Articles

Geographic variation and the evolution of song in Mesoamerican rufous-naped wrens *Campylorhynchus rufinucha*

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Speciation may be influenced by geographic variation in animal signals, particularly when those signals are important in reproductive decisions. Here, we describe patterns of geographic variation in the song of rufous-naped wrens *Campylorhynchus rufinucha*. This species complex is a morphologically variable taxon confined to tropical dry forest areas from Mexico to northwestern Costa Rica. Morphological and genetic analyses suggest that there are at least three partially isolated groups within the complex, including a secondary-contact zone in coastal western Chiapas between the subspecies *C. r. humilus* and *C. r. nigricaudatus*. Based on recordings throughout their geographic range, we investigate the effects of historical isolation on song structure and analyze whether genetic differences or climatic conditions explain observed patterns of variation. Our findings, based on a culturally-transmitted and sexually-selected trait, support the hypothesis that three evolutionary units exist within this taxon. Our results suggest that song differences between genetic groups were influenced by historical isolation. We report a strong relationship between vocal dissimilarity and genetic distance, suggesting that differences in vocal characteristics are probably affected by the same factors that drive genetic divergence. We argue that the evolution of song in this taxon is influenced by vicariant events, followed by accumulation of changes in song structure due to several possible factors: cultural drift in song structure; genetic drift in features related to song production; or natural selection acting on features that influence songs, such as body and beak size.

Understanding patterns of geographic variation in avian acoustic signals is important because these signals have a direct function in mate attraction and territory defense (Wilczynski and Ryan 1999, Bradbury and Vehrencamp 2011). The complex songs of oscine songbirds stand out as a particularly interesting behaviour since their development involves both genetic (innate) and cultural (learned) components (Catchpole and Slater 2008). Divergence in learned songs in a group of organisms may arise through cultural drift, genetic drift, cultural selection, natural selection, or through selection on song as a social and sexual signal (Podos et al. 2004a). As a learned behaviour, song characteristics may evolve by cultural drift when inaccurate copying of tutors' songs leads to changes in songs due to isolation or founder events (so called 'cultural mutations', Koetz et al. 2007, Campbell et al. 2010). Genetic drift could drive changes in morphological features associated with sound production thereby influencing the evolution of vocal traits by limiting song performance (Podos 2001, Podos and Nowicki 2004, Podos et al. 2004a, Odom and Mennill 2012). Specific song characteristics may evolve by cultural selection to suit particular vegetation and climatic conditions, as suggested by the acoustic adaptation hypothesis (Morton 1975), further contributing to song differentiation between populations living in different habitats (Slabbekoorn and Smith 2002, Ruegg et al. 2006, Tubaro and Lijtmaer 2006). Song evolution also can occur when natural selection acts indirectly by modifying the vocal apparatus (Schluter et al. 1985, Podos 2001, Podos et al. 2004b). Variation in social environments may also influence acoustic divergence in songs through intrasexual selection, intersexual selection, or social selection (Tobias and Seddon 2009, Dingle et al. 2010, Tobias et al. 2011).

The rufous-naped wren *Campylorhynchus rufinucha* is a morphologically variable and non-migratory taxon that inhabits tropical dry forest areas from Mexico to northwestern Costa Rica (Selander 1964, Howell and Webb 1995, del Hoyo et al. 2005, Bradley and Mennill 2011). This variation has led several taxonomists to recognize from four to six distinct subspecies (Howell and Webb 1995, Brewer and MacKay 2001, Dickinson 2003, del Hoyo et al. 2005). According to the taxonomy by del Hoyo et al. (2005) there are six subspecies, grouped in three distinct evolutionary units by Vázquez-Miranda et al. (2009): the *rufinucha* group in Veracruz and adjacent areas in the north of Oaxaca (a monotypic lineage); the *humilis* group from Colima east to western Chiapas (a monotypic lineage); and the *capistratus* group from western Chiapas south to

northwestern Costa Rica (Howell and Webb 1995, Brewer and MacKay 2001, Dickinson 2003, del Hoyo et al. 2005). The *capistratus* group includes populations assigned to the subspecies *nigricaudatus*, distributed from the west coast of Chiapas to the east of Guatemala; *capistratus*, distributed from Guatemala to the northwest of Costa Rica; *castaneus*, restricted to interior lands of Guatemala east to Honduras and Nicaragua; and *nicoyae*, restricted to the Nicoya Peninsula in northeastern Costa Rica (Brewer and MacKay 2001, del Hoyo et al. 2005). Several authors suggest that songs also show remarkable geographic variation (Selander 1964, Howell and Webb 1995, Brewer and MacKay 2001, del Hoyo et al. 2005). However, a comprehensive analysis of acoustic variation across the distribution range of rufous-naped wrens has never been conducted.

Interestingly, the rufous-naped wren's range includes a putative secondary-contact zone in coastal western Chiapas between *C. r. humilis* and *C. r. nigricaudatus* (Fig. 1; Selander 1964, 1965). The contact zone occurs in a very restricted area along 30 km in western Chiapas, between Tonalá and Tres Picos (Fig. 1; Selander 1965). In this area, Selander (1964, 1965) found birds with phenotypes clearly assignable to the parental forms based on plumage differences (*humilis* and *nigricaudatus*), as well as phenotypically intermediate forms (*humilis* × *nigricaudatus*). Selander (1964) suggested that the plumage traits have a multifactorial genetic basis, that hybrids are fertile and interbreed with the parental forms, and that they show no preferential mate selection. Hybrids, however, are only found in a restricted area, and no hybrids are found in populations bordering the secondary contact zone (Selander 1964, 1965; but see Vázquez-Miranda et al. 2009). Furthermore, the most variable hybrid individuals, with characters that are intermediate between parental forms, are located in the centre of the hybrid zone. Selander (1964) argued that this evidence suggests that

1) the rates of gene flow between parental forms are approximately equal, and 2) the habitat in the hybrid zone is not more suitable for one form than for the other.

In this study, we ask whether there is variation in songs throughout the range of rufous-naped wrens. We then ask what mechanisms are important in shaping geographic variation in songs of rufous-naped wrens. Our motivation for the first question was to determine whether geographic variation in vocal traits matches with the hypothesis proposed by the genetic research of Vázquez-Miranda et al. (2009) that three evolutionary units exist inside this taxon. For the second question, we test whether vocal signals have been affected by historical geographic isolation, and whether cultural selection, genetic and cultural drift, and morphological constraints can explain patterns of geographic variation in rufous-naped wren songs. If songs have been influenced by isolation, we predicted that differences between closely related populations would be smaller, regardless of the geographic distance between populations. Under the cultural selection model, we predicted that song divergence would be weakly correlated with genetic distance, but strongly correlated with differences in climate (a proxy for ecological differences; Cicero 2004, Ruegg et al. 2006). Under the drift model (cultural or genetic drift), we predicted that acoustic dissimilarity would be positively correlated with genetic distance, whereby acoustic divergence would be greatest between populations that have been isolated by the longest amount of time (Campbell et al. 2010). Under the morphological constraint of vocal performance hypothesis, we predicted that body and beak size would correlate with song structure (Podos 2001, Podos and Nowicki 2004, Podos et al. 2004a). Quantification of acoustic variation informs our understanding of species boundaries, sheds light on the factors involved in the evolution of complex signals, and provides insight into the mechanisms that promote and maintain patterns of biological diversity (Endler 1977, Helbig et al. 1996, Irwin et al. 2001, Päckert et al. 2003, Koetz et al. 2007, Toews and Irwin 2008, Dingle et al. 2010).

