

DIVERSIFICATION OF CONSPECIFIC SIGNALS IN SYMPATRY: GEOGRAPHIC OVERLAP DRIVES MULTIDIMENSIONAL REPRODUCTIVE CHARACTER DISPLACEMENT IN FROGS

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Theoretical models suggest that geographic overlap with different heterospecific assemblages can promote divergence of mate recognition systems among conspecific populations. Divergence occurs when different traits undergo reproductive character displacement across populations within a contact zone. Here, I tested this hypothesis by assessing patterns of acoustic signal divergence in two- and three-species assemblages of chorus frogs (*Pseudacris*), focusing in particular on *P. feriarum* and *P. nigrita*. In addition, I tested one criterion for reinforcement, by examining the evolution of female *P. feriarum* preferences in the contact zone. Patterns of signal evolution indicated that in each of the four sympatric populations studied, only the rarer species displaced substantially (*P. feriarum* in three cases and *P. nigrita* in one instance). Moreover, the three displaced *P. feriarum* populations diverged in different signal traits across the contact zone, evolving in directions that increased the energetic cost of calling relative to the allopatric call, and in ways that maximized differences from the particular heterospecific assemblage present. Consistent with reinforcement, divergence of female preferences in sympatry was estimated to reduce their propensity to hybridize by 60%. Together, signal and preference data suggest that interactions between species can promote diversification within species, potentially contributing to reproductive isolation among conspecific populations.

KEY WORDS: Acoustic signal, contact zone, female preference, multidimensional divergence, *Pseudacris*, reproductive character displacement, reinforcement.

When two or more species come into geographic contact, selection may favor divergence of their reproductive communication systems in sympatry to maintain species integrity and mating efficiency (Howard 1993; Noor 1999). Interference of reproductive signals by other species can decrease the effectiveness of signal propagation and hinder the ability of a receiver to decode information (Gerhardt and Huber 2002). Individuals may waste time,

energy, and gametes attracting, approaching, or mating with heterospecifics. As a result of this interference, species may evolve greater differences in signals and preferences for these signals in sympatry relative to allopatry. This pattern, known as reproductive character displacement (RCD; Brown and Wilson 1956; Howard 1993), may result from at least two processes including selection against hybridization (reinforcement [“broad sense”; Servedio and Noor 2003], Dobzhansky 1937, 1940; e.g., Noor 1995; Sætre et al. 1997; Rundle and Schluter 1998; Higgie et al. 2000; Pfennig 2003) or selection against signal interference by

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neighbors (facilitated RCD, Howard 1993; noisy neighbors hypothesis, Noor 1999; e.g., Amézquita et al. 2006; Cooley et al. 2006; Mullen and Andrés 2007).

Recent theoretical and neural network models (McPeck and Gavrillets 2006; Pfennig and Ryan 2006, 2007) have shown that geographic overlap with other taxa can not only promote divergence of communication systems between species (Gerhardt 1994; Noor 1995; Sætre et al. 1997; Higgin et al. 2000), and between allopatric and sympatric populations of a single species (Littlejohn 1965; Littlejohn and Loftus-Hills 1968; Zouros and d'Entremont 1980; Hoskin et al. 2005), but it can even lead to diversification of signals and preferences among conspecific populations within the zone of overlap. This process can occur when reinforcement leads to reproductive character displacement along different signal axes, potentially as a consequence of differing local selection pressures across populations (Howard 1993). To date, however, no empirical studies have tested this prediction. Studies of RCD generally focus on one or a few sympatric populations within a geographically continuous region where migration can occur among populations (Fouquette 1975; Waage 1975, 1979; Loftus-Hills and Littlejohn 1992). In this situation, gene flow among conspecific populations within the contact zone may overwhelm divergent selection on signals and preferences in different abiotic (Rundle et al. 2005; Seehausen et al. 2008) or biotic environments (McPeck and Gavrillets 2006; Pfennig and Ryan 2006, 2007). The one well-supported study (Hoskin et al. 2005) that examined RCD across discontinuous geographic space found evidence that sympatric populations have become reproductively isolated (divergent signals and preferences) from allopatric conspecific populations. Their work suggests that RCD, when combined with a reduction of gene flow among conspecific populations, can potentially initiate a cascade of speciation events (Howard 1993).

In this study, I test the hypothesis that geographic overlap can generate divergent evolution of signals among conspecific populations in sympatry by assessing patterns of acoustic signal divergence in a contact zone between the chorus frog species *Pseudacris feriarum* and *P. nigrita*. Throughout this paper, "sympatry" refers to areas of overlap between these two species, unless otherwise specified. *P. feriarum* and *P. nigrita* hybridize occasionally in nature and form viable and partially fertile hybrids in the lab (Mecham 1965; E. Moriarty Lemmon, unpubl. data). The taxa come into contact along the boundary between the Piedmont and the Coastal Plain of the southeastern United States, extending from western Alabama to eastern Virginia. Additionally, ranges of these taxa overlap with a third closely related species, *P. brimleyi*, in the northern half of their contact zone (Fig. 1). Chorus frogs occupy a distinctive niche among North American frog fauna in terms of breeding time because they congregate to mate in late winter and early spring when few or no other taxa are present in the breeding ponds. Thus, mating interactions of *Pseudacris*

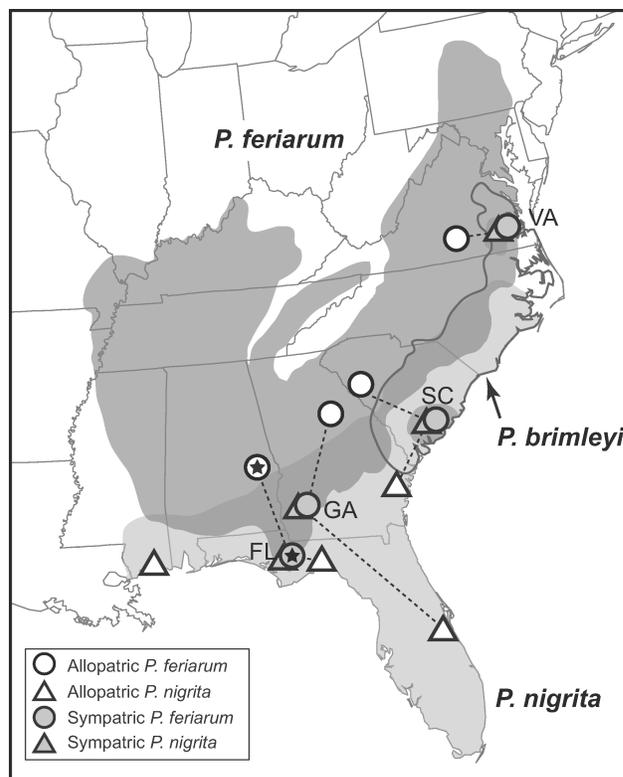


Figure 1. Distributions of *Pseudacris feriarum* and *P. nigrita* in the southeastern United States and populations sampled in this study. Call transects are indicated by dashed lines and state abbreviations. The distribution of *P. brimleyi* is delineated by a solid gray line. Female preference experiments were conducted in the two populations marked with a black star.

exist almost exclusively with congeneric taxa. Phylogenetic and phylogeographic studies indicate that *P. feriarum* and *P. nigrita* diverged during the Miocene approximately eight million years ago, and thus the current contact zone between the two species likely represents an instance of secondary contact. Moreover, the lineage leading to these species diverged from the ancestor of *P. brimleyi* approximately 11.5 million years ago (Moriarty and Cannatella 2004; Lemmon et al. 2007a,b). A previous study of signal structure in this contact zone (a transect of nine allopatric and five sympatric populations total) demonstrated a strong pattern of RCD in populations of *P. feriarum* sympatric with *P. nigrita* in southern Alabama/Georgia and western Florida (Fouquette 1975). The former species has diverged with respect to two of four signal characters examined.

Here, I extend the work of Fouquette (1975) by examining 14 call characters of sympatric and allopatric populations of *P. feriarum* and *P. nigrita* across the entire contact zone, including areas where *P. brimleyi* is present. In particular, I address three questions: (1) Is there heterogeneity in the amount of RCD among sympatric localities? (2) Does RCD exist in both species? (3) Have the same signal traits diverged across the contact zone?

