UNIVERSIDAD NACIONAL AUTÓNOMA DE MÉXICO
POSGRADO EN CIENCIAS BIOLÓGICAS

FACULTAD DE CIENCIAS

BIOLOGÍA EVOLUTIVA

EVOLUCIÓN EN LAS TIERRAS ALTAS DE MESOAMÉRICA,

EL CASO DE "LAS GEMAS DE LAS MONTAÑAS":

***Eugenes fulgens* y *Lamprolaima rhami* (AVES: Trochilidae).**

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QUE PARA OPTAR POR EL GRADO DE:

DOCTORA EN CIENCIAS

PRESENTA:

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TUTORA PRINCIPAL: DRA. BLANCA ESTELA HERNÁNDEZ BAÑOS.

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POSGRADO EN CIENCIAS BIOLÓGICAS
FACULTAD DE CIENCIAS
DIVISIÓN DE ESTUDIOS DE POSGRADO

OFICIO FCIE/DEP/357/2016

ASUNTO: Oficio de Jurado

Dr. Isidro Ávila Martínez
Director General de Administración Escolar, UNAM
P r e s e n t e

Me permito informar a usted que en la reunión ordinaria del Comité Académico del Posgrado en Ciencias Biológicas, celebrada el día **23 de noviembre de 2015**, se aprobó el siguiente jurado para el examen de grado de **DOCTORA EN CIENCIAS** del (la) alumno (a) **ZAMUDIO BELTRÁN LUZ ESTELA** con número de cuenta **509015165** con la tesis titulada: **"Evolución en las tierras altas de Mesoamérica, el caso de "Las Gemas de las Montañas": Eugenes fulgens y Lamprolaima rhami (Aves: Trochilidae)"**, realizada bajo la dirección del (la) **DRA. BLANCA ESTELA HERNANDEZ BAÑOS:**

Presidente:	DRA. MARIA DEL CORO ARIZMENDI ARRIAGA
Vocal:	DR. CARLOS ALBERTO LARA RODRIGUEZ
Secretario:	DR. LUIS ENRIQUE EGUIARTE FRUNS
Suplente:	DRA. LIVIA SOCORRO LEON PANIAGUA
Suplente:	DR. JUAN PABLO JARAMILLO CORREA

Sin otro particular, me es grato enviarle un cordial saludo.

A T E N T A M E N T E
"POR MI RAZA HABLARA EL ESPIRITU"
Ciudad Universitaria, Cd. Mx., a 15 de junio de 2016

DRA. MARÍA DEL CORO ARIZMENDI ARRIAGA
COORDINADORA DEL PROGRAMA

POSGRADO EN CIENCIAS BIOLOGICAS
COORDINACIÓN

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ÍNDICE

RESUMEN GENERAL

Para tener un mejor entendimiento y estimación de la biodiversidad es necesario estudiar los mecanismos que han moldeado dicha variación. Actualmente, estos estudios son el principal objetivo de la biología evolutiva. La diversidad de muchos grupos biológicos aún está subestimada, y se pretende que esto cambie con la incorporación de diferentes tipos de caracteres que faciliten la descripción y clasificación de dicha diversidad. Uno de los grupos de interés en el campo de la ornitología, es el de la familia Trochilidae, ya que comprende una gran diversidad de especies con características únicas. En la presente tesis tuvimos como principales objetivos el proponer una hipótesis evolutiva para el clado de las "Gemas de las Montañas", pertenecientes a la familia Trochilidae, así como dilucidar los patrones de variación genética de dos complejos pertenecientes a dicho clado: *Eugenes fulgens* y *Lamprolaima rhami*. Esto se consiguió mediante el empleo de información múltiple (e.g. caracteres morfológicos, caracteres moleculares, áreas biogeográficas), y con la incorporación de diversos análisis, los cuales incluyeron reconstrucciones filogenéticas, reconstrucciones de áreas ancestrales, estimaciones de tiempos de divergencia, análisis de demografía histórica, proyecciones de distribuciones potenciales, entre otros. Este estudio se ha dividido en cuatro capítulos, los cuales abordan objetivos particulares y en los cuales se discuten los resultados obtenidos.

ABSTRACT

To have a better understanding and estimation of biodiversity is necessary to study the mechanisms that have shaped this variation. Nowadays, these studies are the focus of evolutionary biology. Biological diversity of many groups is still underestimated, and it is intended that this will change with the addition of different types of characters to facilitate the description and classification of biodiversity. One of the groups of interest in ornithology, is the Trochilidae family, and it comprises a great diversity of species with unique characteristics. The main goals of the present study were to propose an evolutionary hypothesis for the clade of "the Mountain Gems", belonging to the Trochilidae family, and elucidate the patterns of genetic variation in two complexes belonging to this clade: the magnificent hummingbird *Eugenes fulgens* and the garnet-throated hummingbird *Lamprolaima rhami*. This was achieved by using multiple information (e.g. morphological characters, molecular markers, biogeographic areas), and with the addition of multiple analyzes, which included phylogenetic reconstructions, reconstructions of ancestral areas, estimates of divergence times, analysis of historical demography , projections of potential distributions, among others. This study is divided into four chapters, which address particular objectives and in which the results are discussed.

INTRODUCCIÓN

Mesoamérica y variación geográfica.

La región geográfica de transición entre la zona Neártica y la zona Neotropical ha sido definida en un contexto biogeográfico con el nombre de Mesoamérica ("Mesoamérica biótica"; Ríos-Muñoz, 2013). Mientras que otros autores reconocen que esta zona está comprendida dentro de una región geográfica de mayor amplitud, entre el sur de Estados Unidos y el Istmo de Darién en Panamá, la cual ha sido acuñada con el término de América Media ("Middle America"; Winker, 2011). Esta región geográfica está asociada en sus tierras altas a diferentes tipos de vegetación como son los bosques de coníferas, bosques de encinos, bosques mesófilos de montaña y matorrales, y en sus tierras bajas se pueden encontrar bosques tropicales caducifolios, subcaducifolios, perenifolios y pastizales (Rzedowski, 1981; Rzedowski, 2001). Se han reconocido cinco provincias biogeográficas para la zona de transición en México (Sierra Madre Occidental, Sierra Madre Oriental, Faja Volcánica Transmexicana, depresión del Balsas y Sierra Madre del Sur) y cuatro provincias para el dominio Mesoamericano (costa del Pacífico Mexicano, costa del Golfo de México, Chiapas, costa este de Centroamérica y oeste del Istmo de Panamá), con base en patrones compartidos de componentes bióticos en cada región (Morrone, 2006; Figura 1).

A pesar de las discrepancias en el nombramiento y delimitación de la zona central del continente americano, no cabe duda que esta región es bien conocida por su historia biogeográfica y por sus altos niveles de biodiversidad. Se ha establecido que Mesoamérica contiene aproximadamente 24,000 especies de plantas y alrededor de 2,859 especies de vertebrados, situándola como una de las regiones con un mayor índice de biodiversidad a nivel mundial y una zona prioritaria para la conservación (Myers et al., 2000). Por consiguiente, Mesoamérica representa una región biogeográfica de gran interés en el estudio de los patrones y los procesos que han moldeado dicha diversidad.

La historia evolutiva en Mesoamérica ha sido descrita en términos de un conjunto de procesos a diferentes escalas espacio-temporales. Estos procesos están relacionados con los principales eventos geológicos que promovieron la formación de las cadenas montañosas iniciando en el Oligoceno (38~25 millones de años; Ferrari et al., 1999), al conjunto de fluctuaciones climáticas ocurridas durante el Pleistoceno (~2 mill. años, Avise et al., 1998), y a los eventos de intercambio faunístico, como el ocurrido durante el cierre del Istmo de Panamá (Great American Biotic Interchange "GABI", 3 mill. años; Coates y Obando, 1996; Smith y Klicka, 2010). El resultado de

estos procesos a diferentes niveles (macro y microevolutivos), ha dado lugar a especies mesoamericanas con altos niveles de variación geográfica.

Figura 1. Provincias biogeográficas propuestas para la zona central de América (Morrone, 2006). 1) Sierra Madre Occidental, 2) Sierra Madre Oriental, 3) Faja Volcánica Transmexicana, 4) depresión del Balsas, 5) Sierra Madre del Sur, 6) costa del Pacífico Mexicano, 7) costa del Golfo de México, 8) Chiapas, 9) costa este de Centroamérica, y 10) oeste del Istmo de Panamá. Imagen tomada y modificada de Ríos-Muñoz, 2013).

La variación geográfica se manifiesta en la diferenciación fenotípica y genotípica entre las poblaciones con relación a su distribución geográfica (Futuyma, 1998; Hillis et al., 1996; Weir, 1996). La estructuración geográfica de una especie puede ser moderada, por ejemplo cuando hay altos niveles de flujo genético entre las poblaciones o debido a un proceso de especiación reciente, o puede ser muy elevada, por ejemplo cuando las poblaciones de una especie han permanecido aisladas durante periodos largos favoreciendo la diferenciación intraespecífica. Los estudios enfocados a nivel poblacional (e.g. estudios filogeográficos) son de gran utilidad para detectar y describir los eventos históricos que han dado lugar a dicha variación (Domínguez-Domínguez y Vázquez-Domínguez, 2009). Los objetivos principales de estos estudios se han centrado en analizar los principios y los procesos que gobiernan la distribución geográfica de los linajes genealógicos, y muchos de ellos se han enfocado en el estudio

de diferentes vertebrados en Mesoamérica. Algunos de estos trabajos han tenido como propósitos el reconocimiento y el establecimiento de los límites entre las especies con implicaciones taxonómicas, aplicaciones en la biología de la conservación y en el manejo de especies (Avise, 2000; Avise et al., 1987; Bryson et al., 2011; Nyári, 2007).

Estudios realizados específicamente en las tierras altas de Mesoamérica muestran que un patrón recurrente en el grupo de las aves es la especiación alopátrica (Arbeláez-Cortés et al., 2010; Barrera-Guzmán et al., 2012). En general, se han detectado barreras geográficas que han sido claves en moldear la variación a nivel intraespecífico, siendo el Istmo de Tehuantepec un ejemplo clásico en estudios filogeográficos de aves en México (Barber y Klicka, 2010). Sin embargo, aún existen algunas interrogantes por esclarecer en éstos estudios, en los cuales no se ha enfatizado la evaluación de los mecanismos promotores de la diferenciación genética y fenotípica, ni se ha analizado el papel que han tenido la deriva génica o la selección natural en el proceso evolutivo. Por otra parte, actualmente en el mundo se están realizando estudios que conjuntan los fundamentos de genética de poblaciones, filogeografía y ecología del paisaje, con el fin de establecer los procesos evolutivos actuales y pasados a una escala fina, en donde se detallan los efectos que tienen las características de cierto hábitat sobre la estructuración poblacional de las especies (Manel et al., 2003).

Gemas de las Montañas.

Los colibríes (familia Trochilidae) es uno de los grupos de aves con mayor número de especies en el continente americano, antecedido sólo por la familia Tyrannidae. Debido a su alto número de especies y sus particulares características morfológicas, fisiológicas y de comportamiento representa un grupo atractivo para estudios de biología evolutiva y comparativa (Altshuler et al., 2004). Los colibríes son uno de los grupos de aves que cuenta con uno de los mayores índices de especialización presentando un gran polimorfismo en ciertas características fenotípicas, especialmente en la forma y tamaño de su pico, y en el color y patrón del plumaje. Habitan ambientes que van desde selvas húmedas a bosques templados, zonas costeras y desiertos, aunque algunas especies se encuentran sólo en áreas geográficas muy específicas y limitadas (Torres-Chávez y Navarro-Sigüenza, 2000).

Estas aves forman un grupo monofilético, con aproximadamente 349 especies descritas en aproximadamente 104 géneros (Bleiweiss et al., 1997; Dickinson, 2003; Gerwin y Zink, 1998; Gill y Gerwin, 1989; Johnsgard, 1984). Gran parte de la clasificación actual de los colibríes está basada en las descripciones originales de sus

caracteres morfológicos, como el pico y el plumaje (Boucard, 1895; Elliot, 1879; Gould, 1861; Hartert, 1900; Peters, 1945; Ridgwary, 1911; Simon, 1921). En algunos estudios, además de los caracteres morfológicos se han incluido caracteres conductuales, sobre todo de forrajeo (Feinsinger y Colwell, 1978).

En un primer estudio empleando técnicas de hibridización de ADN (Bleiweiss et al., 1997), se intentaron resolver las relaciones filogenéticas en 26 especies de colibríes. Se determinaron los principales linajes dentro de la familia, que corresponden a dos grandes subgrupos: Ermitaños (*Hermits*) y los No-ermitaños (*Nonhermits*), y en los que se definieron a su vez 7 clados mayores: 1. Ermitaños, 2. Mangos, 3. Coquetas, 4. Brillantes, 5. Esmeraldas, 6. Gemas de las Montañas, y 7. Abejas. Estos autores propusieron como representantes del clado de las Gemas de las Montañas a las especies *Eugenes fulgens* y *Lampornis clemenciae.*

Un estudio posterior analizó la topología reconstruida anteriormente y estableció una escala de tiempo para la radiación de la familia Trochilidae a partir de las distancias genéticas y datos del registro fósil (Bleiweiss, 1998), estableciendo los principales eventos históricos y fechas aproximadas de sus tiempos de divergencia. Encontró que la separación entre los dos grupos principales (Ermitaños y No-ermitaños) sucedió durante el Mioceno (~17 Millones de años, Ma), mientras que la divergencia de las Gemas de las Montañas y su clado hermano (Abejas) fue mucho más reciente, hace unos 6 Ma. Bleiweiss (1998) también propone algunas hipótesis acerca de la historia evolutiva de la familia y los procesos que favorecieron la diversificación dentro del grupo.

Posteriormente, Schuchmann (1999) define arreglos taxonómicos basados en comparaciones de comportamiento, formas de los nidos y características morfológicas. Sin embargo, señala que estos grupos propuestos deben tomarse como provisionales. Los géneros considerados dentro del grupo de las Gemas de las Montañas son: *Microchera*, *Anthocephala*, *Lampornis*, *Basilinna* (*Hylocharis*) y *Lamprolaima.*

Altshuler et al. (2004) estudió la morfología y los mecanismos de vuelo de la familia Trochilidae en un análisis filogenético multilocus con 43 especies de colibríes. Se encontraron los mismos clados descritos por Bleiweiss et al. (1997). En este estudio se identificaron otras especies dentro del grupo de las Gemas de las Montañas: *Heliomaster longirostris* y *Panterpe insignis*, cercanamente relacionadas con *Eugenes fulgens*.

Renner y Schuchmann (2004) realizaron un estudio sobre la taxonomía del género *Eugenes* y su relación con los géneros monotípicos *Hylonympha* y *Sternoclyta*, ambos con distribuciones restringidas endémicas en Venezuela. Con base en datos

morfológicos y biogeográficos proponen el cambio de los géneros *Hylonympha* y *Sternoclyta* al género *Eugenes*, éste último con prioridad taxonómica por cronología. Con respecto a patrones etológicos, de plumaje y tamaño corporal, se argumenta que el género *Eugenes* mantiene una relación cercana con el género *Heliodoxa*, particularmente con *Heliodoxa schreibersii*. Sin embargo, mencionan la necesidad de investigación adicional con el fin de dilucidar con mayor claridad las relaciones filogenéticas entre los géneros *Eugenes* y *Heliodoxa*.

En una revisión taxonómica del género *Lampornis* (García-Moreno et al., 2006), se hizo una reconstrucción filogenética de 100 especies de colibríes utilizando un gen nuclear (subunidad 5 de Adenilato Quinasa, AK5) y un gen mitocondrial (subunidad 2 de NADH deshidrogenasa, ND2) y se encontró una relación filogenética cercana entre el género *Lampornis* y *Eugenes fulgens*, y este último resultó cercano a *Lamprolaima rhami*.

Uno de los estudios recientes más importantes referentes a las relaciones filogenéticas dentro de la familia Trochilidae fue el realizado por McGuire y colaboradores (2007), en el cual presentan una filogenia más robusta incluyendo 151 especies de colibríes y 12 taxa como grupo externo, empleando dos marcadores nucleares (AK1; Beta Fibrinogeno, Bfib), RNAs de transferencia (tRNAs) y dos genes mitocondriales (ND2 y ND4). En este estudio se identificaron dos clados mayores adicionales a los 7 clados nombrados con anterioridad (Topazas y *Patagona*). El grupo de las Gemas de las Montañas estuvo representado por las especies: *Eugenes fulgens, Heliomaster longirostris, Panterpe insignis, Lampornis hemileucus, Lampornis calolaemus* y *Lampornis castaneoventris.* Con respecto a la relación entre los géneros *Eugenes* y *Heliodoxa*, se presenta evidencia molecular que refuta la hipótesis de Renner y Schuchmann (2004), en la cual se había propuesto una relación cercana entre el género *Eugenes* y el género *Heliodoxa*, en específico con *Heliodoxa shreibersii*, la cual de acuerdo al estudio de McGuire et al. (2007) se aclara al ubicar al género *Heliodoxa* en un grupo por separado (grupo "Brillantes") del grupo donde se ubica al género *Eugenes* (grupo "Gemas de las Montañas").

Y finalmente, el estudio más reciente y completo que explica las relaciones filogenéticas para la familia Trochilidae en un contexto de tasas de especiación, fue el realizado por McGuire et al. (2014). En éste estudio se incluyen las 15 especies reconocidas hasta el momento para el grupo de las Gemas de las Montañas, comprendidas en cinco géneros (Figura 2; *Eugenes*, *Heliomaster*, *Lampornis*, *Lamprolaima* y *Panterpe*). A pesar de la inclusión de todas las especies reconocidas

para es este grupo, las relaciones entre algunas de las especies del género *Lampornis* no fueron esclarecidas en su totalidad, obteniendo bajos valores de soporte entre las especies *L. hemileucus* y *L. sybillae*. Se propone una hipótesis biogeográfica, en la que se reconoce que el ancestro de las Abejas y Gemas de Montañas se estableció en Centroamérica a partir de un evento de dispersión desde Sudamérica. Sin embargo, no se presentan resultados sobre la historia biogeográfica dentro del grupo de las Gemas de las Montañas a detalle.

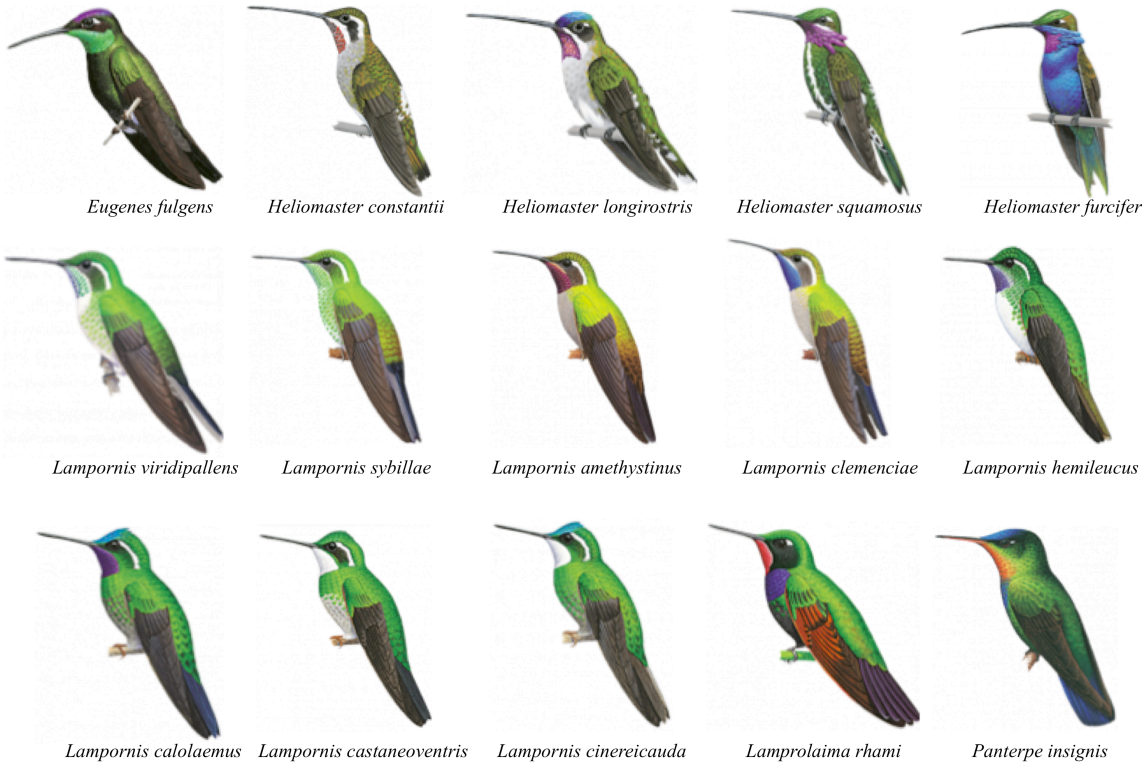

Figura 2. "Gemas de las Montañas". 15 especies reconocidas (Schuchmann, 1999). Ilustraciones tomadas de www.hbw.com.

Eugenes fulgens.

El colibrí magnífico, *Eugenes fulgens* (Swainson, 1827), es una especie que presenta un marcado dimorfismo sexual (Figura 4). Los machos tienen un pico negro, largo y recto, corona violeta iridiscente, una pequeña mancha blanca detrás del ojo, dorso de color verde obscuro, garganta verde iridiscente, mientras que el resto de su cuerpo es negruzco y la cola es de color bronce. En contraste, las hembras carecen de color iridiscente en la garganta, presentan un color bronce en alas y cola, y un color grisáceo en el vientre (Schuchmann, 1999). Esta especie se distribuye por lo general entre los 1500 y los 2500 msnm, asociada a bosques de pino-encino, bosques mesófilos de montaña, áreas riparias, a menudo presente en áreas abiertas con flores (Johnsgard,

1983; Schuchmann, 1999). Su dieta se basa principalmente en el néctar floral y algunos artrópodos. Esta especie es considerada como generalista, y diversas especies de plantas proporcionan el néctar a éste colibrí, como lo son: *Cirsium sp.*, *Penstemon roseus, P. gentianoides*, *Salvia elegans*, *S. mocinoi*, *Castilleja tenuiflora*, *C. scorzonerifolia*, *Bouvardia ternifolia*, *Prunella vulgaris*, *Agave americana*, *A. parryi*, *Bomarea costaricensis*, *Centropogon talamanensis*, *Erythrina corallodendrum*, *Fushsia splendens* y *Lobelia laxifolia* (Johnsgard, 1983; Lara, 2006; Schuchmann, 1999).

El colibrí magnífico se distribuye desde el sur de los Estados Unidos (sureste de Arizona, suroeste de Nuevo México y oeste de Texas), a través de las montañas de México, Guatemala, oeste de El Salvador, Honduras, norte de Nicaragua, hasta las montañas centrales de Costa Rica y Panamá (oeste de Chriquí), tiene una distribución típicamente Mesoamericana (AOU, 1998; Howell y Webb, 1995; Schuchmann, 1999, Figura 3). Las poblaciones al norte y posiblemente las del centro de México presentan movimientos migratorios a inicios de la primavera, hacia el límite norte de su distribución (norte de México y suroeste de USA), mientras que las poblaciones al sur de México y Centroamérica son sedentarias, presentando sólo movimientos altitudinales con base en la disponibilidad de recursos (Schuchmann, 1999).

E. fulgens presenta una distribución geográfica discontinua identificándose variaciones en su morfología (diferencias en sus patrones de coloración y tamaño, Figura 4), para lo cual se han propuesto diversas hipótesis taxonómicas (ver Tabla 1).

Tabla 1. Subespecies y especies propuestas para el género *Eugenes*.

Autor (referencias)	**Hipótesis taxonómicas**
Ridgway, 1911.	*E. fulgens* y *E. spectabilis.*
Peters, 1945.	*E. f. fulgens, E. f. viridiceps* y *E. f. spectabilis.*
Johnsgard, 1983.	*E. f. fulgens* y *E. f. viridiceps.*
American Ornithologists´Union (AOU, 1998).	*E. fulgens.*
Schuchmann, 1999.	*E. f. fulgens* y *E. f. spectabilis.*
Navarro-Sigüenza y Peterson, 2004.	*E. fulgens* y *E. viridiceps.*
Renner y Schuchmann. 2004.	*E. fulgens* y *E. spectabilis.*

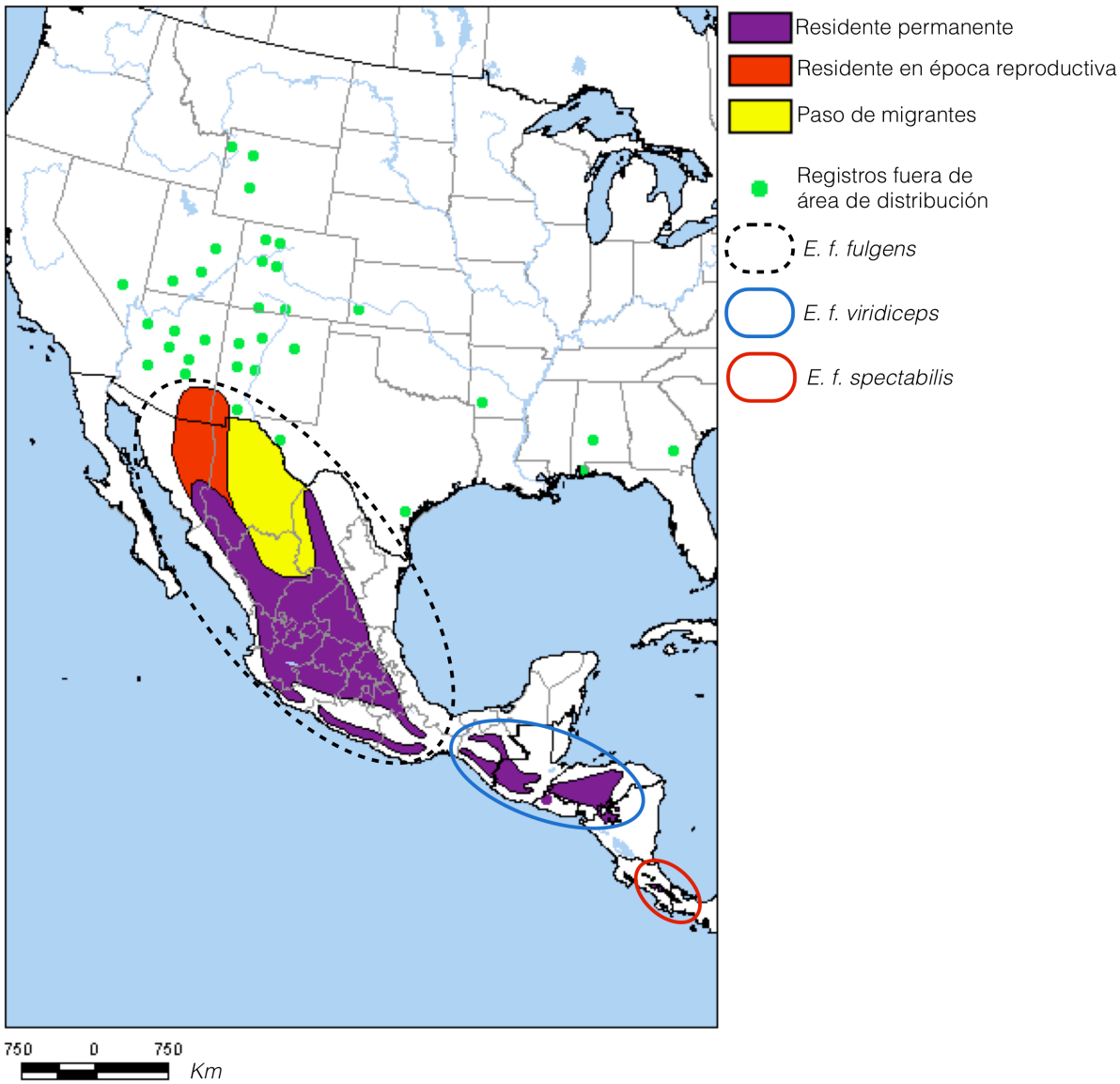

Figura 3. Distribución geográfica de *Eugenes fulgens* (tomado y modificado de NatureServe, www.natureserve.org).

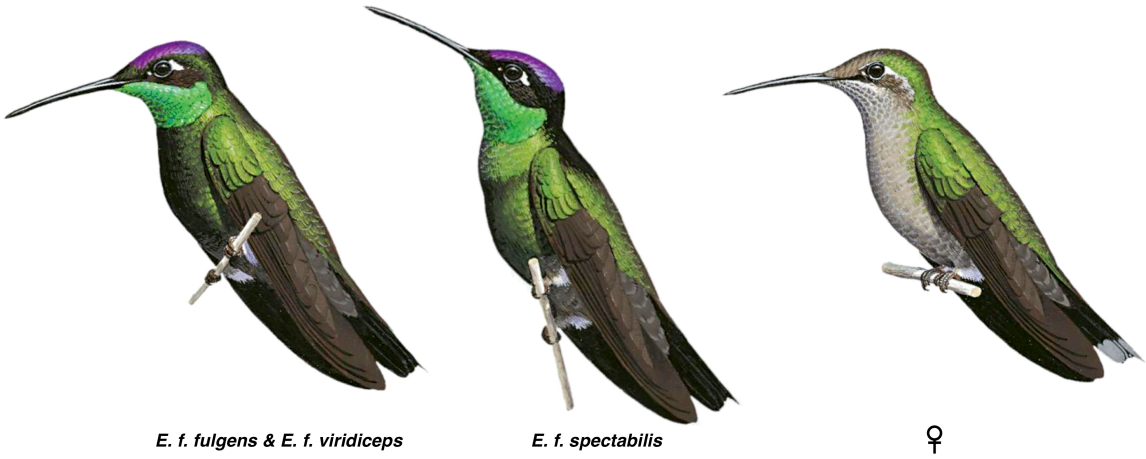

Figura 4. *Eugenes fulgens*: dimorfismo sexual y variación morfológica (Ilustraciones, Del Hoyo et al., 1999).

La descripción de las tres diferentes subespecies que conforman al complejo *Eugenes fulgens* presentan las siguientes correspondencias geográficas:

- *E. f. fulgens* (Swainson, 1827): montañas del sureste de Arizona y suroeste de Nuevo México, hacia el sur a través de las montañas de México hasta el Istmo de Tehuantepec.
- *E. f. viridiceps* (Boucard, 1878): montañas de Chiapas, Guatemala, Honduras, oeste de El Salvador y Nicaragua.
- *E. f. spectabilis* (Lawrence, 1867): bosques de alta montaña (arriba de los 1800m) en Costa Rica y oeste de Panamá.

Lamprolaima rhami.

Conocido como colibrí alicastaño, *Lamprolaima rhami* (Lesson, 1838), presenta un marcado dimorfismo sexual. Los machos tienen pico corto, recto y negro, una mancha postocular blanca, garganta rosa brillante, pecho violeta-azul brillante, dorso verde iridiscente, vientre negruzco, flancos verde moteado, remeras rojizas (“rufous”) con puntas de color café obscuro, cola de color morado obscuro y la punta de las rectrices externas de color gris. Las hembras presentan dorso verde iridiscente, puntos rosas en garganta y la punta de las rectrices externas de color blanco (Schuchmann, 1999; Figura 5).

Es una especie sedentaria, presentando únicamente movimientos a mayores altitudes durante el periodo reproductivo, por arriba de los 1500 msnm. Se alimenta del néctar floral de plantas de matorral, además de las flores presentes en árboles de *Inga* y *Erythrina* (Schuchmann, 1999). Se ha reportado la presencia de ésta especie en la

localidad de El Triunfo, en el Estado de Chiapas, alimentándose de las flores de *Cavendishia bracteata* (Ericaceae), *Clusia sp.* (Guttiferae) y *Clethra mexicana* (Gómez de Silva et al., 1999). En otro estudio llevado a cabo en la Reserva Ecológica Huitepec, en el Estado de Chiapas, se reportó la presencia de *L. rhami* junto con otras tres especies de colibríes (*Basilinna leucotis*, *Lampornis amethystinus* y *Eugenes fulgens*), alimentándose del néctar de las especies *Chirantodendron pentadactylon* y *Passiflora membranacea* (Partida-Lara et al., 2012). En éste estudio, *L. rhami* resultó ser una especie poco abundante en la zona muestreada, y estuvo asociada principalmente al bosque mesófilo de montaña.

L. rhami habita entre los 1200 y 3000 msnm, asociada a bosques tropicales altos, bosques mesófilos de montaña, pino-encino y matorrales, con distribución restringida a las tierras altas de Puebla, el oeste de Veracruz, Guerrero, Oaxaca, Chiapas, Guatemala, El Salvador y Honduras (Figura 6), presentando una distribución geográfica disjunta con poblaciones aisladas y restringidas (Schuchmann, 1999). Debido a las características en su distribución geográfica se han propuesto algunas hipótesis taxonómicas para la especie (ver Tabla 2).

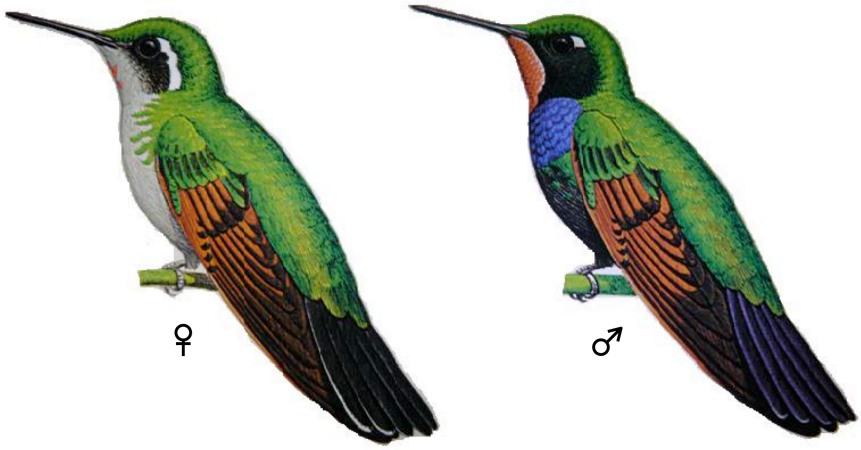

Figura 5. *Lamprolaima rhami*: dimorfismo sexual (Ilustraciones, Del Hoyo et al., 1999).

Tabla 2. Subespecies y especies propuestas para el género *Lamprolaima*.

Autor (referencias)	Hipótesis taxonómicas
Ridgway, 1911.	*L. rhami.*
Peters, 1945.	*L. r. rhami* y *L. r. saturatior.*
AOU, 1998.	*L. rhami.*
Shuchmann, 1999.	Razas: *occidentalis* y *saturatior.*

La descripción de las dos subespecies que conforman al complejo *Lamprolaima rhami* presentan las siguientes correspondencias geográficas:

- *L. r. rhami* (Lesson, 1838): montañas del sur de México: Puebla, Veracruz, Guerrero (raza *occidentalis*; Schuchmann, 1999), Oaxaca y Chiapas, y tierras altas de Guatemala.
- *L. r. saturatior* (Griscom, 1932): montañas de Honduras y del norte de El Salvador.

La propuesta de Schuchmann (1999) se basa en variaciones de coloración y tamaño, sin embargo, menciona que ambos patrones son dependientes de la edad, por lo que su propuesta es categorizar estos morfotipos como "razas", en lugar de ser consideradas bajo el rango taxonómico de subespecie.

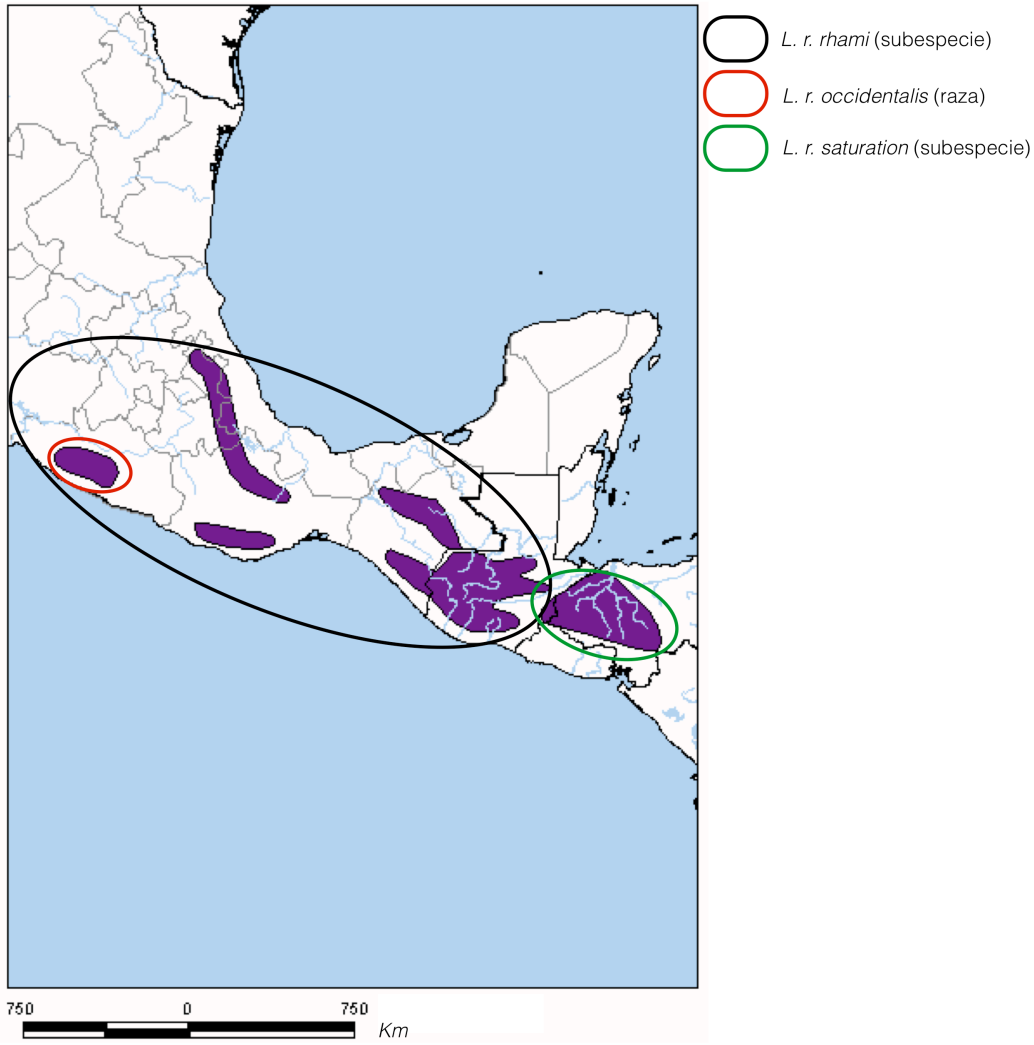

Figura 6. Distribución geográfica de *Lamprolaima rhami* (tomado de NatureServe, www.natureserve.org).

OBJETIVOS DE LA TESIS

- Proponer una hipótesis filogenética para el grupo de las "Gemas de las Montañas" empleando caracteres moleculares.

- Analizar y describir los patrones geográficos de variación genética entre las distintas poblaciones de la especie *Eugenes fulgens*, con base en el análisis de marcadores moleculares mitocondriales (ND2, Región Control) y nucleares (microsatélites) para inferir los posibles escenarios evolutivos para la distribución de los linajes presentes en las tierras altas de Mesoamérica.

- Analizar y describir los patrones geográficos de variación genética entre las distintas poblaciones de la especie *Lamprolaima rhami*, con base en el análisis de marcadores moleculares mitocondriales (ATPasa 6 y 8, Región Control, ND2, ND4) y nucleares (AK1, ODC, MUSK), para inferir los posibles escenarios evolutivos en la distribución de los linajes presentes en las tierras altas de Mesoamérica.

ARTÍCULO 1. HISTORIA EVOLUTIVA Y FILOGENIA DEL CLADO DE LAS GEMAS DE LAS MONTAÑAS (Aves: Trochilidae).

Zamudio-Beltrán, L. E., Smith, L., McGuire, J, Hernández-Baños, B. E.

Resumen.- Las Gemas de las Montañas (Aves: Trochilidae) conforman un grupo monofilético que comprende 15 especies de colibríes, en cinco géneros (*Eugenes*, *Heliomaster*, *Lampornis*, *Lamprolaima* y *Panterpe*), que comparten características en común como diferencias morfológicas marcadas en sus patrones de coloración y tamaño, distribuciones geográficas heterogéneas y por estar presentes en hábitats con diferencias altitudinales. Este grupo habita desde el sur de los Estados Unidos hasta Sudamérica, siendo Mesoamérica una región de sobrelapamiento entre varias de estas especies. Para elucidar los patrones de evolución dentro de este grupo se llevaron a cabo análisis filogenéticos con seis marcadores moleculares (ADN mitocondrial: subunidad 2 de NADH deshidrogenasa *ND2*, subunidad 4 de NADH deshidrogenasa *ND4*; ADN nuclear: Beta Fibrinogeno *BFib*, Ornitina Descarboxilasa *ODC*, Receptor Tirosina Quinasa de Músculo Esquelético *MUSK*, y Adenilato Quinasa AK1). De igual forma, se realizaron estimaciones de tiempos de divergencia y se reconstruyeron las áreas ancestrales. Con esto se propone una hipótesis evolutiva en la que se sugiere que el grupo de las Gemas de las Montañas se originó en la región que comprende el sur de Norteamérica y Centroamérica, durante mediados del Mioceno (~16.15 Ma), seguido de eventos de dispersión hacia Norte y Suramérica. También se encontró evidencia de estructura dentro de algunas especies. Estos eventos de diferenciación ocurrieron durante los periodos Plioceno-Pleistoceno (~5-0.01 Ma). Se sugiere realizar trabajos futuros para esclarecer las relaciones a nivel intraespecífico, y detectar si dicha estructura genética está relacionada con eventos geológicos a gran escala o con fluctuaciones climáticas más recientes (p. e., durante Plio-Pleistoceno).

Palabras clave: Gemas de las Montañas, Trochilidae, Neotrópico, Mesoamérica, Filogenia, Colibríes.

Phylogeny and evolutionary history of the Mountain Gems group (Aves: Trochilidae), a hummingbird clade of Neotropical distribution.

(proposal: Molecular Phylogenetic and Evolution).