Methods

Study species

In this study, as in the genetic study of Vázquez-Miranda et al. (2009), we include most of the groups recognized by del Hoyo et al. (2005), except for the *castaneus* and *nicoyae* forms, for which we were not able to gather recordings. In the sampled populations, careful genetic analysis reveals the existence of three evolutionary units (Vázquez-Miranda et al. 2009): the *rufinucha* group, the *humilis* group, and the *capistratus* group (the latter comprising the *nigricaudatus* and *capistratus* forms; Fig. 1). Furthermore, the genetic data match Selander's (1964) findings based on morphological traits, and match the three main groups described by previous authors (Howell and Webb 1995, Brewer and MacKay 2001, Dickinson 2003, Navarro-Sigüenza and Peterson 2004, del Hoyo et al. 2005). Vázquez-Miranda et al. (2009) did not include the *castaneus* and *nicoyae* forms in their study, and the genetic relationships
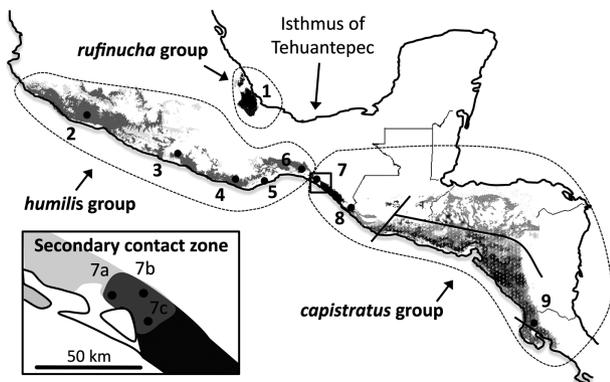

Figure 1. Map of Mesoamerica showing the nine sampled localities of rufous-naped wrens analyzed for patterns of acoustic variation. Shaded regions show the distribution of rufous-naped wrens. Numbers indicate the different sampled localities (details in Table 1). The inset map shows a detailed view of the secondary contact zone in Chiapas, México. Thin dashed lines show the ranges of the three evolutionary units sampled (*rufinucha*, *humilis*, and *capistratus* groups). Thin continuous lines show the ranges of four forms comprising the *capistratus* group: *nigricaudatus*, *capistratus*, *castaneus*, and *nicoyae*. The approximate position of the Isthmus of Tehuantepec is shown.

with *nigricaudatus*, *capistratus* or other forms still unknown. Therefore, we use the term 'capistratus group' (Fig. 1) when referring to the sampling and recording localities in the current study, and compare this group to the *rufinucha* and *humilis* groups (i.e. birds in the recognized monotypic lineages *rufinucha* and *humilis*).

Sampling and general methods

We collected field recordings from rufous-naped wrens in nine localities during 2005, covering most of the distribution for this species in Mexico (sampled localities 1 through 8 in Fig. 1, Table 1). Localities along the Pacific coast were sampled with an approximate separation distance of 200 km. However, in the secondary contact zone, we selected three localities, trying to match the populations in which Selander (1964) found strong evidence of hybridization (sampled localities 7a–c; Fig. 1, Table 1). Despite Selander's findings, all individuals we observed during our field recording sessions appeared to be *C. r. nigricaudatus*, based on plumage patterns described by Selander (1964; all birds we observed had a dark nape, black superciliary stripe, unpatterned back, tail with reduced barred patterns, and underparts lacking barred or spotted patterns). Some birds that we observed, however, had a submalar stripe, a characteristic of the *humilis* form. Although we did not capture and ring the birds recorded in this study, some specimens were collected simultaneously with our recordings at all three locations (i.e. 7a, 7b and 7c), and these specimens were used for the molecular analysis published by Vázquez-Miranda et al. (2009). According to our in-the-field observations, all individuals collected where phenotypically *C. r. nigricaudatus* (with the characteristics describe above), but the genetic analysis suggests the presence of hybrids across the secondary contact zone (Vázquez-Miranda et al. 2009).

To increase the geographic representation in our study, we gathered recordings for nine additional locations from natural sound libraries and private libraries. In total, we gathered songs from 18 localities from across Mesoamerica (Table 1). To establish units for the geographic analyses, we followed the criteria in Vázquez-Miranda et al. (2009). We pooled recording localities into groups and we refer to them as 'sampled localities'. First, we mapped the locations where we recorded birds in 2005 and the locations of the library recordings. Then, we pooled together recordings from locations that fell within a radius of 20 km, following the pooling method in Vázquez-Miranda et al. (2009). This approach yielded nine sampled localities (Fig. 1), representing most of the distribution of the species.

Acoustic sampling

We recorded songs using either a shotgun microphone (model: Audio-Technica AT835) or a dual omnidirectional microphone (model: Pro5 TwinScience) with a parabolic reflector (model: Telinga), and a digital recorder (model Sony Hi-MD MZ-NH700; recording media: MD Golden Premium Sony MiniDiscs). All songs were recorded in WAV format, at 16 bits with sampling rate of 44.1 kHz. Recordings were collected between 07:00 to 11:00 h and 15:00 to 18:00 h. None of the birds recorded were individually marked. To avoid recording the same individuals twice, we moved each day in a different direction within each locality. This species is territorial (Bradley and Mennill 2009a, b, unpubl.), allowing easy recognition of territorial boundaries between neighbors. If there was

Table 1. List of the sampled localities of rufous-naped wrens with their corresponding subspecies and geographic coordinates. The number of songs used per population in the analysis, sound libraries where recordings are archived, equivalent localities used for calculations of genetic distance, and mean body mass is shown.

