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RESEARCH ARTICLE

Habitat connectivity, gene flow, and population genetic structure in a Neotropical understory insectivore, the Rufous-and-white Wren

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ABSTRACT

Among tropical organisms, heightened habitat specialization, limited natal dispersal, and strong philopatry suggest that many species may experience reduced rates of gene flow. Diverse forms of barriers, including geographic, ecological, and behavioral barriers, further promote genetic divergence among tropical bird populations. Here, we extend our comprehension of gene flow in tropical birds by examining population genetic structure in a widespread insectivorous songbird of the Neotropics, the Rufous-and-white Wren (*Thryophilus rufalbus*). We explore the effects of geographic distance and habitat connectivity on genetic structure using 10 microsatellite loci, and nuclear and mitochondrial sequence data. We report high levels of genetic divergence and population structure with reduced contemporary gene flow between populations over a 500-km transect in Nicaragua and Costa Rica. Mitochondrial DNA and nuclear sequence data indicate that 2 distinct mtDNA genetic groups came into contact in northwestern Costa Rica; molecular dating suggests that the genetic patterns arose as a result of Pleistocene glaciations. Geographic distance and habitat connectivity predicted genetic structure but explained a relatively low proportion of the observed contemporary genetic variation. Patterns were similar for both males and females. Our research demonstrates the deep genetic divergence in tropical birds, and that genetic differentiation can occur over a relatively short distance. For tropical birds, strong limits to gene flow likely arise as a result of limited dispersal from natal populations.

Keywords: gene flow, Neotropics, philopatry, Rufous-and-white Wren, *Thryophilus rufalbus*, *Thryothorus rufalbus*

LAY SUMMARY

- Among tropical organisms, heightened habitat specialization and strong philopatry suggest that many species may experience reduced rates of gene flow.
- We explored the effects of geographic distance and habitat connectivity on genetic structure for Rufous-and-white Wrens (*Thryophilus rufalbus*) using 10 microsatellite loci and nuclear and mitochondrial sequence data.
- We observed high levels of population genetic structure and low levels of contemporary gene flow among five populations in Nicaragua and Costa Rica.
- We found two distinct genetic clades present in this region, and divergence times suggest that these clades diverged during the last Pleistocene glaciation.
- Our research demonstrates the deep genetic divergence in tropical organisms, and that genetic differentiation can occur over a relatively short distance; strong limits to gene flow likely arise as a result of strong philopatry.

Conectividad de hábitat, flujo génico y estructura genética poblacional en un insectívoro neotropical de sotobosque, *Thryophilus rufalbus*

RESUMEN

Entre los organismos tropicales, la elevada especialización de hábitat, la limitada dispersión natal y la fuerte filopatría sugieren que muchas especies pueden experimentar tasas reducidas de flujo génico. Diversas formas de barreras,

incluidas las barreras geográficas, ecológicas y de comportamiento, promueven aún más la divergencia genética entre las poblaciones de aves tropicales. En este estudio, ampliamos nuestra comprensión del flujo génico en las aves tropicales mediante el examen de la estructura genética de la población en un ave canora insectívora neotropical ampliamente distribuida, *Thryophilus rufalbus*. Exploramos los efectos de la distancia geográfica y la conectividad de hábitat en la estructura genética utilizando 10 loci de microsatélites y datos de secuencias nucleares y mitocondriales. Reportamos altos niveles de divergencia genética y estructura poblacional con reducido flujo génico contemporáneo entre poblaciones a lo largo de un transecto de 500 km en Nicaragua y Costa Rica. El ADN mitocondrial (ADNmt) y los datos de secuencia nuclear indican que dos grupos genéticos distintos de ADNmt entraron en contacto en el noroeste de Costa Rica; la datación molecular sugiere que los patrones genéticos surgieron como resultado de las glaciaciones del Pleistoceno. La distancia geográfica y la conectividad de hábitat predijeron la estructura genética, pero explicaron una proporción relativamente baja de la variación genética contemporánea observada. Los patrones fueron similares tanto para machos como para hembras. Nuestra investigación demuestra la profunda divergencia genética en las aves tropicales y que la diferenciación genética puede ocurrir sobre una distancia relativamente corta. Para las aves tropicales, probablemente surgen fuertes límites al flujo génico como resultado de la dispersión limitada de las poblaciones natales.

Palabras clave: filopatría, flujo génico, Neotrópico, *Thryophilus rufalbus*

INTRODUCTION

Biodiversity is highest in the tropics, yet diversity remains underestimated because many regions are poorly sampled and many species have not been studied in detail (Redford et al. 1990, Hebert et al. 2004, Lohman et al. 2010, Bálint et al. 2011). Many tropical species exhibit high population genetic structure across geographic space, characterized by deep genetic divergence observed within species complexes (Cadena and Cuervo 2009, Derryberry et al. 2011, González et al. 2011, Isler et al. 2012, Habel et al. 2013, Lougheed et al. 2013, De Camargo et al. 2015, Céspedes et al. 2021, Del-Rio et al. 2021). The observed genetic patterns are often attributed to the genetic legacy of the Pleistocene (Hewitt 2000, Dhorta et al. 2011, Cabanne et al. 2016), or biogeographic barriers (Burney and Brumfield 2009, Huntley and Voelker 2016, Del-Rio et al. 2021), although anthropogenic alterations to habitat, and outright loss of habitat, also influence genetic patterns (Bates 2002, Athrey et al. 2012, Woltmann et al. 2012a, Barr et al. 2015). Examining patterns of gene flow across diverse landscapes provides important insight into the connectivity of habitats, animal movements, and biodiversity.

Tropical birds occupy diverse habitats and often exhibit strong philopatry and niche specialization compared with their temperate counterparts (Russell et al. 2004). Both philopatry and habitat specialization are thought to promote genetic divergence and reduce gene flow between populations and are understood to be important aspects of speciation for birds (Arguedas and Parker 2000, Salisbury et al. 2012, Smith et al. 2014, Peterson et al. 2015, Khimoun et al. 2016). It is widely understood that avian diversity peaks near the equator, but recent research, using the ecological species concept, suggests that avian diversity is severely underestimated (estimates using molecular tools suggest that the number of bird species may be two to two-and-a-half times greater than currently recognized; Barrowclough et al. 2016). In conjunction with the recent description of new species from the Neotropics (e.g., Lara et al. 2012, Seeholzer et al. 2012, Sandoval et al. 2014, 2017), these findings demonstrate the importance of examining population genetic patterns for tropical birds and can help to guide efforts that conserve tropical biodiversity.

