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Diversidad vocal y estructura genética en *Campylopterus curvipennis* (Aves: Trochilidae)

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RESUMEN GENERAL

El estudio de los patrones de divergencia genética entre poblaciones de distintos organismos es importante para evaluar hipótesis acerca de los mecanismos evolutivos que promueven y mantienen la diversidad biológica. En animales, dentro de los mecanismos que pueden estar involucrados en la divergencia genética y especiación no solo son importantes aquellos basados en aspectos geográficos, ecológicos y temporales, sino aquellos de tipo conductual seleccionados sexualmente, la variación geográfica de rasgos involucrados en la elección de pareja y el aprendizaje. Las especies con poblaciones altamente diferenciadas, pueden representar estados tempranos del proceso de especiación, donde la acción tanto de la deriva como de la selección sexual y ecológica pueden contribuir a la divergencia morfológica y conductual rápidamente en alopatría o parapatría. En aves, el papel del canto es particularmente importante por los factores múltiples que influyen en la evolución vocal y el potencial de cambio rápido a través del aprendizaje y la evolución cultural. En esta tesis traté de entender los patrones de divergencia genética y fenotípica (morfológica y acústica) y su relación a lo largo de la distribución de *Campylopterus curvipennis*, un colibrí con una extraordinaria complejidad y variación vocal y con un sistema de apareamiento de leks, evaluando tanto factores geográficos, históricos y climáticos como factores microevolutivos relacionados con su sistema de apareamiento que pudieron haber afectado esta divergencia. Los análisis de dos genes mitocondriales y diez loci de microsatélites para inferir la historia evolutiva de *C. curvipennis* en Mesoamérica (Capítulo 1) mostraron la presencia de tres linajes sin flujo genético contemporáneo, congruentes con dos eventos de diversificación: un primer evento vicariante en el Istmo de Tehuantepec y una separación más reciente durante el Pleistoceno aislando poblaciones de la región de los Tuxtlas. Encontré una divergencia tanto en morfología como en rasgos acústicos muy evidente entre los tres linajes, y los análisis de coalescencia, para examinar los efectos de la deriva y la selección en promover divergencia acústica y morfológica, sugirieron que la selección está conduciendo la evolución del canto, pero la deriva no puede rechazarse como causa de la divergencia morfológica. Contrario a lo que se esperaba los datos mostraron que la divergencia acústica no está correlacionada con la divergencia genética neutral entre leks a lo largo de la distribución de uno de los linajes en la Sierra Madre Oriental (Capítulo 2). Las estimaciones de diferenciación genética y tasas de migración indicaron altos niveles de flujo genético entre leks, sin embargo, encontré una fuerte estructura acústica entre leks. Puesto que los patrones de divergencia vocal fueron explicados

parcialmente por deriva, los resultados apoyan el papel de la selección social en promover convergencia vocal dentro de leks y la selección sexual en promover novedad y diversidad acústica entre leks. Finalmente a nivel local, describí con detalle la variación vocal y temporal dentro de un lek focal de *C. curvipennis* y determiné el potencial de la selección de familia actuando en los leks de esta especie (Capítulos 3 y 4). Encontré tres vecindarios vocales los cuales fueron consistentes año con año en la estructura y composición general de las sílabas además de que los machos pertenecientes a cada vecindario están espacialmente agregados. A pesar de la consistencia de la composición de sílabas entre años, algunas sílabas se ganaron y otras se perdieron durante el periodo de estudio, lo que sugiere la rapidez con que pueden cambiar las señales acústicas y el repertorio silábico en esta especie. Encontré que el coeficiente de parentesco promedio dentro de los leks es bajo y con una varianza muy grande, lo que sugiere que tanto individuos emparentados como no emparentados despliegan juntos en un lek, por lo que la selección de familia no parece ser un factor importante en la formación de leks de esta especie. También analicé estimaciones de parentesco entre los machos de los vecindarios vocales y no encontré evidencia de agrupamientos de parientes dentro de los vecindarios. Esto sugiere que los juveniles que se establecen en los leks no aprenden el canto de sus padres o parientes, sino que lo aprenden probablemente de sus vecinos. Los resultados de esta tesis a una escala geográfica sugieren un patrón de divergencia en alopatría promovida por barreras geográficas o de hábitat donde la deriva y la selección (sexual) han jugado un papel importante en la divergencia fenotípica y probablemente en mantener el aislamiento reproductivo en caso de que ocurra contacto secundario. A una escala local, los datos sugieren que las diferencias vocales entre leks y vecindarios vocales se mantienen cuando los machos aprenden las vocalizaciones después de la dispersión, probablemente para tener mayores probabilidades de establecerse en un territorio, tener éxito en obtener cópulas o tener acceso a grupos sociales. Debido a esto, la estructura genética a lo largo de las “fronteras vocales” y de los vecindarios es muy débil por lo que es muy probable que la transmisión vocal sea de manera horizontal (entre individuos no emparentados). Este trabajo sugiere que la convergencia por cantar un mismo tipo de canto no limita el flujo de genes, lo cual parece ser un patrón común en distintos grupos de aves que son capaces de aprender sus vocalizaciones. A pesar de las predicciones sobre la divergencia de rasgos utilizados en la elección de pareja como promotores de divergencia genética y especiación, existen otros factores relacionados con la fisiología y el apareamiento *asortativo* no tomados en cuenta en este estudio que pueden estar influyendo en la falta de estructura genética local.

INTRODUCCIÓN

Filogeografía y procesos históricos

El estudio de los patrones de divergencia genética entre poblaciones de regiones particulares del planeta es importante para evaluar hipótesis acerca de los mecanismos evolutivos que promueven y mantienen la diversidad biológica. El flujo génico puede mantener a las poblaciones como unidades cohesionadas, mientras que la interacción entre diferentes formas de selección, incluyendo la selección natural y la sexual, la deriva génica así como procesos históricos pueden conducir a la diferenciación genética (Slatkin 1994). Esto puede causar divergencia entre poblaciones, que aunado a la evolución del aislamiento reproductivo eventualmente se separen en especies distintas.

La filogeografía trabaja con herramientas estadísticamente poderosas para inferir los componentes históricos de la distribución espacial actual de linajes de genes utilizando información temporal a partir de marcadores moleculares (Avise 2000). Una de las aplicaciones más importantes de los estudios filogeográficos ha sido el poder determinar el grado de estructuración genética de las poblaciones de especies o complejos de especies a lo largo de su distribución, así como la inferencia de los procesos (tanto históricos como aquellos más recientes) que han determinado dicha estructuración geográfica.

La evaluación tanto de las barreras físicas como de las barreras de hábitat (o climáticas) son importantes en los sistemas biológicos porque pueden promover y mantener la divergencia poblacional y a su vez influir en los patrones históricos de diversificación (Weir 2009). Además, este conocimiento puede servir como una guía para la preservación y manejo de la diversidad genética y endemismos. El Pleistoceno fue una época que inició hace aproximadamente dos millones de años y estuvo caracterizada por oscilaciones importantes en el clima global. Ciclos de 100,000 años enfriaron la Tierra y expandieron los glaciares y posteriormente, condiciones

de calentamiento periódicamente interrumpieron las glaciaciones (Gibbard & Kolfschoten 2004). El efecto de estos cambios climáticos en la distribución geográfica de las especies ha sido substancial. Particularmente en aves las condiciones del Pleistoceno han jugado un papel importante en el proceso de especiación, tanto en la iniciación de separaciones filogeográficas mayores dentro de especies como completando eventos de especiación que pudieron haber iniciado antes, por ejemplo durante el Plioceno (Avise & Walker 1998).

Las regiones Neotropicales son particularmente importantes porque albergan una gran riqueza de especies y un gran porcentaje de taxa endémicos (Myers *et al.* 2000), sin embargo, desafortunadamente son de las menos estudiadas. En la actualidad existen estudios filogeográficos en varios grupos de organismos dentro del Neotrópico que han ayudado a entender los patrones de estructuración genética de las poblaciones de muchos taxa, las fechas aproximadas de diferenciación y el papel de barreras geográficas al flujo genético, con lo cual se han generado hipótesis biogeográficas para entender patrones históricos de diversificación en esta región (revisado en Moritz *et al*. 2000, Barber & Klicka 2010).

El papel del canto en la divergencia genética

La divergencia en caracteres involucrados en la elección de pareja como el canto, plumaje y despliegues de cortejo (en el caso de las aves), es probable que juegue un papel importante en el proceso de aislamiento reproductivo y especiación. En poblaciones altamente diferenciadas genéticamente, la acción tanto de la deriva como de las distintas formas de selección pueden contribuir a la rápida divergencia tanto de rasgos morfológicos como conductuales en poblaciones alopátricas o parapátricas. Sin embargo, estos cambios podrán conducir a la especiación dependiendo del aislamiento reproductivo antes o después del contacto secundario (Edwards *et al.* 2005). La acción única de la deriva y la mutación puede promover diferenciación fenotípica y aislamiento reproductivo sobretodo en poblaciones pequeñas (Clegg *et al*. 2002). En contraste, la selección ecológica puede ocurrir cuando presiones de selección

divergentes actúan generalmente en caracteres morfológicos de especies distribuidas en hábitats heterogéneos donde el flujo genético entre poblaciones adaptadas localmente es reducido (Nosil *et al*. 2007). Aunque la selección en contra de híbridos puede ayudar a mantener las fronteras de las especies, el aislamiento reproductivo depende principalmente de mecanismos precigóticos (Edwards *et al.* 2005).

El papel del canto es particularmente importante por los factores múltiples que influyen en la evolución vocal tales como el control vocal, producción y ontogenia, además del potencial de cambio rápido a través del aprendizaje y la evolución cultural. El canto en muchas aves es aprendido y está sujeto a una rápida evolución cultural, en la cual innovaciones estocásticas errores o innovaciones son comunes cuando los individuos aprenden el canto de sus padres o vecinos mediante imitación. Se ha sugerido que la evolución rápida de los sistemas de comunicación entre emisores y receptores pueden reducir el flujo génico entre poblaciones (Ritchie *et al*. 2007), pudiendo ser facilitada por la variación geográfica de señales involucradas en el apareamiento (Podos & Warren 2007).

La divergencia de rasgos acústicos en muchas ocasiones está moldeada por la selección dependiente del hábitat, ya que sonidos con ciertas características se transmiten mejor en cierto tipo de hábitat (Slabbekoorn & Smith 2002*b*, Patten *et al*. 2004, Seddon 2005, Ruegg *et al.* 2006, Nichols *et al.* 2006). También puede ser resultado de un efecto indirecto de adaptaciones morfológicas, como por ejemplo aquellas relacionadas con el forrajeo (Podos 2001, Seddon 2005), ya que en algunas especies la morfología del pico y las características vocales están correlacionadas debido a restricciones físicas en la producción del sonido. Estas hipótesis sobre los procesos que pueden conducir a la divergencia de señales predicen una dirección en la evolución de acuerdo a la situación particular. Por el contrario, existe otro grupo de hipótesis en las cuales no existe una predicción sobre la dirección de la evolución del rasgo, sino que son procesos estocásticos que pueden causar la divergencia de las señales acústicas (Irwin *et al*. 2001). Dentro de estas hipótesis se encuentra la selección social, la cual se ha

definido como un tipo de selección natural que es causada por miembros de un grupo en competencia por alimento, territorios, dominancia, derechos reproductivos, acceso a apareamientos y otros recursos. (Thornhill & Alcock 1983), y la selección sexual la cual actúa sobre la habilidad de los organismos para obtener cópulas con una pareja (Darwin 1871). Estos dos tipos de selección pueden causar la evolución rápida de señales por la atractividad hacia rasgos novedosos, el potencial de cambio desbocado (runaway) y la ausencia de un óptimo bien definido (West-Eberhard 1983, Andersson 1994). Los cambios estocásticos pueden ocurrir también por mutación cultural y deriva en ausencia de alguna forma de selección.

Se ha sugerido que los rasgos seleccionados sexualmente tienen el potencial de divergir de manera obvia en algunos taxa (Irwin *et al.* 2001). En algunas especies de aves, existe una amplia variación subespecífica en la expresión de caracteres sexuales secundarios, que puede reflejar divergencia por acción de la selección sexual (Johnsen *et al.* 2006). Por lo tanto, la selección sexual puede ser una fuerza poderosa en la divergencia genética entre poblaciones y en eventuales procesos de especiación en parte porque favorece rasgos novedosos o complejos (Darwin 1871, West-Eberhard 1983). Lande (1982) exploró el potencial de la selección sexual para promover especiación a lo largo de un gradiente geográfico, analizando la evolución conjunta de la variación geográfica en la preferencia reproductiva de las hembras y de los rasgos sexuales secundarios de los machos medidos cuantitativamente. Sus modelos mostraron que el aislamiento reproductivo y la divergencia de caracteres pueden originarse rápidamente en un rango geográfico amplio, debido a una inestabilidad genética del sistema de apareamiento en poblaciones locales y a que la evolución de preferencias reproductivas de las hembras puede amplificar la variación geográfica en rasgos sexuales secundarios de los machos sin discontinuidad geográfica, conduciendo a un aislamiento precopulatorio e incluso la especiación parapátrica. Trabajos de revisión más recientes discuten el poder de la selección sexual en conducir cambios en rasgos involucrados en la elección de pareja como una fuerza poderosa en promover especiación (Panhuis *et al.* 2001, Ritchie 2007), los cuales sugieren que

existen modelos que confirman esta predicción, sin embargo el efecto puede ser más fuerte junto con la especialización ecológica.

Selección sexual y estructuración genética entre leks

La expresión de los rasgos sexuales secundarios depende de la intensidad de la selección inter e intrasexual en interacción con las características del sistema de apareamiento (cuidado parental, número de parejas obtenidas; Shuster & Wade 2003). En especies poligínicas donde algunos machos se aparean con muchas hembras, y otros con ninguna, el éxito en la competencia por las parejas es crucial para la adecuación de los machos (Emlen & Oring 1977) y sus armas y/o ornamentos a menudo están muy desarrollados. El potencial de la selección sexual alcanza su máxima expresión en especies con sistemas de leks (Trail 1990). Los leks se han definido como cualquier agregación de machos que las hembras visitan solamente con el propósito de aparearse (Höglund & Alatalo 1995), sin embargo puede distinguirse de otros sistemas de apareamiento en cuanto a que en los leks 1) no hay cuidado parental, ya que los padres solo contribuyen con gametos a la siguiente generación; 2) existe una arena o sitio donde los machos se reúnen y ocurre la mayoría de las cópulas; 3) los sitios de despliegue no contienen recursos tales como comida, agua, lugares de descanso o de anidamiento; 4) las hembras tienen la oportunidad de seleccionar a su pareja cuando visitan la arena. En este sistema de apareamiento el éxito reproductivo varía mucho entre machos y generalmente solo uno o pocos machos obtienen la mayoría de las cópulas (Höglund & Alatalo 1995). Los machos en varias de estas especies tienen ornamentos conspicuos que deben ser el blanco de la elección por hembras, involucrando también la competencia entre machos.

Los leks son sistemas que parecen ofrecer condiciones favorables para la selección sexual ya que la elección de pareja no estaría influenciada por recursos materiales (por ejemplo recursos alimenticios, nidos, etc; Höglund & Alatalo 1995). Estos han sido descritos al menos en 14 diferentes familias de aves y se piensa que han evolucionado independientemente. Por lo

tanto la ocurrencia de los leks está concentrada en muy pocas familias lo que sugiere un fuerte sesgo filogenético en su origen (Höglund & Alatalo 1995). En estos sistemas de apareamiento los machos están sujetos a una fuerte selección sexual, y como este tipo de selección puede promover la evolución de diferentes rasgos de los machos en diferentes poblaciones de la misma especie, pueden ocurrir eventos de especiación muy rápidamente (Lande 1980).

Una predicción del modelo de evolución de leks que está relacionado con la adecuación inclusiva, es que los machos deben estar más relacionados dentro que entre leks (Kokko & Lindstrom 1996). Dentro de los leks la distribución de los apareamientos generalmente es muy sesgada (Semple *et al.* 2001), y la variación en el éxito reproductivo de los machos se ha relacionado con la elección de las hembras. Los machos con rasgos sexuales secundarios elaborados pueden aumentar su éxito reproductivo desplegando en sitios que aumenten la eficacia de la señal. También pueden aumentar su éxito reproductivo vía selección de familia (kin selection), desplegando en sitios donde la población esté estructurada genéticamente (formada por parientes; Madden *et al.* 2004). Los sistemas de apareamiento pueden afectar el tamaño efectivo de la población, particularmente cuando solo algunos individuos contribuyen a la reproducción (como es el caso de los leks), y cuando los individuos reproductivos están relacionados, pueden amplificarse los efectos genéticos estocásticos (Parker & Waite 1997, Bouzat & Johnson 2004). Además de la selección de familia, la fidelidad de sitio y la baja dispersión de algunas especies pueden reducir la variación genética dentro de los leks e incrementar la diferenciación genética entre leks, pudiendo promover diversificación.

Cantos en colibríes

La diversificación de colibríes se ha considerado como un ejemplo clásico de especiación a través de la selección sexual (Darwin 1871; Futuyma 1987). En colibríes, los estudios para evaluar el significado evolutivo de características fenotípicas se han enfocado principalmente en señales visuales ya que en muchas especies los machos despliegan coronas y gargantas

iridiscentes y colas elongadas en despliegues aéreos complejos (Wagner 1954, Ornelas *et al.* 2002, Clark & Dudley 2009, Parra 2009). Sin embargo, también han desarrollado una diversidad de vocalizaciones importante. Por mucho tiempo se pensó que los colibríes tenían cantos o llamados relativamente simples (Johnsgard 1997) y que no eran aprendidos, sin embargo, ahora se sabe que producen cantos aprendidos distintos a los llamados, siendo algunos de ellos muy complejos y variables (Ficken *et al*. 2000, Ornelas *et al*. 2002). El aprendizaje vocal en aves se ha demostrado solamente en tres grupos: aves canoras, pericos y colibríes y se piensa que ha evolucionado independientemente (Jarvis *et al.* 2000). En comparación con otros grupos de aves los repertorios vocales en colibríes han sido muy poco estudiados en cuanto a las características acústicas, estructura y organización, función, variación geográfica y evolución. Una de las consecuencias del aprendizaje vocal es la variación geográfica y la formación de dialectos. La forma en que las aves aprenden a cantar es mediante imitación, por lo tanto se pueden cometer errores en este proceso y como consecuencia pueden surgir nuevas variaciones en el canto, además de la posibilidad de recombinar e innovar elementos. Cuando la variación vocal entre poblaciones vecinas o grupos es mayor que dentro de poblaciones, se les llama dialectos (Nottebohm 1969). Los dialectos vocales se han descrito para numerosas especies de oscines (revisado en Mundinger 1982) y en algunas no paseriformes incluyendo colibríes (Snow 1968, Gaunt *et al.* 1994, Wright 1996, Bradbury *et al.* 2001, Vehrencamp *et al.* 2003, González & Ornelas 2005, Yang *et al.* 2007).

Sistema de estudio

El género *Campylopterus* está compuesto por 10 especies de colibríes, mas tres especies que alguna vez se han clasificado dentro del género *Campylopterus* y que actualmente se encuentran clasificadas dentro de géneros monotípicos (*Phaeochroa, Aphantochroa y Eupetomena*, AOU 1998). Se distribuyen principalmente en Sudamérica (siete especies) y Centroamérica (tres especies) las cuales alcanzan el límite norte de su distribución en México

(*C. curvipennis, C. rufus y C. hemileucurus*). La mayoría de las especies, con excepción de *C. largipennis*, tienen una distribución disyunta y restringida en bosques húmedos y semiáridos (Schuchmann 1999). Este grupo de colibríes, llamados también alas de sable, son grandes (en relación a otros linajes de colibríes) y se caracterizan por tener engrosados y aplanados los raquis de las plumas primarias exteriores. De las cinco especies donde se ha descrito su sistema de apareamiento, todas forman leks (*C. curvipennis*, González & Ornelas 2005; *C. rufus*, Skutch 1972; *C. hemileucurus*, Skutch 1967; *C. ensipennis*, Wilson *et al.* 1997; *C. villaviscencio*). Este grupo de colibríes es interesante ya que la mayoría son dimórficos en tamaño, siendo los machos más grandes que las hembras, lo que puede estar relacionado con su sistema de apareamiento y con la competencia entre machos por las hembras (Payne 1984), y la mitad de las especies presentan desde un dimorfismo en plumaje moderado a muy pronunciado como es el caso de *C. hemileucurus*. Existen tres grupos de *Campylopterus* de acuerdo a su plumaje, los que tienen garganta, pecho y partes frontales grises con poca o ninguna iridiscencia (*C. curvipennis, C. largipennis*), los que tienen gargantas y pechos violeta iridiscente (*C. hemileucurus*, *C. ensipennis, C. falcatus, C. phainopeplus* y *C. villaviscencio*) y los que tienen garganta, pecho y partes frontales café canela (*C. rufus, C. hyperythrus* y *C. duidae*). En cuanto a los cantos aunque no se han estudiado con detalle en varias especies, aparentemente existe una complejidad vocal que va desde cantos relativamente simples y estereotipados en *C. hemileucurus,* que es la especie con un dimorfismo en plumaje más marcado, hasta *C. curvipennis* que tiene cantos muy complejos y versátiles. Debido a la distribución disyunta y restringida de la mayoría de estas especies y la variación en coloración, dimorfismo y complejidad en el canto, este es un sistema interesante para abordar preguntas relacionadas con la historia evolutiva del grupo así como aquellas relacionadas con la evolución de rasgos seleccionados sexualmente y compromisos asociados entre tener cantos o plumajes costosos.

Campylopterus curvipennis (la especie en la que se enfoca este trabajo) es particularmente interesante porque tiene cantos con una estructura silábica muy compleja los cuales varían a diferentes escalas geográficas. Además tienen una amplia distribución de manera disyunta desde el noreste del país (Tamaulipas) hasta Oaxaca, la Sierra de Los Tuxtlas y la Península de Yucatán. De acuerdo con estas características *C. curvipennis* es un sistema ideal para abordar preguntas sobre asociaciones entre estructura genética a varios niveles y evolución cultural.

Objetivos y estructura de la tesis

El objetivo principal de este trabajo fue determinar los patrones de divergencia genética y fenotípica (principalmente vocal) a lo largo de la distribución de *Campylopterus curvipennis*, un colibrí con una extraordinaria complejidad y variación vocal, evaluando tanto factores geográficos, históricos y climáticos como factores microevolutivos relacionados con su sistema de apareamiento que pudieron haber generado esta divergencia. Además, determinar si los patrones de variación vocal, un rasgo transmitido culturalmente, están relacionados con una estructuración genética a nivel local (dentro de leks) y geográfica (entre leks). Los resultados de este trabajo contribuirán a entender el papel que tiene un rasgo aprendido como son las vocalizaciones en la estructuración genética a diferentes escalas, y contribuir al entendimiento de la gran diversificación de especies que existe dentro de la familia.

La tesis está organizada de acuerdo a la escala del estudio, iniciando con la parte a nivel geográfico y terminando con un estudio local. El primer capítulo es uno de los más importantes y extensos de la tesis, y es el estudio filogeográfico del complejo de especies de *C. curvipennis* a lo largo de su distribución desde el sur de Tamaulipas hasta la Península de Yucatán. Se encuentra publicado en BMC Evolutionary Biology (**11**:38, 2011). Los objetivos de este capítulo fueron inferir la historia evolutiva del complejo (específicamente probar el papel del Istmo de Tehuantepec como una barrera) y los procesos que pudieron moldear la distribución

de la variabilidad genética actual, así como determinar el papel de la selección y la deriva sobre la divergencia morfológica y acústica. Las hipótesis fueron 1) que las poblaciones con distribución disyunta son genéticamente divergentes, 2) que el Istmo de Tehuantepec ha actuado como una barrera al flujo genético tanto histórico como contemporáneo, 3) dado que las señales acústicas dentro de este complejo son muy variables y generalmente están involucradas en la elección de pareja y reconocimiento de especies, éstas deben ser resultado de fuerzas estocásticas tales como la selección natural o sexual y no resultado de procesos neutrales. Para esta parte utilicé tanto genes mitocondriales como microsatélites para poder hacer inferencias a dos escalas de tiempo distintas, así como medidas de rasgos fenotípicos (morfología y vocalizaciones) de los tres grupos genéticos inferidos. Encontré tres linajes en el complejo de especies que corresponden a poblaciones disyuntas las cuales carecen de flujo genético contemporáneo y han divergido a distintas escalas de tiempo. En cuanto a los análisis de rasgos fenotípicos, encontré que la selección es responsable de la evolución del canto pero la deriva no puede rechazarse como posibilidad para explicar la divergencia morfológica. En este capítulo hice recomendaciones de conservación en particular para la región de los Tuxtlas, por haber encontrado formas genéticas y morfológicas únicas además de un tamaño efectivo de la población menor que en las otras áreas.

El objetivo del segundo capítulo fue evaluar si la variación geográfica de los cantos de *C. curvipennis* está correlacionada con la divergencia genética entre leks localizados a lo largo de la distribución en la Sierra Madre Oriental de uno de los tres linajes, además de evaluar el papel de la deriva y la selección en la divergencia vocal a esta escala geográfica. La hipótesis fue que los dialectos locales en *C. curvipennis* contribuyen a una estructuración genética entre leks, ya que las preferencias por un dialecto local podrían conducir a una divergencia genética a través del apareamiento *asortativo*, y que la divergencia vocal es resultado de la selección sexual. Los datos de este capítulo mostraron una fuerte estructura acústica entre leks, donde cada uno tiene un ensamble de tipos de sílaba exclusivos, sin embargo, la divergencia genética

neutral y la divergencia vocal no están correlacionadas. Además encontré una baja estructuración genética y altas tasas de migración entre leks. Este capítulo enfatiza el papel del aprendizaje en la rápida evolución que pueden tener las señales acústicas en situaciones con una baja estructura genética, además del papel de la selección social en promover convergencia de cantos dentro de leks y la selección sexual en promover novedades vocales. Este capítulo se enviará al Journal of Evolutionary Biology para su posible publicación.

El objetivo del tercer capítulo fue describir los vecindarios vocales dentro de un lek particular y determinar la variación en el canto de machos de estos vecindarios a lo largo de cuatro años. Este capítulo se encuentra publicado en Condor (**111**:633-640, 2009). Encontré tres vecindarios vocales los cuales fueron consistentes año con año en la estructura y composición general de las sílabas además de que los machos pertenecientes a cada vecindario están espacialmente agregados. A pesar de la consistencia de la composición de sílabas entre años, algunas sílabas se ganaron y otras se perdieron durante el periodo de estudio, lo que sugiere la rapidez con que pueden cambiar las señales acústicas y el repertorio silábico en esta especie.

El objetivo del cuarto capítulo fue inferir el potencial de la selección de familia dentro de leks de *C. curvipennis*, así como investigar si la variación vocal observada dentro de un lek focal (vecindarios vocales) está relacionada con el parentesco entre machos. Las hipótesis fueron: 1) si el éxito reproductivo entre machos está sesgado y los machos que no logran aparearse aumentan su adecuación inclusiva agrupándose en leks, se esperaría que los machos que despliegan en leks estén relacionados genéticamente; 2) si los individuos de esta especie utilizan las características vocales como un mecanismo de reconocimiento entre parientes, debería existir una relación entre el parentesco y los vecindarios vocales. Encontré que el coeficiente de parentesco promedio dentro de los leks es bajo y con una varianza muy grande, lo que sugiere que tanto individuos emparentados como no emparentados despliegan juntos en un lek y en general los machos dentro del lek no están más emparentados que lo que se esperaría al azar. También comparé el parentesco entre los machos de los vecindarios vocales

y no encontré evidencia de agrupamientos de parientes dentro de los vecindarios, donde los machos están igualmente emparentados que con machos de diferentes vecindarios. Esto sugiere que la selección de familia no es importante en esta especie y que los juveniles que se establecen en los leks no aprenden el canto de sus padres o parientes. Este capítulo se enviará a Behavioral Ecology and Sociobiology para su posible publicación.

Por último el objetivo del quinto capítulo fue aislar loci de microsatélites polimórficos para *Campylopterus curvipennis*, que fue uno de los marcadores moleculares que utilicé en el trabajo. Este capítulo se encuentra publicado en Molecular Ecology Resources (**10**:232-236, 2010). Cuando se inició este proyecto no existían reportes en la literatura de microsatélites aislados para ningún colibrí (actualmente hay dos más), por lo que fue necesario su asilamiento. Los 10 loci de microsatélites polimórficos aislados fueron probados en otras especies del mismo género donde la mayoría amplificaron exitosamente. También los probé en otros 5 géneros de colibríes representando a los principales linajes, sin embargo, la amplificación fue menos exitosa.

CAPÍTULO I

Selection and geographic isolation influence hummingbird speciation: genetic, acoustic and morphological divergence in the wedge-tailed sabrewing (*Campylopterus curvipennis*)

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Selection and geographic isolation influence hummingbird speciation: genetic, acoustic and morphological divergence in the wedge-tailed sabrewing (*Campylopterus curvipennis*)

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Abstract

Background: Mesoamerica is one of the most threatened biodiversity hotspots in the world, yet we are far from understanding the geologic history and the processes driving population divergence and speciation for most endemic taxa. In species with highly differentiated populations selective and/or neutral factors can induce rapid changes to traits involved in mate choice, promoting reproductive isolation between allopatric populations that can eventually lead to speciation. We present the results of genetic differentiation, and explore drift and selection effects in promoting acoustic and morphological divergence among populations of *Campylopterus curvipennis*, a lekking hummingbird with an extraordinary vocal variability across Mesoamerica.

Results: Analyses of two mitochondrial genes and ten microsatellite loci genotyped for 160 individuals revealed the presence of three lineages with no contemporary gene flow: *C. c. curvipennis, C. c. excellens*, and *C. c. pampa* disjunctly distributed in the Sierra Madre Oriental, the Tuxtlas region and the Yucatan Peninsula, respectively. Sequence mtDNA and microsatellite data were congruent with two diversification events: an old vicariance event at the Isthmus of Tehuantepec (*c.* 1.4 Ma), and a more recent Pleistocene split, isolating populations in the Tuxtlas region. Hummingbirds of the *excellens* group were larger, and those of the *pampa* group had shorter bills, and lineages that have been isolated the longest shared fewer syllables and differed in spectral and temporal traits of a shared syllable. Coalescent simulations showed that fixation of song types has occurred faster than expected under neutrality but the null hypothesis that morphological divergence resulted from drift was not rejected.

Conclusions: Our phylogeographic analyses uncovered the presence of three Mesoamerican wedge-tailed sabrewing lineages, which diverged at different time scales. These results highlight the importance of the Isthmus of Tehuantepec and more recent Pleistocene climatic events in driving isolation and population divergence. Coalescent analyses of the evolution of phenotypic traits suggest that selection is driving song evolution in wedge-tailed sabrewings but drift could not be rejected as a possibility for morphological divergence.

Background

Mesoamerica is considered one of the largest and most important biodiversity hotspots in the world, based mainly on the global proportion of vertebrate endemism and the loss of the original primary vegetation cover [1]. Nonetheless, the processes influencing the evolutionary history and diversification within the region are not well understood in most taxa. The Mesoamerican region has been a key land bridge for biotic migrations between North and South America (see for example [2]), and a significant pulse of avian interchange and further radiation occurred in concert with the Isthmus of Panama uplift [3,4]. Geographic barriers, by promoting and maintaining population divergence, are expected to have influenced historical patterns of diversification and the evaluation of such patterns can provide guidance for the preservation and management of genetic diversity and endemism in the region [5]. The Mesoamerican

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highlands have featured recently in vertebrate mitochondrial DNA phylogeographic studies [6-11]. These studies observed a point of divergence at the Isthmus of Tehuantepec, most noticeably in the rodents and birds that inhabit the mesic highlands. The Isthmus of Tehuantepec, a geologically complex zone that has undergone continental uplift and sea level oscillation since the late Miocene [12,13], has been considered a major biogeographic barrier [14]. The temporary isolation of populations from either side of the isthmus owing to oceanic incursions, and the emergence of the Sierra de los Tuxtlas in the late Pliocene -a volcanic massif 200-250 km away from the Sierra Madre Oriental- along with changes in local environmental conditions that accompanied late Pleistocene glacial cycles, may also explain patterns of biotic diversification in this region.

The wedge-tailed sabrewing (*Campylopterus curvipennis*), a sexually monochromatic, size dimorphic hummingbird species complex commonly found in montane cloud forests and humid tropical forests [15], offers an excellent system for addressing questions about historical biogeography and speciation of Mesoamerican biota. It is one of the few hummingbird species known for both the lowlands and montane region with a geographical disjunction at the Isthmus of Tehuantepec [15]: populations in the foothills of the Atlantic slope of the Sierra Madre Oriental (from south Tamaulipas to north Oaxaca) (SMO), and the Tuxtlas region (Sierra de los Tuxtlas and Sierra de Santa Marta) and a small area on the Isthmus of Tehuantepec (Jesús Carranza and Uxpanapa) (TUX), are separated from those found from northeastern Chiapas to central-south of the Yucatan Peninsula (YUC) (Table 1) [15]. Across their geographic range, they display subtle variation in plumage coloration and bill and body size, which has lead taxonomists to name three subspecies: *Campylopterus curvipennis curvipennis* (SMO), *C. c. excellens* (TUX) and *C. c. pampa* (YUC) [16]. Wedge-tailed sabrewings are remarkable among hummingbirds both because they are one of *c*. 30 out of 320 extant hummingbird species known as lek breeders [17], and because of their elaborate singing displays [18]. In addition, there are marked regional differences in their vocalizations, most noticeably in the introductory syllable and syllable repertoire [18]. Their complex syllable structure exhibits geographic variation that ranges from differences between neighboring males within a lek to differences between lek members separated by several kilometers [18,19].

Species with highly differentiated populations may represent the early stages of the speciation process. In these populations, drift, ecological selection or both can induce changes in traits involved in mate choice, promoting reproductive isolation between allopatric populations that can eventually lead to speciation [20-23]. The sole action of drift and mutation can promote phenotypic differentiation or reproductive isolation [24]. In contrast, ecological speciation can occur when divergent selection pressures act generally on the

Table 1 Geographic region, coordinates, and altitude of *Campylopterus curvipennis* sampled populations

Locality	Region	Latitude N	Longitude W	Altitude (masl)
	curvipennis group			
1. El Cielo (Ciel)*	nSMO	25° 3'33.66"	99° 12'21.40"	943
2. Gomez Farías (GF)*	nSMO	23° 3'58.26"	99° 10'6.52"	564
3. El Naranjo (Nar)*	nSMO	22° 34'33.33"	99° 21'11.80"	270
4. Aquismón (Aqm)*	cSMO	21° 37'30.87"	99° 1'12.52"	378
5. Xilitla (Xil)*	cSMO	21° 22'39.50"	98° 59'35.77"	637
6. San Bartolo Tutotepec (SBT)	cSMO	20° 21'11.10"	98° 13'10.40"	1155
7. Cuetzalan (Cuet)*	sSMO	20° 0'49.14"	97° 30'21.27"	906
8. Macuiltépetl (Mac)*	sSMO	19° 32'50.51"	96° 55'12.45"	1500
9. Coapexpan (Coap)	sSMO	19° 31'22.90"	96° 58'2.20"	1392
10. Parque Clavijero (Clav)	sSMO	19° 30'47.01"	96° 56'28.64"	1225
11. La Orduña (Ord)*	sSMO	19° 27'50.94"	96° 56'13.05"	1190
12. El Riscal (Risc)	sSMO	19° 28'47.40"	96° 59'51.00"	1586
13. Ursulo Galván (UG)*	sSMO	19° 25'31.48"	96° 58'35.20"	1200
14. Xico (Xico)	sSMO	19° 24'37.99"	96° 59'31.81"	1350
15. Amatlán (Ama)	sSMO	18° 49'51.84"	96° 54'7.78"	720
	excellens group			
16. Los Tuxtlas (Tux)*	TUX	18° 33'29.05"	95° 11'46.00"	998
17. El Nopal (Nop)	TUX	17° 14'23.5"	90°45'40.5"	664
18. Chalchijapa (Chal)	TUX	17°2'4.07"	94°41'57.85"	260
	pampa group			
19. Escárcega (Esc)	YUC	18° 38'12.5"	90° 47'16.89"	60
20. Río Bec (RB)	YUC	18° 24'31.4"	89° 26'37.2"	251
21. Ejido 20 de Noviembre (Nov)*	YUC	18° 25'29.0"	89° 18'37.3"	179
22. Tres Garantías (Gar)	YUC	18° 12'51.2"	89° 02'34.8"	137

Geographic regions correspond to three disjunct areas along the Sierra Madre Oriental, the Tuxtlas region and Yucatan Peninsula (see also Figure 2). Asterisk indicates populations on for acoustic recordings were made.

morphological or behavioral characters of species distributed on heterogeneous habitats, where gene flow among locally adapted populations is reduced [25]. For acoustic traits, microclimate and vegetation structure can be important selective pressures on the transmission of acoustic traits of birds living in different habitats [26,27]. Another possibility is that within populations, stochastic changes in traits driven by processes such as sexual selection promote reproductive isolation between geographically isolated populations where divergent natural selection is not acting [28,29].

