

# GEOGRAPHIC VARIATION IN THE SONGS OF NEOTROPICAL SINGING MICE: TESTING THE RELATIVE IMPORTANCE OF DRIFT AND LOCAL ADAPTATION

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Patterns of geographic variation in communication systems can provide insight into the processes that drive phenotypic evolution. Although work in birds, anurans, and insects demonstrates that acoustic signals are sensitive to diverse selective and stochastic forces, processes that shape variation in mammalian vocalizations are poorly understood. We quantified geographic variation in the advertisement songs of sister species of singing mice, montane rodents with a unique mode of vocal communication. We tested three hypotheses to explain spatial variation in the song of the lower altitude species, *Scotinomys teguina*: selection for species recognition in sympatry with congener, *S. xerampelinus*, acoustic adaptation to different environments, and stochastic divergence. Mice were sampled at seven sites in Costa Rica and Panamá; genetic distances were estimated from mitochondrial control region sequences, between-site differences in acoustic environment were estimated from climatic data. Acoustic, genetic and geographic distances were all highly correlated in *S. teguina*, suggesting that population differentiation in song is largely shaped by genetic drift. Contrasts between interspecific genetic-acoustic distances were significantly greater than expectations derived from intraspecific contrasts, indicating accelerated evolution of species-specific song. We propose that, although much intraspecific acoustic variation is effectively neutral, selection has been important in shaping species differences in song.

**KEY WORDS:** Acoustic adaptation, bird song, character displacement, ecological selection, mammal, speciation.

Understanding the origins of phenotypic diversity is a fundamental goal of evolutionary biology; few phenotypes are as diverse as signals used in intraspecific communication (Endler 1992; Bradbury and Vehrencamp 1998). Acoustic signals make particularly good models for signal evolution because variation is readily quantifiable, and population and species differences can accrue over short evolutionary timescales with significant impact

on receiver response (MacDougall-Shackleton and MacDougall-Shackleton 2001; Mendelson and Shaw 2002; Nelson and Soha 2004; Mendelson and Shaw 2005; Jang and Gerhardt 2006; Pröhl et al. 2006; Klappert et al. 2007; Nicholls 2008). For example, female túngara frogs discriminate against male advertisement calls from geographically disjunct populations (Pröhl et al. 2006), and differences in male pulse rate drive assortative mating in the recent radiation of Hawaiian crickets (Mendelson and Shaw 2005). Given that acoustic signals can act as major isolating mechanisms between young and incipient species, discriminating the processes that shape intraspecific acoustic variation contributes to the long-standing debate over the relative importance of selection and drift

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in the process of speciation (Rice and Hostert 1993; Gavrilets 2003; Coyne and Orr 2004).

Although signal evolution is rarely explained by a single selective force, work in birds and anurans suggests that general aspects of the design and function of acoustic signals may bias sensitivity to different sources of selection. For example, the territorial advertisement songs of male oscine birds are acoustically complex signals that can transmit information about species membership, individual identity, breeding status, and quality to receivers at varying distances (Catchpole and Slater 1995; Mathevon et al. 2008). Work in song birds has emphasized the role of ecological selection in shaping the fit between song and transmission efficiency in different environments, with the expectation that spectral and temporal properties of acoustic signals should covary with environmental attributes that influence sound propagation, such as microclimate, vegetation structure, and ambient noise ("acoustic adaptation," Morton 1975; Wiley and Richards 1982). Although many other factors contribute to song divergence in birds (e.g., correlated evolution with body size, Ryan and Brenowitz 1985; selection for species recognition, Seddon 2005; Kirschel et al. 2009), support for habitat- or climate-driven song differences is prevalent in recent studies (Slabbekoorn and Smith 2002; Baker 2006; Nicholls et al. 2006; Rugg et al. 2006; Dingle et al. 2008; but see Kroon and Westcott 2006; Irwin et al. 2008), suggesting that complex acoustic signals with high information content may be especially prone to environmental degradation.

Relative to oscine bird song, the acoustic structure of anuran advertisement calls is simple and highly stereotyped. Calls function solely in female attraction and male–male competition for mates, and calling males can experience intense competition for acoustic space (Gerhardt 1994). Geographic variation in anuran advertisement calls is rarely correlated with acoustic properties of different environments; signal divergence is better explained by population differences in female mate preferences, geographic overlap with congeners, and clinal variation in body size (Zimmerman 1983; Narins and Smith 1986; Penna and Solís 1998; Kime et al. 2000; Gerhardt and Huber 2002; Pröhl et al. 2006, 2007; Moriarty Lemmon 2009; but see Ryan et al. 1990). Thus, the relatively simpler structure and information content of anuran calls may reduce signal sensitivity to the acoustic properties of different environments, while strong intraspecific competition for acoustically orienting mates enhances response to sexual selection (Boul et al. 2007). Likewise, in multispecies mating assemblages, both interspecific competition for signal space (Chek et al. 2003; Amézquita et al. 2006) and selection against heterospecific mating (Hobel and Gerhardt 2003; Moriarty Lemmon 2009) favor acoustic character displacement.

Although neutral evolution is the fundamental alternative to adaptive hypotheses, the potential contribution of neutral pro-

cesses is not consistently addressed in studies of acoustic variation. Explicit tests of neutral divergence require comparison of acoustic and genetic distance; a strong positive correlation suggests that acoustic divergence is largely stochastic. Of the studies that have incorporated this test, most have found little evidence for divergence due to drift (e.g., Soha et al. 2004; Wright et al. 2005; Nicholls et al. 2006; Pröhl et al. 2006; Rugg et al. 2006; Huttunen et al. 2008). However, recent comparison of patterns of geographic variation in the learned song and innate calls of a passerine bird found a significant positive correlation between genetic and acoustic distances for both types of vocalization indicating that, under some conditions, stochastic processes can play a major role in acoustic divergence (Irwin et al. 2008).

In mammals, the relative contributions of selection and drift to intraspecific acoustic variation are largely unexplored. Patterns of geographic variation in acoustic signals are documented in cetaceans (Helweg et al. 1998; Delarue et al. 2009), primates (Mitani et al. 1992; Delgado 2007; Wich et al. 2008), bats (Law et al. 2002; Aspetsberger et al. 2003; Russo et al. 2007; Yoshino et al. 2008), pikas (Borisova et al. 2008), and in the alarm calls of sciurid rodents (Gannon and Lawlor 1989; Slobodchikoff et al. 1998; Yamamoto et al. 2001; Eiler and Banack 2004). In most cases, however, the processes underlying these patterns are unknown. Cultural transmission is a probable source of geographic variation in whale songs (Deecke et al. 2000; Rendell and Whitehead 2001; Mercado et al. 2005), primate long-distance calls (Mitani et al. 1992), and bat social calls (Esser and Schubert 1998; Yoshino et al. 2008), but the relative roles of cultural and genetic drift remain to be determined (Delarue et al. 2009). Likewise, strong support for the hypothesis that selection for species recognition drives dramatic shifts in echolocation call frequency within and between closely related species of rhinolophoid bats awaits more rigorous tests of alternatives (Heller and Von Helversen 1989; Kingston et al. 2001; Russo et al. 2007).

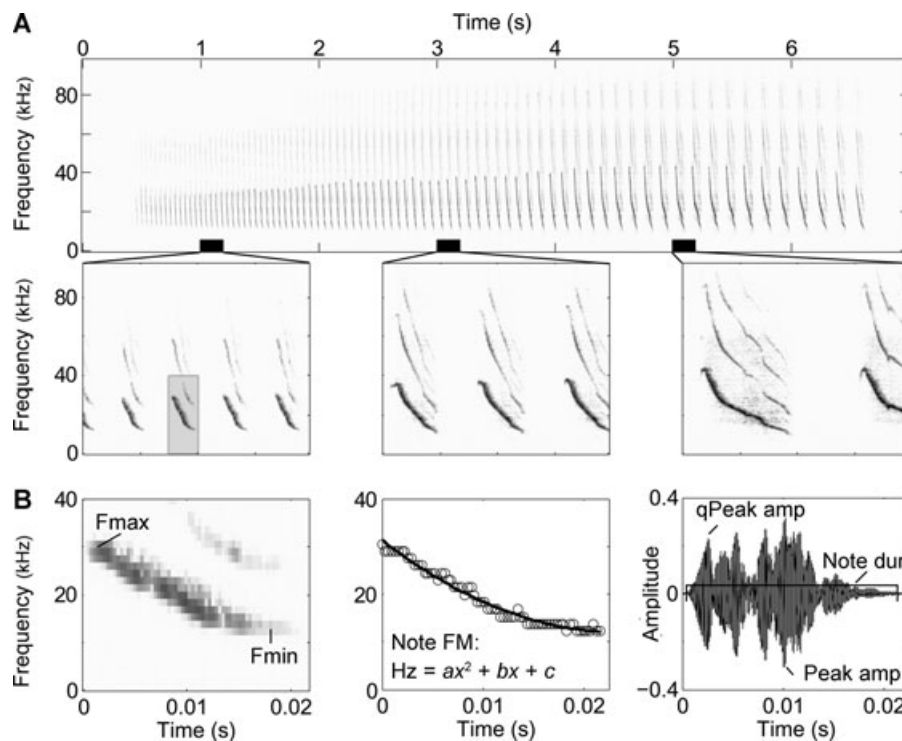
Here, we quantify geographic variation in the advertisement songs of sister species of Neotropical singing mice, *Scotinomys teguina* and *S. xerampelinus*, and ask what processes best explain observed patterns. Singing mouse advertisement songs are highly stereotyped but acoustically complex. Thus, the signal combines characteristics of the two best-studied acoustic communication systems: anuran advertisement calls and bird song. As such, singing mice represent an interesting mammalian system in which to explore the roles of deterministic and neutral processes in shaping signal variation. To our knowledge, this is the first study to evaluate the relative contributions of selection and drift to acoustic divergence in a mammal. Because the mice are a novel system in the study of acoustic communication, we summarize relevant aspects of their ecology, distribution, and vocal behavior below.

