Incipient sexual isolation in *Laupala cerasina*: Females discriminate population-level divergence in acoustic characters

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**Abstract** Sexual selection by female choice can shape the evolution of male traits within populations, since the most attractive males experience an increase in fitness through elevated mating success. Speciation by sexual selection occurs when evolution in traits and preferences within populations causes differentiation among populations, such that females in alternative populations prefer sexual signals of their own population relative to others. Differentiated traits and preferences thereby play an active role in limiting gene flow between divergent populations. The effectiveness of differentiated preferences in maintaining differentiated male signals against the homogenizing effects of gene flow across populations will be limited by both the degree to which females can discriminate against non-local males, and the breeding values of traits and preferences. Populations of the Hawaiian cricket *Laupala cerasina* have diverged in pulse rate, a sexually selected male signal, and female acoustic preference for pulse rate. Gene flow between neighboring populations may be reduced if migrants from sexually diverged populations experience reduced mating success. We show that females discriminate among divergent songs characteristic of neighboring populations, that differences among populations in song and preference breed true in a common environment, and that mean preferences for each population closely match the mean pulse rates. Divergence in preference was observed only between populations that also differed in song. Along with a striking ability to discriminate slight differences in song, correlated evolution of song and preference within populations could be a mechanism that promotes assortative mating among populations, thereby reducing gene flow, and leading to speciation in *Laupala* [Current Zoology 58 (3): 416–425, 2012].

**Keywords** Sexual selection, Phenotypic divergence, Behavioral isolation, Preference functions, Speciation

More than a century ago, Darwin (1871) first observed that secondary sexual characters diverge rapidly among closely related species, suggesting that sexual selection is an important mechanism influencing evolution within species. Much more recently, divergent sexual selection among populations has been proposed as a mechanism that could drive speciation (Coyne and Orr, 2004; Panhuis et al., 2001; Ritchie, 2007; West-Eberhard, 1983). Since sexual selection within species is widely reported (Andersson, 1994), its action within geographically isolated populations may lead to population divergence across a broader geographic range (Endler, 1992; Lande, 1982). Indeed, because isolated populations experience no selection pressure to maintain reproductive compatibility, their divergence is possibly inevitable (Mayr, 1963; Turelli et al., 2001; West-Eberhard, 1983).

While divergence through natural selection (Schluter, 2009; Schluter and Conte, 2009; van Doorn et al., 2009) or the evolution of genetic incompatibilities (Pryke and Griffith, 2009) as by-products may contribute, there are several reasons to suspect that sexual selection actively facilitates speciation. First, reproductive isolation is more likely to result from a divergence in traits directly involved in mate choice, such as sexually selected characters (Panhuis et al., 2001; West-Eberhard, 1983). Second, in several taxonomic groups, behavioral (and especially sexual) isolation appears to evolve at a faster rate than hybrid incompatibilities (Alexander and Breden, 2004; Coyne and Orr, 1997; Mendelson, 2003). Third, comparative studies using various proxies for the intensity of sexual selection have indicated that phylogenetic clades with higher levels of sexual selection (and sexual conflict) tend to be species-rich (Arnqvist et al., 2000; Barraclough et al., 1995; Kraaijeveld et al., 2011; Mitra et al., 1996). Lastly, the rapid evolution of sexual signaling is a key feature associated with diversification in two systems demonstrating accelerated rates of speciation (cichlids, Danley and Kocher, 2001; crickets, Mendelson and Shaw, 2005).
Demonstrating that sexual selection has contributed to speciation requires detailed studies of specific populations in the process of divergence. First, sexual selection within populations must occur or have occurred, resulting in differentiation among populations in the phenotypic targets of selection. Second, differentiation in these phenotypic targets must be substantial enough to actively discourage gene flow between populations. For example, females must be capable of discriminating between males from their own versus phenotypically distinct neighboring populations. Under these circumstances, coevolution of signal and preference is expected to bring about the assortative mating that can close the reproductive boundary between incipient species (Kirkpatrick, 1982; Lande, 1981).