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ABSTRACT

Mountain Gems (Aves: Trochilidae) comprise a small monophyletic group of hummingbird species, characterized by marked morphological differences such as in coloration and size patterns, heterogeneous geographical distributions, and different altitudinal habitats. This group includes 15 species from 5 genera (*Eugenes, Heliomaster, Lampornis, Lamprolaima* and *Panterpe*), distributed from the southern USA to South America, with a significant overlap of species ranges in Mesoamerica. To elucidate patterns of evolution within this group, we performed phylogenetic analysis with six molecular markers (mitochondrial DNA: NADH dehydrogenase subunit 2 *ND2*, NADH dehydrogenase subunit 4 *ND4*; nuclear DNA: Adenylate Kinase *AK1*, Beta Fibrinogen *BFib*, Muscle Sekeletal Receptor Tyrosine Kinase *MUSK*, and Ornithine Decarboxylase *ODC*). We also estimated divergence times between clades and species, and reconstructed ancestral biogeographic areas. Based on our results, we propose a hypothesis for the evolution of this clade where the Mountain Gems group originated in the region between southern North America and Central America, during the middle Miocene (~16.15 Mya), followed by various dispersal events to North and South America. We also found of significant evidence of within-species genetic structure dating back to the Pliocene-Pleistocene (~5-0.01 Mya). Further studies will be needed to clarify such intraspecific differentiations and to detect if this genetic structure is more correlated with major geological events or with more recent climatic fluctuations (e.g., during Plio-Pleistocene).

Keywords: Mountain Gems, Trochilidae, Neotropics, Mesoamerica, Phylogeny, Hummingbirds.

1. Introduction

An increasing number of bird studies have explored the principal drivers of speciation in the Neotropics (Smith et al., 2014; Weir and Hey, 2006). Besides increasing our knowledge of evolutionary biology and biodiversity, studies on patterns of diversification and geographical structure play a key role in conservation efforts (Batalha-Filho et al., 2014; Beckman and Witt, 2015; Pérez-Emán, 2005; Slager et al., 2014).

Several phylogenetic studies of diverse avian families suggest that major geological events and drastic environmental changes were crucial to explain species diversity (Ohlson et al., 2008). Trochilidae, the second species-richest avian family in the Americas is a well studied group, comprising 349 species distributed in 3 subfamilies: Topazinae, Phaethornithinae, and Trochilinae (Gill and Donsker, 2015; http://checklist.aou.org/). Within Trochilidae nine monophyletic clades are included: Topazes, Hermits, Mangoes, Brilliants, Coquettes, *Patagona*, Mountain Gems, Bees and Emeralds (Bleiweiss, 1998a; Bleiweiss et al., 1997). The most accepted evolutionary hypothesis for this group proposes an Andean origin and followed by a radiation with multiple dispersal events to the Caribbean, North America and Central America (McGuire et al., 2007; McGuire et al., 2014).

Most major hummingbird clades share some characteristics, such as wide and disjunct geographical distributions and/or morphological patterns,

while others are far more diverse. The latter is the case with the Mountain Gems group (MGg), one of the smallest clades in terms of number of species within the Trochilidae family. This heterogeneous group is composed of species distributed from North America to Central and South America, with some taxa inhabiting lowlands and some others found in highlands (Del Hoyo et al., 1999).

The Mountain Gems group comprises 15 recognized species distributed in five genera: *Lampornis*, *Eugenes*, *Lamprolaima*, *Heliomaster*, and *Panterpe* (See Table 1). According to morphological traits, two South American species are related to this group: *Hylonympha macrocerca* and *Sternoclyta cyanopectus* (Renner and Schuchmann, 2004). However, no molecular phylogenetic studies have been performed so far for these species, probably because of a lack of samples given their restricted distribution and because one of them is endangered (*Hylonympha macrocerca*, Schuchmann, 1999).

Previous phylogenetic studies have found that all genera comprising the Mountain Gems group are reciprocally monophyletic (McGuire et al., 2007; McGuire et al., 2014). However, the phylogenetic relationships within these genera remain unclear (e.g. *Lampornis* genus). In addition, a general biogeographic hypothesis has been proposed for this group, in which the common ancestor of Mountain Gems and its sister clade (“Bees”) arrived from South America and got established in Central America, where diversification of Mountain Gems took place *in situ* (McGuire et al., 2014).

In this paper by using mitochondrial and nuclear markers and adequate subspecies sampling, we 1) inferred evolutionary processes and divergence

98 times based on multilocus phylogenetic analyses, 2) we proposed a
99 biogeographic hypothesis of ancestral areas of distribution and colonization,
100 and 3) we analyzed the presence of intraspecific genetic structure. Based on
101 previous phylogenetic and biogeographic hypotheses, and by incorporating a
102 broad intraspecific sampling, we hypothesize that the Mountain Gems species
103 originated from a common ancestor in Central America, that their geographic
104 ranges expanded through North America by some dispersal events, followed
105 by few dispersal events promoted that some species got established back into
106 South America. Also, we expect to clarify all interspecific phylogenetic
107 relationships and find evidence of intraspecific structure correlated with
108 vicariant events during Plio-Pleistocene ages.
109
110 **2. Material and Methods**
111 *2.1 Taxon sampling*
112 We obtained tissues samples from 86 individuals representing all 15
113 recognized species of the Mountain Gems group (MGg) (Fig. 1). For six
114 species we included representatives from the different recognized subspecies
115 (see Table 1), and considered at least two individuals for most taxa (nine
116 species). In addition, 17 previously published sequences from the MGg were
117 obtained from GenBank (McGuire et al., 2014). In order to evaluate the
118 phylogenetic relationships of MGg with other hummingbird clades, we
119 included some sequences published in GenBank from the Bees, *Patagona*,
120 Coquettes, Brilliants, Mangoes, Hermits and Topazes groups (Bleiweiss et al.,
121 1997; McGuire et al., 2007). We also included species from Aegothelidae,
122 Hemiproncidae and Apodidae families as outgroups (McGuire et al., 2014).

123 Tissue samples were provided by different collections, including the Museo de
124 Zoología Alfonso L. Herrera (Universidad Nacional Autónoma de México), the
125 Natural History Museum (University of Kansas), The Burke Museum
126 (University of Washington), and the Museum of Natural Science (Louisiana
127 State University). See supplementary material S1 for vouchers information.
128 DNA was extracted with the DNAeasy™ Blood and Tissue kit (Qiagen
129 Inc., Valencia, CA, USA) following manufacturer's protocols. For most
130 samples, we sequenced two mitochondrial genes (NADH dehydrogenase
131 subunit 2: *ND2*, and NADH dehydrogenase subunit 4: *ND4*) and four nuclear
132 genes (adenylate kinase intron 5: *AK1*, beta fibrinogen intron 7: *BFib*, Muscle
133 Skeletal Receptor Tyrosine Kinase exons 4 and 5: *MUSK*, and a segment of
134 the ornithine decarboxylase gene comprising the end of exon 6 to the
135 beginning of exon 8: *ODC*).
136 We amplified these molecular markers with polymerase chain reaction
137 (PCR) using specific primers and protocols (Table S2 in supplementary
138 material). All reactions were a total volume of 12.5μL and contained: 1.25 μL
139 10x buffer (magnesium-free), 0.19 μL dNTPs (10mM each), 0.38 μL MgCl2
140 (50 mM), 0.25 μL of each primer (10 μM), 0.1 μL Invitrogen Taq polymerase
141 (5U/μL), and 0.5 μL of genomic DNA. PCR products were visualized on a 1%
142 agarose gel, and purified using shrimp alkaline phosphatase and exonuclease
143 I (Exo-SAP-IT, Affymetrix).
144
145 *2.2 Sequencing and Evolutionary Models.*
146 Purified PCR products were sequenced using the Big Dye Terminator mix
147 following manufacturer's protocols (Big Dye 3.1, Applied Biosystems). DNA

148 sequence data was collected by the ABI 3730 automated sequencer at the
149 Evolutionary Genetics Laboratory at the Museum of Vertebrate Zoology
150 (University of California, Berkeley).
151 Chromatograms were visualized and edited with Sequencher v4.8
152 (GeneCodes Corporation, Ann Arbor, MI). Heterozygous sites in nuclear
153 markers were coded according with IUPAC ambiguities. Each data matrix was
154 aligned using ClustalX (Thompson et al., 1997), and visually corrected using
155 MacClade 4.06OSX software (Maddison and Maddison, 2000). All new
156 sequences are available in GenBank under accession numbers XXXX-XXXX.
157 We created a unique concatenated data matrix (136 individuals) of
158 3721 bp distributed as follows: ND2, 1041 bp; ND4, 471 bp; AK1, 518 bp;
159 BFib, 776 bp; MUSK, 609 bp; and ODC, 628 bp. For each molecular marker
160 we calculated the evolutionary model that better fit the data using jModeltest
161 0.1.1 (Posada, 2008), based on the Akaike Information Criterion AIC (Akaike,
162 1987).
163
164 *2.3 Phylogenetic analysis.*
165 We used the concatenated data set to estimate the MGg phylogeny and
166 detect intraspecific structure under Bayesian Inference (BI) and Maximum
167 Likelihood (ML) frameworks. For the BI approach, we used Mr. Bayes v3.0
168 (Huelsenbeck and Ronquist, 2002), assigning different evolutionary models to
169 each partition (i. e. gene). We ran four simultaneous chains for each Monte
170 Carlo Markov Chain analysis for 20 million generations, taking samples every
171 250 generations. We eliminated the first 2.5 million iterations as burn-in using
172 Tracer v1.6.0 (Rambaut et al., 2013). The remaining trees were used to

construct a majority rule consensus tree with posterior probability distribution. The final tree was visualized in FigTree v1.2.3 (http//tree.bio.ed.ac.uk/software/figtree/).

The Maximum likelihood analysis was run in RaxML v1.31 (Stamatakis et al., 2008), under the GTRG model. We ran the analysis with 1000 bootstrap replicates.

2.4 Divergence times

Divergence time estimates were obtained using BEAST v1.8.2 (Drummond and Rambaut, 2007; Drummond et al., 2012). We used the concatenated dataset (136 individuals), and assigned the previously determined best-fit model of evolution to each partition. We employed an uncorrelated lognormal relaxed clock, and a Yule speciation model to model the tree prior. We delimitated two major groups as monophyletic (Apodiformes and Trochilidae) according to previous studies (e.g. McGuire et al., 2007), and following our own results. Despite of the lack of fossil records for modern hummingbirds, we assigned calibration nodes based on Old World fossil records and from secondary calibrations. We took into account the following fossil records for Trochilidae group for calibration: *Parargornis messelensis*, 47 Ma (Mayr, 2003); *Eurotrochilus inexpectatus*, 30-40 Ma (Mayr, 2004); *Eurotrochilus noniewiczi*, 31 Ma (Bochenski and Bochenski, 2008) and *Eurotrochilus sp*, 28-34 Ma (Louchart et al., 2008). According to this fossil information, we set the minimum and maximum limits in Trochilidae Crown node as 28.2-46.8 Ma, with a lognormal prior distribution (mean 37.5, SD 4.75). For the limits in the Apodiformes node, we set a minimum age of 58.7 and a maximum of 72 Ma,

198 and a lognormal prior distribution (mean 65.37, SD 3.4). This last time interval
199 was taken from a secondary calibration resulting from a mitogenomics study
200 (Pacheco et al., 2011).
201 We incorporated substitution rates with a normal distribution for our
202 concatenated dataset (see Table 2). This analysis was run for 50 million
203 generations and sampling every 1000 generations. We used Tracer v1.6.0
204 (Rambaut et al., 2013) to visualize the burn-in, and with LogCombiner v1.8.2
205 (Drummond and Rambaut, 2007) the first 25% data was eliminated. We used
206 TreeAnnotator v1.8.2 (Rambaut and Drummond, 2007) to summarize the
207 sampled trees as a maximum clade credibility tree, and to obtain mean
208 divergence times with 95% highest posterior density intervals.
209
210 2.5 *Ancestral area reconstruction*
211 To infer the possible ancestral areas of each clade within MGg, we used the
212 Statistical Dispersal-Vicariance Algorithm (S-DIVA) implemented in RASP
213 v3.2 (Yu et al., 2012). We performed two analyses: 1) according to
214 geographical areas, and 2) according to highland and lowland habitats.
215 For the first analysis, we defined four geographical areas: A. North
216 America (from southern USA to west of the Isthmus of Tehuantepec), B.
217 South North America (from east of the Isthmus of Tehuantepec to the west of
218 the Nicaraguan Depression), C. Central America (from the east of the
219 Nicaraguan Depression to the west of the Panamanian Isthmus), and D.
220 South America (from the east of the Panamanian Isthmus to South America).
221 These areas were selected according to the discontinuous geographical
222 distribution for most of the MGg species, and the existence of major

223 geographical barriers previously observed in other phylogeographic surveys.
224 In a second analysis we classified species according to their distribution in
225 highlands (A) or lowlands (B) (Schuchmann, 1999). The geographic areas
226 and highland/lowland assignation to each species are shown in Table 3.
227 In both analyses we created a new data set that excluded all
228 outgroups. As the S-DIVA algorithm is sensitive to polytomies, we did not
229 include all samples for each species, given that some intraspecific
230 relationships remained unsolved in our phylogenies (see Results).
231 We performed a Bayesian analysis using BEAST v1.8.2 (Drummond
232 and Rambaut, 2007; Drummond et al., 2012), with the same parameters used
233 above, but using 100 million generations, and sampling every 1000
234 generations. After discarding the first 25 million generations as burn-in, we
235 used 75000 trees from MCMC output to build a maximum clade credibility
236 tree. The number of maximum areas was kept as 2.
237
238 **3. Results**
239 3.1 *Phylogenetic analysis*.
240 We obtained a concatenated dataset of 3721 bp for 136 individuals (including
241 outgroups and GenBank sequences). The best-fit models for each molecular
242 marker were as follows: GTR+I+G (ND2, ND4), TIM2+G (AK1), TPM1uf+G
243 (BFib), TPM3uf+G (MUSK), and TrN+G (ODC).
244 In Figure 2, we show the phylogenetic trees resulting from our analyses using
245 Bayesian Inference (BI) and Maximum Likelihood (ML). Both topologies are
246 highly supported and support the monophyly of all the main hummingbird
247 clades (Mountain Gems, Bees, Coquettes, Brilliants, Mangoes, Hermits, and

248 Topazes; See Supplementary Information S3). All MGg genera were
249 monophyletic, and supported by high values of posterior probability (PP≥0.95)
250 and bootstrap replicates (BT≥50). However, the clade containing *L.*
251 *cinereicauda*, *L. calolaemus* and *L. castaneoventris* remained unsolved, in
252 part due to the fact that there is only one sequence per species, but, more
253 importantly, the genetic distances among them are very small, suggesting that
254 they belong to a single species distributed in the highlands of Central America
255 (from Nicaragua to Panama). Some species present evidence of genetic
256 structure that in some cases corresponds to discontinuous geographical
257 distribution, and to proposed subspecies. However, some internal groups
258 presented a few intraspecific differences between phylogenies, such as in
259 *Lampornis amethystinus*, *L. clemenciae*, and *Eugenes fulgens*. In all these
260 cases, our phylogenies do not support the proposed subspecies as
261 monophyletic groups. Nevertheless, these few topological differences had no
262 impact on relationships between species within the MGg.
263 In the case of *Eugenes fulgens*, it is known that genetic structure is
264 related to geography and isolated populations (Zamudio-Beltrán and
265 Hernández-Baños, 2015). Our results show however that this observation can
266 also be expanded to *Lamprolaima rhami*, *Heliomaster constantii*, and *H.*
267 *longirostris*. In the case of *L. rhami*, individuals are nested into two
268 independent and highly supported clades, while for the cases of *H. constantii*
269 and *H. longirostris*, the individuals corresponding to South American
270 subspecies are separated from Northern and Central American individuals.
271 However, in these two species, this genetic differentiation should be taken
272 carefully as we have only one representative of the South American

subspecies. In the case of *L. amethystinus*, we recovered one well supported clade containing all the individuals from *salvini* subspecies, distributed from the highlands of Chiapas and Guatemala to the highlands of Nicaragua, but no genetic pattern was found for the rest of proposed subspecies (*margaritae*, *circumventus* and *amethystinus*).

3.2 *Divergence times*.

The estimation of divergence times reveals that the split between Bees clade and MGg should have occurred during the early Miocene (Node B, ~18.50 Mya) (Figure 3 and Table 4). The major radiation of MGg should have taken place during the middle to late Miocene that originated around ~16.15 Mya (19.08-13.24 Mya) as estimated by BEAST. In addition, the estimation of divergence times for the main Trochilidae clades overlaps with previously reported results (Ornelas et al., 2013a).

As detailed in supplementary material S4, the separation of the owlet-nightjars (Aegotelidae) from the swifts and the hummingbirds occurred at ~72.16 Mya (node I), and the split between hummingbirds and swifts was dated at ~67.14 Mya (node II). The diversification of the hummingbird clade took place around ~36.52 Mya (Node IV), with the subsequent split of the main Trochilidae clades.

3.3 *Ancestral area reconstruction*.

The reconstruction of the most likely ancestral areas is in Fig. 4. Our results suggest the presence of vicariant and dispersal events promoting diversification. In general, we found evidence that suggest that the Most

298 Likelihood State/Area (MLS) for the origin of MGg was not in South America,
299 but southern in North America and/or Central America. In the same way, for
300 both major clades within MGg (*Lampornis* genus and the rest of species) the
301 MLS appeared to be the same, South North-America plus Central America,
302 with evidence of a MGg common ancestor distributed most probably in the
303 highlands. When comparing the results shown in Fig 4a and Fig 4b, the most
304 probable hypotheses of ancestral areas for each genus are as follows: 1) the
305 ancestor of *Lampornis* genera was distributed in the highlands of South North-
306 America and Central America, 2) the ancestor of *Eugenes* was distributed in
307 the highlands of North America, 3) the ancestor of *Lamprolaima* was
308 distributed in the highlands of North America, 4) the ancestor of *Heliomaster*
309 was distributed in the lowlands of Central and South America, and 5) the
310 ancestor of *Panterpe* was distributed in Central America.
311
312 **4. Discussion**.
313 *4.1 Evolutionary history.*
314 In this study, we analyzed the phylogenetic relationships and provided
315 evidence for the reciprocal monophyly of Mountain Gems species, where all
316 genera and main clades were all well supported. We also presented a
317 biogeographic hypothesis of evolutionary history based on estimations of
318 divergence times and reconstruction of ancestral areas to infer the
319 evolutionary history for this clade within the Trochilidae family.
320 Previous works have argued that most of major hummingbird clades
321 originated in South American lowlands (Bleiweiss, 1998a; McGuire et al.,
322 2007). According to our results of ancestral biogeographic areas

323 reconstruction, we can infer that the Mountain Gems higher-level clade
324 originated in an area between southern North America and Central America
325 (Mesoamerican origin), with posterior dispersal events towards North America
326 and South America. This colonization took place during the first two
327 diversification events: 1) the origin of *Lampornis* during middle Miocene, and
328 2) the origin of the clade containing the genus *Eugenes*, *Lamprolaima*,
329 *Heliomaster* and *Panterpe*. The diversification event that occurred during the
330 split between *Panterpe* and *Heliomaster*, was probably the event of
331 recolonization towards South America. Bleiweiss (1998a) proposed that the
332 Mountain Gems originated in North America. However, the absence of South
333 American species could not be explained, because it was believed that the
334 limit of its distribution was Central America. In a posterior study (McGuire et
335 al., 2007), it was found that the Mountain Gems group originated in Central
336 America, with some species that expanded their ranges into North America
337 and South America. Later, McGuire et al. (2014) conclude that the ancestor of
338 “Bees” and “Mountain Gems” groups came from a single invasion from South
339 America around 12 Mya, date that corresponds with Panamanian uplift. Our
340 results showed an older split between “Bees” and “Mountain Gems” (18.5
341 Mya, 21.86-15.30 Mya), inferring that this invasion from South America was
342 carried out through sea before the completion of Panamanian uplift. Also,
343 McGuire et al. (2014) found that the ancestor of the Mountain Gems had a
344 lowland distribution, however, our results reject this hypothesis as a highland
345 ancestry is suggested after the first two events of diversification. The
346 settlement at lowland habitats only should have occurred during the split of
347 *Heliomaster* from the other genera (middle Miocene).

348 According to our results, the colonization from Central America along
349 South America took place during the late Miocene, following the expansion of
350 *H. longirostris*, and the divergence of *H. furcifer* and *H. squamosus*. This
351 indicates that the main events related to species diversification within this
352 group are due to dispersion, one of the principal drivers of speciation in the
353 neotropics (Smith et al., 2014). By contrast, in Central America most
354 divergence events were related to vicariance or allopatric speciation, and
355 were more recent, during the Pliocene and Pleistocene. One of these
356 vicariance events comprises the split between *L. sybillae* (distributed in the
357 highlands of Honduras and northern Nicaragua) from *L. cinereicauda*, *L.*
358 *calolaemus* and *L. castaneoventris* (distributed in the highlands of Costa Rica
359 and Panama). In this study, we dated this split around 3.85 Mya, that could be
360 related with an allopatric speciation favored by the Nicaraguan depression,
361 that has been reported as an important geographic barrier that has favored
362 divergence events during Miocene and Pliocene (e.g. Arbeláez-Cortés et al.,
363 2010).
364 Also, the split between *H. constantii* from the others *Heliomaster*
365 species could be explained as a vicariance event, where *H. constantii* is the
366 unique species from its genus with no presence in South America, limiting its
367 distribution from North America to Central America. According to the
368 reconstructions based on the habitats of highlands and lowlands, our study
369 recovered two major events: 1) the dispersal of the ancestor of *Panterpe* and
370 *Heliomaster* that expanded its range towards the lowlands, and 2) a
371 vicariance event for the establishment of *Panterpe* into the highlands and
372 *Heliomaster* into the lowlands.

373 In general, our estimates of divergence times were congruent with
374 those of a recent work, showing that geological events could be implicated on
375 the radiation of *Amazilia* genus (Ornelas et al., 2013a). The times determined
376 herein (~18.50 Mya, 21.86-15.30 Mya) represent nevertheless older dates for
377 the divergence between the Mountain Gems and the Bees, when compared to
378 those of Bleiweiss (1998b) and McGuire et al. (2014), that dated this split
379 around 5.82-6.11 Mya and ~12 Mya respectively.
380
381 *4.2 Phylogenetic relationships.*
382 Regarding the phylogenetic relationships, our main findings were the
383 monophyly of each genus, the resolution of relationships between most
384 *Lampornis* species, and the presence of variation at an intraspecific level.
385 Previous studies using molecular data, have described the phylogenetic
386 relationships in the Trochilidae family, and indirectly within the Mountain
387 Gems group (Altshuler et al., 2004; Bleiweiss, 1998a, b; Bleiweiss et al.,
388 1997; García-Moreno et al., 2006; McGuire et al., 2007; McGuire et al., 2014;
389 Renner and Schuchmann, 2004; Schuchmann, 1999). However, phylogenetic
390 relationships, mainly between *Lampornis* species, were still ambiguous. In this
391 study, the phylogenetic positions of *L. hemileucus*, *L. sybillae* and *L.*
392 *viridipallens* were clarified, where *L. hemileucus* represents the outermost
393 species in *Lampornis* genus, and *L. sybillae* and *L. viridipallens* are sister
394 groups closely related to the clade that contains *L. castaneoventris*, *L.*
395 *cinereicauda* and *L. calolaemus*. The unsolved clade containing *L.*
396 *castaneoventris-L. calolaemus-L. cinereicauda*, represents a controversial
397 group. Previous studies, trying to elucidate phylogenetic relationships within

398 the Trochilidae family (McGuire et al., 2014), and more specific in the
399 *Lampornis* genus, have not clarified the species limits between these taxa
400 (García-Moreno et al., 2006). In García-Moreno et al. (2006), they used a
401 multilocus database, with both mitochondrial and nuclear markers (mtDNA:
402 ND5, cyt b; nDNA: AK5, cmos). Their sampling for this complex was higher
403 than our study (*L. calolaemus*: 4 individuals, *L. castaneoventris*: 5 individuals;
404 *L. cinereicauda*: 1 individual). Despite the reciprocal monophyly found, this
405 clade showed no resolution among the three taxa. According to our results of
406 divergence times, this complex is the youngest clade within Mountain Gems
407 group (~0.14 Mya). Therefore, this lack of structure is probably due to their
408 recent origin and overlapped geographical distribution, and indicates that this
409 group should be evaluated at a different phylogenetic (e. g. population
410 genetic) level.
411 At the intraspecific level, the genetic structure within *L. amethystinus*,
412 *L. clemenciae*, and *L. rhami*, is not consistent with the subspecies previously
413 proposed (Schuchmann, 1999). For example, the present study shows that
414 within *L. amethystinus* a well supported clade contains all sampled individuals
415 from *L. a. salvini* subspecies. This result confirms the differentiation between
416 individuals east of the Isthmus of Tehuantepec (*salvini* subspecies), whereas
417 the differentiation between *margaritae*, *amethystinus* and *circumventus*
418 subspecies is not clear. Moreover, *L. clemenciae* formed a well supported
419 clade with no resolution at the subspecies level. Previous phylogeographic
420 studies describing the relationships at the population level for this species
421 also showed that genetic differentiation was more related to geography than

422 to morphology (i.e. the characters used for describing subspecies: Cortés-
423 Rodríguez et al., 2008).
424 A similar case was observed for *L. rhami*, where all individuals from the
425 highlands of Chiapas are nested in a well-supported clade. This pattern of
426 geographic correspondence, associated to geographic barriers, has frequently
427 reported (Barber and Klicka, 2010; Rodríguez-Gómez et al., 2013). *L. rhami* is
428 mainly distributed in patches of cloud forests through the highlands of Mexico
429 and Central America (Schuchmann, 1999). The variation found in this study
430 could serve as a first evidence to address further questions about population
431 dynamics and phylogeographic patterns of this species associated to a well
432 known endangered habitat (Mulligan, 2010).
433 Contrary to the cases above, the genetic structure of *E. fulgens*, *H.*
434 *constantii* and *H. longirostris* was according to some of the subspecies
435 proposed. As previously reported (Zamudio-Beltrán and Hernández-Baños,
436 2015) *Eugenes fulgens* represents a species complex, formed by three
437 independent lineages (*fulgens*, *viridiceps* and *spectabilis*), and its taxonomic
438 status should be reevaluated. About *H. constantii and H. longirostris,* there
439 seems to exist variation at intraspecific level, but no final statements could be
440 done as subspecific sampling is inadequate. However, we consider that this
441 possible genetic structure should be explored closely.
442 The number of species in the Mountain Gems is low compared to that
443 of other hummingbird clades, however, the evidence presented herein of
444 within-species structure might imply a higher number of cryptic taxa and
445 increase the number of species within this group. This work emphasizes on
446 the importance of increasing intraspecific sampling in phylogenetic studies,

447 which is crucial to understanding the evolutionary history of differentiation
448 between species in a more general framework, and at lower taxonomic levels.
449 We should consider that the distribution of some Mountain Gems species, is
450 secluded to highland cloud forests (e. g. *L. amethystinus*, *L. rhami*, *P.*
451 *insignis*). This habitat is well known to be formed by isolated island-like
452 patches throughout the main neotropical mountain chains, all with unique
453 characteristics that increases endemisms (Ornelas et al., 2013b; Ponce-
454 Reyes et al., 2013; Ramírez-Barahona and Eguiarte, 2013), and it is possible
455 that species number in this group is underestimated as there is a lack of
456 studies at population level. We must also take into account species that have
457 been considered closely related to *Eugenes* genus (*Hylonympha macrocerca*
458 and *Sternoclyta cyanopectus* (Renner and Schuchmann, 2004)), from which
459 no samples were obtained for this study. Their phylogenetic relationships are
460 unknown, so further work is needed in sampling effort for species that
461 presumably are related to this group.
462

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Tables

Table 1. Mountain Gems species. Asterisks represent sampled subspecies.

Genera	Species	Subspecies
Lampornis (Schuchmann, 1999).	*L. amethystinus*	*amethystinus**, *margaritae**, *circumventus**, *salvini**, *nobilis*.
	L. calolaemus	*pectoralis*, *calolaemus*, *homogenes*.
	L. castaneoventris	
	L. cinereicauda	
	L. clemenciae	*bessophilus**, *phasmorus*, *clemenciae**.
	L. hemileucus	
	L. sybillae	
	L. viridipallens	*amadoni*, *ovandensis*, *viridipallens*, *nubivagus*.
Eugenes (Navarro-Sigüenza and Peterson, 2004; Peters, 1945; Schuchmann, 1999; Zamudio-Beltrán and Hernández-Baños, 2015).	*E. fulgens*	*fulgens**, *viridiceps**, *spectabilis**.
Lamprolaima (Schuchmann, 1999).	*L. rhami*	*occidentalis**, *saturatior*.
Heliomaster (Schuchmann, 1999).	*H. constantii*	*pinicola**, *leocadiae**, *constantii**.
	H. furcifer	
	H. longirostris	*pallidiceps**, *longirostris**, *albicrissa*.
	H. squamosus	
Panterpe (Schuchmann, 1999).	*P. insignis*	*eisenmanni*, *insignis*.

Table 2. Average substitution rates, and Deviation Standar (SD) for the molecular makers used in this study.

Molecular Marker	Average Rate	S.D.	Reference
ND2	0.0068	0.00191	Pacheco et al., 2011
ND4	0.0045	0.00131	Pacheco et al., 2011
AK1	0.00083	0.00017	McGuire et al., 2014
BFib	0.0019	0.0003	McGuire et al., 2014
MUSK	0.00135	0.00021	Ellegren, 2007
ODC	0.0015	0.00024	McGuire et al., 2014

Table 3. Species coding for ancestral areas reconstruction analyses. Geographic area: A. North America, B. South North-America, C. Central America, D. South America. Highland/lowland assignation: A. Highland, B. Lowland.

Genera	Species	Geographic area	Highland/lowland
Lampornis	*L. amethystinus*	AB	A
	L. calolaemus	C	A
	L. castaneoventris	C	A
	L. cinereicauda	C	A
	L. clemenciae	A	A
	L. hemileucurus	C	A
	L. sybillae	B	A
	L. viridipallens	B	A
Eugenes	*E. fulgens*	ABC	A
Lamprolaima	*L. rhami*	AB	A
Heliomaster	*H. constantii*	ABC	B
	H. furcifer	D	B
	H. longirostris	ABCD	B
	H. squamosus	D	B
Panterpe	*P. insignis*	C	A

Table 4. Divergence times, posterior probabilities and 95% confidence intervals (high posterior density, HPD) in millions of years (Mya) for MGg species, and outgroups (Bees clade and *Patagona* genus).

Node	**PP**	**Age Mya (95% of HPD)**
A (*Patagona*/Bees+MGg)	0.99	21.34 (25.16-17.64)
B (MGg/Bees)	0.99	18.50 (21.86-15.30)
C (MGg)	0.99	16.15 (19.08-13.24)
D (*L. hemileucurus*/Other)	0.63	14.21 (17.32-11.20)
E	0.99	14.15 (17.04-11.53)
F (*Panterpe*/Other)	0.98	13.14 (16.00-10.56)
G (*Eugenes*/*Lamprolaima*)	0.99	10.79 (13.73-8.01)
H (Bees)	0.99	10.04 (12.72-7.36)
I	0.99	8.15 (10.17-6.02)
J (*L. clemenciae*/Other)	0.99	8.04 (10.45-5.88)
K (*H. longirostris*/Other)	0.99	6.97 (8.92-5.05)
L (*L. amethystinus*/Other)	0.99	5.19 (6.75-3.71)
M (*H. furcifer*/*H. squamosus*)	0.99	4.48 (6.23-2.76)
N	0.99	3.85 (5.25-2.52)
O (*E. fulgens*)	0.99	3.57 (5.07-2.27)
P (*H. constantii*)	0.99	3.52 (5.20-2.00)
Q (*H. longirostris*)	0.99	3.19 (4.82-1.74)
R (*L. sybillae*/*L. viridipallens*)	0.81	3.22 (4.51-1.98)
S (*L. clemenciae*)	0.99	3.21 (4.66-1.80)
T (*L. amethystinus*)	0.99	2.68 (3.68-1.79)
U (*L. viridipallens*)	0.99	1.42 (2.15-0.76)
V (*Lamprolaima*)	0.99	0.91 (1.47-0.45)
W (*H. furcifer*)	0.99	0.81 (1.58-0.21)
X (*L. hemileucurus*)	0.99	0.52 (1.05-0.10)
Y (*L. calolaemus*/Other)	0.99	0.14 (0.31-0.02)
Z (*L. cinereicauda*/*L. castaneoventris*)	0.18	0.23 (0.50-0.04)

Figure captions

Figure 1. Geographic distribution of species within the Mountain Gems group. Dots on the maps indicate sampled localities. For some species, these sites belong to different subspecies. All distributions based on "Bird species distribution maps of the world" (BirdLife International and NatureServe: http://www.birdlife.org). All illustrations reproduced from the Handbook of the birds of the world, Alive (http://www.hbw.com) with the corresponding permissions. a) *Heliomaster longirostris* (*H. l. longirostris*, *H. l. pallidiceps*), *H. squamosus*, *H. furcifer* (Schuchmann et al., 2013, 2015; Stiles and Boesman, 2013); b) *Lampornis clemenciae* (*L. c. bessophilus*, *L. c. clemenciae*), *L. viridipallens*, *L. hemileucus* (Powers and Boesman, 2013; Stiles and Boesman, 1999); c) *L. amethystinus* (*L. a. amethystinus*, *L. a. margaritae*, *L. a. circumventus*, *L. a. salvini*), *L. sybillae*, *L. castaneoventris* (Chai et al., 2013; Stiles et al., 2015; Züchner and Boesman, 1999); d) *Eugenes fulgens* (*E. f. fulgens*, *E. f. viridiceps*, *E. f. spectabilis*; Powers and Boesman, 1999); e) *H. constantii* (*H. c. pinicola*, *H. c. leocadiae*, *H. c. constantii*), *Panterpe insignis* (Stiles and Boesman, 2014; Stiles et al., 2015); f) *Lamprolaima rhami* (*L. r. rhami*, *L. r. occidentalis*), *Lampornis calolaemus*, *L. cinereicauda* (del Hoyo et al., 2015; Schuchmann and Boesman, 1999).

Figure 2. Phylogenetic relationships of the Mountain Gems group. Topologies were obtained by Bayesian Inference (BI, left) and Maximum Likelihood (ML, right) algorithms. Analyses were based on a concatenated dataset of mitochondrial and nuclear genes (3721 bp). In both topologies, asteriks represent node support: posterior probabilities PP≥0.95 (BI), and bootstrap values BT≥50 (ML). Topology edited from Fig. S3. Subspecies abbreviations: m: *margaritae*, c: *circumventus*/*clemenciae*, a: *amethystinus*, s: *salvini*, b: *bessophilus*, f: *fulgens*, v: *viridiceps*, sp: *spectabilis*, r: *rhami*, o: *occidentalis*, p: *pinicola*, l: *leocadiae*, co: *constantii*, pa: *pallidiceps*, lo: *longirostris*.

Figure 3. Phylogeny illustrating the divergence times for the Mountain Gems group as generated by BEAST. Bars on each node represent 95% of high posterior densities of divergence times (HPD). Letters at nodes correspond to those referred to in Table 3. Topology edited from Fig. S4. Ma = Million of years.

Figure 4. Ancestral area reconstruction generated by RASP under the Statistical Dispersal-Vicariance Algorithm. a) reconstruction of geographic areas (A. North America, B. South North-America, C. Central America, D. South America); b) reconstruction of elevation ranges (A. highlands, B. lowlands). Colors at tips of each taxa represent the current geographical area and elevation range. Letters denote main evolutionary events inferred and discussed in the text. DE: dispersal event, VE: vicariance event, * ambiguous event.

Figure S3. Phylogenetic relationships of the Trochilidae, with emphasis on the Mountain Gems group. Topologies were obtained by Bayesian Inference (BI, left) and Maximum Likelihood (ML, right) algorithms. Analyses were based on a concatenated dataset of mitochondrial and nuclear genes (3721 bp, 136 taxa). In both topologies, asterisks represent node support: posterior probabilities PP≥0.95 (BI), and bootstrap values BT≥50 (ML).

Figure S4. Phylogeny illustrating the divergence times for the Trochilidae, with emphasis on the Mountain Gems group as generated by BEAST. Bars on each node represent 95% of high posterior densities of divergence times (HPD). Numbers at nodes correspond to the divergence times referred in Table S5 (excluding Mountain Gems, Bees and *Patagona*). Ma = Million of years.

Figure 1.

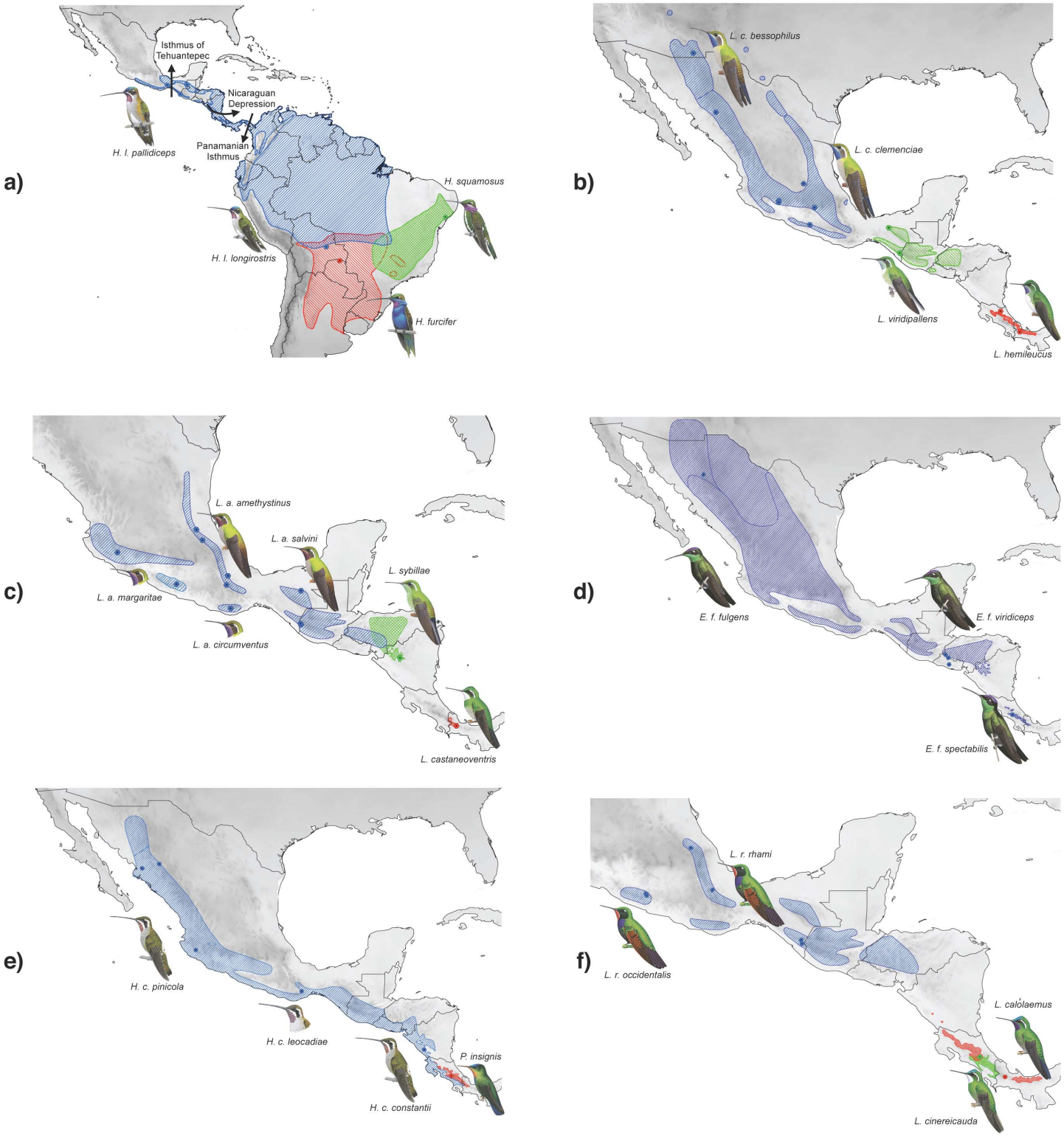

a)
Isthmus of Tehuantepec
Nicaraguan Depression
Panamanian Isthmus
H. l. pallidiceps
H. squamosus
H. l. longirostris
H. furcifer
b)
L. c. bessophilus
L. c. clemenciae
L. viridipallens
L. hemileucus
c)
L. a. amethystinus
L. a. salvini
L. sybillae
L. a. margaritae
L. a. circumventus
L. castaneoventris
d)
E. f. fulgens
E. f. viridiceps
E. f. spectabilis
e)
H. c. pinicola
H. c. leocadiae
P. insignis
H. c. constantii
f)
L. r. rhami
L. r. occidentalis
L. calolaemus
L. cinereicauda

Figure 2.

