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USE OF PHYLOGENETIC ANALYSIS TO IDENTIFY EVOLUTIONARILY SIGNIFICANT UNITS FOR THE ORANGE-FRONTED PARAKEET (*EUPSITTULA CANICULARIS*) IN MEXICO

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Resumen. – Uso del análisis filogenético para la identificación de unidades evolutivamente significativas del Periquito Frente-naranja (*Eupsittula canicularis*) en México. – En biología de la conservación, el concepto de subespecie basado en monofilia recíproca se ha aplicado con éxito para definir poblaciones prioritarias a través de las Unidades Evolutivamente Significativas (ESUs). El Periquito Frente-naranja (*Eupsittula canicularis*) ocupa el primer lugar de sustracción de psitácidos en México. Su distribución en el país abarca desde el Sur de Sonora hasta Chiapas por la vertiente del Pacífico con poblaciones representantes de las tres subespecies descritas para la especie: *E. c. canicularis*, *E. c. eburnirostrum* y *E. c. clarae*. Con la finalidad de identificar y proponer ESUs que auxilien en las propuestas de conservación para diferentes poblaciones, evaluamos la monofilia recíproca subespecífica mediante análisis filogenético y red de haplotipos, con base en caracteres de los genes de ADN mitocondrial, Citocromo Oxidasa I y NADH Deshidrogenasa 2. Se utilizaron muestras de plumas y sangre colectadas de individuos de nidos en 2005 y 2007. Se analizaron un total de cinco especímenes de *E. c. eburnirostrum* de dos localidades en el estado de Michoacán y cuatro especímenes de *E. c. clarae* del estado de Sinaloa y no se incluyó individuos de *E. c. canicularis*. En los análisis se incluyeron secuencias obtenidas por nosotros y las reportadas previamente para *E. aurea*, *E. cactorum*, *E. canicularis*, *E. nana* y *E. pertinax*. Tanto los análisis filogenéticos como las redes de haplotipos sugieren dos grupos que corresponden con dos de las subespecies descritas para *E. canicularis* por sus características morfológicas y distribución geográfica, por lo que se proponen como ESUs independientes para fines de conservación.

Abstract. – In avian conservation biology, the subspecies concept based on reciprocal monophyly has been successfully applied to define priority populations through Evolutionarily Significant Units (ESUs). In México, the Orange-fronted Parakeet (*Eupsittula canicularis*) ranks first in illegal parrot trade. Its distribution ranges from southern Sonora to Chiapas on the Pacific slope, with populations representing three subspecies: *E. c. canicularis*, *E. c. eburnirostrum*, and *E. c. clarae*. To identify and propose ESUs to assist in conservation proposals for different populations, we assessed subspecific reciprocal monophyly via phylogenetic analyses and haplotype networks based on the mitochondrial DNA genes cytochrome oxidase I and NADH dehydrogenase 2. Feather and blood samples from specimens collected from nests in 2005 and 2007 were used. A total of five specimens of *E. c. eburnirostrum* from two localities in the state of Michoacán and four specimens of *E. c. clarae* from the state of Sinaloa were analyzed and no specimens of *E. c. canicularis* were included. The analyses included sequences obtained by us and those previously reported for *E. aurea*, *E. cactorum*, *E. canicularis*, *E. nana*, and *E. pertinax*. Both the phylogenetic

analyses and haplotype networks suggest two groups that correspond to two subspecies of *E. canicularis* based on morphological and geographical evidence. Therefore these two subspecies are proposed as independent ESUs for conservation purposes.

Key words: Conservation, *Eupsittula aurea*, *Eupsittula cactorum*, *Eupsittula canicularis*, *Eupsittula c. clarae*, *Eupsittula c. eburnirostrum*, *Eupsittula nana*, *Eupsittula pertinax*, Evolutionarily Significant Units, molecular phylogeny, Orange-fronted Parakeet, *Psittacidae*, reciprocal monophyly.

