UNIVERSIDAD NACIONAL AUTÓNOMA DE MÉXICO

POSGRADO EN CIENCIAS BIOLÓGICAS

INSTITUTO DE INVESTIGACIONES EN ECOSISTEMAS Y SUSTENTABILIDAD

BIOLOGÍA EVOLUTIVA

VARIACIÓN ECOLÓGICA, FENOTÍPICA Y GENÓMICA EN UN COMPLEJO DE ESPECIES CRÍPTICAS DEL GÉNERO *Hetaerina* (ODONATA)

TESIS

QUE PARA OPTAR POR EL GRADO DE:

DOCTORA EN CIENCIAS

PRESENTA:

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MORELIA, MICHOACÁN, MÉXICO. 2022

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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 ordinaria del Comité Académico del Posgrado en Ciencias Biológicas, celebrada el día **16 de mayo de 2022,** se aprobó el siguiente jurado para el examen de grado de **DOCTORA EN CIENCIAS,** de la estudiante **VEGA SÁNCHEZ YESENIA MARGARITA,** con número de cuenta **514012702** con la tesis titulada**, VARIACIÓN ECOLÓGICA, FENOTÍPICA Y GENÓMICA EN UN COMPLEJO DE ESPECIES CRÍPTICAS DEL GÉNERO *Hetaerina* (ODONATA),** realizada bajo la dirección del **DR. ANTONIO GONZÁLEZ RODRÍGUEZ**, quedando integrado de la siguiente manera:

Presidenta:	DRA. NANCY CALDERÓN CORTÉS
Vocal:	DRA. ELLA GLORIA VÁZQUEZ DOMÍNGUEZ
Vocal:	DR. LUIS FELIPE MENDOZA CUENCA
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Secretario:	DRA. CARLA GUTIÉRREZ RODRÍGUEZ

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"
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COORDINADOR DEL PROGRAMA

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COORDINACIÓN

DR. ADOLFO GERARDO NAVARRO SIGÜENZA

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"Next to music and art, science is the greatest, most beautiful and most enlightening achievement of the human spirit."

Karl Popper

ÍNDICE

RESUMEN 1

ABSTRACT 4

I. INTRODUCCIÓN GENERAL 7

1.1. ESPECIACIÓN 7
1.1.1. MECANISMOS DE ESPECIACIÓN 8
1.1.2. MODELOS DE ESPECIACIÓN; EL PAPEL DEL FLUJO GÉNICO 11
1.2. ESPECIACIÓN EN ODONATOS 11
1.2.1. ESPECIACIÓN ALOPÁTRICA Y EL PAPEL DE LA DERIVA GÉNICA 12
1.2.2. ESPECIACIÓN ECOLÓGICA EN ODONATOS 13
1.2.3. ESPECIACIÓN Y EL PAPEL DE LA SELECCIÓN SEXUAL 14
1.3. ESPECIES CRÍPTICAS EN ODONATOS 15
1.4. GÉNERO *HETAERINA* 17
1.4.1. *HETAERINA AMERICANA* 19

II. CAPÍTULO I: COMPLEX EVOLUTIONARY HISTORY OF THE AMERICAN RUBYSPOT DAMSELFLY, *HETAERINA AMERICANA* (ODONATA): EVIDENCE OF CRYPTIC SPECIATION 22

III. CAPÍTULO II: *HETAERINA CALVERTI* (ODONATA: ZYGOPTERA: CALOPTERYGIDAE) SP. NOV., A NEW CRYPTIC SPECIES OF THE AMERICAN RUBYSPOT COMPLEX 43

IV. CAPÍTULO III: MORPHOLOGICAL VARIATION AND REPRODUCTIVE ISOLATION IN THE *HETAERINA AMERICANA* SPECIES COMPLEX 57

V. CAPÍTULO IV: GENOMIC DIFFERENTIATION IN THE *HETAERINA AMERICANA* CRYPTIC SPECIES COMPLEX 78

VI. DISCUSIÓN GENERAL Y CONCLUSIONES 111

6.1. ¿CUÁNTAS ESPECIES INCLUYE EL COMPLEJO? 111
6.1.1. AISLAMIENTO REPRODUCTIVO EN EL COMPLEJO *HETAERINA AMERICANA* 113
6.1.2. MECANISMOS DE ESPECIACIÓN EN EL COMPLEJO 114

LITERATURA CITADA 117

RESUMEN

La especiación, bajo el concepto biológico de especie, es el proceso por el cual las poblaciones divergen en grupos, los cuales dejan de intercambiar alelos por el establecimiento de aislamiento reproductivo. Este aislamiento está dado por barreras que difieren a lo largo del proceso reproductivo. En odonatos, se ha sugerido que las barreras pre-copulatorias son las que aportan en mayor medida al aislamiento reproductivo total. Se han registrado tanto barreras de aislamiento sexual (por ejemplo, las manchas alares), como barreras de aislamiento mecánico (por ejemplo, la forma de la genitalia primaria y secundaria). Además, se ha sugerido que el principal mecanismo que ha dirigido la evolución de este aislamiento es la selección sexual, principalmente por elección femenina.

Hetaerina americana (Calopterygidae) es un caballito del diablo de amplia distribución que presenta considerable variación en el tamaño de la mancha alar y en la morfología de los apéndices caudales en machos. Debido a esto, se han descrito varias sinonimias para la especie (por ejemplo, *H. pseudoamericana, H. texana, H. californica, H. basalis*). A pesar de que la información sobre los aspectos conductuales de *H. americana* es extensa (por ejemplo, territorialidad y sistema de apareamiento), hasta la fecha estudios en otros campos como la genética, son limitados. Un estudio genético previo incluyó poblaciones a lo largo de la distribución de esta especie y mostró alta diferenciación genética (con base a microsatélites) la cual estuvo asociada a morfotipos con apéndices caudales específicos, en cambio, los datos mitocondriales (un fragmento del gen COI) mostraron resultados discordantes a los microsatélites, así como a la variación morfológica. Estos resultados sugirieron que *H. americana* en realidad representa un complejo de al menos dos especies. Sin embargo, la limitación por el uso de pocos marcadores dificultó el establecer claramente el número de especies, así como determinar cuáles son los mecanismos relacionados con la historia evolutiva del complejo.

En el presente estudio se investigó de manera detallada la variación ecológica, fenotípica y genética del complejo *H. americana* para esclarecer su historia evolutiva. Esperamos que procesos históricos de vicarianza, así como diferentes presiones selectivas, incluyendo la variación en los factores abióticos a lo largo de su distribución y una fuerte selección sexual, hayan contribuido, en diferentes grados, al proceso de especiación en este complejo. El objetivo principal fue determinar

el número de especies dentro del complejo *H. americana* y determinar cuáles mecanismos están relacionados con el proceso de especiación. Para esto, la tesis se dividió en cuatro capítulos.

En el capítulo uno se investigó la variación de marcadores mitocondriales y nucleares, así como de caracteres morfológicos de más de 30 poblaciones de *H. americana.* Los resultados muestran la presencia de cuatro haplogrupos mitocondriales y una fuerte estructura filogeográfica, mientras que los datos de microsatélites indicaron dos grupos genéticos principales altamente diferenciados (F_{ST} > 0.50). Los resultados de la región del ITS1-5.8S-ITS2, sugieren tres grupos genéticos principales, los cuales concuerdan con la sub-estructuración encontrada con los microsatélites. Dos grupos genéticos mostraron una alta congruencia con la variación morfológica de los apéndices caudales de los machos, encontrándose al menos dos morfotipos claramente distinguibles. Estos morfotipos difieren en la forma del lóbulo medio y en la extensión del margen superior. El tercer grupo genético presenta cierta variación en la forma de los apéndices, pero ésta se traslapa con la de uno de los otros grupos.

En el capítulo dos se describe una nueva especie del complejo a partir de la información obtenida en el capítulo uno. Además, se realizó una revisión extensiva de la bibliografía, así como de individuos depositados en diferentes colecciones entomológicas que nos permitió estimar la distribución de las especies y reconocer otros caracteres morfológicos de importancia taxonómica para el grupo. Con base en esto, describimos a *H. calverti.* Esta especie difiere de *H. americana* en la forma de los apéndices caudales en los machos y en la forma de los interesternitos en las hembras. También se encontró que *H. calverti* suele estar en simpatría con *H. americana.*

En el capítulo tres se analizó la existencia de variación genética, morfológica y conductual entre las especies del complejo en una localidad simpátrica. Se encontró que no existe flujo génico, así como altas tasas de interferencia conductual. Los caracteres morfológicos más divergentes fueron los apéndices caudales, la forma de las alas y el tamaño de los individuos. También se encontró que existe un patrón de desplazamiento de caracteres en torno al tamaño de los machos. Con base en esto, se sugiere que los apéndices caudales son la principal barrera pre-copulatoria y que el tamaño tiene un efecto importante en el proceso reproductivo.

En el cuarto capítulo se determinaron los patrones genómicos de diferenciación por medio del análisis de SNPs utilizando una submuestra de 90 individuos de las tres especies putativas. Los resultados mostraron una alta diferencia genómica entre las tres especies potenciales, donde más del 80% de los loci analizados presentan valores de G_{ST} arriba de 0.5. Además, se encontraron loci

que están bajo selección divergente, esto para *H. americana North* y *H. americana South* (ver Capítulo IV). También se encontraron patrones de adaptación local asociados a la variación en la temperatura. Se sugiere que estas tres entidades están aisladas reproductivamente, aunque no es claro cuáles son las barreras presentes entre las especies que presentan poca variación morfológica.

Como conclusiones, se sugiere que el complejo *H. americana* está formado por tres especies: *H. americana sensu stricto*, *H. calverti* y *H*. nov. sp. Estas especies empezaron a divergir hace aproximadamente 26 millones de años y presentan una alta divergencia genética (mayor al 4%). *Hetaerina calverti* y *H. americana*/*H*. nov. sp. están aisladas reproductivamente a través de barreras de aislamiento mecánicas asociadas a los apéndices caudales, mientras que *H. americana* y *H.* nov. sp. parecen estar aisladas por barreras ecológicas. A partir de esto, se sugiere que tanto mecanismos de selección natural como de selección sexual han participado en el proceso de especiación de este complejo.

ABSTRACT

Speciation, under the biological species concept, is the process by which populations diverge into groups, which stop exchanging alleles due to the establishment of reproductive isolation. This isolation is given by barriers that differ throughout the reproductive process. In odonates, it has been suggested that pre-copulatory barriers are the ones that contribute the most to total reproductive isolation. Both sexual isolation barriers (e. g., wing spots) and mechanical isolation barriers (e. g., the shape of primary and secondary genitalia) have been recorded. In addition, it has been suggested that the main mechanism that has driven the evolution of this isolation is sexual selection, mainly by female choice.

Hetaerina americana (Calopterygidae) is a widely distributed damselfly that exhibits considerable variation in wing-spot size and caudal appendage morphology in males. Because of this, several synonyms have been described for the species (e. g. *H. pseudoamericana, H. texana, H. californica, H. basalis*). Although the information on the behavioral aspects of *H. americana* is extensive (e. g., territoriality and mating system), to date studies in other fields such as genetics are limited. A previous genetic study included populations throughout the distribution of this species and showed high genetic differentiation (based on microsatellites) which was associated with morphotypes with specific caudal appendages, however, mitochondrial data (a fragment of the COI gene) showed results discordant to microsatellites, as well as to morphological variation. These results suggested that *H. americana* represents a complex of at least two species. However, the limitation due to the use of few markers made it difficult to clearly establish the number of species, as well as to determine the mechanisms related to the evolutionary history of the complex.

In the present study, the ecological, phenotypic, and genetic variation of the *H. americana* complex was investigated in detail to elucidate its evolutionary history. We expect that historical processes of vicariance, as well as different selective pressures, including variation in abiotic factors throughout its distribution and strong sexual selection, have contributed, to different degrees, to the speciation process in this complex. The main objective was to determine the number of species within the *H. americana* complex and to determine which mechanisms are related to the speciation process. For this, the thesis was divided into four chapters.

In chapter one, the variation of mitochondrial and nuclear markers, as well as morphological characters of more than 30 populations of *H. americana*, was investigated. The results showed the

presence of four mitochondrial haplogroups and a strong phylogeographic structure, while the microsatellite data indicated two highly differentiated main genetic groups ($F_{ST} > 0.50$). The results of the ITS1-5.8S-ITS2 region suggest three main genetic groups, which agree with the sub-structuring found with the microsatellites. Two genetic groups showed a high congruence with the morphological variation of the caudal appendages of the males, finding at least two distinguishable morphotypes. These morphotypes differ in the shape of the median lobe and the extent of the upper margin. The third genetic group shows some variation in the shape of the appendages but this overlaps with that of the other groups.

In chapter two a new species of the complex is described from the information obtained in chapter one. In addition, we made an extensive review of the bibliography, as well as of individuals deposited in different entomological collections, which allowed us to estimate the distribution of the species and recognize other morphological characters of taxonomic importance for the group. Based on this, we describe *H. calverti*. This species differs from *H. americana* in the shape of the caudal appendages in the males and the shape of the intersternites in the females. It was also found that *H. calverti* is often in sympatry with *H. americana*.

In chapter three, the existence of genetic, morphological and behavioral variation among the species of the complex in a sympatric locality was analyzed. It was found that there is no gene flow, as well as high rates of behavioral interference. The most divergent morphological characteristics were the caudal appendages, the shape of the wings and the size of the individuals. It was also found that there is a pattern of character displacement in the size of males. Based on this, it is suggested that the caudal appendages are the main pre-copulatory barrier and that size has an important effect on the reproductive process.

In the fourth chapter, the genomic patterns of differentiation were determined by the analysis of SNPs using a subsample of 90 individuals of the three putative species. The results showed a high genomic difference between the three potential species, where more than 80% of the loci analyzed present G_{ST} values above 0.5. In addition, loci that are under divergent selection were found *for H. americana North* and *H. americana South* (see Chapter IV). Local adaptation patterns associated with temperature variation were also found. It is suggested that these three entities are reproductively isolated, although it is not clear what barriers exist between the species that present little morphological variation.

In conclusions, it is suggested that the *H. americana* complex is made up of three species: *H. americana sensu stricto, H. calverti and H.* nov. sp. These species began to diverge approximately 26 million years ago and present a high genetic divergence (greater than 4%). *Hetaerina calverti* and *H. americana/H.* nov. sp. are reproductively isolated through mechanical isolation barriers associated with the caudal appendages, while *H. americana* and *H*. nov. sp. appear to be isolated by ecological barriers. From this, we can suggest that both natural selection and sexual selection mechanisms have participated in the speciation process of this complex.

I. INTRODUCCIÓN GENERAL

1.1. Especiación

La especiación, bajo el concepto biológico de especie, es el proceso por el cual las poblaciones divergen en grupos, los cuales dejan de intercambiar alelos por el establecimiento de aislamiento reproductivo (Fig. 1A) (Coyne y Orr, 2004; Matute y Cooper, 2021; Turelli et al., 2001). En un modelo simple, el aislamiento reproductivo (AR) se puede definir como la fracción complementaria al factor de flujo génico (m_e/m), donde m representa el número de migrantes por generación y m_e representa la tasa de migración efectiva; es decir, el número de migrantes que contribuyen genéticamente a la siguiente generación (Stankowski y Ravinet, 2021). Esta tasa de migración se vería afectada por las barreras reproductivas, por lo que, dado lo anterior AR= 1 – (m_e/m); sin embargo, definir el AR en la naturaleza, es mucho más complicado.

Las barreras reproductivas representan aquellas características biológicas que están genéticamente determinadas, por lo que el aislamiento geográfico no representa una barrera reproductiva ya que sólo afecta a m y no a m_e (Stankowski y Ravinet, 2021). Las barreras reproductivas reducen el flujo genético potencial y están clasificadas dependiendo a su aparición en el ciclo reproductivo: las barreras pre-copulatorias incluyen barreras ecológicas, sexuales/conductuales y mecánicas, mientras que las que ocurren después de que se dio la cópula (post-copulatorias) pero antes de que se forme el cigoto (pre-cigóticas), incluyen barreras como la incompatibilidad gamética. Finalmente, las barreras que ocurren después de que se formó el cigoto híbrido (post-cigóticas), incluyen esterilidad e inviabilidad (Tabla 1) (Coyne y Orr, 2004).

Tabla 1. Barreras de aislamiento reproductivo.

Barreras pre-copulatorias	**Barreras que impiden el flujo génico antes de que se transfieran los gametos a miembros de otra especie.**
Aislamiento ecológico	Barreras de aislamiento basadas principalmente en diferencias en la ecología de las especies, en este caso, las barreras suelen ser subproductos de la adaptación local.
Aislamiento por hábitat^{+}	Diferencias en el tipo de hábitat reducen posibles encuentros entre parejas potenciales. Este tipo de barreras pueden ser causadas por diferencias en las preferencias de las especies, por competencia o por adaptación diferencial.
Aislamiento temporal^{+}	Diferencias fenológicas reducen posibles encuentros entre parejas potenciales.
Aislamiento sexual*	Diferentes señales y preferencias de apareamiento reducen posibles cruces entre parejas potenciales.
Aislamiento mecánico/sensorial	Incompatibilidad entre las estructuras reproductivas (primarias y secundarias), reduce posibles apareamientos; puede ser estructural y/o sensorial.
Barreras post-copulatorias	Barreras que actúan después de la transferencia de gametos.
Pre-cigóticas	Barreras que actúan antes de la fertilización.

Aislamiento gamético	El mejor rendimiento de los gametos conspecíficos y/o la incompatibilidad entre los gametos evitan la formación del cigoto.
Post-cigóticas	Barreras que actúan después de la formación del cigoto. Pueden ser intrínsecas, es decir, independientes del ambiente o extrínsecas, las cuales pueden depender tanto de los factores bióticos como abióticos.
Inviabilidad de híbridos	Se producen híbridos, pero estos no llegan a la madurez sexual. Intrínseca.
Adecuación de híbridos	Menor adecuación de los híbridos. Extrínseca.
Esterilidad de híbridos	Los híbridos generan gametos inviables. Intrínseca.

*Algunos autores consideran este tipo de barreras como aislamiento conductual. +Estas barreras se suelen incluir dentro del aislamiento ecológico.

El grado de aislamiento reproductivo (i.e., el continuo de especiación; Fig. 1E) se ha descrito como una relación positiva entre el tiempo y la divergencia genética (Coyne y Orr, 2004). Sin embargo, esta relación puede variar según el tipo de barreras reproductivas presentes. Por ejemplo, se ha documentado que, en ciertas especies de cíclidos, el aislamiento está más correlacionado con las preferencias de apareamiento selectivo dependiente del nicho y la morfología de los peces que con la divergencia genética (Stelkens y Seehausen, 2009). Por lo tanto, el grado del aislamiento reproductivo asociado con una mayor divergencia genética no es universal. A pesar de esto, la mayoría de los casos reportados sugieren una relación positiva entre estas variables (Fig. 1D) (Matute y Cooper, 2021).

Que tan rápido evoluciona el aislamiento reproductivo y si las barreras pre- y post-cigóticas evolucionan a la misma tasa han sido algunas de las preguntas principales de la biología evolutiva. Se ha argumentado que el aislamiento reproductivo se acumula a una mayor tasa en simpatría que en alopatría; esta hipótesis es conocida como reforzamiento, según la cual las presiones selectivas pueden acelerar el proceso de especiación a través de la penalización de la hibridación mal-adaptativa (Coyne y Orr, 2004). Por otro lado, se ha documentado que las barreras pre-copulatorias se acumulan de manera más rápida en múltiples taxa. Sin embargo, en otros organismos, las barreras post-cigóticas evolucionan igual o incluso más rápido que las pre-cigóticas (Christianson et al., 2005; Mendelson, 2003). Dado lo anterior, más que del contexto geográfico, la tasa de evolución del aislamiento reproductivo parece estar asociada en mayor medida a los mecanismos de especiación (Fig. 1B y 1C).

1.1.1. Mecanismos de especiación

Las barreras reproductivas pueden evolucionar por varios mecanismos: selección natural divergente, deriva génica, pleiotropía, selección sexual, etc. (Panhuis et al., 2001). La importancia

relativa de estos mecanismos varía entre especies, así como temporal y geográficamente, y generalmente no actúan de manera independiente (Arnqvist, 1997).

La especiación ecológica es el proceso en el que las barreras al flujo génico evolucionan como resultado de la selección natural divergente sobre poblaciones que se encuentran en diferentes ambientes (Nosil, 2015). Frecuentemente, la divergencia en los caracteres bajo selección puede ocasionar la incompatibilidad reproductiva entre las poblaciones; por lo tanto, la especiación

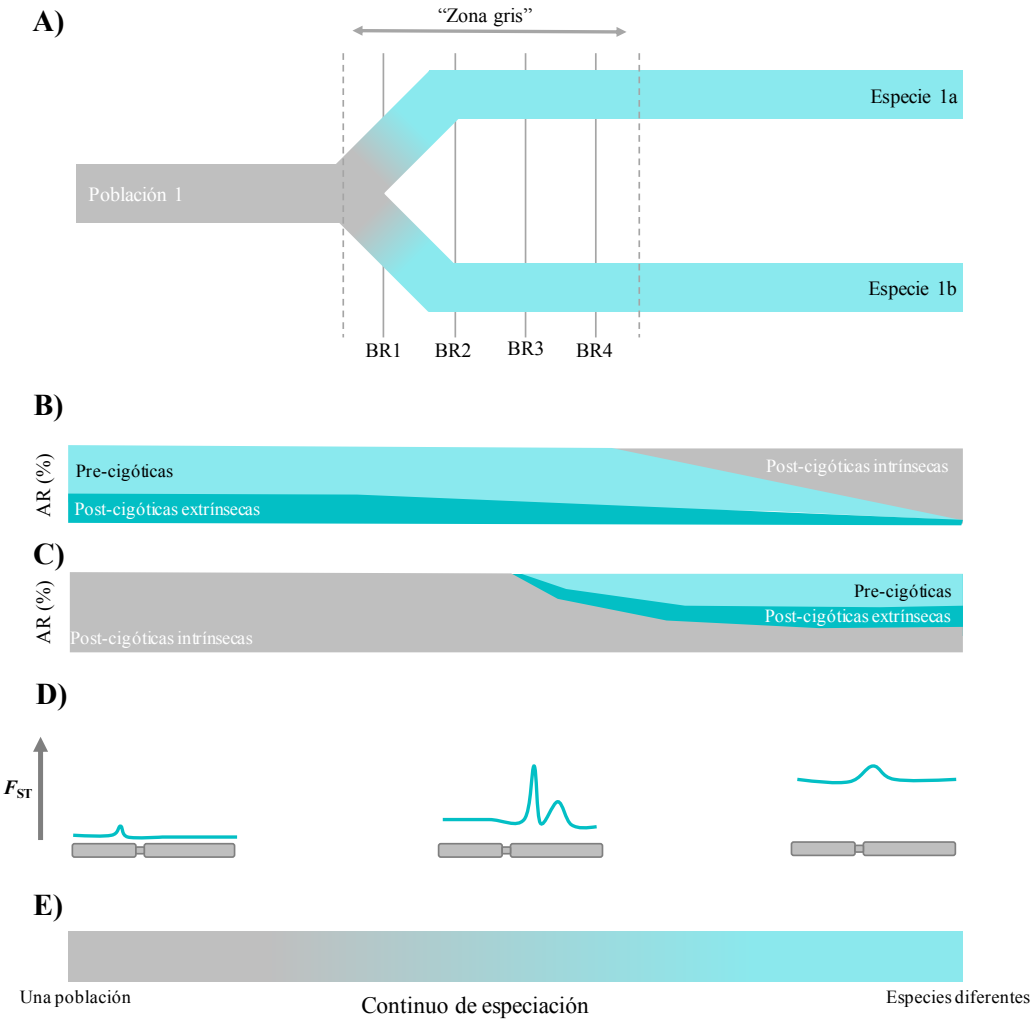

Figura 1. Descripción gráfica del proceso de especiación a lo largo del continuo de especiación. A) Divergencia de una población en dos especies a través de la evolución de barreras reproductivas (BR). **B)** Porcentaje de aislamiento reproductivo (AR) con el que contribuyen los diferentes tipos de barreras reproductivas; en este caso la evolución de éstas está dado por selección divergente. **C)** Porcentaje de aislamiento reproductivo con el que contribuyen las barreras reproductivas, en este caso la evolución está dada por procesos intrínsecos donde la selección no necesariamente está presente. **D)** Variación en el grado de diferenciación genética (F_{ST}) de diferentes loci en un cromosoma a lo largo del proceso de especiación. **E)** Continuo de especiación, que de acuerdo con Stankowski y Ravinet (2021), representa un continuo del grado de aislamiento reproductivo. Figuras modificadas de Feder et al., (2012) y Stankowski y Ravinet (2021).

ecológica puede ocurrir como un producto incidental de la adaptación diferencial, mientras que, en otros casos, la selección divergente puede actuar sobre el aislamiento reproductivo *per se*.

Los factores de la selección natural divergente durante el proceso de especiación ecológica son extrínsecos y pueden incluir factores abióticos y bióticos como el clima o las interacciones interespecíficas incluyendo competencia, enfermedades, etcétera (Coyne y Orr, 2004). Se espera que el aislamiento pre-cigótico evolucione en estas circunstancias a través de mecanismos de reforzamiento (Turelli et al., 2001).

Las barreras reproductivas también pueden evolucionar por selección sexual, ya sea por competencia intrasexual (competencia macho-macho o competencia espermática) o por interacciones intersexuales (elección femenina). Estas interacciones involucran caracteres sexuales primarios y secundarios, y la divergencia de estos caracteres usualmente afecta directamente al sistema de reconocimiento, por lo que la divergencia puede tener como resultado el aislamiento reproductivo. Se ha sugerido que este mecanismo puede tener un efecto rápido en las poblaciones y llevar a la divergencia incluso en ausencia de diferencias ambientales (Panhuis et al., 2001). También se ha sugerido que la variación de los caracteres sexuales secundarios puede ser un subproducto de la divergencia ecológica (Nosil, 2015). Sin embargo, estructuras como la genitalia y otros rasgos que actúan como barreras pre-cigóticas (por ejemplo, el aislamiento gamético por competencia espermática o elección críptica femenina) se relacionan a factores intrínsecos (Turelli et al., 2001). Se espera que las poblaciones bajo fuertes presiones de selección sexual tengan más probabilidad de evolucionar aislamiento reproductivo, ya que la selección sexual afecta directamente los patrones de apareamiento (Kirkpatrick y Ravigné, 2002).

Por último, existen mecanismos de especiación en los que la selección no tiene un papel directo en el establecimiento del aislamiento reproductivo. Por ejemplo, en la especiación por deriva génica, el aislamiento reproductivo es un subproducto de la divergencia genética, la cual ocurre por cambios aleatorios en las frecuencias alélicas y no por adaptación diferencial (Sánchez-Guillén et al., 2014a). La deriva génica es preponderante, sobre todo, en contextos geográficos alopátricos, y se ve acelerada cuando los tamaños poblacionales son reducidos. Si bien este mecanismo puede causar especiación por sí mismo, se ha sugerido que es un evento raro y difícil de analizar (Coyne y Orr, 2004). La especiación por poliploidía, en la que las barreras post-cigóticas evolucionan por incompatibilidades genéticas es otro mecanismo donde la selección no participa en la evolución del aislamiento reproductivo (Nosil, 2015).

1.1.2. Modelos de especiación; el papel del flujo génico

El proceso de especiación también se ve afectado por el componente geográfico. Cuando la divergencia ocurre con aislamiento geográfico estricto (i. e., poblaciones alopátricas), las poblaciones aisladas pueden empezar a divergir de manera independiente por efecto de la selección o por eventos neutrales, en este contexto el proceso se daría sin flujo génico. En cambio, cuando existe flujo génico, ya sea por un contacto secundario entre las poblaciones divergentes, por parapatría o simpatría, éste puede actuar como una fuerza que homogeniza las frecuencias alélicas entre las poblaciones (Coyne y Orr, 2004). Para que el proceso de especiación ocurra con la presencia de flujo génico, la selección tendría que actuar en direcciones contrastantes en las poblaciones para mantener la divergencia (Nosil et al., 2021).

Bajo el modelo alopátrico o sin flujo génico, el aislamiento reproductivo no es producto de la barrera geográfica *per se*, sino que ha evolucionado por una serie de mecanismos (o combinación de estos) como adaptación local, mutación o deriva génica (Gavrilets, 2003), los cuales se pueden ver acelerados con aislamiento geográfico presente.

En el caso de los modelos de simpatría y parapatría, es decir, con flujo génico, la presencia de selección divergente suele ser el mecanismo principal. Por otro lado, existen pocos ejemplos de estos tipos de especiación (Schliewen et al., 1994; Turelli et al., 2001). Sin embargo, se ha debatido que, dado el acomodo geográfico de las poblaciones, sobre todo de aquellas especies que presentan distribuciones amplias, el proceso de especiación se suele dar en parapatría y no alopatría (Bourret et al., 2012; Gavrilets, 2003; Mallet et al., 2009).

1.2. Especiación en Odonatos

El orden Odonata (libélulas y caballitos del diablo) es un grupo relativamente pequeño de insectos hemimetábolos con aproximadamente 6300 especies y está dividido en tres subórdenes: Anisozigoptera (tres especies), Anisoptera (~3100 especies) y Zigoptera (~3200) (Bybee et al., 2021; Paulson et al., 2022). Los odonatos pertenecen a uno de los primeros linajes de insectos voladores y empezaron a divergir en el Triásico (Thomas et al., 2013). Su ciclo de vida incluye una etapa acuática (como huevos y después larvas) y otra terrestre aérea (adultos), por lo que se encuentran íntimamente asociados a cuerpos de agua (Corbet, 1962). En ambas etapas son depredadores voraces; como adultos, usan la visión para capturar a sus presas, la cual se ha reconocido es extraordinaria (Bybee et al., 2012).

El proceso de especiación en odonatos se ha relacionado a su complejo sistema de apareamiento, así como a la rápida respuesta de estos organismos a los cambios climáticos (Damm et al., 2010a). Sin embargo, a la fecha son pocos los estudios que se han enfocado en discernir cuáles mecanismos y modelos de especiación son más comunes en este grupo. Wellenreuther y Sánchez-Guillén (2016) sugieren que el proceso de especiación en zigópteros es predominantemente bajo mecanismos no adaptativos (especiación no-ecológica), con base en información de sólo tres géneros del suborden (Wellenreuther y Sánchez-Guillén, 2016).

1.2.1. Especiación alopátrica y el papel de la deriva génica

Los estudios genéticos y filogeográficos en odonatos en los que se han aportado conclusiones acerca de los patrones evolutivos relacionados con la especiación se han centrado principalmente en especies con distribuciones paleárticas o neárticas, y básicamente sólo en zigópteros (por ejemplo, *Enallagma, Euphaea, Ischnura, Megalagrion, Pyrrhosoma*) (Guan et al., 2013; Huang y Lin, 2011; Jones y Jordan, 2015; Jordan et al., 2005; Lee y Lin, 2012; Sánchez-Guillén et al., 2014b; Turgeon et al., 2005; Turgeon y McPeek, 2002; Watson, 1977). En estos casos, se sugiere que las alteraciones ecológicas y geográficas relacionadas con los ciclos glaciales a lo largo del Neógeno y el Cuaternario provocaron la fragmentación de las áreas de distribución de poblaciones ancestrales, y que junto con diferentes procesos demográficos históricos (por ejemplo, cuellos de botella, efecto fundador) de las poblaciones ya aisladas, provocaron divergencia genética. Como consecuencia de esta divergencia, estructuras importantes en el sistema reproductivo y de reconocimiento empiezan a diferir. En varios de los casos se han descrito patrones de flujo génico interespecífico alto (barreras reproductivas permeables), por lo que se sugiere que procesos como la hibridación o la introgresión también han tenido un papel en el proceso de especiación (por ejemplo, *Mnais, Enallagma*), lo cual no se espera en un contexto alopátrico estricto (Coyne y Orr, 2004; Hayashi et al., 2005; Turgeon et al., 2005).

Dado este patrón, se sugiere que la deriva génica bajo un contexto alopátrico es el motor principal de la especiación en estos grupos. Sin embargo, en estos trabajos no se analizó empíricamente si la deriva génica llevó al proceso de divergencia de las estructuras reproductivas, sólo se hipotetizó que dados ciertos patrones genéticos (baja diversidad genética), el proceso de especiación estuvo probablemente dirigido por deriva génica. A la fecha se conocen pocos casos (ninguno en odonatos) en los que la deriva génica haya sido el único determinante en el proceso de especiación (Coyne y Orr, 2004; Network et al., 2012). En algunos de los casos se sugiere que la

disponibilidad de nichos nuevos también jugó un papel relevante en el establecimiento del aislamiento reproductivo (por ejemplo en *Megalagrion, Enallagma*).

Uno de los pocos casos en los que se han evaluado diferentes barreras reproductivas es el de las especies hermanas del género *Ischnura* (*I. elegans* e *I. graelsii*) (Sánchez-Guillén et al., 2012, 2014b), en el cual se encontró que el aislamiento mecánico/sensorial es la principal barrera y que existe una marcada asimetría en esta barrera. Dada esta asimetría, se propone que las especies evolucionaron por efecto fundador, según la hipótesis de Kaneshiro que sugiere que, en sistemas con elección de pareja por parte de las hembras, los elementos del cortejo masculino podrían perderse en las poblaciones fundadoras debido a la deriva genética junto con la selección sexual relajada. Sin embargo, Eberhard (1985) sugiere que tanto las estructuras reproductivas primarias, así como aquellas estructuras sexuales secundarias (por ejemplo, los apéndices caudales), han evolucionado por elección femenina, actuando como 'un cortejo interno' y que es poco probable que su variación sea resultado de pleiotropía, como se propone en los casos anteriores.

1.2.2. Especiación ecológica en odonatos

Se ha sugerido que existe poca variación ecológica entre las poblaciones de varias especies de odonatos (McPeek y Brown, 2000a; Svensson, 2012). Sin embargo, se han registrado procesos de especiación ecológica en varias especies, en asociación con tres factores ecológicos principales: depredadores, variación en la temperatura y tipo de sistema acuático (sistemas lóticos o lénticos) (Damm et al., 2010a; McPeek et al., 1996).

Uno de los ejemplos clásicos de especiación ecológica asociada a depredadores en odonatos se encuentra en el género *Enallagma* (Zygoptera: Coenagrionidae), en el que se ha descrito que las diferencias ecológicas relacionadas con el tipo de depredador clave presente en las pozas, ya sean peces o larvas de anisópteros, ha llevado a la evolución de diferentes estrategias de escape y como subproducto, aislamiento reproductivo (McPeek, 2000; McPeek y Brown, 2000b). Un caso similar ha sido descrito para *Leucorrhinia* (Anisoptera: Libellulidae), en el que la colonización de nuevos hábitats y la plasticidad de ciertos rasgos de defensa presentes en las larvas pudo haber impulsado la diversificación del género, ya que las larvas pudieron haberse establecido en hábitats con diferentes tipos de depredadores (peces vs invertebrados) (Mikolajewski et al., 2016, 2010; Petrin et al., 2010). En ambos casos, se pueden reconocer dos tipos de barreras pre-copulatorias: el hábitat y las mecánicas/sensoriales relacionadas con los apéndices caudales. Sin embargo, en este estudio no se evaluó el tiempo de aparición ni la intensidad de las barreras.

En el caso de especiación del género hawaiano *Megalagrion* (que como se mencionó antes, también concuerda con un modelo alopátrico) se ha sugerido divergencia por especiación ecológica (radiación adaptativa). Las especies de este género exhiben la capacidad de habitar una gran diversidad de nichos (cuerpos de agua en los que suelen reproducirse): pozas ácidas (*M. paludicola*), pozas y arroyos presentes en las partes bajas de las islas (*M. xanthomelas* y *M. pacificum*), escurrideros en paredes (*M. eudytum*), etc. Esta variación en la capacidad diferencial de habitar un tipo de poza/río específico ha llevado al aislamiento reproductivo. Se concluyó que, probablemente, la combinación de la edad de la isla y la diversidad de hábitats presentes fueron los responsables de la radiación observada en este género (Jordan et al., 2003).

1.2.3. Especiación y el papel de la selección sexual

Varios autores sugieren que el proceso de especiación en odonatos se ajusta al modelo de especiación no adaptativa (i.e., sin divergencia ecológica) y que el principal mecanismo que la dirige es la selección sexual (McPeek y Brown, 2000b; Misof, 2002; Svensson, 2012; Svensson y Waller, 2013; Wellenreuther y Sánchez-Guillén, 2016), donde generalmente, el aislamiento reproductivo precede a la diferenciación ecológica (McPeek et al., 2011; Svensson, 2012). Lo anterior se ha descrito para varios géneros de zigópteros (*Calopteryx* y *Enallagma*) (Svensson et al., 2006), mientras que para los anisópteros sólo se han realizado revisiones generales (Misof, 2002).

Uno de los ejemplos mejor analizados son los calopterígidos, específicamente las especies del género *Calopteryx*, en las cuales se han estudiado ampliamente los patrones ecológicos, genéticos y de selección sexual (Lorenzo-Carballa et al., 2014; Svensson et al., 2004; Wellenreuther y Vercken, 2010). Para estas especies, se ha sugerido que varias características como la territorialidad, la pigmentación y la forma alar intervienen en el proceso de especiación (Anderson y Grether, 2010; Outomuro et al., 2013). Específicamente, la variación de la coloración alar entre especies (la cual está asociada al reconocimiento de especies) representa unas de las principales barreras pre-copulatorias, la cual está asociada a tasas altas de especiación (Svensson y Waller, 2013). Incluso, en poblaciones parapátricas (e. g., *Calopteryx splendens*) se ha observado que la variación interpoblacional de la pigmentación puede generar aislamiento reproductivo parcial debido a selección sexual divergente (Svensson et al., 2006) a pesar de que el flujo genético suele ser alto (Svensson et al., 2004). Por lo tanto, se sugiere que la pigmentación alar está involucrada

directamente en el proceso de especiación en asociación con la selección sexual bajo el modelo de elección femenina (Svensson et al., 2006; Svensson y Waller, 2013).

Se ha demostrado que individuos del género *Calopteryx* que poseen ciertas características en las alas, están también bajo presiones selectivas ecológicas en relación tanto al color (correlacionado con la condición inmunológica) como a la forma (relacionado a vuelos óptimos) (Outomuro et al., 2013; Siva-Jothy, 2000; Svensson et al., 2016, 2004; Svensson y Friberg, 2007). A pesar de esto, la similitud ecológica dentro de este grupo de especies y el marcado aislamiento reproductivo implican que éste último no es resultado de la divergencia de nicho, por lo que mecanismos como competencia macho-macho y la elección femenina han fungido como los principales conductores de especiación en este grupo (Svensson, 2012). En general, los resultados apoyan la noción de que la diversificación del género *Calopteryx* se ajusta mejor a un mecanismo de selección sexual, con un rol más modesto de la selección natural y la divergencia de nicho (Wellenreuther et al., 2012).

1.3. Especies crípticas en odonatos

Las especies crípticas son organismos que muestran poca o nula divergencia morfológica en comparación con especies cercanas (Struck y Cerca, 2019) (Fig. 2) y usualmente sólo se pueden identificar a través del uso de marcadores moleculares (Bickford et al., 2007; Hughes et al., 2018). Estos patrones morfológicos pueden evolucionar por diferentes procesos como: una divergencia temprana, convergencia, paralelismo o estasis morfológica (Struck et al., 2018). Además, se ha sugerido que la especiación críptica está dirigida por mecanismos involucrados en el desarrollo de señales no visuales para el reconocimiento intraespecífico, como pueden ser sonidos, feromonas y señales eléctricas, las cuales no implican cambios morfológicos externos (Bickford et al., 2007). Sin embargo, también se han descrito procesos de especiación críptica por mecanismos ecológicos o biogeográficos (McPeek y Brown, 2000a; Turgeon y McPeek, 2002), los cuales se ven acelerados en un contexto alopátrico.

