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DEMOGRAPHIC HISTORY OF THE ORANGE-FRONTED PARAKEET (*EUPSITTULA CANICULARIS*) IN MEXICO

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Abstract · Molecular analyses can contribute to an understanding of the present and past demography of a species. In this study, we analyzed genetic diversity, intraspecific divergence patterns and historical demography of the Orange-fronted Parakeet (*Eupsittula canicularis*) using molecular data obtained from biological samples collected on the Pacific slope of México from Sinaloa to Guerrero. In addition, we analyzed the possible effect of Quaternary climatic changes on the population of this species. Based on genetic differentiation, and genealogical relationships analysis, we identified three genetic groups with overlapping geographical distributions on the north coast of Michoacán. Apparently, an ancestral group in the Balsas Basin underwent diversification and range expansion, initially towards the north coast of Michoacán and later northward along the Pacific slope to Sinaloa, and southward to the coast of Guerrero. Our results of skyline plot analysis suggest that a population expansion occurred during the Upper Pleistocene. The present analysis contributes to the knowledge of the phylogeographic pattern of *E. canicularis* in the tropical dry forest of western México, and identifies the Balsas Basin as an important center of diversification for the species.

Resumen · Historia demográfica del Periquito Frente-naranja (*Eupsittula canicularis*) en México
Los análisis moleculares pueden contribuir a la comprensión de la demografía actual e histórica de una especie. En este estudio analizamos la diversidad genética, el patrón de divergencia intraespecífica y la demografía histórica del Periquito Frente-naranja (*Eupsittula canicularis*) utilizando datos moleculares obtenidos de muestras biológicas colectadas en la vertiente del Pacífico desde Sinaloa hasta Guerrero, México. Además, analizamos el posible efecto de los cambios climáticos del Cuaternario sobre la población de esta especie. Con base en el análisis de diferenciación genética y relaciones genealógicas, identificamos tres grupos genéticos que sobrelapan su distribución geográfica en la costa del norte de Michoacán. Aparentemente, un grupo ancestral con distribución en la Cuenca del Balsas sufrió diversificación y expansión de rango hacia la costa norte de Michoacán y por la vertiente del Pacífico hacia el norte hasta Sinaloa, y hacia el sur hasta la costa de Guerrero. Los resultados de los análisis de "skyline plot" sugieren una expansión de la población que ocurrió durante el Pleistoceno superior. El presente análisis contribuye al conocimiento del patrón filogeográfico de *E. canicularis* en el bosque tropical seco del oeste de México, e identifica a la cuenca del Balsas como un importante centro de diversificación para la especie.

Key words: Demographic history · *Eupsittula canicularis* · Genealogic analyses · Genetic differentiation · Genetic diversity · Psittacidae · Quaternary · Tropical deciduous dry forest

INTRODUCTION

Based on patterns of distribution and diversity of birds in México, hypothesized factors promoting biodiversity include the following: 1) Historical processes, such as climatic oscillations and habitats refuge; 2) Variety of environments available due to topographical complexity; and 3) Confluence of birds from Nearctic and Neotropical lineages (Escalante-Pliego et al. 1993).

During the Quaternary period (2.58 million years ago until present), climatic oscillations occurred with alternating warmer and cooler conditions. For the last 900,000 years, these large global glacial-interglacial climate oscillations have been recurring with a periodicity of approximately 100,000 years (Berger et al. 1993, Mudelsee & Schulz 1997). These oscillations are proposed as one of the primary factors that influenced the distribution and population dynamics of species and as consequence, the patterns of genetic diversity observed in extant populations (Hewitt 2000, 2004b; Provan & Bennett 2008, Stewart et al. 2010).

In México, the tropical dry forest (TDF) is one of the main types of vegetation (Rzedowski 1978). The TDF extends continuously from 0 to 1900 m a.s.l. along the Pacific slope, from the center of Sonora and southeast of Chihuahua, to Chiapas. It co-

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vers areas of the Balsas and Santiago River Basins and in patches in the extreme south of Baja California, the south of Tamaulipas, southeast of San Luis Potosí, the north and center of Veracruz, northeast of Querétaro, and northern part of the Yucatán Peninsula (Rzedowski 1978). Due to the diversity and richness of species identified in the TDF of México, it has been proposed that the region can be divided into biotic provinces. For example, Becerra (2005) subdivides the TDF into 10 biotic provinces (or subareas) based on previous studies and the distribution pattern of plant species of the genus *Bursera* (Burseraceae). Based on the pattern of distribution and endemism of the birds of México, Escalante-Pliego et al. (1993) propose a subdivision into 35 biotic provinces.

In birds, Pleistocene environments are thought to have promoted genetic diversification and initiated phylogeographic splits in a number of clades (Avise & Walker 1998, Avise 2000, Johnson & Cicero 2004). With the use of molecular data, the origins of current biological diversity can be inferred through the analysis of genetic diversity and the reconstruction of phylogenetic patterns and demographic histories (Hewitt 2004a, Schmitt 2007, Provan & Bennett 2008). Based on DNA data, the pronounced phylogeographic divergences within avian species usually occurred within the last two million years (Avise & Walker 1998, Johnson & Cicero 2004). For birds that inhabit the highlands of México, genetic differentiation among populations revealed by phylogeographic studies may have resulted from habitat fragmentation during climatic changes of the Pliocene and Pleistocene (e.g., Common Bush-tanager *Chlorospingus ophthalmicus*, García-Moreno et al. 2004, Bonaccorso et al. 2008, Maldonado-Sánchez et al. 2016; Emerald Toucanet *Aulacorhynchus prasinus*, Puebla-Olivares et al. 2008; Wedge-tailed Sabrewing *Campylopterus curvipennis*, González et al. 2011). In some species, geographical barriers have been identified that limit the gene flow between populations (for example: Amethyst-throated Hummingbird *Lampornis amethystinus*, Cortés-Rodríguez et al. 2008b, Ornelas et al. 2016; Spot-crowned Woodcreeper *Lepidocolaptes affinis*, Arbeláez-Cortés et al. 2010; Azure-crowned Hummingbird *Amazilia cyanocephala*, Rodríguez-Gómez et al. 2013).

However, for lowland species inhabiting TDF in México, very little information is available. In some taxa, different phylogeographic patterns have been observed. For example, in the Streak-backed Oriole (*Icterus pustulatus*) a shallow divergence with a weak geographical structure attributed to a recent population expansion has been reported (Cortés-Rodríguez et al. 2008a). In the Rufous-naped Wren (*Campylorhynchus rufinucha*), genetically differentiated and geographically structured populations have been observed (Vázquez-Miranda et al. 2009). In the Mexican Sheartail (*Doricha eliza*), genetic differentiation among groups was attributed to a period of isolation by distance and divergence in different environmental conditions (Licona-Vera & Ornelas 2014). Finally, in studies of Golden-cheeked Woodpeker (*Melanerpes chrysogenys*), Orange-breasted Bunting (*Passerina leclancherii*), Russet-crowned Motmot (*Momotus mexicanus*), and Golden Vireo (*Vireo hypochryseus*), where phylogeographic structure was found, patterns may be associated with Pleistocene climatic fluctuations that may have isolated populations by fragmenting and reconnecting the TDF (Arbeláez-Cortés et al. 2014a, 2014b).

Twenty-one species of psittacines inhabit México and are distributed primarily in the south and on Atlantic and Pacific slopes (Forshaw 1989, Escalante-Pliego et al. 1993, Howell & Webb 1995, Chesser et al. 2014). The main vegetation zones they inhabit are the TDF on the Pacific slope and the evergreen tropical forest on the Atlantic slope, although they can also be found in tropical deciduous forest, wet and semi-arid pine-oak forest, pine forest, and mangrove, among others (Rzedowski 1978, Forshaw 1989, Escalante-Pliego et al. 1993, Howell & Webb 1995, Chesser et al. 2014). Studies of Mexican psittacines using molecular data have mainly focused on phylogenetic relationships among a few taxa (Eberhard et al. 2004, 2015; Russello & Amato 2004, Tavares et al. 2006, Latta et al. 2010, Kirchman et al. 2012, Smith et al. 2013). So far, only one study has analyzed the distribution of the genetic diversity of a psittacine (Military Macaw *Ara militaris*) in México (Rivera-Ortiz et al. 2016). Using microsatellites, the study found a genetic break between localities separated by the Central Plateau and the Trans-Mexican Volcanic Belt, suggesting that these features serve as geographic barriers for the species (Rivera-Ortíz et al. 2016).

