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Y ZOOTECNIA

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Estudio de la diversidad genética del Periquito Atolero *Eupsittula canicularis* (Aves: Psittacidae)

TESIS

QUE PRESENTA

M.C. GABRIELA PADILLA JACOBO

PARA OBTENER EL GRADO DE DOCTOR EN CIENCIAS BIOLÓGICAS EN LA OPCIÓN EN BIOTECNOLOGIA MOLECULAR AGROPECUARIA

ASESORA:
DRA. MARÍA GUADALUPE ZAVALA PÁRAMO

Co-ASESOR:
DR. TIBERIO CÉSAR MONTERRUBIO-RICO

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Dedicatoria:

A mis Padres, hijos, esposo y hermanos.

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I. RESUMEN

Eupsittula canicularis es el psitácido que sufre mayor presión de sustracción en nuestro país (23,500 ejemplares sustraídos por año aproximadamente). Para establecer estrategias de conservación adecuadas es necesario reunir información de diferentes fuentes. Una de estas proviene de datos moleculares, ya que a través de ellos es posible proponer poblaciones prioritarias para su conservación. Mediante análisis filogeográficos es posible describir la historia evolutiva de la especie, y considerando este conocimiento se deben plantear estrategias de conservación. En el presente trabajo se realizó un análisis de poblaciones de *E. canicularis* procedentes de la parte centro y norte de su distribución en México. Se estimó la historia demográfica de la especie y su relación con los cambios climáticos del Cuaternario.

Entre las poblaciones de *E. canicularis* distribuidas desde Sinaloa hasta Guerrero existe poca divergencia, una diferenciación genética moderada y una expansión de la población reciente, con muestras de crecimiento ligero pero constante durante el Pleistoceno. También se detectó un ligero descenso de la población durante el Holoceno. Se identificaron tres haplogrupos, asociados a regiones geográficas diferentes, el Haplogrupo I se encuentra desde Sinaloa hasta el norte de Michoacán, el Haplogrupo II exclusivamente en Michoacán y el Haplogrupo III en Michoacán y Guerrero. Con base en datos de diversidad genética, se propone que la cuenca del Balsas pudo ser un refugio ante las fragmentaciones del bosque tropical seco provocadas por los cambios climáticos del Pleistoceno.

Adicionalmente, se realizó el análisis de una población de individuos decomisados para la determinación de la diversidad genética, los sitios de origen más probables y propuesta de sitios de reintroducción, con base en la información de los linajes identificados en campo, por asignación directa y mediante inferencia filogenética. La diversidad genética del grupo de individuos decomisados fue alta, por lo que esta población puede ser usada para establecer una colonia de reproductores. De acuerdo al probable origen de los individuos se propone su reintroducción en un área de bosque tropical seco de Colima, Jalisco o Sur de Nayarit.

Palabras clave: Psitácidos, Marcadores moleculares, Filogeografía de aves neotropicales, Genética de la conservación.

II. SUMMARY

Eupsittula canicularis is a psitacid that undergoes greater pressure of subtraction in our country (23,500 specimens subtracted per year approximately). To establish adequate conservation strategies it is necessary to gather information from different sources. One of these comes from molecular data, since through them it is possible to propose priority populations for their conservation. Through phylogenetic analysis it is possible to have a better understanding of the evolutionary history of the species and considering this knowledge it is possible to propose conservation strategies. In the present work an analysis of populations of *E. canicularis* from the centre and north of their distribution in México was performed. Demographic history of the species and the influence of the climatic changes of the Quaternary were estimated. Between the populations of *E. canicularis* distributed from Sinaloa to Guerrero there are a shallow genetic divergence, a moderate genetic differentiation and a recent population expansion that shows a slight but steady growth. Three haplogroups were identified, Haplogroup I is found from Sinaloa to the north of Michoacán, Haplogroup II exclusively in Michoacán and Haplogroup III in Michoacán and Guerrero. Based on the information gathered, it was established that the climatic changes of the Quaternary did not reduce population size, and it is proposed that the Balsas basin could be a refuge during the fragments of the dry tropical forest.

In addition, an analysis of a population of confiscated individuals was carried out for the determination of genetic diversity, probable sites of origin and proposal of reintroduction sites based on the information of the lineages identified in the field, through direct assignment and through inference phylogenetic. The genetic diversity of the group of individuals confiscated was high, so this population can be used to establish a breeding colony. According to the probable origin of the individuals, their reintroduction is proposed in an area of dry tropical forest of Colima, Jalisco or South of Nayarit.

III. INTRODUCCIÓN GENERAL

3.1. Especie de estudio: *Eupsittula canicularis*

E. canicularis pertenece a la subfamilia Arinae constituida exclusivamente por psitácidos de distribución neotropical. Se acepta ampliamente la monofilia de esta subfamilia y que su origen ocurrió durante el periodo Cretácico en Sudamérica (Tavares *et al.* 2006, Tokita *et al.* 2007, Wright *et al.* 2008, Schweizer *et al.* 2010). Posteriormente, a través de eventos de dispersión y vicarianza algunos taxa representantes del grupo llegan a Norteamérica (Padilla-Jacobo et al., artículo sometido, consultarlo en el apéndice). Las rutas propuestas para su arribo a México, son a través de Centroamérica y la península de Yucatán, aunque cabe mencionar que la especie extinta *Conuropsis carolinensis* que habitaba las Carolinas (EUA) pudo utilizar las Antillas para llegar a la región más septentrional conocida para un psitácido americano. En el caso particular de *E. canicularis*, una vez establecida en Centroamérica continúa hacia Norteamérica utilizando el bosque tropical seco como vía de dispersión durante el Pleistoceno (Padilla-Jacobo *et al*. artículo sometido, consultarlo en el apéndice).

E. canicularis es un perico de tamaño pequeño, que mide de 20 a 24 cm de longitud y pesa entre 74 y 80 gr aproximadamente; en general el color del plumaje es verde brillante; en la garganta y el pecho el tono puede ser igual al resto del cuerpo o diferenciarse en un gradiente hasta el verde olivo pálido; el color de las plumas de la corona y la nuca es azul; y presenta una franja de plumas color naranja en la frente que ocasionalmente se extiende hacia la corona; las plumas secundarias y el extremo de las plumas de la cola son azules; el iris es de color amarillo pálido y el anillo periocular está desnudo y puede tener tonos de amarillo a naranja pálido; el pico es color marfil y en la mandíbula inferior puede presentan un par de manchas café oscuro o negro a cada costado (Forshaw 1989).

Esta especie se encuentra por la vertiente del Pacífico desde el noreste de México hasta el noreste de Costa Rica, y es en nuestro país donde está la mayor parte de su distribución (Forshaw 1989, Howell y Webb 1995, Collar *et al.* 2000). *E. canicularis* es una de las pocas especies de psitácidos que se encuentra en mayores latitudes de América (alcanza la región noroeste de México). Su hábitat principal es el bosque tropical seco al que está fuertemente asociada, aunque también se le puede encontrar en bosque húmedo y sub-húmedo, en bosque rivereño y en zonas agrícolas (Ridgely 1981, Howell y Webb 1995, Stotz *et al.* 1996, Collar *et*

al. 2000). La distribución actual en México es continua y abarca desde el sur de Sonora hasta Chiapas por la vertiente del Pacífico, con incursiones tierra dentro en la cuenca del Balsas en Michoacán y Guerrero (Monterrubio-Rico *et al.* 2016, Collar *et al*. 2017) (Fig. 1).

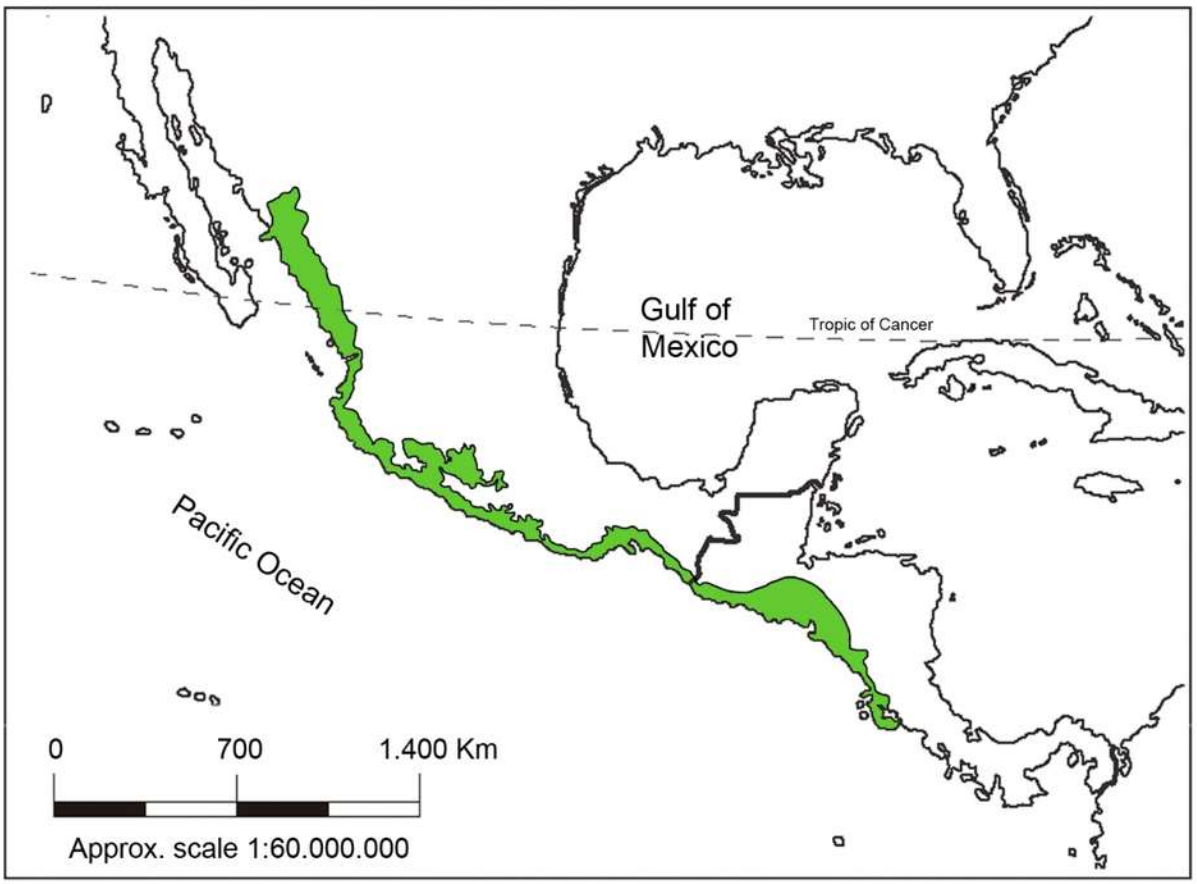

Figura 1. Distribución geográfica de *Eupsittula canicularis*. En verde se muestra la estimación de la distribución actual de la especie. Mapa tomado y modificado de Monterrubio-Rico *et al.* (2016) y Collar *et al*. (2017).

Dentro de *E. canicularis* se han identificado tres subespecies: *E. c. canicularis, E. c. eburnirostrum* y *E. c. clarae,* de acuerdo con diferencias morfológicas y la distribución de sus poblaciones (Forshaw 1989). Los caracteres que distinguen a estas tres subespecies son el tono de verde en la garganta y pecho, el tamaño del manchón de plumas color naranja en la frente, y un par de manchas submandibulares (que pueden estar ausentes, o presentes en color café o casi negro). La subespecie *E. c. canicularis* se ubica en el estado mexicano de Chiapas, y

desde ahí hasta Costa Rica; *E. c. eburnirostrum* se encuentra exclusivamente en los estados mexicanos de Michoacán, Guerrero y Oaxaca; y *E. c. clarae* se encuentra desde el sur de Sinaloa hasta Michoacán en México (mayores detalles en Forshaw 1989).

3.2. Amenazas sobre poblaciones de *E. canicularis*

Las dos amenazas principales sobre las poblaciones de psitácidos a nivel mundial son, en primer lugar la sustracción de individuos con fines comerciales, y en segundo lugar la destrucción del hábitat. En nuestro país estas amenazas también se han identificado como las causas principales de la disminución en el número de individuos de diferentes especies de psitácidos (Iñigo-Elias y Ramos 1991, Collar y Juniper 1992, Cantú-Guzmán *et al.* 2007, Weston y Memon 2009). En las aduanas internacionales, durante los años 2007-2014, individuos de diferentes especies de los géneros *Eupsittula* y *Aratinga* fueron los segundos más decomisados (2149 individuos) (UNODC 2016). De acuerdo con los reportes de UNODC (2016), se ha identificado que África y Latinoamérica son las regiones geográficas de origen de las especies decomisadas. También en este reporte se ha identificado que en dichas regiones se encuentran los países que exportan la mayor cantidad de individuos de manera ilegal. En Latinoamérica, desafortunadamente México ocupa el primer lugar en exportación y tránsito ilegal de psitácidos. En nuestro país, las especies que ocupan los primeros lugares de sustracción son: *Eupsittula canicularis*, *Amazona albifrons*, *Forpus cyanopygius*, *Aratinga nana*, *Amazona autumnalis* y *Amazona finschi* (Cantú-Guzmán *et al.* 2007).

E. canicularis es la especie que más se sustrae en México, se ha estimado que cada año se capturan aproximadamente 23,500 individuos (Cantú-Guzmán *et al.* 2007). Como se mencionó, una parte de los individuos que son sustraídos de las poblaciones silvestres son enviados al comercio internacional, sin embargo la gran mayoría van al mercado local o regional de mascotas (Iñigo-Elias y Ramos 1991, Cantú-Guzmán *et al.* 2007). Lamentablemente, las tasas de mortalidad registradas durante esta práctica (sustracción-venta) son altas, entre el 66 y 83% de los individuos mueren durante este proceso (Iñigo-Elias y Ramos 1991, Enkerlin 2000, Cantú-Guzmán *et al.* 2007). Adicionalmente, se ha analizado la mortalidad por etapas durante la sustracción-venta, y se ha identificado que en el periodo del acopio y estancia ocurre el mayor porcentaje de mortalidad (cerca del 30%).

A pesar de lo anterior, a nivel internacional la IUCN (2017) ubica a la especie *E. canicularis* en la categoría de preocupación menor, en CITES (2017) está en el apéndice II, y en nuestro país bajo la NOM-059-SEMARNAT-2010 (DOF 2010) se encuentra Sujeta a Protección Especial. Esto posiblemente debido a que su población no se ha visto gravemente mermada ya que tiene un éxito reproductivo alto y es capaz de anidar en termiteros ubicados en áreas modificadas (Sánchez-Martínez y Renton 2009).

Una vez que autoridades mexicanas realizan un decomiso, los individuos se depositan en centros de estancia conocidos como Centros para la Conservación e Investigación de la Vida Silvestre (CIVS). Después de mantenerlos en cuarentena, su principal objetivo es realizar reintroducciones. Desafortunadamente, por la naturaleza de la práctica (sustracción-venta) el lugar de origen de los individuos decomisados es desconocido, limitando la realización de reintroducciones adecuadas. Ante la imposibilidad de regresarlos a sus poblaciones silvestres, los grupos de aves permanecen en los CIVS o son enviados a zoológicos durante tiempo indefinido en ambos casos. Se debe destacar que estos organismos representan no solo la pérdida de individuos para sus poblaciones, sino que también son una pérdida para el acervo génico de las poblaciones de origen.

3.3. Filogeografía y conservación

Para proponer estrategias de conservación adecuadas, es indispensable reunir datos de diferentes fuentes que ayuden a incrementar las probabilidades de éxito de cada acción. El establecimiento de las relaciones genealógicas dentro de las especies es especialmente informativo en el contexto de la conservación; la identificación de grupos y la estimación de sus relaciones evolutivas es crucial para el diseño de estrategias eficientes (Avise 2000, Allendorf y Luikart 2007). Por citar un ejemplo puntual, a través de las reconstrucciones genealógicas es posible identificar poblaciones en divergencia, dichas poblaciones representan unidades evolutivas significativas (ESU) con una historia única (Moritz 1994). Las propuestas de conservación basadas en ESU reconocen la historia evolutiva de las poblaciones y plantean manejarlas por separado ya que representan linajes que están evolucionando de manera independiente. Identificar linajes intraespecíficos y proponerlos *per se* para su conservación es un buen inicio pero es simplista. A través de la filogeografía se puede establecer la influencia

de factores geológicos o climáticos históricos que moldearon la distribución de linajes y por lo tanto que nos ayuden a entender los procesos que detonan la diversidad biológica.

El concepto de filogeografía fue propuesto por Avise *et al.* (1987) para referirse al estudio de la relación entre la genealogía y la geografía. A través de los años se ha consolidado como una disciplina que se encarga de explicar los principios y procesos que gobiernan la distribución geográfica de los linajes genéticos, particularmente dentro de las especies o entre especies cercanas (Avise 2000, 2009).

La mayoría de las especies están constituidas por poblaciones geográficamente estructuradas, las cuales pueden experimentar poco o ningún contacto genético por largos periodos de tiempo derivando en divergencia de linajes (Avise 2000). Se espera que exista una estructura filogeográfica entre poblaciones aisladas por largos periodos de tiempo, para lo cual se requieren cientos de generaciones en aislamiento para que mutaciones nuevas se fijen localmente (Allendorf y Luikart 2007). Los patrones filogeográficos basados en datos moleculares sugieren que factores biogeográficos históricos, factores ecológicos contemporáneos y el comportamiento de los organismos son los responsables de moldear los patrones genéticos de las especies existentes (Avise *et al.* 1987).

En el área de la ornitología, Ball y Avise (1992) sugieren que las aves exhiben una gran variedad de historias demográficas y filogenéticas. Por ello es necesario realizar descripciones filogeográficas caso por caso. En México existe una gran diversidad de aves, constituyen aproximadamente el 10% de las especies de aves del mundo (1070 especies), en número superan a las calculadas para Canadá y USA juntos (Escalante-Pliego *et al.* 1993, Navarro y Benítez 1993, Howell y Webb 1995). La tarea de describir historias filogeográficas para México demandará recursos humanos especializados y económicos considerables, por lo cual se debe iniciar con aquellas especies bajo alguna categoría enlistada en la NOM-059-SEMARNAT-2010 (DOF 2010), como en el caso de la especie que nos ocupa, y que adicionalmente es la número uno en sustracción y venta ilegal en México como se ha mencionado con anterioridad.

3.4. Filogeografía en aves Neotropicales

Previo a los análisis filogeográficos, se propuso que los factores promotores de la biodiversidad de aves de México son los procesos históricos (como las oscilaciones climáticas

y los refugios pleistocénicos asociados), la variedad de ambientes promovida por la complejidad topográfica, y la posición geográfica de nuestro país (donde existe la presencia de aves de origen neártico y neotropical) (Escalante-Pliego *et al.* 1993).

Los análisis filogeográficos en aves de distribución Neártica con presencia en México, han permitido establecer que los cambios ambientales del Pleistoceno y la heterogeneidad topográfica promovieron la diversificación genética de las poblaciones e iniciaron la mayoría de las divisiones filogenéticas durante los últimos dos millones de años (Avise y Walker 1998, Johnson y Cicero 2004, Klicka *et al.* 2011).

En general, para aves de distribución Neotropical se ha establecido que los linajes se originan durante el Plioceno y Cuaternario, y se ha propuesto que los patrones genéticos observados son debidos a procesos históricos, a barreras geográficas (ríos, cadenas montañosas, planicies, etc.), a factores idiosincráticos de las especies, o a una combinación de estos. Además, en los patrones filogeográficos descritos para aves neotropicales se han observado linajes de divergencia profunda, somera, poca o nula diferenciación genética entre poblaciones.

Algunos de los trabajos donde se ha determinado que las fluctuaciones climáticas del Plioceno y Pleistoceno provocaron la fragmentación de hábitats promoviendo la diferenciación genética entre poblaciones son en las especies *Xiphorhynchus fuscus* (Cabanne *et al.* 2007, 2008), *Junco phaeonotus* (Milá *et al.* 2007), *Amazona aestiva* (Caparroz *et al.* 2009), *Lepidocolaptes affinis* (Arbeláez-Cortés *et al.* 2010), *Myioborus miniatus* (Pérez-Emán *et al.* 2010), *Sclerurus scansor* (D'Horta *et al.* 2011), *Campylopterus curvipennis* (González *et al.* 2011), *Amazila cyanocephala* (Rodríguez-Gómez *et al.* 2013), *Amazila violiceps* y *A. viridifrons* (Rodríguez-Gómez y Ornelas 2015), *Pyriglena atra* y *P. leucoptera* (Maldonado-Coelho 2012).

Por otro lado, existen investigaciones donde se ha destacado que los grandes ríos de Sudamérica son la causa principal de la diversificación para algunas especies de aves o sus poblaciones. Algunos ejemplos son dados a continuación, entre poblaciones dentro de *Lepidothrix coronata* (Cheviron *et al.* 2005), para las especies *Xiphorhynchus kienerii* y *X. obsoletus* (Aleixo 2006), en especies dentro del género *Psophia* (Ribas *et al.* 2012), o entre taxa de diferentes familias (Naka *et al.* 2012).

De igual manera, existen publicaciones donde se describe que otras barreras como las cadenas montañosas están implicadas en la diversificación de aves, por ejemplo entre poblaciones de

las especies *Adelomyia melanogenys* (Chaves *et al.* 2007), *Leptopogon amaurocephalus* (Bates and Zink 1994), *Amazilia cyanocephala* (Rodríguez-Gómez *et al.* 2013), o entre especies del género *Tangara* (Burns y Naoki 2004).

Adicionalmente, la poca diferenciación genética identificada en aves neotropicales, es atribuida a flujo genético alto, a expansiones poblacionales recientes o a factores idiosincráticos como la habilidad de dispersión, por ejemplo en *Chysomus icterocephalus* (Cadena *et al.* 2011), *Amazilia beryllina*, *A. cyanura*, *A. saucerottei* (Jiménez y Ornelas 2016), *Schiffornis virescens* (Cabanne *et al.* 2013), o como se ha observado entre especies de diferentes familias (Bates *et al.* 2003).

3.4.1. Patrones filogeográficos en aves neotropicales de México

Las descripciones de los patrones filogeográficos en aves neotropicales mexicanas se han realizado principalmente con aquellas que habitan en el bosque mesófilo de montaña y en el bosque tropical seco. Aunque, se acepta que como los hábitats no son estrictos, a las aves del bosque mesófilo de montaña (y/o de pino, encino) también se les conoce como aves de tierras altas o de montaña, y su distintivo es que ocupan hábitats que se encuentran a más de 1500 metros sobre el nivel del mar (msnm); a las aves del bosque tropical seco también se les conoce como aves de tierras bajas, que habitan desde los 0 hasta los 1500 nsnm.

Generalmente, las poblaciones de aves de tierras altas en México están diferenciadas genéticamente y presentan linajes que concuerdan con el rango de las montañas que habitan, algunas de las especies donde se observa esta condición son, *Chorospingus oftalmicus* (García-Moreno *et al.* 2004, Bonaccorso *et al.* 2008, Maldonado-Sánchez *et al.* 2016), *Lampornis amethystinus* (Cortés-Rodríguez *et al.* 2008, Ornelas *et al.* 2016), *Aulacorhynchus prasinus* (Puebla-Olivares *et al.* 2008), *Pharomachrus mocinno* (Solórzano *et al.* 2004), *Amazilia cyanocephala* (Rodríguez-Gómez *et al.* 2013), y *Lepidocolaptes affinis* (Arbeláez-Cortés *et al.* 2010), entre otras.

En detalle, se observan linajes de divergencia profunda en *Chorospingus oftalmicus* (García-Moreno *et al.* 2004, Bonaccorso *et al.* 2008, Maldonado-Sánchez *et al.* 2016), *Lampornis amethystinus* (Cortés-Rodríguez *et al.* 2008, Ornelas *et al.* 2016) y *Aulacorhynchus prasinus* (Puebla-Olivares *et al.* 2008). Además, se ha identificado que poblaciones de *Amazilia*

cyanocephala (Rodríguez-Gómez *et al.* 2013) y *Lepidocolaptes affinis* (Arbeláez-Cortés *et al.* 2010) muestran una diferenciación intermedia y mantienen flujo génico.

Para explicar la diferenciación genética profunda y la estructura filogeográfica, se ha propuesto que son el resultado de fragmentación del hábitat debida a cambios climáticos del Plioceno y Pleistoceno, por ejemplo en *Chorospingus oftalmicus* (García-Moreno *et al.* 2004, Bonaccorso *et al.* 2008, Maldonado-Sánchez *et al.* 2016) y *Aulacorhynchus prasinus* (Puebla-Olivares *et al.* 2008). Además, se ha identificado que una de las barreras geográficas para el intercambio genético de aves de tierras altas es el Istmo de Tehuantepec. Se ha observado que esta barrera limita en mayor o menor medida el flujo génico entre poblaciones de las especies *Amazilia cyanocephala* (Rodríguez-Gómez *et al.* 2013), *Lampornis amethystinus* (Cortés-Rodríguez *et al.* 2008, Ornelas *et al.* 2016), y *Lepidocolaptes affinis* (Arbeláez-Cortés *et al.* 2010). O bien, que como en *Campylopterus curvipennis* (González *et al.* 2011) la divergencia es el resultado de una combinación de una barrera geográfica (Istmo de Tehuantepec) y fragmentación de hábitat debido a cambios climáticos durante el Pleistoceno.