## Study System

*Scotinomys teguina* and *S. xerampelinus*, are small (10–15 g), diurnal, terrestrial murid rodents (Hooper 1972). Although phylogenetic data support a North American origin for the genus (Bradley et al. 2004; Jansa and Weksler 2004), extant populations of singing mice are limited to cool, high-elevation sites in Central America. The distribution of *S. teguina* is extensive but highly fragmented: populations occur at elevations of 1000–2930 m, from Chiapas State in southern Mexico to western Panamá, with major disjunctions across lowland areas in Honduras and Nicaragua. *Scotinomys xerampelinus* is known only from altitudinally isolated populations along the Cordillera de Talamanca in Costa Rica and Panamá, where it is restricted to montane cloud forest and páramo at 2150–3300 m (Hooper and Carleton 1976; Blondel et al. 2009). In this region, *S. xerampelinus* replaces *S. teguina* at elevations above 2300–2900 m. Sympatric populations are mainly altitudinally segregated but come into contact in narrow zones of syntopy, typically less than 1000 m wide. It is thought that these narrow bands of contact are maintained by a combination of interspecific competition and species differences

in thermal tolerance (Hooper and Carleton 1976). There is no evidence for seasonal reproduction in either species; in Panamá and Costa Rica pregnant females are captured throughout the year (Hooper and Carleton 1976).

Adults of both species are highly vocal and produce a variety of frequency-modulated (FM) vocalizations in different social contexts (Miller and Engstrom 2007). Most notable is the highly stereotyped advertisement song, used predominantly in long-distance communication. Advertisement songs comprise a series of rapidly articulated FM sweeps (up to 20 sweeps/sec) that span audible and ultrasonic frequencies (*S. teguina*, 10–43 kHz; *S. xerampelinus*, 10–34 kHz; Hooper and Carleton 1976; Miller and Engstrom 2007). Bandwidth, note duration, internote interval, and amplitude all increase over the course of a song; a representative adult male *S. teguina* advertisement song is shown in Figure 1A. Although the acoustic structure of the two species' advertisement songs is very similar, singing rate is typically higher and song duration is longer in *S. teguina* than in *S. xerampelinus* (Miller and Engstrom 2007). Males of both species sing significantly more than females, male songs are longer, and *S. teguina* male singing



**Figure 1.** The structure of *Scotinomys* advertisement song. (A) Sonogram depicting the frequency modulation of a representative song from *S. teguina*. Lower panels show how notes change over the course of a song. Details correspond to 0.2 sec of song at 1 sec, 3 sec, and 5 sec, indicated by black boxes in the whole-song sonogram. Within the 1-sec detail, the shaded box identifies the note used as an exemplar in panel b. (B) Note parameters measured in this study. The left panel depicts a sonogram detail of a note, with maximum frequency (Fmax) and minimum frequency (Fmin) labeled. The middle panel illustrates how note curves were measured. In each time bin (open circles), the frequency with the highest energy was measured, and a quadratic curve was fit to the resulting data. The right panel shows an oscillogram, with peak amplitude (Peak amp), peak amplitude within the first quarter of a note (qPeak amp), and note duration (Note dur) labeled.

rate is increased by presentation and removal of a female (Miller and Engstrom 2007; M. Fernandez-Vargas, Z. Tang-Martinez, and S. M. Phelps, unpubl. ms.), implicating a role in mate attraction. However, song structure is sexually monomorphic (Miller and Engstrom 2007). Basic features of the songs of both species, such as high repetition rate, high stereotypy, broad bandwidth, and concentration of energy at relatively low frequencies, are characteristic of signals designed to maximize propagation and facilitate localization by conspecifics (Bradbury and Vehrencamp 1998; Miller and Engstrom 2007). Field playback and behavioral experiments in sympatry demonstrate that song is important in male–male competition and plays a role in interspecific interactions (Pasch 2009). Anecdotal evidence for differences in the duration of *S. teguina* advertisement songs between disjunct populations in Nicaragua and Costa Rica (Hooper and Carleton 1976) suggested that a more thorough analysis might reveal additional acoustic differences at finer spatial scales.

The first goal of this study was to determine whether there was detectable geographic variation in the advertisement songs of *S. teguina* and *S. xerampelinus* and, if so, to define acoustic parameters that differentiate populations. Singing mouse advertisement songs were sampled from seven sites along the Cordillera de Talamanca in Costa Rica and Panamá. Our ~200 km transect encompassed the entire known distribution of *S. xerampelinus* and the southeastern limits of the distribution of *S. teguina*. Our second goal was to test the fit between observed patterns of geographic variation in *S. teguina* song and those predicted by each of three hypotheses to explain acoustic divergence: selection for species recognition, acoustic adaptation, and drift. (1) Selection for species recognition, either to avoid heterospecific mating and reduce mate search costs, or to minimize interspecific competition for acoustic call space, should produce a pattern of character displacement (Wiley and Richards 1982; Duellman and Pyles 1983; Gerhardt and Huber 2002), in which acoustic differences between *S. teguina* and *S. xerampelinus* are greater in sympatry than in allopatry. Alternatively, strong selection to maintain territorial boundaries between heterospecific competitors might favor enhanced sensitivity to heterospecific signals (Cody 1969), resulting in a reduction in acoustic differences between the two species in sympatry. In either case, directional selection due to species interactions should reduce overall variance in *S. teguina* songs in sympatry versus allopatry, and patterns of acoustic divergence or convergence should be concordant across replicate sympatric/allopatric sites. (2) FM signals with rapid repetition rates are sensitive to environmental attributes that influence sound propagation (Wiley and Richards 1982; Badyaev and Leaf 1997). If acoustic properties of different environments are important in driving acoustic divergence between *S. teguina* populations, selection to maximize transmission efficiency should produce strong correlations between basic features of the song, such as dominant

frequency and note rate, and differences in environment. (3) In the absence of strong selection, signal divergence should reflect the stochastic process of genetic drift. If geographic variation in *S. teguina* song is largely stochastic, acoustic distance between sites should be significantly correlated with genetic distance. Although we focus on the more widely distributed *S. teguina*, preliminary analyses revealed interesting differences between the two species in the environmental correlates and the identity of acoustic parameters that differentiate populations. Therefore, patterns of acoustic and genetic divergence within *S. xerampelinus* are included for comparative purposes. Finally, to gain a better understanding of how processes acting on acoustic communication within species might shape interspecific signal divergence, we compared acoustic and genetic distance within and between species.

## Materials and Methods

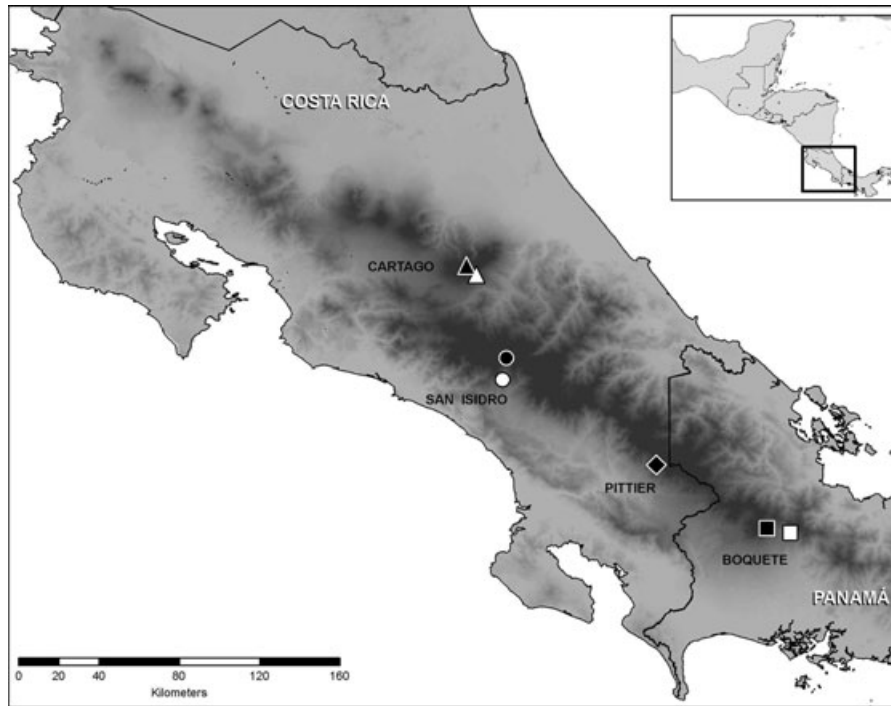
### SAMPLING

Field work for this study was conducted between May 2006 and February 2009. Singing mice were captured in Sherman traps baited with peanut butter and oats and were released on site following data collection. All individuals were weighed to the nearest 0.1 g and hind foot, tail, and male anogenital distance were measured to the nearest 0.1 mm. Toe clips were collected for genetic analysis and to facilitate identification of recaptures.