The Orange-fronted Parakeet (*Eupsittula canicularis*) is strongly associated with the TDF of the Mexican Pacific. Its distribution is continuous along the Pacific slope from southern Sinaloa, México to northern Costa Rica (Forshaw 1989, Howell & Webb 1995, Collar et al. 2000). Although the TDF is preferred by the species, this parrot can also be found in humid and subhumid deciduous forests, riparian forests, and agricultural areas (Ridgely 1981, Howell & Webb 1995, Stotz et al. 1996, Collar et al. 2000). Previously, a phylogenetic analysis and haplotype network of two populations of *E. canicularis* based on the mitochondrial DNA genes cytochrome oxidase I and NADH dehydrogenase II was performed. Using five samples of *E. c. eburnirostrum* from Michoacán and four of *E. c. clarae* from Sinaloa, the study revealed that these two morphologically defined subspecies corresponded to at least two distinct lineages proposed as Evolutionarily Significant Units (ESUs) (Padilla-Jacobo et al. 2016).

In the current study, we used mitochondrial sequence data to analyze the genetic diversity, genetic differentiation, and genealogic relationships of *E. canicularis* in its northernmost distribution in México, with the objective of identifying genetic groups and associating them with possible geographic barriers. Additionally, we used our data to infer the demographic history in order to determine whether demographic changes might have been associated with Quaternary climatic changes.

METHODS

Biological samples. Feather and blood samples collected from nestlings in 2005, 2007, and 2013–2016 were used. Each sample was geo-referenced, and samples were taken under collection permits SGPA/DGVS/06387 and SGPA/DGVS/01893/16. The samples were collected and preserved using the method described by Padilla-Jacobo et al. (2016) and deposited in the wildlife sample collection at the Multidisciplinary Center for Studies in Biotechnology (Centro Multidisciplinario de Estudios en Biotecnología, CMEB) at the Universidad Michoacana de San Nicolás de Hidalgo (UMSNH) in Morelia, Michoacán, México. We collected and analyzed samples from 56 individuals of *E. canicularis* from the Pacific

Table 1. Collection locations for samples of the Orange-fronted Parakeet (*Eupsittula canicularis*) included in this study. Map location numbers (Map loc.) correspond to sites marked on the map in Figure 1. The number of samples collected at each locality (N) and assigned sample identification codes are indicated as well.

Population	Locality	Map loc.	N	Samples
WCM	Sinaloa (Cosala)	1	4	Arca2005-42, Arca2005-45, Arca2005-48, Arca2005-49.
	Sinaloa (Culiacan, Imala)	2	7	Arca2005-01, Arca2005-20, Arca2005-21 Arca2005-22, Arca2005-27, Arca2005-28, Arca2005-29.
	Sinaloa (Mocorito, Badiraguato)	3	4	Arca2005-14, Arca2005-15, Arca2005-16, Arca2005-17.
WCS1	Jalisco (Puerto Vallarta)	4	3	Arca2016-01, Arca2016-02, Arca2016-03.
	Nayarit (Rincón de Guayabitos)	5	1	Arca2013-01.
WCS2	Michoacán (Palos Marías)	6	14	Arca2005-52, Arca2005-53, Arca2005-54, Arca2005-55, Arca2005-57, Arca2005-58, Arca2005-59, Arca2005-60, Arca2005-61, Arca2005-62, Arca2005-63, Arca2006-83, Arca2006-84, Arca2006-85.
	Michoacán (Chorumo, Zapotan)	7	5	Arca2005-64, Arca2005-65, Arca2005-70, Arca2005-74, Arca2005-76.
WCS3	Michoacán (Agua Cola, Zapotillo)	8	6	Arca2007-07, Arca2007-09, Arca2007-10, Arca2007-11, Arca2007-12, Arca2007-13.
WCS4	Guerrero (Tecpan, Atoyac)	9	6	Arca2007-17, Arca2007-18, Arca2007-19, Arca2007-20, Arca2007-21, Arca2007-22.
BBW	Michoacán (Huetamo)	10	5	Arca2014-01, Arca2014-03, Arca2014-04, Arca2014-05, Arca2014-06.
	Michoacán (Palma de Huaro)	11	1	Arca2005-78.
	Total		56	

slope from Sinaloa to Guerrero in México. The samples include representatives from the northernmost third of the species distribution (Table 1, Figure 1). In addition, samples from 80 individuals of unknown origin, confiscated by Mexican authorities and donated by the Procuraduría Federal de Protección al Ambiente (PROFEPA), were included in some analyses.

We assigned the field-collected samples to populations in accordance with the biotic provinces proposed by Escalante-Pliego et al. (1993) and according to the distribution of the vegetation of the region by Rzedowski (1978) as follows: for the population from Sinaloa, West Coast Middle (WCM); from the Jalisco and Nayarit coasts, West Coast South 1 (WCS1); from the north coast of Michoacán, West Coast South 2 (WCS2); from the Balsas Basin, West Coast South 3 (WCS3); from the coast of Guerrero, West Coast South 4 (WCS4); and from the Balsas Basin West (BBW) (Table 1, Figure 1). For the country, Escalante-Pliego et al. (1993) proposed 35 biotic provinces of which *E. canicularis* inhabits at least six. The localities situated in Sinaloa are in the center of province 22 (WCM). The ones identified in Nayarit-Jalisco are in the northern portion of province 21 (WCS1). The populations of the north coast of Michoacán are in the central part of province 21 and the localities of Guerrero are towards the south of the province 21. In particular, the samples of "Agua Cola" (WCS3) are in the limits of the province 21 and 24 (BBW). Finally, the localities sampled towards the interior of the continent are within the Balsas basin in the province 24. Although the WCS3 and BBW samples appear to be spatially close, they are separated by the complex called the Río Grande-Adolfo López Mateos Reservoir (Infiernillo dam).

DNA extraction, PCR amplification, and sequencing of markers. DNA was extracted using the phenol-free method described by FitzSimmons (1997). We amplified the DNA mitochondrial fragments of cytochrome oxidase I (COI), NADH dehydrogenase II (ND2), and cytochrome b (Cytb). The COI fragments from 44 individuals and the ND2 fragments from 37 individuals were amplified. A fragment of the COI gene was amplified using the primers COIarcaD and COIarcaR (Padilla-Jacobo et al. 2016), and a ND2 fragment was amplified using the primers L5215 and HTrpC (Hackett 1996). In addition, the sequences of the COI and ND2 genes of nine individuals sampled in a previous study were included in the analyses (Access: KJ612380-KJ612397) (Padilla-Jacobo et al. 2016). The Cytb amplification was performed for the field-collected samples from 53 individuals, and for 80 samples from confiscated individuals, using the primers CytbD (5'-ATCATCCGCACTATCTGTCCT-3') and CytbR (5'-AATAGCC-TTCGTCTTTTGGTTTA-3'). The Cytb primers were designed for this study using DNASTAR Lasergene software LG10VC (Kumar & Blaxter 2010), based on the Cytb sequences reported in GenBank for species of the genus *Eupsittula* and related genera.