Given the theoretical results of McPeck and Gavrillets (2006) and Pfennig and Ryan (2006, 2007), I predict that calls of the focal species have evolved along signal axes that maximize the acoustic distance from the particular heterospecific assemblage present in the population. One process that may have caused male signal displacement is divergence of the female preference as a result of selection against maladaptive hybridization. Thus, here I also test one criterion for reinforcement (Howard 1993) by examining evolution of female *P. feriarum* preferences at one locality in the sympatric region studied by Fouquette (1975). I address the following questions with respect to female choice: (1) Do females prefer conspecifics? (2) Have female preferences diverged in sympatry? (3) Has the propensity to hybridize been reduced in sympatry? (4) Is the displacement in male signal perceptible to females? Given the pattern of male signal displacement uncovered by Fouquette (1975), I predict that divergence of the female preference has occurred.

Materials and Methods

MALE SIGNAL ANALYSES

Sampling

To examine geographic variation in acoustic signals, male *P. feriarum* and *P. nigrita* were recorded and collected from eight

populations in allopatry (four per species) and eight populations from sympatry (four per species) in the southeastern United States (Fig. 1). These populations corresponded to four rough transects spanning the contact zone; each transect included an allopatric *P. feriarum* population, an allopatric *P. nigrita* population, and sympatric *P. feriarum* and *P. nigrita* populations from the same locality. The transects spanned the following geographic regions: (1) Florida/Alabama (transect studied by Fouquette [1975]), (2) Georgia/Florida, (3) South Carolina/Georgia, and (4) Virginia (Fig. 1). Hereafter, the first state listed will be used as the transect name (i.e., FL, GA, SC, and VA, respectively). Because *P. nigrita* do not exist in allopatry in Virginia and because loss of habitat in eastern North Carolina prevented the discovery of allopatric *P. nigrita* at historic localities, this population was lacking from the Virginia transect. Instead, to increase the number of allopatric *P. nigrita* populations, one was included from southern Mississippi. None of the statistical analyses described below depend on the geographic layout of the transects, which are presented here for visual purposes only. Representative calls of both species are presented in Figure 2.

To ensure that the focal species were monotypic, I previously conducted detailed phylogeographic studies on these taxa (Lemmon et al. 2007a,b). This work revealed that populations of putative *P. feriarum* in Louisiana and Mississippi (referenced in

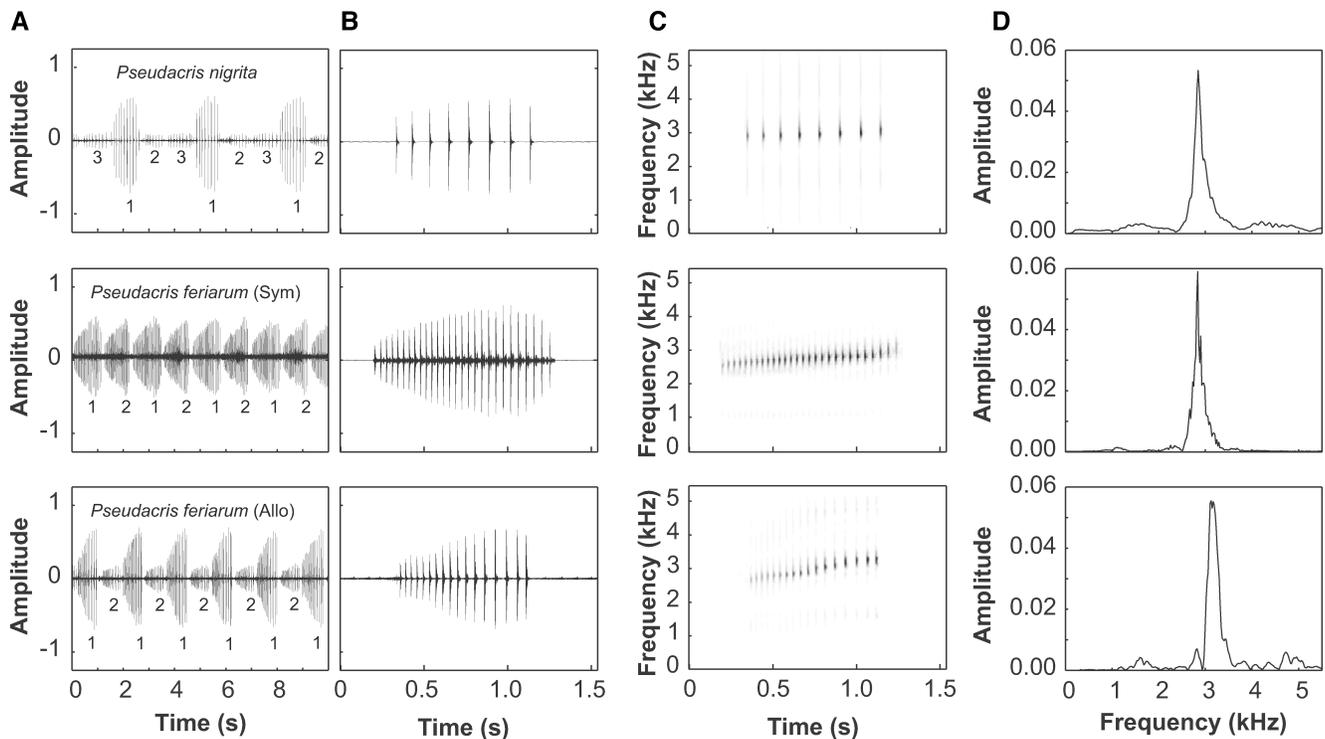


Figure 2. Male acoustic signal structure, represented by oscillograms (column A—10 sec sequence, column B—1.5 sec sequence), spectrograms (column C), and power spectra (column D) for allopatric *P. nigrita*, sympatric *P. feriarum*, and allopatric *P. feriarum* (by row). Oscillograms in column A show multiple individuals calling in sequence; the different individuals are indicated by a number below each call. Calls were recorded between 12.4 and 13.8°C, therefore, temporal differences are not due to temperature variation.

Gerhardt and Huber 2002) were actually an undescribed, cryptic species (*P. fouquettei*, Lemmon et al. 2008) that hybridizes with *P. nigrita* in a narrow contact zone (Gartside 1980). Therefore this region was not included in the present study. Moreover, the Mississippi *P. nigrita* samples that I included here were from well outside of the *P. fouquettei* contact zone.

Areas of sympatry between the two species were located based on published studies, museum databases, personal communications, and field surveys. In the contact zone, *P. feriarum* and *P. nigrita* can be found calling in close proximity, often alternating calls with each other (Crenshaw and Blair 1959; E. Moriarty Lemmon, unpubl. data). The two species show some ecological separation in sympatry, which is more pronounced in the southern part of their range: *P. feriarum* prefers bottomland hardwood swamps (dominated by cypress and gum trees), whereas *P. nigrita* prefers pine flatwoods wetlands (Carr 1940; Crenshaw and Blair 1959). Interaction between the species most often occurs at the interface of these habitats or in artificial habitats, such as roadside ditches. At several of these sites (in FL and VA), putative hybrids have been found that are morphologically and acoustically intermediate between the parental species (Fig. 3; E. Moriarty Lemmon, unpubl. data). The hybrids were not included in the analyses below. Where possible, individuals calling syntopically were sampled; to obtain a large enough sample, however, frogs were collected from some ponds that were dominated by one of the species.

A total of 318 individuals were recorded from the 16 populations. Each population included five to 43 individuals (mean 18 frogs). Between two and 17 calls were sampled per individual (mean 10 calls), depending on the quality of the recording and activity level of the frog (Table S1). In addition, recordings of 17 *P. brimleyi* were obtained from two localities in North Carolina. Calls were recorded onto TDK MA90 metal bias tape cassettes with a Sony stereo cassette-recorder (WM-D6C) using a Sennheiser ME67 directional microphone. The microphone was held approximately one meter from the calling individual during recording. When possible, the frog was then captured. In all cases, the temperature of the frog's calling location (aquatic or terrestrial) was measured. Tissue samples were taken from euthanized frogs (following IACUC protocol 06022701) and voucher specimens were deposited into the Texas Memorial Museum (Austin, TX).

