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TESIS DOCTORAL

Estudio de patrones de diversidad intraespecífica en anfibios (Amphibia: anura, caudata) a través de métodos filogeográficos

MEMORIA PARA OPTAR AL GRADO DE DOCTOR
PRESENTADA POR

Ernesto Recuero Gil

DIRECTORES:

Gabriela, dir Parra Olea
Mario, dir García París

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Ernesto Recuero Gil

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Dpto. Zoología y Antropología Física
Facultad de Ciencias Biológicas
Universidad Complutense de Madrid

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Dpto. Biodiversidad y Biología Evolutiva
Museo Nacional de Ciencias Naturales
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Memoria presentada por ERNESTO RECUERO GIL para optar al grado de Doctor en Biología

V° B° directores de tesis

Dra. Gabriela Parra Olea

Dr. Mario García París

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A mi familia.

Por todo.

Índice

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I. Introducción General

La biología evolutiva aborda el estudio de la evolución desde dos perspectivas. Por un lado trata de reconstruir e interpretar la historia evolutiva de los organismos. Por otro busca elucidar los procesos y mecanismos generadores de dicha historia (Futuyma, 1986; O'hara, 1988). A menudo ambas líneas de estudio se mantienen separadas por las dificultades a la hora de integrar diferentes ramas de investigación, debido principalmente a importantes diferencias en las metodologías y objetivos propios (Schlichting & Pigliucci, 1998). En algunos casos, sin embargo, el estudio de la historia evolutiva y sus procesos causales van unidos de la mano. Así, por ejemplo, el estudio de la diversidad intraespecífica suele implicar una parte descriptiva en la que se establecen las características de los diferentes linajes así como las relaciones entre ellos y otra parte que trata de explicar los procesos que han dado origen a dicha diversidad (Carstens *et al.*, 2009) .

Consecuencia directa de la evolución es la diversidad que caracteriza a los seres vivos. Sin embargo, esta diversidad no siempre resulta evidente. Suele ser así el caso de la existente entre poblaciones de una misma especie o incluso de organismos estrechamente relacionados. El desarrollo de técnicas

moleculares, especialmente desde el último cuarto del siglo XX, ha permitido explorar un vasto campo de diversidad, a menudo críptica, que en muchos casos resultaba inconcebible no hace muchos años (Beheregaray & Caccone, 2007; Trontelj & Fiser, 2009). Se trata de una revolución que ha afectado a todos los niveles y muy especialmente en aspectos básicos como por ejemplo en el eterno debate sobre el concepto de especie (Wiens, 2004a; Vences & Wake, 2007). De este modo el estudio de los patrones de variación genética intraespecífica se ha convertido en un elemento clave que está permitiendo un progreso considerable en nuestros conocimientos sobre los factores que influyen en las estructuras poblacionales, en la definición de linajes y en los procesos de especiación. En las últimas décadas este tipo de estudios se han visto favorecidos notablemente no sólo por el continuo desarrollo de las técnicas moleculares que permiten cada vez más la generación de gran cantidad de datos acordes a los objetivos planteados en cada proyecto, sino también gracias a la aparición de nuevos marcos conceptuales en los que analizar e interpretar dichos resultados (Avise, 2000; Wiens, 2004a; Buckley, 2009; Hickerson *et al.*, 2010).

Filogeografía y diversificación.

La filogeografía es una disciplina que estudia los patrones de distribución geográfica de los linajes genéticos, normalmente a nivel intraespecífico o entre especies próximamente emparentadas, así como los procesos que generan dichos patrones (Avise, 2009; Nielsen & Beaumont, 2009). Al estudiar la diversidad intraespecífica mediante una aproximación filogeográfica, la distribución espacial de los linajes encontrados debe analizarse en un trasfondo causal que permita conjeturar qué procesos históricos y demográficos se encuentran detrás (Buckley, 2009). Desde este punto de vista el objetivo último de la filogeografía puede ser el de aclarar los mecanismos microevolutivos y de especiación en su contexto geográfico o espaciotemporal (Kidd & Ritchie, 2006). Ese ha sido el mayor éxito de esta relativamente reciente disciplina, al trascender la mera descripción de eventos pasados y convertirse en un potente herramienta para recuperar y explicar la historia evolutiva de los organismos.

Los orígenes de la filogeografía comenzaron a forjarse a finales de la década de los 70 del siglo XX, cuando se comenzaron a utilizar marcadores

mitocondriales para establecer las relaciones genealógicas entre individuos de la misma especie (Avise *et al*, 1979a, 1979b). Entre los resultados de estos estudios destacaba la aparición de patrones singulares que asignaban una distribución geográfica estructurada para los diferentes linajes, sugiriendo una conexión efectiva entre genealogía y geografía (Avise, 2000). El bautizo oficial de esta nueva disciplina no se produjo hasta unos pocos años después (Avise et al., 1987) y, a partir de entonces, el término filogeografía se convirtió en la referencia adecuada para cualquier estudio que pretenda explorar procesos microevolutivos a través de las dimensiones espaciales y temporales de genealogías específicas (Avise, 2009).

La filogeografía se centra principalmente en el estudio de patrones y procesos a nivel intraespecífico, lo que la aproxima en cierto modo a la genética de poblaciones. Sin embargo, la genética de poblaciones clásica se centra básicamente en el estudio de frecuencias alélicas, mientras que en filogeografía se trabaja en torno a genealogías, utilizando con frecuencia métodos filogenéticos para generar hipótesis de trabajo (Avise, 2009). La diferencia básica entre filogenia y filogeografía reside en el enfoque de esta última hacia la historia poblacional y demográfica de la especie en estudio (Avise, 2009).

Diversidad intraespecífica en anfibios: patrones filogeográficos y procesos de diferenciación.

Los anfibios se han convertido en uno de los grupos protagonistas en los estudios filogeográficos. Este auge puede parecer extraño si se piensa que durante un largo periodo de tiempo ha sido una clase relativamente marginada en comparación a otros grupos de vertebrados como las aves y los mamíferos. Esto puede explicarse de forma general por diferentes ventajas que aportan a la hora de plantear ese tipo de trabajos. Por ejemplo, los anfibios suelen ser organismos que presentan como norma general una vagilidad limitada y a menudo una marcada filopatría hacia los lugares de reproducción (Blaustein *et al*, 1994; Beebee, 1996). Como consecuencia suele observarse una fuerte estructuración genética poblacional incluso a escalas geográficas pequeñas, quedando habitualmente señales patentes acerca de los eventos históricos que han moldeado la distribución actual de los linajes. Por este motivo los patrones observados pueden diferir marcadamente de los de organismos más móviles, especialmente especies animales voladoras o plantas que producen semillas capaces de dispersarse a largas distancias (Zeisset & Beebee, 2008). Otros aspectos resultan ventajosos desde un punto de vista más práctico. Por

ejemplo, suele resultar sencillo delimitar las poblaciones a estudiar, al asociarse de forma más o menos estricta a los puntos de reproducción y su entorno, especialmente en ciertos ambientes como pueden ser muchos ecosistemas mediterráneos, en los que a menudo los puntos de agua se encuentran de forma aislada.

La progresiva acumulación de trabajos concernientes al estudio de la historia evolutiva de las especies de anfibios permite tener una idea general sobre algunos de los procesos de diferenciación que caracterizan la generación de nuevos linajes así como de la existencia de patrones biogeográficos comunes en algunas regiones del Planeta (Vences & Wake, 2007; Conn, 2009). En su revisión sobre los procesos de diferenciación y especiación en anfibios, Vences & Wake (2007) hacen especial hincapié en las diferencias entre los procesos vicariantes y los procesos adaptativos. Los primeros se refieren a la divergencia producida por el aislamiento físico, generalmente por cuestiones geográficas, de diferentes grupos poblacionales que eventualmente terminarán por constituir linajes independientes. Estos procesos alopátricos se subdividen a su vez en dicopátricos (Bush, 1994) y peripátricos (Mayr, 1954). En los procesos dicopátricos se produce una fragmentación de la distribución de un linaje que previamente presentaba una distribución continua, por

ejemplo por un evento orogénico o un cambio en las condiciones ecológicas. Los procesos peripátricos se dan cuando se produce la ocupación de nuevos territorios con los cuales se interrumpe el flujo génico, como puede ser por ejemplo la colonización puntual de una isla. Los procesos adaptativos permiten la divergencia de linajes pero manteniendo la posibilidad de interacciones genéticas. Estos procesos pueden ocurrir en parapatría pero también serían los responsables de la especiación en situaciones de simpatría (Bush, 1994; Crow *et al.*, 2010) e incluiría también la formación de nuevos linajes por procesos de hibridación.

A menudo los linajes formados por alguno de los procesos mencionados están sometidos a episodios continuos de aislamiento y recontacto, estableciéndose zonas de contacto secundario, también llamadas zonas de sutura (Remington, 1968, Hewitt, 2001, Swenson & Howard, 2004). La hibridación en anfibios es un fenómeno habitual y que ocurre incluso entre linajes antiguos, con una edad estimada de 21 millones de años (Wilson *et al.*, 1974; Prager & Wilson, 1975), por lo que la existencia de zonas de hibridación no necesariamente indica tiempos de divergencia recientes. De hecho, en ocasiones es posible observar huellas de eventos antiguos de hibridación en los patrones genéticos, como queda reflejado por ejemplo por la introgresión de

haplotipos mitocondriales entre especies (Bryson *et al.*, 2010). Aunque pueden resultar problemáticos desde un punto de vista taxonómico los procesos de hibridación a menudo representan excelentes modelos para estudios de especiación (Schwenk *et al.*, 2008).

Los datos disponibles relativos a anfibios indican que la diferenciación de linajes se produce principalmente por procesos vicariantes, principalmente dicopátricos (Vences & Wake, 2007). Esta idea se ve reforzada por la habitual distribución alopátrica tanto de linajes intraespecíficos como de especies hermanas (Watson & Littlejohn, 1985; Lynch, 1989; Vences & Wake, 2007; Zeisset & Beebee, 2009). En el modelo típico de diferenciación dicopátrica el aspecto clave es la interrupción del flujo génico en relación a la capacidad de dispersión de cada organismo. Al emplear marcadores moleculares es frecuente encontrar entre los anfibios elevados niveles de diferenciación geográfica. Por el contrario se conocen pocos casos en los que exista flujo génico entre todas las poblaciones de una especie. El grado de diferenciación a menudo se ve favorecido por ciertos rasgos particulares. la combinación de factores neutrales y selectivos que actúan durante largos periodos de tiempo. Es el caso en varios grupos de anfibios caracterizados por poseer desarrollo directo, como por ejemplo las salamandras del género *Thorius* en México

(Hanken & Wake, 1994, 1998) o las ranas del género *Philautus* en Sri Lanka (Manamendra & Pethiyagoda, 2005; Meegaskumbura & Manamendra-Arachchi, 2005), que presentan una elevada diversidad específica aparentemente asociada a una gran heterogeneidad ambiental pero también a una considerable antigüedad de sus linajes. Sin embargo este tipo de procesos diferenciadores también ocurren en organismos de amplia de distribución, con estrategias reproductivas comunes en anfibios y con tamaños poblacionales grandes, como puede ser el caso de los complejos *Pseudacris regilla* o *Bufo viridis* en anuros (Recuero *et al.*, 2006; Stöck *et al.*, 2006) o de *Salamandra salamandra* dentro del orden Caudata (García-París *et al.*, 2003).

Los datos obtenidos a lo largo de los últimos años apoyan la idea generalizada de que los anfibios constituyen un grupo de organismos con una pobre capacidad de dispersión y un considerable grado de filopatría (Blaustein *et al.*, 1994; Beebee, 1996) y que estos rasgos tienen una relación directa con los frecuentemente marcados patrones filogeográficos. Las diferencias observadas suelen ser el resultado de periodos relativamente largos de aislamiento. Por este motivo, la estructura geográfica más fuerte se observa en los lugares con poblaciones más estables a lo largo del tiempo. Por el contrario, en los casos de orígenes recientes, como por ejemplo el de

poblaciones introducidas por el hombre, la estructura suele ser uniforme (Slade & Moritz, 1998; Recuero *et al.* 2007). Esto no implica, sin embargo, que se conozcan casos de expansiones rápidas en anfibios. Por ejemplo, buena parte de las especies presentes hoy en día en el centro y norte de Europa han colonizado estas áreas desde refugios localizados al sur del continente, aprovechando las condiciones favorables aparecidas después de la última glaciación y estableciendo nuevas poblaciones caracterizadas por una baja diferenciación genética (ver por ejemplo Larson *et al.*, 1984; Palo *et al.*, 2004; Babik *et al.*, 2005; Stöck *et al.*, 2008). Sin embargo, es habitual que las recolonizaciones estén protagonizadas por un número pequeño de especies. Según Wiens (2004b), un componente principal en la formación de linajes por alopatría es el mantenimiento de los nichos ecológicos ancestrales, ya que la falta de capacidad para adaptarse a nuevos ambientes es un factor que favorece el aislamiento geográfico de grupos poblacionales, dificultando la conexión entre núcleos fragmentados y limitando la dispersión hacia nuevos territorios. Eso implica que las barreras al flujo génico entre poblaciones no son necesariamente accidentes geológicos, sino que también puede ser consecuencia de factores ecológicos, como por ejemplo la presencia de una especie competidora que impida la conexión entre poblaciones fragmentadas.

Los procesos adaptativos pueden, a su vez, generar algún tipo de barrera más o menos permeable al flujo génico. En algunos casos el aislamiento reproductivo puede llegar a ser total, permitiendo incluso procesos simpátricos de especiación (Bush, 1994; Crow et al., 2010), aunque generalmente es difícil establecer si los mecanismos de aislamiento reproductivo han actuado como agentes diferenciadores o si han surgido después de una divergencia previa en alopatría. Existen numerosos aspectos que presentan un considerable potencial adaptativo a la hora de generar nuevos linajes (Vences & Wake, 2007), incluyendo diferencias en la preferencia de hábitats, selección de caracteres sexuales particulares, alocronía en los periodos reproductivos, pedomorfosis, etc. Este último factor, por ejemplo, podría ser uno de los factores desencadenantes de radiaciones adaptativas como en el casos del complejo de especies de *Ambystoma mexicanum-tigrinum* (Shaffer & McKnight, 1996; Weisrock *et al.*, 2006; Parra-Olea *et al.*, 2007; Recuero *et al.*, 2010), un grupo de especies de origen relativamente reciente en el que se pueden observar notables niveles de diferenciación ecológica, presentando intrincados patrones de especiación e introgresión. Algunas de las especies de este grupo (*Ambystoma andersoni*, *A. taylori*, *A. dumerilii* y *A. mexicanum*) son estrictamente pedomórficas, mientras que en otros casos se observan frecuentes casos de pedomorfosis facultativa (Shaffer & Voss, 1996).

El estudio de los procesos evolutivos implicados en la formación de este tipo de complejos representa a menudo una tarea complicada, especialmente en los casos en los que el conocimiento disponible sobre los organismos implicados es limitado, a lo que hay que sumar a menudo situaciones críticas en el estado de conservación además de cierta incertidumbre taxonómica.

Al plantear un estudio mediante una aproximación filogeográfica nos cuestionamos qué procesos han tenido lugar durante la evolución de un determinado linaje, así como cuándo y dónde han ocurrido. De esta forma se pretende establecer la historia evolutiva del organismo en cuestión, situándola en un marco temporal y causal. Los patrones observados pueden ser comparados directamente con los datos acumulados para otros organismos en las mismas regiones geográficas, lo que se ha convertido en uno de los puntos fuertes de los estudios filogeográficos y ha derivado en la llamada filogeografía comparada (Bermingham & Moritz, 1998). A través de estas comparaciones se puede llegar a un conocimiento más profundo sobre los procesos que han modelado la diversidad en regiones concretas. El análisis filogeográfico de diferentes especies de anfibios en diferentes regiones presenta por tanto el potencial para obtener información sobre los procesos

evolutivos y biogeográficos, especialmente los ocurridos desde finales del Terciario y a lo largo del Cuaternario (Zeisset & Beebee, 2008).

Objetivos y estructura de la tesis

Esta tesis representa el compendio de publicaciones producidas durante el desarrollo de distintos proyectos de investigación sobre la sistemática molecular de diferentes especies de anfibios y que tienen como objetivo común profundizar en el conocimiento de la historia evolutiva de estos grupos. Las publicaciones elegidas se centran en estudios a nivel intraespecífico o bien incluyen diferentes especies cercanamente emparentadas. Nos encontramos de este modo con una serie de trabajos que analizan desde una perspectiva eminentemente filogeográfica los patrones de diversificación.

El primer capítulo se titula "**Filogeografía de *Pseudacris regilla* (Anura: Hylidae) en el oeste de Norte América, con una propuesta para un nuevo ajuste taxonómico**" y se trata de un artículo publicado en el número 39 de la revista *Molecular Phylogenetics and Evolution*. La región de la costa oeste de Norteamérica está ocupada por lo que tradicionalmente han sido consideradas dos especies pertenecientes al género *Pseudacris*. Una de ellas,

Pseudacris cadaverina, presenta una distribución extremadamente reducida que se limita al extremo norte de Baja California (México) y al sur de la Alta California (Estados Unidos). Por el contrario, *Pseudacris regilla* se encuentra ampliamente distribuida, desde las zonas tropicales y desiertos de Baja California hasta los bosques de coníferas de la Columbia Británica (Canadá), caracterizándose por una gran plasticidad ecológica así como por una considerable variabilidad que se traduce en la descripción de numerosas subespecies.

Debido a su amplia distribución se trata de un organismo ideal para estudiar los procesos históricos que han moldeado la diversidad de esta región. De especial interés resultan las poblaciones existentes en las zonas desérticas de Baja California, las cuales presentan un grado alto de aislamiento poblacional y representan un caso especial, ya que se trata junto a *Bufo punctatus* de las únicas especies de anfibios ampliamente distribuidas por esta interesante región biogeográfica.

En este estudio se analizan secuencias de ADN mitocondrial de 114 individuos procedentes de 48 poblaciones que cubren la totalidad del área de distribución, utilizando métodos de análisis filogenéticos, filogeográficos y de

demografía histórica para determinar la historia evolutiva de sus linajes. Además, la comparación de nuestro resultados con datos publicados correspondientes a marcadores nucleares nos permite plantear una propuesta taxonómica para este grupo.

Como segundo capitulo se incluye el artículo "**Diferenciación mitocondrial y biogeografía de *Hyla meridionalis* (Anura: Hylidae): un patrón filogeográfico inusual**", publicado en el número 34 de la revista *Journal of Biogeography*. En este trabajo se discuten los patrones de variabilidad genética así como los procesos y eventos históricos que han moldeado la distribución de esta especie, así como su diversidad intraespecífica. Su distribución actual abarca buena parte del oeste de la región mediterránea, con presencia tanto en el norte de África como en el suroeste de Europa. Existen también poblaciones insulares, tanto en islas continentales (Menorca), como en islas oceánicas (Canarias). Se trata, por consiguiente, de un caso ideal para estudiar fenómenos como el papel del Estrecho de Gibraltar como barrera a la dispersión de esta especie, la colonización de islas oceánicas por parte de organismos con una tolerancia osmótica limitada, o el papel de la actividad humana como vector de dispersión para la fauna.

Para este trabajo se han caracterizado, mediante diferentes secuencias de ADN mitocondrial, 112 individuos colectados de 36 poblaciones diferentes y representativas de la distribución total de la especies, permitiendo la reconstrucción de las relaciones filogenéticas entre los linajes observados y el establecimiento del grado de divergencia entre los mismos. Los patrones observados ofrecen además una interesante perspectiva sobre los eventos de colonización reciente de esta especie.

El capítulo número tres, titulado "**Historia evolutiva de *Lissotriton helveticus*: evaluación multilocus de la colonización ancestral o reciente de la Península Ibérica**", ha sido enviado a revisión a la revista *BMC Evolutionary Biology*. *Lissotriton helveticus* es un salamándrido cuya distribución actual se restringe a Europa Occidental, extendiéndose por el oeste hasta la isla de Gran Bretaña y Portugal y hacia el este hasta la República Checa. En la Península Ibérica su distribución se concentra básicamente en la región Eurosiberiana, con escasa penetración hacia ambientes mediterráneos. Toda su área de distribución ha sido fuertemente afectada por los periodos glaciales acaecidos durante el Pleistoceno, por lo que se trata de un caso de estudio realmente apropiado para estudiar el efecto de estos sucesivos cambios climáticos en la distribución y diversidad de los organismos afectados.

Además, otra peculiaridad de esta especie es su antigüedad, pues se estima que su origen, así como el del resto de especies del género *Lissotriton*, se remonta al Mioceno. La comparación de patrones entre especies estrechamente emparentadas y sometidas a los mismos eventos paleoclimáticos puede aportar luz sobre las respuestas alternativas que cada linaje presenta ante cambios similares.

Para conocer los patrones de diversidad genética en *Lissotriton helveticus* se han empleado secuencias de genes mitocondriales y nucleares. Se han analizado un total de 100 ejemplares procedentes de 35 poblaciones. Los resultados obtenidos nos ofrecen una visión general del efecto de las glaciaciones en esta especie y en la medida de lo posible han sido comparados con los disponibles para otras especies del género *Lissotriton* además de otras especies con distribuciones similares, de forma que se pueda establecer qué factores pueden controlar la respuesta especifica a grandes cambios en las condiciones ambientales.

Los capítulos cuarto, quinto y sexto presentan una aproximación al estudio de un grupo de especies de compleja situación sistemática como es el de las especies mexicanas del género *Ambystoma*. El capítulo cuarto, titulado

"**Hábitats acuáticos urbanos y conservación de especies gravemente amenazadas: el caso de *Ambystoma mexicanum* (Caudata, Ambystomatidae)**", ha sido publicado en el número 47 de la revista *Annales Zoologici Fennici*. El ajolote (*Ambystoma mexicanum*) es una especie de salamandra caracterizada por su neotenia estricta y endémica del Valle de México. Su estado actual de conservación en estado silvestre es crítico, con únicamente dos poblaciones salvajes conocidas, ambas amenazadas de forma alarmante por factores como el aislamiento poblacional, la contaminación acuática o la introducción de especies exóticas entre otros.

No muy lejos de las poblaciones salvajes conocidas, en el parque urbano de Chapultepec, se confirmó la existencia de una población reproductora de salamandras del género *Ambystoma* en el denominado lago Viejo, al aparecer durante las tareas de limpieza del lago ejemplares adultos así como algunas puestas. El examen morfológico de los ejemplares permitió su identificación como *Ambystoma mexicanum*, procediéndose posteriormente a su caracterización desde un punto de vista genético utilizando marcadores mitocondriales. Nuestros resultados confirman la identificación morfológica, pero también confirman la generalizada falta de monofilia dentro de este grupo de especies, por lo que se plantea la necesidad de desarrollar nuevos

marcadores que permitan la caracterización de estas especies y poblaciones a escala más fina.

En este contexto se presenta el quinto capitulo, "**Marcadores microsatélites polimórficos para salamandras mexicanas del género *Ambystoma***", publicado en el número 7 de la revista *Molecular Ecology Notes.* Las especies mexicanas del género *Ambystoma* forman un complejo de difícil estudio por diferentes motivos. Por un lado se trata de una radiación cuyos linajes tienen un origen relativamente reciente pero a menudo con fuertes diferencias ecológicas entre ellos, lo que se traduce en complejos patrones filogenéticos. Además se caracteriza por la adquisición de manera repetida de desarrollo pedomórfico estricto. Por último, en buena parte de los casos las poblaciones están más o menos aisladas y afrontan graves amenazas de conservación. Estos rasgos convierten a este grupo en un complicado pero especialmente interesante objeto de estudio tanto desde un punto de vista evolutivo como de conservación.

El desarrollo de marcadores moleculares altamente variables es fundamental para el estudio de complejos como éste y determinar los grados de conectividad, flujo génico y estructura poblacional. En este caso se han

caracterizado varios microsatélites polimórficos que pueden ser empleados en diferentes especies del complejo, lo que permitirá su empleo no sólo en estudios de genética de poblaciones, sino futuras aproximaciones para clarificar la situación taxonómica del grupo.

El sexto capítulo se titula "**Aproximación a la genética de la conservación de poblaciones amenazadas de *Ambystoma* (Caudata: Ambystomatidae) de México**", manuscrito actualmente en preparación. Como ya hemos comentado, este grupo de especies se caracteriza por un preocupante estado de conservación. El grado de amenaza es especialmente crítico para las especies estrictamente pedomórficas. Estos animales presentan distribuciones muy restringidas y se encuentran de forma aislada en lagos que de forma generalizada sufren fuertes perturbaciones derivadas de la actividad humana. Por ejemplo, la continua transformación del sistema lacustre del Valle de México ha reducido el hábitat actualmente disponible para *Ambystoma mexicanum* a apenas un 1% de su extensión histórica. Además, lo poco que queda se encuentra en pobres condiciones, lo que no permitirá mantener poblaciones viables de esta especie a largo plazo si no se toman medidas efectivas al respecto. La caracterización de los patrones de diversidad genética de especies y poblaciones amenazadas resulta de vital importancia a la hora de

diseñar planes de conservación y determinar las prioridades de acción.

En este estudio se caracterizan, mediante el uso de microsatélites, ocho poblaciones correspondientes a seis especies del género *Ambystoma*. Cuatro de ellas corresponden a especies con un ciclo biológico típico, es decir, presentan metamorfosis: *A. velasci*, *A. granulosum*, *A. rivulare* y *A. altamirani*. Las otras dos especies son estrictamente pedomórficas: *A. mexicanum* y *A. andersoni*. Al analizar poblaciones con diferentes ciclos biológicos se puede comparar el efecto de los mismos sobre la estructura genética de las mismas, más allá de los condicionantes locales que puedan sufrir cada una de ellas.

El conjunto de estos seis capítulos pone de manifiesto la existencia de una serie de problemas que generalmente pasan desapercibíos en estudios de carácter biogeográfico o de biología de la conservación. En primer lugar destaca el problema de las colonizaciones recientes y su importancia en la constitución de la fauna de una determinada región biogeográfica, lo que a menudo conlleva problemas a la hora de aplicar criterios de conservación (tanto en el caso de *Hyla meridionalis* como de *Ambystoma mexicanum*). Desde un punto de vista biogeográfico destaca el problema de los movimientos a gran escala de especies sujetas a cambios climáticos, con la existencia de

extinciones y recolonizaciones en áreas de ocupación ancestral. Las consecuencias de estos cambios demográficos dependen marcadamente de la brevedad del periodo disponible par la recolonización lo que en ocasiones supone una pérdida significativa de diversidad genética, como en el caso de *Lissotriton helveticus*, y en otros la formación de linajes genéticamente diferenciados, como en el caso de *Pseudacris regilla*.

El establecimiento de generalizaciones sobre estos procesos requiere la estudio comparado de numerosos casos singulares, pero el paso inicial es sin duda la puesta de relieve de la existencia de los mismos y la formulación de hipótesis demostrables, objetivo fundamental de esta tesis.

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II. Capítulo 1

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Phylogeography of *Pseudacris regilla* (Anura: Hylidae) in western North America, with a proposal for a new taxonomic rearrangement

Ernesto Recuero [a], Íñigo Martínez-Solano [a,b], Gabriela Parra-Olea [c,*], Mario García-París [a]

[a] *Museo Nacional de Ciencias Naturales, CSIC, José Gutiérrez Abascal, 2. 28006 Madrid, Spain*
[b] *Museum of Vertebrate Zoology, University of California, Berkeley, 3101 Valley Life Sciences Building, 94720 Berkeley, CA, USA*
[c] *Instituto de Biología, UNAM. AP 70-153, CP 04510, Ciudad Universitaria, México D.F., Mexico*

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Abstract

The Baja California populations of *Pseudacris regilla*, a widespread species in Western North America ranging from British Columbia to southern Baja California, are characterized by extensive geographic fragmentation. We performed phylogeographic and historical demographic analyses on 609 bp of the cytochrome *b* mitochondrial gene of 110 individuals representing 28 populations to determine the relative influences of current and historical processes in shaping the present distribution of genetic diversity on the Baja California Peninsula. Haplotypes from this area were nested in a clade with three well-differentiated groups. Two of these groups are from Baja California Sur and another is from California and Baja California. The estimated date for the split of these groups, between 0.9–1 Ma, fits with previously proposed hypotheses of vicariance due to different transpeninsular seaways, although successive population fragmentation and expansion due to climatic oscillations during Pleistocene glaciations cannot be discarded. Historical demographic analyses detected signs of past population expansions, especially in the southernmost group. With respect to populations north of this region, two older clades were identified, one with haplotypes mainly distributed in central California, and the other corresponding to the northern half of the species range, in what apparently is a recurrent pattern in the Pacific coast of North America. Based on the concordance between mt-DNA and available allozyme data indicating that these species have a long independent evolutionary history, we propose to consider the three major clades as distinct species: *P. regilla*, *P. pacifica*, and *P. hypochondriaca*.

Keywords: Phylogeography; Historical demography; *Pseudacris regilla*; *Pseudacris pacifica*; *Pseudacris hypochondriaca*; mt-DNA; Midpeninsular seaway

1. Introduction

The Baja California Peninsula (BCP) is characterized by a number of historical peculiarities that have long attracted the interest of biologists. This narrow peninsula, over 1200 km long, is characterized by great ecological and geological complexity as well as a high biological diversity, and thus has been the subject of several biogeographic studies (Durham and Allison, 1960; Grismer, 1994a; Johnson and Ward, 2002; Murphy and Aguirre-León, 2002; Savage, 1960; Taylor and Regal, 1978; Wiggins, 1960; Wiggins, 1999).

For vertebrate taxa, three main biogeographical models have been postulated to explain the current distribution and diversity of Baja California's fauna. The first model, a late Quaternary dispersal related to climatic changes associated with glacial cycles (Orr, 1960; Savage, 1960) is based on the successive change of ecological conditions of the area. A second model postulates vicariant events during the Pliocene and Pleistocene due to different geographical barriers that isolated populations multiple times, the results of which are reflected in the diversity patterns found in different organisms that exhibit well-differentiated north vs. south intraspecific lineages

* Corresponding author.
E-mail address: gparra@ibiologia.unam.mx (G. Parra-Olea).

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(Aguirre et al., 1999; Riddle et al., 2000a; Upton and Murphy, 1997). A third model would include, as a causal factor to explain current patterns, the late Miocene vicariant event associated with separation of the BCP from mainland Mexico, which seems to explain the patterns observed in several other species (Grismer, 1994a).

To test some of these hypotheses, Riddle et al. (2000b) analyzed mitochondrial DNA (mt-DNA) variation of several vertebrate species and found similar patterns in most of the analyzed taxa, supporting the existence of past barriers to gene flow, the most common pattern being two well-differentiated north-south clades, whose divergence presumably dated to the Pleistocene.

Unlike other vertebrate groups, the amphibian community of BCP presents a pattern that apparently fits with the existence of a peninsular effect (Busack and Hedges, 1984), with species richness decreasing from the north to the south of the peninsula, and only three species (*Scaphiopus couchii*, *Bufo punctatus*, and *Pseudacris regilla*) widely distributed along the entire peninsula. Interestingly, *B. punctatus*, the only amphibian species included in the analysis of Riddle et al. (2000b), does not display substantial mitochondrial variation along the BCP, but presents a pattern conforming to the third hypothesis (Jaeger et al., 2005).

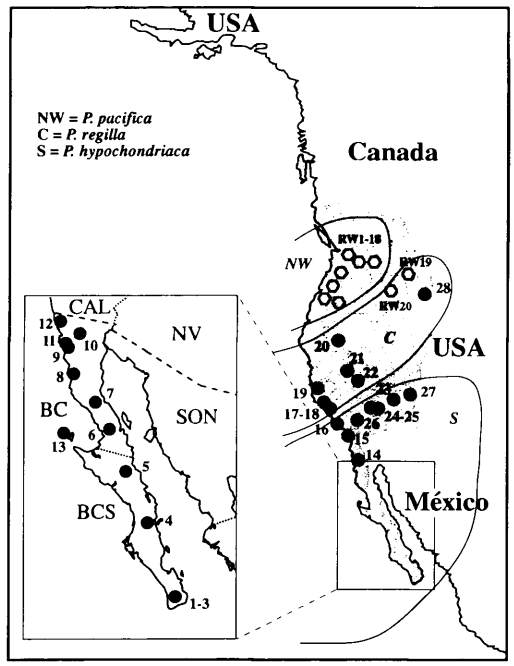

Fig. 1. Distribution of *P. regilla* in western North America, showing the new taxonomic proposal (the three groups delimited by solid lines), based on allozyme (Case et al., 1975) and mt-DNA data (Ripplinger and Wagner, 2004; this study). Populations sampled for mt-DNA are marked with hexagons (Ripplinger and Wagner, 2004) and black dots (this study, numbers refer to Table 1). Sampled populations in the Baja California Peninsula are highlighted. CAL = California, NV = Nevada, BC = Baja California, BCS = Baja California Sur, and SON = Sonora.

Pseudacris regilla is a widespread species present along the Pacific coast of North America from southern British Columbia to the southern tip of the BCP (Stebbins, 1985), being the most abundant and ubiquitous amphibian in western North America (Brattstrom and Warren, 1955; Matthews et al., 2001). Studies of mating call (Snyder and Jameson, 1965), morphology (Jameson et al., 1966), allozymes (Case et al., 1975), and mt-DNA (Ripplinger and Wagner, 2004) have revealed intraspecific variability, and several subspecies have been described, of which seven are currently recognized (Crother et al., 2000; Duellman, 1970). In BCP, *P. regilla* can be found in montane and mesic areas as well as in desert oases (Grismer, 2002a). According to the latest taxonomic revision, two subspecies are present in BCP: *P. r. hypochondriaca*, in the northern portion of the Peninsula; and *P. r. curta*, endemic to the area south to the Vizcaíno desert (Duellman, 1970) (Fig. 1).

In the present study, we used mt-DNA to determine the geographic patterns of genetic variation among the southern (BCP) populations of *P. regilla*. We performed phylogeographic and historical demographic analyses to determine such patterns and to postulate a solid hypothesis for the evolutionary history of this species in the BCP. Our results are discussed in the context of our current knowledge about general biogeographic patterns in the BCP, with implications for the taxonomy and conservation of the species in Baja California.

2. Materials and methods

2.1. Sampling

We obtained tissue samples from 77 individuals, adults and tadpoles, from 12 populations on the BCP. The sampling was completed with 33 individuals from the Museum of Vertebrate Zoology tissue bank (University of California, Berkeley) corresponding to 16 additional populations from Baja California, California (Alta California), Nevada and Montana (Fig. 1; Table 1). *Pseudacris cadaverina*, the sister taxon to *P. regilla* (Moriarty and Cannatella, 2004), was used as outgroup.

2.2. Mitochondrial DNA amplification and sequencing

Total genomic DNA was extracted from ethanol-preserved tissues (muscle, liver, and tail fin from tadpoles) using a phenol–chloroform protocol (Sambrook et al., 1989), preceded by a digestion with proteinase K. Polymerase chain reaction (PCR) was used to amplify 609 bp of the mitochondrial cytochrome *b* gene (*cytb*), using the primers MVZ15 and MVZ18 (Moritz et al., 1992). PCRs were performed in a total volume of 25 μl, including 1 U *Taq* polymerase (Biotools, 5 U/ml), 1.0 μl of each primer (10 μmol/L), 0.4 mM dNTPs (10 nmol/L), 1.5 μl $MgCl_2$ (25 mmol/L), and 67 mM of a reaction buffer (Tris–HCl, pH 8.3, Biotools).

Table 1
Populations sampled in this study

ID	Locality	Latitude	Longitude	*n*	Haplotypes
1	México: Baja California: Sierra de la Laguna (1)	23° 31.711′ N	110° 01.591′ W	5	XIX, XX, XXI, XXII, XX
2	México: Baja California: Sierra de la Laguna (2)	23° 33.019′ N	109° 59.500′ W	3	XXIII, XXIV
3	México: Baja California: Sierra de la Laguna (3)	23° 32.783′ N	109° 58.464′ W	7	XIX, XXII, XXIII, XXV, XXVI
4	México: Baja California: Las Parras	25° 58.655′ N	111° 27.888′ W	9	I
5	México: Baja California: San Ignacio	27° 17.098′ N	112° 53.934′ W	15	II, III, IV, V, VI
6	México: Baja California Norte: La Ciénaga	28° 36.841′ N	114° 02.699′ W	5	VII, VIII, IX
7	México: Baja California Norte: Cataviña	29° 43.555′ N	114° 42.772′ W	7	VII, X
8	México: Baja California Norte: San Telmo	30° 58.554′ N	116° 05.882′ W	6	XI, XII, XIII, XIV
9	México: Baja California Norte: Ojos Negros	31° 52.795′ N	116° 21.609′ W	4	XIII, XV, XVI
10	México: Baja California Norte: Las Huertas	32° 00.089′ N	115° 57.034′ W	5	XI, XII, XIV, XVI
11	México: Baja California Norte: El Tigre	31° 57.150′ N	116° 44.048′ W	7	XI, XIII, XVII
12	México: Baja California Norte: El Descanso	32° 11.899′ N	116° 53.341′ W	7	XV, XVII, XVIII
13	México: Baja California Norte: Isla Cedros	28° 06.666′ N	115° 10.666′ W	2	XXVIII
14	USA: California: San Diego Co., Pala junction on Hwy. 76	33° 21.918′ N	117° 01.926′ W	1	XI
15	USA: California: Los Angeles Co., Santa Monica Mountains	34° 06.705′ N	118° 46.345′ W	1	XI
16	USA: California: Santa Barbara Co., Buellton	34° 36.656′ N	120° 11.951′ W	2	XV, XXIX
17	USA: California: San Luis Obispo Co., Los Padres Ntl. Forest	35° 17.626′ N	120° 19.327′ W	1	XXXIII
18	USA: California: San Luis Obispo Co., Santa Margarita	35° 25.487′ N	120° 34.052′ W	1	XI
19	USA: California: Monterey Co., McClusky Slough	36° 50.356′ N	121° 47.387′ W	2	XXXVII, XXXVIII
20	USA: California: Shasta Co., Shingleton	40° 28.077′ N	121° 53.082′ W	1	XI
21	USA: California: Alpine Co., Highland Lakes	38° 29.446′ N	119° 48.159′ W	2	XXXV, XXXVI
22	USA: California: Tuolumne Co., Kennedy Meadows	38° 19.634′ N	119° 39.418′ W	1	XXXIX
23	USA: California: Inyo Co., Surprise Canyon	36° 06.747′ N	117° 10.473′ W	4	XI
24	USA: California: Inyo Co., Little Lake	35° 56.218′ N	117° 54.343′ W	4	XXX, XXXII, XXXIII
25	USA: California: Inyo Co., Indian Joe Canyon	35° 49.772′ N	117° 23.620′ W	2	XI
26	USA: California: Kern Co., Bakersfield	35° 31.909′ N	118° 38.806′ W	1	XXX
27	USA: Nevada: Nye Co., Beatty	36° 54.500′ N	116° 45.500′ W	3	XXXI
28	USA: Montana: Missoula Co., Clark Fork River	46° 49.017′ N	113° 42.153′ W	2	XXXIV

Geographical coordinates, number of individuals sampled (*n*) and haplotypes found (see also Figs. 2 and 4).

PCRs consisted of 35 cycles with a denaturing temperature of 94 °C (1 min), annealing at 56 °C (1 min), and extension at 72 °C (1 min). Double-strand templates were cleaned using sodium acetate and ethanol to precipitate the PCR products and then re-suspended in 22 μl of ddH_2O. Sequencing reactions were performed for both strands and sequenced on an ABI PRISM 3700 DNA sequencer following the manufacturer's instructions.

2.3. Sequence alignment and phylogenetic analyses

All sequences were compiled using Sequence Navigator version 1.0.1 (Applied Biosystems) and aligned manually. Thirty-seven additional haplotypes obtained from GenBank (Accession Nos. AY363181–AY363219, Ripplinger and Wagner, 2004), corresponding to 20 populations from Washington, Oregon, and Idaho were added to the final alignment for the phylogenetic analyses. Genetic divergence (*p*-uncorrected and maximum likelihood (ML)-corrected sequence divergence) in pairwise comparisons were calculated using the software PAUP*4.0b10 (Swofford, 2002). Mean sequence divergence between groups was calculated with MEGA2 (Kumar et al., 2001).

Phylogenetic analyses including all haplotypes were performed with PAUP. Maximum parsimony (MP) phylogenies were estimated using the heuristic search algorithm with TBR branch swapping and 10 random addition sequence replicates. Each base position was treated as an unordered character with four alternative states. We used nonparametric bootstrapping (1000 pseudoreplicates) to assess the stability of internal branches in the resulting topologies (Felsenstein, 1985).

Data were analyzed with the software ModelTest 3.6 (Posada and Crandall, 1998) to determine the substitution model that best fit our data for subsequent maximum likelihood analyses (ML, Felsenstein, 1981) and to calculate the transition/transversion ratio. ML analyses were performed using the heuristic search algorithm in PAUP with model parameters estimated with Modeltest. We used nonparametric bootstrapping (100 pseudoreplicates) to assess the stability of internal branches.

Bayesian phylogenetic analyses were conducted with MrBayes 3.0 (Huelsenbeck and Ronquist, 2001). Analyses were initiated with random starting trees and run for 2,500,000 generations, sampling every 100 generations. Of the resulting 25,000 trees, 2000 were discarded as "burnin." Posterior clade probabilities were used to assess nodal support.