In the current study, we focused on Rufous-and-white Wrens (*Thryophilus rufalbus*), a nonmigratory, understory insectivore with a widespread distribution in the Neotropics. The range of Rufous-and-white Wrens extends southward from southern Mexico, along the Pacific coast through Central America, into northern Colombia and northwestern Venezuela (Figure 1). Five subspecies of Rufous-and-white Wrens are recognized (Valderamma et al. 2007). This species has been included in many phylogenetic studies examining relationships among wrens (Barker 2004, Mann et al. 2006, Lara et al. 2012), yet little is known about genetic variation within this species. Although most wrens (Family: Troglodytidae) are characterized as having a plain appearance, and most exhibit limited plumage variation across geographic space, genetic studies have revealed deep genetic divergence and high population structure within the wrens and highlighted substantial cryptic variation (Barker 2004, Vázquez-Miranda et al. 2009, Lara et al. 2012, Saucier et al. 2015, Camacho-Alpízar et al. 2018). Previous research on Rufous-and-white Wrens has revealed limited dispersal among populations (Graham et al. 2018b), but to date little is known about population genetic structure.

We explored population genetic structure and phylogeographic structure in the Rufous-and-white Wren along a 500-km transect in Central America. We collected blood samples from 5 populations in Nicaragua and Costa Rica and examined population structure using sequence data (both nuclear and mitochondrial DNA [mtDNA]) and microsatellite data. In addition to characterizing population structure, we assessed the level of gene flow between populations. We examined the effect of habitat connectivity on genetic divergence, and whether habitat connectivity influences genetic patterns for males and females differently. Given that many tropical birds exhibit limited natal dispersal and high site fidelity, we examined the effect of geographic

distance on genetic patterns as well. If population genetic patterns arise due to limited dispersal between sites, then we would expect a weak relationship between genetic distance and geographic distance and habitat connectivity.

METHODS

We studied Rufous-and-white Wrens living in 5 populations along a 500-km gradient from Nicaragua to Costa Rica (Figure 1; Table 1). Currently, 5 subspecies of Rufous-and-white Wrens are recognized; the populations we studied are all found in the distribution range of the *castanonotus* subspecies. At each of the 5 populations, we captured birds using mist nets and banded each bird with a unique combination of leg bands that included 3 color bands and a numbered aluminum band. We collected a small blood sample (~100 µL) from the brachial vein of each bird and stored blood samples in 95% ethanol or Queen's Lysis Buffer (Seutin et al. 1991). Individuals were sexed based on the presence of a brood patch (females) and by singing behavior (sexes can be distinguished based on fine structural differences in songs, as described in Mennill and Vehrencamp 2005, Harris et al. 2016).

We extracted DNA from blood samples using a Wizard Extraction Kit (Promega) and genotyped 211 birds (129 males and 81 females plus 1 individual from Nicaragua whose sex was unknown) at 10 microsatellite loci: *ThPl 14, ThPl 20, ThPl 30* (Brar et al. 2007), *RWWR 2c, Tru 08, Tru 11, Tru 18, Tru 20, Tru 24,* and *Tru* 25 (Graham et al. 2018a). Polymerase chain reaction (PCR) amplification protocols followed those outlined in Graham et al. (2018a). We tested for deviations from Hardy–Weinberg Equilibrium (HWE) and linkage disequilibrium to ensure that all loci met Hardy–Weinberg expectations and also to ensure that none of the loci examined were linked with each other. All tests were conducted in GenePop, version 4.0.10 (Raymond and Rousset 1995), and corrected for multiple tests using sequential Bonferroni corrections (Rice 1989).

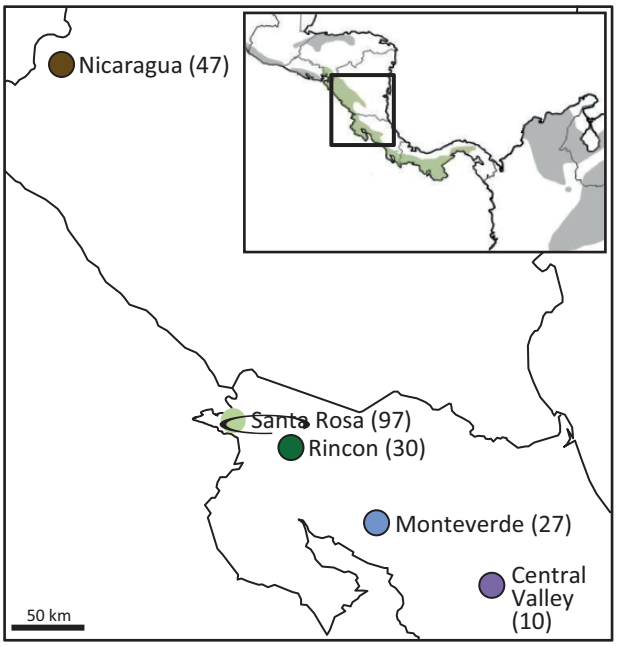

FIGURE 1. Map of the 5 populations of Rufous-and-white Wrens (*Thryophilus rufalbus*) in Nicaragua and Costa Rica where genetic samples were collected, with sample sizes for microsatellite analyses listed in brackets. Inset: Range map of Rufous-and-white Wrens, with the range of the subspecies *T. r. castanonotus* shown in green. The box in the inset map shows the position of the larger map.

We calculated allelic richness (A_R), and observed (H_O) and expected heterozygosity (H_E), using FSTAT version 2.9.2.3 (Goudet 1995).

To examine mtDNA divergence, we analyzed the mitochondrial gene NADH dehydrogenase 2 (ND2; 1,041 base pairs [bp]) using previously designed primers (ND2 primers: L5215 and H1064; Hackett 1996). From each population, we tried to analyze at least 10 individuals (range: 9–13; Table 1) to determine the level of population differentiation. Given that mitochondrial and nuclear genes can potentially show contrasting patterns (Zink and

TABLE 1. Population locations and genetic statistics for the 5 populations of Rufous-and-white Wrens (*Thryophilus rufalbus*) examined in this study. N_{mast} is the number of individuals examined for microsatellite analysis. Numbers in parentheses are the number of males and females examined in each population; A_r, allelic richness; H_o, observed heterozygosity; H_e, expected heterozygosity. F_{IS} represents the inbreeding coefficient. N_{mtDNA} is the number of individuals genotyped for the ND2 mtDNA gene; H, number of haplotypes; H_d, haplotype diversity; π, nucleotide diversity.; N_{NucMt} is the total number of individuals sequenced for the 2 mtDNA and 3 nuclear genes used in the IMa2 analyses.