Mice were sampled at four main localities in Costa Rica and Panamá, selected to maximize coverage of the region where *S. teguina* and *S. xerampelinus* are locally sympatric (Fig. 2). Sample sizes for acoustic and genetic data, and geographic coordinates and mean elevations of trapping transects are provided in Table 1. Within three localities (Cartago, San Isidro, and Boquete), we paired sites where *S. teguina* is replaced by *S. xerampelinus* at higher elevations with nearby lower altitude sites where only *S. teguina* occurs (hereafter, sympatric and allopatric sites, respectively). This sampling design was chosen to test for evidence of selection for species recognition in the advertisement song of *S. teguina*, while controlling for divergence due to significant barriers to gene flow or an ecological cline. At a fourth locality, Pittier, *S. teguina* was sampled at the sympatric site only. *Scotinomys xerampelinus* was captured at Pittier but songs were recorded from only one individual and are not included in the analyses described below.

### ACOUSTIC DATA COLLECTION AND MEASUREMENT

At each field site, individuals selected for recording were housed singly in 28 × 28 × 28 cm<sup>3</sup> PVC-coated wire mesh cages and provided with sunflower seeds, cat chow, and water ad lib. Cages were placed inside recording chambers constructed from expanded PVC and lined with anechoic foam. In most cases, our goal sample size of a minimum of three songs per individual was reached



**Figure 2.** Sample sites for *Scotinomys* in along the Cordillera de Talamanca (dark shading) in Costa Rica and Panamá. Filled symbols represent sympatric sites, open symbols represent allopatric sites where only *S. teguina* occurs. See Table 1 for locality data and sample sizes.

within two to three days of capture, after which the mice were released on site. Only adult male songs were used in this study.

Songs were sampled at a rate of 195 kHz, 32 bits with an ACO Pacific microphone, and Tucker-Davis RX6 hardware. Songs were downloaded onto a laptop computer with RPvdsEx software (version 6.4, Tucker-Davis Technologies, Alachua, FL). Sound file quality was evaluated at the time of recording by visual examination of spectrograms; low-quality recordings were marked and excluded from analyses.

The spectral properties of advertisement songs were measured using an automated routine in MatLab (version 6.1; code available on request from S. M. Phelps). Dominant frequency, song duration, and note number were quantified as single measures from each song, and note rate was obtained from note number/song duration. Each song is a trill comprised of a series of frequency sweeps that change systematically over the course of the song (Fig. 1A). An individual note has a fundamental frequency sweep best described by the quadratic function,

**Table 1.** Locality data, mean altitude, and sample sizes for *S. teguina* (*St*) and *S. xerampelinus* (*Sx*) sampled at seven sites in Costa Rica (CR) and Panamá (Pan).

Sites	Location, province, country	Lat (N)	Long (W)	Mean altitude (m)		Songs (Males)		mtDNA	
				<i>St</i>	<i>Sx</i>	<i>St</i>	<i>Sx</i>	<i>St</i>	<i>Sx</i>
Cartago sym	Volcan Irazu, Cartago, CR	9°58'43"	83°51'10"	2900	3186	69 (21)	21 (11)	6	9
Cartago allo	La Carpintera, Cartago, CR	9°53'19"	83°58'19"	1785	–	51 (12)	–	8	–
San Isidro sym	Cerro Cuerici, San Jose, CR	9°33'18"	83°40'04"	2687	2864	75 (17)	21 (9)	8	7
San Isidro allo	Cerro Gomez, San Jose, CR	9°31'02"	83°39'54"	1775	–	75 (14)	–	9	–
Pittier sym	Cerro Pittier, Puntarenas, CR	9°01'49"	82°57'42"	1800	2368	26 (9)	–	9	–
Boquete sym	Volcan Baru, Chiriquí, Pan	8°47'51"	82°29'51"	1940	3003	58 (16)	32 (10)	7	6
Boquete allo	Peterson's Nature Reserve, Chiriquí, Pan	8°46'50"	82°23'46"	1763	–	35 (18)	–	6	–

sym=sympatric; allo=allopatric.

$f(x) = ax^2 + bx + c$ , where  $f(x)$  is the frequency of the note at time  $x$ ,  $a$  is the curvature of the fundamental frequency sweep,  $b$  is the starting slope of the sweep, and  $c$  is the starting frequency (Fig. 1B). We refer to these parameters as FMa, FMb, and FMc. To characterize the amplitude envelope of a note, we measured the peak amplitude in the first quarter of the note, and of the entire note (Fig. 1B). We also measured the duration of the note, the internote interval, and the minimum and maximum frequencies (Fig. 1B). We measured each of these parameters for every note in the trill, and then used a quadratic function to summarize the change in each measurement over the course of a song, with time  $x$  defined as the number of the note (Fig. S1). Because we used quadratic functions to describe both within-note fundamental frequency, and within-song changes in note structure, we use lower-case parameters  $a$ ,  $b$ , and  $c$  to refer to the frequency modulation of a note, and upper-case  $A$ ,  $B$ , and  $C$  to refer to changes that occur in note parameters across a song. For example, FMc describes the starting frequency of a note, FMc-C is the starting frequency of the first note in a song, FMc-B corresponds to the change in starting frequency between the first and second notes, and FMc-A describes how rapidly note-to-note differences in starting frequency change over the course of a song.

#### MULTIVARIATE ANALYSIS OF SONG VARIATION

In our final dataset, each song was described by 30 variables: three descriptors of the whole song, 12 descriptors of amplitude modulation, and 15 descriptors of frequency modulation (Table 2). Multivariate analysis of variance (MANOVA) on individual male means for all song variables was used to identify the acoustic measures that differentiate populations and species (*S. teguina* split by sample site, *S. xerampelinus* split by sample, site and *S. teguina* vs. *S. xerampelinus*; Bonferroni-corrected  $\alpha = 0.0017$ ). Variables with uncorrected  $P$ -values  $\leq 0.05$  were selected for principal components analysis (PCA).

Intraspecific acoustic distances between sites were calculated as Mahalanobis distances from individual PC scores, summed across the first four factors for *S. teguina* and the first three factors for *S. xerampelinus*. In choosing how many factors to include in the distance calculations, we attempted to maximize the percentage of variance captured, without including factors with low contribution to the total variance (i.e.,  $<10\%$ ).

To test for an effect of sympatry on *S. teguina* song, we used individual scores from the first three axes of the interspecific PCA to calculate Euclidean distances from Cartago, San Isidro, and Boquete *S. teguina*, to the corresponding local mean for *S. xerampelinus* (Pittier was excluded from this analysis because only sympatric *S. teguina* were sampled at this locality). We performed an analysis of variance (ANOVA) on individual Euclidean distances with ecological context (sympatry, allopatry) and locality (Cartago, San Isidro, Boquete) as covariates. The

expectation that directional selection on *S. teguina* song in sympatry should reduce acoustic variance was tested with ANOVA; within-site variance was calculated from PC scores. Multivariate analyses were conducted in SYSTAT (version 11, SYSTAT Software Inc.); Mahalanobis distances were calculated in XLSTAT (version 2009.2.03, Addinsoft).

#### GENETIC DATA COLLECTION AND ANALYSIS

We used mitochondrial sequence data to estimate genetic distances within and between *S. teguina* and *S. xerampelinus*. Genomic DNA was extracted from toe clips preserved in 95% ethanol using the DNeasy Tissue Kit (Qiagen, Valencia, CA). The D-loop of the mitochondrial control region was amplified using primers located in tRNA<sup>Thr</sup> (5' ATAAACATTACTCTGGTCTT GTAAAC 3') and the Central Domain of the control region (5' CACAGTTATGTTGRTCATGG 3'; Bellinvia 2004). Amplifications were carried out in a volume of 25  $\mu$ l containing 12.5  $\mu$ l of GoTaq Green Master Mix (Promega, Madison, WI) and 5  $\mu$ M of each primer, with an annealing temperature of 58°C for *S. teguina* and 52°C for *S. xerampelinus*. PCR products were purified using the QIAquick PCR Purification Kit (Qiagen) and cycle sequencing reactions were carried out using ABI BigDye Terminator (version 3.1, Applied Biosystems, Foster City, CA). Sequencing reaction products were run on an ABI Prism 3100 automated capillary sequencer (Applied Biosystems). Sequences were aligned and edited in SEQUENCHER (version 4.5, GeneCodes Corp., Ann Arbor, MI).

Intraspecific haplotype diversity ( $h$ ) and nucleotide diversity ( $\pi$ ) were calculated in DnaSP (version 4.10; Rozas et al. 2003). We tested the assumption of neutral evolution for control region sequences with Tajima's  $D$  (Tajima 1989), implemented in Arlequin (version 3.1; Excoffier et al. 2005). The best-fit model of sequence evolution was estimated in ModelTest (version 3.7; Posada and Crandall 1998) for *S. teguina* and *S. xerampelinus* control region alignments separately, and combined. Phylogenetic relationships between *S. teguina* and *S. xerampelinus* mitochondrial lineages were evaluated in PAUP\* (version 4.0b10; Swofford 2000) under distance criteria with maximum likelihood settings from ModelTest (see Fig. S2 caption for details). Genetic distances were calculated in MEGA (version 3.1; Kumar et al. 2004). Intraspecific relationships between genetic and geographic distances were evaluated with Mantel tests in XLSTAT. Significance of association between matrices was assessed with 10,000 permutations and Bonferroni-corrected alpha values ( $\alpha_{teguina} = 0.0024$ ;  $\alpha_{xerampelinus} = 0.017$ ). Geographic distance was estimated as a linear distance between sites.

