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Does character displacement initiate speciation? Evidence of reduced gene flow between populations experiencing divergent selection

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divergent selection;
ecological speciation;
gene flow;
reproductive isolation.

Abstract

Character displacement – trait evolution stemming from selection to lessen resource competition or reproductive interactions between species – has long been regarded as important in finalizing speciation. By contrast, its role in initiating speciation has received less attention. Yet because selection for character displacement should act only where species co-occur, individuals in sympatry will experience a different pattern of selection than conspecifics in allopatry. Such divergent selection might favour reduced gene flow between conspecific populations that have undergone character displacement and those that have not, thereby potentially triggering speciation. Here, we explore these ideas empirically by focusing on spadefoot toads, *Spea multiplicata*, which have undergone character displacement, and for which character displacement appears to cause post-mating isolation between populations that are in sympatry with a heterospecific and those that are in allopatry. Using mitochondrial sequence data and nuclear microsatellite genotypes, we specifically asked whether gene flow is reduced between populations in different selective environments relative to that between populations in the same selective environment. We found a slight, but statistically significant, reduction in gene flow between selective environments, suggesting that reproductive isolation, and potentially ecological speciation, might indeed evolve as an indirect consequence of character displacement. Generally, character displacement may play a largely underappreciated role in instigating speciation.

Introduction

Character displacement arises when selection acting to lessen resource competition or reproductive interactions causes sympatric species to diverge in traits associated with resource or mate acquisition (Brown & Wilson, 1956; Grant, 1972; Howard, 1993; Schluter, 2000; Dayan & Simberloff, 2005). Character displacement has been documented in diverse organisms (Grant, 1972; Schluter, 2000; Dayan & Simberloff, 2005), and it is regarded as a general and important adaptive explanation for why interacting species typically differ (Pfennig & Pfennig, 2009).

Although character displacement is widely viewed as a mechanism for explaining why species differ (and thereby coexist), it can also play a key role in the formation of new species (specifically, 'ecological' speciation; e.g. see Rundle & Nosil, 2005; Schluter, 2009). Indeed, character displacement has long been viewed as vital in completing the process of speciation (reviewed in Schluter, 2000; Coyne & Orr, 2004; Rundle & Nosil, 2005; Grant & Grant, 2008; Price, 2008). For example, after ecological character displacement has occurred (reviewed in Schluter, 2000; Grant & Grant, 2008), populations that evolve to specialize on alternate resources might experience reduced contact, thereby allowing for the accumulation

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of genetic differences between them that enhance isolation (reviewed in Rundle & Schluter, 2004). Moreover, if individuals from two such divergent populations mate and produce offspring of low fitness, reproductive character displacement should cause these populations to diverge in reproductive traits such that they no longer interbreed (i.e. through reinforcement; Dobzhansky, 1940; Howard, 1993; Servedio & Noor, 2003).

Less widely recognized is the possibility that character displacement might also *initiate* ecological speciation (but see Hoskin *et al.*, 2005; Pfennig & Ryan, 2006; Pfennig & Rice, 2007). In particular, because selection for character displacement should act only where species co-occur, character displacement causes populations in sympatry with a heterospecific to diverge from conspecific populations in allopatry. By creating such divergence, character displacement might indirectly instigate speciation through the evolution of either post-mating or premating barriers to gene flow between sympatric and allopatric populations (Pfennig & Rice, 2007; Pfennig & Pfennig, 2009). Post-mating barriers might arise when, as an indirect consequence of either ecological or reproductive character displacement between species, offspring created by matings between sympatric and allopatric parents express an intermediate phenotype that is poorly adapted to *either* selective environment (Rice & Hostert, 1993; Hatfield & Schluter, 1996, 1999; Vamosi & Schluter, 1999; Pfennig & Rice, 2007; van der Sluijs *et al.*, 2008; Svedin *et al.*, 2008). Moreover, once such isolation evolves, populations in divergent selective environments might begin to evolve independently of each other and thereby accumulate alleles that are incompatible with genomes from the alternative environment (reviewed in Coyne & Orr, 2004). Premating barriers might also arise when, as a consequence of reproductive character displacement, female preferences or male traits become so divergent that females in one selective environment (i.e. sympatry or allopatry) fail to recognize allopatric males from the other selective environment as acceptable mates (Hoskin *et al.*, 2005; Pfennig & Ryan, 2006). Likewise, ecological character displacement can contribute to premating barriers between populations in different selective environments if shifts in habitat or resource use preclude matings between such populations (reviewed in Rundle & Schluter, 2004).

Given that conspecific populations in sympatry with a heterospecific can become isolated from those in allopatry as an indirect consequence of character displacement, it is important to resolve whether character displacement actually initiates ecological speciation. One way to do so is to determine whether gene flow is reduced between sympatric and allopatric populations. Finding such a reduction is critical, because the process of speciation is thought to begin when gene flow is disrupted between populations (Coyne & Orr, 2004). Yet no study to date has shown that gene flow is reduced because of character displacement.

Here, we test for a reduction in gene flow between populations of spadefoot toads, *Spea multiplicata*, that are experiencing divergent selection following character displacement. As we describe in detail later, this species has undergone both ecological and reproductive character displacement in areas where it co-occurs with a heterospecific, *S. bombifrons*. Moreover, previous work suggests that character displacement has resulted in post-mating isolation between populations of *S. multiplicata* that are in sympatry with *S. bombifrons* and those that are in allopatry. We therefore asked whether gene flow is reduced between such populations.