Population	Latitude	Longitude	N_{msat}	A_r	H_o	H_e	F_{IS}	N_{mtDNA}	H	H_d	π	N_{NucMt}
Nicaragua	13.27	–86.31	47(25/22)	6.29	0.61 ± 0.09	0.65 ± 0.09	0.06 ± 0.05	12	9	0.94	0.004	5
Santa Rosa	10.85	–85.60	97(65/32)	7.00	0.56 ± 0.09	0.65 ± 0.10	0.16 ± 0.03	13	13	1.00	0.010	5
Rincon	10.78	–85.35	30(20/10)	6.37	0.56 ± 0.09	0.64 ± 0.10	0.10 ± 0.04	10	9	0.98	0.010	6
Monteverde	10.28	–84.80	27(17/10)	6.47	0.62 ± 0.08	0.69 ± 0.08	0.12 ± 0.06	13	8	0.91	0.003	7
Central Valley	9.90	–84.25	10(6/4)	6.06	0.60 ± 0.09	0.67 ± 0.09	0.08 ± 0.06	9	5	0.72	0.002	7

Barrowclough 2008), we amplified sequences from 4 other gene regions for a subset of individuals (Table 1): 1 mitochondrial gene cytochrome oxidase I (COI, 914 bp), and 3 nuclear genes: the fourth intron of the β-Fibrinogen region, Fib 4, (600 bp); the recombination activity gene, RAG-1; (1,006 bp); and the third exon of the avian c-myc gene, c-myc; (493 bp), using COI primers: LTyr and Coi907aH2 (Tavares et al. 2011); Fib 4: FIB3 and FIB4 (Barker 2004); RAG-1 primers: R17 and R22 (Groth and Barrowclough 1999); and C-myc primers: mycEX3A and RmycEX3A (Ericson et al. 2000). Whereas we used the larger ND2 dataset for all population differentiation analyses, we used the smaller 5-gene dataset to estimate gene flow and divergence times (see below). All PCR reactions were conducted in 25 μL reactions with 1 μL of genomic DNA. PCR cocktails contained 2.5 μL of 10× PCR Buffer (Applied Biosystems), 1.0 μL of $MgCl_2$ (2.5 mM), 0.9 μL of dioxynucleotide triphosphate (dNTP) (0.2 mM), 1 μM each of the forward and reverse primer, and 1.0 U of Taq (Genscript, Applied Biosystems). PCR thermocycler conditions used the following conditions: one cycle of 94.0°C for 3 min, followed by 35 cycles of 94.0°C for 40 s, 50.0°C for 40 s, 72.0°C for 1 min, followed by a final extension cycle of 72°C for 3 min. PCR amplicons were sequenced using the forward primers at the McGill University and Génome Quebec Innovation Center. Sequences were aligned to reference sequences available on GENBANK and trimmed to their respective lengths in Mega 5.0 (Tamura et al. 2011). In addition, we calculated the number of haplotypes, haplotype diversity (H_d), and nucleotide diversity (π) using DNAsp 5.0 (Librado and Rozas 2009; Table 1).

Population Structure

We used several approaches to examine population differentiation and describe population structure. First, we calculated pairwise F_{ST} and F'_{ST} (microsatellites), and θ_{ST} (mitochondrial) comparisons. We calculated F_{ST} and F'_{ST} values using GENODIVE version 3.04 (Meirmans and Van Tienderen 2004) and θ_{ST} values using ARLEQUIN version 3.11 (Excoffier et al. 2005); deviations from zero were determined using 10,000 permutations for both sets of comparisons. All pairwise tests were corrected using sequential Bonferroni tests (Rice 1989). We chose to calculate both F_{ST} and F'_{ST} over other measures like G_{ST} or G'_{ST} based on the recommendation of Meirmans and Hedrick (2011). F'_{ST} complements the traditional F_{ST} as it represents the normal F_{ST} standaridized to the maximum possible value. Additionally, F'_{ST} is more robust for making inferences on the effects of drift and migration on population differentiation (Meirmans and Hedrick 2011).

Second, to describe population structure, we conducted an analysis of molecular variance (AMOVA) using GenAlEx 6.5 (for microsatellites; Peakall and Smouse 2012) and ARLEQUIN (for mtDNA; 10,000 permutations). We examined both our microsatellite and ND2 dataset to determine if there was a hierarchical structure among groups, among populations within groups, and within populations. In this analysis, our goal was to describe population structure and determine the relationship among populations.

We used the Bayesian clustering model STRUCTURE, version 2.3.3 (Pritchard et al., 2000) to investigate microsatellite population structure. For all runs, we used the admixture model with the correlated allele frequencies model setting but did not use sampling location as a prior. Each run consisted of a burn-in of 100,000 chains followed by 500,000 chains; we ran 5 iterations for each K, and K ranged from 1 to 6 (the maximum number of populations plus one). To determine the true K, we used the ΔK method (Evanno et al. 2005) as implemented in STRUCTURE HARVESTER (Earl and vonHoldt 2012). Following this initial run, we ran STRUCTURE (using the same settings) on the individual clusters containing more than one locality to examine if there was a hierarchical population structure within the recovered clusters.

To visualize the relationships among mtDNA and nuclear haplotypes, we constructed a haplotype network for each gene using TCS, version 1.21 (Clement et al. 2000).

Estimates of Divergence and Gene Flow

We estimated divergence times in the program IMa2 (Hey, 2010). For this analysis, we examined a subset of individuals (n = 30; Table 1) for the 2 mitochondrial genes and 3 nuclear genes. We ran *JModelTest*, version 0.1.1 (Posada, 2008) for each locus to determine the best-model fit for each of our 4 loci (mtDNA combined and the 3 nuclear loci). The best-fit model for the combined mtDNA and nuclear sequence dataset was a GTR G + I, and, therefore, we used the HKY model in IMa2 as this was the closest model available in IMa2. Tests on individual nuclear and mtDNA loci revealed that a GTR G + I or HKY model best fit the individual datasets, further justifying our use of an HKY model in the IMa2 analysis (model corrected substitution distances for the ND2 and COI mtDNA genes can be found in Supplementary Material Table S2). For our analysis, we used a 2-population model. Given that IMa2 requires that paired populations have a significant F_{ST} between them, we pooled our Santa Rosa and Rincon populations together (hereafter referred to as "Northwestern Costa Rica") and our Monteverde and Central Valley populations together (hereafter referred to as "Central Costa Rica") to meet this criterion. We converted all parameters to demographic units, using a generation time of 2 years and a substitution rate of 2.0×10^{-8} substitutions site^{-1} year^{-1} (Fleischer et al. 1998, Weir and Schluter 2008). Although this rate is lower than recent estimates for the ND2 gene (Lerner et al. 2011), we chose a more conservative substitution rate because

we concatenated both mtDNA genes and treated them as a single locus for this analysis. We calculated length scaled mutation rates for each of the 4 loci (3 nuclear and 1 mtDNA) and used a different mutation rate for each locus. Finally, we performed 3 replicates for each pairwise combination, employing a geometric heating scheme (20 MCMC chains, $ha = 0.98$, $hb = 0.92$) for >17 million steps following the burn-in. Upon completion, results from each replicate were combined and analyzed in L-mode within IMa2.

