prediction referred to as *rarer-female effect*. This prediction states that in sympatry, females of the smaller

population should evolve stronger premating isolation compared to females of the bigger population (Noor 1995; Bordenstein et al. 2000; Hoskin et al. 2005).

To test this second prediction of reinforcement, I determined the relative range sizes of sympatric species pairs (Table 2, also see Table S5 for details). Asymmetry in range size was assumed if species ranges differed by 5% or greater. Out of the 11 sympatric pairs with symmetrical postzygotic isolation, but asymmetrical range sizes, 9 pairs (82%) showed greater premating isolation in the reciprocal mating with females of the smaller range size species (Binomial: one-tailed sign test: $P < 0.033$; Table 2). The fact that we also see this pattern in other organisms (Bordenstein et al. 2000; Hoskin et al. 2005; Cooley 2007), suggests that this pattern may be fairly common in nature. The implication of this finding is that the selection pressure to evolve premating isolation in sympatry may indeed be stronger for females than for males and may depend on how often females encounter potential foreign mates.

COMPUTER SIMULATIONS OF ASYMMETRICAL REINFORCEMENT AND OF TEMPLETON EFFECT

Below I modeled the evolution of asymmetrical reinforcement using individual-based computer simulations. The goal was to determine if and when can reinforcement generate the observed patterns in sympatric *Drosophila*. Briefly, the model assumed two discrete “island” populations that come back into secondary contact after some allopatric divergence (see *Methods* for details). It contains four diploid loci, which are as follows: two fitness loci *A* and *B* cause Dobzhansky-Muller epistatic incompatibility between differentially fixed alleles of the two populations. The offspring of the two reciprocal matings can experience asymmetrical D-M fitness consequences. The third locus *C* represents a “magic trait”, which is differentially fixed for two alternative alleles between populations that serve as a male sexual mating cue and a trait involved in local ecological adaptation (see Servedio et al. 2011). The fourth locus *D* is a female mating preference trait. The females are initially carrying random mating allele(s) in each population. *D1D1* females prefer to mate with *C1C1* males while *D2D2* females prefer to mate with *C2C2* males (i.e. a “two-allele” preference model). I also explored a “one-allele” assortative mating model. The increase in frequency of these alleles in each population is monitored to study speciation.

The Evolution of Concordant Isolation Asymmetries. First, I assumed a “two-allele” mating preference model. Simulations showed that whenever there was substantial intrinsic postzygotic isolation, female mating preference alleles, $D1$ in population 1 and $D2$ in population 2, increased in frequency over the random mating allele $D3$, resulting in reinforcement (i.e. completing premating isolation between the populations; Fig. 3). Moreover, whenever intrinsic postzygotic isolation was asymmetrical between the two reciprocal matings, the preference allele that avoided the stronger cost of hybridization nearly always evolved faster relative to the preference allele that avoided the weaker cost of hybridization, resulting in the evolution of *concordant isolation asymmetries* (Fig. 3A). This asymmetry evolved fairly rapidly upon secondary contact, was most pronounced at intermediate levels of premating isolation, and lasted until both preference alleles reached fixation (Fig. 3). Remarkably, even when premating isolation asymmetries were made to be initially discordant upon secondary contact, selection rapidly reversed the direction of premating isolation in agreement with the direction of selection pressures, resulting in concordant isolation asymmetries (Fig. 3B). This shows that concordant isolation asymmetries can be favored in secondary contact largely independent of the initial premating isolation asymmetry. Such a reversal in premating isolation asymmetry from allopatry to sympatry appears to have occurred between *Drosophila recens* and *D. subquinaria* (Jaenike et al. 2006).

To further understand which conditions favor the evolution of concordant isolation asymmetries, I performed a sensitivity analysis. Table 3 summarized these results by showing how robust the evolution of concordant isolation asymmetries is to changes in various assumptions of the basic model. As long as there is substantial asymmetry in the cost of hybridization between matings, virtually all other parameters of the model can be relaxed and still favor concordant isolation asymmetries (Table 3). For instance, concordant isolation asymmetries evolve even when genetic penetrance of preference alleles is fairly low (e.g. 0.5) and recombination rate between loci is fairly high (e.g. 0.25, which is maximum known for *Drosophila*). Moreover, I also found that these asymmetries are equally likely to evolve when sexual isolation is based on a “one-allele” assortative mating model rather than the “two-allele” mating preference model (second to last row in Table 3). In other words, the genetic architecture of the sexual mating behavior appears to have little influence on these results. Indeed, the sensitivity analysis shows that under the above assumptions, as long as $s1 > s2$ (*asymmetrical*

intrinsic cost of hybridization) and $s1 > s4$ (*maximum intrinsic selection $s1$ must be greater than ecological selection against hybrids $s4$*), concordant isolation asymmetries readily evolve by reinforcement (Table 3). The fact that we see this asymmetrical pattern in sympatric *Drosophila* suggests that these conditions are often met in this genus.

The Evolution of the Rarer-Female Effect. I then asked if reinforcement can also generate the observed pattern predicted by the *rarer-female effect*, which showed that the cross with females of the rarer species had stronger premating isolation than the reciprocal cross (see Table 2). To test this scenario theoretically, I assumed that population 1 is 50% smaller than population 2, while making postzygotic isolation between the reciprocal matings symmetrical (Fig. 4). Finally, I increased divergent ecological selection ($s3$) against homozygote migrants (*C1C1* in pop.2 and *C2C2* in pop.1) from 0.6 to 0.8. This was necessary because with asymmetrical population sizes, the smaller population is more vulnerable to extinction (see Paterson 1978). Increasing $s3$ reduced those chances of extinction dramatically.

First I found that migration rate from population 2 to population 1 was much higher as a result of population size asymmetry (Fig. 4). This generated higher selection pressure on females of the smaller population 1 to evolve the mating preference allele *D1* relative to females from the larger population to evolve allele *D2* (Fig. 4). This led to the expected evolution of concordant isolation asymmetries, predicted by the rarer-female effect.