To better understand the patterns of diversification and endemism in Mesoamerica, we use mitochondrial (mtDNA) and nuclear (microsatellites) DNA data to infer the processes behind the evolutionary history of *C. curvipennis*. The use of both bi-parentally inherited molecular markers with different mutation rates is important because it allows us to make inferences at different temporal scales and provides more reliable insight into the historical processes involved in the evolution of taxa than studies based on the analyses of a single marker do. In addition to genetic data (mtDNA and microsatellites as a proxy for neutral processes; [30]), we used morphological and acoustic data along with information about the habitat (climate and topography) to examine the relative roles of drift and selection in driving population divergence. The specific goals of this study were to: (i) examine the patterns of genetic variation and demographic history of *C. curvipennis* populations, (ii) infer the processes behind its evolutionary history, specifically assessing the role of the Isthmus of Tehuantepec as a barrier, and (iii) evaluate the role of drift and selection in driving phenotypic (morphology and song) divergence in wedge-tailed sabrewings. Patterns resulting from intraspecific geographical variation in phenotypic traits and genetic markers should provide insights into the factors driving population differentiation and ultimately speciation in Mesoamerica.

Results

Sequence variation and phylogenetic analyses

From 160 wedge-tailed sabrewings we obtained mtDNA sequences that contained 81 polymorphic sites (38 in the ATPase and 43 in the control region) of the 1,407 bp of the genes analyzed. No insertions or deletions were present; therefore, variants were identified based solely on nucleotide substitutions. Sixty-three haplotypes were identified for the 22 wedge-tailed sabrewing populations. Haplotype and nucleotide diversity are summarized in Table 2. Haplotype proportion relative to the number of samples per population was more than 50% in most cases, indicating high haplotype diversity. No differences between populations were evident. Nucleotide diversity was low, indicating little variation between sequences from the same population.

The consensus tree obtained from Bayesian inference clustered the haplotypes into two main well-supported clades (posterior probabilities of 1.0), corresponding to disjunct western (*curvipennis*, SMO) and eastern (*pampa*, YUC) groups on either side of the Isthmus of Tehuantepec (Figure 1). Haplotypes of the *excellens* group from the Tuxtlas region were clustered in a well-supported clade nested within the poorly resolved SMO clade.

Assuming a constant molecular clock and rates of 2 and 5% divergence per My, for the ATPase coding region BEAST estimated that the SMO and TUX clades diverged from the YUC clade 1.47 (0.35-3.42) and 0.52 (0.13-1.21) Mya, respectively. These results suggest that the split between the Sierra Madre Oriental and Yucatan Peninsula clades may have occurred during the mid-Pleistocene. With respect to SMO, for the TUX clade the constant clock TMRCA estimated divergence times of 614,000 (0.12-1.55 Mya) and 202,000 (0.05-0.47 Mya) years ago for 2 and 5% substitution/My, respectively. This implies that the split between the TUX and SMO clades may have occurred more recently in the late Pleistocene.

Phylogeographic and genetic structure

The haplotype network showed a strong phylogeographic structure among three groups (SMO, TUX, YUC), but not among populations or areas within the SMO (nSMO, cSMO, sSMO) (Figure 2). Of the 63 haplotypes obtained, 44 were private to SMO populations. The most frequent haplotype was shared by samples from all of the SMO populations, and the rest were low frequency haplotypes (Figure 2). Seven haplotypes were private to the Tuxtlas region, and are separated from the SMO haplotypes by five mutational steps. The remaining haplotypes (12) were exclusively found in populations from the Yucatan Peninsula, and formed a separate network (Figure 2).

The AMOVA results revealed significant genetic differentiation at every hierarchical level. When grouped by geographic areas (nSMO, cSMO, sSMO, TUX, and YUC; see Table 1 and field procedures for the definitions) most of the variation (85%) was explained by differences among areas, whereas variation among populations within areas (0.92%) and variation within populations (13.94%) only explained a small percentage of the total variation (Table 3). The same pattern was observed when grouped by subspecies (*curvipennis*, SMO; *excellens*, TUX; and *pampa*, YUC) with the highest percentage of the variation being explained by differences among groups (90.41%) and the lowest by differences among populations within groups (0.55%; Table 3). Φ_{CT} values for both analyses were very high (Table 3) indicating high levels of genetic differentiation

Table 2 Population genetic variability of *Campylopterus curvipennis*

		Microsatellite DNA				Mitochondrial DNA			
Locality	***n***	**Mean alleles /locus**	**Allelic Richness**	H_O	H_E	**H**	**S**	***Hd***	**π**
curvipennis group									
El Cielo	18	5.6	1.60	0.47*	0.59	6	9	0.83	0.0021
Gomez Farías	4	3.6	1.61	0.65	0.61	2	1	0.5	0.0035
El Naranjo	6	3.6	1.58	0.55	0.57	6	8	1	0.0020
Aquismón	4	3.5	1.63	0.55	0.63	2	1	0.5	0.0003
Xilitla	8	5	1.64	0.49*	0.64	6	7	0.92	0.0013
S. B. Tutotepec	1	1.4	1.40	-	-	1	0	-	-
Cuetzalan	27	6.2	1.62	0.57	0.62	17	20	0.92	0.0020
Macuiltépetl	3	2.66	1.55	0.55	0.57	3	5	1	0.0023
Coapexpan	6	4.5	1.65	0.18	0.30	6	11	1	0.0027
Parque Clavijero	7	4.1	1.60	0.46	0.60	7	8	1	0.0023
La Orduña	22	6.2	1.63	0.49*	0.63	11	14	0.819	0.0020
El Riscal	2	2.3	1.60	0.45	0.60	2	1	1	0.0007
Ursulo Galván	13	5.3	1.60	0.48†	0.60	6	8	0.85	0.0014
Xico	4	2.8	1.61	0.50	0.61	2	8	0.83	0.0033
Amatlán	3	3.1	1.62	0.47	0.62	3	6	1	0.0028
excellens group									
Los Tuxtlas	10	4.7	1.61	0.51	0.61	5	5	0.66	0.0007
El Nopal	1	1.3	1.30	-	-	1	0	-	-
Chalchijapa	1	1.7	1.70	-	-	1	0	-	-
pampa group									
Escárcega	1	1.5	1.50	-	-	1	0	-	-
Río Bec	2	1.9	1.45	0.45	0.45	2	2	1	0.0014
20 de Noviembre	15	5.5	1.57	0.46*	0.57	11	12	0.96	0.0027
Tres Garantías	3	2.7	1.56	0.4	0.56	2	5	0.66	0.0020

Estimates based on ten microsatellite loci, and 1407 bp of the control region and ATPase 6-8 mtDNA genes. *n* = sample size, observed (H_O) and expected heterozygosity (H_E), H = number of haplotypes observed, S = number of polymorphic sites, *Hd* = haplotype diversity, and π = nucleotide diversity. Asterisks indicate significant departure ($P < 0.05$, after a sequential Bonferroni correction) from Hardy-Weinberg equilibrium for locus CACU13-2 and † for locus CACU13-7.

among areas and groups. Pairwise comparisons of F_{ST} among sampling localities ranged from 0.003 for Aqm/Ama to 0.95 for UG/Gar and were significant in most cases between sampling localities from the TUX region and the other localities, and for the YUC region and the other localities [Additional file 1]. Comparisons between sites located in the same geographic area (SMO, TUX, YUC) were not significant [Additional file 1].

Microsatellite markers

Across all sampling localities, the number of alleles per locus varied from 4 to 20, and within localities across all loci this number varied from 13 to 62 (mean alleles per locus, 1.3-6.2) (Table 2). Observed heterozygosity values did not consistently deviate from H-W equilibrium. Only four localities were not in H-W equilibrium after Bonferroni corrections at locus CACU13-2 and one locality at locus CACU13-7 (Table 2) probably due to the presence of null alleles. No significant linkage disequilibrium was detected in any of the population-loci comparisons after Bonferroni corrections.

Significant genetic subdivision was detected among sampling localities (global R_{ST} estimate ± SE, 0.085 ± 0.0021, $P < 0.0001$), but not among sampling localities within the fragmented areas of the SMO (global R_{ST} estimate ± SE, -0.020 ± 0.0021, $P > 0.05$). Pairwise F_{ST} and R_{ST} were quantitatively similar but we only report R_{ST} values because the stepwise mutation model implemented in this estimate is more appropriate for microsatellites [Additional file 1]. Pairwise R_{ST} values among sampling localities ranged from 0.001 for Xil/Ord to 0.42 for Risc/Nov. As with the pairwise F_{ST} values for the mitochondrial genes, estimates of R_{ST} were significant for almost all comparisons between localities from TUX *versus* the rest of the localities and comparisons between localities from YUC *versus* the rest after Bonferroni corrections. Values from comparisons between sampling localities within SMO were not statistically

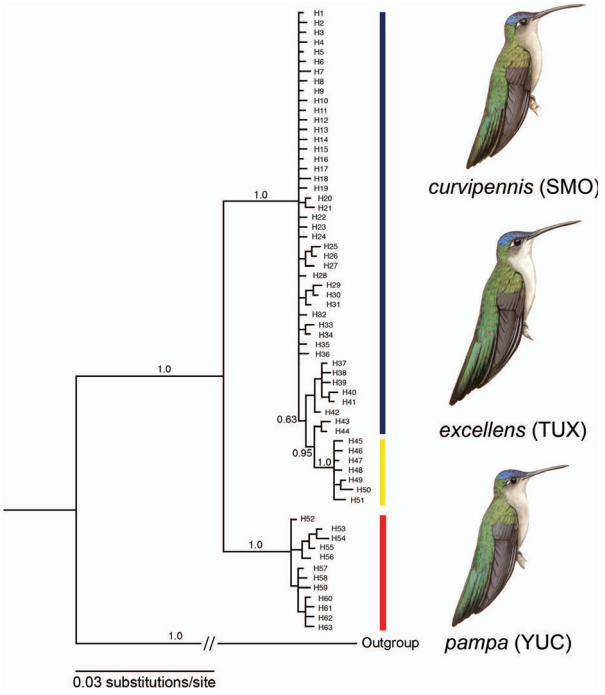

Figure 1 Phylogenetic relationships among mtDNA haplotypes based on Bayesian inference. Values above branches denote posterior probabilities, and numbers at the tip of the branches indicate distinct haplotypes. Outgroups were *Campylopterus rufus*, *C. hemileucurus*, and *C. largipennis*, and are shown collapsed into a single branch. Hummingbird illustrations were taken from color plates in the *Handbook of the Birds of the World* [32].

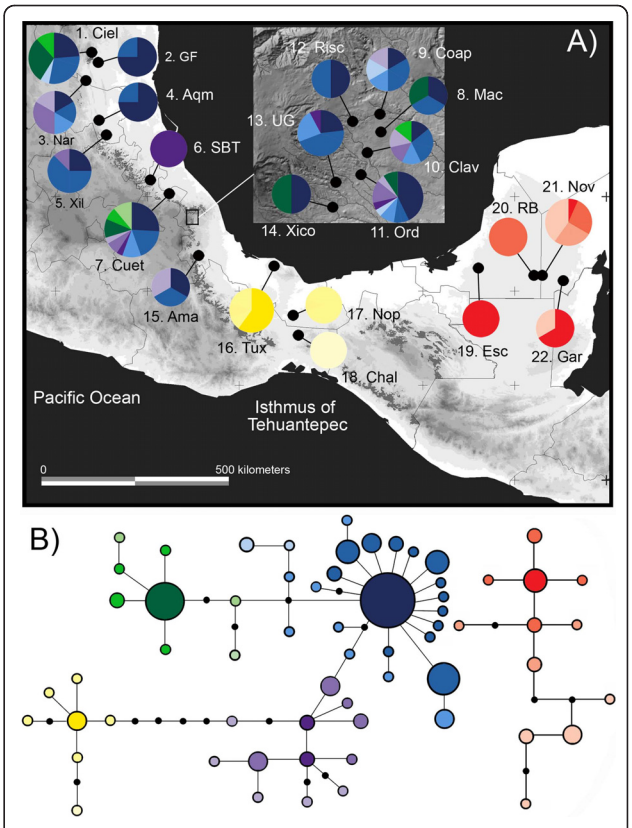

Figure 2 Sampling localities, geographic distribution, and genealogical relationships of mtDNA haplotypes. **A**. Pie charts indicate the frequency of occurrence of haplotypes in each population, and colors correspond to those shown in the haplotype network below. The current natural range of cloud forest (indicated by dark gray shading) is overlaid on a relief map of eastern Mexico. **B**. Statistical parsimony haplotype network derived from a combined matrix of mitochondrial genes (ATPase 6-8 and control region). Each circle represents a haplotype and its size is proportional to its frequency. Lines represent a single mutational change and black circles correspond to unsampled haplotypes.

significant. Comparisons between samples from Bec and Gar (from the YUC region) *versus* the rest were not significant in most cases after Bonferroni corrections probably due to sample size, affecting statistical power.

The phenogram constructed with pairwise R_{ST}, recovered sampling localities from the YUC group clustering together in a basal position followed by the TUX and the SMO groups [Additional file 2]. Within the SMO group, samples from the northern limit of the distribution (Tamaulipas) are clustered together, as are samples from San Luis Potosi (central distribution). However, localities from the southern distribution (Veracruz and Puebla) are scattered throughout the tree [Additional file 2].

Results of the STRUCTURE analysis corroborated population substructure. Using all genotypes together the log likelihood was highest for K = 6; however, when ΔK is calculated the break in the slope of the distribution of $L(K)$ was at K = 2 (Figure 3). One cluster includes populations from the Yucatan Peninsula (YUC), and the other includes samples from populations from the TUX and SMO groups (Figure 3). However, in the clustering pattern at K = 3, samples were assigned with high probabilities to three clusters, corresponding to the

Table 3 Analysis of molecular variance (AMOVA) for the control region and ATPase 6-8 mtDNA genes of *Campylopterus curvipennis*

Structure	Source of variation	Variation (%)	Fixation indices
Five areas	Among areas	85.13	Φ_{CT} 0.85**
	Among populations within areas	0.92	Φ_{SC} 0.06**
	Within populations	13.94	Φ_{ST} 0.86**
Three groups	Among groups	90.41	Φ_{CT} 0.90**
	Among populations within groups	0.55	Φ_{SC} 0.05**
	Within populations	9.05	Φ_{ST} 0.91**

Data were grouped by geographic area and subspecies. The five areas are nSMO, cSMO, sSMO, TUX and YUC. Groups are *curvipennis* (SMO), *excellens* (TUX), and *pampa* (YUC). ** $P < 0.001$.

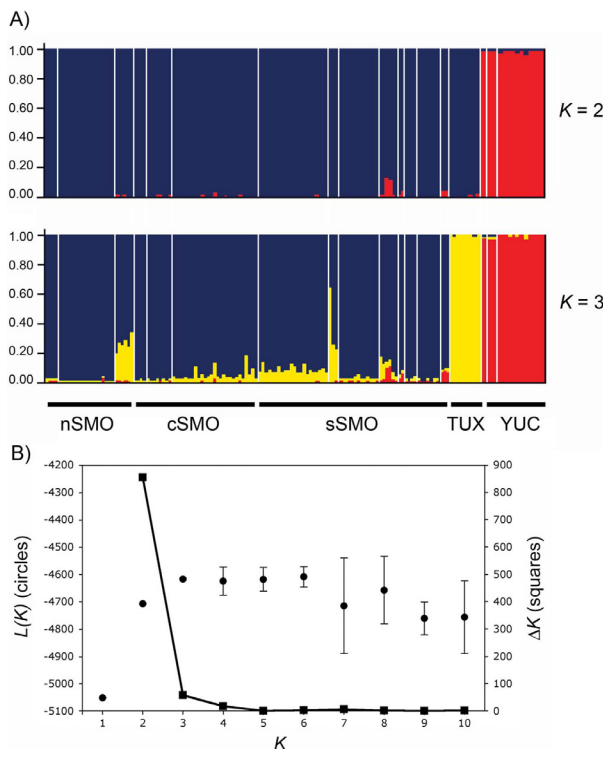

Figure 3 Spatial genetic structure determined by a Bayesian assignment analysis using STRUCTURE. **A**. Posterior assignment probabilities of 160 individuals of *Campylopterus curvipennis* to an optimal number of $K = 2$ and $K = 3$. Each individual is represented by a vertical line that is partitioned into K colored sections, with the length of each section proportional to the estimated membership coefficient. **B**. Mean log probability of the data ($L(K) \pm SD$) over 10 runs for each K value (left Y axis) and values of ΔK calculated according to Evanno *et al.* [90] (right Y axis).

YUC, TUX and SMO groups, which is congruent with the sequence mtDNA data results. In further analyses of these data from which the YUC samples were first excluded, STRUCTURE assigned samples to two groups (TUX and SMO); in the analysis where the TUX samples were excluded two clusters were detected for the SMO samples with no evidence of genetic clustering at this level. These results suggest a hierarchical clustering pattern where YUC samples are highly divergent and samples from TUX are sub-structured in a cluster that contains samples from SMO.

Among-group comparisons of gene flow (M) from the MIGRATE analysis ranged from 0.49 (TUX to YUC) to 1.98 (TUX to SMO), however, all comparisons were less than or not significantly greater than 1.0 (Table 4). None of the comparisons among genetic groups indicate contemporary gene flow, suggesting that the Isthmus of Tehuantepec is preventing gene flow between populations on either side of the isthmus, and that the more recently divergent populations from the Tuxtlas region

Table 4 Estimates of M (mutational corrected migration) from the MIGRATE analysis of microsatellites among genetic groups

	SMO	TUX	YUC
SMO	-	1.48 (0.58 - 4.22)	0.58 (-0.1 - 1.56)
TUX	1.98 (0.54 - 3.81)	-	0.49 (-0.68 - 3.39)
YUC	0.91 (0.03 - 2.29)	0.63 (-0.25 - 1.58)	-

Donor populations are in the first column. Estimates given are followed by 95% confidence intervals and none of the comparisons was significantly greater than 1.

and SMO also have interrupted gene flow. Estimates of M among populations were only significantly greater than 1.0 between those within the SMO (M values ranged between 1.4 and 2.3) and the direction of gene flow was asymmetric in most cases. The direction was mainly northwards from sSMO to cSMO, and only in few cases did gene flow reached populations on the northern limit of the distribution (Ciel, GF). Estimates of gene flow between populations of different genetic groups were not significantly greater than 1.0.

Our simulations to test whether genetic groups might have originated in the face of gene flow were consistent across replicates, and produced confident posterior probability peaks for the parameters estimated. When testing for migration following the split between the SMO and TUX groups, migration rates were higher than 1 in the SMO-TUX direction ($m = 1.416$, 90% highest posterior density (HPD), 0.004-4.21) and close to 1 in the opposite direction TUX-SMO ($m = 0.91$, 90% HPD, 0.007-4.47), suggesting that divergence took place in the presence of gene flow from the SMO to the Tuxtlas region. In contrast, low migration rates following the split were estimated for populations east and west of the Isthmus of Tehuantepec (west: $m = 0.52$, 90% HPD, 0.003-1.27; east: $m = 0.36$, 90% HPD, 0.002-1.23), indicating that the population split occurred in the absence of gene flow, i.e. this geographic barrier was not permeable to migrants. Assuming a mutation rate for microsatellites of 2.96×10^{-3}, estimates of effective population size indicate that the ancestral population was significantly larger (3,924 individuals, 90% HPD, 2,598-19,005) than the populations after divergence. The TUX group had the lowest population size (103.88 individuals 90% HPD, 38.01-259.3) followed by YUC (175.67 individuals 90% HPD, 96.28-282.93) and SMO (326.01 individuals 90% HPD, 203.54-474.66).

Morphological and acoustical variation

The MANOVA showed significant lineage differences in male morphology (Wilks' Lambda, $F_{6,222} = 33.77$, $P = 0.0001$), and these differences were significant for each morphological variable (one-way ANOVAs: wing chord

$F_{2,113}$ = 16.71, P = 0.0001; tail length $F_{2,113}$ = 48.45, P = 0.0001; culmen $F_{2,113}$ = 57.58, P = 0.0001, SMO n = 106, TUX n = 6, YUC n = 16; Figure 4). The relationship between culmen length and wing chord (as a measure of body size; r = 0.38, P < 0.0001) was further examined for possible allometric effects using the residuals of this relationship, and the differences between lineages were maintained ($F_{2,113}$ = 48.33, P < 0.0001). For females the MANOVA also showed significant lineage differences (Wilks' Lambda, $F_{6,42}$ = 3.89, P < 0.005), and the results of the univariate ANOVAs were all significant (wing chord $F_{2,23}$ = 10.17, P < 0.001; tail length $F_{2,23}$ = 11.94, P < 0.0005; culmen $F_{2,23}$ = 4.65, P < 0.05, SMO n = 21, TUX = 11, YUC n = 5; Figure 4). Overall these results are congruent with previous subspecific designation based on bill and body size [31,32], indicating that individuals from the *excellens* group (TUX) are significantly larger than those of the *curvipennis* (SMO) and *pampa* (YUC) groups, and that *pampa* individuals have shorter bills.

A total of 344 syllable types were detected across populations. Songs were very versatile and no successive syllable repetitions were detected except for vocalizations recorded in the YUC region from the *C. c. pampa* lineage, where individuals commonly repeated one of the syllables three times in succession (Figure 5). Based on vocal similarity measures (syllable type sharing), individuals from each sampling locality were clustered accordingly (Figure 5). In addition, the analysis showed individuals from the YUC lineage group together in a basal position followed by individuals from the TUX and SMO lineages (Figure 5). On average populations from SMO shared a lower proportion of syllable types with the TUX and YUC lineages (0.062 ± 0.018), than within any other locality from the SMO (0.145 ± 0.037). Despite the great syllable diversity observed across populations and regions, there were some syllables shared by all recorded individuals, whereas other were shared only by members from populations from the SMO. Regarding acoustic measurements of the common syllable, the first three PCs accounted for 53% of variation (PC1 = 24.4%, PC2 = 18.2%, PC3 = 10.4%). One-way ANOVAs yielded significant group differences in the first PC (PC1, $F_{2,74}$ = 107.05, P < 0.0001), but not in the second and third (PC2, $F_{2,74}$ = 2.13, P > 0.05; PC3, $F_{2,74}$ = 1.01, P > 0.05 SMO n = 66, TUX = 6, YUC n = 5). PC1 was mainly explained by minimum and peak frequency of note 1, duration of note 3, and frequency range, peak frequency and duration of note 4.

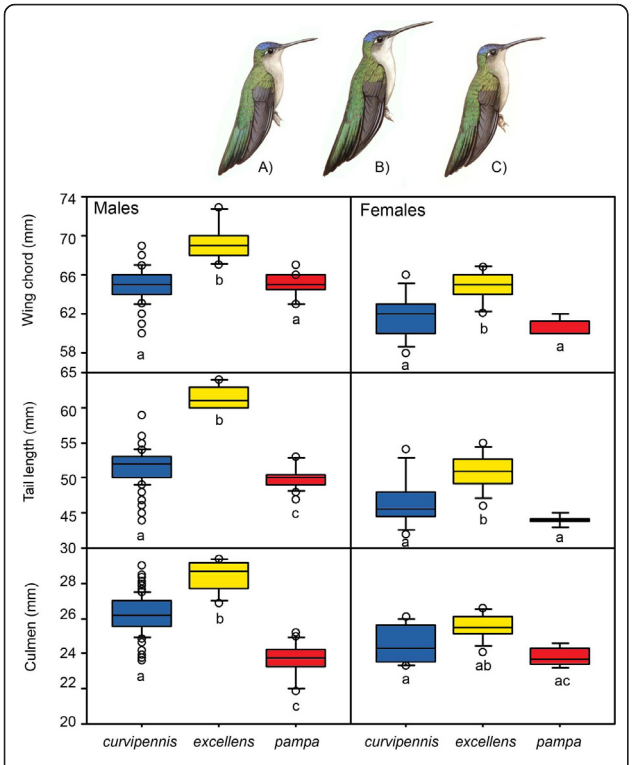

Figure 4 Morphological traits taken for males and females of *C. curvipennis* lineages. Boxplots show the 10th, 25th, 50th (median), 75th, and 90th percentiles. Values above the 90th and below the 10th percentile are plotted as open circles. Illustrations correspond to *C. c. curvipennis* (**A**), *C. c. excellens* (**B**) and *C. c. pampa* (**C**) taken from the *Handbook of the Birds of the World* [32].

Comparisons between morphological, acoustic, habitat-related, and genetic distances

Mantel tests showed a strong positive correlation between genetic (pairwise F_{ST}) and morphological distances for males (r = 0.42, P < 0.05), even when controlled for geographic distance (r = 0.38, P < 0.05). However, the relationships between pairwise R_{ST} and morphology were not significant. In the case of females none of the relationships were significant. Regarding acoustic distances, Mantel test showed a strong positive correlation between genetic and song sharing distance (F_{ST}: r = 0.72, P < 0.05; R_{ST}: r = 0.71, P < 0.05), and also for the genetic and common syllable distance (F_{ST}: r = 0.72, P < 0.01; R_{ST}: r = 0.72, P < 0.01). These relationships were maintained when geographic distance was accounted for (song sharing: F_{ST}, r = 0.50, P = 0.05; R_{ST}, r = 0.51, P < 0.05; common syllable: F_{ST}, r = 0.44, P < 0.05, R_{ST}, r = 0.44, P = 0.01). These analyses indicate that more genetically divergent lineages shared fewer syllable types, and differed acoustically in the common syllable, independently of distance. Lastly, we did not find a significant relationship between morphological and acoustic distances *versus* habitat-related (climate and topography) distances for either males or females.

The role of drift and selection: a coalescent test

Coalescent simulations [30] of morphology and mtDNA sequence data from three populations with large sample

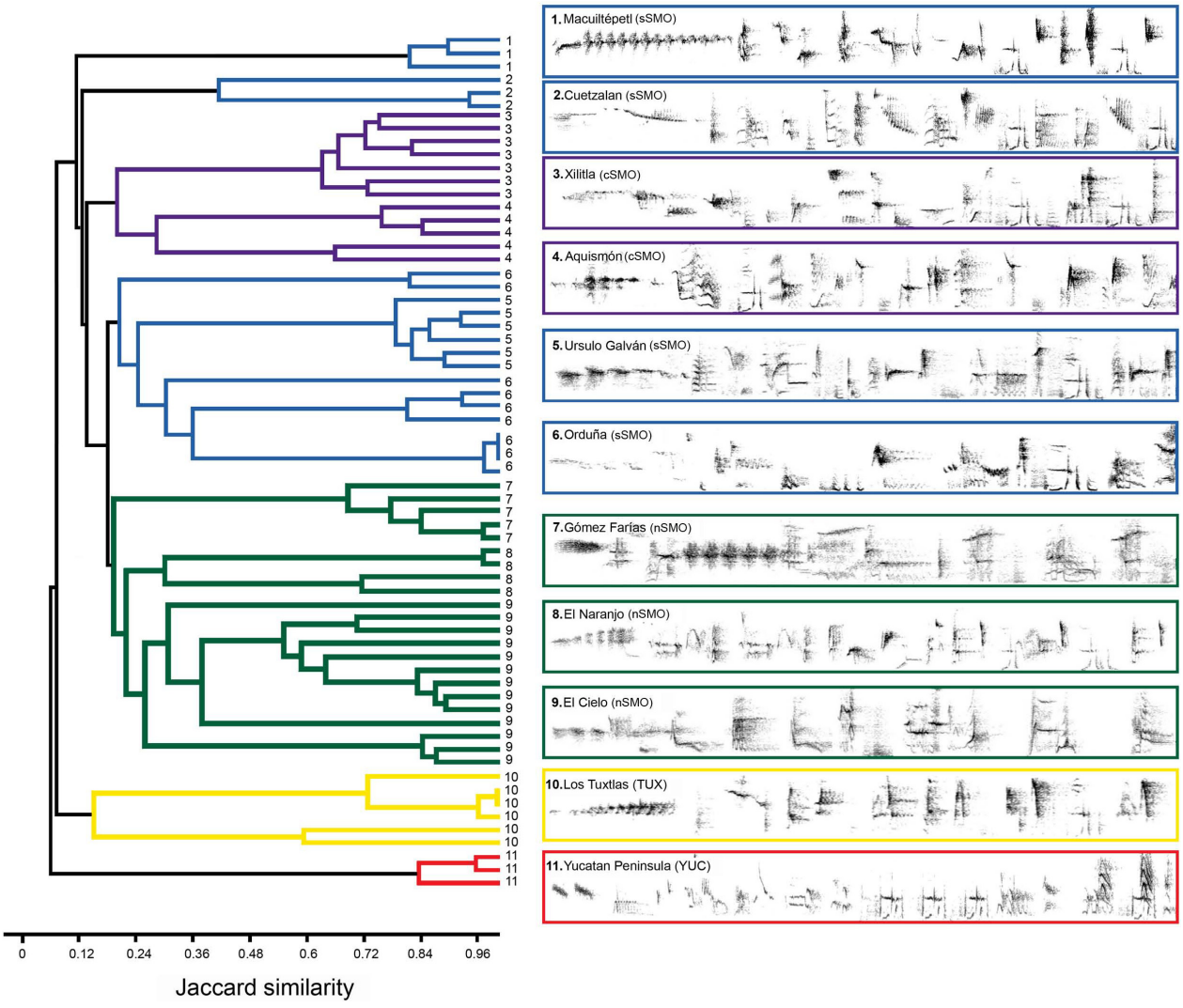

Figure 5 Dendrogram generated by cluster analysis of a presence/absence matrix of syllable types from recordings of individuals. Colored lines correspond to individuals recorded at different geographic locations: green, purple and blue correspond to sites located in the northern, central and southern parts of the Sierra Madre Oriental respectively (nSMO, cSMO, and sSMO) for the *C. c. curvipennis* lineage, yellow corresponds to the Tuxtlas region for the *C. c. excellens* lineage and red corresponds to the Yucatan Peninsula for the *C. c. pampa* lineage. Attached to the dendrogram fragments of vocalizations (4 sec) representing each sampled site are shown.

sizes of sequences corresponding to the three genetic groups in which morphological characters are fixed (Tux, Ciel, Nov) were used to differentiate the roles of selection and genetic drift in morphology evolution. If morphological characters, assumed to be encoded by nuclear genes, have sorted significantly faster than mtDNA haplotypes, then the hypothesis of divergent selection rather than that of drift is supported. The observed *s* value for reconstructed trees was equal to 2 when considering three populations for morphology characters. The upper 95% CL for time since population divergence was $3N_e$ generations assuming a dichotomous branching model of divergence and $3.3N_e$ generations assuming a simultaneous model. When branch lengths were reduced four times to mimic nuclear genes, an *s* value of 2 occurred in one third of the simulated trees for dichotomous branching, and in one fifth of the simulated trees for simultaneous branching. There is a high probability ($P > 0.05$) that nuclear genes would be fixed in these populations under neutrality, suggesting that drift cannot be rejected as a possibility for morphological divergence [Additional file 3].

The coalescent simulations of the song and mtDNA sequence data from four populations with large sample sizes of sequences in which song characters are fixed (Ciel, Ord, Tux and Nov) were used to differentiate the

roles of selection and genetic drift in song evolution. The observed *s* value for reconstructed trees was equal to 8 when considering four populations for vocal characters. The upper 95% CL for time since population divergence was $0.48N_e$ generations assuming a dichotomous branching model of divergence and $0.68N_e$ generations assuming a simultaneous model. When branch lengths were reduced four times, an *s* value of 3 occurred in none of simulated trees, suggesting that there is a very low probability ($P < 0.0001$) that nuclear genes would be fixed in different populations under neutrality [Additional file 3]. In this case the hypothesis of divergent selection rather than that of drift is supported.

Discussion

Phylogeography and evolutionary history

Phylogeographic analyses on mtDNA sequence data and microsatellites were highly congruent identifying three wedge-tailed sabrewing lineages: the *pampa* group in the Yucatan Peninsula, the *curvipennis* group along the Sierra Madre Oriental, and the *excellens* group in the Tuxtlas region. The patterns of genetic differentiation inferred here for *C. curvipennis* are consistent with the results for other codistributed avian species in montane cloud forests of the Sierra Madre Oriental and the Tuxtlas region such as *Chlorospingus ophthalmicus* [9] and *Buarremon* finches [11]. Even though the TUX mtDNA haplotypes formed a clade nested within the more diverse haplotype clade of the Sierra Madre Oriental, these haplotypes are not shared with individuals from any other population, and are separated by several mutational steps from the SMO haplotypes. This suggests that not enough time has elapsed for lineages to sort out the formation of two reciprocally monophyletic clades [33]. Similarly, the cluster analysis with microsatellites identified the *pampa* group when $K = 2$ and *excellens* when $K = 3$, indicating a lesser degree of divergence between the TUX and SMO genotypes in comparison to those of YUC. Consistent with the lack of haplotype sharing and high pairwise R_{ST} values, the results of gene flow measures based on microsatellites indicate that the SMO, TUX and YUC populations exchange no migrants. Both mtDNA and biparentally inherited microsatellite loci are spatially structured between allopatric populations of wedge-tailed sabrewings suggesting that gene flow is restricted and that *C. c. curvipennis, C. c. excellens*, and *C. c. pampa* constitute genetically unique populations.