Debido a su elaborado comportamiento de apareamiento, se ha sugerido que la especiación críptica en odonatos es poco común (Cordero-Rivera y Lorenzo-Carballa, 2010), aunque se han reportado algunos casos (Damm et al., 2010b; Dow et al., 2015; Feindt et al., 2014; Stoks et al., 2005; Yong et al., 2015). El primer caso de especiación críptica reportado para Odonata fue para el género *Enallagma* (Stoks et al., 2005). Este género de distribución holártica está conformado por 38 especies e incluye dos grupos de especies crípticas que pueden ser separadas por la forma de los apéndices caudales: el grupo *cyathigerum*-tipo, el cual incluye las especies neárticas *E.*

annexum y *E. vernale* y la especie paleártica *E. cyathigerum*. El otro grupo, *boreale*-tipo, incluye la especie neártica *E. boreale* y las especies paleárticas *E. circulatum* (Japón), *E. risi* (Asia central) y *E. deserti* (norte de África). A partir de estudios filogenéticos moleculares, se han reconocido dos clados bien soportados los cuales tiene un origen biogeográfico diferente (Paleártico y Neártico). Estos clados no son congruentes con los grupos asignados por la morfología de los apéndices caudales (Callahan y McPeek, 2016). La evolución de este patrón, es decir, morfotipos similares entre especies distantes filogenéticamente, puede ser resultado de evolución paralela en los caracteres reproductivos de los adultos (Stoks et al., 2005). Por otro lado, en algunos casos en los que especies con apéndices caudales similares presentan un traslape en su distribución, por ejemplo, entre *E. vernale* y *E. annexum*, la evolución del aislamiento reproductivo ha sido relacionada con la divergencia del hábitat, específicamente, con diferentes tipos de depredadores de los estadios inmaduros (McPeek, 2000). Esta diferencia ecológica ha resultado en la variación

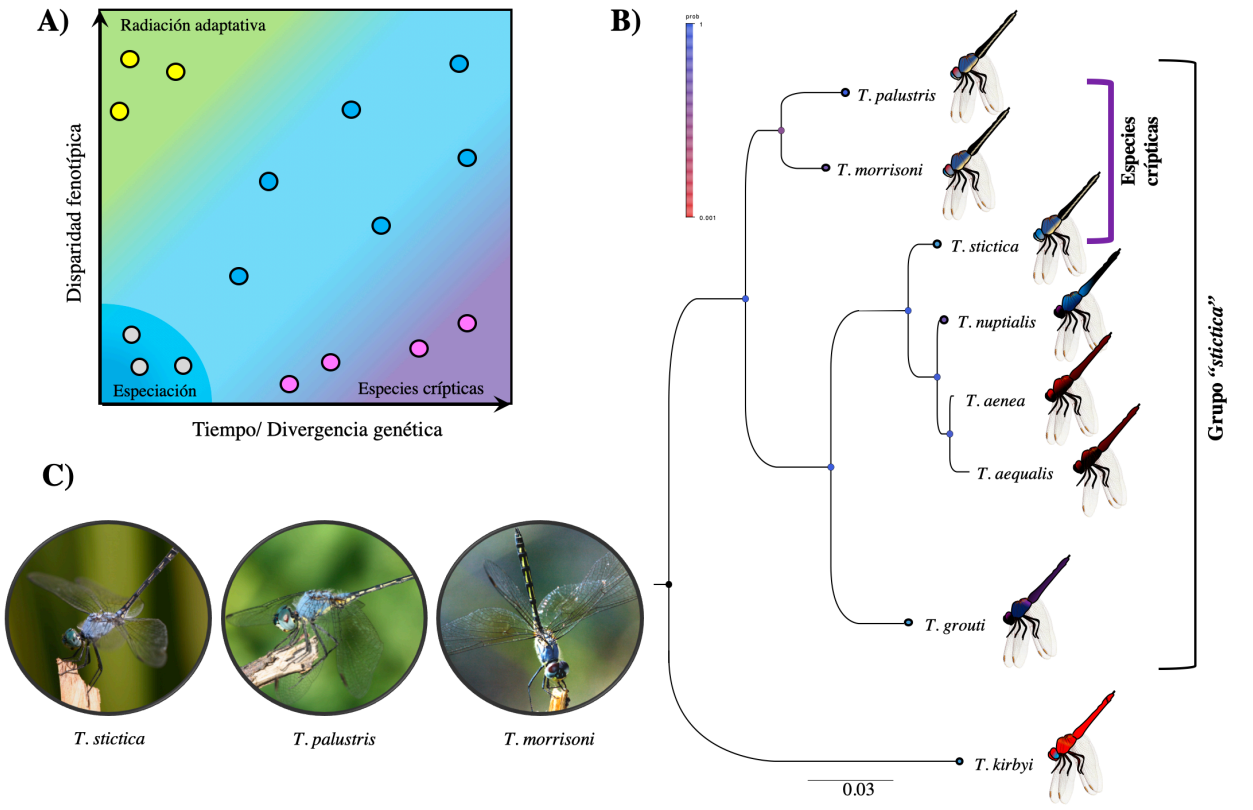

Figura 2. Especies crípticas. A) Definición de especies crípticas de acuerdo con Struck et al. (2018). **B)** Relaciones filogenéticas de algunas especies del género *Trithemis* (reanalizado de Damm et al. 2010); se observa la poca variación morfológica entre *T. palustris* y *T. morrisoni* en comparación con cualquier otro par de especies hermanas. **C)** Fotografías de las especies crípticas: *T. stictica* por Hans-Joachim Clausnitzer ©, *T. palustris* y *T. morrisoni* por Jens Kipping©.

de la conducta y en la morfología de las larvas, pero la estasis morfológica permanece en los estadios adultos.

Otro caso de especiación críptica se ha reportado para anisópteros del género *Trithemis* (Damm et al., 2010b, 2010a). Estos estudios se basaron tanto en análisis genéticos (usando dos genes mitocondriales) como en análisis de la variación morfológica (coloración, forma del edeago, etc.) de la especie africana común *Trithemis stictica* (Fig. 2). A través de análisis filogenéticos, se encontró que varios de los individuos de esta especie putativa no eran monofiléticos cuando se incluían otras especies del género en el análisis. Además, se encontró que no había variación clara en atributos morfológicos entre los individuos a pesar de ser distantes filogenéticamente. Basado en estos resultados, *T. stictica* fue dividida en tres especies: *T. stictica* y las especies hermanas *T. palustris* y *T. morrisoni*. El único carácter morfológico importante para distinguir *T. stictica* de las otras dos especies fue la coloración de los ojos y la forma del edeago, mientras que las diferencias entre *T. palustris* y *T. mossisoni* fueron crípticas (excepto por la pequeña diferencia en el tamaño de los individuos) (Damm y Hadrys, 2009). El tiempo de divergencia entre estas dos especies fue estimado en un millón de años, lo que sugiere estasis morfológica en comparación a otras especies de *Trithemis* (Damm et al., 2010a).

1.4. Género *Hetaerina*

Los odonatos del género *Hetaerina* Hagen in Selys, 1853 comprenden un grupo de 38 especies pertenecientes a la familia Calopterygidae, el cual presenta una distribución principalmente Neotropical, desde Canadá hasta Argentina (Fig. 3) (Garrison, 1990). Las especies de este género suelen estar asociadas a sistemas lóticos, así como a una gran variedad de ecosistemas (e. g. selva alta perennifolia, selva baja caducifolia, bosque mesófilo de montaña, bosque de coníferas, etcétera (Garrison, 19909). En cuanto a su taxonomía, la forma de los apéndices caudales es el principal carácter usado para una correcta identificación de los machos, mientras que en el caso de las hembras se han reconocido pocos caracteres para la identificación de las especies, entre ellos está la coloración del tórax y la forma de los interesternitos (Garrison, 1990) (Fig. 4C).

Los machos de *Hetaerina* se caracterizan por presentar una mancha roja en la base de las alas (Fig. 4A y B), así como un sistema de apareamiento tipo lek, es decir, los machos defienden territorios, donde se reúnen, exhiben y esperan el arribo de las hembras, mientras que éstas sólo llegan a estas áreas con el propósito de copular y no reciben otros recursos de los machos (al menos no previamente a la cópula) (Córdoba-Aguilar et al., 2009). Se ha sugerido que la mancha roja

presente en los machos ha evolucionado por competencia intrasexual, ya que las hembras no seleccionan a los machos con base a este carácter (Drury y Grether, 2014). De acuerdo con esto, la presencia de manchas más grandes aumenta la probabilidad de que un macho obtenga y mantenga por más tiempo el territorio, lo cual tiene un efecto positivo en su adecuación (Contreras-Garduño et al., 2008; Grether, 1997, 1996). Debido a lo anterior, se ha sugerido que la mancha es un indicador honesto de la calidad del macho.

Recientemente, se ha documentado que existen altas tasas de interferencia conductual entre algunas especies de *Hetaerina* (Drury et al., 2019, 2015; Grether et al., 2015; 2017), debido a que varias especies se suelen encontrar en simpatría y las hembras presentan coloraciones de las alas similares (Fig. 4A). Esto resulta en que los machos no puedan diferenciar entre las hembras, y por lo tanto la competencia por éstas se dé de manera interespecífica (Drury et al., 2019). La interferencia reproductiva tiene un efecto negativo en la adecuación de al menos una de las especies involucradas en las interacciones (Gröning y Hochkirch, 2008). Para *Hetaerina*, se han

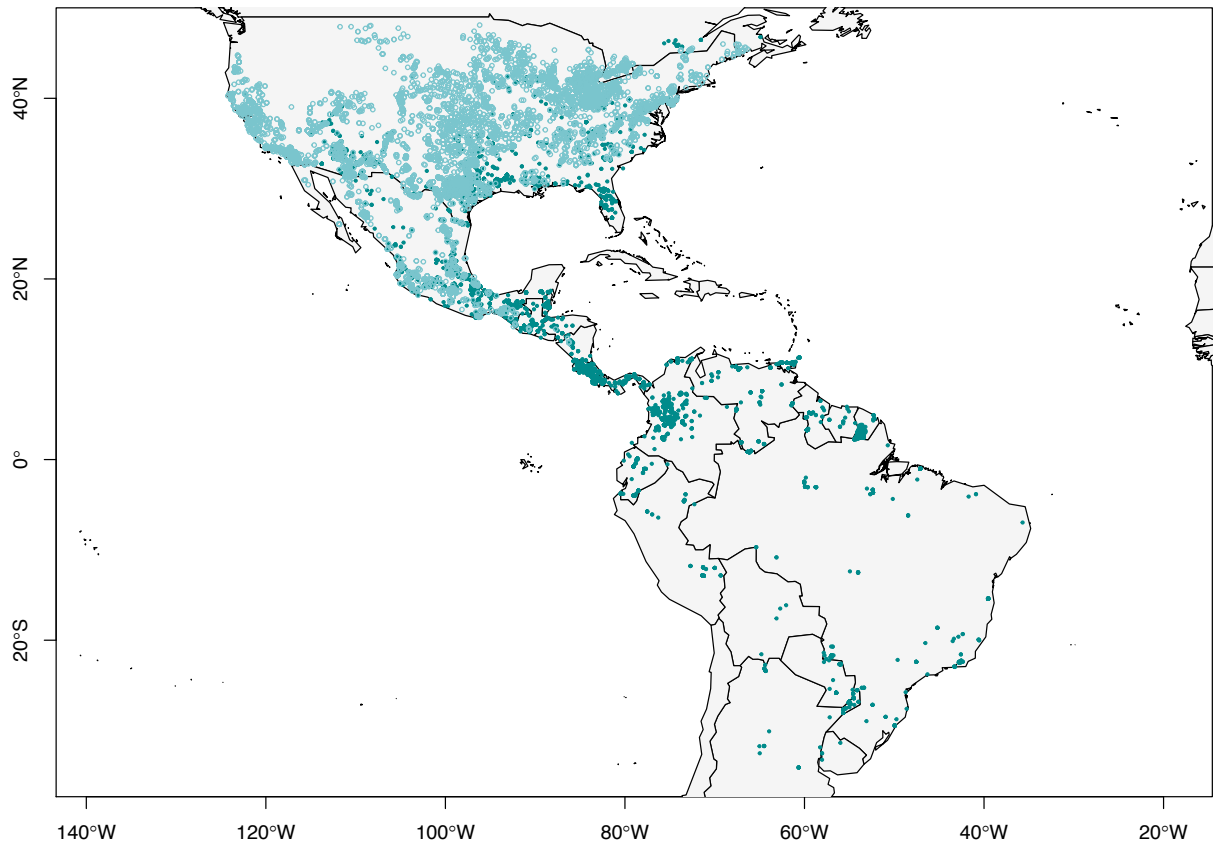

Figura 3. Distribución del género *Hetaerina*. Los círculos claros representan la distribución de *H. americana* y los puntos obscuros representan registros de otras especies *Hetaerina.* Datos disponibles en el Global Biodiversity Information Facility (GBIF).

documentado varias rutas para evitar o disminuir este tipo de interacciones negativas, como lo es la evolución de coloración contrastante entre los machos (i.e., *H. americana* y *H. titia*), a lo que Grether et al. (2014) llamaron desplazamiento de caracteres agonístico (Drury and Grether, 2014). También se ha documentado que especies que presentan coloraciones similares, por ejemplo, *H. americana* y *H. occisa* pueden optar por establecer territorios en microhábitas diferentes lo que evita la interacción entre los machos de estas especies (Anderson y Grether, 2011).

1.4.1. Hetaerina americana

Hetaerina americana es una de las especies más conocidas del género, y ha sido ampliamente utilizada como modelo en estudios conductuales (Contreras-Garduño et al., 2007; Córdoba-Aguilar et al., 2009; Grether, 1996; Raihani et al., 2008). Esta especie tiene un sistema de apareamiento tipo lek con machos territoriales y no territoriales, y además presenta una conducta adicional que sólo se ha descrito para esta especie del género, la cual consiste en machos que cambian entre conductas territoriales y no territoriales (switchers) (Raihani et al., 2008).

Hetaerina americana presenta una de las distribuciones más amplias en el género, abarcando desde Nicaragua hasta el sureste de Canadá (Fig. 3), por lo que aparentemente tiene una gran

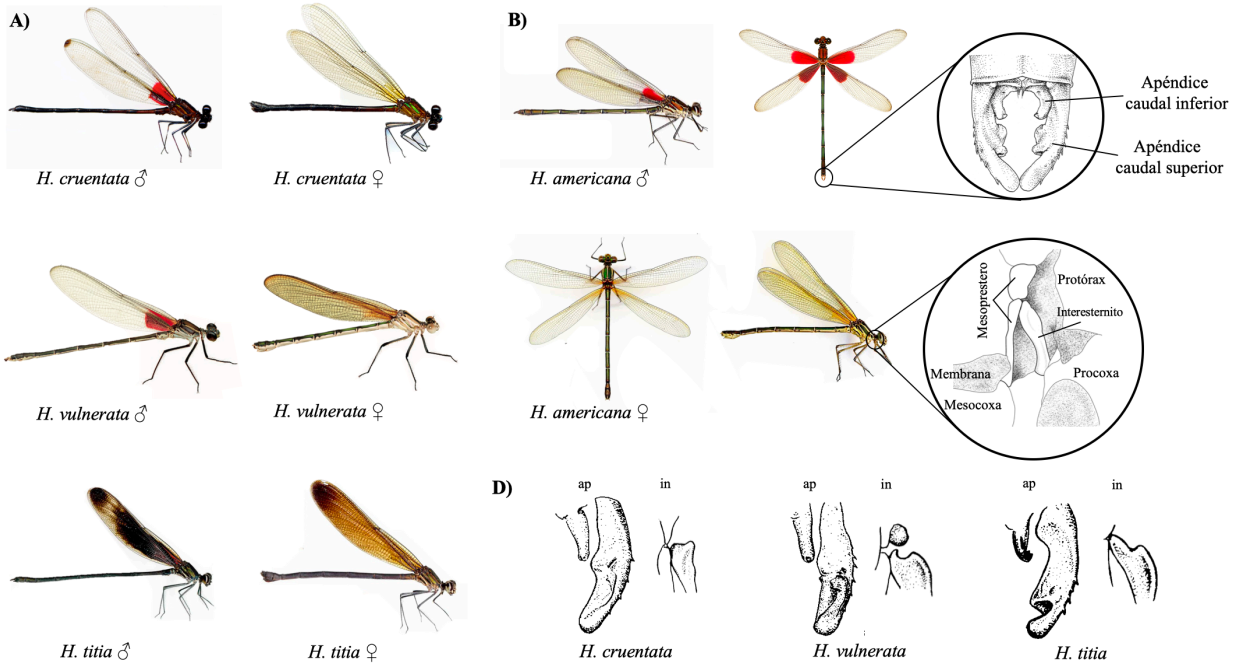

Figura 4. Variación morfológica del género *Hetaerina*. A) Variación de la coloración de tres especies del género, se resalta la variación en los machos y hembras de *H. titia*. **B)** Macho y hembra de *H. americana* en vista dorsal y lateral y ampliación de los apéndices caudales y el interesternito. **C)** Apéndices caudales (ap) e interesternitos (in) de varias especies del género (modificado de Garrison, 1990).

capacidad para colonizar una gran diversidad de hábitats que van desde los bosques de coníferas hasta las selvas tropicales (Garrison, 1990). *Hetaerina americana* es morfológicamente variable en la forma de los apéndices caudales y en el tamaño de la mancha alar, y debido a esto, se había separado en varias especies y razas (por ejemplo, *H. pseudoamericana, H. texana, H. californica*, etc.) (Garrison, 1990). Sin embargo, tras un muestreo más intensivo Calvert (1901) sugirió que *H. americana* representa sólo una especie altamente variable.

La información acerca de los aspectos conductuales de esta especie es extensa, sin embargo, los estudios en otros campos, como la genética, son limitados. En el estudio más reciente, Vega-Sánchez (2016) analizó genéticamente 220 individuos adultos de *H. americana* de 31 localidades situadas desde Guatemala hasta Canadá, usando un fragmento del gen mitocondrial citocromo oxidasa I y seis loci de microsatélites nucleares. En este mismo estudio se analizó la morfología de los apéndices caudales usando técnicas de morfometría geométrica. Los resultados mostraron la presencia de cuatro haplogrupos mitocondriales y una fuerte estructura filogeográfica, mientras que los datos de microsatélites indicaron dos grupos genéticos principales altamente diferenciados. Estos grupos genéticos mostraron una alta congruencia con la variación morfológica de los apéndices caudales de los machos, encontrándose al menos dos morfotipos claramente distinguibles, que difieren en la forma del lóbulo medio y en la extensión del margen superior. Además, dentro de cada grupo genético nuclear se encontraron varios subgrupos genéticos que presentaban forma de apéndices caudales variables, aunque menos diferenciables. Sin embargo, estos patrones de subestructura no fueron claramente definidos.

Dada la importancia de los apéndices caudales en el proceso de reconocimiento intraespecífico durante el proceso reproductivo en estas especies y la evidencia genética, la autora sugirió que *H. americana* representa un complejo de especies crípticas que están aisladas reproductivamente. Sin embargo, la variación del ADN mitocondrial solo mostró una congruencia parcial con la morfología y con los microsatélites nucleares, lo cual podría estar relacionado con un proceso de sorteo incompleto de linajes, que es común en especies que han divergido recientemente. Después de realizar una prueba de selección de los marcadores mitocondriales, se encontró que la región del código de barras del gen COI se encuentra bajo selección purificadora, lo cual concuerda con un patrón geográfico-ambiental de los diferentes haplogrupos encontrados, es decir, se sugiere que el genoma mitocondrial se comparte entre las especies debido a las presiones selectivas asociadas al clima en las diferentes áreas de distribución.

La limitación del estudio mencionado arriba con respecto al uso de pocos loci dificultó establecer los límites entre las especies en el complejo, así como saber cuáles son los mecanismos relacionados con su historia evolutiva. Se ha sugerido que muestreos que analicen de manera más exhaustiva la variación genética entre las especies, como los escaneos genómicos, pueden revelar patrones de diferenciación genética a un nivel más fino, incluyendo además el análisis de loci que están bajo selección (Chattopadhyay et al., 2016). Este tipo de escaneos pueden ayudar a establecer cuáles factores, ya sean ecológicos, geográficos o incluso fenotípicos, están relacionados al proceso de especiación.

En el presente estudio analizamos detalladamente la variación ecológica, fenotípica y genómica con el objetivo de esclarecer la historia evolutiva del complejo *H. americana,* así como determinar el número de especies dentro del complejo y definir qué mecanismos están relacionados con el proceso de especiación. Para esto, se describieron los patrones de diferenciación genética y morfológica en el complejo de *H. americana* a lo largo de su distribución geográfica (Capítulo I). Además, se describe una de las especies crípticas del complejo *H. americana* (Capítulo II), y se analizó en condiciones de simpatría la diferenciación fenotípica y los niveles de flujo génico entre las especies del complejo (Capítulo III). Finalmente, se determinaron los patrones de diferenciación genómica entre poblaciones y especies del complejo *H. americana* mediante secuenciación de nueva generación (Capítulo IV).

II. Capítulo I: Complex Evolutionary History Of The American Rubyspot Damselfly, *Hetaerina americana* (Odonata): Evidence Of Cryptic Speciation

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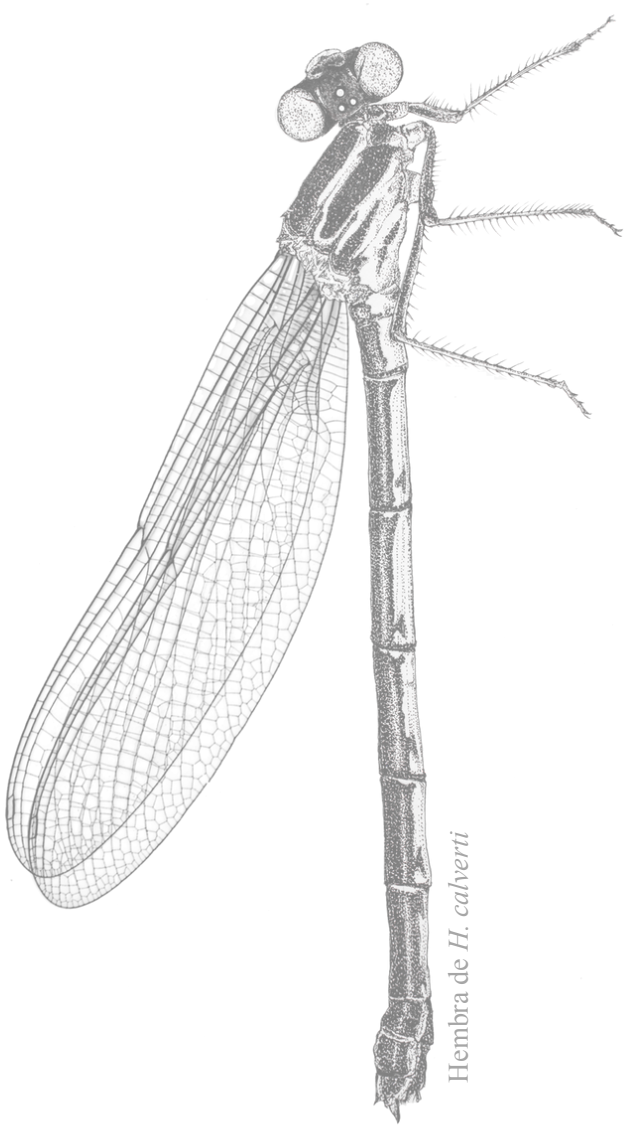

Hembra de *H. calverti*

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Complex evolutionary history of the American Rubyspot damselfly, *Hetaerina americana* (Odonata): Evidence of cryptic speciation

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ABSTRACT

Analyzing the magnitude and distribution of genetic variation within and among populations allows for hypothesis testing about historical demographic size changes, secondary contacts, refugia, and speciation patterns. Species distribution and genetic structure are greatly influenced by the complex life cycle and behavior of odonates. *Hetaerina americana* has been widely used as a model system in behavioral studies, but its population genetic structure has not been analyzed, except for a single study that included only three populations but identified the presence of markedly differentiated genetic groups, suggesting the existence of cryptic species. Here, we tested this hypothesis by assessing throughout the distribution range of *H. americana* the patterns of genetic and morphological variation in the male caudal appendages, due to the great importance of these structures in mate recognition. As molecular markers we used sequences of the mitochondrial cytochrome oxidase I (COI) gene and the nuclear internal transcribed spacer (ITS) region, as well as six nuclear microsatellites. We found very high population genetic differentiation ($\Phi_{ST} > 0.51$) in the three sets of markers but with strong mitonuclear discordance. A neutrality test suggested that the mitochondrial genome might be under purifying selection in association to climatic variables (temperature seasonality). The assignment of individuals to nuclear genetic groups showed little admixture and complete congruence with morphological differentiation in the male caudal appendages. Hence, the results suggest that *H. americana* represents at least two different cryptic species which are isolated reproductively.

1. Introduction

The current distribution of species is a result of several historical and contemporary factors, including dispersal, vicariance and gene flow (Avise, 2000). Analyzing how the distribution of evolutionary lineages changes over time allows us to test hypotheses about historical demographic size changes, secondary contacts, refugia and speciation patterns (Futuyma and Kirkpatrick, 2017). In odonates, understanding the factors that have shaped species distribution and genetic structure may be difficult because of their complex life cycle and behavior. For example, previous studies in this insect group have shown that some species are constituted by a single panmictic population at a global scale (Troast et al., 2016), while others show isolation by distance within few kilometers (Watts et al., 2004). These differences seem to be related to the dispersal capacity of the individuals of each species as well as to their territorial or migratory behavior. Also, the effects of particular physical barriers and the Quaternary glacial cycles have also been documented for some species (Callahan and McPeek, 2016; Jones and Jordan, 2015).

Genetic divergence can also be related to the evolution of reproductive isolation barriers. In odonates, the most common pre-zygotic barriers are sexual and mechanical or sensory isolation (Battin, 1993; Sánchez-Guillén et al., 2014a, 2014b; Svensson and Waller, 2013). Sexual isolation has been associated with the coloration of individuals, such as in *Calopteryx* (Svensson et al., 2016; Tynkkynen et al., 2008), *Mnais* (Hayashi et al., 2005) and *Argia* (Nava-Bolaños et al., 2016), in which differences in wing and body coloration result in assortative mating, leading to genetic differentiation even among populations within species (Svensson et al., 2004). In turn, mechanical or sensory isolation is mainly related to the morphological configuration of clasping structures in males and the prothorax or head in females. The clasping structures (i.e. the caudal appendages) are structures found at the end of the abdomen of the males and used to grasp females and form the tandem position during copulation (Corbet, 1962). It has

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been suggested that besides the mechanical compatibility, there must be tactile stimuli for the female to accept to copulate (Barnard et al., 2017). The shape of the caudal appendages is well known to be species-specific in several genera of zygoptera (Barnard et al., 2017; Sánchez-Guillén et al., 2014b), but the role of intraspecific variation of these structures on mating patterns, genetic differentiation and incipient speciation processes has been barely analyzed (Barnard et al., 2017).

Despite that existence of more than 5000 species of odonates, to date the analyses of genetic differentiation and phylogeographic patterns are limited to a few species, mostly Palearctic and Nearctic species (Ferreira et al., 2016; Kohli et al., 2018; Swaegers et al., 2014; but see Feindt et al., 2014; Fincke et al., 2018; Sánchez-Herrera et al., 2010). In this study, we focused on *Hetaerina americana* (Calopterygidae), a broadly distributed species with a range from southeastern Canada to Nicaragua. Species of *Hetaerina* (commonly known as rubyspots), are characterized by the presence of a red spot at the base of the wings of male individuals and a territorial behavior. Most rubyspots are found along the neotropical region, with the highest species richness being found in South America (Garrison, 1990). Species identification within this genus has been problematic since many of them are very similar in wing and body coloration, body size, and are usually sympatric. Therefore, the main traits that allows an unambiguous species recognition are the morphology of the male caudal appendages (Garrison, 1990).

Hetaerina americana has been reported to occur in a variety of habitats, from temperate to tropical forests, and frequently is the most abundant species when it is in sympatry with other rubyspot species. *Hetaerina americana* is morphologically variable in the shape of the caudal appendages of the males and other traits such as the presence or absence of the pterostigma (i.e. a specialized colored cell in the outer wings, related to gliding control during flight) and the relative size of the red spots in the wings. Due to this variability, several synonymous taxa have been described (for example, *H. pseudoamericana, H. texana, H. californica,* etc.) (Garrison, 1990).

Hetaerina americana has been widely used as a model system in behavioral studies due to its complex mating system (Contreras-Garduño et al., 2008; Grether, 1996; Raihani et al., 2008). Males are territorial and display a lekking behavior (Córdoba-Aguilar et al., 2009), in which groups of males defend territories in the riverbanks and perform flying displays while females visit these areas to mate and do not receive other resources from males (nuptial gifts, oviposition sites). However, the population genetic structure of this species has not been studied and therefore no information is available regarding gene flow patterns and population history throughout its range. The only previous study was performed by Vega-Sánchez (2013), in which three widely separated populations of the species were analyzed using nuclear DNA sequences of the internal transcribed spacer 1, the 5.8S ribosomal RNA gene and the transcribed spacer internal 2 (ITS1-5.8S-ITS2) region. The populations analyzed were from Colorado in USA, and from Veracruz and Oaxaca in Mexico. The results showed a very high genetic differentiation among the three populations ($\Phi_{ST} > 0.60$) and, remarkably, the presence of two similarly highly differentiated sets of individuals within the Veracruz population (Vega-Sánchez, 2013). Even though geographic isolation may partially explain these results, the considerable genetic differentiation among individuals within the Veracruz population indicates that other mechanisms of genetic isolation may be operating at a local level and suggesting that *H. americana* may be a complex of cryptic species. To test this hypothesis, in this study we performed a detailed phylogeographic and genetic structure analysis of *H. americana* throughout its distribution range using sequences of a fragment of the mitochondrial gene cytochrome oxidase I (COI) and the nuclear ITS1-5.8S-ITS2 region, as well as six nuclear microsatellites. Additionally, we employed geometric morphometric techniques to analyze and compare the shape variation of the male caudal appendages. The specific questions addressed were (i) what are the patterns of population genetic structure in *H. americana* throughout its distribution range? (ii) is the morphological variation of the male caudal appendages congruent with genetic differentiation patterns? (iii) does the evidence support the cryptic speciation hypothesis in *H. americana*?

2. Materials and methods

2.1. Sampling

Two hundred and twenty adult individuals of *H. americana* were directly collected or obtained through donations, representing a total of 31 localities (Supplementary Table 1), from Guatemala to the United States, covering most of its distribution range.

2.2. DNA extraction, amplification, sequencing and genotyping

Genomic DNA was isolated from thoracic muscle with the Pure Link Genomic DNA Mini Kit (Invitrogen®) following the protocol of the manufacturer. A fragment of the cytochrome oxidase I (COI; 658 pb) gene was amplified using the ODO_HCO2198d and ODO_LCO1490d primers (Dijkstra et al., 2014). We also amplified the nuclear region of the internal transcribed spacers (ITS1 and ITS2) and the 5.8S coding sequence (hereafter referred to as "ITS"; 600 pb) for a subsample of eleven populations, using the primers 18S rDNA (Weekers et al., 2001) and 28R1 (Dumont et al., 2010). Reactions were prepared on a final volume of 25 μL using 10 μL of *Taq* PCR Master Mix Kit 2x (Qiagen®), 1 μL of each primer (13 pmol/μL) and 1 μL of template DNA (10 ng/μL). The amplification protocol consisted of an initial denaturation step of three minutes at 94 °C, followed by 30–35 cycles of one minute at 94 °C, one minute at 50–58 °C and one minute at 72 °C, with a final extension at 72 °C for three minutes. The annealing temperature and the number of cycles depended on the DNA quality, see Supplementary Table 2 for details. PCR products were sent for sequencing to Macrogen Company (Rockville, MD, USA) using ODO_LCO1490d primer for the COI region and 18S primer for the nuclear region. Additionally, six nuclear microsatellite loci previously characterized for *H. americana* (Anderson and Grether, 2013) were amplified (H3, H8, H11, H15, H17 and H22) using a final volume of 10 μL with 5 μL of Platinum Multiplex PCR Master Mix (Applied Biosystems®), 1 μL of each primer (13 pmol/μL) and 1 μL of template DNA (10 ng/μL). We grouped the primers in multiplex reactions; the first group was formed by primer pairs H8, H11 and H22; the second group by H15 and H17, and the H3 locus was amplified alone. The amplification protocol consisted of an initial denaturation step of three minutes at 94 °C followed by 30–35 cycles of one minute at 94 °C, one minute at 54–58 °C and one minute at 72 °C, with a final extension at 72 °C for three minutes (see Supplementary Table 2 for details). The fragments were analyzed on an ABI 3300 Avant-PRIMS sequencer (Applied Biosystems®) and the size of the fragments was determined in Peak Scanner v. 2.0 (Applied Biosystems®) using as reference of size the marker Liz GeneScan 600 (Qiagen®).

2.3. Analyses of DNA sequences

Both sets of sequences, COI and ITS, were manually aligned using MEGAX (Kumar et al., 2018). For the COI alignment we added 11H. *americana* sequences obtained from the barcode of life data System (BOLD), which came from five localities in the United States and from three localities in Canada (Supplementary Table 1). For the ITS, sequences were first analyzed with the phase algorithm in DnaSP v. 5 (Librado and Rozas, 2009) to reconstruct haplotypes, and were tested for recombination using IMgc (Woerner et al., 2007). Haplotype (Hd) and nucleotide (π) diversity, the rarefacted haplotype richness (h) and Tajima's D and Fu's Fs were obtained for each population using DnaSP v. 5 (Librado and Rozas, 2009). For some of these analyses, populations with small sample sizes were grouped with others according to their

geographic proximity (see Supplementary Tables 2 and 3). Additionally, the overall average rate of synonymous (d_S) and non-synonymous (d_N) mutations in the COI sequences were estimated using the Nei and Gojobori's (1986) method with the Jukes and Cantor (1969) correction and both estimates were compared with a Z-test, to evaluate the null hypothesis of strict neutrality of this gene ($d_S = d_N$). The variance of the difference between the two estimates was computed using the bootstrap method with 1000 replicates. The MEGAX software was used for this analysis.

Population genetic differentiation was evaluated with analyses of molecular variance (AMOVA), using matrices of pairwise differences among haplotypes. Statistical significance of the differentiation estimates was determined with 10,000 permutations. These analyses were performed for individuals grouped according to their population of origin and grouped according to haplogroups (see Results section). The Arlequin v. 3.5 software (Excoffier and Lischer, 2010) was used for these analyses.

Haplotype networks were constructed for both COI and ITS datasets based on statistical parsimony in TCS v. 1.2.1 (Clement et al., 2000), using a connection limit of 90% probability. Additionally, phylogenetic relationships among haplotypes were analyzed using a Bayesian approach. Both datasets were analyzed in JModelTest v. 2.1.6 (Darriba et al., 2012) to determine the appropriate substitution model using the Bayesian Information Criterion (BIC). Then, phylogenetic relationships were estimated in MrBayes v. 3.2 (Ronquist et al., 2012) based on the GTR + G + I substitution model for COI and GTR + G for ITS. For each data set, two runs of 50 million generations were made, with trees sampled every 1000 generations. The convergence of the runs was evaluated through examination of ESS values in Tracer v. 1.7.0 (Rambaut et al., 2018). The initial 10% of reconstructions were discarded as burn-in to obtain a consensus tree. The tree was visualized in FigTree v. 1.4.3 (http://tree.bio.ed.ac.uk/software/figtree/). As outgroups, sequences of three other *Hetaerina* species (*H. laesa*, *H. sanguinea* and *H. titia*) were included in the COI phylogeny, of which the first two were obtained from GenBank and the third was generated for this study using the methods described above. In the case of the ITS phylogeny, we obtained sequences for *H. cruentata*, *H. medinai* and *H. titia* from GenBank (Supplementary Table 1).

For the ITS dataset, we estimated the divergence times among haplotypes with a Bayesian approach in BEAST v. 2.5 (Bouckaert et al., 2014) using the GTR + I + G model of molecular evolution, under an uncorrelated lognormal relaxed clock and the Yule speciation process. For tree calibration, we added to the alignment several species of Calopterygidae and two species of Amphipterygidae as outgroups (Supplementary Table 1). The crown node ages of these additional species were obtained from Dumont et al. (2005) and used as secondary calibrations: for *Hetaerina* we assigned a mean age of 60.1 million years ago (Mya) (SD = 1.2 Mya), for *Calopteryx* 21 Mya (SD = 3 Mya), for *Phaon* 31.9 Mya (SD = 3.4 Mya), for *Vestalis* 68.6 Mya (SD = 6.1 Mya), and for *Umma-Sapho* we used 53.1 Mya (SD = 2.1 Mya). Finally, we assigned a mean age of 151.5 Mya (SD = 5.8 Mya) for the divergence of Calopterygidae. A normal distribution was used for every calibration prior. We performed two independent runs with 200^6 of generations, sampling every 2000 generations. The convergence was evaluated in Tracer v. 1.7.0, and both runs were summarized using LogCombiner v. 1.8.4 and TreeAnnotator v. 1.8.4 (Drummond and Rambaut, 2007). All analyses were run on the CIPRES Science Gateway portal (Miller, 2010). A similar analysis was not conducted for the COI dataset because of the low resolution of the haplotype phylogeny.

2.4. Analyses of microsatellites

Microsatellites were analyzed in SPAGeDi v. 1.5 (Hardy and Vekemans, 2002) to estimate the mean number of alleles per locus (N_A), the mean effective number of alleles (N_{EA}), the rarefacted mean allelic richness ($AR_{K=2}$), and the average observed and expected heterozygosity (H_O and H_E, respectively). Deviations from Hardy-Weinberg equilibrium (HWE) were evaluated by calculating the inbreeding coefficient (F) and its significance in Arlequin v. 3.5.

A Bayesian clustering analysis was performed in STRUCTURE v. 2.3.4 (Pritchard et al., 2000), using the admixture model with correlated allele frequencies, without prior population information. The program was set to run with values of assumed genetic clusters (K) from 1 to 10 with a burn-in of 10,000 steps and chain length of 100,000. Ten independent runs were performed for each value of K. The most likely number of genetic clusters was determined using the method of Evanno (ΔK) (Evanno et al., 2005) in the STRUCTURE HARVESTER website (Earl and vonHoldt, 2012). Subsequent analyses in STRUCTURE were performed to assess substructure within the two main genetic clusters (see in Results) using similar program settings. Runs were summarized and plotted using CLUMPAK (Kopelman et al., 2015).

The magnitude of genetic differentiation among populations and among the genetic clusters identified in STRUCTURE were determined with analyses of molecular variance (AMOVA) in Arlequin v. 3.5 with 10,000 permutations.

2.5. Morphological data

To evaluate variation in the shape of the male caudal appendages we employed geometric morphometric techniques (Zelditch et al., 2004). We photographed the superior caudal appendages of 101 males under a stereoscopic microscope with a scale reference. On each image we superimposed a 'fan' on the right superior caudal appendage with the MakeFan v. 6 software (Zelditch et al., 2004) and then digitized six homologous points (i.e. landmarks *sensu* Bookstein, 1991) and 22 semilandmarks using the TpsDig v. 2 software (Rohlf, 2004). These landmarks and semilandmarks adequately describe the contour shape of the right superior caudal appendage in dorsal view (Supplementary Fig. 1). We performed a semilandmark superimposition using the Semiland module of Coordgen v.7 software (Zelditch et al., 2004) to minimize bending energy of the curves (Bookstein, 1991), and we also performed a superimposition Procrustes analysis using the CoordGen v. 7 software to evaluate appendage shape variation without the effect of size.

Principal component analyses (PCA) using Procrustes coordinates as shape variables were performed in PAST v. 3 (Hammer et al., 2001). We assigned the individuals to different nominal clusters based on the results of the genetic structure analysis for both nuclear and mitochondrial data. Shape variation in the caudal appendages between the different groups was visualized through a "thin-plate" analysis in Tgroup v. 7 software (Zelditch et al., 2004).

3. Results

3.1. Mitochondrial genetic diversity and neutrality tests

Two-hundred and sixteen sequences of the COI region were obtained and deposited at GenBank (see Supplementary Table 1 for accession numbers). In total, 61 haplotypes were observed with high haplotypic and nucleotide diversity (see Supplementary Table 3). The haplotype network based on statistical parsimony showed four major haplogroups (Fig. 1A). The first one (in green) included haplotype H9, which had the highest frequency (81 individuals), and other 26 haplotypes, which are singletons or have a low frequency and are separated from haplotype H9 by one to four mutational steps. This haplogroup was found in 20 populations from northern Mexico to Guatemala with different frequencies. This haplogroup is connected to a second haplogroup (in red; Fig. 1A) through ten mutational steps. In this second haplogroup there was also a central high frequency haplotype (H2, found in 40 individuals) and eighteen singletons. This haplogroup occurred in 19 populations with a distribution from Canada to northern Mexico. Another set of nine haplotypes (in blue in Fig. 1A), closely

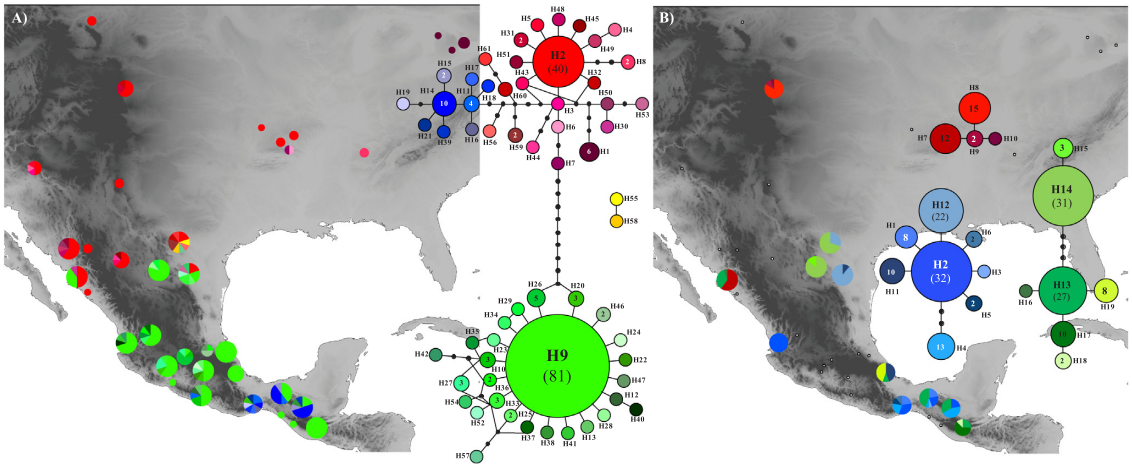

Fig. 1. Haplotype networks. (A) Geographic distribution and statistical parsimony network of 61 COI haplotypes of *H. americana*. (B) Geographic distribution and statistical parsimony network of 19 ITS haplotypes in a subsample of populations of *H. americana*. Pie charts represent frequencies of the haplotypes found in each sampling locality and the size of the chart is proportional to sample size. In the map, dark gray color represents higher altitudes. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

related to the red haplogroup, was found exclusively in four populations (20 individuals) of southern Mexico. Finally, two individuals from Zaragoza, Coahuila, harbored haplotypes (in yellow; Fig. 1A) that were a single mutational step from each other but disconnected from the rest of the network.