For our analysis, we used unlinked neutral markers to test for reduced gene flow between selective environments. Although many studies have used such markers to evaluate whether divergent selection reduces gene flow, the results from such studies have been mixed in that some have found the expected signature (Lu & Bernatchez, 1999; Ogden & Thorpe, 2002; Berner *et al.*, 2009), whereas others have not (Crispo *et al.*, 2006; Crispo & Chapman, 2008). Such mixed results might arise because (i) divergent selection sometimes does and sometimes does not trigger speciation (see Berner *et al.*, 2009; Hendry, 2009; Nosil *et al.*, 2009b), or (ii) the conceptual foundation of the particular test is uncertain. Regarding the latter possibility, some authors have argued that unlinked neutral markers can flow almost freely between selective environments (Gavrilets & Vose, 2005), whereas others have argued for a generalized barrier across the genome (Gavrilets, 2004; Grahame *et al.*, 2006; Nosil *et al.*, 2007). Recently, Thibert-Plante & Hendry (2009) concluded that finding evidence of reduced gene flow between selective environments is probably a good indicator of ecological speciation. However, they also maintained that failing to detect such a signature does not imply that ecological speciation is not occurring. They suggested that the expected signature is most likely to emerge when divergent selection and assortative mating are both strong (Thibert-Plante & Hendry, 2009), and levels of gene flow are intermediate (Thibert-Plante & Hendry, in revision). In sum, although the use of neutral markers to test whether divergent selection reduces gene flow comes with caveats, for the reasons we describe in the next section, our system is a likely candidate to find such reduced gene flow between selective environments.

Study system

Two species of spadefoot toads, *Spea multiplicata* and *S. bombifrons*, have undergone ecological character displacement and reproductive character displacement in south-eastern Arizona and south-western New Mexico, USA. In this region of north-south trending valleys and mountain ranges, both species are generally present in the valleys (such sites are designated as 'sympatry'). However, only *S. multiplicata* breeds in ponds above

1350 m, and only *S. bombifrons* breeds in the lowest elevation playas (temporary ponds that form in the lowest areas of valleys; the latter two sites are designated as 'allopatry'; Pfennig *et al.*, 2006).

The two species have undergone ecological character displacement in tadpole trophic phenotype (Pfennig & Murphy, 2000, 2002, 2003; Pfennig *et al.*, 2006, 2007; Rice *et al.*, 2009). In allopatric ponds, both species produce similar, intermediate frequencies of two trophic phenotypes: an omnivore morph, which feeds mostly on organic detritus on the pond bottom; and a large-headed carnivore morph, which specializes on anostracan fairy shrimp in the water column (Pomeroy, 1981; Pfennig, 1990, 1992). However, in nearby sympatric ponds, *S. multiplicata* produces almost entirely omnivores, and *S. bombifrons* produces almost entirely carnivores (Pfennig & Murphy, 2000, 2002, 2003; Pfennig *et al.*, 2006, 2007). Allopatric and sympatric *S. multiplicata* differ in tadpole morph production even when reared under common conditions (Pfennig & Murphy, 2002).

These canalized shifts in morph production likely evolved because of selection acting to lessen interspecific resource competition; i.e. they likely reflect ecological character displacement (Pfennig & Murphy, 2000, 2002, 2003; Pfennig *et al.*, 2007; Rice *et al.*, 2009). In particular, because *S. bombifrons* is the superior competitor for fairy shrimp (the resource on which the carnivore morph specializes), sympatric *S. multiplicata* is under strong selection to produce only omnivores (Pfennig *et al.*, 2007). By contrast, in allopatry, disruptive selection favours production of both morphs as a means of lessening intraspecific competition for food (Pfennig *et al.*, 2007; Martin & Pfennig, 2009).

Sympatric and allopatric *S. multiplicata* may experience ecologically dependent post-mating isolation. Offspring produced by matings between individuals from *different* selective environments (i.e. sympatry vs. allopatry; hereafter 'between-selective-environment offspring' or BSE offspring) grow less well than offspring produced by matings of individuals from the *same* selective environment (hereafter 'within-selective-environment offspring' or WSE offspring), in part because BSE offspring are competitively inferior to WSE offspring in both the allopatric and sympatric selective environments (Pfennig & Rice, 2007). Thus, sympatric and allopatric populations of *S. multiplicata* may be reproductively isolated from one another as an indirect by-product of ecological character displacement with *S. bombifrons*.

Spea multiplicata and *S. bombifrons* have also undergone reproductive character displacement. In particular, female *S. multiplicata* from allopatry prefer males with faster call rates in contrast to females in sympatry, which prefer males with slower calls (Pfennig, 2000, 2008; Pfennig & Pfennig, 2005). Presumably, female *S. multiplicata* from sympatry prefer slow-calling males as mates to avoid costly hybridization with fast-calling *S. bombifrons* males (Simovich *et al.*, 1991; Pfennig & Simovich, 2002). Because *S. multiplicata* males from sympatry produce slower calls on average than *S. multiplicata* males in allopatry (Pierce, 1976; Pfennig, 2000), this difference in female mate preferences is likely to result in assortative mating within each selective environment.

As noted earlier, reduced gene flow between selective environments is best detected when overall levels of gene flow are intermediate, and *S. multiplicata* in the southwestern USA appear to meet this requirement. On the one hand, gene flow between populations is likely not high. Adult *Spea* spend much of the year underground, emerging only during the summer rainy season to breed and feed (Bragg, 1944, 1945). Opportunities for dispersal are thus rare. Indeed, previous work has shown that both species exhibit significant population genetic structure (Rice, 2008; Rice & Pfennig, 2008). On the other hand, gene flow is likely not negligible. Because there are no physical barriers to gene flow between populations, and because running water from heavy rains can sweep *S. multiplicata* from one population to another (A. Rice, pers. obs.), some gene flow between populations is possible.