Como se ha expuesto, en general las aves de tierras altas de México están estructuradas geográficamente y la divergencia entre poblaciones es ocasionada principalmente por la fragmentación del hábitat (que ocurrió como consecuencia de cambios climáticos históricos), pero también las tierras bajas como el Istmo de Tehuantepec han sido identificadas como barreras geográficas para estas especies.

Por otro lado, las aves de tierras bajas en México muestran patrones filogeográficos diversos, se han observado poblaciones diferenciadas genéticamente y geográficamente estructuradas como en *Campylorhynchus rufinucha* (Vázquez-Miranda *et al.* 2009), o con linajes someros en *Doricha eliza* (Licona-Vera y Ornelas 2014) *Melanerpes chrysogenys*, *Passerina leclancherii* y *Momotus mexicanus* (Arbeláez-Cortés *et al.* (2014). O bien, se han observado grupos de divergencia intermedia como en el caso de *Vireo hypochryseus* (Arbeláez-Cortés *et al.* (2014), o *Icterus pustulatus* (Cortes-Rodríguez *et al.* 2008). Cabe mencionar que en las especies analizadas aún no se ha observado una divergencia de linajes profunda como en las aves de tierras altas. Los detalles de estos ejemplos son descritos en la siguiente sección, debido a que se distribuyen de manera similar a *E. canicularis* en el bosque tropical seco de México.

3.4.2. Patrones filogeográficos en aves del BTS de México

E. canicularis está fuertemente asociada al bosque tropical seco del Pacífico mexicano. El bosque tropical seco (BTS) es uno de los principales tipos de vegetación en México (Rzedowski 1978). Está constituido por una alta diversidad de plantas, en su mayoría son caducifolias que pierden sus hojas durante la época seca del año (durante 5-6 meses recibe menos de 100 mm), aunque también tiene especies suculentas y perenes (Pennington *et al.* 2009). En México, el BTS se extiende de manera continua desde los 0 hasta los 1900 msnm, se encuentra por la vertiente del Pacífico abarcando el centro de Sonora y sureste de Chihuahua hasta Chiapas. Cubre zonas de la cuenca del Río Balsas y Santiago, y también tiene presencia en manchones en el extremo sur de Baja California, en el sur de Tamaulipas, sureste de San Luis Potosí, norte y centro de Veracruz, noreste de Querétaro y norte de la península de Yucatán; adicionalmente tiene presencia discontinua en Centroamérica y Sudamérica (Fig. 1A, apéndice) (Rzedowski 1978, Becerra 2005, Pennington *et al.* 2009).

Las investigaciones que describen historias filogeográficas de aves que habitan en el BTS de México son escasas. Hasta donde conocemos existen publicados cinco artículos que describen patrones en las especies *Melanerpes chrysogenys*, *Passerina leclancherii*, *Momotus mexicanus*, *Icterus pustulatus*, *Vireo hypochryseus*, *Campylorhynchus rufinucha* y *Doricha eliza*. Estos documentos son descritos brevemente a continuación.

En *Icterus pustulatus*, Cortes-Rodríguez *et al.* (2008) han reportado que existe una divergencia somera, con estructura geográfica débil y determinan que puede ser debida a una expansión reciente de la población. Además, identifican tres grupos genéticos, el grupo A está desde Guerrero hasta Honduras, encontraron al grupo B desde Sonora hasta Guerrero y hacia el interior del país en Jalisco, Zacatecas y Puebla, y finalmente el grupo C está en el centro de México, en Michoacán, Guerrero, Puebla y Oaxaca.

La especie *Campylorhynchus rufinucha* que se encuentra en poblaciones disyuntas por la vertiente del Pacífico y del Golfo de México fue analizada por Vázquez-Miranda *et al.* (2009), en su estudio incluyen individuos desde México hasta Costa Rica. En México encuentran cuatro haplogrupos: Veracruz, Guerrero-Oaxaca, Chiapas, y en los límites de Oaxaca y Chiapas encuentran un haplotipo compartido entre poblaciones a cada lado del Istmo de Tehuantepec. En este trabajo, no especifican factores que propician la diversificación.

Arbeláez-Cortés *et al.* (2014), analizan el patrón filogeográfico de tres especies pertenecientes a diferentes órdenes de aves que habitan la parte norte del BTS: *Melanerpes chrysogenys*, *Passerina leclancherii*, y *Momotus mexicanus*. Encuentran estructura filogeográfica a pesar de que estas especies habitan en un área relativamente pequeña (<200,000 km^2), que ocupan rangos aparentemente continuos donde no existen barreras geográficas evidentes y que poseen habilidad de dispersión por vuelo. Mediante la descripción del patrón filogenético de cada especie, identifican grupos que coinciden con áreas geográficas, y encuentran que las discontinuidades están en Guerrero-Oaxaca, y Jalisco-Michoacán. Sugieren que este patrón puede estar asociado con la historia paleoecológica de la región, es decir, que probablemente las fluctuaciones climáticas del Pleistoceno fragmentaron y reconectaron el BTS causando el aislamiento de poblaciones de estas aves.

En otro trabajo, Arbeláez-Cortés *et al.* (2014) analizan individuos de *Vireo hypochryseus*, y encuentran dos grupos diferenciados, el grupo 1 está en las Islas Marías y en Sinaloa y el grupo 2 se encuentra desde Jalisco hasta Oaxaca y en Hidalgo. Igual que en el artículo anterior, proponen que probablemente la fragmentación de hábitat debida a oscilaciones climáticas de Pleistoceno causó la diversificación dentro de la especie.

Licona-Vera y Ornelas (2014), analizan a la especie *Doricha eliza*, cabe mencionar que esta no habita por la vertiente del Pacífico, sus poblaciones están restringidas al BTS de Yucatán y Norte de Quintana Roo y al BTS de Veracruz. Encuentran dos grupos diferenciados genéticamente, con flujo génico restringido. Explican que sus poblaciones pasaron por un período de aislamiento por distancia y divergencia en condiciones ambientales distintas.

Finalmente, para *E. canicularis* no existen trabajos previos que involucren análisis de diversidad genética o que describan los patrones filogeográficos de sus poblaciones. Por lo anterior, en el presente trabajo se propuso realizar una descripción de los patrones filogeográficos de *E. canicularis* en la parte norte de su distribución, y determinar el efecto de los cambios climáticos del Cuaternario en la historia demográfica de la especie. Adicionalmente, recordando la condición vulnerable de la especie, se propuso utilizar la información genealógica y filogeográfica para establecer estrategias de conservación, en particular identificar unidades evolutivas significativas y sugerir posibles sitios de reintroducción para un grupo de individuos decomisados.

IV. HIPÓTESIS

4.1. Es posible identificar unidades evolutivas significativas en la especie *E. canicularis* a través de monofilia recíproca con secuencias del ADNmt.

4.2. El patrón filogeográfico de *Eupsittula canicularis* en el centro y norte de su distribución mostrará discontinuidades genéticas similares a otras especies distribuidas en tierras bajas del bosque tropical seco.

4.3. Los cambios climáticos del Cuaternario afectaron de manera negativa el crecimiento poblacional de la especie.

4.4. Los datos genéticos obtenidos servirán para proponer sitios de reintroducción para la especie.

V. OBJETIVOS

OBJETIVO GENERAL

Evaluar la diversidad genética, describir la historia genealógica y demográfica de *Eupsitula canicularis*.

OBJETIVOS ESPECÍFICOS

1. Describir el patrón filogeográfico de *Eupsittula canicularis* en el centro y norte de su distribución.
2. Analizar la historia demográfica de la especie y determinar si tiene relación con los cambios climáticos del Cuaternario.
3. Identificar unidades evolutivas significativas y proponerlas como grupos para su conservación.
4. Con base en la información establecida, determinar sitios de reintroducción para individuos decomisados.
5. Describir la diversidad genética contenida en individuos decomisados.

VI. RESULTADOS

CAPÍTULO I

ARTÍCULO 1

De la aplicación de herramientas filogenéticas para la identificación de unidades evolutivas significativas a través de monofilia recíproca en *E. canicularis*, y su valor en la conservación de la especie. Artículo publicado: Ornitología Neotropical 26, 297-307, 2015.

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

Gabriela Padilla-Jacobo[1], Tiberio C. Monterrubio-Rico[2], Horacio Cano-Camacho[1] and María G. Zavala-Páramo[1]

[1]Centro Multidisciplinario de Estudios en Biotecnología, FMVZ, Universidad Michoacana de San Nicolás de Hidalgo (Padilla-Jacobo, Cano-Camacho and Zavala-Páramo); [2]Laboratorio de Ecología de Vertebrados Terrestres Prioritarios, Facultad de Biología, Universidad Michoacana de San Nicolás de Hidalgo, Morelia, Michoacán, México (Monterrubio-Rico).

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 Mexico, 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 y 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. canicularis*, *E. nana*, *E. pertinax* and *E. cactorum*. 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 c. canicularis*, *Eupsittula c. clarae*, *Euspsittula c. eburnirostrum*, *Eupsittula nana*, *Eupsittula pertinax*, Evolutionarily Significant Units, molecular phylogeny, Orange-fronted Parakeet, Psittacidae, reciprocal monophyly.

Resumen

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 análizaron 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. canicularis, E, nana, E. pertinax* y *E. cactorum*. 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 ESU's independientes para fines de conservación.

Palabras clave: Conservación, *Eupsittula aurea*, *Eupsittula cactorum*, *Eupsittula c. canicularis*, *Eupsittula c. clarae*, *Euspsittula c. eburnirostrum*, *Eupsittula nana*, *Eupsittula pertinax*, Unidades Evolutivas Significativas, filogenia molecular, Perico Frente-naranja, Psittacidae, monofilia recíproca.

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). *E. 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 forecrown, pale olive throat and chest, and horn-colored bill. *E. c. eburnirostrum* is found only in Mexico 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 km^2 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 km^2 (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 (Avise 2000).

Although the relation between molecular characteristics and subspecific determinations in bird species has been discussed (Avise & 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 (Avise & 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 the 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 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.

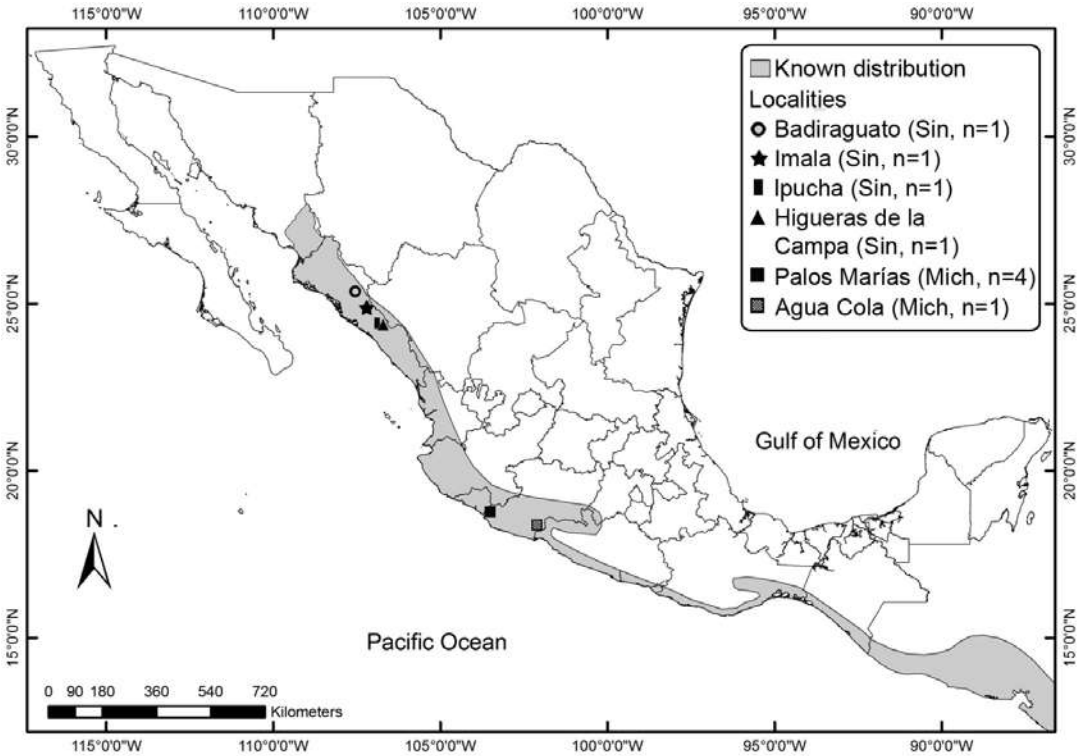

FIG. 1. Geographic distribution (northern range) and sampling localities (México) of the Orange-fronted Parakeet *Eupsittula canicularis*. Agua Cola, Michoacán (18º20'N, 102º05'W), Badiraguato, Sinaloa (25º28'N, 107 º37'W), Higueras de la Campa, Sinaloa (24º31'N, 106º47'W), Imala, Sinaloa (24º51' N107º12'W), Ipucha, Sinaloa (24º20'N, 106º45'W), Palos Marías, Michoacán (18º49'N 103º34'W).

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 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 the phenotypic descriptions and distributions 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'-TATCGGGCCCATACCCCGAATA-3') and HTrpC (5'-CGGACTTTAGCAGAAACTAAGAG-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 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 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) 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 branches-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. nana* (GenBank Access: GU826184 and HQ270490), *E. pertinax* (GenBank Access: JQ174079.1 and EU327600.1) and *E. cactorum* (AF370750). 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 (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 $1x10^6$ 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 haplotipic diversity (Hd) of 0.944 and nucleotidic diversity (Pi) of 0.004. The ND2 gene had five haplotypes (1_{acND2} - 5_{acND2}) with nine polymorphic sites (Hd = 0.722 and Pi=0.002).

The phylogenetic analysis of the *E. canicularis* and the congeners shows that EcNCBI clearly groups with *E. c. clarae* and *E. c. eburnirostrum* indicating that it belongs to the species *E. canicularis* (Fig. 2A). Since the analysis shows weak bootstrap support for the separating *E. canicularis* and *E. nana*, this can be explained by the data/taxa lack.

In the trees built to determine intraspecific relations in *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. c. eburnirostrum* of Michoacán.

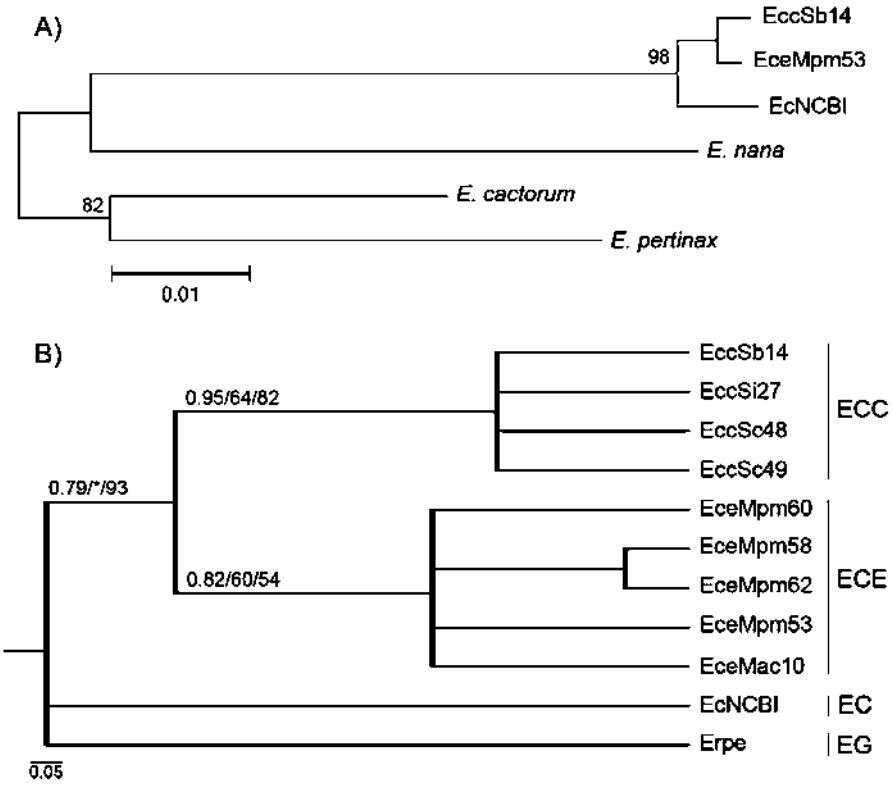

FIG. 2. A) Phylogenetic tree showing the relationships among species of *Eupsittula*, constructed with 1607 bp of the concatenated sequences of COI and ND2; bootstrap values are displayed on the branches. B) Three showing relationship among subspecies of *E. canicularis* constructed with 1545 bp of the 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 branches length scale. The length of the branches is proportional to the amount of genetic change.

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

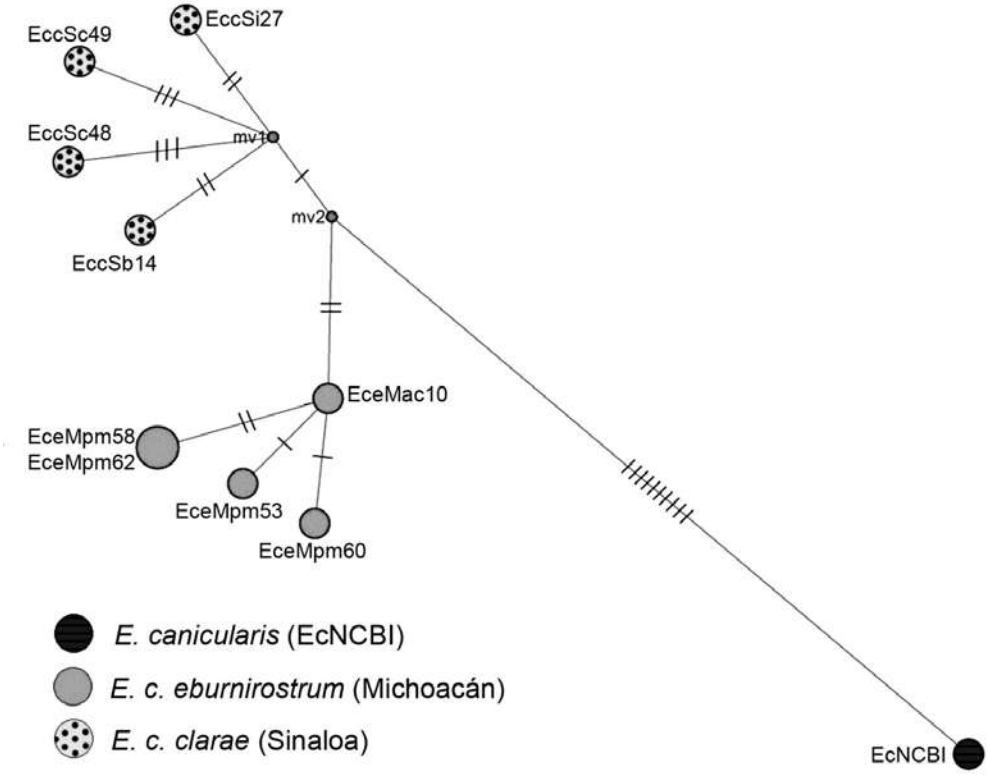

FIG. 3. Median-joining haplotype network showing the relationships between subspecies of *Eupsittula canicularis*, based on 1545 bp of the concatenated sequences of the COI and ND2 markers. The circle size is proportional to the number of individuals sharing the haplotype, but the length of the branches 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)**.**

Discussion

Phylogenetic relationships were analyzed for two of the three subspecies of *E. canicularis*. As a result, in all trees constructed two clades can be identified that likely correspond to the recognized subspecies *E. c. clarae* and *E. c. eburnirostrum*. The clades likely correspond to diverging subpopulations, 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, providing 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; *Aegotheles wallacii,* 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 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 are sympatric populations in Michoacán, or if there is hybridization between *E. c. clarae* and *E. c. eburnirostrum* in the region, collection should be expanded to additional areas in Michoacán and neighboring regions in addition to the use of additional markers that would allow identification of these population phenomena.

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 individual is from captivity and its true geographic origin is unknown (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 likely

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 would be necessary to use polymorphic markers of nuclear DNA (nDNA) 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*) mDNA differentiation is substantial both among local colonies and among distant geographical regions; however, the differentiation among colonies is much less pronounced at microsatellites 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 likely valid ESU's is of conservation importance for each of these units. The species occur in at least 11 protected areas of México, covering 2,303 km^2, which represent only 1.6% of the species total distribution in the country (Marín-Togo *et al.* 2012). However, 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 the protected areas to assess future conservation priorities and to ensure the conservation of the species as a whole.

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CAPÍTULO II

ARTÍCULO 2

Descripción de la distribución de linajes en *E. canicularis* en la parte centro y norte de su distribución en México. Considera también la estimación de la historia demográfica de la especie, y su relación con cambios climáticos del Cuaternario. Artículo sometido a IBIS Journal de British Ornithologists´ Union.

The effect of Quaternary climate changes on diversification and demographic history of Orange-Fronted parakeet *Eupsittula canicularis*

Gabriela Padilla-Jacobo[1], Tiberio C. Monterrubio-Rico[2], Horacio Cano-Camacho[1] and María G. Zavala-Páramo[1]

[1]Centro Multidisciplinario de Estudios en Biotecnología, FMVZ, Universidad Michoacana de San Nicolás de Hidalgo (Padilla-Jacobo, Cano-Camacho and Zavala-Páramo); [2]Laboratorio de Ecología de Vertebrados Terrestres Prioritarios, Facultad de Biología, Universidad Michoacana de San Nicolás de Hidalgo, Morelia, Michoacán, México (Monterrubio-Rico).

Abstract

The effect of Quaternary climate changes on bird populations inhabiting lowland dry tropical forests in the northernmost part of their distribution has been the focus of little research. Molecular analyses can contribute to an understanding of the present and historic states of a species. In this study, we analyzed the genetic diversity, the intraspecific divergence pattern and the historical demography of the psittacine *Eupsittula canicularis* using molecular data. Moreover, we analyzed the possible effect of the climatic changes of the Quaternary on the populations of this species. The results suggest a population growth from a small effective size, a recent expansion occurred during the Upper Pleistocene and a slight decrease for the approximately 19,000 years until the present time. We identified three genetic lineages denominated North, Balsas and South consistent with their genealogic relations and geographic distributions. The Balsas lineage in the western Balsas Basin was the apparent origin of the North (with individuals located from the north coast of Michoacan to Sinaloa) and South lineages (constituted by individuals of the coasts of Michoacán and Guerrero). Given the absence of obvious geographical barriers in the region, we propose that the observed pattern is a result of climatic changes, and we suggest that the Balsas Basin could be a refuge.

Keywords: Genetic diversity, Quaternary, tropical deciduous dry forests, Psittacidae.

Resumen

Existe poca investigación sobre el impacto de los cambios climáticos del Cuaternario en las poblaciones de aves que habitan tierras bajas del bosque tropical seco en la parte más norteña de su distribución. Los análisis moleculares pueden contribuir al conocimiento del estado actual e histórico de las especies. En este estudio, analizamos la diversidad genética, el patrón de divergencia intraespecífica y la demografía histórica del psitácido *Eupsittula canicularis* usando datos moleculares. Por otra parte, analizamos el posible impacto de los cambios climáticos del Cuaternario. Los resultados sugieren un crecimiento demográfico a partir de un tamaño efectivo de la población pequeño, una expansión reciente que ocurrió durante el Pleistoceno Superior y un ligero descenso de la población desde hace aproximadamente 19 mil años hasta la actualidad. Identificamos tres linajes genéticos denominados Norte, Balsas y Sur

de acuerdo a sus relaciones genealógicas y distribución geográfica. Aparentemente el linaje Balsas ubicado en la cuenca Oeste del Balsas es el origen de los linajes Norte (con individuos localizados desde la costa Norte de Michoacán hasta Sinaloa) y Sur (constituido por individuos de la costa de Michoacán y Guerrero). Ante la ausencia de barreras geográficas evidentes en la región, proponemos que el patrón observado es resultado de los cambios climáticos y sugerimos que la cuenca del Balsas pudo ser un refugio.
Palabras clave: Diversidad genética, Cuaternario, bosque tropical seco, Psittacidae.

Introduction

The Quaternary period includes the Pleistocene and Holocene epochs, beginning 2.58 million years ago and extending to the present (IUGS 2014). During this period, climatic oscillations occurred between warmer and cooler conditions. These large global interglacial-glacial-interglacial climate oscillations have been recurring at approximately 100,000 years periodicity for the last 900,000 years (Berger et al. 1993; Mudelsee and Schulz 1997). These climatic changes are proposed as one of the primary factors that influenced the distributions and population dynamics of species and as consequence, the patterns of genetic diversity (Hewitt 2000, 2004a; Provan and Bennett 2008; Stewart et al. 2010). In birds, Pleistocene environments promoted genetic diversification and initiated phylogeographic splits (Avise et al. 1987; Avise and Walker 1998; Johnson and Cicero 2004). Based on DNA data, the pronounced phylogeographic separations within avian species usually occurred within the last two million years (Avise and Walker 1998; Johnson and Cicero 2004).

