UNIVERSIDAD NACIONAL AUTÓNOMA DE MÉXICO

POSGRADO EN CIENCIAS BIOLÓGICAS

INSTITUTO DE BIOLOGÍA

BIOLOGÍA EVOLUTIVA

LOS FACTORES HISTÓRICOS Y AMBIENTALES COMO PROMOTORES DE VARIACIÓN ACÚSTICA EN RANAS DE CRISTAL (ANURA: CENTROLENIDAE)

TESIS

QUE PARA OPTAR POR EL GRADO DE:

DOCTORA EN CIENCIAS

PRESENTA:

ÁNGELA MARÍA MENDOZA HENAO

TUTORA PRINCIPAL DE TESIS: DRA. GABRIELA PARRA OLEA
INSTITUTO DE BIOLOGÍA, UNAM
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Ciudad Universitaria, CD. MX., Agosto, 2020.

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COORDINACIÓN DEL POSGRADO EN CIENCIAS BIOLÓGICAS
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ASUNTO: Oficio de Jurado

M. en C. Ivonne Ramírez Wence
Directora General de Administración Escolar, UNAM
P r e s e n t e

Me permito informar a usted que en la reunión del Subcomité por Campo de Conocimiento de Ecología, Manejo Integral de Ecosistemas, Biología Evolutiva y Sistemática del Posgrado en Ciencias Biológicas, celebrada el día 24 de febrero de 2020, se aprobó el siguiente jurado para el examen de grado de **DOCTORA EN CIENCIAS** de la alumna **MENDOZA HENAO ANGELA MARIA** con número de cuenta **512451060** con la tesis titulada: **"Los factores históricos y ambientales como promotores de variación acústica en ranas de cristal (Anura: Centrolenidae)",** realizada bajo la dirección de la **DRA. GABRIELA PARRA OLEA,** quedando integrado de la siguiente manera:

Presidente:	DR. OSCAR ALBERTO FLORES VILLELA
Vocal:	DR. JUAN PABLO JARAMILLO CORREA
Secretario:	DRA. ELLA GLORIA VÁZQUEZ DOMÍNGUEZ
Suplente:	DR. LUIS ENRIQUE EGUIARTE FRUNS
Suplente	DR. ALEJANDRO EMMANUEL GONZÁLEZ VOYER

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

A T E N T A M E N T E
"POR MI RAZA HABLARÁ EL ESPÍRITU"
Cd. Universitaria, Cd. Mx., a 10 de agosto de 2020

COORDINADOR DEL PROGRAMA

DR. ADOLFO GERARDO NAVARRO SIGÜENZA

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A mi mamá, a mi abuelita y a mi tío Jaime (Q.E.P.D.)

que los quiero demasiado

… y los extraño muchísimo

ÍNDICE

RESUMEN

La diversidad biológica es afectada por diferentes procesos evolutivos, incluyendo a la deriva génica (procesos neutrales y estocásticos), la selección (relación entre los rasgos y el entorno), la dispersión (combinación de procesos estocásticos y procesos dependientes de rasgos), la especiación (que moldea los conjuntos de especies en las comunidades) y la extinción. Por ejemplo, las barreras geográficas pueden afectar significativamente el flujo genético, tanto generando la especiación alopátrica por vicarianza, como reduciendo la dispersión y colonización de nuevas áreas. Este proceso de aislamiento de los linajes independientes puede producir eventos de especiación, reflejando o no cambios detectables a nivel fenotípico. Las señales acústicas involucradas en la reproducción se han utilizado en ocasiones para resolver casos de diversidad críptica en diferentes grupos de animales, como en aves, anfibios e insectos; por ello, es fundamental comprender los procesos que determinan la diferenciación de estas señales. El objetivo de esta tesis es evaluar la interacción entre el ambiente físico, la estructura filogenética y filogeográfica y las divergencias fenotípicas (señales acústicas) en ranas de cristal (familia Centrolenidae), una familia diversa cuyos miembros puedes ser encontrados en las quebradas y riachuelos del neotrópico. A lo largo del proyecto se lograron resolver varios interrogantes relacionados con el impacto de elementos bióticos y ambientales en la historia evolutiva de este grupo de anfibios de enorme diversidad. Este estudio proporciona información valiosa que revela la importancia de las barreras geográficas y las divergencias del canto como motores evolutivos en ranas de cristal. En el Capítulo I detectamos por medio de una aproximación filogeográfica y con base en secuencias mitocondriales cómo algunas barreras geográficas a lo largo de Centro América como la falla de Motagua y la cordillera de Talamanca han tenido impacto en la estructura genética de poblaciones de una especie de amplia distribución (*Hyalinobatrachium fleischmanni*). Estas barreras geográficas aislaron las poblaciones desde el Plioceno y Pleistoceno. En el Capítulo II observamos, desde una aproximación de taxonomía integrativa, cómo este aislamiento está generando procesos de especiación, al comparar las diferencias moleculares y en atributos clave (canto y morfología) en el comportamiento reproductivo entre linajes aislados. En este capítulo delimitamos a *H. fleischmanni* y a su especie hermana (*H. tatayoi*) y sugerimos la resurrección de una especie sinónima (*H. viridissimum*) considerada sinónima hasta el momento. Finalmente, en el Capítulo III realizamos una aproximación comparativa filogenética tanto a nivel intraespecífico como entre especies, para entender el impacto de la transmisión efectiva de las señales acústicas, dada su importancia en el reconocimiento de especies y las presiones de selección a las cuales están expuestas. Encontramos que el ambiente físico puede generar un impacto en la selección de las señales, donde sin embargo los patrones no coinciden enteramente con las hipótesis tradicionales sobre la transmisión efectiva de las señales.

ABSTRACT

Biologic diversity is affected by different processes, including drift (neutral and stochastic processes), selection (relationship between traits and environment), dispersion (combination of stochastic processes and trait-dependent processes), speciation (which shapes species clusters in communities) and extinction. For example, geographic barriers can significantly affect gene flow, generating both allopatric speciation by vicariance and dispersion to colonization of new areas. This process of lineage isolation can result in speciation, reflecting or not detectable changes in phenotypic differentiation. The acoustic signals involved in reproduction have sometimes been used to resolve cases of cryptic diversity in different groups such as birds, amphibians and insects; therefore, it is essential to understand the processes that determine the differentiation of these signals. The objective of this thesis was to evaluate the interaction between the physical environment, phylogenetic and phylogeographic structure and phenotypic divergences (acoustic signals) in glassfrogs (Centrolenidae family), a diverse family whose members are found in streams and rivers of the Neotropics. Throughout the project, several questions related to the impact of biotic and environmental elements in the evolutionary history of this group of amphibians of enormous diversity were solved. This study provides valuable information that reveals the importance of geographic barriers and call divergences as evolutionary drivers in glassfrogs. In Chapter I, using a phylogeographic framework on mitochondrial DNA data, we were able to detect how some geographic barriers throughout Central America, including the Motagua fault and the Talamanca range had great impact on the genetic structure of populations of a widely distributed species (*Hyalinobatrachium fleischmanni*). Those barriers isolated populations since Pliocene and Pleistocene. In Chapter II, we observed, from an integrative taxonomy approach, how isolation resulted in speciation by comparing molecular differences and key attributes (calls and morphology) in reproductive behavior between isolated lineages. In this chapter we delimited *H. fleischmanni* and its sister species (*H. tatayoi*) and suggest the resurrection of a synonymous species (*H. viridissimum*). Finally, in Chapter III, we used a comparative phylogenetic approach, both at intra-specific level and between species, to understand the impact of effective transmission of acoustic signals, given their importance in the recognition of species and the selection pressures to which they are exposed. We found that the physical environment can impact selection of these signals, where patterns do not coincide entirely with the traditional hypotheses of the effective signal transmission.

INTRODUCCIÓN

Los sistemas montañosos, los valles, los ríos y los mares han sido identificados como barreras o corredores para diferentes especies (Beheregaray 2008, Bagley & Jonhson 2014). El impacto de estos elementos en la conectividad de las poblaciones puede variar en función de la biología de cada especie y su estudio constituye la base de la biogeografía (Coz et al. 2016). Si las poblaciones de un taxón se muestrean intensamente a lo largo de su área de distribución, es posible estudiar sus patrones filogeográficos por medio de marcadores moleculares (Avise, 2000). Cuando las barreras que separan las poblaciones limitan el flujo génico de manera constante durante un número considerable de generaciones, las poblaciones separadas por la barrera empiezan a experimentar estructuración (diferenciación) genética (Lande 1980). Estos patrones de estructura genética son comúnmente detectados en grupos de baja vagilidad, como los anuros (ej: Crawford et al 2007: Gehara et al 2014; García-R et al. 2012), en comparación con algunos organismos que presentan capacidades de dispersión mayores (ej. algunas aves y mamíferos grandes), que les permite una alta conectividad entre individuos de poblaciones relativamente alejadas (Figura 1A; Lenormand 2002, Statham et al. 2014, Lougheed 2013).

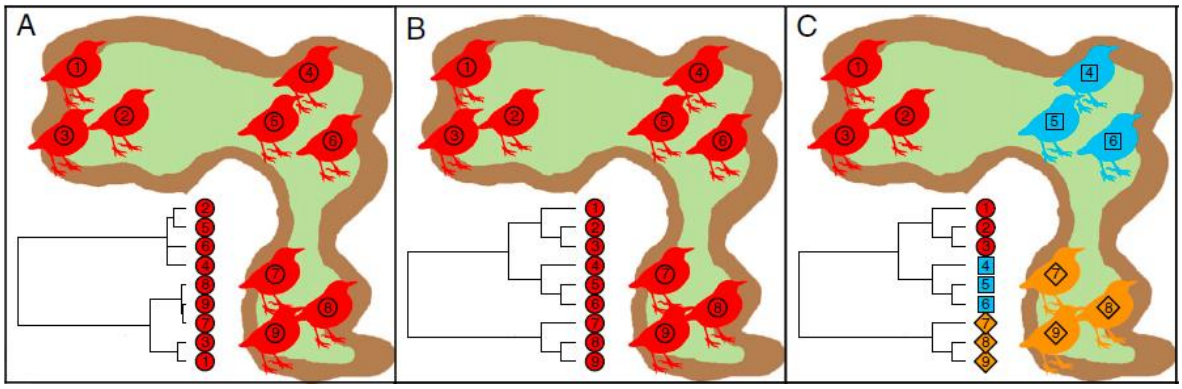

Figura 1. Patrones potenciales de estructura genética y diversidad fenotípica entre linajes. A) Fenotipos uniformes sin estructura genética, B) fenotipos uniformes con estructura genética C) fenotipos agrupados geográficamente con estructura genética [Modificado de Zamudio et al. 2016].

Este proceso de aislamiento de linajes independientes puede tener consecuencias en la diferenciación fenotípica. Es decir, los linajes estarán sujetos a presiones de selección y deriva independientes, que impulsan el ritmo y el modo de dicha diferenciación (Zamudio et al. 2016). En algunos casos, la diferenciación fenotípica es nula o indetectable (Figura 1B), pero en otros pueden actuar procesos como la divergencia neutral, la adaptación local y la selección sexual

divergente que permiten que la estructura genética y la variación fenotípica coincidan (Figura 1C, Zamudio et al. 2016).

Cuando esta diferenciación lleva al asilamiento de los linajes evolutivos, podemos hablar de procesos generación de nuevas especies o especiación. Históricamente han surgido múltiples conceptos de especie basados en diferentes propiedades de interés para los biólogos con diferentes preocupaciones (Ereshefsky, 1992; Mayden, 1997; de Queiroz, 1998). Entre estas, el concepto biológico de Mayr (1942) es la definición más ampliamente adoptada en zoología, con el aislamiento reproductivo sirviendo un doble papel como concepto y criterio, y la formación de especies fue vista como un proceso gradual iniciado desde la alopatría. Según de Queiroz (2005) más que conceptos, estas definiciones previas son propiedades contingentes (pero no necesarias) de los linajes evolutivos, es decir, son atributos que los linajes evolutivos adquieren por separado a medida que cambian a través del tiempo. Para solucionar el dilema entre concepto y propiedad de especie, el consenso actual ha implementado el concepto unificado de especie como “segmentos de linajes evolutivos a nivel de metapoblación” (de Queiroz 1998). Con esta aproximación, los desacuerdos sobre los límites entre las especies no se derivarán del desacuerdo sobre el concepto de especie como tal.

La especiación por fragmentación geográfica y genética con mínima variación detectable en el fenotipo es tan común en los anfibios tropicales (Fouquet et al. 2007, Páez-Vacas et al. 2010, Funk et al. 2012, Guarnizo et al. 2015, Lyra et al. 2017), que con frecuencia se detectan altos niveles de diversidad críptica lo que resulta en una subestimación de la riqueza de especies (Wynn & Heyer 2001). Considerando que muchos de los estudios taxonómicos tradicionales han estado basados en una sola fuente de evidencia (variación morfológica), no es de extrañar que la diversidad pueda estar oculta bajo un único nombre científico para múltiples linajes (i.e. especies crípticas, Dayrat 2005, Bickford et al. 2007). Esto se debe en parte a que algunos aspectos clave para la independencia de los linajes, como su ecología, sistema de reconocimiento y selección de pareja, sean independientes de las características morfológicas usadas tradicionalmente en la descripción de especies (Stuart et al. 2006). Revelar especies ocultas, identificar sus límites de distribución y sus relaciones con otras especies es fundamental en estudios donde se evalúen patrones de diversidad (Padial et al. 2010). Para resolver casos complejos de diversidad críptica se ha propuesto la necesidad de integrar fuentes de evidencia múltiples, incluyendo la diferenciación genética, la morfológica, la conductual y la ecológica (Padial et al. 2010). Las diferencias en estos caracteres podrían ser una fuerte evidencia de flujo génico reducido o nulo (Wiens & Servedio 2000); por lo tanto, el

consenso entre los resultados representa evidencia de independencia entre los linajes (de Queiroz 2007, Padial et al. 2010).

Entre las herramientas moleculares para la delimitación de especies vale la pena mencionar algunos métodos automatizados de diferenciación como el *General Mixed Yule-Coalescent* (GMYC, Pons et al., 2006), el *Poisson Tree Processes* (PTP, Zhang et al. 2013, Kapli et al., 2017) y el *Bayesian Phylogenetics and Phylogeography* (BPP, Yang & Rannala, 2010, 2015). El método GMYC infiere el punto de transición entre la cladogénesis (proceso de Yule) y el proceso de coalescencia alélica a nivel de la población, utilizando el supuesto de que la cladogénesis se producirá a una tasa mucho menor que la coalescencia (Carstens et al., 2013; Tang et al., 2014). Para ello utiliza estadísticas de máxima verosimilitud y toma una estimación de la genealogía ultramétrica como punto de referencia (Pons et al., 2006; Fujisawa & Barraclough, 2013). Por otra parte, los métodos PTP usan el número de sustituciones para modelar los eventos de especiación y coalescencia y con eso identificar cambios significativos en la tasa de ramificación en un árbol filogenético (Zhang et al., 2013). En este caso, se utilizan algoritmos heurísticos para identificar la clasificación más probable de las ramas en procesos a nivel de poblaciones y especies (Tang et al., 2014). Finalmente, el método BPP explora explícitamente el espacio de delimitación considerando diferentes combinaciones de poblaciones preespecificadas como especies candidatas mediante cadenas de Markov-Monte Carlo de salto reversible (rjMCMC) (Zhang & Rannala 2015).

Estos métodos han sido de gran utilidad sobre todo en la detección de especies crípticas (ej. Hedin 2015, Toussaint et al. 2015). Sin embargo, estos métodos han sido fuertemente criticados por ignorar la complejidad de los procesos de diferenciación de poblaciones que pueden tener las especies (Kuchta & Wake 2016), de modo que se les dificulta diferenciar procesos de especiación y estructura genética poblacional (Sukumaran & Knowles, 2017). La división excesiva de poblaciones pequeñas y aisladas basada en datos genéticos podría ser perjudicial durante el diseño de estrategias de conservación, así como en la estimación de diversidad en estudios macroecológicos o en análisis de redes alimenticias (Isaac et al. 2004, Frankham et al. 2012, Hambäck et al. 2013).

Por otra parte, las señales acústicas son un atributo de las especies cada vez más utilizado en estudios de ecología y en sistemática para la identificación de especies crípticas de anuros (Funk et al. 2008, 2011). Estas señales constituyen un elemento importante en la reproducción y en el reconocimiento de conespecíficos (Gerhard 1994), las cuales están vinculadas con el éxito reproductivo y, por lo tanto, están asociadas a presiones de selección

sexual y natural (Reznick 1985). La selección sexual puede promover aislamiento conductual entre poblaciones y, en última instancia, la formación de nuevas especies (Lande 1982; West-Eberhard 1983). Debido a su gran importancia como carácter taxonómico en la identificación de especies (Köhler et al. 2017), es indispensable también comprender los procesos que determinan su diferenciación.

Al igual que otros atributos fenotípicos de las especies, parte de la variación en las señales acústicas depende del tiempo de divergencia. Las especies y poblaciones evolutivamente distantes tienen más tiempo acumulando cambios de manera independiente que aquellas más estrechamente emparentadas, de modo que los organismos tienden a presentar rasgos más o menos disímiles en función de su cercanía filogenética (Pagel 1999; Revell et al. 2008). Del mismo modo, la comunicación es eficaz sólo cuando el receptor recibe la señal sin ningún tipo de alteración (Shannon & Weaver 1949). La adaptación local debería dar lugar a señales variables, dependientes del hábitat y de la comunidad circundante, de modo que se maximice la fiabilidad de la transmisión a los receptores (Endler 1992). La fidelidad de la transmisión puede ser afectada por perturbaciones (ruido) que tienden a enmascarar la información, mientras que el ambiente donde se comunica un individuo puede generar interferencia, impidiendo el desplazamiento de la señal (Goutte *et al.* 2013). Por otra parte, las señales pueden cambiar limitando la similitud entre los competidores, de la misma manera que la competencia por los recursos ecológicos fomenta la división del espacio de los nichos (Schmidt et al. 2013). Muchas especies de una misma comunidad utilizan canales similares para la comunicación, lo que limita el “espacio” que puede ocupar cada una de ellas (Tobias *et al.* 2014a,b). Además de las presiones directas en las señales acústicas, las diferencias entre especies también pueden ser promovidas por restricciones anatómicas y fisiológicas que pueden ser independientes de la selección de las señales como tal.

El estudio de la evolución de las señales acústicas presenta un problema de gran relevancia: la imposibilidad de obtener evidencia tangible del comportamiento en tiempos pasados puesto que difícilmente quedan rastros del comportamiento de las especies en el registro fósil (Rogers & Kaplan 2002). Afortunadamente, algunas áreas de investigación en historia evolutiva han sido beneficiadas gracias a los nuevos enfoques estadísticos entre los que se pueden mencionar los estudios comparativos. El método comparativo es una herramienta que usa correlaciones y regresiones para evaluar qué características de los organismos (como el tamaño, la forma, el ciclo de vida y el comportamiento) cambian respecto a otras características o aspectos de su entorno (Olson & Arroyo-Santos 2015). De este modo

se puede proporcionar evidencia del orden temporal de los cambios en dos rasgos, sugiriendo caminos causales probables (Pagel 1999). Los métodos comparativos permiten investigar el significado adaptativo en los patrones de la evolución de los rasgos de los organismos al comparar las especies contemporáneas (Pagel 1993). Los métodos comparativos filogenéticos integran las relaciones filogenéticas entre las especies y usan un modelo de evolución de caracteres que especifica la distribución esperada bajo ciertos modelos de evolución, lo que incrementa la rigurosidad estadística al probar la evolución correlacionada de los atributos de las especies (Freckleton et al. 2006). Desde la publicación del artículo de Pagel (1994), el número de artículos usando métodos filogenéticos comparativos se ha incrementado considerablemente, pasando de 13 artículos en 1993 hasta 265 en 2017 (Fuente: Web of Science).

Teniendo en cuenta lo anterior, el objetivo principal de esta tesis fue evaluar la interacción entre el ambiente físico, la estructura filogenética y la filogeográfica y las divergencias fenotípicas (señales acústicas). Es decir, mi proyecto buscó identificar cómo las barreras geográficas y diferencias ecológicas y la divergencia en factores relacionados con el aislamiento reproductivo influyen en la estructuración genética en ranas de cristal (familia Centrolenidae), y finalmente evaluar cómo estos patrones inciden en su evolución y divergencia a diferentes escalas evolutivas.

Organismo de estudio (Ranas de Cristal)

Para abordar estos temas, se utilizó como organismo de estudio las ranas de cristal (familia Centrolenidae); familia que incluye 12 géneros y 157 especies (Guayasamin *et al.* 2009, Frost 2019). Su área de distribución abarca el sur de México, América Central y los Andes desde Venezuela hasta Bolivia, la cuenca del río Orinoco, la región del Escudo Guayanés y los bosques atlánticos de Brasil y Argentina. Ocupan hábitats fluviales, sotobosque y dosel en bosques húmedos, bosques nublados y páramos (Cisneros Heredia & McDiarmid 2007), aunque la mayor diversidad está concentrada en los bosques tropicales del norte de los Andes (Ruiz-Carranza & Lynch, 1991).

Los miembros de la familia son nocturnos y generalmente arbóreos. Respecto a la capacidad de dispersión de las ranas de cristal, se sabe que están restringidas a los hábitats de arroyos durante la época reproductiva (Ruiz-Carranza y Lynch, 1991), donde depositan sus huevos fuera del agua, en la vegetación sobresaliente en arroyos como hojas, musgo o ramas, o desde las rocas de las paredes de las cascadas o cerca de ellas donde alcanza a llegar el rocío (Delia et al. 2017). Ello se traduce en baja movilidad, con subdivisión genética potencial y

flujo genético restringido. Por ejemplo, en un estudio sobre dispersión efectiva en *Espadarana prosoblepon* encontró que el flujo génico entre poblaciones es limitado (Robertson et al. 2008), mientras que un estudio de fidelidad de percha en *Hyalinobatrachium aureoguttatum* (Valencia-Aguilar et al. 2012) reportó que cada macho usa repetidamente la misma hoja para posarse, cantar, aparearse y asistir las posturas de huevos.

A pesar de que no son muchos los estudios sobre el aporte de los anfibios en los ecosistemas en el neotrópico (Valencia-Aguilar et al. 2013), algunos estudios han encontrado que las ranas de cristal son elementos de gran importancia en el funcionamiento de los ecosistemas acuáticos (Connelly *et al*. 2011; Whiles *et al.* 2006). Por ejemplo, en Panamá, los renacuajos de *E. prosoblepon*, *Sachatamia albomaculata*, *H. colymbiphyllum* y *Centrolene spp*. son clave para el ciclo de los nutrientes en los ecosistemas acuáticos, ya que estimulan la actividad fúngica en el lecho de los ríos (Connelly *et al.* 2011), y aumenta la concentración de nutrientes en los sistemas acuáticos a través de sus excreciones. La ausencia de estas especies puede alterar la estructura y las funciones del sistema (Brenes et al. 2006, Whiles *et al.* 2006) y llevar a la extinción local de especies depredadoras como es el caso de la reciente desaparición de serpientes posterior al declive de anfibios en Panamá (Zipkin *et al.* 2020).

Las relaciones filogenéticas entre las especies de la familia han sido evaluadas principalmente a partir de caracteres morfológicos altamente homoplásicos (Ruiz-Carranza & Lynch 1991), como la presencia de espinas humerales o la carencia de pigmentación ventral, lo que implica un escaso conocimiento de las relaciones entre especies y llevó a modificaciones constantes en la clasificación taxonómica a nivel genérico e infragenérico (ej. Savage 2002, Frost et al. 2006, Cisneros-Heredia & McDiarmid 2007).

Desde el trabajo realizado por Guayasamin *et al.* (2008, 2009), quienes generaron la primera filogenia molecular extensiva de ranas de cristal a partir de genes mitocondriales y nucleares, se cuenta con una filogenia robusta y una clasificación taxonómica estable sobre todo a nivel genérico. La subsecuente generación de árboles filogenéticos cada vez más completos ha permitido que las ranas de cristal sean un grupo reconocido para responder diferentes preguntas en biología evolutiva de organismos neotropicales. Estos estudios se han evaluado los patrones de diversidad altitudinal (Hutter et al., 2013), la biogeografía histórica y diversificación (Castroviejo-Fisher et al. 2014), los patrones de diversidad y endemismo filogenético (Mendoza & Arita 2014), la influencia de la energía ambiental en la tasa de evolución molecular (Dugo-Cota et al. 2015), la evolución del cuidado parental (Delia et al.

2017) y, más recientemente, la relación entre algunos caracteres sexuales, morfológicos y ecológicos de las especies (Escalona-Sulbarán et al. 2018).

Aunque la mayoría de las ranas de cristal presentan distribuciones restringidas (sobre todo aquellas distribuidas en los Andes), existen algunas como *Hyalinobatrachium fleischmanni* (*sensu lato*) que tiene una de las distribuciones más amplias entre las ranas de cristal. Se encuentra desde los estados de Guerrero y Veracruz en México, pasando por las tierras bajas de América Central, hasta el límite más meridional de su distribución en Ecuador (Cruz et al., 2017). Similar a las demás especies de la familia, esta especie exhibe fidelidad de percha y cuidado parental por parte de los machos, quienes pueden asistir más de una puesta al mismo tiempo (Delia et al., 2010; Savage, 2002; Barrera-Rodríguez, 2000). A lo largo de su distribución, *H. fleischmanni* es un organismo ideal para estudiar el papel que las barreras geográficas han jugado en los patrones de dispersión en anfibios y su impacto en ciertos caracteres, como el canto, sujetos a presiones de selección y con impacto en procesos de especiación.

Estructura de la tesis

La presente tesis está estructurada en tres capítulos (Figura 2). En el primer capítulo titulado "The role of Central American barriers in shaping the evolutionary history of the northernmost glassfrog, *Hyalinobatrachium fleischmanni* (Anura: Centrolenidae)" se describe la estructura filogeográfica de una rana de cristal de amplia distribución. En el segundo capítulo titulado "Phylogeny-based species delimitation and integrative taxonomic revision of the *Hyalinobatrachium fleischmanni* species complex, with resurrection of *H. viridissimum* (Taylor, 1942)" se integran los datos genéticos obtenidos en el capítulo anterior con datos bioacústicos y morfológicos, para así ayudar a resolver el estatus taxonómico de las especies. Finalmente, en el tercer capítulo titulado "Environment rather than Character Displacement explains call evolution in glassfrogs" se integran los resultados obtenidos en los dos capítulos anteriores, junto con datos bioacústicos de las otras especies de la familia para evaluar la relevancia de los posibles promotores de la variación observada en el canto.

Adicionalmente, gracias al esfuerzo de trabajo en campo y laboratorio, se logró la publicación de varios artículos (Anexos) reportando entre otras cosas, la reaparición de la especie introducida en México *Eleutherodactylus planirostris* (Contreras-Calvario et al. 2016) y la extensión del área de distribución de una especie fácilmente confundible con *H. fleischmanni*, (*H. chirripoi*, Mendoza-Henao et al. 2019a) por medio de identificación molecular; también reportamos la reaparición de una rana de cristal *Centrolene huilensis* (Mendoza-Henao et al.

2019b) y la descripción del canto de dos especies mexicanas (*Plectrohyla avia*, Barrio-Amorós et al. 2016 y *Eleutherodactylus rubrimaculatus*, Mendoza-Henao et al. 2020) que fueron observadas en las localidades de trabajo.

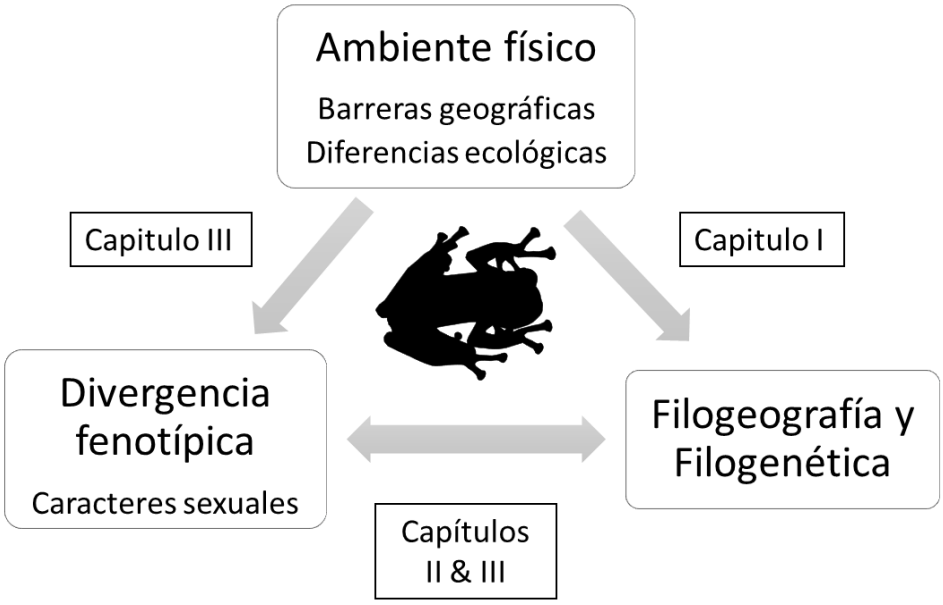

Figura 2. Síntesis de las relaciones entre el ambiente, el aislamiento genético y la divergencia fenotípica (caracteres sexuales) entre especies y poblaciones de ranas de cristal (Centrolenidae) que se evaluaron en esta tesis.

CAPÍTULO I

The role of Central American barriers in shaping the evolutionary history of the northernmost glassfrog, *Hyalinobatrachium fleischmanni* (Anura: Centrolenidae)

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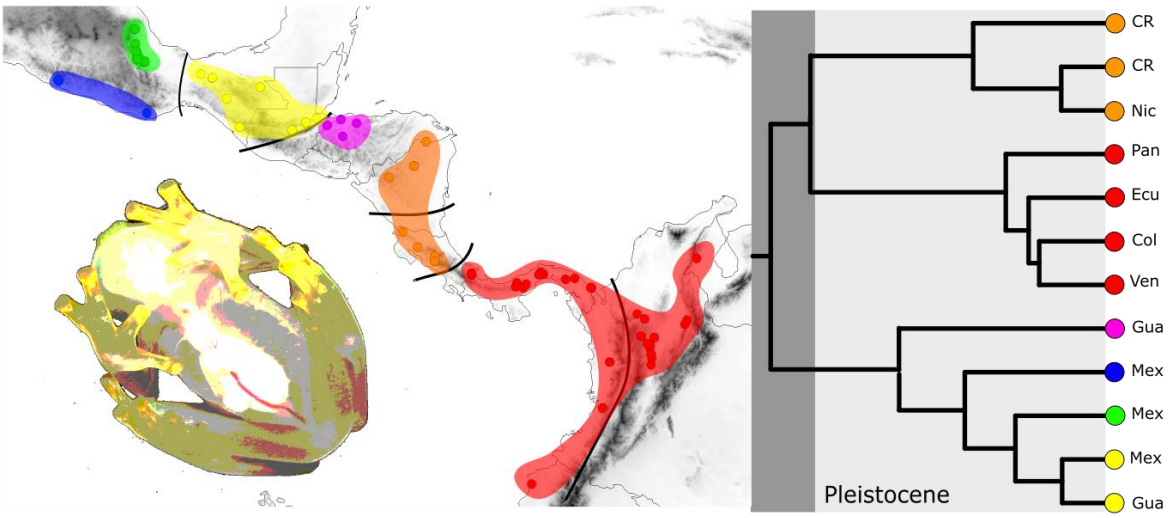

The role of Central American barriers in shaping the evolutionary history of the northernmost glassfrog, *Hyalinobatrachium fleischmanni* (Anura: Centrolenidae)

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ABSTRACT

The complex geological history of Central America has been useful for understanding the processes influencing the distribution and diversity of multiple groups of organisms. Anurans are an excellent choice for such studies because they typically exhibit site fidelity and reduced movement. The objective of this work was to identify the impact of recognized geographic barriers on the genetic structure, phylogeographic patterns and divergence times of a wide-ranging amphibian species, *Hyalinobatrachium fleischmanni*. We amplified three mitochondrial regions, two coding (COI and ND1) and one ribosomal (16S), in samples collected from the coasts of Veracruz and Guerrero in Mexico to the humid forests of Chocó in Ecuador. We examined the biogeographic history of the species through spatial clustering analyses (Geneland and sPCA), Bayesian and maximum likelihood reconstructions, and spatiotemporal diffusion analysis. Our data suggest a Central American origin of *H. fleischmanni* and two posterior independent dispersals towards North and South American regions. The first clade comprises individuals from Colombia, Ecuador, Panama and the sister species *Hyalinobatrachium tatayoi*; this clade shows little structure, despite the presence of the Andes mountain range and the long distances between sampling sites. The second clade consists of individuals from Costa Rica, Nicaragua, and eastern Honduras with no apparent structure. The third clade includes individuals from western Honduras, Guatemala, and Mexico and displays deep population structure. Herein, we synthesize the impact of known geographic areas that act as barriers to glassfrog dispersal and demonstrated their effect of differentiating *H. fleischmanni* into three markedly isolated clades. The observed genetic structure is associated with an initial dispersal event from Central America followed by vicariance that likely occurred during the Pliocene. The southern samples are characterized by a very recent population expansion, likely related to sea-level and climatic oscillations during the Pleistocene, whereas the structure

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of the northern clade has probably been driven by dispersal through the Isthmus of Tehuantepec and isolation by the Motagua–Polochic–Jocotán fault system and the Mexican highlands.

Subjects Biogeography, Zoology
Keywords Amphibian, Biogeography, *Hyalinobatrachium*, Pliocene, Centrolenidae, Pleistocene, Central America

INTRODUCTION

Historical biogeography focuses on the role of the geographic space as a driver of biological processes such as speciation, extinction, and diversification (*Cox, Ladle & Moore, 2016*). Areas with a complex geological history are characterized by the appearance and disappearance of multiple barriers and corridors in their history. These barriers may significantly affect the gene flow of resident species, leading to allopatric speciation by vicariance, whereas corridors may lead to species dispersal and colonization of new areas (*Noss, 1991*). The use of molecular data for the reconstruction of species relationships, the development of new methods for biogeographic analyses, and the increase in geological studies in complex regions have revolutionized the understanding of such biological processes (*Ronquist & Sanmartín, 2011*). Biogeographic studies have integrated information regarding the relationships within or between closely related taxa, providing valuable opportunities to understand how patterns of biodiversity may have been shaped, even at short time scales (*Crawford, Bermingham & Polanía-S, 2007*; *Streicher et al., 2014*).

Central America is a region with a rather complex biogeographic history and high diversity of habitats and species (*Myers et al., 2000*; *Cavers, Navarro & Lowe, 2003*; *Iturralde-Vinent, 2006*; *Daza, Castoe & Parkinson, 2010*). The region is delimited to the north by the Isthmus of Tehuantepec (IT) in Mexico and to the south by the Andes in Colombia (*Gutiérrez-García & Vázquez-Domínguez, 2013*). The geological landscape of Central America has been continuously modified, especially during the last 15 million years (Ma), by major events including the emergence of the Panama Arc (13–15 Ma, *Montes et al., 2015*), the posterior closure of the Panama Isthmian land bridge when it ceased to function as a seaway (~9–10 Ma, *Montes et al., 2012a*; *Montes et al., 2012b*; *Ramírez et al., 2016*), and the posterior global climatic transitions during the Plio-Pleistocene (*Montes et al., 2015*). These events triggered the Great American Biotic Interchange, or GABI, involving the replacement of native taxa (extinctions) and the establishment and diversification of colonizing taxa (speciation) on both continents (*Marshall et al., 1982*; *Stehli & Webb, 1985*). Ample phylogeographic research in this region has allowed the effects of geomorphology, topographic barriers, volcanic activity, large climate changes, intermittent connections, and corridors on the biota to be described, aiding in our understanding of the influence of past events on the patterns of genetic structure and the geographic distribution of birds (*García-Moreno et al., 2004*; *Cadena, Klicka & Ricklefs, 2007*; *Arbeláez-Cortés, Nyári & Navarro-Sigüenza, 2010*), plants (*Cavers, Navarro & Lowe, 2003*; *Ornelas, Ruiz-Sánchez & Sosa, 2010*; *Cavender-Bares et al., 2011*), reptiles (*Hasbún*

et al., 2005; *Venegas-Anaya et al., 2008*), mammals (*Eizirik et al., 2001*; *Ordóñez Garza et al., 2010*; *Pérez-Consuegra & Vázquez-Domínguez, 2017*), and amphibians (*Mulcahy, Morrill & Mendelson, 2006*; *Crawford, Bermingham & Polanía-S, 2007*; *Wang, Crawford & Bermingham, 2008*; *Hauswaldt et al., 2011*). As a result, diverse geological factors and major barriers have been more frequently correlated with the evolutionary history and dispersal of species (*Bagley & Johnson, 2014*).

Amphibians are excellent systems for studies in which geological and environmental histories are inferred at fine scales, due to their ecology, particularly regarding their terrestrial habits, intolerance to salt water (*Beebee, 2005*), and marked niche conservatism (*Smith, Stephens & Wiens, 2005*; *Wiens et al., 2006*), as well as the restriction of the particular habitats of many species (e.g., *Savage, 2002*). However, evaluation of the impact of barriers on the phylogeographic patterns of this taxon, extending across the entire Central American region, has been precluded because most amphibians have small ranges.

Glassfrogs (Centrolenidae) comprise a diverse family endemic to the Neotropics, with numerous species and high levels of endemism, mainly distributed among the Northern Andes and Central America regions (*Guayasamin et al., 2009*; *Castroviejo-Fisher et al., 2014*; *Mendoza & Arita, 2014*). Studies on the dispersal capability of glassfrogs are limited, but these frogs are known to be restricted to streamside habitats (*Ruiz-Carranza & Lynch, 1991*) and to show site fidelity (*Valencia-Aguilar, Castro-Herrera & Ramírez-Pinilla, 2012*) and low mobility, with potential genetic subdivision and restricted gene flow (*Delia, Bravo-Valencia & McDiarmid, 2017*; *Robertson, Lips & Heist, 2008*). The glassfrog *Hyalinobatrachium fleischmanni* (Boettger, 1893) has one of the widest distributions, ranging from Guerrero and Veracruz states in Mexico through the lowlands of Central America, to the southernmost limit of its distribution in Ecuador (*Cruz et al., 2017*). Males of the species call from vegetation along the margins of streams, and egg masses are usually laid on the underside of leaves over a stream. This species exhibits site fidelity and parental care by males, who attend one or more clutches at the same time (*Delia et al., 2010*; *Savage, 2002*; *Barrera-Rodríguez, 2000*). Tadpoles fall from vegetation into the water, where they develop; they are apparently fossorial, living buried in the leaf litter and bank substrate of streams (*Villa & Valerio, 1982*). Related species (i.e., *Hyalinobatrachium tatayoi*, *H. carlesvilai*, *H. mondolfii*, *H. kawense*, and *H. munozorum*) are distributed in different regions of South America, including the northern and central Andes, Guiana shield, and Amazon basin, where previous analyses have suggested an Andean origin for *H. fleischmanni* (*Castroviejo-Fisher et al., 2014*).

Considering its wide distribution, coupled with its site fidelity, *H. fleischmanni* is an ideal organism for studying the role that Central American geographic barriers have played in the dispersal patterns of lowland glassfrog species. In the present study, we had the following objectives: (1) to reconstruct the historical biogeography that has shaped the evolutionary history of *H. fleischmanni*, including dispersal or vicariance events and time of divergence; (2) to evaluate the possible presence of multiple isolated lineages within *H. fleischmanni*; and (3) to identify the impact of recognized geographic barriers on the genetic structure and phylogeographic patterns of *H. fleischmanni* over time. Based on known information about the species, we tested the hypothesis that *H. fleischmanni* had

a South American origin and subsequently dispersed into the Central American lowlands after the closure of the Isthmus of Panama. Additionally, we hypothesized that the dispersal of this species in Central America has been limited by various high mountain ranges acting as barriers and that changes in sea level during the Pleistocene had an impact on the genetic structure of the lowland populations (*Bagley & Johnson, 2014*). Hence, our prediction is that the current genetic structure of *H. fleischmanni* reflects patterns of vicariance events driven by dispersal barriers.

MATERIAL AND METHODS

Tissue sampling

Genetic material was obtained across the entire distribution area of the species, from both museum collections and fieldwork (Fig. 1). Fieldwork was performed during the rainy season, in which at least three individuals were collected at each locality. Specimen collection permits were provided by the Ministerio de Medio Ambiente, Colombia (Resolution 120 of 24 August 2015) and the Secretaría del Medio Ambiente y Recursos Naturales, Mexico (office number 00947/16). Captured specimens were euthanized with a 20% lidocaine hydrochloride (Xylocaine) injection, and all efforts were made to minimize suffering. Liver or muscle tissues were collected in the field and were stored in an RNAlater solution until their use in the laboratory. Specimens were fixed with 10% formalin, stored in 70% ethanol and deposited in biodiversity collections at public research institutions in each country.

Molecular techniques

DNA was obtained from muscle and liver tissues following the phenol-chloroform extraction protocol (*Sambrook & Russell, 2006*). The quantity and quality of the DNA were verified in 1% agarose gels and by measuring absorbance using a NanoDrop spectrometer (Thermo Fisher Scientific Inc., Wilmington, DE, USA). Amplification of the mitochondrial COI (658 bp), 16S (895 bp), and ND1 (961 bp) genes was performed following the protocols described by *Guayasamin et al. (2008)*. PCR products were visualized with agarose gels and purified according to the EXO-SAP protocol (GE Healthcare, Chicago, IL, USA). DNA sequences were obtained with the BigDye Terminator Cycle Sequencing kit (Applied Biosystems). Sequences from *H. tatayoi* from Venezuela (*Guayasamin et al., 2008*; *Castroviejo-Fisher, Ayarzagüena & Vila, 2007*) were also included. The sequences were assembled, manually edited and aligned with Geneious 9.1.2., and the three genes were concatenated by using the *cbind* function in the *ape* package in R (*Paradis, Claude & Strimmer, 2004*).

We are aware that analyses based on mitochondrial DNA (mtDNA) provide a limited view of species evolution (i.e., matrilineal inheritance), but the available genetic material from nuclear Central America is very scarce since there has been limited or no sampling in the region (due to a lack of funding as well as logistical and even bureaucratic difficulties) compared to that in northern or southern countries. Therefore, we chose to compile a database including gene sequences available from GenBank and BOLD databases with our own sequences to perform our analyses. In this way, our study encompasses the entire

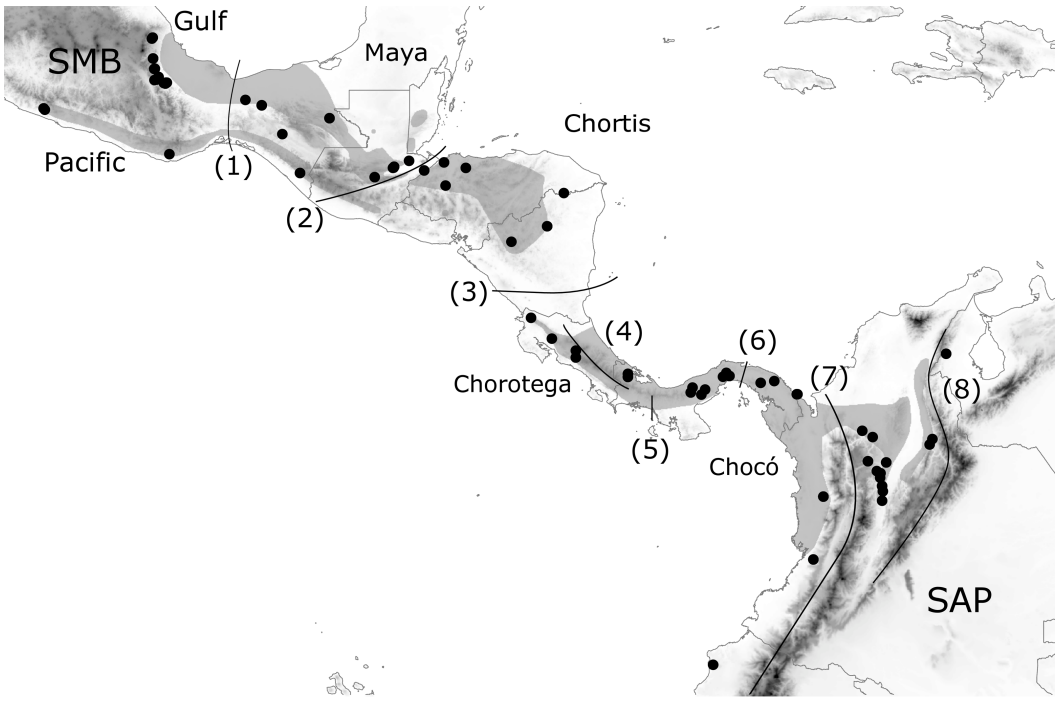

Figure 1 Geographic distribution of *H. fleischmanni* samples. Geographic distribution of *H. fleischmanni* samples. Main geological blocks, delimited by geological barriers, are shown. Sample origins are indicated by black dots, while gray polygons show the species distribution according to IUCN. SMB, South Mexican block, SAP, South American Plate. The evaluated barriers are numbered as follows: 1, Isthmus of Tehuantepec, 2, Motagua-Polochic-Jocotán fault system, 3, Hess Escarpment, 4, Talamanca Range, 5, Western Panama Isthmus, 6, Central Panama Isthmus, 7, Northern Andes and 8, Eastern Cordillera.
Full-size DOI: 10.7717/peerj.6115/fig-1

geographic distribution of *H. fleischmanni*, allowing us to evaluate the events shaping the evolutionary history of the species.

Additionally, mtDNA is a robust indicator of population structure and historical biogeographic barriers and can be used to detect cryptic diversity (*Gehara et al., 2014*; *Dudoit et al., 2018*; *Lait & Hebert, 2018*). Mitochondrial DNA markers have been used in a variety of Central American studies to decipher genetic structuring (for a review, see *Bagley & Johnson, 2014*), allowing us to make direct comparisons with previous studies from this region.

Data analysis

Identification of landscape barriers to dispersal

We defined *a priori* a set of possible geographic elements based on the four geological blocks across Central America (Maya, Chortis, Chorotega, and Chocó) (*Marshall, 2007*; *Gutiérrez-García & Vázquez-Domínguez, 2013*) as well as their northern and southern limits: the southern Mexico Block (SMB) west of the IT and the South American Plate (SAP) east of the Andes, respectively. The effects on species dispersal were evaluated for three highland barriers: the Motagua–Polochic–Jocotán (MPJ) fault system, the Talamanca Cordillera, and the Andes range, which separates the Northern Andes (Western and Central

Cordilleras) and the Eastern Cordillera (including Serranía del Perijá). Three previously recognized barriers for lowland amphibians (the Hess Escarpment, HE; western Panama Isthmus, WPI; and central Panama Isthmus, CPI) were also tested as possible factors during Pleistocene sea-level oscillations (Fig. 1). Each barrier was tested by four spatial and non-spatial analyses, and supported barriers were defined as those detected by at least three of the analyses.

The first step was to perform spatial and clustering analyses that are commonly applied to mtDNA sequences. The spatial locations of genetic discontinuities were estimated with Geneland (*Guillot, Mortier & Estoup, 2005*), which estimates the number of populations within the geographical area of interest, maps borders between populations, assigns individuals to populations, and detects possible migrants. We ran the model in R under the correlated allele frequency model, without uncertainty regarding spatial locations. We generated 10^5 iterations to a thinning of 100, with the maximum rate of the Poisson process fixed as the number of individuals, and the generated map borders were compared with our hypothesized barriers. Additionally, we performed a spatial principal component analysis (sPCA; *Jombart et al., 2008a*) using the *adegenet* package in R (*Jombart, 2008b*) for which we constructed a neighbor-distance net among all coordinates and tested for significant, geographically correlated genetic structures along the main axis based on a global randomization test. We extracted the values of the first two sPCA components and generated polygons for each interbarrier set of individuals to test if the populations adjacent to each barrier were clustered (non-overlap) or displayed as a continuum (partially or completely overlapping). Previous studies have suggested multivariate ordination analyses as an alternative to Bayesian algorithms because they do not make any assumptions about the underlying population genetic model (*Jombart, Devillard & Balloux, 2010*). Additionally, we performed a non-spatial test by estimating the Nei's pairwise F_{ST} value between adjacent regions and estimated significance by a Monte Carlo test based on 999 permutations with the *hierfstat* package (*Goudet, 2005*); because we did not have data for the three mitochondrial genes for all samples in all localities, these analyses included missing values. Additionally, we used the alignments per gene to calculate diversity indices and to perform additional clustering analyses that do not support missing values in the concatenated sequences (see Supplemental Information 1).

Phylogenetic analyses

Since phylogeographic breaks can be detected in the form of phylogenetic splits between mostly distinct geographical lineages (*Bagley & Johnson, 2014*), the sequences of all genes were concatenated, and a phylogenetic tree was estimated at the intraspecific level by implementing both likelihood analysis in RAxML (*Stamatakis, 2006*) and a Bayesian inference approach in MrBayes (*Ronquist & Huelsenbeck, 2003*). We rooted our phylogeny using the species *Hyalinobatrachium carlesvilai, H. mondolfii, H. chirripoi* and *H. colymbiphyllum* as outgroups. A list of the specimens and GenBank accession numbers included in this study is presented in Table S1. The best evolutionary model for each noncoding region (16S) and for the coding genes (COI and ND1) was evaluated using PartitionFinder 2 software (*Lanfear et al., 2016*). Maximum likelihood analysis

was conducted using 10,000 rapid bootstrap analyses, the GTR+Γ evolution model and summarized support for the best tree. For Bayesian inference, we ran two independent analyses for 12 million generations, sampling trees and parameter values every 1,000 generations. Burn-in was set to 25%, and the first 3 million generations were therefore discarded.

Divergence times and Bayesian spatiotemporal diffusion analyses

To estimate diversification times for the different *H. fleischmanni* mitochondrial lineages, we employed Beast 1.6.2 (*Drummond & Rambaut, 2007*). The time to the most recent common ancestor (MRCA) for the main lineages was calculated via Bayesian Markov chain Monte Carlo (MCMC) searches. The ultrametric tree was inferred *de novo* using the same partition substitution models. In the absence of a fossil record for glassfrogs, we based our analysis on previously published divergence times. We used three stem ages for *Hyalinobatrachium* species as calibration constraints, following *Castroviejo-Fisher et al. (2014)*. The most recent calibration point was placed at 2.42 Ma (confidence interval CI [1.63–3.37]), representing the divergence between *H. fleischmanni* (USNM 559092) and *H. tatayoi* (MHNLS 17174). The following calibration node was placed at 7.65 Ma (CI [5.93–9.63]), representing the divergence between *H. fleischmanni-H. tatayoi* and *H. carlesvilai*, and the most ancient calibration point corresponded to the divergence between *H. fleischmanni* and *H. mondolfii* (8.4 Ma, CI [6.68–10.52]). The calibration points on inner nodes in *Castroviejo-Fisher et al. (2014)* were based on a geological vicariance-based strategy, which requires additional precautions (*Kodandaramaiah, 2011*) when compared to the fossil-based calibration approach. For more detail, see the section on divergence time estimates and Appendix S1 in *Castroviejo-Fisher et al. (2014)*. We implemented an uncorrelated lognormal relaxed molecular clock, and trees were sampled every 1,000th iteration for 100,000,000 generations, with 20% of the initial samples being discarded as burn-in, after empirical assessment of appropriate chain convergence and mixing with Tracer 1.7 (*Rambaut et al., 2018*). We constructed the historical demography of the major clades obtained from the phylogenetic results, using Bayesian skyline plots that estimate the posterior distribution of population sizes (*Drummond et al., 2005*).

To reconstruct the ancestral distribution and spatial dispersal of the species, we performed a Bayesian spatiotemporal diffusion analysis in BEAST (v.1.8.4), assuming continuous spatial diffusion with a time-heterogeneous random walk model (''Relaxed Random Walk'', RRW, (*Lemey et al., 2010*). For this analysis, we used a subset of 34 samples with data for all three genes plus samples lacking some genes but originating from intermediate localities, encompassing the entire distribution of the species. We applied a normally distributed diffusion rate, a coalescent Bayesian Skyride model, and SRD06 substitution models (*Shapiro, Rambaut & Drummond, 2005*). We used the jitter option under the TraitLikelihood statistic with a parameter value of 0.1. To summarize the posterior distribution of ancestral ranges using the RRW model, we annotated nodes in a maximum clade credibility tree (MCC) using the program TreeAnnotator v1.7.5. This tree was then used as an input in SpreaD3 (*Bielejec et al., 2016*) to reconstruct the pattern of spatial diffusion and to visualize lineage diversification across the landscape.

Table 1 Detection of geographic barriers for *Hyalinobatrachium fleischmanni* by multiple approaches based on genetic structure. (1) Monte Carlo test of Nei's pairwise *Fst* based on 999 replicates, (2) agreement between the border maps generated with Geneland for the considered barriers, (3) degree of overlap between the two first sPCA components between adjacent populations, and (4) supported monophyletic lineages between distinct geographical regions.

Geographic barrier	Adjacent regions	F_{ST} ($n = 115$)	Geneland ($n = 60$)	sPCA ($n = 115$)	Phylogeny ($n = 115$)
Eastern Cordillera	Serranía del Perijá/Magdalena	0.0543	no agreement	complete overlap	not monophyletic
Andes Range	Magdalena/Chocó	**0.3624****	no agreement	complete overlap	not monophyletic
Central Panama Isthmus	Chocó/Central Panama	**0.3452****	no agreement	complete overlap	not monophyletic
Western Panama Isthmus	Central Panama/ North Talamanca Range	**0.7509*****	**agreement**	**no overlap**	not monophyletic
Talamanca Range	North Talamanca Range/ South Talamanca Range	**0.9071****	**agreement**	**no overlap**	**monophyletic**
Hess Escarpment	South Talamanca Range/Chortis block	0.4055	no agreement	partial overlap	not monophyletic
MPJ fault system	Chortis block/Maya block	**0.5262****	**agreement**	**no overlap**	**monophyletic**
Isthmus of Tehuantepec	Maya block/southern Mexico block	**0.3733****	**agreement**	**low overlap**	not monophyletic

Notes.
Bold values show the support of tested barriers for each analysis.
** p-value ≤ 0.01.
*** p-value ≤ 0.001.

If *H. fleischmanni* had a South American origin and subsequently dispersed to Central America after the closure of the Isthmus of Panama as we hypothesized, we would expect that the MRCA of all *H. fleischmanni* would be located in South America, and the more recent nodes would represent dispersal events into North America.

RESULTS

We generated a final alignment of 2,036 base pairs (bp) for 123 samples from 9 countries, including 13 sequences obtained from the GenBank and BOLD system databases (Table S1). The obtained sequences, representing unique haplotypes of the single genes, are available via GenBank (accession numbers: MG944443–MG944695; Supplementary Material 2). We did not detect any stop codons in protein-coding genes (COI, ND1). We obtained 25 haplotypes with an overall haplotypic diversity (h) = 0.863 and a nucleotide diversity (π) = 1.282 for the 16S gene. In contrast, we found 63 haplotypes for COI, with $h = 0.979$ and $\pi = 0.044$, and 45 haplotypes for ND1, with $h = 0.991$ and $\pi = 0.042$ (Table S1.2).

Spatial clustering analysis

The Nei's pairwise F_{ST} results showed significant differences between adjacent regions for all barriers except for the HE and the Eastern Cordillera (Table 1). The Geneland map of population membership for the concatenated genes revealed nine clusters that partially coincided with our hypothesized barriers, depicting the IT, the MPJ fault system, the Talamanca Range and the WPI as barriers. Additionally, three non-considered barriers were indicated between samples from west of the IT and on the Chortis block, between samples from both sides of the Darien and between the Ecuadorian and Colombian samples (Fig. 2A).

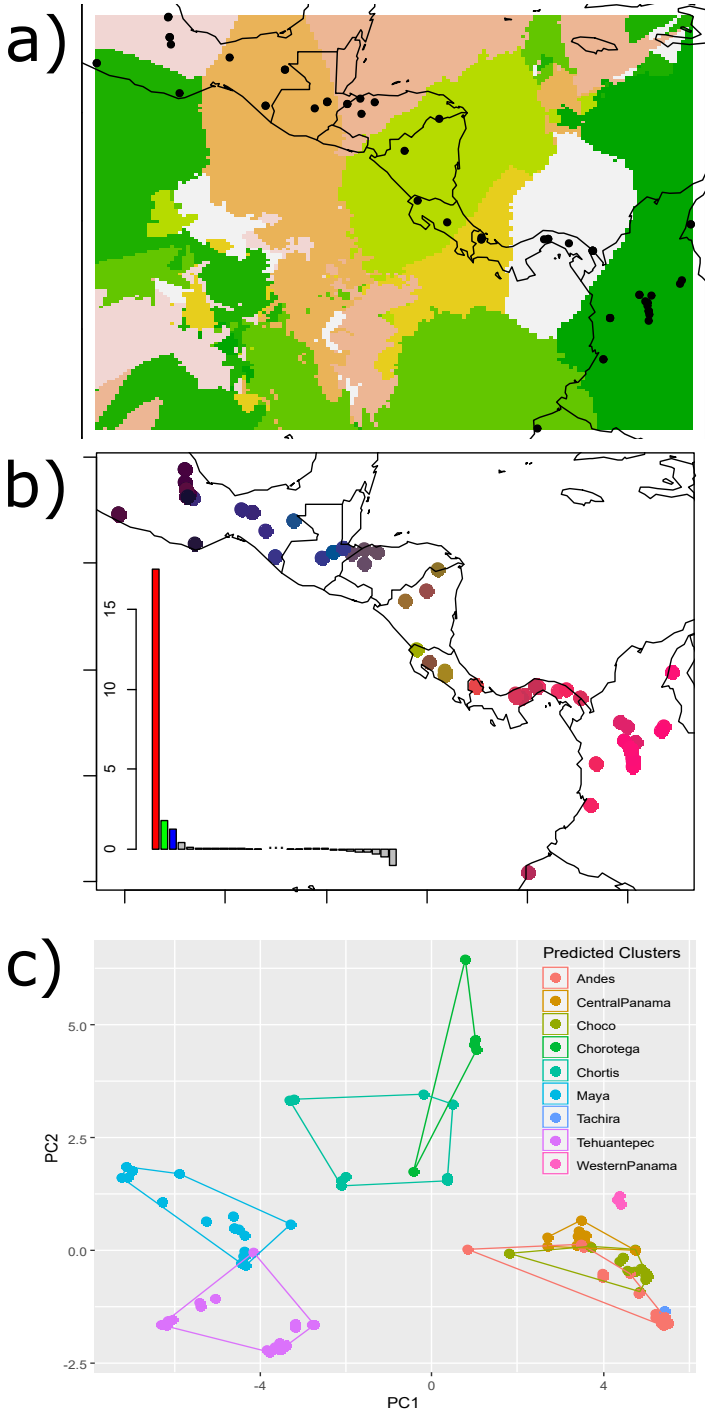

Figure 2 Results of the (A) Bayesian (Geneland) and (B–C) multivariate spatial analyses (sPCA) for *H. fleischmanni* population clustering based on mtDNA sequences. Results of the (A) Bayesian (Geneland) and (B–C) multivariate spatial analyses (sPCA) for *H. fleischmanni* population clustering based on mtDNA sequences. For the sPCA analysis, the color of each point in (B) is determined in the red-green-blue (RGB) system based on the score of each individual on the first (translated to a red channel) and second axes (translated to green) of the sPCA. The points of (C) represent the values of the two first sPCA components, and the colors indicate the predicted clusters resulting from the hypothesized barriers.

Full-size DOI: 10.7717/peerj.6115/fig-2

sPCA performed on individual genotypes revealed a significant, geographically correlated genetic structure for all three genes (nper = 999, $P = 0.001$). Eigenvalues indicated a higher spatial genetic structure for the main axis, related to the global structure. The positive axis of the first sPCA (regional scale) exhibited the greatest variation in genetic distance in relation to the distance network (Fig. 2B). The samples separated by the WPI, the Talamanca Range and the MPJ fault system did not overlap based on the two sPCA first components, while the samples separating the IT and HE exhibited partial overlap (Fig. 2C).

Phylogenetic patterns, times of divergence, and demography

The PartitionFinder output indicated that HKY+I+G, GTR+G, and GTR +G+I were the best models for 16S, COI, and ND1 respectively. The phylogenetic relationships based on the Bayesian and maximum likelihood approaches for the concatenated genes indicated three main, well-supported clades, although their relative positions were not fully resolved (Fig. 3). The first clade, designated the "Northern clade", was divided into two lineages: a large lineage containing all samples from the SMB and Maya regions (pp = 1) and a smaller one from the western Chortis region (pp = 1). The second clade, the "Central clade", was comprised of samples from the eastern Chortis and Chorotega regions (pp = 1). The third clade, the "Southern clade", consisted of samples from the Chocó and SAP regions, including the species *H. tatayoi* from Venezuela (pp = 1). The Southern clade did not show any structure, displaying a polytomic topology.

The divergence time estimation results showed a pattern of divergence among the three main clades occurring during the Pliocene (~3.40 Ma, HPD = 2.25–4.56 Ma; Fig. 3). With respect to the Northern clade, the split between the lineage from West Chortis and the remaining samples also occurred during the Pleistocene in the Gelasian age (~2.19 Ma, HPD = 1.38–3.53), while separation between samples from the Pacific and Gulf+Maya regions occurred at the beginning of the Calabrian age (~1.76 Ma, HPD = 0.86–2.23). Finally, the split between lineages from the Gulf and Maya regions occurred near the end of the Calabrian age (~1.51 Ma, HPD = 0.62–1.73). The divergence between the Central and Southern clades occurred at the end of the Pliocene (~2.64 Ma, HPD = 1.50–3.68), while splits within each clade began at the end of the Calabrian age for the Central clade (~0.81 Ma, HPD = 0.12–0.71) and during the Gelasian age (~1.64 Ma, HPD = 0.63–1.89) for the Southern clade (Fig. 3).

The 95% CIs of the effective population size (BSP results) overlapped along the entire time period in the Northern clade (Fig. 4A). However, the ancient and recent effective population sizes of the Southern clade did not overlap (by 95% CI of the Bayesian posterior probability), providing significant support for a change in population size and suggesting that the clade exhibited a constant population size and posterior expansion at approximately 0.1–0.3 Ma (Fig. 4B).

The results regarding Bayesian spatiotemporal diffusion (Fig. 5) highlight the Chorotega and West Chortis region as the most likely ancestral geographic area for *H. fleischmanni*, suggesting two subsequent dispersal events, in which the MRCA of the Northern clade was distributed in the environs of the Chortis and Maya regions, whereas that of the Southern clade (stem node) was distributed around the Chorotega and Chocó regions.

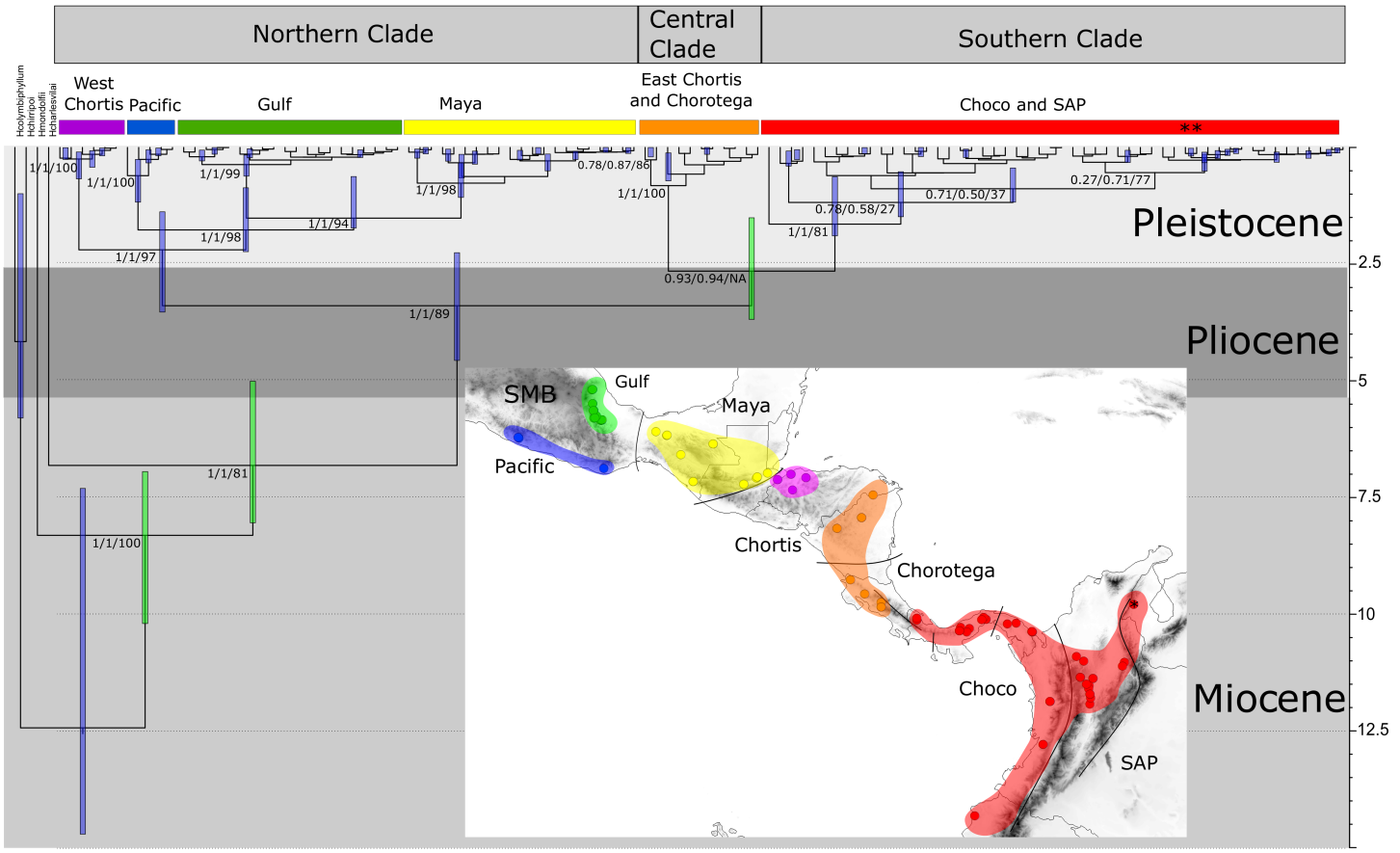

Figure 3 Time-calibrated tree of *H. fleischmanni* unique haplotypes. Time-calibrated tree of *H. fleischmanni* unique haplotypes, inferred from BEAST based on the combined ribosomal (16S) and protein-coding (COI, ND1) mitochondrial sequences, with calibration on three nodes indicated by green bars (see 'Materials and Methods' for details). Blue rectangles over key nodes indicate the 95% highest posterior densities (HPD) of the estimated times of divergence events (in Ma). Clade support is indicated by *posterior* BI values in BEAST and MrBayes and by RAxML Bootstrap analysis and is presented in this order separated by a slash. Asterisks at tips represent *H. tatayoi* samples included in the analysis. The inner map shows the geographic locations of haplotype lineages. Each color in the map coincides with the haplogroup obtained in the phylogenetic reconstruction.

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Our results also reflect independent dispersal for samples west of the MPJ fault system and later divergence of the three remaining clusters in the Northern clade around the IT. For the Central clade, an initial range in the Chorotega region south of the Talamanca range was observed, with posterior dispersal towards eastern Chortis. Furthermore, an ancestral range was detected in the Chocó block within the Southern clade with subsequent dispersal towards the south and east, reaching the lowlands east of the Andes range to the south, while two lineages dispersed northwards independently, reaching the southern limit of the Chocó region.

