RESEARCH ARTICLE

Geographic variation in the duets of the Rufous-naped Wren (*Campylorhynchus rufinucha*) complex

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ABSTRACT
Acoustic signals used in animal communication play a key role in mate attraction, species recognition, and territory defense. Variation in acoustic signals may reflect population structure, lack of gene flow, and phylogenetic relationships. In birds, the study of geographic variation in acoustic signals has been useful for elucidating potential factors involved in phenotypic divergence and for establishing species limits. However, most of the studies on geographic variation have focused on calls and solo songs, with few studies focusing on complex behaviors such as duets. In this study, we investigated the variation in the duets of the Rufous-naped Wren (*Campylorhynchus rufinucha*). Our results suggest that duets vary across the distribution range of the species complex, matching the 3 taxonomic groups previously suggested (*rufinucha*, *humilis*, and *capistratus*). We also found a marked song sexual dimorphism in 2 groups, *rufinucha* and *humilis*, that is lacking in *capistratus*. At the local level, we found that duets also vary among sites within groups, but less than between groups. Our results suggest that groups *rufinucha* and *humilis* sing the most similar duets, whereas *capistratus* performs the most divergent duets of the 3 groups. Further, when comparing duet variation across geography, we found that birds living closer to each other sing more similar duets. We suggest that the existence of 3 vocal lineages is probably the result of historical geographic isolation followed by other selective (i.e. sexual selection, social selection, habitat structure) and/or non-selective factors (i.e. drift, isolation by distance), and that variation found within groups may be the result of cultural drift or social selection.

Keywords: acoustic divergence, allopatry, song sexual dimorphism, song variation, species limits

Variación geográfica en los duetos de la Matraca Nuquirrufa (*Campylorhynchus rufinucha*)

RESUMEN
Las señales acústicas usadas en la comunicación animal juegan un papel importante en la atracción de pareja, el reconocimiento de especies y la defensa del territorio. La variación de las señales acústicas puede reflejar la estructura poblacional, ausencia de flujo génico y relaciones filogenéticas entre especies. En aves, el estudio de los patrones de variación geográfica de las señales acústicas ha sido útil para definir límites de especies. Sin embargo, la mayoría de los estudios sobre variación geográfica se han centrado en llamados y cantos "solos", con pocos estudios centrados en comportamientos complejos como los duetos. En este estudio, investigamos la variación en los duetos de la Matraca Nuquirrufa (*Camplorhynchus rufinucha*). Los resultados sugieren que lo duetos varían a lo largo del rango de distribución del complejo, coincidiendo con los tres grupos taxonómicos sugeridos previamente (*rufinucha*, *humilis* y *capistratus*). Encontramos un marcado dimorfismo sexual en los duetos de los grupos *rufinucha* y *humilis*, y la falta de dimorfismo sexual en los duetos de *capistratus*. A nivel local, encontramos que los duetos varían entre sitios dentro de grupos, pero menos que entre grupos. Nuestros resultados sugieren que los grupos *rufinucha* y *humilis* producen los duetos más similares, mientras que *capistratus* produce los duetos más divergentes de los tres grupos. Además, al comparar la variación de los duetos a lo largo de la geografía, encontramos que las parejas que viven más cerca producen los duetos más similares. Sugerimos que la existencia de los tres linajes vocales es quizás el resultado del aislamiento geográfico histórico seguido de otros factores selectivos (i.e. selección sexual, selección social, estructura del hábitat) y/o no selectivos (i.e. deriva, aislamiento por distancia), y que la variación de los duetos dentro de los grupos pueden ser el resultado de deriva cultural o selección sexual.

Palabras clave: alopatría, aves, dimorfismo sexual del canto, divergencia acústica, límite de especies, variación del canto

INTRODUCTION

Many animal groups produce acoustic signals that are used in mate attraction, species recognition, and territory defense (Bradbury and Vehrencamp 2011). Therefore, acoustic signals are under strong selection pressures (Podos et al. 2004), and their spatial variation (hereafter referred as "geographic variation") has been well documented in several taxa, such as primates (Burton and Nietsch 2010), bats (Barclay 1999, Sun et al. 2013), anurans (Narins and Smith 1986, Pröhl et al. 2007), insects (Symes 2018), cetaceans (Cerchio et al. 2001), and birds (Leger and Mountjoy 2003, Benedict and Bowie 2009, Budka and Osiejuk 2017, Villegas et al. 2018). Particularly in birds, the study of patterns of geographic variation in acoustic signals has been useful for understanding factors and mechanisms involved in speciation processes (Catchpole and Slater 2008).

Geographic variation in acoustic signals can occur at the micro-geographic or macro-geographic levels (Mundinger 1982). At the micro-geographic level, some song properties may differ among neighboring populations that are close enough for individuals to interact socially or interbreed (Mundinger 1982). For example, the dialects in the White-crowned Sparrow (*Zonotrichia leucophrys*) around San Francisco Bay suggest micro-geographic variation because neighboring populations show differences for several song traits, but similarities within populations (Marler and Tamura 1962). On the other hand, at the macro-geographic level, differences in song properties are found among populations separated by great distances or barriers that prevent either the gene flow or the transmission of cultural traditions among populations (Mundinger 1982). The song variation among 2 subspecies of Swamp Sparrow (*Melospiza g. georgiana* and *M. g. nigrescens*) in western Maryland and coastal Delaware that are separated by over 320 km is an example of macro-geographic variation, given that songs of both subspecies differed in some acoustic characteristics, including frequency bandwidth and trill rate (Liu et al. 2008).

In non–vocal learners, species divergence among isolated populations could emerge from selective forces such as ecology and mate choice, or could arise as a result of non-selective forces such as genetic drift (Jones 1997, Podos et al. 2004, Tobias and Seddon 2009, Wilkins et al. 2013). When song learning is present, however, other factors such as cultural selection and cultural drift might play an important role in the origin of vocal divergence (Podos et al. 2004, Wilkins et al. 2013). Thus, the interpretation of geographic variation in learned acoustic signals is complex and its description is fundamental to elucidate the factors involved in its diversification (Derryberry et al. 2012, Wilkins et al. 2013).

Compared to the study of geographic variation in male solo songs, less attention has been paid to patterns of geographic variation in complex vocalizations such as duets (Odom and Mennill 2012). A duet is a vocal display produced by 2 birds—usually members of a pair—where their vocalizations may overlap or alternate between both pair members and are often accompanied by coordinated visual displays (Farabaugh 1982, Hall 2004). These duets could be polyphonal (non-matching notes generally produced at the same time), simultaneous (similar notes generally produced at the same time), or antiphonal (alternating notes between male and female; Dahlin and Benedict 2014). The few studies that focus on the analysis of geographic variation of bird duets (e.g., Bretagnolle and Lequette 1990, Mennill and Rogers 2006, Dahlin and Wright 2009, Odom and Mennill 2012) suggest the existence of variation in complex signals in non-passerine and passerine birds. However, further studies are needed to better understand the processes involved in the origins and diversity of complex vocal signals.