According to the AMOVA, genetic differentiation among populations was very high (Φ_{ST} = 0.65, p-value < 0.0001). Differentiation among haplogroups was higher (Φ_{CT} = 0.89, p-value < 0.0001) with also a considerable differentiation among populations within haplogroups (Φ_{SC} = 0.35, p-value < 0.0001) and very high overall differentiation (Φ_{ST} = 0.93, p-value < 0.0001) (Table 1).

The consensus phylogenetic tree based on haplotypes showed poor resolution, but the same four groups identified in the haplotype network can be distinguished (Fig. 2A). Nevertheless, the blue and red haplotypes did not form monophyletic clades, and the blue haplotypes appeared as basal. The yellow and green haplotypes formed a clade derived from the red group, with yellow haplotypes at the base (Fig. 2A).

In several populations the values of Tajima's D and Fu's Fs were negative and significant, suggesting a selective sweep or a recent population expansion. Furthermore, the d_N/d_S test for the whole dataset (Z-stat = −4.5; p-value < 0.0001) indicated deviation from neutrality, suggesting purifying selection acting on the COI gene. To further assess if this selection could be due to climatic differences among the geographic areas where the different haplogroups are distributed, we performed a principal components analysis on the values of the 19 bioclimatic variables corresponding to each population downloaded from the WorldClim database (Fick and Hijmans, 2017; http://www.worldclim.org/). The results are presented in Supplementary Fig. 2, with the populations corresponding to each of the haplogroups shown in their respective colors. The first component (PC1) explained 95.6% of the total variance and the second component (PC2) 3.6%, and mainly corresponded to temperature seasonality and annual precipitation, respectively. The climatic differentiation between the areas where the red and green + blue haplogroups occur is clear along the PC1 and was further supported by an ANOVA, performed with haplogroups as factors and population scores along the PC1 as dependent variable (F = 43.96, p-value < 0.0001). Meanwhile, the blue haplogroup was nested within the space of the green haplogroup.

3.2. Nuclear sequence variation and divergence times

For the ITS, we obtained 101 sequences (see Supplementary Table 1 for GenBank accession numbers), and after the phase and recombination analyses, a final alignment of 202 sequences with a length of 528 pb was used. In total, there were 19 haplotypes with high genetic diversity within populations and high positive and significant values of Tajima's D and Fu's Fs in some populations (Supplementary Table 4). The degree of population genetic differentiation according to the AMOVA was high (Φ_{ST} = 0.61; p-value < 0.0001), as well as differentiation among haplogroups (Φ_{CT} = 0.92, p-value < 0.0001) (Table 1). The haplotype network showed three disconnected haplogroups (Fig. 1B). The first haplogroup (in blue; Fig. 1B) was found distributed from southern (Chiapas) to northern Mexico (Coahuila) in seven populations with the most frequent haplotype in this haplogroup being H2 (17 individuals). The second haplogroup (in red; Fig. 1B) had only four haplotypes distributed in two populations in the north of Mexico (Sinaloa) and in the USA (Colorado). In this case, the most frequent haplotype was H8 (8 individuals). Finally, the third haplogroup (in green; Fig. 1B) had seven haplotypes present in seven populations from Guatemala to the northwest of Mexico, with two highly frequent haplotypes, H14 (16 individuals) and H13 (13 individuals). Most of the populations show a mixture of different haplogroups.

The consensus phylogenetic tree of ITS haplotypes showed three highly supported monophyletic clades (Fig. 2B) corresponding to the three haplogroups in the haplotype network. According to this tree, the red and the blue haplogroups are sister clades while the green haplogroup is basal to them. The BEAST dated tree (Fig. 3) suggests that the divergence between the blue + red and the green haplogroups was about 24.4 Mya (95% HPD 12.1–38.2 Mya; Fig. 3), and the divergence between the blue and the red clades was about 16.6 Mya (95% HPD 6.9–27.9 Mya).

3.3. Microsatellites genetic structure

Microsatellites showed low genetic diversity with a mean number of alleles per locus (N_A) from 1.0 to 3.0 across populations, and values of H_O and H_E between 0 and 0.37 and 0 and 0.55, respectively (Supplementary Table 5). Several populations showed deviations from HWE with high positive and significant *F* values, indicating a deficiency

Table 1
Analyses of molecular variance for COI and ITS sequences and for microsatellites. See text for details.

Loci	Group	Source of variation	d. f.	Sum of squares	Variance components	Percentage of variation	
COI	Global	Among populations	35	682.75	3.02	65.34	$\Phi_{ST} = 0.65^{**}$
		Within populations	180	288.38	1.6	34.66	
		Total	215	971.12	4.62		$\Phi_{ST} = 0.65^{**}$
	Haplogroups	Among groups	3	762.37	6.55	88.91	$\Phi_{CT} = 0.89^{**}$
		Among populations within groups	25	64.29	0.28	3.87	$\Phi_{SC} = 0.35^{**}$
		Within populations	176	93.6	0.53	7.22	
		Total	204	920.25	7.37		$\Phi_{ST} = 0.93^{**}$
ITS	Global	Among populations	10	1320.48	6.95	61.29	$\Phi_{ST} = 0.61^{**}$
		Within populations	191	838.98	4.39	38.71	
		Total	201	2159.47	11.35		$\Phi_{ST} = 0.61^{**}$
	Haplogroups	Among groups	3	1722.11	6.55	88.91	$\Phi_{CT} = 0.92^{**}$
		Among populations within groups	13	142.86	0.28	3.87	$\Phi_{SC} = 0.78^{**}$
		Within populations	186	46.78	0.53	7.22	
		Total	202	1911.75	7.36		$\Phi_{ST} = 0.98^{**}$
Microsatellites	Global	Among populations	23	181.61	0.43	51.16	$\Phi_{ST} = 0.51^{**}$
		Within populations	392	162.32	0.42	48.54	
		Total	415	343.94	0.85		$\Phi_{ST} = 0.51^{**}$
	Among genetic groups (K = 2)	Among groups	1	90.97	0.53	45.36	$\Phi_{CT} = 0.45^{**}$
		Among populations within groups	30	137.44	0.34	28.77	$\Phi_{SC} = 0.53^{**}$
		Within populations	384	115.52	0.3	26.86	
		Total	415	343.93	1.17		$\Phi_{ST} = 0.74^{**}$
	Among genetic groups (K = 2) for GG1	Among groups	1	74.9	0.5	50.4	$\Phi_{CT} = 0.50^{**}$
		Among populations within groups	24	24.56	0.05	5.19	$\Phi_{SC} = 0.10^{**}$
		Within populations	282	122.95	0.44	44.41	
		Total	307	222.41	0.99		$\Phi_{ST} = 0.56^{**}$
	Among genetic groups (K = 2) for GG2	Among groups	1	20.58	0.3	26.95	$\Phi_{CT} = 0.27^{*}$
		Among populations within groups	11	29.95	0.3	27.24	$\Phi_{SC} = 0.38^{**}$
		Within populations	95	47.88	0.5	45.63	
		Total	107	98.41	1.1		$\Phi_{ST} = 0.54^{**}$

d.f. = degrees of freedom. * = p-value $<$ 0.01; ** = p-value $<$ 0.0001.

of heterozygotes (Supplementary Table 5).

The STRUCTURE analysis supported the presence of two clearly delineated main genetic groups based on the ΔK method (K = 2; Fig. 4). The first genetic group (GG1) is mainly distributed from the United States to Chiapas in Southern Mexico. The second genetic group (GG2) was found in the population of Guatemala, two populations of Chiapas and the populations along the Gulf of Mexico coast and eastern Mexico plus a population in northwestern Mexico. It is important to mention that both genetic groups were found together in six populations (Fig. 4). In general, there was very little evidence of admixture between these two genetic groups. Individual assignments to one or the other genetic groups were higher than 90% in 95% of the cases and only 11 individuals showed evidence of mixed ancestry.

When we analyzed the substructure within each of these groups (Janes et al., 2017), we found two further subgroups within each of the two main groups (Supplementary Figs. 3 and 4). Within GG1 we found a genetic structure that strongly matched the latitudinal location of the populations (Supplementary Fig. 3). The first subgroup (GG1A) had a frequency higher than 90% in the United States and northern Mexico populations, and a lesser frequency (34 and 14%) in two central-western Mexico populations. The second subgroup (GG1B) was present mostly in central Mexico and southern populations. Only a few individuals (11) showed evidence of mixed ancestry between GG1A and GG1B, particularly in the population Chupícuaro in central-western Mexico. In the case of the second main genetic group (GG2) the analysis of substructure also revealed two subgroups (GG2A and GG2B) but with a less distinct geographical distribution and with higher levels of admixture (Supplementary Fig. 4).

The magnitude of genetic differentiation among populations was high ($\Phi_{ST} = 0.51$; p-value $<$ 0.0001) as well as the differentiation between the GG1 and GG2 main genetic groups ($\Phi_{CT} = 0.45$; p-value $<$ 0.0001) (Table 1). Differentiation between the GG1A and GG1B was similarly high ($\Phi_{ST} = 0.50$; p-value $<$ 0.0001), while differentiation between the GG2A and GG2B genetic groups was somewhat lower ($\Phi_{ST} = 0.27$; p-value $<$ 0.0001) (Table 1).

3.4. Morphological variation

The PCA based on the morphometric description of the shape of the superior caudal appendage revealed two clearly distinct morphological groups (Fig. 5). Remarkably, these two morphological groups perfectly matched the individual genetic assignment into the two main groups obtained in STRUCTURE, based on the nuclear microsatellites. It was also possible to observe a partial morphological differentiation between the GG1A and GG1B genetic subgroups (Fig. 5A). In contrast, there was no evidence of morphological differentiation between the GG2A and GG2B genetic subgroups. Also, there was no morphological differentiation corresponding to the COI haplogroups (Fig. 5B).

The deformation grids showed that the major variation between the two main morphological groups (GG1 and GG2) is in the median lobe and the distal part of the appendage. In the morphological group GG1, the median lobe is much flatter than the median lobe of group GG2, while the distal part of the appendage is shorter (Fig. 5B). Variation in shape between the genetic subgroups GG1A and GG1B was mainly in the median lobe (Fig. 5D).

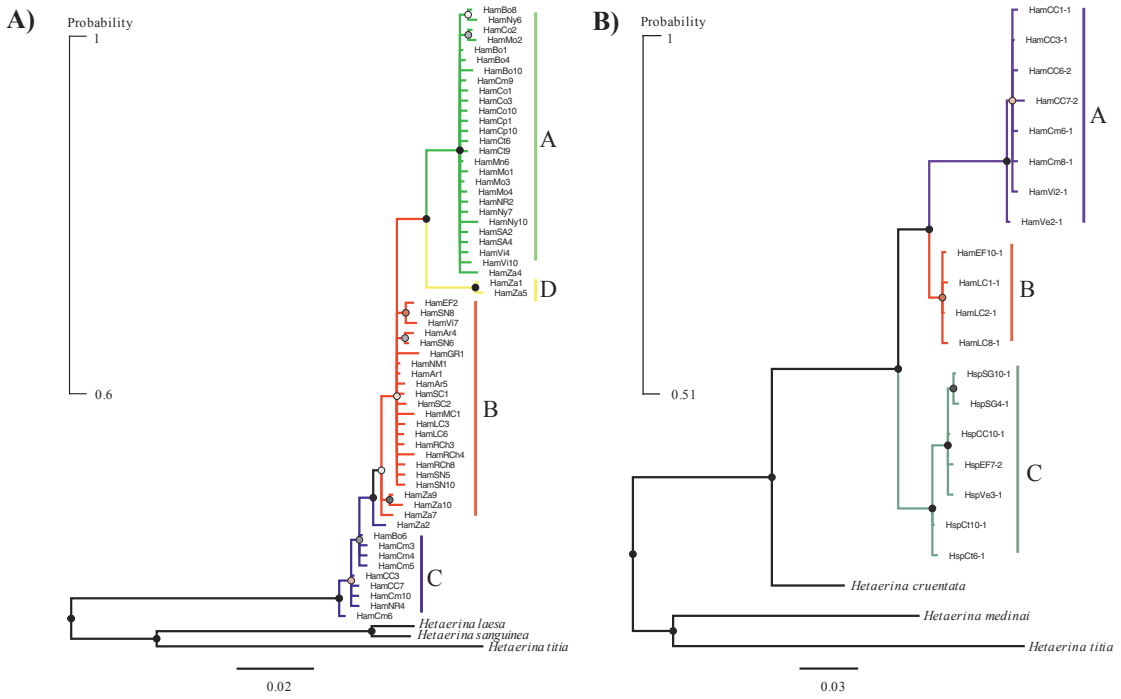

Fig. 2. Bayesian inference of haplotype relationships. (A) Gene tree of 61 haplotypes of the COI region. (B) Gene tree of 19 haplotypes of the ITS nuclear region. Bayesian posterior probability values are shown with colors in the node circles; the colors of the clades represent the different haplogroups found (see Fig. 1). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

4. Discussion

The results of this study show complex patterns of genetic and morphological variation within *H. americana*. Levels of genetic differentiation were very high for the three sets of molecular markers used (mitochondrial COI and nuclear ITS and microsatellites), but the COI variation was strongly discordant with the pattern shown by both nuclear markers. On the other hand, morphological variation in the male caudal appendages was completely congruent with nuclear genetic variation. Taken together, these results are consistent with recent views on speciation (Seehausen et al., 2014) and strongly suggest that *H. americana* is actually a complex of cryptic species at various stages of the speciation continuum

4.1. Mitonuclear discordance

There could be several explanations for incongruence between mitochondrial and nuclear genetic variation, including neutral demographic processes related to population history or differential dispersal and introgression patterns between the two sexes (since the mitochondria is maternally inherited). In our case, it also should be acknowledged that the number of nuclear sequences obtained was smaller than the number of COI sequences, therefore limiting a more detailed comparison between both datasets. However, the results of the d_N/d_S test strongly suggest that the COI region might be under purifying selection. This type of selection acting on mtDNA has been recurrently reported in many different animal species (Camus et al., 2017; Fontanillas et al., 2005; Quintela et al., 2014) and has been considered a feasible explanation for mitonuclear discordance (Morales et al., 2015). In most of these cases, the geographic distribution of haplotypes has been associated to temperature gradients (Fontanillas et al., 2005; Camus et al., 2017). Therefore, the influence of selection on the mitochondrial genome of the rubyspots studied here could also be the reason for the lack of congruence between the genealogical relationships between haplogroups and their geographic distribution, which is particularly clear in the case of the blue haplogroup, which is genealogically closer to the red haplotypes but overlaps in geographic distribution with the green haplotypes. This hypothesis is also supported by the significant climatic differences in temperature seasonality between the areas where the green + blue and the red haplotypes occur; but should be more carefully tested in further niche differentiation and ecophysiological studies.

4.2. Cryptic speciation in Hetaerina americana

In contrast to the mitochondrial DNA variation, the two sets of nuclear DNA markers (microsatellites and ITS sequences) were largely congruent with each other and with the morphological variation in the male caudal appendages. The main groups recognized (here called GG1 and GG2) were highly differentiated and showed little evidence of admixture even in sympatry. Remarkably, within each of these two main groups there was significant substructuring, detectable by both morphology and genetic variation, which was more pronounced within the GG1 than within the GG2. This evidence suggests that *H. americana* is a complex of biological entities at different degrees of divergence along the speciation continuum (i.e. from weakly reproductively isolated populations to irreversibly isolated species) (Seehausen et al., 2014), since we found three situations: (i) population groups with low genetic and morphological divergence (e. g. groups GG2A and GG2B), (ii) population groups with high genetic divergence and an incipient morphological divergence (GG1A and GG1B) and finally (iii) population groups with high genetic divergence and clear (i.e. non overlapping) morphological differentiation (GG1 and GG2).

The morphology of the caudal appendages is one of main sources of

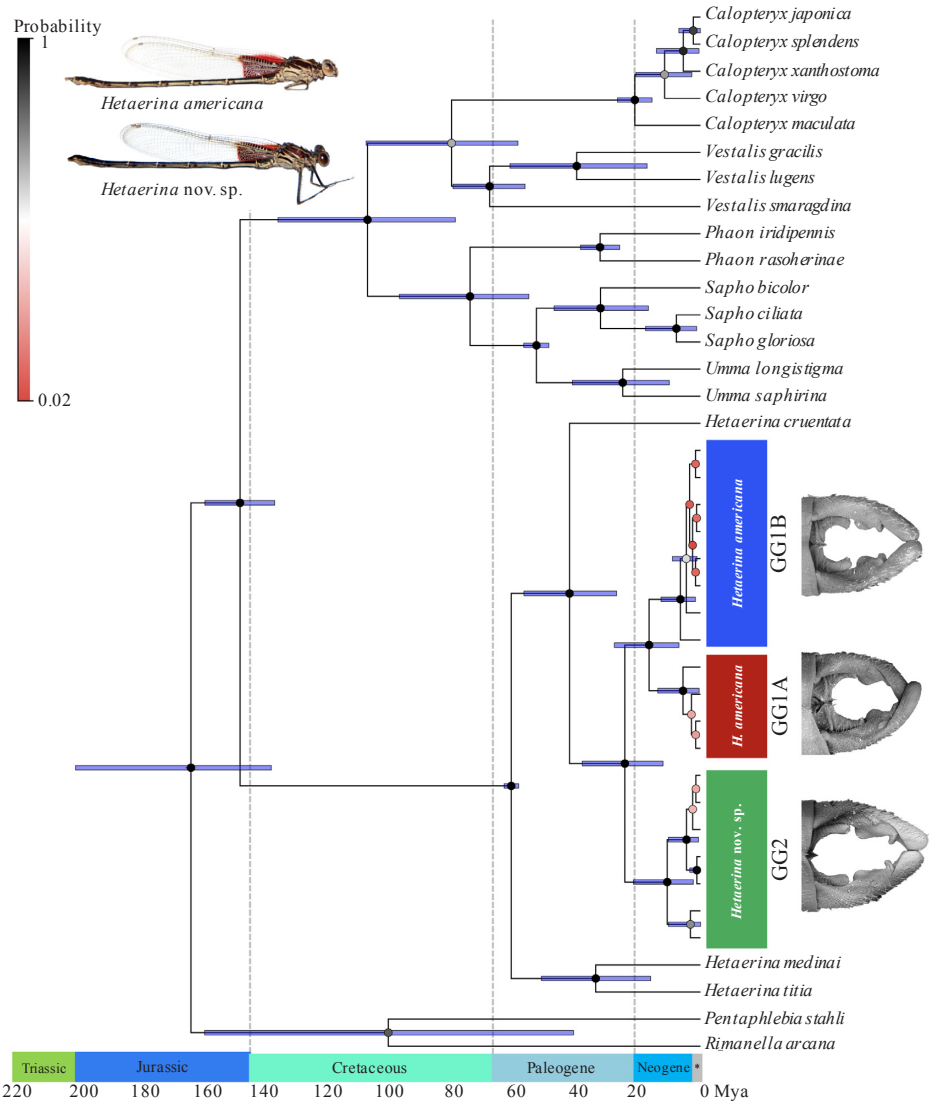

Fig. 3. Time calibrated tree based on ITS haplotypes. Bayesian posterior probabilities are shown with colors in the node circles. The node bars show the 95% Highest Posterior Density of node ages. The microsatellite genetic groups are signaled in their corresponding ITS clades. The morphological variation of caudal appendages is shown in electronic microscopic photographs (60 X) and the coloration pattern and morphology of male adults of *H. americana* and *H.* nov. sp. are illustrated. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

characters used by taxonomists for the recognition of species within some groups of Odonata, even in the case of phylogenetically closely related and ecologically similar lineages (such as in the genera *Ischnura* and *Enallagma*) (McPeek et al., 2011; Monetti et al., 2002). The divergence of these morphological structures is related to their important role in mate recognition during reproduction, functioning as a mechanical/sensory isolation mechanism (Barnard et al., 2017; Sánchez-Guillén et al., 2014). This role has been demonstrated through the experimental modification of these structures in *Enallagma* species, which results in the rejection of conspecific males by females (Robertson and Paterson, 1982). Thus, it has been suggested that mechanical/sensory isolation is, if not the most important, one of the main reproductive isolating barriers in odonates (Paulson, 1974).

Due to the variation found in the morphology of the caudal appendages in this study, and the congruence with the nuclear genetic groups, we can suggest that reproductive isolation exists even in localities where the groups occur in sympatry. This very low gene flow level is also evidenced by the minimal number of individuals that presented mixed ancestry (less than 10% of the sample). These results differ from those found in other odonates, for example, in the genus *Ischnura*, in which some of the taxonomically recognized species show low genetic differentiation related to high rates of hybridization (Sánchez-Guillén et al., 2011). This pattern is presumably observable when the divergence between species is recent and the species present incomplete reproductive barriers (Sánchez-Guillén et al., 2014, 2011). In contrast, in the case of the *H. americana* complex, the divergence among the genetic groups is probably old as observed in the dated phylogeny of the ITS haplotypes and suggested by the strong

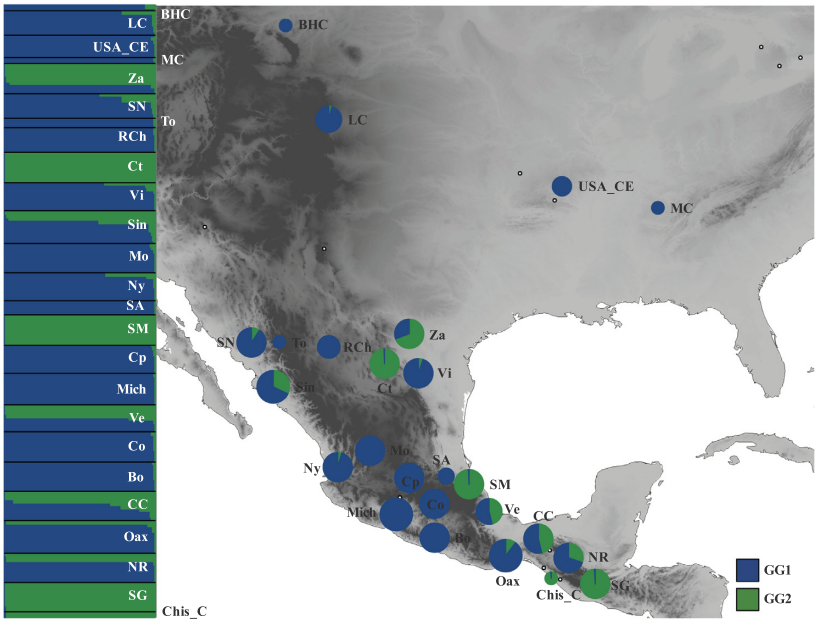

Fig. 4. STRUCTURE assignments for K = 2 in populations of *H. americana*. Individuals are represented by thin vertical lines, which are partitioned into K shaded segments representing each individual's estimated membership fraction; the black lines separate sampling sites. Pie charts represent the proportion and distribution of the two genetics groups in the populations. The size of pay charts represents the sample size analyzed. In the map, dark gray color represents higher altitudes. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

reproductive isolation. Even though divergence times should be taken with caution given the various possible sources of uncertainty, in general the results of our analysis are congruent with a recently published large time-calibrated phylogeny for all odonates (Waller and Svensson, 2017) in which and age of c.a. 60 my was also obtained for the divergence of the genus *Hetaerina*.

Overall, the results of this study allow us to propose that the *H. americana* complex includes, at least, two different cryptic species. The first one is *H. americana* Fabricius 1798, that shows the morphology described by Garrison (1990) and corresponds to the GG1, with a distribution from southern Mexico through almost the whole country to the United States (Colorado). The second species is *Hetaerina* nov. sp. that corresponds to the GG2 and presents a morphology of the superior caudal appendage that has not been described before (Fig. 3). This species is distributed from Guatemala to the north of Mexico but mostly along the Gulf of Mexico slope (but present in some populations in northwestern Mexico, what suggests that more extensive surveys are needed to clearly establish the geographic distribution of the two species). An interesting point is the stage of speciation between the GG1A and GG1B groups, which show morphological differentiation as well, but with a greater level of overlap between shapes, possibly related to a more recent divergence.

The detection of cryptic speciation is quite frequent (especially since the boom of molecular data) (Bickford et al., 2007). However, the evolutionary reasons for the retention of similar phenotypes among divergent lineages are not well understood (Fišer et al., 2018; Struck et al., 2018). Three main explanations have been suggested, which are recent divergence, morphological convergence and niche conservatism (Fišer et al., 2018). As previously mentioned, the first does not seem to be the case in the *H. americana* complex, given a probably old divergence and strong reproductive barriers. Second, the *H. americana* complex seems to be monophyletic according to the phylogenetic trees presented here and to a genus level phylogeny (unpublished data); thus, discarding the morphological convergence explanation. The last hypothesis, niche conservatism, seems more probable given the frequent sympatry between the GG1 and the GG2 and the lack of obvious differentiation in climatic niche across their distribution. The result of this conservatism could be a constrained evolution of external morphological features (as body size, shape and coloration), from processes such as stabilizing selection. In this way, by selecting against extreme phenotypic traits, stabilizing selection prevents morphological divergence, and distinct populations will remain similar over long periods of time (Fišer et al., 2018; Smith et al., 2011). In fact, it has been suggested that the process of diversification in zygopterans is mainly "non-adaptive", that is, occurs most often through non-ecological speciation (Rundell and Price, 2009; Czekanski-Moir and Rundell, 2019). This is because niche conservatism is common among closely related damselfly species and divergence is generally associated to traits involved in mate recognition system, such as coloration and the shape of the caudal appendages, which are traits whose evolution is usually driven by sexual selection (Wellenreuther and Sánchez-Guillén, 2016).

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ympev.2019.106536.

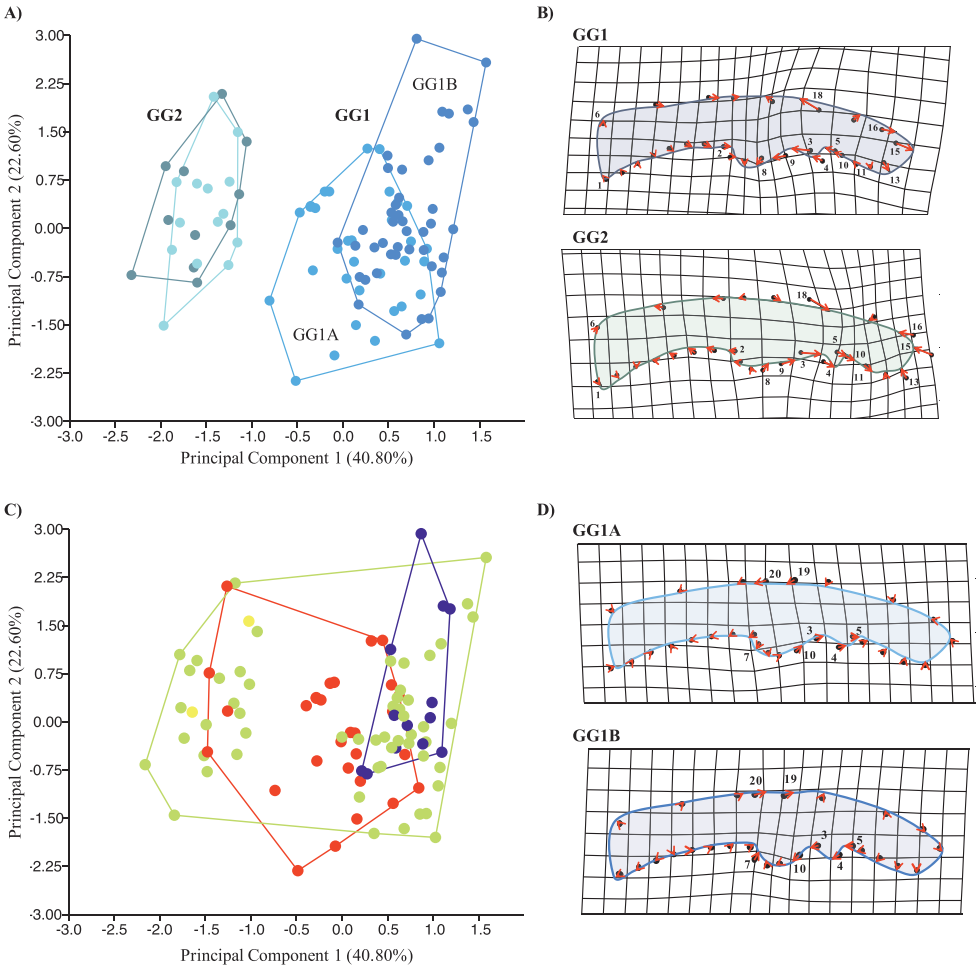

Fig. 5. Differences in shape of the superior caudal appendage. (A) Principal components analysis based on the coordinates that describe the shape of the superior caudal appendage; the color of the circles represents the nuclear genetic group based on microsatellites. (B) Superior caudal appendage shapes: deformation grids represent the mean shape for each morphological-nuclear genetic group; the size and direction of the arrows represent the deformation degree of each morphological group with respect to each other. (C) Principal component analysis; the color of the circles represents the mitochondrial (COI) haplogroups. (D) Superior caudal appendage shapes: deformation grids represent the mean shape for each morphological-nuclear genetic subgroup (GG1A and GG1B); the size and direction of the arrows represent the deformation degree of each morphological group with respect to the other. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

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Supplementary Table 1. Species, sampling localities and country, sample size for molecular analysis (N), longitude and latitude, and accession number.

Species	Locality	Country	N	Longitude	Latitude	Accession numbers
H. americana	Grand River, Ontario (GR)	Canada	1	-80.348093	43.382217	AAG0949*
	Saugeen River, Ontario (SR)	Canada	1	-81.353847	44.41793	AAG0949*
	Urban Park, Ontario (UP)	Canada	4	-79.183184	43.83428	AAG0949*
	Sacapulas, Quiché (SG)	Guatemala	10	-91.089086	15.290081	
	Apazapan, Veracruz (Ve)	Mexico	9	-96.721044	19.327447	ITS: MH090377-MH090477; COI: MH084732-MH084741, and MH090478-MH090669
	Arroyo Frío, Michoacán (AF)	Mexico	1	-101.45799	19.163265	
	Camichines, Nayarit (Ny)	Mexico	10	-104.688813	21.345635	
	Carretera Culiacán-Guamuchil, Sinaloa (Cu)	Mexico	1	-107.855344	25.048578	
	Chiapa de Corzo, Chiapas (CC)	Mexico	10	-93.013803	16.715415	
	Chupícuaro, Guanajuato (Cp)	Mexico	10	-100.687669	20.072121	
	Cocoyotla, Morelos (Co)	Mexico	10	-99.46094	18.75354	
	Comitancillo, Oaxaca (Cm)	Mexico	10	-95.167071	16.490491	
	Cuatro Ciénegas, Coahuila (Ct)	Mexico	10	-102.12426	26.92852	
	El Borbollón, Guerrero (Bo)	Mexico	10	-99.21634	17.412114	
	El Fuerte, Sinaloa (EF)	Mexico	10	-108.621821	26.424746	
	La Mintzita, Michoacán (Mn)	Mexico	10	-101.274796	19.645084	
	Momax, Zacatecas (Mo)	Mexico	10	-103.31071	21.94449	
	Nuevo Recuerdo, Chiapas (NR)	Mexico	10	-91.99499	15.90103	
	Puente Madera, Chiapas (PM)	Mexico	2	-93.33325	15.76587	
	Río Chonchos, Chihuahua (RCh)	Mexico	8	-105.187726	27.71242	
	Río Cuyamiapa, Chiapas (RC)	Mexico	1	-92.437407	15.117529	
	San Agustín, Hidalgo (SA)	Mexico	4	-98.6425	20.52911	
	San Marcos, Puebla (SM)	Mexico	10	-97.87192	20.39233	
	San Nicolás, Sonora (SN)	Mexico	10	-109.191303	28.429343	
	Tomochic, Chihuahua (To)	Mexico	2	-107.853641	28.353155	
	Villaldama, Nuevo León (Vi)	Mexico	10	-100.43224	26.49984	
	Zaragoza, Coahuila (Za)	Mexico	10	-100.91071	28.48658	
	Big Horn County, Montana (BHC)	USA	2	-107.45367	45.568608	
	Crawford County, Kansas (CrC)	USA	1	-94.65174	37.46924	
	Douglas County, Missouri (DC)	USA	2	-92.18117	36.8493	
	Lincoln County, New Mexico (NM)	USA	2	-105.439	33.526	
	Maury County, Tennessee (MC)	USA	2	-86.87155	35.571	
	Ozark County, Missouri (OC)	USA	2	-92.28699	36.73524	
	Searcy County, Arkansas (SC)	USA	2	-92.74495	35.98511	
	Larimer County, Colorado (LC)	USA	9	-105.019276	40.557465	AAG0949*
	Yavapai County, Arizona (Ar)	USA	6	-112.024376	34.527138	AAG0949*
H. cruentata			1			KM383820.1
H. laesa	Sipaliwini	Surinam	1			KF369394
H. medinai	Sierra de Lema	Venezuela	1			AJ459229.1
H. sanguinea	Reserva Tanshiyacu-Tahuayo	Peru	1	-73.402	-4.57	KF369395.1
H. titia	Puente Madera, Chiapas (PM)	Mexico	1	-93.33325	15.76587	MK577430
H. titia	Arkansas	USA	1			AJ458990.1
Phaon iridipennis	Mbalmago forest	Cameroon	1			AJ459225
Rimanella arcana	Sierra de Lema	Venezuela	1			AJ746323
* = Accession numbers (BIN) from BOLD.						

Supplementary Table 2. PCR conditions for the different markers.

Loci	Annealing temperature	Cycles
COI	50ºC-58ºC	35
ITS	58ºC	30
H3	54ºC	35
Group 1: H8, H11 and H22	57ºC	35
Group 2: H15 and H17	56ºC	30

Supplementary Table 3. Summary of genetic diversity and neutrality tests for each population based on mitochondrial (COI) data.

Population	N	h	Hd (SD)	π (SD)	Tajima's D	Fu's Fs
Apazapan, Veracruz	9	1	0 (0)	0 (0)	0	0
El Borbollón, Guerrero	10	1.76	0.76 (0.13)	0.006921 (0.004)	-1.81742**	1.07182
Camichines, Nayarit	10	1.53	0.53 (0.18)	0.002357 (0.002)	-1.83913**	-0.17467
Canada	6	1	0 (0)	0 (0)	0	0
Chiapa de Corzo, Chiapas	10	1.73	0.73 (0.10)	0.015638 (0.009)	2.14702	5.25295
Chihuahua	10	1.32	0.53 (0.18)	0.002020 (0.002)	-1.79631**	-0.49677
Chupícuaro, Guanajuato	8	1.64	0.64 (0.18)	0.002044 (0.002)	0.20364	-0.84351
Cocoyotla, Morelos	10	1.78	0.78 (0.14)	0.001684 (0.001)	-1.74110*	-3.8770**
Comitancillo, Oaxaca	9	1.97	0.97 (0.06)	0.008698 (0.005)	-1.78825**	-2.62984
Cuatro Ciénegas, Coahuila	10	1.38	0.38 (0.18)	0.000673 (0.001)	-1.40085	-1.16394*
Michoacán	11	1.6	0.60 (0.15)	0.001592 (0.001)	-0.26331	-0.85694
Momax, Zacatecas	10	1.67	0.67 (0.16)	0.001684 (0.001)	-1.74110**	-2.2598**
Sacapulas, Puente Madera and Río Cuyamiapa.	12	1	0 (0)	0 (0)	0	0
Nuevo Recuerdo, Chiapas	10	1.76	0.76 (0.13)	0.014366 (0.008)	0.9732	3.11897
San Agustín, Hidalgo	4	1	0.83 (0.22)	0.002806 (0.002)	0.16766	-0.13331
San Marcos, Puebla	10	1.83	0 (0)	0 (0)	0	0
San Nicolás, Sonora	10	1.67	0.67 (0.16)	0.002020 (0.002)	-1.79631**	-1.8027*
Sinaloa	11	1.62	0.62 (0.10)	0.013590 (0.008)	1.75048	7.53769
USA_CE	9	1.26	0.69 (0.15)	0.004022 (0.003)	-0.32331	0.7754
USA_CN	10	1.305	0.51 (0.16)	0.000935 (0.00)	-0.69098	-0.59381
USA_SO	8	1.4	0.64 (0.18)	0.001684 (0.001)	-1.53470*	-1.23563
Villaldama, Nuevo León	10	1.93	0.93 (0.06)	0.013618 (0.008)	0.67767	0.38293
Zaragoza, Coahuila	10	1.96	0.96 (0.06)	0.018257 (0.010)	-0.04985	-0.13057

N = Sample size; h = rarefacted haplotype richness; Hd = haplotypic diversity; π = nucleotide diversity. * = p-value < 0.05, ** = p-value < 0.02. Canada = includes populations: GR, SR and UP. Chihuahua = includes populations: To and RCh. Michoacán = includes populations: Mn and AF. Sinaloa = includes populations: EF and Cu. USA_CE = includes populations: SC, CrC, MC, DC and OC. USA_CN = includes populations: LC and BHC. USA_SO = includes populations: Ar and NM.

Supplementary Table 4. Summary of genetic diversity and neutrality tests for each population based on ITS data.

Population	N	h	Hd (SD)	π (SD)	Tajima's D	Fu's Fs
Apazapan, Veracruz	9	3	0.63 (0.06)	0.040 (0.021)	3.24***	18.58***
Camichines, Nayarit	9	1	0	0	0	0
Chiapa de Corzo, Chiapas	9	5	0.74 (0.07)	0.040 (0.021)	2.72**	12.44***
Comitancillo, Oaxaca	9	5	0.81 (0.05)	0.004 (0.002)	0.26	0.53
Cuatro Ciénegas, Coahuila	10	2	0.27 (0.11)	0.001 (0.001)	-0.09	0.38
El Fuerte, Sinaloa	10	3	0.54 (0.08)	0.025 (0.013)	2.83**	14.75***
Larimer County, Colorado	9	3	0.31 (0.13)	0.001 (0.001)	-0.74	-0.68
Nuevo Recuerdo, Chiapas	9	3	0.68 (0.06)	0.036 (0.019)	2.28*	17.35***
Sacapulas, Quiché	8	3	0.57 (0.11)	0.001 (0.001)	0.13	0.06
Villaldama, Nuevo León	9	2	0.21 (0.12)	0.001 (0.001)	-0.68	1.01
Zaragoza, Coahuila	10	2	0.44 (0.09)	0.032 (0.012)	2.25*	21.37***

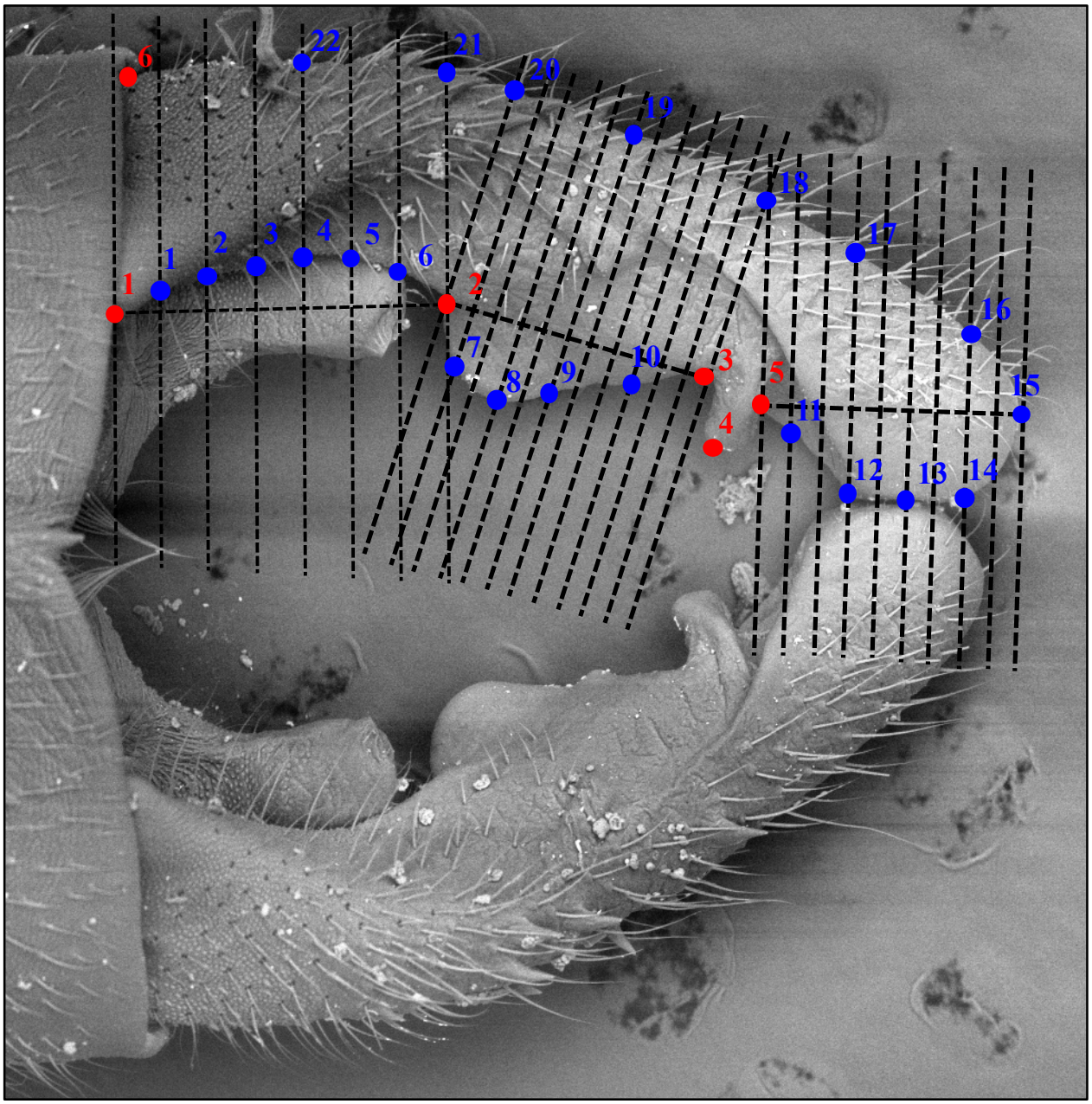
N= Sample size; h= haplotype richness; Hd= haplotypic diversity; π = nucleotide diversity; * = p-value < 0.05, ** = p-value < 0.01, *** = p-value < 0.001.