Thus, *S. multiplicata* has undergone both ecological and reproductive character displacement with *S. bombifrons*. Moreover, ecological character displacement appears to have resulted in the evolution of post-mating reproductive isolation between sympatric and allopatric populations of *S. multiplicata* (Pfennig & Rice, 2007). Finally, levels of gene flow between populations of *S. multiplicata* are likely intermediate. We therefore predicted that gene flow (measured with neutral markers) should be reduced between populations in these two distinct selective environments.

Methods

The overall goal of this study was to determine whether gene flow is reduced between populations of *S. multiplicata* inhabiting *different* selective environments (i.e. sympatric with *S. bombifrons* vs. allopatric) relative to that between populations inhabiting the *same* selective environment. To estimate gene flow between populations, we used indirect DNA-based methods. We specifically used neutral DNA markers both in nuclear genes and in a maternally inherited mitochondrial gene to estimate the degree of population genetic structure between populations in the same vs. different selective environments (reviewed in Bohonak, 1999; Crispo *et al.*, 2006). Using this approach, we first sought to determine whether the population structure of *S. multiplicata* is consistent with reduced gene flow between selective environments. We then sought to determine whether there is a significant correlation between selective environment and population structure after controlling for geographic distance between populations.

Sampling, sequencing and genotypes

We used previously generated mitochondrial DNA sequence data and nuclear microsatellite genotypes from wild-collected *S. multiplicata* tadpoles. We collected *S. multiplicata* tadpoles during summers 1999–2004 in south-eastern AZ and south-western NM. Tadpoles were sampled seven to 15 days post-hatching from random sites throughout natural, temporary ponds using a hand-held dip net. We sampled ten allopatric ponds and eight sympatric ponds (Fig. 1; Table S1). Within a few hours of collection, tadpoles were killed by immersion in a 0.1% aqueous solution of tricane methanesulfonate (MS 222) and preserved in 95% ethanol. For each pond site, we used Google Earth version 4.2.0198.2451 (beta) to determine latitudinal and longitudinal coordinates. The geographic coordinates were used to calculate geographic distance between each pair of pond sites using the great circle formula as implemented by the GPS WAYPOINT REGISTER'S distance calculator (http://www.gpswaypoints.co.za/Downloads/distcalc.xls). Using the procedures outlined in Rice & Pfennig (2008), we generated sequences of a 663 bp portion of the cytochrome *b* (cyt *b*) gene from the mitochondrial genome from a total of 275 individuals (8–28 individuals per pond; Table S1; GenBank accession nos. EU285643-EU285652, EU285654, EU285657). For eight to ten individuals per pond (Table S2), eight previously published microsatellite loci were genotyped using the protocols published by Rice *et al.* (2008) (GenBank Accession Numbers EU285444–EU285445, EU285450–EU285452, EU285454-EU285456). With the exception of the genotypes from individuals collected in Post Office Canyon pond (PO; Fig. 1, Table S1), these data were used in previously published analyses (Rice *et al.*, 2009); however, the analyses presented here are new. Moreover, because previous studies had found a high degree of population genetic structure among the *S. multiplicata* in our study area (Rice, 2008; Rice & Pfennig, 2008), we regarded different ponds as representing separate populations.

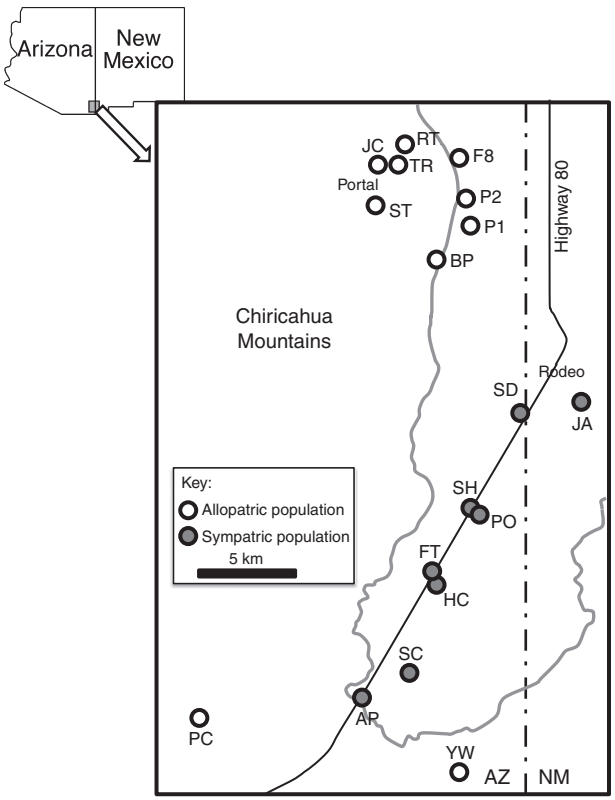

Fig. 1 Map of the study area, showing approximate locations of ponds (populations) in which only *Spea multiplicata* is present (i.e. allopatric ponds) and ponds in which both *S. multiplicata* and *S. bombifrons* are present (i.e. sympatric ponds). Exact geographical coordinates for each population are given in Table S1. The dashed line is the boundary between the states of Arizona and New Mexico. The solid black line is Highway 80, and the solid gray line follows the 1350 m elevation contour.