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INTRODUCTION

The Orange-fronted Parakeet (*Eupsittula canicularis*) inhabits the Pacific slope from México to Costa Rica (Forshaw 1989, Howell & Webb 1995, Collar *et al.* 2000). In México, three subspecies have been identified based on morphological differences and geographic range (Bangs & Peters 1928, Moore 1937, Forshaw 1989). *Eupsittula c. canicularis*, distributed from the northwest of Costa Rica to Chiapas, México, has a broad orange frontal band extending down to the lores, a dull blue fore-crown, pale olive throat and chest, and horn-colored bill. *E. c. eburnirostrum* is found only in México from Oaxaca to Michoacán. It has a narrow orange frontal band, the lower throat and breast are greener, and it has brownish spots on each side of base of lower mandible. *E. c. clarae* ranges from Michoacán to Sinaloa, México. It has a significantly reduced orange frontal band, so that blue of crown continues in front of eye and down to the lores. Lower throat and breast are greener, less yellowish-olive and the dark spots on the side of the lower mandible are more blackish than in *E. c. eburnirostrum* (Bangs & Peters 1928, Moore 1937, Forshaw 1989).

The species' habitat includes humid and sub-humid deciduous forests, tropical deciduous dry forests, riparian forests, and agricultural areas (Ridgely 1981, Howell & Webb 1995, Stotz *et al.* 1996, Collar *et al.* 2000). Based on specimens from scientific collections, ecological niche modeling, and land use-change analysis, the original distribution

of *E. canicularis* was estimated at 155,940 km² for México, with an estimated 37.6% of its potential habitat having been lost (Ríos-Muñoz & Navarro-Sigüenza 2009). In another study based on fieldwork and ecological niche models, it was estimated that the species' current distribution covers 140,938 km² (Marín-Togo *et al.* 2012). The species has a wide distribution and it is the parrot with the highest frequency of extraction for illegal trade in México (Cantú-Guzmán *et al.* 2007). The Official Mexican Norm NOM-059 (DOF 2010) listed the species in the special protection category, even though at the international level it is still regarded as a species of Least Concern (BirdLife International 2015). In México, only 1.6% of its distribution falls within Protected Areas (Marín-Togo *et al.* 2012), and the situation at the subspecies level is unknown.

Subspecific taxonomic determination includes DNA sequence analysis of variation, particularly of mitochondrial DNA (mtDNA), which allows determination of whether a subspecies is evolving independently, whether individuals are exchanged between populations, or whether there is an intermediate level of isolation (Wiens 1982, Zink & Barrowclough 2008, James 2010). Based on this type of analysis, and from a phylogenetic point of view, a subspecies can be identified through the concept of reciprocal monophyly (Avice 2000).

Although the relation between molecular characteristics and subspecific discrimination in birds has been discussed (Avice & Nelson

1989), the subspecies concept remains useful as it identifies priority populations and provides information on genetic diversity that should be considered in management and conservation plans (Avice & Nelson 1989, Solórzano *et al.* 2004, Zink 2004, Johnson *et al.* 2005, Phillimore & Owens 2006, Pruett & Winker 2010). The concept of Evolutionarily Significant Units (ESUs; Moritz 1994) is intended to aid in recognizing and protecting the evolutionary heritage of natural populations. Through the conservation of ESUs processes can be preserved to ensure that species retain their ability to persist and evolve (Moritz 1994, 1995, 2002).

Molecular markers can be used to distinguish different degrees of population differentiation, which is essential for identification and proposal of ESUs as phylogenetically important units with conservation priority (Haig *et al.* 2001, Pitra *et al.* 2004, Solórzano *et al.* 2004, Barry & Tallmon 2010, Wu *et al.* 2012, Hill *et al.* 2012). One method to identify ESUs refers to test for the reciprocal subspecific monophyly of subpopulations (Russello *et al.* 2010, Wenner *et al.* 2012). Hence the objective of this analysis was to determine whether *E. c. clarae* and *E. c. eburnirostrum* form monophyletic clades in gene trees and divergent groups in haplotype networks, respectively, as prerequisite for their recognition as Evolutionarily Significant Units.

METHODS

Sampling. Feather and blood samples from specimens collected from nests in 2005 and 2007 were used. Each sample was geo-referenced and samples were taken under collection permits number SGPA/DGVS/06387. In order to avoid the collection of samples of related individuals that shared the same maternal line, only one chick per nest was used. From each individual we collected a sample of growing feathers with tissue or

blood at the base and placed it in a 2 ml vial with 0.5 ml of storage and lysis buffer (100 mM Tris pH 8.0, 100 mM EDTA pH 8.0, 10 mM NaCl and 2% SDS) (Dutton 1996). Samples placed in the solution were stored at room temperature during transport for a week and subsequently stored at 4°C until further analysis.