To estimate rates of contemporary gene flow, we calculated rates of gene flow among populations using our microsatellite dataset in the program BAYESASS+, version 3.0; BAYEASS+ is understood to produce values that are indicative of gene flow over the last few generations (Wilson and Rannala 2003). We ran 10 replicates for 1×10^7 with a burn-in of 1×10^6; we altered mixing parameters to ensure that allele frequency, inbreeding coefficients, and migration rates fell within the 20%–60% acceptance rate suggested by Wilson and Rannala (2003). Lastly, we calculated Bayesian deviance values (Spiegelhalter et al. 2002) in R using the script provided in Meirmans (2014) to determine the best-fit run; results presented for BAYEASASS+ are from this best-fit run (Chiucchi and Gibbs 2010).

Habitat Connectivity Measurements

We measured habitat connectivity among 211 Rufous-and-white Wren individuals following the approach of Hindley et al. (2018). We used the R package *gdistance*, version 1.3-6 (van Etten and Hijmans 2010), which uses graph theory to calculate the distance and route used to travel between each unique sample comparison. This method used raster cell values to represent suitable tree-habitat and create a least-cost resistance matrix between each pair of individuals based on their geographic location. Suitable forest habitat data was obtained from the global map of tree diversity (Crowther et al. 2015).

We used distance-based redundancy analyses and partial-distance-based redundancy analysis (Legendre and Legendre 1998) to examine the effects of isolation by distance and habitat connectivity on contemporary genetic patterns. Although Mantel tests are commonly used to measure the relationship between geographic distance and habitat connectivity with genetic distance, this approach has been criticized due to its inability to detect the appropriate amount of variation explained by a given variable (reviewed in Legendre and Fortin 2010). Therefore, we chose to use redundancy analysis because it allowed us to test the relationship of multiple factors on genetic distance, control for various factors, as well as partition the variance explained by each factor, allowing us to more directly compare the effect of each factor. For our redundancy analysis, we included all 211 individuals and we included geographic distance, habitat connectivity sex, latitude, longitude, and population as the factors in our model. We ran several partial-distance-based redundancy analyses where we controlled for the effects of several factors on the influence of a main factor. For example, we controlled for sex in our analyses of geographic distance and habitat connectivity because sex-biased dispersal is common in birds (Greenwood 1980, Greenwood and Harvey 1982) and because other studies have shown that males and females respond differently to habitat connectivity (Wang et al. 2012, Amos et al. 2014). For our analysis of geographic distance, we controlled for habitat connectivity and performed the reciprocal analysis where we controlled for geographic distance while testing for the effect of habitat connectivity. Following these initial analyses, we analyzed males and females separately to examine the effect of geographic distance and habitat connectivity on microsatellite genetic patterns for each sex. As in the previous analysis, we ran a partial-distance-based redundancy analysis to control for the effects of geographic distance and habitat connectivity on each other.

We ran all redundancy and partial-redundancy models in R using the *Vegan* package, version 2.5-7 (Oksanen et al. 2020). For our genetic distance, we calculated the Cavalli-Sforza chord distance between all individuals, males, and females using GENODIVE (Meirmans and Van Tienderen 2004). We used the decimal latitude and longitude point for each individual to calculate the geographic distance between individuals. To quantify habitat connectivity, we used a principal coordinate analysis (PCA) in GenAlEx to transform the habitat connectivity distances to eigenvectors to express these values as coordinate explanatory variables following the protocol outlined by Oksanen et al. (2020).

RESULTS

Genetic Variation

Genetic analyses of Rufous-and-white Wrens revealed high sequence diversity for our analyses of the full ND2 gene (1,042 bp). We identified 39 haplotypes with 37 variable sites (31 of which were parsimony informative). Haplotypes are separated geographically, with 2 distinct haplogroups (North and South) identified based on our statistical parsimony network (Figure 2). Both haplotype and nucleotide diversity were high (0.978 and 0.01, respectively; Table 1), although we did see some variation among populations. The Central Valley had the lowest haplotype and nucleotide diversity (0.72 and 0.002, respectively), whereas Rincón and Santa Rosa had the highest haplotype (0.97 and 1.00) and nucleotide diversity (0.01 at both sites); haplotypes from both the North and South were present at these 2 populations, which explains why haplotype and nucleotide diversity were higher. Haplotype and nuclear diversity were also high for our analysis of COI and RAG-1

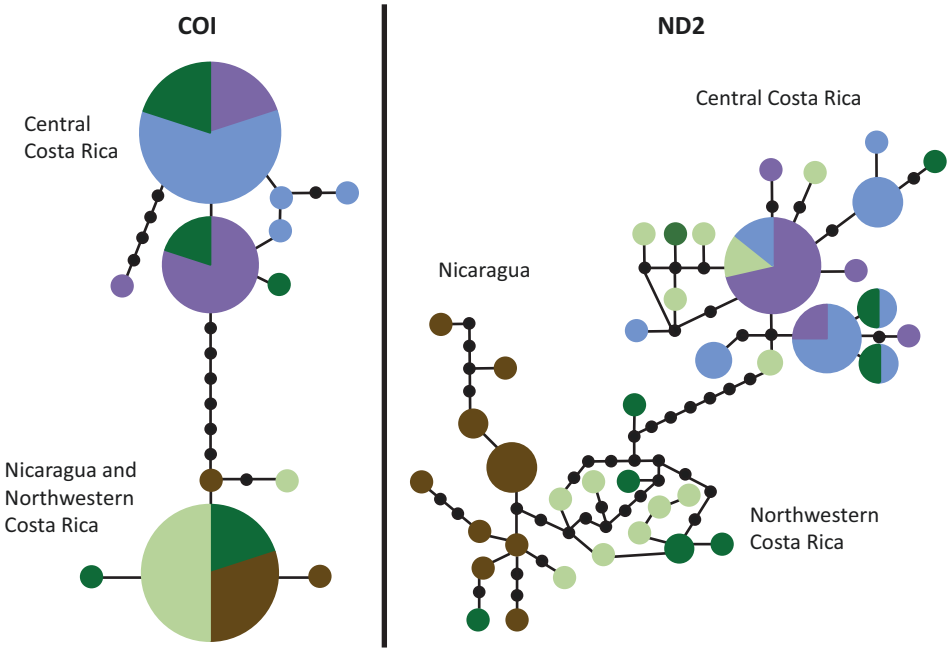

FIGURE 2. Statistical parsimony network showing the relationship between 34 individuals from 5 populations using a 914-bp sequence of the COI gene (A; at left), and 57 individuals using a 1,041-bp sequence of the ND2 gene (B; at right). Colored circles represent the number of individuals with the same haplotype, whereas the small black circles represent inferred/missing haplotypes.

sequences, but relatively low for MYC and FIB 4 sequences (Supplementary Material Table S1).