With phylogenetic and genetic diversity analyses, phylogeographic patterns and demographic histories can be reconstructed, which provide support to establish the origins of current biological diversity (Hewitt 2004b; Schmitt 2007; Provan and Bennett 2008). For bird distributions in Mexico, these types of analyses are scarce in proportion to the diversity of birds in the country. For the distributions of Neotropical birds in highlands, García-Moreno et al. (2004), Cadena et al. (2007), McCormack et al. (2008), Navarro-Sigüenza et al. (2008) and Puebla-Olivares et al. (2008) provide some examples. These analyses identify well differentiated intraspecific clades, some with deep divergence, which are attributed to geographical barriers and climatic changes of the Quaternary. However, for lowland Neotropical species inhabiting dry tropical forests in Mexico, very little information is

available. In several taxa analyzed in west Mexican tropical dry forests, some species have been discovered that show divegent lineages associated with several biogeographic breaks (Cortés-Rodríguez et al. 2008a; Arbeláez-Cortés et al. 2014a, 2014b).

A group of Neotropical birds that deserve particular attention are the psittacines. In America, these birds constitute the Arini tribe of Neotropical origin and are one of the groups of birds that suffer the most from subtractive pressure for their illegal trade in the world. Regarding the origin of the Arini tribe, the monophyly of the American parakeets has been widely accepted (Tavares et al. 2006; Tokita et al. 2007; Wright et al. 2008; Schweizer et al. 2010), and the origin and divergence of the Arini tribe are proposed to have begun in South America (Tavares et al. 2006). Later, through dispersal events, some representative taxa arrived in North America, with the primary routes that could be followed those from Central America to the northeast and northwest and through the Caribbean (Manuscript submitted). The highest latitude reached by current psittacines on the American continent is in Mexico (Howell and Webb 1995). Twenty-one species of psittacine inhabit Mexico and are distributed primarily in the south and on the slopes of the Atlantic and Pacific (Forshaw 1989; Escalante-Pliego et al. 1993; Howell and Webb 1995; Chesser et al. 2014). The type of vegetation that inhabits the side of the Pacific slope is primarily dry tropical forest (Howell and Webb 1995).

The analyses that consider molecular data involving Mexican psittacines are primarily dedicated to establishing phylogenetic relationships among a few taxa and are not intended to describe origins of genetic diversity (Eberhard et al. 2004, 2015; Russello and Amato 2004; Tavares et al. 2006; Latta et al. 2010; Kirchman et al. 2012; Smith et al. 2013). Although some published sequences are found in databases, no articles that analyze the distribution of the genetic diversity of psittacines in Mexico have been published. Orange-Fronted parakeet *Eupsittula canicularis* is one of the species of American psittacines that reaches greater latitudes in America. The distribution of the species is continuous from south of Sinaloa in Mexico on the slope of the Pacific to the north of Costa Rica (Forshaw 1989; Howell and Webb 1995; Collar et al. 2000). The lowlands of the tropical deciduous dry forests are the habitat of the species, but this parakeet can also be found in humid and subhumid deciduous forests, riparian forests, and agricultural areas (Ridgely 1981; Howell and Webb 1995; Stotz et al. 1996; Collar et al. 2000). The arrival of *E. canicularis* to Central America occurred posterior to the last closure of the Isthmus of Panama (3.5 millions of years ago), and later by

dispersion, the parakeet reached the north of Mexico (Manuscript submitted). With this distribution pattern, *E. canicularis* represents an appropriate species to establish the effects of climatic changes of the Quaternary on the demography of a Neotropical species in its most northern distribution.

For *E. canicularis*, at least two genetic groups occur separated by geographical distance in the northern region of the distribution (Padilla-Jacobo et al. 2016). However, the low number of samples and the use of mitochondrial markers exclusively limited interpretation of this analysis.

The purpose of this study was to describe the phylogeographic pattern of *E. canicularis* in the center-north of the distribution, with molecular data and using phylogenetic approaches. We also explored the historical demography to determine whether changes occurred in the population that might be associated with the climatic changes of the Quaternary.

Materials and Methods

Biological samples

Feather and blood samples from specimens collected from nests in 2005, 2006, 2007, 2013, 2014 and 2016 were used. 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 and subsequently stored at 4°C. These samples were deposited in the wildlife sample collection at the Multidisciplinary Center for Studies in Biotechnology (Centro Multidisciplinario de Estudios en Biotecnologia, CMEB, for initials in Spanish) at Universidad Michoacana de San Nicolas de Hidalgo (UMSNH, for initials in Spanish) in Morelia, Michoacan, Mexico. We analyzed individuals from the central and northern regions of the distribution of *E. canicularis*. A total of 63 specimens of *E. canicularis* were collected (Table 1).

The euclidean distance between 25 points of the distribution of *E. canicularis* was approximately 3,338 km along the Pacific coast, and we analyzed approximately a third of the distribution (1,282 km euclidean distance of nine points; Figure 2 Appendix), which included

localities in the Mexican states of Sinaloa, Nayarit, Jalisco, Michoacan and Guerrero (Figure 1, Table 1). Additionally, biological samples of 80 individuals of unknown origin donated by Procuraduria Federal de Proteccion al Ambiente (acronym in Spanish PROFEPA) were integrated into some analyses.

Table 1. Collection localities of the biological samples. Abbreviations and numbers in Abb map correspond to sites marked on the map, and (n) is the number of samples.

Group	Locality	Abb map	n	Samples
WCM	Cosala	1	5	Arca2005-41, Arca2005-42, Arca2005-45, Arca2005-48 Arca2005-49.
	Culiacan, Imala	2	7	Arca2005-01, Arca2005-20, Arca2005-21 Arca2005-22, Arca2005-27, Arca2005-28, Arca2005-29.
	Mocorito, Badiraguato	3	4	Arca2005-14, Arca2005-15, Arca2005-16, Arca2005-17.
WCS1	Puerto Vallarta	4	3	Arca2016-01, Arca2016-02, Arca2016-03.
	Rincon de Guayabitos	5	1	Arca2013-01.
WCS2	Palos Marias	6	15	Arca2005-52, Arca2005-53, Arca2005-54, Arca2005-55, Arca2005-57, Arca2005-58, Arca2005-59, Arca2005-60, Arca2005-61, Arca2005-62, Arca2005-63, Arca2005-69, Arca2005-83, Arca2005-84,
	Chorumo, Zapotan	7	7	Arca2005-85. Arca2005-64, Arca2005-65, Arca2005-70, Arca2005-74, Arca2005-75, Arca2005-76, Arca2005-77.
WCS3	Agua Cola, Zapotillo	8	7	Arca2007-07, Arca2007-08, Arca2007-09, Arca2007-10, Arca2007-11, Arca2007-12, Arca2007-13.
WCS4	Tecpan, Atoyac	9	6	Arca2007-17, Arca2007-18, Arca2007-19, Arca2007-20, Arca2007-21, Arca2007-22.
BBW	Huetamo	10	6	Arca2014-01, Arca2014-02, Arca2014-03, Arca2014-04, Arca2014-05, Arca2014-06.
	Palma de Huaro	11	2	Arca2015-78, Arca2015-79.
	Total		63	

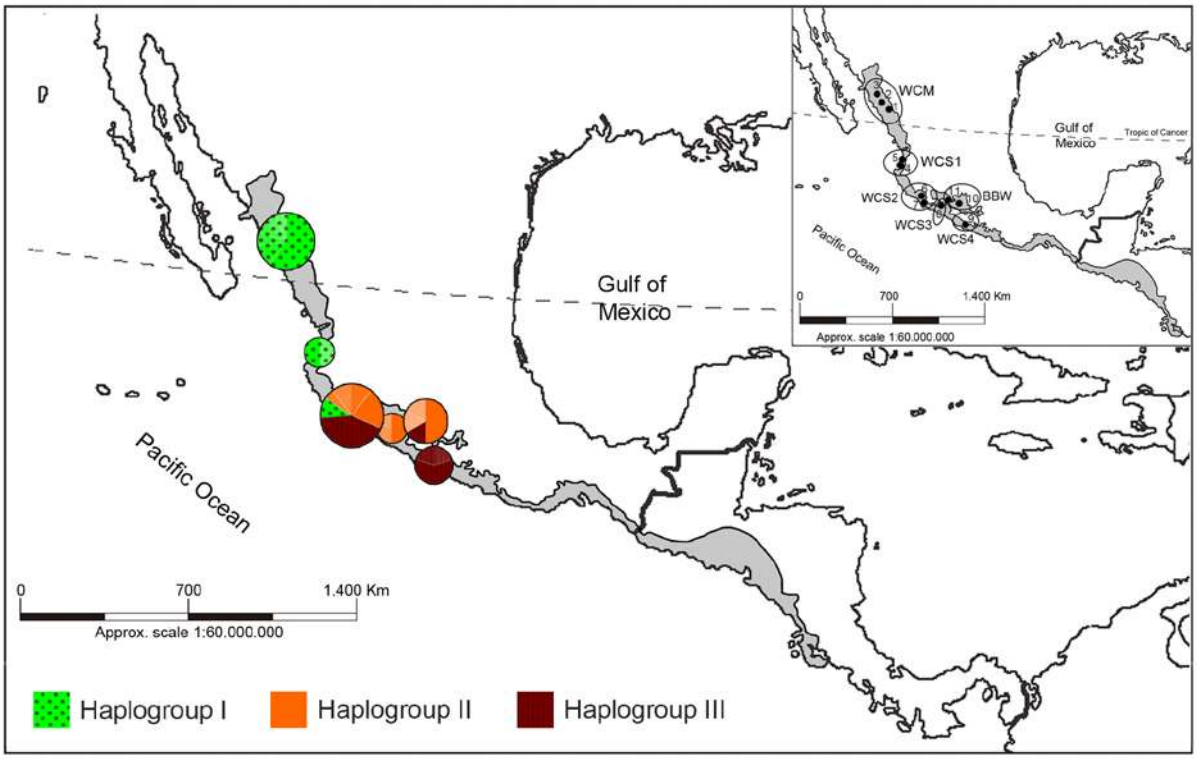

Figure 1. Geographic distribution and sampled regions of *E. canicularis*. The map is modified according to Monterrubio-Rico et al. (2016) and Collar et al. (2017). The shaded areas represent the distribution of the species. Points represent the sampling locations; for the numbers and abbreviations of each point, see Table 1.

DNA extraction and sequencing

DNA was extracted using the phenol-free method described by FitzSimmons (1997). We amplified three mitochondrial (COI, ND2 and Cytb) and two nuclear (CRYAA and TROP) sequences. To obtain a fragment of approximately 590 bp of the COI gene, we used the oligonucleotides COIarcaD (5'-CTACCACGCGGGCAAAAA-3') and COIarcaR (5'-CCCAATGGAGGATAAAGTGTT-3') (Padilla-Jacobo et al. 2016). For the amplifications of the ND2 gene, we used L5215 (5'-TATCGGGCCCATACCCCGAATA-3') and HTrpC (5'-CGGACTTTAGCAGAAACTAAGAG-3') (Hackett 1996) oligonucleotides to obtain a fragment of approximately 1140 bp. The Cytb sequence of approximately 1140 bp was obtained using the oligonucleotides CytbD (5′-ATCATCCGCACTATCTGTCCT-3′) and CytbR (5′- AATAGCCTTCGTCTTTTGGTTTA-3′) designed using DNASTAR Lasergene

software LG10VC (Kumar and Blaxter 2010), based on the Cytb sequences reported in GenBank for species of the genus *Eupsittula* and related genera.

After obtaining amplifications of the mtDNA, we amplified fragments of the nuclear genes CRYAA (crystallin alpha A) and TROP (tropomyosin alpha-1) of six individuals of distant localities. Oligonucleotides were designed with DNASTAR Lasergene software LG10VC (Kumar and Blaxter 2010) using sequences from the database of *Melopsittacus undulatus* (NW_004848230.1) to design TROP and of *Cacatua sulphurea* (EU737657.1) to design CRYAA. To obtain the CRYAA sequence, we used CRYAAL (5'-TCCTGCCTTTGTTCTCCTCCACTA-3') and CRYAAH (5'-GTCATCAATAATCTTCACGCTCAG-3') oligonucleotides, which produced a fragment of approximately 1100 bp. The TropoD (5'- TGGCAGTTCACCGTTTGTTC-3) and TropoR (5'-CAGCGACTTCAGGTTGTTGG-3) oligonucleotides were used for the amplifications of an approximately 650 bp fragment of the TROP gene.

The PCR reactions were performed in a total volume of 25 µl with the following components: 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, Grand Island, NY, USA), and 50 ng of DNA. The reactions were placed in a thermocycler (Gene Amp 2700; Applied Biosystems, Foster City, CA, USA) and amplified under the following conditions: 94°C for 5 min; followed by 30 cycles of 94°C for 40 s, 55-57°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 dideoxy method (Sanger et al. 1977); services were provided by Macrogen (Rockville, MD, USA).

Structure and genetic diversity analysis

Sequence editing, alignments, and the construction of data matrices were conducted with Sequencher v.4.1 (Gene Codes Corporation, Ann Arbor, Michigan, 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 revised with ARLEQUIN v3.1 (Excoffier et al. 2005). In the analyses of genetic diversity, we grouped the samples in six regions consistent with the sampling locations, which were the following: West Coast Middle (WCM), West Coast South 1 (WCS1), West Coast South 2 (WCS2), West Coast South 3

(WCS3), West Coast South 4 (WCS4) and Balsas Basin West (BBW) (details in Table 1, Figure 1).

To determine the variation distribution and genetic differentiation among groups, an analysis of molecular variance (AMOVA) (Excoffier et al. 2005) with 10,000 permutations was performed. To perform AMOVAs, we grouped the samples according to the following: 1) without a priori grouping, and 2) North (WCM and WCS1), Balsas (WCS3 and BBW) and South (WCS2 and WCS4). Additionally, computed pairwise comparisons of F_{ST} values with 1,000 permutations were obtained with ARLEQUIN v3.1 (Excoffier et al. 2005).

Relationships among haplotypes and phylogenetic analysis

To review frequencies of haplotypes and relationships among them, haplotype networks under the median-joining method with the software NETWORK v5 (Fluxus Technology Ltd. 2017) and data of Cytb were constructed.

Genealogic relationship reconstructions were generated using Maximum Likelihood (ML) and Bayesian Inference (BI) frameworks. The matrix for these analyses included haplotypes obtained in this study, sequences of 11 individuals reported in GenBank (Access: KJ612380-KJ612397; Euca34417: KJ142289.1, KJ142251.1; and Euca9252: HQ629753.1, HQ629718.1) and *Eupsittula pertinax* (Access: KJ142289.1) was included as the out-group. Molecular evolution models were constructed with jModelTest v2.1.1 (Posada 2008) and selected using the corrected Akaike Information Criterion (AICc) (Alfaro and Huelsenbeck 2006). The best model for COI was HKY (Hasegawa et al. 1985), for ND2, TrN+I (Tamura and Nei 1993) + Invariant sites) and for Cytb, TrN (Tamura and Nei 1993), and the best model was TIM3+I+G (Transition model, -Posada 2003- + Invariant sites + Gamma distribution) for all sequence data after concatenation.

The ML and BI reconstructions were performed using RAxML v7.8 (Stamatakis 2014) and MrBayes v3.2 (Ronquist and Huelsenbeck 2003) software. 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 with sampling 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

Bayesian skyline plot (Drummond et al. 2005) and mismatch distribution (Rogers and Harpending 1992) methods were used to infer demographic history. Bayesian skyline plot (Drummond et al. 2005) was performed with the program BEAST v1.7.4 (Drummond and Rambaut 2007). This analysis is sensitive to the number of samples, and for that reason, the 80 sequences of Cytb of individuals captured from unknown locations were included. The time axis was scaled using the rate of 2.7×10^{-3} substitutions per site per million years for Cytb reported for Psittaciformes by Pacheco et al. (Pacheco et al. 2011). Five independent runs with 30 million generations were performed. The model of substitution TrN with empirical base frequencies was utilized, 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 1,000 iterations, with a burn-in of 10%. The results of every run were combined in LogCombiner v1.7.4 (Drummond and Rambaut 2007). The result was visualized using TRACER 1.5 (Rambaut and Drummond 2007). Mismatch distributions were obtained in ARLEQUIN (Excoffier et al. 2005). The sudden expansion model (Schneider and Excoffier 1999) with 1,000 parametric bootstraps was used. Sum of squared deviations (SSD) and Harpending´s raggedness index (Hri) were determined to validate the sudden expansion assumption.

Results

Genetic diversity analysis and differentiation

From the DNA samples of *E. canicularis*, amplifications of the 53 COI fragments of 552 to 587 bp, 46 ND2 fragments of 1022 to 1100 bp and 53 Cytb gene sequences of 994 to 1161 bp were obtained and registered in GenBank (Access: MF441253-MF441296, MF441297-MF441333, KU532328-KU532329 and MF441340-MF441470). To obtain the metrics of genetic diversity, in the alignments, we used the lengths reported in table 2. We obtained 13 (ND2), 14 (COI) and 18 (Cytb) haplotypes when all samples were analyzed as a single group

and detected high haplotype diversity (Hd = 0.799-0.894) and low nucleotide diversity (Pi = 0.00198-0.00216) with the mitochondrial markers. In the TROP sequences obtained from six individuals and deposited in GenBank (Access: MF441334-MF441339), we detected a single allele of TROP, and therefore, the genetic diversity was zero for the analyzed samples (Table 2). In the CRYAA sequences we find at least ten alleles with polymorphic sites in the intron sequence, where transitions were the most frequent changes. Because of this polymorphism, the CRYAA sequences cannot be used in the analyses.

Table 2. Genetic diversity indices obtained for *Eupsittula canicularis*.

Molecular marker	n	nt	H/A	S	Hd	Pi	D_T
COI	53	567	14	13	0.894	0.00350	-0.9126 (NS)
Cytb	53	1068	18	24	0.880	0.00216	-1.989 (*)
ND2	46	1010	13	16	0.799	0.00198	-1.4138 (NS)
Trop	6	594	1	0	0	0	-

n= number of individuals, nt = number of characters considered in the matrix; H/A = Haplotypes or allele number; S = Polymorphic sites; Hd = Haplotype diversity; Pi = Nucleotide diversity; D_T = D-Tajima. NS = Not significant, $P > 0.10$. *Statistical significance: $P < 0.05$.

With the best representation among our samples, the analysis for each group was performed with the Cytb marker. All the groups exhibited more than one haplotype, even those with a small sample size (WCS1 and WCS3) (Table 3). We found moderate haplotype diversity in the groups WCM (Hd = 0.648), WCS1 (Hd = 0.667) and WCS4 (Hd = 0.700) and high diversity in WCS2 (Hd = 0.784), WCS3 (Hd = 0.833) and BBW (Hd = 0.8). The nucleotide diversity (Pi) was low for all groups, with the minimum value in BBW (Pi = 0.00125) and the maximum in WCS1 (Pi = 0.00268; Table 3).

Pairwise comparison of F_{ST} values showed moderate to low differentiation between groups (Table 4). The lowest value was between the groups BBW and WCS3 (F_{ST} = 0.00557). Both groups were located in the Bajo Balsas Basin, separated by the Rio Balsas and the Infiernillo reservoir. Additionally, these two groups had less genetic differentiation with the WCS2 group (coast of Michoacan) than with the other groups. The greatest differentiation between groups was between WCS3 and WCS4 (F_{ST} = 0.49735). As mentioned above, WCS3 was in the Bajo

Balsas Basin and WCS4 was in Guerrero. Additionally, WCM presented low values with WCS1 and WCS2 and moderate with the other groups. Thus, the groups from the north coast of Michoacan to Sinaloa were less genetically differentiated among themselves than with the other groups. Finally, the WCS4 group had low values of differentiation with WCS2 and moderate with the others, which indicated a greater genetic proximity of the groups of the coasts of Guerrero and Michoacan (Table 4).

Table 3. Genetic diversity indices obtained by groups.

Group	n	H	S	Hd	Pi	D_T	Fu´s F_S	Haplotypes
WCM	15	6	10	0.648	0.00163	-1.75529 (NS1)	-1.82341	H1(9), H2(2), H3(1), H4(1), H5(1), H6(1)
WCS1	4	2	4	0.667	0.00268	2.08033 (NS2)	2.19722	H1(2), H18(2)
WCS2	19	6	5	0.784	0.00141	0.17000 (NS2)	-1.14469	H1(2), H7(4), H8(8), H9(2), H10(1), H11(2)
WCS3	4	3	3	0.833	0.00140	-0.75445 (NS2)	-0.28768	H7(2), H12(1), H13(1)
WCS4	5	3	3	0.700	0.00206	-0.56199 (NS2)	0.80363	H8(1), H14(3), H15(1)
BBW	6	4	4	0.800	0.00125	-1.29503 (NS2)	-1.25217	H7(3), H8(1), H16(1), H17(1)
Total	53	18	24	0.880	0.00217	-1.98938(*)	-10.937	-

NS1 = Statistical significance: Not significant, $0.10 > P > 0.05$. NS2 = Statistical significance: Not significant, $P > 0.10$. *Statistical significance: $P < 0.05$.

Table 4. Pairwise genetic differentiation (F_{ST}) of groups. Fort the abbreviations in the graph see the text and Table 1.

	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. Summary AMOVA, 5A) without *a priori* defined groups, 5B) 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 indices
5A) Without a priori grouping					
Among groups	5	16.459	0.31347	28.87	
Within subspecies/population	47	36.296	0.77225	71.13	F_{ST} = 0.28872
Total	52	52.755	1.08572	100	
5B) North/Balsas/South					
Among groups	2	10.003	0.11537	10.44	F_{ST} = 0.30120
Among populations within groups	3	6.456	0.21749	19.68	F_{SC} =0.21975
Within populations	47	36.296	0.77225	69.88	F_{CT} = 0.10439
Total	52	52.755	1.10511	100	

Based on the AMOVA results without *a priori* defined groups, 71.13% of the genetic variation was explained by the differences within groups, 28.87% by the differences among groups, and the fixation index was low (F_{ST} = 0.2887; Table 5). When the groups were analyzed as North (WCM and WCS1), Balsas (WCS3 and BBW) and South (WCS2 and WCS4), the results indicated that most of the variation was explained by differences within groups 69.88%, and the F_{ST} value increased slightly (F_{ST} = 0.3012; Table 5). Thus, some differentiation occurred among the groups of the North (Sinaloa, Nayarit and Jalisco), Balsas (Michoacan) and the South (Michoacan-Guerrero).

Relationships among haplotypes and their geographic distribution

The haplotype network showed the relationships among the 18 haplotypes and the frequencies found in 53 individuals (Figure 2). In the network, few mutations separated the haplotypes and three haplogroups could be distinguished (HGI, HGII and HGIII; Figure 2). HGI had a dominant and widespread haplotype (H1). H1 was shared by 24.52% of individuals and was found in the groups WCM, WCS1 and WCS2, which represented the entire coast from Sinaloa to Michoacan (Figure 1). From H1, seven haplotypes were derived separated by one to three mutational steps. The frequency and the wide distribution of H1 suggest that this haplotype

represent an ancestral condition. The haplotypes of HGII were found exclusively in groups WCS2, WCS3 and BBW from Michoacan (Figures 1 and 2). A central haplotype (H7) and six derivatives separated by one or two mutational steps integrated HGII. The third most abundant haplotype was H7 with 16.98% of the samples. In HGIII, the central haplotype H8 represented 18.86% of the samples and was in the groups WCS2, WCS4 and BBW, primarily on the coasts of Guerrero and Michoacan (Figures 1 and 2). The HGI and HGII haplogroups showed the typical star-like genealogy that indicates a population in expansion. Moreover, both had a central haplotype and six derived haplotypes with few separating mutational steps (Figure 2).

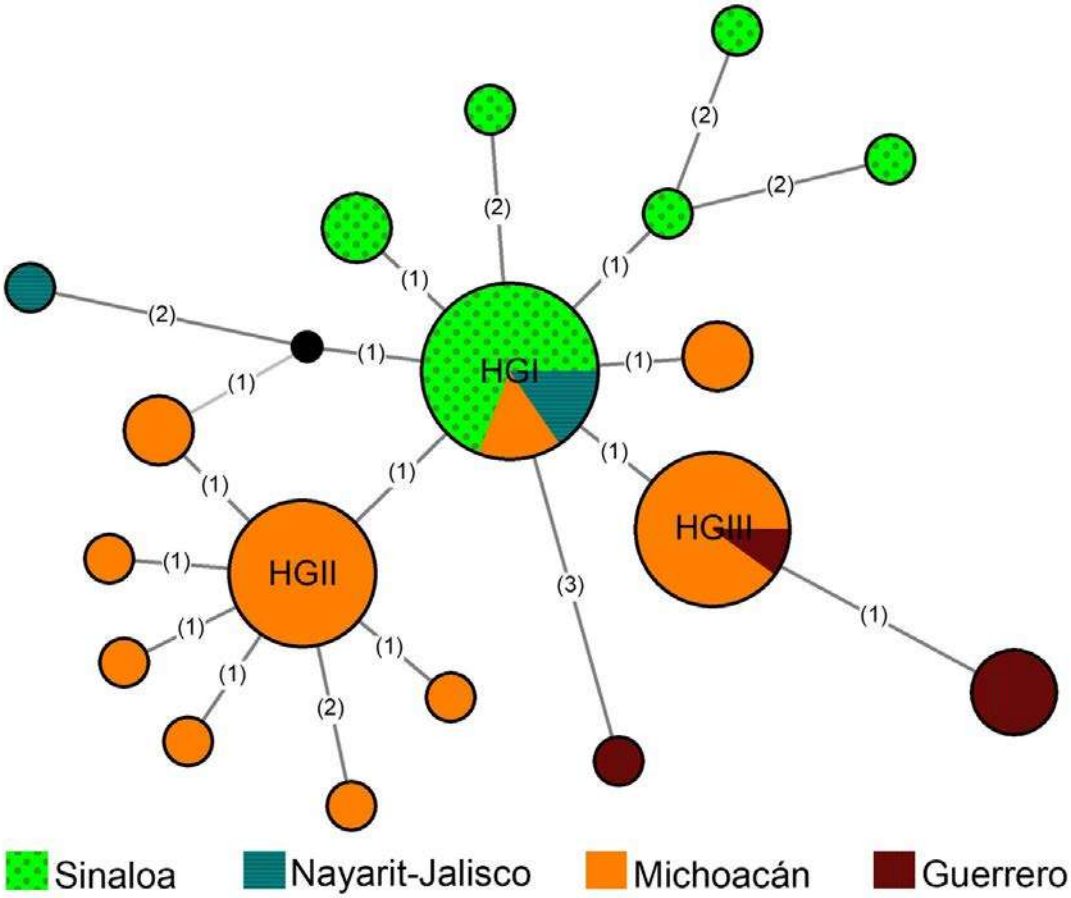

Figure 2. Median-joining haplotype network showing the relationships among 18 haplotypes of Cytb from 53 individuals of *E. canicularis*. The size of circles is proportional to the haplotype frequency; the length of the branches is not proportional to the number of mutations. The numbers on the branches represent mutations between haplotypes. HGI, HGII, and HGII correspond to haplogroups I, II and III, respectively.

