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**“EXPLORANDO LA ECOLOGÍA QUÍMICA, CONDUCTA QUIMIOSENSORIAL E
HISTORIA EVOLUTIVA DE *Aspidoscelis lineattissimus*”**

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RESUMEN

En animales, las señales químicas son importantes para establecer y modular una amplia variedad de relaciones intra e interspecíficas. Habilidades sensoriales como el reconocimiento químico entre especies y entre poblaciones puede formar una barrera de aislamiento reproductivo y facilitar los procesos de especiación. En esta tesis se examinó el tipo de señales químicas que produce la especie de lagartija *Aspidoscelis lineattissimus* que habita los ambientes tropicales de México con el fin de: a) identificar variaciones interspecíficas en la composición y abundancia de compuestos químicos producidos por las glándulas femorales de los machos de una comunidad de lagartijas de hábitos arbóreos y terrestres; b) evaluar las variaciones poblacionales en las señales químicas que produce la lagartija tropical *A. lineattissimus* y su efecto sobre conductas intra e intersexuales de reconocimiento químico entre machos y hembras, así como la conducta femenina para la elección parcial de territorios; y c) examinar desde un enfoque molecular las diferencias genéticas entre las poblaciones y subespecies de la lagartija *A. lineattissimus* con el fin de explorar sus relaciones filogenéticas y comprender su historia evolutiva. Adultos de lagartijas tropicales fueron capturados en distintos sitios de una región tropical del Centro-Occidente de México, se colectaron muestras de tejido de cada animal y las secreciones femorales para análisis de laboratorio y para experimentos de conducta en campo. En general se encontró que: 1) existe una diversidad importante de compuestos químicos presentes en las secreciones de las glándulas femorales de los machos; 2) en todas las especies, ácidos carboxílicos y ésteres fueron los grupos químicos principales, seguidos de escualeno; 3) diferencias significativas interspecíficas e interpoblacionales en la composición química entre grupos químicos; 4) existe una definida y divergente mezcla de compuestos químicos de origen lipídico en cuatro distintas poblaciones de machos de la lagartija *A. lineattissimus*; 5) existe un mayor reconocimiento químico entre individuos de la misma población de origen que entre individuos de distintas poblaciones; 6) la elección de las hembras por los territorios marcados con señales químicas de los machos fue distinta entre poblaciones de dos distintas regiones; 7) un grupo monofilético es identificado en *A. lineattissimus* con cinco definidos linajes y 8) tres linajes fueron soportados como unidades evolutivas independientes. Finalmente, las variaciones químicas, conductuales y genéticas registradas aquí, sugieren un alto grado de divergencia de especies en el complejo *A. lineattissimus*. Por lo que, en un futuro será necesario considerar una reclasificación taxonómica de la especie y con ello garantizar nuevas estrategias de conservación.

PALABRAS CLAVE: biología sensorial, conducta sexual, ecología animal, evolución, señales químicas.

ABSTRACT

In animals, chemical signals are important for establish and modulate a wide variety of intra and interspecific relationships. Sensory abilities such as the chemical recognition between species and between populations, can form a reproductive isolation barrier and facilitate speciation processes. In this thesis we examine the type of chemical signals that produces the lizard species *Aspidoscelis lineattissimus* that inhabits the tropical environments from Mexico in order to: a) to identify interspecific variations in the composition and abundance of chemical compounds produced by the femoral glands of males of a community of lizards with arboreal and terrestrial habits; b) to evaluate the population variations in the chemical signals produced by the tropical lizard *A. lineattissimus* and their effect on intra and intersexual behaviors of chemical recognition between males and females, as well as female behavior for the partial choice of territories; and c) to examine from a molecular approach the genetic differences between populations and subspecies of the lizard *A. lineattissimus* in order to explore their phylogenetic relationships and to understand their evolutionary history. Adult tropical lizards were captured at different sites in a tropical region from Central-Western Mexico, tissue samples from each animal and femoral gland secretions were collected for laboratory analysis and for field behavior experiments. In general, we find that: 1) there is an important diversity of chemical compounds present in the femoral gland secretions males; 2) in all species, carboxylic acids and esters were the main chemical groups, followed by squalene; 3) significant interspecific and interpopulation differences in chemical composition between chemical groups; 4) there is a defined and divergent mixture of chemical compounds of lipid origin in four different populations of male lizard *A. lineattissimus*; 5) there is greater chemical recognition between individuals from the same population of origin than between individuals from different populations; 6) the female choice by the territories marked with male chemical signals was different between populations of two different regions; 7) a monophyletic group is identified in *A. lineattissimus* with five defined lineages and 8) three lineages were supported as independent evolutionary units. Finally, the chemical, behavioral, and genetic variations recorded here suggest a high degree of species divergence in the *A. lineattissimus* complex. Therefore, in the future it will be necessary to consider a taxonomic reclassification of the species and thereby ensure new conservation strategies.

INTRODUCCIÓN GENERAL

La mayoría de los procesos naturales que ocurren en la biosfera de nuestro planeta son sistemas muy complejos de entender (Levin 1998, 2005), debido a esto, en la actualidad son necesarias las actividades de investigación inter y multidisciplinarias para llenar vacíos de información. Por ejemplo, procesos biológicos como la comunicación animal siempre han despertado la curiosidad e interés del propio ser humano, los estudios sobre el tema del intercambio de información entre miembros del reino animal ha permitido ampliar nuestro entendimiento de la función de múltiples conductas y estrategias evolutivas que adoptan las especies en los diversos contextos en los que se desarrollan sus actividades diarias (McGregor 2005, Wiley 2006, Seyfarth *et al.* 2010, Stevens 2013, Bradbury y Vehrencamp 2014).

Los canales de transmisión de la información pueden llegar a ser multi-sensoriales en muchas especies (Partan 2004, Higham y Hebets 2013). Sin embargo, esta característica es altamente dependiente de la estructura biológica y complejidad evolutiva de cada ser vivo (Smith y Evans 2013, Hebets *et al.* 2016). En este sentido, podemos destacar que los canales de comunicación animal más conocidos y estudiados en la naturaleza son los que involucran el uso de señales visuales y acústicas (Schaefer 2010, Chen y Wiens 2020). La comunicación química es otro medio de transferencia de información complejo, antiguo y diverso en los seres vivos (Steiger *et al.* 2011, Stökl y Steiger 2017, Baeckens 2019). En cierta parte, los procesos como la comunicación química han recibido menos atención que la comunicación acústica o visual. Esta diferencia relativa en su investigación probablemente sea debida a una combinación de dificultades metodológicas para evaluar la diversidad química en el ambiente y la complejidad de las señales químicas junto con los mecanismos de percepción en relación con los sistemas acústicos y visuales (Yohe y Brand 2018).

La comunicación química implica que los organismos tengan la capacidad de producir, detectar y responder a estímulos químicos de su misma o diferente especie y el medio ambiente (Moulton 1968, Müller-Schwarze 2006, Yohe y Brand 2018). Sensor “señales químicas” no es un proceso fácil para los animales y requiere una gran cantidad de energía, el proceso implica tanto el uso de habilidades conductuales y estructuras fisiológicas especializadas para una efectiva transducción de un amplio rango de estímulos tanto olfativos como gustativos del ambiente a señales neurológicas interpretadas por el sistema nervioso de cada organismo (Müller-Schwarze 2006, Yohe y Brand 2018), así como su correcta filtración

o discriminación de los químicos no esenciales que generan ruido de fondo en el ambiente (Yohe y Brand 2018).

Como resultado de los procesos de quimiosensación, los vínculos e interacciones que se pueden formar a través de las señales químicas son ampliamente diversos en la naturaleza, este tipo de señales pueden regular diferentes comportamientos en los animales que son esenciales para su sobrevivencia y reproducción, como las actividades de forrajeo (Tomba *et al.* 2001, Hodgkison *et al.* 2007, Bucciarelli y Kats 2015), detectar y evitar depredadores (Hazlett 2010, McCoy *et al.* 2012, Hettyey *et al.* 2015), identificar a conoespecíficos (Ibáñez *et al.* 2012, Vicente y Halloy 2016, Campos *et al.* 2017), cuidar de las crías (Mathews 2011, Leigh y Smiseth 2012, Amo *et al.* 2014) y buscar pareja (Chen *et al.* 2012, Fassotte *et al.* 2016, Billeter y Wolfne 2018), por mencionar solo algunos ejemplos.

El reto de comprender como funcionan estos estímulos químicos tiene sus orígenes a finales del siglo XIX en el grupo de los insectos, principalmente con mariposas y abejas como modelos de investigación (Harborne 2001, Bergström 2007). Los semioquímicos producidos por los organismos para regular las interacciones intraespecíficas reciben el nombre de feromonas y los que regulan las interacciones interespecíficas reciben el nombre de aleloquímicos (Law y Regnier 1971, Nordlund y Lewis 1976). Los avances tecnológicos y técnicas de análisis desarrolladas en las últimas décadas, han permitido identificar y caracterizar los semioquímicos que son responsables de diversas conductas y funciones biológicas en una gran cantidad de especies animales (Burger 2005, Pickett *et al.* 2014, Schulte 2016, Wyatt 2017, El-Sayed 2019). La composición y estructura química de estos compuestos puede llegar a ser de naturaleza simple o muy compleja según sea el caso de cada especie o grupo de organismos (Wyatt 2014). Por ejemplo, el bombicol fue la primera feromona sexual descrita en hembras de la mariposa de la seda *Bombyx mori*, feromona que por sí sola provoca la atracción de los machos para iniciar el apareamiento (Karlson y Butenandt 1959). Por otro lado, en animales que hacen jerarquías sociales como las abejas, no es solo un tipo de feromona sino la contribución sinérgica de varios tipos de feromonas (ej. Secreciones de glándulas mandibulares) las que modulan la conducta de dominancia reproductiva que ejercen las reinas sobre las demás clases sociales que integran la colmena (Slessor *et al.* 2005, Pirk *et al.* 2011).

En reptiles, la ecología química ha sido relativamente poco estudiada en comparación con un amplio grupo de invertebrados (Martín y López 2015). Por ejemplo, hasta hace más de una década los estudios con feromonas en reptiles representaban sólo el 1% de todos los artículos publicados sobre este tema (Symonds y Elgar 2008). Actualmente se sigue ampliando de forma constante el conocimiento sobre el origen, función y significancia de las señales químicas implicadas en la fisiología y conducta de muchos reptiles (Weldon *et al.* 2008, Houck 2009, Mason y Parker 2010, Martín y López 2011, Martín y López 2015, García Roa *et al.* 2017). En varias especies de lagartijas y serpientes, se ha demostrado que las señales químicas liberadas en el periodo de reproducción pueden ser la base para el reconocimiento de especies, sexos, elección de pareja, reconocimiento de rivales, marcaje de territorio etc. (Cooper y Pérez-Mellado 2002, Aragon *et al.* 2008, Bresciani *et al.* 2011, Shine y Mason 2012; Scott *et al.* 2013; Martín y López 2015).

Por todo lo anterior, es claro que en la naturaleza existe una gran diversidad de compuestos químicos activos relacionados con muchas conductas quimiosensoriales que probablemente determinan los diferentes niveles de adecuación en los animales. Esta característica es importante porque se sugiere está involucrada en los procesos adaptativos y evolutivos que influyen sobre la diversificación de las especies (Kirkpatrick y Ravigné 2002, Shaw y Parsons 2002). Se ha documentado que la quimiosensación es caracterizada por tener rápidas tasas de evolución y con frecuencia se le relaciona con adaptaciones sensoriales a nuevos ambientes en numerosas poblaciones, especies y linajes (Nei *et al.* 2008, Brand *et al.* 2015, Bear *et al.* 2016).

Los complejos de especies son sistemas biológicos de particular interés para investigar las estrategias de adaptación local o mecanismos de evolución. Por ejemplo, la abeja de la miel *Apis mellifera* es un complejo taxonómico de por lo menos 26 subespecies con variaciones geográficas en su distribución, genéticas y aspectos fenotípicos de su morfología y conducta (Han *et al.* 2012, Wallberg 2014, Cridland *et al.* 2017). Adicionalmente, variaciones químicas encontradas en el espectro y composición de las secreciones de las glándulas mandibulares de algunas subespecies de abejas de la miel *A. mellifera*, ha demostrado que la composición de las feromonas puede llegar a ser una característica subespecie-específica y contribuir a mantener un aislamiento reproductivo (Pankiw *et al.* 1996, Yusuf *et al.* 2015).

En grupos de vertebrados tan biodiversos como lo son los reptiles, estudios han reportado variaciones tanto interespecíficas como interpoblacionales en las señales químicas usadas para modular las relaciones sociales y reproductivas en varias especies de lagartijas (Escobar *et al.* 2001, 2003, Gabirot *et al.* 2010, García Roa *et al.* 2016, Khannoon 2016, Khannoon *et al.* 2013), y en un menor número de especies de serpientes (LeMaster y Mason, 2003, Shine *et al.* 2002), así como cambios significativos en sus conductas de reconocimiento y discriminación química (Cooper y Vitt 1986, Cooper 1987, Shine *et al.* 2002, 2004, Barbosa *et al.* 2006, Labra 2011, Khannoon *et al.* 2010, 2011, LeMaster y Mason, 2001, 2002, Gabirot *et al.* 2012). Sin embargo, en estos vertebrados poco se ha precisado en el conocimiento de la magnitud, relevancia e importancia de la segregación de señales químicas sobre el aislamiento etológico (tipo de aislamiento reproductivo precopula) y sus implicaciones sobre los procesos evolutivos como la divergencia de especies (Runemark *et al.* 2011, Gabirot *et al.* 2012, Martín *et al.* 2016). Actualmente existe un acalorado debate y un especial interés en comprender la contribución relativa de las conductas sexuales que favorecen aislamiento etológico mediante señales químicas y su implicación en los procesos de especiación (Heathcote *et al.* 2016, MacGregor *et al.* 2017, McLean *et al.* 2020, Irwin 2020, van Schooten *et al.* 2020).

En el contexto anterior, los objetivos de la presente tesis son: (1) caracterizar la composición química de las secreciones de las glándulas femorales que regulan las interacciones sociales y reproductivas entre especies y entre poblaciones de lagartijas de ambientes tropicales, (2) usar como modelo biológico a la lagartija *Aspidoscelis lineattissimus* para evaluar el efecto de las señales químicas sobre las conductas precopulatorias de reconocimiento y discriminación sexual entre diferentes poblaciones, (3) evaluar la influencia del olor sobre la elección de pareja por parte de las hembras y finalmente, (4) establecer las relaciones genéticas y evolutivas que existen en el complejo *A. lineattissimus* desde un punto de vista sistemático-filogenético. Para cumplir con los anteriores objetivos se desarrollaron tres capítulos que se describen a continuación.

En el capítulo I se explora las diferencias que existen en la abundancia y composición química de las secreciones de las glándulas femorales de una comunidad de cinco especies de lagartijas con dos diferentes tipos de hábitat en una zona tropical del occidente de México. El objetivo principal fue (1) identificar los principales compuestos y grupos químicos que

conforman las secreciones femorales de cada especie de lagartija, y (2) identificar las diferencias químicas entre especies arbóreas y terrestres.

Las variaciones geográficas producidas en las señales químicas de las poblaciones animales pueden afectar la dinámica evolutiva de las especies, esto según el contexto de aislamiento reproductivo propiciado por una disrupción en las habilidades de reconocimiento y preferencias químicas entre individuos de distintas poblaciones (Smadja y Butlin 2009). En el capítulo II se presenta un estudio que explora si las variaciones poblacionales en las señales químicas que producen las secreciones femorales de la lagartija tropical *Aspisdocelis lineattissimus* afectan de manera significativa las conductas pre-copulatorias de reconocimiento, discriminación y preferencia química entre machos y hembras de cuatro poblaciones de distintas áreas geográficas. En particular probamos las siguientes hipótesis (1) la abundancia y composición química de las secreciones femorales de los machos es diferente entre poblaciones con aislamiento geográfico, (2), machos y hembras reconocen y discriminan correctamente entre señales químicas de individuos de su misma y diferente población, y (3) las hembras de cada población prefieren visitar los territorios marcados con las señales químicas de los machos de su misma población sobre los territorios marcados por los machos de otras poblaciones.

Finalmente, en el capítulo III usando marcadores moleculares se explora la sistemática y las relaciones filogenéticas entre poblaciones de la lagartija *A. lineattissimus*. En particular se buscó evidencia genética que soporte la posible divergencia de especies previamente sugerida por los estudios morfológicos y ahora también por los resultados de los estudios químicos y conductuales de la presente tesis. Por lo que los objetivos fueron los siguientes: (1) identificar las relaciones filogenéticas en el complejo *A. lineattissimus*, (2) determinar la probabilidad de especiación en el complejo y (3) identificar los tiempos de divergencia entre los linajes de *A. lineattissimus*.

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CAPÍTULO I

Chemical differences in femoral gland secretions of arboreal and terrestrial tropical lizards from Western Mexico

Manuscrito enviado a *Salamandra*

Chemical differences in femoral gland secretions of arboreal and terrestrial tropical lizards from Western Mexico

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Abstract

Chemical signals in the animals stimulate different links and relationships. In lizards, chemical signals play an essential role in reproductive and social communication. Although an important diversity of lizard species inhabits tropical ecosystems, the chemical composition of the femoral gland secretions that tropical lizards use for communication is poorly known. In this work, we used gas chromatography-mass spectrometry (GC-MS) to determine and compare the main compounds contained in the femoral gland secretions of males of three species of arboreal lizards, *Hemidactylus frenatus*, *Sceloporus melanorhinus*, and *Urosaurus bicarinatus*; and of two species of terrestrial lizards *Aspidoscelis lineattissimus* and *Sceloporus pyrocephalus*. We identified 12 lipophilic compounds in femoral gland secretions of males, corresponding to fatty acids esters (~58%), carboxylic fatty acids (~25%), and other compounds (~17%). Carboxylic acids, mainly with esters content showed significant differences among lizard species. The potential role of these chemical compounds to social and sexual behaviours is discussed.

Keywords. Chemical signals, femoral glands, interspecific variation, tropical lizards.

Introduction

Chemical signals are pivotal information resources for communication in several animal groups (MÜLLER-SCHWARZE 2006, WYATT 2014). The use of chemical signals in both inter and intraspecific interactions are of valuable interest in the study of animal behaviour (MARTÍN & LÓPEZ 2014, WYATT 2017). In reptiles, for example, some of these semiochemicals contribute to regulate social and reproductive behaviours (HOUCK 2009, MASON & PARKER 2010, MARTÍN & LÓPEZ 2011).

In recent years, lizards have become a popular model group to study the composition and functional of vertebrate chemical signals (e.g. BAECKENS 2019). Lizards produce and release several chemical compounds through the skin or specialized body glands (WELDON et al. 2008). For example, many species have femoral, pre-cloacal or pre-anal glands (MARTÍN & LÓPEZ 2011, KHANNOON 2012, VALDECANTOS et al. 2014). These glands produce holocrine secretions that are released through epidermal pores, and are constituted mainly by lipids and proteins (MARTÍN & LÓPEZ 2015, MANGIACOTTI et al. 2019). Both male and female lizards have femoral (or the analogous pre-cloacal or pre-anal) pores, but in many species secretions are only observed in males, which have more abundant secretions during the mating season (MARTÍN & LÓPEZ 2015).

Although tropical ecosystems are home to an important diversity of lizard species (PIANKA & VITT 2003), information about semiochemicals that many lizards use for communication is understudied compared to Lacertid species from Mediterranean environments (GARCÍA-ROA et al. 2016, BAECKENS et al. 2018).

Here we identified the lipophilic fraction of chemical compounds in the femoral secretions of males of three arboreal and two terrestrial lizard species from a tropical area of western Mexico, and evaluated the existence of interspecific differences in the relative abundance of each group of chemical compounds. We discuss our results with previously reported chemical compounds in other species under similar or different environmental conditions.