Genetic diversity and structure

Our results provide evidence of four of the hypothesized barriers (WPI, Talamanca Range, MPJ fault system and IT) and identified two additional barriers: one east of the IT and one within the Chortis block. Hence, we identified seven genetically homogeneous

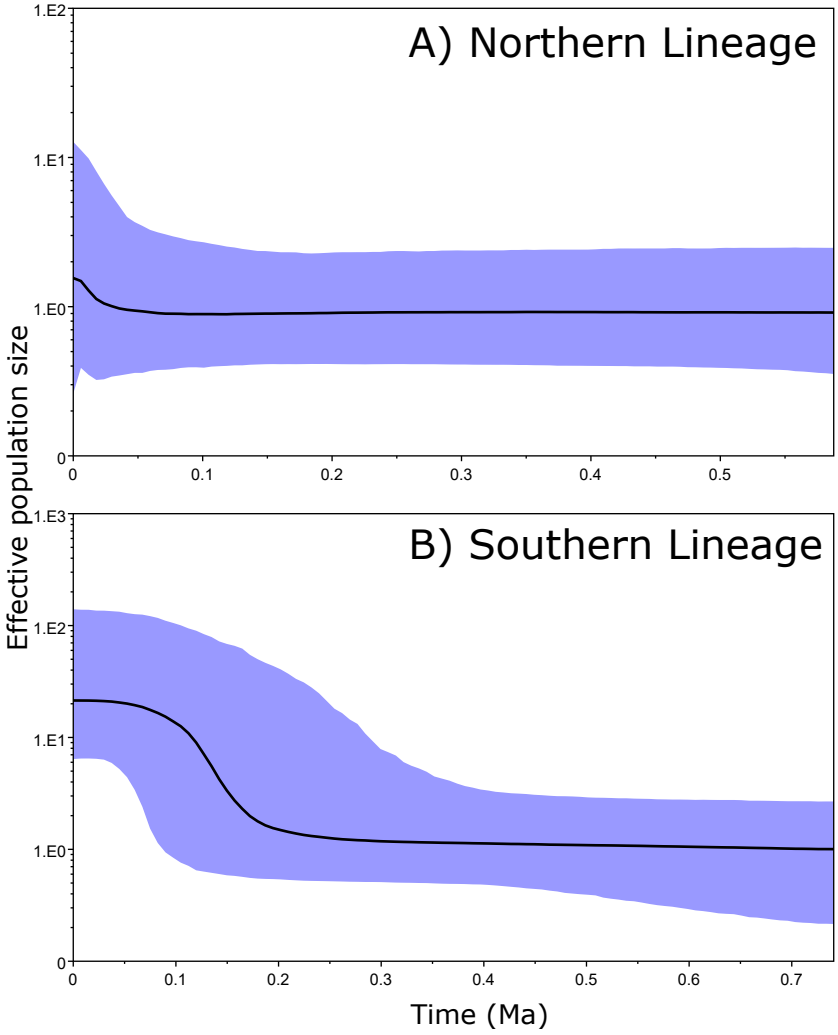

Figure 4 Bayesian skyline plots. Bayesian skyline plots for Northern (A) and Southern (B) clades generated through phylogenetic reconstruction.

Full-size DOI: 10.7717/peerj.6115/fig-4

regions: North American Pacific, Gulf of Mexico, Maya block, western Chortis, eastern Chortis-South Talamanca Range, North Talamanca Range and Chocó-SAP. A minimum genetic distance (Table 2) was exhibited between regions for North Talamanca Range and Chocó-SAP (K2P = 1.6%), while the maximum was observed between eastern Chortis and North Talamanca Range (K2P = 6.0%). Significant Nei's F_{ST} indices between regions were obtained for almost all combinations (F_{ST} >0.4), except between North Talamanca Range and Chocó-SAP (Table 2).

DISCUSSION

The complex geologic and geographic history of Central America has long intrigued researchers, who have aimed to decipher how different features that act as barriers to or corridors for dispersal have affected the distribution and diversity of multiple taxa

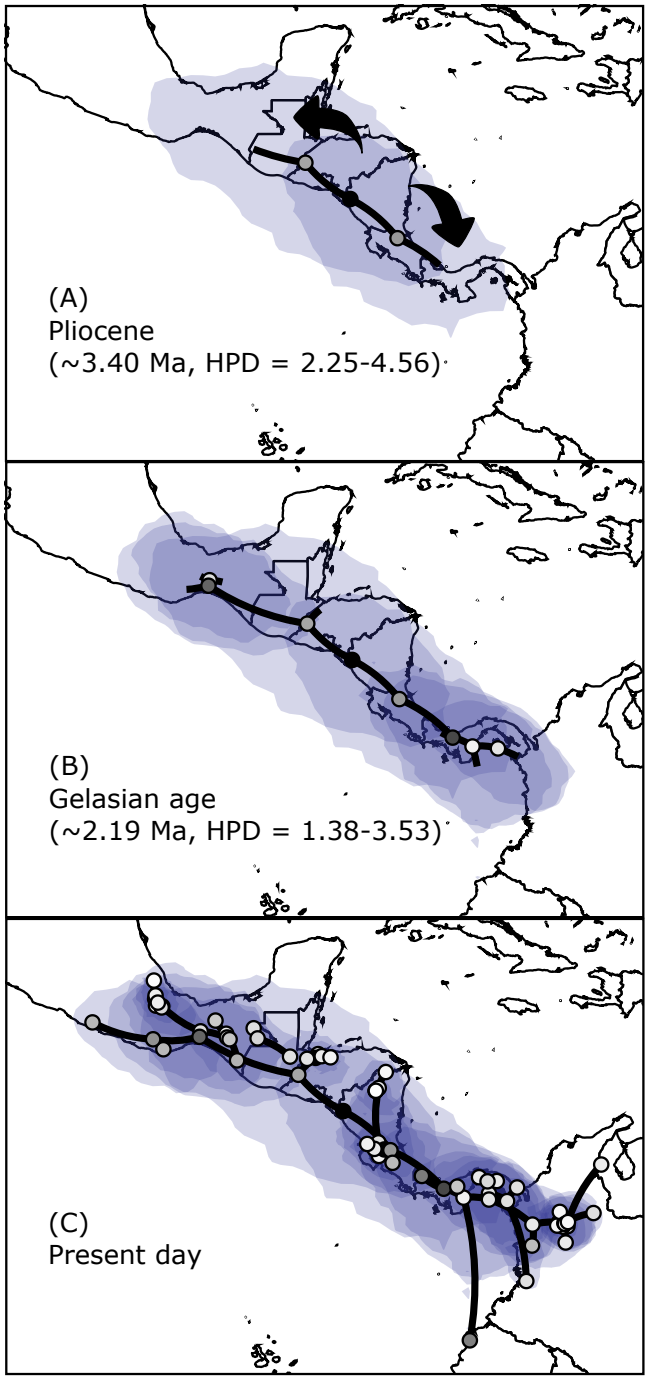

Figure 5 Spatial projection of the Bayesian spatiotemporal diffusion analysis of *H. fleischmanni*. Spatial projection of the Bayesian spatiotemporal diffusion analysis of *H. fleischmanni* lineages for three time-points, based on the maximum clade credibility (MCC) tree estimated with a "Relaxed Random Walk" model. Lines represent branches of the MCC tree; shaded areas indicate the 95%-HPD uncertainty for the ancestral branches; the shading gradient indicates older (lighter) versus younger (darker) events; and dot color represents the ages of older nodes (darker) and younger tips (lighter).

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Table 2 Mean K2P distances within and among populations (lower) for all tree mitochondrial genes and Nei's pairwise F_{ST} indices per pair of populations (upper). Significance of F_{ST} indices was estimated by a Monte Carlo test based on 999 permutations.

	K2P Mean Within distance	Chorotega	Chortis	Gulf	Maya	Pacific	South America	Western Panama
Chorotega	0.005	–	0.922***	0.845***	0.766***	0.879***	0.452**	0.886***
Chortis	0.004	0.048	–	0.801***	0.687***	0.876***	0.432**	0.950***
Gulf	0.005	0.046	0.04	–	0.545**	0.635***	0.659***	0.815**
Maya	0.009	0.049	0.045	0.023	–	0.471**	0.648**	0.694***
Pacific	0.004	0.047	0.036	0.022	0.023	–	0.413*	0.848***
South America	0.009	0.035	0.057	0.046	0.051	0.043	–	0.138 ns
Western Panama	0.004	0.037	0.060	0.049	0.054	0.046	0.016	–

Notes.
ns, non-significant; *, p-value <0.05; **, p-value ≤ 0.01; ***, p-value ≤ 0.001.

(*Gutiérrez-García & Vázquez-Domínguez, 2013*; *Bagley & Johnson, 2014*). Our results show a deep phylogenetic structure of *H. fleischmanni*, which has differentiated as three well-supported clades, revealing old divergence events dating back to the Pliocene and younger divergence events within clades during the Pleistocene (Fig. 3). Additionally, our results agree with those of previous studies showing *H. fleischmanni* to be paraphyletic with *H. tatayoi* (*Guayasamin et al., 2008*; *Castroviejo-Fisher et al., 2011*; *Delia, Bravo-Valencia & Warkentin, 2017*), and we found no differences between the *H. tatayoi* and the *H. fleischmanni* samples within the South American clade.

The Southern clade

The Southern clade encompasses samples from the Chocó and SAP regions, including the species *H. tatayoi* from Venezuela. Samples ranging from Panama to Venezuela and Ecuador grouped together, with no clear phylogenetic separation among them. However, haplotype networks and spatial clustering analyses for coding genes allowed us to identify a partition on both sides of the Northern Andes (Supplemental Information 1 and Fig. S3). The lack of significant structure for the Southern clade is remarkable, considering that the geographic distances between populations reach 1,600 km. In addition, the CPI and the Northern Andes, which are widely recognized as speciation drivers for both highland and lowland species (*Bagley & Johnson, 2014*; *Mendoza et al., 2015*), do not seem to have exerted any effect on the genetic structure of this clade. Notably, the genetic distance observed on both sides of the Northern Andes (COI K2P = 1.9%; Table S1.1) is lower than the distances reported for lowland species with a higher dispersal capacity from similar localities, such as the hummingbird *Amazilia amabilis* (COI K2P = 2.06%, *Mendoza et al., 2016*). These results contrast with previous knowledge of the ecology of glassfrog species, which have been found to be characterized by site fidelity (*Valencia-Aguilar, Castro-Herrera & Ramírez-Pinilla, 2012*), low mobility and restricted gene flow even at local scales (*Delia, Bravo-Valencia & McDiarmid, 2017*; *Robertson, Lips & Heist, 2008*). However, most of the previous research on this frog has focused on calling males and reproductive behavior (*Delia, Bravo-Valencia & McDiarmid, 2017*), while the dispersal capability of females and tadpoles, which can have a significant impact on mtDNA genetic structure, is still unknown.

Thus, it is possible that the Chocó and SAP regions present adequate conditions for tadpole dispersal, allowing range expansion. However, this hypothesis needs to be evaluated based on additional demographic studies and a greater sample size per site.

Our results do not support our initial hypothesis that the species was originally from South America and then dispersed through the Isthmus of Panama. Indeed, the Southern clade is rather young, and the various populations it encompasses differentiated during the last million years (middle Pleistocene). Our results show that this clade has experienced a recent population expansion during the last 100,000–300,000 years, reaching a relatively final stable population size, exhibiting a dispersal route from Central Panama to South America (Fig. 5). Based on the genetic and phylogenetic results for this clade, we suggest that its dispersal towards South America and on both sides of the Northern Andes occurred very recently, likely as a consequence of climatic oscillations during glacial periods (*Smith, Amei & Klicka, 2012*). Under this scenario, there has been insufficient time for effective genetic differentiation to occur, and the phylogenetic reconstruction therefore failed to resolve the divergence detected in the spatial analysis.

In particular, the lack of differentiation between sequences from east of Northern Andes (in the Magdalena Valley) and *H. tatayoi* in Serranía del Perijá is remarkable: sequences were identical with extremely low divergence ($F_{ST} = 0.0543$, Table 1), supporting a recent dispersal through the Eastern Cordillera. In contrast, we detected a slight structure for the Ecuadorian record (Geneland; Fig. 2B) based on a single specimen (QCAZ-22303). We acknowledge that a more detailed genetic structure analysis of the Colombian and Ecuadorian populations is needed, including samples from the southern departments in Colombia, and the use of additional genetic markers (e.g., microsatellites or SNPs) is suggested.

The Northern clade

Unlike the Southern clade, the Northern clade shows significant genetic structure in four different lineages, ranging from western Chortis (Central America) to lowland forests in Veracruz and Guerrero (Mexico). One remarkable finding was the split between samples from either side of the MPJ fault system, where individuals separated by only 60 km exhibit great genetic distances (K2P = 4.5% for all three genes, Table 1), even reaching the limit of the 6% barcode gap for Neotropical amphibians in COI (5.2% for COI, Table S1.2; *Lyra, Haddad & Azeredo-Espin, 2017*). The calibration results showed that samples from these localities have been isolated since the Gelasian age (early Pleistocene; ~2.19 Ma, HPD = 1.38–3.53). The MPJ fault system has been recognized as the main barrier to dispersal for multiple species ranging from the Maya to the Chortis blocks (*Ornelas, Ruiz-Sánchez & Sosa, 2010*; *Barrera-Guzmán et al., 2012*; *Rovito et al., 2015*). Our results indeed support the hypothesis that MPJ has effectively acted as a barrier for *H. fleischmanni* dispersal.

On the other hand, the Geneland and phylogenetic results showed a more complex scenario, in agreement with the presence of three well-defined lineages within the Northern clade, one of which is located in the north of the SMB, another southward the SMB, and the last in the Maya block (Fig. 2B). This genetic structure is very similar to that observed for

the brush-finch *Arremon brunneinucha*, distributed in humid montane forests (*Navarro-Sigüenza et al., 2008*). Samples from the Gulf of Mexico and Pacific are clearly isolated by the highlands of the Sierra Madre Oriental and the Sierra Madre Occidental (Fig. 1), indicating a divergence pattern that is frequently detected for lowland species (*Mulcahy, Morrill & Mendelson, 2006*; *Rivera-Ortíz et al., 2016*) and species groups (*Streicher et al., 2014*; *Palacios et al., 2016*).

Within this clade, we found that samples from SMB do not group as a single lineage but instead display a paraphyletic position in relation to the lineage from the Maya region. These two regions present the lowest K2P among all comparisons performed in this analysis, except for samples separated by the WPI. Importantly, unlike the known impact of the WPI and CPI on lowland species, the effect of the IT has mostly been defined in relation to montane species (*Bryson, García-Vázquez & Riddle, 2011*; *Jiménez & Ornelas, 2016*), for which it represents a dispersal-limiting barrier. Currently, the IT is occupied by dry, scrubby coastal plains that are very different from the moist areas on either side (*Rodríguez-Gómez, Gutiérrez-Rodríguez & Ornelas, 2013*), so one plausible explanation for this finding is that the dry forests along the IT did not always act as a barrier to *H. fleischmanni*. Instead, considering that those clades were likely isolated between 1.76 to 2.19 Ma (HPD = 0.86–3.53), it is likely that the successive glacial cycles during the Pleistocene could have enabled the montane forests to reach lower elevations through the impact of climate change (*Barber & Klicka, 2010*), thus creating a temporal corridor and allowing the species to disperse through the region. Hence, for species like *H. fleischmanni*, the IT probably acted as both a corridor and a barrier, which is supported by our Bayesian spatiotemporal diffusion results (Fig. 5).

The Central clade

Our results revealed a Central clade without any deep geographic structure expanding across the HE and separated from the Southern clade around the end of the Pliocene (~2.64 Ma). We did not find any evidence suggesting differentiation between samples from the Chortis and Chorotega regions, leading us to reject the hypothesis that the HE acted as a barrier. However, we must consider the small sample sizes from Honduras and Nicaragua ($n = 10$ samples), which likely limits detailed structural evaluation for this region. Most phylogeographic studies performed in the region known as nuclear Central America face similar problems, with limited or null sampling from northern Honduras (*Castoe et al., 2003*; *Crawford & Smith, 2005*; *Mulcahy, Morrill & Mendelson, 2006*) or sampling that is biased towards the dry forests of the Pacific coast (*Parkinson, Zamudio & Greene, 2000*; *Hasbún et al., 2005*; *Vázquez-Miranda, Navarro-Sigüenza & Omland, 2009*; *Poelchau & Hamrick, 2011*), where *H. fleischmanni* has not been recorded. Our Bayesian spatiotemporal diffusion results showed rapid dispersal from the Chorotega to Chortis blocks, with no apparent impact on the genetic structure of these populations (Fig. 5). Nevertheless, additional work is needed to confirm whether the main geographic features present in this region have driven the dispersal of low-mobility species such as *H. fleischmanni* in humid forests.

Phylogeographic patterns

The three main clades that we identified for *H. fleischmanni* show deep intraspecific divergence, with genetic distance values (K2P) greater than 3% (Table 2). Indeed, the landscape analysis, Bayesian spatiotemporal diffusion analyses, and estimated divergence times revealed interesting patterns that allowed us to reconstruct the historical biogeography of these frogs and identify the impact of different geographic barriers on the genetic structure and phylogeographic patterns of *H. fleischmanni*. Although the main phylogenetic topology and the three major clades were well supported in Bayesian analyses, the maximum likelihood phylogenetic reconstruction did not resolve these relationships (see Supplemental Information 1).

The Bayesian spatiotemporal diffusion analysis did not support the hypothesis proposed by *Castroviejo-Fisher et al. (2014)* of a South American origin and subsequent dispersal to Central America. In contrast, *H. fleischmanni* appears to have originated in the region encompassing the Chorotega and eastern Chortis elements. Interestingly, we found that *H. fleischmanni* has undergone two dispersal events: one southwards towards the Chocó region and one northwards, reaching the Maya region, followed by vicariance events driven by the effect of the Chortis highlands and the Talamanca Range. Considering that all species that are closely related to *H. fleischmanni* are endemic to South America and that the divergence times among the three clades are similar (i.e., the isolation of the Northern clade occurred ca. 3.40 Ma (HPD = 2.25–4.56 Ma), while that between the two other clades occurred 2.64 Ma (HPD = 1.50–3.68)), it is likely that the MRCA ancestor of *H. fleischmanni* and *H. carlesvilai* was located in South America around 7.65 Ma (CI = 5.93–9.63). Later, the ancestor of *H. fleischmanni* arrived in Central America during the Pliocene, soon after the closure of the Isthmus of Panama (*Montes et al., 2015*), whereas the current South American populations are descendants of a second migration from Central to South America. Accordingly, the dispersal-vicariance events among the main clades potentially occurred simultaneously or over a very short time, which might explain why the position of the clades and their internal structure were not consistent between the Bayesian and maximum likelihood approaches.

Regarding the vicariance events for the Central and Southern clades, multiple elements need to be revised. The samples from each cluster that are geographically closest are located on opposite sides of the Talamanca Cordillera in the Chorotega block. The time of divergence of the MRCA for these clades (3.28 Ma, HPD = 1.59–3.86) coincides with the estimated age of the intervening mountains (1–2 Ma; *Denyer, Alvarado & Aguilar, 2000*; *Marshall et al., 2003*, which are recognized as a main driver of speciation (*Savage, 2002*). The time of divergence also coincides with the rise of the sea level during the mid-late Pliocene (~3.5–3 Ma), which generated a seaway, likely reinforcing the WPI break and therefore acting as a barrier across the Pacific region (*Cronin & Dowsett, 1996*; *Bagley, Hickerson & Johnson, 2018*). Hence, the central mountain ranges on Costa Rica and Panama and the eustatic sea levels around the WPI might have increased divergence, as documented for multiple spatial divergence patterns of amphibian species (*Crawford, Bermingham & Polanía-S, 2007*; *Wang, Crawford & Bermingham, 2008*; *Bagley, Hickerson & Johnson, 2018*).

The isolation of the Northern clade from the other two does not entirely correspond to our hypothesis of geographical barriers. Populations from both the Northern and Central clades are distributed throughout the Chortis block, indicating that the MPJ fault system was not the main driver of divergence between clades. On the other hand, our structure (Geneland) results suggest a frontier at the center of the Chortis block, near northeastern Honduras (Fig. 2B). Similar divergence patterns have been observed between two water-dependent subspecies of *Caiman crocodilus* (*Venegas-Anaya et al., 2008*), in agreement with the eastern limit of the Chortis highlands (*Morrone, 2014*; *Townsend, 2014*). Here, the complex topography resulting from multiple volcanic activities along the Chortis highlands during the last 2 Ma and the presence of dry habitats in the Pacific region (*Savage, 2002*) may have isolated the *H. fleischmanni* populations during the late Pliocene. This hypothesis is in agreement with the high species endemism recognized for the region (*Anderson et al., 2010*; *Townsend et al., 2012*), in which intensive study is required to evaluate the underestimated regional taxonomic diversity (*Townsend & Wilson, 2016*).

Taxonomic implications

Previous studies have suggested that *H. fleischmanni* is a paraphyletic species in relation to its sister species *H. tatayoi* (*Castroviejo-Fisher et al., 2011*; *Delia, Bravo-Valencia & Warkentin, 2017*). Here, we confirm the paraphyly of the species, as the *H. tatayoi* samples are grouped within the Southern clade, lacking significant genetic differences from the western Andes samples. Furthermore, our overall Bayesian topology agrees with the results obtained by *Delia, Bravo-Valencia & Warkentin (2017)* for 12S sequences. We identified three main isolated lineages with large genetic distances that likely include cryptic diversity within Central America. To confirm the existence of cryptic—and potential candidate—species, different lines of evidence must be obtained; in fact, we are conducting a follow-up study of the integrative taxonomy of this species complex that includes genetic, morphologic and acoustic data (Mendoza et al., 2018, unpublished data) to describe lineage divergence and determine the identities and geographic distributions of all valid species (*Padial et al., 2010*).

CONCLUSIONS

We have conducted the most comprehensive analysis of genetic variation and divergence within *H. fleischmanni* to date, producing one of the few phylogenetic and phylogeographic studies for glassfrogs, with the exception of a few studies from Guyana (*Castroviejo-Fisher et al., 2011*; *Jowers et al., 2015*), and this is the first such study of a Central American species. Moreover, our results aided in the successful reconstruction of the historical biogeography of these frogs and dispersal and vicariance events during the history *H. fleischmanni* lineages, revealing a higher complexity for the species than expected, especially for the Northern lineage, in which significant population structure was found. Indeed, our results support the Talamanca range, the MPJ fault system, and the Chortis highlands as significant factors exerting effects on the dispersal of lowland amphibians during the late Pliocene and early Pleistocene. Additionally, we suggest that the IT acted as both a corridor and a barrier for *H. fleischmanni* during the early Pleistocene, while the HE and the Andes Range

did not act as significant barriers. The complementary use of phylogenetic and landscape analyses allowed us to perform an adequate evaluation of dispersal patterns and potential barriers within this region; hence, our approach can be applied in biogeographic and phylogeographic studies of different taxa.

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Competing Interests

Gabriela Parra Olea is an Academic Editor for PeerJ.

Author Contributions

- Angela M. Mendoza conceived and designed the experiments, performed the experiments, analyzed the data, contributed reagents/materials/analysis tools, prepared figures and/or tables, authored or reviewed drafts of the paper, approved the final draft.
- Wilmar Bolívar-García and Roberto Ibáñez contributed reagents/materials/analysis tools, authored or reviewed drafts of the paper, approved the final draft.
- Ella Vázquez-Domínguez and Gabriela Parra Olea conceived and designed the experiments, contributed reagents/materials/analysis tools, authored or reviewed drafts of the paper, approved the final draft.

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Data Availability

The following information was supplied regarding data availability:

Sequences are provided in Table S1.

Supplemental Information

Supplemental information for this article can be found online at http://dx.doi.org/10.7717/peerj.6115#supplemental-information.

REFERENCES

Anderson DL, House P, Hyman RE, Steiner R, Hawkins HR, Thorn S, Rey MJ, Espinal MR, Marineros LE. 2010. Rediscovery of the Honduran Emerald *Amazilia luciae* in western Honduras: insights on the distribution, ecology, and conservation of a 'Critically Endangered' hummingbird. *Bird Conservation International* **20(3)**:255–262 DOI 10.1017/S0959270910000389.

Arbeláez-Cortés E, Nyári ÁS, Navarro-Sigüenza AG. 2010. The differential effect of lowlands on the phylogeographic pattern of a Mesoamerican montane species (*Lepidocolaptes affinis*, Aves: Furnariidae). *Molecular Phylogenetics and Evolution* **57(2)**:658–668 DOI 10.1016/j.ympev.2010.06.013.

Bagley JC, Hickerson MJ, Johnson JB. 2018. Testing hypotheses of diversification in Panamanian frogs and freshwater fishes using hierarchical approximate Bayesian computation with model averaging. *PeerJ Preprints* **6**:e26623v1 DOI 10.7287/peerj.preprints.26623v1.

Bagley JC, Johnson JB. 2014. Phylogeography and biogeography of the lower Central American Neotropics: diversification between two continents and between two seas. *Biological Reviews* **89(4)**:767–790 DOI 10.1111/brv.12076.

Barber BR, Klicka J. 2010. Two pulses of diversification across the Isthmus of Tehuantepec in a montane Mexican bird fauna. *Proceedings of the Royal Society of London B: Biological Sciences* **277**:2675–2681 DOI 10.1098/rspb.2010.0343.

Barrera-Guzmán AO, Milá B, Sánchez-González LA, Navarro-Sigüenza AG. 2012. Speciation in an avian complex endemic to the mountains of Middle America (*Ergaticus*, ves: Parulidae). *Molecular Phylogenetics and Evolution* **62(3)**:907–920 DOI 10.1016/j.ympev.2011.11.020.

Barrera-Rodríguez M. 2000. Estudio Anatómico de cuatro especies de ranitas de cristal del género *Hyalinobatrachium* Ruíz and Lynch 1991 grupo *fleischmanni* (Amphibia: Anura: Centrolenidae). *Revista de la Academia Colombiana de Ciencias Exactas, Físicas y Naturales* **23**:245–260.

Beebee TJC. 2005. Conservation genetics of amphibians. *Heredity* **95(6)**:423–427 DOI 10.1038/sj.hdy.6800736.

Bielejec F, Baele G, Vrancken B, Suchard MA, Rambaut A, Lemey P. 2016. SpreaD3: interactive visualisation of spatiotemporal history and trait evolutionary processes. *Molecular Biology and Evolution* **33(8)**:2167–2169 DOI 10.1093/molbev/msw082.

Bryson RW, García-Vázquez UO, Riddle BR. 2011. Phylogeography of Middle American gophersnakes: mixed responses to biogeographical barriers across the Mexican Transition Zone. *Journal of Biogeography* **38(8)**:1570–1584 DOI 10.1111/j.1365-2699.2011.02508.x.

Cadena CD, Klicka J, Ricklefs RE. 2007. Evolutionary differentiation in the Neotropical montane region: molecular phylogenetics and phylogeography of *Buarremon* brushfinches (Aves-Emberizidae). *Molecular Phylogenetics and Evolution* **44(3)**:993–1016 DOI 10.1016/j.ympev.2006.12.012.

Castoe TA, Chippindale PT, Campbell JA, Ammerman LK, Parkinson CL. 2003. Molecular systematic of the middle American jumping pitvipers (genus *Atropoides*) and phylogeography of the *Atropoides nummifer* complex. *Herpetologica* **59(3)**:421–432 DOI 10.1655/01-105.2.

Castroviejo-Fisher S, Ayarzagüena J, Vila C. 2007. A new species of *Hyalinobatrachium* (Centrolenidae: Anura) from Serrania de Perijá, Venezuela. *Zootaxa* **1441(1)**:51–62 DOI 10.11646/zootaxa.1441.1.4.

Castroviejo-Fisher S, Guayasamin JM, Gonzalez-Voyer A, Vila C. 2014. Neotropical diversification seen through glassfrogs. *Journal of Biogeography* **41(1)**:66–80 DOI 10.1111/jbi.12208.

Castroviejo-Fisher S, Vila C, Ayarzagueena J, Blanc M, Ernst R. 2011. Species diversity of *Hyalinobatrachium* glassfrogs (Amphibia: Centrolenidae) from the Guiana Shield, with the description of two new species. *Zootaxa* **3132**:1–55 DOI 10.5281/zenodo.200895.

Cavender-Bares J, González-Rodríguez A, Pahlich A, Koehler K, Deacon N. 2011. Phylogeography and climatic niche evolution in live oaks (*Quercus* series Virentes) from the tropics to the temperate zone. *Journal of Biogeography* **38(5)**:962–981 DOI 10.1111/j.1365-2699.2010.02451.x.

Cavers S, Navarro C, Lowe AJ. 2003. Chloroplast DNA phylogeography reveals colonization history of a Neotropical tree, *Cedrela odorata* L., in Mesoamerica. *Molecular Ecology* **12(6)**:1451–1460 DOI 10.1046/j.1365-294X.2003.01810.x.

Cox CB, Ladle R, Moore PD. 2016. *Biogeography: an ecological and evolutionary approach*. Oxford: Blackwell Science Ltd.

Crawford AJ, Bermingham E, Polanía-S C. 2007. The role of tropical dry forest as a long-term barrier to dispersal: a comparative phylogeographical analysis of dry forest tolerant and intolerant frogs. *Molecular Ecology* **16(22)**:4789–4807 DOI 10.1111/j.1365-294X.2007.03524.x.

Crawford AJ, Smith EN. 2005. Cenozoic biogeography and evolution in direct-developing frogs of Central America (Leptodactylidae: *Eleutherodactylus*) as inferred from a phylogenetic analysis of nuclear and mitochondrial genes. *Molecular Phylogenetics and Evolution* **35(3)**:536–555 DOI 10.1016/j.ympev.2005.03.006.

Cronin TM, Dowsett HJ. 1996. Biotic and oceanographic response to the Pliocene closing of the Central American Isthmus. In: Jackson JBC, Budd AF, Coates A, eds. *Evolution and environment in tropical America*. Chicago: The University of Chicago Press, 76–104.

Cruz FK, Urgiles VL, Sánchez-Nivicela JC, Siddons DC, Cisneros-Heredia D. 2017. Southernmost records of *Hyalinobatrachium fleischmanni* (Anura: Centrolenidae). *Check List* **13(4)**:67–70 DOI 10.15560/13.4.67.

Daza JM, Castoe TA, Parkinson CL. 2010. Using regional comparative phylogeographic data from snake lineages to infer historical processes in Middle America. *Ecography* **33(2)**:343–354.

Delia J, Bravo-Valencia L, McDiarmid RW. 2017. Notes on paternal behavior in *Hyalinobatrachium* glassfrogs (Anura: Centrolenidae). *Phyllomedusa* **16(1)**:101–107 DOI 10.11606/issn.2316-9079.v16i1p101-107.

Delia J, Bravo-Valencia L, Warkentin KM. 2017. Patterns of parental care in Neotropical glassfrogs: fieldwork alters hypotheses of sex-role evolution. *Journal of Evolutionary Biology* **30(5)**:898–914 DOI 10.1111/jeb.13059.

Delia J, Cisneros-Heredia DF, Whitney J, Murrieta-Galindo R. 2010. Observations on the reproductive behavior of a Neotropical glassfrog, *Hyalinobatrachium fleischmanni* (Anura: Centrolenidae). *South American Journal of Herpetology* **5(1)**:1–12 DOI 10.2994/057.005.0101.

Denyer P, Alvarado GE, Aguilar T. 2000. Historia Geológica. In: Denyer P, Kussmaul S, eds. *Geología de Costa Rica*. San José: Editorial Tecnológica de Costa Rica, 155–167.

Drummond AJ, Rambaut A. 2007. BEAST: Bayesian evolutionary analysis by sampling trees. *BMC Evolutionary Biology* **7(1)**:214 DOI 10.1186/1471-2148-7-214.

Drummond AJ, Rambaut A, Shapiro B, Pybus OG. 2005. Bayesian coalescent inference of past population dynamics from molecular sequences. *Molecular Biology and Evolution* **22(5)**:1185–1192 DOI 10.1093/molbev/msi103.

Dudoit AA, Iacchei M, Coleman RR, Gaither MR, Browne WE, Bowen BW, Toonen RJ. 2018. The little shrimp that could: phylogeography of the circumtropical Stenopus

hispidus (Crustacea: Decapoda), reveals divergent Atlantic and Pacific lineages. *PeerJ* **6**:e4409 DOI 10.7717/peerj.4409.

Eizirik E, Kim JH, Menotti-Raymond M, Crawshaw Jr PG, O'Brien SJ, Johnson WE. 2001. Phylogeography, population history and conservation genetics of jaguars (*Panthera onca*, Mammalia, Felidae). *Molecular Ecology* **10**(**1**):65–79 DOI 10.1046/j.1365-294X.2001.01144.x.

García-Moreno J, Navarro-Sigüenza AG, Peterson AT, Sánchez-González LA. 2004. Genetic variation coincides with geographic structure in the common bush-tanager (*Chlorospingus ophthalmicus*) complex from Mexico. *Molecular Phylogenetics and Evolution* **33**(**1**):186–196 DOI 10.1016/j.ympev.2004.05.007.

Gehara M, Crawford AJ, Orrico VGD, Rodríguez A, Lötters S, Fouquet A, Barrientos LS, Brusquetti F, De La Riva I, Ernst R, Urrutia GG, Glaw F, Guayasamin JM, Hölting M, Jansen M, Kok PJR, Kwet A, Lingnau R, Lyra M, Moravec J, Pombal JP, Rojas-Runjaic FJM, Schulze A, Señaris JC, Solé M, Rodrigues MT, Twomey E, Haddad CFB, Vences M, Köhler J. 2014. High levels of diversity uncovered in a widespread nominal taxon: continental phylogeography of the neotropical tree frog *Dendropsophus minutus*. *PLOS ONE* **9**:e103958 DOI 10.1371/journal.pone.0103958.

Goudet J. 2005. hierfstat, a package for r to compute and test hierarchical F-statistics. *Molecular Ecology Notes* **5**(**1**):184–186.

Guayasamin JM, Castroviejo-Fisher S, Ayarzagüena J, Trueb L, Vilà C. 2008. Phylogenetic relationships of glassfrogs (Centrolenidae) based on mitochondrial and nuclear genes. *Molecular Phylogenetics and Evolution* **48**(**2**):574–595 DOI 10.1016/j.ympev.2008.04.012.

Guayasamin JM, Castroviejo-Fisher S, Trueb L, Ayarzagüena J, Rada M, Vilá C. 2009. Phylogenetic systematics of Glassfrogs (Amphibia: Centrolenidae) and their sister taxon *Allophryne ruthveni*. *Zootaxa* **2100**:1–97.

Guillot G, Mortier F, Estoup A. 2005. GENELAND: a computer package for landscape genetics. *Molecular Ecology Notes* **5**(**3**):712–715 DOI 10.1111/j.1471-8286.2005.01031.x.

Gutiérrez-García TA, Vázquez-Domínguez E. 2013. Consensus between genes and stones in the biogeographic and evolutionary history of Central America. *Quaternary Research* **79**(**3**):311–324 DOI 10.1016/j.yqres.2012.12.007.

Hasbún CR, Gómez A, Köhler GG, Lunt DH. 2005. MitochondrialDNA phylogeography of the Mesoamerican spiny-tailed lizards (*Ctenosaura quinquecarinata* complex): historical biogeography, species status and conservation. *Molecular Ecology* **14**(**10**):3095–3107 DOI 10.1111/j.1365-294X.2005.02665.x.

Hauswaldt JS, Ludewig AK, Vences M, Pröhl H. 2011. Widespread co-occurrence of divergent mitochondrial haplotype lineages in a Central American species of poison frog (*Oophaga pumilio*). *Journal of Biogeography* **38**(**4**):711–726 DOI 10.1111/j.1365-2699.2010.02438.x.

Iturralde-Vinent MA. 2006. Meso-Cenozoic Caribbean paleogeography: implications for the historical biogeography of the region. *International Geology Review* **48**(**9**):791–827 DOI 10.2747/0020-6814.48.9.791.

Jiménez RA, Ornelas JF. 2016. Historical and current introgression in a Mesoamerican hummingbird species complex: a biogeographic perspective. *PeerJ* **4**:e1556 DOI 10.7717/peerj.1556.

Jombart T. 2008b. adegenet: a R package for the multivariate analysis of genetic markers. *Bioinformatics* **24(11)**:1403–1405 DOI 10.1093/bioinformatics/btn129.

Jombart T, Devillard S, Balloux F. 2010. Discriminant analysis of principal components: a new method for the analysis of genetically structured populations. *BMC Genetics* **11(1)**:94.

Jombart T, Devillard S, Dufour A-B, Pontier D. 2008a. Revealing cryptic spatial patterns in genetic variability by a new multivariate method. *Heredity* **101(1)**:92–103 DOI 10.1038/hdy.2008.34.

Jowers MJ, Lehtinen RM, Downie RJ, Georgiadis AP, Murphy JC. 2015. Molecular phylogenetics of the glass frog *Hyalinobatrachium orientale* (Anura: Centrolenidae): evidence for Pliocene connections between mainland Venezuela and the island of Tobago. *Mitochondrial DNA* **26(4)**:613–618 DOI 10.3109/19401736.2014.880888.

Kodandaramaiah U. 2011. Tectonic calibrations in molecular dating. *Current Zoology* **57(1)**:116–124 DOI 10.1093/czoolo/57.1.116.

Lait LA, Hebert PD. 2018. Phylogeographic structure in three North American tent caterpillar species (Lepidoptera: Lasiocampidae): *Malacosoma americana*, *M. californica*, and *M. disstria*. *PeerJ* **6**:e4479 DOI 10.7717/peerj.4479.

Lanfear R, Frandsen P, Wright AM, Senfeld T, Calcott B. 2016. PartitionFinder 2: new methods for selecting partitioned models of evolution for molecular and morphological phylogenetic analyses. *Molecular Biology and Evolution* **34(3)**:772–773 DOI 10.1093/molbev/msw260.

Lemey P, Rambaut A, Welch JJ, Suchard MA. 2010. Phylogeography takes a relaxed random walk in continuous space and time. *Molecular Biology and Evolution* **27(8)**:1877–1885 DOI 10.1093/molbev/msq067.

Lyra ML, Haddad CF, Azeredo-Espin AML. 2017. Meeting the challenge of DNA barcoding Neotropical amphibians: polymerase chain reaction optimization and new COI primers. *Molecular Ecology Resources* **17(5)**:966–980 DOI 10.1111/1755-0998.12648.

Marshall JS. 2007. The geomorphology and physiographic provinces of Central America. *Central America: Geology, Resources and Hazards* **1**:75–121 DOI 10.1201/9780203947043.pt2.

Marshall JS, Idleman BD, Gardner TW, Fisher DM. 2003. Landscape evolution within a retreating volcanic arc, Costa Rica, Central America. *Geology* **31(5)**:419–422 DOI 10.1130/0091-7613(2003)031<0419:LEWARV>2.0.CO;2.

Marshall LG, Webb SD, Sepkoski JJ, Raup DM. 1982. Mammalian evolution and the great American interchange. *Science* **215(4538)**:1351–1357 DOI 10.1126/science.215.4538.1351.

Mendoza ÁM, Arita HT. 2014. Priority setting by sites and by species using rarity, richness and phylogenetic diversity: the case of neotropical glassfrogs (Anura:

Centrolenidae). *Biodiversity and Conservation* **23(4)**:909–926 DOI 10.1007/s10531-014-0642-5.

Mendoza ÁM, Ospina OE, Cárdenas-Henao H, García-R JC. 2015. A likelihood inference of historical biogeography in the world's most diverse terrestrial vertebrate genus: diversification of direct-developing frogs (Craugastoridae: *Pristimantis*) across the Neotropics. *Molecular Phylogenetics and Evolution* **85**:50–58 DOI 10.1016/j.ympev.2015.02.001.

Mendoza ÁM, Torres MF, Paz A, Trujillo-Arias N, López-Alvarez D, Sierra S, Forero F, González MA. 2016. Cryptic diversity revealed by DNA barcoding in Colombian illegally traded bird species. *Molecular Ecology Resources* **16(4)**:862–873 DOI 10.1111/1755-0998.12515.

Montes C, Bayona G, Cardona A, Buchs DM, Silva CA, Morón S, Hoyos N, Ramírez DA, Jaramillo CA, Valencia V. 2012b. Arc-continent collision and orocline formation: closing of the Central American seaway. *Journal of Geophysical Research: Solid Earth* **117(B4)** DOI 10.1029/2011JB008959.

Montes C, Cardona A, Jaramillo C, Pardo A, Silva JC, Valencia V, Ayala C, Pérez-Angel LC, Rodriguez-Parra LA, Ramirez V, Niño H. 2015. Middle Miocene closure of the Central American seaway. *Science* **348(6231)**:226–229 DOI 10.1126/science.aaa2815.

Montes C, Cardona A, McFadden R, Morón SE, Silva CA, Restrepo-Moreno S, Ramírez DA, Hoyos N, Wilson J, Farris D, Bayona GA, Jaramillo CA, Valencia V, Bryan J, Flores JA, Bayona GA. 2012a. Evidence for middle Eocene and younger land emergence in central Panama: implications for Isthmus closure. *Bulletin* **124(5–6)**:780–799 DOI 10.1130/B30528.1.

Morrone JJ. 2014. Biogeographical regionalisation of the Neotropical region. *Zootaxa* **3782(1)**:1–110 DOI 10.11646/zootaxa.3782.1.1.

Mulcahy DG, Morrill BH, Mendelson JRI. 2006. Historical biogeography of lowland species of toads (*Bufo*) across the Trans-Mexican Neovolcanic Belt and the Isthmus of Tehuantepec. *Journal of Biogeography* **33(11)**:1889–1904 DOI 10.1111/j.1365-2699.2006.01546.x.

Myers N, Mittermeier RA, Mittermeier CG, Da Fonseca GA, Kent J. 2000. Biodiversity hotspots for conservation priorities. *Nature* **403(6772)**:853–858 DOI 10.1038/35002501.

Navarro-Sigüenza AG, Peterson AT, Nyari A, García-Deras GM, García-Moreno J. 2008. Phylogeography of the *Buarremon* brush-finch complex (Aves, Emberizidae) in Mesoamerica. *Molecular Phylogenetics and Evolution* **47(1)**:21–35 DOI 10.1016/j.ympev.2007.11.030.

Noss RF. 1991. *Landscape connectivity: different functions at different scales. Landscape linkages and biodiversity*. Washington, D.C.: Island Press.

Ordóñez Garza N, Matson JO, Strauss RE, Bradley RD, Salazar-Bravo J. 2010. Patterns of phenotypic and genetic variation in three species of endemic Mesoamerican *Peromyscus* (Rodentia: Cricetidae). *Journal of Mammalogy* **91(4)**:848–859 DOI 10.1644/09-MAMM-A-167.1.

Ornelas JF, Ruiz-Sánchez E, Sosa V. 2010. Phylogeography of *Podocarpus matudae* (Podocarpaceae): pre-Quaternary relicts in northern Mesoamerican cloud forests. *Journal of Biogeography* **37(12)**:2384–2396 DOI 10.1111/j.1365-2699.2010.02372.x.

Padial JM, Miralles A, De la Riva I, Vences M. 2010. The integrative future of taxonomy. *Frontiers in Zoology* **7(16)**:1–14 DOI 10.1186/1742-9994-7-16.

Palacios M, Voelker G, Rodriguez LA, Mateos M, Tobler M. 2016. Phylogenetic analyses of the subgenus *Mollienesia* (Poecilia, Poeciliidae, Teleostei) reveal taxonomic inconsistencies, cryptic biodiversity, and spatio-temporal aspects of diversification in Middle America. *Molecular Phylogenetics and Evolution* **103**:230–244 DOI 10.1016/j.ympev.2016.07.025.

Paradis E, Claude J, Strimmer K. 2004. APE: analyses of phylogenetics and evolution in R language. *Bioinformatics* **20**:289–290 DOI 10.1093/bioinformatics/btg412.

Parkinson CL, Zamudio KR, Greene HW. 2000. Phylogeography of the pitviper clade *Agkistrodon*: historical ecology, species status, and conservation of cantils. *Molecular Ecology* **9(4)**:411–420 DOI 10.1046/j.1365-294x.2000.00854.x.

Pérez-Consuegra SG, Vázquez-Domínguez E. 2017. Intricate evolutionary histories in montane species: a phylogenetic window into craniodental discrimination in the *Peromyscus mexicanus* species group (Mammalia: Rodentia: Cricetidae). *Journal of Zoological Systematics and Evolutionary Research* **55(1)**:57–72 DOI 10.1111/jzs.12155.

Poelchau MF, Hamrick JL. 2011. Comparative phylogeography of three common Neotropical tree species. *Journal of Biogeography* **40(4)**:618–631 DOI 10.1111/j.1365-2699.2011.02599.x.

Rambaut A, Drummond AJ, Xie D, Baele G, Suchard MA. 2018. Posterior summarisation in Bayesian phylogenetics using Tracer 1.7. *Systematic Biology* **67(5)**:901–990 DOI 10.1093/sysbio/syy032.

Ramírez DA, Foster DA, Min K, Montes C, Cardona A, Sadove G. 2016. Exhumation of the Panama basement complex and basins: implications for the closure of the Central American seaway. *Geochemistry, Geophysics, Geosystems* **17(5)**:1758–1777 DOI 10.1002/2016GC006289.

Rivera-Ortíz FA, Solórzano S, Arizmendi MDC, Dávila-Aranda P, Oyama K. 2016. Genetic diversity and structure of the Military Macaw (*Ara militaris*) in Mexico: implications for conservation. *Tropical Conservation Science* **10**:1–12 DOI 10.1177/1940082916684346.

Robertson JM, Lips KR, Heist EJ. 2008. Fine scale gene flow and individual movements among subpopulations of *Centrolene prosoblepon* (Anura: Centrolenidae). *Revista de Biologia Tropical* **56(1)**:13–26.

Rodríguez-Gómez F, Gutiérrez-Rodríguez C, Ornelas JF. 2013. Genetic, phenotypic and ecological divergence with gene flow at the Isthmus of Tehuantepec: the case of the azure-crowned hummingbird (*Amazilia cyanocephala*). *Journal of Biogeography* **40**:1360–1373 DOI 10.1111/jbi.12093.

Ronquist F, Huelsenbeck JP. 2003. MRBAYES 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics* **19(12)**:1572–1574 DOI 10.1093/bioinformatics/btg180.

Ronquist F, Sanmartín I. 2011. Phylogenetic methods in biogeography. *Annual Review of Ecology, Evolution, and Systematics* **42**:441–464 DOI 10.1146/annurev-ecolsys-102209-144710.

Rovito SM, Vásquez-Almazán CR, Papenfuss TJ, Parra-Olea G, Wake DB. 2015. Biogeography and evolution of Central American cloud forest salamanders (Caudata: Plethodontidae: *Cryptotriton*), with the description of a new species. *Zoological Journal of the Linnean Society* **175(1)**:150–166 DOI 10.1111/zoj.12268.

Ruiz-Carranza PM, Lynch JD. 1991. Ranas Centrolenidae de Colombia I: Propuesta de una nueva clasificación genérica. *Lozania* **57**:1–30.

Sambrook J, Russell DW. 2006. Purification of nucleic acids by extraction with phenol: chloroform. *Cold Spring Harbor Protocols* **2006(1)**: pii: pdb.prot4455 DOI 10.1101/pdb.prot4455.

Savage JM. 2002. *The amphibians and reptiles of costa rica: a herpetofauna between two continents, between two seas.* Chicago: University of Chicago press.

Shapiro B, Rambaut A, Drummond AJ. 2005. Choosing appropriate substitution models for the phylogenetic analysis of protein-coding sequences. *Molecular Biology and Evolution* **23(1)**:7–9 DOI 10.1093/molbev/msj021.

Smith BT, Amei A, Klicka J. 2012. Evaluating the role of contracting and expanding rainforest in initiating cycles of speciation across the Isthmus of Panama. *Proceedings of the Royal Society of London B: Biological Sciences* **279(1742)**:3520–3526 DOI 10.1098/rspb.2012.0706.

Smith SA, Stephens PR, Wiens JJ. 2005. Replicate patterns of species richness, historical biogeography, and phylogeny in Holarctic treefrogs. *Evolution* **59(11)**:2433–2450 DOI 10.1111/j.0014-3820.2005.tb00953.x.

Stamatakis A. 2006. RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. *Bioinformatics* **22(21)**:2688–2690 DOI 10.1093/bioinformatics/btl446.

Stehli FG, Webb SD. 1985. A kaleidoscope of plates, faunal and floral dispersals, and sea level changes. In: Stehli FG, Webb SD, eds. *The great American biotic interchange.* Boston: Springer, 3–16.

Streicher JW, García-Vázquez UO, Ponce-Campos P, Flores-Villela O, Campbell JA, Smith EN. 2014. Evolutionary relationships amongst polymorphic direct-developing frogs in the *Craugastor rhodopis* species group (Anura: Craugastoridae). *Systematics and Biodiversity* **12(1)**:1–22 DOI 10.1080/14772000.2014.882428.

Townsend JH. 2014. Characterizing the Chortís block biogeographic province: geological, physiographic, and ecological associations and herpetofaunal diversity. *Mesoamerican Herpetology* **1(2)**:204–252.

Townsend JH, Wilson LD. 2016. Amphibians of the Cordillera Nombre de Dios, Honduras: COI barcoding suggests underestimated taxonomic richness in a threatened endemic fauna. *Mesoamerican Herpetology* **3(4)**:910–927.

Townsend JH, Wilson LD, Medina-Flores M, Aguilar-Urbina E, Atkinson BK, Cerrato-Mendoza CA, Contreras-Castro A, Gray LN, Herrera-B LA, Luque-Montes IR, McKewy-Mejía M, Alionso Portillo-Avilez A, Stubbs AL, McKewy-Mejía M. 2012.

A premontane hotspot for herpetological endemism on the windward side of Refugio de Vida Silvestre Texíguat, Honduras. *Salamandra* **48(2)**:92–114.

Valencia-Aguilar A, Castro-Herrera F, Ramírez-Pinilla MP. 2012. Microhabitats for oviposition and male clutch attendance in *Hyalinobatrachium aureoguttatum* (Anura: Centrolenidae). *Copeia* **2012(4)**:722–731 DOI 10.1643/CE-11-173.

Vázquez-Miranda H, Navarro-Sigüenza AG, Omland KE. 2009. Phylogeography of the rufous-naped red (*Camphylorhynchus rufinucha*): speciation and hybridization in Mesoamerica. *The Auk* **126(4)**:765–778 DOI 10.1525/auk.2009.07048.

Venegas-Anaya M, Crawford AJ, Escobedo-Galván AH, Sanjur OI, Densmore LD, Bermingham E. 2008. Mitochondrial DNA phylogeography of *Caiman crocodilus* in Mesoamerica and South America. *Journal of Experimental Zoology Part A: Ecological and Integrative Physiology* **309(10)**:614–627 DOI 10.1002/jez.502.

Villa J, Valerio CE. 1982. Red, white and brown: preliminary observation on the color of the centrolenid tadpole (Amphibia: Anura: Centrolenidae). *Brenesia* **1982(19/20)**:1–16.

Wang IJ, Crawford AJ, Bermingham E. 2008. Phylogeography of the pygmy rain frog (*Pristimantis ridens*) across the lowland wet forest of isthmian Central America. *Molecular Phylogenetics and Evolution* **47(3)**:992–1004 DOI 10.1016/j.ympev.2008.02.021.

Wiens JJ, Graham CH, Moen DS, Smith SA, Reeder TW. 2006. Evolutionary and ecological causes of the latitudinal diversity gradient in hylid frogs: treefrog trees unearth the roots of high tropical diversity. *The American Naturalist* **168(5)**:579–596 DOI 10.1086/507882.

Supplementary Material 1.

We performed separate analysis per gene to include all samples available avoiding the difficulties caused by missing data. First, we conducted a Bayesian analysis of population structure with BAPS v6.0 (Corander et al., 2008), using the spatial clustering of individuals, considering that the spatial prior may strengthen inferences for sparse molecular data. Considering that the pool of samples was slightly different for each gene and BAPS was very sensitive to missing data, BAPS analysis was run independently for each gene, with a maximum *k* value of 10 populations per analysis. Geneland and sPCA analyses as those generated for the concatenated sequences in the main text were also performed per gene. Additionally, a median-joining haplotype network for each gene was constructed using PopArt (French et al., 2014).

We finally defined genetically homogeneous regions as those obtained through all spatial, and non-spatial analyses for diversity index estimation. We calculated haplotype (h) and nucleotide diversity (π), globally and by region, with DNAsp (Libardo & Rozas, 2009). Additionally, the distribution of genetic variability at hierarchical levels was estimated using analysis of molecular variance (AMOVA). Genetic differentiation among all regions was again estimated based on the Nei's pairwise F_{ST} statistic with the *hierfstat* package (Goudet & Jombart 2015) and used corrected distances according to the K2P parameter (Kimura, 1980) in MEGA v.7 (Kumar, Strecher & Tamura 2016).

Results.

The BAPS results depicted six clusters for 16S, seven for COI and five for ND1. Although the number of clusters varied for the northern and southern regions, three clusters in western Chortis, Chorotega and eastern Chortis were consistently recovered. The 16S sequences separated the northern clusters, while the COI sequences could differentiate the southern clusters, east-west of the Andes range (Fig. S1.1).

Geneland analysis per gene showed six, seven and six clusters for 16S, COI and ND1, respectively. Both coding genes showed two clusters on both sides of the Andes range. Separation between the Choco and Chorotega samples was found in all cases (Fig. S1.1).

Overall, the haplotype networks for the three genes were concordant, with higher diversity and structure being revealed for COI and ND1 than for 16S. Four mitochondrial haplotype groups were detectable among the entire distribution (Fig. S3), where the concordance between the haplotype network and the species distribution suggested a deep pattern of geographic structuring and differentiation across the complete range. The SAP and Choco regions shared the same 16S haplotype but showed differences in the COI and ND1 coding genes.

The minimum genetic distance between regions was obtained for SAP-Choco and WPI (K2P = 0.007, 0.018 and 0.019 for 16S, COI and ND1 respectively) and the maximum between North American Pacific and WPI (K2P=0.029) for 16S, between Maya and SAP for COI (K2P= 0.078), and between SAP and Chorotega for ND1 (K2P=0.077) (Table S1.1). The F_{ST} indices between regions ranged between 0.29 (SAP and Chorotega) and 0.9208 (WPI and Chorotega) in almost all combinations except between SAP and WPI for COI and ND1 (Table S1.1).

The AMOVA results for all genes indicated that 79-88% of the observed genetic variability was partitioned between regions, compared with 12-20% within regions (all P <0.001; Table S1.2).

When comparing the diversity per cluster, the Chorotega samples showed the highest haplotypic diversity for all genes, while the Maya populations exhibited the highest nucleotide diversity for coding genes. The Gulf population showed the lowest haplotypic diversity, and SAP exhibited the lowest nucleotide diversity (Table S1.3).

References non-included in main text

Corander J, Marttinen P, Sirén J, Tang J. 2008. Enhanced Bayesian modelling in BAPS software for learning genetic structures of populations. BMC bioinformatics 9(1):539

French N, Yu S, Biggs P, Holland B, Fearnhead P, Binney B, Fox A, Grove-White D, Leigh JW, Miller W, Muellner P, Carter P, 2014. Evolution of Campylobacter species in New Zealand, In: Sheppard SK, Méric G. eds. Campylobacter Ecology and Evolution. Caister Academic Press., Norfolk, pp. 221–240.

Goudet, J., & Jombart, T. (2015). hierfstat: estimation and tests of hierarchical F-statistics. R package version 0.04-22.

Kamvar ZN, Tabima JF, Grünwald NJ. 2014. Poppr: an R package for genetic analysis of populations with clonal, partially clonal, and/or sexual reproduction. PeerJ 2:e281 http://dx.doi.org/10.7717/peerj.281

Kimura M. 1980. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. Journal of molecular evolution 16(2):111-120

Kumar S, Stecher G, Tamura K. 2016. MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. Molecular biology and evolution 33(7):1870-1874
Libardo P, Rozas J, 2009. DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. Bioinformatics 25(11):1451-1452

Table S1.1. Mean K2P distances within and among populations (lower) for all tree mitochondrial genes and F_{ST} indices (upper) per pair of populations detected.

Block	Intra-population	SAP-Choco	WPI	Chorotega	Chortis	Maya	Gulf	Pacific
16S								
SAP-Choco	0.002	---	0.4507	0.8347	0.7467	0.8560	0.7347	0.9065
WPI	0.001	0.007	---	0.8641	0.9591	0.6505	0.7226	0.8119
Chorotega	0.003	0.021	0.021	---	0.8981	0.7693	0.7962	0.8822
Chortis	0	0.02	0.021	0.031	---	0.4594	0.5762	0.6343
Maya	0.003	0.03	0.03	0.033	0.016	---	0.3925	0.4923
Gulf	0.002	0.023	0.023	0.03	0.011	0.008	---	0.4951
Pacific	0.003	0.028	0.029	0.041	0.019	0.022	0.015	---
COI								
SAP-Choco	0.009	---	0.0938	0.3840	0.5046	0.6661	0.4360	0.7214
WPI	0.008	0.018	---	0.8761	0.5226	0.5219	0.8986	0.6609
Chorotega	0.006	0.054	0.044	---	0.5266	0.6292	0.9120	0.7610
Chortis	0.005	0.071	0.067	0.06	---	0.5158	0.4754	0.6003
Maya	0.015	0.078	0.068	0.071	0.052	---	0.3509	0.6048
Gulf	0.006	0.068	0.062	0.067	0.047	0.038	---	0.5880
Pacific	0.004	0.064	0.051	0.058	0.037	0.028	0.032	---
ND1								
SAP-Choco	0.01	---	0.1793	0.2944	0.3648	0.5668	0.3608	0.6451
WPI	0.013	0.019	---	0.9208	0.6556	0.6666	0.8510	0.8557
Chorotega	0.003	0.077	0.071	---	0.6556	0.6666	0.8510	0.8557
Chortis	0.003	0.05	0.052	0.052	---	0.5215	0.5548	0.6610
Maya	0.016	0.068	0.065	0.07	0.061	---	0.3349	0.3723
Gulf	0.005	0.073	0.069	0.067	0.059	0.021	---	0.5774
Pacific	0.017	0.074	0.071	0.065	0.059	0.032	0.03	---

Table S1.2. AMOVA result for each mitochondrial gene per populations detected.

Source of variation	**d.f.**	**Sum of squares**	**Mean Squares**	**Sigma**	**Percentage of variation**	**Φ statistics**
16S						
Between regions (ΦST)	6	322.53	53.75	3.64	88.12	0.88
Within regions (ΦCT)	102	50.03	0.49	0.49	11.88	
Total	108	372.57	3.45	4.13	100	
COI						
Between regions (ΦST)	6	1102.41	183.73	11.54	85.85	0.85
Within regions (ΦCT)	109	207.43	1.90	1.90	14.15	
Total	115	1309.84	11.39	13.45	100	
ND1						
Between regions (ΦST)	6	676.78	112.80	15.42	79.65	0.79
Within regions (ΦCT)	47	185.17	3.94	3.93	20.34	
Total	53	861.96	16.26	19.36	100	

Table S1.3. Genetic polymorphism data for mitochondrial DNA sequences from the *Hyalinobatrachium fleischmanni* populations detected in this study.

	N	h	S	Hd	π	Tajima's D	Fu Fs	Rozas R Fs
16S								
Total	109	24	35	0.844	0.01554	0.16824	-2.097	0.0906
SAP-Choco	47	9	9	0.414	0.00120	-2.13240*	-8.151	0.0471
WPI	3	2	1	0.667	0.00159	NA	NA	0.4714
Chorotega	8	4	3	0.821	0.00266	-0.1774	-1.029	0.1804
Chortis	3	1	0	0	0	NA	NA	NA
Maya	20	4	7	0.431	0.0023	-1.78918	0.122	0.1659
Pacific	5	2	3	0.4	0.00288	-1.04849	1.688	0.4
Gulf	23	4	5	0.438	0.00156	-1.52991	-0.853	0.1307
COI								
Total	116	63	110	0.979	0.04416	0.25275	-11.326	0.1043
SAP-Choco	46	25	37	0.939	0.01270	-0.70179	-8.259	0.0812
WPI	3	3	4	1	0.00505	NA	NA	0.3118
Chorotega	7	6	8	0.952	0.00569	-0.9744	-2.238	0.2203
Chortis	8	4	6	0.785	0.00454	0.15875	0.522	0.1794
Maya	21	14	30	0.957	0.01388	-0.47131	-2.568	0.1098
Pacific	5	4	5	0.900	0.00417	-0.56199	-0.848	0.2408
Gulf	36	7	24	0.809	0.00601	-1.80729*	0.759	0.148
ND1								
Total	54	45	141	0.99	0.04257	-0.36248	-9.584	0.1092
SAP-Choco	28	22	57	0.97	0.01341	-1.31381	-7.189	0.0764
WPI	3	3	5	1	0.00436	NA	NA	0.3399
Chorotega	3	3	3	1	0.00262	NA	NA	0.2722
Chortis	2	2	2	1	0.00262	NA	NA	0.5
Maya	8	7	42	0.964	0.01578	-1.45862	-0.108	0.1981
Pacific	2	2	13	1	0.01702	NA	NA	0.5
Gulf	8	6	14	0.892	0.00528	-1.28604	-0.94	0.1455

N = number of sequences, h = number of haplotypes, S = segregating sites, Hd = haplotype diversity, π = nucleotide diversity.

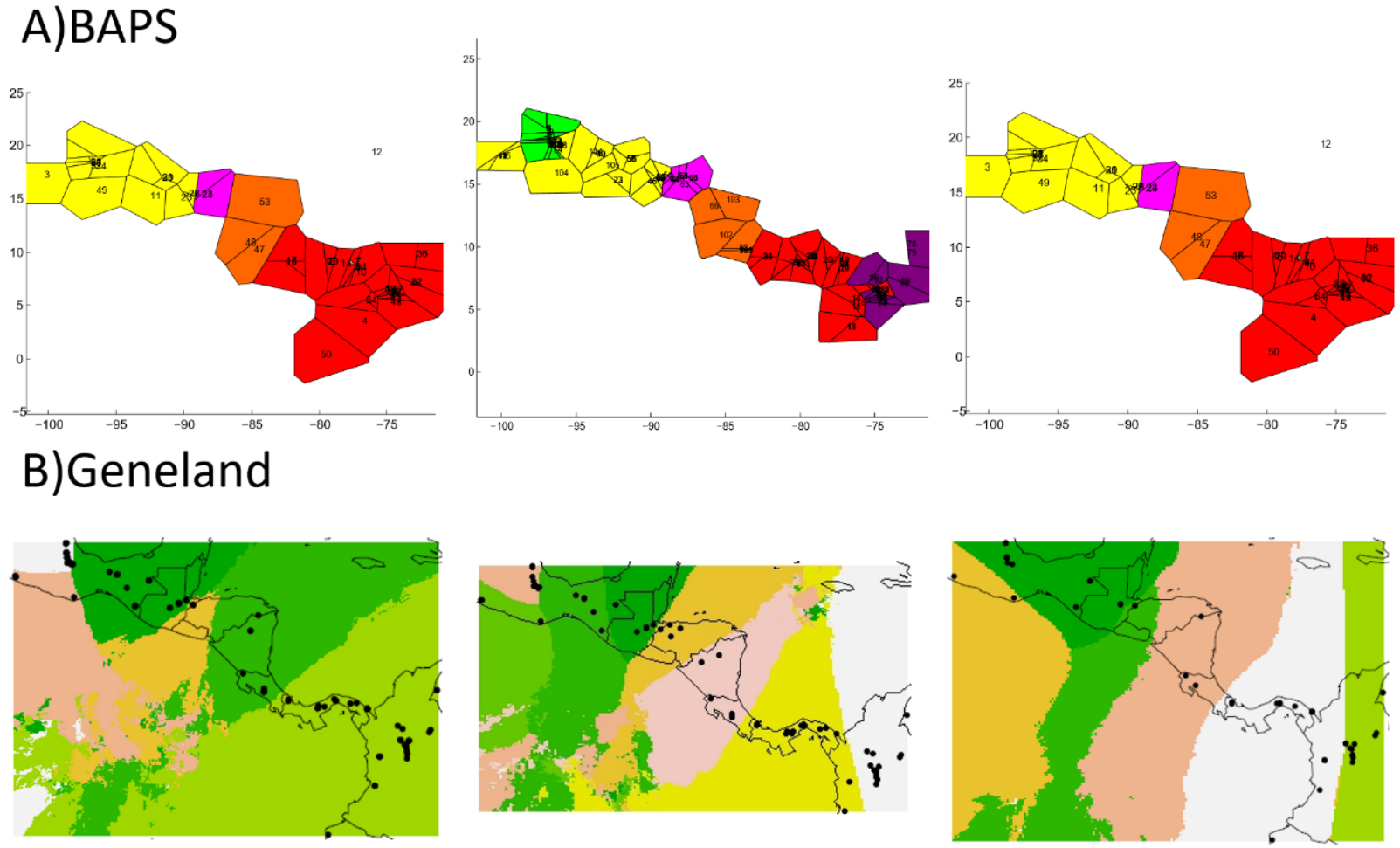
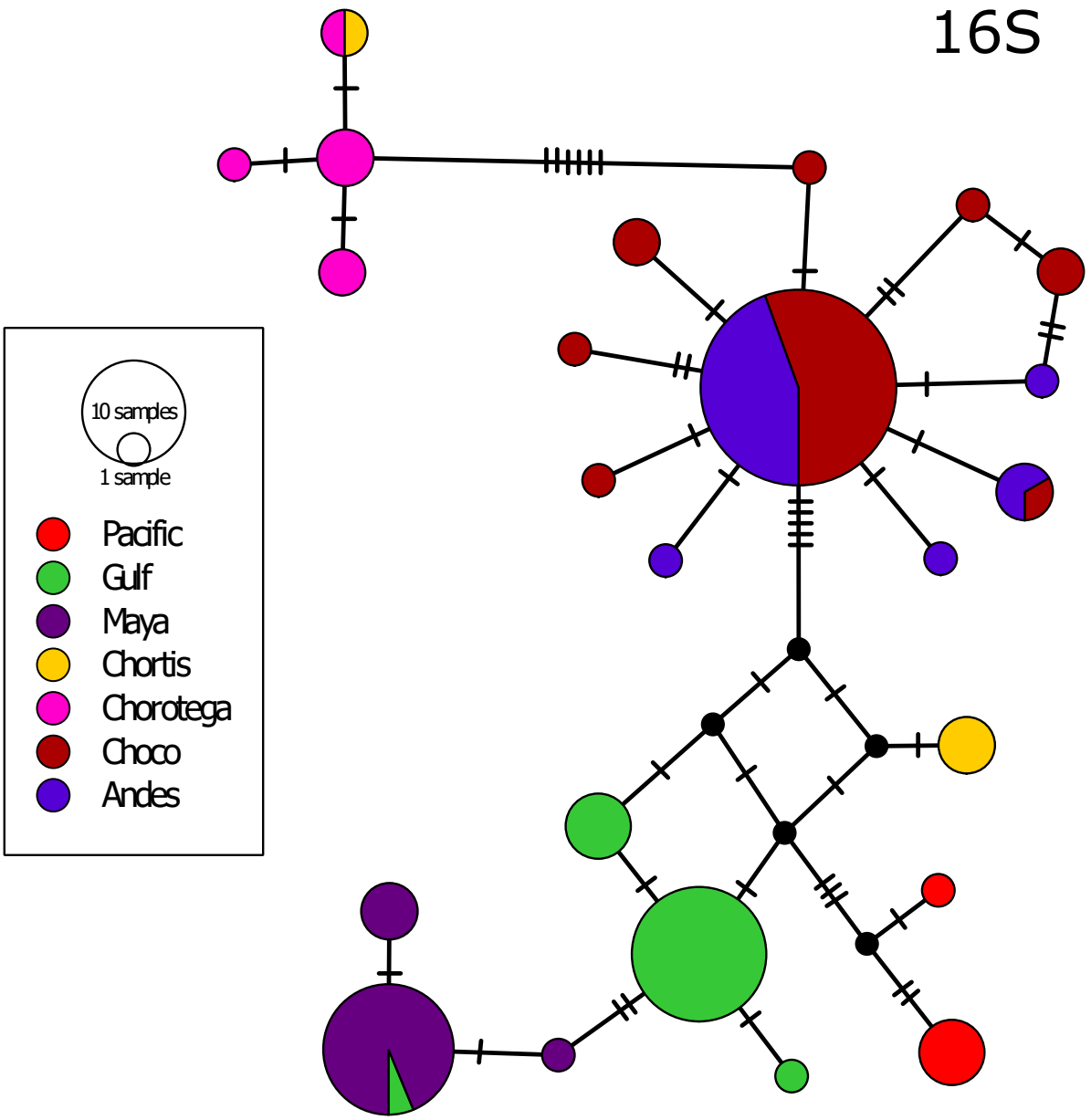
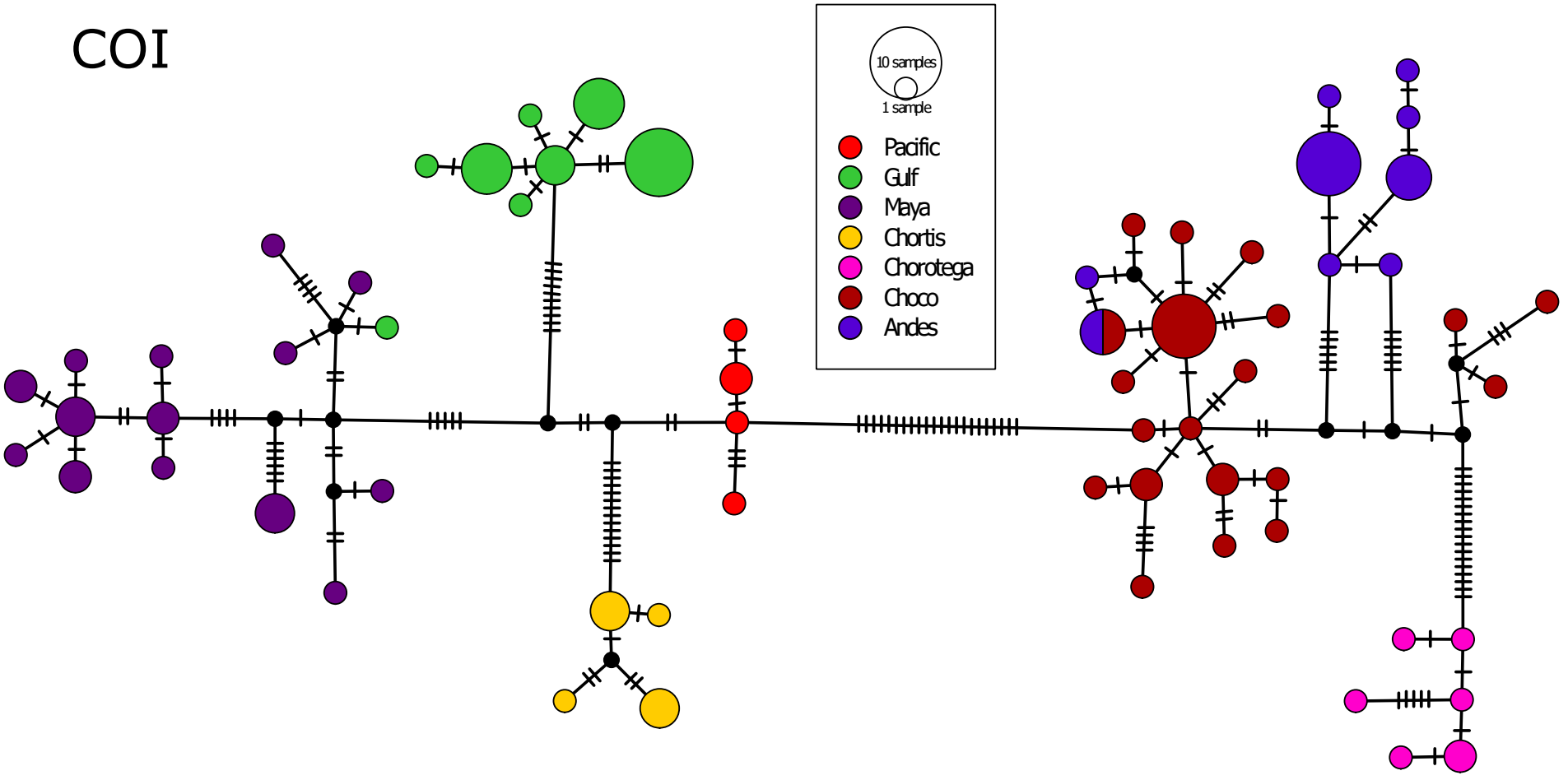
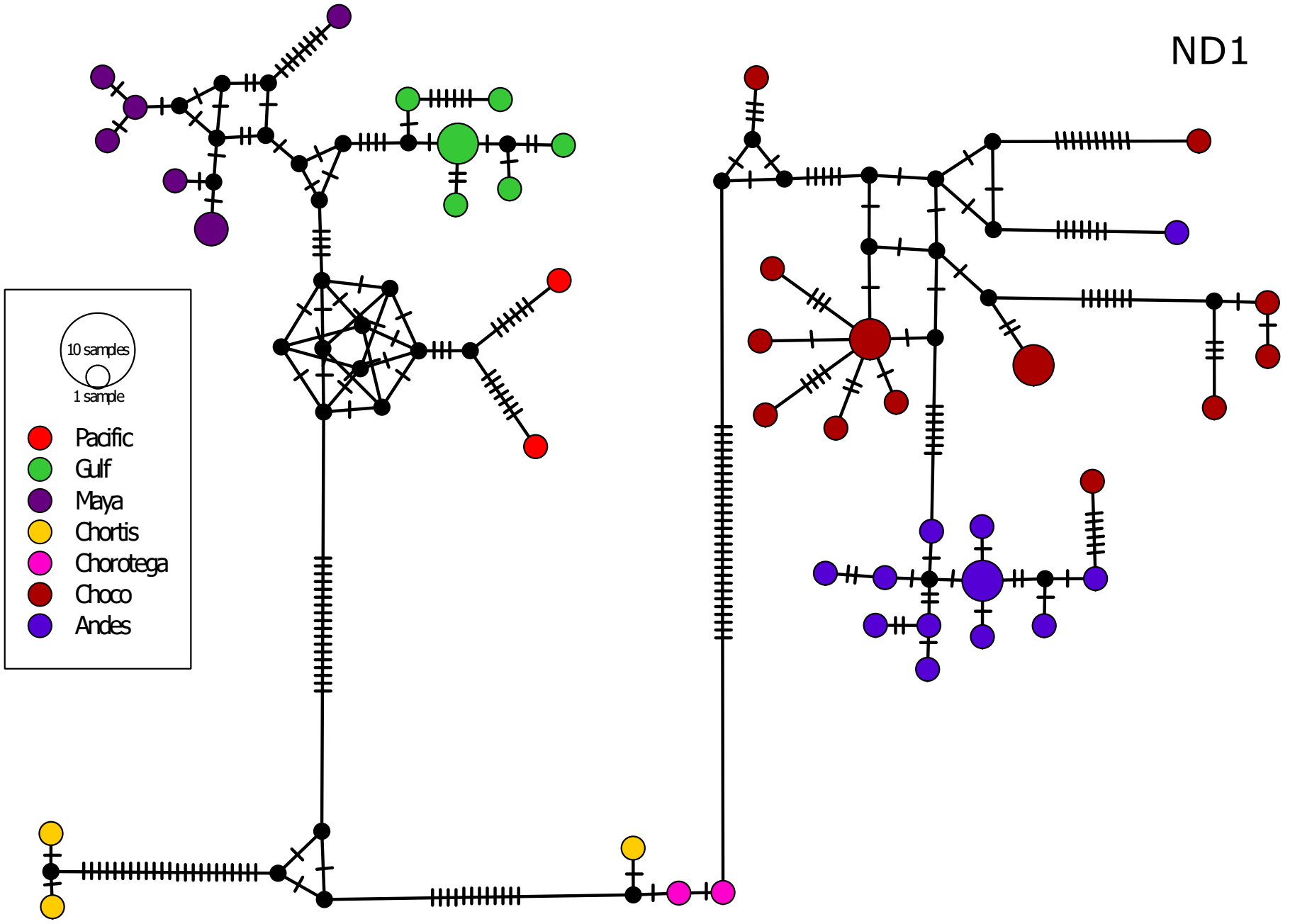


Fig. S1.1. Results of the Bayesian for *H. fleischmanni* population clustering A) BAPS and B) Geneland based on 16S (first column), COI (second column) and ND1 (third column) sequences.


