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VARIABILIDAD GENÉTICA POBLACIONAL DE ANFIBIOS EN HÁBITATS URBANOS Y RURALES. APLICACIONES PARA LA CONSERVACIÓN

(POPULATION GENETIC VARIABILITY OF AMPHIBIANS IN URBAN AND RURAL HABITATS. APPLICATIONS FOR CONSERVATION)

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I believe a leaf of grass is no less than the journey-work of the stars,
And the pismire is equally perfect, and a grain of sand, and the egg of the wren,
And the tree toad is a chef-d’oeuvre for the highest,
And the running blackberry would adorn the parlours of heaven.

Walt Whitman, “Song of Myself”

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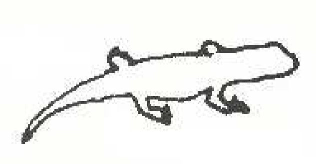


Introducción

FACTORES DE RIESGO ACTUALES PARA LOS ANFIBIOS

Los anfibios son el taxón de vertebrados más amenazado por las condiciones actuales de cambio climático y las modificaciones antropogénicas de sus hábitats naturales (Stuart *et al.*, 2004; Beebee y Griffiths, 2005). Desde la fragmentación del hábitat (Cushman, 2006) a la desecación y aumento de los riesgos de depredación (Rohr y Madison, 2003), las amenazas a los anfibios han ido aumentando en las últimas décadas. La identificación y protección de los lugares clave para su reproducción son cruciales para su supervivencia; entre ellos, los hábitats terrestres asociados a puntos de agua aislados son elementos esenciales para mejorar la biodiversidad anfibia (Marsh y Trenham, 2001; Gibbons, 2003).

Algunas de las propiedades comunes a todos los anfibios son su dependencia del agua y de la humedad, sus ciclos de vida complejos y su sensibilidad fisiológica a condiciones ambientales, debido a su piel extremadamente permeable. Todas estas condiciones contribuyen a que este grupo de animales sea uno de los primeros en ser afectados por alteraciones ambientales (Wake, 1991). Además de su vulnerabilidad fisiológica, su ecología también contribuye a su sensibilidad a alteraciones del hábitat. Sus ciclos de vida transcurren en zonas bien diferenciadas de su entorno porque tienen una etapa terrestre y otra acuática, de manera que su condición y abundancia podría reflejar los efectos combinados o sinérgicos de interacciones entre ecosistemas. Se pueden ver afectados desde sus primeros estadíos de vida, ya que los huevos de la mayor parte de las especies no tienen una cubierta dura que pueda bloquear la radiación, los rayos UV o la contaminación. Por todo esto, pueden servir como indicadores de salud ambiental (Blaustein y Wake, 1995) y su declive se considera una advertencia de degradación ambiental significativa. Además, los anfibios adultos juegan un rol ecológico muy importante en las comunidades biológicas, con implicaciones para la salud humana, por ejemplo mediante el control de las poblaciones de insectos, particularmente mosquitos (Becker, 2006; Chase y Shulman, 2009).

La emergencia de nuevas enfermedades puede afectar a las poblaciones de anfibios rápidamente, provocando repentinos declives poblacionales e incluso la extinción (UICN, 2004). Dos de las enfermedades que más están afectando a los anfibios son un hongo Chitridiomiceto, *Batrochochytrium dendrobatis*, que está afectando a poblaciones de todo el mundo e incluso causando extinciones de algunas especies como *Taudactylus acutirostris*

(Berger *et al.*, 1998; Bosch *et al.*, 2001). La otra es una patología recientemente descrita en Asturias, la infección por ranavirus, que fue documentada en 2007 en larvas de sapo partero *Alytes obstetricans* de Picos de Europa (Balseiro *et al.*, 2009a) y en 2008 en la misma zona en individuos juveniles de tritón alpino, *Mesotriton alpestris cyreni* (Balseiro *et al.*, 2009b).

Además de las patologías emergentes que afectan a los anfibios, la reducción del hábitat se postula como la principal causa para su declive en la Península Ibérica (Stuart *et al.*, 2004). La conectividad juega un papel fundamental para la viabilidad de las poblaciones de anfibios (Cushman, 2006). Su baja capacidad de dispersión (Bowne y Bowers, 2004) favorece los procesos de fragmentación de poblaciones y pérdida de hábitat. La comprensión de los procesos que llevan a la fragmentación de hábitats y al establecimiento de barreras es fundamental para una correcta conservación, pero está limitada por la falta de conocimiento de las necesidades y limitaciones propias de cada especie.

Los declives en la población antes mencionados y la fragmentación de poblaciones pueden ser causantes de una pérdida en la diversidad genética (Frankham *et al.*, 2002), debido a una disminución de la riqueza alélica (número de alelos o variantes genéticas) y una caída de la heterocigosidad, con una mayor pérdida de alelos raros o poco comunes (Nei *et al.*, 1975). Como recomendaciones para una mejor gestión y manejo poblacional, se ha sugerido comenzar con estudios especie-específicos, empleando técnicas moleculares o de simulación (Cushman, 2006).

IMPACTOS ANTROPOGÉNICOS SOBRE EL HÁBITAT DE LOS ANFIBIOS

Un número creciente de estudios sugiere que el declive de las poblaciones de anfibios a nivel mundial es debido principalmente a factores antropogénicos (Wake, 1991; Pechmann y Wake, 1997; Semlitsch, 2003). La urbanización es, en la actualidad, una de las causas más generalizadas de perturbaciones del ecosistema natural en todo el mundo, y por lo tanto, representa una gran amenaza para la biota (Czech *et al.*, 2000; Miller y Hobbs, 2002). La urbanización amenaza actualmente a más de un tercio de las especies conocidas de anfibios en el mundo (Hamer y McDonnell, 2008) y, además, se trata de un proceso en expansión, lo que incrementa la importancia de conocer sus efectos actuales para poder prevenir futuras intensificaciones de su alcance. Los principales efectos de la antropización para los anfibios son la pérdida y fragmentación del hábitat (McKinney, 2002, 2006), la contaminación (Paul y Meyer, 2001; Miltner *et al*., 2004), el abandono y destrucción de puntos de agua (Dodd y Smith, 2003) y la transformación del suelo para usos agrícolas (Houlahan y Findlay, 2003). Además de estos impactos directos, se están identificando factores asociados a la actividad humana que también contribuyen al declive de los anfibios, como un incremento de actividades recreativas (Rodriguez-Prieto y Fernandez-Juridic, 2005), la introducción de especies invasoras (Knapp y Matthews, 1996; Kats y Ferrer, 2003) o la iluminación artificial (Baker y Richardson, 2006).

La antropización es un proceso en expansión que afecta a los ecosistemas de forma global. La expansión de las ciudades es un fenómeno acelerado en el último siglo, lo que ha llevado a un aumento exponencial de las zonas urbanas y suburbanas. Pero la acción humana no sólo afecta a las zonas urbanas, sino que también está presente en zonas rurales. En Asturias (Noroeste de la Península Ibérica) se han producido importantes cambios socioeconómicos, con una tendencia al abandono del campo y a la modificación de las técnicas agrícolas y ganaderas empleadas tradicionalmente (Dopico *et al.*, 2009). Los anfibios pueden, sin embargo, seguir habitando sistemas fuertemente antropizados, en parte debido a algunos elementos construidos por los humanos. En regiones donde la densidad de charcas naturales es baja, las construcciones agrícolas como charcas artificiales o bebederos para el ganado pueden representar una importante alternativa como hábitats de reproducción y cría de anfibios (Baker y Halliday, 1999). Sin embargo, algunos autores han encontrado que la riqueza de especies disminuye en charcas agrícolas expuestas a agricultura intensiva (Beja y Alcazar, 2003), muchas veces relacionado con las altas concentraciones de productos químicos agrícolas como pesticidas o fertilizantes (revisado por Mann *et al.*, 2009).