Sampled locality	Location	Group	Lat N	Long W	Song[a]	GD[b]	Body mass[c]
1	La Mancha, Veracruz, México	*rufinucha*	19°35′52.7″	96°25′29.9″	19	VER	30.2 ± 0.7 (7)
2	Rancho Los Cirios, Guerrero, México	*humilis*	17°31′44.3″	101°18′57.6″	17	PET	23.0 ± 1 (12)
3	Tecomate, Guerrero, México	*humilis*	16°42′36″	99°21′35.9″	13	TEC	24.8 ± 1.1 (11)
4	Manialtepec, Guerrero, México	*humilis*	15°58′51.5″	97°14′20.4″	16	MAN	23.5 ± 1.3 (8)
5	Salina Cruz, Oaxaca, México‡	*humilis*	16°11′2.4″	95°11′41.9″	2	NA	NA
	Tehuantepec, Oaxaca, México‡	*humilis*	16°19′58.7″	95°13′58.8″	2		
6	Tapanatepec, Oaxaca, México‡	*humilis*	16°20′56.4″	94°12′0″	1	TAP	22.1 ± 2 (3)
	Rancho las Minas, Oaxaca, México	*humilis*	16°23′45.6″	94°8′27.6″	16		
7a	Rancho La Industria, Chiapas, México	secondary contact zone	16°2′42″	93°41′41.9″	8	JOY	29.0 ± 0.6 (15)
	Tonalá, Chiapas, México‡	secondary contact zone	16°4′58.7″	93°45′57.6″	1		
7b	La Polka, Chiapas, México	secondary contact zone	15°57′10.7″	93°40′4.8″	12		
7c	Tres Picos, Chiapas, México‡	secondary contact zone	15°51′57.5″	93°31′58.8″	1		
	Los Patos, Chiapas, México‡	secondary contact zone	15°49′58.8″	93°27′0″	1		
	El Llano, Chiapas, México	secondary contact zone	15°54′32.4″	93°33′7.2″	20		
8	Puerto Madero, Chiapas, México‡	*capistratus*	14°44′16″	92°24′32″	1	TUX	30.9 ± 1 (9)
	Rancho el Porvenir, Chiapas, México	*capistratus*	14°51′25.1″	92°11′52.7″	13		
9	Santa Rosa, Costa Rica∗	*capistratus*	10°39′57.6″	85°30′0″	6	NIC	34.5 ± 1.2 (9)

[a]Number of songs from different birds used in the analysis.
[b]Acronyms of the localities where birds were collected for the molecular analysis. Values of genetic distance are depicted in Table 2 in Vázquez-Miranda et al. (2009).
[c]Body mass in grams ± SE, number in parenthesis show number of birds measured.
NA denotes the sampled locality for which genetic and mass data were not available.
Libraries: (‡) Macaulay Library of Natural Sounds, (∗) songs provided by D. Bradley.

ambiguity about whether multiple recordings came from the same individual, we used songs only from the first recording and did not use subsequent recordings from that site.

We generated spectrograms of songs using Raven 1.2.1 software (Cornell Bioacoustics Research Program 2004; window type: Hanning; overlap: 80%; transformation frequency: 512; filter bandwidth: 64 ms). We selected one song per bird recorded, choosing the best quality song present in the recording (i.e. songs with the highest signal-to-noise ratio).

Experiments involving playback of song to rufous-naped wrens confirm that their songs are territorial signals (Bradley and Mennill 2009b). Prior research demonstrates that territorial birds produce both solo songs and vocal duets. We focus our analysis of geographic variation on solo songs, which are the most common type of vocalization (Bradley and Mennill 2009a). Bradley and Mennill (2009a) also found that, on average, pairs of rufous-naped wrens have a song repertoire of 20 phrase types. Since our objective was to determine the geographic variation in songs in terms of fine structural characteristics, we included one song per individual in our analysis. We also include anecdotal observations of the duets that we recorded in the nine sampled localities, with a focus on apparent patterns of geographic variation in duetting behaviour.

Fine structure measurements

We measured a total of 12 fine structural variables to describe the frequency and temporal characteristics of the songs. Rufous-naped wren songs are composed of phrases of up to five notes, which are sung in repetition (Bradley and Mennill 2009a). Seven of our measurements describe features of the entire song: 1) song length (s), 2) minimum frequency (Hz), 3) maximum frequency (Hz), 4) frequency bandwidth (maximum minus minimum frequency; Hz), 5) number of notes in the song, 6) number of phrases in the song (a phrase is defined as series of notes which occur together in a particular pattern, Catchpole and Slater 2008), and 7) number of notes per second. Our remaining five measurements describe features of individual phrases: 8) phrase length (s), 9) bandwidth of the note with highest frequency (Hz), 10) bandwidth of the note with lowest frequency (Hz), 11) number of notes in the phrase, and 12) number of notes per second in the phrase. We used the third phrase in the song to describe phrase structure; successive repetitions of phrases usually increase in amplitude, and the third repetition typically had the highest signal-to-noise ratio.

Climatic and genetic data

Climate plays a role in determining the structure and density of vegetation (Bachelet et al. 2001). Abiotic factors such as precipitation are closely related to biotic habitat structure, particularly in ecosystems where seasonality is defined by changes in vegetation, such as the Neotropical dry forest (Stotz et al. 1996). Vegetation density has an important influence on sound transmission and the acoustic structure of animal vocalizations (Morton 1975, Wiley and Richards 1982, Ryan and Brenowitz 1985, Tubaro and Lijtmaer 2006, Barker 2008). Furthermore, abiotic factors including

humidity and temperature influence sound transmission (Bradbury and Vehrencamp 2011). For example, high frequency sounds attenuate more in hot and humid localities and in dense vegetation compared to low frequency sounds (Catchpole and Slater 2008, Bradbury and Vehrencamp 2011).

To understand the relationship between rufous-naped wren songs and habitat, we used climatic variables as a proxy for environmental variation. We extracted climatic variables for every sampled locality using Worldclim, a global climate database based on data collected between 1950 and 2000, with ~1 km of spatial resolution (Hijmans et al. 2005). We summarized climatic conditions by extracting both temperature and precipitation variables. We used 10 temperature variables (annual mean temperature, mean temperature of warmest quarter, mean temperature of coldest quarter, mean diurnal range temperature, temperature seasonality, maximum temperature of warmest month, minimum temperature of coldest month, temperature annual range [maximum temperature of warmest quarter minus the minimum temperature of coldest quarter], mean temperature of wettest quarter, mean temperature of driest quarter), and eight precipitation variables (annual precipitation, precipitation of wettest month, precipitation of driest month, precipitation seasonality, precipitation of wettest quarter, precipitation of driest quarter, precipitation of warmest quarter, precipitation of coldest quarter). Following Ruegg et al. (2006) and Campbell et al. (2010), we make the assumption that locations with higher precipitation have denser vegetation than locations with lower precipitation. Moreover, the use of temperature variables allowed us to assess the effects of the differences in the environment over the fine structure characteristics of songs (Ruegg et al. 2006, Barker 2008). Temperature and humidity affect sound transmission in complex way, and the effect varies with the frequency of the sound. For example, many birds sing at frequencies around 4 kHz, and at this frequency, sounds have high attenuation when the humidity is low and temperature is high (Catchpole and Slater 2008). We extracted the climatic profile (i.e. the 18 climatic variables) for every recording location and calculated the average of each at the nine sampled localities.

Genetic distance values were extracted from Table 2 in Vázquez-Miranda et al. (2009) from sampled localities that matched our vocal dataset (Table 1). Our field recordings were collected simultaneously with the genetic sampling by Vázquez-Miranda et al. (2009) for 8 of the 9 sampled localities, so that there is a near-perfect temporal match between the acoustic and genetic data for this analysis.