Many species exhibit minor inter-populational differences in male sexual signals (Andersson, 1994; Gerhardt and Huber, 2002); however, geographic variation in sexual signals may not always reflect a correlated divergence in female preference for those signals (Hill, 1994; van der Sluijs et al., 2010; Zuk et al., 2006). Several studies of various frog species have found that divergence in male acoustic characters among populations may have been the result of divergent sexual selection (Boul et al., 2007; Prohl et al., 2006); however, these studies have not addressed differences in the shape of female preference functions in populations in the process of divergence. In contrast, many studies of divergent sexual selection in crickets do indicate divergent preference functions (Gray and Cade, 2000; Shaw, 2000b); however, these studies document differences at the interspecific level. In the cricket *Teleogryllus oceanicus*, populations vary in the shape of the female preference function but these differences are not correlated with male song, which is constrained by strong natural selection from predators (Simmons et al., 2001). Understanding the shape of female preference functions among populations in the early stages of divergence is critical, because this is the time when these initially minor differences have the greatest potential to either accelerate or hinder the rate of population divergence by impacting the degree of gene flow.

The Hawaiian cricket genus *Laupala* makes an ideal candidate for studying questions of sexual selection and speciation. The 38 constituent species evolved recently and are morphologically cryptic (Mendelson and Shaw, 2005; Otte 1994; Shaw, 2000a), yet distinctive in pulse rate of the male calling song. Male song is easily quantified and synthesized (Otte, 1994; Shaw, 1996) and females readily respond to songs in the course of locating potential mates. Furthermore, the interspecific differences in both pulse rate and preference have a genetic basis (Oh et al., in press; Shaw and Lesnick, 2009; Shaw et al., 2007; Wiley and Shaw, 2010).

Within the species *Laupala cerasina*, pulse rate has been shown to be a sexually selected trait that varies extensively among populations, with females exhibiting correspondingly variable preferences for pulse rates at or near the male population mean (Grace and Shaw, 2011; Mendelson and Shaw, 2002; Shaw, 2000b; Shaw and Herlihy, 2000). In addition, evidence supports a genetic basis to intraspecific variation in song and preference in *L. cerasina* (Grace and Shaw, 2011; Otte, 1994; Shaw, 1999). Because the within-species divergence in these sexual communication traits mirrors the differences between species, albeit to a lesser degree, we hypothesized that *L. cerasina* acoustic variation reflects incipient speciation by sexual selection. In the present study, we test whether females are capable of distinguishing among the slight differences in pulse rate that characterize geographically adjacent populations (~ 0.2 pulses per second, pps) across the range of *L. cerasina* on Hawaii Island (known as the Big Island of Hawaii). If females are to influence the reproductive fate of migrant males from phenotypically distinct populations, they are expected to exhibit biased phonotactic responses to songs that differ by this order of magnitude. Furthermore, when neighboring populations are compared, females should express divergent preferences, thus favoring songs representative of homotypic over heterotypic males. Finally, if coordinated changes arise due to selection within populations, we expect to see evidence of this in the form of genetic correlations between song and preference within families.

1 Materials and Methods

*Laupala cerasina* were collected from eight locations on the Big Island of Hawaii in July 2005, hereafter referred to as populations. The collecting sites cover the majority of the range of *L. cerasina*, both geographically and phenotypically, and included the following paired comparisons: A) Muliwai Plateau (MP) vs. Waimea Reservoir (WM) (7.55 km apart), B) Kalopa Park (KP) vs. Laupahoehoe (LP) (21.26 km apart), C) Eucalyptus Toe (ET) vs. Kawaiwiki (KW) (4.08 km apart), and D) Glenwood Road (GR) vs. Naulu Trail (NT) (12.46 km apart). Specific geographic coordinates are reported in Grace and Shaw (2011). These pairings were designated *a priori* because a previous study of wild-caught individuals found that two of these popula-
tion pairs showed significant difference in pulse rate (B and D) and two did not (A and C) (Grace and Shaw 2011). Likewise, wild-caught females showed a significant difference in preference in two population pairs (B and D), while two did not (A and C), albeit based on a course-grained preference experiment (see ‘Quantifying female discrimination’ below).