La pérdida de hábitat ha sido considerada como la principal causa del declive a escala global de los anfibios (Stuart *et al.*, 2004; Gardner *et al.*, 2007). Su necesidad de dos hábitats diferentes, uno acuático y otro terrestre, los hace doblemente vulnerables, ya que las alteraciones en ambos medios pueden afectarlos. Su hábitat acuático es fundamental; es el lugar para la reproducción, puesta de huevos y crecimiento de la progenie durante su vida acuática hasta la metamorfosis (Duellman y Trueb, 1994; Griffiths, 1996). Como la supervivencia de anfibios está íntimamente ligada con la disponibilidad de agua, las alteraciones antropogénicas de la hidrología pueden desencadenar incrementos de mortalidad que aceleren el declive de las poblaciones por la combinación de sucesos como la degradación del hábitat, el cambio climático y la aparición de enfermedades (Beebee, 1995; Berguer *et al.*, 1998; Kiesecker *et al.*, 2001). Diversos estudios han tratado de determinar qué factores afectan a la presencia de anfibios en hábitats acuáticos y se han identificado como muy importantes la presencia de especies predadoras (Bradford *et al.*, 1994; Knapp y Matthews, 1996; Orizaola y Braña, 2006), la vegetación acuática (Joly *et al.*, 2001), la profundidad del agua (Joly *et al.*, 2001; Denoël y Ficetola, 2008) y, como se ha comentado antes, la contaminación (Bridges y Semlitsch, 2000; Paul y Meyer, 2001; Miltner *et al.*, 2004).

A pesar de su importancia en la funcionalidad del ecosistema y de la disminución generalizada de muchas especies, los anfibios son uno de los grupos taxonómicos menos estudiados en áreas urbanas y suburbanas (Pickett *et al*., 2001). En Asturias (Norte de España), ha sido en las franjas costera y central donde han sufrido el impacto de la actividad humana de un modo más continuado y de forma más extensa. En cambio, en las zonas de montaña han estado menos expuestos. Así, zonas como Somiedo, Muniellos o Picos de Europa, al ser considerados espacios naturales protegidos, se han visto menos afectadas por la actividad del hombre. De ahí que las poblaciones de anfibios de estas zonas sean más estables y estén mejor caracterizadas (Braña *et al.*, 1996; Veith *et al.*, 2002). Sin embargo, el trabajo central de esta Tesis es el estudio de impactos antropogénicos en anfibios. Por este motivo son las zonas no montañosas, por su mayor nivel de antropización y cuyas poblaciones no han sido estudiadas genéticamente hasta ahora, las que resultan de especial interés.

REFUGIOS PARA ANFIBIOS EN ZONAS ANTROPIZADAS

Como consecuencia de la creciente urbanización, las charcas naturales y los humedales están desapareciendo, con la consiguiente pérdida de hábitats adecuados para la reproducción de anfibios. Para la conservación de estas especies, parece imprescindible identificar posibles refugios existentes en zonas de actividad humana y resaltar su valor e incluso convertirlos en figuras de protección ambiental. En medios rurales se ha documentado que los canales agrícolas artificiales pueden suponer una alternativa como hábitat acuático para anfibios (Beebee, 1997; Knutson *et al*., 2007; Denoël y Ficetola, 2008). Algunos de los elementos empleados en la ganadería tradicional, como los bebederos para el ganado, podrían constituir un refugio para diversas especies de anfibios. La exploración de estos refugios será una parte importante de la presente Tesis.

El mayor grado de antropización corresponde a las zonas urbanas. Pese a su apariencia inhóspita para la vida salvaje, también albergan anfibios. Generalmente, las zonas urbanas no se tienen en consideración a la hora de establecer planes para la conservación de especies. Se suele partir de la idea de que las ciudades no suponen un hábitat rico en especies y que por ello los esfuerzos en conservación no serían eficaces. Sin embargo, en áreas con altos niveles de urbanización las charcas urbanas pueden considerarse como el último reducto para los anfibios. Pocos estudios al respecto se pueden encontrar y además con resultados contradictorios. Algunos autores han descrito una menor riqueza de especies de anfibios en hábitats urbanos (Hitchings and Beebee, 1997; Arens *et al.*, 2007; Gagne y Fahrig, 2007; Noël *et al.*, 2007), minimizando su importancia como posibles figuras de protección, mientras que otros sí que destacan su valor como hábitat para anfibios (Parris, 2006; Gledhill *et al.*, 2008; Birx-Raybuck *et al.*, 2010) Al no haber sido evaluados en profundidad, su valor ecológico está aún por determinar.

MARCADORES MOLECULARES Y SU USO EN GENÉTICA DE POBLACIONES DE ANFIBIOS.

El estudio de la variación genética ha contribuido en gran medida a la comprensión de los procesos evolutivos y de las relaciones entre organismos, tanto a nivel interespecífico como intraespecífico. Los estudios de la variación en el ADN mitocondrial (ADNmt) son una poderosa fuente de información en genética de poblaciones (Ballard, 2000) y en estudios de filogeografía (Avise 2000, 2004). Las características del ADN mitocondrial que han contribuido a esta situación incluyen la facilidad de extracción y recuperación de información, la alta tasa de mutación en comparación con los genes nucleares, la ausencia de recombinación para la mayoría de las especies (aunque este punto ha sido fuente de discusión, ver Rokas *et al.*, 2003) y el hecho de que la herencia sea uniparental y haploide (Avise, 2000).

Las mitocondrias suelen encontrarse en el citoplasma en gran número y, excepto en casos muy concretos, presentan exactamente la misma secuencia en todos los tejidos. Por tanto, en una célula animal existen múltiples genomas mitocondriales, todos ellos iguales, por lo que es muy fácil obtener ADN a partir de cantidades muy pequeñas de tejido conservado en alcohol durante muchos años (Carranza *et al.*, 1999; Carranza *et al.*, 2001) e incluso a partir de material degradado o subfósil (Austin y Arnold, 2001).

El ADNmt se transmite, en la mayoría de animales, de forma no mendeliana por línea materna. Aunque machos y hembras lo tienen, son únicamente las hembras quienes lo transmiten a la descendencia (Avise, 2000; Hurst y Jiggins, 2005) y por eso este tipo de ADN no sufre recombinación. Esto permite que, salvo excepciones, las diferencias encontradas entre individuos se deban única y exclusivamente a fenómenos de mutación y no sean el resultado de recombinación. El modo de transmisión uniparental de ADNmt se refleja a la hora de calcular el tamaño efectivo de la población, es en promedio cuatro veces menor que la de los genes nucleares, y resulta por tanto un marcador molecular más sensible a eventos estocásticos y la deriva genética (Moore, 1995).

Bajo condiciones neutrales (no selectivas), la cantidad de polimorfismo de una población es directamente proporcional al producto del tamaño efectivo de la población por la tasa de mutación (Ballard y Whitlock, 2004). Es importante señalar que, por su herencia materna, el ADNmt es un tipo de ADN muy útil para evaluar efectos sobre el tamaño efectivo de una población, ya que al tener la mitad del tamaño efectivo que el ADN nuclear, puede verse afectado por procesos naturales como los efectos de fragmentación, puesto que es relativamente más sensible a efectos demográficos como los cuellos de botella, incrementando el efecto de la deriva génica y la tasa de renovación génica dentro de las poblaciones (Avise, 1994). Además, este último factor, junto con una elevada tasa de mutación y su tasa de evolución que es mucho más rápida, de 5 a 10 veces más rápida que la de genes nucleares de copia única (Ballard y Whitlock, 2004; Hurst y Jiggins, 2005), aumentan el nivel de variabilidad entre poblaciones al hacer desaparecer más rápido los polimorfismos ancestrales que en los genes nucleares. Esta característica de rápida evolución sumada a la herencia materna, recombinación limitada y gran robustez, hacen que el ADN mitocondrial sea un marcador ideal para la solución de muchas preguntas a nivel de especie (Avise *et al.*, 1987).