Acoustic analyses

Recordings were digitized using SoundEdit16 version 2 (Macromedia) with a sample size of 16 bits at a sampling rate of 44,100 Hz. Calls were analyzed using SoundRuler version 0.941 (<http://soundruler.sourceforge.net/>; reviewed by Bee 2004). Frequency measurements were taken from spectrograms generated with fast fourier transform (FFT) length of 1024 and 900 samples

of overlap among subsequent FFTs. The values of call variables were taken directly from or calculated from SoundRuler's raw data output. A total of 3046 calls were measured for within-call data and 2751 for across-call data (Table S1). For details of the acoustic analyses, see Supporting Information.

A total of 14 call variables were examined to explore patterns of evolution in acoustic signals. These variables were chosen for two reasons: (1) they showed high interspecific and low intrapopulation variation and/or (2) they are known to be important for species recognition in other frogs (Loftus-Hills and Littlejohn 1971; Klump and Gerhardt 1987; Gerhardt 1991; Gerhardt 1994; Gerhardt 1996; Murphy and Gerhardt 2000; Gerhardt and Huber 2002). The call variables, described in Table S2, include both spectral characters and temporal characters.

Several components of the frog's acoustic signal are influenced by changes in temperature (Gerhardt and Huber 2002). To control for this effect, I tested for correlations between temperature and each call variable. If the effect of temperature was strong ($P < 0.01$), I adjusted the variable to a common temperature of 14°C, using species-specific slopes. Regression slopes used in the corrections are shown in Table S3.

Statistical analyses

Randomization tests were performed to address two questions: (1) Is there variation in the amount of divergence between sympatric *P. feriarum* and *P. nigrita* among localities? and (2) In each sympatric locality, does reproductive character displacement exist in *P. feriarum* and/or *P. nigrita*? Normality of the 14 signal variables was first assessed using a Shapiro–Wilk's test in R version 1.16 (R Foundation for Statistical Computing), then a principal components analysis (PCA) was conducted for all individuals using JMP 5.1 (SAS Institute Inc., Cary, NC). To address the first question, the standard deviation in level of divergence between sympatric populations was calculated and compared to a null distribution. For details of randomization test 1, see Supporting Information.

To address the second question, the difference between the allopatric calls of the two species was compared to the difference between the allopatric call of one species and the sympatric call of the other species. This measure quantified how much the signal of a species has changed (displaced) since secondary contact relative to the precontact state. Allopatric individuals were pooled by species for this test because I wished to avoid relying on transect identity. To assess the robustness of the results, however, I also performed these analyses by transect (without pooling allopatric populations). The test was performed for each of the four sympatric populations, for each of the two species, and along the first three PC axes for a total of 24 tests. An example of one of these tests is given in the Supporting Information under randomization test 2. A table-wide sequential Bonferroni correction was applied

to correct for multiple tests here and in subsequent tests (Rice 1989).

A discriminant function analysis (DFA) of the 14 call variables was performed to test the null hypothesis that the sympatric populations diverged with respect to the same components of the acoustic signal. The analysis was performed on the eight *P. fe-*

riarum populations using JMP 5.1, in which call variables were stepped into the model until the next variable had a *P*-value >0.05. Seven call variables were thus included in the analysis (pulse rate, pulse number, pulse duration, call rise time, call fall time, call duration, and dominant frequency peak). Scores on the first two canonical axes (CVs) were saved for further analysis.

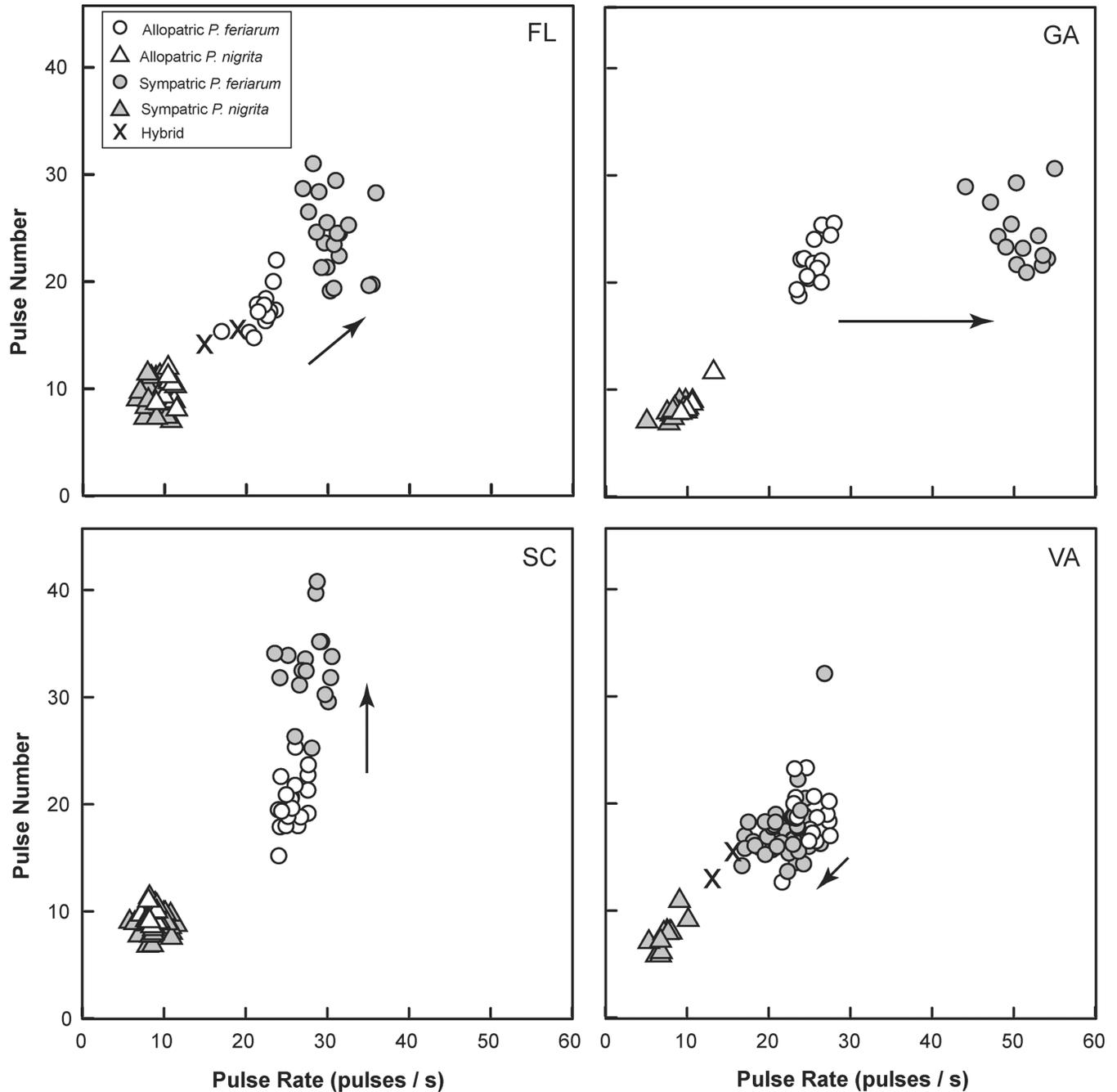


Figure 3. Signal divergence in the four call transects: Florida (FL), Georgia (GA), South Carolina (SC), and Virginia (VA). Each symbol represents an individual male. Putative hybrids collected in sympatry are denoted with a black "X." Due to habitat destruction, an allopatric *P. nigrita* population from the Virginia transect could not be located, so an allopatric Mississippi population (not shown) was substituted in the statistical analyses. Black arrows show the general direction of signal divergence in sympatric *P. feriarum* relative to the nearest allopatric population. Note that evolution occurred in different directions in the four populations.