In the geographical distribution of haplotypes, from the northern coast of Michoacan to Sinaloa, at least one haplotype (H1) was shared (Figure 1), and the individuals of Sinaloa, Nayarit and Jalisco were in haplogroup I. Similarly, we observed the shared haplotype H8 in the groups of Guerrero and Michoacan and the haplogroup II in the Balsas Basin and on the northern coast of Michoacan (Figure 1).

Phylogenetic analysis

The tree constructed under BI coincided with the topology observed in the tree obtained with ML. At the base of both trees, we observed individuals who are reported in NCBI and have unknown origin (Figure 3). From their position in the trees, we propose that the places of origin of these individuals could be the south of Mexico or Central America. In the present work, four individuals from the BBW group (Balsas Basin West) were sister taxa of the other individuals. The others, which represented most of the individuals analyzed, were integrated in a large clade forming a polytomy (CI) (Figure 3), although within the CI, four subclades were distinguished (SCI, SCII, SCII and SCIV). Although individuals from all groups were included in SC1, samples of WCM (15 individuals from Sinaloa localities), WCS1 (four individuals from Jalisco-Nayarit) and WCS2 (four individuals from the north coast of Michoacan) primarily composed this subclade. SCII was exclusively integrated with four individuals from WCS2. SCIII grouped individuals of the groups WCS2 (eight individuals) and WCS4 (four individuals from Guerrero localities), although one from BBW was also included (Balsas Basin West). SCIV also included individuals from the WCS2 group (Figure 3).

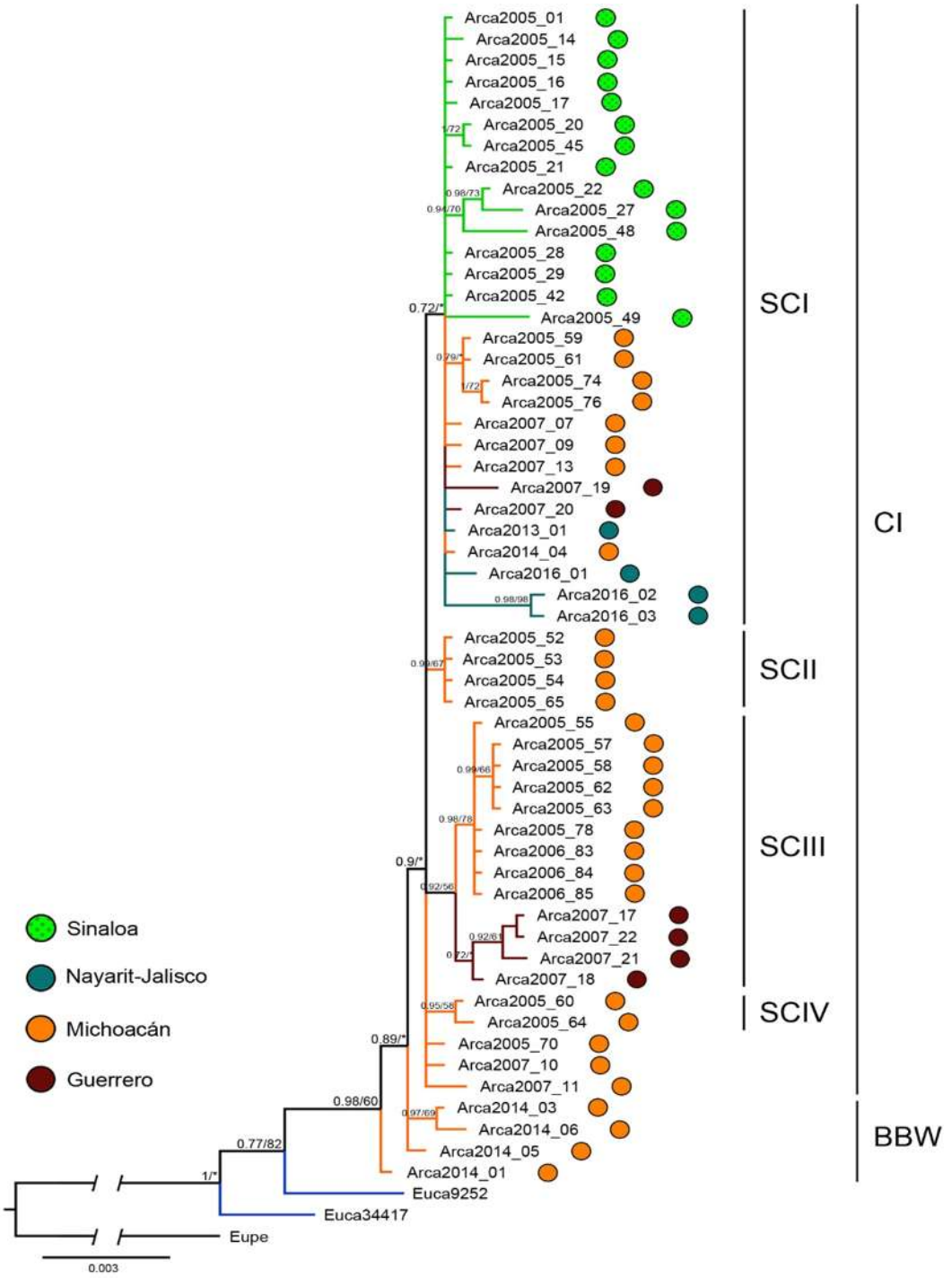

Figure 3. Consensus tree showing genealogic relationships among haplotypes of *E. canicularis* obtained with Bayesian inference (BI) and Maximum likelihood (ML) analyses. Estimates were performed with 2,645 characters of 58 taxa of Cytb, ND2 and COI (DNAmt). The sister species *Eupsittula pertinax* was the out-group. Values over the branches represent posterior probabilities and bootstrap values (PP/BP). (*) Value inferior at PP = 0.5 or PB = 50.

Demographic history

The mismatch distribution graph (Figure 4A) showed a positive asymmetric distribution representing groups with few mismatches, which is interpreted as the result of closely related lineages. Additionally, plots resulting from the skyline analyses showed the effective size of the population (Ne_{τ}) over the last 238.7 thousand years ago (kya) (Figure 4B). During the Middle and Upper Pleistocene, the population showed constant growth from 238.7 kya, with a peak reached at 19 kya. However, a slight decline of the population occurred to reach the population size of today. Therefore, we established that climatic oscillations of the Quaternary did not dramatically reduce the size of the populations of *E. canicularis* in the northern part of their distribution. 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 slight recent expansion of the population (Figure 4 and Table 2).

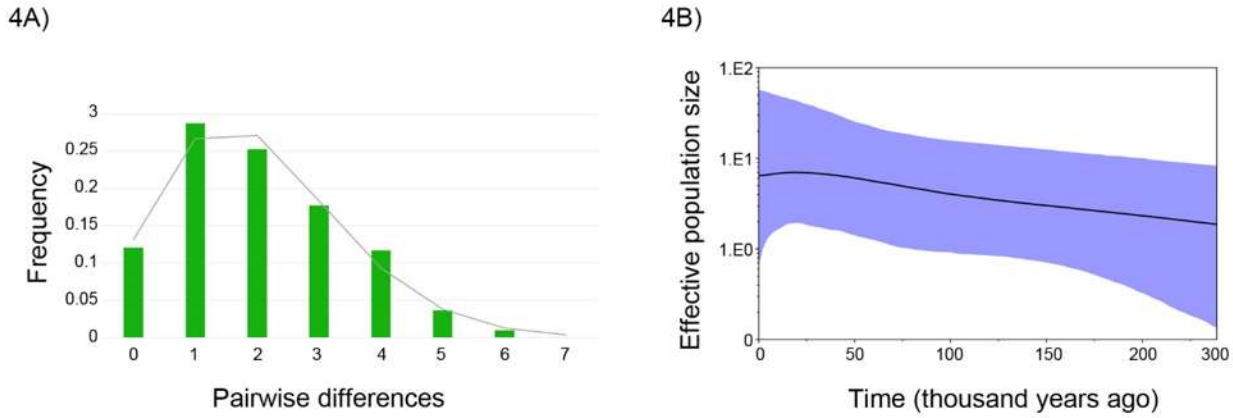

Figure 4. Mismatch distribution (4A) and Bayesian skyline plots (4B) for all samples. The histograms correspond to observed frequencies; the lines represent expected frequencies under a sudden expansion model. Bayesian skyline plots showing population history of *E. canicularis*, with black lines indicating median population size estimates expressed in $N_e.\tau$ through time; shaded areas represent 95% HPD intervals.

Discussion

The analysis of genetic diversity showed moderate to high haplotype diversity (Hd) and low nucleotide diversity (Table 2). According to Grant and Bowen (1998), these results indicate a population growth from a small effective size of the population. Additionally, when all samples were analyzed together, the values of D_T and Fu´s F_S for Cytb were negative, which also indicated a recent expansion, according to Hedrick (2011) and Hamilton (2011) (Table 3). The results of the haplotype network, mismatch graph and skyline plot corroborated and supported the population growth observed in genetic diversity metrics and neutrality tests (Figures 2 and 4). Moreover, the skyline plot showed constant population growth from 238.7 to 19 kya. Based on these results, we propose that the northern population of *E. canicularis* was not negatively affected by the climatic changes of the Upper and Middle Pleistocene. Only at the Upper Pleistocene-Holocene boundary, a slight decline in population was initiated that coincided with the Last Glacial Maximum (LMG, approximately 23-18 kya).

Our results showed the formation of three lineages. Individuals from Sinaloa, Nayarit, Jalisco and the north coast of Michoacan formed the North lineage. The Balsas lineage was composed of individuals from the Balsas Basin West, and individuals from the coasts of Michoacan and Guerrero formed the South lineage. Pairwise genetic differentiation (F_{ST}) between groups and phylogenetic trees showed less genetic differentiation among the groups from Sinaloa to Guerrero than with the group located in the Balsas Basin West (Table 4, Figure 3).

No apparent geographical barriers can be associated with the diversification observed in *E. canicularis*. However, some phylogeographic breaks have been identified in birds with distributions similar to that of *E. canicularis*. Based on the phylogeographic patterns described for three different species that inhabit the tropical dry forest, Arbeláez-Cortés et al. (2014a) identified phylogeographic breaks at the Guerrero-Oaxaca border and between Michoacan and Guerrero. These authors report a division for *Melanerpes chrysogenys* and *Momotus mexicanus* at Michoacan-Guerrero. In our results for *E. canicularis*, no phylogeographic break was observed between Michoacan and Guerrero, because at least one haplotype was shared among individuals from the coast, and therefore, less genetic differentiation was observed among those groups than with that from Balsas Basin West. In another publication, Arbeláez-Cortés et al. (2014b) identified two lineages in *Vireo hypochryseus*, one associated with Sinaloa-Marias Islands and another associated with the Oaxaca-Guerrero and Jalisco-

Hidalgo states. Unfortunately, the absence of information for Michoacan in that report makes it impossible to determine whether the pattern observed in the present work is repeated in *V. hypochryseus*. Finally, the species *Icterus pustulatus* also has a geographical distribution similar to that of *E. canicularis*. Cortés-Rodríguez et al. (2008a) found three primary haplogroups in *I. pustulatus*, with the north haplogroup with individuals from Sinaloa to Michoacan; the center haplogroup with representative individuals in Michoacan, Guerrero, Chiapas and central Mexico; and the south haplogroup with individuals from Oaxaca, Chiapas and Central America. In this study, we also found a haplogroup in the north with a presence from Michoacan to Sinaloa (HGI). However, Cortés-Rodríguez et al. (2008a) did not identify a haplogroup that relates individuals from the coasts of Michoacan and Guerrero as was found in this study. We also emphasize that these authors found two haplogroups in Michoacan, particularly in the Balsas Basin West, and in the present study, we also found that this region had two haplogroups and the greatest genetic diversity.

In *E. canicularis*, a lineage divergence occurred during the Quaternary. In the relationships established in the trees, a shallow divergence was observed represented by different subclades in a polytomy, a typical topology in analyses with groups of closely related haplotypes. Our results differ from those published in which a profound divergence of lineages occurs in birds of Neotropical distribution (García-Moreno et al. 2004; Cadena et al. 2007; Nyári 2007; McCormack et al. 2008; Ortiz-Ramírez et al. 2016). Nevertheless, the results do coincide with the observations of bird distributions in temperate regions (Zink et al. 2001, 2005, 2010; Alexander and Burns 2006; Cortés-Rodríguez et al. 2008b; Johnson 2008; White et al. 2013). We emphasize that the lineage in Balsas Basin West (BBW) is ancestral to the other individuals analyzed, including those of Guerrero (Figure 3). Additionally, in CI, haplotypes of individuals from WCS3 and WCS2 groups formed a polytomy with four subclades (Figure 3). This result indicated that the area of the Balsas Basin West was the origin of the rest of the analyzed groups and that it could have been a refuge during the climatic changes of the Quaternary. Moreover, the groups WCS3 and BBW had the highest genetic diversity, which is consistent with the refuge scenario (Table 3). Although our results are not conclusive and must be interpreted with caution, they are a signal that the analysis must continue with the inclusion of samples from the Michoacan-Guerrero region. In the southeast of Mexico, different regions have been proposed as a forest refuge; for example, for birds of arid regions on the Pacific

side, the Balsas Basin and the Pacific side of the Isthmus of Tehuantepec have been proposed as refuges (Howell and Webb 1995).

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CAPÍTULO III

ARTÍCULO 3

Sobre el uso de herramientas moleculares en el diseño de estrategias de conservación para *Eupsittula canicularis*. Por medio de asignación directa de individuos y de inferencia filogenética se identifican posibles áreas de captura y reintroducción de individuos decomisados. Artículo sometido a Tropical Conservation Science.

Applying molecular markers and phylogenetic inference to identify areas of intensive poaching and for potential reintroduction of the Orange-fronted parakeet, *Eupsittula canicularis*

Gabriela Padilla-Jacobo[a], Tiberio C. Monterrubio-Rico[b], Horacio Cano-Camacho[a] and María G. Zavala-Páramo[a]

[a]*Centro Multidisciplinario de Estudios en Biotecnología, FMVZ, Universidad Michoacana de San Nicolás de Hidalgo, Km. 9.5 Carretera Morelia-Zinapecuaro, Posta Veterinaria Morelia, Michoacán, México. Tel/Fax +52 443 2958029*

[b]*Laboratorio de Ecología de Vertebrados Terrestres Prioritarios, Facultad de Biología, Edificio R, CU, Universidad Michoacana de San Nicolás de Hidalgo, Morelia, Michoacán, México.*

Abstract

One concern in conservation biology is how to adequately reintroduce confiscated parrots because the poached population's site of origin is unknown. Among the Mexican species of Psittacidae, the Orange-fronted parakeet, *Eupsittula canicularis*, is the species most in demand for the illegal pet trade, and it suffers the heaviest poaching pressure. In this study, both direct assignation and assignation by phylogenetic inference of confiscated individuals were obtained using genetic diversity and phylogenetic analyses with the molecular marker Cytb in a confiscated population of 80 individuals and a population of *E. canicularis* field individuals that were properly geo-referenced. Additionally, we used rarefaction and extrapolation curves to estimate the richness and impact on the genetic diversity subtraction. The confiscated group showed a high genetic diversity (Hd = 0.851), sufficient to establish a potentially viable breeding colony or to reinforce local populations. The place of origin of 50 confiscated individuals (63%) was identified, and any area with suitable tropical forests from the north of Michoacán to the south of Nayarit, México would serve as a potential reintroduction region. The rarefaction curve shows a light but constant growth, with a potential high diversity that has not yet been detected in field populations of *E. canicularis*. Thus, this study also allowed the observation of the impact of the species subtraction from the genetic pool and emphasizes the importance of the use of molecular tools in such cases.

Keywords: *Eupsittula canicularis*, Haplotypes, Poached parakeets, Reintroduction

Introduction

The Orange-fronted parakeet, *Eupsittula canicularis*, inhabits the Pacific slope from the north of México to the north of Costa Rica (Collar et al., 2000; Forshaw, 1989; Howell and Webb, 1995) (Figure 1). The species habitat includes humid and sub-humid deciduous forests, tropical deciduous dry forests, riparian forests, and agricultural areas (Collar et al., 2000; Howell and Webb, 1995; Ridgely, 1981; Stotz et al., 1996). Based on fieldwork and ecological niche models, the species' current distribution has been estimated to include an area that covers 247,312 km^2 along the Pacific coast of México (Monterrubio-Rico et al., 2016). Due to its extensive distribution and the low height of the termitaria where it nests, this parrot species suffers the highest degree of extraction for the illegal parrot trade in México (Cantú-Guzmán et al., 2007; Iñigo-Elias and Ramos, 1991). From 2007–2014, parakeets from the genus *Eupsittula* and the related *Aratinga* were the second-most poached parrot group in the world (2149 individuals) according to official statistics (UNODC, 2016). The actual number of individuals poached each year remains a mystery. Locally, the official Mexican normativity NOM-059 (DOF, 2010) listed the species in the special protection category because of the pet trade's increasing impact, even though at the international level it is still considered a species of Least Concern (BLI, 2015). In México, only 1.6% of its distribution corresponds to Protected Areas (Monterrubio-Rico et al., 2016). Two causes are mentioned as the driving forces of the distribution contraction and decline of parakeets in México: habitat destruction and illegal trade (Cantú-Guzmán et al., 2007; Collar and Juniper, 1992; Iñigo-Elias and Ramos, 1991; Weston and Memon, 2009). In México, illegal trade supplies the domestic market, where they are sold as pets (Cantú-Guzmán et al., 2007; Iñigo-Elias and Ramos, 1991). Although some routes are known to be used by poachers to mobilize parakeet shipments to their markets (Cantú-Guzmán et al., 2007), parakeet origin is particularly difficult to determine because parakeets from different regions and localities are housed together. In addition, obtaining information from the arrested poachers is not practical, since the illegal trade process involves different people from poaching in rural areas to collection, transportation and sale, covering multiple nesting localities across entire regions. During the poaching-purchase process, many poached individuals die. The mortality rate during the trading process depends on the species captured and the techniques used; however, different authors have estimated that mortality rates range from approximately 66% to 77% or 83%

during the poaching-purchase process (Cantú-Guzmán et al., 2007; Enkerlin, 2000; Iñigo-Elias and Ramos, 1991). Based on estimates of the individuals captured and the mortality rate, 50,050 to 60,445 individuals are estimated to die every year (Cantú-Guzmán et al., 2007). Although the laws concerning the illegal trade of Psittacidae in México imply severe penalties and imprisonment, eradicating rural poaching is difficult because enforcement is lax and the practice persists widely. When a confiscation occurs, the specimens are housed in state zoos or private aviaries under quarantine or in the few Centers for Research and Conservation of Wildlife (CIVS, Spanish acronym). Although the main objective of these centers is to rehabilitate the confiscated parrot for future reintroduction, because they lack monetary resources and precise information about the origin of the confiscated individuals, the parakeets generally remain in the zoos indefinitely.

To improve the success of reintroductions, it has been suggested that the confiscated individuals be returned to their original habitat as soon as possible (Frankham et al., 2004). However, the limiting factor in the reintroduction process is the lack of information concerning the originating population of the poached individuals that were confiscated. However, the use of molecular markers offers an opportunity to reverse this situation. With these markers, the population of origin can be identified provided reference data exists from sampled individuals across their range. Currently, this tool is used to assign individuals to their natural populations (*Pan troglodytes*, Ghobrial et al., 2010; *Pantera pardus*, Mondol et al., 2015; *Ursus americanus*, Puckett and Eggert, 2016; *Epicrates subflavus*, Tzika et al., 2009). In one of the few cases involving Psittacidae, specifically Hyacinth macaws (*Anodorhynchus hyacinthinus*), by using molecular data from pre-existing sampled individuals from their original populations in the wild, researchers were able to successfully identify the origin of poached individuals (Presti et al., 2015). Based on their results, the authors propose the use of molecular data for selecting the release site to increase the chances of successfully reintroducing confiscated individuals. In addition, molecular markers have proven their usefulness in assessing the genetic diversity of populations or groups selected for reintroduction, either to reinforce the population or to establish a new population. In birds, we can mention a few examples (Lesser kestrel, *Falco naumanni*; Peregrine falcon, *Falco peregrinus*; Trumpeter swan, *Cygnus buccinator*) in which the genetic diversity of pre-release and post-release populations were evaluated (Alcaide et al., 2010; Jacobsen et al., 2008;

Ransler et al., 2011). From the samples analyzed in the confiscated individuals, some other advantages include the use of baseline information to survey the potential loss of genetic diversity because of intensive poaching or management, the designing of strategies to avoid the negative consequences of population inbreeding in protected areas, or the avoidance of bottleneck or founder effects in released and/or recipient populations (Alcaide et al., 2010; Jacobsen et al., 2008; Ransler et al., 2011).

In the present study, we aimed to analyze the molecular characteristics of confiscated individuals of *E. canicularis*, to compare the characteristics with molecular data from samples obtained from wild field populations in parts of their range and to determine the appropriate reintroduction regions by examining the potential allocation of individuals through two methods: direct assignment and assignment through phylogenetic inference. We also aimed to provide a description of the genetic diversity of the confiscated population, estimating the genetic richness found in the illegal trade through rarefaction and extrapolation curves. More specific and concrete goals include the following: 1) to propose reintroduction sites based upon samples of individuals confiscated by Mexican authorities and 2) to describe the genetic diversity in the sample collected by the illegal trade. The results could become baseline information allowing us to measure the degree of genetic diversity lost because of poaching, establishing the precedent for this type of analysis in México and for the Neotropical Parakeet species for potential reintroductions.

Methods

Biological samples

The biological samples were obtained from 80 parrots confiscated by Mexican authorities in the city of Tlajomulco de Zuñiga Jalisco, Mexico (20°28´34´´N, 103°27´00´´W) in August 2014 (Figure 1). Sample collection and storage were performed as follows: feathers of 80 *E. canicularis* individuals were collected, and each sample was placed in a 2 ml vial with 0.5 ml of a 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 and were subsequently stored at 4°C. These samples were deposited in the wildlife sample collection at the Multidisciplinary Center for Studies in Biotechnology

(Centro Multidisciplinario de Estudios en Biotecnología) at Universidad Michoacana de San Nicolás de Hidalgo in Morelia, Michoacán, México.

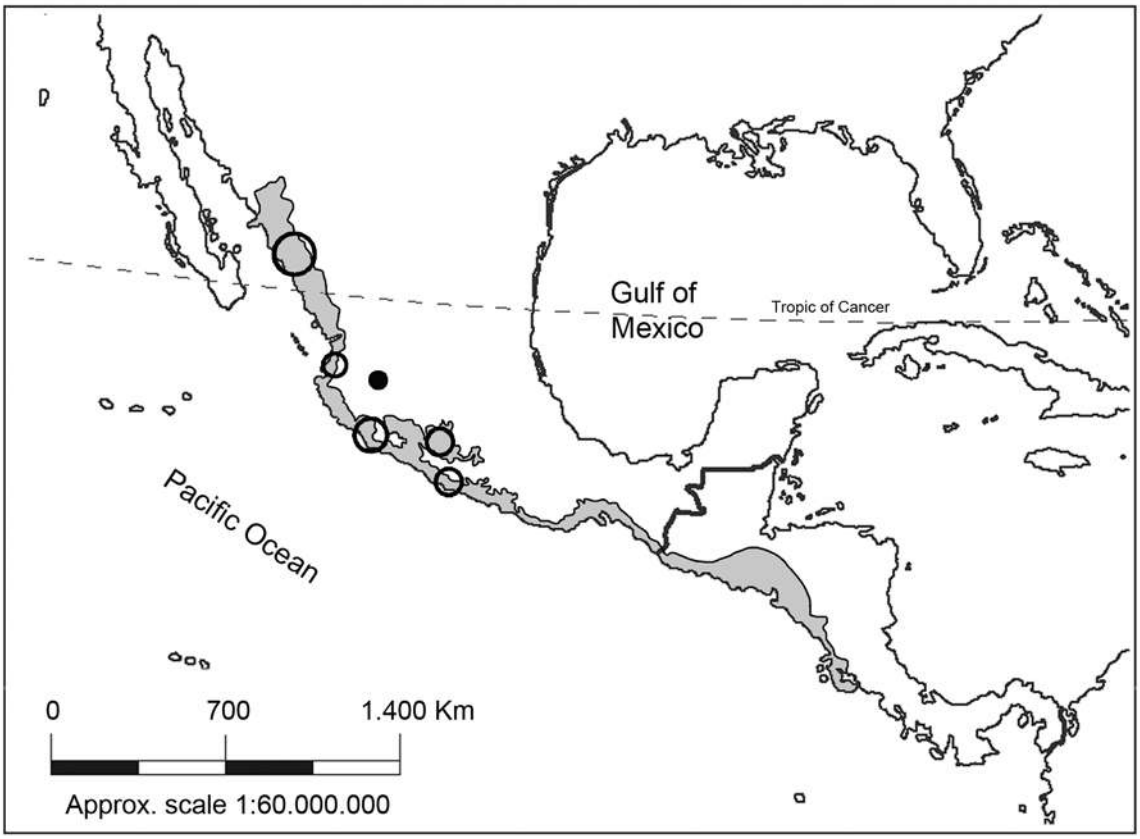

Figure 1. Geographic distribution and sampling regions of *E. canicularis*. The map is adapted from Monterrubio-Rico et al. (2016) and Collar et al. (2017). The shaded areas represent the potential distribution of the species. Solid circles indicate localities with confiscated individuals (Tlajomulco de Zúñiga Jalisco, Mexico). Open circles indicate the sampling localities for field individuals.