1.1 Rearing conditions
Crickets collected from Hawaii as adults were housed individually to allow collection of eggs produced from natural matings prior to capture. Wild-caught nymphs were reared to maturity and then randomly paired with a mate from the same population to generate offspring. Eggs collected from wild-inseminated adult females and through laboratory matings of wild-caught individuals were reared to maturity in the laboratory at the University of Maryland. Individuals were reared under a 12:12 light/dark cycle in a temperature-controlled room maintained at 20°C. Small nymphs were reared in 1-quart glass jars with no more than 20 individuals per jar, and larger nymphs and adults were kept in plastic specimen cups, with no more than three per cup, separated by sex. Individuals were provided with moistened kimwipes for moisture and fresh cricket chow (Fluker Farms, Louisiana) treated with the mold inhibitor methyl paraben (Tegosept, Fisher Scientific) once per week. These first generation lab-reared offspring were used in the experiments described below.

1.2 Quantifying song differentiation
To record songs, males were placed beneath clear plastic cups fitted with mesh covers in a temperature-controlled room maintained at 20°C. Upon singing, males were recorded onto cassette tapes using a Sony Professional Walkman (Model WM-D6) and Sony condenser microphone. A female from the same population was introduced if the male did not sing spontaneously (Laupala males produce only one type of song in both long and short distance contexts). The songs of 260 first generation lab-reared males were recorded and digitized using Raven 1.2 (Cornell University, Ithaca, NY). The pulse period was determined for each male by measuring the duration between the beginning of one pulse and the beginning of the next. Five estimates of pulse period were obtained per male, using five independent pairs of pulses within a single recording. These values were averaged and then transformed to calculate pulse rate (the inverse of the pulse period), which is the primary song feature that differs among species of Laupala and among populations of L. cerasina. The coefficient of variation (CV) was calculated for each individual by dividing the standard deviation by the mean and multiplying by 100. Pulse rates and CVs have previously been reported for wild-caught L. cerasina individuals from these populations (Grace and Shaw 2011). Planned comparisons were conducted between the songs of males from neighboring populations (population pairs A–D) using t-tests.

1.3 Quantifying female discrimination
Prior work has demonstrated that females of L. cerasina show geographic variation in pulse rate preference that matches geographic variation in pulse rate (Grace and Shaw 2011). However, this prior work did not test whether females are capable of fine-scale pulse rate discrimination on the order of population-level differences in song. Our objective was to determine the extent to which females can discriminate between the minor pulse rate differences that characterize neighboring populations in this species, and whether females exert divergent preferences as a result of these perceived differences in song. We simultaneously evaluate whether females prefer males from their local population.

Virgin females approximately 20–25 days past the final molt were used in phonotaxis trials. Females of this age show a high level of responsiveness to synthesized cricket songs (Grace and Shaw, 2011). Since several adjacent populations of L. cerasina express a song divergence of around 0.2 pulses per second, our experimental design tested for this level of discrimination. To test for female discrimination and preference differentiation, six trials were conducted, spanning the range (c. 2.3–2.9 pps at 20°C) of variation in L. cerasina, and differing in the pulse rate choices presented to the female: 2.0 vs. 2.2, 2.2 vs. 2.4, 2.4 vs. 2.6, 2.6 vs. 2.8, 2.8 vs. 3.0, and 3.0 vs. 3.2 pps. All parameters other than pulse rate of the synthetic songs were held constant at values that approximate the natural parameters found in Laupala species: pulse duration, 40 ms and carrier frequency, 5 kHz (Shaw and Herlihy 2000). A total of 233 females were used, once in each trial (six trials total per female).