The PCR reactions for amplification of COI and ND2 were carried out according to Padilla-Jacobo et al. (2016). The PCR reactions for amplification of Cytb were performed in a total volume of 25 µl as follows: 20 mM Tris-HCl pH 8.4, 50 mM KCl, 1.5 mM $MgCl_2$, 200 mM of each dNTP, 10 pmol of each oligonucleotide, 1.5 U Platinum Taq polymerase (Invitrogen, Grand Island, NY, USA), and 50 ng of DNA. The reaction mixtures were placed in a thermocycler (Gene Amp 2700; Applied Biosystems, Foster City, CA, USA) under the following conditions: 94°C for 5 min; followed by 30 cycles of 94°C for 40 s, 57°C for 40 s, and 72°C for 1.5 min; and a final extension at 72°C for 5 min.

The sequencing of both DNA strands was performed using the amplification primers and the dideoxy method

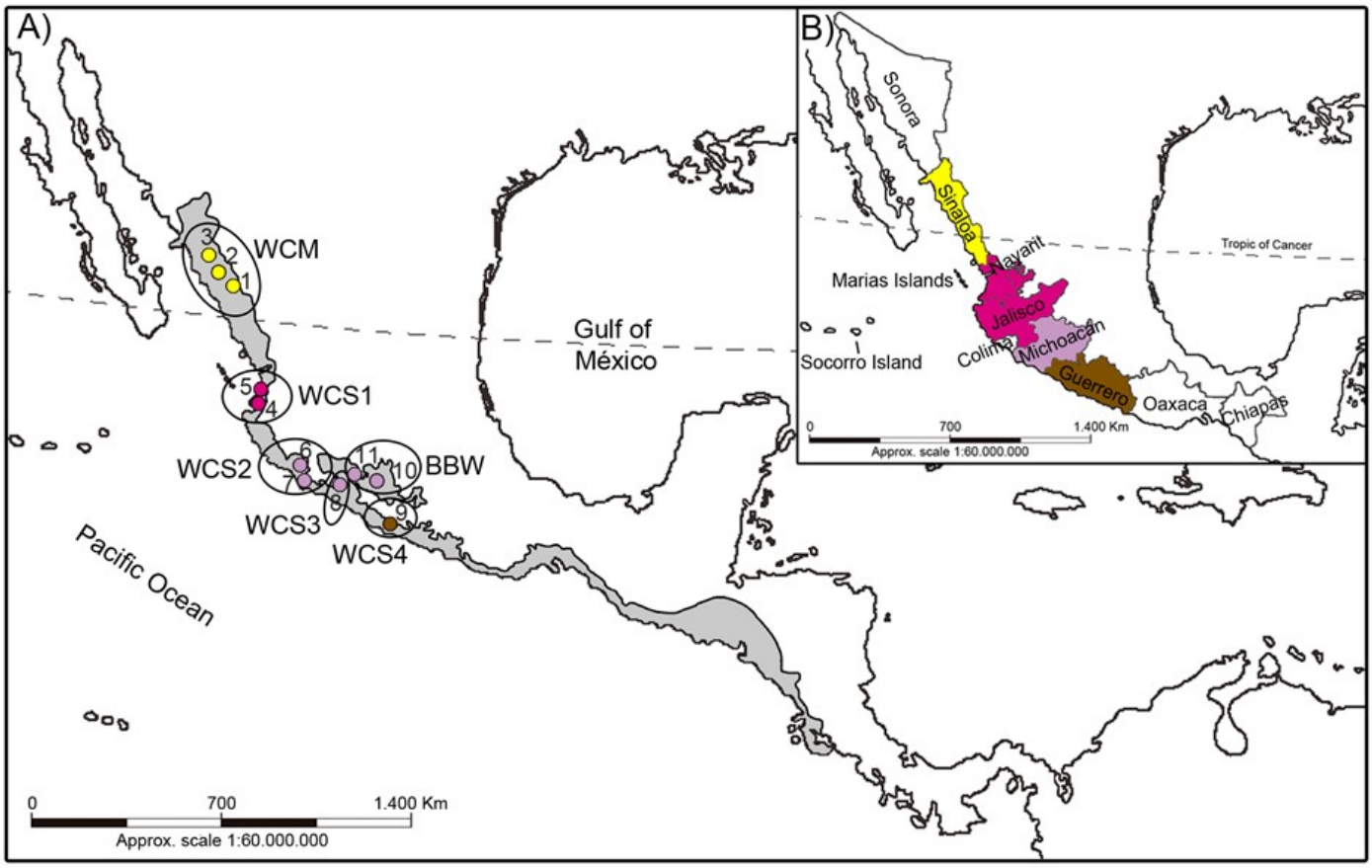

Figure 1. Geographic distribution and sampled regions of the Orange-fronted Parakeet (*Eupsittula canicularis*). A) The map is modified according to Monterrubio-Rico et al. (2016) and Collar (2017). The shaded areas represent the distribution of the species. Points represent the sampling locations. For the numbers and abbreviations of each point and group, see Table 1. B) Map of the political division of México, shows the states to the west of the country. The colored states show the collection region of the individuals considered in the present analysis.

(Sanger et al. 1977); service was provided by Macrogen (Rockville, MD, USA).

Sequence analysis. Sequence editing, alignments, and the construction of data matrices were conducted using Sequencher v.4.1 (Gene Codes Corporation, Ann Arbor, MI, USA) and PhyDE (Müller et al. 2005). The number of haplotypes (H), polymorphic sites (S), the nucleotide (Pi) and haplotype (Hd) diversity, and the neutrality test were estimated using ARLEQUIN v3.1 (Excoffier et al. 2005). In the analyses of genetic diversity, we used the samples from populations in the six regions consistent with the sampling locations (Table 1, Figure 1).

An analysis of molecular variance (AMOVA) (Excoffier et al. 2005) was carried out to determine if there was any geographic structure in the distribution of variation and genetic differentiation among the groups. The AMOVA (Excoffier et al. 2005) was performed with 10,000 permutations. To carry out the analysis, we grouped the samples as follows: 1) without *a priori* grouping, and 2) with samples grouped by their distribution in biotic provinces (Escalante-Pliego et al. 1993), North (WCM and WCS1), Balsas (WCS3 and BBW), and South (WCS2 and WCS4) (Table 1, Figure 1). Additionally, we computed pairwise comparisons of F_{ST} values with 1,000 permutations using ARLEQUIN v3.1 (Excoffier et al. 2005). To test whether the isolation-by-distance hypothesis could account for differentiation among the populations analyzed, we performed a Mantel correlation test using XLSTAT software (SARL 2017). For this test, we used genetic distance matrix and geographic Euclidean distance matrix among the populations.

Relationships among haplotypes and genealogic analyses. To examine frequencies of haplotypes and relationships among them, we used Cytb sequences from 53 of the field-collected samples to construct haplotype networks under the median-joining method with the software NETWORK v5 (Fluxus Technology Ltd. 2017).

Genealogic relationship reconstructions were generated using Maximum Likelihood (ML) and Bayesian Inference (BI) frameworks. The matrix for these analyzes included 53 sequences of COI, 46 of ND2, and 53 of Cytb from field-collected samples. We also included sequences of COI, ND2, and Cytb from two individuals of unknown origin deposited in Genbank (Access: Euca34417: KJ142289.1, KJ142251.1; and Euca9252: HQ629753.1, HQ629718.1). Sequences of *Eupsittula pertinax* (Access: HM640208.1) were included as the out-group. Molecular evolution models were estimated using jModelTest v2.1.1 (Posada 2008) and selected using the corrected Akaike Information Criterion (AICc) (Alfaro & Huelsenbeck 2006). The best model for COI was HKY (Hasegawa et al. 1985), for ND2, TrN+I (Tamura & Nei 1993 + Invariant sites) and for Cytb, TrN (Tamura & Nei 1993), and the best model for the concatenated sequences (COI + ND2 + Cytb) was TIM3+I+G (Transition model-Posada 2003 + Invariant sites + Gamma distribution).