We tested the null hypothesis of clocklike rates in our sequence dataset with a likelihood ratio test (Felsenstein, 1981) as implemented in ModelTest 3.6.

2.4. Molecular diversity, genetic structure, and phylogeographic analyses

Estimates of mean nucleotide and haplotype diversities within the main mt-DNA lineages identified by the

previous analyses within *P. regilla* were calculated with DNASP 4.0 (Rozas and Rozas, 1999). We also used analysis of molecular variance (AMOVA, Excoffier et al., 1992) to characterize patterns of genetic variation at different hierarchical levels (individuals, populations, and the main mt-DNA lineages identified by phylogenetic analyses) as implemented by Arlequin v. 2000 (Schneider et al., 2000). Levels of significance of statistics characterizing variation at different hierarchical levels were assessed through 100,000 permutations.

Phylogeographic analyses were based on a nested cladistic analysis (NCA) of haplotype data. We constructed a haplotype network from mt-DNA sequences using the software TCS 1.18 (Clement et al., 2000), which follows the statistical parsimony algorithm described in Templeton et al. (1992). Then, a nested statistical design was used following the general guidelines provided by Templeton et al. (1995). Finally, we tested for the existence of geographical associations of the different clades by means of: (i) a categorical test, in which clades showing genetic and/or geographic variation are tested against their geographical location (permutational contingency analysis, see Templeton et al., 1995); and (ii) a second test that incorporates the information on geographical distances and relative positions among the sampled populations. These tests were performed with the GeoDis 2.2 software package (Posada et al., 2000, 1,000,000 permutations), and the evolutionary patterns were identified following the inference key (updated 14th July 2004) provided by these authors with GeoDis 2.2.

2.5. Historical demography

To explore the demographic histories of the main mt-DNA lineages within *P. regilla*, mismatch analysis of mt-DNA sequences within each group was performed with Arlequin v. 2000 (Schneider et al., 2000). This analysis compares the frequency distribution of pairwise differences between haplotypes with that expected under a model of population expansion. The fit of observed versus modeled distributions is assessed by a goodness-of-fit statistic (p), whose significance is tested using a bootstrap approach (1000 replicates). The frequency distribution is usually unimodal for lineages that have undergone recent population expansions and multimodal for lineages whose populations are either subdivided or in equilibrium.

A complementary approach to analyze the historical demography of the main lineages within sampled populations of *P. regilla* was based on the coalescent-based method of Kuhner et al. (1998). This method calculates maximum likelihood estimates of theta (θ_{ML}), where θ equals twice female effective population size (Ne) times mutation rate (μ), and an exponential growth parameter (g). Both parameters and their standard deviations were calculated using the software Fluctuate 1.4 (Kuhner et al., 1998). Each Markov chain Monte Carlo run consisted of 10 short chains (with sampling increments of 10; 1000 steps/chain) and 10 long chains (sampling increment: 10; 20,000 steps). A neighbor-joining tree based on uncorrected distances was used as a starting tree. We used several different starting values for g and performed different replicates with different seed numbers to check for convergence of results. We followed the criterion of Lessa et al. (2003) for the interpretation of results and assumed population growth if g was consistently higher than three times its standard deviation (SD).

Additionally, Fu's tests of neutrality (Fu, 1997) were performed for sequences within each of the previously identified mt-DNA lineages. Significant negative values of Fu's statistics can be interpreted (in the absence of selection, as it is assumed to be the case for mt-DNA) as a signature of population expansion. This statistics was calculated with Arlequin v. 2000 and its significance was assessed through 10,000 simulations.

3. Results

3.1. Phylogenetic analyses

No insertions or deletions were present in the sequences obtained. Fifty-nine variable positions were found among the 110 sequences of *cytb* in the samples of *P. regilla* analyzed, defining 39 different haplotypes (Fig. 2; and Table 1). These mutations involved nine non-synonymous substitutions. The TrN + I + G model of evolution was selected by AIC in ModelTest 3.6. The ML-estimated transition–transversion ratio was 20.6. All sequences were deposited in GenBank under Accession Nos. DQ195169–DQ195207.

The phylogenetic analyses recovered well-structured trees with three main groups (Figs. 2 and 3): a "northwestern" group present in Washington and Oregon; a "central" group, distributed from Central California through eastern Oregon and Idaho to western Montana; and a "southern" group, distributed in the southern half of California and in the BCP. The "southern" group includes the populations of *P. r. hypochondriaca* and those corresponding to *P. r. curta*, which are in turn separated into two clades corresponding to the populations from the Sierra de la Laguna ("Laguna" group) and populations from oases south to the Vizcaíno Desert (Las Parras and San Ignacio, "Oases" group). In general, bootstrap values are moderate to low in the "*hypochondriaca*" group, probably due to the overall low number of variable characters in the dataset (Figs. 2 and 3). ML-corrected sequence divergence values ranged from 0.17 to 1.63% between samples from Sierra de la Laguna, 0.17 to 0.69% between samples from Las Parras-San Ignacio, and 0.17 to 1.87% between samples within *P. r. hypochondriaca*. Within *P. r. curta*, haplotypes from La Laguna and Las Parras-San Ignacio differed by 1.26 to 2.99%. Differences between groups ranged from 1.26 to 3.98% between *P. r. curta* and *P. r. hypochondriaca*, and 3.96 to 8.50% between both subspecies and samples from the "central" group. Mean p-uncorrected distances between groups were 4.18% between the "*curta*" and "central" clades, 3.39% between "*hypochondriaca*" and "central," 1.91% between

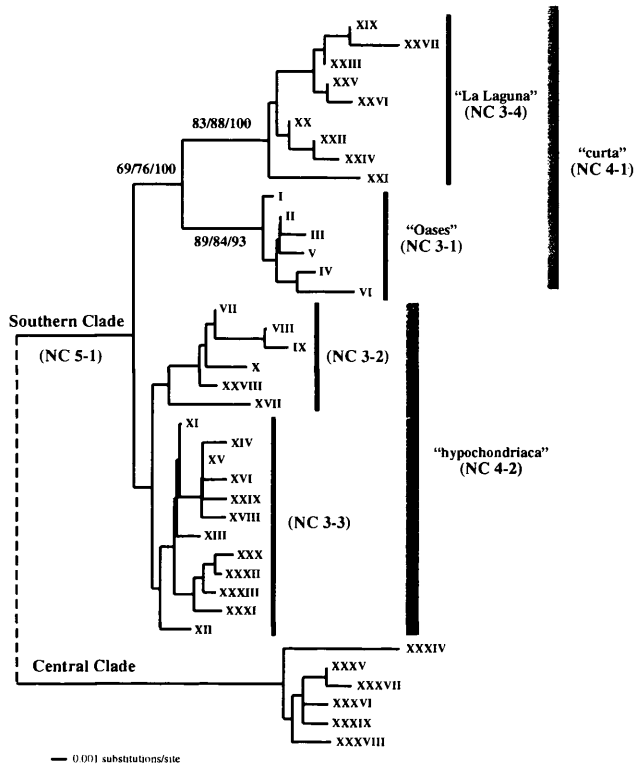

Fig. 2. Neighbor-joining tree based on maximum-likelihood corrected genetic distances depicting relationships between *cytb* haplotypes (Table 1) from the "Southern" and "Central" clades in this study. Bootstrap values (MP, ML, and % Bayesian posterior probabilities) at relevant nodes are shown. The main groups used in the nested clade analysis of sequence data are also shown (see also Fig. 4). Haplotype XI also occurs in some Central Clade populations.

"*hypochondriaca*" and "*curta*," and 1.71% within the "*curta*" clade. Distances between the "northwestern" group and the other two ranged from 5 to 6.5%.

The results of the likelihood ratio test showed no significant differences in the likelihood scores when comparing trees estimated with or without enforcing a molecular clock (with: $-\ln L = 1972.2613$; without, $-\ln L = 1929.5946$, ratio = 85.333496; df = 77; $p = 0.24133$). Thus, the null hypotheses of homogeneous evolutionary rates among sequences cannot be rejected.

Subsequent analyses were performed on the three main mt-DNA lineages recovered within "southern" populations: the "La Laguna" group (populations 1–3), the "oases" group (populations 4–5), and the "*hypochondriaca*" group (populations 6–18 and 22–26).

3.2. Molecular diversity

Values of nucleotide and haplotype diversity are presented in Table 2. In general, the values of haplotypic diversity observed are high (0.78–0.90) and similar between the main mt-DNA lineages, although it was always highest in the *hypochondriaca* group, where up to 18 haplotypes were observed, and lowest in the "oases" group (six haplotypes).

Results from AMOVA indicate that most of the observed variation among mt-DNA lineages is related to differences between groups (68.03% of the total variance observed). Lower values were observed for variance related to differences among populations within groups (15.50%) and within populations (16.47%). All hierarchical components of genetic variation were highly significant ($p < 0.0001$).

3.3. Phylogeography

Haplotypes from the "central" and "northwestern" groups fall outside the 95% confidence limit for the maximum parsimony connection of haplotypes, which was fixed at 10 mutational steps. The remaining 33 haplotypes were arranged in a nested design within a single five-step clade (Fig. 4). The three main mt-DNA groups recovered in the phylogenetic analyses of haplotypes ("Laguna," "Oases," and "*hypochondriaca*") corresponded to three-step clades 3–4, 3–1, and 3–2 + 3–3, respectively.

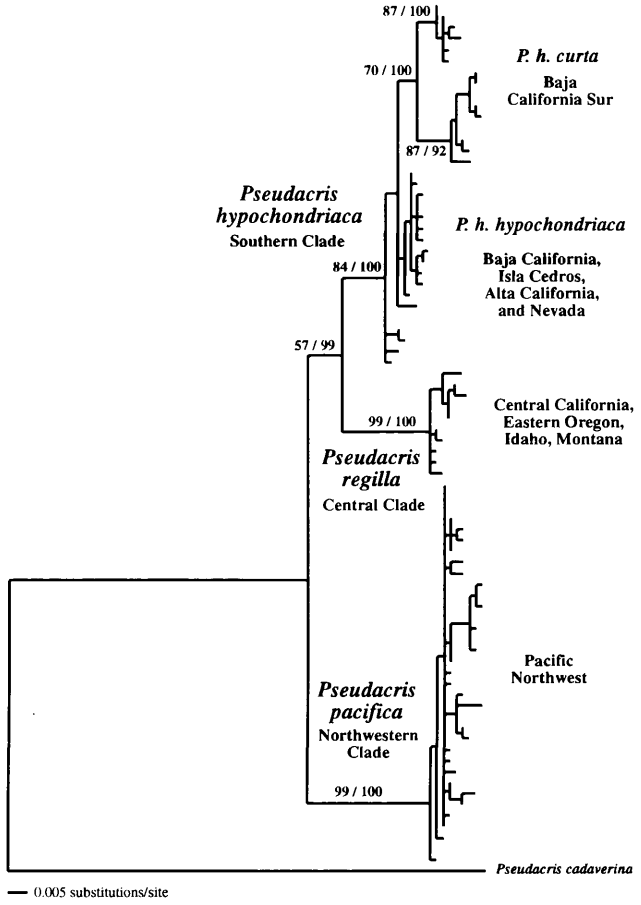

Fig. 3. Maximum likelihood tree ($-\ln L = 1937.4627$) of *cytb* sequences (Ripplinger and Wagner, 2004, this study) showing the new proposal of taxonomic rearrangement for populations formerly included within of *P. regilla*. Bootstrap values (MP and % Bayesian posterior probabilities) at relevant nodes are shown.

Table 2
Molecular diversity (number of haplotypes, nucleotide and haplotype diversity), Fu's *Fs* statistic and maximum likelihood historical demographic parameters: θ (1/sites*generation) and g ($1/\mu$*generations) in the main mt-DNA lineages within *P. regilla* identified in the present study

mt-DNA lineage	*N*	No. of haplotypes	Nucleotide diversity (SD)	Haplotype diversity (SD)	Fu's Fs	*g* (SD)	θ (SD)
hypochondriaca	64	18	0.006 (0.003)	0.904 (0.025)	−4.56 ns	319.681 (150.158)	0.015 (0.002)
Oases	24	6	0.003 (0.002)	0.790 (0.051)	−0.31 ns	248.484 (334.163)	0.003 (0.001)
Laguna	15	9	0.005 (0.003)	0.886 (0.069)	−2.87*	857.048 (229.564)	0.027 (0.010)

Standard deviations (SD) in parentheses *N* = sample size.
* significant.

We found significant geographical association between clades and their geographical locations at all nesting levels (Table 3). Hypotheses derived from the interpretation of Dc and Dn values according to Templeton's (2004) updated inference key are also shown in Table 3. For clade 1–1, including haplotype I (characterizing all individuals from the population of Las Parras, see Table 1), and the haplotypes II, IV, and VI (found only in the population of San Ignacio), the analysis could not discriminate between long distance movements and the combined effects of gradual movement during a past range expansion and subsequent fragmentation. The same inference was produced for clades 3–2 (haplotypes from southernmost populations of the *hypochondriaca* group—including Isla Cedros—vs. haplotypes from some northern populations of *hypochondriaca*) and clade 4–2 (haplotypes nested within clade 3–2 vs.

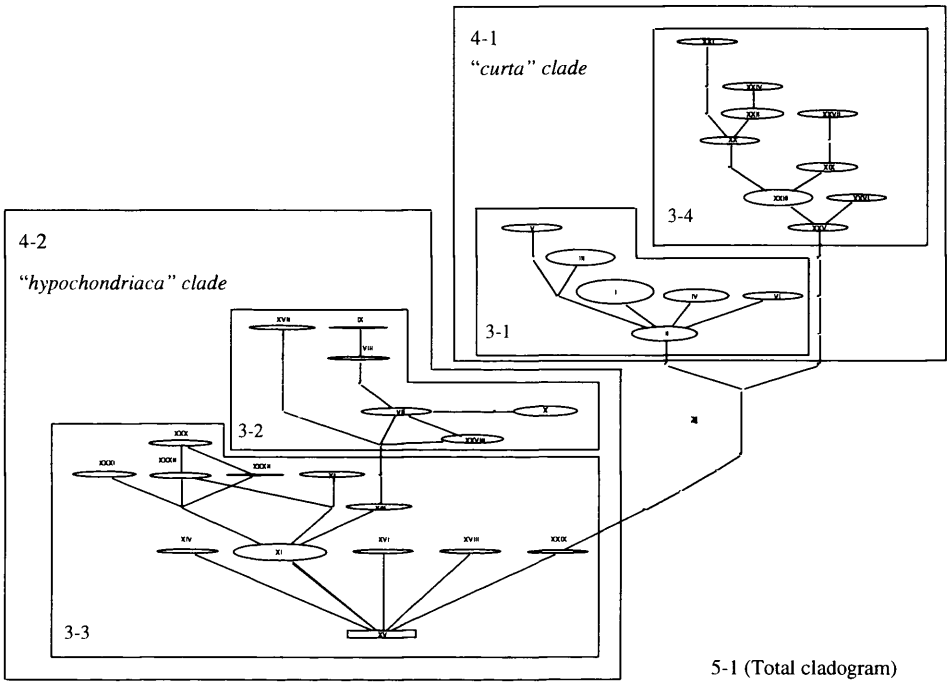

Fig. 4. Nested design for the statistical parsimony haplotype network of *cytb* sequences from *P. regilla* in this study (see haplotype designation in Table 1).

Table 3
Results of the nested cladistic analysis of haplotypes of *P. regilla*

Haplotype group	Chi-square statistic	Inference chain	Inferred event
Clade 1–1	18.00 ($p < 0.0001$)	1–2–3–5–15–21	Insufficient genetic evidence to discriminate between long distance movements and the combined effects of gradual movement during a past range expansion and fragmentation (Las Parras, S. Ignacio)
Clade 1–18	8.00 (ns)	1–19	Allopatric fragmentation (Isla Cedros, La Ciénaga-Cataviña)
Clade 2–3	9.30 (ns)	1–2–3–4	Restricted gene flow with isolation by distance (Isla Cedros, La Ciénaga, Cataviña)
Clade 2–4	21.61 ($p < 0.01$)	1–2–11–17–4	Restricted gene flow with isolation by distance (San Telmo-Las Huertas, Nevada-S. California)
Clade 3–1	4.80 (ns)	1–2–3–4	Restricted gene flow with isolation by distance (S. Ignacio, S. Ignacio-Las Parras)
Clade 3–2	18.00 ($p < 0.001$)	1–19–20–2–11–12–13–21	Insufficient genetic evidence to discriminate between long distance movements and the combined effects of gradual movement during a past range expansion and fragmentation (La Ciénaga-Cataviña-Isla Cedros, El Tigre-El Descanso)
Clade 4–1	32.57 ($p < 0.0001$)	1–19	Allopatric fragmentation (La Laguna, Las Parras-S. Ignacio)
Clade 4–2	51.27 ($p < 0.0001$)	1–2–11–12–13–21	Insufficient genetic evidence to discriminate between long distance movements and the combined effects of gradual movement during a past range expansion and fragmentation (La Ciénaga-Cataviña-Isla Cedros-El Tigre-El Descanso, Nevada-S. California, BCN)
Total cladogram	104,00 ($p < 0.0001$)	1–19	Allopatric fragmentation (*curta, hypochondriaca*)

For each clade showing geographic and/or genetic variation, results of the categorical χ^2 test, and inference chain and inferred events after the geographical distances tests are presented.

haplotypes from all other populations of *hypochondriaca*). Allopatric fragmentation was inferred for clades 1–18 (including haplotype XXVIII, exclusive of individuals from Isla Cedros, vs. haplotypes from inland southernmost populations of *hypochondriaca*), 4–1 (haplotypes found in La Laguna vs. those found in Las Parras and San Ignacio), and the total cladogram (haplotypes from *P. r. curta vs. P. r. hypochondriaca*). Finally, restricted gene flow with isolation by distance was inferred for clades 2–3 (haplotypes nested within 1–18 vs. haplotypes found in the population of La Ciénaga vs. haplotypes found in Cataviña), 2–4 (haplotypes from San Telmo and Las Huertas vs. haplotypes from

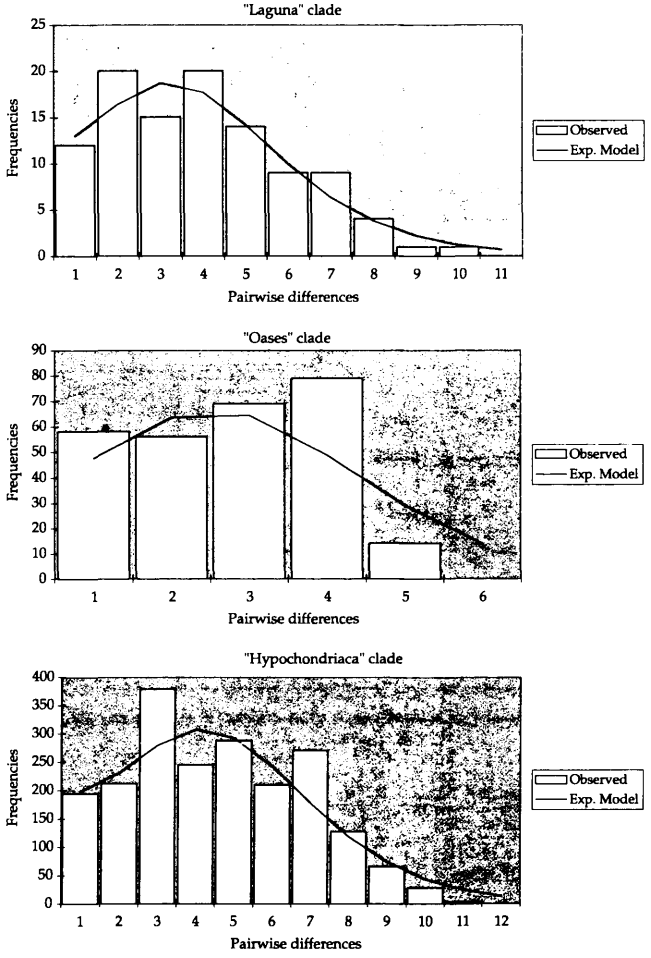

Fig. 5. Mismatch distributions of *cytb* sequences in the three mt-DNA lineages identified in this study. Solid bars represent the observed frequencies of pairwise differences between haplotypes; open bars represent those expected under the model of population expansion.

Nevada and southern California), and 3–1 (haplotypes III and V, exclusive from S. Ignacio, vs. haplotypes nested within 1–1).

3.4. Historical demography

The mismatch distributions for the three main mt-DNA lineages identified are presented in Fig. 5. The population expansion model was not rejected in either case ($p = 0.20$, 0.54, and 0.93, respectively).

Female effective population size (θ) and growth (g) were estimated by ML for the three "southern" lineages. The results are presented in Table 2. The values of θ obtained in different simulations were always higher in the "Laguna" group, being on average more than two times the values observed for the "*hypochondriaca*" group and almost 10 times higher than those from the "Oases" clade, suggesting differences in ancestral population sizes in these lineages. ML estimates of g for each of these groups showed the same trends, being highest in the "Laguna" group and lowest in the "Oases" group. The only lineage that consistently showed high and positive values of g and where $g > 3$ SD (g) was the "Laguna" group. The "*hypochondriaca*" group always had positive values of g, but different runs produced inconclusive results on the basis of the criterion employed ($1 < g > 4$ SD (g) in different simulations). Finally, the "Oases" group did not show evidence of demographic expansion, with g values associated with high standard errors.

The results of Fu's neutrality tests were non significant for the "Oases" (Fu's $Fs = -0.31$) and "*hypochondriaca*"

groups ($Fs = -4.56$), whereas they were significant for the "Laguna" group ($Fs = -2.87$, $p < 0.05$) (Table 2).

4. Discussion

4.1. Evolutionary history and taxonomy of P. regilla

The results of this study show three main haplotype clades ("northwestern," "central," and "southern") congruent with allozyme-based phenogram of Case et al. (1975) (see their Fig. 1). Case et al.'s (1975) allozyme analysis revealed three major groups within *P. regilla*, characterized by high genetic distances (mean D_{Nei} values ranging from 0.18 to 0.21). Our "southern" (*hypochondriaca* + *curta*) clade would correspond to the southern California-Baja California group in Case et al. (1975), while our "central" clade (including central California, Idaho, eastern Oregon and Montana) would correspond to their central California group. Finally, our "northwestern" group, with populations from Washington and Oregon corresponds with the third group in Case et al. (1975), which included populations from Oregon. Levels of genetic diversification within the "northwestern" clade are, however, much lower than those observed in southern populations. Thus, there is concordance between nuclear (allozymes) and mt-DNA, although the latter suggests substructuring within the southern California–Baja California group. Unfortunately, the analysis of Case et al. (1975) lacked samples from the southernmost population in BCP (Los Cabos region, La Laguna), and thus more samples would be needed to confirm this pattern.

Among the haplotypes analyzed from populations on the BCP, there is a clear division between southern and northern populations. This pattern has been observed in several vertebrates in the region (see Riddle et al., 2000b), leading to the resurrection of an old biogeographical hypothesis consisting of vicariance due to a midpeninsular seaway (Johnson, 1924; Nelsen, 1921). This seaway was thought to exist approximately 1–1.6 Ma (Riddle et al., 2000b; Upton and Murphy, 1997), although there is no geological evidence for a seaway at any time in the past.

To elucidate the possible events that caused the observed patterns, it is important to determine the age of the split, but the application of molecular clocks is not always straightforward. For *cytb* in amphibians, published sequence divergence rates range from 0.8% (Tan and Wake, 1995) to 3.6% (Babik et al., 2004). Ripplinger and Wagner (2004) used a divergence rate for *P. regilla* of 2%, which lies within the mentioned range for amphibians and is consistent with data for other vertebrates (Wilson et al., 1985). According to this calibration, the split between "*hypochondriaca*" and "*curta*" occurred around 1 Ma, and subsequently, populations of "*curta*" fragmented about 0.9 Ma. This estimation would be in close agreement with the postulated midpeninsular seaway, 1–1.6 Ma. However, to explain the phylogeographic structure observed within the "*curta*" clade would also require another barrier to dispersal located in the area of the Isthmus of La Paz. Aguirre et al. (1999) studied patterns of genetic structure in *Urosaurus* and suggested this additional seaway. These authors dated both seaways around 1.5 Ma. Assuming that there was a sea-level increase that flooded part of the central Peninsula (see Haq et al., 1988), it would not be unlikely that it happened somewhere else, especially in the area of the Isthmus, where maximum elevation is about 10 m above sea level (Grismer et al., 2002b; see also Murphy and Aguirre-León, 2002). However, at present, there is no geological evidence for the existence of two or more transpeninsular seaways in Baja California in the Pleistocene and the inferred locations vary over a wide latitudinal range of about 650 km depending on the taxonomic group examined.

Alternatively to the midpeninsular seaway hypothesis, the observed pattern might be associated with glacial events during the Pleistocene. In that period, environmental conditions in BCP were more mesic than at present (Upton and Murphy, 1997). Based on these glacial events, Savage (1960) proposed a general biogeographical scenario for the BC herpetofauna involving a succession of fragmentations of the distributional ranges of mesophilic species related to aridification processes during interglacial periods, followed by range expansions during glacial maxima. Our data also fit this model, which is similar to those proposed for other peninsulas affected by Pleistocene glaciations (see for example Taberlet et al., 1998).

The deep divergence between the three main clades suggests some vicariant events dating at least from the Pliocene that affected the Pacific coast of North America. Ripplinger and Wagner (2004) related the differentiation of their "Coastal" and "Inland" clades with the orogeny of the Cascade Mountains, which began approximately 4 Ma ago and drastically changed the environmental conditions of the area. Apparently, this event has caused a genetic division in other taxa from this region (Carstens et al., 2004; Good, 1989; Howard et al., 1993; Nielson et al., 2001; Steele et al., 2005) and together with the uplift of other mountain chains on the Pacific Coast has produced repetitive patterns of genetic diversification across multiple taxa (Calsbeek et al., 2003; Shaffer et al., 2004).

4.2. Evolutionary history of P. regilla in Baja California

The highest level in our nested cladistic analysis consists of two main four-step clades, one restricted to southern BCP and the other distributed in Baja California, California, and western Nevada. These clades, according to Templeton (2004) inference key, formed after allopatric fragmentation of the ancestral stock for both groups. As stated above, fragmentation between *hypochondriaca* and *curta* would have occurred around 1 Ma ago, while fragmentation of *curta* into the "Laguna" and "Oases" groups occurred around 0.9 Ma. Unfortunately, we lack samples from lowlands in the Cape region that would provide additional information on the events that affected the "*curta*" clade, and to discriminate the possible alternative evolutionary scenarios.

Samples within the "*hypochondriaca*" group were clustered into two third-level clades. One of them included the populations from the southern part of the distribution, associated mainly with arid regions (Grismer, 1994b), and also the population from Isla Cedros. Between these two groups, the inference key offers two alternative hypotheses to explain the observed pattern: long distance movements or a gradual range expansion followed by fragmentation. The limited dispersal abilities of this species, and of amphibians in general (Smith and Green, 2005), make unlikely the existence of long distance movements, and thus we favor the hypothesis of range expansion and subsequent fragmentation of the populations due to recent desertification. We also favor the same hypothesis to explain the pattern observed in the clade containing the populations from Las Parras and San Ignacio (the "Oases" group), which at present are also surrounded by unsuitable habitat (Grismer, 1994b).

Allopatric fragmentation was inferred for the clade including the haplotype found in individuals from Isla Cedros and those from La Ciénaga and Cataviña. Isla Cedros was once a prolongation of the Vizcaíno Peninsula (Grismer et al., 1994), but has been isolated from the mainland during the last 9000–15,000 years (Murphy et al., 1995; Wilcox, 1978). Other species have relict populations on this island (Grismer and Mellink, 1994; Grismer et al., 1994) and some of them were formerly described as endemic taxa (Grismer, 1988; Grismer et al., 1994; Montanucci, 2004; Murphy et al., 1995).

There are signs of demographic growth in the three main mt-DNA lineages, but the sign is strongest in populations from the Sierra de La Laguna. The "*hypochondriaca*" group is characterized by relative demographic stability, but there are also some restrictions to gene flow, especially apparent in populations from arid areas between the southernmost populations within this group and also between these and populations from more mesic habitats north to El Rosario.

Within the "Oases" clade, populations are prone to isolation due to the arid conditions of the surrounding area, and frogs are confined to areas where water is available (Grismer, 2002a). The results of the nested cladistic analysis for this clade indicate restrictions to gene flow between sampled populations.

We found some differences between the results of the different tests used to infer historical demographic trends in the three main groups detected. In general, Fu's tests and ML estimates of demographic parameters appear more conservative than mismatch distributions. In any case, congruence between different tests with regard to the historical demography of the "Laguna" group points to this region as an important refugial area for *P. regilla* in Baja California. Further studies are, however, required to confirm other refugia along the BCP. The analysis of more variable nuclear markers, such as microsatellites, would provide very useful information to ascertain present demographic trends and to contrast them to the historical events inferred from mt-DNA analyses.

4.3. Taxonomic implications

According to the results of the phylogeographic analyses, the "southern" clade includes two population groups that appear to constitute well-differentiated, independent evolutionary lineages currently in allopatry. Genetic divergence between "*curta*" and "*hypochondriaca*" suggests an older split of the two groups than was suggested by Jameson et al. (1966) in their taxonomic revision of the species, where they explained the significant morphometric differences between populations from different regions throughout the species range on the basis of the climatic oscillations in the last 11,000 years.

The "central" clade is represented in our sample by populations corresponding to *P. r. palouse* from Montana, *P. r. sierrae* from the Sierra Nevada of California, and *P. r. regilla* from Central California. The samples from Idaho and Eastern Oregon included by Ripplinger and Wagner (2004) in their "inland clade" are also included in this clade. All other samples used by these authors and included in their "coastal clade" form a basal highly differentiated "northwestern" clade, which is basically concordant with the range of *P. r. pacifica*.

From the allozyme data published by Case et al. (1975), Highton (2000) rejected the idea of *P. regilla* representing a single species. The information provided by the work of Case et al. (1975) is not complete due to sampling constraints. However, both allozyme and mt-DNA are congruent, suggesting a division of *P. regilla* into three independent groups that deserve taxonomic recognition at the species level. *P. regilla* (Baird and Girard, 1852) corresponds to the populations ranging from Central California to Montana. Populations from the northwest should be regarded as *Pseudacris pacifica* (Jameson et al., 1966) *stat. nov.* Southern populations, from Nevada and southern California to the Cape region in Baja California, would take the name *Pseudacris hypochondriaca* (Hallowell, 1854) *stat. nov.*, with two different subspecies, *P. h. hypochondriaca* from the Vizcaíno desert to the north and *P. h. curta* (Cope, 1867) distributed south of the Vizcaíno Desert to the southern tip of BCP. Additional studies on variation in nuclear markers will be helpful to determine the precise distribution of the three lineages and to delimit possible contact zones.

4.4. Conservation implications

Our results depict two well-differentiated lineages among populations of *P. h. curta* in southern Baja California. At present, both groups face very different situations from a conservation perspective. On the one hand, *P. h. curta* from the Sierra La Laguna apparently presents large, well-preserved populations distributed over a large (over 110,000 ha) and strongly protected area (Reserve of the Biosphere "Sierra de la Laguna"). On the other hand, populations of *P. h. curta* from the "Oases" clade are characterized by extensive isolation, and as a consequence they are very vulnerable to disturbance, such as the introduction of

exotic species. The presence of introduced *Rana catesbeiana* is displacing native *P. hypochondriaca* in the oasis of San Ignacio (Grismer and McGuire, 1993; personal observations). Conservation measures to preserve the oases as well as other breeding sites, such as traditional water reservoirs for cattle will help to preserve populations of *P. hypochondriaca* and other species in the region.

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Corrigendum

Corrigendum to "Phylogeography of *Pseudacris regilla* (Anura: Hylidae) in western North America, with a proposal for a new taxonomic rearrangement" [Mol. Phylogenet. Evol. 39 (2006) 293–304]

Ernesto Recuero [a], Íñigo Martínez-Solano [a,b], Gabriela Parra-Olea [c,*], Mario García-París [a]

[a] *Museo Nacional de Ciencias Naturales, CSIC, José Gutiérrez Abascal, 2. 28006 Madrid, Spain*
[b] *Museum of Vertebrate Zoology, University of California, Berkeley, 3101 Valley Life Sciences Building, Berkeley, CA 94720, USA*
[c] *Instituto de Biología, UNAM. AP 70-153, CP 04510, Ciudad Universitaria, México D.F., Mexico*

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Two of the names we proposed for newly recognized species in the *Pseudacris regilla* complex are incorrect. The synonymy of the *P. regilla* complex is summarized in Frost (2004), including the list of correct available names. Jameson, Mackey, and Richmond (Jameson et al., 1966) designated the specimen USNM 9182 as a lectotype of *Hyla regilla*. The type locality of this taxon (Puget Sound) is within the range of the "Northern Clade" we described and therefore should take the name *Pseudacris regilla* (Baird and Girard, 1852), and not *P. pacifica* as we suggested. Among the available names for the "Central Clade," all with the same date of publication (Jameson et al., 1966), and on the basis of the First Revisor Principle, we propose the use of *Hyla regilla sierra* Jameson et al., 1966, whose type locality, "1 1/4 miles SSE of Tioga Pass Ranger Station (east of entrance to Yosemite National Park)," is included within the geographic range of the Central Clade that we described. The available name for the Central Clade should be thus *Pseudacris sierra* (Jameson et al., 1966), and not *P. regilla* as we indicated. The name for the "Southern Clade" remains *Pseudacris hypochondriaca* (Hallowell, 1854).

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* Corresponding author.
E-mail address: gparra@ibiologia.unam.mx (G. Parra-Olea).

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II. Capítulo 2

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ORIGINAL ARTICLE

Mitochondrial differentiation and biogeography of *Hyla meridionalis* (Anura: Hylidae): an unusual phylogeographical pattern

Ernesto Recuero[1]*, Ainhoa Iraola[1,2], Xabier Rubio[2], Annie Machordom[1] and Mario García-París[1,2]

[1]*Museo Nacional de Ciencias Naturales (CSIC), C/José Gutiérrez Abascal, 2, 28006 Madrid, Spain and* [2]*Sociedad de Ciencias Aranzadi, Zorroagagaina, 11, 20014 Donostia, Spain*

ABSTRACT

Aim To study the patterns of genetic variation and the historical events and processes that influenced the distribution and intraspecific diversity in *Hyla meridionalis* Boettger, 1874.

Location *Hyla meridionalis* is restricted to the western part of the Mediterranean region. In northern Africa it is present in Tunisia, Algeria and Morocco. In south-western Europe it is found in the south of France, north-western Italy and north-eastern and south-western Iberian Peninsula. There are also insular populations, as in the Canaries and Menorca.

Methods Sampling included 112 individuals from 36 populations covering the range of the species. We used sequences of mitochondrial DNA *Cytochrome Oxidase I* (*COI*) for the phylogeographical analysis (841 bp) and *COI* plus a fragment including part of *tRNA lysine*, *ATP synthase subunits 6* and *8* and part of *Cytochrome Oxidase III* for phylogenetic analyses (2441 bp). Phylogenetic analyses were performed with PAUP*4.0b10 (maximum likelihood, maximum parsimony) and MRBAYES 3.0 (Bayesian analysis). Nested clade analysis was performed using TCS 1.18 and GEODIS 2.2. A dispersal-vicariant analysis was performed with DIVA 1.0 to generate hypotheses about the geographical distribution of ancestors.

Results We found little genetic diversity within samples from Morocco, south-western Europe and the Canary Islands, with three well-differentiated clades. One is distributed in south-western Iberia and the High Atlas, Anti-Atlas and Massa River in Morocco. The second is restricted to the Medium Atlas Mountains. The third one is present in northern Morocco, north-eastern Iberia, southern France and the Canaries. These three groups are also represented in the nested clade analysis. Sequences from Tunisian specimens are highly divergent from sequences of all other populations, suggesting that the split between the two lineages is ancient. DIVA analysis suggests that the ancestral distribution of the different lineages was restricted to Africa, and that an explanation of current distribution of the species requires three different dispersal events.

Main conclusions Our results support the idea of a very recent colonization of south-western Europe and the Canary Islands from Morocco. South-western Europe has been colonized at least twice: once from northern Morocco probably to the Mediterranean coast of France and once from the western coast of Morocco to southern Iberia. Human transport is a likely explanation for at least one of these events. Within Morocco, the pattern of diversity is consistent with a model of mountain refugia during hyperarid periods within the Pleistocene. Evaluation of the phylogenetic relationships of Tunisian haplotypes will require an approach involving the other related hylid taxa in the area.

*Correspondence: Ernesto Recuero, Museo Nacional de Ciencias Naturales (CSIC), C/José Gutiérrez Abascal, 2, 28006 Madrid, Spain.
E-mail: erecuero@mncn.csic.es

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Canary Islands, dispersal-vicariant analysis, human transport, *Hyla meridionalis*, mtDNA, nested clade analysis, phylogeography, western Mediterranean.

INTRODUCTION

Since its formation in the Tertiary (Krijgsman, 2002), the Mediterranean Basin has been characterized by a complex geological and climatic history that has shaped the distribution and diversity of the biota present in this region, resulting in the formation of several areas of endemism and hot spots (Oosterbroek & Arntzen, 1992; De Jong, 1998; Sanmartín, 2003). Some of the more dramatic events occurred at the end of the Miocene, directly affecting the biogeographical history of the western Mediterranean region. During the Tortonian, 11–7 million years ago (Ma), the Mediterranean was connected to the Atlantic Ocean by two seaways, the Betic and the Riffean corridors (Barbadillo *et al.*, 1997; Duggen *et al.*, 2003). These gateways suffered a progressive closing process that resulted, about 5.9–5.3 Ma, in the isolation of the Mediterranean from the Atlantic Ocean and the beginning of the Messinian salinity crisis (Krijgsman *et al.*, 1999; Duggen *et al.*, 2003). The disappearance of these two major barriers allowed the exchange of terrestrial biota between north-western Africa and south-western Europe across the Betic–Riffean Massif (Busack, 1986; Benammi *et al.*, 1996; Garcés *et al.*, 1998). About 5.3 Ma the Atlantic Ocean reconnected with the Mediterranean Basin through the Strait of Gibraltar (Krijgsman *et al.*, 1999). From this time no other terrestrial connection between north-western Africa and south-western Europe is known and the Strait has apparently been a strong barrier to dispersal and gene flow between the two regions since the Pliocene. Its presence has long been hypothesized to be the ultimate cause for the presence of many vicariant lineages on both sides (Busack, 1986; Palmer & Cambefort, 2000; Sanmartín, 2003; Juste *et al.*, 2004; Martínez-Solano *et al.*, 2004).

Several species show disjunct distributions with populations on both sides of the Strait of Gibraltar. In some cases, the application of molecular techniques has revealed the existence of deep divergences between the populations settled in both regions, which are now considered different species. This applies to amphibians as in the genera *Alytes* (Martínez-Solano *et al.*, 2004) and *Discoglossus* (García-París & Jockusch, 1999) and also to several other vertebrate taxa, e.g. *Lacerta* (Busack, 1987), *Blanus* (Busack, 1988; Albert *et al.*, in press), *Parus* (Salzburger *et al.*, 2002) and *Plecotus* (Juste *et al.*, 2004). In others, those techniques suggest the existence of effective dispersal across the Strait, not only in flying organisms such as *Gypaetus barbatus* (Godoy *et al.*, 2004) or *Galerida cristata* (Guillaumet *et al.*, 2006) but also in groups with limited overseas dispersal capacities such as the newt *Pleurodeles waltl* (Carranza & Arnold, 2003; Veith *et al.*, 2004), the lizards *Chamaeleo chamaeleon* (Paulo *et al.*, 2002) and *Podarcis vaucheri* (Pinho *et al.*, 2006), the snakes *Malpolon monspessulanus* and *Hemorrhois hippocrepis* (Carranza *et al.*, 2006) and micromammals such as *Apodemus sylvaticus* (Michaux *et al.*, 2003) and *Crocidura russula* (Cosson *et al.*, 2005).

Hyla meridionalis Boettger, 1874, is the only hylid species present in Africa. It ranges from the Atlantic coasts of Morocco to northern Tunisia, although its distribution is still poorly known in the region and the species has not been reported from most parts of Algeria (Bons & Geniez, 1996; Salvador, 1996; Schleich *et al.*, 1996). In Europe, it is present in the Iberian Peninsula, with two main distributional areas, south-western and north-eastern, plus a few more or less isolated and scattered populations in the south-east and the Basque Country. It is also present in southern France and from there it extends eastwards to the Liguria and Piedmont regions in north-western Italy. There are reports from Madeira and it can be found in Menorca (one of the Balearic Islands) and in all of the Canary Islands (Arnold, 2003; García-París *et al.*, 2004; Emanueli & Salvidio, 2006).

Amphibians have generally been considered to be poor dispersers (Smith & Green, 2005) and for this reason the presence of *H. meridionalis* in oceanic islands such as Madeira and the Canary Islands has usually been explained as the result of human transport (Pleguezuelos, 2002). The distribution in both shores of the western Mediterranean could be explained without the necessity for overseas dispersal by range expansion during the Mediterranean closure at the end of the Miocene. The formation of the Strait of Gibraltar fragmented the distributional range of the species, isolating African populations from European ones. In this case, if the Strait has been an efficient barrier to gene flow for this species, a vicariant pattern is expected between south-west Europe and north-west Africa. Populations from both areas should present a high genetic differentiation, predictably higher than within European or African populations (Busack, 1986; Gantenbein, 2004).