The Rufous-naped Wren (*Campylorhynchus rufinucha*) complex is known to produce duets throughout the year (Skutch 1935, Bradley and Mennill 2009). They are distributed in Neotropical dry forests, ranging from central Veracruz and Colima in Mexico south to northwestern Costa Rica (Selander 1964). This taxon shows remarkable morphological variation throughout its distribution (Selander 1964, Howell and Webb 1995), which has led several authors to recognize 5–9 subspecies (Peters 1960, Brewer and Mackay 2001, Dickinson 2003). However, some studies show that these subspecies can be grouped into 3 main groups based on morphology, genetics, and behavior (Selander 1964, Vázquez-Miranda et al. 2009, Sosa-López et al. 2013; Figure 1); these are (1) the *rufinucha* group, isolated in central Veracruz in Mexico; (2) the *humilis* group, ranging from Colima to western Chiapas along the Pacific slope of Mexico; and (3) the *capistratus* group, ranging from western Chiapas to northwestern Costa Rica. Further, several taxonomic authorities suggest that the 3 groups in fact represent different species (Ridgway 1904, Selander 1964, Brewer and Mackay 2001, Navarro-Sigüenza and Peterson 2004, Howell and Webb 1995, Clements 2009, del Hoyo et al. 2016). Indeed, Vázquez-Miranda et al. (2009), using a molecular phylogenetics approach, suggested that differences between the 3 groups might have arisen through a series of geographic isolation events during the Middle to Late Pleistocene, with *rufinucha* and *humilis* being sister taxa, and *capistratus* the sister group of the *rufinucha–humilis* clade.

In this study, we analyzed the geographic variation in duets of Rufous-naped Wren across its distribution range and address 3 questions: (1) Do the Rufous-naped Wrens' duets vary along the species' distribution? (2) Does duet variation match the 3 previously described groups within the
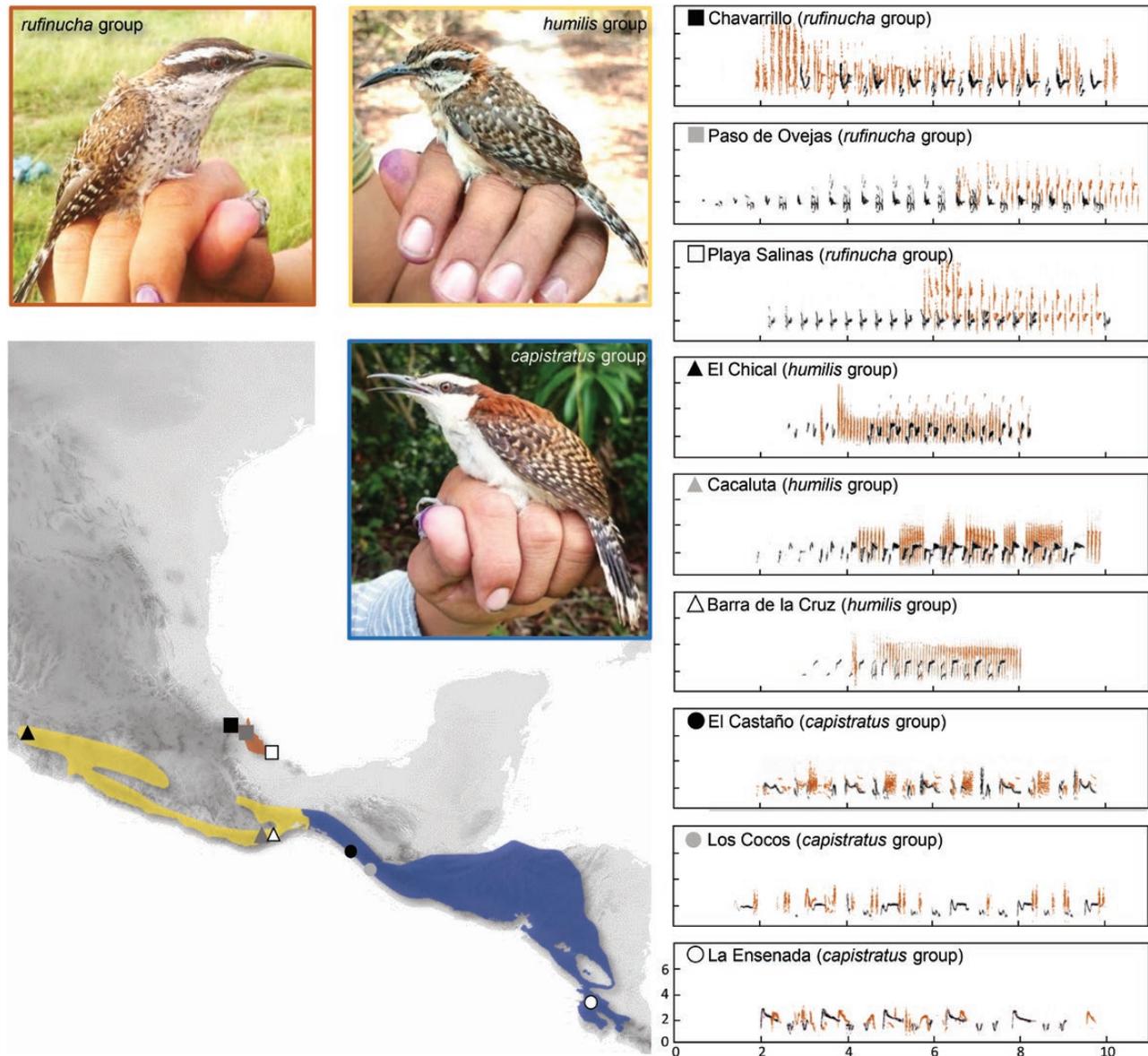
FIGURE 1. Map of Mesoamerica showing the distribution of the 3 Rufous-naped Wren groups. The yellow-shaded area shows the distribution of the *humilis* group, blue-shaded area shows the distribution of the *capistratus* group, and light-red area shows the distribution of the *rufinucha* group; *humilis* and *capistratus* groups have a contact zone in northwest Chiapas. Spectrograms depict common duets recorded at the 9 localities. Within the *rufinucha* and *humilis* groups, orange-colored elements in the spectrograms represent the contribution of females and black-colored elements represent the contribution of males. Symbols (squares, triangles, circles) depict the recording locations of each duet in the map. The *x*-axis tick marks of spectrograms show increments of 2 s, whereas the *y*-axis tick marks show increments of 2 kHz.

complex (*capistratus*, *rufinucha*, and *humilis* groups)? and (3) Does duet similarity between groups reflect the phylogenetic relationship among the groups proposed by Vázquez-Miranda et al. (2009)? To answer these questions, we first describe the fine structural characteristics of the duets. Then, we test whether variation and similarity in duets reflect the 3 proposed groups and the phylogenetic relationship previously proposed. If historical geographic isolation has promoted variation in the fine structural characteristics

of the duets, we predict differences in duets between closely related groups (*humilis* vs. *rufinucha*) to be smaller than between distantly related groups (*humilis* vs. *capistratus* or *rufinucha* vs. *capistratus*). Conversely, if variations in the fine structural characteristics are not related to geographic isolation, we predict that duet similarities will not mirror phylogenetic relationships. This study seeks to describe the variation of complex signals across geographic space to highlight potential factors driving the evolution of such behaviors.

