

Mate choice and courtship signal differentiation promotes speciation in an Amazonian frog

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Female mate choice influences the evolution of male courtship signals and may promote speciation when those sexually selected traits also have a function in species discrimination. Here, we assess interpopulation female mate choice by conducting phonotaxis experiments on a population of the Amazonian frog *Engystomops petersi* in Puyo, Ecuador. Our results show very strong behavioral isolation relative to 1 of 2 foreign populations. Puyo females strongly discriminate against La Selva in favor of Puyo or Yasuní signals. In contrast, Puyo females do not discriminate against signals from Yasuní, which are similar in frequency. Behavioral isolation was stronger than expected because Puyo females were unable to recognize La Selva courtship signals as belonging to conspecific males. Overall, female mate choices are consistent with male courtship signal differentiation among populations but inconsistent with geographic or genetic distances. Simulations under a null model of undirected evolution (Brownian motion) suggest directional selection on courtship signals at La Selva. Based on our results, we hypothesize that sexual selection and/or reinforcement is driving speciation between *E. petersi* populations. *Key words*: discrimination, Ecuador, *Engystomops petersi*, *Physalaemus*, recognition, reinforcement, sexual selection. [*Behav Ecol* 19:1128–1135 (2008)]

Tropical rain forests are highly diverse ecosystems where efforts to understand the origin and maintenance of diversity began more than 150 years ago (e.g., Wallace 1852) and until now have been inconclusive. High species diversity should be expected when speciation rates are high and/or extinction rates are low. Thus, the study of processes that can result in high speciation rates can be crucial to understand the origin of these rich communities. One of such processes is sexual selection because it can lead to rapid speciation (e.g., Panhuis et al. 2001; Mendelson and Shaw 2005). Therefore, the study of sexual selection can contribute to explain tropical rain forest diversity.

Because traits that inform mate choice also allow females to discriminate among conspecific and heterospecific males, sexual selection can incidentally promote speciation (i.e., origination of new species) by generating prezygotic isolation (see Panhuis et al. 2001 for a review). Prezygotic isolation can also occur via reinforcement, a process that favors divergence in mate recognition traits and/or mate preferences in closely related species that interbreed. Reinforcement occurs as a result of natural selection against maladaptive gene combinations present on hybrid offspring (Dobzhansky 1951).

Sexual selection and reinforcement are not mutually exclusive processes, and both should generate a rapid rate of evolution in mate recognition traits (West-Eberhard 1983; Andersson 1994; Coyne and Orr 1998). Sexual selection will do so because of the potential for mutually accelerating social evolution of preference and trait (West-Eberhard 1983; Andersson 1994). Reinforcement will generate rapid diver-

gence because of the strong selective pressure to avoid heterospecific matings (Howard 1993; Coyne and Orr 1997).

THE STUDY SYSTEM

Frogs are model organisms to study sexual selection and reinforcement because female mate choice, particularly in nocturnal species, is mainly based on characteristics of the advertisement male call (Blair 1964; Ryan 1985). This study focuses on *Engystomops petersi*, a leptodactylid frog widely distributed in the Amazon Basin. Its typical advertisement call has 2 obligatory components: the prefix and the whine. The prefix consists of 1 or 2 short (20–70 ms) amplitude-modulated pulses, whereas the whine is a downward frequency sweep (150–290 ms, Figure 1). At some populations (e.g., Yasuní), males can add a facultative higher frequency suffix (squawk) after the whine (Cannatella et al. 1998; Boul and Ryan 2004). The suffix has been shown to be under sexual selection in *Engystomops pustulosus* and at least one population of *E. petersi* (Ryan 1985; Boul et al. 2007). Hereafter, we refer to calls containing the squawk as “complex”. “Simple” calls only consist of the prefix and the whine.

In a recent study, Boul et al. (2007) demonstrated the existence of strong female preferences for local calls in 2 populations (Yasuní and La Selva). Because the squawk is present in Yasuní but not in La Selva, they hypothesized that behavioral isolation between both populations has been a by-product of sexual selection for the squawk in Yasuní. Genetic data were consistent with the proposed scenario because it rejected random drift as a significant cause of divergence in calls of *E. petersi* (Boul et al. 2007). Microsatellite markers showed low gene flow and failed to find evidence of hybridization. These findings were interpreted as inconsistent with divergence resulting from reinforcement as primary force of speciation (Boul et al. 2007).

Herein, we present female preference data from a third population to assess the potential role of sexual selection in speciation in *E. petersi*. We analyzed interpopulation differentiation in call-acoustic structure, mitochondrial DNA, and body size