Materials and Methods

Study area and lizard species

During field-work, carried out in may-june 2017, coinciding with lizards' mating seasons (RAMÍREZ-BAUTISTA et al. 1995, 2000, 2006, RAMÍREZ-BAUTISTA & OLVERA-BECERRIL 2004, DÍAZ et al. 2017), we captured by loop adult males of five species in the vicinity of Maruata bay in the Michoacan State on the west coast of Mexico (18.271944° N, -103.355556° W). The area is characterized by a dry tropical climate with an average annual temperature of 28°C and a long annual dry season from November through June (ALVARADO et al. 1995). The dominant vegetation is dry deciduous forest (RZEDOWSKI 1978, ALVARADO et al. 1995). There are also patches of xerophilus vegetation, herbaceous communities in coastal dunes, and stands of introduced palms species and banana trees. Ten common house geckos *Hemidactylus frenatus* with snout vent length (SVL)= 53.1 ± 1.2 mm, weight= 4.2 ± 0.2 g, seven black-nosed lizards *Sceloporus melanorhinus* (SVL= 73 ± 3.6 mm, weight= 18.6 ± 2.7 g) were captured on coconut palms (*Cocos nucifera*) and oil palms *Elaeis guineensis*; five tropical tree lizards *Urosaurus bicarinatus* (SVL= 44.2 ± 1.3 mm, weight= 3.5 ± 0.1 g) were captured on guanacaste trees (*Enterolobium cyclocarpum*) and fallen trunks. Fifteen terrestrial many-lined whiptail lizards *Aspidoscelis lineattissimus* (SVL= 90.7 ± 1.5 mm, weight= 22.5 ± 1.2 g) were captured in open areas of tropical deciduous forest and xerophilous vegetation where *Bursera* spp, *Cercidium* spp, *Prosopis* spp, *Acacia* spp, *Opuntia* spp and *Stenocereus* spp were the predominant plant genera. Additionally, nine terrestrial red-headed spiny lizard *Sceloporus pyrocephalus* (SVL= 66.6 ± 1.5 mm, weight= 14.8 ± 0.8 g) were captured around saxicolous habitats and rock quarries with little or no xerophilous vegetation present (Fig. 1).

Analyses of lizard femoral gland secretions

Immediately after capture, femoral secretions were obtained by gently squeezing the femoral pores with forceps. Forceps were washed with alcohol and distilled water between extractions. Femoral secretions were collected with glass inserts and immediately put inside sterilized glass vials that were closed with Teflon-lined stoppers. New glass inserts were used for every sample. Vials were stored at -20°C until analysis. We also used the same procedure, but without collecting secretion, to obtain blank control vials that were treated with the same procedure to compare with the femoral secretion samples, and to be able to exclude contaminants from the handling procedure or from the environment, and for further examining impurities in the solvent.

Solvent extraction of the solid samples was performed adding 200 μ l of dichloromethane (Sigma). Samples were placed in vortex for 5 min, then centrifuged by 5 min for particle precipitated elimination (insoluble portion) and stored at -20°C . The solvent-extract phase was collected and transferred to appropriate vials for GC-MS analysis.

The samples were analysed by GC-FID and GC-MS (GC; Agilent 7890A, equipped with MSD 5975C and FID detectors; Agilent technologies), fitted with a fused-silica capillary column (Zebron ZB-FFAP, 30 m length X 0.25 mm ID, 0.25- μ m film thickness; Phenomenex) with helium as the carrier gas. In splitless mode were injected 2 μ l of each sample dissolved in dichloromethane. Sample injection was performed at splitless temperature of 280°C and FID detector temperature of 300°C . Oven temperature was programed to start at 50°C , maintained in isothermal for 5 min, then increased to 280°C at a rate of $10^{\circ}\text{C}/\text{min}$, and then isothermal 280°C for 5 min. To analyse using both the MS and FID detectors, at the exit of the capillarity column, a continuous-flow particle separation (50:50) was installed. The mass spectrometer was operated at an ionization voltage of 70 eV and with scanning between m/z 30-500 at 3.9 scans/s. Quantitation was performed using the relative values of the peak areas in chromatograms obtained by using FID detector. Compounds were identified by comparison with the mass spectral library (NIST/EPA/NIH, ChemStation, Agilent Technologies Rev. D.04.00 2014). The synthesized compounds used for GC-MS identification consisted of fatty acids (palmitic and stearic acids) and esters (n-butyl acetate) was acquired from Sigma-Aldrich. Other synthesized compounds as the esters isobutyl acetate, methyl laurate, isopropyl myristate, methyl palmitate, methyl palmitoleate, and methyl oleate were obtained by chemical synthesis in our laboratory using the respective chemical precursors. Precursor compounds were acetic acid, methanol, ethanol, isopropanol, isobutanol, lauric acid, myristic acid, palmitic acid, palmitoleic acid, oleic acid, and stearic acid (all from Sigma-Aldrich, Merck or T.J. Baker). Briefly, esters synthesis was carried out by esterification of their respective alcohols with fatty acids treated with H_2SO_4 and KMnO_4 at 130°C for 30 min (CAMPOS-GARCÍA et al. 2018). All the esters compounds were extracted with dichloromethane solvent, evaporated, weighed, and dissolved in dichloromethane for GC-MS analysis. Additionally, FAME Mix C4-C24, catalog no. 18919 (Supelco) were used as standards.

Statistical analyses

The proportion of each peak identified in the GC chromatograms were determined by calculating the relative proportion of each compound (area under the peak) with respect to the total peak area for all chemicals (IBÁÑEZ et al. 2017). Then, relative areas of the peaks were transformed following Aitchison's formula: $[Z_{ij} = \ln(Y_{ij}/g(Y_j))]$, where Z_{ij} is the standardized peak area i for individual j , Y_{ij} is the peak area i for individual j , and $g(Y_j)$ is the geometric mean of all peaks for individual j (AITCHISON 1986, DIETEMANN et al. 2003). Then, we calculated euclidean distances between every pair of individual samples to produce a resemblance matrix that formed the basis of the analyses. We used a single factor permutational multivariate analysis of variance test PERMANOVA (ANDERSON 2001, MCARDLE & ANDERSON 2001) based on the euclidean resemblance matrix using 999 permutations to analyse whether the composition of the femoral secretions varied between the five species. All unidentified and identified compounds were included in the analysis. Differences between species were further investigated using canonical analysis of principal coordinates (CAP) (ANDERSON & WILLIS 2003). The software PRIMER V6.1.13 (CLARKE & GORLEY 2006) with the PERMANOVA V1.0.3 add-on package (ANDERSON et al. 2008) was used to investigate differences between chemical profiles. In addition, we assessed if there were statistical differences in the relative amount (area under the peak) of each compound class among species by fitting negative binomial GLM analysis (GARDNER et al. 1995). Pairwise comparisons were performed with Tukey HSD test. All statistical analyses were performed with the interface Rstudio in R software version 3.3.2 (R DEVELOPMENT CORE TEAM 2013).

Results

Chemical profiles of lizard species

We found 8 lipophilic compounds in femoral gland secretions of male lizards *H. frenatus* (Table 1), which was constituted mainly by fatty acid esters corresponding to 4.4% of total area from all found peaks, three fatty acids (acetic, palmitic, and stearic acids) corresponding to 60.6%, and 35% of squalene. The three most abundant chemicals, which together comprised >90% of the total peak areas were palmitic and stearic acid and squalene (Table 1).

Femoral gland secretions of male *S. melanorhinus* contained a mixture of 9 compounds that can be identified by mass fragmentation profiles (Table 1). Overall, these compounds mainly consisted of esters 40.9%. These were mainly of two types: esters of acetate such as isobutyl acetate and esters from fatty acids, such as methyl laurate. The three most abundant chemicals, which together comprised >70% of the total peak areas were methyl laurate, palmitic acid and squalene (Table 1).

In the gland secretions of *U. bicarinatus* males, 10 compounds including esters 17.6%, three carboxylic acids 43.4% and others such as squalene 38% were found. The three most abundant chemicals, which together comprised >90% of the total peak areas were isobutyl acetate, palmitic acid and squalene (Table 1).

Femoral gland secretions of *A. lineattissimus* males contained a mixture of 12 compounds, including esters 38%, carboxylic acids 38%, and others such as squalene and dihydro-lanosterol 24%. The five most abundant chemicals, which together comprised >90% of the total peak areas were isobutyl acetate, methyl oleate, palmitic acid, stearic acid and squalene (Table 1).

We registered the presence of 5 compounds in the femoral gland secretions of *S. pyrocephalus* males, including three esters (2.4%) and two carboxylic acids 97.6%. The most abundant chemical was palmitic acid (Table 1).

Chemical variation among species

The PERMANOVA analysis based on the resemblance matrix comparing individuals of each species showed significant differences in the proportion of compounds among the five evaluated species (pseudo $F_{4,41} = 7.35$, $p = 0.001$). The pairwise comparisons showed significant differences in all cases (permutation tests, $2.06 < t < 3.60$, $p = 0.001$ for all). The CAP analysis assembled 100% of the chemical profiles into the correct species using leave-one-out cross-validation and $m=6$ axes (permutational test, $\delta_1^2 = 0.982$, $p = 0.001$, Fig. 1). Considering the five species, carboxylic acids, fatty acid esters and squalene were the most abundant chemical types, being the palmitic acid the main and most abundant compound. We found significant differences in the relative proportions of carboxylic acids among species (GLM; $\chi^2 = 9.63$, $df=4$, $p < 0.001$) but not in palmitic acid ($\chi^2 = 1.81$, $df = 4$, $p = 0.14$). Pairwise comparisons showed that *H. frenatus* and *S. pyrocephalus* have lower proportions of carboxylic acids than *A. lineattissimus* and *S. melanorhinus* (Tukey's test, $p <$

0.01 for all), but *U. bicarinatus* not differed significantly of any other species ($p= 0.15$). Squalene, although absent in *S. pyrocephalus* was the second most abundant compound (29 %) in the other species. However, we found no significant differences in the relative proportions of squalene among species ($\chi^2 = 2.10$, $df= 3$, $p= 0.11$). Regarding esters, we found significant differences among species ($\chi^2 = 9.79$, $df=4$, $p< 0.001$), comparisons showed that *S. melanorhinus*, *U. bicarinatus* and *A. lineattissimus* have higher proportions of esters than *H. frenatus* and *S. pyrocephalus* ($p< 0.01$ for all), other comparisons were not significant.

We found some differences in chemical profiles among arboreal and terrestrial species (Table 1). Arboreal lizards (*H. frenatus*, *S. melanorhinus*, and *U. bicarinatus*) shared eight compounds among them, whereas the terrestrial lizards (*A. lineattissimus* and *S. pyrocephalus*) shared five compounds. Exclusive compounds were found only in *A. lineattissimus* (methyl oleate and dihydro-lanosterol), while the remaining species did not show exclusive compounds. Both groups of species, arboreal and terrestrial shared four compounds; two esters and, two carboxylic acids (Table 1).

Discussion

Our study reports fatty acids, esters, and squalene as main chemical compounds registered in femoral glands secretions of five tropical lizard species. This composition is similar to that registered in other lizard's species. For example, hexadecanoic acid (palmitic acid) was the main fatty acid and the most abundant compound present in both terrestrial and arboreal lizards. This result is similar as described for other tropical lizard species (e.g. Fam. Agamidae, Crotaphytidae and Iguanidae) (ALBERTS et al. 1992, MARTÍN et al. 2013 a,b, IBÁÑEZ et al. 2017). Other carboxylic acids of long-chain such as octadecanoic acid (stearic acid) was found in all studied species, except *S. pyrocephalus*; but palmitic acid was found in higher proportions than in other lizards. Fatty acids of long-chain and very-long-chain types have been reported in other lizards from arid and desert ecosystems (LÓPEZ & MARTÍN 2005a, MARTÍN et al. 2012) and have a functional role as territorial scent marks (KHANNOON 2016). It is probable that one or more carboxylic acids in the lizard secretions of our study facilitate the development of similar social contexts, but today it's an information gap that needs to be evaluated.

Some lizards inhabiting high mountain areas with low temperatures and high humidity, frequently have greater relative proportions of esters in their femoral gland secretions than lizards inhabiting low elevation areas (GABIROT et al. 2012, MARTÍN et al. 2017). This ester variation is explained as an adaptation to maximize efficacy of substrate scent marks under different microclimatic conditions (MARTÍN et al. 2015, BAECKENS et al. 2018). In our study, lizards from tropical climates with high temperature and humidity produced relatively higher amounts of esters (5-42%) than the high mountain lizards *Psammodromus algirus* (5.5% of esters) from Mediterranean climates (MARTÍN et al. 2017). Possibly based on its chemical properties, esters are more volatile and prone to oxidation than carboxylic acids in environments with tropical climates. Therefore, it is possible but not conclusive that more humid habitats influence higher proportions of esters due to its accelerated hydrolysis.

Although in relatively low proportions, squalene is a common compound in femoral secretions of many lizard species (WELDON et al. 2008). This chemical is considered as an antioxidant that enhances the signalling function of other compounds (MARTÍN & LÓPEZ 2014). As squalene might stabilize other lipid compounds by limiting oxidation (GABIROT et al. 2008), its abundant presence in four of the five studied species may be an adaptation to the usually high humidity of tropical environment. Squalene role as a pheromone has only been tested in fossorial amphisbaenians, and it has been considered an important compound in agonistic and sex discrimination behaviours (LÓPEZ & MARTÍN 2005b). Although the presence of diverse steroids in femoral secretions of lizards is frequent (WELDON et al. 2008), in our study only one steroid, the lanost-8-en-3 β -ol (dihydro-lanosterol) was registered in one terrestrial species (*A. lineattissimus*). Cholesterol and some of its derivatives are the most important steroids recorded in almost all lizard species (MARTÍN et al. 2015). However, there are other steroids of plant origin (phytosterols), example of this is the lanosterol, abundant steroid in femoral gland secretions of the green iguana, *Iguana iguana*, suggesting a possible relationship between diet and chemical characteristics of gland secretions (ALBERTS et al. 1992, BAECKENS et al. 2017). Similar to lanosterol, dihydro-lanosterol, present in *A. lineattissimus*, is a phytosterol and may be could have some relationships with the food spectrum of this species.

In conclusion, we identify a significant diversity of lipophilic compounds in femoral gland secretions of arboreal and terrestrial species of tropical lizards showed clear interspecific differences in fatty acids and esters. Some of the compounds reported in this study have been described as semiochemicals with signaling function (pheromones) involved in the sexual communication of some lizard's species, but their behavioural role in tropical lizards remains to be confirmed in future research.

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Figure and table legends:

Fig. 1. Species of lizards photographed on their substrate habitat in the tropical Maruata bay. (A) *Hemidactylus frenatus*, (B) *Sceloporus melanorhinus*, (C) *Urosaurus bicarinatus*, (D) *Aspidoscelis lineattissimus*, and (E) *Sceloporus pyrocephalus*.

Fig 2. Canonical analysis of principal coordinates (CAP) showing classification of chemicals identified on femoral secretions of three arboreal *H. frenatus*, *S. melanorhinus*, and *U. bicarinatus*; and two terrestrial *A. lineattissimus* and *S. pyrocephalus* lizard species

Table 1. Chemical compounds identified from femoral gland secretions of arboreal and terrestrial lizard species. Relative proportions (mean \pm SE of the percentage of the total peak areas from FID detector). nd, not detected compound. RT retention time and retention index are showed. Compounds without standard confirmation (*).

Fig. 1

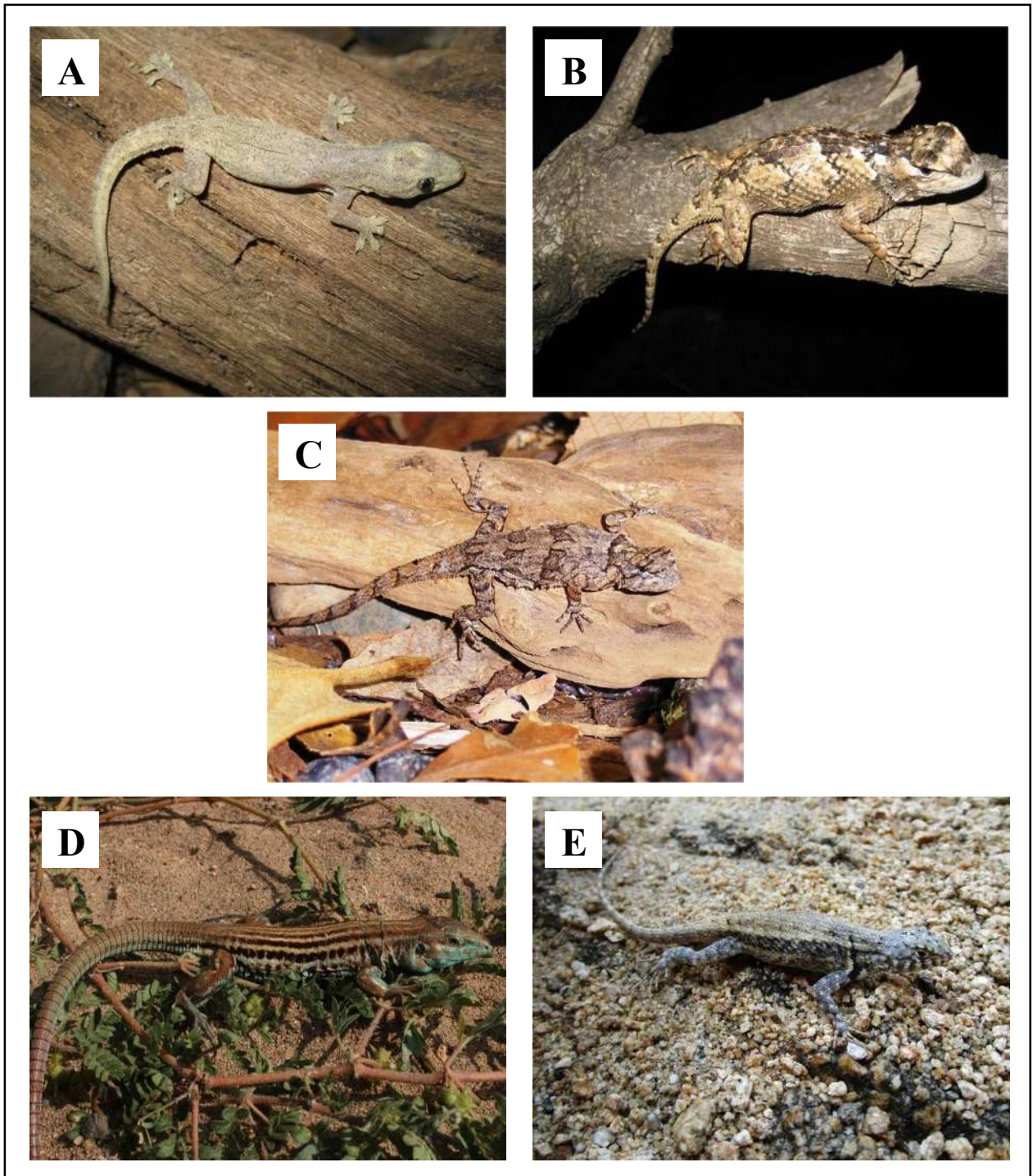


Fig. 2

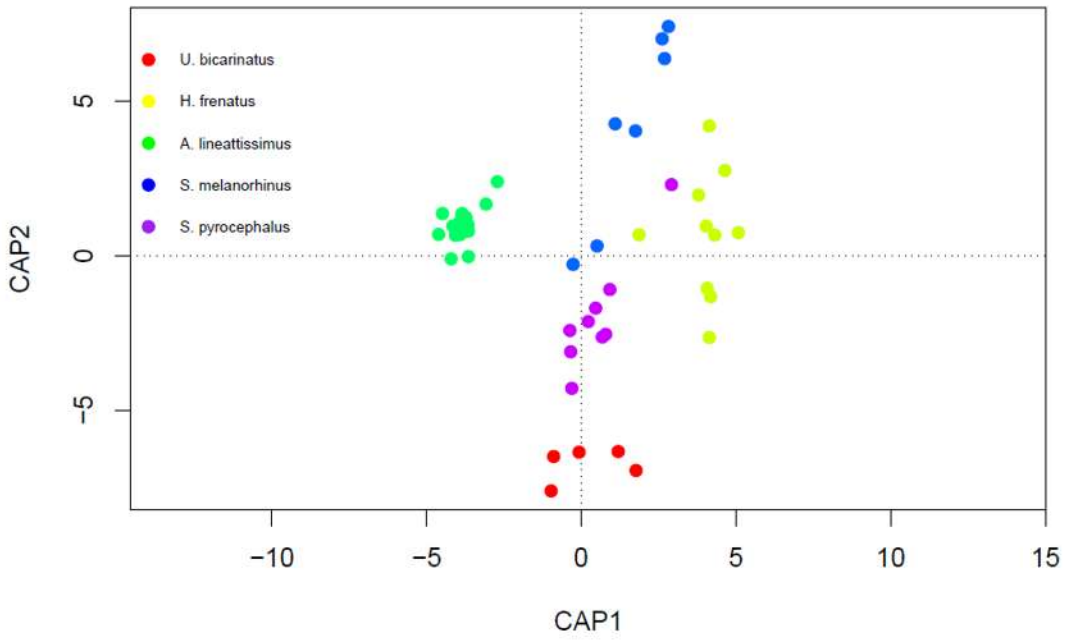


Table 1

RT (min)	Compound name	R. Index	Arboreal lizards			Terrestrial lizards	
			<i>H. frenatus</i>	<i>S. melanorhinus</i>	<i>U. bicarinatus</i>	<i>A. lineattissimus</i>	<i>S. pyrocephalus</i>
<i>Esters</i>							
5.2	Isobutyl acetate	1012	0.55 ± 1.32	7.01 ± 9.78	13.27 ± 4.69	9.61 ± 1.65	nd
6.4	n-Butyl acetate	1074	nd	nd	0.22 ± 3.70	0.15 ± 1.05	0.24 ± 5.51
19.3	Methyl laurate	1804	1.46 ± 1.43	31.39 ± 6.33	1.52 ± 5.00	1.28 ± 0.74	1.32 ± 5.00
22.3	Isopropyl myristate	2027	0.21 ± 2.32	0.20 ± 6.19	0.42 ± 1.40	0.36 ± 2.13	0.80 ± 17.77
24.5	Methyl palmitate	2208	nd	0.23 ± 3.59	0.97 ± 2.14	0.56 ± 1.03	nd
24.7	Methyl palmitoleate	2240	2.19 ± 2.69	2.02 ± 8.32	1.23 ± 8.54	1.12 ± 0.65	nd
27.0	Methyl oleate	2434	nd	nd	nd	25.64 ± 1.2	nd
<i>Carboxylic acids</i>							
14.1	Acetic acid	1449	1.35 ± 1.17	0.64 ± 3.70	1.52 ± 7.67	1.18 ± 0.22	4.96 ± 1.59
31.4	Palmitic acid	2931	39.95 ± 3.04	25.44 ± 6.44	42.62 ± 8.83	25.98 ± 2.50	92.68 ± 5.50
33.6	Stearic acid	3134	19.27 ± 2.46	11.35 ± 7.13	0.21 ± 1.10	10.89 ± 0.90	nd
<i>Others</i>							
33.1	t-Squalene*	2865	35.02 ± 3.17	21.72 ± 7.00	38.02 ± 8.56	22.93 ± 2.10	nd
41.3	Dihydro-lanosterol*	3823	nd	nd	nd	0.29 ± 2.15	nd