La mayoría de polimorfismos mitocondriales son sustituciones puntuales y permiten identificar diferentes haplotipos dentro de una población. A nivel de población, secuencias completas de ADN mitocondrial no resultan prácticas debido al elevado número de muestras a analizar y a la presencia de regiones del ADN mitocondrial con poca variabilidad. Por tanto, suelen definirse los haplotipos mitocondriales existentes en las regiones más variables, y por tanto más informativas, posibilitando el análisis de un número elevado de individuos (Avise *et al.*, 1987). En anfibios, una gran mayoría de los estudios moleculares publicados hasta ahora están basados en polimorfismos de diferentes regiones del ADN mitocondrial. Por ejemplo, en la Península Ibérica, utilizando los genes *nad*4 y región control para *L. boscai*, se ha descrito una red de poblaciones interconectadas con flujo génico entre ellas (Martínez-Solano *et al.*, 2006) y en *L. helveticus* mediante COI y región control, fenómenos de expansión poblacional (Recuero y García-París, 2011). En *D. galganoi* fue detectado flujo génico limitado y aislamiento por distancia empleando los genes citocromo b y *nad*4 (Martínez-Solano, 2004a).

Por otra parte, existen algunos inconvenientes que se deben tener en cuenta al trabajar con ADN mitocondrial. Entre ellos se encuentra la integración de copias de ADN mitocondrial en el ADN nuclear. De esta manera pueden evolucionar como pseudogenes nucleares o Numts (*Nu*clear copies of mitocondrial DNA, *mt*DNA) a un ritmo más lento que sus homólogos mitocondriales (Bensasson *et al.*, 2001; Willams y Knowlton, 2001; Ballard y Whitlock, 2004) y pueden aparecer cambios en sitios al azar, generando rupturas en la pauta de lectura o aparición de inserciones o deleciones (indels). La presencia de estos fragmentos puede dificultar la amplificación de secuencias exclusivamente mitocondriales y por tanto las conclusiones pueden ser descritas de manera errónea (Bensasson *et al.*, 2001; Ballard y Whitlock, 2004; Frézal y Leblois, 2008). Otro inconveniente inherente a su modo de herencia consiste en su haploidía, que los hace ineficaces en estudios de genealogías y de familias. Los genes nucleares son mucho más adecuados para estos objetivos.

Los genes autosómicos nucleares son una fuente importante de información para comprender los procesos históricos, demográficos y selectivos que determinan la arquitectura genética de las especies (Hare, 2001). Sin embargo, su baja tasa de mutación y su alto tamaño efectivo, a menudo responsables del mantenimiento de polimorfismos ancestrales, y la existencia de recombinación, podrían dificultar o sesgar las inferencias obtenidas mediante estos marcadores (Zhang y Hewitt, 2003). Esto se ha podido comprobar en anfibios. Por ejemplo, el intrón 7 del gen beta-fibrinógeno (*Fib*), comparado con el gen mitocondrial citocromo oxidasa b, resulta de 3,7 a 5 veces menos variable en *Discoglossus* (Velo-Antón *et al*., 2008) y de 2,5 a 7,6 veces en *Alytes* (Martínez-Solano *et al.*, 2004b; Gonçalves *et al*., 2007). De similar manera, el análisis de tres marcadores nucleares (*Tva4*, *Cxcr4* y *Fib*) en *Lissotriton helveticus* no evidencia estructuración geográfica, que sí puede ser apreciada con los genes mitocondriales citocromo c oxidasa I y D-loop (Recuero y García-París, 2011). Por tanto, este tipo de marcadores no parece resultar muy apropiado en estudios a nivel regional, donde serían precisos marcadores más variables y con mayor poder resolutivo.

Los microsatélites pueden resolver este problema. Consisten en repeticiones en tándem de motivos nucleótidos cortos, de dos a seis pares de bases, que, debido a su alta tasa de mutación, de polimorfismo y por su modo de transmisión mendeliana simple, permiten el estudio de aspectos relacionados con la historia evolutiva más reciente de las poblaciones (Jarne y Lagoda, 1996; Angers y Bernatchez, 1998), así como inferir la existencia de cuellos de botella o expansiones poblacionales (Goldstein y Schlotterer, 1999; Leblois *et al*. 2000; Jehle y Arntzen, 2002). Si bien los loci microsatélite han resultado ser una herramienta útil para estudios poblacionales, en el momento de elaboración de esta tesis, que comenzó en 2007, no se disponía de esta herramienta para las especies modelo, que ha sido desarrollada posteriormente: para tritón palmeado (*Lissotriton helveticus*) en 2009 (Johanet *et al*., 2009), y para sapo partero (*Alytes obstetricans*) en 2011 (Agata *et al*., 2011). Por este motivo, se han empleado polimorfismos en el ADN mitocondrial como marcadores genéticos en poblaciones de estas dos especies.

TRATAMIENTO METAGENOMICO DE MARCADORES DE ADN

El concepto de “diversidad” en biología ha sido a menudo objeto de controversia. Tradicionalmente, se emplean diversos índices para estimar la diversidad ecológica, como pueden ser la riqueza de especies o los índices de Shannon (Shannon, 1948; Magurran, 1988) o Simpson (Simpson, 1949), que caracterizan la diversidad en función del número de especies presentes (riqueza de especies) y la distribución del número de individuos por especie (equitatividad de especies).

Recientemente se ha acuñado el concepto de metágenómica, que incluye tanto la diversidad entre como dentro de especies y aporta por tanto una visión holística de la diversidad. La metagenómica consiste en el análisis conjunto de secuencias del genoma de los diferentes organismos que componen una comunidad, extrayendo y analizando su ADN de forma global. La primera aplicación fue dirigida a la evaluación de las funciones metabólicas a nivel de comunidad en procariotas (Kennedy *et al*., 2008). El ADN del metagenoma se definió originalmente como la representación de todos los genomas de las bacterias que configuran la comunidad (Gill *et al*., 2006). Así, el análisis metagenómico proporcionó una estrategia para identificar nuevos organismos y para aislar genomas completos de especies no cultivadas que están presentes en muestras ambientales (Rusch *et al*., 2007). Un metagenoma también se podría definir como un conjunto de genomas que ocupa un nicho ecológico.

Desarrollado originalmente para procariotas, este tipo de estudio es difícil de extrapolar a eucariotas, debido al elevado tamaño de sus genomas. Actualmente es aún muy caro y laborioso abordar investigaciones de genética de poblaciones, y más aún de comunidades de eucariotas, a nivel de genomas completos. Pero es posible comenzar con unos pocos genes. Como gen modelo se ha propuesto la subunidad I del gen citocromo oxidasa c (Ardura *et al*., 2011). Este gen es utilizado por el proyecto BOLD (The Barcode of Life Data Systems), una ambiciosa iniciativa para la caracterización de seres vivos a través de su ADN (Hebert *et al*., 2003). En su aplicación en eucariotas, la metagenómica trataría de medir la diversidad filogenética (Mouillot *et al*., 2011; April *et al*., 2011) o las diversidades nucleotídica y haplotídica y el número correspondiente de proteínas (Ardura *et al*., 2011) en una comunidad, independientemente de las especies que se encuentren, tratando todo el conjunto como una sola unidad genética. Esta metodología novedosa se aplicará en esta Tesis para evaluar diversidades de anfibios en distintos hábitats.

BARRERAS PAISAAJÍSTICAS EN ESPECIES DE ANFIBIOS

El conocimiento de la genética de poblaciones explica la dinámica de extinciones debido a procesos de fragmentación, que hacen que las poblaciones más pequeñas y menos estables vayan perdiendo variabilidad genética debido a la endogamia y el aumento de la deriva genética (Avise *et al*., 1987; Young *et al*., 1996; Saccheri *et al*., 1998; Reed y Frankham, 2003). Los procesos de dispersión son fundamentales para la persistencia de las poblaciones a largo plazo (Marsh y Trenham, 2001; Cushman, 2006); por tanto, el conocimiento de las barreras al flujo genético es esencial en los programas de conservación. Pero, muchas veces, la capacidad de dispersión de unas especies de anfibios no son las mismas que las de otras y es necesario conocer las dinámicas poblacionales de cada una y adaptar las medidas de conservación a la estructura poblacional para un manejo más adecuado.