It is possible that allopatric fragmentation has affected the genetic structure of wedge-tailed sabrewings. Although the existence of several mutational steps in a haplotype network is indeed indicative of allopatric fragmentation, it could also result from non-sampled haplotypes from intermediate populations. The latter is not the case here, as we sampled populations covering the entire geographical range of the species complex [Additional file 4]. In addition, the possibility that allopatric fragmentation has been a historical process causing the pronounced mtDNA divergence between the *pampa* and the *curvipennis-excellens* groups is consistent with the hypothesis that the Isthmus of Tehuantepec was a major vicariant event and has been a significant habitat barrier to dispersal, as observed in previous mtDNA phylogeographical studies with montane bird species [7,9-11]; however, our study is the first to document the impact of this barrier in a species complex with lowland populations on either side of the isthmus. The isthmus, formed by the superposition of three distinct tectonic episodes, has been exposed to continental uplift and sea level oscillation since the late Miocene (*c.* 6 Ma; [12]), periodically encroaching upon or retreating from the coastal plains, with the peak in sea level occurring at the end of the Pleistocene [34]. The phylogeographic breaks in our genetic data suggest a split between the SMO-TUX and YUC clades in the mid-Pleistocene (1.4 - 0.56 Ma) toward the end of the Isthmus of Tehuantepec's process of formation. Despite the variability in date estimation using the coalescence approach, our divergence time estimates point to the Pleistocene epoch, and these estimates are consistent with the timing of one of the two pulses of diversification proposed by Barber and Klicka [14] across the isthmus for a community of montane bird species. Our results from gene flow and isolation with migration (IMa) support the role of the isthmus as an effective barrier in which no migration occurred during genetic divergence.

Regarding the most recent divergence between the SMO and TUX populations, the oscillations in climate during the Pleistocene caused a considerable expansion in the range of highland habitats and their descent to lower elevations [35,36]. These repeated altitudinal up- and downslope migrations of montane forest during glacial periods probably connected the cloud forests of the SMO with the TUX montane region, and as temperatures increased, the redistributed forested habitat caused the isolation of the TUX populations. This idea is supported by our results from IMa, which suggests that this vicariant event occurred in the face of gene flow, from populations of the SMO to those of the Tuxtlas region, but not in the opposite direction, indicating that the connection of the forests allow individuals to disperse towards the Tuxtlas region. However, estimates of contemporary migration rates indicate that contemporary gene flow is restricted between these two groups. The current distribution of coastal plain along the Gulf of Mexico, which surrounds and isolates the Tuxtlas region

from the Sierra Madre Oriental, could be a dispersal barrier between wedge-tailed sabrewing populations. Because microsatellites have a faster mutation rate than mitochondrial genes and therefore these markers have very different temporal scales of inference, our measures of gene flow based on microsatellite loci indicate that contemporary gene flow is prevented by both barriers (the isthmus and the coastal plain). The absence of suitable habitat on the coastal plains along with the presence of pastures and savanna-like habitats in the isthmus region likely prevents population expansion that could result in secondary contact between the SMO, TUX and YUC populations. Hence, gene flow would require long distance dispersal for wedge-tailed sabrewings to overcome these barriers.

Morphological and acoustical variation: the role of drift and selection

Morphological data for both males and females indicated that individuals from TUX were significantly larger than those from SMO and YUC, and individuals from SMO and TUX had longer bills than those from YUC. The lack of correspondence between climate and topographic conditions and morphological traits suggests that weak habitat selection pressures have been shaping morphological divergence. This inference should be made with caution, considering that habitat structure was characterized using only ground-based interpolated climate and topography data. Information about vegetation structure obtained using satellite and airborne remotely sensed images would offer a more powerful way for testing adaptive and nonadaptive hypotheses concerning the evolutionary processes that operate across the environmentally heterogeneous space occupied by wedge-tailed sabrewings. However, these data require substantial processing for applications in spatial analyses and are sensitive to cloud contamination [37]; these data may not be useful for regions with cloud forest and rainforest.

When conducted a coalescent-based test using mtDNA sequence data to evaluate whether the observed phenotypic differentiation can be attributed to drift or some form of selection, we could not reject the possibility of drift in driving morphological differentiation of the SMO (*C. c. curvipennis*), TUX (*C. c. excellens*), and YUC (*C. c. pampa*) populations. Besides, independently of geographic distance, a significant positive relationship between genetic and morphological distances was found highlighting the idea that drift might facilitate phenotypic divergence when acting in concert with selection [38,39]. The increase in body size in small peripheral populations can be driven by random genetic drift [38-40]. In addition, the effective population size of wedge-tailed sabrewings in the TUX region is one-third and one-half smaller compared to the SMO and YUC groups, respectively. Another possible explanation for the increased body size of the TUX individuals is the relaxed competition for resources that is expected on islands [40] or in isolated populations such as those in the TUX region, which are competing for resources with fewer hummingbird species. If so, this would suggest that individuals from the TUX region have evolved independently and unconstrained by the evolutionary events that occurred in the other regions [38].

The increase in culmen length in the SMO and TUX regions may indicate selection-driven divergence, and the effect of adaptation to local resources (e.g. flower size and shape) through natural selection. Differences in culmen length are congruent with the differences in habitat characteristics of the Yucatan Peninsula (drier deciduous tropical forests at a much lower elevation) compared to the higher altitude and wetter habitats of the Sierra Madre Oriental and the Tuxtlas region, suggesting that populations adapted to different ecological conditions, including perhaps the presence of flowers with longer corollas. Differences in environmental variables provide the potential for ecological differentiation where different selection pressures might act to shape the relationship between pollinator and plant species [41]. However, a comparative study involving traits of flowers with hummingbird pollination syndrome occurring in each region and the culmen characteristics of hummingbirds using those resources need to be done.

The divergence of mating-related acoustic signals has been described as being shaped by habitat-dependent selection in many bird species [42-47], because sounds with certain features are better transmitted in some environments than others. In wedge-tailed sabrewings, some populations live in different habitats with divergent environmental conditions, most markedly in the *pampa* lineage from the Yucatan Peninsula whose individuals live in deciduous tropical forest at a lower altitude and much drier conditions than populations from the other lineages, which inhabit cloud or tropical forests at higher altitudes that have wetter conditions. However, we found no significant correlations between acoustic and habitat-related distances (climate and topographic variables), which suggest that these conditions play a minor role in shaping song divergence.

In contrast, we found a strong acoustic divergence pattern in syllable sharing between SMO populations and also between geographic areas, the latter corresponding to the genetic differentiation among the three lineages of wedge-tailed sabrewings. Each lineage had an exclusive assemblage of syllable types, and song sharing was lower between than within lineages. Also, the acoustic traits of a common syllable were more divergent between than within lineages. The coalescent

analysis to evaluate whether vocal differentiation can be attributed to drift or selection, suggested that the fixation of song types assumed to be encoded by nuclear genes, has occurred faster than expected by genetic drift, providing evidence that selection is driving song evolution in wedge-tailed sabrewings. In lek-breeding systems where male birds are exposed to strong sexual selection pressures, the use of certain song types over others could increase their adaptation to social conditions and/or reproductive success. Although song could potentially play a role in increasing genetic polymorphisms and generating reproductive isolation and speciation through sexual selection [21,48,49], further evidence of female preference for local male signals over foreign ones is needed to fully demonstrate that sexual selection is causing speciation in wedge-tailed sabrewings (see [50]). An alternative cause of divergence of songs in wedge-tailed sabrewings is reinforcement selection against maladaptive hybridization, being song a sexually selected mechanism to maintain reproductive isolation in case of secondary contact [51]. However, it is unlikely that the wedge-tailed sabrewings from these populations come into contact owing to the lack of contemporary gene flow shown in this study, which indicates a historically low potential for hybridization.

The positive relationship between genetic and acoustic distances suggests that independently of geographic distance, the most genetically distant lineages shared fewer vocal elements and differed in the acoustic traits of the common syllable. This relationship is expected under a drift model of song evolution, where acoustic divergence is higher between populations that have been genetically isolated for the longest time [45]. As in songbirds, song is learned in hummingbirds [52]. Likely song traits are culturally transmitted through imitation, and learning errors or innovations (new syllables) across generations can provide an important source of variation [21,53] affected by processes similar to those driving genetic variation, such as drift or selection [54]. In the case of drift, dialects or song variation at spatial scales could arise from a process of cultural diversification where random song mutations are produced by copy errors [55]. It has also been suggested that song learning may increase the rate at which genetic predisposition to learn or prefer certain songs evolves in allopatry [56]. Our data suggest that besides the role of selection in song divergence, these long time isolated lineages may have limited acoustic contact, probably resulting in a low level of repertoire syllable sharing. Despite the long isolation, individuals of the three lineages share some syllable types suggesting that their complex songs have conservative vocal elements retained through generations, and that non-shared syllables were gained or lost during the isolation and selection process.

Conservation recommendations

The information generated by our study allows us to make the following conservation recommendation. The genetic differentiation among the three genetic groups revealed from the mtDNA analyses suggests that these groups have been genetically isolated for a long period of time. This genetic isolation and the lack of contemporary gene flow among these groups were also supported by the microsatellite data. In addition, the ancestral effective population size was larger than the size of either genetic group after splitting as revealed by IMa analysis. Because SMO, YUC and TUX are distinct genetic groups with no genetic connection among them, conservation plans should consider the sites within each of them as independent, and future management plans should focus on conserving the genetic diversity of the three genetic groups.

Conservation of the Tuxtlas region is particularly important due to the restricted distribution of the *excellens* group, the relatively small effective population size (one-third of that from SMO), and because the accelerated deforestation rates in the Tuxtlas region [57] is a threat to the unique genetic diversity of this group and possibly that of other endemic forms. Although populations within each of the genetic groups showed similar levels of genetic diversity, erosion of genetic variation is more likely to occur in the TUX group and thus conservation plans are of particular relevance for this geographic area. The mountains of this region, isolated from other mountain systems, arose from volcanic activity in the Oligocene, where oceanward orographic uplift produced one of the wettest climates in Mesoamerica [58]. This region contains one of the most diverse avifaunas in the northern Neotropics and, due to physical isolation it has been considered an important region of endemism [59]. Considering the alarming rate of deforestation in the Neotropics, the preservation of the montane and tropical forest in this region is urgently needed. To this end, the study of other co-distributed taxa to cover the phylogeography of Mesoamerica is warranted. This would shed light on any shared patterns of colonization and isolation in Mesoamerica and contribute to our understanding of the processes and responses of organisms to the environmental changes that drive population differentiation in this region.

Conclusions

The genetic and phylogeographic analyses of this study, based on mtDNA and microsatellites, uncover the presence of three lineages of Mesoamerican wedge-tailed sabrewings that exhibit no contemporary gene flow. These correspond to the disjunct distribution of populations at the Sierra Madre Oriental, the Tuxtlas region, and the Yucatan Peninsula. Our results highlight the

importance of the Isthmus of Tehuantepec in generating population divergence *c*. 1.4 million years ago without gene flow during the process of divergence, as well as more recent climate events during the Pleistocene in driving isolation and population divergence of wedge-tailed sabrewings with gene flow from the SMO to the Tuxtlas populations. Coalescent analyses of the evolution of phenotypic traits suggest that the fixation of song types has occurred faster than would be expected by genetic drift, suggesting that the action of selection is driving song evolution in wedge-tailed sabrewings. However, the role of drift in driving morphological divergence could not be rejected. Finally, considering the accelerated deforestation rates in the Neotropics the conservation of the montane and tropical forest in this region is needed, and special attention must be paid to small isolated areas with genetic uniqueness such as the Tuxtlas region.

Methods

Field procedures and feather sampling

For genetic analysis, based on the disjunct geographical distribution of the species complex [15,32], we sampled 160 individuals from 22 populations during the 2006, 2007 and 2008 *Campylopterus curvipennis* breeding seasons, covering the entire geographical range of the species complex: (*i*) 15 populations of *C. curvipennis curvipennis* (SMO), (*ii*) three populations of *C. curvipennis excellens* (TUX), and (*iii*) four populations of *C. curvipennis pampa* (YUC) (Table 1 Figure 2). The populations of the SMO group were sampled from three disjunct areas along the Sierra Madre Oriental: (*i*) three populations from southern Tamaulipas and northern San Luis Potosí (nSMO herein); (*ii*) three populations from southern San Luis Potosí and Hidalgo (cSMO herein); and (*iii*) nine populations from Puebla and central Veracruz (sSMO herein) (Table 1 Figure 2). Most of the *curvipennis* SMO populations are located in the understory of cloud forest or second-growth vegetation at a higher elevation (270-1,500 m above sea level) where the temperature is lower. The *excellens* TUX populations are located in wetter semideciduous tropical forests at a lower elevation (*c*. 260-1,000 m above sea level), and those of the *pampa* YUC populations are in drier deciduous tropical forests at a much lower elevation (*c*. 60-250 m above sea level) and higher temperatures (Table 1). Birds were captured in mist nets and the two outer tail rectrices were collected for subsequent genetic analysis before released. Research reported here was performed with the approval of the Mexican government (INE, SEMARNAT, SGPA/DGVS/02038/07), the UNAM Graduated Studies Committee (Doctorado en Ciencias Biomédicas), and followed the Guidelines for the Use of Wild Birds in Research proposed by the Ornithological Council. We also obtained tissue samples from three congeners to be used as outgroups in the phylogenetic analysis (*Campylopterus rufus*, *C. hemileucurus* and *C. largipennis*) (see Acknowledgments for tissue loans).

Three body measurements were obtained from mist-netted hummingbirds using a dial caliper to an accuracy of 0.1 mm and a wing ruler: exposed culmen (from the base of the bill to the tip of the upper mandible), wing chord (the distance from the carpal joint to the tip of the longest primary), and tail length (from the base of the uropygial gland to the tip of the longest rectrix). All measurements were taken by CG. Sex determination was carried out in the laboratory by means of a polymerase chain reaction (PCR) using primers 2550F [60] and MSZ1R [61]. González and Ornelas [19] have further detailed amplification conditions. Because males were not captured in the Tuxtlas region, we used data measurements taken by Lowery and Dalquest [62] from six males at the same collecting sites. To confirm the feasibility of these data and the possibility of combining them with ours, we compared the measurements in males and females from San Luis Potosí taken by these authors with data taken by us at the same locality. Also we compared the published measurements taken of females from those in the Tuxtlas region with our own data. As we did not detect significant differences between the two sources of measurements (data not shown) we used the published measurements of males from the Tuxtlas region in subsequent analyses.

Acoustic recordings were made for 64 males (557 recordings, ca. 9 recordings per bird) at 11 of the 22 sampled populations (Table 1). Song recordings were made with a Marantz PMD660 portable solid-state recorder and a Sennheiser MKH-70 directional microphone.

Mitochondrial DNA sequencing and microsatellite genotyping

We extracted genomic DNA from one of the feather samples using chelex (5%, [63]), and that from tissue samples using the DNA easy blood and tissue kit (Qiagen, Inc.), following the recommended protocol. We used PCR to amplify two mitochondrial genes. The ATPase 6 and 8 coding region (875 bp), which includes two partially overlapping mitochondrial genes, was amplified using primers L8929 and H9947 [64]. The first domain of the control region (532 bp) was amplified using primers ARCO1F (5' AATTTTATGGTGTTT GTGTGTGAA 3') and ARCO1R (5' ACCCTAGCA-CAACTCGCACT 3') designed for this study from an *Archilochus colubris* D-loop and the complete tRNA-Phe gene sequence, and a partial 12 S ribosomal RNA gene sequence (GenBank accession EF520732.1).

Amplification reactions (25 μl total volume) contained 0.72× buffer, 3.5 mM $MgCl_2$, 0.14 mM of each dNTP, 0.2 μg/μl of BSA, 0.3 μM of each primer, 0.05 U *Taq* (Promega), and 2-5 μl of genomic DNA. PCR reactions were performed in a 2720 thermal cycler (Applied Biosystems) or in an Eppendorf Mastercycler thermocycler with the following temperature profile: initial denaturation at 94°C for 5 min; 35 cycles consisting of denaturation at 94°C for 1.5 min, annealing at 53-60°C for 1 min and an extension of 1.5 min at 72°C; and a final extension of 72°C for 7 min. PCR products were visualized on a 1% agarose gel stained with ethidium bromide, purified with the QIAquick PCR purification kit (Qiagen, Inc.) and sequenced using the Big Dye cycle terminator kit. Sequences were visualized in a 310 automated sequencer (Applied Biosystems) and by Macrogen Inc. ATPase genes were sequenced in both directions and the control region only in the reverse direction using primer ARCO1R except when ambiguities were present in the sequences. Sequences were edited using SEQUENCHER 4.8 demo (Gene Codes) and then manually aligned with SE-AL 2.0a11 [65]. All unique sequences used in this study have been deposited in the GenBank under accession nos. HQ380686-HQ380755.

Samples were genotyped at 10 polymorphic microsatellite loci designed specifically for *Campylopterus curvipennis* ([66], GeneBank accession nos. GQ294539-GQ294550). PCR conditions and fragment sizing are fully described in Abdoullaye et al. [66].

Analysis of mtDNA sequence data

We reconstructed intraspecific phylogenetic relationships among haplotypes using Bayesian inference in MRBAYES 3.12 [67]. Data were partitioned into two matrices, one corresponding to 875 bp of ATPase 6 and 8 and the other to 532 bp of control region. MODELTEST 3.7 [68] was run for each partition to choose the model of molecular evolution that best fit our sequence data. The best model for ATPase under the Bayesian information criterion (BIC) was HKY + G (base frequencies: A, 0.3588; C, 0.1664; G, 0.2373; T, 0.2376; gamma distribution shape parameter = 0.1825; transition/tranversion ratio = 6.22), and for the control region was TrN + I (base frequencies: A, 0.3688; C, 0.1564; G, 0.2470; T, 0.2278; proportion of invariable sites = 0.6869). We ran the analysis for 10 million generations using four chains (twice), sampling every 100th generation. We plotted the number of generations versus likelihood scores to check stationarity. Trees prior to stationarity were discarded as burn-in (25%), and the remaining were used to generate a consensus tree, later visualized in FIGTREE 1.2.3. We used three congeners as outgroups (*C. rufus, C. hemileucurus*, and *C. largipennis*) to root the tree. Alignment for the phylogenetic analyses is included as an additional file [Additional file 5].

In the absence of appropriate internal calibration points for many groups of birds, the 2% divergence-per-My clock calibration has been widely used. However, the degree of heterogeneity of molecular evolution rates across lineages and genetic loci could confound the accuracy of divergence time estimates, making the use of the 2% rule controversial [69-71]. In a more recent study, however, Weir and Schluter [72] cross-validated 90 avian clock calibrations (including one for hummingbirds) for cytochrome *b* obtained from fossil records and biogeographical events, demonstrating support for the 2% rule across taxonomic orders. Similar substitution rates have been described for other protein-coding mitochondrial DNA markers such as ATPase and ND2 [5,73-75]. Nevertheless, in order to minimize potential biases of this calibration, we calculated divergence times using two rates for the ATPase coding region data (0.02 and 0.05 substitutions/site/My) as suggested by Tarr and Fleischer [76], Milá et al. [77], and Barber and Klicka [14]. Uncertain homology arising from hypervariability or heteroplasmy in non-protein coding mitochondrial control region could confound measures of molecular clock estimates [77,78], so we did not estimate divergence time for that locus. Time to the most recent common ancestor (TMRCA) was estimated for the resulting clades using a Bayesian MCMC sampling approach with BEAST 1.4.2 [79]. TMRCA estimates were obtained using a relaxed clock model with log-normally distributed and uncorrelated rates of substitution between branches. No topological constraints were used allowing topological uncertainty to be taken into account. The model of evolution determined by MODELTEST under BIC (HKY+G for ATPase), was used for the analyses, and all other priors were set as default values in the program BEAUTI 1.4.7 that was used to create the XML files for input into BEAST. We ran BEAST for 10 million generations sampled every 1,000 generations, with the first 10 percent of the sampled points removed as burn-in. We obtained TMRCA estimates employing a range of 2 - 5% My to convert TMRCA to millions of years ago.

A statistical parsimony haplotype network was obtained using the program TCS 1.2.1 [80] using the 95% connection probability limit. Some ambiguities were detected in the networks (loops), and were broken according to three criteria (frequency, topology and geography), as proposed by Pfenninger and Posada [81].

We assessed the genetic variation within populations by calculating the haplotype diversity (*Hd*) and nucleotide diversity (π). To estimate population genetic structure we conducted an analysis of molecular variance (AMOVA). We used the model of molecular evolution of Tamura and Nei with a gamma shape parameter = 1.2757 for the combined data set, to estimate genetic

distances. A total of 16,000 permutations were estimated to determine the AMOVA's significance. Two AMOVAs were run, grouping sampling sites based on the fragmented geographical range of wedge-tailed sabrewings (Table 1 Figure 2), and grouping sampling sites into subspecific groups (SMO, TUX, and YUC). Genetic differentiation among populations was determined with Φ statistics and pairwise F_{ST}. Differences between populations were tested using 10,000 permutations among populations with Fisher's exact test. Genetic diversity estimates, AMOVAs and pairwise F_{ST} were performed in ARLEQUIN 2.0 [82].

Analysis of microsatellite data

Expected and observed heterozygosity and mean number of alleles per locus (allelic diversity) were calculated in GENEPOP 3.4 [83]. Allelic richness, a measure of the number of alleles per locus among populations independent of sample size, was calculated with the program FSTAT 2.9.3. Microsatellite genotypes were tested for linkage disequilibrium between pairs of loci and for departures from Hardy-Weinberg equilibrium (HWE) within populations and loci in GENEPOP. Bonferroni corrections were applied to correct for multiple simultaneous comparisons [84].

To investigate population genetic structure we calculated global and pairwise R_{ST} [85] and pairwise F_{ST} [86] in RSTCALC 2.2 [87] and ARLEQUIN, respectively. Differences between populations were tested using 10,000 permutations with a Fisher's exact test. To represent the relationships between localities we constructed a neighbor-joining tree using R_{ST} pairwise values in PAUP 4.0 [88].

To examine geographic patterns of population genetic substructure, we performed Bayesian genetic clustering using STRUCTURE 2.2.3 [89], which is used to infer the most likely number of genetic clusters (K) through the multilocus genotypic data. We ran STRUCTURE without population information under the admixture model with correlated allele frequencies. Ten independent chains were run for each K, from $K = 1$ to $K = 10$. The length of the burn-in was 500,000 and the number of Markov chain Monte Carlo (MCMC) replications after the burn-in was 1,000,000. To determine an accurate number of clusters we calculated the statistic ΔK based on the rate of change in the log probability of data between successive K values following Evanno et al. [90].

Contemporary gene flow (M) among populations, and groups of populations, defined by mtDNA and microsatellite analyses, was estimated with a maximum likelihood coalescent approach using microsatellite data and MIGRATE v. 3 [91]. The first genealogy was started with a random tree, and initial theta and migration rate (M) parameters were obtained from F_{ST} calculations. We ran 10 short (30,000 genealogies sampled) and three long chains (800,000 genealogies sampled), after discarding the first 20,000 genealogies as a burn-in.

Both mtDNA and microsatellite genetic analyses indicated three genetically diverged groups. We investigated whether these groups of populations had diverged in the face of gene flow by using IMa [92]. We performed two different tests, one involving populations from more recently diverged SMO and TUX populations, and the other involving populations east and west of the Isthmus of Tehuantepec. IMa is based on a Bayesian coalescent implementation of a model in which an ancestral population splits into two populations that may exchange genes in both directions at unequal rates during divergence. We performed preliminary simulations to specify the appropriate priors for each analysis, and ran three replicates with different random seed numbers with burn-in periods of 3,000,000 steps and 50,000,000 steps in chain following burn-in. We obtained estimates of migration rates between populations (m_1, m_2), and effective population sizes (q) from the ancestral population and after the split occurred between the two groups in both analyses. Estimates of migration rates were converted to the effective number of immigrants per generation by using estimates of theta. For estimates of effective population sizes of each genetic group we assumed a mutation rate per generation of 2.96×10^{-3} [93].

Morphological and acoustical variation

To examine differences in morphological variation between groups of the *C. curvipennis* complex, we performed a multivariate analysis of variance (MANOVA) with the three measurements taken as dependent variables, and lineages (see below) as fixed factors. One-way ANOVAs followed the MANOVA to determine lineage differences for each variable. To meet parametric analysis assumptions, variables were log transformed, but untransformed data are reported in figures. These analyses were performed for each sex separately due to size dimorphism using SUPERANOVA (Abacus Concepts, Inc).

We generated spectrograms of recordings using RAVENPRO V. 1.4 http://www.birds.cornell.edu/raven with the parameters fully described in González and Ornelas [19]. We used syllabic units throughout the wedge-tailed sabrewing repertoire for population comparisons, and syllables were visually classified by structure on printed spectrograms by CG (see [19] for details). To assess acoustic structure among groups, we performed hierarchical cluster analyses based on a presence/absence matrix of syllable types for each of 64 individuals, where each entry consists of 0 or 1. We used the unweighted pair group method of arithmetic averages (UPGMA) and the Jaccard similarity measure

to construct a dendrogram. As syllabic composition between individuals and localities is extremely variable [18,19], we took measurements from a common syllable emitted by all individuals from all localities to compare the temporal and spectral traits of song. The common syllable was randomly chosen from each individual and the duration (s), minimum frequency (kHz), bandwidth (kHz) and peak frequency (kHz) were measured. As this syllable has several elements (notes), we took the same measures for two of their elements (numbers 1 and 2, Figure 6), and the duration of another element (number 3, Figure 6). Principal component analysis (PCA) reduced the 13 variables taken from this syllable, and further one-way ANOVAs with uncorrelated factor scores were used to determine group differences for the first three PC components. PCAs and cluster analysis were performed in SPSS v.11.0.2 (SPSS, Inc.), and ANOVAs in STATVIEW (Abacus Concepts, Inc).

Comparisons between morphological, acoustic, habitat-related, and genetic distances

We used climate and topographic variables across all 22 sampling localities to test for habitat selection acting on morphology and acoustic traits. We used these variables as a broad level approximation of habitat and ecological variation potentially related to availability of floral nectar resources (see also [41,45]). We extracted 19 climate variables from WorldClim, a global climate database with a spatial resolution of *c.* 1 km^2 [94], in combination with elevation, slope, and the compound topographic index (CTI; a function of upstream contributing area and slope that reflects tendency to pool water), all from the Hydro-1K dataset [95] for each geographic point where samples were obtained for genetic analysis. The WorldClim database provides high-resolution climate matrices that capture climate variability and also extreme or limiting climate factors.

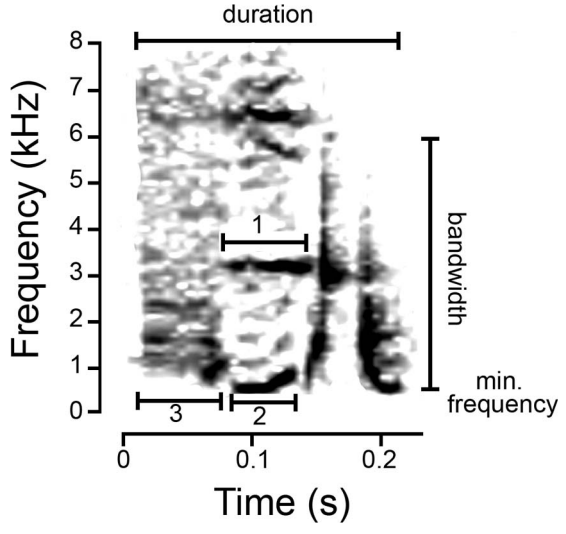

Figure 6 Measurements taken from a common syllable emitted by every individual recorded.

We followed Ruegg et al. [45] to examine the relationships between morphology, acoustic, habitat-related (climate) and genetic distances. Namely, we performed a series of simple and partial Mantel tests using IBD [96], assessing the significance levels of association between matrices with 1,000 randomizations. Morphological and habitat-related (climate) distances between populations were calculated as a dissimilarity matrix with Euclidean distance scaling to values between 0 and 1. Acoustic distance matrices were built as follows. For song sharing data (presence/absence syllable types), we estimated the pairwise Jaccard similarity coefficient between individuals among sampling localities. This coefficient was calculated in ESTIMATES v. 8.0.0 [97] and values were subtracted from 1 to change it to a dissimilarity matrix. For the distance matrix of the common syllable measurements, we first performed a discriminant function analysis (DFA) using locality as the grouping variable, and the distance between localities was estimated as the Euclidean distance between group centroids for the first discriminant functions, and variables scaled to values between 0 and 1. Dissimilarity matrices and DFA were performed in SPSS 11.0.2. To test for the vicariance-drift model, we compared the matrices of morphology (for males and females separately) and acoustic distances (syllable sharing and acoustic measurements distance matrices) *versus* genetic distances (F_{ST} for sequence data and R_{ST} for microsatellites), controlling for geographic distance with partial Mantel tests. To test for the habitat-selection model, we compared matrices of morphology and acoustic distances (acoustic measurements) with habitat-related (climate and topography) distances.

The role of drift and selection: a coalescent test

In order to test whether the degree of male phenotypic differentiation (vocal and morphological characters) was caused by drift or divergent selection, we performed an indirect coalescent-based simulation method developed by Masta and Maddison [30]. Because sexual selection by female choice reduces the amount of time needed for fixation of male phenotypic characters, without reducing the effective population size of neutral mitochondrial genes, Masta and Maddison's method tests statistically whether the rate of fixation of mitochondrial sequences differ from those of male phenotypic characters. This method assumes that nuclear genes code for the phenotypic character of interest (in our case, body size measurements and predisposition to learn certain song type), and asks whether the degree of fixation of phenotypes observed is likely to occur under the assumption

of neutrality (see also [50,98]). We performed the HKA neutrality test for each set of sequences, to determine whether the genes were appropriate neutral markers.

In order to implement this method with our data, we performed two different analyses: in the first analysis for morphological characters we considered three representative populations (a higher number of populations increases the complexity of simulations; [30]) corresponding to the three genetic groups (Tux, Ciel and Nov). Because the use of continuous characters in phylogenetic studies can be problematic, we converted the morphological measurements into discrete characters states for this analysis. We constructed the character states of each of the continuous variables (wing chord, tail length and culmen) using the gap-weighting method in which the observed variation is divided into a number of segments of equal size (five character states), giving large weights to large differences in individual measures and small weights to small differences [99]. Continuous characters were coded as follows: wing chord, 0: 62-63 mm, 1: 64-66 mm, 2: 67-68 mm, 3: 69-71 mm, 4: 72-74 mm; tail length, 0: 47-49 mm, 1: 50-53 mm, 2: 54-55 mm, 3: 60-61 mm, 4: 63-64 mm; culmen, 0: 20-22 mm, 1: 23-24 mm, 2: 25-27 mm, 3: 27.5-28 mm, 4: 28.5-30 mm. The character states were fixed for the three populations considered: Nov, intermediate in size with short bill (wing chord: 0, 1; tail lengh: 0, 1; culmen: 0,1); Ciel, intermediate in size (wing chord: 0, 1; tail lengh: 1, 2; culmen: 2, 3); Tux, large in size (wing chord: 2, 3, 4; tail lengh: 3, 4; culmen: 3, 4). In the second analysis for vocal variation, we considered four populations in which vocal characters are fixed (Ciel, Ord, Tux and Nov); vocal traits were coded as presence/absence of introductory syllable type. In both analyses tests were performed alternatively by means of two assumptions of population divergence, dichotomous branching and simultaneous divergence.

We first calculated the *s* statistic of Slatkin and Maddison [100], a measure of incomplete lineage sorting among populations, for maximum parsimony trees inferred from the analysis of mtDNA sequences (results not shown); larger *s* values indicate greater levels of incomplete lineage sorting, suggesting a more recent population divergence, and smaller *s* values indicate lower levels of incomplete lineage sorting suggesting older population divergence. Then, we compared the observed *s* value against *s* values obtained from simulated trees to estimate time since population divergence. This comparison was performed with computer simulations generating 10,000 gene trees within a population tree by gene coalescence. We set N_e = 500 in each population (as suggested by Masta and Maddison, [30]), and through simulations the upper 95% confidence limit was estimated for the number of generations since population divergence that would be expected to give the observed *s* value. Finally, simulations of nuclear gene trees were run to estimate the probability of complete fixation of morphological (*s* = 2) and vocal (*s* = 3) characters. Estimates of generations since population divergence were divided by four considering that nuclear genes have four times the population size of mitochondrial genes. Estimates of *s*, branch lengths and coalescent simulations were performed in MESQUITE v. 2.73 [101].

Additional material

Additional file 1: Pairwise comparisons between populations of *Campylopterus curvipennis*. Pairwise F_{ST} values (above diagonal) of mtDNA and pairwise R_{ST} values (below diagonal) of microsatellites between populations. Values statistically significant at $P < 0.001$ are indicated in bold.

Additional file 2: Neighbour-joining tree of sampled locations based on pairwise R_{ST}. Analysis obtained from microsatellite data showing YUC group clustering together in a basal position, and sampling localities of SMO group clustering in a geographically unresolved clade. Sampled locations with only one individual were excluded from the analysis.

Additional file 3: Results of the coalescent-based simulation on phenotypic traits. Estimates of *s* of Slatkin and Maddison from 10,000 coalescent simulated gene trees to test the probability of complete sorting of vocal characters (A and B) and morphological characters (C and D) in wedge-tailed sabrewing populations. Regarding vocal characters, there is a low probability of fixation in nuclear genes under neutrality regardless of the assumption of dichotomous branching (A) or the simultaneous model of divergence (B). In contrast, the probabilities that nuclear genes would be fixed under neutrality are high for morphological characters, regardless of the assumption of dichotomous branching (C) or the simultaneous model of divergence (D). This suggests that divergent selection has caused the pattern of vocal variation among populations, but the null hypothesis that morphological divergence resulted from drift was not rejected. Arrows indicate the expected value of *s* in a completely sorted tree.

Additional file 4: Geographic distribution of *Campylopterus curvipennis* species complex. Distribution of *Campylopterus curvipennis* species complex based on museum (MZFC) and bibliographic records [32,59], showing the disjunct distribution of three subspecies. Blue = *C. c. curvipennis*, yellow = *C. c. excellens*, red = *C. c. pampa*.

Additional file 5: Alignment of ATPase 6-8 and control region mtDNA sequences. Alignment of the concatenated ATPase 6-8 (1-875 bp) and control region (876-1407 bp) mtDNA sequences for 160 individuals of *Campylopterus curvipennis* and three outgroups used in the phylogenetic analyses.

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Authors' contributions
CG and JFO conceived of and designed the study. CG carried out the fieldwork, generated the molecular data, and conducted all genetic and phenotypic analyses with input from CGR and JFO. CG and JFO wrote the manuscript with contributions from all authors, who read and approved the final manuscript.