Supplementary table 5. Summary of genetic diversity and inbreeding coefficient for six microsatellite loci.

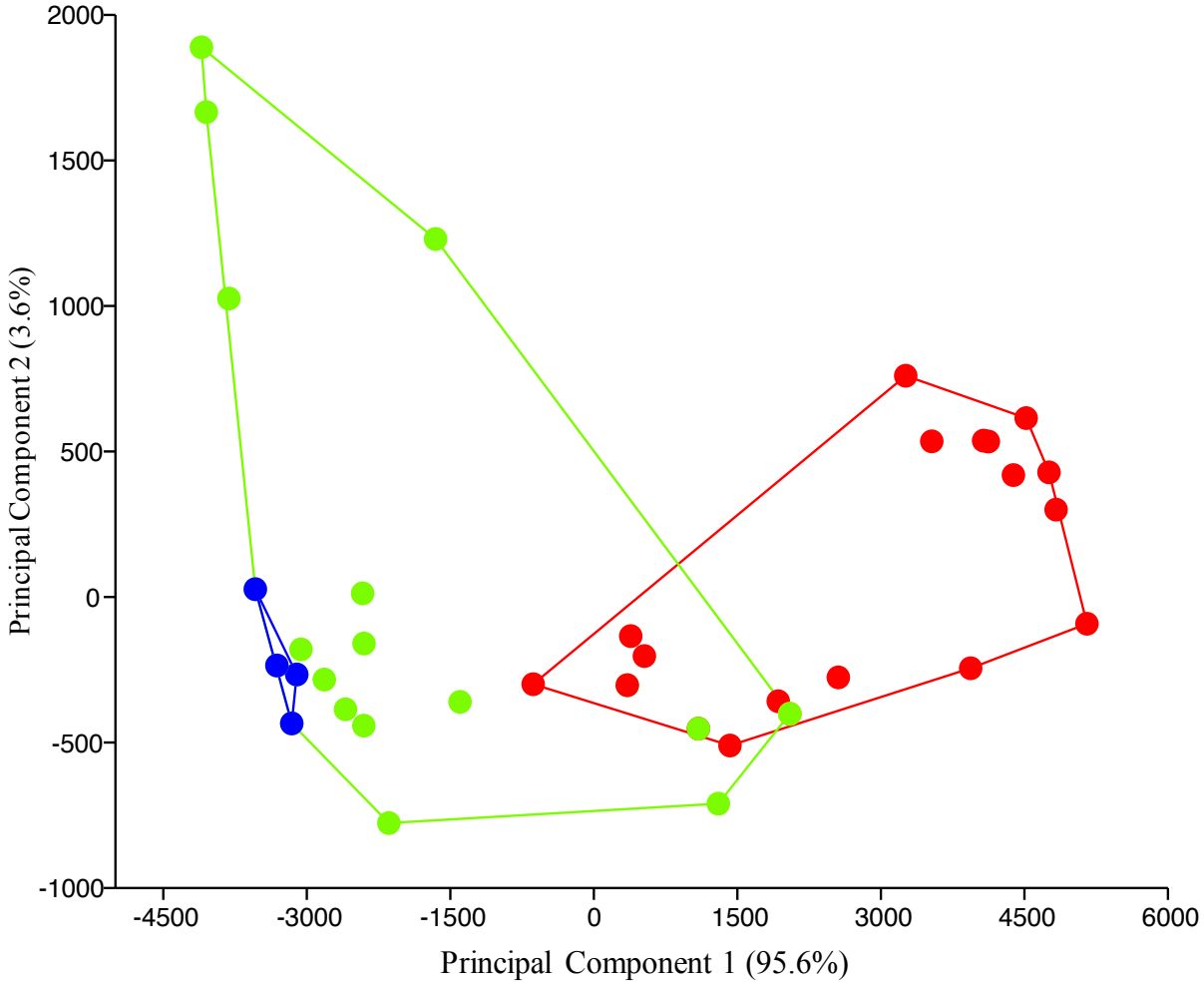
Locality	N	N_A (SD)	N_{EA} (SD)	AR $_{(K=2)}$	H_E (SD)	H_O (SD)	F
Apazapan, Veracruz	9	2.67 (0.49)	2.02 (0.33)	1.45	0.45 (0.11)	0.15 (0.09)	0.81**
El Borbollón, Guerrero	10	2.33 (0.49)	1.58 (0.26)	1.3	0.30 (0.11)	0.25 (0.09)	0.17
Camichines, Nayarit	10	2.67 (0.56)	1.58 (0.27)	1.3	0.30 (0.11)	0.10 (0.05)	0.39*
Chiapa de Corzo, Chiapas	10	2.83 (0.40)	1.86 (0.21)	1.45	0.45 (0.06)	0.25 (0.08)	0.60**
Chupícuaro, Guanajuato	10	2.17 (0.31)	1.42 (0.17)	1.26	0.27 (0.08)	0.07 (0.03)	0.6
Cocoyotla, Morelos	10	3.00 (0.62)	1.90 (0.39)	1.36	0.36 (0.14)	0.37 (0.14)	–0.27
Chiapas coast	2	1.50 (0.22)	1.37 (0.17)	1.28	0.28 (0.13)	0.33 (0.18)	0
Cuatro Ciénegas, Coahuila	10	1.50 (0.22)	1.36 (0.20)	1.2	0.20 (0.10)	0.27 (0.18)	–1
Maury County, Tennessee	2	1.16 (0.17)	1.10 (0.10)	1.08	0.08 (0.08)	0.08 (0.08)	NP
Michoacán	11	2.33 (0.49)	1.55 (0.24)	1.29	0.29 (0.11)	0.31 (0.13)	0.04
Momax, Zacatecas	10	2.50 (0.43)	1.58 (0.30)	1.29	0.29 (0.11)	0.19 (0.07)	0.23
Nuevo Recuerdo, Chiapas	10	2.67 (0.49)	1.65 (0.20)	1.36	0.36 (0.10)	0.24 (0.12)	0.49*
Oaxaca	11	2.83 (0.48)	1.50 (0.21)	1.29	0.29 (0.09)	0.26 (0.13)	0.04
Río Chonchos, Chihuahua	8	1.17 (0.17)	1.02 (0.02)	1.02	0.02 (0.02)	0.02 (0.02)	0
Sacapulas, Quiché	10	1.00 (0.26)	0.85 (0.17)		0.02 (0.02)	0.02 (0.02)	0
San Agustín, Hidalgo	4	1.83 (0.40)	1.47 (0.22)	1.27	0.27 (0.12)	0.21 (0.10)	–0.09
San Marcos, Puebla	10	1.83 (0.40)	1.27 (0.16)	1.17	0.17 (0.09)	0.25 (0.16)	–0.67
San Nicolás, Sonora	10	1.67 (0.56)	1.09 (0.26)		0.16 (0.09)	0.12 (0.07)	–0.08
Sinaloa (Sin)	11	2.67 (0.42)	1.84 (0.23)	1.42	0.42 (0.11)	0.30 (0.11)	0.53**
Tomochic, Chihuahua	2	1.00 (0)	1.00 (0)	1	0.00 (0.00)	0.00 (0.00)	NP
USA_CE	7	1.16 (0.17)	1.05 (0.05)	1.04	0.04 (0.04)	0.00 (0.00)	1
USA_CN	11	2.33 (0.33)	1.19 (0.06)	1.16	0.16 (0.04)	0.17 (0.05)	–0.13
Villaldama, Nuevo León	10	1.33 (0.21)	1.12 (0.80)	1.09	0.09 (0.06)	0.03 (0.03)	–0.06
Zaragoza, Coahuila	10	3.00 (0.37)	2.22 (0.23)	1.55	0.55 (0.05)	0.25 (0.07)	0.5**

N= sample size; N_A= number of alleles; N_{EA}= effective number of alleles; AR= Alleles richness corrected by the sample size; H_O= Heterozygosity observed; H_E= Heterozygosity expected corrected by the sample size; F = Inbreeding coefficient. * = p-value < 0.05; ** = p-value < 0.01; NP = Analysis not performed due size sample. Chiapas coast = includes populations: PM and RC. Michoacán = includes populations: Mn and AF. Oaxaca = includes populations: Cy and Cm. USA_CE = includes populations: CrC, SC, DC and OC. USA_CN = includes populations: LC and BHC.

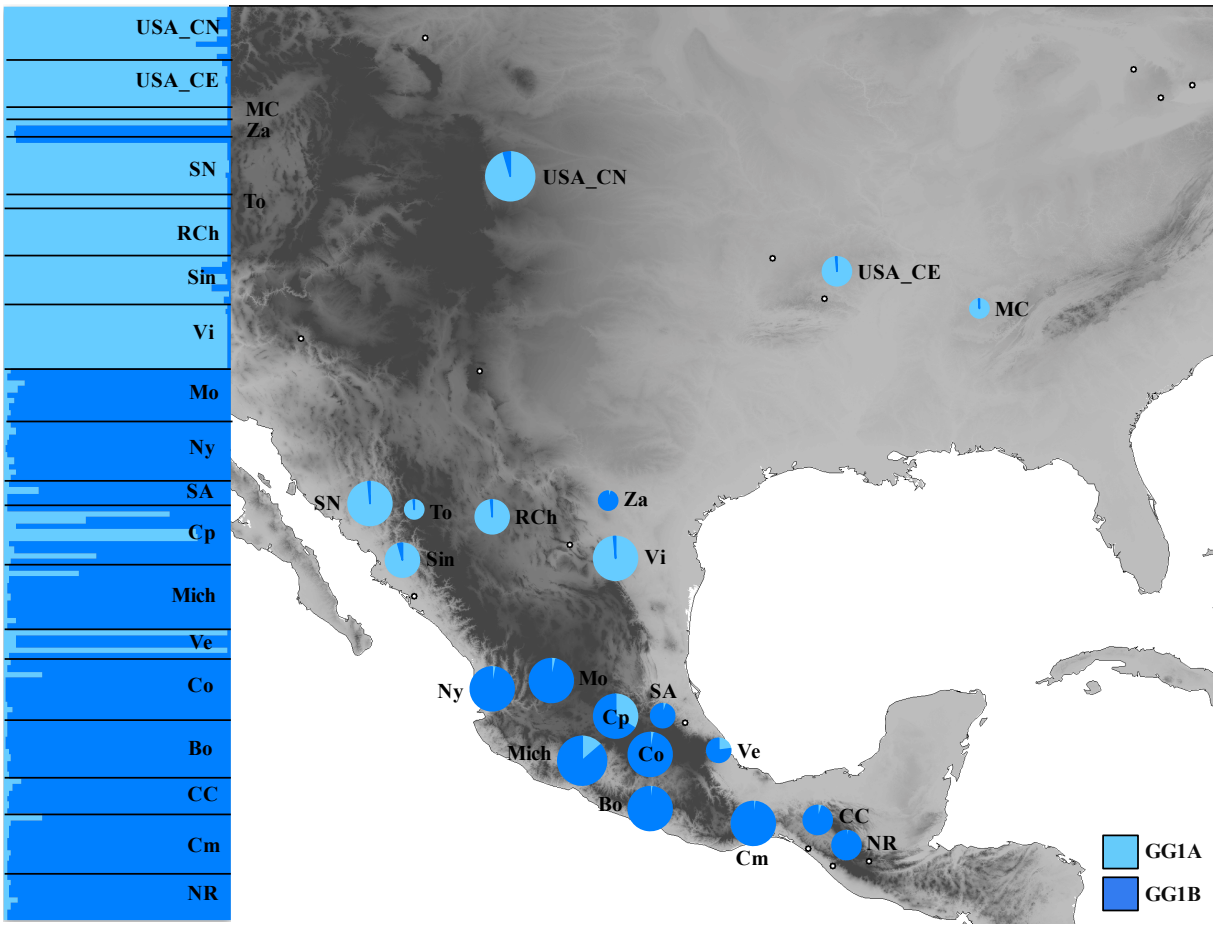
Supplementary Fig. S1. Fan (punctuated lines), landmarks (red dots) and semilandmarks (blue dots) used for geometric morphometric analyses of the right superior caudal appendage.



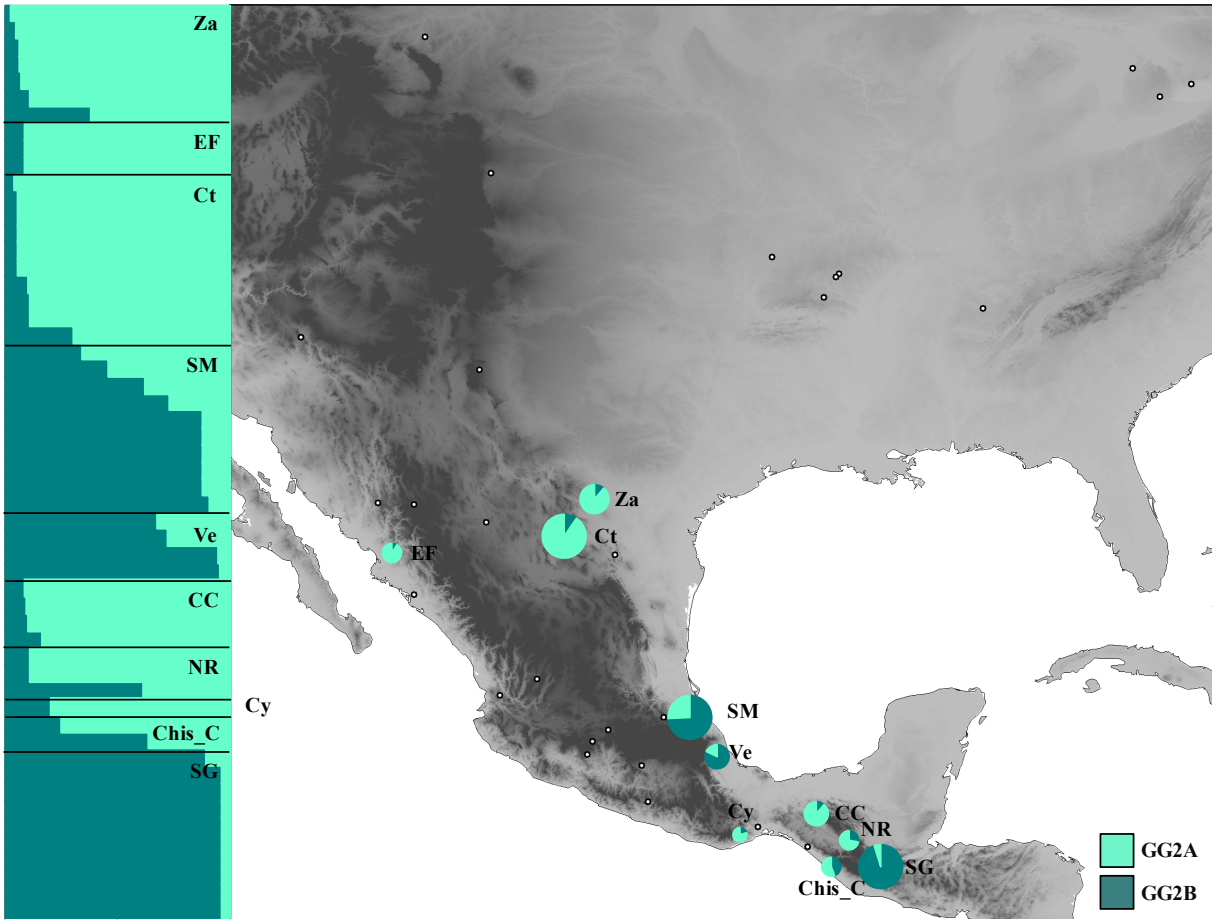
Supplementary Fig. S2. PCA analysis of 19 bioclimatic variables for each population. Each circle represents a population and the color represents the mitochondrial haplogroup present in the population.



Supplementary Fig. S3. Bayesian STRUCTURE analysis of assignment probabilities for K = 2 within populations of *H. americana* (GG1). Individuals are represented by thin vertical lines, which are partitioned into K shaded segments representing each individual's estimated membership fraction; the black lines separate sampling sites. Pie charts represent the proportion and distribution of the two genetic groups in the populations. The size of pie charts represents the sample size analyzed. In the map, dark gray color represents higher altitudes. See text for details.



Supplementary Fig. S4. Bayesian STRUCTURE analysis of assignment probabilities for K = 2 within populations of *H. nov*. sp. (GG2). Individuals are represented by thin vertical lines, which are partitioned into K shaded segments representing each individual's estimated membership fraction; the black lines separate sampling sites. Pie charts represent the proportion and distribution of the two genetics groups in the populations. The size of pie charts represents the sample size analyzed. In the map, dark gray color represents higher altitudes.



III. CAPÍTULO II: *HETAERINA CALVERTI* (ODONATA: ZYGOPTERA: CALOPTERYGIDAE) SP. NOV., A NEW CRYPTIC SPECIES OF THE AMERICAN RUBYSPOT COMPLEX

Yesenia Margarita Vega-Sánchez, Luis F. Mendoza-Cuenca y Antonio González-Rodríguez

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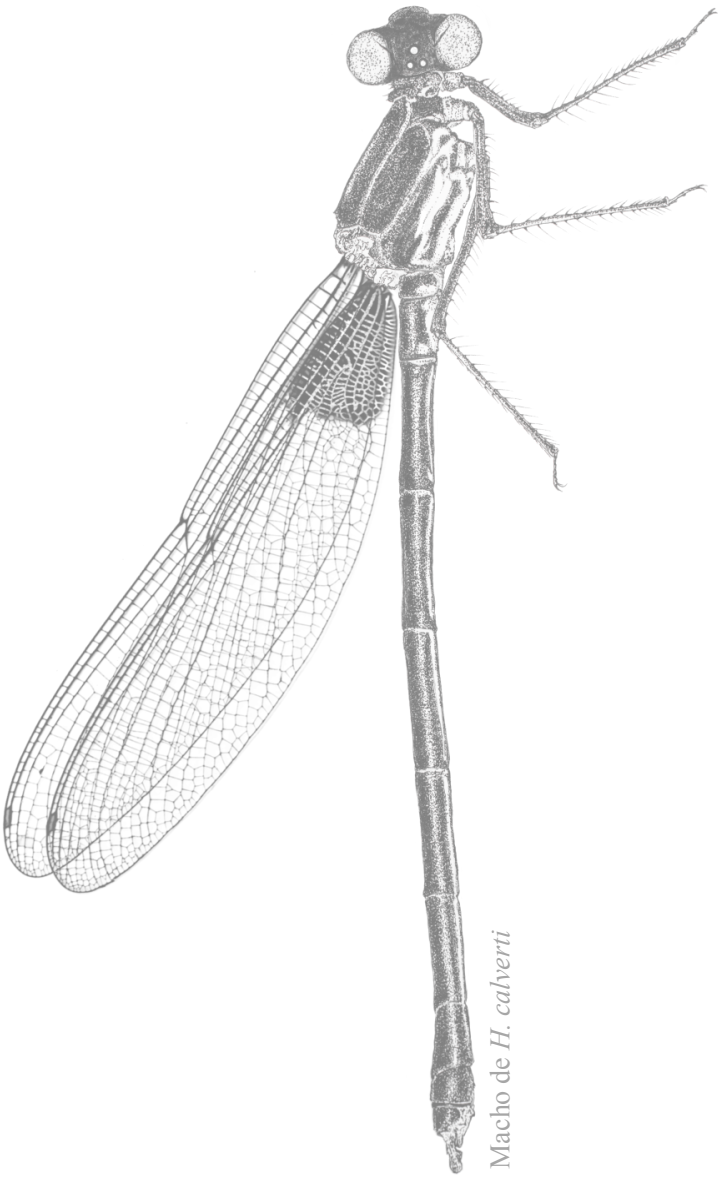

Macho de *H. calverti*

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Hetaerina calverti (Odonata: Zygoptera: Calopterygidae) sp. nov., a new cryptic species of the American Rubyspot complex

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Abstract

Hetaerina americana Fabricius, 1798 has a long and irresolute taxonomic history. Several synonyms have been suggested (*H. californica* Hagen *in* Selys-Longchamps, 1859, *H. basalis* Hagen *in* Selys-Longchamps, 1859, *H. texana* Walsh, 1863, *H. scelerata* Walsh, 1863, etc.), related to the variation in the size of the wing spots as well as to the morphology of the male cerci. However, Calvert (1901) suggested that *H. americana* represents one variable species. Nevertheless, Vega-Sánchez *et al.* (2019) through a genetic and morphological analysis presented evidence to propose that *H. americana* represents a species complex. In the present work, we describe a new species that belongs to this complex: *H. calverti* **sp. nov.** The morphological characteristics by which males and females of *H. calverti* differ from *H. americana* are highlighted. The most important character for the differentiation of males is the shape of the cerci and the size of the individuals (when the two species are in sympatry). In females, the main differences are in the shape of the intersternites and the medio-dorsal carina of the last segment of the abdomen. Some generalities about the biology of the species are presented, including geographical distribution patterns and genetic divergence data. [urn:lsid:zoobank.org:act:F5C329AE-7A00-4979-8A0D-A13D869E54B1]

Keywords: *Hetaerina americana*, damselfly, genetic divergence, morphological differentiation

Resumen

Hetaerina americana Fabricius, 1798 presenta una larga e irresoluta historia taxonómica. Varias sinonimias han sido propuestas (*H. californica* Hagen *in* Selys-Longchamps, 1859, *H. basalis* Hagen *in* Selys-Longchamps, 1859, *H. texana* Walsh, 1863, *H. scelerata* Walsh, 1863, etc.), asociadas con la variación en el tamaño de las manchas alares, así como con la morfología de los cercos de los machos. Sin embargo, Vega-Sánchez *et al.* (2019), a través de una análisis genético y morfológico, evidenciaron que *H. americana* representa un complejo de especies crípticas. En este trabajo, describimos una especie nueva que pertenece a este complejo: *H. calverti* **sp. nov.** Señalamos las características morfológicas en las que machos y hembras de *H. calverti* difieren de *H. americana*. El carácter más importante para la diferenciación de los machos es la forma de los cercos y el tamaño de los individuos (cuando las especies están en simpatría). En las hembras, la diferencia principal está en la forma de los interestenitos y la carina medio dorsal del último segmento del abdomen. Se describen algunas generalidades de la biología de las especies, incluyendo los patrones de distribución, así como datos de divergencia genética.

Palabras clave: *Hetaerina americana,* caballitos del diablo, divergencia genética, diferenciación morfológica

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1. Introduction

The genus *Hetaerina* Hagen *in* Selys, 1853 is distributed from Canada to Argentina and reaches its highest diversity in South America (Garrison 1990). Males of this genus are characterized by the presence of a red spot at the base of each wing as well as their territorial behavior. Currently, the genus comprises 38 species (Schorr & Paulson 2020), but many of them are similar in wing coloration and geographic distribution. Therefore, for most species, the shape of the male cerci provides the primary characters for identification (Garrison 1990; Garrison *et al.* 2010). The identification of females is more complicated and few characters, such as the patterns of body coloration and the shape of the intersternites allow correct identification. However, in some species including *H. capitalis* Selys, 1873, *H. caja* Drury, 1773 and *H. americana* Fabricius, 1798 there is great variation in these characters (coloration, cerci and intersternites) and as a result, additional species have been described that are now considered synonyms.

Hetaerina americana presents one of the broadest geographical distributions in the genus; it is present from Canada to Nicaragua and has been widely used as a model species in behavioral ecology and evolution (e.g. Grether 1996; Serrano-Meneses *et al.* 2018). Also, this species has a long taxonomic history, being one of the first species of the genus and an early species of odonate to be described (*Agrion americana* Fabricius, 1798). Since then, and especially at the end of the 19th century, additional species were named based on variation in two main characters: the size of the red spots on the wings and the shape of the cerci; these are now considered synonyms and are listed below. However, Calvert (1901) and Garrison (1990) suggested that *H. americana* represents only one highly variable species. After these publications, subsequent taxonomic revisions in the genus have been focused mainly on species with southern distributions.

Vega-Sánchez *et al.* (2019) carried out the first genetic analysis for *H. americana*. They also analyzed the variation of the cerci using geometric morphometric techniques. The authors, based on several loci and an extensive sampling, found strong evidence indicating that *H. americana* actually represents a complex of cryptic species with at least two species highly differentiable based on genetic and morphometric data (see Fig. 5 in Vega-Sánchez *et al.* 2019). While the variation in other male characters such as the coloration of the basal wing spots was not concordant with the proposed cryptic species, these groups can be clearly separated based on the morphology of the cerci. On the other hand, in that study females were not analyzed.

Therefore, the purposes of this work were: 1) to review the literature and establish if the new species suggested by Vega-Sánchez *et al.* (2019) is valid, 2) to analyze the morphological variation of the females, and 3) to describe, including morphological, genetic and life history data, the male and female of the new species.

2. Materials and methods

2.1. Literature review

The literature on the taxonomy of *H. americana* was reviewed. This review included the original description of *H. americana* (Fabricius 1798) as well as most documents in which the synonymized species were described up to 1901: *H. californica* and *H. basalis* (Selys-Longchamps 1859), *H. pseudoamericana, H. texana* and *H. scelerata* (Walsh 1863). These documents were reviewed on the Biodiversity Heritage Library page (https://www.biodiversitylibrary.org). We did not review the types of the synonyms because they were not available and suspected to be lost (Garrison 1990).

2.2. Material studied

Type specimens of the new species were collected in Apazapan, Veracruz, Mexico, on July 3rd, 2019 and in El Platanal, Xichú, Guanajuato, Mexico on April 5th, 2019. The holotype, allotype and one paratype were deposited at the Colección Nacional de Insectos (CNIN), Instituto de Biología, UNAM (IBUNAM). Three more paratypes were deposited at the Genoteca y Colección Entomológica del Laboratorio de Ecología de la Conducta de la Facultad de Biología, Universidad Michoacana de San Nicolás de Hidalgo (GCEUMSNH).

Additionally, we examined genotyped individuals that were previously included in the genetic analysis of Vega-Sánchez *et al.* (2019), 34 males and 19 females deposited at GCEUMSNH. We also examined 108 male specimens

deposited at CNIN (see below) (Appendix 1).

The illustrations were obtained with the help of a stereoscopic microscope and made by hand.

We followed Garrison (1990) and Garrison *et al.* (2010) for wing venation and body terminology. All measurements are in millimeters (mm) and were obtained analyzing photographs and using ImageJ software (Schneider *et al.* 2012). Total length and length of the abdomen include cerci. Abbreviations for structures used throughout the text are as follows: S1–10: abdominal segments from 1 to 10, FW: fore wing, HW: hind wing.

The photographs in free life were taken with a Canon ® T7i camera at Apazapan, Mexico.

In addition, for the genetic analysis, we sampled one male and one female of each species present at Apazapan (i.e. *H. americana* and *H.* **nov. sp.**).

2.3. Review of specimens and differences in distribution and body size

We reviewed all the physical specimens previously classified as *H. americana* that were registered in the database of the CNIN (Departamento de Zoología 2019) (https://datosabiertos.unam.mx/biodiversidad/) and confirmed their identity or assigned them to the new species based on the shape of the cerci (Vega-Sánchez *et al.* 2019). We only considered information from males since the females may require molecular confirmation. The coordinates of the collection localities of these specimens and those records from GCEUMSNH were used to produce a map of the distribution of *H. americana* and the new species.

To analyze the variation in the body and wing size of both species, we photographed each specimen from both collections and obtained the total length and HW length using the ImageJ software. Two-way ANOVAs were carried out using as independent categorical variables the species and whether they co-occur in the localities or not (i.e., sympatry *vs* allopatry), and total length and HW length as response variables.

2.4. Analysis of genetic distances between species

We obtained DNA from one male and one female from each species (i.e. *H. americana* and *H.* **nov. sp.**). DNA extractions were made from two legs of the individuals and we amplified and sequenced the nuclear region ITS1-5.8S-ITS2 of approximately 600 base pairs following the protocol of Vega-Sánchez *et al.* (2019). The sequences were deposited in the GenBank database (Table 1).

TABLE 1. Species and accession number of individuals used for genetic divergence analysis.

Species	Abbreviation	Sex	Latitude, longitude	GenBank accession number
Rimanella arcana	*Rar*			AJ746323.1
Euthore fasciata	*Efa*			AJ746325.1
Phaon iridipennis	*Pir*			AJ459225.1
Hetaerina americana	*Ham*F	♀	19.327, -96.721	MN381736
Hetaerina americana	*Ham*M	♂	19.327, -96.721	MN381737
Hetaerina calverti	*Hcl*F	♀	19.327, -96.721	MN381738
Hetaerina calverti	*Hcl*M	♂	19.327, -96.721	MN381739
Hetaerina capitalis	*Hca*		18.657, -95.151	KM383813
Hetaerina cruentata	*Hcr*		18.371, -95.001	KM383818
Hetaerina miniata	*Hmi*		10.989, -84.334	KM383801
Hetaerina occisa	*Hoc*		18.359, -95.000	KM383809
Hetaerina pilula	*Hpi*		18.371, -95.001	KM383804
Hetaerina sempronia	*Hse*		18.592, -95.084	KM383814
Hetaerina titia	*Hti*		10.989, -84.334	KM383799
Hetaerina vulnerata	*Hvu*		31.480, -110.337	KM383821

To determine the degree of divergence, we estimated the genetic distances between the species using the MEGAX program (Kumar *et al.* 2018). We used a p-distances model treating the gaps as missing data. For this analysis we included the sequences obtained in this study and the sequences of other *Hetaerina* species as well as three species of other genera obtained from GenBank (Table 1).

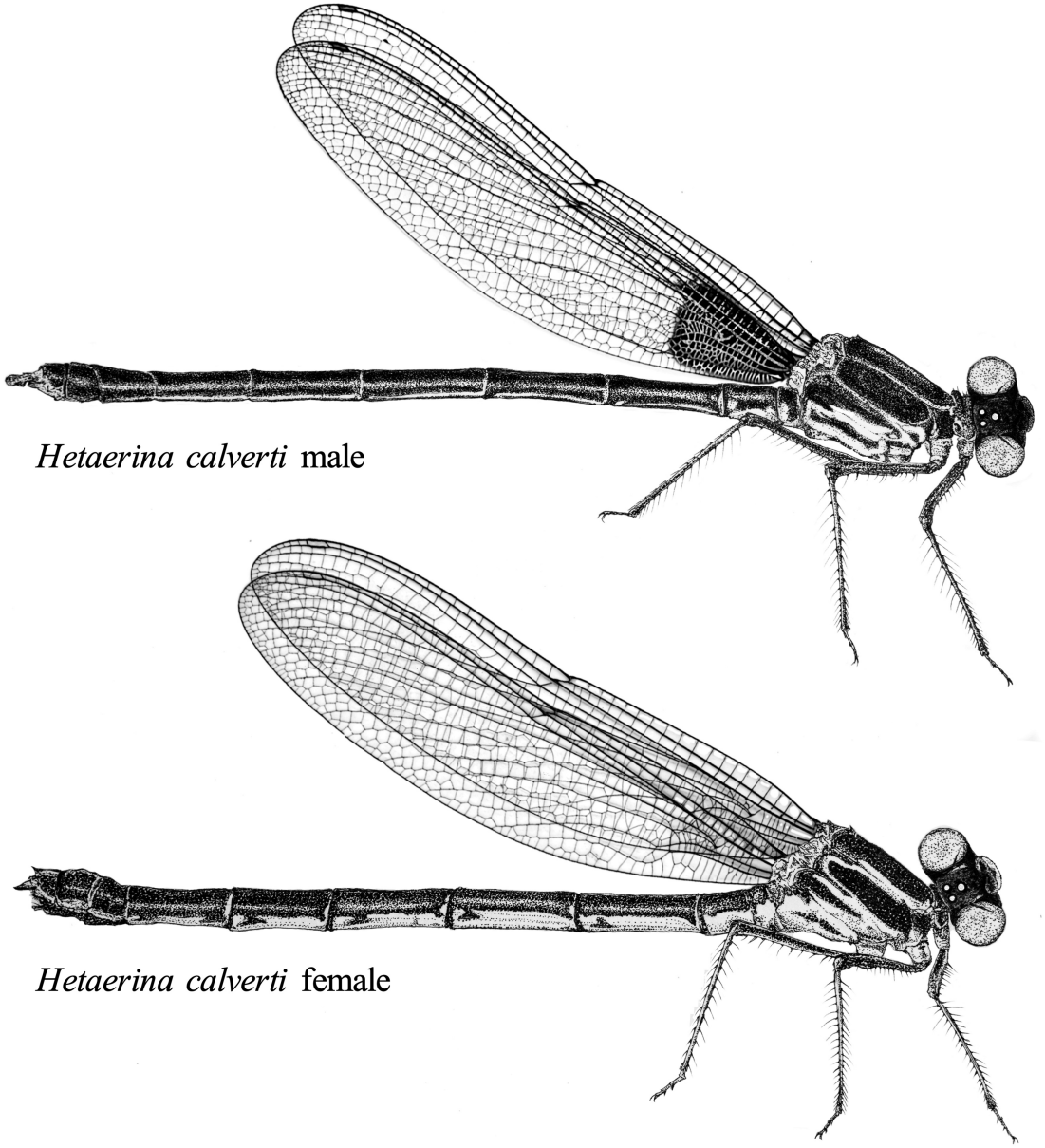

FIGURE 1. Male (above) and female (bottom) of *Hetaerina calverti* **sp. nov**. The body coloration patterns are shown. In the male, the dark zones represent metallic red color and the clear zones represent whitish color. In female, the dark zones represent metallic green color and the clear zones represent whitish color. The illustrations are not at scale.

3. Results

3.1. *Hetaerina americana* taxonomic history

The original description of *H. americana* (and until 1853) only included few aspects of the male coloration (Fabricius 1789). Selys-Longchamps (1853, 1854) made a more precise description of the species including a small description of the females. The first illustration of the cerci of *H. americana* was included in Selys-Longchamps (1854; Plate 12, Fig. 3, pp. 331). The author described the appendages as follows:

"*Anal appendages yellow, black at their extremities; the superiors cylindrical, arched, little toothed on the outside; the superior ridge ends in a semicircle before the tip, which is a little thicker, cylindrical, obtuse, a little depressed on the inner lower edge that offers, from the middle to a little before the tip, a very strong dilatation flattened, which is preceded by an excavation below. This dentate dilation in the middle, in profile view, is a fairly large obtuse medial tooth, ending in a very small and rounded tooth.*

Inferior appendages are shorter than half of the superior ones, straight, cylindrical, obtuse, separated; its internal angle is weak, with a hair brush".

After these descriptions, Hagen *in* Selys-Longchamps (1859) delved into the variation existing within *H. americana* and suggested two new species: *H. californica* and *H. basalis*. The first species differs in the shape of the cerci, which is quadrangular, and also in that the pterostigma is absent. This species is restricted to Northern California. The second species, *H. basalis*, is characterized by having a much larger red wing spot than Hagen's definition of *H. americana* (i.e. reaching the node) and a triangular median lobe in the cerci. Hagen suggested that this is a 'race' of *H. americana.*

Walsh (1863) suggested three new species from the review of several specimens of *H. americana*: *H. texana, H. scelerata* and *H. pseudoamericana*. In general, these species differ, according to Walsh, in small variations of the cerci as well as in the extension and coloration of the red spot and in the presence and coloration of the pterostigma. It should be noted that all the individuals reviewed by Walsh were from USA populations.

Finally, Calvert (1901) included the first specimens from Mexico and Guatemala, and in his Plate II, figures 6, 11 and 15, drew the appendages of what we suggest is the here described new species. Calvert commented that, in a population from Tamaulipas he examined individuals that showed cerci of this shape, as well as others such as those shown in figure 16 that correspond to *H. americana sensu stricto* (i.e. they were in sympatry). The difference between individuals was only recognized by this character, since the coloration and extension of the red spot in the two types were identical.

Calvert suggested that the variation in the shape of the cerci was not correlated with the geographical location or the season in which the individuals were collected. Finally, Calvert suggested that all the aforementioned species belong to a single *H. americana* species, which is very variable.

From this review, we found no evidence that any of the synonyms that were described for *H. americana* match the new species suggested by Vega-Sánchez *et al.* (2019). The only report that included illustrations of the cerci of this new species is the work of Calvert (1901), nevertheless it is in this paper that the author synonymized all species in *H. americana.*

Taxonomy

Hetaerina calverti **sp. nov.**
(Figs. 1–3)

Type specimens

Holotype. ♂ (CNIN), tributary stream of Los Pescados River in Apazapan, Veracruz, Mexico (19.3250389, -96.7248972, 305 m asl), 3 July 2019. L.F. Mendoza-Cuenca *leg*. The male was collected in tandem position with the female allotype (Fig. 1).

Allotype. ♀ (CNIN). A mature female that was collected in the same locality, date and collector as the holotype (Fig. 1).

Paratypes. 4 ♂♂ (CNIN and GCEUMSNH). A mature male, same locality and date as the holotype. Y.M. Vega-Sánchez *leg.* (Fig. 2), and three mature males from El Platanal, Xichú, Guanajuato, Mexico (21.465478, -

99.832283, 700 m asl), 5 April 2019. Y.M. Vega-Sánchez *leg*.

Etymology. This species was named in honor to Dr. Philip P. Carlvert because he was the first scientist to analyze and illustrate the shape of the cerci of the new species and for his prominent contribution to the odonatology of the Neotropics.

FIGURE 2. Paratype male of *H. calverti* (left) and young male of *H. americana* (right) from Apazapan, Veracruz, Mexico.

Description of holotype

Head. Metallic red.

Thorax. Prothorax mostly metallic red. Pterothorax mainly red, with metallic brown flashes; the sutures—humeral, interpleural and metapleural—are whitish. Mesepisternum completely metallic red except for white anterior humeral margin; mesepimeron mainly red-brown with metallic green flashes; metepisternum with an elongated metallic brown hook-shaped spot that invades the metepimeron at its superior margin and is surrounded by pale whitish coloration (Fig. 1). Metepimeron surrounded by pale whitish color and with an elongate reddish spot in the center. Legs dark brown.

Wings. All wings with a red spot at the base, extending up to four crossveins beyond the quadrangle. All veins on HW spot white below. All wings lacking apical spots. Pterostigma present in all wings, dark brown.

Abdomen. Metallic green on dorsum becoming metallic brown from S7–10. S1 with pale spots on sides.

Caudal appendages. Cerci yellowish with the median lobe widened and flattened at the beginning and with a prominent tooth at the end. The transverse ridge connects at 45° with the superior ridge, which is beyond three fourths of the length of the cercus. Paraprocts cylindrical and one third the length of the cerci, with a pair of spines at the tip; yellowish at the base, the distal part dark brown (Fig. 3).

Measurements. Total length 47; abdomen 37.5; HW 25.7; FW 27.1.

Variation in male paratypes

The male paratype deposited at CNIN has pale brown humeral, interpleural and metapleural sutures (Fig. 3). In the paratypes deposited at GCEUMSNH, the red spot at the base extends up to two crossveins beyond the quadrangle.

Measurements. Total length 39.65–45.9 (41.69); abdomen 31.76–36.7 (33.56); HW 23.79–25.1 (24.18); FW 26–27 (26.3).