METHODS

Sampling and General Methods

We collected recordings from 9 sites within the distribution range of the groups from Mexico to Costa Rica (Table 1), sampling 3 sites per group, as suggested by Selander (1964), Vázquez-Miranda et al. (2009), and Sosa-López et al. (2013). For all 3 groups, we tried to include samples from the most distant sites belonging to the same group (Figure 1), allowing us to test the effect of isolation-by-distance within and between groups (Koetz et al. 2007).

Duet Recording

We recorded duets with a FOSTEX digital recorder (model DC-R302) using either a shotgun Sennheiser (model ME67/K6) or an omnidirectional Sennheiser microphone (model ME62/K6) mounted in a parabolic reflector (Telinga Universal), or a Tascam digital recorder (model DR-70D) with a shotgun Sennheiser microphone (model ME67/K6). All recordings were made in WAV format, 24 bits and a sample rate of 44.1 kHz. Recordings were collected from 0630 to 1100 hours and from 1600 to 1900 hours.

Most of the duets included in the analysis occurred naturally, with only 18 of 101 territories recorded after simulating territorial intrusion using other duets in a playback (3 territories belonging to the *rufinucha* group and 15 belonging to the *capistratus* group). During the recording sessions, we noted the birds' behavior in order to differentiate duets (both members of the pair less than 1 m apart, flapping their wings, spreading their tail feathers, and raising their beaks at almost a vertical position) from each individual's vocalizations overlapping randomly (songs produced when both individuals were more than 1 m apart, without flapping their wings, spreading their tail feathers, and/or raising their beaks). This criterion for inclusion is conservative, given that it may exclude some duets performed by pairs singing more than 1 m apart, as some duets may be performed from a location where the partner birds are out of visual contact of the other. However, this criterion excludes recordings that might represent random overlap in songs rather than true duets.

To identify pairs and members within a pair, we used mist nets to capture and color-band most of the birds included in the study. Each individual received a unique combination of colored plastic bands. Whenever possible, we sexed birds based on the presence of a brood patch or cloacal protuberance. We captured and banded 18 individuals from the *capistratus* group (7 males, 7 females, and 4 not sexed), 29 from the *rufinucha* group (16 males and 13 females), and 30 from *humilis* group (3 males, 3 females, and 24 not sexed). From a total of 101 recorded pairs, in 3 territories we banded 3 birds (an adult female, an adult male, and a helper); in 28 territories we banded both adults (one female and one male); in 12 territories we banded only one adult (either female or male); and in 58 territories the birds were unbanded. When individuals of a pair could not be banded, we identified territories by location, as this taxon is highly territorial, and individuals stay in the same territory for several months (Rabenold 1990). The presence of cooperative breeding in the *capistratus* group raises the possibility that some recordings represent helper/adult duets; however, we recorded in 2 territories where we color-banded 2 adults and a helper and never observed a helper duetting with one of the adults.

Acoustic Measurements and Analysis

To obtain the fine structural characteristics of the duets, we randomly chose up to 5 duets from each recorded pair by selecting high-quality recordings with no overlapping background sounds and high signal-to-noise ratio. In some cases, we did not have 5 recordings for analysis, so we included all available high-quality duets for that specific pair. First, we pre-selected all duet recordings and assigned them a consecutive number, then we generated 5 random numbers by pair to select the duets to be included in the analysis. We generated spectrograms for each selected duet with Raven Pro software (version 1.5 Beta; Cornell Bioacoustics Program 2010) using the following parameters: Hamming window, DFT 512, and 80% overlap. Measurements were taken using the threshold method, which involves the use of the power spectrum to measure frequency-related traits (Podos 1997,

TABLE 1. Localities, group membership, recording dates, and number of Rufous-naped Wren pairs recorded.

Locality	Group	Date	Coordinates	Pairs recorded
Barra de la Cruz, Oaxaca, Mexico	*humilis*	April 25 to June 16, 2018	15.8406°N, −95.9706°W	10
Cacaluta, Oaxaca, Mexico	*humilis*	May 10–23, 2018	15.7428°N, −96.1500°W	15
El Chical, Colima, Mexico	*humilis*	April 19 to June 27, 2018	19.2363°N, −103.8485°W	10
Chavarrillo, Veracruz, Mexico	*rufinucha*	July 6–21, 2017	19.4260°N, −96.7933°W	13
Paso de Ovejas, Veracruz, Mexico	*rufinucha*	May 29 to July 3, 2018	19.2934°N, −96.4482°W	13
Playa Salinas, Veracruz, Mexico	*rufinucha*	June 25 to July 4, 2017	18.9071°N, −95.9435°W	15
El Castaño, Chiapas, Mexico	*capistratus*	June 9–21, 2018	15.2904°N, −92.9688°W	3
La Ensenada, Puntarenas, Costa Rica	*capistratus*	June 30 to July 8, 2018	10.1378°N, −85.0404°W	12
Los Cocos, Chiapas, Mexico	*capistratus*	May 25 to June 2, 2017	14.7581°N, 92.4044°W	10

Ríos-Chelén et al. 2017). Frequency variables were calculated using the power spectrum and a threshold setting of −30 dB related to the song's peak amplitude, including all the peaks that exceeded the threshold. We also used the waveform to determine the beginning and ending time of each element within the duet.

We measured a total of 13 fine structural characteristics to describe the spectral–temporal traits and coordination of duets: (1) delay (s): elapsed time from the end of the first element of the first contributor to the onset of the first element of the second contributor; (2) song duration difference (s): calculated as the difference between the song duration of the first contributor and the song duration of the second contributor; (3) maximum frequency difference (Hz): the difference between the mean highest frequencies of the songs of duet contributors; (4) minimum frequency difference (Hz): the difference between the mean lowest frequencies of the songs of both duet contributors; (5) frequency bandwidth difference (Hz): the difference between the mean frequency bandwidths of the songs of both duet contributors; (6) entropy difference (μ): difference between the mean entropy values of the song of both duet contributors; (7) peak frequency difference (Hz): difference between the mean frequencies with the maximum amplitude of the song of both duet contributors; (8) maximum frequency (Hz): the maximum frequency defined by the −30 dB threshold; (9) minimum frequency (Hz): the minimum frequency defined by the −30 dB threshold; (10) frequency bandwidth (Hz): calculated as the difference between minimum and the maximum frequency in the song; (11) peak frequency (Hz): the frequency with the maximum amplitude within the duet; (12) entropy (μ): defined as a measure of energy dispersion in a sound, with continuous values ranging from zero to infinite, where pure-tone sounds have values closer to zero while noisy sounds have higher values; and (13) rate: number of notes per second within the duet.