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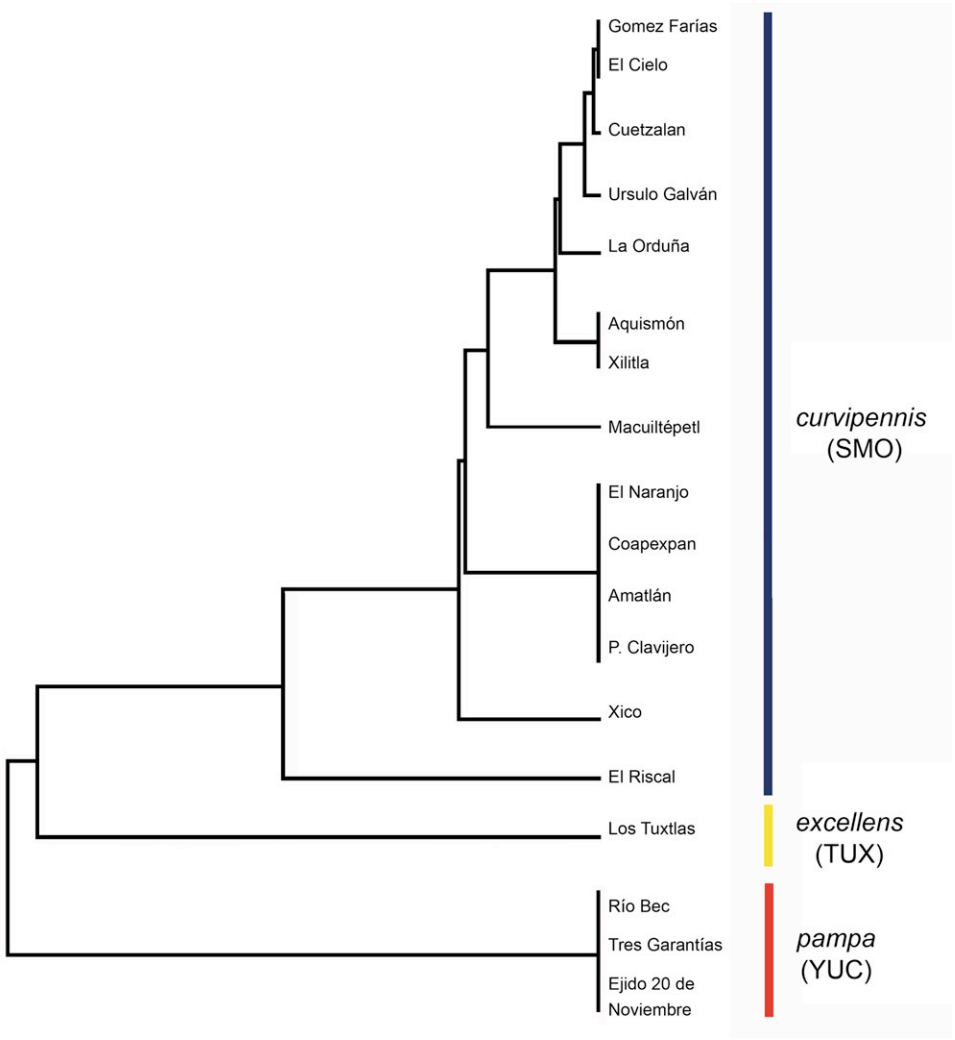
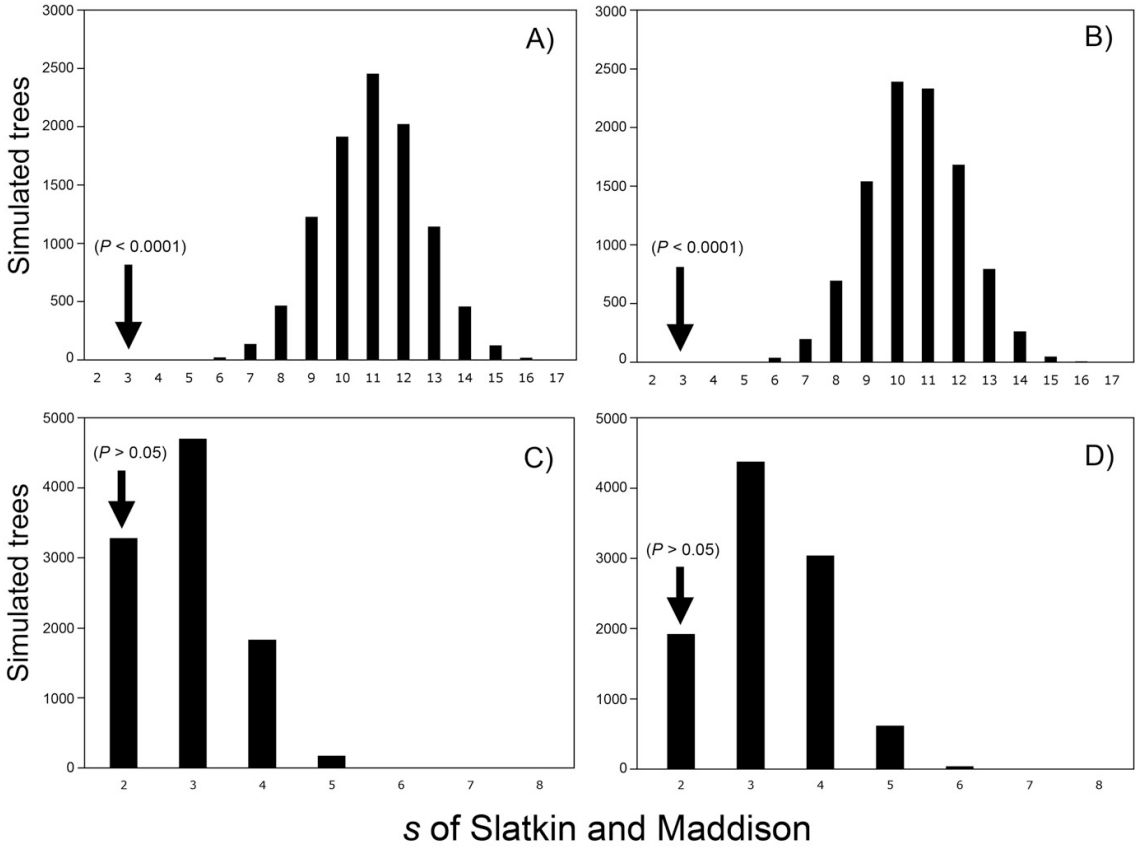
Additional file 1 Supplemental Table S1. Pairwise comparisons between populations of *Campylopterus curvipennis*.

	SMO														TUX	YUC		
	GF	Ciel	Nar	Aqm	Xil	Ord	Mac	UG	Coap	Risc	Cuet	Xico	Ama	Clav	Tux	Bec	Gar	Nov
GF	–	0.141	0.199	0	-0.045	0.029	0.079	0.007	-0.043	0.113	0.019	0.308	0.079	0.175	**0.899**	0.982	0.966	**0.934**
Ciel	-0.025	–	0.249	0.1159	0.169	0.111	-0.138	0.159	0.097	0.077	0.003	0.185	0.036	0.155	**0.746**	**0.940**	**0.938**	**0.932**
Nar	0.031	0.083	–	0.2010	0.169	0.030	0.105	0.234	0.052	0.025	0.105	0.048	-0.065	-0.035	**0.795**	0.929	0.926	**0.921**
Aqm	0.016	0.053	0.043	–	-0.092	0.030	0.079	0.065	-0.040	0.110	-0.002	0.308	0.003	0.155	**0.899**	0.982	**0.966**	**0.934**
Xil	0.003	-0.017	0.024	-.0310	–	0.029	0.051	0.026	-0.041	-0.097	0.043	0.282	0.011	0.155	**0.845**	0.960	0.954	**0.935**
Ord	0.022	0.021	0.079	.0393	0.001	–	-0.037	0.095	-0.027	-0.063	0.009	0.137	-0.087	0.042	**0.749**	0.941	**0.939**	**0.933**
Mac	0.016	0.027	0.069	.0778	0.004	0.068	–	0.057	-0.094	-0.114	-0.135	0.026	-0.139	0.034	0.827	0.941	**0.933**	**0.924**
UG	-0.053	0.010	0.072	.0072	0.008	0.008	0.078	–	0.047	-0.042	0.074	0.332	-0.019	0.187	**0.823**	0.958	**0.954**	**0.939**
Coap	0.013	0.012	-0.012	-.0204	-0.015	0.035	0.005	0.032	–	-0.176	0.003	0.111	-0.137	0.058	**0.757**	0.927	0.924	**0.923**
Risc	0.047	0.089	0.169	.0350	0.046	0.165	0.060	0.117	0.056	–	-0.063	0.133	-0.132	0.065	0.887	0.969	0.947	0.925
Cuet	-0.023	0.002	0.045	.0340	-0.006	0.009	0.033	0.005	-0.002	0.125	–	0.119	-0.065	0.057	**0.702**	**0.931**	**0.930**	**0.928**
Xico	-0.002	0.058	0.068	.1129	0.061	0.031	0.139	0.038	0.043	0.252	0.007	–	0.025	0.015	**0.793**	0.916	0.914	**0.918**
Ama	0.069	0.151	-0.022	.0342	0.091	0.135	0.155	0.102	-0.055	0.279	0.087	0.094	–	-0.067	0.798	0.930	**0.924**	**0.922**
Clav	-0.037	**0**.072	-0.005	.0102	0.040	0.059	0.099	0.006	-0.001	0.207	0.025	0.010	-0.048	–	**0.789**	0.935	0.931	**0.924**
Tux	**0.212**	**0.238**	**0.239**	**.2367**	**0.194**	**0.223**	0.094	**0.203**	0.139	**0.301**	**0.222**	**0.312**	**0.295**	**0.248**	–	0.979	0.974	**0.950**
Bec	0.209	**0.351**	0.215	**.**3000	0.327	**0.322**	0.302	0.246	0.153	0.525	**0.302**	0.304	0.113	0.128	**0.318**	–	-0.202	0.015
Gar	0.140	**0.226**	0.134	.2099	0.217	**0.208**	0.215	0.171	0.089	0.421	**0.196**	0.180	0.046	0.070	**0.316**	-0.158	–	-0.128
Nov	**0**.**201**	**0.296**	**0.172**	**.2611**	**0.277**	**0.289**	**0.237**	**0.253**	0.124	**0.428**	**0.266**	**0.269**	0.114	**0.150**	**0.360**	-0.020	-0.068	–

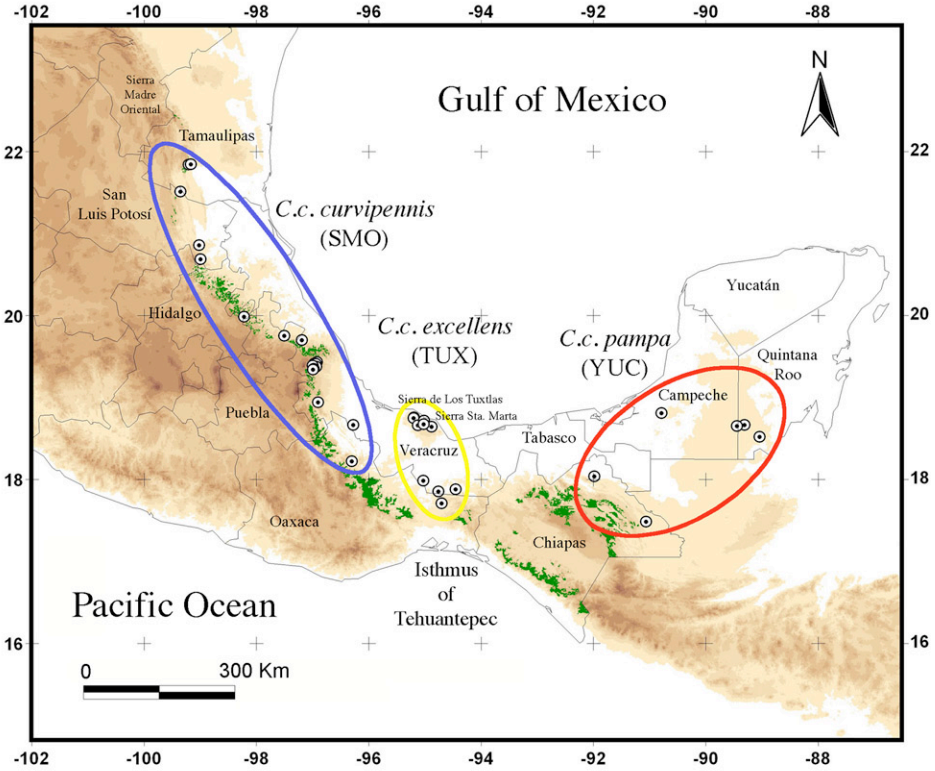
Pairwise F_{ST} values (above diagonal) of mtDNA and pairwise R_{ST} values (below diagonal) of microsatellites between populations. Values statistically significant at $P < 0.001$ are indicated in bold.



Additional file 2 Supplemental Figure S1. Neighbour-joining tree of sampled locations based on pairwise R_{ST}. Analysis obtained from microsatellite data showing YUC group clustering together in a basal position, and sampling localities of SMO group clustering in a geographically unresolved clade. Sampled locations with only one individual were excluded from the analysis.



Additional file 3 Supplemental Figure S2. Results of the coalescent-based simulation on phenotypic traits. Estimates of *s* of Slatkin and Maddison from 10,000 coalescent simulated gene trees to test the probability of complete sorting of vocal characters (A and B) and morphological characters (C and D) in wedge-tailed sabrewing populations. Regarding vocal characters, there is a low probability of fixation in nuclear genes under neutrality regardless of the assumption of dichotomous branching (A) or the simultaneous model of divergence (B). In contrast, the probabilities that nuclear genes would be fixed under neutrality are high for morphological characters, regardless of the assumption of dichotomous branching (C) or the simultaneous model of divergence (D). This suggests that divergent selection has caused the pattern of vocal variation among populations, but the null hypothesis that morphological divergence resulted from drift was not rejected. Arrows indicate the expected value of *s* in a completely sorted tree.



Additional file 4- Supplemental Figure S3. Geographic distribution of *Campylopterus curvipennis* species complex. Distribution of *Campylopterus curvipennis* species complex based on museum (MZFC) and bibliographic records [32, 59], showing the disjunct distribution of three subspecies. Blue =*C. c. curvipennis*, yellow =*C. c. excellens*, red =*C. c. pampa.*

CAPÍTULO II

Genes and culture: decoupled patterns of genetic and acoustic divergence in a lekking hummingbird species (Aves: *Campylopterus curvipennis*)

We evaluate if geographic variation of hummingbird song is correlated with genetic divergence, and test for the effects of drift and selection shaping song geographical variation. Hummingbirds have developed the ability of song learning, and this trait can generate the transmission of copy errors or novelties contributing to geographic variation and dialect formation. Our study compared male song divergence with genetic divergence (using microsatellites) of wedge-tailed sabrewings from leks throughout eastern Mexico. Acoustic characteristics of their song were analyzed using a multivariate and a Bayesian approach to test for among-lek differences. We also explored the relative exchange of migrants between leks along their distribution. Contrary to expectations, our data revealed that neutral genetic divergence and song divergence were not correlated, and measures of genetic differentiation and migration rates indicated high levels of gene flow between leks. However, we found acoustic structure between leks, where leks had an exclusive assemblage of syllable types, and differed in spectral and temporal measurements of song. Because patterns of song divergence were partially explained by drift (isolation-by-distance), our results support the role of social selection in promoting song convergence within leks and sexual selection in promoting novelty and vocal diversity among leks.

KEY WORDS: Gene flow, geographic variation, hummingbirds, sexual selection, song.

Geographic divergence in acoustic signals involved in mate choice and species recognition is often the first step toward speciation. It can potentially facilitate reproductive isolation through assortative mating (females that may preferentially mate with males from their own region and dialect; Searcy and Yasukawa 1996) and reduce dispersal among dialects, affecting survival due to ecological specialization, success in finding mates, and/or access to social groups (Wright et al. 2005). These factors potentially reduce gene flow between dialects, promoting neutral genetic differentiation (Baker 1982; Irwin et al. 2001; Podos et al. 2004; Patten et al. 2004). Despite the appeal of this prediction, most studies have found no correlation between dialects boundaries and genetic divergence (Soha et al. 2004; Wright et al. 2005; Leader et al. 2008; but see MacDougall-Shackleton and MacDougall-Shackleton 2001; Irwin et al. 2008), making this a controversial issue (Zink and Barrowclough 1984). An alternative explanation for these mixed results is that geographic song variation and dialect formation do not promote genetic differentiation as individuals learn their vocalizations after dispersal, probably to facilitate territory establishment or the access to social groups. Under this scenario, variation in vocalizations could be maintained without a concordant differentiation in neutral genetic markers (Payne 1981; Ellers and Slabbekoorn 2003; Nicholls et al. 2006).

The diversification of hummingbirds has long been portrayed as a classical example of speciation via sexual selection (Darwin 1871; Futuyma 1987). The sexual dimorphism, conspicuousness of their iridescent signals, flashing crowns and gorgets, other head ornaments, and elongated tails have presumably arisen because females favor males with such traits (but see Bleiweiss 2008). In hummingbirds, attention to understanding the evolutionary significance of such phenotypic traits has been focused almost exclusively on visual signals because in many species males display various of these ornaments in complex aerial displays (Wagner 1954; Ornelas et al. 2002; Clark and Dudley 2009; Parra 2009) and spectacular dive displays accompanied by non-vocal sounds (Clark 2008; Clark and Feo 2008). Nonetheless,

hummingbirds have also developed a remarkable diversity of vocalizations. Although most hummingbirds species sing simple one- or two- syllable songs (Atwood et al. 1991; Kroodsma et al. 1996), there are species intermediate in vocal complexity (Baptista and Schuchman 1990; Gaunt et al. 1994; Kroodsma et al. 1996), species with intricate and extraordinarily complex vocal signals (Kroodsma et al. 1996; Ornelas et al. 2002; Ficken et al. 2000), and species that stand out as versatile singers, producing many syllable types which can turn from one type to another in a song sequence (Jarvis et al. 2000; González and Ornelas 2005; Ferreira et al. 2006). Like humans, hummingbirds have also developed the trait of vocal learning (Jarvis et al. 2000), where the potential for rapid change implies a fundamental role in avian speciation (Nottebohm 1969, Slabbekoorn and Smith 2002*a*, Lachlan and Servedio 2004, Edwards et al. 2005; but see Baptista and Trail 1992). Vocal learning through imitation can generate the rapid transmission of new acoustic elements, contributing to geographic variation and dialect formation (reviewed in Slabbekoorn and Smith 2002*a*). During the learning process “copy errors” can occur and, as a consequence, novel variations and the possibility of recombination and innovation of elements introduce variation in song.

Few phenotypic traits used in intraspecific communication are as diverse as acoustic signals, and understanding the causes of signal evolution can provide insight into how biodiversity arises (Campbell et al. 2010). Because acoustic signals can act as an isolating mechanism between populations and incipient species, it is important to discriminate between the relative contribution of drift and selection in the process that shape intraspecific communication (González et al. 2011). Several processes driving song variation among populations include (1) selection of particular acoustic traits due to habitat characteristics, where different frequencies of sound travel better in different environments (Slabbekoorn and Smith 2002*b*; Patten et al. 2004; Seddon 2005; Nichols et al. 2006; Ruegg et al. 2006; Dingle et al. 2008), (2) stochastic forms of selection such as sexual or social selection, producing random

geographic variation of male songs (Irwin et al. 2008), and (3) cultural drift, which occurs in the absence of selection because of genetic and cultural mutation (Mundinger 1982; Baker et al. 2006; Förschler and Kalko 2007). Under cultural drift, song similarity would be expected to decrease with distance due to the accumulation of small differences across space (isolation by distance; Koetz et al. 2007), producing most dramatic changes between populations further apart. Studies relating intraspecific genetic divergence and learned vocal traits have featured mainly oscine passerines (e.g., Soha et al. 2004; Ruegg et al. 2006; Irwin et al. 2008), a suboscine passerine (Saranathan et al. 2007), and parrots (e.g., Wright et al. 2005), but no studies with hummingbirds in which vocal learning is thought to have evolved independently (Jarvis et al. 2000). Geographic song variation has been documented in some hummingbird species (see González and Ornelas 2005 for more references), however, the range of variation among individuals, and among populations of the same species at micro and macrogeographic scales is poorly documented, and detailed studies of song divergence combined with neutral molecular markers could yield insights into patterns of drift, selection, and finally speciation.

The wedge-tailed sabrewing, *Campylopterus curvipennis*, is a sexually monochromatic, size dimorphic hummingbird with a broad distribution through the cloud and humid tropical forests along the eastern rim of the Sierra Madre Oriental in eastern Mexico (Howell and Webb 1995). Males are polygynous and during the breeding season they congregate in leks attended for several months (from January to June), performing elaborate singing displays to females. Individual territories are established in close proximity (2-5 m between singing perches) in dense vegetation where males usually perch on exposed twigs and advertise to females (González and Ornelas 2005). Their songs are loud, high-pitched, and are composed of 30-60 discrete units (syllables), with a highly variable acoustic structure given at a high-speed rate (González and Ornelas 2005). These elaborate acoustic signals are intriguing because most lekking hummingbird species acoustically studied in detail have relatively simple and

stereotyped songs. The complex syllable structure varies geographically, most notably the introductory syllable and the syllable repertoire, ranging from differences between neighboring males within a lek (song neighborhoods) to differences between lek members separated by ≤ 20 kilometers (González and Ornelas 2005, 2009). In addition to this site-related variation, there is some lek- and time-related variation within leks. Males can cluster in song neighborhoods, and the clustering pattern is quite consistent from year to year (González and Ornelas 2009). But whether song variation bears a relationship to the geographic proximity of leks at larger geographic scales or in the entire distribution of a species has not been reported on this or any other hummingbird species.

The broad-scale phylogeography of this species complex has been examined using sequences of two mitochondrial DNA genes and 10 polymorphic microsatellite loci. These data showed the distinctiveness of the three acoustically and morphologically divergent and allopatrically distributed subspecies (*C. c. curvipennis*, along the Sierra Madre Oriental, *C. c. excellens* in the Sierra de los Tuxtlas, *C. c. pampa* in the Yucatan Peninsula; González et al. 2011). Here we quantify vocal variation and song divergence across leks of the lineage continuously distributed along the Sierra Madre Oriental, from Tamaulipas to southern Veracruz, *Campylopterus curvipennis curvipennis*. Specifically, we test whether vocal divergence is correlated with genetic divergence, using leks distributed throughout the subspecies entire range, and test for the possible effects of drift and selection shaping the geographical variation in song. To accomplish this, we first derived estimates of lek genetic structuring from microsatellite loci to be contrasted with measures of vocal divergence derived from recordings of wedge-tailed sabrewings singing displays at the same leks. If vocal variation is affected by processes of random drift we expect vocal divergence to be positively correlated with genetic lek distances, and leks would be acoustically different. A pattern of cultural drift can also be detected if vocal divergence increases with geographical separation between leks along the

geographic range with no apparent barriers to gene flow (isolation by distance). On the other hand, if the patterns of vocal variation are more strongly affected by some kind of selection, we expect a lack of relationship between vocal and genetic distances, because phenotypic traits are expected to evolve faster than neutral markers.

Materials and Methods

DNA SAMPLING

Feather samples were collected from 105 live wedge-tailed sabrewings captured at nine leks located throughout the hummingbird's distribution during the breeding season of the years 2006-2008, with 3-27 individuals sampled per lek (Table 1). Birds were captured in mist nets at leks and the two outer tail rectrices were collected for subsequent genetic analyses before release. Sampled leks were categorized in three areas: (*i*) northern limit of the distribution, from southern Tamaulipas and north of San Luis Potosí (nSMO herein, *n* = 3 leks); (*ii*) central part of the distribution, from south of San Luis Potosí (cSMO herein, *n* = 2 leks); and (*iii*) southern limit of the distribution, from Puebla and Veracruz (sSMO herein, *n* = 4 leks) (Table 1).

MICROSATELLITES GENOTYPING AND GENETIC ANALYSES

DNA extraction was made from the calamus of one of the feathers with chelex (5%) according to Morin et al. (1994), and samples were genotyped at 10 polymorphic microsatellites loci designed specifically for *Campylopterus curvipennis* (Abdoullaye et al. 2010, GeneBank accession nos. GQ294539– GQ294550). PCR conditions and fragment sizing are fully described in Abdoullaye et al. (2010). Observed and expected heterozygosity and mean number of alleles per locus were calculated in GENEPOP 3.4 (Raymond and Rousset 1995). Microsatellite genotypes were tested for departures from Hardy-Weinberg equilibrium and for linkage disequilibrium between pairs of loci within leks and loci in GENEPOP. The presence of

null alleles was tested using MICROCHECKER 2.2.3 (Van Oosterhout et al. 2004). MICROCHECKER infers the presence of a null allele when significant excess homozygosity is distributed evenly across all of the alleles at a locus. To investigate lek genetic structure we calculated global R_{ST} (Slatkin 1995) in RSTCALC 2.2 (Goodman 1997) adapted to microsatellites stepwise mutation model, and pairwise R_{ST} to make comparisons with vocal variation.

To examine geographic patterns of population genetic structure, we performed Bayesian genetic clustering using STRUCTURE 2.2.3 (Pritchard et al. 2000), which is used to infer the most likely number of genetic clusters (K) through the multilocus genotypic data. We ran STRUCTURE without population information under the admixture model with correlated allele frequencies. Ten independent chains were run for each K, from K = 1 to K = 12. The length of the burn-in was 500,000 and the number of Markov chain Monte Carlo (MCMC) replications after the burn-in was 1,000,000. To determine an accurate number of clusters we calculated the statistic ΔK based on the rate of change in the log probability of data between successive K values following Evanno et al. (2005).

Gene flow (4Nm) among leks was estimated with a maximum likelihood coalescent approach using genotypic data and Migrate v. 3 (Beerli and Felsenstein 2001). The first genealogy was started with a random tree, and initial theta and migration rate (xNm) parameters were obtained from F_{ST} calculations. We ran 5 short and 3 long chains (200,000 genealogies sampled), after discarding the first 5000 genealogies as a burn-in.

ACOUSTIC ANALYSES

Songs were recorded from territory-holding males at the nine leks at which feather samples were taken. Between 3 and 13 birds were recorded at each lek, with 5 to 19 song recordings obtained from each of 56 males (500 recordings, c. 9 recordings per bird). Songs recordings

were made with a Marantz PMD660 portable solid-state recorder and a Sennheiser MKH-70 directional microphone. Recordings were digitized at a sampling rate of 44100 Hz and stored as 16-bit samples. Spectrograms of recordings from all individuals were generated with a 349.7 Hz filter bandwidth and a frame length of 512 points (=11.6 ms) using RavenPro 1.4 (www.birds.cornell.edu /raven).

We used three different data sets for acoustic analyses: two related with syllabic variation and song sharing, and a third related to acoustic traits of two comparable syllables. For syllabic variation and song sharing data sets, we used syllabic units throughout the wedge-tailed sabrewing repertoire. Syllables were visually classified by structure on printed spectrograms by CG, and assigned a letter or combination of letters for identification (see González and Ornelas 2009 for details). We screened the 500 recordings and built a presence/absence matrix of syllable types for each of the 56 individuals, where each entry consists of 0 or 1, and a matrix with the relative frequency of each syllable per song averaged by bird. Finally for the data set related with acoustic traits, we took spectral and temporal measurements of two selected syllables to allow comparisons: the introductory syllable (all individuals emit one at the beginning of the song bout), and a shared syllable found across all leks (emitted at least once in the songs and shared by all recorded individuals). For the introductory syllable, we measured the duration (s), minimum frequency (kHz), bandwidth (the difference between maximum and minimum frequency, kHz), peak frequency (frequency at which maximum power occurs, kHz), total number of elements, and the number of different elements (Fig. 1). For the shared syllable we measured the duration (s), minimum frequency (kHz), bandwidth (kHz), and the peak frequency (kHz). Because the shared syllable has several elements (notes), we took the same measures on two of their elements (numbers 1 and 2, Fig. 1), and the duration of another element (number 3, Fig. 1). In total 13 measures were taken on the shared syllable.

To evaluate song divergence among leks of wedge-tailed sabrewings, we performed a hierarchical cluster analysis using the unweighted pair-group method with arithmetic mean (UPGMA) and the Euclidean distances to construct a dendrogram in SPSS (SPSS Inc.) and also a principal coordinate analysis using PAST (Hammer et al. 2001), both analyses based on the binary presence/absence matrix of syllable types. As vocal variation can be affected by processes similar to those driving genetic variation (Koetz et al. 2007), we additionally run STRUCTURE to graphically represent geographic patterns of acoustic structure and compare with those of genetic structure. The Bayesian clustering technique has been usually applied to most commonly used genetic markers, however, STRUCTURE is also applicable to analyses based on phenotypic data, such as analysis of human language (Reesink et al. 2009). In the Bayesian clustering method implemented in STRUCTURE algorithm, individuals are probabilistically assigned to one or more populations, and also the most likely number of groups is determined, in this case based on the syllabic structure of songs. We used the presence/absence matrix of the syllable types (where each syllable type is treated as different loci, and the values is the equivalent of the genetic alleles) to determine the most likely number of acoustic clusters (*K*). The ploidy option was set at 1, and ten independent chains were run for each *K,* from *K* = 1 to *K* = 12. Length of the burn-in was 500,000 and the number of Markov chain Monte Carlo (MCMC) replications after the burn-in was 1,000,000.

Measurements of the introductory and the shared syllable were tested for significant vocal differences among leks performing analyses of multivariate variance (MANOVA). One-way ANOVAs followed the MANOVA to determine lek differences for each variable. Finally, we used the relative frequency of syllables, and the measurements of the introductory and shared syllable as predictors in a discriminant function analysis (DFA) for each data set to examine whether individuals could be classified according to their lek of origin.

COMPARISON BETWEEN ACOUSTIC, GENETIC AND GEOGRAPHIC DISTANCES

Acoustic distance matrices regarding syllable variation and song sharing data were built by estimating the pairwise Jaccard similarity coefficient between individuals among leks. This coefficient was calculated in EstimateS 8.0.0 (Colwell 2006) and values were subtracted from 1 to make a dissimilarity matrix. Distance matrices for the introductory syllable and the shared syllable measurements were built as the Euclidean distance between the group centroids of the first discriminant functions using SPSS, in which variables were scaled to values between 0 and 1. We followed Ruegg et al. (2006) to examine relationships between acoustic, genetic and geographic distances. Namely, we performed a series of simple and partial Mantel tests using IBD (Bohonak 2002), assessing significance levels of association between matrices with 1000 randomizations. To address whether acoustic distance is correlated with genetic distance we compared the song sharing distance and the introductory and shared syllable distance matrices with pairwise R_{ST} matrix. This relationship was controlled for potential effects of geographic distance through partial Mantel tests. Finally, to test for the effect of isolation-by-distance we looked at the relationship between acoustic and geographic distances, controlling for genetic distances.

Results

GENETIC STRUCTURE AMONG LEKS

Number of alleles per locus varied from 3 to 15, and observed heterozygosity values showed no consistent deviations from H-W equilibrium. Only four leks were not in H-W equilibrium after Bonferroni corrections in locus CACU13-2, two leks in locus CACU5-7 and one lek in locus CACU13-7 (Table 2). MICROCHECKER identified that these loci were possibly affected by the presence of null alleles. No significant linkage disequilibrium was detected in any of the population-loci comparisons after Bonferroni corrections.

We did not detect significant genetic subdivision among leks (global R_{ST} estimate ± SE, -0.032 ± 0.0017, $P > 0.05$), indicating that leks are not genetically structured. Results of STRUCTURE analysis showed a weak genetic structure among leks. The average over 10 runs of the log likelihood was highest at $K = 5$; however, when ΔK is calculated the break in slope of the distribution of $L(K)$ was at $K = 2$ (Fig. 2). At this optimum we did not observed a clear pattern of genetic clustering with a lek or geographic correspondence. Only the lek from Cuet appears to be different from the rest. However when $K = 4$, we observed that Ord and UG leks could be classified in a single cluster, Ciel, GF, Nar, Aqm, Xil and Mac leks in another, and Cuet in the last, though it is a weak pattern of genetic structure because they have an admixtured ancestry.

Estimates of gene flow showed that in general there is a high asymmetric interchange of immigrants per generation among leks (Table 3), but we did not find a clear geographic pattern of gene flow. There are very low migration rates from all leks towards the Ciel lek, which is in the northern limit of the distribution. Cuet is the lek that receives the lower level of gene flow but it is the lek that donates more migrants per generation. This corresponds to the pattern observed in STRUCTURE analysis, where Cuet appears to be genetically differentiated. Finally, Aqm and Mac leks receive the higher levels of gene flow, however, estimates of gene flow can be overestimated by the low sample size of these leks.

ACOUSTIC VARIATION AND SONG DIVERGENCE

Songs of wedge-tailed sabrewings were composed of a long series of structurally complex syllables emitted at a high pitch given at high rates. They also emitted an introductory syllable characteristic of individuals from each lek, although sometimes more than one introductory syllable is emitted because of the existence of song neighborhoods (González and Ornelas 2009). In total we detected 294 syllable types across recorded leks, and songs were very versatile (birds turn from one syllable type to another in a song sequence, with no successive

syllable repetitions detected). The dendrogram derived from the cluster analysis based on the presence/absence of syllable types showed that in general individuals within the same lek are more similar than between leks, and most of them are clustered in different groups, except leks from Ciel and Ord (Fig. 3). This could be an effect of song neighborhoods described earlier at Ord lek and maybe in the Ciel lek. In addition, we observed a song sharing geographic pattern: leks from the northern (GF, Ciel, Nar; nSMO), central (Aqm and Xil; cSMO) and southern limit of the distribution (Cuet, Ord, UG and Mac; sSMO) are clustered together in different groups, except three individuals from the Orduña lek, which are basal in the dendrogram. On average, the proportion of syllables shared between individuals within leks was 0.60 ± 0.18 (mean ± SD), whereas the proportion of syllables shared among leks was 0.15 ± 0.04 (Table 4). Despite the great syllable diversity observed across leks, there were some syllables extensively shared, whereas only members from closely distributed leks shared others. A principal coordinates analysis based on the presence/absence of syllable types revealed a marked pattern of song structure between leks, consistent with their geographic origin. A plot of the two principal coordinates (accounting for 23.5% and 17% of the variance, respectively), showed a clear separation between leks from the northern and central part of the distribution along the first coordinate, and also a clear separation between those from the leks in the southern part of the distribution along the second coordinate (Fig. 4).

The results of the STRUCTURE analysis showed that structural features of song could be used to help clarify lek relationships. The average over 10 runs of the log likelihood was highest for K = 7, but we present results from K = 2-9 (Fig. 5). At K = 7 the Xil and Aqm leks emerge as a solid cluster (light green), some individuals of leks from Cuet, Mac, Ord and UG were put in the same cluster as Nar (orange), and some individuals from Mac lek were put in Ord cluster (light blue). The rest of individuals were assigned to different lek clusters. At K = 9, which is the number of leks sampled, all leks are assigned probabilistically to different clusters

except Cuet, which individuals were assigned to the Nar and UG leks, and Mac, which individuals were assigned to the Nar and Aqm leks. This could be an effect of low sample sizes in those leks. In the case of the Ord lek, four clusters emerged coinciding with the song neighborhoods described in an earlier study (González and Ornelas 2009).

Regarding spectral and temporal measurements, introductory syllables were distinct at each lek (Wilks' Lambda, $F_{48,269} = 4.71$, $P < 0.0001$; Fig. 6), and lek distinctiveness was significant for all six acoustic variables (one-way ANOVAs: duration, $F_{8,59} = 4.39$, $P = 0.0003$; minimum frequency, $F_{8,59} = 5.5$, $P < 0.0001$; bandwidth, $F_{8,59} = 2.37$, $P = 0.03$; peak frequency, $F_{8,59} = 2.8$, $P = 0.01$; number of elements $F_{8,59} = 9.34$, $P < 0.0001$; number of different elements $F_{8,59} = 5.63$, $P < 0.0001$) (Table 5). Regarding measurements of the shared syllable, the MANOVA with the 13 variables also showed significant lek differences (Wilks' Lambda, $F_{104, 320}$= 2.55, $P < 0.0001$), but these differences were only significant for seven acoustic variables (one-way ANOVAs: minimum frequency, $F_{8,57} = 16.82$, $P < 0.0001$; bandwidth, $F_{8,57} = 2.29$, $P = 0.03$; and peak frequency of the element 1, $F_{8,57} = 6.35$, $P < 0.0001$; bandwidth of the element 2, $F_{8,57}$ = 2.24, $P < 0.05$; duration of the element 3, $F_{8,57} = 3.6$, $P = 0.001$, duration $F_{8,57} = 2.42$, $P < 0.05$; and bandwidth of the complete syllable, $F_{8,57} = 24.78$, $P < 0.0001$) (Table 6).