#### CLIMATIC DATA COLLECTION AND ANALYSIS

We used climatic data for each site to summarize environmental features relevant to sound propagation. Although climate is a

**Table 2.** Results of multivariate analysis of variance of 30 acoustic variables for *S. teguina* (*St*) and *S. xerampelinus* (*Sx*) split by population, and *S. teguina* versus *S. xerampelinus*.

Acoustic variable	<i>St</i> population		<i>Sx</i> population		<i>St</i> vs. <i>Sx</i>	
	$F_{6,102}$	<i>P</i>	$F_{2,26}$	<i>P</i>	$F_{1,127}$	<i>P</i>
Song length (sec)	22.82	< <b>0.0001</b> *	1.71	0.20	162.47	< <b>0.0001</b> *
Note rate (notes/sec)	13.20	< <b>0.0001</b> *	2.10	0.14	8.11	<b>0.005</b>
Dominant frequency (kHz)	1.83	0.10	11.41	< <b>0.0001</b> *	80.78	< <b>0.0001</b> *
Amplitude						
Note dur-A	1.04	0.40	0.24	0.79	289.76	< <b>0.0001</b> *
Note dur-B	1.96	0.08	0.08	0.93	398.66	< <b>0.0001</b> *
Note dur-C	11.82	< <b>0.0001</b> *	3.80	<b>0.04</b>	15.70	< <b>0.0001</b> *
Internote-A	3.50	<b>0.003</b>	0.04	0.96	122.48	< <b>0.0001</b> *
Internote-B	5.89	< <b>0.0001</b> *	0.60	0.56	42.07	< <b>0.0001</b> *
Internote-C	6.59	< <b>0.0001</b> *	2.28	0.12	0.24	0.63
Peak amp-A	0.73	0.63	0.48	0.62	186.80	< <b>0.0001</b> *
Peak amp-B	1.65	0.14	0.54	0.59	350.55	< <b>0.0001</b> *
Peak amp-C	2.08	0.07	0.15	0.86	32.00	< <b>0.0001</b> *
qPeak amp-A	2.90	<b>0.01</b>	0.75	0.48	142.81	< <b>0.0001</b> *
qPeak amp-B	3.48	<b>0.004</b>	1.28	0.30	218.42	< <b>0.0001</b> *
qPeak amp-C	3.09	<b>0.008</b>	0.005	0.99	14.90	< <b>0.0001</b> *
Frequency						
F $M_a$ -A	0.21	0.97	0.78	0.47	0.26	0.61
F $M_a$ -B	0.64	0.70	0.72	0.50	3.06	0.08
F $M_a$ -C	1.96	0.08	1.95	0.16	20.17	< <b>0.0001</b> *
F $M_b$ -A	1.23	0.30	0.37	0.70	0.01	0.91
F $M_b$ -B	1.02	0.42	0.48	0.62	5.01	<b>0.03</b>
F $M_b$ -C	3.51	<b>0.003</b>	5.62	<b>0.009</b>	91.15	< <b>0.0001</b> *
F $M_c$ -A	0.83	0.55	0.64	0.54	28.06	< <b>0.0001</b> *
F $M_c$ -B	1.7	0.13	1.12	0.34	10.91	<b>0.001</b> *
F $M_c$ -C	4.8	< <b>0.0001</b> *	11.61	< <b>0.0001</b> *	53.73	< <b>0.0001</b> *
F $max$ -A	1.04	0.41	0.77	0.47	28.95	< <b>0.0001</b> *
F $max$ -B	1.39	0.23	0.32	0.73	13.52	< <b>0.0001</b> *
F $max$ -C	4.08	<b>0.001</b> *	6.45	<b>0.005</b>	31.33	< <b>0.0001</b> *
F $min$ -A	2.84	<b>0.01</b>	0.42	0.66	15.92	< <b>0.0001</b> *
F $min$ -B	3.87	<b>0.002</b>	1.00	0.39	5.23	<b>0.02</b>
F $min$ -C	0.96	0.46	6.60	<b>0.005</b>	20.20	< <b>0.0001</b> *

Note: dur, note duration; internote, internote interval; Peak amp, peak amplitude within note; qPeak amp, peak amplitude in first quarter of note; FM, frequency modulation; F $max$ , maximum frequency; F $min$ , minimum frequency; significant at  $P \leq 0.05$  in bold; \*significant after Bonferroni correction ( $\alpha$ , 0.0017).

coarse proxy for vegetation composition and density, temperature and humidity also have direct influence on sound transmission. Moreover, the use of climatic descriptors allowed us to address the possibility that acoustic divergence among localities is driven by a geographic cline in temperature or precipitation. Although we did not select sites based on clear environmental differences, Neotropical mountains are characterized by steep gradients in temperature, precipitation, and vegetation composition (Janzen 1967; Terborgh 1977). Thus, we expected that between-site and -species variation in altitude should be associated with differences in microclimate and habitat structure.

Six descriptors of temperature (annual mean temperature, mean diurnal temperature, maximum temperature in warmest month, minimum temperature in coldest month, mean temperature in wettest and driest quarters) and three descriptors of precipitation (annual precipitation, precipitation in wettest and driest quarters) were extracted from WorldClim, a global database with a spatial resolution of  $\sim 1$  km, covering the years 1950–2000 (Hijmans et al. 2005). GPS inputs for each site were taken near the mid-elevation of trapping transects for each species, resulting in species-specific climatic information for sympatric sites. We tested for pairwise correlations between the nine climatic variables

and the song variables that differed between sites, within species (intraspecific MANOVAs,  $P \leq 0.05$ ). Climatic distances were summarized with dissimilarity matrices (Euclidean distances), calculated in XLSTAT.

### TESTS FOR ENVIRONMENT-MEDIATED SELECTION

We used pairwise Mantel tests to test for intraspecific correlations between acoustic distance (Mahalanobis distances from intraspecific PCA scores), and climatic, genetic and geographic distances. Significance was assessed as for the genetic versus geographic distance comparison. Partial Mantel tests were used to test for association between acoustic and climatic distances after removing the effects of genetic or geographic distance. All Mantel tests were implemented in XLSTAT.

### COMPARISON OF WITHIN VERSUS BETWEEN SPECIES ACOUSTIC DIVERGENCE

Under the expectations of neutrality, polymorphism within species should be proportional to divergence between species (Kimura 1968; Hudson et al. 1987). Using mitochondrial sequence divergence as a metric of neutral evolution, we asked whether the relation between acoustic and genetic divergence between *S. teguina* and *S. xerampelinus* was significantly different from that observed within each species. To express intraspecific acoustic distances for both species on the same scale, we used the factor coefficients from the *S. teguina* PCA to calculate PC scores for individual *S. xerampelinus*. This allowed us to compare population pairwise acoustic distances within *S. teguina* and *S. xerampelinus* to acoustic distances between interspecific population pairs. We regressed intraspecific acoustic and genetic distances, and recorded the residual acoustic distance after correcting for genetic distance. Using the same regression line, we calculated residual acoustic distances between heterospecific populations. If equivalent processes drive song divergence at both evolutionary scales, acoustic distances between *S. teguina* and *S. xerampelinus* populations should fall about the same regression line calculated for intraspecific comparisons, and the resulting residuals should have a mean near zero. Because each population was represented in multiple contrasts, we estimated the null distribution using a randomization procedure. We randomly assigned each population pair the status of an “intraspecific” or “interspecific” comparison, preserving the number of intra- and interspecific contrasts observed in the original data. We then regressed intraspecific acoustic distances against genetic distances, obtained residuals for intraspecific and interspecific acoustic distances, and calculated the resulting  $t$ -statistic for this randomized dataset. We repeated this randomization procedure 10,000 times to generate a null distribution of the  $t$ -statistic that avoided assumptions about the independence of population contrasts. We used the randomization results to calculate a  $P$ -value. Because the best-fit model of nucleotide substitution differed between species (see below), we repeated

the entire analysis twice, with genetic distances estimated under either *S. teguina*, or *S. teguina* plus *S. xerampelinus* model parameters.

## Results

### PATTERNS OF GEOGRAPHIC VARIATION IN ADVERTISEMENT SONGS

Significant geographic variation was detected in the songs of both *S. teguina* and *S. xerampelinus* (Table 2). In both species, descriptors of frequency modulation and bandwidth were important in defining population differences in song. There were, however, notable interspecific differences in other acoustic variables that distinguished populations: amplitude modulation was highly variable in *S. teguina*, whereas *S. xerampelinus* populations differed in dominant frequency. Population means for selected song variables are shown in Table 3. (See also Table S1.)