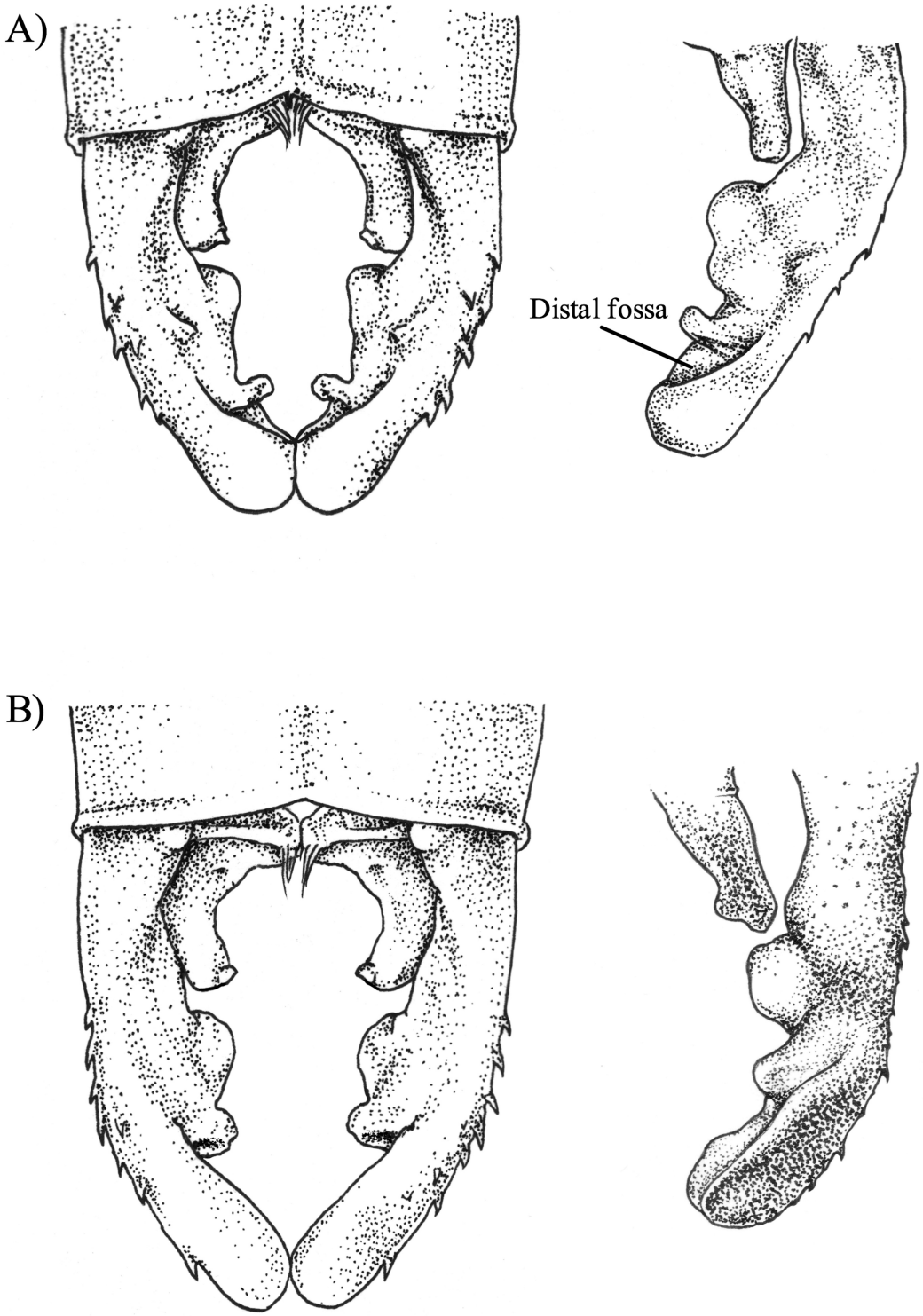

FIGURE 3. Variation between *H. calverti* and *H. americana* males. Male cerci (dorsal and mediodorsal view) of *H. calverti* (A) and *H. americana* (B). The illustrations are not at scale.

Description of allotype

Head. Frons mainly metallic reddish with green flashes. Postclypeus metallic red in the center, margins white. Labrum mainly whitish. First segment of antennas white, remainder black (Fig. 1).

Thorax. Prothorax metallic brown-green; intersternites linear, without anterior shoulder. Pterothorax mainly metallic brown-green with whitish humeral, interpleural and metapleural sutures. Mesepisternum completely metallic brown-green except for white humeral margin; mesepimeron mainly metallic brown-green, metepisternum surrounded by whitish color, with an elongated metallic brown hook-shaped spot that invades the metepimeron at its superior margin (Fig. 1). Metepimeron with an elongated brown spot in the center, surrounded by whitish coloration. Coaxes pale yellowish, rest of legs dark brown.

Wings. All wings with an amber spot that lightens towards the apex. Veins on HW spot white. All wings lacking apical spots. Pterostigma present in all wings, white.

Abdomen. Each segment metallic green above, except the last three segments metallic brown, in addition, all segments with a thin pale medio-dorsal line. Ventral part of the abdomen pale. Medio-dorsal carina of S10 ends in a prominent spine that extends beyond the rest of the segment (Fig. 1 and 3).

Measurements. Total length 36.3; abdomen 28.1; HW 24.5; FW 26.1.

Diagnosis

The male of *H. calverti* is distinguished from other species of the genus by the shape of the cerci. The median lobe extends from one third to beyond two thirds of the appendage and is widened and flattened in the middle part and ends in an acute projection. In contrast, the median lobe in *H. americana* is more bilobed (Fig. 3).

Additionally, in the distal part of the cerci, after the transverse ridge the distal fossa is short and represents a quarter or less of the total length of the appendage. This is the principal difference with *H. americana*, in which the distal fossa is larger and represents more than a quarter of the cercus (Fig. 3).

The female of *H. calverti* is characterized by having a linear intersternite. The medio-dorsal carina of S10 forms a prominent spine that extends beyond the margin of the segment. In *H. americana*, the intersternite presents an anterior shoulder and the medio-dorsal carina of S10 forms a spine but no as prominent as in *H. calverti* (Fig. 4).

3.3. Distribution, ecology and variation in size

Based on 1071 specimens reviewed in both collections (CNIN and GCEUMSNH), we found 621 individuals with georeferenced information (principally males), of which 457 belong to *H. americana* and 164 to *H. calverti* (Supplementary table 1).

Hetaerina calverti is found from Guatemala to northern Mexico (Fig. 5). It is distributed in shallow streams and small rivers in tropical dry forests principally, but in the northern part of its distribution it can be found in desert shrub and grass vegetation types. It is usually in sympatry with *H. americana* (Fig. 4). Like other species of *Hetaerina*, the males are territorial and perform synchronized flights. The females are usually far from the territories of the males and only approach these in order to copulate. After copulation, the tandem pair leaves the territory of the male to look for oviposition sites.

On the other hand, we found differences in the total length between species when they are in sympatry (Species: $F= 54.8$; p-value< 0.0001; Locality type: $F= 1.15$; p-value$= 0.28$; Interaction: $F= 8.76$; p-value$= 0.003$), being *H. calverti* larger. Differentiation in HW was somewhat less pronounced (Species: $F= 12.95$; p-value $= 0.0003$; Locality type: $F= 0.15$; Interaction: $F= 3.29$; p-value $= 0.07$).

3.4. Genetic distances

Nuclear DNA (ITS region) data show a 5% divergence between *H. calverti* and *H. americana*. Therefore, *H. calverti* can be distinguished without hesitation by using this region of the nuclear genome. The distances between other species of the genus range from 2% to 12% (Table 2).

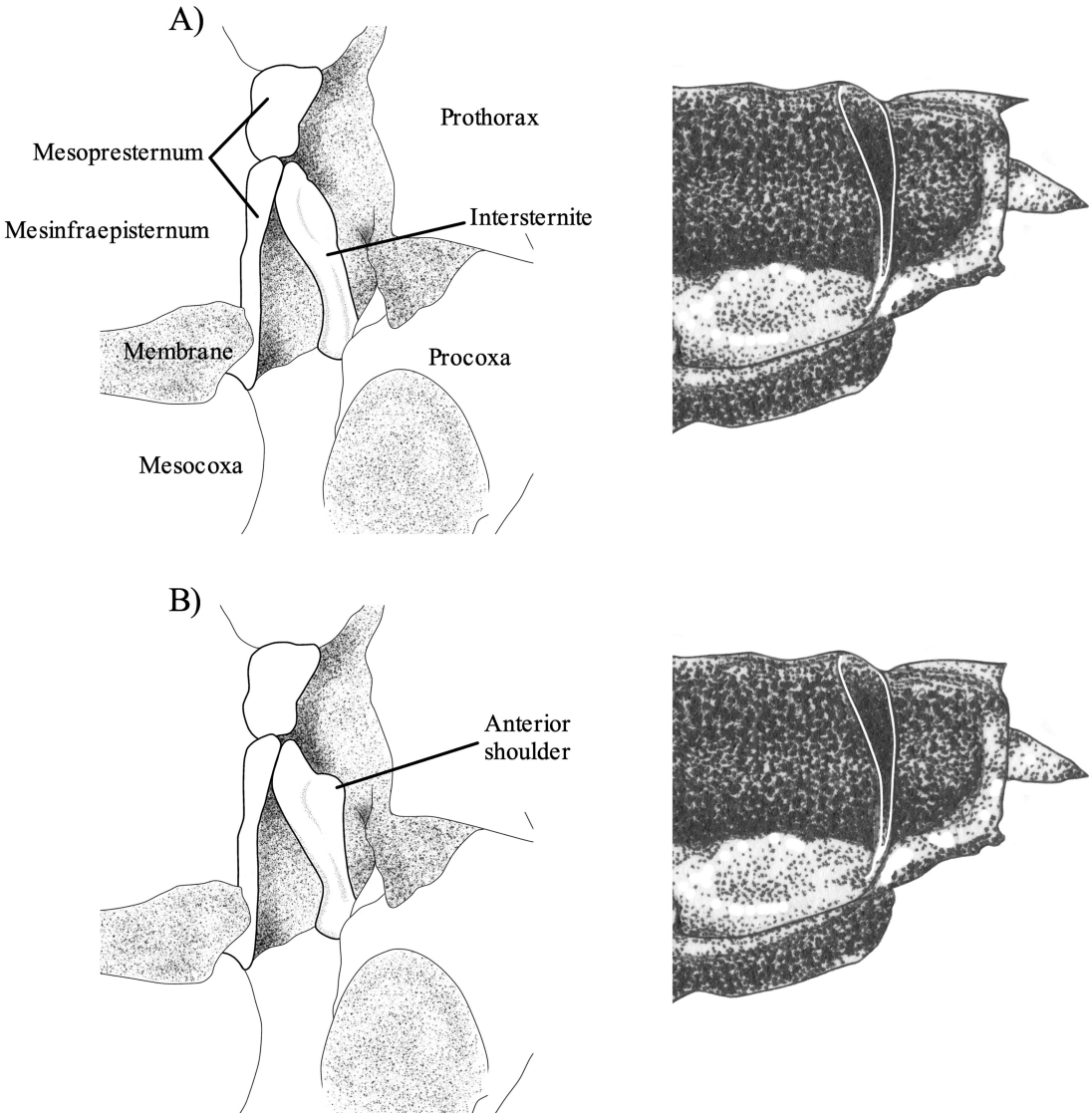

FIGURE 4. Variation between *H. calverti* and *H. americana* females. Intersternite and S10 of *H. calverti* (A) and *H. americana* (B). The illustrations are not at scale.

4. Discussion

4.1. Comparison with *H. americana*

From the description of *H. americana* in 1798 until 1901, the variability of this "species" was the subject of discussion among taxonomists (Selys-Longchamps 1853, 1854; Calvert 1901). But in 2019, the use of molecular markers provided the opportunity to categorize this variability, resulting in the proposal of a new cryptic species: *H. calverti*.

We suggest that this new species does not belong to any of the synonyms described in the last century. This conclusion is based on the revision of the original descriptions (despite being a little vague), in which the diagnostic characters do not resemble those of *H. calverti*. Even in Calvert's (1901) review, he illustrated some specimens that

may be what Walsh (1863) suggested as *H. basalis* or *H. texana* but none of these were *H. calverti* specimens (as can be judged based on the illustrations of the cerci). Another fact that supports that *H. calverti* is a valid name is that all specimens in which synonyms were based were from USA where, at least from our data, *H. calverti* is not distributed.

TABLE 2. Estimates of genetic divergence between species. The percentage divergence between ITS sequences are shown. Species abbreviations follow Table 1.

Species	*Rar*	*Efa*	*Pir*	*Ham*F	*Ham*M	*Hcl*F	*Hcl*M	*Hca*	*Hcr*	*Hmi*	*Hoc*	*Hpi*	*Hse*	*Hti*	*Hvu*
Rar	0.0														
Efa	39.2	0.0													
Pir	31.3	38.8	0.0												
*Ham*F	29.4	38.6	30.1	0.0											
*Ham*M	29.4	38.6	30.1	0.0	0.0										
*Hcl*F	29.0	39.0	28.1	5.3	5.3	0.0									
*Hcl*M	29.0	39.0	28.1	5.3	5.3	0.0	0.0								
Hca	31.8	38.0	29.2	17.1	17.1	16.6	16.6	0.0							
Hcr	29.2	38.2	27.7	11.3	11.3	9.8	9.8	14.7	0.0						
Hmi	31.6	37.3	30.5	19.8	19.8	21.1	21.1	21.5	19.4	0.0					
Hoc	29.6	38.0	29.2	17.7	17.7	16.8	16.8	20.0	17.3	19.4	0.0				
Hpi	31.6	37.3	28.8	22.4	22.4	21.5	21.5	21.7	20.7	21.5	19.2	0.0			
Hse	30.9	39.7	30.1	17.3	17.3	17.1	17.1	17.9	13.9	19.0	18.3	21.1	0.0		
Hti	31.1	37.3	28.4	21.5	21.5	21.3	21.3	23.0	19.8	16.8	20.5	23.2	20.7	0.0	
Hvu	27.7	39.2	27.3	11.3	11.3	9.8	9.8	15.1	2.1	19.0	16.2	20.0	12.8	19.2	0.0

These early descriptions as in Walsh (1863), included coloration and the lack of pterostigma as diagnostic characters to differentiate among taxa, but these traits are geographic and seasonally variable (Garrison 1990; Contreras-Garduño *et al*. 2008). Therefore, we suggest that the only reliable character for the correct differentiation between *H. americana* and *H. calverti*, in males, is the shape of the cerci (Fig. 3). In females, the variation in potential distinguishing characters has not been analyzed over their whole distribution, and due to this we suggest that the use of genetic markers is currently the most reliable method for their correct taxonomic recognition.

The distribution of each species (Fig. 5) suggests that *H. calverti* has a more limited distribution compared to *H. americana*. *Hetaerina calverti* frequently is found at lower altitude (i.e. < 900 m asl) and in the neotropical region. In contrast, *H. americana* extends its distribution to Canada and can be found also at higher elevations (> 900 m asl) in Mexico (e.g. along the Trans-Mexican Volcanic Belt).

Another interesting fact is the difference in size between species, which is maximized when they are in sympatry. This possible character displacement pattern may be related to the cryptic speciation process in this complex.

Finally, the genetic divergence between both species is about 5% for the ITS1-5.8S-ITS2 nuclear region. It has been suggested that this magnitude of genetic divergence predicts reproductive isolation (Sánchez-Guillén *et al.* 2014). Moreover, it is interesting that this degree of divergence is even larger than between *H. cruentata* Rambur, 1842, and *H. vulnerata* Hagen *in* Selys, 1853*,* two species that differ in color patterns as well as in the shape of the cerci (Garrison 1990).

4.2. *Hetaerina calverti*, a cryptic species

Genetic and morphological data of both females and males suggest that *H. calverti* is a new species that is reproductively isolated from *H. americana*. However, although the differences in morphological structures such as the cerci in males and the intersternites in females differentiate the species, it is important to mention that interspecific variation in other characters such as wing-spot and body coloration is practically absent and, as we have already mentioned, this has been one of the biggest problems in the identification of possible new species within "*H. americana*".

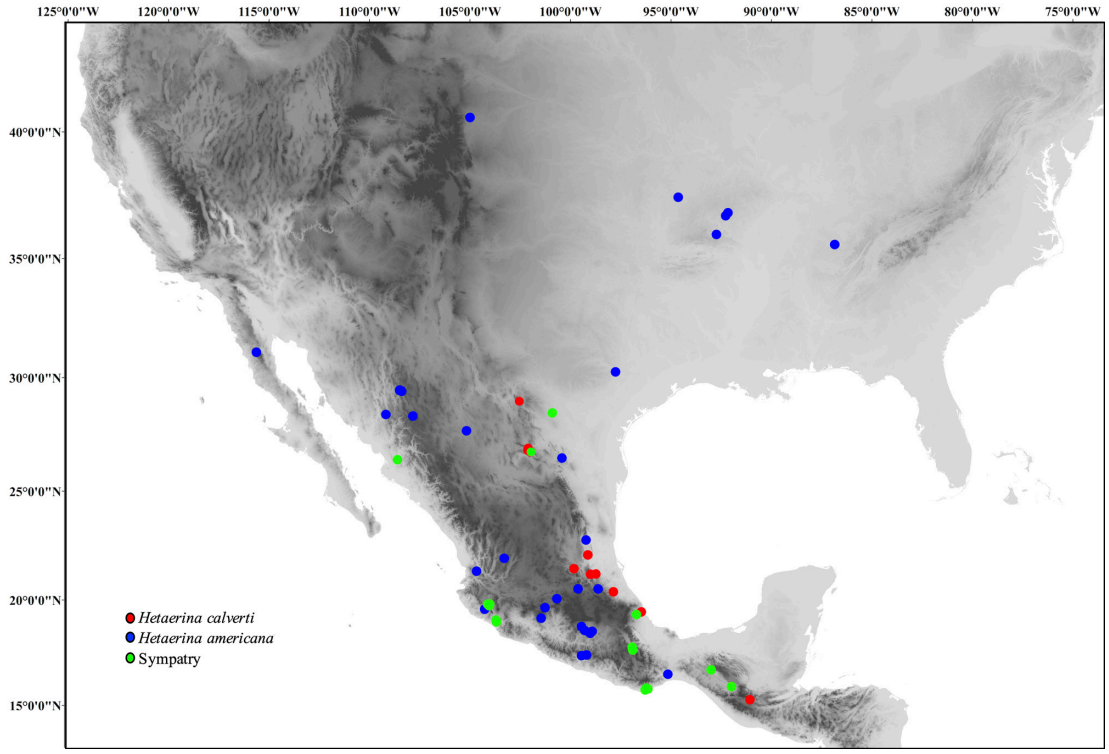

FIGURE 5. Distribution of the American Rubyspot Complex based on coordinates from the CNIN and GCEUMSNH collections.

In recent years, the discovery of cryptic species in different groups, including odonates, has been increasing (Damn *et al.* 2010). However, the causes of morphological stasis in these species remain unknown. Due to this, new questions arise such as: Are we underestimating the diversity of odonates? What is the importance of the reproductive system in cryptic speciation? And, bearing in mind that *H. americana* is one of the first species of damselflies described in North America (i.e. more than 200 years ago) and one of the best studied, it would not be surprising if this phenomenon is very common in other less studied odonates. It is also important to mention that in the work of Vega-Sánchez *et al.* (2019) the sampling was mainly in Mexico, hence it is possible that other cryptic species are found in northern and southern parts of the distribution, for example in California, Guatemala, El Salvador, Honduras and Nicaragua.

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Supplementary table 1. Collection data and measurements obtained in this study.
Excel file.

Appendix 1. *Hetaerina calverti* material examined.

Guatemala, Quiché State: Río Negro, Sacapulas, 20.IV.2014, Y.M. Vega-Sánchez *leg.* (10 ♂♂, GCEUMSNH). **Mexico, Chiapas State:** Chiapa de Corzo, 20.X.2013, Y.M. Vega-Sánchez *leg.* (4 ♂♂, GCEUMSNH); Nuevo Recuerdo, 22.X.2013 (3 ♂♂, GCEUMSNH); **Coahuila State:** Campo Uno, Área de Protección de Flora y Fauna Maderas del Carmen, 18.VI.2007, González *leg.* (1 ♂, CNIN); La Poza Azul, Cuatro Ciénegas, 29.IX.2011, M. Trujano *et al. leg.* (1 ♂, CNIN); Poza Churince, Churince, 01.X.2011, M. Trujano *et al. leg.* (1 ♂, CNIN); Antiguos Mineros del Norte, Las Teclas, 02.X.2011, H. Ortega *et al. leg.* (1 ♂, CNIN); Poza Churince, Churince, 23.II.2012, M. Trujano *et al. leg.* (2 ♂♂, CNIN); Antiguos Mineros del Norte, Las Teclas, 24.II.2012, M. Trujano *et al. leg.* (1 ♂, CNIN); Antiguos Mineros del Norte, Las Teclas, 04.IV.2012, M. Trujano *et al. leg.* (1 ♂, CNIN); Poza Churince, Churince, 02.IX.2012, M. Trujano *et al. leg.* (1 ♂, CNIN); Rancho Orozco, Poza Escobedo, 30.IX.2012, H. Ortega *et al. leg.* (2 ♂♂, CNIN); Antiguos Mineros del Norte, Las Teclas, 24.II.2013, H. Ortega and E. González *leg.* (3 ♂♂, CNIN); La Poza Azul, La Poza Las Tortugas, 13.V.2013, M. Trujano *et al. leg.* (2 ♂♂, CNIN); Poza Churince, Churince, 09.VII.2013, H. Ortega-Salas *et al. leg.* (1 ♂, CNIN); Antiguos Mineros del Norte, Las Teclas, 30.X.2013, M. Trujano *et al. leg.* (1 ♂, CNIN); Arroyo, Poza Azul, Cuatro Ciénegas, 06.III.2014, Y.M. Vega-Sánchez *leg.* (5 ♂♂, 5 ♀♀, GCEUMSNH); Zaragoza, 10.III.2014, Y.M. Vega-Sánchez *leg.* (6 ♂♂, 1 ♀, GCEUMSNH); **Colima State:** La Presa Derivadora Las Trancas, 28.VI.2006, E. González *leg.* (1 ♂, CNIN); La Presa, Arroando El Salado, 28.VIII.2006, E. González *leg.* (1 ♂, CNIN); **Guanajuato State:** El Platanal, 05.IV.2019, Y.M. Vega-Sánchez *leg.* (7 ♂♂, GCEUMSNH); **Guerrero State:** Quelchutenango, Balneario El Borbollón, 29.III.1987, E. González and C. Deloanda *leg.* (1 ♂, CNIN); Las Juntas, 27.X.2008, E. González-Soriano *leg.* (1 ♂, CNIN); **Hidalgo State:** Pisaflores, 17.IV.2000. P. Alonso-Eguíalis *leg.* (2 ♂♂, CNIN); **Jalisco State:** San Buenaventura, 08.I.1997, A. Morales *leg.* (1 ♂, CNIN); San Buenaventura, 07.III.1997, E. González and A. Morales *leg.* (1 ♂, CNIN); San Buenaventura, 31.III.1997, E. González and A. Morales *leg.* (1 ♂, CNIN); El Limón, 02.IV.1997, E. González and A. Morales *leg.* (1 ♂, CNIN); San Buenaventura, 02.IV.1997 E. González and A. Morales *leg.* (1 ♂, CNIN); El Limón, 04.IV.1997, E. González and A. Morales *leg.* (1 ♂, CNIN); San Buenaventura, 02.V.1997 E. González and A. Morales *leg.* (1 ♂, CNIN); San Buenaventura, 05.VI.1997, M. Guardado and M. Sarmiento *leg.* (2 ♂♂, CNIN); El Limón, 05.VI.1997, L.F. Novelo *leg.* (1 ♂, CNIN); San Buenaventura, 06.VI.1997, E. González and A. Morales *leg.* (1 ♂, CNIN); El Limón, 30.VI.1997, E. González and A. Morales *leg.* (4 ♂♂, CNIN); El Limón, 01.VIII.1997, E. González and A. Morales *leg.* (1 ♂, CNIN); El Limón, 04.VIII.1997, E. González and A. Morales *leg.* (1 ♂, CNIN); El Limón, 02.IX.1997, E. González and A. Morales *leg.* (1 ♂, CNIN); San Buenaventura, 02.X.1997, E. González and A. Morales *leg.* (1 ♂, CNIN); El Limón, 08.X.1997, A. Morales *leg.* (1 ♂, CNIN**); Morelos State:** Arroando Quilamula, 07.X.1996, E. González *leg.* (1 ♂, CNIN); **Oaxaca State:** Santiago Dominguillo, 23.XI.1997, E. González-Soriano *leg.* (2 ♂♂, CNIN); Santiago Dominguillo, 23.XI.1997, E. González-Soriano *leg.* (2 ♂♂, CNIN); Santiago Dominguillo, 25.XI.1997, E. González-Soriano *leg.* (2 ♂♂, CNIN); Santiago Dominguillo, 22.I.1998, E. González-Soriano and N. Sandoval *leg.* (5 ♂♂, CNIN); Río Grande Presa Matambo, 23.I.1998, E. González-Soriano and N. Sandoval *leg.* (5 ♂♂, CNIN); Santiago Dominguillo, 24.III.1998, E. González-Soriano *leg.* (4 ♂♂, CNIN); San Pedrito Chicozapote, 25.III.1998, E. González-Soriano *leg.* (2 ♂♂, CNIN); Santiago Dominguillo, 20.IV.1998, E. González-Soriano *leg.* (1 ♂, CNIN); Santiago Dominguillo, 23.V.1998, E. González-Soriano *leg.* (2 ♂♂, CNIN); Santiago Dominguillo, 24.V.1998, E. González-Soriano *leg.* (1 ♂, CNIN); Santiago Dominguillo, 25.V.1998, E. González-Soriano *leg.* (1 ♂, CNIN); Santiago Dominguillo, 20.IX.1998, E. González-Soriano *leg.* (6 ♂♂, CNIN); Santiago Dominguillo, 21.IX.1998, E. González-Soriano *leg.* (3 ♂♂, CNIN); Puente Arroando Xuchitl, 09.VII.2005, A. González and E. González-Soriano *leg.* (1 ♂, CNIN); Puente Arroando Xuchitl, 02.IX.2005, E. González-Soriano *leg.* (2 ♂♂, CNIN); Puente Arroando Guajiniquil, 04.IX.2005, E. González-Soriano *leg.* (2 ♂♂, CNIN); Puente Arroando Guajiniquil, 04.XI.2005, E. González-Soriano *leg.* (2 ♂♂, CNIN); Puente Arroando Xuchitl, 06.XI.2005, E. González-Soriano *leg.* (1 ♂, CNIN); **Puebla State:** Río San Marcos, 15.III.2014, Y.M. Vega-Sánchez *leg.* (3 ♂♂, 7 ♀♀, GCEUMSNH); **Querétaro State:** Arroyo Seco, Puente Ayutla, 21.IV.2014, H. Ortega-Salas *leg.* (3 ♂♂, CNIN); Puente Ayutla, 22.VII.2014, H. Ortega-Salas and E. González-Soriano *leg.* (4 ♂♂, CNIN); **San Luis Potosí State:** Río Huichihuayán, 18.VII.1999 (1 ♂, CNIN); Cascada de Micos, 22.VII.1999 (4 ♂♂, CNIN); Río Claro, Tamazunchale, 23.II.2000, P. Alonso-Eguíales *leg.* (1 ♂, CNIN); **Sinaloa State:** El Fuerte, 08.VI.2014, Y.M. Vega-Sánchez *leg.* (3 ♀♀, GCEUMSNH); **Veracruz State:** Apazapan, 07.IV.2010, Y.M. Vega-Sánchez *leg.* (2 ♂♂, GCEUMSNH); Apazapan, 03.VII.2019, L.F. Mendoza Cuenca and Y.M. Vega-Sánchez *leg.* (2 ♂♂, 1 ♀, CNIN).

IV. Capítulo III: Morphological Variation And Reproductive Isolation In The *Hetaerina americana* Species Complex

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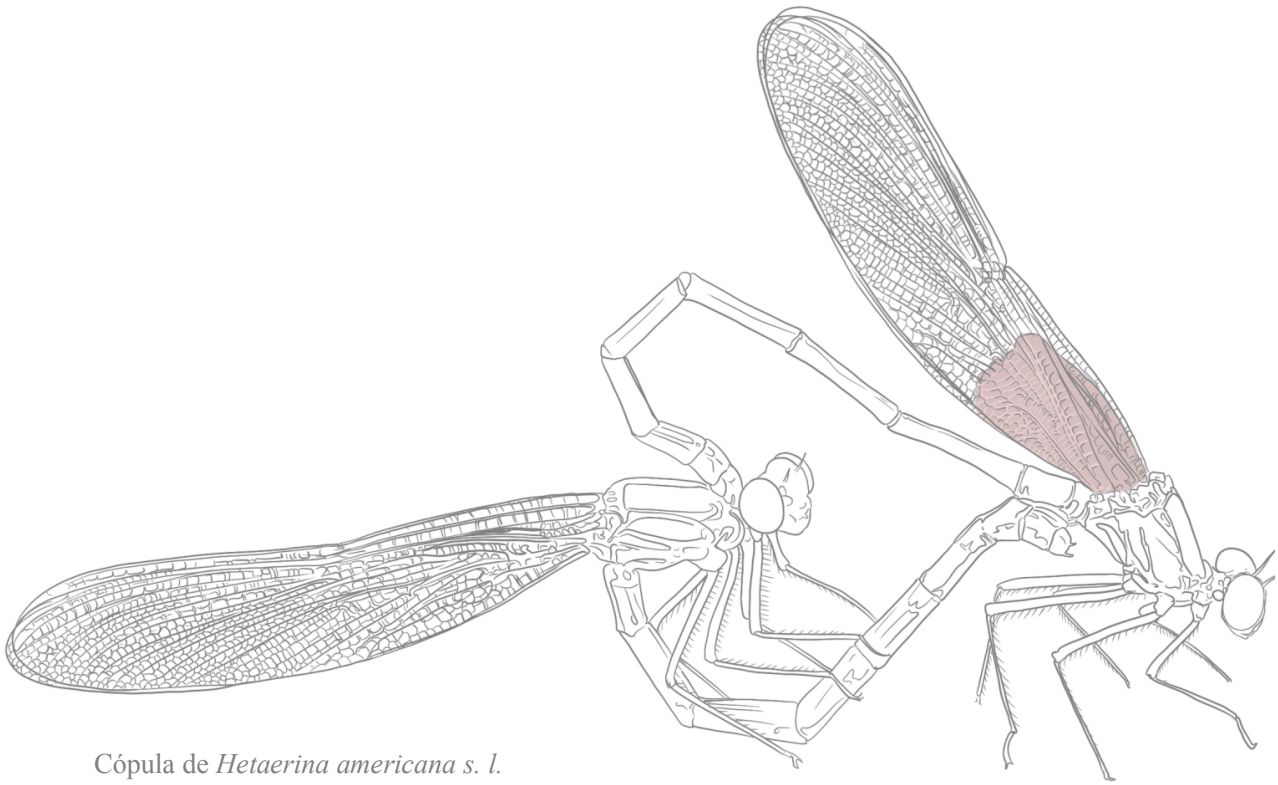

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Morphological variation and reproductive isolation in the *Hetaerina americana* species complex

Yesenia Margarita Vega-Sánchez[1]✉, Luis Mendoza-Cuenca[2] & Antonio González-Rodríguez[1]

Incomplete premating barriers in closely related species may result in reproductive interference. This process has different fitness consequences and can lead to three scenarios: niche segregation, sexual exclusion, or reproductive character displacement. In morphologically cryptic species, isolation barriers can be difficult to recognize. Here, we analyzed the morphological, behavioral, and genetic differences between two sympatric cryptic species of the genus *Hetaerina* to determine the characters that contribute the most to reproductive isolation and the effect of the high rates of behavior interference between the species. We found complete genetic isolation and significant differences in the morphometry of caudal appendages and wing shape, as well as body size variation between species. In contrast, we did not find clear differences in the coloration of the wing spot and observed high rates of interspecific aggression. Our results suggest that divergence in the shape of the caudal appendages is the principal pre-mating barrier that prevents interspecific mating. Moreover, a scenario of character displacement on body size was found. Nevertheless, size could play an important role in both inter- and intrasexual interactions and, therefore, we cannot differentiate if it has resulted from reproductive or aggressive interference.

In sympatric closely related species, there may be costs associated with incomplete premating barriers[1]. When there is incomplete species or mate recognition, processes of reproductive interference can occur. Reproductive interference is any interspecific intersexual interaction with a negative effect on the fitness of the species involved[1,2,3], resulting from wasted time, energy, nutrients or gametes. These fitness consequences depend on the type of reproductive interference: signal jamming, misdirected courtship, heterospecific mating attempts, erroneous female choice, heterospecific mating and hybridization[2]. Moreover, similar to competition, reproductive interference is density-dependent and can lead to three principal scenarios: niche segregation, sexual exclusion or reproductive character displacement[2–5]. Niche segregation refers to temporal or spatial habitat partitioning to avoid interspecific interactions[2,6], while sexual exclusion is the demographic displacement of one of the involved species (local extinction)[7]. Finally, reproductive character displacement is a divergence in traits related to the species recognition systems in sympatric conditions[2,8]. The last two processes may be a consequence of reproductive interference in a longer evolutionary lapse[7].

Hetaerina is a new world genus where all male individuals present a red wing spot on the base of each wing and display territorial behavior, characteristics that are absent in females[9]. Since *Hetaerina* females do not select males, and territorial males (i.e., sexually mature adults) do not respond aggressively to immature males, this strongly suggests that the evolution of wing pigmentation is driven by recognition between male competitors[10–14].

Moreover, reproductive interference (i.e., heterospecific mating attempts) has been documented to occur at high rates between some *Hetaerina* species (e.g., *H. americana*, *H. occisa*, *H. cruentata*) due to both a high frequency of species in sympatry and the similarity of the wing coloration of the females[11–13]. Also, aggressive interference is common in *Hetaerina* males[13,15]. Unlike reproductive interference, aggressive interference includes interspecific aggressive interactions such as fights, displays and territoriality, and can drive different evolutionary scenarios such as agonistic character displacement or competitive exclusion[3]. For *Hetaerina*, when aggressive interference occurs between species with contrasting wing pigmentation as with *H. titia* and *H. americana*, a process of agonistic character displacement has been suggested as a way to avoid these interspecific interactions[13].

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Species	N	Na (SE)	Ne (SE)	Ho (SE)	He (SE)	uHe (SE)	F
H. calverti	100	4 (0.89)	1.29 (0.01)	0.11 (0.05)	0.21 (0.06)	0.21 (0.07)	0.836*
H. americana	57	2 (0.55)	1.36 (0.22)	0.12 (0.11)	0.19 (0.11)	0.19 (0.11)	0.035

Table 1. Summary of genetic diversity estimators for *H. calverti* and *H. americana* individuals in sympatry at Apazapan, Veracruz. N = sample size; Na = mean number of different alleles; Ne = mean number of effective alleles; Ho = mean observed heterozygosity; He = mean expected heterozygosity; uHe = mean unbiased expected heterozygosity; F = mean fixation index; SE = standard error. *p values < 0.0001 after 10,000 permutations.

However, interspecific competition among *Hetaerina* males mostly involves species with similar wing spot coloration (e.g., *H. americana, H. occisa, H. cruentata*)[10].

Hetaerina americana had been recognized as a widely distributed species until very recently, when a cryptic species complex was suggested for the American rubyspot, possibly integrated by three species[16]. Moreover, this "species" has been extensively used as an ecological model for behavioral studies and diverse mating tactics have been described. In this paper, we studied two cryptic species of the American rubyspot complex that are formally described: *H. americana* and *H. calverti*. *Hetaerina americana* presents an extensive distribution from Chiapas in Mexico to Canada; in contrast, *H. calverti* is distributed in Honduras, El Salvador and Guatemala to the north of Mexico. The two species frequently occur in sympatry[16,17]. Furthermore, these species have indistinguishable coloration patterns to the naked eye and the diagnostic character for males is the shape of the superior caudal appendages; for females, morphological differentiation is less clear, and a genetic assignment is needed[17]. Therefore, these species are an excellent model to assess the effects of reproductive interference and make some inferences about the mechanisms that could maintain reproductive isolation through the detailed evaluation of differentiation in morphological characters such as wing coloration and the shape and size of caudal appendages, which are characters that contribute to species recognition in other odonate species[18–20].

Our main questions were (1) Is there complete reproductive isolation between the two cryptic species when they occur in sympatry? (2) If this is the case, how is this reproductive isolation maintained? (3) Which morphological characters are important for reproductive isolation? And (4) what is the evolutionary effect of interference between these two species? To assess these questions, we explored whether there is behavioral and morphological variation between *H. americana* and *H. calverti* and tested the possibility of hybridization with genetic markers, all under sympatric conditions.

Results

Genotyping and genetic diversity. Microsatellite data were obtained from 162 individuals. However, the H17 locus had to be discarded because it did not amplify in most of the individuals of *H. calverti*. In total 14 tandems were collected: 11 for the year 2017, and three for the year 2018. Of these, eleven corresponded to male individuals of *H. calverti* and three to male individuals of *H. americana*. The genotyping corroborated that none of these tandems corresponded to heterospecific mating.

The genetic diversity estimators showed similar values between species (Table 1). The inbreeding coefficient (F) was very high and significant in *H. calverti* and nonsignificant in *H. americana* (Table 1). According to FreeNA, there was evidence of null alleles at three loci of *H. calverti*, which could explain the high F values. Genetic differentiation was very high between the two species ($F_{ST} = 0.72$; $p < 0.0001$) without an important effect of null alleles on the estimation (corrected $F_{ST} = 0.76$).

For the genetic assignment, only 10 collected females corresponded to *H. americana* and 27 to *H. calverti* for the year 2017 and seven to *H. americana* and 33 to *H. calverti* for the year 2018. Three females were not identified due to problems with DNA quality. Individual *q-values* showed that mixed ancestry is almost nonexistent (only one individual showed 90% of one genetic group and 10% of the other). Therefore, if hybrid individuals are present, they should be at a very low frequency. The PCoA analysis obtained based on the genetic distances among all individuals also showed that there are two highly differentiated groups, which agree with the genetic groups suggested by STRUCTURE (Fig. 1).

Abundance and behavioral interactions. For the year 2017, 220 individuals were marked, and differences in the abundance of the two cryptic species were observed. In total, 60 males of *H. americana* and 160 males of *H. calverti* were marked. In the year 2018, a lower abundance of both species was found: 146 individuals were marked in total, although the proportions were more equitable: 54 males of *H. americana* and 92 of *H. calverti*.

We found interspecific differences in the number of days that males maintain a territory but only in one of the years. The following results are presented as mean ± SE. Males of *H. americana* maintained their territories for more days, during the year 2017 but not in the year 2018 (Supplementary Fig. S1; year 2017: *H. americana* 4.09 ± 0.53, *H. calverti* 1.44 ± 0.29, $X^2 = 22.11$, $p < 0.0001$; year 2018: *H. americana* 2.14 ± 0.14, *H. calverti* 2.36 ± 0.43, $X^2 = 0.07$, $p = 0.80$). We also found differences between both species in the average time duration of aggressive interactions depending on the opponent species and the year. In the year 2017, for the territorial males of *H. calverti*, aggressive intraspecific interactions were longer than interspecific interactions with males of *H. americana* or *H. occisa* (species also present at this site) ($X^2 = 8.33$, $p = 0.017$; Supplementary Fig. S1; Supplementary Table S1). In the case of territorial males of *H. americana*, aggressive interactions were longer with males of *H. calverti* than with males of their species or males of *H. occisa* ($X^2 = 7.20$, $p = 0.027$; Supplementary Fig. S1;

Journal : SREP 41598 | Article No : 14866 | Pages : 10 | MS Code : 14866 | Dispatch : 15-6-2022

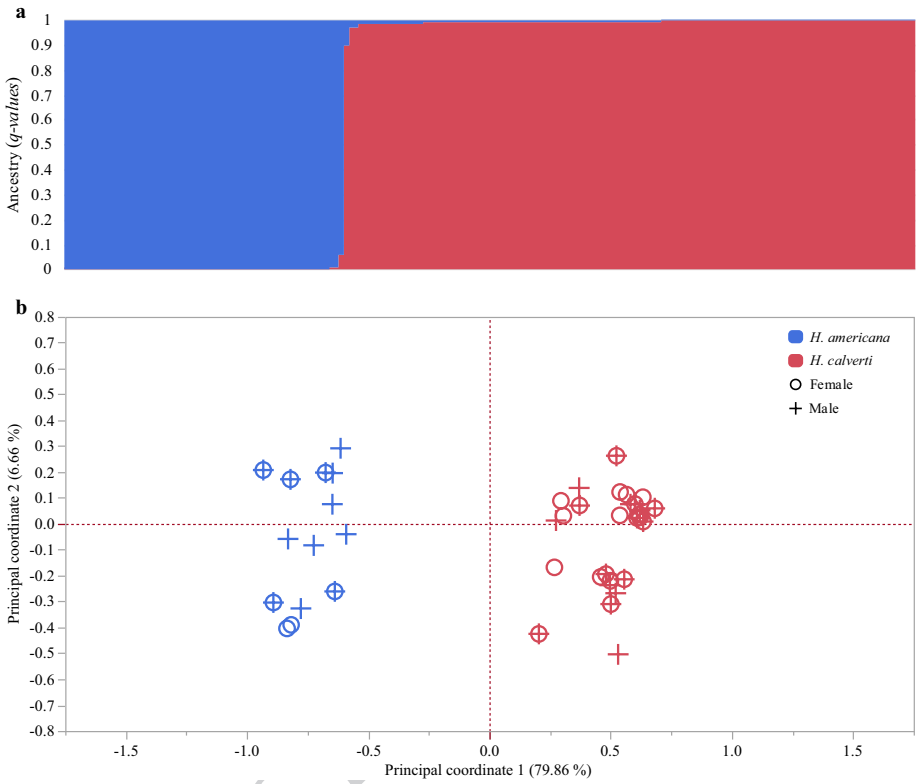

Figure 1. Genetic structure analyses. (**a**) STRUCTURE assignment analysis for K = 2 from five nuclear microsatellites for sympatric *H. calverti* and *H. americana* individuals in Apazapan, Veracruz. In the bar plot, individuals are represented by thin vertical lines, which are partitioned into K shaded segments representing each individual's estimated membership fraction. (**b**) Principal coordinate analysis (PCoA) for females and males collected in both years.