La mayoría de los estudios de especies de anfibios publicados hasta el momento se centran en análisis filogenéticos (Martínez-Solano *et al.*, 2004b; Martínez-Solano *et al*., 2006; Gonçalves *et al*., 2007), pero pocos autores estudian genética de poblaciones a escala regional. Cuando la información básica sobre la variación genética y los patrones de flujo génico entre las poblaciones son desconocidas, es difícil la adopción de medidas de conservación. Esta Tesis aborda precisamente este tema.

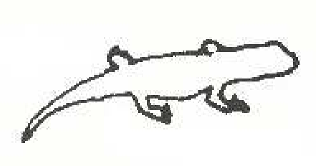
Las características del paisaje modelan la estructura genética de las poblaciones mediante la interrupción de los patrones de dispersión (Duellman y Trueb, 1994; Lynch y Duellman, 1997; Funk *et al*., 2005; Spear *et al*., 2005; Vences y Wake, 2007). El Norte de la Península Ibérica es una zona recorrida por numerosos ríos y éstos han sido descritos como potenciales barreras para el flujo de anfibios (Zeisset y Beebee, 2008). Parece que determinan la estructura poblacional de algunas especies de ranas como *Rana tungara* (Lampert *et al*., 2003), *R. luteiventris* (Funk et al., 2005), *R. cascadae* (Monsen y Blouin, 2003) y la salamandra *Chioglossa lusitanica* (Alexandrino *et al*., 2000) entre otras. Sin embargo, los ríos no parecen ser los principales determinantes de las actuales estructuras de otras poblaciones de anfibios y su papel como barrera se ha discutido en especies Sudamericanas (por ejemplo, Gascon *et al*., 2000).

Algunas barreras son antrópicas, por ejemplo, las redes de carreteras que atraviesan el territorio suponen obstáculos para el movimiento de animales, muertes por atropello, perturbaciones debidas al ruido o a los contaminantes de los vehículos y fragmentación del hábitat (revisado por Forman y Alexander, 1998; Trombulak y Frissell, 2000). El tráfico puede afectar a las poblaciones animales de manera directa induciendo mortalidad, o indirectamente, mediante la fragmentación de poblaciones (Hels y Buchwald, 2001). El impacto de las carreteras en la dispersión o en la estructura genética poblacional ha sido estudiado en diferentes grupos de animales, como por ejemplo en insectos (Keller y Largiadèr, 2003), aves (Develey y Stouffer, 2001), anfibios (DeMaynadier y Hunter, 2000; Carr y Fahrig, 2001) o mamíferos (Clarke *et al*., 1998; Gerlach y Musolf, 2000; Wang y Schreiber, 2001; Barrientos y Bolonio, 2009). Los anfibios son especialmente sensibles a las carreteras debido a que su capacidad de dispersión, generalmente, es baja (Gibbs, 1998; DeMaynedier y Hunter, 2000; Joly *et al*., 2001; Diego-Rasilla y Luengo, 2007) y presentan altos costes de locomoción (Bennett y Licht, 1974; Gatten *et al*., 1992). Estos efectos negativos de las carreteras en anfibios han sido comprobados en varias especies de anuros como *Rana arvalis* (Vos y Chardon, 1998; Hels y Buchwald, 2001), *R. temporaria* (Reh y Seitz, 1990; Hels y Buchwald, 2001), *Pelobates fucus*, *Bufo bufo* (Hels y Buchwald, 2001), y de urodelos como *Triturus cristatus* y *T. vulgaris* (Hels y Buchwald, 2001).

Debido al crecimiento constante del tráfico a nivel mundial se espera un incremento aún mayor de los efectos negativos de las carreteras (Peden *et al.*, 2004; Kirchner *et al.*, 2005; Vold, 2006). Una red de carreteras rurales intricadas puede tener importantes efectos ecológicos, especialmente relacionados con las perturbaciones y efectos de barrera, que juegan un papel muy importante en la pérdida de conectividad entre poblaciones (Jaarsma y Willems, 2002; van Langevelde *et al*., 2009). El concepto espacial de área rural “con tráfico calmado” (TCRA, en inglés), consiste en concentrar los flujos difusos de las carreteras rurales en unas pocas autopistas o vías rápidas elevadas o circunvalatorias para una disminución de los volúmenes de tráfico dentro de la región a nivel local (Jaarsma, 1997; Jaarsma *et al.*, 2002). El “calmar el tráfico” ha sido propuesto como una solución para mitigar los efectos negativos de las redes de carreteras en los espacios rurales. La idea de mitigación del tráfico se acuñó inicialmente para designar áreas urbanas residenciales con acceso restringido para el tráfico motorizado y un diseño específico a bajas velocidades. La extensión de esta idea a zonas rurales (Jaarsma, 1997) permitiría disminuir la intensidad de tráfico en estas áreas y minimizar sus efectos negativos al concentrarlo en unas pocas autopistas que recojan el flujo de vehículos. La eficacia de estas medidas ha sido evaluada positivamente en mamíferos (Jaarsma *et al.*, 2006), y los modelos indican que mitigar el tráfico puede aumentar la supervivencia en poblaciones de algunas especies, incluidas especies de anfibios en lugares con una red densa de carreteras rurales (van Langevelde y Jaarsma, 2009).

Finalmente, en proyectos de conservación de vida salvaje uno de los factores que más cuenta a la hora de llevarlos a la práctica es la limitación de la financiación. Un análisis económico de coste-beneficio puede ayudar a justificar, evaluar y optimizar los recursos empleados en conservación, especialmente para especies que no son consideradas un recurso natural de interés, como pueden ser los anfibios (Shwiff *et al*., 2007). Estos animales, salvo excepciones, no representan recursos alimenticios ni suponen beneficios aparentes para las poblaciones humanas que pueden traducirse fácilmente en resultados económicos, salvo el posible papel antes citado de control de poblaciones de mosquitos (Becker, 2006; Chase y

Shulman, 2009). El análisis coste-beneficio (Engeman *et al*., 2002, 2003; Shwiff *et al*., 2003) consiste en estimar el valor económico de los beneficios, medidos a partir del valor económico que se asigna a los animales conservados, frente a los costos que suponga la conservación de los mismos. Una forma de cuantificar en precio el valor de cada anfibio puede ser el uso de las multas o penalizaciones. Éste es el método utilizado por los autores antes citados (Engeman *et al*., 2002, 2003; Shwiff *et al*. 2003), en otros países como Estados Unidos. Es España podría aplicarse la Ley de Protección de los Animales (Decreto Legislativo 2/2008, 15 de Abril 2008; puede ser consultada en la página web: http://noticias.juridicas.com/base_datos/CCAA/ca-dleg2-2008.t7.html), en la que se establecen penalizaciones económicas por la posesión o muerte de determinados animales. Para completar el trabajo de la presente Tesis doctoral, se hará un ensayo de estimación de coste-beneficio en alguno de los estudios de caso concretos, con la intención de sugerir ideas que puedan contribuir a la conservación de estas especies tan vulnerables.



Objetivos

Los objetivos de esta Tesis son:

1. Evaluar el papel de hábitats acuáticos antropogénicos como refugio para anfibios en zonas rurales.

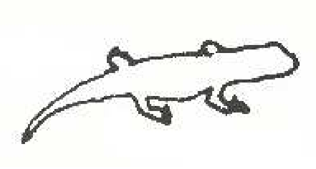
2. Estudiar el papel de las charcas urbanas en la conservación de especies modelo de anfibios empleando herramientas de genética de poblaciones.

3. Estimar el valor de los puntos de agua urbanos para el mantenimiento de la diversidad genética en anfibios empleando un enfoque metagenómico.

4. Estudiar el impacto de construcciones viarias en la conectividad poblacional de *Lissotriton helveticus* y *Alytes obstetricans*.