Statistical analyses

We normalized continuous data using log and square-root transformations. We reduced the number of variables in our analyses of songs by conducting principal components analysis on all of the song measurements (PCA$_{song}$), using the correlation matrix and Varimax factor rotation. We report all factors with eigenvalues >1. We described the geographic variation in rufous-naped wren songs using a multivariate analysis of variance (MANOVA) on population

Table 2. Principal component analysis of the rufous-naped wren songs based on twelve fine structural features. Acoustic variables with the strongest loadings are indicated with asterisks.

	$PC1_{song}$	$PC2_{song}$	$PC3_{song}$	$PC4_{song}$
Eigenvalue	4.11	2.95	1.88	1.19
Variance explained	34.3%	24.6%	15.6%	9.9%
Factor loadings:				
Song length	−0.43	−0.16	0.83*	−0.20
Low frequency	0.20	−0.34	−0.10	0.58*
Frequency bandwidth of song	0.25	0.89*	0.15	0.12
Number of notes in song	0.37	−0.21	0.87*	0.15
Number of phrases in song	0.77*	−0.17	0.49	−0.28
Number of notes per second in the song	0.94*	−0.06	−0.07	0.07
Bandwidth of the note with highest frequency	−0.18	0.79*	0.11	0.05
Bandwidth of the note with lowest frequency	−0.02	0.76*	0.04	−0.28
Number of notes in the phrase	−0.65*	0.01	0.26	0.58*
High frequency	0.31	0.83*	0.13	0.28
Phrase length	−0.95*	−0.00	0.17	0.17
Number of notes per second in the phrase	0.84*	−0.05	0.03	0.37

mean principle component factors. We computed a post hoc Tukey test in order to describe significant differences in songs between localities. Given that the populations we sampled in the secondary contact zone (i.e. populations 7a, 7b, 7c) may contain hybrids, we ran our analyses twice: once including the birds in the secondary contact zone, and once excluding the birds in the secondary contact zone.

To understand the song variation across the secondary contact zone, we plotted the location of each individual's PC_{song} score. We used PC_{song} with significant differences in the aforementioned post hoc test (i.e. $PC1_{song}$ and $PC2_{song}$, see Geographic variation and isolation section), and calculated the distance of every individual to the centre of the hybrid zone. According to Selander's (1964) study, Río Agua Dulce, Chiapas, México (16°1′36.9″N, 93°43′59.2″W) is located in the midway of the secondary contact zone. Therefore, we used this position as the centre of the hybrid zone. We included in the analysis all sampled localities in the secondary contact zone (i.e. 7a, 7b and 7c), as well as the closest population at each side of the zone (i.e. 6 and 8).

To analyze climatic variables, we reduced the number of variables extracted from Worldclim using a principal component analysis ($PCA_{climate}$), including both precipitation and temperature variables in a single analysis (again using the correlation matrix and Varimax rotation).

To conduct vocal divergence analyses, we calculated average vocal and climatic values by sampled locality, and then calculated pair-wise dissimilarity values between sampled localities using Euclidean distances (sample size = 8 sampled localities where we had both genetic and acoustic data, Table 1). As result, we obtained two matrices, one for vocal characteristics, and a second for climatic characteristics, with pair-wise values of dissimilarity between sampled localities. We calculated the vocal matrix using principal components that could statistically distinguish between vocal groups (i.e. $PC1_{song}$ and $PC2_{song}$, see Geographic variation and isolation), and climatic matrix using all first four principal components. We created a matrix with pair-wise geographic distances in km between populations using the software Geographic Distance Matrix Generator (ver. 1.2.3; Ersts 2012). To test cultural or genetic drift, we compared the vocal and genetic matrices using a Mantel test. We then performed a partial Mantel test controlling for geographic distance between populations, because closer populations tend to have songs that are more similar. To test cultural selection, we compared the vocal and climatic matrices using a Mantel test. We then performed a partial Mantel test controlling for genetic distance and geographic distance between populations. Mantel tests and partial Mantel tests were computed using the software PASSaGE (ver. 2; Rosenberg and Anderson 2011) with 10 000 randomizations.

To test whether song structure is related to morphology, we performed a linear regression analysis using bird body mass as an independent variable and the PC_{song} calculations as dependent variables. We used mass as a proxy for body size. We also consider mass an indicator of beak size; Selander (1964) showed that larger rufous-naped wrens have larger beaks. Only those PC_{song} with significant differences in the post hoc test in our MANOVA (above) were included. Body mass values were obtained in field from birds collected for the genetic study by Vázquez-Miranda et al. (2009); D. Bradley provided additional mass measurements from birds of Costa Rica (Table 1). The regression analysis was based on means of sampled populations. All analyses were computed in PASW (ver. 18; SPSS), unless stated otherwise above.

Results

Geographic variation and isolation

We analyzed songs from 149 rufous-naped wrens (134 individuals from our field recordings and 15 individuals from sound libraries) from nine sampled localities distributed across the species' geographic range (Fig. 1). Rufous-naped wren songs showed high levels of variation (Fig. 2).