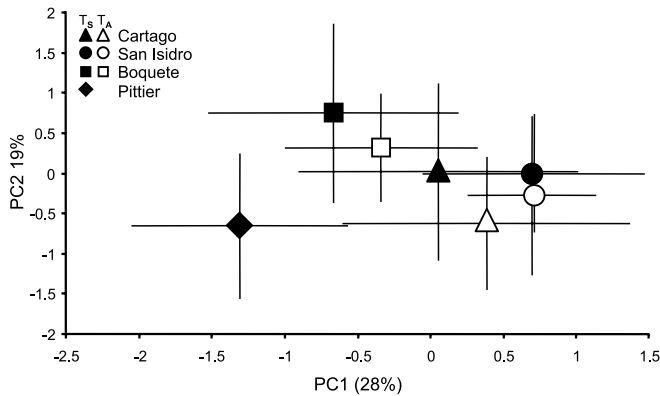
In *S. teguina*, population differences in the amplitude envelope included song length, note rate, note duration-*C*, internote interval-*B* and -*C* ( $P \leq 0.0017$ ), internote interval-*A*, and peak amplitude in the first quarter of each note (-*A*, -*B* and -*C*;  $P \leq 0.05$ ). Among spectral parameters, FM*C-C* and maximum frequency-*C*, were significant at  $P \leq 0.0017$ ; FM*B-C* and minimum frequency-*A* and -*B* were significant at  $P \leq 0.05$  (Table 2). Seventy-five percent of the total variance described by these 14 variables was distributed across the first four factors of the *S. teguina* PCA (Table S2). The distribution of population means on the first two factors is shown in Figure 3. Focusing on those variables that loaded strongly ( $>0.50$ ) and uniquely on a factor, PC1 described aspects of the amplitude envelope: note duration, song length, and changes of the internote interval over the course of the song. PC2

**Table 3.** Means and standard deviations for song length, note rate and dominant frequency, split by species and population.

	Song length (s)	Note rate (n/sec)	Song DF (kHz)
<i>S. teguina</i>			
Cartago sym	5.37 (0.95)	13.3 (1.5)	25.53 (3.81)
Cartago allo	7.09 (1.6)	11.1 (1.7)	23.47 (3.00)
San Isidro sym	6.60 (1.00)	12.1 (1.4)	23.36 (1.86)
San Isidro allo	7.16 (0.96)	11.7 (1.2)	23.57 (2.14)
Pittier sym	4.44 (1.10)	12.4 (1.9)	23.93 (4.54)
Boquete sym	4.13 (0.70)	15.0 (1.3)	24.06 (3.07)
Boquete allo	4.34 (0.93)	14.2 (1.4)	22.49 (2.13)
<i>S. xerampelinus</i>			
Cartago sym	1.77 (0.41)	14.6 (1.0)	16.36 (1.70)
San Isidro sym	1.78 (0.28)	14.5 (1.7)	18.24 (1.44)
Boquete sym	2.03 (0.30)	13.3 (1.6)	20.43 (2.16)

sym, sympatric; allo, allopatric; DF, dominant frequency.





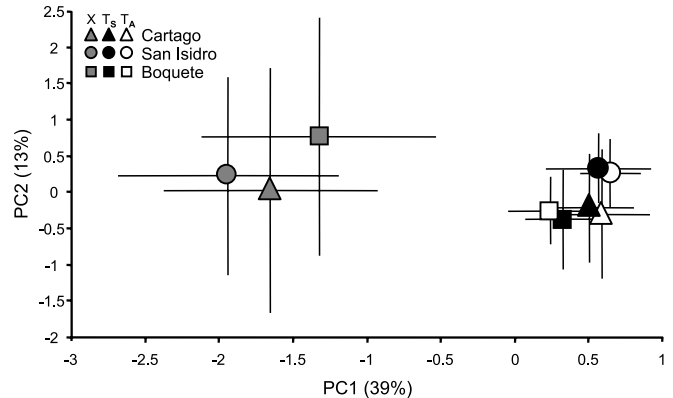
**Figure 3.** Geographic variation in *S. teguina* advertisement songs. Plot of population mean scores from first (PC1) and second (PC2) principle components axes for *S. teguina* from sympatric ( $T_S$ , filled symbols) and allopatric ( $T_A$ , open symbols) sites at Cartago (triangles), San Isidro (circles), Boquete (squares) and Pittier (diamond). PC1 explains 28% of the total variance in songs, PC2 explains 19%. Error bars show standard deviations.

described variance in early note amplitude at the start of a song, PC3 described change in early note amplitude over the course of a song, whereas PC4 described variation in starting internote interval. Population means for each of the four factors are given in Table S2.

In *S. xerampelinus*, dominant frequency and starting frequency (FMc-C), were significantly different between populations at  $P \leq 0.0017$ , and note duration-C, FMb-C, maximum frequency-C, and minimum frequency-C were significant at  $P \leq 0.05$  (Table 2). In the *S. xerampelinus* PCA, 87% of the total variance was distributed on the first three factors (Table S3). All variables excepting starting minimum frequency (Fmin-C) were strongly loaded on PC1. Fmin-C and dominant frequency were strongly loaded on PC2, whereas PC3 described the rate of change in frequency at the start of songs (FMb-C). Population means for each factor are reported in Table S3.

### INTERSPECIFIC DIFFERENCES IN ADVERTISEMENT SONG

Significant differences between the advertisement songs of *S. teguina* and *S. xerampelinus* were detected for 26 of 30 acoustic variables; 23 of these were significant at  $P < 0.0017$  (Table 2). In the interspecific PCA, major differences between the two species were concentrated on the first axis, which accounted for 39% of the total variance (Fig. 4; Table S4). Of the large number of variables with high loadings on PC1, descriptors of amplitude modulation were more important than spectral features: *S. teguina* was strongly differentiated from *S. xerampelinus* by greater increase in note duration and peak amplitude over the course of longer songs with more evenly spaced notes. Acoustic variance within and between *S. xerampelinus* populations was high relative to *S. teguina*



**Figure 4.** Geographic variation and species differences in *S. xerampelinus* and *S. teguina* advertisement songs. Plot of population mean scores from first (PC1) and second (PC2) principle components axes for *S. xerampelinus* (X) and *S. teguina* from sympatric ( $T_S$ , filled symbols) and allopatric ( $T_A$ , open symbols) sites at Cartago (triangles), San Isidro (circles), and Boquete (squares). PC1 explains 39% of the total variance in songs, PC2 explains 13%. Error bars show standard deviations.

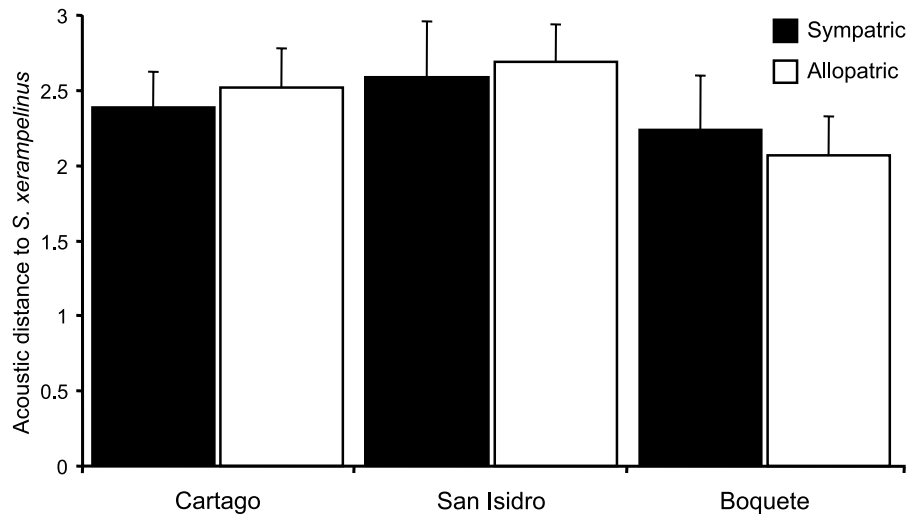
(Fig. 4). Although within-population variation in *S. xerampelinus* may be explained by smaller sample sizes (see Table 1), the wider spread of populations indicates that this species has more extensive geographic differentiation in its song. Strikingly, of the seven variables with strong loadings ( $>0.50$ ) on the first axis of the *S. teguina* PCA, five also loaded highly on the first axis of the interspecific PCA (Tables S2 and S4). This result suggests that acoustic differences between *S. teguina* populations recapitulate major dimensions of interspecific divergence.

### TEST FOR SELECTION FOR SPECIES RECOGNITION IN SYMPATRY

Differences between *S. teguina* songs from paired sympatric/allopatric sites were not explained by the presence or absence of *S. xerampelinus* ( $F_{1,94} = 0.09$ ,  $P = 0.76$ ), and acoustic variance was not reduced in sympatry ( $F_{1,4} = 0.12$ ,  $P = 0.76$ ). Moreover, acoustic distances were slightly less in sympatry at Cartago and San Isidro, but slightly greater at Boquete (Fig. 5). Thus, there was no evidence that the addition of more sampling localities would uncover a meaningful pattern of either displacement or convergence in *S. teguina* songs.

### GENETIC VARIATION

Amplification of approximately 495 base pairs spanning the D-loop of the mitochondrial control region recovered 19 haplotypes for 53 *S. teguina* from seven sites, and 12 haplotypes for 22 *S. xerampelinus* from three sites (GenBank accession nos., GQ502732-GQ502762). Overall haplotype ( $h$ ) and nucleotide ( $\pi$ ) diversity were lower in *S. teguina* ( $h = 0.896 \pm SD 0.024$ ,  $\pi = 0.04038 \pm SD 0.0013$ ) than in *S. xerampelinus* ( $h = 0.918 \pm SD 0.036$ ,



**Figure 5.** Acoustic distance from *S. teguina* to *S. xerampelinus* in sympatry (filled bars) versus allopatry (open bars) at Cartago, San Isidro, and Boquete. Geographic variation in *S. teguina* advertisement songs is not explained by the presence or absence of *S. xerampelinus* ( $F_{1,94} = 0.09, P = 0.76$ ). Error bars show standard deviations.