Phonotaxis trials were conducted in a circular arena (radius 47 cm) housed in a temperature-regulated (20°C), acoustically buffered sound chamber (Acoustic systems) illuminated by red gel-filtered lights. Two speakers (Radio Shack 40-1218) broadcast programmable, digitally synthesized, pulsed sinusoidal tones created using custom-designed software (T.G. Forrest, Univ. of North Carolina, Asheville) from opposite ends of the arena. A 16-bit digital/analog converter (Tucker-Davis Technologies) and 10 kHz filter (Krohn-Hite 3322)
processed the acoustic output. A digital attenuator (Brüel and Kjaer 4155) was used to monitor and equalize sound pressure levels between speakers.

For each trial, playbacks occurred for an acclimation period of five minutes, during which time a female was restrained beneath a plastic cup in the center of the arena. Once the cup was raised, she received an additional five minutes to respond to the acoustic stimuli. A 10-cm score zone was designated around each speaker (as in Fig. 2 of Shaw and Herlihy, 2000). Once a female entered the score zone associated with a speaker, the trial was ended. She received a score of one for approaching the faster song, or zero for the slower song. If the female did not approach either score zone after five minutes, no score was recorded for that trial. A random number generator was used to determine the order of presentation and the speaker assigned to play the faster/slower song. Females were used in two trials per day over three consecutive days to complete the series of six trials.

To determine whether females discriminate between songs differing by 0.2 pulses per second, the proportion of females that approached the faster song of a given pair was compared to the null expectation under a model of random choice, where the probability of choosing either song is 0.5. The 95% confidence intervals for each proportion were calculated from the binomial distribution. If these confidence intervals excluded 0.5, the population showed a significant response bias towards either the fast or the slow song of a given trial.

To determine whether neighboring populations differ in their song preferences, female preference data were further analyzed using the statistical program R. To estimate the mean female preference for a population, all female phonotaxis data from a given population were analyzed simultaneously using logistic regression. The point at which females are equally likely to choose either the fast or the slow song (probability of 0.5) was determined for each population, providing an estimate of the mean pulse rate preference (Grace and Shaw, 2004, 2011; Shaw and Herlihy, 2000). Standard errors for this estimate were calculated for each population by bootstrap analysis, which randomly resampled the data with replacement, while maintaining the original sample size, for 1000 replicates (Grace and Shaw, 2011). For each replicate, the mean pulse rate preference was estimated, allowing a calculation of the standard deviation of the means (i.e., the standard error). Planned comparisons between neighboring population pairs were also performed using the Wald $\chi^2$ test (Grace and Shaw, 2004).

### 1.4 Song and preference covariation across time and space

Spearman correlation analyses were used to compare the mean population estimates for pulse rate and pulse rate preference obtained here for first-generation laboratory-reared individuals with previously published population means (Grace and Shaw, 2011) for these traits obtained from wild-caught crickets from the same populations. Additionally, the mean estimates for pulse rate and preference obtained in this study were used in another Spearman correlation analysis to determine whether the correlation between song and preference was maintained across populations following one full generation in a common-garden environment.

### 1.5 Quantifying genetic correlations within populations

In addition to investigating population-level differences in pulse rate and pulse rate preference, the genetic covariance among song and preference was examined for families within populations. Means for pulse rate and pulse rate preference were estimated from full sibs within families for each family in the data set for which both song and preference data were available. Because the number of families meeting this sampling requirement within each population was typically small (4 to 11 families per population), the residuals of the family means from the population means were calculated to adjust for population differences. A Spearman correlation analysis was performed using the residuals.

### 2 Results

#### 2.1 Male song variation

Populations that showed evidence of pulse rate differentiation in the wild-caught generation (KP-LP and GR-NT; Grace and Shaw 2011) also showed significant pulse rate differentiation between males of the first lab generation (first generation comparisons: KP-LP: $t=-12.21$, $P<0.0001$; GR-NT $t=8.36$, $P<0.0001$; MP-WM: $t=0.10$, $P=0.92$; ET-KW: $t=1.68$, $P=0.095$; Table 1). The average coefficient of variation for pulse rate measurements within individuals was 0.75%, which corresponds to about 0.03 pulses per second.