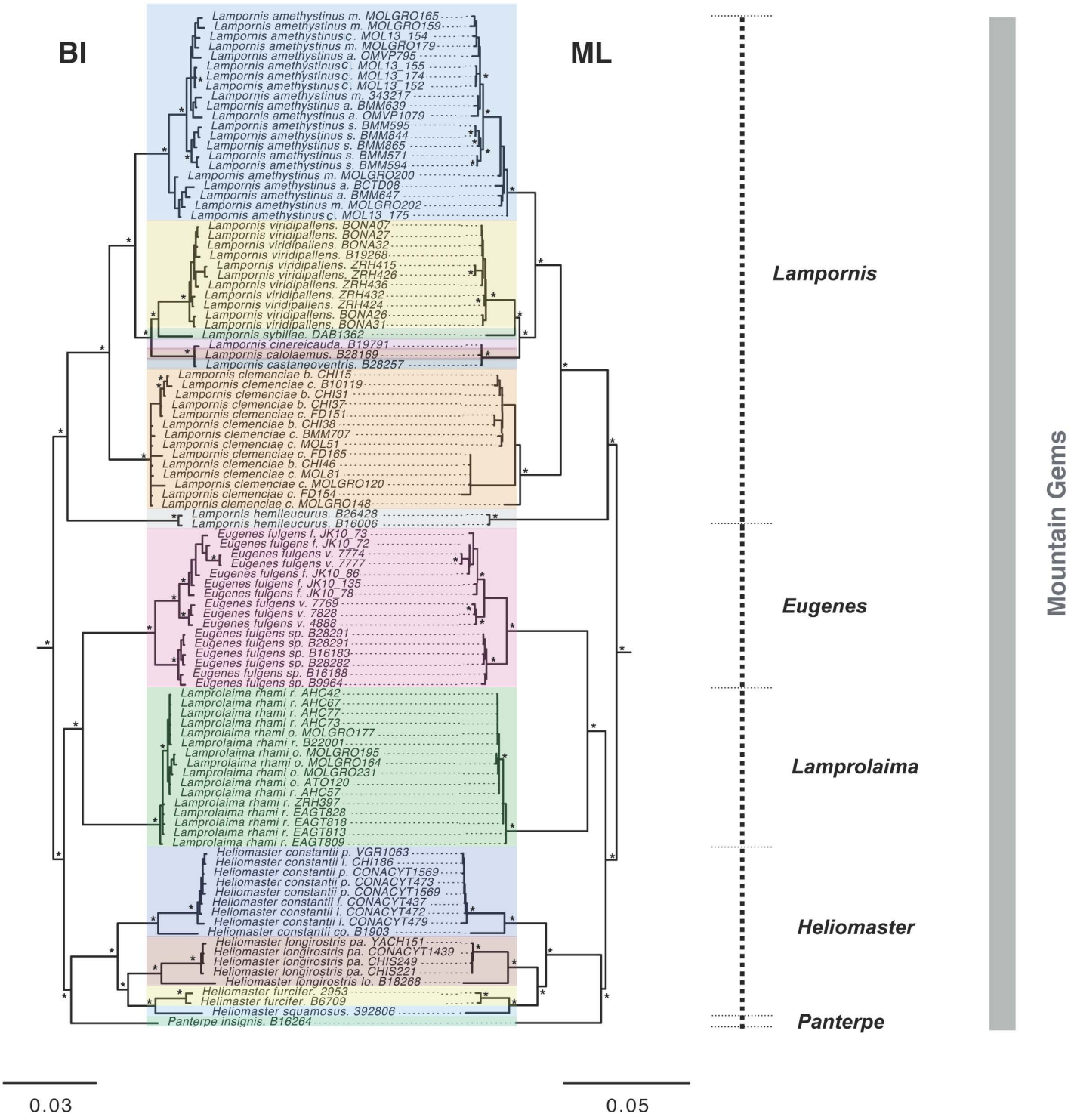
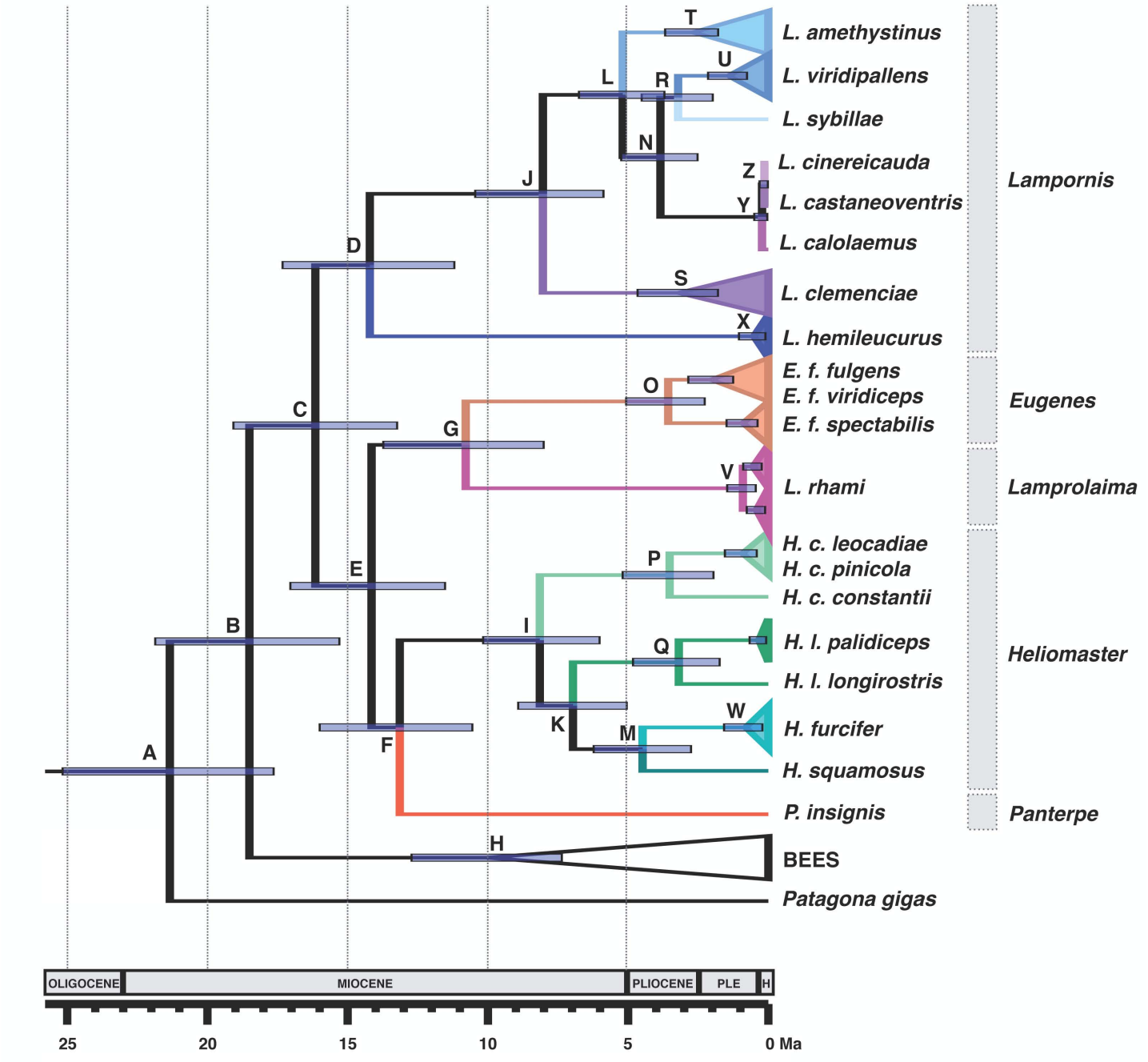

BI
ML
Lampornis amethystinus m. MOLGRO165
Lampornis amethystinus m. MOLGRO159
Lampornis amethystinus c. MOL13_154
Lampornis amethystinus m. MOLGRO179
Lampornis amethystinus a. OMVP795
Lampornis amethystinus c. MOL13_155
Lampornis amethystinus c. MOL13_174
Lampornis amethystinus c. MOL13_152
Lampornis amethystinus m. 343217
Lampornis amethystinus a. BMM639
Lampornis amethystinus a. OMVP1079
Lampornis amethystinus s. BMM595
Lampornis amethystinus s. BMM844
Lampornis amethystinus s. BMM865
Lampornis amethystinus s. BMM571
Lampornis amethystinus s. BMM594
Lampornis amethystinus m. MOLGRO200
Lampornis amethystinus a. BCTD08
Lampornis amethystinus a. BMM647
Lampornis amethystinus m. MOLGRO202
Lampornis amethystinus c. MOL13_175
Lampornis viridipallens. BONA07
Lampornis viridipallens. BONA27
Lampornis viridipallens. BONA32
Lampornis viridipallens. B19268
Lampornis viridipallens. ZRH415
Lampornis viridipallens. ZRH426
Lampornis viridipallens. ZRH436
Lampornis viridipallens. ZRH432
Lampornis viridipallens. ZRH424
Lampornis viridipallens. BONA26
Lampornis viridipallens. BONA31
Lampornis sybillae. DAB1362
Lampornis cinereicauda. B19791
Lampornis calolaemus. B28169
Lampornis castaneoventris. B28257
Lampornis clemenciae b. CHI15
Lampornis clemenciae c. B10119
Lampornis clemenciae b. CHI31
Lampornis clemenciae b. CHI37
Lampornis clemenciae c. FD151
Lampornis clemenciae b. CHI38
Lampornis clemenciae c. BMM707
Lampornis clemenciae c. MOL51
Lampornis clemenciae c. FD165
Lampornis clemenciae b. CHI46
Lampornis clemenciae c. MOL81
Lampornis clemenciae c. MOLGRO120
Lampornis clemenciae c. FD154
Lampornis clemenciae c. MOLGRO148
Lampornis hemileucurus. B26428
Lampornis hemileucurus. B16006
Eugenes fulgens f. JK10_73
Eugenes fulgens f. JK10_72
Eugenes fulgens v. 7774
Eugenes fulgens v. 7777
Eugenes fulgens f. JK10_86
Eugenes fulgens f. JK10_135
Eugenes fulgens f. JK10_78
Eugenes fulgens v. 7769
Eugenes fulgens v. 7828
Eugenes fulgens v. 4888
Eugenes fulgens sp. B28291
Eugenes fulgens sp. B28291
Eugenes fulgens sp. B16183
Eugenes fulgens sp. B28282
Eugenes fulgens sp. B16188
Eugenes fulgens sp. B9964
Lamprolaima rhami r. AHC42
Lamprolaima rhami r. AHC67
Lamprolaima rhami r. AHC77
Lamprolaima rhami r. AHC73
Lamprolaima rhami o. MOLGRO177
Lamprolaima rhami r. B22001
Lamprolaima rhami o. MOLGRO195
Lamprolaima rhami o. MOLGRO164
Lamprolaima rhami o. MOLGRO231
Lamprolaima rhami o. ATO120
Lamprolaima rhami r. AHC57
Lamprolaima rhami r. ZRH397
Lamprolaima rhami r. EAGT828
Lamprolaima rhami r. EAGT818
Lamprolaima rhami r. EAGT813
Lamprolaima rhami r. EAGT809
Heliomaster constantii p. VGR1063
Heliomaster constantii l. CHI186
Heliomaster constantii p. CONACYT1569
Heliomaster constantii p. CONACYT473
Heliomaster constantii p. CONACYT1569
Heliomaster constantii l. CONACYT437
Heliomaster constantii l. CONACYT472
Heliomaster constantii l. CONACYT479
Heliomaster constantii co. B1903
Heliomaster longirostris pa. YACH151
Heliomaster longirostris pa. CONACYT1439
Heliomaster longirostris pa. CHIS249
Heliomaster longirostris pa. CHIS221
Heliomaster longirostris lo. B18268
Heliomaster furcifer. 2953
Helimaster furcifer. B6709
Heliomaster squamosus. 392806
Panterpe insignis. B16264
Lampornis
Eugenes
Lamprolaima
Heliomaster
Panterpe
Mountain Gems
0.03
0.05

Figure 3.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A
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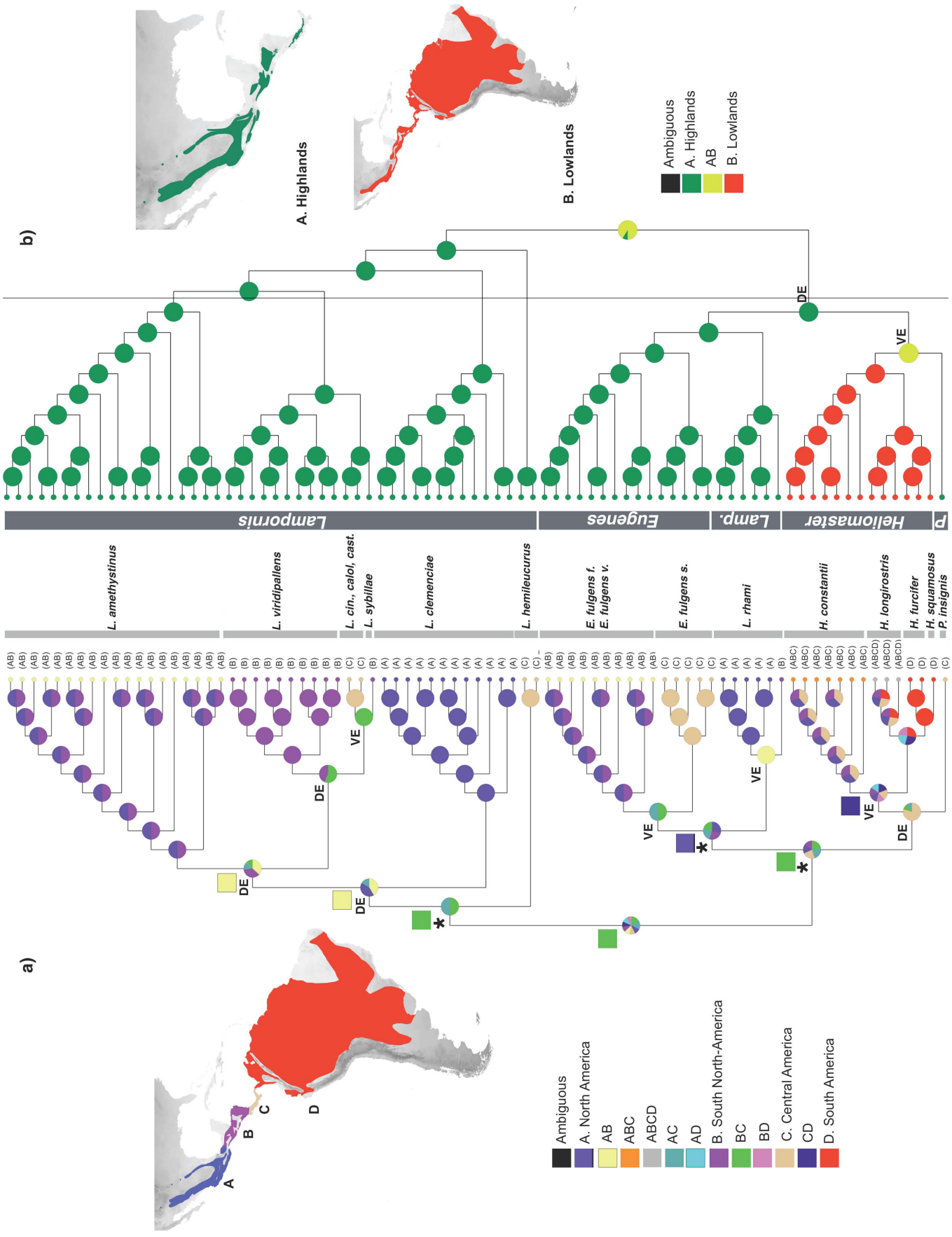
H
L. amethystinus
L. viridipallens
L. sybillae
L. cinereicauda
L. castaneoventris
L. calolaemus
L. clemenciae
L. hemileucurus
E. f. fulgens
E. f. viridiceps
E. f. spectabilis
L. rhami
H. c. leocadiae
H. c. pinicola
H. c. constantii
H. l. palidiceps
H. l. longirostris
H. furcifer
H. squamosus
P. insignis
BEES
Patagona gigas
Lampornis
Eugenes
Lamprolaima
Heliomaster
Panterpe
OLIGOCENE
MIOCENE
PLIOCENE
PLE
H
25
20
15
10
5
0 Ma

Figure 4.


a)
A
B
C
D
Ambiguous
A. North America
AB
ABC
ABCD
AC
AD
B. South North-America
BC
BD
C. Central America
CD
D. South America
b)
A. Highlands
B. Lowlands
Ambiguous
A. Highlands
AB
B. Lowlands
Lampornis
Eugenes
Lamp.
Heliomaster
P.
L. amethystinus
L. viridipallens
L. cin., calol, cast.
L. sybillae
L. clemenciae
L. hemileucurus
E. fulgens f.
E. fulgens v.
E. fulgens s.
L. rhami
H. constantii
H. longirostris
H. furcifer
H. squamosus
P. insignis
DE
VE

SUPPLEMENTARY MATERIAL S1

Collection numbers, localities, georeferences and species used in this study. We included information about the scientific biological collections that tissues were come from.

BC: Biological Collection.
UNAM: Universidad Nacional Autónoma de México, Museo de Zoología Alfonso L. Herrera.
KU: The University of Kansas, Natural History Museum.
LSU: Louisiana State University, Museum of Natural Science.
UW: University of Washington, The Burke Museum.
FMNH: Field Museum of Natural History.

Mountain Gems Group (Sequenced tissues samples for this study).

#	Collection No.	*Genus*	*Species*	*Subspecies*	ND2	ND4	BFIB	ODC	MUSK	AK1	BC	Longitude	Latitude
1	BCTD008	*Lampornis*	*L. amethystinus*	*amethystinus*	•	•	•	•	•	x	UNAM	-98.2216	20.3383
2	BMM571	*Lampornis*	*L. amethystinus*	*salvini*	x	•	x	•	•	x	UNAM	-92.0833	17.1833
3	BMM594	*Lampornis*	*L. amethystinus*	*salvini*	•	•	x	•	•	x	UNAM	-92.0833	17.1833
4	BMM595	*Lampornis*	*L. amethystinus*	*salvini*	x	x	x	•	•	x	UNAM	-92.0833	17.1833
5	BMM639	*Lampornis*	*L. amethystinus*	*amethystinus*	•	•	•	•	•	•	UNAM	-98.6066	20.985
6	BMM647	*Lampornis*	*L. amethystinus*	*amethystinus*	x	•	•	•	•	•	UNAM	-98.6066	20.985
7	BMM844	*Lampornis*	*L. amethystinus*	*salvini*	•	•	•	•	•	•	UNAM	-92.0833	15.0666
8	BMM865	*Lampornis*	*L. amethystinus*	*salvini*	•	•	•	•	•	•	UNAM	-92.1083	15.1316
9	MOL13-152	*Lampornis*	*L. amethystinus*	*circumventus*	•	•	•	•	•	•	UNAM	-96.4797	16.0891
10	MOL13-154	*Lampornis*	*L. amethystinus*	*circumventus*	•	•	•	•	•	•	UNAM	-96.4797	16.0891
11	MOL13-155	*Lampornis*	*L. amethystinus*	*circumventus*	•	•	•	•	•	•	UNAM	-96.4797	16.0891
12	MOL13-174	*Lampornis*	*L. amethystinus*	*circumventus*	•	•	•	•	•	•	UNAM	-96.4797	16.0891
13	MOL13-175	*Lampornis*	*L. amethystinus*	*circumventus*	x	•	•	•	•	x	UNAM	-96.4797	16.0891
14	MOLGRO159	*Lampornis*	*L. amethystinus*	*margaritae*	•	•	•	•	•	•	UNAM	-99.8370	17.5866
15	MOLGRO165	*Lampornis*	*L. amethystinus*	*margaritae*	•	•	•	•	•	•	UNAM	-99.8370	17.5866

#	Collection No.	*Genus*	*Species*	*Subspecies*	ND2	ND4	BFIB	ODC	MUSK	AK1	BC	Longitude	Latitude
16	MOLGRO179	*Lampornis*	*L. amethystinus*	*margaritae*	•	•	•	•	•	•	UNAM	-99.8370	17.5866
17	MOLGRO200	*Lampornis*	*L. amethystinus*	*margaritae*	•	•	•	•	•	•	UNAM	-99.8370	17.5866
18	MOLGRO202	*Lampornis*	*L. amethystinus*	*margaritae*	•	•	•	•	•	•	UNAM	-99.8370	17.5866
19	OMVP1079	*Lampornis*	*L. amethystinus*	*amethystinus*	•	•	•	•	•	x	UNAM	-96.64	18.1116
20	OMVP795	*Lampornis*	*L. amethystinus*	*amethystinus*	x	•	x	•	•	•	UNAM	-96.74	17.5433
21	BONA07	*Lampornis*	*L. viridipallens*	*ovandensis*	x	•	•	•	•	•	UNAM	-93.2	17.3138
22	BONA26	*Lampornis*	*L. viridipallens*	*ovandensis*	•	•	•	•	•	•	UNAM	-93.2	17.3138
23	BONA27	*Lampornis*	*L. viridipallens*	*ovandensis*	•	•	•	•	•	•	UNAM	-93.2	17.3138
24	BONA31	*Lampornis*	*L. viridipallens*	*ovandensis*	•	•	•	•	•	•	UNAM	-93.2	17.3138
25	BONA32	*Lampornis*	*L. viridipallens*	*ovandensis*	x	x	•	•	•	•	UNAM	-93.2	17.3138
26	ZRH415	*Lampornis*	*L. viridipallens*	*ovandensis*	•	•	•	•	•	•	UNAM	-92.3033	15.2374
27	ZRH424	*Lampornis*	*L. viridipallens*	*ovandensis*	•	•	•	•	•	x	UNAM	-92.3033	15.2374
28	ZRH426	*Lampornis*	*L. viridipallens*	*ovandensis*	•	•	•	•	•	•	UNAM	-92.3033	15.2374
29	ZRH432	*Lampornis*	*L. viridipallens*	*ovandensis*	•	•	•	•	•	•	UNAM	-92.3033	15.2374
30	ZRH436	*Lampornis*	*L. viridipallens*	*ovandensis*	•	•	•	•	•	•	UNAM	-92.3033	15.2374
31	BMM707	*Lampornis*	*L. clemenciae*	*clemenciae*	x	x	x	•	•	•	UNAM	-100.1883	20.9316
32	CHI015	*Lampornis*	*L. clemenciae*	*bessophilus*	•	x	•	•	•	•	UNAM	-107.3786	26.8380
33	CHI031	*Lampornis*	*L. clemenciae*	*bessophilus*	•	x	•	•	•	•	UNAM	-107.3786	26.8380
34	CHI037	*Lampornis*	*L. clemenciae*	*bessophilus*	•	•	•	•	•	•	UNAM	-107.3786	26.8380
35	CHI038	*Lampornis*	*L. clemenciae*	*bessophilus*	•	x	•	•	•	x	UNAM	-107.3786	26.8380
36	CHI046	*Lampornis*	*L. clemenciae*	*bessophilus*	•	•	•	•	•	•	UNAM	-107.3786	26.8380
37	FD151	*Lampornis*	*L. clemenciae*	*clemenciae*	•	•	•	•	•	•	UNAM	-99.29333	18.9716
38	FD154	*Lampornis*	*L. clemenciae*	*clemenciae*	x	•	•	•	•	•	UNAM	-99.29333	18.9716
39	FD165	*Lampornis*	*L. clemenciae*	*clemenciae*	x	x	x	•	•	•	UNAM	-99.29333	18.9716
40	MOL051	*Lampornis*	*L. clemenciae*	*clemenciae*	•	x	x	•	•	•	UNAM	-102.2319	19.5449
41	MOL081	*Lampornis*	*L. clemenciae*	*clemenciae*	x	x	•	•	•	•	UNAM	-102.2226	19.4029
42	MOLGRO120	*Lampornis*	*L. clemenciae*	*clemenciae*	•	•	•	•	•	•	UNAM	-99.67883	17.5578
43	MOLGRO148	*Lampornis*	*L. clemenciae*	*clemenciae*	•	•	•	•	•	•	UNAM	-99.67883	17.5578

#	Collection No.	*Genus*	*Species*	*Subspecies*	ND2	ND4	BFIB	ODC	MUSK	AK1	BC	Longitude	Latitude
44	AHC042	*Lamprolaima*	*L. rhami*	*rhami*	•	•	x	•	•	•	UNAM	-97.69386	19.8868
45	AHC057	*Lamprolaima*	*L. rhami*	*rhami*	•	•	x	•	•	x	UNAM	-97.6938	19.8868
46	AHC067	*Lamprolaima*	*L. rhami*	*rhami*	•	•	x	•	•	x	UNAM	-97.6938	19.8868
47	AHC073	*Lamprolaima*	*L. rhami*	*rhami*	•	•	x	•	•	•	UNAM	-97.6938	19.8868
48	AHC077	*Lamprolaima*	*L. rhami*	*rhami*	•	•	•	•	•	•	UNAM	-97.6938	19.8868
49	ATO120	*Lamprolaima*	*L. rhami*	*occidentalis*	•	•	•	•	•	•	UNAM	-99.8819	17.6761
50	EAGT809	*Lamprolaima*	*L. rhami*	*rhami*	•	x	•	•	•	•	UNAM	-92.3426	15.4262
51	EAGT813	*Lamprolaima*	*L. rhami*	*rhami*	•	x	•	•	•	•	UNAM	-92.3426	15.4262
52	EAGT818	*Lamprolaima*	*L. rhami*	*rhami*	•	•	•	•	•	x	UNAM	-92.3426	15.4262
53	EAGT828	*Lamprolaima*	*L. rhami*	*rhami*	•	•	•	•	•	•	UNAM	-92.3033	15.2374
54	MOLGRO164	*Lamprolaima*	*L. rhami*	*occidentalis*	•	•	•	•	•	•	UNAM	-99.8370	17.5866
55	MOLGRO177	*Lamprolaima*	*L. rhami*	*occidentalis*	•	•	•	•	•	•	UNAM	-99.8370	17.5866
56	MOLGRO195	*Lamprolaima*	*L. rhami*	*occidentalis*	•	•	•	•	•	•	UNAM	-99.8370	17.5866
57	MOLGRO231	*Lamprolaima*	*L. rhami*	*occidentalis*	•	•	x	•	•	•	UNAM	-99.8370	17.5866
58	ZRH397	*Lamprolaima*	*L. rhami*	*rhami*	•	•	•	•	•	•	UNAM	-92.3426	15.4262
59	4888	*Eugenes*	*E. fulgens*	*viridiceps*	•	•	•	•	•	x	KU	-89.12	14.38
60	7769	*Eugenes*	*E. fulgens*	*viridiceps*	•	•	•	•	•	•	KU	-88.84	13.6
61	7774	*Eugenes*	*E. fulgens*	*viridiceps*	•	•	•	•	•	x	KU	-88.84	13.6
62	7777	*Eugenes*	*E. fulgens*	*viridiceps*	•	•	x	•	•	x	KU	-88.84	13.6
63	7828	*Eugenes*	*E. fulgens*	*viridiceps*	•	•	•	•	•	x	KU	-88.91	14.13
64	B9964	*Eugenes*	*E. fulgens*	*spectabilis*	•	•	•	•	•	x	LSU	-83.79	9.64
65	16183	*Eugenes*	*E. fulgens*	*spectabilis*	•	•	•	•	•	•	LSU	-83.79	9.64
66	16188	*Eugenes*	*E. fulgens*	*spectabilis*	•	•	•	•	•	•	LSU	-83.79	9.64
67	28282	*Eugenes*	*E. fulgens*	*spectabilis*	•	•	•	•	•	•	LSU	-83.79	9.64
68	28291	*Eugenes*	*E. fulgens*	*spectabilis*	•	•	•	•	•	•	LSU	-83.79	9.64
69	JK10-072	*Eugenes*	*E. fulgens*	*fulgens*	•	•	•	•	•	x	UW	-108.2689	28.6329
70	JK10-073	*Eugenes*	*E. fulgens*	*fulgens*	x	•	•	•	•	•	UW	-108.2689	28.6329

#	Collection No.	*Genus*	*Species*	*Subspecies*	ND2	ND4	BFIB	ODC	MUSK	AK1	BC	Longitude	Latitude
71	JK10-078	*Eugenes*	*E. fulgens*	*fulgens*	•	•	•	•	•	•	UW	-108.2689	28.6329
72	JK10-086	*Eugenes*	*E. fulgens*	*fulgens*	•	x	•	•	•	•	UW	-108.2689	28.6329
73	JK10-135	*Eugenes*	*E. fulgens*	*fulgens*	•	•	•	•	•	•	UW	-108.2689	28.6329
74	CHI186	*Heliomaster*	*H. constantii*	*pinicola*	•	•	•	•	•	•	UNAM	-107.3085	26.6646
75	CONACYT04-037	*Heliomaster*	*H. constantii*	*leocadiae*	•	•	•	•	•	•	UNAM	-95.8296	16.4495
76	CONACYT04-072	*Heliomaster*	*H. constantii*	*leocadiae*	•	•	•	•	•	•	UNAM	-95.8296	16.4495
77	CONACYT04-073	*Heliomaster*	*H. constantii*	*leocadiae*	•	•	•	•	•	•	UNAM	-95.8296	16.44958
78	CONACYT04-079	*Heliomaster*	*H. constantii*	*leocadiae*	•	•	•	•	•	•	UNAM	-95.8296	16.4495
79	CONACYT1569	*Heliomaster*	*H. constantii*	*pinicola*	•	•	•	•	•	•	UNAM	-104.3683	19.77
80	CONACYT1573	*Heliomaster*	*H. constantii*	*pinicola*	•	•	•	•	•	•	UNAM	-104.3683	19.77
81	VGR1063	*Heliomaster*	*H. constantii*	*pinicola*	•	•	•	•	•	•	UNAM	-108.6983	26.3033
82	CHIS221	*Heliomaster*	*H. longirostris*	*palidiceps*	•	•	•	•	•	•	UNAM	-92.3372	14.9230
83	CHIS249	*Heliomaster*	*H. longirostris*	*palidiceps*	•	•	•	•	•	•	UNAM	-92.3372	14.9230
84	CONACYT1439	*Heliomaster*	*H. longirostris*	*palidiceps*	•	•	•	•	•	•	UNAM	-95.0425	16.7924
85	YACH151	*Heliomaster*	*H. longirostris*	*palidiceps*	•	•	•	•	•	•	UNAM	-90.9827	16.9058
86	2953	*Heliomaster*	*H. furcifer*		•	•	•	•	•	x	KU	-59.265	-19.5866

Mountain Gems Group (Already published in GenBank, McGuire et al. 2014).

#	Collection No.	*Genus*	*Species*	*Subspecies*	ND2	ND4	BFIB	ODC	MUSK	AK1	BC	Longitude	Latitude
87	B9964	*Eugenes*	*E. fulgens*	*spectabilis*	•	•	•	•	•	•	LSU	-83.79	9.64
88	B28291	*Eugene*	*E. fulgens*	*spectabilis*	•	•	•	•	•	•	LSU	-83.79	9.64
89	DAB1903	*Heliomaster*	*H. constantii*	*constantii*	•	•	•	•	•	•	UW	-85.958	11.76
90	B6709	*Heliomaster*	*H. furcifer*		•	•	•	•	•	•	LSU	-62.075	-16.747
91	B18268	*Heliomaster*	*H. longirostris*	*longirostris*	•	•	•	•	•	•	LSU	-62.075	-16.747
92	392806	*Heliomaster*	*H. squamosus*		•	•	•	•	•	•	FMNH	-37.385	-10.574
93	343217	*Lampornis*	*L. amethystinus*	*margaritae*	•	•	•	•	•	•	FMNH	-103.513	19.572
94	B28169	*Lampornis*	*L. calolaemus*		•	•	•	•	•	•	LSU	-82.432	8.7745
95	B28257	*Lampornis*	*L. castaneoventris*		•	•	•	•	•	•	LSU	-82.432	8.7745
96	B19791	*Lampornis*	*L. cinereicauda*		•	•	•	•	•	•	LSU	-83.6773	9.7539
97	B10119	*Lampornis*	*L. clemenciae*	*bessophilus*	•	•	•	•	•	•	LSU	-109.2317	31.6887
98	16006	*Lampornis*	*L. hemileucurus*		•	•	•	•	•	•	LSU	-84.0167	10.4735
99	26428	*Lampornis*	*L. hemileucurus*		•	•	•	•	•	•	LSU	-82.432	8.7745
100	DAB1362	*Lampornis*	*L. sybillae*		•	•	•	•	•	•	UW	-85.924	13.015
101	B19268	*Lampornis*	*L. viridipallens*	*ovandensis*	•	•	•	•	x	•	LSU		
102	B22001	*Lamprolaima*	*L. rhami*	*rhami*	•	•	•	•	•	•	LSU	-96.6456	17.856
103	B16264	*Panterpe*	*P. insignis*	*insignis*	•	•	•	•	•	•	LSU	-83.7993	9.64786

Outgroups (Sequences from GenBank, McGuire et al. 2014).

#	***Species***	**Clade/Family**	**ND2**	**ND4**	**BFIB**	**ODC**	**MUSK**	**AK1**
104	*Tilmatura dupontii*	Bees	•	•	•	•	•	•
105	*Myrtis fanny*	Bees	•	•	•	•	•	•
106	*Microstilbon burmeisteri*	Bees	•	x	•	•	•	•
107	*Calliphlox bryantae*	Bees	•	x	•	•	•	•
108	*Doricha eliza*	Bees	•	•	•	•	•	•
109	*Calypte costae*	Bees	•	x	•	•	•	•
110	*Atthis heloisa*	Bees	•	x	•	•	•	•
111	*Selasphorus ardens*	Bees	•	x	•	•	•	•
112	*Selasphorus rufus*	Bees	•	x	•	•	•	•
113	*Selasphorus sasin*	Bees	•	x	•	•	•	•
114	*Patagona gigas*	Patagona	•	x	•	•	•	•
115	*Sephanoides fernandensis*	Coquettes	•	x	•	•	•	•
116	*Lophornis delattrei*	Coquettes	•	•	•	•	•	•
117	*Discosura popelairii*	Coquettes	•	•	•	•	•	•
118	*Heliangelus micraster*	Coquettes	•	•	•	•	•	•
119	*Adelomyia melanogenys*	Coquettes	•	•	•	•	•	•
120	*Eriocnemis derbyi*	Brilliants	•	•	x	x	x	x
121	*Aglaeactis cupripennis*	Brilliants	•	•	•	•	•	•
122	*Urosticte benjamini*	Brilliants	•	•	•	•	x	•
123	*Heliodoxa gularis*	Brilliants	•	•	•	•	•	•
124	*Coeligena coeligena*	Brilliants	•	•	•	•	•	•
125	*Doryfera ludovicae*	Mangoes	•	•	•	•	•	•
126	*Colibri thalassinus*	Mangoes	•	•	•	•	x	•
127	*Anthracothorax mango*	Mangoes	•	•	•	•	•	•
128	*Eulampis jugularis*	Mangoes	•	•	•	•	•	•
129	*Eutoxeres condamini*	Hermits	•	•	•	•	•	•
130	*Phaethornis longirostris*	Hermits	•	•	•	•	•	•

#	***Species***	**Clade/Family**	**ND2**	**ND4**	**BFIB**	**ODC**	**MUSK**	**AK1**
131	*Florisuga fusca*	Topazes	•	•	•	•	•	•
132	*Topaza pella*	Topazes	•	•	•	•	•	•
133	*Hemiprocne mystacea*	Hemiprocnidae	•	•	•	•	•	•
134	*Streptoprocne zonaris*	Apodidae	•	•	•	•	•	•
135	*Aerodramus salangana*	Apodidae	•	x	•	•	x	x
136	*Aeronautes saxatalis*	Apodidae	•	x	•	•	x	x
137	*Chaetura pelagica*	Apodidae	•	•	•	•	x	•
138	*Aegotheles insignis*	Aegothelidae	•	•	•	•	•	•

SUPPLEMENTARY MATERIAL S2

Primers and PCR protocols used in this study.

Gene	Primer name	Primer sequence	References	PCR protocol		
				Denaturation	Annealing (35x)	Extension
ND2	L5219	CCCATACCCCGAAAATGATG	Sorenson *et al.*, 1999.	1x 03:00(94°C)	00:30(94°C), 00:30(54°C), 00:45(72°C)	1x 10:00(72°C)
ND2	H6313	CTCTTATTTAAGGCTTTGAAGGC				
ND4	ND4	CACCTATGACTACCAAAAGCTCATGTAGAAGC	Arévalo *et al.*, 1994.	1x 05:00(94°C)	00:30(95°C), 00:30(55°C), 00:45(72°C)	1x 07:00(72°C)
ND4	LEU	CATTACTTTTACTTGGATTTGCACCA				
AK1	AK5b^{+}	ATTGACGGCTACCCTCGCGAGGTG	Shapiro & Dumbacher, 2001.	1x 05:00(94°C)	00:45(92°C), 01:00(54°C), 01:00(72°C)	1x 07:00(72°C)
AK1	AK6c^{-}	CACCCGCCCGCTGGTCTCTCC				
BFib	BFib-17L2	TGGGAGGTGAAGCAGCTAAGAAAAACAA	Prychitko & Moore, 1997.	1x 10:00(94°C)	01:00(92°C), 01:00(50°C), 01:00(72°C)	1x 07:00(72°C)
BFib	BFib-17U2	CATCCATGCAGTTCTGGCAATTC				
MUSK	MUSK-F3	GCTGTACTTCCATGCACTACAATG	McGuire *et al.*, 2014.	1x 05:00(95°C)	00:25(95°C), 00:25(50°C), 01:00(72°C)	1x 07:00(72°C)
MUSK	MUSK-R3	ATCCTCAAATTTCCCGAATCAAG				
ODC	ODC-2F	GCGTGCAAAAGAACTTGACC	McGuire *et al.*, 2014.	1x 03:00(94°C)	00:30(94°C), 00:30(57°C), 00:30(72°C)	1x 05:00(72°C)
ODC	ODC-2R	AGCCACCACCAATATCAAGC				

Arévalo, E., Davis, S.K. & Sites, J.W. (1994) Mitochondrial DNA sequence divergence and phylogenetic relationships among eight chromosome races of the Sceloporus grammicus complex (Phrynosomatidae) in central Mexico. *Syst Biol*, **43**, 387-418.

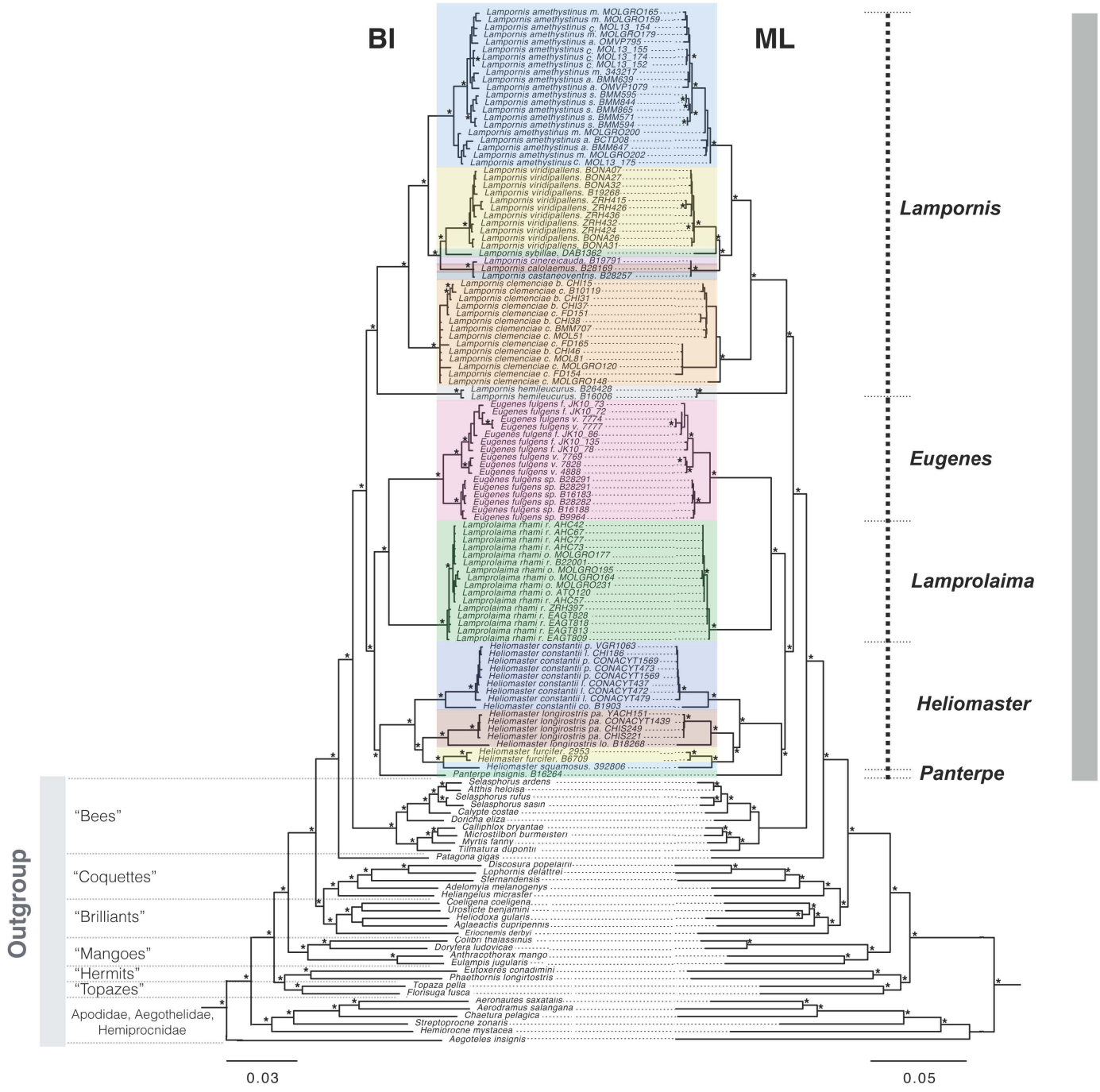
McGuire, J.A., Witt, C.C., Remsen Jr, J., Corl, A., Rabosky, D.L., Altshuler, D.L. & Dudley, R. (2014) Molecular phylogenetics and the diversification of hummingbirds. *Current Biology*, **24**, 910-916.

Prychitko, T.M. & Moore, W.S. (1997) The utility of DNA sequences of an intron from the β-fibrinogen gene in phylogenetic analysis of woodpeckers (Aves: Picidae). *Molecular Phylogenetics and Evolution*, **8**, 193-204.

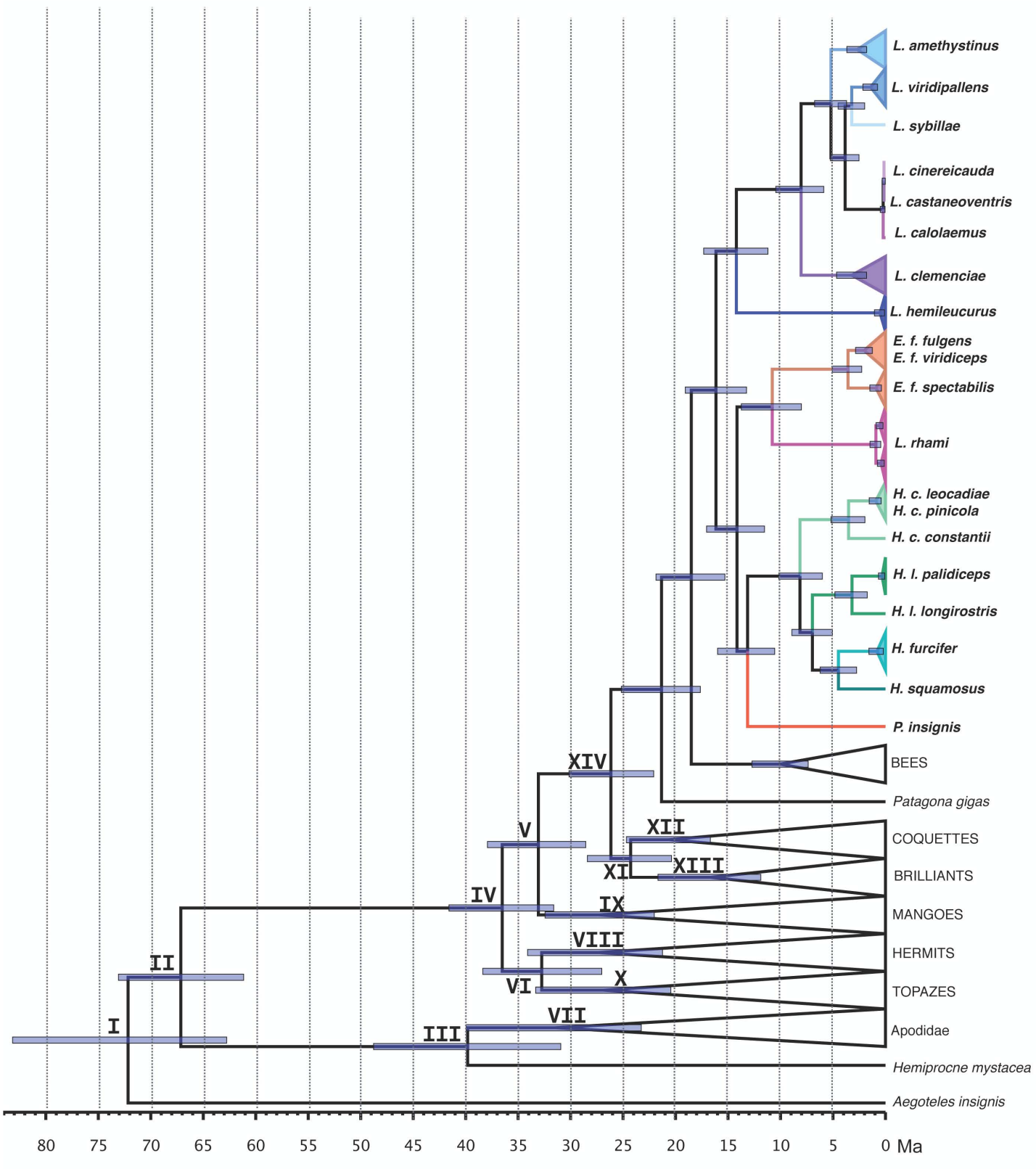
Shapiro, L.H. & Dumbacher, J.P. (2001) Adenylate kinase intron 5: a new nuclear locus for avian systematics. *The Auk*, **118**, 248-255.