The samples were deposited in the Collection of Wildlife Biological Samples of the Multidisciplinary Center for Studies in Biotechnology (CMEB) of the Michoacán University of San Nicolás de Hidalgo (UMSNH, for its initials in Spanish). The tested individuals were assigned to subspecies based on morphology and sampling locality according to phenotypic descriptions and distributional data published for these subspecies by Forshaw (1989). A total of five specimens of *E. c. eburnirostrum* from two localities in the state of Michoacán (Palos Marias [n = 4] and Agua Cola [n = 1]), and four specimens of *E. c. clarae* from the state of Sinaloa (Badiraguato [n = 1], Higeras de la Campa [n = 1], Imala [n = 1], and Ipucha [n = 1]) were analyzed (Fig. 1). No specimens of *E. c. canicularis* were included.

DNA extraction and PCR amplification of markers.

DNA was extracted from tissue or blood in feathers using the phenol-free method described by FitzSimmons (1997). Two sequences of mtDNA were amplified: NADH dehydrogenase-2 (ND2) and cytochrome oxidase 1 (COI). The ND2 gene sequence of approximately 1140 bp was obtained using the oligonucleotides L5215 (5'-TATCGGGCCCCATACCCCGAATA-3') and HTrpC (5'-CGGACTTTAGCAGAACTAAGAG-3') (Hackett 1996, Smithsonian TR Inst.). For the COI amplification of approximate 590 bp, we used the set of oligonucleotides COI_{arcaD} (5'-CTACCACGCGGGCAAAAA-3') and COI_{arcaR} (5'-CCCAATGGAGGATAAAGTGTT-3') designed in this study using the DNASTAR

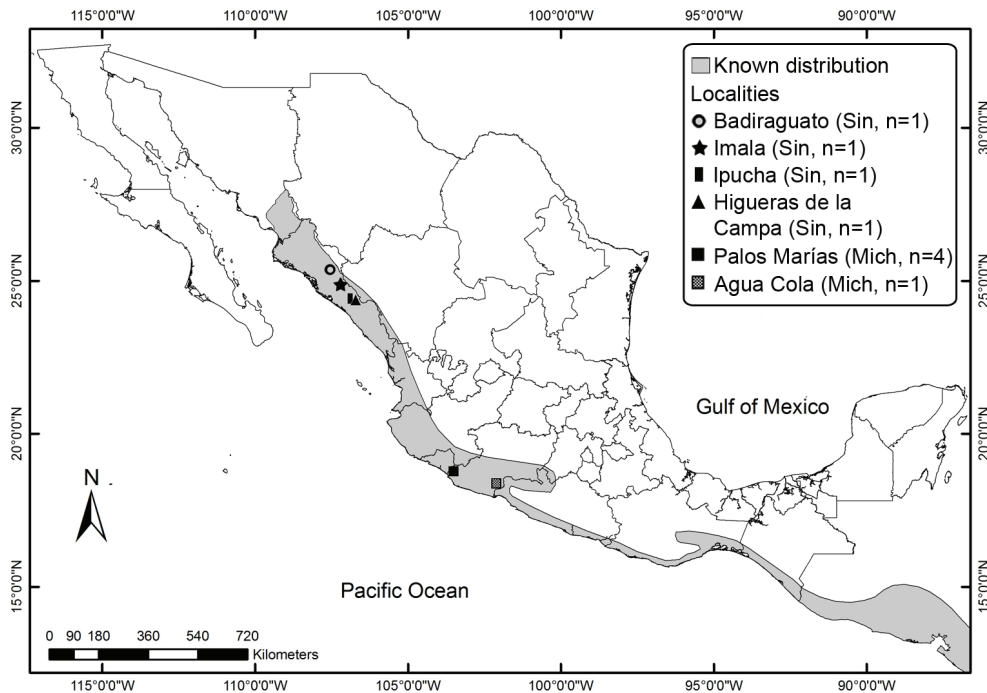


FIG. 1. Geographic distribution (northern range) and sampling localities (México) of the Orange-fronted Parakeet *Eupsittula canicularis*. Agua Cola, Michoacán (18°20'N, 102°05'W), Badiraguato, Sinaloa (25°28'N, 107°37'W), Higueras de la Campa, Sinaloa (24°31'N, 106°47'W), Imala, Sinaloa (24°51'N, 107°12'W), Ipucha, Sinaloa (24°20'N, 106°45'W), Palos Marias, Michoacán (18°49'N, 103°34'W).