$\pi = 0.04367 \pm SD 0.0051$ ). A single *S. teguina* haplotype was recovered from both Boquete sites; no other haplotypes were shared between sites in either species. Phylogenetic analysis recovered two geographically concordant clades within each species: haplotypes from Cartago and San Isidro were monophyletic with respect to haplotypes from Pittier and Boquete (Fig. S2). There was no evidence of mitochondrial introgression between species. Weakly positive, nonsignificant values of Tajima’s *D* were consistent with neutral evolution of control region sequences (*S. teguina*,  $D = 2.02, P = 0.99$ ; *S. xerampelinus*,  $D = 1.04, P = 0.88$ ).

The likelihood ratio test statistic identified the HKY model (Hasegawa et al. 1985) with A-T-biased base frequencies as the best-fit model of nucleotide substitution for *Scotinomys* control region sequences. Rates among sites were gamma-distributed for *S. teguina* ( $\alpha = 0.95$ ) and for the two species combined ( $\alpha = 0.073$ ), and equal for *S. xerampelinus*. Genetic distances were estimated under the Tamura three-parameter model (Tamura 1992)

with the gamma distributions specified above. We chose this model because it extends that of Hasegawa et al. (1985) to account for unequal base frequencies (Tamura 1992).

Genetic distances ranged from 0 to 0.081 in *S. teguina* (Table 4) and 0.034 to 0.066 in *S. xerampelinus*. There was a highly significant positive correlation between genetic and geographic distance in both *S. teguina* ( $r = 0.93, P < 0.0001$ ) and *S. xerampelinus* ( $r = 0.99, P < 0.0001$ ).

**CLIMATIC VARIATION**

There was an imperfect NW to SE cline for all temperature variables at *S. teguina* sympatric sites: values were lowest at Cartago, intermediate at San Isidro, highest at Pittier, but slightly lower at Boquete (Table S5). However, this pattern could be explained by between-site differences in elevation (see Table 1); across all *S. teguina* sample sites correlations between elevation and temperature variables were negative and highly significant (all  $r \leq -0.95$ ,

**Table 4.** Genetic distances within *S. teguina* (below diagonal) and *S. xerampelinus* (above diagonal), estimated from mitochondrial control region sequences.

	Cartago sym	Cartago allo	San Isidro sym	San Isidro allo	Pittier sym	Boquete sym	Boquete allo
Cartago sym	0	–	0.0357	–	–	0.0580	–
Cartago allo	0.0145	0	–	–	–	–	–
San Isidro sym	0.0204	0.0175	0	–	–	0.0576	–
San Isidro allo	0.0163	0.0126	0.0187	0	–	–	–
Pittier sym	0.0688	0.0648	0.0653	0.0593	0	–	–
Boquete sym	0.0805	0.0769	0.0777	0.0717	0.0190	0	–
Boquete allo	0.0805	0.0769	0.0777	0.0717	0.0190	0.0000	0

sym, sympatric; allo, allopatric.

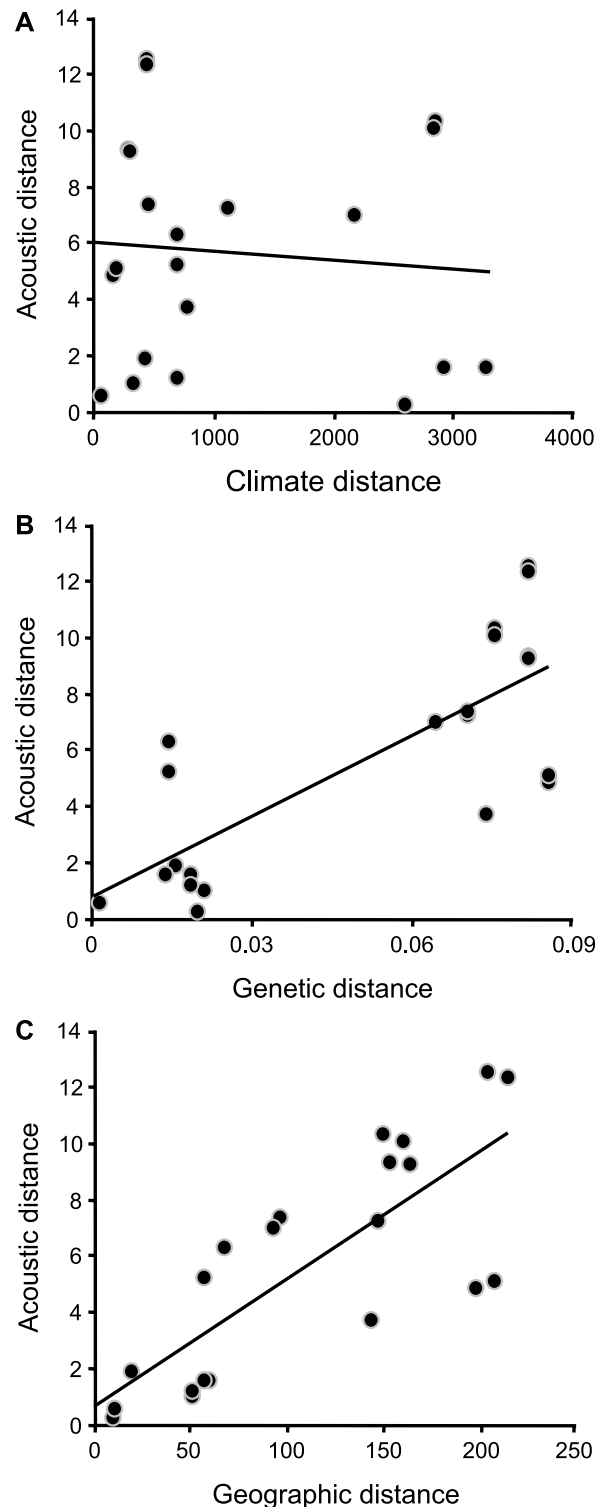
all  $P < 0.0001$ ). There were no geographic trends in precipitation across *S. teguina* sites, and the relationship between elevation and precipitation variables was weakly negative and nonsignificant ( $-0.38 \leq r < 0$ , all  $P \geq 0.41$ ).

For *S. xerampelinus*, all temperature and precipitation variables were lowest at Cartago (Table S5). Mean diurnal temperature increased from Cartago to San Isidro to Boquete, whereas annual precipitation and precipitation in the driest quarter decreased along the same NW to SE cline. These patterns were not strongly related to elevation (mean diurnal temp.,  $r = -0.47$ ,  $P = 0.69$ ; annual precip.,  $r = -0.74$ ,  $P = 0.47$ ; precip. in driest quarter,  $r = -0.13$ ,  $P = 0.92$ ). There was, however, a strong negative relationship between elevation, and temperature in the wettest and driest quarters ( $r = -0.997$ ,  $P = 0.05$ ;  $r = -0.998$ ,  $P = 0.04$ , respectively).

### CORRELATES OF SONG DIVERGENCE

None of the individual climatic descriptors for each site were significantly correlated with acoustic variables that differentiated *S. teguina* songs among sites ( $-0.56 \leq r \leq 0.71$ , all  $P \geq 0.08$ ). Likewise, there was no correlation between climatic and acoustic distance ( $r = -0.09$ ,  $P = 0.65$ ; Fig. 6A). In contrast, there were highly significant positive correlations between genetic and acoustic distance ( $r = 0.77$ ,  $P < 0.0001$ ; Fig. 6B), and geographic and acoustic distance ( $r = 0.78$ ,  $P < 0.0001$ ; Fig. 6C). Removing the effects of either genetic or geographic distance did not significantly alter the relation between climatic and acoustic distance ( $r = 0.08$ ,  $P = 0.36$ ;  $r = 0.13$ ,  $P = 0.29$ , respectively).

In *S. xerampelinus*, two of the six acoustic variables that differentiate populations, dominant frequency, and note duration-*C*, were strongly correlated with mean diurnal temperature ( $r = 0.997$ ,  $P = 0.05$ ;  $r = 1.0$ ,  $P = 0.01$ , respectively). A strong negative relationship between dominant frequency and body size is common in birds and frogs (Ryan and Brenowitz 1985; Pröhl et al. 2007), and body size is negatively correlated with ambient temperature in a wide range of endotherms (Brown and Lee 1969; Olson et al. 2009). Thus, the correlation between *S. xerampelinus* population differences in dominant frequency and diurnal temperature could be a byproduct of ecological selection on body size. We tested this possibility, using population mean adult male body mass as a proxy for size (Cartago,  $n = 9$ , mean =  $14.2 \pm SD 1.79$ ; San Isidro,  $n = 17$ , mean =  $13.1 \pm SD 1.73$ ; Boquete,  $n = 12$ , mean =  $15.0 \pm SD 2.20$ ). Population differences in mass were nonsignificant ( $F_{2,35} = 0.38$ ,  $P = 0.69$ ) and trends were opposite to those expected if song divergence were driven by morphological adaptation: dominant frequency increased with mass ( $r = 0.98$ ,  $P = 0.17$ ), and mass increased with diurnal temperature ( $r = 0.88$ ,  $P = 0.17$ ).



**Figure 6.** Correlates of acoustic divergence in *S. teguina*. (A) Acoustic and climate distance ( $r = -0.09$ ,  $R^2 = 0.008$ ,  $P = 0.65$ ), (B) acoustic and genetic distance ( $r = 0.77$ ,  $R^2 = 0.60$ ,  $P < 0.0001$ ), (C) acoustic and geographic distance ( $r = 0.78$ ,  $R^2 = 0.61$ ,  $P < 0.0001$ ). Genetic distance (Tamura 1992) estimated from mitochondrial control region sequences; geographic distance estimated as linear distance in kilometers.