Genetic variation, Hardy-Weinberg equilibrium, null alleles and linkage disequilibrium

We characterized the genetic diversity at each locus using standard tests (see Supporting Information). Using the probability test in Genepop 4.0.6 (Rousset, 2008), we tested each locus for Hardy-Weinberg Equilibrium (HWE). Statistical significance was estimated using the Markov chain method, with 10 000 dememorizations, 1000 batches and 10 000 iterations per batch. Because tests for HWE were performed for each population-locus combination, we assessed the results both with and without sequential Bonferroni corrections (Rice, 1989) to the α-value for each locus. Because some population-locus combinations were not in HWE (see Results, Table S3), we used Micro-Checker (van Oosterhout *et al.*, 2004) to test for the possible presence of null alleles, which is one likely cause for departure from HWE. Significance was estimated using 1000 randomizations and Bonferroni-corrected significance levels. Two loci (*Sm4* and *Sb15*) exhibited signatures of a null allele (see Results). Null alleles might affect the accuracy of estimates of population structure and gene flow (Selkoe & Toonen, 2006). Two ways to deal with null alleles are to exclude them from analyses or to correct for their presence statistically. Because one of the loci that exhibited signatures of a null allele was also one of our most variable loci (*Sm4*, see Table S2), and therefore valuable for detecting subtle population structure, we chose the latter approach. Specifically, we used the Oosterhout correction algorithm in Micro-Checker (van Oosterhout *et al.*, 2004) to generate corrected genotype and allele frequencies for use where possible. Although our power to detect linkage disequilibrium was likely limited, we used Fisher's global test in Genepop

4.0.6 (Rousset, 2008) to test for linkage disequilibrium between all pairs of loci across all populations.

Testing for reduced gene flow between selective environments

Hierarchical population structure

If gene flow is reduced between populations in contrasting selective environments, then populations within each environment should be more similar to each other in genotype frequencies than they are to populations in the opposite environment. To test this prediction, we used an Analysis of Molecular Variance (AMOVA) in ARLEQUIN 3.11 (Schneider *et al.*, 2000) to calculate hierarchical F-statistics for the microsatellite data and the analogue for the cyt *b* sequence data: hierarchical Φ-statistics. We defined two population groups: allopatry and sympatry (Fig. 1, Table S2). We calculated F_{SC} (or Φ_{SC} for the cyt *b* sequences) as an indication of the average gene flow among populations within each selective environment. We then calculated F_{CT} (or Φ_{CT} for the cyt *b* sequences) as an indication of whether gene flow is reduced between selective environments relative to the gene flow within each environment. To determine the effect of null alleles at *Sm4* and *Sb15*, we used a locus-by-locus AMOVA to estimate F_{SC} and F_{CT} for each locus individually, using both the original and the corrected genotype frequencies. We also re-ran the first AMOVA without data from *Sm4* and *Sb15*. Significance of the F- and Φ-statistics (against the null hypothesis of zero) was estimated using 50 000 permutations of the data.

Genetic clustering

We used the Bayesian approach implemented in STRUCTURE 2.3.1 (Pritchard *et al.*, 2000; Falush *et al.*, 2003; Hubisz *et al.*, 2009) to determine whether population structure was present in our microsatellite data without incorporating any prior assumptions about the form or patterns of the structure. We were unable to use data that were corrected for the presence of null alleles, because the corrected genotypes for each locus did not correspond to particular individuals, but instead to population-wide genotype frequencies. Once we determined whether any genetic clusters were present in our data, we asked whether allopatric populations were more likely to cluster with other allopatric populations vs. with sympatric populations, and vice versa for sympatric populations. Such a pattern of clustering would suggest that gene flow was reduced between contrasting selective environments.

To first determine the most likely number of genetic clusters in our data, we performed 20 runs of STRUCTURE at each assumed number of genetic clusters (*K*) from 1 to 18 (the number of populations we sampled). Each run consisted of 25 000 steps after a burnin of 100 000 steps. We used the admixture model with allele frequencies correlated among populations. Additionally, we used the new option in version 2.3.1, which allows the use of sampling locations as priors for when the signal of population structure is weak (Hubisz *et al.*, 2009). For each run, STRUCTURE provides an estimate of the posterior probability of the data given the assumed K (L(K); Evanno *et al.*, 2005). We calculated the mean and standard deviation of L(K) for each K (Fig. S1). Following the method of Evanno *et al.* (2005), we calculated ΔK, and graphically identified the mode of the ΔK distribution (Fig. S2), which represented the most likely number of genetic clusters (Evanno *et al.*, 2005). We then performed a longer run of STRUCTURE, with a run length of 250 000 after a burnin of 200 000 steps, using this most likely value of K ($K = 6$; see Results). Moreover, because our ΔK mode and the differences in L(K) between adjacent values of K were small (Figs. S1, S2), suggesting the presence of only weak structure in our data, we also performed long runs of STRUCTURE for each value of K below 6. We visualized the results using the program *distruct* (Rosenberg, 2004).

The results from our STRUCTURE analysis indicated that the most likely number of genetic clusters was six; however, because the differences in L(K) among different values of K were relatively small, suggesting that the presence of six genetic clusters was not much more likely than fewer clusters, we decided to more closely examine the STRUCTURE results when K was set at 2. We chose this value because our hypothesis that gene flow should be reduced between the two selective environments relative to gene flow within each environment would predict that allopatric and sympatric populations would be assigned to two genetic clusters. Moreover, according to the distribution of ΔK (Fig. S2), the most likely number of clusters when $K < 6$ was two. Using the posterior probabilities of population assignment to either of the two genetic clusters output from STRUCTURE (see Results; Table 3), we tested this prediction in two ways. First, we classified each population as belonging to one of the two clusters based on the highest probability of assignment. Then, we performed a chi-square analysis to determine whether populations in the two selective environments were nonrandomly assigned to the two genetic clusters. Second, we used a *t*-test to compare the arcsine square-root transformed probabilities of assignment to cluster 1 between populations in the two selective environments. The residuals from this analysis were normally distributed. We used JMP 8 (SAS Institute, Cary, NC, USA) for both of these analyses.