Three of 50 (6%) locus × population comparisons showed departures from HWE, while only 1 of 225 (0.004%) locus × population comparisons showed evidence of linkage disequilibrium. Two of the 3 locus × population combinations that were not in HWE were found at Santa Rosa; to ensure that departures from HWE were not driving the observed patterns, we performed our analysis with all 10 loci and then repeated the analyses without the 2 loci that showed departures from HWE at Santa Rosa (*ThPl-14* and *ThPl-30*). We used the full microsatellite dataset for all analyses, given that removal of these loci did not change our results. We found no differences in genetic diversity among populations; allelic richness (mean: 6.19 ± 0.42; $\chi^2 = 0.47$, $P = 0.98$; Table 1), H_o (0.58 ± 0.04; $\chi^2 = 0.90$, $P = 0.92$), and H_e (0.65 ± 0.04; $\chi^2 = 0.93$, $P = 0.92$) were comparable across populations. Furthermore, F_{is} was relatively low and comparable across populations (0.10 ± 0.02; $\chi^2 = 4.06$, $P = 0.40$).

Pairwise F_{ST}, F'_{ST}, and θ_{ST} comparisons indicated distinct population differentiation among 5 populations along a transect in Nicaragua and Costa Rica. All 10 of our F_{ST} pairwise comparisons, and 8 of 10 θ_{ST} pairwise comparisons, provide evidence of population differentiation (Table 2; F_{ST} values ranged from 0.03 to 0.10; θ_{ST} ranged from 0 to 0.81). The Nicaraguan population differed from all Costa Rican populations for both F_{ST} and θ_{ST} comparisons. Two pairwise comparisons (Monteverde vs. Central Valley, and Santa Rosa vs. Rincon) showed contrasting patterns between markers, and in both cases, θ_{ST} pairwise comparisons were not different for these 2 pairs of populations (θ_{ST} equaled 0.02 and 0, respectively).

The results of our AMOVA suggest that populations form 3 separate mitochondrial groups ($F_{CT} = 0.50$, $P < 0.001$; Table 3); Nicaragua is distinct from all Costa Rican populations, while Rincon and Santa Rosa group together (hereafter: "Northwestern Costa Rica") and the Central Valley and Monteverde group together (hereafter: "Central Costa Rica") as distinct mitochondrial groups. Using our microsatellite dataset, AMOVA also indicates 3 distinct genetic groups ($F_{CT} = -0.03$, $p = 0.01$); again Nicaragua is distinct from all Costa Rican populations, while Rincon and Santa Rosa group together and the Central Valley and Monteverde group together form distinct genetic clusters.

Our analysis using STRUCTURE also revealed population structure among populations of Rufous-and-white Wrens (Figure 3). Using the ΔK method suggests that $K = 3$ is the optimal K ($\Delta K = 84.39$); at $K = 3$, STRUCTURE recognized Santa Rosa and Nicaragua as separate clusters, and Rincon, Monteverde, and Central Valley as a single cluster. Hierarchical analysis of the third cluster (i.e. Rincon, Monteverde, and the Central Valley) using STRUCTURE revealed that $K = 2$ was the optimal K ($\Delta K = 0.48$), separating Rincon as a unique genetic cluster from Monteverde and the Central Valley. In our initial

TABLE 2. Population pairwise F_{ST}/F'_{ST} (below diagonal) and θ_{ST}/geographic distance (above diagonal) for 5 Rufous-and-white Wren (*Thryophilus rufalbus*) populations.

	Nicaragua	Santa Rosa	Rincon	Monteverde	Central Valley
Nicaragua	–	0.39/281 km	0.41/297 km	0.77/372 km	0.81/438 km
Santa Rosa	0.05/0.14	–	0/29 km	0.29/82 km	0.31/182 km
Rincon	0.05/0.14	0.04/0.12	–	0.32/110 km	0.35/155 km
Monteverde	0.09/0.27	0.07/0.21	0.04/0.13	–	0.02/73 km
Central Valley	0.10/0.31	0.10/0.29	0.06/0.19	0.03/0.10	–

TABLE 3. Hierarchical AMOVA describing population structure for Rufous-and-white Wren populations in central America based on mtDNA ND2 gene and microsatellite results; *K*, number of groups tested for each analysis; F_{CT}, variation among groups; F_{SC}, variation among populations; $F_{ST,}$ variation within populations; $F_{IS,}$ variation among individuals.

Groups	*K*	Variation (%)	Fixation index	*P*-value
mtDNA				
Group 1: *Nicaragua* vs. Group 2: *Central Valley, Monteverde, Rincon, and Santa Rosa*	2	46.0	$F_{CT} = 0.46$	<0.001
		14.0	$F_{SC} = 0.26$	<0.001
		39.9	$F_{ST} = 0.60$	<0.001
Group 1: *Nicaragua, Rincon de la Vieja, and Santa Rosa* vs. Group 2: *Central Valley and Monteverde*	2	35.6	$F_{CT} = 0.36$	<0.001
		17.2	$F_{SC} = 0.27$	<0.001
		47.2	$F_{ST} = 0.53$	<0.001
Group 1: *Nicaragua* vs. Group 2: *Central Valley, and Monteverde* vs. Group 3: *Rincon, and Santa Rosa*	3	49.9	$F_{CT} = 0.50$	<0.001
		0.7	$F_{SC} = 0.01$	<0.001
		49.5	$F_{ST} = 0.51$	<0.001
Microsatellites				
Group 1: *Nicaragua* vs. Group 2: *Central Valley, Monteverde, Rincon, and Santa Rosa*	2	0.0	$F_{CT} = 0.00$	0.796
		5.6	$F_{SC} = 0.06$	0.001
		16.5	$F_{ST} = 0.05$	0.001
		77.9	$F_{IS} = 0.18$	0.001
Group1: *Nicaragua* vs. Group 2: *Central Valley, and Monteverde* vs. Group 3: *Rincon, and Santa Rosa*	3	2.6	$F_{CT} = 0.03$	0.001
		3.4	$F_{SC} = 0.04$	0.001
		16.4	$F_{ST} = 0.06$	0.001
		77.6	$F_{IS} = 0.18$	0.001
Group 1: Nicaragua vs. Group 2*: Central Valley, Monteverde, and Rincon;* Group 3: *Santa Rosa*	3	1.5	$F_{CT} = 0.02$	0.001
		4.1	$F_{SC} = 0.04$	0.001
		16.5	$F_{ST} = 0.06$	0.001
		77.9	$F_{IS} = 0.18$	0.001