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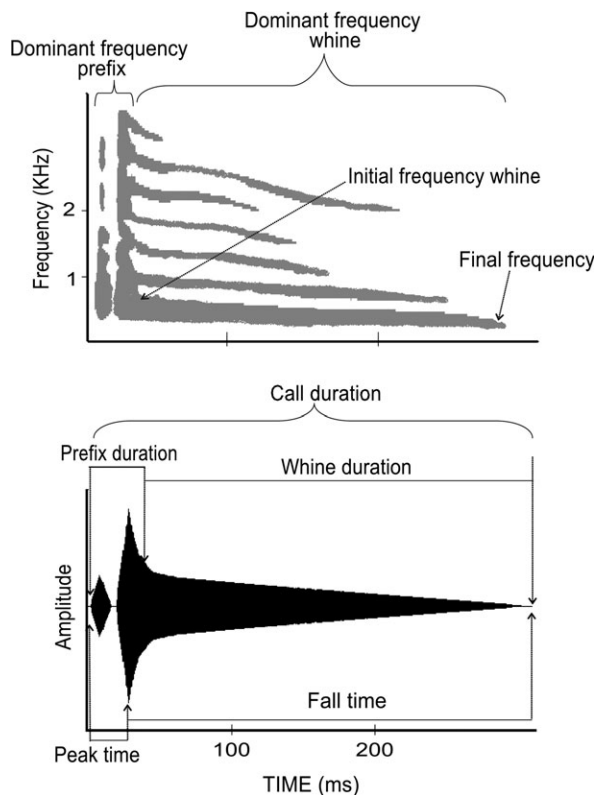


Figure 1
Advertisement call of *Engystomops petersi* from Puyo (spectrogram above, oscillogram below) showing the analyzed variables.

among these populations. Calls evolving by random processes, like genetic drift, should fit a model of evolution without directional change and constant variance. We tested whether interpopulation call variation departed from this null model of evolution by simulating calls evolving under undirected change. We also assessed interpopulation female choice in one of those localities to assess patterns of behavioral isolation. We posed 2 central questions: 1) do females discriminate and recognize the advertisement calls of other populations? and 2) what properties of the advertisement calls are likely to explain interpopulation female preferences?

METHODS

The studied populations are in Amazonian Ecuador. We conducted the female choice study 5 km northeast of Puyo, on the road Puyo-Tena (hereafter, Puyo), Pastaza province (1.4431°S, 77.9967°W; altitude 900 m; Figure 2). Calls were compared between Puyo and 2 populations in the vicinities of the Napo River: 1) La Selva Lodge, Napo province (0.4982°S, 76.3738°W; altitude 250 m; Figure 2) and 2) Yasuní Scientific Research Station Pontificia Universidad Católica del Ecuador, Orellana province (hereafter, Yasuní; 0.6743°S, 76.3971°W; altitude 250 m; Figure 2). Yasuní and La Selva are on opposite sides of the Napo River, which is 1–2 km wide in that region.

Interpopulation divergence in the sexually selected male trait

At Puyo, we recorded advertisement calls using a Sony Digital Audio TCD-D8 tape recorder and a Sony Walkman WM-D6C professional stereo cassette recorder with Sennheiser SE 66-67 microphones. We digitized the calls with Cool Edit

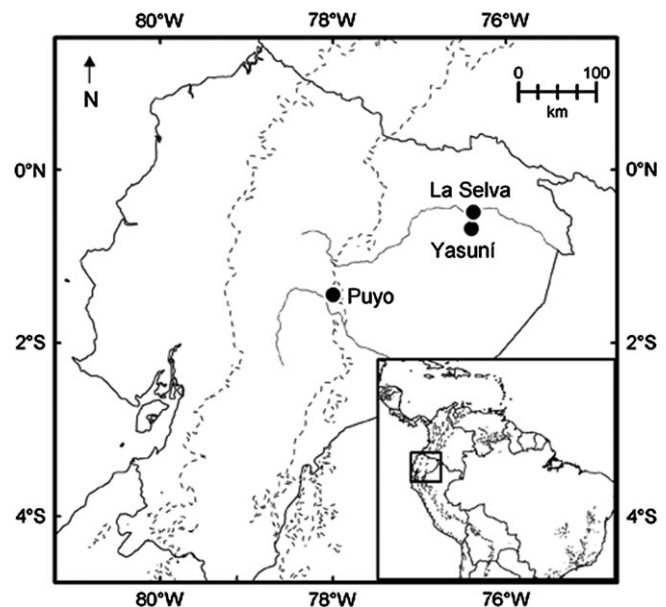


Figure 2
Map of Ecuador with localities of *Engystomops petersi* (circles). The dashed line marks the 1000 m of elevation limit.

pro 2.0 (Syntrillium Software Corporation) (sampling rate = 22050 Hz, resolution = 16 bits). Simple calls from Puyo, Yasuní, and La Selva were analyzed using Sound Analysis Software Canary 1.2.4 (Charif et al. 1995). We used a Fast Fourier Transformation of 1024 points. Measured parameters are presented in Table 1.

We applied principal components analysis (PCA) to 9 acoustic variables to explore patterns of call differentiation among populations. The PCA (on correlations) was carried out on averages (from 5 to 6 males) from each of 3 *E. petersi* populations and *E. pustulosus* (from Gamboa, Panama). In addition, we used squared changes parsimony (Maddison 1991; as implemented in software Mesquite 1.06, Maddison 1991; Maddison and Maddison 2005a) to reconstruct ancestral character states of the resulting PC scores along the maximum likelihood tree topology (including branch lengths) presented by Ron et al. (2006).