5. Identificar el impacto de los ríos en la conectividad poblacional de seis especies de anfibios: los anuros *Alytes obstetricans*, *Bufo bufo* y *Discoglossus galganoi*, y los urodelos *Lissotriton boscai*, *Lissotriton helveticus* y *Triturus marmoratus*.

6. Analizar el coste-beneficio de distintas estrategias de manejo de charcas urbanas para la conservación de anfibios.



Material y Métodos

En este capítulo se describen de forma global las muestras analizadas y las técnicas utilizadas. En cada capítulo de Resultados se especifica la metodología correspondiente de una forma detallada, en cada sección de Material y Métodos y se incluyen los análisis estadísticos requeridos para cada artículo.

ZONA DE ESTUDIO

El estudio se llevó a cabo en el norte de la Península Ibérica, principalmente en la región de Asturias (Fig. 1). La zona cantábrica se caracteriza por el clima oceánico, que se define por precipitaciones abundantes, nubosidad persistente y, por tanto, escasa aridez. Las temperaturas son suaves tanto en invierno como en verano, con una media anual de 12,9º C y con fluctuaciones de temperatura día-noche suaves, favorecidas por la proximidad al mar. Los ecosistemas típicos de esta región del Arco Atlántico son el bosque dominado por árboles caducifolios como el castaño (*Castanea sativa*), el roble (*Quercus robur*) y el haya (*Fagus sylvatica*). Hay numerosos ríos de curso corto surcando valles profundos, y abundan las pequeñas granjas familiares dedicadas a la agricultura (maíz *Zea mays*, habas *Phaseolus vulgaris*, manzanos *Malus domestica*) y la ganadería, principalmente la cría de vacas *Bos taurus*, ovejas *Ovis orientalis aries*, y caballos *Equus caballus ferus*.

Figura 1. Mapa de Europa con la región de Asturias resaltada.

Dentro del contexto europeo, España es el tercer país con mayor riqueza de especies anfibias, con 34 especies (Temple y Cox, 2009). La región asturiana alberga 14 especies reconocidas de anfibios: 6 urodelos y 8 anuros (Martínez-Solano *et al.*, 2001), situándose dentro de las áreas más ricas de Europa (Temple y Cox, 2009) (Fig. 2).

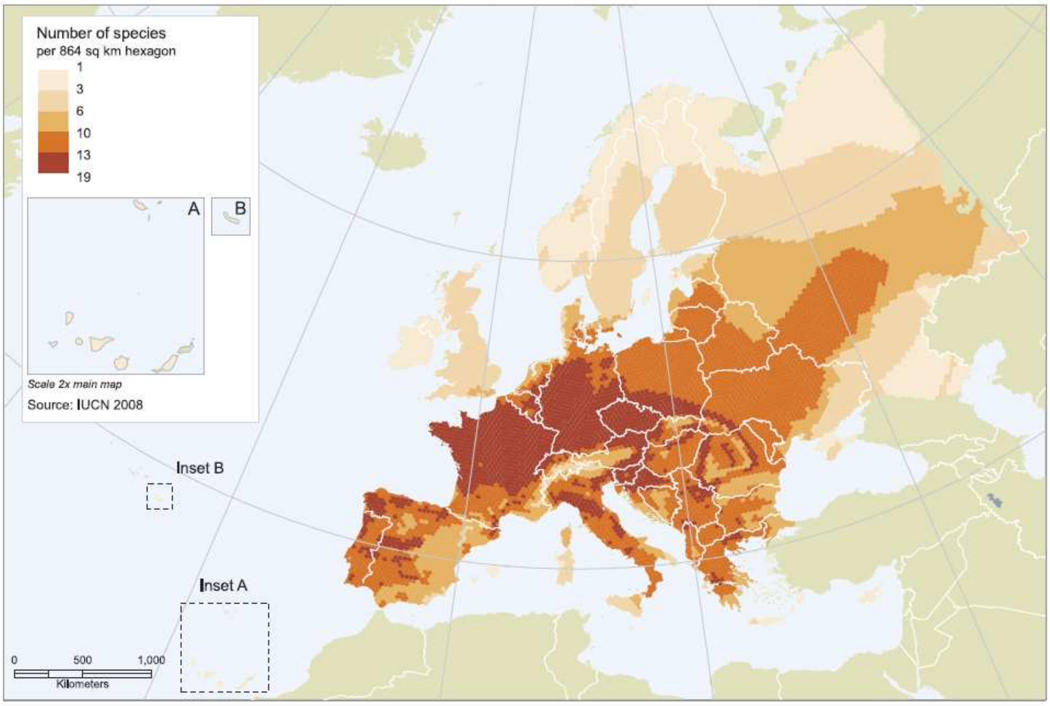


Figura 2. Riqueza de especies en anfibios europeos. Fuente: European Red List of Amphibians (Temple y Cox, 2009).

El estado de conservación de las especies de anfibios en Asturias presenta ciertas diferencias dependiendo de la escala a la que sean consideradas. A nivel internacional, según la Lista Roja de especies redactada por la IUCN (International Union for Conservation of Nature) sólo una especie de urodelos, la salamandra colilarga, *Chioglossa lusitanica*, aparece catalogada como Vulnerable; y otra de anuros, la rana patilarga, *Rana iberica*, como Casi Amenazada (Tabla 1). A nivel estatal la *R. iberica* estaría considerada Vulnerable y se incluye también al urodelo, el tritón alpino, *Messotriton alpestris cyreni,* en esta categoría. Como especie Casi Amenazada se incluye al sapo partero, *Alytes obstetricans*. Según el Proyecto de Real Decreto para el desarrollo del Listado de Especies Silvestres en Régimen de Protección Especial, y del Catálogo Español de Especies Amenazadas de la Asociación Herpetológica Española (AHE), se incluye una categoría de especies denominada “De interés especial”. Engloba a aquellas que, sin estar en las categorías antes establecidas, son merecedoras de una atención particular en función de su valor científico, ecológico, cultural o por su singularidad (Tabla 1). A su vez, desde la AHE se propone incluir a *A. obstetricans* como especie Vulnerable e incluir a las especies sapo común, *Bufo bufo*, rana patilarga, *R. perezi*, y salamandra común, *Salamandra salamandra*, en el Listado de Especies Silvestres en Régimen de Protección Especial. A nivel regional, en el Libro Rojo de la fauna asturiana (Nores *et al.*, 2007) se incluyen una especie de anuros, ranita de San Antón (*Hyla arborea*) y otra de urodelos, tritón alpino (*M. alpestres cyreni*) en la categoría de especies vulnerables y tres especies de anuros: sapo corredor, rana patilarga y rana común (*B. calamita*, *R. iberica* y *R. perezi*) y una de urodelos, la salamandra patilarga (*C. lusitanica*) catalogados como Casi Amenazados.

Tabla 1. Especies de anfibios presentes en Asturias (Martínez-Solano *et al.*, 2001), categorías establecidas por IUCN (v.2011.1), clasificación nacional, autonómica y propuesta de la AHE. LC, preocupación menor; NT, casi amenazada y V, vulnerable.

