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**Maestría en Ciencias en Conservación y
Aprovechamiento de Recursos Naturales
(Biodiversidad Del Neotrópico)**

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

**T E S I S
QUE PARA OBTENER EL GRADO DE:
MAESTRO EN CIENCIAS**

**PRESENTA:
BIOL. WILIAM KU PERALTA**

**DIRECTOR:
DR. JOSÉ ROBERTO SOSA LÓPEZ**

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ACTA DE REVISIÓN DE TESIS

En la Ciudad de Oaxaca siendo las 12:00 horas del día 9 del mes de noviembre del 2018 se reunieron los miembros de la Comisión Revisora de la Tesis, designada por el Colegio de Profesores de Estudios de Posgrado e Investigación de CIIDIR OAXACA para examinar la tesis titulada:
"Variación geográfica en los duetos de la Matraca Nuquirrufa (*Campylorhynchus rufinucha*)"

Presentada por el alumno:

Ku Peralta
Apellido paterno Apellido materno
Nombre(s) Wiliam

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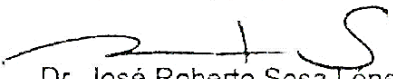
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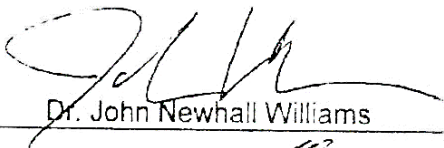
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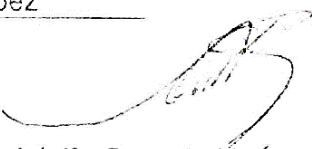
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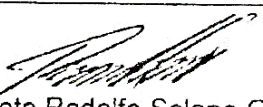
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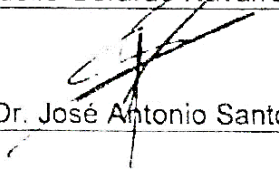
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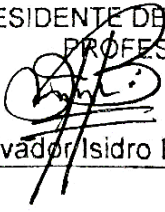

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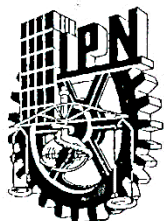

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**Variación geográfica en los duetos de la Matraca Nuquirrufa
(*Campylorhynchus rufinucha*)**

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

RESUMEN

La comunicación animal mediante señales acústicas tiene un papel importante en varias actividades primordiales de las especies como la atracción de pareja, reconocimiento de especies y la defensa del territorio. En muchos casos, la variación de las señales acústicas refleja estructuras poblacionales, ausencia de flujo génico o historias filogenéticas. En aves, el estudio de la variación geográfica de las señales acústicas ha sido útil para resolver problemas taxonómicos. Sin embargo, la mayoría de los estudios se enfocan al estudio de la variación en cantos o llamados, con un puñado de estudios enfocándose en conductas complejas como los duetos. En este estudio investigo la variación de las características de los duetos de la Matraca nuquirrufa (*Campylorhynchus rufinucha*). Debido a la gran variación presente en caracteres morfológicos de la Matraca nuquirrufa, varios autores reconocen entre cinco y nueve subespecies dentro del complejo, agrupadas en tres grupos taxonómicos. Los resultados sugieren que los duetos varían a lo largo de la distribución de este complejo, concordando con los tres grupos taxonómicos (*rufinucha*, *humilis* y *capistratus*) propuestos previamente. A nivel local, encontré que los duetos también varían entre sitios dentro de los grupos. Mis resultados sugieren que la existencia de los tres linajes vocales es probablemente el resultado de un aislamiento geográfico histórico, seguido de otros factores selectivos y/o no selectivos. Además, argumento que la variación dentro de los grupos puede ser el resultado del de aislamiento por distancia o debida a formación de dialectos. Estos resultados respaldan la hipótesis—apoyada por estudios morfológicos, genéticos y comportamentales—que sugiere que los tres grupos representan unidades taxonómicas distintas.

ABSTRACT

Acoustic signals used in animal communication play a key role in important species behaviours such as mate attraction, species recognition and territory defense. In many cases, variation in acoustic signals reflects population structure, lack of gene flow and phylogenetic histories. In birds, the study of geographic variation in acoustic signals has been useful to address taxonomic problems. However, most of the studies on geographic variation have focused on calls and solo songs, with a just a few studies focusing on complex behaviors such as duets. In this study, I investigated the variation in the traits of the Rufous-naped Wrens' (*Campylorhynchus rufinucha*) duets. Due to enormous variation present in morphological traits of the Rufous-naped Wrens, some authors have recognized between five and nine subspecies within the complex, grouped into three taxonomical groups. The results of this study suggest that duets vary across the distribution range of the complex, matching the three taxonomical groups (*rufinucha*, *humilis* and *capistratus*) previously suggested. At the local level, I found that duets also vary among sites within groups. My results suggest that the existence of three vocal lineages is probably the result of historical geographic isolation followed by other selective or non selective factors. Furthermore, I argue that variation found within groups may be the result of isolation-by-distance or dialect formation. These results support the hypothesis—in line with morphological, genetic and behavioral studies—suggesting that the three groups should represent three distinct taxonomical units.

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Director:

Dr. J. Roberto Sosa López

Comité tutorial:

Dr. José Antonio Santos Moreno

Dr. Aniceto Rodolfo Solano Gómez

Dr. John Newhall Williams

Dr. Adolfo Gerardo Navarro Sigüenza

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Víctor Hugo Vargas Herrera

M. V. Estefanía Meza montes

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"I am happy being me, although sometimes I would like to be a bird, so that I could fly"

—Joy Fielding

Introduction

Many vertebrates and invertebrates produce acoustic signals used in mate attraction, species recognition and territory defense (Catchpole and Slater 2008). These acoustic signals are under strong selection pressures (Podos et al. 2004), as a result they exhibit enormous variation in their structure even within species. Spatial variation in acoustic signals (hereafter referred as “geographic variation”) has been well documented in a diversity of acoustic signals in several animal groups as primates (Burton and Nietsch 2010), bats (Barclay et al. 1999, Sun et al. 2013) anurans (Narins and Smith 1986, Pröhl et al. 2007), insects (Symes 2018) and birds (Leger and Mountjoy 2003; Sosa-López et al. 2012, Sosa-López et al. 2013; 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 to address taxonomic problems and to better understand which mechanisms are involved in speciation processes (Toews and Irwin 2008, Sosa-López et al. 2012, Sosa-López et al. 2016).