Testing if the Templeton Effect can explain concordant isolation asymmetries in sympatry. Instead of reinforcement selection, it is possible that a bias in species survival from allopatry could generate the pattern we see in sympatry (referred to as the Templeton effect). I asked whether such a scenario is likely using computer simulations. This hypothesis suggests that species pairs with initially concordant isolation asymmetries should survive better than species pairs with initially discordant isolation asymmetries upon secondary contact. To do this, I assumed that divergent ecological selection against homozygote migrants is either zero or weak (i.e. $s3 < 0.30$). This was necessary so as to increase the chances of studying genetic extinctions, since higher levels of ecological selection strongly reduce the chances of such extinctions (see Table 3 above).

Interestingly, my results showed the very opposite pattern to expectations (Table 4). I found that starting out with initially concordant isolation asymmetries substantially increased the chances of one of the populations going extinct compared to starting with initially discordant

isolation asymmetries under virtually all conditions where extinctions were likely (Table 4). The average difference in the probability of extinction between these scenarios was 40%. However, the speed to genetic extinction was not very different (Table 4).

The reason why initially concordant asymmetries typically increased the probability of genetic extinction is because under this scenario, the more favored preference allele is also initially more frequent as well. Thus it is able to rapidly spread and dominate in both populations when divergent ecological selection is zero or weak (data not shown). Because the random mating allele *D3* is still present, individuals from the rarer second population keep mating with those of the first, and thus produce *more* unfit hybrids which ultimately cause it to go extinct (data not shown, also see Paterson 1978). This phenomenon is much less likely to occur when populations initially have discordant isolation asymmetries because the more favored preference allele is now initially *less* common relative to the other. Thus, the more favored allele has a much harder time dominating in both populations. Instead, both alleles are more likely to increase in frequency and eliminate the random mating allele altogether to complete the speciation process (data not shown). In conclusion, this evidence strongly suggests that we can eliminate the Templeton effect as an alternative explanation for the pattern of concordant isolation asymmetries in sympatry.

ESTIMATING THE IMPACT OF REINFORCEMENT ON SYMPATRIC *DROSOPHILA*

Finally, using the phylogenetic approach of Noor (1997) and the new asymmetrical patterns in sympatry, I estimated the overall impact of reinforcement on sympatric *Drosophila*. First, I identified a total of 23 phylogenetically informative cases where the difference in premating isolation between allopatry and sympatry can be assessed (Table S6). Some cases were between lesser and greater sympatry. Remarkably, I found that 19 (83%) of these species pairs showed enhanced premating isolation in sympatry (Binomial: one-tailed sign test: $P < 0.0013$). Interestingly, this estimated range is consistent with Howard's (1993) non-phylogenetic estimate of enhanced premating isolation across many different taxa, which he calculated to be 69%. Noor's (1997) phylogenetic estimate was more conservative at 21%, but because his study was only based on 6 informative species pairs, he concluded that the true estimate is likely to be much higher.

Next, I gathered all cases where the directionality of isolation asymmetry can be assessed in sympatry. This resulted in only 10 informative cases in sympatry (Table S6). Even among this small number of cases, eight (80%) showed concordant isolation asymmetries in sympatry (Binomial: one-tailed sign test: $P = 0.05$). Since both patterns may be considered as evidence for reinforcement, I combined both criteria in order to determine the most likely impact of reinforcement on sympatric *Drosophila* species. Six pairs (or 60% of all informative cases) showed both concordant isolation asymmetries and enhanced isolation in sympatry (Table 5). Even more striking, four of these pairs showed a shift towards greater isolation and greater concordant isolation asymmetry in the sympatric pair relative to its allopatric sister pair (Table 5). While still based on a small sample size, this analysis does provide a preliminary estimate suggesting that reinforcement appears to enhance premating isolation in 60% to 83% of all sympatric *Drosophila* species pairs.

Moreover, comparing the average level of premating isolation of these cases showed that the actual impact on premating isolation was substantial. I found that the net average gain in premating isolation in sympatry for all 19 cases of enhanced isolation was 0.26 (st. dev. = 0.162; 26% on a scale of 0 to 1), and was 0.182 (st. dev. = 0.12; 18.2%) for those 6 cases with both criteria satisfied (Table 5). These results suggest that selection in sympatry appears to dramatically increase the level of premating isolation in nature.

Discussion

Reinforcement speciation theory has historically been one of the most contentious subjects in evolutionary biology (e.g. Servedio and Noor 2003; Coyne and Orr 2004). Arguably, a major turning point for the theory was the discovery of a pattern of enhanced premating isolation in sympatry (Dobzhansky et al. 1968; Coyne and Orr 1989, 1997). While this pattern has been instrumental in stimulating more research on reinforcement, it is well known that reinforcement is not the only mechanism that can generate enhanced isolation in sympatry (e.g. Coyne and Orr 2004).

ALTERNATIVE EXPLANATIONS FOR ENHANCED ISOLATION IN SYMPATRY

Enhanced isolation in sympatry has alternatively been explained by what is known as the “Templeton effect”, which states that enhanced isolation arises because strongly prezygotic

isolated allopatric pairs survive better in secondary contact than weakly isolated allopatric pairs (Patterson 1978; Templeton 1981). While this argument is theoretically sound, it assumes that such strongly prezygotic isolated taxa already exist at very early stages of allopatric divergence (Noor 1997). The first goal in this new meta-analysis was to determine if this was indeed the case. After gathering information on 129 allopatric species pairs, my results suggest a negative answer. No young allopatric species pair contained premating isolation above the index of 0.6. Indeed, the data suggests a definable limit to how fast premating isolation can evolve in allopatry that no documented species pair has been able to cross. While this new evidence has made the Templeton effect unlikely, other modes of speciation, such as sympatric and parapatric speciation that can also accelerate evolution of premating isolation in sympatry cannot yet be ruled out. Thus I asked if reinforcement makes any other predictions that can be tested using the *Drosophila* data set.

TWO NOVEL PREDICTIONS OF REINFORCEMENT SPECIATION

It has been argued throughout the paper that reinforcement does indeed make two related predictions that so far have never been theoretically explored or empirically tested. Broadly, reinforcement predicts that selection pressure to evolve premating isolation should be a function of the cost of hybridization. Thus, whenever the costs of hybridization can be shown to be asymmetrical between reciprocal matings, we can ask if premating isolation reflects this asymmetry in nature (i.e. *concordant isolation asymmetries*). Asymmetrical costs of hybridization can result either from asymmetries in intrinsic postzygotic isolation (see Turelli and Moyle 2007) or in the relative sizes of hybridizing populations, where we would expect stronger selection on females of the rarer population (i.e. *rarer-female effect*).