Results of the DFA based on the relative frequency of syllables, acoustic measurements of the introductory and the shared syllable, showed that the 100%, 70% and 82% (respectively) of the individuals were correctly classified by lek membership (Fig. 6). In the DFA with the relative frequency of syllables the first three discriminant functions recovered 74.2% of the variation (function 1: eigenvalue 360.4, 43% variance; function 2: eigenvalue 149.4, 17.7% variance; function 3: eigenvalue 116.2, 14% variance). In DFA with acoustic measurements of the introductory syllables, the first three discriminant functions recovered 89.3% of the variation (function 1: eigenvalue 3.25, 36% variance; function 2: eigenvalue 1.18, 27% variance; function 3: eigenvalue 0.90, 17% variance). Lastly in DFA with acoustic measurements of the shared

syllable, the first two discriminant functions recovered 77.7% of the variation (function 1: eigenvalue 5.9, 67.6% variance; function 2: eigenvalue 0.88, 10.1% variance).

COMPARISON BETWEEN ACOUSTIC, GENETIC AND GEOGRAPHIC DISTANCES

There was no evidence for a positive relationship between pairwise measures of acoustic distances and R_{ST} values among leks (Mantel test: song sharing, r = -0.06, P > 0.05; introductory syllable r = 0.06; P > 0.05; shared syllable, r = 0.13, P > 0.05). Acoustic distance and geographic distance were significantly correlated when using the song sharing distance matrix (r = 0.23, P < 0.05) but not significantly correlated when using the shared syllable (r = -0.006, P > 0.05), or introductory syllable (r = 0.09, P > 0.05) distance matrices. After controlling for the effects of genetic distance the positive relationship between geographic and song sharing distances was not affected (partial Mantel test, r = 0.23, P < 0.05). Therefore, these results indicate that song sharing decreases with increased geographic distance.

Discussion

Our study reveals that vocal divergence and neutral genetic divergence are decoupled among wedge-tailed sabrewing leks. The lack of association between vocal and genetic divergence seems an ubiquitous pattern among vocal learners such as songbirds and parrots, and the evidence presented here adds a hummingbird species, providing independent support for the idea that dialect formation does not limit the movement of genes (Wright et al. 2005). Measures of migration rates, genetic differentiation and population structure indicated high levels of gene flow among leks and low levels of genetic structure. Similar values of genetic differentiation measures (F_{ST}), using microsatellites or mtDNA, have been observed in songbirds and parrots exhibiting vocal dialects, but lacking any dialect-based genetic differentiation (Wright and Wilkinson 2001; Soha et al. 2004; Wright et al. 2005). Although song traits play a potential role

in increasing genetic polymorphisms and generating reproductive isolation and speciation in sympatry through sexual selection (Nottebohn 1969; Vaneechoutte 1997; Edwards et al. 2005), our data showed that the strong acoustic divergence occurs among leks that experience high levels of gene flow.

ACOUSTIC STRUCTURE AND SONG LEARNING

A strong pattern of acoustic structure with sharp boundaries between leks was observed in wedge-tailed sabrewings, where each lek had an exclusive assemblage of syllable types, and song sharing was lower between than within leks. Also, acoustic traits of two types of comparable syllables (introductory and shared syllables) were more divergent between than within leks. Results from the STRUCTURE analyses based on vocal characters also detected the strong pattern of acoustic structure, congruent with the results of the multivariate analyses. The STRUCTURE analysis, a genetic clustering method based on a Bayesian algorithm, gives an alternative approach to multivariate classification analyses for phenotypic data (Reesink et al. 2009). Importantly for study of geographic song variation, our study demonstrates that the STRUCTURE analysis can be applied to this type of data particularly for studying complex acoustic signals even where the processes of learning and transmission are not yet understood.

The decoupled patterns between the neutral genetic and vocal datasets highlights the role that learning may play in the formation of lek-level vocal structure in wedge-tailed sabrewings. As in songbirds and parrots, song learning in hummingbirds (Jarvis et al. 2000) is culturally transmitted through imitation and the process of learning across generations provides an important source of variation (Mundinger 1980; Edwards et al. 2005). However, the extent of dispersal and the capacity of learning postdispersal are critical in determining the degree of genetic divergence between populations with divergent songs, where song divergence in predispersal learners can most likely lead to a higher genetic subdivision than song divergence

in open-ended learners (Ellers and Slabbekoorn 2003; Leader et al. 2008). Dispersal patterns of male hummingbirds are not known, however, we suspect that their polygamous mating system and their associated lack of paternal care behaviors lead the offspring to have almost no chances to learn the complex vocalizations from their fathers or relatives without a recognition mechanism among them, an thus suggesting that the transmission of vocal elements is between unrelated individuals. The sensitive phase of learning in hummingbirds is not known with certainty, but it might be sufficiently extended to allow acquisition of the song before or at the time of territory establishment because we have occasionally observed, and recorded, presumably young males emitting imperfect and very quiet songs at leks before territory establishment. Besides, song neighborhoods within leks are not static over time because new local or foreign syllables are incorporated each year, and apparently some syllables go extinct (González and Ornelas 2009). Although nothing is known about the specific vocal learning process in this species that contribute to this variation, our previous work indicates that males alter their singing display over time (González and Ornelas 2009), suggesting that vocal learning in this species may be open-ended.

SONG DIVERGENCE AND CULTURAL DRIFT

The strong acoustic variation in wedge-tailed sabrewings can be affected by processes similar to those driving genetic variation, such as drift or selection (Koetz et al. 2007). In the case of drift, dialects or song variation at different levels as that shown here for wedge-tailed sabrewings could be originated through a process of cultural diversification where random song mutations are produced by copy errors (Podos et al. 2004). Although no relationship between genetic and vocal divergence in the wedge-tailed sabrewing was found along the Sierra Madre Oriental, the vocal characters show the effect of isolation. The increased vocal divergence with increased geographic distance (isolation-by-distance) suggests that vocal divergence is partially

explained by drift, where small differences in song accumulate along a geographical gradient due to the reduced probability of interaction among individuals from more distant leks. Although the degree of syllable sharing between individuals of a particular lek was always higher than between individuals from different leks, numbers of syllable types shared between leks decreased with geographic distance. Regarding acoustic measures of the introductory syllable and the shared syllable, no significant correlations between these acoustic measures and geographic distance were detected. The lack of association is consistent with the observation that the introductory syllable is a vocal signature of group membership among members of song neighborhoods within leks (González and Ornelas 2009) and among lek members at various spatial scales (González and Ornelas 2005; González et al. 2011; this study), probably facilitating the learning of the lek song (see also Soha and Marler 2000).

SONG DIVERGENCE AND SELECTION

Adaptation of sound transmission by microclimate and vegetation structure can be important selection pressures of birds living in different habitats (Irwin 2000; Slabbekoorn et al. 2002). However, our studied leks were located in habitats with very similar climatic conditions and no significant relationships were found between acoustic distance measures *versus* habitat-related (climate and topography) distance measures (González et al. 2011), so we think habitat-selection is not an important pressure in shaping song divergence in wedge-tailed sabrewings. In a recent study of the *Campylopterus curvipennis* complex, where allopatric populations of three subspecific lineages without contemporary gene flow were involved, González et al. (2011) found that lineages isolated the longest shared fewer syllables and differed in spectral and temporal traits of syllables, suggesting an effect of drift in acoustic divergence. However, coalescent simulations of the evolution of phenotypic traits suggested that the fixation of song

types has occurred faster than would be expected by genetic drift, providing evidence that selection has shaped the variation in their complex and versatile songs (González et al. 2011).

In our study, besides a partial effect of cultural drift, the lack of correlation between genetic and acoustic distances and the strong pattern of vocal structure observed, imply a strong role of selection in promoting song divergence. Social selection or adaptation to social conditions, could be selective forces operating in song divergence among wedge-tailed sabrewings leks, where song sharing among neighbors confers social benefits that could possibly increase their reproductive success (Soha et al. 2004). Under this scenario, the adaptive significance of song learning implies that vocal learning evolved to allow individuals to adapt to each other in an immediate social context. Males could obtain reproductive advantages from the vocal imitation of an established and successful conspecific, such as obtaining a singing territory. Payne (1982) has explicitly referred to situations in which young males attempting to establish territories for the first time copy the song of neighbors, or of an adult whom they eventually replace. This social selection hypothesis also implies that song sharing and matching during vocal interactions are effective strategies for deterring strangers and controlling aggression levels from closest neighbors (Catchpole and Slater 1995). On the other hand, in lek-breeding systems males are subject to strong sexual selection pressures promoting the evolution of different traits, which in turn can lead to speciation events (Emlen and Oring 1977; Lande 1980). Because wedge-tailed sabrewings are monochromatic and do not display aerially at leks, it is likely that their complex and variable songs have also resulted from strong sexual selection pressures, causing the sharp acoustic structure in parapatric populations despite the high levels of gene flow. Social selection can also be operating to promote convergence within a lek; especially as such convergence would be maintaining cultural differences in the face of gene flow. Our results would then be congruent with the idea that both sexual and social selection cause rapid evolution of complex signals in which sexual selection is

promoting novelty (versatile singing) and the potential for runaway selection (West-Eberhard 1983; Anderson 1994) at the same time that there is social selection for convergence (sharing a group of syllables including the introductory syllable).

CONCLUSIONS

Our study clearly demonstrates the independence between acoustic and neutral genetic divergence in wedge-tailed sabrewings, contrary to predictions from the hypothesis that avian dialects contribute to genetically structure populations. In this continuously distributed species, with no apparent geographic barriers, genetic differentiation appears to be restricted by homogenizing gene flow, and vocal variation was not related to restricted gene flow between leks. However, the highly structured and marked song divergence between leks, only partially explained by cultural drift, is more likely explained by stochastic processes such as social and/or sexual selection. Our results suggest that vocal diversity and novelty are promoted by sexual selection at the same time that there is social selection for vocal convergence.

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Table 1. Localities, geographic location and altitude of wedge-tailed sabrewing sampled leks. Regions correspond to north, central and south of the Sierra Madre Oriental (nSMO, cSMO and sSMO).

Location	Region	Latitude/longitude	Altitude (masl)
1. El Cielo, Tamaulipas (Ciel)	nSMO	25º 3'33.66"N, 99º 12'21.40"W	943
2. Gomez Farías, Tamaulipas (GF)	nSMO	23º 3'58.26"N, 99º 10'6.52"W	564
3. El Naranjo, San Luis Potosí (Nar)	nSMO	22º 34'33.33"N, 99º 21'11.80"W	270
4. Aquismón, San Luis Potosí (Aqm)	cSMO	21º 37'30.87"N, 99º 1'12.52"W	378
5. Xilitla, San Luis Potosí (Xil)	cSMO	21º 22'39.50"N, 98º 59'35.77"W	637
6. Cuetzalan, Puebla (Cuet)	sSMO	20º 0'49.14" N, 97º 30'21.27"W	906
7. Macuiltépetl, Veracruz (Mac)	sSMO	19º 32'50.51"N, 96º 55'12.45"W	1500
8. La Orduña, Veracruz (Ord)	sSMO	19º 27'50.94"N, 96º 56'13.05"W	1190
9. Ursulo Galván, Veracruz (UG)	sSMO	19º 25'31.48"N, 96º 58'35.20"W	1200

Table 2. Population genetic variability in wedge-tailed sabrewing leks based on ten microsatellites loci: *n* = sample size, H_O = observed, and H_E = expected heterozygosity; * indicates significant departure ($p < 0.05$, after sequential Bonferroni correction) from Hardy-Weinberg equilibrium for locus CACU13-2,† for locus CACU13-7, and ζ for locus CACU5-7.

Lek	*n*	Mean alleles /locus	H_O	H_E
El Cielo	18	5.6	0.47*	0.59
Gomez Farías	4	3.6	0.65	0.61
El Naranjo	6	3.6	0.55	0.57
Aquismón	4	3.5	0.55	0.63
Xilitla	8	5	0.49*	0.64
Cuetzalan	27	6.2	0.57	0.62
Macuiltépetl	3	2.66	0.55	0.57
La Orduña	22	6.2	0.49*	0.63
Ursulo Galván	13	5.3	0.48*†ζ	0.60

Table 3. Estimates of the gene flow parameter 4Nm generated in Migrate analysis among leks from 10 microsatellite genotypes. Donor populations are on vertical, recipient populations are on the horizontal. Estimates are given followed by 95% confidence intervals in parentheses.

	Ciel	GF	Nar	Aqm	Xil	Cuet	Mac	Ord	UG
Ciel	—	3.58 (2.24–5.32)	0.58 (0.28–1)	17.62 (12.96–23.24)	6.23 (4.76–7.96)	0.25 (0.14–0.4)	69.2 (46.2–98.7)	0.32 (0.18–0.52)	1.4 (0.78–1.6)
GF	0.05 (0.004–0.17)	—	0.16 (0.04–0.44)	4.95 (2.71–8.2)	0.15 (0.02–0.55)	0.05 (0.01–0.13)	10.93 (3.52–24.8)	0.24 (0.01–0.17)	0.22 (0.08–0.44)
Nar	0.09 (0.02–0.25)	1.19 (0.48–2.28)	—	1.65 (0.53–3.76)	0.3 (0.07–0.8)	0.1 (0.04–0.21)	10.93 (3.52–24.8)	0.19 (0.09–0.35)	0.27 (0.12–0.52)
Aqm	0.14 (0.04–0.32)	0.24 (0.24–0.88)	0.25 (0.08–0.56)	—	0.46 (0.14–1.04)	0.05 (0.01–0.13)	3.64 (0.38–13.28)	0 (0–0.51)	0.11 (0.03–0.29)
Xil	0.37 (0.2–0.64)	1.91 (1–3.24)	0.66 (0.34–1.12)	2.75 (1.19–5.28)	—	0.07 (0.02–0.17)	14.57 (5.6–30.04)	0.06 (0.01–0.17)	0 (0–0.07)
Cuet	1.31 (0.92–1.76)	4.53 (3.04–6.48)	1.81 (1.24–2.52)	11.01 (7.44–15.56)	4.26 (3.06–5.72)	—	58.28 (37.44–85.6)	1 (0.73–1.32)	0.71 (0.43–1.08)
Mac	0.14 (0.04–0.32)	0.48 (0.11–1.28)	0 (0–0.11)	0.55 (0.06–2.0)	0.46 (0.14–1.04)	0.02 (0.00–0.09)	—	0.06 (0.01–0.17)	0 (0–0.07)
Ord	0.61 (0.36–0.92)	2.15 (1.16–3.56)	1.11 (0.64–1.64)	5.5 (3.12–8.88)	0.61 (0.23–1.25)	0.68 (0.49–0.92)	61.92 (40.36–90)	—	0.43 (0.23–0.74)
UG	0.19 (0.07–0.36)	1.67 (0.84–2.92)	0.91 (0.52–1.64)	2.75 (1.19–5.28)	1.82 (1.08–2.83)	0.33 (0.2–0.92)	21.49 (12.72–44.8)	0 (0–0.04)	—

Table 4. Similarity matrix based on the presence/absence of syllable types among leks. Values in bold indicate comparisons between individuals within the same lek.

	Ciel	GF	Nar	Aqm	Xil	Cuet	Mac	Ord	UG
Ciel	**0.437**								
GF	0.191	**0.771**							
Nar	0.218	0.189	**0.481**						
Aqm	0.144	0.144	0.163	**0.471**					
Xil	0.112	0.111	0.178	0.199	**0.673**				
Cuet	0.102	0.126	0.129	0.119	0.109	**0.459**			
Mac	0.099	0.125	0.111	0.091	0.099	0.090	**0.840**		
Ord	0.174	0.190	0.203	0.135	0.120	0.146	0.162	**0.424**	
UG	0.175	0.160	0.214	0.139	0.176	0.176	0.137	0.235	**0.823**

Table 5. Mean ± stdev of spectral and temporal measurements of introductory syllables across leks of wedge-tailed sabrewings. N = number of individuals, n = number of songs.

Lek	N	n	Duration (s)	Minimum frequency (kHz)	Bandwidth (kHz)	Peak Frequency (kHz)	No. elements	No. different elements
1. Ciel	13	143	0.4 ± 0.12	2.97 ± 0.5	2.19 ± 0.87	4.09 ± 0.35	3.85 ± 1.57	1.61 ± 1.04
2. GF	5	25	0.305 ± 0.04	4.43 ± 0.19	2.12 ± 0.22	5.51 ± 1.12	1 ± 0	1 ± 0
3. Nar	4	17	0.55 ± 0.07	3.15 ± 0.34	3.2 ± 0.5	4.31 ± 0.09	7 ± 1.09	2 ± 0
4. Aqm	5	21	0.74 ± 0.23	2.66 ± 0.53	3.1 ± 0.54	3.77 ± 0.5	6.6 ± 1.35	2 ± 0
5. Xil	7	33	0.73 ± 0.15	2.81 ± 0.49	3.25 ± 0.69	4.12 ± 0.32	9.28 ± 1.98	2.86 ± 0.38
6. Cuet	3	27	0.76 ± 0.3	3.28 ± 0.46	2.51 ± 0.62	3.96 ± 0	5.67 ± 0.58	2.67 ± 0.58
7. Mac	3	43	0.86 ± 0.61	2.47 ± 1.22	2.81 ± 0.92	4.43 ± 0.78	11.5 ± 7.23	1.75 ± 0.5
8. Ord	10	149	0.54 ± 0.19	2.73 ± 0.6	2.78 ± 1.1	4.24 ± 0.21	5.75 ± 2.41	2.12 ± 0.34
9. UG	6	42	0.55 ± 0.09	3.23 ± 0.48	2.1 ± 0.58	4.11 ± 0.46	4.6 ± 0.84	2.2 ± 0.42

Table 6. Mean ± stdev of spectral and temporal measurements of the shared syllable and three of its elements, across leks of wedge-tailed sabrewings (see Fig. 1). N = number of individuals, n = number of songs.

Lek	N	n	Duration (s)	Minimum frequency (kHz)	Bandwidth (kHz)	Peak frequency (kHz)	Duration (s)	Minimum frequency (kHz)	Bandwidth (kHz)	Peak frequency (kHz)	Duration (s)	Duration (s)	Minimum frequency (kHz)	Bandwidth (kHz)	Peak frequency (kHz)
			element 1				element 2				element 3	complete syllable			
1. Ciel	13	143	0.07 ± 0.00	3.17 ± 0.05	0.37 ± 0.05	3.30 ± 0.11	0.05 ± 0.00	0.40 ± 0.03	0.56 ± 0.06	0.78 ± 0.13	0.07 ± 0.00	0.20 ± 0.00	0.39 ± 0.04	6.10 ± 0.10	4.56 ± 0.77
2. GF	5	25	0.07 ± 0.01	3.36 ± 0.05	0.40 ± 0.05	3.56 ± 0.10	0.05 ± 0.01	0.39 ± 0.05	0.64 ± 0.06	0.77 ± 0.12	0.06 ± 0.00	0.20 ± 0.01	0.37 ± 0.05	6.52 ± 0.08	4.75 ± 1.25
3. Nar	4	17	0.07 ± 0.00	2.89 ± 0.05	0.43 ± 0.07	3.16 ± 0.16	0.05 ± 0.01	0.37 ± 0.01	0.57 ± 0.04	0.73 ± 0.15	0.07 ± 0.00	0.21 ± 0.00	0.37 ± 0.02	5.65 ± 0.15	3.06 ± 1.24
4. Aqm	5	21	0.07 ± 0.00	2.89 ± 0.06	0.31 ± 0.06	3.13 ± 0.11	0.05 ± 0.00	0.43 ± 0.02	0.57 ± 0.07	0.72 ± 0.22	0.08 ± 0.00	0.21 ± 0.01	0.41 ± 0.03	5.42 ± 0.29	4.55 ± 0.99
5. Xil	7	33	0.07 ± 0.00	2.97 ± 0.07	0.43 ± 0.09	3.19 ± 0.17	0.05 ± 0.00	0.43 ± 0.06	0.59 ± 0.07	0.69 ± 0.26	0.07 ± 0.00	0.20 ± 0.00	0.42 ± 0.05	5.67 ± 0.11	3.87 ± 1.45
6. Cuet	3	27	0.07 ± 0.00	3.01 ± 0.18	0.32 ±0.06	3.13 ± 0.22	0.05 ± 0.00	0.47 ± 0.04	0.57 ± 0.00	0.95 ± 0.09	0.07 ± 0.00	0.21 ± 0.00	0.46 ± 0.02	5.72 ± 0.20	3.87 ± 2.03
7. Mac	3	43	0.07 ± 0.00	3.26 ± 0.11	0.41 ± 0.05	3.47 ± 0.08	0.05 ± 0.00	0.39 ± 0.06	0.63 ± 0.11	0.80 ± 0.11	0.07 ± 0.01	0.20 ± 0.01	0.39 ± 0.07	6.32 ± 0.19	4.28 ± 1.80
8. Ord	10	149	0.07 ± 0.00	3.14 ± 0.10	0.38 ± 0.05	3.34 ± 0.13	0.05 ± 0.00	0.41 ± 0.03	0.63 ± 0.05	0.85 ± 0.19	0.07 ± 0.00	0.20 ± 0.00	0.40 ± 0.04	6.09 ± 0.17	4.29 ± 1.36
9. UG	6	42	0.07 ± 0.01	3.09 ± 0.11	0.36 ± 0.06	3.24 ± 0.13	0.05 ± 0.00	0.41 ± 0.04	0.65 ± 0.07	0.75 ± 0.19	0.07 ± 0.00	0.20 ± 0.00	0.40 ± 0.03	5.89 ± 0.19	3.79 ± 1.79

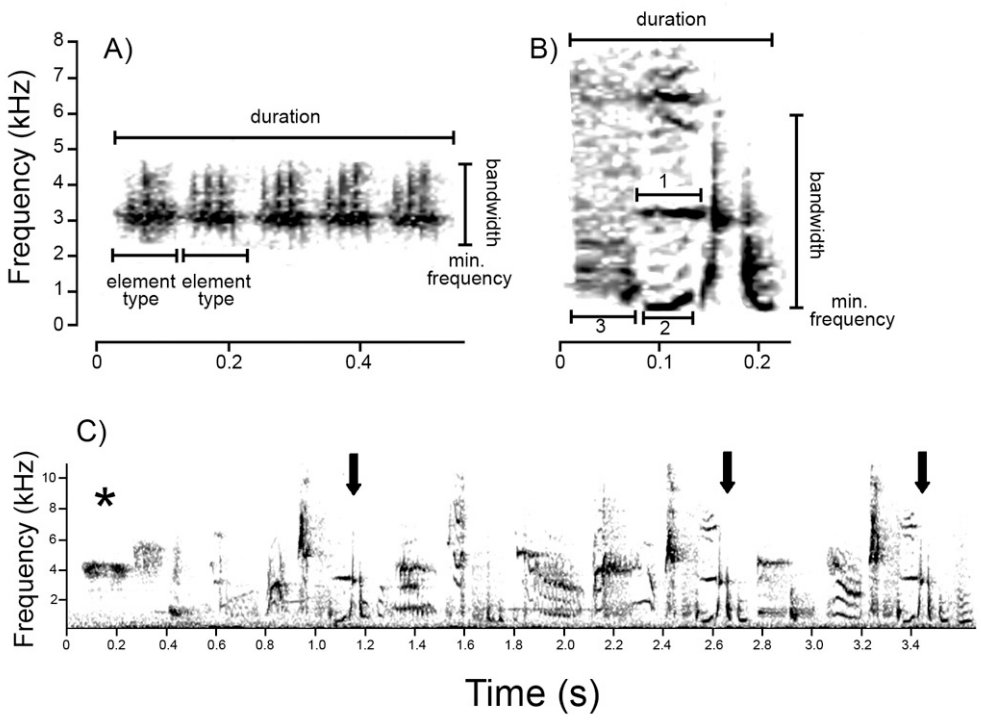

Figure 1. Measurements taken from the A) introductory, and B) shared syllable emitted by every recorded individual. Numbers in B) refer to three elements where the same measures as the complete syllable were taken. A fragment of a song bout is shown C) indicating the introductory syllable with an asterisk and the shared syllable with arrows.

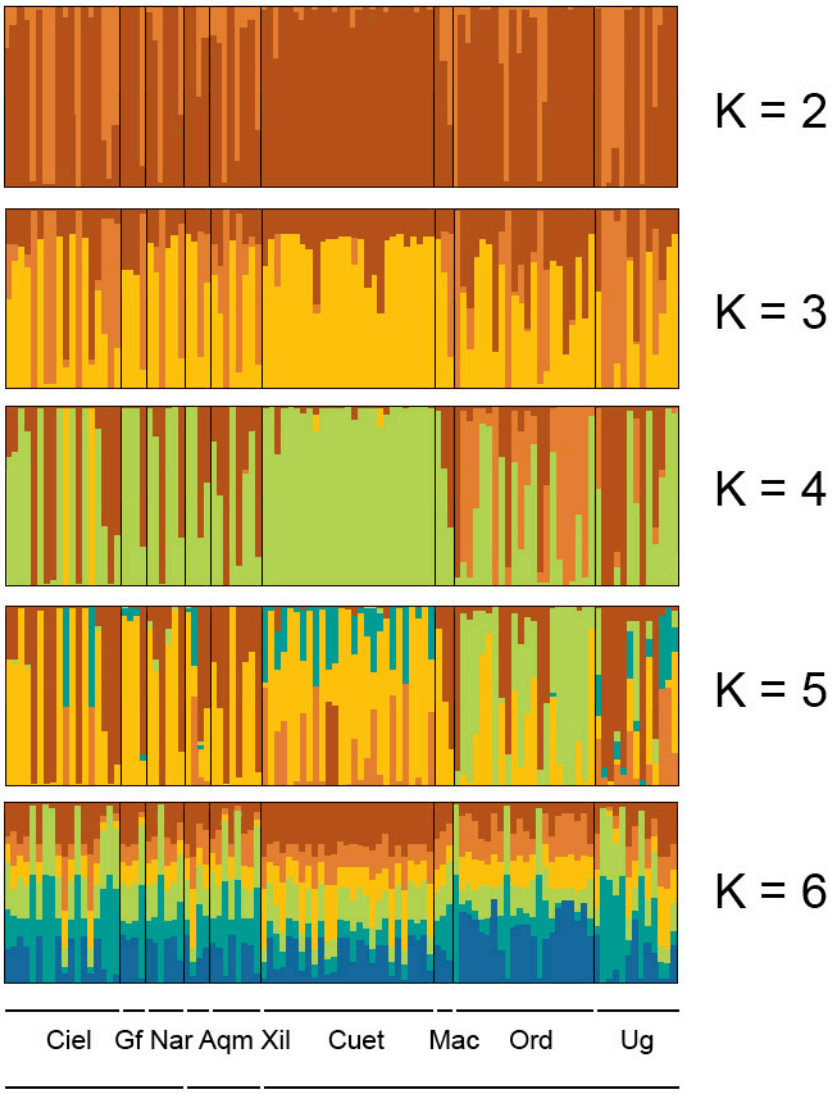

Figure 2. Geographic patterns of genetic structure based on Bayesian assignment analysis in STRUCTURE. Posterior assignment probabilities of 105 individuals of *C. curvipennis* show a weak genetic structure, with an optimal number of *K* = 2, although clusters from *K* = 2 to *K* = 6 are shown. Each individual is represented by a vertical line that is partitioned into *K* colored sections, with the length of each section proportional to the estimated membership coefficient.

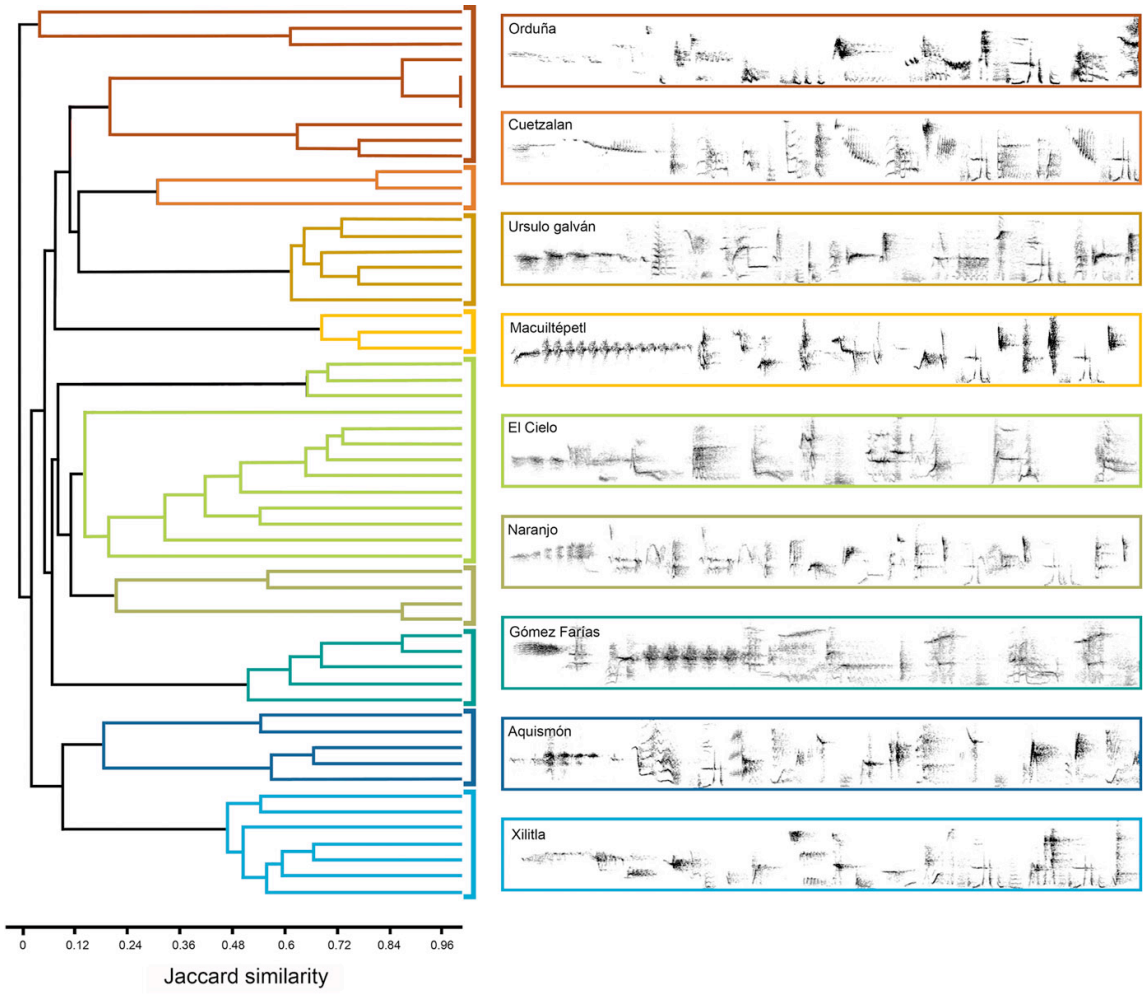

Figure 3. Dendrogram generated by cluster analysis of a presence/absence matrix of syllable types from individuals recorded. Each of nine leks is located in a different cluster and represented by a different colored line. Green, blue and red-orange lines correspond to leks from the north, central and south part of the Sierra Madre Oriental respectively (nSMO, cSMO, and sSMO). Attached to the dendrogram, 4 sec fragments of vocalizations representing syllable variation of each lek are shown.

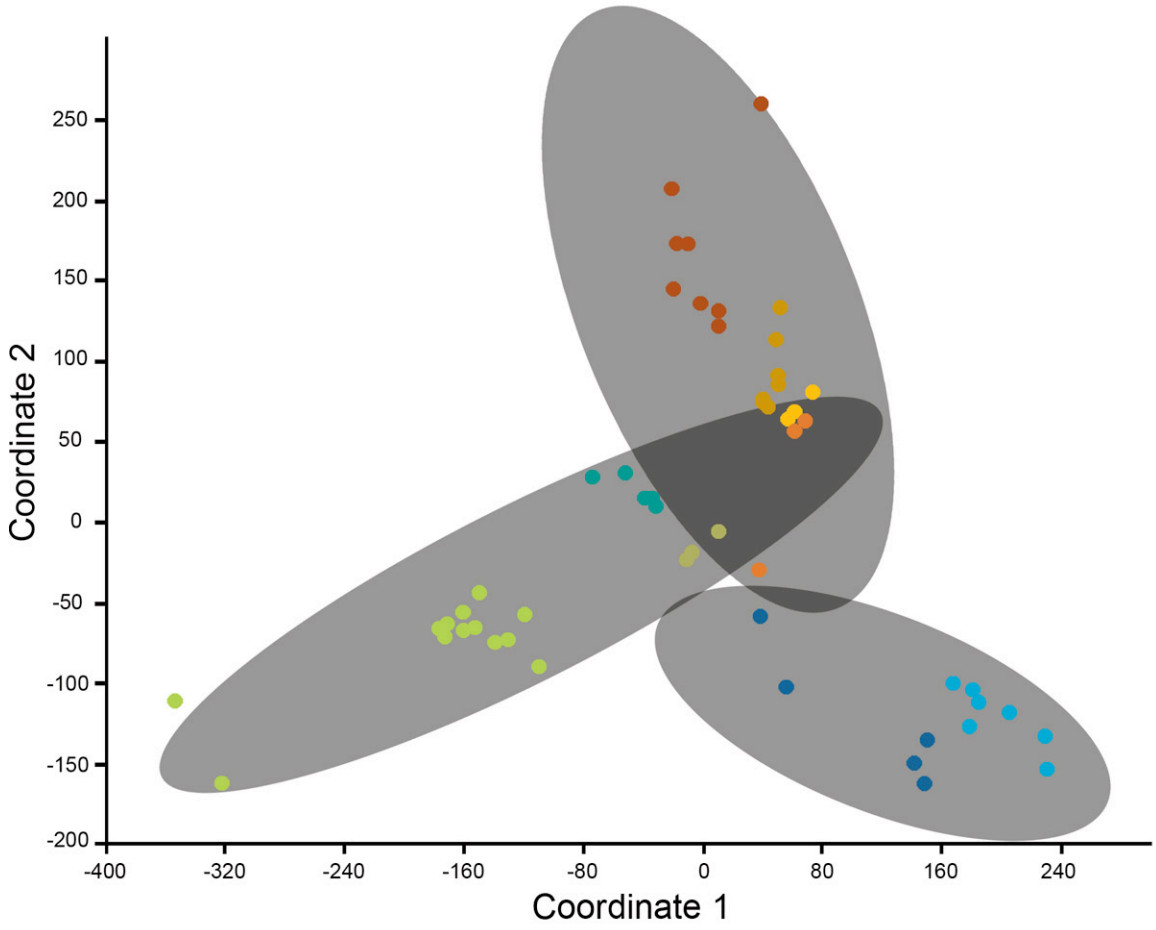

Figure 4. Plot of the first two principal coordinates of acoustic variation based on the binary presence/absence matrix of syllable types. Individuals from each lek are in separate clusters (indicated by circles with the same colors as dendrogram). A clear separation between leks from the northern, central and southern part of the distribution is also shown (ellipses).