Geographic variation in acoustic signals occurs at two distinct scales: micro-geographic and macro-geographic level (Benedict and Bowie 2009, Sosa-López et al 2013). Dialects are an example of micro-geographic variation in which limits of vocalizations produced by individuals of adjacent populations are well defined (Catchpole and Slater 2008). This is best illustrated by the case of the Splendid Sunbird (*Cynniris coccinigaster*), where dialects are shared between five to nine males within an area less than 1 km² (Payne 1978). At the macro-geographic level, spatial patterns of variation are broader and do not necessarily show discrete limits between them (Mundinger 1982, Catchpole and Slater 2008). Some Costa Rican populations of the Orange-Fronted Parakeets (*Eupsittula canicularis*) exhibit significant variation in the contact calls along the geography, however, variation occurs gradually without showing a discrete pattern (i.e., clinal variation; Bradbury et al. 2001). Conversely, macro-geographic variation can also occur among populations isolated by great distances or physical barriers which prevent migration between populations (Catchpole and Slater 2008). For example, the Tawny Owl (*Strix aluco*) has two

subspecies, the *Strix aluco* subsp. *aluco* inhabits East and Central Europe and North Italy and the *Strix aluco* subsp. *sylvatica* inhabits Britain, West France and Iberia. The subspecies are separated by approximately 450 km and showed a marked difference in “hoot” vocalization, a typical call of the species.

Divergence in acoustic signals also mirrors phylogenetic relationships among species (De Kort and Ten Cate 2001). Evidence of songs reflecting phylogenetic relationships comes from some species of doves in the genus *Streptopelia* and species of wrens in the genus *Troglodytes*. These studies shown that acoustic similarity is negatively related to phylogenetic distance, which in turn predicts the intensity of behavioral response to this signals; being the similarity among focal individual songs and simulated intruders’ songs a good predictor of the intensity of the response (De Kort and Ten Cate 2001, Sosa-López et al. 2016). Thus, these studies suggest that geographic variation in acoustic signals is also influenced by the phylogenetic history and demonstrate that these variations are accurate indicators for defining species limits. Geographic variation in acoustic signals also could arise from other selective forces promoting phenotypical variations such as ecological, sexual and cultural selection, or non-selective forces like cultural and genetic drift (Jones 1997, Podos et al. 2004, Catchpole and Slater 2008, Tobias and Seddon 2009, Wilkins et al. 2013). The study of patterns of geographic variation in acoustic signals is fundamental to elucidate which of these factors drive the diversification of acoustic signals (Perreault-Derryberry 2012, Wilkins et al. 2013).

In birds, geographic variation in male solo songs has been well studied, however, less attention has been paid to patterns of geographic variation in more complex behaviors such as duets (Odom and Mennill 2012). A duet is a vocal display produced by members of a pair or an extended family group, where their vocalizations overlap or occur with high levels of alternation and low variation coefficients in the interval between each vocalization’s member and often accompanied by coordinated visual displays (Farabaugh 1982). There are only four studies that focus in the analysis of geographic variation on bird duets (Bretagnolle and Lequette 1990, Mennill and Rogers 2006, Dahlin and Wright 2009, Odom and

Mennill 2012). These studies suggest the existence of variation in complex signals in non-passerine and passerine birds. However, more studies on the patterns of variation in this complex signals among widely distributed passerine birds are needed in order better understand which processes are involved in the origin of variation across the geography in complex signals as duets.

Here, I analyze the geographic variation in the duets of Rufous-naped Wrens complex (*Campylorhynchus rufinucha*). This is a social bird which produces duets throughout the year (Skutch 1935, Bradley and Mennill 2009). Rufous-naped Wrens are found in neotropical dry forest ranging from central Veracruz and Colima in México to Northwestern Costa Rica (Selander 1964). The species shows remarkable morphological variation along its distribution (Selander 1964, Howell and Webb 2005), that has led to several authors to recognize one species with between five, seven or nine subspecies (Peters 1960, Dickinson 2003, Brewer and Mackay 2001). However, some authors agree that this subspecies can be grouped into three main groups based on their morphology and behavior (Selander 1964, Brewer and MacKay 2001, Howell and Webb 2005; Del Hoyo et al. 2005, Clements 2009, Vázquez-Miranda 2009, Sosa-López et al. 2012): the *rufinucha* group, isolated in central Veracruz in Mexico; the *humilis* group ranging from Colima to western Chiapas in Mexico; and the *capistratus* group, from western Chiapas to northwest Costa Rica. Many authors agree that the three suggested groups should represent different species (Ridgway 1904, Navarro-Sigüenza and Peterson 2004, Vázquez-Miranda 2009, Del Hoyo et al. 2005, Sosa-López et al. 2012, Del Hoyo et al. 2017), and Vázquez-Miranda et al. (2009) suggested a phylogenetic relationship in which *rufinucha* and *humilis* are sister taxa, whit *capistratus* as the ancestral group. The hypothesis of the existence of three different taxonomical units has been supported by morphological, genetic and behavioral studies (Selander 1964, Vázquez-Miranda 2009, Sosa-López et al. 2012).

In this study, I analyze the duets of Rufous-naped Wrens across the species range and address two questions: (1) do the Rufous-naped Wrens' duets vary along the species distribution? And if so, (2) does duet variation matches the three previously described groups within the complex (*capistratus*,

rufinucha and *humilis* groups)? To answer these questions, first I describe the fine structural characteristics of the duets. Then, I test whether variation matches the three proposed groups. If historical geographic isolation has promoted variation in the fine structural characteristics of the duets, I predict that differences in duets among closely related groups (*humilis* vs. *rufinucha*) to be smaller than between distantly related groups (*humilis* vs. *capistratus* or *rufinucha* vs. *capistratus*) regardless the geographic distance among populations within the same group (i.e., isolation-by-distance). Our study will add to our knowledge of the patterns of geographic variation of complex signals and will provide evidence to support or reject the hypothesis of three independent taxonomical units within the Rufous-naped Wren complex.

Methods

Sampling and general methods

To describe the fine-structural characteristics of Rufous-naped Wrens' duets, I selected six sample sites within the groups proposed by Selander (1964), Vázquez-Miranda et al. (2009) and Sosa-López et al. (2012). I sampled two sites per group. Sampled sites were located in a way that the existing distance among sites belonging to different groups should be smaller than the distance among sites belonging to the same group (Fig. 1). This experimental design allowed the comparison of duets within and between groups, controlling for the effect of isolation-by-distance (i.e. lineal increasing differences in song characteristics owing to the accumulation of differences throughout the space; Koetz et al. 2007). The field sites and sampling dates were: (1) La Ensenada, Puntarenas Province, Costa Rica from 30 June to 8 July and (2) Los Cocos, Chiapas, Mexico from 25 May to 2 June 2017, both populations belonging to *capistratus* group; (3) Chavarrillo, Veracruz, Mexico from 6 July to 21 July 2017 and (4) Playa Salinas, Veracruz, Mexico from 25 June to 4 July 2017, both belonging to *rufinucha* group; (5) Cacaluta, Oaxaca, Mexico from 10 May to 23 May 2017 and (6) El Chical from 19 April to 27 April 2018, Colima Mexico belonging to the *humilis* group (Fig. 1).