Out of the total 630 species pairs studied, only those species pairs that contained complete information on both premating and postzygotic isolation indexes were considered. I then focused on sympatric species pairs that showed asymmetries in either intrinsic postzygotic isolation or in geographic range sizes, implying asymmetrical costs of hybridization between reciprocal matings. For these pairs, I asked if premating isolation was concordant to this asymmetry in sympatry. Remarkably, I found that 94% of all asymmetrical cases of intrinsic postzygotic isolation and 82% of all asymmetrical cases of geographical range sizes showed concordant premating isolation asymmetries in sympatry. On the other hand, in allopatry,

species pairs showed an equal number of concordant and discordant isolation asymmetries, implying that these isolating mechanisms evolve independently in allopatry. The overwhelming enrichment of concordant isolation asymmetries in sympatry appears to extend to other taxa where postzygotic isolation is asymmetrical (e.g. Bordenstein et al. 2000; Pfennig and Simovich 2002; Hoskin et al. 2005). Importantly, these patterns could not have been generated by publication bias since the original data was collected without any intent of relating these asymmetries to each other.

I then created a model to study the theoretical plausibility and generality of this process. Similar to previous models of reinforcement (e.g. Liou and Price 1994; Servedio and Kirkpatrick 1997; Servedio 2000), individual-based simulations were used with two “island” populations that come into secondary contact after accumulating Dobzhansky-Muller genetic incompatibilities in allopatry. Reciprocal matings were allowed to have asymmetrical costs of hybridization by assuming, for instance, that the D-M incompatibilities are influenced by maternal effects (see Turelli and Moyle 2007). Theoretical results demonstrated that reinforcement can in principle produce asymmetrical patterns of speciation as a function of different selection pressures on the two reciprocal matings. Moreover, the evolution of concordant isolation asymmetries was highly robust to changes in biological assumptions, with a few key exceptions. This analysis provided the theoretical argument that the patterns observed in *Drosophila* are broadly expected in nature.

ARE CONCORDANT ISOLATION ASYMMETRIES IN SYMPATRY UNIQUE TO REINFORCEMENT?

First, I determine if a modified version of the Templeton effect can explain the observed pattern of concordant isolation asymmetries in sympatry. Simulations were used to ask if initially starting out in allopatry with concordant isolation asymmetries would increase the species pair survival upon secondary contact compared to starting out with discordant isolation asymmetries. Results showed the very opposite pattern, where species pairs that initially had discordant isolation asymmetries survived on average 40% better in secondary contact relative to initially concordant isolation asymmetries. If this type of Templeton effect was frequently operating in nature, it would have produced a bias in discordant isolation asymmetries in sympatry. This

strongly argues against the idea that concordant isolation asymmetries are a result of the Templeton effect.

Other alternatives also seem highly unlikely. One hypothesis is that concordant isolation asymmetries in sympatry arise because selection favors the greater reduction of postzygotic isolation in that cross that had initially lower pre-mating isolation. While this hypothesis is plausible, it is unlikely. First, it is well appreciated that intrinsic postzygotic isolation is very difficult to reverse in nature (see Coyne and Orr 2004). Second, this hypothesis explicitly predicts that sympatric species will evolve lower postzygotic isolation compared to allopatric species. This prediction is falsified by the available data (Wilcoxon-Kruskal-Wallis test on corrected data: avg. $\text{index}_{\text{Sympatry}} = 0.45$ (st. dev. = 0.38), $n = 37$; avg. $\text{index}_{\text{Allopatry}} = 0.33$ (st. dev. = 0.29), $n = 40$; Z -value = 1.58, $P = 0.11$; same result in uncorrected data; not shown).

Another hypothesis can be called “directional pleiotropy” (e.g. Templeton 1981). This hypothesis suggests that genes controlling postzygotic isolation also control pre-mating isolation. Thus the level of one index should reflect the level of the other as they should evolve in concert. However, this also appears invalid, since it cannot explain why only sympatry is enriched for concordant isolation asymmetries as we would expect the same pleiotropic effects in both sympatric and allopatric species pairs. Further, pre-mating and postzygotic isolation are unlikely to have a wide-ranging pleiotropic genetic basis (see Coyne and Orr 2004).

Other hypotheses, such as ecological character displacement, runaway sexual selection or sympatric and parapatric speciation, do not make any predictions about the reciprocal nature of the relationship between prezygotic and postzygotic isolation. Therefore, I conclude that the observed patterns of concordant isolation asymmetries in sympatry are very difficult to explain by any other process and thus appear to represent unique signatures of reinforcement.

IMPLICATIONS OF NOVEL PATTERNS OF REINFORCEMENT AND FUTURE DIRECTIONS

First, the identification of a consistent pattern of concordant isolation asymmetries in sympatry on its own suggests that reinforcement seems to occur widely in at least this group of organisms. This evidence supports the original notion of Dobzhansky (1940) that reinforcement is playing a significant role in the later stages of speciation in sympatric *Drosophila*.

Second, we can use these novel patterns together with the pattern of enhanced isolation to estimate the frequency of reinforcement and its effect on the level of sexual isolation in sympatry. The above phylogenetic analysis showed that reinforcement appears to enhance premating isolation in 60% to 83% of all sympatric *Drosophila* species pairs and increases it by a dramatic 18% to 26% compared to phylogenetically allopatric sister taxa. These estimates suggest that reinforcement is more general and is more influential than previously thought (see Howard 1993; Noor 1997 for previous estimates).

Third, we can begin to look at other organisms for these patterns. Some have already noted these asymmetries (e.g. Bordenstein et al. 2000; Pfennig and Simovich 2002; Hoskin et al. 2005). More systematic approaches are necessary to determine the generality of these patterns in other taxa. We can also compare allopatric versus sympatric local populations of the same species pair for concordant isolation asymmetries (previously referred to as Asymmetrical Reproductive Character Displacement or ARCD; see Cooley 2007). The above theoretical results demonstrated that selection can reverse the direction of isolation asymmetries from being discordant in allopatric populations to being concordant in sympatric populations. This appears to have occurred in at least one well documented system in *Drosophila* (Jaenike et al. 2006), and several other examples are consistent with this occurrence (see Table 5 above).