Sorenson, M.D., Ast, J.C., Dimcheff, D.E., Yuri, T. & Mindell, D.P. (1999) Primers for a PCR-based approach to mitochondrial genome sequencing in birds and other vertebrates. *Molecular Phylogenetics and Evolution*, **12**, 105-14.

SUPPLEMENTARY MATERIAL S3



SUPPLEMENTARY MATERIAL S4



ARTÍCULO 2. REEVALUACIÓN TAXONÓMICA DEL COMPLEJO *Eugenes fulgens* (Aves: Trochilidae).

Zamudio-Beltrán, L. E. & Hernández-Baños, B. E. (2015).

Resumen.- El estatus taxonómico de las subespecies en el estudio de la sistemática es un tema controversial. Estudios recientes han empleando secuencias de ADN para evaluar el estatus de las subespecies en complejos de especies**,** además de establecer los límites entre ellas. En el presente artículo analizamos las relaciones filogenéticas, el estatus taxonómico de las subespecies propuestas, y los límites de especies para el género monotípico de colibrí *Eugenes* (*E. fulgens* y sus subespecies reconocidas: *E. f. fulgens*, *E. f. viridiecps*, y *E. f. spectabilis*), empleando marcadores nucleares (Beta Fibrinogeno *BFib*, Ornitina Descarboxilasa *ODC*, y Receptor Tirosina Quinasa de Músculo Esquelético *MUSK*), así como marcadores mitocondriales (subunidad 2 de NADH deshidrogenasa *ND2*, subunidad 4 de NADH deshidrogenasa *ND4*, y Región Control *CR*). Llevamos a cabo análisis filogenéticos y análisis de límites de especies (Bayesian Phylogenetics and Phylogeography BPP), y encontramos evidencia de diferenciación genética entre los tres grupos. Se sugiere la existencia de dos especies crípticas (*E. fulgens* y *E. viridiceps*) y el reconocimiento de una especie fenotípicamente diferenciada (*E. spectabilis*). Nuestros análisis muestran que *E. fulgens* y *E. viridiceps* son dos grupos estrechamente relacionados, y estos a su vez se relacionan con *E. spectabilis*.

Palabras clave: Colibríes, *Eugenes fulgens*, *viridiceps*, *spectabilis*, sistemática, límites de especies.

A multilocus analysis provides evidence for more than one species within *Eugenes fulgens* (Aves: Trochilidae)

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ABSTRACT

The status of subspecies in systematic zoology is the focus of controversy. Recent studies use DNA sequences to evaluate the status of subspecies within species complexes and to recognize and delimit species. Here, we assessed the phylogenetic relationships, the taxonomic status of the proposed subspecies and the species limits of the monotypic hummingbird genus *Eugenes* (*fulgens*, *viridiceps* and *spectabilis*), by using nuclear and mitochondrial markers. We found genetic differences between these group, suggesting the existence of two cryptic species (*E. fulgens* and *E. viridiceps*) and one phenotypically differentiated species (*E. spectabilis*). Our analyses show that the *E. viridiceps* and *E. fulgens* groups are more closely related between them than with *E. spectabilis*.

Keywords: Hummingbirds; *Eugenes fulgens*; *viridiceps*; *spectabilis*; systematics; species limits; speciation.

1. Introduction

For many years, there has been an important debate about the use of the subspecies concept to describe geographic variation in birds (Patten, 2010; Zink, 2004). The use of different species concepts has been one of the main reasons for this controversy. Whereas some authors defend the application of the biological species concept, others argue that taxonomic decisions should be done exclusively under a framework of independence in evolutionary histories (James, 2010).

In an influential paper, De Queiroz (2007) proposed that different types of data (morphological, ethological, ecological, molecular, etc.) are needed to operationally determine if the studied lineages are evolving separately, i.e. if they can be considered different species. De Queiroz (2007) also argues that species differentiation is affected by the time elapsed since the diversification event, a problem that must be taken into account in species delimitation studies.

Much research in recent years has focused on implementing molecular tools to support morphological variation and geographic isolation in taxonomic evaluations of bird species complexes (Navarro-Sigüenza et al, 2001; Cadena et al., 2007; Bnaccorso et al., 2008). The systematic situation of several bird families remains unsolved, and such is the case of the Trochilidae. The number of studies describing the phylogenetic relationships of different taxa within this family has increased recently (e.g. García-Moreno et al., 2006; McGuire et al., 2007, 2009, 2014), but only a few of them deal with the taxonomic status of proposed subspecies using molecular markers (Arbeláez-Cortés and Navarro-Sigüenza, 2013; Cortés-Rodríguez et al., 2008; García-Deras et al., 2008). The monotypic genus *Eugenes* (Gould 1861) represents an interesting model to explore this basic taxonomic issue.

Eugenes fulgens (Swainson, 1827) is a sexually dimorphic hummingbird species that shows phenotypic differences across its geographic range, that goes from southeastern USA to the Central American highlands (Howell and Webb, 1995; Schuchmann, 1999). In particular, differences in color and body size between populations have led to four different hypotheses on the subdivision of *E. fulgens*. The first hypothesis considers that *E. fulgens* consists of two subspecies: *E. f. fulgens* (Swainson 1827), distributed from southeastern USA to Nicaragua highlands, and *E. f. spectabilis* (Lawrence, 1867), distributed in the highlands of Costa Rica and Panama (Johnsgard, 1984; Schuchmann, 1999). The second hypothesis proposes a third subspecies, *E. f. viridiceps* (Boucard, 1878, cited in Schuchmann, 1999), distributed from the highlands of Chiapas to Nicaragua (Peters, 1945). Other researchers propose the existence of more than one species within *E. fulgens*. Renner and Schuchmann (2004) propose two species, *E. fulgens* (including *viridiceps*) and *E. spectabilis* whereas a fourth hypothesis suggests the existence of three full species: *E. fulgens, E. viridiceps* and *E. spectabilis* (Navarro-Sigüenza and Peterson, 2004; Ridgway, 1911).

According to Renner and Schuchmann (2014), the males of the original *fulgens* and *viridiceps* subespecies have the feathers of the chest, breast and abdomen completely black, in contrast to those of *spectabilis* which are green (Fig. 1-II). Additionally, Schuchmann (1999) mentions that the gorget of *fulgens* and *viridiceps* is green, while that of *spectabilis* is blue green (Fig. 1-II). The females of *spectabilis* are in general more yellow-green than those of *fulgens* and *viridiceps*, and the tips of the rectrices of *fulgens* and *viridiceps* are less white (Renner and Schuchmann, 2014). No colour differences between *viridiceps* and *fulgens* have been reported and their separation is based in their non-overlapping geographic ranges (Navarro-Sigüenza and Peterson, 2004).

78 The purpose of the present work is to test the above mentioned hypotheses by
79 evaluating the phylogenetic relationships between the subspecies proposed for *E.*
80 *fulgens*, and to provide its systematic reevaluation using a multilocus molecular dataset.
81
82 **2. Methods**
83 *2.1. Taxon sampling and laboratory procedures*
84 We used tissues samples from *E. fulgens* voucher specimens from the collections
85 of the Museo de Zoología Alfonso L. Herrera (Universidad Nacional Autónoma de
86 México), Natural History Museum (University of Kansas), The Burke Museum
87 (University of Washington), and Museum of Natural Science (Louisiana State
88 University).
89 We isolated genomic DNA from tissues using Qiagen DNAeasy™ kit (Qiagen
90 Inc., Valencia, CA, USA) following the manufacturer's protocol. We sequenced 34
91 individuals collected in 16 localities throughout the entire range of *E. fulgens* (Table 1;
92 Fig. 1-I), including individuals representing three geographic regions: (1) northern and
93 central Mexico, (2) southern Mexico and northern Central America, and (3) southern
94 Central America. We defined these geographic regions according to the three
95 subspecies proposed for this complex (*fulgens, viridiceps* and *spectabilis*). We included
96 five samples from *Lamprolaima rhami*, the sister group of *Eugenes* (García-Moreno et
97 al., 2006), and two samples from *Heliomaster constantii* as outgroup (McGuire et al.,
98 2014).
99 We obtained molecular data of two types: maternally inherited mitochondrial
100 DNA (ND2, ND4 and CR) and biparentally inherited nuclear DNA (BFib, ODC and
101 MUSK). We amplified these six molecular markers via polymerase chain reaction
102 (PCR) in 12.5 μl reactions. For the amplification of NADH dehydrogenase subunit 2

103 (ND2) we used primers L5219 and H6313 (Sorenson et al., 1999); we amplified the
104 NADH dehydrogenase subunit 4 (ND4) using primers ND4 and LEU (Arévalo et al.,
105 1994), and the mtDNA Control Region (CR) using primers ARCOIF and ARCOIR
106 (González et al., 2011). For the amplification of beta-fibrinogen intron 7 (BFib) we used
107 the primers BFibU and BFibL; we amplified the ornithine decarboxylase gene (ODC)
108 with primers ODC2-F and ODC2-R, and the z-linked muscle skeletal receptor tyrosine
109 gene (MUSK) by using MUSK R3 and MUSK F3 (McGuire et al., 2007, 2014). We
110 used the following annealing temperatures: 54°C (ND2), 55°C (ND4), 50°C (CR, BFib,
111 MUSK), and 57°C (ODC). PCR products were visualized on a 1% agarose gel. DNA
112 sequencing was performed in the High-Throughput Genomics Unit Service of the
113 University of Washington. We edited and aligned chromatograms with the Sequencher
114 v4.8 software (GeneCodes Corporation, Ann Arbor, MI). We deposited all sequences in
115 the GenBank under accession numbers KR149831-KR150022.
116
117 *2.2 Phylogenetic analyses*
118 We performed a multilocus analysis using a concatenated dataset of 3721 bp
119 distributed among genes as follows: ND2: 881 bp; ND4: 484 bp; CR: 501 bp; BFib: 746
120 bp; ODC: 568 bp, and MUSK: 541 bp. We obtained 246 sequences representing 89 %
121 of the ideal total data, as we could not obtained some reliable sequences form some
122 individuals. We estimated the best-fitting model of molecular evolution for each
123 molecular marker with the jModeltest 0.1.1 software (Posada, 2008), and conducted the
124 analyses by using the Akaike Information Criterion AIC (Akaike, 1973). We obtained
125 the phylogenetic tree with the Mr. Bayes v3.0 software (Huelsenbeck and Ronquist,
126 2002), by running four simultaneous chains for each Monte Carlo Markov Chain
127 analysis for 10 million generations, sampling every 500 generations. The number of

128 burn-in was determined by using Tracer v1.5 (Rambaut and Drummond, 2009), and a
129 majority rule consensus was calculated.
130 Finally, we used MEGA v5.05 (Tamura et al., 2007) to estimate the pairwise
131 genetic distances between the proposed subspecies from each type of molecular marker
132 (mitochondrial *vs.* nuclear).
133
134 *2.3. Tests of species delimitation*
135 We used the multilocus dataset to assess the limits between the three groups
136 (*fulgens*, *viridiceps*, *spectabilis*). We used a coalescent approach implemented in the
137 Bayesian Phylogenetics and Phylogeography software (BPP; Rannala and Yang 2003;
138 Yang and Rannala, 2010). We estimated a species tree topology and the uncertainty
139 associated to each group with the *BEAST software (Drummond and Rambaut, 2007;
140 Drummond et al., 2012; Heled and Drummond, 2010). We assigned the three
141 recognized subspecies as OTUs (*fulgens*, *viridices* and *spectabilis*) in the species tree
142 estimation approach. We ran 100 million generations, sampling every 10 thousand, and
143 discarding the first 20% as burn-in. The convergence was visualized in the program
144 Tracer v1.5. This tree was used as guide tree in BPP analysis (three species hypothesis:
145 *fulgens*, *viridiceps* and *spectabilis*). For BPP runs, we used the graphics user interface
146 plataform called BPPX (http://abacus.gene.ucl.ac.uk/software) and the command line
147 interface.
148 First, we performed multiple analyses using both algorithms (0, 1) with different
149 seed numbers, and changing parameters ε and prior τ, as suggested by Yang and
150 Rannala (2010). The posterior probability values obtained showed consistency between
151 runs. For the next analyses we used algorithm 0, with values of $\varepsilon = 5$ and prior $\tau = 10$
152 25000.

153 We conducted the first set of analyses using parameter finetune = 1. This setting
154 implies that the program makes automatic adjustments to prior parameters. As it has
155 been reported that different values of θ can result in different posterior probabilities for
156 the same guide tree (Leaché et al., 2010; McKay et al., 2013), we evaluated the results
157 using three different values of θ: 0.01, 0.001, and 0.0001. For the second set of analyses
158 we used the finetune parameters estimated before and the same θ values (0.01, 0.001,
159 0.0001). For each analysis, we ran the reversible-jump Markov chain Monte Carlo
160 algorithm (rjMCMC) for 100 thousand generations, sampling every 5, and discarding
161 50 thousand generations as burn-in period. The delimitation analysis performed in BPP
162 estimates the posterior distribution for species delimitation models, and assigns values
163 of speciation probabilities in the guide tree (Rannala and Yang, 2003; Yang and
164 Rannala, 2010).
165
166 **3. Results**
167 We obtained the following best-fit models for each molecular marker: TIM3+I
168 (ND2), HKY+G (ND4), HKY+I+G (CR), TPM2uf (BFib), TPM1uf (ODC) and HKY
169 (MUSK).
170
171 *3.1 Phylogenetic analyses*
172 In Fig. 1-III we show the phylogenetic tree resulting from the multilocus dataset
173 analysis under the Bayesian inference criterion. The analysis revealed a well-supported
174 topology for *E. fulgens*. We recovered two main monophyletic groups (A+B and C in
175 Fig. 1-III). One group (A+B) contains the individuals from the original *fulgens* and
176 *viridiceps* subspecies, and the second group (C) corresponds to the *spectabilis*
177 subspecies. The *spectabilis* subspecies form a monophyletic group with high posterior

178 probability values. Within the group formed by *fulgens* and *viridiceps*, all *fulgens*
179 individuals are nested in a monophyletic group, whereas the *viridiceps* individuals are
180 grouped in different clades, three of which have high posterior probability values (the
181 clade formed by three individuals from Chiapas and one specimen from El Salvador,
182 and two clades formed by two and three individuals from El Salvador; see Fig. 1-I). A
183 group that includes the above mentioned clade of two individuals from El Salvador plus
184 one individual from Chiapas (MOL13-017) is closely related to the monophyletic
185 *fulgens* clade.

186 We present the pairwise genetic distances in Table 2. Both mitochondrial and
187 nuclear markers showed significant genetic differentiation between *fulgens*, *viridiceps*
188 and *spectabilis*, although, as predicted, genetic distances were lower for nuclear than for
189 mitochondrial markers (Table 2). The genetic differentiation values between *fulgens*
190 and *viridiceps* were lower than those between *spectabilis* and *fulgens*, and between
191 *spectabilis* and *viridiceps* (Table 2).

192

193 *3.2 Species delimitation*

194 We present the species tree obtained with *BEAST in Figure 3a. The topology
195 and posterior probability values support the independence of the three different lineages
196 within the *E. fulgens* complex, with a clade formed by *fulgens* and *viridiceps* and
197 *spectabilis* as the sister group.

198 In Fig. 2b and c we show the Bayesian species delimitation results using BPP. In
199 all but one case, the speciation probabilities values for each node were 1.0; the only
200 exception (0.77 for the clade formed by *fulgens* and *viridiceps*) occurred when we
201 performed the analysis using $\theta = 0.0001$ and the program estimated the finetune

parameters (Figure 2b). The first analyses performed to confirm consistency between runs also resulted in probabilities of speciation values of 1.0 for each node.

4. Discussion

We provide multiple evidence (multilocus phylogenetic reconstruction, genetic distances, and species delimitation analyses) indicating that the *Eugenes fulgens* complex should be considered a species complex, not a taxa with different subspecies. Our multilocus evaluation of the phylogenetic relationships between the three proposed subspecies of *E. fulgens* unveiled the presence of two independent lineages within this group: *fulgens* and *spectabilis* (Fig. 1-III); this result was supported by both mitochondrial and nuclear molecular markers. However, the genetic distances values (Table 2) suggest a degree of differentiation between *fulgens* and *viridiceps* that could be evidence of incomplete lineage sorting (Peters et al., 2007). Furthermore, the Bayesian species delimitation analyses (Fig. 2) provided compelling evidence for the existence of three species, corresponding to each of three groups traditionally considered as subspecies. Overall, we think that our results support the proposal that the *E. fulgens* complex is formed by three full species (Navarro-Sigüenza and Peterson, 2004; Ridgway, 1911).

The separation of *spectabilis* as a distinct species is not surprising considering its clear colour differences (see Introduction), together with the results of a recent morphometric study of the complex across its geographical range that showed differences between *spectabilis* and the rest of the individuals (Tovilla-Sierra, 2012), the absence of colour differences between *fulgens* and *viridiceps* suggests that these could be cryptic species. It will be interesting to perform detailed morphological studies

of *fulgens* and *viridiceps* as these two lineages appear to be the product of a recent diversification event.

In conclusion, our results suggest that *Eugenes fulgens* is a species complex composed of three different species corresponding to the three subspecies previously considered by some authors (see Introduction); two of these lineages (*fulgens* and *viridiceps*) are apparently cryptic species as no morphological or colour difference between them has been reported, while the third lineage (*spectabilis*) is clearly distinct from the other two. Thus, our results have a considerable impact on the systematics of the genus (considered until now as monotypic) and could have conservation implications since the distribution range of each of the lineages distinguished is much smaller than that of the genus.

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251

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345

Tables

Table 1. List of individuals sequenced, localities and georeferences. The numbers in column # correspond to the localities mapped in Figure 1.

#	Taxon (subspecies)	Collection number	Country	State/Province	Latitude	Longitude
1	*E. f. fulgens*	jk10-72, 278, 86, 135, 136	MEXICO	Chihuahua	28.63	-108.26
2		mm634, 639, 649, 651, 719	MEXICO	Nuevo Leon	24.88	-100.22
3		jk03-198, 271-273, 350	MEXICO	Jalisco	21.86	-103.86
4		BMM631, 644, 645, 777, MFOR09	MEXICO	Hidalgo	21.00	-98.72
5		AMAQ03, 05, 07, 20	MEXICO	Morelos	18.97	-99.03
6		jk04-125, 134, 170, 175, 342	MEXICO	Guerrero	17.67	-99.88
7		brb1110, 1111, 1136	MEXICO	Oaxaca	17.32	96.46
8	*E. f. viridiceps*	MOL13-04, 06, 16, 17, 70	MEXICO	Chiapas	16.72	-92.69
9		gav2374, jk02-085, 077	GUATEMALA	Quezaltenango	14.71	-91.52
10		4888	EL SALVADOR	Chalatenango	14.38	-89.12
11		7828	EL SALVADOR	Chalatenango	14.13	-88.91
12		7766, 7769, 7774, 7777	EL SALVADOR	San Vicente	13.6	-88.84
13	*E. f. spectabilis*	9950, 9953, 9964, 9977, 9978, 9979, 9988, 16183, 16188	COSTA RICA	San Jose	9.64	-83.79
14		28254, 28262	PANAMA	Chiriquí	8.89	-82.61
15		28282	PANAMA	Chiriquí	8.84	-82.53
16		28291	PANAMA	Chiriquí	8.77	-82.43

Table 2. Pairwise genetic distances among groups in *Eugenes* complex. a) Mitochondrial markers, b) Nuclear markers.

	fulgens	*viridiceps*	*spectabilis*	*L_rhami*
Panel (a)				
fulgens				
viridiceps	0.008**			
spectabilis	0.060*	0.061*		
L_rhami	0.246*	0.248*	0.248*	
H_constantii	0.316*	0.317*	0.296*	0.211*
Panel (b)				
fulgens				
viridiceps	0.000**			
spectabilis	0.002**	0.002**		
L_rhami	0.009**	0.009**	0.011**	
H_constantii	0.011**	0.011**	0.014**	0.011**

* $P < 0.05$.
** $P < 0.005$.

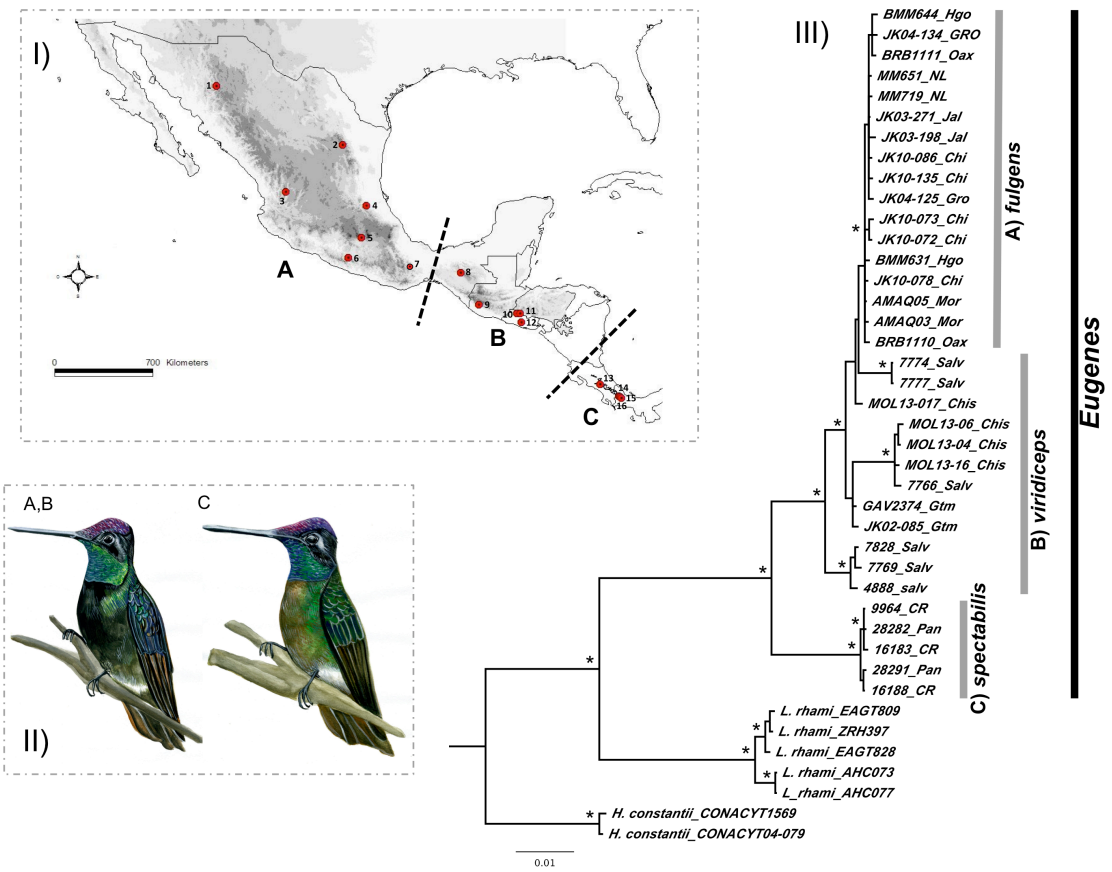
352 **Figure captions**

353 Figure 1. I) Sampled localities for this study (see Table 1). II) The morphological
354 variation is illustrated according with previous reported descriptions; the first morpho
355 corresponds to fulgens and viridiceps subspecies (A, B), and the second one
356 corresponds to spectabilis subspecies (C). III) Phylogenetic Bayesian Inference
357 reconstruction from 34 individuals from *Eugenes fulgens* complex using mitochondrial
358 and nuclear markers (ND2, ND4, RC, Bfib, MUSK, and ODC). Posterior probabilities
359 P≥0.95 are shown (*). Above right is represented the main different groups recovered
360 on the phylogenetic reconstruction acording to their geographic distribution and the
361 subspecies proposed (A: *fulgens*, B: *viridicpes*, C: *spectabilis*).

362

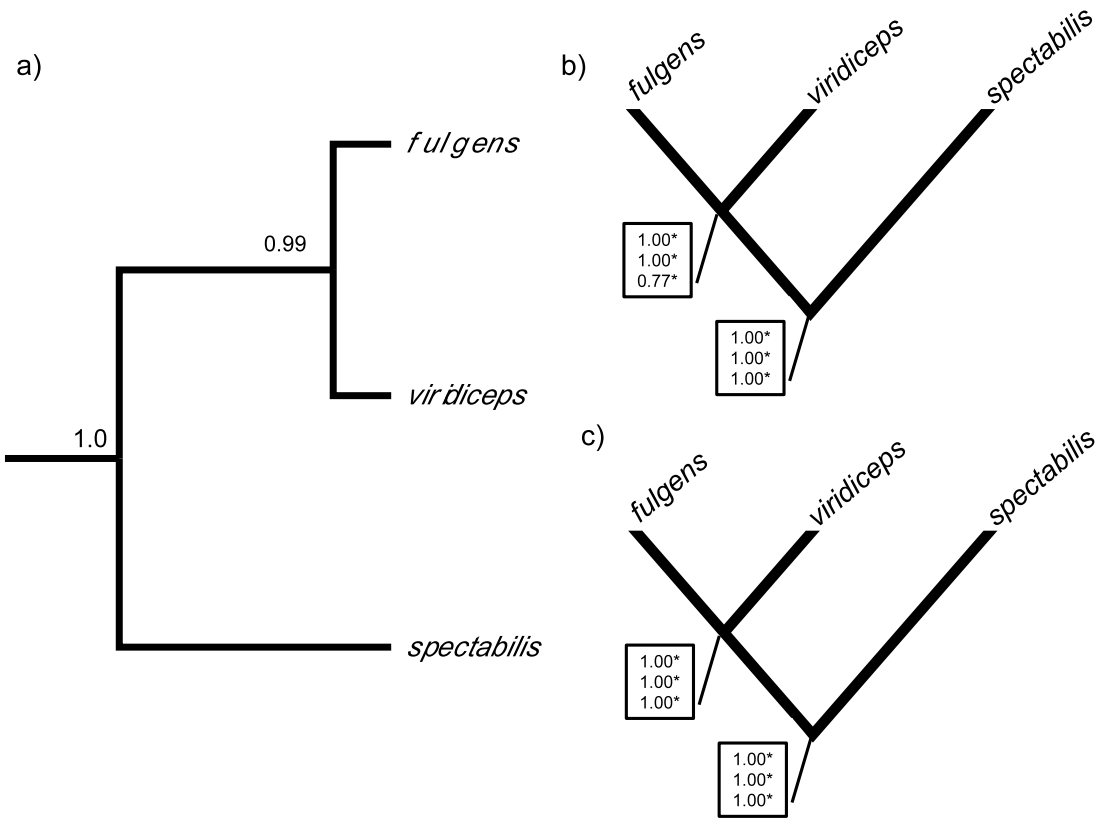
363 Figure 2. a) Bayesian species tree topology and posterior probability values using
364 *BEAST. b) Bayesian species delimitation results (finetune=1; top, θ=0.01; middle,
365 θ=0.001; bottom, θ=0.0001). c) Bayesian species delimitation results (finetune=0,
366 parameters estimated previously; top, θ=0.01; middle, θ=0.001; bottom, θ=0.0001).

367


I)
A
B
C
0
700 Kilometers
A,B
C
II)
III)
BMM644_Hgo
JK04-134_GRO
BRB1111_Oax
MM651_NL
MM719_NL
JK03-271_Jal
JK03-198_Jal
JK10-086_Chi
JK10-135_Chi
JK04-125_Gro
JK10-073_Chi
JK10-072_Chi
BMM631_Hgo
JK10-078_Chi
AMAQ05_Mor
AMAQ03_Mor
BRB1110_Oax
7774_Salv
7777_Salv
MOL13-017_Chis
MOL13-06_Chis
MOL13-04_Chis
MOL13-16_Chis
7766_Salv
GAV2374_Gtm
JK02-085_Gtm
7828_Salv
7769_Salv
4888_salv
9964_CR
28282_Pan
16183_CR
28291_Pan
16188_CR
L. rhami_EAGT809
L. rhami_ZRH397
L. rhami_EAGT828
L. rhami_AHC073
L_rhami_AHC077
H. constantii_CONACYT1569
H. constantii_CONACYT04-079
0.01
A) fulgens
B) viridiceps
C) spectabilis
Eugenes

368

369


a)
fulgens
viridiceps
spectabilis
0.99
1.0
b)
fulgens
viridiceps
spectabilis
1.00*
1.00*
0.77*
1.00*
1.00*
1.00*
c)
fulgens
viridiceps
spectabilis
1.00*
1.00*
1.00*
1.00*
1.00*
1.00*

370

ARTÍCULO 3. VARIACIÓN GENÉTICA Y MORFOLÓGICA DEL COLIBRÍ MAGNÍFICO *Eugenes fulgens* (*E. f. fulgens* and *E. f. viridiceps*, Aves: Trochilidae)

Zamudio-Beltrán, L. E., Ornelas, F., Hernández-Baños, B. E.

Resumen.- Mesoamérica es una región biogeográfica con uno de los niveles más altos de biodiversidad de América. La variación genética y los estudios filogeográficos han sido cruciales para entender los mecanismos que promueven la especiación en esta región. En este estudio analizamos la variación genética y filogeográfica, junto con la variación morfológica y la historia demográfica del complejo del colibrí magnífico *Eugenes fulgens*. Esta especie de amplia distribución geográfica habita en las tierras altas de México y el norte de América Central. Recientemente este complejo fue reevaluado taxonómicamente, y se distinguen dos subespecies como evidencia de la variación geográfica de esta especie (*E. f. fulgens y E. f. viridiceps*). La distribución geográfica de estas dos subespecies está definida por la presencia de una barrera geográfica: el Istmo de Tehuantepec (oeste: *fulgens*, y este: *viridiceps*). Analizamos la variación genética mediante el uso de ADN mitocondrial (NADH deshidrogenasa subunidad 2 ND2, y Región Control CR) y nuclear (microsatélites), para 129 y 85 individuos, respectivamente. También estudiamos la variación morfológica en 465 especímenes, se infirió la historia demográfica, se obtuvieron estimaciones de tiempos de divergencia y se construyeron modelos de proyecciones ancestrales para el complejo. Nuestros resultados sugieren que el complejo *Eugenes fulgens* está conformado por dos grupos diferenciados genéticamente, con sutiles diferencias morfológicas. Los resultados de historia demográfica bajo un modelo de expansión, las estimaciones de tiempos de divergencia y las proyecciones ancestrales confirman que esta separación se debe a un evento de especiación alopátrica a ambos lados del Istmo de Tehuantepec, debido a las fluctuaciones climáticas durante el Pleistoceno.

Palabras clave: *Eugenes fulgens*, Trochilidae, colibríes, filogeografía, Mesoamérica.

ORIGINAL ARTICLE

Genetic and morphological variation of the Magnificent Hummingbird *Eugenes fulgens* complex (*E. f. fulgens* and *E. f. viridiceps*, Aves: Trochilidae)

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Keywords: Eugenes fulgens, Trochilidae, Hummingbirds, Phylogeography, Mesoamerica.

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ABSTRACT

Mesoamerica is a biogeographic region with one of the highest levels of biodiversity in America. Genetic variation and phylogeographic studies have been crucial to understand the mechanisms promoting speciation in this region. Here we performed genetic variation and phylogeographic analyses, and evaluated the morphological differentiation and demographic history of the Magnificent Hummingbird, *Eugenes fulgens,* complex. This widely distributed species inhabits the highlands of Mexico and northern Central America. This complex was recently taxonomically reevaluated and now comprises only two subspecies (*E. f. fulgens* and *E. f. viridiceps*). Their geographic distribution is divided by the Isthmus of Tehuantepec (west region: *fulgens*, and east region: *viridiceps*). We analyzed the genetic variation of this complex by surveying the mitochondrial DNA (NADH dehydrogenase subunit 2 ND2, and Control Region CR) and nuclear DNA (microsatellites) variation of 129 individuals and 85 individuals, respectively. We also analyzed the morphological variation from 465 voucher specimens, inferred the demographic history, estimated divergence times and carried out projections of ancestral niche models for this complex. Our results suggest that *Eugenes fulgens* is conformed by two genetically differentiated groups with subtle morphological differences. Further demographic, divergence times and niche analyses confirmed that these groups diverged through allopatric speciation across the Isthmus of Tehuantepec due to Pleistocene climatic fluctuations.

1. Introduction

Studies of genetic variation and phylogeography are key tools to understanding how populations are geographically structured and shaped through time (Avise, 1998), representing the first steps for species delimitation (Camargo et al., 2010; Fraser and Bernatchez, 2001). Inferences regarding species boundaries based on genetic data alone are likely insufficient (Carstens et al., 2013). An alternative approach used by several phylogeographers is to integrate, if applicable, behavioral, morphological and environmental variation and species distribution modeling in the context of species delimitation from genetic data (e.g., González et al., 2011; Rodríguez-Gómez and Ornelas, 2015; Ornelas et al., 2016). When divergence patterns from these kinds of data and methods of analysis are not fully congruent with those based on genetic data, it is reasonable to exercise caution in delimiting evolutionary lineages while allowing the possibility of phenotypically cryptic species (Carstens et al., 2013). The incorporation of different methods into phylogeographic studies is thus helping to evaluate historical patterns of diversification more accurately (Latta, 2004; Manel et al., 2003), and to more closely understand the role of different evolutionary forces in population dynamics and species formation (Avise, 2000; Hewitt, 2001; Carstens et al., 2013).

In the Mesoamerican highlands, phylogeographic studies in birds have shown two general patterns of diversification, with either high levels of variation and geographic structure linked to old separation and geographic isolation (Bonaccorso et al., 2008; McCormack et al., 2008; Arbeláez-Cortés et al., 2010; Barrera-Guzmán et al., 2012; Maldonado-Sánchez et al., 2016; Ortíz-Ramírez et al., 2016) or low levels of genetic differentiation and geographic structure due to higher levels of gene flow and population connection during recent events of expansion and secondary contact, and dispersal ability through permeable geographical barriers (e.g., van Els et

al., 2014). These contrasting results found in recent studies in Mesoamerican species of Trochilidae make them an ideal biological reference for phylogeographic studies. Some of those have found high levels of genetic structure in species inhabiting fragmented habitats such as cloud forests (González et al., 2011; Ornelas et al., 2016), while others in general showed lower phylogeographic structure and higher levels of gene flow (Miller et al., 2011; Rodríguez-Gómez and Ornelas, 2015), except for groups of populations separated by major geographic barriers such as the Isthmus of Tehuantepec (Cortés-Rodríguez et al., 2008; Rodríguez-Gómez et al., 2013; Malpica and Ornelas, 2014; Jiménez and Ornelas, 2016).

The Magnificent Hummingbird *Eugenes fulgens* (Swainson, 1827), a sexually dimorphic species complex, has a geographic distribution that includes both slopes of the Sierra Madre Occidental and the Sierra Madre Oriental, the highlands of Southern Mexico, Guatemala, Honduras, El Salvador, and Nicaragua (AOU, 1998; Howell and Webb, 1995). This species inhabits pine-oak, cloud and evergreen forests between 1500 and 2500 m above sea level and desert grasslands with flowering agaves in migration (Johnsgard, 1984; Howell and Webb, 1995; Schuchmann, 1999). Recently, this complex was taxonomically reevaluated according to morphological and genetic evidence (Zamudio-Beltrán and Hernández-Baños, 2015). It was shown that *E. spectabilis* distributed in the highlands of Costa Rica and Panama (Gill, 2015) and previously recognized as an *E. fulgens* subspecies, represents an independent species. In contrast, the phylogenetic relationships between the two remaining subspecies of *Eugenes fulgens* (*E. f. fulgens* and *E. f. viridiceps,* Boucard, 1878; Swainson, 1827) are more closely related with one another than to *E. spectabilis* but the significant geographic structure of few samples indicates that species limits should be evaluated at the population level with a throughout geographic sampling.

The purpose of this study is to describe genetic variation within the *Eugenes fulgens* complex by using molecular markers (mitochondrial and nuclear DNA) as to examine morphological divergence, demographic history, and divergence times and ancestral geographic distributions of the resulting lineages, in order to propose a hypothesis of evolutionary history for this species. According to phylogeographic patterns found in Mesoamerican bird species, we expect to find moderate levels of geographic structure in disjunt populations. This moderate structure is expected because in general Trochilidae species present high dispersal abilities that could be reflected in high levels of gene flow (Schuchmann, 1999). If geographic structure is found, we expect that this would be more related with recent climatic shifts than with older events, such as Mesoamerican mountain uplift, based on biogeographic studies and time estimation approaches in Trochilidae (McGuire et al., 2014).

2. Materials and methods

2.1. Taxon sampling and sequencing

We used tissue and feather samples from 129 individuals of *E. fulgens* collected at 45 localities covering most of its geographic range (see Supplementary Information S1 and S2). We defined seven groups corresponding to each of the main mountain ranges where the species complex is distributed according to biogeographic areas previously proposed in biogeographic studies (Morrone, 2006, 2010): Sierra Madre Occidental, SMOc; Sierra Madre Oriental, SMOr; Trans-Mexican Volcanic Belt, TMVB; Sierra Madre del Sur, SMS; north of Sierra Madre del Sur, SMSn; and north and south of populations at east of Isthmus of Tehuantepec: EITn, and EITs (see Supplementary Information S2). Tissue samples were provided by the Museo de Zoología Alfonso L. Herrera (Universidad Nacional Autónoma de México), Natural History

Museum (University of Kansas), Burke Museum (University of Washington), and the Museum of Natural Science (Louisiana State University).

DNA was extracted with the DNAeasy™ kit (Qiagen Inc., Valencia, CA, USA) and following manufacturer's protocols. DNA was then amplified for both mitochondrial (mtDNA) and nuclear (nDNA) markers. The mtDNA included the NADH dehydrogenase subunit 2 (ND2), and the Control Region (CR), which were amplified using previously reported primers: L5219 and H6313 (Sorenson et al., 1999), and ARCOIF and ARCOIR (González et al., 2011), respectively. All PCR reactions contained 10× of buffer (1.25 μL), 10mM of each dNTP (0.19 μL), 50 mM of $MgCl_2$ (0.38 μL), 10 μM of each primer (0.25 μL), 0.1 μL of *Taq* (INVITROGEN), and 0.5 μL of genomic DNA (12.5 μL total volume). Protocols for PCR reactions and for sequencing the PCR products are described elsewhere (González *et al*., 2011; Zamudio-Beltrán and Hernández-Baños, 2015). PCR products were visualized on a 1% agarose gel, and sequencing was performed at the High-Throughput Genomics Unit Service (University of Washington). Chromatograms were edited and aligned with Sequencher v4.8 (GeneCodes Corporation, Ann Arbor, MI) and sequences were deposited in GenBank under accession numbers XXXX–XXXX.

Samples from 85 individuals were genotyped for six polymorphic microsatellite loci designed for *Campylopterus curvipennis* (Cacu13-7, Cacu16-1; Molecular Ecology Resources Primer Development Consortiium et al., 2010), *Amazilia cyanocephala* (Acya-10-9, Acya-4-1; Molecular Ecology Resources Primer Development Consortiium et al., 2013), and *Selasphorus platycercus* (HumB2, HumB9, HumB11; Oyler-McCance et al. 2011). A full description of the development protocol for the loci, PCR conditions, and fragment sizing of microsatellites can be found at the Molecular Ecology Resources Database (http://tomato.biol.trinity.edu/), González et

al. (2011), Oyler-McCance et al. (2011) and Gutiérrez-Rodríguez et al. (2013). Chromatograms were visualized and genotyped using GeneMapper v4.1 (Applied Biosystems, GeneMapper®).

2.2. Population structure

In order to identify the number of mtDNA haplotypes and the relationships between them, we constructed a statistical parsimony haplotype network using the program TCS v1.21 (Clement et al., 2000) with a 95% in connection probability limit. Connections ambiguities were resolved using criteria reported elsewhere (Pfenninger and Posada, 2002). Networks were constructed for each mitochondrial marker and for both concatenated markers. Then, estimates of haplotype diversity, nucleotide diversity, mean number of pairwise differences, and population F_{ST} values were determined for each marker by performing an analysis with 1000 replicates implemented in Arlequin v3.11 (Excoffier et al., 2005). Using the same program, we conducted two analyses of molecular variance (AMOVA; Excoffier et al., 1992) to detect structure between populations, based on comparisons between groups defined geographically, and between groups at both sides of the Isthmus of Tehuantepec (IT).