Especies	Taxonomía	IUCN Estatus/ Tendencia poblacional	Catálogo Nacional	Categoría Autonómica	AHE: Propuesta
Alytes obstetricans	Anuro	Estatus: LC Pob.: en disminución	NT De interes especial	LC	V
Bufo bufo	Anuro	Estatus: LC Pob.: estable	LC		Protección Especial
Epidalea calamita	Anuro	Estatus: LC Pob.: en disminución	LC De interes especial	NT	
Chioglossa lusitanica	Urodelo	Estatus: V B2ab(ii,iii,iv) Pob.: en disminución	V De interes especial	NT	
Discoglossus galganoi	Anuro	Estatus: LC Pob.: en disminución	LC De interes especial	LC	
Hyla arborea	Anuro	Estatus: LC Pob.: en disminución	LC De interes especial	V	
Rana iberica	Anuro	Estatus: NT Pob.: en disminución	V A2ce	LC	V
Rana perezi	Anuro	Estatus: LC Pob.: estable	LC	LC	Protección Especial
Rana temporaria	Anuro	Estatus: LC Pob.: estable	LC De interes especial		
Salamandra salamandra	Urodelo	Estatus: LC Pob.: en disminución	LC		Protección Especial
Mesotriton alpestris	Urodelo	Estatus: LC Pob.: en disminución	V	V A1ac+2c	
Lissotriton boscai	Urodelo	Estatus: LC Pob.: estable	LC De interes especial		
Lissotriton helveticus	Urodelo	Estatus: LC Pob.: estable	LC De interes especial		
Triturus marmoratus	Urodelo	Estatus: LC Pob.: en disminución	LC De interes especial	LC	

ESPECIES ESTUDIADAS

Las principales especies estudiadas en esta Tesis son:

Alytes obstetricans

Se trata de un sapo de pequeño tamaño, generalmente menos de 6 cm, perteneciente a la familia Alytidae (Fig. 3A). Su área de distribución comprende parte de Suiza, Bélgica y

Alemania, casi toda Francia y gran parte de la Península Ibérica. Su alimentación se basa principalmente en lombrices y artrópodos en su vida adulta y vegetales, carroña y larvas de insectos en su fase larvaria.

Es conocido como sapo partero porque es el macho el potador de los huevos, exhibiendo cuidado paterno de los descendientes. Una vez realizada la fertilización externa de los huevos, que quedan unidos por un cordón, la hembra se los cede al macho. Éste los acumula y los sujeta a su parte posterior, entre sus patas traseras. Un solo macho puede llevar las puestas de varias hembras diferentes. Tras 30 días, se introduce en el agua para liberar a las larvas. Pueden pasar largo tiempo en fase larvaria, llegando a alcanzar hasta 8 cm. Suele transcurrir un año hasta experimentar la metamorfosis.

Bufo bufo

También conocido como sapo común, es un anfibio de gran tamaño (Fig. 3B). Los machos alcanzan 8 cm y las hembras 13 cm. Sus larvas son pequeñas y alcanzan un máximo de 35 mm. Se trata del anuro con mayor distribución Paleártica, que se encuentra en casi toda Europa, excepto Irlanda y la mayor parte de las islas mediterráneas. Las larvas son detritívoras y los adultos se alimentan de pequeños invertebrados.

Son fieles a los puntos de agua que utilizan para reproducirse año tras año, produciendo una elevada cantidad de huevos que depositan en cordones en plantas acuáticas o en los fondos. Sus larvas eclosionan entre 5 y 15 días tras la fertilización y la metamorfosis tiene lugar a los 2 a 4 meses.

Discoglossus galganoi

Este sapo de pequeño tamaño, entre 4,5 y 7,5 cm, es conocido como sapo pintojo y también pertenece a la familia Alytidae (Fig. 3C). Se trata de un endemismo ibérico. Su área de distribución comprende Portugal y la mitad occidental de la España peninsular. Se alimentan de artrópodos, moluscos y oligoquetos. El apareamiento tiene lugar en el agua, durante el verano, las hembras depositan los huevos en el fondo de las charcas, que eclosionan a los diez días, y la metamorfosis la efectúan a los 20-55 días.

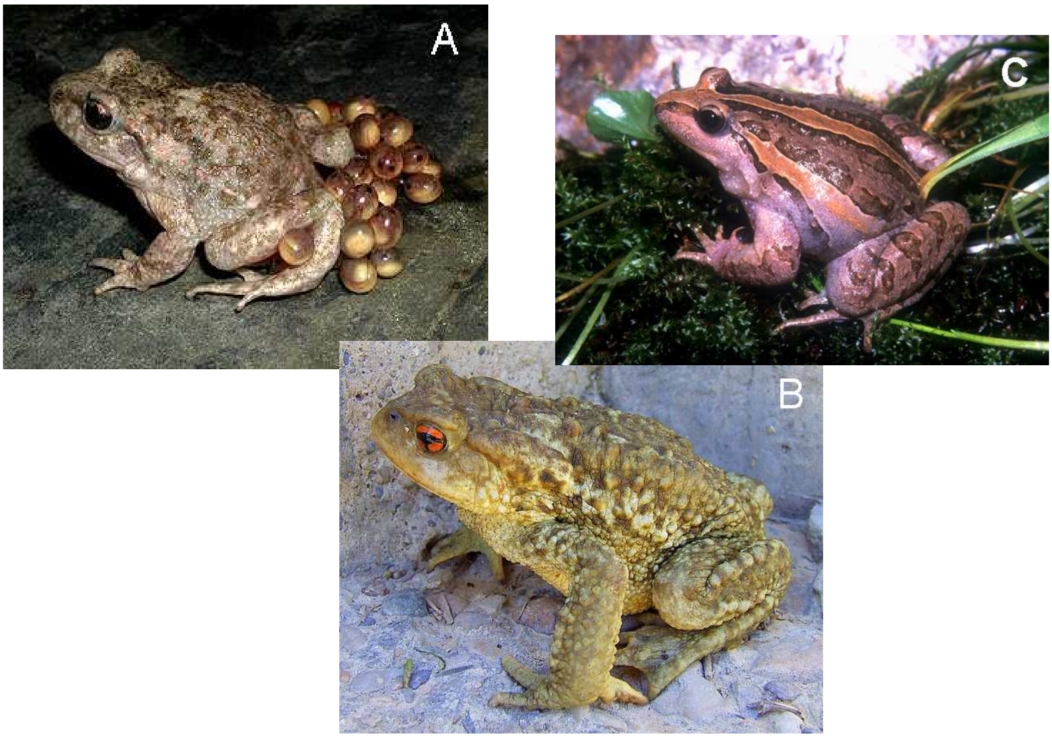


Figura 3. A) *Alytes obstetricans*, B) *Bufo bufo* y C) *Discoglossus galganoi*. Imágenes A y C tomadas de *La enciclopedia virtual de los vertebrados españoles* (http://www.vertebradosibericos.org) y la imagen B fue tomada de la web http://www.sekano.es.

Lissotriton boscai

Conocido como tritón ibérico, se trata de un endemismo de la mitad noroccidental de la Península Ibérica (Fig. 4A). Pertenece a la familia Salamandridae, tiene tamaño pequeño, entre 7 y 10 cm, y su vientre presenta una coloración anaranjada o rojiza. Se alimenta de gusanos, insectos y pequeños crustáceos. Su período reproductivo se extiende de noviembre a julio, de manera más temprana hacia el sur de su distribución. La puesta de huevos tiene lugar en el agua, de uno en uno, y la hembra los va pegando a la vegetación acuática ayudándose de sus miembros inferiores. En algunas poblaciones, los tritones tienen actividad terrestre manteniendo también una fase acuática asociada a la reproducción, mientras que en otras poblaciones se pueden observar individuos en fase acuática durante todo el año.

Lissotriton helveticus

Se trata también de un tritón de tamaño pequeño, entre 7,5 y 9,2 cm, perteneciente a la familia Salamandridae, con el vientre amarillento o anaranjado (Fig. 4B). Se distribuye por casi toda Europa occidental y en la Península Ibérica se encuentra en toda la franja norte. Los adultos se alimentan principalmente de artrópodos y crustáceos. Las larvas se alimentan de crustáceos, aunque también pueden consumir larvas de dípteros, e incluso larvas de urodelos, incluidas las de su misma especie. Es comúnmente conocido como tritón palmeado por la forma de sus patas traseras, ya que en la fase de celo el macho presenta las patas posteriores palmeadas, y el extremo terminal de la cola finaliza en un delgado filamento de unos 3 a 5 mm.

La reproducción tiene lugar en al agua entre los meses de febrero y mayo, y en la puesta cada huevo es envuelto individualmente en la vegetación acuática. La metamorfosis dura unos 4 meses, o más en zonas altas. Por lo general, abandonan el agua entre junio y agosto, una vez transcurrido el período reproductor, pero en regiones de clima suave algunos ejemplares adultos pueden permanecer en el agua mucho más tiempo, incluso durante todo el año.