One interesting question is whether the difference in the directionality of asymmetrical patterns between allopatric and sympatric species pairs will hold once we systematically study allopatric versus sympatric populations across many taxa. This may not necessarily follow since asymmetrical gene flow between allopatric and sympatric local populations of the two hybridizing species may lead to unexpected asymmetries in RCD (e.g. Noor 1997; Cooley 2007). For instance, if the first species experienced greater cost of hybridization in sympatry also happened to experience greater levels of gene flow between sympatric and allopatric populations, it is possible that the second species may actually have greater RCD from allopatry to sympatry compared to the first species, even though the first species would have evolved greater overall premating isolation. Thus, this case would exhibit concordant isolation asymmetries at the species level, but not at the population level.

In conclusion, the discovery of these novel signatures of reinforcement should further advance our continuing pursuit of understanding when and how natural selection directly favors speciation in nature.

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Table 1. Concordant and discordant isolation asymmetries in pre mating and postzygotic isolation between reciprocal matings of allopatric and sympatric species pairs.

Species 1	Species 2	Concordant = 1, Discordant = 0		Direction of Pre-mating Isol. asymmetries	Direction of Postzygotic Isol. asymmetries	% Sympatry	Pre-mating Isolation	Postzygotic Isolation	Pre-mating Isolation (sp1♀ x sp2♂)	Pre-mating Isolation (sp1♂ x sp2♀)	Postzygotic Isolation (sp1♀ x sp2♂)	Postzygotic Isolation (sp1♂ x sp2♀)
		1	0									
Allopatric species												
<i>sericeo</i>	<i>richardsoni</i>	1	0	-0.05	-0.50	0	0.78	0.75	0.75	0.80	0.50	1.00
<i>vittis</i>	<i>montana</i> (<i>montana</i> sub-species)	1	0	-0.06	-1.00	0	0.66	0.50	0.63	0.69	0	1.00
<i>popuensis-like</i>	<i>parpalillidosa</i>	0	0	-0.07	0.25	0	0.96	0.13	0.93	1.0	0.25	0
<i>williston quechua</i>	<i>williston willistoni</i>	1	0	0.08	0.50	0	0.33	0.25	0.37	0.29	0.50	0
<i>huacensis</i>	<i>rtluca</i>	0	0	0.12	-0.10	0	0.84	0.55	0.90	0.78	0.50	0.60
<i>pullidosa</i>	<i>parapallidosa</i>	0	0	-0.16	0.50	0	0.16	0.25	0.08	0.24	0.50	0
<i>sulflrigaster albostrigata</i>	<i>sulflrigaster blimbua</i>	1	0	0.19	0.50	0	0.43	0.25	0.53	0.34	0.50	0
<i>gambhavi</i>	<i>palipes</i>	1	0	-0.19	-0.17	0	0.48	0.25	0.38	0.57	0.17	0.33
<i>birehiti Papua New Guinea</i>	<i>birehiti australia</i>	0	0	-0.22	0.50	0	0.13	0.25	0.02	0.24	0.50	0
<i>triaroria</i>	<i>quad-aria</i>	0	0	0.25	-0.50	0	0.09	0.25	0.22	-0.03	0	0.50
<i>arizonae</i>	<i>mojavensis baja (baja California: race B2)</i>	1	0	0.26	0.31	0	0.73	0.35	0.86	0.60	0.50	0.19
<i>arizonae</i>	<i>mojavensis wrigleyi (California Isl.: race C)</i>	1	0	0.31	0.25	0	0.77	0.38	0.92	0.61	0.50	0.25
<i>peruana</i>	<i>hiti-like (ogunai + obnisiiti)</i>	0	0	-0.31	1.00	0	0.84	0.50	0.69	1.0	1.00	0
<i>recess</i>	<i>subquitarica race A (allopatric)</i>	0	0	0.33	-0.45	0	0.68	0.73	0.85	0.51	0.50	0.95
<i>eradockae</i>	<i>palipes</i>	0	0	0.38	-0.17	0	0.47	0.25	0.66	0.28	0.17	0.33
<i>buzzatii</i>	<i>richardsoni</i>	1	0	0.42	0.50	0	0.64	0.75	0.85	0.43	1.00	0.50
<i>sahans</i>	<i>proslans</i>	0	0	-0.51	0.50	0	0.27	0.25	0.01	0.52	0.50	0
<i>pallidosa-like Wai:</i>	<i>parapallidosa</i>	1	0	0.61	0.25	0	0.68	0.38	0.99	0.38	0.50	0.25
<i>arizonae</i>	<i>mojavensis mojavensis (California: race A)</i>	1	0	0.62	0.40	0	0.48	0.30	0.79	0.17	0.50	0.10
<i>sulflrigaster blimbua</i>	<i>sulflrigaster neomusua</i>	0	0	0.74	-0.25	0	0.60	0.38	0.97	0.23	0.25	0.50
Sympatric species												
<i>malerkoiliana</i>	<i>pseudonanassae</i>	1	0	0.05	0.10	0.98	0.95	0.95	0.98	0.93	1.00	0.90
<i>pallidosa-like Wai:</i>	<i>pallidosa-like</i>	1	0	0.07	0.25	0.52	0.96	0.13	1.0	0.93	0.25	0
<i>anamassae</i>	<i>pallidosa-like Wai</i>	0	0	-0.10	0.50	0.50	0.95	0.25	0.90	1.0	0.50	0
<i>equinoxialis</i>	<i>palistorum</i>	1	0	-0.12	-0.50	0.75	0.94	0.75	0.88	1.0	0.50	1.00
<i>recess</i>	<i>subquitarica race S (sympatric)</i>	1	0	-0.14	-0.45	0.56	0.93	0.73	0.86	1.0	0.50	0.95
<i>malerkoiliana</i>	<i>parapalillidosa</i>	1	0	-0.22	-0.25	0.93	0.85	0.63	0.74	0.96	0.50	0.75
<i>arizonae</i>	<i>talera</i>	1	0	0.24	0.50	0.44	0.56	0.25	0.68	0.44	0.50	0
<i>sternerii</i>	<i>venezolana</i>	1	0	0.25	0.50	1.0	0.79	0.75	0.92	0.67	1.00	0.50
<i>vittis</i>	<i>montana</i> (<i>ovivororum</i> sub-species)	1	0	-0.27	-1.00	0.33	0.79	0.50	0.65	0.92	0	1.00
<i>macropsina macropsina</i>	<i>macropsina himpensis</i>	1	0	-0.30	-0.38	0.05	0.09	0.19	-0.06	0.24	0	0.38
<i>arizonae</i>	<i>tritararia</i>	1	0	0.33	1.00	0.71	0.80	0.50	0.96	0.63	1.00	0
<i>surtavanti</i>	<i>milleri</i>	1	0	-0.33	-0.50	0.50	0.68	0.25	0.52	0.85	0	0.50
<i>hitoralis</i>	<i>montana</i>	1	0	-0.37	-0.50	0.24	0.78	0.75	0.60	0.97	0.50	1.00
<i>buzzatii</i>	<i>keopjevae</i>	1	0	0.42	0.50	0.20	0.77	0.75	0.98	0.56	1.00	0.50
<i>palistorum</i>	<i>willistoni</i>	1	0	0.46	0.25	0.90	0.72	0.88	0.94	0.49	1.00	0.75
<i>anamassae</i>	<i>parapallidosa</i>	1	0	0.47	0.50	0.52	0.72	0.25	0.96	0.49	0.50	0