For microsatellites, we obtained values of observed heterozigosity, expected heterozigosity and mean number of alleles with 1000 replicates in Arlequin v3.11 (Excoffier et al., 2005). The extent of linkage disequilibrium between loci, and departures from Hardy-Weinberg equilibrium were calculated using default parameters in Genepop v4.2 web server (Raymond and Rousset, 1995; Rousset, 2008). These markers were further used to examine geographic structure patterns and to detect the number of hypothetical populations within *E. fulgens* complex with the program STRUCTURE v2.3.4 (Falush et al., 2003; Hubisz et al., 2009; Pritchard et al., 2000). We performed 20 independent runs under the admixture model with

154 correlated allele frequencies for each *K* value, ranging from one to seven. The burn-in period was
155 500 000, followed by 1 000 000 additional Monte Carlo Markov Chains (MCMC) replicates.
156
157 *2.3. Demographic analyses*
158 To evaluate the demographic history of *E. fulgens* populations, we used the mtDNA data
159 set and estimated Tajima's *D* and Fu's *Fs* using Arlequin (Excoffier et al., 2005), with 1000
160 replicates. We also estimated values and reconstructed graphics of mismatch distributions under
161 an expansion model for each defined geographic group, and for groups at both sides of the IT,
162 using the same program and parameters. In order to obtain significance in statistical values, we
163 calculated the Harpending's Raggedness Index (HRI) for each mismatch reconstruction
164 (Harpending, 1994; Rogers and Harpending, 1992; Slatkin and Hudson, 1991). To analyze
165 variation in effective population size through time, we used Bayesian skyline plots (BSP;
166 Drummond et al., 2005) performed in BEAST v1.6.0 (Drummond and Rambaut, 2007), with 10
167 million steps for mtDNA, using a mean rate of 0.026 substitutions per site per lineage per million
168 years (s/s/l/My), according to ND2 and Control Region estimates (Lerner et al., 2011).
169
170 *2.4. Divergence times*
171 Divergence time estimates were obtained using BEAST v1.6.0 (Drummond and
172 Rambaut, 2007). We used the mtDNA data set and included data from *Eugenes spectabilis*,
173 *Lamprolaima rhami*, *Heliomaster longirostris*, *Lampornis clemenciae*, *Atthis heloisa*, *Doricha*
174 *eliza*, and *Tilmatura dupontii* as outgroups. For each partition (ND2+CR), we calculated the
175 evolutionary model that better fit the data using jModeltest v0.1.1 (Posada, 2008), and based on
176 the Akaike Information Criterion AIC (Akaike, 1987). We employed an uncorrelated lognormal
177 relaxed clock, and a Yule speciation model to model the tree prior. We assigned a calibration

178 node based on a secondary calibration obtained for the split between the "Mountain Gems" (*E.*
179 *fulgens*, *E. spectabilis*, *L. rhami*, *H. longirostris,* and *L. clemenciae*) and "Bees" (*A. heloisa*, *D.*
180 *eliza*, and *T. dupontii*; 18.5 Mya, 95% highest posterior density intervals 21.86–15.30 Mya;
181 Zamudio-Beltrán et al., *in prep*.). We incorporated mean substitution rates, with a normal
182 distribution, of 0.0068 (s/s/l/My) for ND2 (Pacheco et al., 2011), and 0.023 (s/s/l/My) for Control
183 Region (Lerner et al., 2011). This analysis was run for 100 million generations, sampling every
184 1000 generations, with a burn-in period of 20%. We used TreeAnnotator v1.8.2 (Rambaut and
185 Drummond, 2007) to summarize the sampled trees as a maximum clade credibility tree, and to
186 obtain mean divergence times with 95% highest posterior density (HPD) intervals.
187
188 *2.5. Present and past geographic distribution*
189 We performed present and past reconstructions of potential distributions for *E. fulgens*
190 complex, under three different scenarios: two for the Last Glacial Maximum (LGM), 21 000–18
191 000 years ago (using both MIROC and CCSM models), and one for the Last Interglacial (LIG),
192 120 000–140 000 years ago. We used a data set of 472 records provided by the Museo de
193 Zoología Alfonso L. Herrera (UNAM) from georeferenced museum specimens for the "Atlas de
194 Aves de México" (Navarro et al., 2003). We used 19 bioclimatic layers from the WorldClim
195 database (BIO1–BIO19; Hijmans et al., 2005), for each georeferenced individual, using the
196 software ArcView v3.2 (ESRI, Redlands, CA, USA). We performed a Principal Components
197 Analysis (PCA) using R statistical software (Ripley, 2001), to detect correlation between
198 variables and to choose the most informative bioclimatic variables.
199 Ancestral and present reconstructions were performed using the maximum entropy
200 algorithm implemented in Maxent v1.0 (Phillips and Dudík, 2008). Default parameters were

used, with 80% of the data for training the model and 20% for testing it. Climate estimations for each reconstruction were projected using ArcView v3.2 (ESRI, Redlands, CA, USA).

2.6. Morphological variation

To examine morphological variation within the *E. fulgens* complex, we took five measures from 465 voucher specimens from different biological collections: Museo de Zoología Alfonso L. Herrera (UNAM), Museum of Comparative Zoology (MCZ), the American Museum of Natural History (AMNH), the Bird and Mammal Collection (UCLA), and the Moore Lab of Zoology (MLZ). These included: (1) bill length (from the upper base of the bill to the tip of the upper mandible), (2) bill width (width by the location of the nostrils), (3) bill depth (from the upper mandible to the base of the bill by the location of the nostrils), (4) wing chord (the distance from the carpal joint to the tip of the longest primary), and (5) tail length (the distance from the uropygial gland to the tip of the longest rectrix). All measures were taken using a dial calliper with a precision of 0.1 mm or a millimetric ruler (for the last measurement). We performed statistical analysis (*t*-Student test) to detect significant differences between males and females with the statistical software STATISTICA v7 (StatSoft, 2004). Subsequently using the same program, we performed an analysis of variance (ANOVA) comparing the seven geographical groups defined above, considering males and females separately, and performed a post-hoc analysis (Fisher's Least Significant Difference Test, LSD; Williams and Abdi, 2010) to detect significant differences between groups.

We then carried out a further discriminant analysis by using all morphological measures as independent variables, and using either the seven geographical groups or low more general groups (east and west of the IT) as clustering variables to predict category classifications. Results

were plotted in R statistical software (Ripley, 2001), using the package ggplot2 (Wickham, 2009).

3. Results

3.1. Genetic diversity and population structure

We obtained 129 mtDNA sequences that were concatenated in dataset of 1405 bp (877 bp for ND2, and 528 bp for CR), containing111 polymorphic sites, 81 parsimony informative sites and 86 haplotypes (45 for ND2 and 52 for CR). Only 21 of these haplotypes were shared between two or more populations. Haplotype diversity was high in all geographic groups (Table 1), but nucleotide diversity (π) was low.

As shown in Fig. 1, the haplotype networks revealed different groupings within the complex. However, there seems to be a separation and geographic correspondence of populations separated by the IT (i.e. orange/yellow haplotypes vs. other colors in Fig. 1). Indeed, some of the haplotypes found east of the IT could not be reliably linked to the main network with the distinct datasets. The most common haplotypes were shared by all locations west of the Isthmus of Tehuantepec. Pairwise F_{ST} values (Table 2) revealed a significant genetic structure between some groups, particularly when comparing TMVB and EITs with the other *a priori* defined population sets.

The mean number of alleles for the microsatellite locus ranged from 4.6 to 13.33 (Table 3). Across sampled localities, this number varied from 4 (HUMB9) to 43 (Acya-10-9). No linkage disequilibrium was detected among locus across populations, and only one locus departed from H-W equilibrium (Cacu16-1). The STRUCTURE results revealed that the highest value of likelihood was obtained for K=5, although there was no correspondence with the

248 geographical groups defined *a priori*, excepting for some concordance with one locality sampled
249 at the highlands of El Salvador (EITs). AMOVA results indicated that the highest genetic
250 variation was observed among populations, and it was even higher when grouping populations at
251 both sides of the IT, 70.30% (P<0.0001, Table 4).
252
253 *3.2. Demographic analyses*
254 According to neutrality test values (Table 1), the populations that presented signs of
255 demographic expansion, when grouping by geographic region, were SMOc, SMO, SMS, and
256 EITn; and only population west of the IT was significant when grouping by populations at both
257 sides of the IT. Mismatch distributions revealed demographic expansion signals in most
258 populations, except in TMVB and EITs (see supplementary information S3). The BSPs indicated
259 that populations west of the IT experienced a demographic expansion, supported also by
260 mismatch distribution curves, starting around 30,000 years ago, while demographic stability was
261 found for the east group (Fig. 3).
262
263 *3.3. Divergence times*
264 Our results showed that the split between *E. fulgens* complex and its sister group (*E.*
265 *spectabilis*) occurred at ca. 5.57 Ma (7.12–3.79 Ma, Fig. 5). Phylogenetic relationships recovered
266 from BEAST analysis showed that the group east of IT does not represent a monophyletic group,
267 while few individuals were grouped within the west group. Diversification time for the split
268 between these two clades was dated around 2.56 Ma (3.27–1.61 Ma), which coincides with the
269 beginning of the Pleistocene. Main west group was dated at ca. 1.93 Ma (2.48–1.14 Ma), and the
270 east group around 1.02 Ma (1.32–0.47 Ma).
271

3.4. Present and past geographic distribution

Among the 19 variables for performing species distribution modelling, only five were retained according to PCA analysis: BIO6 (min temperature of coldest month), BIO5 (max temperature of warmest month), BIO4 (temperature seasonality), BIO15 (precipitation seasonality), and BIO2 (Mean Diurnal Range). Models for the present were consistent with the known geographic distribution of the species (values of AUC>0.96), excepting the predicted area in Baja California (Fig. 4). The projections to the LGM (MIROC and CCSM) predicted a similar geographic area than at present. In contrast, projections into the LIG predicted a narrower geographic distribution, with no presence of the species northern of the Sierra Madre Oriental, and lower probability of presence across TMVB and the Sierra Madre Occidental.

3.5, Morphological variation

Tests between males and females of *E. fulgens* revealed significant differences between sexes for all evaluated variables. Differences were also between geographic groups (see Supplementary Information S4). Females showed differences for all variables excepting for wing chord ($F = 0.633$, $P = 0.70$), while in the males the only variable that did not show differences between groups was the tail length ($F = 1.78$, $P = 0.10$). LSD test showed significant differences between groups, principally in populations at east of IT (EITn, EITs). Results from SMSn population were not taking into account for discussion due to their limited number of samples.

In the discriminant analyses, for females the first two canonical variables explained the 87% of data, with bill depth and bill length being the most informative variables for these roots. For males, the first two canonical variables only explained 53% of total variation, which were loaded by wing chord and bill length variation, respectively. As illustrated by Fig. 6, plots were projected by geographical groups defined *a priori* and by groups at both sides of Isthmus of

296 Tehuantepec. Significant differences were found between groups at east of IT for males and
297 females.
298
299 **4. Discussion**
300 Here, we present full evidence for the evolutionary history of the magnificent
301 hummingbird *Eugenes fulgens*. Morphology and mtDNA variation suggest an incipient process
302 of diversification between populations at both sides of the IT. In contrast, microsatellite data
303 revealed high levels of gene flow and no geographic correspondence was found, excepting for a
304 southern coastal population limited by the Polochic-Motagua fault system. Divergence time
305 estimates suggest that the split between east and west populations occurred during early
306 Pleistocene (~2.56 Mya), and that climatic changes during this period have influenced population
307 dynamics of expansion on western (~30,000 ya) and stability on eastern populations.
308
309 *4.1. mtDNA and microsatellites variation*
310 mtDNA results confirm the previously observed high levels of genetic variation of the
311 *Eugenes fulgens* complex, and the existence of two main groups, corresponding to the *fulgens*
312 and *viridiceps* subspecies, as previously reported (Zamudio-Beltrán and Hernández-Baños,
313 2015). Our haplotype networks reconstructions, F_{ST} values and AMOVA estimates revealed that
314 southern populations in Mesoamerica are genetically distinct from those west of the IT,
315 explained by the presence of this geographic barrier limiting gene flow (Barber and Klicka,
316 2010), and promoting isolation between populations, a pattern that has been found in other
317 Mesoamerican birds (Arbeláez-Cortés et al., 2010; Barber and Klicka, 2010; Barrera-Guzmán et
318 al., 2012), and hummingbirds (Cortés-Rodríguez et al., 2008; González et al., 2011; Malpica and
319 Ornelas, 2014; Rodríguez-Gómez et al., 2013; Jiménez and Ornelas, 2016).

320 There is concordance between the IT separation of populations and taxonomic proposals
321 (*E. f. viridiceps* from Chiapas to Nicaragua and *E. f. fulgens* from SW USA to Oaxaca), which
322 argue that populations east of the IT should be taxonomically reevaluated (Navarro-Sigüenza and
323 Peterson, 2004). In a recent study, enough multilocus genetic differences were found for most of
324 sampled individuals of *E. f. viridiceps* subspecies (Zamudio-Beltrán and Hernández-Baños,
325 2015) to suggest that it should be considered as a cryptic species resulted by a recent allopatric
326 speciation process, and that shared haplotypes could be the result of incomplete lineage sorting
327 (Maddison and Knowles, 2006; Peters et al., 2007).
328 In contrast, the STRUCTURE analysis with six microsatellites did not identify the seven
329 geographic groups defined *a priori*, or the disjunction between locations east and west of the IT.
330 The only exception observed was for the last cluster, where one sampled locality at EITs group
331 was partially separated from the rest. This sampled population from El Salvador (San Vicente) is
332 located on a coastal mountain range that could be isolated from nearer populations.
333 Differentiation between coastal volcanoes and main cordilleras has been reported before for an
334 Andean hummingbird, where population differentiation was found due to environmental
335 conditions at each site (*Adelomyia melanogenys*; Chaves et al., 2007). Also, this genetic
336 differentiation on southernmost population could be influenced by the Polochic-Motagua fault
337 system, a geographic barrier located in Guatemala that extends along ca. 400 km from the
338 Caribbean Sea to the Pacific Coast (Lyon-Caen et al., 2006). This fault system, conformed by
339 three tectonic plates (Polochic, Motagua and Jocotán), was originated as a result of plate
340 convergence and ocean closure since late Cretaceous (Donnelly, 1977; Lawrence, 1976;
341 Schwartz et al., 1979). Several studies have reported that this system fault could promote
342 isolation among populations from different taxa at different temporal scales (Puebla-Olivares et

al., 2008; Rovito et al., 2012; Villalobos, 2013; Malpica and Ornelas, 2014; Rodríguez-Gómez and Ornelas, 2014).

The relationships between *E. fulgens* haplotypes were different depending on the molecular marker used (mtDNA vs. microsatellites). Microsatellites signal was low compared to that in mtDNA, which was unexpected given that microsatellites are subject to higher mutation rates (Chistiakov et al., 2006). However, these differences could be more related to inheritance (maternally or biparentally signal) plus dispersal movements. Northern and possibly central populations of *E. fulgens* presents breeding movements to extreme north Mexico and southwest USA, and populations in south Mexico and Central America are sedentary (Schuchmann, 1999). The high levels of gene flow found for most populations, and the high genetic differentiation between southernmost populations could be linked to these migration movements. This signal of dispersal is expected since movement abilities are well known in Trochilidae, and also considering that *E. fulgens* is a generalist hummingbird that feeds from a wide variety of resources, and presents low territorial behavior (Lara, 2006). Higher geographic structure found in mtDNA should suggest male-biased dispersal, and a higher site fidelity by females that remain closer to their territories, probably due to their breeding biology, where females could limiting longer movements due to exclusive parental care, a documented behavior in hummingbirds (Pitelka, 1942; Wolf, 1969).

4.2. Demographic history and biogeographic hypothesis

Population dynamic analyses (mismatch and BSPs), and models of ancestral distributions projections provide a general overview of demography history of *E. fulgens*. When we compared geographic groups, the expansion model was the more common pattern found in five of the seven groups. This has been a common pattern found in widely distributed species, as is *E. fulgens* case

(Hewitt, 2000; Milá et al., 2007). Given that the genetic variation was best explained when grouping populations at both sides of the IT (AMOVA, 70.3% among populations), we decided to evaluate the demographic patterns on those two main groups. In this case, widespread populations at the west presented a pattern of demographic expansion (ca. 30 000 years ago) that coincides closely to the LGM (ca. 20 000 years ago), while eastern populations remained stable over time, which is consistent with the geographic structure previously discussed.

Accordingly, our results show that *E. fulgens* complex and the split into the two lineages separated by the IT occurred about 2.56 Mya. Taking into account these estimates, it is clear that the geologic origin of the IT that started during the Late Miocene (ca. 6 Mya, Barrier et al., 1998), was not the primary cause of *E. fulgens* divergence. One of the most accepted hypotheses of geographic differentiation is that Pleistocene climatic fluctuations promoted the so-called Pleistocene refugia (Hewitt, 1996; Sánchez-González et al., 2008; Smith et al., 2011). Our divergence time estimation combined with our demography results suggest that *E. fulgens* complex was established during early Pleistocence, and that posterior climatic fluctuations and changes in seaways around the IT could have fractured habitats (Barber and Klicka, 2010), and promoted the divergence of these main two lineages: *viridiceps* and *fulgens*, which might represent an incipient process of speciation.

The projections into ancestral conditions during LIG (~140 000 years ago) and LGM (~20 000 years ago), showed that in both cases there were favorable climatic conditions for the species, but the ecological climatic conditions were more suitable during LGM period, possibly promoting the recent demographic expansion found for western populations, and having no significant effect on eastern populations. This expansion of territories and contraction of them, during interglacial periods, could lead on mixing of populations east and west during glacial

cycles (i.e. secondary contact), explaining the presence of shared haplotypes between both lineages.

The phylogeographic split between populations separated by the IT was also supported by data of morphological variation. Despite the fact that we found overlap in measures for some geographic groups, the statistically significant values confirm that there exists a pattern of phenotypic separation between those populations. When plotting values of discriminant analyses, significant differences between eastern populations were also revealed, which suggest that southernmost populations do present isolation limited probably by the Motagua system zone. Some studies have analyzed ecological and environmental factors to explain morphological differentiation as consequence of natural selection (Chaves et al., 2007; De Leon et al., 2010; McCormack et al., 2008). For evaluating the selection as a driver of differentiation, comparisons on environmental conditions and morphological traits should be done between the different areas to corroborate whether morphological differences are either related to ecological adaptation or random genetic drift under isolation.

The present work allowed us to infer about the phylogeographic pattern and the evolutionary history of *E. fulgens* in the highlands of Mesoamerica. This study revealed the presence of two main lineages, one of which has remained demographically stable and geographically isolated by the Isthmus of Tehuantepec and/or by the Motagua-Polochic system fault. An increasing in sampling effort around this area (Central America) should help to closely evaluate the genetic variation and the dynamics of gene flow around this region.

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432

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Author Contributions

L. E. Zamudio-Beltrán designed the research, performed research, analyzed data, and wrote the paper. J. F. Ornelas designed the research, and wrote the paper. B. E. Hernández-Baños designed the research, conducted fieldwork and wrote the paper.

Table 1. Statistical parameters of genetic diversity, population structure and population demography for mtDNA data, n: number of sequences used, h: number of haplotypes, Hd: haplotype diversity, π: nucleotide diversity, Pi: mean number of pairwise differences.

GROUP	n	h	Hd	π	Pi (theta)	Tajima's D	Fu's Fs Test
(a) Biogeographic region							
SMOc	38	31	0.99	0.0024	3.559	-1.88 (P=0.01)	-26.02 (P=0.00)
SMO	22	20	0.99	0.0026	3.80	-1.59 (P=0.04)	-22.69 (P=0.00)
TMVB	26	20	0.95	0.0018	11.73	-0.82 (P=0.22)	-3.04 (P=0.04)
SMS	20	17	0.97	0.0022	3.62	-1.53 (P=0.05)	-17.99 (P=0.00)
SMSn	5	5	1.0	0.0018	2.60	-0.67 (P=0.37)	-2.52 (P=0.02)
EITn	12	10	0.97	0.0099	3.04	-1.95 (P=0.006)	-25.26 (P=0.00)
EITs	6	5	0.93	0.0192	27.5	-0.09 (P=0.54)	0.88 (P=0.42)
(b) Isthmus of Tehuantepec							
WEST	111	72	0.97	0.0022	3.11	-2.37 (P=0.00)	-26.37 (P=0.00)
EAST	18	14	0.97	0.0184	25.9	1.25 (P=0.93)	-4.89 (P=0.02)

Table 2. Population pairwise F_{ST} mtDNA

	SMOc	SMO	TMVB	SMS	SMSn	EITn	EITs
SMOc	----						
SMO	-0.008	----					
TMVB	0.829*	0.807*	----				
SMS	-0.014	-0.020	0.799*	----			
SMSn	-0.095	-0.076	0.743*	-0.081	----		
EITn	0.009	0.016	0.821*	0.0146	-0.065	----	
EITs	0.705*	0.655*	0.467*	0.637*	0.466*	0.677*	----

*P<0.05

Table 3. Statistical parameters for microsatellites data, Ho: observed heterozigosity, He: expected heterozigosity.

GROUP	n	Mean alleles/ locus	Ho	He
SMOc	31	13.33	0.62	0.75
SMO	15	9.67	0.54	0.74
TMVB	9	8.00	0.55	0.83
SMS	8	8.50	0.63	0.78
SMSn	5	5.33	0.57	0.76
EITn	11	8.83	0.60	0.80
EITs	6	4.6	0.46	0.75

Table 4. AMOVA results on *Eugenes fulgens* populations defined according to biogeographic regions, and grouped into groups separated by the Isthmus of Tehuantepec.

	d.f.	Sum of squares	Variance components	Percentage of variation	Fixation indices
Biogeographic region					
Among populations	6	301.22	2.74	51.17	
Within populations	122	319.22	2.62	48.83	
Total	128	620.44	5.36		F_{ST}=0.51***
Isthmus of Tehuantepec					
Among populations	1	229.07	7.29	70.30	
Within populations	127	391.37	3.08	29.70	
Total	128	620.44	10.38		F_{ST}=0.70***

***P<0.0001

Figure captions

Figure 1. Statistical parsimony haplotype networks for 129 individuals of *E. fulgens*, constructed with three different databases: ND2, CR and concatenated mtDNA markers. Different colors in networks correspond to the different geographic groups in the map. Size of each circle is proportional to the number of individuals carrying each haplotypes.

Figure 2. Posterior assignment probabilities of 85 individuals of *E. fulgens* to an optimal number of K=5 (top). Each individual is represented by a vertical rectangle. Proportions of each hypothetical cluster to each sampled site are drawn on the map. Size of each circle is proportional to the number of individuals at each locality.

Figure 3. Mismatch distributions and Bayesian skyline plots for each group at both sides of the Isthmus of Tehuantepec. In mismatch distributions, solid lines indicate the observed distributions of pairwise differences, and dotted lines represent simulated distributions under a model of population expansion. In Bayesian skyline plots, solid lines represent median estimates and shaded areas represent 95% confidence intervals.

Figure 4. Species distribution model for *E. fulgens* complex at Last Interglacial (LIG, ~140,000 Ya), at Last Glacial Maximum (LGM, ~21,000 Ya) under two different ancestral models (CCSM, MIROC), and at present.

Figure 5. Phylogeny illustrating divergence times for *Eugenes fulgens* complex as generated by BEAST. Bars on each node represent 95% of high posterior density of divergence times (HPD). Numbers below nodes represent posterior probability values of principal clades in topology. Ma (Million years).

Figure S2. Geographic distribution of *Eugenes fulgens* complex. Red hexagons represent sampled localities corresponding to tissues used in this study. Geographic groups defined *a priori* are drawn by different colors and represented by different letters on the map. Geographic groups: SMOc (Sierra Madre Occidental), SMO (Sierra Madre Oriental), TMVB (Trans Mexican Volcanic Belt), SMS (Sierra Madre del Sur), SMSn (north from Sierra Madre del Sur), ETIn (north from east of the Isthmus of

Tehuantepec), EITs (south from east of the Isthmus of Tehuantepec). The illustration corresponds to a male from *E. fulgens*.

Figure S3. Mismatch distributions for each geographic group of *Eugenes fulgens*. Solid lines indicate the observed distributions of pairwise differences, and dotted lines represent simulated distributions under a model of population expansion. Geographic groups are represented in different colors according to the geographic regions on the map. The *y* axis is distribution frequency, and the *x* axis represent pairwise differences.

Figure S4. Morphological characters taken for males and females of *E. fulgens* groups. Boxplots show the percentiles of 25%, 50% (median), and 75%, upper and lower whisker show quartiles of 25%. Geographic groups are represented in different colors according to geographic regions. Numbers above or below each boxplot represent sampled individuals. Statistical differences between groups are represented with an asterisk (*P<0.05).

Figure S5. Discriminant analysis for males and females of *E. fulgens*. A) Plots representing geographic groups in different colors, mean values are represented by a black dot for each group. B) Plots by geographic groups differentiating populations at east and west from the Isthmus of Tehuantepec. C) Plots representing populations at east and west from the Isthmus of Tehuantepec. Statistical differences between groups are represented with an asterisk (*P<0.05).

Figure 1.

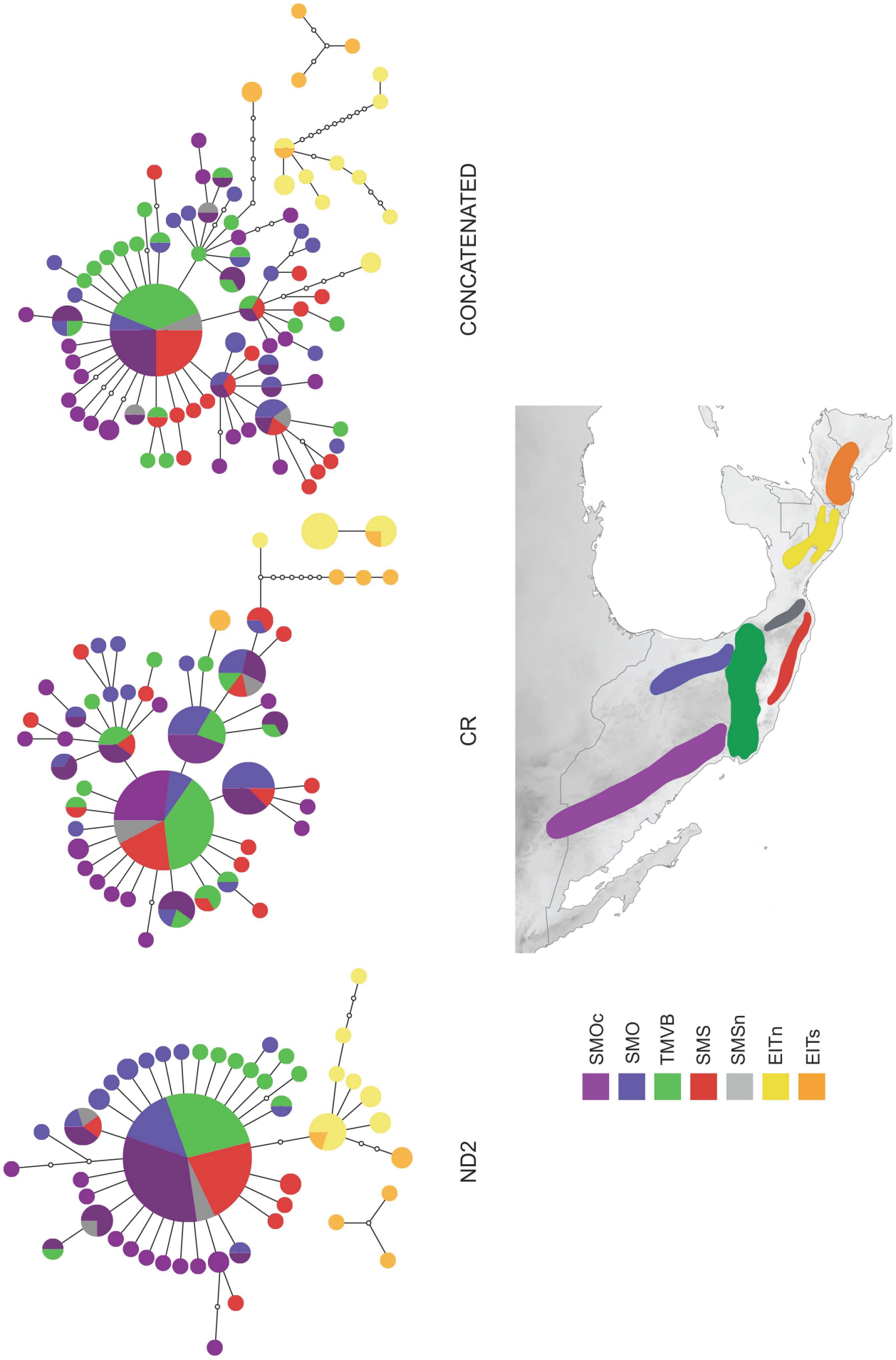


Figure 2.

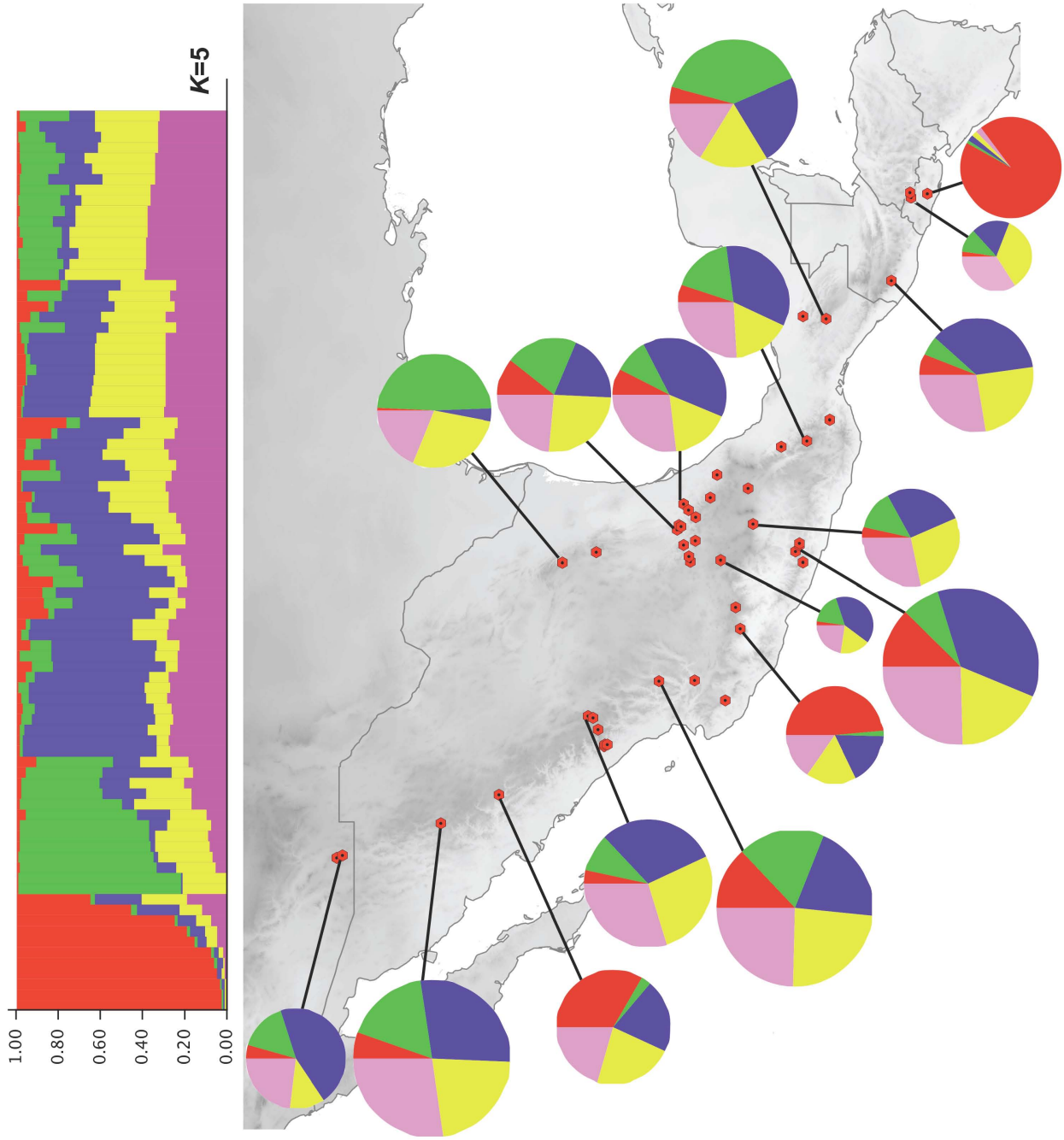


Figure 3.

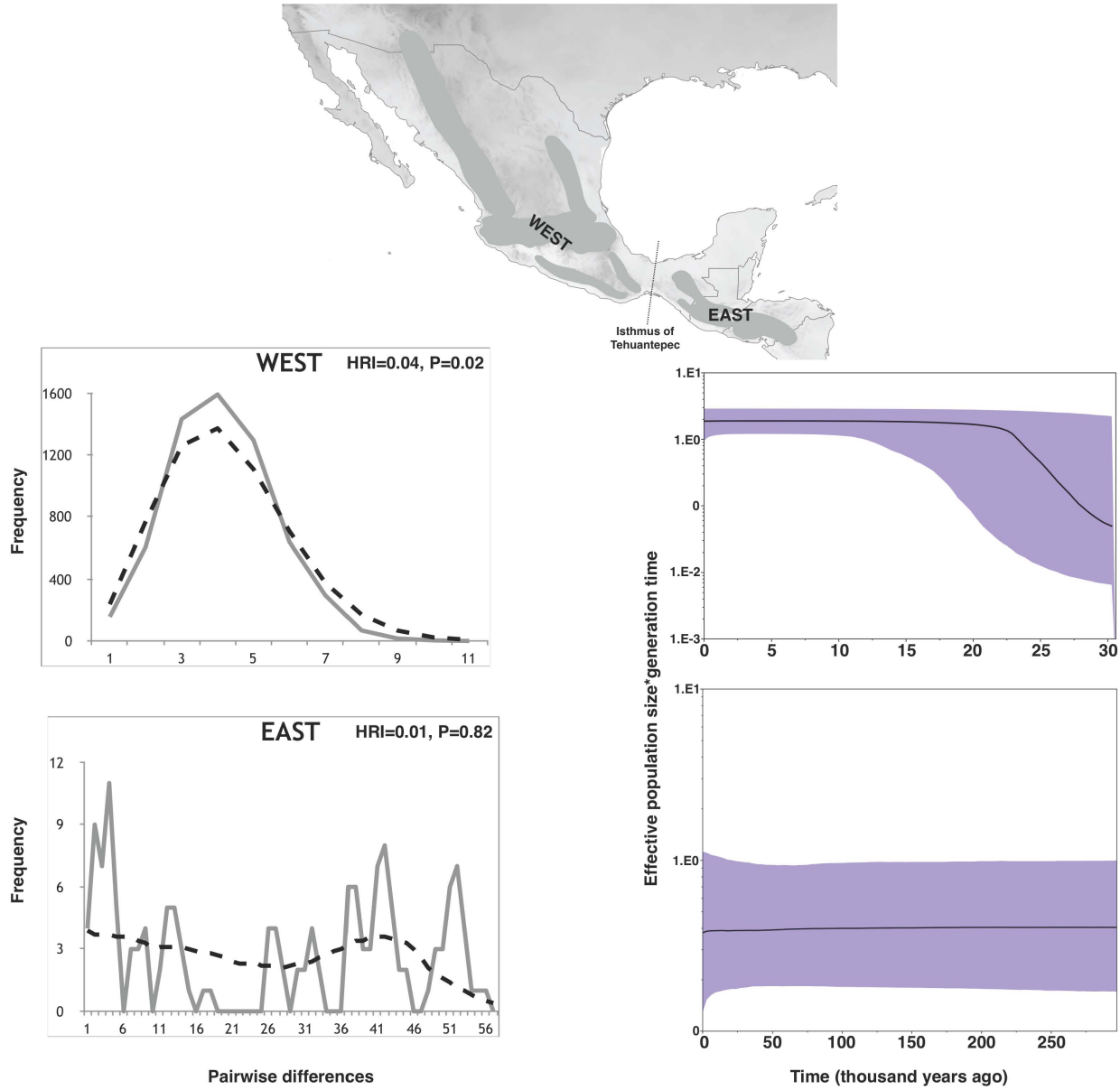

WEST
EAST
Isthmus of Tehuantepec
WEST HRI=0.04, P=0.02
Frequency
1600
1200
800
400
0
1 3 5 7 9 11
EAST HRI=0.01, P=0.82
Frequency
12
9
6
3
0
1 6 11 16 21 26 31 36 41 46 51 56
Pairwise differences
Effective population size*generation time
1.E1
1.E0
0
1.E-2
1.E-3
0 5 10 15 20 25 30
1.E1
1.E0
0
0 50 100 150 200 250
Time (thousand years ago)

Figure 4.

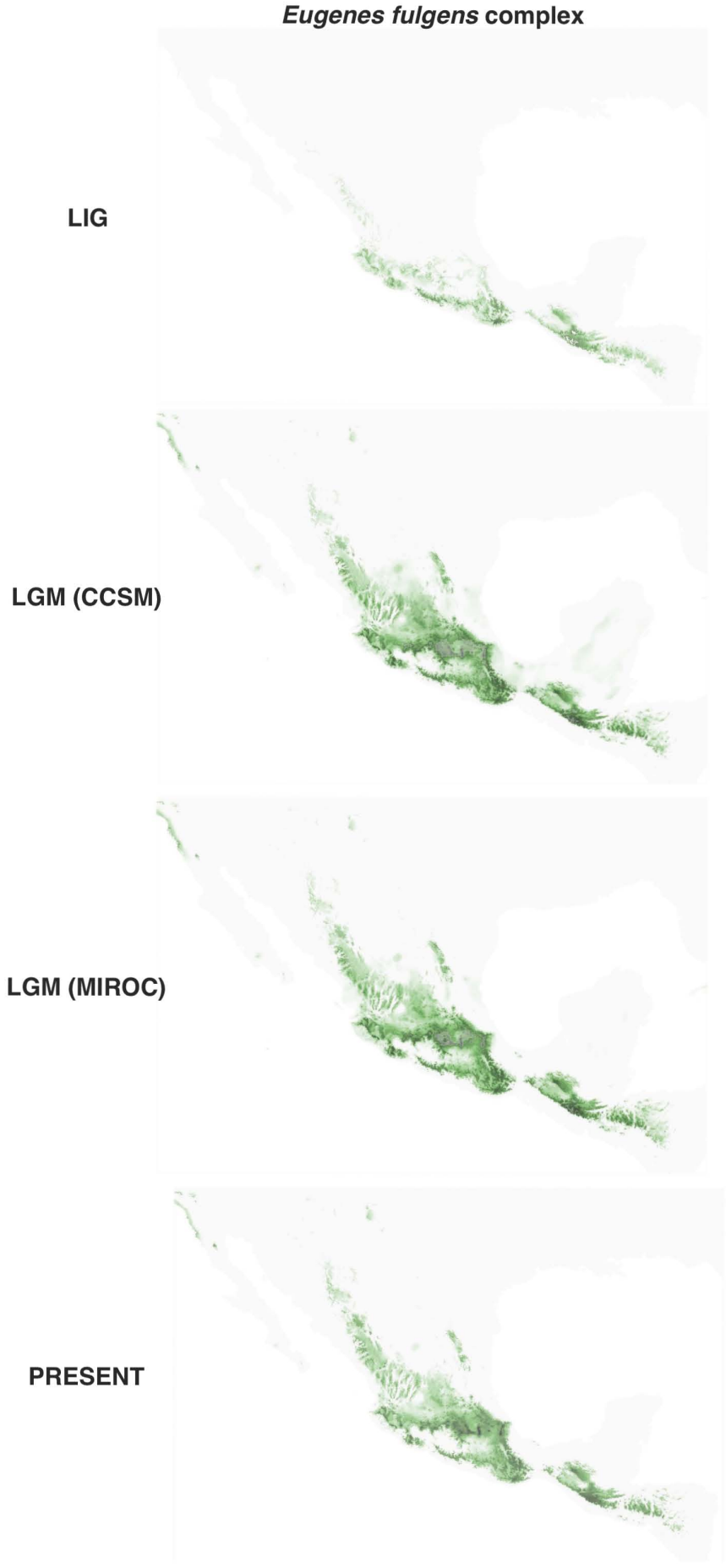
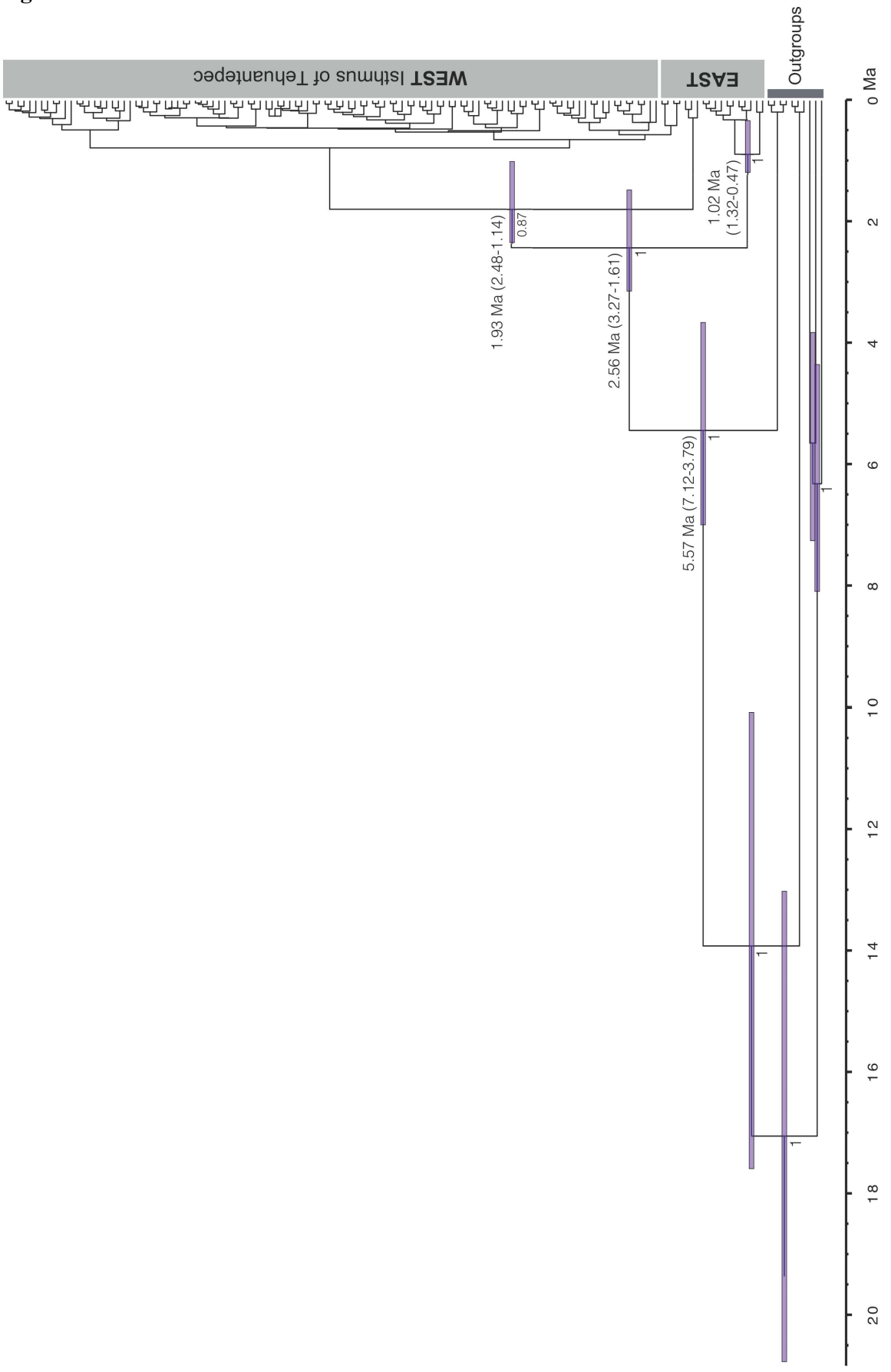


Figure 5.