DNA extraction, PCR amplification of markers and sequencing

DNA was extracted using the phenol-free method described by FitzSimmons (1997). The Cytb sequence of approximately 1140 bp was obtained using the oligonucleotides CytbD (5′-ATCATCCGCACTATCTGTCCT-3′) and CytbR (5′- AATAGCCTTCGTCTTTTGGTTTA-3′) designed in this study using the DNASTAR Lasergene software LG10VC (Kumar and

Blaxter, 2010), based on the Cytb sequences reported in GenBank for species of the genus *Eupsittula* and related genera.

The PCR reactions were performed in a total volume of 25 µl with the following components: 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 DNA. The reactions were placed in a thermocycler (Gene Amp 2700, Applied Biosystems, Foster City, CA, USA) and amplified under the following conditions: 94°C for 5 min; followed by 30 cycles of 94°C for 40 s, 55–57°C for 40 s, and 72°C for 90 s; and a final extension at 72°C for 5 min. The sequencing of both DNA strands was performed using the dideoxy method (Sanger et al., 1977); services were provided by Macrogen (Rockville, MD, USA).

Genetic diversity analysis

Sequence editing, alignments, and the construction of data matrices were carried out with Sequencher v.4.1 (Gene Codes Corporation, Ann Arbor, Michigan, USA) and PhyDE (Müller et al., 2005). The number of haplotypes (H), polymorphic sites (S), and the nucleotide (Pi) and haplotype (Hd) diversity were revised with ARLEQUIN v3.1 (Excoffier et al., 2005). Because it is not possible to collect and analyze samples of all individuals from the illegal trade, and with the aim of estimating the impact of the illegal trade on the genetic diversity of the *E. canicularis* natural population, we relied on a methodology frequently used in ecological analyses. Rarefaction and extrapolation curves are ecological analyses used to estimate species richness in an area based on a limited local sample, and several methods have been developed for this purpose (Colwell et al., 2012; Chao et al., 2005). To calculate the diversity of haplotypes in confiscated individuals, we implemented the individual-based method (Coleman method) and assumed that each haplotype represents a “species” to calculate extrapolation and rarefaction curves using EstimateS 9.1.0 software (Colwell, 2013).

Allocation from individuals

The allocation from individuals to a geographic region followed two methodologies: direct assignment and phylogenetic inference. Direct assignment consisted of comparing haplotypes from confiscated individuals with haplotypes previously identified from field surveys to

generate a haplotype network. The reconstruction of genealogical relationships between haplotypes proposes the assignment of individuals through phylogenetic inference.

To review frequencies of haplotypes and relationships between them, a haplotype network was constructed under the median-joining method with the software NETWORK v5 (Fluxus Technology Ltd.). To associate these haplotypes to particular regions, we included sequences from 53 field individuals that were properly geo-referenced (GenBank Access: KU532328-KU532329 and MF441340-MF441390) (Manuscript submitted). We also included a sequence of Cytb reported for a specimen identified as *E. canicularis* (EcNCBI hereinafter) (GenBank Access: KJ142251.1). In total, the data matrix included 134 sequences, of which 53 sequences were from field individuals, one was from GenBank, and 80 were from confiscated individuals.

The assignment of individuals by phylogenetic inference was performed under the criteria of Maximum Likelihood (ML) and Bayesian Inference (BI). This data matrix included *Eupsittula pertinax* as an outgroup (Access: KJ142289.1). Molecular evolution models were constructed using jModelTest 2.1.1 (Posada, 2008) and selected using the corrected Akaike Information Criterion (cAIC) (Alfaro and Huelsenbeck, 2006). The best model obtained using this criterion was TrN (Tamura and Nei, 1993). The ML and BI reconstructions were performed using RAxML v7.8 (Stamatakis, 2014) and MrBayes v3.2 (Ronquist and Huelsenbeck, 2003) software. The branches-support values were estimated using a bootstrap analysis (BP) of 500 replicates and posterior probabilities (PP). MrBayes runs were performed using the following parameters: four independent runs of four chains each (one cold chain and three hot chains) for 10 million generations with sampling every 1000 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).

Results

Genetic diversity analysis

From the DNA samples of *E. canicularis*, amplifications of the 80 Cytb gene sequences of 1088 to 1151 bp were obtained and registered in GenBank (Access: MF441391-MF441490). The sequence matrix was analyzed to diversity constituting 984 characters. The data revealed

23 haplotypes with 955 invariable sites, 29 polymorphic sites, of which 17 are singleton variable sites and 12 are parsimony informative sites (Table 1).

Table 1. Haplotypes and variable sites in 984 pb of Cytb gene from confiscated *E. canicularis* population.

Haplotype	Alignment site																													
	29	52	83	89	131	160	172	198	307	352	379	400	418	442	574	609	646	667	670	680	730	781	814	905	945	957	988	967	968
H1	A	C	T	A	G	T	A	A	A	G	A	A	T	T	G	C	T	G	C	C	T	G	C	A	T	T	A	C	T
H2	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.
H3	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H4	.	.	.	.	.	.	G	.	.	A	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.
H5	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.
H6	.	.	.	.	.	.	.	.	.	A	.	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	T	.
H7	.	.	.	.	.	.	.	.	.	A	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H8	.	.	.	.	.	.	.	.	.	A	G	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H9	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	T	.	.	.	.	.	A	.	.	.	.	.	.	.
H10	.	T	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H11	.	.	.	.	.	.	.	G	G	A	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H12	.	.	.	.	.	.	.	.	.	A	.	.	C	.	.	.	.	.	.	T	.	.	.	C	.	G	.	.	.
H13	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.
H14	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	G	.	.	.	.
H15	.	.	.	.	A	.	.	.	.	A	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.
H16	.	.	.	.	.	.	.	.	.	A	.	.	C	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.
H17	.	.	.	.	.	.	.	.	.	A	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H18	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.	.	.	.	.
H19	.	.	.	.	.	.	.	.	.	A	G	.	.	.	.	.	.	.	.	.	C	.	.	.	.	.	.	.	.
H20	G	.	C	.	.	.	.	.	.	A	.	.	.	C	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H21	.	.	.	.	.	C	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
H22	.	.	.	C	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	T	.	.	.	.	.	.
H23	.	.	.	.	.	.	.	.	.	A	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	C

The mutations were 25 transitions and four transversions; no indels were observed. A high haplotype diversity (Hd) of 0.851 (+/-0.032) and a low nucleotide diversity (Pi) of 0.00178 (+/-0.00018) were detected (Table 2). The composition of bases was adenine (A) 27.62%, thymine (T) 24.68%, cytosine (C) 35.28% and guanine (G) 12.42%.

When the field survey samples were incorporated, the resulting data yielded 36 haplotypes with 887 invariable sites and 44 polymorphic sites, of which 25 are singleton variable sites and 19 are parsimony informative sites (Table 2). The mutations were 39 transitions and five transversions; no indels were observed. A high haplotype diversity (Hd) of 0.8783 (+/-0.0226)

and a low nucleotide diversity (Pi) of 0.002067 (+/-0.001310) were detected (Table 2). The composition of bases was adenine (A) 26.83%, thymine (T) 25.11%, cytosine (C) 35.04% and guanine (G) 13.02%.

Table 2. Genetic diversity indices in Cytb gene from *E. canicularis* samples. Shows numbers of individuals (n), Haplotypes numbers (H), Haplotype diversity (Hd), Standard deviation (SD), Nucleotide diversity (Pi) and Polymorphic sites (S).

Group	Samples (n)	Alignment	H	Hd (SD)	Pi (SD)	S
Captured	80	984	23	0.851 (+/-0.032)	0.00178 (+/-0.00018)	29
Field	53	931	18	0.879 (+/-0.026)	0.00222 (+/-0.00140)	25
Total	133	931	36	0.878 (+/-0.022)	0.00206 (+/-0.00131)	44

The rarefaction curve shows the mean of expected values for the richness, and a slight but constant growth is observed proportional to the number of samples without reaching the asymptote up to 240 samples. The estimated extrapolation curve of captured individuals shows approximately 36 haplotypes (95% CI Bound = 21.76–50.29) for the 160 individuals sampled and 47 haplotypes (95% CI Bound = 25.95–68.64) for a group of 240 individuals (Figure 2).

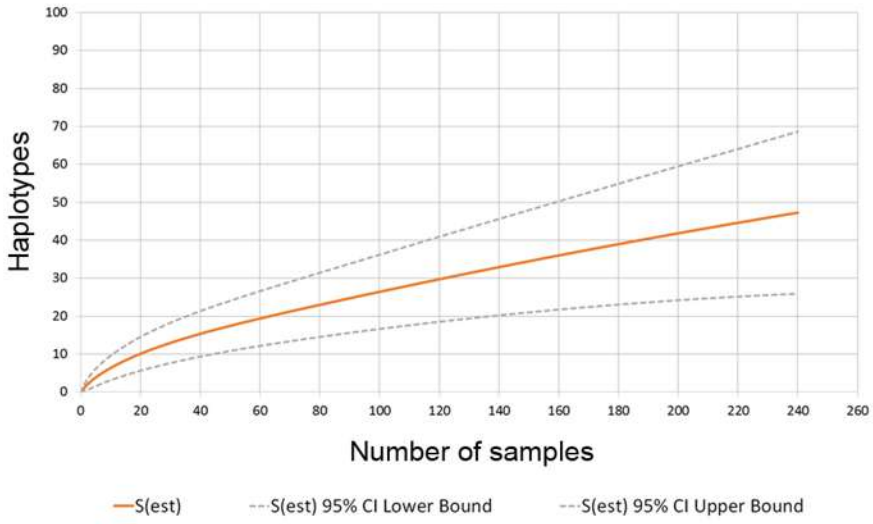

Figure 2. Rarefaction curves based on Cytb data showing the expected values of diversity in *E. canicularis*.

When extrapolation was performed up to 320 individuals, the CI values widen and become less reliable (mean = 57, 95% CI Bound = 27.34–86.76, not shown in the graph).

Direct assignment of individuals

In the haplotype network with 36 haplotypes and 133 individuals, a maximum of three mutational steps were observed between haplotypes. The network shows the typical star shape of expanding populations, and we distinguished four haplogroups (Figure 3).

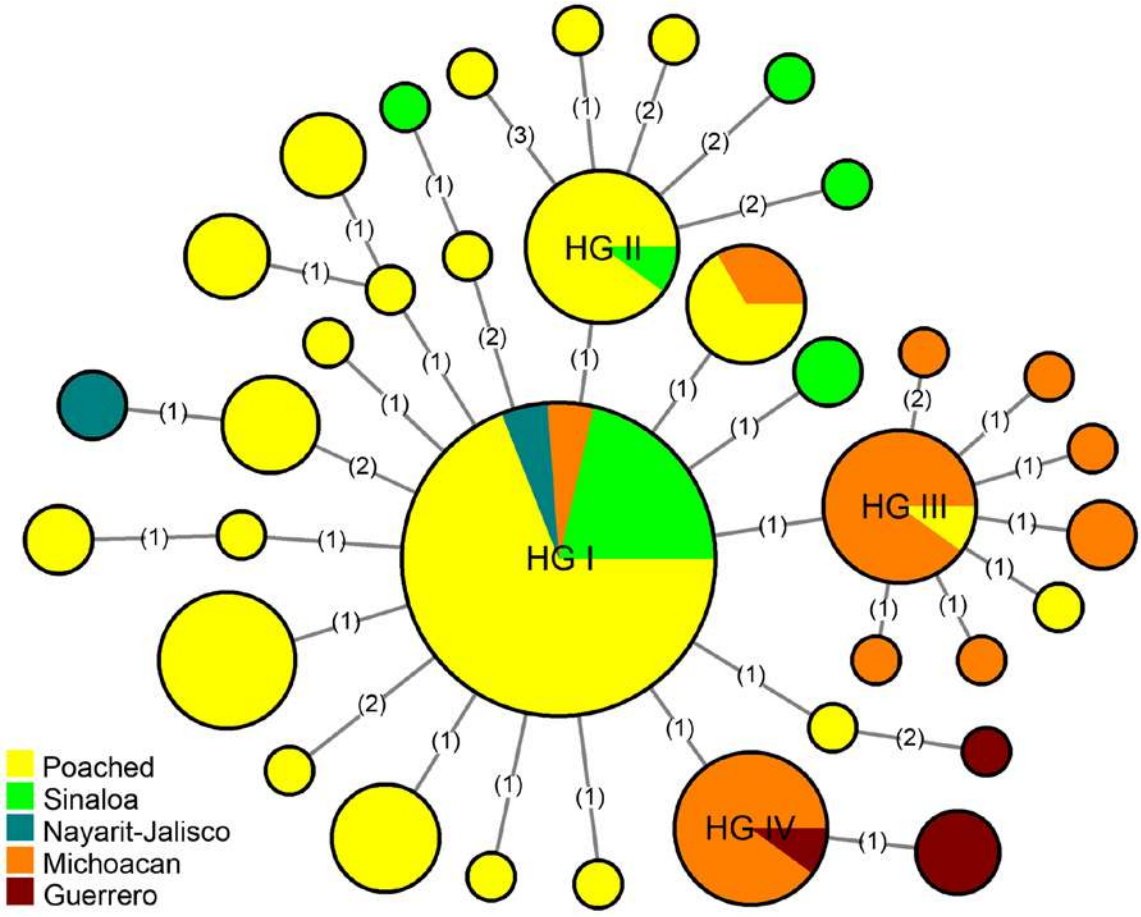

Figure 3. Median-joining haplotype network illustrating the relationships among 36 haplotypes from 133 individuals of *E. canicularis* based on Cytb. Circle size is proportional to haplotype frequency; branch length is not proportional to the number of mutations. Numbers in the branches represent mutations between haplotypes. HGI, HGII, HGIII and HGIV correspond to haplogroups I, II, III and IV, respectively.

In haplogroup I (HGI) a central haplotype (H1) was observed with 16 directly connected peripherals, of which 11 haplotypes were exclusively from captured individuals. H1 is widely distributed and is shared by the confiscated individuals and the field survey samples from Sinaloa mostly, as well as with a smaller proportion in individuals from Michoacán and Nayarit. H1 was not detected in localities of Guerrero. In haplogroup II (HGII) a central haplotype (H3) is shared by confiscated individuals and individuals from Sinaloa; from this derive five haplotypes (two from Sinaloa and three confiscated). Haplogroup III (HGIII) includes individuals from Michoacán. The central haplotype (H7) is shared with individuals from Michoacán and one confiscated individual, from which seven peripheral haplotypes are derived (six from Michoacán and one confiscated). Finally, we consider haplotype H8 and its derivative haplotype H14 as haplogroup IV (HGIV), and we speculate that more haplotypes are derived from H8 that were not sampled.

Under direct assignment, we observed that four haplotypes are shared with those from geo-referenced individuals. Haplotypes H1, H3, H7 and H11 were located in Sinaloa, Michoacán and Nayarit but were not found in Guerrero localities. These four haplotypes represent 54.0% of the individuals captured.

Assignment by phylogenetic inference

The matrix for phylogenetic reconstruction included 1025 characters of the single haplotypes, with 92 polymorphic sites, of which 64 were singleton variable sites and 28 were parsimony informative sites. The phylogenetic consensus tree constructed under ML and BI is illustrated in Figure 4. In both types of analysis, the same topology is observed. Although some nodes do not have wide PP and BP values, the topology presented in these analyses should be considered only for the purpose of revising phylogenetic affinity clusters. The EcNCBI sample shows early divergence and is sister to the rest of the analyzed taxa. In a consensus tree, a smooth polytomy is observed, although it is possible to detect that a group from Sinaloa and some poached individuals (CII) separate early from the main clade (CI) integrated by samples from Sinaloa, Nayarit, Michoacán, and Guerrero. Although this clade also constitutes a polytomy, some subclades are detected that are particularly interesting for the present analysis.

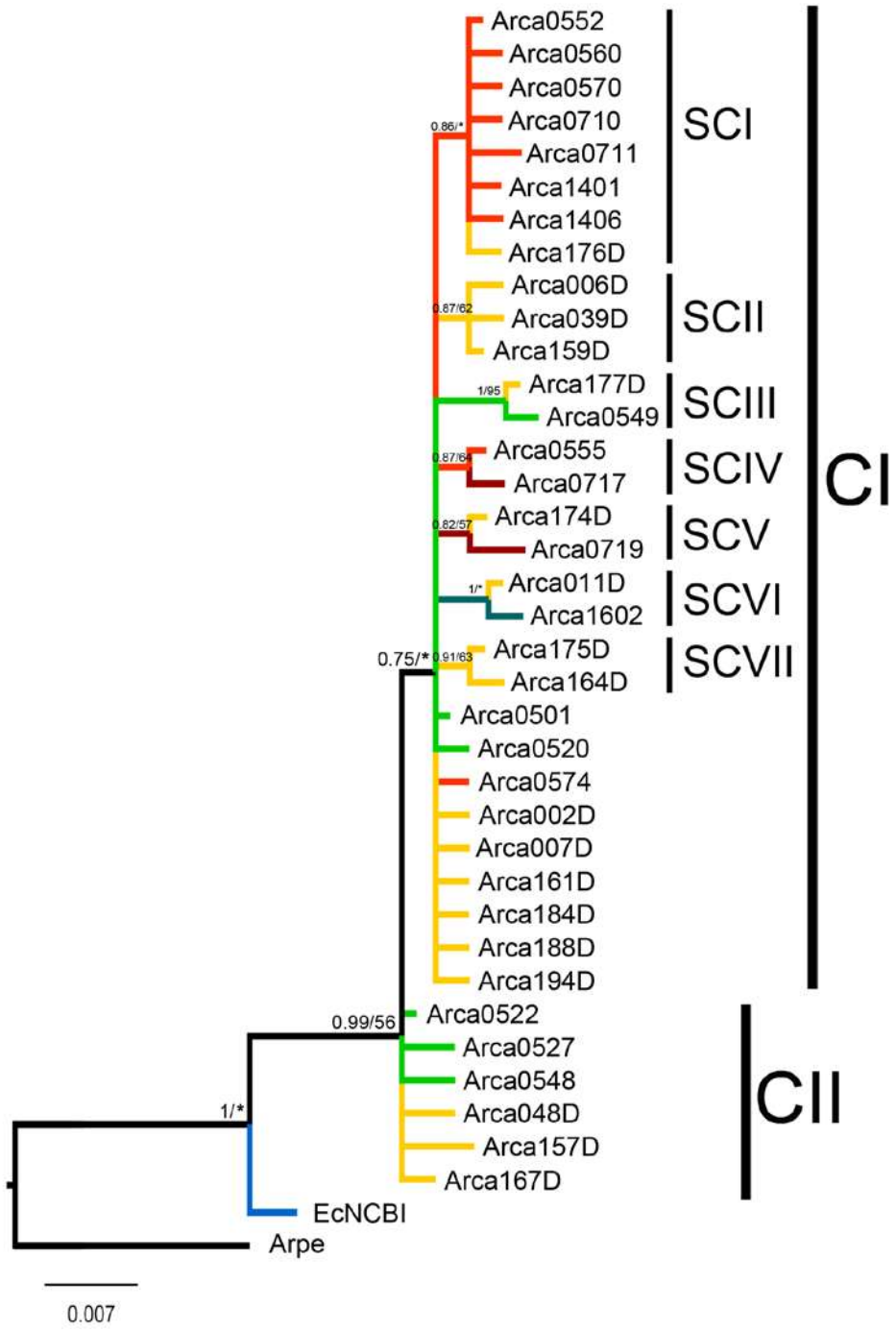

Figure 4. Consensus tree showing the genealogic relationships among haplotypes of *E. canicularis* obtained using Bayesian inference (BI) and Maximum likelihood (ML) analyses. Estimates were made with 1025 characters of 36 haplotypes of Cytb (DNAmt). The out-group is the sister species *Eupsittula pertinax*. Values over the branches represent posterior probabilities and bootstrap values (PP/BP). (*) Value inferior at PP = 0.5 or BP = 50.

The subclade SCI is composed mainly of haplotypes present in Michoacán and one poached individual; in this subclade the field sample Arca2005–52 is shared with a confiscated individual.The SCII and SCVII clades have only haplotypes from confiscated individuals. Clade SCIII has representative haplotypes of Sinaloa as well as from one poached individual. SCIV has haplotypes of Michoacán and Guerrero without confiscated individuals. SCV has one individual from Guerrero and one confiscated. Finally, SCVI has one haplotype with two individuals from Jalisco and four poached individuals. By phylogenetic inference, 63.0% of individuals were assigned with support.

Proposed reintroduction sites

Based on the results of the direct allocation and phylogenetic methods, we propose two regions with reintroduction potential. Considering that the central haplotype H1 presents the highest relative frequency in confiscated individuals and is shared with some individuals from several localities in the central and northern region of the *E. canicularis* range, the origin of the southernmost limit of the confiscated individuals may be the Northwest area of coastal Michoacán. No haplotypes of captured individuals were detected from the Guerrero coast. The central haplotype (H3) in haplogroup II is shared with confiscated individuals, suggesting that the capture was carried out in the coastal region corresponding to the states of Colima, Jalisco and southern Nayarit; therefore, we consider that range for reintroductions.

Discussion

The main threat to *E. canicularis* is poaching for the illegal trade. Despite efforts to reverse this practice, the trade persists. Hundreds of parakeets are confiscated each year and are finally deposited in zoos for an indefinite period of time. Ideally, these organisms should be reintroduced into their wild populations to assist in their recovery. Although the task is not easy because of the lack of accurate information, the use of molecular information can play a central role in designing reintroduction strategies. Molecular information is also useful in assessing the current genetic diversity of the wild populations because these constitute random sub-samples, allowing us to determine which areas deserve priority attention for reintroductions (Favé and Turgeon, 2008; Frankham et al., 2004; Nabholz et al., 2009; Pelletier et al., 2009; Tzika et al., 2009).

If the purpose of reintroduction is to strengthen local populations, the evaluated organisms are suitable because the results indicate they have high haplotype diversity (Table 2). The use of confiscated individuals should be carefully considered because captive-bred individuals may accumulate genetic deterioration as result of stochastic processes in small populations, reducing the probability of success in reintroduction programs. However, a carefully designed program considering some unsuitable individuals for reintroduction may help increase individual genetic diversity in captive-breeding programs because using populations with high genetic diversity may mitigate the effects of these phenomena (Araki et al., 2007; Frankham, 2008; Smith and Hughes, 2008). Nevertheless, economic constraints may be considered because in determining regional differences with some degree of precision, the financial cost of captive-breeding programs may be greater than surveying wild parakeet populations. Moreover, released individuals must be carefully chosen to maintain a high local diversity in wild populations (Hedrick and Fredrickson, 2008; Smith and Hughes, 2008; Zeng et al., 2007). The confiscated group of *E. canicularis* analyzed in this work showed a high genetic diversity sufficient to establish a potentially viable breeding colony or to reinforce the local population. Using the molecular marker Cytb and through direct assignment and phylogenetic inference, we could determine the origin of approximately 63% of the confiscated individuals. Total individual identification was not possible because more field samples are required for several regions of the country. Direct allocation results indicate that the majority of the confiscated individuals (54.0%) share a widely distributed haplotype from northern Michoacán to Sinaloa. However, the absence of confiscated individual haplotypes that were shared or related with haplotypes from Guerrero and the Balsas in Michoacán narrowed the selection of potential reintroduction areas (Figure 3). The relationship among haplotypes observed in the network was corroborated by phylogenetic analyses (Figure 4). Phylogenetic analyses showed that EcNCBI is the individual with a greater genetic distance. In phylogenetic trees, this haplotype shows an early divergence with respect to the rest of the analyzed individuals. Previously, we reported a similar relation Padilla-Jacobo (2016); therefore, we assume that this haplotype may potentially correspond to an individual from a population distributed south of the Tehuantepec Isthmus. This haplotype is not shared with any haplotype in the confiscated group.