CAPÍTULO II

**Chemical signal divergence among populations influences behavioral discrimination
in the whiptail lizard *Aspidoscelis lineattissimus* (Squamata: Teiidae)**

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Chemical signal divergence among populations influences behavioral discrimination in the whiptail lizard *Aspidoscelis lineattissimus* (squamata: teiidae)

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Abstract. Signal divergence and sensory preferences may lead to sexual isolation and eventually promote speciation between animal populations. However, few studies have quantified the degree of chemical signal divergence and scent-mediated sexual isolation in lizard populations. Geographic and ecological variation among populations of the whiptail lizard *Aspidoscelis lineattissimus* suggest that there might be chemical signal divergence among these populations. Here we used gas chromatography-mass spectrometry to

characterize and compare the chemical composition of the femoral gland secretions of male whiptail lizards of four populations from a western region of Mexico, and through behavioral experiments explored the effects of lizard scents on precopulatory behaviors and intrasexual male-male chemical recognition among populations. Our results showed that males of each population contain a divergent mixture of compounds in their femoral gland secretions. Differential chemosensory behavior indicated that male and female lizards discriminated and were more attracted to scents of lizards from their same population. Although females also seem to discriminate male scents between populations, their associated preference to territories scent-marked by males of their own population is different between regions (eastern vs western) and not between populations. We suggest that between some populations of *A. lineattissimus* there may be partial premating isolation mediated by chemical signals and behavioral divergence.

Significance statement

Geographic variation in sexual signals can strongly affect discrimination and recognition abilities among reproductive individuals from divergent populations, resulting in sexual isolation and speciation. Studies suggest that reproductive isolation and speciation in lizard systems may be mediated by chemical signals, male mate preferences and male-male interactions but not by female mate preferences. Using gas chromatography-mass spectrometry techniques and behavioral experiments, we found that chemical divergence in femoral gland secretions of male *Aspidoscelis lineattissimus* influences behavioral discrimination among four distinct populations. Males and females recognized and responded more toward lizard scents from their own population. In addition, some female populations were able to discriminate between territories scent marked by males from different populations. We suggest that chemical and behavioral differences between populations may influence partial premating isolation, which can be mediated by inter and intrasexual interactions.

Keywords for indexing: chemical cues, chemosensory recognition, femoral glands, lizards, female preferences, premating isolation.

Declarations:

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Conflicts of interest/Competing interests. The authors declare that they have no conflict of interest.

Ethics approval. This study was carried out in strict accordance with the guidelines for use of live amphibians and reptiles in field research compiled by the American Society of Ichthyologists and Herpetologists (ASIH). In the research protocol, adult lizards were visually detected and captured without any harm. During the experimental trials all experimental organisms were maintained under optimal conditions with access to water and food *ad libitum*. At the end of the experiments, these individuals were returned in good health to the original capture sites. All procedures with animals were carried out in accordance with the standards of bioethics and biosafety of the Universidad Michoacana de San Nicolás de Hidalgo.

Availability of data and material. The datasets generated and/or analysed during the current study are available from the corresponding author on reasonable request.

Authors' contributions. Conceptualization: ERG, ISO; Methodology: ERG, JM, JCG; Formal analysis and investigation: ERG, JCG, JM; Writing - original draft preparation: ERG; Writing - review and editing: ISO, JAD, EMR, JM, JCG; Funding acquisition: ISO; Resources: ISO, JCG; Supervision: ISO, JAD, EMR.

Introduction

Speciation involves the evolution of reproductive isolation between diverging populations (Mayr 1963; Nosil et al. 2007; Barton and De Cara 2009). Understanding speciation thus requires determining which reproductive barriers initially reduced gene flow between

populations and the evolutionary forces producing them (Coyne and Orr 2004; Sobel et al. 2010). The evolution of premating isolation caused by divergent mating signals and preferences (sexual isolation, hereafter) appears to be an important component of speciation in many taxa (Coyne and Orr 2004; Safran et al. 2013). Many selective processes can affect the evolution of sexual isolation, but their relative contributions are poorly understood (Boughman et al. 2005; Nosil et al. 2007; Schluter 2009).

Mating signal divergence among populations has been studied relatively more in visual and acoustic sensory systems than chemosensory systems (Tobias et al. 2010; Wilkins et al. 2013; Schaefer and Ruxton 2015). Chemical signals are widely used as phenotypic traits in social and reproductive communication among several animal groups (Müller-Schwarze 2006; Wyatt 2014). Interpopulation differences in chemical traits can be amplified by sexual selection, leading to differences in mate preferences and chemosensory recognition (Boughman 2001, 2002; Panhuis et al. 2001; Ritchie 2007). Therefore, chemical variations are of particular interest to understand premating reproductive isolation and speciation processes (Symonds and Elgar 2008; Smadja and Butlin 2009).

In many lizards, sexual and social behaviors are partially based on the use of chemical cues secreted by femoral glands of males (Mason and Parker 2010; Martín and López 2011, 2014). These secretions are deposited on substrates and used as reliable scent marks to convey information (e.g. health state, social status, and competitive ability) to conspecifics (Martín and López 2006, 2015). These scent marks may be important to intrasexual (e.g. male-male rival recognition) and intersexual relationships (e.g. potentially mate preference) (Olsson et al. 2003; López et al. 2006; Carazo et al. 2008; López and Martín 2011; Martín and López 2015). Interspecific and interpopulation chemical signal variation has been reported in reptile species (Cooper and Vitt 1986; Shine et al. 2002; Barbosa et al. 2006; Gabirot et al. 2010a, b, 2012). There are relatively few studied lizard systems using information of geographic variation of chemical traits affecting sexual isolation among divergent populations in an experimental setting (Runemark et al. 2011; Gabirot et al. 2012, 2013; Martín et al. 2016; MacGregor et al. 2017). Therefore, we do not have precise knowledge of the magnitude, causes and the relevance of sexual isolation mediated by chemical signals in lizard evolution (Martín and López 2015). Thus, we consider important for speciation research to understand how evolutionary processes

mediated by chemical signals occur in lizard systems with early signs of species divergence.

The many-lined whiptail lizard *Aspidoscelis lineattissimus* is a medium-sized (67-110 mm snout-vent length) teiid lizard that inhabits open areas of tropical deciduous forest and xerophilous vegetation in the middle western coast of Mexico (Ramírez-Bautista et al. 2000). Taxonomic status of *A. lineattissimus* species complex is uncertain and molecular and phylogeographic information is limited (Castiglia et al. 2010). However, Duellman and Wellman (1960) considered that there are currently four subspecies within this complex. There is some information about reproductive and thermal ecology (Ramírez-Bautista et al. 2000; Navarro-García et al. 2008), dietary habits (Güizado-Rodríguez and Casas-Andreu 2011), and morphology between continental and insular populations (Walker 1970; Hernández-Salinas et al. 2014).

Within the wide geographic distribution of this species there are allopatric populations separated by geographical barriers (e.g. ocean stretch, mountain system). These barriers also contribute to the existence of habitats with contrasting environmental characteristics (e.g. temperature, humidity, vegetation type). These features resulted in populations with a marked ecological and geographical isolation (Duellman and Wellman 1960; Wang and Bradburd 2014). Additionally, there are other *A. lineattissimus* populations with broad and continuous distributions (parapatric populations) (Duellman and Wellman 1960).

Chemosensory recognition is well developed in the genus *Aspidoscelis* and individuals can differentiate between chemical cues of plant food and arthropod prey (Cooper et al. 2000). They can also identify chemical cues of different predators, such as snakes and lizards (Punzo 2007, 2008). Several factors suggest that *Aspidoscelis* lizards might rely on chemicals for intraspecific communication. For example, 1) they have femoral glands that are the source of chemical signals in many species of lizards (Duellman and Wellman 1960; Martín and López 2014), 2) observations have revealed a “cloacal rubbing” behavior in *Aspidoscelis* lizards (Carpenter 1962), 3) it has been observed that male lizards release chemical signals (femoral secretions) onto substrates making scents marks (Ribeiro et al. 2011), and 4) *Aspidoscelis* lizards display tongue flicking behavior

when exploring substrates and conspecifics (Ribeiro et al. 2011). Tongue flicking is a chemoreceptive response in many reptiles (Cooper 1998; Cooper and Burghardt 1990).

These features of *Aspidoscelis* species and the geographic and ecological conditions of *A. lineattissimus* suggest that there might exist a chemical signal divergence among populations. These chemosensory differences might influence variation in the sexual interactions and precopulatory behaviors of *A. lineattissimus*. Here we explored how chemical signal divergence affects the initial recognition and attraction between sexes and female choice of male territories. To test this, 1) we analyzed the possible existence of chemical variation in femoral gland secretions of males from four different populations, 2) we evaluated the intersexual and intrasexual chemosensory recognition responses of male and female lizards to chemical scents of their own and different population, and 3) we tested if females preferred territories with chemical scents of males of their own or of different population. We hypothesized that chemical variation in femoral gland secretions could explain differences in sexual discrimination and female choice of male territories in populations of *A. lineattissimus*. These chemical and behavioral differences could affect reproductive isolation to future stages of secondary contact between at least the two most geographically isolated populations of *A. lineattissimus*.

Materials and methods

Study populations and animal maintenance

During May-June 2018, we captured by noosing and pitfall traps adult male and female *A. lineattissimus* lizards from four localities in middle western Mexico (Fig. 1). Within this region two populations were located in the most western area and are separated by an ocean stretch (“Perula” and “Island” populations), two other populations were located in the most eastern area and were separated by the mountain system “Sierra Madre del Sur” (“Capirio”, and “Maruata” populations), and two populations (“Maruata” and “Perula”) were located between eastern and western areas and are not separated by a geographical barrier (parapatric distribution). These populations (both allopatric and parapatric) show differences in morphology and coloration (Duellman and Wellman 1960; Hernández-Salinas et al. 2014; Fig. 2). In the western area, we captured 15 male and 15 female lizards from “Perula population” in abandoned agricultural fields and thorny scrub vegetation (Fig.

2) in Jalisco state (19.589167° N, -105.126111° W; 10 m altitude; Fig. 1). We also captured 17 male and 13 female lizards from “Island population” inhabiting tropical deciduous forest and xeric vegetation (Fig. 2) on Cocinas island from the bay of Chamela in Jalisco state (19.546637° N, -105.109577° W; 40 m altitude; Fig. 1). In the eastern area we captured 21 male and 14 female lizards from “Capirio population” in gallery forest adjacent to the rivers in the locality of Múgica within the Balsas-Tepalcatepec Depression (Michoacán state, 18.8500266° N, -102.1351157° W; 220 m altitude; Fig. 1). Finally, we collected 20 male and 15 female lizards from “Maruata population” in the coast of Michoacán state (18.271944° N, -103.355556° W; 20 m altitude; Fig. 1). These lizards occupy open areas of tropical deciduous forest and xerophilous vegetation in the vicinity of Maruata beach (Fig. 2).

To evaluate differences in sexual behavior, mediated by scent marks, lizards collected from the four studied populations were transported to Maruata in the coast of Michoacan state. Lizards were individually housed at Centro Ecoturístico Ayult in indoor 60 X 40 X 30 cm PVC terraria containing a coconut fiber substratum and a plywood refuge for cover. Every day, lizards were fed mealworm larvae (*Tenebrio molitor*) and crickets (*Acheta domesticus*) and water was provided *ad libitum*. At the end of experiments lizards were returned in good health to the original capture sites.

Analysis of femoral gland secretions

Immediately after capture, femoral secretions were obtained by gently squeezing the femoral pores with forceps. Forceps were washed with alcohol and distilled water between extractions. Femoral secretions were collected with glass inserts and immediately put inside sterilized glass vials that were closed with Teflon-lined stoppers. New glass inserts were used for every sample. Vials were stored at -20°C until analysis. We also used the same procedure, but without collecting secretion, to obtain blank control vials that were treated with the same procedure to compare with the femoral secretion samples, and to be able to exclude contaminants from the handling procedure or from the environment, and for further examining impurities in the solvent.

Solvent extraction of the solid samples was performed by adding 200 µl of dichloromethane (Sigma). Samples were placed in vortex for 5 min, then centrifuged for 5

min for particle precipitated elimination (insoluble portion) and stored at -20°C. The solvent-extract phase was collected and transferred to appropriate vials for GC-MS analysis.

The samples were analyzed by GC-FID and GC-MS (GC; Agilent 7890A, equipped with MSD 5975C and FID detectors; Agilent technologies), fitted with a fused-silica capillary column (Zebron ZB-FFAP, 30 m length X 0.25 mm ID, 0.25- μ m film thickness; Phenomenex) with helium as the carrier gas. In splitless mode we injected 2 μ l of each sample dissolved in dichloromethane. Sample injection was performed at splitless temperature of 280 °C and FID detector temperature of 300 °C. Oven temperature was programmed to start at 50 °C, maintained in isothermal for 5 min, then increased to 280°C at a rate of 10°C/min, and then isothermal 280°C for 5 min. To analyze using both the MS and FID detectors, at the exit of the capillary column, a continuous-flow particle separation (50:50) was installed. The mass spectrometer was operated at an ionization voltage of 70 eV and with scanning between m/z 30-500 at 3.9 scans/s. Quantification was performed using the relative values of the peak areas in chromatograms obtained by using FID detector. Compounds were identified by comparison with the mass spectral library (NIST/EPA/NIH, ChemStation, Agilent Technologies Rev. D.04.00 2014) and standard compounds. The standard compounds used for GC-MS identification were acquired from Sigma-Aldrich, Merck or T.J. Baker, and FAME Mix C4-C24, catalog no. 18919 (Supelco).

The proportion of each chemical component was determined by calculating the relative proportion of each compound (area under the peak) with respect to the total peak area for all chemicals (Ibáñez et al. 2017). Then, relative areas of the peaks were transformed following Aitchison's formula: $[Z_{ij} = \ln(Y_{ij}/g(Y_j))]$, where Z_{ij} is the standardized peak area i for individual j , Y_{ij} is the peak area i for individual j , and $g(Y_j)$ is the geometric mean of all peaks for individual j (Aitchison 1986; Dietemann et al. 2003). Then, we calculated euclidean distances between every pair of individual samples to produce a resemblance matrix that formed the basis of the analysis. We used a single factor permutational multivariate analysis of variance test PERMANOVA (Anderson 2001; McArdle and Anderson 2001) based on the euclidean resemblance matrix using 999 permutations to analyze whether the composition of the femoral secretions varied between

the four populations. All identified compounds were included in the analysis. Differences between populations were further investigated using canonical analysis of principal coordinates (CAP) (Anderson and Willis 2003). The software PRIMER V6.1.13 (Clarke and Gorley 2006) with the PERMANOVA V1.0.3 add-on package (Anderson et al. 2008) was used to determinate differences between chemical profiles.

In addition, we assessed if there were statistical differences in the relative amount (area under the peak) of five major compounds and compound classes (e.g. esters, carboxylic acids). among populations by fitting negative binomial GLM analysis (Gardner et al. 1995). When the number of zeros (compound absence) was large in many samples, we adjusted GLM models to fit zero-inflated models (Heilbron 1994; Tu 2014). We conducted the zero inflated and negative binomial GLMs analysis using the `zeroinfl` function in `pscl` package (Jackman et al. 2011) in R software ver. 2.13.0 (R Core Team 2013). For GLMs, we used χ^2 tests using the ANOVA function to evaluate the significance of terms in the model.

Experimental design for behavioural discrimination

Chemosensory recognition

To test for chemosensory discrimination among populations, we made comparisons of tongue flick (TF) rate by lizards (male and female) in response to chemical stimuli (lizard scents) from males or females from each of the four different populations (Perula, Island, Capirio, and Maruata) or with deionized water (odorless control). We obtained lizard scents from the femoral pores of males by squeezing femoral pores with forceps (they were cleansed between extractions) and secretion was collected with a cotton applicator, whereas female secretions were obtained by rubbing a cotton applicator moistened with distilled water over the cloacal area. Because *Aspidoscelis* lizards swiftly escape in the researcher presence, lizard scents were presented on small ceramic tiles (15 X 15 cm) without the intervention of the researcher (Cooper et al. 2000). Tiles were prepared by rubbing the lizard scents on them with the aid of a cotton swab (above, same swab of collection). We used a new applicator and tile in each trial.

We tested each individual under three sets of chemical discrimination experiments to evaluate intrasexual recognition (male-male) and intersexual recognition (male-female,

and female-male) among individuals. Each set of experiments considered the four study populations, all individuals were exposed to lizard scents from their own and from all different populations (treatments). In the three experimental sets we also tested responses to deionized water (odorless control), which was used to gauge baseline TF's rates in the experimental situation (Cooper and Burghardt 1990). Each individual participated in one trial (one scent) per day consecutively until the end of each set of experiments. Trials were conducted in outdoor conditions in June, which coincided with the mating season of lizards in their original natural habitats (Ramirez-Bautista et al. 2000), and between 11:00 and 13:00 (GMT) when lizards were fully active and container achieved a temperature of 31°C. Scent treatments and individuals were assigned under a randomized block design before initiating an experiment. In each trial, the same person in all tests (ERG) slowly approached the terrarium and carefully placed the tile with the lizard scent in the center of the terrarium. The observer was blind respect to the lizard scents tested. Water control was first tested in all cases to eliminate the potential confounding effects of the presence of tile within the container. The total number of TFs directed at the tile were recorded for 10 min beginning with the first TF.