However, as Busack (1986) suggested, dispersal of *H. meridionalis* across the Strait is also possible and we cannot discard the possibility that either European or African populations were founded after the formation of the Strait. According to this hypothesis we would expect a complex genetic structure within the source population group, while genetic variation within the newly originated group will depend on its age and number of founders.

An alternative to natural dispersal implies humans to be the passive or active dispersal vector for species across the Mediterranean. This area has supported intense human activity

for the last 3000 years. This translates not only into severe habitat alterations, but also into the translocation of many species from different points of the region to others. Introductions and translocations have contributed to the composition of communities both in islands and continental areas (Dobson, 1998; Corti *et al.*, 1999; Pleguezuelos, 2002; Cosson *et al.*, 2005; Cucchi *et al.*, 2005). Again we expect that, due to their recent origin and a probable founder effect, populations originated from human introductions will present no or very limited mitochondrial DNA (mtDNA) haplotype diversification.

The distribution of *H. meridionalis* in the western Mediterranean, as well as its presence in both continental (Menorca) and oceanic islands (Canary Islands and Madeira), makes this species an excellent model to test such biogeographical hypotheses. In this work we analyse sequences of mtDNA to study the geographical pattern of genetic variation within the range of *H. meridionalis* in order to test and postulate new hypotheses for the evolutionary history of this species in the region. Our results are discussed in the context of our current knowledge of general biogeographical patterns in the western Mediterranean.

MATERIAL AND METHODS

Sampling and sequencing

We obtained tissue samples from 75 individuals, adults and tadpoles, from 23 populations along the range of the species. The sampling was completed with 12 specimens from the Museum of Vertebrate Zoology Tissue Bank (University of California, Berkeley), representing five additional populations, and 25 samples from eight populations from the Tissue and DNA Collection of the Museo Nacional de Ciencias Naturales (CSIC), for a total of 112 specimens and 36 populations (Fig. 1 & Table 1).

Total genomic DNA was extracted from ethanol-preserved tissues (muscle, liver and tail fin from tadpoles) using a phenol–chloroform protocol (Sambrook *et al.*, 1989), preceded by a digestion with proteinase K. Polymerase chain reaction (PCR) was used to amplify 841 bp of the mitochondrial *Cytochrome Oxidase I* gene (*COI*), using the primers Amp-P3F and Amp-P3R (San Mauro *et al.*, 2004). Additionally, for 38 individuals covering the whole area of distribution and in order to improve the resolution of the phylogenetic analyses, a fragment of 1599 bp including part of the *tRNA Lysine* (*Lys*), *ATP synthase subunits 6* and *8* (*ATP6*, *ATP8*) and part of the *Cytochrome Oxidase III* gene (*COIII*) was sequenced, with the primers pairs 8.2L8331 (San Mauro *et al.*, 2004) plus P5R′ (5′-GCAATTTCTAGTATAGTTAA-3′) and P5F′ (5′-CAGCTACCCTAGCCCTACTAT-3′) plus MNCN-*COIII* R (San Mauro *et al.*, 2004). Polymerase chain reactions were performed in a total volume of 25 μl, including 1 unit of Taq polymerase (Biotools, Madrid, Spain, 5 U μl^{-1}), 2.5 μM of each primer, 0.4 mM of dNTPs, 1.5 mM of $MgCl_2$ and 67 mM of reaction buffer (Tris–HCl, pH 8.3, Biotools). Polymerase chain reactions consisted of 35 cycles with a denaturing temperature of 94°C (1 min), annealing at 42°C (Amp-P3F + Amp-P3R primer pair) or 48.5°C (8.2L8331 + P5′r and P5′f + MNCN-*COIII* R primer pairs) (1 min), and extension at 72°C (1 min). Double-strand templates were cleaned using sodium acetate and ethanol to precipitate the PCR products and then resuspended in 22 μl of double-distilled H_2O. Sequencing reactions were performed for both strands and sequenced on an ABI PRISM 3730 DNA sequencer following the manufacturer's instructions.

Sequence alignment and phylogenetic analyses

All sequences were compiled using SEQUENCE NAVIGATORTM version 1.0.1 (Applied Biosystems) and aligned manually. *Hyla chinensis* was included as an outgroup (Zhang *et al.*, 2005; GenBank accession number AY458593). Genetic divergence in pairwise comparisons was calculated using the software PAUP*4.0b10 (Swofford, 2002). Mean sequence divergence between groups was calculated with MEGA2 (Kumar *et al.*, 2001).

Phylogenetic analyses were performed with PAUP. Maximum parsimony (MP) phylogenies were estimated using the heuristic search algorithm with tree bisection–reconnection (TBR) branch swapping and 100 random addition sequence replicates. Each base position was treated as an unordered character with four alternative states. We used nonparametric bootstrapping (1000 pseudoreplicates) to assess the stability of internal branches in the resulting topologies (Felsenstein, 1985).

Data were analysed with the software MODELTEST 3.6 (Posada & Crandall, 1998) in order to determine the substitution model that best fits our data for subsequent maximum likelihood analyses (ML; Felsenstein, 1981). ML analyses were performed using the heuristic search algorithm in PAUP with model parameters estimated with MODELTEST. We used nonparametric bootstrapping (100 pseudoreplicates) to assess the stability of internal branches.

Bayesian phylogenetic analyses were conducted with MRBAYES 3.0 (Huelsenbeck & Ronquist, 2001). Analyses were initiated with random starting trees and run for 2,500,000 generations, sampling every 100 generations. Generations sampled before the chain reached stationarity (200,000) were discarded ('burn-in'). Posterior clade probabilities were used to assess nodal support.

We tested the null hypothesis of clocklike rates in our sequence data set with a likelihood ratio test (Felsenstein, 1981) as implemented in MODELTEST 3.6.

Biogeographical and phylogeographical analyses

To test among competing biogeographical hypotheses we used a dispersal–vicariant optimization method proposed by Ronquist (1997), which was performed with the program DIVA 1.0 (Ronquist, 1996). The dispersal–vicariant analysis does not consider area cladograms and can be used in the case of reticulate biogeographical scenarios (Ronquist, 1997), and is

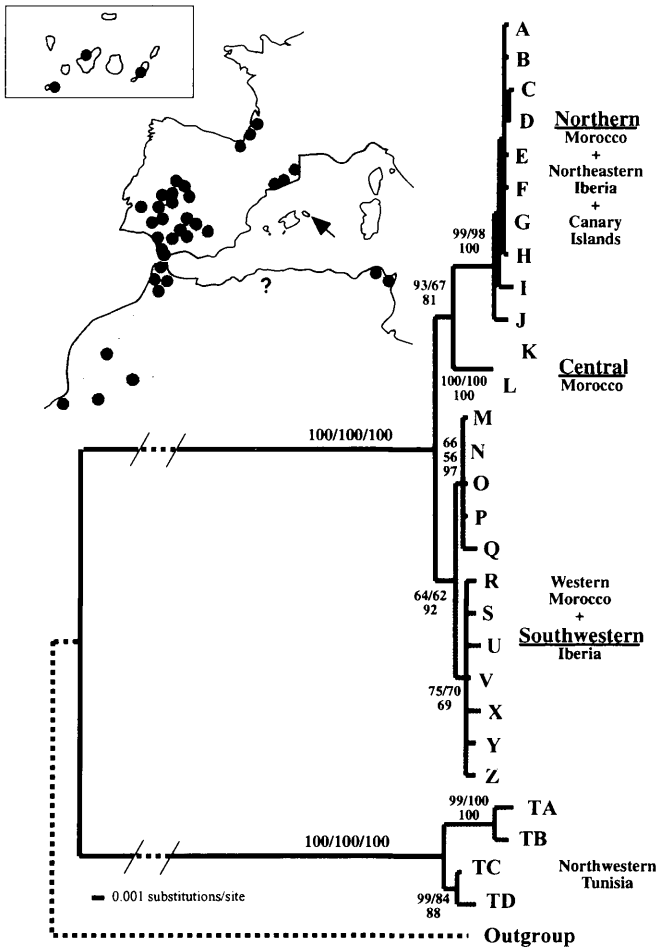


Figure 1 Distribution of *H. meridionalis* (populations sampled for the study are marked with dots) and maximum likelihood tree (−ln *L* = 4444.69816) of the combined data set showing the different lineages found in the phylogenetic analyses. Bootstrap values (MP, ML and % Bayesian posterior probabilities) at relevant nodes are shown. Colours correspond to the different groups found in the phylogenetic analyses: green = Tunisian group; red = Northern subgroup; yellow = Central subgroup; blue = Southwestern subgroup. The Canary Islands and Madeira are represented in a box outside their geographical location.

thus considered appropriate for phylogeographical interpretations.

For the analysis is necessary to define unit areas where the lineages are present. In our case we defined four different areas corresponding to Tunisia (A), Morocco (B), south-west Europe (C) and the Canary Islands (D). The analysis was performed using the main lineages defined by phylogenetic analyses and with no restrictions regarding the number of possible ancestral areas.

Phylogeographical analyses were based on a nested cladistic analysis (NCA) of the *COI* haplotype data set. We constructed a haplotype network using the software TCS 1.18 (Clement *et al.*, 2000), which follows the statistical parsimony algorithm described in Templeton *et al.* (1992). Then, a nested statistical design was carried out following the general guidelines provided by Templeton *et al.* (1995). Finally, we tested for the existence of geographical associations of the different clades by means of: (1) a categorical test, in which clades showing genetic and/or geographical variation are tested against their geographical location (permutational contingency analysis; see Templeton *et al.*, 1995) and (2) a second test that incorporates the information on geographical distances and relative positions among the sampled populations. These tests were performed with the GEODIS 2.2 software package (Posada *et al.*, 2000, 1,000,000 permutations), and the evolutionary patterns were sorted out following the inference key (updated 11 November 2005) provided by these authors with GEODIS 2.2.

RESULTS

Phylogenetic analyses

The combination of the *COI* and the *Lys–COIII* fragments resulted in a total of 28 haplotypes. Including the outgroup,

Table 1 Populations sampled in this study. Geographical coordinates, number of individuals sampled (*n*) and haplotypes found for the combined data set (used in the phylogenetic analyses; see Fig. 1) and for the *COI* sequences (used for NCA; see Fig. 3). A dash indicates populations not available for the combined data set.

ID	Locality	Latitude	Longitude	*n*	COI haplotypes	Combined data set haplotypes
1	Real de San Vicente, Toledo, Spain	40°07′60N	04°40′60W	1	VII	–
2	Logrosán, Cáceres, Spain	39°19′60N	05°28′60W	1	VII	–
3	Donostia, Guipúzcoa, Spain	43°19′00N	01°58′60W	16	II	A
4	Puerto de la Cruz, Tenerife, Spain	28°22′60N	16°33′00W	5	II	G
5	Cabezarrubias, Ciudad Real, Spain	38°37′00N	04°10′60W	1	VII	–
6	Villanueva del Río, Sevilla, Spain	37°37′00N	05°40′60W	2	VII	–
7	Cañamero, Cáceres, Spain	39°22′60N	05°22′60W	2	VII	–
8	Alconchel, Badajoz, Spain	38°31′00N	07°04′00W	1	VII	–
9	Vega del Río Palmas, Fuerteventura, Spain	28°22′60N	14°04′60W	2	II	–
10	Calañas, Huelva, Spain	37°38′60N	06°52′60W	6	VII	–
11	Pallares, Badajoz, Spain	38°07′00N	06°09′00W	4	VII	–
12	Aldeaquemada, Jaén, Spain	38°23′60N	03°22′00W	1	VII	–
13	Rubí, Barcelona, Spain	41°28′60N	02°01′60E	1	II	A
14	Garrovillas, Cáceres, Spain	39°43′00N	06°32′60W	3	VII	M
15	Facinas, Cádiz, Spain	36°07′60N	05°42′00W	6	VII, IX	Q
16	Tarnos, Aquitaine, France	43°31′60N	01°28′00W	1	II	–
17	Biarritz, Aquitaine, France	43°28′60N	01°34′00W	6	II	A
18	Monte Claro, Portalegre, Portugal	39°31′60N	07°43′00W	1	VII	N
19	Oukaimeden, Marrakech, Morocco	31°12′21N	07°51′51W	2	XI	R
20	Tingis, Tanger, Morocco	35°47′05N	05°48′46W	2	II, IV	C, I
21	Chefchaouen, Chaouen, Morocco	35°10′17N	05°16′11W	2	II, III	B, E
22	Ait Oufella, Khenifra, Morocco	32°55′54N	05°05′07W	2	VI	K, L
23	Asilah, Tanger, Morocco	35°27′56N	06°02′25W	2	II, IV	D, F
24	Ksar el Kebir, Larache, Morocco	35°03′20N	05°54′15W	3	I, V	H, J
25	Tarifa, Cádiz, Spain	36°00′45N	05°36′20W	1	IX	Q
26	Córdoba, Córdoba, Spain	37°52′60N	04°46′00W	4	VII	–
27	Candeleda, Ávila, Spain	40°08′60N	05°13′60W	5	VII	N
28	El Rocío, Huelva, Spain	37°07′60N	06°29′16W	4	VII, VIII, X	N, O, P
29	San Andrés, El Hierro, Spain	27°46′00N	17°56′60W	4	II	–
30	Garraf, Barcelona, Spain	41°15′00N	01°53′60E	1	II	–
31	Santa Coloma de Farnés, Girona, Spain	41°52′00N	02°40′00E	1	II	–
32	Tagounit, Taroudant, Morocco	29°48′33N	09°02′51W	5	XI, XIII, XV	S, U, V, X
33	Oued Massa, Tiznit, Morocco	29°53′20N	09°35′32W	1	XIV	Y
34	Taznakht, Ouarzazate, Morocco	30°41′43N	07°16′13W	3	XI, XII	Z
35	Lebna, Nabeul, Tunisia	36°46′56N	10°59′15E	6	XVI, XVII, XVIII	TA
36	Tabarka, Jendouba, Tunisia	36°57′16N	08°45′29E	4	XVIII, XIX, XX, XXI	TB, TC, TD

the data matrix presented 2451 bp, of which 502 (20.5%) sites were variable and 182 (7.4%) were parsimony informative. Mean nucleotide frequencies were 27.7% for A, 25.3% for C, 14.2% for G and 32.8% for T. The GTR + G model of evolution was selected by AIC in ModelTest 3.6. We found no insertions or deletions among the sequences corresponding to *H. meridionalis*. The outgroup, *H. chinensis*, presented an insertion of three codons at the beginning of the *ATP6* with respect to the sequences of *H. meridionalis*. Sequences were deposited in GenBank under accession numbers DQ996400–DQ996457.

The *H. meridionalis* haplotypes analysed were separated in two well-supported clades (Fig. 1), with average *p*-uncorrected distances of 6.4% (7.1% for the *COI* sequences alone): a 'Tunisian' group, including all haplotypes found in samples from Tunisian populations, and a 'Western' group, distributed over the westernmost Mediterranean region, that included all samples from France, Spain, Portugal and Morocco, but also the samples from the Canary Islands. Divergences within clades were relatively shallow. The 'Tunisian' group presented two clades not geographically structured and with a mean uncorrected distance of 0.8% (1.1% for *COI*). The 'Western' group was also divided into three different clades that we call 'South-western', 'Central' and 'Northern' subgroups. The 'South-western' subgroup included the Moroccan samples from the High Atlas, the Anti-Atlas and the Massa River populations (the southernmost populations of the species), as well as all the samples from south-western Iberian populations (Portugal and south-western Spain). The 'Central' subgroup corresponded to the single population analysed from the Moroccan Medium Atlas Mountains. The 'Northern' subgroup is distributed in the Rif Mountains and the Tingitane region of Morocco, and also

includes all populations from northern Spain, southern France and the Canary Islands. The mean *p*-uncorrected distance among 'South-western', 'Central' and 'Northern' subgroups was around 1.0%. Main clades presented high bootstrap values for both ML and MP analyses, as well as high posterior probability values from the Bayesian analysis, with the exception of the 'South-western' subgroup (Fig. 1). The results of the likelihood ratio test showed no significant differences in the likelihood scores when comparing trees estimated with or without enforcing the molecular clock (with: $\ln L = -5943.88336$; without, $\ln L = -5926.31748$, ratio = 35.131836; d.f. = 27; $P = 0.135558$). Thus, the null hypotheses of homogeneous evolutionary rates among sequences cannot be rejected.

Biogeographical and phylogeographical analyses

The vicariance–dispersal analysis performed with DIVA on the main lineages in Fig. 1 resulted in two equally parsimonious possibilities for the ancestral distribution of two of the nodes (Fig. 2). All possible hypotheses required three dispersals to explain the present distribution of lineages. After a DIVA exact search we found two hypotheses for the distributions of the ancestor of the entire *H. meridionalis* clade: Tunisia + Morocco or Tunisia + Morocco + south-west Europe. For the ancestor of the 'Western' group again we found two different hypothetical distributions: Morocco or Morocco + south-west Europe. The hypothesized ancestral distribution for Tunisian lineages is Tunisia, for the 'South-western' subgroup it is Morocco + south-west Europe, while the ancestral distribution for the 'Central' and 'Northern' subgroups is Morocco (Fig. 2). The ambiguous hypotheses are discussed below.

For the NCA we analysed 112 sequences of *COI*, including 841 bp with 181 variable positions (73 parsimony informative)

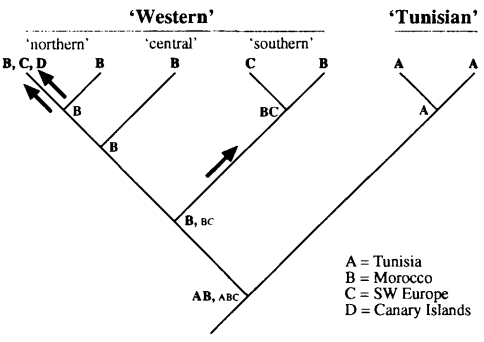
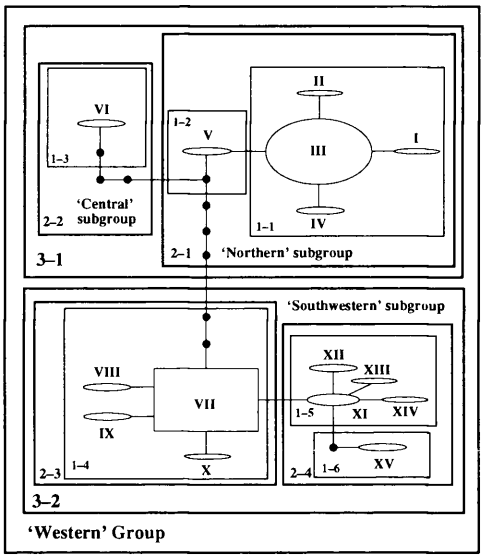


Figure 2 Summary of the optimal reconstructions of ancestral distributions for the main clades of *H. meridionalis* as proposed by dispersal–vicariance analysis (DIVA). Optimal distribution is given at each node. When more than one alternative exist they are presented separated by a comma. The favoured alternatives according to the phylogenetic analyses, are highlighted.



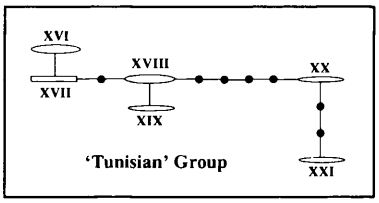


Figure 3 Nested design for the statistical parsimony haplotype network of *COI* sequences from *H. meridionalis*.

defining 21 different haplotypes (Fig. 3 & Table 1). Sequences were deposited in GenBank under accession numbers DQ996400–DQ996429. In this analysis we found that haplotypes corresponding to individuals from Tunisia fall outside the 95% confidence limit for the maximum parsimony connection of haplotypes, fixed at 12 mutational steps. The 15 haplotypes from Morocco, south-western Europe and the Canary Islands were joined in a nested design within a single four-step clade (Fig. 3). Haplotypes from the 'Central' + 'Northern' and 'South-western' subgroups are included within three-step clades 3-1 and 3-2 respectively.

The third and fourth nesting levels presented significant association between clades and their geographical position (Table 2). Templeton's (2004) inference key, used to interpret *Dc* and *Dn* values, (clade and nested clade distances respectively) generated the hypotheses shown in Table 2. For clade 3-2 (haplotypes VII–XV) the inference is past fragmentation and/or long-distance colonization. For clade 3-1 (haplotypes I–VI) and clade 4–1 (total cladogram) the hypothesis inferred is allopatric fragmentation. We found no significant results for the Tunisian haplotypes.

Table 2 Results of the NCA of haplotypes of *H. meridionalis*. Results of the categorical chi-square test, and inference chain and inferred events after the geographical distances tests are presented for each clade showing geographical and/or genetic variation.

Haplotype group	Chi-square statistic	Inference chain	Inferred event
Clade 1-1	65.45 (n.s.)	1–2–3–4	Restricted gene flow with isolation by distance
Clade 1-4	55.54 (n.s.)	1–2–3–5–6–13–14	Sampling design inadequate to discriminate between contiguous range expansion, long distance colonization, and past fragmentation
Clade 2-1	21.95 (n.s.)	1–2–11–12	Contiguous range expansion
Clade 3-1	48.00 ($P < 0.01$)	1–19	Allopatric fragmentation
Clade 3-2	53.00 ($P < 0.0001$)	1–19–20–2–3–5–15	Past fragmentation and/or long distance colonization
Total cladogram	101.00 ($P < 0.0001$)	1–19	Allopatric fragmentation

DISCUSSION

Phylogeographical hypotheses for Moroccan and south-west European populations

Populations of the 'Western' clade of *H. meridionalis* (Morocco, south-western Europe and the Canary Islands) are characterized by a poor mitochondrial diversification, lower than usually found among amphibians with comparable distributional ranges (García-París *et al.*, 2003; Martínez-Solano *et al.*, 2004; Zangari *et al.*, 2006). We found few, little-differentiated mitochondrial haplotypes grouped in three clades (Fig. 1), with some of the haplotypes present simultaneously on both sides of the Strait of Gibraltar. This reduced mitochondrial variation is in part consistent with the allozyme data published by Busack (1986), and also resembles the patterns found for some other terrestrial vertebrates, such as the colubrid *Macropotodon brevis* (Carranza *et al.*, 2004) and the soricid *C. russula* (Cosson *et al.*, 2005). Had the opening of the Strait of Gibraltar around 5.3 Ma divided the distribution of *H. meridionalis* and acted as a physical barrier to dispersal and gene flow between Europe and Africa, then the expected pattern is the presence of vicariant, well-differentiated clades on both sides of the Strait (Busack, 1986; Castella *et al.*, 2000; Sanmartín, 2003; Gantenbein, 2004). However, the presence of closely related or even identical haplotypes of *H. meridionalis* in Morocco and south-west Europe suggests the existence of dispersal events rather than vicariance since the opening of the Strait. Busack (1986) had previously suggested that the Strait of Gibraltar had not been an impermeable barrier for several species, including *H. meridionalis*. Since there is no evidence of further closure of the Strait after the Messinian, any latter dispersal movement implies dispersion over the sea. In our case we found higher haplotype diversity within Moroccan than within European population groups. Apparently, this is the opposite case to the pattern described for *P. waltl* (Carranza & Arnold, 2003), with Moroccan populations recently originated from the Iberian Peninsula, but similar to those described for *M. brevis* (Carranza *et al.*, 2004) and *C. russula* (Cosson *et al.*, 2005), where recent colonization occurred from Morocco to Europe.

Regarding the ancestral distribution hypotheses obtained with DIVA, the pattern of variation present within *H. meridionalis* argues against a widespread ancestral distribution that included south-west Europe and/or the Canary Islands. Hence, from the different proposed hypotheses, we suggest that lineages differentiated from an African ancestor. The ancestor of the 'Western' group was distributed in Morocco, from where the species moved into Europe and probably the Canary Islands. However, the haplotypes present in Morocco, south-west Europe and the Canaries (Fig. 1) present a geographical distribution that can hardly be explained by a single dispersal event but appears to require at least three, as suggested by the DIVA analysis (Fig. 2).

All sequences obtained from specimens of northern Iberian and southern French (NI-SF) populations share a single haplotype (A in Table 1), also present in northern Morocco, which clusters with the other haplotypes from northern Morocco in a clade (Fig. 1). This pattern of extreme low diversity suggests a very recent colonization of NI-SF populations by individuals probably from the northern coast of Morocco or western Algeria. As has been proposed for other species (Carranza & Arnold, 2003; Carranza *et al.*, 2004; Cosson *et al.*, 2005), two alternative hypotheses can explain this distribution. On the one hand, a possible natural origin of European populations would imply rafting for hundreds of kilometres on some kind of natural support across the Mediterranean Sea. This kind of dispersal is considered highly infrequent for other taxa (Heaney, 1986; Dobson, 1998) and so we consider it in this case, in view of the long distance from the suggested point of origin. On the other hand, passive or active transportation of frogs by humans from northern Africa to Europe can be considered plausible. The effect of human activity on the distribution of taxa has been especially strong in the Mediterranean Basin (Dobson, 1998; Corti *et al.*, 1999), with several cases of species arriving in Europe from Africa (Dobson, 1998; Martínez-Solano, 2004; Cosson *et al.*, 2005) and in Africa from Europe (Dobson, 1998; Libois *et al.*, 2001; Pleguezuelos, 2002; Michaux *et al.*, 2003), including translocation of amphibians in the western Mediterranean in different historical moments (Hemmer *et al.*, 1981; Corti *et al.*, 1999; Llorente *et al.*, 2002a,b).

We can determine neither the date nor the point of origin, natural or human-mediated, of NI-SF populations with our data. The geographical distribution of the NI-SF populations ranges from the single population in the Basque country across

southern France to Liguria, with the area south to the Pyrenees restricted to the north-eastern extreme of the Iberian Peninsula (García-París, 1997) (Fig. 1). If colonization took place from a single point it would be reasonable to expect this point to be centrally located with respect to the whole distributional range. For these populations of *H. meridionalis* we would consider as a probable starting point some locality in the Mediterranean coast of France (in coincidence with the starting point of the recent introduction of *Discoglossus pictus* in western Europe; Knoepffler, 1962; Llorente *et al.*, 2002b). From there it has spread eastward, westward and southward. Further northward and eastward expansion would have been prevented by the absence of ecologically favourable habitats and/or the presence of *Hyla arborea* and *Hyla intermedia*, which present a mostly parapatric distribution with *H. meridionalis* in the zone. The origin of the introduced populations could be any coastal point in Morocco, where the haplotype is mixed with other different ones.

Our results argue against the suggested presence of *H. meridionalis* in the Pleistocene of Great Britain (Holman, 1992). Holman (1992) suggested a possible colonization of Great Britain from France during the Ipswichian interglacial stage, about 150,000–115,000 years ago. Had the species been in the area for that time we should have found some variation across sequences of the 26 specimens analysed from northern Iberia and southern France. The remains found there probably belong to *H. arborea*, which is osteologically difficult to separate from *H. meridionalis*, as is known for all species of the *H. arborea* group (Delfino, 2006).

The 'South-western' subgroup is represented by different haplotypes from Morocco and south-western Iberia (Fig. 1). Samples from the 'South-western' subgroup are characterized by a reduced degree of mitochondrial diversification, perhaps caused by a massive extinction process followed by a reduced number of founder specimens. Again, haplotypic diversity is higher for this subgroup in Morocco than in the Iberian Peninsula, where we find a single, widespread haplotype (N), with the others mostly restricted to the southern coast. However, we found no shared haplotypes between Morocco and the Iberian Peninsula, and the overall diversity is not as limited as it is in NI-SF populations.

The geographical distribution of the 'South-western' subgroup is not continuous, as populations included in it are not only separated by the Strait of Gibraltar but also by a broad area in northern Morocco where populations present haplotypes of the 'Northern' subgroup (Fig. 1). It is possible that in the recent past the 'South-western' subgroup occupied most of the western Moroccan territory, from the Tingitane Peninsula to the Anti-Atlas Mountains, which is compatible with a natural colonization of southern Iberia by rafting across the Strait of Gibraltar. In this scenario, after the Iberian arrival, the species became extinct from northern Morocco. During the Pleistocene, ecological conditions in northern Africa suffered changes including alternating arid and wet periods (Jamet, 1991). A hyperarid period affected the Maghreb until about 12,000 yr BP (Dobson, 1998). The subsequent desertification could have caused the extinction of the species in the lowlands of northern Morocco. After that, a wetter period, between 12,000–4000 yr BP (Dobson, 1998), allowed recolonization of the area probably from some refugia in the Rif Mountains.

Again the alternative hypothesis would be human translocation of individuals from the western coast of Morocco to southern Iberia. In this case is hard to tell whether the haplotypes currently present in Iberia differentiated from the Moroccan ones after the arrival, or whether we failed to find them in Morocco. In both situations a very plausible arriving point for south-west Iberian populations is the extreme southern tip. This is supported by the restricted distribution in this area of most of the south-west Iberian haplotypes. From this point the species moved rapidly westward and northward, reaching the southern slopes of the Sistema Central Mountains, which apparently act as a barrier to northward dispersal (Merchán *et al.*, 2005). In this region we find some restricted sympatry between *H. meridionalis* and *H. arborea* and a few cases of hybridization have been reported (Oliveira *et al.*, 1991; Barbadillo & Lapeña, 2003). The presence of the latter species can be also a limiting factor for the northward expansion of *H. meridionalis*.

The results of the NCA analyses are statistically significant for high-level clades. The inferences obtained from Templeton's (2004) updated key for third- and fourth-level clades are concordant with our conclusions. For clade 3-1, representing the 'Northern' and 'Central' subgroups, the results suggest allopatric fragmentation between the populations from the Medium-Atlas Mountains (Central) and the remaining populations (Northern). The same inference is proposed for the total clade, comparing the 'Northern', 'Central' and 'South-western' subgroups. As we proposed before, climatic changes during the Quaternary could have led to a situation of multiple refugia in the different mountain chains in Morocco, the Rif, the Medium-Atlas and the High-Atlas and the Anti-Atlas, where populations were isolated long enough to differentiate in allopatry. For clade 3-2, comparing the south-western Iberian haplotypes with those from the Anti-Atlas, the inferred processes are past fragmentation and/or long-distance colonization, which are necessary to explain the colonization of the peninsula across the Strait of Gibraltar without human intervention.

Colonization of Atlantic islands

The single haplotype (G) found in the Canary Islands is very similar (only one base pair change in the *ATP6* region) to those present in northern Morocco and NI-SF populations. *Hyla meridionalis*, together with *Rana perezi*, are the only amphibians present in this volcanic archipelago, and both are traditionally considered to be introduced species (Pleguezuelos, 2002). Our results seem to confirm the introduction hypothesis, but yield little information on the date and geographical origin for these populations. For *R. perezi* it has been proposed that the species arrived after colonization by the Europeans (Pleguezuelos, 2002). The first human settlements

in the Canary Islands are dated to about 2500 yr BP and originated in the Maghreb (Navarro, 2001). Sporadic new arrivals from north-western Africa appeared in some islands, although there was little contact among islands (Navarro, 2001). Canarian settlers transported with them cargos such as cattle or seeds, and could have carried other species intentionally or passively, as happened with *Mus musculus* (Cucchi *et al.*, 2005). However a more recent origin cannot be ruled out. At present the species is often associated with banana plantations and movements among islands may have been linked to past expansion of this activity.

The use of additional markers, such as microsatellites, would be very helpful in order to elucidate the routes and times of colonization between Morocco, south-western Europe and the Canary Islands.

Evolution of *Hyla meridionalis* in northern Africa

The two main lineages present in northern Africa are characterized by a deep genetic divergence and correspond to two different areas, one mainly in Morocco and the other in northern Tunisia and probably north-eastern Algeria. This pattern of distribution can be found in some other amphibian species, such as *Pleurodeles waltl/poireti/nebulosus*, *Discoglossus scovazzi/pictus* or *Rana saharica*, with different biogeographical scenarios proposed to explain each. Carranza & Arnold (2003) proposed that differentiation between *P. waltl* (Iberia and Morocco) and *P. poireti/nebulosus* (north-east Algeria and Tunisia) was favoured by the formation of the Strait of Gibraltar. They suggested that the desiccation of the Mediterranean during the Messinian Crisis allowed the ancestor of the *poireti/nebulosus* clade to reach northern Africa, from the Iberian Peninsula to Algeria. Moroccan populations of *P. waltl* originated very recently from the Iberian Peninsula, possibly due to accidental human translocation (Carranza & Arnold, 2003). If we apply this scenario to the mitochondrial data for *H. meridionalis*, then the mean divergence rate estimated for COI would be 1.34% Myr^{-1}, which doubles the proposed rates for this gene in anurans (Macey *et al.*, 1998; Weigt *et al.*, 2005). Additionally, our data suggest an older presence of the species in Morocco than in Europe, without any trace of an older lineage of the species in the Iberian Peninsula.

Buckley *et al.* (1994, 1996) and Arano *et al.* (1998) found a remarkable genetic differentiation between Moroccan and Algerian/Tunisian populations of *R. saharica*. Apparently this species was separated from *R. perezi* from Iberia by the opening of the Strait of Gibraltar (Arano *et al.*, 1998). Later, *R. saharica* experienced the differentiation that was dated by Arano *et al.* (1998) as about 2 Ma, around the time of the Pliocene–Pleistocene boundary. This was associated with a marine transgression occupying part of the current Moulouya River basin (Arano *et al.*, 1998). Other vertebrates, such as *C. russula*, present a similar pattern, with divergences among clades dated about 2.2 Ma and explained on the basis of rapid alternation of dry and humid periods in the region (Cosson *et al.*, 2005). However, the suggested hypothesis for *R. saharica* implies again a very high mean COI divergence rate in *H. meridionalis*, 3.5% Ma^{-1}, again far higher than estimated rates for anura (Macey *et al.*, 1998; Weigt *et al.*, 2005).

A much older scenario is proposed for *Discoglossus* by Fromhage *et al.* (2004). These authors dated the separation of the Iberian + Moroccan (western) lineages from those from Sardinia, Sicily and Tunisia (eastern) to about 12–10 Ma, during the structuring of the Neo-Pyrenees. According to Fromhage *et al.* (2004), *Discoglossus* entered Morocco from the Iberian Peninsula and the western lineage subsequently differentiated into *D. scovazii* from Morocco and *Discoglossus galganoi* and *Discoglossus jeanneae* from the Iberian Peninsula. In the eastern lineage, separation of the Calabro-Pelorian massif from Sardinia resulted in the differentiation of *Discoglossus sardus* from *D. pictus*, the species currently present in Sicily, Algeria and Tunisia. In the case of *H. meridionalis*, the temporal frame proposed in this hypothesis is concordant with mean divergence rates proposed for other hyloid anurans, such as *Bufo*, with 0.69% Ma^{-1} (Macey *et al.*, 1998), or *Physalaemus*, with 0.43–0.49% Ma^{-1} (Weigt *et al.*, 2005). However, this scenario implies that populations from Morocco and Tunisia might not be sister lineages. A key to test the validity of this hypothesis for *H. meridionalis* will be the resolution of the phylogenetic position of *Hyla sarda*, an endemic hylid from Corsica and Sardinia. For a long time this species was included within the widespread taxon *H. arborea*, as was *H. meridionalis*. Unfortunately we lack a good phylogenetic framework involving Eurasian hylids in general and Mediterranean ones in particular. A sister relationship between *H. sarda* and Tunisian *H. meridionalis* would support the hypothesis of an old evolutionary history of what could be called *meridionalis* species group.

CONCLUSIONS

Hyla meridionalis, to date the only hylid species present in Africa, has spread recently, colonizing south-western Europe and the Canary Islands from Africa. The populations from south-western Europe present at least two different and independent origins with identical or very similar haplotypes on both sides of the Strait of Gibraltar suggesting recent, overseas dispersal routes probably associated with human movements. Populations from the Canary Islands have no genetic variation within islands, indicating a single arrival from north-west Africa with subsequent expansion inside the archipelago, again associated with human activity. However, populations from Tunisia present a high genetic divergence within *H. meridionalis* and must be analysed together with other related taxa from the western Mediterranean. Our results show the importance of human activity as a biogeographical factor, especially in areas that have long been populated by humans, such as the Mediterranean Basin.

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BIOSKETCHES

Ernesto Recuero is a doctoral student at the MNCN interested in biogeography, evolutionary biology and systematics.

Ainhoa Iraola is a doctoral student at the ASC and the MNCN interested in phylogeography and conservation of amphibians.

Xabier Rubio is a biologist at the ASC interested in conservation biology of amphibians.

Annie Machordom is senior scientist of the CSIC at the MNCN, and is an evolutionary biologist with particular interests in evolutionary processes and systematics.

Mario García-París is senior scientist of the CSIC at the MNCN, with particular interests in speciation processes, systematics and evolution of Amphibia and Coleoptera.

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II. Capítulo 3

Evolutionary history of *Lissotriton helveticus*: multilocus assessment of ancestral vs. recent colonization of the Iberian Peninsula.

Abstract

Background

The Pleistocene was characterized by climatic changes that greatly altered the distribution of organisms, with special incidence in the Holartic region. Population extinctions, bottlenecks, isolation, range expansions and contractions were often associated with glacial periods, leaving their signatures in the spatial patterns of genetic diversity across species. *Lissotriton helveticus* belongs to a Pan-European lineage of newts that were strongly affected by glaciations, and thus represent an excellent model to analyse the effect of generalized climatic changes in phylogeographic patterns.

Results

We studied the genetic diversity of the species using data from two mitochondrial and three nuclear genes analyzed in a Bayesian phylogenetic framework. Phylogeographic and historical demographic analyses were

performed on mtDNA sequences from 100 individuals from 35 populations covering the whole range of *L. helveticus* to investigate the historical processes shaping spatial patterns of genetic diversity. Mitochondrial haplotypes cluster in four different groups, all of them present in the Iberian Peninsula and of Pleistocene origin, probably by allopatric fragmentation. Nuclear genes present no obvious patterns of geographic structure, suggesting gene flow and generalized incomplete lineage sorting. Populations north of the Pyrenees are closely related to those from northeastern Iberia, suggesting recent range expansion from this region. Historical demographic analyses indicate a considerable demographic expansion starting about 100,000 years ago followed by more recent population declines.

Conclusions

Compared to other *Lissotriton* species, *L. helveticus* includes only relatively young genetic lineages, suggesting a Central European pre-Pleistocene distribution followed by complete extirpation of the species during glaciations in that area. Historical demographic trends in the Iberian Peninsula are reversed with respect to the more Mediterranean species *L. boscai*, indicating different responses of both species to climate changes. Diversity patterns among *Lissotriton* species seem to be defined by four main factors: ancestral

distributions, colonization capabilities, interactions with other species and effective population sizes. Differences in these factors define two types of species, referred to as "R" (refugia) and "S" (sanctuaries) that explain part of the diversity in patterns of genetic diversity created by glaciations in Western Europe.

Introduction

Climatic oscillations during the Quaternary have been crucial in the determination of present patterns of biological diversity, especially in the Holarctic region (Hewitt, 1996, 1999, 2000, 2001, 2004; Taberlet *et al.*, 1998). On one hand they produced successive contractions and expansions of populations of different organisms, causing extinctions across broad parts of their ranges (Hewitt, 2000, 2004). Since the origins of many groups and species are much older, predating the Pleistocene (Zink & Slowinski, 1995; Klicka & Zink, 1997; Ribera & Vogler, 2004), glacial periods often acted as an impoverishing force that reduced intraspecific diversity by population extinction and range reduction. On the other hand, glaciations favoured geographical isolation of populations in refugia, prompting lineage differentiation by allopatric fragmentation. During interglacial periods, more favourable environmental conditions allowed the expansion of quartered populations from their refugia into new territories, originating areas of secondary contact (e. g. Martínez-Solano *et al.*, 2006; Babik *et al.*, 2005; Sequeira *et al.*, 2005). Although some generalized patterns have been depicted, including identification of common recolonization routes and clustering of secondary contact zones shared across studied taxa (Taberlet *et al.*, 1998;

Hewitt, 2004), some species present specific responses to climate oscillations that are usually reflected in peculiarities either in the recolonization processes or in the genetic and demographic structure within the main refugial areas.

The accumulation and comparison of numerous phylogeographic studies in a given area provides a valuable framework to infer common historical patterns, but also the processes and events that give rise to patterns of intraspecific genetic diversity (Gómez & Lundt, 2006; Buckley, 2009). In this context, the compilation of data from closely related species that, sharing a common temporal evolutionary timescale, have been historically constrained by the same global events, is critical to determine the relative importance of species-specific responses in shaping patterns of intraspecific genetic structure.

The salamandrid genus *Lissotriton* is a group of five species of small newts distributed in the western Palaearctic, from the Iberian Peninsula and the British Islands to western Siberia. Species in this genus are old, dating back to at least the Miocene (Babik *et al.*, 2005; Rafinski & Arntzen, 1987; Steinfartz *et al.*, 2007), so all of them have been under the effect of Quaternary climate changes, in a way or another. To date, and based on different molecular markers, there is detailed information about the evolutionary history of each of

these species (Ragghianti & wake, 1986; Babik *et al.*, 2005; Martinez-Solano *et al.*, 2006) except *L. helveticus*.