To test whether the divergence in advertisement calls (the sexually selected male trait) observed between *E. petersi* populations was different from the divergence expected from a character evolving without directional change and with constant variance through time (Brownian motion model), we conducted 10 000 simulations of a character evolving along the phylogeny under Brownian motion (Figure 3A; from Ron et al. 2006). Under this model, the expected divergence from an ancestor is distributed normally with mean equal to 0 and variance proportional to branch length. Thus, trait similarity between 2 species is inversely proportional to the time since they shared a common ancestor. Under this model, calls of the more closely related Yasuní and La Selva populations would be expected to be more similar to each other than either is to Puyo (Figure 3A). Specifically, we tested whether the Euclidean distances of the acoustic variables between local and foreign populations significantly departed from the Brownian motion model. We tested the null hypothesis (compliance with Brownian model) with the ratio $a/(a + b)$ where a is the Euclidean distance between Puyo and Yasuní and b is the Euclidean distance between Puyo and La Selva. The ratio varies between 0 and 1; a value of 0.5 would indicate that calls from La Selva and Yasuní have diverged by the same amount

Table 1
Acoustic variables of the advertisement call of *Engystomops petersi* considered in this study

Parameter	Description
Call duration	Time from the beginning to the end of the call
Call dominant frequency	Frequency containing the most energy across the call
Call shape	Rise time/call duration
Fall time	Time from the point of maximum amplitude of the call to the end of the call
Final frequency	Frequency at the end of the first harmonic
Rise time	Time from the beginning of the call to the point of its maximum amplitude
Prefix duration	Time from beginning to the end of the prefix (Figure 1)
Prefix dominant frequency	Frequency containing the most energy in the prefix
Whine duration	Time from the beginning to the end of the whine (Figure 1)
Whine dominant frequency	Frequency containing the most energy in the whine
Whine frequency decrease	Difference in frequency between the initial frequency and final frequency of the first harmonic of the whine
Whine initial frequency	Frequency at the beginning of the fundamental frequency of the whine

from Puyo (although not necessarily in the same direction). H_0 was rejected if the observed ratio was lower than the ratio of 95% of the simulations or more. We applied this test to the population averages of 9 acoustic variables (Table 2) and to the PC scores of the first 2 components from a PCA of these same 9 variables. Interpretations for the rejection of H_0 would suggest directional or stabilizing selection in one or more of the 3 populations. Hansen and Martins (1996) demonstrated that random drift and fluctuating directional selection result in a macroevolutionary pattern not significantly different from Brownian motion. Thus, acceptance of H_0 would pertain to either of these processes. The simulations were implemented in Mesquite v.1.06 (Maddison and Maddison 2005a) with the StochChar module (Maddison and Maddison 2005b).

We tested for differences in call parameters between Puyo-Yasuní and Puyo-La Selva with *U* Mann–Whitney test. We did not consider temperature as a factor in our call analyses because all recordings were made within the range of 21–24 °C (S.R.R, W.C. Funk, and K.E. Boul field notes).

We did not apply the Bonferroni corrections because its use is problematic (Moran 2003; Nakagawa 2004). Instead, we report the probability of finding the observed number of significant tests by chance (Moran 2003).

Female choice experiments

Experiments were carried out at Puyo. Experiments were of 2 types, recognition and discrimination. In the recognition experiments, the female was given a choice between a simple call and white noise (with the amplitude envelope and duration of an average simple call). Recognition experiments assess whether the call is identified as signaling an appropriate mate. In the discrimination experiments, the female was given a choice between 2 different calls. Discrimination experiments determine the capacity of the female to make a distinction between 2 signals, showing preference for one of them.

We tested females that were collected in amplexus in the field between 2000 and 0130 h, from April to December of 2004. Experiments consisted in 2-choice trials following the methodology used by Ryan and Rand (1990). We performed the experiments using an acoustic chamber (180 × 105 × 100 cm) with walls covered with padded foam to reduce acoustic reverberance. All experiments were performed under darkness. The behavior of the females was observed in real time through a television connected to a digital video camera Sony DCR-TV70 equipped with an infrared light. We placed 2 speakers SME-AFS Saul-Minneroff one at each end side of the arena, facing the center. The distance between the center of the arena and each speaker was 78 cm. At the beginning of the experiments with each female, we adjusted the

sound pressure level from each speaker to the center of the arena at 80 dB (reference: 20 µPa) using a Radioshack No. 33–2055 SPL-meter. The sound used to calibrate each speaker was a 600-Hz pure tone with the same peak amplitude of the calls used in the experiments. We broadcasted the stimuli from a Toshiba Satellite 1100-s101 computer using Cool Edit pro 2.0.

We edited the calls with Cool Edit 2.0. Each experiment consisted of 2 stimuli. Both stimuli were normalized to the same peak amplitude. Each stimulus was played from a different channel of a stereo sound file, and they were emitted antiphonally through the 2 speakers, each at a rate of 2.5 s (maximum call repetition rate of the local population). To avoid biases, we randomized the side from which the stimuli were broadcasted in all trials. Temperature inside the chamber was maintained between 22.3 and 24.6 °C, and its floor was kept wet.

Females were tested on the same night of capture except for few females that did not respond to the first control and were kept in captivity and tested the following night. Before each trial, the female was placed under a plastic funnel in the center of the arena. We broadcasted the stimuli for 3 min, then the funnel was raised, and the female was free to move inside the chamber. A no response was scored if the female stayed motionless in the center of the arena more than 5 min or if she stayed motionless at any place of the chamber more than 2 min or has not chosen any of the stimuli for 15 min. A positive response was scored if the female had approached less than 10 cm to one of the speakers (modified from Ryan and Rand 1993).