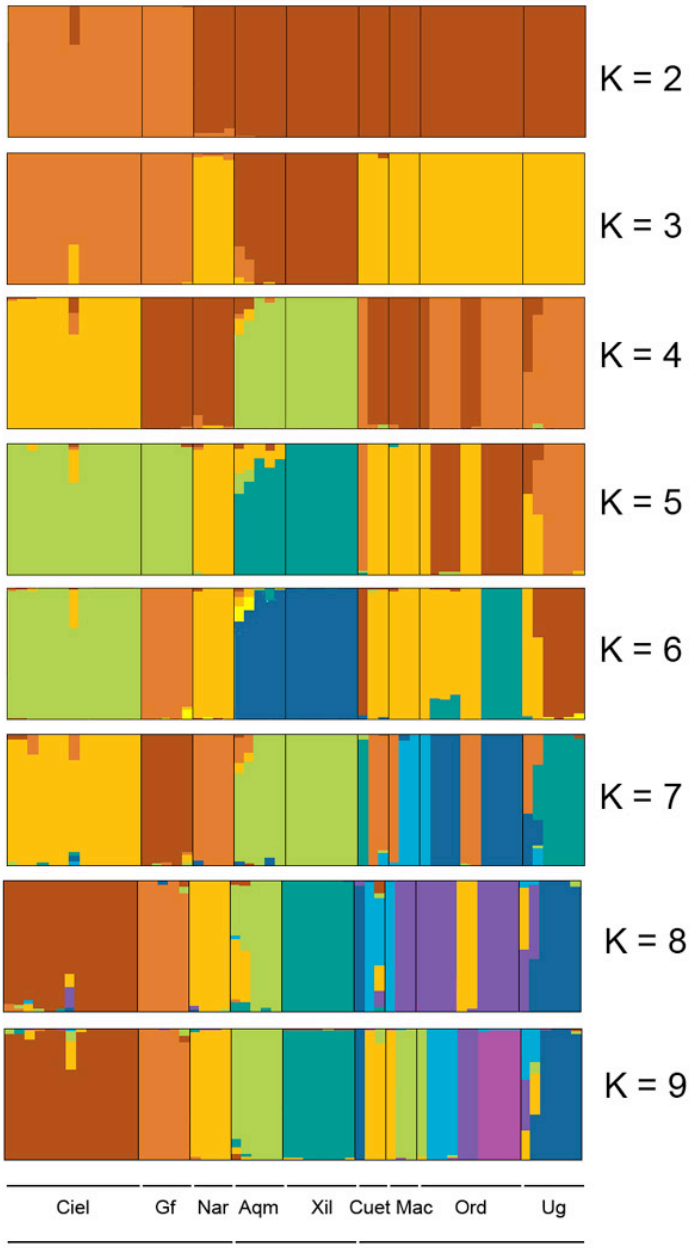

Figure 5. Geographic patterns of acoustic structure based on Bayesian assignment analysis in STRUCTURE. Posterior assignment probabilities of 56 individuals of *C. curvipennis* show a strong acoustic structure with the maximum probability at $K = 7$, although clusters from $K = 2$ to $K = 9$ are shown. Each individual is represented by a vertical line that is partitioned into K colored sections, with the length of each section proportional to the estimated membership coefficient.

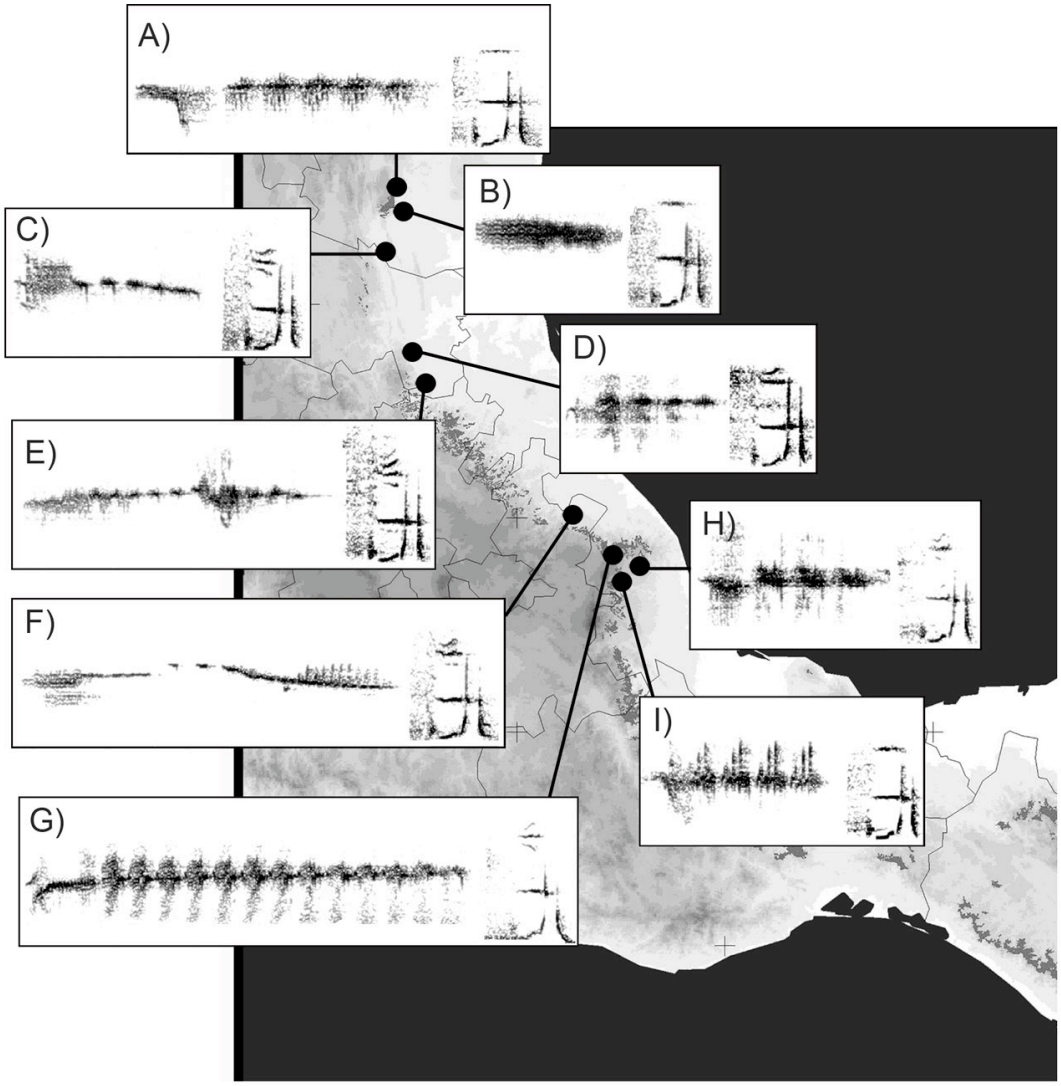

Figure 6. Spectrograms of introductory and shared syllables placed on to a map of the geographic distribution of leks. Both syllables are typical from each lek, most notably the introductory syllable, although the 70 and 82% of the individuals were correctly classified in their lek of origin accordingly to measurements from the introductory and shared syllables, respectively.

CAPÍTULO III

Song variation and persistence of song neighborhoods in a lekking hummingbird

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SONG VARIATION AND PERSISTENCE OF SONG NEIGHBORHOODS IN A LEKKING HUMMINGBIRD

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Abstract. Recent studies reveal that hummingbirds' songs are more variable and complex than previously realized. The Wedge-tailed Sabrewing (*Campylopterus curvipennis*) forms leks composed of small adjacent territories defended individually from intruders. When females visit these territories, males emit long songs composed of structurally complex syllables. Their elaborate acoustic signals are intriguing because most species of lekking hummingbirds studied in detail have relatively simple and stereotyped songs. Here we report song variation among territorial males at one lek over 4 years. Despite variation in syllable composition, cluster analyses classified songs of territorial males into three groups ("song neighborhoods"), and the pattern of clustering was consistent over time. Song neighborhoods were also clustered spatially. The syllabic composition of songs from members of a given neighborhood was relatively constant from year to year, suggesting site and territory fidelity, and introductory syllables were emitted consistently as the signature of a group. Our findings demonstrate the existence (and persistence) of song neighborhoods in a lekking hummingbird. This spatial structure might result from reduced competition among males to occupy a territory for singing, kinship between members of a neighborhood, and/or as a consequence of sexual selection. Therefore, documentation of song neighborhoods should be considered more often in future studies of song variation in hummingbirds that breed in leks.

Key words: Campylopterus curvipennis, *dialects*, *dialect persistence*, *lek*, *syllable composition*, *temporal variation*; *Wedge-tailed Sabrewing.*

Variación en el Canto y Persistencia de Vecindarios Vocales en un Colibrí que Forma Asambleas de Cortejo

Resumen. Estudios recientes han revelado que los cantos en colibríes son más variables y complejos de lo que se pensaba anteriormente. En *Campylopterus curvipennis* se forman asambleas de cortejo compuestos por pequeños territorios adyacentes defendidos individualmente de los intrusos. Cuando las hembras visitan estos territorios, los machos emiten cantos largos compuestos por sílabas estructuralmente complejas. Sus señales acústicas elaboradas son interesantes porque la mayoría de las especies de colibríes que forman asambleas que han sido estudiadas con detalle tienen cantos relativamente simples y estereotipados. Aquí reportamos la variación del canto entre machos territoriales en una asamblea de cortejo durante cuatro años. A pesar de que la composición de sílabas fue muy variable, los análisis de agrupamiento clasificaron los cantos de los machos territoriales en tres vecindarios vocales y el patrón de agrupamiento fue consistente en el tiempo. Los vecindarios vocales también se agruparon espacialmente. La composición de las sílabas de los cantos de miembros de los vecindarios fue relativamente constante entre años, lo que sugiere fidelidad al sitio y a los territorios, y las sílabas introductorias fueron consistentemente emitidas como señales de grupo. Nuestros resultados demuestran la existencia (y persistencia) de vecindarios vocales en una asamblea de cortejo de una especie de colibrí. Esta estructura espacial podría ser el resultado de una competencia reducida entre machos para ocupar un territorio de canto, de ayuda entre miembros de un vecindario vinculados por parentesco o de la selección sexual. Por lo tanto, la documentación de la existencia de vecindarios vocales debe ser considerada en estudios futuros de variación en el canto en colibríes que forman asambleas.

INTRODUCTION

Microgeographic song variation has been documented in some species of lekking and nonlekking hummingbirds (Snow 1968, Vielliard 1983, Gaunt et al. 1994, González and Ornelas 2005, Yang et al. 2007). These studies addressed leks or populations separated by few or several kilometers, and most of them focused on song variation as a consequence of song learning in hummingbirds. Among lek-breeding hummingbirds, song neighborhoods (within leks) have been rarely documented (Wiley 1971, Stiles and Wolf 1979). In the hermits *Phaethornis superciliosus* and *P. longuemareus* acoustic differences among song neighborhoods are slight because, as in most lekking hummingbirds studied, these hermits have monosyllabic calls or songs with few syllables repeated stereotypically (Wiley 1971, Stiles and Wolf 1979). Nevertheless,

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acoustic variation in hummingbirds includes differences in the structure of songs (Wiley 1971, Gaunt et al. 1994, Pizo and Silva 2001, Ferreira et al. 2006), variability of syllable types (Yang et al. 2007), or differences in the frequency of harmonics (MacDougall-Shackleton and Harbison 1998).

One of the consequences of song learning is that dialects may arise if birds learn their songs where they (later) breed. When members of a group share features of their vocalizations and these features differ discretely (in structure) from vocal characteristics of other groups, the shared acoustic elements are called a dialect (Marler and Tamura 1962). The term dialect is commonly used for macro- or microgeographic scales of vocal variation among populations (Baker and Cunningham 1985) in which a whole population shares a dialect with well-defined boundaries (Mundinger 1982). Dialects of varying sizes have been described in many passerines (reviewed by Mundinger 1982) and a few nonpasserines (e.g., Wright 1996, Wright and Wilkinson 2001, Bradbury et al. 2001, Vehrencamp et al. 2003, Wright et al. 2005). However, the existence of dialects has rarely been reported from areas as small as a lek, where selection on acoustic divergence would act at the level of the individual (Slater 1986). In this context the term "song neighborhood" is better because connotes song variation among subgroups within a geographically distinct population, whereas the dialect is a population-level phenomenon (Podos and Warren 2007).

Documenting the persistence of dialects or song neighborhoods, i.e., whether most individuals sing songs with the same acoustic structure from year to year, is an important first step to understanding the dynamics of social interactions (among leks) and the cultural evolution of songs (Podos et al. 2004). Variation across time in acoustic signals has not been analyzed thoroughly in nonpasserine birds (Lengagne 2001). In some passerines rates of song modification over time in terms of structure and temporal and frequency patterns of elements are high (e.g., Borror 1965, Trainer 1988, Forstmeier and Leisler 2004, Gammon and Baker 2004, Runciman et al. 2005), whereas in others they are low (e.g., Harbison et al. 1999, Kopuchian et al. 2004). Some long-term studies of dialect persistence, however, have focused on changes in the geographic distribution of dialect areas (Harbison et al. 1999) rather than on temporal changes in song structure within and among dialects (Nelson et al. 2004, Baker and Gammon 2006, 2008).

The Wedge-tailed Sabrewing (*Campylopterus curvipennis*) is a hummingbird monomorphic in plumage but dimorphic in size. It is common in montane cloud and tropical forests in eastern Mexico and the Yucatan Peninsula (Howell and Webb 1995). During the breeding season males congregate in leks attended for several months (from January to June), and it is common to find leks every year in the same locations. At our study location, the lek (~2500 m^2) is composed of approximately 50 individually defended territories (10–20 m^2), although at other locations the number of established territories is lower (C. González, pers. obs.). Individual territories are established in close proximity (2–5 m between perches) in dense second growth (González and Ornelas 2005). When advertising to females, males usually perch on exposed twigs in their territories, emitting just the introductory syllable of the song and rapidly moving their heads from side to side. When females visit these territories, males leave their perches to get into the dense vegetation, and both males and females perform short zigzag flights in front of each other while males emit their song (González and Ornelas 2005). Occasionally, males emit the same song while flying around their territory and from perches. Territorial males also sing when other males trespass their territorial boundaries, the usual site of chases and physical contact.

Songs of the Wedge-tailed Sabrewing have a complex acoustical structure, rivaling those of some passerine birds with complex signals (e.g., Mundinger 1982, Leitner et al. 2001, Griessmann and Naguib 2002, Briefer et al. 2008). Their songs are loud, high-pitched (>8 kHz), and long sounds (8–10 sec) composed of more than 45 rapidly emitted discrete units (syllables) with a highly variable and elaborate acoustic structure (Fig. 1). Each male's songs varies from rendition to rendition.

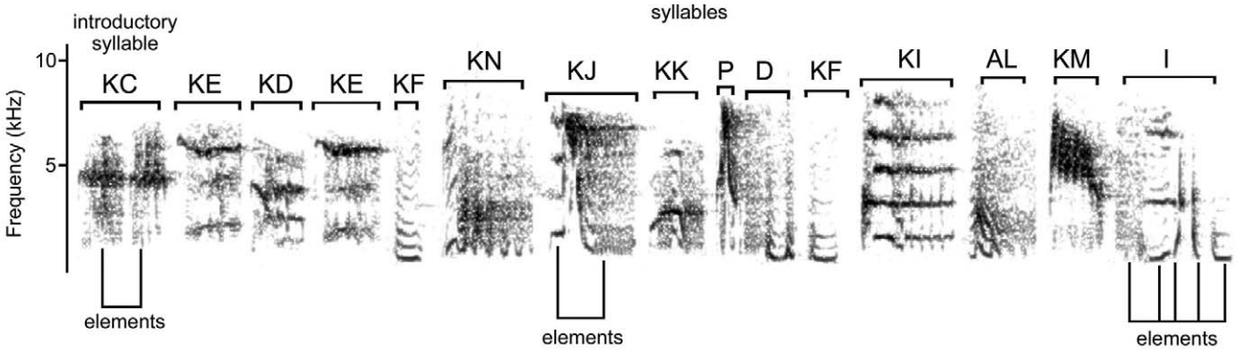

FIGURE 1. Spectrogram of a Wedge-tailed Sabrewing song fragment indicating the units of song production used in the analysis. Letter codes were assigned to identify syllables according to different traces on spectrograms and have no biological meaning.

Substantial vocal variation between groups of Wedge-tailed Sabrewings has been found on a microgeographic scale (10–20 km^2), individuals of the same locality sharing 50% of the syllables, individuals at different localities only 10% (González and Ornelas 2005). In this study we document the existence of song neighborhoods at one lek in central Veracruz, Mexico. We then compare the syllabic composition of their elaborate acoustic signals over time, discussing general implications for the adaptive significance of song neighborhoods, if any. Of the three avian groups clearly documented to have evolved the capacity to learn vocalizations (songbirds, parrots, and hummingbirds; Gahr 2000), hummingbirds are the least studied.

METHODS

SONG RECORDINGS AND GENETIC SEXING

Our study took place over 4 years at one lek in central Veracruz, Mexico, near the city of Coatepec (19°, 26′ N, 96°, 57′ W; 1150 m above sea level), in an area of tangled second growth near coffee and sugar-cane plantations. We mist-netted and color-marked males at the lek every year. Marks consisted of small colored circles made of sheets foam, attached with nontoxic fast-drying hard glue (Kola Loka, E. I. du Pont de Nemours and Company, Edo. de México) to the back of the sabrewings. Caught individuals were handled gently and were not harmed during mist netting and color marking. Marked individuals were observed defending territories over several weeks after manipulation; no birds died during the study. Each individual carried an exclusive color combination for further identification. We were able to locate, identify, and record the songs of 8 to 19 color-marked territorial males between April and June of each year (10 in 2004, 8 in 2005, 12 in 2006, and 19 in 2007). Because banding the hummingbirds was not feasible, and color marks are not permanent, it was not possible to know if same individuals occupied the same territories every year. For this reason we consider the marked individuals as 49 samples rather than as 49 individuals. Most of the recordings were made in the same territories each year, however, sample sizes each year varied because some of the territories were not easily reached because of the density of the vegetation.

To verify that singing territorial individuals were males, we sexed the marked individuals genetically with primers 2550F (Fridolfsson and Ellegren 1999) and MSZ1R (Sehgal et al. 2005). With this pair of primers, two bands of DNA are expected for females, one for males, because female birds are heterogametic (ZW) and males are homogametic (ZZ). We had successful amplifications of the W chromosome for known female sabrewings only (ca. 400 bp), as observed for other species of hummingbirds (Chaves et al. 2007, pers. comm.). To confirm that the lack of amplification in samples from marked individuals was because they were males and not because of poor DNA quality, we used a second pair of primers that amplifies the first domain of the control region of the Wedge-tailed Sabrewing specifically (ca. 500 bp; González et al., unpubl. data.). We extracted DNA from tail feathers by a standard method with Chelex (5%) and amplified it by the polymerase chain reaction (PCR) according to standard protocols. PCR reactions were performed in a 2720 thermal cycler (Applied Biosystems) under the following conditions: an initial denaturing step at 94 °C for 5 min was followed by 35 cycles of 94 °C for 1.5 min, 45–48 °C for 1 min, 72 °C for 1.5 min, and a final run of 72 °C for 7 min. PCR products were separated by electrophoresis in a 1% agarose gel stained with ethidium bromide. Successful amplifications of the control region of all recorded individuals confirmed they were male.

Vocalizations of marked territorial males were tape recorded approximately twice a week with a digital Tascam DA-P1 tape recorder and a Sennheiser MKH-70 shotgun microphone. Spectrograms of recordings of all individuals were generated with Canary 1.2.4 (Charif et al. 1995). Recordings were digitized at a sampling rate of 44 100 Hz and stored as 16-bit samples. Spectrograms were produced with a 349.7-Hz filter bandwidth and a frame length of 512 points (= 11.6 msec). The temporal and frequency-grid resolution of the spectrograms were 64 points (= 1.45 msec) and 2.69 Hz, respectively. For analyses we used 600 songs (149 in 2004, 107 in 2005, 146 in 2006, and 198 in 2007). In most cases we obtained 15 full songs per individual (range 5–20).

ACOUSTIC AND STATISTICAL ANALYSES

In birds with complex and variable songs, such as the Wedge-tailed Sabrewing, it is difficult to choose an appropriate level for comparisons of acoustic structure among individuals. In this case, proper identification of minimal units of song production (syllables) may be necessary to measure the repertoire for comparison. For this reason, González first categorized syllables visually by structure on printed spectrograms and assigned a letter code to each syllable (Fig. 1). A syllable was defined as an element or several elements always grouped together in a fixed composition. The visual inspection of spectrograms and visual classification of syllable types were done without knowledge of the outcome of the cluster analyses (see below). The classification of syllable types is comparable to the approach taken in previous visual classifications of acoustic signals (e.g., Searcy et al. 1985, Podos et al. 1992). Classification was not difficult, as syllables differ consistently in their acoustic structure in obvious ways. We believe that our sampling of syllable types each year was reasonably complete. In previously studied leks of this species the repertoire of an individual male reached an asymptote of 30–40 syllable types with 10–15 sampled songs, a sample size and syllable repertoire similar to ours (see Results).

To document the existence of song neighborhoods and their persistence over the years, we performed hierarchical cluster analyses based on a matrix of syllable types for each of 49 cases, where each entry consists of the relative frequency of 103 syllable types. A separate cluster analysis was done on

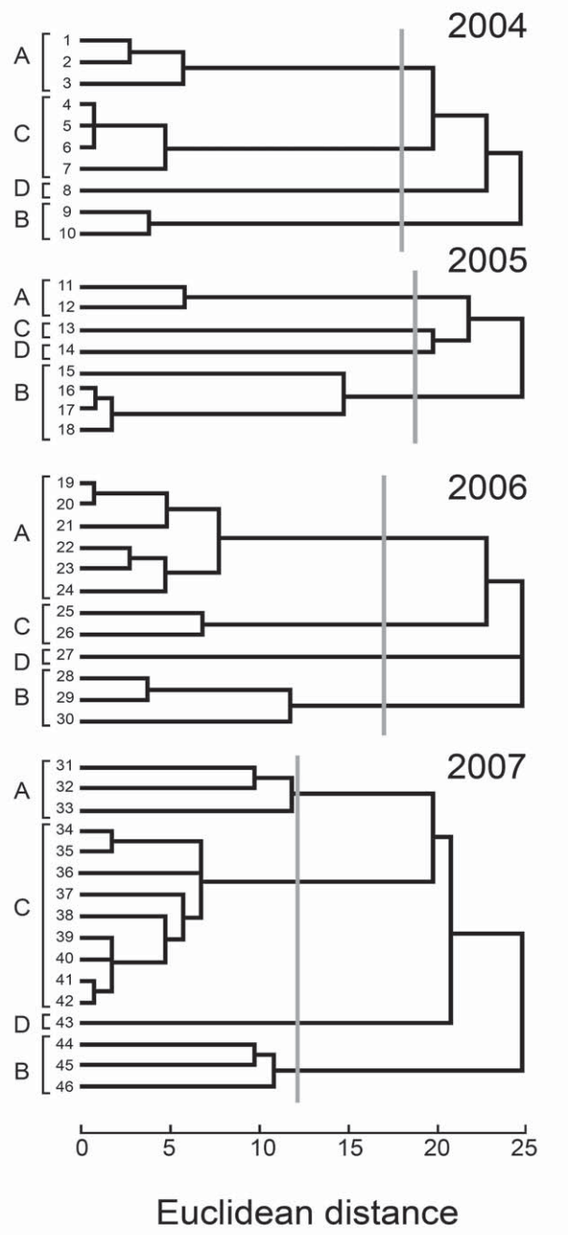

FIGURE 2. Dendrograms generated by cluster analysis of the relative frequency of syllable types from recordings of individuals by year. Syllable types were classified into song neighborhoods (indicated by letters to the left) by determining the clustering level at which the moat index reached its maximum (indicated by vertical gray lines). The moat index was calculated at multiple levels of clustering, verifying the existence of three clusters and one outlier. The values of the moat index gave a cutoff point out of the Euclidean distance range in the dendrograms, except in 2007, where the moat index peaked at two clusters, regardless of the clear existence of more clusters beyond that point. Three individuals were eliminated from dendrogram construction in 2007 because of small sample size ($n < 5$ songs per individual). Lines and numbers indicate individuals (although same individuals could be represented in more than one year).

the individuals recorded in each year (10 in 2004, 8 in 2005, 12 in 2006, and 19 in 2007). In constructing the dendrograms we used the unweighted pair-group method of arithmetic averages (UPGMA) and the Euclidean distance measure. Because the determination of clusters might be considered arbitrary, the numbers of clusters suggested by the cluster analyses were identified with an independent variable. We calculated "moat" indices (Wirth et al. 1966) for all possible levels of clustering of syllable types from each dendrogram, where the moat index describes the degree to which cluster groups are separated from each other. Podos et al. (1992) detailed calculation of the moat index further. Our assignments of individuals to song neighborhoods each year corresponded to the level of clustering at which the moat index reached its maximum value. We used SPSS version 11.0.2 (SPSS 2002) for all statistical analyses.

RESULTS

We detected a total of 103 syllable types in 600 songs of 49 samples (at least 19 different individuals) made at the lek over the 4 years. On average, each individual (sample per year) had 36 syllable types in its repertoire (range 27–45). The composition of the syllables, however, varied greatly, both within one male's repertoire and from male to male.

Within the lek, individuals clustered in three song neighborhoods, and the clustering pattern was consistent over the 4 years of the study (Fig. 2, 3). Every year, the moat indices

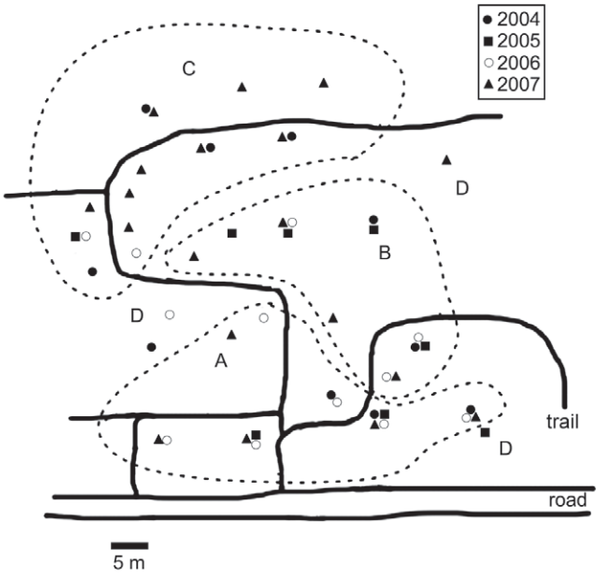

FIGURE 3. Spatial distribution of 49 samples of displaying males recorded at one lek over 4 years. Areas defined with dashed lines correspond to song neighborhoods defined by cluster analyses. Symbols close to each other were presumably of the same individual recorded in the same territory in multiple years. The four points outside these areas represent an outlier (labeled as D), probably the same individual recorded at different locations each year. Solid black lines represent the main road and trails.

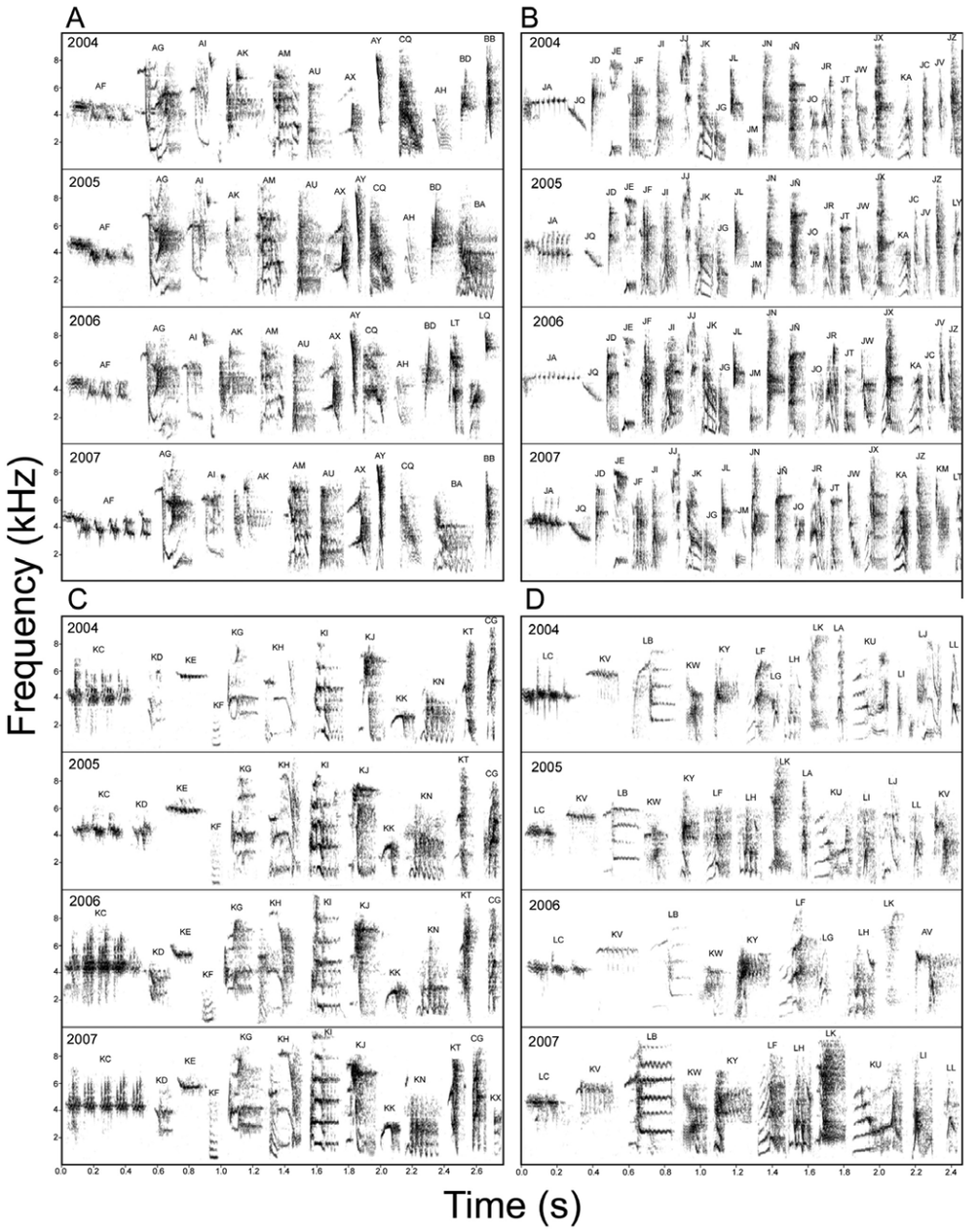

FIGURE 4. Composite spectrograms representing syllables found exclusively in song neighborhoods by year. Within each spectrogram, all syllables were taken from the song of one individual. Introductory syllables (AF, JA, KC, and LC) were also exclusive of each song neighborhood and constant over time.

revealed three clusters with one outlier, labeled D (Fig. 2). Based on song similarity, neighborhood associations were as follows: neighborhoods C and A clustered together (more similar), and this association with the outlier (D) formed another cluster. Finally, neighborhood B was the most distant cluster (most dissimilar). Our analysis of syllables within each cluster indicates that each neighborhood had basically the same syllable assemblage each year (Fig. 4).

The total number of syllables per song, the number of different syllable types per song, and the total number of syllables types recorded in a song neighborhood varied greatly by neighborhood and year (Table 1). Between 27% and 64% of

TABLE 1. Variation over time in acoustic characteristics of song neighborhoods of the Wedge-tailed Sabrewing. Numbers represent means ± standard deviations, with ranges in parentheses.

Song neighborhood and year	No. of syllables emitted in a song		No. of syllable types emitted in a song		No. of syllable types emitted in a song recorded in a neighborhood		Exclusive syllable types in a neighborhood per year
A							
2004	65 ± 27.8	(16–135)	26.4 ± 5.7	(12–35)	36.6 ± 1.7	(34–37)	35%
2005	49.2 ± 26.5	(19–117)	21.8 ± 6.5	(11–36)	35.5 ± 2.1	(34–37)	42%
2006	60.3 ± 23.2	(22–125)	27.3 ± 5.7	(14–41)	37.3 ± 3.3	(34–43)	45%
2007	56.2 ± 27.1	(16–119)	24.1 ± 5.6	(11–33)	33.8 ± 3.9	(27–37)	38%
B							
2004	51.5 ± 23.2	(25–134)	25.5 ± 4.3	(18–37)	39 ± 0	(39–39)	61%
2005	59.5 ± 30.3	(18–149)	26.4 ± 6.1	(13–38)	37 ± 4	(31–39)	64%
2006	69.5 ± 31.4	(19–133)	29.7 ± 6.1	(17–38)	39.3 ± 0.6	(39–40)	63%
2007	46.5 ± 23.7	(18–111)	23.6 ± 5	(14–33)	38.2 ± 6.5	(30–44)	57%
C							
2004	57.6 ± 29.7	(13–131)	25 ± 6	(12–34)	34 ± 0	(34–34)	38%
2005	67.2 ± 10.2	(55–79)	28.2 ± 2.2	(25–30)	34 ± 0	(34–34)	32%
2006	59 ± 24.5	(14–91)	25 ± 7.6	(10–34)	34.5 ± 0.7	(34–35)	28%
2007	58.7 ± 27	(19–168)	24.5 ± 5.1	(12–33)	33.4 ± 0.9	(32–35)	35%
D							
2004	58.6 ± 27	(28–125)	27.1 ± 6.4	(18–39)	43 ± 0	(43–43)	37%
2005	74 ± 31.5	(24–146)	31.7 ± 6.4	(17–42)	45 ± 0	(45–45)	37%
2006	56.5 ± 37.5	(30–83)	27 ± 7.1	(22–32)	34 ± 0	(34–34)	38%
2007	68.7 ± 31	(23–124)	28.3 ± 8.3	(13–41)	44 ± 0	(44–44)	27%

syllables were recorded in only one neighborhood in a given year, and song neighborhood B had the highest proportion of unique syllables (Table 1). Song neighborhoods were not static over time because new local or foreign syllables were incorporated each year, and apparently some syllables went extinct. Members of a neighborhood incorporated in their repertoires on average 2.4 (range 0–5) new local syllables per year (syllables new to a song neighborhood but previously present in another neighborhood) and 0.9 (0–6) new foreign syllables per year (syllables new to a song neighborhood not previously recorded in other neighborhoods). On average 2.8 (0–7) syllables recorded in one year were not recorded in subsequent years. Interestingly, through the study, song neighborhoods shared not only a large number of syllable types but also the same introductory syllable (Fig. 4).

DISCUSSION

SONG NEIGHBORHOODS AND PERSISTENCE OVER TIME

The elaborate and variable acoustic signals of the Wedge-tailed Sabrewing are intriguing because most lekking hummingbirds studied in detail have relatively simple and stereotyped songs (Snow 1968, Wiley 1971, Stiles and Wolf 1978, Atwood et al. 1991, MacDougall-Shackleton and Harbison 1998, Pizo and Silva 2001). Individual differences found in the songs of other lekking hummingbirds are variation in the structure of a single modulated note in *Eupetomena macroura* (Pizo and Silva 2001), the harmonic frequency of monosyllabic songs in *Phaethornis guy* (MacDougall-Shackleton and Harbison 1998), and in the use of song types in *Phaethornis superciliosus, Colibri coruscans*, and *C. thalassinus* (Stiles and Wolf 1979, Gaunt et al. 1994). Although songs of male Wedge-tailed Sabrewings are highly variable in syllable composition, the proportion of syllable types used in the full song was useful in identifying neighborhoods.

The existence of song neighborhoods in a lek was evident in the variation among neighbors within a lek. The spatial segregation within the lek by distinctive vocal characteristics and the temporal persistence of syllable composition constitute to our knowledge the first report for a lekking hummingbird species with complex song. A high proportion of unique syllables and, as a result, low song sharing characterized song neighborhoods of the Wedge-tailed Sabrewing. Surprisingly, song neighborhoods were quite stable over time in terms of syllable composition and spatially aggregated in the lek every year, where territories and singing perches remained constant over time (Fig. 3). Despite our failure to identify individuals in successive years (color marks were lost during the annual molt), consistent occupancy of territories and perches from year to year by birds with identical syllable proportions provides some circumstantial evidence that the individuals were the same.