Lissotriton helveticus (Razoumovski, 1789) is a western European newt present from the northern half of the Iberian Peninsula to the West of the Czech Republic and also in most part of Great Britain (Raffaëlli, 2007). It presents sympatric populations with other *Lissotriton* species: with *L. boscai* in northwestern Iberia and, more widely, with *L. vulgaris* in Great Britain and Central Europe. Hybrids with the latter species have been found sporadically but interspecific crossings are apparently rare in the wild (Arntzen *et al.*, 1998). On the basis of morphological differentiation, including secondary-sexual characters and variation in coloration patterns, different subspecies were originally described from the Iberian Peninsula. *Lissotriton h. alonsoi* (Seoane, 1885) from northwestern Spain, *L. h. sequeirai* (Wolterstorff, 1905) from northern Portugal and considered a synonym of the former by Salvador (1973), and *L. h. punctillatus* (Schmidtler, 1970), with a very restricted distribution in the "Pozo Negro" glacial lake in northern Burgos. North of the Pyrenees the species presents much less morphological variation, with the exception of paedomorphic populations (García-París *et al.*, 2004). However, more recent morphological surveys from populations in northern Iberia reject

the validity of these taxa, suggesting, instead, a pattern of variation among populations related to elevation (Galán, 1985). No genetic studies are so far available to evaluate the degree of morphological diversification and the genetic structure of this species in an explicit historical (phylogeographic) framework.

In general terms, *Lissotriton* species present a common phylogeographic pattern of both, deep intraspecific lineages in the southern parts of their ranges, areas that supposedly included several glacial refugia, and relatively homogeneous patterns of genetic variation in northern populations, probably as a result of recent recolonization events (Ragghianti & wake, 1986; Babik *et al.*, 2005; Martinez-Solano *et al.*, 2006). If this was the case of *L. helveticus*, it would predictably fit the model of "refugia within refugia" described for several Iberian species, characterized by the persistence of ancient lineages through Pleistocene glacial cycles (Gómez & Lundt, 2006). However, other alternatives are plausible. Compared to its wide distribution in Western Europe north of the Pyrenees, its presence in the Iberian Peninsula is limited and basically restricted to typically Euro-Siberian habitats with little presence in the Mediterranean biogeographic region outside the mountains. This situation, as in *L. montandoni*, is compatible with the possible existence of northern

refugia for the species (Stewart & Lister, 2001). In this case, the absence of competing species could have allowed its expansion into the Iberian Peninsula, occupying territories with favourable environmental conditions during the Quaternary period.

In this study we analyse patterns of genetic variation along the range of *L. helveticus*, with special emphasis on populations in the Iberian Peninsula, and discuss the phylogeographic structure of the species in relation to its evolutionary history. A comparative study of our results relative to published data from other closely related *Lissotriton* species, as well as with other similarly distributed organisms, serves as an evaluative tool to assess factors that have shaped the genetic structure and the current distribution of species in a region recurrently affected by climatic oscillations in recent geological times. Based on these comparisons we propose the existence of two distinct types of glacial refugia with different evolutionary outcomes.

Methods

Sampling and sequencing

We obtained tissue samples from a total of 100 individuals from 35 populations along the whole species' range. Eight of these populations are represented by a single sample, whereas for the rest we sampled 2-7 individuals, trying to represent most of the mitochondrial DNA (mtDNA) haplotype variation on a broad geographical scale (fig. 1; table 1). A subset of this sampling was used to obtain nuclear sequences from three different genes to compare with mtDNA genealogies.

Total genomic DNA was extracted from ethanol preserved and frozen tissues (tail tips and liver) using a phenol-chloroform protocol (Sambrook & Russell, 2001), preceded by a digestion with proteinase K. Part of the samples corresponded to long time frozen (-80°C), homogenized tissues used in a previous allozyme electrophoresis study (Montori, Llorente & Arano, unpublished data) that were extracted under the same protocol. Polymerase chain reaction (PCR) was used to amplify a total of 1355 base pairs (bp) of mtDNA corresponding to 644 bp of the mitochondrial *Cytochrome Oxidase I*

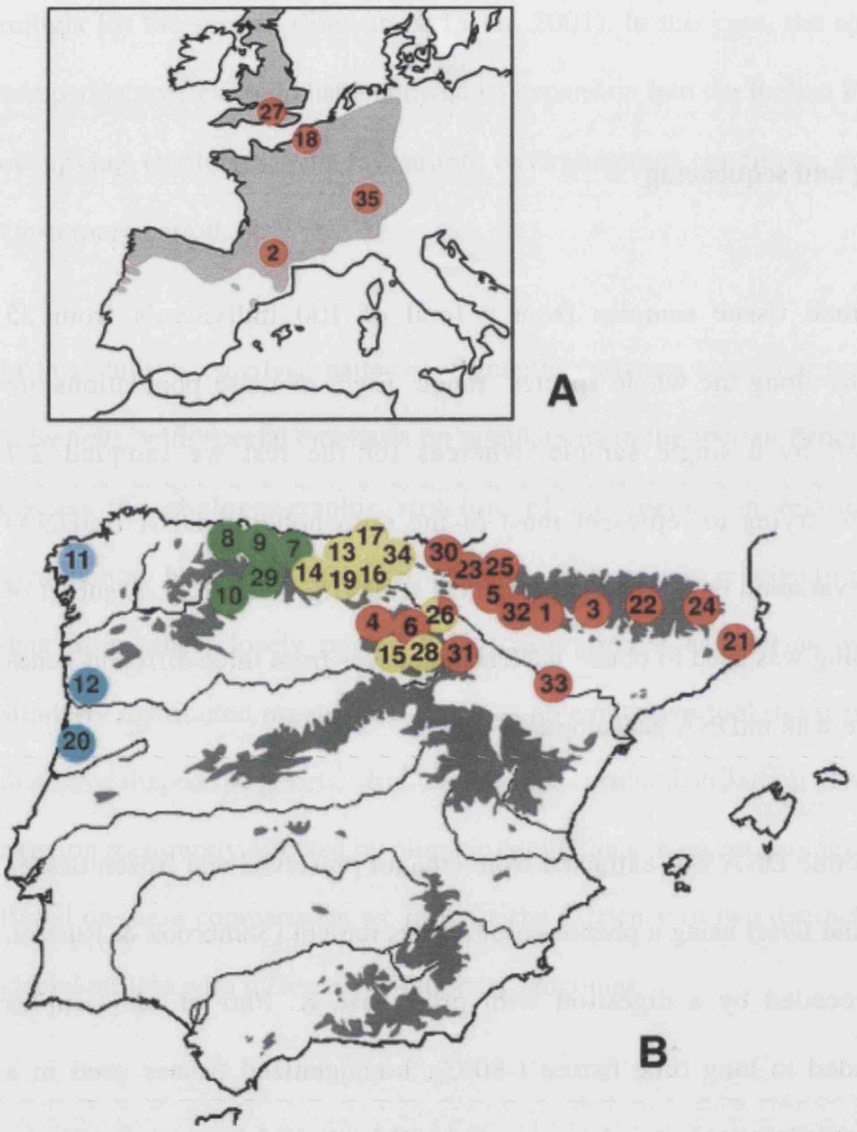

Figure 1. A: geographic distribution of *Lissotriton helveticus* and localities sampled for this study North of the Pyrenees. B: localities sampled in the Iberian Peninsula. Populations are identified with numbers (see table 1). Colours represent the four main mtDNA groups identified (blue: Northwestern; green: Asturian; yellow: East Cantabrian; red-orange: Northeastern).

gene (*COX1*), using the primers LCO1490 (Folmer *et al.*, 1994) and COI-H (Machordom *et al.*, 2003), and 711 bp of the mitochondrial *control region* (*D-loop*) using the primers PRO and PHE (Martínez-Solano *et al.*, 2006). With respect to nuclear markers, we amplified 453 bp of the seventh intron of the beta *fibrinogen* gene (*Fib*) using the primers Fib-F and Fib-R (Nadachowska & Babik, 2009), 493 bp of the *Chemokine receptor 4* (*Cxcr4*) gene using the primers Cxcr4-F and Cxcr4-R (Nadachowska & Babik, 2009) and 742 bp of an anonymous noncoding genomic DNA fragment (*Tva4*) using primers Tva4-F and Tva4-R (Nadachowska & Babik, 2009). For some samples we used internal primers to amplify 554 bp of the latter fragment. These primers are Tva4I-F (5'-CCAGGTCAGCAAGAGCCTAT-3') and Tva4I-R (5'-TGGGTGGCTCAGTCTTCTCT-3').

PCR reactions were performed in a total volume of 25ml, including one unit of Taq polymerase (Biotools, 5 U/ml), 2.5mM of each primer, 0.4 mM of dNTPs, 1.5 mM of $MgCl_2$ and 67 mM of a reaction buffer (Tris-HCl, pH=8.3, Biotools). PCR reactions consisted of 35 cycles with a denaturing temperature of 94 °C (45 seconds), annealing at 48 °C (*COX1*), 56°C (*D-loop*), 58°C (*Tva4*), 67°C (*Cxcr4*) or 60°C (*Fib*) (45 seconds), and extension at 72 °C (45 seconds). Double strand templates were cleaned using sodium acetate and

ethanol to precipitate the PCR products and then re-suspended in 22 ml of ddH_2O. Sequencing reactions were performed for both strands and sequenced on an ABI PRISM 3700 DNA sequencer (Applied Biosystems) following the manufacturer's instructions and the conditions described in Martínez-Solano *et al.* (2006).

Sequence alignment and phylogenetic analyses

Two closely related species, *Lissotriton vulgaris* and *L. boscai* were chosen as outgroups. Sequences of *L. vulgaris* were obtained in the laboratory while those of L. boscai were obtained from GenBank (accession numbers DQ491890 (Martínez-Solano *et al.*, 2006) and EF525956 (Smith *et al.*, 2008). Sequences were compiled and revised using Sequence Navigator™ version 1.0.1 (Applied Biosystems) and aligned manually except the D-loop sequences that were aligned using the online version of MAFFT v.6 (Katoh & Kuma, 2002). Several nuclear sequences resulted in two alleles that differed at one or more sites. In these cases, we used the software Phase v2.1 (Stephens *et al*, 2001) to infer haplotype phase.

We performed Bayesian phylogenetic analyses with MrBayes v3.1.2 (Huelsenbeck & Ronquist, 2001; Ronquist & Huelsenbeck, 2003). The optimal substitution models for each gene and partition were selected with ModelTest v3.7 (Posada & Crandall, 1998). Mitochondrial genes were analyzed together considering three different partition designs. First with two partitions using the best models for both genes. Second with three partitions using the best model estimated for D-loop, the best model for first and second codon positions of *COX1* and the best model for the third positions of *COX1*. Third with four partitions using the best models estimated for D-loop and each codon positions of *COX1*. The Bayes factor for each analysis was calculated with Tracer 1.3 (Rambaut & Drummond, 2007) to select the best data partitioning scheme. Nuclear genes were analyzed independently considering the best model estimated for each one. We initiated the analysis with random starting trees and ran four Metropolis coupled Monte Carlo Markov chains (three heated, one cold) for 20 millions of generations, sampling every 1000 generations. We checked for stationarity and convergence of the chains with TRACER 1.5 (Rambaut & Drummond, 2007) and discarded 2000 trees as burn-in. Posterior clade probabilities were used to assess nodal support. Additionally we constructed a haplotype network using the software TCS 1.2.1 (Clement *et al.*, 2000), which follows the statistical parsimony algorithm described in

Templeton *et al.* (Templeton, 1992). Ambiguities in the network were resolved after the recommendations in (Pfenninger & Posada, 2002).

We used BEAST v1.5.4 (Drummond & Rambaut, 2007) to estimate divergence times among clades. We used sequences of *Lissotriton vulgaris*, *L. boscai* and *Mertensiella caucasica* [GenBank accession number EU880319 (Zhang *et al.*, 2008)] to calibrate the nodes. The age of the *Lissotriton* clade was set in 21 Mya with normal distribution and 1.0 as standard deviation (Martínez-Solano *et al.*, 2006). The age of the root of the whole tree was set in 48 Mya with lognormal distribution and 1.0 as standard deviation (Martínez-Solano *et al.*, 2006). The analyses were run under an uncorrelated lognormal relaxed clock model and the Birth and Death process speciation tree prior with a length of chain of 250 millions sampled every 1000 generations. The analysis was performed twice and the results combined in tracer with a burnin for each run of 25000 generations.

Phylogeographic and historical demographic analyses

We tested the possible existence of "isolation by distance" (IBD) processes in the different mitochondrial lineages. For this we performed Mantel's tests

(Mantel, 1967) to analyse the relationships between genetic and geographical distances considering samples and groups of samples, in our case the main clades recovered in the phylogenetic and phylogeographic analyses. The matrices of geographical (km) and genetic (ML-corrected) distances were generated with Geodis v2.4 and PAUP v4.0b10 respectively (Posada *et al.*, 2000; Swofford, 2002). To perform Mantel's tests we used GENALEX version 6, with 999 permutations to estimate the 95% upper tail probability of the matrix correlation coefficients. In the cases we found significant associations between genetic and geographical distances, the corresponding scatter plots were visually analysed, since its patterns are indicative of the possible effects of random genetic drift and gene flow in the geographical genetic structure (Hutchison & Templeton, 1999).

We used analysis of molecular variance [AMOVA, (Excoffier *et al.*, 1992)] to characterize patterns of genetic variation at different hierarchical levels (individuals, populations, and the main groups characterized by their general geographic distribution and mtDNA lineages identified by phylogenetic analyses) as implemented by Arlequin v. 3.11 (Excoffier *et al.*, 2005). Levels of significance of statistics characterizing variation at different hierarchical levels were assessed through 10000 permutations.

The demographic history of the different *L. helveticus* lineages was inferred using different approaches. For a Bayesian coalescent approach (Drummond *et al.*, 2005) we chose a Bayesian Skyline Plot (BSP) as our initial demographic model. BSP estimates a posterior distribution of effective population size through time using standard Markov chain Monte Carlo (MCMC) sampling procedures, calculating directly from the sequence data rather than from a predetermined genealogy, and offering credibility intervals for the given estimates (Drummond *et al.*, 2005). This method was performed as implemented in BEAST v1.5, running 50 millions of generations. Burn-in and final BSP was determined with TRACER v1.5. The second approach were mismatch-distribution analyses of pairwise mtDNA differences (Slatkin & Hudson, 1991), comparing the observed distributions with that expected under a model of populations expansion, which are usually unimodal compared with the multimodal distributions of populations in equilibrium or that are subdivided (Martínez-Solano *et al.*, 2007). The analyses were performed with Arlequin v3.11 (Excoffier *et al.*, 2005) and the goodness-of-fit of the compared models was tested with 1000 bootstrap replicates. Finally we also used Fu's test of neutrality (Fu, 1997), searching for signals of population

expansions of the analysed lineages, as implemented in DNASP v4.20.2 (Rozas *et al.*, 2003).

Results

Phylogenetic analyses

A total of 100 specimens of *L. helveticus* were studied through DNA sequencing. The combination of mtDNA sequences of *COX1* and *D-loop* data, yielded an ingroup dataset of 1355 base pairs (bp), of which 1302 were constant and 35 were parsimony informative. When outgroups are included the database size increases (because of insertions) to 1388 bp, with 1055 constant characters and 136 parsimony informative. We found 47 haplotypes among the studied sequences. Some insertions and deletions were observed between *L. helveticus* and the outgroups in the non-coding *D-loop* sequences. Mean nucleotide frequencies in *L. helveticus* D-loop/COX1 sequences were 29.18/24.93% for A, 21.28/26.88% for C, 15.88/18.86% for G and 33.66/29.33% for T. The best models selected by the Akaike information criterion (AIC) were TIM+G and TrN+I for *D-loop* and *COX1* respectively. For *COX1* sequences we also estimated the best substitution models for the different codon positions: TrN for the first positions, F81 for the second positions, TrN+I for first and second positions together and TrN for third

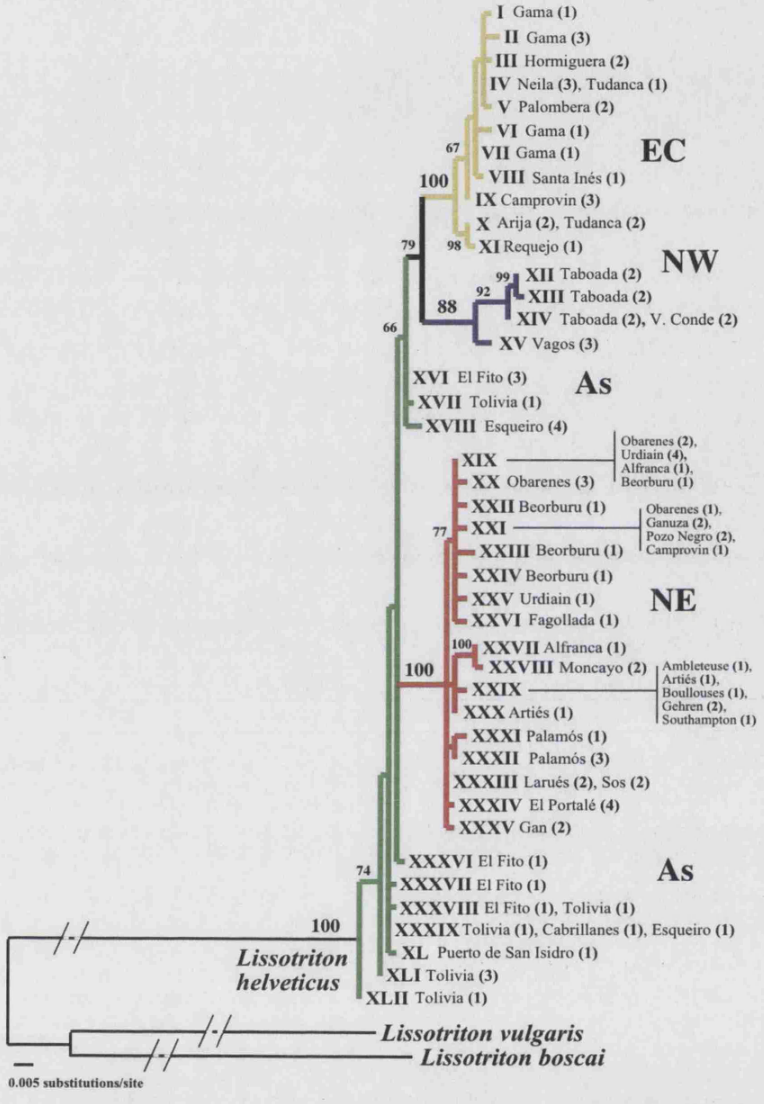

Figure 2. Bayesian phylogenetic reconstruction of the mitochondrial haplotypes found in *Lissotriton helveticus*. Posterior probability values are given for main clades.

positions. Sequences were deposited in GenBank and accession numbers are given in table 1 [to be added upon manuscript acceptance].

For nuclear genes we analyzed sequences from a subset of 39 individuals, with 453 bp for *Fib*, 493 bp for *Cxcr4* and 742 bp for *Tva4*. All three genes are characterized by reduced variation in *L. helveticus* except for a few divergent haplotypes. In *Fib* sequences, 431 characters were constant, with 21 parsimony informative characters. The best substitution model under AIC was F81 and base frequencies were 30.59% for A, 20.34% for C, 16.74% for G and 32.33% for T. The *Cxcr4* sequences presented 484 constant characters and only 4 were parsimony informative. The best model was K81 and bases presented equal frequencies. In *Tva4* sequences 693 characters were constant and 43 were parsimony informative, with HKY as the best substitution model and base frequencies of 35.53%, 20.63%, 19.74% and 24.1% for A, C, G, and T respectively.

The different partition schemes tested in the phylogenetic analyses of the mtDNA data produced identical topologies. The observed structure in our phylogenetic reconstructions presents a peculiar distribution of haplotypes. On one hand we obtained three well-supported clades with a, in general, well-defined geographic

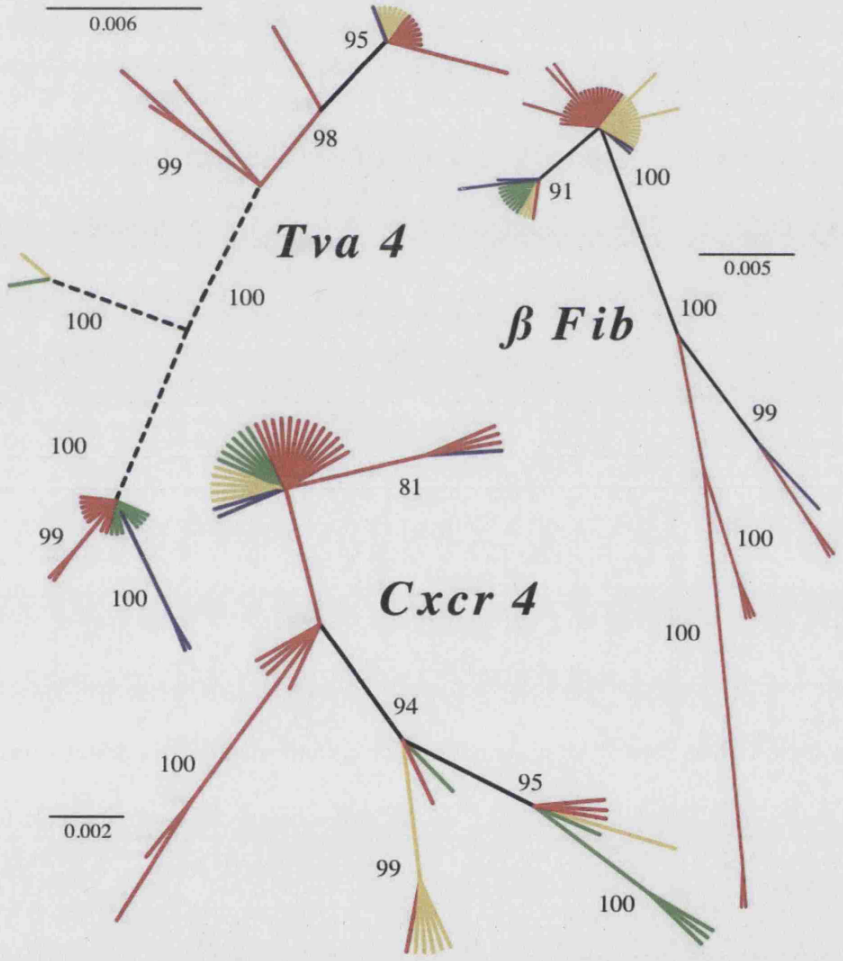

Figure 3. Midpoint rooted Bayesian phylogenetic reconstructions of the three nuclear genealogies in *Lissotriton helveticus*. Posterior probability values are given for main clades.

structure (figs. 1, 2, 4). On the other hand we find a group of haplotypes, all coming from populations from Asturias and northern León, that are not clustered together, but are distributed along the basal structure of the whole *L. helveticus* clade (figs. 1, 2) and are central in the haplotype network (fig. 4). Based on these results we defined four different geographic groups: the "Northwestern" group (blue in figures 1, 2, 3, 4), which includes samples from the north of Portugal and Galicia; the "Asturian" group (green), formed by haplotypes found in Asturias and northern León; the "East Cantabrian" group (yellow), distributed mainly in Cantabria, Palencia and Burgos, but also in La Rioja and Soria; and finally, the "Northeastern" group (red), found in the Northeast of the Iberian Peninsula and north of the Pyrenees to Great Britain and Germany. The "Northwestern", "East Cantabrian" and "Northeastern" clades were well supported by posterior probability values (fig. 2). The divergence time estimates for mtDNA clades suggest a Pleistocene origin of the four main groups (all of them are recovered as well supported clades in BEAST analyses). The earliest split probably occurred around 1.28 (0.24-2.76) million years ago (Mya) and most of the nodes originated in the lower-middle Pleistocene. Age of the most recent common ancestor for the "Northwestern" clade dates back to about 0.42 (0.04-1.01) Mya, 0.55 (0.11-1.16) Mya for the "Cantabrian" group, 0.48 (0.1-1.05) Mya for the "Northeastern" and 1.0 (0.19-2.14) Mya for the "Asturian" group.

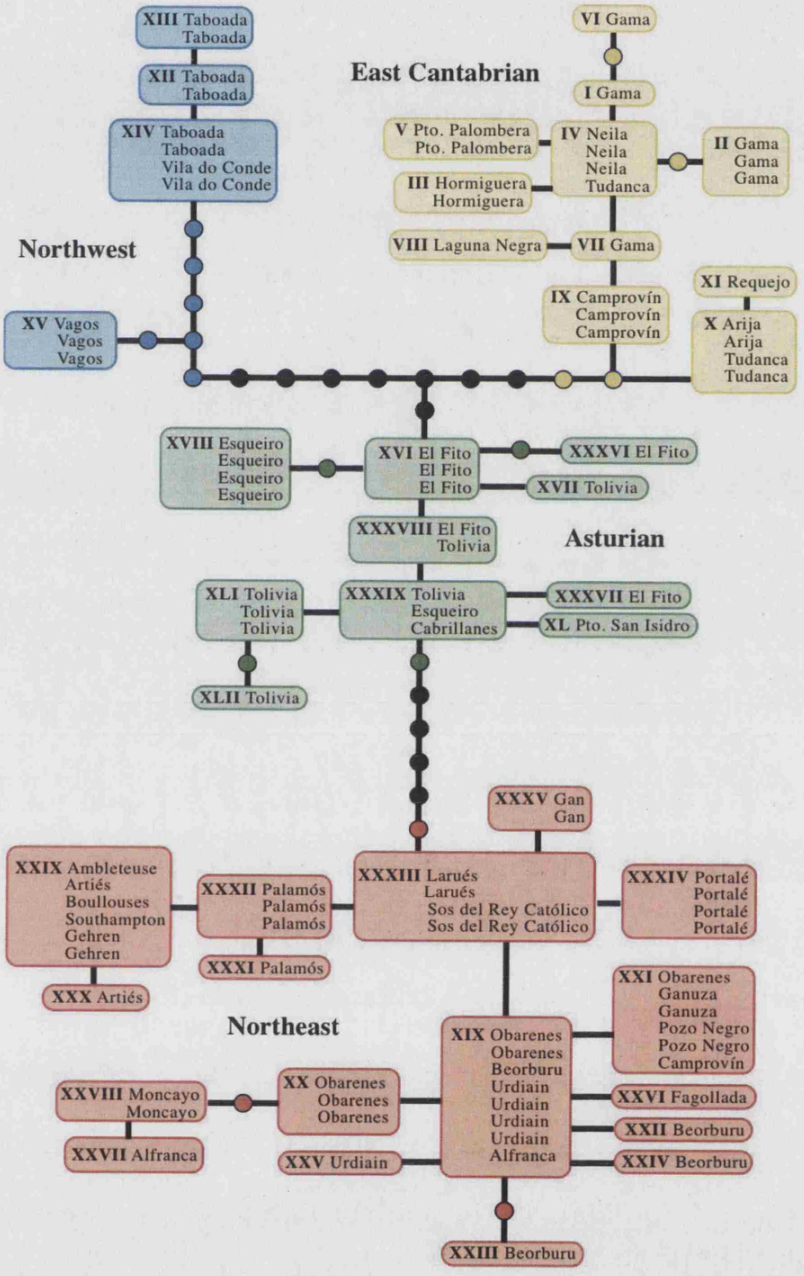

Figure 4. Statistical parsimony haplotype network of mtDNA sequences from *L. helveticus*. Colours conform to the main groups as defined in figs. 1 and 2.

The topologies obtained with the three nuclear markers are conditioned by their low intraspecific variation and are characterized by a generalized pattern of incomplete lineage sorting. The *Tva4* topology presents three main clades (fig. 3). One of them is formed by a single haplotype present in two not very distant populations, one form the "Asturian" group and other from the "Eastern Cantabrian" group. This clade is the sister of a three haplotype clade where the rest of the "Asturian" populations cluster together with the Portuguese ones and with some of the "Northeastern" populations. The third clade is formed by haplotypes present both in the "East Cantabrian" and the "Northeastern" groups, although the most common haplotype is also present in a very distant population from Galicia.

The *Cxcr4* topology presents two main clades (fig. 3) with no evident geographic pattern. The most common haplotype is present across the whole geographic range of the species, from Portugal to Germany.

The *Fib* topology presents three main clades (fig. 3). One of them is formed by low frequency haplotypes present in "Northeastern" populations, while the other two clades present a mixed geographical composition. There are two predominant haplotypes. One is characteristic from "Asturian" populations, but

is also present in Galicia and Cantabria. The other is widely distributed, from Portugal to southern Great Britain.

Although there is no evident geographic structure observed in nuclear sequences, there seems to be a weak pattern of incipient sorting of haplotypes in some of the "Northeastern" populations (fig. 3).

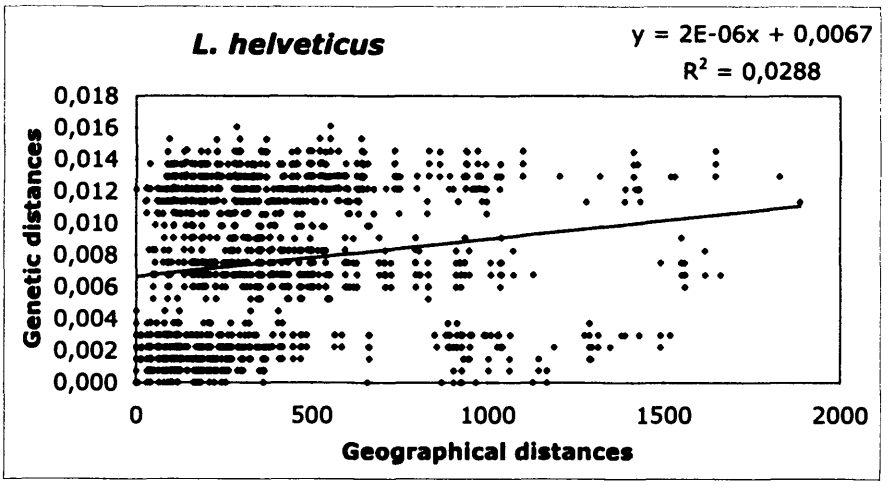

Figure 5. Isolation by distance (IBD) plots (uncorrected genetic distances –p– vs. geographical distances, in kilometres) for the total sample dataset and including regression equations and correlation coefficients. Main groups show similar patterns when analyzed independently except the "East Cantabrian" group, with non-significant results (see table 2).

Phylogeographic and historical demographic analyses

Except for the "East Cantabrian" group, we found significant relationships between geographical and genetic distances in all Mantel tests performed, both for all individuals of *L. helveticus* and for the three remaining major lineages separately (table 2). However, in all cases correlations are weak with coefficients ranging from 0.03 to 0.40, being highest in the "Northwestern" group that, with few samples included in the analysis, probably offers an incomplete picture of the population variation and structure in that region. Additionally, the scatter plots (fig. 5) reflect a generalized lack of regional equilibrium, with drift apparently more influential than gene flow, similar to pattern III in Hutchison & Templeton (1999), suggesting that other processes apart from isolation by distance have affected the genetic structure of the populations.

Results from AMOVA indicate that most of the observed variation among mtDNA lineages is related to differences between groups (76.16% of the total variance observed). Lower values were observed for variance related to differences among populations within groups (12.69%) and within populations

(11.15%). All hierarchical components of genetic variation were highly significant (p<0.0001).

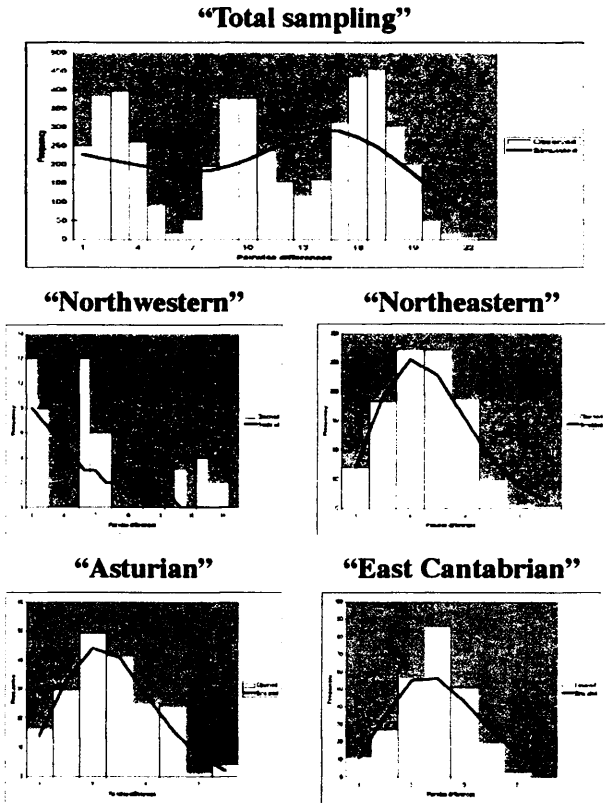

Figure 6. Mismatch distributions of sequences in the total sample dataset and the four main mitochondrial lineages identified in this study. White bars represent the observed frequencies of pairwise differences between haplotypes; black lines represent those expected under the model of population expansion.

Mismatch distributions were multimodal for the total data set and the "Northwestern" group, suggesting a history of population stability or subdivision, whereas they were clearly unimodal for the "Northeastern", "Asturian" and "East Cantabrian" groups, which is indicative of population expansion (fig. 6). Accordingly, Fu's Fs test was significant, again indicating population expansion, for the total data set and all groups except the "Northwestern" (table 2). The result of the historical demographic reconstruction under the BSP model for the total sample dataset is shown in fig. 7. When all individuals are analyzed together, we see that for the last million years the population size remained constant until about 100,000 years ago, when the populations started to experience a considerable demographic expansion that lasted for several thousands of years until it started to decrease in recent times. When we analyze the different groups separately, we find similar patterns of demographic expansions in all groups but the "Northwestern" one, which presents a relatively small but constant population decrease, although the small sample size in this particular group makes the results unreliable.

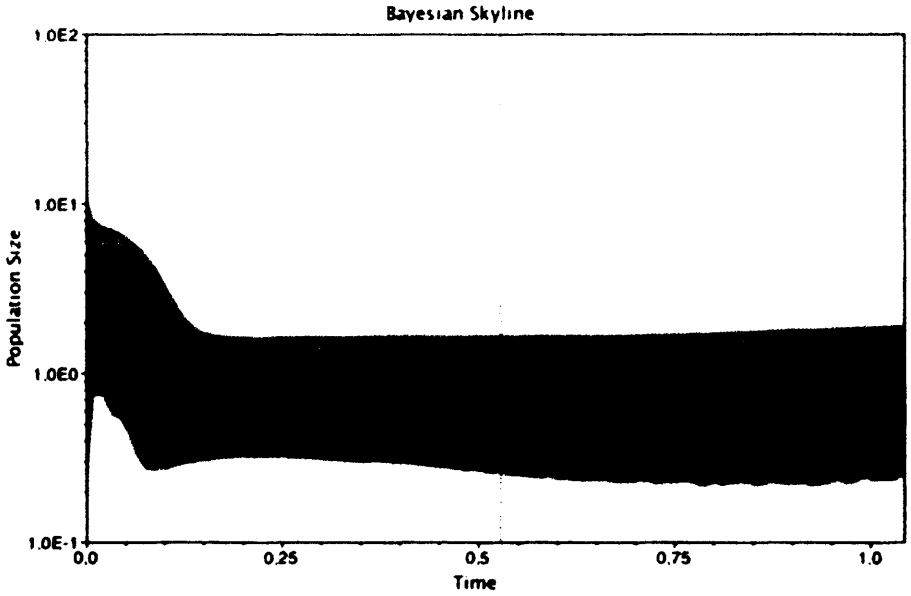

Figure 7. Bayesian Skyline Plot (BSP) showing historical demographic trends in *Lissotriton helveticus*; x-axis: time in millions of years before present; y-axis: estimated population size [units = Net, the product of effective population size and generation length in years (log transformed)]. BSPs for the main groups, when analyzed independently, show similar trends except the "Northwestern", which shows a slight and continuous population decrease but with little resolution due to low sample size.

Discussion

Evolutionary history of Lissotriton helveticus

The phylogeographic structure of *Lissotriton helveticus* in the Iberian Peninsula presents a pattern that is superficially similar to the recurrent model of "refugia within refugia" (Gómez & Lundt, 2006) often found in the Mediterranean peninsulas. Climatic fluctuations during the Quaternary probably played a key role in the origin of the main lineages *in L. helveticus* by favouring isolation and subsequent allopatric differentiation, as our results suggest. Based on our divergence time estimates, the original fragmentation of the main mtDNA clades in *L. helveticus* occurred around 400,000 to 1 million years before the present, from Lower to Middle Pleistocene. These are relatively recent events if we consider that the age of this species was estimated in about 20 millions of years (Babik *et al.*, 2005) and that other *Lissotriton* species such as *L. boscai and L. vulgaris* present much older lineages that originated during the Pliocene and even the Miocene (Babik *et al.*, 2005; Martínez-Solano *et al.*, 2006), as is also the case in several other taxa with comparable distributions, e. g. *Chioglossa lusitanica* (Alexandrino *et al.*, 2000; Alexandrino *et al.*, 2002), *Salamandra salamandra* (García-París *et al.*,

1998, 2003), *Alytes obstetricans* (Martínez-Solano *et al.*, 2004; Gonçalves *et al.*, 2006), and *Lacerta schreiberi* (Paulo *et al.*, 2001). This relatively shallow differentiation has relevant implications for the evolutionary history of *L. helveticus*, and represents the basic difference with the mentioned "refugia within refugia" model.

One possible hypothesis to explain this pattern is that ancient lineages in the Iberian Peninsula have become extinct. There are alternative hypotheses, however, to consider in order to explain the absence of Pliocene or older Iberian lineages. *Lissotriton helveticus* could have speciated during the Lower Miocene somewhere in Central or Western Europe outside the Iberian Peninsula. This situation is compatible with the fossil record, which includes remains assigned to the species in Middle and Upper Pleistocene deposits from Central Europe and Great Britain (Ashton *et al*, 1994; Holman, 1998, 2000; Green *et al.*, 2006; Ivanov, 2007) and even Miocene remains found in sites north of the Pyrenees that have been identified as, or at least *affinis* to, *L. helveticus* (Rage & Bailon, 2005).

Following this scenario, the Iberian Peninsula would have been colonized later, at least not before the generalized cooling of the weather that started in

the Pleistocene, probably at the same time as other species, like *Mesotriton alpestris* (Sotiropoulos *et al.*, 2007) or *Rana temporaria* (Veith *et al.*, 2003). The oldest fossil remains in this region come from Middle Pleistocene deposits (Sanchiz, 1987). The colonization of the Iberian Peninsula probably was facilitated not only by the changes in environmental conditions but also by the retreat of a competing species such as *L. boscai* to southwestern regions. The phylogeographic pattern found in *L. helveticus* could therefore be explained assuming that one or several extinction events took place, probably associated with glacial maximum times, which eliminated most of the species' populations except those present in refugial areas in the northern third of the Iberian Peninsula. The approximated location of these areas is coincident with refugia proposed for other species (Gómez & Lundt, 2006). One has been proposed along the Atlantic coast of the Peninsula, most likely in the northern half of Portugal, where refugia for species such as *Chioglossa lusitanica longipes* (Alexandrino *et al.*, 2000, 2002), *Lissotriton boscai* (Martínez-Solano *et al.*, 2006), *Lacerta schreiberi* (Paulo *et al.*, 2001) or *Podarcis bocagei* (Pinho *et al.*, 2007) existed. Another one would be located in the western Cantabrian mountains, as hypothesized also for other taxa like *Salamandra s. bernardezi* (García-París *et al.*, 2003), *Mesotriton alpestris cyreni* (Sotiropoulos *et al.*, 2007) or *Lepus castroviejoi* (Pérez-Suarez *et al.*, 1994). A

third refugia would be in the northeastern part of the Iberian Peninsula, maybe along the Ebro basin and the Mediterranean coast, or maybe associated to the complex orography of the Pre-Pyrenean ranges, as it is probably the case for species of the genus *Calotriton* (Carranza & Amat, 2005) and Pyrenean *Iberolacerta* (Carranza *et al.*, 2004). A fourth one could have existed in the Sistema Ibérico Mountains, again a very complex area that served as a refugium for species like *Pinus pinaster* (Salvador *et al.*, 2000) or *Nebrioporus croceus* (Ribera, 2003).

Molecular data allow us to determine the amount of genetic diversity that has persisted along a period of extinctions and, together with comparable data from closely related taxa, give us an idea of how much has been lost through the Pleistocene climatic change cycles. However it gives us little hints of the evolutionary history of the species in areas where it completely disappeared at a given time, as seems to be the case for *L. helveticus*. As it has been recently proposed (Araújo *et al.*, 2008), species richness among amphibians and reptiles in Europe is strongly influenced by historic climate changes, which have affected specially narrow-ranging species probably because of their reduced dispersal capacity that prevents them to track climate changes, favouring endemicity in areas with more stable conditions (Jansson, 2003). It is logical to

suppose that the southward expansion of Polar conditions erased an amount of diversity at least equal to the diversity present today in the southern European peninsulas.