#### 2.2 Female discrimination

All eight populations showed a similar response pattern. At the low end of the trial series, females responded preferentially to the faster song of the pair, and at the high end, they responded preferentially to the slower song (Fig. 1). In the eight *L. cerasina* populations examined, females showed biased responses to the
Fig. 1  Geographic variation in female phonotaxis responses

The y-axis shows the proportion of females responding to the faster pulse rate in a two-choice trial, along with the 95% confidence intervals. The dashed line at 0.5 indicates no preference for a given trial; populations whose confidence intervals for a given trial exclude 0.5 show significant preference for one of the two pulse rates in the trial. Trials in which the confidence intervals of one population exclude the mean preference of the other population have significantly different preferences for that trial. Plots are organized according to planned population comparisons: A) Muliwai Plateau and Waimea Reservoir B) Kalopa Park and Laupahoehoe C) Eucalyptus Toe and Kaiwiki D) Glenwood Road and Naulu Trail. Below each panel, the closed and open bars indicate the 95% confidence intervals for male pulse rate for each population. In the center, a topographic map of the Big Island of Hawaii shows the collecting locations, where boxes indicate the planned pairwise comparisons between neighboring populations.
slow or the fast song offered (thus differing significantly from a null response probability of 0.5) in at least two of the six trials (average: 4; range: 2 to 6; Fig. 1). Biased responses in these trials are indicative of females’ ability to distinguish two songs differing by 0.2 pulses per second.

2.3 Female preference variation

We used the results from all six trials together to estimate the mean pulse rate preference for each of the eight populations. In each population, a single transition in response from the faster (at the low end of the trial range) to the slower (at the fast end of the trial range) song was used to estimate the inflection point wherein response to the faster song equaled that of the slower song. We estimated the inflection point using logistic regression (Table 1) and equated this estimate to the mean preference for the population (Fig. 1). Standard errors were calculated using the bootstrapping procedure outlined above. When preferences were compared across populations, two of the four planned comparisons (KP-LP and GR-NT) showed significantly different female preference functions (KP-LP: Wald $\chi^2=12.12$, $P=0.0005$; GR-NT: Wald $\chi^2=11.49$, $P=0.0007$; Fig. 1; Table 1). These were the same populations that differed significantly in pulse rate (Table 1). Populations that did not differ significantly in pulse rate also did not differ significantly in preference (MP-WM: Wald $\chi^2=0.57$, $P=0.45$; ET-KW: Wald $\chi^2=0.10$, $P=0.75$; Fig. 1; Table 1).

Table 1  Population variation in male song and female song preference estimated from first lab generation individuals

| Pair | Population | PR ± SE, n | PR Pref ± SE, n |
|------|------------|------------|----------------|
| A    | MP         | 2.604±0.019, 33 | 2.439±0.123, 30 |
|      | WM         | 2.602±0.016, 33 | 2.541±0.054, 30 |
| B    | KP         | 2.421±0.014, 46 | 2.325±0.097, 28 |
|      | LP         | 2.695±0.015, 33 | 2.693±0.091, 32 |
| C    | ET         | 2.903±0.016, 35 | 2.830±0.057, 28 |
|      | KW         | 2.852±0.030, 15 | 2.851±0.096, 25 |
| D    | GR         | 2.683±0.018, 33 | 2.673±0.065, 30 |
|      | NT         | 2.479±0.017, 32 | 2.354±0.099, 30 |

Mean pulse rate (PR; pulses per second) of male song and mean female pulse rate preference (PR Pref; pulses per second) are shown, along with sample size (N) and one standard error (SE). The letters A, B, C and D refer to the planned comparisons (see figure 1) between population pairs.