Note: Allopatric and sympatric species pairs sorted separately, each set by increasing premating isolation asymmetry. The sign of each asymmetry (positive or negative) was arbitrarily determined by taking the left reciprocal mating (sp.1♀ x sp.2♂) minus the right reciprocal mating (sp.1♂ x sp.2♀); it has no biological significance and was only used to determine whether asymmetries are concordant or discordant. Details of premating and postzygotic isolation can be found in Table S2. Phylogenetically corrected data can be found in Table S3.

Species 1	Species 2	Concordant = 1, Discordant = 0		Direction of premating isol. asymmetries	Direction of relative range sizes	% Sympatry	Premating Isolation (sp1 x sp2)	Premating Isolation (sp1 x sp2)	Absolute Range (sp1)	Absolute Range (sp2)	Absolute Range Overlap
		Direction of premating isol. asymmetries	Direction of relative range sizes								
parishorum/Andean-Borealian	parishorum/Transsional	1	0	-0.51	-0.12	0.06	0.302	0.814	12,595,370	3,583,74	45,000
parishorum/Andean	parishorum/Andean-Borealian	0	0	-0.09	0.38	0.56	0.818	0.908	3,975,847	12,595,370	5,376,754
parishorum/Andean	parishorum/Centroamerican	1	1	-0.10	-0.06	0.04	0.905	1.000	3,975,847	4,541,975	20,682
yucahu	sancticola	1	1	-0.15	-0.58	0.29	0.768	0.916	10,884,567	860	500
peruvianus	perubolobocera	1	1	0.24	0.87	0.56	0.905	0.665	624,127	4,869,262	624,127
parishorum/Andean-Borealian	parishorum/Criocan	1	1	-0.27	-0.42	0.26	0.727	1.000	12,595,370	1,310,386	630,547
parishorum/Andean-Borealian	parishorum/Centroamerican	1	1	-0.32	-0.06	0.03	0.526	0.842	12,595,370	4,541,975	20,682
borealiana	paraborealiana	1	1	-0.73	-0.24	0.88	0.186	0.914	8,630,082	6,517,563	6,517,563
mekongensis (Africa only)	sinuatschikensis (Africa only)	1	1	-0.34	-0.28	0.70	0.707	0.947	14,133,545	9,394,818	7,932,640
borealiana	maderelliana	1	1	0.22	0.18	0.94	0.946	0.731	8,630,082	10,500,000	8,630,082
parishorum	propeusis/propeusis	0	0	0.37	-0.08	0.81	0.722	0.350	14,200,194	13,319,437	11,261,252

Table 2. The relationship between the directionality of premating isolation asymmetry and range size asymmetry in sympatry, referred to as the *rarer-female effect* (see text).

Note: Asymmetry in relative population sizes of species pair is defined as having geographical range size differences between species being greater than 5%. See *Methods* on how geographical ranges were estimated. Asymmetry in premating isolation is defined above. Absolute geographic range of each species is described in square kilometers. The direction and % of range size asymmetry is based on the absolute ranges (see text for calculations).