Lasergene software LG10VC (Kumar & Blaxter 2010), based on the COI sequence reported in GenBank for *E. canicularis* (GenBank Access: HQ629753.1) and other species of the genus *Eupsittula*.

The PCR reactions were performed in a total volume of 25 μ l as follows: 20 mM Tris-HCl pH 8.4, 50 mM KCl, 1.5 mM MgCl₂, 200 mM of each dNTP, 10 pmol of each oligonucleotide, 1.5 U Platinum Taq polymerase (Invitrogen) and 50 ng of DNA. The reaction mixtures were placed in a thermocycler (Gene Amp 2700, Applied Biosystems) under the following amplification conditions: 94°C for 5 minutes, followed by 30 cycles of 94°C for 40 seconds, 55–56°C for 40 seconds and 72°C for 2 minutes, and finally an extension of 72°C for 5 minutes.

Sequencing and analysis. DNA sequencing was performed using the dideoxy technique on both strands (Sanger *et al.* 1977) by means of the commercial service of Macrogen USA. Electropherograms and their sequences were analyzed, edited, and aligned with BioEdit 7.09 software (Hall 1999). The number of haplotypes and polymorphic sites, and the nucleotide and haplotype diversity were revised with DnaSP v.5 (Librado & Rozas 2009).

Phylogenetic analysis. From the alignments, phylogenetic trees were constructed for the combined data with the available sequences. Trees were obtained based on the criteria of Maximum Likelihood (ML), Maximum Parsimony (MP), and Bayesian Inference (BI), and were

constructed with the software programs MEGA 5.05 (Tamura *et al.* 2011), PAUP 4.0b10 (Swofford 2002), and MrBayes v.3.1 (Ronquist & Huelsenbeck 2003). The branch-support values were estimated with bootstrap analysis (500 replicates) and posterior probability.

For the analysis of interspecific genetic relations of the genus *Eupsittula*, the alignment included 1607 pb of the concatenated sequences of COI and ND2 markers from *E. c. clarae* and *E. c. eburnirostrum* obtained in this work and sequences previously reported for *E. aurea* (GenBank Access: GU826175 and HQ270482), *E. cactorum* (GenBank Access: AF370750), *E. nana* (GenBank Access: GU826184 and HQ270490) and *E. pertinax* (GenBank Access: JQ174079.1 and EU327-600.1). We also included sequences of COI and ND2 markers reported for a specimen identified as *E. canicularis* (EcNCBI hereinafter) (GenBank Access: HQ629753.1 and HQ629718.1). This specimen is a female obtained from captivity and deposited in the American Museum of Natural History of New York (AMNH registration no.: DOT9252; collector no.: PRS-160). In the tree construction, ML algorithm under the model of molecular evolution GTR+G+I (General Time Reversible + Gamma distributed + Invariable sites), chosen under the corrected Akaike Information Criterion (AICc) was used (Alfaro & Huelsenbeck 2006).

For the analyses of intraspecific genetic relations of *E. canicularis* the alignments included 1545 bp of the concatenated sequences obtained from COI and ND2 markers from *E. c. clarae* and *E. c. eburnirostrum* in this work and those reported for EcNCBI. As outgroup we included sequences from *E. pertinax*. To reconstruct phylogenetic relationships using ML we used the molecular evolution model TIM + I (Transitional Model + Invariable sites, Tavaré 1986) chosen based on the corrected Akaike Information Criterion

(AICc) (Alfaro & Huelsenbeck 2006). For the Bayesian phylogenetic inference we used the model HKY + I (Hasegawa-Kishino-Yano-1985 + Invariable sites) determined by the best Bayesian Information Criteria (BIC), both with the ModelTest v.3.7 program (Posada & Crandall 1998). The Bayesian analyses were run for 1×10^6 generations using two Monte Carlo Markov Chains (MCMCs) with the default options in Mr. Bayes v.3.1. The trees were sampled every 1000 generations, discarding 10% to reach a majority consensus tree. The relationship between haplotypes was determined by constructing haplotype networks under the median-joining method with the software NETWORK v4.6.0.0 (Fluxus Technology 2012).

RESULTS

From the DNA samples of *E. canicularis*, amplifications of the nine COI fragments of 552 to 598 bp and nine ND2 gene sequences of 1056 to 1100 bp were obtained and registered in GenBank (Access: KJ612380 to KJ612397). The analyzed COI fragments of *E. canicularis* revealed seven haplotypes (1_{acCOI} – 7_{acCOI}) with eight polymorphic sites with a haplotypic diversity (Hd) of 0.944 and nucleotide diversity (Π) of 0.004. The ND2 gene had five haplotypes (1_{acND2} – 5_{acND2}) with nine polymorphic sites (Hd = 0.722, Π = 0.002).