Cantú-Guzmán et al. (2007) described how local poachers in Nayarit state sell the captured parakeets to traders in Guadalajara. Our results corroborate that the captures for sale in the domestic market include parakeets from different localities in different states. The poaching range established in the sample examined extends from Nayarit to the north of Michoacán, where the number of poachers involved is unknown. We found no evidence that poachers mixed individuals from international trafficking in the sampled individuals (Cantú-Guzmán et al., 2007); in this group, only closely related haplotypes were detected.

Additionally, we observed an impact on the diversity richness by subtraction through rarefaction and extrapolation curves. The application of these tools is especially useful in the area of ecology and conservation biology, because through extrapolation the species richness in an area can be estimated (Colwell and Coddington, 1994; Colwell et al., 2004, 2012; Chao, 2005; Chao et al., 2009). Recently, estimates based on rarefaction and extrapolation curves have been applied to the analysis of DNA sequences, in particular in the estimation of bacterial and sea slug diversity, or prey preference (Feng et al., 2009; Ibáñez et al., 2016; Salinas-Ramos et al., 2015; Wilson et al., 2009). To our knowledge, this is the first study where mtDNA sequences were used in time rarefaction and extrapolation curves to estimate haplotype richness in psittacine populations.

The graph shows a light but constant growth directly proportional between the haplotype diversity and the number of samples without reaching the asymptote up to 240 samples. With these estimates, we can speculate that wild populations of *E. canicularis* experience a high diversity that has not yet been related to their geographical location. Moreover, when we compared the number of haplotypes detected in the field group (H = 18) against the number of haplotypes in the confiscated group (H = 23), the impact of the subtraction on the genetic pool of the species was evident. It is therefore advisable to return the confiscated individuals to their original populations. We highlight the utility of molecular tools in cases such as the one described in this study.

The Psittacidae family in México includes 21 species and represents the terrestrial bird group under the greatest poaching pressure, causing the family to present the highest number of species at risk (AOU, 1998; Cantú-Guzmán et al., 2007; Collar and Juniper, 1992; Howell and Webb, 1995; Iñigo-Elias and Ramos, 1991; Weston and Memon, 2009). According to the official Mexican norm NOM-059-SEMARNAT-2010, *Amazona auropalliata*, *A. farinosa*, *A.*

finschi, *A. oratrix*, *A. viridigenalis*, *Ara macao*, *A. militaris*, *Pyrilia haematotis*, *Rhynchopsitta pachyrhyncha*, and *R. terrisi* are endangered; and *Amazona xantholora*, *Psittacara holochlora*, *P. strenuus*, *Bolborhynchus lineola*, *Brotogeris jugularis* and *Pionus senilis* are facing a threatened status.

Therefore, a strategy should be designed to evaluate the genetic diversity of each species across their ranges in México and neighboring countries. Samples in the field must be collected from individuals during the nesting season, ideally from chicks in their nests, using non-invasive techniques. Information provided by the molecular data after sampling constitutes the most valuable information for conservation purposes, as demonstrated in the present work. To make this information more efficient, molecular data thus obtained can help in conservation strategies when collaborations are established between the seizure institutions and the research centers.

Conclusion

The confiscated group of 80 *E. canicularis* individuals showed a high genetic diversity, sufficient to establish a potentially viable breeding colony or to reinforce a local population. Direct assignment by comparing haplotypes with field samples allowed identification of 43 confiscated individuals, and an additional seven were identified through phylogenetic inference. The place of origin of 50 confiscated individuals was identified for a potential reintroduction region on any area with suitable tropical forests from the north of Michoacán to the south of Nayarit, México. The rarefaction curve shows a light but constant growth, with a potential high diversity that has not yet been detected in wild field populations of *E. canicularis*.

Conflict of interest

The authors declare that they have no conflict of interest.

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VII. DISCUSIÓN GENERAL

Como se ha mencionado, los psitácidos son un grupo de aves que sufre la mayor presión de sustracción tanto a nivel mundial como en nuestro país. Por esta razón es necesario establecer estrategias de conservación valorando diferentes aspectos, uno de los más importantes a considerar es la historia evolutiva que se conoce a través de la información genética.

En el presente trabajo se analizaron datos moleculares de *E. canicularis* ya que es el psitácido que sufre mayor presión de sustracción en México; se reunió información a nivel intraespecífico proveniente de secuencias de ADNmt y ADNnu, y se revisaron desde una perspectiva evolutiva con fines de conservación.

Ante la ausencia de información de datos moleculares, se realizó una exploración inicial donde se identificaron y se obtuvieron marcadores del ADNmt útiles para realizar análisis de diversidad genética intraespecífica en *E. canicularis*. Una vez que se definieron secuencias específicas del ADNmt (Cytb, ND2, COI), se utilizaron herramientas filogenéticas y de poblaciones para establecer unidades evolutivas significativas (ESU), identificadas a través de monofilia recíproca. Con estos datos y herramientas, en el primer acercamiento se reconocieron dos grupos genéticos que correspondían a la parte norte y centro de la distribución de la especie. Dichos grupos fueron propuestos como ESU para su conservación. Sin embargo, en el análisis no se incluyeron datos del ADNnu y la muestra analizada fue modesta.

En el avance de la investigación en una fase subsecuente, se buscaron y se obtuvieron secuencias nucleares y se consideró una muestra mayor. Con esta información se realizó la descripción de la historia evolutiva intraespecífica de *E. canicularis* abarcando localidades ubicadas en la parte norte de su distribución, esto es, desde Michoacán hasta Sinaloa, lo que representa más de un tercio del total de la distribución de la especie. En este análisis se detectaron señales genéticas de un crecimiento poblacional reciente. Aunque el patrón es claro en análisis de redes de haplotipos, mismatch y skiline plots, en otros análisis (D_T por ejemplo) no son contundentes, sino sugerentes; adicionalmente falta incluir muestras del sur, y de tierra dentro (en la cuenca del Balsas) con la finalidad de identificar si existen otros grupos y barreras geográficas asociadas a las posibles discontinuidades genéticas. Es necesario el uso

de herramientas diversas (software) para analizar los datos moleculares adicionales. Esto con la finalidad de observar la historia evolutiva "completa" de *E. canicularis*.

Respecto a la historia demográfica de la especie, se debe destacar que los resultados de las estimaciones demográficas muestran un crecimiento durante el Pleistoceno Medio y Superior, y un ligero descenso durante el Holoceno, estos resultados pueden ser complementados incluyendo individuos de la cuenca del Balsas, porque como sugieren los resultados del capítulo II, hay una señal de refugio por lo que podemos estar ante la posibilidad de identificar y proponer que la Cuenca del Balsas fue un refugio del pleistoceno.

En este contexto, los resultados obtenidos en el presente trabajo contribuyen al conocimiento de la historia evolutiva de aves del bosque tropical seco de México. Al respecto, la información es escasa y aún no se logra ver si existe o no un patrón filogeográfico que permita identificar los factores y el grado de su influencia en el modelado de las historias evolutivas y demográficas de las aves. Aunque son pocos ejemplos, se logra observar que las aves que habitan este tipo de vegetación no muestran divergencias profundas y que *E. canicularis* coincide en que la diferenciación genética es somera.

Por otro lado, como parte de los resultados obtenidos se muestra una propuesta que auxilia en la conservación de la especie. Se utilizó la información proveniente de la descripción de las relaciones genealógicas para establecer una recomendación de sitios para la reintroducción de individuos decomisados. Hasta donde conocemos, en nuestro país este es el primer trabajo que utiliza datos moleculares y herramientas filogenéticas para precisar posibles lugares de origen de los individuos sustraídos y áreas de reintroducción, por supuesto respetando la historia evolutiva de las poblaciones regionales. Además de identificar un posible sitio de reintroducción, en ese trabajo se logra corroborar puntos interesantes como el hecho de que los sitios de captura están relativamente cercanos a los centros de venta, en este caso para el mercado de mascotas en Jalisco.

Regresando a la propuesta de sitios de reintroducción, aparentemente el área propuesta aún es extensa, se sugiere el uso de microsatélites con la finalidad de limitar el área de reintroducción para propuestas posteriores. También es el primer trabajo en psitácidos donde se utilizan curvas de rarefacción y extrapolación con datos moleculares provenientes de individuos decomisados para estimar la diversidad de haplotipos no detectada durante el muestreo de campo.

VIII. PERSPECTIVAS Y/O RECOMENDACIONES

La demanda de psitácidos en el mundo provoca que este grupo de aves sea vulnerable. En México, existen 21 especies de psitácidos y todos están en la NOM-059-SEMARNAT-2010. Existe ausencia de información sobre las historias evolutivas intraespecíficas de especies de psitácidos en México; sus historias filogeográficas y demográficas sirven y deberían ser consideradas para plantear estrategias de conservación en estas especies.

En el presente trabajo se analizó a la especie *E. canicularis* mediante datos moleculares (secuencias nucleares y mitocondriales), con esos datos se describió la diversidad genética, la diferenciación genética entre grupos, la distribución de grupos genéticos en la parte centro y norte de la distribución de la especie, se revisaron sus relaciones genealógicas, se estimó su historia demográfica, y se utilizó esta información para realizar propuestas de conservación.

Con ello se establecieron las bases para ampliar y continuar con la descripción y el análisis filogeográfico de la especie, lo cual va a permitir esclarecer el grado de la influencia de diferentes factores sobre la estructura genética de la especie. En esta parte, se recomienda completar el trabajo incluyendo muestras de individuos que pertenezcan a poblaciones localizadas en el centro y sur de la distribución. Se debe prestar atención particular e incrementar el esfuerzo de colecta en las regiones donde otros autores han identificado discontinuidades genéticas que propician divergencia de linajes o diferenciación entre poblaciones de aves (por ejemplo a cada lado del Istmo de Tehuantepec, o entre Guerrero y Oaxaca). Los resultados obtenidos en este trabajo revelaron que entre individuos de localidades de la cuenca del Balsas existe alta diversidad genética (aun cuando se consideraron pocos organismos), por lo tanto en subsecuentes análisis también se deberá ampliar la muestra en esta región.

Adicionalmente, se deberá incluir la información proveniente de marcadores nucleares que presenten mayor tasa de mutación, por ejemplo microsatélites. Nuestros resultados muestran una ligera pero constante diferenciación entre poblaciones con diferentes tipos de análisis. Al usar la información proveniente de microsatélites se puede establecer si existe una divergencia marcada entre los grupos detectados, si existe migración entre ellos, si los machos o hembras son filopátricos, y desde un punto de vista de la conservación si se diferencian en ESU’s o MU’s.

Existe un conjunto de trabajos importantes que describen los posibles efectos de los cambios climáticos del Cuaternario en las estructuras genéticas de aves de tierras altas. Sin embargo, para aves de tierras bajas (o como en este caso del bosque tropical seco de México) las investigaciones son escasas, de tal manera que aún no existen propuestas sobre cuáles son los principales factores que pudieron o pueden estar modelando la estructura genética de las aves que habitan este tipo de vegetación.

La descripción inicial de los patrones filogeográficos que se observen en estas aves enriquece el conocimiento de la historia evolutiva del ecosistema, pero además tienen una aplicación práctica, para especies endémicas, vulnerables o en peligro de extinción pueden ser auxiliares para plantear estrategias de conservación que tengan mayor probabilidad de éxito. En el presente trabajo, para *E. canicularis* se muestra cómo los datos moleculares pueden ser útiles en la identificación de áreas de origen para individuos decomisados. En los individuos incautados identificamos alta diversidad genética; es ampliamente conocido que a través de la evaluación de la diversidad genética es posible plantear la recuperación de poblaciones con baja diversidad o establecer colonias reproductoras en cautiverio con alta diversidad genética. Destacamos la necesidad de establecer convenios con las instituciones nacionales encargadas de la conservación de especies con la finalidad de acercar este tipo de herramientas que ayudan a la solución de problemas específicos como las reintroducciones o la evaluación de la diversidad genética.

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X. APÉNDICE

ARTÍCULO 4

Enfocado al género *Psittacara*, pero en el contexto de la biogeografía histórica el siguiente artículo muestra las relaciones filogenéticas de *E. canicularis*, dentro de su género y con especies de géneros cercanos. Se establecen el área de origen más probable y los tiempos de divergencia para la especie que nos ocupa. Artículo sometido a Genetica, Springer.

Origin and diversification of the genus *Psittacara* and related genera (Aves: Psittacidae)

Gabriela Padilla-Jacobo[1], Tiberio C. Monterrubio-Rico[2], Horacio Cano-Camacho[1] and María G. Zavala-Páramo[1]

[1]Centro Multidisciplinario de Estudios en Biotecnología, FMVZ, Universidad Michoacana de San Nicolás de Hidalgo, Km. 9.5 Carretera Morelia-Zinapecuaro, Posta Veterinaria Morelia, Michoacán, México

[2]Laboratorio de Ecología de Vertebrados Terrestres Prioritarios, Facultad de Biología, Edificio R, CU, Universidad Michoacana de San Nicolás de Hidalgo, Morelia, Michoacán, México.

Abstract

In this study, divergence times and ancestral areas of *Psittacara* and related genera were estimated to propose hypotheses of diversification and dispersal of groups under a Bayesian inference framework, based on mitochondrial molecular markers. Five monophyletic clades were identified; a) The basal Lepthosittaca-group denominated thus by its basal species *Lepthosittaca branickii*, b) *Aratinga*, c) Orthopsittaca-group designated thus by its basal species *Orthopsittaca manilatus*, d) *Eupsittula*, and e) *Psittacara.* In agreement with results of phylogenetic analyses, we recommended the formal recognition of *Psittacara acuticaudatus* as *Thectocercus acuticaudatus*, *Conuropsis carolinensis* as *Aratinga carolinensis*, and *Psittacara h. brevipes* as *Psittacara brevipes.* Under the hypothesis of the Cretaceous or Paleogene origin, the diversification of the analyzed groups took place during the Miocene and Pliocene periods, mainly through dispersal events. Biogeographic reconstruction suggests that the most likely origin is the Amazonian-Caribbean region. Diversification of these groups is related to geo-climatic events associated with the uplift of the central and northern portions of the Andes and the closure of the Isthmus of Panama. There is no coincidence with the hypotheses Pliocene-Pleistocene refuges. We propose dispersion routes from south to north in the Neotropic and also the use of the Greater and Lesser Antilles as a northward path.

Keywords: Psittaciformes, Arini, Bayesian inference, historical biogeography, phylogeny, Neotropic.

Introduction

It has been established that geo-climatic events promoted the diversification of different tetrapod's lineages during the Miocene-Pliocene in the Neotropic. These events include the isolation and reconnection of South America, the uplift of the Andes, the marine incursions and extensive flood-basin system in the Amazonian, the formation of Orinoco and Amazon drainages, and the dry-wet climate cycles of the Pliocene-Pleistocene (Haffer 1997; Cortés-Ortiz et al. 2003; Johnson et al. 2006; Patterson and Velazco 2008; Santos et al. 2009; Hoorn and Wesselingh 2010; Leigh et al. 2014). The Neotropics present the greatest diversity of birds in the world with approximately 36% of all know land-bird species, showing 90 families, of which 28 are endemic (Stotz et al. 1996; Newton 2003). The monophyletic Arini tribe consists of Neotropical parrots, with approximately 149 species distributed from México to the south of South America (Forshaw 1989; Collar 1997). For some parrot

species has been established that the triggers of speciation were the complex of geo-tectonics events, marine incursions, and river dynamic (Ribas and Miyaki 2004; Eberhard and Bermingham 2005; Ribas et al. 2005), but analysis of this type are scarce or inexistent for parrots of northern distributions.

Into the Arini tribe, the genus *Psittacara* and related groups are a good example to observe diversification and dispersal patterns of birds from south to north in the Neotropic, because they are widely distributed with species present in México, Central America and the Caribbean islands, and South America. The gradual increase in available information and revisions of taxonomic relations have made possible to generate hypotheses that explain the origin and diversification of the natural groups. To propose divergence times and ancestral areas under Bayesian Inference (BI) is necessary to have a strong phylogenetic reconstruction. A valuable basis for proposals of nomenclatural changes has been established for the genus *Psittacara* and related genera.

Psittacara was first described by Vigors (1825), who referred to the genus as Parakeet-Macaws due to the morphological characters ranked among the macaws and the long-tailed parakeets. Previously, species inside this genus were described for the genera *Psittacus*, *Sittace*, *Psittacara*, and *Conurus*. However, Salvadori (1891) included these species within the genus *Conurus* (28 recognized species). Later, Peters (1937) placed these species in the genera *Aratinga* and *Nandayus*. For the genus *Aratinga*, the classification proposed by Peters (1937) included 21 species (Table 1). However, based on evidence demonstrated by phylogenetic reconstructions from molecular data, some species have been reclassified into different genera (Chesser et al. 2014; del Hoyo et al. 2014). *Aratinga* was initially proposed to be a polyphyletic group that included the monotypic genus *Nandayus* (Ribas and Miyaki 2004). Furthermore, *Nandayus nenday* was reclassified as a sister taxa to *Aratinga solstitialis.* In other studies, *Aratinga* was split into three clades, and the genus *Rhynchopsitta* was proposed as the putative sister group to all *Aratinga* (Tavares et al. 2006; Kirchman et al. 2012). More recently, a suggestion was made to divide *Aratinga sensu* Peters (1937) into four genera: *Aratinga*, *Eupsittula*, *Psittacara*, and *Thectocercus* (Remsen et al. 2013). BirdLife International -BLI- (2015) and del Hoyo et al. (2014), however, have formally recognized only *Aratinga*, *Eupsittula*, and *Psittacara* (Table 1).

On the other hand, the origin of the Psittaciformes into the neornithine crown group is under discussion. There are two points of view: a) The Paleogene origin hypothesis sustained by fossil records (Dyke and Mayr 1999; Mayr 2002, 2014), and b) A Cretaceous origin based on molecular data analyses and biogeographical inferences (Cracraft 1973; Forshaw 1989; Tavares et al. 2006; Wright et al. 2008).

Table 1 Contrasting classifications of the genus *Psittacara*

Salvadori (1891)		**Peters (1937)**		**Remsen et al. (2013)**		**Chesser et al. (2014)**		**del Hoyo et al. (2014); BLI (2015)**			**This study**	
Conurus	*acuticaudatus*	*Aratinga*	*acuticaudata*	*Aratinga*	*auricapillus*	*Aratinga*	*auricapillus*	*Aratinga*	*auricapillus*		*Aratinga*	*auricapillus*
	aztec		*astec*		*jandaya*		*jandaya*		*carolinensis**			*carolinensis*
	aureus		*aurea*		*maculata*		*maculata*		*jandaya*			*jandaya*
	auricapillus		*auricapillus*		*nenday*		*nenday*		*maculata*			*maculata*
	cactorum		*cactorum*		*solstitialis*		*solstitialis*		*nenday*			*nenday*
	canicularis		*canicularis*		*weddellii*		*weddellii*		*solstitialis*			*solstitialis*
	chloropterus		*chloroptera*						*weddelli*			*weddellii*
	rubrolarvatus		*erythrogenys*	*Eupsittula*	*aurea*	*Eupsittula*	*astec*	*Eupsittula*	*astec*		*Eupsittula*	*aurea*
	euops		*euops*		*cactorum*		*aurea*		*aurea*			*cactorum*
	finschi		*finschi*		*canicularis*		*cactorum*		*cactorum*			*canicularis*
	guarouba		*guarouba*		*nana*		*canicularis*		*canicularis*			*nana*
	holochlorus		*holochlora*		*pertinax*		*nana*		*nana*			*pertinax*
	jendaya		*jandaya*				*pertinax*		*pertinax*			
	leucophthalmus		*leucophthalmus*	*Thectocercus*	*acuticaudatus*						*Thectocercus*	*acuticaudatus*
	mitratus		*mitrata*			*Psittacara*	*acuticaudatus*	*Psittacara*	*acuticaudatus*			
	nanus		*nana*	*Psittacara*	*chloropterus*		*chloropterus*		*chloropterus*		*Psittacara*	*brevipes*
	pertinax		*pertinax*		*erythrogenys*		*erythrogenys*		*erythrogenys*			*chloropterus*
	solstitialis		*solstitialis*		*euops*		*euops*		*euops*			*erythrogenys*
			strenua		*finschi*		*finschi*		*finschi*			*euops*
	wagleri		*wagleri*		*holochlorus*		*frontatus*		*frontatus*			*finschi*
	weddelli		*weddellii*		*leucophthalmus*		*holochlorus*		*holochlorus*	*P.h. brevipes* *P.h. strenuus*		*holochlorus*
					mitratus		*leucophthalmus*		*labati**			*leucophthalmus*
	nenday	*Nandayus*	*nenday*		*strenuus*		*mitratus*		*leucophthalmus*			*mitratus*
	brevipes				*wagleri*		*rubritorquis*		*mitratus*			*rubritorquis*
	frontatus						*strenuus*		*rubritorquis*			*wagleri*
							wagleri		*wagleri*			

*Only considered by BLI (2015)

On the Paleogene origin hypothesis, a diverse array of putative stem group representatives of Psittaciformes is known from Eocene-Paleogene fossil sites (Mayr 2014). Moreover, although with some discrepancies, recently some authors support the idea of the most recent origin of Psittaciformes (Jarvis et al. 2014; Claramunt and Cracraft 2015). Under the hypothesis of Cretaceous origin, the Psittaciformes occurred in Gondwana during the Cretaceous Period after the separation of Africa from the Indian/Madagascar block, and subsequent diversification of the groups occurred through dispersal and vicariance (Wright et al. 2008).

Psittacara, *Aratinga*, and *Eupsittula* are a noteworthy case due to their wide distribution, and they have representing species in North America and South America. However, until now no studies have been conducted to test any hypotheses concerning the origin and diversification of these genera. Determining their origin and diversification helps to reinforce the general hypothesis of dispersal patterns of fauna from South America to North America. The first aim of this study was to establish for the first time the divergence times of *Psittacara* and related genera under the hypothesis that considers the origin of Psittaciformes during the Cretaceous or Paleogene. A second objective was to propose the most likely ancestral areas where these groups originated and how they diversified and dispersed.

Materials and methods

Sequences and biological samples

Sequences for *Psittacara* and related genera were obtained from the National Center for Biotechnology Information (NCBI) GenBank database. Using the species included in the phylogenetic reconstruction by Kirchman et al. (2012) and data available in GenBank, 37 taxa were compared via two mitochondrial DNA (mtDNA) molecular markers: cytochrome c oxidase subunit 1 (COI) and NADH dehydrogenase subunit 2 (ND2) (Table 2). In addition, biological samples were collected from two *Eupsittula canicularis* individuals found in two localities in Mexico: Palos Marías (Michoacán) and Badiraguato (Sinaloa). In the field, we collected a sample of growing feathers with tissue or blood at the base from each individual and placed the sample 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 for a week during transport and subsequently stored at 4°C.