A principal components analysis of 12 structural features produced four principal components that accounted for the 84.5% of the measured variation in the songs (Table 2). MANOVA of the population mean principal component scores revealed significant vocal differences between sampled localities in both of the models we used to quantify geographic variation (hybrid sampled localities included: Pillai's Trace = 1.4, $F_{32,552}$ = 9.308, $p \leq 0.001$; hybrid sampled localities excluded: Pillai's Trace = 1.5, $F_{28,384}$ = 8.379, $p \leq 0.001$). In both models, post hoc tests revealed significant differences between three groups of sampled localities that corresponded with geography and the three established genetic groups: 1) the *rufinucha* group, 2) the *humilis* group, and 3) the *capistratus* group (Fig. 3; data with all hybrid sampled localities are shown).
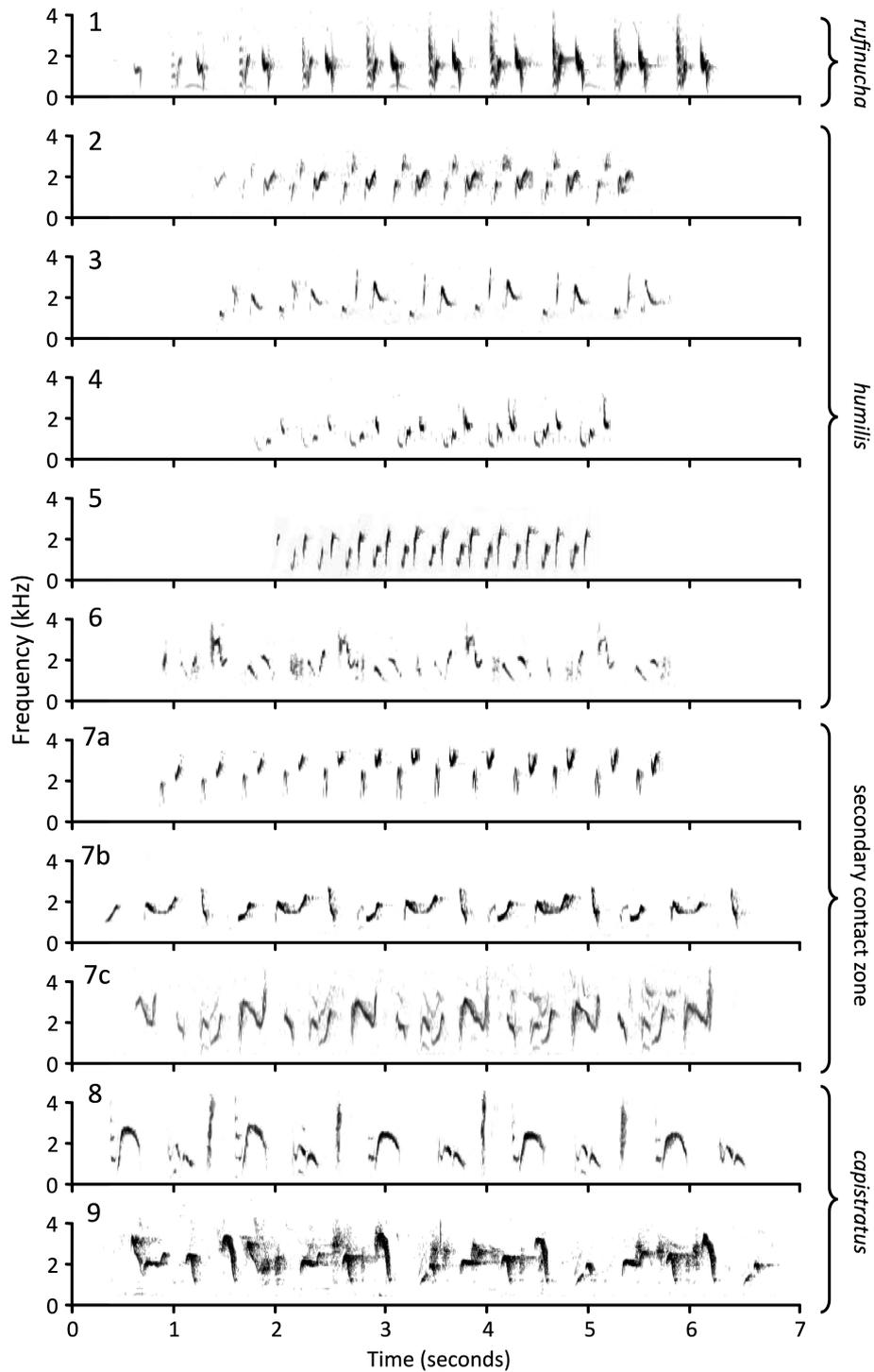
Figure 2. Spectrograms of rufous-naped wrens songs showing the variation between sampled localities across the geographic range of the species. Numbers refer to sampled localities indicated in Fig. 1.

Variation in PC1$_{song}$ showed significant differences between sampled localities on either side of the secondary contact zone (Fig. 3a); birds from sampled localities 1 through 6 (i.e. *rufinucha* and *humilis* groups) had significantly higher PC1$_{song}$ scores than birds in sampled localities 7 through 9 (i.e. *capistratus* group), reflecting more phrases per song, more notes per second in the song, fewer notes per phrase, and shorter phrase lengths (Table 2). Variation in PC2$_{song}$ showed significant differences between sampled locality 1 (i.e. *rufinucha* group) and the other eight sampled localities (i.e. *humilis* and *capistratus* groups; Fig. 3b), reflecting a broader bandwidth of the song, a broader bandwidth of the note with the highest and lowest frequency, and a higher maximum frequency in the song (Table 1). The
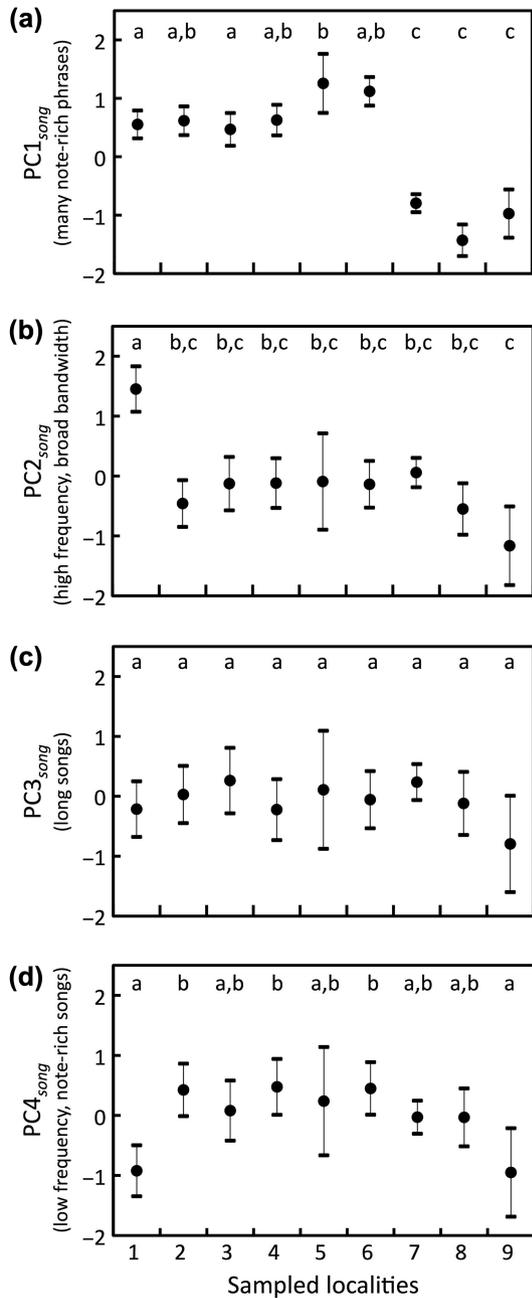
Figure 3. Plots of the principal component scores summarizing acoustic variation in the songs of rufous-naped wrens: (a) $PC1_{song}$; (b) $PC2_{song}$; (c) $PC3_{song}$; and (d) $PC4_{song}$. Points refer to population mean principal component scores, error bars show 95% confidential intervals. Numbers refer to sampled localities indicated in Fig. 1. Mean populations that not differ from each other are shown with the same letter, and subsets that are significant different at $p < 0.05$ are shown with different letters according to the Tukey post hoc test.

remaining components, $PC3_{song}$ and $PC4_{song}$, did not statistically distinguish between any of the sampled localities (Fig. 3c, d).