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Scenario	Explored Parameters	$D1^{pre}/D1^{post}$	$D2^{pre}/D2^{post}$	$D3^{pre}/D3^{post}$	$D4^{pre}/D4^{post}$	$D5$	$s1$	$s2$	$s3$	$s4$	rec.	lec.	panc.	migr.	robustness threshold	alternative outcome(s) outside threshold
Basic Assumptions (see Fig. 3A)	-	0.100	0.010	0.900/90	-	-	1	0	0.6	0.2	0.05	7	0.95	0.05	robust 90%	loss of one of both preference alleles
Reducing Parentage	0.1, 0.2, 0.3, 0.4	0.100	0.010	0.900/90	-	-	1	0	0.6	0.2	0.05	7	VAR	0.05	(0.3 > - - - - - 1.0)	loss of both pref. alleles or random asymmetry
Increasing Reinforcement	0.15, 0.25, 0.3, 0.4, 0.5	0.100	0.010	0.900/90	-	-	1	0	0.6	0.2	VAR	7	0.95	0.05	(0.2 < - - - - - 0.5)	random asymmetry or genetic extinction of population
Increasing Migration	0.15, 0.2, 0.25, 0.5	0.100	0.010	0.900/90	-	-	1	0	0.6	0.2	0.05	7	VAR	(0.05 > - - - - - 1.0)	population #2 goes genetically extinct	
Reducing Initial Freq. of Preference Alleles	see right	0.05, 0.070	0.05, 0.07	0.95, 0.90/95, 0.93	-	-	1	0	0.6	0.2	0.05	7	0.95	0.05	(0.2 > - - - - - 1.0)	loss of one of both pref. alleles
Changing Ecological Horiz. Selection $s3$	0.2, 0.3, 0.8, 1	0.100	0.010	0.900/90	-	-	1	0	VAR	0.2	0.05	7	0.95	0.05	(0.2 > - - - - - 1.0)	population #2 goes genetically extinct
Changing Ecological Hybrid Selection $s4$	0.5, 0.8, 1	0.100	0.010	0.900/90	-	-	1	0	0.6	VAR	0.05	7	0.95	0.05	(0.1 > - - - - - 1.0)	no asymmetry or random asymmetry
Changing Postzygotic Isolation $s1 = 0.5, s2 = 0$	see right	0.100	0.010	0.900/90	-	-	0.5	0	0.6	0.2	0.05	7	0.95	0.05	robust 80%	no asymmetry or random asymmetry
Changing Postzygotic Isolation $s1 = 1.0, s2 = 0.5$	see right	0.100	0.010	0.900/90	-	-	1	0.5	0.6	0.2	0.05	7	0.95	0.05	robust 80%	no asymmetry or random asymmetry
Reducing Fecundity	1, 2, 5	0.100	0.010	0.900/90	-	-	1	0	0.8	0.2	0.05	VAR	0.95	0.05	(1.2 > - - - - - 7)	no asymmetry or random asymmetry
Initially Discontin. Asymmetries (see Fig. 3B)	see right	0.100	0.050	0.900/70	-	-	1	0	0.8	0.2	0.05	7	0.95	0.05	robust 70%	retains discordant asymmetry
Different random mating allelic backgrounds	see right	0.100	0.010	0.900/90	-	-	1	0	0.8	0.2	0.05	7	0.95	0.05	robust 90%	loss of one of both preference alleles
Asymmetric mating allelic backgrounds	see right	0.100	0.010	0.900/90	-	-	1	0	0.8	0.2	0.05	7	0.95	0.05	robust 90%	loss of one of both preference alleles
Rarer female effect (see 11.5.9% of pop. 2)	see right	0.100	0.010	0.900/90	-	-	0.5	0.5	0.8	0.2	0.05	7	0.95	0.05	robust 90%	loss of one of both preference alleles

Table 3. Sensitivity analysis of the evolution of *concordant isolation asymmetries* and the *rarer-female effect* after secondary contact.

Note: Sensitivity analysis is based on changing specific parameters while holding all other variables constant based on the *Basic Assumptions* scenario (see first row). The specific parameters explored are either designated by VAR in bold and given in each row under the

"Explored Parameters" column or simply shown in the appropriate rows. For each major scenario (e.g. *Basic Assumptions*), 10 replicates were run to determine the probability of observing the evolution of concordant isolation asymmetry (shown as "robust %"). For scenarios where multiple parameters were explored (shown as VAR), each sub-scenario with unique parameters was run 3 times. This generated an approximate "robustness threshold" which shows below or above which conditions all 3 replicates result in the evolution of concordant isolation asymmetry. I also describe alternative outcomes that occur outside the threshold values with probability greater than zero. Raw data is available upon request.

Table 4. Probability and speed of genetic extinction of one of the populations after secondary contact.

Ecological Selection (s_3)	Initial Isolation Asymmetry (initial freq. difference of D_1 and D_2 pref. alleles = 0.2)			
	Probability (Concordant)	Probability (Discordant)	Speed (Concordant)	Speed (Discordant)
0	1.0	0.6	46.0	70.0
0.05	1.0	0.2	62.0	50.0*
0.1	0.6	0.2	70.0	60.0*
0.2	0.2	0.0	70.0*	-
0.3	0.0	0.0	-	-
average	0.6	0.2	62	60

Note: Based on 5 replicates per scenario. Speed is in generations. *Based on only a single replicate that showed extinction. As indicated in Table 3, the probability of genetic extinction approaches zero under the *Basic Assumptions* scenario when $s_3 > 0.25-0.3$ and are thus not explored in this context. All other parameter values follow the *Basic Assumptions* scenario in Table 3.

Table 5. Species pairs that show both *concordant isolation asymmetries* and enhanced pre mating isolation in sympatry relative to their allopatric sister species pair.

outgroup species	sympatric species	allopatric species(s)	enhanced pre mating isolation in sympatry
<i>recens</i>	<i>subquinaria</i> race <i>S</i>	<i>subquinaria</i> race <i>A</i>	0.25
<i>virilis</i>	<i>montana ovivororum</i>	<i>montana montana</i>	0.12
<i>parapallidosa</i>	<i>ananassae</i>	<i>pallidosa-like Wau, pallidosa, pallidosa-like</i>	0.28*
<i>persimilis</i>	<i>pseudoobscura USA</i>	<i>pseudoobscura Bogota</i>	0.05, for <i>persim. females only</i>
<i>melanogaster</i>	<i>simulans</i>	<i>sechellia, mauritiana</i>	0.06*
<i>auraria</i>	<i>triauraria</i>	<i>quadraria</i>	0.33

Note: Values indicate the gain in pre mating isolation index from allopatric species pair to sympatric species pair. The first four species pairs in addition show increased concordant isolation asymmetries in sympatry relative to allopatry. *Averaged over the multiple allopatric species pairs. *Persimilis-pseudoobscura USA* and *melanogaster-simulans* sympatric species pairs indicate concordant isolation asymmetries based solely on asymmetrical range sizes since their postzygotic isolation is symmetrical (see Table S5). The three other informative sympatric species pairs with concordant isolation asymmetries (not shown here) did not have enhanced pre mating isolation relative to allopatry: 1) *pallidosa-like Wau - pallidosa-like*, 2) *azteca - tolteca*, and 3) *paulistorum - tropicalis tropicalis*.

Figure 1. Relationship between preminging isolation and genetic distance (Nei's D) in (A) 129 allopatric species pairs and (B) 125 sympatric species pairs. Data is shown as phylogenetically uncorrected for the explicit purpose of showing a clear pattern of constraint on how fast preminging isolation is accumulated in allopatry as compared to sympatry (corrected data for non-independence shows the same constraint with fewer points; see Fig. S1). Insert in panel A shows two fitted regressions on the maximum rate of accumulation of preminging isolation in allopatry with the exponential curve best fitting the data (see insert). The greatest difference between allopatry and sympatry occurred during incipient stages of speciation (for $D < 0.25$: avg. index_{allopatry} = 0.283, st.dev. = 0.156, avg. index_{sympatry} = 0.757, st.dev. = 0.263, Wilcoxon-Kruskal-Wallis test: Z -value = -4.91, $P < 0.0001$; compare Figs. 1A and 1B), but remains significantly different even for cases with $D > 0.25$ (avg. index_{allopatry} = 0.798, st.dev. = 0.233, avg. index_{sympatry} = 0.890, st.dev. = 0.182, Wilcoxon-Kruskal-Wallis test: Z -value = 3.48, $P = 0.0005$).