analysis ($K = 3$), 18 of 211 (8.5%) individuals were assigned to another cluster outside of their home cluster ($Q > 0.5$), and 5 individuals showed evidence of admixture (where the maximum Q to one cluster <0.50). Taken together, these results suggest potential gene flow among populations.

Gene Flow

Our genetic divergence analysis suggests that Nicaragua and Central Costa Rica diverged from each other ~160,000 years ago (95% highest posterior parameter lower and upper bound (HPD): 43,885–1,139,293). By comparison, estimates suggest that Northwestern Costa Rica diverged recently from both Central Costa Rica and Nicaragua, ~38,000 years ago (95% HPD: 10,829–1,138,185) and 16,500 yr ago (95% HPD: 2,850–1,139,293), respectively.

We found limited evidence of contemporary gene flow among Rufous-and-white Wren populations based on our analysis of microsatellite data in BAYEASS+ (Figure 4). The majority of estimates (15 of 20) were very low (<0.03), with only a single comparison distinguishable from estimates generated with uninformative data. Our estimates indicate that there is high gene flow from Monteverde into the Central Valley (0.24 ± 0.04). Overall, our results suggest that dispersal events are difficult to detect in our dataset, given the short time period when samples were collected.

A relatively small percentage of the observed genetic variation was explained by geographic distance (2.8%) and habitat connectivity (2.1%) in our full model examining both males and females (Table 4). The percent of genetic variation explained by geographic distance and habitat connectivity slightly decreased, although it remained low (1.6% and 0.9%, respectively) for our partial redundancy models, which accounted for each variable. Sex (0.5%) explained a small portion of population genetic variation. Separate analyses of the sexes showed similar results to

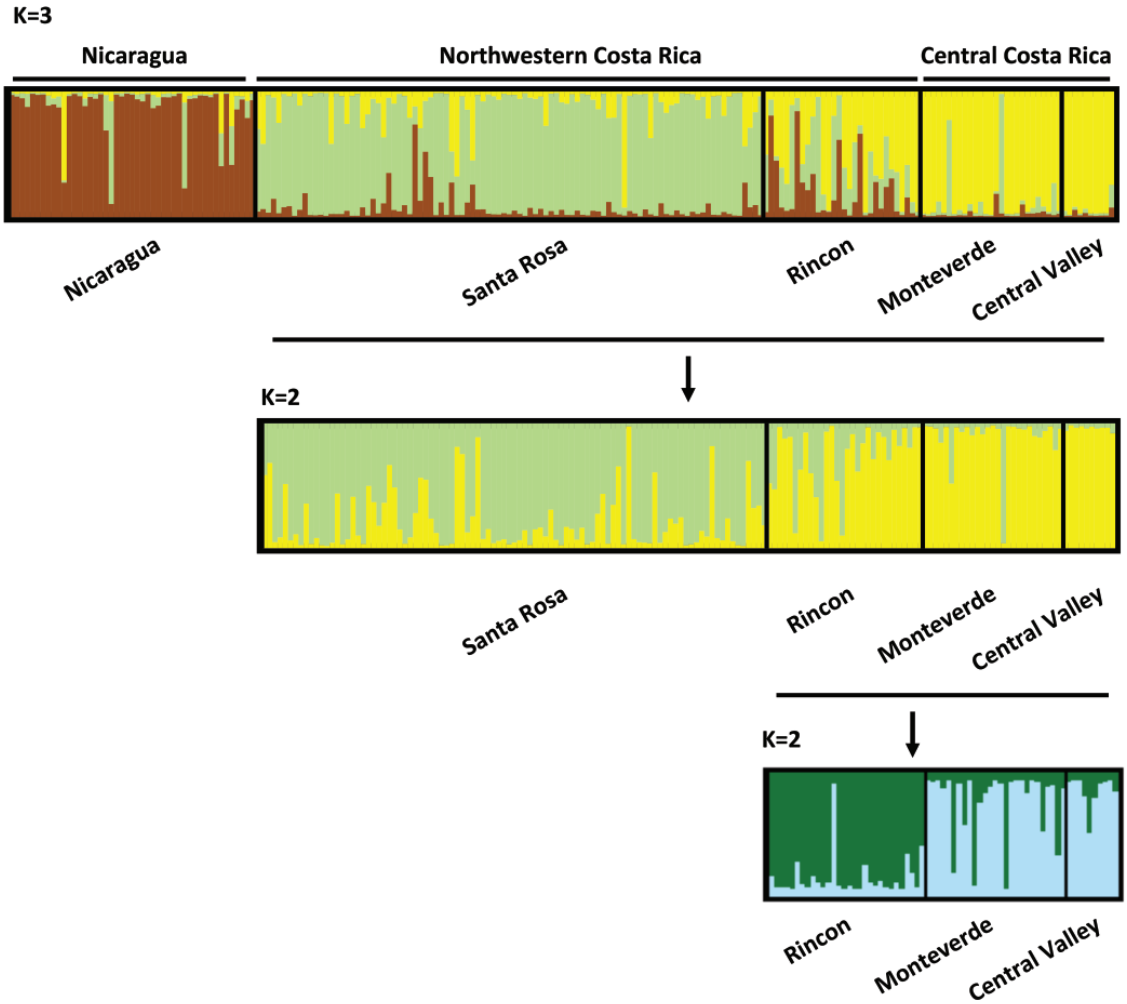

FIGURE 3. (*Top*) Individual membership probabilities for 211 Rufous-and-white Wrens (*Thryophilus rufalbus*) from 5 populations in Nicaragua and Costa Rica analyzed in STRUCTURE at $K = 3$. (*Bottom*) Hierarchical analysis of STRUCTURE ($K = 2$) for Santa Rosa, Rincon, Monteverde, and Central Valley ($n = 1,674$), and for Rincon, Monteverde, and Central Valley ($N = 67$). MtDNA groups are listed above the STRUCTURE histogram, as a reference for similarities and differences between microsatellite and mtDNA patterns.

our full model; geographic distance and habitat connectivity explained similar proportions of genetic variation for both males and females; however, our partial-redundancy models showed contrasting patterns between males and females; geographic distance and habitat connectivity accounted for a greater proportion of the genetic variation for females (2.6% and 1.6%, respectively) compared with males (2.1% and 1.2%, respectively).