Although post hoc analyses following MANOVA (above) revealed no statistical difference between sampled locality 7 (the secondary contact zone) versus localities 8 and 9, a comparison of all individuals in the secondary contact zone against the populations on either side reveals that the range
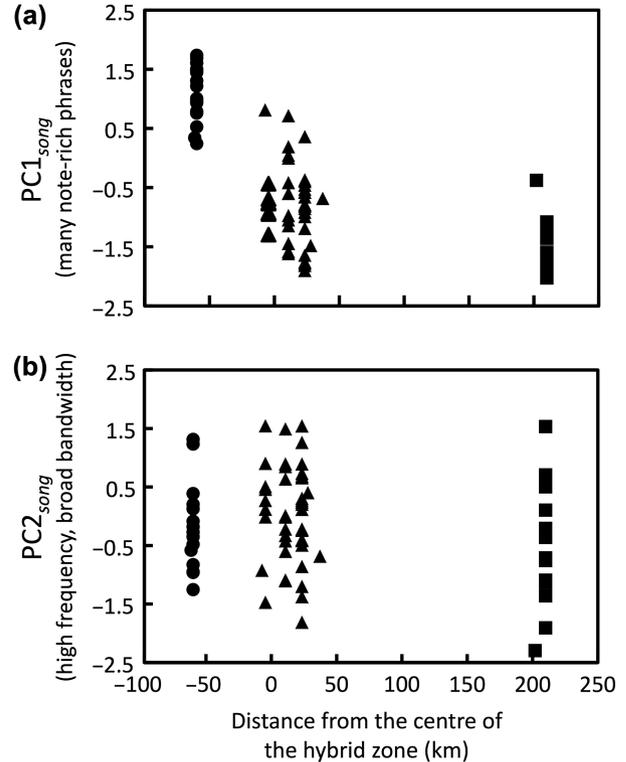

Figure 4. Plot of each individual's (a) $PC1_{song}$ and (b) $PC2_{song}$ score with geography at the secondary contact zone. Negatives and positives values in the x axis refer to the relative position of the localities in km; negative values indicate localities located west to the center, whereas positive values indicate localities located east to center of the secondary contact zone. Distance zero refers to Río Agua Dulce, Chiapas, México, which is located in the midway of the secondary contact zone. Circles show individuals at sampled locality 6, triangles show individual at sampled locality 7, and squares show individuals at sampled locality 8.

of sample locality 7 includes variation that is intermediate between localities 6 and 8 for $PC1_{song}$ (Fig. 4a), but not for $PC2_{song}$ (Fig. 4b). This analysis suggests that the acoustic variation in the secondary contact zone is intermediate, where both *humilis* and *capistratus* types of song are present, being the latter more frequent than the former.

Climatic variation

Principal components analysis of 18 climatic variables produced four principal components that explained 89.5% of the variation in temperature and precipitation measurements (Table 3). $PC1_{climate}$ related to higher mean temperatures and low precipitation in the coldest periods; $PC2_{climate}$ related to higher annual precipitation and high precipitation in the wettest periods; $PC3_{climate}$ also related to high temperatures in the driest and coldest period and low precipitation in the driest periods; $PC4_{climate}$ was influenced by precipitation of warmest quarter (Table 3).

Vocal divergence

To understand the forces driving vocal differences between populations, we tested whether vocal dissimilarity relates to

Table 3. Principal component analysis of 18 climatic variables for all localities in where rufous-naped wrens were recorded. Acoustic variables with the strongest loadings are indicated with asterisks. Climatic variables were extracted from BIOCLIM database.

	$PC1_{climate}$	$PC2_{climate}$	$PC3_{climate}$	$PC4_{climate}$
Eigenvalue	5.1	4.9	4.5	1.4
Variance explained	28.8%	27.7%	25.0%	8.0%
Factor loadings:				
Annual mean temperature	0.83*	−0.02	0.53	−0.02
Mean diurnal range temperature	0.56	0.64	0.12	−0.02
Temperature seasonality	−0.09	−0.44	−0.80*	−0.11
Max temperature of warmest month	0.81*	0.41	0.22	−0.22
Min temperature of coldest month	0.31	−0.20	0.86*	−0.04
Temperature annual range	0.43	0.56	−0.59	−0.17
Mean temperature of warmest quarter	0.77*	−0.57	0.08	0.19
Mean temperature of driest quarter	0.60	−0.19	0.73*	0.07
Mean temperature of wettest quarter	0.87*	−0.16	0.24	−0.08
Mean temperature of coldest quarter	0.58	0.27	0.74*	−0.08
Annual precipitation	−0.25	0.94*	−0.11	−0.03
Precipitation of wettest month	−0.06	0.95*	−0.05	−0.05
Precipitation of driest month	−0.19	0.35	−0.70*	0.40
Precipitation seasonality (coefficient of variation)	0.64	−0.56	0.14	−0.15
Precipitation of wettest quarter	−0.16	0.95*	−0.12	−0.05
Precipitation of driest quarter	0.06	0.28	−0.80*	0.44
Precipitation of warmest quarter	−0.21	−0.21	−0.13	0.90*
Precipitation of coldest quarter	−0.73*	0.31	0.09	0.29

climatic differences and genetic distance using Mantel tests. The analysis showed a significant relationship between vocal dissimilarity and genetic distance (Mantel test: r = 0.6, p < 0.001); this held true even when controlling for geographic distance (partial Mantel test: r = 0.5, p = 0.01). Conversely, vocal dissimilarity and climatic differences showed no significant relationship (Mantel test: r = 0.2, p = 0.1); this held true when controlling for genetic and geographic distances (partial Mantel test: r = −0.05, p = 0.8). Therefore, genetic relatedness explained similarities in vocalizations between sampled localities, whereas climate did not.

Linear regression analysis showed a significant relationship between body mass and $PC1_{song}$ ($r^2 = 0.65$, β = −0.8, DF = 6, p = 0.01), but no significant relationship with $PC2_{song}$ ($r^2 = 0.13$, β = −0.1, DF = 6, p = 0.7). In other words, mass was a reliable predictor of variation in length of the song and the number of notes per second, but not the frequency of the song. Based on the morphological constraint of vocal performance hypothesis (Podos 2001), we computed a further linear regression analysis using mass as an independent variable and number of notes per second in the phrase as dependent variable, revealing a strong relationship between variables ($r^2 = 0.76$, β = −0.8, DF = 6, p = 0.005). Therefore, body size explained a significant amount of variation in the number of notes per second in a wren's song.

Geographic variation in duets

We recorded vocal duets (two birds producing song simultaneously) in all sampled localities, although solo songs were more common than duets in all sampled localities (sign test: p = 0.004, n = 9). The recorded birds were not individually marked, yet duet partners appeared to be two birds of the same family group; we assumed that they were the reproductive pair of the group, based on their close physical proximity, and the similarity in physical behaviour compared

to the formal description of the duetting behaviour of this species (Bradley and Mennill 2009a).

Duets appeared to vary substantially between vocal groups (i.e. *rufinucha*, *humilis* and *capistratus* groups), but not substantially within groups. This difference stemmed primarily from variation in duet contributions. In some populations, duets included matched, overlapping tonal phrases by both duetting birds (Fig. 5a); in others, duets included overlapping tonal phrases where each bird sang non-matching phrase types (Fig. 5b); and in others, duets included tonal phrases by one bird that were overlapped by harsh, staccato notes by the second bird (Fig. 5c). The matched, overlapping tonal duets were only recorded from the secondary contact zone southwards, in the *capistratus* group (i.e. recorded in sampled localities 7 through 9). We only encountered duets with tonal phrases overlapping harsh, staccato notes in the *humilis* group, north of the secondary contact zone; these duets were never recorded south of the secondary contact zone, or in La Mancha, Veracruz (i.e. these were recorded exclusively in sampled localities 2 through 6). The overlapping, two-type tonal phrase duet was recorded only in La Mancha, Veracruz (i.e. *rufinucha* group) and we never heard another type of duet during our stay at that locality. Whenever we were able to observe the birds performing the duets, they engaged in intricate visual displays while they produced song (see detailed description in Bradley and Mennill 2009a). Consequently, our anecdotal observations of vocal duets suggest that rufous-naped wrens also have geographic differences in duetting behaviour. Further studies are needed to quantify this variation.