The ML and BI reconstructions were performed using RAxML v7.8 (Stamatakis 2014) and MrBayes v3.2 (Ronquist &

Huelsenbeck 2003) software, respectively. The branch-support values were estimated by bootstrap analysis (BP) of 500 replicates and by posterior probabilities (PP). MrBayes runs were performed using the following parameters: four independent runs for each of four chains (one cold chain and three hot chains) for 10 million generations, sampling one tree every 1,000 generations. Trees and parameters were summarized after discarding 25% of the data (burn-in). The remaining trees were summarized as a majority consensus tree. Trees were visualized using FigTree v1.4.0 (Rambaut 2012).

Demographic history. To infer demographic history we used the Cytb dataset in a Bayesian skyline plot analysis (Drummond et al. 2005) and using mismatch distribution methods (Rogers & Harpending 1992). The Bayesian skyline plot (Drummond et al. 2005) analysis was performed using the program BEAST v1.7.4 (Drummond & Rambaut 2007). This analysis is sensitive to the number of samples. According to Grant (2015) sample sizes of individuals greatly affect power to reconstruct historical demographics; even analyses with 50 individuals often fail to capture a population expansion when it has occurred. For that reason, we included the 80 Cytb sequences obtained from samples collected from confiscated individuals. A haplotype network analysis revealed a close relationship between haplotypes of the confiscated individuals with those of field-collected samples from the northern Michoacán to southern Nayarit (Padilla-Jacobo et al. in prep.). We based our time estimates on the rate of 2.7×10^{-3} substitutions per site per million years for Cytb reported for Psittaciformes by Pacheco et al. (2011). Five independent runs with 30 million generations were performed. We used the TrN substitution model with empirical base frequencies; the clock model was uncorrelated, lognormal relaxed; and a piecewise-constant coalescent Bayesian skyline tree prior with 10 starting groups was used. Trees and parameters were sampled every 1000 iterations, with a burn-in of 10%. The results of every run were combined in LogCombiner v1.7.4 (Drummond & Rambaut 2007). Results and convergence testing for Bayesian analyses (ESS) were visualized using TRACER 1.5 (Rambaut & Drummond 2007). Mismatch distributions were obtained in ARLEQUIN v3.1 (Excoffier et al. 2005). The sudden expansion model (Schneider & Excoffier 1999) with 1000 parametric bootstraps was used. The sum of squared deviations (SSD) and Harpending´s raggedness index (Hri) were determined to evaluate the sudden expansion assumption.

RESULTS

Analyses of diversity and genetic differentiation. From the DNA samples of *E. canicularis*, 44 COI and 37 ND2 sequences of were obtained. Although we obtained amplification products of COI and ND2 from 44 biological samples, after sequencing seven sequences of ND2 were discarded due to their low quality. From the 53 DNA samples of field-collected individuals and 80 samples from confiscated individuals, 133 Cytb gene sequences of 994–1161 bp were obtained. All sequences were deposited in GenBank (Access: MF441253-MF441296, MF441297-MF441333, KU532328-KU532329, and MF441334-MF441470). To obtain the metrics of genetic diversity we used COI, ND2, and Cytb sequences from field-collected individuals. Also, we included the sequences of COI and ND2 genes from nine field-collected individuals in a previous study (Access: KJ612380-KJ612397) (Padilla-Jacobo et al. 2016). Table 2 shows the total number of samples per marker and the sequence lengths used in the alignments.

We detected 13 ND2, 14 COI, and 18 Cytb haplotypes, and when all samples were analyzed as a single group, a high diversity of haplotypes (Hd = 0.799–0.894) and low nucleotide diversity (Pi = 0.00198–0.00350) were found. Because our dataset was most complete for Cytb, we performed the analysis of genetic diversity for each population using this marker. All the populations exhibited more than one haplotype, even those with a small sample size (WCS1 and WCS3) (Table 3), and high haplotype diversity was detected in all populations, ranging from 0.648 (WCM) to 0.8 (BBW) (Table 3). The nucleotide diversity was low for all populations, with the lowest estimated for BBW (Pi = 0.00125) and the highest for WCS1 (Pi = 0.00268) (Table 3). When all samples were pooled, the haplotype diversity estimate was highest (Pi = 0.88) and the nucleotide diversity was lowest (Pi = 0.00216).

Pairwise comparison of F_{ST} values showed moderate to low differentiation among populations (Table 4). The comparison of Balsas Basin populations (BBW and WCS3) showed the lowest F_{ST} value (0.00557). Comparison between the Balsas Basin and Guerrero coast populations (WCS3 and WCS4) revealed the greatest differentiation (F_{ST} = 0.49735). In addition, the population of Sinaloa (WCM) presented low values of differentiation in comparison with the populations of the Jalisco, Nayarit, and northern Michoacán coasts (WCS1 and WCS2), and the greatest differentiation with a population in the Balsas Basin (WCS3). Finally, the population on the coast of Guerrero (WCS4) had low values of differentiation compared to that of the coast of Michoacán (WCS2) and moderate compared to the other populations (Table 4). According to the Mantel test, the obtained correlation index, r = 0.25 (P = 0.036) rejected the hypothesis of isolation by distance.

As is mentioned in methods, to carry out the AMOVA analysis, we grouped the samples as follows: 1) without *a priori* grouping, and 2) with samples grouped by their distribution in biotic provinces (Escalante-Pliego et al. 1993), North (WCM and WCS1), Balsas (WCS3 and BBW), and South (WCS2 and WCS4) (Table 1, Figure 1). Based on the results without *a priori* defined groups, 71% of the genetic variation was explained by the differences within populations and 29% by the differences among populations, suggesting genetic structure (Table 5A). When the populations were analyzed as the groups in the North (WCM and WCS1), Balsas Basin (WCS3 and BBW), and South (WCS2 and WCS4), the results indicated that most of the variation (70%) was explained by differences within populations. The difference among groups accounted for 10% of the variation, and the variation among populations within the groups accounted for 20%. The fixation index F_{CT} showed a moderate value (F_{CT} = 0.10439) indicating moderate genetic differentiation among groups (Table 5B).

Relationships between haplotypes and their geographical distribution. The haplotype network showed the relationships among the 18 haplotypes and their frequencies found in 53 individuals (Figure 2). In the network, the haplotypes are separated by few mutations and three haplogroups (HG) could be distinguished, HGI, HGII, and HGIII (Figure 2). HGI

Table 2. Genetic diversity indices obtained for the Orange-fronted Parakeet (*Eupsittula canicularis*). Nt = number of characters considered in the matrix; H = number of haplotypes; S = Polymorphic sites; Hd = Haplotype diversity; Pi = Nucleotide diversity; D_T = D-Tajima. * Statistically significant at $p < 0.05$.

Molecular marker	N	Nt	H	S	Hd	Pi	D_T (*p* value)
COI	53	567	14	13	0.894	0.00350	-0.9126 (0.182)
Cytb	53	1068	18	24	0.880	0.00216	-1.989 (0.0059)*
ND2	46	1010	13	16	0.799	0.00198	-1.4138 (0.051)

Table 3. Genetic diversity indices obtained for each population of the Orange-fronted Parakeet (*Eupsittula canicularis*). * Statistically significant at $p < 0.05$.

Population	N	H	S	Hd	Pi	D_T (*p* value)	Fu's F_S	Haplotypes
WCM	15	6	10	0.648	0.00163	-1.75529 (0.025)*	-1.82341	H1(9), H2(2), H3(1), H4(1), H5(1), H6(1)
WCS1	4	2	4	0.667	0.00268	2.08033 (0.983)	2.19722	H1(2), H18(2)
WCS2	19	6	5	0.784	0.00141	0.17000 (0.601)	-1.14469	H1(2), H7(4), H8(8), H9(2), H10(1), H11(2)
WCS3	4	3	3	0.833	0.00140	-0.75445 (0.246)	-0.28768	H7(2), H12(1), H13(1)
WCS4	5	3	5	0.700	0.00206	-0.56199 (0.383)	0.80363	H8(1), H14(3), H15(1)
BBW	6	4	4	0.800	0.00125	-1.29503 (0.077)	-1.25217	H7(3), H8(1), H16(1), H17(1)
Total	53	18	24	0.880	0.00216	-1.98938(0.0059)*	-10.937	

Table 4. Pairwise genetic differentiation (F_{ST}) between populations of the Orange-fronted Parakeet (*Eupsittula canicularis*).