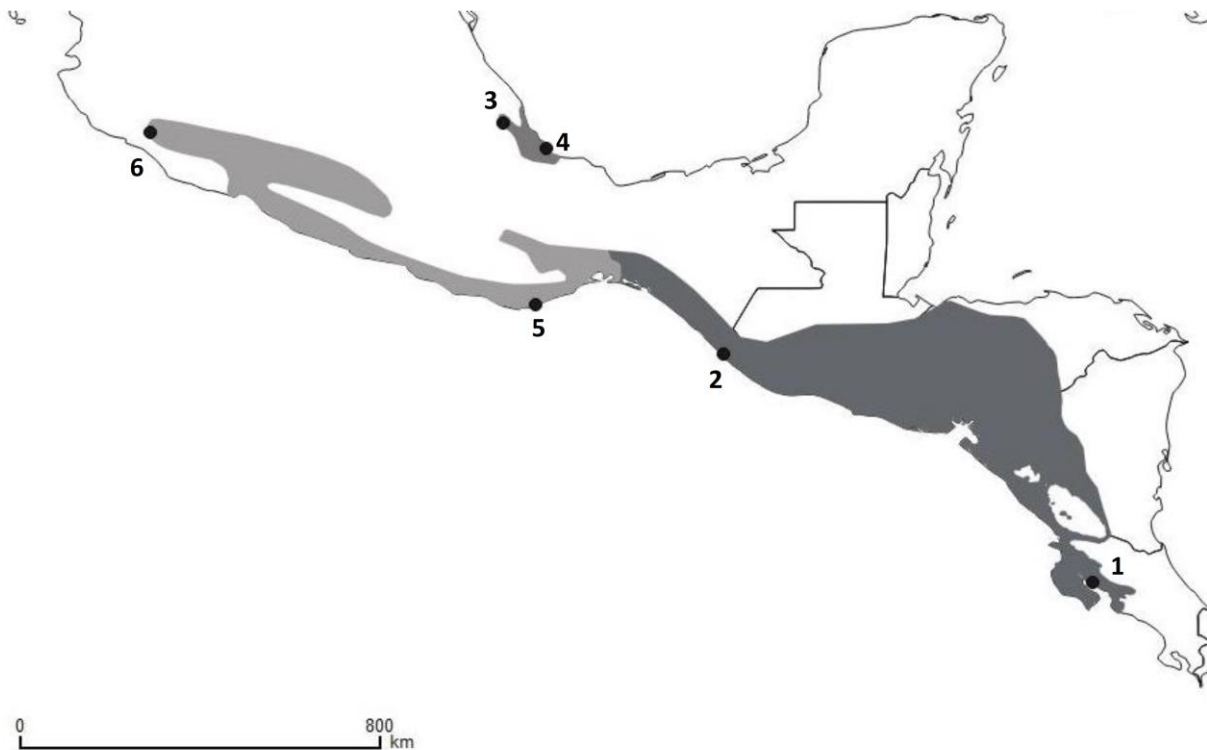


Figure 1. Map of Mesoamerica showing the distribution of the three Rufous-naped Wren groups proposed by Selander (1964), Vázquez-Miranda et al. (2009) and Sosa-López et al. (2012). Open circles depict sampled sites for each group: 1) La Ensenada, Puntarenas Province, Costa Rica and 2) Los Cocos, Chiapas, Mexico from *capistratus* group; 3) Chavarrillo, Veracruz, Mexico and 4) Playa Salinas Veracruz, Mexico from *rufinucha* group; 5) Cacaluta, Oaxaca, Mexico and 6) El Chical, Colima, Mexico from *humilis* group.

Duet recording

I 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) with a parabolic universal reflector (model: Telinga Universal), or a Tascam digital recorder (model: DR-70D) with a Shotgun Sennheiser (model: ME67/K6). All recordings were made in WAV format and 32 bits with a sample rate of 44.1 kHz. Recordings were collected from 06:30 to 11:00 h and from 16:00 to 19:00 h in order to collect as many duets as possible. During the recording sessions, I registered the birds' behavior in order to differentiate duets from both individual's vocalizations overlapping randomly. To identify pairs

and members within a pair, I captured using mist-net and color-banded most of the birds included in the study. Each individual received a unique combination of colored plastic bands. I took standard morphological measurements. Whenever was possible, I sexed birds on the basis of brood patch and cloacal protuberance. A brood patch was distinguishable by the presence of an area of thickened and vascularized skin, while the cloacal protuberance was distinguishable by the enlargement of this body region (Pyle 1997). When individuals of a pair were not marked, I identified territories by location as this species is highly territorial, pairs always join each other to produce their duets and pairs have little or non-overlap in their territories. Thus determining territories in the field was straightforward (Bradley and Mennill (2009).

Acoustic measurements and analysis

To obtain the fine structural characteristics of the duets, I randomly chose five duets (whenever was possible) from each recorded pair by selecting a subgroup of high quality recordings, with no overlapping background sounds, and low ambient noise. These selected duets were numbered and five random numbers were chosen. Then, I generated spectrograms for each selected duet using Raven Pro software (Version 2.5 Beta; Cornell Bioacoustics Program 2010) using the following parameters: Hamming window, DFT 512 and 80 % overlap. Measurements were taken following Podos (2001) suggested measure technique. Briefly, the technique involved the use of the waveform to measure time-related traits and power spectrum to measure frequency-related traits. By using the waveform, I was able to determine the beginning and end time of each element within the duet. Frequency variables were calculated using the power spectrum and using a threshold setting of 30 dB in relation to the song's peak amplitude, including all the peaks that exceeded the threshold.