16S
10 samples
1 sample
Pacific
Gulf
Maya
Chortis
Chorotega
Choco
Andes


COI
10 samples
1 sample
Pacific
Gulf
Maya
Chortis
Chorotega
Choco
Andes


ND1
10 samples
1 sample
Pacific
Gulf
Maya
Chortis
Chorotega
Choco
Andes

CAPÍTULO II

Phylogeny-based species delimitation and integrative taxonomic revision of the *Hyalinobatrachium fleischmanni* species complex, with resurrection of *H. viridissimum* (Taylor, 1942)

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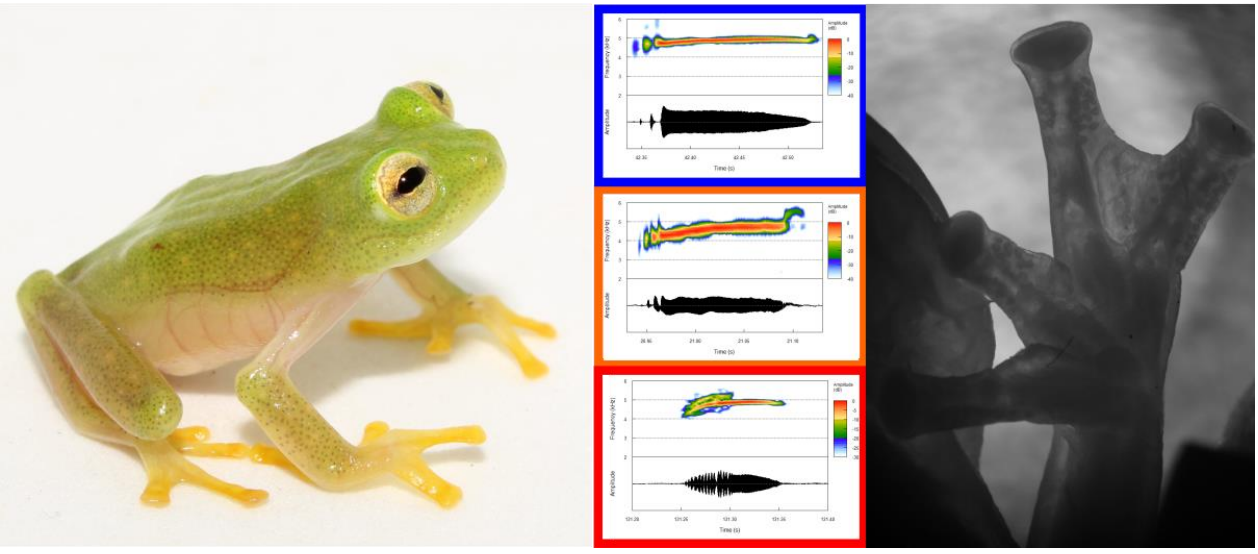

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Research Article

Phylogeny-based species delimitation and integrative taxonomic revision of the *Hyalinobatrachium fleischmanni* species complex, with resurrection of *H. viridissimum* (Taylor, 1942)

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Hyalinobatrachium fleischmanni is one of the widest ranging glassfrog species, occurring in the lowlands from Mexico through Central America to Ecuador. Despite its conservative morphology, previous studies suggested that the species is comprised of multiple lineages. Here we test the hypothesis of cryptic species within *H. fleischmanni* by means of morphology, morphometrics, bioacoustics, and molecular analysis. Molecular delimitation based on mitochondrial and nuclear genes detected 17 candidate species within *H. fleischmanni* but combined with other sources of evidence, we support the recognition of at least three different species within the name *H. fleischmanni*. The identity of *H. fleischmanni* sensu stricto is supported for populations from Costa Rica to eastern Honduras while the name *Hyalinobatrachium tatayoi* corresponds to the southern lineages in Costa Rica and South America. Those two species differ in the note duration of the advertisement call and in the absence of nuptial pads in the hand webbing of *H. fleischmanni* males. Populations from Mexico and Guatemala represent a third species to which we assign the available name *H. viridissimum* **comb. nov.** *Hyalinobatrachium viridissimum* differs from *H. fleischmanni* and *H. tatayoi* in mitochondrial DNA divergence, variation in peak frequency, and note duration of the advertisement call. A divergent lineage from western and central Honduras is tentatively assigned to *H. viridissimum*. Based on these results, we provide updated information for each species.

http://www.zoobank.org/urn:lsid:zoobank.org:pub:96D65174-4F8B-4E4B-8C85-967767311255

Key words: Bioacoustics, Centrolenidae, glassfrogs, integrative taxonomy, species delimitation

Introduction

Hyalinobatrachium is a taxonomically stable genus of glassfrogs (family Centrolenidae) with 33 currently recognized species (Frost, 2019; Guayasamin et al., 2009), distributed in tropical Mexico and Central America, the tropical Andes, the Coastal Cordillera of Venezuela, Tobago, the Amazon Basin, and the Guiana Shield (Guayasamin et al., 2009). Due to the morphological similarities among the species within the genus (Señaris & Ayarzagüena, 2005), species identification and delimitation has been challenging, and taxonomic studies have required a molecular approach (Castroviejo-Fisher et al., 2011; Mendoza et al., 2019b).

The type species of the genus, *Hyalinobatrachium fleischmanni*, is one of the most studied centrolenid frogs (e.g., Delia et al., 2010; Starrett & Savage, 1973; Villa, 1984) due to its putatively widespread distribution from Mexico to northern South America. The limited morphological variation for certain characters

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(colouration, morphometry) combined with the high variation of other characters (guanophores of the pericardium, interdigital webbing; Cisneros-Heredia & McDiarmid, 2007) led to the recognition of a single species with a very large geographic distribution. Through time, multiple names have been synonymized with *H. fleischmanni*, including *Hylella chrysops* (Cope, 1894), *Cochranella decorata* (Taylor, 1958) and *Cochranella millepunctata* (Taylor, 1958) from Costa Rica, and *Centrolenella viridissima* (Taylor, 1942) from Mexico. On the other hand, some populations previously considered to represent *H. fleischmanni* have been recognized as distinct species, such as *H. guairarepanense* in Venezuela (Barrio-Amorós, 2004). Duellman and Tulecke (1960) stated that all Mexican material represented a single species, and that the characteristics used by Taylor (1942) for the description of *C. viridissima* were variable and overlapped to a considerable extent with Costa Rican *H. fleischmanni* but nonetheless recognized *C. viridissima* as tentatively distinct. Later, Starrett and Savage (1973) synonymized *C. viridissima* with *H. fleischmanni*, arguing that populations considered to represent both taxa are connected by a continuous series of populations through Central America.

Castroviejo-Fisher et al. (2007) described *H. tatayoi* from a single locality in the Serranía del Perijá in Venezuela, based on subtle differences in morphology and acoustics with respect to Central American populations of *H. fleischmanni*. Subsequent molecular studies (Castroviejo-Fisher et al., 2009; Delia et al., 2017; Guayasamin et al., 2008) suggested that *H. fleischmanni* was paraphyletic with respect to *H. tatayoi.* Recent phylogeographic analysis of *H. fleischmanni* based on mitochondrial DNA across its entire distribution identified three large genetically divergent clusters (Mendoza et al., 2019a). Samples from the type locality of *H. fleischmanni* are clustered in a Central American small-range clade (the Central clade of Mendoza et al., 2019a) that includes populations from southern Costa Rica, Nicaragua, and eastern Honduras. The populations from Mexico, Guatemala, and west Honduras formed a second clade (the Northern clade), formed by four allopatric lineages, referred to as the Maya, Gulf, Pacific, and West Chortis clades by Mendoza et al. (2019a). Finally, populations from Panama and South America, including those assigned to *H. tatayoi*, formed a third clade, the Southern clade.

Herein, we re-evaluate the taxonomy of *H. fleischmanni* and *H. tatayoi* by combining three lines of evidence: genetic (nuclear and mitochondrial), acoustics, and morphology to detect lineage divergence and to determine the identities and geographic distribution of each valid species. Based on our results, we assign the name *H. tatayoi* to the Southern clade from Costa Rica and South America, and we resurrect the name *C. viridissima* (as *H. viridissimum* **comb. nov.**) and assign it to all four lineages of the Northern clade, with the caveat that further investigation is needed to determine the distinctiveness and taxonomic status of each of the four lineages within the Northern clade.

Materials and methods

Species criterion

Our definition of species follows the general metapopulation lineage species concept (deQueiroz, 2007; Simpson, 1951). We recognize a species when there is evidence for the separation of metapopulation lineages, preferably based on multiple lines of evidence, following the consensus protocol for integrative taxonomy (Dayrat, 2005; Padial et al., 2010). We used two criteria to delimit species boundaries using molecular data: reciprocal monophyly and genetic distances (reviewed in Vences & Wake, 2007). The first criterion is based on the assumption that coalescent patterns in gene genealogies are related to historical processes that originate separate lineages (e.g., Avise, 2000; Knowles & Carstens, 2007). The second assumes that genetic divergence between populations within a species tends to be relatively small because of gene flow, whereas divergence between species increases with time.

Phylogenetic analysis

We used a subset of 56 previously published samples from the entire distribution of *H. fleischmanni* and *H. tatayoi* and four outgroups for phylogenetic reconstructions (Mendoza et al., 2019a). This subset included sequences for three mitochondrial genes (16S, COI, and ND1). Since an analysis based only on the mitochondrial genome may display differences between gene and species trees as a result of distinct biological processes, such as mitochondrial DNA introgression, a nuclear gene (POMC) was included for 38 of those samples, following laboratory protocols by Guayasamin et al. (2008). Mitochondrial and nuclear sequences were concatenated to perform new Maximum likelihood (RAxML) and Bayesian reconstructions (Beast and MrBayes) following the methods outlined in Mendoza et al. (2019a). The new sequences were deposited in GenBank (accession numbers: MK817126–MK817164).

Analysis of molecular species delimitation

From the phylogenetic trees obtained with the entire set of sequences (nuDNA + mtDNA), we used three approaches to test species boundaries. First, we employed the general mixed Yule-coalescent (GMYC, Pons et al., 2006) model to estimate species boundaries using the ultrametric tree. The GMYC method (Fujisawa & Barraclough, 2013) uses a speciation and a neutral coalescent model. It strives to maximize the likelihood score by separating/classifying the branches of an ultrametric tree (in units of absolute or relative ages) into two processes; within and between species, searching the transition point between species-level and population-level variability based on a shift in the rate of an ultrametric tree branching (Pons et al., 2006). These analyses were performed with single and multiple thresholds for the ultrametric tree in the splits package (Ezard et al., 2009) in R version 3.5.1.

Second, we used the Bayesian Poisson tree process (bPTP; Zhang et al., 2013) and the multi-rate PTP (mPTP) model (Kapli et al., 2017) in the web servers (https://species.h-its.org/ptp/ and https://mptp.h-its.org respectively) on multilocus phylogenetic trees inferred by MrBayes and BEAST. The bPTP was run for 100,000 generations, with a thinning of 100 and a 10% burn-in. These two analyses use the number of substitutions to identify significant changes in branching rates in a phylogeny, thereby they do not depend on the accuracy of ultrametric tree estimations. In addition, it may outperform other species delimitation methods when evolutionary distances are small (Malavasi et al. 2016; Pons et al., 2006). In particular, mPTP incorporates different coalescence rates between clades, allowing for different levels of intraspecific genetic diversity (Kapli et al., 2017).

Third, we used a coalescent-based modelling approach called Bayesian Phylogenetics and Phylogeography (BP&P v.2.0; Yang & Rannala, 2010) to generate the posterior probabilities of species assignments taking into account uncertainties due to unknown gene trees and the ancestral coalescent process. BPP evaluates speciation models using a reverse jump Markov chain Monte Carlo (rjMCMC) algorithm to determine whether to collapse or retain branches throughout the phylogeny.

Following Leaché and Fujita (2010) we applied the following assumptions on population sizes and divergences: (1) Large ancestral population sizes and deep divergences (theta ~ G(1, 10) and t0 ~ G(1, 10), both with a prior mean = 0.1 and variance = 0.01; (2) Relatively small ancestral population sizes and shallow divergences among species theta ~ G(2, 2000) and t0 ~ G(2, 2000), with a prior mean = 0.001 and variance = 5×1027); and (3) Large ancestral populations sizes (theta ~ G(1, 10) and relatively shallow divergences among species t0 ~ G(2, 2000). Analyses were run for 500,000 generations (first 10,000 were burn-in), with a sampling interval of 5. We used the BEAST topology as a guide species tree, and the number of candidate species was based on the PTP results as input. A conservative approach was used for the BP&P and bPTP analysis; we required strong support (pp $\geq$ 0.95) across all runs to retain a given branch (i.e., indicating lineage splitting).

Finally, we compared the genetic distances from the lineages obtained in the delimitation analysis (based on strict consensus) with those from recent studies in glassfrogs (i.e., Amador et al., 2018; Rada et al., 2017) and DNA barcoding in anurans (Lyra, Haddad, & de Azeredo-Espin, 2017). To do so, we calculated the uncorrected p-distances for 16S and COI genes in MEGA version 7 (Kumar et al., 2016).

Morphology

Following Castroviejo-Fisher et al. (2011), we considered morphology to be divergent if a taxon exhibits at least one fixed (qualitative) or non-overlapping (quantitative) character or a unique combination separating it from each of the other taxa. The underlying assumption is that fixed differences in heritable morphological traits might be strong evidence of reduced or absent gene flow (Wiens & Servedio, 2000); thus, constituting evidence of independent lineages (deQueiroz, 2007).

We examined 174 specimens from the following institutions: Museo de Herpetología de la Universidad de Antioquia (MHUA-A, $n = 43$), American Museum of Natural History (AMNH, $n = 36$), Museo de Zoología de la Pontificia Universidad Católica del Ecuador (QCAZ, $n = 19$), Universidad de Costa Rica (UCR, $n = 10$), Colección Nacional de Anfibios y Reptiles, Instituto de Biología, Universidad Nacional Autónoma de México (CNAR-IBH, $n = 24$), and Museo de Zoología de la Facultad de Ciencias (MZFC, $n = 42$). All specimens were measured by the same person (AMMH) except for those from Universidad de Costa Rica (EA). A list of material examined is provided in Appendix S1.

Each locality from museum databases was carefully checked (lat–long coordinates) to correct for imprecise geo-references in decimal degrees, based on the WGS 1984 datum. Ten standardized morphological measurements for centrolenids were selected following Cisneros-Heredia and McDiarmid (2007): Snout-vent length (SVL); Head length (HL); Head width (HW); Inter-orbital distance (IOD); Horizontal eye diameter (ED); Eye-nostril distance (ES); Width of disc on the

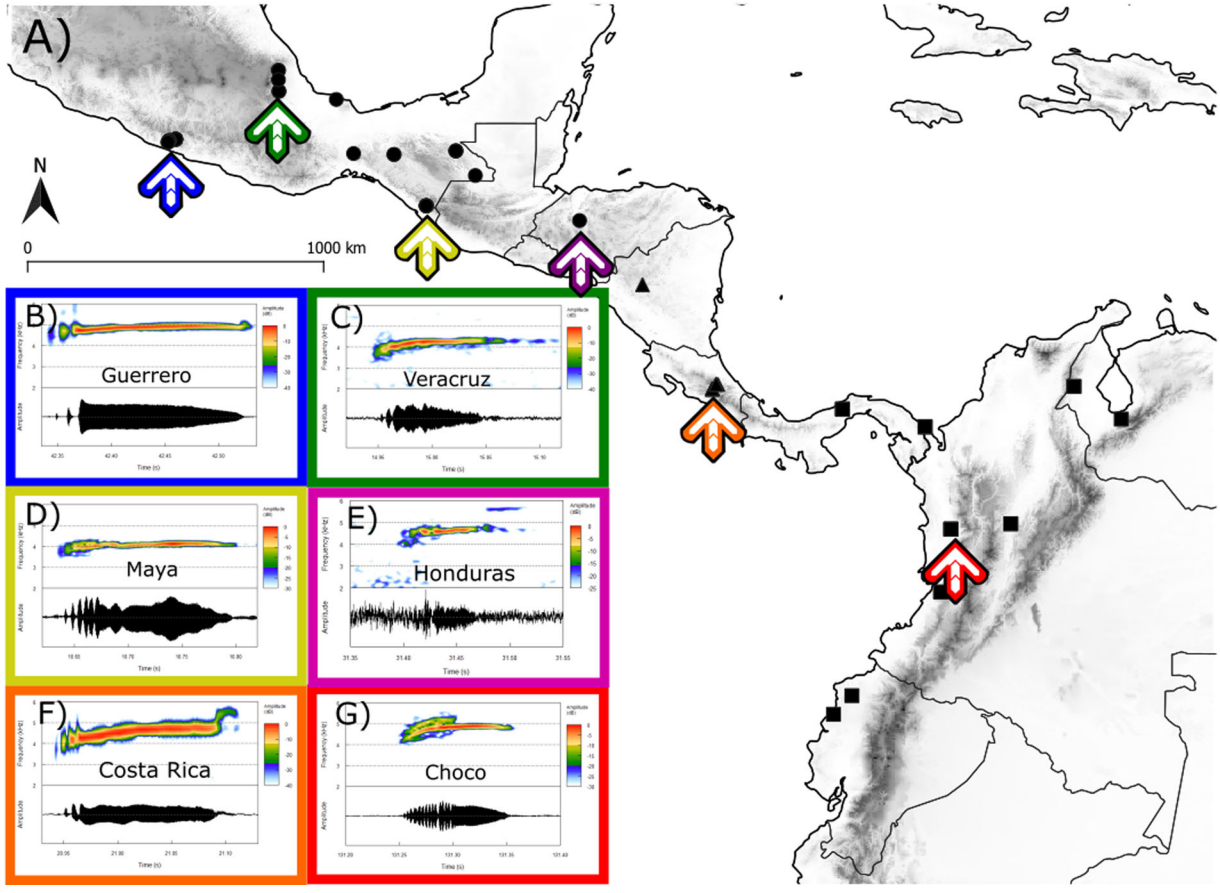

Fig. 1. (A) Location of the 64 calls for *Hyalinobatrachium fleischmanni* species complex analysed in this study. Each shape represents the candidate species tested (circle = *H. viridissimum* **comb. nov.**, triangle = *H. fleischmanni* and square = *H. tatayoi*), the colour of the arrow indicates the location of the corresponding spectrogram. (B–G) Spectrogram and sonogram of the advertisement call from some localities evaluated.

third finger (FIII); Femur length (FL); Tibia length (TL); Foot length (FL). The measurements were made with a digital calliper and rounded to the nearest 0.1 mm. Since our data do not show homogeneity of variances (based on a Levene's test), we compared morphometric variables between lineages with a Wilcoxon rank test. Subsequently, the dimensions of the scaled measurements were reduced by means of a Principal Component Analysis (PCA) on the residuals of the linear regressions between the SVL and the morphometric variables (to remove the effect of body size) and the groups generated from the first two components were visualized in a plot.

Additionally, 10 categorical characters suggested by Cisneros-Heredia and McDiarmid (2007) were also evaluated: Snout form at dorsal and lateral view, tympanum visibility, dorsal skin texture, cloacal ornamentation, colour of peritonea, shape of liver, hand webbing, iris colouration, and type of nuptial pads.

Bioacoustics

We employed differences in advertisement call since they are usually interpreted as evidence of lineage divergence that can be used to separate species (Bickford et al., 2007; Köhler et al., 2017; Padial et al., 2008; Vences & Wake, 2007). We considered that advertisement calls strongly indicate the existence of lineage divergence when they do not overlap in quantitative parameters, since vocalizations directly tied to mate recognition and sexual selection can be considered a prezygotic reproductive barrier, establishing and maintaining reproductive isolation (Vences & Wake, 2007).

Recordings of advertisement calls were obtained throughout the entire distribution of the species (Fig. 1) from collections and researchers (unpublished data) and were complemented by field recordings made in Colombia and Mexico through a Sennheiser unidirectional microphone (ME66/K6) connected to a Tascam DR-40 recorder. The recording from Honduras was

made using a Sennheiser MKE 400 microphone and Olympus WS-823 digital recorder.

At least 10 calls per individual were recorded in .wav format at a sampling frequency of 44.1 kHz and an amplitude resolution of 16 bits. The sound files were analysed in the software Raven Pro 1.4 (Cornell University, Ithaca, NY, USA). Voucher specimens were fixed with 10% formalin and deposited at the Colección Nacional de Anfibios y Reptiles (CNAR), Instituto de Biología, Universidad Nacional Autónoma de México (UNAM) under the numbers IBH-31786 to 13809 (for the Mexican specimens) and at Colección de Anfibios from Instituto de Investigación de Recursos Biológicos Alexander von Humboldt under the numbers IAvH-Am-14756 to 14770 (for the Colombian specimens). The recordings were deposited in Colección de Sonidos Ambientales (CSA) of Humboldt Institute and the Colección digital de cantos grabados of Museo de Zoología de la Facultad de Ciencias, UNAM.

The spectrogram of each call was obtained from the Fourier transformation with a Blackman type window of 5 ms, with 80% superposition and a DFT of 1024 in the software Raven Pro 1.4 (Bioacoustics Research Program, 2013), and the following parameters taken: Peak frequency (frequency at maximum amplitude), length of the note (length in milliseconds of a note, where a note is a discrete series of pulses) and frequency bandwidth. We generated call figures using Seewave v. 1.6 package (Sueur et al., 2008) and in WarbleR (Araya-Salas & Smith-Vidaurre, 2017) in R (version 3.5.1) (R Core Team, 2019). The differences in the components of the song between the lineages were evaluated with a Kruskal–Wallis test and by a Wilcoxon rank-sum test. Subsequently, a 2D plot of spectral and temporal domain (peak frequency vs. call duration) was constructed as a visual inspection of acoustic space isolation.

Results

Phylogenetic analysis

The best-fit model for our dataset was HKY + I + G for 16S, GTR + G for COI, GTR + G + I for the first codon of POMC, TRNEF for the second codon of POMC, and HKY + X for the third codon for POMC. Our analysis with nuclear and mitochondrial genes clearly shows that *H. fleischmanni* is differentiated into genetic groups (Fig. 2). Two main, well-supported clades were recovered (pp > 0.95 for most cases). The first clade was divided into two lineages: a large lineage containing all samples from Mexico and Guatemala (divided into three subgroups) and a smaller one containing samples from western Honduras and a small region of eastern Guatemala. The second clade is formed by two lineages, one includes samples from east Honduras to Costa Rica (including the type locality of *H. fleischmanni*) and the second with samples from Panama to Ecuador, including the type locality of *H. tatayoi* from Venezuela. Monophyly of these groups is supported by posterior probability (PP) > 0.98 and is consistent with Bayesian and Maximum likelihood analyses of concatenated sequences.

Molecular delimitation

GMYCs and GYMCm approaches applied to the MrBayes reconstruction, suggested 17 candidate species from the *H. fleischmanni* sensu lato matrix (Fig. 2). The northern samples were grouped as six different candidate species. The likelihood value was higher in both approaches than the value for the null model (ML single = 164.22; ML multiple = 164.91; null L = 161.38), but the results of the likelihood ratio tests was significant only for the multiple analysis (LRTest = 0.058 ns for GYMCs and 0.029* for GYMCm) suggesting that multiple thresholds are necessary in different parts of the tree to delimit species.

The ML implementation of PTP and the Bayesian implementation of the method (bPTP) applied to the MrBayes tree delimited 8–11 putative species with high support values (0.99–1), for Beast and MrBayes reconstruction. The northern samples were recovered in at least four distinct lineages in all analysis and the results with Beast tree split the southern clade in three to five different species. Bayesian species delimitation on the concatenated data sets of all genes for all three scenarios support the guide tree with eight species with speciation probabilities of 1.0.

Thus, based on strict consensus of all molecular delimitation analyses here tested, our phylogenetic approach demonstrates that *H. fleischmanni* is genetically well structured into up to eight distinct lineages: (1) Chortis region of eastern Guatemala and western Honduras, (2) Pacific Mexico, (3) Gulf of Mexico, (4) Mayan region in Mexico and Guatemala, (5) Costa Rica, Honduras, and Nicaragua, (6) Ecuador, (7) western Panama, and (8) eastern Panama, Colombia, and Venezuela (Fig. 2). The uncorrected p-genetic distances among these lineages (Table 1) show values between 0.5% and 4.1% for 16S and between 2.1% and 6.5% for COI.

Morphology

The morphometric variation among lineages is reported in Table 2. For statistical analysis, we excluded the

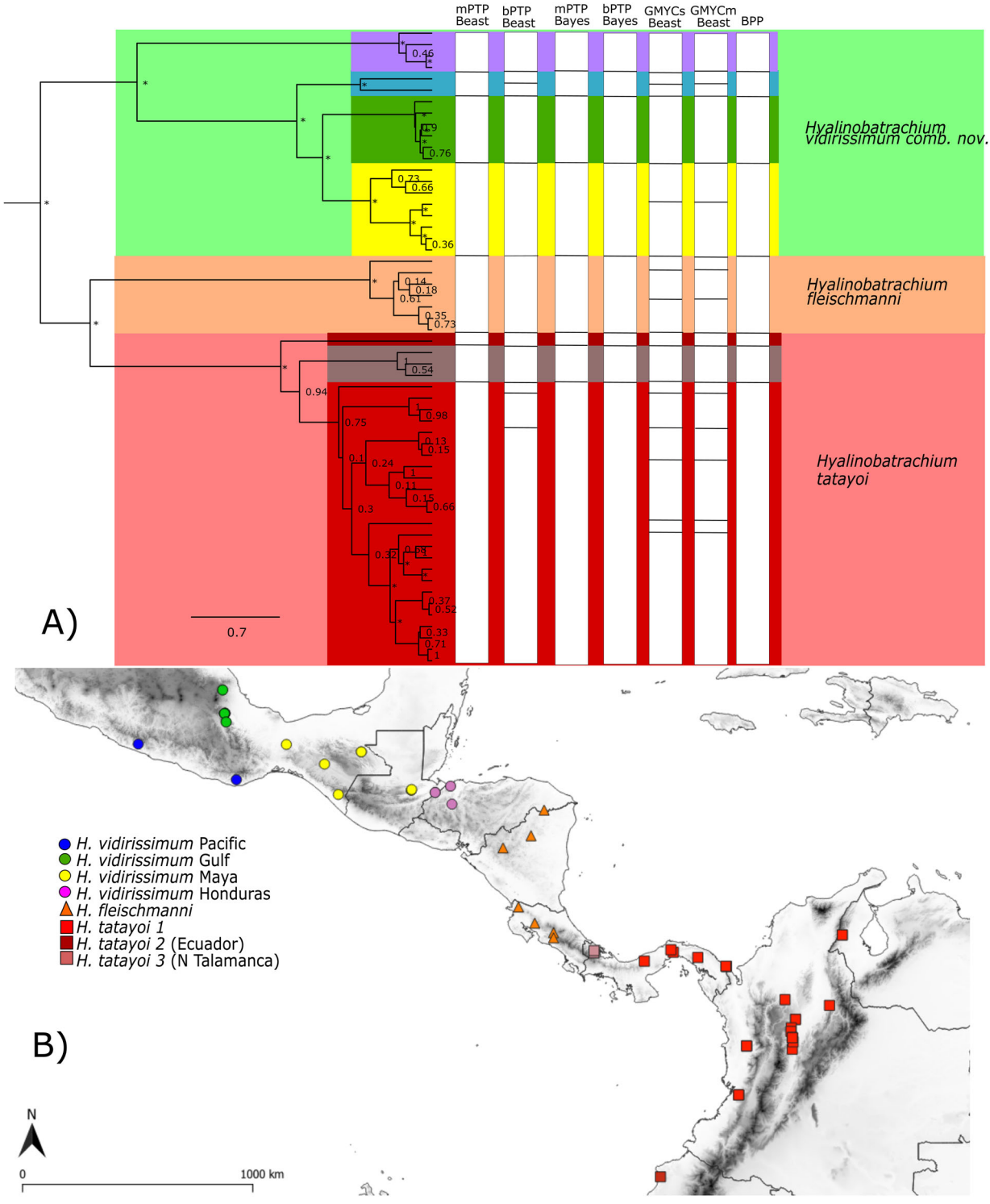

Fig. 2. (A) Ultrametric Bayesian reconstruction based on mitochondrial (16S, COI and ND1) and nuclear (POMC) sequences for *Hyalinobatrachium fleischmanni* species complex and results of molecular species delimitation by multiple approaches (GMYC multiple and single, mPTP, bPTP and BP&P). Asterisks on branches represent Bayesian posterior probabilities equal or greater than 0.95. Topologies were equal in MrBayes and BEAST. Scale bar below tree represents 0.7 million years. (B) Localization of the sequences included in molecular species delimitation analysis. Each shape represents the candidate species tested as in Figure 1.

Table 1. Pair of uncorrected p-distances for COI (upper) and 16S (lower) among the eight lineages gathered by molecular delimitation analysis.

	1	2	3	4	5	6	7	8
1- Gulf of Mexico	–	0.033	0.029	0.043	0.058	0.056	0.058	NA
2- Mayan region in Mexico and Guatemala	0.008	–	0.026	0.039	0.054	0.052	0.053	NA
3- Pacific Mexico	0.013	0.021	–	0.049	0.065	0.063	0.062	NA
4- Eastern Guatemala and Honduras	0.010	0.017	0.018	–	0.063	0.064	0.055	NA
5- Eastern Panama, Colombia and Venezuela	0.022	0.03	0.028	0.02	–	0.021	0.047	NA
6- Western Panama	0.028	0.036	0.034	0.026	0.007	–	0.041	NA
7- Costa Rica, eastern Honduras, and Nicaragua	0.03	0.033	0.041	0.03	0.021	0.027	–	NA
8- Ecuador	0.027	0.034	0.033	0.024	0.005	0.011	0.023	–

Table 2. Morphological measurements (in mm) of the lineages suggested by molecular delimitation (mean ± SD). * = metrics were not taken for the entire number of individuals. See Supplemental Material for statistical comparisons among groups.

			***H. viridissimum* comb. nov.**			
	H. fleischmanni	***H. tatayoi***	**Honduras**	**Gulf**	**Pacific**	**Maya**
N	19	85	1	28	23	12
Snout-vent length	21.67 ± 1.70	21.55 ± 1.57	21.09	23.67 ± 1.45	21.83 ± 1.03	24.11 ± 1.15
Head length	6.33 ± 0.71	6.20 ± 0.61	5.6	6.93 ± 0.56	6.21 ± 0.47	7.07 ± 0.83
Head width	8.41 ± 1.01	8.21 ± 0.63	7.77	8.97 ± 0.50	8.20 ± 0.53	9.08 ± 0.46
Interorbital distance*	3.88 ± 0.41	3.75 ± 0.50	3.66	3.98 ± 0.27	3.79 ± 0.24	4.27 ± 0.27
Eye length	2.28 ± 0.15	2.39 ± 0.29	2.48	2.38 ± 0.28	2.32 ± 0.15	2.37 ± 0.14
Eye nostril	1.86 ± 0.15	2.05 ± 0.34	1.98	2.52 ± 0.23	2.13 ± 0.31	2.48 ± 0.38
Finger III width*	1.24 ± 0.20	1.09 ± 0.16	1.16	1.25 ± 0.15	1.17 ± 0.13	1.10 ± 0.20
Femur length*	11.36 ± 0.51	12.05 ± 0.74	11.52	12.94 ± 0.79	12.40 ± 0.54	13.54 ± 0.53
Tibia length	12.08 ± 0.84	11.98 ± 0.76	11.22	12.96 ± 0.55	12.10 ± 0.75	13.34 ± 0.48

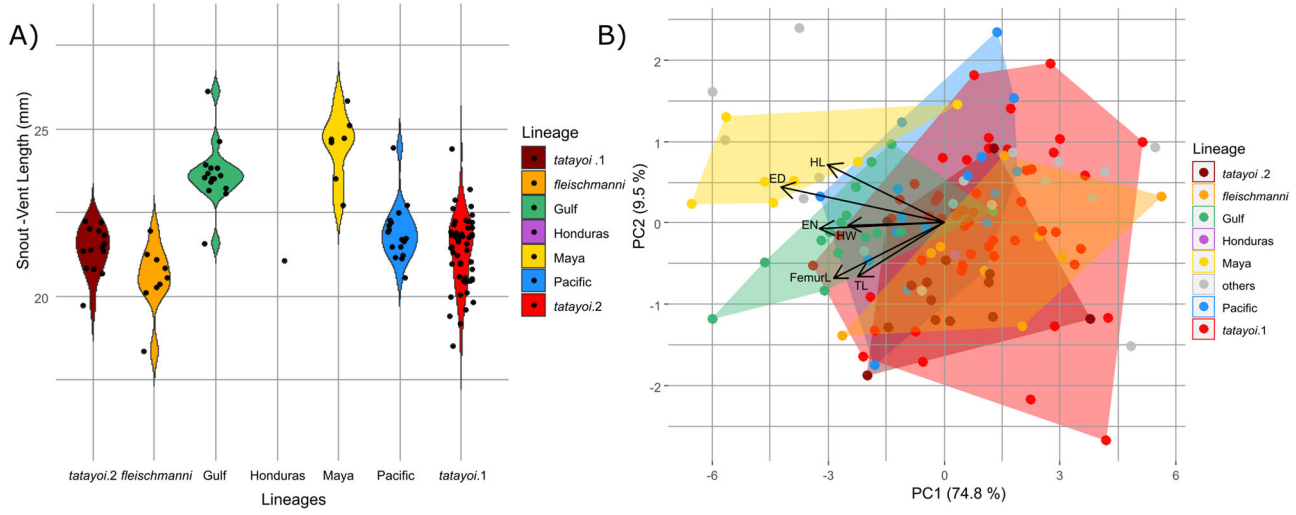

Fig. 3. (A) Violin plot of snout–vent length for males of candidate species, with Kruskal–Wallis test results per pair of lineages (statistical results are provided in Supplemental Material). (B) Principal component analyses of the residuals of the morphometric measurements against SVL for 132 males of *H. fleischmanni* complex plus other species within Hyalinobatrachium.

Chortis lineage due to the small sample size in this study (a single individual: AMNH-54777). The non-parametric tests show that males from the Mexican Gulf and Maya lineages have larger SVL compared with the other clades (Kruskal $Y = 48.055$, $P > 0.001$, $n = 115$, Fig. 3a). Similar results were encountered for the remaining metrics (Table S1). The morphospace generated by the two first components of the PCA with and without SVL correction showed no differences between the genetic groups (Fig. 3b). In both cases, the first component explains most (60.6% and 74.8%) of the variation and no metric was dominant in the contribution of the component. This absence of differentiation was also observed when including other known

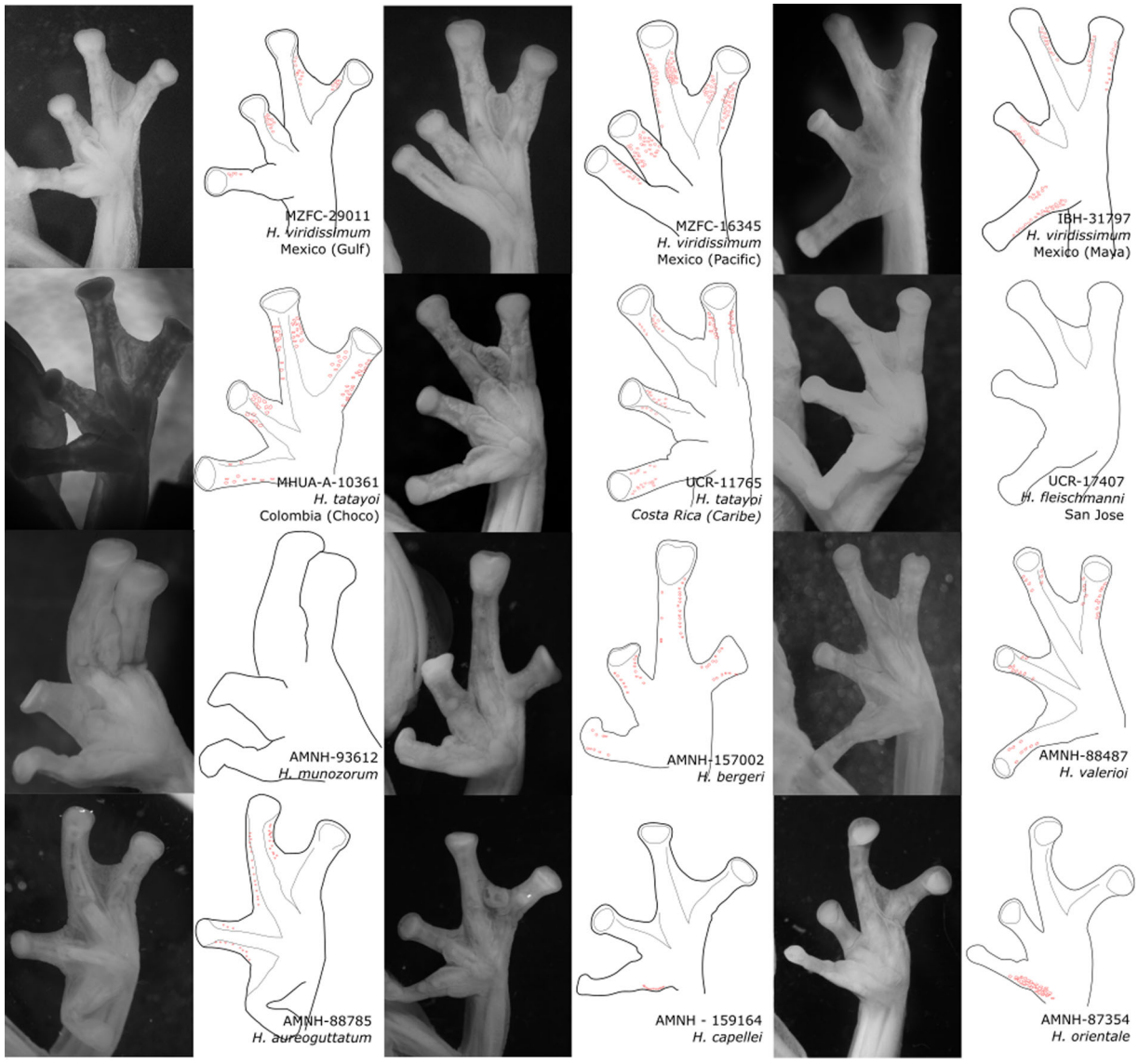

Fig. 4. Nuptial pads variation (or lack of) in some Hyalinobatrachium species. Type V forming glandular clusters in the webbing of all fingers in *H. viridissimum* **comb. nov.** (MZFC-25001, MZFC-16345, IBH-31797) and *H. tatayoi* (MHUA-A-10361, UCR-11765); absent in *H. fleischmanni* (UCR-17407) and *H. munozorum* (AMNH-93612); Type V as a discrete line in the webbing of all fingers in *H. bergeri* (AMNH-157002), *H. valerioi* (AMNH-88487), and *H. aureoguttatum* (AMNH-88785); a single line in thumb in *H. capellei* (AMNH-15916); type I-like in *H. orientale* (AMNH-87354).

Hyalinobatrachium species for the comparison (grey points).

Regarding categorical variables, the nuptial pads was the only character that shows enough variations among lineages. Males from Colombia, Ecuador, Venezuela, Panama, and the Caribbean region in Costa Rica show abundant warts on the webbing of hands (nuptial pads type V following Cisneros-Heredia & McDiarmid, 2007 categories) while males from San Jose (Costa Rica) lack warts (Fig. 4). Additionally, males from Mexican lineages show multiple states of warts within the same locality.

Bioacoustics

We obtained recordings for 64 individuals (Fig. 5). The pairwise comparisons using Wilcoxon rank sum test for each of the call variables according to the genetic clusters, showed significant differences of the Gulf and Mayan lineages with respect to the remaining lineages for the peak frequency (see Supplemental Material). There was also a significant difference in the duration of the call between the *fleischmanni* and *tatayoi* clades and among the three Mexican lineages. The frequency bandwidth showed significant differences between the lineages of the two big clades (Pacific, Gulf, and Maya

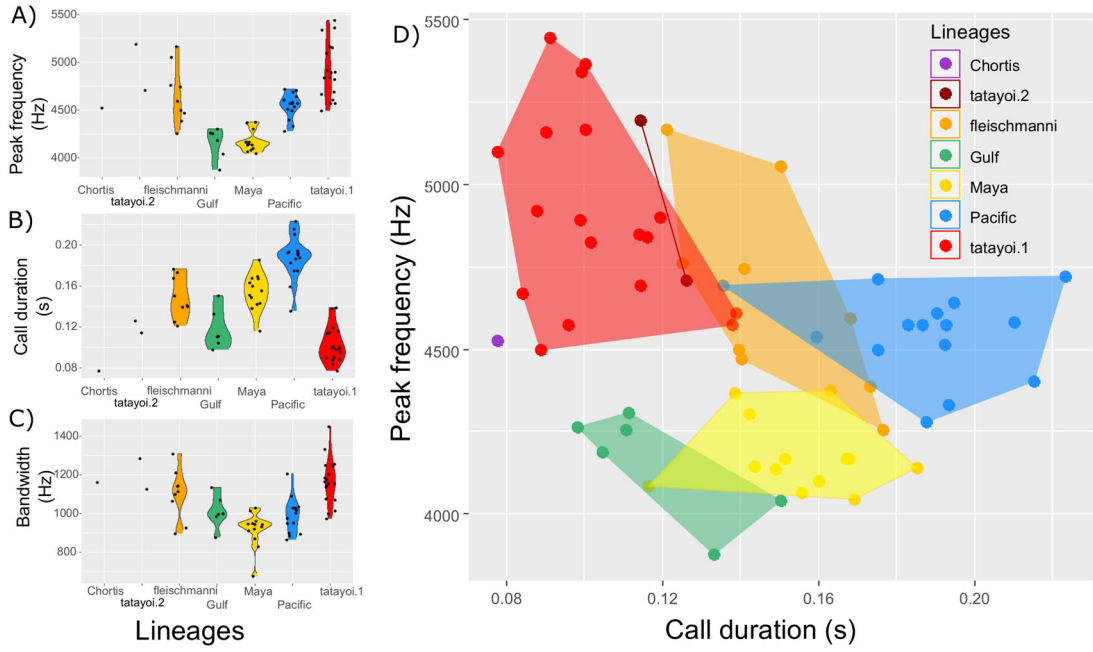

Fig. 5. (A–C) Violin plots of call variation per lineages for the three metrics analysed and (D) Acoustic space (in peak frequency vs. call duration) of the species hypothesized as hidden inside *H. fleischmanni*.

vs *fleischmanni* and *tatayoi*). In the visual inspection of acoustic space (Fig. 5D), there is an overlap between calls recorded for the two suggested lineages within *tatayoi* (*tatayoi.1* and *tatayoi.2*), calls from the Maya and Gulf lineages partially overlap, and calls from *tatayoi* and *fleischmanni* show marginal overlap in call duration. On the other hand, calls of the Pacific lineage and the single call obtained for the Chortis clade in Honduras do not overlap with any of the sister lineages (Maya and Gulf).

Taxonomic account

Based on the available evidence and following the consensus protocol for integrative taxonomy (Padial et al., 2010), the differences in molecular, bioacoustic, morphological and geographic information suggest at least three distinct species (Table 3). Therefore, we update the distribution range of two formerly described species (*H. fleischmanni* and *H. tatayoi*) and we resurrect a synonym for *H. fleischmanni* (*Hyalinobatrachium viridissimum* **comb. nov.**) to apply to the four lineages of the Northern clade. Additional information of the taxonomic conclusion is provided in the Discussion.

Species accounts

Hyalinobatrachium fleischmanni (Boettger, 1893)

Synonyms. *Hylella fleischmanni*: Boettger, 1893: 252; *Hylella chrysops*: Cope, 1894: 196**;** *Hyla fleischmanni*: Nieden, 1923: 225; *Centrolenella fleischmanni*: Noble, 1924: 69; *Centrolenella viridissima*: Taylor, 1942: 74; *Cochranella chrysops*: Taylor, 1951: 35; *Cochranella*: *fleischmanni* Taylor, 1951: 34; *Cochranella decorata*: Taylor, 1958: 50; *Cochranella millepunctata* Taylor, 1958: 53; *Cochranella fleishmanni* [*sic*]: Rivero, 1961: 153; *Centrolenella fleischmanni*: Goin, 1964: 1; *Hyalinobatrachium fleischmanni*: Ruiz-Carranza & Lynch, 1991, 24.

Holotype. Forschungsinstitut und Natur-Museum Senckenberg (SMF) 3760

Type locality. 'San José, [Cantón de San José, Provincia San José,] Costa Rica'.

Taxonomic history. *Hyalinobatrachium fleischmanni* was originally described by Boettger (1893) based on two syntypes from Canton de San José, San José Province, in Costa Rica, ~1,180 m asl (Mertens, 1967). The species was included in the *H. fleischmanni* group by Ruiz-Carranza and Lynch (1991). Starrett (1960) described the eggs and tadpole based on 16 specimens from San Jose de la Montaña, Heredia Province, Costa Rica. Savage (2002) redescribed the species including the information of the species biology, tadpole, and

Table 3. Multiple sources of evidence used in species delimitation for sister lineages inside the *H. fleischmanni* complex.

	Pairs of sister lineages			
Source of evidence	***fleischmanni / tatayoi***	***tatayoi* Colombia / Ecuador**	***viridissimum* comb. nov. Pacific / Honduras**	***viridissimum* comb. nov. Pacific / Maya**
Genetic p-distances (16S / COI)	2.1 % / 4.7 %	1.1 % / NA	1.8 % / 4.9 %	2.1 % / 2.6 %
Phylogeny-based species delimitation	delimited	delimited	delimited	delimited
Advertisement call differences	significant differences	no differences	differences (few data)	significant differences
Nuptial pads	two fixed states	one single state	unknown	highly variable
Morphometry	no differences	no differences	unknown	significant differences
Speciation event	yes	no	unknown	unknown

advertisement call description. He stated that *H. fleischmanni* is among the most studied Central American amphibians. Kubicki (2007) provides a description of the species, including a detailed range map for Costa Rica.

New data

Diagnosis. (1) Vomerine teeth absent; (2) snout rounded to semi-rounded in dorsal and truncated lateral profiles with region about nostrils slightly elevated, with a slight depression between them; (3) tympanum concealed, indistinct, lack of supratympanic fold; (4) dorsal skin shagreen; (5) ventral skin granular, transparent; (6) parietal peritoneum transparent, visceral and pericardium peritoneum covered by iridophores; (7) liver bulbous; (8) humeral spines absent; (9) hand webbing II 2+ — 3 III 2 — 2 IV, absent between fingers I and II; (10) webbing between toes I (I 1 — 2 II 1 — 2 III 1+ — 2- IV 2 1/2- — 1+ V; (11) ulnar and tarsal fold absent; (12) no nuptial pads in adult males; prepollex concealed; (13) finger I slightly larger than finger II; (14) disc of finger III width about 54% of eye diameter; (15) colour in life, dorsum lime green with yellow spots; colour of bones white; (16) colour in preservative, dorsum cream with black spots; (17) iris colouration in life golden to with dark reticulations; (18) dorsal surfaces of fingers and toes lacks melanophores; (19) males usually call below leaves (20); eggs greenish white, deposited on underside of leaves over streams as a monolayer in a laminar array; (23) snout–vent length (SVL) in males 21.7 mm ± 1.6 ($n = 15$), 26.1 females ($n = 1$).

Distribution and habitat. The species is widely distributed from 25 to 1,740 m asl in the Lowland Moist Forest, Lowland Wet Forest, Premontane Pine-Oak Forests, Premontane Wet Forest, and marginally into Lower Montane Wet Forest from the Mosquitia region of eastern Honduras and Nicaragua and southwards to the Tilarán range and Central Valley in Costa Rica (McCranie & Wilson, 2002; Sunyer et al., 2014). The species is ubiquitous and often common even in pastures and other cleared sites, where it occurs along small streams or areas with remnant riparian vegetation with an overstorey.

Advertisement call. Here we update the information of the advertisement call and the reproductive behaviour based on our results and former descriptions. The call consists of a single tonal note preceded by a short series of 3–7 pulses at a mean rate of 176.9 pulses/s (Fig. 1d). The overall call has a peak frequency of 4,659.6 ± 366.2 Hz and a duration of 148.3 ± 24.9 ms (N = 20). Males observed in Gaucimal River in Monteverde, Puntarenas and from San José (Costa Rica) call from the undersides of leaves from dusk (1800 h), with higher activity until 2100 h and decreasing until dawn (Gutiérrez-Vannucchi et al., 2019; Jacobson, 1985)

Comparisons. *Hyalinobatrachium fleischmanni* can be differentiated from most species of the genus by its visceral and pericardium peritoneum covered by iridophores. Among the species with the same condition (i.e., *H. tatayoi*, *H. viridissimum* **comb. nov.**, *H. bergeri*, *H. mondolfii*), *H. fleischmanni* differs by the lack of nuptial pads in males and by its advertisement call (Table 4) and by lack of white enamelled glands delimiting the jaw (presence of white enamelled glands delimiting the jaw in *H. viridissimum* **comb. nov.** and *H. tatayoi*). In particular, the peak frequency of *H. fleischmanni* is lower than those of *H. munozorum* which lacks nuptial pads too (4,659.6 ± 303.4 Hz and 5,011.5 ± 16.0 Hz respectively).

Conservation status. Even though the range of the species is now more restricted than before, the species can still be considered as Least Concern (LC) following IUCN (2017) categories, due to its wide distribution (more than 20,000 km^2) and high tolerance to disturbed habitats.

Table 4. Main acoustic parameters of the advertisement call of *H. fleischmanni* complex plus other closely related *Hyalinobatrachium* species (mean ± SD). Call data were obtained from the following sources: *H. carlesvilai* and *H. bergeri* (Castroviejo-Fisher et al., 2009), *H. mondolfii* and *H. kawense* Castroviejo-Fisher et al. (2011).

Species or lineage	Distribution	Call duration (s)	Peak frequency (Hz)
H. fleischmanni	Lowlands in Costa Rica and Nicaragua	0.148 ± 0.020	4659.6 ± 303.4
H. tatayoi	Widespread along Choco-Magdalena region and eastern slope of Merida range	0.105 ± 0.017	4916.2 ± 287.9
H. viridissimum **comb. nov.** Gulf	Veracruz state in Gulf of Mexico	0.118 ± 0.020	4154.5 ± 165.5
H. viridissimum **comb. nov.** Pacific	Pacific region of Madre del Sur, Mexico	0.188 ± 0.022	4549.5 ± 130.0
H. viridissimum **comb. nov.** Maya	South-eastern Chiapas of Mexico and Guatemala	0.155 ± 0.017	4174.0 ± 108.1
H. viridissimum **comb. nov.** Honduras	Nuclear Central America in Honduras	0.209	4048.2
H. carlesvilai	Amazonian Andean slopes of Peru and Bolivia	0.134 ± 0.013	4837.9 ± 85.8
H. mondolfii	Amazonas, Colombia and Pará, Brazil	0.190 ± 0.010	5011.5 ± 16.0
H. munozorum	Upper Amazon Basin in Ecuador, Colombia, and northern Bolivia	0.134 ± 0.027	5037.9 ± 244.0
H. kawense	French Guiana	0.090 ± 0.01	5285.5 ± 117.9
H. bergeri	Amazonian slopes of Andes in Peru and Bolivia	0.154 ± 0.019	4599.08 ± 69.9

Hyalinobatrachium tatayoi Castroviejo-Fisher et al., 2007

Holotype. Museo de Historia Natural La Salle (MHNLS) 17174

Type locality

'A stream near Tokuko (09°50′30.6″N, 72°49′13.6‴W; 301 m asl), Estado de Zulia, Venezuela.'

New data

Diagnosis. (1) Vomerine teeth absent; (2) snout semi-round to round in dorsal and truncate to round lateral profiles; (3) tympanum covered by skin; (4) dorsal skin shagreen; (5) ventral skin granular, transparent; (6) parietal peritoneum transparent, visceral and pericardium peritoneum covered in white iridophores; (7) liver bulbous; (8) humeral spines absent; (9) hand webbing II 2 — $3^{1/2}$ III 2 — 2 IV, absent between fingers I and II and basal between fingers II and III; (10) webbing between toes 1 — 2 II 1— 2 III 1^{-}— 2 IV 2 — 1 V; (11) enamelled ulnar and tarsal fold present; (12) nuptial pads in adult males; (13) finger I almost as long as finger II; (14) disc of finger III width about 56% of eye diameter; (15) colour in life, dorsum dark apple green with small pale yellow spots; colour of bones white; (16) colour in preservative, dorsum cream with purple melanophores; (17) iris colouration in life yellow with black flecks more concentrated towards the pupil creating a horizontal band that connects the pupil with the lateral edges of the eye; (18) dorsal surfaces of fingers lacks melanophores; small melanophores reaching the last phalange of the fifth toe; (19) males call above and below leaves (20); eggs deposited on underside of leaves over streams; (23) snout–vent length (SVL) in males 21.3 ± 1.4 mm, 21.9 ± 0.9 mm in females.

Distribution and habitat. *Hyalinobatrachium tatayoi* ranges from the Venezuelan Cordillera de Perijá, in the northern border between Colombia and Venezuela, through dry forests in middle and upper Magdalena valley including the xeric scrub ecoregion in Caribbean lowlands (Acosta-Galvis, 2012) up to the department of Tolima (Rada & Guayasamin, 2008), from the Isthmian-Atlantic moist forests (Caribbean slope of Talamanca range) in Panama and Costa Rica and from the Choco-Darien moist forests through Colombia to Esmeraldas province in Ecuador. The type locality is in submontane rainforest. The southernmost record to date is from the western Ecuador, at Cerro de Hayas (−02.7299, −79.6297, 127 m), ∼20 km south-west of Naranjal, province of Guayas, in fragments of riparian vegetation near paddocks and pastures (Cruz et al., 2017). The species ranges from sea level to 1,640 m asl.

Advertisement call. The call consists of a single note with a peak frequency of 4,916.2 ± 287.9 Hz ($n = 20$) and a duration of 104 ± 17 ms ($n = 20$). The first third of the call is pulsar composed by a series of 5–18 pulses at a mean rate of 258.6 pulses/s. The remaining two-thirds of the note are tonal (Fig. 1e-f). Calling activity began at dusk (1910 h) and decreased near midnight for Chocó and Magdalena populations in Colombia. In Ecuador, multiple males called from the upper surfaces of leaves at dusk, followed by a retreat to the undersides of the leaves after dark (Delia et al., 2010). During heavy rains, the species can be heard calling also between 0440 and 0530 h (L. Coyazos et al., unpubl. data).

Greer and Wells (1980) described the encounter and courtship calls based on individuals recorded in Barro Colorado (Panama). They described these calls in two

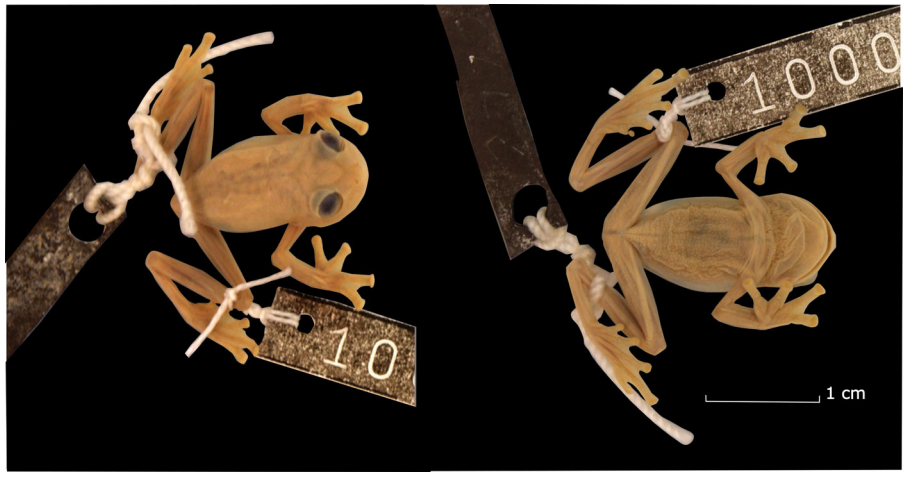

Fig. 6. Dorsal and ventral view of holotype of *Hyalinobatrachium viridissimum* **comb. nov.** (FMNH 100093). © Field Museum of Natural History. CC-BY-NC. a2dbe194-2421-472e-bb93-7290b2666a5b https://fm-digital-assets.fieldmuseum.org/426/161/C_viridissima100093d.jpg; https://fm-digitalassetsfieldmuseum.org/426/162/C_viridissima100093v.jpg (accessed 6 May 2019).

types: mews and chirps. Mew-type calls had a duration of 0.45 s and a range frequency between 4,238 and 4,852 Hz (during encounters) or a duration of 0.27 s and a range frequency between 4,194 and 4,546 Hz (during courtship). Chirp-type calls emitted during courtship had a duration of 0.10 s and a range frequency between 4,002 and 4,387 Hz.

Comparisons. *Hyalinobatrachium tatayoi* differs from most species of the genus by its visceral and pericardium peritoneum covered by iridophores. Among the species with the same condition (i.e., *H. viridissimum* **comb. nov.**, *H. bergeri*, *H. mondolfii*), *H. tatayoi* can be differentiated by its advertisement call, being shorter than all species except *H. kawense* (104 ± 17 ms for *H. tatayoi* and 0.090 ± 0.01 for *H. kawesne*, Table 4). *Hyalinobatrachium tatayoi* differs from *H. viridissimum* **comb. nov.** in the advertisement call, higher frequency (4,916.2 ± 287.9 Hz) in *H. tatayoi* (4,549.5 ± 130.0 Hz for *H. viridissimum* **comb. nov.**), and shorter duration 104 ± 17 ms in *H. tatayoi* (187.6 ± 21.6 ms for *H. viridissimum* **comb. nov.**). In this case, both species differs by the dorsal melanophores of two different sizes in *H. kawense* (small and minute), being absent or very small in *H. tatayoi*. Finally, the species differs from *H. fleischmanni*, *H. bergeri*, and *H. munozorum* by the presence of abundant nuptial pads type V in the hand webbing of males (*H. fleischmanni* and *H. munozorum* lack nuptial pads and those for *H. bergeri* form a single line; Fig. 4) and by the presence of white enamelled glands delimiting the jaw (white enamelled glands delimiting the jaw absent in *H. fleischmanni*).

Natural history. The species is found on leaves over small and big streams, calling in vegetation up to 10 metres above stream water, found also in the vegetation along big rivers such as the Atrato River in Choco (∼200 m width). The species is highly tolerant to water pollution, having been observed even downstream of mining camps. In Magdalena populations in La Dorada municipality (Caldas, Colombia), males were found during dry (February) and humid (May) seasons calling from the underside of leaves (Araceae and Heliconiaceae) and have been observed with up to three clutches. Eggs are predated by katydids and predatory wasps, but males show defensive behaviour by knocking the predator with their hind legs (Delia et al., 2010). Males show venter-to-venter combat which starts with both males dangling upside down while holding vegetation with their hind limbs and lasting for ∼20 minutes until one frog was knocked from the leaf (Delia et al., 2010). The embryonic development from cleavage to hatching was described by Salazar-Nicholls and del Pino (2015) based on samples from Ecuador.

Conservation status. The species could be considered as Least Concern (LC) following IUCN (2017) categories, due to its wide distribution and high tolerance to disturbed habitats. The species is found inside protected areas such as Katios National Park (Burbano et al., 2015) and Farallones de Cali National Park in Colombia, and in Barro Colorado Island National Monument, Panama (Greer & Wells, 1980).

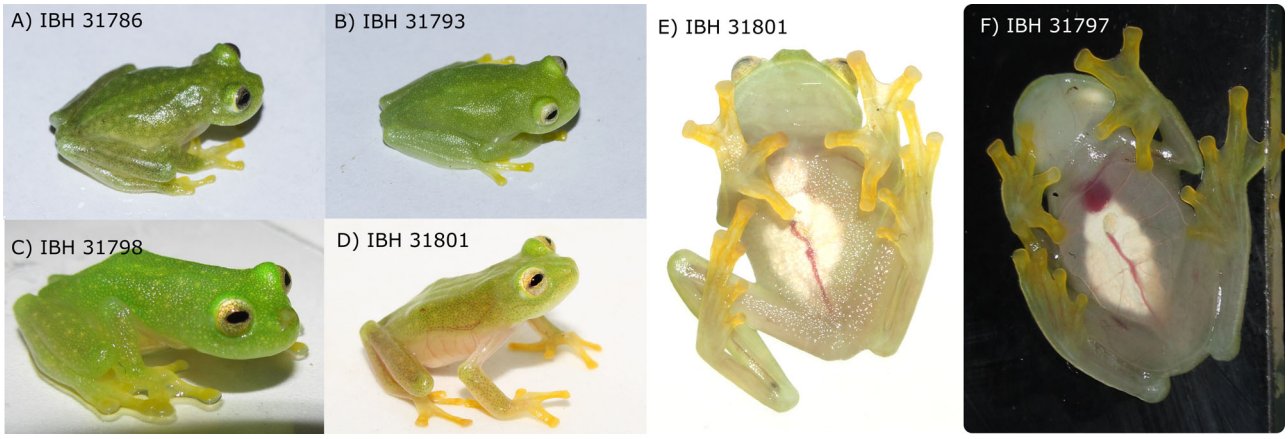

Fig. 7. Colour in life of *Hyalinobatrachium viridissimum* **comb. nov.** dorsal (A–D) and ventral (E–F), highlighting variation of iridophores in pericardium between populations.

Fig. 8. Detail of enamelled jaw and anal decoration of *Hyalinobatrachium viridissimum* **comb. nov.** (voucher MZFC-18408).

Hyalinobatrachium viridissimum **comb. nov.** (Taylor, 1942) (Figs 6, 7, 8)

Synonyms. *Centrolenella viridissima*: Taylor, 1942: 74; *Cochranella viridissima*: Taylor, 1951: 34.

Holotype. Edward H. Taylor–Hobart M. Smith collection (EHT-HMS) No. 27725, now Field Museum/FMNH 100093, Fig. 6), male, 2 August 1941, collected and described by Edward H. Taylor.