Given our limited sampling outside the Iberian Peninsula we cannot reject the hypothesis of the persistence of some northern refugia for the species. However our data seems to indicate that populations of *L. helveticus* north of the Pyrenees originated from a rapid range expansion from the Iberian Northeast, most probably during the Holocene as a consequence of the amelioration of climatic conditions. This is in accordance with previously proposed biogeographic hypotheses for this species (Zuiderwijk, 1980) and also fits the general model proposed for postglacial recolonization of Western Europe (Taberlet *et al.*, 1998; Schmitt, 2007). If we consider the limited dispersal ability of the species, this process probably took place as a contiguous range expansion. Again considering the poor capabilities of amphibians in general for oversea dispersal, the most likely way in which Great Britain was colonized was before the land bridge with the continent totally disappeared under the English Channel, 7,500 years ago at the very latest (Lambeck, 1997; Sanchiz, 2002; Martínková *et al.*, 2007), which is completely compatible with the Holocene expansion hypothesis. A full

characterization, using fast evolving markers such as microsatellites, of the genetic diversity of *L. helveticus* populations outside the Iberian Peninsula is needed to complete our knowledge on the colonization routes and mechanisms of expansion of the species north of the Pyrenees.

Phylogeographic implications

The possibility that *L. helveticus* has expanded its distribution range in thousands of square kilometres in about 10,000 years raises several questions, including: why didn't they expand to the south as well? Why is there so little introgression/admixture among mtDNA lineages? The answer to the first question may be related to the ecological requirements of the species, which include the cool and humid conditions typical of the Euro-Siberian phyto-climatic region and mountain habitats. Only a few populations of *L. helveticus* are known in Mediterranean habitats. These are often isolated and in precarious state of conservation and many populations from the Northern Plateau in central Spain have recently disappeared (Barbadillo, 2002). However in areas of favourable habitat, such as most of Galicia and northern Portugal, the species is not as common as expected. Here there is sympatry with the other Iberian species of the genus, *L. boscai*, which usually presents

higher densities and is more ubiquitously found. Apparently there is a progressive replacement of both species: as we move westwards and southwards *L. helveticus* is rarer than *L. boscai*, while *L. helveticus* becomes the dominant species as we move eastwards along the Cantabrian region. A similar pattern has been described between *L. helveticus* and *L. vulgaris* in Central Europe (Zuiderwijk, 1980), which could indicate there is some kind of competitive interaction among these species that prevents further expansions of their distributions, or at least not as fast as if there were no other competing species. The existence of wide areas of overlap between *L. helveticus* and *L. vulgaris* could be associated with the timing of the range expansion process. If Central Europe and Great Britain were colonized by these two species at similar times, low densities in the newly formed populations probably allowed the simultaneous colonization and largely sympatric distribution of both species in these regions.

The observed lack of admixture between mtDNA lineages is probably associated to the peculiarities of this molecular marker rather than to actual restrictions to gene flow among the groups. At present there is a continuous area with propitious habitat from the Pyrenees to northern Portugal and in these conditions moderately high levels of gene flow among populations is

expected. A similar pattern has been described for *Salamandra salamandra* in the same geographical area (García-París *et al.*, 2003). According to mtDNA data, *S. salamandra* populations are strongly structured along the Cantabrian region, but this structure is lost when analyzed with nuclear markers. The persistence of ancestral mtDNA lineages in these situations of high nuclear admixture is usually explained by differences in the evolutionary dynamics between maternally inherited molecular markers such as mtDNA genes from those in the nuclear genome (Birky *et al.*, 1983, 1989). The effective population size for mtDNA is about four times smaller than for nuclear DNA when sex ratio is 1:1 and if the sex ratio is skewed towards males the mtDNA effective population size is progressively reduced (Birky *et al.*, 1983). As a consequence, processes such as genetic drift are more marked at the mitochondrial level. The migration of mtDNA haplotypes could then be affected by the existence of large and already settled populations, especially if colonization of new areas is the result of contiguous range expansions, as our results suggest. In our case, the only population with mtDNA haplotypes from two different lineages is Camprovín, in the medium course of the Ebro River, giving additional support to the important role of this river as a dispersion vector for this species, allowing relatively long distance colonization and the admixture of ancestral lineages. Our nuclear results show a generalized lack of

geographic structure among the observed variation. This is probably explained by incomplete lineage sorting in these markers but also by the existence of considerable gene flow among populations. There is a shallow signal of lineage sorting in some populations of the "Northeastern" group, but with differences among the three studied genes. Gene flow reduction, small population size, bottlenecks, founder effects, could be some of the triggers for this incipient sorting. The results found in *L. helveticus* and other species (Irwin, 2002; Martínez-Solano *et al.*, 2007), with differentiated mitochondrial lineages along continuously populated areas argues against the often urging necessity to invoke the presence of physical barriers to explain the persistence in time of such patterns.

Within *L. helveticus*, estimated population sizes seem to have remained constant for most part of the last million years (fig. 7). The absence of dramatic population size reductions could have also favoured the integrity of ancestral lineages. Our results from historical demographic analyses suggest the existence of a population size expansion that started about 100,000 years ago, roughly corresponding with the last glacial age. This could be related, as suggested for other amphibians such as *Hyla intermedia* (Canestrelli *et al.*, 2007), to an increase of favourable habitat for *L. helveticus*, but also with a

retreat of potentially competing species like *L. boscai*, which, compared with *L. helveticus*, presents a reversed historical demographic model [as represented in fig. 3 in Martínez-Solano *et al* (2006)]. The only group not showing expansion, but rather a progressive population size reduction is the Northwestern lineage, which supposedly shared refugia with *L. boscai*. The end of the last glacial period apparently affected population sizes in *L. helveticus* negatively in the Iberian Peninsula but favoured *L. boscai* (Martínez-Solano *et al.*, 2006), which is probably associated to an expansion of Mediterranean conditions in the region and indicates the importance of autoecological traits in demographic responses to climate changes.

Biogeographic implications

The Iberian Peninsula is the tip of the large European Peninsula and, following the principles of the Peninsular Effect (Simpson, 1964), should present a reduced diversity compared to areas closer to the "main continent" (Baquero & Tellería, 2001). However Iberia presents a high degree of endemicity and important diversity hotspots when compared with most parts of Europe, which contradicts this idea. The arrival of African taxa alone could hardly explain

these differences so the question is why are endemicity and diversity higher in the Southern Peninsulas?

The phylogeographic patterns observed in the two Iberian *Lissotriton* species have been shaped by processes associated to climate changes during the Pleistocene. The resulting specific patterns conform to two different models (fig. 8) that can be roughly applicable to many other European species and that are identifiable by their genetic signatures in mitochondrial lineages. Species such as *L. boscai*, represent old inhabitants of the Peninsulas that persistently occupied at least parts of their ancestral ranges through the glacial cycles. In these cases, the existing populations would present traces of their long, independent evolutionary histories in that area, with deep phylogenetic lineages and genetic variation markedly structured geographically. Under this model, Pleistocene glaciations probably caused fragmentations and favoured lineage sorting, but allowed preservation of at least part of the accumulated ancestral diversity, creating, rather than refugia, sanctuaries of biological diversity. We refer to taxa under this model as type "S" (sanctuary) species. Other well-studied cases that fit this model include both geographically restricted species like *Chioglossa lusitanica* (Alexandrino *et al.*, 2000, 2002) and widespread taxa like *Salamandra salamandra* (Steinfartz *et al.*, 2000;

García-París *et al.*, 2003) or *Alytes obstetricans* (Martínez-Solano *et al.*, 2004; Gonçalves *et al.*, 2006). For most cases in model "S" species there are evidences of recent, big or small, range expansions after the last glacial maximum, but there are no evidences for range contractions during the Pleistocene, suggesting a certain stability of populations with migrations and range expansions limited geographically, maybe to altitudinal movements.

Species such as *L. helveticus*, represent taxa that colonized Iberia during the Quaternary in times of generalized climate changes. The cooler conditions characterizing the Pleistocene triggered southward range expansions in many Central and Northern European species. The newly colonized areas resulted in the precursors of truly glacial refugia, where only a small part of the populations could survive to generalized extinctions and most of the ancestral intraspecific diversity was lost under the ice, with the current diversification resulting from Pleistocene fragmentation and isolation in the refugia. We call them type "R" (refugia) species. In these cases a much shallower mitochondrial diversification is expected. Lineages would not probably be older than one million years and, in the case of widely distributed species, with large areas of genetically homogeneous populations. Several European taxa

can be ascribed to this model, for example *Bufo calamita* (Rowe *et al.*, 2006) or *Apodemus sylvaticus* (Michaux *et al.*, 2003).

The complexity generated by the presence of sets of species representing each model in Iberia and other Mediterranean peninsulas results in a markedly higher diversity when compared to other regions in Central and northern Europe and gave rise to the "refugia within refugia theory" which, in fact, can be misleading. The relative contribution to the diversity of a region of "R" and "S" species can be a more useful indicator of the incidence of past glaciations and of the levels of biodiversity lost by climate changes.

It has already been suggested that neutral genetic diversity in populations is more affected by its position relative to historical refugia than to the core of the current range (Garner *et al.*, 2004) and our data support this idea. Populations from recently colonized areas are often considered marginal and are neglected in conservation efforts because of their reduced genetic variation (Lesica & Allendorf, 1995). Nevertheless these populations can be precursors of future refugia under future global changes. Current trends in climate change

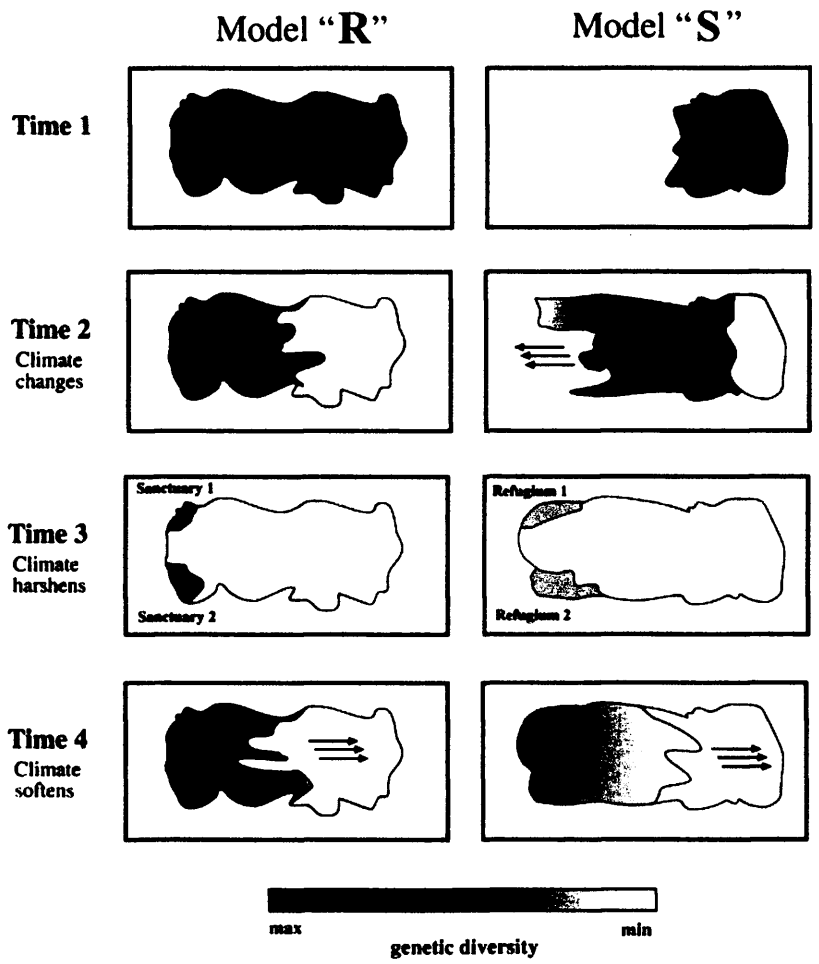

Figure 8. Graphical representation of the phylogeographic models described in the text, from time 1, before climate changes, to time 4, when phylogeographic studies take place. Model "S" conforms to persistence of ancestral populations in areas within the original (ancestral) territory (represented by a grey-black zone). In this case, reduction of the distribution area and posterior range expansion is not necessarily mandatory. Model "R" conforms to species that suffered extinction in all ancestral territories. Past contraction of their distribution range is mandatory but not the posterior range expansion. Genetic diversity, larger in older populations, is represented by the intensity of dark colour. Arrows indicate direction of range expansion and white areas denote population extirpation due to climate change. High biodiversity levels in the European southern Peninsulas are promoted by the persistence of "S" and "R" species together, while only "R" species are present in central-northern Europe.

threaten the persistence of many species in the Mediterranean region (Araújo *et al.*, 2006) and the species that managed to expand their ranges further North may have more chances of survival in the near future. These "S" and "R" models can be applied not only to the Iberian but also to other Mediterranean peninsulas, suggesting that these areas acted not only as glacial refugia but as sanctuaries for diversity whose conservation is more affected by the recent action of humans than by millions of years of ecological changes.

Diversity patterns in Lissotriton

Among the five species of *Lissotriton* we find three that present a restricted distribution, a priori reduced to the areas that served as refugia during the Quaternary: *L. montandoni* in the Carpathian Mountains, *L. italicus* in the southern half of the Italian Peninsula and *L. boscai* in the western half of the Iberian Peninsula (Raffaëlli, 2007). A common pattern among these species is the lack of intraspecific morphological differentiation in their populations, which, together with their reduced distributions, could also imply a homogeneous genetic structure (Karron, 1987). However, *L. italicus* and specially *L. boscai* present deep patterns of genetic variation associated to past fragmentation of their ranges (Ragghianti & Wake, 1987; Martínez-Solano *et*

al., 2006). *Lissotriton montandoni*, in contrast, is characterized based on mtDNA [11] by extensive introgressive hybridization with its sister species, *L. vulgaris* (Kotlík & Zavaldil, 1999; Babik et al., 2003; Litvinchuk et al., 2003; Babik & Rafinski, 2004; Mikulícek & Zavaldil, 2008), although they are well differentiated when analyzed with nuclear markers, morphology and sexual behaviour (Babik *et al.*, 2005 and references therein).

The other two species of the genus present much wider geographical distributions. *Lissotriton vulgaris* (Linnaeus, 1758) ranges from western Siberia and the Caucasus to Western Europe, including the British Islands but excluding the Iberian Peninsula and southern France (Raffaëlli, 2007). Through its range, high levels of morphological diversity have been described, concentrated mainly in the southern part of its distribution, a region where several glacial refugia could have existed, and seven or eight subspecies are usually recognized (Raxworthy, 1990; Schmidtler & Frantzen, 2004; Raffaëlli, 2007). The characters used to describe this intraspecific diversity correspond basically to secondary-sexual characters, which are supposed to be directly influenced by sexual selection (Halliday, 1990). The existence of different phenotypic groups goes along with the existence of a high genetic diversity and a relatively deep phylogeographic structure, although it is not always

concordant with the morphological variation, which can be explained by the existence of local selective processes (Babik *et al.*, 2005). In contrast, *L. helveticus* concentrates most of its intraspecific variation in a small part of its range corresponding to the Iberian Peninsula populations.

Allopatry seems to be the principal force in the genesis of species within *Lissotriton*, as in most amphibians (Vences & Wake, 2007). Given the observed patterns of diversity, the current distributions, and the fossil record, the most likely origin of *L. boscai*, *L. italicus* and *L. vulgaris* is located in the Mediterranean peninsulas, while *L. helveticus* and *L. montandoni* originated most probably in Central Europe. Allopatric processes are also the main factor in generating the observed intraspecific diversity patterns (Ragghianti & Wake, 1987, Babik *et al.*, 2005; Martínez-Solano *et al.*, 2006).

Patterns of diversity, however, are also governed by additional factors. One of them is the persistence of ancestral populations. Diversity, both at specific and intraspecific levels, is concentrated in particular areas that represent a small part of the entire range of the genus. These areas correspond to sanctuary regions were global changes like glaciations had little influence on the biological communities. In these cases, long term stability of population

distribution and size allow the maintenance of high levels of diversity, as in *L. boscai* or *L. vulgaris* (Babik *et al.*, 2005; Martínez-Solano *et al.*, 2006).

Another factor is the capability of colonization of new territories. After Pleistocene glaciations much of Europe became a territory with optimal ecological conditions for *Lissotriton* species and virtually free of competitors. Under this situation both *L. helveticus* and *L. vulgaris* were able to expand their respective geographic ranges. In the case of *L. helveticus* this was probably a recolonization of ancestral territories for the species, while *L. vulgaris* probably profited from the absence of other *Lissotriton* species to expand outside the Balkans (Babik *et al.*, 2005).

Another important factor is the level of interaction between species. The presence of a species in a given area seems to prevent the expansion of other species into that region. For example, the presence of *L. vulgaris meridionalis* in the northern half of Italy might prevent the expansion of *L. italicus* from the southern half. *Lissotriton montandoni* is probably confined to its small range because its populations are surrounded by large populations of *L. vulgaris*. In the case of *L. helveticus*, further expansion both to the south and to the east

might have been prevented by the presence of *L. boscai* and *L. vulgaris* respectively.

Effective population size is another factor that can directly affect all other factors. High population sizes will facilitate the persistence of populations and recolonization processes and will prevent possible replacement by other species. For example, a strong reduction of population size in *L. montandoni* could be related to the deep introgression with *L. vulgaris*, including the almost complete replacement of its mitochondrial genome (Babik *et al.*, 2005). In this case, the evolution of reproductive isolation mechanisms could have prevented the complete extirpation of *L. montandoni.* On the contrary, large population sizes would have helped the persistence of old lineages in *L. boscai* (Martínez-Solano *et al.*, 2006).

The different combinations of these four factors will ultimately define the two types of species described in this work, which should be in general applicable to most organisms present in the western Paleartic.

Conclusions

Almost all of the genetic diversity in *Lissotriton helveticus* is concentrated in the Iberian Peninsula. The analysis of mtDNA sequences reflects a geographically structured pattern that is maintained in spite of their continuous distribution in northern Iberia and the inferred existence of gene flow among the four main groups as indicated by nuclear markers.

All lineages in *L. helveticus* originated during the Pleistocene, probably during the last million years. These lineages are relatively recent if we consider that the origin of this species is dated about 20 Mya and that within other Mediterranean species of the genus *Lissotriton,* lineages originated during the Pliocene or even the Miocene. Our interpretation is that *L. helveticus* was originally a Central European species that colonized the Iberian Peninsula in the Middle Pleistocene, probably favoured by the generalized climate cooling. Glaciations eventually eradicated all ancestral lineages from its original geographical distribution but persistence of populations in southern refugia allowed the survival of the species with recent range expansions into favourable areas. The current distribution of the species in areas north of the

Pyrenees is probably a consequence of a rapid Holocene expansion from the Iberian Peninsula.

Lissotriton helveticus belongs to a group of taxa that we denominate type "R" species, which endured changes during glacial periods sheltered in peripheral or even marginal populations in meridional areas and, consequently, lost a big part of their ancestral intraspecific diversity. In contrast, we also define a group of type "S" species, as *Lissotriton boscai*, whose ancestral distribution areas acted as sanctuaries and were not as severely affected by climatic changes as in type "R" species and that could thus retain most of their long time accumulated diversity. These types are defined by four main factors that govern the patterns of diversity observed among *Lissotriton* species: persistence of ancestral populations, capability of colonization, interspecific interactions and effective population sizes. These factors, and hence their associated species types can be generalized to many organisms in the Western Paleartic.

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Table 1. Sampled localities, sample size and GenBank accession numbers (to be added upon manuscript acceptance) for sequences obtained for this study.

Population ID	Locality	Sample size	GenBank Accesion numbers				
			COX1	D-loop	Fib	Cxcr4	Tva4
1	Larués, Huesca, Spain	2	----	----	----	----	----
2	Gan, Aquitaine, France	2	----	----	----	----	----
3	Pto. del Portalé, Huesca, Spain	4	----	----	----	----	----
4	Obarenes-Encio, Burgos, Spain	6	----	----	----	----	----
5	Ganuza, Navarra, Spain	2	----	----	----	----	----
6	Pozo Negro, Burgos, Spain	2	----	----	----	----	----
7	El Fito, Asturias, Spain	6	----	----	----	----	----
8	Esqueiro, Soto de Luiña, Asturias, Spain	5	----	----	----	----	----
9	Tolivia, Asturias, Spain	7	----	----	----	----	----
10	Cabrillanes, León, Spain	1	----	----	----	----	----
11	Taboada, A Coruña, Spain	6	----	----	----	----	----
12	Vila do Conde, Porto, Portugal	2	----	----	----	----	----
13	Requejo, Cantabria, Spain	1	----	----	----	----	----
14	Pto. de Palombera, Cantabria, Spain	2	----	----	----	----	----
15	Neila, Burgos, Spain	3	----	----	----	----	----
16	Arija, Burgos, Spain	2	----	----	----	----	----
17	Gama, Cantabria, Spain	6	----	----	----	----	----
18	Ambleteuse, Alsace, France	1	----	----	----	----	----
19	Quintanas de Hormiguera, Palencia, Spain	2	----	----	----	----	----
20	Vagos, Aveiro, Portugal	3	----	----	----	----	----

21	Palamós, Girona, Spain	4	----	----	----	----	----
22	Artíes, Lleida, Spain	2	----	----	----	----	----
23	Beorburu, Navarra, Spain	4	----	----	----	----	----
24	Bouillouses, Pyrénées-Orientales, France	1	----	----	----	----	----
25	Urdiáin, Navarra, Spain	5	----	----	----	----	----
26	Camprovín, La Rioja, Spain	4	----	----	----	----	----
27	Southampton, England, UK	1	----	----	----	----	----
28	Santa Inés, Soria, Spain	1	----	----	----	----	----
29	Pto. de San Isidro, Asturias, Spain	1	----	----	----	----	----
30	Fagollaga, Guipúzcoa, Spain	1	----	----	----	----	----
31	Moncayo, Zaragoza, Spain	2	----	----	----	----	----
32	Sos del Rey Católico, Zaragoza, Spain	2	----	----	----	----	----
33	Alfranca, Zaragoza, Spain	2	----	----	----	----	----
34	Tudanca, Cantabria, Spain	3	----	----	----	----	----
35	Gehren, Thuringia, Germany	2	----	----	----	----	----
	Total:	100					

Table 2. Fu's Fs statistic and Mantel's tests results for the main mt-DNA lineages within *Lissotriton helveticus*.

Group	Fs	p	r	p
Total	-11.1743	**<0.05**	0.170	**<0.005**
Northeastern	-9.0064	**<0.0005**	0.335	**<0.005**
East Cantabrian	-4.2135	**<0.05**	0.094	>0.05
Asturian	-3.2640	**<0.05**	0.316	**<0.005**
Northwestern	2.1678	>0.05	0.634	**<0.005**

II. Capítulo 4

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Urban aquatic habitats and conservation of highly endangered species: the case of *Ambystoma mexicanum* (Caudata, Ambystomatidae)

Ernesto Recuero[1], Juan Cruzado-Cortes[2], Gabriela Parra-Olea[3,*] & Kelly R. Zamudio[4]

[1)] *Museo Nacional de Ciencias Naturales, CSIC, José Gutiérrez Abascal 2, 28006 Madrid, Spain*
[2)] *Instituto de Ecología, UNAM, Tercer Circuito exterior s/n, Cd. Universitaria, C.P. 04510, México, D. F., México*
[3)] *Instituto de Biología, UNAM, Tercer Circuito exterior s/n, Cd. Universitaria, C.P. 04510, México, D. F., México (*corresponding author's e-mail: gparra@ibiologia.unam.mx)*
[4)] *Department of Ecology and Evolutionary Biology, Cornell University, Ithaca, New York 14853, USA*

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Species with highly restricted distributions are vulnerable to extinction, and modification of natural habitats within their small ranges is a primary threat to their persistence. Expansion of urban development significantly impacts natural habitats and, therefore, threatens local diversity. The Mexican axolotl, *Ambystoma mexicanum*, is a strictly aquatic species that persists currently in two highly threatened and isolated populations. The current habitat remaining for these species are remnants of a historically extensive lacustrine system that occupied the entire Valley of Mexico, but has been destroyed by the growth of Mexico City. Unexpectedly, a third viable population of axolotls has been found in Chapultepec Park, a public recreational area in the heart of Mexico City. Phylogenetic and haplotype network analyses of mitochondrial DNA sequences confirmed low genetic differentiation and a recurrent lack of monophyly in many of the taxa belonging to the *Ambystoma tigrinum* species group, including *A. mexicanum*, but clustered the Chapultepec samples with other *A. mexicanum* samples. Our data revealed higher haplotypic diversity in *A. mexicanum* populations than previously recorded, due to new haplotypes from Chapultepec Park. We found high incidence of parasites and deformities among individuals in this population, which could negatively impact their viability. Our results emphasize the important role that artificial or semi-natural urban habitats can play in the conservation of highly threatened species.

Introduction

Amphibian populations are declining worldwide (Houlahan *et al.* 2000, Stuart *et al.* 2004, Young *et al.* 2004) and various factors such as climate change, emergent diseases, and introduced species have been implicated as threats to remaining species and populations (Alford & Richards

1999, Blaustein & Kiesecker 2002, Lips *et al.* 2006, Pounds & Puschendorf 2004, Pounds *et al.* 2006). Habitat loss and fragmentation are two important factors threatening amphibians (Dodd & Smith 2003, Stuart *et al.* 2004) because they potentially lead to reduced population sizes, isolation and loss of metapopulation dynamics, habitat split, and increased susceptibility to edge effects (Petranka *et al.* 1993, Marsh & Trenham 2001, Andersen *et al.* 2004, Homan *et al.* 2004, Cushman 2006, Gagné & Fahrig 2007, Becker *et al.* 2007). Urban development, especially in cities with high population densities, eliminates habitat required for local species and reduces chances for dispersal and recolonization of habitat patches that remain (Marzluff 2005, Husté & Boulinier 2007, Hahs *et al.* 2009). Aquatic habitats are especially vulnerable, because in addition to becoming isolated, they also often serve as final disposal points for urban pollutants that affect the viability of biological communities (Wolter *et al.* 2000). The threat of extinction due to urban development will be particularly high for locally endemic aquatic species that have small geographic ranges and therefore no assurance of population survival outside of the urbanized region. Management of narrow endemic species is a difficult task, and requires preservation of as much native habitat as possible, restoration of historical habitats, careful planning of new urban development, and attention to potential alternate solution such as the use of non-native habitats and *ex-situ* conservation programs (Gordon *et al.* 2009, Hostetler & Drake 2009).

The Valley of México is an inland hydrographic basin that was partially covered by a widespread complex of several large, interconnected lakes (Armillas 1971, Berres, 2000). These lakes were the original habitat of a rich biological community that has been exploited by humans since prehistoric times (Niederberger 1979). Modification of the lacustrine complex started in pre-Hispanic times with the construction of dykes separating Lago de México from Lagos de Xochimilco, Chalco and the saline Lago Texcoco (Armillas 1971, Alcocer-Durand & Escobar-Briones 1992). Several artificial drainages were constructed from the 17th to the 20th centuries in attempts to dry the lake systems for urban and agricultural development and to prevent frequent floods in the area (Aréchiga Córdoba 2004). Currently, the lakes are reduced to only a few small and highly perturbed remnants and are isolated by Mexico City, one of the world's largest metropolitan areas. Not surprisingly, the native biota of the valley lakes is now impoverished; habitat loss and alterations have led to the extinction of approximately one third of the aquatic plant species in Xochimilco (Novelo & Gallego 1988) as well as endemic taxa such as four species of the cyprinid fish in the genus *Evarra* (Méndez-Sánchez *et al.* 2002) and Tlaloc's Leopard Frog, *Lithobates tlaloci* (Santos-Barrera & Flores-Villela 2004). Chapultepec Park is the largest green area in Mexico City, occupying approximately 670 hectares. Chapultepec harbors three artificial lakes (Alcocer *et al.* 1988), and one of them, Lago Viejo, still harbors a substantial number of species of the historical aquatic biota of the Valley of México (Alcocer-Durand & Escobar-Briones 1992, Ceballos *et al.* 2005), including several protected species including the atherinid *Chirostoma jordani*, the goodeid *Girardinychthys viviparus*, and the amphibians *Rana montezumae* and *Ambystoma* salamanders (Alcocer & Lugo 1995).

Ambystoma mexicanum is an obligate paedomorphic species, endemic to the Valley of México. It is widely used as a model organism in evolutionary and developmental biology, and is thus commonly maintained in captivity, with several breeding colonies around the world (Malacinski & Able 1989). Currently, only two wild populations persist: one in the channels of Xochimilco and a second in the remnants of Lago de Chalco, both in southern México City (Fig. 1; Zambrano *et al.* 2004). The continued persistence of this species in the wild is uncertain due to population isolation, water pollution and eutrophication, introduction of exotic species and overharvesting. In addition, hormonal disruption caused by chemical contaminants can cause abnormal reproductive development in amphibians that disrupt recruitment in natural populations (Hayes *et al.* 2002, Reeder *et al.* 1998, 2005, Petterson & Berg 2007). Hormonal disruption has been proposed as a cause of sex ratio biases in the Xochimilco population (Griffiths *et al.* 2003). Water pollution can also

negatively affect amphibian immune systems (Gilbertson *et al.* 2003) leading to higher levels of parasitism and susceptibility to infectious diseases. Combined, these factors have raised concerns for the persistence of the Mexican axolotl and led to various initiatives to preserve remaining natural populations (Graue *et al.* 1998, Griffiths *et al.* 2003, 2004, Zambrano 2006, Bride *et al.* 2008). In an effort initiated in 2004, the Mexico City government began a program to eradicate exotic species from Chapultepec Park (Ceballos *et al.* 2005). Lago Viejo was drained and 26 adults and three clutches of *Ambystoma* were found. The adults were identified as *A. mexicanum* based on morphology (Taylor 1939). The presence of *Ambystoma* in the Chapultepec lakes had been previously reported (Alcocer-Durand & Escobar-Briones 1992); however, species identity was uncertain, and the specimens were tentatively assigned either to *A. mexicanum* or to the widespread *A. velasci* (Zambrano *et al.* 2006, Stuart *et al.* 2008).

Here, we characterize the *Ambystoma* population from Chapultepec Park on the basis of mtDNA sequence data, and examine the genetic relationships among individuals in this artificial lake and the two remaining natural populations. We also report on deformities and parasites found in the Chapultepec population. Our study highlights the persistence of a highly threatened species in an artificial and highly urbanized environment. We discuss the possible role of this and other similar populations as reservoirs that guard against species extinction and/or reductions in the genetic diversity of wild populations.

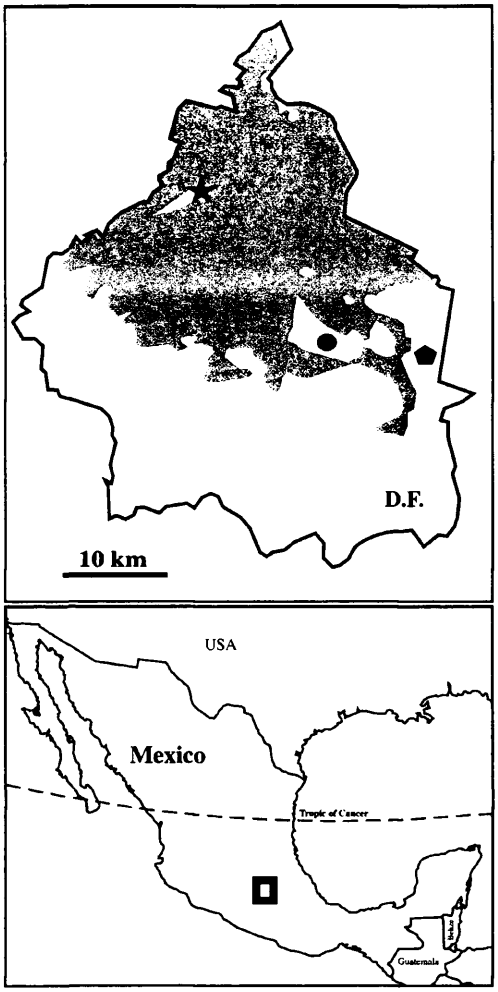

Fig. 1. Location of the three remaining populations of *Ambystoma mexicanum*. Lower map indicates the position of Distrito Federal, México (square). Upper map shows the political boundaries of the Distrito Federal and the location of the three known *Ambystoma mexicanum* populations: Xochimilco (circle), Chalco (pentagon), and Chapultepec (star). The extent of urban development in México City is represented by the gray shaded area.

Material and methods

Study site

Chapultepec Park is located in the western section of Mexico City (between 19°24′ and 19°26′N, 99°11′ and 99°13′W) at an elevation of 2240 m (Fig. 1). Currently, three lakes exist in the park: Lago Viejo, with a surface area of 6 ha and a maximum depth of 1.8 m; Lago Mayor, which is 5.8 ha in area and with a 1.3 m maximum depth; and Lago Menor, which is 2.8 ha in area and with a 1.2 m maximum depth (Lugo *et al.* 1998). These three lakes are widely used for recreational purposes and are mainly filled with treated wastewater, resulting in high degree of eutrophication (Alcocer-Durand & Escobar-Briones 1992, Lugo *et al.* 1998). The three Chapultepec lakes are approximately 27 and 20 km distant from Lago de Chalco and

Xochimilco, respectively, the two sites that harbor natural populations of *A. mexicanum*. Although the Chapultepec Lakes are artificial, they occur very near the historical range of the lacustrine system that occupied the Valley of México and still harbor a number of aquatic organisms that were present in the ancient lake complex (Alcocer-Durand & Escobar-Briones 1992).

Sampling

All individuals in this study were collected from Lago Viejo in the same series of lake drainages in 2005. The lake was partially drained multiple times for cleaning and removal of non-native fauna. Native vertebrate and macroinvertebrate species were captured with nets and maintained in outdoor tanks. Once the lake was cleaned and refilled, the animals were released at the site of capture. Tissues were collected from adult *Ambystoma* by clipping a small portion of the tip of the tail.

Mitochondrial DNA amplification and sequencing

We extracted total genomic DNA from 11 *A. mexicanum* individuals from Chapultepec and six individuals from Xochimilco. We digested ethanol-preserved tissues in lysis buffer and Proteinase K, followed by standard organic purification with phenol-chloroform (Sambrook & Russell 2001). Polymerase chain reaction (PCR) was used to amplify a fragment of 1029 base pairs, including the tRNA-proline and the mitochondrial control region (D-loop), using the primers THR and 651 (Shaffer & McKnight 1996). PCR reactions were performed in a total volume of 25 μl, including one unit of *Taq* polymerase (Biotools, 5 U ml^{-1}), 2.5 μM of each primer, 0.4 mM of dNTPs, 1.5 mM of $MgCl_2$, and 67 mM of PCR buffer (Tris-HCl, pH = 8.3, Biotools). PCR reactions consisted of 35 cycles with a denaturing temperature of 94 °C (1 min), an annealing temperature of 50 °C (1 min) and an extension temperature of 72 °C (1 min). Double-stranded templates were cleaned using sodium acetate and ethanol precipitation, and PCR products were then re-suspended in 22 μl of ddH_2O. Sequencing of the amplified segments followed Martínez-Solano *et al.* (2006). The resulting sequences were deposited in GenBank (accession nos. HM142769–HM142771).

Sequence alignment and phylogenetic reconstruction

Sequences were read and aligned by eye using Sequence Navigator™ ver. 1.0.1 (Applied Biosystems). In our phylogenetic analyses, we also included published sequences from 17 additional *Ambystoma* species from GenBank (number of haplotypes included in the analyses are given in parentheses): *Ambystoma mexicanum* (4), *A. velasci* (14), *A. ordinarium* (9), *A. flavipiperatum* (1), *A. taylori* (1), *A. andersoni* (2), *A. amblycephalum* (1), *A. granulosum* (2), *A. lermaense* (1), *A. altamirani* (2), *A. rivulare* (2), *A. dumerilii* (3), *A. rosaceum* (2), *A. tigrinum* (4), *A. m. mavortium* (4), *A. mavortium melanostictum* (4) and *A. mavortium nebulosum* (6). The California tiger salamander, *A. californiense*, was used as an outgroup (Table 1).

We applied a neighbor-joining (NJ) analysis under the Minimum Evolution objective function with uncorrected ("p") distances; ties were broken systematically. We used nonparametric bootstrapping (1000 pseudoreplicates) to assess the stability of internal branches in the resulting topologies. Neighbor-joining analyses were performed with PAUP* 4.0b10 (Swofford 2002). We also performed Bayesian phylogenetic analyses using MrBayes ver. 3.1.2 (Ronquist & Huelsenbeck 2003). We initiated the analysis with random starting trees and ran four Metropolis coupled Monte Carlo Markov chains (three heated, one cold) for 10^7 generations, sampling every 1000 generations. We checked for stationarity and convergence of the chains with Tracer ver. 1.3 (Rambaut & Drummond 2004) and discarded 10^4 trees as burn-in. Posterior clade probabilities were used to assess nodal support. In addition, we constructed a haplotype network from mtDNA sequences using the software TCS ver. 1.18 (Clement *et al.* 2000), which implements the statistical parsimony algorithm

Table 1. Species, collection localities, and sequences included in this study. Numbered localities, and GenBank accession numbers for each haplotype are from Shaffer and MacKnight (1996), Weisrock *et al.* (2006), Arnason *et al.* (2004), and Samuels *et al.* (2005) indicated in the table's locality and reference columns with A, B, C, and D, respectively. Haplotype numbers are those represented in Figs. 2–4.

Species	Locality	Haplotype	GenBank accession number	References
A. altamirani	Locality 64 (A): México, México	XLIII	DQ241130	A, B
A. altamirani	Locality 65 (A): Morelos, México	XLII	DQ241131	A, B
A. amblycephalum	Locality 58 (A): Michoacán, México	XII	DQ241132	A, B
A. andersoni	Locality 55 (A): Michoacán, México	XXIII, XXIV	DQ241134, DQ241133	A, B
A. californiense	Locality 2 (A): California, USA	LXIV	DQ241127	A, B
A. dumerilii	Lake Patzcuaro, Michoacán,México	XLVIII, L, LI	DQ241203, DQ241202, DQ241137	A, B
A. flavipiperatum	Locality 54 (A): Jalisco, México	XVII	DQ241138	A, B
A. granulosum	Locality 61 (A): México, México	XLV	DQ241139	A, B
A. granulosum	Locality 62 (A): México, México	XL	DQ241140	A, B
A. lermaense	Locality 63 (A): México, México	XLI	DQ241142	A, B
A. m. mavortium	Locality 32 (A): Nevada, USA	XXVI	DQ241143	A, B
A. m. mavortium	Locality 33 (A): Colorado, USA	XXXV	DQ241144	A, B
A. m. mavortium	Locality 34 (A): Nuevo Mexico, USA	XXXVII	DQ241145	A, B
A. m. mavortium	Locality 35 (A): Texas, USA	XXXVIII	DQ241146	A, B
A. m. melanostictum	Locality 15 (A): California, USA	XXXVI	DQ241147	A, B
A. m. melanostictum	Locality 16 (A): Washington, USA	XXXII	DQ241148	A, B
A. m. melanostictum	Locality 18 (A): Montana, USA	XXXIII	DQ241150	A, B
A. m. melanostictum	Locality 25 (A): Wyoming, USA	XXXIV	DQ241154	A, B
A. m. nebulosum	Locality 11 (A): Colorado, USA	XXX	DQ241163	A, B
A. m. nebulosum	Locality 6 (A): Utah, USA	XXVIII	DQ241158	A, B
A. m. nebulosum	Locality 7 (A): Utah, USA	XXXI	DQ241159	A, B
A. m. nebulosum	Locality 8 (A): Utah, USA	XXVII	DQ241160	A, B
A. m. nebulosum	Locality 9 (A): Arizona, USA	XXIX	DQ241161	A, B
A. mexicanum	captive animals, source population unknown	II, III	AJ584639, AY659991	C, D
A. mexicanum	Locality 68 (A): Chalco, D.F., México	II	DQ241155, DQ241156	A, B
A. mexicanum	Lago Viejo, Parque Chapultepec, D.F., México	I, IV, V	HM142769–HM142771	this paper
A. mexicanum	Lago Xochimilco, D.F., México	I, IV	HM142769–HM142770	this paper
A. nebulosum	Locality 14 (A): Colorado, USA	LIX	DQ241167	A, B
A. ordinarium	Locality 1 (B): Michoacán, México	XLVII	DQ240926	B
A. ordinarium	Locality 15 (B): Michoacán, México	VI	DQ241073	B
A. ordinarium	Locality 16 (B): Michoacán, México	VII	DQ241084	B
A. ordinarium	Locality 17 (B): Michoacán, México	VIII	DQ241096	B
A. ordinarium	Locality 18 (B): Michoacán, México	IX	DQ241106	B
A. ordinarium	Locality 19 (B): Michoacán, México	LIV	DQ241115	B
A. ordinarium	Locality 20 (B): Michoacán, México	XV	DQ241125	B
A. ordinarium	Locality 4 (B): Michoacán, México	XLIX	DQ240967	B
A. ordinarium	Locality 60 (A): Michoacán, México	LIII	DQ241169	A, B
A. rivulare	Res. Mariposa Monarca, Michoacán, México	XLIV, XLVI	DQ241217, DQ241215	B
A. rosaceum	Locality 51 (A): Durango, México	LXII	DQ241170	A, B
A. rosaceum	Locality 52 (A): Chihuahua, México	LXIII	DQ241171	A, B
A. taylori	Locality 76 (A): Puebla, México	XXII	DQ241173	A, B

continued

Table 1. Continued.