Female choice of male territories

We performed this experiment at the end of June, coinciding with the mating season of this species (Ramírez-Bautista et al. 2000). We ensured that lizards were in reproductive state, selecting only females with developed ovarian egg follicles detectable by abdominal palpation (the onset of sexual activity) but had not yet mated (i.e. mating scars were absent). Selected males had developed blue or red breeding coloration and presented abundant femoral secretions. Prior to experiments, we had placed in males' terraria several absorbent paper strips (25 × 10 cm) fixed to the floor, and left them there for one week to allow males to scent-mark the substrates. Female cages were virtually divided in three areas of equal surface area (20 × 40 cm each). With sterilized gloves and before the females were active (08:00 h, GMT), we placed two treated paper strips, one on each side of each female's container; a paper strip with the male scent from of their own population and in the opposite side of the cage other paper strip with the male scent of different population, leaving a neutral intermediate zone. Therefore, two different scented papers from each male

were used in different choice tests with different females. Testing order and paper position were randomized. Each female participated in one trial with scented papers from two different males (same vs. different population) per day consecutively until the end of the experiment. Each trial lasted 6 h (from 9:00 h GMT, shortly after females emerged from refuges and until 15:00 h GMT). Females were observed every 15 min from a hidden point (i.e., a total of 24 observations). If a female was located active on an area containing a paper strip, she was designated as having chosen temporarily that particular area, whereas if she was located in the intermediate neutral area without paper strips, she was designated as having made no choice (Martín and López 2000, 2006; Olsson et al. 2003). We also registered observations in which the female was not seen active on any of the areas because she was hidden in the refuge. We recorded the number of times that females were observed active on each of the areas containing scent marked papers and in the non-choice area. At the end of the trials, we moved the female to another clean cage, removed the papers, and thoroughly rinsed the experimental cage with clean water and let it dry outdoors before using it in another test. We did not use commercial detergents to avoid contamination of the cage with artificial scents.

Behavioural data and analysis

Our analysis was focused to test the effects of scent on intra and intersexual chemosensory discrimination, and on female choice of male territories in different populations of *A. lineattissimus*.

Chemosensory recognition data among individuals (intrasexual recognition: male-male; intersexual recognition: male-female, and female-male) and female choice data (same territory, different territory, neutral) were analyzed as discrete variables (e.g. number of tongue flicks, and number of observations in each territory, respectively) by fitting generalized linear mixed models (GLMMs) using a negative binomial distribution (Bates et al. 2012). Fixed factors for chemosensory recognition were the following: i) Population (P, with four levels: capirio, maruata, perula, and island), ii) Scent (S, with five levels: capirio, maruata, perula, island, and water), and the interaction between these two factors (P:S). The effect of each individual (1 | lizard id) and the nested design of repeated measures of “lizard id within scent” (1 | scent : lizard id) were included as a random effect (Faraway 2005;

Pinheiro and Bates 2006; Crawley 2013). Fixed factors for female choice were the following: i) Population (P, with four levels: capirio, maruata, perula, and island), ii) Scent mark (SM, with three levels: same population, different population, and neutral), and the interaction between these two factors (P:SM). We included variance associated with “female id” nested within scent mark (SM : female id) as random effect.

We conducted the analysis using the lme4 package (Bates et al. 2012) in R software ver. 2.13.0 (R Core Team 2013). Saturated models, which included the main terms and the two-way interactions were fitted. For GLMMs, we used the glmer.nb function and maximum likelihood ratio tests (for fixed effects) were obtained using the ANOVA function. We used this function to evaluate the significance of terms in the model. The reported likelihood ratio test ($LR\chi^2$), degrees of freedom, and p-values are those from the likelihood ratio tests (Faraway 2005; Pinheiro and Bates 2006).

Results

Chemical profiles of lizard populations

We found 25 lipophilic compounds in femoral gland secretions of males from the four lizard populations (Table 1), which was constituted mainly by three carboxylic acids corresponding to 35.3% of total area from all found peaks, 17 esters (35.2%), squalene (16.6%), one steroid (9.7%) and other compounds (3.2%). The five most abundant chemicals, which together comprised 74.1% of the total peaks areas were methyl oleate, palmitic and stearic acid, dihydro-lanosterol and squalene (Table 1).

There were 17 chemical compounds shared by lizards from all populations and the presence/absence of eight compounds of which there were two exclusive compounds; methyl myristate and methyl margarate for Capirio and Island population, respectively (Table 1). The chemical profiles of the four populations were significantly different (PERMANOVA: pseudo $F_{3,69} = 10.66$, $p < 0.001$). The CAP analysis classified 100 % of the chemical profiles into the correct population using leave-one-out cross-validation (Permutational test, $\delta_1^2 = 0.98$, $p = 0.001$, $m = 2$ axes) (Fig. 3). In the pairwise comparisons there were clear differences in chemical composition of femoral gland secretions among populations ($p = 0.001$ for all).

Comparing the main classes of compounds among populations, there were differences in the relative proportions of carboxylic acids (GLM; $\chi^2 = 2.88$, $df = 3$, $p = 0.031$). Pairwise comparisons showed that Island and Perula populations differed significantly in possessing relatively higher proportions of carboxylic acids than Capirio population ($p = 0.003$ and $p = 0.048$, respectively), but other population comparisons were not significant ($p \geq 0.092$).

There were also significant differences in the relative proportions of esters among populations (GLM; $\chi^2 = 5.25$, $df = 3$, $p < 0.001$). Capirio population had significantly more esters than the Island, the Maruata and the Perula populations ($p < 0.001$ for all). Other population comparisons were not significant ($p \geq 0.099$, all cases).

When comparing the relative proportions of the five most abundant shared compounds, there were significant differences in squalene (GLM; $\chi^2 = 9.24$, $df = 3$, $p = 0.026$) and methyl oleate (GLM; $\chi^2 = 13.93$, $df = 3$, $p = 0.002$) among populations. Capirio population had significantly more squalene but less methyl oleate than Perula population ($p = 0.008$ and $p < 0.001$, respectively). We did not find differences in other abundant compounds (Table 1).

Chemosensory recognition

Males and females usually explored the tile repeatedly by tongue flicking or ignored it after the first TFs. In all cases, lizards directed TFs to the tile in all conditions.

Male-male responses

All males directed TFs to the scented tiles in all conditions (Fig. 4A). We found significant differences in overall male responses of *A. lineattissimus* to male scents (Table 2). There were significant differences in male responses to male scents among each lizard population (Table 2). Male-male chemical discrimination was different in all populations (Fig. 4A), responding with significantly higher TFs to male scents of their same population than male scents from different populations (Fig. 4A).

Male-female responses

All males directed TFs to the tile in all conditions (Fig. 4B). There were significant differences in overall male responses of *A. lineattissimus* to female scents (Table 2). Male

responses to female scents were different among populations (Table 2). Males from all populations discriminated among different female scents (Fig. 4B). Males from Capirio and Island populations responded with significantly higher TFs to female scents from their own population than to female scents from different populations (Fig. 4B), whereas males from Maruata and Perula populations responded differentially to female scents of their own population and female scents from Capirio population; other discriminations were not significant (Fig. 4B).

Female-male responses

All females directed TFs to scented tiles in all conditions (Fig. 4C). There were significant differences in overall female responses of *A. lineatissimus* to male scents (Table 2).

Female responses to male scents were different among populations (Table 2). Females of all populations discriminated among different male scents (Fig. 4C). Most females responded with significantly higher TFs to male scents from their own population than male scents from different populations (Fig. 4C), except for females from Capirio population that did not discriminate between male scents from their own population and male scents from Perula population (Fig. 4C).

Female choice of male territories

There were significant differences in female choice of chemically marked territories among populations (Table 3). Females from Capirio population were seen more often on territories scented marked by males of their own population than in territories scented marked by males from Maruata and Island populations, except for territories scented marked by males from Perula population (Fig. 5A). Females from Maruata population discriminated between spending more time on territories scented marked by males from their own population than in territories scented marked by males from different populations (Fig. 5B). Choice of male territories by females from Perula population did not differ between territories scented marked by males from their own population or territories scented marked by males from different populations (Fig. 5C). Finally, females from Island population did not discriminate between territories scented marked by males from their own population and territories scented marked by

males from Maruata and Perula populations, while territories scent marked by males from Capirio population were discriminated (Fig. 5D).

Discussion

Our results showed that male *A. lineattissimus* of each population contains a well-defined and divergent mixture of compounds in their femoral gland secretions. In general, female and male lizards discriminated and were more attracted to lizard scents from their own population than to lizard scents from different populations. In addition, although females also seem to discriminate male scents between populations, their associated preferences for territories scent-marked by males of their own population is different between regions (i.e. eastern vs western) and not among all populations. This clear ability of both sexes of *A. lineattissimus* to discriminate and to choose between some members of the opposite sex from different populations suggest the existence of a partial premating isolation (to future zones of secondary contact) mediated by the role of chemical signals and possible species divergence.

Chemical variation

Chemical analysis showed that, similarly to other tropical lizard species, femoral gland secretions of *A. lineattissimus* have carboxylic acids and esters as predominant components (Weldon et al. 2008; Martín et al. 2011, 2013; Ibáñez et al. 2017). However, compounds found in femoral gland secretions of male *A. lineattissimus* varied in composition and proportion among populations, and these chemical differences allowed the significant allocation of secretions to their population of origin. Our results support the idea that a high degree of divergence in chemical signals and discriminatory behaviors could inform us about the speciation process and suggest that infochemicals and behavioral patterns are a useful trait to integrate into species delimitation in divergent populations or cryptic lineages (Zozaya et al. 2019). Additionally, these interpopulation differences in chemical cues could be due to local adaptations to environmental conditions (Alberts 1992; Martín and López 2013; Martín et al. 2015, Baeckens et al. 2018), different diets or availability of food resources (García-Roa et al. 2017; Henneken et al. 2017), genetic drift (Runemark et al. 2011) or sexual selection (Martín and López 2000, 2015), but further studies are needed to evaluate these conditions.

Intra and inter sexual behavior

Based on their scent alone, males were able to discriminate between rival males from all populations, although the response was stronger towards males from their own population. The importance of chemical signals for rival recognition and rival assessment between males has already been shown in other lizard species (López and Martín 2002, 2011; Carazo et al. 2007, 2008; Martín and López 2007; Heathcote et al. 2016). In some species (e.g. *Podarcis hispanicus*), these chemical signals can be even more relevant during agonistic relationships than visual signals (López et al. 2002). However, in other lizard species (e.g. *Cnemaspis mysoriensis*) visual cues are more relevant in the modulation of male-male interactions (Kabir et al. 2019). Differential chemosensory responses male-male among all *A. lineattissimus* populations, suggested that chemical divergence of femoral gland secretions promotes discrimination among males of different populations. In general, males were less interested in exploring male scents from other populations, suggesting that they may not perceive all males as potential rivals.

Our results suggested that intersexual recognition of male-female interactions in *A. lineattissimus* is at least partially mediated by chemical signals. Both lizard sexes were apparently able to discriminate scents of individuals of the opposite sex from their own and from at least a different population. Other studies have also found that intersexual recognition among populations may be modulated by differences in chemical signals in both sexes (Gabirot et al. 2012; Martín et al. 2016). Additionally, as a by-product of chemical discrimination, males might choose females with certain morphological characters, such as larger body size. This trait might be an indicator of chemical differences of cloacal secretions among females (Heathcote et al. 2016; Martín et al. 2016).

Female choice behavior

Several studies document that lizard female choice of male territories (association preferences) is not affected by interpopulation variations in male chemical cues (Gabirot et al. 2013; Heathcote et al. 2016; Martín et al. 2016). In concordance with these studies, females of two allopatric, western populations (Perula and Island) of *A. lineattissimus* showed no preferences towards territories scent marked by males, regardless of the males' population. However, our results also showed that in allopatric, eastern populations (Capirio and Maruata) there was a higher female preference for visiting territories scent

marked by males of their own population than for territories scent marked by males from different populations.

This behavioural mismatch in female choice of male territories suggests that there are different levels of chemical cues relevance in mate preferences. For example, females *A. lineattissimus* from Perula and Island populations might be able to respond to the interindividual variation of some compounds released by the males of their own population, as has been reported in other lizard species (Martín et al. 2007; López et al. 2009; Gabirot et al. 2013). Our observations that females of Capirio and Maruata populations spent more time in territories scent marked by males from their own population suggest that this behavior might be part of an initial process of premating isolation. However, in other lizard species, females that have chosen territories of males from their population might mate with males from other populations, as a possible consequence of higher dominance levels shown by these males (MacGregor et al. 2017).

Evolutionary ecology consequences

Our study shows evidence from chemical signal analysis and behavioral experiments that chemical discrimination between populations of *A. lineattissimus* occurs and could be relevant for cases of secondary contact between populations by preventing population admixture and further strengthening sexual isolation. These results seem concordant with the previous description of subspecies of *A. lineattissimus* complex using morphological data (Duellman and Wellman 1960) and genetic data using mitochondrial and nuclear DNA (ERG unpubl. data) that showed that these populations of *A. lineattissimus* form a set of defined and divergent lineages. It has been suggested that reproductive isolation and speciation in lizard populations may be mediated by male preferences and intrasexual relationships between males (e.g. rival recognition,) rather than by female mate choice (Gabirot et al. 2012; Heathcote et al. 2016; Martín et al. 2016). Contrarily, MacGregor et al. (2017) suggest that these sexual mechanisms are of little relevance in the evolution of reproductive isolation in lizards. Our results suggest that intrasexual chemosensory recognition and precopulatory behaviors (intersexual chemical recognition and female association preferences) in *A. lineattissimus* are important in the process of ethological isolation among different populations. However, the magnitude of this process tends to be

different depending on the particular pair of geographic populations and the precopulatory behaviors analyzed.

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Figures legends:

Fig. 1 Geographic locations of the four studied populations and distribution range (yellow) of whiptail lizard *Aspidoscelis lineattissimus* in a western region of Mexico

Fig. 2 Habitat types and phenotypic variation observed in female and male lizards *Aspidoscelis lineattissimus* of four studied populations

Fig. 3 Representation of the two first axes of the canonical analysis of principal coordinates (CAP) showing classification of femoral secretions of *Aspidoscelis lineattissimus* populations

Fig. 4 Chemosensory recognition by males and females *Aspidoscelis lineattissimus* lizards in response to scents of different populations (x-axis represents the population of origin; Capirio, Maruata, Perula, and Island). (A) Tongue flicks by males of four populations in response to control deionized water, male scents of their same and different population (mean \pm SE). (B) Tongue flicks by males of four populations in response to control deionized water, female scents of their same and different population (mean \pm SE). (C) Tongue flicks by females of four populations in response to control deionized water, male scents of their same and different population (mean \pm SE). Different letters above the bars indicate statistically significant differences among scents according to GLMMs

Fig. 5 Number of observations (mean \pm SE) of females *Aspidoscelis lineattissimus* from (A) Capirio, (B) Maruata, (C) Perula, and (D) Island populations on territories scent marked by males of their own (Sa) and different population (Ca: Capirio, Ma: Maruata, Pe: Perula, and Is: Island) and one neutral territory (Ne). Different letters above the bars indicate statistically significant differences among territories according to GLMMs