Type locality. Agua del Obispo, Guerrero, Mexico.

Generic placement. The species is placed in the genus *Hyalinobatrachium* (Ruiz-Carranza & Lynch, 1991, as modified by Guayasamin et al., 2009) on the basis of morphological and molecular data. The main diagnostic phenotypic traits of *Hyalinobatrachium* are: (1) ventral parietal peritoneum completely transparent; (2) digestive tract and bulbous liver covered by iridophores; (3) humeral spines absent; (4) white bones in life; and (5) males call from the undersides of leaves. All the aforementioned characteristics are shared by the species. Additionally, analyses of three mitochondrial genes place the species as a close relative of other *Hyalinobatrachium* species (Mendoza et al., 2019a); thus, generic placement in *Hyalinobatrachium* is unambiguous.

Diagnosis. (1) Vomerine teeth absent; (2) snout rounded to semi-rounded in dorsal and truncated lateral profiles with region about nostrils slightly elevated, with a slight depression between them; (3) tympanum concealed, indistinct, lack of supratympanic fold; (4) dorsal skin smooth; (5) ventral skin granular, transparent; (6) parietal peritoneum transparent, visceral and pericardium peritoneum covered by iridophores; (7) liver bulbous; (8) humeral spines absent; (9) hand webbing II 2+ — 31/2 III 2+ — 2- IV, absent between fingers I and II; (10) webbing between toes I 1- — 2 II 1- — 2 III 1— 3 IV 2+ — 1 V; (11) ulnar fold present, low; tarsal fold absent; (12) nuptial excrescences unpigmented between

hand webbing, Type V, prepollex concealed; (13) finger I slightly larger than finger II; (14) disc of finger III width about 51% of eye diameter; (15) colour in life, dorsum lime green with yellow spots; colour of bones white; (16) colour in preservative, dorsum cream to dark cream with black spots (in shape of asterisk and/or points) and white specks (sometimes the white sparks disappear) in visible areas of the body in rest position; (17) iris colouration in life golden to with dark flecks and reticulations and with a conspicuous silvery-white crescent-shaped mark; (18) dorsal surfaces of fingers and toes lacks melanophores; (19) males usually call below leaves; advertisement call consisting of single tonal note, with duration of 187.6 ± 21.6 ms and dominant frequency at 4,549.5 ± 130.0 Hz (20); eggs pale cream, deposited on underside of leaves over streams as a monolayer in a laminar array; (21) snout–vent length (SVL) in males 21.7 mm ± 1.6 ($n = 19$), 22.4 ± 0.6 on females ($n = 4$). The diagnosis refers to the Pacific lineage, associated with the type locality

Comparisons with other species. Most *Hyalinobatrachium viridissimum* **comb. nov.** can be differentiated from most species of the genus by its visceral and pericardium peritoneum covered by iridophores (except for some Mexican populations in Chiapas and Oaxaca with transparent peritoneum). Among the species with the same condition, *H. viridissimum* **comb. nov.** differs in having lower frequency (4,154.5 ± 165.5 Hz for populations from the Mexican Gulf and 4,549.5 ± 130.0 Hz for populations from Mexcian Pacific, Table 4). Also, *H. viridissimum* **comb. nov.** differs from *H. fleischmanni* and *H. munozorum* by the presence of Type V nuptial pads along the hand webbing (*H. fleischmanni* and *H. munozorum* lack nuptial pads).

Colour in life. Dorsum light green with yellow spots; concealed parts of limbs and ventral surfaces transparent; parietal peritoneum translucent, visceral peritoneum white and pericardium covered by iridophores (Fig. 7). Fingers and toes yellow. Jaw and folds in hand and feet and cloacal decoration enamelled (shiny white) (Fig. 8). Iris cream to yellow with dark points connected by a golden reticulation, being more intense near the horizontal pupil. The iridophores in pericardium are reduced or absent in some Mexican populations detected in San Gabriel Mixtepec, Oaxaca (Twomey et al., 2014).

Distribution. We consider *H. viridissimum* **comb. nov.** to be restricted to moist and cloud forest in the Pacific region in Madre del Sur subprovince (Morrone, 2017) in the Mexican states of Oaxaca and Guerrero from 20 to 1,275 m asl. We tentatively assign populations of the moist and cloud forests in Mexico in the states of Chiapas, Oaxaca, Veracruz, Puebla, and Tabasco, as well as those on both sides of the Motagua-Polochic-Jocotán fault system through lowlands of Guatemala and Honduras to this taxon, but further work is necessary to identify the species status of those populations.

Colour in preservative. Dorsum of head, body and limbs light cream to dark cream with black spots as points or small asterisks. Heart and all other visceral peritonea covered by white pericardium (Fig. 6).

Advertisement call. The call of Guerrero populations consists of a single and high-pitched tonal note with a peak frequency of 4,549.5 ± 130.0 Hz and a duration of 187.6 ± 21.6 ms without no major frequency modulation. The call shows a slight pulsar region in the first 28.1 ms (Fig. 1a) composed by a series of 1–6 pulses at a mean rate of 117.4 pulses/s ($n = 15$). Males usually call from underside of the leaves from 1 metre to more than 10 metres above water, although in a few cases they are observed calling from the upper-side of the leaves (Delia et al., 2010).

We also recorded the courtship call (Fig. 9) of a male (IBH-31806) from the underside of a Myrtaceae leaf at 2.5 m above the running water at 1940 h (before sunset) in San Vicente de Benitez municipality (Guerrero, Mexico). The call was emitted when another individual (likely a female) approached from the upper-side of the leaf. This call consists of 1–9 individual pulses followed sometimes by a long note, and it was emitted in call groups with short silent intervals of 536.3 ± 209.2 ms between calls. The courtship call shows a mean peak frequency of 4,441.4 Hz (± 88.8 Hz, $n = 88$), high variable duration (110.1 ± 47.5 ms, $n = 88$, T° = 17 °C) and significant complexity and frequency modulation compared with the advertisement call.

Natural history. The species can be seen in vegetation along small and medium streams in conserved and disturbed habitats, including coffee plantations. Males can be heard calling from low to extremely high altitudes from the running water from dusk (1800–1900 h) and call rate goes down after midnight.

In Atoyac de Alvarez municipality (Guerrero), the species is syntopic with *Agalychnis moreletii* (Duméril, 1853), *Hypopachus variolosus* (Cope, 1866), *Tlalocohyla smithii* (Boulenger, 1902), *Leptodactylus melanonotus* (Hallowell, 1861), *Lithobates sierramadrensis* (Taylor, 1939), *Chaladrahyla pinorum* (Taylor, 1937), *Ptychohyla leonhardschultzei* (Ahl, 1934),

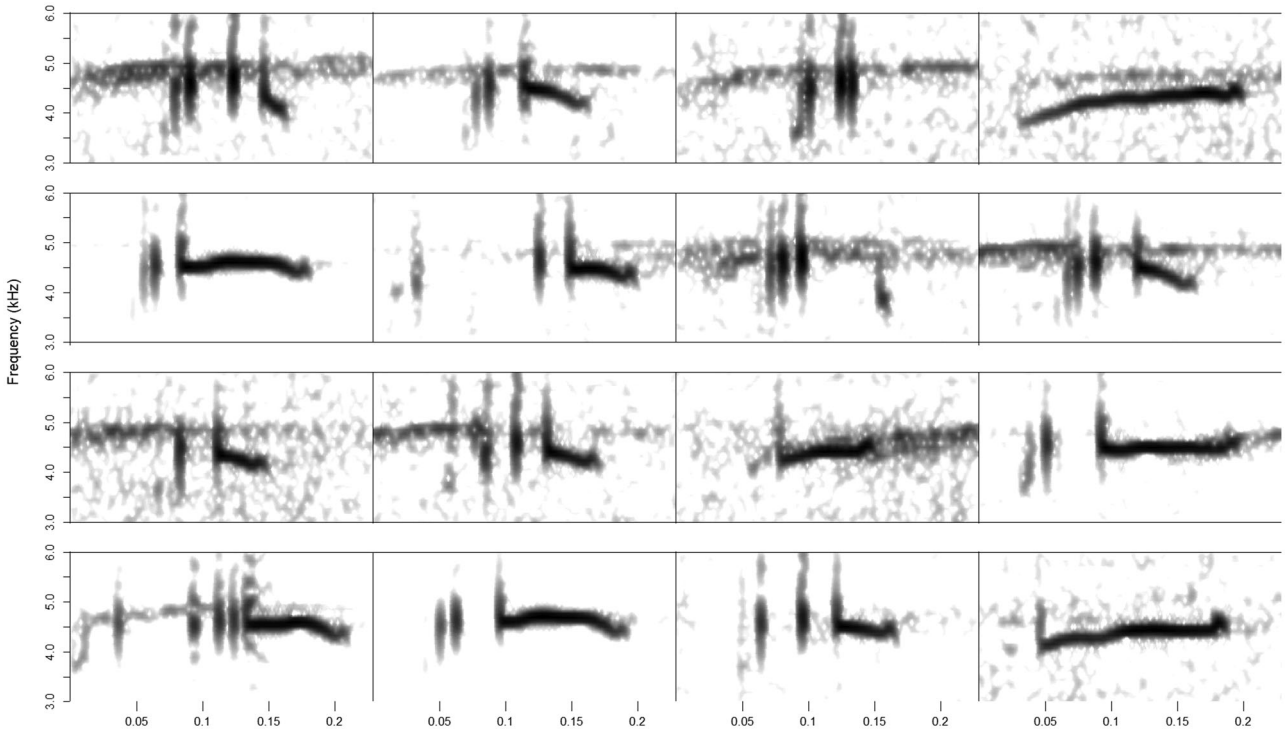

Fig. 9. Spectrograms of variation in courtship calls of *Hyalinobatrachium viridissimum* **comb. nov.** Voucher IBH-31806, T = 17 °C, SVL = 22.7 mm.

Rhinella horribilis (Wiegmann, 1833), and *Sarcohyla pentheter* (Adler, 1965).

Eggs are usually deposited in the underside of leaves above running water (Delia et al., 2013; Delia et al., 2010). Clutches have 22–23 pale-green eggs in a monolayer. The tadpole was described by Duellman and Tulecke (1960) based on individuals collected from the type locality in Agua del Obispo. The species shows parental care with brooding behaviour, males sleep next to the egg mass during the daytime and return to brood eggs three to five times in a single evening.

Etymology. The original name *viridissima* was adjusted to fit with the neutral gender of *Hyalinobatrachium* according to the Code (ICZN 1999). The name is derived from the Latin *viridis* meaning 'green' with the neutral superlative *-imum* to refer to 'the most' in reference to its bright green colour.

Conservation status. Considering that to date there is inadequate information to make a direct, or indirect, assessment of its risk of extinction based on its distribution and/or population status, the species could be considered as Data Deficient (DD) following IUCN (2017) categories.

Discussion

Neotropical anurans including glassfrogs frequently have high levels of cryptic diversity, and species richness is commonly underestimated (Fouquet et al., 2007; Funk et al., 2012; Guarnizo et al., 2015; Lyra et al., 2017; Paez-Vacas et al., 2019). The more frequent use of molecular data in taxonomy, plus DNA barcoding techniques, and automatized species delimitation analysis have increasingly revealed previously hidden species (Amador et al., 2018; Gehara et al., 2014; Ortega-Andrade et al., 2015). Unfortunately, some of these studies lack the integration of morphological, behavioural variation, and multilocus genetic data to most effectively test taxonomic hypotheses and to formally recognize cryptic species (Vences et al., 2013). In the past decade, the incorporation of molecular, morphological, and bioacoustic characters has been very useful to discover and describe new hidden species of glassfrogs for the Guiana Shield (Castroviejo-Fisher et al.,

2011), northern Peru (Twomey et al., 2014), and Colombia (Rada et al., 2019).

Our integrated study of species delimitation within the *H. fleischmanni* complex will be useful for future studies of the diversity and conservation of these frogs (Bickford et al., 2007; Funk et al., 2012). Similar to many amphibian species and in particular for *Hyalinobatrachium*, the species complex *H. fleischmanni* has been treated as a single species, due to its superficially uniform morphology across its distribution, even though Taylor & Tulecke (1960) found differences in tadpoles from Guerrero and Costa Rica, while Delia et al. (2010) found differences in the reproductive behaviour of Mexican and Ecuadorian lineages. Despite the apparent morphological conservatism in *H. fleischmanni*, here we reported differences on genetic, morphological, and acoustic characters.

Although molecular delimitation analysis has been used for the detection of cryptic species in amphibians and reptiles (e.g. Blair & Bryson Jr, 2017; Kuchta et al., 2016), it has been questioned when used as a definitive evidence of speciation processes since it assumes no population structure within lineages after speciation occurred (see Jackson et al., 2017; Sukumaran & Knowles, 2017). Therefore, molecular delimitation analysis tends to misidentify population structure as species boundaries. In populations with limited dispersal capacity or isolated by non-permeable barriers to gene flow, the phylogeographic structure is commonly pronounced (Avise, 2000; Hickerson et al., 2010), generating independence among populations (Hey & Pinho, 2012), sometimes congruent with phenotypic variation (Zamudio et al., 2016). Therefore, a rigorous interpretation of all sources of evidence is mandatory to identify if the differences detected by those methods actually correspond with speciation.

In our case, despite the fact that species delimitation analysis split *H. tatayoi* in three candidate species (Fig. 2), genetic distances with other populations were very low (16S = 0.8%, COI < 2.0%) compared with the candidate species thresholds suggested by Vences et al. (2005) (5% divergence for 16S and 10% for COI), Fouquet et al. (2007) (3% for 16S in Neotropical frogs), and Crawford et al. (2010) (8% for COI and 2% for 16S). As for molecular species delimitation, genetic distances alone must be used with caution, always with a geographic, phylogenetic, and integrative framework.

Furthermore, advertisement calls, morphometry, and nuptial pads of Ecuadorian populations show no differences regarding those from the remaining *H. tatayoi* populations. Therefore, we apply the name of *H. tatayoi* to all populations from Colombia, Venezuela, Panama, Ecuador, and Caribbean slope of Costa Rica.

On the other hand, we face a complex situation regarding divergence among allopatric lineages herein tentatively assigned to *H. viridissimum* **comb. nov.**. Molecular delimitation analysis suggested four lineages within the *H. viridissimum* **comb. nov.** clade, and bioacoustic analysis shows differences in acoustic space occupied by some of the genetic clusters (Fig 4.c). The most divergent populations (from the Chortis region, western Honduras, and eastern Guatemala) are separated from the remaining by the Motagua depression, an important geographic barrier delimiting distinct communities of vertebrates (Barrera-Guzmán et al., 2012; Ornelas et al., 2010; Rovito et al., 2015; Hofmann & Townsend, 2017). The remaining three lineages within the *H. viridissimum* clade show relatively low genetic distances (Table 1), even compared with values obtained in other sister glassfrog species (Amador et al., 2018; Rada et al., 2017; Twomey et al., 2014). We are in the process of acquiring additional morphological and acoustic data for Honduran specimens to evaluate the distinctiveness of this most-divergent lineage of the Northern clade. Until the status of Chortis populations can be resolved, the differences in call and morphology of the remaining inner clusters cannot be determined as products of rapid speciation events or structured populations showing local adaptation or divergence. Meanwhile, we suggest that the different populations of *H. viridissimum* **comb. nov.** at minimum constitute early stages of allopatric speciation and should be handled as independent evolutionarily significant units (ESUs) for conservation strategies (Moritz, 1994).

Populations formerly designated as *H. fleischmanni* in El Salvador have been registered in Cantón Montenegro, Metapán municipality, Santa Ana department (Köhler et al., 2005), in the Área Natural Rio Sapo, Arambala municipality, Morazán department (Henríquez & Greenbaum, 2014), and recently in the Area Natural Bosque de Cinquera in the Cabañas department (Segura et al., 2018). Due to its geographic location adjacent to both *H. fleischmanni* and *H. viridissimum* **comb. nov.**, it is necessary to obtain genetic, morphological, and/or bioacoustic information to compare with those species to determine to which clade these populations would be assigned.

The identity of *H. fleischmanni* sensu stricto was strongly supported for populations from east of Chortis highlands of Honduras to the Tilarán range and Central Valley of Costa Rica based on the uniformity of the morphological and bioacoustic traits (Figs 3 and 5), together with the absence of significant genetic isolation among populations (Fig. 2). Although the Talamanca range might be isolating *H. fleischmanni* and *H. tatayoi* populations (Mendoza et al., 2019a), considering the

wide distribution of both lineages in Costa Rica, it is necessary to perform an exhaustive revision of individuals and if possible some controlled reproductive experiments to identify if there are regions of secondary contact and hybridization between both species.

Further sampling at under-represented areas (i.e., Chortis region) is imperative in this case, and species delimitation by next-generation sequencing can provide robust phylogenies based on a massive number of independent loci. Speciation is not an instantaneous event and it ranges from continuous variation to population differentiation, ecotype formation, speciation, and post-speciation divergence (Nosil et al., 2009). Correct species delineation is fundamental to the discovery of life's diversity (Dayrat, 2005), but is not trivial given the biological, climatic, and geological changes and complexities such as those from Central American region (Bagley & Johnson, 2014). This study exemplifies how different lines of evidence can be integrated as a powerful tool to solve longstanding taxonomic problems and to discover cryptic lineages within diverse and taxonomically complex groups such as *Hyalinobatrachium*.

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Disclosure statement

No potential conflict of interest was reported by the authors.

Supplemental data

Supplemental data for this article can be accessed here: https://dx.doi.org/10.1080/14772000.2020.1776781.

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References

Acosta-Galvis, A. (2012). First record of Hyalinobatrachium fleischmanni (Boettger, 1893) (Anura: Centrolenidae) from the Caribbean region of Colombia. *Check List, 8*, 794–795.

Amador, L., Parada, A., D'Elía, G., & Guayasamin, J. M. (2018). Uncovering hidden specific diversity of Andean glassfrogs of the *Centrolene buckleyi* species complex (Anura: Centrolenidae). *PeerJ.*, *6*, e5856. https://doi.org/10.7717/peerj.5856

Araya-Salas, M., & Smith-Vidaurre, G. (2017). warbleR: An R package to streamline analysis of animal acoustic signals. *Methods in Ecology and Evolution*, *8*, 184–191.

Avise, J. C. (2000). *Phylogeography. The history and formation of species*. Harvard University Press.

Barrera-Guzmán, A. O., Milá, B., Sánchez-González, L. A., & Navarro-Sigüenza, A. G. (2012). Speciation in an avian complex endemic to the mountains of Middle America (*Ergaticus*, Aves: Parulidae). *Molecular Phylogenetics and*

Evolution, *62*, 907–920. – https://doi.org/10.1016/j.ympev.2011.11.020

Barrio-Amorós, C. L. (2004). Amphibians of Venezuela systematic list, distribution and references, an update. *Revista Ecología Latino Americana*, *9*, 1–48.

Bagley, J. C., & Johnson, J. B. (2014). Phylogeography and biogeography of the lower Central American Neotropics: diversification between two continents and between two seas. *Biological Reviews, 89*, 767–790.

Bickford, D., Lohman, D. J., Sodhi, N. S., Ng, P. K. L., Meier, R., Winker, K., Ingram, K. K., & Das, I. (2007). Cryptic species as a window on diversity and conservation. *Trends in Ecology & Evolution*, *22*, 148–155. https://doi.org/10.1016/j.tree.2006.11.004

Bioacoustics Research Program. (2013). *Raven Pro: Interactive Sound ANalysis Software* (Version 1.5) [Computer Software]. The Cornell Lab of Ornithology.

Blair, C., & Bryson, R. W. Jr, (2017). Cryptic diversity and discordance in single-locus species delimitation methods within horned lizards (Phrynosomatidae: *Phrynosoma)*. *Molecular Ecology Resources*, *17*, 1168–1182. https://doi.org/10.1111/1755-0998.12658

Boettger, O. (1893). Ein neuer Laubfrosch aus Costa Rica. *Bericht über die Senckenbergische Naturforschende Gesellschaft in Frankfurt am Main, 1893*, 251–252.

Burbano, C., Bolívar, W., & Giraldo, A. (2015). Anura assemblages in three areas with human intervention in the National Natural Park Los Katíos (Colombia). *Boletín Científico. Centro de Museos. Museo de Historia Natural*, *19*, 157–170. https://doi.org/10.17151/bccm.2015.19.1.11

Castroviejo-Fisher, S., Ayarzagüena, J., & Vilà, C. (2007). A new species of *Hyalinobatrachium* (Centrolenidae: Anura) from Serrania de Perijá, Venezuela. *Zootaxa*, *1441*, 51–62. https://doi.org/10.11646/zootaxa.1441.1.4

Castroviejo-Fisher, S., Guayasamin, J. M., & Kok, P. J. R. (2009). Species status of *Centrolene lema* Duellman and Señaris, 2003 (Amphibia: Centrolenidae) revealed by Integrative Taxonomy. *Zootaxa*, *1980*, 16–28. https://doi.org/10.11646/zootaxa.1980.1.2

Castroviejo-Fisher, S., Moravec, J., Aparicio, J., Guerrero-Reinhard, M., & Calderon, G. (2011). DNA taxonomy reveals two new species records of *Hyalinobatrachium* (Anura: Centrolenidae) for Bolivia. *Zootaxa*, *2798*, 64–68. https://doi.org/10.11646/zootaxa.2798.1.6

Cisneros-Heredia, D. F., & McDiarmid, R. W. (2007). Revision of the characters of Centrolenidae (Amphibia: Anura: Athesphatanura), with comments on its taxonomy and the description of new taxa of glassfrogs. *Zootaxa*, *1572*, 1–82. https://doi.org/10.11646/zootaxa.1572.1.1

Cope, E. D. (1894). Third addition to a knowledge of the Batrachia and Reptilia of Costa Rica. *Proceedings of the Academy of Natural Sciences of Philadelphia*, *46*, 194–206.

Crawford, A. J., Lips, K. R., & Bermingham, E. (2010). Epidemic disease decimates amphibian abundance, species diversity, and evolutionary history in the highlands of central Panama. *Proceedings of the National Academy of Sciences*, *107*, 13777–13782. https://doi.org/10.1073/pnas.0914115107

Cruz, F. K., Urgiles, V. L., Sánchez-Nivicela, J. C., Siddons, D. C., & Cisneros-Heredia, D. (2017). Southernmost records of *Hyalinobatrachium fleischmanni* (Anura: Centrolenidae). *Check List*, *13*, 67–70. https://doi.org/10.15560/13.4.67

Dayrat, B. (2005). Towards integrative taxonomy. *Biological Journal of the Linnean Society*, *85*, 407–417. https://doi.org/10.1111/j.1095-8312.2005.00503.x

Delia, J., Bravo-Valencia, L., & Warkentin, K. M. (2017). Patterns of parental care in Neotropical glassfrogs: fieldwork alters hypotheses of sex-role evolution. *Journal of Evolutionary Biology*, *30*, 898–914. https://doi.org/10.1111/jeb.13059

Delia, J., Cisneros-Heredia, D. F., Whitney, J., & Murrieta-Galindo, R. (2010). Observations on the reproductive behavior of a Neotropical glassfrog, *Hyalinobatrachium fleischmanni* (Anura: Centrolenidae). *South American Journal of Herpetology*, *5*, 1–12. https://doi.org/10.2994/057.005.0101

Delia, J. R., Ramírez-Bautista, A., & Summers, K. (2013). Parents adjust care in response to weather conditions and egg dehydration in a Neotropical glassfrog. *Behavioral Ecology and Sociobiology*, *67*, 557–569. https://doi.org/10.1007/s00265-013-1475-z

deQueiroz, K. (2007). Species concepts and species delimitation. *Systematic Biology*, *56*, 879–886.

Duellman, W. E., & Tulecke, J. B. (1960). The distribution, variation, and life history of the frog *Cochranella viridissima* in Mexico. *American Midland Naturalist*, *63*, 392–397. https://doi.org/10.2307/2422800

Ezard, T., Fujisawa, T., & Barraclough, T. G. (2009). Splits: species' limits by threshold statistics. *R Package Version*, *1*, r29.

Fouquet, A., Gilles, A., Vences, M., Marty, C., Blanc, M., & Gemmell, N. J. (2007). Underestimation of species richness in Neotropical frogs revealed by mtDNA analyses. *PLoS One*, *2*, e1109. https://doi.org/10.1371/journal.pone.0001109

Frost, D. R. (2019). Amphibian Species of the World: an Online Reference. Version 6.0 (Accessed 5 Aug 2019). Electronic Database accessible at http://research.amnh.org/herpetology/amphibia/index.html. American Museum of Natural History, New York, USA.

Fujisawa, T., & Barraclough, T. G. (2013). Delimiting species using single-locus data and the Generalized Mixed Yule Coalescent approach: a revised method and evaluation on simulated data sets. *Systematic Biology*, *62*(5), 707–724. https://doi.org/10.1093/sysbio/syt033

Funk, W. C., Caminer, M., & Ron, S. R. (2012). High levels of cryptic species diversity uncovered in Amazonian frogs. *Proceedings of the Royal Society B: Biological Sciences*, *279*, 1806–1814. https://doi.org/10.1098/rspb.2011.1653

Gehara, M., Crawford, A. J., Orrico, V. G. D., Rodríguez, A., Lötters, S., Fouquet, A., Barrientos, L. S., Brusquetti, F., De la Riva, I., Ernst, R., Urrutia, G. G., Glaw, F., Guayasamin, J. M., Hölting, M., Jansen, M., Kok, P. J. R., Kwet, A., Lingnau, R., Lyra, M., … Köhler, J. (2014). High levels of diversity uncovered in a widespread nominal taxon: continental phylogeography of the neotropical tree frog *Dendropsophus minutus*. *Public Library of Science One*, *9*, e103958https://doi.org/10.1371/journal.pone.0103958

Greer, B. J., & Wells, K. D. (1980). Territorial and reproductive behavior of the tropical American frog *Centrolenella fleischmanni*. *Herpetologica*, *36*, 318–326.

Guarnizo, C. E., Paz, A., Muñoz-Ortiz, A., Flechas, S. V., Méndez-Narváez, J., & Crawford, A. J. (2015). DNA barcoding survey of anurans across the Eastern Cordillera of Colombia and the impact of the Andes on cryptic

diversity. *Public Library of Science One*, *10*, e0127312https://doi.org/10.1371/journal.pone.0127312

Guayasamin, J. M., Castroviejo-Fisher, S., Ayarzagüena, J., Trueb, L., & Vilà, C. (2008). Phylogenetic relationships of glassfrogs (Centrolenidae) based on mitochondrial and nuclear genes. *Molecular Phylogenetics and Evolution*, *48*, 574–595. https://doi.org/10.1016/j.ympev.2008.04.012

Guayasamin, J. M., Castroviejo-Fisher, S., Trueb, L., Ayarzagüena, J., Rada, M., & Vilà, C. (2009). Phylogenetic systematics of Glassfrogs (Amphibia: Centrolenidae) and their sister taxon *Allophryne ruthveni*. *Zootaxa*, *2100*, 1–97. https://doi.org/10.11646/zootaxa.2100.1.1

Gutiérrez-Vannucchi, A. C., Rodríguez-Umaña, P., Sandoval, L., & García-Rodríguez, A. (2019). Variación en la actividad acústica de *Hyalinobatrachium fleischmanni* (Anura: Centrolenidae) en un ambiente urbano con diferentes niveles de ruido antropogénico. *Revista de Biología Tropical*, *67*, S249–S258. https://doi.org/10.15517/rbt.v67i2SUPL.37237

Henríquez, V., & Greenbaum, E. (2014). Geographic distribution: *Hyalinobatrachium fleischmanni* (Fleischmann's Glass Frog). *Herpetological Review*, *45*, 87.

Hey, J., & Pinho, C. (2012). Population genetics and objectivity in species diagnosis. *Evolution; International Journal of Organic Evolution*, *66*, 1413–1429. https://doi.org/10.1111/j.1558-5646.2011.01542.x

Hickerson, M. J., Carstens, B. C., Cavender-Bares, J., Crandall, K. A., Graham, C. H., Johnson, J. B., Rissler, L., Victoriano, P. F., & Yoder, A. D. (2010). Phylogeography's past, present, and future: 10 years after Avise, 2000. *Molecular Phylogenetics and Evolution*, *54*, 291–301. https://doi.org/10.1016/j.ympev.2009.09.016

Hofmann, E. P., & Townsend, J. H. (2017). Origins and biogeography of the Anolis crassulus subgroup (Squamata: Dactyloidae) in the highlands of Nuclear Central America. *BMC Evolutionary Biology*, *17*, 267.

Jackson, N. D., Carstens, B. C., Morales, A. E., & O'Meara, B. C. (2017). Species delimitation with gene flow. *Systematic Biology*, *66*, 799–812. https://doi.org/10.1093/sysbio/syw117

Jacobson, S. K. (1985). Reproductive behavior and male mating success in two species of glass frogs (Centrolenidae). *Herpetologica*, *41*, 396–404.

Kapli, P., Lutteropp, S., Zhang, J., Kobert, K., Pavlidis, P., Stamatakis, A., & Flouri, T. (2017). Multi-rate Poisson tree processes for single-locus species delimitation under maximum likelihood and Markov chain Monte Carlo. *Bioinformatics (Oxford, England))*, *33*, 1630–1638. https://doi.org/10.1093/bioinformatics/btx025

Knowles, L. L., & Carstens, B. C. (2007). Delimiting species without monophyletic gene trees. *Systematic Biology*, *56*, 887–895. https://doi.org/10.1080/10635150701701091

Köhler, J., Jansen, M., Rodríguez, A., Kok, P. J. R., Toledo, L. F., Emmrich, M., Glaw, F., Haddad, C. F. B., Rödel, M.-O., & Vences, M. (2017). The use of bioacoustics in anuran taxonomy: theory, terminology, methods and recommendations for best practice. *Zootaxa*, *4251*(1), 1–124. https://doi.org/10.11646/zootaxa.4251.1.1

Köhler, G., Vesely, M., & Greenbaum, E. (2005 "2006"). *The Amphibians and Reptiles of El Salvador*. Krieger Publishing Company.

Kubicki, B. (2007). *Ranas de vidrio de Costa Rica*. Instituto Nacional de Biodiversidad INBio.

Kuchta, S. R., Brown, A. D., Converse, P. E., & Highton, R. (2016). Multilocus phylogeography and species delimitation in the Cumberland Plateau Salamander, *Plethodon kentucki*: Incongruence among data sets and methods. *Public Library of Science One*, *11*, e0150022https://doi.org/10.1371/journal.pone.0150022

Kumar, S., Stecher, G., & Tamura, K. (2016). MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Molecular Biology and Evolution*, *33*, 1870–1874. https://doi.org/10.1093/molbev/msw054

Leaché, A. D., & Fujita, M. K. (2010). Bayesian species delimitation in West African forest geckos (*Hemidactylus fasciatus*). *Proceedings of the Royal Society B: Biological Sciences*, *277*, 3071–3077. https://doi.org/10.1098/rspb.2010.0662

Lyra, M. L., Haddad, C. F., de Azeredo-Espin, A. M. L. (2017). Meeting the challenge of DNA barcoding Neotropical amphibians: polymerase chain reaction optimization and new COI primers. *Molecular Ecology Resources*, *17*, 966–980. https://doi.org/10.1111/1755-0998.12648

Malavasi, V., Škaloud, P., Rindi, F., Tempesta, S., Paoletti, M., & Pasqualetti, M. (2016). DNA-based taxonomy in ecologically versatile microalgae: a re-evaluation of the species concept within the coccoid green algal genus Coccomyxa (Trebouxiophyceae, Chlorophyta). *PLoS One, 11*, e0151137.

McCranie, J .R., & Wilson, L. D. (2002). *The amphibians of honduras. Society for the study of amphibians and reptiles*, 625.

Mendoza, A. M., Bolivar-G, W., Vázquez-Domínguez, E., Ibañez, R., & Parra-Olea, G. (2019a). The role of Central American barriers in the phylogeographic structure of the northern glass frog *Hyalinobatrachium fleischmanni* (Anura: Centrolenidae). *PeerJ*, *7*, e6115. https://doi.org/10.7717/peerj.6115

Mendoza-Henao, A. M., Márquez, R., Molina-Zuluaga, C., Mejía-Vargas, D., & Palacios-Rodríguez, P. (2019b). New records and distribution extension of the rare glassfrog *Hyalinobatrachium chirripoi* (Anura: Centrolenidae) throughout the Chocó-Magdalena region in Colombia. *Amphibian & Reptile Conservation*, *13*, 145–151.

Mertens, R. (1967). Die herpetologische Sektion des Natur-Museums und Forschungs-Institutes Senckenberg in Frankfurt a. M. nebst einem Verseichnis ihrer Typen. *Senckenbergiana Biologica*, *48*, 1–106.

Moritz, C. (1994). Defining 'evolutionarily significant units' for conservation. *Trends in Ecology & Evolution*, *9*, 373–375. https://doi.org/10.1016/0169-5347(94)90057-4

Morrone, J. J. (2017). Biogeographic regionalization of the Sierra Madre del Sur province, Mexico. *Revista Mexicana de Biodiversidad*, *88*, 710–714. https://doi.org/10.1016/j.rmb.2017.07.012

Nosil, P., Harmon, L. J., & Seehausen, O. (2009). Ecological explanations for (incomplete) speciation. *Trends in Ecology & Evolution*, *24*, 145–156. https://doi.org/10.1016/j.tree.2008.10.011

Ornelas, J. F., Ruiz-Sánchez, E., & Sosa, V. (2010). Phylogeography of *Podocarpus matudae* (Podocarpaceae): pre-Quaternary relicts in northern Mesoamerican cloud forests. *Journal of Biogeography*, *37*, 2384–2396. https://doi.org/10.1111/j.1365-2699.2010.02372.x

Ortega-Andrade, H. M., Rojas-Soto, O. R., Valencia, J. H., de los Monteros, A. E., Morrone, J. J., Ron, S. R., &

Cannatella, D. C. (2015). Insights from integrative systematics reveal cryptic diversity in *Pristimantis* frogs (Anura: Craugastoridae) from the Upper Amazon Basin. *Public Library of Science One*, *10*, e0143392https://doi.org/10.1371/journal.pone.0143392

Padial, J. M., Köhler, J., Muñoz, A., & De la Riva, I. (2008). Assessing the taxonomic status of tropical frogs through bioacoustics: geographical variation in the advertisement calls in the *Eleutherodactylus discoidalis* species group (Anura). *Zoological Journal of the Linnean Society*, *152*, 353–365. https://doi.org/10.1111/j.1096-3642.2007.00341.x

Padial, J. M., Miralles, A., De la Riva, I., & Vences, M. (2010). The integrative future of taxonomy. *Frontiers in Zoology*, *7*, 16. https://doi.org/10.1186/1742-9994-7-16

Paez-Vacas, M. I., Coloma, L. A., & Santos, J. C. (2019). Systematics of the *Hyloxalus bocagei* complex (Anura: Dendrobatidae), description of two new cryptic species, and recognition of *H. maculosus*. *Zootaxa*, *2711*, 1–75. https://doi.org/10.11646/zootaxa.2711.1.1

Pons, J., Barraclough, T. G., Gomez-Zurita, J., Cardoso, A., Duran, D. P., Hazell, S., Kamoun, S., Sumlin, W. D., & Vogler, A. P. (2006). Sequence-based species delimitation for the DNA taxonomy of undescribed insects. *Systematic Biology*, *55*, 595–609. pmid:16967577 https://doi.org/10.1080/10635150600852011

R Core Team. (2019). R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.

Rada, M., Dos Santos Dias, P. H., Pérez-Gonzalez, J. L., Anganoy-Criollo, M., Rueda-Solano, L. A., Pinto-E, M. A., Quintero, L. M., Vargas-Salinas, F., & Grant, T. (2019). The poverty of adult morphology: Bioacoustics, genetics, and internal tadpole morphology reveal a new species of glassfrog (Anura: Centrolenidae: *Ikakogi*) from the Sierra Nevada de Santa Marta, Colombia. *Public Library of Science One*, *14*, e0215349https://doi.org/10.1371/journal.pone.0215349

Rada, M., & Guayasamin, J. M. (2008). Redescripción de *Cochranella megista* (Rivero, 1985) y ampliación de la distribución de nueve ranas de cristal (Anura: Centrolenidae) en Colombia. *Papéis Avulsos de Zoologia*, *48*, 89–101.

Rada, M., Jeckel, A. M., Caorsi, V. Z., Barrientos, L. S., Rivera-Correa, M., & Grant, T. (2017). A Remarkable New White-Eyed Glassfrog Species of Sachatamia from Colombia (Anura: Centrolenidae), with Comments on the Systematics of the Genus. *South American Journal of Herpetology*, *12*, 157–174. https://doi.org/10.2994/SAJH-D-16-00041.1

Rovito, S. M., Parra-Olea, G., Recuero, E., & Wake, D. B. (2015). Diversification and biogeographical history of Neotropical plethodontid salamanders. *Zoological Journal of the Linnean Society*, *175*, 167–188. https://doi.org/10.1111/zoj.12271

Ruiz-Carranza, P. M., & Lynch, J. D. (1991). Ranas Centrolenidae de Colombia 1: propuesta de una nueva clasificación genérica. *Lozania*, *57*, 1–30.

Salazar-Nicholls, M. J., & del Pino, E. M. (2015). Early development of the glass frogs *Hyalinobatrachium fleischmanni* and *Espadarana callistomma* (Anura: Centrolenidae) from cleavage to tadpole hatching. *Amphibian and Reptile Conservation*, *8*, 89–106.

Savage, J. M. (2002). *The amphibians and reptiles of Costa Rica: a herpetofauna between two continents, between two seas*. University of Chicago press.

Segura, J., Aguirre, C., Pineda, R., & Pineda, L. (2018). Ampliación de la distribución geográfica de la ranita de vidrio *Hyalinobatrachium fleischmanni* (Anura: Centrolenidae) en El Salvador. *Acta Zoológica Mexicana (N.S.).)*, *34*, 1–4. https://doi.org/10.21829/azm.2018.3412146

Señaris, J. C., & Ayarzagüena, J. (2005). *Revisión taxonómica de la Familia Centrolenidae (Amphibia; Anura) de Venezuela*. Publicaciones del Comité Español del Programa Hombre y Biosfera – Red IberoMaB de la UNESCO.

Simpson, G. G. (1951). The species concept. *Evolution*, *5*, 285–298. https://doi.org/10.1111/j.1558-5646.1951.tb02788.x

Starrett, P. H. (1960). Description of tadpoles of Middle American frogs. *Miscellaneous Publications of the Museum of Zoology University of Michigan*, *110*, 1–37.

Starrett, P. H., & Savage, J. M. (1973). The systematic status and distribution of Costa Rican glass-frogs, genus *Centrolenella* (Family Centrolenidae), with description of a new species. *Bulletin of the Southern California Academy of Sciences*, *72*, 57–78.

Sueur, J., Aubin, T., & Simonis, C. (2008). Seewave, a free modular tool for sound analysis and synthesis. *Bioacoustics*, *18*, 213–226. https://doi.org/10.1080/09524622.2008.9753600

Sukumaran, J., & Knowles, L. L. (2017). Multispecies coalescent delimits structure, not species. *Proceedings of the National Academy of Sciences of the United States of America*, *114*, 1607–1612. https://doi.org/10.1073/pnas.1607921114

Sunyer, J., Martínez-Fonseca, J. G., Salazar-Saavedra, M., Galindo-Uribe, D. M., & Obando, L. A. (2014). Range extensions and new departmental records for amphibians in Nicaragua. *Mesoamerican Herpetology*, *1*, 164–175.

Taylor, E. H. (1942). New tailless Amphibia from Mexico. *University of Kansas Science Bulletin*, *28*, 67–89.

Taylor, E. H. (1958). Notes on Costa Rican Centrolenidae with descriptions of new forms. *University of Kansas Science Bulletin*, *39*, 41–68.

Twomey, E., Delia, J., & Castroviejo-Fisher, S. (2014). A review of Northern Peruvian glassfrogs (Centrolenidae), with the description of four new remarkable species. *Zootaxa*, *3851*, 1–87. https://doi.org/10.11646/zootaxa.3851.1.1

Vences, M., Guayasamin, J. M., Miralles, A., & De La Riva, I. (2013). To name or not to name: criteria to promote economy of change in Linnaean classification schemes. *Zootaxa*, *3636*, 201–244. https://doi.org/10.11646/zootaxa.3636.2.1

Vences, M., Thomas, M., Bonett, R. M., & Vieites, D. R. (2005). Deciphering amphibian diversity through DNA barcoding: chances and challenges. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *360*, 1859–1868. https://doi.org/10.1098/rstb.2005.1717

Vences, M., & Wake, D. B. (2007). Speciation, species boundaries and phylogeography amphibians. In: Heatwole, H. & Tyler, M.J., Eds., *Amphibian Biology, Volume 7, Systematics*. (pp. 2613–2671). Surrey Beatty and Sons.

Villa, J. (1984). Biology of a neotropical glass frog, *Centrolenella fleischmanni* (Boettger), with special reference to its frogfly associates. *Milwaukee Public Museum Contributions in Biology and Geology*, *55*, 1–60.

Wiens, J. J., & Servedio, M. R. (2000). Species delimitation in systematics: inferring diagnostic differences between species. *Proceedings of the Royal Society of London. Series*

B: Biological Sciences, *267*, 631–636. https://doi.org/10.1098/rspb.2000.1049

Yang, Z., & Rannala, B. (2010). Bayesian species delimitation using multilocus sequence data. *Proceedings of the National Academy of Sciences*, *107*, 9264–9269. https://doi.org/10.1073/pnas.0913022107

Zamudio, K. R., Bell, R. C., & Mason, N. A. (2016). Phenotypes in phylogeography: Species' traits, environmental variation, and vertebrate diversification. *Proceedings of the National Academy of Sciences*, *113*, 8041–8048. https://doi.org/10.1073/pnas.1602237113

Zhang, J., Kapli, P., Pavlidis, P., & Stamatakis, A. (2013). A general species delimitation method with applications to phylogenetic placements. *Bioinformatics (Oxford, England)*, *29*, 2869–2876. https://doi.org/10.1093/bioinformatics/btt499

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APPENDIX I. Specimens examined.

Hyalinobatrachium bergeri PERU: San Pedro (AMNH-A 157002)

Hyalinobatrachium capellei: VENEZUELA: Amazonas, Cerro Yutaje (AMNH-A 159164)

Hyalinobatrachium aureoguttatum: COLOMBIA: Cauca, Quebrada Guangui (AMNH-A 88785); Antioquia, San Francisco, Rio Santo Domingo (MHUA-A 7675-7); Antioquia, San Carlos, Cano Borboyones (MHUA-A 7075-6)

Hyalinobatrachium chirripoi: COLOMBIA: Choco, Nuqui (MHUA-A 5150-2); Antioquia, San francisco (MHUA-A 6650)

Hyalinobatrachium collymbiphyllum: COLOMBIA: Choco, Nuqui, (MHUA-A 05149); PANAMA: Chiriqui, Quebrada de arena (AMNH-A 124170-77, 124187)

Hyalinobatrachium esmeralda: COLOMBIA: Norte de Santander, Cucutilla (MHUA-A 1673)

Hyalinobatrachium fleischmanni: COSTA RICA: Alajuela, Alajuela, Sarapiqui (UCR 5394-5); Alajuela, Grecia, Tacares (UCR 6559); Cartago, Cartago, Quebradilla (UCR 6563); Cartago, Paraíso, Orosi (UCR 6211); Limón, Siquirres, Siquirres (UCR 6639); Puntarenas, Puntarenas, Monte Verde (UCR 4960); San José, Montes de Oca, San Pedro (AMNH-A 71609-71613, 1425652-1425654); San José, Vázquez de Coronado, Dulce Nombre (UCR 5444-5, 7676); HONDURAS: El Paraiso (AMNH-A 54815)

Hyalinobatrachium munozorum: ECUADOR: Napo, Santa Cecilia (AMNH-A 93162)

Hyalinobatrachium orientale: TRINIDAD AND TOBAGO (AMNH-A 87347-58)

Hyalinobatrachium tatayoi: PANAMA. Chiriqui, 9 km airline SSE el volcan (AMNH-A 124178-80); Cocle (AMNH-A 52743, 59594, 59595); Panama, Canal Zone, Barro Colorado (AMNH-A 40456-7, 69755-60); COLOMBIA: Antioquia, Amalfi, Fca. La Picardia (MHUA-A 01349), Antioquia, Anori, Liberia, Casa de Maquinas campamento mineros providencia (MHUA-A 08529); Antioquia, Caracoli, Qda.Charco Azul (MHUA-A 06490); Antioquia, Maceo, Hda Sra Barbara (MHUA-A 4072-4, 4347, 4352, 4355, 4882); Antioquia, Gomez Plata, La Clara, Hda. Vegas de La Clara (MHUA-A 5085-6, 7143, 8167); Antioquia, Ituango, Rio sinitare (MHUA-A 6911); Antioquia, San Carlos, Juanes, Casino Viejo (MHUA-A 07198); Antioquia, San Carlos, Puerto Garza, Limones, Deposito proyecto Porvenir II (MHUA-A 07659); Antioquia, San Francisco (MHUA-A 06650); Antioquia, San Luis, Reserva Natural El Refugio (MHUA-A 04908); Antioquia, San Rafael, La Granja, , (MHUA-A 09749); Antioquia, Sonson, San Miguel, Parcelas, San Antonio (MHUA-A 08405-6, 8420); Antioquia, Taraza, Rayo, Bosque ripario Rio Rayo (MHUA-A 07522); Antioquia, Hidroelectrica Porce II (MHUA-A 01158); Antioquia, El Huevo, Porce II (MHUA-A 01348); Cauca, Rio Patia (AMNH-A 85890, 85893, 85895, 85916, 85924,

88789, 88790); Choco, Acandi, Capurgana (MHUA-A 9379, 9383, 10361); Santander, Giron, Villa Leiva (MHUA-A 10139); ECUADOR: Esmeraldas, Carchi Goaltal; Quebrada Golondrinas (QCAZ-A-64612, 64616, 64619), Esmeraldas aproximadamente a 4 km al oeste de Durango (QCAZ-A-23549), Esmeraldas Caimito (QCAZ-A-55032-33), Esmeraldas Durango San Francisco (QCAZ-A-27679, 27695), Esmeraldas En la interseccion de la vía Caimito - Tachihue (QCAZ-A-37308), Esmeraldas La Tola (QCAZ-A-22301, 22303), Esmeraldas Reserva Tesoro Escondido. Río Camarón (QCAZ-A-65393), Esmeraldas San Francisco, 2.2km este, Vía a Durango (QCAZ-A-32107); Guayas Cerro de Hayas, Siete Cascadas, Cooperativa 23 de Noviembre (QCAZ-A-71828-29, 71832, 71833); Manabí Reserva Ecológica Jama Coaque (QCAZ-A-51000, 52705)

Hyalinobatrachium valerioi: COLOMBIA: Antioquia, Anori (MHUA-A 2044, 2045); Valle del Cauca, 13km NW Dagua (AMNH-A 88487)

Hyalinobatrachium viridissima: MEXICO: Chiapas, Nahá (MZFC 28314); Guerrero, San Vicente de Benitez (MZFC 16128-30, 16345, 18095, 19312, 19328, 19354); Oaxaca, 12-15 rd km W Jalapa de Díaz (MZFC 5227-30); Oaxaca, 13.1 km W Jalapa de Diaz, Hwy 150 W of Tuxtepec (MZFC 15717-9); Oaxaca, Cabaña Sedesol, camino a nueva Guadaupe (MZFC 18407-9); Oaxaca, Carretera San Jose Pacifico-Candelaria Loxicha, 480m (MZFC 19389, 19395); Oaxaca, Cerro Rabón (MZFC 29010); Oaxaca, Ejido Clemencia (MZFC 29011); Oaxaca, San Jerónimo Tecoatl (MZFC 29007-8); Oaxaca, Pluma Hidalgo, Río Juquilita (MZFC 22561); Puebla, Comunidad de la Aurora, ladera del rio Maloapan (MZFC 22676);

Puebla, Ejido Tepequezquiapan, Carr. Eloxochitlan-Tlacotepec de Diaz (MZFC 20509, 21498, 21949, 21950); Puebla, Paso real (MZFC 22675); Puebla, Xonotipan de Juárez, Carr. Azumbilla-Tlacotepec de Díaz (MZFC 30103-4); Tabasco, Camino hacia Ejido Francisco J. Mujica, 1.5 km al NE del pueblo (MZFC 30403-5); Tabasco, Northern foothills of Cerro Las Flores, road to Ejido Francisco J. Mujica (MZFC 30406); Veracruz, Ejido Piedritas (MZFC 28393);

Supplementary Material 1. Results of the pairwise comparisons using Wilcoxon rank sum test of Snout-vent length and the size-residuals for the remaining morphometric parameters evaluated among lineages within *Hyalinobatrachium fleischmanni* complex.

	tatayoi.2	*fleischmanni*	Golfo	Honduras	Maya	Pacifico
Snout-vent length						
fleischmanni	0.0569	-	-	-	-	-
Golfo	0.0000	0.0000	-	-	-	-
Honduras	0.8485	0.9333	0.2188	-	-	-
Maya	0.0001	0.0006	0.2040	0.3684	-	-
Pacifico	0.2276	0.0022	0.0001	0.3457	0.0002	-
tatayoi.1	1.0000	0.0904	0.0000	0.8755	0.0002	0.2040
Tibia Length						
fleischmanni	0.1356	-	-	-	-	-
Golfo	0.0216	0.0135	-	-	-	-
Honduras	0.7368	0.6462	0.6562	-	-	-
Maya	0.1556	0.1452	0.7674	0.6562	-	-
Pacifico	0.7042	0.5026	0.0216	0.7368	0.1452	-
tatayoi.1.	0.5026	0.5026	0.0021	0.6562	0.0563	0.9500
Head Length						
fleischmanni	0.8291	-	-	-	-	-
Golfo	0.0026	0.0082	-	-	-	-
Honduras	0.5935	0.5935	0.8167	-	-	-
Maya	0.0014	0.0029	0.0174	0.6176	-	-
Pacifico	0.1023	0.1431	0.1224	0.8167	0.0050	-
tatayoi.1	0.6248	0.5935	0.0026	0.5935	0.0016	0.1633
Head Width						
fleischmanni	0.9030	-	-	-	-	-
Golfo	0.0940	0.1220	-	-	-	-
Honduras	0.9330	1.0000	0.9030	-	-	-
Maya	0.1220	0.1970	0.4980	0.9260	-	-
Pacifico	0.7180	0.6000	0.0940	1.0000	0.1970	-
tatayoi.1	0.6000	0.9890	0.0100	0.9030	0.0430	0.2660
Eye Diameter						
fleischmanni	0.0870	-	-	-	-	-
Golfo	0.0000	0.0000	-	-	-	-
Honduras	0.7778	0.8201	0.1750	-	-	-
Maya	0.0001	0.0005	0.1166	0.3088	-	-
Pacifico	0.1111	0.0016	0.0001	0.2917	0.0001	-
tatayoi.1	0.8201	0.1111	0.0000	0.7949	0.0001	0.0383
Eye-Nostril						
fleischmanni	0.2152	-	-	-	-	-

Golfo	0.0088	0.0028	-	-	-	-
Honduras	0.8750	1.0000	0.3500	-	-	-
Maya	0.0127	0.0088	0.2152	0.3500	-	-
Pacific	0.9849	0.2152	0.0371	0.9608	0.0114	-
tatayoi.1	0.0534	0.9904	0.0002	0.9849	0.0021	0.0534
Femur Length						
fleischmanni	0.1825	-	-	-	-	-
Golfo	0.1615	0.0235	-	-	-	-
Honduras	0.6588	1.0000	0.5662	-	-	-
Maya	0.1129	0.0493	0.5696	0.6563	-	-
Pacific	0.1129	0.7430	0.0064	1.0000	0.0075	-
tatayoi.1	0.0145	0.7430	0.0007	0.5662	0.0064	0.2289

Supplementary Material 2. Kruskall-Wallis and Wilcoxon-rank tests for the advertisement calls within *H. fleischmanni* complex (N = 61, excluding two calls from Ecuador and one call from Honduras). Bold values represent significative differences for each analysis.

	Call Duration	**Peak Frequency**	**Bandwidth**
Kruskall Wallis rank sum test			
chi squared	47.115***	43.415***	30.061***
Pairwise comparisons between pair of lineages using Wilcoxon rank sum test			
fleischmanni - Gulf	0.05837	**0.0048**	0.2531
fleischmanni - Pacific	**0.00142**	0.67172	0.0741
fleischmanni - Maya	0.56621	**0.00124**	**0.0292**
fleischmanni - *tatayoi*	**0.000036**	0.1078	0.3127
Gulf - Pacific	**0.00039**	0.93991	0.8742
Gulf - Maya	**0.00663**	**0.00247**	0.1083
Gulf - *tatayoi*	0.21818	**0.00016**	**0.0176**
Maya - Pacific	**0.00075**	**0.00016**	0.1083
Maya - *tatayoi*	**0.0000012**	**0.000065**	**0.0000061**
Pacific - *tatayoi*	**0.00000016**	**0.00217**	**0.0017**

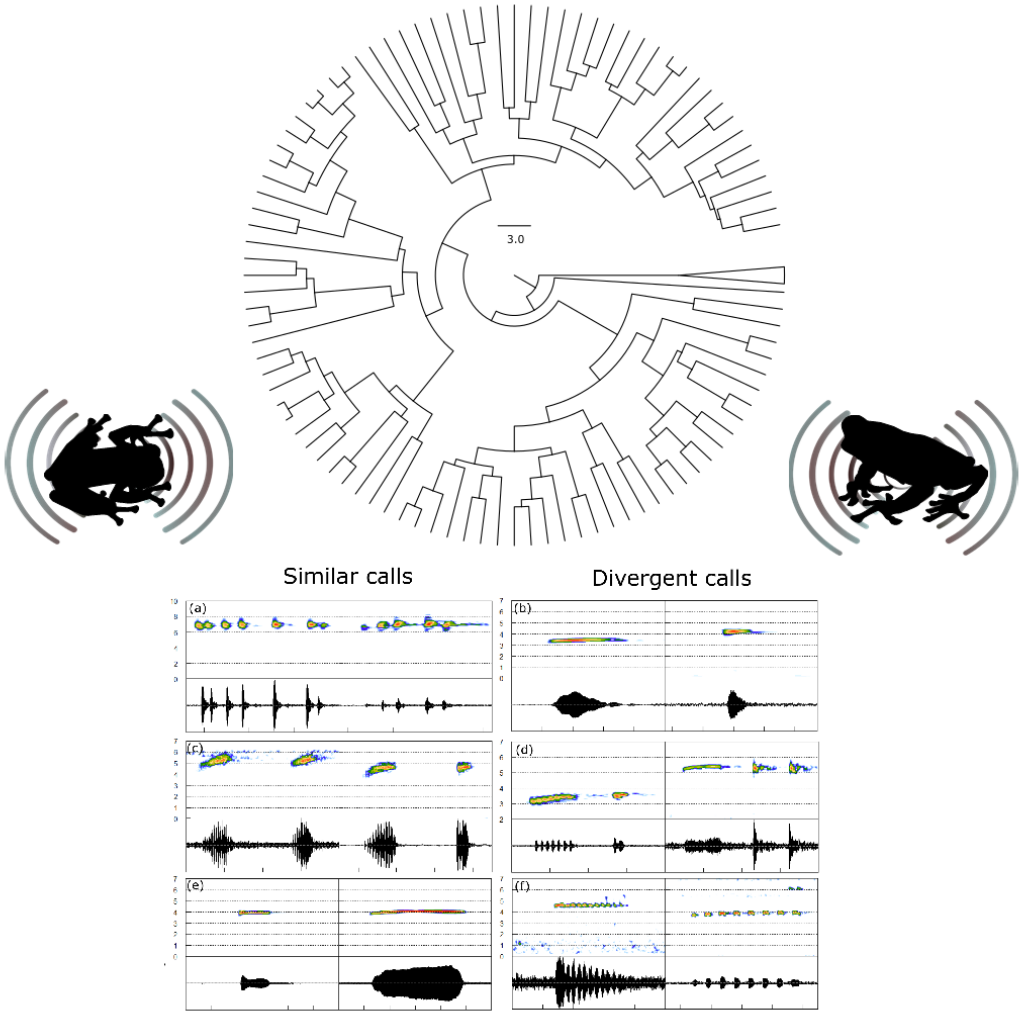
Supplementary Material 3. Full body view dorsal (A) and ventral (B) of *Hyalinobatrachium viridissimum* comb. nov. From left to right: MZFC-16130 (Pacific), MZFC-18407 (Oaxaca), MZFC-15718 (Oaxaca), MZFC-29006 (Gulf) and MZFC-30406 (Maya).

CAPÍTULO III

Environment rather than Character Displacement explains call evolution in glassfrogs

Autores: Angela M. Mendoza-Henao, Kelly R. Zamudio, Juan M. Guayasamin, Moisés Escalona-Sulbarán, Gabriela Parra-Olea

Sometido a: Evolution



Environment rather than character displacement explains call evolution in glassfrogs

Short running title: Environment explains glassfrog calls

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Data Accessibility Statement: Call descriptions and list of georeferenced localities used in this article are deposited on Dryad. Phylogenetic tree nexus file is deposited on TreeBASE.

Conflict of interest: The authors do not have a conflict of interest to declare.

Environment rather than character displacement explains call evolution in glassfrogs

Abstract. The Acoustic Adaptation Hypothesis (AAH) and Ecological Character Displacement (ECD) are two potential mechanisms shaping call evolution and predict opposite trends on differentiation of signals. The AAH posits that signals evolve to minimize degradation in the environment and maximize detection against background noise, and thus predicts call homogenization due to environmental constraints on acoustic propagation. In contrast, ECD predict greater differences in traits of closely-related taxa in sympatry because of selection against competitive masking. We test the strength of antagonistic selective mechanisms (environment measured by vegetation and temperature, and community composition) on the evolution of advertisement calls in glassfrogs. Environmental characteristics had a larger effect on call evolution than community composition. As expected under the AAH, temporal call parameters are correlated with vegetation density, but an unexpected correlation between spectral call parameters and temperature was also observed. We detected convergence among co-occurring species and among different populations co-occurring with different glassfrogs communities, indicating that character displacement is not common and call convergence could be attributed to habitat filtering. The differences in the mechanisms acting among species and among populations confirms that the strength of drivers shaping call evolution might differ depending on phylogenetic scale, and time since divergence among interacting species.

Key Words: trait evolution, advertisement calls, community phylogenetics, adaptation, character displacement, Centrolenidae

Introduction

The relative importance of environmental and biotic selection in trait variation is a key question for understanding the origin and maintenance of biodiversity (Wellborn et al. 1996; Indermaur et al. 2010). Environmental selection operates on phenotypic variation of individuals in populations, yet interactions of species within communities also shape the selective landscape. Acoustic signals are the main type of communication in several animal species (Bradbury and Vehrencamp 2012) and this phenotype potentially evolves in response to both environmental and biotic selection (West-Eberhard 1983; Endler 1992; Chek et al. 2003). Acoustic signals are subject to temporal and spectral degradation, and attenuation of calls in a certain environment depends on its sound frequency (pitch) (Wiley and Richards 1982). The environment can generate interference or "noise" by distorting signals or impeding detection by the intended receiver (Goutte et al. 2013), affecting transmission fidelity, and generating suboptimal communication (acoustic masking). The Acoustic Adaptation Hypothesis (AAH) posits that signals evolve to minimize degradation in the environment and maximize detection against background noise (Ey and Fischer 2009). In general, slow-paced and low-pitched signals travel more effectively in closed habitats (such as rainforests), while faster-paced and higher-frequency signals suffer greater attenuation and reverberation (Marten and Marler 1977; Wiley 1991; Tobias et al. 2010). In addition to the effect of habitat on call propagation, the role of environmental temperature in acoustic communication has been well characterized in anurans (Gerhardt 1994; Gerhardt and Huber 2002). While note rate and pulse rate (repetitions in a period of time) increase as temperature increases, call duration is reduced under the same circumstances (Gerhardt and Huber 2002; Köhler et al. 2017). These environmentally-induced changes in call traits have consequences for signal recognition by conspecifics (Gerhardt and Mudry 1980; Bosch 2001).

Ecological Character Displacement (ECD) is a process of trait evolution caused by exploitative competition with one or more sympatric species resulting in shifts in traits that affect resource use (Brown and Wilson 1956). In particular, Reproductive Character Displacement (RCD) causes greater differences in the reproductive traits (e.g. mating

signals) of closely-related taxa in sympatry than in allopatry because of selection in sympatry against costly mating mistakes (e.g., reduced viability or fitness of hybrids, wasted gametes, or missed mating opportunities) (13, 19–22, but see 23). Because ecological character displacement causes changes in acoustic signals, and potentially reinforces reproductive isolation, both kinds of character displacement have been suggested as drivers of call variation in anurans (Villanueva-Rivera 2014; Garey et al. 2018). However, it has been notoriously difficult to rigorously exclude alternative processes that might lead to those patterns resembling ECD or RCD, such as variation in environmental factors, shifts in resource use, species sorting, or even random evolution (Stuart and Losos 2013). While ECD/RCD predict differentiation in acoustic emission due to potential competition for acoustic space, the AAH predicts call homogenization due to environmental constraints on acoustic propagation and, thus, on signal transmission (Gasc et al. 2017), but the joint effect of these interacting drivers remains poorly studied.

To uncover selective mechanisms underlying call evolution, it will be necessary to examine trait evolution in a phylogenetic context at different levels of phylogenetic divergence within communities. By quantifying call heterogeneity and phylogenetic distances of species at a given locality, we can compare three scenarios to test the strength of those mechanisms (Fig. 1). In the first scenario, the differences among calls are independent of phylogenetic community heterogeneity and the evolution of calls among species shows convergence due to other factors (Fig 1A). This is the pattern expected if species are optimizing acoustic space independent of phylogenetic relationships among community members. In the second scenario (Fig 1B), the divergence of calls is positively correlated to phylogenetic distance among community members. This pattern is expected if calls are evolving due to neutral variation since the split from the common ancestor, or due to weak episodes of selection in varying directions, such that call evolution approximates pure neutral variation. Finally, in the last scenario (Fig 1C), species living in communities composed by very closely related taxa tend to have more dissimilar calls than expected under common ancestry (a signature of character displacement), while the species that live in communities

composed of distantly-related taxa will show convergence in their calls (potentially because of acoustic adaptation to similar environmental selective pressures).

If traits diverge continuously from individuals, to populations, to the level of species, we might expect similar selective pressures and outcomes, independent of evolutionary scale (Steppan et al. 2002). However, the response to selective pressures may not be equal for species within all communities because communities are composed of species of different ages, with varying levels of divergence, and with different degrees of overlapping traits. Although the differences in micro and macroevolutionary signatures of selection have been widely discussed (Hansen and Martins 1996; Arnold et al. 2001; Uyeda et al. 2011), few empirical studies have examined environmental and biotic drivers of selection at multiple evolutionary scales (Grant and Grant 2002; Brawand et al. 2014; Riesch et al. 2017; Jonathan et al. 2018).

Glassfrogs (Centrolenidae) are a highly diverse Neotropical family (153 species, (Guayasamin et al. 2020)) ideal for testing the relative roles of the Acoustic Adaptation Hypothesis and Character Displacement in call evolution. A well-established phylogeny of glassfrogs (Guayasamin et al. 2008, 2020; Castroviejo-Fisher et al. 2014; Twomey et al. 2014) provides the framework for testing hypotheses about historical biogeography and diversification (Hutter et al. 2013; Castroviejo-Fisher et al. 2014; Guayasamin et al. 2020), patterns of phylogenetic diversity and endemism (Mendoza and Arita 2014; Guayasamin et al. 2020), molecular evolution (Dugo-Cota et al. 2015), parental care (Delia et al. 2017), and call evolution (Escalona Sulbarán et al. 2019). Also, a recent phylogeographic study of the widespread *Hyalinobatrachium fleischmanni* species complex (Mendoza et al. 2019) provides an excellent opportunity to test the same hypotheses about signal evolution among deeply divergent conspecific lineages (Padial et al. 2010).

Using comparative and community phylogenetic methods, we tested three hypotheses for the evolution of call signals: 1) Environmental conditions (temperature and vegetation) act as drivers for call evolution in glassfrogs (Acoustic Adaptation), 2) Glassfrog community composition shapes call evolution (Ecological and Reproductive Character Displacement) and, 3) The strength of mechanisms leading to call

differentiation will differ at different phylogenetic depths because different mechanisms operate from incipient species (deep conspecific lineages) to family levels. This phylogenetic approach allows us to investigate the relative roles of environmental and biotic selection (or lack thereof) on the evolution of acoustic signals at multiple evolutionary scales, to disentangle the selective context and mechanisms contributing to differentiation of the complex trait.

Materials and Methods

Evolutionary patterns for call traits. We reconstructed a phylogeny for glassfrogs, using all available sequences from GenBank plus additional sequences obtained for this study (Table S4). The final dataset included 6304 bp of three mitochondrial (12S (941 bp), 16S (871 bp), ND1 (961 bp) and seven nuclear loci (POMC (604 bp), BDNF (700 bp), C-MYC (406 bp), CXCR (356 bp), RAG (456 bp), SLC8A1 (542 bp), and SLC8A3 (467 bp) for 97 species in 12 glassfrog genera (ingroup) and three species of *Allophryne* (outgroup). Sequences of each gene were aligned with ClustalW (Thompson et al. 1994) in Mega7 (Kumar et al. 2016); concatenated sequences were used in phylogenetic Bayesian reconstruction in BEAST2 (Bouckaert et al. 2014) using the CIPRES Science Gateway (Miller et al. 2010). We used Partition Finder 2.1.1 (Lanfear et al. 2016) to choose the best model of evolution for each gene. We then inferred ultrametric phylogenies using a lognormal relaxed-clock model and a Yule speciation process (Drummond et al. 2006). We ran two independent analyses for 50 million generations, sampling trees and parameter values every 1000 generations. Burn-in was set at 25%, and the first 12.5 million generations were discarded. A maximum clade credibility (MCC) tree was generated in TreeAnotator 1.8.4 (Bouckaert et al. 2014). For the *H. fleischmanni* complex, we reconstructed a phylogeographic topology for 16 populations sampled throughout the range of the species complex (Mendoza et al. 2019) and we extracted the MCC tree and 100 randomly sampled post burn-in trees for comparative analyses using the MCMCglmm function in the mulTree library (Guillerme and Healy 2014).

We gathered all available glassfrog calls from the following sound collections: Fonoteca Zoológica of the Museo Nacional de Ciencias Naturales-CSIC (Spain),

Cornell's Macaulay Library (USA), Museo de Zoología of the Pontificia Universidad Católica del Ecuador, Fonoteca Neotropical Jacques Villar, UNICAMP (Brazil), and Laboratorio de Biología Evolutiva of the Universidad San Francisco de Quito. We supplemented this data set with contributions by researchers, call descriptions available in literature, and our own recordings obtained during field work. Whenever possible we obtained up to ten calls per species to account for individual variation. For comparisons among conspecific lineages, we recorded a minimum of three calls, and a maximum of 10 for each locality sampled in the *H. fleischmanni* phylogeography (Mendoza et al. 2019).

We measured call parameters from the wave format files in Raven Pro v.1.5 (Cornell Lab of Ornithology, Ithaca, NY, USA) with a Blackmann window of 5 ms, an 80% overlap and a DFT of 1024. Two spectral and four temporal parameters of the call were obtained: dominant frequency or peak frequency (frequency at which the highest amplitude peak is found), frequency bandwidth (the difference between upper and lower frequency bounds of notes, as measured 20 dB below the peak frequency), note duration (length in milliseconds of a note, measured from beginning to end of the note), notes per call, pulse rate (number of pulses in a note minus 1, divided by the length of the note; with this formula, purely tonal calls always have a pulse rate of zero) and note rate (count of notes in a call minus 1, divided by the length of the call). We followed recommendations in (Köhler et al. 2017) and defined pulsed notes as those comprised by multiple short undividable sound units having a duration less than 50ms including spaced pulses with silent intervals. Note series are separated from other such groups by periods of silence of longer duration than the inter-note intervals in a regular pattern. The first three parameters were also measured for *H. fleischmanni* complex. Given that peak frequency is strongly dependent on body size (Gingras et al. 2013; Escalona Sulbarán et al. 2019; Tonini et al. 2020) we extracted the mean snout-vent length (SVL) for each species from the literature and colleagues, and size-corrected the spectral attributes with the phyl.resid function in Phytools (Revell 2009).

The call metrics were reduced in a phylogenetic Principal Component Analysis (Revell 2009). Before analysis, call parameters were standardized to a mean of zero

and standard deviation of one. In addition, we performed two phylogenetic PCAs for the temporal and spectral variables to evaluate the impact of the potential drivers affecting each set of traits. We quantified the acoustic divergence between species as the Euclidean distance between the two first principal components of each phylogenetic PCA. In the case of *H. fleischmanni* lineages, the Euclidean distances were constructed between the calls of the *H. fleischmanni* from each locality against the call of all other glassfrog species (N = 13), and we separated these values into two categories depending on whether the species co-occurred at each locality or not.