	WCM	WCS1	WCS2	WCS3	WCS4	BBW
WCM	-					
WCS1	0.28505	-				
WCS2	0.18716	0.32021	-			
WCS3	0.42657	0.46154	0.27103	-		
WCS4	0.38787	0.39739	0.26119	0.49735	-	
BBW	0.33784	0.43071	0.11976	0.00557	0.43369	-

Table 5. Results of molecular variance analysis (AMOVA) for the Orange-fronted Parakeet (*Eupsittula canicularis*). A) without *a priori* defined groups, B) North (WCM and WCS1), Balsas (WCS3 and BBW), and South (WCS2 and WCS4).

Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation	Fixation index	*P*-value
A) Without *a priori* grouping						
Among populations	5	16.459	0.31347	28.87		
Within populations	47	36.296	0.77225	71.13	F_{ST} = 0.28872	0.000
Total	52	52.755	1.08572	100		
B) North/Balsas/South						
Among groups	2	10.003	0.11537	10.44	F_{CT} = 0.10439	0.068
Among populations within groups	3	6.456	0.21749	19.68	F_{SC} = 0.21975	0.000
Within populations	47	36.296	0.77225	69.88	F_{ST} = 0.30120	0.000
Total	52	52.755	1.10511	100		

included individuals of Sinaloa (WCM), Nayarit+Jalisco (WCS1), and Michoacán populations (WCS2) with a widely distributed dominant haplotype (H1) shared by 25% of individuals (Figures 1–3). Derived from H1, seven haplotypes were found, each separated by one to three mutational steps. The haplotypes in HGII were found exclusively in the populations of Michoacán coast and Balsas Basin (WCS2, WCS3, and BBW) (Figures 1–3), with a dominant haplotype (H7) and six derivatives separated by one or two mutational steps. The haplotype H7, shared by 17% of the samples, was the third most abundant and is mainly distributed on the Michoacán coast. In HGIII, the dominant haplotype H8 represented 19% of the samples and is in the population of the Michoacán and Guerrero coasts, and the Balsas Basin (WCS2, WCS4, and BBW), mainly on the coast of Michoacán (Figures 1–3). The HGI and HGII haplogroups showed the typical star-like genealogy that indicates a population in expansion (Bandelt et al. 1995).

Genealogic analysis. Phylogenetic analyses were performed to estimate the genealogical relationships between the haplogroups detected. The tree constructed under BI coin-

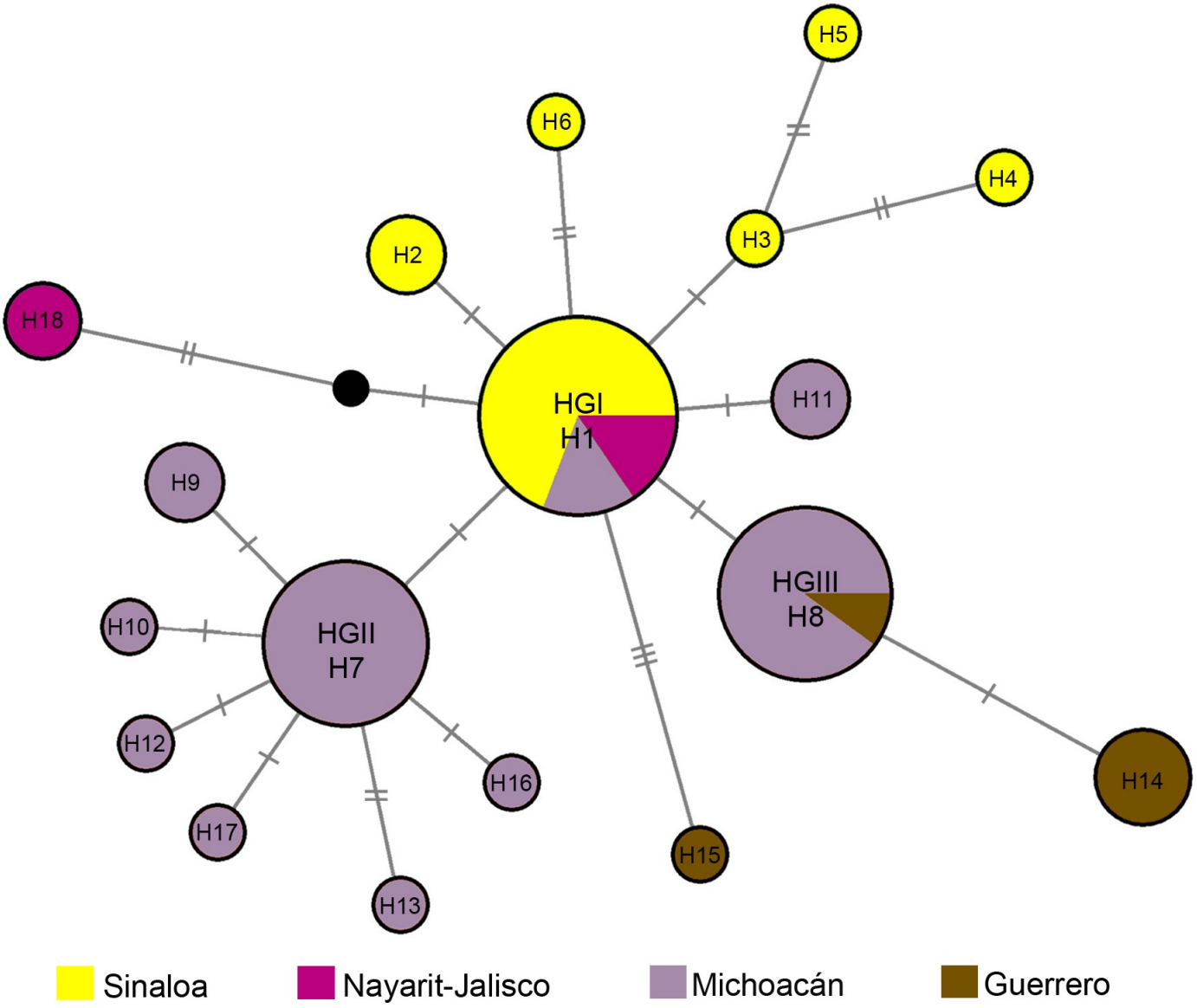

Figure 2. Median-joining haplotype network showing the relationships among 18 haplotypes of Cytb from 53 individuals of the Orange-fronted Parakeet (*Eupsittula canicularis*). The size of each circle is proportional to the haplotype frequency; the length of the branches is not proportional to the number of mutations; transverse lines on the branches represent mutations between haplotypes. Black circles are non-sampled haplotypes. HGI, HGII, and HGII correspond to haplogroups I, II and III, respectively.

cided with the topology obtained with ML. At the base of the consensus tree, we observed individuals who are reported in NCBI and have unknown origin (Figure 4). From their position in the tree, we propose that the places of origin of these individuals could be the south of México (Chiapas) or Central America. Four individuals from the haplogroup HGII and in the Balsas Basin West population (BBW) were sister taxa of the other individuals. The others, which represented most of the individuals analyzed, formed a large clade with a polytomy (CI) and four subclades. The SCI subclade included individuals from the haplogroup HGI in the network, who belong to the populations of Sinaloa (WCM, 15 individuals), Jalisco-Nayarit (WCS1, four individuals), the north coast of Michoacán (WCS2, seven individuals), and the Guerrero coast (WSC4, two individuals). SCI also included individuals from the haplogroup HGII, belonging to the populations of the Balsas Basin (WSC3, three individuals, and BBW, one individual). The inclusion of an individual (Arca2014-04) with the haplotype H7 in the subclade SCI, whose geographical origin is the Balsas Basin (BBW), can be attributed to the retention of ancestral polymorphisms (Avise et al. 1983, Freeland 2005). SCII comprised individuals of the dominant haplotype H7 (HGII) belonging to the Michoacán coast (WCS2). SCIII included individuals from the haplogroup HGIII, who belong to the populations in the Michoacán and Guerrero coasts (WCS2, nine individuals and WCS4, four individuals), although one from the Balsas Basin (BBW) was also included. The individual (Arca2005-78) with the haplotype H8, included in the SCIII subclade and belonging to the Balsas Basin (BBW), also revealed retention of ancestral polymorphisms in this population. SCIV included individuals of the haplotype H9 belonging to the Michoacán coast population (WCS2) (Figures 3, 4). In the polytomy (identified as CI), the samples Arca2007_10, Arca2007_11, and Arca2005_70 from the Balsas Basin population (WSC3) were also included.