Species	Locality	Haplotype	GenBank accession number	References
A. tigrinum	Locality 41 (A): Tennessee, USA	LVI	DQ241179	A, B
A. tigrinum	Locality 42 (A): South Carolina, USA	LVIII	DQ241180	A, B
A. tigrinum	Locality 43 (A): Georgia, USA	LVII	DQ241181	A, B
A. tigrinum	Locality 44 (A): Florida, USA	LV	DQ241182	A, B
A. velasci	Locality 45 (A): Chihuahua, México	XXV	DQ241183	A, B
A. velasci	Locality 46 (A): Chihuahua, México	LX, LXI	DQ241184, DQ241185	A, B
A. velasci	Locality 47 (A): Durango, México	XIV	DQ241186	A, B
A. velasci	Locality 48 (A): Nuevo León, México	XIX	DQ241187	A, B
A. velasci	Locality 49 (A): San Luís Potosí, México	XVI	DQ241188	A, B
A. velasci	Locality 50 (A): Guanajuato, México	XVIII	DQ241189	A, B
A. velasci	Locality 53 (A): Jalisco, México	LII	DQ241190	A, B
A. velasci	Locality 57 (A): Michoacán, México	XIII	DQ241191	A, B
A. velasci	Locality 66 (A): México, México	XXXIX	DQ241192	A, B
A. velasci	Locality 67 (A): México, México	XI	DQ241193	A, B
A. velasci	Locality 69 (A): Hidalgo, México	X	DQ241194	A, B
A. velasci	Locality 70 (A): Puebla, México	XX	DQ241195	A, B
A. velasci	Locality 77 (A): Veracruz, México	XXI	DQ241201	A, B

described in Templeton *et al.* (1992) and more accurately represents the genealogical relationships of haplotypes that are recently diverged. We ran the analysis under a 95% probability connection limit considering gaps as a fifth state. Our network included all *A. mexicanum* haplotypes and exemplar haplotypes of an additional seven closely associated species, based on the results of our phylogenetic analyses.

Malformations and parasites

All adult *Ambystoma* captured at Chapultepec were measured and visually inspected for deformities. We recorded the presence of *Lernaea*, an ectoparasitic copepod commonly found on amphibians (Green *et al.* 2002) and *Saprolegnia*, a pathogenic fungus that also infects amphibians (Blaustein *et al.* 1994). Three of the adult salamanders died in captivity, allowing for post mortem examination and detection of internal parasites.

Results

A total of 26 adult *Ambystoma* and three egg masses were collected from the Lago Viejo. All individuals and egg masses were removed from the lake and maintained in plastic containers at the facilities of the Chapultepec Zoological Garden while the lake was cleaned. Once the process was completed, the animals were released into the lake. One of the egg masses hatched, but two did not develop to hatching.

Genetic variation

Our results indicate low overall genetic variation among the sequences analyzed. Of the total of 1085 characters, only 86 were parsimony-informative (including the out-group). Among sequences of *Ambystoma mexicanum*, we found five haplotypes (Table 1, Figs. 2 and 3). Two of them were found both in Xochimilco and Chapultepec (Haplotypes I and IV); one was unique to Chapultepec (Haplotype V); the other two were found in the Chalco population (Haplotype II) or in the samples of unknown origin (Haplotypes II and III) (Figs. 2 and 3). Three of the GenBank sequences have a deletion at position 13 of the amplified fragment, in the threonine-proline intergenic spacer; all remaining individuals have cytosine at that position. The other variable positions among *A. mexicanum* sequences were located in the control region.

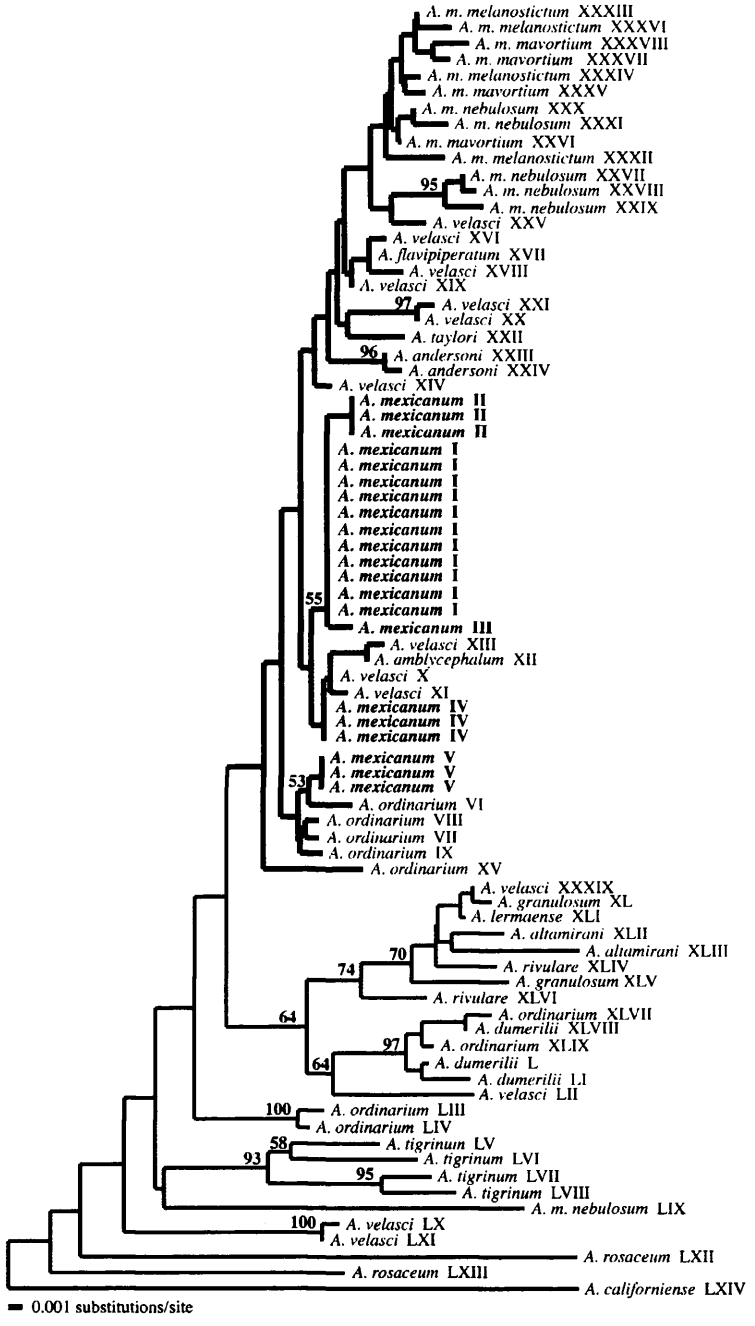

Fig. 2. Neighbor-Joining topology showing relationships among haplotypes of species in the *A. tigrinum* complex. Geographic origin of each haplotype is given in Table 1. Haplotypes in the *A. mexicanum* clade (indicated by thicker lines) are represented in a haplotype network in Fig. 4. Numbers on branches are bootstrap values of nodal support.

When all 80 sequences were analyzed together, we found low divergence among species. Both NJ (Fig. 2) and Bayesian (Fig. 3) reconstructions resulted in poorly supported topologies, as expected in view of the low number of variable positions in the sequence data and previously published phylogenies (Shaffer & McKnight 1996). The Bayesian consensus tree

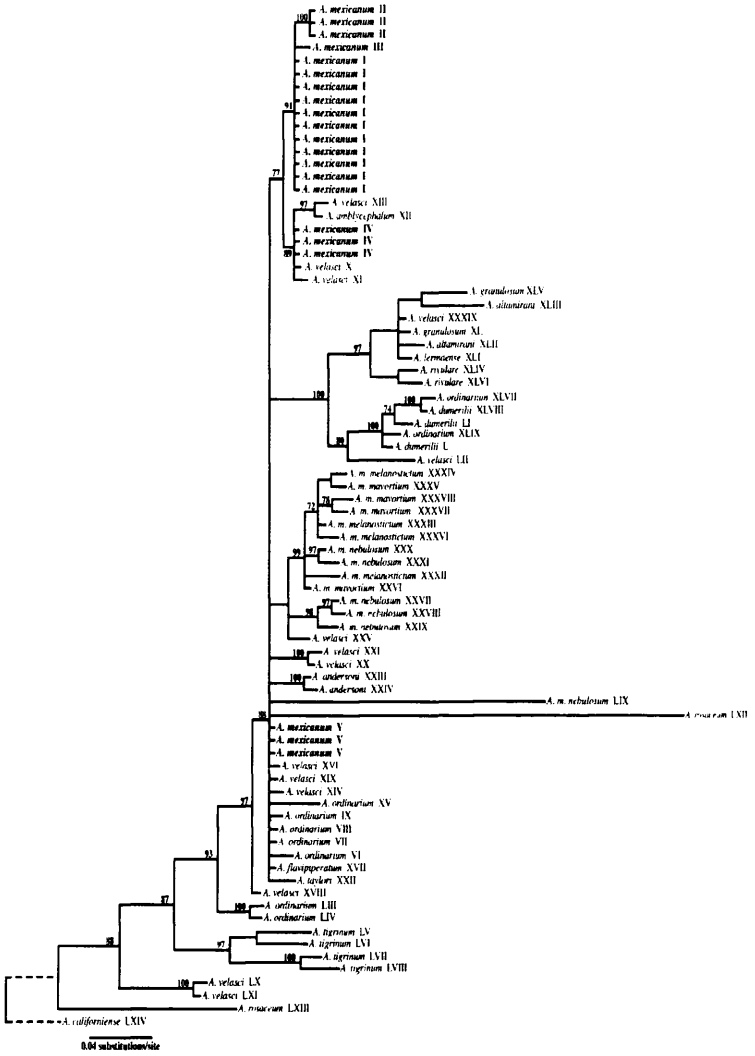

Fig. 3. Bayesian consensus tree showing relationships among haplotypes of species in the *A. tigrinum* complex. Number along branches are posterior probabilities values from the Bayesian analyses.

(Fig. 3) is characterized by polytomy with only a few distinct lineages, including an *A. tigrinum* clade, a second clade composed primarily of *A. mavortium* haplotypes, a third clade containing several Mexican species (*A. dumerilii*, *A. rivulare*, *A lermaense* or *A. granulosum*), and a fourth clade composed primarily of *A. mexicanum* haplotypes. These groups are also found in the NJ tree (Fig. 2), with some changes. For example, the NJ topology has a clade formed by *A. mexicanum* haplotype V and haplotypes from *A. ordinarium*. The most remarkable result in both analyses is the lack of monophyly for most taxa represented by more than one haplotype: *A. velasci* and *A. ordinarium* haplotypes are distributed in at least three different clades. *Ambystoma mexicanum* haplotypes I, II, III and IV are grouped in a single clade together with *A. velasci* and *A. amblycephalum* haplotypes. Haplotype V, as mentioned before, is grouped with *A. ordinarium* haplotypes in the NJ tree (Fig. 2), but forms part of a basal polytomy in the Bayesian topology (Fig. 3).

We reconstructed a haplotype network including all five *A. mexicanum* haplotypes plus 33 additional *Ambystoma* haplotypes, for a total

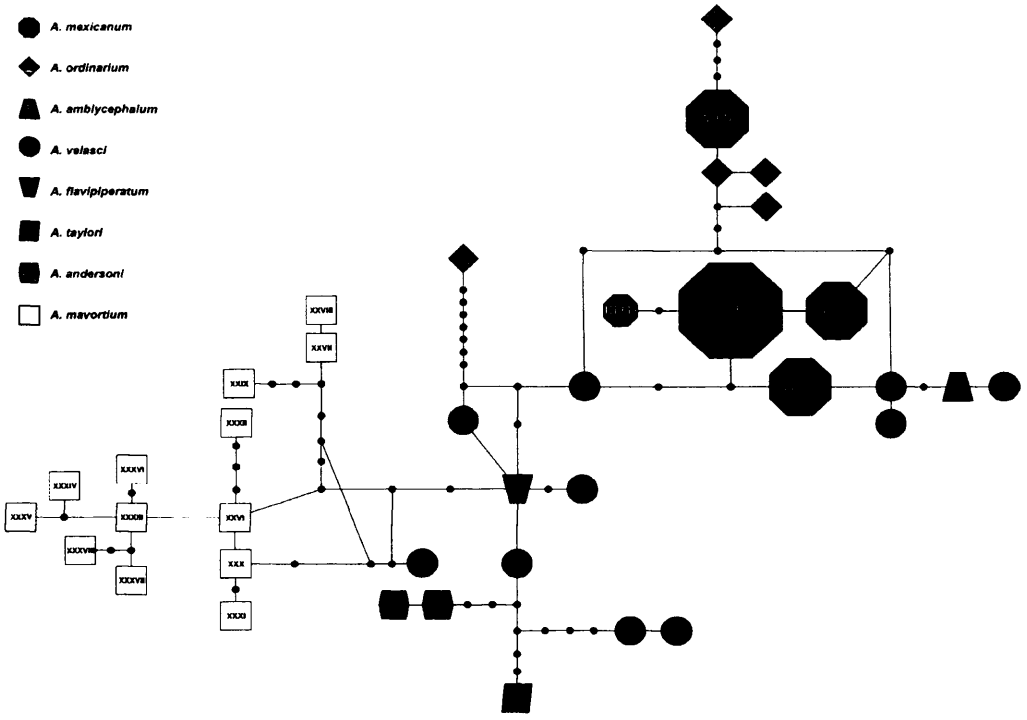

Fig. 4. Haplotype network showing position and distances among haplotypes in the *A. mexicanum* clade inferred in our phylogenetic analysis. Haplotype frequencies for *A. mexicanum* haplotypes are indicated in parentheses. For simplicity we included only unique haplotypes for the remaining species in the clade.

of 8 species (Fig. 4). This network includes representatives of all taxa in the *A. mexicanum* clade found in our NJ topology (Fig. 2). The resulting network reflects the low genetic differentiation among Mexican species of *Ambystoma* and corroborates results from the phylogenetic analyses. Despite the small number of mutations separating named species, our network does reveal some clustering among regional groups. For example, the Mexican species are generally more closely related to each other than to the North American samples (*A. mavortium*). Within the group of haplotypes from Mexican endemics, it is noteworthy that *A. velasci* haplotypes are spread throughout the network; this taxon is a metamorphic species widespread throughout the range of other *Ambystoma* species in southern México. The five haplotypes from *A. mexicanum* differ by only one or two mutational steps and are associated primarily with haplotypes of *A. velasci*, *A. amblycephalum*, and *A. ordinarium*. Haplotype V, from the Chapultepec samples of *A. mexicanum*, is an exception. This haplotype differs by five mutational steps from the remaining *A. mexicanum* haplotypes, and may reflect the isolation of that population; however, this level of intraspecific differentiation is not unusual among other taxa in our sample.

Malformations and parasites

Twenty adult specimens were visually inspected for external deformities and parasites (Table 2). All observed deformities were present in the limbs. Eight specimens were polydactylous, four specimens had missing toes, two had fused toes and one had toes reduced in size. Three adults were dissected at the time of death and all hosted larval forms of the nematode *Eustrongylides* sp. The nematodes were found tightly coiled, encapsulated, and attached to the mesentery in the

coelomic cavity, liver, and subepidermal tissue; the latter formed bumps that were visible externally so the presence of this parasite was also detected in six additional specimens. The pathogenic fungus *Saprolegnia* spp. was found on two adult specimens. Approximately 60% of the individuals had the parasitic copepod *Lernaea*, which was found mostly in the cephalic area, but also on other parts of the body.

Discussion

Systematics of *Ambystoma* and species identification.

All Mexican species of *Ambystoma*, including *A. mexicanum*, belong to a taxonomic group defined as the *Ambystoma tigrinum* complex (Shaffer & McKnight 1996). Systematics of this species complex has been problematic due to the scarcity of reliable morphological characters (Shaffer 1984a, 1984b, Irschick & Shaffer 1997). Allozyme and mitochondrial DNA studies (Shaffer 1984a, Shaffer & McKnight 1996) revealed low genetic differentiation among species, and poorly resolved phylogenetic trees with several apparently non-monophyletic taxa (Shaffer 1984a, Shaffer & McKnight 1996, Weisrock *et al.* 2006). This lack of monophyly could be the result of incomplete lineage sorting, which precludes the recovery of a resolved phylogenetic tree (Avise 2000). This is a common difficulty in studies of recently radiated species groups (Takahashi *et al.* 2001) and occurs due to large effective population sizes and short periods of time between speciation events that favor the retention of ancestral polymorphisms (Pamilo & Nei 1988). An alternate explanation for our results is that gene flow occurs among morphologically and ecologically differentiated *Ambystoma* species. Interbreeding among paedomorphic and metamorphic species produces fertile offspring under captive conditions (Brandon 1972, 1977, Voss 1995), thus hybridization in the wild among some species may be feasible.

Previous phylogenetic studies of this group included a maximum of two samples of *A. mexicanum* that showed no genetic differentiation (Shaffer 1984a, Shaffer & McKnight 1996, Weisrock *et al.* 2006). Our dataset included 19 individuals from three different populations

Table 2. Data on sex, snout-vent length (SVL), tail length (TL), toe abnormalities, and presence of *Eustrongylides* cysts for 20 specimens of *Ambystoma mexicanum* collected in Chapultepec Park.

ID	Sex	SVL (mm)	TL (mm)	Polydactyly	Toe deformities	Nematode cysts
1	Female	128.0	92.0	Present	None	Present
2	Female	130.9	86.7	Absent	Missing	Present
3	Female	154.9	104.0	Absent	None	Present
4	Male	139.4	120.0	Absent	None	no data
5	Male	131.1	88.3	Absent	Missing	no data
6	Female	127.1	94.6	Absent	Reduced	no data
7	Male	105.4	109.0	Absent	None	no data
8	Female	138.2	101.0	Present	None	no data
9	Female	127.2	78.1	Absent	Fused	no data
10	Male	131.3	111.0	Absent	Missing	Present
11	Female	130.0	94.0	Present	None	no data
12	Male	91.6	126.0	Absent	None	no data
13	Male	111.7	76.9	Absent	None	no data
14	Female	120.0	87.4	Present	None	Present
15	Male	105.9	146.0	Present	None	Present
16	Female	119.2	78.8	Absent	None	no data
17	Female	127.7	83.8	Present	None	Present
18	Female	132.6	83.4	Absent	Fused	Present
19	Female	119.0	79.0	Present	None	no data
20	Female	102.7	64.1	Present	Missing	Present

(plus two captive specimens of unknown origin) and we found five haplotypes with extremely low genetic divergences. However, the haplotypes did not form a well-supported clade. *Ambystoma mexicanum* haplotypes cluster with samples of *A. velasci*, *A. amblycephalum* , and *A. ordinarium*. Of these species, *A. amblycephalum* and *A. ordinarium* occur in regions that are relatively far from the current distribution of *A. mexicanum*, thus, incomplete lineage sorting might explain their association with *A. mexicanum* (Weisrock *et al.* 2006). In contrast, *A. velasci* is a widely distributed species, occurring across most of the Trans-Mexican Volcanic Belt of Central México (Frost 2009) and although this species has not been collected near Xochimilco, we cannot exclude the possibility that its presence may have gone unnoticed, and the possibility of hybridization with paedomorphic forms.

The most divergent haplotype of *A. mexicanum* collected at Chapultepec population clusters with *A. ordinarium*. This might lead us to infer the introduction of *A. ordinarium* in Chapultepec, however, that species is only narrowly distributed in the state of Michoacán, rarely metamorphoses, and is usually found in clean mountain streams (Weisrock *et al.* 2006, Stuart *et al.* 2008). It is unlikely that a species with such narrow ecological requirements could survive and reproduce in a highly eutrophic lake such as Chapultepec.

Clearly, the taxonomic issues in this clade will require additional studies, especially those focusing on species limits in the recently diverged Mexican *Ambystoma*. These studies should include measures of inter- and intrapopulation morphological diversity, autoecological and distributional data and in particular, an emphasis on population genetic approaches to better understand current and historical introgression among populations that might be species or incipient species.

Parasites and disease

We recorded the presence of different parasites affecting adult *Ambystoma* in Chapultepec. *Eustrongylides* is a nematode parasite with a complex life cycle that usually infects fish as second intermediate hosts (Pérez-Ponce de León *et al.* 1996, Coyner *et al.* 2002), but it has also been reported in the amphibians *Ambystoma dumerilii* and *Rana dunni* (García-Altamirano *et al.* 1993). Outbreaks of this parasite often occur with anthropogenic alterations in aquatic systems that lead to high densities of first intermediate hosts. Thus, this parasite is considered an indicator of polluted aquatic environments (Measures 1988, Spalding *et al.* 1993, Franson & Custer 1994, Frederick *et al.* 1996). This nematode has been associated with high mortality and serious population declines in its final host, fish-eating birds (Spalding *et al.* 1993, 1994), but to date it has not being reported as a factor in amphibian declines.

We detected only two cases of *Saprolegnia* infection from the *Ambystoma* collected at Chapultepec. This fungus is a pathogen of fish and attacks eggs, larvae and adults. It has also an important pathogen of amphibian eggs in species such as *Ambystoma maculatum* (Bragg 1962), *Rana montezumae* (Frías 2005), *Bufo boreas*, and *Rana cascadae*, among others (Kiesecker & Blaustein 1995). Several studies have shown that a synergistic effect between *Saprolegnia* and other stressors, such as UV-B radiation and pollutants, can cause massive mortalities in wild amphibian populations (Kiesecker & Blaustein 1995, Blaustein *et al.* 1994, Lizana & Pedraza 1998). In our study, however, the fungus infected adult specimens that were probably already weakened by other diseases or by the polluted environment. The incidence of *Saprolegnia* in the lake must be monitored, as it can ultimately affect the viability of the population.

A very common parasite found in Chapultepec is the copepod *Lernaea*. Although it usually infects fish, it has been reported in aquatic amphibians, including several species of *Ambystoma* (Huacuz 2002) and anuran larvae (Martins & Souza 1996, Leong 2001). In a few cases, the presence of the copepod has been associated with amphibian limb abnormalities (Leong 2001), but this does not seem to be the case in Chapultepec axolotls. The high frequency of malformations axolotls from Chapultepec could again be a side effect result of water pollution. An abnormally high prevalence of malformed amphibians has been associated with outbreaks

of *Ribeiroia ondatrae*, a trematode parasite, due to the increased density of intermediate hosts in highly eutrophic lakes and ponds (Johnson & Chase 2004). This parasite was not found in the Chapultepec population, but Lago Viejo receives a large amount of nutrients from its water source (Alcocer *et al.* 1988, Alcocer-Durand & Escobar-Briones 1992, Lugo *et al.* 1998) and thus is at potential risk for parasites outbreaks.

Conservation implications for *A. mexicanum*

Ambystoma mexicanum is listed as Critically Endangered by the IUCN because its total area of occupancy is estimated to be less than 10 km^2. The aquatic habitat in which it evolved is highly reduced; of the original 180 km of channels in Xochimilco, less than 10 km still retain conditions required for survival of axolotls (e.g., high transparency, appropriate salinity and a lower density of exotic species) and within that area, the habitat is severely fragmented (Zambrano 2006).

Pollution, and introduced species are two of the main factors that have reduced habitat suitability in Xochimilco for *A. mexicanum*. The current and projected declines in the extent and quality of the habitat (Zambrano *et al.* 2006) paint a dire scenario for the two remaining wild populations, and in fact, rapid population reductions in axolotl densities have been observed in the last few years (Zambrano *et al.* 2004, Zambrano 2006). The major threats to remaining axolotl populations are clear (Sciences-Hernández *et al.* 2006, Zambrano *et al.* 2004, 2007, Zambrano 2006), but difficult to mitigate. In Chalco, a restoration program has increased the flooded surface in recent years (Zambrano 2006). In Xochimilco, natural water income has been adapted for human use, and the system is therefore now fed mainly with treated water and precipitation during the rainy season (Solís *et al.* 2006). Eutrophication levels are extremely elevated, chemical pollutants are present in excess, and infectious bacteria including *Pseudomonas* and *Aeromonas* are present in much higher concentrations than are recommended (Zambrano *et al.* 2004). In addition to habitat deterioration, illegal harvest of *A. mexicanum* for food and medicinal purposes has also been a threat to the continued persistence of the species. Captive-breeding efforts and the inclusion of this species in appendix II of CITES have reduced the number of captures of wild specimens, but adult axolotls are still illegally captured today, both for international and local trade (Zambrano *et al.* 2004).

Given the threats to wild *A. mexicanum* populations, its confirmed presence in Chapultepec Park should be used as an opportunity to improve conservation of the species in its native range. The long-term persistence of this population is threatened by several factors similar to those in Xochimilco, but which may be easier to overcome because of the smaller size of the lake. If the lakes of Chapultepec are to serve a role in conservation of this species, it will be important to estimate the population size in Lago Viejo, explore other lakes in the park to detect the presence of axolotls, perform surveys to quantify population sizes and life stage distributions, and manage the lakes to promote restoration and survival of the native species living there.

In México, the coordination of researchers and institutions currently working on the conservation biology of *A. mexicanum* has yielded encouraging results in recent years, and culminated in the creation of several captive colonies in México and abroad, and the organization of a network of researchers (GIA-X, http://ajolote.ibiologia.unam.mx/) that promote multidisciplinary investigation of wild and captive populations, and conservation measures to preserve all paedomorphic *Ambystoma* species and their habitats (Graue *et al.* 1998, Griffiths *et al.* 2003, 2004, 2008, Griffiths & Bride 2005, Zambrano 2006, Zambrano *et al.* 2006, Bride *et al.* 2008). However, although the preservation of axolotl species may be guaranteed due to captive breeding colonies (Malacinski & Able 1989, Zambrano *et al.* 2006), wild populations and their habitats require urgent conservation measures to save them from extinction in their natural habitats (Sciences-Hernández *et al.* 2006, Zambrano *et al.* 2007). Aside from the challenges associated with restoration of habitat, other factors need to be considered before re-introduction of captive-bred animals into wild populations. The propagation of diseases in captivity has played a key role in spreading epidemics such as amphibian chytridiomycosis (Daszak

et al. 2003, Weldon *et al.* 2004). Most individuals in the captive colony of *Ambystoma mexicanum* at the Instituto de Biología (UNAM) were infected by *Batrachochytrium dendrobatidis*, the parasitic chytrid fungus that causes chytridiomycosis. However, none of the captive individuals show signs of disease (Frías-Álvarez *et al.* 2008), thus it is possible that this fungus is prevalent in other colonies without being detected. Researchers must ensure that all colonies are disease-free before reintroducing individuals to natural or restored populations. Our study also revealed population genetic diversity among local natural populations of *A. mexicanum* that was previously unknown. We identified five haplotypes among the 19 samples sequenced thus far. The spatial distribution of those haplotypes among localities is not yet clear, therefore, we recommend genetically characterizing all colonies, with emphasis on wild caught breeding adults, to maximize our potential of maintaining local variants and maximizing any potential natural genetic variability of the species.

Maintenance of the Chapultepec population is an alternative to *ex-situ* conservation in aquaria and terrariums, and may offer benefits because individuals experience natural seasonal dynamics and breed naturally in the lakes. This semi-natural setting might prevent some of the negative effects of captivity, such as loss of fitness due to small breeding groups and artificial selection for traits favored in captivity. A healthy breeding population in Chapultepec would also facilitate future reintroduction programs and serve as a source of acclimatized animals. Finally, the location of the population and the number of visitors to Chapultepec provide a suitable environment for the development of conservation and educational programs. Green areas such as large urban parks can be of great conservation value as reserves for local biodiversity and for education of the public about environmental challenges facing local flora and fauna.

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II. Capítulo 5

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PRIMER NOTE

Polymorphic microsatellite markers for Mexican salamanders of the genus *Ambystoma*

G. PARRA-OLEA,* E. RECUERO† and K. R. ZAMUDIO‡
*Instituto de Biología, Universidad Nacional Autónoma de Mexico, Tercer Circuito Exterior s/n AP 70-153 Ciudad Universitaria Distrito Federal CP04510, Mexico, †Departamento de Diversidad y Biología Evolutiva, Museo Nacional de Ciencias Naturales, Madrid, 28006, Spain, and ‡Department of Ecology and Evolutionary Biology, Cornell University, Ithaca, New York 14853, USA

Abstract

We screened a partial genomic library enriched for microsatellites and characterized nine loci for the Mexican species of *Ambystoma* for studies of population structure. We tested marker variability in two metamorphic (*A. granulosum, A. altamirani*) and two paedomorphic (*A. andersoni, A. mexicanum*) species of the *A. tigrinum* complex. Our microsatellites were developed from pooled genomic DNA from three species, and may work on all species in the *A. tigrinum* complex in Mexico. These markers will be important for studies of conservation genetics in this radiation.

Keywords: Ambystomatidae, axolotl, Mexico, microsatellites, paedomorphosis, salamander

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The Transmexican Volcanic Belt, with varied topography and complex barriers to dispersal is one of the regions of Mexico with the highest diversity and endemism (Ochoa-Ochoa & Flores-Villela 2006). This region is also the most populated of Mexico, encompassing large metropolitan areas such as Mexico City. Species of *Ambystoma* in this region are threatened by habitat modification; therefore, understanding connectivity, gene flow, and population structure is important for their conservation and management. Mexican *Ambystoma* are recently diverged yet highly ecologically differentiated and show complex patterns of speciation and historical introgression (Weisrock *et al.* 2006). In addition, four species (*Ambystoma andersoni, A. taylori, A. dumerilii*, and *A. mexicanum*) are obligate paedomorphs (Shaffer & Mcknight 1996), each inhabiting only one or very few lakes. Because of threats to remaining populations, complex taxonomy, and the repeated evolution of paedomorphosis, the Mexican *Ambystoma* are an ideal system for population genetic studies.

We cloned loci from an enriched partial genomic library (Hamilton *et al.* 1999) prepared with DNA from four individuals: two *A. tigrinum* from the states of Jalisco and Michoacán (MVZ 173473–173474), one *A. granulosum* (*GP769*), and one *A. altamirani* (*GP770*). Genomic DNA was extracted using a QIAGEN DNeasy tissue kit, digested with *Alu*I and *Hae*III (New England Bio Laboratories), size selected for fragments 500–700 bp in length, and ligated to SNX linkers using T4 DNA Ligase (New England Bio Laboratories). Linked genomic fragments were enriched for microsatellites with biotinylated dimer, trimer, and tetramer probes bound to streptavidin-coated magnetic beads (Dynabeads, Dynal Biotech). DNA fragments containing microsatellites were captured magnetically and amplified via polymerase chain reaction (PCR) with linker-specific primers. Amplification products were digested with *Nhe*I (New England Bio Laboratories), cloned into pUC19 vector, and transformed using DH5α competent cells (Invitrogen). Colonies were grown on X-Gal/IPTG-coated agar plates and transferred to Magna Lift nylon membranes (Osmonics) that were probed with the same series of di-, tri-, and tetra-nucleotide radiolabelled repeats. Positive clones were cultured and plasmid DNA was extracted with QIAGEN miniprep columns. We sequenced template DNA directly with vector-specific primers (M13 F and R) using dGTP BigDye terminator cycle sequencing components on an ABI 3100 Genetic Analyser (Applied Biosystems).

We designed PCR primers in the flanking regions of 49 microsatellites using PRIMER SELECT (DNA Star software

Correspondence: Gabriela Parra-Olea, Fax: 52-55-55500164; E-mail: gparra@ibiologia.unam.mx

Table 1 Repeat motif, primer sequences, amplicon size, and annealing temperatures (T_a) for nine microsatellite loci developed for the Mexican species of *Ambystoma*. Sequences of original clones have been accessioned in GenBank (EF062516–EF0625525)

Locus	Repeat motif	Primer sequence (5′– 3′)	Size range	T_a (°C)
Atig52.143	$(TC)_9GCTCAC(TC)_6AC(TC)_3TT(TC)_5TA(TC)_7$	F: TCAGGCATCAGATTCGTTGTTA R: TGTTTGTCGGATTTCGTTGTG	309–359	57
Atig52.115	$(TC)_{15}$	F: AGCACAAGTTCTGAACCTTTCAC R: CCGATCACTCGGGTTACTGT	223–297	59
At52.1	$(GA)_{15}GT(GA)_2GTGAGT(GA)_{27}$	F: GACACCCACAATGCATTTCTACACC R: GCTCTGGCCTTACCCTGCTATCC	381–465	60
At60.3	$(AG)_{30}(TG)_{13}(AG)_1(TG)_{25}$	F: TTTGCCAATGTTTACCTGCCTGAAT R: TGAGTCATGCCTTTCCTGGTGTAA	216–297	62
At52.2	$(GATT)_8$	F: GGGGAGAGCCAGCCACAGAGTAT R: CCCTTTGCCACAGTTAATTGCTTTTT	221–269	60
At52.20	$(TC)_{17}$	F: TTCCTCTTCCCACTTCTCGTTCTGTATT R: TTTCGAGGGTAAGGGGTCTATTGATTC	267–341	54
At52.6	$(ATGT)_3(ATCT)(ATGT)_5$	F: TTACTCAATATCAGACTCCCCAAATGT R: CCTATCCCTTCCCCAGCACTCC	151–163	58
At52.10	$(CT)_6CGCC(CT)_{10}TT(CT)_7$	F: GGTGCAACGAGGCAGTTTTTACCTATTT R: GTCGCTCCTTTCCCTAAGCAAACTGAT	405–435	56
At52.34	$(AG)_4AA(AG)_{21}$	F: TGTACAGACAGGCAAGAGGTATTGACAGT R: GTCTCCCACTTTAATTTCCCTCAGTTTTT	373–457	64

version 5.05) and amplified 24 individual *Ambystoma* (including representatives of each of the four target species) for an initial test of amplification reliability. Following optimization, nine loci yielded specific PCR product of good concentration and showed polymorphism in our pilot samples (Table 1). We expanded our data set to include 154 individuals across all four species (22 *A. altamirani*, 60 *A. granulosum*, 24 *A. mexicanum*, and 48 *A. andersoni*). Each species was sampled at an independent locality: *A. granulosum* — Presa Ignacio Ramirez, Estado de México; *A. altamirani* — Peña de lobos, Estado de México; *A. mexicanum* — Lago de Xochimilco, Distrito Federal; *A. andersoni* — Lago Zacapu, Michoacán.

Ambystoma tissues were extracted in 150 μL of a 5% Chelex solution (Chelex-100, Bio-Rad) with proteinase K by incubation at 55 °C for 180 min and 99 °C for 10 min; the supernatant was used directly as template in PCRs. Each PCR consisted of a total volume of 10 μL including 1 μL of DNA template, 0.05 U *Taq* polymerase (Applied Biosystems), 1× PCR buffer with MgCl, 0.4 mM dNTPs, and 0.1 μM of each primer. Forward primers were 5′-labelled with a fluorescent dye (VIC, PET, 6-FAM, or NED). Loci were amplified in an MJ Research PTC100 or a Hybaid PCR Express thermalcycler under the following conditions: 5 min initial denaturation at 94 °C; 35 cycles of 1 min denaturing at 94 °C, 1 min annealing at the locus-specific temperature, 1 min extension at 72 °C; and a final extension of 72 °C for 30 min. Loci Atig52.115, Atig52.143, At52.6 and At52.2 amplified best with a slightly shorter annealing time of 45 s. Amplified products with different labels or nonoverlapping size ranges were multiplexed and electrophoresed on an ABI 3100 capillary genetic analyser. Fragment sizes were determined with the GENESCAN LIZ-500 standard using GENEMAPPER version 3.5 (Applied Biosystems).

We tested for evidence of linkage disequilibrium and departures from Hardy–Weinberg equilibrium using the software GENEPOP on the web version 3.4 (Raymond & Rousset 1995). A Markov chain method (Guo & Thompson 1992) with 10 000 dememorization steps and 1000 batches of 10 000 iterations per batch was used to determine significance. We found no evidence of linkage disequilibrium among any pairs of loci across all populations (*P* values were all nonsignificant, ranging from 0.142 to 1.0). Overall observed heterozygosities were slightly lower than expected (Table 2) which may be a result of Wahlund effects due to unknown pooling of distinct genetic demes in our samples, or due to drift in isolated populations. Within-population tests of departure from HWE were nonsignificant for most loci and most populations after Bonferroni correction for multiple comparisons; however, four of the nine loci showed significant deviation in *A. granulosum*. This pattern of deviation at these loci in only one of the tested species suggests that this may be due to demographic patterns or population genetic structure within that specific population, rather than a null allele.

Our preliminary results suggest that these loci will be useful for studies of population structure, gene flow among and within populations/species, and development of conservation strategies for these endemic taxa.

Table 2 Number of genotyped samples (N), number of alleles (A), and observed and expected heterozygosities (H_O/H_E) for nine microsatellite loci amplified in four populations of Mexican species of *Ambystoma*. P values are from exact tests of deviation from Hardy–Weinberg equilibrium for each locus within each of the four genotyped species; values in bold are those that exceed the Bonferroni-corrected value ($P < 0.0016$, 31 pairwise comparisons) for a table-wide significance level of 0.05. Alleles fixed in species are denoted by †

	A. granulosum				*A. altamirani*				*A. mexicanum*				*A. andersoni*			
Locus	N	A	H_O/H_E	*P*	N	A	H_O/H_E	*P*	N	A	H_O/H_E	*P*	N	A	H_O/H_E	*P*
At52.143	57	6	0.789/0.751	0.809	18	5	0.611/0.705	0.663	23	6	0.478/0.569	0.101	41	1†	0.000/0.000	—
At52.115	50	10	0.560/0.844	**< 0.001**	10	6	0.100/0.655	**< 0.001**	18	9	0.556/0.710	0.042	38	6	0.447/0.538	0.080
At52.1	35	17	0.714/0.852	0.099	6	7	0.667/0.833	0.021	22	2	0.045/0.044	—	42	3	0.238/0.251	0.599
At60.3	57	10	0.088/0.689	**< 0.001**	22	7	0.773/0.844	0.064	22	5	0.591/0.580	0.061	48	1†	0.000/0.000	—
At52.2	53	7	0.679/0.718	0.245	21	8	0.333/0.635	0.002	23	3	0.174/0.392	0.002	45	2	0.111/0.105	1.000
At52.20	48	14	0.458/0.828	**< 0.001**	22	11	0.864/0.881	0.547	19	8	0.421/0.776	**< 0.001**	42	2	0.143/0.133	1.000
At52.6	59	1†	0.000/0.000	—	22	3	0.455/0.430	0.817	23	2	0.391/0.466	0.411	47	2	0.340/0.282	0.320
At52.10	36	8	0.194/0.711	**< 0.001**	7	2	0.286/0.245	1.000	23	1†	0.000/0.000	—	41	2	0.000/0.048	0.012
Atig52.34	56	14	0.786/0.798	0.059	22	10	0.909/0.782	0.003	19	10	0.632/0.832	0.007	38	5	0.763/0.731	0.060

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II. Capítulo 6

Insight on the conservation genetics of threatened populations of Mexican *Ambystoma* (Caudata: Ambystomatidae)

Abstract

The evolution of larval reproduction has consequences for the persistence and conservation of paedomorphic populations and species. Paedomorphic species are often narrowly distributed and may have reduced genetic diversity due to bottlenecks and low gene flow among populations. These characteristics potentially increase susceptibility to environmental disturbance, inbreeding, and erosion of genetic diversity. A number of salamanders in the genus *Ambystoma* endemic to Mexico are paedomorphic. Here, we assay neutral genetic diversity in paedomorphic and transforming species and characterize the population genetic patterns in those that are threatened or endangered. We found significantly reduced genetic diversity in paedomorphic species, but also populations of transforming species that were equally genetically depauperate. We term these populations 'effective paedomorphs' because despite their ability to metamorphose, other factors must be reducing their genetic diversity. We found signatures of genetic bottlenecks in paedomorphic and metamorphic

species, suggesting that all may have suffered historical population declines. However, we found evidence of higher relatedness within paedomorphic populations than most metamorphic populations; therefore, these species may be especially susceptible to the negative effects of inbreeding with continued declines in population size. All species of *Ambystoma* are now protected by law; nonetheless many axolotls captured by fishermen are still sold illegally at markets. We analyzed samples purchased from a public market in the state of Michoacán, to test the utility of our markers for assigning samples to their population/species of origin. We could not determine the source population but we were able to ascertain that they did not correspond to the highly endangered paedomorphic species. We discuss attributes of the historical landscape and habitat change that may impact population connectivity, and evaluate the prospects for continued genetic health of *Ambystoma* populations in Mexico.