Correlations between gene flow and selective environment

Because our hierarchical population structure analysis indicated that gene flow is reduced between populations in contrasting selective environments (see Results), we predicted that for pairs of populations separated by a given geographical distance, population structure should tend to be higher, and therefore gene flow lower, between populations in different environments vs.

between populations in the same environment. To test this prediction, we used partial Mantel tests (Smouse *et al.*, 1986) in Arlequin 3.11. This method tests for partial correlations among distance matrices by creating a null distribution of correlation coefficients from permutations of the data. We performed separate partial Mantel tests for pairwise F_{ST} values based on all eight microsatellite loci (calculated with MSA 4.05; Dieringer & Schlötterer, 2003) and pairwise Φ_{ST} values (calculated in Arlequin 3.11) based on the cyt *b* sequence data. We chose to include all eight microsatellite loci because the presence of null alleles at *Sm4* and *Sb15* did not appear to affect population structure estimates greatly (see Results).

The first predictor matrix included log-transformed geographic distances (km) between populations. The second predictor matrix was categorical and coded for the environment comparison between populations (i.e. 0 = both populations are either allopatric or sympatric, 1 = one population is allopatric and one is sympatric). To estimate significance, we used 100 000 permutations. Because the hierarchical population structure analysis indicated a general reduction in gene flow between selective environments (see Results), we report one-tailed *P*-values.

Results

Genetic variation, Hardy-Weinberg equilibrium, null alleles and linkage disequilibrium

Population-specific genetic variation measures for the cyt *b* locus and the eight microsatellite loci are listed in Tables S1 and S2, respectively. Most population-loci combinations did not significantly depart from HWE (Table S3); however, *Sm4* departed from HWE in 15 of the 18 populations, whereas *Sb15* showed departure from HWE in 17 of the 18 populations (both before Bonferroni correction of α-values). Similarly, when we tested for the presence of null alleles, these two loci exhibited significant signatures of a null allele in at least 50% of the populations (Table S3). No loci were in linkage disequilibrium ($P > 0.37$ for all loci pairs across all populations), suggesting that the different loci can be treated as independent.

Testing for reduced gene flow between contrasting selective environments

Hierarchical population structure

For all eight microsatellite loci combined, significant population structure was evident ($F_{SC} = 0.040$, $P < 0.0001$; Table 1), suggesting that gene flow among populations within each selective environment (i.e. allopatry or sympatry) is lower than expected under panmixia. This significant population structure was evident across all loci except *Sb15* (Table 1), whether

Table 1 Hierarchical population structure results. Values in bold indicate loci showing significant ($P < 0.05$) population structure.

	Sm1	*Sm4*	*Sm14*	*Sm20*	*Sm23*	*Sm25*	*Sb15*	*Sb28*	Combined
F_{SC}-uncorrected microsatellite data (*P*-value)	**0.051 (0.0005)**	**0.073 (0.0001)**	**0.022 (0.0023)**	**0.058 (< 0.0001)**	**0.021 (0.0051)**	**0.043 (0.0005)**	0.016 (0.6081)	**0.037 (0.0027)**	**0.040 (< 0.0001)**
F_{SC}-corrected allele frequency data (*P*-value)	**0.049 (0.0004)**	**0.063 (< 0.0001)**	**0.022 (0.0083)**	**0.064 (< 0.0001)**	**0.021 (0.0064)**	**0.043 (0.0001)**	−0.005 (0.6610)	**0.037 (0.0049)**	
F_{CT}-uncorrected microsatellite data (*P*-value)	−0.006 (0.7049)	**0.015 (0.0399)**	0.006 (0.0932)	0.009 (0.1080)	**0.016 (0.0089)**	−0.002 (0.5587)	−0.002 (0.5801)	−0.008 (0.8934)	**0.005 (0.0296)**
F_{CT}-corrected allele frequency data (*P*-value)	−0.007 (0.7452)	0.012 (0.0844)	0.005 (0.1124)	0.010 (0.1010)	**0.016 (0.0116)**	−0.002 (0.5560)	−0.003 (0.7859)	−0.008 (0.8914)	

analysed as microsatellite genotypes or as allele frequencies corrected for the presence of null alleles. Significant population structure was also evident at the cyt *b* locus (F_{SC} = 0.162, P < 0.00001).

In the microsatellite data, we found evidence that a slight, but significant, proportion of the variance in genotypes can be explained by the allopatric and sympatric population groupings (F_{CT} = 0.005, P = 0.0296; Table 1), consistent with a slight reduction in gene flow between populations in contrasting selective environments. This pattern is driven by differentiation at the *Sm23* locus, with slight contributions from the *Sm4*, *Sm14* and *Sm20* loci (although the F_{CT}s for these loci are marginally nonsignificant when allele frequencies are corrected; Table 1). In contrast, these groupings do not explain any of the variance in the cyt *b* sequences (F_{CT} = −0.016, P = 0.81).

Null alleles at the *Sm4* and *Sb15* loci do not appear to have a large effect on our estimates of F_{SC} or F_{CT}. Estimates of F_{SC} based on allele frequency data corrected for the presence of null alleles are similar in magnitude and significance to estimates based on the uncorrected genotype data (Table 1). Likewise, although the F_{CT} value for *Sm4* became marginally nonsignificant, the F_{CT} estimates based on the corrected data are similar overall to the estimates from the uncorrected data (Table 1). When we removed *Sm4* and *Sb15* from the AMOVA analysis, both F_{SC} and F_{CT} were similar in magnitude when calculated with all loci, but the F_{CT} value became marginally nonsignificant (F_{SC} = 0.038, P < 0.000001; F_{CT} = 0.004, P = 0.0934).