I measured a total of 13 fine structural characteristics to describe the spectral-temporal characteristics and coordination of duets. The first set of variables described spectral-temporal traits of

duets as a whole, without taking into account the contribution by male and female. I measured the following variables: (1) maximum frequency (Hz): the maximum frequency defined by the 30 dB threshold. (2) Minimum frequency (Hz): the minimum frequency defined by the 30 dB threshold. (3) Frequency bandwidth (Hz): calculated as the difference between minimum and the maximum frequency in the song. (4) Peak frequency (Hz): the frequency with the maximum amplitude within the duet. (5) Entropy: defined as a measure of energy dispersion in a sound, with continuous values ranging from 0 to infinite, where pure-tone sounds have values closer to 0 while noisy sounds have higher values. (6) Number of notes per second within the duet. The second set of variables estimated the degree of coordination among paired individuals by quantifying differences among their contributions. I measured the following variables: (7) Delay time of response (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. (8) Difference between song durations (s): calculated as the difference between the song duration of the first contributor and the song duration of the second contributor. (9) Difference between maximum frequencies (Hz): the difference between the mean highest frequencies of the songs of duet contributors. (10) Difference between minimum frequencies (Hz): the difference between the mean lowest frequencies of the songs of both duet contributors. (11) Difference between frequency bandwidths (Hz): the difference between the mean frequency bandwidths of the songs of both duet contributors. (12) Difference between entropy: difference between the mean entropy values of the song of both duet contributors. (13) Difference between peak frequencies (Hz): difference between the mean frequencies with the maximum amplitude of the song of both duet contributors.

Statistical analyses

I conducted a principal component analysis (PCA) using the correlation matrix and Varimax factor rotation with all the 13 variables and report all the components with eigenvalues ≥ 1 . The principal component analysis retained five principal components which altogether explained 79.3% of the total variance (Table

1). The first component was strongly associated to variables related to duet coordination in terms of frequency (i.e., difference between maximum frequencies, difference between bandwidths and difference between entropies). The second component was strongly associated to coordination in terms of time (i.e., delay time of response and difference between the song duration of both contributors). The third component was strongly associated to spectral variables of the duet as a whole (i.e., maximum frequency, frequency bandwidth and entropy). The fourth component was strongly associated to minimum frequency, peak frequency and number of notes per second of the whole duet. The fifth component was strongly associated to differences between minimum frequencies and differences between peak frequencies in the songs of both contributors.

Table 1. Factor loadings for the five main components extracted of the principal component analysis for the 13 variables measured from 316 Rufous-naped Wrens' duets. Eigenvalues and percentage of variance explained are presented for each component. Variables with the higher loadings are highlighted in bold.

	PC 1	PC 2	PC 3	PC 4	PC 5
Eigenvalues	4.30	1.94	1.77	1.22	1.10
Variance explained (%)	33.06	14.89	13.63	9.35	8.46
Factor loadings					
Maximum frequency	0.33	-0.07	0.88	0.01	0.08
Minimum frequency	0.16	0.04	-0.43	0.64	0.00
Frequency bandwidth	0.29	-0.07	0.91	-0.10	0.07
Peak frequency	-0.11	0.00	0.32	0.73	-0.16
Entropy	0.00	-0.20	0.69	0.28	0.20
Number of notes per second	0.26	-0.21	0.05	0.74	0.15
Delay time of response	-0.02	0.88	-0.19	-0.05	0.03
Difference between song durations	-0.06	0.92	-0.03	-0.08	0.00
Difference between maximum frequencies	0.79	-0.02	0.35	-0.05	0.39
Difference between minimum frequencies	0.00	0.01	0.05	0.04	0.92
Difference between bandwidths	0.89	-0.02	0.32	0.03	0.02
Difference between entropy	0.81	-0.08	-0.04	0.31	0.03
Difference between peak frequencies	0.45	0.03	0.26	-0.09	0.70

I tested whether the duets of Rufous-naped Wrens diverged among the three proposed groups, among sites (two sites per group), and among pairs (between three and five duets per pair) using a three-level nested ANOVA. To build the nested ANOVA model, I used the five principal components extracted from the PCA as dependent variables (one analysis per component, for a total of five analysis), with pairs nested into sampling sites, sampling sites nested into groups and groups as categorical variables. I treated group as a fixed factor and sampling sites and pairs as random factors. To control for the potential effect of using different microphone types (shotgun vs. parabola), I included the variable “Microphone type” as a covariate in the model (see Table 2). I performed post-hoc tests at each level of the model (group, sampling sites and pairs) with each of the performed ANOVAs. I applied a square root transformation to non-normal distributed variables, to improve normality, meet the assumption of homogeneity of variance, and reduce outliers’ effects.

Table 2. Localities and their respective coordinates. The number of pairs recorded in each site for each group is shown and microphone systems used during the recording sessions for Rufous-naped Wrens’ duets in each sampled site.

Number of locality	Site name	Group	Coordinates	Pairs recorded	Microphone type
1	La Ensenada, Puntarenas, Costa Rica	Capistratus	10° 8' 16.354", 85° 2' 25.609"	7	Shogun ME67/K6
2	Los Cocos, Chiapas, Mexico	Capistratus	14° 45' 29.38", 92° 24' 16.11"	10	Shogun ME67/K6
3	Chavarrillo, Veracruz, Mexico	Rufinucha	19° 25' 33.629", 96° 47' 35.966"	13	Shogun ME67/K6
4	Playa Salinas, Veracruz, Mexico	Rufinucha	18° 54' 25.574", 95° 56' 36.913"	15	Shogun ME67/K6
5	Cacaluta, Oaxaca, Mexico	Humilis	15° 44' 34.112", 96° 9' 0.115"	15	Shogun ME67/K6
6	El Chical, Colima, Mexico	Humilis	19° 14' 10.756", 103° 50' 54.801"	10	Shotgun ME62/K6; Telinga Universal Parabola

Results

During the field work I was able to observe several instances where the male and female joined to create duets. According to my field experience, when I was able to capture and sex both birds from a pair, I captured and banded both male and female or either one or another. I never captured just two females

or two males. Furthermore, as this species is highly territorial, it is unlikely that two birds from the same sex duetted together. Despite, the site or group, the physical behavior accompanying the duets was highly similar. Duet performance consisted in both birds generally vocalizing from the same perch performing movements such as spreading the tail feathers, flapping their wings and raising their beaks at about 25 degrees. When birds performed the duet from different perches, the physical behavior was similar.