Supplementary Table S1). In the year 2018, no significant differences were found in the duration of aggressive territorial interactions in either *H. calverti* or *H. americana* (Supplementary Fig. S1; Supplementary Table S1).

Variation in secondary sexual traits. *Differences in size between species.* The two species differed significantly in size (Fig. 2; Supplementary Table S2 and S3). Males and females of *H. calverti* had larger wings and greater body length than males and females of *H. americana* regardless of the year of sampling (Fig. 2). Moreover, we found that there is an effect of the year of sampling on the female body length since individuals collected in the year 2017 were larger than those collected in the year 2018 (Supplementary Tables S2 and S3), but differences between species remained.

Additionally, the linear mixed effect model for comparing the body length of both species between localities under conditions of sympatry or allopatry showed significant differences (Species: Estimate = 0.79, E.E. = 0.34, t-value = 1.14, $p < 0.0001$; Locality type: Estimate = 0.44, E.E. = 0.56, t-value = − 0.78, $p = 0.89$; Interaction Species*Locality type: Estimate = 1.51, E.E. = 0.72, t-value = 2.07, $p = 0.037$). We found that differences in body length are enhanced in sympatry, with males of *H. calverti* being larger (41.04 ± 0.17 mm) than males of *H. americana* (38.90 ± 0.13 mm) (Supplementary Fig. S2).

Wing morphology and coloration patterns of wing spots. The Procrustes ANOVA conducted to determine shape variation both for hindwing (HW) and forewing (FW) in males showed significant differences between years of sampling and species but not for the interaction (Supplementary Table S3). Post-hoc multiple comparisons showed significant differences between species both in the same year of sampling and between years. The Procrustes ANOVA analysis used to determine interspecific shape variation in females was also significant for the year of sampling and species for both HW and FW (Supplementary Table S4). Post-hoc multiple comparisons only showed significant intra- and inter-year differences between species. The principal component analyses showed that the species present different wing shapes for both sexes (Supplementary Fig. S3).

Regression results showed no allometric effect on wing shape for males (*H. calverti* HW, $R^2 = 0.013$, $p = 0.713$; *H. calverti* FW, $R^2 = 0.042$, $p = 0.06$; *H. americana* HW, $R^2 = 0.043$, $p = 0.104$; *H. americana* FW, $R^2 = 0.045$,

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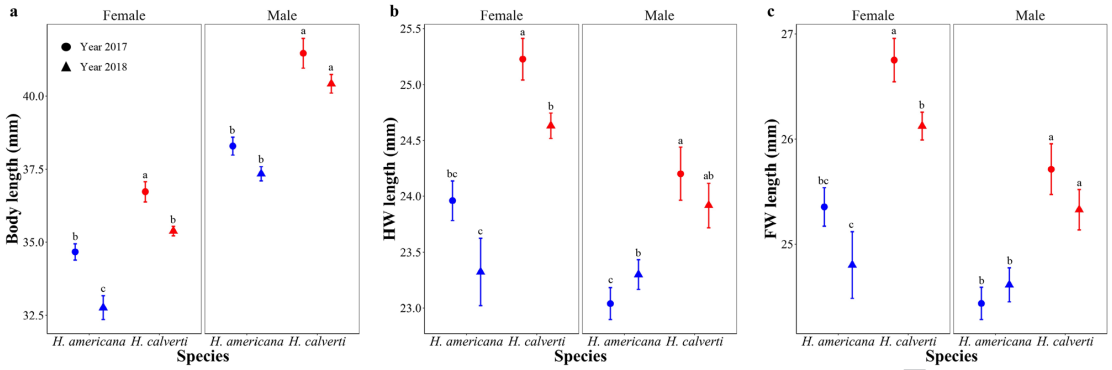

Figure 2. Variation in size between species. Differences in body length (**a**), hind wing length (**b**) and fore wing length (**c**) between species and years of sampling by sex. Means and standard errors are shown, different letters represent significant differences.

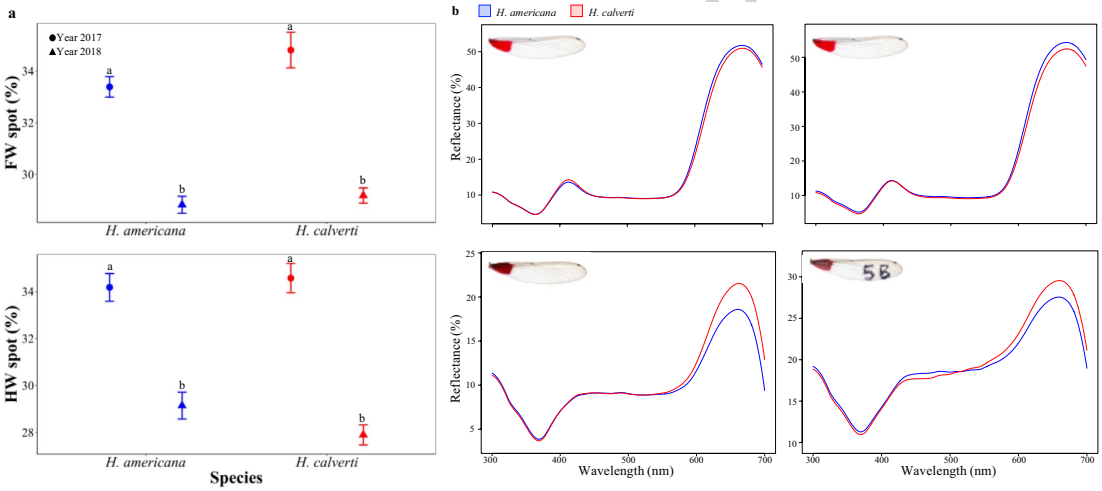

Figure 3. Variation in wing spots between species. (**a**) Variation in the percentage of FW (above) and HW (bottom) spots between species and years. (**b**) Reflectance patterns of FW (above) and HW (bottom), including inner (left) and outer (right) parts.

$p = 0.086$) nor females (*H. calverti* HW, $R^2 = 0.013$, $p = 0.713$; *H. calverti* FW, $R^2 = 0.044$, $p = 0.172$; *H. americana* HW, $R^2 = 0.024$, $p = 0.171$; *H. americana* FW, $R^2 = 0.063$, $p = 0.06$).

Related to coloration, we found no differences in the percentage of the wing spot with respect to the total wing area between species (Fig. 3a), but we observed a great difference in the percentage of the wing spot related to the year of sampling, with larger spots in the year 2017 (Supplementary Table S3; Fig. 3a). The comparison of reflectance spectra between the species showed an almost complete overlap for the FW, while, for the HW, it is possible to observe a subtle variation, especially in the peak of the long wave (670 nm) spectral region (Fig. 3b). Concordantly, our color analysis predicted that the vision system of *Calopteryx splendens* is not capable of discriminating chromatic differences between *H. calverti* and *H. americana* males. The JND values comparing both species were not above the discrimination threshold (i.e., JND > 1) for the *Calopteryx* vision model for either FW ($JND_{inner} = 0.262$, $JND_{outer} = 0.123$) and HW ($JND_{inner} = 0.432$, $JND_{outer} = 0.433$).

Variation in superior caudal appendages. The two first principal components of the PCA analysis using the Procrustes coordinates that describe the shape of the superior caudal appendages recovered 59.7% of the variation and showed two distinct groups, representing each species (Fig. 4a). These groups are also different in the discriminant analysis (Wilks' Lambda: $F = 73.82$, $p < 0.0001$).

We also found a positive relationship between male body length and the size of caudal appendages (Fig. 4b) (*H. americana*: $R^2 = 0.21$, $F = 7$, $p = 0.0134$; *H. calverti*: $R^2 = 0.17$, $F = 5.19$, $p = 0.0315$).

Journal : SREP 41598 | Article No : 14866 | Pages : 10 | MS Code : 14866 | Dispatch : 15-6-2022

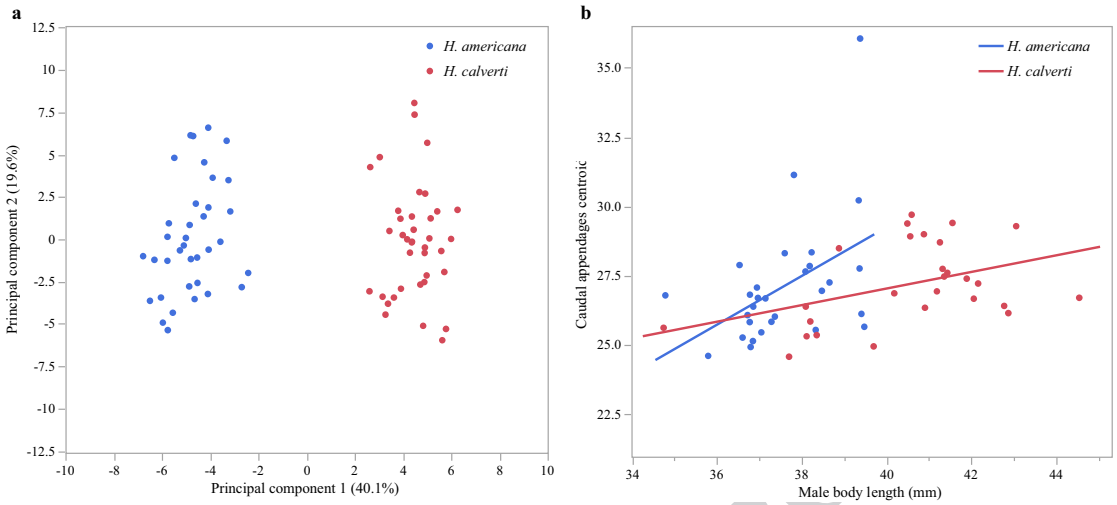

Figure 4. Differences in shape and size of caudal appendages. (**a**) Principal components analysis for the shape of superior caudal appendages of 77 males. (**b**) Relationship between the body length and the size of caudal appendages for each species.

Discussion

The mate/species recognition systems in Odonata are complex, and usually several sexual characters are involved. These characters could act as barriers (that are mostly pre-zygotic) hence affecting the reproductive isolation process. These characters could be body and wing coloration (i.e., behavioral/sexual isolation, e.g., *Calopteryx*), the shape of the male caudal appendages and the female prothorax (i.e., mechanical/sensorial isolation, e.g., *Enallagma*, *Ischnura*) and even the shape of the secondary male genitalia (i.e., mechanical/sensorial isolation, e.g., *Calopteryx*, *Polythore*, *Chalcopteryx*, etc.)[19–21]. Besides these characters, different behaviors such as territoriality and complex courtships can be involved in the mating process and species recognition systems[22].

In this study, the high genetic differentiation and the results of the Bayesian genetic assignment suggest that there is no hybridization between the two cryptic species and, therefore, that reproductive barriers are effective. It has been proposed that in sympatric species with overlapping mating seasons and that interact with each other (e.g., competition for territories), but do not hybridize, the existence of behavioral/sexual reproductive isolation could be inferred[1]. However, our results indicate that reproductive isolation between *H. calverti* and *H. americana* is not related to wing coloration which supports the idea that the wing spots have evolved through male–male competition.

Then, how is the reproductive isolation between these species maintained? The process of choosing a mate in *Hetaerina* involves two main phases: (1) the male's choice, which begins when a female flies into a male's territory and ends when a male grasps the female in flight without prior courtship. There is a high degree of reproductive interference (heterospecific mating attempts) between *Hetaerina* species, especially when the females have similar wing colorations (e. g. females of *H. occisa* and *H. americana*), but lessened interference when females are contrasting in wing coloration (i.e. females of *H. titia*, smoked-wing morphotype)[23], suggesting that males cannot discriminate similar females. Although in our case the females of *H. calverti* and *H. americana* are very similar in color patterns, we cannot discard that males may be able to differentiate heterospecific females. This is partially supported by the absence of heterospecific tandems in our observations, which suggests that another character could be involved in the recognition process when the males try to grasp a female in flight. However, the number of tandems analyzed is low and, thus, more data are necessary to make more robust inferences about possible male mate recognition prior to clasping. (2) The female's choice, which starts when the male has already chosen a female and the couple is in the tandem position. For other calopterygid species, wing coloration is the main character by which females recognize males[24], but this does not occur in *Hetaerina*[13]. The slight variation in wing spot coloration between these cryptic species supports this notion. Another character involved in species recognition and mate choice in odonates is the shape of the caudal appendages, and the females decide whether to copulate depending on the mechanical stimulation of these structures[18,22,25], and it is precisely this character that presents the greatest divergence between these cryptic species. Mechanical/sensorial isolation barriers contribute most to reproductive isolation in some odonates, especially in species that do not exhibit courtship or territorial behavior, such as many coenagrionids, although this barrier rarely leads to complete isolation by itself[19]. Other pre- and postzygotic barriers such as gametic and genetic incompatibility could also contribute to the complete reproductive isolation, which can be expected in highly diverged species like in this case[17]. Nevertheless, to evaluate these aspects, experimental analyses with different crosses are needed.

Even though behavioral/sexual isolation based on male wing coloration has been ruled out for *Hetaerina*[10,13], our results suggest that characters other than color must be involved in the reproductive isolation of these cryptic species. For example, body size was previously reported as a strong premating barrier in different animal

Journal : SREP 41598 | Article No : 14866 | Pages : 10 | MS Code : 14866 | Dispatch : 15-6-2022

groups[26–28]. In this context, male assortative mating by size has been described in *H. americana sensu lato*[29], and even though we cannot ensure interspecific discrimination of females based on their size, it has been widely recognized that assortative mating can promote or reinforce divergence even in incipient species and in the presence of gene flow[30,31]. Assortative mating in males of *H. americana* y *H. calverti* could have two causes: first, males prefer mates with specific trait values, in this case larger females because they may be more fertile (and not because they discriminate between species). Second, mating with females that match their own phenotype, for example, large or small males may be able to mate only with females of a similar size due to the isometric relationship between body size and the size of the caudal appendages, since, although the male may be capable of forming the tandem with the female and the shape of the caudal appendages may be adequate, the variation in size between these structures could modify the stimulation in the females and result in an unsuccessful mating attempt[25]. Moreover, males cannot force females to copulate[32]. Also, during the whole mating process, males should avoid harassment from other males (they attempt to break up the tandem and steal the female) and take the female to oviposition sites. This may be more likely if there is not a big difference in the body size between individuals.

Moreover, size has an important effect on competition between males[33]. Larger males can maintain a territory longer and therefore have more mating opportunities[34,35]. However, all studies to date have considered *H. americana* as a single species[29,35–37] and it is unknown which is the effect of the possible presence of cryptic species at the localities where the studies were carried on. We suggest that body size may be an important character that usually is not taken into account in analyses of interspecific interactions, but experimental studies would be needed to define its role in both inter- and intrasexual interactions.

Wing shape and body size have a critical aerodynamic effect on flight performance[38–40]. The variation in flight performance has been related to behaviors such as territoriality and courtships, and despite the fact that in *Hetaerina* there is no courtship, this wing shape variation could be associated with the recognition of conspecifics in flight (e.g., before forming a tandem).

Behavioral interference (i.e., reproductive and aggressive interference[3]) can lead to several patterns, such as species segregation into particular habitats, reproductive or agonistic character displacement, and sexual or competitive exclusion[3]. Drury et al.[11] suggested the catch-22 hypothesis, which states that if the females of sympatric species are similar phenotypically (in terms of coloration), males will be unable to discriminate between them. Therefore, the interference will remain indefinitely since there is no selection on trait divergence related to the recognition of mates by males. However, our results indicate phenotypic variation and even a character displacement pattern associated with body size.

The character displacement pattern on body size could help to reduce interference but in the case of heterospecific rivalry interactions, results suggest that differences in body size do not necessarily reduce the intensity of aggressive interspecific interactions. Then, the variation on body size may be related to reduce reproductive interference, but this needs to be tested.

Other effects of behavior interference such as niche segregation or sexual or competitive exclusion may have evolved in these species. Niche segregation has been reported for *H. cruentata* and *H. occisa* in sympatry with *H. americana* because the former prefers shadier territories than the latter, avoiding the interspecific encounters[6].

Finally, a latent exclusion (the demographic displacement of one of the involved species; local extinction) could be taking place because *Hetaerina americana* has the lowest abundance at the site, even lower than *H. occisa*, which could be as common as *H. calverti* (Y. M. Vega-Sánchez, personal observation). In addition, *Hetaerina americana* males are smaller, and males of *H. calverti* could displace them because body size is an important character that predicts males' success in maintaining a territory and therefore of their fitness. Interestingly, we observed that males of *H. americana* remained in territories for a longer time (although only in year 2017). However, it has been suggested that this may happen when the territories vary in quality[41], for example, males established in shadier territories that have fewer passing females should experiment less aggressive interactions than males established in better territories and then remain longer in their territories[6,33]. We need to analyze more sympatric localities to test if this process, a possible competitive exclusion, is occurring.

In conclusion, we found, for the cryptic species studied here, that caudal appendages seem to be the principal trait related to the avoidance of heterospecific mating. However, understanding why other traits such as coloration (as in other *Hetaerina* species) have not diverged between cryptic species remains an unanswered question. Moreover, we showed that body size also may be an important character that could enhance reproductive isolation, reducing heterospecific mating. Besides, body size could play an important role in intrasexual aggressive interactions. Nevertheless, more data are needed to analyze the evolutionary and ecological importance of size in these organisms.

Materials and methods

Sampling. The study was conducted at Apazapan, Veracruz, Mexico (19°19′30.14″ N, 96°43′29.63″ W), in a perennial tributary stream of the Pescados River. Using mark-recapture techniques, we studied both species in two field campaigns, the first in April–May 2017 (hereafter year 2017) and the second in February 2018 (hereafter year 2018), on average 12 days per year. We marked all male individuals and assigned them to one of four age categories: teneral, young, mature and old; these categories are determined by the appearance of the wings (stiffness, brightness, damage)[42]. Moreover, we captured individuals that were found in tandem and that had copulated. At the end of each campaign, we collected 20 male individuals of each species and 40 females (females cannot be separated into species due to the lack of diagnostic characters). We used all these individuals to analyze genetic and morphological variation (see below).

Genetic diversity and gene flow. We genotyped all males and females (including tandems) collected at the end of each campaign to determine if there is evidence of genetic exchange between the two cryptic species. The genetic analysis was performed using the same six microsatellite loci and protocol used by[16]. With this data, we obtained different genetic diversity estimators: number of alleles, number of effective alleles, observed and expected heterozygosity, unbiased expected heterozygosity and the interbreeding coefficient using GenAlex v. 6.0[43]. We also estimated the null allele frequencies for each locus and species in FreeNA v.1[44].

To estimate the degree of genetic differentiation among the two species we performed an analysis of molecular variance (AMOVA) in Arlequin v.3.5[45]. Additionally, Bayesian analysis in STRUCTURE[46] was performed, which allowed to determine the genetic ancestry (*q-value*) for each individual and evaluate the possibility of genetic admixture, which would support the occurrence of hybridization between the two species. The genetic relationships between individuals were also visualized by a Principal Coordinates Analysis (PCoA) using the GenAlEx program.

Behavioral interactions between males. We located the territories of all males of both species along one kilometer of the river and marked males on the hindwing with a consecutive number and assigning a letter "A" for *H. calverti* and "B" for *H. americana* (on the basis of the diagnostic characters described by[17]), using a permanent marker. Marked individuals were observed daily, and those males that were present for at least 3 days in the same part of the river were considered territorial[33]. Then, we selected 15 territorial males of each species (i.e., defender individuals) to determine the frequency and duration of their aggressive interactions (intra- and interspecific) during observation periods of 30 min. Non-parametric Kruskal–Wallis, Wilcoxon and Chi-square tests were carried out to assess differences in the duration of the interactions between species and the number of days the males maintain a territory. These analyses were performed for each year separately and carried out in JMP v.15 (SAS Institute Inc).

Analyses of secondary sexual traits. *Variation in size.* The males and females collected at the end of each campaign were photographed and their four wings were scanned along with a scale. Then, the total body length and the forewings and hindwings length were measured using the Image J software[47]. The total body length was estimated as the straight line from head to the caudal appendages for males and from head to the last abdominal segment for females. Wing length was estimated from the initial part of the costal vein (C) to the end of the radial vein 1 (R1) for both forewing (FW) and hindwing (HW). For these data, two-way analyses of variance (two-way ANOVA) were performed using species and the sampling year as independent variables. The analyses were performed separately for each sex. Post-hoc multiple comparisons were performed using Tukey HSD analyses for each data set.

Moreover, we analyzed the variation in body length for the two species in conditions of sympatry and allopatry through their geographical range using the complete data set of[17] plus the data obtained in this study since a possible process of reproductive character displacement has been suggested for these species[17]. In total, we measured 602 individuals distributed in 27 allopatric and 12 sympatric localities for *H. americana* and 9 allopatric and 10 sympatric localities for *H. calverti* (Supplementary Table S5). With these data, we performed a linear mixed effects model in the *lme4* package[48] in the R software, using body length as the response variable, species and locality type (allopatric or sympatric) as fixed effects and the locality as random effect.

Variation in wing morphology and coloration. We analyzed wing morphology for all males and females using geometric morphometric techniques. We used the images of individuals that had complete left FW and HW to compare wing shape between males and females of both species in two consecutive years. Ten anatomical marks (i.e., landmarks) were placed on the outline of the FW and HW on the intersection with principal veins (Supplementary Fig. S4), which are homologous landmarks on all individuals of both sexes[40]. Two points were also placed on the millimetric ruler as a scale factor, using Geomorph program version 3.3.2[49]. A Generalized Procrustes Analysis was conducted to obtain the coordinates that were used as variables of shape. We performed Procrustes ANOVA with 9999 permutations, including year and species as independent variables to compare for each sex the shape of FW and HW. We also performed a Principal Components Analysis of the variance–covariance matrix to visualize the wing shape variation separately for each sex.

Multivariate regression was performed to evaluate size effects on wing shape (allometry) using shape as the dependent variable and the natural logarithm of the centroid size as the independent variable for each species, sex, and both wings[50].

We also compared the percentage area covered by the wing spots with respect to the total wing area, using the ImageJ program for both FW and HW. With these data, we performed two-way ANOVAs in JMP v.15 (SAS Institute Inc) to test for differences between the two species and between years.

The spectral signature of the wing spots of both species was characterized using an Ocean Optics USB2000 spectrophotometer equipped with a xenon pulse lamp (PX2) in a UV–VIS range of 300–750 nm. The spectrometer was calibrated with a diffuse reflectance white standard from Ocean Optics (WS-1). We put the wings on a ColourWorker (X-rite) 80% grey standard chart and obtained these measurements on a 45° angle using an RHP probe support (Ocean Optics, Dunedin, FL). We made four measurements, including the outer and inner part of the right FW spot and the outer and inner part of the right HW spot. These data were obtained for the 28 males collected in the year 2017. We used the package *pavo2* v. 2.6.1 to analyze reflectance spectra[51]. The software allows to manage, process and visualize reflectance spectra in color spaces and psychophysical models for color discrimination, which allows the estimation of potential receptor quantum catch of the wing-spot coloration (i.e., Receptor Noise Limited Model[52]).

Journal : SREP 41598 | Article No : 14866 | Pages : 10 | MS Code : 14866 | Dispatch : 15-6-2022

In order to evaluate how different both *Hetaerina* species are in terms of their wing spot coloration, we evaluated the ability of an Odonata visual system to discriminate between *H. calverti* and *H. americana* species by their wing spot. Since information about the spectral sensitivities, receptor proportions and receptor noise of *Hetaerina* is not available, we seek the species most closely related to *Hetaerina* for which these data are available. We used pooled sensitivity data of *Calopteryx splendens* (λ_{max} 366, 480, 552 and 640 nm[53]), a receptor proportion of 3:2:2:1 (i.e., ultraviolet, shortwave, mediumwave, and longwave[54]) and receptor noise was adjusted to 0.2 as for other Odonata visual systems[53]. As information for the illuminants, we used the environment "bluesky"[55] included in the package to model the potential quantum catch of the species. The data for the receptor quantum catch was obtained from fitting a psychophysical visual model of the reflectance spectra of the inner and outer parts of the wings.

We analyzed chromatic contrasts by determining the probability that the *Calopteryx* visual model could discriminate between *H. calverti* and *H. americana* male FW and HW (including inner and outer parts) wing spots. Based on a discrimination threshold of 1 Just Noticeable Difference (JND) tridimensional space (where JND values < 1 imply that the two species are indistinguishable), as commonly used in studies of color discrimination[56], taking a conservative approach to the interpretation of psychophysical discrimination relative to known discrimination thresholds[57].

The shape of the caudal appendages. To analyze the variation in the shape of the caudal appendages, we took photographs of the superior caudal appendages in a dorsal view of 80 males (20 per species and year) using a stereoscopic microscope with a scale. First a "fan" was superimposed on each image to guide the placement of semilandmarks using MakeFan v.6 program[58]. Then, *x* and *y* coordinates were digitalized at six homologous points of the superior caudal appendage and 22 semilandmarks using TpsDig v.2 program[59] (Supplementary figure 1 in[16]). These anatomical marks describe the contour shape of the superior caudal appendage in a dorsal view. Once the landmarks and semilandmarks were placed, a Procrustes superimposition was performed using CoorGen v.7 program[58]. Once the coordinates were obtained, they were used as morphological variables to perform principal components and discriminant analyses in JMP v.15 (SAS Institute Inc).

Moreover, we tested for isometric allometry between the male body length and the size of superior caudal appendages using the centroid; we performed a linear regression in JMP v.15 (SAS Institute Inc) for each species.

Data availability

The datasets generated and analyzed during the current study are available from the corresponding author upon request.

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Author contributions

Y.M.V.S., L.M.C and A.G.R. designed the study. Y.M.V.S collected the samples and behavioral data and performed lab work. Y.M.V.S., L.M.C. and A.G.R. performed statistical and morphometric analyses. Y.M.V.S. drafted the manuscript. All authors completed and approved the final manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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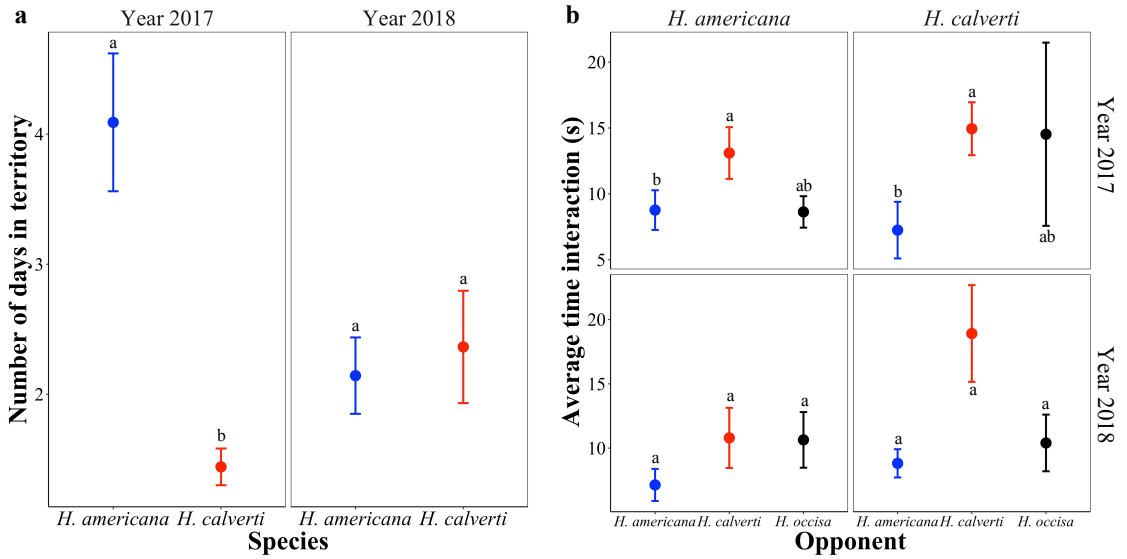
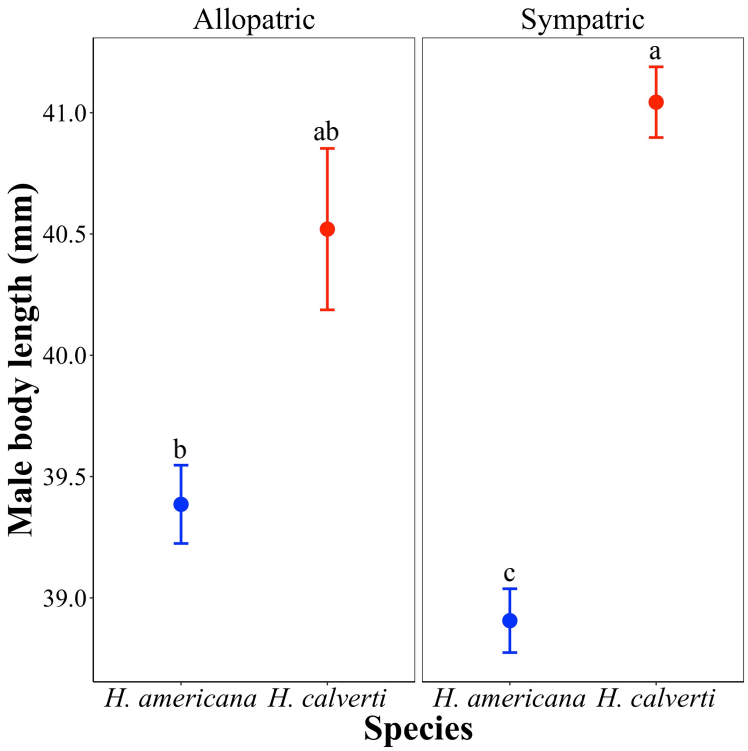
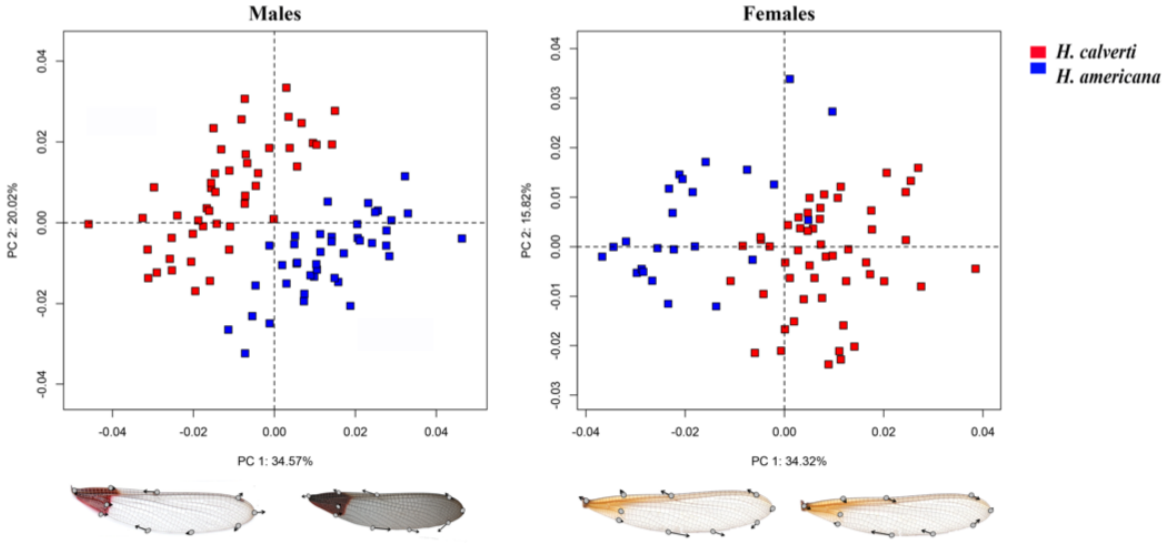
Supplementary Figure S1. Territorial behavioral variation between species. a) Number of days that males retain a territory. b) Average time duration of aggressive interactions that males of *H. americana* and *H. calverti* spend with conspecific or heterospecific males for the two sampling years. Means and standard errors are show, different letters represent significant differences.



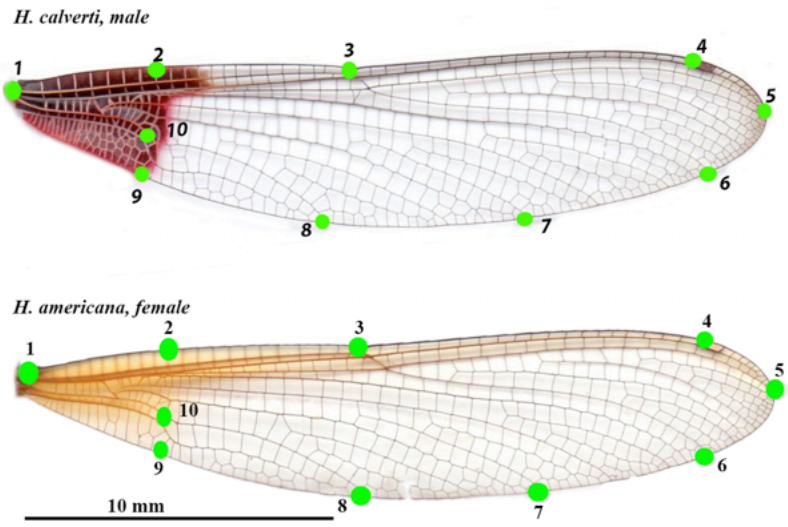
Supplementary Figure S2. Change in male body length between species in allopatry and sympatry. Means and standard errors are show, different letters represent significant differences.



Supplementary Figure S3. Principal component analyses for the wing shape for males and females.



Supplementary Figure S4. Landmarks (green dots) used for geometric morphometric analyses of the wing shape.



Supplementary Table S1. Non-parametric Wilcoxon analyses for time of interactions between species pair.

Defender	Year	Comparisons	Z	P value
H. americana	2017	*H. americana vs H. calverti*	2.589	0.0096*
	2017	*H. americana vs H. occisa*	1.229	0.2192
	2017	*H. calverti vs H. occisa*	-1.625	0.1042
	2018	*H. americana vs H. calverti*	0.8406	0.4006
	2018	*H. americana vs H. occisa*	1.130	0.2548
	2018	*H. calverti vs H. occisa*	0.4255	0.6704
H. calverti	2017	*H. americana vs H. calverti*	2.8763	0.0040*
	2017	*H. americana vs H. occisa*	1.5712	0.1161
	2017	*H. calverti vs H. occisa*	-0.5315	0.5951
	2018	*H. americana vs H. calverti*	1.9558	0.0505
	2018	*H. americana vs H. occisa*	0.1749	0.8611
	2018	*H. calverti vs H. occisa*	-1.4539	0.1460
*P values < 0.01				

Supplementary Table S2. Summary of wings and body size means and standard errors (SE) for females and males of each species.

Species	Sex	Year	Number of samples	Length (mm)	SE (mm)
Hindwings					
H. americana	Female	2017	10	23.96	0.18
H. americana	Female	2018	7	23.32	0.30
H. americana	Male	2017	20	23.04	0.14
H. americana	Male	2018	17	23.30	0.13
H. calverti	Female	2017	26	25.22	0.19
H. calverti	Female	2018	33	24.63	0.11
H. calverti	Male	2017	17	24.20	0.24
H. calverti	Male	2018	23	23.92	0.20
Forewings					
H. americana	Female	2017	10	25.35	0.18
H. americana	Female	2018	7	24.80	0.32
H. americana	Male	2017	20	24.44	0.15
H. americana	Male	2018	17	24.61	0.16
H. calverti	Female	2017	26	26.75	0.21
H. calverti	Female	2018	33	26.12	0.13
H. calverti	Male	2017	17	25.71	0.24
H. calverti	Male	2018	23	25.33	0.19
Body size					
H. americana	Female	2017	10	34.67	0.28
H. americana	Female	2018	7	32.76	0.41
H. americana	Male	2017	20	38.19	0.31
H. americana	Male	2018	17	37.35	0.24
H. calverti	Female	2017	26	36.73	0.35
H. calverti	Female	2018	33	35.38	0.16
H. calverti	Male	2017	17	41.46	0.51
H. calverti	Male	2018	23	40.42	0.32

Supplementary Table S3. Two-way ANOVA of the differences in the body length, wing length and percentage of wing spots for species, sex and season. d.f.= degrees of freedom; FW=forewing; HW= hindwing.

Variables	d.f.	Sum of squares	F	Prob> F
Body length for females				
Species	1	70.063453	41.5948	<0.0001
Year	1	33.888257	20.1185	<0.0001
Interaction	1	1.005582	0.597	0.4423
FW length for females				
Species	1	23.769169	31.9748	<0.0001
Year	1	4.456791	5.9954	0.0168
Interaction	1	0.018217	0.0245	0.876
HW length for females				
Species	1	21.297432	35.9259	<0.0001
Year	1	4.877674	8.228	0.0054
Interaction	1	0.005624	0.0095	0.9227
Body length for males				
Species	1	184.639	77.9211	<0.0001
Year	1	18.72907	7.904	0.0063
Interaction	1	0.04357	0.0184	0.8925
FW length for males				
Species	1	18.786383	27.0476	<0.0001
Year	1	0.210241	0.3027	0.5839
Interaction	1	1.506546	2.169	0.1451
HW length for males				
Species	1	15.020281	22.6839	<0.0001
Year	1	0.002902	0.0044	0.9474
Interaction	1	1.403016	2.1189	0.1498
Percentage of FW spot				
Species	1	0.00959683	1.794	0.1838
Year	1	0.79971331	149.492	<0.0001
Interaction	1	0.01131587	2.1153	0.1493
Percentage of HW spot				
Species	1	0.01169479	3.5197	0.0639
Year	1	0.60164035	181.0734	<0.0001
Interaction	1	0.00283769	0.854	0.3579

Supplementary Table S4. Procrustes ANOVA results for species, sex and year wing shape variation. d.f. = degrees of freedom; FW=forewing; HW= hindwing; SS= sum if squares; MS= mean square value; Rsq= R-square.

Factor	d.f.	SS	MS	Rsq	F	Z	Prob >F
Male HW							
Year	1	0.003	0.003	0.036	4.25	2.70	0.004
Species	1	0.019	0.019	0.237	27.86	6.96	0.001
Year*species	1	0.0009	0.0009	0.011	1.33	0.71	0.24
Residuals	84	0.059	0.0007	0.715			
Total	87	0.083					
Male FW							
Factor	d.f.	SS	MS	Rsq	F	Z	Pr(>F)
Year	1	0.004	0.003	0.045	5.36	3.39	0.001
Species	1	0.020	0.020	0.227	27.03	6.63	0.001
Year*species	1	0.0009	0.0009	0.010	1.28	0.68	0.265
Residuals	84	0.062	0.0007	0.716			
Total	87	0.087					
Female HW							
Factor	d.f.	SS	MS	Rsq	F	Z	Pr(>F)
Year	1	0.044	0.004	0.073	6.83	3.83	0.001
Species	1	0.008	0.009	0.148	13.78	5.70	0.001
Year*species	1	0.0009	0.001	0.016	1.51	1.04	0.157
Residuals	72	0.046	0.0006	0.762			
Total	75	0.604					
Female FW							
Factor	d.f.	SS	MS	Rsq	F	Z	Pr(>F)
Year	1	0.005	0.006	0.090	8.59	4.25	0.001
Species	1	0.009	0.009	0.144	13.83	5.05	0.001
Year*species	1	0.0006	0.0007	0.011	1.006	0.242	0.406
Residuals	72	0.0487	0.0006	0.754			
Total	75	0.646					

Supplementary Table S5. Summary of body length means and standard errors (SE) for locality and locality type by species. N= Sample size. Data obtained from [14].