Genetic clustering

The most likely number of genetic clusters in our data was six, which had both the highest mean L(K) (−4871.24; overall range of mean L(K): −5178.98 to −4871.24; Fig. S1) and was the location of the mode for the ΔK distribution (Fig. S2; Evanno *et al.*, 2005). The small observed differences between mean L(K) values for each value of K, along with the low height of the mode of ΔK, suggest that only weak population structure is present among our populations. Results from the longer runs of STRUCTURE show similarly small differences between L(K) for values of K between 2 and 6 (Table 2). When K was set at six, most individuals did not show a clear assignment to any particular genetic cluster, nor did most of our sampling locations (Fig. 2, Table 3). However, two of the allopatric populations, BP and F8, did show some evidence of differentiation (Fig. 2).

When K was set at two, populations within each selective environment were more likely to be assigned to the same genetic cluster as other populations from the same environment (χ^2 = 8.1, d.f. = 1, P = 0.004).

Table 2 Posterior probability of the data given the assumed K, estimated from the longer STRUCTURE runs.

K	L(K)
2	−5059.9
3	−5030.0
4	−4979.2
5	−4981.5
6	−4978.0

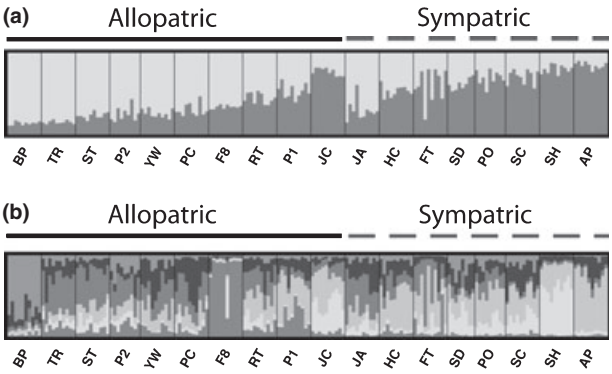

Fig. 2 Structure membership assignments to genetic clusters. (a) Two genetic clusters assumed. (b) Six genetic clusters assumed. Individuals from each population are grouped together, with each vertical bar representing one individual. Each shade represents a different genetic cluster, and the proportion of each bar that is a given shade represents the posterior probability of that individual belonging to that cluster. Population labels along the bottom of each graph refer to Fig. 1 and Table S1.

Table 3 Posterior probabilities of the assignment of each population to alternative genetic clusters as estimated by STRUCTURE, assuming the presence of two and six clusters, respectively. Populations are listed on the left (see Table S1). The first 10 populations are allopatric, and the last 8 are sympatric.

	Cluster		Cluster					
	1	2	1	2	3	4	5	6
Allopatric								
BP	0.137	0.863	0.032	0.017	0.051	0.066	0.154	0.679
F8	0.337	0.663	0.835	0.065	0.019	0.032	0.037	0.011
JC	0.769	0.231	0.054	0.366	0.353	0.131	0.063	0.033
PC	0.301	0.699	0.101	0.068	0.145	0.295	0.359	0.032
P1	0.530	0.470	0.254	0.028	0.408	0.082	0.184	0.043
P2	0.250	0.750	0.148	0.090	0.148	0.299	0.118	0.198
RT	0.445	0.555	0.106	0.116	0.287	0.268	0.172	0.050
ST	0.232	0.768	0.076	0.150	0.171	0.436	0.115	0.052
TR	0.151	0.849	0.125	0.083	0.080	0.431	0.203	0.078
YW	0.257	0.743	0.095	0.021	0.220	0.276	0.292	0.096
Sympatric								
AP	0.848	0.152	0.020	0.047	0.662	0.066	0.182	0.023
FT	0.606	0.394	0.103	0.111	0.367	0.285	0.122	0.012
HC	0.533	0.467	0.061	0.044	0.453	0.160	0.197	0.084
JA	0.296	0.704	0.022	0.117	0.161	0.387	0.256	0.057
PO	0.693	0.307	0.023	0.163	0.399	0.185	0.140	0.090
SH	0.801	0.199	0.017	0.591	0.283	0.033	0.069	0.007
SC	0.709	0.291	0.037	0.208	0.311	0.048	0.283	0.113
SD	0.617	0.383	0.024	0.249	0.244	0.104	0.219	0.160

Moreover, the posterior probability of assignment to cluster 1 differed significantly between contrasting competitive environments ($t_{16} = 3.36$, $P = 0.004$; Table 3).

Correlations between gene flow and selective environment
In the microsatellite data set, we found a marginally nonsignificant positive correlation between type of environment comparison and pairwise F_{ST}, which was stronger after we controlled for the effect of geographic distance between populations (without controlling for distance: $r = 0.06$, $P = 0.11$; controlling for distance: $r = 0.11$, $P = 0.07$). This result suggests that there might be a slight reduction in gene flow between sympatry and allopatry. In contrast, for the cyt *b* sequence data, we found no relationship between type of environment comparison and pairwise Φ_{ST} (without controlling for distance: $r = -0.06$, $P = 0.90$; controlling for distance: $r = -0.08$, $P = 0.88$).

Discussion

We sought to evaluate the hypothesis that character displacement can initiate speciation (Hoskin *et al.*, 2005; Pfennig & Ryan, 2006; Pfennig & Rice, 2007; Pfennig & Pfennig, 2009). To do so, we tested for a reduction in gene flow between populations of spadefoot toads, *Spea multiplicata*, that are experiencing divergent selection following character displacement. As we described in the *Study System*, this species has undergone both ecological and reproductive character displacement in areas where it co-occurs with a heterospecific, *S. bombifrons*. Moreover, previous work revealed that character displacement has resulted in post-mating isolation between populations of *S. multiplicata* that are in sympatry with *S. bombifrons* and those that are in allopatry. Thus, this system is a good candidate to evaluate character displacement's role in initiating speciation. We therefore evaluated whether gene flow is reduced between populations of *S. multiplicata* that inhabit divergent selective environments.