To test whether females can recognize each population's calls, we conducted recognition experiments with the simple calls from: 1) Puyo (local call), 2) Yasuní, and 3) La Selva. In each experiment, the female had to choose between white noise and the call. Lack of choice on a trial could result from lack of recognition or motivation. To eliminate trials with responses due to lack of motivation, recognition trials were followed by a control trial consisting of a choice between white noise versus the simple local call. If the female did not choose the simple call, the antecedent recognition trial was eliminated from the analysis.

In the recognition experiments, we used a nonparametric Fisher's exact test to evaluate the null hypothesis of no recognition. The null expectation is the probability of approaching a silent speaker when it is paired with a white noise stimulus (2 out of 20 females in *E. pustulosus*; Ryan and Rand 2001). The use of a null expectation of 0.5:0.5 (results not shown) yielded similar results as the null expectation of 0.1:0.9 (2:18).

To test interpopulation female preferences at Puyo, we carried out 3 discrimination experiments between the following simple calls: 1) local call versus Yasuní, 2) local call versus

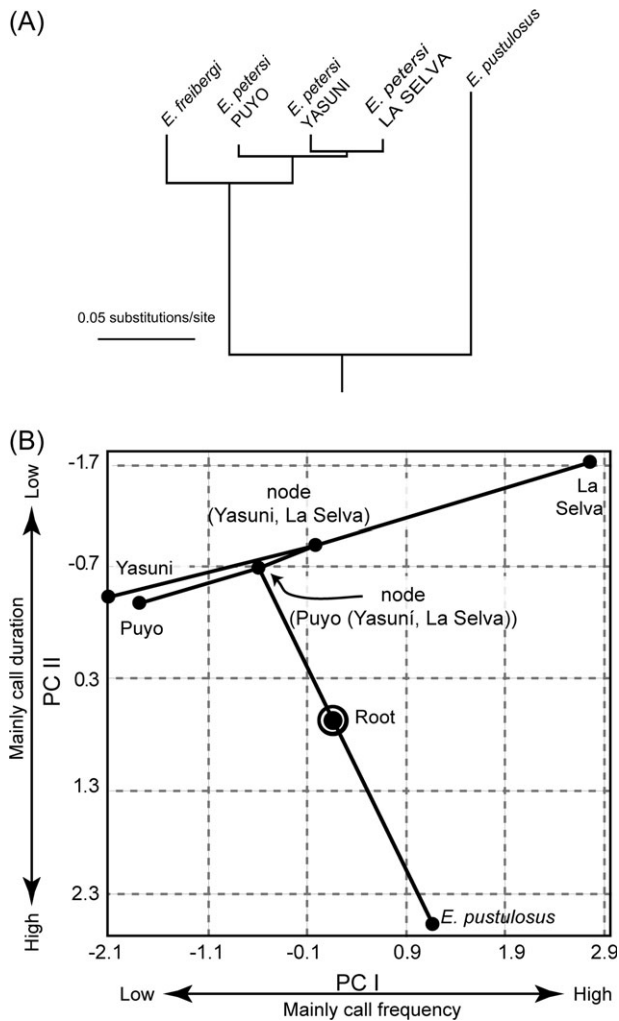


Figure 3
 Call differentiation among populations of *Engystomops petersi*. (A) Phylogram depicting relationships based on mitochondrial DNA (from Ron et al. 2006); (B) axes I and II from PCA based on 9 acoustic variables of the advertisement call of *Engystomops pustulosus* and 3 populations of *E. petersi*. The connecting lines correspond to the topology shown in the phylogram. Nodal values were reconstructed with squared changes parsimony. Note that the calls from La Selva have diverged significantly from Yasuni, Puyo, and the common ancestor of *E. petersi*.

La Selva, and 3) Yasuni versus La Selva. Boul et al. (2007) hypothesized that Yasuni and La Selva populations are prezygotically isolated because sexual selection for complex calls at Yasuni has resulted in divergence of the simple call. La Selva has simple calls and lacks preference for complex calls. To test the hypothesis of preference for complex calls at Puyo, we conducted a discrimination experiment on which females had to choose between a simple call from the local population and the same call with the squawk from Yasuni appended.

In all the discrimination experiments, nonparametric binomial tests were applied to test the null hypothesis of no preference for either stimuli (0.5:0.5). We used 1-tailed tests only in the local call versus foreign call tests because there was an a priori prediction about the directionality of the response (preference for local). We applied 2-tailed tests in all other experiments. Tests were implemented in the Statistical Package for Social Science v.13.0 (SPSS 2004).

Table 2

Character loadings, eigenvalues, and percentage of explained variance from PCA applied to 9 acoustic variables of the advertisement call of 3 populations of *Engystomops petersi*

	PC I	PC II
Call duration	-0.03122	0.54635
Call shape	0.18012	-0.49791
Call dominant frequency	0.40946	-0.07531
Final frequency	0.41856	-0.07908
Rise time	0.32832	0.34829
Prefix dominant frequency	0.42052	0.07588
Whine duration	-0.08884	0.53753
Whine initial frequency	0.42314	0.02463
Whine frequency decrease	-0.39009	-0.15829
Percent of variance explained	61.6267	36.7041
Eigenvalue	5.5464	3.3034

Each female was tested in more than 1 experiment (usually 6) but only once in each experiment to avoid pseudoreplication. The number of trials performed by each female varied according to her responsiveness. For each female, the first trial was a control. The control was repeated once every 3 trials to ensure that the female was motivated and not behaving randomly. The order and type of experiments varied between females. To avoid pseudoreplication, we tested females with calls randomly sampled from a pool of calls from 10 individuals from the local population, 5 from Yasuni, and 3 from La Selva.