We estimated the best model of evolution for each call trait by comparing the AIC scores for 5 models models with the fitContinuous function in geiger (Harmon et al. 2007): Brownian motion (BM), Pagel lambda (λ) Ornstein–Uhlenbeck (OU), Kappa (κ) or White Noise (WN). We reconstructed the evolutionary history of acoustic traits using Bayesian MCMC to infer the transition of character states at internal nodes in the tree with the function anc.Bayes in phytools (Revell 2012) to infer the transition of character states at internal nodes in the tree. We ran each MCMC chain for 10 million generations with 10% excluded as burn-in and we check the convergence with the coda package (Plummer et al. 2006), checking that the effective sample size for the character state at each interior node over 200.

Testing the Acoustic Adaptation hypothesis. We obtained georeferenced records as a to infer the distribution for all glassfrog species included in this study from the database generated by Hutter et al. (Hutter et al. 2013), which was then manually refined, species names were updated, and supplemented with recent data (final database comprised a total of 2819 records, *SI Appendix*, Table S4). For each record we extracted the environmental temperature, estimated from the mean temperature of the wettest quarter (WorldClim BIO8) as a proxy for the temperature during the rainy season, when reproductive activity is peaks. We also extracted vegetation density, taken from the Enhanced Vegetation Index (EVI) proposed by the MODIS Land Discipline Group (Liu and Huete 1995). In this case, we obtain the mean value per cell for 204 layers with monthly data between 2000 and 2017.

We tested the correlation between environmental temperature and the vegetation density against each acoustic trait by mean of Markov chain Monte Carlo (MCMC) Sampler for Multivariate Generalized Linear Mixed Models from the MCMCglmm package (Hadfield 2010). MCMCglmm uses a MCMC estimation approach and accounts for non-independence among closely related species by including the degree of phylogenetic relatedness in the variance-covariance matrix. Because vegetation density and air temperature are highly correlated (0.60 Pearson's correlation coefficient, t = 7.342, df = 95, p-value <0.001), each metric was included as a random effect in the MCMC model of the another.

Testing the Character displacement hypothesis. Here, we used two approaches, one using species pairs, and the second using overall-community composition. For the species pairs analysis, we created a presence-absence matrix using a grid with cells of 0.05x0.05 degrees over the georeferenced records and we then estimated the overlap in distribution for each pair of species calculated as the proportion of the smaller range that overlaps within the larger range (Chesser and Zink 1994) using the lets.overlap function of the letsR package. We followed an approach similar to that used for bird communities (Tobias et al. 2014) to test the direct impact of coexistence in call divergence correcting by common ancestry. To do so, we ran an MCMCglmm between call divergence (Euclidean distances in the PCAs) and coexistence (range overlap) including the phylogenetic relationships among species in the variance-covariance matrix. In this case, since we have multiple metrics per species, we treated the species names as random effect. We need to be cautious of the results here obtained since our data include assumptions that may affect interpretations of evolutionary processes. The main one is the choice of proxy for historical species interactions (Harmon et al. 2019). Co-occurrence in the present day is a common proxy for interactions, but does not necessarily imply long-term co-occurrence (Mayfield and Levine 2010). Here we measured species co-occurrence through information from geo-referenced museum specimen records. Although this approach is still far from perfect to assess the historical pattern of interactions among species (Losos and Glor 2003), it allow us to detect some evolutionary patterns at large geographic scales more accurately that using the overlap of polygons (Peterson et al. 2018).

For the overall-community analysis, we calculated community phylogenetic heterogeneity with the Mean Pairwise Phylogenetic Distance (MPD), measured as the mean of all pairwise branch lengths for a group of species present in a community (Webb et al. 2002). The MPD, derived in the MetricTester package (Miller et al. 2017), assesses if individual species coexist with closely related, distantly related, or a random set of species. For call divergence, we constructed an Acoustic Dispersion index for each species as a metric of the call heterogeneity to which the species is exposed. To do so, we first measured the average distance to the centroid of all calls within each cell of the presence-absence matrix by calculation the Functional Dispersion index (Laliberté and Legendre 2010) of the phylogenetic PCA of the calls. Later, we obtained a value of the overall acoustic structure per species averaging the Functional Dispersion of all sites in which a particular species occurs, following the Qr-mode suggested by Arita et al. (Arita et al. 2008). Thus, species living with species whose calls are more different from each other will show higher Acoustic Dispersion while those species living with species having similar calls will show lower Acoustic Dispersion. Here, we performed a MCMCglmm between the Acoustic Dispersion and the community phylogenetic heterogeneity per species, adding temperature and vegetation as random effects.

Testing mechanisms of selection at different phylogenetic scales. To evaluate if call evolution in response to environmental and biotic selection occurs similarly at different phylogenetic levels, we used a deconstructive approach (Marquet et al. 2004): for the analyses among species, we performed analyses at the family level, subfamily level, and lastly in four diverse clades (Fig. 3). For the analyses within *H. fleischmanni* complex, we analyzed the entire group of populations within *H. fleischmanni.*

Finally, to test if the Reproductive Character Displacement was evident only at sister species level, we extracted the pairs of sister species (N = 26) and divided those into two groups, one composed of pairs of species with range overlap (N = 7). and those with no range overlap (N = 19). These pairs of sister species with no range overlap were split into those separated geographically by a well-defined barrier without differences in environmental conditions between them (N = 8) and those living in localities with different environmental conditions (e.g. different altitudinal range) (N = 11). We pruned

the phylogenetic tree to include one terminal per pair of sister species and the call divergence among these three groups was compared using a Phylogenetic ANOVA (Revell 2012).

Results

Overall evolutionary patterns for call traits. First, we established the historical context for call evolution by examining the phylogenetic signal, the best-fit model for character evolution, and the ancestral state of the call traits of interest. Our phylogenetic tree included 97 glassfrogs species for which we had advertisement call data, covering 63.4 % of species diversity in the family and representatives from all twelve described genera. The phylogeny is consistent with previous studies (Guayasamin et al. 2008, 2020; Castroviejo-Fisher et al. 2014; Twomey et al. 2014; Delia et al. 2017), and shows strong support for the monophyly of glassfrog genera and their relationships (posterior probabilities > 0.95 for most nodes) (*SI Appendix*, Figure S1).

The dominant frequency of calls ranged from 2713.2 Hz (*Ikakogi tayrona*) to 7407.4 Hz (*Chimerella corleone*) with a mean value of 4950.6 Hz. *Centrolene bacatum* showed the shortest note duration (3.6 ms) and *Centrolene condor* the longest (601.0 ms). More than half of the species (N = 62) displayed only one note per call while the remaining (N = 35) display between two and nine notes. Forty-three species showed a tonal call (each note contains a single frequency component at any time instant) and the remaining 52 showed pulsed calls (each note contains series of energy bursts separated from each other by distinctly reduced amplitude). The pulse rate ranged from 19.8 pulses/s (*Hyalinobatrachium tricolor*) to 309.9 pulses/s (*Sachatamia ilex*). The first two components of the phylogenetic PCA for all call traits explained 57.4% of the variance (Fig. 2). Number of notes had the highest loading for the first component, while note rate, note duration, and peak frequency had higher loadings in the second component.

The calls of *H. fleischmanni* individuals showed no variation in the number of notes or note rate among 16 localities but did have variation in peak frequency (4112.8 to 5343.8 Hz), note duration (0.089 to 0.159 ms), and frequency bandwidth (864.1 to 1189.9 Hz). Complete call metrics for all species and for populations within *H. fleischmanni* are provided in *SI Appendix* (Table S1).

By comparing the evolutionary model that best fit call traits based on the AIC criterion (Table 1 and *SI Appendix*, Table S2), we found that Pagel's lambda is the best-fit model of evolution for peak frequency, frequency bandwidth, note duration, and number of notes, suggesting that those call traits follow a Brownian Motion model in a scenario with fewer phylogenetic structure (given by the lambda value). In contrast, Ornstein–Uhlenbeck (OU) was the best-fit model of evolution for note rate, pulse rate, and the integration of all temporal variables in the first component of PCA, indicating that these parameters are constrained around an adaptive optimum over time. Pulse rate had the highest instantaneous rate of change (sigma square) while note duration and bandwidth had the lowest. Among populations within the *H. fleischmanni* complex, Pagel's lambda was the best-fit model of evolution for peak frequency, OU model for note duration, and Brownian Motion for frequency bandwidth.

Finally, ancestral state reconstructions (Fig. 3) revealed an evolutionary trend toward higher peak frequencies within all genera except *Nymphargus* (within Clade F, Fig. 3*A*). A few other species showed independent changes from medium to low peak frequencies and from medium to low frequency bandwidth values. In all species of the subfamily Hyalinobatrachinae (Clade B) the number of notes is one, and thus the note rate is zero (Fig. 3*C-D*); for Centroleninae (Clade D), we found multiple cases of evolution from low to higher number of notes along the tree (Fig. 3*C*). Pulse rate was the trait with most variation among glassfrogs (Fig. 3f); while some subclades of *Hyalinobatrachium*, *Nymphargus*, *Teratohyla,* and *Vitreorana* show relatively low change in pulse rate among species, the remaining clades in the family had highly variable pulse rates even between sister species.

Acoustic Adaptation. A correlation between vegetation density and temperature with many call parameters was found at multiple evolutionary levels in the glassfrog phylogeny, indicating that the environment plays an important role in call evolution. The Generalized Linear Mixed Models using Markov chain Monte Carlo methods (MCMCglmm), accounting for the statistical non-independence of closely related taxa (Fig. 4*A*, details in *SI Appendix*, Table S3), detected a positive correlation between peak frequency (after accounting for body size) and both environmental variables at almost all

levels. The exceptions Hyalinobatrachinae, where a positive correlation was found only between peak frequency and temperature. Pulse rate and note rate were negatively and positively correlated with vegetation density within the *Centrolene* and *Nymphargus*, respectively. At the family level, the number of notes was negatively correlated with vegetation density. For all Centrolenidae and Cochranellini, bandwidth was positively correlated with vegetation density. For populations within the *H. fleischmanni* complex the results were opposite, note duration was the only call parameter inversely correlated to vegetation and temperature.

Character Displacement vs Acoustic Adaptation. We found a convergence of calls in species with higher range-overlap, a pattern opposite that expected under character displacement. A negative correlation of spectral parameters and range-overlap was found for the entire family (post.mean = -0.309, 95% CI = -0.579 – -0.035, pMCMC = 0.027) and for the Centroleninae subfamily (post.mean = -0.626, 95% CI = -0.975 – -0.291, pMCMC = 0.0006) accounting for the statistical non-independence of closely related taxa. Details of tests are provided in *SI Appendix*, Table S2.

In the overall-community approach (Fig. 4*B*), the phylogenetic heterogeneity of the community was positively correlated with the acoustic dispersion for spectral parameters (similarity by phylogenetic relatedness, scenario B, Fig. 1) at the family level (post.mean = 3.467, 95% CI = 0.288 – 6.786, pMCMC = 0.038) and for Hyalinobatrachinae (post.mean = 13.097, 95% CI = 1.086 – 24.763, pMCMC = 0.033). However, we found a negative correlation between phylogenetic heterogeneity of the community and acoustic dispersion for temporal parameters in the analysis restricted to *Centrolene* + *Nymphargus* (post.mean = -20.37, 95% CI = -36.764 – -4.215, pMCMC = 0.017) suggesting both character displacement and environmental pressures (scenario C, Fig. 1) for this subclade.

Character displacement of sister species and deep conspecific lineages. The phylogenetic ANOVA did not detect significant differences among call divergence of pairs of sister species of three categories based on range-overlap and environment similarity (allopatric species in different environments vs species with range-overlap: t-val = -0.005, p = 0.997; allopatric species in different environments vs allopatric species

in similar environments: t-val = -1.798, p = 0.303; species with range overlap vs allopatric species in similar environments: t-val = -1.610, p = 0.303). However, the lowest call divergences were found between allopatric sister-species in similar environments and the highest call divergences were observed in sister-species from different environments (Fig. 5*A*), suggesting that the environment might exert more pressure on calls of sister-species than the community call composition.

In the MCMCglmm analysis of coexistence versus call divergence for populations in the *H. fleischmanni* complex, the overall call of *H. fleischmanni* was more similar to the call of species present at each locality (post.mean = -0.110, 95% CI = -0.215 – -0.008, pMCMC = 0.036), suggesting a convergence of the calls at this scale (Fig. 5*B*). The Euclidean distance of calls between *H. fleischmanni* complex and most species was shorter when they are registered in the same locality. The distances of *H. fleischmanni* complex against *H. collymbiphyllum* and *H. valerioi* appears to be higher when they are registered in the same locality, but the number of calls sampled was too low to perform a statistical comparison restricted only to these species.

Discussion

We implemented recent advances in phylogenetic comparative methods and community phylogenetics in an integrative study of call evolution in a family of Neotropical anurans. Our study specifically addresses whether signal evolution responds primarily to environmental or biotic selective pressures and the role of these selective mechanisms at different evolutionary scales.

Acoustic adaptation. If the advertisement calls of glassfrogs follow the AAH, we expect spectral parameters to be driven by vegetation density (species in denser vegetation will have low-pitched calls), and temporal parameters to be driven by both vegetation (pulse rate will be lower and note duration will be longer at denser vegetation) and temperature (note rate and pulse rate will be higher and note duration will be shorter at warmer sites) (Ey and Fischer 2009). Interestingly, some of our results for spectral parameters show the opposite pattern than expected under the Acoustic Adaptation Hypotheses. Glassfrog species living in localities with denser vegetation have significantly higher peak frequencies, which, in theory, will suffer greater attenuation and reverberation

under such circumstances (Fig 4*A*) (Wiley and Richards 1982). Likewise, species within Cochranellini show broad bandwidth frequencies for species in localities with denser vegetation, opposite to the expected in terms of call attenuation (Wiley and Richards 1978). The Acoustic Adaptation predictions were also not supported for call variation within the *H. fleischmanni* complex. We predicted that low-pitched and longer (slower) calls would be more common at denser vegetation sites to increase the likelihood of their signals reaching the intended receiver (Ey and Fischer 2009); however, peak frequency did not show any correlation with vegetation and note duration was inversely correlated with vegetation density (Fig. 3).

Some predictions of the AAH were supported for temporal parameters. We found that for some clades, species living in habitats with denser vegetation have fewer number of notes and fewer pulse rates, as expected under selection for efficiency of sound transmission (Fig. 4*A*). A lower repetition rate reduces overlap with reverberated waves in closed habitats (Ey and Fischer 2009). The fact that temporal call parameters show this association with potential environmental drivers is corroborated by our finding that temporal traits best fit an Ornstein-Uhlenbeck model, indicating that these parameters are constrained around an adaptive optimum over time (i.e., stabilizing selection, (Hansen 1997), but see (Cooper et al. 2016)). In addition, the temporal parameters exhibit the highest evolutionary rates (sigma square) possibly because they are rapidly responsive to selection in relation to sound transmission efficiency. For anuran calls, it is not surprising that temporal parameters are more subject to environmental selection than spectral parameters (Gerhardt and Mudry 1980), because the latter are more constrained by morphology (Escalona Sulbarán et al. 2019).

Tests of the AAH in anurans have similarly found contradictory results. Zimmerman (Zimmerman 1983) found that lower frequency calls for Amazonian frogs living in closed habitats were due to differences in body size and phylogenetic relationships, Bosch & De la Riva (Bosch and De la Riva 2004) found no relationship between macrohabitat features and advertisement calls for Bolivian frogs, and a study on multiple Asian anurans did not detect a significant correlation among call parameters and canopy coverage (Goutte et al. 2018). In contrast, a study at the intraspecific level

revealed a strong effect of habitat structure on the temporal call parameters among individuals of *Boana pulchella* (Ziegler et al. 2016). Here, we support the conclusion of Zimmerman that the theory relating acoustic signals to habitat structure may not be entirely transferable to frogs (Zimmerman 1983). Although we found an impact of vegetation on the evolution of temporal call parameters as predicted by the AAH, spectral parameters showed an opposite effect, indicating that those traits might respond to vegetation density via a different unknown mechanism.

We also did not find the expected relationships between temperature and call parameters for species comparisons. Typically, temporal parameters but not spectral parameters of calls increase with temperature (Gerhardt and Huber 2002; Ziegler et al. 2016), yet we found that species with calls at higher peak frequencies inhabit warmer regions (after accounting for the effect of phylogeny, body size, and vegetation). Only the call for the *H. fleischmanni* complex follows the common trend for anurans: warmer sites effectively show shorter (faster) note durations (Fig. 4A). These results indicate that only at microevolutionary levels does the air temperature have the expected impact on advertisement calls of glassfrogs. This suggests that once lineages get past speciation, other selective drivers arise, that over longer timeframes promote call evolution, and the signal of metabolic rate becomes less evident beyond speciation events in anurans.

In summary, air temperature and vegetation density have the opposite effect than expected as drivers of call evolution for spectral parameters (at all evolutionary levels) and for temporal parameters (among species) in glassfrogs. What might be the cause of this pattern? Highland species (in colder sites with lesser vegetation) call at the lowest peak frequencies, contrary to what we expected if stream noise is higher in highlands because of steeper terrain and higher gradient streams; therefore, this pattern of peak frequencies must be responding to a different factor. Considering that peak frequency is strongly related to body size (Goutte et al. 2016; Escalona Sulbarán et al. 2019; Tonini et al. 2020), selection for size differences in anurans at different temperatures that are independent of sound transmission properties (i.e., Bergmann's Rule) might be the primary selective mechanism (Bergmann 1847; Ashton 2002; Ziegler et al. 2011;

Gouveia et al. 2013). However, the fact that we found a weak relation between body size and temperature (post.mean = -0.040, l-95% CI = -0.083 – 0.001, pMCMC = 0.058) but a significant relationship of peak frequency and temperature even after accounting for the effect of body size (*SI Appendix*, Table S2) indicates that this trend is not a secondary consequence of body size.

Another possibility is the temperature-dependent signal perception of one of the two hearing organs in the anuran inner ear, the amphibian papilla (Mohneke and Schneider 1979; Narins and Meenderink 2014). If the inner-ear of females plays a role in the evolution of spectral traits, frequency preferences (and selection) could be positively correlated to temperature (Meenderink et al. 2010; Narins and Meenderink 2014; Humfeld and Grunert 2015). Future studies on inner-ear anatomy and function are needed to test the possible mechanisms underlying the relationship between call pitch and temperature.

Ecological and Reproductive Character displacement. If community composition acts as a driver for call evolution among glassfrog species, we would expect differences in species calls to be greater when their range overlap is higher, but in most cases, advertisement calls from glassfrogs exhibit the exact opposite pattern. We found call convergence in species pairs with higher range overlap for all Centrolenidae, for the subfamily Centroleninae and for comparisons at different *H. fleischmanni* populations (Fig. 4B, *SI Appendix*, Table S3). The convergence of call parameters has also been observed in Amazonian bird communities (Tobias et al. 2014) and usually is attributed to habitat similarity (Cardoso and Price 2010) and use of the same optimal resource, often referred to as ‘habitat filtering’ (Webb et al. 2002). The higher similarity in calls of species with higher range overlap supports the hypothesis that environmental factors are driving the species in each locality towards an acoustic optimum. In this case, the call of all species in a given community would be constrained by the vegetation density and air temperature and, likely, other factors we did not test here.

Extreme convergence of calls could have disadvantages for species in the community (29, but see 38). In cases of trait convergence, we would expect species to avoid competition through other behavioral and ecological strategies. For instance, the

use of different streams in the same locality, fine temporal partitioning during the night or during the rainy season, the use of different perching sites at different heights above the water (Gottsberger and Gruber 2004; Blair and Doan 2009). Glassfrogs share the same macro-habitat, they all call at night and because their close proximity of streams, they experience noise-masking for low-frequencies by flowing water, but it is possible that the acoustic space is still broad enough to avoid niche overlap in species assemblages, or that the competition is not strong enough to demand partitioning of the acoustic space (Chek et al. 2003). A recent study (Escalona Sulbarán et al. 2019) detected strong phylogenetic signal in glassfrogs advertisement calls and found that call evolution can be constrained by body size, calling site, and parental care. A fine-scale comparison of microhabitats and timing among species and populations would be very useful to test both the effect of physical factors at small scales and micro-allopatry. But this would require a large effort in documenting the natural history of co-occurring species because those parameters can experience high variation among species and even among individuals at the same locality.

Because competition can arise from very similar signals due to recent shared ancestry (Stuart and Losos 2013), studies of RCD focus on pairs of closely related species (Höbel and Gerhardt 2003; Lemmon 2009; Grossenbacher and Whittall 2011; Jansen et al. 2016). If RCD at the time of speciation plays a role in call divergence, then we predict that sister species with no overlap in their geographic ranges and without change of environment will not require divergence in traits conferring reproductive isolation (Mayr 1942). The phylogenetic ANOVA for call divergence in species with different distributions and same habitat types showed no significant differences among groups. However, when we compare the pattern in call divergence of sister species belonging to three distribution and habitat use categories, we did observe the lowest divergence values for allopatric species (Fig. 5*A*) and the highest divergence values for sister species experiencing geographic isolation with environmental divergence (e.g. living at different altitudinal bands, *SI Appendix*, Figure S2). This indicates that environmental divergence might exert higher selection for differences among advertisement calls than interspecific competition for acoustic space. If that is the case, further studies discriminating how speciation mode and environment interact to drive call

divergence will require a rigorous evaluation of niche divergence at macrohabitat and microhabitat levels.

Integrating Acoustic Adaptation and Character Displacement at different phylogenetic depths. Our results indicate that the call of all species in any given community is associated with the vegetation density (driving some temporal parameters) and in some cases with air temperature (driving spectral parameters) and these factors promote trait convergence among species at the same locality. This pattern was detected between species and for deep conspecific lineages. Also, we found that vegetation density as a driver of call evolution is observed only between species while the effect of temperature on temporal parameters is only observed among conspecific lineages. We found evidence suggesting that differences in environmental selective mechanisms can drive call divergence between pairs of sister species more than Reproductive Character Displacement.

Call evolution in response to selective pressures may be evident at some levels and not others because selection might depend on the divergence among species. Comparing the community results in our deconstructive approach (Fig. 4*B*, *SI Appendix*, Table S3) we found that patterns in call divergence differ substantially among and within clades. In most cases, we found no correlation of call divergence and community heterogeneity (scenario A; Fig.1) where the differentiation is not a by-product of neutral evolution and community composition is also not driving call divergences among species, so environmental selective mechanisms are acting independently of community heterogeneity. Only comparisons of the entire family Centrolenidae and for the Hyalinobatrachinae subfamily showed a positive correlation of call divergence and community heterogeneity, suggesting that at these levels, most trait differentiation is a by-product of neutral evolution (Scenario B, Fig 1). However, the analysis for Centroleninae subfamily showed the opposite pattern, suggesting that for this subclade, communities composed of closely related species experience character displacement while heterogeneous communities experience trait convergence due to habitat filtering (supporting the result obtained in the pairwise species analysis; Scenario 1C, Fig 1). Therefore, different subclades respond in different ways to external pressures and by

decomposing the patterns into smaller subsets within the family, we get better insight into the mechanisms that underlie trait changes. Those differences might be related to the overall variation in call traits, which is higher for Centroleninae than for Hyalinobatrachinae (Fig 2), notably, Centroleninae also showed a higher impact of environment on call evolution (Fig 4A). These results, along with the fact that *H. fleischmanni* populations show higher divergence in sympatry than in allopatry with other *Hyalinobatrachium* species corroborates the hypothesis that character displacement occurs only in few cases and is primarily detected for the most closely-related species (Brown and Wilson 1956). Overall, evolution of calls responds more frequently the environmental pressures and that effect can be detected across multiple phylogenetic levels and for different independent clades.

Literature Cited

Arita, H. T., J. A. Andre, P. Rodrıguez, and J. Soberon. 2008. Species Diversity and Distribution in Presence-Absence Matrices: Mathematical Relationships and. Am Nat 172:519–532.

Arnold, S. J., M. E. Pfrender, and A. G. Jones. 2001. The adaptive landscape as a conceptual bridge between micro- and macroevolution. Genetica 112–113:9–32.

Ashton, K. G. 2002. Do amphibians follow Bergmann's rule? Can J Zool 80:708–716. NRC Research Press.

Bergmann, C. 1847. Über die Ver-hältnisse der wärmeokönomie der Thierezu ihrer Grösse. Göttinger Studien.

Blair, C., and T. M. Doan. 2009. Patterns of Community Structure and Microhabitat Usage in Peruvian Pristimantis (Anura: Strabomantidae). Copeia 2009:303–312. The American Society of Ichthyologists and Herpetologists.

Bosch, J. 2001. Female reciprocal calling in the Iberian midwife toad (Alytes cisternasii) varies with male call rate and dominant frequency: implications for sexual selection. Naturwissenschaften 88:434–437.

Bosch, J., and I. De la Riva. 2004. Are frog calls modulated by the environment? An analysis with anuran species from Bolivia. Can J Zool 82:880–888.

Bouckaert, R., J. Heled, D. Kühnert, T. Vaughan, C.-H. Wu, D. Xie, M. A. Suchard, A. Rambaut, and A. J. Drummond. 2014. BEAST 2: A Software Platform for Bayesian

Evolutionary Analysis. PLOS Comput Biol 10:e1003537. Public Library of Science.

Bradbury, J. W., and S. L. Vehrencamp. 2012. Principles of Animal Communication, Second Edition.

Brawand, D., C. E. Wagner, Y. I. Li, M. Malinsky, I. Keller, S. Fan, O. Simakov, A. Y. Ng, Z. W. Lim, E. Bezault, J. Turner-Maier, J. Johnson, R. Alcazar, H. J. Noh, P. Russell, B. Aken, J. Alföldi, C. Amemiya, N. Azzouzi, J.-F. Baroiller, F. Barloy-Hubler, A. Berlin, R. Bloomquist, K. L. Carleton, M. A. Conte, H. D'Cotta, O. Eshel, L. Gaffney, F. Galibert, H. F. Gante, S. Gnerre, L. Greuter, R. Guyon, N. S. Haddad, W. Haerty, R. M. Harris, H. A. Hofmann, T. Hourlier, G. Hulata, D. B. Jaffe, M. Lara, A. P. Lee, I. MacCallum, S. Mwaiko, M. Nikaido, H. Nishihara, C. Ozouf-Costaz, D. J. Penman, D. Przybylski, M. Rakotomanga, S. C. P. Renn, F. J. Ribeiro, M. Ron, W. Salzburger, L. Sanchez-Pulido, M. E. Santos, S. Searle, T. Sharpe, R. Swofford, F. J. Tan, L. Williams, S. Young, S. Yin, N. Okada, T. D. Kocher, E. A. Miska, E. S. Lander, B. Venkatesh, R. D. Fernald, A. Meyer, C. P. Ponting, J. T. Streelman, K. Lindblad-Toh, O. Seehausen, and F. Di Palma. 2014. The genomic substrate for adaptive radiation in African cichlid fish. Nature 513:375–381. The Author(s).

Brown, W. L., and E. O. Wilson. 1956. Character Displacement. Syst Zool 5:49–64. [Oxford University Press, Society of Systematic Biologists, Taylor & Francis, Ltd.].

Cardoso, G. C., and T. D. Price. 2010. Community convergence in bird song. Evol Ecol 24:447–461.

Castroviejo-Fisher, S., J. M. Guayasamin, A. Gonzalez-Voyer, and C. Vilà. 2014. Neotropical diversification seen through glassfrogs. J Biogeogr 41:66–80.

Chek, A. A., J. P. Bogart, and S. C. Lougheed. 2003. Mating signal partitioning in multi-species assemblages: A null model test using frogs. Ecol Lett 6:235–247.

Chesser, R. T., and R. M. Zink. 1994. Modes of speciation in birds: A test of Lynch's method. Evolution (N Y) 48:490–497. John Wiley & Sons, Ltd (10.1111).

Cooper, N., G. H. Thomas, C. Venditti, A. Meade, and R. P. Freckleton. 2016. A cautionary note on the use of Ornstein Uhlenbeck models in macroevolutionary studies. Biol J Linn Soc 118:64–77. Oxford University Press.

Delia, J., L. Bravo-Valencia, and K. M. Warkentin. 2017. Patterns of parental care in Neotropical glassfrogs: fieldwork alters hypotheses of sex-role evolution. J Evol Biol

30:898–914.

Drummond, A. J., S. Y. W. Ho, M. J. Phillips, and A. Rambaut. 2006. Relaxed Phylogenetics and Dating with Confidence. PLOS Biol 4:e88. Public Library of Science.

Dugo-Cota, Á., S. Castroviejo-Fisher, C. Vilà, and A. Gonzalez-Voyer. 2015. A test of the integrated evolutionary speed hypothesis in a Neotropical amphibian radiation. Glob Ecol Biogeogr 24:804–813.

Endler, J. A. 1992. Signals, signal conditions, and the direction of evolution. Am Nat 139:S125–S153.

Escalona Sulbarán, M. D., P. Ivo Simões, A. Gonzalez-Voyer, and S. Castroviejo-Fisher. 2019. Neotropical frogs and mating songs: The evolution of advertisement calls in glassfrogs. J Evol Biol 32:163–176.

Ey, E., and J. Fischer. 2009. The “acoustic adaptation hypothesis”—a review of the evidence from birds, anurans and mammals. Bioacoustics 19:21–48.

Garey, M. V., D. B. Provete, T. Gonçalves-Souza, L. S. Ouchi-Melo, C. F. B. Haddad, and D. C. Rossa-Feres. 2018. Phylogenetic and adaptive components of the anuran advertisement call correlate with temporal species co-occurrence. Biol J Linn Soc 125:292–301.

Gasc, A., D. Francomano, J. B. Dunning, and B. C. Pijanowski. 2017. Future directions for soundscape ecology: The importance of ornithological contributions. Auk 134:215–228.

Gerhardt, H. C. 1994. The evolution of vocalization in frogs and toads. Annu Rev Ecol Syst 25:293–324. Annual Reviews.

Gerhardt, H. C., and F. Huber. 2002. Acoustic communication in insects and anurans. Common problems and diverse solutions. University.

Gerhardt, H. C., and K. M. Mudry. 1980. Temperature effects on frequency preferences and mating call frequencies in the green treefrog, Hyla cinerea (Anura: Hylidae). J Comp Physiol 137:1–6.

Gingras, B., E. Mohandesan, D. Boko, and W. T. Fitch. 2013. Phylogenetic signal in the acoustic parameters of the advertisement calls of four clades of anurans. BMC Evol Biol 13:1. BMC Evolutionary Biology.

Gottsberger, B., and E. Gruber. 2004. Temporal partitioning of reproductive activity in a neotropical anuran community. J Trop Ecol 20:271–280. Cambridge University Press.

Goutte, S., A. Dubois, S. D. Howard, R. Marquez, J. J. L. Rowley, J. M. Dehling, P. Grandcolas, X. Rongchuan, and F. Legendre. 2016. Environmental constraints and call evolution in torrent-dwelling frogs. Evolution (N Y) 70:811–826.

Goutte, S., A. Dubois, S. D. Howard, R. Márquez, J. J. L. Rowley, J. M. Dehling, P. Grandcolas, R. C. Xiong, and F. Legendre. 2018. How the environment shapes animal signals: a test of the acoustic adaptation hypothesis in frogs. J Evol Biol 31:148–158.

Goutte, S., A. Dubois, and F. Legendre. 2013. The Importance of Ambient Sound Level to Characterise Anuran Habitat. PLoS One 8:e78020.

Gouveia, S. F., R. Dobrovolski, P. Lemes, F. A. S. Cassemiro, J. A. F. Diniz-filho, D. De Ecologia, I. Icb, U. Federal, and D. G. Ufg. 2013. Environmental steepness , tolerance gradient , and ecogeographical rules in glassfrogs (Anura : Centrolenidae). 773–783.

Grant, P. R., and B. R. Grant. 2002. Unpredictable Evolution in a 30-Year Study of Darwin's Finches. Science (80-) 296:707–711.

Grether, G. F., N. Losin, C. N. Anderson, and K. Okamoto. 2009. The role of interspecific interference competition in character displacement and the evolution of competitor recognition. Biol Rev 84:617–635.

Grossenbacher, D. L., and J. B. Whittall. 2011. Increased floral divergence in sympatric monkeyflowers. Evolution (N Y) 65:2712–2718.

Guayasamin, J. M., S. Castroviejo-fisher, J. Ayarzagüena, L. Trueb, and C. Vilà. 2008. Molecular Phylogenetics and Evolution Phylogenetic relationships of glassfrogs (Centrolenidae) based on mitochondrial and nuclear genes. Mol Phylogenet Evol 48:574–595.

Guayasamin, J. M., S. Castroviejo-Fisher, L. Trueb, J. Ayarzagüena, M. Rada, C. Vilà, J. Ayarzagüena, M. Rada, and C. Vilà. 2009. Phylogenetic systematics of Glassfrogs (Amphibia: Centrolenidae) and their sister taxon Allophryne ruthveni. Zootaxa 2100:1–97.

Guayasamin, J. M., D. F. Cisneros-Heredia, R. W. McDiarmid, P. Peña, and C. R. Hutter. 2020. Glassfrogs of Ecuador: Diversity, Evolution, and Conservation. Diversity 12:222.

Guillerme, T., and K. Healy. 2014. mulTree: a package for running MCMCglmm analysis on multiple trees.

Hadfield, J. D. 2010. MCMC Methods for Multi-Response Generalized Linear Mixed Models: The MCMCglmm R Package. J Stat Softw 033:1–22.

Hansen, T. F. 1997. Stabilizing selection and the comparative analysis of adaptation. Evolution (N Y) 51:1341–1351.

Hansen, T. F., and E. P. Martins. 1996. Translating Between Microevolutionary Process and Macroevolutionary Patterns: The Correlation Structure of Interspecific Data. Evolution (N Y) 50:1404–1417.

Harmon, L. J., C. S. Andreazzi, F. Débarre, J. Drury, E. E. Goldberg, A. B. Martins, C. J. Melián, A. Narwani, S. L. Nuismer, M. W. Pennell, S. M. Rudman, O. Seehausen, D. Silvestro, M. Weber, and B. Matthews. 2019. Detecting the macroevolutionary signal of species interactions. J Evol Biol 1–14.

Harmon, L. J., J. T. Weir, C. D. Brock, R. E. Glor, and W. Challenger. 2007. GEIGER: investigating evolutionary radiations. Bioinformatics 24:129–131.

Höbel, G., and H. C. Gerhardt. 2003. Reproductive character displacement in the acoustic communication system of green tree frogs (Hyla cinerea). Evolution (N Y) 57:894–904.

Humfeld, S. C., and B. Grunert. 2015. Effects of temperature on spectral preferences of female gray treefrogs (Hyla versicolor). Herpetol Conserv Biol 10:1013–1020.

Hutter, C. R., J. M. Guayasamin, and J. J. Wiens. 2013. Explaining Andean megadiversity : the evolutionary and ecological causes of glassfrog elevational richness patterns. Ecol Lett 16:1135–1144.

Indermaur, L., B. R. Schmidt, K. Tockner, and M. Schaub. 2010. Spatial variation in abiotic and biotic factors in a floodplain determine anuran body size and growth rate at metamorphosis. Oecologia 163:637–649.

Jansen, M., M. Plath, F. Brusquetti, and M. J. Ryan. 2016. Asymmetric frequency shift in advertisement calls of sympatric frogs. Amphibia-Reptilia 37:137–152.

Jonathan, R., S. Daniele, L. Glenn, F. Laurélène, S. Nicolas, N. Salamin, L. Faye, D. Silvestro, J. Rolland, and G. Litsios. 2018. Clownfishes evolution below and above the species level. Proc R Soc B Biol Sci 285:20171796. Royal Society.

Köhler, J., M. Jansen, A. Rodríguez, P. J. R. Kok, L. F. Toledo, M. Emmrich, F. Glaw, C. F. B. Haddad, M. O. Rödel, and M. Vences. 2017. The use of bioacoustics in anuran taxonomy: Theory, terminology, methods and recommendations for best practice. Zootaxa 4251:1–124.

Kumar, S., G. Stecher, and K. Tamura. 2016. MEGA7: Molecular Evolutionary Genetics Analysis Version 7.0 for Bigger Datasets. Mol Biol Evol 33:1870–1874.

Laliberté, E., and P. Legendre. 2010. A distance-based framework for measuring functional diversity from multiple traits. Ecology 91:299–305. John Wiley & Sons, Ltd.

Lanfear, R., P. B. Frandsen, A. M. Wright, T. Senfeld, and B. Calcott. 2016. PartitionFinder 2: New Methods for Selecting Partitioned Models of Evolution for Molecular and Morphological Phylogenetic Analyses. Mol Biol Evol 34:772–773.

Lemmon, E. M. 2009. Diversification of conspecific signals in sympatry: geographic overlap drives multidimensional reproductive character displacement in frogs. Evolution (N Y) 63:1155–1170. John Wiley & Sons, Ltd (10.1111).

Liu, H. Q., and A. Huete. 1995. A feedback based modification of the NDVI to minimize canopy background and atmospheric noise. IEEE Trans Geosci Remote Sens 33:457–465.

Losos, J. B., and R. E. Glor. 2003. Phylogenetic comparative methods and the geography of speciation. Trends Ecol Evol 18:220–227.

Marquet, P. A., M. Fernández, S. A. Navarrete, and C. Valdovinos. 2004. Diversity emerging: towards a deconstruction of biodiversity patterns. Pp. 191–209 *in* M. Lomolino and L. Heansey, eds. Frontiers in Biogeography: New directions in the Geography of Nature. Sunderland MA.

Marten, K., and P. Marler. 1977. Sound transmission and its significance for animal vocalization. Behav Ecol Sociobiol 2:271–290.

Mayfield, M. M., and J. M. Levine. 2010. Opposing effects of competitive exclusion on the phylogenetic structure of communities. Ecol Lett 13:1085–1093. John Wiley &

Sons, Ltd (10.1111).

Mayr, E. 1942. Systematics and the Origin of Species. Columbia University Press, New York.

Meenderink, S. W. F., M. Kits, and P. M. Narins. 2010. Frequency matching of vocalizations to inner-ear sensitivity along an altitudinal gradient in the coqui frog. Biol Lett 6:278–281. Royal Society.

Meier, J. I., D. A. Marques, S. Mwaiko, C. E. Wagner, L. Excoffier, and O. Seehausen. 2017. Ancient hybridization fuels rapid cichlid fish adaptive radiations. Nat Commun 8:1–11. Nature Publishing Group.

Mendoza, A. M., and H. T. Arita. 2014. Priority setting by sites and by species using rarity, richness and phylogenetic diversity: The case of neotropical glassfrogs (Anura: Centrolenidae). Biodivers Conserv 23:909–926.

Mendoza, A. M., W. Bolívar-García, E. Vázquez-Domínguez, R. Ibáñez, and G. Parra Olea. 2019. The role of Central American barriers in shaping the evolutionary history of the northernmost glassfrog, Hyalinobatrachium fleischmanni (Anura: Centrolenidae). PeerJ 7:e6115.

Miller, E. T., D. R. Farine, and C. H. Trisos. 2017. Phylogenetic community structure metrics and null models: a review with new methods and software. Ecography (Cop) 40:461–477.

Miller, M. A., W. Pfeiffer, and T. Schwartz. 2010. Creating the CIPRES Science Gateway for inference of large phylogenetic trees. Pp. 1–8 *in* 2010 Gateway Computing Environments Workshop (GCE).

Mohneke, R., and H. Schneider. 1979. Effect of temperature upon auditory thresholds in two anuran species, Bombina v. variegata and Alytes o. obstetricans (Amphibia, Discoglossidae). J Comp Physiol 130:9–16.

Narins, P. M., and S. W. F. Meenderink. 2014. Climate change and frog calls: long-term correlations along a tropical altitudinal gradient. Proc R Soc B Biol Sci 281:20140401.

Padial, J. M., A. Miralles, I. D. la Riva, and M. Vences. 2010. The integrative future of taxonomy. Front Zool 7:1–14.

Peterson, A. T., A. G. Navarro-Sigüenza, and A. Gordillo. 2018. Assumption-versus

data-based approaches to summarizing species' ranges. Conserv Biol 32:568–575. John Wiley & Sons, Ltd (10.1111).

Pfennig, K. S., and D. W. Pfennig. 2009. Character Displacement: Ecological And Reproductive Responses To A Common Evolutionary Problem. Q Rev Biol 84:253–276.

Plummer, M., N. Best, K. Cowles, and K. Vines. 2006. CODA: convergence diagnosis and output analysis for MCMC. R News 6:7–11.

Revell, L. J. 2012. phytools: an R package for phylogenetic comparative biology (and other things). Methods Ecol Evol 3:217–223. John Wiley & Sons, Ltd (10.1111).

Revell, L. J. 2009. Size-correction and principal components for interspecific comparative studies. Evolution (N Y) 63:3258–3268.

Riesch, R., M. Muschick, D. Lindtke, R. Villoutreix, A. A. Comeault, T. E. Farkas, K. Lucek, E. Hellen, V. Soria-Carrasco, S. R. Dennis, C. F. de Carvalho, R. J. Safran, C. P. Sandoval, J. Feder, R. Gries, B. J. Crespi, G. Gries, Z. Gompert, and P. Nosil. 2017. Transitions between phases of genomic differentiation during stick-insect speciation. Nat Ecol Evol 1:82. Macmillan Publishers Limited, part of Springer Nature.

Steppan, S. J., P. C. Phillips, and D. Houle. 2002. Comparative quantitative genetics: evolution of the G matrix. Trends Ecol Evol 17:320–327.

Stuart, Y. E., and J. B. Losos. 2013. Ecological character displacement: Glass half full or half empty? Trends Ecol Evol 28:402–408. Elsevier Ltd.

Thompson, J. D., D. G. Higgins, and T. J. Gibson. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 22:4673–4680.

Tobias, J. A., J. Aben, R. T. Brumfield, E. P. Derryberry, W. Halfwerk, H. Slabbekoorn, and N. Seddon. 2010. Song divergence by sensory drive in Amazonian birds. Evolution (N Y) 64:2820–2839. John Wiley & Sons, Ltd (10.1111).

Tobias, J. A., R. Planque, D. L. Cram, and N. Seddon. 2014. Species interactions and the structure of complex communication networks. Proc Natl Acad Sci 111:1020–1025.

Tonini, J. F. R., D. B. Provete, N. M. Maciel, A. R. Morais, S. Goutte, L. F. Toledo, and R. A. Pyron. 2020. Allometric escape from acoustic constraints is rare for frog calls. Ecol Evol 10:3686–3695. Wiley Online Library.

Twomey, E., J. Delia, and S. Castroviejo-fisher. 2014. A review of Northern Peruvian glassfrogs (Centrolenidae), with the description of four new remarkable species. Zootaxa 3851:1–87.

Uyeda, J. C., T. F. Hansen, S. J. Arnold, and J. Pienaar. 2011. The million-year wait for macroevolutionary bursts. Proc Natl Acad Sci 108:15908–15913.

Villanueva-Rivera, L. J. 2014. Eleutherodactylus frogs show frequency but no temporal partitioning: implications for the acoustic niche hypothesis. PeerJ 2:e496.

Webb, C. O., D. D. Ackerly, M. A. McPeek, and M. J. Donoghue. 2002. Phylogenies and Community Ecology. Annu Rev Ecol Syst 33:475–505.

Wellborn, G. A., D. K. Skelly, and E. E. Werner. 1996. Mechanisms creating community structure across a freshwater habitat gradient. Annu Rev Ecol Syst 27:337–363. Annual Reviews.

West-Eberhard, M. J. 1983. Sexual Selection, Social Competition, and Speciation. Q Rev Biol 58:155–183. The University of Chicago Press.

Wiley, R. H. 1991. Associations of Song Properties with Habitats for Territorial Oscine Birds of Eastern North America. Am Nat 138:973–993. The University of Chicago Press.

Wiley, R. H., and D. G. Richards. 1982. Adaptations for acoustic communication in birds: sound transmission and signal detection. Pp. 131–181 *in* D. Kroodsma and E. Miller, eds. Acoustic Communication in Birds. Academic Press.

Wiley, R. H., and D. G. Richards. 1978. Physical Constraints on Acoustic Communication in the Atmosphere: Implications for the Evolution of Animal Vocalizations. Behav Ecol Sociobiol 3:69–94.

Wilkins, M. R., N. Seddon, and R. J. Safran. 2013. Evolutionary divergence in acoustic signals: Causes and consequences. Trends Ecol Evol 28:156–166. Elsevier Ltd.

Ziegler, L., M. Arim, and F. Bozinovic. 2016. Intraspecific scaling in frog calls: the interplay of temperature, body size and metabolic condition. Oecologia 181:673–681.

Ziegler, L., M. Arim, and P. M. Narins. 2011. Linking amphibian call structure to the environment: the interplay between phenotypic flexibility and individual attributes. Behav Ecol 22:520–526.

Zimmerman, B. L. 1983. A Comparison of Structural Features of Calls of Open and Forest Habitat Frog Species in the Central Amazon. Herpetologica 39:235–246.

Figures and Tables

Figure 1. Expected evolution of advertisement calls under different dominant scenarios: (A) Environmental selection is dominant, (B) neutral evolution is dominant, and (C) character displacement is dominant. The Mean Pairwise Phylogenetic Distance (MPD) is the mean of all pairwise branch lengths for a set of species in a community and the Acoustic Dispersion is the mean distance to the centroid in a phylogenetic PCA of calls from all localities where a species occurs. Species with larger Acoustic Dispersion are exposed to more different calls than species with lower Acoustic Dispersion. Species with high values of MPD live in heterogeneous communities (e.g. communities composed of species of different genera). For details see Main Text.

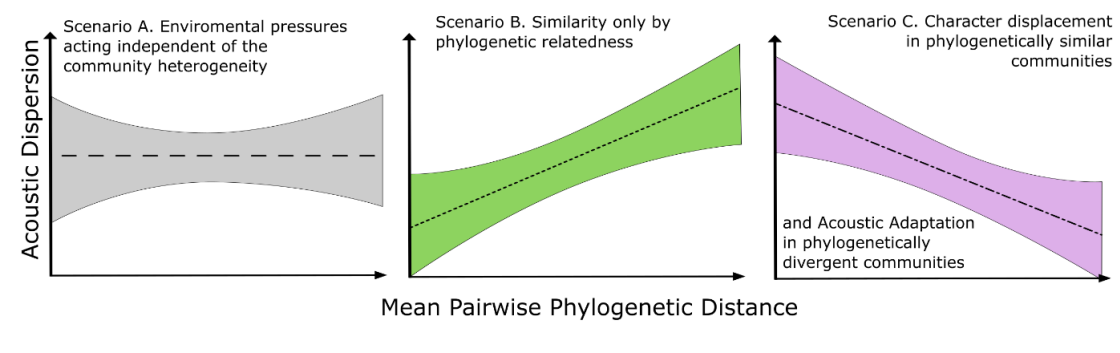

Figure 2. Call variation among glassfrogs species, displayed with the phylogenetic PCA of six call parameters analyzed in this study. Insets are examples of spectrograms and sonograms for eight species with highly divergent calls. PeakFreq = Peak Frequency, BandW = Frequency Bandwidth, Nrate= Note rate, MNotes = Maximum number of notes, PR = Pulse rate, NDur = Note duration.

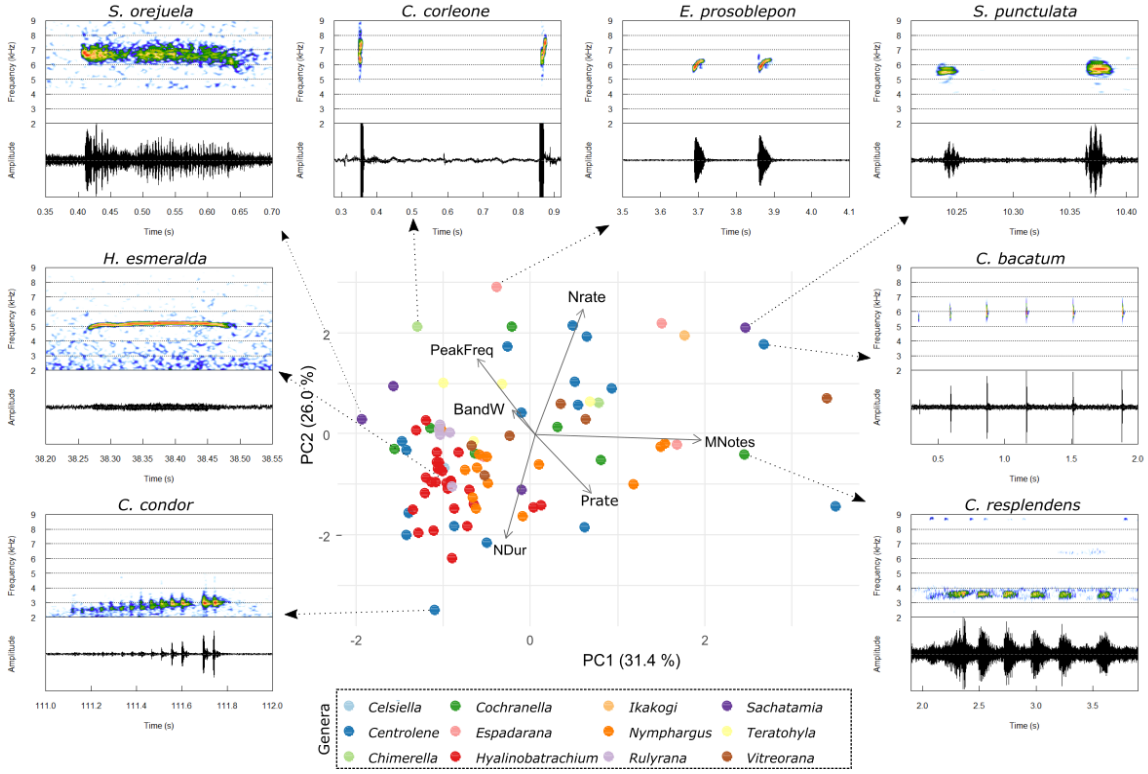

Figure 3. Ancestral state reconstruction of six call traits in glassfrogs. A) Peak frequency (Hz), B) Frequency bandwidth (Hz), C) Number of notes, D) Note rate, E) Note duration (ms) and F) Pulse rate. Color legend below each tree denotes minimum and maximum values for each trait. Letters at nodes identify the same clades in Supplementary Figure S2 tree. Trees for these analyses were pruned depending on the availability of trait data for each species

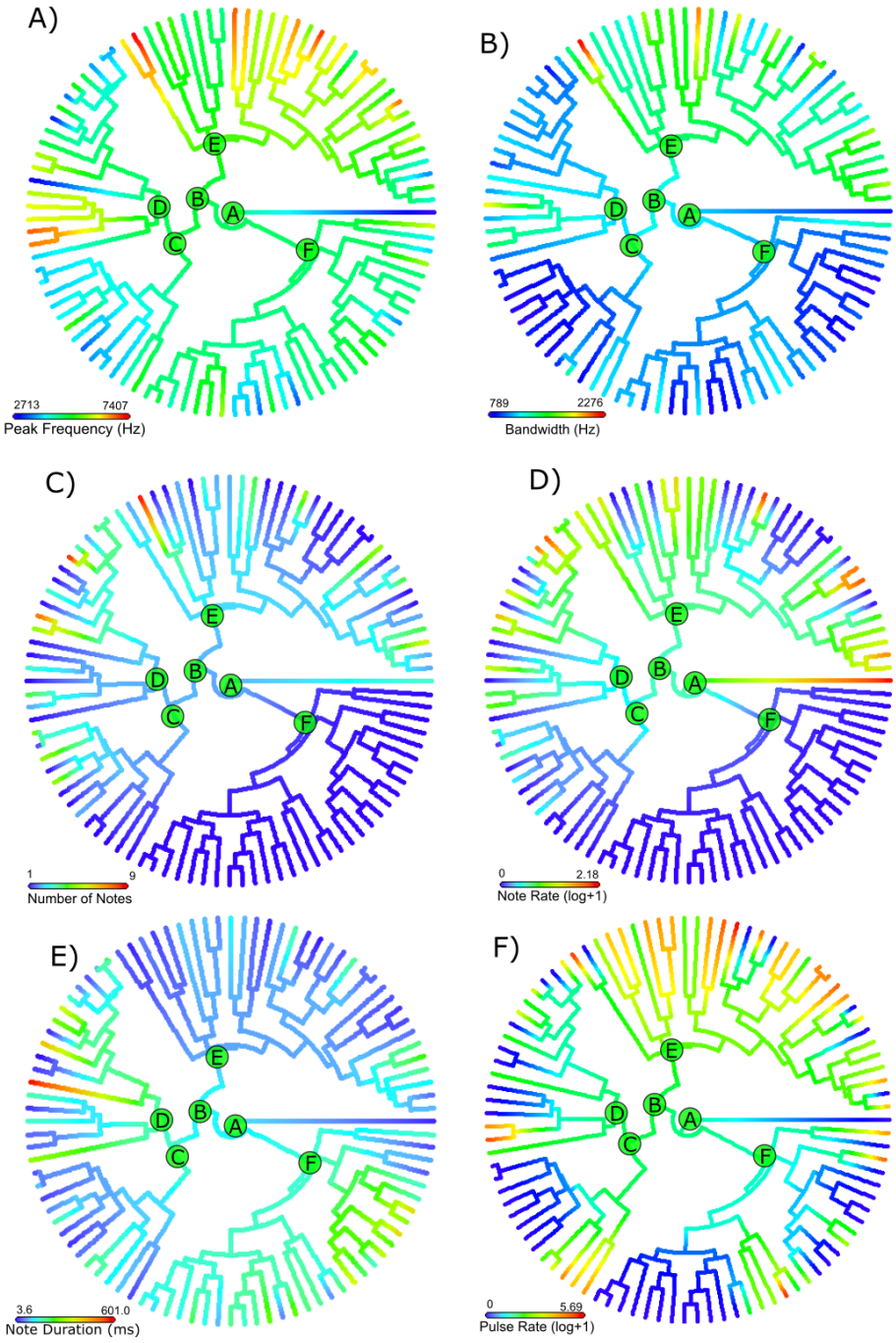

Figure 4. Summary of the results for Acoustic Adaptation (A) and combined Acoustic Adaptation / Character Displacement (B) at different phylogenetic levels. Horizontal bar represents posterior mean (slope) values and their 95% compatibility intervals (CI) for each test through Bayesian mixed-model approach. Overlap of horizontal line with the vertical black dashed line indicates non-significant correlation among variables. A = Centrolenidae, B = Hyalinobatrachinae, C = Centroleninae, D = *Centrolene* + *Nymphargus*, E = *Centrolene*, F = Cochranellini. A-F letters shows the position of each clade in the Supplementary Figure 2 tree. Details of the analyses are provided in *SI Appendix*, Table S3.

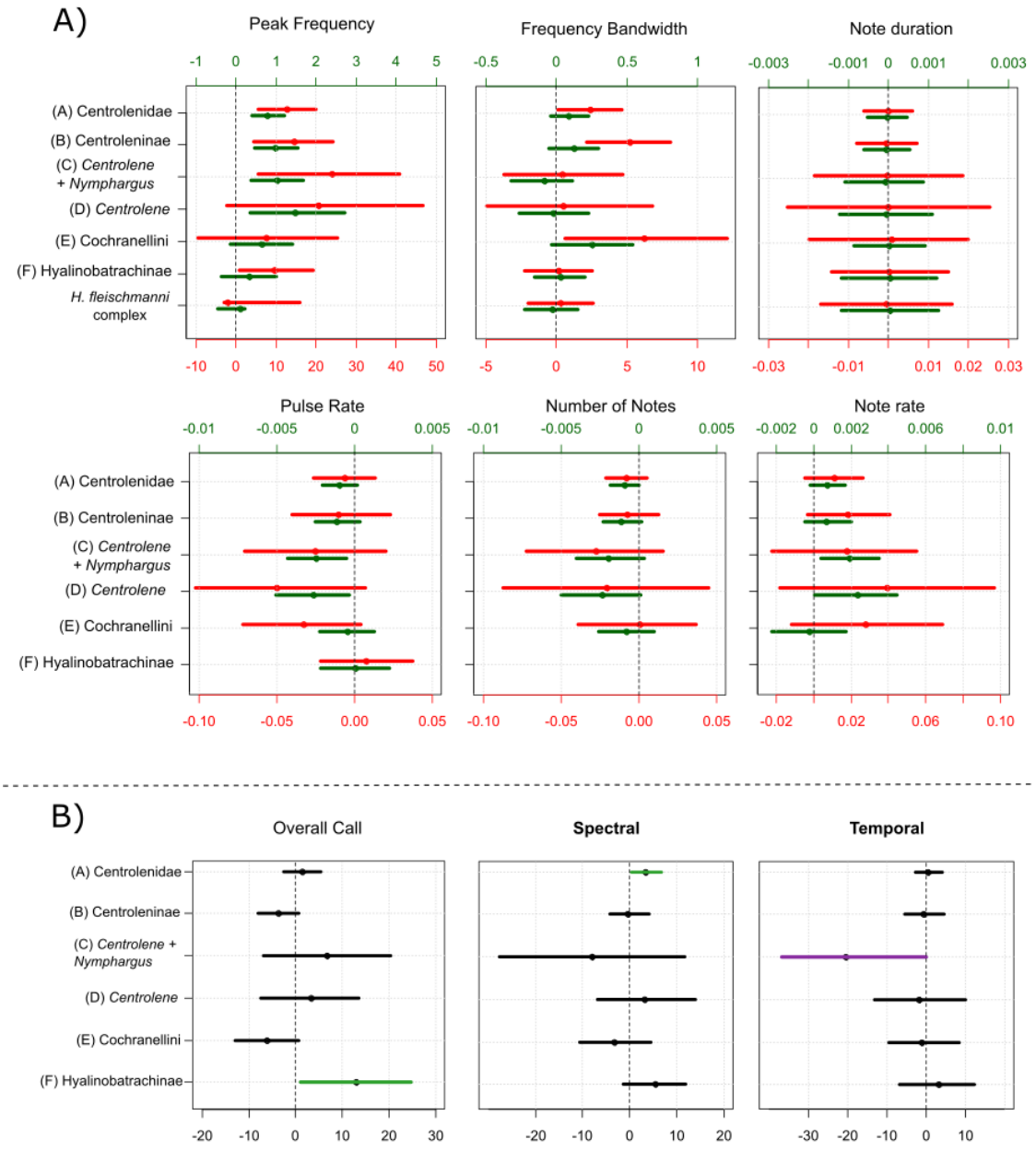

Figure 5. Violin plot of call divergence values between pairs of species. A) Call divergence for 26 pairs of sister species across three categories: Allopatric species in similar environments (gray), allopatric species in different environments and (yellow), and sister species with range overlap (blue). B) Euclidean distances of the call of *Hyalinobatrachium fleischmanni* complex against each glassfrog species in localities sympatry (blue) and allopatry (yellow).

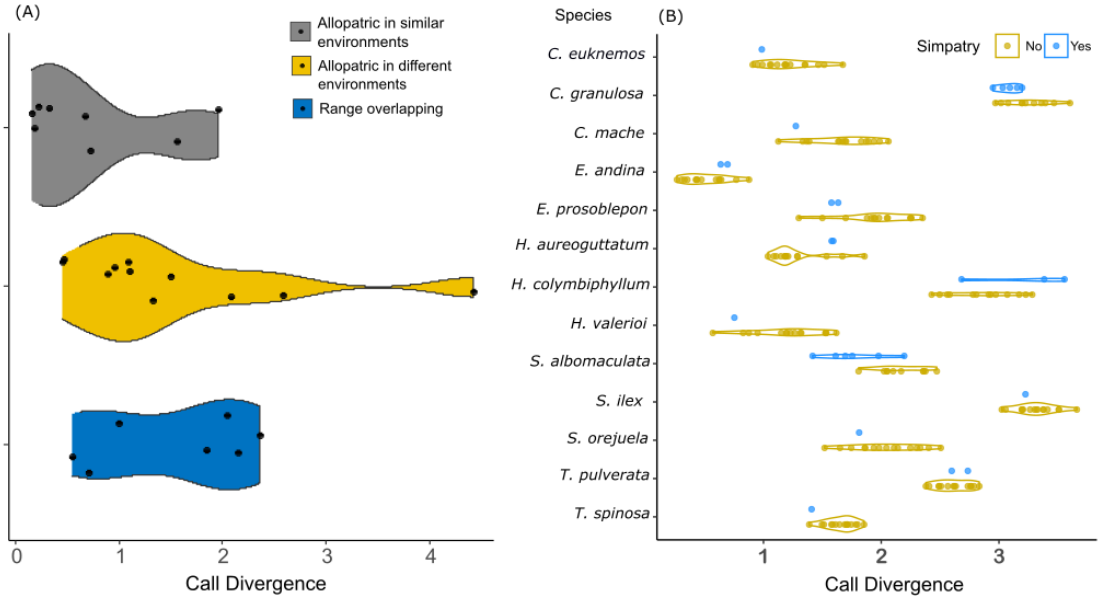

Table 1. Best fit model of evolution for each acoustic trait in glassfrogs. α = constraint parameter, λ = Lambda Pagel, σ2 = rate parameter and Z = starting value.

Variables		Best bit Model	Model metrics			
All glassfrog species			α	λ	σ2	Z
Spectral	Peak Frequency	Lambda Pagel	--	0.846	13.11	4.65
	Frequency Bandwidth	Lambda Pagel	--	0.796	0.99	1.17
Temporal	Number Notes	Lambda Pagel	--	0.216	27.19	1.99
	Note Duration	Lambda Pagel	--	0.690	0.16	0.11
	Note Rate	Ornstein-Uhlenbeck	27.92	--	23.37	0.45
	Pulse Rate	Ornstein-Uhlenbeck	36.46	--	236.40	1.01
H. fleischmanni complex						
Spectral	Peak Frequency	Lambda Pagel	--	0.688	0.05	4.66
	Frequency Bandwidth	Brownian Motion	--	--	0.01	1.06
Temporal	Note Duration	Ornstein-Uhlenbeck	0.89	--	0.0016	0.13

Supplementary Material

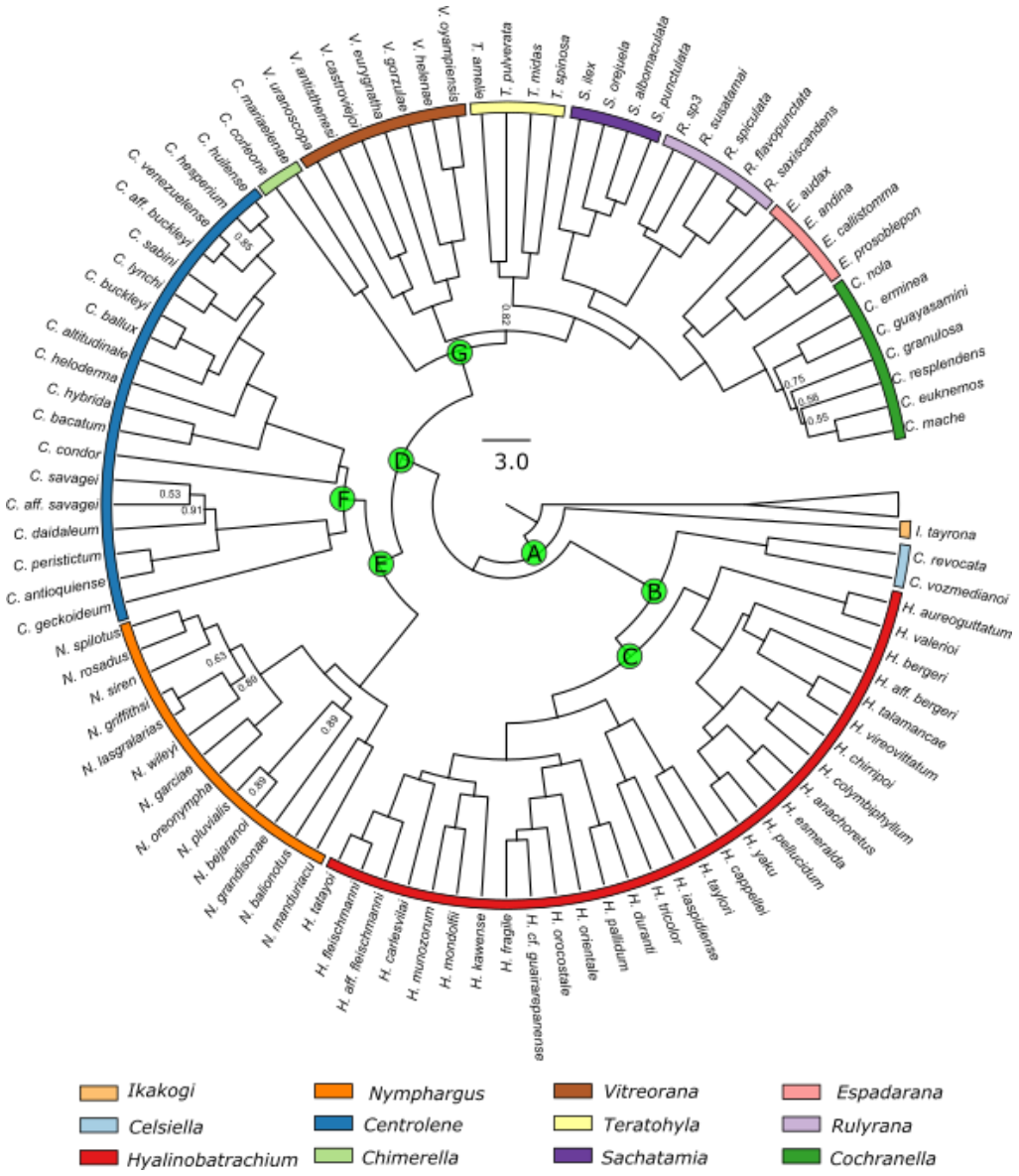

Fig. S1. Bayesian phylogenetic reconstruction of 97 glassfrog species based on DNA sequences from three mitochondrial (12S, 16S and ND1) and seven nuclear (POMC, BDNF, C-MYC, CXCR, RAG, SLC8A1, and SLC8A) markers (6304 bp). Allophrynidae species (gray triangle) were used as outgroup. Scale bar show mean calibration in million years. Green circles identify the crown node of Centrolenidae and analyzed subclades. A = Centrolenidae, B = Centroleninae, C = *Centrolene* + *Nymphargus,* D = *Centrolene*, E = Cochranellini, F = Hyalinobatrachinae*.* Different colors at the tips of the tree represent genera. Clade support < 0.95 is indicated by posterior probability values from Bayesian Inference.