Introduction

The potential loss of genetic diversity in small or isolated populations is a common concern for threatened or endangered species (Daniels *et al.*, 2000; Sherwin & Moritz, 2000). Populations isolated by natural or anthropogenic barriers to gene flow will lose genetic diversity due to reduced genetic exchange with neighboring populations. This genetic erosion will be exacerbated in cases where isolated populations are small (Keller & Waller, 2002), as is often the case with threatened vertebrates in disturbed habitats (Banks *et al.*, 2005; Andersen *et al.*, 2004; Stow & Briscoe, 2005). Small, isolated populations are susceptible to the negative effects of inbreeding and genetic drift, resulting in lower heterozygosity and allelic diversity (Frankham *et al.*, 2002; Reed & Frankham, 2003). This loss of genetic diversity potentially decreases fitness, and results in lowered adaptive potential and increased probability of population extinction (Keller, 1998; Keller & Waller, 2002; O'Grady *et al.*, 2006; Slate *et al.*, 2000). Species or populations with life history attributes that result in small populations and/or low levels of gene flow will be particularly susceptible to decreases in genetic diversity, even in the absence of anthropogenic habitat change. One such group is the *Ambystoma tigrinum* species complex, which includes a number of species and

populations that are facultative or obligate paedomorphs (Shaffer, 1984; Shaffer, 1994; Shaffer & McKnight, 1996). Seventeen species of the *A. tigrinum* complex are endemic to México, and most of these occur in the Trans-Mexican Volcanic Belt (TVB) of central México, a region with extremely high biodiversity and endemism (Flores-Villela *et al.*, 2010; Ochoa-Ochoa & Flores-Villela, 2006) and an equally high degree of habitat alteration due to urban and agricultural development (García-Romero, 2002; Hernández-García & Granados-Sanchez, 2006). The *Ambystoma* endemic to México are a recently derived complex (Shaffer, 1984; Shaffer, 1994) yet the group displays the entire range of developmental pathways, including obligate larval reproduction (*A. mexicanum, A. andersoni, A. dumerilii*), facultative paedomorphosis (*A. granulosum, A. ordinarium*), and obligate metamorphic forms (*A. rosaceum, A. velasci*).

Paedomorphosis is a dramatic shift in life history, in which larval traits are retained in the adults (Gould, 1977) that may have significant consequences for the origin of genetic diversity. Most evolutionary models predict the maintenance of paedomorphosis in favourable aquatic habitats when surrounded by hostile terrestrial environments and therefore the potential for dispersal is limited (Denoel, 2003). Obligate paedomorphosis can substantially

reduce the effective population size and the geographic range of a species, because breeding populations and even entire species become restricted to a single or small number of lakes (Shaffer & Breden, 1989). Reduced population sizes due to obligate aquatic habitats with finite carrying capacity, and possible bottlenecks associated with the founding of paedomorphic populations or species, can reduce overall genetic diversity in paedomorphic taxa, thus increasing the probability of inbreeding and further loss of variability.

Shaffer (1984) proposed that the fixation of larval reproduction in Mexican ambystomatids tended to lead to increased genetic divergence among paedomorphic populations presumably due to restricted gene flow between isolated non-transforming populations. In addition, Shaffer and Breden (1989) present evidence that paedomorphic species or populations of salamanders have on average less allozyme diversity than metamorphic species or populations, and that there is a strong relationship between larval reproduction and genetic variation: larval reproducers are less variable on average than metamorphosing salamander species. In addition to the genetic consequences of larval reproduction, the restricted distributional ranges of paedomorphic species also increase their vulnerability to anthropogenic habitat modification. The obligate paedomorphic Mexican *Ambystoma* (*A. mexicanum, A. dumerilii,*

A. andersoni and *A. taylori*) inhabit single isolated lakes or small lake systems and therefore are more vulnerable to extirpation due to water quality changes, pollution, and other altered environmental conditions. For example, recent studies show that *A. mexicanum* has experienced effective population size reductions as a result of over-exploitation, habitat destruction or modification, and population fragmentation (Zambrano *et al.*, 2007). For over 500 years, humans have modified the lake system that once occupied the entire high-elevation basin to which this species is endemic (Legorreta, 2006; UNESCO, 2006), reducing it to an area close to 1% of its historical size (Fox, 1965). Reductions in population size and persistent isolation for many generations result in two genetic threats. First, as alleles are randomly fixed or lost from the population by drift, levels of genetic variation erode. Second, excessive mating among close relatives increases the proportion of homozygotes, resulting in inbreeding depression and in extreme cases, lowered individual fitness, decreasesd population growth rates and population extinction (O'Grady *et al.*, 2006; Vilas *et al.*, 2006). Genetic erosion can be a gradual process, and thus may not threaten populations in the short term (Lande, 1995; Lynch *et al.*, 1995). However, inbreeding can act swiftly if small populations are composed primarily of related individuals (Keller & Waller, 2002).

Conservation programs for threatened species will benefit from a clear understanding of the genetic diversity within and among populations (Frankham *et al.*, 2002). The evolutionary history of paedomorphic *Ambystoma*, combined with reduction of their habitat, pollution of water bodies, overharvesting, and introduction of exotic species has most likely already had a negative effect on the genetic diversity of these species. In this study, we focus our attention on the conservation genetic status of two of the paedomorphic *Ambystoma* endemic to Mexico, *A. mexicanum* and *A. andersoni*, and compare them to populations of facultative and obligate transforming species to ask the following questions: i) do paedomorphic species show lower genetic diversity than transforming species, and can this be attributed to population bottlenecks, drift and/or lack of gene flow; ii) is there any evidence of gene flow among transforming and paedomorphic populations that may contribute to the maintenance of diversity in species with either life history; iii) do paedomorphic species show evidence of inbreeding and high within-population relatedness?; and iv) given the differences in distribution of genetic diversity in paedomorphic and transforming populations, can we use molecular markers to assign illegally harvested individuals to their populations of origin? Characterizing genetic diversity of species within this complex will help identify mechanisms with the potentially largest negative effects on

remaining populations, prioritize conservation efforts, and provide a method for identifying populations threatened by illegal harvest. We interpret our results in light of what is known about historical (Shaffer, 1984, 1994; Shaffer & McKnight, 1996) and recent changes (Zambrano *et al.*, 2007) in population size and habitat availability.

Materials and Methods

Population sampling and laboratory protocols

We sampled 298 individual *Ambystoma* from eight independent populations along the Trans-Mexican Volcanic Belt (TVB; Figure 1). Our samples included individuals from 5 populations of four metamorphic species (*Ambystoma velasci*, *A. granulosum*, *A. rivulare*, and *A. altamirani*) and 3 populations of two obligate paedomorphic species (*Ambystoma mexicanum* and *A. andersoni*). We focused on natural populations for which we could obtain sufficiently large sample sizes for genotyping; the mean (± SD) sample size per population was 33.1 (± 12.0) individuals and population samples ranged from 21-57 individuals. To avoid problems associated with missing data, we included in the final dataset only individuals with complete genotypes for at least five of the nine loci used in our study. In addition to the eight natural populations, we also included a sample of 38 individuals obtained at the public market of Morelia in the state of Michoacán. At the time of purchase, these individuals were large aquatic forms (mean body size approximately 25 cm) but of unknown population origin and/or species

identity. Exact collection localities, sample sizes, and voucher specimens, when available, are listed in the Appendix.

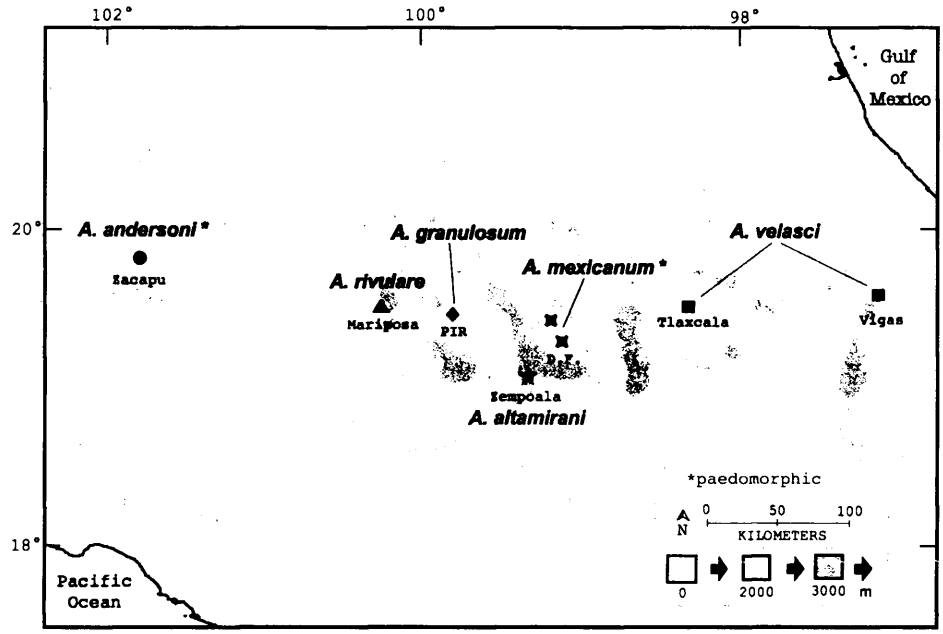

Figure 1. Topographic map of the Trans-Mexican Transvolcanic Belt, with collection localities for the eight *Ambystoma* populations sampled for this study.

Ambystoma samples consisted of liver or tissue clips from tail fins or gills. Samples were extracted in 250ul 5% (w/v) Chelex and 0.20 mg proteinase K (Roche). Samples were incubated at 55 °C for 180 minutes, followed by a 10

min denaturation step at 99 °C (Sambrook & Russell 2001). Supernatant containing genomic DNA was used directly as template for microsatellite amplification. Microsatellite loci for these species were developed and characterized previously (Parra-Olea *et al.*, 2007) and we employed nine of those loci (Atig52.143, Atig52.115, At52.1, At60.3, At52.2, At52.20, At52.6, At52.10, At52.34) in this study. Forward primers were 5'-labelled with a fluorescent dye (VIC, PET, 6-FAM, or NED) and loci were amplified in a Hybaid PCR Express thermal cycler following previously published amplification protocols (Parra-Olea *et al.*, 2007). Amplified products with different colored labels or non-overlapping size ranges were multiplexed and electrophoresed on an ABI 3100 Genetic Analyzer. Fragment sizes were determined with a LIZ-500 standard using GENEMAPPER v. 3.5.

Characterizing population genetic variability

We estimated genetic variability within populations as the number of alleles (A), number of private alleles (P), observed (Ho), and expected (He) heterozygosities. Population indices of diversity where calculated in GenAlex v. 6 (Peakall & Smouse, 2006) or GenePop on the Web v. 3.4 (Raymond & Rousset, 1995). We tested for significant deviation from Hardy-Weinberg

expectations in the program GenePop using the exact test of Guo and Thomson (1992) with 10,000 dememorization steps and 1000 batches of 10,000 iterations per batch. Statistical significance values were corrected using the sequential Bonferroni method (Rice, 1989) for a tablewide significance value of 5%. After an initial analysis, heterozygous deficiency was detected at one locus (At52.10), but not at the other eight markers, suggesting the possible existence of null alleles at that marker (Brookfield, 1996; Hedrick, 1999). The data were then analyzed using Micro-Checker v 2.2.1 (van Oosterhout *et al.*, 2004) for the presence of null alleles at each of the nine loci. For loci with significant probability of null alleles, we followed the corrective procedures described in Chapuis and Estoup (2007) using the program FreeNA. The corrected frequencies were then used to recalculate the number of homozygotes and heterozygotes in each population sample, for comparison with values derived from the original dataset. The corrected and "null allele free" dataset was used to estimate F_{ST} according to Chapuis and Estoup (2007). Comparing F_{ST} values derived from both datasets indicates the effects of presumptive null allele at these loci on estimates of genetic diversity. We tested for linkage disequilibrium between all pairs of loci across all populations in our sample using an exact test (Raymond & Rousset, 1995)

implemented in GenePop on the Web v.3.4, with 10,000 dememorization steps and 1000 batches of 10,000 iterations per batch.

Genetic divergences among all pairs of populations were estimated using the fixation index F_{ST} (Weir & Cockerman, 1984). Statistical significance of divergence was assessed using 90,000 permutations over loci in the program ARLEQUIN V. 2.0 (Schneider *et al.*, 2000). Pairwise significance tests for F_{ST} values were performed by permutation and resampling of multilocus genotypes among pairs of samples. Performing 90,000 randomizations allowed for a table-wide significance at the 5% nominal level after Bonferroni corrections (adjusted p-value=0.00066).

Isolation by distance (IBD) was tested including all populations, except Market, in Genalex v. 6 (Peakall & Smouse, 2001; 9,999 permutations), where the correlation between pairwise F_{st} and geographical distances was analyzed using the Mantel test (Mantel, 1967).

Population structure of paedomorphic and metamorphic populations

We used a Bayesian model-based clustering method to infer population structure and assign genotyped individuals to populations. The computer program STRUCTURE v. 2.2 (Pritchard *et al.*, 2000) identifies clusters of genetically similar diploid individuals from multilocus genotypes without prior knowledge of their population affinities. The model assumes K genetic clusters, each characterized by a set of allele frequencies at each locus. The admixture model then probabilistically estimates the proportion of individuals with ancestry in each cluster. We estimated the $log\ P(D|M)$, where D refers to the data and M refers to the model under different assumptions of K. We assumed a model allowing admixture and independence among our loci, for K between 1 (the expected value if all populations belonged to the same breeding deme) and 15 (the maximum possible number of populations plus six). The upper limit of K=15 was chosen because it allows for the potential case where each sampled population represents a single deme (9 demes) and in addition, can accommodate up to 6 independent genetic demes present within the potentially admixed market sample. We ran a series of pilot runs to estimate the number of generations required for stationarity. Using the option to ignore population affiliation when clustering individuals, we ran 20 independent runs

of 3,000,000 iterations (following a burn-in period of 1,000,000) for each value of *K*. Posterior probabilities for choice of *K* can be misleading in cases where *Log P(D|M)* increases continuously (Pritchard *et al.*, 2000), resulting in overestimates of the number of genetic demes in the sample (Pritchard *et al.*, 2007). Therefore, we evaluated our results in two ways: first we examined the plot of *log P(D|M)* against *K*, and sought the range of *K* along the inflection point of the curve (Pritchard *et al.*, 2007). Within this range is the smallest value of K that captures the most structure in the data. Second, we also applied the ΔK method (Evanno *et al.*, 2005), that estimates the plateau in *log P(D|M)* by comparing second derivatives for various values of *K*. ΔK is most useful in cases where increases in *log P(D|M)* are incremental, and the variance among runs is relatively uniform, yielding a single mode for ΔK that can be interpreted as the true number of genetically distinct demes.

In each STRUCTURE run, individuals were assigned to a cluster based on their estimated membership coefficients; individual and population membership coefficients were plotted graphically in the program DISTRUCT v. 1.0 (Rosenberg, 2004).

Bottlenecks, inbreeding, and relatedness

We used the method described by Cornuet and Luikart (1996) and implemented in the software BOTTLENECK to detect the occurrence of bottlenecks in our sampled populations. This method exploits the fact that allelic diversity is reduced faster than heterozygosity during a bottleneck, because rare alleles are lost rapidly and have little effect on heterozygosity, thus producing a transient excess in heterozygosity relative to that expected in a population of constant size with the same number of alleles (Cornuet & Luikart, 1996). We carried out 1,000,000 replicates and assumed that all loci follow the two-phase mutation model (TPM) in which 90% of mutations are one-step and 10% are multistep (Di Rienzo *et al.*, 1994). Probability values of heterozygosity excess or deficit were estimated for each population by comparison with the simulated null distribution and a Wilcoxon signed-rank test to evaluate significance. Also, using BOTTLENECK the allele frequency distribution of our loci were examined for a mode shift (Luikart & Cornuet, 1998), which may indicate if a recent genetic bottleneck has occurred.

M-ratios were calculated using the software M_P_Val (Garza & Williamson, 2001). Three parameters are needed for this program: theta ($\theta = 4*N_e*\mu$);

percentage of mutations greater than one step (P_s), and average size of mutations that are not one-step (Δ_g). The significance of an observed M-value is determined by comparing it to a distribution of M-values calculated from theoretical populations in mutation-drift equilibrium. The test is significant if more than 95% of the simulated values are superior to the observed value. The critical value of M (M_c) is set at the lower 5% tail of this distribution. The program CRITICAL_M (Garza & Williamson, 2001) generates M_c thresholds, allowing users to modify three TPM parameters (θ, P_s, Δ_g) that approximate the mutation process in real populations.

Since N_e and μ are typically unknown, most studies base their significance criteria on a wide range of biologically plausible θ values (Abdelkrim *et al.*, 2005; Busch *et al.*, 2007). We chose to use general and species-specific estimates of θ. First we estimated θ for each site using MIGRATE 2.1.3 (Beerli & Felsenstein, 1999, 2001). We used the SMM (Ohta & Kimura, 1973) model and applied the following run conditions: 10 short chains of 1,000 genealogies sampled every 50 trees, followed by 5 short chains of 10,000 genealogies sampled every 50 trees. The first 10,000 trees were discarded as burn-in. This gave us the smaller value of θ used for the estimation of M. We also used a generic N_e value of 5000, and a common estimate of microsatellite

mutation rate suggested by Garza and Williamson (2001):5.0 x 10^{-4} mutants/generation/locus (Weber & Wong, 1993). Substituting these values in θ gives a broad range of 0.2-10, allowing us to compare the effect of uncertainty in this parameter on the significance of our bottleneck tests. We used default values for the remaining two parameters needed for the TPM (P_s=0.12 Δ_g=2.8) (Garza & Williamson, 2001). Because the origin of the individuals in our market sample is not known and may include more than one locality, we excluded the market population from both bottleneck tests.

Finally, we also investigated how paedomorphosis may have affected genetic variability within populations, by estimating the coefficient of genetic relatedness, *r* (Queller & Goodnight, 1989) in the software GENALEX v. 6 (Peakall & Smouse, 2006). Expected relatedness values (*r*) are 0.5 among full sibs, 0.25 among half sibs and 0 among unrelated individuals. Elevated relatedness within populations can result from inbreeding or the preponderance of closely related individuals in reduced populations. Thus, intra-population relatedness should be significantly higher in populations that were reduced due to severe bottlenecks, or in paedomorphic populations founded by a small number of individuals. To test for differences in relatedness among samples, we bootstrapped allelic data within populations 999 times to derive 95%

confidence intervals for the mean r estimates for each population; localities with non-overlapping bootstrap intervals are considered statistically distinct. To test for differences among our sampled populations in comparison to all other populations, we permuted genotypes from all populations 999 times and derived upper and lower 95% confidence intervals (CI) for the expected range of r, based on all populations. These intervals represent the range of *r* expected if random mating occurs across all populations. Population *r* values that fall above the upper bound of the 95% CI indicate that reproductive skew, inbreeding, or drift are increasing relatedness, despite potential gene flow among some localities.

Results

Characterizing population genetic variability

All nine microsatellite loci showed polymorphism in most populations of *Ambystoma* sampled, with the number of alleles per locus ranging from four (At52.6) to 34 (At52.1). Our sampled populations varied in the number of polymorphic loci. Three of the transforming populations (*A. rivulare* and the two populations of *A. velasci*) showed polymorphism at all loci, as did the sample of illegally harvested animals from the Morelia market. The three paedomorphic populations (*A. andersoni* from Lago de Zacapu, and *A. mexicanum* from Xochimilco and Chapultepec) were monorphic at one or two of the loci genotyped. Finally, *A. altamirani* and *A. granulosum* were also monomorphic at one locus each. Averaging across all loci, the allelic diversity in each population follows the same pattern (Figure 2). Transforming species generally show higher genetic diversity, with the exception of the populations in Zempoala (*A. altamirani*) and Vigas (*A. velasci*). These two populations share the characteristics of paedomorphic species with lower number of alleles overall, but similar numbers of private alleles. Finally, our market sample shows very high genetic diversity, higher than other populations of

transforming *Ambystoma*, suggesting this sample is likely a mixture of individuals from different populations.

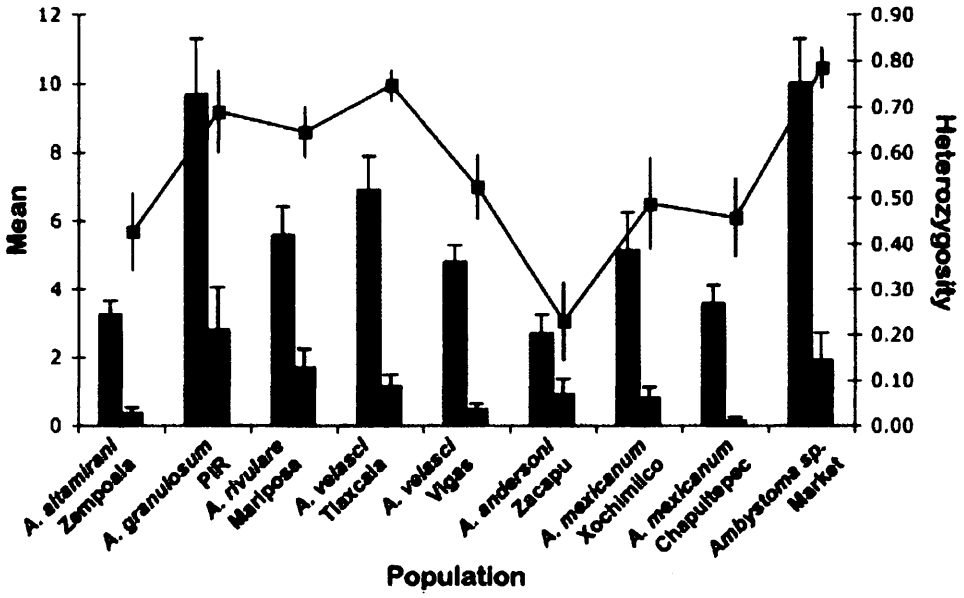

Figure 2. Patterns of allelic richness and heterozygosity in nine sampled populations of *Ambystoma* genotyped at nine microsatellite loci. Bars represent mean ± SD number of alleles (gray bars) and mean ± SD number of private alleles (black bars). Mean ± SD heterozygosities for each population (across all loci) are represented by the black line.

Randomization tests of H-W equilibrium indicated heterozygous deficit at one locus (At52.10) for four of the natural populations (Table 1). The market population showed evidence of departure from equilibrium at eight of nine loci, likely as a result of the pooled distinct genetic demes in that sample. Test for null alleles using Micro-Checker were significant at loci At52.10 (for six populations) and At52.115 (for five populations). For those two loci, we derived the corrected frequencies from FreeNA and used those to recalculate the number of homozygotes and heterozygotes in each population sample, for comparison with values derived from the original dataset. Comparing F_{ST} values derived from both datasets (Table 2) indicates that the presumptive null allele at these loci has only a marginal effect on estimates of genetic diversity; therefore, we used the original dataset for all subsequent analyses.

We found no evidence of linkage disequilibrium among any pair of loci across the eight natural populations (Bonferroni corrected. *P*-values ranged from 0.0047 to 1.0). All of the pairwise comparisons (37) among loci were significant for the market population, corroborating the likely mixed nature of this sample.

Population structure of paedomorphic and metamorphic populations

Pairwise F_{ST} values ranged from 0.04 to 0.64 among all population pairs (Table 2). These values represent high levels of population differentiation. All of our pairwise population comparisons were significant. In general, the paedomorphic species *A. andersoni* exhibited the greatest divergences from all other populations (F_{ST} ranging from 0.37 to 0.64). Limited divergence was evident only for comparisons between the two populations of *A. mexicanum* (Xochimilco and Chapultepec) with an F_{ST} =0.04. We sampled two populations of *Ambystoma mexicanum*, one is the type locality of the species (Xochimilco) and the second (Chapultepec) is found in an artificial pond in Parque Chapultepec, a large recreational area in the heart of Mexico city. These introduced neotenic *Ambystoma mexicanum* in the Chapultepec lakes were reported previously (Alcocer-Durand & Escobar-Briones, 1992); however, until recently species identity of these populations has not been certain (Recuero *et al.*, 2010). The low F_{ST} value found between the Xochimilco and this introduced population corroborate the identity of the Chapultepec population as *A. mexicanum*.

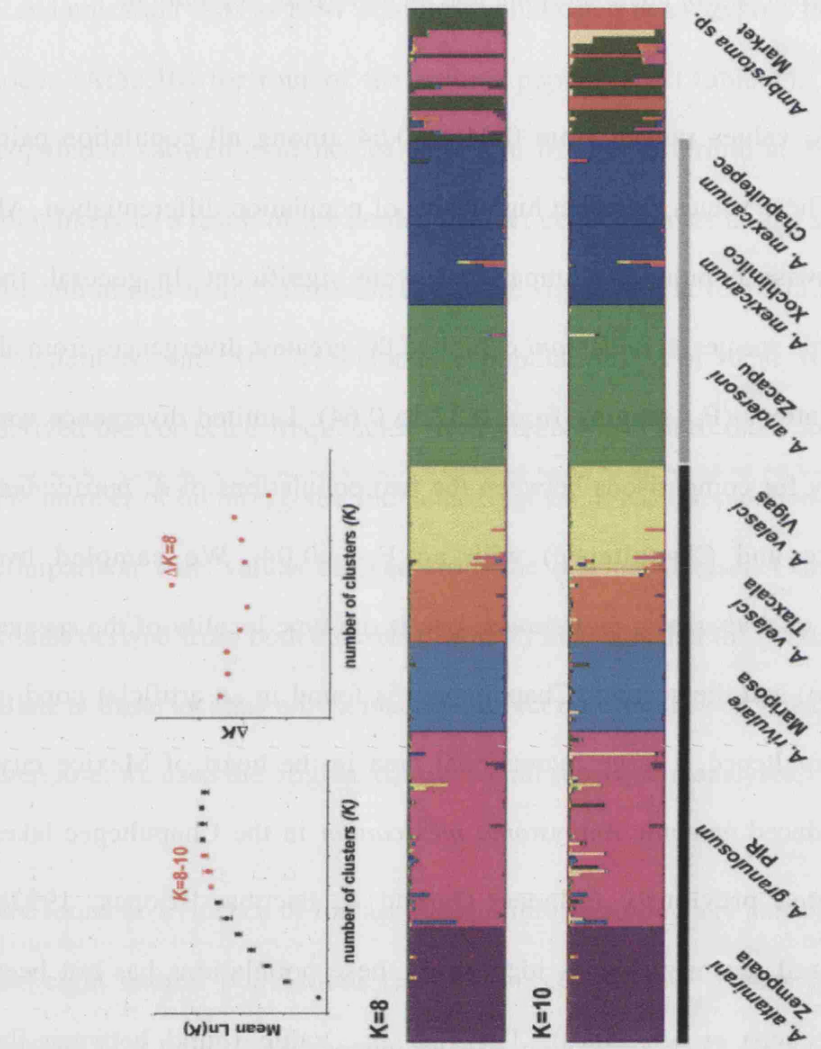

Figure 3. Population structure inferred by Bayesian assignment of 298 individuals of paedomorphic and metamorphic *Ambystoma* populations and/or species endemic to Mexico. The top left graphs show Mean L(*K*) and SD for 20 runs for each *K* value between 1 and 14; the top right graph shows ΔK calculated as ΔK = m|L__(*K*)|/ s[L(*K*)]. The modal value of this distribution is the true or the uppermost level of structure. The plateau on the L(K) curve occurs between K=8 and 10, and ΔK suggests that sampled populations can be assigned to eight geographical genetic demes. The color-coded assignment graphs represent the mean membership coefficient for individuals in each sampled population to one of eight or 10 inferred genetic demes; increasing the assumed value of *K* results in increased admixture only in the market population. Populations marked with a gray bar are obligate paedomorphs and show significantly lower admixture than most transforming populations (black bar).

Mantel tests showed a non-significant correlation (r=0.410, p=0.104) between estimated pairwise F_{st} and geographical distances for the 8 populations analyzed. Thus we cannot assume that IBD is the cause, or at least the principal cause, for the genetic differentiation found among populations.

Results of the distance-based F_{ST} analyses are corroborated by Bayesian assignment tests; the probability of the data under models with increasing K shows the characteristic incrementally increasing curve with an inflection point in the range of K=8-11. The ΔK method confirms eight genetic demes or clusters among our samples (Figure 3). In most cases these demes correspond to individual sampling localities, underscoring the independent history of many of these *Ambystoma* populations. All populations sampled in the wild show high membership coefficients to their own clusters (mean membership coefficients range= 0.932 to 0.980). The three populations of paedomorphic species have some of the highest membership coefficients, and relatively low variance among individuals in genetic admixture (*A. mexicanum* Chapultepec:Q=0.974 ± 0.040; *A. mexicanum* Xochimilco: Q=0.951 ± 0.110; *A. andersoni*: Q=0.979 ± 0.034). Two populations of transforming species show similarly high levels of membership to one genetic deme: *A. altamirani* from Zempoala and *A. velasci* from Las Vigas. The creeks surrounding the

Lagunas de Zempoala harbor a population of *A. altamirani*, a facultatively transforming species that inhabits creeks and streams in lake drainages (Aguilar-Miguel, 1997; Castro-Franco *et al.*, 2006). *A. altamirani* presents a wide distribution in central Mexico, however, the population from Lagunas de Zempoala was once described as a paedomorphic form, *A. zempoalense,* by Taylor and Smith (1945), but was later synonymized with *A. altamirani* by Reilly and Brandon (1994). The sample we obtained from that locality has the highest assignment values of all the populations we sampled (Q=0.980 ± 0.021). Our data underscore that even among metamorphic forms, populations show genetic patterns consistent with prolonged isolation.

Bayesian assignment tests of the market samples underscore their diversity and mixed origin. In the model with eight genetic demes, the market sample shows low membership coefficients to two independent demes (q1=0.390 and q2=0.507). Some individuals show high genetic similarity with individuals from Presa Ignacio Ramirez, a large population of *A. granulosum*. The other genetic deme found in this sample is unique to the market population, and thus likely represents a species or population that we did not sample. Individual assignments using models with higher K do not change the results significantly for the natural populations, but do change the deme assignments for the market

population. If we assume 10 demes, market individuals are assigned to these two additional demes (Figure 3).

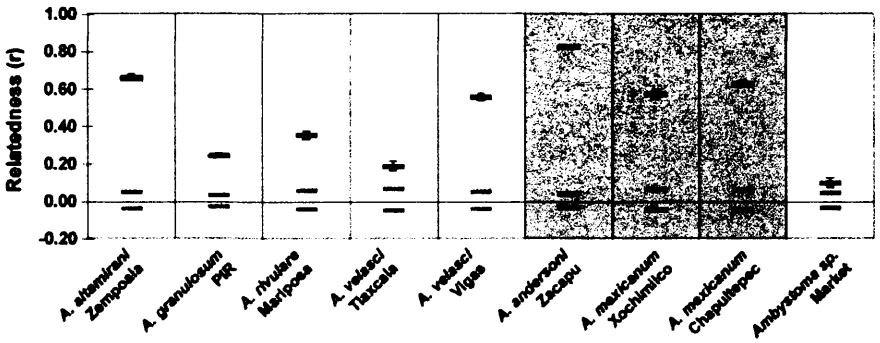

Figure 4. Mean within-population pairwise relatedness for nine *Ambystoma* populations included in this study. Gray bars are 95% upper and lower expected values for a null distribution generated from 999 permutations of data from all populations, and enclose the values expected if breeding were panmictic across all populations; relatedness in all sampled populations fell outside of the range expected under panmixia. Black bars represent the observed mean relatedness in each population or species, and the upper and lower bootstrap value for each population. The three populations shaded in gray are the obligate paedomorphic species/populations with the highest degree of inbreeding. Populations of *A. altamirani* (Zempoala) and *A. velasci* (Las Vigas) show surprisingly high levels on intra-population relatedness for metamorphic populations.

Bottlenecks, inbreeding and relatedness

We examined allele frequencies within populations for signatures of historical changes in population size using the program BOTTLENECK (Cornuet & Luikart, 1996; Piry *et al.*, 1999) which computes for each population and locus the distribution of the expected heterozygosity and compares it to the observed heterozygosity under the assumption of mutation-drift equilibrium. BOTTLENECK did not detect significant evidence of a recent bottleneck (excess of heterozygosity) under the two-phase mutation model (Table 3) for any of the sampled populations. In contrast, M-ratio values were low for all populations, ranging from 0.4164 to 0.6805, independent of the assumed θ included in the model. In every case M-ratio estimates were lower than the critical value for individual sites calculated by M_c (Table 3).

Average pairwise relatedness (r) within populations was generally high for all populations, but was significantly higher in paedomorphic species (Figure 4). Relatedness in all populations fell outside of the 95% CI for the permutations assuming random mating across all populations. The paedomorphic species *A. andersoni* is the most inbred with a mean r=0.825 (95% CI =0.817-0.832); likewise, the two paedomorphic and isolated populations of *A. mexicanum* also showed high degree of relatedness (Xochimilco: 0.571 [0.550-0.592];

Chapultepec: 0.626 [0.611 – 0.643]). The mean inbreeding coefficient across all paedomorphic populations is 0.674 (± 0.133), significantly higher than the inbreeding coefficients across the five sampled metamorphosing populations (r=0.4 ± 0.202). Despite this significant difference, two populations of transforming *Ambystoma* show surprisingly high levels of intra-population relatedness; *A. altamirani* from Zempoala, and the population of *A. velasci* from Las Vigas, show levels of relatedness comparable to the paedomorphic species sampled in this study (Figure 4), suggesting that some of the same processes increasing the likelihood for inbreeding might be acting in otherwise isolated metamorphic populations as well.

Relatedness among individuals from the market corroborates our previous finding that those individuals likely do not represent a single locality; relatedness in that sample was lower than in any other sampled natural population (r=0.098, 95% CI=0.074-0.123) and the range of values from the bootstrapped confidence intervals approximates the range of values expected under the null distribution assuming panmictic breeding among individuals from all populations (Figure 4).

Discussion

Mexican axolotls were well known to the Aztec people and held an important position in their mythology as one of the Gods that generated the modern era and the fifth sun (Fernandez, 2006; Ingham, 1984); the axolotl is also a model organism for studies of embryology and developmental biology. It is thus ironic and unfortunate that an organism that has historically played an iconic cultural role and is a model organism for modern science is now endangered in nature. Currently, many communities exploit the axolotl and other paedomorphic *Ambystoma* for food and as a remedy for respiratory infections. However, the largest threat to wild populations of paedomorphic salamanders (*A. mexicanum, A. dumerilii, A. andersoni*) are anthropogenic changes in their habitats, including pollution, altered lake hydrology, introduced exotic species, and over-exploitation. As a result paedomorphic *Ambystoma* are today some of Mexico's most threatened species and are listed as critically endangered species on the IUCN red data list of species, each with an current population area of less than 10km (Griffiths & Bride, 2005).

The axolotl (*Ambystoma mexicanum*) was once abundant in the Xochimilco and Chalco lakes even with the creation of the Chinampas, man-made landfill

mounds created for agriculture, that began with the Aztecs. The modification of the Chinampas in the 50's reduced exchange among different channels of this lake system and interrupted natural hydrological patterns that renewed water flow into the lake system as a whole (Espinoza *et al.*, 2006; Legorreta, 2006). Continued modification of hydrological patterns over the last 50 years further reduced the lake system; the original lake system occupied an area of 240 km^2 in the valley now occupied by Mexico City; this area has been reduced to 2.3 km^2, or to 1% of its original size (Fox, 1965). The Chalco and Xochimilco remnant populations are now completely isolated form each other (Alcocer-Durand & Escobar-Briones, 1992). Chalco has been completely dried out many times and Xochimilco has suffered from radical changes in its hydrology, decreasing the water quality of all the system (Mazari *et al.*, 2006). Today, most of the water in the system comes from water treatment plants instead of springs from the water table (Solís *et al.*, 2006). As a consequence, *A. mexicanum* populations have dramatically declened over five years from 0.006 m^2 to 0.0012 m^2 (Zambrano *et al.*, 2007). Over the last decades agricultural practices in the areas surrounding the lakes have intensified with high-yield production in greenhouses with high use of fertilizers and pesticides (Zambrano *et al.*, 2009). These changes have elevated nutrient concentrations (particularly in nitrogen in form of ammonia) to levels that are toxic for

axolotls (Contreras, 2006). Nutrient runoff has also caused proliferation of algae in the water column and increased turbidity of the water. Axolotls are mostly visual predators, thus increased turbidity may reduce their feeding capacities. Combined, these habitat threats reduce and isolate populations, and because paedomorphic populations are expected to harbor lower genetic diversity, they may be especially vulnerable to extinction in changing environments.

The other two obligate paedomorphic species, *A. andersoni* (Zacapu Lake, Michoacán) and *A. dumerilii* (Pátzcuaro Lake, Michoacán) suffer from many of the same threats as *A. mexicanum* in Xochimilco. Population growth in the towns around Zacapu and Pátzcuaro has been exponential in the last decades (Fernández & Miranda, 1998), with the lakes functioning as the final point for sewage, and in the case of Zacapu Lake, waste from local livestock production (Fernandez & Miranda, 1998). Population growth has resulted in high levels of pollution, reduction of lake size, increases in water temperature increased due to local landfills (Huacuz, 2001). At the same time, these two species in particular have been overharvested for food and medicinal purposes and the dramatic decrease in their abundance now ranks *A. dumerilii* as one of the most threatened species of *Ambystoma* (Huacuz, 2001).

The lakes where Mexican paedomorphic species of ambystomatid salamanders are found are isolated from each other by considerable distances of inhospitable terrestrial habitat. Therefore, gene flow, if it occurs, could be mediated by admixture with facultatively paedomorphic forms rather than direct movement of peadomorphic species among lakes. Our data show clearly that the two paedomorphic forms in our sample show low admixture with other species, corroborating data from other markers showing that paedomorphic populations in different lakes have evolved independently (Shaffer, 1994). The expected genetic consequences of isolation via the evolution of paedomorphosis are lower genetic diversity due to founder effects and reduced connectivity among populations due to cessation of immigration among sites. We found patterns in genetic diversity within paedomorphic populations that are consistent with these expectations: the two paedomorphic species in our sample showed lower allelic richness, lower heterozygosity, and higher rates of inbreeding. These two species also show the genetic signature of bottlenecks; however, we detected a similar genetic bottleneck in some of the metamorphosing populations of *Ambystoma* as well. Genetic signatures of bottleneck and founder events are expected to disappear after many generations following the reduction in population size (Keller *et al.*, 2001).

Most tests for bottlenecks measure the heterozygosity excess at independent loci that is expected immediately after the bottleneck because allelic diversity is lost faster than heterozygosity (Cornuet & Luikart, 1996; Nei et al., 1975) Additionally, the range of allelic frequencies will change in a characteristic manner immediately after the bottleneck, and this shift in allelic frequencies is transient and will revert to the expected frequency ranges in subsequent generations (Luikart & Cornuet, 1998).

Methods classically used to detect genetic bottlenecks rely on different statistics, and can therefore favor different time scales for detection. The M-ratio method is expected to detect older events because of the longer time needed for the M statistic to reach equilibrium (Garza & Williamson, 2001). Our results are in accordance with this expectation; the M-ratio method detected a significant reduction in population size for all populations studied. Our estimates ranged from 0.416 to 0.680, values that are in the range of those inferred for taxa known to have experienced a reduction in population size (Garza & Williamson, 2001). The other methods we applied did not detect bottleneck in any of the sampled populations.

We found evidence for high relatedness within populations of paedomorphic forms, suggesting a higher degree of inbreeding within those populations. Although inbreeding can lead to reduced fitness, the degree to which populations suffer from inbreeding depression can vary widely depending on population history, the trait examined, lineage effects, and the environment (Keller & Waller, 2002). If inbreeding accumulates gradually in populations, natural selection may have higher chances to remove deleterious alleles from populations than in cases where all individuals in the population are the result of breeding between close relatives (Keller & Waller, 2002). Our data suggest that the paedomorphic species *Ambystoma mexicanum* and *A. andersoni* have evolved in isolation, and show high levels of relatedness among individuals, but we have no evidence that this is associated with decreases in fitness due to inbreeding depression. This may be due to the fact that high relatedness in this population has evolved gradually, making the species less susceptible to inbreeding depression because deleterious alleles should be rare in these breeding systems (Keller & Waller, 2002). This pattern does not mean that inbreeding does not threaten these species; overexploitation of paedomorphic populations that significantly reduces population sizes could potentially increase inbreeding above a threshold, causing reduced population fitness.

We found two populations of the metamorphic species *A. velasci* (Las Vigas) and *A. altamirani* (Zempoala) that showed patterns of genetic diversity and relatedness similar to paedomorphs; we refer to these as "effectively paedomorphic" because population dynamics in these two cases must be restricting genetic exchange among populations and limiting population sizes in a manner similar to paedomorphic populations. *Ambystoma velasci* is a widely distributed species that ranges from Northwestern Chihuahua and along the eastern slope of the Sierra Madre Occidental and southern Nuevo León in the Sierra Madre Oriental, and reaches the TVB of central México at the southernmost extent of its range. Previous molecular studies using mtDNA and allozymes indicate that *A. velasci* is a distinct species from *A. tigrinum* (Irschick & Shaffer, 1997; Shaffer & McKnight, 2006). However, current data suggest that *A. velasci* is a paraphyletic complex of metamorphic populations, with some populations more closely related to paedomorphic species than to the rest of the populations across México. Our population from Las Vigas is the eastern and southernmost population of the *Ambystoma tigrinum* complex and of the genus *Ambystoma* in Mexico, and the population genetic signatures we identified may be the result of isolation in this population at the edge of the species distribution. A comparison of the population dynamics in this and other *A. velasci* populations more central to the species range will provide an

interesting comparison of how gene flow and drift shape the distribution of metamorphic species in this complex landscape.