To determine which sympatric populations differ significantly from each other, Tukey-type randomization tests were conducted. The number of tests was reduced by ranking populations by mean canonical score and comparing only rank neighbors. For details of this test (randomization test 3), see Supporting Information. To quantify how important a call variable was in discriminating among groups (as the standardized coefficients from the discriminant analysis), the canonical vector coefficients were multiplied by the pooled standard deviation within groups for each variable (equivalent to root mean square error). These coefficients were calculated for CV1 and CV2.

FEMALE PREFERENCE TESTS

To determine whether female preference evolution is driving male signal divergence, phonotaxis experiments were conducted on *P. feriarum* females from one allopatric and one sympatric population (Macon/Lee Cos., Alabama, and Liberty Co., FL, respectively; Table S4; Fig. 1). Future studies will be conducted to examine geographic variation in preferences throughout the range of *P. feriarum*. Following the general methodology of Ryan and Rand (1999), I performed three binary mate-choice experiments on *P. feriarum* females. The females were given a choice between two acoustic stimuli in each experiment as follows: (A) sympatric *P. feriarum* versus *P. nigrita*, (B) allopatric *P. feriarum* versus *P. nigrita*, and (C) sympatric *P. feriarum* versus allopatric *P. feriarum*. Sympatric *P. nigrita* calls were used because preliminary analyses indicated that sympatric calls did not differ from allopatric calls in Florida. Female preferences were not tested in *P. nigrita* in this study for the same reason. Tests were presented in random order for each female. Natural calls from these populations are shown in Figure 2.

Construction of acoustic stimuli

The three synthetic acoustic stimuli were constructed based on natural male calls from the local populations of females. Individuals used for constructing the stimuli include: (1) allopatric *P. feriarum*, 101 calls from 13 individuals, mode number pulses = 17; (2) sympatric *P. feriarum*, 178 calls from 18 individuals, mode number pulses = 29; (3) *P. nigrita*, 205 calls from 23 individuals, mode number pulses = 10 (Table S1). For details of how stimuli were constructed, see Supporting Information.

Experimental conditions

Experiments involving allopatric females were conducted at Auburn University (Auburn, AL), and experiments on sympatric females were performed at the Florida Department of Environmental Protection research station (Eastpoint, FL) in 2004 and 2005. Amplexed or gravid single females were collected from breeding ponds and tested within 48 h, although the majority of females were tested on the night of capture. For details of the phonotaxis experiment procedure, see Supporting Information.

Statistical analyses

Four questions were addressed; (1) Do females prefer conspecific males? (2) Has the female preference diverged in sympatry? (3) Has the propensity to hybridize been reduced? (4) Is the displacement in male calls perceptible to the females? This last question was derived from a criterion of Howard (1993) for demonstrating that reproductive character displacement is due to reinforcement.

To answer the first question, one-tailed exact binomial tests were conducted on the results of experiments A and B under the null hypothesis of no preference (proportion = 0.5). One-tailed tests were employed because the a priori expectation was that females would choose the conspecific stimulus. To answer the second question, Fisher's exact test was used to compare the proportion of allopatric females and sympatric females that chose the sympatric *P. feriarum* stimulus in test A. This test was also performed for experiment B. To address the third question, Fisher's exact test was performed to compare the proportion of sympatric females that chose the conspecific stimulus in experiment A to the proportion of allopatric females that chose the conspecific signal in experiment B. To address the last question, a two-tailed exact binomial test was conducted on the results of experiment C. A sequential Bonferroni correction was applied to correct for multiple (9) tests (Rice 1989).

Results

MALE SIGNAL VARIATION

The goal of the first randomization test was to assess whether the level of RCD was consistent across the contact zone. This test showed significant heterogeneity in the magnitude of displacement among sympatric localities of *P. feriarum* and *P. nigrita* along the first three principal component axes (PC1, $P < 0.00001$; PC2, $P = 0.00007$; PC3, $P = 0.00021$). PC loadings are given in Table 1. The goal of the second randomization test was to identify which species had displaced in each of the four populations. This test indicated that *P. feriarum* displaced in three populations and *P. nigrita* displaced in one population. Substantial RCD has occurred along PC1 in sympatric *P. feriarum* from Florida, Georgia, and South Carolina, but not Virginia. Additionally, some divergence has occurred along PC3 in Georgia and Virginia. Conversely, in the only sympatric population (VA) in which *P. feriarum* showed little (PC3) to no (PC1, PC2) divergence in call variables, *P. nigrita* instead exhibited strong character displacement (PC1; Table 2). Because a randomization test comparing variation among allopatric populations of each species was significant (Table S5), randomization test 2 was also conducted by transect, without pooling allopatric populations. The results were qualitatively similar, showing strong divergence of *P. feriarum* in Florida, Georgia, and South Carolina, and divergence of *P. nigrita* in Virginia (Table S6).

Table 1. Loadings for the first five principal components from the multivariate analysis of 14 call variables.

Call variables	I	II	III	IV	V
Call duration	-0.119	0.346	0.527	-0.173	0.210
Call duty cycle	0.350	0.288	0.111	-0.096	0.225
Call fall time	-0.239	-0.148	0.161	-0.067	0.697
Call rate	0.402	-0.002	-0.253	0.063	0.077
Call rise time	0.074	0.458	0.387	-0.140	-0.306
Dominant frequency peak	-0.177	0.269	-0.197	0.282	0.056
Pulse duration	0.217	-0.383	0.405	0.052	-0.111
Pulse duty cycle	0.435	-0.042	-0.071	0.042	0.111
Pulse fall time	0.217	-0.330	0.323	-0.119	-0.056
Pulse number	0.369	0.225	0.030	-0.126	0.184
Pulse rate	0.419	0.065	-0.175	0.037	0.152
Pulse rise time	0.105	-0.302	0.297	0.355	-0.147
Pulse shape offset	0.048	0.294	0.100	0.513	-0.269
Pulse shape onset	-0.003	0.047	0.171	0.655	0.372
Eigenvalue	4.994	2.456	1.891	1.267	1.024
Percent of variation	35.672%	17.541%	13.508%	9.049%	7.311%
Cumulative percent	35.672%	53.213%	66.722%	75.771%	83.081%

The four sympatric *P. feriarum* populations separated along both the first and second canonical axes (which together explain 93% of the variation), as shown by the discriminant analysis (Table 3). The standardized coefficients indicate that pulse rate contributed substantially to the first axis (compared to other call variables), but contributed little to the second axis. In contrast, pulse number loaded heavily on the second axis compared to other call variables, but contributed little to the first (Table 4).

The sympatric populations have diverged along different axes of the acoustic signal, as indicated by the multiple compari-

son randomization test (Fig. 3). Along the pulse rate-dominated axis (CV1) all four populations differed significantly from each other, in descending order Georgia > Florida > South Carolina > Virginia (Table 5). In contrast, along the pulse number-dominated axis (CV2), three of the four populations differed significantly, in descending order South Carolina > Florida > Virginia > Georgia (Table 5). Raw temperature-corrected pulse rate and pulse number data are presented in Table S7.

Table 2. Results of randomization test for detecting reproductive character displacement in different sympatric populations (allopatric populations pooled by species). Populations that were significant after sequential Bonferroni correction are denoted with an asterisk.

Axis	Population	<i>P. feriarum</i> P-value	<i>P. nigrita</i> P-value
PC1	Florida	<0.00001*	0.01456
PC1	Georgia	<0.00001*	0.00608
PC1	South Carolina	0.00004*	0.62421
PC1	Virginia	1	<0.00001*
PC2	Florida	0.85151	0.99837
PC2	Georgia	0.94572	0.99950
PC2	South Carolina	0.00390	0.93119
PC2	Virginia	0.99818	0.97145
PC3	Florida	0.54770	0.20655
PC3	Georgia	0.00052*	0.89440
PC3	South Carolina	0.01160	0.48610
PC3	Virginia	0.00036*	0.77278

FEMALE PREFERENCE TESTS

The female preference has evolved in sympatry, and this divergence has led to reduced likelihood of hybridization by *P. feriarum*

Table 3. Loadings for the first three canonical variates axes. These are unstandardized eigenvectors. Note that variables were stepped into the model until the next variable had a P-value > 0.05, resulting in the inclusion of seven total variables in the analysis.