The phylogenetic analysis of *E. canicularis* and its congeners shows that EcNCBI clearly groups with *E. c. clarae* and *E. c. eburnirostrum*, indicating that it should be included in *E. canicularis* (Fig. 2A). In the trees built to determine intraspecific relations within *E. canicularis*, three lineages were observed: the monophyletic clade of ECC, the ECE clade, and the specimen EcNCBI located basal to both clades. The clade ECC grouped sequences belonging to the four individuals of *E. c. clarae* for the state of Sinaloa, and the clade ECE grouped the five individuals of *E.*

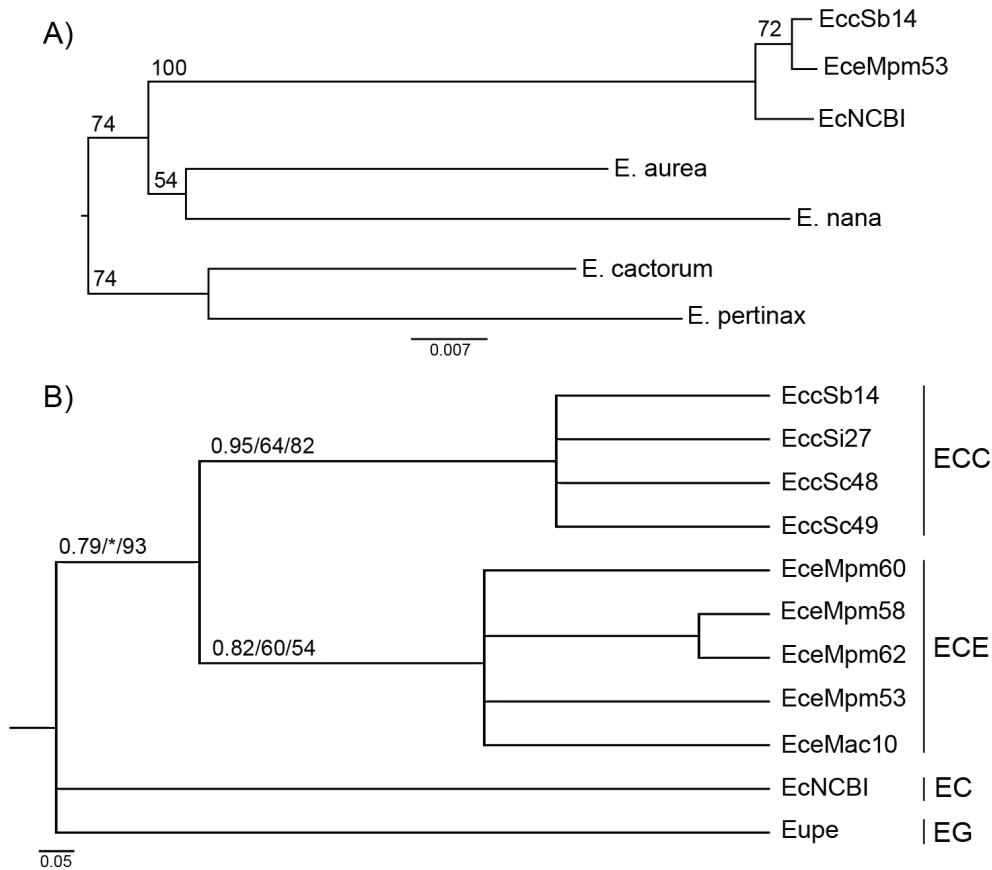


FIG. 2. A) Phylogenetic tree showing the relationships among species of *Eupsittula*, constructed with 1607 bp of the concatenated sequences of COI and ND2; bootstrap values are displayed on the branches. B) Tree showing relationship among subspecies of *E. canicularis* constructed with 1545 bp of the concatenated sequences of COI and ND2. Posterior probability and bootstrap values are displayed on the branches in the following order: BI, ML and MP; (*) value not obtained. ECC (Sinaloa samples): *Eupsittula canicularis clarae*; ECE (Michoacán samples): *Eupsittula canicularis eburnirostrum*; EC (unknown origin): *Eupsittula canicularis* (EcNCBI); EG (Guyana, West Bank Berbice River, Dubulay Ranch sample [Kirchman *et al.* 2012]): Outgroup (*Eupe* = *Eupsittula pertinax*). The bar under each tree depicts branch length scale. The length of the branches is proportional to the amount of genetic change.