Fig. 1

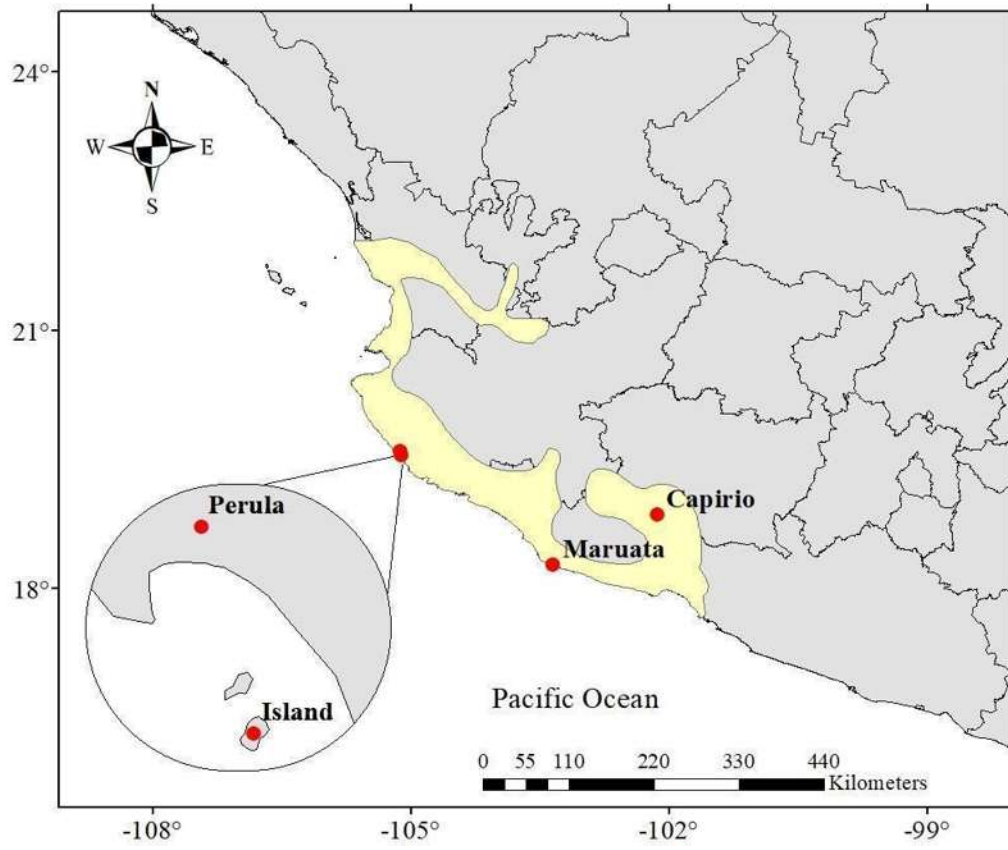


Fig. 2

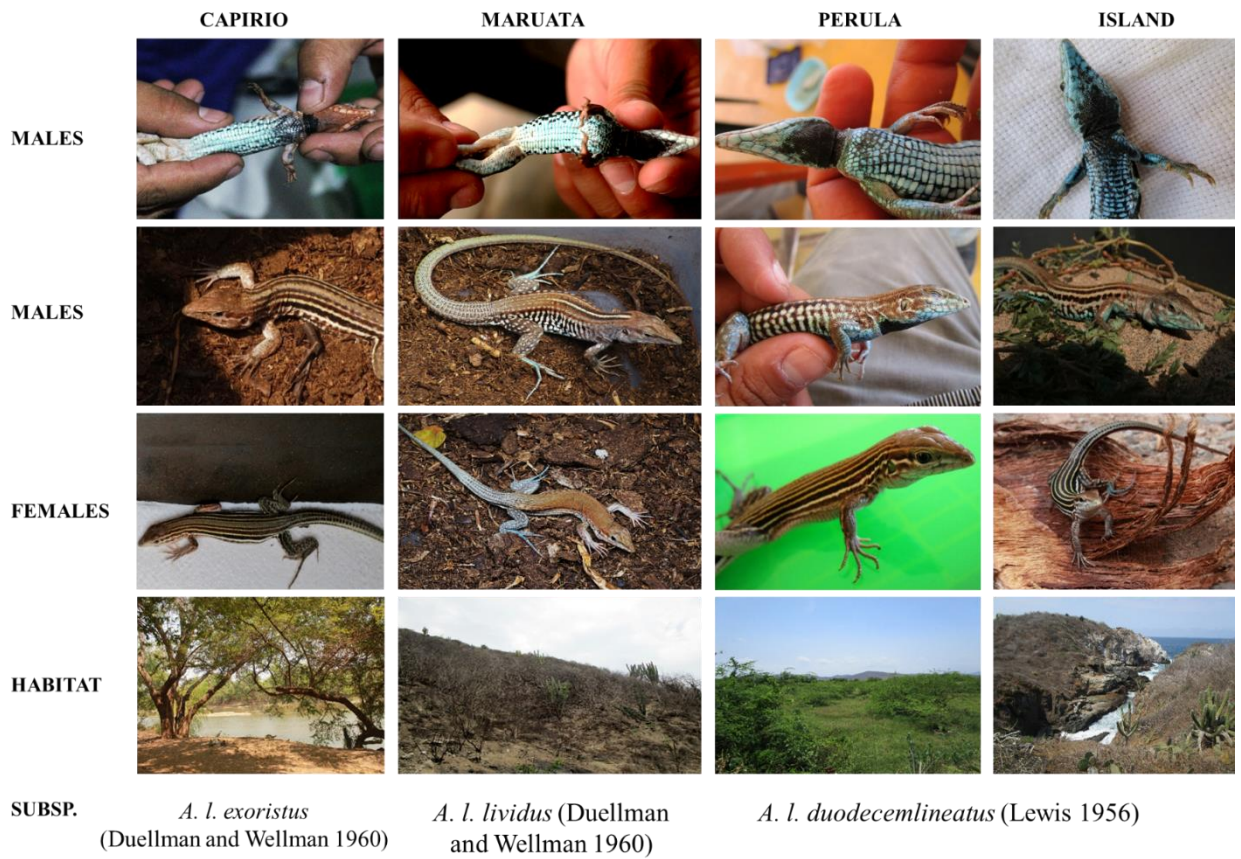


Fig. 3

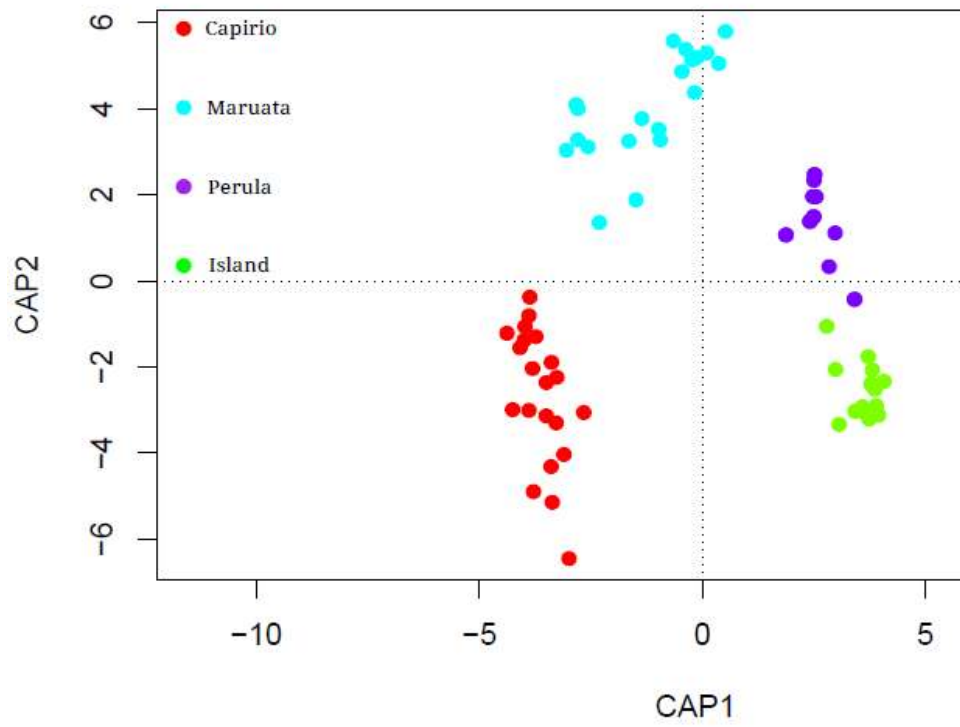


Fig. 4

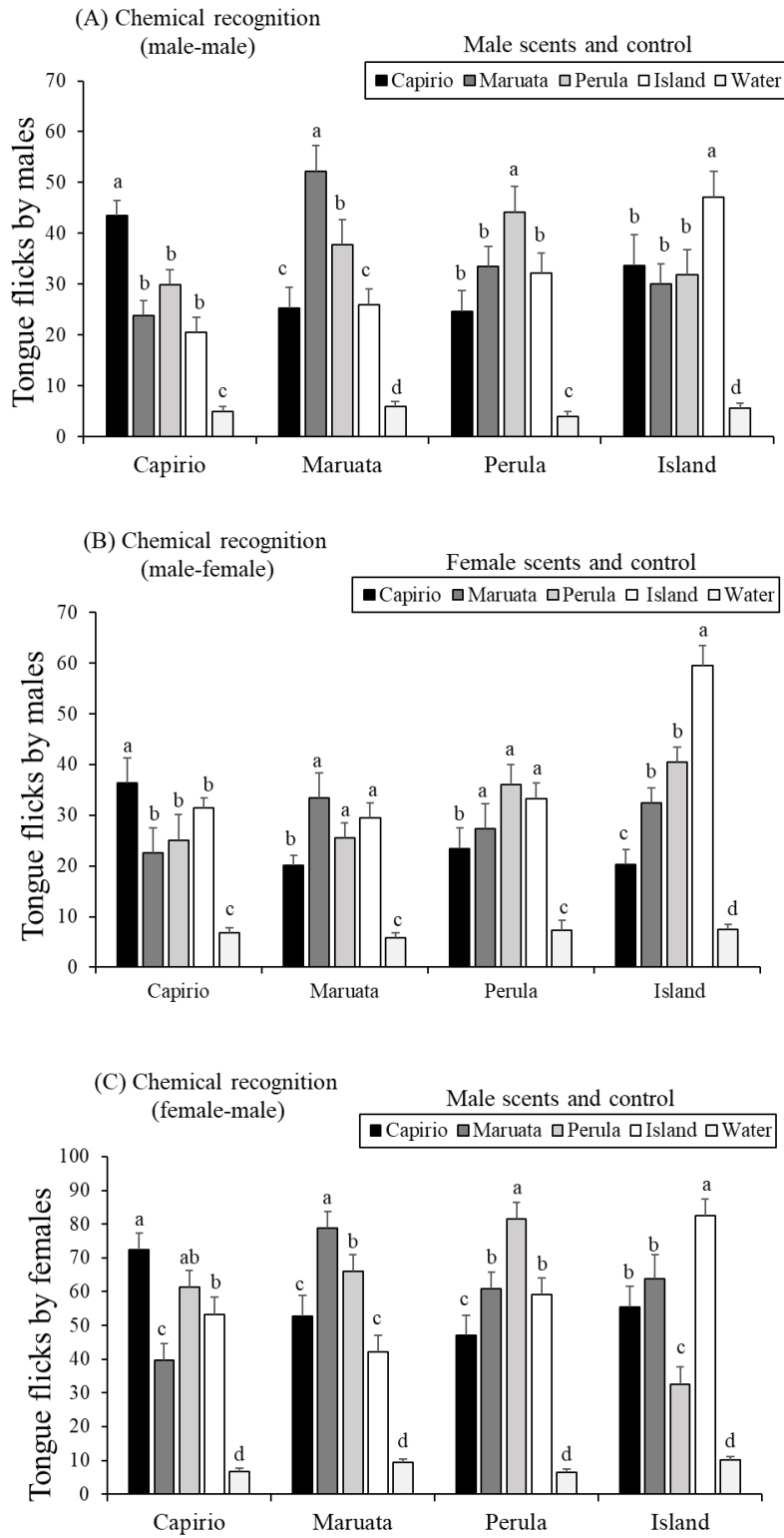


Fig. 5

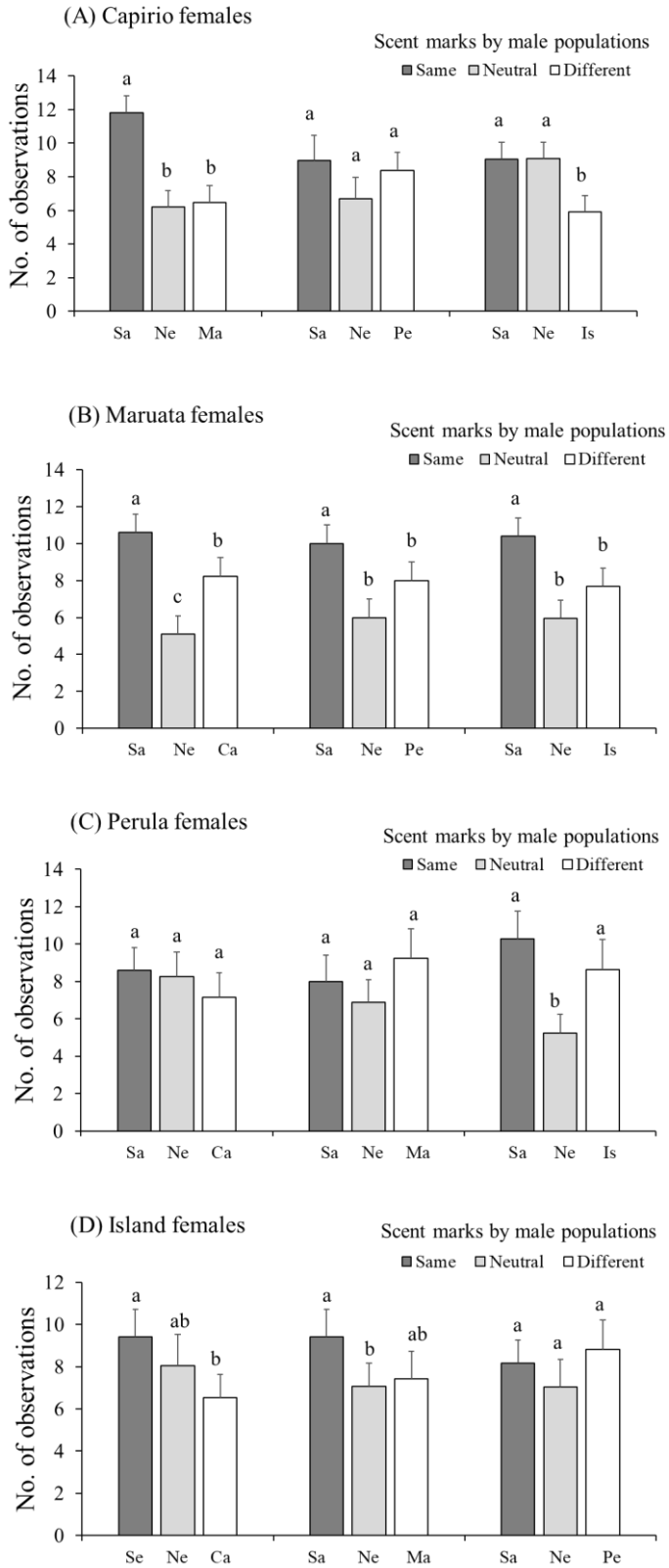


Table 1 Chemical compounds identified from femoral gland secretions of male lizards *Aspidoscelis lineattissimus* from four distinct populations (Capirio, Maruata, Perula, and Island)

RT (min)	Compound name	R. Index	Capirio	Maruata	Perula	Island
<i>Esters</i>						
5.2	Isobutyl acetate	1012	6.89 ± 1.45	8.80 ± 1.89	1.02 ± 1.34	0.44 ± 1.35
6.4	n-Butyl acetate	1074	0.02 ± 0.03	0.14 ± 1.11	0.09 ± 2.91	0.06 ± 1.59
9.1	β-Terpenyl acetate	1622	0.07 ± 8.23	0.89 ± 1.97	4.29 ± 1.93	3.17 ± 2.13
19.3	Methyl laurate	1804	2.89 ± 1.26	1.17 ± 1.73	nd	1.01 ± 0.69
19.8	Isopropyl laurate	1827	1.03 ± 1.10	0.83 ± 0.74	0.37 ± 1.25	nd
22.0	Methyl myristate	2005	19.96 ± 1.50	nd	nd	nd
22.3	Isopropyl myristate	2027	0.09 ± 7.41	0.31 ± 3.28	0.07 ± 8.22	0.07 ± 2.54
23.3	Methyl pentadecanoate	2108	1.28 ± 1.80	0.61 ± 0.86	0.62 ± 1.87	0.59 ± 5.84
24.5	Methyl palmitate	2208	1.20 ± 3.45	0.51 ± 1.03	0.28 ± 1.30	nd
24.7	Methyl palmitoleate	2240	1.10 ± 0.98	1.03 ± 0.65	1.53 ± 4.88	0.20 ± 6.92
24.9	Ethyl palmitate	2251	1.07 ± 1.11	1.04 ± 0.81	1.85 ± 6.53	1.62 ± 1.82
25.6	Methyl margarate	2309	nd	nd	nd	7.54 ± 0.87
26.7	Methyl stearate	2418	3.69 ± 0.47	0.45 ± 0.96	0.21 ± 5.34	0.10 ± 3.25
27.0	Methyl oleate	2434	0.33 ± 3.27	23.48 ± 2.29	8.27 ± 3.67	22.22 ± 2.50
27.5	Ethyl linoleate	2521	0.07 ± 2.34	0.22 ± 2.80	nd	nd
28.8	Methyl arachidate	2639	3.87 ± 9.66	0.05 ± 1.47	nd	nd
30.8	Methyl docosanoate	2750	0.38 ± 0.75	0.59 ± 0.63	0.95 ± 2.46	0.49 ± 1.13
<i>Carboxylic acids</i>						
14.1	Acetic acid	1449	1.50 ± 1.19	1.08 ± 0.62	1.04 ± 1.05	0.62 ± 0.74

31.4	Palmitic acid	2931	21.14 ± 1.69	23.79 ± 2.29	20.88 ± 6.94	23.35 ± 2.54
33.6	Stearic acid	3134	10.06 ± 1.65	9.97 ± 1.00	14.23 ± 1.19	13.49 ± 1.80
<i>Others</i>						
6.78	Undecane	1100	2.06 ± 1.06	1.99 ± 0.72	2.06 ± 2.05	1.10 ± 1.50
11.4	Trimethyl dodecane*	1320	1.01 ± 5.73	1.59 ± 0.59	1.40 ± 0.97	0.69 ± 1.07
25.0	α-Tocopherol*	3149	0.12 ± 4.19	0.19 ± 1.49	0.26 ± 2.68	0.40 ± 3.86
33.1	t-Squalene*	2865	20.16 ± 1.62	21.00 ± 2.31	7.10 ± 2.69	18.00 ± 2.50
41.3	Dihydro-lanosterol*	3823	nd	0.27 ± 2.96	31.02 ± 2.93	7.32 ± 4.95

Relative proportions of 25 compounds (mean ± SE of the percentage of the total peak areas from FID detector). nd, not detected compound. RT, retention time and retention index are shown. Compounds without standard confirmation (*)

Table 2 Results of the GLMM's for the chemosensory recognition behaviors in the studied populations of *Aspidoscelis lineattissimus*. The statistics for fixed effects from the GLMM are shown: $LR\chi^2$ (likelihood ratio Chi square), df (degrees of freedom) and p-value. The GLMM's considered the nesting design as a random effect (lizard id within scent)

	Intrasexual chemical recognition			Intersexual chemical recognition					
	Male-Male			Male-Female			Female-Male		
Fixed factor	$LR\chi^2$	df	p	$LR\chi^2$	df	P	$LR\chi^2$	df	p
Population (P)	3.6	3	0.297	12.3	3	0.006	1.5	3	0.666
Scent (S)	249.3	4	<0.001	411.3	4	<0.001	810.3	4	<0.001
P:S	57.9	12	<0.001	61.4	12	<0.001	131.8	12	<0.001

Table 3 Results of the GLMM's for the female choice of male territories in the studied populations of *Aspidoscelis lineattissimus*. The statistics for fixed effects from the GLMM are shown: LR χ^2 (likelihood ratio Chi square), df (degrees of freedom) and p-value. The GLMM's considered the nesting design as a random effect (female id within scent mark)

Fixed factor	Female choice of male territories		
	LR χ^2	df	p
Population (P)	0.03	3	0.998
Scent mark (SM)	176	2	<0.001
P:SM	48.3	6	<0.001

CAPÍTULO III

Phylogenetic relationships and species boundaries of the *Aspidoscelis lineattissimus* complex (Squamata: Teiidae) from western Mexico

Manuscrito en preparación

Phylogenetic relationships and species boundaries of the *Aspidoscelis lineattissimus* complex (Squamata: Teiidae) from western Mexico

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Abstract

The systematics of the genus *Aspidoscelis* is one of the most intricate among reptiles due to the high morphological variation and complex evolutionary history. The whiptail lizard *Aspidoscelis lineattissimus* is a polymorphic species that inhabit tropical ecosystems in a wide region at western Mexico, and currently is classified as four subspecies *A. l. exoristus*, *A. l. lineattissimus*, *A. l. lividus* and *A. l. duodecemlineatus*. In this study, we used phylogenetic and coalescent-based approaches to disentangle the phylogenetic relationships among the *A. lineattissimus* subspecies and to assess the differentiation level of these sub-specific taxa through a species delimitation approach. Four molecular markers were used, three mitochondrial genes (ND4, 12S, and 16S) and one nuclear exon (BACH1), along with a comprehensive sampling that included individuals from the four subspecies. Phylogenetic analyses support the monophyly of this whiptail lizard and show five well-supported clades. Three well-differentiated lineages were supported as independent evolutionary units corresponding to *A. l. exoristus*, *A. l. lineattissimus*+*A. l. lividus* and *A. duodecemlineatus*, recovering partially the recognized subspecies based on classical taxonomy. In addition to the morphological previous classification, the phylogenetic and coalescent criteria provide new insight into the current classification and evolutionary history of this species complex, suggesting to elevate these three independent evolutionary units at species level.

Keywords *Aspidoscelis*. Genetic structure. Phylogeny. Species complex. Whiptail lizards.

Introduction

North American whiptail lizards of *Aspidoscelis* genus form a well-defined monophyletic group of gonochoric and unisexual species (Reeder et al. 2002) integrated by around 44 species and 51 subspecies recognized (Uetz et al. 2019). The *Aspidoscelis* species have been classified into five groups: *cozumela*, *deppii*, *sexlineata*, *tessellata*, and *tigris* (Burt 1931; Lowe et al. 1970) according to their shared morphological characteristics and phylogenetic relationships (Reeder et al. 2002; Manríquez-Morán et al. 2014). Unfortunately, because of the complex systematics and taxonomy of the group (Duellman and Wellman 1960) still uncertain the number of valid species and their phylogenetic relationship (Zweifel 1961; Reeder et al. 2002; Taylor et al. 2005; Manriquez-Moran 2007).

Great morphological complexity is present in most of the whiptail species such as the intraspecific variation in polymorphism change in the pattern color in the different life stages, or the sexual dimorphism and the scalation variation among populations (Walker 1970; Elizalde-Rocha et al. 2008; Cruz-Elizalde et al. 2014). These variations could mask processes like the hybridization, introgression, and undescribed lineages, how have been evidenced in the *Aspidoscelis sexlineatus* species group (sexlineata group) using a molecular approach (Barley et al. 2019).

The many lined whiptail lizard *Aspidoscelis lineattissimus* is a bisexual species endemic to the western region of Mexico and distributed in tropical areas of the states of Michoacán, Colima, Jalisco, and Nayarit (García and Ceballos 1994). The early taxonomic and morphological study by Duellman and Wellman (1960) classified *A. lineattissimus* within the deppii group and recognized four subspecies: *A. l. exoristus*, *A. l. lividus*, *A. l. lineattissimus* and *A. l. duodecemlineatus*. The subspecies are grouped based on their phenotypic variations of coloration pattern, size, disposition of the supraocular granules, including meristic characteristics such as the number of femoral pores, mid-dorsal scales, and preanal scales (see, Duellman and Wellman 1960). In addition to polymorphism register in this whiptail lizard, the complex topography and wide geographical distribution have favored the isolation of some populations both mainland and island habitats (Hernández-Salinas et al. 2014). However, these elements have been insufficient to delimit the boundaries among the subspecies of *A. lineattissimus* (Duellman 1965).

The high taxonomic complexity and the need to delimit the entities in *A. lineattissimus* lizards make it necessary to evaluate the phylogenetic relationship of its subspecies since a molecular approach. We consider that incorporating mitochondrial and nuclear markers can be an appropriate criterion for establishing a systematic classification of this taxon. In the present study we carried out a phylogenetic hypothesis and species delimitation test, in order to obtain the evolutionary relationships within this whiptail lizard group and to assess its taxonomic status.

Materials and methods

Study area and sampling

The sampling took place in the western region of Mexico in the states of Michoacan, Colima, Jalisco, and Nayarit (Fig. 1). Whiptail lizards were captured by noosing and pitfall traps between 2017 and 2018. Fifty-seven specimens from *A. lineattissimus* complex were collected from eight localities to cover the distribution of the four subspecies (Table 1). Furthermore, individuals of *A. calidipes*, *A. communis*, and *A. gularis* whiptail lizards were collected in the same region to be included as outgroup in the phylogenetic analyses. Taxonomic identification of the specimens was based on a revision of the specialized literature (Duellman and Wellman 1960; Duellman and Zweifel 1962). Tissue samples were taken of lizard's tail fragment less than 3 cm long that was then stored in 96% ethanol at -20 °C. Most lizards were released at the collection site and no more than 10 lizards per locality were sacrificed by an injection of sodium pentobarbital. All tissue samples and voucher specimens were properly curated and deposited at the herpetological collection of Instituto de Investigaciones sobre los Recursos Naturales, Universidad Michoacana de San Nicolas de Hidalgo (CHUM-DF-CC-292-15) (Table S1).