Call variable	I	II	III
Call duration	1.501	-2.807	-6.741
Call fall time	0.808	0.818	4.174
Call rise time	1.632	2.754	8.218
Dominant frequency peak	0.001	-0.003	0.004
Pulse duration	82.729	-143.586	-364.454
Pulse number	-0.096	0.343	-0.045
Pulse rate	0.525	-0.103	0.011
Eigenvalue	16.091	2.900	0.881
Percent of variation	78.899%	14.220%	4.322%
Cumulative percent	78.899%	93.119%	97.441%
Canonical correlation	0.970	0.862	0.684

Table 4. Standardized coefficients for three canonical axes from the discriminant analysis. Variables that are more important for distinguishing groups have higher values (positive or negative, in bold). The percent of variation explained by each axis is listed below the call variables.

Call variable	St. Coeff. 1	St. Coeff. 2	St. Coeff. 3
Call duration	0.257	-0.481	-1.155
Call fall time	0.119	0.120	0.613
Call rise time	0.304	0.513	1.532
Dominant frequency peak	0.136	-0.468	0.619
Pulse duration	0.107	-0.185	-0.470
Pulse number	-0.279	0.997	-0.129
Pulse rate	1.136	-0.223	0.023
Percent of variation	78.899%	14.220%	4.322%

females (Fig. 4). In experiments A and B, female *P. feriarum* were given a choice between a heterospecific and a conspecific male signal. These experiments indicated that allopatric and sympatric *P. feriarum* females preferred conspecific signals to heterospecific signals (experiment A, allopatric females, $P = 0.005$; sympatric females, $P < 0.00001$; experiment B, allopatric females, $P = 0.024$; sympatric females, $P < 0.00001$). Sympatric females chose the conspecific stimulus significantly more often than allopatric females (experiment A, proportion 0.87 vs. 0.67, $P = 0.015$; experiment B, proportion 0.88 vs. 0.63, $P = 0.004$). The propensity of females to hybridize has been substantially reduced from 37% in allopatric females (experiment B) to 13% in sympatric females (experiment A; $P = 0.004$). In experiment C, female *P. feriarum* were given a choice between an allopatric and a sympatric conspecific male signal. This test indicated that displacement of the signal in sympatry was perceptible to females: sympatric females had a strong preference for the sympatric signal ($P < 0.00001$), whereas allopatric females exhibited a weak preference for the

Table 5. Pairwise comparisons of populations along canonical axes 1 and 2 with randomization tests. For each canonical axis, populations were compared in rank order from low to high mean canonical scores. Populations that were significant after sequential Bonferroni correction are denoted with an asterisk.

Axis	Comparison	P-value
CV1	Virginia versus South Carolina	<0.00001*
CV1	South Carolina versus Florida	0.00307*
CV1	Florida versus Georgia	<0.00001*
CV2	Georgia versus Virginia	0.19632
CV2	Virginia versus Florida	<0.00001*
CV2	Florida versus South Carolina	<0.00001*

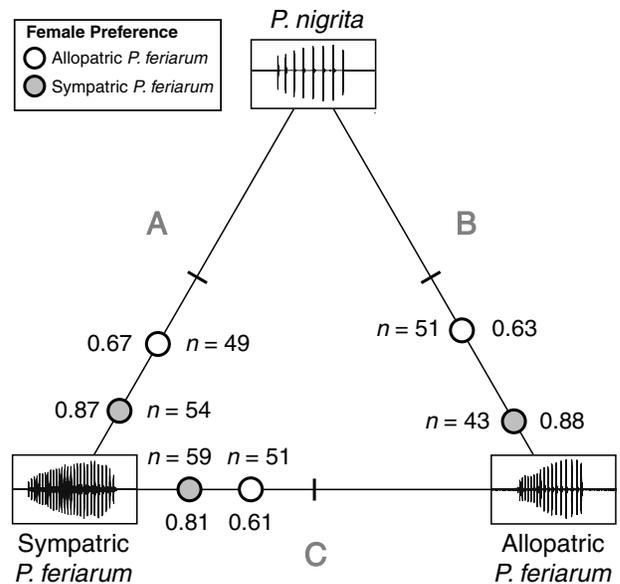


Figure 4. Summary of female preference results from the three experiments (A, B, and C). An oscillogram of a natural call from the respective populations is shown in the box at each vertex of the triangle. The proportion of females that chose the more popular stimulus is shown outside the triangle and the sample size is indicated inside the triangle next to each dot. Black tick marks indicate the expectation under no preference. Note that the propensity of females to hybridize has been reduced in sympatry. Also note that sympatric females have the same preference in tests A and B, regardless of the conspecific call presented.

sympatric signal ($P = 0.046$, not significant after sequential Bonferroni correction; Fig. 4). Of all females tested in this study, 75% of sympatric (64 of 85) and 65% of allopatric (54 of 83) females responded in at least one experiment. Approximately equal proportions of allopatric females responded in the three experiments (A: 59%, B: 61%, and C: 61%). In contrast, a smaller proportion of sympatric females responded in test B (A: 64%, B: 51%, and C: 69%), in which the only conspecific option was the nondisplaced allopatric signal.

Discussion

I have shown that interactions between species can drive diversification of reproductive signals within species. In addition, I have shown evidence that female preference evolution in sympatry has driven this divergence, as suggested by experiments from one site. As an indirect effect of the evolution of increased between-species discrimination, females have also evolved increased within-species discrimination. This divergence of the female preference has set the stage for intensified sexual selection on males for more energetically costly signals (discussed below). Male signal data are consistent with the idea that geographic variation in the local heterospecific assemblage may have influenced