DISCUSSION

In our genetic analysis of 5 populations of Rufous-and-white Wrens, we observed distinct population genetic differentiation and structure based on mtDNA and microsatellite data over a relatively short distance (~500 km). Contemporary analyses of gene flow, using microsatellite data, indicate that there is limited gene flow among most of the populations we examined. Analysis of mitochondrial and nuclear sequences revealed 2 distinct lineages that come into contact in northwestern Costa Rica, and divergence times indicate that these genetic lineages diverged from each other during the Pleistocene. Geographic distance and habitat connectivity accounted for a small portion of the observed genetic variation, indicating that other factors, including natal dispersal and drift, may be more important contributors to the observed genetic patterns, as has been reported for other tropical birds (Pérez-Emán 2005, Castoe et al. 2009, Cadena and Cuervo 2009, González et al. 2011, Lougheed et al. 2013). Overall, our results provide further evidence for deep genetic divergence in populations of tropical birds.

Analysis of mitochondrial and nuclear genes revealed 2 distinct clades, and secondary contact between these 2 clades, in northwestern Costa Rica. Divergence times

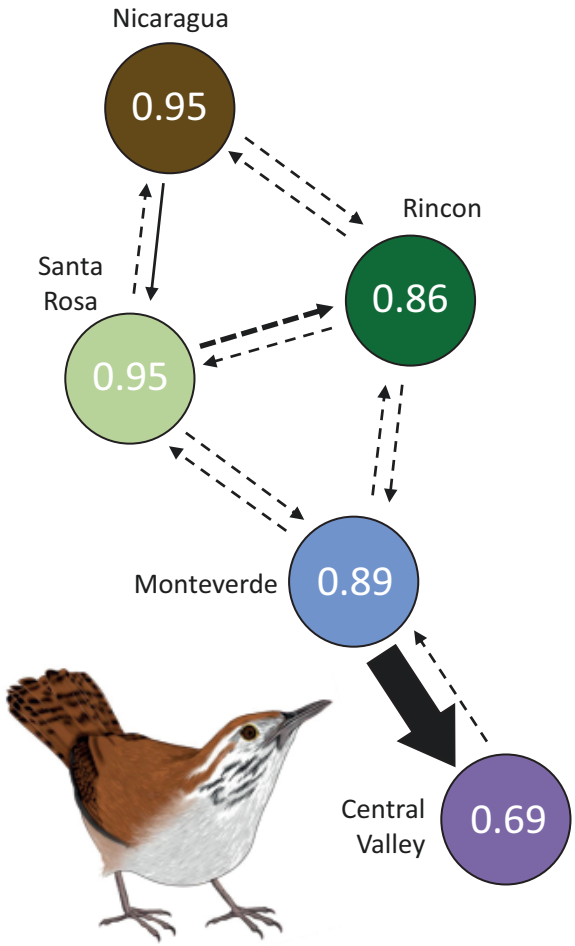

FIGURE 4. Estimates of gene flow among the 5 populations using microsatellite markers. Thin lines represent migration estimates < 0.05; medium line indicates migration rates between 0.05 and 0.10; thick line indicates migration rates between 0.20 and 0.25. Values on each circle represent the proportion of individuals recruited within each population. For simplicity, we only show estimates between adjacent populations. For all comparisons not shown, migration estimates are <0.03. Dashed lines indicate estimates that were not distinguishable from estimates generated with uninformative data, whereas bold lines indicate lines that were distinguishable from estimates generated with uninformative data.

between clades (~162 kya) coincide with Pleistocene climate changes, when temperatures cooled and conditions were drier, especially in the lowland tropical regions where forested habitats were replaced with dry open "savannah" habitat (Piperno and Jones 2003). Rufous-and-white Wren populations became isolated, as a result of Pleistocene glaciations, due to habitat fragmentation, and secondary contact between the 2 clades has occurred more recently in northwestern Costa Rica. Populations in northwestern Costa Rica separated from central Costa Rica during the last glacial maximum (~40 kya), whereas they became separated from Nicaragua at the end of the last glacial maximum (~16.5 kya) when forested habitat returned to the lowlands of Central America (~20 kya; Piperno and Jones 2003). Our samples were collected exclusively from the known distribution range of the *castanonotus* subspecies. The *rufalbus* subspecies, found northwest of our most northerly sampling location, is genetically distinct from the *castanonotus* subspecies (Lara et al. 2012), and introgression from *rufalbus* populations into Nicaragua could contribute to the genetic structure within the populations we sampled. Further research is required to delineate genetic structure and differentiation across all subspecies of Rufous-and-white Wrens.

Microsatellite analyses indicate substantial contemporary genetic divergence among Rufous-and-white Wren populations. Similar to the mtDNA genetic patterns, populations in Nicaragua and central Costa Rica (Monteverde and the Central Valley) are distinct from each other, but we also found population differentiation between populations within northwestern Costa Rica. Genetic differentiation between Santa Rosa and Rincon occurs despite the close proximity of these populations (these populations are 30 km apart), although similar patterns of genetic variation over relatively small areas have been revealed for many tropical species (Bates 2002, Francisco et al. 2007, Chavarría-pizarro et al. 2010, Woltmann et al. 2012a, Caro et al. 2013, Vangestel et al. 2013, Husemann et al. 2015, Del-Rio et al. 2021).

Santa Rosa represents a lowland population (~300 meters above sea level [m.a.s.l.]), whereas the remaining 3 Costa Rica populations represent higher elevation populations (>1,000 m.a.s.l.). Recent work has highlighted the differences between lowland and highland populations, where breaks in habitat and fragmentation have been shown to limit dispersal (Francisco et al. 2007; but see Van Houtan et al. 2007). The Santa Rosa population is located in Neotropical Dry Forest, and prior to conservation efforts, this area was fragmented from other forested areas. Therefore, the biogeographic history of this area may explain population genetic patterns. Other studies (Caro et al. 2013) have suggested that genetic differentiation may arise due to separate colonization events, and that behavioral barriers (such as acoustic differences) may lead to reproductive isolation between populations. It is noteworthy that we have observed acoustic differences between these Costa Rican populations (Graham et al. 2018a), although a previous playback study revealed that birds from other Costa Rican populations do respond to song playback of Santa Rosa Wren songs (Hick et al. 2015).