As predicted, relative to the level of gene flow between *S. multiplicata* populations in the same selective environment, gene flow between populations in different selective environments was reduced. In particular, our microsatellite data indicated that sympatric and allopatric population groupings explained a significant portion of the variance in genotype frequencies ($F_{CT} = 0.005$, $P = 0.0296$; Table 1). This suggests that the populations within each selective environment were more similar in genotype frequencies than they were to populations in the other environment, which is the expected pattern if gene flow was reduced between environments relative to gene flow within environments. Moreover, because it might be impossible for estimates of F-statistics based on highly variable loci, such as microsatellites, to reach the theoretical maximum value of one (Nagylaki, 1998; Hedrick, 1999), our estimate likely understates the true F_{CT} value. Hedrick (1999) proposed an equation that allows a correction, given the level of variation and number of populations sampled (see equation 2a in Hedrick, 1999). When our F_{CT} value is thereby corrected, allopatric and sympatric groupings account for even more of the variance in genotype frequencies (1.6%).

Likewise, our genetic clustering analysis suggested that populations within the same selective environment were more likely to belong to the same cluster. Although the STRUCTURE results revealed only weak population structure, our microsatellite data were explained better by models incorporating some degree of genetic differentiation ($K > 1$), which is evident from the steadily increasing mean L(K) from $K = 1$ to $K = 6$ (Fig. S1). The most likely number of genetic clusters was six (Figs S1 and S2, Table 2), but these six clusters do not show any clear patterns of assignment to one selective environment vs. the other (Table 3; Fig. 2b), as we would predict if gene flow was reduced between allopatric and sympatric populations. When we set K equal to two, however, we found support for our prediction that populations within the same selective environment should be more likely to belong to the same cluster (Fig. 2a; $\chi^2 = 8.1$, d.f. = 1, $P = 0.004$). The significant difference in the probability of assignment to the first genetic cluster between allopatry and sympatry also supported this prediction (Table 3; $t_{16} = 3.36$, $P = 0.004$).

Furthermore, when controlling for differences in geographic distance, we found a marginally nonsignificant relationship between population pairwise F_{ST} and the type of environment comparison ($r = 0.11$, $P = 0.07$) in our microsatellite data, suggesting that differentiation between populations in different selective environments was slightly higher (and gene flow lower) than between populations in the same selective environment. Although this correlation was marginally nonsignificant, this result is consistent with the predicted trend and in accord with the results from the aforementioned hierarchical population structure analysis. Indeed, the fact that allopatric populations from very different locations (Fig. 1) clustered together in our genetic clustering analysis further supports these arguments (e.g. the allopatric populations YW and PC from southern sites clustered with allopatric populations from the northern sites; Fig. 2). At the same time, the fact that one allopatric population (JC) did not cluster with other, nearby allopatric populations (Fig. 2), suggests that although, on average, divergent selection might be important in reducing gene flow, local nuances might be important as well.

Thus, our microsatellite data suggest that gene flow between contrasting selective environments is slightly reduced, consistent with the possibility that character displacement may initiate speciation. Several limitations in our data set, however, require that some caution be used when drawing conclusions. First, to allow greater sampling of populations within our two environments, which is important for maximizing the power to detect structure between population groups (Fitzpatrick, 2009),

we limited the number of individuals we sampled per population to 10 (Table S2). Small sample sizes per population may decrease the ability to accurately estimate allele frequencies and genetic diversity and provide insufficient power to reject HWE within each population. Such sample sizes are therefore not ideal, but they can still be useful for measuring genetic diversity and genetic distance (Kalinowski, 2005; Pruett & Winker, 2008). A second limitation is that our data do not allow us to rule out possible environmental differences other than the presence or absence of *S. bombifrons*, such as the spatial arrangement or elevational differences between sympatric and allopatric populations, as causes of the observed reduction in gene flow (see also the previous paragraph). However, using the partial Mantel test, we did control for differences in geographical distances between populations in the two environments (see above). Moreover, although other such environmental differences may exist between sympatric and allopatric populations, prior experimental work in this system has repeatedly demonstrated the existence of strong divergent selection and its outcomes between these environments resulting from differences in interactions with *S. bombifrons* (see Study system). These studies, together with earlier data demonstrating ecologically dependent post-mating isolation (Pfennig & Rice, 2007), led us to predict the observed pattern of reduced gene flow between these contrasting environments.

Although the magnitude of the observed reduction in gene flow is not great, several factors could explain why we might not expect to see a large reduction in this, and perhaps most other systems (see also the Introduction). First, as noted in the *Study System*, opportunities for gene flow between populations exist in this system: there are no physical barriers to gene flow between sympatric and allopatric populations; and following heavy rains, *S. multiplicata* can be swept by running water from allopatric to sympatric sites (sympatric sites, which can be within a few kilometers of allopatric sites, generally occur at lower elevations; see Fig. 1). Second, and related to this lack of physical barriers, temporal variation in the direction of selection acting on populations at the sympatry–allopatry boundary might have dampened any signal of reduced gene flow between selective environments. Twenty-five years ago (e.g. about a dozen *Spea* generations), *S. bombifrons* was observed in populations that are now classified as allopatric (e.g. ponds P1, P2, and F8 in Fig. 1; see Simovich, 1985). These populations would potentially have had less time to adapt to the current selective environment than other populations located further from the boundary between selective environments. We cannot evaluate the importance of this temporal variation in selection, but work on other systems has demonstrated that such variation can impact patterns of genetic differentiation (Crispo & Chapman, 2010).