Table 2 List of the species (and/or subspecies) used in this study, including author, locality, identification, GenBank accession numbers, and sequence author

Specie	Authors	Locality	Museum/ collection (identification)	GenBank Access		Sequence Author
				COI	ND2	
Aratinga nenday	(Vieillot, 1823)	Captive, Spain, Canary Islands, Tenerife, Loro Parque	LPF(LP07-23)	EU621632	EU327636	Kirchman, et al. (2012)
Aratinga solstitialis	(Linnaeus, 1758)	Captive, locality unknown	NMNH(B06816)	GU826185.1	HQ270491.1	Kirchman, et al. (2012)
Aratinga auricapillus	(Kuhl, 1820)	Captive, U.S.A., Texas, Clarence Killian Aviary	LSU(B-29884)	GU826176.1	HQ270483.1	Kirchman, et al. (2012)
Aratinga weddellii	(Deville, 1851)	Brazil, Acre	LGEMA(2085)	---	AY669445.1	Ribas, et al. (2005)
Aratinga jandaya	(Gmelin, 1788)	Locality unknown	Unknown	---	JX645194.1	Urantowka, et al. (2013)
Aratinga maculata	(Statius Müller, 1776)	Monte Alegre PA (Naturaleza)	LGEMA(9158)	---	KJ142296.1	Freddi, et al. (2015)
Eupsittula nana	(Vigors, 1830)	Panamá, Bocas del Toro, San Cristobal I, Bocatorito	NMNH(B00465)	GU826184.1	HQ270490.1	Kirchman, et al. (2012)
Eupsittula canicularis	(Linnaeus, 1758)	Captive, locality unknown	AMNH(DOT9252)	HQ629753.1	HQ629718.1	Schrizinger et al. 2012
Eupsittula canicularis	(Linnaeus, 1758)	Palos Marías, Michoacan, Mexico	CMEB(AceMpm53)	KJ612381	KJ612390	This study
Eupsittula canicularis	(Linnaeus, 1758)	Badirahuato, Sinaloa, Mexico	CMEB(AccSb14)	KJ612385	KJ612394	This study
Eupsittula aurea	(Gmelin, 1788)	Brazil, Para, Altamira	NMNH(b07010)	GU826175.1	HQ270482.1	Kirchman, et al. (2012)
Eupsittula pertinax	(Linnaeus, 1758)	Venezuela, Caracas, Phelps Ornithological Collection	POC(ML683)	NC_015197.1	NC_015197.1	Kirchman, et al. (2012)
Eupsittula cactorum	(Kuhl, 1820)	Tocantins, Brazil	LGEMA(0992)	AF370750.1	---	Tavares, et al. (2004)
Thectocercus acuticaudatus	(Vieillot, 1818)	Captive, locality unknown	Unknown	NC_020325	NC_020325	Urantowka, et al. (2013)
Psittacara holochlorus	(Sclater, 1859)	Captive, USA, Texas	LSU(B-23651)	GU826181.1	HQ270487.1	Kirchman, et al. (2012)
Psittacara h. brevipes	(Lawrence, 1871)	Captive, Mexico, Puebla, African Safari Parque	Unknown	NC_021764.1	NC_021764.1	Urantowka, et al. (2014)
Aratinga h. rubritorquis	(Sclater, 1887)	Captive, Spain, Canary Islands, Tenerife, Loro Parque	Unknown	JX524614.1	JX524614.1	Urantowka, et al. (2014)
Psittacara wagleri	(G.R. Gray, 1845)	Captive, Spain, Canary Islands, Tenerife, Loro Parque	LPF(LP07-14)	GU826186.1	HQ270492.1	Kirchman, et al. (2012)
Psittacara mitratus	(von Tschudi, 1844)	Captive, Spain, Canary Islands, Tenerife, Loro Parque	LPF(LP07-12)	GU826183.1	HQ270489.1	Kirchman, et al. (2012)

Psittacara finschi	(Salvin, 1871)	Panamá, Bocas del Toro, San Cristobal I, Bocatorito	NMNH(B00402)	GU826180.1	HQ270486.1	Kirchman, et al. (2012)
Psittacara leucophthalmus	(Statius Muller, 1776)	Argentina, Corrientes, 45 km S, Manuel Derqui	NMNH(B05906)	GU826182.1	HQ270488.1	Kirchman, et al. (2012)
Psittacara euops	(Wagler, 1832)	Captive, Spain, Canary Islands, Tenerife, Loro Parque	LPF(LP07-25)	GU826179	HQ270485	Kirchman, et al. (2012)
Psittacara chloropterus	(Souancé, 1856)	Captive, Spain, Canary Islands, Tenerife, Loro Parque	LPF(LP07-26)	GU826178	HQ270484	Kirchman, et al. (2012)
Psittacara erythrogenys	(Lesson, 1844)	Rio Zoo RJ.Cautiverio	LGEMA(9166)	---	KJ142291.1	Freddi, et al. (2015)
Anodorhynchus hyacinthinus	(Latham,1790)	Captive, locality unknown	LSU(B100020/B-13478)	GU826173.1	HQ270480.1	Kirchman, et al. (2012)
Ara glaucogularis	(Dabbene, 1921)	Locality unknown	NC_026029.1	NC_026029.1	NC_026029.1	Kirchman, et al. (2012)
Ara macao	(Linnaeus, 1758)	Captive, USA, Iowa, Blank Park Zoo	Unknown	CM002021.1	CM002021.1	Kirchman, et al. (2012)
Cacatua sulphurea	(J. F. Gmelin, 1788)	Captive, USA, Florida, Miami, Miami Zoo	Unknown	EU621602.1	EU327605.1	Wright, et al. (2008)
Conuropsis carolinensis	(Linnaaeus, 1758)	USA, Florida, Manatee County	NYSM(9421)	GU826189	HQ270495	Kirchman, et al. (2012)
Cyanopsitta spixii	(Wagler, 1832)	Captive, Spain, Canary Islands, Tenerife, Loro Parque	LPF(Cyspi01)	EU621610.1	EU327614.1	Kirchman, et al. (2012)
Diopsittaca nobilis	(Linnaaeus, 1758)	Guyana, Karaudanawa	NMNH B12392	EU621614.1	EU327618.1	Kirchman, et al. (2012)
Guaruba guarouba	(J. F. Gmelin, 1788)	Captive, locality unknown	NMNH(B-06578)	EU621624.1	EU327628	Kirchman, et al. (2012)
Leptosittaca branickii	(Berlepsch and Stolzmann, 1894)	Ecuador, Jimbura; E Slope Cord. Lagunillas	ANSP(4966)	EU621626.1	EU327630.1	Kirchman, et al. (2012)
Orthopsittaca manilatus	(Boddaert, 1783)	Captive, locality unknown	Unknown	KJ579139.1	KJ579139.1	Kirchman, et al. (2012)
Primolius couloni	(Sclater, 1876)	Captive, locality unknown	Unknown	NC_025742.1	NC_025742.1	Kirchman, et al. (2012)
Rhynchopsitta pachyrhyncha	(Swainson, 1827	Captive, locality unknown	NMNH(B08714)	EU621661.1	EU327665.1	Kirchman, et al. (2012)
Rhynchopsitta terrisi	(Moore, 1947)	Captive, Mexico, Puebla, African Safari Parque	Unknown	NC_021771	NC_021771	Urantowka, et al. (2013)

Abbreviations for collections: AMNH, American Museum of Natural History; ANSP, Academy of Natural Sciences, Philadelphia; CMEB, Centro Multidisciplinario de Estudios en Biotecnología, Universidad Michoacana de San Nicolás de Hidalgo; LGEMA Laboratorio de Genetica e Evolucao Molecular Universidade de Sao Paul, Brazil; LPF Loro Parque Fundación Tenerife, Spain; LSU = Louisiana State University Museum of Natural Science; NMNH, Smithsonian National Museum of Natural History; NYSM = New York State Museum; POC = Phelps Ornithological Collection

These samples were deposited under the identifiers AccSb14 and AceMpm53 in the wildlife sample collection at the Multidisciplinary Center for Studies in Biotechnology (Centro Multidisciplinario de Estudios en Biotecnologia) at Universidad Michoacana de San Nicolas de Hidalgo in Morelia, Michoacan, Mexico.

DNA extraction and sequencing

DNA was extracted using the phenol-free method described by FitzSimmons (1997). Two sequences of mtDNA were amplified: ND2 and COI. The ND2 sequence of approximately 1100 bp was obtained using the oligonucleotides L5215 (5′-TATCGGGCCCATACCCCGAATA-3′) and HTrpC (5′-CGGACTTTAGCAGAAACTAAGAG-3′) (Hackett 1996). For the CO1 amplification of about 550 bp, we used the oligonucleotides COI_{arcaD} (5′-CTACCACGCGGGCAAAAA-3′) and COI_{arcaR} (5′-CCCAATGGAGGATAAAGTGTT-3′). These oligonucleotides were designed using Lasergene 10 software (DNASTAR, Madison, WI, USA) (Kumar and Blaxter 2010), based on the COI sequences from *E. canicularis* and other species of the genus *Eupsittula*.

PCR was performed in a total volume of 25 µl with the following components: 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 reactions were placed in a thermocycler (Gene Amp 2700, Applied Biosystems, Foster City, CA, USA) and amplified under the following conditions: 94°C for 5 min; followed by 30 cycles of 94°C for 40 s, 55-56°C for 40 s, and 72°C for 2 min; and a final extension at 72°C for 5 min.

The sequencing of both DNA strands was performed using the dideoxy method (Sanger et al. 1977); services were provided by Macrogen (Rockville, MD, USA).

Phylogenetic analysis

Sequence editing, alignments, and the construction of the data matrices were carried out with Sequencher v.4.1 (Gene Codes Corporation, Ann Arbor, MI, USA) and PhyDE (Müller et al. 2005). Genetic distance was estimated under the K2P model of MEGA 5.05 (Tamura et al. 2011), and separate analyses were run for each genetic marker where the sequences with “N” were excluded. Models of molecular evolution were constructed with jModelTest 2.1.1 (Posada 2008) and selected using the corrected Akaike Information Criterion (cAIC)

(Alfaro and Huelsenbeck 2006). The best models obtained using this criterion was the COI (HKY+I+G), ND2 (TIM2+I+G), and TrN+I+G for all the sequence data after concatenation.

Phylogenetic reconstructions were generated using maximum likelihood (ML) and Bayesian inference (BI) frameworks. The ML and BI reconstructions were performed using GARLI (Zwickl 2006) and MrBayes v3.2 (Ronquist and Huelsenbeck 2003) software. The branch support values were estimated by the bootstrap analysis of 250 replicates and by posterior probabilities (PP). MrBayes runs were performed using the following parameters: four independent runs of four chains each (one cold chain and three hot chains) for 10 million generations with sampling every 1000 generations. Trees and parameters were summarized after discarding 25% of the data (burn-in). The remaining trees were summarized as a majority consensus tree. Separate analyses were run for each genetic marker and the concatenated sequences.

Bayesian inference of divergence time

To estimate divergence times for the genus *Psittacara* and related taxa, we used two different calibration dates. Because of the absence of fossil records containing available specimens for our analysis (www.fossilworks.org), we considered data from previous calibrations performed by Claramunt and Cracraft (2015) and Tavares et al. (2006). Claramunt and Cracraft (2015) dates are consistent with the Psittaciformes Paleogene origin hypothesis, and Tavares et al. (2006) date considering a Cretaceous origin. In detail, Claramunt and Cracraft (2015) propose estimates made under Bayesian inference with nuclear sequences of two recombination-activating genes (*Rag1* and *Rag2*) for 230 species representing 202 families and all avian orders. Twenty-four calibrations based on estimates likelihood distributions of fossil age were used. The divergence date estimate by Tavares et al. (2006) was made under two methods (penalized likelihood and Bayesian inference) with nuclear sequences of *Rag1* gene and sequences of mitochondrial DNA of 29 species in the Arini tribe, one individual of Strigopidae, one Cacatuidae, and one Psittaculidae. They calibrated the node that separates the taxon *Strigops* from New Zeland of the other parrots at 85-82 Mya because this split can be a vicariant precedent (isolation of New Zeland).

In this study, divergence times were estimated using BEAST v1.7.4 (Drummond and Rambaut 2007). To establish the divergence age by to the Paleogene origin hypothesis, we used one calibration point using a normal distribution for the root of the tree (average = 33.5 Mya, SD = 0. 5). To establish the divergence age by the hypothesis of a Cretaceous origin, we used constraints for two calibration points using normal distributions. One

point at the root of the tree (average = 67.6 Mya, SD = 0.5) and the other one at the Nenday-Solstitialis-Auricapillus node (average = 5.5 Mya, SD = 0.5). With both data sets, the following specifications were used: an uncorrelated lognormal relaxed clock model was selected with a GTR+G+I selection model. We used a Yule-type speciation model because it is appropriate for the analysis of sequences obtained from different species (Yule 1924; Aldous 2001). In addition, constraints were set for four clades in the crown group. Markov Chain Monte Carlo (MCMC) analyses were run for 10 million generations with sampling every 1000 generations. The results were summarized using TreeAnnotator v1.7.4 (Drummond and Rambaut 2007). After 10% of the trees were discarded, the remaining trees were summarized as a maximum clade credibility tree, including the average divergence times and their associated 95% high posterior densities (HPDs). Trees were visualized using FigTree v1.4.0 (Rambaut 2012).

Reconstruction of ancestral areas

Reconstruction of ancestral areas was performed using 10,001 trees generated by BEAST (Drummond and Rambaut 2007). Dispersal-vicariance analysis was performed using the S-DIVA tool in RASP v 3.0 (Yu et al. 2014). S-DIVA analyses are advantageous because they provide statistical support for the ancestral area reconstructions (Yu et al. 2010). Four defined zones according to the Neotropical subregions proposed by Morrone (2001) were included in these analyses. The following subregions were included. The Caribbean subregion (A), which comprises central and southern México, Central America, the Antilles and northwestern South America, Ecuador, Colombia, Venezuela, and Trinidad and Tobago. The Amazonian subregion (B), which is the largest in the Neotropical region and extends through most of Brazil and the Guyanas and parts of Venezuela, Colombia, Ecuador, Peru, Bolivia, Paraguay, and Argentina. The Chaco subregion (C), which covers northern and central Argentina, southern Bolivia, western and central Paraguay, and central and northwestern Brazil. The Parana subregion (D), which comprises northwestern Argentina, the eastern region of Paraguay, and the extreme southern and eastern areas of Brazil. The distributions of the genera were established in geographic maps according to records produced by different authors and summarized in del Hoyo et al. (2014) (Table 3).

Table 3 Species distribution according to del Hoyo et al. (2014) and codes assigned to the distribution areas

Specie	Distribution	Area code
Aratinga nenday	Bolivia, Brazil, Paraguay, Argentina	BC
Aratinga solstitialis	Brazil, Guiana	B
Aratinga auricapillus	Brazil	B
Aratinga weddellii	Colombia, Ecuador, Peru, Brazil, Bolivia	B
Aratinga jandaya	Brazil	BC
Aratinga maculata	Brazil	B
Eupsittula nana	Mexico, Jamaica	A
Eupsittula canicularis	Mexico, Costa Rica	A
Eupsittula canicularis	Mexico, Costa Rica	A
Eupsittula canicularis	Mexico, Costa Rica	A
Eupsittula aurea	Suriname, Brazil, Peru, Bolivia, Paraguay, Argentina	BCD
Eupsittula pertinax	Panama, Colombia, Venezuela, Antillas, Guiana, Brazil	AB
Eupsittula cactorum	Brazil	BCD
Thectocercus acuticaudatus	Colombia, Venezuela, Brazil, Bolivia, Paraguay, Argentina	ABCD
Psittacara holochlorus	Mexico, Nicaragua	A
Psittacara h. brevipes	Mexico	A
Aratinga h. rubritorquis	Guatemala, El Salvador, Honduras, Nicaragua	A
Psittacara wagleri	Colombia, Venezuela	A
Psittacara mitratus	Peru, Bolivia, Argentina	BC
Psittacara finschi	Nicaragua, Costa Rica, Panama	A
Psittacara leucophthalmus	Colombia, Ecuador, Peru, Brazil, Guianas, Venezuela, Bolivia, Paraguay, Argentina, Uruguay	ABCD
Psittacara euops	Cuba	A
Psittacara chloropterus	I. Hispaniola	A
Psittacara erythrogenys	Ecuador, Peru	A
Anodorhynchus hyacinthinus	Brazil, Bolivia, Paraguay	BC
Ara glaucogularis	Bolivia	B
Ara macao	Mexico, Central America , Colombia, Venezuela, Guianas, Brazil, Ecuador, Peru, Bolivia	AB
Cacatua sulphurea	-	E
Conuropsis carolinensis	USA	A
Cyanopsitta spixii	Brazil	C
Diopsittaca nobilis	Venezuela, Guianas, Brazil	AB
Guaruba guarouba	Brazil	B
Leptosittaca branickii	Colombia, Ecuador, Peru	A
Orthopsittaca manilatus	Colombia, Ecuador, Peru, Bolivia, Venezuela, Trinidad, Guianas, Brazil	BCD
Primolius couloni	Peru, Brazil, Bolivia	B
Rhynchopsitta pachyrhyncha	Mexico	A
Rhynchopsitta terrisi	Mexico	A

Results

Phylogenetic analyses

Two COI fragments of 552 bp and 576 bp and two ND2 gene sequences of 1055 bp and 1066 bp were amplified from the DNA samples of *E. canicularis* (AceMpm53 and AccSb14 in trees) and registered in GenBank (accession numbers: KJ612381, KJ612385, KJ612390, and KJ612394). The sequences of the 550-bp and 1037-bp fragments were used for the sequence alignments of COI and ND2, respectively. The alignments included 1610 bp from 37 taxa, with 904 invariable characters, 535 parsimony-informative characters, and 171 variable but uninformative characters.

BI and ML analyses produced trees with similar topologies. Overall, the PP and BP values provided sufficient support for the phylogenetic relationships established within each group (Fig. 1).

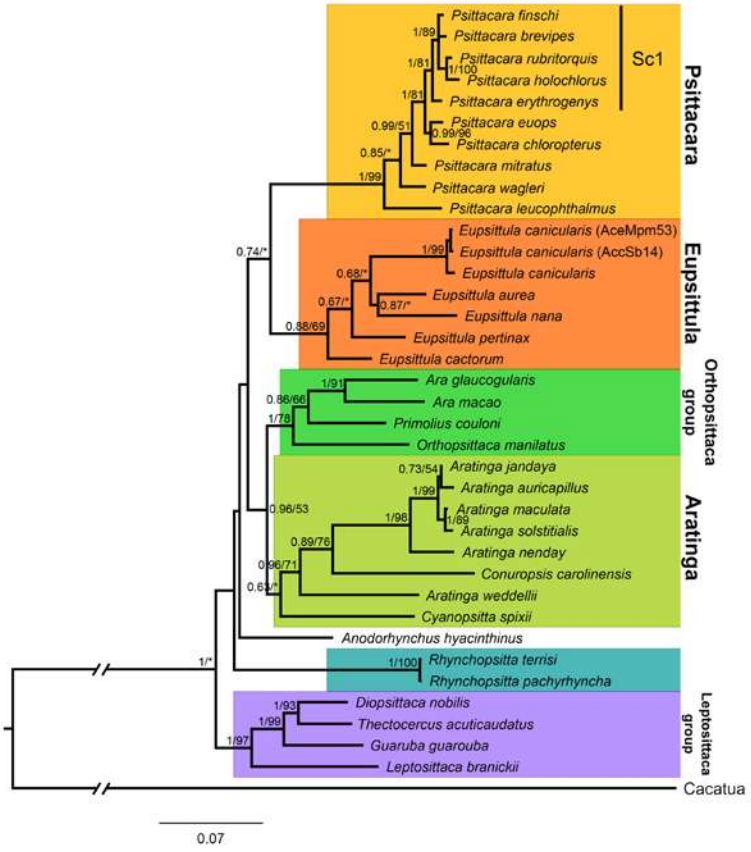

Fig. 1 Phylogram of the genus *Psittacara* and related genera. Values over the branches represent posterior probabilities obtained by Bayesian inference analysis and maximum likelihood bootstrap values (PP/BP). (*) Value inferior at PP = 0.5 or PB = 50

We found five monophyletic clades within the analyzed taxa. The Leptosittaca-group, which we denominated thus by its basal species *Leptosittaca branickii*, is sister to all remaining taxa. The genus *Rhynchopsitta* is sister to the crown group and *Anodorhynchus hyacinthinus*. The relations among some clades were not well supported, so they must be taken with caution. However, BI and ML analyses show the genus *Aratinga* as the sister of the Orthopsittaca-group, which we denominated thus by its basal species *Orthopsittaca manilatus*, and the genus *Eupsittula* as the sister of the *Psittacara*. Our results from the taxa classification are summarized in Table 1.

In our analyses, *Psittacara acuticaudatus*, proposed by Remsen et al. (2013) as *Thectocercus acuticaudatus* is placed within the Leptosittaca-group clade formed by *Diopsittaca nobilis*, *Guaruba guarouba*, and *Leptosittaca branickii* with a support of PP = 1.00 and BP = 97. The clade *Aratinga* contains eight taxa (including the extinct *Conuropsis carolinensis*). The position of *Cyanopsitta spixii* is weakly supported, placing that position in doubt. The rest of the clade is supported with PP = 0.96 and PB = 71. The relation of the Orthopsittaca-group to the *Aratinga* in the Bayesian tree is supported by PP = 0.96 and, in the ML tree, is supported by PB = 53. The *Eupsittula* clade (PP = 0.88; BP = 69) contains five species (*E. aurea*, *E. cactorum*, *E. canicularis*, *E. nana*, and *E. pertinax*). *Eupsittula aurea* and *E. nana* form a sister clade with *E. canicularis*, although relations within this genus are not well supported.

Within the *Psittacara* clade (PP = 1.0; BP = 99), we analyzed 10 taxa. The subclade (Sc1) (PP = 1.0; BP = 81) (Fig. 1) includes *P. erythrogenys*, *P. finschi*, *P. holochlorus brevipes*, *P. rubritorquis*, and *P. holochlorus*. The topology of the BI and ML trees shows *P. holochlorus* and *P. rubritorquis* as sister species (PP = 1; PB = 100) and as closely related to *P. h. brevipes* (*P. brevipes* in the tree) and *P. finschi* (PP = 1; PB = 89). We corroborated the relation of *P. chloropterus* and *P. euops* (PP = 0.99; PB = 96) as sister species (Collar et al. 2016a). In addition, *P. leucophthalmus*, *P. wagleri*, and *P. mitratus* indicate an early divergence within the *Psittacara* clade. The greatest genetic distance between the closely related species in this genus was 0.071 (with ND2), and the least distance was 0.006 (with COI) (Table 4).

Table 4 Pairwise genetic distance between *Psittacara* species, estimated under K2P model using ND2 (above diagonal) and COI (below diagonal)

	P. leucophthalmus	*P. wagleri*	*P. mitratus*	*P. euops*	*P. chloropterus*	*P. erythrogenys*	*A. h. rubritorquis*	*P. holochlorus*	*P. finschi*	*P. h. brevipes*
P. leucophthalmus		0.051	0.061	0.060	0.066	0.067	0.071	0.070	0.070	0.063
P. wagleri	0.053		0.030	0.044	0.045	0.039	0.043	0.047	0.041	0.045
P. mitratus	0.043	0.026		0.029	0.028	0.027	0.032	0.040	0.029	0.034
P. euops	0.053	0.033	0.029		0.022	0.025	0.033	0.041	0.025	0.029
P. chloropterus	0.058	0.036	0.031	0.015		0.027	0.034	0.042	0.027	0.030
P. erythrogenys	-	-	-	-	-		0.018	0.025	0.015	0.020
A. h. rubritorquis	0.063	0.041	0.036	0.019	0.022	-		0.016	0.013	0.019
P. holochlorus	0.066	0.043	0.038	0.022	0.024	-	0.006		0.021	0.024
P. finschi	0.064	0.041	0.036	0.019	0.022	-	0.013	0.011		0.011
P. h. brevipes	0.061	0.038	0.029	0.017	0.020	-	0.011	0.008	0.006	

(-) No available Data

Bayesian inference of divergence times and reconstruction of ancestral areas

The estimated node ages and biogeographic reconstruction are shown in Figure 2 and Table 5. Because the group ages were calculated based on secondary calibrations, these results should be considered with caution. To our knowledge, no previous proposals exist about the regions of origin and estimated ages for the genus *Psittacara*.

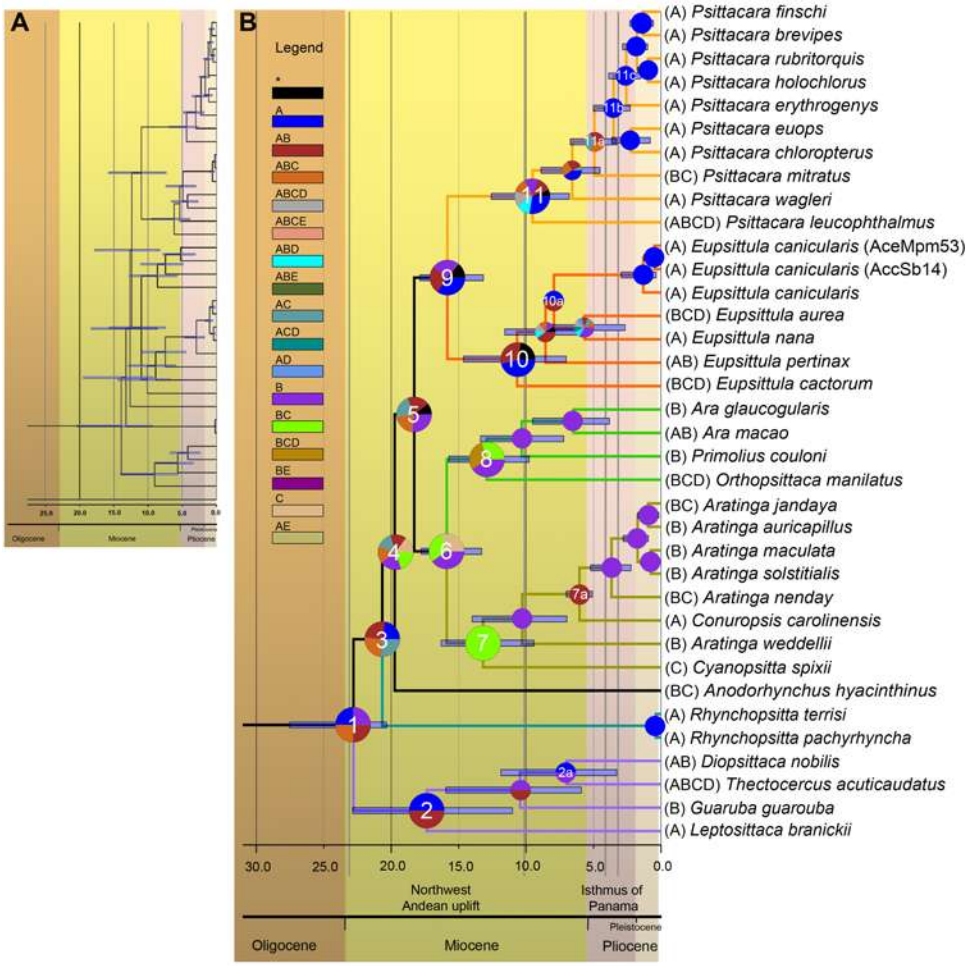

Fig. 2 Chronogram with divergence times and ancestral distribution of the genus *Psittacara* and related genera. A) Chronogram with divergence times under the hypothesis of Paleogene origin of Psittaciformes. B) Chronogram with divergence times under the hypothesis of Cretaceous origin of Psittaciformes and ancestral areas. Bars indicate 95% confidence intervals (high posterior densities, HPD) for the estimation of node age. In B), the labels at the nodes represent ancestral zone probabilities. The legend to the left summarizes the colors and codes that represent geographic zones associated with the labels: (A) Caribbean subregion, (B) Amazonian subregion, (C) Chaco subregion, and (D) Parana subregion

Table 5 Divergence times of the genus *Psittacara* and related groups

	Cretaceous origin		Paleogene origin	
Node	Mean	HPD 95%	Mean	HPD 95%
1	22.8	20.34-27.53	13.91	9.39-20.43
2	17.37	11.00-22.86	9.03	5.65-14.10
2a	7.00	3.28-11.87	4.20	2.41-6.83
3	20.66	19.75-21.55	13.24	9.25-19.50
4	19.74	18.39-20.96	12.65	8.59-18.36
5	18.29	17.42-19.18	12.37	8.26-17.86
6	15.88	13.29-17.79	10.99	7.47-15.70
7	13.19	9.42-16.31	10.11	6.80-14.42
8	12.91	9.77-15.74	8.63	5.76-12.74
9	15.84	13.16-17.88	10.99	7.32-15.81
10	10.67	7.02-14.65	6.19	3.90-9.49
11	9.52	6.79-12.58	4.38	2.68-6.96
11b	3.51	2.28-5.02	1.63	1.20-5.40
11c	2.57	1.50-3.89	1.17	0.68-1.83

The values are in millions of years. The nodes correspond to the chronogram in figure 2; HPD (high posterior densities) indicate 95% confidence intervals for the estimation of node age

The results of our analyses show that under the hypothesis of the origin Cretaceous or Paleogene, most genera underwent diversification during the Miocene period. However, there are differences in the ages of divergence. According to Paleogene origin, the divergence of the genus *Rhynchopsitta* is in middle Miocene. Orthopsittaca-group, Leptosittaca-group, *Aratinga*, and *Eupsittula* diverge at late Miocene, and the age estimated for the divergence of the genus *Psittacara* is in Pliocene (Fig. 2A). On the other hand, in concordance with a Cretaceous origin, the dates of divergence of the groups are previous: The genus *Rhynchopsitta* in early Miocene, Leptosittaca-group, Orthopsittaca-group and *Aratinga* in middle Miocene, and *Eupsittula* and *Psittacara* in late Miocene (Fig. 2B).