Ambystoma altamirani is distributed in the high mountains south and west of the Valley of Mexico in the central state of México, southern Distrito Federal, and northwestern Morelos. The population we sampled from streams that feed the 'Lagunas de Zempoala' was once described as a distinct species, *A. zempoalense* by Taylor and Smith (1945). This taxonomic status as a distinct species has now been synonimized (Maldonado-Koerdell, 1947; Reilly & Brandon, 1994) and is referred to as *A. altamirani.* Our data suggest that the population from Lagunas de Zempoala is a closed system without genetic exchange with other populations of *A. altamirani.* It is clear from this first assessment of population genetic variation of *Ambystoma* populations along the TVB that restrictions to gene flow can play an important role in the history of both paedomorphic and transforming species of this complex. Given the high levels of inbreeding found in our results, further population genetic comparisons among populations of *A. altamirani* will help guide conservation efforts for this species.

Current conservation protection measures for widespread metamorphic taxa such as *A. velasci* and *A. altamirani* are not as stringent as those for the paedomorphic species, because they are considered less endangered due to larger distributional ranges. However, if many populations within those widespread species are effectively paedomorphic, and each one is threatened due to decreased population sizes and other anthropogenic factors, then a species wide collapse could occur due to the absence of any possible rescue effect (Waite *et al.*, 2005). Migration among effectively paedomorphic populations is so low that localized extinctions will never be recolonized in the classic metapopulation sense. Therefore, we suggest that a more thorough evaluation of genetic patterns among populations of the wide-ranging metamorphic species will be an important step in decisions for their conservation.

The conservation genetics literature is replete with examples of inbred populations that show reduced reproductive fitness as a consequence of inbreeding depression (Bijlsma *et al.*, 2000; Crnokrark & Roff, 1999; Frankham *et al.*, 2002; Reed *et al.*, 2002, 2003). Erosion of genetic variability has also been implicated in decreased resistance to diseases, including various cases where endangered species were threatened with extinction as a result of a

disease outbreak (Acevedo-Whitehouse *et al.*, 2003; Hedrick *et al.*, 2001; Real, 1996; Spielman *et al.*, 2004). The link between inbreeding, reduced genetic diversity, and the lowered ability of populations to evolve in response to new pests or pathogens is particularly problematic for endangered amphibians. Amphibians are declining worldwide and a number of emergent pathogens have been implicated in population declines even in habitats that are protected and undisturbed (Lips *et al.*, 2005, 2005a, 2006; Wake & Vredenburg, 2008). Populations of *Ambystoma tigrinum* in the northern parts of the range are susceptible to infection by *Ranavirus* (Brunner *et al.*, 2007; Picco *et al.*, 2007), thus, it is possible that lowered genetic diversity may further increase susceptibility to disease outbreaks in the southern populations we study. Given the low levels of genetic diversity and high inbreeding we found among populations of paedomorphic and some metamorphic populations in this complex, future studies should focus on the interaction between population genetic diversity and disease susceptibility in these populations.

Our results also highlight the utility of high-resolution markers in more practical conservation efforts to identify and curb the illegal harvest of endangered species. The sample we obtained for sale at the Morelia market showed unambiguously that the microsatellite markers we used in this study

can distinguish among species and even populations of *Ambystoma* in the TVB. We could not identify the exact origin of the market samples, most likely because that population was not represented in our reference samples. However, given high assignments probabilities for all specimens of both paedomorphic species, we can conclusively determine that the market individuals were not collected from the paedomoprhic populations of *A. mexicanum* and *A. andersoni*. We also found that the market individuals represented a mixed sample collected from at least two localities belonging to two independent genetic demes. The resolution of our markers to distinguish finer-scale population differentiation (such as regional or population level differences within species) should be tested in studies including more reference populations and species; however, it is clear from our results that they can clearly differentiate among most species, and some populations, tested thus far.

The Trans-mexican Volcanic Belt is a mountainous system with a large longitudinal range, and has been identified as one of the major barriers in North America because of its position at the limit between the Neartic and the Neotropics (Marshall & Liebherr, 2000). This biogeographic province, although relatively recent (Ferrusquía-Villafranca, 1993) is home to a large

number of species and is also an important zone of endemism for many groups (Fa, 1989; Fa & Morales, 1991; Escalante *et al.*, 2002; Monroy-Vilchis *et al.*, 1999). The Mexican *Ambystoma* are a recently derived species complex (Shaffer, 1994; Weisrock *et al.*, 2006) that radiated approximately 10-12 mya (Shaffer, 1984) and was presumably affected by the uplift of the TVB (Shaffer, 1984). The group reaches its greatest taxonomic and metamorphic diversity in this region where 15 of the 17 species are present, adding to the rich fauna present in the TVB. Our results underscore how genetic isolation, population sizes, and life history evolution contribute to species diversification over relatively short evolutionary time scales. Loss of diversity and the habitats that contribute to this evolutionary potential may also result in reduced diversification of species in this complex in the future (Johanson *et al*, 2007; Reed & Frankham, 2003). The TVB is a biogeographic region with extremely high degree of habitat alteration due to urban and agricultural development. Our data show that paedomorphic and transforming populations of *Ambystoma* have persisted despite the genetic effects of isolation imposed by the landscape. However, anthropogenic changes, if sufficiently severe, could reduce population sizes to the point that this promotes the negative effects of genetic erosion in these populations that already show reduced genetic diversity and low inter-population connectivity. Conservation measures to

maintain the persistence of *Ambystoma* endemic to Mexico must take into account detailed population diversity, and the importance of population-level evolutionary processes for the continued persistence of each unique population.

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Table 1. Tests for deviation from Hardy-Weinberg equilibrium at each locus in the nine samples populations of *Ambystoma* from Central Mexico. Observed and expected heterozygosity (Ho/He) and associated *P*-values are reported for each locus and population. *P*-values in bold exceed the Bonferroni-corrected value ($P<0.00066$, 75 comparisons) for a table-wide significance level of 0.05.

	A. altamirani **Zempoala**	***A. granulosum*** **PIR**	***A. rivulare*** **Mariposa**	***A. velasci*** **Tlaxcala**	***A. velasci*** **Las Vigas**	***A. andersoni*** **Zacapu**	***A. mexicanum*** **Xochimilco**	***A. mexicanum*** **Chapultepec**	***Ambystoma* sp.** **Market**
52.143	0.333/0.346	0.778/0.741	0.880/0.790	0.810/0.795	0.862/0.721	0.000/0.000	0.500/0.587	0.478/0.411	0.829/0.836
	(0.0323)	(0.7259)	(0.5753)	(0.2130)	(0.3379)	---	(0.1331)	(0.6148)	**(0.0003)**
52.115	0.000/0.500	0.563/0.843	0.381/0.580	0.467/0.836	0.667/0.603	0.447/0.538	0.556/0.710	0.444/0.656	0.686/0.907
	(0.3332)	**(0.0003)**	(0.0067)	**(0.0005)**	(0.1846)	(0.0797)	(0.0413)	(0.0085)	**(0.0001)**
52.1	0.484/0.544	0.714/0.852	0.739/0.727	0.450/0.655	0.333/0.319	0.238/0.251	0.045/0.044	0.000/0.000	0.553/0.785
	(0.2720)	(0.1000)	(0.5060)	(0.1190)	(0.6757)	(0.5997)	---	---	**(0.000)**
60.3	0.697/0.711	0.093/0.696	0.840/0.778	0.857/0.836	0.967/0.618	0.000/0.000	0.571/0.573	0.591/0.569	0.684/0.832
	(0.5773)	**(0.000)**	(0.9687)	(0.6625)	**(0.000)**	----	(0.0670)	(1)	**(0.000)**
52.2	0.286/0.492	0.679/0.718	0.000/0.444	0.600/0.673	0.633/0.634	0.091/0.087	0.174/0.392	0.417/0.451	0.737/0.654
	(0.0000)	(0.2435)	**(0.0000)**	(0.1368)	(0.2110)	(1)	(0.0027)	(0.6362)	(0.7465)
52.20	0.630/0.718	0.458/0.828	0.619/0.764	0.412/0.711	0.679/0.605	0.146/0.136	0.421/0.776	0.667/0.601	0.429/0.847
	(0.0908)	**(0.0000)**	(0.0401)	(0.0012)	(0.1674)	(1)	**(0.0003)**	(1)	**(0.0000)**
52.6	0.000/0.000	0.000/0.000	0.538/0.473	0.571/0.591	0.100/0.095	0.311/0.263	0.391/0.466	0.625/0.520	0.053/0.497
	----	----	(0.6861)	(0.7897)	(1)	(0.5700)	(0.4104)	(0.4123)	**(0.0000)**
52.10	0.063/0.061	0.194/0.711	0.125/0.420	0.400/0.711	0.167/0.391	0.000/0.048	0.000/0.000	0.130/0.122	0.405/0.809
	(1)	**(0.0000)**	(0.0006)	**(0.0000)**	**(0.0000)**	(0.0123)	----	(1)	**(0.0000)**
52.34	0.394/0.451	0.786/0.798	0.769/0.814	0.857/0.895	0.655/0.722	0.763/0.731	0.632/0.832	0.917/0.780	0.947/0.881
	(0.0620)	(0.0578)	(0.6210)	(0.1298)	(0.7752)	(0.0599)	(0.0067)	(0.0018)	**(0.0001)**

Table 2. Pairwise estimates of population differentiation for nine population of paedomorphic and transforming *Ambystoma* species and/or populations. We estimated pairwise F_{ST} for each pair of populations and tested for significance using permutation tests in the program ARLEQUIN (90000 randomization) All p-values were smaller than 0.001

		1	2	3	4	5	6	7	8	9
1	***A. altamirani*** **Zempoala**	—								
2	***A. granulosum*** **PIR**	**0.29**	—							
3	***A. rivulare*** **Mariposa**	**0.39**	**0.24**	—						
4	***A. velasci*** **Tlaxcala**	**0.35**	**0.15**	**0.22**	—					
5	***A. velasci*** **Las Vigas**	**0.43**	**0.24**	**0.33**	**0.24**	—				
6	***A. andersoni*** **Zacapu**	**0.64**	**0.43**	**0.54**	**0.50**	**0.51**	—			
7	***A. mexicanum*** **Xochimilco**	**0.55**	**0.37**	**0.38**	**0.29**	**0.43**	**0.64**	—		
8	***A. mexicanum*** **Chapultepec**	**0.55**	**0.35**	**0.38**	**0.29**	**0.42**	**0.61**	**0.04**	—	
9	***Ambystoma*** **sp. Market**	**0.24**	**0.11**	**0.14**	**0.12**	**0.23**	**0.37**	**0.29**	**0.28**	—

Table 3. Results based on heterozygosity excess test, allele frequency analysis and M-ratios. For heterozygosity excess test we used a two-phase mutation model with 10% multistep mutations. Ex HE: Expected number of loci with heterozygosity excess. Obs HE: Observed number of loci with heterozygosity excess. Wilcoxon test probability (one tail for H excess). Normal allele frequency means that the majority of alleles had frequencies less than 0.1. M-ratio average were calculated across loci with the program M_Val_ P. Mc is the critical M-value calculated through the M-crit program developed by Garza and Williamson (2001). θ* is the effective population sizes estimated by MIGRATE for each population. θ^ is the generic value used of Ne=5000 for all populations.

	Expected HE	**Observed HE**	**P-value**	**Allele Freq**	**M**	**Mc**	**θ *= (P-value)**	**P-value θ^ =10**
***A. altamirani* Zempoala**	4.28	4.0	0.32031	Normal	0.6180	0.8302	0.211 (0.0)	0.03
***A. granulosum* PIR**	4.75	3	0.87500	Normal	0.4664	0.816	0.503 (0.0)	0.00
***A. rivulare* Mariposa**	5.05	6	0.12500	Normal	0.5456	0.8304	0.314 (0.0)	0.00
***A. velasci* Tlaxcala**	5.34	8	0.06445	Normal	0.4939	0.8333	0.211 (0.0)	0.00
***A. velasci* Las Vigas**	5.21	4	0.71484	Normal	0.4164	0.8388	0.212 (0.0)	0.00
***A. andersoni* Zacapu**	3.50	2	0.94531	Normal	0.4694	0.8238	0.267 (0.0)	0.00
***A. mexicanum* Xochimilco**	4.46	3	0.87500	Normal	0.6805	0.8191	0.300 (0.71)	0.03
***A. mexicanum* Chapultepec**	4.55	4	0.37109	Normal	0.465	0.8229	0.279 (0.0)	0.00

Appendix. Populations of *Ambystoma* sampled for this study. We sampled a total of 298 individuals from 9 populations and 6 species, including two obligate paedomorphic species (*A. andersoni* and *A. mexicanum*). Sampling locality "market" was obtained from the San Juan market in Morelia, Michoacán; these larval samples are of unknown origin and species identity.

Locality	State	GPS coordinates	Species	Paedomorphic	Sample
Lagunas de Zempoala	Morelos	19.052999, -99.310997	*altamirani*	no	33
Presa Ignacio Ramirez	Mexico	19.448, -99.788002	*granulosum*	no	57
Reserva de la Mariposa Monarca	Michoacan	19.523001, -100.252998	*rivulare*	no	26
Laguna San Fernando, Hueyotlipan	Tlaxcala	19.509359, -98.303998	*velasci*	no	21
Road to Microondas station 'Las Lajas' near Las Vigas	Veracruz	19.57333, -97.09833	*velasci*	no	30
Laguna de Zacapu	Michoacán	19.824433, -101.787685	*andersoni*	yes	46
Lago Xochimilco	Distrito Federal	19.288, -99.102997	*mexicanum*	yes	23
Laguna Vieja, Parque Chapultepec	Distrito Federal	19.422001, -99.184998	*mexicanum*	yes	24
Morelia Public Market	—	—	unknown	unknown	38

III. Discusión General

La aplicación de técnicas moleculares ha revolucionado numerosos aspectos dentro de áreas como la sistemática, la biología evolutiva o la biología de la conservación, al generar nuevos conceptos o disciplinas como la filogeografía o la genética de la conservación. La nueva perspectiva que otorgan estos nuevos campos de estudio permiten la reinterpretación y validación de hipótesis previamente establecidas. En este sentido, por ejemplo, podemos referirnos al fuerte efecto que la sistemática molecular ha tenido y tiene sobre las decisiones taxonómicas tomadas a cualquier nivel jerárquico (Caterino *et al.*, 2000). Si consideramos una designación taxonómica como una hipótesis a comprobar, es habitual hoy en día recurrir a datos moleculares para confirmar o poner en duda la validez de la misma. De este modo se puede determinar, pongamos el caso, si un género representa una unidad monofilética o si una especie representa efectivamente un linaje independiente y genéticamente aislado de sus congéneres. Al igual que el uso de marcadores moleculares se usa regularmente para comprobar decisiones taxonómicas, también se ha convertido en una herramienta clave a la hora de cuestionarnos prácticamente cualquier otro tipo de hipótesis: biogeografía, procesos adaptativos, procesos de especiación... (Avise, 2000; Vences &

Wake, 2007; Wiens, 2004).

Al igual que los resultados obtenidos mediante el empleo de marcadores moleculares pueden ser una herramienta perfecta para el estudio de hipótesis e ideas previamente establecidas, también se han convertido en un importante generador de nuevas hipótesis de estudio. Por ejemplo, como sugiere Buckley (2009), las inferencias filogeográficas no son ni mucho menos el punto y final de este tipo de investigaciones, si no que constituyen hipótesis evolutivas que deben ser comprobadas en el campo, al igual que sus predicciones y los modelos planteados. Así se consigue nueva información que eventualmente podrá ser integrada en el análisis de datos, reevaluándose y replanteándose las hipótesis analizadas. De esta forma se pretende poder caracterizar de forma completa la complejidad de los sistemas estudiados. Entender esta complejidad es esencial, ya que la evolución de estos sistemas no responde a leyes deterministas si no más bien a leyes no prescriptivas que definirán la esfera de posibilidades dentro de la cual el sistema puede evolucionar, existiendo por tanto más de un camino o alternativa posible para dicha evolución. La única forma de llegar a una comprensión profunda de los procesos evolutivos será desentrañar estos factores de complejidad e impredecibilidad para poder incorporarlos en los modelos de estudio.

La definición de los patrones de diversidad constituye por lo tanto el primer paso en el estudio de la historia evolutiva de los organismos. Estos patrones son especialmente informativos cuando pueden comparase con los obtenidos a través de diferentes marcadores. En el caso de *Pseudacris regilla* (capítulo I) nuestros datos permiten identificar la existencia de tres clados principales que denominamos "noroccidental", "central" y "meridional". Estos tres linajes, definidos mediante secuencias mitocondriales, presentan una distribución geográfica concordante con los grupos previamente propuestos a través del análisis de aloenzimas (Case *et al.*, 1975). La congruencia observada entre los resultados nucleares y mitocondriales supone un sólido apoyo a la credibilidad de los patrones definidos, ya que frecuentemente se encuentran fuertes discordancias entre marcadores, generalmente fruto de la estocasticidad de los procesos de coalescencia y de las peculiaridades de cada marcador. Por ese motivo puede ocurrir que la interpretación de diferentes marcadores proponga diferentes versiones de la historia evolutiva de los organismos estudiados, por lo que a menudo se suele plantear la distinción entre "árboles de genes" y "árboles de especies" (Maddison, 1997). En nuestro caso, la similitud en los patrones observados a través de marcadores nucleares y mitocondriales nos permite asumir que los resultados obtenidos son en

buena parte consecuencia de la historia evolutiva de *P. regilla.*

Dentro de los tres clados principales observados en esta especie se observa una mayor subestructuración dentro del clado "meridional", presente en la península de Baja California (México), así como en el sur del estado de California y en Nevada (Estados Unidos). El estudio de patrones filogeográficos en diversos vertebrados ha puesto al descubierto la existencia de patrones aparentemente vicariantes entre las mitades sur y norte de Baja California (Riddle *et al.* 2000). Esta repetición de patrones ha conllevado el desarrollo de una hipótesis biogeográfica según la cual está península habría estado partida en dos por la existencia de un brazo de mar hace aproximadamente entre 1-1.6 millones de años (Upton & Murphy, 1997; Riddle *et al.*, 2000). Nuestros datos, teniendo en cuenta la datación aproximada de los clados, se aproximan bastante a estas edades, ya que la separación de la subespecie "*hypochondriaca*" (mitad norte) y la subespecie "*curta*" (mitad sur) se habría producido según nuestras estimaciones en torno a un millón de años atrás. Adicionalmente dentro de "*curta*" se encuentra una nueva división cuya antigüedad estimamos en 0.9 millones de años y que exigiría la existencia de una barrera adicional en la zona del istmo de La Paz. Dada la escasa altitud de este área sería asumible la existencia aquí de otro

brazo marino, como ya ha sido sugerido para explicar el patrón de diversidad genética observado en saurios del género *Urosaurus* (Aguirre *et al.*, 1999). Sin embargo, no se han encontrado evidencias geológicas que apoyen estas hipótesis, por lo que deben considerarse alternativas para estudiar los procesos que han generado estos patrones. Una de estas alternativas podría ser la alternancia de condiciones áridas con otras más moderadas (Savage, 1960), que habría producido sucesivas contracciones y expansiones de la distribución de las diferentes especies en función de sus requerimientos ecológicos, favoreciendo el aislamiento de ciertos grupos poblacionales y la formación de patrones vicariantes.

Más allá de los eventos biogeográficos que han afectado a las poblaciones en Baja California, la diferenciación de los tres linajes principales habría sido relativamente antigua y promovida por la compleja actividad orogénica acaecida en la zona durante el Plioceno, como parece haber sido el caso en un buen número de organismos existentes en la región del Pacífico de Norteamérica (Brunsfeld *et al.*, 2001; Calsbeek *et al.*, 2003).

Taxonómicamente hablando, *Pseudacris regilla* constituía una especie politípica con numerosas subespecies descritas pero pobremente definidas

(Jameson *et al.*, 1966). En nuestro caso, teniendo en cuenta la congruencia observada entre los patrones mitocondriales y aloenzimáticos, proponemos un ajuste taxonómico por el cual *Pseudacris regilla s. l.* queda dividida en tres especies correspondientes a los tres linajes principales observados a nivel nuclear y mitocondrial. El grupo "noroccidental" mantiene el nombre de *Pseudacris regilla*, el grupo "central" pasa a llamarse *P. sierra* y finalmente el grupo "meridional" toma el nombre de *P. hypochondriaca.*

En nuestro estudio sobre la historia evolutiva de *Hyla meridionalis* (capítulo II) partíamos de una hipótesis de partida clara, según la cual el Estrecho de Gibraltar actúa como una barrera efectiva que limita el flujo génico entre poblaciones ibéricas y norteafricanas. En esta caso también existían algunos datos previos al respecto. En concreto Busack (1986) había estudiado poblaciones de varias especies a ambos lados del Estrecho mediante el análisis de varios loci aloenzimáticos. En el caso de *Hyla meridionalis* ya observó la existencia de flujo génico reciente, pero sus datos resultaban insuficientes para detallar más en profundidad los procesos acaecidos en los últimos 5.3 millones de años desde la reapertura del Estrecho. En este caso los resultados aloenzimáticos no son directamente comparables con nuestros datos mitocondriales, aunque las inferencias que se obtienen en ambos casos

son similares en cuanto a que el Estrecho no ha actuado como una barrera completamente impermeable a la dispersión de la especie entre los dos continentes, como implica la existencia de alelos y haplotipos idénticos o muy parecidos en poblaciones tanto ibéricas como marroquíes. La mayor variabilidad genética dentro de las poblaciones africanas indica que las poblaciones europeas son producto de colonizaciones recientes de la Península Ibérica, un patrón ya observado en otros organismos de la zona (Carranza *et al.*, 2004; Cosson *et al.*, 2005; Pleguezuelos *et al.*, 2008).

Las poblaciones de *Hyla meridionalis* del norte de la Península Ibérica y del sur de Francia comparten un único haplotipo mitocondrial, el cual está también presente en poblaciones del norte de Marruecos. Este patrón de extrema homogeneidad genética sugiere una colonización muy reciente del norte de la Península probablemente desde la costa norte de Marruecos o, quizás, de Argelia, lo que implicaría la dispersión de la especie a través de cientos de kilómetros de mar, algo que, de forma natural, se considera poco frecuente (Heany, 1986; Dobson, 1998), especialmente en anfibios, debido a su poca tolerancia a situaciones de alta salinidad (Vences *et al.*, 2004). Como alternativa se plantea, como posible origen de estas poblaciones, el transporte humano, pasivo o activo, de ejemplares de *Hyla meridionalis* que se habría

aclimatado y expandido por los nuevos territorios. El efecto de la actividad humana en la distribución y expansión de especies es especialmente fuerte en la cuenca mediterránea, con numerosos casos de especies que llegan a Europa desde África y viceversa, incluyendo varios casos conocidos de traslocación de anfibios en el Mediterráneo Occidental en varios momentos históricos (Hemmer *et al.*, 1981; Corti *et al.*, 1999; Llorente *et al.*, 2002).

En el caso de las poblaciones presentes en el suroeste peninsular también observamos un diversidad haplotípica reducida frente a las poblaciones marroquíes, con haplotipos muy similares presentes en ambas regiones pero ninguno compartido, aunque esto puede ser consecuencia del limitado número de muestras procedentes de Marruecos. De nuevo en este caso las hipótesis explicativas de este patrón serían bien un origen natural, atravesando el Estrecho en alguna balsa de vegetación a la deriva, bien un origen humano, voluntario o no.

Las poblaciones Europeas de la especie, después de su llegada reciente, parecen haberse expandido con rapidez, aunque parecen existir algunos factores que limitan su avance. Por ejemplo, el Sistema Central aparentemente actúa como una barrera que limita la dispersión hacia el norte de la especie

(Merchán et al., 2005). Además, la presencia de posibles competidores como *Hyla arborea* o *H. intermedia* podría ser un factor limitante para la expansión de *H. meridionalis*, lo que explicaría la distribución prácticamente parapátrica de estas especies tanto en la Península Ibérica como en el Sur de Francia e Italia.

Entre las poblaciones marroquíes se observa la existencia de al menos tres clados bien diferenciados que probablemente se originarían alopátricamente en refugios situados en los diferentes sistemas montañosos durante los cambios climáticos del Pleistoceno.

En cuanto a las poblaciones presentes en el archipiélago canario la hipótesis generalmente reconocida es que son fruto de una introducción humana, ya que se considera muy poco probable la existencia de anfibios autóctonos en islas oceánicas (Vences *et al.*, 2004). Nuestros resultados apoyan esta hipótesis, sin embargo no aportan suficiente información sobre el origen concreto de esta introducción, que podría haberse realizado tanto desde la Península Ibérica como desde Marruecos. Sería recomendable aplicar otro tipo de marcador de evolución más rápida, por ejemplo microsatélites, para profundizar en las raíces de este asunto.

Uno de los temas que más interés ha despertado en el campo de la filogeografía es el del efecto de las glaciaciones pleistocénicas en los patrones de diversidad de los organismos (Taberlet, 1998; Hewitt, 2000). La acumulación de estudios particulares con diferentes tipos de organismos han llevado a la definición de algunos modelos generales como por ejemplo el de "refugios dentro de refugios" (Gómez & Lundt, 2006) descrito para la Península Ibérica. En el caso de *Lissotriton helveticus* (capítulo III) los cambios climáticos asociados a los ciclos glaciales han sido determinantes a la hora de establecer los actuales patrones de diversidad genética. Los linajes mitocondriales observados se habrían diferenciado según nuestras estimas durante el Pleistoceno medio y superior, probablemente por alopatría como consecuencia del aislamiento poblacional favorecido por los mencionados cambios climáticos, traducidos en una fragmentación del hábitat óptimo para la especie. Podemos considerar, sin embargo, que estos linajes pleistocénicos son relativamente jóvenes, si tenemos en cuenta que la edad de esta especie se sitúa en torno a 20 millones de años (Babik *et al.*, 2005) y que otras especies del género, como *L. boscai* y *L. vulgaris* presentan linajes diferenciados desde el Plioceno e incluso el Mioceno (Babik *et al.*, 2005; Martínez-Solano *et al.*, 2006).

La ausencia de linajes antiguos, pre-pleistocénicos, representa una diferencia notable en comparación con los organismos típicamente incluidos dentro del modelo "refugios dentro de refugios" (Gómez & Lundt, 2006). Esta ausencia podría haberse producido simplemente por la extinción de los linajes ancestrales. Otra alternativa es que, en realidad, la colonización de la Península Ibérica por parte de *Lissotriton helveticus* tuvo lugar, favorecida por el progresivo enfriamiento del clima, durante el Pleistoceno, motivo por el cual estas poblaciones no pueden albergar linajes más antiguos, que de haber sobrevivido, deberían encontrarse en la zona de especiación de *L. helveticus*, probablemente situada en algún lugar en el centro o el noroeste de Europa, como sugiere el registro fósil de la especie (Holman, 1998; Rage & Bailon, 2005; Ivanov, 2007). Sin embargo estas regiones fueron barridas por las glaciaciones, provocando la extinción de las poblaciones más antiguas de *L. helveticus* y una depauperación de su diversidad intraespecífica, de la que sólo persiste la acumulada desde el Pleistoceno en el tercio norte de la Península. Los genes nucleares analizados muestran una variabilidad genética aún menor que los mitocondriales y además no presentan una clara estructura geográfica. Este patrón seguramente es consecuencia de la lenta tasa de substitución de estos genes y también de la existencia de cierto nivel de flujo génico entre los

diferentes grupos, algo lógico si pensamos que se trata de una especie de distribución prácticamente continua.

En la actualidad *Lissotriton helveticus* se extiende ampliamente por el norte de los Pirineos, hasta el este de Alemania y la República Checa, así como por la mayor parte de la isla de Gran Bretaña. Nuestros datos sugieren que la recolonización de estos territorios es muy reciente, acaeciendo muy probablemente durante el Holoceno a consecuencia de la suavización de las condiciones climáticas generales y se habría producido, dadas las características de dispersión de este tipo de especies, como una expansión continua del área de distribución desde las poblaciones ibéricas. La recolonización de Gran Bretaña se debió producir antes de la completa apertura del Canal de la Mancha, hace unos 7500 años (Sanchiz, 2002).

Resulta sorprendente que, en vista de la capacidad de dispersión mostrada por esta especie, no haya sido capaz de expandirse más ampliamente en la Península Ibérica, ocupando zonas de hábitat favorable como podría ser el Sistema Central. Esto podría explicarse en parte por su limitada capacidad para medrar en medios típicamente mediterráneos. Sin embargo, en zonas favorables como Galicia o el norte de Portugal se trata de una especie mucho

más escasa de lo esperable. Se trata de una zona de simpatría entre *Lissotriton helveticus* y *L. boscai*, especies que presentan un patrón de reemplazo progresivo. Hacia el oeste y el sur *L. helveticus* se va rarificando hasta desaparecer, quedando sólo *L. boscai*. Al contrario, al moverse hacia el este a lo largo de la Cornisa Cantábrica *L. helveticus* va convirtiéndose en la especie dominante y es *L. boscai* la especie que acaba desapareciendo. Este patrón de reemplazo se observa también entre *L. helveticus* y *L. vulgaris* en el centro de Europa (Zuiderwijk, 1980), lo que parece indicar la existencia de algún tipo de interacción competitiva entre especies del género *Lissotriton*.

La diferenciación de las especies de *Lissotriton* parecen deberse principalmente a procesos alopátricos, como parece ser la norma general en la mayor parte de casos dentro de la clase Amphibia (Vences & Wake, 2007). Sin embargo, los patrones de diversidad observados en este grupo de tritones parecen responder además a una serie de factores adicionales. Entre éstos destacarían la persistencia de poblaciones ancestrales, la capacidad de colonización de nuevos territorios, el grado de interacción competitiva con otras especies y por supuesto los tamaños poblacionales efectivos, algo que puede ser determinante para los otros factores.

Los patrones de diversidad genética observados en *Lissotriton* nos permiten plantear dos modelos que caracterizan a las especies presentes en Europa, que referimos como especies tipo "S" y especies tipo "R". Las primeras se ajustan al patrón observado en *L. boscai* (Martínez-Solano *et al.*, 2006) y se trata de especies que han mantenido su presencia en al menos parte de su distribución ancestral, zonas que actúan como santuarios, por lo que se caracterizarían por la persistencia de linajes filogenéticos profundos y una variabilidad genética fuertemente estructurada. Dentro de este tipo de especie podemos encontrar ejemplos como *Chioglossa lusitanica*, *Salamandra salamandra* o *Alytes obstetricans* (Alexandrino *et al.*, 2000; García-París *et al.*, 2003; Martínez-Solano *et al.*, 2004). Las especies de tipo "R" se ajustan al patrón observado en *L. helveticus* y se trata de especies que han sufrido drásticas extinciones en sus áreas de distribución ancestrales, perviviendo sólo en zonas periféricas que han actuado como refugios. Como consecuencia de esas extinciones estas especies presentan una variabilidad genética reciente y a menudo poco estructurada. Como ejemplos de este tipo de especies podemos citar casos como el de *Bufo calamita* o *Apodemus sylvaticus* (Michaux *et al.*, 2003; Rowe *et al.*, 2006).

Uno de los proceso evolutivos más interesantes y a la vez difícil de

estudiar es el de las radiaciones, en las cuales la diferenciación de linajes y la especiación ocurre en un breve intervalo de tiempo, por lo que resulta extremadamente difícil reconstruir de forma fehaciente las relaciones filogenéticas, encontrando frecuentemente problemas como falta de monofilia en grupos bien caracterizados morfológicamente o también observar fuertes discrepancias en los patrones resultantes al analizar diferentes marcadores. Los estudios filogeográficos en este contexto son de difícil interpretación y especialmente difíciles de plantear en el caso de especies amenazadas y cuya distribución natural ha sido profundamente alterada por acción del hombre. Este es el caso de *Ambystoma mexicanum* y demás congéneres mexicanos del complejo *A. tigrinum* (capítulos IV, V y VI). La sistemática de este grupo de especies ha sido siempre problemática debido a la escasez de caracteres morfológicos adecuados y a la escasa diferenciación genética observada tanto a nivel mitocondrial como en aloenzimas. Todos los intentos por resolver las relaciones filogenéticas de estas especies han dado pobres resultados (Shaffer, 1984; Shaffer & McKnight, 1996; Weisrock *et al.*, 2006). Uno de los principales problemas es la falta de monofilia de muchas de las especies que integran el complejo. Este hecho, como ocurre en otras radiaciones de especies (Takahashi *et al.*, 2001) podría ser una consecuencia de un ordenamiento de linajes incompleto. Sin embargo también podría ser el

resultado de la existencia de flujo genético entre especies bien diferenciadas desde un punto de vista morfológico y ecológico.

En el caso concreto de *Ambystoma mexicanum*, nuestros datos mitocondriales incrementan considerablemente los datos conocidos para esta especie, al incluir ejemplares de todas las poblaciones silvestres conocidas. Estos nuevos datos reflejan que la falta de monofilia, a nivel mitocondrial, afecta también a esta especie, ya que los cinco haplotipos encontrados en especimenes de *A. mexicanum* se colocan en distintos puntos del árbol filogenético del grupo de especies, asociándose con haplotipos de *A. velasci*, *A. amblycephalum* y *A. ordinarium*. Debido a la disparidad de estos patrones y las reducidas diferencias genéticas observadas es lógico achacar estas variadas relaciones entre especies a un incompleto ordenamiento de linajes, pero no sería descartable la existencia de poblaciones simpátricas de *A. mexicanum* y *A. velasci* y por tanto de procesos de hibridación entre estas especies.

Para profundizar en este asunto se plantea la necesidad de una completa caracterización de las poblaciones, para lo cual ha sido necesario el desarrollo de marcadores moleculares apropiados, en nuestro caso varios loci de microsatélites que son aplicables no sólo a las poblaciones de *A.*

mexicanum si no también a las de especies relacionadas. Nuestros resultados indican que tanto *A. mexicanum* como las otras especies pedomórficas estudiadas presentan poblaciones aisladas genéticamente y que la introgesión con otras especies no pedomórficas es muy reducida, reforzando la hipótesis de una evolución independiente en cada lago promovida posiblemente por la fijación de las formas neoténicas (Shaffer, 1994). Sin embargo, el patrón de baja diversidad genética esperable en la poblaciones pedomórficas está también presente en otras especies con metamorfosis, indicando también un cierto grado de aislamiento con otras poblaciones y pequeños tamaños poblacionales. Las poblaciones estudiadas se sitúan bien en zonas áridas, bien en zonas de alta montaña. La existencia de amplias zonas intermedias con condiciones ambientales no propicias podría impedir un flujo génico continuo con otras poblaciones.

Nuestros resultados tienen relevancia en lo concerniente a la conservación de estas especies. Si el aislamiento y empobrecimiento genético se trataran de rasgos generalizables al resto de poblaciones y especies, habría que tenerlos muy en cuenta en cualquier estrategia de conservación, ya que cualquier agresión sobre estas poblaciones podría llevar a su completa desaparición, sin esperanzas de una futura recolonización natural desde otras

poblaciones. Además, la ampliación de este tipo de estudio a otras poblaciones y especies permitirán definir de forma más precisa los procesos evolutivos, adaptativos y alopátricos, que han generado la diversidad de este grupo.

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IV. Conclusiones

A continuación se resumen las principales conclusiones extraídas de los diferentes estudios presentados en esta tesis doctoral. El orden en que se presentan respeta el orden establecido por los diferentes capítulos.

Filogeografía de *Pseudacris regilla* (Anura: Hylidae)

1- *Pseudacris regilla s. l.* representa en realidad un complejo de especies distribuido a lo largo de buena parte de la costa oeste de Norteamérica. Estas especies son *P. regilla* en el norte, *P. sierra* en la zona centro y *P. hypochondriaca* en el sur y han sido definidas a partir de la concordancia observada entre datos mitocondriales y nucleares.

2- La diferenciación de los tres linajes se habría producido por fragmentación alopátrica, a consecuencia de la orogénesis de los sistemas montañosos de la zona durante el Plioceno.

3- Las poblaciones de Baja California presentan una estructuración genética más definida que el resto, con linajes diferenciados durante el Pleistoceno. Los patrones observados en esta zona son compatibles con la antigua presencia de hipotéticos brazos de mar dividiendo la península y que explicarían la continua aparición de patrones

vicariantes especialmente en el centro de la misma. Esta hipótesis, sin embargo, requiere de más estudio y no deben descartarse otras posibilidades, como el efecto de los cambios climáticos pleistocénicos en esta zona.

Filogeografía de *Hyla meridionalis* (Anura: Hylidae)

4- La especie se caracteriza por la existencia de dos linajes antiguos: un linaje oriental formado por las poblaciones de la especie localizadas en el Este de su distribución, en Túnez, y un linaje occidental formado por las poblaciones presentes en Europa y Marruecos, así como en las Islas Canarias. La extensión de ambos linajes en Argelia es aún desconocida.

5- Dentro del grupo occidental se distinguen tres linajes más recientes probablemente originados en alopatría durante el Pleistoceno, como consecuencia de los cambios climáticos y el acantonamiento de la especie en refugios situados en los sistemas montañosos marroquíes.

6- El origen de las poblaciones Europeas es reciente e independiente de los avatares históricos del Estrecho de Gibraltar. Muy probablemente la colonización de estos territorios habría sido facilitada por la acción humana, bien por introducciones voluntarias bien accidentales.

7- Las poblaciones ibéricas están separadas geográficamente en dos grupos, uno en el noreste y otro en el suroeste. Ambos grupos poblacionales tienen orígenes independientes, como demuestran su adscripción a diferentes clados mitocondriales.

8- Las poblaciones canarias también son de origen reciente, pero no podemos determinar si provienen de Marruecos o de la Península Ibérica. Nuestros resultados apoyan la hipótesis de una introducción humana, posiblemente activa dada su presencia en todas las islas del archipiélago.

9- La expansión de *Hyla meridionalis* en Europa parece estar condicionada por la presencia de poblaciones asentadas de otras especies del género, como *H. arborea* e *H. intermedia*. En la actualidad estas tres especies presentan distribuciones básicamente parapátricas con pequeñas áreas puntuales de simpatría.

Filogeografía de *Lissotriton helveticus* (Caudata: Salamandridae)

10- *Lissotriton helveticus* presenta una diversidad genética intraespecífica relativamente reciente en comparación con lo observado en otras especies del mismo género. Dentro de *L. helveticus* se pueden distinguir varios linajes mitocondriales cuya antigüedad se remonta al Pleistoceno Medio y Superior.

11- La ausencia de linajes ancestrales en *L. helveticus* marca las diferencias principales con las especies típicamente incluidas en el modelo de "refugios dentro de refugios". Encuadramos a *L. helveticus* dentro de las especies tipo "R", aquellas cuya reducida diversidad actual es la que se localiza en poblaciones periféricas que han actuado como auténticos refugios. Las especies tipo "S", como por ejemplo *L. boscai*, incluyen aquellas en las que se ha conservado su diversidad ancestral en áreas estables que actúan como santuarios.

12- Los patrones observados para los genes nucleares difieren de los obtenidos a través de genes mitocondriales. Estas diferencias podrían estar causadas por la lenta tasa de substitución de los genes nucleares y su incompleta ordenación de linajes, además de un cierto grado de flujo génico entre los grupos poblacionales.

13- *Lissotriton helveticus* presenta áreas de simpatría con *L. boscai* y *L. vulgaris*, en las que se observa un progresivo reemplazo de especies, sugiriendo la existencia de algún tipo de interacción competitiva entre éstas. Los patrones de diversidad en las especies de *Lissotriton* estarían condicionados por factores tales como la persistencia de poblaciones ancestrales, la capacidad de colonización de nuevos territorios, el grado de competencia con otras especies y el tamaño poblacional efectivo.

Filogeografía de *Ambystoma mexicanum* (Caudata: Ambystomatidae)

14- Se confirma la existencia de una tercera población silvestre de *Ambystoma mexicanum*, en un sistema acuático seminatural ubicado en el Bosque de Chapultepec, dentro del entorno urbano del Distrito Federal, México.

15- La diversidad mitocondrial de la especie es muy reducida y aparece de forma polifilética en las reconstrucciones filogenéticas. Esta falta de monofilia es habitual en las radiaciones evolutivas y parece consecuencia de una ordenación incompleta de linajes.

16- La utilización de microsatélites permite una caracterización detallada de la estructura genética poblacional y pueden ser aplicados para

profundizar en los procesos evolutivos, la sistemática y la biología de la conservación del grupo.

17- Las poblaciones pedomórficas se caracterizan por una reducida diversidad genética consecuencia de su aislamiento y de cuellos de botella poblacionales.

18- Las poblaciones no pedomórficas incluidas en el estudio presentan una diversidad genética equivalente a la de las especies pedomórficas, indicando un notable aislamiento poblacional posiblemente causado por las condiciones ambientales y también por la alteración del hábitat.

Conclusiones generales. El aporte de datos moleculares a escala poblacional permite:

19- Resolver problemas taxonómicos derivados de la falta de caracteres morfológicos claramente diagnósticos o, en el caso contrario, de la existencia de una elevada diversidad fenotípica.

20- Determinar el origen y antigüedad de las poblaciones y detectar poblaciones introducidas, lo que puede resultar de gran relevancia a la hora de desarrollar planes de gestión y protección de especies.

21- Desarrollar modelos generales explicativos sobre los patrones de diversidad genética.

22- Caracterizar los grados de aislamiento y conectividad entre poblaciones, las zonas de contacto y grado de introgresión entre linajes, los tiempos de diversificación de las especies, etc. Estos aspectos son una parte esencial en estudios de biología evolutiva, biogeografía, sistemática, biología de la conservación, etc.