Demographic history. The mismatch-distribution graph (Figure 5A) showed a positive asymmetric distribution representing groups with few mismatches, which is interpreted as the result of closely related lineages. The values recovered

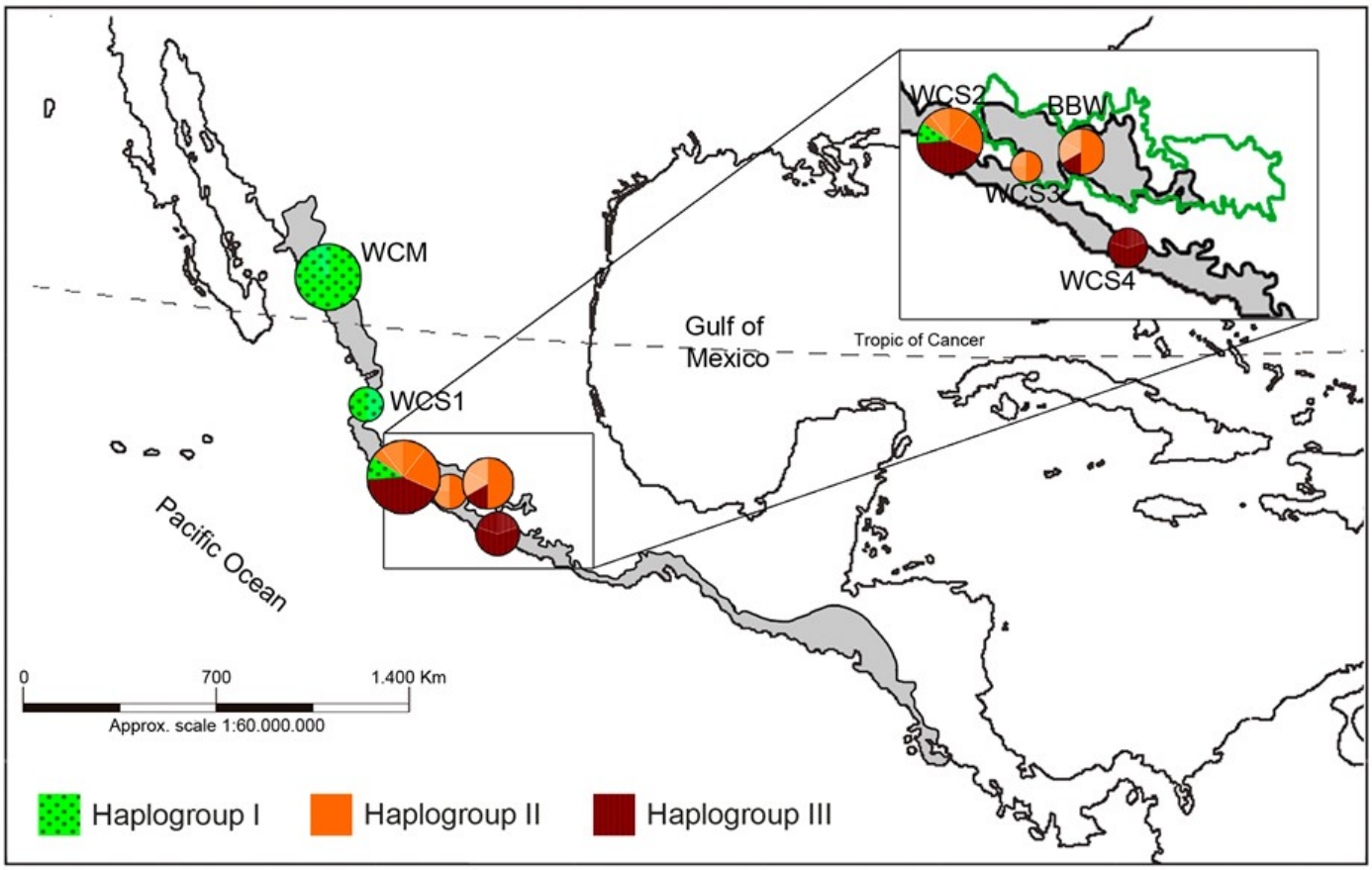

Figure 3. Geographic distribution of haplotypes from the Orange-fronted Parakeet (*Eupsittula canicularis*). The map is modified according to Monterrubio-Rico et al. (2016) and Collar (2017). Circles indicate the frequency of occurrence of haplotypes in each population. The colors correspond to the three haplogroups detected. The area outlined in green (top right) indicates the limits of the Balsas Basin. For abbreviations, see Table 1.

from SSD (0.00149, P = 0.5660) and Hri (0.04569, P = 0.4670) indicate that the analyzed data fit the sudden expansion model (Schneider & Excoffier 1999) (Figure 5A). On the other hand, the combined results of the five runs in BEAST v1.7.4 (Drummond & Rambaut 2007) showed different values of ESS, all greater than 400, validating the convergence in analysis of skyline plots. Plots resulting from the skyline analyses showed the effective size of the population (N_eT) over the last 238.7 thousand years ago (kya) (Figure 5B). During the Middle and Upper Pleistocene, the population showed constant growth from 238.7 kya, with a peak reached at 19 kya followed by a slight decline to the current population size. The analyses of skyline plots and mismatch distribution were consistent with one another and with the results of D_T (-1.989) and Fu's F_S (-10.937) values, which indicated a recent expansion of the population (Table 2, Figure 5).

DISCUSSION

Genetic diversity and differentiation. According to Grant & Bowen (1998), a moderate to high diversity of haplotypes and low diversity of nucleotides, as found in the population of *E. canicularis* analyzed, indicate growth from a small effective population size. Additionally, according to Hedrick (2011) and Hamilton (2011), the negative values of D_T and Fu's F_S for Cytb in the population analyzed suggest a recent expansion (Table 3).

Analysis of genetic differentiation showed that, although the populations in the Balsas Basin (WCS3 and BBW) are located in two different biotic provinces, there is minimal genetic differentiation between them, which suggests that they form an Evolutionarily Significant Unit (ESU) as proposed by Padilla-Jacobo et al. (2016). Additionally, we identified that the population located on the coast of Guerrero (WCS4) has the highest values of genetic differentiation with respect to the populations of the Balsas Basin (WCS3 and BBW) (Table 4, Figure 3). Both observations suggest *E. canicularis* may have expanded its range through the lowlands of the Balsas Basin. Individuals from the coast of Guerrero and those from the Balsas Basin are separated by the Sierra Madre del Sur where elevations surpass 2000 m a.s.l.. This elevation is an effective barrier for these parakeets, which are not typically found at elevations above 1500 m a.s.l. (Forshaw 1989).