Although duets in Rufous-naped Wrens are the result of the combination of male and female solo songs, as in the majority of duetting species, they are likely nonrandomly produced and the contributions by both female and male may follow several rules as in other duetting wren species (e.g., Canebrake Wren [*Cantorchilus zeledoni*], Rivera-Cáceres 2015; Black-bellied Wren [*Pheugopedius fasciatoventris*], Logue 2006; Rufous-and-White Wren [*Thryophilus rufalbus*], Mennill and Vehrencamp 2004). Therefore, it is expected that if different rules play a main role in duet formation across groups, the first set of variables (1 to 7) may describe such differences. The second set of variables (8 to 13) describes the spectral–temporal traits of duets as single vocalizations, similar to the measurements that are used to describe solo songs and other vocalizations produced by a single individual.

These measurements are useful because they allow comparisons between species within the genus or phylogenetic reconstructions (e.g., "*Thryothorus*" wrens; Mann et al. 2009). We did not include the contribution by the sexes in the analysis, as we were not able to assign with confidence male and female contributions in the *capistratus* group due to the dense vegetation that prevented us from making reliable observations during duet performance, and because of the song similarity among members of a pair (Figure 1).

Statistical Analyses

We conducted a principal component analysis (PCA) using the correlation matrix (Supplementary Material Table S1) and varimax rotation with all 13 variables and report all the components with eigenvalues ≥1. The PCA retained 5 main components that combined explained 78.6% of the total variance (Table 2). The first component was strongly associated with variables related to duet coordination in terms of frequency (i.e. maximum frequency difference, frequency bandwidth difference, and entropy difference). The second component was associated with coordination in terms of time (i.e. the delay time of response and difference between the song duration of the contributors). The third component was strongly associated with spectral variables of the duet as a whole (i.e. minimum frequency, peak frequency, and rate). The fourth component was strongly associated with maximum frequency, frequency bandwidth, and entropy of the whole duet. The fifth component was strongly associated with minimum frequency difference and peak frequency difference of the songs of the contributors.

We tested whether the duets of Rufous-naped Wrens diverged among the 3 proposed groups, among sites (3 sites per group) and pairs (between 2 and 5 duets per pair) using a 3-level nested analysis of variance (ANOVA). To build the nested ANOVA model, we used the 5 main components extracted from the PCA as dependent variables (one analysis per component, for a total of five analyses), with sampling sites nested within groups and pairs nested within sampling sites by groups as our categorical variables. The variable group was treated as a fixed factor, while sampling sites nested within groups and pairs nested within the sampling site by groups were treated as random factors. We then performed Fisher LSD post hoc tests at each level of the ANOVA model (i.e. group, sampling site within groups, and pair within sampling sites by group). We used p values in combination with Cohen's d values to interpret the data (following Nakagawa 2004). Prior to the analyses, we applied a square root transformation to all the variables, improving normality and reducing outliers' effects (residuals for each nested ANOVA model were normally distributed; Quinn and Keough 2002).

TABLE 2. Score loadings for the 5 main components extracted from the PCA using 13 acoustic variables measured from 442 Rufous-naped Wren duets of 101 pairs (territories). Eigenvalues and percentage of variance explained are presented for each factor. Loadings contributing to each component are shown in bold.

Variables	First	Second	Third	Fourth	Fifth
Eigenvalues	4.2	1.9	1.8	1.3	1.1
Variance explained (%)	32.5	14.3	13.5	9.8	8.5
Delay	0.0	−**0.9**	0.0	−0.2	0.0
Song duration difference	−0.1	−**0.9**	0.0	0.0	0.0
Maximum frequency difference	**0.8**	0.0	0.0	0.3	0.3
Minimum frequency difference	0.0	0.0	0.1	0.0	**0.9**
Frequency bandwidth difference	**0.9**	0.0	0.0	0.3	0.0
Entropy difference	**0.8**	0.1	0.4	0.0	0.0
Peak frequency difference	0.5	−0.1	−0.1	0.3	**0.6**
Maximum frequency	0.3	0.1	0.0	**0.9**	0.1
Minimum frequency	0.1	−0.1	**0.7**	−0.3	0.1
Frequency bandwidth	0.3	0.1	−0.1	**0.9**	0.0
Peak frequency	−0.1	0.0	**0.7**	0.3	−0.1
Entropy	0.0	0.2	0.2	**0.8**	0.1
Rate	0.3	0.2	**0.8**	0.2	0.1

Kaiser-Meyer-Olkin measure indicated that the sample was optimum to conduct the analyses (KMO = 0.6) and Bartlett's test of sphericity was highly significant (χ^2 = 4970.6, df = 78, $P < 0.05$).

We calculated Cohen's d as an estimator of the magnitude of divergence in the duets between pairs of groups (e.g., *humilis* vs. *capistratus*) and between pairs of sites within groups (e.g., Barra de La Cruz vs. Cacaluta). To compute Cohen's d values, we used the 5 principal components extracted from the PCA. We calculated Cohen's d as the differences between the means of 2 groups divided by the pooled standard deviations of the same groups (Cohen 1992). According to Sawilowsky (2009), Cohen's d = 0.2 represents a small effect size; Cohen's d = 0.5 represents a medium effect size; Cohen's d = 0.8 represents a large effect size; and Cohen's d = 1.2 or 2 represent very large and huge effects, respectively.

To explore duet similarity between sampled sites and between the 3 groups, we averaged component scores by pairs and by sites. Then, we performed 2 hierarchical cluster analyses (i.e. one for pairs and one for sites) using Euclidian distances and the unweighted pair group method (UPGMA) as the clustering method using the 5 components as dependent variables (Quinn and Keough 2002).

We then conducted a discriminant function analysis (DFA) to test the odds of correctly assigning a taxonomic group based on the duet traits. For the DFA we used the 5 averaged component scores by pairs used in the hierarchical cluster analysis as dependent variables and using the leave-one-out cross-validation for the classification.