DNA extraction and sequence data

Genomic DNA for all specimens was extracted using the phenol-chloroform method (Sambrook et al., 1989). Sequences from three mitochondrial and one nuclear region were obtained from most of the individuals including in the analyses. The genes sequenced were the mitochondrial NADH dehydrogenase subunit 4 and associated tRNAs (ND4), 12S rRNA (12S), and 16S rRNA (16S), plus the nuclear gene of the transcription factor BTB and CNC homology 1 (BACH1). Primers ND4 (Manríquez-Morán et al. 2014) and LEU (Arevalo et al. 1994) were used to amplify the ND4 gene; the universal primers L1091 and H1478 (Kocher et al. 1998) were used to amplify the 12S; primers 16Sc and 16Sd (Reeder 1995) were used to amplify the 16S; the specified primers for Squamata BACH_F1 and BACH1_R2 (Townsend et al. 2008) were used to amplify the BACH1. To obtain the BACH1 sequences from La Huacana population of *A. lineattissimus*, and all the individuals of *A. gularis*, *A. communis*, and *A. calidipes* we designed the internal primers BACH2F (5'-AGA AGT GTT GCA AGT CAC GTT GT-3') and BACH2R (5'-CGC TGA TTT GTC TCA GCT TCC-3'), they were used in the amplification and sequencing process.

PCR reactions consisted of a volume of 12.5 µl with a final concentration of 0.2 µM of each primer, 0.25 mM of each dNTP, 1.5 mM of MgCl₂, and 1 unit of Taq DNA Polymerase

(Invitrogen). Amplification conditions for ND4 region consisted of an initial denaturation at 94° C for 3 min followed by 32 cycles of 94° C for 30 s, 50° C for 45 s, 72° C for 60 s, with a final extension of 72° C for 5 min. The 12S conditions consisted of an initial denaturation at 94° C for 3 min followed by 32 cycles of 94° C for 30 s, 55.7° C for 45 s, 72° C for 60 s, with a final extension of 72° C for 5 min. The 16S amplification consisted of an initial denaturation at 94° C for 3 min followed by 32 cycles of 94° C for 30 s, 57° C for 45 s, 72° C for 60 s, with a final extension of 72° C for 5 min. Finally, for the BACH_1F Y BACH_1R primers the PCR profile consisted of an initial denaturation at 94° C for 5 min followed by 35 cycles of 94° C for 30 s, 60.2° C for 40 s, 72° C for 90 s, with a final extension of 72° C for 10 min. For the BACH2F and BACH2R, the PCR profile consisted of an initial denaturation at 94° C for 5 min followed by 35 cycles of 94° C for 40 s, 52° C for 40 s, 72° C for 60 s, with a final extension of 72° C for 10 min. PCR products were visualized by electrophoresis on agarose gel 1.5%, then purified with ExoSAP-IT (USB Corp.) according to the manufacture instruction, and sequenced by Macrogen Inc (Korea).

Sequence alignment

The chromatograms of the mitochondrial and nuclear genes sequences were visualized and manually aligned in MEGA v7 (Kumar et al. 2016). All genes were translated into amino acids to verify for stop codons and confirm the absence of pseudogenes in exons. For the nuclear gene BACH1, the heterozygotes were manually coded according to the international IUPAC code. Sequences obtained were deposited in GenBank under accession numbers XXX-XXX for ND4, XXX-XXX for 12S, XXX-XXX for 16S, and XXX-XXX for BACH1 (Table S1).

Phylogenetic analyses

The evolutionary model selection was implemented by the Akaike information criterion and optimal partition setting analyses using PartitionFinder v1.1.0 (Lanfear et al. 2012) computed in the CIPRES Science Gateway v3.3 portal (Miller et al. 2010). We defined the blocks from each gene, only the ND4 gene was separated from its associated tRNAs, testing five blocks in the partition scheme: ND4, tRNAs associated with ND4, 12S, 16S, and BACH1. The best-

fitted scheme showed that the best substitution model was the HKY+G model for ND4; GTR+G for tRNAs associated with ND4, 12S and 16S; and HKY+I+G for BACH1.

To evaluate the potential effect of genes on tree topology and the support of branches, we performed independent analyses for each gene and one concatenated data that includes all genes using two phylogenetic reconstruction methods. We implemented Bayesian Inference (BI) analyses in MrBayes v3.2.5 (Ronquist et al. 2012) using the best substitution model partition obtained by PartitionFinder for each corresponding gene. The BI analyses consisted of two runs each with four Markov chains, and 5 million generations sampling every 500 generations. After verifying the convergence and stationarity within chains in Tracer v1.7 (Rambaut et al. 2018), we discarded 10% of sampled trees as burn-in to generate a majority-rule consensus tree. We also run Maximum Likelihood (ML) analyses in RaxmlGUI v1.3 (Silvestro and Michalak 2012) using the GTR+I+G substitution for all the genes and datasets. The node support in the ML analyses was assigned by bootstrap through 1000 pseudoreplicates. We used as the outgroup the species *A. gularis*, *A. communis*, and *A. calidipes* in all the phylogenetic inferences with both phylogenetic methods and the different gene datasets. All phylogenetic analyses were computed in CIPRES.

Genetic differentiation and structure

We calculated the evolutionary distance independently for each gene through the mean uncorrected p distance, and assessed its standard deviation through 10 000 bootstrap replications, between recognized subspecies and principal clades identified in the phylogenetic analysis in MEGA v7. To assess the genetic differentiation among subspecies and principal clades we computed the Φ_{ST} pairwise values for each gene independently, and testing their significance implementing 10 000 permutations in DNAsp v5.10 (Librado and Rozas 2009). Then, the significance of Φ_{ST} values was adjusted by Bonferroni correction (Weir and Cockerham 1984). Only for the nuclear gene BACH1, the sequences were phased in DNAsp V5.10 to conduct the pairwise Φ_{ST} values.

Species delimitation

Bayesian multi-locus species delimitation analysis was conducted in the program BPP v.3.4 (Yang 2015). This program uses a Bayesian framework and multispecies coalescent model to compare the posterior probabilities of different species delimitation models (Yang and Rannala 2010; Rannala and Yang 2013) and to accommodate the gene tree-species conflict due to incomplete lineage sorting (Yang and Rannala 2010, 2014). The following set parameters were specified: fixed guide tree; a Dirichlet distribution ($\alpha=2$) to account for variation in mutation rates among loci; in order to discern how the effective ancestral population size and the divergence influenced the results, an inverse-gamma prior (IG) was used to specify the population size parameter θ and root age τ_0 of the species tree considering three different scenarios of prior sets: 1) large ancestral population sizes and deep divergences (IG $\theta=3, 0.2$, IG $\tau_0 = 3, 0.4$); 2) small ancestral population sizes and shallow divergences (IG $\theta=3, 0.002$, IG $\tau_0 = 3, 0.004$); and 3) large ancestral population sizes and shallow divergences (IG $\theta=3, 0.2$, IG $\tau_0 = 3, 0.004$). Reversible jump (rj) MCMC was run for 100,000 generations, with a burn-in of 8000 and a sampling frequency of two. We performed this twice to confirm convergence between the runs. The A10 (species delimitation using a fixed guide tree) and A11 (joint species delimitation and species-tree estimation) analyses were implemented using the same above prior sets. The BPP analysis was conducted separately for three species delimitation models: (1) proposal of 3 independent taxonomic units, (2) 4 independent taxonomic units, and (3) 5 independent taxonomic units.

Divergence times analysis

A divergence time analysis was carry out for 48 individuals, including individuals of all four sub-species, *A. l. exoristus*, *A. l. lividus*, *A. l. lineattissimus* and *A. l. duodecemlineatus* and individuals of *A. calidipes*, *A. communis*, and *A. gularis* as outgroup, using BEAST v.1.8.4 (Drummond et al. 2012). We use the best fit model of nucleotide substitution estimated for each locus (ND4, 12S and 16S: GTR+G; BACH1: HKY+I+G), implementing a relaxed clock with uncorrelated lognormal model, and a yule speciation process. To calibrate we use the substitution rate for ND4 in the genus *Podarcis* of 1.15% (0.12% SD) per million years (Salvi et al. 2014). We conduct two independent analyses of 100,000,000 generations sampling each 1,000 generations. Chains convergence was assessed by

visualizing the sampled parameter values in Tracer 1.5 (<http://BEAST.bio.ed.ac.uk/Tracer>). Ten percent of the generations were discarded as burn-in and pooled using the module Log-Combiner in the BEAST package. The maximum clade credibility species tree was obtained by the module Tree-Annotator in the BEAST package. The analysis was performed in BEAST 2 (Heled et al. 2013) implemented on the web server CIPRES Science Gateway v. 3.3. (Miller et al. 2010).

Results

We sequenced four genes for 53 individuals with a total of 207 sequences, corresponding to individuals of the four subspecies of *A. lineatissimus* complex and 5 individuals for the species *A. calidipes*, *A. communis*, and *A. gularis* used as the outgroups. For the *A. lineatissimus* complex we sequenced 48 individuals getting 187 sequences for the four genes that span 2933 pairs of bases (pb). In the mitochondrial genes, we sequenced 47 samples of ND4 (844 pb) with 719 conserved sites, 122 variable sites, 109 parsimony informative sites and 13, singleton sites; for 12S were sequenced 48 individual recovering 407 pb with 360 conserved sites, 45 variable sites, 42 parsimony informative sites and 3 segregate sites, and for 16S 45 individuals were sequenced recovering 770 pb with 703 conserved sites, 65 variable sites, 59 parsimony informative sites and 6 singleton sites. Finally, for the nuclear gene BACH1 was recovered 912 pb with 904 conserved sites, 8 variable sites, 6 parsimony informative sites and 2 segregate sites.

Phylogenetic analyses

The phylogenetic inference based on the concatenated dataset (ND4, 12S, 16S, and BACH1) showed five well-supported clades for the *A. lineatissimus* complex (Fig. 2). The individuals recognized as *A. l. exoristus* from Capirio, La Huacana, and Churumuco localities within the Balsas-Tepalcatepec Valley formed the clade I (BI=100, ML=100). The clade II (BI=100, ML=100) included the samples from Maruata locality belonging to the subspecies *A. l. lividus* and from Boca de Apiza locality recognized as *A. l. lineatissimus*. The populations from the subspecies *A. l. duodecemlineatus* were structured in three clades (Clade III to Clade V). The clade III (BI=100, ML=91) is represented by individuals from Perula locality, the clade IV (BI=89, ML=81) is represented by

individuals from Cocinas Island, both localities are located in Chamela bay in the Jalisco coast (Fig.1). Finally, the clade V (BI=89, ML=81) is represented by individuals from San Blas locality. The clade I was the deepest lineage within this complex, whereas the clades II to V were phylogenetic closely related.

The phylogenetic inference individually reconstructed for mitochondrial genes (ND4, 12S, and 16S) recovered the five clades with a similar topology such as the concatenated inference; but, for the relationship among the clades II, III, IV, and V that varied, and in some cases unresolved or poorly supported for BI and ML (Fig. S1-S3). For nuclear gene BACH1, the relationships among the subspecies still unresolved in a basal polytomy (Fig. S4).

Genetic differentiation and structure

The mean uncorrected distances p between the *A. lineattissimus* complex and the outgroups for the mitochondrial genes ranged from 12.16% to 15.6% for ND4, from 10.74% to 11.79% for 12S, from 5.23% to 6.38% for 16S, and for the nuclear gene, BACH1 ranged from 0.18% to 0.53%. Between the clades recognized within the *A. lineattissimus* complex higher mean p distances were observed among the clade I (*A. l. exoristus*) with the clade II (*A. l. lividus* + *A. l. lineattissimus*) and the clades III, IV and V (*A. l. duodecemlineatus*) for all genes; ND4, 9.03% to 10.53%; 12S, 8.31% to 9.18%; 16S, 4.62% to 5.27%; BACH1, 0.18% to 0.19%. While the lowest genetic distance was observed between the clades II, III, IV and V; ND4, 2.65% to 3.08%; 12S, 0.46% to 1.40%; 16S, 0.52% to 1.36%; BACH1, 0% to 0.02% (Table 2).

The higher pairwise Φ_{ST} values were observed in 16S comparisons, for both subspecies and lineages arrangements, while lower values were observed in BACH1 comparisons (Table S2). Pairwise Φ_{ST} values for comparisons among *A. l. exoristus* with *A. l. lividus* + *A. l. lineattissimus* and *A. l. duodecemlineatus*, and *A. l. duodecemlineatus* with *A. l. lividus* were significant for mitochondrial genes. For the nuclear gene BACH1, pairwise Φ_{ST} values for all comparisons were significant among subspecies. Among lineages, pairwise Φ_{ST} values were significant for most of the comparisons in each gene, except between the clade III and clade IV for the genes ND4, 12S and BACH1.

Species delimitation

BPP A10 and A11 analyses did not support the hypotheses of five and four species (Table 3). Instead, these analyses using the three different scenarios of prior sets supported the hypothesis of three independent taxonomic units matching with the recognized subspecies *A. l. exoristus*, *A. l. lividus*+ *A. l. lineattissimus* and *A. l. duodecemlineatus* (speciation probabilities >0.95, table 3, figure 2). Overall, the A10 and A11 of BBP analyses produced similar posterior probabilities (Table 3), indicating that the estimated species did not differ from the inferred Bayesian phylogeny. Posterior probabilities for the A10 and A11 among clades III, IV, and V of *A. l. duodecemlineatus* were markedly lower (Table 3).

Divergence times

The first split within the *lineattissimus* complex was ca. 3.03 Myr (95% HPD 1.43-5.24 Myr) that separated to *A. l. exoristus* from remaining members of the complex (Fig. 3). Second split occurred ca. 1.3 Myr (95% HPD 0.67-2.16 Myr) between the clade *A. l. lividus*+ *A. l. lineattissimus* and *A. l. duodecemlineatus* (Fig. 3). Even within latter clade, there were two other separations at ca. 0.7 Myr (95% HPD 0.34-1.20 Myr) and 0.8 Myr (95% HPD 0.43-1.44 Myr) (Fig. 3).

Discussion

Our study, together with the most recent phylogenetic research of the *sexlineata* group (Barley et al. 2019), suggest that it is necessary to do a comprehensive taxonomic and systematic revision of the *Aspidoscelis* genus because the number of species could be underestimated by their high morphological variation and complex evolutionary history. Our findings, based on phylogenetic analyses using mitochondrial and nuclear data, clearly show that the subspecies recognized within the *Aspidoscelis lineattissimus* complex form a monophyletic group structured in five well-supported clades, four of them phylogenetic closely related (Clade II, III, IV, and V) and the other one deeply divergent and differentiated from the rest (Clade I).

Taxonomic implications in *lineattissimus* complex

The species delimitation analyses supported three independent taxonomic units considered as valid species, this result is partially coincident with the early morphological

classification established by Duellman and Wellman (1960); a geographically restricted, *A. l. exoristus* from the Balsas-Tepalcatepec Valley (Clade I), a coastal lizard *A. l. lividus*+ *A. l. lineatissimus* from Michoacan and Colima Pacific Coastal Plain (Clade II), and the wide distributed *A.l. duodecemlineatus* from the Jalisco and Nayarit Coastal Plain (Clade III, IV, and V). In accordance with above, besides of the species richness, the level of endemism of the genus *Aspidoscelis* in Mexico increases. Therefore, the taxonomic classification of the *lineatissimus* complex and its distribution pattern would be as follows:

Aspidoscelis exoristus. Microendemic species from Balsas-Tepalcatepec Valley and more divergent species that is characterized by have a lower number of dorsal granules than other species in the complex *lineatissimus* (Duellman and Wellman 1960). *A. exoristus* differs from *A. duodecemlineatus* in have a greater number of femoral pores, and differs from *A. lividus*+ *lineatissimus* in have a lower average number of preanal scales (Duellman and Wellman 1960).

Aspidoscelis lividus+*lineatissimus*. Species restricted to the coasts of Michoacán and Colima and can be distinguished from *A. exoristus* by having greater numbers of dorsal granules (Duellman and Wellman 1960). *A. lividus*+ *lineatissimus* differ from *A. duodecemlineatus* in have series of supraorbital granules incomplete (Duellman and Wellman 1960).

Aspidoscelis duodecemlineatus. The most coastal and northern distributed species in Jalisco and Nayarit is characterized by have a lower number of femoral pores than other species of the complex *lineatissimus* (Duellman and Wellman 1960). Differs from *A. exoristus* in having a greater number of dorsal granules and differ from *A. lividus*+ *lineatissimus* in have series of supraorbital granules predominantly completes (Duellman and Wellman 1960).

Evolutionary history of the *lineatissimus* complex

The topology and divergence times we estimated suggest different and early cladogenetic events in the *A. lineatissimus* complex, started during the late Pliocene with the split of *A. exoristus* from other members of the complex and ending on the middle Pleistocene with the common ancestor of the insular and continental clades of the complex along West Pacific Coast of Mexico.

Although the origin and evolution of the Mexican herpetofauna has been influenced by different historical dispersal and vicariance processes (Flores-Villela and Martínez-Salazar, 2009; Ramírez-Reyes et al. 2017). Our analysis showed that the diversification of the *A. lineattissimus* complex occurred during the transition Plio-Pleistocene, supports two biogeographic explanations: (a) that the climate changes during the Plio-Pleistocene likely effect on the speciation processes of these lizard complex, and (b) that geological or vicariance processes prior the Pliocene no favored immediately the speciation of *A. lineattissimus* in Mexico.

Evolutionary history needs to be inspected in the actual endemic species, geomorphologic units as the *A. exoristus* in Balsas-Tepalcatec basin and *A. lividus-lineattissimus* in coastal plains from Colima and Michoacán, and *A. duodecemlineatus* in coastal plains from Jalisco and Nayarit.

In the first case, the Balsas-Tepalcatepec region has experienced a complex geological history and exhibits high levels of endemism in both plants and animals (Flores-Villela et al., 2010). Probably many of these endemisms are due to the separation of Balsas-Tepalcatepec basin and coastal plain from Michoacán during orogeny of “Sierra Madre del Sur” (ca. 35-20 Myr) (Morán-Zenteno et al. 1999, 2000). However, despite the orogeny of this geographic barrier, the cladogenesis in *A. lineattissimus* was a more recent event probably influenced by the climatic changes that arose during the Plio-Pleistocene transition. Probably the beginning of the cladogenesis was favored over a period of 300,000 years from 3.3 to 3 Myr. known as "Optimal Climate of the Middle Pliocene". During this time, a warmer climate was predominating and sea levels then rose about thirty meters above the current coast line (Uriarte-Cantolla 2003).

The second cladogenetic event occurred 1.3 mya ago splitting *A. lividus+lineattissimus* and *A. duodecemlineatus* coinciding with the early Pleistocene where a series of extreme climatic events with rapid alternation occurred (glacial and interglacial episodes, ~40,000 years) that probably influenced the final divergence of these two coastal species.

Islands are natural laboratories of evolution where populations of terrestrial vertebrates encounter a physical impediment to gene flow between populations (Whittaker and Fernández-Palacios 2007; Losos and Ricklefs 2009), and high degrees of population divergence are expected (Mayr 1963; Whittaker et al. 2008). Our findings show that *A.*

duodecemlineatus form Cocinas island and Perula (mainland) is grouped in different clades (Clade III and IV), but with low genetic distance and species differentiation. Although a low level of genetic differentiation of insular populations respect to the mainland populations is unexpected, this pattern has been found in other species of lizards distributed in small islands and the mainland (Senczuk et al. 2014, Silicio-Cantero et al. 2016, Martín et al. 2017; Pérez-Cembranos et al. 2020). This pattern may be explained by the ocean stretch separation of no more than three kilometers among Cocinas Island and the continent (CONANP 2008). Besides, Cocinas island and other surrounding islands were formed 18,000-7000 years ago (Ramírez-Herrera et al. 2004), being this frame the most ancient limit for the age of this isolated population of *A. duodecemlineatus*. However, cladogenetic processes between mainland and island populations of *A. duodecemlineatus* occurred about 700-800 K years ago, therefore, this suggests that the recent oceanic isolation seems to be not yet involved in their genetic divergence.

Declarations

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Conflicts of interest/Competing interests. The authors declare that they have no conflict of interest.

Authors' contributions. All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by [Ernesto Raya-García], [Ana Berenice García-Andrade], [Rodolfo Pérez-Rodríguez], and [Ileri Suazo-Ortuño]. The first draft of this manuscript was written by [Ernesto Raya-García] and [Ana Berenice García-Andrade]. All authors commented on previous versions of the manuscript, read and approved the final version.

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Figure and table legends:

Table 1. Sampling localities for *Aspidoscelis lineattissimus* complex and sequence information for the four molecular markers used in this study.