The correlation between total climatic and acoustic distance among *S. xerampelinus* populations was positive but nonsignificant ( $r = 0.61$ ,  $P = 0.17$ ), whereas the correlations between genetic and acoustic, and geographic and acoustic distances were positive and significant ( $r = 0.68$ ,  $P < 0.0001$ ;  $r = 0.86$ ,  $P < 0.0001$ , respectively). Removing the contribution of either genetic or geographic distance resulted in a strong, significant correlation between climatic and acoustic distance (both,  $r = 1.0$ ,  $P < 0.0001$ ).

### PATTERNS OF SONG DIVERGENCE WITHIN VERSUS BETWEEN SPECIES

The relationships between acoustic and genetic distances were sharply discordant within versus between species. This result was consistent whether genetic distances were estimated under the *S. teguina* ( $\alpha_T$ ) or combined species ( $\alpha_{T+X}$ ) gamma distributions; acoustic-genetic contrasts with  $\alpha_{T+X}$  are shown in Figure 7. Whereas intraspecific contrasts between acoustic and genetic distances were positive and highly significant ( $\alpha_T$ ,  $t = 6.36$ ;  $\alpha_{T+X}$ ,  $t = 5.84$ ; both,  $P < 0.0001$ ), the relationship between interspecific acoustic and genetic distances was weak and negative ( $\alpha_T$ ,  $t = -2.23$ ,  $P = 0.04$ ;  $\alpha_{T+X}$ ,  $t = -1.97$ ,  $P = 0.06$ ). Genetic distances were continuously distributed; the highest genetic distances between *S. teguina* populations were comparable to the lowest distances between *S. teguina* and *S. xerampelinus*. In contrast, species differences in song were greater than predicted from genetic-acoustic relationships within species: acoustic distances corrected for genetic distances were much higher for between species contrasts ( $\alpha_T$ , mean residual acoustic distance (MRAD) =

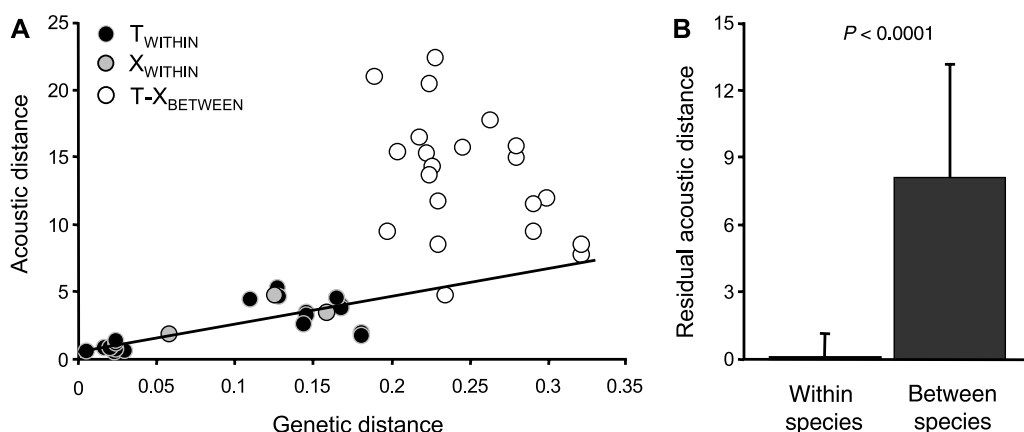
8.87,  $SD = 4.81$ ;  $\alpha_{T+X}$ , MRAD = 8.11,  $SD = 5.02$ ) than for within-species contrasts (MRAD = 0.00,  $\alpha_T$ ,  $SD = 1.03$ ;  $\alpha_{T+X}$ ,  $SD = 1.08$ ; Fig. 7B). Randomization tests revealed that this difference was highly significant ( $\alpha_T$ ,  $t = -5.33$ ;  $\alpha_{T+X}$ ,  $t = -5.06$ ; both, two-tailed  $P < 0.0001$ ).

### Discussion

The fundamental question we sought to answer in this study is whether drift or selection has played the major role in shaping geographic variation in a mammalian acoustic signal used in long-distance communication. We found that a significant proportion of variation in the advertisement song of a Neotropical murid rodent, *S. teguina*, may be explained by drift alone. Results for a small number of populations of a codistributed congener, *S. xerampelinus*, suggest that both drift and local adaptation contribute to acoustic variation in this species. Strikingly, evidence that the songs of *S. teguina* and *S. xerampelinus* are more different from each other than can be explained by genetic differences alone suggests that the advertisement song of one or both species has been subject to strong directional selection in the past. These findings are discussed in relation to the distinct ecologies of singing mice, and the evolution of their unique vocal behavior.

### EVIDENCE FOR STOCHASTIC DIVERGENCE IN *S. TEGUINA* SONG

Both spectral and temporal attributes of *S. teguina* advertisement songs were significantly differentiated between populations; a strong positive relationship between acoustic and genetic distance



**Figure 7.** Population pairwise acoustic-genetic distances, within versus between *S. teguina* and *S. xerampelinus*. (A) Linear contrasts between acoustic and genetic distance within *S. teguina* ( $T_{\text{WITHIN}}$ , black circles) and *S. xerampelinus* ( $X_{\text{WITHIN}}$ , gray circles), and between species ( $T-X_{\text{BETWEEN}}$ , open circles). Genetic distance (Tamura 1992), estimated with the gamma distribution fit to mitochondrial control region sequences for *S. teguina* and *S. xerampelinus* combined ( $\alpha = 0.073$ ). The regression line was calculated from intraspecific contrasts; the resulting equation was used to calculate residuals, but was not directly used in hypothesis testing. (B) Residual acoustic distances within versus between species ( $t = -5.06$ ,  $P < 0.0001$ ); the null distribution of the  $t$ -statistic and resulting  $P$ -value were obtained using a randomization procedure, described in Methods. Error bars depict standard deviations.

supports the hypothesis that the observed pattern of geographic variation is largely due to stochastic processes. Although cultural transmission and copying errors are major drivers of stochastic divergence in learned vocal signals (e.g., bird song, Lynch 1996; whale song, Rendell and Whitehead 2001), we think it unlikely that singing mouse songs are subject to these sources of variation. Both *Scotinomys* species produce clear precursors of the adult song from birth (Miller and Engstrom 2007), and the songs of laboratory-reared *S. teguina* retain acoustic characteristics of the population of origin, despite exposure to the songs of conspecifics derived from different populations (P. Campbell, pers. obs.). Thus, divergence due to genetic drift is unlikely to be confounded with cultural drift.

The significant positive correlation between genetic and geographic distance in *S. teguina* (isolation by distance) is consistent with equilibrium between migration and drift in continuously distributed populations (Slatkin 1993). However, the sharp increase in genetic distance for all Irazu/Cartago versus Pittier/Boquete population comparisons does not reflect geographic distance (Table 4; Fig. S2), suggesting either colonization from different source populations or historic barriers to gene flow. Moreover, although acoustic distances are highly correlated with both genetic and geographic distances, the relationship with geography is slightly stronger. A reasonable interpretation of these results is that recent or ongoing gene flow that is not captured by mitochondrial sequence divergence contributes to patterns of geographic variation in *S. teguina* song.

We note that inferences based on a single, maternally inherited locus should be treated with caution, and that genome-wide nuclear markers such as microsatellites or AFLPs would provide a more complete picture of population differentiation, and the potential for ongoing gene flow (e.g., Irwin et al. 2005; Mila et al. 2009). Likewise, in a topographically and ecologically complex landscape such as the Talamancas, linear distance may underestimate actual dispersal distances between neighboring populations (Vignieri 2005; Wang et al. 2008). However, given the strength of the positive correlations between acoustic distance within *S. teguina* and our estimates of both genetic and geographic distance, we think it unlikely that inclusion of faster evolving nuclear loci or landscape-based distance measures would alter the conclusion that song divergence between *S. teguina* populations is largely a function of isolation by distance.

#### DOES ECOLOGY CONTRIBUTE TO DIVERGENCE IN *S. TEGUINA* SONG?

We tested two adaptive hypotheses for acoustic divergence in *S. teguina*, but found no evidence that geographic variation in this species' advertisement song reflects response to selection under different ecological conditions. First, the lack of any pattern of song displacement or convergence in sympatry with

*S. xerampelinus* strongly refutes the hypothesis that selection due to species interactions contributes to current variation in *S. teguina* song. This result suggests that interspecific song divergence, sufficient to maintain territorial and reproductive boundaries, took place earlier in the evolutionary history of the genus and prior to colonization of the region sampled in this study. Second, there was no relationship between population differences in advertisement song and variation in acoustic environment, estimated from local climatic data. Given the substantial contribution of drift to song divergence in *S. teguina*, one interpretation of this result is that ecological selection is unimportant in the evolution of this species' advertisement song. We stress, however, that our data do not rule out an ecological contribution to population differences in song.