Discussion

Detailed acoustic analyses of recordings collected throughout the range of rufous-naped wrens reveal significant vocal
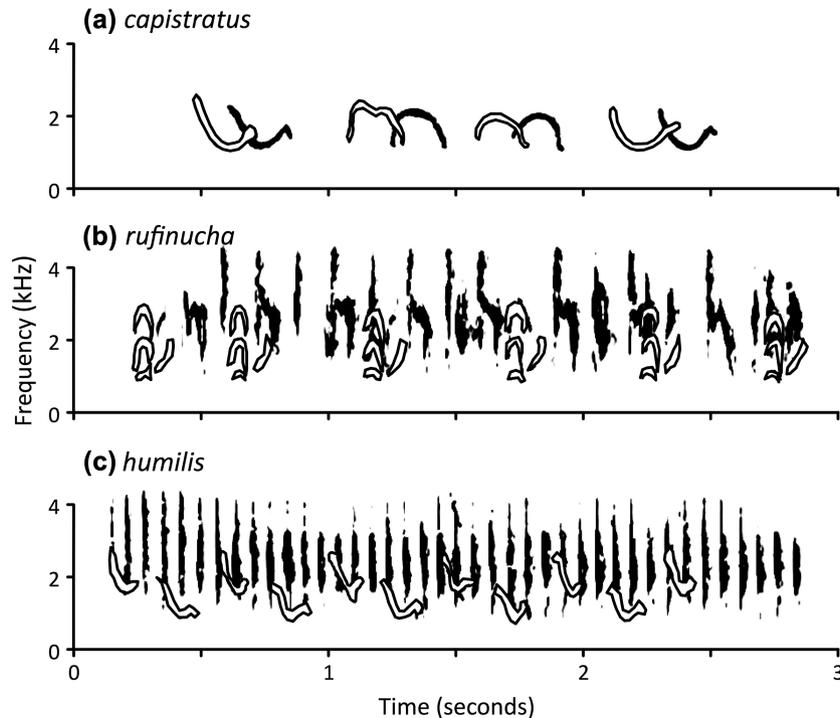
Figure 5. Three representative spectrogram tracings of the duets of rufous-naped wrens: (a) duet in which both individuals sing the same tonal phrase type, (b) duet in which both individuals sing different tonal phrases; (c) duet composed of tonal phrases produced by one bird and repeated atonal phrases by the second bird.

differences between populations of this charismatic Mesoamerican bird. Our results suggest that the discontinuous variation between the three evolutionary units (*rufinucha*, *humilis*, and *capistratus* groups) likely arose due to geographical isolation. We report a strong relationship between vocal divergence and genetic distance, but not between vocal divergence and climatic differences. Body size and the rate of note delivery show a significant relationship, suggesting that body morphology influences song structure. Our results have important implications for the taxonomy of rufous-naped wrens and, more broadly, for our understanding of the processes that contribute to geographic variation in animal signals.

We found that most geographic variation in rufous-naped wren songs is attributable to differences between the *rufinucha*, *humilis*, and *capistratus* groups. Therefore, variation in vocal traits in rufous-naped wrens matches variation in genetic structure, which in turn reflects historical isolation due to physical barriers (Vázquez-Miranda et al. 2009). These congruent patterns in vocal variation and genetic variation (as well as variation in morphological and plumage traits, as described by Selander 1964), and geographic isolation have been reported in several bird species (e.g. *Phylloscopus collybita* complex, Helbig et al. 1996; *Phylloscopus trochiloides*, Irwin et al. 2001; *Regulus* genus, Päckert et al. 2003; *Orthonyx spaldingii*, Koetz et al. 2007; *Troglodytes troglodytes pacificus* vs *T. t. hiemalis*, Toews and Irwin 2008; *Henicorhina l. leucophrys* and *H. l. hilaris*, Dingle et al. 2010).

The simplest explanation for the large-scale variation in vocal and genetic traits between the *rufinucha*, *humilis*, and *capistratus* groups is presented by Vázquez-Miranda et al.

(2009). They suggest that these populations were isolated by the Middle to Late Pleistocene water channel that existed during the formation of the Isthmus of Tehuantepec, approximately 3 million yr ago. This isolation likely promoted differences in morphology and plumage (Selander 1964), genetics (Vázquez-Miranda et al. 2009), and vocal characters (current study), explaining the consistent pattern of variation in these traits. The existence of a seaway barrier across the Isthmus of Tehuantepec is controversial, but additional evidence from studies of rodents and toads provide support for this idea (Sullivan et al. 2000, Mulcahy et al. 2006). Evidence of lineage separation due to paleogeographic events has been suggested previously for the genus *Campylorhynchus* across the Isthmus of Panama (Barker 2007).

We found a strong relationship between vocal dissimilarity and genetic distance, but not with climatic differences. This finding suggests that acoustic differences mirror genetic differences. According to Lynch (1996), in the absence of environmental changes or other selective factors, songs of populations will tend to diverge randomly. The logic behind this is that cultural transmission plays a weaker role in neutralizing the accumulation of new variation due to cultural mutation (Lynch 1996). Thus, a tentative explanation for the pattern of variation we described in the songs of rufous-naped wrens is that songs were influenced by vicariant events in the past, followed by accumulation of random changes in the fine structural characteristics of songs through errors during the learning process (cultural drift; Lynch 1996), or were influenced by random changes or natural selection on mechanisms involved in the production of sounds (genetic drift or indirect natural selection; Podos et al. 2004a).

The strong negative correlation between body mass and number of notes per second provides some support for the latter idea. Body size and beak size are known to limit a bird's ability to produce different types of sounds (Podos 2001, Podos et al. 2004a). For example, studies of Darwin's finches revealed that beak size correlates negatively with both vocal performance (Podos 2001, Huber and Podos 2006) and positively with bite force (Herrel et al. 2005); bite force, in turn, correlates negatively with jaw movement velocity (Herrel et al. 2009); and increasing beak size and strength is thereby expected to constrain maximum speeds of beak movement while singing (Podos and Nowicki 2004). The marked differences in beak size between rufous-naped wren groups (Selander 1964), suggest that beak size could constrain beak movement during song, limiting the number of notes that a rufous-naped wren can sing. Whether changes in beak size are caused by genetic drift or natural selection is an interesting question; we are not aware of any study reporting song evolution through genetic drift of the vocal apparatus (Podos et al. 2004a). As such, natural selection driving changes in morphology serves as a possible mechanism driving acoustic variation in song by constraining the rate of note repetition throughout the evolution of beak size, as shown in Darwin's finches (Schluter et al. 1985, Podos 2001, Herrel et al. 2005, 2009, Huber and Podos 2006).