WEST Isthmus of Tehuantepec
EAST
Outgroups
0 Ma
2
4
6
8
10
12
14
16
18
20
1.93 Ma (2.48-1.14)
0.87
1
2.56 Ma (3.27-1.61)
1
1.02 Ma
(1.32-0.47)
1
5.57 Ma (7.12-3.79)
1
1
1
1

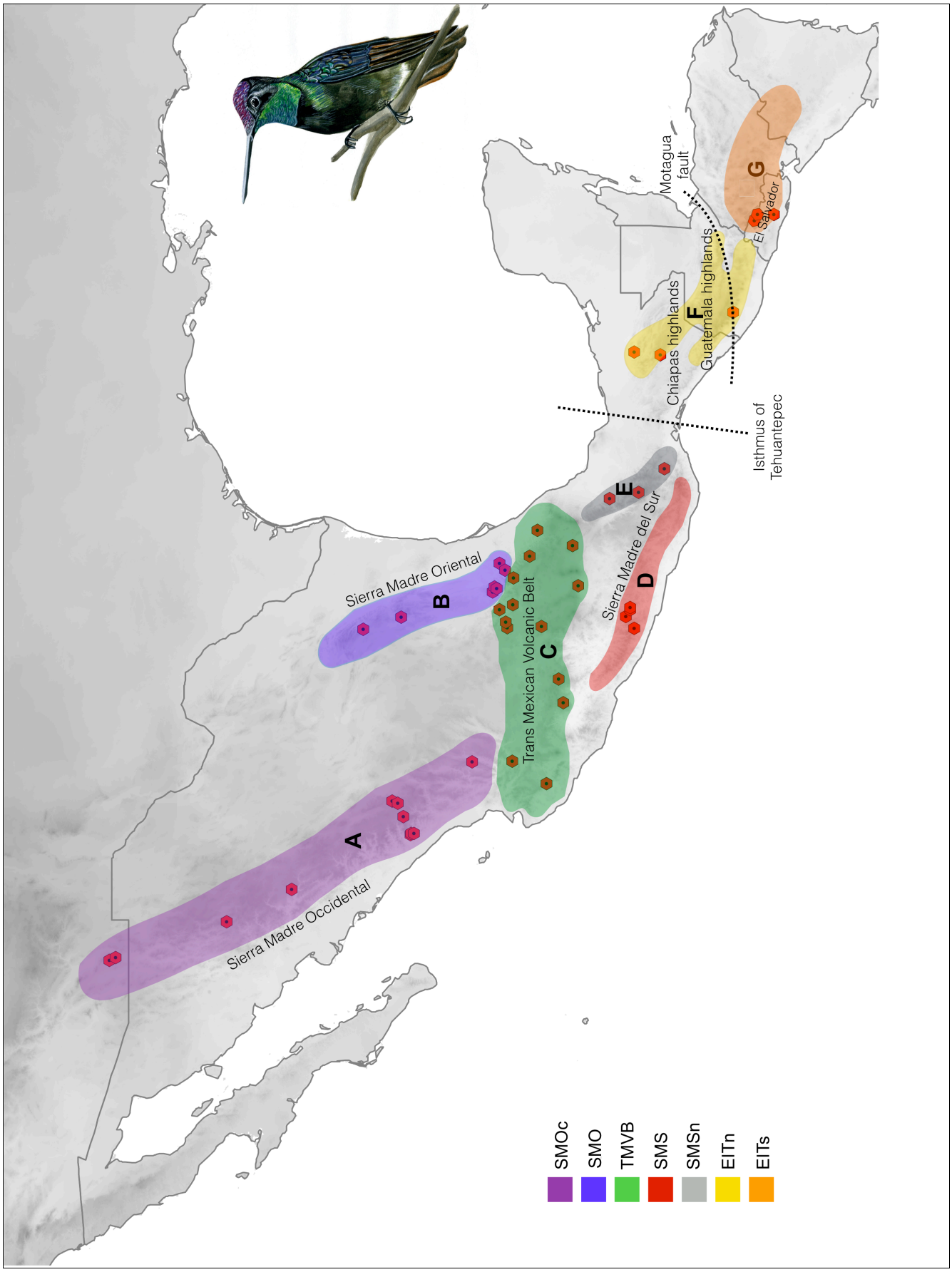
Supplementary material S1. Localities, geographic groups (GG), number of sequences, coordinates and biological collections of *Eugenes fulgens* tissue samples.

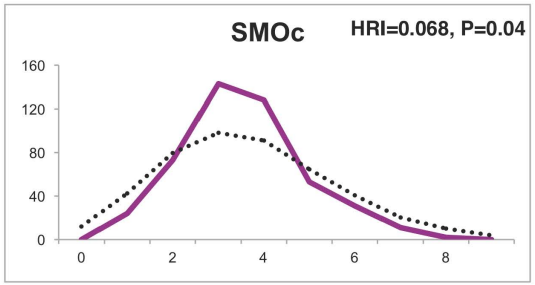
#ID	Localities	**GG**	mtDNA (ND2, CR)	nDNA (microsatellites)	Latitude	Longitude	BC
1	USA, Arizona, Chiricahua mountains.	**SMOc**	2	2	31.84	-109.32	UW
2	USA, Arizona, Price Canyon.	**SMOc**	2	2	31.68	-109.23	LSU
3	Chihuahua, Madera	**SMOc**	9	8	28.63	-108.26	UW
4	Chihuahua, Cumbres de Güerachi.	**SMOc**	5	5	26.83	-107.37	UNAM
5	Durango, Salvador Allende.	**SMOc**	1	0	24.08	-104.93	UW
6	Durango, Mimbres.	**SMOc**	6	6	23.93	-104.99	UNAM
7	Durango, El Salto.	**SMOc**	1	0	23.77	-105.36	UNAM
8	Sinaloa, Chara Pinta.	**SMOc**	2	0	23.59	-105.87	UNAM
9	Sinaloa, El Palmito.	**SMOc**	1	0	23.56	-105.82	UNAM
10	Sinaloa, La Laguna.	**SMOc**	1	0	23.48	-105.83	UNAM
11	Jalisco, Sierra de Bolaños	**SMOc**	9	8	21.88	-103.86	UW
12	Nuevo León, Galeana	**SMO**	5	5	24.88	-100.22	UW
13	Nuevo León, Peña Nevada	**SMO**	4	0	23.84	-99.89	UW
14	Querétaro, Tres Lagunas	**SMO**	2	2	21.33	-99.20	UNAM
15	Querétaro, Río Tancuilín	**SMO**	1	0	21.27	-99.06	UNAM
16	Querétaro, El Pemoche	**SMO**	3	3	21.22	-99.10	UNAM
17	Hidalgo Huejutla	**SMO**	2	0	21.14	-98.41	UNAM
18	Hidalgo, Tlalchinol	**SMO**	5	5	20.98	-98.60	UNAM
19	Jalisco, Sierra Cacoma	**TMVB**	1	0	19.84	-104.45	UNAM
20	Jalisco, Tequila	**TMVB**	6	0	20.78	-103.84	UW
21	Michoacán, Nuevo San Juan	**TMVB**	2	3	19.38	-102.24	UW
22	Michoacán, Patzcuaro	**TMVB**	1	1	19.51	-101.58	UNAM
23	Querétaro, El Zamorano	**TMVB**	1	0	20.93	-100.18	UNAM

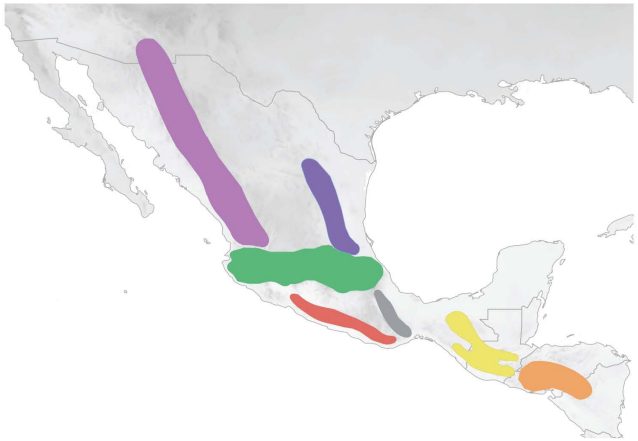
24	Michoacán, Contepec	**TMVB**	1	1	19.97	-100.15	UNAM
25	Querétaro, Ojo de Agua	**TMVB**	1	0	20.97	-100.02	UNAM
26	Querétaro, San Gaspar	**TMVB**	1	0	21.13	-99.68	UNAM
27	Querétaro, Chavarrías	**TMVB**	2	0	20.77	-99.55	UNAM
28	Morelos, Amatlán de Quetzalcóatl	**TMVB**	4	4	18.97	-99.03	UNAM
29	Hidalgo, Eloxochitlán	**TMVB**	2	0	20.74	-98.81	UNAM
30	Hidalgo, El Potrero	**TMVB**	1	0	20.31	-98.22	UNAM
31	Puebla, Acajete	**TMVB**	1	0	19.14	-97.92	UNAM
32	Puebla, Cuitchat	**TMVB**	2	0	20.09	-97.51	UNAM
33	Guerrero, Carrizal de Bravo.	**SMS**	12	8	17.67	-99.88	UNAM
34	Guerrero, Omiltemi	**SMS**	5	0	17.55	-99.67	UNAM
35	Guerrero, Xocomanatlán	**SMS**	2	0	17.55	-99.63	UNAM
36	Guerrero, El Molote.	**SMS**	1	0	17.44	-100.2	UNAM
37	Oaxaca, Teotitlán	**SMSn**	1	1	18.11	-96.64	UW
38	Oaxaca, Ixtlán Las Nubes	**SMSn**	3	3	17.32	-96.46	UNAM
39	Oaxaca, Cerro Piedra Larga	**SMSn**	1	1	16.60	-95.8	UNAM
40	Chiapas, Cerro Mozotal	**EITn**	1	0	17.43	-92.60	UNAM
41	Chiapas, Cerro Huitepec	**EITn**	6	6	16.73	-92.68	UNAM
42	Guatemala, Quezaltenango, Sta. Ma. de Jesús	**EITn**	5	5	14.71	-91.52	UW
43	El Salvador, Chalatenango	**EITs**	1	1	14.38	-89.12	KU
44	El Salvador, Chalatenango	**EITs**	1	1	14.13	-88.91	KU
45	El Salvador, San Vicente	**EITs**	4	4	13.6	-88.84	KU

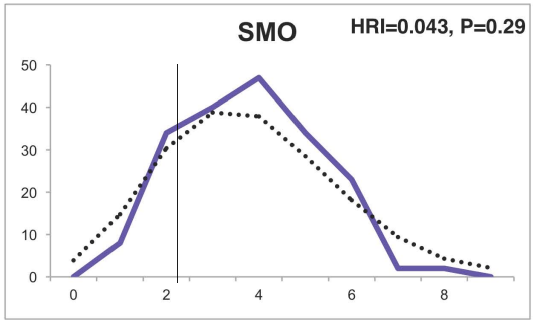
BC: Biological Collection.
UNAM: Universidad Nacional Autónoma de México, Museo de Zoología Alfonso L. Herrera.
KU: The University of Kansas, Natural History Museum.
LSU: Louisiana State University, Museum of Natural Science.
UW: University of Washington, The Burke Museum.

SUPPLEMENTARY MATERIAL S2




SMOc
HRI=0.068, P=0.04
160
120
80
40
0
0
2
4
6
8

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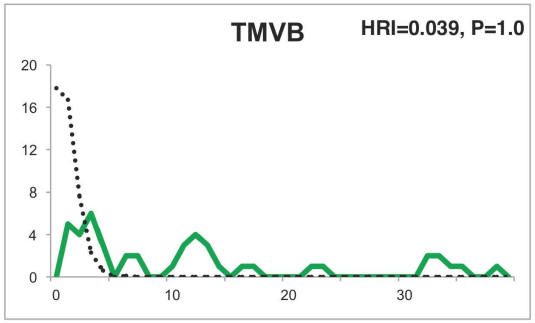
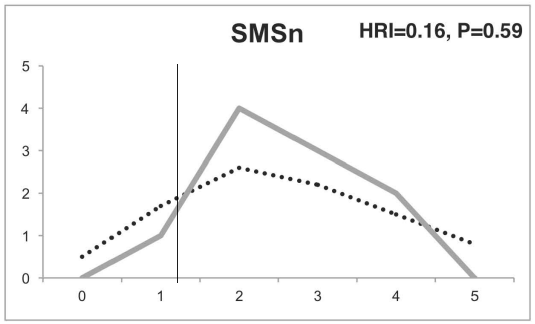
HRI=0.043, P=0.29
50
40
30
20
10
0
0
2
4
6
8


TMVB
HRI=0.039, P=1.0
20
16
12
8
4
0
0
10
20
30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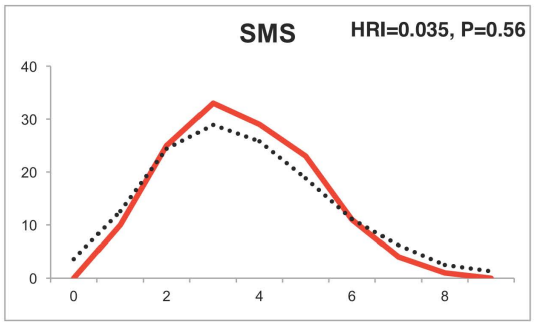
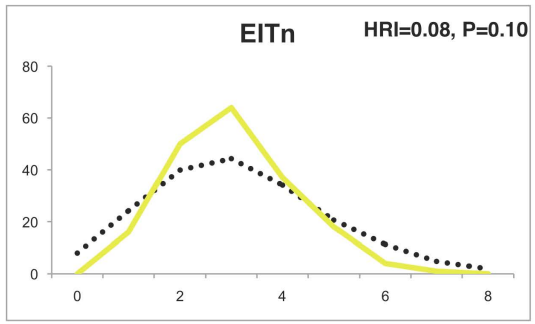
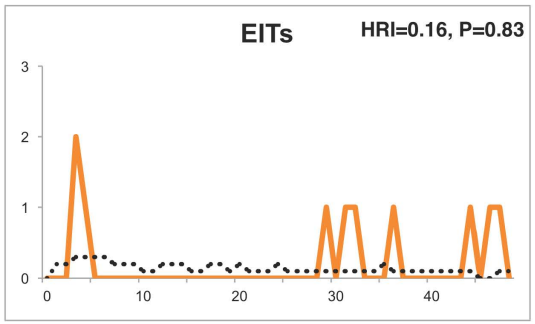
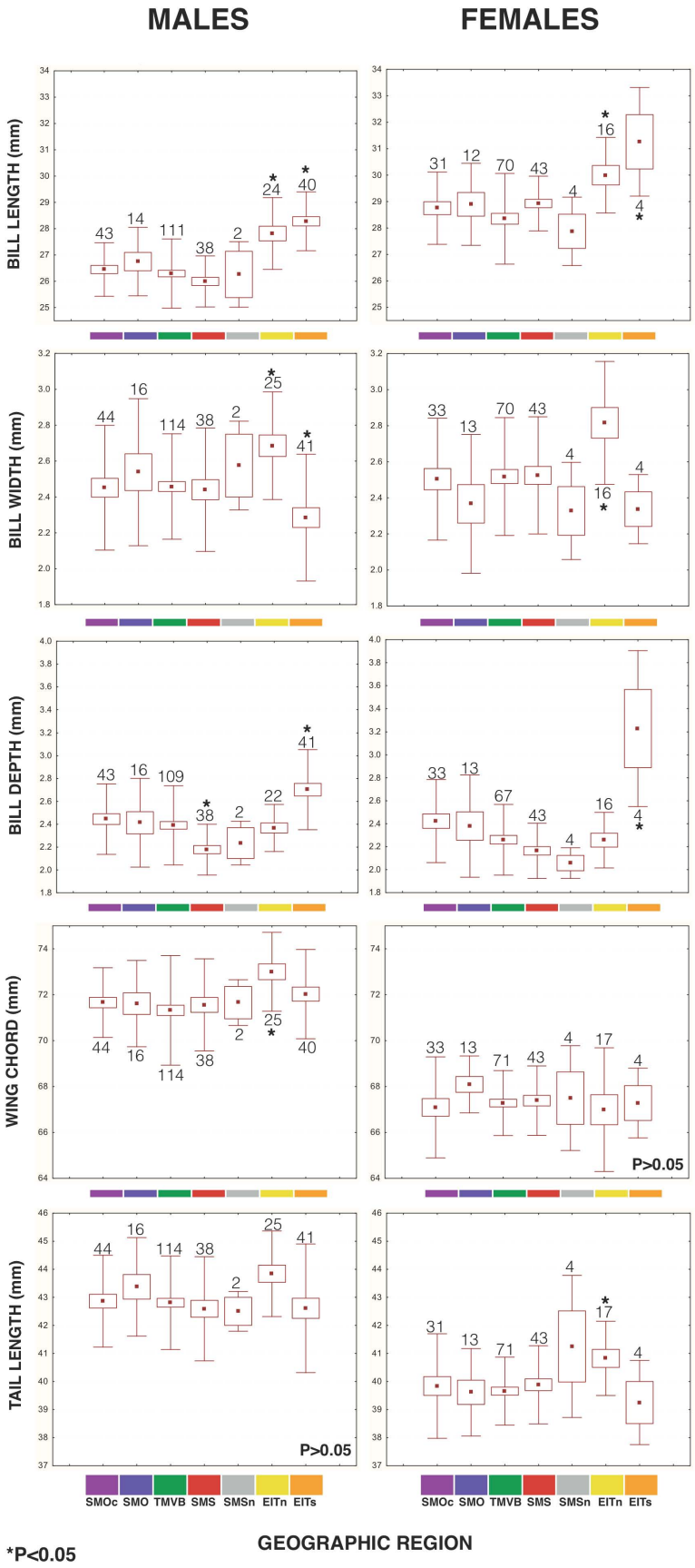
HRI=0.16, P=0.59
5
4
3
2
1
0
0
1
2
3
4
5


SMS
HRI=0.035, P=0.56
40
30
20
10
0
0
2
4
6
8

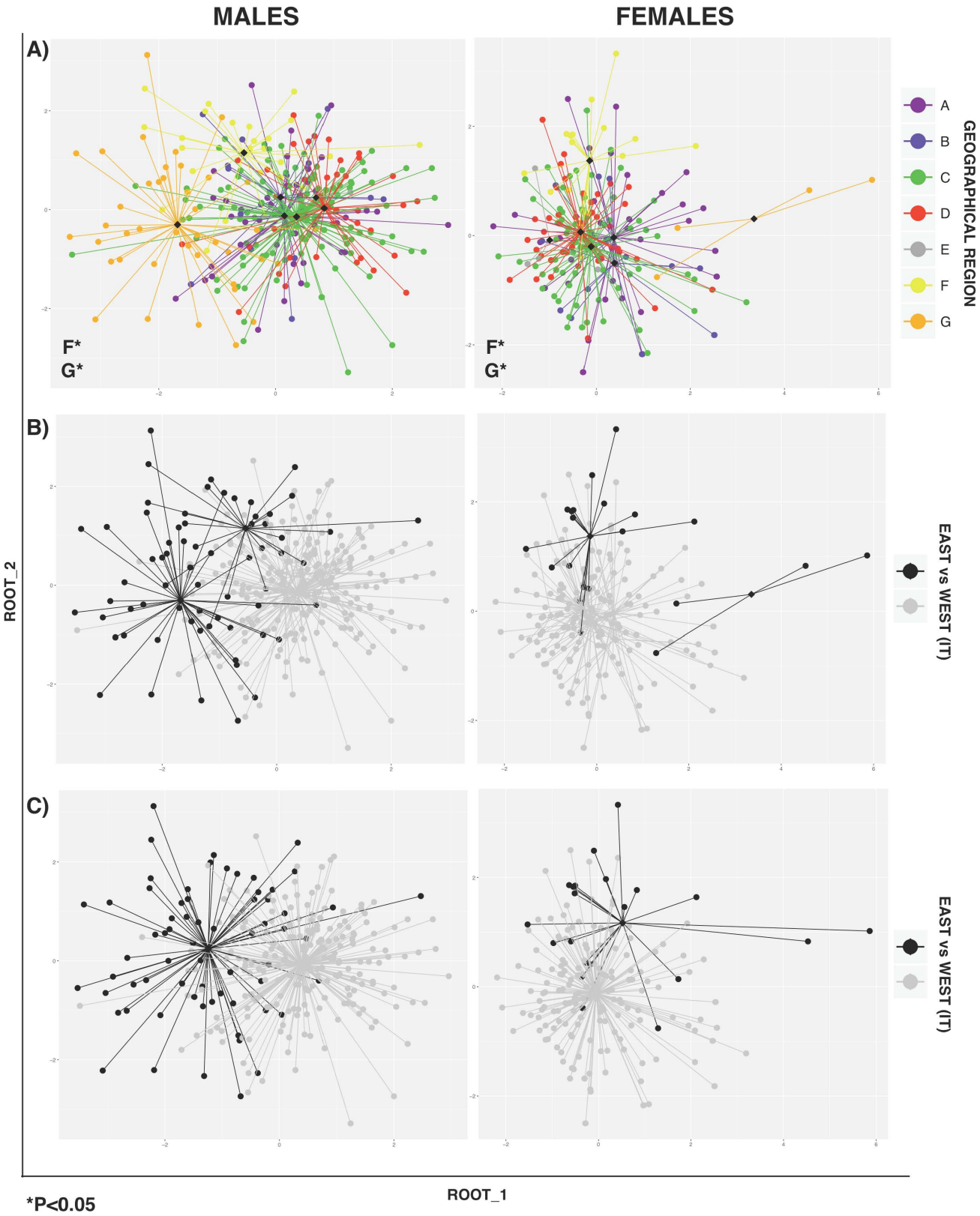

EITn
HRI=0.08, P=0.10
80
60
40
20
0
0
2
4
6
8


EITs
HRI=0.16, P=0.83
3
2
1
0
0
10
20
30
40

SUPPLEMENTARY MATERIAL S4



SUPPLEMENTARY MATERIAL S5



ARTÍCULO 4. VARIACIÓN GENÉTICA Y FENOTÍPICA DEL COLIBRÍ *Lamprolaima rhami* (Aves: Trochilidae).

Zamudio-Beltrán, L. E., & Hernández-Baños, B. E.

Resumen.- Los bosques mesófilos de montaña, también conocidos como bosques de niebla, son uno de los ecosistemas más amenazados en América y uno de los de mayor biodiversidad en el mundo. Las especies que habitan estos bosques se caracterizan por presentar altos niveles de estructuración geográfica. Aquí analizamos si las poblaciones del colibrí *Lamprolaima rhami* (Garnet-throated Hummingbird) presentan éste mismo patrón, y describimos la importancia de este estudio a nivel poblacional en esta especie con una distribución geográfica altamente restringida en Mesoamérica. Esta especie habita principalmente en los bosques mesófilos de montaña, sin embargo, su rango altitudinal abarca los hábitats comprendidos entre los 1200 y los 3000 msnm, como los bosques de pino-encino, los bosques tropicales y los matorrales. Algunas propuestas taxonómicas han descrito tres grupos diferentes basados en sutiles diferencias morfológicas: 1) *L. r. rhami* (descrita como una subespecie), presenta una distribución geográfica restringida en las tierras altas de México (Puebla, Veracruz, Oaxaca, Chiapas) y Guatemala, 2) *L. r. occidentalis* (descrita como una raza), distribuida en Guerrero, y 3) *L. r. saturatior* (descrita como subespecie), distribuida en las tierras altas de Honduras y El Salvador. Empleamos ADN mitocondrial (subunidades 6 y 8 del gen ATPasa y la Región Control) con el fin de analizar la variación genética de 52 individuos. También evaluamos la variación morfológica en 213 ejemplares de colección, analizamos la historia demográfica del complejo, estimamos tiempos de divergencia y llevamos a cabo una reconstrucción filogenética multilocus con el fin de proponer una reevaluación taxonómica para la especie. En general, encontramos altos niveles de diferenciación genética, además de variación a nivel morfológico que coincide con las regiones geográficas de distribución de la especie. Recomendamos considerar a todos los grupos acomo unidades de manejo independientes tomando en cuenta el aislamiento geográfico, las diferencias morfológicas significativas y la evidencia multilocus de diferenciación.

Palabras clave: Gemas de las Montañas, Trochilidae, Neotrópico, Mesoamérica, Filogenia, Colibríes.

1 Genetic and phenotypic divergence in the Garnet-Throated Hummingbird *Lamprolaima*
2 *rhami* (Aves: Trochilidae).

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16
17 **Author Contributions**
18 L. E. Zamudio-Beltrán designed the research, performed research, analyzed data, and wrote
19 the paper. B. E. Hernández-Baños design the research, conducted field work and wrote the
20 paper.
21

ABSTRACT

Cloud forests is one of the most endangered ecosystems in the Americas, but also one of the richest in biological diversity in the world. The species inhabiting these forests are characterized by high levels of geographic structure. Here, we analyzed if this pattern is also present in populations of the Garnet-Throated Hummingbird *Lamprolaima rhami*, and describe the importance of this study at the population level for a species with a highly restricted distribution in Mesoamerica. This species mainly inhabits cloud forest, but can also be found in all habitats at elevations between 1,200 and 3,000 m above the sea level, such as forest edges, pine-oak forests, upper tropical forests and scrub. Three taxa have been described based on subtle morphological differences: 1) *L. r. rhami* (described as subspecies) restricted to the Mexican highlands (Puebla, Veracruz, Oaxaca, Chiapas and Guatemala), 2) *L. r. occidentalis* (described as race) distributed in Guerrero, 3) *L. r. saturatior* (subspecies), distributed in the highlands from Honduras and El Salvador. We used mitochondrial DNA (subunits 6 and 8 of ATPase, and Control Region) to analyze the genetic variation of 52 individuals. We also evaluated morphological variation in 213 specimens, analized demographic history, estimated divergence times, and built a multilocus phylogeny to propose a taxonomic reevaluation for the complex. We found high levels of genetic differentiation, and significant morphological variation that corresponded with the geographic distribution for the species. We recomended considering all groups as independent management units taking into account geographic isolation, significant morphological differences and multilocus evidence of diferentiation.

Keywords: *Lamprolaima rhami*, Trochilidae, Mesoamerica, Cloud Forest, Hummingbirds.

1. Introduction

Cloud forests is one of the mostly threatened and biodiverse habitats in the world (Hamilton, 1995; Mulligan, 2010). In Mesoamerica, that biologically represents the transition zone between Nearctic and Neotropical region (Morrone, 2006; Ríos-Muñoz, 2013), the cloud forests are restricted to a highland particular sector between 600 and 3000 m above sea level (Foster, 2001). Several studies have tried to describe the evolutionary proceses that have shaped the enormous diversity of cloud forests, concluding that species show high levels of isolation and population differentiation when compared to those geographically interconnected habitats (Ataroff and Rada, 2000; de Barcellos Falkenberg and Voltolini, 1995; Ornelas et al., 2013). Lack on population differentiation studies could result in underestimating biodiversity as discussed elsewhere (Arbeláez-Cortés and Navarro-Sigüenza, 2013; Bonaccorso et al., 2008; McCormack et al., 2008).

Recently, different studies have focused in describing historical patterns and recognizing new species (Cortés-Rodríguez et al., 2008; González et al., 2011; González-Rodríguez et al., 2004; Ornelas et al., 2010). However, the number of species that inhabit these forests are far from been fully studied, while the pace at which these forests are disappearing due to anthropogenic causes is pushing researches to study more species before the damage would be irrevocable (Martínez-Morales, 2005; Martínez-Morales, 2005; Olander et al., 1998).

The Trochilidae family is a well known reference in biological studies containning interesting species models for evolutonary studies (Bleiweiss, 1998a; McGuire et al., 2007), although only a few of them focus on species inhabiting cloud forests (Bleiweiss, 1998b; Chaves et al., 2007; Chaves and Smith, 2011; Cortés-Rodríguez et al., 2008). The Garnet-

Throated Hummingbird, *Lamprolaima rhami,* is one of such taxa. It has a restricted Mesoamerican distribution mainly inhabiting patches of cloud forests, although its altitudinal range (between 1200 and 3000 m) aslo can comprise other kind of habitats, like pine-oak forests, upper tropical forests, forest edges and scrub (Howell and Webb, 1995; Schuchmann, 1999). Previous surveys propose three different taxa for this species based on subtle morphological differences: 1) *L. r. rhami* (described as subspecies), restricted to the highlands of central and southern Mexico (in the states of Puebla, Veracruz, Oaxaca and Chiapas) and Guatemala (Lesson, 1838); 2) *L. r. occidentalis* (described as a race), which can only be found in the state of Guerrero in south-western Mexico (Schuchmann, 1999); and 3) *L. r. saturatior* (described as subspecies), distributed in the highlands of Honduras and El Salvador (Griscom, 1932).

Being a resident of high fragmented cloud forests with unique bioclimatic characteristics, *Lamprolaima rhami* represents an interesting model to assess evolutionary hypotehses about geographic structure and the recognition of independent lineages that should be considered in conservation plans. Hence, the main objectives of this paper were: 1) to evaluate the genetic and morphological variation of the *Lamprolaima rhami* complex, 2) to describe the phylogenetic relationships within *L. rhami* using a multilocus dataset, and 3) to identify possible independent lineages within this complex. Based on cloud forest characteristics and site fidelity of this hummingbird species, we would expect to find high levels of genetic structure supported by congruence in morphological variation within *L. rhami* complex. Thus and according to patterns described on phylogeography (Avise et al., 1987), phylogenetic discontinuities and spatial separation are expected rather than phylogenetic continuity and lack of spatial separation.

2. Material and methods

2.1 Taxon sampling and sequencing.

We obtained tissues from 52 individuals of *L. rhami* from 13 localities across most of its geographic range (See supplementary information S1, S2). We defined 5 groups *a priori*, corresponding to as many isolated geographic regions: 1) the Sierra Madre Oriental and the northern portion of Sierra Madre del Sur (SMO&nSMS), 2) the Sierra Madre del Sur (SMS), 3) the Sierra Sur de Oaxaca (SSO), 4) highlands of Chiapas and Guatemala (EITn), and 5) highlands of Honduras and El Salvador (EITs). Tissues samples were obtained just for three geographic groups (SMO&SMSn, SMS, EITn), and provided by different biological collections, inlcuding the Museo de Zoología Alfonso L. Herrera (Universidad Nacional Autónoma de México), the Museum of Natural Science (Lousiana State University), and the Museum of Vertebrate Zoology (MVZ).

DNA was extracted using the DNAeasy™ kit (Qiagen Inc., Valencia, CA, USA), and following manufacturer's protocols. For evaluating the general genetic variation of the complex, two mitochondrial markers were obtained for the totality of samples (Control Region, *CR*; and subunits 6 and 8 from ATPase gene, *ATPase 6 & 8*), while for evaluating the phylogenetic relationships between groups, two additional mitochondrial markers and three nuclear regions were surveyed for a subsample of 15 individuals (NADH dehydrogenase subunit 2, *ND2*; NADH dehydrogenase subunit 4, *ND4*; the 7th intron of the beta fibrinogen gene, *BFib*, the regions between exons 4 and 5 of the Muscle Skeletal Receptor Tyrosine Kinase gene, *MUSK*; and a segment comprising the end of exon 6 and the beginning of exon 8 of the Ornithine Decarboxylase gene, *ODC*). These 15 individuals were chosen to have at least five individuals per geographic group (three sampled groups), and analyze phylogenetic

117 relationships by increasing loci number. Also, we included sequences from these molecular
118 markers available in GenBank for *Eugenes fulgens* and *Heliomaster constantii*, as sister group
119 and outgroup respectively (McGuire et al., 2007; Zamudio-Beltrán and Hernández-Baños,
120 2015).

121 We amplified these molecular markers via the polymerase chain reaction (PCR) using
122 specific primers and protocols (See supplementary information S2). Reactions contained 10X
123 buffer (1.25 μL), 10mM dNTP (0.19 μL), 50 mM $MgCl_2$ (0.38 μL), 10 μM of each primer
124 (0.25 μL), 0.1 μL of *Taq* (INVITROGEN), and 0.5 μL of genomic DNA (12.5 μL total
125 volume). PCR products were visualized on a 1% agarose gel, and DNA sequencing was
126 performed in the High-Throughput Genomics Unit Service of the University of Washington.
127 We edited and aligned chromatograms with Sequencher v4.8 (GeneCodes Corporation, Ann
128 Arbor, MI). All sequences were deposited in GenBank under accession numbers XXXX-
129 XXXX.

130

131 *2.2 Population structure.*

132 To evaluate the number of haplotypes and their relationships, statistical parsimony
133 haplotype networks were constructed for each mitochondrial marker (CR and ATPase 6 & 8)
134 and for their concatenated dataset using the program TCS v1.21 (Clement et al., 2000).

135 To analyze genetic diversity and genetic structure, we obtained values of haplotype
136 diversity, nucleotide diversity, mean number of pairwise differences, and population F_{ST}
137 values. This analysis was performed with 1000 replicates, using the program Arlequin v3.11
138 (Excoffier et al., 2005). Using the same program, we conducted an analysis of molecular
139 variance (AMOVA; Excoffier et al., 1992) to detect structure between populations, based on
140 comparisons between groups defined geographically.

To evaluate the isolation by distance among geographic regions, we performed a Mantel Test with 1000 iterations, comparing matrices of genetic and geographic distances, using the program zt v1.1 (Bonnet and de Peer, 2002).

2.3 Demographic analyses.

To evaluate demography and population stability, we obtained Tajima's *D* and Fu's *Fs* values, using Arlequin v2.11 (Excoffier et al., 2005), with 1000 replicates. Using the same program and parameters, we further evaluated the historical demography of each group under an expansion model with a MISMATCH distribution test and estimated its significance with the raggedness index (Harpending, 1994; Rogers and Harpending, 1992; Slatkin and Hudson, 1991). To analyze variation in effective population size through time, we used Bayesian skyline plots (BSP; Drummond et al., 2005) performed in BEAST v1.6.0 (Drummond and Rambaut, 2007), with 10 million steps for mtDNA, using a mean rate of 0.023 substitutions per site per lineage per million years (s/s/l/My), according to Control Region and ATPase estimates (Lerner et al., 2011).

2.4 Evolutionary Models and Phylogenetic analyses.

We created four databases for the 15 individuals subset of *L. rhami*: 1) mtDNA (ATPase 6 and 8, CR, ND2, ND4), 2) nDNA (BFib, ODC), 3) nuclear Z-linked (MUSK), and 4) concatenated markers. These arrangements were made to analyze topologies separately, as different markers could lead on different genetic histories (maternally, paternally or biparentally). Heterozygous sites in nuclear markers were coded according with IUPAC ambiguities. The allele phase of each nuclear locus was resolved using PHASE v2.1 (Stephens et al., 2001), and seqPHASE web server for file conversions (Flot, 2010). We used

one randomly chosen haplotype from the two generated if it was the case, for further analyses. For each molecular marker, we calculated the evolutionary model that better fit the data using jModelTest 0.1.1. (Posada, 2008), based on the Akaike Information Criterion AIC (Akaike, 1987). We performed phylogenetic reconstructions with the Bayesian Inference (BI) approach available in Mr. Bayes v3.0 (Huelsenbeck and Ronquist, 2002). We assigned different evolutionary models to each gene partition for our four phylogenetic reconstructions. We ran four simultaneous chains for each Monte Carlo Markov Chain analysis for 10 million generations, and sampling every 250 generations. We determined the burn-in value using Tracer v1.6.0 (Rambaut et al., 2013), and eliminated the initial 15% of generations. The remaining trees were used to construct a majority rule consensus tree with posterior probability distributions, which was visualized using the program FigTree v1.2.3 (http//tree.bio.ed.ac.uk/software/figtree/).

2.5 Divergence times.

Divergence time estimates were obtained using BEAST v1.6.0 (Drummond and Rambaut, 2007). We used the concatenated data set and included data from *Eugenes fulgens, E. spectabilis*, *Heliomaster longirostris*, *Atthis heloisa*, *Doricha eliza*, and *Tilmatura dupontii* as outgroups. For each partition, we assigned previous selected evolutionary model . We employed a uncorrelated lognormal relaxed clock, and a Yule speciation model to model the tree prior. We assigned a calibration node based on a secundary calibration obtained for the split between the "Mountain Gems" (*L. rhami*, *E. fulgens*, *E. spectabilis*, and *H. longirostris*) and "Bees" (*A. heloisa*, *D. eliza*, and *T. dupontii*; 18.5, 21.86-15.30 Mya; Zamudio-Beltrán et al., *in prep*.). We incorporated mean substitution rates reported previously (ATPase 6 and 8, ND2, ND4: Pacheco et al., 2011; CR: Lerner et al., 2011; BFib, MUSK, ODC: McGuire et

al., 2014). This analysis was run for 100 million generations, sampling every 1000 generations, with a burnin period of 20%. We used TreeAnnotator v1.8.2 (Rambaut and Drummond, 2007) to summarize the sampled trees as a maximum clade credibility tree, and to obtain mean divergence times with 95% highest posterior density intervals.

2.6 Morphological variation.

To examine morphological variation between groups of *L. rhami*, we took five measures from 213 voucher specimens corresponding to four or the five geographic groups defined *a priori* (SMO&SMSn, SMS, EITn, EITs). These specimens were available from different biological collections, incluiding the Museo de Zoología Alfonso L. Herrera (UNAM), the Museum of Comparative Zoology (MCZ), the American Museum of Natural History (AMNH), the Bird and Mammal Collection (UCLA), and the Moore Lab of Zoology (MLZ).

Measures for bill length (from the upper base of the bill to the tip of the upper mandible), bill width (width by the location of the nostrils), bill depth (from the upper mandible to the base of the bill by the location of the nostrils), and wing chord (the distance from the carpal joint to the tip of the longest primary) were taken with a dial calliper with a precision of 0.1 mm , while the tail length (the distance from the uropigial gland to the tip of the longest rectrix) was determined with a milimetric ruler. We performed statistical analysis (*t*-student test) to detect significative differences between males and females using the statistical software STATISTICA v7 (StatSoft, 2004). Subsequently usign the same program, we performed an analysis of variance (ANOVA) comparing four groups defined *a priori* for each variable, treating males and females separately, and performed a post-hoc analysis

212 (Fisher's Least Significant Difference Test, LSD; Williams and Abdi, 2010) to detect
213 significant differences between groups.
214 A category classification was performed with a discriminant analysis using the five
215 morphological measurements as independent variables, and the geographic groups defined as
216 grouping variable. In a second discriminant analysis we used as grouping variable the
217 populations east and west of the Isthmus of Tehuantepec (IT). The results of discriminant
218 analysis were plotted in R statistical software (Ripley, 2001), using the package ggplot2
219 (Wickham, 2009).
220
221 **3. Results**
222
223 *3.1 Genetic diversity and population structure*.
224 We obtained a concatenated dataset of 1402 bp for 52 individuals (527 bp of the CR
225 and 875 bp of ATPase 6 & 8). The complementary dataset of five molecular markers for 15
226 individuals included 863 bp of ND2, 536 bp of ND4, 686 bp of BFib, 620 bp of MUSK, and
227 581 bp of ODC. The initial dataset included 30 haplotypes (22 found with CR and 14 with
228 ATPase 6 & 8), nine of which were shared between populations. Estimates of haplotype and
229 nucleotide diversity can be found in Table 1. Overall, high values of haplotype diversity, and
230 low levels of nucleotide diversity were observed within groups (SMO&SMSn, SMS, EITn).
231 Haplotype networks revealed a significant population structure within the *L. rhami*
232 complex (Fig. 1). There was a clear separation between populations at both sides of the IT,
233 which were separated by three to twelve mutation steps depending on the dataset, while the
234 localities of the SMS were closely linked to those of the SMO&SMSn. In general, the most
235 frequent haplotype was present in populations from the Sierra Madre Oriental and the

236 northern portion of the Sierra Madre del Sur group (SMO&SMSn). In Table 2, we can
237 observe that F_{ST} values confirm high levels of geographic structure between these two groups,
238 although all values are significant. This further translated into a significant correlation
239 between the genetic distance and the geographic distance matrices, according to the Mantel
240 test, thus suggesting isolation by distance between groups ($r=0.87$, $p<0.005$).
241 AMOVA results indicated that the highest genetic variation was observed among
242 populations, with similar percentages when grouping populations according to geographic
243 region or at both sides of the IT, 76.41% and 78.74% respectively ($P<0.0001$, Table 3).
244
245 *3.2 Demographic analyses*
246 The different methods used to evaluate demographic history resulted in ambiguous
247 results. The occurence of historical population expansion was supported by negative and
248 significant values of neutrality tests, except that for Tajima's *D* statistic in SMO&SMSn and
249 SMS populations (Table 1). Mismatch distribution unimodal curve was recovered only for
250 EIT population, but no significant values of raggedness index indicated possible demographic
251 expansion in all populations as curves under the expansion model did not deviate from a
252 unimodal distribution. BSP estimates revealed that effective population size was flat across
253 time for SMS. This pattern was also found for EIT, however, higher posterior density low
254 interval presented a growing demographic tendency, and subtle demographic expansion is
255 recovered in SMO&SMS population (Fig. 2).
256
257 *3.3 Evolutionary models and Phylogenetic analyses.*

We obtained a concatenated dataset of 4679 bp. The best-fit models for each molecular marker were as follows: HKY (ATPase 6 & 8, MUSK), HKY+I (CR), TNR (ND2), GTR (ND4), and TPM1uf (BFib, ODC).

We recovered different topologies when using different kind of molecular marker dataset (see Supplementary Information S4). Phylogenetic relationships using mtDNA resulted in two main clades, one of them corresponding to all individuals from EIT group (PP>0.95). Two main clades were recovered when using Z-linked dataset, one including individuals from all different populations and one containing three of the five individuals from EIT group. The hypothesis obtained with nDNA resulted in a topology where four of the five individuals from SMO&SMSn were grouped in a well supported clade that belonged to a bigger clade that contained individuals from all different populations, out of this main clade three of the five individuals from SMS group were nested. To get a multilocus phylogenetic approach, we obtained a topology using concatenated dataset, the phylogenetic reconstruction showed a marked differentiation between groups at both sides of the IT (Fig. 3), although the separation between the SMO & SMSn and the SMS groups could not be recovered. Most of clades are supported by high posterior probabilities (PP>0.95).

3.4 Divergence times.

Our divergence time estimations (Fig. 4, Table 4) showed that the split between *L. rhami* complex and its sister group (*E. fulgens*) was dated around 10.46 Mya (12.66-8.32 Mya). Time estimation for *L. rhami* complex was dated ca. 0.68 Mya (0.93-0.45 Mya), that corresponded to the divergence between populations at both sides of the IT. Group at west from the IT (SMO&SMSn and SMS) was dated at ~0.28 Mya (0.42-0.16 Mya), and east group (EITn) around 0.21 Mya (0.33-0.10 Mya).