Most evidence for acoustic adaptation in birds comes from studies in which populations or species were sampled across habitat types, or forest strata, with clear differences in vegetation composition and degree of openness (e.g., coniferous vs. temperate rainforest, Ruegg et al. 2006; ecotonal vs. rainforest, Slabbekoorn and Smith 2002; midstory vs. forest floor, Seddon 2005). Although many of the climatic variables differed between *S. teguina* sample sites, the habitat occupied by *S. teguina* was heterogeneous within sites: the mice were captured in both fallow pasture near secondary forest edges where ground cover is dense, and in primary montane forest where ground cover is comparatively sparse. Finally, although temperature and humidity have direct influence on sound transmission, climate is an indirect measure of habitat structure and does not account for other environmental features that impact sound propagation, such as ambient noise and air turbulence (Morton 1975; Ryan and Brenowitz 1985; Dingle et al. 2008). Thus, although the results of this study demonstrate that geographic variation in *S. teguina* song is not driven by between-site differences in temperature and precipitation, a more complete understanding of the fit between signal and acoustic environment awaits more precise vegetative sampling, and acoustic ranging experiments in structurally distinct habitats.

The broad distribution of populations in acoustic space, together with considerable intrapopulation variation (Fig. 3), suggests that *S. teguina* song may be an example of a signal that is broadly tuned to the average characteristics of a variable environment (i.e., Endler 1992). Nonetheless, we expect ecology and physiology to place constraints on signal divergence, such that some song variants are either less effective or too costly or both. Closer examination of Figure 3 provides some clues as to where these constraints may lie. Populations are characterized by long songs with long notes (PC1, reflected in loadings on song length and Note dur-C) or shorter songs with loud opening notes with higher bandwidth and a faster note rate (PC2, reflected in loadings on song length, qPeak amp-C, FMc-C, Fmax-C, Fmin-C), but these sets of acoustic characteristics are not observed in combination. Although the absence of populations with high positive

scores on both factors may be due to sampling error or random loss of song types, there are biologically feasible explanations for this result. For example, the song length evident on PC1 and the loud initial amplitude and high bandwidth evident on PC2 may be equivalent means of making a signal more detectable; combining these features may raise costs due to predation or energetics beyond some allowable maximum (e.g., Ryan 1988; Eberhardt 1994; McCarty 1996). Thus, although observed patterns of geographic variation in *S. teguina* song seem to be shaped by drift, selection may act to remove song variants with combinations of features that lower individual fitness.

### CORRELATES OF GEOGRAPHIC VARIATION IN *S. XERAMPELINUS* SONG

As in *S. teguina*, acoustic variation within *S. xerampelinus* was positively correlated with both genetic and geographic distances, suggesting an important role for genetic drift in song divergence. However, a perfect correlation between acoustic and climatic distance, after controlling for genetic distance, suggests that ecological selection contributes to geographic variation in this species' advertisement song. Given the small sample size for *S. xerampelinus*, these inferences are speculative. However, strong geographic differentiation in key features of song across three populations suggests that additional sampling may strengthen the proposed relation between song and ecology.

Most notably, mean dominant frequency decreased by 4 kHz from Boquete to Cartago (Table 3), tracking a reduction in mean diurnal temperature, annual precipitation and precipitation in the driest quarter (Table S5). This result raises the possibility that change in frequency in *S. xerampelinus* song is driven by a geographic cline in temperature and rainfall. A similar pattern of reduction in frequency with distance from the equator is found in a New World passerine and several species of frogs (Nottebohm 1975; Ryan et al. 1996; Pröhl et al. 2007). For example, the dominant call frequency of a Neotropical frog, *Dendrobates pumilio*, decreases through the range of the species, from SE Panamá to NW Costa Rica (Pröhl et al. 2007). However, geographic variation in *D. pumilio* call frequency and body size is negatively correlated, suggesting that frequency reduction in this species is a byproduct of morphological adaptation to cooler environments (i.e., larger frogs produce lower frequency calls; Pröhl et al. 2007). The absence of a relationship between body mass and dominant frequency in *S. xerampelinus* is therefore striking, and consistent with the hypothesis that population differences in this acoustic parameter are adaptive.

Lower frequency signals are less sensitive to degradation in dense vegetation and attenuate less with distance (Bradbury and Vehrencamp 1998). There are many examples of negative associations between habitat closure and frequency in bird song (Price 2008, Table 12.1 and references therein). However, there were no

obvious differences in vegetation structure among *S. xerampelinus* sample sites and advertisement song frequency was lowest at Cartago, where cooler, drier conditions are unlikely to increase vegetation density. An alternative that is more parsimonious with climatic data is that population variation in social spacing due to different resource distributions selects for song transmission over different distances. Thus, sparser social spacing under relatively harsher conditions should favor lower frequency signals, a hypothesis that could be tested with comparison of population densities and spectral properties of advertisement songs across climatically distinct sites.

### THE EVOLUTION OF ADVERTISEMENT SONG IN SINGING MICE

Acoustic divergence between *S. teguina* and *S. xerampelinus* was significantly greater than that predicted from variation within either species (Fig. 7). Thus, although patterns of geographic variation in *S. teguina* song are consistent with stochastic evolution, divergence between species suggests that strong directional selection has shaped interspecific differences in song. We propose that both ecological and sexual selection have been important in the evolution of genus- and species-specific features of advertisement song.

The closest extant relatives of singing mice, *Baiomys musculus* and *B. taylori* occupy dry, open, lowland habitats in the southwestern United States and Mexico (Packard and Montgomery 1978; Eshelman and Cameron 1987; Bradley et al. 2004). Both species are crepuscular and both produce vocalizations that resemble *Scotinomys* advertisement songs in general structure, but are shorter, less stereotyped, and span a narrower frequency range that is entirely ultrasonic (Miller and Engstrom 2007). Thus, altitudinal specialization in moist, closed habitats, diurnality, and longer, lower frequency vocalizations with broader and more elaborate FM components are all shared, derived characteristics of singing mice. In the absence of ecological or acoustic intermediates, the order of character evolution in the common ancestor of *S. teguina* and *S. xerampelinus* cannot be reconstructed. However, one reasonable scenario is that competitive release in a novel environment facilitated energy investment in a more elaborate form of acoustic communication, whereas release from nocturnal, acoustically orienting predators permitted use of lower frequencies, better adapted to long-distance transmission through forest understory.

If ecological release contributed to ancestral features of *Scotinomys* song, what processes catalyzed the apparently rapid rate of acoustic divergence between *S. teguina* and *S. xerampelinus*? Relative to *S. xerampelinus*, *S. teguina* song is characterized by greater increase in amplitude and note duration over the course of a longer song with more notes. In combination, these acoustic features create a larger, more elaborate sensory stimulus, a hallmark of signals subject to sexual selection by female choice (Ryan

and Keddyhector 1992; Tuckerman et al. 1993; Neubauer 1999; Nolan and Hill 2004). Species differences in altitudinal distribution are consistent with an ecological potential for stronger sexual selection in *S. teguina*; altitudinal gradients in sexual selection intensity are documented in birds, with higher sexual selection in species or populations breeding at lower, more resource-rich elevations (Zang 1980; Badyaev and Ghalambor 2001). As in the contrast between *S. teguina* and *S. xerampelinus*, low-elevation finch species sing longer songs with more notes relative to high-altitude sister species (Snell-Rood and Badyaev 2008).

Although altitudinal gradients in temperature and resource distributions may mediate the strength of sexual selection on advertisement song, it is likely that ecological selection in different environments also contributes directly to species differences in song. For example, the greater energetic demands of a colder, higher altitude environment may place an upper limit on song length in *S. xerampelinus*. Likewise, reduction in dominant frequency relative to *Baiomys* is greater in *S. xerampelinus* than in *S. teguina* (Miller and Engstrom 2007, Table 1; this study, Table 3), and our preliminary evidence for environmental effects on dominant frequency within *S. xerampelinus* supports a past history of ecological selection on this acoustic character.

Finally, given the proposed North American origin of *Scotinomys*, the extensive but highly fragmented distribution of *S. teguina*, and the complex topography of the region where *S. teguina* and *S. xerampelinus* currently co-occur, cycles of population isolation and reconnection have almost certainly influenced acoustic diversification in singing mice. The absence of an effect of sympatry on *S. teguina* song indicates that interspecific acoustic differences sufficient for coexistence were established before populations reached their current geographic configuration. Whether acoustic divergence between species was driven entirely by processes acting in isolated lineages, whether interactions in an earlier period of contact contributed to species differences in song, or whether song itself was an important isolating mechanism during the process of speciation are all open questions that will be challenging to answer. However, parallels between axes of acoustic variation within *S. teguina* and the major axis of divergence between species point to similar alternative solutions to producing an effective acoustic signal. Thus, stochastic variation within species can provide the raw material for adaptive divergence in deeper evolutionary time.

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## Supporting Information

The following supporting information is available for this article:

**Figure S1.** Measures of notes change systematically over the course of a song.

**Figure S2.** Unrooted phylogram for mitochondrial control region haplotypes from *S. xerampelinus* (orange) and *S. teguina* (blue).

**Table S1.** Population means on measured acoustic variables.

**Table S2.** Principle component (PC) axis loadings and population means for 14 acoustic variables with significant contribution to geographic variation in *S. teguina* (*St*) advertisement songs.

**Table S3.** Principle component (PC) axis loadings and population means for six acoustic variables with significant contribution to geographic variation in *S. xerampelinus* (*Sx*) advertisement songs.

**Table S4.** Principle component (PC) axis loadings and population means for 26 acoustic variables with significant contribution to species differences in *S. teguina* (*St*) vs. *S. xerampelinus* (*Sx*) advertisement songs.

**Table S5.** Climate data extracted from WorldClim (Hijmans et al. 2005) for *S. teguina* (*St*) and *S. xerampelinus* (*Sx*) sample sites.

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

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