Whether differences in song reflect taxonomic boundaries is another important question. Three independent lines of evidence suggest there are at least three evolutionary units within the rufous-naped wren complex that correspond to the three main recognized taxa. First, our observations of variation in song fine structural characteristics and duetting styles are consistent with the general pattern of genetic traits and morphological traits between subspecies (Selander 1964, Vázquez-Miranda et al. 2009). Second, acoustic differences are correlated with genetic distance. Third, the discontinuous pattern of song variation indicates that transfer of vocal traits through learning between taxa is likely to be low, reflecting geographic isolation (Lynch 1996). Species and subspecies differences have been documented in other members of the family Troglodytidae using acoustic characters (e.g. *Hylorchilus navai* vs *H. sumichrasti*, Gómez de Silva 1997; *Thryothorus nicefori* vs *T. rufalbus*, Valderrama et al. 2007; *Troglodytes pacificus* vs *T. hiemalis*, Toews and Irwin 2008; *Henicorhina l. leucophrys* vs *H. l. hilaris*, Dingle et al. 2010), showing that songs can reveal species limits, even in song-learning species that have highly complex vocalizations.

Song, duets and the hybrid zone

The best explanation for the origin of the hybrid zone is secondary contact between the *humilis* and *capistratus* groups following the formation of the Isthmus of Tehuantepec (Selander 1964, Vázquez-Miranda et al. 2009). Anthropogenic habitat modification may have created additional habitat appropriate for rufous-naped wrens, further promoting population expansion and contact between birds in the hybrid zone area (Selander 1964, 1965, Vázquez-Miranda et al. 2009). Under the scenario of secondary contact and hybridization between these two groups, two outcomes in song variation are possible. If exchange of cultural traits between forms is low or absent, then we would expect a discontinuous pattern of song variation between forms. Under this scenario, all birds would sing either *humilis* or *capistratus* types of songs, and birds with mixed repertoires (i.e. repertoires containing song types from both parental populations) or mixed songs (i.e. songs that share characteristics with both parental populations) would be rare or absent. Conversely, if cultural traits are transferred between isolated populations after contact, through song learning, we would expect to find individuals singing mixed repertoires or mixed songs. The first scenario would result in most birds in the contact zone (sampled locality 7) producing either *humilus*-like or *capistratus*-like songs. The second scenario would result in birds in the contact zone producing mixed songs that are different from other *humilus* and *capistratus* populations (e.g. sampled localities 6 or 8), or resulting in an intermediate song variation if producing mixed repertoires. Our analysis showed that songs in the secondary contact zone are significantly different from *humilis* songs, but not statistically different from the *capistratus* songs (Fig. 3). Our results also revealed that acoustic variation in the secondary contact zone is intermediate, with more birds singing *capistratus*-like song type songs (Fig. 4), and spectrograms reveal that songs resemble either *humilis* (e.g. Fig. 2, 7a) or *capistratus* (e.g. Fig. 2, 7b, c). Therefore, our findings do not support Selander's (1964) suggestion that mixed songs occur at the secondary contact zone. We, however, cannot confirm the presence of birds singing mixed repertoires since we only included one song per individual. Further research that includes exhaustive sampling of the repertoires of birds in and around the hybrid zone may provide a definitive answer to this question.

Our analysis revealed a relationship between song characters and body mass. In rufous-naped wrens, birds with larger bodies also have larger beaks (Selander 1964). Selander (1964) showed a marked cline in beak and body mass in rufous-naped wrens at the secondary contact zone (i.e. sampled locality 7). Therefore, if morphology has a major influence on song structure, it is to be expected that songs would show a similar pattern in the secondary contact zone. In line with this expectation, our results show that there is a pronounced correlation between body mass and number of notes per second (a variable with high loading on $PC1_{song}$). Thus, given the size differences between *humilis* and *capistratus*, the pattern of variation in $PC1_{song}$ at the secondary contact zone could be related to the cline in body size.

Sexual selection may also contribute to the observed pattern of acoustic variation across the secondary contact zone. For example, if hybrids suffer from genetic deficiencies or poor fit to their ecological niche, then individuals that select con-subspecific mates, rather than hetero-subspecific mates, would be favoured by sexual selection. Our observations of duetting behaviour support this idea. It is thought that separation between populations or closely-related species could be an important component of sexual selection through duetting (Diamond and Terborgh 1968). Allopatric populations in the *rufinucha* and *humilis* groups show modest variation (in relation to the *capistratus* group) and have similar but distinctive duets. Conversely,

differences in the duets of the *humilis* versus *capistratus* groups, which interbreed in the secondary contact zone, appear to be pronounced. A similar pattern is seen in duets of black-collared barbets *Lybius torquatus*, which show little variability when compared to allopatric populations of black-billed barbets *Lybius guifsobalito*, but high variability in comparison to sympatric populations of red-faced barbets *L. rubrifacies* (Short and Horne 1983). Selander (1964) reported patterns of variation in *Campylorhynchus* duets that match our observations, but we are aware that these observations are anecdotal and further quantitative analyses are needed to describe this species duets in more detail (for example, whether the duet contributions of males and females might show different geographic patterns; whether the harsh staccato notes might be alarm calls rather than songs; and whether duets vary seasonally). These studies are fundamental to confirming whether duets correspond to genetic differences in rufous-naped wrens, as do our analyses of solo songs. Collecting recordings for quantitative comparisons will be challenging research, because duets are highly complex vocal behaviours and they are produced less often than solo vocalizations.

Our results show that *capistratus*-like songs are more common in the secondary contact zone than *humilis*-like songs. This pattern may reflect a female preference for *capistratus*-like songs (but see Selander 1964). An alternative explanation for this finding is nonrandom acoustic sampling. We believe this is unlikely because our recordings were collected while trying to sample birds in all directions in every locality, recording all individuals in each transect. Similar to our findings, Vázquez-Miranda et al. (2009) reported that all specimens collected at the secondary contact zone showed *capistratus*-like plumage. Therefore, the secondary contact zone appears to feature more *capistratus*-like birds, both in plumage (Vázquez-Miranda et al. 2009) and in song (current study), arguing that our results do not arise through nonrandom sampling, but that hybrid zone birds do indeed share more features with the *capistratus* group.

In summary, our study of acoustic variation in the songs of rufous-naped wrens reveals patterns of geographic variation that match closely with previous morphological and genetic studies, which showed the presence of at least three evolutionary units inside this complex taxon (Selander 1964, Navarro-Sigüenza and Peterson 2004, Vázquez-Miranda et al. 2009). Our analysis also indicates that large-scale processes affecting variation in vocal traits are similar to those driving variation in genetic and morphological traits. We suggest that the variation in the songs of the rufous-naped wren is affected, at least partially, by vicariant events followed by direct factors like accumulation of changes in song as result of cultural drift, or indirect factors like genetic drift or/and natural selection acting on the vocal apparatus of the rufous-naped wren.

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