Species	Locality	Locality type	N	Mean body length (mm)	SE (mm)
Hetaerina americana	Acahuizotla	Allopatric	26	39.38	0.340
Hetaerina americana	Amacuahutitlan	Allopatric	10	41.23	0.448
Hetaerina americana	Arroando Ajuchitlan	Allopatric	11	38.64	0.401
Hetaerina americana	Arroando Quilamula	Allopatric	32	38.02	0.360
Hetaerina americana	Camichines	Allopatric	9	38.34	0.230
Hetaerina americana	Chupicuaro	Allopatric	10	37.85	0.662
Hetaerina americana	Cocoyotla	Allopatric	7	41.99	0.309
Hetaerina americana	Comitancillo	Allopatric	6	39.89	0.846
Hetaerina americana	El Borbollon	Allopatric	13	37.20	0.538
Hetaerina americana	El Jagueand	Allopatric	7	41.03	0.474
Hetaerina americana	El Limon de Cuauchichinola	Allopatric	5	39.64	0.949
Hetaerina americana	La Mintzita	Allopatric	8	40.28	0.430
Hetaerina americana	La Palma, Huatulco	Allopatric	3	39.42	0.453
Hetaerina americana	La Poza Madre	Allopatric	2	42.94	0.150
Hetaerina americana	Larimer County	Allopatric	3	46.28	0.277
Hetaerina americana	Las Juntas	Allopatric	5	41.37	1.053
Hetaerina americana	Los Yesos	Allopatric	5	41.73	0.738
Hetaerina americana	Momax	Allopatric	7	40.29	0.737
Hetaerina americana	Norte de la posada de la Paz	Allopatric	6	38.02	0.444
Hetaerina americana	Rancho Lo de Campa	Allopatric	10	37.78	0.337
Hetaerina americana	Rio Ayutla, Ejido Santa Maria de los Coocs	Allopatric	2	43.30	1.075
Hetaerina americana	Rio Conchos	Allopatric	7	41.19	0.242
Hetaerina americana	San Agustin	Allopatric	3	35.70	0.734
Hetaerina americana	San Nicolas	Allopatric	8	38.65	0.491
Hetaerina americana	San Pedro Martir	Allopatric	4	36.15	0.926
Hetaerina americana	Tomochic	Allopatric	2	38.83	0.835
Hetaerina americana	Villaldama	Allopatric	8	42.44	0.153
Hetaerina americana	Antiguos Mineros del Norte Las Teclas	Sympatric	2	41.06	2.905
Hetaerina americana	Apazapan	Sympatric	40	37.80	0.199
Hetaerina americana	Chiapa de Corzo	Sympatric	3	38.10	0.456
Hetaerina americana	El Fuerte	Sympatric	2	39.58	0.220
Hetaerina americana	El limon	Sympatric	55	39.38	0.315
Hetaerina americana	Ixtlahuacan	Sympatric	9	39.87	0.516
Hetaerina americana	Jiliotupa	Sympatric	4	40.92	1.058
Hetaerina americana	Nuevo Recuerdo	Sympatric	6	39.90	0.451
Hetaerina americana	Puente Ayutla	Sympatric	2	40.92	0.135
Hetaerina americana	Puente, Arroando Guajiniquil	Sympatric	5	39.90	0.738
Hetaerina americana	San Pedrito Chicozapote	Sympatric	11	39.19	0.667
Hetaerina americana	Santiago Dominguillo	Sympatric	72	38.63	0.183
Hetaerina calverti	Arroyo, Poza Azul	Allopatric	4	37.17	0.868
Hetaerina calverti	Cascada de Micos al NO de Cd. Valles	Allopatric	4	42.18	0.560
Hetaerina calverti	Churince Poza Churince	Allopatric	5	39.55	0.549
Hetaerina calverti	El Platanal	Allopatric	7	40.74	0.357
Hetaerina calverti	La Poza Azul	Allopatric	3	40.12	0.471
Hetaerina calverti	Pisaflores	Allopatric	2	41.76	0.390
Hetaerina calverti	Rancho Orozco Poza Escobedo	Allopatric	2	41.29	2.800
Hetaerina calverti	Rio Negro, Sacapulas	Allopatric	5	40.82	0.410

Hetaerina calverti	Rio San Marcos	Allopatric	3	42.43	1.272
Hetaerina calverti	Antiguos Mineros	Sympatric	7	40.32	0.827
Hetaerina calverti	Apazapan	Sympatric	42	40.84	0.278
Hetaerina calverti	Chiapa de Corzo	Sympatric	2	40.79	0.435
Hetaerina calverti	El limon	Sympatric	20	41.90	0.444
Hetaerina calverti	Nuevo Recuerdo	Sympatric	3	42.74	0.739
Hetaerina calverti	Puente Ayutla	Sympatric	7	41.83	0.554
Hetaerina calverti	Arroando Guajiniquil	Sympatric	7	40.42	0.363
Hetaerina calverti	San Pedrito Chicozapote	Sympatric	2	39.64	0.545
Hetaerina calverti	Santiago Dominguillo	Sympatric	41	40.80	0.234
Hetaerina calverti	Zaragoza	Sympatric	6	41.63	0.282

V. CAPÍTULO IV: GENOMIC DIFFERENTIATION IN THE *HETAERINA AMERICANA* CRYPTIC SPECIES COMPLEX

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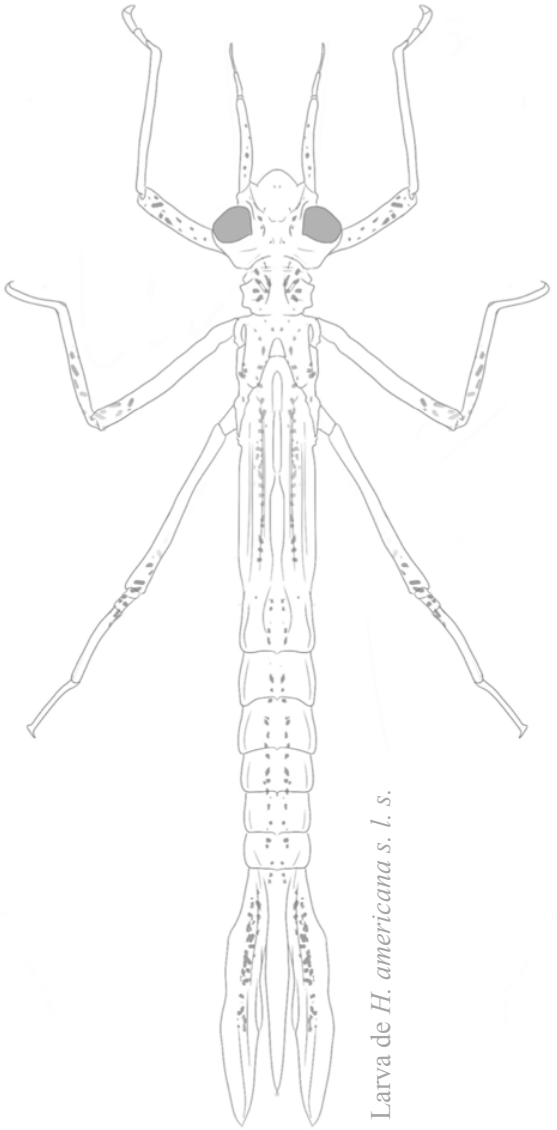

Larva de *H. americana s. l. s.*

Genomic differentiation in the *Hetaerina americana* cryptic species complex

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Abstract

The evolution of reproductive barriers, that is, the speciation process, implies limitation of gene flow between populations. This limitation can generate different patterns of genomic differentiation throughout the speciation continuum that, when analyzed, may provide insight into causal evolutionary forces such as, for instance, natural selection. In this study, we analyzed a complex of cryptic species of the genus *Hetaerina*. This complex includes *H. americana* and *H. calverti*; however, in *H. americana* two highly differentiated genetic groups have been previously detected, which, we hypothesize, may correspond to different species with low morphological variation. We used next-generation sequencing to obtain SNP information for 90 individuals belonging to the different potential species in the complex and carried out differentiation tests to identify divergent genetic groups. The results from STRUCTURE and discriminant analysis of principal components (DAPC), based on almost 5,000 SNPs, indicated the presence of three highly differentiated groups. More than 80% of the loci presented G_{ST} values above 0.5 in pairwise comparisons, which suggests a considerable degree of genetic isolation among the suggested species. We also identified several loci under selection among the putative species, which were associated with temperature and precipitation variables. Based on these results, we suggest that *H. americana* is constituted by two cryptic species, which may be reproductively isolated by ecological barriers related to local adaptation; morphological variation is minimal and therefore mechanical barriers are probably less effective compared to other species as *H. calverti*.

Keywords: Speciation, divergent selection, Odonata, ecological speciation, reproductive isolation

1. Introduction

The evolution of reproductive isolation, a key aspect of speciation, starts with the limitation of gene flow among populations caused by different types of barriers (Stankowski and Ravinet, 2021). These barriers can act at any stage in the lifecycle of an organism and can be intrinsic (e.g., gamete incompatibility) or extrinsic (e.g., different habitat preferences) (Coyne and Orr, 2004; Stankowski and Ravinet, 2021).

The speciation process could also be promoted by the geographic distributi0on of populations. When divergence occurs under geographic isolation (i. e., allopatric populations), isolated populations may experience differentiation at many genomic regions by the combination of divergent natural selection and genetic drift. Unlike, when divergence occurs in the presence of gene flow (under geographical situations of secondary contact, parapatry or sympatry), differentiation is expected to occur mostly at the loci that are targets of divergent selection and linked genomic regions (Nosil et al., 2021).

To date, most cases suggest a positive correlation between the strength of reproductive isolation and the genetic distance between species pairs (Matute and Cooper, 2021; Roux et al., 2016; Sánchez-Guillén et al., 2014). Therefore, comparing multiple closely related populations or species that exhibit different levels of genomic divergence can help to unravel the factors determining how far speciation has proceeded (Nosil et al., 2021). However, genetic divergence can also be strongly impacted by factors other than those related to reproductive isolation, such as differences in population size, isolation by distance, local populations bottlenecks, the selection at non-barrier loci or by geographic isolation (Stankowski and Ravinet, 2021).

In the suborder Zygoptera (Odonata), this positive correlation between genetic distances and reproductive isolation has been suggested (Sánchez-Guillén et al., 2014) and the reproductive isolation usually is related to sexual and mechanical barriers (Svensson et al., 2006; Sánchez-Guillén et al., 2012). Also, the speciation process could be driven, mainly, by "nonadaptive" forces as sexual selection (Wellenreuther and Sánchez-Guillén 2016). Nevertheless, some cases of ecological speciation have been described (Damm et al., 2010; Jordan et al., 2003; McPeek et al., 1996), where variation in ecological factors as predators, temperature and water body type can drive the evolution of reproductive isolation.

Hetaerina (Calopterygidae) is a mainly Neotropical genus of damselflies, commonly known as rubyspots, comprising 39 species. Rubyspots are characterized by their territorial behavior and,

when two or more species occur in sympatry, high degrees of reproductive interference have been observed (Drury et al., 2015). The most emblematic member of the genus is "*H. americana"* (the American Rubyspot), which has been considered to have a wide distribution from Nicaragua to Canada and therefore it is capable to inhabit a great variety of biomes as temperate forests, tropical dry forests, desert scrubs, etc. However, Vega-Sánchez et al. (2019) reported the existence of three differentiated groups within this taxon based on genetic and morphological data and suggested that these groups may correspond to different species. One group is distributed in the United States to the north of Mexico (*H. americana North* hereafter), a second group is mainly distributed in the center and south of Mexico (*H. americana South* hereafter), and a third group is found to occur from northern Mexico to Guatemala. This last group was later described as a new species, *H. calverti* (Vega-Sánchez et al., 2020). *Hetaerina americana North* and *South* can be found in sympatry with *H. calverti* but there is no record of sympatric localities for *H. americana North* and *South*. Also, evidence of balancing selection on the mitochondrial gene COI was found, possibly related to the gradient of temperature found along the whole distribution of the three groups (Vega-Sánchez et al., 2019).

Additional analyses indicated that *H. calverti* is probably isolated from the other two groups by mechanical barriers related to the shape of caudal appendages (Vega-Sánchez et al. *in press*) but morphological variation on this trait between *H. americana North* and *South* is less significant, suggesting that the three entities represent different stages of the speciation continuum and that other isolation mechanisms besides mechanical barriers are present. Based on this, we hypothesized that niche differences also have contributed to the isolation between these putative species.

In this study we implemented genomic analyses to unravel the evolutionary history of this species complex and try to respond several questions:1) how many species can be recognized based on the genomic information? 2) is divergent selection or neutral processes the driving forces involved in the speciation of this group? 3) did the speciation process occur in allopatry followed by a secondary contact or under constant gene flow?

2. Materials and methods

2.1. Sampling and sequencing

We analyzed a subsample of 95 individuals from Vega-Sánchez et al. (2019). Twenty-five individuals belonged to *H. americana North*, 39 to *H. americana South* and 31 to *H. calverti*. These individuals were sampled in different populations (8-10) for each of the three species (Fig. 1).

We extracted genomic DNA from the thoracic muscle of each individual using the Pure Link Genomic DNA mini kit (Invitrogen) and followed the manufacturer's protocol. The DNA was quantified using the Qubit fluorometer system (High Sensitivity DNA kit, Life Technologies), and samples were standardized to a final concentration of 40-50 ng/µL of DNA. Library preparation and sequencing were performed using the 3RAD method (Bayona-Vásquez et al., 2019) with the *Msp*I and *Cla*I enzymes, at the University of Georgia (Georgia, US).

2.2. SNP calling

We used STACKS ver. 2.54 (Rochette et al., 2019) to identify SNPs. First, we ran *process_radtags* to demultiplex, quality filter and trim the sequence data. Then, we used *clone_filter* on demultiplexed reads to identify PCR clones, using Illumina iTru5 barcodes. After filtering the data, five samples were removed due the low quality of the data, the final number of individuals analyzed was 90.

After cleaning, we performed seven different SNP callings with different sample sets to try to enhance the number of SNPs obtained and to answer different questions for each set. To analyze genetic structure among species, the set 1 included all individuals (90) divided into three groups (*H. calverti*, *H. americana North* and *H. americana South*; Fig. 1). Secondly, to analyze genetic variation within each putative species, we carried out three SNP callings: set 2 included only individuals of *H. calverti* (n= 31), set 3 included individuals of *H. americana North* (n=24) and set 4 included individuals of *H. americana South* (n=35). Finally, to compare between species pairs, we performed three more SNP callings: set 5 included individuals of *H. calverti* and *H. americana North*, set 6 included individuals of *H. calverti* and *H. americana South* and set 7 included individuals of *H. americana North* and *South*. The parameters *m* (minimum deep coverage), *n* (distance allowed between catalog loci) and *M* (distance allowed between stacks) for de novo assembly were chosen based on the tests of a subset of samples (r80 method) as suggested by Paris et al. (2017). The final STACKS parameters (*m*, *n*, *M*) used in each set and the total number of SNPs are in Table 1.

In the *populations* module of STACKS, the parameters were constant for every set: *-r* 0.5, *-p* 2, *--min-maf* 0.05, *--max-obs-het* 0.6 and, to avoid linked loci, we used *--write-single-snp*. The

parameter *-r* is the minimum percentage of individuals in a population required to process a locus for that population, *-p* is the minimum number of populations a locus must be present in to process a locus; *--min-maf* specify a minimum minor allele frequency required to process a nucleotide site at a locus and *--max-obs-het* specify a maximum observed heterozygosity required to process a nucleotide site at a locus. Finally, we used a *max-missing* of 0.7 in VCFtools (Danecek et al., 2011).

2.3. Genomic diversity and differentiation analyses

We analyzed genetic diversity by calculating heterozygosity, nucleotide diversity and the inbreeding coefficient for the 1-4 data sets in Arlequin v.3.5.2.2 (Excoffier and Lischer, 2010). For the genetic structure, we performed AMOVA analyses with 10000 permutations in Arlequin and estimated the F_{ST} values between putative species and whitin species between populations. Moreover, we estimated the genomic divergence through G_{ST} values for every locus between pairs of species in GENODIVE v.3.0 (Meirmans, 2020).

We estimated the number of genetic clusters through Bayesian analyses in STRUCTURE (Pritchard et al., 2000). These analyses we performed for the set 1, for each putative species separately (sets 2, 3 and 4), and for set 7 to evaluate the differentiation between *H. americana North* and *South* (see below). The program was set to run with values of assumed genetic clusters (*K)* from 1 to 4, 9 or 10 (depending on the number of populations in each data set) with a burn-in of 10,000 steps and chain length of 100,000 using the admixture model with correlated allele frequencies, without prior population information. Ten independent runs were performed for each value of *K*. The most likely number of genetic clusters was determined using the method of Evanno (ΔK) (EVANNO et al., 2005) in the STRUCTURE HARVESTER website (Earl and vonHoldt, 2012). Runs were summarized using CLUMPAK (Kopelman et al., 2015) and plotted in R with the *ggplot2* package (Wickham, 2011). We separated the outlier SNPs (see below) from the neutral ones to assess the relative contribution of genetic drift and selection in shaping patterns of genetic structure. Specifically, we estimated the genetic structure for neutral loci using the program STRUCTURE for sets 2, 4 and 7 which presented outlier loci (see Results).

We also performed principal component analyses (PCA) in PLINK v.1.90 (Chang et al., 2015) to visualize genetic structure for data sets 1-4 and 7. Moreover, we carried out a discriminant analysis of principal components (DPCA) (Jombart et al., 2010) in *adegenet* v.2.1.4 in R (Jombart, 2008) for set 1 to test the presence of three putative species, this method maximizes the variance among groups while minimizing the variation within groups. To assess the optimal group number,

we tested for *K*=3 and to estimate the number of principal components (PCs) to be retained, the cross-validation method was used.

We estimated the phylogenetic relationships between and within the putative species (sets 1-4). We used the one consensus sequence per locus (that include all variant and invariant sites) per population *(--phylip-all-var* in *populations* module). We estimated the mutation model with ModelFinder (Kalyaanamoorthy et al., 2017) for every partition and then a maximum likelihood approximation with IQ-TREE (Nguyen et al., 2015) was used to establish the phylogenetic relationships. We also performed 10000 bootstrap alignments using the ultrafast bootstrap method (Hoang et al., 2018); all these analyses were run in the IQ-TREE server (Trifinopoulos et al., 2016). The trees were visualized and edited with iTOL v.5 (Letunic and Bork, 2021).

2.4. Candidate loci under selection

To identify candidate loci under selection we performed several outlier analyses in BayeScan v.2 (Foll and Gaggiotti, 2008), for SNP sets 2-7. BayeScan is a Bayesian approach that estimates the probability that each locus is subject to selection using differences in allele frequencies between populations. The priors used were: 100 Posterior Odds for the neutral model and included 20 pilot runs with 5000 interactions and a burn-in length of 50000. The outlier loci having a false discovery rate (FDR) of 0.05 (*q-value* lower than 5%), were considered under divergent selection or balancing selection when Alpha values were negative.

2.5. Environmental association analyses

To identify SNPs associated with environmental variables, we used redundancy analysis (RDA). RDA is a multivariate constrained ordination technique which can analyze multiple loci and multiple environmental predictors simultaneously. This method uses multiple regression with all loci and all environmental variables to generate fitted genetic values and then, performs PCA with the fitted values. Loci with extreme loading values on the constrained ordination axes are identified as outliers. RDA is more powerful than univariate techniques for detecting weak, multilocus selection (Forester et al., 2018).

RDA was conducted with *vegan* v.2.5-7 package (Oksanen et al., 2020) in R, following the protocol described in Kamvar et al., (2017). We used data set 1 for this analysis, missing genotypes were imputed by replacement with the most common genotype at each SNP across all individuals. As independent variables, we used six bioclimatic variables obtained from Worldclim (resolution = 2.5 minutes; Fick & Hijmans, 2017): Annual Mean Temperature (AMT), Min Temperature of

the Coldest Month (MnTCM), Max Temperature of the Warmest Month (MxTWaM), Precipitation Seasonality (PS) and Precipitation of the Driest Quarter (PDQ). These variables were selected because they contributed the most for prediction of the potential distribution of each putative species (unpublished data). On the other hand, to control for population structure, we conducted a principal components analysis for the genetic data and retained the first PC and this was used as conditional variable in the RDA analysis (Capblancq and Forester, 2021).

The full RDA model and each constrained axis were considered significant if *p-value* < 0.01 under the null hypothesis that no linear relationship exists between SNP data and the environmental predictors, for this we used *anova.cca* function with 999 permutations. To identify redundant predictors, we calculated the Variance Inflation Factors using *vif.cca* function and kept those variables with values below 10.

A SNP was considered an outlier if its loading in the ordination space was greater than 2.5 (two-tailed *p-value* = 0.012) standard deviations from the mean on a significant constrained axis (Kamvar et al., 2017). To illustrate these environment-SNP associations, we created triplots of most important RDA axes using *vegan* package. For the triplots, symmetrical scaling was used for both SNP and individual scores.

3. Results

3.1. Genomic diversity and structure

After filtering steps, a range of 4908 to 14581 SNPs was obtained for the different sets (Table 1). In general, we found low levels of observed heterozygosity for the groups. For the populations within putative species, the observed heterozygosity ranged from 0.33 to 0.53 (Table 2). Positive and significant values of F_{IS} were found for each species, being *H. americana North* the one that presents the highest levels of inbreeding (Table 2).

3.1.1. Genomic differentiation between putative species

The AMOVA analysis showed that the overall genetic structure is high between the three putative species (F_{ST} = 0.78, p < 0.001) (Table 3). The highest pairwise F_{ST} values were found between *H. calverti* and *H. americana South* (F_{ST} = 0.83, p < 0.0001) and between *H. calverti* and *H. americana South* (F_{ST} = 0.79, p < 0.0001) while the F_{ST} value for *H. americana North* and *H. americana South* was 0.57 (p < 0.0001) (Suppl. Table 1). We also found that most of the loci present high values of genetic differentiation (G_{ST}) between species pairs (Fig. 2).

STRUCTURE analyses supported the presence of two main genetic groups based on the ΔK method (*K*=2) for set 1, grouping *H. calverti* and *H. americana North* plus *H. americana South* (Fig. 3). Nevertheless, in the plot for *K*=3, it is evident that the different clusters show very low admixture (Fig. 3C) and *H. americana North* and *South* formed different groups, which was also evident in the PCA analysis (Fig. 3B). When we estimated the structure for data without *H. calverti* (Suppl. Fig. 1A and B) and with and without outlier loci (see below) (Suppl. Fig. 1C), we found that the most likely value of *K* was 2, separating *H. americana North* and *H. americana South*, with only a few individuals with an admixture between groups, from populations SN and EF.

The DAPC analysis that included all individuals, retained 10 PCs (based on the lowest MSE value from the cross-validation); three groups were found that correspond to the potential species (Suppl. Fig. 2). The maximum likelihood tree showed three different clades that correspond to the putative species (Fig. 3A) with bootstrap values close to 100.

3.1.2. Genomic differentiation between populations within putative species.

The AMOVA analyses showed high and significant differentiation values for populations within each group (*H. calverti*: F_{ST} =0.47, $p < 0.0001$; *H. americana* North: F_{ST} =0.62, $p < 0.0001$; *H. americana* South: F_{ST} =0.42, $p < 0.0001$) (Suppl. Table 1). For pairwise F_{ST} population values, we found that most population pairs have high and significant values of differentiation in *H. calverti* (Suppl. Fig. 3) with the highest differentiation observed between SG and Ct ($F_{ST} = 0.75$, $p < 0.01$). For *H. americana North*, the highest differentiation was between SP and EF ($F_{ST} = 0.78$, $p < 0.05$) and for *H. americana South*, the highest differentiation was between Ve and CC ($F_{ST} = 0.75$, $p < 0.05$). In contrast to the other groups, in *H. americana South* most of populations from the center of Mexico were no differentiated (Suppl. Fig. 3).

The STRUCTURE analysis suggested that the most likely number of genetic clusters (*K*) for *H. calverti* was 2. The first group included the north-east populations Ct and Za and the second group included the rest of populations (Fig. 4C). Nevertheless, the PCA analysis suggested that SG is a different group separated by the PC2 (Fig. 4B). This is also supported by the maximum likelihood tree, which showed four different clades (Fig. 4A). It is interesting to observe that the population EF (north-west) is more related to the populations of the south (NR and CC) than to the populations of the north-east (Ct, Za).

For *H. americana North,* STRUCTURE analysis suggested the presence of three clusters (*K*=3), the first group included the populations BHC, DC, LC and MC, distributed in the center-east of the

US. The second group included populations EF, SN and RCh, which are distributed in the north of Mexico and, finally, the last group included only the population SP, from the Baja California Peninsula (Fig. 5D). The PCA showed a similar pattern which is also congruent with the maximum likelihood tree (Fig. 5C, 5A).

In the case of *H. americana South K* was 2, the first group included populations Ve, Vi and Za, which are distributed in the north-east of Mexico, the second group included populations CC, NR and Cm from the south of Mexico, and the rest of the populations (Bo, Co, Mo, and Ny) showed a high degree of admixture between the two genetic clusters (Fig. 6D). The PCA showed the same pattern, with two highly differentiated groups, north-east and south, and an intermediate group of populations from the center of Mexico (Fig. 6C). The phylogenetic relationships between these populations are less clear than in the other species, only being monophyletic for the east-north group (Fig. 6A).

3.3. Candidate loci under selection

BayeScan identified a different number of SNPs under selection for the different data sets (Fig. 7). For *H. calverti*, 0.4% (n= 52) of the loci were identified for divergent selection. In *H. americana North*, none of the loci was found to be under divergent selection. Finally, for *H. americana South*, only 0.08% (n= 10) were identified under potentially divergent selection.

When we performed pairwise comparisons among the three groups, BayeScan identified 148 loci under balancing selection for *H. americana North* and *H. calverti* and 244 loci under balancing selection for *H. americana South* and *H. calverti*. Finally, for *H. americana North* and *H. americana South*, 38 loci were identified for balancing selection and 37 loci under divergent selection (Fig. 8).

3.4. Environmental associated loci

We found that the variables AMT and TAR were highly correlated, therefore, they were removed of the RDA analysis. The final analysis included two temperature variables (MxTWaM and MnTCM) as well as two precipitation variables (PS and PDQ). Based on the ANOVA (Suppl. Table 1), the full model significantly explained the genetic variation with an adjusted R^2 of 0.10 (*p-value* < 0.001). Also, RDA axes were significant (*p-value* < 0.001 for the three first axes and *p-value* < 0.003 for the last one). The first RDA axis explained 45% of the variation, the second and third axes explained 20% each and the last axis explained 10% (Fig. 9). For the first axis, the

variable MnTCM showed a strong association with the genetic variation of the putative species; individuals of *H. americana North* inhabit localities with colder temperatures than *H. americana South* and *H. calverti* (except for the Za and Ct populations) (Fig. 9A). Precipitation seasonality also was an important variable, populations of *H. americana South* and *H. calverti* presented higher percentages of variability on precipitation than populations of *H. americana North*. For the second axis, both PDQ and MxTWaM were associated with the genetic variation, being the populations of *H. americana South* those that distribute in habitats with driest conditions. The third axis also showed an association between MxTWaM and the genetic data of populations with highest temperatures (EF, RCh, SN).

By using the SNPs loadings for each RDA axis, we found 635 outliers associated to the four climatic variables. Two hundred and ninety-eight loci were correlated with MxTWaM, 154 with MnTCM, 88 with PDQ and 85 with PS (Fig. 9B).

4. Discussion

4.1. How many species are in the *Hetaerina americana* complex?

The first remarkable result is the high degree of differentiation between the three species basically for every locus (Fig. 2). Based on this, we can suggest that most of the genome is already differentiated, and that gene flow is null among the three groups even when there is sympatric co-occurrence (as it occurs in EF, Za, Ve, CC, NR). These results evidenced that the divergence of *H. calverti* is higher with respect to the two potential species within *H. americana*, as was also shown by the phylogenetic three. Also, the differentiation in the shape of the male caudal appendages is more conspicuous in *H. calverti* (Vega-Sánchez et al., 2020). Morphological variation in caudal appendages is known to play an importante role as a premating mechanical isolation barrier in odonates as *Enallagma*, *Ischnura* and in *Hetaerina* (McPeek et al., 2011; Sánchez-Guillén et al., 2012).

In the case of *H. americana North* and *South*, there is a high degree of genomic differentiation, which suggests that the gene flow is very low or null in some populations (for example in EF and SN). Nevertheless, unlike with *H. calverti*, the isolation barriers between these two groups seem to be different, since the divergence in the shape of caudal appendages is subtler (Vega-Sánchez et al., 2019). Another premating barriers that have been reported in odonates with not courtship, is habitat isolation (Sánchez-Guillén et al., 2012). In this sense, we found that there are SNPs under

selection (Fig. 8, 9) that may be related to local adaptation to differences in temperature and precipitation. For example, minimal temperatures for populations of *H. americana North* range from 8.5ºC to -14.4ºC meanwhile the populations of *H. americana South* range from 19.4ºC to 3.7ºC (Fig. 1). Moreover, during winter season in northern populations, all adult individuals die and only immature stages remain in latency until the next spring season; meanwhile, in southern populations both adults and immatures stages remains active during the whole year. These differences in the ability to deal with very low temperatures, in special of immatures stages, could be associated to the outlier loci found in both BayeScan and RDA analyses. Therefore, populations of *H. americana North* could be adapted to more extreme cold temperatures than populations of *H. americana South*. This adaptation to different habitats could limit the number of effective migrants and therefore, the gene flow between species. In other species of odonates it has been described that temperature is the principal environmental factor that shapes the distribution in these ectotherm animals (Dudaniec et al., 2018).

Under this scenario of habitat isolation, we could expect incomplete mechanical barriers and then hybrid zones; for example, in the north of Mexico where there is a transition to extremely cold temperatures. Based on this evidence, we suggest that *H. americana* should be divided into two valid species which are reproductively isolated.

4.2. Speciation by neutral or divergent selection process?

The divergence of *H. calverti* with the ancestor of *H. americana sensu lato* has been estimated for 24 million years ago approximately, and the divergence between *H. americana North* and *South* was estimated in 16 million years ago, based on ITS1-5.8S-ITS2 sequences (Vega-Sánchez et al., 2019). These dates, plus the genomic patterns of differentiation described here, indicate that the speciation process is very old. Figuring out which mechanisms were involved is difficult since the process of speciation is basically completed, which is particularly evident for *H. calverti*. However, intraspecific differentiation patterns may provide some insight into the mechanisms involved in the speciation process of these organisms.

First, high intraspecific differentiation was found within the three species (Suppl. Fig. 3). We did not find evidence that selection is shaping these patterns of intraspecific genetic differentiation among populations, then they seem to be related to neutral process such as genetic drift. Moreover, even with the high potential dispersion of these species, their territorial behavior could limit it, and result in a low migration rate between populations, enhancing the genetic differentiation. Based on

this, we could suggest that the divergence between the three species started in an allopatric setting. This agrees with the patterns found for other damselflies where "nonadaptive processes" drive the speciation (Wellenreuther and Sánchez-Guillén, 2016). Nevertheless, natural or sexual selection can act after a secondary contact and maintain the genetic divergence if the cross between diverged individuals is maladaptive; this is what we expect that is happening in *H. americana North* and *South*. Finally, understanding why in some species the divergence of morphological traits as the caudal appendages have evolved and not in others is an outstanding question.

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Table 1. Number of samples and values for de novo SNP calling in STACKS for each data set.

Data set	Individuals included	N	*m*	*M*	*n*	Depth of coverage	SNPs
1	All individuals	90	3	8	9	18.6X	4908
2	*H. calverti*	31	3	8	9	20.5X	14581
3	*H. americana North*	24	3	8	9	15.6X	9956
4	*H. americana South*	35	3	6	7	17.7X	12731
5	*H. americana North + H. calverti*	55	3	8	9	18.4X	6827
6	*H. americana South + H. calverti*	66	3	8	9	19X	7193
7	*H. americana North + H. americana South*	59	3	8	9	16.9X	9296
N= Number of samples; *m*, *M* y *n* = STACKS priors.							

Table 2. Summary of genetic diversity estimators for each data set.

Data set	Group/Pop	N	H_O	S.D.	H_E	S.D.	Pi	S.D.	F_{IS}
1	*H. americana North*	24	0.1041	0.0992	0.323	0.1574	0.06195	0.02991	0.5766**
	H. americana South	35	0.1426	0.1096	0.2775	0.1693	0.04859	0.02331	0.3544**
	H. calverti	31	0.1647	0.1222	0.3416	0.1434	0.06778	0.03255	0.3951**
3	BHC	2	0.5278	0.2831	0.5539	0.078	0.11359	0.07418	-0.0133
	DC	2	0.5327	0.2888	0.5562	0.0788	0.08424	0.05503	-0.03824
	EF	3	0.4228	0.2547	0.4752	0.1131	0.14028	0.08103	0.06665
	LC	4	0.4057	0.2353	0.4273	0.1203	0.09658	0.05276	-0.35744
	MC	2	0.5240	0.2532	0.5430	0.0730	0.07735	0.05053	-0.07769
	RCh	2	0.4513	0.2734	0.4399	0.1259	0.10414	0.05686	-0.20382
	SN	3	0.4447	0.2682	0.4874	0.1136	0.12673	0.07321	-0.03733
	SP	4	0.4370	0.2410	0.4621	0.1138	0.04327	0.02364	0.03384
2	CC	4	0.3693	0.2306	0.4225	0.1238	0.13344	0.07283	-0.02982
	Ct	4	0.4082	0.2392	0.4239	0.1293	0.08352	0.04559	-0.0446
	EF	3	0.4401	0.2602	0.4838	0.114	0.11484	0.06633	-0.01581
	NR	3	0.4088	0.2563	0.4701	0.1157	0.14752	0.08520	0.08153
	SG	4	0.3766	0.2355	0.4253	0.1280	0.08533	0.04658	0.06041
	SM	4	0.3748	0.2265	0.4252	0.1216	0.13500	0.07368	-0.06612
	Ve	4	0.3414	0.2253	0.4142	0.1247	0.16881	0.09212	0.15328*
	Za	5	0.3263	0.2048	0.3622	0.1327	0.15026	0.0794	0.05514
4	Bo	3	0.4672	0.2629	0.5080	0.1057	0.12383	0.07155	-0.26588
	CC	4	0.4168	0.2367	0.4291	0.1242	0.13305	0.07262	-0.0598
	Cm	2	0.5123	0.2721	0.5494	0.0761	0.17996	0.11757	-0.76305
	Co	3	0.4563	0.2551	0.4896	0.1103	0.14260	0.08237	-0.06979
	Mo	4	0.3633	0.2274	0.4128	0.1252	0.21200	0.11568	0.08398
	NR	4	0.4048	0.2322	0.4268	0.1246	0.12887	0.07034	-0.15087
	Ny	4	0.4016	0.2369	0.4154	0.1243	0.17242	0.09409	-0.14192
	Ve	4	0.4239	0.2365	0.4301	0.1234	0.06221	0.03397	-0.00962
	Vi	4	0.3666	0.2227	0.4037	0.1277	0.13174	0.07190	0.07267
	Za	3	0.4095	0.2467	0.4637	0.1133	0.11932	0.06892	0.12082

N= Number of samples; Ho= Observed heterozygosity; He= Expected heterozygosity; S.D. = Standard deviation; Pi= Nucleotide diversity; F_{IS}= Inbreeding coefficient; ** p<0.00001; *p<0.01.

Supplementary table 1. Analyses of molecular variance for different data sets.

** $p < 0.0001$.

Data set	Source of variation	d.f.	Sum of squares	Variance components	Percentage of variation
1	Among populations	2	60022.9	502.51	77.64
	Among individuals within populations	87	18033.4	63.58	9.67
	Within individuals	90	7391	82.12	12.69
	Total	179	85447.23	647.21	
	Fixation indices: F_{IS}=0.43**, F_{ST}= 0.78**, F_{IT}= 0.87**				
2	Among populations	7	49039.01	789.06	47.30
	Among individuals within populations	23	20959.17	32.26	1.93
	Within individuals	31	26249	846.74	50.76
	Total	61	96247.19	1668.07	
	Fixation indices: F_{IS}=0.04, F_{ST}= 0.47**, F_{IT}= 0.49**				
3	Among populations	7	30761.27	678.3	62.37
	Among individuals within populations	16	5969.88	-36.20	-3.33
	Within individuals	24	10692.5	445.52	40.96
	Total	47	47423.65	1087.62	
	Fixation indices: F_{IS}= -0.09, F_{ST}= 0.62**, F_{IT}= 0.59**				
4	Among populations	9	42072.39	563.17	42.18
	Among individuals within populations	25	18715.96	-23.37	-1.75
	Within individuals	35	27838	795.37	59.57
	Total	69	88626.34	1335.17	
	Fixation indices: F_{IS}= -0.03, F_{ST}= 0.42**, F_{IT}= 0.40**				

Figure 1. Distribution of samples. Each circle represents a locality/population for every putative species. Circles with two colors represent sympatric localities. Blue gradient represents the variation of the MnTCM along distribution.

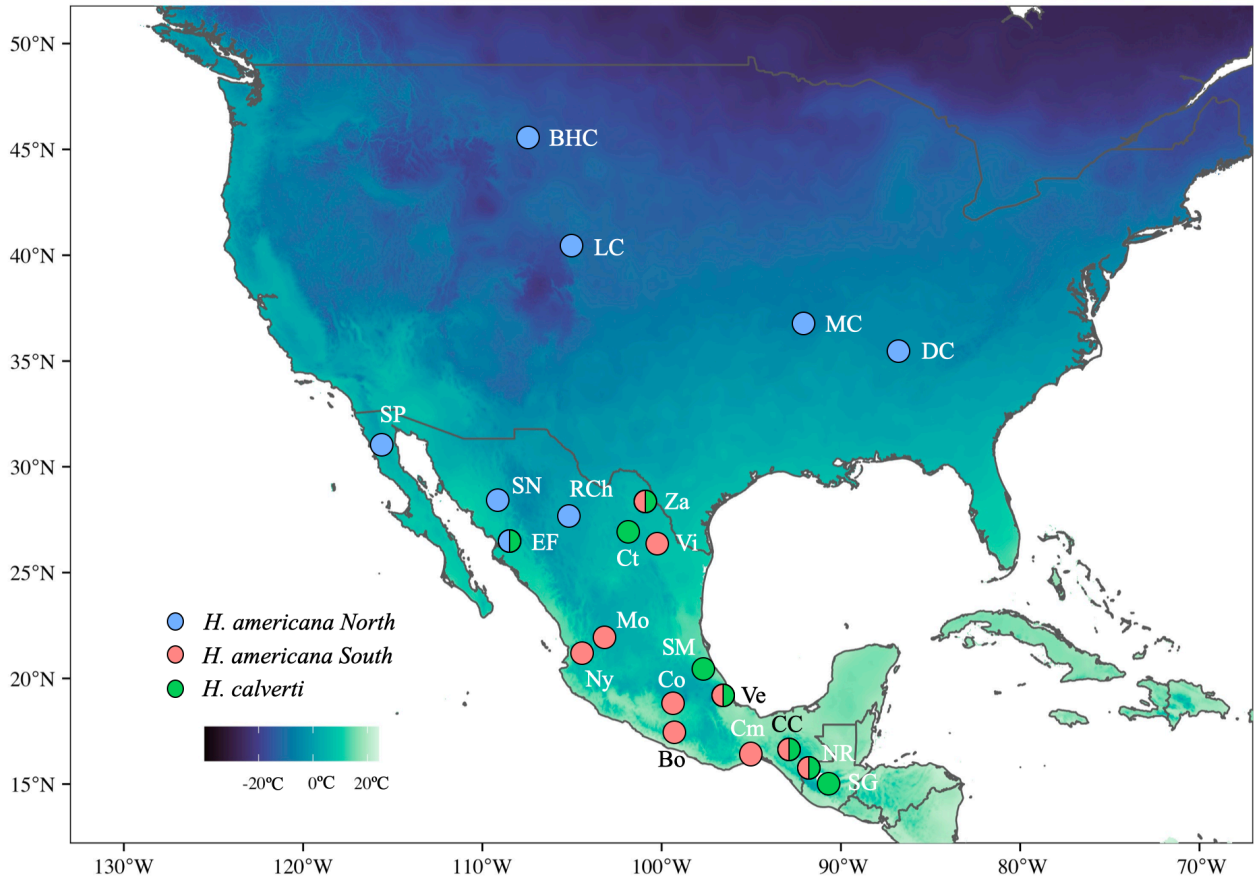


Figure 2. Patterns of genomic differentiation between putative species. The frequency of G_{ST} values for every locus are showed and the mean for each comparison.

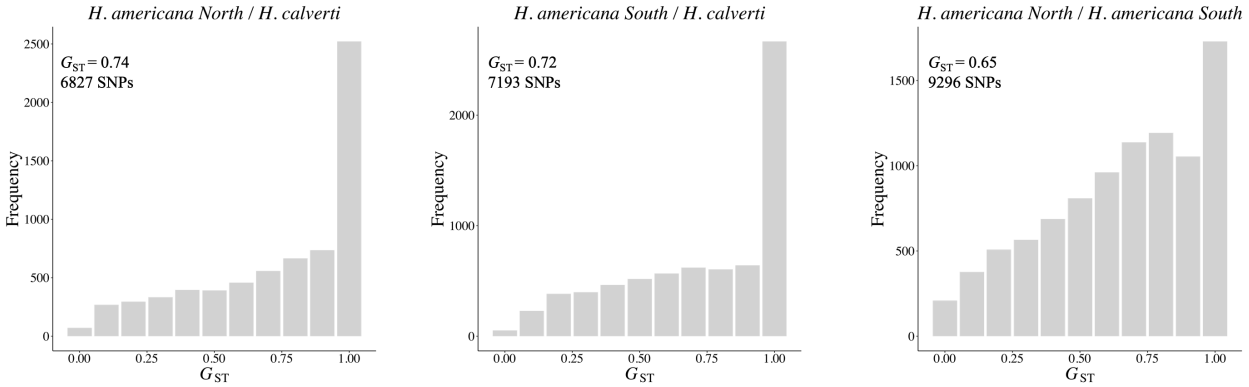


Figure 3. Genomic structure patterns for the putative species. A) Maximum likelihood tree estimated based on one consensus sequence per population per putative species, bootstrap values are displayed in circles. **B)** Principal component analysis for the 90 individuals. **C)** STRUCTURE analyses for K=3 (up) and for K=2 (bottom). Every bar represents an individual.