We conducted a Mantel test to explore whether acoustic divergence relates to geographic distance (i.e. the isolation-by-distance hypothesis). For the analysis, we used 2 matrices: the first included the geographic distance between territories (i.e. the linear distance between territory centers

obtained using a Garmin GPS model GPSMAP 64 with a precision of 5 m); the second matrix included the Euclidean similarity distance between pairs (all duets per pair were averaged), which were obtained using the first 5 factors produced by the PCA analysis mentioned above. The Mantel test was conducted using 9,999 permutations. The PCA and ANOVA analyses were conducted in Statistica 8.0, Mantel Test and Cluster Analyses were conducted in Past 3.25 statistical software, and the DFA was conducted in IBM SPSS Statistics 24. Values are presented as average ± SD.

RESULTS

During the fieldwork, we observed several instances where males and females joined to perform duets. Despite the distances between recording sites, the physical behavior accompanying the duets was highly similar. Duet performance consisted of both birds generally vocalizing from the same perch and performing movements, such as spreading the tail feathers, flapping their wings, and raising their beaks at ~25°.

By contrast, our visual analysis of the duets suggests that the contribution in terms of song type of both females and males varies among the 3 groups (Figure 1). In particular, the duets from the *capistratus* group were unique and very different from the other 2 groups. Birds from the *capistratus* group sang 2 types of duets (Figure 1): one type where both female and male contribute with the same song type, sung almost simultaneously but not necessarily in the same frequencies (showing lack of sexual dimorphism), and a second duet type where one of the contributors

(presumably the male) sings a tonal, melodious song made of multiple elements, while the second contributor (presumably the female) produces harsh, noisy calls. Birds from the *rufinucha* group sang duets where male and female songs overlap, producing polyphonal duets (duets made of tonal but different song types; Figure 1) where males produced complex songs made of different elements while females produced simple trill-like songs made of one element type. Birds from the *humilis* group sang duets where male and female songs overlapped, but sexes contributed with different song types to create the duet (Figure 1); males produced complex songs made of different elements, however, unlike the *rufinucha* group, females produced a non-tonal song made of one harsh-like element. Both *humilis* and *rufinucha* produced duets with marked sexual dimorphism. Overall, birds from the *capistratus* group sang duets with the lowest maximum frequency, lowest minimum frequency, and narrowest frequency bandwidth (Supplementary Material Table S2).

For the quantitative analyses, we included a total of 442 duets belonging to 101 pairs (4.3 ± 1.1 songs per pair) across 9 sampled sites. For the first, third, and fourth components, the results showed statistically significant differences between groups, among sampled sites and pairs (Table 3). For the second component, the results showed non–statistically significant differences between groups but revealed a significant difference among sites within groups, and among pairs within sites by groups (Table 3). For the fifth component, the results showed statistically significant differences between groups and among sites by group, but not among pairs within sites by group (Table 3).

At the group level, the post hoc test revealed pairwise differences between groups in 4 of 5 components (Figure 2A–E; Table 4). The first component—explaining duet coordination in terms of frequency—separated *capistratus* from both *humilis* and *rufinucha* groups, while *humilis* and *rufinucha* were not different from each other (Figure 2A). The *capistratus* group had the lowest component scores, indicating that the songs used by both birds to create the duets were more similar in terms of frequency, with the lowest differences for maximum frequency, frequency bandwidth, and entropy. The second component—explaining duet coordination in terms of time, so the 3 groups produce duets with similar coordination between paired individuals—did not differentiate among groups (Figure 2B). The third component—explaining variation in minimum frequency, peak frequency, and rate—separated *humilis* from both *rufinucha* and *capistratus* groups, while *rufinucha* and *capistratus* were not different from each other (Figure 2C). The fourth component—explaining maximum frequencies, bandwidth and entropy in the whole duet—separated *capistratus* from *humilis* and *rufinucha* groups, while *humilis* and *rufinucha* were not different from each other,

TABLE 3. Results from the nested ANOVA using the 5 components extracted from the PCA. Statistically significant results (*P* < 0.05) are shown in bold.

Components	F	df	P
First component [a]			
Intercept	3.8	1 and 341	**0.04**
Group	35.9	2 and 341	**0.06E−13**
Site (Group)	2.8	6 and 341	**0.01**
Pair (Group+Site)	1.3	92 and 341	**0.02**
Second component [b]			
Intercept	0.4	1 and 341	0.52
Group	1.5	2 and 341	0.22
Site (Group)	5.7	6 and 341	**0.00001**
Pair (Group+Site)	1.5	92 and 341	**0.0002**
Third component [c]			
Intercept	7.2	1 and 341	**0.007**
Group	366.4	2 and 341	**<0.01E−5**
Site (Group)	10.9	6 and 341	**0.03E−9**
Pair (Group+Site)	1.7	92 and 341	**0.0001**
Fourth component [d]			
Intercept	0.5	1 and 341	0.44
Group	12.5	2 and 341	**0.05E−4**
Site (Group)	3.1	6 and 341	**0.005**
Pair (Group+Site)	1.8	92 and 341	**0.0002**
Fifth component [e]			
Intercept	9.54	1 and 341	**0.002**
Group	47.0	2 and 341	**<0.01E−5**
Site (Group)	7.9	6 and 341	**0.04E−6**
Pair (Group+Site)	1.2	92 and 341	0.06

[a] Component associated to maximum frequency difference, frequency bandwidth difference, and entropy difference.
[b] Component associated to delay time of response and difference between the song duration of both contributors.
[c] Component associated to minimum frequency, peak frequency, and rate.
[d] Component associated to maximum frequency, frequency bandwidth, and entropy.
[e] Component associated to minimum frequency difference and peak frequency.

with *capistratus* having the lowest factor scores for maximum frequency, frequency bandwidth, and entropy (Figure 2D). The fifth component—explaining minimum frequency difference and peak frequency difference in the songs of both contributors—separated all 3 groups, with *capistratus* having the lowest factor scores, *humilis* intermediate factor scores, and *rufinucha* the highest values for differences between minimum frequencies and differences between peak frequencies (Figure 2E). These results suggest that duets have particular frequency and temporal characteristics that can be used to distinguish each of the groups.

At the site level, the post hoc test revealed pairwise differences among sites within groups in all 5 components (Table 4). Within the *humilis* group the first, second, fourth, and fifth components showed differences among sites (Figure 2A, B, D, and E). Within the *rufinucha*

group, the second, third, fourth, and fifth components showed differences among sites (Figure 2B–E). Within the *capistratus* group, the third component showed differences among sites (Figure 2C). At the pair level, the post hoc test revealed significant pairwise differences among 320 of 2,824 (11%) possible pair-wise comparisons within sites, with no clear pattern of duet differences between pairs, suggesting that Rufous-naped Wrens do not have a signature in their duets (see Supplementary Material Table S3).

As variation in duet structure among sites within groups could be due to differences in recording dates, we conducted additional quadratic regressions testing the relationship between Julian date and 2 variables related to coordination among duetters in time (i.e. delay time of response and difference between songs). We found no significant association between Julian date and duet coordination in any of the groups (see Supplementary Material Table S4).