After the female was tested, we took digital photos of her venter. The female was released the next day at the capture site. We used the ventral pattern to individually identify females and avoid testing females more than once.

RESULTS

Interpopulation divergence in the sexually selected male trait

Acoustic parameters of simple calls from the 3 populations are presented in Table 3. Mann-Whitney *U* test shows that most call parameters of the local population (Puyo) differ significantly with those from La Selva; in contrast, only final frequency and whine frequency decrease are significantly different between Puyo and Yasuni (Table 3). Male body size (snout-vent length) is significantly different between Puyo and both Yasuni and La Selva (Table 3).

La Selva calls are characterized by a significantly higher frequency (Table 3, Figure 3B). Body size and call frequency are usually negatively correlated in frogs (Gerhardt and Huber 2002), and differences in frequency could be a by-product of body size differences. However, the difference in frequency between La Selva and Puyo cannot be explained by body size differences because La Selva females are larger (frequency should be lower).

PCA of 9 acoustic variables resulted in 2 components with eigenvalues >1 that explained 98.3% of the variance (Table 2). Character loadings indicate that PC I mainly characterizes spectral properties of the call while PC II mainly characterizes temporal properties. In the acoustic space defined by both PCs, ancestral character reconstruction indicates that calls from La Selva are at a large acoustic distance from the calls of the common ancestor of *E. petersi* and the calls of Puyo and Yasuni (Figure 3B). Divergence is substantial especially along PC I (call frequency).

Simulations of character evolution show that the divergence in the male sexually selected trait between Yasuni-Puyo and La Selva-Puyo populations was significantly different from a

Table 3

Means and ranges for snout-vent length and acoustic variables of the advertisement call of *Engystomops petersi* at Puyo, Yasuní, and La Selva populations

Parameter	Puyo	Yasuní	La Selva	Puyo versus Yasuní <i>P</i>	Puyo versus La Selva <i>P</i>
Number of individuals	6	5	5		
Snout-vent length (mm)	24.1 (23.1–25.7)	29.7 (26.9–31.6)	26.1 (25.4–26.6)	0.001	0.011
Call duration (ms)	285.05 (241.93–321.99)	275.09 (260.74–298.98)	246.32 (217.10–278.06)	0.584	0.068
Call dominant frequency (Hz)	627.65 (524.87–877.48)	541.99 (439.45–585.94)	836.21 (785.96–925.93)	0.272	0.045
Call shape	76.68 (62.08–93.19)	73.99 (61.56–87.40)	120.82 (89.13–176.01)	0.584	0.018
Fall time (ms)	263.16 (225.76–295.15)	254.80 (239.03–277.30)	217.24 (178.89–253.28)	0.584	0.045
Final frequency (Hz)	372.72 (344.53–409.10)	332.03 (317.38–341.80)	596.47 (559.86–646.00)	0.005	0.006
Rise time (ms)	21.90 (16.16–26.84)	20.29 (17.04–23.09)	29.08 (22.60–38.21)	0.584	0.100
Prefix dominant frequency (Hz)	718.75 (602.93–829.03)	717.77 (634.77–830.08)	957.15 (845.18–1033.59)	0.855	0.006
Prefix duration (ms)	32.35 (26.53–41.30)	27.99 (22.12–36.01)	53.47 (36.69–68.54)	0.201	0.011
Whine dominant frequency (Hz)	517.15 (473.73–544.48)	502.93 (439.45–537.11)	760.12 (678.30–839.79)	0.359	0.006
Whine duration (ms)	252.70 (212.09–281.56)	247.10 (232.25–262.97)	192.85 (148.56–228.03)	0.715	0.028
Whine frequency decrease (Hz)	186.95 (150.73–226.10)	234.37 (195.31–292.97)	360.68 (274.55–409.13)	0.044	0.006
Whine initial frequency (Hz)	559.67 (516.80–592.16)	566.41 (512.70–610.35)	959.6 (845.18–1033.59)	0.583	0.006

Probability values are from Mann–Whitney *U* test (2 tails). Bold numbers indicate significant differences.

Brownian motion model of evolution ($P = 0.035$ for PC I, $P = 0.025$ for PC II; Table 4; Figure 4). The observed trend is unlikely to be an artifact of multiple tests. The chance probability of obtaining 2 significant results out of 2 tests at $\alpha = 0.05$ is 0.002. The null hypothesis was also rejected for call shape, whine dominant frequency, whine duration, and whine initial frequency (Table 4). These results are unlikely to be an artifact either. The chance probability of obtaining 4 significant results out of 10 tests is 0.001.

Female interpopulation preference and recognition

We completed 144 trials with 36 females (each female tested only once on each experiment). The results for the female choice experiments are summarized in Table 5. Females recognized the Puyo (local) and Yasuní calls ($P < 0.001$ in both tests); the ratio of recognition of the Yasuní call versus noise (16:0) was not significantly different from that of Puyo call versus noise (34:2; Fisher's $P > 0.999$). Females did not show any preference between Puyo versus Yasuní ($P = 0.50$; Table 5).