However, a visual analysis of the duets suggests that the acoustic structure and contribution of both females and males vary among groups. In particular, the duets from the *capistratus* group were unique and very different to the other two groups. The *capistratus* group sang two types of duets: one type where both contributors, female and male, sing the same song type sometimes matching the elements (Fig. 2a), and a second duet type where one of the contributors (presumably the male) sings a tonal, melodious song while the second contributor (presumably the female) vocalize calls (Fig. 2b). On average, *capistratus* duets (69 duets from 17 pairs analyzed) had a maximum frequency of 4.10 ± 1.05 kHz, minimum frequency of 0.63 ± 0.22 kHz and a frequency bandwidth of 3.46 ± 1.13 kHz. Conversely, birds from the *rufinucha* group sang duets (129 duets from 28 pairs analyzed) where male and female produce tonal non-matched vocalizations (Fig. 2c). Birds from the *rufinucha* group had duets with maximum frequency of 5.3 ± 1.15 kHz, minimum frequency of 0.64 ± 0.17 kHz and frequency bandwidth of 4.73 ± 1.21 kHz. Birds from the *humilis* group sang duets (118 duets from 25 pairs analyzed) where the male produced tonal vocalizations overlapped by the non-tonal vocalizations of the female (Fig. 2d). Duets from the *humilis* group had a maximum frequency of 5.02 ± 1.34 kHz, a minimum frequency of 0.83 ± 0.21 kHz and a frequency bandwidth of 4.18 ± 1.21 kHz.

For the analysis, I included a total of 316 Rufous-naped Wren duets belonging to 70 pairs (4.5 ± 1.1 songs per pair) across six sampled sites. For the first, fourth and fifth principal components the results showed statistically significant differences between groups, among sampled sites and among pairs (Table 3). For the second principal component the results showed non-statistically significant differences among

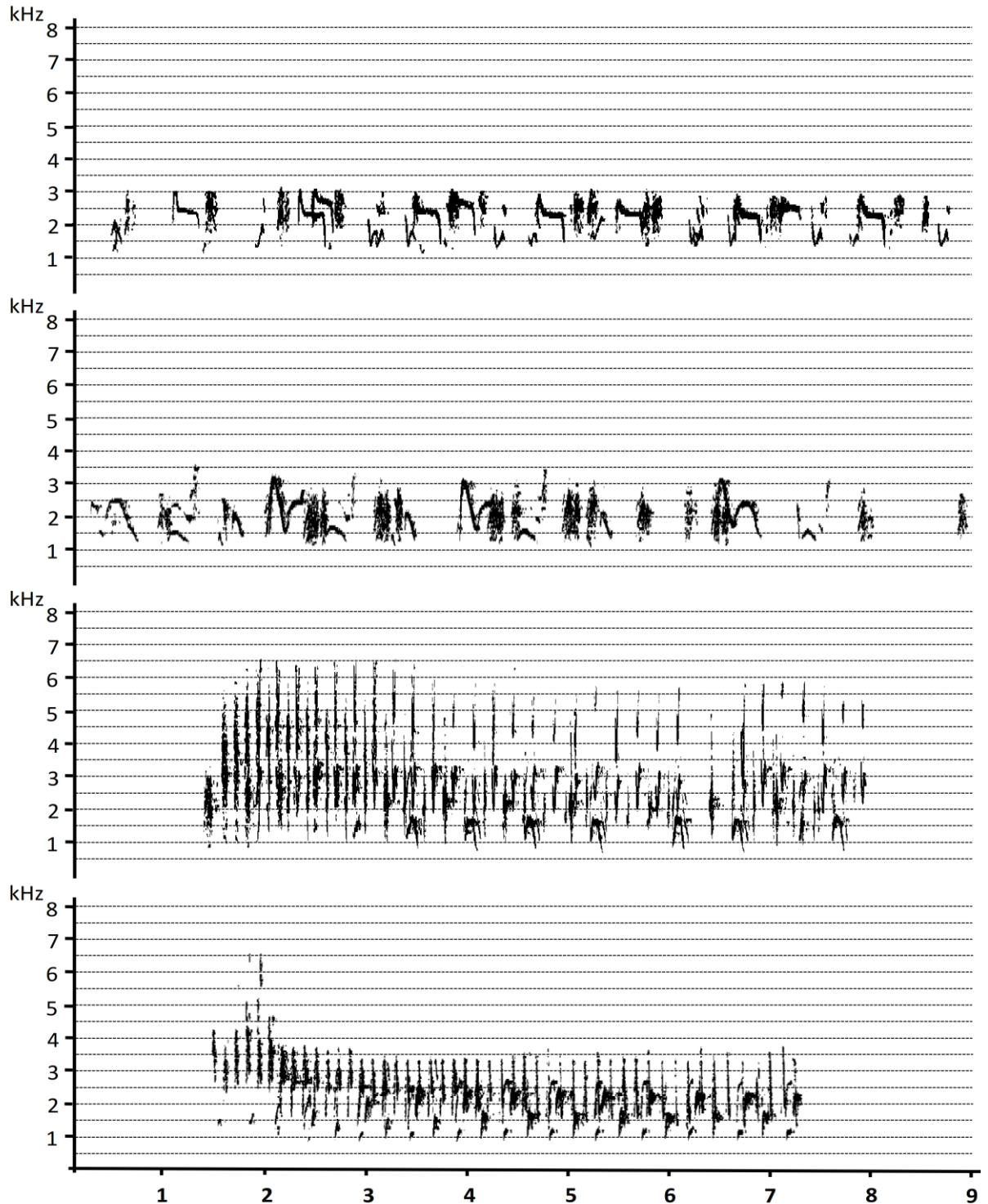


Figure 2. Spectrograms depicting four representative duets of Rufous-naped Wrens: (a) duet of the *capistratus* group in which both members of the pair sing the same song type, (b) duets of the *capistratus* group in which one bird sings tonal songs and the second bird vocalize calls, (c) duets of the *rufinucha* group in which both birds produce non-matched vocalizations and (d) duets from the *humilis* group in which one bird produce tonal vocalizations overlapped by the non-tonal vocalization of the second bird.

groups nor among pairs, but a significant difference among sampled sites (Table 3). For the third principal component the results showed statistically significant differences between groups and among pairs, but not among sampled sites (Table 3).

At group level, the post-hoc test revealed pairwise differences between groups in four of five components. The first principal component separated *capistratus* from both *humilis* and *rufinucha* groups, but *humilis* and *rufinucha* were not different from each other (Fig. 3a; see appendix for the PCA biplot). Component scores were lower for *capistratus* indicating that the songs used by both birds to create the duets were more similar, with the lower differences in maximum frequencies, lower differences in frequency bandwidths and lower differences in entropy. The third principal component separated *rufinucha* from both *capistratus* and *humilis*; but *capistratus* and *humilis* were not different from each other (Fig. 3e; see appendix for the PCA biplot). Component scores were higher for *rufinucha* indicating that their duets had higher maximum frequency, broader bandwidth and higher entropy levels. The fourth principal component separated *humilis* from both *capistratus* and *rufinucha*; but *capistratus* and *rufinucha* were not different from each other (Fig. 3g; see appendix for the PCA biplot). Component scores were higher for *humilis* indicating that duets of this group had higher minimum frequency, higher peak frequency and higher rate (notes per second). The fifth principal component separated all three groups (Fig. 3i; see appendix for the PCA biplot). Component scores were lower for *capistratus*, higher for *rufinucha* and medium for *humilis*. Thus indicating lower differences between minimum frequencies and lower differences between peak frequencies for *capistratus* group, higher differences between minimum frequencies and higher differences between peak frequencies for *rufinucha* group and medium differences between minimum frequencies and lower differences between peak frequencies for *humilis* groups. Second principal component did not differentiate between groups (Fig. 3c; see appendix for the PCA biplot). These results suggest that the duets of each group have particular fine-structural traits than can be used to distinguish each one of the groups.