Taxon	Biogeographic region	Locality	n	Sequences per-gene			
				ND4	12S	16S	BACH1
<i>A. l. exoristus</i>	Balsas-Tepalcatepec Valley	1. Capirio, La Huacana, Mich.	10	10	10	10	10
		2. Churumuco, Churumuco, Mich.	1	1	1	1	1
		3. La Huacana, La Huacana, Mich.	7	6	7	6	7
<i>A. l. lividus</i>	Michoacan Coastal Plain	4. Maruata, Aquila, Mich.	10	10	10	8	10
<i>A. l. lineattissimus</i>	Colima Coastal Plain	5. Boca de Apiza, Tecoman, Col.	1	1	1	1	1
<i>A. l. duodecemlineatus</i>	Jalisco Coastal Plain	6. Cocinas Island, La Huerta, Jal.	5	5	5	5	5
		7. Pérula, La Huerta, Jal.	5	5	5	5	5
	Nayarit Coastal Plain	8. San Blas, San Blas, Nay.	9	9	9	9	8
Total			48	47	48	45	47

Table 2. Pairwise mean uncorrected *p* distances among lineages recognized within the *Aspidoscelis lineattissimus* complex. Genetic distances are presented in percentages.

	ND4	SE	12S	SE	16S	SE	BACH1	SE
Among lineages								
Clade I vs Clade II	9.40	0.99	8.31	1.33	4.62	0.79	0.19	0.16
Clade I vs Clade III	9.03	0.94	8.69	1.36	5.27	0.87	0.18	0.16
Clade I vs Clade IV	9.55	0.96	8.64	1.37	4.91	0.83	0.18	0.16
Clade I vs Clade V	10.53	1.07	9.18	1.41	4.75	0.81	0.18	0.16
Clade III vs Clade II	2.65	0.51	0.91	0.43	1.36	0.45	0.02	0.02
Clade III vs Clade IV	2.85	0.53	0.46	0.32	0.52	0.28	0.00	0.00
Clade III vs Clade V	2.85	0.57	0.59	0.43	0.85	0.34	0.00	0.00
Clade IV vs Clade II	3.08	0.57	0.86	0.42	1.16	0.42	0.02	0.02
Clade IV vs Clade V	2.78	0.56	1.05	0.49	0.65	0.30	0.00	0.00
Clade V vs Clade II	2.79	0.53	1.40	0.56	1.16	0.41	0.02	0.02

SE = standard error, ND4= NADH dehydrogenase subunit 4 and associated tRNAs, 12S

=12S rRNA, 16S = 16S rRNA, BACH1 = transcription factor BTB and CNC homology 1.

Table 3. Posterior probabilities from both BPP analyses (A10 | A11) for the candidate clades for supported and not supported species under three different runs of BPP analysis and three species hypotheses. A10 = species delimitation on a guide tree; A11 = joint species delimitation and species tree estimation. Shaded colors indicate clades tested for speciation probabilities.

Hypothesis	Candidates for valid species			Unsupported candidates			
	Clade I (<i>exoristus</i>)	Clade II (<i>lividus-lineattissimus</i>)	Clade III, IV, V (<i>duodecemlineatus</i>)	Clade III (<i>d. coastal</i>)	Clade IV (<i>d. island</i>)	Clade V (<i>d. northern</i>)	Clade IV, V (<i>duodecemlineatus</i>)
three species	1.0 1.0	1.0 1.0	1.0 1.0	–	–	–	–
three species	1.0 1.0	1.0 1.0	1.0 1.0	–	–	–	–
three species	1.0 1.0	1.0 1.0	1.0 1.0	–	–	–	–
four species	1.0 1.0	1.0 0.98	–	0.03 0.14	–	–	0.03 0.16
four species	1.0 1.0	1.0 1.0	–	1.0 1.0	–	–	1.0 1.0
four species	1.0 1.0	1.0 0.97	–	0.05 0.39	–	–	0.05 0.42
five species	1.0 1.0	1.0 0.99	–	0.02 0.01	0.00 0.01	0.00 1.0	–
five species	1.0 1.0	1.0 1.0	–	1.0 1.0	1.0 1.0	1.0 1.0	–
five species	1.0 1.0	1.0 0.99	–	0.04 0.04	0.00 0.04	0.00 0.99	–

Fig. 1. Sampling localities and distribution for the *Aspidoscelis lineattissimus* complex from western Mexico. The geographical distribution of *A. lineattissimus* complex was obtained from IUCN, the International Union for Conservation. Figures represent the localities and subspecies described in the table 1.

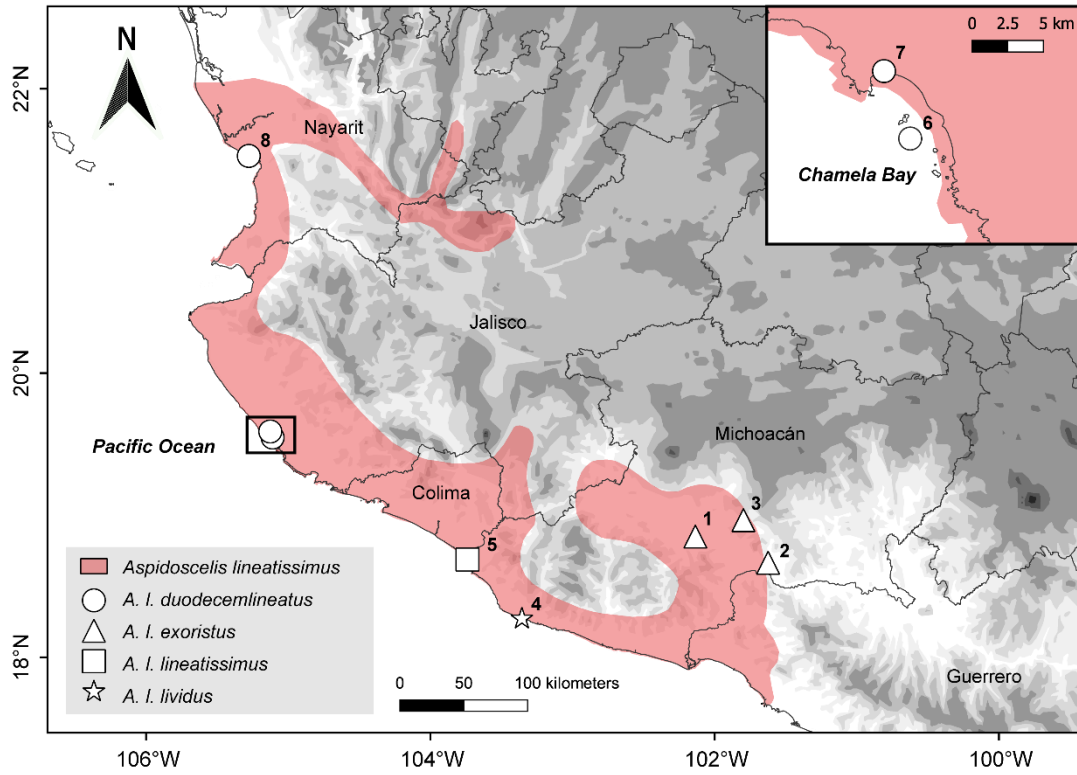
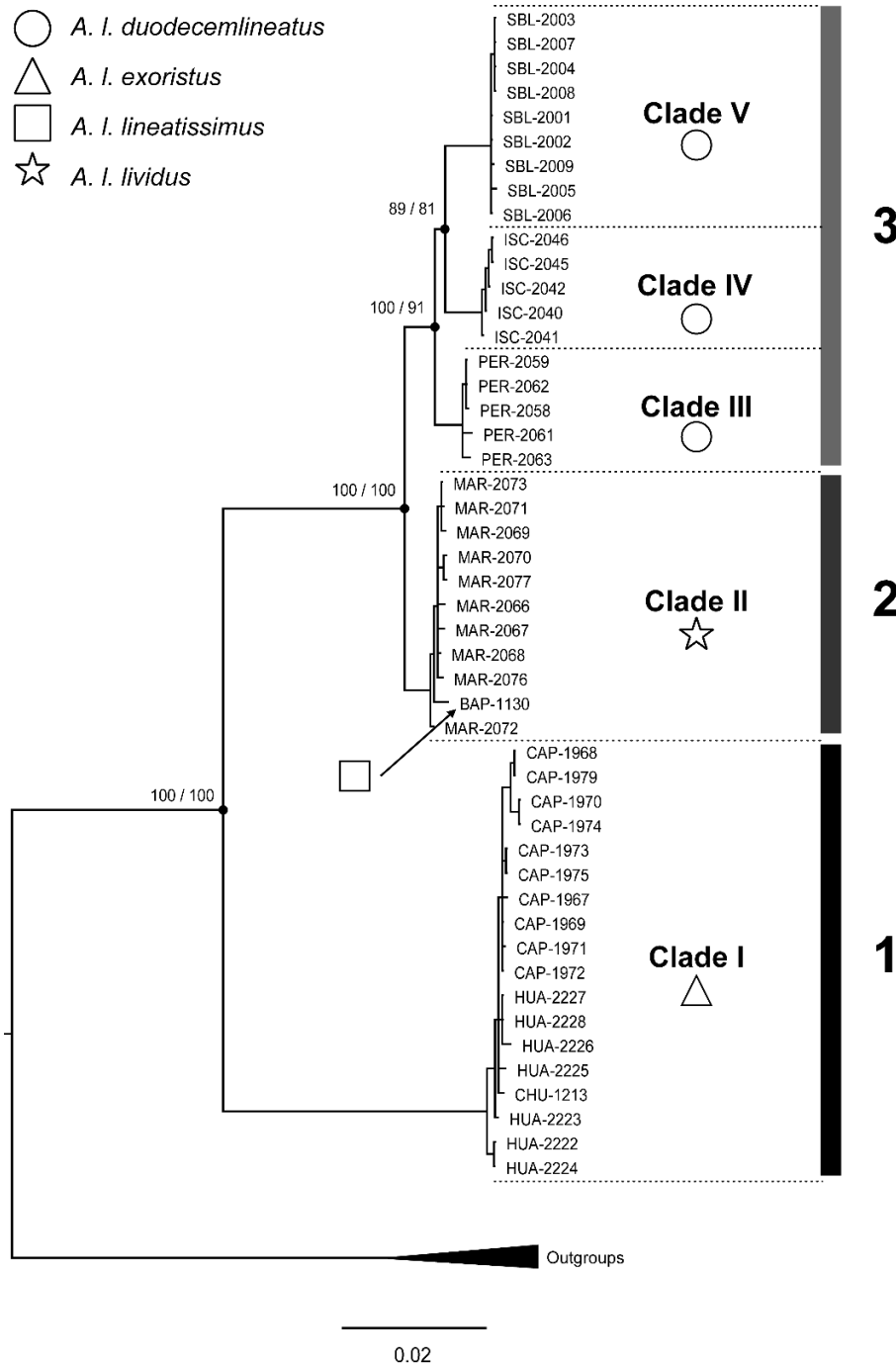


Fig. 2. Phylogenetic tree of the *Aspidoscelis lineatissimus* complex obtained for the concatenated data set of four genes (ND4, 12S, 16S, and BACH1) and 2,933 pair of bases. Posterior probabilities and bootstrap values are only shown in the principal nodes (BI/ML). Vertical bars indicate the different species detected by the BPP analysis.



Material suplementario

Table S1. Vouchers and Genbank numbers for molecular markers sequences generated in this study for *Aspidoscelis lineatissimus* complex and the outgroups used in the phylogenetic analyses.

Biogeographic region	Locality	GPS coordinates		Taxon	Voucher	Genbank number			
		Lat	Long			ND4	12S rRNA	16S rRNA	BACH1
Balsas-Tepalcatepec Valley	Capirio, La Huacana, Mich.	18.55	102.14	<i>A. l. exoristus</i>	INIRENA-1967	2-capirio	2-capirio	2-capirio	2-capirio
					INIRENA-1968	3-capirio	3-capirio	3-capirio	3-capirio
					INIRENA-1969	4-capirio	4-capirio	4-capirio	4-capirio
					INIRENA-1970	5-capirio	5-capirio	5-capirio	5-capirio
					INIRENA-1971	6-capirio	6-capirio	6-capirio	6-capirio
					INIRENA-1972	7-capirio	7-capirio	7-capirio	7-capirio
					INIRENA-1973	8-capirio	8-capirio	8-capirio	8-capirio
					INIRENA-1974	9-capirio	9-capirio	9-capirio	9-capirio
					INIRENA-1975	11-capirio	11-capirio	11-capirio	11-capirio
					INIRENA-1976	12-capirio	12-capirio	12-capirio	12-capirio
Churumuco, Churumuco, Mich.	18.67	101.62	<i>A. l. exoristus</i>	INIRENA-1213	line1213	line1213	line1213	line1213	
La Huacana, La Huacana, Mich.	18.97	101.80	<i>A. l. exoristus</i>	INIRENA-2223	line2223	line2223	line2223	line2223	
				INIRENA-2222	2222deppei	2222deppei		2222deppei	
				INIRENA-2224	deppei1h	deppei1h	deppei1h	deppei1h	
				INIRENA-2225	deppei2h	deppei2h	deppei2h	deppei2h	
				INIRENA-2226		deppei4h	deppei4h	deppei4h	
				INIRENA-2227	deppei5h	deppei5h	deppei5h	deppei5h	
				INIRENA-2228	deppei6h	deppei6h	deppei6h	deppei6h	
A. communis	INIRENA-2221	2221communis	2221communis	2221communis	2221communis				

	Lombardia, Gabriel Zamora, Mich.	19.16	102.05	<i>A. calidipes</i>	INIRENA- 2176	2176calidi pes	2176calidi pes	2176calidi pes	2176calidi pes
Michoacan Coastal Plain	Maruata, Aquila, Mich.	18.27	103.36	<i>A. l. lividus</i>	INIRENA- 2073	1-maruata	1-maruata	1-maruata	1-maruata
					INIRENA- 2066	3-maruata	3-maruata	3-maruata	3-maruata
					INIRENA- 2072	5-maruata	5-maruata	5-maruata	5-maruata
					INIRENA- 2070	7-maruata	7-maruata	7-maruata	7-maruata
					INIRENA- 2071	8-maruata	8-maruata	8-maruata	8-maruata
					INIRENA- 2067	9-maruata	9-maruata		9-maruata
					INIRENA- 2068	10- maruata	10- maruata	10- maruata	10- maruata
					INIRENA- 2069	11- maruata	11- maruata		11- maruata
					INIRENA- 2076	13- maruata	13- maruata	13- maruata	13- maruata
					INIRENA- 2077	14- maruata	14- maruata	14- maruata	14- maruata
Colima Coastal Plain	Boca de Apiza, Tecoman, Col.	18.69	103.74	<i>A. l. lineattiss imus</i>	INIRENA- 1130	1-colima	1-colima	1-colima	1-colima
Jalisco Coastal Plain	Cocinas Island, La Huerta, Jal.	19.55	105.11	<i>A. l. duodece mlineatu s</i>	INIRENA- 2040	3-isla	3-isla	3-isla	3-isla
					INIRENA- 2046	5-isla	5-isla	5-isla	5-isla
					INIRENA- 2041	7-isla	7-isla	7-isla	7-isla
					INIRENA- 2042	8-isla	8-isla	8-isla	8-isla
					INIRENA- 2045	11-isla	11-isla	11-isla	11-isla
	Pérula, La Huerta, Jal.	19.59	105.13	<i>A. l. duodece mlineatu s</i>	INIRENA- 2061	2-perula	2-perula	2-perula	2-perula
					INIRENA- 2059	3-perula	3-perula	3-perula	3-perula
					INIRENA- 2063	3-perula-2	3-perula-2	3-perula-2	3-perula-2
					INIRENA- 2062	6-perula	6-perula	6-perula	6-perula
					INIRENA- 2058	8-perula	8-perula	8-perula	8-perula

Nayarit Coastal Plain	San Blas, San Blas, Nay.	21.53	105.28	<i>A. l. duodecemlineatus</i>	INIRENA-2001	40-SB	40-SB	40-SB	40-SB
					INIRENA-2002	44-SB	44-SB	44-SB	44-SB
					INIRENA-2003	46-SB	46-SB	46-SB	46-SB
					INIRENA-2007	48-SB	48-SB	48-SB	48-SB
					INIRENA-2004	50-SB	50-SB	50-SB	50-SB
					INIRENA-2008	53-SB	53-SB	53-SB	53-SB
					INIRENA-2009	56-SB	56-SB	56-SB	
					INIRENA-2005	59-SB	59-SB	59-SB	59-SB
					INIRENA-2006	60-SB	60-SB	60-SB	60-SB
					<i>A. communis</i>	INIRENA-2011	2011communis	2011communis	2011communis
Mexican Transversal Axis	Morelia, Morelia, Mich. Charo, Charo, Mich.	19.70	101.19	<i>A. gularis</i>	INIRENA-2220	2220gularis	2220gularis	2220gularis	2220gularis
					INIRENA-595	595gularis	595gularis	595gularis	595gularis

INIRENA=Instituto de Investigaciones sobre los Recursos Naturales

Table S2. Pairwise Φ_{ST} values among lineages recognized within the *Aspidoscelis lineattissimus* complex and the taxonomic subspecies. Significant values are adjusted by Bonferroni correction.

	ND4	12S	16S	BACH1
Among subspecies				
<i>exoristus</i> vs <i>lividus</i>	0.97	0.96	1.00	0.43
<i>exoristus</i> vs <i>lineattissimus</i>	0.96	0.95	1.00	0.60
<i>exoristus</i> vs <i>duodecemlineatus</i>	0.88	0.94	0.95	0.43
<i>duodecemlineatus</i> vs <i>lividus</i>	0.55	0.63	0.74	0.28
<i>duodecemlineatus</i> vs <i>lineattissimus</i>	0.35	0.54	0.69	0.48
<i>lineattissimus</i> vs <i>lividus</i>	0.34	0.52	0.60	0.65
Among lineages				
Clade I vs Clade II	0.96	0.96	0.99	0.42
Clade I vs Clade III	0.96	0.96	1.00	0.41
Clade I vs Clade IV	0.97	0.96	1.00	0.42
Clade I vs Clade V	0.97	0.96	1.00	0.62
Clade III vs Clade II	0.94	0.82	0.94	0.20
Clade III vs Clade IV	0.95	0.89	0.94	0.01
Clade III vs Clade V	0.95	0.91	0.97	0.48
Clade IV vs Clade II	0.94	0.84	0.95	0.26
Clade IV vs Clade V	0.90	0.97	1.00	0.63
Clade V vs Clade II	0.94	0.92	0.96	0.52

The bold format indicates significant pairwise Φ_{ST} values

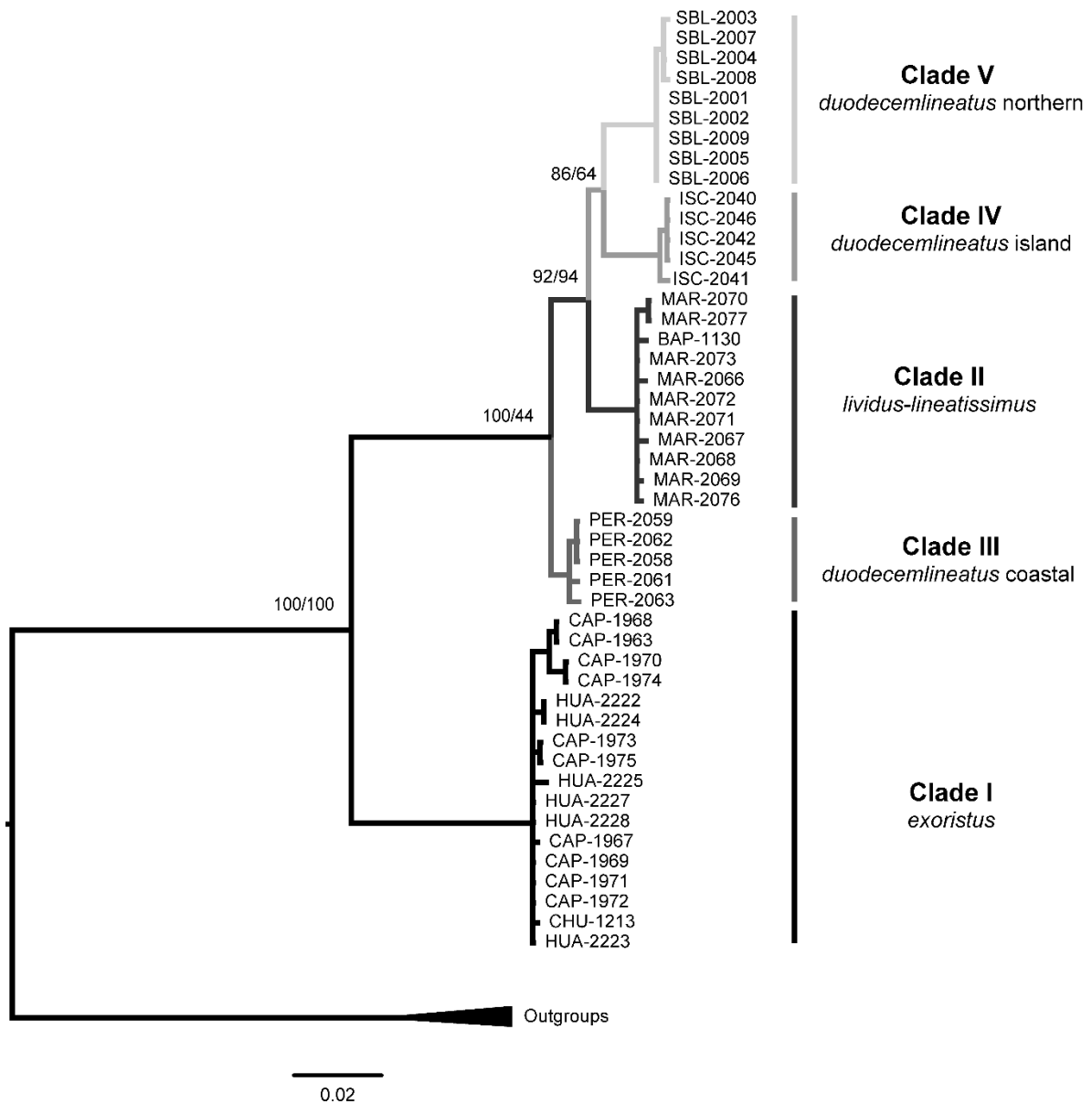


Figure S1. Phylogenetic tree of the *Aspidoscelis lineatissimus* complex obtained for 844 pairs of bases of mitochondrial gene ND4. Posterior probabilities and bootstrap values are only shown in the principal nodes (BI/ML). Clade names correspond to the recognized clades in the concatenated dataset.