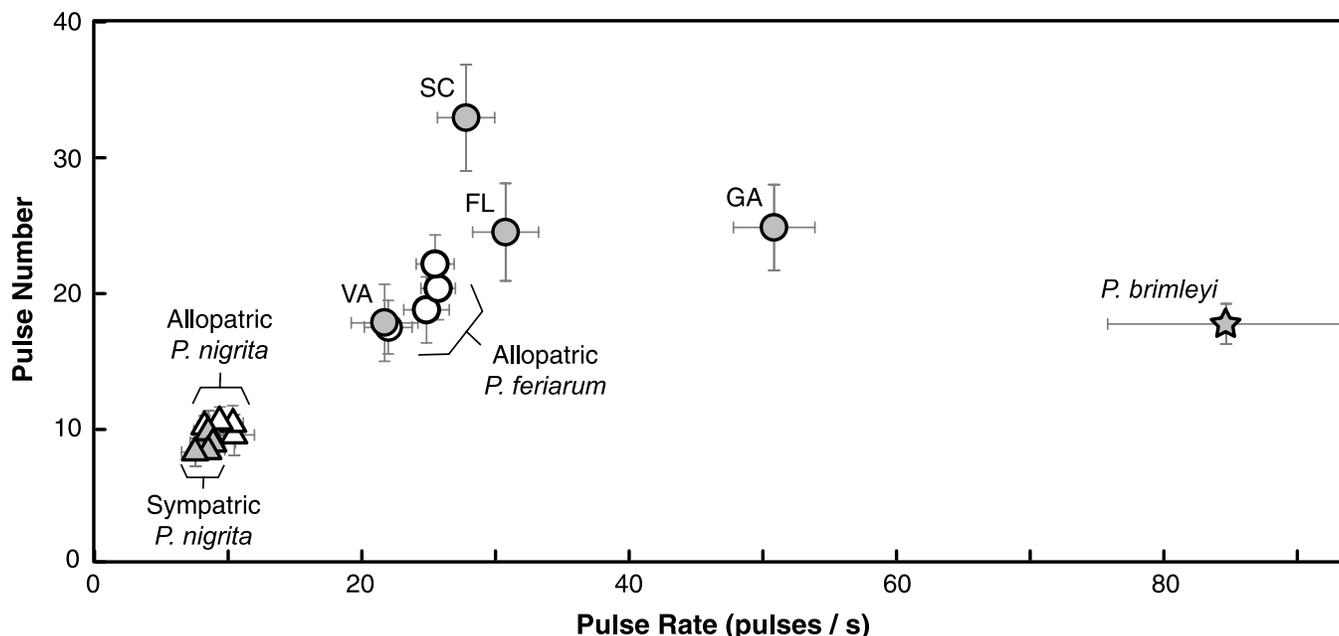


Figure 5. Acoustic signal variation in *P. feriarum*, *P. nigrita*, and *P. brimleyi* populations with respect to pulse rate and pulse number. Means (symbols) and standard deviations (error bars) are shown. *P. feriarum* is represented by circles, *P. nigrita* by triangles, and *P. brimleyi* by a star. Gray symbols indicate sympatric populations and white symbols indicate allopatric populations. Note that the amount of variation among sympatric *P. feriarum* populations is substantially greater than variation among allopatric populations, although geographic distances among populations within each group are similar. In the two populations (FL and GA) in which *P. feriarum* is sympatric with a single heterospecific (*P. nigrita*), the species displaced primarily in pulse rate, and to a lesser degree in pulse number. In the single displaced population (SC) in which *P. feriarum* overlaps with two heterospecifics (*P. nigrita* and *P. brimleyi*), the species displaced only in pulse number. Note that the ancestral (allopatric) call of *P. feriarum* was intermediate in pulse rate (x-axis) but not pulse number (y-axis; greater than *P. nigrita*, but equal to *P. brimleyi*) with respect to the two heterospecifics. This initial state presumably led to *P. feriarum* displacing only in pulse number where it occurs with the other two taxa to avoid signal interference and potentially hybridization.

behavioral evolution. Further research is required, however, to rigorously test this hypothesis.

WHY MULTIDIMENSIONAL SIGNAL DIVERGENCE IN SYMPATRY?

The pattern of signal diversification among populations of sympatric *P. feriarum* could potentially result from stochastic genetic processes or from differing local selection pressures across the distribution of this species. For example, local selection pressures may be exerted by the abiotic environment, leading to sensory drive (Boughman 2001, 2002; Seehausen et al. 2008), or by the biotic community, resulting in character displacement (Brown and Wilson 1956; Howard 1993). Alternatively, behavioral diversification may derive from variation in the intensity and direction of sexual selection across populations (Boul et al. 2007). Although the proposed hypotheses need to be tested thoroughly, some evidence supports the role of variable community interactions in behavioral diversification of chorus frogs. The directions and axes of signal evolution in sympatric *P. feriarum* are generally consistent with predictions from theoretical and neural network models of one- and two-taxon heterospecific interactions (McPeck and

Gavrilets 2006; Pfennig and Ryan 2006, 2007). Although the number of populations examined in the present study is admittedly small, in the two divergent populations in which a single heterospecific is present (*P. nigrita*; GA and FL), *P. feriarum* signals displaced away from the heterospecific, either primarily along one axis (pulse rate) or to a lesser degree along multiple axes (pulse rate and pulse number; Fig. 5). In the single divergent population in which two heterospecifics are present (*P. brimleyi* and *P. nigrita*; SC), *P. feriarum* signals diverged along an axis (pulse number) that increased the distance from both heterospecifics. In South Carolina, evolution along the pulse rate axis in either direction would have led to increased interference with the other two species in the community (Fig. 5). This particular pattern of trait divergence is predicted by the model of McPeck and Gavrilets (2006). Reproductive interaction of *P. brimleyi* with *P. feriarum* is likely because breeding occurs syntopically, mating call differences are similar to other hybridizing *Pseudacris* species pairs (E. Moriarty Lemmon, unpubl. data), and postzygotic isolation is incomplete (Mecham 1965).

A limitation of the models of McPeck and Gavrilets (2006) and Pfennig and Ryan (2006, 2007) is that they make the implicit

assumption that the displaced sympatric signal or preference has an equal direct cost to the individual as the nondisplaced allopatric state. This assumption is not valid for chorus frogs. The two call variables found to be most divergent among sympatric *P. feriarum* populations, pulse rate and pulse number, were positively correlated with two other call variables measured here, call rate ($r^2 = 0.744$, $P < 0.0001$) and call duration ($r^2 = 0.573$, $P < 0.0001$), respectively. Studies of frog metabolic rates during signaling indicate that increasing the duration of the call without a proportional decrease in the rate of calling and vice versa results in greater expenditure of energy (Wells 2007, pp. 202–220, and references therein). In all three populations in which RCD has occurred, sympatric *P. feriarum* populations have increased calling effort during signaling compared to the pooled allopatric *P. feriarum* sample (measured in terms of call duty cycle; same procedure as in randomization test 2 described above, Florida: $P = 0.00576$, Georgia: $P < 0.00001$, South Carolina: $P < 0.00001$, Virginia: $P = 0.05113$, two-tailed test; Fig. 6). In contrast, all four sympatric *P. nigrata* populations have decreased calling effort compared to allopatric conspecifics (FL: $P < 0.00001$, GA: $P < 0.00001$, SC: $P = 0.00361$, VA: $P < 0.00001$; Fig. 6). Although these patterns need to be studied more thoroughly through metabolic experiments, preliminary data suggest that in the presence of heterospecifics, *P. feriarum* signals diverged in a manner that is more metabolically expensive than the allopatric state, whereas *P. nigrata* signals shifted to a slightly less costly state in sympatry (Fig. 6). One explanation for the evolution of costly signals in *P. feriarum* is an increase in the intensity of sexual selection exerted by females in sympatry. This increase may occur if females evolve greater within-species discrimination ability as an indirect consequence of greater between-species discrimination. Available female preference data (see below) are consistent with this hypothesis.

The production of more costly signals directly affects male fitness (Ryan 1988), and can lead to changes in other mating behaviors. For example, males may have to signal for shorter periods of time, thus reducing the length of the breeding season and leading to more explosive breeding (Wells 2007). This narrowing of the breeding window may then lead to greater temporal isolation from heterospecific taxa, thereby further reducing the opportunity for hybridization. Alternatively, males may adopt other energy-conserving strategies for obtaining mates (satellite behavior, Roble 1985; scramble competition, Byrne and Roberts 2004; Wells 2007; egg clutch piracy, Vieites et al. 2004). These three shifts in mating behaviors, however, are expected to reduce the efficiency of female choice (Kokko and Rankin 2006; but see Head et al. 2007), and therefore hinder the evolution of reproductive isolation between species. Further work is needed to understand the interactions of these different processes.

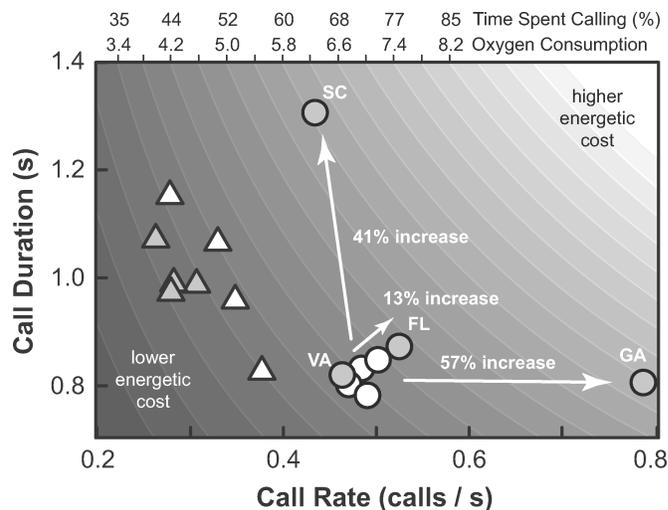


Figure 6. Increased energy expenditure during signaling in sympatric *P. feriarum*. Population means (symbols) are shown. *P. feriarum* is represented by circles and *P. nigrata* by triangles. Gray symbols indicate sympatric populations and white symbols indicate allopatric populations. Each point along a given curved line represents equal calling effort (holding call-duty cycle constant), with a trade-off between call rate and call duration. An increase in one of these two variables requires a concomitant decrease in the other to maintain equal energetic output. Lines toward the lower left corner indicate lower energetic costs, whereas those in the upper right corner indicate higher energetic costs. The scales at the top of the figure indicate the percentage of time spent calling (calculated as [call duration/call period] \times 100) and rate of oxygen consumption (in units of ml O₂ per gram per hour; calculated based on equation O₂ consumption = 9.642 \times call duty cycle, which was estimated from Wells and Taigen 1986 for *Hyla versicolor*). Here, the three acoustically displaced sympatric *P. feriarum* populations have significantly increased the proportion of time spent calling (call duty cycle; see discussion) relative to allopatric populations (by 13–57%).