Table 3. Results of the nested ANOVAS performed with the main five components extracted from the principal component analysis. Statistically significant values ($p < 0.05$) for each level of the nested ANOVA are shown in bold.

Source	df	SS	MS	F	P
Principal component 1					
Group	2	26.3230	13.16152	17.27368	0.000000
Site (Group)	3	7.3907	2.46356	3.23327	0.022993
Pair (Group+Site)	64	74.1515	1.15862	1.52061	0.012886
Error	245	186.6754	0.76194		
Principal component 2					
Group	2	4.2600	2.129978	2.428917	0.090252
Site (Group)	3	22.3540	7.451328	8.497109	0.000022
Pair (Group+Site)	64	74.9294	1.170772	1.335087	0.062771
Error	245	214.8466	0.876925		
Principal component 3					
Group	2	11.2400	5.619997	7.036880	0.001068
Site (Group)	3	1.0890	0.363001	0.454519	0.714337
Pair (Group+Site)	64	99.8546	1.560229	1.953585	0.000151
Error	245	195.6690	0.798649		
Principal component 4					
Group	2	148.8862	74.44310	228.2557	0.000000
Site (Group)	3	14.6798	4.89326	15.0036	0.000000
Pair (Group+Site)	64	33.7341	0.52710	1.6162	0.005209
Error	245	79.9041	0.32614		
Principal component 5					
Group	2	67.5495	33.77474	52.74133	0.000000
Site (Group)	3	12.4475	4.14915	6.47915	0.000309
Pair (Group+Site)	64	63.8350	0.99742	1.55754	0.009139
Error	245	156.8942	0.64038		

At site level, the post-hoc test revealed pairwise differences among sites within groups in four of five principal components. The first, second and fifth principal components showed differences in the duets among sites within the *humilis* group, however, did not differentiate among sites within *capistratus* and *rufinucha* groups (Fig. 3b, d, j). The first principal component had higher scores for el Chical, indicating higher differences between maximum frequencies, higher difference between bandwidths and higher differences between peak frequencies than Cacaluta. Second principal component also had higher scores

for El Chical, indicating a longest delay time of response and higher differences between song durations than Cacaluta. Conversely, the fifth principal component had lower scores for el Chical, indicating lower differences between minimum frequencies and lower differences between peak frequencies than Cacaluta. The fourth principal component showed differences in the duets among sites within the *capistratus* group, however, did not differentiate among localities within *humilis* and *rufinucha* groups (Fig. 3h). Component scores were higher for Los Cocos, indicating a higher minimum frequency, higher peak frequency and higher rate (notes per second) than La Ensenada. These results show that there is an enormous variation in some duets' traits among sites within the *humilis* group, and in a lesser degree among localities within the *capistratus* group, suggesting that distance could be playing a key role in such differentiation.

At the pair level, the post-hoc test revealed pairwise differences among pairs within sites in two of five principal components. The first and third principal components showed differences among pairs within the Playa Salina site from the *rufinucha* group. The First principal component differentiated among four pairs and third principal component only differentiated among two pairs. These results suggest that duets within sites do not show enough variation in their structure to distinguish between pairs from the same site.

Discussion

In this study, I investigated whether Rufous-naped Wrens' duets vary across its distribution range. In particular, I explored whether variation matches the three different groups suggested by some taxonomic approaches based on research on morphology, genetic variation, and behavior by previous authors (Selander 1964, Brewer and MacKay 2001, Howell and Webb 2005; Del Hoyo et al. 2005, Clements 2009,