In contrast, females did not recognize the call from La Selva ($P = 0.064$). They approached to La Selva call in 7 trials, but twice as frequently they did not respond to any signal or approached to the noise stimulus. In addition, females strongly discriminated against La Selva either in favor of Puyo or Yasuní calls (Table 5). Discrimination between Puyo versus La Selva (17:1) was not significantly different from discrimination between Yasuní versus La Selva (14:1; Fisher's $P > 0.999$) or recognition in the control experiment (Puyo vs. white noise, 34:2; Fisher's $P > 0.999$).

Finally, we found no statistically significant preferences for simple versus complex calls ($P = 0.134$; Table 5). However, twice as many females chose the complex call over the simple call and there are no significant differences in female preferences between Puyo (15 of 22 preferred complex calls) and Yasuní (13 of 15 preferred complex calls, Boul et al. 2007), Fisher's exact test $P = 0.26$.

DISCUSSION

Signal divergence and premating isolation

Our results suggest that female preferences for male calls are promoting premating isolation among populations of *E. petersi*. Interestingly, the pattern of discrimination is more

consistent with divergence in the acoustic properties of male advertisement calls than with the genetic divergence among populations.

We found significant differences in the acoustic properties of La Selva calls compared with Puyo and Yasuní. The observed level of differentiation is striking because the divergence in acoustic space of La Selva is comparable to that observed between the Puyo population and *E. pustulosus* (Figure 3B), a species that diverged from *E. petersi* approximately 12 million years ago (Weigt et al. 2005).

Signal differentiation by itself is insufficient to demonstrate prezygotic isolation. To provide compelling evidence, one must also show that differences are salient to females and that they influence the likelihood of mate choice as a function of signal value. Our results demonstrate both for Puyo population (relative to La Selva). At Puyo, female preference for the local call (versus La Selva) was almost unanimous. If females have a

Table 4

Interpopulation Euclidean distances for acoustic variables and body size in *Engystomops petersi*

	Distance Puyo–Yasuní	Distance Puyo–La Selva	<i>P</i>
Snout-vent length (mm)	5.59	2.00	0.791
PC I	0.3232	4.5456	0.035
PC II	0.0660	1.3113	0.025
Call dominant frequency (Hz)	85.65	208.56	0.206
Call duration (ms)	0.0100	0.0387	0.130
Call shape	0.0031	0.0412	0.0365
Final frequency (Hz)	40.69	223.74	0.0930
Rise time (ms)	0.0016	0.0072	0.112
Whine dominant frequency (Hz)	14.22	242.97	0.029
Whine duration (ms)	0.0056	0.0598	0.046
Whine initial frequency (Hz)	6.73	399.98	0.009
Whine frequency decrease (Hz)	47.42	173.73	0.137

PC I and PC II were derived from a PCA of 9 acoustic variables (Table 3). The *P* values are from a test comparing the observed Euclidean distances with those derived from simulations of 10 000 characters evolving under a Brownian motion model of evolution. Significant values (bold) indicate that the divergence between La Selva and Yasuní populations (relative to Puyo) has been greater than expected from characters evolving under Brownian motion.

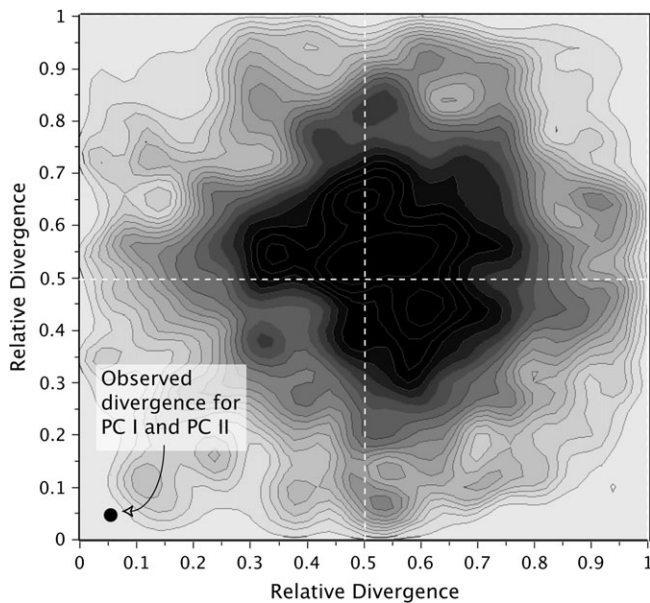


Figure 4 Frequency distribution of expected divergence, under a Brownian motion model of evolution, among calls from the Yasuní and La Selva populations relative to Puyo in *Engystomops petersi*. The distribution was obtained from 10 000 simulations (5 000 per axis) of an hypothetical character evolving under Brownian motion along the phylogeny (Figure 3A). Relative divergence is the ratio $a/(a + b)$ where a is the Euclidean distance between Puyo and Yasuní and b the Euclidean distance between Puyo and La Selva (equal divergence of Yasuní and La Selva from Puyo is shown by white dashed lines). Contour lines are 5% nonparametric density quantiles (darker regions represent more characters). The black circle shows the observed ratios (based on call distances for PC I and PC II; Table 4). Note that most simulations fall into a region where divergence from Yasuní and La Selva is similar. However, the actual advertisement calls show a significantly higher divergence of La Selva (outside the 95% quantile).

choice between males from both populations, they will prefer the local males. This evidence, however, cannot rule out the choice of foreign males in the absence of local males.