The effect size analysis showed that differences between groups ranged from 0 to 2.6 (Table 4). The effect size for *humilis* vs. *capistratus* was on average 1.2, while *rufinucha* vs. *humilis* and *rufinucha* vs. *capistratus* was on average 0.8. This result suggest that the effect size varied from medium to large, suggesting that the differences between groups are probably big enough to be distinguished with the "naked eye" (Cohen 1992).

The cluster analysis resulted in 3 main branches that consistently resemble the 3 proposed groups for the Rufous-naped Wren complex (Figure 3; Supplementary Material Figure S1). These results suggest that *rufinucha* and *humilis* groups have the most similar duets, while *capistratus* group has the most divergent duets within the complex. The discriminant function analysis resulted in 2 canonical discriminant functions and correctly classified 98.0% of the pairs to the original taxonomical group with a correct classification higher than expected by chance ($\chi^2 = 259.1$, df = 10, $P < 0.0001$; Figure 4).

The Mantel test showed that similarity in duets among pairs decrease with geographic distance ($r = -0.35$, $P = 0.001$). However, the analysis for each group separately showed that similarity also decreases with distance for 2 groups (*capistratus* [$r = -0.10$, $P = 0.03$] and *humilis* [$r = -0.15$, $P = 0.02$]) but not for *rufinucha* ($r = 0.04$, $P = 0.16$).

DISCUSSION

In this study, we investigated whether the Rufous-naped Wren's duets vary across its distribution range and explored whether duet variation matches the 3 groups suggested by some taxonomic frameworks (Selander 1964, Brewer and MacKay 2001, Howell and Webb 1995, Clements 2009, del Hoyo et al. 2016). Our results revealed significant differences between groups, among sampled
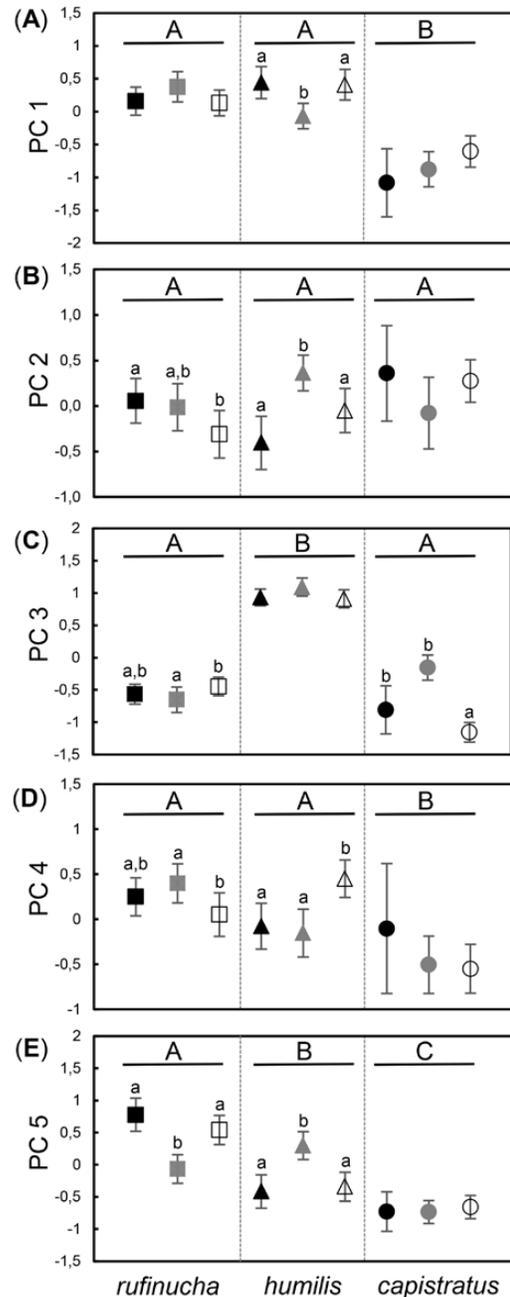
FIGURE 2. Plots depicting the variation of Rufous-naped Wren duets in the 5 main components. Graphs show comparisons between groups and among sites within groups. Squares, triangles, and circles correspond to *rufinucha*, *humilis*, and *capistratus*, respectively. Black-filled squares correspond to Chavarrillo, gray-filled squares correspond to Paso de Ovejas, open squares correspond to Playa Salinas; black-filled triangles correspond to El Chical, gray-filled triangles correspond to Cacaluta, open triangles correspond to Barra de la Cruz; black-filled circles correspond to El Castaño, gray-filled circles correspond to Los Cocos, and open circles correspond to La Ensenada. Results of the post hoc tests are indicated for differences between groups with uppercase letters and with lowercase letters for differences among sites (sites with no lowercase letters were not significantly different). Error bars denote 95% confidence intervals.

TABLE 4. *P* values and Cohen's *d* scores for each pair of comparisons. Cohen's *d* values were calculated based on the 5 components extracted from the PCA. Values for each comparison between groups and between sampled sites within groups are shown. Cohen's $d = 0.02$ represent a small effect size; $d = 0.5$ represent a medium effect size; $d = 0.8$ represent a large effect size; and $d = 1.2$ or 2 represent large and huge effect sizes, respectively. Cohen's values are presented as absolute values. Statistically significant results ($P < 0.05$ and Cohen's $d > 0.2$) are shown in bold.

	First		Second		Third		Fourth		Fifth	
	P	*d*	*P*	*d*	*P*	*d*	*P*	*d*	*P*	*d*
Groups										
humilis vs. *capistratus*	**0.02E–14**	**1.1**	0.36	NA	**<0.01E–14**	**2.6**	**0.00003**	**0.5**	**0.02E–6**	**0.8**
rufinucha vs. *humilis*	0.98	0.0	0.15	NA	**<0.01E–14**	**2.6**	0.07	0.2	**0.01E–6**	**0.5**
rufinucha vs. *capistratus*	**0.01E–14**	**1.1**	0.03	NA	**0.01**	**0.3**	**0.08E–8**	**0.7**	**<0.01E–14**	**1.4**
Sites within groups										
humilis										
Barra de La Cruz vs. Cacaluta	**0.003**	**0.6**	**0.01**	**0.5**	0.07	0.3	**0.0002**	**0.6**	**0.00004**	**0.7**
Barra de la Cruz vs. El Chical	0.85	0.0	0.06	0.4	0.84	0.0	**0.003**	**0.6**	0.66	0.1
Cacaluta vs. El Chical	**0.002**	**0.6**	**0.01E–3**	**0.8**	0.12	0.3	0.64	0.1	**0.000008**	**0.8**
rufinucha										
Chavarrillo vs. Paso de Ovejas	0.19	0.2	0.69	0.1	0.40	0.1	0.38	0.2	**0.0000002**	**0.9**
Chavarrillo vs. Playa Salinas	0.85	0.0	**0.02**	**0.4**	0.22	0.2	0.20	0.2	0.10	0.2
Paso de Ovejas vs. Playa Salinas	0.12	0.2	0.08	0.3	**0.04**	**0.3**	**0.03**	**0.6**	**0.00008**	**0.7**
capistratus										
El Castaño vs. La ensenada	0.11	0.7	0.78	0.1	0.07	0.6	0.14	0.4	0.81	0.1
El Castaño vs. Los Cocos	0.51	0.3	0.18	0.4	**0.0008**	**1.1**	0.19	0.3	0.97	0.0
La Ensenada vs. Los Cocos	0.15	0.3	0.08	0.3	**0.01E–13**	**1.7**	0.81	0.0	0.67	0.1