Triturus marmoratus

También perteneciente a la familia Salamandridae, se trata de un tritón de mayor tamaño que los anteriores, entre 11 y 16 cm (Fig. 4C). Su coloración de fondo es negra con jaspeado verde, de ahí que se le conozca como tritón jaspeado. Durante el periodo de celo los machos presentan una cresta dorsal muy desarrollada con líneas verticales blancas, negras y anaranjadas. La hembra carece de cresta dorsal y presenta un pequeño surco en su lugar de color amarillento anaranjado. Se haya presente en el norte, centro y este de la Península Ibérica, centro y oeste de Francia y en la costa bretona. Se alimentan de larvas de insectos, caracoles, babosas y gusanos. La reproducción tiene lugar en el agua y los huevos son depositados de forma individual en la vegetación sumergida doblando las hojas si es posible.

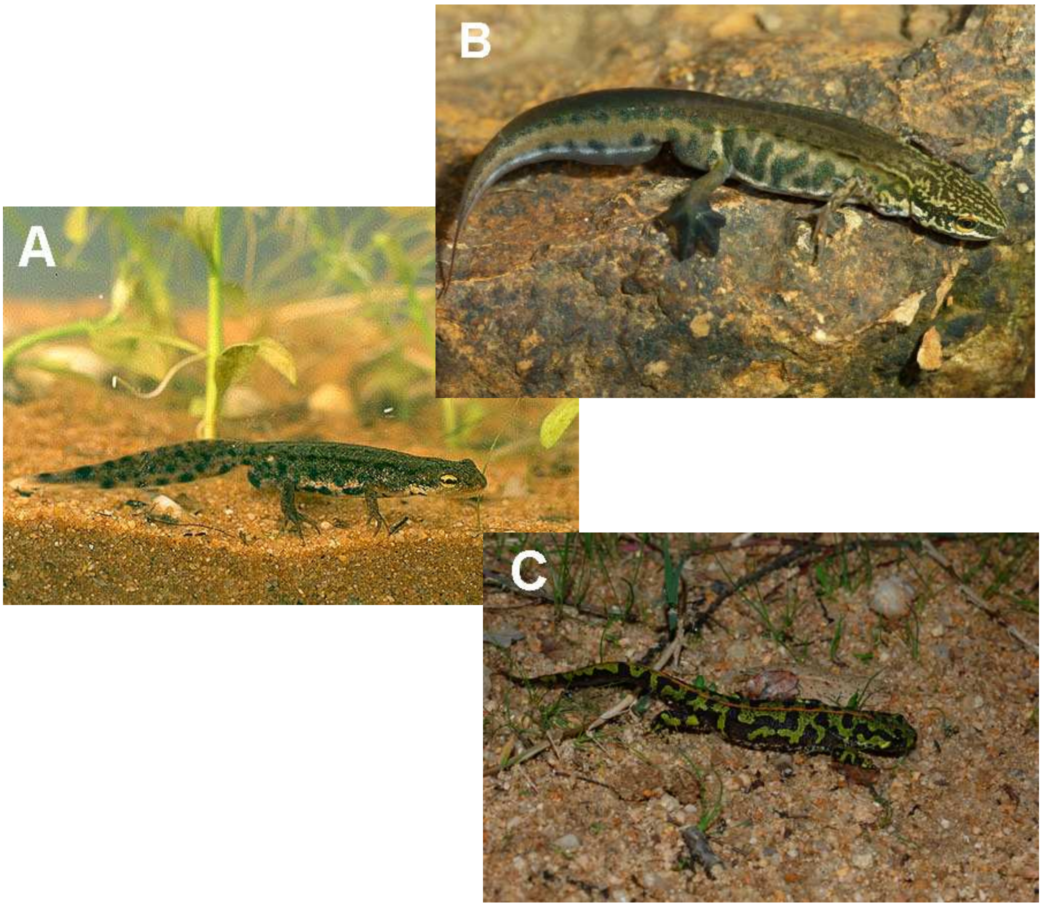


Figura 4. A) *Lissotriton boscai*, B) *L. helveticus* y C) *Triturus marmoratus*. Imágenes tomadas de *La enciclopedia virtual de los vertebrados españoles* (http://www.vertebradosibericos.org).

TIPO DE MUESTREO

Se obtuvieron muestras tanto de urodelos adultos como de larvas de anuros y urodelos. Se trató de buscar el método que fuera menos invasivo; para ello se tomó un pequeño fragmento de cola de 1 mm para la extracción de ADN. Este método de muestreo fue escogido por ser el menos invasivo, debido a la rápida capacidad de regeneración de la cola de adultos y larvas (Arntzen *et al.*, 1999). Las muestras de tejido se obtuvieron con ayuda de tijeras y pinzas de disección esterilizadas para evitar la dispersión de enfermedades, y se conservaron en etanol absoluto hasta su análisis en el laboratorio.

EXTRACCIÓN DE ADN

La extracción de ADN total se realizó empleando la resina Chelex® (Bio-Rad Laboratories), siguiendo el protocolo descrito por Estoup *et al.*, 1996. Este método consiste en añadir al fragmento de tejido 500 µl de una solución de esta resina al 10% y 7 µl de proteinasa k (20 mg/ml). Se incuba durante una hora a 55 ºC, agitando cada 10 minutos. Finalmente, se inactiva la proteinasa incubando en un horno a 100 ºC durante 20 minutos. El ADN queda flotando en el sobrenadante y la resina precipita en el fondo del tubo. Para la conservación de las muestras, a corto plazo se guardan a 4 ºC y a largo plazo a –20 ºC.

AMPLIFICACIÓN DE ADN

La amplificación mediante PCR fue llevada a cabo en un volumen final de 40 µl, conteniendo 50 ng de ADN, Promega Buffer 5x, 250 µM de cada dNTP, 2.5 mM de MgCl, 1 µM de cada cebador, 1 unidad de Taq ADN polimerasa de Promega y completando hasta el volumen final con agua bidestilada.

La reacción de amplificación se llevó a cabo en un termociclador GeneAmp® PCR System 2700 (Applied BioSystem). El programa estándar de amplificación fue de 5 minutos a 95º C, seguido de 35 ciclos de 30 segundos a 95º C, 30 segundos a la temperatura adecuada según cebador (Tabla 2), 30 segundos a 72º C, para finalizar con un periodo de extensión final de 15 minutos a 72º C.

Tabla 2. Características de los cebadores. L y F = directo, H y R = reverso, referencia bibliográfica, secuencia en sentido 5'-3' y temperatura de hibridación (Ta).

Gen	Nombre	Referencia	Secuencia 5'-3'	Ta
16S	16s-L	Palumbi *et al.*,	GCCTGTTTATCAAAAACAT	53º C
	16s-H	1991	CCGGTCTGAACTCAGATCACG	
COI	COI-F	Ward *et al.*,	TCAACCAACCACAAAGACATTGGCAC	58º C
	COI-R	2005	TAGACTTCTGGGTGGCCAAAGAATCA	
Cyt b	Cytb-F	Moritz *et al.*,	GAACTAATGGCCCACACWWTACGNAA	53º C
	Cytb-R	1992	AAATAGGAARTATCAYTCTGGTTTRAT	

Para la especie *T. marmoratus* no se obtuvieron amplificaciones positivas con los cebadores para COI descritos por Ward *et al.* (2005). Un nuevo conjunto de cebadores fue diseñado para esta especie de la misma región COI, empleando el programa Primer 3 v.0.4. (Rozen y Skaletsky, 2000). BioEdit software (Hall, 1999) fue empleado para alinear nuestras secuencias COI de anfibios y la secuencia completa del gen COI de *T. marmoratus* de GenBank EU880337.1 y de este modo seleccionar la misma región de la COI para todas las especies de anfibios. Los cebadores diseñados fueron los siguientes COI Tmar-F (5’-TCATAAAGATATTGGCACCCTCT-3’) y COI Tmar-R (5’-AAGAATATAGACCTCGGGGTGA-3’), con temperatura de hibridación a 59º C.