A more convincing indication of prezygotic isolation is given by recognition tests because they evaluate whether a signal belongs to a valid mate (Ryan and Rand 2001). Lack of rec-

ognition for calls from La Selva suggests that females from Puyo do not consider La Selva males to be appropriate mates. This is the first indication of lack of recognition of conspecific signals in *Engystomops*. Considering that *E. pustulosus* females can still recognize calls from congeners from which diverged during the Miocene (Ryan and Rand 1993; Ryan and Rand 2001; Weigt et al. 2005), this result was unexpected.

In contrast, females from Puyo recognized the Yasuní calls and consider them as attractive as the local calls. Thus, prezygotic isolation via mate choice is unlikely between these 2 populations. Overall, results from recognition and discrimination experiments are congruent with interpopulation call divergence (Figure 3B). Previous mate choice experiments in Yasuní and La Selva show a similar pattern because both populations have a large divergence in acoustic space (Figure 3B) and strong preference for the local call (Boul et al. 2007; Figure 5).

Both genetic and geographic distances would suggest that Puyo females should respond similarly to Yasuní and La Selva male signals. The genetic distance between Puyo and Yasuní (0.0169) is only slightly higher than that between Puyo and La Selva (0.0146; distances are corrected P from sequences published by Ron et al. 2006). Geographically, Puyo is at similar distances from both populations (198 km from Yasuní and 209 km from La Selva). Female preferences show, however, a marked asymmetry in the direction of the preferences that parallels the asymmetry observed in call divergence.

Is selection driving signal divergence?

The results from the character simulations indicate that directional and/or stabilizing selection have disrupted the pattern of call divergence expected from random drift in one or more of the 3 populations. Scenarios that could explain the observed pattern include the following: 1) La Selva have evolved predominantly by directional selection, whereas Puyo and Yasuní have diverged by random drift; 2) Puyo and Yasuní have experienced predominantly stabilizing selection, whereas La Selva have diverged by random drift or directional selection; or 3) all populations have evolved predominantly by directional selection with convergence between Puyo and Yasuní. External evidence favors the first scenario because interpopulation differences between the common ancestor of *E. petersi* and La Selva are as great as differences between the common ancestor of *E. petersi* and *E. pustulosus* (Figure 3B). This large acoustic distance suggests the occurrence of strong directional selection at La Selva. In addition, calls from La Selva

Table 5 Number of choices between 2 alternative advertisement male calls in female *Engystomops petersi* during phonotaxis experiments at Puyo

Experiments	Number of female responses		Number of females tested	P (binomial)
	(A)	(B)		
Control ^a				
(A) Simple call Puyo versus (B) white noise	34	2	36	<0.001 (1 tail)
Interpopulation call discrimination				
(A) Simple call Yasuní versus (B) simple call La Selva	14	1	15	<0.001 (2 tails)
(A) Simple call Puyo versus (B) simple call Yasuní	8	9	17	0.5 (1 tail)
(A) Simple call Puyo versus (B) simple call La Selva	17	1	18	<0.001 (1 tail)
Simple call versus complex call				
(A) Simple call Puyo versus (B) simple call Puyo + squawk Yasuní	7	15	22	0.134 (2 tails)
Interpopulation call recognition				P (Fisher)
(A) Simple call La Selva versus (B) white noise + no response	7	13	20	0.064 (1 tail)
(A) Simple call Yasuní versus (B) white noise + no response	16	0	16	<0.001 (1 tail)

^a Only includes the first response of each female.

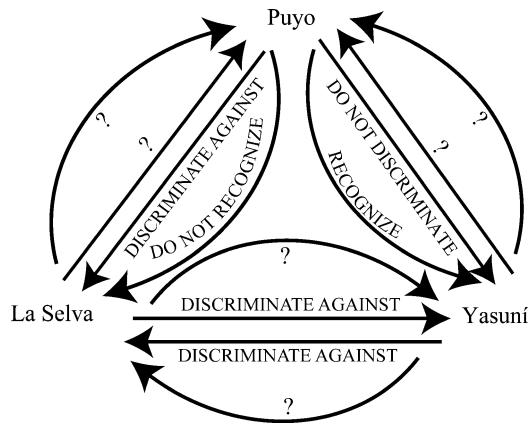


Figure 5

Summary of *Engystomops petersi* female phonotaxis experiments at Yasuní, La Selva, and Puyo. Straight lines = female recognition experiments; curved lines = discrimination experiments (between local and foreign call). Data for La Selva and Yasuní are from Boul et al. (2007). Question marks indicate that tests have yet not been conducted.

represent an extreme phenotype among extant *E. petersi* populations because they have the highest known dominant frequency (Funk CW, personal communication). Thus, the pattern of divergence is more consistent with the occurrence of rapid directional selection at La Selva.

Three alternative, nonmutually exclusive, hypotheses that could explain the observed interpopulation call divergence and behavioral isolation are 1) natural selection for local adaptation, 2) sexual selection, or 3) reinforcement between Yasuní and La Selva. Below we address each of these possibilities.

Is behavioral isolation a product of natural selection for local adaptation?