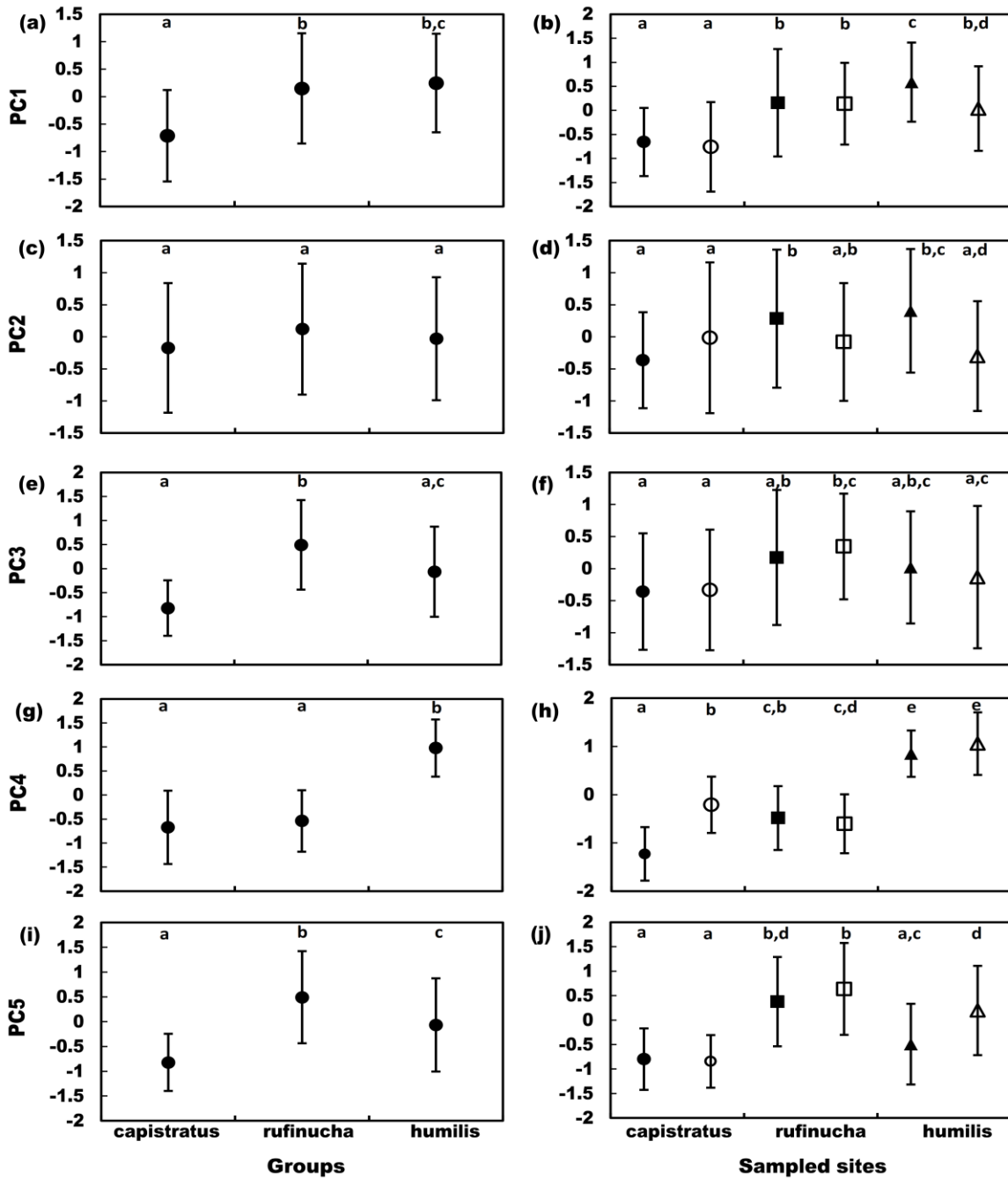


Figure 3. Plots of the five components by groups and sampled localities showing the variation in the duets of Rufous-naped Wrens. Black-filled circles correspond to mean value scores for La Ensenada, Puntarenas province; open circles correspond to mean value scores for Los Cocos, Chiapas, Mexico; Black-filled squares correspond to mean value scores for Playa Salinas, Veracruz, Mexico; open squares correspond to mean value scores for Chavarrillo, Veracruz, Mexico ;Black-filled triangles correspond to mean value scores for El Chical, Colima, Mexico and open triangles correspond to mean value scores for Cacaluta, Oaxaca, Mexico. Bars represent standard deviations. Post-hoc statistically significant differences among groups and sampled localities are showed with different letters.

Vázquez-Miranda et al. 2009, Sosa-López et al. 2012). Results revealed differences between groups, among sampled sites within groups and among pairs within sites. The results suggest that duets are different between all the three groups, despite the geographic distance between sampled sites. Duets also varied among sampled sites. Particularly, duets showed marked differences among sites within the groups with the larger distribution range (*humilis* and *capistratus*), but still this difference is smaller than those observed between groups. All these results suggest that there are differences in the duets between groups, that there is significant variation between the duets from different sites within, and that it is not possible to distinguish among pairs within the same site by using the duets.

The *capistratus* group had the lowest scores for all the principal components. This fact may reflect that birds from the *capistratus* group produce tonal duets with the lowest maximum frequency, narrow bandwidth and low levels of entropy. Conversely, birds from *rufinucha* and *humilis* groups sang relative noisier duets (or the least tonal duets), showing the higher values of maximum frequency, the broader bandwidth and higher values of entropy. Duets from the *capistratus* group were also the most coordinated, with both contributors –male and female- vocalizing similar frequencies (i.e. least difference between maximum frequencies, between minimum frequencies, between frequency bandwidths, between peak frequencies and between entropy of both individuals' song). While coordination in frequency parameters was low for both the *rufinucha* and *humilis* groups, with higher differences in entropies, in maximum frequencies and in bandwidth. The *humilis* group had intermediate coordinated duets in terms of differences in minimum frequencies and differences in peak frequencies. Birds from the *humilis* group sang duets with the high number of notes per second, high minimum frequencies and high peak frequency. Thus, *capistratus* produce the most tonal, low rate (notes per second), and frequency coordinated duets, *humilis* produced noisy, high rate (notes per second), intermediate frequency coordinated duets, and *rufinucha* produced noisy, low rate (notes per second), and low frequency coordinated duets.

High coordination among contributors in a duet has been reported in other species such as Purple-crowned Fairy Wrens (*Malurus coronatus*; Hall and Peters 2008), Plain Wrens (*Cantorhilus modestus* subsp. *zeledoni*; Rivera-Cáceres 2015), Gray-breasted Wood Wren (*Henicorhina leucophrys*; Dingle and Slabbekoorn 2018) and Magpie Lark (*Grallina cyanoleuca*; Hall and Magrath 2007). These studies suggest that highly coordinated duets might signal pair bond strength and might serve as an effective mechanism to defend territories and resources. However, playback studies simulating different degrees of coordination are needed to test these non-mutually exclusive hypotheses within this species complex.

Coordination in time did not vary with geography in the Rufous-naped Wrens' duets. Some studies have demonstrated that temporal coordination in duets show high levels of plasticity (Sandoval et al. 2015, Trejos-Araya and Barrantes 2017). Also, it has been proposed that temporal coordination in duets depends upon how long the birds have been together (Hall and Magrath 2007, Rivera-Cáceres 2017). It is possible that laying eggs, incubating and feeding the chicks during breeding season affect the birds' physical condition reducing their attentiveness to their partners' song. Another alternative explanation to high variation in time coordination is that during the start of the breeding season, it is possible to find several recently formed pairs. As coordination may require some degree of practice, new couples may show less time-coordinated duets. Thus, the great variation in temporal coordination observed between sites may be the result of my sample design (where I sampled different sites in different breeding stages). A more finely designed study is necessary to test whether temporal coordination in duets vary between breeding and non-breeding seasons, whether birds' physical conditions affect the rate of duet production and the temporal coordination and whether recently formed pairs produce less coordinated duet than relatively older pairs in this taxon.

My results suggest that Rufous-naped Wrens duets divergence mirrors the variation described in morphological, genetics and other behavioral traits (Selander 1964, Vázquez-Miranda et al. 2009, Sosa-López et al. 2012). A variety of studies have shown congruent variation across different traits within other

bird species complex. For instance, 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) found that genes, morphology and songs of the Sharp-beaked ground finch (*Geospiza difficilis*) vary among the Galápagos Islands. Also, a comprehensive study of songs and morphology in the genus *Troglodytes* showed that traits of populations living in allopatry usually diverge in tandem (Sosa-López et al. 2014). Studies such as these are wide spread in the literature suggesting that allopatric population with distinctive characteristics should be recognized as different species (Grant et al. 2000, Uy et al. 2009, Sosa-López 2014, Cooper and Cuervo 2017, Nwankwo et al. 2018, Van Doren et al. 2018, Wei et al. 2018). However, whether differences in traits or whether the variables analyzed in these studies are relevant for the species is still an open question and further studies should consider how individuals perceive such variation by applying experimental playbacks.