3.5 Morphological variation.

Dimorphism tests between males and females revealed significant differences for all variables. Females showed differences for all variables excepting for bill lenght (F=2.35, p=0.07), while in the males all variables showed significant statistically differences between groups (see supplementary information S5). In all cases, except in female bill lenght comparison, statistical differences between groups were detected when LSD test was performed. Such differences were further observed in the discriminant analyses using only the first two cannonical roots (Fig. 4). For both sexes the most informative variables were bill depth and bill lenght. Comparisons between groups were significant in all cases except between group A (SMO&SMSn) and D (EITn).

4. Discussion

Our study provides evidence of high levels of genetic differentiation and geographic correspondence among isolated populations in the *L. rhami* complex, according to haplotype network reconstructions, and high F_{ST} values, which confirm to isolation by distance test. The three sampled geographic regions (genetic data; SMO & SMSn, SMS, and EITn) mostly contained unique haplotypes that showed correspondence with the significant morphological differences found between them, so that allopatric divergence represents an important factor promoting genetic and morphological structure.

According to the original descriptions of phylogeographic patterns, the genetic variation found in *L. rhami* corresponds to a phylogenetic discontinuity and spatial vicariance pattern (Avise et al., 1987), which is the result of long-term isolation, and restricted gene flow among groups, probably promoted by geographic barriers. This pattern of high genetic

306 differentiation has been found in many Mesoamerican species of animals and plants
307 (Arbeláez-Cortés et al., 2014; Barber, 1999; Bonaccorso, 2009; Bryson et al., 2011;
308 Castañeda-Rico et al., 2014; Smith et al., 2011; Zarza et al., 2008). As expected, levels of
309 genetic variation were correlated with a pattern of isolation by distance associated with
310 disjunt distribution of cloud forests, where particular environmental characteristics have been
311 reported as drivers of differentiation between populations (Ramírez-Barahona and Eguiarte,
312 2014). In the case of populations west of the Isthmus of Tehuantepec, geographic structure
313 could be explained by limited gene flow between regions (SMO & SMSn, SMS, and EITn)
314 due to this isolation more than the existence of geographic barriers. In contrast, the genetic
315 separation between populations at both sides of the Isthmus of Tehuantepec is certainly
316 influenced by this geographic barrier plus the distance variable. High levels of geographic
317 structure have been found on other hummingbird species, related to differences on present or
318 historical ecological conditions (*Adelomyia melanogenys*: Chaves et al., 2007; *Lampornis*
319 *amethystinus*: Ornelas et al., 2016). Also, moderated levels of differentiation have been found
320 on hummingbird species codistributed in Mesoamerican cloud forests (*Campylopterus*
321 *curvipennis*: González et al., 2011; *Amazilia cyanocephala*: Rodríguez-Gómez et al., 2013).
322 Despite the well-known movement abilities of Trochilidae species, some studies have
323 found that geographical barriers are crucial in promoting high levels of differentiation and in
324 the diversification of independent evolutionary lineages in various regions, such as the Andes
325 region (e.g. *Adelomyia melanogenys*, Chaves and Smith, 2011), Mesoamerica (Ornelas et al.,
326 2016), the Motagua fault region (Rodríguez-Gómez and Ornelas, 2014), and the Isthmus of
327 Tehuantepec (González et al., 2011). By contrast, there is a hypothesis suggesting that the
328 high levels of intraspecific diversification found mostly on lowland Neotropical birds, are
329 related to limited dispersal ability (Burney and Brumfield, 2009). *L. rhami* exhibits some

altitudinal movements related with the presence of resources available along elevational gradients (Schuchmann, 1999). However, long dispersal movements have not been reported for this species, so both factors could be influencing the geographic separation observed herein (geographic barriers and limited longitudinal and latitudinal migration movements).

Divergence time estimations provided evidence of a Pleistocene origin of *L. rhami* complex (0.68 Mya, 0.93-0.45 Mya), followed by a subsequent separation of populations across the Isthmus of Tehuantepec. Moreover, demographic history was evaluated under different methods (neutrality tests, mismatch distributions and BSP). The demographic analyses presented herein showed ambiguous patterns of populations dynamics, and despite that range expansion is revealed in some tests (Tajima's *D* and/or Fu's *Fs*), no considerable population size changes through time can be detected by the BSP approach. Mismatch analysis recovered a unimodal curve, representing a possible demographic expansion on EIT population, the most genetically differentiated region. It has been reported that climatic fluctuations occurred during the Pleistocene could affect highland distributed species, expanding and contracting their ranges, promoting allopatric differentiation (Still et al., 1999).

Concordance among genetic structure and morphological variation was found. Our results of phenotypic variation for 213 voucher specimens of *L. rhami* showed geographic structure between all populations. Despite that the group sampled at the east of the Isthmus of Tehuantepec (EITn) was the most genetically differentiated, the morphological results showed that groups at Sierra Madre del Sur (SMS), and the southern group east of the Isthmus of Tehuantepec (EITs), were the most differentiated according to phenotypic traits. Unfortunately, we had no access to samples from ETIs region, so we could not confirm if this morphological variation is consistent at genetic level. Also, we neither had access to enough voucher specimens from SSO to conduct a reasonable statistical analysis.

354 According to our multilocus phylogenetic approach, *L. rhami* is a complex conformed
355 by two reciprocal monophyletic groups (full evidence, concatenated dataset). The use of
356 different kinds of molecular markers resulted in different structured phylogenetic hypotheses,
357 due to inheritance factors (e.g. recombination) and/or the differences on nucleotide
358 substitution rates. However, all the hypotheses recovered monophyletic groups associated to
359 an allopatric geographic region.
360 The large evidence of population differentiation for each isolated geographic region,
361 must be taking into account to make taxonomic reevaluations for this complex. The case of
362 *Eugenes fulgens* complex, the sister group of *L. rhami*, is one example of taxonomic
363 reevaluation based on a multilocus analysis (Gill, 2015.; Zamudio-Beltrán and Hernández-
364 Baños, 2015). In this particular case, the subspecies recognition was underestimating
365 biodiversity at the intraspecific level. The problem of an incorrect placement of subspecies is
366 that this could promote mismanagement in conservation efforts (Zink, 2004). Taking into
367 account the level of threat that is reported in cloud forests in Mesoamerica, and the reduced
368 geographic distribution of this species, we suggest considering populations inhabiting each
369 geographic region as separate management units. Moreover, multiple evidence of
370 differentiation presented here must be taking into account to consider populations at both
371 sides of the Isthmus of Tehuantepec, as full species.
372 Even though we found distinguishable lineages, further work is needed, including a
373 larger sampling effort in the southern highlands of Oaxaca (Mexico), and Central American
374 highlands (Hounduras and El Salvador). Probably, despite of this study is still
375 underestimating the number of independent units within *L. rhami* complex.
376
377

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Table 1. Statistical parameters of genetic diversity, population structure and population demography for mtDNA. n: number of sequences used, h: number of haplotypes, Hd: haplotype diversity, π: nucleotide diversity, Pi: mean number of pairwise differences.

GROUP	n	h	Hd	π	Pi(theta)	Tajima's D	Fu's Fs Test
SMO&SMSn	22	9	0.81	0.0022	4.22	-0.559 (P=0.30)	-5.505 (P=0.003)
SMS	9	6	0.89	0.0026	3.73	-0.886 (P=0.22)	-2.77 (P=0.022)
EIT	21	15	0.94	0.0021	3.50	-1.988 (P=0.012)	-14.93 (P=0.000)

Table 2. Population pairwise F_{ST} mtDNA

	SMO&SMSn	SMS	EIT
SMO&SMSn	----		
SMS	0.176*	----	
EIT	0.769*	0.784*	----

*P<0.05

Table 3. AMOVA results on *Lamprolaima rhami* populations defined according to biogeographic regions, and grouped into groups separated by the Isthmus of Tehuantepec.

	d.f.	Sum of squares	Variance components	Percentage of variation	Fixation indices
Biogeographic region					
Among populations	2	167.63	5.04	76.41	
Within populations	49	76.23	1.56	23.59	
Total	51	243.87	6.59		F_{ST}=0.76***
Isthmus of Tehuantepec					
Among populations	1	159.05	6.28	78.74	
Within populations	50	84.82	1.70	21.26	
Total	51	243.86	7.98		F_{ST}=0.79***

Table 4. Divergence times, posterior probabilities and 95% confidence intervals (high posterior density, HPD) in millions of years (Mya) in *L.rhami* complex.

Node	PP	Age Mya (95% of HPD)
A	1.00	14.02 (16.56-11.50)
B	0.71	12.79 (15.37-10.35)
C (*E.fulgens*/*L.rhami*)	1.00	10.46 (12.66-8.32)
D	1.00	5.83 (7.09-4.59)
E	1.00	4.54 (5.61-3.50)
F	1.00	3.82 (4.83-2.91)
G	1.00	0.69 (1.02-0.4)
H (*L.rhami*)	1.00	0.68 (0.93-0.45)
I	1.00	0.28 (0.42-0.16)
J	1.00	0.21 (0.33-0.10)
K	0.90	0.21 (0.31-0.12)
L	0.27	0.16 (0.26-0.07)
M	0.41	0.15 (0.24-0.06)
N	0.61	0.14 (0.23-0.06)
O	0.30	0.12 (0.21-0.03)
P	0.66	0.09 (0.17-0.02)
Q	0.98	0.08 (0.14-0.02)
R	0.97	0.07 (0.15-0.01)
S	0.87	0.04 (0.11-0.00)
T	1.00	0.02 (0.06-0.00)
U	1.00	0.02 (0.05-0.00)

Figure captions

Figure 1. Statistical parsimony haplotype networks for 52 individuasl of *L. rhami*, constructed with three different databases: ATPase 6 and 8, CR and concatenated mtDNA markers. Different colors in networks correspond to the different geographic groups on the map. Size of each circle is proportional to the number of individuals carrying each haplotypes.

Figure 2. Mismatch distributions and Bayesian skyline plots for each geographic group of *L. rhami*. In mismatch distributions, solid lines indicate the observed distributions of pairwise differences, and dotted lines represent simulated distributions under a model of population expansion. In Bayesian skyline plots, solid lines represent median estimates and shaded areas represent 95% confidence intervals. Geographic groups are represented in different colors according to the geographic regions on the map.

Figure 3. Phylogenetic Bayesian Inference reconstruction from 15 individuals of *L. rhami* complex using mitochondrial and nuclear markers (ATPase 6 and 8, CR, ND2, ND4, MUSK, BFib, ODC). Posterior probabilities PP>0.95 are shown (*). Different colors represent different groups according to the geographic regions on the map. Dotted line represents the separation at both sides of the Isthmus of Tehuantepec.

Figure 4. Phylogeny illustrating the divergence times for *L. rhami* complex as generated by BEAST. Bars on each node represent 95% of high posterior densities of divergence times (HPD). Letters at nodes corresponds to those referred in Table 4. Color bars represent groups defined *a priori*: SMO & SMSn (Sierra Madre Oriental and north from Sierra Madre del Sur), SMS (Sierra Madre del Sur), and ETIn (north from east of the Isthmus of Tehuantepec), Ma (Million years).

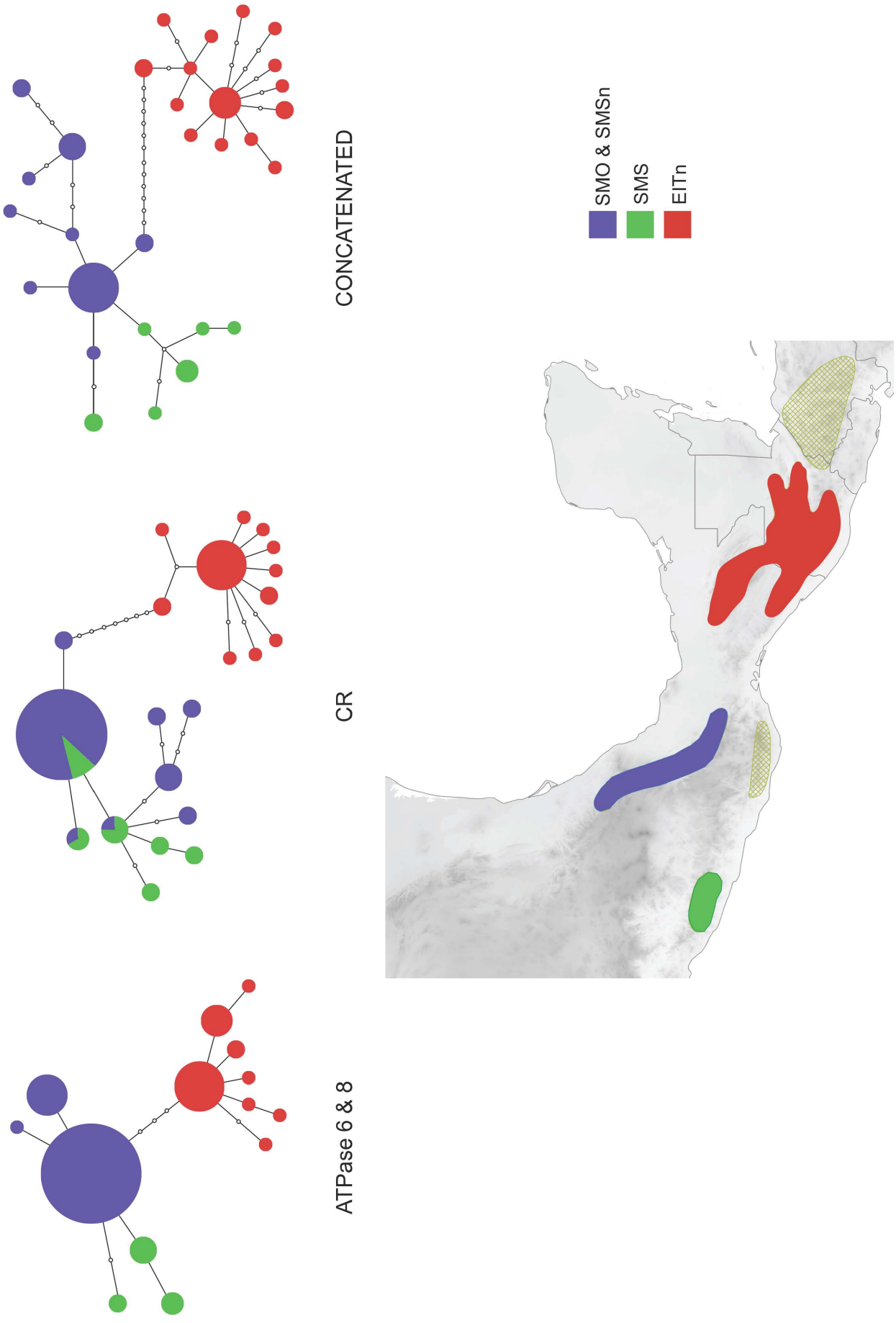
Figure 5. Discriminant analysis for males and females of *L. rhami*. A) Plots representing geographic groups in different colors, mean values are represented by a black dot for each group. B) Plots by geographic groups differentiating populations at east and west from the Isthmus of Tehuantepec. C) Plots representing populations at east and west from the Isthmus of Tehuantepec. Statistical differences between groups are represented with an asterisk (*P<0.05).

Figure S2. Geographic distribution of *Lamprolaima rhami* complex. Red hexagons represent sampled localities corresponding to tissues used in this study. Geographic groups defined *a priori* are drawn by different colors and represented by different letters on the map. Geographic groups: SMO & SMSn (Sierra Madre Oriental and north from Sierra Madre del Sur), SMS (Sierra Madre del Sur), SSO (south from Sierra de Oaxaca), ETIn (north from east of the Isthmus of Tehuantepec), EITs (south from east of the Isthmus of Tehuantepec). Illustrations were obtained from Handbook of the birds of the world, Alive (http://www.hbw.com) with the corresponding permission.

Figure S4. Phylogenetic Bayesian Inference reconstructions for: a) mitochondrial DNA (ATPase 6 and 8, CR, ND2, ND4), b) Z-linked marker (MUSK), and c) concatenated nuclear markers (BFib, ODC) in *L. rhami* complex. Posterior probabilities PP>0.95 are shown (*). Different colors represent different groups according to the geographic regions on the map.

Figure S5. Morphological characters taken for males and females of *L. rhami* groups. Boxplots show the percentiles of 25%, 50% (median), and 75%, upper and lower whisker show quartiles of 25%. Geographic groups are represented in different colors according to geographic regions. Numbers above or below each boxplot represent sampled individuals. Statistical differences between groups are represented with an asterisk (*P<0.05).

Figure 1.



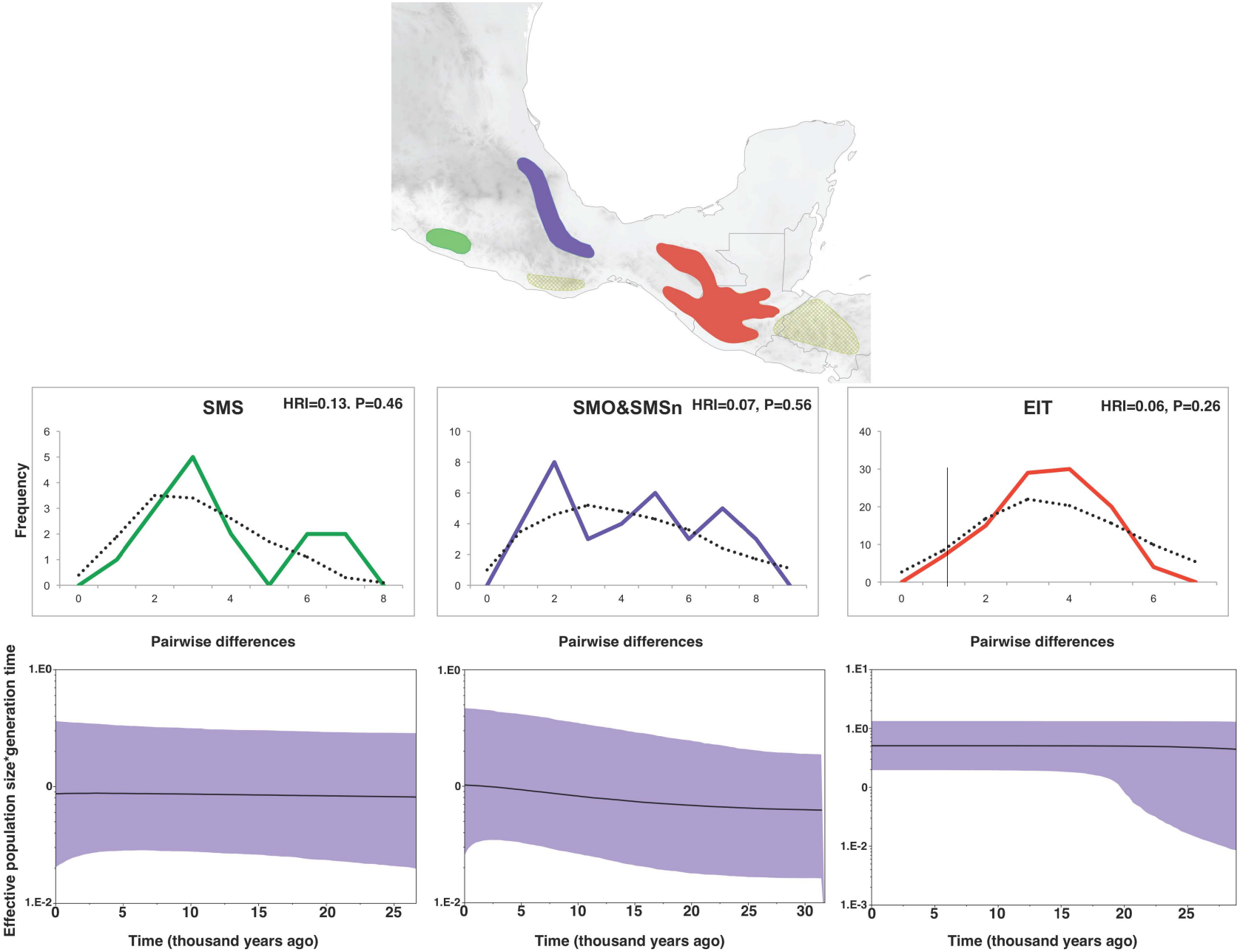

SMS
HRI=0.13, P=0.46
SMO&SMSn
HRI=0.07, P=0.56
EIT
HRI=0.06, P=0.26
Frequency
Pairwise differences
Pairwise differences
Pairwise differences
Effective population size*generation time
1.E0
0
1.E-2
1.E1
1.E-3
Time (thousand years ago)
Time (thousand years ago)
Time (thousand years ago)

Figure 2.

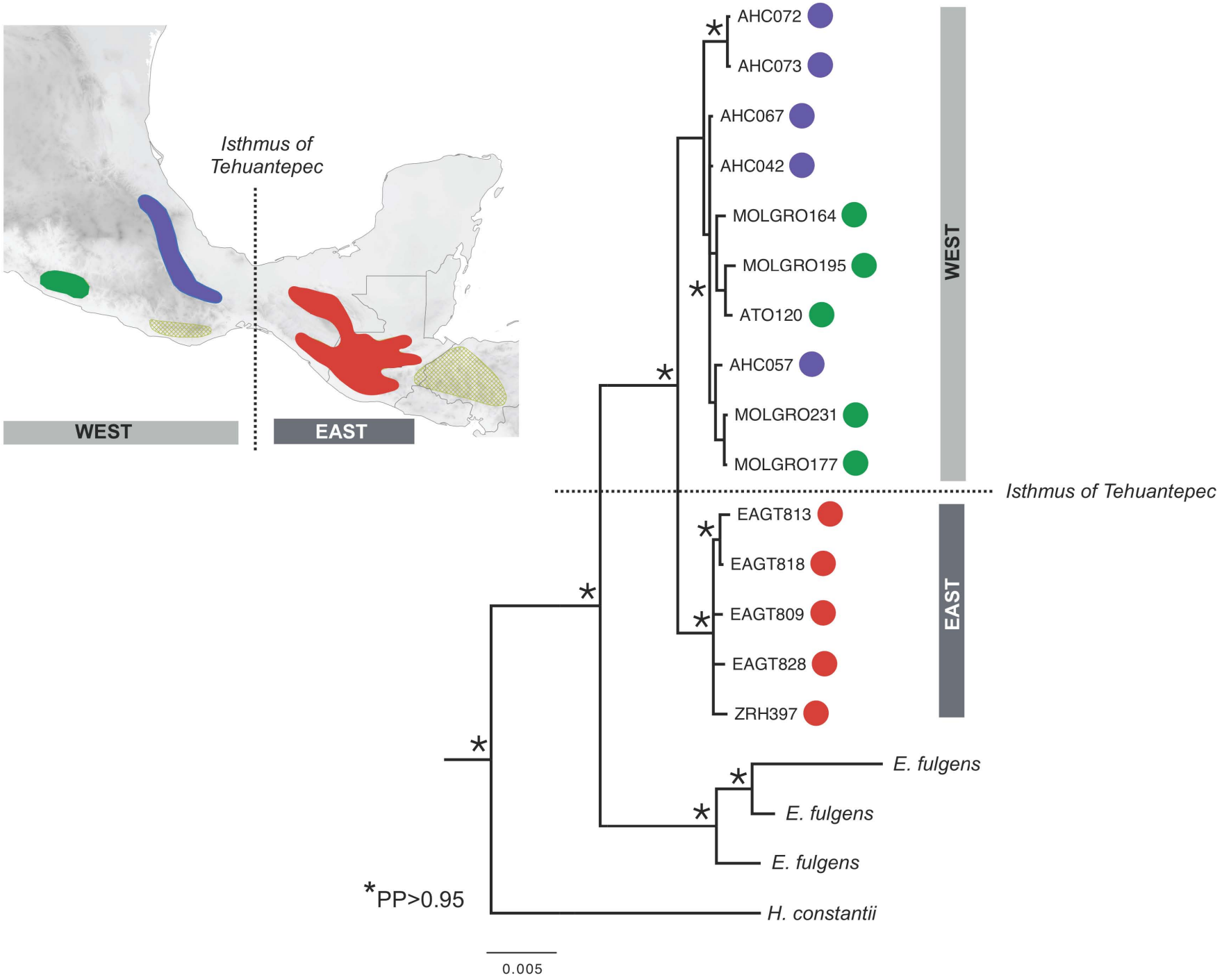

Isthmus of Tehuantepec
WEST
EAST
AHC072
AHC073
AHC067
AHC042
MOLGRO164
MOLGRO195
ATO120
AHC057
MOLGRO231
MOLGRO177
WEST
Isthmus of Tehuantepec
EAGT813
EAGT818
EAGT809
EAGT828
ZRH397
EAST
E. fulgens
E. fulgens
E. fulgens
H. constantii
*PP>0.95
0.005

Figure 3.

Figure 4.

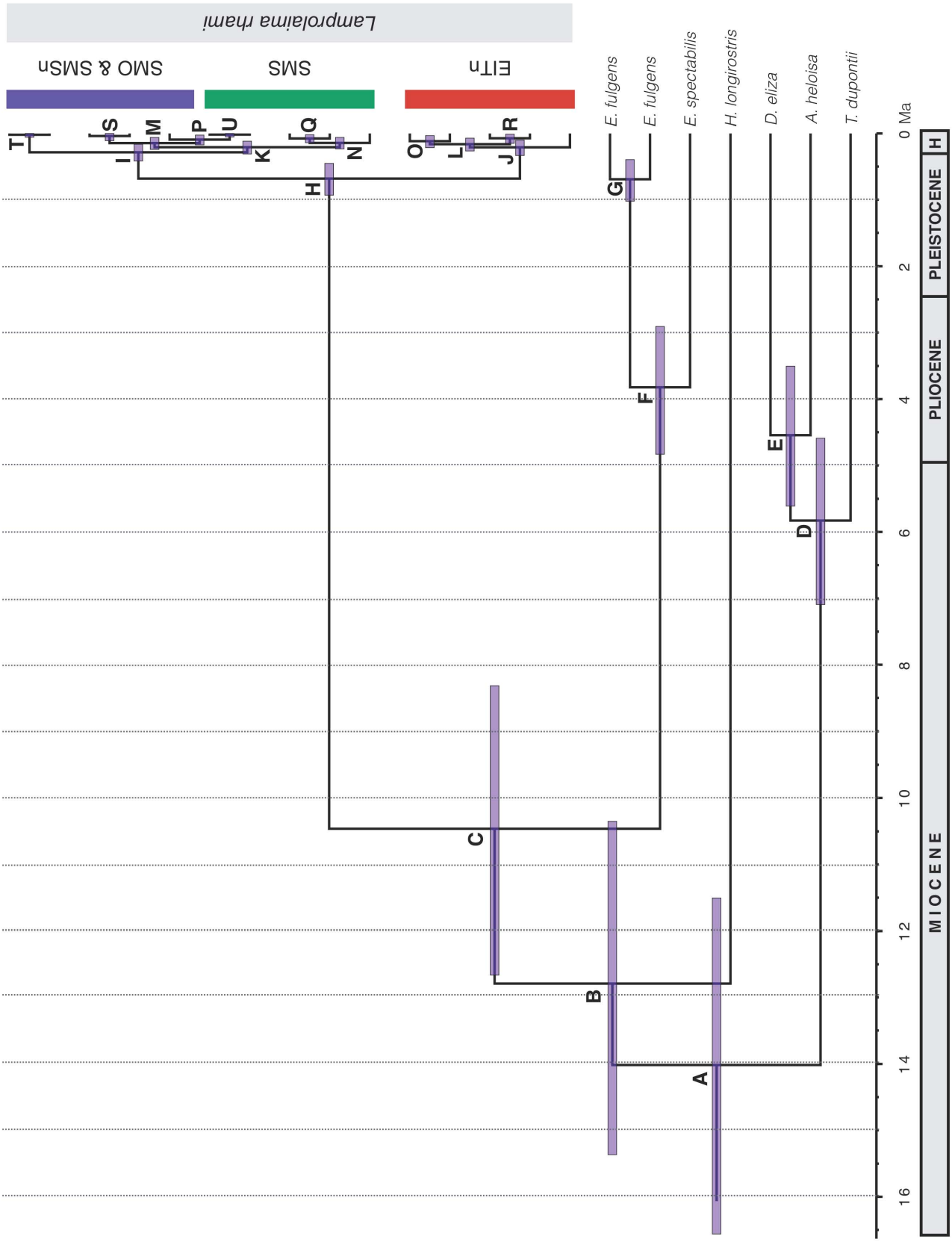
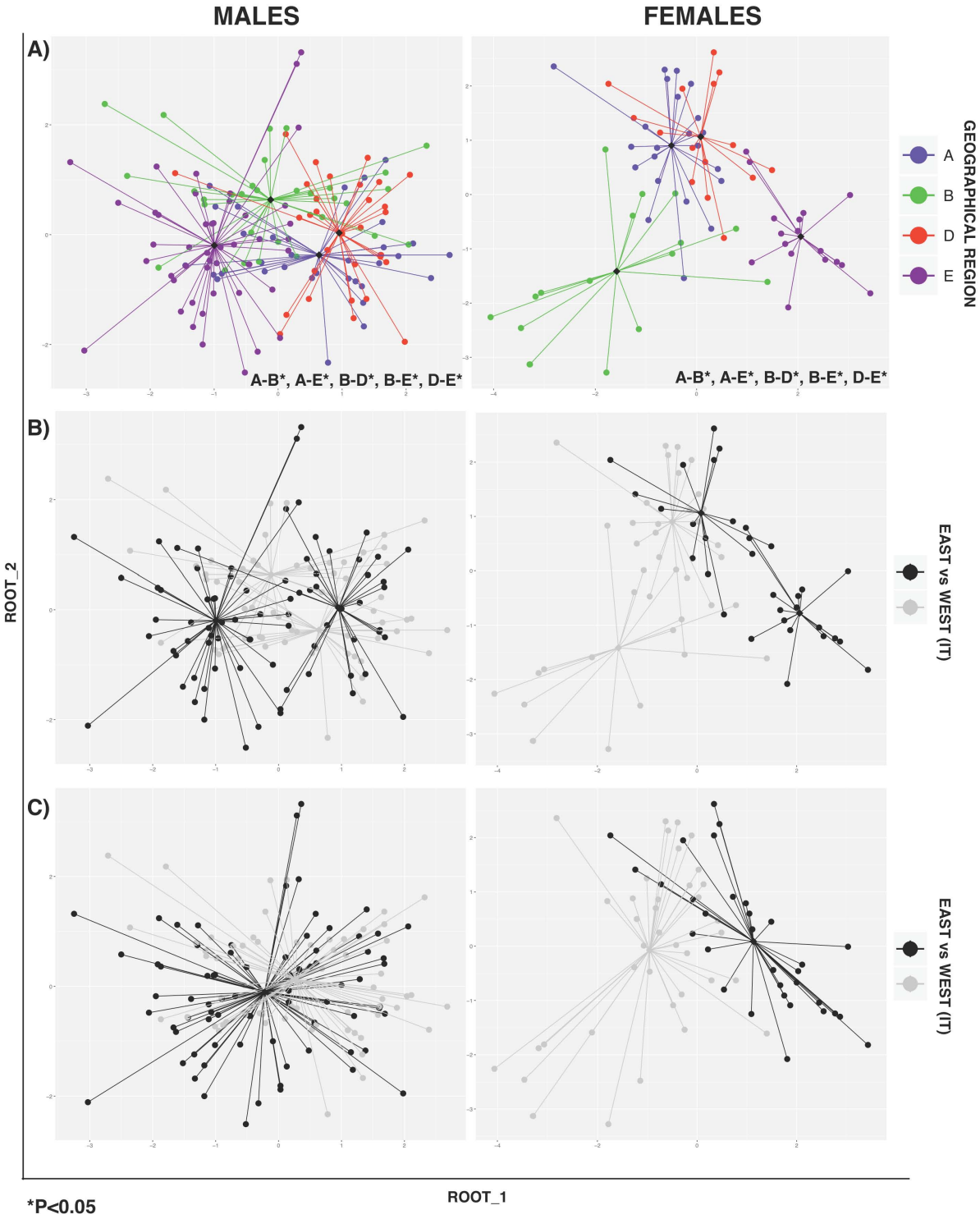

Lamprolaima rhami
SMO & SMSn
SMS
EITn
T
S
M
P
U
I
K
Q
N
H
O
L
J
R
E. fulgens
E. fulgens
E. spectabilis
H. longirostris
D. eliza
A. heloisa
T. dupontii
G
F
E
D
C
B
A
0 Ma
2
4
6
8
10
12
14
16
MIOCENE
PLIOCENE
PLEISTOCENE
H

Figure 5.


MALES
FEMALES
A)
A-B*, A-E*, B-D*, B-E*, D-E*
A-B*, A-E*, B-D*, B-E*, D-E*
GEOGRAPHICAL REGION
A
B
D
E
B)
C)
EAST vs WEST (IT)
EAST vs WEST (IT)
ROOT_2
ROOT_1
*P<0.05

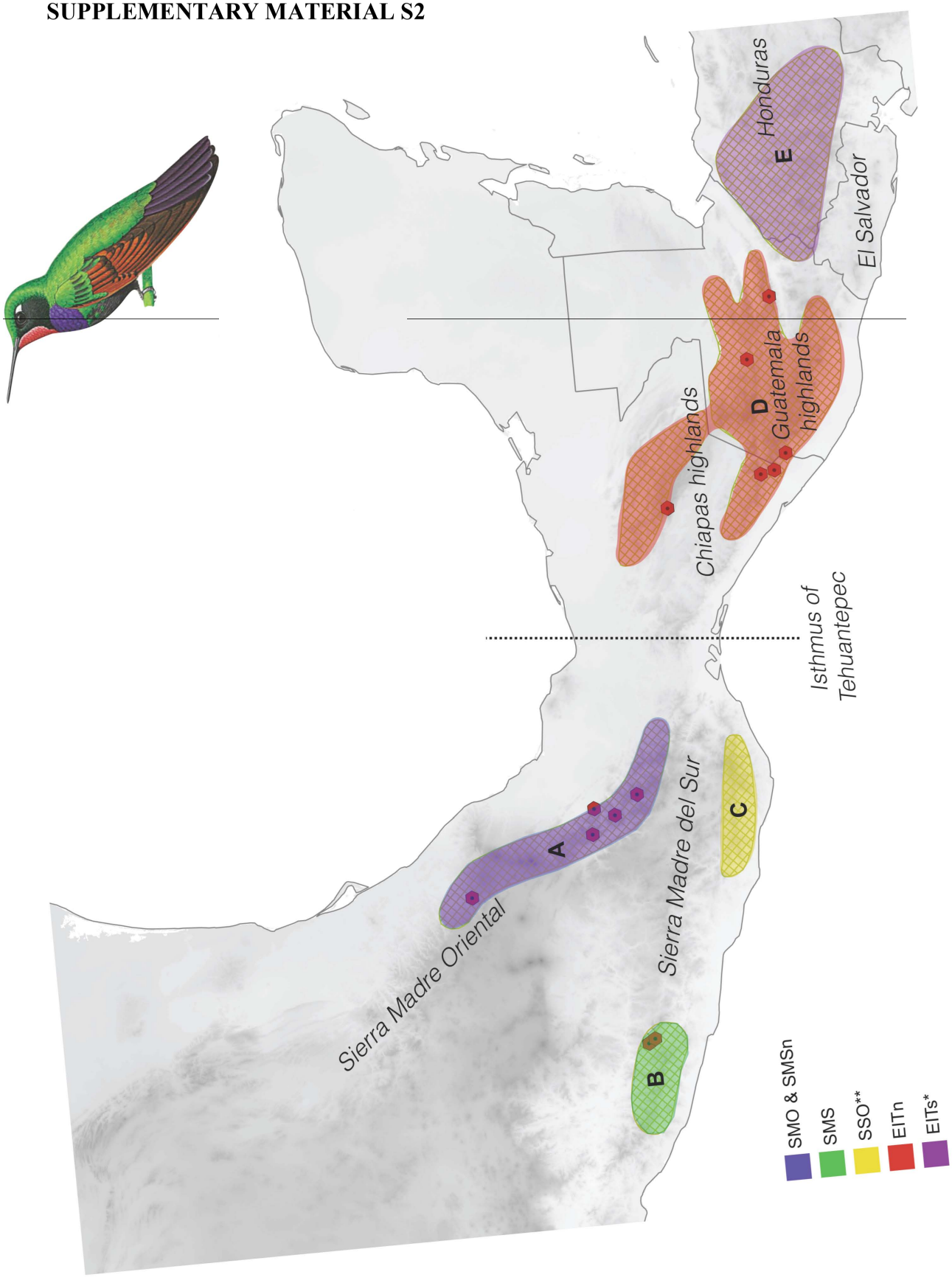
Supplementary material S1

Localities, geographic groups (GG), number of sequences, coordinates and biological collections of *Lamprolaima rhami* tissue samples.

#ID	Localities	**GG**	mtDNA		Latitude	Longitude	BC
			CR	ATPase 6 &8			
1	Puebla, Tetela.	**SMOr**	5	5	19.88	-97.69	UNAM
2	Oaxaca, Puerto de La Soledad.	**SMSn**	9	9	18.16	-96.99	UNAM
3	Oaxaca, La Esperanza.	**SMSn**	1	1	17.51	-96.50	UNAM
4	Oaxaca, Distrito de Cuicatlán.	**SMSn**	3	3	17.84	-96.75	UNAM/LSU
5	Oaxaca, San Martín Caballero.	**SMSn**	4	4	18.11	-96.64	UNAM
6	Guerrero, Carrizal de Bravo.	**SMS**	5	5	17.67	-99.88	UNAM
7	Guerrero, Carrizal de Bravo	**SMS**	4	4	17.58	-99.83	UNAM
8	Chiapas, Cerro Huitepec	**EITn**	5	5	16.73	-92.68	UNAM
9	Chiapas, Cerro Mozotal	**EITn**	5	5	15.42	-92.34	UNAM
10	Chiapas, Cerro Boquerón	**EITn**	4	4	15.23	-92.30	UNAM
11	Chiapas, Volcán Tacaná	**EITn**	3	3	15.06	-92.08	UNAM
12	Guatemala,	**EITn**	2	2	15.08	-89.94	UCB
13	Guatemala	**EITn**	2	2	15.46	-90.77	UCB

BC: Biological Collection.
UNAM: Universidad Nacional Autónoma de México, Museo de Zoología Alfonso L. Herrera.
LSU: Louisiana State University, Museum of Natural Science.
UCB: University of California Berkeley, Museum of Vertebrate Zoology.

SUPPLEMENTARY MATERIAL S2

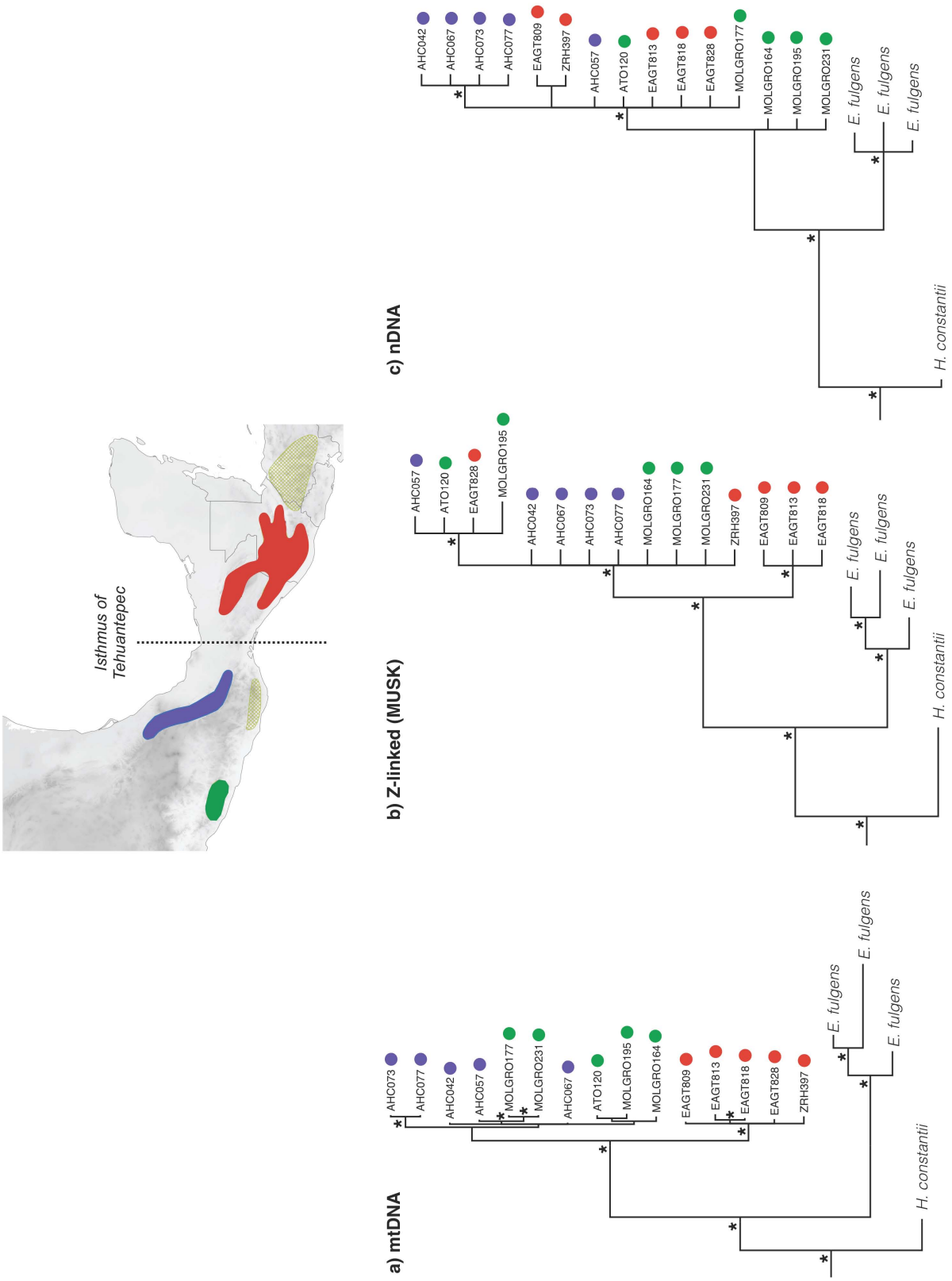


Supplementary material S3.