The most likely origin of the clades in this study was the Amazonian-Caribbean subregion (node 1, probability [P] = 1, node frequency [F] = 1) at 22.8-13.91 Mya, a time corresponding to Miocene. Overall, the speciation of these groups was associated with dispersal. The S-DIVA analysis showed 18 events of dispersal; however, six vicariance events were observed in nodes 3, 5, 7, 7a, 10a, and 11a (data not shown).

For the genus *Rhynchopsitta*, with an origin of approximately 20.66-13.24 Mya, the origin region not was determinate, but the results suggest a possible origin in the Caribbean-Amazonian subregion (node 3, PAC = 0.25, PABC = 0.25, PAB = 0.25, PA = 0.22, F = 1) (Figs. 2B and 3F).

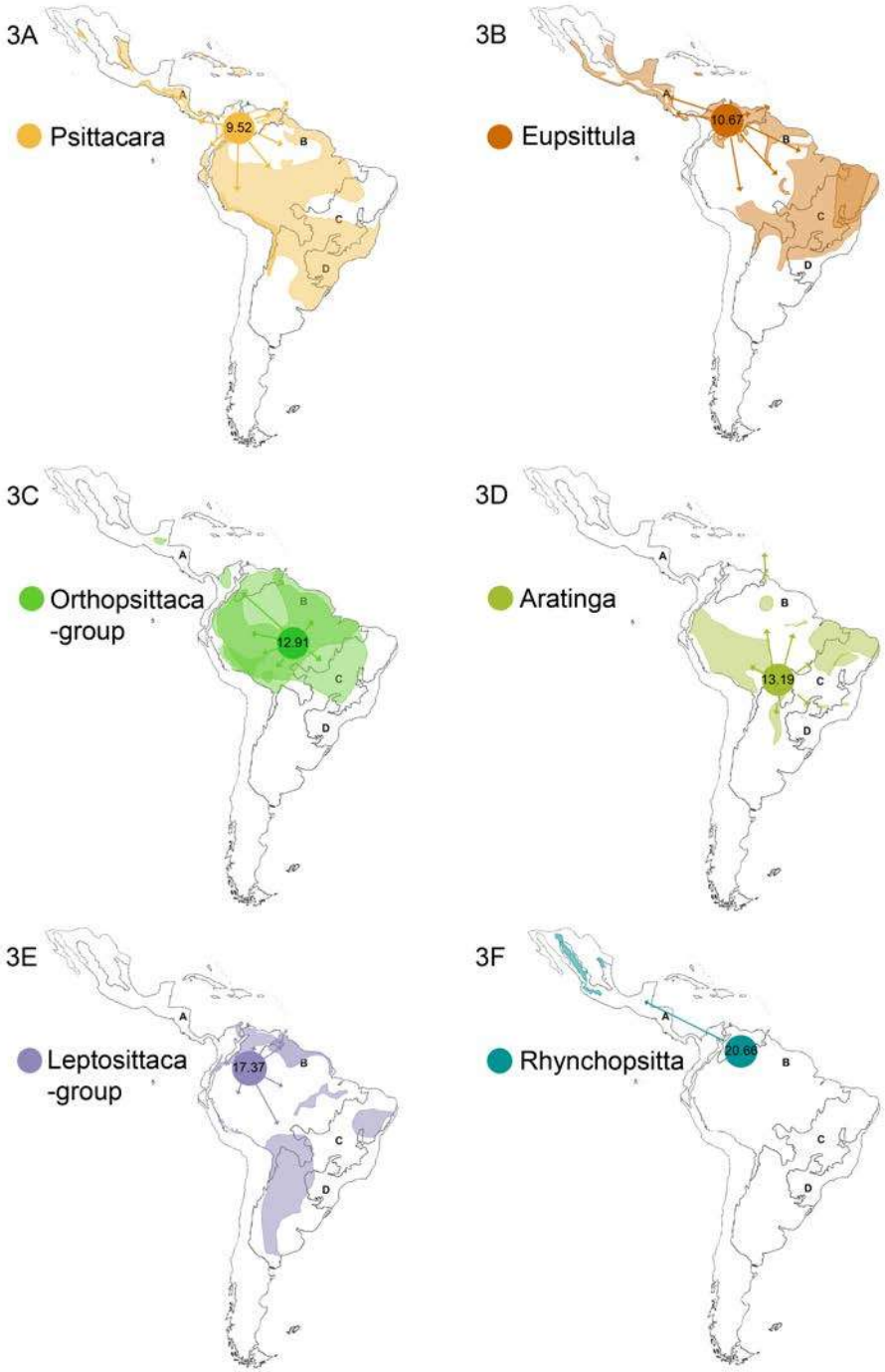

Fig. 3 Maps of ancestral areas and possible routes of dispersion for each group. Letters outside the maps correspond to clades in the crown group: 3A) *Psittacara*, 3B) *Eupsittula*, 3C) Orthopsittaca-group, 3D) *Aratinga*, 3E) Leptosittaca-group, and 3F) *Rhynchopsitta*. The circles and the numbers inside represent the area and origin in millions of years. The letters A, B, C, and D within the maps represent the four Neotropical subregions (details in the text). The shaded areas represent the current distribution of the species of each genus or group included in this analysis

The S-DIVA analysis did not show dispersion or vicariance events in *Rhynchopsitta*. The ancestral area of Leptosittaca-group is assigned to the Caribbean or Amazonian-Caribbean subregions (node 2, PA = 0. 5, PAB = 0. 5, F = 1) at 17.37-9.03 Mya (Figs. 2B and 3E). The most recent common ancestor (MRCA) of *Thectocercus* and *Diopsittaca* (node 2a, PB = 0.5, PA = 0.5, F = 1) is assigned to the Amazon or Caribbean subregions at 7.00-4.2 Mya. The ancestral area of *A. hyacinthinus* does not have a determined subregion at 19.74-12.65 Mya (node 4, Fig. 2B).

The *Aratinga* and Orthopsittaca-group clades shared a common ancestor at approximately 15.88-10.99 Mya, which may have inhabited the Amazonian subregion (node 6, PB = 0.4, F = 1, Figs. 2B). The estimated node age for the Orthopsittaca-group clade is 12.91-8.63 Mya, and the most likely ancestral area is the Amazonian subregion (node 8, PB = 0.4, F = 1) (Figs. 2B and 3C). This group diversified through dispersal events. The genus *Aratinga* originated approximately 13.19-10.11 Mya. According to the biogeographic reconstruction obtained with S-DIVA, this clade (node 7, PBC = 1, F = 0.98) originated in the Amazonian-Chaco subregion (Figs. 2B and 3D). This group diversified via dispersal, except for *Cyanopsitta spixii* and *Conuropsis carolinensis*, which showed vicariance.

The MRCA of the genera *Psittacara* and *Eupsittula* occurred approximately 15.84-10.99 Mya. The origins of these genera may be assigned to the Caribbean subregion (node 9, PA = 0.34, PAB = 0.25, PB = 0.25, F = 1). The genus *Eupsittula* was estimated to have originated 10.67-6.19 Mya; however, its region of origin is the Caribbean or Caribbean-Amazonian subregion (node 10, PA = 0.46, PAB = 0.31, F = 1) (Figs. 2B and 3B).

Psittacara constitutes the most recent clade, with an estimated node age of approximately 9.52-4.38 Mya. The results do not show determined subregions for the origin of this clade (node 11, PA = 0.27, PABD = 0.13, PABCD = 0.13, PABC = 0.13, PB = 0.13, PAB = 0.13, F = 1). However, the most likely states appear in the Amazonian subregion (Figs. 2B and 3A). Within this group, the species *P. erythrogenys*, *P. finschi*, *P. h. brevipes*, *P. rubritorquis*, *P. holochlorus*, *P. chloropterus* and *P. euops* dated to approximately 3.51-1.63 Mya and diversified in the Caribbean subregion (node 11b, PA = 1, F = 1). Although dispersal events seemed to be the most likely cause of diversification of *Psittacara* and *Eupsittula*, two vicariance events were observed in nodes 10a and 11a.

Discussion

The topology of the BI and ML trees place *Rhynchopsitta*, the Leptopsittaca-group, and *A. hyacinthinus* as lineages with early divergence. Some uncertainties remain within the genus *Psittacara* in the relatedness among the species. We recommended the formal recognition of *Psittacara acuticaudatus* as *Thectocercus acuticaudatus*, which has been proposed by Remsen et al. (2013). For some authors, *P. finschi* and *P. chloropterus* are conspecifics of *P. leucophthalmus*, and *P. rubritorquis* has been suggested to be conspecific with *P. holochlorus* (Collar et al. 2016b; Collar and Sharpe 2016). However, in our results, the phylogenetic trees demonstrate that *P. leucophthalmus* is basal and distant from *P. finschi* and *P. chloropterus*, similar to the observed by Kirchman et al. (2012). Additionally, *P. holochlorus* is conspecific with *P. rubritorquis* (Fig. 1, Table 4). Moreover, *P. h. brevipes* (restricted to the Revillagigedo Islands of Mexico) clearly is not a subspecies of *P. holochlorus* (distributed from the eastern, and south of Mexico to the north of Nicaragua), as traditionally has been considered (del Hoyo et al. 2014; BLI 2015). The genetic distance of *P. h. brevipes* is greater with *P. holochlorus* than with *P. finschi* (distributed in Nicaragua, Costa Rica, and Panamá) (Table 4). Here we propose the formal recognition of *P. h. brevipes* as *P. brevipes* (Fig. 1), in particular, because of their conservation implications; the taxa present the greater vulnerability into *Psittacara* since it is in imminent threat of extinction due to its remoteness, low potential habitat availability and small overall population size (Rodriguez-Estrella et al. 1992, 1995). In the trees, *P. finschi* and *P. brevipes* are located in an unresolved subclade (PB = 1, PP = 0.89). However, the inclusion of another taxon and character could resolve this uncertainty.

In the case of the *Eupsittula* clade, BLI (2015) and del Hoyo et al. (2014) also recognize *E. astec* as a species in the genus *Eupsittula*. This clade include to *E. aurea, E. nana,* and *E. pertinax*, as observed by Kirchman et al. (2012). Our results also corroborate the Remsen et al. (2013) proposal, which placed *E. aurea* and *E. cactorum* in this genus. However, our results contrast those of Kirchman et al. (2012), who proposed *R. pachyrhyncha* as a sister group of *Eupsittula*. Additionally, within the *E. canicularis* grouping, a separation was observed between the NCBI GenBank specimen and the two samples obtained in this study (AccSb14, AceMpm53). *Eupsittula canicularis* is a species distributed throughout the Pacific slope from northern México, its main region of occurrence, to northern Costa Rica (Forshaw 1989; Howell and Webb 1995; Collar et al. 2000, 2014). Although the origin of the NCBI GenBank specimen is unknown (Schirtzinger et al. 2012), according to the genetic distance shown by this sample (Table 4), an origin in southern Mexico or Central America, where *E. c.*

canicularis occurs, is possible. A study of samples from the extreme southern portion of the distribution is needed to corroborate this.

The *Aratinga* clade is sister to the Orthopsittaca-group, a relation previously observed by Kirchman et al. (2012). The placement of the extinct *Conuropsis carolinensis* into *Aratinga* remains the same as that proposed by Kirchman et al. (2012). Thus we recommended its formal reclassification as *Aratinga carolinensis*. Additionally, in this study, *A. weddellii* and *A. jandaya* were also included in this group.

Bayesian inference of divergence times and the reconstruction of ancestral areas

Age estimations of species of the genus *Psittacara* are presented for the first time. According to our results, *Psittacara* is a recent genus whose divergence is located in late Miocene or Pliocene. Despite the discrepancies in divergence times estimates, we propose that related groups to *Psittacara* have their origin and diversification in the Miocene period. The present study also show the reconstruction of ancestral areas, allowing us to establish that the Caribbean and Amazonian subregions are the most likely areas of origin for the studied taxa. On the other hand, the early divergence of groups in our results do not coincide with diversification due to the dry-wet climate cycles of the Pliocene-Pleistocene (Pleistocene refuges hypothesis) as propose Haffer (1969). In concordance with other authors, much of this early divergence speciation is previous to the Pleistocene in different taxa (Cortés-Ortiz et al. 2003; Burns and Naoki 2004; Barker 2007; Brumfield and Edwards 2007; Brumfield et al. 2007; Lim 2007; Antonelli et al. 2009; Santos et al. 2009).

Two geological events that have been associated with the diversification of species in South and Central America are the formation of the central and northern parts of the Andes (65-34 and 23-10 Mya, respectively) and the closure of the Isthmus of Panama (approximately 3.5 Mya) (Hoorn et al. 2010; Leigh et al. 2014). The formation of the Andes changed the landscape, hydrologic systems, climate, and the Amazonian biota (Hoorn et al. 1995; Wesselingh et al. 2002; Antonelli et al. 2009; Hoorn and Wesselingh 2010; Latrubesse et al. 2010; Poulsen et al. 2010; Shephard et al. 2010). Whereas the closure of the Isthmus of Panama facilitated the phenomenon known as “The Great American Biotic Interchange,” which allowed the dispersion and diversification of taxa (Leigh et al. 2014).

Overall, the initial diversification of these clades began at the same time or after changes in the Amazonian region occurred due to the formation of the northern parts of the Andes (23-10 Mya).

Also, a series of events that influenced the diversification within different clades occurred between 5 and 10 Mya. In this period, the western Amazonian region changed from a lacustrine to a fluvial or fluvio-tidal system. This "Acre" system is represented by an environmental model that includes grassland and river swamp and lake-side gallery forest, which were subject to a fluctuating water level in a seasonally flooded tropical to subtropical wet-dry climate (Latrubesse et al. 2010). The Acre system declined with the disappearance of mega-wetlands in western Amazonia at 7 Mya. It is possible that the establishment of terrestrial conditions in western Amazonia could be important for the diversification of the current biota of this region (Hoorn et al. 2010). At least some species in the *Psittacara* and *Eupsittula* clades seemed to diversify relatively quickly and occupy areas to the south favored by these conditions. Moreover, the ancestor of *Thectocercus* (7.00-4.2 Mya) occupied the Amazonian or Caribbean subregions and later dispersed to the Chaco and Parana subregions, where some of their populations are current inhabitants. The genus *Rhynchopsitta* currently inhabits North America, but its area of origin is in the southern subregions. This difference between the current location and the area of origin indicates a possible ancestral dispersion event, perhaps before the closure of the Isthmus of Panama.

For the Orthopsittaca-group (12.91-8.63 Mya), which probably occupied central-eastern Amazonia, the dispersal occurred in the direction of central-western Amazonia and central-northern Amazonia (Fig. 3C).

In the genus *Aratinga* (13.19-10.11 Mya), the Amazonia-Chaco is the center of dispersal that took place in southeastern, western, and eastern Amazonia and northward. *Conuropsis carolinensis* (*P. carolinensis*) could have used the Greater and Lesser Antilles as part of its route northward (Fig. 3D).

For *Eupsittula* (10.67-6.19 Mya), the ancestral area is most likely south of the Caribbean subregion, and the dispersion occurred northward of this subregion and into the Amazonian subregion. Currently, *Eupsittula* is found to the south of the Amazonian subregion (*E. aurea*, *E. cactorum*, and *E. pertinax*) and North America (*E. canicularis* and *E. nana*) (Fig. 3B). This genus also arrived at the Chaco and Parana subregions (*E. aurea* and *E. cactorum*) (Fig. 3B). As mentioned before, this group may have benefitted from the biotic and abiotic changes that caused the disappearance of Pebas Lake and the establishment of Acre system conditions.

The genus *Psittacara* is widely distributed across all the geographic subregions, with species inhabiting areas from North to South America. The genus is the largest and experienced the most rapid diversification, which was initiated 9.5-4.38 Mya, coinciding with the disappearance of the Acre system and the establishment of modern drainage in the Amazon basin (Hoorn et al. 2010). The dispersal began south-central and southeast of the

Amazon subregion. The diversification of the subclade Sc1 most likely occurred in Central America, where 85.71% of its species occur. The diversification of this subclade occurred after the establishment of the Isthmus of Panama 3.5 Mya (Leigh et al. 2014), which suggests *in situ* speciation. As with *Conuropsis carolinensis* (*P. carolinensis*), *P. chloroptera*, and *P. euops* likely could have used the Lesser Antilles as a dispersal route.

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Conflict of interest

The authors declare that they have no conflict of interests.

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APÉNDICE

FIGURAS

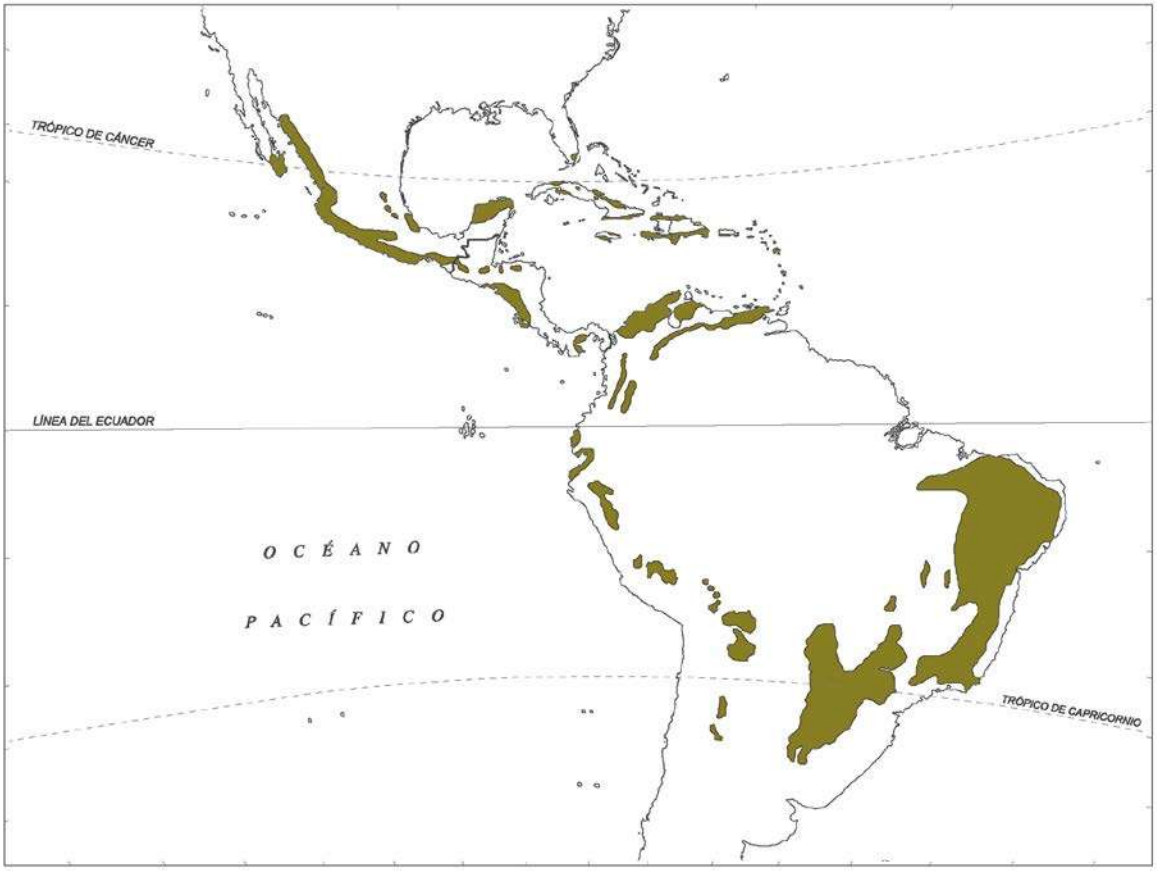

Figura 1A. Mapa de distribución del Bosque tropical seco caducifolio en América. Tomado y modificado de Pennington *et al*. 2009.

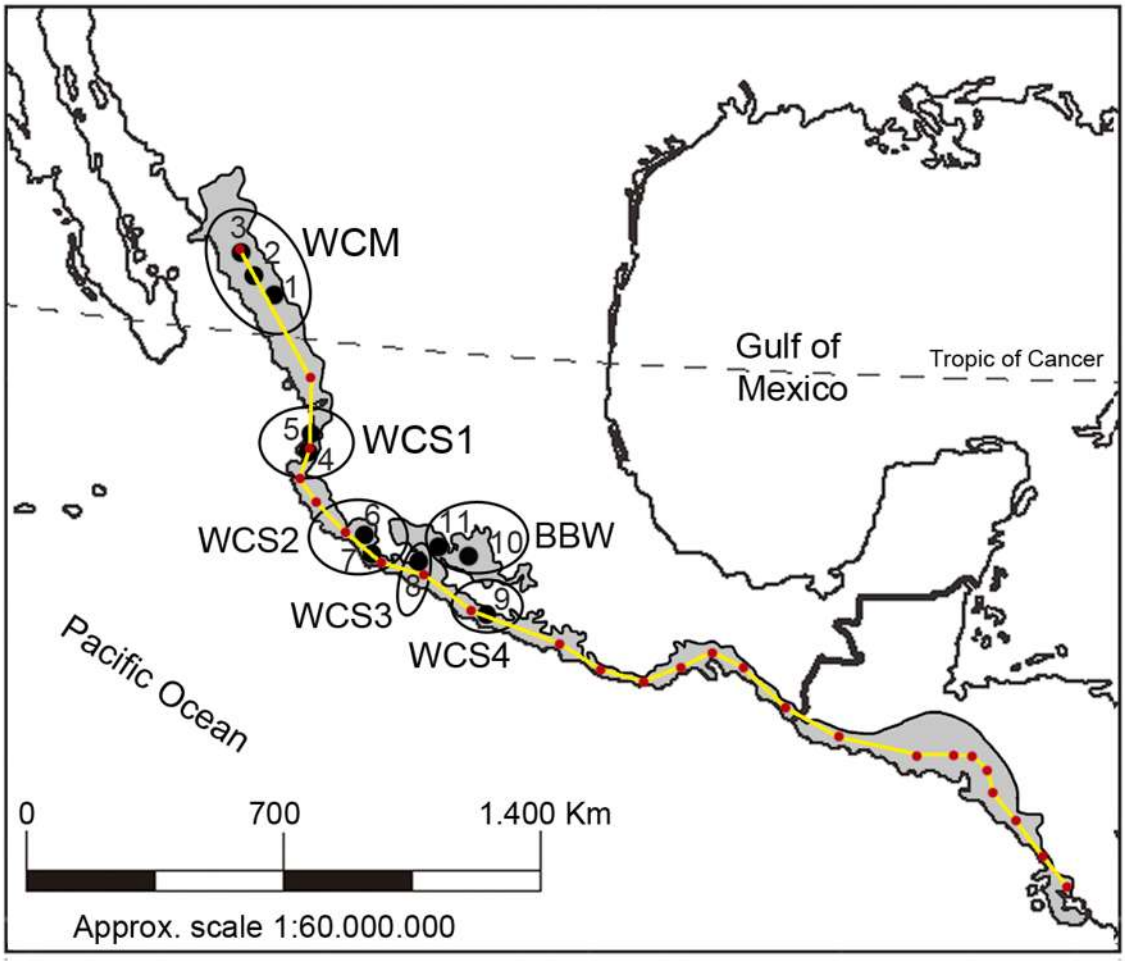

Figura 2A. Mapa que muestra los 25 puntos considerados para calcular la distancia euclidiana de la distribución de *E. canicularis* (3,338 km aproximadamente). De esta manera se estimó que un poco más de un tercio de la distribución de la especie (1,282 km) fue considerada para los análisis.