WHY DOES THE DEGREE OF SYMPATRIC SIGNAL DISPLACEMENT VARY GEOGRAPHICALLY?

The pattern of geographic variation in the degree of RCD among sympatric populations may be due to a variety of causes alone or in combination, including (1) geographic variation in hybrid fitness (Parris 2001; Sweigart et al. 2007), (2) variation in the distance of populations from allopatry (Littlejohn 1965), (3) differences in the relative abundance of each species (Howard 1993; Noor 1995; Servedio and Kirkpatrick 1997; Nosil et al. 2003; Servedio and Noor 2003; Peterson et al. 2005), (4) variation in the degree of ecological overlap between species (Gow et al. 2006; Taylor et al. 2006), and (5) differences in the timing of secondary contact across the contact zone (Borge et al. 2005). Available data for chorus frogs lend support for several hypotheses (2, 3, and 5) that are not mutually exclusive. Within the Florida transect, Fouquette

(1975) found increasing displacement with distance from allopatry in *P. feriarum*. Further, in the southern part of the contact zone, *P. feriarum* is relatively rarer than *P. nigrita*, whereas in the northern region, *P. nigrita* is the relatively rarer species (E. Moriarty Lemmon, unpubl. data). In the present study, only the rarer species diverged in all populations. Data implicating the timing of secondary contact hypothesis are the strongest. Phylogeographic and population genetic data indicate that *P. feriarum* expanded into the northern part of its range, probably in response to climate change since the last glacial maximum (Lemmon et al. 2007b; Lemmon and Lemmon 2008). Therefore, the two species came into contact more recently in Virginia, whereas they have been interacting for a longer period in the southern part of the range. These data suggest that speciation-in-action may be observed in this system, in which populations are in an earlier stage of divergence in northern areas and in a later stage in the southern region.

WHY IS REPRODUCTIVE CHARACTER DISPLACEMENT ASYMMETRIC?

The pattern of asymmetric signal divergence in sympatry is a pattern found frequently in taxa that have undergone RCD (Fouquette 1975; Butler 1988; Loftus-Hills and Littlejohn 1992; Marshall and Cooley 2000; Smadja and Ganem 2005; Cooley et al. 2006; Jaenike et al. 2006). This pattern may result from several individual factors or combination of factors including (1) asymmetric postzygotic isolation between taxa (Pfennig and Simovich 2002; Gabor and Ryan 2001), (2) asymmetric prezygotic isolation (but equal postzygotic isolation), (3) differences in relative abundances of species (Howard 1993; Peterson et al. 2005), (4) different costs or constraints on trait evolution in each species, and (5) the spread of divergent characters into allopatry in one species, thus obscuring RCD in that taxon (Howard 1993). In *Pseudacris*, there is some evidence supporting hypothesis (5). The ancestor of *P. feriarum* and *P. nigrita* speciated in the late Miocene, ~8 million years ago (Lemmon et al. 2007b). Since this time, multiple sea level fluctuations have covered much of the current distribution of *P. nigrita* throughout the Coastal Plain (Dowsett and Cronin 1990), likely forcing *P. nigrita* inland toward *P. feriarum* and causing extinction in allopatry (Lemmon et al. 2007b; Lemmon and Lemmon 2008). It is plausible that after sea level recession, sympatric *P. nigrita* recolonized coastal regions, spreading the diverged signal through allopatry. Additional work is needed to elucidate the role of other processes in promoting asymmetric signal divergence.

WHY HAS THE FEMALE PREFERENCE EVOLVED?

The preferences of female *P. feriarum* have undergone reproductive character displacement, such that sympatric females made significantly fewer mating mistakes than allopatric females. The

propensity to hybridize has been reduced through evolution of the female preference in sympatry and not through evolution of the male trait itself. The reduction can be seen by the fact that whether females were given allopatric or sympatric conspecific calls paired with the heterospecific call, they made the same number of mistakes. This finding begs the question, “Why then did the male signal evolve?” The answer can be found in experiment C in which sympatric females strongly preferred the sympatric to the allopatric signal, suggesting that sympatric females exerted directional sexual selection on the male call, causing it to diverge from that of *P. nigrita*. Interestingly, this result contrasts with several frog studies in which a pattern of RCD of the female preference but not the male signal was detectable (Gerhardt 1994; Höbel and Gerhardt 2003). Because the experiments in this study were done in a single pair of populations (sympatric and allopatric), the argument could be made that female preference displacement has not occurred, rather, females simply preferred their local conspecific stimulus due to clinal variation in preference (Gerhardt 1974). This idea is not supported by the data: first, allopatric females did not prefer their local male signal—in fact, they had a weak preference for the sympatric signal (experiment C; Fig. 4). Incidentally, the pattern is consistent with the signal energetic cost hypothesis described above—allopatric females prefer the more energetically expensive signal, as found in other frog studies (Klump and Gerhardt 1987; Welch et al. 1998; Gerhardt et al. 2000). Second, the significant increase in the level of species discrimination in sympatric compared to allopatric females indicates more than just a preference for local signals. Future studies will explore geographic variation in female preferences throughout the species’ distribution (Gerhardt 1999).

An important question is whether the information contained in the diverged signal (pulse rate and pulse number) is salient to females for species recognition. Although not yet established for *P. feriarum*, extensive behavioral and neurological studies of *P. regilla* (formerly *Hyla regilla*), a congener that has a structurally similar signal, indicate that these two characters are both necessary and sufficient for species recognition in this taxon (Straughn 1975; Brenowitz and Rose 1994; Rose and Brenowitz 1997; Alder and Rose 1998; Rose and Brenowitz 2002). In a detailed study of auditory neuron responses in the *P. regilla* midbrain, Edwards et al. (2002) found that these neurons actually count the pulses (specifically, interpulse intervals) contained within the call, and only fire after reaching a specified number. Additionally, if the temporal spacing between pulses (pulse rate) becomes too large or small, the neuron resets the counting process. If the neural circuitry of *P. regilla* is similar to that of *P. feriarum*, these results could have important implications for the transfer and reception of signal information in chorus frogs.

Evidence to date supports that reinforcement is driving divergence in chorus frogs. First, putative hybrids, which are

morphologically and acoustically intermediate (Fig. 3), have been found in nature. Second, comparative analyses of acoustic signal evolution across the entire genus *Pseudacris* indicate that pulse rate and pulse number are uncorrelated with body size (Lemmon et al., unpubl. data), which is related to the ecological niche in frogs (Parmelee 1999). Moreover, within the pooled allopatric sample of *P. feriarum* in this study, there is no correlation between body size and pulse rate ($r^2 = 0.027$, $P = 0.3962$) or pulse number ($r^2 = 0.004$, $P = 0.7347$). In nearly every study of RCD and reinforcement in anurans, the character that displaced is dominant frequency, a call character that is negatively correlated with body size (Blair 1955; Pierce 1976; Loftus-Hills and Littlejohn 1992; Höbel and Gerhardt 2003; Hoskin et al. 2005; Pfennig and Pfennig 2005). This correlation makes it more difficult to disentangle the effects of ecological selection from reproductive selection on signals (Pfennig and Pfennig 2005). In the present study, characters other than dominant frequency diverged, thereby supporting the idea that signal displacement is not merely a by-product of ecological character displacement. Third, *P. feriarum* raised in isolation in the laboratory produce calls that fall within the distribution of field-recorded males, indicating that this trait is inherited rather than learned (E. Moriarty Lemmon, unpubl. data). Finally, laboratory experiments indicate that hybrids have lower viability, and males are partially sterile (E. Moriarty Lemmon, unpubl. data). Data on the degree of natural and sexual selection against hybridization and on the frequency of hybridization in the field will be presented elsewhere.