Divergence in allopatry could result from pleiotropic effects from natural selection for adaptation to different ecological conditions. Available phylogenetic data show that Puyo belongs to a clade primarily distributed in the Andean foothills forest, above 400 m of altitude (Ron et al. 2006; Funk et al. 2007). This clade is sister to a clade that includes Yasuní and La Selva, which is distributed in Amazonian tropical rain forest at an altitude of ~250 m. Thus, both clades are allopatric (Funk et al. 2007). Because Yasuní and La Selva are at similar elevations and separated by only 20 km, they experience a similar environment. In contrast, Puyo is in a different biogeographic region with lower ambient temperatures and higher precipitation (annual precipitation at Yasuní = 2823 mm, at Puyo = 4537 mm). If divergence were a by-product of adaptation to the environment, it would be more likely to expect calls and preferences from La Selva and Yasuní to be similar to each other and different from Puyo. The observed pattern do not fit these expectations.

Size influences the food niche of Amazonian frogs (Parmelee 1999), and thus, size differences can be indicative of niche divergence. Although size was significantly different between populations (Table 3), it did not depart from expectations under a Brownian motion model of evolution (Table 4). Furthermore, La Selva was intermediate in size. An alternative effect of body size could result from its negative relationship with call frequency (Gerhardt and Huber 2002). This explanation is unlikely as males from Puyo are significantly smaller than those from La Selva and would be expected to have higher frequency calls; the observed divergence is the oppo-

site. Thus, ecological divergence seems unlikely to explain the observed divergence in calls and female preferences.

Is behavioral isolation a product of sexual selection?

Sexual selection can generate rapid change in display traits (Fisher 1930; Kirkpatrick 1982; West-Eberhard 1983). Because these traits also play a role in species recognition, sexual selection could result in speciation (Panhuis et al. 2001). Although theoretical models indicate the plausibility of this process (Turelli et al. 2001), compelling evidence is limited to few examples (e.g., Uy and Borgia 2000; Masta and Maddison 2002; Mendelson and Shaw 2005).

The combined data (herein and in Boul et al. 2007) strongly suggest that sexual selection could be driving interpopulation behavioral isolation. Critical support for this process is already in place with 1) interpopulation differentiation in calls, 2) behavioral reproductive isolation based on call attributes, and 3) presumptive directional selection at one of the populations.

Our data suggest that call-based female mate choice has resulted in behavioral isolation. In addition, we demonstrate that interpopulation isolation can be much deeper than previously documented and can involve not only strong discrimination but also lack of recognition for foreign signals.

Boul et al. (2007) hypothesize a mechanism for divergence under sexual selection: Yasuní females prefer complex calls, and call complexity is negatively correlated with whine frequency. By selecting for greater complexity, females indirectly selected for lower frequency whines, which resulted in behavioral isolation. If low frequency whines are a result of female preference for complex calls, we would expect to find female preferences for complex calls at Puyo. Our results suggest that females do not prefer complex calls, lacking consistency with Boul et al. (2007) hypothesis because Puyo have simple calls, and yet, its whine-dominant frequency is closer to Yasuní (complex call) than La Selva (simple call).

Is behavioral isolation a product of reinforcement?

The presented evidence is also compatible with reinforcement, a process that favors divergence in mate recognition traits and/or mate preferences in closely related species that interbreed. This hypothesis is supported by the geographic pattern of call divergence because there is large differentiation in calls and preferences between geographically adjacent populations (Yasuní and La Selva are separated by only 20 km). Thus, periodic contact between both lineages could allow hybridization. Hybrids with lower fitness would favor divergence in mate choice traits and preferences. Interestingly, the behavioral isolation between Puyo and La Selva would be an incidental by-product of reinforcement between La Selva and Yasuní. This form of rapid allopatric speciation as indirect consequence of reinforcement has been documented in other systems (Zouros and d'Entremont 1980; Hoskin et al. 2005).

A potential caveat on this hypothesis is the fact that Yasuní and La Selva are on opposite sides of the Napo River (width 1–2 km), which could act as a barrier preventing frequent hybridization. Funk et al. (2007) found no support for the Napo River as a barrier generating the basal divergence among populations in the northwest Amazon Basin and the lower Napo. However, the possibility of the Rio Napo acting as a barrier between more closely related populations have been not tested.

Boul et al. (2007) did not find hybrids among 90 individuals collected at Yasuní, La Selva, and Tiputini (a population ~30 km south from Yasuní and genetically similar to Yasuní). However, the absence of La Selva × Yasuní or La Selva × Tiputini

hybrids was expected because the putative hybridizing parental types are not known to coexist at either of those 3 sites. The exact boundaries between La Selva and Yasuní genetic groups are unknown, and it is unclear if a contact zone exists.

CONCLUSIONS

We present evidence that divergence of the mate recognition system promotes speciation in the frog *E. petersi*. Our study documents significant interpopulation differences in the structure of courtship signals and strong behavioral isolation between 2 populations. Signal differentiation cannot be readily explained by geographic or genetic distances between populations but instead is consistent with the pattern of female mate choice. In addition, we present evidence implying that interpopulation divergence in signal structure is inconsistent with divergence by genetic drift. With the combined evidence, we propose 2 nonexclusive hypotheses to explain behavioral isolation and incipient speciation in this system: sexual selection and reinforcement. Sampling of call variation and female choice in other populations is necessary to complement available data and test conclusively these hypotheses.

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