Both geographic distance and habitat connectivity explained population genetic distance, but both factors accounted for a low proportion of the observed genetic variation. Several studies have found that isolation by distance and habitat fragmentation influence population genetic patterns for tropical bird species (Arguedas and

TABLE 4. Results of Redundancy and partial-redundancy models examining the relationship between genetic variation and geographic distance, habitat connectivity, population, and sex for Rufous-and-white Wrens (*Thryophilus rufalbus)*. Inertia represents the amount of variation in the dataset; $\%R^2$ represents the proportion of variance explained by a variable; *F* represents the calculated *F* score; *P* represents the *P*-value.

	Females and males combined				Females				Males			
Variable	Inertia	$\%R^2$	*F*	*P*	Inertia	$\%R^2$	*F*	*P*	Inertia	$\%R^2$	*F*	*P*
Geographic distance	3.23	2.84	6.11	0.001	1.62	3.58	3.05	0.001	2.06	3.05	3.89	0.001
Habitat connectivity	2.41	2.11	4.52	0.001	1.14	2.53	1.14	0.001	1.49	2.20	2.78	0.001
Geographic distance | habitat connectivity	1.83	1.61	3.47	0.001	1.19	2.64	2.25	0.001	1.39	2.06	2.64	0.001
Habitat connectivity | geographic distance	1.00	0.90	1.91	0.001	0.72	1.59	1.36	0.001	0.82	1.21	1.55	0.001
Sex	0.55	0.48	1.01	0.446								
Geographic distance | Sex	3.21	2.81	6.05	0.001								
Habitat connectivity | Sex	2.39	2.09	4.47	0.001								

Parker 2000, Francisco et al. 2007, Woltmann et al. 2012a), although others have found a limited relationship between geographic distance and genetic variation (Peterson et al. 1993, Brown et al. 2004). One reason for the limited influence of geographic distance is that natal dispersal is limited in tropical birds; many bird species establish breeding territories relatively short distances from their natal territories (Martin and Bucher 1993, Woodworth et al. 1998, Sharp et al. 2008, Woltmann et al. 2012b). Both natal dispersal and genetic drift are viewed as key components of speciation in birds, with the effects of genetic drift on genetic differentiation especially enhanced when gene flow becomes restricted (Smith et al. 2014).

Limited dispersal distances have been described for diverse wren species (Arguedas and Parker 2000, Robinson 2000, Yáber and Rabenold 2002, Gill and Stutchbury 2010, Barr et al. 2015), and our pairwise genetic comparisons, Bayesian STRUCTURE results, and analyses of gene flow with microsatellite markers all indicate that Rufous-and-white Wrens exhibit limited dispersal. Dispersal appears to play an important role in the genetic differentiation of populations within this species, given that studies within the same region of bird species with greater dispersal behavior than wrens have observed comparably lower genetic differentiation (McDonald et al. 2001, Wright et al. 2005). Previous long-term analysis of natal and breeding dispersal for Rufous-and-white Wrens at the Santa Rosa population found that, on average, birds disperse ~1.3 km from natal territories, postnatal dispersal is infrequent, and that these movements between breeding territories are short (birds usually moved to new breeding territories just 1 or 2 territories away; Graham et al. 2017). In the current study, we show that gene flow among populations is relatively low, although we did observe substantial gene flow between Monteverde and the Central Valley, and this likely reflects the recent expansion of Rufous-and-white Wrens into this area (Sandoval 2004). Taken together, our previous findings and our current findings indicate that high site fidelity, together with limited natal and postnatal dispersal, limits gene flow among populations, which in turn leads to high population differentiation among Rufous-and-white Wrens, even across relatively short distances.

We found no differences in population genetic patterns between males and females. Although dispersal is female-biased for Rufous-and-white Wrens (Graham et al. 2017), as in many bird species (Greenwood 1980; Greenwood and Harvey 1982), geographic distance and habitat connectivity exhibited a comparable influence on genetic patterns for both sexes. Long-term analysis at Santa Rosa indicates that females disperse over twice as far as males from natal territories; however, these movements are still small-scale, local movements (males: 675 ± 190 m; females: 1644 ± 397 m; Graham et al. 2017). In the context of these previous and current analyses, distance and habitat connectivity exhibit a comparable influence on males and females because both sexes disperse relatively short distance from natal territories and dispersal beyond natal populations is infrequent (Graham et al. 2017, Woodworth et al. 2018).

Our detailed genetic analyses provide insight into the population genetic differentiation and phylogeography of Rufous-and-white Wrens. Although this species has been included in many phylogenetic studies to resolve taxonomic relationships between wren species, little was known about the genetic variation within this species. Our investigation was conducted across a limited part of this species' geographic range but demonstrates the high level of genetic divergence present within this species as well as the magnitude of genetic differentiation that can occur across short distances. Our findings provide a framework for future genetic studies of Rufous-and-white Wrens to examine

genetic patterns across their full range. We found evidence for limited gene flow between populations, and we suggest that this pattern is likely due to Rufous-and-white Wrens exhibiting limited natal dispersal and strong philopatry to breeding populations. Overall, natal dispersal appears to be one of the key factors promoting genetic divergence for this species, although geographic distance and habitat connectivity likely influence genetic variation for this species as well as others. Given the persistent fragmentation of many tropical habitats, it is likely that deforestation as well as other human-mediated landscape changes will influence many aspects of the life history, ecology, and evolution of terrestrial species. We hope that the findings of this study highlight the biodiversity in tropical habitats and demonstrate the value of using genetic markers to quantify biodiversity in the tropics, especially for those species that show limited phenotypic variation across their range.

SUPPLEMENTARY MATERIAL

Supplementary material is available at *Ornithology* online.

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Ethics statement: This research was reviewed and approved by the Animal Care Committee at the University of Windsor and the Government of Costa Rica (MINAE).

Author contributions: B.A.G. conceived of and designed the study. B.A.G. conducted all genetic analyses in collaboration with D.D.H. and R.P.W. B.A.G., M.M., and D.J.M. collected field data. B.A.G. wrote the manuscript in collaboration with D.J.M., and B.A.G., D.D.H., R.P.W., P.C.P., and D.J.M. shared in the editing and revising the manuscript.

Conflict of interest statement: The authors have no conflict of interest to declare.

Data availability: Analyses reported in this article can be reproduced using the data provided by Graham et al. (2022). All ND2 and COI sequences have been deposited in GenBank (ND2 Accession number: ON938107–ON938163; COI Accession number: 870805–ON870838).

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