How the differences in the duets of this polytypic taxon originated is still not clear, but my results align with findings previously reported. Based on genetic evidence, Vázquez-Miranda et al. (2009) suggested that differences between the three groups arose through a geographic isolation event 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 three groups and promoting genetic and behavioral differentiation (Vázquez-Miranda et al. 2009, Sosa-López et al. 2012). Compelling available evidence supports the hypothesis that vicariant events across the Isthmus of Tehuantepec likely isolated a great number of taxa, originating the enormous diversity we observe nowadays (Mulcahy et al. 2006; Barber and Klicka, 2010; González et al. 2011; Ortiz-Ramírez et al. 2016; Hernández-Soto et al. 2018). Furthermore, a great body of 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. 2012, Roach and Phillmore, 2017). Whether other selection pressures such as social or ecological or

cultural drift are contributing to the vocal differentiation among groups is still to be answered, however, Sosa-López et al. (2012) find no association between song and ecological variables. Sosa-López et al. (2012) study, nonetheless, was conducted using proxy ecological variables and further studies using direct habitat measurements should be done to corroborate this hypothesis. Despite our gap of knowledge about how duet diversity within Rufous-naped Wrens arose (and other traits such as morphology and songs), the results of this and previous studies (Selander 1964, Vázquez-Miranda et al. 2009, Sosa-López et al. 2012) support the hypothesis of isolation as a primary factor promoting such diversity in acoustic signals.

In addition to the differences found between the three groups, the analysis also suggests the existence of significant variation among sampled sites within groups, particularly within the *humilis* and *capistratus* groups. Within the *humilis* group, duets from Cacaluta were more coordinated in some spectro-temporal variables than duets from El Chical. And birds from Los Cocos in Chiapas Mexico, sang duets with higher minimum frequencies, higher frequencies with the maximum amplitude and more notes per second than birds from La Ensenada in Cost Rica. To record the duets, different types of microphones (shotgun vs parabola) were used at different sites. My experience indicates that recordings of duets made with parabolas have a better signal-to-noise ratio than shotgun systems, and these differences could affect frequency measurements. This variation was not observed within the *rufinucha* group isolated in Central Veracruz, Mexico, where I used only shotgun systems. To rule out the potential effect of different microphone systems on the results, I included microphone type as a covariate in the statistical analysis. Thus, the observed variation within groups it is possible due to isolation-by-distance or the presence of dialects (Baptista and Morton 1982, Mundinger 1982, Catchpole and Slater 2008). I suggest that historical geographic isolation resulted in vocal differentiation among groups, while isolation-by-distance could be acting within groups, promoting variation among populations within the *capistratus* and *humilis* groups. A similar pattern has been reported in four species of Fairy Wrens of the genus *Malurus* (Yandell et al.

2017). Another recent study strongly supports the key role of isolation-by-distance in the origin of differences in acoustic signals (Graham et al. 2018). Whether duet variation within groups is due to an accumulation of cultural changes along the distribution (Koetz et al. 2007) or whether Rufous-naped Wrens present dialects (Baptista and Morton 1982, Mundinger 1982, Catchpole and Slater 2008) is still an open question and further studies analyzing micro-geographic variation should be conducted.

Patterns of variation found in this study suggest that *capistratus* produces the most divergent duets from all the three groups, while *humilis* and *rufinucha* produce more similar duets. This finding are in line with the phylogenetic analysis proposed by Vázquez-Miranda et al. (2009) in which *Campylorhynchus rufinucha* and *C. humilis* are sister taxa and *C. capistratus* is the sister group of *C. rufinucha* - *C. humilis* group. Thus, this results reinforce the idea that similitude in avian vocalizations parallels the phylogenetic relationships among taxa (De Kort and Ten Cate, 2001; Sosa-López et al. 2016; Price and Lanyon, 2002). However, whether similitude in duets (and solo songs) is perceived by individuals it is unknown, but a preliminary playback experiment performed on these three groups suggest that members from the *capistratus* group discriminate between conspecific songs and allopatric songs (*rufinucha* and *humilis*), whereas members from *rufinucha* and *humilis* groups discriminate between conspecific and *capistratus* group songs, but neither *rufinucha* or *humilis* distinguish between *rufinucha* and *humilis* songs (Sosa-López in. prep.). This suggest that in fact, birds from the Rufous-naped Wrens complex perceive the differences in the fine-structural traits in this complex signals.

Why duets of the parapatric *humilis* and *capistratus* group are so different? Reproductive character displacement is a possible mechanism to explain this phenomenon. Thus, once acoustic divergence has arisen in secondary contact zones; selection should favor the increasing divergence in acoustic signals, if they compete for resources (Wilkins et al 2013). Several studies have provided evidence of the reproductive characters' displacement in a wide variety of taxa (Hoskins et al. 2005; Wallin 2008; Kirschel et al. 2009; Grant and Grant 2010; Wilkins et al. 2013; Scordato 2018; Nwankwo 2018; but see Seddon

and Tobias 2010). Conversely, duets may converge if a secondary contact zone has intermediate characteristics, promoting high fitness of the hybrids. According to Vázquez-Miranda et al. (2009), there is evidence of hybrid birds along the secondary contact zone between *humilis* and *capistratus* groups and Sosa-Lopez et al. (2012) found that songs in the secondary contact zone vary gradually, suggesting that reproductive character displacement is not occurring at the secondary zone.

Conclusions

In conclusion, this study on Rufous-naped Wrens provide evidence of the presence of geographic variation in complex signals, supporting the existence of three vocal lineages within the complex, also suggesting that divergence in duets probably is the result of vicariant events, followed by other selective and/or non-selective factors. Moreover, I also demonstrate that there exists variation between sites within the same group, may be as the result of isolation-by-distance or dialect formation. Based on the multiple evidence now available, I suggest that the three groups represent distinct taxonomical units, hence, adding to the idea of three different species within the complex as proposed by other authors (Ridgway 1904, Navarro-Sigüenza and Peterson 2004, Vázquez-Miranda 2009, Del Hoyo et al. 2017, Sosa-López et al. 2012).

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Appendix

Biplots of component scores extracted from the principal component analysis performed from the correlation matrix with varimax rotation factor. Blue-filled circles correspond to duet scores from *capistratus* group; yellow-filled diamonds correspond to duet scores from *humilis* group and red-filled triangles correspond to due scores from *rufinucha* group. Black-filled circles represent the centroid scores from *capistratus* group; black-filled diamond represent the centroid scores from *humilis* group and black-filled triangles represent the centroid scores from *rufinucha* group.