2.4 Song and preference covariation across time and space

Both preference and pulse rate means were significantly correlated between the wild-caught generation and the laboratory generation (preference: Spearman $r=0.929$, $P=0.0009$; pulse rate: Spearman $r=0.976$, $P<0.0001$; Fig. 2). There was also a significant correlation between preference and pulse rate means in the laboratory generation (Spearman $r=0.952$, $P=0.0003$; Fig. 2), consistent with the pattern found in the wild-caught generation (Grace and Shaw 2011).
2.5 Genetic correlations between song and preference within populations

Song and preference data were available for 57 families. After accounting for population differences through the calculation of residuals, pulse rate and preference were significantly correlated across families (Spearman $r=0.357$, $P=0.0064$; Fig. 3).

![Fig. 3 Genetic correlations within populations](image)

Population-adjusted residuals of full sibling female pulse rate preference means plotted against male pulse rate means for 57 families are shown (Spearman $r=0.357$, $P=0.0064$). The linear best-fit curve was fitted to the data to illustrate the slope of the relationship ($y = -8.6 \times 10^{-11} + 0.15x$).

3 Discussion

During the process of speciation, divergent sexual selection among populations should eventually result in reproductive isolation among species, but the factors that facilitate this transition are poorly understood (Panhuis et al., 2001; Ritchie, 2007). If divergent sexual selection among populations drives speciation by causing assortative mating and sexual isolation, females (or males, when choosey) from the divergent populations must have evolved distinctive preferences and display the ability to discriminate between the signals of local (homotypic) versus non-local (heterotypic) males. Under these conditions, sexual selection should play an active role in limiting gene flow among populations and thereby causally be involved in speciation. The resolution of the evolutionary trajectories of signal and preference traits can allow us to demonstrate whether divergence in sexual communication is a cause or consequence of the differentiation leading to speciation and whether gradual or significant change is required to limit gene flow.

In *Laupala cerasina*, females from neighboring populations showing small but significant differences in pulse rate displayed preferential phonotaxis toward songs of their local population over the adjacent population (pairs B and D). As expected, females from neighboring populations that did not differ in pulse rate likewise did not differ in pulse rate preference (pairs A and C). In all populations, however, female preference means corresponded to male pulse rate means (Fig. 2), corroborating and extending previous findings (Grace and Shaw, 2011). The fact that we made these observations in first-generation laboratory-reared crickets, and that they match wild-caught values (Fig. 2) suggests a genetic basis to differentiation (although maternal effects cannot be ruled out in the present experimental design, previous work suggests that they do not account for such differences; Shaw and Lesnick, 2009; Grace and Shaw, 2011). Based on the results presented here, we predict that even small differences in song and preference are sufficient to initiate a pattern of assortative mating across the geographic range of *L. cerasina*, supporting an important feature of the hypothesis of incipient speciation by sexual selection. Further divergence in female acoustic preference in any particular population could result in complete sexual isolation and speciation.

While this study does not address the hypothesis that divergent signaling leads to variable reproductive success among males from the divergent populations, extending this type of analysis to include assortative mating trials, including closely related sister species, is underway (Grace and Shaw, in prep.).

Although there is no evidence for differentiation by distance with respect to pulse rates across the entire range of *L. cerasina* (Grace and Shaw, 2011), such a pattern might pertain over the shorter distances such as those involved in this study. Limited sample size precludes a test of this hypothesis here, but it is worth noting that the two population pairs that showed the least divergence in song and preference (pairs A and C) also happened to be separated by the shortest geographic distances. In addition to potentially experiencing more similar selection pressures, geographically closer populations are also expected to exchange a higher frequency of migrants, all else being equal, and this exchange might prevent differentiation due to higher levels of gene flow, providing a counterweight to divergent sexual selection.