The nested ANOVA for the second component was not significant among groups (see Table 3); thus, Cohen's *d* values among groups are not shown.
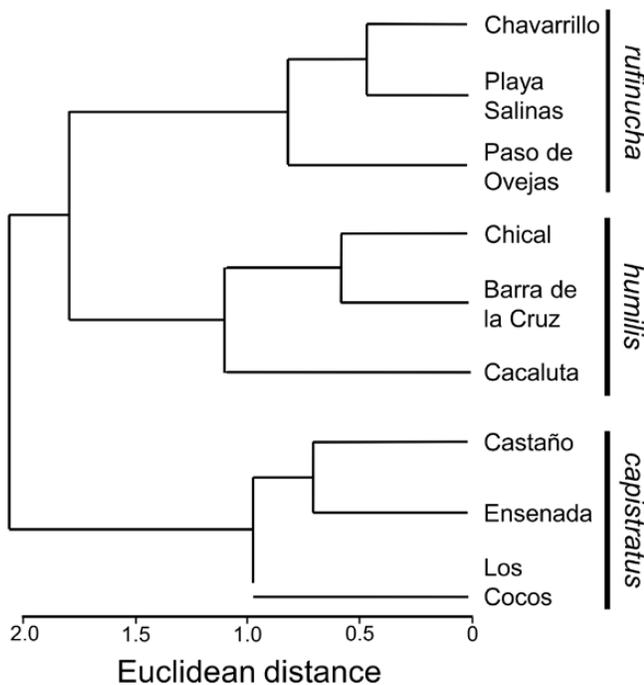
sites within groups, and among pairs within sites. Duets are different between the 3 groups, with a marked song sexual dimorphism in *rufinucha* and *humilis* that is lacking in *capistratus*, supporting the hypothesis of the existence of 3 vocal lineages. At the site level, duets showed marked differences among sites within *humilis* and *rufinucha* groups, and to a lesser degree among sites within the *capistratus* group; still, these differences are smaller than those observed between groups. Further, both the cluster analysis and DFA show that duets from *rufinucha* and *humilis* groups are more similar, while *capistratus* duets are the most divergent, paralleling the genetic and vocal results reported by Vázquez-Miranda et al. (2009) and Sosa-López et al. (2013). Overall, duet similarity between pairs decreases with distance; however, within groups the pattern holds only for *humilis* and *capistratus*, but not for *rufinucha*.

The *capistratus* group was the most different in terms of frequency and temporal characteristics because it produces the most tonal duets with the lowest values of entropy, the lowest maximum and minimum frequency, narrowest bandwidth, lowest peak frequency, and the slowest pace. Conversely, birds from the *rufinucha* and *humilis* groups sang the most similar duets, producing duets with higher values of entropy, higher values of maximum frequency, and broader bandwidth. Both *humilis* and *rufinucha* groups had differences between them—*humilis* sang duets with highest values of minimum frequency, peak frequency, and pace, and *rufinucha* had intermediate values. Duets from the *capistratus* group were also the most coordinated, with both contributors—male and female—vocalizing similar frequencies. Coordination in frequency parameters was relatively low for both groups *rufinucha* and *humilis*, with the *humilis* group showing intermediate coordinated duets and the *rufinucha* group showing the least coordinated duets. We acknowledge that many recordings from the *capistratus* group (15 of 25 pairs recorded) resulted from playback, so differences observed might be attributable, at least in part, to the different recording contexts. We believe this is an unlikely explanation, as the differences between groups we found in the songs produced during duets are very similar to the solo songs differences between groups reported by Sosa-López et al. (2013). Further, female song contributions in *capistratus* are usually complex songs, while *humilis* and *rufinucha* female songs are much simpler. Thus, birds from the *capistratus* group produce the most tonal, slow-paced, and high frequency–coordinated duets, generating simultaneous duets. By contrast, birds from the *humilis* group produced noisy, fast-paced, and intermediate frequency–coordinated duets. Meanwhile, birds from the *rufinucha* group produced noisy, intermediate-paced, and low frequency–coordinated duets. Both *humilis* and *rufinucha* produced polyphonal duets.

Our results suggest that divergence in Rufous-naped Wrens' duets mirrors the variation described in morphological, genetic, and solo songs (Selander 1964, Vázquez-Miranda et al. 2009, Sosa-López et al. 2013). A variety of studies have shown congruent variation across different traits within other bird species complexes. For instance,

FIGURE 3. Dendrogram resulted from the cluster analysis at the level of sites. Values on the horizontal axis represent the average Euclidian distance (similitude) between sites.

Van Doren et al. (2018) found that genes, morphology, and songs of the Rusty-breasted Antpitta (*Grallaricula ferrugineipectus*) vary across its distribution range. In another example, Grant et al. (2000) also found that genes, morphology, and songs of the Sharp-beaked Ground Finch (*Geospiza difficilis*) vary among the Galápagos Islands. A similar study in the Timberline Wren (*Thryorchilus browni*) showed that traits of populations living in allopatry usually diverge in tandem (Camacho-Alpízar et al. 2018). Studies such as these are widespread in the literature, sometimes suggesting that allopatric populations with distinctive song characteristics should be recognized as different species (Grant et al. 2000, Uy et al. 2009, Sosa-López and Mennill 2014, Cooper and Cuervo 2017, Nwankwo et al. 2018, Van Doren et al. 2018, Wei et al. 2019).