Following Escalante-Pliego et al. (1993), *E. canicularis* populations on the Mexican Pacific slope belong to two different biotic provinces. Nevertheless, despite being separated by a large geographical distance, we found little differentiation among the populations of Sinaloa, Jalisco-Nayarit, and the coast of Michoacán (WCS, WCS1, and WCS2), and low differentiation between the populations on the coasts of Michoacán and Guerrero (WCS2 and WCS4). These findings suggest a second lineage or ESU as proposed by Padilla-Jacobo et al. (2016) (Table 4, Figure 3). These results are in agreement with the distribution of genetic variation detected by AMOVA analysis, where the species shows genetic structure with moderate differentiation (Table 5), an indication of moderate proportion of unshared haplotypes among the populations (Bird et al. 2011).

From a conservation perspective, our results support the establishment of complementary conservation areas. In the

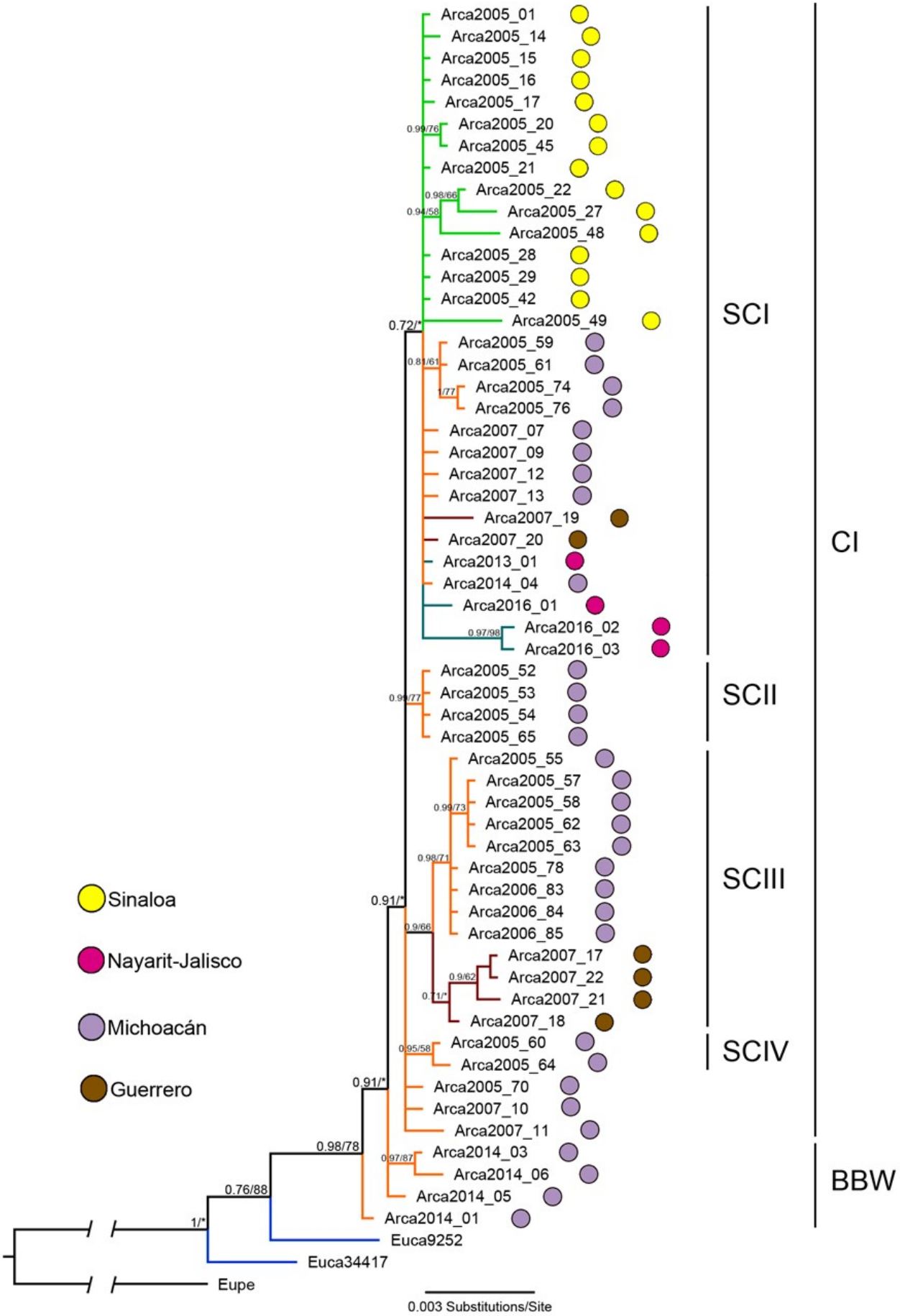

Figure 4. Consensus tree showing genealogic relationships among haplotypes of the Orange-fronted Parakeet (*Eupsittula canicularis*) individuals obtained with Bayesian inference (BI) and maximum likelihood (ML) analyses. Estimates were performed with 2,645 nt of concatenated Cytb, ND2, and COI sequences from 59 individuals. The sister species *Eupsittula pertinax* (Eupe) served as the out-group to root the tree. Values at the nodes represent posterior probabilities and bootstrap values (PP/BP). (*) Values below PP = 0.5 or PB = 50. The scale bar under each consensus tree is a reference to length of the branches and branch length is proportional to the amount of evolutionary change.

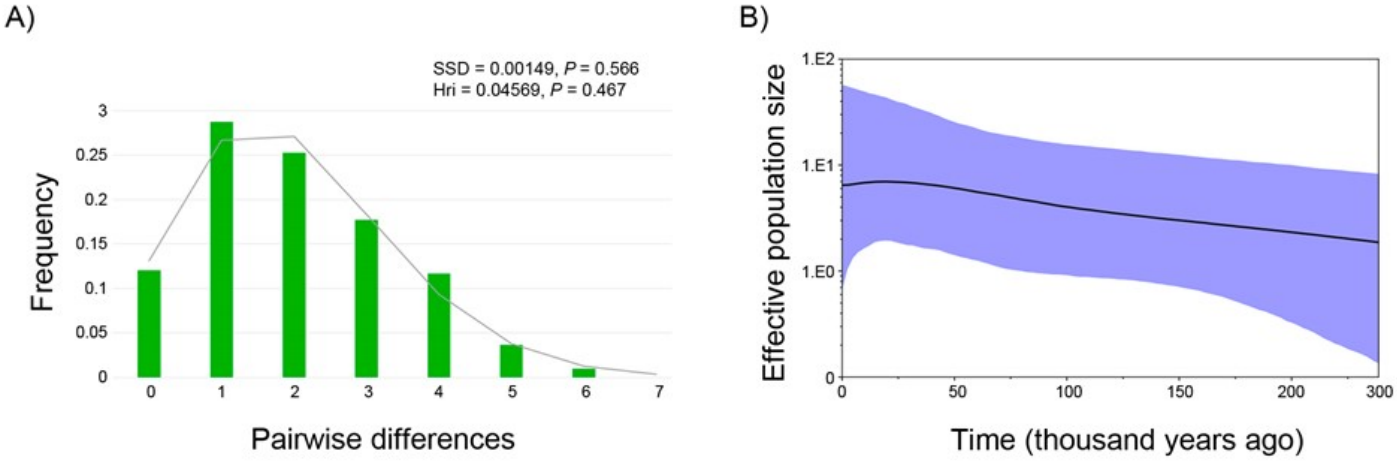

Figure 5. Mismatch distribution (A) and Bayesian skyline plots (B) for all samples. A) Bars correspond to observed frequencies; line represents expected frequencies under a sudden expansion model. B) Skyline plot showing the population history of the Orange-fronted Parakeet (*Eupsittula canicularis*), with the black line indicating median population size estimates expressed in N_eT through time; shaded areas represent 95% HPD intervals.

present analysis we identify that the populations in WCS2, WCS3, and BBW have the greatest genetic diversity. According to the argument of focusing efforts on the conservation of as much genetic diversity as possible, we propose the consideration of two areas: one located on the north coast of Michoacán and the other in the southeast region of Huetamo within the Balsas Basin. Because the populations of the Balsas Basin have the greatest genetic diversity, we recommend additional genetic studies in the biotic province Balsas Basin East (belonging to the state of Guerrero) to assess the importance of establishing protected areas in that region.