The pulse rate discrimination ability of females in this study is striking. Indeed, it seems possible that fe-
males are capable of even finer pulse rate distinctions than can be inferred here, given that statistically distinct pulse rate means can occur when songs differ by as little as 0.1 pulses per second (e.g., Waimea vs. Laupahoehoe; $t = -4.25, P < 0.0001$). However, with the phonotaxis preference design used here, our inference is limited to concluding that females can detect and discriminate pulse rate differences at least as small as 0.2 pulses per second.

An explanation for the resolution of female discrimination ability could derive from multiple levels of analysis, including genetic, physiological and/or evolutionary mechanisms. Quantitative trait locus studies in the closely related species pair Laupala paranigra and L. kohalensis demonstrate that the phenotypic effect sizes of preference alleles range from 0.11-0.239 pulses per second (Wiley et al., 2012), roughly corresponding to the magnitude of differentiation examined in this study. Thus, single mutational effects alone may result in phenotypic variation that contributes to or dictates discrimination ability. It is not presently known, however, if QTL effect sizes are due to single mutations, nor whether QTL in these other Laupala species are common to those underlying song and preference divergence in L. cerasina. The resolution of discrimination may also be dictated by the physiological or psychoacoustic mechanism that underlies temporal pattern generation and recognition. Mechanistic hypotheses of male and female traits frequently involve linkage through common neuronal oscillators or functionally coupled neuronal filters (Hedwig, 2006; Hoy, 1992). Likewise, the discrimination ability of females may depend on the ratio, rather than the difference, of two stimuli (Akre et al., 2011). The ability of females to consistently discriminate very similar pulse rates and prefer the pulse rates characteristic of their own population may also be the result of selection in, say, the context of acoustical interference in the natural environment. Laupala cerasina typically occurs with congeners whose songs are quite similar in all respects but pulse rate (Otte, 1994). Selection may favor discrimination abilities that reduce acoustical interference between species of Laupala. These are not competing explanations but address different facets of the question of what determines discrimination ability. Further investigations into the causes of female discrimination ability should be both feasible and rewarding.

For sexual selection to drive speciation, female discrimination and preference for local over foreign signals must evolve, implying a genetic component. Our finding that covariation in song and preference exists among families (Fig. 3) provides further evidence that a mechanism of sexual selection has caused the geographical correspondence between preference and signal values. Few studies have demonstrated this pattern, even in systems where sexual selection acts on traits used in sexual communication. In the Amazonian frog Physalaemus petersi, females prefer the songs of local males to those of foreign males, and song divergence is correlated with reduced gene flow (Boul et al., 2007). However, a separate study in the closely related congener P. pastulosus found that females prefer the calls of local males over those of foreign con specifics only one-third of the time in choice trials (Pfennig and Ryan, 2007). All of these studies were conducted under field conditions in the natural environment, so a genetic basis is difficult to infer. The use of laboratory-born individuals in this experiment provides evidence that the observed differences in song and preference among populations of Laupala have a genetic basis. The environmental variation that might contribute to total phenotypic variation is reduced, since these individuals experienced a common environment throughout their lives.

In summary, the differentiation of sexual traits in L. cerasina is a predicted precursor to sexual isolation that characterizes distinct species in this genus. Because females demonstrated the ability to discriminate the pulse rates characteristic of neighboring populations, divergence in pulse rate of the male calling song together with divergent preferences has the potential to reduce gene flow, since rare male migrants between distinct populations should have difficulty attracting mates. In L. cerasina and other Laupala, acoustic preference is only the first opportunity for sexual selection to influence mate choice, and further studies are needed to specifically address the hypothesis that divergent acoustic preference predicts assortative mating. In addition, the influence of variation in pulse rate relative to other male song characters (e.g., pulse duration, carrier frequency) in determining overall song attractiveness has not been assessed in Laupala, but could have further consequences for female preference evolution (Gerhardt, 2008; Gerhardt and Brooks, 2009). The finding that females can discriminate among songs so precisely goes a long way towards demonstrating that these subtle differences in song are maintained by sexual selection, and ultimately, that sexual selection may be driving speciation in this group.
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