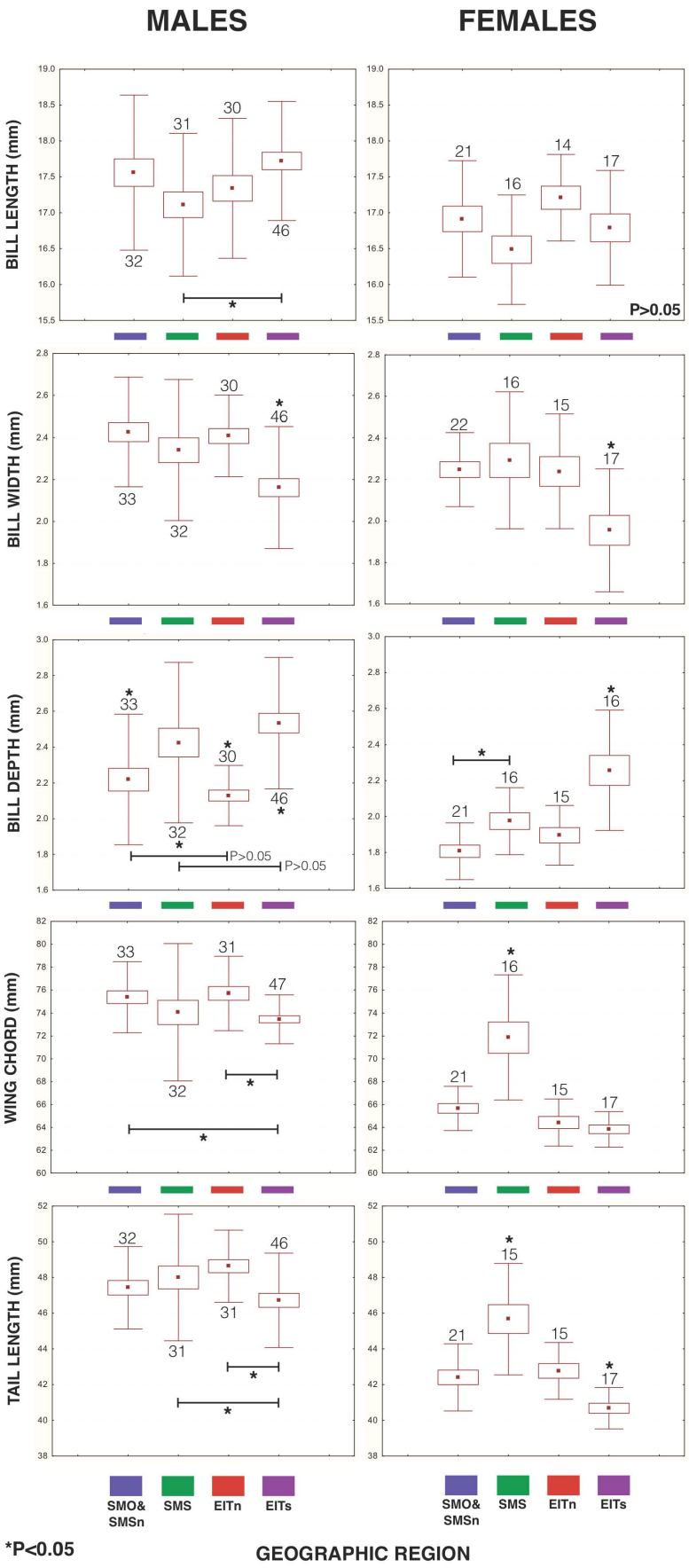
Primers and PCR protocols used in this study.

Gene	Primer name	Primer sequence	References	PCR protocol		
				Denaturation	Annealing (35X)	Extension
ND2	L5219	CCCATACCCCGAAAATGATG	Sorenson *et al.*, 1999.	1x 03:00(94°C)	00:30(94°C), 00:30(54°C), 00:45(72°C)	1x 10:00(72°C)
ND2	H6313	CTCTTATTTAAGGCTTTGAAGGC				
ND4	ND4	CACCTATGACTACCAAAAGCTCATGTAGAAGC	Arévalo *et al.*, 1994.	1x 05:00(94°C)	00:30(95°C), 00:30(55°C), 00:45(72°C)	1x 07:00(72°C)
ND4	LEU	CATTACTTTTACTTGGATTTGCACCA				
ATPase6 and 8	CO2GQL	GGACAATGCTCAGAAATCTGCGG	Eberhard et al., 2004	1x 03:00(94°C)	00:30(94°C), 00:30(58°C), 00:45(72°C)	1x 10:00(72°C)
ATPase6 and 8	CO3HMH	CATGGGCTGGGGTCRACTATGTG				
CR	ARCOIF	AATTTTATGGTCTTTGTGTGTGAA	González et al. 2011	1x 03:00(94°C)	00:30(94°C), 00:30(50°C), 00:45(72°C)	1x 10:00(72°C)
CR	ARCOIR	ACCCTAGCACAACTCGCACT				
BFib	BFib-17L2	TGGGAGGTGAAGCAGCTAAGAAAAACAA	Prychitko & Moore, 1997.	1x 10:00(94°C)	01:00(92°C), 01:00(50°C), 01:00(72°C)	1x 07:00(72°C)
BFib	BFib-17U2	CATCCATGCAGTTCTGGCAATTC				
MUSK	MUSK-F3	GCTGTACTTCCATGCACTACAATG	McGuire *et al.*, 2014.	1x 05:00(95°C)	00:25(95°C), 00:25(50°C), 01:00(72°C)	1x 07:00(72°C)
MUSK	MUSK-R3	ATCCTCAAATTTCCCGAATCAAG				
ODC	ODC-2F	GCGTGCAAAAGAACTTGACC	McGuire *et al.*, 2014.	1x 03:00(94°C)	00:30(94°C), 00:30(57°C), 00:30(72°C)	1x 05:00(72°C)
ODC	ODC-2R	AGCCACCACCAATATCAAGC				

SUPPLEMENTARY MATERIAL S4



SUPPLEMENTARY MATERIAL S5



DISCUSIÓN GENERAL

En el presente estudio proponemos una hipótesis filogenética para el clado de las "Gemas de las Montañas", perteneciente a la familia Trochilidae, en un contexto biogeográfico y evolutivo en la región Neotropical. Además de dilucidar los patrones que han favorecido a la actual distribución de dos especies pertenecientes a este grupo y que son consideradas grupos hermanos: *Eugenes fulgens* y *Lamprolaima rhami*, a partir del análisis de su estructuración geográfica. Para este análisis se tomaron en cuenta varios aspectos como la variación a nivel genético y morfológico, los tiempos de divergencia y la dinámica histórica poblacional en cada uno de ellos. En ambos casos proponemos una historia evolutiva que está situada en las tierras altas de Mesoamérica.

Con base en los resultados obtenidos a partir del análisis filogenético y biogeográfico para el grupo de las "Gemas de las Montañas", sugerimos que este grupo no tuvo un origen Sudamericano (Bleiweiss, 1998a; McGuire et al., 2007), sino que surgió en la región sur de Norteamérica y Centroamérica, con eventos de dispersión hacia el norte y sur, posteriores a su establecimiento en la región central de América. Previamente se había sugerido un origen Norteamericano para las "Gemas de las Montañas" (Bleiweiss, 1998a). Sin embargo, las conclusiones hechas en dicho estudio no contemplaban la incorporación de especies sudamericanas, ya que se creía que el límite de distribución del grupo era Centroamericano. Los trabajos más recientes sobre la historia evolutiva de la familia Trochilidae apoyan la hipótesis de un origen Centroamericano para el grupo de las "Gemas de las Montañas" (McGuire et al., 2007; McGuire et al., 2014). En dichos estudios se plantea que el ancestro del clado de las "Abejas" y de las "Gemas de las Montañas" llegó a Centroamérica durante un evento único de dispersión desde Sudamérica, hace alrededor de 12 Ma, fecha que sugieren que coincide con el cierre del Istmo de Panamá. Nuestros resultados muestran una separación entre el clado de las "Abejas" y de las "Gemas de las Montañas" más antiguo (18.5 Ma, 21.86-15.30 Ma), lo cual indica que la invasión desde Sudamérica se llevó a cabo a través del mar, antes del término del cierre del Istmo de Panamá. Nuestras estimaciones de tiempos de divergencia concuerdan con las estimaciones encontradas recientemente en un estudio llevado a cabo para describir las relaciones y la historia biogeográfica del género *Amazilia* (Ornelas et al., 2013), las cuales sugieren eventos de especiación más antiguos que los propuestos con anterioridad. La separación del grupo de las "Gemas de las Montañas" y de su grupo hermano, las "Abejas", se llevó a cabo durante el Mioceno, periodo durante el cual se tiene descrita la mayor radiación para la familia Trochilidae (Bleiweiss, 1998b).

La expansión del rango geográfico en el grupo se llevó a cabo durante los primeros dos eventos de diversificación, que contemplaron el origen del género *Lampornis* y el origen del clado perteneciente a *Eugenes*, *Lamprolaima*, *Heliomaster* y *Panterpe*, definidos principalmente por múltiples eventos de dispersión, siendo éste mecanismo uno de los factores reportados más importantes en la diversificación a lo largo del Neotrópico (Smith et al., 2014). Durante estos primeros eventos de dispersión se llevó a cabo un establecimiento a lo largo de las tierras altas. El evento de recolonización hacia Sudamérica ocurrió durante la separación de *Heliomaster* y *Panterpe,* evento durante el cual también se llevó a cabo la recolonización hacia las tierras bajas a mediados del Mioceno (13.14 Ma, 16.00-10.56 Ma).

A pesar de que en general la radiación del grupo de las "Gemas de las Montañas" se debió a procesos de dispersión, se obtuvo evidencia de que para los eventos de divergencia más recientes, los procesos de vicarianza fueron más comunes y ocurrieron durante los periodos del Plioceno y Pleistoceno, donde se logran ubicar eventos de separación a nivel intraespecífico en algunas de las especies. Algunos eventos de especiación y variación genética a nivel intraespecífico en el grupo de las aves, se han relacionado principalmente con cambios en los rangos de distribución (contracciones y expansiones) durante los periodos glaciales (Hewitt, 1996). Sin embargo, este patrón no es consistente para todos los linajes, ya que varios grupos de ésta región presentan divergencias más antiguas al Pleistoceno (Ericson, 2008). Uno de los eventos de vicarianza que logramos identificar fue el de la separación de *L. sybillae* (distribuído en las tierras altas de Honduras y el norte de Nicaragua) y el grupo conformado por *L. cinereicauda*, *L. calolaemus* y *L. castaneoventris* (distribuídos en las tierras altas de Costa Rica y Panamá). Este evento de especiación alopátrica con fecha aproximanda de 3.85 Ma, posiblemente está relacionado con la presencia de una importante barrera geográfica, la depresión de Nicaragua, la cual ha sido reportada como promotora de eventos de divergencia durante el Mioceno y Plioceno (e. g. Arbeláez-Cortés et al., 2010).

Las "Gemas de las Montañas" es un grupo perteneciente a la región Neotropical, la cual comprende la región geográfica delimitada a partir de las tierras altas de México hacia Sudamérica, donde Mesoamérica ha sido considerada como una zona biogeográfica de transición entre la región Neártica y la región Neotropical (Ríos-Muñoz, 2013; Weir, 2009). Con respecto a los mecanismos más importantes en la especiación del grupo de las aves, se sabe que diversos factores han influido en los procesos de especiación en el Neotrópico, los cuales combinan en general factores ecológicos, geográficos, geológicos y climáticos, a diferentes escalas espacio-temporales. Aunado a esto, importantes eventos geológicos han sido estudiados y considerados cruciales en la historia de

la diversificación de aves del Neotrópico. Un ejemplo de esto es el Gran Intercambio Biótico Americano (GABI; Webb, 2006), en el que se produjo un intercambio de especies distribuidas al norte y al sur de América, a través del puente creado por el cierre del Istmo de Panamá, hace alrededor de 3.5 Ma. Principalmente éste intercambio en el grupo de las aves ocurrió de sur a norte, aumentando en gran medida la tasa de intercambio entre grupos de aves altamente especializadas, a diferencia de grupos con tendencias más generalistas (Weir et al., 2009). Se considera que este evento unificó las avifaunas del norte y sur de América en una nueva región biogeográfica, la región del Neotrópico (Smith y Klicka, 2010). Otro evento importante, precedido al gran intercambio biótico, fue la formación de la Cordillera de los Andes, su arreglo geográfico conformado por montañas aisladas y valles con la presencia de gran variedad de hábitats, ha promovido importantes eventos de diversificación en el grupo de las aves, acentuando que el evento de establecimiento en América y de mayor radiación en la familia Trochilidae se llevó a cabo en ésta región (~22 My, McGuire et al., 2014).

Con respecto a las reconstrucciones filogenéticas, estudios previos han propuesto hipótesis generales de dichas relaciones para la familia Trochilidae (Altshuler et al., 2004; Bleiweiss, 1998b; Bleiweiss et al., 1997; McGuire et al., 2007). Sin embargo, uno de nuestros principales objetivos fue proponer una hipótesis detallada para el grupo de las "Gemas de las Montañas", incrementando el muestreo a nivel intraespecífico, con el propósito de esclarecer en su totalidad las relaciones filogenéticas y detectar la presencia de estructuración genética a nivel poblacional. En primer lugar se apoyó la monofilia recíproca para cada género. En cuanto a las relaciones filogenéticas y a pesar de los múltiples antecedentes de estudios previos, el género *Lampornis* permanecía sin ser esclarecido. La posición filogenética de *L. hemileucus*, *L. sybillae* y *L. viridipallens* fue resuelta, siendo *L. hemileucus* la especie basal del género *Lampornis*, y *L. sybillae* y *L. viridipallens* resultaron ser especies hermanas, directamente relacionadas con el clado perteneciente a las especies centroamericanas: *L. castaneoventris*, *L. cinereicauda* y *L. calolaemus*.

El clado conformado por *L. cinereicauda*, *L. castaneoventris* y *L. calolaemus* permanece aún sin ser esclarecido. Previamente, en el estudio filogenético llevado a cabo para el género *Lampornis* (García-Moreno et al., 2006), se había incluido un mayor número de especies que el incluido en el presente estudio, encontrándose un grupo monofilético sin resolución dentro del clado. De acuerdo a nuestras estimaciones de tiempos de divergencia, encontramos que éste grupo es el de más reciente diversificación dentro de las "Gemas de las Montañas", por lo que sugerimos que dicha falta de resolución filogenética puede ser el resultado de un origen reciente, además de que estas tres especies tienen distribuciones geográficas que se sobrelapan, lo que podría estar

favoreciendo eventos de hibridación entre especies, un fenómento que ha sido estudiado en aves, incluyendo a la familia Trochilidae (Banks and Johnson, 1961; Gill, 1998; Gill et al., 1973; Wells et al., 1978). Actualmente se reconoce que este grupo es inestable en cuanto a su clasificación, considerándose en ocasiones como conespecíficos (AOU, 1998). Sin embargo, a pesar de que las hembras de este grupo no presentan claras diferencias morfológicas en cuanto a su coloración, los machos son clamarmente diferenciables y presentan segregación altitudinal, por lo que la asignación taxonómica de especie para cada morfotipo ha sido lo más conveniente (Schuchamann, 1999).

Se detectó estructuración dentro de las poblaciones en algunas de las especies del grupo. En el caso de *L. amethystinus, L. clemenciae* y *L. rhami*, esta separación a nivel poblacional no coincide con la taxonomía subespecífica descrita para cada especie (Schuchmann, 1999). Acerca de *L. amethystinus*, nuestros resultados apoyan la existencia del grupo "*salvini*", distribuido al este del Istmo de Tehuantepec y reconocido hasta ahora como una subespecie. En un estudio previo, en el cual se evaluó la variación genética y de coloración de ésta especie, se encontró diferenciación genética entre los grupos a ambos lados del Istmo, sin la presencia de variación morfológica, haciendo mención sobre una posible propuesta de considerar al grupo "*salvini*" como un linaje independiente (Cortés-Rodríguez et al., 2008). En nuestro estudio, la separación del resto de subespecies (*margaritae*, *amethysinus* y *circumventus*) no es clara. Sin embargo, recientemente se ha descrito la presencia de altos niveles de diferenciación genética y morfológica en *L. amethystinus*, con la incorporación de múltiples caracteres, identificándose cuatro grupos con correspondencia geográfica y que son congruentes con las subespecies propuestas para el complejo (Ornelas et al., 2016).

Es importante mencionar que un estudio basado en un análisis de caracteres morfológicos y morfométricos, sugiere que dos especies con distribuciones restringidas en Sudamérica (*Hylonympha macrocerca* y *Sternoclyta cyanopectus*) podrían estar cercanamente emparentadas con la especie *Eugenes fulgens* (Renner y Schuchmann, 2004), con base en semejanzas morfológicas. Esto podría indicar que tal vez el número de especies que conforman el grupo de las "Gemas de las Montañas" es mayor que las que actualmente se reconocen. La restricción en la distribución de estas especies y su posición en categorías de vulnerabilidad, han dificultado la obtención de muestras, lo cual sería de gran importancia para corroborar si dicha propuesta de parentesco se sustenta a nivel molecular. El clado de las "Gemas de las Montañas" es un grupo con una gran diversidad morfológica, con una amplia distribución geográfica y con representantes tanto en tierras altas como en tierras bajas en el Neotrópico (Schuchmann, 1999). A pesar de contar con

esta diversidad de características, es uno de los clados dentro de la familia Trochilidae con menor número de especies, apenas precedido por el género monotípico *Patagona* y el clado de los "Topacios". Hasta el momento se han reconocido 15 especies dentro de éste grupo, distribuidas en cinco géneros (McGuire et al., 2014). La evidencia presentada en éste estudio, incrementando el muestreo a nivel intraespecífico, muestra la existencia de estructuración geográfica y no debe descartarse la posibilidad de la presencia de complejos de especies crípticas que no hayan sido delimitadas hasta el momento.

Sobre la variación genética hallada previamente en *Eugenes fulgens*, colibrí de distribución Mesoamericano, se llevaron a cabo diferentes análisis con el objetivo de confirmar o rechazar las señales de estructura genética encontradas. Se realizó un análisis genético multilocus y se obtuvieron resultados importantes que tuvieron repercusiones a nivel taxonómico. Se evaluaron las diferencias a nivel genético mediante reconstrucciones filogenéticas y se llevaron a cabo análisis de delimitación de especies. Proponemos que *Eugenes fulgens* es un complejo de especies y que las subespecies descritas deberían ser taxonómicamente reevaluadas al nivel de especie (Zamudio-Beltrán y Hernández-Baños, 2015). La propuesta de reconocer a la subespecie *E. f. spectabilis* en el rango de especie se había hecho anteriormente por diversos autores (History, 1918; Ridgway, 1911; Stiles y Skutch, 1989). Sin embargo, nuestro trabajo proporciona las primeras evidencias a nivel genético que apoya esta hipótesis basada en diferencias morfológicas (diferencias en tamaño y en coloración). El Comité Internacional en Ornitología (IOC) ha tomado en cuenta nuestro estudio como referencia en la reevaluación taxonómica de *E. spectabilis* como la especie hermana de *E. fulgens* (Gill, 2015). Por otra parte, las relaciones filogenéticas entre *E. fulgens* y la subespecie *E. f. viridiceps* sugieren una reciente especiación alopátrica, sin evidencia clara de diferenciación morfológica. Sin embargo, no se presenta monofilia recíproca para cada grupo, por lo que se analizó la variación genética de este complejo (*fulgens*-*viridiceps*) a nivel poblacional, para evaluar si dicha separación genética es congruente a nivel morfológico y así poder discutir sobre los posibles factores que han promovido dicha diferenciación.

En general, el colibrí magnífico *Eugenes fulgens* presentó un patrón moderado de discontinuidades filogenéticas y separación espacial (Avise et al., 1987). La variación morfológica y mitocondrial encontrada sugiere un proceso incipiente de diversificación a ambos lados del Istmo de Tehuantepec. En contraste, la información obtenida a partir del ADN nuclear (microsatélites) sugiere altos niveles de flujo genético a lo largo de toda la distribución geográfica, con la identificación de una sola población con correspondencia geográfica (El Salvador, población costera). Las estimaciones en los tiempos de divergencia sugieren una separación entre las

poblaciones a ambos lados del Istmo de Tehuantepec a principios del Pleistoceno (~2.56 Ma), periodo durante el cual se promovieron dinámicas poblacionales de expansión (oeste del Istmo, ~30,000 años) y de estabilidad (este del Istmo), favorecidas por los cambios climáticos ocurridos durante este periodo.

La información obtenida a partir del ADN mitocondrial apoya la existencia de dos grupos principales, que tienen correspondencia con las dos subespecies propuestas (*fulgens* y *viridiceps*), como se sugirió con anterioridad (Zamudio-Beltrán y Hernández-Baños, 2015). Las redes de haplotipos y las estimaciones de varianza molecular AMOVA, confirman que las poblaciones al sur de Mesoamérica son genéticamente distintas de aquellas ubicadas al oeste del Istmo de Tehuantepec. La influencia de esta barrera geográfica ha sido ampliamente estudiada y se ha confirmado que su presencia ha limitado el flujo genético entre especies distribuidas en las tierras altas a ambos lados de esta región, en la que se han identificado dos etapas con altos índices de diversificación, promovidos por oscilaciones climáticas durante el Pleistoceno y cambios en los niveles marítimos que han tenido como consecuencia transgresiones en esta región, fragmentando los hábitats alrededor (Barber y Klicka, 2010). Este aislamiento geográfico ha sido encontrado repetidamente en el grupo de las aves (Arbeláez-Cortés et al., 2008; Barrera-Guzmán et al., 2012) y en particular en algunos colibríes (González et al., 2011; Malpica y Ornelas, 2014; Rodríguez-Gómez et al., 2013; Jiménez y Ornelas, 2016).

En contraste, la información obtenida a partir del ADN nuclear (microsatélites) no recuperó la diferenciación de grupos a ambos lados del Istmo de Tehuantepec. Sin embargo, si se identificó un grupo con correspondencia geográfica en las tierras altas de El Salvador, localizado en una cadena montañosa del lado de la costa del Pacífico (San Vicente). En un estudio llevado a cabo en la Coordillera de los Andes, se reportó diferenciación genética en el colibrí *Adelomyia melanogenys*, en una población costera con respecto a las poblaciones ubicadas al interior de la cadena montañosa, debido a la variación en las condiciones ambientales en ambos sitios a pesar de la aparente cercanía geográfica (Chaves et al., 2007). Dicha variación en características ambientales podría estarse presentando de igual forma en la localidad de San Vicente, lo cual podría ser evaluado analizando la diferenciación de condiciones climáticas y ambientales a una escala regional. Por otro lado, la presencia de un sistema de fallas geológicas ubicadas en el Valle Motagua (Polochic-Motagua fault), localizado en Guatemala y que se extiende unos 400 km desde el mar Caribe hasta la costa del Pacífico (Lyon-Caen et al., 2006), podría estar limitando el flujo genético de ésta población, situada en la parte sur de esta región. Este sistema de fallas está conformado por tres placas tectónicas (Polochic, Motagua y Jocotán), y se originó como resultado

de su unión y del cierre oceánico en el Cretácico tardío (Donnelly, 1977; Lawrence, 1976; Schwartz et al., 1979). Varios estudios han reportado que éste sistema probablemente ha promovido el aislamiento poblacional en diferentes taxa, a diferentes escalas temporales (Puebla-Olivares et al., 2008; Rovito et al., 2012; Villalobos, 2013; Malpica y Ornelas, 2014; Rodríguez-Gómez y Ornelas, 2014). El patrón filogeográfico de separación entre los grupos situados al este y oeste del Istmo de Tehuantepec fue congruente con la variación morfológica encontrada. Además de la variación reconocida entre los dos principales filogrupos, se recuperó el grupo correspondiente a las tierras altas de El Salvador, el cual ya había sido parcialmente identificado con base en el análisis del ADN nuclear y que coincide con un posible aislamiento promovido por el sistema de fallas en el valle del Río Motagua.

La señal obtenida a partir de los diferentes marcadores moleculares (ADN mitocondrial y nuclear) mostró variaciones en los resultados de estructuración genética para *E. fulgens*. Los microsatélites mostraron una resolución menor que el ADN mitocondrial, lo cual fue inesperado debido a tasas mutacionales de mayor magnitud reportadas en microsatélites (Chistiakov et al., 2006). Estas diferencias encontradas podrían estar relacionadas de igual forma a las distintas vías de herencia de cada marcador (vía materna y biparental) y a la variación en movimientos de dispersión que se pudiera estar presentando entre sexos. Las poblaciones norteñas y posiblemente las del centro de México presentan movimientos migratorios hacia el extremo norte de México y el suroeste de USA, mientras que las poblaciones al sur de México y Centroamérica son sedentarias (Schuchmann, 1999). En general *E. fulgens* presentó evidencia de contar con altos niveles de flujo genético, exceptuando las poblaciones más sureñas, lo cual es congruente con los patrones migratorios reportados para la especie. Se sabe que las especies pertenecientes a la familia Trochilidae cuentan con una gran habilidad de dispersión, además se debe tomar en cuenta que *E. fulgens* es un colibrí de hábitos generalistas, que se alimenta de una gran variedad de recursos florales, lo cual podría significar que para ésta especie no existe una restricción a permanecer en sitios específicos con recursos exclusivos, además de que presenta conductas de territorialidad bajas (Lara, 2006).

El patrón de estructuración geográfica recuperada fue la base para analizar la historia demográfica de los grupos definidos a ambos lados del Istmo de Tehuantepec. El grupo conformado por las poblaciones al oeste presentó un patrón claro de expansión demográfica (ca. 30 000 años), el cual se situó temporalmente cerca del último periodo glacial (ca. 20 000 años), mientras que las poblaciones al este presentaron un patrón poblacional de estabilidad, que coincide con los altos niveles de estructuración geográfica previamente descritos. La separación ocurrida entre los dos

grupos principales se llevó a cabo hace alrededor de 2.56 Ma. El origen geológico del Istmo de Tehuantepec, el cual inició durante el Mioceno tardío (ca. 6 Ma, Barrier et al., 1998), no puede señalarse como la causa primaria de la divergencia del complejo. Por otro lado, una de las hipótesis más aceptadas para explicar la variación geográfica, es aquella sobre los refugios promovidos durante las fluctuaciones climáticas ocurridas en el Pleistoceno y los cambios en la redistribución altitudinal de los bosques templados durante los periodos glaciales e interglaciales en esta época (Hewitt, 1996; Sánchez-González et al., 2008; Smith et al., 2011). Las estimaciones de tiempos de divergencia y la dinámica poblacional presentada en éste trabajo sugiere que el complejo *E. fulgens* se estableció a inicios del Pleistoceno y los posteriores cambios en los niveles del mar y de clima alrededor del Istmo de Tehuantepec fracturaron el habitat (Barber y Klicka, 2010), promoviendo la divergencia de los dos principales linajes. Las proyecciones de distribuciones potenciales ancestrales (LIG, ~140 000 años; LGM, ~20 000 años), mostraron que las condiciones climáticas eran favorables para ambos grupos del complejo, las cuales durante el último máximo glacial (LGM) cubrían una región más extensa al oeste del Istmo comparada con la región disponible durante el último interglacial (LIG), lo cual confirma la señal de expansión demográfica encontrada en el grupo oeste. Los eventos de expansión y contracción pudieron favorecer el contacto o la dispersión entre las poblaciones a ambos lados del Istmo (i.e. contacto secundario), una posible causa de la presencia de haplotipos compartidos entre estos grupos.

En cuanto a los recientes cambios taxonómicos, validando al taxón "*spectabilis*" (subespecie propuesta anteriormente) como una especie (IOC, Gill 2015), actualmente no se reconocen de manera oficial otros taxa que describan la variación geográfica a lo largo de la distribución del complejo *Eugenes fulgens*. Sin embargo, se ha propuesto que las poblaciones al este del Istmo de Tehuantepec, correspondientes al grupo "*viridiceps*" (Boucard 1878), deberían ser reconocidas como un linaje evolutivo independiente (Navarro-Sigüenza y Peterson, 2004). Como se concluyó en el presente estudio, los grupos *viridceps* y *fulgens* deberían ser reevaluadas como especies crípticas en un proceso de especiación incipiente (~1.93 Ma), cuyos haplotipos compartidos pueden ser explicados como resultado de una separación incompleta de linajes (Maddison y Knowles, 2006; Peters et al., 2007). Asociado a esto, se ha evaluado el efecto que tiene la temporalidad en algunas especies de reciente origen, concluyéndo que es posible hacer una identificación precisa de estas, a pesar de no contar con evidencia de monofilia recíproca durante el evento de diversificación, capturándose diferentes momentos en dicho proceso (Knowles y Carstens, 2007).

Con una distribución geográfica más restringida, pero presente de igual forma en las tierras altas de Mesoamérica, se encuentra el colibrí alicastaño *Lamprolaima rhami*. El patrón evolutivo encontrado en esta especie difiere del de su grupo hermano (complejo *Eugenes*) con respecto a los niveles de estructuración geográfica encontrados, ya que *L. rhami* presentó mayores índices de diferenciación a nivel poblacional. Comparando sus distribuciones, *E. fulgens* sólo presentó correspondencia geográfica a ambos lados del Istmo de Tehuantepec, mientras que en *L. rhami*, cuya distribución es menor en extensión, se obtuvo estructuración geográfica para cada región muestreada, además de que se mantuvo el patrón de diferenciación a ambos lados del Istmo de Tehuantepec. La evaluación de datos morfológicos sustenta las diferencias genéticas encontradas en cada región, apoyando la hipótesis de divergencia alopátrica en éste complejo. Las estimaciones de los tiempos de divergencia ofrecen indicios de un origen Pleistocénico para el complejo *L. rhami* (0.68 Ma, 0.93-0.45 Ma), seguido de una posterior separación poblacional a ambos lados del Istmo de Tehuantepec. La población al este del Istmo, genética y morfológicamente diferenciada del resto, presentó evidencia de una posible expansión demográfica (curva unimodal, Mismatch), que al situarla en el tiempo estimado de su divergencia (0.21 Ma, 0.33-0.10 Ma) coincide con la temporalidad aproximada de ocurrencia del último máximo glacial (LGM, 20 000 Ma), lo cual indica que los cambios climáticos ocurridos durante éste periodo influyeron posiblemente en su diferenciación.

Genéticamente se evaluaron tres de las cinco regiones geográficas en *L. rhami*. De acuerdo a las descripciones originales de los patrones filogeográficos y al igual que en *E. fulgens*, *L. rhami* presentó un patrón de discontinuidades filogenéticas y separación espacial (Avise et al., 1987). Estos altos niveles de variación genética se correlacionaron con un patrón de aislamiento por distancia asociado a la distribución alopátrica de los bosques mesófilos de montaña, habitat preferido de *L. rhami*. Las condiciones ambientales características en estos bosques han sido reportadas como promotores de diferenciación poblacional (Ornelas et al., 2013; Ramírez-Barahona y Eguiarte, 2014). La diferenciacion encontrada entre las regiones de la Sierra Madre Oriental y norte de Oaxaca (SMO&SMS) y la Sierra Madre del Sur (SMS) están más relacionadas a un patrón de aislamiento por distancia que a un patrón de aislamiento promovido por la presencia de barreras geográficas que limiten el intercambio genético. En contraste, la presencia del Istmo de Tehuantepec ha promovido la diferenciación entre poblaciones a ambos lados de ésta región.

Retomando el tema de la vagilidad y dispersión, las habilidades de movimiento que pueden presentar algunas especies de la familia Trochilidae son bien conocidas (e.g. los grandes movimientos migratorios en el grupo de las “Abejas”). A pesar de esto, varios estudios en la familia

Trochilidae, han encontrado que la presencia de barreras geográficas ha sido crucial en procesos de diversificación sin que influya la aparente cercanía geográfica, como en la región de los Andes (*Adelomyia melanogenys*, Chaves y Smith, 2011), Mesoamérica (*Lampornis amethystinus*, Ornelas et al., 2016), la región del Valle Motagua (*Amazilia cyanocephala*, Rodríguez-Gómez y Ornelas, 2014) y el Istmo de Tehuantepec (*Campylopterus curvipennis*, González et al., 2011). En contraste, existe una hipótesis que sugiere que los altos niveles de diversificación a nivel poblacional, encontrados en su gran mayoría en aves neotropicales de tierras bajas, están relacionados a habilidades limitadas de dispersión (Burney y Brumfield, 2009). Al respecto, algunas especies presentan gran afinidad por sus sitios y sus movimientos migratorios son limitados, como es el caso de *L. rhami*. Se han reportado movimientos altitudinales en ésta especie, relacionados con la presencia de recursos en diferentes épocas del año, pero no se han reportado movimientos migratorios hacia regiones distantes en su distribución geográfica (Schuchmann, 1999), por lo que ambos factores (presencia de barreras geográficas y limitaciones en movimientos de dispersión) podrían estar influenciando la marcada separación geográfica encontrada.

Con base en la evidencia completa de diferenciación poblacional y de acuerdo al análisis filogenético multilocus llevado a cabo, se recuperaron dos grupos monofiléticos que coinciden con los grupos a ambos lados del Istmo de Tehuantepec. Además de encontrar agrupaciones correspondientes a cada región muestreada dependiendo del marcador molecular empleado. Tomando en cuenta esto, uno de los aspectos más importantes a considerar para *L. rhami* es su limitada distribución geográfica, restringida a los pocos fragmentos de bosques mesófilos de montaña aún existentes en Mesoamérica. Se sabe que estos bosques, ricos en diversidad biológica, están altamente amenazados (Hamilton, 1995; Martínez-Morales, 2005; Mulligan, 2010). La evidencia presentada en éste último estudio podría ser tomada como referencia que tuviera implicaciones a nivel taxonómico, como se consiguió anteriormente para *Eugenes* (Zamudio-Beltrán y Hernández-Baños, 2015). El riesgo de una incorrecta clasificación a nivel de especie repercute directamente en el manejo y en los esfuerzos de conservación (Zink, 2004). Es por esto que sugerimos que las poblaciones de *L. rhami*, presentes en cada región geográfica, sean tratadas como unidades de manejo independientes, sin descartar la posibilidad de que las poblaciones a ambos lados del Istmo de Tehauntepec sean reconocidas como especies válidas. A pesar de esto, aún queda pendiente la incorporación de información de las dos regiones que no pudieron ser muestreadas correspondientes a la Sierra Sur de Oaxaca y a la región comprendida en las tierras altas de El Salvador, Honduras y Nicaragua.

Existen diversos estudios que han tratado de abordar los patrones filogeográficos para las especies de aves que habitan las tierras altas de la región Mesoamericana (Tabla 1), sin tener un patrón exclusivo que defina de forma general su historia evolutiva.

Tabla 1. Generalidades de algunos estudios filogenéticos y a nivel poblacional de aves en Mesoamérica.

Taxón	Patrón evolutivo
Amazilia cyanocephala (Rodríguez-Gómez et al., 2013; Rodríguez-Gómez y Ornelas, 2013).	• Diferenciación a ambos lados del Istmo de Tehuantepec, en un evento de diversificación reciente en presencia de flujo genético. • Diferenciación entre grupos al sur de México y norte de Centroamérica, asociada a un aislamiento promovido por el sistema de fallas Motagua-Polochic-Jocotán.
Aulacorhynchus prasinus (Puebla-Olivares et al., 2008).	• Se identificaron siete grupos diferenciados genéticamente con correspondencia a rompimientos geográficos en Mesoamérica y Sudamérica, sugiriendo la reevaluación taxonómica de estos grupos.
Aphelocoma ultramarina (McCormack et al., 2008).	• Divergencia entre los principales grupos durante el Pleistoceno (~0.7 Ma) y después dl periodo de mayor actividad geológica en México, descartando éste último evento como promotor en su diversificación. • Los principales linajes tienen correspondencia con las principales cadenas montañosas en México (Sierra Madre Oriental, Sierra Madre Occidental y Faja Volcánica Transmexicana).
Buarremon (Navarro-Sigüenza et al., 2008).	• Se identifican seis grupos con marcada estructura geográfica en Mesoamérica correspondientes a las principales cordilleras.
Campylopterus curvipennis (González et al., 2011).	• Dos principales eventos de diversificación promovieron la diferenciación entre grupos: un evento de vicarianza a través del Istmo de Tehuantepec (~1.4 Ma) y el aislamiento de la región de Los Tuxtlas (México) en el Pleistoceno.
Certhia americana (Manthey et al., 2011).	• Se identificaron seis clados bien diferenciados y estructurados geográficamente. • Se sugiere que la alopatría es uno de los factores principales en la diferenciación de los grupos.
Chlorospingus ophthalmicus (Sosa-López et al., 2013).	• Variación en cantos para dos grupos con correspondencia y aislamiento geográfico. • Tres grupos sin variación en cantos, pero con variación en morfología y flujo genético restringido.
Dendroica coronata (Milá et al., 2007).	• Los grupos migratorios y los sedentarios se diferenciaron a inicios del Pleistoceno. • Se identificaron dos grupos para las poblaciones sedentarias, se presume que su diferenciación fenotípica ocurrió durante el Holoceno como el resultado del aislamiento geográfico y una expansión demográfica desde la última glaciación.
Doricha eliza (Licona-Vera y Ornelas, 2014).	• Se encontraron marcadas diferencias genéticas, con bajos niveles de flujo genético y una historia de aislamiento hace alrededor de 120,000 años entre poblaciones disjuntas.
Ergaticus	• Patrón filogeográfico de separación espacial, promovido por barreras en

(Barrera-Guzmán et al., 2012).	las tierras bajas (e.g. Istmo de Tehuantepec) durante el Pleistoceno (~1.06-0.21 Ma).
Lampornis amethystinus (Cortés-Rodríguez et al., 2008)	• Diferenciación genética de las poblaciones a ambos lados del Istmo de Tehuantepec, sin correspondencia con variación en la morfología.
Lampornis amethystinus (Ornelas et al., 2016).	• Se identifican seis grupos genéticos con correspondencia geográfica (Sierra Madre del Sur, Faja Volcánica Transmexicana, Sierra Madre Oriental, este del Istmo de Tehuantepec). • El evento de separación basal se sitúa durante el Pleistoceno (2.39-0.57 Ma) y corresponde a la diferenciación a ambos lados del Istmo de Tehuantepec).
Lepidocolaptes affinis (Arbeláez-Cortés et al., 2010).	• Se detectaron dos barreras geográficas importantes para la diferenciación de grupos genéticos: el Istmo de Tehuantepec y la depresión de Nicaragua.
Myioborus miniatus (Peréz-Emán et al., 2010).	• Se identificaron cuatro grupos, tres de ellos coinciden con algunas subespecies propuestas. • Evidencia de expansión demográfica durante el Pleistoceno.
Pharomachrus mocinno (Solórzano et al., 2009).	• Se identificaron tres grupos genéticos, con correspondencia geográfica: tierras altas de México, tierras altas del norte de Centroamérica y las tierras altas de Panamá.
Selasphorus platycercus (Malpica y Ornelas, 2014).	• Hay diferenciación entre poblaciones migratorias-sedentarias y exclusivamente sedentarias en éste complejo. • Evidencia de reciente conducta migratoria durante los ciclos glaciales.

Entender los mecanismos de evolución y especiación es un tema central en los estudios clásicos y contemporáneos de biología evolutiva (Barton y Partridge, 2000; Coyne, 1992; Walsh et al., 2002; Yeaman y Otto, 2011), al igual que la evaluación del papel de las distintas fuerzas evolutivas en el proceso adaptativo y en el mantenimiento de la diversidad. Aunado a esto, se ha incrementado el número de métodos para su detección conjugando fundamentos básicos (como por ejemplo los descritos en genética de poblaciones) y se ha incorporado el uso de múltiples datos (e.g. nuevos marcadores moleculares), dando como resultado una amplia gama de posibilidades para estudiar estos cambios a nivel intraespecífico. A pesar de todos estos avances, los patrones que moldean dicha variación en la naturaleza no han sido esclarecidos en su totalidad. Tal es el caso de la historia evolutiva en las tierras altas de Mesoamérica y en general de la región Neotropical.

Para esclarecer los patrones evolutivos, es importante tomar en cuenta un gran número de factores como son los caracteres morfológicos, los caracteres moleculares, los procesos históricos y los temporales, entre otros. Una de las repercusiones que tienen estos estudios es que sirven de referencia para tomar decisiones con implicaciones en la taxonomía y en la conservación de poblaciones altamente diferenciadas. Sin embargo, estas decisiones se basan en el uso de diferentes

conceptos de especie. Mientras algunos autores toman como referencia conceptos como el biológico de especie, el cual define a las especies con base en su aislamiento reproductivo (Mayr, 2000), otros argumentan que la clasificación de los sistemas biológicos deberían realizarse con base en la independencia de sus historias evolutivas (De Queiroz, 2007; James, 2010). Este tipo de estudios cobran importancia cuando incorporan información múltiple de cada sistema biológico, ya que se puede tener una estimación y descripción de la biodiversidad más precisa, lo cual tendría importantes repercusiones en su conservación.

CONCLUSIONES

- El grupo de las "Gemas de las Montañas" se originó en las tierras altas del área comprendida entre el sur de Norteamérica y Centroamérica, a mediados del Mioceno, hace aproximadamente 16.15 Ma (19.08-13.24 Ma).
- Posteriores eventos de dispersión hacia el norte y sur ampliaron el rango de distribución del grupo, favoreciendo procesos de especiación en el Neotrópico.
- Dichos eventos de dispersión fueron los más importantes en cuestión de diversificación a nivel específico.
- Algunos procesos de diversificación, relacionados a eventos ocurridos durante el Plioceno-Pleistoceno, apoyan a la vicarianza como el promotor más probable de variación a nivel poblacional.
- Ocho de las quince especies dentro del grupo de las "Gemas de las Montañas" presentaron evidencia de variación genética a nivel poblacional, detectada a partir de la incorporación de un mayor muestreo a nivel intraespecífico.
- Se lograron esclarecer las relaciones filogenéticas de las especies *L. hemileucus*, *L. viridipallens* y *L. sybillae*.
- El grupo conformado por *L. calolaemus*, *L. castaneoventris* y *L. cinereicauda* es un complejo taxonómicamente inestable, ya que su resolución filogenética permanece sin ser esclarecida.
- La evidencia de variación intraespecífica encontrada podría estar revelando la posible presencia de especies crípticas que no hayan sido reconocidas.
- Se ha confirmado la presencia de una especie críptica que era considerada una subespecie del complejo *Eugenes fulgen*s, la cual ahora es reconocida como especie válida: *Eugenes spectabilis* (tierras altas de Costa Rica y Panamá).

- El complejo *E. fulgen*s y *L. rhami* presentaron un patrón filogeográfico de discontinuidades filogenéticas y separación espacial.
- Dos linajes principales se reconocen en *E. fulgens,* los cuales tienen correspondencia geográfica a ambos lados del Istmo de Tehuantepec y sugieren ser parte de un proceso incipiente de diversificación.
- Cuatro linajes principales se reconocen en *L. rhami* con correspondencia geográfica, los cuales se sugiere deberían ser tratados como linajes evolutivos independientes.
- En ambas especies (*E. fulgens* y *L. rhami*) la influencia del Istmo de Tehuantepec ha sido relevante en su diferenciación.
- Los tiempos de divergencia estimados en ambos grupos sitúan el origen y diversificación de estos grupos en el Pleistoceno, por lo que las oscilaciones climáticas durante éste periodo han afectado probalemente sus dinámicas poblacionales.
- Mientras que *E. fulgens* presentó altos niveles de flujo genético en la mayor parte de su distribución geográfica, *L. rhami* presentó poblaciones diferenciadas genética y morfológicamente asociado al efecto del aislamiento por distancia en ausencia de flujo genético.
- Remarcamos la importancia de la incorporación de información múltiple en la evaluación de la historia evolutiva de los complejos biológicos, para su correcta identificación.

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