In a previous study we documented microgeographic song variation in the Wedge-tailed Sabrewing, comparing the songs

of groups of individuals from both leks and "feeding groups" (group of individuals defending flowers while singing) separated by no more than 20 km (González and Ornelas 2005). In that study we also recorded individuals from "feeding groups" for two consecutive years and found an inconsistency in syllable composition between the years, concluding that this species did not have geographically distinct dialects that persist over time. However, this conclusion should be taken with caution because in that study comparisons involved only individuals from "feeding groups" and recordings from leks were not analyzed for more than one consecutive year. It is likely that "feeding groups" were composed of different individuals each year. A less likely possibility is that the same individuals adopted another song type from year to year, which contradicts the consistency found in leks. The syllable composition of song neighborhoods might be affected over time by invasion and/or extinction of syllables from local or foreign dialects. Because we found that changes in syllable composition (adopted new syllables or extinctions) were relatively few and discrimination of song neighborhoods over time was possible, this culturally transmitted signal may change slowly.

Although individual differences in syllable-type composition exist, only members of each song neighborhood shared introductory syllables, and this pattern was consistent over 4 years (Fig. 4). In a study of the White-crowned Sparrow (*Zonotrichia leucophrys*), Soha and Marler (2000) showed that the introductory syllable served as a cue for selective song learning and for facilitating learning of the species' song. In the Wedge-tailed Sabrewing, the constancy of introductory syllables among members of a neighborhood suggests that these signatures may play a role in neighbor–neighbor and neighbor–stranger discrimination (see also Briefer et al. 2008).

ADAPTIVE EXPLANATIONS FOR THE FUNCTION OF SONG NEIGHBORHOODS

The mechanism of formation of song neighborhoods in Wedge-tailed Sabrewing leks is uncertain. The song variation observed (González and Ornelas 2005, this study) implies that the song of this species is learned and thus that song neighborhoods may be maintained by juveniles copying the song type of their neighbors as they settle within a lek. For the function of song neighborhoods we suggest the following adaptive explanations for Wedge-tailed Sabrewing leks. First, singing territories are gained through the vocal imitation of an established and successful member of the lek (Payne 1982), allowing individuals to adapt to each other in an immediate social context. Second, song neighborhoods are determined by kinship among members, i.e., leks are composed of clusters of related kin (Höglund et al. 1999, Shorey et al. 2000, Francisco et al. 2007; but see Gibson et al. 2005, Loiselle et al. 2007). Third, the elaborate or complex vocal features that challenge males' developmental or performance capacities or that enable increased precision in communication in male–male interactions (reviewed by Podos and Warren 2007) may be the consequence of strong sexual selection, in which song elaboration replaces plumage characteristics as the target of sexual selection (Darwin 1871). Investigation into whether acoustic similarity of neighboring male Wedge-tailed Sabrewings is correlated with their relatedness is warranted.

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CAPÍTULO IV

Low levels of relatedness within wedge-tailed sabrewings leks and song neighborhoods

ABSTRACT

Leks are male aggregations visited by females for the only purpose of mating. The social courtship displays of lekking birds are though to be the result of strong sexual selection, and because of these selection pressures that males are subject to, only one or few individuals obtain most copulations. This skewed reproductive success leads unsuccessful males to join related successful males to increase their inclusive fitness because their genes would be transmitted indirectly to the next generation. Kin selection theory therefore predicts that males might be more related within leks than males between leks. The wedge-tailed sabrewing (*Campylopterus curvipennis*) is a hummingbird species that congregates at leks where males perform elaborate singing displays to females. Their complex songs exhibit marked differences between neighboring males within a lek, forming song neighborhoods spatially clustered according to their introductory syllable and syllable composition. We used allele frequencies at 10 microsatellite loci to investigate the potential for kin selection in wedge-tailed sabrewings leks by estimating pairwise relatedness among males from one focal lek over a 3-year period, and compared the results with those from five leks located along the distribution range of the species. We also asked if the vocal variation observed within the focal lek (song neighborhoods) is related with kinship using previously described data of song similarity. We found low levels of relatedness and a high variance of the observed mean relatedness within leks, which suggests that both related and unrelated males are displaying together. We also found no evidence for local clustering of kin within song neighborhoods, where males are equally related than males between neighborhoods. Our results indicate that kin selection might not be important in this hummingbird species, and that juveniles attempting to join a lek learn the song of their fathers or relatives, and settle close to them, is unlikely.

INTRODUCTION

Males of several bird species congregate at leks to perform mating displays and females visit for the only purpose of mating (Höglund and Alatalo 1995). The formation of leks offers favorable conditions for sexual selection because mate choice would not be influenced by material resources such as food, nests, or territories (Payne 1984). Because sexual selection can promote the evolution of different male traits in different populations of the same species, speciation events can occur rapidly (Lande 1980) leading to the diversification of bird families that exhibit lekking behavior. Males of lekking species have often conspicuous ornaments or behaviors that must be the target of female choice, involving male-male competition (Anderson 1994). For example, some of the male traits selected by female grouses are the display rate, lek attendance, call parameters and rate of production (Gibson and Bradbury 1985, Kruijt and de Vos 1988, Alatalo et al. 1991); the length of head ornaments, the probability and rate of displays, and male dominance in birds of paradise (LeCroy et al. 1980, Pruett-Jones and Pruett-Jones 1991); and in manakins the display rate, dance quality (McDonald 1989), and mechanical sounds during displays (Fusani et al. 2007).

Group displays have been shown to increase the encounter rate of males with females in several species (Bradbury 1981, Bradbury et al. 1986, Beehler and Foster 1988), however, mating distribution within leks is generally skewed due to the strong sexual selection pressures that males are subject to; only one or few individuals obtain most copulations, whereas most of them do not or get copulations in a lower frequency (Höglund and Alatalo 1995, Semple et al. 2001). According to kin selection theory, this biased reproductive success lead non-preferred males to display joined with preferred related males to increase their inclusive fitness, so that the non-preferred males would transmit their genes to the next generation because they share a proportion of their genome with the preferred male due to their kinship (Kokko and Lindstrom 1996, Höglund et al. 1999). Kin selection therefore predicts that males within leks might be more related than males between leks (Kokko and Lindstrom 1996).

Inclusive fitness benefits mediated through kin selection have been suggested to be involved in the evolution of lekking behavior (Kokko and Lindstrom 1996, Höglun et al. 1999, Petrie et al. 1999). Some studies have described that lekking males are related in some species of grouses and peafowls (*Tetrao tetrix*, Höglund et al. 1999, Lebigre et al. 2008; *T. urogallus*, Regnaut et al. 2006, Segelbacher et al. 2007; *Tympanuchus pallidicinctus*, Bouzat and Johnson 2004; *Pavo cristatus*, Petrie et al. 1999; *Meleagris gallovapo*, Krakauer 2005), and manakins (*Manacus manacus*, Shorey et al. 2000, Höglund and Shorey 2003), whereas other studies have found that lekking males are not related in other species of grouses (*Centrocercus urophasianus*, Gibson et al. 2005), manakins (*Chiroxiphia linearis*, McDonald and Potts 1994; *Pipra filicauda*, *P. pipra*, *Lepidothrix coronata*, *Chiroxiphia pareola*, Loiselle et al. 2007), and bowerbirds (*Chlamydera maculata*, Madden et al. 2004). Leks have been described in at least 14 bird families and it is thought that lekking behavior has evolved independently (Höglund and Alatalo 1995), however, detailed studies describing the potential for kin selection have been concentrated in Phasianidae and Pipridae but not Trochilidae in which *c.* 30 of 320 extant hummingbird species are known as lek breeders (Snow 1968, Stiles and Wolf 1979, Payne 1984, Atwood et al. 1991, Bleiweiss 1997, Pizo and Silva 2001, González and Ornelas 2005).

In cooperative-breeding systems, due to the apparent acquisition of indirect fitness benefits, it has been suggested that there must be some recognition mechanism between kin and non-kin group members (Komdeur and Hatchwell 1999). The possibility that some kind of kin-recognition mechanism plays an active role in the spatial distribution of relatives within leks and not simply a process produced by absence of natal dispersal, has been suggested in *Pavo cristatus* (Petrie et al. 1999) and *Manacus manacus* (Shorey et al. 2000). The most likely recognition mechanism between both juvenile floaters and established territorial males before settling on a lek, might be some kind of phenotype matching, which involves the learning and assessment of phenotypes (e.g. vocalizations, plumage, size, dominance) of particular individuals (Komdeur and Hatchwell 1999, Hauber and Sherman 2001). Vocal variation, in

particular, could play a role as a “family distinctive” trait allowing recognition of individuals with various degrees of kinship (Krebs and Kroodsma 1980). Nevertheless, encounter and interaction probabilities between relatives imply that dispersal might be limited at some point. Lekking behavior can promote male philopatry trough kinship selection (Kokko and Lindström 1996, Höglund et al. 1999), however, intersexual and parent-offspring competition can cause male bias dispersal. Therefore, dispersal evolution it the result of complex interactions between factors such as inbreeding, kinship, and dispersal abilities.

Wedge-tailed sabrewing, *Campylopterus curvipennis,* is one of the hummingbird species whose males congregate at leks performing elaborate singing displays to females. Detailed studies of the vocalizations of this species have determined geographical variation, most notably in the introductory syllable and the syllable repertoire between lek members (González and Ornelas 2005, González and Ornelas, unpublished data). Besides geographic variation among leks, we have described noticeable within-lek according to their introductory syllable and syllable composition, with a spatial male clustering pattern of four song neighborhoods consistent from year to year (González and Ornelas 2009). The observed geographic patterns of song divergence are only partially explained by drift, which suggests a role of social selection in promoting song convergence within leks and sexual selection in promoting vocal novelty and remarkable song variation among leks (González et al. 2011, González and Ornelas, unpublished data).

In this study we investigate the potential for kin selection to operate in leks of wedge-tailed sabrewings by estimating relatedness between lekking males, and ask if the observed vocal variation within a lek (song neighborhoods) is associated with kinship between territorial males. Because local levels of relatedness could vary among years (Piertney et al. 2008), we calculated relatedness between males one focal lek during three consecutive years, and then compared that with the relatedness values obtained from other leks. To address this, we used allele frequencies at 10 microsatellite loci and acoustic data on song neighborhoods described

in a previous study (González and Ornelas 2009). We expect that relatedness of males displaying on leks is higher within than among leks, and that the genetic substructure would correspond with vocal variation patterns in the lek (song neighborhoods). If wedge-tailed individuals use vocal features as a recognition mechanism between relatives, a relationship between kinship and vocal similarity is expected.

METHODS

Study system and sampling

Wedge-tailed sabrewing, *Campylopterus curvipennis*, is a sexually monochromatic, size dimorphic hummingbird species distributed along the Sierra Madre Oriental in eastern Mexico (Howell and Webb 1995). Males are polygynous and during the breeding season they congregate in leks performing elaborate singing displays to females. These male aggregations are characterized as a typical lek (Payne 1984) since their territories do not contain any resources required by females except the males themselves, and nearly all-foraging activity occurs away from the lek. Leks are attended for several months (from January to June), and in the study location the lek is composed of more than 50 individually defended territories (10–20 m^2) established in close proximity to each other in dense vegetation areas of second growth (González and Ornelas 2005), allowing acoustical contact between neighbouring territory owners. Males usually perch on exposed twigs emitting the introductory syllable of the song and rapidly moving their heads from side to side (González and Ornelas 2005). When females visit these territories, males leave their perches and both males and females perform short zigzag flights in front of each other in the dense vegetation while males emit their complex songs (González and Ornelas 2005). Territorial males also sing when other males trespass their territorial boundaries. Thus, their singing behavior apparently has both an intersexual (courtship display) and an intrasexual (male-male competition) function. There is evidence that in leks of hummingbirds *Phaethornis guy* and *P. superciliosis*, song output is related with the territory

position in such a way that individuals with a central position of the territory sings more and obtain more matings than the males with a peripheral territory (Stiles and Wolf 1979, MacDougall-Shackleton and Harbison 1998). Although there is not evidence of biased male reproductive success at leks of wedge-tailed sabrewings, we have observed more singing activity and, therefore, more courtship displays to females and territorial aggressions in territories situated at central positions on leks, than on those in the periphery of the lek.

Our study was mainly conducted at one focal lek (Orduña) located in central Veracruz, Mexico (19º, 26′ N, 96º, 57′ W; 1150 m above sea level), in an area of second-growth tangled vegetation near coffee and sugarcane plantations, where the study of song neighborhoods was performed. We mist-netted individuals at the lek during three consecutive years (2005-2007) between April and June, and collected two outer rectrices for genetic analyses. For further acoustic and relatedness comparisons we used recordings of 22 males, caught and color marked during the same dates (6 birds from song neighborhood A, 6 from B, 8 from C and 2 from D; González and Ornelas 2009). To allow comparisons with other leks, we used male genotypic data from five leks (Cielo, Naranjo, Xilitla, Cuetzalan and Ursulo Galván) located on the entire distributional range of wedge-tailed sabrewings (González et al. 2011).

Molecular sexing and microsatellite genotyping

DNA was extracted from the calamus of one of the feathers with a standard protocol with chelex 5% (Morin et al. 1994). Sex determination from individuals sampled was carried out in the laboratory by polymerase chain reaction (PCR) using primers 2550F (Fridolfsson and Ellegren 1999) and MSZ1R (Sehgal et al. 2005, see González and Ornelas 2009 for detailed amplification conditions). Samples were genotyped at 10 polymorphic microsatellite loci that were cloned specifically for *Campylopterus curvipennis* (Abdoullaye et al. 2010; GeneBank accession nos. GQ294539– GQ294550). PCR conditions and fragment sizing are fully described in Abdoullaye et al. (2010).

We estimated expected and observed heterozygosity and mean number of alleles per locus, and tested each locus for departures of Hardy-Weinberg equilibrium (HWE) and tested for linkage disequilibrium between pairs of loci using GENEPOP 3.4 (Raymond and Rousset 1995). We also tested for the presence of null alleles using MICROCHECKER 2.2.3 (Van Oosterhout et al. 2004).

Data analysis

To determine if male philopatry resulted in greater kinship within leks, we first determined patterns of sex biased dispersal. Because hummingbird banding was not feasible and color marks are not permanent (González and Ornelas 2009), we identified individuals with the same genotype obtained with microsatellites along the three years of sampling. Then, we estimated coefficients of relatedness (*r*) for pairs of males and females within years using the program KINGROUP v2 (Konovalov et al. 2004). Relatedness measures the proportion of alleles shared between individuals that are identical by descent (Queller and Goodnight 1989). Mean estimates of relatedness between males and females were compared with a one-way ANOVA using STATVIEW (Abacus Concepts, Inc).

To obtain an estimate of family structure within the Orduña lek over the three years of the study, we calculated the likelihood of pedigree relationships between pairs of individuals. Pedigree relationship refers to a particular category of genealogical relationship, such as parent-offspring, full-sib or half-sib. In ML-RELATE (Kalinowski et al. 2006) we calculated the likelihood of four pedigree relationships (parent-offspring, full-sib, half-sib and not-closely related). ML-RELATE calculates the likelihood of each relationship for each pair of individuals and shows a matrix with the relationship with the maximum likelihood for each comparison.

To investigate whether males attending leks were more related than expected by chance we first computed coefficients of relatedness (*r*) for pairs of males within the Orduña lek and years using KINGROUP v2, and then compared the samples mean to a null expectation of zero with a

t-test using STATVIEW. These estimates were also performed for the additional five leks. Estimations of male relationship within all leks were also made as described above. Both relatedness and relationship coefficients gives complementary estimates on kinship structure.

To establish if song neighborhoods are determined by kinship between members, i.e., song neighborhoods are composed of clusters of related kin, we compared estimates of relatedness between males established on territories within and between song neighborhoods. Then, we compared the samples mean to a null expectation of zero using a *t*-test, and performed a one-way ANOVA to determine within and between pairwise relatedness comparisons differences. To assess kinship structure between song neighborhoods, we performed a principal coordinates analysis on a genetic distance matrix generated from the genotypes of individuals belonging to each neighborhood as implemented in GENALEX 6.4 (Peakall and Smouse 2006). To determine if the acoustic similarity of neighboring males is correlated with their relatedness (r), we calculated the Jaccard similarity coefficient between pairs of birds based on a matrix of presence/absence of syllable types and correlated with pairwise relatedness using a simple Mantel test, assessing significance levels of association between matrices with 1000 randomizations.

RESULTS

Microsatellite analysis

The number of alleles per locus varied from 2 to 14. Values of observed heterozygosity did not deviate consistently from H-W equilibrium across sampled years, except for locus CACU4-6 and CACU13-2 after Bonferroni corrections (Table 1) due probably to the presence of null alleles as suggested by MICROCHECKER. To assess the possible influence of these null alleles we performed analyses with and without these loci and results were affected only by locus CACU13-2, therefore this loci were excluded from final analyses. No significant linkage

disequilibrium was detected in any of the population-loci comparisons after Bonferroni corrections.

Site fidelity

The number of birds caught during the three years of the study at Orduña was 126 (53 in 2005, 32 in 2006, and 41 in 2007). Out of these, 104 were males (44 in 2005, 24 in 2006, and 36 in 2007), and 22 females (9 in 2005, 8 in 2006, and 5 in 2007).

Comparing microsatellite genotypes of the total individuals caught, 25 were found to have the same genotype as other individuals captured the following years, indicating that these individuals were recaptured between years. Three male sabrewings were captured along the three years of study, 3 were captured in 2005 and then in 2006, 8 males and one female were captured in 2005 and latter in 2007, and finally 9 males and one female were captured in 2006 and then in 2007. Because our sampling effort was not the same between years, we cannot discard that individuals caught in 2005 and 2007 were present in 2006. In general these results indicate that males return each year to display at this lek, and in a much lower frequency females visit the same lek each year (only two recaptures). Mean genetic relatedness was slightly higher for males (mean ± SD r = -0.001 ± 0.2 in 2005; r = 0.04 ± 0.22 in 2006; and r = -0.016 ± 0.21 in 2007) than for females (mean ± SD r = 0.0008 ± 0.18 in 2005; r =- 0.0066 ± 0.23 in 2006; and r = -0.0081 ± 0.25 in 2007) but they were not significantly different ($F_{1,3850}$ = 0.56, P = 0.453). However, recapture data suggest that it is likely that males but not females are faithful to displaying sites.

Patterns of relatedness and relationship within leks

Individual pairwise relatedness values within all leks ranged from -0.016 to 0.87. Mean coefficients of male relatedness within leks were low, and not statistically different than zero, except for the Orduña lek in 2006 and Ursulo Galvan (Table 2).

Using ML-RELATE, maximum likelihood estimates of relationship showed that out of the 4750 possible individual comparisons of 98 different individuals across years at the Orduña lek, 836 were assigned with precision to half-sib or greater (Fig. 1). Of these, 95 comparisons were parent-offspring, 96 were full-sib and 645 were half-sib. However, 82% of the possible comparisons excluded any relationship other than NCR (not-closely related). Considering the six leks, relationship comparisons within leks also showed pairs of closely related birds, however, these relationships were not common. Out of 5244 comparisons within leks, less than 20% were half-sib or greater and only the 2% were parent-offspring and full-sib relationships.

Kinship in song neighborhoods

Mean coefficients of male relatedness within song neighborhoods ranged from -0.46 to 0.45 (mean ± SD, 0.016 ± 0.21), and between song neighborhoods ranged from -0.42 to 0.58 (mean ± SD, 0.0408 ± 0.21, Table 3). Relatedness coefficients were statistically different than zero in three comparisons: within song neighborhood A, between A and B, and between A and C, indicating that males within song neighborhood A are related, but these males are also related with individuals from song neighborhood B and C (Table 3, Fig. 2). However, the ANOVA yielded no significant differences between comparisons of relatedness within and between song neighborhoods ($F_{2,229} = 0.56$, $P = 0.454$). These results indicate that males within song neighborhoods are equally related than males do between neighborhoods. Similarly, the principal coordinate analysis did not show any structure between song neighborhoods, and the Mantel tests did not show a significant correlation between pairwise relatedness and song similarity between males of the song neighborhoods ($r = -0.09$, $P > 0.05$).

DISCUSSION

A previous study on wedge-tailed sabrewings showed that leks have a very weak genetic structure at the entire distribution of the species, and therefore high levels of gene flow between leks (González and Ornelas, 2011). We observed low levels of relatedness within males and females in wedge-tailed sabrewings leks, and females are slightly less related than males. Lower female relatedness suggests that wedge-tailed sabrewing females disperse more than males, which are either philopatric or disperse shorter distances. Thus, the high gene flow estimated between leks might be due to females dispersing more than males. The female sex-biased dispersal is common in birds (Greenwood and Harvey 1982). In the case of male philopatry, the familiarity with resources could be advantageous, and in dispersing females the possibility to choose among males and their resources (Greenwood and Harvey 1982). Besides, the higher level of female-mediated gene flow could be preventing the loss of genetic variation (Pierney et al. 1998).

One possible explanation for the evolution of male aggregations that display to females is the kin selection theory (Kokko and Lindström 1996). This theory predicts that in lek systems where reproductive success is generally highly skewed, unsuccessful males join their relatives to increase their inclusive fitness, implying that males that attend leks are more related than expected by chance. In our study, we observed low levels of relatedness and a high variance of the observed within-lek mean relatedness, which suggests that both related and unrelated males are displaying together. The low levels of mean relatedness observed within leks is likely due to the presence of many unrelated lekking males and a few relatives. It has been suggested that local levels of relatedness among years could vary (Piertney et al. 2008). In our focal lek we observed different levels of relatedness among the three years of study, and only in 2006 males are more related than expected by chance. This variation could be result of differences in sampling effort or variation in the demographic characteristics of the studied populations (Lebigre et al. 2008).

The mean low levels and high variance of relatedness between lekking males may be due to a lack of mating skew or this is not as strong as in other species (Widemo and Owens 1995), such as grouses (Alatalo et al. 1992, Semple et al. 2002), peafowls (Krakauer 2005), and manakins (McDonald and Potts 1994; Loiselle et al. 2007). Although we have observed more singing activity and territorial chases in central positions of the lek, it is possible that males with central territories obtain more matings, or peripheral males trespass in to territories of preferred males and steal matings. Another alternative that has not been tested is the possibility of multiple paternity which is common in birds. All hummingbird species have generally two eggs but they can have two clutches in the same season, so females could copulate with more than one male in the same breeding season. Besides, not all reproductive males congregate at leks in a breeding season. Some of them remain on food resources (flower patches) along the reproductive season (González and Ornelas 2005), where it is likely to obtain copulations. A high variance in male relatedness is also expected if there is a rapid turnover of the males that obtained most of the copulations in a given breeding season (Lebigre et al. 2008) because these males do not retain their dominant status from year to year (Rintamäki et al. 1995).

In addition to the possibilities of mating skew lacking and turnover of dominant males, the most likely explanation for the observed pattern in wedge-tailed sabrewings is that males are highly philopatric to displaying sites (males at La Orduña and at El Cielo leks have at least 15 years displaying at the same site). Natal philopatry is unlikely because females nest outside the lek, maybe at long distances, making unfeasible that males will join the lek at which their fathers or relatives display, or the probability depends on the distance of nesting sites. This later is also supported by analyses of relatedness in song neighborhoods (see below). Although juveniles attempting to join a lek, where their parents or relatives are already settled could gain indirect benefits, the presence of many unrelated males on the leks do not support the potential for kin selection and, therefore, this is not a strong explanation for the maintenance of male aggregations in wedge-tailed sabrewings. In this species the maintenance of male aggregations

is likely due to direct rather than indirect benefits as suggested by kin selection theory; there must be some benefit in terms of reproductive success to congregate at leks, and if so, males attending leks must have higher reproductive success over their life span than solitary males (Höglund and Alatalo 1995). Among the direct benefits that males could obtain displaying in aggregations are a reduction of predation risk (Boyko et al. 2004), and the intensification of the display signals to attract more females and also the non-attractive males to be noticed by females. In the case of wedge-tailed sabrewings, aggregations of males could increase the intensity of the singing displays, and make more likely to be detected by females. It has been tested in some species that larger leks are more preferred by females than smaller ones (Alatalo et al. 1992). Thus, although the intrasexual competition and agonistic interactions that can occur between territorial males, it is likely that leks function as a cooperative system as a whole.

The potential for kin selection has been demonstrated in some but no all lekking species studied (Höglund et al. 1999, Petrie et al. 1999, Shorey et al 2000, Höglund and Shorey 2003, Bouzat and Johnson 2004, Krakauer 2005, Regnaut et al. 2006, Segelbacher et al. 2007, Lebigre et al 2008, but see McDonald and Potts 1994, Madden et al. 2004, Gibson et al 2005, Loiselle et al 2007). This suggests that kin selection might be important in some species but not in others. However, although some of these studies showed that lekking males are more related than expected by chance, it does not necessarily imply that low-ranking males have higher inclusive fitness when displaying with kin than displaying alone or with unrelated individuals, except for wild turkeys (Krakauer 2005).

Some studies have suggested that in some species where a fine-genetic structuring within leks exists, there must be a recognition mechanism between relatives to display in family groups (Höglund et al. 1999, Petrie et al. 1999, Shorey et al. 2000). In wedge-tailed sabrewings, there is a strong vocal structure within leks (song neighborhoods), and it is possible to think that this vocal structure is the mechanism of phenotype matching to allow males join groups of relatives.

Our results indicated that in only one case (song neighborhood A) males are more related than expected by chance. However, when comparing relatedness between males of different song neighborhoods, we observed that males of neighborhood A were equally related with males of the other neighborhoods. This indicates that males may join song neighborhoods by chance, and it is unlikely that males actively choose or prefer to display close to relatives or close to unrelated individuals. The results of this study confirm that wedge-tailed sabrewings do not learn the song of their fathers or relatives, in which case song dialects are not acting as a recognition mechanism between kinship. Adult or juveniles that join a lek for the first time have to interact with males established on territories to get access, and it is more likely their acceptance if a relative has already settle down in a territory. That is why it has been proposed to be recognition mechanisms between relatives. However, our data suggest that juveniles attempting to settle in a lek must learn the song (either from related or unrelated males) to get access to territories. Thus, it seems to be that some males within a song neighborhood are more related than others only by chance.

In conclusion, our data indicate that both male and female wedge-teiled sabrewings have low values of relatedness, and males do not display with relatives. Also, members of song neighborhoods are not close kin. The low values of relatedness among lekking males might be the result of a lacking of skewed reproductive success, and/ore multiple paternity. Large male aggregations in this species rather than be the result of kin selection, it would be to reduce predation risks or to intensify acoustic signals for females. Finally, the lack of kin structure within song neighborhoods suggests that song sharing is not a recognition mechanism between relatives, but learning the song of a lek or neighborhood likely facilitates the joining to the lek.

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Table 1. Characteristics of microsatellite loci used in this study.

	2005			2006			2007		
Locus	Alleles	H_O	H_E	Alleles	H_O	H_E	Alleles	H_O	H_E
CACU1-10	4	0.70	0.58	3	0.50	0.51	3	0.61	0.57
CACU8-1	4	0.41	0.43	4	0.37	0.49	6	0.32	0.53*
CACU7-5	2	0.21	0.25	2	0.12	0.12	2	0.10	0.22
CACU16-1	9	0.87	0.82	8	0.75	0.77	9	0.71	0.80
CACU4-6	3	0.04	0.11*	3	0.09	0.25*	3	0.05	0.18*
CACU13-2	8	0.51	0.85*	9	0.59	0.83*	9	0.58	0.81*
CACU13-7	14	0.87	0.89	11	0.84	0.86	12	0.78	0.85
CACU4-8	9	0.79	0.77	8	0.66	0.76	8	0.61	0.75
CACU5-7	9	0.66	0.81	8	0.47	0.75*	9	0.49	0.78*
CACU17-2	10	0.77	0.81	11	0.69	0.70	11	0.76	0.73

Observed heterozygosity, H_O; expected heterozygosity, H_E; * indicates significant departure ($p < 0.05$, after sequential Bonferroni correction) from Hardy-Weinberg equilibrium.

Table 2. Mean coefficients of relatedness within leks. One sample *t*-test was compared to a null expectation of zero.

Lek	*n*	Mean relatedness ± SD	Range of pairwise relatedness	One-sample *t*-test
Orduña 2005	44	-0.001 ±0.205	-0.63–0.8	t_{1891} = -0.22, NS
Orduña 2006	24	0.043 ± 0.22	-0.5–0.64	t_{170} = 4.58, ***
Orduña 2007	36	-0.0165 ± 0.21	-0.55–0.87	t_{1259} = -2.73, NS
El Cielo	16	0.036 ± 0.21	-0.38–0.55	t_{119} = 1.91, NS
El Naranjo	6	0.094 ±0.22	-0.35–0.5	t_{14} = 1.68, NS
Xilitla	8	-0.061 ± 0.23	-0.42–0.45	t_{27} = -1.41, NS
Cuetzalan	24	-0.016 ± 0.23	-0.42–0.76	t_{275} = -1.17, NS
Ursulo Galván	11	0.079 ± 0.23	-0.38–0.42	t_{54} = 2.52, *

SD = standard deviation; *** P<0.0001, * P<0.05; NS = no significantly different from zero.

Table 3. Mean coefficients of relatedness within and between song neighborhoods. One sample *t*-test were compared to a null expectation of zero.

Song neighborhood comparison	No. comparisons (*n*)	Mean relatedness ± SD	Range of pairwise relatedness	One-sample *t*-test
A–A	15 (6)	0.204 ±0.11	0.04–0.38	t_{14} = 6.89, ***
B–B	15 (6)	-0.005 ± 0.18	-0.36–0.21	t_{14} = -0.11, NS
C–C	28 (8)	-0.084 ± 0.20	-0.46–0.45	t_{27} = -2.19, NS
D–D	1 (2)	0.352 ± 0	0.35–0.35	——–
A–B	36	0.144 ±0.18	-0.30–0.47	t_{35} = 4.75, ***
A–C	48	0.098 ± 0.19	-0.33–0.58	t_{47} = 3.54, **
A–D	12	0.068 ± 0.26	-0.42–0.32	t_{11} = -0.89, NS
B–C	48	0.004 ± 0.21	-0.39–0.52	t_{47} = -0.14, NS
B–D	12	0.021 ± 0.22	-0.33–0.38	t_{11} = 0.32, NS
C–D	17	-0.133 ± 0.17	-0.42–0.12	t_{15} = -3.12, NS

SD = standard deviation; *** P<0.0001, ** P<0.001; NS = no significantly different from zero.

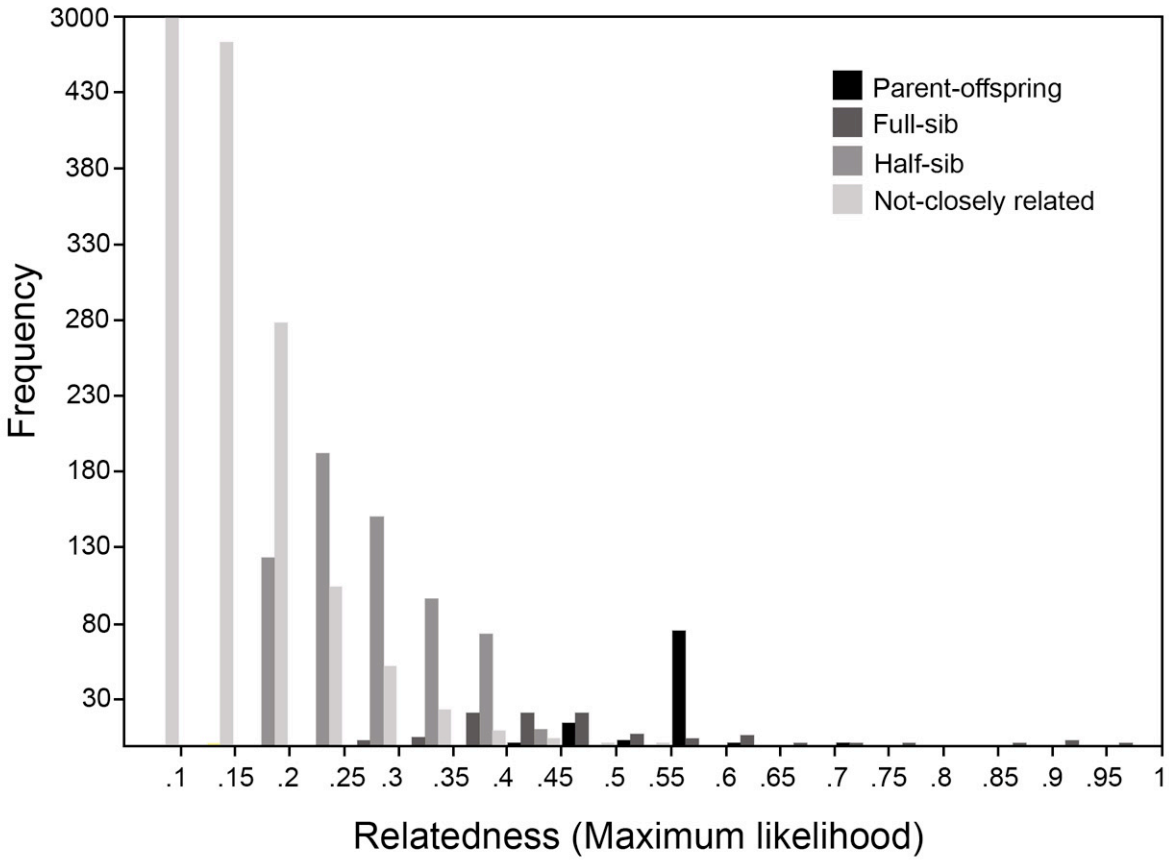

Figure 1. Frequency distribution of maximum likelihood relatedness estimates for pairs of wedge-tailed sabrewings of the Orduña lek using M-RELATE. Parent-offspring, full-siblings, half-siblings and not-closely related pedigree categories are shown.

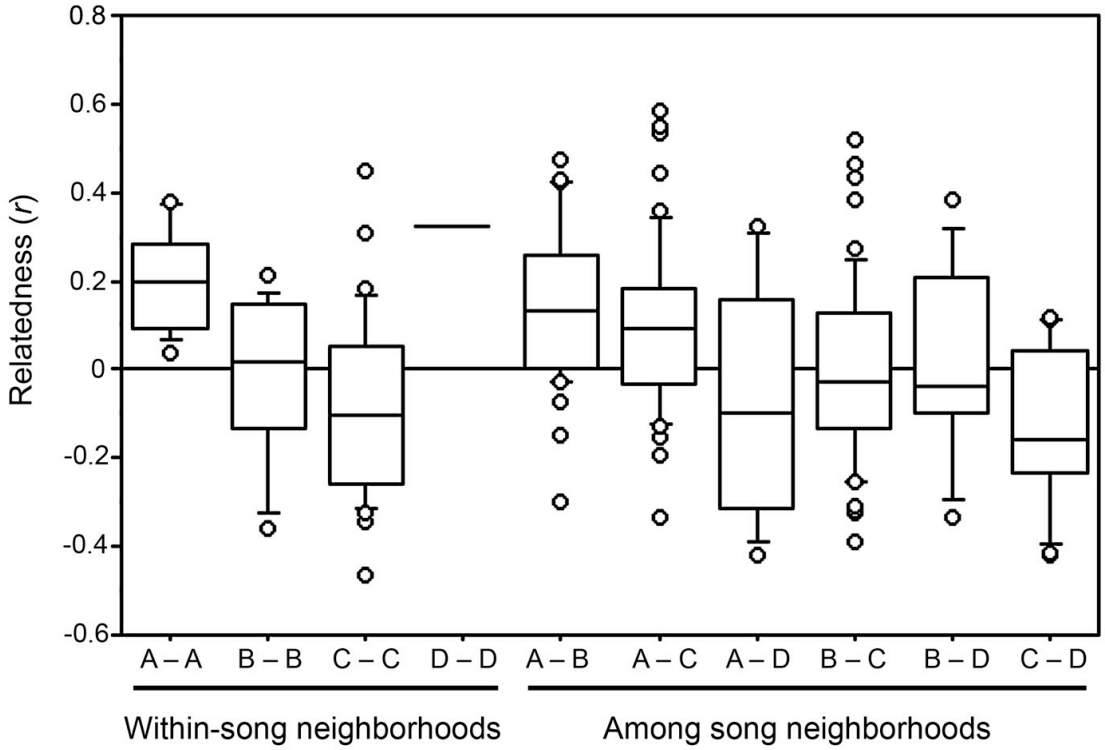

Figure 2. Boxplot of genetic relatedness (*r*) within and between males of song neighborhoods. The box represents the 10th, 25th, 50th (median), 75th, and 90th percentiles. Values above the 90th and below the 10th percentile are plotted as open circles.