Third, as we described in the Introduction, recent theoretical (Gavrilets & Vose, 2005; Thibert-Plante & Hendry, 2009) and empirical studies (e.g. Emelianov *et al.*, 2004; Turner *et al.*, 2005; Via & West, 2008) suggest that randomly chosen neutral markers, such as those we employed, might often fail to detect signatures of ecological speciation. As populations adapt to divergent environments while some level of gene flow persists, genetic divergence might occur only at loci under selection and those closely linked to them (reviewed in Nosil *et al.*, 2009a). Indeed, future studies may address the possibility that the *Sm23* locus, which showed the strongest signature of reduced gene flow between selective environments (Table 1), is linked to a locus under selection. In general, because there is a low probability that any of our markers were linked to a locus under selection, our measures of reduced gene flow were likely conservative. Nevertheless, if divergent selection is sufficiently strong (as seems to be the case in our system; see Pfennig & Rice, 2007), and if mating preferences act against individuals from the alternative selective environment (as also seems to be the case in our system; Pfennig, 2007), then neutral markers should ultimately reflect a reduction in gene flow between environments (Thibert-Plante & Hendry, 2009). In such situations, local adaptation can decrease gene exchange across the entire genome (Nosil *et al.*, 2009a; Thibert-Plante & Hendry, 2009). Therefore, because a failure to find a reduction in gene flow between divergent environments using neutral markers might still be consistent with ecological speciation, our evidence of a slight reduction in gene flow between sympatric and allopatric populations of *S. multiplicata* – in accord with predictions based on previous work (see Study system) – likely reflects an early stage in the speciation process.

The precise selective agent(s) that favour the observed reduction in gene flow between *S. multiplicata* inhabiting contrasting selective environments is unclear. This reduction might stem from selection acting on larvae to minimize costly resource competition with larval *S. bombifrons* (Pfennig & Murphy, 2000, 2002; Pfennig *et al.*, 2007). Alternatively, decreased gene flow might result from selection acting on adults to lessen costly reproductive interactions with *S. bombifrons* (Pfennig, 2000, 2007, 2008; Pfennig & Pfennig, 2005). Although these two agents of selection are not mutually exclusive, either agent acting alone should, in theory, suffice to favour a reduction in gene flow between populations in different selective environments.

Additionally, we have not identified the proximate causes of the diminished gene flow. Such a diminution could arise from a lack of recruitment of population hybrids into either sympatry or allopatry (population hybrids are poorly adapted to either selective environment; see Study system). Alternatively, reduced gene flow might reflect the mate choice decisions of individuals as they select against mates from the alternative selective environment (Pfennig, 2000, 2007, 2008; Pfennig & Pfennig, 2005). Further studies are needed to determine whether either, or both, proximate

mechanisms explain the attenuation of gene flow between sympatry and allopatry. Generally, ecological and reproductive character displacement may often combine to initiate speciation between populations in sympatry and allopatry (Pfennig & Pfennig, 2009).

Finally, our results suggest that character displacement can lead to reduced gene flow and thereby possibly initiate speciation, but is there any evidence that different species of *Spea* (or any other species) have arisen through this process? In answering this question, it is important to note that we tested for a reduction in gene flow within a *single* metapopulation. Varying degrees of progress toward, or completeness of, ecological speciation are likely present in different metapopulations (e.g. see Berner *et al.*, 2009; Hendry, 2009; Nosil *et al.*, 2009b). Generally, speciation should be the most complete (i.e. gene flow most reduced) between populations that have experienced divergent selection for a long period of time, and this might explain why we detected only a slight reduction in gene flow in our study. Population genetic surveys (Rice & Pfennig, 2008) indicate that *S. bombifrons* has undergone a widespread range expansion out of its ancestral range in the southern Great Plains, and that this species is a recent invader into our study area (Fig. 1), where *S. multiplicata* was already resident (in contrast to *S. bombifrons*, *S. multiplicata*'s range appears to have been stable; Rice & Pfennig, 2008). Indeed, *S. bombifrons* has recently increased in frequency in our study area (Pfennig, 2003), suggesting that the *S. multiplicata* in our sympatric populations may have only recently experienced significant selective pressures from *S. bombifrons*. Recent contact might therefore explain why the reduction in gene flow between sympatric and allopatric populations in our study area is not great. By contrast, we might expect to observe a much greater diminution in gene flow between sympatric and allopatric populations in the southern Great Plains, where *S. multiplicata* has likely experienced selection from *S. bombifrons* for a prolonged period of evolutionary time. Such comparative studies of different populations that vary in the length of time that they have experienced divergent selection are ideal for testing the hypothesis that character displacement initiates speciation.

In sum, our results demonstrate how reproductive isolation, and potentially ecological speciation, might evolve as an indirect by-product of selection acting to reduce costly interspecific interactions. Future work with additional species will be necessary to establish how frequently character displacement initiates speciation and to clarify how ecological character displacement and reproductive character displacement interact to start speciation.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Appendix S1 Molecular diversity calculation methods.

Table S1 Pond geographic locations and cytochrome *b* sample sizes and variation summaries.

Table S2 Summary of genetic variation at each microsatellite locus for each pond site.

Table S3 Significance levels (*P*-values) from tests for departure from Hardy-Weinberg Equilibrium and the presence of null alleles.

Figure S1 Mean and standard deviations of the posterior probabilities of the data for 20 runs of STRUCTURE at values of K ranging from 1 to 18.

Figure S2 The ΔK distribution for our STRUCTURE runs, calculated following the method of Evanno *et al.* (2005).

Figure S3 STRUCTURE membership assignments to genetic clusters for $K = 2$ through $K = 6$.

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