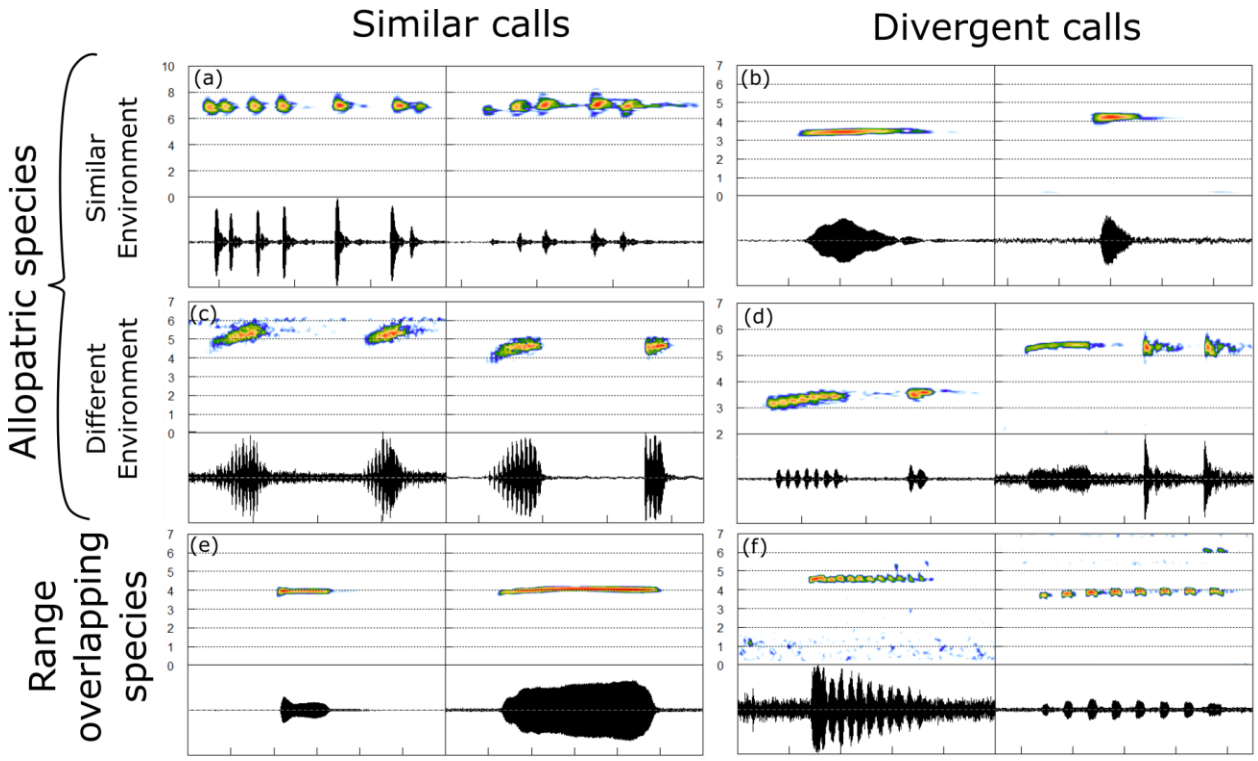

Fig. S2. Exemplar spectrograms and sonograms of two pairs of sister species in sympatry and four pairs of sister species in allopatry with and without environmental differences. a) *Teratohyla spinosa / T. midas,* b) *Nymphargus garciae / N. oreonympha,* c) *Vitreorana ritae / V. helenae,* d) *Centrolene sabini / C. lynchi*, e) *N. rosadus / N. spillotus,* f) *H. chirripoi / H. colymbiphyllum.*

Table S1. Call metrics of all species. False = non available

Species	Peak Freq	Bandwidht	No. Notes	Note Duration	note rate	pulses/note	pulse rate
Celsiella revocata	4084.1	false	1	0.0252	0	1	0
Celsiella vozmedianoi	6098.22	1338.86	1	0.156	0	21.25	130
Centreolene aff savagei	5964.887	1079.907	1	0.173	0	1	0
Centrolene aff buckleyi	3301.4	1103.3	3	0.27	0.8787	24.9	88.519
Centrolene altitudinale	4737.3	1042	3	0.0087	3.0181	1	0
Centrolene antioquiense	7000	1320.2	4	0.0416	1.2513	6.62	135.096
Centrolene bacatum	5900	1127.4	8	0.0036	3.9216	1	0
Centrolene ballux	5240.62	1113.84	1	0.366	0	8.2	19.672
Centrolene buckleyi	3046.9	1109.7	1	0.279	0	18.3	62
Centrolene condor	2896.175	1225.425	1	0.601	0	13	19.967
Centrolene daidaleum	6210.173	1101.107	1	0.157	0	1	0
Centrolene geckoideum	3874.4	1004.6	1	0.2997	0	8.37	24.591
Centrolene heloderma	4921.9	1170.85	1	0.438	0	10	20.548
Centrolene hesperium	3630	false	false	false	false	false	false
Centrolene huilense	4858.3	881.7	2	0.0291	6.4267	1	0
Centrolene hybrida	6093.1	942.6	5	0.0176	1.5868	1	0
Centrolene lynchi	5264.162	1032.118	3	0.055	5.5928	1	0
Centrolene peristictum	6871.97	1446.073	2	0.042	1.5406	9.45	201.190
Centrolene sabini	3374.603	1057.717	9	0.16	0.5408	11.8	67.500
Centrolene savagei	6214	1901	3	0.0166	2.6233	1	0
Centrolene venezuelense	4316.2	false	4	0.0473	2.4492	false	false
Chimerella corleone	7407.4	2275.7	2	0.0105	1.9342	1	0
Chimerella mariaelenae	6365.22	1391.02	3	0.0342	2.8297	8.44	217.544
Cochranella erminea	5286.433	1512.2	2	0.2445	1.4728	10.5	38.855
Cochranella euknemos	4269.3	1532.587	2	0.0857	2.8297	10.45	110.268
Cochranella granulosa	4242.05	1370.175	4	0.193	1.8622	10	46.632
Cochranella guayasamini	6129.817	1770.233	1	0.195	0	9.5	43.590

Cochranella mache	5426.4	1806.3	2	0.046	5.0000	false	0
Cochranella nola	5357.48	1266.08	1	0.044	0.0000	1	0.000
Cochranella resplendens	3574.5	1501.4	6	0.1232	2.8417	16.5	125.812
Espadarana andina	5196.667	1363.167	1	0.029	0	5.04	139
Espadarana audax	5693.38	1005.64	5	0.039	1.2594	7.9	176.923
Espadarana callistomma	5578.1	1211.8	4	0.026	6.5359	3.25	86.538
Espadarana prosoblepon	6933.7	1456.5	2	0.016	5.5866	1	0
Hyalinobatrachium aff bergeri	4510.493	810.393	1	0.166	0	1	0
Hyalinobatrachium anachoretus	4742.713	947.938	1	0.365	0	16.5	42.47
Hyalinobatrachium aureoguttatum	5880.1	1044.1	1	0.0334	0	1	0
Hyalinobatrachium bergeri	4684.199	991.369	1	0.153	0	1	0
Hyalinobatrachium cappellei	4523.162	1210.321	1	0.199	0	8.5	37.688
Hyalinobatrachium carlesvilai	4659.787	1263.147	1	0.134	0	1	0
Hyalinobatrachium cf guairarepanense	3923	false	1	0.195	0	1	0
Hyalinobatrachium chirripoi	4437.5	991.9	1	0.25	0	12.5	46.000
Hyalinobatrachium colymbiphyllum	3570.4	853.367	1	0.406	0	8.4	18.227
Hyalinobatrachium duranti	4216.21	1208.375	1	0.054	0	1	0
Hyalinobatrachium esmeralda	5154.4	960.1	1	0.2132	0	1	0
Hyalinobatrachium fleischmanni	4659.6	1099.9	1	0.1483	0	1	0
Hyalinobatrachium fragile	3824.28	820.14	1	0.149	0	1	0
Hyalinobatrachium iaspidiense	4531.293	993.01	1	0.064	0	16	234
Hyalinobatrachium kawense	5831.2	1195.64	1	0.087	0	1	0
Hyalinobatrachium mondolfii	5057.069	834.945	1	0.216	0	1	0
Hyalinobatrachium munozorum	5073.9	873.1	1	0.134	0	1	0
Hyalinobatrachium orientale	5025.84	880.69	1	0.265	0	1	0
Hyalinobatrachium orocostale	3436.68	862.03	1	0.181	0	1	0
Hyalinobatrachium pallidum	3329.02	1040.353	1	0.267	0	5.5	17
Hyalinobatrachium pellucidum	5057.367	827.707	1	0.157	0	1	0
Hyalinobatrachium talamancae	4600	false	1	0.3	0	1	0
Hyalinobatrachium tatayoi	4916.1	1169.3	1	0.1048	0	1	0

Hyalinobatrachium taylori	4758.85	1191.017	1	0.187	0	9	43
Hyalinobatrachium tricolor	4755.595	1139.9	1	0.202	0	4	14.851
Hyalinobatrachium valerioi	3884.48	992.352	1	0.077	0	14.5	175.325
Hyalinobatrachium vireovittatum	4770.8	794.505	1	0.405	0	1	0.000
Hyalinobatrachium viridissima	4337.2	968.5	1	0.1633	0	1	0
Hyalinobatrachium yaku	5211	840.4	1	0.344	0	1	0
Ikakogi tayrona	2713.2	873.9	3	0.012	7.8740	1	0
Nymphargus balionotus	5196.7	1173.6	1	0.023	0	4	130.435
Nymphargus bejaranoi	3602.016	905.29	1	0.1951	0	6	25.628
Nymphargus garciae	3375	823.9	2	0.0482	0.5759	1	0
Nymphargus grandisonae	3798.42	855.2	1	0.125	0	18.8	142.400
Nymphargus griffithsi	4089.247	922.271	1	0.095	0	1	0
Nymphargus lasgralarias	3702.629	872.948	5	0.0235	1.1223	1	0
Nymphargus manduriacu	4306.6	1206.4	1	0.096	0	10.75	101.563
Nymphargus oreonympha	3995.9	842.8	1	0.015	0	1	0
Nymphargus pluvialis	3881.367	1366.967	1	0.171	0	12.4	66.667
Nymphargus rosadus	3923.7	803.1	1	0.0598	0	1	0
Nymphargus siren	5469.4	898.767	1	0.027	0	1	0
Nymphargus wileyi	3824.2	808.5	5	0.0236	0.8951	1	0
Rulyrana flavopunctata	6525	1559.52	1	0.0369	0	6.69	154.201
Rulyrana pezzutti	6361.8	1439.2	1	0.029	0	4	103.448
Rulyrana saxiscandens	6447.82	1868.692	1	0.046	0	7.8	147.826
Rulyrana spiculata	4602.725	893.825	1	0.183	0	4.9	21.311
Rulyrana susatamai	6075.2	1781.8	1	0.056	0	7.86	122.500
Sachatamia albomaculata	7192.08	1191.04	1	0.006	0	1	0
Sachatamia ilex	6129.8	1073.95	1	0.0715	0	22.16	295.944
Sachatamia orejuela	6775.8	1894.1	1	0.176	0	1	0
Sachatamia punctulata	5618	1150.5	5	0.0144	7.2939	3.48	172.222
Teratohyla amelie	5953.1	1652.9	1	0.011	0	3	181.818
Teratohyla midas KHJ	6672.578	1524.47	3	0.0854	1.7612	3.77	32.436

Teratohyla pulverata	5828.333	2093.2	4	0.039	1.8018	6.4	138.462
Teratohyla spinosa	7019.8	1618.4	2	0.1215	1.7513	4.5	28.807
Vitreorana antisthenesi	5431.5	false	1	0.04	0	5	100.000
Vitreorana castroviejoi	5267.03	1313.515	1	0.02	0	3.09	104.500
Vitreorana eurygnatha	4556.27	1271.33	2	0.0952	1.3617	3.18	22.899
Vitreorana gorzulae	4926.8	1242.44	1	0.085	0	false	131.940
Vitreorana helenae	4597.325	1602.2	2	0.0546	3.3818	7.5	119.048
Vitreorana ritae	4786.36	1767.76	3	0.089	2.7541	12.65	130.899
Vitreorana uranoscopa	4746.656	1342.215	9	0.048	3.0581	3	41.667
Nymphargus spilotus	4094.03	788.811	5	0.173	0.5045	1	0

Table S2.1 Results of MCMCglmm between call and environmental metrics (temperature and vegetation density).

Centrolenidae	**post.mean**	**l-95% CI**	**u-95% CI**	**eff.samp**	**pMCMC**
Peak frequency					
(Intercept)	767.3224	-1443.0295	2959.2083	7808	0.495687
EVI	0.7864	0.4003	1.2002	9048	0.000401
(Intercept)	2034.955	246.888	3766.744	9970	0.022066
Temperature	12.863	5.469	19.879	9970	0.000401
Note Duration					
(Intercept)	0.2117	-2.203	2.696	9970	0.869
EVI	-0.00001736	-0.0005312	0.00047	9970	0.95
(Intercept)	0.138	-1.19	1.368	9970	0.827
Temperature	-0.00004551	-0.006134	0.005934	9970	0.985
Bandwidht					
(Intercept)	743.32311	43.37073	1450.07845	6812	0.0413
EVI	0.08699	-0.04114	0.22603	7792	0.2022
(Intercept)	684.7	159	1225	9062	0.0122
Temperature	2.372	0.08273	4.625	8979	0.0427
Pulse Rate (log)					
(Intercept)	6.9246807	1.1468359	12.9181075	9055	0.0237
EVI	-0.0009658	-0.0020739	0.0001855	8798	0.1015
(Intercept)	3.483676	-0.834773	8.104759	6619	0.125
Temperature	-0.006269	-0.026038	0.013531	5823	0.535
Number of Notes					
(Intercept)	6.504	2.065	11.1	9385	0.00642
EVI	-0.0009231	-0.001815	3.957E-06	9970	0.04754
(Intercept)	3.647984	0.689821	6.505821	9970	0.0189
Temperature	-0.008006	-0.02128	0.005611	9970	0.2481
Note rate					
(Intercept)	-1.8582498	-6.5290421	2.7273685	9970	0.443
EVI	0.0007407	-0.0001797	0.0016726	9603	0.116
(Intercept)	-0.526782	-4.013785	2.88856	9183	0.774
Temperature	0.011049	-0.004921	0.026457	8333	0.168

Centroleninae	post.mean	l-95% CI	u-95% CI	eff.samp	pMCMC
Peak frequency					
(Intercept)	473.5186	-2448.7461	3099.1219	216.7	0.705717
EVI	0.9838	0.4551	1.5528	219.4	0.000201
(Intercept)	2300.29	176.066	4486.656	8890	0.03952
Temperature	14.485	4.428	24.156	7689	0.00502
Note Duration					
(Intercept)	0.3207	-2.515	3.006	10940	0.813
EVI	-0.00004489	-0.0006115	0.0005321	10664	0.878
(Intercept)	0.218273	-1.298524	1.733651	9970	0.776
Temperature	-0.000582	-0.00785	0.007078	9970	0.877
Bandwidth					
(Intercept)	693.75653	-158.58102	1577.41707	8118	0.117
EVI	0.12823	-0.05002	0.29994	8850	0.14
(Intercept)	257.148	-313.872	926.437	959.3	0.40602
Temperature	5.197	2.157	8.068	1003.5	0.00321
Pulse Rate (log)					
(Intercept)	8.3868694	1.1817164	15.4959224	6959	0.0243
EVI	-0.0011386	-0.0025254	0.0003567	6758	0.127
(Intercept)	4.99053	-1.85915	11.21083	2785	0.151
Temperature	-0.01032	-0.04011	0.02294	2640	0.538
Number of Notes					
(Intercept)	7.7440649	1.4864046	13.4850316	8636	0.00903
EVI	-0.0011081	-0.0023042	0.0001646	8464	0.08084
(Intercept)	3.9368721	-0.0003336	7.7257248	9337	0.0485
Temperature	-0.0075656	-0.0250597	0.0128884	9348	0.406
Note rate					
(Intercept)	-1.9676279	-8.1075734	4.0211505	7478	0.54
EVI	0.0006787	-0.0004833	0.0019975	7571	0.278
(Intercept)	-2.370164	-6.921443	2.161297	5970	0.2987
Temperature	0.018235	-0.003083	0.040684	6028	0.0859

Centrolene + Nymphargus	post.mean	l-95% CI	u-95% CI	eff.samp	pMCMC
Peak frequency					
(Intercept)	-111.9545	-3179.1213	2994.4207	48712	0.93972
EVI	1.0337	0.3861	1.6746	48051	0.00248
(Intercept)	491.554	-2766.816	3746.076	674.5	0.7815
Temperature	23.914	5.481	40.837	605	0.0191
Note Duration					
(Intercept)	0.4996	-3.873	5.163	10369	0.837
EVI	-0.00007976	-0.001093	0.0008806	10388	0.875
(Intercept)	0.1909621	-3.055	3.265736	9970	0.911
Temperature	-0.0003562	-0.0185034	0.0185722	9970	0.972
Bandwidth					
(Intercept)	1502.29276	481.12381	2528.34744	6334	0.00401
EVI	-0.08758	-0.32321	0.11471	5564	0.40622
(Intercept)	1026.9397	265.2484	1761.0747	4794	0.00863
Temperature	0.4175	-3.7007	4.714	5333	0.85256
Pulse Rate (log)					
(Intercept)	13.5328585	4.2300604	22.1776834	9970	0.00702
EVI	-0.0024427	-0.0043184	-0.0004922	9970	0.01805
(Intercept)	6.54484	-1.28769	14.99639	7622	0.112
Temperature	-0.02512	-0.07055	0.02032	8414	0.272
Number of Notes					
(Intercept)	11.2088065	1.1152738	21.3414817	7658	0.0341
EVI	-0.0019246	-0.0040095	0.0003767	7175	0.0909
(Intercept)	7.10145	-0.30721	14.73254	9970	0.0616
Temperature	-0.02769	-0.07197	0.01561	9970	0.2124
Note rate					
(Intercept)	-8.007	-15.5	-0.8015	8759	0.0387
EVI	0.001918	0.0003726	0.003481	9202	0.0213
(Intercept)	-2.2125	-9.03609	4.51003	7927	0.529
Temperature	0.01801	-0.02227	0.0551	7967	0.35

Centrolene	post.mean	l-95% CI	u-95% CI	eff.samp	pMCMC
Peak frequency					
(Intercept)	-1786.5051	-7366.3769	3391.6863	1605	0.4939
EVI	1.4837	0.3551	2.7221	1440	0.0144
(Intercept)	1449.469	-3081.41	5645.945	172.5	0.5511
Temperature	20.732	-2.333	46.625	151.3	0.0939
Note Duration					
(Intercept)	0.4343	-4.647	5.834	9970	0.855
EVI	-0.00005784	-0.001207	0.001085	9970	0.905
(Intercept)	0.1786	-3.805	4.478	9970	0.936
Temperature	-0.00002051	-0.02531	0.02528	9970	0.99
Bandwidth					
(Intercept)	1281.78515	116.06913	2396.27291	2332	0.0431
EVI	-0.02366	-0.26304	0.22564	2501	0.7757
(Intercept)	1091.2692	66.2874	2072.1707	353.2	0.0439
Temperature	0.5031	-4.9131	6.8246	351.6	0.9174
Pulse Rate (log)					
(Intercept)	14.2596507	3.5731521	25.5096194	9926	0.015
EVI	-0.0026301	-0.0050217	-0.0003347	9862	0.0323
(Intercept)	10.429923	0.874548	19.620051	7808	0.0291
Temperature	-0.049636	-0.101969	0.006966	8216	0.0748
Number of Notes					
(Intercept)	13.2275895	1.5748346	25.3985658	9671	0.0369
EVI	-0.0023608	-0.0049653	0.0001503	8763	0.0746
(Intercept)	5.89085	-4.68016	17.6493	9970	0.272
Temperature	-0.02024	-0.08701	0.04522	9970	0.523
Note rate					
(Intercept)	-9.618	-19.87	1.022	8845	0.0762
EVI	0.002371	0.00005473	0.004484	8965	0.0429
(Intercept)	-5.27546	-15.30773	4.44363	6956	0.288
Temperature	0.03947	-0.01837	0.09678	7254	0.173

Cochranellini	post.mean	l-95% CI	u-95% CI	eff.samp	pMCMC
Peak frequency					
(Intercept)	2486.9335	-1385.5553	6455.1677	18093	0.2053
EVI	0.6471	-0.1447	1.4086	24624	0.0993
(Intercept)	3963.333	-426.263	7690.265	1517	0.0698
Temperature	7.593	-9.6	25.308	1739	0.3783
Note Duration					
(Intercept)	-0.001376	-4.52	4.42	9970	0.998
EVI	0.00001446	-0.0008706	0.0009182	9970	0.978
(Intercept)	-0.1083769	-4.6261703	4.5070502	9970	0.958
Temperature	0.0007744	-0.0197821	0.0200531	10258	0.931
Bandwidth					
(Intercept)	233.8	-1222	1613	638.6	0.7334
EVI	0.2534	-0.03088	0.543	506.7	0.0722
(Intercept)	68.0882	-1246.9774	1391.5457	10258	0.9161
Temperature	6.2236	0.6255	12.0997	10302	0.0355
Pulse Rate (log)					
(Intercept)	6.0391632	-2.9271771	14.9309268	9970	0.181
EVI	-0.0004594	-0.0022402	0.0013058	9970	0.611
(Intercept)	11.20923	2.63688	20.31598	9970	0.0136
Temperature	-0.03261	-0.07189	0.00441	9970	0.0909
Number of Notes					
(Intercept)	6.3640004	-2.3504697	15.3747029	9970	0.15
EVI	-0.0007977	-0.0025552	0.0009798	9970	0.364
(Intercept)	2.2241477	-6.5359763	10.9075091	9970	0.611
Temperature	0.0007905	-0.0388087	0.0366729	9970	0.969
Note rate					
(Intercept)	2.769848	-7.3241453	12.4907072	9970	0.568
EVI	-0.0002304	-0.0022287	0.0017065	9970	0.813
(Intercept)	-4.73609	-14.20151	4.58358	9970	0.303
Temperature	0.02789	-0.01188	0.06909	9970	0.17

Hyalinobatrachinae	**post.mean**	**l-95% CI**	**u-95% CI**	**eff.samp**	**pMCMC**
Peak frequency					
(Intercept)	3126.999	-508.0613	6583.0865	48125	0.0823
EVI	0.3222	-0.3772	1.0151	48105	0.352
(Intercept)	2556.0799	361.8401	4633.8094	8479	0.0191
Temperature	9.6535	0.8031	19.3129	9561	0.0411
Note Duration					
(Intercept)	-0.028001	-5.858109	6.027696	10260	0.987
EVI	0.000043	-0.00117	0.001212	10260	0.945
(Intercept)	0.1339001	-3.0671355	3.5025173	9970	0.938
Temperature	0.0002343	-0.0141363	0.0150742	9970	0.982
Bandwidth					
(Intercept)	928.38711	13.42505	1796.91452	9970	0.0419
EVI	0.02749	-0.15223	0.19998	9970	0.746
(Intercept)	1026.7036	434.796	1569.571	2999	0.001
Temperature	0.1737	-2.2741	2.5156	9970	0.879
Pulse Rate (log)					
(Intercept)	1.563	-9.583	12.63	10282	0.78
EVI	0.00004602	-0.002149	0.002237	9970	0.962
(Intercept)	0.0712	-6.54487	6.9566	9970	0.978
Temperature	0.00776	-0.0215	0.0375	9970	0.598

Hyalinobatrachium	**post.mean**	**l-95% CI**	**u-95% CI**	**eff.samp**	**pMCMC**
Peak frequency					
(Intercept)	3801.2831	259.5424	7189.066	49970	0.0341
EVI	0.1681	-0.5191	0.8603	49970	0.6266
(Intercept)	2385.3707	144.1499	4577.8654	12379	0.0335
Temperature	9.7971	0.2931	19.3908	13298	0.047
Note Duration					
(Intercept)	-0.06282	-6.407	5.68	9970	0.98
EVI	0.00005047	-0.00118	0.001249	9970	0.928
(Intercept)	0.282138	-3.423232	4.115845	9970	0.892
Temperature	-0.000388	-0.016995	0.015835	9970	0.969
Bandwidth					
(Intercept)	1152.32471	222.97196	2093.07249	2854	0.0146
EVI	-0.02978	-0.22506	0.15412	2625	0.7653
(Intercept)	947.0511	378.1763	1466.1528	5365	0.001
Temperature	0.2824	-1.9904	2.6137	5463	0.797
Pulse Rate (log)					
(Intercept)	4.2187893	-6.9392639	14.979281	10511	0.444
EVI	-0.0005207	-0.0027249	0.00166	10657	0.629
(Intercept)	0.923142	-6.125842	8.363842	9970	0.807
Temperature	0.003025	-0.026963	0.034863	9970	0.851

Table S2.2 Results of MCMCglmm between Acoustic Dispersion and Phylogenetic Heterogeneity.

	Mvalue	**post.mean**	**l-95% CI**	**u-95% CI**	**eff.samp**	**pMCMC**	
Centrolenidae							
Overall Call	(Intercept)	0.8702	0.2619	1.4761	9120	0.00782	**
	MPD	1.4514	-2.476	5.5073	9459	0.47623	
Temporal	(Intercept)	0.8183	0.3155	1.3576	9331	0.0016	**
	MPD	0.5793	-2.6958	4.0815	9485	0.7294	
Spectral	(Intercept)	0.3407	-0.1538	0.834	9970	0.1685	
	MPD	3.4671	0.2881	6.7859	9970	0.0385	*
Centroleninae							
Overall Call	(Intercept)	1.5801	1.0207	2.1872	9970	<1e-04	***
	MPD	-3.5981	-7.9359	0.7325	9970	0.0985	.
Temporal	(Intercept)	0.9285	0.2543	1.6025	9970	0.00682	**
	MPD	-0.5821	-5.4485	4.5741	9970	0.81324	
Spectral	(Intercept)	0.8456	0.2762	1.3939	9970	0.00381	**
	MPD	-0.3136	-4.2583	4.142	9970	0.88385	
Centrolene+Nymphargus							
Overall Call	(Intercept)	0.1807	-1.6068	1.9787	99970	0.838	
	MPD	6.7918	-6.8161	20.4606	99970	0.315	
Temporal	(Intercept)	3.541	1.408	5.672	9970	0.0012	**
	MPD	-20.37	-36.764	-4.215	9970	0.0173	*
Spectral	(Intercept)	2.038	-0.4433	4.7369	9970	0.115	
	MPD	-7.9257	-27.6407	11.7981	9970	0.408	
Centrolene							
Overall Call	(Intercept)	0.5444	-0.635	1.7672	99970	0.317	
	MPD	3.2917	-7.3571	13.6772	99970	0.484	
Temporal	(Intercept)	1.193	-0.139	2.495	9970	0.0682	.
	MPD	-1.736	-13.112	10.021	9970	0.7308	
Spectral	(Intercept)	0.5821	-0.6416	1.7527	9970	0.278	
	MPD	3.1628	-6.8775	13.978	9970	0.483	
Cochranellini							
Overall Call	(Intercept)	1.9256	1.1593	2.6818	99970	<1e-05	***
	MPD	-6.1425	-12.9754	0.6521	100268	0.0768	.
Temporal	(Intercept)	1.04298	0.01279	2.01161	9970	0.0421	*
	MPD	-1.05094	-9.38009	8.42114	9970	0.806	
Spectral	(Intercept)	1.2941	0.4567	2.1686	9970	0.00502	**
	MPD	-3.303	-10.7044	4.6181	9970	0.38837	
Hyalinobatrachinae							
Overall Call	(Intercept)	-0.1305	-1.2525	1.0248	90604	0.814	
	MPD	13.097	1.0866	24.7639	87354	0.0329	*

Temporal	(Intercept)	0.5932	-0.3083	1.5405	9970	0.197	
	MPD	3.2497	-6.7453	12.3715	9970	0.486	
Spectral	(Intercept)	0.3544	-0.2848	0.9739	9970	0.254	
	MPD	5.4088	-1.3083	11.8866	9970	0.102	
Hyalinobatrachium							
Overall Call	(Intercept)	-0.09599	-1.2458	1.05051	94900	0.8624	
	MPD	12.99143	0.65689	25.35186	94807	0.0407	*
Temporal	(Intercept)	0.4514	-0.4166	1.2967	47695	0.284	
	MPD	4.9924	-4.5626	14.1122	49970	0.276	
Spectral	(Intercept)	0.60416	0.06843	1.15998	50791	0.0332	*
	MPD	2.91156	-2.93528	8.77896	48219	0.3074	

Table S3. GenBank sequences used in this study for the phylogenetic reconstruction.

Species	Voucher	12S	16S	ND1	BDNF	c-myc	CXCR4	POMC	RAG1	SLC8A1	SLC8A3
Allophryne relicta	CRBH 29909	KF582053.1	KF582053.1	NA	NA	NA	NA	NA	NA	NA	NA
Allophryne resplendens	MZUNAP-01-605	JQ436697.1	JQ436698.1	NA	NA	NA	NA	NA	NA	NA	NA
Allophryne ruthveni	MAD 1852	AY819328	NA	AY819458	NA	AY819162	NA	AY819077	NA	NA	NA
Allophryne ruthveni	MAD 1857	NA	EU662973	NA	NA	NA	KF534373	NA	EU663432	KF534112	KF534194
Celsiella revocata	MHNLS 17319	EU663379	EU663019	EU663113	KF534278	EU663281	KF534374	EU663204	EU663479	KF534113	KF534195
Celsiella vozmedianoi	MHNLS 17877*	EU663385	EU663025	EU663163	NA	EU663324	NA	EU663247	EU663531	NA	NA
Centrolene aff. buckleyi	MAR 371 (170)	EU663339	EU662980	EU663069	KF534279	EU663254	KF534376	EU663170	EU663438	KF534115	KF534196
Centrolene aff. savagei1	MAR 2071	KM068295	KM068295	NA	NA	NA	NA	NA	NA	NA	NA
Centrolene altitudinale	MHNLS 17194*	EU663333	EU662974	EU663070	KF534280	EU663249	KF534377	EU663165	NA	KF534116	KF534197
Centrolene antioquiense	NRPS 014	EU663336	EU662977	EU663073	KF534281	EU663251	KF534378	EU663167	EU663436	KF534117	KF534198
Centrolene bacatum	QCAZ 22728	EU663337	EU662978	EU663074	NA	EU663252	KF534379	EU663168	EU663437	KF534118	KF534199
Centrolene ballux	QCAZ 40196*	KF639754	NA	HG764783	NA	NA	NA	NA	NA	NA	NA
Centrolene buckleyi	KU 178031	EU663338	EU662979	EU663075	KF534282	EU663253	KF534380	EU663169	NA	NA	KF534200
Centrolene condor	QCAZ 44896	KF639755	JX126955	JX187513	NA	NA	NA	NA	NA	NA	NA
Centrolene daidaleum	MHUA 3271*	EU663366	EU663007	EU663101	NA	EU663272	KF534381	EU663192	EU663465	KF534119	KF534201
Centrolene geckoideum	KU 178015	EU663341	EU662982	EU663077	NA	NA	KF534382	NA	EU663440	KF534120	KF534202
Centrolene heloderma	QCAZ 40200	KF639757	JX126956	JX187509	NA	NA	NA	NA	NA	NA	NA
Centrolene hesperium	MHNSM 25802	EU663345	EU662986	EU663081	KF534284	EU663258	KF534383	KF639777	EU663444	KF534121	KF534203
Centrolene hybrida	MAR 347	EU663346	EU662987	EU663082	KF534285	EU663259	KF534384	EU663175	EU663445	KF534122	KF534204
Centrolene lynchi	QCAZ 40191*	NA	JX126957	JX187508	NA	NA	NA	NA	NA	NA	NA
Centrolene peristictum	QCAZ 22312	EU663352	EU662993	EU663088	KF534288	EU663266	KF534387	EU663181	EU663451	KF534124	KF534207
Centrolene sabini	MUSM 28018	NA	JX126960	JX187511	NA	NA	NA	NA	NA	NA	NA
Centrolene savagei	MHUA 4094	EU663380	EU663020	EU663114	KF534290	EU663282	KF534389	EU663205	EU663480	KF534126	KF534209
Centrolene venezuelense	EBRG 5244; MHNLS / ADN 17340*	NA	NA	NA	KF534291	NA	KF534390	EU663186	NA	KF534127	KF534210
Chimerella corleone	CORBIDI 10475	KF639761	KF534359	NA	NA	NA	NA	NA	NA	NA	NA
Chimerella mariaelenae	QCAZ 31729	EU663350	EU662991	EU663086	KF534292	EU663263	KF534391	EU663179	EU663449	KF534128	KF534211
Cochranella erminea	MHNC 7247	KF639762	KF534360	HG764786	KF534293	KF534460	KF534392	KF639780	NA	KF534129	KF534212
Cochranella euknemos	CH 5109	EU663367	EU663008	EU663102	NA	KF534461	KF534393	EU663193	EU663466	NA	KF534213

Cochranella granulosa	AJC 1152*	NA	NA	NA	KF534294	NA	KF534394	KF639781	NA	KF534130	KF534214
Cochranella guayasamini	MHNC 13929	KF639764	KF534362	NA	NA	NA	NA	NA	NA	NA	NA
Cochranella mache	QCAZ 27747	EU663373	EU663013	EU663107	KF534296	EU663277	KF534396	EU663198	EU663472	KF534132	KF534216
Cochranella nola	CBG 1096	EU663381	EU663021	EU663115	KF534297	EU663283	KF534397	EU663206	EU663481	KF534133	KF534217
Cochranella resplendens	QCAZ 38088	KF639763	KF534361	HG764787	NA	NA	NA	NA	NA	NA	NA
Espadarana andina	JMG 366*	EU663335	EU662976	EU663072	NA	EU663250	NA	EU663166	EU663435	NA	NA
Espadarana audax	QCAZ 23910*	KF639753	NA	NA	NA	NA	NA	NA	NA	NA	NA
Espadarana callistomma	QCAZ 28555	EU663340	EU662981	EU663076	KF534299	EU663255	KF534399	EU663171	EU663439	KF534135	KF534219
Espadarana prosoblepon	UCR 17102*	EU663354	EU662995	NA	NA	NA	KF534400	NA	EU663453	KF534136	KF534220
Hyalinobatrachium aff. bergeri	MTD 46305*	EU663393	EU663026	EU663119	NA	EU663290	NA	EU663210	EU663485	NA	NA
Hyalinobatrachium anachoretus	CORBIDI 10472	KM068254	KM068300	NA	NA	NA	NA	NA	NA	NA	NA
Hyalinobatrachium aureoguttatum	QCAZ 32105	EU663391	EU663032	EU663124	KF534302	EU663288	KF534403	EU663214	EU663491	KF534139	KF534223
Hyalinobatrachium bergeri	MHNC 5676 MNCN/ADN 5547	EU663392	EU663033	EU663125	KF534303	EU663289	KF534404	EU663215	EU663492	KF534140	KF534224
Hyalinobatrachium cappellei	MHNLS 16475*	EU663401	EU663040	EU663132	NA	EU663297	NA	EU663222	EU663499	NA	NA
Hyalinobatrachium carlesvilai	CBG 1099	EU663388	EU663030	EU663122	KF534305	EU663291	KF534405	EU663212	EU663489	KF534142	KF534226
Hyalinobatrachium cf. guairarepanense	MIZA 0281	KF639765	KF534363	HG764788	KF534306	KF534463	KF534406	KF639782	KF639786	KF534143	KF534227
Hyalinobatrachium chirripoi	UCR 17424	EU663398	EU663037	EU663129	KF534307	EU663294	KF534407	EU663219	EU663496	KF534144	KF534228
Hyalinobatrachium colymbiphyllum	UCR 17423*	EU663400	EU663039	EU663131	NA	EU663296	NA	EU663221	EU663498	NA	NA
Hyalinobatrachium duranti	MHNLS 16493*	EU663402	EU663041	EU663133	NA	EU663298	NA	EU663223	EU663500	NA	NA
Hyalinobatrachium fleischmanni	USNM 559092	EU663406	EU663045	EU663137	KF534310	EU663300	KF534410	EU663225	EU663504	KF534147	KF534231
Hyalinobatrachium fleischmanni MX	JAC 21365	DQ283453	DQ283453	NA	NA	NA	NA	NA	NA	NA	NA
Hyalinobatrachium fragile	MHNLS 17161	EU663407	EU447286	EU663138	KF534311	EU663301	KF534411	EU663226	EU663505	KF534148	KF534232
Hyalinobatrachium iaspidiense	MHNLS 17126*	EU663408	EU663047	EU663139	NA	EU663302	KF534412	NA	EU663506	KF534149	KF534233
Hyalinobatrachium kawense	MNHN 2011.0119*	EU663387	EU663029	EU663121	NA	NA	NA	NA	NA	NA	NA
Hyalinobatrachium mondolfii	MHNLS 17119	EU663411	EU663050	EU663142	KF534315	EU663305	KF534415	EU663229	EU663509	KF534152	KF534236
Hyalinobatrachium munozorum	QCAZ 31056	EU663395	EU663034	EU663126	NA	KF534464	KF534416	EU663216	EU663493	NA	KF534237
Hyalinobatrachium orientale	MHNLS 17878*	EU663413	EU447289	EU663144	NA	EU663306	NA	EU663230	EU663511	NA	NA
Hyalinobatrachium orocostale	MHNLS 17247	EU663414	EU447284	EU663145	KF534317	EU663307	KF534418	EU663231	EU663512	KF534154	KF534239
Hyalinobatrachium pallidum	MHNLS 17238*	EU663415	EU663052	EU663146	KF534318	NA	KF534419	NA	EU663513	KF534155	KF534240
Hyalinobatrachium pellucidum	QCAZ 29438	EU663397	EU663036	EU663128	KF534319	EU663293	KF534420	EU663218	EU663495	KF534156	KF534241

Hyalinobatrachium talamancae	CH 5330	EU663418	EU663054	EU663149	KF534321	EU663313	KF534422	EU663233	EU663516	KF534158	KF534243
Hyalinobatrachium tatayoi	MHNLS 17174	EU663419	EU663055	EU663150	KF534322	EU663310	KF534423	EU663234	EU663517	KF534159	KF534244
Hyalinobatrachium taylori	MHNLS 17141	EU663420	EU663056	EU663151	KF534323	EU663311	KF534424	EU663235	EU663518	KF534160	KF534245
Hyalinobatrachium tricolor	MNHN 2011.0116*	EU663386	EU663027	NA	KF534324	EU663328	KF534425	NA	EU663486	KF534161	KF534246
Hyalinobatrachium valerioi	UCR 17418	EU663421	EU663058	EU663152	KF534325	EU663312	KF534426	EU663236	EU663519	KF534162	KF534247
Hyalinobatrachium vireovittatum	CH 6443	NA	KF604303	NA	NA	NA	NA	NA	NA	NA	NA
Ikakogi tayrona	MAR 544	EU663356	EU662997	EU663091	KF534326	EU663330	KF534427	EU663183	EU663455	KF534163	KF534248
Nymphargus bejaranoi	CBG 1488	EU663422	EU663059	EU663155	KF534328	EU663314	KF534429	EU663239	EU663522	KF534165	KF534250
Nymphargus garciae	KU 202796	AY326022	AY326022	NA	NA	NA	NA	NA	NA	NA	NA
Nymphargus grandisonae	QCAZ 22310	EU663344	EU662985	EU663080	KF534330	EU663257	KF534431	EU663174	EU663443	KF534167	KF534252
Nymphargus griffithsi	QCAZ 31768	EU663426	EU663062	EU663157	KF534331	EU663318	KF534432	EU663241	EU663524	KF534168	KF534253
Nymphargus pluvialis	MNCN/ADN 5004*	NA	NA	NA	KF534334	NA	KF534436	NA	NA	KF534172	KF534257
Nymphargus rosadus	MHUA 4308	EU663429	EU663066	EU663161	KF534335	EU663322	KF534438	EU663245	EU663529	KF534174	KF534258
Nymphargus siren	KU 179171	EU663430	EU663067	EU663162	KF534336	EU663323	KF534439	EU663246	EU663530	KF534175	KF534259
Nymphargus wileyi	QCAZ 27435	EU663431	EU663068	EU663164	KF534338	EU663325	KF534441	EU663248	EU663532	KF534177	KF534261
Rulyrana flavopunctata	QCAZ 32265	EU663368	EU663009	EU663103	KF534340	EU663273	KF534443	EU663194	EU663467	KF534179	KF534263
Rulyrana saxiscandens	CORBIDI-HE-2012-14153 (MNCN/ADN 51737)	KF639772	KF534369	NA	NA	NA	NA	NA	NA	NA	NA
Rulyrana spiculata	MHNSM 24867	EU663382	EU663022	EU663116	KF534341	EU663284	KF534444	EU663207	EU663482	KF534180	KF534264
Rulyrana susatamai	MAR 337	EU663384	EU663024	EU663118	KF534342	EU663286	KF534445	EU663209	EU663484	KF534181	KF534265
Sachatamia albomaculata	USNM 534151*	EU663362	EU663003	EU663097	NA	EU663270	NA	EU663188	EU663461	NA	NA
Sachatamia ilex	UCR 16861	EU663347	EU662988	EU663083	KF534344	EU663260	KF534447	EU663176	EU663446	KF534183	KF534267
Sachatamia punctulata	MHUA 4071	EU663378	EU663018	EU663112	KF534345	EU663280	KF534448	EU663203	EU663477	KF534184	KF534268
Teratohyla amelie	MHNC 5646 / MNCN ADN 20619	EU663365	EU663005	EU663099	KF534347	EU663327	KF534450	EU663190	EU663463	KF534186	KF534270
Teratohyla midas	KHJ	EU663374	EU663014	EU663108	NA	EU663278	NA	EU663199	EU663473	NA	NA
Teratohyla pulverata	USNM 538588*	EU663416	EU663053	EU663147	NA	EU663308	NA	EU663232	EU663514	NA	NA
Teratohyla spinosa	USNM 538863	EU663383	EU663023	EU663117	KF534349	EU663285	KF534452	EU663208	EU663483	KF534188	KF534272
Vitreorana antisthenesi	MHNLS 17909*	EU663390	EU663031	EU663123	NA	EU663287	NA	EU663213	EU663490	NA	NA
Vitreorana castroviejoi	MHNLS 16446*	EU663363	EU663004	EU663098	NA	EU663271	NA	EU663189	EU663462	NA	NA
Vitreorana eurygnatha	CFBH 5729	AY843595	AY843595	EU663135	NA	NA	NA	NA	AY844383	NA	NA
Vitreorana gorzulae	MHNLS 16036*	EU663343	EU662984	EU663079	NA	EU663256	NA	EU663173	EU663442	NA	NA

Vitreorana helenae	MHNLS 17139	EU663372	EU663012	EU663106	KF534353	EU663276	KF534456	EU663197	EU663471	KF534192	KF534276
Vitreorana oyampiensis	MB 165	NA	EU663017	NA	KF534354	NA	KF534457	NA	NA	KF534193	KF534277
Vitreorana uranoscopa	UFRGS 4381*	KF639776	NA	NA	NA	NA	NA	NA	NA	NA	NA

DISCUSION GENERAL

A lo largo de este proyecto se logró avanzar en varios interrogantes relacionados con el impacto de elementos bióticos y ambientales en la historia evolutiva de un grupo de anfibios de enorme interés dada su gran diversidad. Así mismo, este trabajo proporciona información valiosa que revela la importancia de las barreras geográficas y las divergencias del canto como motores evolutivos en las ranas de cristal.

Gracias a los análisis filogeográficos a partir de información de secuencias mitocondriales realizados en el Capítulo I, encontramos patrones interesantes que nos permitieron reconstruir la historia biogeográfica de *H. fleischmanni* e identificar el impacto de las diferentes barreras geográficas sobre la estructura genética y los patrones espaciales de los linajes internos de este clado. Encontramos que algunas barreras geográficas, pero no todas las esperadas, tuvieron un impacto en el aislamiento de poblaciones actuales de *H. fleischmanni*. La cordillera de Talamanca y el sistema de fallas de Motagua-Polochic-Jocotán fueron barreras geográficas significativas para esta especie, a diferencia de la escarpada de Hess y la cordillera de los Andes. Así mismo, gracias al uso de análisis Bayesiano de difusión temporal se pudo identificar a Centro América como el origen geográfico de los linajes que componen al complejo *H. fleischmanni* (Figura 5, Capítulo I), contrario al origen suramericano esperado considerando a la distribución en Suramérica de las demás especies con las que están cercanamente emparentadas.

El tiempo ocurrido desde el aislamiento de estas poblaciones (entre 0.6 y 4.5 Ma) ha sido suficiente para generar diferenciación detectable en el fenotipo (Zamudio et al. 2016), que puede incluso resultar en procesos de especiación. Sin embargo, la estructura filogeográfica profunda obtenida en el ADN mitocondrial por sí sola no es evidencia suficiente de eventos de especiación (Will et al. 2005, Mendoza et al. 2016) y debe ser confirmada por el ADN nuclear como un paso inicial en la delimitación de especies (Singhal & Moritz 2013).

Distinguir entre linajes a nivel de especie y de población es fundamental para comprender el proceso de especiación en sí mismo (Dynesius & Jansson 2014). Siguiendo a Kuchta & Wake (2016) la evolución de los linajes a nivel de metapoblación pueden verse como un río trenzado, con canales que se bifurcan y conectan en el espacio y el tiempo. De esta manera, la independencia de linajes es análoga a la evolución del aislamiento reproductivo en el sentido de que es un continuo. Muchos complejos de especies (como *H. fleischmanni*) presentan situaciones difíciles en las que la evidencia de divergencia de linajes es ambigua. Los

linajes pueden estar parcialmente separados, variar en sus componentes a lo largo del tiempo, o estar anidados dentro de otros linajes (de Queiroz, 2005, 2007, Figura 1). Así mismo, eventos de hibridación pueden dar lugar a asilamiento y especiación en muy pocas generaciones (Lamichhaney et al. 2018) y cuando coincide con la oportunidad ecológica, puede facilitar una rápida especiación y una amplia radiación adaptativa (Meier et al. 2017). Esta ambigüedad no es un problema para el estudio de la evolución; es más bien un subproducto esperado del cambio evolutivo gradual.

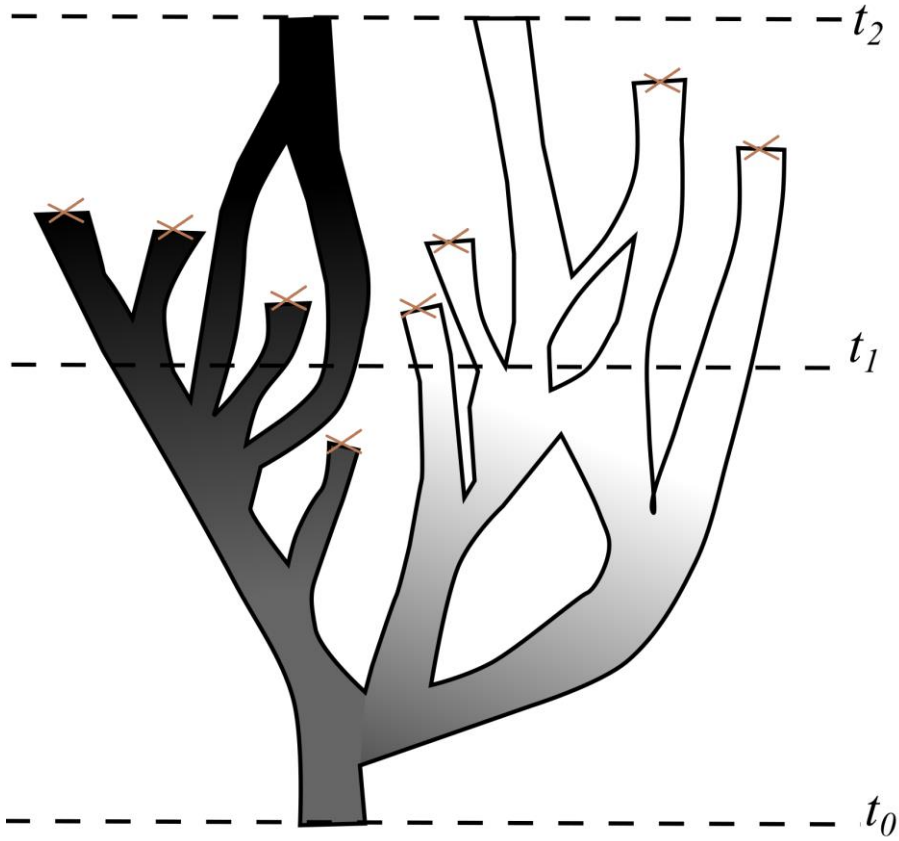

Figura 1. Diagrama de separación de linajes en un complejo que presenta un arreglo de linajes con diferentes grados de independencia evolutiva. Mientras en algunos tiempos la interpretación es sencilla (ej. t2), la naturaleza continua de la separación de linajes dificulta la determinación del número de especies por reconocer (ej. t1). Modificado de Kuchta & Wake (2016).

Considerando lo anterior, en el Capítulo II evaluamos las diferencias genéticas encontradas en el Capítulo I usando varios métodos de delimitación molecular de especies desarrollados recientemente (GMYC, PTP y BPP) e integramos esos resultados con variación en morfología y en atributos de gran importancia en el comportamiento reproductivo entre los linajes aislados. La delimitación molecular de especies es uno de los campos de mayor

desarrollo en sistemática (Sites & Marshall 2003). Aunque la efectividad de los métodos en identificar especies continúa bajo discusión por la posibilidad de confundir estructuración intraespecífica y especiación (Sukumaran & Knowles 2017), el uso de estos métodos puede ser útil como una primera aproximación para reconocer las especies candidatas que posteriormente deberán ser confirmadas con otras líneas de evidencia. En especial para aquellos grupos morfológicamente conservados como lo es el género *Hyalinobatrachium* (Castroviejo-Fisher et al. 2009) en el que el uso de información molecular y acústica es cada vez más frecuente para la identificación de las especies (ej. Castroviejo-Fisher et al. 2011, Guayasamín et al. 2017, Apéndice V).

Al realizar una aproximación integrativa para delimitar las especies, concluimos que han ocurrido al menos dos eventos de especiación entre los linajes de este clado. En muchas ocasiones, los estudios moleculares que revelan diversidad criptica sólo se quedan en sugerir clados como especies potenciales, pero carecen de la integración de datos de otras fuentes para probar las hipótesis taxonómicas y reconocer formalmente dichas especies (Vences et al. 2013). Aquí, realizamos un estudio completo para la delimitación de especies del complejo *H. fleischmanni*, la cual es una tarea importante para incorporar los resultados en estudios adicionales sobre la diversidad y conservación de las especies (Bickford et al. 2007; Funk et al., 2012).

Entre las diferencias que encontramos entre los linajes delimitados destacamos las diferencias significativas en los atributos de las señales acústicas. Por ejemplo, mientras *H. fleischmanni sensu stricto* presenta un canto corto de alta frecuencia, *H. viridissimum* presenta un canto más grave de mayor duración (Figura 1, Capitulo II). Se ha encontrado en algunas especies de anuros que hay una percepción categórica de las señales acústicas, favoreciendo aquellas emitidas por conespecíficos (Marshall *et al.* 2006). La percepción efectiva (por ejemplo, el intervalo de frecuencias detectadas por el oído del receptor) y el reconocimiento de la señal (determinado también por las variaciones en los parámetros temporales) pueden afectar los procesos de especiación (Marshall *et al.* 2006, Tobias *et al.* 2011). Por lo tanto, la relevancia de los cantos en los anuros ha sido cada vez más valorada (Köhler et al. 2017) y las descripciones de los cantos de las especies representan un aporte valioso en estudios de sistemática, ecología y evolución de las especies (Apéndices I y VI).

La teoría de la especiación plantea que el aislamiento reproductivo aumenta con el tiempo de divergencia (Coyne & Orr 1989, Sasa et al. 1998), dependiendo a su vez de la interacción de la selección, la deriva y el flujo génico (Gourbiere & Mallet 2009). Las

adaptaciones locales en señales de reconocimiento de parejas también pueden promover el aislamiento reproductivo entre las poblaciones y, por lo tanto, pueden tener el potencial de iniciar, facilitar o impulsar la especiación (Picq et al. 2016, Boughman 2002). En el Capítulo III encontramos que la diversidad de señales acústicas en las ranas de cristal no se explica únicamente por las barreras físicas que limitan la dispersión de las especies o de las poblaciones. El efecto indirecto de ambientes heterogéneos, que generan altos niveles de aislamiento, ciertamente acelera los procesos de evolución independiente (Bagley & Johnson, 2014). Procesos estocásticos (incluyendo evolución neutral y deriva genética), efectos pleiotrópicos y selección natural pueden actuar a nivel local y afectar la evolución acústica en las poblaciones aisladas (Vellend 2010). La heterogeneidad de la selección divergente, ya provenga de presiones ecológicas o sexuales, también puede alterar la relación entre el aislamiento reproductivo y el tiempo de divergencia (Gourbiere & Mallet 2009).

Dada la importancia de dichas señales en el reconocimiento de especies y las presiones de selección a las cuales están expuestas (Gerhardt & Huber 2002), nuestros resultados del Capítulo III ayudan a entender la variación de las señales acústicas a diferentes escalas filogenéticas en ranas de cristal, sobre todo el papel de la selección respecto la transmisión efectiva de las mismas. A partir de métodos comparativos filogenéticos, en este capítulo logramos identificar cómo la variación de estos atributos no es sólo producto de variación neutral asociada a las diferencias genéticas producto del aislamiento y evolución independiente entre clados y especies (Tabla 2, Capítulo III). Mientras algunos parámetros del canto de las ranas de cristal (p.ej., frecuencia dominante y duración de la nota) presentaron poca variabilidad entre especies cercanas, otros aspectos del canto como la tasa de los pulsos exhiben una tasa de cambio extremadamente alta. Encontramos también que varios aspectos tanto físicos como bióticos influyen de cierto modo en la variación del canto en estas especies (Figura 3 y Tablas 1 y 2; Capitulo III). Nuestros resultados sugieren que el desplazamiento de caracteres reproductivos es un proceso que ha ocurrido pocas veces en ranas de cristal, mientras que los factores ambientales pueden estar generando un mayor impacto. Por lo tanto, pudimos identificar algunos de los procesos evolutivos que afectan los parámetros del canto en esta familia de anuros.

Es muy importante analizar en qué circunstancias los cantos de las ranas son más similares o más divergentes respecto a presencia o no de barreras físicas que aíslan poblaciones (especiación alopátrica). Se puede esperar que las especies hermanas con especiación alopática, sin cambios de su nicho ecológico no requieran una fuerte divergencia en los rasgos que confieren aislamiento reproductivo (Mayr 1942). En el Capítulo III pudimos

observar una tendencia en estas ranas del Nuevo Mundo, en la que los pares de especies simpátricas o bajo diferentes presiones ambientales experimentan mayor divergencia de los cantos que aquellos pares de especies hermanas que sólo están aisladas por una barrera geográfica (Figura 5, Cap. III).

Conclusiones

- Se reconstruyó la historia biogeográfica del complejo *H. fleischmanni* incluyendo el impacto de las diferentes barreras geográficas sobre la estructura genética y los patrones espaciales de sus linajes internos.
- Algunas barreras geográficas, pero no todas las esperadas, tuvieron un impacto en el aislamiento de poblaciones actuales del complejo *H. fleischmanni.*
- Centro América fue identificada como el origen geográfico de los linajes que componen al complejo *H. fleischmanni.*
- Han ocurrido al menos dos eventos de especiación entre los linajes del complejo *H. fleischmanni* que corresponden a por lo menos tres especies diferenciables en sus señales acústicas, su morfología y su genética.
- La variación de las señales acústicas no es sólo producto de variación neutral asociada a la evolución independiente entre especies, sino que varios aspectos físicos y bióticos influyen de cierto modo en la variación del canto en estas especies.

Perspectivas

Los resultados obtenidos en el presente trabajo abren muchas puertas a futuros estudios en biología evolutiva en este grupo de anfibios. Por ejemplo, recientemente han surgido aproximaciones muy interesantes para detectar el papel de algunos rasgos fenotípicos en el incremento o decremento de las tasas de diversificación de las especies (Rabosky et al. 2014, Rabosky & Huang 2015, Herrera-Alsina et al. 2018). Las comparaciones de clados hermanos que difieren en rasgos asociados con la interacción de especies (Mitter et al. 1988; Farrell et al. 1991) sugieren que la evolución de estos rasgos puede estar asociada con mayores tasas de diversificación de linajes. Se ha sugerido también que rasgos asociados al éxito reproductivo, como lo son las señales acústicas, podrían acelerar las tasas de diversificación (Gonzalez-Voyer & Kolm, 2011; Wilkins et al. 2013). Considerando los datos obtenidos en este trabajo,

podría ser de gran interés evaluar si algunos de los parámetros del canto de estas especies estarían relacionados con procesos de mayor o menor especiación y/o extinción.

La especiación no es un evento instantáneo y sus diferentes etapas van desde la variación continua hasta la diferenciación de la población, formación de ecotipos y divergencia post-especiación, y estos tiempos pueden ser muy variables y complejos (Avise et al. 1998; Nosil *et al.* 2009). En el Capítulo II encontramos evidencia para determinar que algunas poblaciones han experimentado aislamiento suficiente para ser consideradas como linajes evolutivos independientes (deQueiroz 2007). Un caso de interés para futuros estudios es el aislamiento de las poblaciones al norte y sur de la cordillera de Talamanca que representan dos especies diferentes: *H. fleischmanni* y *H. tatayoi.* Considerando su amplia distribución y abundancia en Costa Rica, es indispensable realizar una revisión exhaustiva de los individuos, para así identificar si hay regiones de contacto secundario y de posible hibridación entre ambas especies. Este escenario es una buena oportunidad para evaluar la adecuación de los cantos de las dos especies respecto a la discriminación de conespecíficos, y en caso de que existan híbridos fértiles, cuál puede ser el desempeño de dichos híbridos en términos de atracción de parejas (ej. Lamichhaney et al. 2018).

Por otra parte, nuestros resultados sugieren aislamiento reciente entre los cuatro linajes internos dentro de lo que llamamos *H. viridissimum*. A pesar de los bajos valores de distancias genéticas (menos de 2% en COI y menos de 3% en 16S), estos subclados aislados geográficamente presentan una fuerte estructuración tanto en rasgos morfométricos como bioacústicos que pueden sugerir independencia de linajes (deQueiroz 2007). Singhal & Moritz (2013) sugieren que, para abordar estos temas, se debe evaluar si existe un aislamiento reproductivo sustancial entre estos linajes. En tal caso, al relacionar estos resultados con el tiempo de divergencia (entre 1.51 y 2.19 Ma; Figura 3, Capítulo I), posiblemente estaríamos observando un caso de rápida especiación en comparación con los tiempos de divergencia encontrados hasta el momento para las demás especies de la familia (Hutter *et al.* 2013, Castroviejo-Fisher *et al.* 2014).

Finalmente, en el estudio de la evolución de señales acústicas se han propuesto otras fuentes de variación que no pudieron ser abarcadas en este trabajo. Algunos rasgos de la comunicación pueden depender de otros factores más difíciles de evaluar como el contexto de la señal, la motivación del emisor, las variaciones a corto plazo del entorno social o del nivel de ruido ambiental (e.g. Endler, 1992, Forrest 1994, Goutte et al. 2013). El equilibrio entre enviar la señal más visible y permanecer oculto a los depredadores también es afectado por los atributos

del hábitat (Rogers & Kaplan 2002). Adicionalmente, la comunicación acústica no está aislada de otros tipos de comunicación, y en ocasiones su evolución está estrechamente vinculada (e.g. Santos et al. 2014). El reto ahora consiste en poder integrar cada vez más todos estos elementos en un análisis riguroso de la evolución de estos rasgos.

Afortunadamente, las herramientas moleculares y bioinformáticas cada vez son más accesibles, y el análisis masivo de secuencias e incluso de genomas completos puede proveernos de información adicional sobre los loci específicos que están bajo selección (Narum & Hess 2011).

REFERENCIAS BIBLIOGRÁFICAS

Avise J, Walker D, Johns G. 1998 Speciation durations and Pleistocene effects on vertebrate phylogeography. Proc. R. Soc. Lond. B 265, 1707–1712. (doi:10.1098/rspb.1998.0492)

Avise, J. C. (2000). Phylogeography: the history and formation of species. Harvard university press.

Bagley, J. C., & Johnson, J. B. (2014). Phylogeography and biogeography of the lower Central American Neotropics: diversification between two continents and between two seas. Biological Reviews, 89(4), 767-790.

Barrera-Rodríguez, M. (2000). Estudio Anatómico de cuatro especies de ranitas de cristal del género Hyalinobatrachium Ruíz and Lynch 1991 grupo fleischmanni (Amphibia: Anura: Centrolenidae). Revista de la Academia Colombiana de Ciencias Exactas, Físicas y Naturales, 23, 245-260.

Beheregaray, L. B. (2008). Twenty years of phylogeography: the state of the field and the challenges for the Southern Hemisphere. Molecular Ecology 17, 3754–3774

Bickford, D., Lohman, D. J., Sodhi, N. S., Ng, P. K., Meier, R., Winker, K., ... & Das, I. (2007). Cryptic species as a window on diversity and conservation. Trends in ecology & evolution, 22(3), 148-155.

Boughman JW (2002) How sensory drive can promote speciation. Trends Ecol Evol 17: 571–577

Brenes, R., ... & Montgomery, C. (2006). The effects of amphibian population declines on the structure and function of Neotropical stream ecosystems. Frontiers in Ecology and the Environment, 4(1), 27-34.

Castroviejo-Fisher, S., Guayasamin, J. M., Gonzalez-Voyer, A., & Vila, C. (2014). Neotropical diversification seen through glassfrogs. Journal of Biogeography, 41(1), 66-80.

Castroviejo-Fisher, S., Moravec, J. I. Ř. Í., Aparicio, J., Guerrero-Reinhard, M., & Calderon, G. (2011). DNA taxonomy reveals two new species records of Hyalinobatrachium (Anura: Centrolenidae) for Bolivia. Zootaxa, 2798(1), 64-68.

Cisneros-Heredia, D. F., & Mcdiarmid, R. W. (2007). Revision of the characters of Centrolenidae (Amphibia: Anura: Athesphatanura), with comments on its taxonomy and the description of new taxa of glassfrogs. Zootaxa, 1572 1-82.

Connelly S, Pringle CM, Bixby RJ, Brenes R, Whiles MR, Lips KR, Kilham S, Huryn AD (2008) Changes in stream primary producer communities resulting from large-scale catastrophic

amphibian declines: can small-scale experiments predict effects of tadpole loss? Ecosystems 11:1262-1276.

Cox, C. B., Moore, P. D., & Ladle, R. (2016). Biogeography: an ecological and evolutionary approach. John Wiley & Sons.

Coyne, J. A., & Orr, H. A. (1989). Patterns of speciation in Drosophila. Evolution, 43(2), 362-381.

Crawford, A. J., Bermingham, E., &, Polanía-S, C. (2007). The role of tropical dry forest as a long-term barrier to dispersal: a comparative phylogeographical analysis of dry forest tolerant and intolerant frogs. Molecular Ecology, 16(22), 4789-4807.

Cruz, F. K., Urgiles, V. L., Sánchez-Nivicela, J. C., Siddons, D. C., & Cisneros-Heredia, D. (2017). Southernmost records of Hyalinobatrachium fleischmanni (Anura: Centrolenidae). Check List, 13, 67.

Dayrat, B. (2005). Towards integrative taxonomy. Biological journal of the Linnean society, 85(3), 407-417.

De Queiroz, K. (2007). Species concepts and species delimitation. Systematic biology, 56(6), 879-886.

Delia, J., Bravo-Valencia, L., & Warkentin, K. M. (2017). Patterns of parental care in Neotropical glassfrogs: fieldwork alters hypotheses of sex-role evolution. Journal of evolutionary biology, 30(5), 898-914.

Delia, J., Cisneros-Heredia, D. F., Whitney, J., & Murrieta-Galindo, R. (2010). Observations on the reproductive behavior of a Neotropical glassfrog, Hyalinobatrachium fleischmanni (Anura: Centrolenidae). South American Journal of Herpetology, 5(1), 1-13.

deQueiroz, K. (2007) Species concepts and species delimitation. Systematic Biology, 56, 879–886.

Dugo-Cota, Á., Castroviejo-Fisher, S., Vilà, C., & Gonzalez-Voyer, A. (2015). A test of the integrated evolutionary speed hypothesis in a N eotropical amphibian radiation. Global ecology and biogeography, 24(7), 804-813.

Dynesius, M., & Jansson, R. (2014). Persistence of within-species lineages: a neglected control of speciation rates. Evolution, 68(4), 923-934.

Endler, J. A. (1992). Signals, signal conditions, and the direction of evolution. The American Naturalist, 139, S125-S153.

Escalona Sulbarán, M. D., Ivo Simões, P., Gonzalez-Voyer, A., & Castroviejo-Fisher, S. (2019). Neotropical frogs and mating songs: The evolution of advertisement calls in glassfrogs. Journal of evolutionary biology, 32(2), 163-176.

Farrell, B. D., D. E. Dussourd, and C. Mitter. 1991. Escalation of Plant Defense: Do Latex and Resin Canals Spur Plant Diversification? The American naturalist 138:881–900.

Forrest, T. G. (1994). From sender to receiver: propagation and environmental effects on acoustic signals. American zoologist, 34(6), 644-654.

Fouquet, A., Gilles, A., Vences, M., Marty, C., Blanc, M., & Gemmell, N. J. (2007). Underestimation of species richness in Neotropical frogs revealed by mtDNA analyses. PLoS one, 2(10), e1109.

Frankham, R., Ballou, J. D., Dudash, M. R., Eldridge, M. D., Fenster, C. B., Lacy, R. C., ... & Ryder, O. A. (2012). Implications of different species concepts for conserving biodiversity. Biological Conservation, 153, 25-31.

Freckleton, R. P., Harvey, P. H., & Pagel, M. (2002). Phylogenetic analysis and comparative data: a test and review of evidence. The American Naturalist, 160(6), 712-726.

Frost, D. R., Grant, T., Faivovich, J., Bain, R. H., Haas, A., Haddad, C. F., ... & Raxworthy, C. J. (2006). The amphibian tree of life. Bulletin of the American Museum of natural History, 1-291.

Frost, Darrel R. 2019. Amphibian Species of the World: an Online Reference. Version 6.0. Electronic Database accessible at http://research.amnh.org/herpetology/amphibia/index.html. American Museum of Natural History, New York, USA.

Funk, W. C., Angulo, A., Caldwell, J. P., Ryan, M. J., & Cannatella, D. C. (2008). Comparison of morphology and calls of two cryptic species of Physalaemus (Anura: Leiuperidae). Herpetologica, 64(3), 290-304.

Funk, W. C., Caminer, M., & Ron, S. R. (2011). High levels of cryptic species diversity uncovered in Amazonian frogs. Proceedings of the Royal Society B: Biological Sciences, 279(1734), 1806-1814.

García-Navas, V., & Blumstein, D. T. (2016). The effect of body size and habitat on the evolution of alarm vocalizations in rodents. Biological Journal of the Linnean Society, 118(4), 745-751.

García-R, J. C., Crawford, A. J., Mendoza, Á. M., Ospina, O., Cardenas, H., & Castro, F. (2012). Comparative phylogeography of direct-developing frogs (Anura: Craugastoridae: Pristimantis) in the southern Andes of Colombia. PLoS One, 7(9), e46077.

Gehara, M., Crawford, A. J., Orrico, V. G., Rodríguez, A., Lötters, S., Fouquet, A., ... & Urrutia, G. G. (2014). High levels of diversity uncovered in a widespread nominal taxon:

continental phylogeography of the neotropical tree frog Dendropsophus minutus. PloS one, 9(9), e103958.

Gerhardt, H. C. (1994). The evolution of vocalization in frogs and toads. Annual review of ecology and systematics, 25(1), 293-324.

Gerhardt, H. C., & Huber, F. (2002). Acoustic communication in insects and anurans: common problems and diverse solutions. University of Chicago Press.

Gonzalez-Voyer, A., den Tex, R. J., Castelló, A., & Leonard, J. A. (2013). Evolution of acoustic and visual signals in Asian barbets. Journal of Evolutionary Biology, 26, 647–659. https://doi.org/10.1111/jeb.12084

Gould, S. J., & Johnston, R. F. (1972). Geographic variation. Annual review of ecology and systematics, 3(1), 457-498.

Gourbiere S, Mallet J. 2009 Are species real? The shape of the species boundary with exponential failure, reinforcement and the 'missing snowball'. Evolution 64, 1–24. (doi:10.1111/j.1558-5646.2009. 00844.x)

Goutte, S., Dubois, A., & Legendre, F. (2013). The importance of ambient sound level to characterise anuran habitat. Plos one, 8(10), e78020.

Goutte, S., Dubois, A., & Legendre, F. (2013). The importance of ambient sound level to characterise anuran habitat. Plos one, 8(10), e78020.

Guarnizo, C. E., Paz, A., Muñoz-Ortiz, A., Flechas, S. V., Méndez-Narváez, J., & Crawford, A. J. (2015). DNA barcoding survey of anurans across the Eastern Cordillera of Colombia and the impact of the Andes on cryptic diversity. PLoS One, 10(5), e0127312.

Guayasamin, J. M., Castroviejo-Fisher, S., Ayarzagüena, J., Trueb, L., & Vilà, C. (2008). Phylogenetic relationships of glassfrogs (Centrolenidae) based on mitochondrial and nuclear genes. Molecular Phylogenetics and Evolution, 48(2), 574-595.

Guayasamin, J. M., Castroviejo-Fisher, S., Trueb, L., Ayarzagüena, J., Rada, M., & Vilà, C. (2009). Phylogenetic systematics of Glassfrogs (Amphibia: Centrolenidae) and their sister taxon Allophryne ruthveni.

Guayasamin, J. M., Cisneros-Heredia, D. F., Maynard, R. J., Lynch, R. L., Culebras, J., & Hamilton, P. S. (2017). A marvelous new glassfrog (Centrolenidae, Hyalinobatrachium) from Amazonian Ecuador. ZooKeys, (673), 1.

Hamback PA, et al. (2013) Bayesian species delimitation reveals generalist and specialist parasitic wasps on Galerucella beetles (Chrysomelidae): Sorting by herbivore or plant host. BMC Evol Biol13(1):92

Hedin, M. (2015). High-stakes species delimitation in eyeless cave spiders (Cicurina, Dictynidae, Araneae) from central Texas. Molecular ecology, 24(2), 346-361.

Herrera-Alsina, L., van Els, P., & Etienne, R. S. (2018). Detecting the dependence of diversification on multiple traits from phylogenetic trees and trait data. Systematic biology, 68(2), 317-328.

Hutter, C. R., Guayasamin, J. M., & Wiens, J. J. (2013). Explaining Andean megadiversity: the evolutionary and ecological causes of glassfrog elevational richness patterns. Ecology letters, 16(9), 1135-1144.