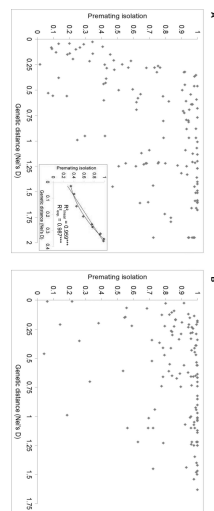


Figure 2. Relationship between asymmetrical premating and postzygotic isolation across each pair of reciprocal matings. Data from Table 1. Concordant isolation asymmetries are labeled as solid lines and discordant isolation asymmetries as broken lines. (A) Allopatric pairs: Spearman's Rho = 0.168, $P = 0.30$ (NS). (B) Sympatric pairs: Spearman's Rho = 0.55, $P = 0.0011$.

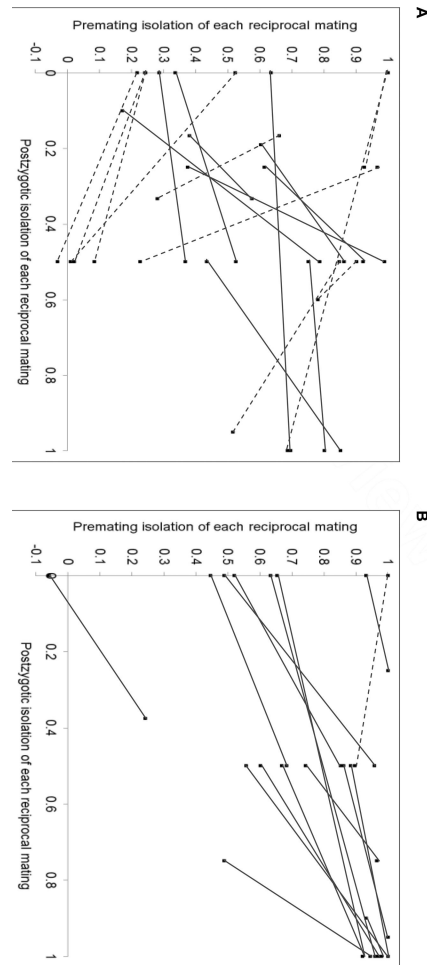


Figure 3. Computer simulation results of the evolution of asymmetrical premating isolation resulting in *concordant isolation asymmetries* during secondary contact. (A) The evolution of concordant isolation asymmetries starting from symmetrical and low frequency of mating preference alleles in the two populations (for detailed parameters see Table 3: *Basic Assumptions* scenario). (B) The evolution of concordant isolation asymmetries starting from discordant isolation asymmetries (for parameters, see Table 3: *Initially Discordant Asymmetries* scenario). Each plot represents average values across five independent replicates. X-axis is number of generations of secondary contact. Y-axis is the average allele frequency of various alleles shown in the graph. Blue line represents premating isolation asymmetry resulting from mating preference allele *D1* increasing in frequency faster than mating preference allele *D2*. Positive values indicate *concordant* asymmetry and negative values indicate *discordant* asymmetry (i.e. asymmetry = *D1* freq. – *D2* freq.). Red line represents total premating isolation averaged between both populations (i.e. total isolation = 1 – *D3* random mating allele frequency). Green and purple lines represent the average local frequencies of fitness (*A1* and *B1*) and sexual cue (*C1*) alleles in population 1 and *A2*, *B2*, and *C2* alleles in population 2, respectively. Their frequencies are less than 1 because migration introduces the alternative alleles every generation (see Table 3 for details).

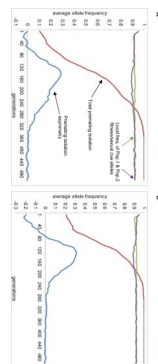
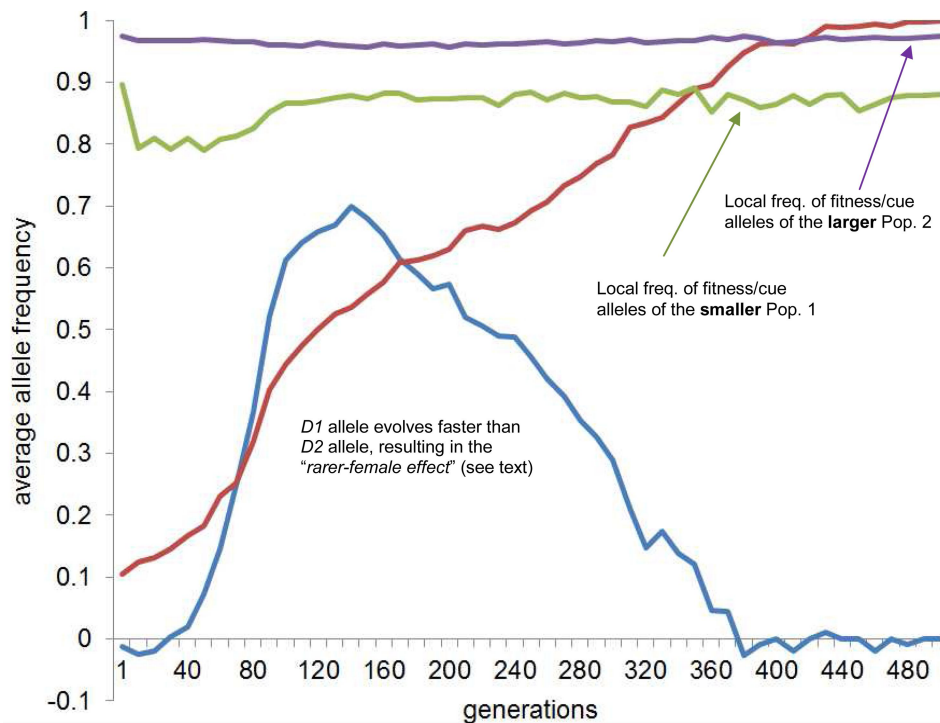


Figure 4. Computer simulation results of the evolution of the *rarer-female effect* during secondary contact. Here the sizes of the two populations are 50% different (Pop. 1 = 1,500 individuals/gen., Pop. 2 = 3,000 individuals/gen.). I also assume that the fitness of hybrids between the two reciprocal matings is symmetrical at $s1 = s2 = 0.5$, and that divergent ecological selection $s3 = 0.8$ (see text for explanations). All other parameters of the simulations are the same as in *Basic Assumptions* in Table 3. Lines shown above represent the same processes as in Fig. 3 (see legend for details).