Relationships among haplotypes and their geographical distribution. We found 18 haplotypes that differ by a small number of mutations. Three haplogroups, each with a dominant haplotype (H1, H7, and H8) were detected. The fact that the dominant haplotypes H1 and H7 and their peripheral haplotypes form stars is typical of population expansions from a small number of founders (Bandelt et al. 1995, Grant & Bowen 1998). This coincides with the genetic diversity results, where moderate to high Hd, low Pi, and negative values of D_T and Fu´s F_S for Cytb were detected (Hamilton 2011, Hedrick 2011). We found that the geographic distribution of the three haplogroups explains the differentiation levels detected among the populations. The haplogroup HGI with the dominant haplotype H1 and its peripheral haplotypes is distributed from the north coast of Michoacán to the coast of Sinaloa. The haplogroup HGII, with the dominant haplotype H7 and its peripheral haplotypes, characterizes individuals from the Michoacán coast (WCS2) and the Balsas Basin (WCS3 and BBW). Finally, haplogroup HGIII with the dominant haplotype H8 is found on the coasts of Michoacán and Guerrero (WCS2 and WCS4) as well as the Balsas Basin (BBW) (Figures 1, 2). The Michoacán coast population includes haplotypes of the three haplogroups, mainly the dominant haplotypes H7 and H8. Based on these observations, we propose that *E. canicularis* diversified and expanded its range from the Balsas Basin to the coast of Michoacán, and then to the north. In addition, we note that the private haplotypes (unique for each population) detected in the populations (Table 3) indicate a diversification *in situ* (Freeland 2005, Chassaing et al. 2011).

For the populations located along the coast, no apparent geographical barriers are associated with the diversification observed in the individuals of *E. canicularis* analyzed. In particular, no phylogeographic break was observed between the coasts of Michoacán and Guerrero, because at least one haplotype was shared among individuals from the coast, and less genetic differentiation was observed among these populations. Cortés-Rodríguez et al. (2008a, 2008b) have proposed that, given the lack of phylogeographic breaks associated with geographic barriers (mountains or rivers) within this TDF area, Pleistocene climatic fluctuations may have fragmented and reconnected TDF causing the isolation of populations. However, although some phylogeographic breaks have been identified in birds that inhabit the TDF in México (*Campylorhynchus rufinucha*, Vázquez-Miranda et al. 2009; *Melanerpes chrysogenys*, *Momotus mexicanus*, and Orange-breasted Bunting *Passerina leclancherii*, Arbeláez-Cortés et al. 2014a), a pattern unifying the evolutionary histories has not yet been distinguished.

Genealogic analysis. In the relationships established in the consensus tree for the three haplogroups identified, a shallow divergence was observed represented by different subclades in a polytomy, a typical topology in analyses with groups of closely related haplotypes within a species (Figure 4) (Freeland 2005). We emphasize that the genetic group HGII belonging to the population in the west of the Balsas Basin (BBW) is ancestral to the other individuals analyzed, including those of Guerrero (Figure 4). The occurrence of the ancestral and dominant haplotypes H7 and H8 in individuals from the populations of the Balsas Basin and the coasts of Michoacán and Guerrero supports our proposal that the Balsas Basin was an important center for the diversification of *E. canicularis*. We hypothesize a diversification in the ancestral genetic group HGII (H7) in the Balsas Basin that gave rise to the genetic groups HGI (H1) and HGIII (H8). A range expansion of the population with haplotypes from HGII and HGI towards the coast of Michoacán, and with haplotypes from HGIII towards the coast of Guerrero would have followed. Additional diversification and range expansion would have continued northward up the coast of Michoacán to the coast of Sinaloa and southward to coast of Guerrero. However, we

must emphasize that the sample sizes that we were able to obtain from populations in the Balsas Basin and in Guerrero were limited, and further analyses with larger sample sizes may modify this hypothesis.

Demographic history. Haplotype network, mismatch graph, and skyline plot are all consistent with the population expansion suggested by metrics of genetic diversity and neutrality tests (Figures 2 and 5). Moreover, the skyline plot showed a slight population growth from 238.7 to 19 kya (Figure 5B). Based on these results, we conclude that the *E. canicularis* population distributed from Sinaloa to Guerrero was not negatively affected by the climatic changes of the Upper and Middle Pleistocene. Only at the Upper Pleistocene-Holocene boundary, it may have leveled off or showed a slight decline around the time of the Last Glacial Maximum (LMG, approximately 23-18 kya).

Two hypotheses or scenarios that are not mutually exclusive may explain the observed patron, i.e., that the species was not negatively affected by the climatic changes, and the presence of three genetic groups detected: 1) *E. canicularis* could have survived in one or several refuges throughout its range, and 2) the behavior of the species. According to several authors, the genetic signals that are expected under a refuge scenario are: shallow clades, unimodal mismatch analyses, star-like networks, genetic diversity declines towards areas of postglacial colonization and large geographic areas mainly harbor a single haplotype, private haplotypes, and genetic differentiation (Hewitt 2000, 2004a, 2004b; Maggs et al. 2008, Provan & Bennett 2008). Some of our data are consistent with the key signals of the refuge scenario. For example, in the genealogic tree, shallow clades containing polytomies that indicate the close relationship among individuals could be observed. In this analysis, it was also possible to detect some genetic differentiation among the populations of the Balsas Basin and the rest of the individuals analyzed, a pattern that was also seen in the F_{ST} analysis (Table 4, Figure 4). In addition, the diversity of haplotypes decreases towards the north in populations distributed in Nayarit-Jalisco and Sinaloa where a dominant haplotype (H1) is observed (Figures 2, 3). On the other hand, *E. canicularis* tends to form large flocks that move together over short distances, without long-distance migrations (Forshaw 1989), which could explain the overlapped distribution of the three genetic groups and the diversification *in situ* observed. However, since the H1 haplotype was not detected in the Balsas Basin or on the Guerrero coast, its absence can be related to the size of the sample considered in those localities. For future analyses, we recommend expanding the sampling to include other biotic provinces, particularly within the Balsas Basin, and extending it in the states of Guerrero, Oaxaca, Chiapas, and other parts of Central America.

Some authors have proposed that areas under stable environmental conditions could act as climatic refuges (Toledo 1981, Becerra 2005, Espinosa et al. 2006, Becerra & Venable 2008). Given that the phylogeographic pattern of *E. canicularis* suggests that its population survived and diverged, respectively, in areas under suitable environmental conditions despite the climatic oscillations of the Quaternary, this suggests the existence of a climatic refuge. According to Becerra (2005), Sierra Madre Occidental and Trans-Mexican Volcanic Belt have often been considered crucial because they provide the ecological conditions for long-term persistence of the tropical dry forest by moderating cold storms and northern winds.

Although our data suggest diversification under a refuge scenario, we must maintain a certain degree of uncertainty in absence of genetic evidence from other taxa. For example, the phylogeographic analyses of other species in the Balsas Basin show little correlation with the paleogeographic history of the region, among them plants, such as *Caesalpinia hintonii* (Leguminosae) (Sotuyo et al. 2007), and animals such as leopard frog (*Rana berlandieri*) (Zaldívar-Riverón et al. 2004), Western lyre snake (*Trimorphodon biscutatus*) (Devitt 2006), and Mexican black iguana (*Ctenosauria pectinata*) (Zarza et al. 2008, 2011). Given that the Balsas region has been described as the richest in endemic species for México (Ceballos et al. 2002, García-Trejo et al. 2004, Rzedowski et al. 2005, García 2006, Navarro-Sigüenza et al. 2014), the area provides a great opportunity to continue with phylogeographic analyses in different species of birds that inhabit the TDF.

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