Kapli, P., Lutteropp, S., Zhang, J., Kobert, K., Pavlidis, P., Stamatakis, A. & Flouri, T. (2017). Multi-rate Poisson tree processes for single-locus species delimitation under maximum likelihood and Markov chain Monte Carlo. Bioinformatics, 33, 1630–1638.

Köhler, J., Jansen, M., Rodriguez, A., Kok, P. J., Toledo, L. F., Emmrich, M., Glaw F., Haddad CFB, Rödel M.O., & Vences, M. (2017). The use of bioacoustics in anuran taxonomy: theory, terminology, methods and recommendations for best practice. Zootaxa, 4251(1), 1-124.

Lande R (1980) Genetic variation and phenotypic evolution during allopatric speciation. Am Nat 116(4):463–479.

Lenormand T (2002) Gene flow and the limits to natural selection. Trends Ecol Evol 17(4):183–189.

Lougheed, S. C., Campagna, L., Dávila, J. A., Tubaro, P. L., Lijtmaer, D. A., & Handford, P. (2013). Continental phylogeography of an ecologically and morphologically diverse Neotropical songbird, Zonotrichia capensis. BMC Evolutionary Biology, 13(1), 58.

Lyra, M. L., Haddad, C. F., & de Azeredo-Espin, A. M. L. (2017). Meeting the challenge of DNA barcoding Neotropical amphibians: polymerase chain reaction optimization and new COI primers. Molecular Ecology Resources, 17(5), 966-980.

Marshall, V. T., Schwartz, J. J., & Gerhardt, H. C. (2006). Effects of heterospecific call overlap on the phonotactic behaviour of grey treefrogs. Animal Behaviour, 72(2), 449-459.

Mayr, E. (1942). Systematics and the Origin of Species. Columbia University Press, New York.

Mendoza, A. M., & Arita, H. T. (2014). Priority setting by sites and by species using rarity, richness and phylogenetic diversity: the case of neotropical glassfrogs (Anura: Centrolenidae). Biodiversity and conservation, 23(4), 909-926.

Mendoza, Á. M., Torres, M. F., Paz, A., Trujillo-Arias, N., López-Alvarez, D., Sierra, S., ... & Gonzalez, M. A. (2016). Cryptic diversity revealed by DNA barcoding in Colombian illegally traded bird species. Molecular ecology resources, 16(4), 862-873.

Mitter, C., B. Farrell, and B. Wiegmann. 1988. The Phylogenetic Study of Adaptive Zones: Has Phytophagy Promoted Insect Diversification? The American naturalist 132:107–128

Narum, S. R., & Hess, J. E. (2011). Comparison of FST outlier tests for SNP loci under selection. Molecular ecology resources, 11, 184-194.

Nosil, P., Harmon, L. J., & Seehausen, O. (2009). Ecological explanations for (incomplete) speciation. Trends in ecology & evolution, 24(3), 145-156.

Olson, M. E., & Arroyo-Santos, A. (2015). How to study adaptation (and why to do it that way). The Quarterly review of biology, 90(2), 167-191.

Padial, J. M., Miralles, A., De la Riva, I., & Vences, M. (2010). The integrative future of taxonomy. Frontiers in zoology, 7(1), 16.

Paez-Vacas, M. I., Coloma, L. A., & Santos, J. C. (2010). Systematics of the Hyloxalus bocagei complex (Anura: Dendrobatidae), description of two new cryptic species, and recognition of H. maculosus. Zootaxa, 2711(1), 1-75.

Pagel, M. (1993). Seeking the evolutionary regression coefficient: an analysis of what comparative methods measure. J. Theor. Biol. 164, 191–205

Picq, S., Alda, F., Bermingham, E., & Krahe, R. (2016). Drift-driven evolution of electric signals in a Neotropical knifefish. Evolution, 70(9), 2134-2144.

Pons, J., Barraclough, T.G., Gomez-Zurita, J., Cardoso, A., Duran, D.P., Hazell, S., ... Vogler, A.P. (2006). Sequence-based species delimitation for the DNA taxonomy of undescribed insects. Systematic Biology, 55, 595–609. pmid:16967577

Rabosky, D. L., & Huang, H. (2015). A robust semi-parametric test for detecting trait-dependent diversification. Systematic Biology, 65(2), 181-193.

Rabosky, D. L., F. Santini, J. T. Eastman, S. A. Smith, B. L. Sidlauskas, J. Chang, and M. E. Alfaro. (2013). Rates of speciation and morphological evolution are correlated across the largest vertebrate radiation. Nature Communications DOI: 10.1038/ncomms2958.

Rabosky, D. L., Grundler, M., Anderson, C., Title, P., Shi, J. J., Brown, J. W., ... & Larson, J. G. (2014). BAMM tools: an R package for the analysis of evolutionary dynamics on phylogenetic trees. Methods in Ecology and Evolution, 5(7), 701-707.

Revell, L. J., Harmon, L. J., & Collar, D. C. (2008). Phylogenetic signal, evolutionary process, and rate. Systematic Biology, 57(4), 591-601.

Reznick, D. (1985). Costs of reproduction: an evaluation of the empirical evidence. Oikos, 257-267.

Robertson, J. M., Lips, K. R., & Heist, E. J. (2008). Fine scale gene flow and individual movements among subpopulations of Centrolene prosoblepon (Anura: Centrolenidae). Revista de biologia tropical, 56(1), 13-26.

Rogers, L. J., & Kaplan, G. T. (2002). Songs, roars, and rituals: Communication in birds, mammals, and other animals. Harvard University Press.

Ruiz-Carranza, P.M. & Lynch, J.D. (1991) Ranas Centrolenidae de Colombia 1: propuesta de una nueva clasificación genérica. Lozania 57, 1–30

Ryan, M. J., Rand, A. S., & Weigt, L. A. (1996). Allozyme and advertisement call variation in the túngara frog, Physalaemus pustulosus. Evolution, 50(6), 2435-2453.

Santos, J. C., Baquero, M., Barrio-Amorós, C., Coloma, L. A., Erdtmann, L. K., Lima, A. P., & Cannatella, D. C. (2014). Aposematism increases acoustic diversification and speciation in poison frogs. Proceedings of the Royal Society of London B: Biological Sciences, 281(1796), 20141761.

Sasa M, Chippindale P, Johnson N. (1998) Patterns of postzygotic reproductive isolation in frogs. Evolution 52, 1811–1820. (doi:10.2307/2411351)

Savage, J. M. (2002). The amphibians and reptiles of Costa Rica: a herpetofauna between two continents, between two seas. University of Chicago press.

Schmidt AKD, Römer H, Riede K (2013) Spectral niche segregation and community organization in a tropical cricket assemblage. Behav Ecol 24(2):470–480.

Shannon, C. E., & Weaver, W. (1949). The mathematical theory of communication–Univ. Illinois press, Urbana, I, 11, 117.

Singhal, S., & Moritz, C. (2013). Reproductive isolation between phylogeographic lineages scales with divergence. Proceedings of the Royal Society B: Biological Sciences, 280(1772), 20132246.

Statham, M. J., Murdoch, J., Janecka, J., Aubry, K. B., Edwards, C. J., Soulsbury, C. D., ... & Tomsett, L. (2014). Range-wide multilocus phylogeography of the red fox reveals ancient continental divergence, minimal genomic exchange and distinct demographic histories. Molecular Ecology, 23(19), 4813-4830.

Stuart, B. L., Inger, R. F., & Voris, H. K. (2006). High level of cryptic species diversity revealed by sympatric lineages of Southeast Asian forest frogs. Biology letters, 2(3), 470-474.

Tang, C. Q., Humphreys, A. M., Fontaneto, D., & Barraclough, T. G. (2014). Effects of phylogenetic reconstruction method on the robustness of species delimitation using single-locus data. Methods in Ecology and Evolution, 5(10), 1086-1094.

Tobias JA, Gamarra-Toledo V, Garcia-Olaechea D, Pulgarin PC, Seddon N. (2011). Year round resource defense and the evolution of male and female song in suboscine birds: social armaments are mutual ornaments. J Evol Biol 24:2118–2138.

Tobias, J. A., Cornwallis, C. K., Derryberry, E. P., Claramunt, S., Brumfield, R. T., & Seddon, N. (2014a). Species coexistence and the dynamics of phenotypic evolution in adaptive radiation. Nature, 506(7488), 359.

Tobias, J. A., Planqué, R., Cram, D. L., & Seddon, N. (2014b). Species interactions and the structure of complex communication networks. Proceedings of the National Academy of Sciences, 111(3), 1020-1025.

Toussaint, E. F., Morinière, J., Müller, C. J., Kunte, K., Turlin, B., Hausmann, A., & Balke, M. (2015). Comparative molecular species delimitation in the charismatic Nawab butterflies (Nymphalidae, Charaxinae, Polyura). Molecular Phylogenetics and Evolution, 91, 194-209.

Valencia-Aguilar, A., Castro-Herrera, F., & Ramírez-Pinilla, M. P. (2012). Microhabitats for oviposition and male clutch attendance in Hyalinobatrachium aureoguttatum (Anura: Centrolenidae). Copeia, 2012(4), 722-731.

Vellend, M. (2010). Conceptual synthesis in community ecology. The Quarterly review of biology, 85(2), 183-206. (doi:10.1086/652373)

Whiles, M. R., Lips, K. R., Pringle, C. M., Kilham, S. S., Bixby, R. J., Brenes, R., ... & Montgomery, C. (2006). The effects of amphibian population declines on the structure and function of Neotropical stream ecosystems. Frontiers in Ecology and the Environment, 4(1), 27-34.

Wiens, J. J., & Servedio, M. R. (2000). Species delimitation in systematics: inferring diagnostic differences between species. Proceedings of the Royal Society of London. Series B: Biological Sciences, 267(1444), 631-636.

Wilkins, M. R., Seddon, N., & Safran, R. J. (2013). Evolutionary divergence in acoustic signals: Causes and consequences. Trends in Ecology & Evolution, 28, 156–166. https://doi.org/10.1016/j.tree.2012.10.002

Will KW, Mishler BD, Wheeler QD (2005) The perils of DNA barcoding and the need for integrative taxonomy. Systematic Biology, 54, 844–851.

Wynn, A., & Heyer, W. R. (2001). Do geographically widespread species of tropical amphibians exist? An estimate of genetic relatedness within the neotropical frog Leptodactylus fuscus (Schneider 1799)(Anura Leptodactylidae). Tropical Zoology, 14(2), 255-285.

Zamudio, K. R., Bell, R. C., & Mason, N. A. (2016). Phenotypes in phylogeography: Species' traits, environmental variation, and vertebrate diversification. Proceedings of the National Academy of Sciences, 113(29), 8041-8048.

Zang Z, Rannala B. (2014) Unguided species delimitation using DNA sequence data from multiple loci. Mol. Biol. Evol. 31, 3125–3135

APÉNDICES

APENDICE I

Notes on natural history and call description of the Critically Endangered *Plectrohyla avia* (Anura: Hylidae) from Chiapas, Mexico.

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Notes on natural history and call description of the Critically Endangered *Plectrohyla avia* (Anura: Hylidae) from Chiapas, Mexico

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Plectrohyla avia Stuart, 1952 was described on the basis of a single male from Granja Lorena, Quetzaltenango, Guatemala (Stuart 1952). Since then, only a few observations have been made of this species, expanding the distribution from the vicinity of the El Triunfo Biosphere Reserve in the Sierra Madre del Sur de Chiapas southeast across the Volcán Tacaná of Chiapas and the Volcán Tajumulco of southwestern Guatemala to the highlands of the Quetzaltenango District of Guatemala (Duellman and Campbell 1992). Only general information on morphology and distribution has been published, and the natural history of this species remains unknown (Duellman 2001; Kohler, 2010).

Plectrohyla avia is the largest member of its genus; males reach 90.4 mm, and females 70.4 mm. None of the large species of *Plectrohyla* (*P. avia*, *P. teuchestes* Duellman and Campbell 1992*; P. exquisita* McCrannie and Wilson, 1998; *P. hartwegi* Duellman 1968) have been tested molecularly (Faivovich 2005; Frost 2006; Pyron and Wiens 2011; Duellman et al. 2016), and thus it is not possible to establish relationships with the smaller species of the genus. The recent split of *Plectrohyla*, describing *Sarcohyla* for the *bistincta* group (Duellman et al. 2016) and retaining *Plectrohyla* for the *guatemalensis* group, helps to clarify the taxonomic panorama. All four species of large *Plectrohyla* are considered Critically Endangered by the IUCN (Acevedo and Smith 2004; Cruz et al. 2010; Santos-Barrera et al. 2004, 2006). While they are likely all closely related, *P. avia* stands out within the group as it has a single prepollical spine (bifid on the others) and very protruding teeth.

No detailed natural history observations on any of the larger species of *Plectrohyla* (*P. avia*, *P. teuchestes*, *P. exquisita*, and *P. hartwegi*) have been published (Duellman and Campbell 1992; Duellman 2001; Köhler 2010). We herein report observations on the reproductive activity and natural history of *P. avia* in its habitat.

On May 5th, 2016, we arrived at the small village of Mirador Chiquihuites, Municipality of Unión Juárez, Chiapas, at ±1700h, just as it was starting to rain (amongst the first rains of the season, according to the locals). Several *Plectrohyla* cf. *sagorum* Hartweg, 1941 were calling from a small ravine below the road. The ravine was located at 2015 m asl at the following coordinates: 15.095167°, -92.106669° datum = WGS84.

About 150 m upstream, within a narrow ravine

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Fig. 1. Aquatic axillary amplexus of *Plectrohyla avia*. (Right top). *Photo by César L. Barrio-Amorós*. **Fig. 2.** Male of *Plectrohyla avia* grasping the female's head with its long and curved teeth. (Left top). *Photo by César L. Barrio-Amorós*. **Fig. 3.** Amplectant pair of *Plectrohyla avia*, two motionless males and eggs over the pool. (Bottom left). *Photo by César L. Barrio-Amorós*. **Fig. 4.** Reproductive male of *Plectrohyla avia* inside a hole on the waterfall side, from where it calls. (Bottom right). *Photo by César L. Barrio-Amorós*.

covered by dense canopy, we heard a low but distinctive call (see below). Upon closer examination, we observed five large green frogs in a small pool. The pool was approximately one square meter in area and located at the base of a three m high waterfall with a very light flow. The five large green frogs (four adult males and one adult female) were *Plectrohyla avia*. Upon discovery all five animals were apart from each other and below the surface of the water. Two males were noticeably larger than the other two, and at one point engaged in what appeared to be male-male combat. Two of us (CG and HF) saw one male grasping the other and though not very clear (too dark), we saw how the largest embraced the smaller and forced it to escape. After this we filmed a short video (https://youtu.be/aa2O-BguqOY). Shortly after the combat, the largest male embraced the only female in an axillar amplexus (Fig. 1), while the other three males remained inactive under water. This male was significantly larger than the female, as has been reported for some large *Plectrohyla* (Duellman and Campbell 1992; Köhler 2010). During the first minutes the female moved around the pool while in amplexus with the inactive male (Fig. 2). The female appeared to be trying to escape from the male who remained in strong amplexus. After some minutes swimming across the pool, beneath the surface of the water at all times, the female stopped on the edge of the pool. The amplectant male remained motionless. Another male began calling underwater (sec 16 of video; the male was not seen calling directly but was the only close male and it was under water; other underwater calls were heard afterwards occasionally); immediately thereafter the amplectant male began scratching the top of the female's head with the long teeth that protrude from the upper jaw. The female tried to release herself from the amplecting male and again began swimming, but the male continued to move his head laterally scratching the female's head. While being passed by the swimming female, the other males remained motionless (Fig. 3). The pair again stopped at the other edge of the pool. The rocky bed and walls of the pool were covered by large eggs, all underwater, probably from earlier amplectant pair(s). We did not see the female laying eggs.

Above the small pool with the five adults, we observed three more adults. Two males were calling from within small holes in the splash-zone of waterfalls varying from one to four m in height (Fig. 4). Another adult male was perched on the wet wall of the higher waterfall (Fig. 5). Adult males called from within holes, crevices or beneath the surface of the water.

Two kinds of eggs were observed in the pool. A smaller one in dense quantities with pigmented pole, and other bigger and unpigmented, more scattered and in a much lower density. We cannot rely of which one was laid by *P. avia*, as we did not see directly the female laying eggs. *Plectrohyla* cf. *sagorum* was present on the ravine as well (we saw several calling males and

Fig. 5. Active male of *Plectrohyla avia* on a waterfall wall, the only male we saw outside the water or holes. *Photo by César L. Barrio-Amorós*.

one amplectant pair) and we cannot be sure to which species the eggs belong. Only unpigmented eggs have been reported for *Plectrohyla* (Duellman and Campbell 1992). An alternative explanation could be that the larger unpigmented eggs belong to different stages of development of the same species.

Vocalization

Recording specifications: The first call (Fig. 6) was recorded with a Sony RX10 camera in HD 1080, and sound extracted in a WAV file, 48 kHz of sample rate, 16 bit signed. The second call (Fig. 7) was recorded with a Nikon d5100 camera and sound extracted in a WAV file, 44.1kHz of sample rate, 16 bit signed. Recordings were analyzed in Raven Pro 1.5 Beta (Bioacoustics Research Program 2013), with a Hann function window, FFT 1024 samples, and 50% overlap). A summary of spectral and temporal features of vocalizations of *Plectrohyla avia* is in Table 1, and details of each pulse of the calls are in Table 2.

Vocalization description: The male advertisement call of *P. avia* is a brief, rapid, trill. The first call (Fig 6) consists in a series of seven consecutive pulses at a mean frequency of 561.5 Hz (541-572 Hz). The duration of the call is of 0.28 sec and duration between pulses of 0.020 sec. The initial and final pulses show the lesser intensity, lower than 80 dB, while the medium pulses have amplitude above 85 dB (Table 2, Fig. 6A).

The second call (Fig. 7) has nine consecutive pulses at a mean dominant frequency of 349.5 Hz (348-353 Hz). This call is longer than the first one (0.405 sec) and we are not sure if was emitted by the same individual. Each pulse has a mean duration of 0.032 sec and the duration between pulses is 0.011 sec. Like the first call, the first and last pulse were lower in intensity (under 60 dB), while the medium pulses presented amplitude above 62 dB (Table 2, Fig. 7B).

Discussion

The first exceptional thing about these observations is that this is the first case of underwater breeding behavior among hylids (with exception of Pseudinae, of which two genera are highly adapted to aquatic life).

A second noteworthy observation is that combat behavior has never been observed in *Plectrohyla*.

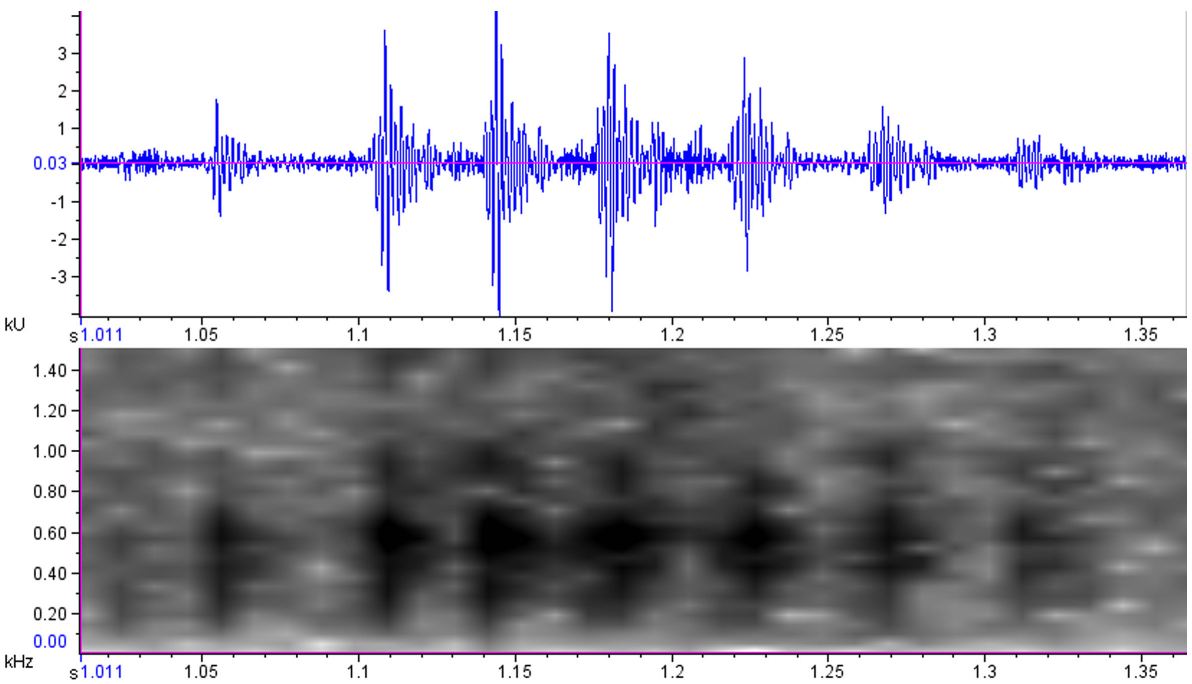

Fig. 6. Waveform (above) and spectrogram (below) of the first subaquatic call analyzed of *Plectrohyla avia*.

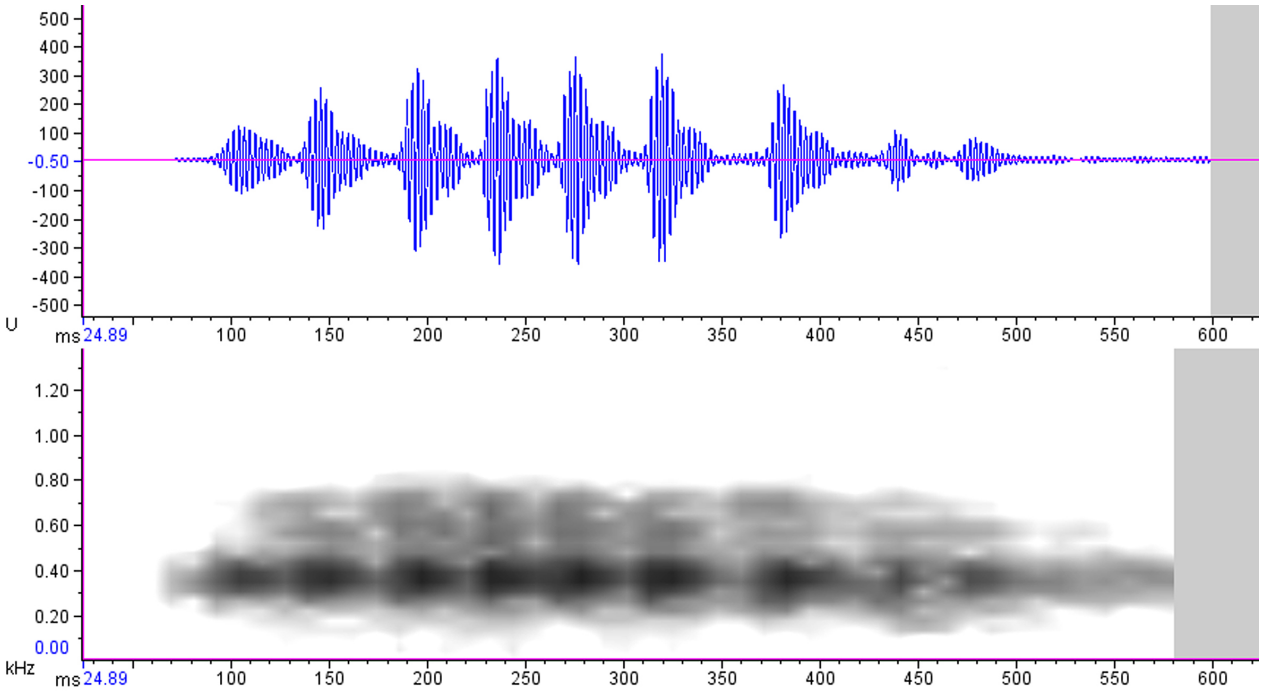

Fig. 7. Waveform (above) and spectrogram (below) of the second subaquatic call analyzed of *Plectrohyla avia*.

Dermal scratches, however, have been reported in some species (like *P. teuchestes* and *P. harwegi*; Duellman and Campbell 1992), and these observations suggest that they are caused by the prepollical spine used in some kind of combat. While large but stout teeth have been reported for *P. exquisita*, a very similar species (McCrannie and Wilson 1998), the teeth in the upper jaw in a breeding-condition male of *P. avia* (MZFC [Museo de Zoología, Facultad de Ciencias, Universidad Nacional Autonoma de México, Mexico] 29273) are extraordinarily long, protruding from the upper jaw and curved (Fig. 8). This may have an important function to promote the female sexual reception or ovulation, as the male was seen apparently using them to scratch the female's head. However, we did not find any trace of mental gland on the reproductive male collected, and the function must not be similar to that in plethodontid salamanders, in which males also scratch female's head with its premaxillary teeth inoculating secretions from the mental glands (Arnold 1977; Duellman and Trueb 1994). Another possible explanation is that during the aquatic period, the individuals eat freshwater hard-shelled insects (Notonectidae, Hydrophilidae, and Gyrinidae). We did not observe any expansion of the throat on the calling male. Another striking feature was the presence of very well developed lateral skin soft folds (Fig 9), that may help in dermal respiration, as in high Andean frogs of the family Telmatobiidae (Duellman and Trueb 1994).

All reproductive activity that we witnessed was from 1715h to 1830h, when it was still light outside the dark ravine. We collected one male and came back after dark at 2200h, but not a single individual was observed in the area.

In the same ravine, many *Plectrohyla* cf. *sagorum* were heard and seen, including one amplectant pair. This species is smaller (males up to 45.5 mm, females up to 51.9 mm; Kohler 2010) and no individual was observed in the water; several males were calling from rocks facing the ravine, and an amplectant pair was on the wall of a small waterfall. In a nearby larger stream, with many *P. matudai* (including recent metamorphs) but not *P. avia*, we found four *Plectrohyla hartwegi*, close to a four m high waterfall, all were perched on stems and ferns in or close to, the spray zone. This species has very flared lips but no protruding teeth.

Fig. 8. Detail of the long and curved teeth protruding from the upper maxilla of a preserved reproductive male MZFC 29273. *Photo by Chris I. Grünwald*.

Fig. 9. Lateral view of a reproductive male MZFC 29273, showing the lateral skin folds. *Photo by César L. Barrio-Amorós*.

Conservation status

In less than two hours we saw eight *Plectrohyla avia* in a section of less than 20 m in the ravine. At the small pool and inside the holes in the wall of the canyon were many eggs attached to the rock or stems, some freshly laid, some with white embryos visible, and already some tadpoles recently hatched (Fig 10). We cannot be absolutely sure that all those eggs belong to *P. avia*, as we never saw females laying. This species, however, was the dominant in that sector of the ravine and occupied the lower pool (five individuals using the pool for reproductive purposes), and though no adults were seen in the upper pool (Fig. 10), two adult males were very close. On the upper pool only unpigmented eggs were seen, very likely belonging to *P. avia*.

Fig. 10. Pool full of eggs in different stages of *Plectrohyla avia*. *Photo by César L. Barrio-Amorós*.

We queried local inhabitants about the frogs, and all them recognized the species, and said they are common. One boy told us that he enjoyed killing them. While the IUCN (Santos-Barrera et al. 2006) and Stuart et al. (2008) consider this species to be in the maximum category of vulnerability (CR A3e), Johnson et al (2015) consider the EVS score as 13 –of 20- (medium category). Probably the species is more widely distributed and more abundant than expected, but with a short period of activity at the beginning of the rainy season. More information is needed to establish a definitive category of conservation.

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Literature Cited

Acevedo M,Smith E. 2004. *Plectrohyla teuchestes*. The IUCN Red List of Threatened Species 2004: Available:

e.T55887A11372355.http://dx.doi.org/10.2305/IUCN.UK.2004.RLTS.T55887A11372355.en [Accessed:12 May 2016].

Arnold SJ. 1977. The evolution of courtship behavior in New World salamanders with comments on Old World salamandrids. Pp. 141–183 In: Editors, Taylor DH, Guttman. *The Reproductive Biology of Amphibians.* Plenum Press, New York, New York, USA. 475 p.

Cruz G, Wilson LD, Castañeda F, Mendelson III J, Kolby JE. 2010. *Plectrohyla exquisita*. The IUCN Red List of Threatened Species 2010: e.T55874A11366549. Available: http://dx.doi.org/10.2305/IUCN.UK.2010-2.RLTS.T55874A11366549.en [Accessed:12 May 2016].

Duellman WE, Campbell JA. 1992. Hylid frogs of the genus *Plectrohyla*: Systmatics and phylogenetic relationships. *Miscellaneous Publications, Museum of Zoology, University of Michigan* 181: 1–32.

Duellman WE. 2001. *The Hylid Frogs of Middle America*. Society for the Study of Amphibians and Reptiles, Ithaca, New York, USA. 1,180 p.

Duellman WE, Marion AE, Hedges BS. 2016. Phylogenetics, classification, and biogeography of the treefrogs (Amphibia: Anura: Arboranae). *Zootaxa*. 4104: 1–109.

Duellman WE, Trueb L. 1994. *Biology of Amphibians*. Johns Hopkins University Press, Baltimore, Maryland, USA. 670 p.

Faivovich J, Haddad CFB, Garcia PCA, Frost DR, Campbell JA, Wheeler WC. 2005. Systematic review of the frog family Hylidae, with special reference to Hylinae: a phylogenetic analysis and taxonomic revision. *Bulletin of the American Museum of Natural History* 294:1–240.

Frost, DR, Grant T, Faivovich J, Bain RH, Haas A, Haddad CFB, de Sá RO, Channing A, Wilkinson M, Donnellan SC, Raxworthy CJ, Campbell JA, Blotto BL, Moler PE, Drewes RC, Nussbaum RA, Lynch JD, Green DM, Wheeler WC. 2006. The Amphibian Tree of Life. *Bulletin of the American Museum of Natural History* 297: 1–370.

Johnson JD, V Mata-Silva, LD Wilson. 2015. A conservation reassessment of the Central American herpetofauna based on the EVS measure. Amphibian & Reptile Conservation 9(2): 1–94 (e100).

Köhler G. 2011. *The Amphibians of Central America*. Herpeton, Verlag Elke Köhler, Offenbach, Germany (www.herpeton-verlag.de). 379 p.

McCranie JR, Wilson LD. 1998. Specific status of the the Honduran frogs formerly referred to *Plectohyla teuchestes* (Anura: Hylidae). *Journal of Herpetology* 32: 91–101.

Pyron, RA, Wiens JJ. 2011. A large-scale phylogeny of Amphibia including over 2800 species, and a revised classification of advanced frogs, salamanders, and caecilians. *Molecular Phylogenetics and Evolution* 61: 543–583.

Santos-Barrera G, Canseco-Márquez L, Acevedo M. 2004. *Plectrohyla hartwegi*. The IUCN Red List of Threatened Species 2004. Available: e.T55877A11367812.http://dx.doi.org/10.2305/IUCN.UK.2004.RLTS.T55877A11367812.en [Accessed:12 May 2016].

Santos-Barrera G, Canseco-Márquez L, Acevedo M, Muñoz A, Mendelson III J. 2006. *Plectrohyla avia*. The IUCN Red List of Threatened Species 2006: Available: e.T55871A11365383.http://dx.doi.org/10.2305/IUCN.UK.2006.RLTS.T55871A11365383.en [Accessed: 12 May 2016].

Stuart LC. 1952.Some new amphibians from Guatemala. *Proceedings of the Biological Society of Washington* 65: 1–12.

César L. Barrio Amorós is an anthropologist who has worked with herpetofauna in Spain, Venezuela, Peru, Costa Rica, and Mexico. His research interests include the biogeography and systematics of Amphibia and Reptilia, with emphasis in Dendrobatoidea and Terrarana from Venezuela, especially of the Guiana Shield. Now a resident of Costa Rica, he is a free-lance investigator and photographer. César has authored or co-authored more than 200 papers, including the description of 50 new species of amphibians and reptiles.

Christoph I. Grünwald is a German-Mexican herpetologist who for the last 15 years has led field expeditions to study herpetofauna. A specialist in Mexican biogeography, he has been involved with the discovery of over 100 range extensions and dozens of state records for amphibians and reptiles from around the country. Christoph specializes in rattlesnakes and pitvipers, and many important discoveries have involved this group. Currently he is leading research expeditions on Mexican rattleless pitvipers and direct-developing frogs. A co-founder of Biodiversa, A.C., an anti-extinction non-profit organization, Chris currently is developing a system of "micro-reserves," aimed at preserving the most vulnerable, high-endemism localities in Mexico.

Hector Franz-Chávez was born in Guadalajara, Mexico, and has had a passion for herpetology since childhood. He is a student of biology at the Universidad de Guadalajara (CUCBA), and his main interests include biogeography, natural history, and ecology of the herpetofauna of Mexico. He also is an avid nature photographer, and has collaborated in various herpetological inventories in different parts of Jalisco, and currently is working on an inclusive project on Mexican direct-developing frogs and several potentially new species of pitvipers. Hector has traveled extensively in the Sierra Madre Occidental and the Sierra Madre del Sur, where he has collected numerous specimens of interest.

Ángela M. Mendoza is a biologist from Universidad del Valle (Colombia) with a M.S. in biological sciences and is currently a Ph.D. student at the Universidad Nacional Autónoma de México (UNAM). Her work has focused mainly in the application of molecular tools in solving questions in ecology and conservation, with an emphasis in terrestrial vertebrates, mainly Neotropical amphibians.

Brandon La Forest was born in Phoenix, Arizona. He has been interested in herps, particularly vipers, as far back as he can remember. He studied Ecology and Evolutionary Biology at the University of Arizona in Tucson. A life long enthusiast, he enjoys traveling abroad to document reptiles and amphibians. Mexico has always been a special place for him; where he spends over a month each year collaborating with different biologist to catalog undocumented, undescribed, and under sampled reptiles and amphibians.

APENDICE II

New record of the introduced species *Eleutherodactylus planirostris* (Anura: Eleutherodactylidae) in the state of Veracruz, Mexico.

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SHORT NOTE

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New record of the introduced species *Eleutherodactylus planirostris* (Anura: Eleutherodactylidae) in the state of Veracruz, Mexico

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Numerous direct developing species of the genus *Eleutherodactylus* native to the Caribbean Islands have been introduced outside its natural range by human activities. The greenhouse frog, *Eleutherodactylus planirostris* is native to Cuba and the Bahamas and has been introduced to many parts of the world. Here, we report the rediscovery of *E. planirostris* in the Mexican Gulf. The species was not reported in the region since 1974. Molecular identification of the species was possible by comparing 16S and COI sequences with samples from the type locality, five introduced populations and 20 other *Eleutherodactylus* species. The species was also verified by morphological characters. By means of phylogenetic reconstruction we propose that its introduction in Veracruz is independent to the Mexican Caribbean event. This is the first record of the species in a small rural region from Veracruz, and thus a comprehensive evaluation of the distribution of the species in Mexico is needed.

Key words: Introduced species, DNA barcoding, Mexico, *Eleutherodactylus*

Introduced species have been considered among the main threats for the preservation of biodiversity worldwide (Bellard et al., 2016). Among the amphibians, several Antillean *Eleutherodactylus* species have been introduced to new areas mainly through the trade of ornamental plants (Kraus et al.,1999; Kaiser et al., 2002). For example, *Eleutherodactylus coqui* is listed as one of the world's worst invasive alien species by IUCN (Lowe et al., 2000), and *Eleutherodactylus johnstonei* has been widely introduced, currently present in the Caribbean countries and recently recorded in Brazil (Kaiser, 1997; Ernst et al., 2011; Melo et al., 2014).

The greenhouse frog (*Eleutherodactylus planirostris*) has been introduced to many regions throughout the world such as continental and island USA (including Hawaii), Hong Kong, Guam, Philippines, Jamaica, Honduras, Panama and Suriname (McCrane et al., 2008; Crawford et al., 2011; Heinicke et al., 2011; Olson et al., 2012; McCranie & Valdés-Orellana 2014; Lee et al., 2016). In Mexico, it was reported on one occasion for the state of Veracruz, 43 years ago (Schwartz, 1974) at the port of Veracruz (Flores-Villela & MCoy, 1993), and recently in the Yucatan Peninsula (Cedeño-Vázquez et al., 2014; García-Balderas et al., 2016; Pavón-Vázquez et al., 2016; Gómez-Salazar & Cedeño-Vázquez, 2017; Ortíz-Medina et al., 2017). Molecular data from specimens collected in the Mexican Caribbean showed they are closely related to populations from Philippines and Panama (Cedeño-Vázquez et al., 2014).

As shown by Crawford et al. (2011), "the identification of invasive species in new localities may be difficult, especially when local knowledge and comparative material of the invader may be limited". The identification of introduced Caribbean *Eleutherodactylus* species is a challenge, considering their huge diversity (191 species to date) and low morphological variation. In this case, DNA sequencing provided characters to assist in species identification. Here, we report the rediscovery of *E. planirostris* in the Mexican Gulf, and we discuss the application of molecular tools in the detection of introduced species.

During field surveys performed on Ejido La Laja (Cuichapa Municipality) in Veracruz, Mexico (18°45.17' N, 96°47.13' W, 423 m. Figure 1A), we recorded an *Eleutherodactylus* population in the leaf litter and under trunks in a house garden. The morphology of the individuals did not coincide with any of the species known for the region. The individuals were found in the same site by visual and acoustic records in April, September and November 2016. Six individuals were captured and voucher specimens were deposited in the Colección Nacional de Anfibios y Reptiles (CNAR), Instituto de

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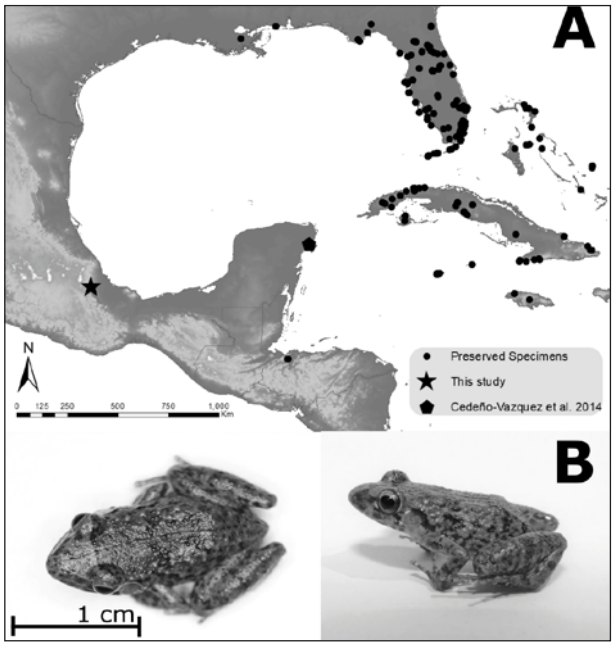

Figure 1. (A) Map with localities recorded for *E. planirostris* near the Gulf of Mexico. The record of Schwartz (1974) from Veracruz, Mexico has no locality details so it was omitted in the map. **(B)** Specimen of *E. planirostris* (IBH-31562) captured in Ejido La Laja, Municipality Cuichapa, Veracruz, México.

Biología, UNAM. Liver and muscle tissue were collected for two individuals (IBH-31562-63) and stored in RNA-laterTM Tissue Storage Reagent (Ambion).

We extracted DNA using the modified protocol of phenol-chloroform (Sambrook & Russell, 2006). For molecular identification, we amplified the mitochondrial genes COI and 16S. PCR amplifications were performed using the primers and procedures detailed in Mendoza et al. (2012) for 16S and Hebert et al. (2004) for COI. We included sequences from GenBank and BOLD databases for 21 species of *Eleutherodactylus* with available information for COI gene and/or species recorded for Mexico (*E. cystignathoides*, *E. marnockii*, *E. nitidus*, *E. pipilans* and *E. planirostris*). Those species are distributed in the states of Veracruz, Queretaro, Guerrero, Oaxaca and Chiapas (Flores-Villela & McCoy, 1993; Lemos-Espinal & Smith, 2007). The sequences obtained were aligned with Geneious using default settings and verified visually. We calculated pairwise distances using the Kimura 2 Parameter model in MEGA7 (Kumar et al., 2016) and performed a Bayesian phylogenetic analysis using the program MrBayes 3.2.2 (Ronquist & Huelsenbeck, 2003; Ronquist et al., 2012). The models of nucleotide substitution were defined following Crawford et al. (2010): one data partitions scheme for 16S (model GTR+I+Γ) and three data partitions for codon positions 1 through 3 in COI (models GTR+I, GTR+Γ, and GTR+I+Γ, respectively). Rates of evolution were allowed to vary across partitions using a rate multiplier. We ran two independent analyses for 20 million generations, each sampling trees and parameter values every 1000 generations. Burn-in was set to 25% and thus the first 5 million generations were discarded. A sequence of *Diasporus quitiddus* (AJC-1789) recovered from GenBank was used as outgroup. Considering that available COI and 16S sequences for *Eleutherodactylus* belong to different sets of species, we generated two analyses (one per gene). Sequences obtained were deposited in the Genbank repository (accession numbers MF374458-MF374461).

The BLAST of both genes matched with *E. planirostris* sequences, with a 99% identity, 0% gaps and an e-value of 0.0. Online BOLD identification generated a match with *E. planirostris* for COI sequences with 99.84% identity. Bayesian trees for both genes corroborate this result, placing the sequences obtained in the *E. planirostris* clade (Figure 2). The K2P estimated distances show a low divergence of our two sequences to all other *E. planirostris* sequences (0.000-0.016 for 16S and 0.002-0.017 for COI, minimum interspecific distance was 0.045 for 16S and 0.040 for COI). COI tree shows a closer relationship between our samples and those from Cuba than to samples than to samples reported by Cedeño-Vázquez et al. (2014) in the Mexican Caribbean (not available for 16S). To confirm the molecular results, we verified the morphological characters of the species, which coincided with the species description (snout-vent length less than 34 mm, finger discs slightly expanded and absence of interdigital webbing in toes; Schwartz, 1974; Köhler, 2010). *Eleutherodactylus planirostris* is native to the Caribbean islands including Bahamas, Cayman Islands Caicos Islands and Cuba (Lee et al., 2016). This species appears to be a generalist, occupying a diversity of habitats including mesic and xeric broadleaf forest as well as secondary forests, shrub land, agricultural fields, near fishponds, urban gardens and parks, and near human settlements (Lee et al., 2016; García-Balderas et al., 2016; Gómez-Salazar & Cedeño-Vázquez, 2017; Ortíz-Medina et al., 2017).

New records of *E. planirostris* have been achieved by help of molecular tools. The species was first reported in Panama (Crawford et al., 2011) and Hong Kong (Lee et al., 2016) through DNA barcoding. In Honduras, molecular data from three specimens showed them to be genetically identical to the Florida-western Cuba populations (McCrane et al., 2014). Cedeño-Vázquez et al. (2014) identified it on the Mexican Caribbean through molecular and morphological information. In the Mexican Gulf, there are no more records of the species after the record of Schwartz (1974) despite being a region constantly studied. It is possible that this previous record was a non-successful introduction. Here, we report the presence of a population 1 000 km west of the recent observation (Cedeño-Vasquez et al., 2014, Figure 1) and 83 km from the record by Schwartz (1974), and we may infer by the Bayesian tree that both populations are coming from independent introduction events. The invasion in Mexican Caribbean in the Yucatan Peninsula may be related to the same dispersal event that occurred for Philippines and Panama, while the Veracruz samples are more related to Cuban populations. Thus, *E. planirostris* is being introduced into Mexico from different source populations at different times.

In general, the introduced species of *Eleutherodactylus* have been reported in big cities or in localities with high commercial trade. *Eleutherodactylus planirostris* has not been recorded for the Veracruz state in the last 43 years. Thus, the presence of *E. planirostris* in Cuichapa is an

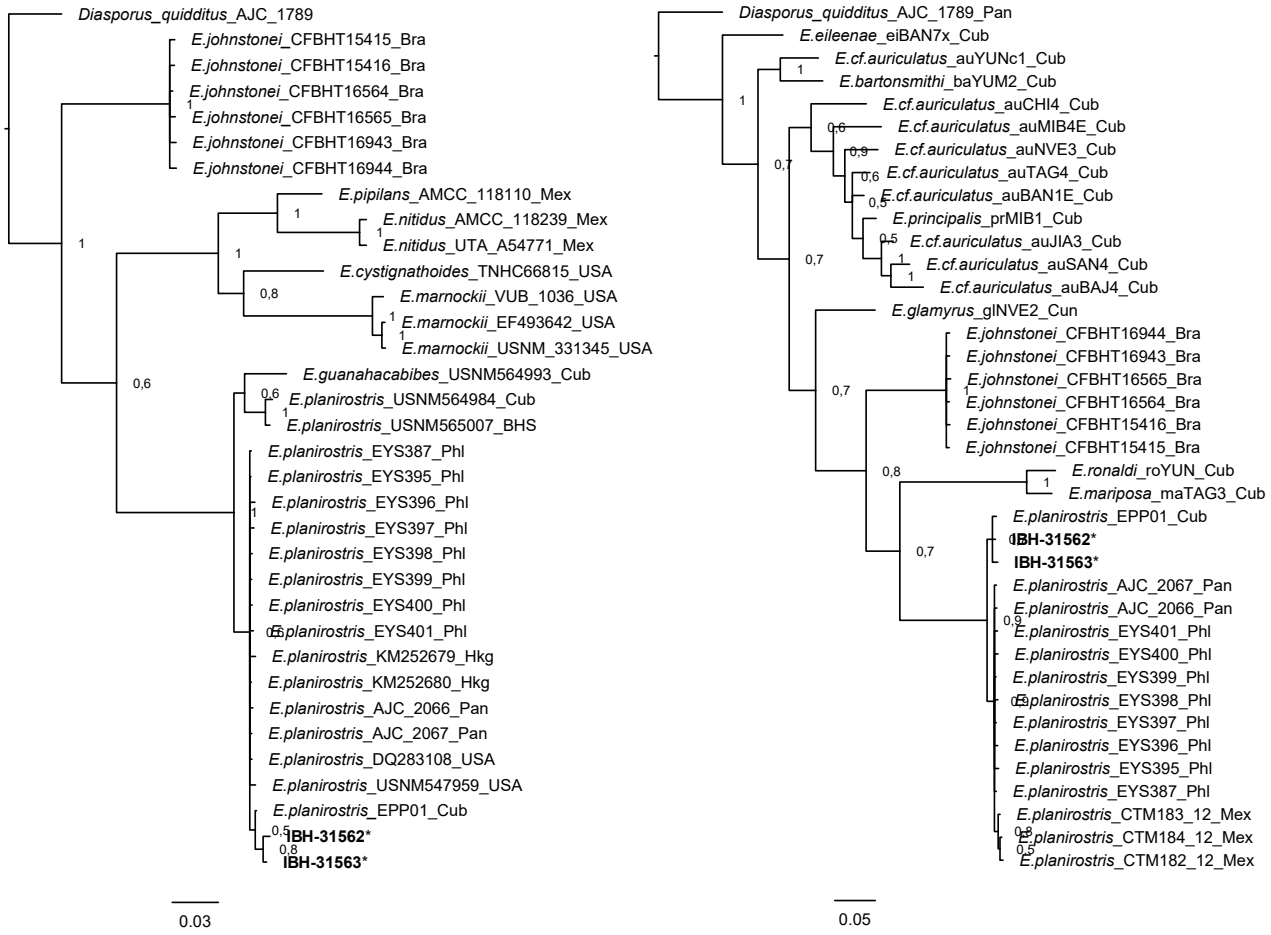

Figure 2. Phylogenetic position of two samples of *E. planirostris* from Veracruz, Mexico in a phylogenetic tree of this species as inferred from Bayesian Analyses (16S rRNA left and COI right). The samples with code IBH (in bold) were obtained for this study, while the other sequences were obtained from GenBank. Country of origin is by the corresponding ISO 3166-1 3-letter country code.

important record for expansion of this invasive species in Mexico. Cuichapa is a small rural municipality with 12,375 inhabitants according to the Instituto Nacional de Estadística y Geografía (INEGI) census (INEGI, 2009). The presence of the species in small rural regions (likely by ornamental plant trade) implies an extensive route of dispersal likely with multiple halfway localities where the species can also establish itself.

When introduced species become established, they feed, compete for food, transform and destroy the habitat, and carry transmissible diseases and parasites, capable of exterminating whole populations of native species (Williamson, 1996). Kraus et al. (1999) and Kraus & Campbell (2002) suggest that invasive populations of *E. planirostris* in Hawaii may be a serious threat to native arthropods, generating a new predation pressure primarily for insects and spiders. In Hawaii, specimens of *E. planirostris* with a population density of 12,522 frogs/ha were found to primarily consume leaf-litter invertebrates and were estimated to consume up to 129,000 invertebrates ha^{-1} $night^{-1}$ (Olson & Beard, 2012). The only population survey of this species in Mexico recorded a density of 20.3 individuals/km in Playa del Carmen, Quintana Roo (Gómez-Salazar & Cedeño-Vasquez, 2017), thus demonstrating that the species was effectively established in the sampled area. The reappearance of the species in the Mexican Gulf implies that an evaluation of the population status and the threats for biodiversity in Mexico is urgently needed, including population density, encounter frequency, spread to nearby localities, and arthropod species consumed.

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REFERENCES

Beard, K. H., Price, E. A., & Pitt, W. C. (2009). Biology and Impacts of Pacific Island Invasive Species. 5. *Eleutherodactylus coqui*, the Coqui Frog (Anura: Leptodactylidae) 1. *Pacific Science* 63, 297-316.

Bellard, C., Cassey, P., & Blackburn, T. M. (2016). Alien species as a driver of recent extinctions. *Biology Letters* 12, 20150623.

Cedeño-Vázquez, J. R., González-Vázquez, J., Martínez-Arce, A., & Canseco-Márquez, L. (2014). First record of the

invasive greenhouse frog (*Eleutherodactylus planirostris*) in the Mexican Caribbean. *Revista Mexicana de Biodiversidad* 85, 650-653.

Crawford, A. J., Lips, K. R., & Bermingham, E. (2010). Epidemic disease decimates amphibian abundance, species diversity, and evolutionary history in the highlands of central Panama. *Proceedings of the National Academy of Sciences* 107, 13777-13782.

Crawford, A. J., Alonso, R., Jaramillo, C.A., Sucre, S. & Ibáñez, R. (2011). DNA barcoding identifies a third invasive species of *Eleutherodactylus* (Anura: Eleutherodactylidae) in Panama City, Panama. *Zootaxa* 2890, 65-67.

Ernst, R., Massemin, D., & I. Kowarik. (2011). Non-invasive invaders from the Caribbean: the status of Johnstone's Whistling frog (*Eleutherodactylus johnstonei*) ten years after its introduction to Western French Guiana. *Biological Invasions* 13, 1767–1777.

Flores-Villela, O., & McCoy C. J. (1993) Herpetofauna Mexicana, Lista anotada de las especies de anfibios y reptiles de México, cambios taxonómicos recientes y nuevas especies. *Carnegie Museum of Natural* History, 17.

García-Balderas, C. M., Cedeño-Vázquez, J. R., & Mineros-Ramírez, R. (2016). *Eleutherodactylus planirostris*. Distribution Notes. *Mesoamerican Herpetology* 3, 173.

Gómez-Salazar, A. A., & Cedeño-Vázquez, J. R. (2017). Distribución y abundancia de la rana de invernadero, *Eleutherodactylus planirostris* (Cope, 1862), en Playa del Carmen, Quintana Roo, México. *Teoría y Praxis* 21, 55-66.

Hebert, P. D., Penton, E. H., Burns, J. M., Janzen, D. H., & Hallwachs, W. (2004). Ten species in one: DNA barcoding reveals cryptic species in the neotropical skipper butterfly *Astraptes fulgerator*. *Proceedings of the National Academy of Sciences of the United States of America* 101, 14812-14817.

Heinicke, M. P., Diaz, L. M., & Hedges, S. B. (2011). Origin of invasive Florida frogs traced to Cuba. *Biology Letters* 7, 407-410.

INEGI. (2009). Instituto Nacional de Estadística, Geografía e Informática. México. Http://www.inegi.org.mx/inegi/default.aspx [accessed 1 June 2017].

Kaiser, H. (1997). Origins and introductions of the Caribbean frog, *Eleutherodactylus johnstonei* (Leptodactylidae): management and conservation concerns. *Biodiversity and conservation* 6, 1391-1407.

Kaiser, H., Barrio-Amorós, C. L., Trujillo, J. D., & Lynch, J. D. (2002). Recent population changes-expansion of *Eleutherodactylus johnstonei* in Northern South America: Rapid Dispersal Through Human Interactions. *Herpetological Review* 33, 290-293.

Köhler, G. (2011). Amphibians of Central America, Offenbach: Herpeton.

Kraus, F., Campbell, E. W., Allison, A., & Pratt, T. (1999). *Eleutherodactylus* frog introduction to Hawaii. *Herpetological Review* 30, 21-25.

Kraus, F., & Campbell, E. W. (2002). Human-mediated escalation of a formerly eradicable problem: the invasion of Caribbean frogs in the Hawaiian Islands. *Biological Invasions* 4, 327-332.

Kumar, S., Stecher, G., & Tamura, K. (2016). MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. *Molecular Biology and Evolution* 33, 1870-1874.

Lee, W. H., Lau, M. W. N., Lau, A., Rao, D. Q., & Sung, Y. H. (2016). Introduction of *Eleutherodactylus planirostris* (Amphibia, Anura, Eleutherodactylidae) to Hong Kong. *Acta Herpetologica* 11, 85-89.

Lemos-Espinal, J. A., & H. M. Smith. (2007). Anfibios y Reptiles del Estado de Coahuila, México. México, D.F.: Comisión Nacional para El Conocimiento y Uso de la Biodiversidad, Universidad Nacional Autónoma de México.

Lowe, S., Browne, M., Boudjelas, S., & De Poorter, M. (2000). 100 of the world's worst invasive alien species: a selection from the global invasive species database (Vol. 12). Auckland: Invasive Species Specialist Group.

McCranie, J. R., & Valdés-Orellana, L. (2014). New island records and updated nomenclature of amphibians and reptiles from the Islas de la Bahía, Honduras. *Herpetology Notes* 7, 41-49.

McCrane, J. R., Collart, J. R., Castañeda, F. E., & Solis, J. M. (2008). Geographic distribution: *Eleutherodactylus* (Euhyas) planirostris. *Herpetological Review* 39, 362.

Melo, M. A., Lyra, M. L., Brischi, A. M., Geraldi, V. C., & Haddad, C. F. (2014). First record of the invasive frog *Eleutherodactylus johnstonei* (Anura: Eleutherodactylidae) in São Paulo, Brazil. *Salamandra* 50, 177-180.

Mendoza, A. M., García-Ramírez, J. C., & Cárdenas-Henao, H. (2012). Blood puncture as a nondestructive sampling tool to obtain DNA in frogs: comparison of protocols and survival analysis. *Molecular Ecology Resources* 12, 470-475.

Olson, C. A., & Beard, K. H. (2012). Diet of the introduced greenhouse frog in Hawaii. *Copeia* 2012, 121-129.

Ortiz-Medina, J. A., Nahuat-Cervera, P. E., Cabrera-Cen, D., Vega-Marcin, A. & Cedeño-Vázquez, J. R. (2017). First records for the invasive Greenhouse Frog, *Eleutherodactylus planirostris* (Cope, 1862) (Anura: Eleutherodactylidae), in the state of Yucatán, Mexico. Distribution Notes. *Mesoamerican Herpetology* 4, 191-193.

Pavón-Vázquez, C. J., Gray, L. N., White, B. A., García-Vázquez, U. O., & Harrison, A. S. (2016). First records for Cozumel Island, Quintana Roo, Mexico: *Eleutherodactylus planirostris* (Anura: Eleutherodactylidae), *Trachycephalus typhonius* (Anura: Hylidae), and *Indotyphlops braminus* (Squamata: Typhlopidae). Distribution Notes. *Mesoamerican Herpetology* 3, 531-533.

Ronquist, F., & Huelsenbeck, J. P. (2003). MrBayes 3: Bayesian phylogenetic inference under mixed models. *Bioinformatics*, 19, 1572-1574.

Ronquist, F., Teslenko, M., Van Der Mark, P., Ayres, D. L., Darling, A., Höhna, S., Larget, B., Liu, L., Suchard, M. A. & Huelsenbeck, J.P. (2012). MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. *Syst. Biol*, 61: 539-542.

Sambrook, J., & Russell, D. W. (2006). Purification of nucleic acids by extraction with phenol: chloroform. Cold Spring Harbor Protocols, 2006, pdb-prot4455.

Schwartz, A. (1974). *Eleutherodactylus planirostris*. Catalogue of *American Amphibians and Reptiles*, 154.1-154,4.

Williamson, M. (1996) Biological invasions. Chapman and Hall, New York. 244 p.

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APENDICE III

Empowering Latina scientists

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Madagascar's ploughshare tortoise faces extinction in the wild.

PHOTO: BRENT STEPHENSON/MINDEN PICTURES

Edited by **Jennifer Sills**

Madagascar: Crime threatens biodiversity

Madagascar's new president, Andry Rajoelina, was elected on a promise to improve living standards for the millions who live in poverty (*1*). To achieve this goal, he must address the declining rule of law. Madagascar fell eight places in the Rule of Law Index between 2016 and 2018 (*2*), and it is 155th of 180 countries listed in the Corruption Perceptions Index (*3*). Weak governance slows development by reducing the willingness of citizens and foreign companies to invest (*4*). Since his election, President Rajoelina has expressed a desire to make Madagascar a model of conservation and a destination for ecotourism (*5*). The solutions to the country's poverty—strengthening Madagascar's government and reducing crime—are also key to turning around the country's precipitous loss of biodiversity.

The threats faced by Madagascar's protected areas and species are increasingly linked to criminal networks and corruption (*6*, *7*). Illegal extraction of high-valued timber from protected areas greatly increased a decade ago (*8*). Repeated gem mining "rushes" and gold mining threaten the integrity of protected areas in the east (*9*); in the west, migrants escaping drought in the south are rapidly clearing theoretically protected forests for large-scale cultivation (*10*). Many species are illegally traded internationally (*7*) [with the ploughshare tortoise facing imminent extinction in the wild (*11*)].

Madagascar, like all nations, has the right to use its natural resource wealth, but the increasing exploitation of protected areas and species without regard to national laws does not benefit the country. Illegal activities, especially mining, are often linked to local violence and insecurity (*12*), discouraging legitimate investment. If urgent action is not taken, some of Madagascar's most iconic habitats and species may reach a point of no return. By restoring the rule of law, President Rajoelina would help deliver a Madagascar with both an inclusive, growing economy and effective biodiversity conservation.

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REFERENCES

1. Initiative Emergence Madagascar, Message de SEM Andry Rajoelina (http://iem-madagascar.com/).
2. Rule of Law Index 2017–2018 (The World Justice Project, Washington, DC, 2018); https://worldjusticeproject.org/our-work/wjp-rule-law-index/wjp-rule-law-index-2017–2018.
3. Corruption Perceptions Index 2017 (Transparency International, Berlin, Germany, 2018); www.transparency.org/news/feature/corruption_perceptions_index_2017#table.
4. D. Rodrik *et al.*, *J. Econ. Growth* **9**, 131 (2004).
5. J. Tollefson, *Nature* **565**, 407 (2019).
6. R. Moore Gerety, "Conservation in a weak state: Madagascar struggles with enforcement," *Mongabay* (2017).
7. European Parliament, "Motion for a resolution on Madagascar–B8-0641/2017" (2017).
8. H. Randriamalala, Z. Liu, *Madagascar Conserv. Dev.* **5**, 1 (2010).
9. M. Cabeza *et al.*, *Anim. Conserv.* 10.1111/acv.12475 (2019).
10. U.N. Environment News, "Secrets of the baobabs: Lifeline for a forest on the edge" (2018).
11. A. R. Mandimbihasina *et al.*, *Oryx* 10.1017/S0030605317001880 (2017).
12. J. P. G. Jones, "Madagascar: Fear and violence making rainforest conservation more challenging than ever," *The Conversation* (2018).

10.1126/science.aaw6402

Empowering Latina scientists

The #MeToo movement and other women's empowerment movements have raised awareness about hostile conditions for women scientists, stimulating revisions of norms of conduct for scientific societies and institutions (*1*, *2*). Specific problems confronted by female researchers, however, are often deeply rooted in national and regional culture. Latin American women scientists, for example, are immersed in a society where culturally ingrained masculine pride ("machismo") is

normalized (*3*, *4*) and deeply intertwined with the scientific endeavor.

Dismissal of women's contributions, patronizing behavior, and objectification of women's bodies are entrenched attitudes in Latin American society (*5*), often extending into academic settings (*6*). Machismo promotes sexist attitudes that often pass unnoticed. Latina scientists grow accustomed to unfair working conditions, where they must guard themselves from unwanted sexual advances and risk retaliation and intimidation from colleagues they do not appease (*7*). These factors contribute notably to Latinas leaving academia (*8*).

Allowing constructive conversations is a foundational issue for improving conditions for Latina scientists. For example, when a scientist known for sexist behavior (*9*) was invited as plenary speaker at a Colombian scientific conference, there was no avenue for Latina researchers to express their concerns and know they would be respected. Blatant sexism that arises from machismo precludes discussions to promote inclusion.

Latinas are striving to be heard, as reflected by an unprecedented nationwide movement to demand nonsexist education in Chile (*10*) and a surge of Women in Science symposia across the region [e.g., (*11*, *12*)]. We urge scientists and institutions across Latin America to be aware of the damage that machismo, and its denial, inflicts on women and the enterprise of science as a whole and to be proactive about recognizing, confronting, and penalizing inappropriate behaviors. Latinas and their allies in science, technology, engineering, and mathematics demand changes to promote a respectful environment for all.

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REFERENCES

1. Council of Evolution Societies, SAFE EVOLUTION (www.evolutionmeetings.org/safe-evolution.html).
2. National Science Foundation, "NSF announces new measures to protect research community from harassment" (2018).
3. V. L. Ortiz, "The [not so] invisible machismo in Latin America," *The Yale Globalist* (2008).
4. W. J. Villemez, J. G. Touhey, *Psychol. Rep.* **41**, 411 (1977).
5. C. Schaefer, *Lat. Am. Res. Rev.* **40**, 230 (2005).
6. K. Englander, C. Yañez, X. Barney, *J. Int. Women Stud.* **13**, 65 (2012).
7. J. Meléndez, A. Ilizaliturri, D. Pérez Damasco, F. Pagola, La Colaboración de la #RedDL, "Violencia sexual en las universidades de América Latina: Omisiones, obstáculos y opacidad," *Distintas Latitudes* (2016) [in Spanish].
8. C. M. Franco-Orozco, B. Franco-Orozco, *Front. Astron. Space Sci.* **5**, 24 (2018).
9. S. Mervosh, "Turtle researcher's award rescinded after he uses racy photos of women in presentation," *New York Times* (2018).
10. "Diputada: Chile debe poner fin a la educación sexista y conservadora," *Sputnik Mundo* (2018) [in Spanish].
11. "Simposio Mujeres herpetólogas en Latinoamérica: Logros y desafíos," XI Congreso Latinoamericano de Herpetología, Puce, Ecuador (2017) [in Spanish].
12. Ministerio de Educacion, Cultura, Ciencia y Tecnologia de Argentina, "Sobre las mujeres en ciencia y tecnología" (2018) [in Spanish].

SUPPLEMENTARY MATERIALS

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Full list of signatories

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Assessing cell-based animal proteins

As our ability to produce meat ("Agencies carve up cultured meat," News In Brief, 30 November 2018, p. 977) and seafood by cell culture (*1*) has increased, the U.S. Food and Drug Administration (FDA) has been called upon to provide stronger and clearer guidelines to biotechnology start-ups on safety and ethical concerns (*2*–*5*). In a 2017 report, the National Academy of Sciences named cell-based animal proteins as an area with "high growth potential" in the field of biosciences and recommended a regulatory framework to govern the new industry (*6*). However, the FDA did not highlight cell-based ingredients for direct consumption as a new case for regulatory approval; instead, the agency determined that the existing guidelines thoroughly covered adventitious agents, cell-based ingredients, and novel manufacturing processes (*7*). This puts the onus on the scientific community to determine the safety of these products.

Evaluating the safety of lab-grown meat, like this burger made from cultured beef, falls to scientists.

According to the FDA (*7*), both cultured cells, as constituents of food, and their corresponding metabolites have been inspected with well-established tests and have a long history of safe consumption. Examples of the former ingredients include the direct consumption of cultured bacterial, fungal, and algal cells (*7*). As suggested by the FDA, most cultured cells and metabolites are certified through the Generally Recognized as Safe (GRAS) notification program. Rather than direct submission to the FDA, the GRAS program relies on the scientific community to show expert consensus on the safety of the ingredients' intended use (*7*).

It is, therefore, both an opportunity and a responsibility for the scientific community to explore and investigate related frameworks for the rigorous evaluation of potential hazards and benefits of cell-based animal proteins. Future research on the foreseeable risks and preventive controls could focus on the possible metabolites of concern and potential microbial contamination, with the aid of quantitative modeling and structural simulation. As part of their safety review, scientists should find ways to determine whether cell-based meat and fish meet the criteria for "substantial equivalence" to or "no material difference" from conventional food products; these concepts (*8*) are accepted by policy-makers such as the FDA (*9*), the World Health Organization (*10*), the Food and Agriculture Organization of the United Nations (*10*), and the Organisation for Economic Co-operation and Development of Europe (*11*).

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REFERENCES

1. N. Jones, *Nature* **468**, 752 (2010).
2. K. Servick, *Science* **360**, 695 (2018).
3. K. Servick, *Science* 10.1126/science.aau7753 (2018).
4. K. Servick, *Science* 10.1126/science.aat2684 (2018).
5. E. Devitt, *Science* 10.1126/science.aah7217 (2017).
6. National Academies of Sciences, Engineering, and Medicine, *Preparing for Future Products of Biotechnology* (National Academies Press, 2017).
7. C. Rottenberg *et al.*, "The use of cell culture technology to develop products derived from livestock and poultry" (USDA/FDA Joint Public Meeting, Day One Presentation, 2018).
8. E. Millstone *et al.*, *Nature* **401**, 525 (1999).
9. FDA, "Statement of policy—Foods derived from new plant varieties" (1992).
10. FAO/WHO, "Joint FAO/WHO expert consultation on biotechnology and food safety" (1990).
11. OECD, "Safety evaluation of foods derived by modern biotechnology: Concepts and principles" (1993).

10.1126/science.aau3905

APENDICE IV

A morphological database for Colombian anuran species from conservation-priority ecosystems.

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A morphological database for Colombian anuran species from conservation-priority ecosystems

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Abstract. Species traits provide a strong link between an organism's fitness and processes at community and ecosystem levels. However, such data remain scarce for amphibians in the Neotropics. Colombia is the country with the highest number of threatened amphibians and the second greatest number of amphibian species worldwide. We present a data set containing eight morphological traits for 4,623 museum specimens of the seven largest collections in the country corresponding to 293 species of 14 families. The number of measured specimens per species ranged from 1 to 118 individuals with a median of 8 individuals per species. Overall, this database gathered morphological information for 37.6% of Colombian anuran diversity. Species measured were mainly distributed in the high Andean forest, the páramo, and wetland ecosystems, and was part of a national initiative led by the Instituto Alexander von Humboldt. The morphological traits were selected on the basis of their role in species' responses to environmental variability and their contributions to ecosystem processes. These traits were related to habitat use, (forearm length, tibia length, femur length, foot length, and foot webbing), predation and food chains (head width and mouth width), and nutrient recycling (snout–vent length). We expect this data set will be used in studies on functional diversity in amphibians and the development of conservation planning for these taxa. No copyright or proprietary restrictions are associated with the use of this data set other than citation of this Data Paper.

Key words: *amphibians; Andes; Colombia; frogs; functional diversity; morphology; toads; traits.*

The complete data sets corresponding to abstracts published in the Data Papers section in the journal are published electronically as Supporting Information in the online version of this article at http://onlinelibrary.wiley.com/doi/10.1002/ecy.2685/suppinfo

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APENDICE V

New records and distribution of the rare glassfrog *Hyalinobatrachium chirripoi* throughout the Chocó-Magdalena region in Colombia.