Supplementary Information

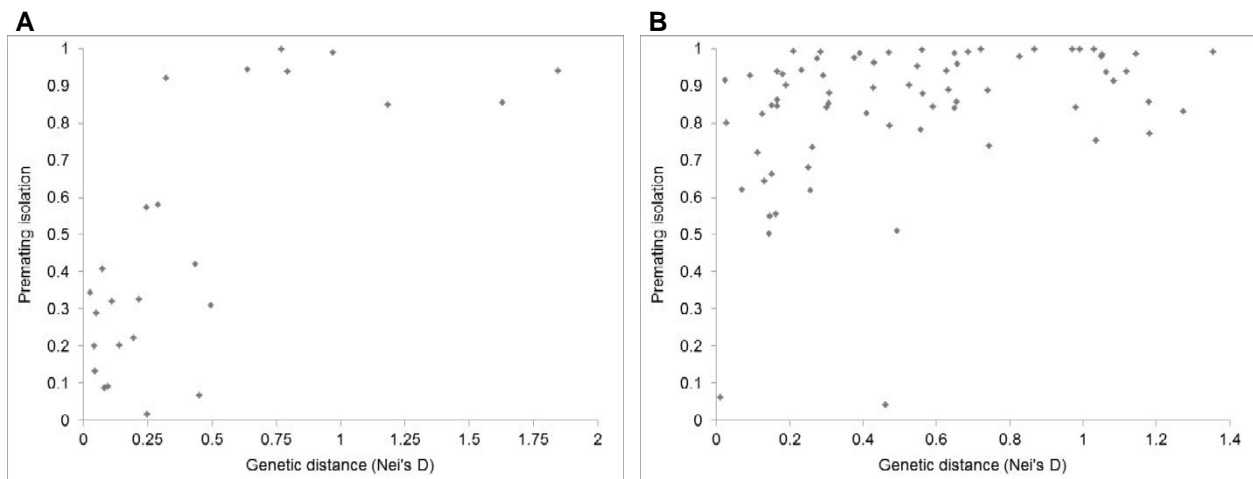


Figure. S1. Relationship between pre-mating isolation and genetic distance (Nei's D) in (A) 26 allopatric species pairs and (B) 70 sympatric species pairs. Data is phylogenetically corrected (uncorrected data is shown in Fig.1). Results remains qualitatively same as in the uncorrected data. For $D < 0.25$: avg. index_{allopatry} = 0.240, st.dev. = 0.149, avg. index_{sympatry} = 0.750, st.dev. = 0.217, Wilcoxon-Kruskal-Wallis test: Z -value = -4.40, $P < 0.0001$. For cases with $D > 0.25$ (avg. index_{allopatry} = 0.735, st.dev. = 0.312, avg. index_{sympatry} = 0.88, st.dev. = 0.160, Wilcoxon-Kruskal-Wallis test: Z -value = -1.17, $P = 0.235$ (ANOVA: F -value = 5.30, $P = 0.023$).

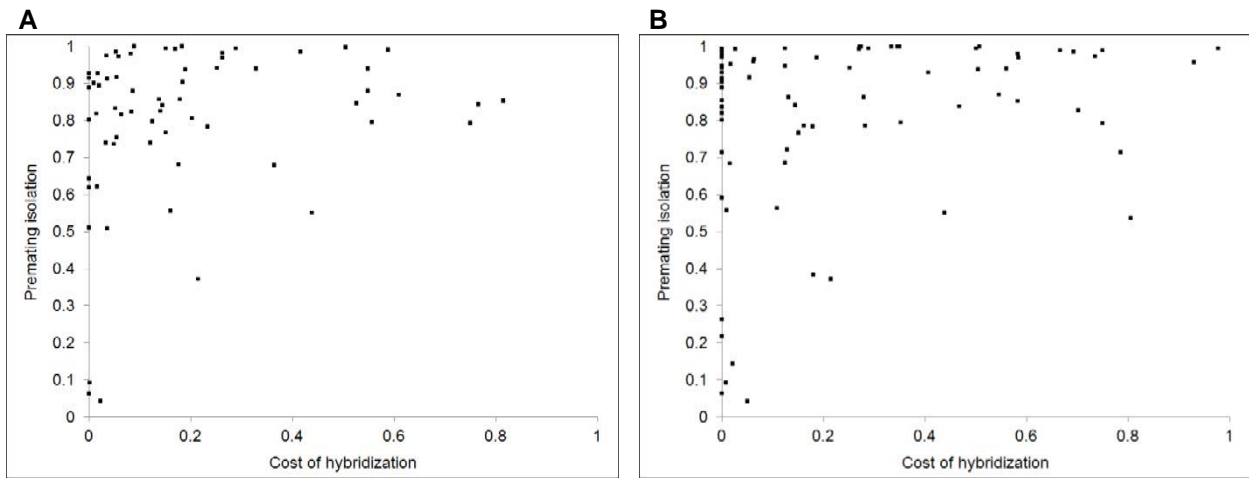


Figure. S2. Relationship between premating isolation and average cost of hybridization across sympatric species pairs. Average cost of hybridization is equal to postzygotic isolation * percent geographical overlap between species. (A) Spearman's $Rho = 0.264$, $P < 0.034$ for 67 phylogenetically corrected species pairs. (B) Spearman's $Rho = 0.283$, $P < 0.014$ for 75 phylogenetically uncorrected species pairs.

Supplementary Tables and Data

(Excel Spreadsheet: “Supplementary Tables and Data”).

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