c. eburnirostrum of Michoacán. The Bayesian tree showed posterior probabilities for the ECE and ECC clades of 0.82 and 0.95 reliability, respectively, and these two clades form a monophyletic unit with a posterior probability of 0.79 (Fig. 2B). However, the ML and MP trees show lower bootstrap support values, which can be attributed to

the small sample sizes used (Fig. 2B). Nevertheless, the three methods yielded similar topology.

The haplotype network constructed based on the concatenated sequences revealed two groups that match the geographical distribution of the subspecies. No shared haplotypes were observed between individuals of differ-

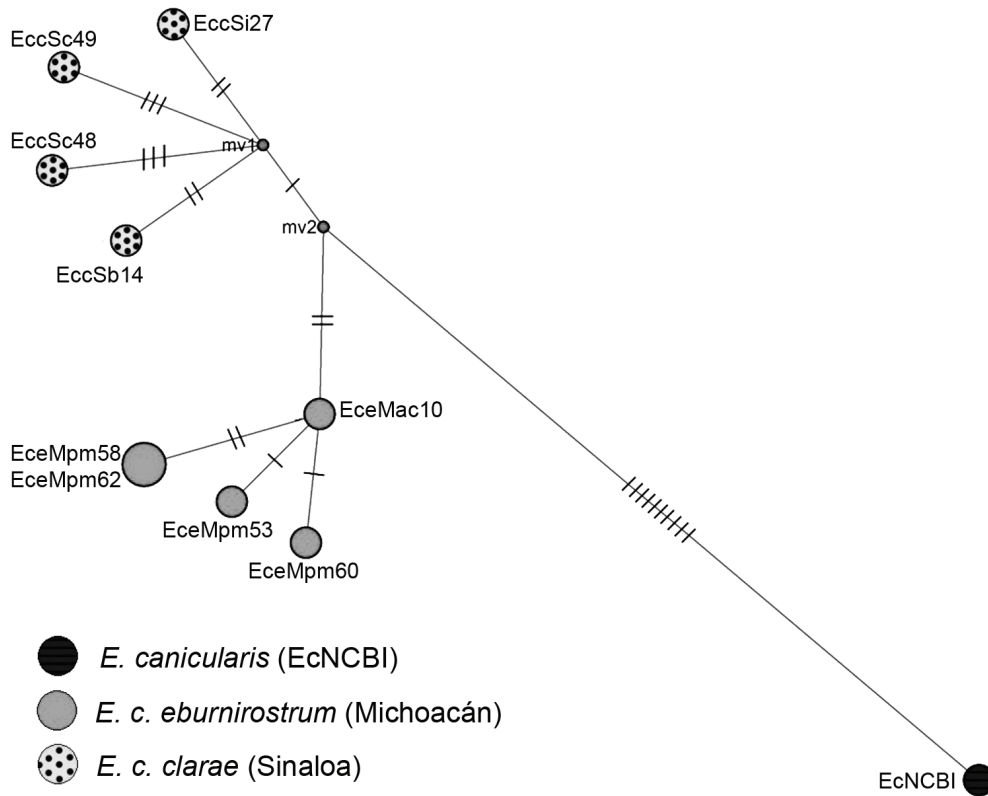


FIG. 3. Median-joining haplotype network showing the relationships between subspecies of *Eupsittula canicularis*, based on 1545 bp of the concatenated sequences of the COI and ND2 markers. The circle size is proportional to the number of individuals sharing the haplotype, but the length of the branches is not proportional to the number of mutations. The transverse lines of the branches represent the number of mutations between haplotypes and the mv1-mv2 circles represent hypothetical haplotypes that were not found (or possible ancestral haplotypes).

ent groups. The number of mutations separating the subspecies *E. c. clarae* and *E. c. eburnirostrum* was moderate (5 to 8), whereas the hypothetical ancestor of these two subspecies (mv2) and EcNCBI were separated by 10 mutations (Fig. 3).