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New records and distribution extension of the rare glassfrog *Hyalinobatrachium chirripoi* (Anura: Centrolenidae) throughout the Chocó-Magdalena region in Colombia

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Keywords. Amphibia, Andes Mountains, DNA barcoding, South America, rainforest, range extension

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Hyalinobatrachium is the most diverse glassfrog genus (family Centrolenidae) with 32 species described to date, ranging from Mexico to Argentina (Guayasamin et al. 2009; Frost 2019). Given the low levels of morphological differentiation within this genus, species identification is sometimes difficult, and requires the use of alternative sources of evidence such as molecular phylogenetics and DNA barcoding (Castroviejo-Fisher et al. 2009). *Hyalinobatrachium chirripoi* (Taylor 1958) is a seldom observed species found in forests under 600 m elevation from Honduras, along the Chocó-Darien to the Esmeraldas Province in north Ecuador (Kubicki 2007; Guayasamin et al. 2016). Here new records of *H. chirripoi* are reported which extend the distribution of this species into the Andean foothills of the central Chocó and, for the first time, into the Magdalena Valley of Colombia. An overview of the known distribution *H. chirripoi* is presented, including previous museum records and the new data.

Specimens examined. One individual was collected in 2010 at Vereda El Porton, in San Francisco, Antioquia, Colombia (5.9015, -74.96925, 589 m asl; Fig. 1), in the Magdalena River drainage. The individual was found at night, calling on the underside of a *Heliconia* leaf overhanging a small stream in a secondary forest, and was euthanized with an overdose of 2% Roxicaine and fixed in 10% formalin. A liver sample was preserved in 99% ethanol. The specimen was deposited in the Museo de Herpetología, Universidad de Antioquia, Colombia (voucher MHUA-A 06650; originally misidentified as *H. fleischmanni*). In 2016, two other individuals were collected during nocturnal surveys performed at 20 km SW of Condoto, Chocó, Colombia, on the western versant of the Cordillera Occidental (5.02078, -76.51633, 423 m asl; Fig. 1A). They were found calling from the upper side of leaves in a tree hanging above a small river (Fig. 1B). They were captured and euthanized with an overdose of topical lidocaine hydrochloride (Xylocaine). Muscle samples were stored in 100% ethanol. Specimens were then fixed with 100% ethanol and deposited in the herpetology collection of the Natural History Museum at Universidad de los Andes, Colombia (vouchers ANDES-A3738 and ANDES-A3739). Other individuals at the same locality were observed on leaves and fronds of Araceae, Musaceae (*Heliconia*), and ferns (Polypodiaceae), perched ~3–8 m off the ground. Both localities (Vereda El Porton at the Magdalena River drainage, and 20 km SW of Condoto in the Chocó) are classified as tropical wet forest biome (bh-T, Holdridge 1964).

Morphological and molecular identification. These samples were identified as *H. chirripoi* based on light dorsal spots, significant webbing between Fingers II and III, clear parietal peritoneum, bare heart condition (i.e., iridophores covering all visceral peritonea except for the urinary bladder and pericardium), tympanum visible, and a truncate snout in sagittal view (Taylor 1958; Ruiz-Carranza and Lynch 1998; Savage 2002, Fig. 2). To

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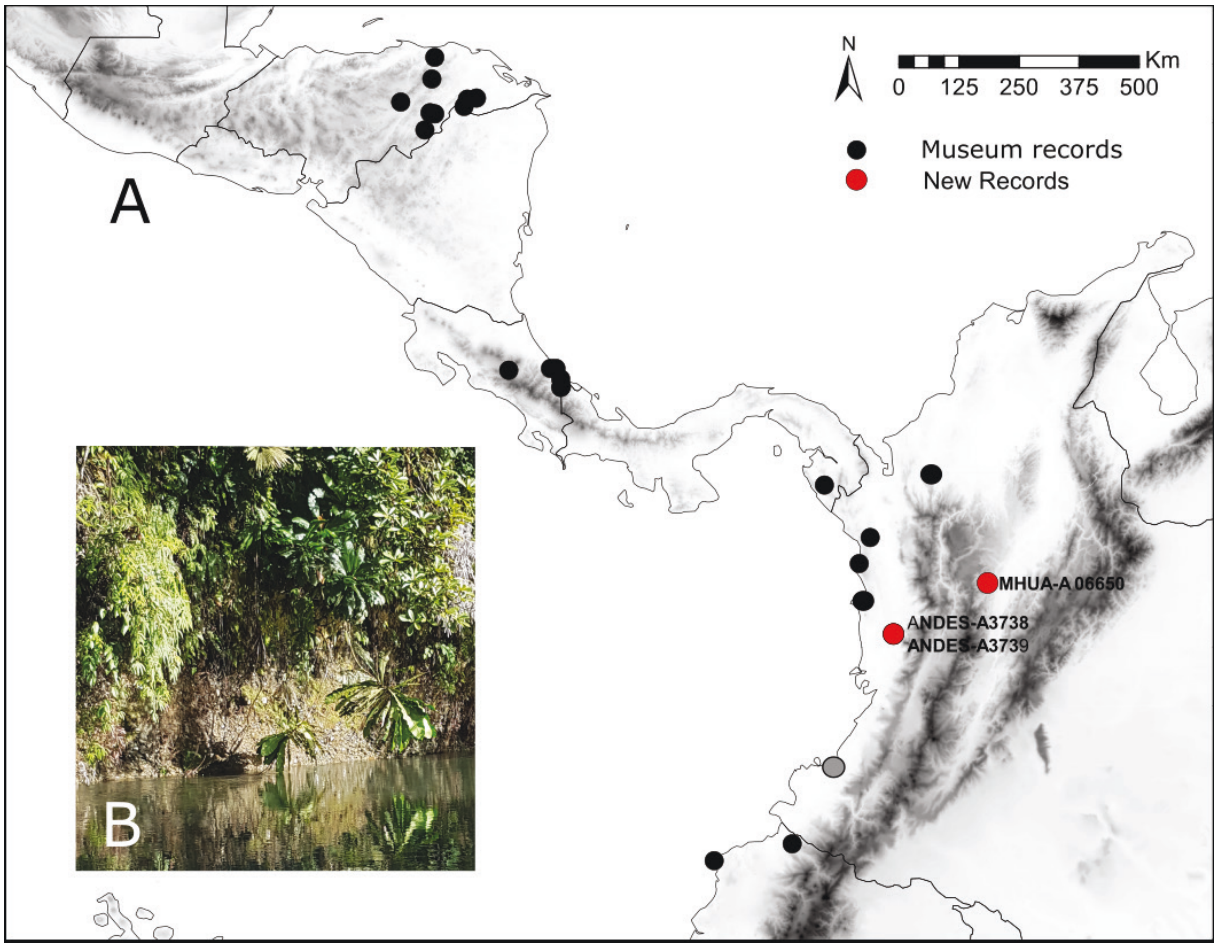

Fig. 1. **(A)** Localities of museum specimens (black dots) and new records (red dots) for *Hyalinobatrachium chirripoi.* Coordinates for specimen IAvH-A-4311 (gray dot) were approximated to the urban area of Río Guapi, Cauca, since precise coordinates of the collection site were lacking. **(B)** Habitat where the ANDES-A individuals were encountered.

further corroborate morphological diagnoses, mtDNA barcoding was used. DNA was extracted following Ivanova et al. (2006) for specimens ANDES-A3738 and ANDES-A3739, or using the Thermo Scientific DNA extraction kit for specimen MHUA-A 06650. Amplification of 16S (567 bp) and COI (609 bp) loci was as described by Guayasamin et al. (2008), and Mendoza et al. (2016; primers from Meyer et al. 2005), respectively. Purified products were Sanger-sequenced in both directions. Sequences obtained are deposited in GenBank under accession numbers MH129045–49.

Sequences of both genes were blasted against the GenBank non-redundant database using megaBLAST. COI sequences were also used as input for the BOLD DNA barcoding system (Ratnasingham and Hebert 2007). In addition, Kimura-two-parameter (K2P; Kimura 1980) pairwise distances between sequences of closely related *Hyalinobatrachium* species available in GenBank (Table 1) were calculated using MEGA7 (Kumar et al. 2016) and maximum likelihood and Bayesian mtDNA genealogies were built with RAxML v.8.2.10 (Stamatakis 2006, 2014), and MrBayes 3.2.2 (Ronquist and Huelsenbeck 2003, Ronquist et al. 2012), respectively.

Maximum likelihood searches used the rapid hill-climbing algorithm and 10,000 rapid bootstrap pseudo-replicates to assess nodal support. In MrBayes two independent 2,000,000 generation analyses were run, sampling every 1,000 generations, and with 20% burn-in. The best models for molecular evolution for the 16S and for each codon position of the COI gene were selected using PartitionFinder 2 (Lanfear et al. 2016).

Mitochondrial sequences unambiguously confirmed the identity of the specimens as *H. chirripoi*. All BLAST searches against GenBank returned *H. chirripoi* sequences as the top hit, with 99% identity and e-values of zero. Online BOLD identification searches matched the COI sequences to *H. chirripoi* with 99.3–99.5% identity. On the other hand, *H. fleischmanni* sequences matched the query sequences with 83.8% similarity (BOLD) and 83.6% identity (GenBank). Maximum likelihood and Bayesian trees corroborated these results, with the query sequences nested within a well-supported clade that includes all the other *H. chirripoi* (Fig. 3). Finally, K2P distances among *H. chirripoi* samples averaged 0.006 (range = 0.002–0.009) for 16S and 0.027 (0–0.039) for COI, while the mean distance with *H. colymbiphyllum*, its sister species, was 0.021 (0.018–0.024) for 16S and 0.081 (0.059–0.093) for COI.

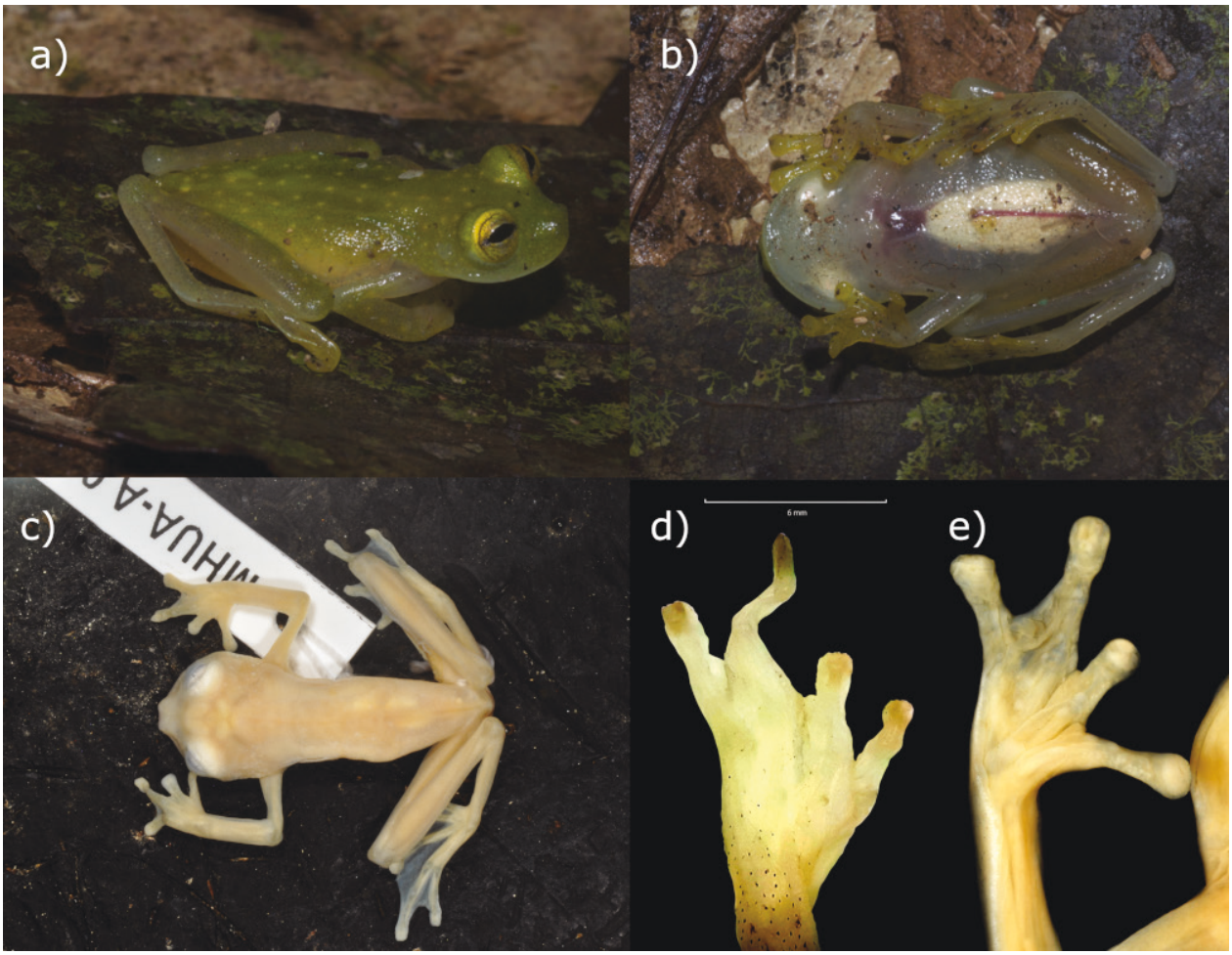

Fig. 2. Dorsal **(a)** and ventral **(b)** view of live specimen from Chocó-Darien (ANDES-A-3738). Dorsal view **(c)** of specimen collected in Madgalena basin (MHUA-A-6650). Details of hand webbing of specimens collected in Chocó-Darien **(d)** and Madgalena basin **(e)**.

Distribution and conservation implications. The records of *H. chirripoi* since its rediscovery by Kubicki (2004) are very scarce. In Colombia there have been very few isolated records of the species (Hayes and Starret 1980; Romero-Martínez et al. 2008; Ruiz-Carranza and Lynch 1998). Most previous records for the species in Colombia are restricted to the Northwest Chocó-Darien region close to Panama, in Nuquí (MHUA-A 5150-53), and in Bahía Solano (ICN 40270-314) (Fig. 1). One additional specimen was collected in 1987 further south, near Río Guapi, Cauca (specimen IAvH-Am-4311) with no georeferenced locality (gray circle in Fig. 1) and the southernmost specimens were collected from Esmeraldas Provinces in Ecuador (QCAZ-A 48271, QCAZ-A 66603, Guayasamin et al. 2016). The new records reported here fill the gap in the Chocó-Darien between the Bahia Solano and Rio Guapi records, extending the distribution of this species 70 km into the Chocoan mainland and into the foothills of the Western Andes (400 m asl).

These records also extend the distribution of *H. chirripoi* into the Magdalena basin, across the Andes from all previous records of this species. Previously, the closest record of *H. chirripoi* to the Magdalena basin was from the Cerro Murrucucú in Tierralta, Córdoba, within the Parque Nacional Natural Paramillo (ICN 39129–30), an intermediate zone between Chocó-Darien rainforests and Magdalena basin. This region is included in the Sinú-San Jorge District, characterized by a biota with common elements, including several amphibian species of the Chocoan, Amazonian, and Magdalenian regions (Henao-Sarmiento et al. 2008; Hernández-Camacho et al. 1992a; Marquez et al. 2017; Romero-Martínez et al. 2008; Vasquez and Serrano 2009), and is considered as a transition zone between the Chocó-Darien, Caribbean, and Magdalena bioregions (Romero-Martínez et al. 2008). Congruently, the Magdalena basin record reported here lies within the Nechí District, for which the biological elements have affinity with those from the upper Sinú and high San Jorge drainages, as well as the Chocó-Darien region (Hernández-Camacho et al. 1992b).

With these new records of *H. chirripoi*, the Magdalena basin and Chocó-Darién regions in Colombia share a total of five species of the genus (including *H. fleischmanni*, *H. colymbiphyllum, H. aureoguttatum*, and *H. valerioi*). *Hyalinobatrachium aureoguttatum* and *H. valerioi* are easily differentiable by the dorsal coloration (large yellow round spots on a green background), but

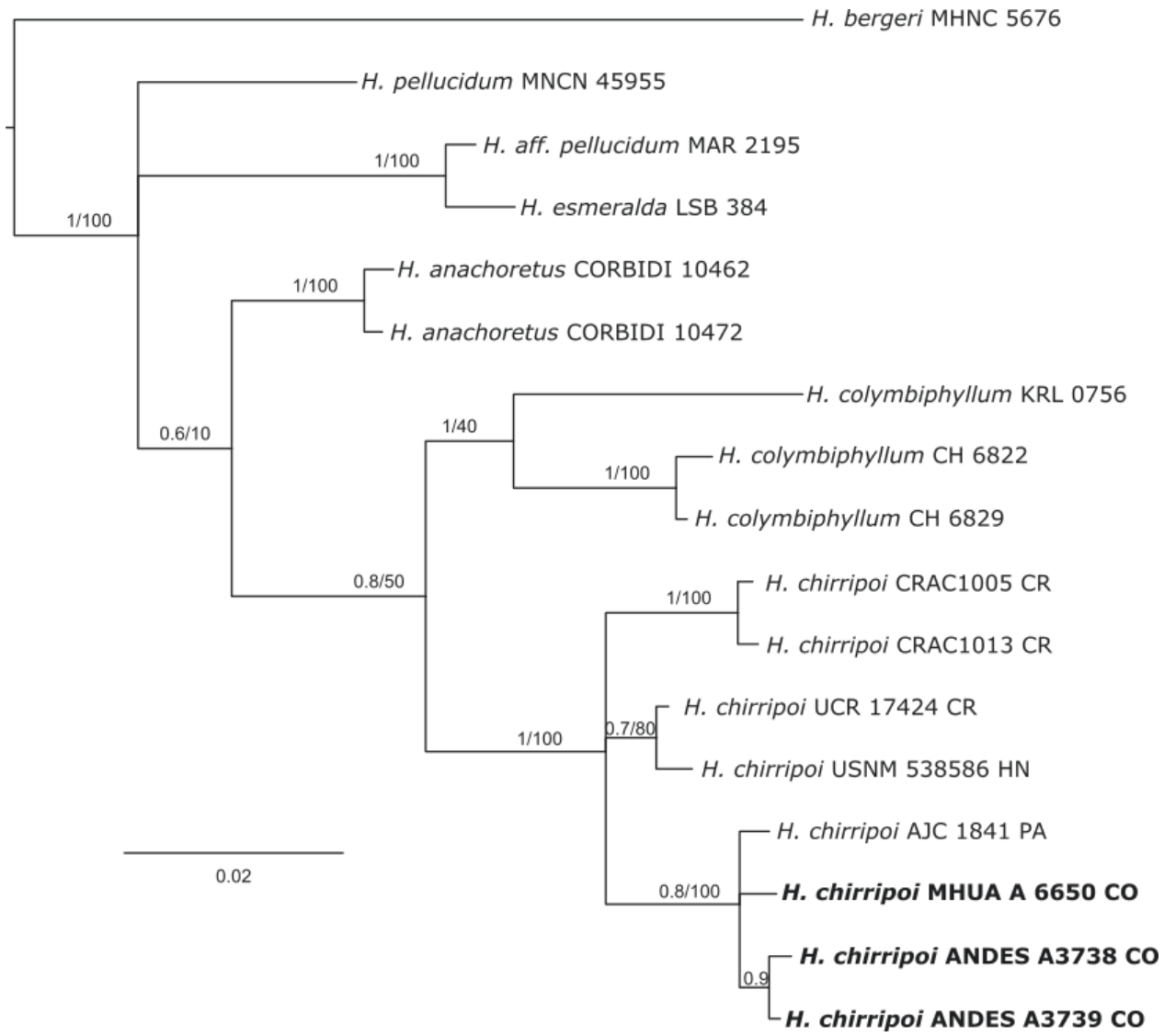

Fig. 3. Phylogenetic positions of three *Hyalinobatrachium chirripoi* samples from Chocó and Antioquia, Colombia, in a Bayesian mtDNA tree inferred from 16S rRNA and COI sequences. The chosen models of evolution using Partition finder were: 16S: GTR+I, COI position 1: GTR+I, position 2: SYM+G, and position 3: F81+I. Samples in bold are from this study, while the others are from GenBank. Posterior probability and bootstrap support values (from a maximum likelihood analysis) are indicated in front of the corresponding node as PP/Bootstrap. Two letter country codes provided for *H. chirripoi* samples follow the International Organization for Standardization: CO = Colombia, PA = Panama, HN=Honduras, CR = Costa Rica.

misidentification is common for the other three species (Kubicki 2004). The most relevant external feature for differentiating *H. chirripoi* is the extensive webbing between Fingers II–III (*H. colymbiphyllum* and *H. fleischmanni* have little webbing between Fingers II–III); additionally *H. fleischmanni* has iridophores covering the pericardium, while *H. chirripoi* and *H. colymbiphyllum* lack iridiophores in the pericardial peritonea (Savage 2002; Starret and Savage 1973, but check Cisneros-Heredia and McDiarmid 2007). After a detailed revision of the *H. fleischmanni* specimens for the Magdalena basin stored in the Museo de Herpetología of Universidad de Antioquia, no additional misidentified *H. chirripoi* were found. However, this work highlights the importance of carefully inspecting museum specimens of *Hyalinobatrachium* (and other taxa with low morphological differentiation between species) when using such specimens for biogeographic and conservation work, in order to avoid errors associated with misidentification.

A shortage of information still remains on the amphibian diversity in Chocó-Darien rainforest and Magdalena basin, both of which are increasingly threatened by human activities such as mining, habitat loss, fragmentation, and other forms of landscape transformation (Etter and van Wyngaarden 2000; Rangel 2004). Indeed, according to the IUCN Red List, certain populations of *H. chirripoi* in Panama and Colombia are threatened by habitat loss, due to increasing agricultural activity and logging (Solís et al. 2008). The new records presented here provide additional information about the distribution of this rare species, and highlight the

Table 1. Sequences for mitochondrial regions 16S and COI of *Hyalinobatrachium chirripoi* and related species used in this study.

Species	Voucher	16S	COI
H. chirripoi	ANDES-A3738	MH129045	MH129047
H. chirripoi	ANDES-A3739	MH129046	MH129048
H. chirripoi	MHUA-A-6640	MH129049	NA
H. chirripoi	UCR 17424	EU663037	NA
H. chirripoi	USNM 538586	EU663038	NA
H. chirripoi	AJC 1841	KF604299	KF604294
H. chirripoi	CRAC1005	NA	KJ703104
H. chirripoi	CRAC1013	NA	KJ703105
H. bergeri	MHNC 5676	EU663033	NA
H. pellucidum	MNCN 45955	KM068262	NA
H. esmeralda	LSB 384	KP149361	KP149161
H. colymbiphyllum	KRL 0756	FJ784359	NA
H. colymbiphyllum	CH 6829	KR863254	KR862999
H. colymbiphyllum	CH 6822	KR863256	KR863001
H. anachoretus	CORBIDI 10462	KM068268	NA
H. anachoretus	CORBIDI 10472	KM068300	NA
H. aff. *pellucidum*	MAR-2195	KM068296	NA

importance of using an integrative taxonomic approach at the junction between these two bioregions in terms of biodiversity conservation, as well as the need for continued documentation of their biological richness.

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Literature Cited

Castroviejo-Fisher S, Vilá C, Ayarzagueena J, Blanc M, Ernst R. 2011. Species diversity of *Hyalinobatrachium* glassfrogs (Amphibia: Centrolenidae) from the Guiana Shield, with the description of two new species. *Zootaxa* 3132: 1–55.

Cisneros-Heredia DF, McDiarmid RW. 2007. Primer registro de *Hyalinobatrachium ruedai* (Amphibia: Centrolenidae) en Ecuador, con notas sobre otras especies congenéricas. *Herpetotrópicos* 3: 21–28.

Etter A, Wyngaarden W. 2000. Patterns of landscape transformation in Colombia, with emphasis in the Andean region. *AMBIO: A Journal of the Human Environment* 29: 432–439.

Frost DR. 2019. Amphibian Species of the World: an Online Reference. Version 6.0. Available: http://research.amnh.org/herpetology/amphibia/index.html [Accessed: 28 July 2019].

Guayasamin JM, Castroviejo-Fisher S, Ayarzagüena J, Trueb L, Vilà C. 2008. Molecular phylogenetics and evolutionary phylogenetic relationships of glassfrogs (Centrolenidae) based on mitochondrial and nuclear genes. *Molecular Phylogenetics and Evolution* 48: 574–595.

Guayasamin JM, Castroviejo-Fisher S, Trueb L, Ayarzagüena J, Rada M, Vilà C. 2009. Phylogenetic systematics of glassfrogs (Amphibia: Centrolenidae) and their sister taxon *Allophryne ruthveni*. *Zootaxa* 2100: 1–97.

Guayasamín JM, Varela-Jaramillo A, Frenkel C, Pazmiño-Armijos G. 2016. *Hyalinobatrachium chirripoi*. Unpaginated In: *Anfibios del Ecuador*. Version 2018.0. Museo de Zoología, Pontificia Universidad Católica del Ecuador, Quito, Ecuador. Available; https://bioweb.bio/faunaweb/amphibiaweb/FichaEspecie/Hyalinobatrachium%20chirripoi [Accessed: 20 November 2018].

Hayes MP, Starrett PH. 1980. Notes on a collection of centrolenid frogs from the Colombian Chocó. *Bulletin of the Southern California Academy of Sciences* 79: 89–96.

Henao-Sarmiento JE, Cárdenas-Torres MA, Fajardo-Patiño A. 2008. Zonificación ambiental de la zona de reserva forestal del Pacífico en jurisdicción del departamento de Córdoba, Caribe Colombiano. *Colombia Forestal* 11: 175–200.

Hernández-Camacho J, Hurtado-Guerra A, Ortiz-Quijano R, Walschburger T. 1992a. Centros de endemismo en Colombia. Pp. 175–190 In: *La Diversidad Biológica de Iberoamérica I*. Acta Zoológica Mexicana. CYTED-B, Instituto de Ecología, Xalapa, Veracruz, México. 389 p.

Hernández-Camacho J, Hurtado-Guerra A, Ortiz-Quijano R, Walschburger T. 1992b. Unidades biogeográficas de Colombia. Pp. 105–152 In: *La Diversidad Biológica de Iberoamérica I*. Acta Zoológica Mexicana. CYTED-B, Instituto de Ecología, Xalapa, Veracruz, México. 389 p.

Holdridge L. 1964. *Life Zone Ecology.* Tropical Science Center, San José, Costa Rica. 206 p.

Ivanova NV, deWaard JR, Hebert PDN. 2006. An inexpensive, automation-friendly protocol for recovering high-quality DNA. *Molecular Ecology Notes* 6: 998–1,002.

Kimura M. 1980. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *Journal of Molecular Evolution* 16: 111–120.

Kubicki B. 2004. Rediscovery of *Hyalinobatrachium chirripoi* (Anura: Centrolenidae) in southeastern Costa Rica. *Revista de Biología Tropical* 52: 215–218.

Kubicki B. 2007. *Ranas de Vidrio Costa Rica/Glass Frogs of Costa Rica*. Instituto Nacional de Biodiversidad, INBio, Santo Domingo de Heredia, Costa Rica. 312 p.

Kumar S, Stecher G, Tamura K. 2016. MEGA7: Molecular Evolutionary Genetics Analysis version 7.0 for bigger datasets. *Molecular Biology and Evolution* 33: 1,870–1,874.

Lanfear R, Frandsen PB, Wright AM, Senfeld T, Calcott B. 2016. PartitionFinder 2: new methods for selecting partitioned models of evolution for molecular and morphological phylogenetic analyses. *Molecular Biology and Evolution* 34: 772–773.

Marquez R, Mejia-Vargas D, Palacios-Rodriguez P, Ramirez-Castaneda V, Amezquita A. 2017. A new species of *Andinobates* (Anura: Dendrobatidae) from the Urabá region of Colombia. *Zootaxa* 4290: 531–546.

Mendoza ÁM, Torres MF, Paz A, Trujillo-Arias N, López-Alvarez D, Sierra S, Forero F, Gonzalez MA. 2016. Cryptic diversity revealed by DNA barcoding in Colombian illegally traded bird species. *Molecular Ecology Resources* 16: 862–873.

Meyer CP, Geller JB, Paulay G. 2005. Fine scale endemism on coral reefs: archipelagic differentiation in turbinid gastropods. *Evolution* 59: 113–125.

Rangel CJO. 2004. *Colombia Diversidad Biotica IV. El Choco Biogeografico/Costa Pacifica*. Universidad Nacional de Colombia, Bogotá, Colombia. 997 p.

Ratnasingham S, Hebert PD. 2007. BOLD: The Barcode of Life Data System (http://www.barcodinglife.org). *Molecular Ecology Resources* 7: 355–364.

Romero-Martínez HJ, Vidal-Pastrana CC, Lynch JD, Dueñas PR. 2008. Preliminary study of the amphibian fauna of the Cerro Murrucucú, Parque Natural Nacional Paramillo, Tierralta, Córdoba, Colombia. *Caldasia* 30: 209–229.

Ronquist F, Teslenko M, Van Der Mark P, Ayres DL, Darling A, Höhna S, Larget B, Liu L, Suchard MA, Huelsenbeck JP. 2012. MrBayes 3.2: efficient Bayesian phylogenetic inference and model choice across a large model space. *Systematic Biology* 61: 539–542.

Ruiz-Carranza PM, Lynch JD. 1998. Ranas Centrolenidae de Colombia XI. Nuevas especies de ranas cristal del género *Hyalinobatrachium*. *Revista de la Academia Colombiana de Ciencias Exactas, Físicas y Naturales* 22: 571–586.

Savage JM. 2002. *The Amphibians and Reptiles of Costa Rica: a Herpetofauna between Two Continents, between Two Seas*. University of Chicago Press, Chicago, Illinois, USA. 954 p.

Solís F, Ibáñez R, Chaves G, Savage J, Jaramillo C, Fuenmayor Q, Castro F, Grant T, Wild E, Kubick B. 2008. *Hyalinobatrachium chirripoi*. The IUCN Red List of Threatened Species 2008: e.T55006A11235906. Available: http://dx.doi.org/10.2305/IUCN.UK.2008.RLTS.T55006A11235906.en [Accessed: 14 December 2017].

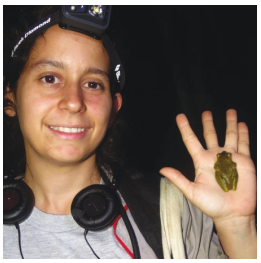
Stamatakis A. 2006. RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. *Bioinformatics* 22: 2,688–2,690.

Stamatakis A. 2014. RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. *Bioinformatics* 30: 1,312–1,313.

Starrett PH, Savage JM. 1973. The systematic status and distribution of Costa Rican glass-frogs, genus *Centrolenella* (family Centrolenidae), with description of a new species. *Bulletin of the Southern California Academy of Sciences* 72: 57–78.

Taylor EH. 1958. Notes on Costa Rican Centrolenidae with descriptions of new forms. *University of Kansas Science Bulletin* 39: 41–68.

Vásquez VH, Serrano MA. 2009. *Las Áreas Naturales Protegidas de Colombia*. Conservación Internacional Colombia y Fundación Biocolombia, Bogotá, Colombia. 696 p.



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APENDICE VI

Description of two calls of *Eleutherodactylus rubrimaculatus* (Anura: Eleutherodactylidae) in Chiapas, Mexico

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Description of two calls of *Eleutherodactylus rubrimaculatus* (Anura: Eleutherodactylidae) in Chiapas, Mexico

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Frogs of the genus *Eleutherodactylus* are direct developing frogs grouped into five subgenera and 192 species, with a geographic distribution primarily centered in the Caribbean (Padial *et al.* 2014). *Eleutherodactylus* species inhabit a variety of environments such as tropical and temperate forests, and scrub, where they occupy different microhabitats including caves, floors rich in leaf litter, cracks and cavities of limestone and volcanic outcrops (Reyes-Velasco *et al*. 2015). Mexico harbors 33 species of *Eleutherodactylus* (AmphibiaWeb 2018), most of them distributed in central-western and southwestern Mexico (Reyes-Velasco *et al*. 2015).

The Red-spotted Chirping Frog or Dusky Chirping Frog *Eleutherodactylus rubrimaculatus* (Taylor & Smith 1945) is restricted to extreme south eastern Chiapas, Mexico, a region considered one of the areas with a highest alpha diversity of amphibians in Mexico and high degree of endemisms (Campbell 1999; Pineda & Lobo 2009). *Eleutherodactylus rubrimaculatus* can be found from 0–700 masl, inhabiting cloud forests but is also known to occur in modified habitats including palm groves and grasslands (Santos-Barrera & Canseco-Márquez 2004). Despite the fact that is locally abundant, this species is considered Vulnerable according to IUCN (Santos-Barrera & Canseco-Márquez 2004) due to its small range. Although it was recently included in a study about the molecular systematics of continental *Eleutherodactylus* (Grünwald *et al.* 2018), to date there are no studies of its biology, ecology, and behavior.

The taxonomy of *Eleutherodactylus* species is still problematic due to their conserved external morphology (Padial *et al.* 2014), hence, detailed descriptions of advertisement calls may aid integrative approaches, targeted at clarifying their taxonomic status (e.g. Padial & De La Riva 2009). Anuran calls are important in species recognition and reproduction and are a valuable tool for taxonomic identification (Köhler *et al.* 2017). However, most studies in anuran acoustics are focused on the advertisement call, and less information is available for other calls such as courtship, territorial, or alarm calls. Less than 1% of the known species of frogs and toads do females vocalize in reproductive contexts (Gerhardt & Bee 2007) and reports on this phenomenon have mostly been anecdotal. Herein, we describe the previously unknown advertisement calls and a second calls of *E. rubrimaculatus*.

Individuals of *E. rubrimaculatus* were detected by acoustic and visual surveys at night in cloud forest in the Escuintla municipality in the state of Chiapas, Mexico (15°25’7” N, 92°34’45.6” W; 562 masl) on June 2017 and June 2018, in vegetation on the side of a dirt road. The species could be unambiguously identified by its size and the dorsal reddish spots on the dorsum of the specimens (Fig. 1a). Between 3 and 12 calls (mean = 7.9) per individual were recorded at 1-meter distance, with a digital recorder (Tascam DR-40) and a unidirectional microphone (Sennheisser K6/ME 66) and it was stored as wave files at a sampling rate of 44.1 kHz and an amplitude resolution of 16 bits. We measured air temperature shortly after the individuals were recorded with a digital thermometer (Benetech GM300, resolution 0.1°C). The air temperature was measured as close as possible at the same heights as the calling sites of the individual recorded. Voucher specimens were fixed with 10% formalin and deposited at the Colección Nacional de Anfibios y Reptiles (CNAR), Instituto de Biología, UNAM under the numbers IBH31782—31784, and the recordings were deposited in both the Colección de cantos of CNAR and the Colección digital de cantos grabados of Museo de Zoología de la Facultad de Ciencias, UNAM with the same IBH voucher number.

Calls were identified using the software Raven Pro 1.4 (Bioacustics Research Program, 2011) and the spectrogram was constructed with a Blackman algorithm and a windows size of 5 ms and 80% overlap. We generated call figures us-

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ing Seewave v. 1.6 package (Sueur *et al.* 2008). From the spectrogram, we obtained the dominant frequency, bandwidth, and the mean frequency of the first harmonic. Temporal parameters were measured from the waveform. Additionally, to include information about call complexity, important for species recognition (Ryan 1983), we used the package warbleR (Araya-Salas & Smith-Vidaurre 2017) to extract two additional parameters of the individual calls as a quantitative metric of the modulation: *dfslope* and *modindx*. The df slope is calculated as the change in dominant frequency through time in Hz/s. It gives us an idea of the frequency spectrum based on the start and end of the signal. The modulation index is the cumulative absolute difference between adjacent measurements of dominant frequencies divided by the dominant frequency range (1 means the signals is not modulated). For call terminology we followed Köhler *et al.* (2017).

The frogs were observed calling on the shrubs between 20 and 100 cm above the ground, at a mean air temperature of 22.3 ± 1.2 °C (mean ± SD). The vocal display in *E. rubrimaculatus* included two types of auditory signals (Fig. 1b–c). The first type of signal (advertisement call) was recorded in ten individuals (mean 7.8 calls per individual, mean snout-vent length = 26.69 ± 1.70 mm) and consisted of a single note similar to a "beep", which lasted 87.8 ± 14.5 ms and whose dominant frequency was 3169.92 ± 141.33 Hz (N = 78) and the first harmonic has a peak frequency of 5159.34 ± 805.15 Hz (N = 61). Most of energy in the call (90%) was concentrated within 2601.64 ± 165.61 Hz and 3290.68 ± 122.88 Hz. The dominant frequency shows a slope of 12.90 ± 2.73 Hz/s and a mean modulation index of 1.64 ± 1.15.

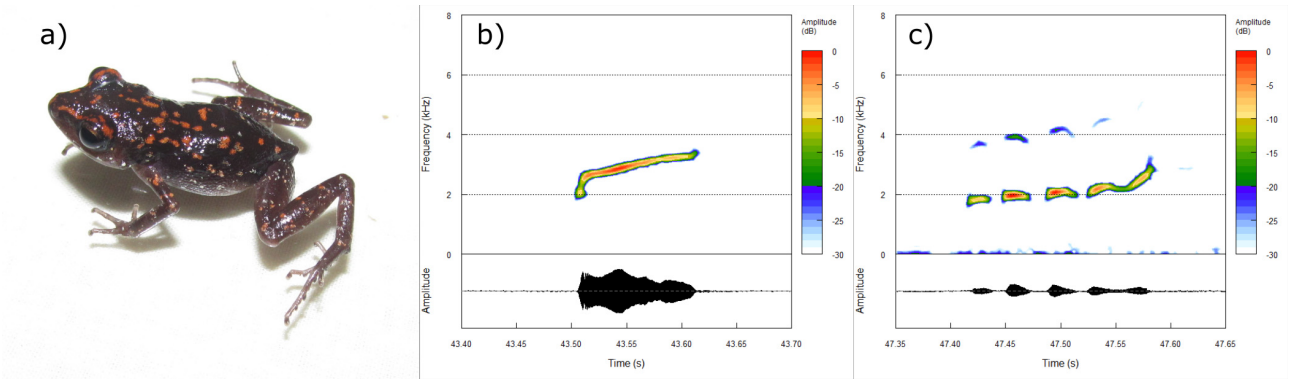

FIGURE 1. Male of *Eleutherodactylus rubrimaculatus* (a). Advertisement call (b) and a second type of call (c) of *E. rubrimaculatus* recorded at Escuintla municipality in Chiapas state, Mexico. Air temperature at time of recording = 21.8°C, mean snout-vent length = 26.69 ± 1.70 mm. Upper graphs: spectrograms. Lower graphs: oscillograms.

The second type of signal was heard less frequently throughout the field samplings than the advertisement call. We observed a couple in amplexus, likely male and female based on size differences (see Supplementary Video). After a few minutes, the amplexus was broke-off with no egg deposition and both individuals emitted this second call. This signal was recorded for a total of three individuals (mean 8.5 calls per individual) and is composed by 3 to 5 short notes (mean 4.1 notes). Each note lasted on average 29.5 ± 12.1 ms (47.0 ± 13.3 ms for the last tone and 24.1 ± 0.3 ms for the remaining) and was followed by short silent intervals of 1.3 ± 0.4 ms on average; the mean call duration was 167.8 ± 21.72 ms and the mean dominant frequency was 2339.37 ± 435.24 Hz (N = 25), the first harmonic had a peak frequency of 3974.77 ± 350.79 Hz (N = 17). As observed in the spectrogram (Fig. 1c) the second call, shows a higher complexity in comparison with the first one, it shows a lower slope for the dominant frequency of 4.73 ± 3.32 Hz/s and it has a higher modulation index (8.76 ± 5.91, N = 25).

This study comprises one of the few call descriptions with temporal and spectral features measured for continental *Eleutherodactylus*. Few *Eleutherodactylus* species have had their advertisement call described and most are restricted to the Caribbean island of Hispaniola (Galvis *et al.* 2016). Among the Mexican species with call description are *E. marnockii*, *E. pipilans*, and *E. nitidus* (Fouquette 1960), *E. grandis* (Serrano 2016), and *E. cystignathoides* (Serrano & Penna 2018). Calls of *E. grunwaldi* and *E. wixarika* were described in the species description (Reyes-Velasco *et al.* 2015) and recently Grünwald *et al.* (2018) gave a brief description of the call of six new species. When comparing the parameters of the calls (Table 1), we found a clear differentiation for *E. rubrimaculatus* calls by the dominant frequency and call duration in both call-types regards previously described ones.

Although female calling is very rare in anurans (Goyes Vallejos *et al.* 2019), female reproductive vocalizations have been reported before in various *Eleutherodactylus* species such as *E. angustidigitorum*, *E. nitidus*, *E. guanahacabibes* and *E. cystignathoides* (Serrano & Penna 2018). Reciprocal calling by female has been reported in some continental and Caribbean species (*E. angustidigitorum, E. podiciferus* and *E. coqui;* Reyes-Velasco *et al*. 2015). In the case of the second

call of *E. rubrimaculatus* we could not verify the sex of the individuals recorded so further evidence must be obtained to verify that female calls on this species. Therefore, our work not only provides new information of the acoustic communication signals for taxonomy purposes but also opens a new opportunity to understand the complexity of reproductive and calling behavior on Neotropical anurans.

TABLE 1. Comparison of calls described among Mexican *Eleutherodactylus* species (Mean ± SD). * = Dominant frequency estimated as the mean value based on range frequency reported. (♂ = male, ♀ = female). NA = Not available.

Species	Dominant frequency (Hz)	**Call Length (ms)**	**Call rate (/m)**	**Source**
E. rubrimaculatus ♂	3169.9 ± 141.3	87.8 ± 14.5	2.91 ± 3.3	**This study**
E. rubrimaculatus ♂ ♀ (?)	2339.3 ± 435.2	167.8 ± 21.7	12.4 ± 9.1	**This study**
E. grunwaldi ♂	2130 ± 0.02	70 ± 10	6.13 ± 1.3	Reyes-Velasco *et al*. 2015
E. wixarika ♂	2750 ± 0.04	130 ± 40	6.13 ± 2.7	Reyes-Velasco *et al*. 2015
E. grandis ♂	2238.8 ± 1.6	169.7 ± 7.3	3.9	Serrano 2009
E. colimotl ♂	3058.7 ± 210.3*	105.0 ± 28.3	NA	Grünwald *et al*. 2018
E. erendirae ♂	3581.2 ± 129.0*	166.0 ± 83.4	NA	Grünwald *et al*. 2018
E. floresvillelai ♂	3700*	210	NA	Grünwald *et al*. 2018
E. jaliscoensis ♂	2625	166	NA	Grünwald *et al*. 2018
E. manantlanensis ♂	2665*	220	NA	Grünwald *et al*. 2018
E. nietoi ♂	3610*	257	NA	Grünwald *et al*. 2018
E. cystignathoides ♂	3880.2 ± 270.1	317.5 ± 196.8	6.01 ± 3.6	Serrano & Penna 2018
E. cystignathoides ♀	3996.3 ± 343.0	383 ± 79	3.82 ± 2.3	Serrano & Penna 2018

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References

AmphibiaWeb (2018) AmphibiaWeb species lists. University of California, Berkeley, CA, USA. Available from: https://amphibiaweb.org (accessed 11 December 2018)

Araya-Salas, M. & Smith-Vidaurre, G. (2016) warbleR: an R package to streamline analysis of animal acoustic signals. *Methods in Ecology and Evolution*, 8, 184–191. https://doi.org/10.1111/2041-210X.12624

Bioacoustics Research Program. (2011) Raven Pro: Interactive Sound Analysis Software. Version 1.4. Computer software. The Cornell Lab of Ornithology, Ithaca, New York. Available from: http://www.birds.cornell.edu/raven (accessed 11 December 2018)

Campbell, J.A. (1999) Distribution patterns of amphibians in Middle America. *In*: Duellman, W.E. (Ed.), *Patterns of Distribution of Amphibians. A Global Perspective*. Johns Hopkins University Press, Baltimore, Maryland, pp. 111–210.

Fouquette, M. (1960) Call structure in frogs of the family Leptodactylidae. *Texas Journal of Science*, 12, 201–215.

Galvis, P.A., Caorsi, V.Z., Sánchez-Pacheco, S.J. & Rada, M. (2016) The advertisement calls of three *Eleutherodactylus* species from Hispaniola (Anura: Eleutherodactylidae). *Bioacoustics*, 27, 1–12. https://doi.org/10.1080/09524622.2016.1260053

Gerhardt, H.C. & Bee, M.A. (2007) Recognition and localisation of acoustic signals. *In*: Narins, P. M., Feng, A.S., Fay, R.R. & Popper, A.N. (Eds.), *Hearing and sound communication in amphibians*. Springer, New York, pp. 113–146. https://doi.org/10.1007/978-0-387-47796-1_5

Goyes Vallejos, J., Grafe, T.U., Ahmad-Sah, H.H. & Wells, K.D. (2017) Calling behaviour of males and females of a Bornean frog with male parental care and possible sex-role reversal. *Behavioral Ecology and Sociobiology*, 71, 95. https://doi.org/10.1007/s00265-017-2323-3

Grünwald, C.I., Reyes-Velasco, J., Franz-Chávez, H., Morales-Flores, K., Ahumada-Carrillo, I.T., Jones, J.M. & Boissinot, S. (2018) Six new species of *Eleutherodactylus* (Anura: Eleutherodactylidae: subgenus *Syrrhophus*) from Mexico, with a

discussion of their systematic relationships and the validity of related species. *Mesoamerican Herpetology*, 5, 7–83.
Köhler, J., Jansen, M., Rodriguez, A., Kok, P.J., Toledo, L.F., Emmrich, M., Glaw, F., Haddad, C.F.B., Rödel, M.O. & Vences, M. (2017) The use of bioacoustics in anuran taxonomy: theory, terminology, methods and recommendations for best practice. *Zootaxa*, 4251 (1), 1–124.
https://doi.org/10.11646/zootaxa.4251.1.1
Padial, J.M. & De la Riva, I. (2009) Integrative taxonomy reveals cryptic Amazonian species of Pristimantis (Anura: Strabomantidae). *Zoological Journal of the Linnean Society*, 155, 97–122.
https://doi.org/10.1111/j.1096-3642.2008.00424.x
Padial, J.M., Grant, T. & Frost, D.R. (2014) Molecular systematics of terraranas (Anura: Brachycephaloidea) with an assessment of the effects of alignment and optimality criteria. *Zootaxa*, 3825 (1), 1–132.
https://doi.org/10.11646/zootaxa.3825.1.1
Pineda, E. & Lobo, J.M. (2009) Assessing the accuracy of species distribution models to predict amphibian species richness patterns. *Journal of Animal Ecology*, 78, 182–190.
https://doi.org/10.1111/j.1365-2656.2008.01471.x
Reyes-Velasco, J., Ahumada-Carrillo, I., Burkhardt, T.R. & Devitt, T.J. (2015) Two new species of *Eleutherodactylus* (subgenus *Syrrhophus*) from western Mexico. *Zootaxa*, 3914 (3), 301–317.
https://doi.org/10.11646/zootaxa.3914.3.4
Ryan, M.J. (1983) Frequency modulated calls and species recognition in a neotropical frog. *Journal of comparative physiology*, 150, 217–221.
https://doi.org/10.1007/BF00606371
Santos-Barrera, G. & Canseco-Márquez, L. (2004) *Eleutherodactylus rubrimaculatus*. The IUCN Red List of Threatened Species 2004: e.T56928A11556146.
https://doi.org/10.2305/IUCN.UK.2004.RLTS.T56928A11556146.en
Serrano, J.M. (2016) El canto de anuncio de la rana endémica del Pedregal de la ciudad de México. *Revista mexicana de biodiversidad*, 87, 535–539.
https://doi.org/10.1016/j.rmb.2016.03.003
Serrano, J.M. & Penna, M. (2018) Sexual monomorphism in the advertisement calls of a Neotropical frog. *Biological Journal of the Linnean Society*, 123, 388–401.
https://doi.org/10.1093/biolinnean/blx141
Sueur, J., Aubin, T. & Simonis, C. (2008) Seewave, a free modular tool for sound analysis and synthesis. *Bioacoustics*, 18, 213–226.
https://doi.org/10.1080/09524622.2008.9753600
Taylor, E.H. & Smith, H.M. (1945) Summary of the collections of amphibians made in México under the Walter Rathbone Bacon traveling scholarship. *Proceedings of the United States National Museum*, 95, 521–613.
https://doi.org/10.5479/si.00963801.95-3185.521

APENDICE VII

Centrolene huilensis (Ruiz-Carranza & Lynch, 1995)

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ANFIBIOS Y REPTILES DE COLOMBIA

Centrolene huilensis Ruíz-Carranza y Lynch, 1995

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Taxonomía y sistemática

Centrolene huilensis fue descrita por Ruíz-Carranza y Lynch (1995) a partir de siete individuos colectados en los alrededores del municipio de Isnos (anteriormente San José de Isnos), en el departamento del Huila, Colombia. Inicialmente, *C. huilensis* fue ubicada en el grupo *prosoblepon*, dentro del género *Centrolene* (Ruíz-Carranza y Lynch 1995), y luego su estatus fue considerado como *incertae sedis* dentro de la subfamilia Centroleninae por Guayasamin et al. (2009), ya que los autores no contaron con material genético de la especie que permitiera inferir su posición filogenética. Posteriormente, Catenazzi et al. (2012) incluyeron a *C. huilensis* dentro del género *Centrolene,* con base en dos individuos de la Reserva Yanayacu en la Provincia del Napo en Ecuador. Aunque Catenazzi et al. (2012) recuperaron a *C. heloderma* como especie hermana de *C. huilensis* por medio de un análisis de máxima verosimilitud, el valor de soporte de esta relación fue bajo (menor al 50% de bootstrap). Estudios posteriores por medio de criterios de optimización diferentes, parsimonia e inferencia bayesiana, y con un mayor número de terminales, indicaron a *C. muelleri* como la especie más próxima a *C. huilensis* con soportes relativamente altos.

Debido a que la raíz del nombre genérico es femenina en griego (*kéntron* - pico, espuela - y *ōlénē* - codo), el epíteto fue corregido por Barrio-Amorós et al. (2019), siguiendo lo establecido por el Código Internacional de Nomenclatura Zoológica (ICZN 1999) de neutro (*C. huilense*) a femenino (*C. huilensis*).

Descripción morfológica

Los individuos adultos de *C. huilensis* son de tamaño mediano en comparación a otras especies del género; la longitud rostro-cloacal (LRC) de los machos adultos se encuentra entre 23,6-26,7 mm, en tanto que la única hembra conocida (depositada en la Colección de Herpetología del Instituto de Biodiversidad de la Universidad de Kansas, USA; voucher KU-169722) presenta una longitud rostro-cloaca de 31,0 mm. La coloración general del dorso es verde con puntos y manchas verdes oscuras de diferentes tamaños, entremezclados con puntos pequeños de color blanquecino o crema con ligera tonalidad verde (Fig. 1A). Los huesos son verdes en vida; el labio superior, los bordes ulnares, tarsales y el área supracloacal son demarcados por una línea blanca. El iris es crema con reticulaciones negras evidentes. El peritoneo parietal que se extiende sobre la mitad anterior del abdomen es parcialmente blanco, mientras que el peritoneo visceral es translúcido (Fig. 1B).

Según la descripción original, *C. huilensis* presenta el rostro subacuminado en vista dorsal y moderadamente inclinado ventro-lateralmente en vista lateral. La especie presenta la piel dorsal levemente granular y los dientes vomerinos ausentes; el tímpano es grande (1,2 mm de diámetro anteroposterior) y en los machos adultos el húmero presenta una espina visible externamente, laminar y curvada hacia atrás (Fig. 1B). Por último, la palmeadura manual está presente pero reducida entre los dedos externos III y IV de la mano (Fig. 1B). Morfológicamente, *C. huilensis* y *C. muelleri* son muy similares, no obstante, *C. huilensis* se diferencia por la presencia de tubérculos pronunciados en la ulna y el tarso (presentes, pero de menor tamaño en *C. muelleri*; ver figura en Duellman y Schulte 1993). Además, *C. muelleri* se ha registrado sólo en las cuencas de los ríos Huallaga y Marañón en la ladera amazónica en Perú, entre los 1830-2050 m s. n. m. (Twomey et al. 2014).

Distribución geográfica

En Colombia, *C. huilensis* es conocida para dos localidades en la vertiente oriental de la cordillera Central

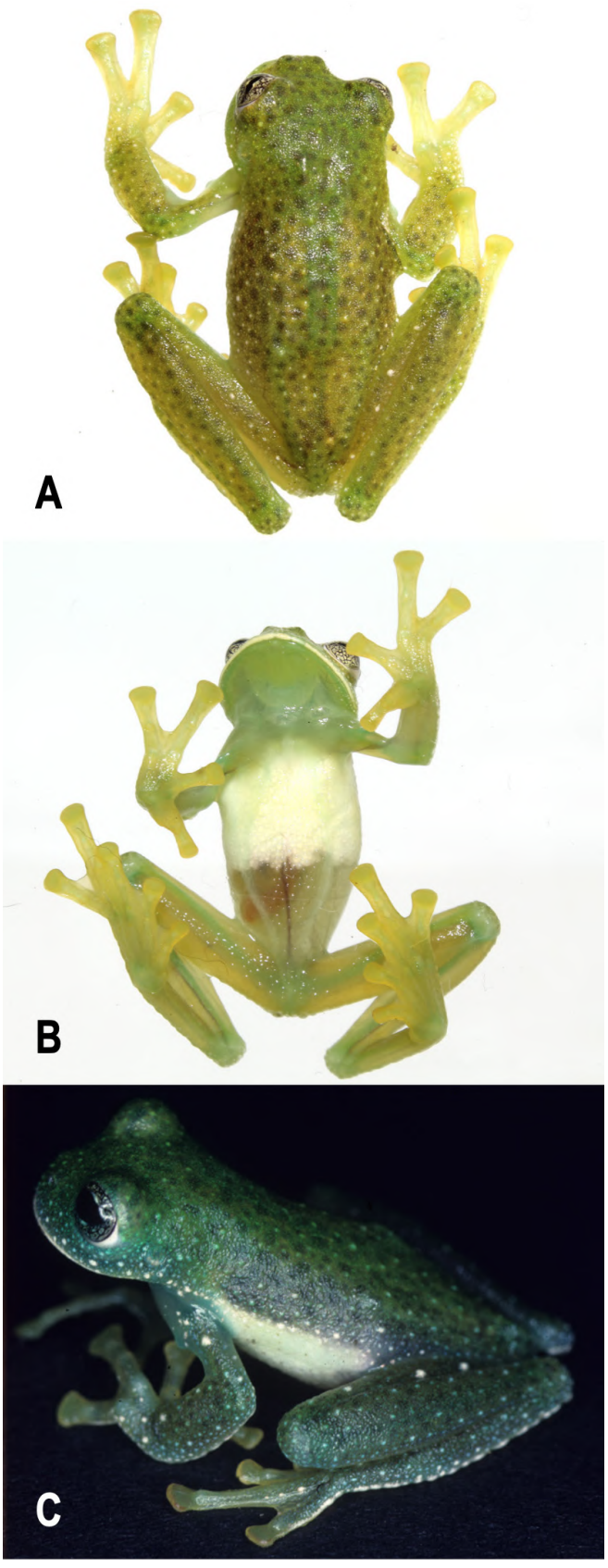

Figura 1. Adulto de *Centrolene huilensis* (AMMH-174), A: vista dorsal, B: vista ventral y C: vista lateral del paratipo (ICN-7455). Fotografías: A y B: Angela M. Mendoza-Henao y C: John D. Lynch.

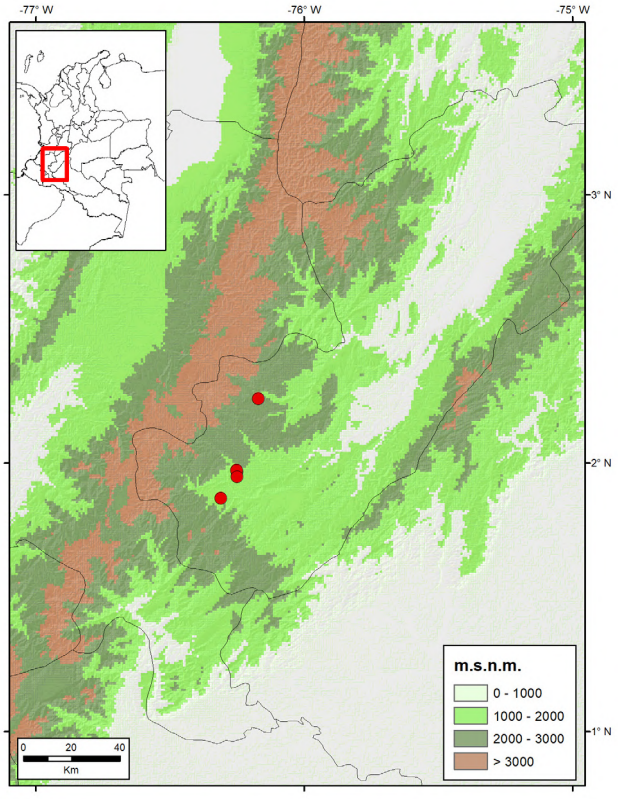

Figura 2. Mapa de distribución de *Centrolene huilensis* en Colombia.

en el municipio de Isnos (kilómetros 1 y 7,4 al noroeste del municipio), departamento del Huila, a una elevación de 1970-2190 m s. n. m (Ruíz-Carranza y Lynch 1995). Adicional a estos registros, en esta revisión reportamos tres nuevas localidades de distribución para la especie. La primera corresponde al km 3 al suroeste del municipio de San Agustín, departamento del Huila (KU 169720-47); la segunda es en el municipio de Santa Leticia, departamento del Cauca (KU 144129-30); y la tercera, corresponde a una población a 6,9 km al noroeste del municipio de Isnos, sobre la carretera que conduce de Isnos a Popayán, vereda Los Hornitos, quebrada Los Hornos (afluente de la quebraba El Helechuzal, 1990 m s. n. m. AMMH 174-178) (Fig. 2, Apéndice I).

Historia natural

La historia natural para *C. huilensis* es en gran parte desconocida, y la información aquí proporcionada corresponde a observaciones directas por parte de los autores de esta ficha.

Centrolene huilensis es una rana de actividad nocturna, asociada a la vegetación en los márgenes de los arroyos en bosques subandinos y andinos; los individuos se observan entre 1-1,5 m de altura (Fig. 3). Los machos adultos en estado reproductivo vocalizan entre las 19:00-02:00 h. El canto de anuncio de *C. huilensis* se compone por dos notas rápidas tonales agudas (obs. pers.), aunque no se cuenta con grabaciones del canto para una descripción cuantitativa del mismo. Algunos individuos son observados cerca (alrededor de 1 m) de algunas masas de huevos, sin embargo, no exhiben comportamientos de atención hacia las posturas (no se posan directamente sobre los huevos). Las masas de huevos son depositadas sobre las hojas que cuelgan por encima de los arroyos (promedio de 46,6 huevos por postura; 44-49; n=3), lo que favorece que los renacuajos caigan directamente en el agua cuando eclosionan los huevos. Los huevos y embriones en sus primeras fases (hasta el estadio 17 *sensu* Gosner 1960) son de color crema y verde claro (vitelo) y se encuentran cubiertos por una gelatina translúcida ligeramente blanquecina (Fig. 4A). A partir del estadio 18 los embriones adquieren un color gris claro, mientras que la gelatina que los recubre se torna de color más blanquecina e incrementa en volumen (Fig. 4 B-C). En los municipios de Isnos y San Agustín, *C. huilensis* es sintópica con la rana de cristal *Espadarana audax*.

Figura 3. Hábitat de *Centrolene huilensis*. Quebrada los Hornos, municipio de Isnos, departamento del Huila, Colombia. Fotografía: Angela M. Mendoza-Henao.

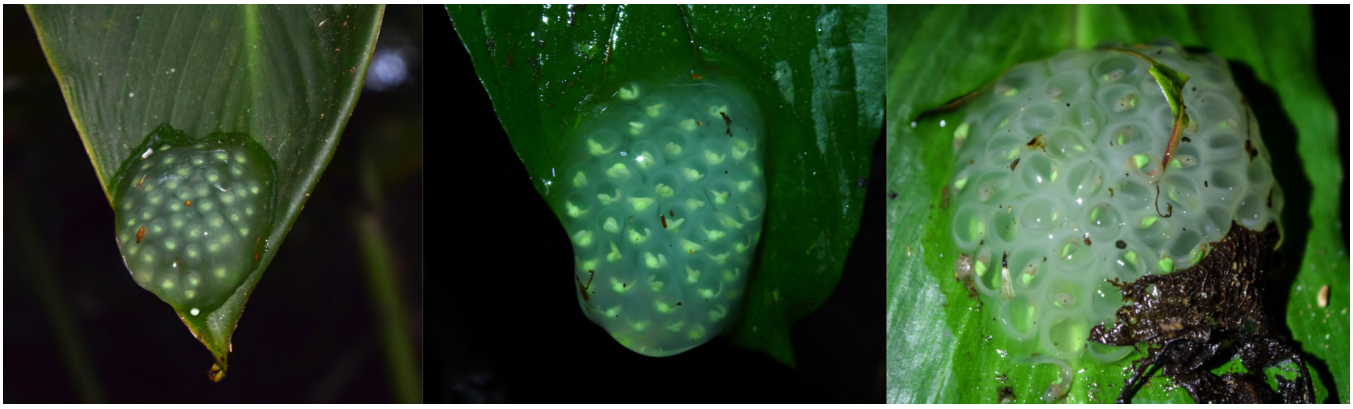

Figura 4. Masas de huevos (no colectadas) de *Centrolene huilensis*, en diferentes estadios de desarrollo (17 a 20 *sensu* Gosner 1960). Vereda Hornitos, municipio de Isnos, departamento del Huila, Colombia; 1990 m s. n. m. Fotografías: José Criollo y Jorge Luis Peña-Núñez.

Amenazas

Las principales amenazas para *C. huilensis* son la destrucción y degradación del hábitat debido a la expansión de actividades pecuarias (ganadería) y la contaminación del agua (Wild y Lynch 2004). Esta especie requiere de un bosque de galería para su reproducción y, por lo tanto, puede ser muy sensible a la destrucción de este hábitat.

Estado de conservación

En Colombia, *C. huilensis* no se encuentra catalogada bajo alguna de las categorías de riesgo de conservación (Rueda-Almonacid et al. 2004), mientras que en la lista roja de la IUCN está bajo la categoría de En Peligro (EN) (IUCN 2019). A la fecha, *C. huilensis* no se encuentra incluida en ningún apéndice CITES (www.cites.org).

Perspectivas para la investigación y conservación

Son necesarios estudios de campo para conocer el estado de las poblaciones de *C. huilensis,* su historia de vida, aspectos reproductivos y preferencias de hábitat. Lo anterior permitirá establecer su estado de conservación, sus amenazas y facilitará planear futuras estrategias de manejo.

El único registro de *C. huilensis* para Colombia en los últimos 30 años es en un bosque de galería en la microcuenca de la quebrada El Helechuzal, la cual surte de agua al acueducto municipal de Isnos, departamento del Huila, Colombia. A pesar de la importancia de dicha quebrada, esta no se encuentra bajo ninguna condición de conservación y está rodeada de potreros utilizados para ganadería (Fig. 3), lo que puede limitar la dispersión de los individuos y la conectividad con otras poblaciones. Considerando que el nombre de la especie (su epíteto corresponde al gentilicio del departamento del Huila) y su grado de endemismo en el país, *C. huilensis* podría ser usada como especie emblemática de las fuentes de agua que surten los acueductos del municipio de Isnos y así incentivar la conservación de los bosques y del recurso hídrico en la región.

Por último, resulta interesante el registro para la especie en la ladera amazónica del Ecuador por parte de Catenazzi et al. (2012), ampliando el área de distribución geográfica de *C. huilensis* en 300 km. Este raro patrón interandino-amazónico ha sido reportado previamente para algunas especies de ranas de cristal (p. ej. *Centrolene geckoideum, C. buckleyi*, "*C*". *medemi, Nymphargus posadae, Cochranella resplendens*; Molina-Zuluaga et al. 2017) y *Espadarana audax*. La distancia geográfica de los registros colombianos y ecuatorianos, el patrón inusual de distribución, y los resultados filogenéticos reportados por Twomey et al. (2014; ver sección taxonomía y sistemática) sugieren una diversidad no descrita entre las poblaciones colombianas y las peruano-ecuatorianas de *C. huilensis*. Por lo tanto, se recomienda colectar material de la localidad tipo o sus alrededores para que nuevos análisis complementen los resultados filogenéticos obtenidos por Twomey et al. (2014) y ayuden a determinar los límites de la especie, así como sus implicaciones taxonómicas.

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Literatura citada

Barrio-Amorós, C. L., F. J. M. Rojas-Runjaic, y J. C. Señaris. 2019. Catalogue of the amphibians of Venezuela: Illustrated and annotated species list, distribution, and conservation. Amphibian & Reptile Conservation 13: 1-198.

Catenazzi, A., R. von May, L. A. G. Gagliardi-Urrutia, E. Lehr y J. M. Guayasamin. 2012. A new, high-elevation glassfrog (Anura: Centrolenidae) from Manu National Park, southern Peru. Zootaxa 3388: 56-68.

Delia, J., L. Bravo-Valencia y K. M. Warkentin. 2017. Patterns of parental care in Neotropical glassfrogs: fieldwork alters hypotheses of sex-role evolution. Journal of Evolutionary Biology 30: 898-914.

Duellman, W. E. y R. Schulte. 1993. New species of centrolenid frogs from northern Peru. Occasional papers of the Museum of natural history. The University of Kansas 155: 1-33.

Gosner, K. L. 1960. A simplified table for staging anuran embryos and larvae with notes on identification. Herpetologica 16: 183-190.

Guayasamin, J. M., S. Castroviejo-Fisher, L. Trueb, J. Ayarzagüena, M. Rada y C. Vilà. 2009. Phylogenetic systematics of Glassfrogs (Amphibia: Centrolenidae) and their sister taxon *Allophryne ruthveni*. Zootaxa 2100: 1-97.

ICZN. 1999. International Code of Zoological Nomenclature = ICZN (4th edition). International Trust for Zoological Nomenclature, London, United Kingdom. 106 pp.

IUCN. 2019. The IUCN Red List of Threatened Species. Electronic database accessible at http://www.iucnredlist.org/search. Acceso 12 de septiembre de 2019.

Molina-Zuluaga, C., E. Cano, A. Restrepo, M. Rada y J. M. Daza. 2017. Out of Amazonia: the unexpected trans-Andean distribution of *Cochranella resplendens* (Lynch and Duellman, (1978) (Anura: Centrolenidae). Zootaxa 4238: 268-274.

Rueda-Almonacid, J. V., J. D. Lynch y A. Amézquita. 2004. Libro Rojo de los Anfibios de Colombia. Serie Libros de Especies Amenazadas de Colombia. Instituto de Ciencias Naturales-Universidad Nacional de Colombia, Ministerio del Medio Ambiente, Bogotá, DC-Colombia. 384 pp.

Ruíz-Carranza, P. M. y J. D. Lynch. 1995. Ranas Centrolenidae de Colombia VIII. Cuatro nuevas especies de *Centrolene* de la cordillera Central. Lozania 65: 1-16.

Twomey, E., J. Delia y S. Castroviejo-Fisher. 2014. A review of Northern Peruvian glassfrogs (Centrolenidae), with the description of four new remarkable species. Zootaxa 3851: 1-87.

Wild, E. y J. D. Lynch. 2004. *Centrolene huilense*. En: IUCN 2018. IUCN Red List of Threatened Species. Versión 2018.1. Electronic database accessible at www.iucnredlist.org. Acceso el 19 de julio de 2018.

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Apéndice I. Localidades donde se ha registrado *Centrolene huilensis* en Colombia. Referencias: (1) este manuscrito, (2) Ruíz-Carranza y Lynch 1995, (3) especímenes en University of Kansas Biodiversity Institute Herpetology Collection. Siglas: AMMH = número de campo de Angela M. Mendoza-Henao, ICN = Instituto de Ciencias Naturales, KU = Kansas University.

Departamento	Municipio	Localidad	Año de colecta	Voucher	Elevación (m s. n. m.)	Latitud	Longitud	Referencia
Huila	Isnos	6,9 km al noroeste de la cabecera municipal de Isnos, vereda Los Hornitos, quebrada Los Hornos (afluente de la Qda. El Helechuzal), departamento del Huila, Colombia.	2018	AMMH 174-178	1990	1,9687	-76,2491	1
Huila	Isnos	7,4 km al noroeste de la cabecera municipal de Isnos, departamento del Huila, Colombia.	1980	ICN 7454-57	1970	1,9731	-76,2513	2
Huila	Isnos	1 km al noroeste de la cabecera municipal de Isnos, departamento del Huila, Colombia.	1980	ICN 7461-63	2190	1,95	-76,25	2
Cauca	Santa Leticia	Santa Leticia, departamento del Cauca, Colombia.	1971	KU 144129-30	2170	2,2403	-76,1702	3
Huila	San Agustín	3 km al suroeste de San Agustín, quebrada Lavapatas dentro del Parque Arqueológico. departamento del Huila, Colombia.	1975	KU 169720-47	1780	1,869	-76,309	3