Our findings show that birds from the *capistratus* group produce the most divergent duets within the complex, while *humilis* and *rufinucha* sing more similar duets. This result strongly supports the phylogenetic hypothesis proposed by Vázquez-Miranda et al. (2009) in which *C. rufinucha* and *C. humilis* are sister taxa, and *C. capistratus* is the sister group to these two. Thus, our results also support the idea that similarity in avian vocalizations parallels the phylogenetic proximity among taxa (de Kort and ten Cate 2001, Price and Lanyon 2002, Sosa-López et al. 2016). This supports the findings of Freeman and Montgomery (2017), who reported that individuals of the *capistratus* group from Costa Rica discriminate foreign songs (*humilis* group) in 57% of their experiments (that is 12 territories from 21). Future studies should carry on playback experiments on *rufinucha* and *humilus* groups to test whether they also can discriminate among groups.
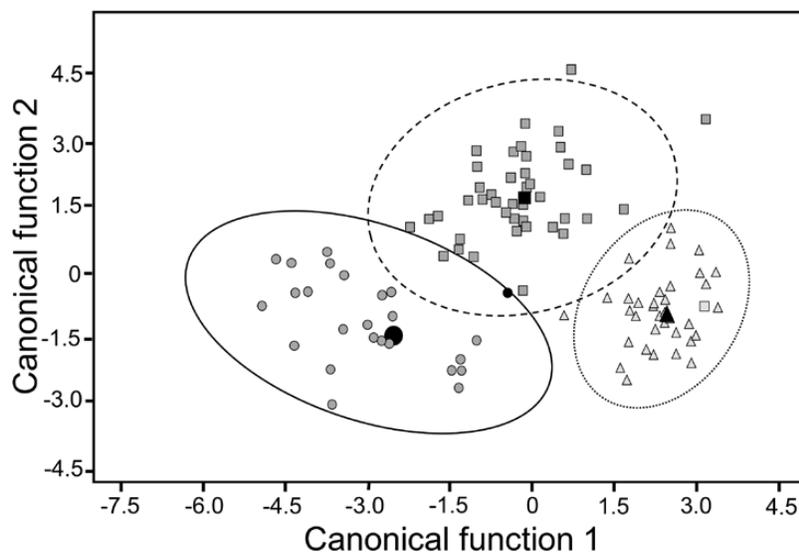
FIGURE 4. Plot of the 2 canonical functions from the discriminant function analysis based on the 5 main components extracted using a PCA. Squares, triangles, and circles correspond to *rufinucha*, *humilis*, and *capistratus*, respectively. Black-filled symbols (square, triangle, and circle) represent the centroids for each group. Ellipses represent the 95% of confidence intervals.

How the differences in the duets of this polytypic taxon originated is still not clear. Based on genetic evidence, Vázquez-Miranda et al. (2009) suggested that genetic differences between the 3 groups arose through a series of geographical isolation events during the Middle to Late Pleistocene. According to this hypothesis, a marine barrier could have separated an ancestral population of this highly sedentary taxon across the Isthmus of Tehuantepec, isolating all 3 groups and promoting genetic differentiation (Vázquez-Miranda et al. 2009). Compelling available evidence supports the hypothesis that vicariant events across the Isthmus of Tehuantepec likely isolated a great number of taxa, originating the high diversity we observe today (Mulcahy et al. 2006, Barber and Klicka 2010, González et al. 2011, Ortiz-Ramírez et al. 2016, Hernández-Soto et al. 2018). Furthermore, evidence shows that geographical isolation is a major phenomenon promoting divergence in biologically relevant characters such as song (Westcott and Kroon 2002, Koetz et al. 2007, Cortés-Rodríguez et al. 2008, Burton and Nietsch 2010, Sosa-López et al. 2013, Roach and Phillmore 2017, Camacho-Alpízar et al. 2018).

With a heavily learned component, oscine songs are subject to drastic evolutionary changes through cultural mutations and drift (Podos et al. 2004). Similarly, social selection could also produce rapid and non-predictable evolutionary changes (West-Eberhard 1983). However, if selection or drift occurs at a relatively constant rate, it would lead to a correlation between signal divergence and genetic divergence as in Greenish Warblers (*Phylloscopus trochiloides*; Irwin et al. 2008). In our study, we did not test the association between genetic and acoustic distances; however, we found that duet similarity decreases with geographic distance providing some support to the hypothesis of cultural drift or social selection as the main factor driving the evolution of duets in Rufous-naped Wrens in the *capistratus* and *humilis* groups (as pointed out by Wilkins et al. 2013), which could result in geographical patterns such as isolation-by-distance or to the presence of dialects (Baptista and Morton 1982, Mundinger 1982, Catchpole and Slater 2008). In line with this finding, Sosa-López et al. (2013) found a relationship among vocal divergence and genetic divergence in Rufous-naped Wren solo songs, but no associations between the fine-structural characteristics of the song with ecological variables, showing that changes in acoustic traits accrue gradually with time. Whether song and duets are under similar evolutionary pressures is still an open question, but there is evidence that suggests that songs and calls could be exposed to different evolutionary drives (e.g., Irwin et al. 2008). Future studies should test the effects of natural and sexual selections on Rufous-naped Wren duets to determine whether drift is a major evolutionary factor in duet divergence.

The present study also suggests the existence of significant variation among sampled sites within groups,

particularly within the *humilis* and *rufinucha* groups, and to a lesser degree, within the *capistratus* group. Within groups, duets differed in some traits such as coordination in frequency and fine spectral–temporal traits of the whole duets. A possible explanation is that variation in duet structure among sites within groups is owed to differences in recording dates; however, the *humilis* group had the most divergent recording dates, while the *capistratus* group had the most divergent duet structure. We tested the relationship between Julian date and 2 variables related to coordination among duetters in time and found no significant association for any of the groups, ruling out the effects of recording dates in the differences between groups.

We suggest that historical geographic isolation resulted in vocal differentiation among groups, while isolation-by-distance—or dialect—could be acting within groups, explaining variation among sites within the groups. A similar pattern has been reported in 4 species of fairy wrens of the genus *Malurus* (Yandell et al. 2018). Another recent study strongly supports the key role of isolation-by-distance in the origin of differences in acoustic signals (Graham et al. 2018). Future studies should analyze whether variation within Rufous-naped Wren groups is graduated or clinal (Koetz et al. 2007), or discrete (dialects; Baptista and Morton 1982, Mundinger 1982, Catchpole and Slater 2008), by describing the acoustic structure of song and duets across populations within the groups.

In conclusion, this study on Rufous-naped Wrens provides evidence of the presence of geographic variation in complex acoustic signals such as duets. These findings support the existence of 3 vocal lineages within the complex, suggesting that divergence in duets may be the result of vicariant events, followed by other selective and/or nonselective factors. Moreover, we also demonstrate that there exists variation among sites within groups and suggest that this variation may be the result of isolation-by-distance or dialect formation. Based on the multiple evidence now available, we suggest that the 3 groups should represent distinct taxonomical units, hence, adding to the idea of 3 different species within the complex as proposed by other authors (Ridgway 1904, Navarro-Sigüenza and Peterson 2004, Vázquez-Miranda et al. 2009, Sosa-López et al. 2013, del Hoyo et al. 2016).

SUPPLEMENTARY MATERIAL

Supplementary material is available at *The Auk: Ornithological Advances* online.

ACKNOWLEDGMENTS

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