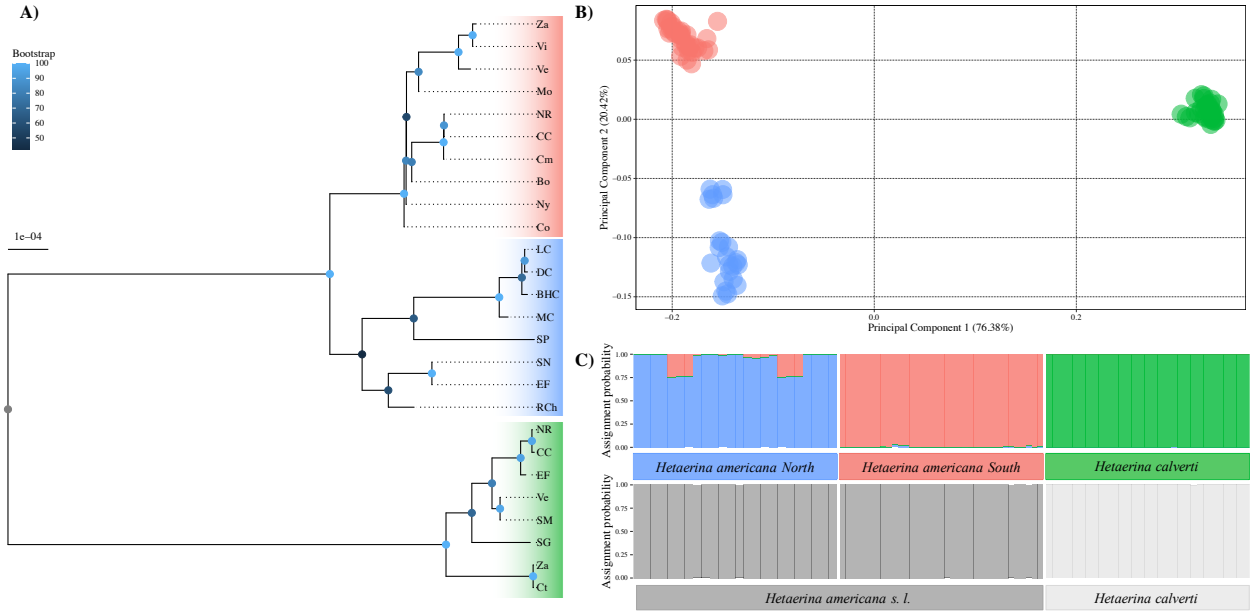


Figure 4. Population structure in *H. calverti*. A) Maximum likelihood tree, bootstrap values are showed. **B)** Populations distribution of *H. calverti*. **C)** Plot of the two principal components for 31 individuals with 14581 SNPs, each color represents a different population plotted on the map. **D)** STRUCTURE analysis for K=2, each bar represents an individual.

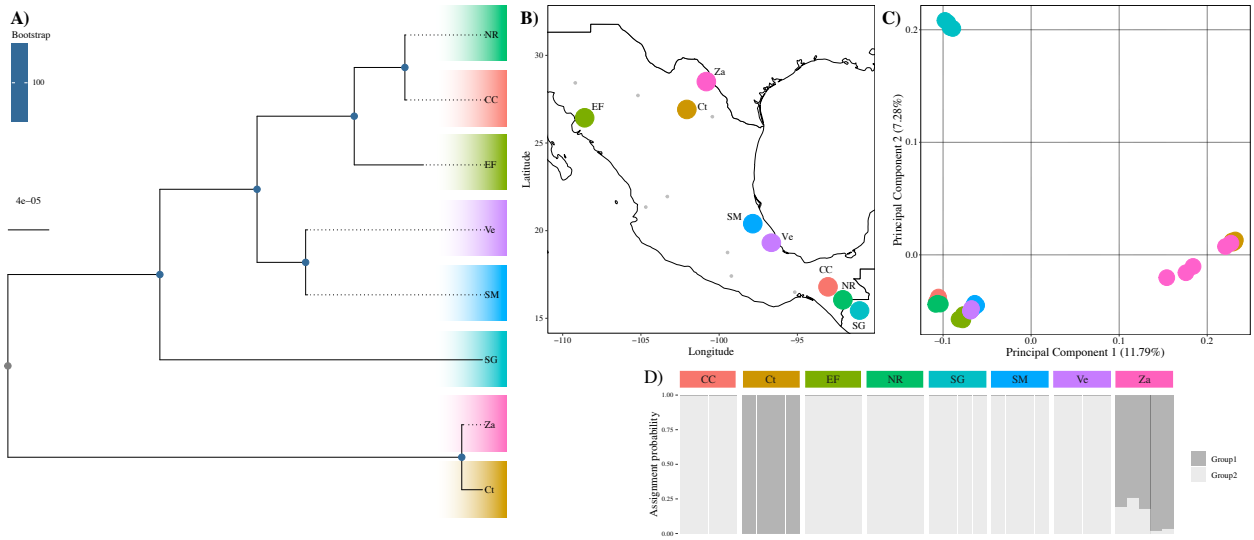


Figure 5. Population structure in *H. americana North*. A) Maximum likelihood tree, bootstrap values are showed. B) Populations distribution of *H. americana North*. C) Plot of the two principal components for 24 individuals with 9956 SNPs, each color represents a different population plotted on the map. D) STRUCTURE analysis for K=3, each bar represents an individual.

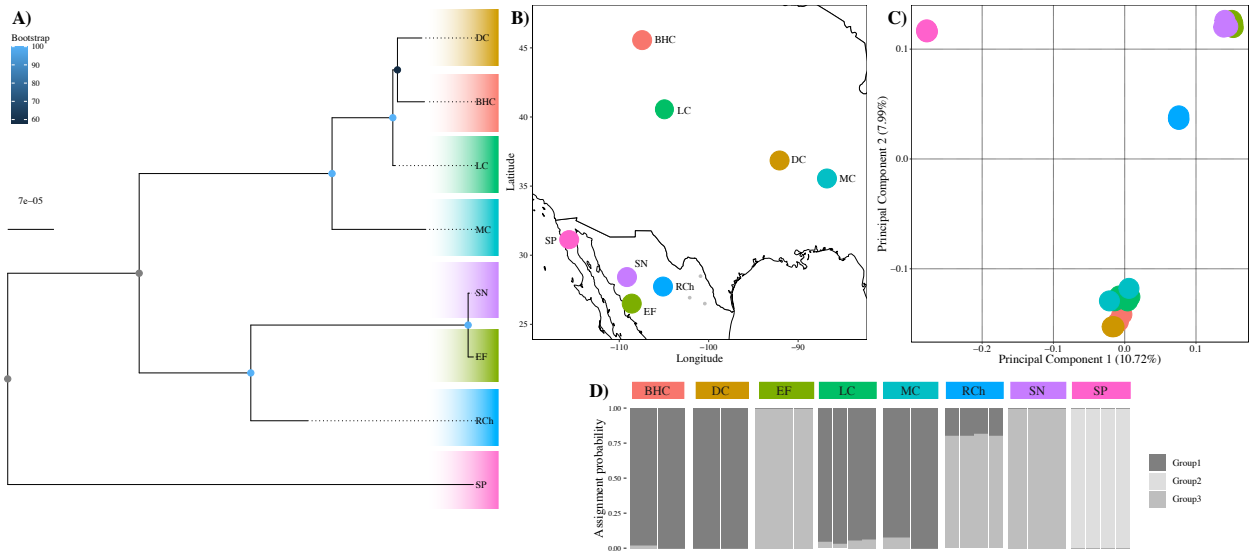


Figure 6. Population structure in *H. americana South*. A) Maximum likelihood tree, bootstrap values are showed. **B)** Populations distribution of *H. americana South*. **C)** Plot of the two principal components for 35 individuals with 12731 SNPs, each color represents a different population plotted on the map. **D)** STRUCTURE analysis for K=2, each bar represents an individual.

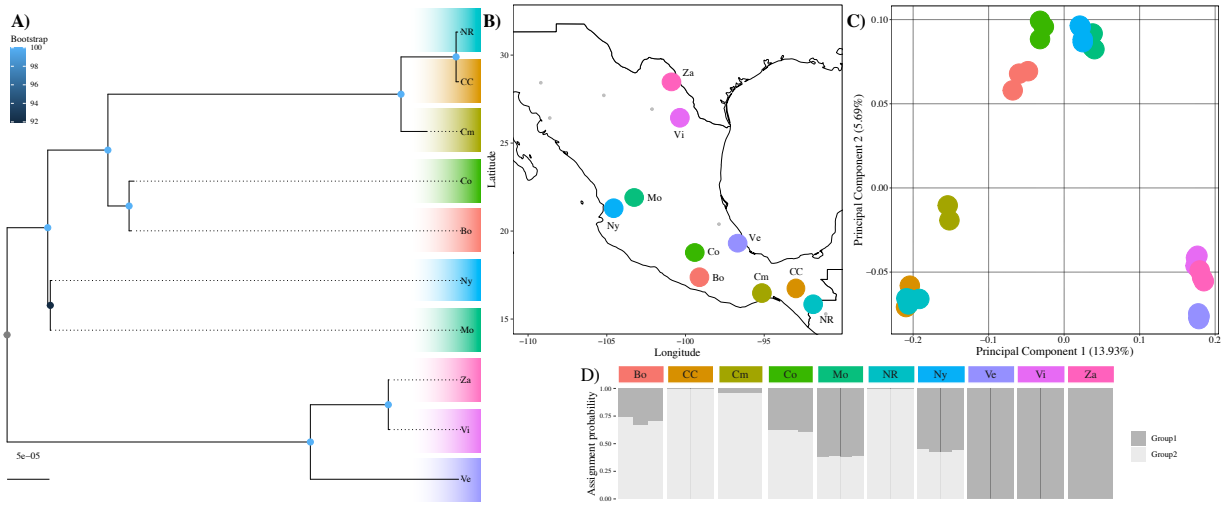


Figure 7. BayeScan analyses for the different putative species.

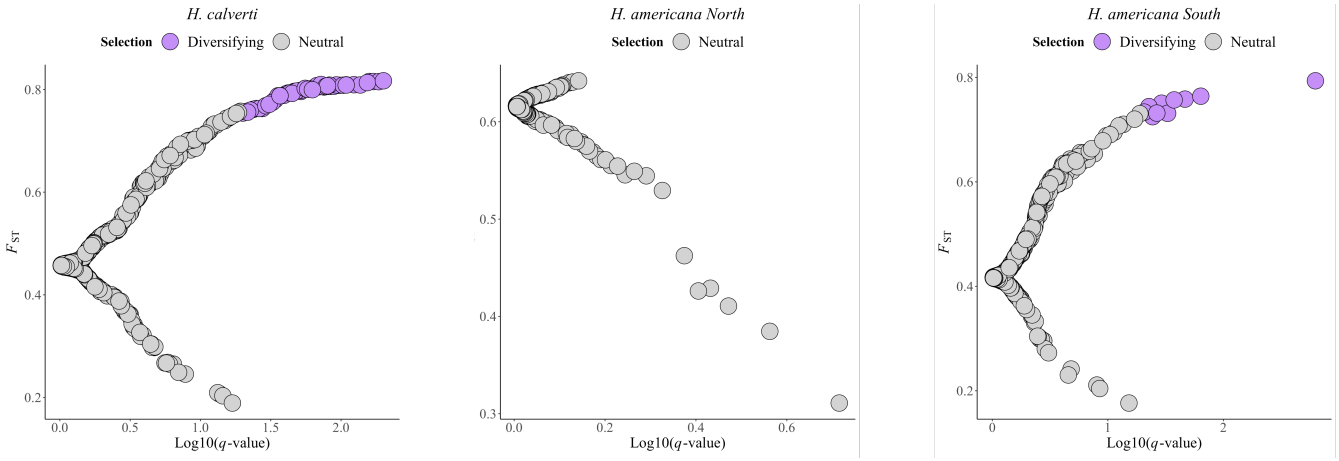


Figure 8. BayeScan analyses between species pairs.

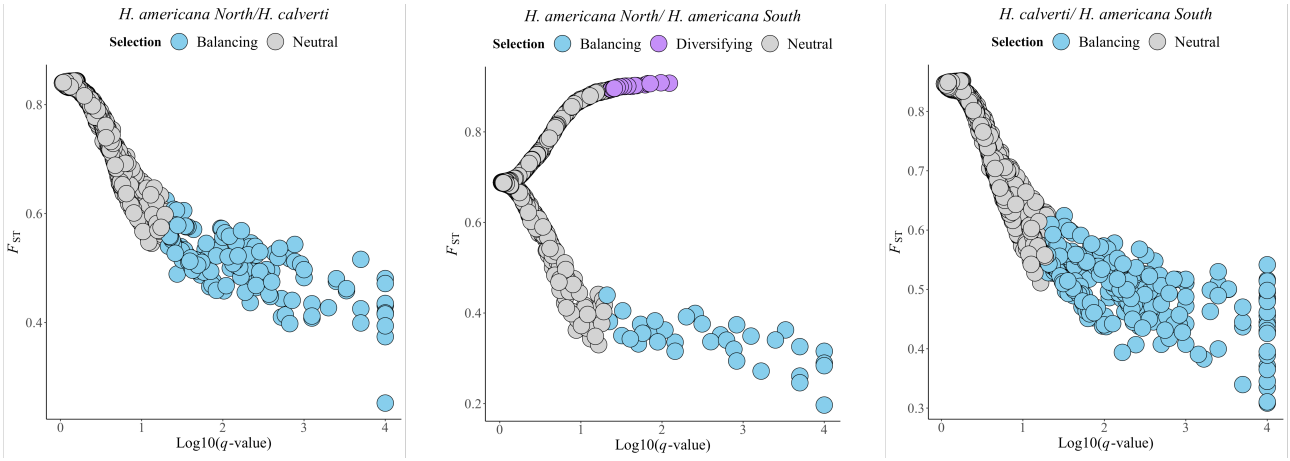
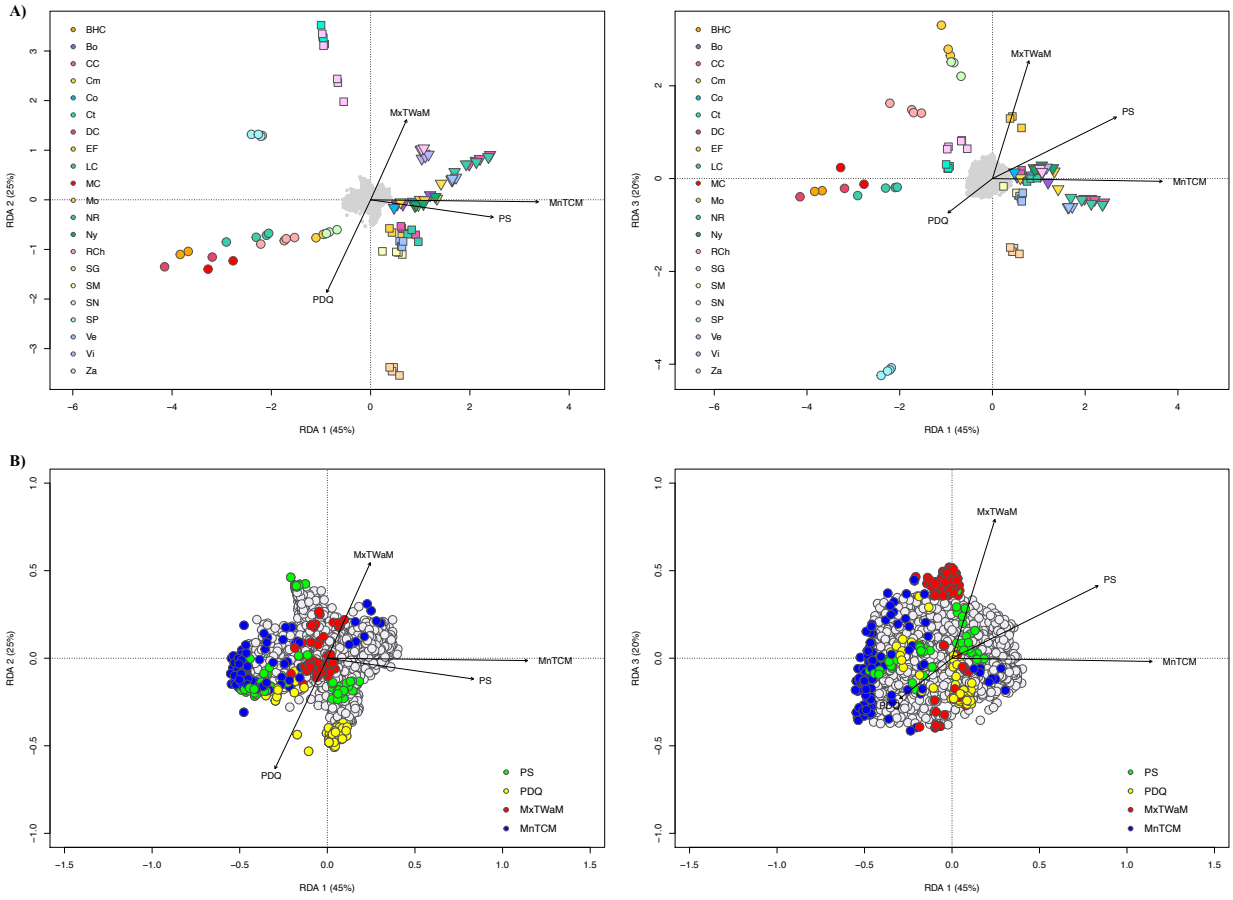
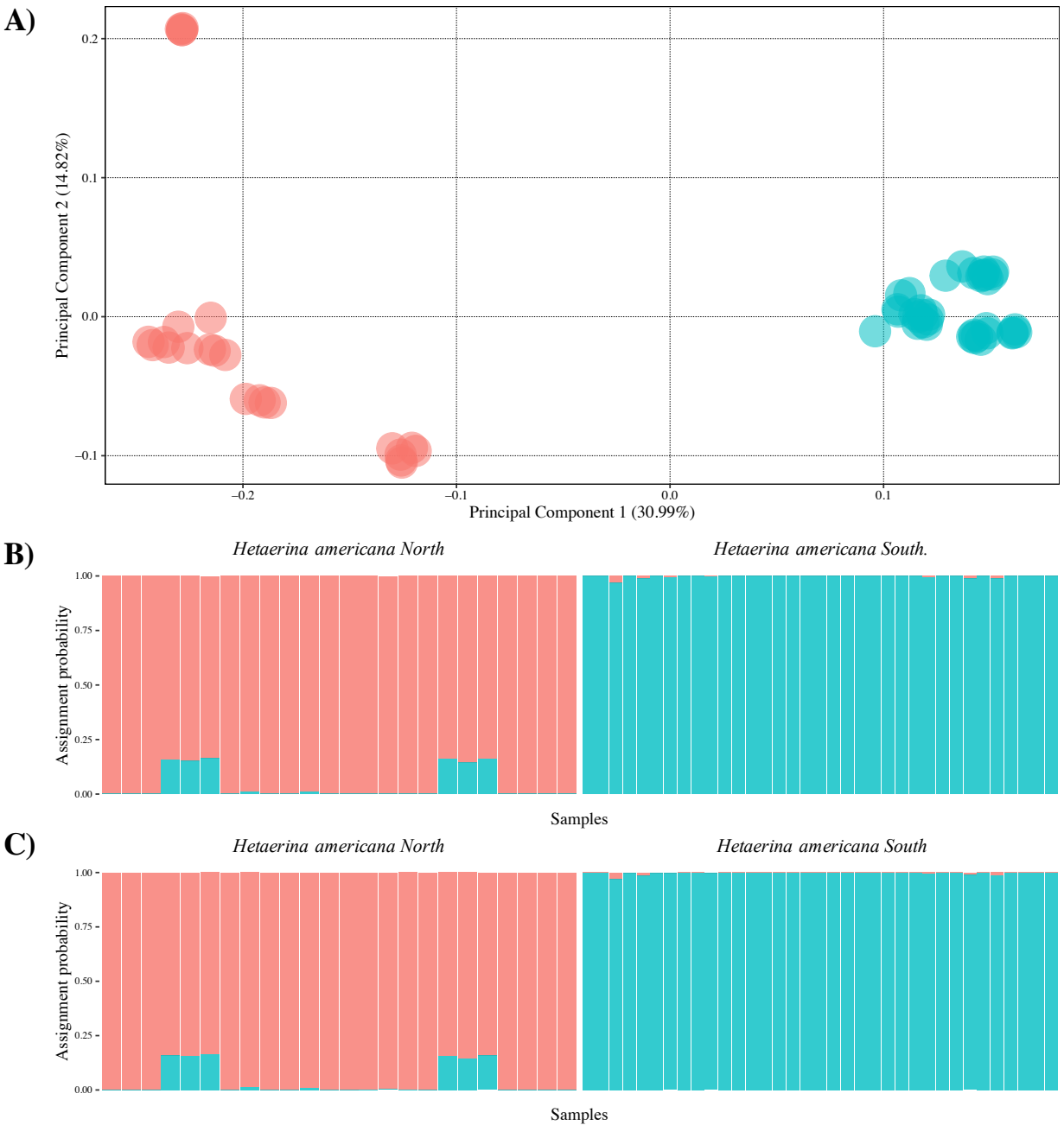


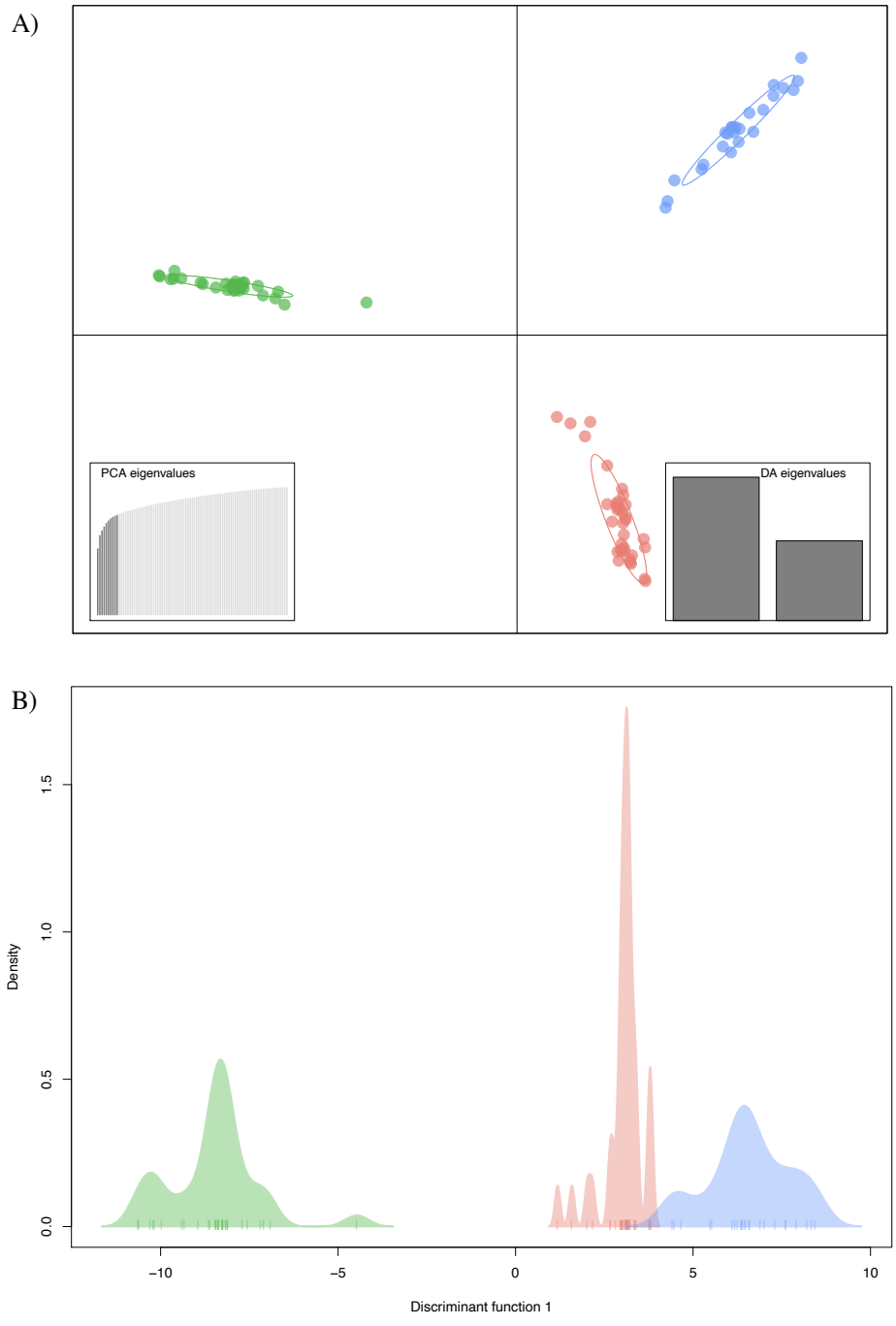
Figure 9. Redundancy analysis (RDA). A) Biplots showing environmental associations with outlier loci (RDA axes 1-2: left; and 1-3: right). The gray dots located at the center of the plot represent SNPs, the colored points refer to individuals (colors represent populations where were sampled), and black vectors represent environmental variables. SNPs and individual RDA scores are scaled by the square root of their eigenvalues. The direction of the arrows indicates the correlation of the environmental variable with each axis. Circles represent individuals of *H. americana North*, triangles *H. americana South* and squares *H. calverti.* **B)** Biplots of 1-2 (left) and 1-3 (right) RDA axes show SNPs identified as outliers (colored circles) as well as all other SNPs (gray circles). Black vectors represent environmental variables elevation. The color of circles corresponds to which environmental variable had the highest correlation coefficient with each SNP.



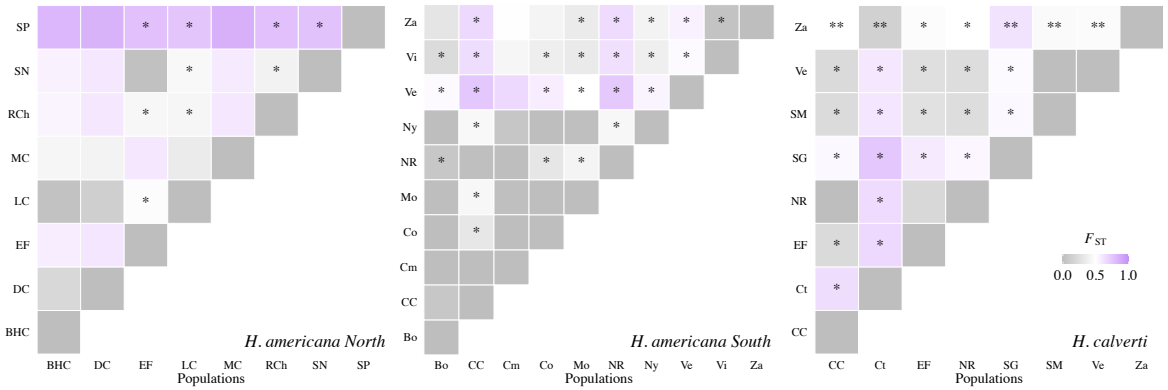
Supplementary figure 1. Genetic structure analyses for *H. americana North* plus *H. americana South.* **A)** Principal component analysis for 59 individuals and 9296 SNPs. **B)** STRUCTURE analysis for *K*=2 that included all SNPs. **C)** STRUCTURE analysis for *K*=2 without outlier *loci*. Every bar represents an individual.



Supplementary figure 2. Discriminant analysis for the tree putative species. A) DAPC scatterplot; each dot representing a single individual. 10 PCs and two discriminant functions (dimensions) were retained during the analysis. **B)** Plots of putative species distributions based on the first discriminant function. Each color represents a different species as in the Figure 1.



Supplementary figure 3. Pairwise F_{ST} values between populations for each species. * p-value < 0.05, ** p-value <0.001.



VI. DISCUSIÓN GENERAL Y CONCLUSIONES

6.1. ¿Cuántas especies incluye el complejo?

Hetaerina americana sensu lato es uno de los primeros odonatos en ser descritos en el continente en 1798 (entonces, *Agrion americana*) (Fabricius, 1798). Si bien se resaltó la variación morfológica presente en la especie (de Sélys-Longchamps, 1859; Walsh, 1863), incluyendo tanto la variación en la forma de los apéndices caudales como la variación en el tamaño de la mancha alar, el color y presencia del pterostigma, etcétera, es evidente que la falta de análisis genéticos impedía dilucidar si esta variación estaba asociada a otros aspectos como la presencia de diferentes especies, sobre todo porque estos morfotipos se encontraban en simpatría (Calvert, 1901).

De acuerdo con el primer análisis genético (Vega-Sánchez, 2016 y Cap. II), se pudo sustentar que parte de la variación morfológica estaba asociada al aislamiento reproductivo generando grupos genéticos altamente diferenciados, pero, ¿cuántas mutaciones generan una especie nueva? La respuesta es desconocida (Nosil et al., 2021) ya que esto puede variar ampliamente entre taxa. En uno de los meta-análisis más completos donde se revisó el grado de divergencia genética a lo largo del continuo de especiación y que incluyó 61 eventos de especiación independientes, se encontró que la especiación estaba completa a partir de un margen superior al 2% de divergencia (Roux et al., 2016), y que el rango donde las especies no han complementado el aislamiento total (i. e., existen barreras permeables), puede ir desde el 0.05% al 2% de divergencia, en este punto conocido como "zona gris" (Fig. 1), definir especies es controversial taxonómicamente. En este caso, encontramos una alta divergencia genética entre los tres grupos. Si bien la diferencia entre *H. americana North* y *H. americana South* (Cap. IV) es un poco menor respecto a la diferencia entre estos taxones y *H. calverti*, más de la mitad de los SNPs entre las primeras especies mencionadas superó los valores de G_{ST} de 0.5 (Cap. IV; Fig. 2). Además, si observamos las distancias genéticas entre las especies del complejo y otras especies del género, podemos ver que la divergencia entre *H. americana North* y *South* es mayor que entre especies con morfología diferente y taxonómicamente reconocidas como *H. cruentata* y *H. vulnerata* (Fig. 4, Tabla 2).

Dado lo anterior, se sugiere que el complejo *H. americana* está formado por tres especies diferentes: *H. calverti*, *H. americana* (que representaría al grupo *H. americana North*) y *Hetaerina* nov. sp., que incluiría a las poblaciones de *H. americana South*.

Tabla 2. Distancias genéticas pareadas en porcentajes entre especies del género *Hetaerina*. Distancias basadas en el fragmento de ITS1-5.8S-ITS2, a partir de secuencias obtenidas en GenBank. Se incluyeron secuencias de dos individuos por especie. HamN = *H. americana Norte*; HamS = *H. americana Sur*; Hcl = *H. calverti*; Hca= *H. capitalis*; Hcr = *H. cruentata*; Hoc = *H. occisa*; Hti = *H. titia*; Hvu = *H. vulnerata*.

Especie	HamN	HamN	HamS	HamS	Hcl	Hcl	Hca	Hcr	Hcr	Hoc	Hoc	Hti	Hti	Hvu
HamN	*													
HamN	0	*												
HamS	**4.7**	**4.7**	*											
HamS	**4.1**	**4.1**	0.8	*										
Hcl	**3.5**	**3.5**	**5.3**	**5.5**	*									
Hcl	**3.5**	**3.5**	**5.3**	**5.5**	0	*								
Hca	19.9	19.9	19.9	20.3	19.5	19.5	*							
Hcr	10.8	10.8	12.8	12.8	10.6	10.6	17.5	*						
Hcr	10.8	10.8	12.8	12.8	10.6	10.6	17.5	0	*					
Hoc	17.3	17.3	18.3	18.5	17.7	17.7	20.5	18.3	18.3	*				
Hoc	17.1	17.1	18.3	18.5	17.5	17.5	20.9	18.1	18.1	0.4	*			
Hti	21.7	21.7	23.2	23.4	22.2	22.2	24.8	19.5	19.5	21.5	21.3	*		
Hti	22.2	22.2	24	24.2	23	23	25.4	19.5	19.5	21.7	21.5	1.8	*	
Hvu	11.6	11.6	13.6	13.6	11.4	11.4	18.9	**3.3**	**3.3**	16.9	16.7	19.7	19.7	*
Hvu	11.4	11.4	13.4	13.4	11.2	11.2	18.7	**3**	**3**	16.9	16.7	19.7	19.7	0.2

Hetaerina calverti es la especie hermana de las dos restantes en el complejo, y su divergencia se dio hace aproximadamente 24 millones de años (Cap. I; Fig. 3). Esta especie presenta una forma de apéndices caudales particular que permite identificarla fácilmente por medio de la morfología (Cap. II; Fig. 3), y se ha podido registrar en simpatría con las otras dos especies (Cap. II; Fig. 5). También existe diferencia en la forma de las alas y el tamaño corporal (Cap. III; Fig. Suppl. 2 y 3) con respecto a *H.* nov. sp. (i. e., *H. americana South*). El tamaño corporal sólo varía en simpatría, lo que se sugiere un posible proceso de desplazamiento de caracteres (Cap. III). La distribución geográfica de esta se especie abarca desde el sureste de Estados Unidos y a lo largo de todo México y hacia Centroamérica en Nicaragua, Honduras y Guatemala; sobre todo a altitudes menores a los 900 m s.n.m (Cap. II).

Hetaerina americana sensu stricto (i.e., *H. americana North*) es especie hermana de *H.* nov. sp., y divergieron hace aproximadamente 16 millones de años (Cap. I; Fig. 3). La forma de los apéndices caudales no ha divergido de manera contrastante, aunque se puede apreciar cierta diferencia sobre todo en la parte del lóbulo medio, el cual suele ser más redondeado en *H*. nov. sp. (Cap. I; Fig. 5). La distribución geográfica de esta especie va desde el norte de México hasta el norte de Estados Unidos y posiblemente Canadá (Cap. I).

Con base en nuestro muestreo, *H.* nov. sp. tiene una distribución restringida al territorio mexicano, y no se han registrado localidades en donde se encuentren en simpatría con *H.*

americana, aunque este patrón podría cambiar al muestrear más localidades, sobre todo en el norte del México.

6.1.1. Aislamiento reproductivo en el complejo Hetaerina americana

En odonatos, específicamente en zigópteros, el aislamiento reproductivo está asociado a dos tipos de barreras principales; aislamiento sexual y aislamiento mecánico, el primero suele estar relacionado a la coloración tanto del cuerpo como de las alas (Svensson et al., 2006) mientras que el segundo se asocia a la forma de los apéndices caudales (Sánchez-Guillén et al., 2012b; Willkommen et al., 2015). También se ha registrado la importancia del aislamiento temporal, donde éste puede contribuir de manera significativa al aislamiento reproductivo total (Sánchez-Guillén et al., 2012b).

El aislamiento sexual se observa en menor medida en especies que no presentan cortejos complejos ni caracteres sexuales secundarios elaborados (*Ischnura*, *Enallagma*) (McPeek et al., 2011; Sánchez-Guillén et al., 2014b). A pesar de que de las especies del complejo *H. americana* presentan territorialidad y caracteres sexuales secundarios elaborados (i.e., mancha alar), la falta de un cortejo como el que se observa en el género *Calopteryx* puede evitar la evolución del aislamiento sexual basado en la mancha alar; sin embargo, el aislamiento sexual también podría darse por la variación en el tamaño (Cap. III).

Para *H. calverti* encontramos que la principal barrera pre-copulatoria es el aislamiento mecánico/sensorial relacionado con la forma de los apéndices caudales, mientras que en *H. calverti vs H. nov. sp.* el tamaño podría funcionar también como una barrera pre-copulatoria (Cap. III). No es claro en qué momento del proceso reproductivo es efectiva esta barrera, si como parte de la elección del macho para intentar la formación del tándem (i.e., aislamiento sexual) y/o como parte del aislamiento mecánico/sensorial, ya que estructuras de tamaños divergentes evitarían el correcto estímulo para propiciar la cópula. La fuerte interferencia conductual registrada en el género y el patrón de desplazamiento de caracteres sobre el tamaño corporal encontrada entre estas especies (Cap. III) apoya la hipótesis de que el tamaño corporal puede ser una barrera de aislamiento sexual. De esta forma, el efecto del desplazamiento de caracteres reproductivo sería la disminución de interacciones heteroespecíficas que pueden ser costosas para los machos en este punto del proceso reproductivo, por ejemplo, al dejar el territorio. Además, se ha registrado que existe apareamiento selectivo sobre este carácter en el complejo (Serrano-Meneses et al., 2018), lo que sugiere que no sólo ha divergido el carácter, sino también la preferencia, lo cual es primordial para la evolución

de las barreras de aislamiento sexual. Aunque no analizamos la presencia de barreras post-copulatorias pre y post-cigóticas, la ausencia de híbridos y el alto grado de divergencia genómica entre estas especies, sugiere que es probable que éstas estén presentes, sin embargo, esto es difícil de poner a prueba experimentalmente ya que el manejo de estas especies en el laboratorio es poco viable.

Entre *H. americana* y *H.* nov. sp. es menos claro identificar cuáles son las barreras al flujo génico. La baja divergencia en la forma de los apéndices caudales (Cap. I; Fig. 5) sugiere que este carácter no funciona como barrera pre-copulatoria, sin embargo, es necesario un análisis morfológico más extensivo, como el realizado en el Capítulo III, y que incluya más localidades. Otro tipo de barreras que no necesariamente implican divergencia morfológica pueden estar presentes, por ejemplo barreras de aislamiento ecológico, ya sea por aislamiento de hábitat o aislamiento temporal. Un aspecto fenológico importante para resaltar en *H. americana* es la temporada de vuelo de los adultos (y por lo tanto la época reproductiva) la cual se ve reducida en relación con la latitud. Por ejemplo, en poblaciones de Texas los adultos sólo están presentes y se reproducen en los meses de febrero a noviembre; mientras que en las poblaciones de Montana la temporada de vuelo se reduce de junio a agosto y el resto del año sólo las larvas quedan en latencia (Abbott, 2011). En este sentido, *H.* nov. sp. presenta una distribución más sureña y por lo tanto se reproduce a lo largo de todo el año. Si bien los individuos pueden traslapar sus épocas reproductivas, puede ser que las larvas no necesariamente puedan establecerse en localidades donde las condiciones climáticas de temperatura cambian abruptamente en comparación con las localidades del sur. Esto último pueden estar asociado a los loci outliers encontrados en el Capítulo IV, los cuales están asociados a la temperatura mínima del mes más frío. Sin embargo, es necesario analizar más localidades en el norte de la distribución, donde probablemente existan zonas de contacto donde se esperarían eventos de reforzamiento.

6.1.2. Mecanismos de especiación en el complejo

Como se ha mencionado, se ha sugerido que la selección sexual es el principal motor de especiación en odonatos debido a que las barreras surgen independientemente de las diferencias ecológicas, específicamente aquellas barreras pre-copulatorias como el aislamiento sexual y mecánico/sensorial (Wellenreuther y Sánchez-Guillén, 2016). La selección sexual actúa por medio de la elección femenina en caracteres sexuales secundarios, como las manchas alares, o por la elección críptica femenina sobre estructuras de la genitalia (primarias o secundarias) (Eberhard,

1985). También se ha registrado que ciertas interacciones antagonistas y el conflicto sexual pueden influir en la evolución de la variación en este tipo de estructuras (Cordero-Rivera y Córdoba-Aguilar, 2010).

En el caso donde el aislamiento mecánico/sensorial está determinado por la forma de los apéndices caudales (i.e., en *H. calverti* vs *H. americana/H.* nov. sp.), es complicado asociar el proceso de especiación a procesos biogeográficos y/o de selección sexual, ya que la divergencia parece ser muy antigua. Sin embargo, hay resultados interesantes que pueden ayudar a hacer conjeturas. Primero, el desplazamiento de caracteres reproductivos con base en el tamaño corporal sugiere que la distribución en simpatría es debida a un contacto secundario, por lo que las especies divergieron en alopatría. Segundo, que la hembra sea la que decide copular (cuando están en tándem y sólo hay contacto de los apéndices caudales y los interesternitos) sugiere que la elección de la hembra tiene un papel fundamental en la divergencia de estas estructuras, lo cual se ha descrito ampliamente en diferentes grupos de insectos (Eberhard y Lehmann, 2019). La divergencia en la preferencia de las hembras en un contexto alopátrico podría acelerar el proceso de especiación en este grupo.

Dado lo anterior, resulta importante analizar la variación poblacional de los apéndices caudales a lo largo de la distribución y determinar si los patrones de alta diferenciación genética encontrados entre las poblaciones de *H. calverti* podrían estar relacionados con ésta, o si en cambio, están asociados a barreras geográficas o incluso a cuestiones conductuales como la territorialidad.

Por otro lado, el por qué no han divergido los apéndices entre *H. americana* y *H.* nov sp. es una pregunta surgente. La evidencia obtenida en esta tesis sugiere que otros mecanismos están actuando entre este par de especies, específicamente, las presiones de selección natural local, lo cual se ve respaldado en parte por los análisis genómicos donde se encontraron varias regiones bajo selección divergente. Es necesario obtener información de la estructura y función del genoma para poder rastrear qué genes pueden estar divergiendo.

Si bien se ha sugerido que la selección sexual es el principal mecanismo de especiación en este grupo (zigópteros), existen varios casos de especiación ecológica en el orden (Brown et al., 2000; Jordan et al., 2003). La divergencia en el nicho (diferencias en temperatura y precipitación) sumado a la evolución de latencia de las larvas podrían ser factores involucrados en la especiación en este grupo. Aumentar el muestreo, sobre todo en zonas del norte de México, así como realizar un

análisis exhaustivo de la variación morfológica de estas entidades es el siguiente paso para entender la historia evolutiva del complejo.

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