CAPÍTULO V

Isolation, characterization and cross species amplification of microsatellite loci in a lek-breeding hummingbird (*Campylopterus curvipennis*, Trochilidae)

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Abstract

This article documents the addition of 238 microsatellite marker loci and 72 pairs of Single Nucleotide Polymorphism (SNP) sequencing primers to the Molecular Ecology Resources Database. Loci were developed for the following species: ***Adelges tsugae, Artemisia tridentata, Astroides calycularis, Azorella selago, Botryllus schlosseri, Botrylloides violaceus, Cardiocrinum cordatum*** **var.** ***glehnii, Campylopterus curvipennis, Colocasia esculenta, Cynomys ludovicianus, Cynomys leucurus, Cynomys gunnisoni, Epinephelus coioides, Eunicella singularis, Gammarus pulex, Homoeosoma nebulella, Hyla squirella, Lateolabrax japonicus, Mastomys erythroleucus, Pararge aegeria, Pardosa sierra, Phoenicopterus ruber ruber*** **and** ***Silene latifolia.*** **These loci were cross-tested on the following species:** ***Adelges abietis, Adelges cooleyi, Adelges piceae, Pineus pini, Pineus strobi, Tubastrea micrantha,*** **three other** ***Tubastrea*** **species,** ***Botrylloides fuscus, Botrylloides simodensis, Campylopterus hemileucurus, Campylopterus rufus, Campylopterus largipennis, Campylopterus villaviscensio, Phaethornis longuemareus, Florisuga mellivora, Lampornis amethystinus, Amazilia cyanocephala, Archilochus colubris, Epinephelus lanceolatus, Epinephelus fuscoguttatus, Symbiodinium*** **temperate-A clade,** ***Gammarus fossarum, Gammarus roeselii, Dikerogammarus villosus*** **and** ***Limnomysis benedeni.*** **This article also documents the addition of 72 sequencing primer pairs and 52 allele specific primers for** ***Neophocaena phocaenoides.***

This article documents the addition of 238 microsatellite marker loci and 72 pairs of Single Nucleotide Polymorphism (SNP) genotyping primers to the Molecular Ecology Resources Database. Table 1 contains information on the focal species, the number of loci developed, any other species the loci were tested in and the accession numbers for the loci in both the Molecular Ecology Resources Database and GenBank. The authors responsible for each set of loci are listed in the final column. Table 2 presents information on SNP genotyping resources added to the MER database, and presents data on the focal species, the number of sequencing primer pairs, the observed number of SNPs, other species the loci were tested in, and the number of allele specific primers or probes. The MER database and Genbank accession numbers and the authors responsible are also listed. A full description of the development protocol for the loci presented here can be found on the Molecular Ecology Resources Database (http://tomato.biol.trinity.edu/).

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Table 1 Information on the focal species, the number of loci developed, any other species the loci were tested in and the accession numbers for the loci in both the Molecular Ecology Resources Database and GenBank. The authors responsible for each set of loci are listed in the final column

Species	No. of primers developed	Other species tested	MER database no.	GenBank Accession no.	Authors
Adelges tsugae	16	*A. abietis*, *A. cooleyi*, *A. piceae*, *Pineus pini*, *P. strobi*	37980–37995	GQ368549–GQ368564	Nathan P. Havil Adalgisa Caccone
Artemisia tridentata	8	n/a	41279–41286	AB488553–AB488560	S. Ishizaki S. Kubota K. Shiojiri R. Karban M. Ohara
Astroides calycularis	13	*Tubastrea micrantha*, *Tubastrea sp. 1*, *Tubastrea sp. 2*, *Tubastrea sp. 3*	41292–41304	GQ292717–GQ292725, GQ496302–GQ496305	P. Casado-Amezúa I. Acevedo R. García-Jiménez A. Machordom
Azorella selago	8	n/a	42478–42485	GQ3651674–GQ3651681	Céline Born Mélodie A. McGeoch Bettine Jansen van Vuuren
Botryllus schlosseri, *Botrylloides violaceus*	28	*Botrylloides fuscus*, *Botrylloides simodensis*	38070–38097	GQ272527–GQ272554	Dan G. Bock Abisola A. Adebayo Emmanuel E. Egbosimba Melania E. Cristescu
Cardiocrinum cordatum var. glehnii	13	n/a	41315–41327	AB512096–AB512108	M. Nishizawa S. Kubota M. Ohara
Campylopterus curvipennis	10	*C. hemileucurus*, *C. rufus*, *C. largipennis*, *C. villaviscensio*, *Phaethornis longuemareus*, *Florisuga mellivora*, *Lampornis amethystinus*, *Amazilia cyanocephala*, *Archilochus colubris*	41305–41314	GQ294539–GQ294550	Clementina Gonzalez Carla Gutierrez-Rodriguez Juan Francisco Ornelas
Colocasia esculenta	19	n/a	38144–38162	FJ895330–FJ895348	Wansha Li Yan Zhou Yongping Yang Xiangyang Hu
Cynomys ludovicianus, *C. leucurus*, *C. gunnisoni*	9	n/a	38175–38184	FJ971631–FJ971639, FJ997263, FJ980459–FJ980464	Loren C. Sackett Lianna K. Etchberger Maxwell N. Mazzella Douglas D. Lim Andrew P. Martin
Epinephelus coioides	14	*Epinephelus lanceolatus*, *Epinephelus fuscoguttatus*	37966–37979	GQ267993–GQ267993 GQ381271, GQ429007–GQ429009	Le Wang Zining Meng Bin Fan Qing Sang Yayan Luo Yong Zhang Xiaochun Liu Haoran Lin

Table 1 (*Continued*).

Species	No. of primers developed	Other species tested	MER database no.	GenBank Accession no.	Authors
Eunicella singularis	12	*Symbiodinium* temperate-A clade	41272–41291	FJ917540–FJ917550, FJ919777	J. Cataneo M. F. Ortu P. Furla D. Forcioli
Gammarus pulex	8	*Gammarus fossarum*, *Gammarus roeselii*, *Dikerogammarus villosus*, *Limnomysis benedeni*	41336–41343	EH268406, EH269344, EH271322, EH271465, EH271889, EH272785, EH274528, EH275159	René Gergs Karl-Otto Rothhaupt Jasminca Behrmann-Godel
Homoeosoma nebulella	9	n/a	38098–38106	GQ150803–GQ150811	Ling-Zhen Cao Qin Ren Xiang-Li Xu Qing-Wen Zhang
Hyla squirella	11	n/a	42486–42496	GQ438807–GQ438817	Tyler D. Hether Eric A. Hoffman
Lateolabrax japonicus	11	n/a	42459–42469	GQ455996 GQ455997 GQ456002 GQ456006 GQ456007 GQ456013 GQ456018 GQ456019 GQ456022 GQ456032 GQ456037	Y. Zhao X. S. Ji H. Wang Y. Q. Zeng J. T. Wang
Mastomys erythroleucus	12	n/a	38163–38174	GQ406216–GQ406227	Philippe Gauthier Patricia O'Brien Laurent Granjon Doukary Abdoullaye Carine Brouat Gauthier Dobigny
Pararge aegeria	10	n/a	41344–41353	FJ899644–FJ899647, FJ899649–FJ899651, GQ847528–GQ847530	P. Helsen S. Vandewoestijne S. Van Dongen E. Matthysen
Pardosa sierra	10	n/a	37996–38005	EU580603–EU580608, FJ975139–FJ975142	M. M. Correa-Ramirez F. J. Garcia de Leon M. L. Jimenez
Phoenicopterus ruber ruber	9	n/a	38108–38116	GQ219786–GQ219790, GQ379053–GQ379055, GQ221667	R. Kapil G. M. Sawyer L. Preston R. C. Benjamin
Silene latifolia	8	n/a	41328–41335	FJ573199, FJ573200, FJ573202–FJ573204, FJ573206, FJ573207, FJ573209	Peter D. Fields Stephen R. Keller Pär K. Ingvarsson Amy B. Pedersen Douglas R. Taylor

Table 2 Information on the focal species, the sequencing primer pairs developed, the number of single nucleotide polymorphisms observed and any other species the loci were tested in. The next columns contain the number of allele specific primers and probes developed, and the Molecular Ecology Resources database and GenBank accession numbers, respectively. The authors responsible for each set of loci are listed in the final column

Species	No. of primer pairs	No. of SNPs in sequence	No. of allele specific primers/probe	MER database no.	Genbank Accession no.	Authors
Neophocaena phocaenoides	72	137	52	38006–38040	FI592654, FI592658–FI592662, FI592665, FI592667, FI592668, FI592670, FI592671, FI592673, FI592678, FI592680–FI592688, FI592690, FI592691, FI592693–FI592697, FI592699–FI592704, FI592706–FI592711, FI592713, FI592714, FI592717	Shuzhen Li Heyi Ji Guang Yang

Isolation, characterization and cross species amplification of microsatellite loci in a lek-breeding hummingbird (*Campylopterus curvipennis*, Trochilidae)

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Keywords: Lek, hummingbirds, sabrewings, SSRs, polymorphic

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Running title: Polymorphic microsatellites in hummingbirds

Abstract

***Campylopterus curvipennis*, a lek-breeding hummingbird characterized by a mating system where males compete for females by singing elaborate displays. To determine the distribution of genetic diversity among and within leks, we isolated and characterized 10 polymorphic microsatellite loci. These loci displayed an average of 6.1 alleles per locus (range 2–13) and average observed and expected heterozygosities of 0.529 and 0.610, respectively. Most loci amplified successfully in four *Campylopterus* species, however, the amplification quality was lower in five additional hummingbird genera. These microsatellites will be useful tools for genetic analyses in *Campylopterus* species and, to a lesser extent, other hummingbird genera.**

The wedge-tailed sabrewing (*Campylopterus curvipennis*) is discontinuously distributed from the mountains of the Sierra Madre Oriental in eastern Mexico to the lowlands of the Yucatan Peninsula. Sabrewings have a polygynous lek-breeding system known from only 30 of 320 hummingbird species (Atwood *et al.* 1991, Höglund & Alatalo 1995, Pizo & Silva 2001). The leks of *C. curvipennis* are composed of small adjacent territories defended individually from conspecific males. When females visit these territories, males perform courtship displays that involve the most elaborate acoustic signal known for hummingbirds (González & Ornelas 2005). The complex syllable structure of the songs shows many levels of geographic variation from differences between neighboring males to differences between leks (Gonzalez & Ornelas 2005). However, little is known about the mechanisms that generate and maintain the acoustic variation over space.

The lack of microsatellites for this and most hummingbird species (a set of microsatellites were described for *Trochilus* hummingbirds; Lance *et al*. 2009) makes difficult the study of phenomena that require highly variable molecular markers. To provide tools for studies investigating distribution of genetic diversity, behavior in lek-breeding systems, and song evolution, we have designed a set of microsatellites for *C. curvipennis* and assessed their amplification in other hummingbird species.

We extracted genomic DNA from heart muscle of a male collected in Tamaulipas, Mexico using the Puregene DNA Extraction from Animal Tissue Kit (Gentra Systems). The extracted DNA was digested with *Rsa*I (Invitrogen), and the fragments ligated to a double-stranded SuperSNX24 linkers (Forward 5´GTTTAAGGCCTAGCTAGCAGAATC, and Reverse 5´GATTCTGCTAGCTAGGCCTTAAACAAAA) (Glenn & Schable 2005). Linker-ligated DNA

was hybridized to two biotinylated microsatellite oligonucleotide mixes: 1) [$(AG)_{12}$, $(TG)_{12}$, $(AAC)_6$, $(AAG)_8$, $(AAT)_{12}$, $(ACT)_{12}$, $(ATC)_8$], 2) $(AAAC)_6$, $(AAAG)_6$, $(AATC)_6$, $(AATG)_6$, $(ACAG)_6$, $(ACCT)_6$, $(ACTC)_6$, $(ACTG)_6$] (Invitrogen). Microsatellite containing DNA fragments were captured with streptavidin-coated Magnetic Dynabeads (Dynal). Enriched DNA was amplified by PCR using the SNX forward primer. Amplified products were cloned using the TOPO TA Cloning Kit (Invitrogen), and transformed into *E. coli* following the kit protocol. Colonies were grown on Luria-Beltrami (LB) ampicillin plates with X-Gal and IPTG overnight. Ten-to-twenty positive colonies per plate were randomly selected and grown overnight in LB media. Plasmid DNA was isolated using the QIAprep Miniprep Kit (QIAGEN) and digested with *Eco*RI (Invitrogen). Sequencing of 134 inserts displaying different digestion banding patterns was performed by Macrogen Inc., Korea using the M13 primers.

We identified 42 perfect and imperfect microsatellites from the sequenced inserts. Primers were designed from 19 microsatellites that had sufficient flanking regions using the program Primer 3 (Rozen & Skaletsky 2000). Gradient PCR reactions with unlabeled primers were conducted using five individuals from different populations to determine optimal conditions for subsequent amplifications. DNA of these and subsequent samples was extracted from a tail feather using a standard protocol with chelex 5% (Morin *et al*. 1994). PCRs were performed in 14 µl reactions contained 0.72X buffer, 2.5–3.5 mM $MgCl_2$, 0.14 mM of each dNTP, 0.23µM of each primer (Invitrogen), 0.05 U *Taq* (Promega), and 2 µl of genomic DNA. Amplifications were performed in an Eppendorf Mastercycler gradient thermocycler with the following parameters: initial denaturation at 94°C for 5 min; 35 cycles consisting of denaturation at 94°C for 1.5 min, annealing at 56–63°C (Table 1) for 1 min and an extension at 72°C for 1.5 min; and

a final extension at 72°C for 7 min. PCR products were visualized on a 1% agarose gels stained with ethidium bromide using a 100 bp ladder. From the 19 tested primers, 15 successfully amplified and were used to test levels of polymorphism in 27 individuals from a single population in Puebla, Mexico. We conducted PCR reactions separately for each locus using fluorescently labeled forward primers and unlabelled reverse primers using the same PCR and cycling conditions used during the initial screening of the primers. Amplified products were diluted 1:30 in distilled water, co-loaded in two sets of 3 loci (CACU1-10, CACU7-5, and CACU8-1; CACU4-8, CACU5-7, and CACU17-2) and one set of 4 loci (CACU4-6, CACU13-2, CACU13-7, CACU16-1) on an ABI-PRISM 310 Genetic Analyzer including the GeneScan-600 LIZ size standard (Applied Biosystems). Fragment sizing was performed in Genemapper version 3.2 (Applied Biosystems). Of 15 microsatellites tested, 10 were polymorphic. Characteristics of microsatellite loci are shown in Table 1.

We estimated observed and expected heterozygosity (H_O and H_E), and tested for deviations from Hardy-Weinberg equilibrium (HWE), and linkage disequilibrium (LD) using GENEPOP version 3.4 (Raymond & Rousset 1995). No showed significant deviations from expectations under HWE, and no linkage disequilibrium among 45 paired loci comparisons were detected after Bonferroni corrections for multiple comparisons.

Cross-species and cross-genera amplifications of microsatellites were performed in one individual from four additional species of the same genus and five species of different hummingbird genera. Most primers amplified successfully across *Campylopterus* species and *Amazilia cyanocephala*, however the quality of the amplifications was lower in other genera (Table 2).

The set of microsatellites described here will allow for studies in *C. curvipennis* and other species of hummingbirds, involving genetic relatedness, parentage and population structure. We are currently investigating the distribution of genetic diversity among and within leks and its effects on the evolution of complex acoustic display signals.

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Table 1. Characterization of microsatellite loci in *Campylopterus curvipennis*. Listed are locus name, repeat motif (interrupted microsatellites are indicated by a (...) between repeats), forward (F) and reverse (R) primers, allele size range, annealing temperature (T_a), optimized $MgCl_2$ concentration, primer fluorescent label, number of alleles (A), observed heterozygosity (H_O), expected heterozygosity (H_E), and GenBank Accession numbers.

Locus	Repeat motif	Primer sequence 5'-3'	Allele size range (bp)	T_a (ºC)	[$MgCl_2$]	Label	A	H_O	H_E	GenBank Accession no.
Cacu1-10	$(CT)_7...(CT)_{10}$	F: CAGGGGTGAATCCTAACCAA	176-180	62	3.5 mM	6-FAM	3	0.59	0.57	GQ294539
		R: CAAGGCAACGAGGAGTAAAA								
Cacu4-6	$(GTTT)_6$	F: CCTGAAAGCCCAAATGTTGT	145-153	60	3 mM	6-FAM	2	0.07	0.07	GQ294540
		R: GCAGCTCTCCCCTTCTTTCT								
Cacu4-8	$(CT)_{17}$	F: TAGGATGCTGCCTGTTCCTT	132-150	60	3.5 mM	6-FAM	8	0.78	0.81	GQ294541
		R: GGACCTGCAGACCAATGAAG								
Cacu5-7	$(AC)_{23}$	F: GTCCAAGCCCTTGACAGAAA	200-218	56	3.5 mM	VIC	8	0.52	0.79	GQ294542
		R: ACTAAACAGGCGGAGCTGAA								
Cacu7-5	$(AC)_{12}$	F: AAGGATGGAAACTTGCCTCA	231-235	62	3.5 mM	VIC	3	0.15	0.20	GQ294543
		R: AATTTTATGGGGGCTGCAA								
Cacu8-1	$(GAAA)_2GAA(GAAA)_2$	F: CGTGGGTGAGATGAGTTATTACC	366-394	62	3.5 mM	NED	3	0.52	0.59	GQ294544
	$(GAAAA)(GAAA)_2$	R: CGTTCAAGAAAAGTCAGCTTGC								
Cacu13-2	$(TG)_8AGTGTC$	F: GGGATGGAGGAGAAGGAGAG	214-224	63	2.6 mM	PET	5	0.50	0.72	GQ294546
	$(TG)_4TC(TG)_3$	R: GCCCATAAATTGTTCGCTGT								
Cacu13-7	$(TG)_{15}$	F: TGGAGGATCCATGAGTGGTC	196-228	56	2.5 mM	NED	13	0.70	0.82	GQ294547
		R: GTGACAATGAGGTGGCAATG								
Cacu16-1	$(TC)_{14}$	F: ATCTGTCCAGGGCTTTTCCT	213-237	63	3.5 mM	VIC	8	0.63	0.76	GQ294548
		R: ACTGACTCCACACGCCACTA								
Cacu17-2	$(AC)_{12}$	F: CAGAGCTAAGGGTGGGACAG	234-262	63	3 mM	VIC	8	0.83	0.77	GQ294550
		R: CCATTTTATAGCGGGCACTT								

Table 2. Cross-species and genera amplifications of microsatellite primers designed from *Campylopterus curvipennis*. Symbols indicate successful amplification (✓), weak or multiple bands amplification (~), or failed amplification (×).

	Cacu1-10	Cacu4-6	Cacu4-8	Cacu5-7	Cacu7-5	Cacu8-1	Cacu13-2	Cacu13-7	Cacu16-1	Cacu17-2
C. hemileucurus	✓	✓	✓	×	✓	~	✓	✓	✓	✓
C. rufus	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓
C. largipennis	~	✓	✓	×	✓	✓	✓	~	✓	✓
C. villaviscensio	✓	✓	✓	✓	✓	×	✓	✓	✓	✓
Phaethornis longuemareus	×	✓	✓	×	×	×	×	✓	✓	×
Florisuga mellivora	×	✓	✓	×	✓	~	✓	~	✓	×
Lampornis amethystinus	×	~	×	×	×	~	✓	✓	✓	✓
Amazilia cyanocephala	✓	✓	✓	×	✓	~	✓	✓	✓	✓
Archilochus colubris	×	✓	×	×	✓	~	✓	~	✓	✓

Museum catalogue numbers and locations of specimens: *C. hemileucurus* (INECOL-VER-114) Mexico: Veracruz; *C. rufus* (INECOL-CHIS-112) Mexico: Chiapas; *C. largipennis* (ANSP-2657) Ecuador: Morona Santiago; *C. villaviscensio* (ANSP-4852) Ecuador: Pasohurco; *Phaethornis longuemareus* (INECOL-CHIS-111) Mexico: Chiapas; *Florisuga mellivora* (MZFC-CHIMA-425) Mexico: Oaxaca; *Lampornis amethystinus* (INECOL-VER-132) Mexico: Veracruz; *Amazilia cyanocephala* (INECOL-VER-154) Mexico: Veracruz; *Archilochus colubris* (INECOL-VER-189) Mexico:Veracruz. INECOL: Instituto de Ecología, A.C. ANSP: Academy of Natural Sciences of Philadelphia. MZFC: Museo de Zoología de la Facultad de Ciencias UNAM. One individual per species was used for cross-amplification tests.

DISCUSIÓN GENERAL

La intención principal de este trabajo fue estudiar múltiples factores a distintas escalas que pudieran explicar los patrones de variación genética y fenotípica de un rasgo involucrado en la elección de pareja (vocalizaciones). Para esto tomé en cuenta tanto factores filogeográficos, históricos y ambientales, así como factores locales relacionados con el sistema social y de apareamiento de un colibrí (*Campylopterus curvipennis*) que tiene vocalizaciones complejas y variables.

En general se ha sugerido que los factores biogeográficos del Pleistoceno (o del Plioceno tardío) han promovido diversificación genética substancial en aves a una escala microevolutiva (Avise & Walter 1998, Barber & Klicka 2010). Los datos de este trabajo sugieren un papel importante del aislamiento geográfico durante el Pleistoceno en la estructura genética de la especie o complejo de especies a lo largo de su distribución geográfica. Esta estructura pudiera ser el resultado de eventos vicariantes por el efecto de barreras geográficas o de hábitat. En el caso de la divergencia de las poblaciones distribuidas en la Península de Yucatán con respecto a la Sierra Madre Oriental y la región de Los Tuxtlas, los datos sugieren un efecto del Istmo de Tehuantepec (lo cual se ha reportado para varias especies tanto de aves como otros grupos de vertebrados; Mulcahy *et al.* 2006, Cadena *et al.* 2007, León-Paniagua *et al.* 2007, Bonaccorso *et al.* 2007, Cortés-Rodríguez *et al.* 2008, Navarro-Sigüenza *et al.* 2008, Barber & Klicka 2010). El Istmo de Tehuantepec estuvo sujeto a oscilaciones en el nivel del mar a distintos tiempos, provocando contracciones y expansiones del territorio. Se ha sugerido que los efectos del Istmo han sido responsables de la divergencia de poblaciones en varias especies de montaña, donde el Istmo actúa como una barrera de tierras bajas entre sistemas montañosos. En el caso de *C. curvipennis*, es probable que la barrera no sean las tierras bajas del Istmo (ya que en la península se encuentran distribuidas muy cerca del nivel del mar) en cuyo caso pudiera ser el

cambio en el tipo de hábitat que ocurre en esta zona que va desde selvas bajas y altas perennifolias y subcaducifolias a un tipo de hábitat de sabana. En el caso de la divergencia de las poblaciones de la región de Los Tuxtlas con respecto a las de la Sierra Madre Oriental (SMO) también es posible que la barrera sea de hábitat, ya que es probable que los bosques mesófilos de la SMO alguna vez estuvieron conectados en el pasado con los bosques de la región de Los Tuxtlas; sin embargo, para probar todas estas hipótesis es necesario construir modelos de predicción de nicho ecológico hacia el pasado.

El estudio de los patrones de variación genética a nivel geográfico es la base para entender la historia evolutiva de muchas especies, sin embargo, la combinación de mecanismos históricos y ecológicos, así como los patrones de variación fenotípica es esencial para inferir procesos evolutivos en poblaciones naturales (Zink & Remsen 1986). En cuanto a los patrones de variación fenotípica en este estudio, en el caso de la morfología los análisis de coalescencia sugirieron que no se puede rechazar la hipótesis de que la variación en el tamaño ha evolucionado bajo neutralidad. No puedo asegurar con estos datos y análisis que la deriva es la causante de la divergencia morfológica que encontré. Sin embargo, la poblaciones de Los Tuxtlas, al estar aisladas de otras poblaciones y con tamaños efectivos de población más pequeños es probable que el efecto de la deriva sea más fuerte que en las otras poblaciones y esto ocasione una evolución rápida e independiente en relación a las otras regiones.

A pesar de que no encontré evidencia de que la divergencia morfológica esté moldeada por la selección dependiente del hábitat, se necesitan explorar otros posibles factores que no se descartan como explicaciones a esta divergencia. Por ejemplo, las diferencias del largo del pico encontradas entre el grupo distribuido en la península (pico corto) y el resto (pico largo), sugiere que características ecológicas tales como forma y longitud de la corola de las plantas que visitan en ambos ambientes pueden diferir, y en este caso estaría actuando la selección a favor de hacer más eficiente la obtención de néctar.

En el caso de las vocalizaciones, los análisis de coalescencia sugieren un papel importante de alguna forma de selección en la divergencia de señales acústicas. Existen muchos trabajos recientes donde encuentran que la divergencia del canto a nivel intraespecífico está correlacionada con las propiedades acústicas del hábitat. Las presiones de selección ambientales pueden modificar las características físicas del canto con respecto a su estructura espectral y temporal, ya que las propiedades de transmisión del sonido están asociadas con la estructura de la vegetación, el microclima, la interferencia de señales y los patrones de ruido ambiental (Wiley 1991). Sin embargo, en este trabajo a pesar de que el complejo *C. curvipennis* se encuentra distribuido bajo condiciones ambientales y de hábitat muy distintas (sobre todo contrastando las condiciones de la Península de Yucatán con las de la Sierra de Los Tuxtlas o la Sierra Madre Oriental), no encontré evidencia de la influencia de las características ambientales de los hábitats distintos en la divergencia acústica. Contrario a lo que se ha pensado sobre la importancia de la selección ecológica (Slabbekoorn & Smith 2002*a*), los datos sugieren que las diferencias en el hábitat no han sido una presión selectiva importante en la divergencia vocal de esta especie, sin embargo, no se descarta completamente al no tener información sobre la estructura de la vegetación obtenida de imágenes de satélite.

A lo largo del estudio encontré muchos niveles de variación en los cantos de *Campylopterus curvipennis* que van desde diferencias muy pronunciadas entre los tres linajes hasta diferencias menos pronunciadas entre vecindarios vocales dentro de un mismo lek, pasando por la variación entre leks de un mismo linaje a lo largo de su distribución. Este es el primer estudio donde se investiga la variación vocal de un colibrí a diversas escalas geográficas y se relaciona con la variación genética. Los resultados sugieren que fuerzas selectivas estocásticas tales como la selección sexual y social, han promovido la divergencia vocal en las diferentes escalas. A una escala geográfica tomando en cuenta los tres linajes, aunque la divergencia vocal y la selección sexual pudieron no ser la causa de la divergencia genética, ya que los datos sugieren que factores vicariantes tales como barreras geográficas o de hábitat como en el caso del Istmo

de Tehuantepec y la Planicie Costera del Golfo de México propiciaron la separación de los tres linajes, la selección tanto del canto como de la morfología pueden jugar un papel importante en la evolución del aislamiento reproductivo en caso de que ocurra contacto secundario. Aunque la variación de rasgos involucrados en la elección de pareja como es el canto tiene el potencial de contribuir a la divergencia entre poblaciones en simpatría o parapatría, los resultados de este trabajo apoyan un modelo clásico de divergencia alopátrica. La evidencia de casos de divergencia de poblaciones de aves en simpatría son escasos aún (Via 2001), por lo que la divergencia de poblaciones en alopatría parece ser el modelo dominante de especiación en aves (Mayr 1942).

El potencial que pueden tener las vocalizaciones como mecanismo de divergencia poblacional en simpatría, sin embargo, depende de factores como por ejemplo la capacidad de aprendizaje y dispersión. En muchas especies de aves el canto es aprendido, y debido a los errores e innovaciones de elementos vocales durante la imitación, está sujeto a una rápida evolución lo cual puede estar implicado en la divergencia genética entre poblaciones y en eventos de especiación (Nottebohm 1969, Slabbekoorn & Smith 2002*a*, Lachlan & Servedio 2004, Edwards *et al.* 2005). Cuando existen dialectos bien definidos, se ha sugerido que los juveniles aprenden a producir o reconocer el canto a una edad temprana mientras se encuentran en su región natal, y cuando son adultos, utilizan el canto como un mecanismo de apareamiento *asortativo* (Nottebohn 1969). Si estas condiciones se cumplen, se esperaría que los dialectos inhibieran el flujo génico entre poblaciones de coespecíficos en cierto grado (MacDougall-Shackleton & MacDougall-Shackleton 2001). Sin embargo, el nivel de aprendizaje después de la dispersión es crítico en la determinación de la divergencia genética entre poblaciones vocalmente divergentes (Ellers & Slabbekoorn 2003).

Debido a la rápida evolución de señales acústicas a través del aprendizaje, se ha sugerido que éste ha facilitado la divergencia genética en aves paserinas (Raikow 1986), contribuyendo a la gran diversidad que existe dentro de ese grupo de aves. Sin embargo, el papel del

aprendizaje vocal en la divergencia reproductiva ha sido tema de controversia, ya que Baptista & Trail (1992) encontraron una correlación muy débil entre la diversidad de especies y las capacidades de aprendizaje, sugiriendo un papel limitado del aprendizaje en promover especiación. Sin embargo, esto debe probarse de manera más cuidadosa controlando posibles efectos filogenéticos.

A pesar de que el aprendizaje vocal puede promover la divergencia genética entre poblaciones, en algunas ocasiones puede inhibir el aislamiento reproductivo si existe una mínima divergencia en la capacidad de aprender cantos particulares (Slabbekoorn & Smith 2002*a*). Es posible que esto último sea una de las causas por las cuales no encontré una correspondencia entre la estructura genética y la variación vocal a lo largo de la distribución del linaje de *C. curvipennis* en la Sierra Madre Oriental, ni tampoco diferencias entre el parentesco de los machos pertenecientes a los vecindarios vocales, a pesar de existir una estructura vocal muy fuerte en ambas escalas.

Por otro lado, nuestros datos sugieren que la selección de familia (kin selection) no está implicada en la agregación social de machos de *C. curvipennis*, ya que no encontré un patrón contundente de parentesco entre los machos dentro de leks. Encontré que básicamente tanto machos altamente emparentados como aquellos no emparentados pueden desplegar en el mismo sitio, lo cual puede ser resultado del azar o de las distancias de dispersión por parte de las madres o los mismo machos, que no necesariamente tienen que estar relacionadas con la localización de los leks, sino puede ser resultado de la búsqueda de recursos florales. Este mismo patrón se ha encontrado en varias especies de saltarines (McDonald & Potts 1994, Loiselle *et al.* 2007) y en una especia de urogallo (Gibson *et al.* 2005). Se han propuesto hipótesis alternativas a la selección de familia para explicar por qué los machos se reúnen en sitios de despliegue cuando las oportunidades de reproducirse pueden ser muy bajas. Entre las posibles explicaciones se encuentran la hipótesis de “hot-spot” la cual sugiere que los machos se agrupan en áreas con alta densidad de hembras o movimientos de hembras (Bradbury &

Gibson 1983). La hipótesis de “hot-shot” sugiere que los machos subordinados se establecen cerca de machos dominantes con un alto éxito reproductivo, en este caso se considera que la elección de la hembra es menos importante comparada con las interacciones de dominancia entre machos (Beehler & Foster 1988). Por ultimo, la hipótesis de beneficios tardíos sugiere que los machos subordinados reciben adecuación directa más tarde cuando reemplazan a machos con un mayor rango (McDonald & Potts 1994). Cualquiera de estas hipótesis pueden ser posibles en el caso de *C. curvipennis* y falta mucho por estudiar cuestiones relacionadas con la jerarquía social, visitas y elección de las hembras, dinámicas de obtención y mantenimiento de territorios, etc.

En *C. curvipennis* encontré un patrón vocal interesante que no se había descrito en alguna otra especie que se congregue en leks, y esto es la existencia de vecindarios vocales dentro de un mismo lek. Los análisis de parentesco tampoco mostraron un patrón de parentesco entre los machos pertenecientes a cada vecindario, igualmente tanto machos emparentados como no emparentados pueden constituir un mismo vecindario, incluso hubo casos en los que los machos estaban más emparentados entre distintos vecindarios que dentro del mismo.

En conclusión los resultados de este trabajo a una escala geográfica sugieren un patrón de divergencia en alopatría promovida por barreras geográficas o de hábitat donde la deriva y la selección (sexual) han jugado un papel importante en la divergencia fenotípica y probablemente en mantener el aislamiento reproductivo en caso de que ocurra contacto secundario. A una escala geográfica más pequeña y local, los datos sugieren que las diferencias vocales entre leks y vecindarios vocales se mantienen cuando los machos aprenden las vocalizaciones después de la dispersión (ya sea de sus madres o de ellos mismos) probablemente para tener mayores probabilidades de establecerse en un territorio, tener éxito en obtener cópulas o tener acceso a grupos sociales (en este caso los leks). Por lo anterior, la estructura genética a lo largo de las “fronteras vocales” y de los vecindarios es muy débil por lo que es muy probable que la transmisión vocal sea de manera horizontal (entre individuos no emparentados). A esta

escala es probable que la selección sexual favorezca rasgos novedosos y variables y la selección social promueva convergencia vocal en los machos pertenecientes a un mismo lek.

Finalmente, este trabajo sugiere que la variación acústica o la existencia de dialectos, no parecen tener una relación con la distribución de la variación genética. Es decir que la similitud vocal, o la convergencia por cantar un mismo tipo de canto no limita el flujo de genes. Esto parece ser un patrón común en distintos grupos de aves, tanto en la mayoría de paseriformes que se han estudiado como en pericos y este trabajo aporta un patrón similar en colibríes. Esto debe ser resultado del grado de la dispersión pre y post natal, y cuestiones relacionadas con el apareamiento *asortativo* y la elección de machos con cierto tipo de canto por parte de las hembras. Por otro lado, los datos del trabajo sugiere un papel importante de fuerzas estocásticas tales como la selección sexual y social en promover la gran diversidad acústica que se encontró. Es probable que en un contexto filogenético, la existencia de esta gran diversidad vocal, originada a través de la selección sexual en algunos grupos de colibríes, haya contribuido junto con la evolución del plumaje y la capacidad de explotar recursos florales, a la gran diversificación que existe dentro de la familia, sin embargo esto debe ser estudiado.

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