CAN INTERACTIONS BETWEEN SPECIES PROMOTE REPRODUCTIVE ISOLATION WITHIN SPECIES?

Howard (1993) first suggested that reproductive character displacement driven by reinforcement could initiate a cascade of speciation events, resulting in reproductive isolation between sympatric and allopatric populations (Hoskin et al. 2005), and even among sympatric conspecific populations that diverge in different traits. Although theoretical models suggest the latter scenario is entirely possible when different heterospecific assemblages exist across populations (McPeck and Gavrillets 2006; Pfennig and Ryan 2006, 2007), empirical examples are scarce. Hints that this process may be operating exist in the literature (e.g., Plethodontid salamanders, Adams et al. 2007; walking stick insects, Nosil et al. 2003), but these examples implicate variation in abiotic rather than biotic conditions as the primary factor promoting diversification. In the one empirical example, to my knowledge, in which variation in the heterospecific assemblage present has been shown to influence the direction of signal evolution, speciation has occurred as a result of these differing selection pressures (*Heliconius* butterflies, Jiggins et al. 2001; Jiggins et al. 2004). This example differs from the present study in that the signals of the focal species have converged upon (through mimicry) rather than

diverged from those of two sympatric heterospecifics, thereby leading to speciation. Collectively, both theoretical and empirical studies suggest that reproductive interactions with the local species assemblage may constitute a potentially powerful evolutionary force driving behavioral divergence not only between but also within species.

Is the behavioral diversification in sympatric *P. feriarum* adequate to initiate speciation? Because data are not yet available for geographic variation in female preference, this discussion is primarily limited to the male signal, which is likely a reflection of the female preference. The level of difference observed here in pulse rate is of the magnitude required by other frog species to coexist with little reproductive interaction (Straughn 1975; Littlejohn 2001; Gerhardt and Huber 2002; Gerhardt 2005; Wells 2007). Moreover, several studies indicate that females can discriminate between signals that differ in pulse rate by a factor as low as 1.2–2.0 (Littlejohn 1965, 1969; Loftus-Hills and Littlejohn 1971; Gerhardt 2005). In the present study, the most different sympatric populations (GA and SC) differ by a factor of 1.8, which is above the discrimination threshold for other species (Figs. 5 and 7). In addition, these populations differ along a second axis, pulse number. Given the finding that female within-species discrimination ability has increased in sympatry, it is possible that the differences in male signals observed here may indirectly contribute to reproductive isolation among conspecific populations and provide the substrate for further divergence should the populations meet (Liou and Price 1994; Kelly and Noor 1996). Moreover, if the intensity of sexual selection within populations has increased in sympatry, as suggested by the signal energetics data above, speciation among conspecific populations may be even more likely (Boul et al. 2007). Additional preference experiments must be conducted to assess these hypotheses. Whether behaviorally divergent populations eventually become species will depend also on the existence of some form of postzygotic isolation (Servedio and Kirkpatrick 1997; Kirkpatrick 2000; Servedio 2000, 2001, 2004; Ritchie 2007). In *P. feriarum*, the process of speciation may be further aided by geographic isolation of coastal South Carolina frogs from other sympatric populations in a “island” situation, in which there is little or no gene flow into this region (Schwartz 1957; Fig. 1). As in the Hoskin et al. (2005) example, the combination of reinforcement and geographic isolation (in their example, isolation between allopatric and sympatric populations), not only facilitates the independent evolution of signals and (potentially) preferences among populations, but also perhaps keeps these populations apart until postzygotic isolation evolves.

Conclusion

This study demonstrates that reproductive character displacement in *P. feriarum* has resulted in diversification of signal traits across

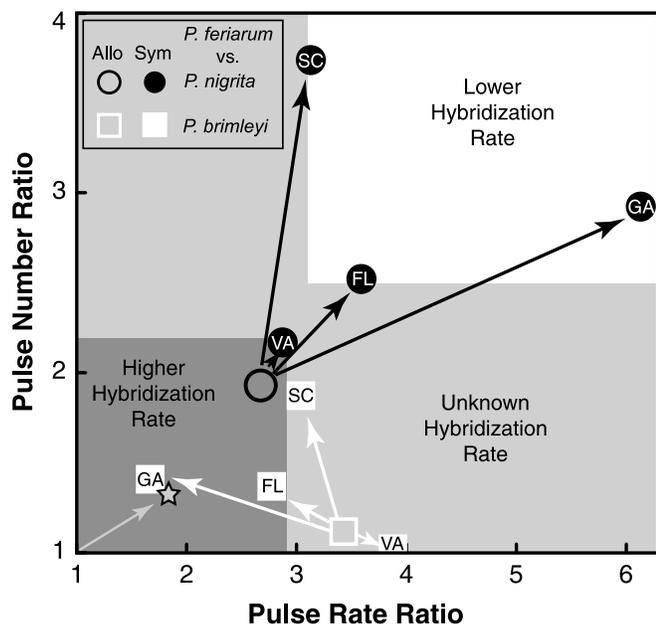


Figure 7. Levels of signal differentiation among species pairs and population pairs. Each point represents the ratio of mean pulse rates and pulse numbers for a given pair of taxa. For the point labeled SC, for example, the x-value was calculated by dividing mean pulse rate of sympatric South Carolina *P. feriarum* by mean pulse rate of sympatric South Carolina *P. nigrita* and the y-value was calculated as the same ratio with respect to pulse number. Black symbols indicate ratios between *P. feriarum* and *P. nigrita*. White symbols indicate ratios between *P. brimleyi* and *P. feriarum*. Hollow symbols represent the amount of differentiation between allopatric populations of the species pairs (precontact signals). Black arrows indicate the increase in call differentiation between *P. feriarum* and *P. nigrita* from the allopatric to sympatric state. White arrows indicate the decrease in call differentiation between *P. brimleyi* and *P. feriarum* as a result of displacement in the former species pair, which could potentially lead to higher hybridization rates should the latter species pair come into contact. Only divergence in sympatric *P. feriarum* from South Carolina increases differentiation from both heterospecifics. The gray arrow indicates the change in amount of differentiation from allopatric *P. feriarum* population pairs (ratio of ~ 1) to sympatric Georgia/South Carolina *P. feriarum* population pairs (star symbol; ratio of ~ 1.8 in pulse rate and ~ 1.3 in pulse number). Field observations and female preference tests (Fig. 4) suggest that hybridization between *P. feriarum* and *P. nigrita* is rare in South Carolina, Florida, and Georgia, and more common in Virginia and in allopatric populations of these species (preference tests). These data were used to delineate predicted regions of lower (white space) and higher (dark gray space) hybridization rates. This figure assumes that the amount of male signal differentiation alone determines the likelihood of hybridization. Given the results of the female preference tests (see text; Fig. 4), evolution of the female preference function could contribute significantly to further reproductive isolation among species and populations.

sympatry. Moreover, as predicted by reinforcement, female preferences have also displaced in the one sympatric population tested, resulting in reduced probability of hybridization and greater discrimination among conspecific signals. Additionally, divergence of the female preference rather than the male signal has reduced the likelihood of hybridization between species. Together, signal and preference data suggest that female preference evolution in sympatry has driven divergence of the male signal, and as a result of variable selection pressures across the contact zone, signals have displaced along different, uncorrelated axes, potentially leading to reproductive isolation among conspecific populations.

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Supporting Information

The following supporting information is available for this article:

Table S1. List of male *Pseudacris feriarum*, *P. nigrata*, and putative hybrids examined for signal variation or used to construct female preference stimuli.

Table S2. Definitions of 14 call characters examined across *Pseudacris*.

Table S3. Correlations between temperature and call variables.

Table S4. Female sympatric (FL) and allopatric (AL) *P. feriarum* tested in the preference experiments.

Table S5. Results of randomization tests comparing allopatric populations within species.

Table S6. Results of randomization test for detecting reproductive character displacement in different sympatric populations (allopatric populations not pooled).

Table S7. Temperature-corrected raw data for two call variables.

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

(This link will take you to the article abstract).

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