DISCUSSION

Phylogenetic relationships were analyzed for two of the three subspecies of *E. canicularis*. As a result, in all trees two clades of *E. canicularis* can be identified that likely correspond to

the recognized subspecies *E. c. clarae* and *E. c. eburnirostrum*. This indicates potentially diverging subpopulations, a finding, which would be consistent with Zink *et al.* (2010), who state that a well-supported geographically-based mtDNA tree provides sufficient evidence to define independently evolving units. Additionally, the haplotype network revealed a correspondence between the geographical distribution of subspecies (Bangs & Peters 1928, Moore 1937) and polymorphism in mtDNA sequences. Our analysis identified *E. c. clarae* and *E. c. eburnirostrum* as ESUs, provid-

ing evidence to consider each unit independently for conservation. Similar relationships have been observed in other bird species, where a genetic differentiation between individuals of distinct subspecies was observed (*Thalassarche melanophris*, Burg & Croxall 2001; *Aegothelus nallacii*, Dumbacher *et al.* 2003; *Nectarina humbloti*, Warren *et al.* 2003; *Chlamydotis undulata*, Pitra *et al.* 2004; *Pharomachrus mocinno*, Solórzano *et al.* 2004), although this is not necessary the case for all species of birds analyzed (Ball & Avise 1992, Guerrini *et al.* 2007, Draheim *et al.* 2010, Wu *et al.* 2012).

According to Forshaw (1989), *E. c. eburnirostrum* and *E. c. clarae* may be sympatric in Michoacán, but there is no detailed description of the distribution of the subspecies in this region. In this study, we found no morphological and genetic evidence for the existence of *E. c. clarae* at the two sampling sites in Michoacán; there were no shared haplotypes between the Michoacán samples and the *E. c. clarae* samples from Sinaloa. For future studies focused on determining whether there is sympatric occurrence or hybridization (i.e., phenotypic or genotypic intergradation) between *E. c. clarae* and *E. c. eburnirostrum* in Michoacán, collecting should be expanded to additional areas in Michoacán and adjacent regions. Depending on these results, consequently a subsequent re-evaluation of the status of both taxa would be required.

The sample EcNCBI showed a clear divergence in the phylogenetic and the haplotype network analyses (Figs 2, 3), and was separated by 10 mutations from the remaining individuals suggesting that this specimen belongs to an independent ESU. Although this sample belongs to a captive individual of unknown geographic origin (Schirtzinger *et al.* 2012), according to genetic information it may come from a distant location in southern México or Central America, where only *E. c. canicularis* occurs. While the genetic distance found is insufficient to warrant specific status,

additional study of individuals from the southern part of the range should be conducted to confirm this hypothesis.

Although our study is based on few specimens per subspecies and involves only mtDNA characters the results can be valuable for future studies. These will require increased sampling and additional collection sites, and should include supplementary molecular data to determine: 1) the continuity of distribution among subpopulations, 2) their degree of isolation, and 3) potential barriers involved. In order to corroborate this it will be necessary to use polymorphic nuclear DNA (nDNA) markers that allow the measurement of gene flow among (sub-)populations. It has been observed that evolutionary history reconstructed for mtDNA is not always the same as that of the nDNA, since mtDNA only reflects the maternal history of an individual while nDNA reflects the evolutionary history of both parents (Zink & Barrowclough 2008). For example, in populations of the Common Eider (*Somateria mollissima*) mtDNA differentiation is substantial both among local colonies and distant geographical regions; however, the differentiation among colonies is much less pronounced at microsatellite loci (nDNA) (Tiedemann *et al.* 2004). Male-mediated gene flow and strong female philopatry may explain the differing patterns of nuclear and mitochondrial variation (Jones *et al.* 2005).

From a conservation perspective, it is important to uncover the distribution of each of the three subspecies of *E. canicularis* to identify and establish complementary conservation areas. Moreover, the finding that at least two of the currently recognized subspecies are potentially valid ESUs is of conservation importance for each of these units. The species occurs in at least 11 protected areas of México, covering 2303 km², which represent only 1.6% of the species total distribution in the country (Marín-Togo *et al.* 2012). How-

ever, protected area availability for each ESU requires further study. Within the potential range of *E. c. clarae*, there are a total of seven protected areas, in that of *E. c. eburnirostrum* there are three protected areas, and in the potential range of *E. c. canicularis*, there are five large protected areas. The data suggest that *E. c. eburnirostrum* may be underrepresented in protected areas, and therefore it will be important to collect samples of each subspecies in these areas to assess future conservation priorities and to ensure the conservation of the species as a whole.

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