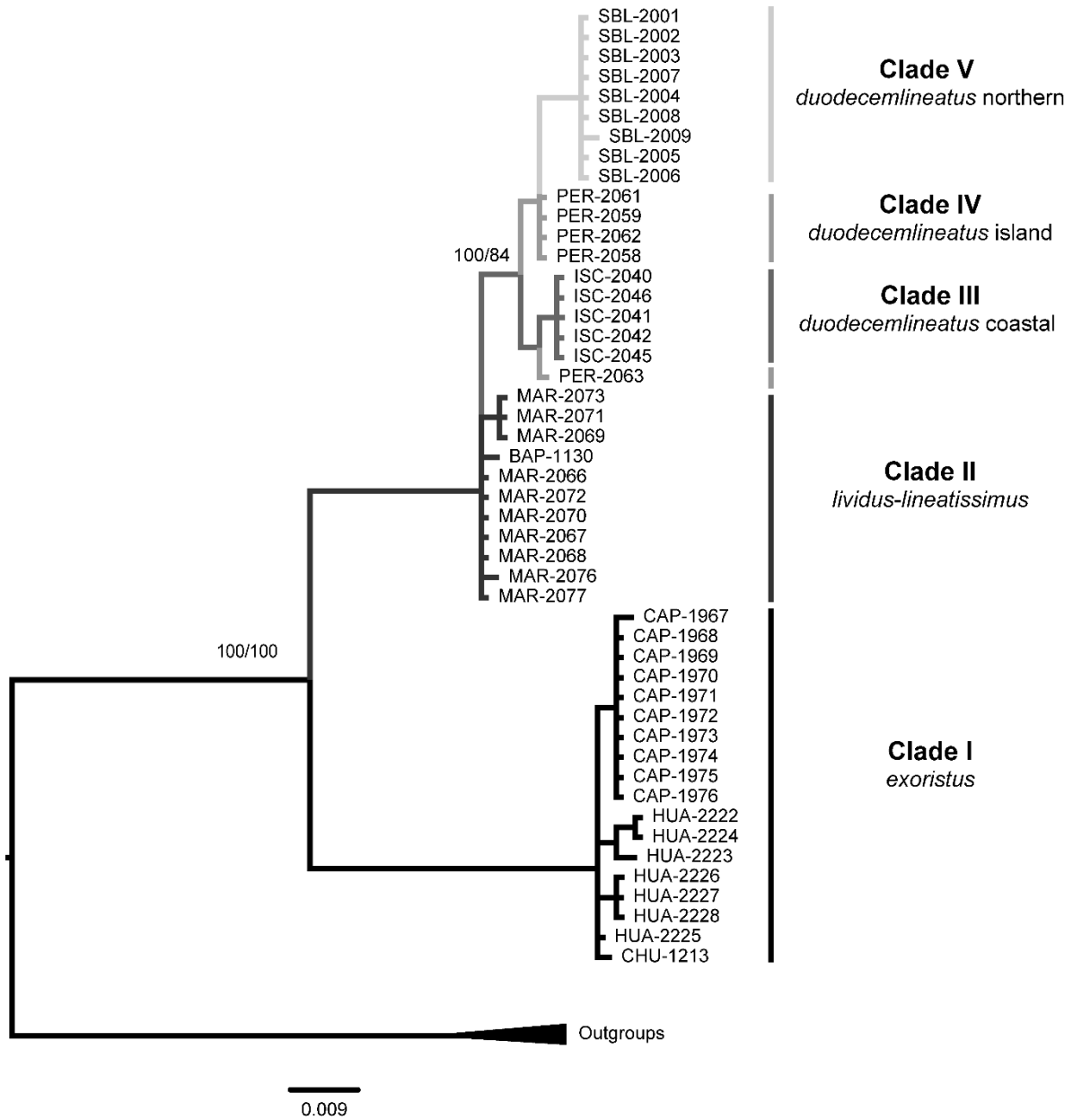


Figure S2. Phylogenetic tree of the *Aspidoscelis lineatissimus* complex obtained for 407 pairs of bases of mitochondrial gene 12S. Posterior probabilities and bootstrap values above 80 are shown in the principal nodes (BI/ML). Clade names correspond to the recognized clades in the concatenated dataset.

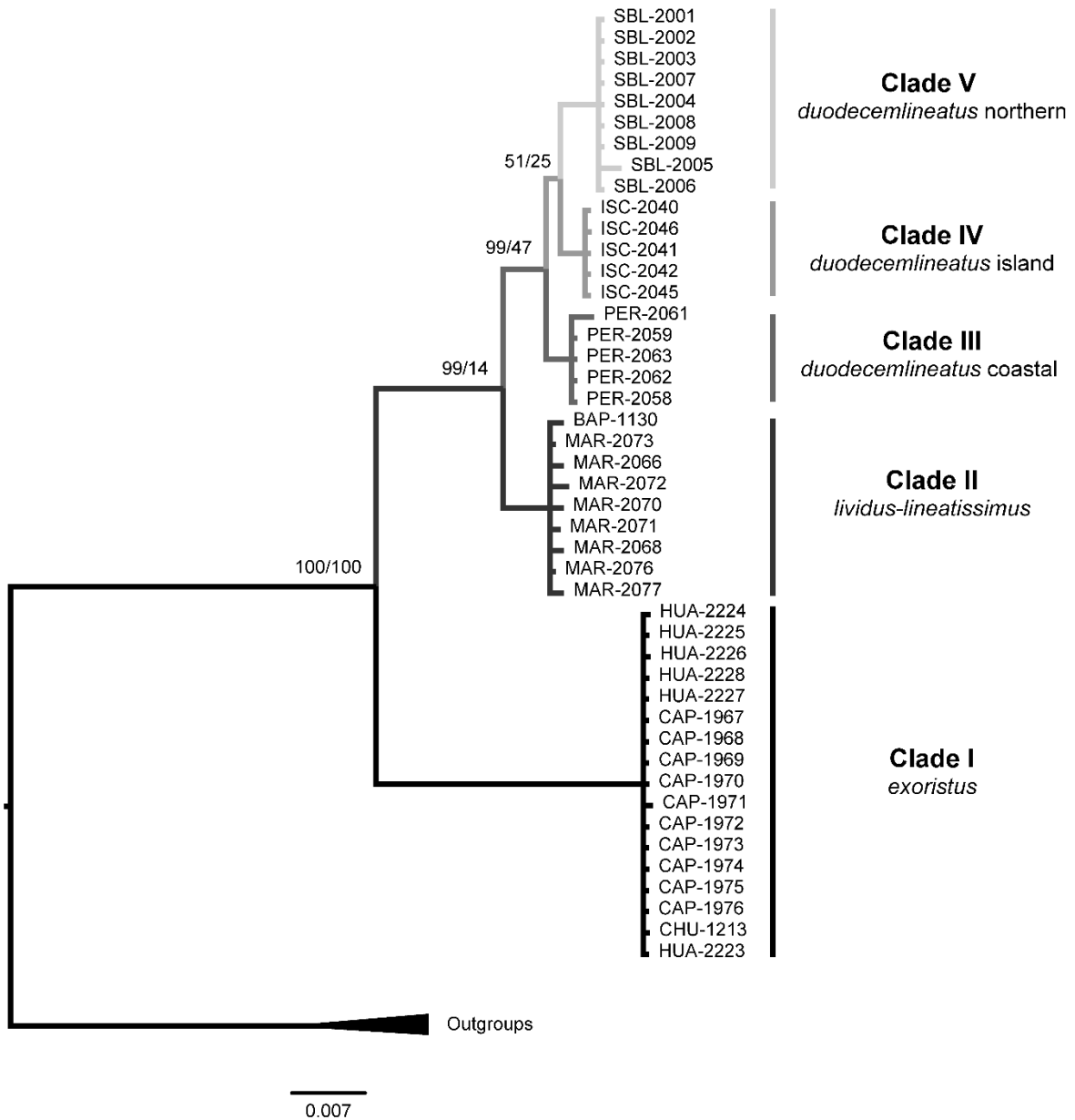


Figure S3. Phylogenetic tree of the *Aspidoscelis lineatissimus* complex obtained for 770 pairs of bases of mitochondrial gene 16S. Posterior probabilities and bootstrap values are only shown in the principal nodes (BI/ML). Clade names correspond to the recognized clades in the concatenated dataset.



Figure S4. Phylogenetic tree of the *Aspidoscelis lineatissimus* complex obtained for 912 pairs of bases of the nuclear gene BACH1.

DISCUSIÓN GENERAL

Históricamente hablando, insectos y otros invertebrados han sido los grupos modelo de estudio y de referencia para la investigación sobre infoquímicos y conducta animal (Harborne 2001). Sin embargo, en los últimos años (15-20 aprox.), las lagartijas se han convertido en un popular modelo para estudiar la composición y el funcionamiento de las señales químicas de vertebrados terrestres (Baeckens 2019). Aunque todavía siguen existiendo vacíos del conocimiento sobre muchos aspectos de la ecología química y comportamiento de vertebrados, es probable que en un futuro no muy lejano esta línea de investigación se consolide y los estudios que generen conocimiento aplicado sean más frecuentes, esto debido a los significativos esfuerzos de distintos equipos de investigación que trabajan actualmente sobre diferentes aspectos relacionados a la comunicación química en animales.

La importancia de las señales químicas para la modulación de las conductas sexuales y sociales en lagartijas ha sido analizada en varias especies provenientes de ambientes mediterráneos y también de algunas zonas desérticas (Khannoon *et al.* 2011, Martín y López 2015), pero en mucho menor grado para las especies tropicales o de zonas templadas (Ibañez *et al.* 2017; Campos *et al.* 2020). Aunque el conocimiento generado en estas especies puede ayudar a comprender más a fondo la función de los semioquímicos en diferentes procesos del comportamiento animal, la diversidad de especies de lagartijas tropicales posee asociaciones y atributos muy particulares que las hacen biológicamente interesantes.

El tema central de esta tesis ha sido sobre el papel de las señales químicas que modulan los comportamientos sociales y reproductivos en especies de lagartijas, evaluando las variaciones interespecíficas en las señales químicas de un grupo de lagartijas tropicales y además utilizamos como modelo biológico a la lagartija *A. lineattissimus* para explorar las variaciones poblacionales en estas mismas señales químicas y su efecto sobre las conductas de reconocimiento y discriminación química. Además, caracterizamos las relaciones genéticas y evolutivas que existen en el complejo *A. lineattissimus* desde un punto de vista filogenético.

Como se expuso en el capítulo I, la lagartija *A. lineattissimus* presenta variación química en las secreciones de sus glándulas femorales (SGF) cuando se compara con las SGF de otras especies de lagartijas que habitan un mismo ambiente tropical.

Esta variación interspecífica en rasgos químicos ha sido explorada en pocas comunidades de lagartijas (Escobar *et al.* 2001; Khannoon 2012; Baeckens *et al.* 2015), en cambio en la literatura son más comunes las descripciones químicas por taxón y las comparaciones químicas entre especies hermanas (Weldon *et al.* 1990; Alberts 1993; Gabirot *et al.* 2008; Martín *et al.* 2011, 2013b; García-Roa *et al.* 2016b). La lagartija *A. lineattissimus* es un forrajero activo con hábitos terrestres y tiene una mayor cantidad de compuestos químicos presentes en sus SGF en comparación con las SGF de otras especies tanto terrestres como arbóreas que cohabitan en una misma zona tropical costera. Estas diferencias interspecíficas están asociadas a grupos de compuestos químicos como ácidos carboxílicos y ésteres, siendo estos últimos los compuestos más abundantes en todas las especies estudiadas. En este estudio algunas de las diferencias observadas en la composición química de las SGF posiblemente estén asociadas al tipo de hábitat, aunque sería importante seguir analizando las SGF de más especies para evaluar si el tipo de hábitat tiene un papel significativo sobre su variación química. Las condiciones climáticas del tipo de microhábitat o la dieta de estas especies podrían ser entonces factores ambientales importantes implicados en determinar las diferencias registradas en las SGF de las especies de lagartijas estudiadas aquí (Baeckens *et al.* 2017, 2018).

Los resultados del capítulo II sugieren que *A. lineattissimus* también tiene una alta variación poblacional en la composición química de sus SGF. Estas variaciones en las SGF son esenciales para el reconocimiento químico entre individuos de distintas poblaciones y la elección parcial de territorios para encontrar una potencial pareja. La importancia de estas conductas varía con las SGF de cada población de origen. Así, para las poblaciones con distribución hacia el oriente y parcialmente aisladas por el sistema montañoso Sierra Madre del Sur (poblaciones de Michoacán, México), tanto machos y hembras se reconocen químicamente entre ellos y otros miembros de su misma población de origen, por lo que las señales químicas de individuos de otras poblaciones son eficientemente discriminadas. Adicionalmente, las hembras de *A. lineattissimus* de las poblaciones del estado de Michoacán, eligen pasar más tiempo sobre los territorios marcados con los aromas o señales químicas de los machos de su misma población de origen en comparación con los territorios de otros machos de distinta población.

Por otro lado, las poblaciones con distribución más hacia el occidente de la distribución de *A. lineattissimus* y geográficamente aisladas entre una zona continental y otra insular (Bahía de Chamela Jalisco, México), muestran un eficiente reconocimiento intersexual entre machos y hembras adultos con procesos de discriminación química entre poblaciones de manera muy similar como lo hacen las poblaciones de Michoacán, pero a diferencia de estas últimas poblaciones, las hembras *A. lineattissimus* del estado de Jalisco no logran discriminar entre territorios marcados con las señales químicas de machos de su misma o diferente población, por lo que no muestran una preferencia clara por ningún territorio de los machos.

El reconocimiento y discriminación sexual entre poblaciones y el patrón asimétrico detectado en el comportamiento de asociación de las hembras de *A. lineattissimus*, nos sugiere que la existencia de un aislamiento etológico entre poblaciones modulado por perfiles de divergencia en las señales químicas. Por lo tanto, es evidente que el aislamiento etológico producido tiene diferentes niveles de importancia y su implicación evolutiva para un evento de especiación podría estar siendo sujeto, al menos parcialmente, por distintos procesos de selección sexual actuando dentro y entre las diferentes poblaciones de *A. lineattissimus* en los ambientes tropicales costeros del Centro-Occidente de México.

Actualmente existe un interesante debate sobre las pautas de la conducta que pueden estar implicadas en modular los procesos de aislamiento reproductivo y que favorecen la especiación en vertebrados como las lagartijas. Conductas sexuales como la competencia entre machos y la elección de los machos por las hembras, están siendo hoy frecuentemente reconocidas por tener un mayor efecto sobre el aislamiento reproductivo más que la elección de las hembras por los machos (e.g. Gabirot *et al.* 2012, Heathcote *et al.* 2016, Martín *et al.* 2016, MacGregor *et al.* 2017). Esto es un punto interesante, ya que, en nuestro estudio de señales químicas, los machos (y en muchas otras especies de lagartijas) son los responsables de elaborar y producir la mayoría de ornamentos químicos esenciales para mostrar las señales honestas de sus cualidades como potencial pareja. Y aunque efectivamente, en unas poblaciones nosotros no encontramos evidencias de una preferencia femenina, en otras si hubo una respuesta selectiva de las hembras por las señales químicas de los machos. Lo que demuestra, que actualmente estamos desestimando las conductas de elección de pareja de las hembras en muchos estudios de selección sexual y aislamiento reproductivo entre

poblaciones y especies de lagartijas. Mas investigación es necesaria para evaluar qué factores podrían estar determinando la “aparente” ausencia o presencia de las conductas de elección de pareja en hembras de distintas especies de lagartijas, ya que como en muchas otras especies de vertebrados, las hembras tienen mucho más que perder o mucho más que ganar en términos tanto genéticos como energéticos si las señales químicas no están transmitiendo efectivamente la información entre emisor y receptor.

En el capítulo III se muestra que la lagartija *A. lineattissimus* conforma un complejo de especies lo cual en cierta forma explica los resultados obtenidos en el capítulo II. Cuatro subespecies con variaciones morfológicas y fenotípicas habían sido previamente descritas para este taxón (Duellman y Wellman 1960). Este capítulo aporta nueva información obtenida de análisis filogenéticos y evolutivos de marcadores nucleares y mitocondriales donde se ha podido detectar que por lo menos tres de las actuales subespecies pueden ser tratadas como linajes independientes. Una alta diversidad genética y las probabilidades de especiación encontradas en este complejo de especies sugieren una reestructuración del estatus taxonómico de la lagartija *A. lineattissimus* donde anteriormente era reconocida una sola especie, ahora podrían ser consideradas por lo menos tres distintas entidades taxonómicas con procesos evolutivos modulados desde su divergencia genética, morfológica y ahora también con su comunicación química durante las relaciones intra e intersexuales donde procesos como la selección sexual también podría estar teniendo un papel clave para mantener esta divergencia de especies.

La divergencia en señales químicas de las SGF en las poblaciones de *A. lineattissimus* parece parcialmente coincidir con la divergencia genética encontrada en las subespecies de este complejo taxonómico. Esto es importante para el reconocimiento taxonómico integrativo de nuevas especies, no solo con definidas características genéticas, morfológicas y ecológicas, sino a ahora también con un nivel de perfiles químicos especie-específicos que podrían ser utilizados en futuros estudios para delimitar las entidades biológicas con incertidumbres taxonómicas o con pobres criterios de diferenciación (ver, Zozaya *et al.* 2019).

Dos de las especies propuestas que divergen de este complejo taxonómico (*A. lividus* y *A. dudodecemlineatus*), parecen seguir respondiendo con los mismos atributos generalistas y funcionales de *A. lineattissimus* en sus hábitats tropicales (Raya-García, observación

personal), mostrando una abundancia en sus poblaciones y ciertos niveles de resiliencia al disturbio antropogénico (ver, Suazo-Ortuño *et al.* 2008). Por lo que, no parece extraño que sean especies exitosas debido a sus capacidades evolutivas y de adaptación en hábitats tropicales con características heterogéneas. Es probable que al igual que *A. lineattissimus* otras especies de lagartijas con atributos funcionales generalistas (por ejemplo, algunas especies de *Anolis*, *Holcosus*, *Sceloporus*) pueden estar siendo favorecidas por procesos evolutivos dejando una divergencia y radiación importante de especies. Conservar este tipo de especies con altas cualidades de resiliencia es clave para mantener los procesos evolutivos y ecológicos que ocurren dentro de los ambientes tropicales. Es recomendable que futuros estudios valoren el estatus de conservación de las nuevas especies identificadas en este estudio.

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