PURIFICACIÓN DE ADN Y SECUENCIACIÓN DE FRAGMENTOS

Los productos de PCR fueron separados mediante electroforesis en geles de agarosa al 2% conteniendo bromuro de etidio en tampón TBE (45 mM Tris, 45 mM ácido bórico, 1 mM EDTA) y visualizados bajo luz U.V. Las bandas correspondientes al tamaño de amplificación esperado fueron cortadas y congeladas hasta su purificación, que se realizó mediante el kit Wizard® SV Gel and PCR Clean-Up System (Promega).

Los productos de la purificación fueron cuantificados y se realizó la reacción de pre-secuenciación usando el Kit Big-Dye 3.1 Terminator (Applied Biosystems). La electroforesis capilar fue llevada a cabo por el Laboratorio de Secuenciación que forma parte de la Unidad de Citometría y Secuenciación de los Servicios Científico-Técnicos de la Universidad de Oviedo, en un secuenciador automático ABI PRISM 3130 Genetic Analyzer (Applied Biosystems).

MARCADORES GENÉTICOS EMPLEADOS

Las características que presenta el ADN mitocondrial hacen que sea una de las herramientas más adecuadas para la comprensión de los procesos evolutivos y las relaciones entre organismos, tanto a nivel interespecífico como intraespecífico (Cook *et al.*, 2002; Hurwood *et al.*, 2003). Para el estudio de la variabilidad genética se han elegido tres secuencias de genes mitocondriales:

16S: Gen del RNA ribosómico 16S.

Este marcador genético fue utilizado para la asignación de especie siguiendo los criterios establecidos por Hebert *et al.* (2003). Es un marcador suficientemente variable para discriminar entre especies, pero suficientemente conservado para ser menos variable dentro de especies que entre especies. La zona de unión a los cebadores está lo bastante conservada para no obtener falsos negativos de las amplificaciones. Es suficientemente informativo filogenéticamente para asignar las especies a los principales taxones usando aproximaciones filogenéticas simples y es repetible en la amplificación y secuenciación. Es posible el alineamiento de secuencias pertenecientes a taxones filogenéticamente distantes.

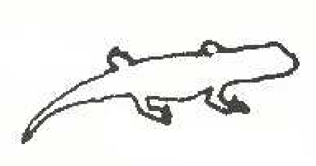
Basándose en estos criterios, se considera al gen 16S como el más indicado para la identificación de especie en anfibios (Vences *et al.*, 2005). Esta técnica es necesaria para clasificar correctamente las muestras de juveniles, cuya morfología no permite identificar la especie visualmente en muchos casos.

COI: Gen de la citocromo oxidasa c, subunidad I.

Para el análisis poblacional se eligió este gen por ser ampliamente utilizado en análisis filogenéticos y poblacionales (Weisrock *et al.*, 2006). Se caracteriza por tener una tasa de sustitución relativamente alta, aunque ésta es variable en función del organismo estudiado. Además, este gen fue el elegido por la iniciativa internacional Barcoding (Hebert *et al.*, 2003), que se basa en el uso de una región estandarizada para catalogar a todas las especies.

Cyt b: Gen citocromo b.

Para completar los análisis poblacionales también se analizó este gen, que ha sido ampliamente empleado en estudios poblacionales y filogenéticos de anfibios (Alexandrino *et al.*, 2000; Vences *et al.*, 2003; Glaw and Vences, 2006).



Resultados

Capítulo 1. Garcia-Gonzalez C, Garcia-Vazquez E. 2011. The value of traditional troughs as freshwater shelters for amphibian diversity. *Aquatic Conservation* **21**: 74-81.

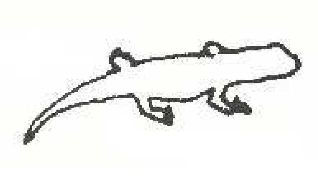
Capítulo 2. Garcia-Gonzalez C, Garcia-Vazquez E. 2011. Urban ponds, neglected Noah’s ark for amphibians. *Journal of Herpetology*. In press.

Capítulo 3. Garcia-Gonzalez C, Garcia-Vazquez E. Amphibian metagenomics, a new approach for assessing the ecological value of anthropogenic habitats. *Biodiversity and Conservation*. Under revision.

Capítulo 4. Garcia-Gonzalez C, Campo D, Pola IG, Garcia-Vazquez E. 2012. Rural Road Networks as Barriers to Gene Flow for Amphibians: Species-Dependent Mitigation by Traffic Calming. *Landscape and Urban Planning* **104**: 171-180.

Capítulo 5. Garcia-Gonzalez C, Garcia-Vazquez E. Species-dependent impact of rivers on amphibian population connectivity at regional scale: implications for conservation. *Landscape Ecology*. Under revision.

Capítulo 6. Garcia-Gonzalez C, Garcia-Vazquez E. 2011. Reasonable Economic Costs of Amphibian Conservation in Urban Environments: A Case Study in North Spain. *Human Ecology* **39**: 807-812.



Capítulo 1

AQUATIC CONSERVATION: MARINE AND FRESHWATER ECOSYSTEMS

Aquatic Conserv: Mar. Freshw. Ecosyst. **21**: 74–81 (2011)

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The value of traditional troughs as freshwater shelters for amphibian diversity

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ABSTRACT

1. Changes in agricultural activities in the Trubia valley (North Iberia, south Europe), mainly substituting husbandry by rural tourism, are modifying trough function: from cattle watering to abandonment or transformation into decorative elements. In the valley, natural ponds are disappearing by desiccation and are scarce or absent.

2. In the present troughs, constructed by artificial deviation of small streams or fountains, eight amphibian species have been found, representing 57% of the amphibian species recorded in the region. Seven of them were in larval development stages.

3. Moderate management, presence of sediments in the bottom and abundant floating vegetation were identified as the main factors contributing to amphibian species richness. Trough management significantly correlated with species richness and also with the presence and the genetic population variation of the model species *Lissotriton helveticus* (palmate newt). Most amphibian species cannot develop in excessively cleaned troughs devoid of vegetation and invertebrates.

4. In the absence or decline of natural ponds and other water points, traditional troughs can be considered refuges for amphibians. Traditional management actions such as moderate cleaning and protecting vegetation and bottom sediments from these freshwater points are recommended. Promoting the traditional management of troughs is easy and, together with the protection of natural ponds, can help to prevent the decline of amphibian species.

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KEY WORDS: agricultural change; amphibians; conservation; freshwater points; management; species richness; troughs

INTRODUCTION

Amphibians are considered to be the most threatened vertebrates under the current conditions of climate change and human modifications of their natural habitats (Stuart *et al.*, 2004; Beebee and Griffiths, 2005). From habitat fragmentation (Cushman, 2006) to increased desiccation and predation risks (Rohr and Madison, 2003), threats to amphibians have increased in recent decades. Identification and protection of key sites for their reproduction is crucial for their survival, in recognition that terrestrial habitats associated with isolated wetlands are essential elements for enhancing biodiversity (Marsh and Trenham, 2001; Gibbons, 2003). Among these reproduction sites, some artificial facilities, such as farm ponds (Beebee, 1997; Pechmann *et al.*, 2001; Knutson *et al.*, 2004; Hartel *et al.*, 2009), have been identified as suitable habitats for amphibian reproduction. Most of these habitats are generally associated with traditional agriculture and husbandry and their persistence may be endangered by changes in land use to modern agriculture.

In the south European Atlantic Iberian region (Figure 1), warming temperatures are significantly affecting climate-sensitive species such as Atlantic salmon (Valiente *et al.*, 2010). Amphibians, which depend on freshwater habitats, are also at risk in this area, where their breeding habitats are mostly temporary ponds. This region contains the greatest diversity of amphibians in southern Europe with 14 species recorded (Table 1). The region is also interesting because it represents the eastern edge of some Iberian endemic species (*Lissotriton boscai*, *Chioglossa lusitanica*) and the western edge of the central-European species *Mesotriton alpestris*.

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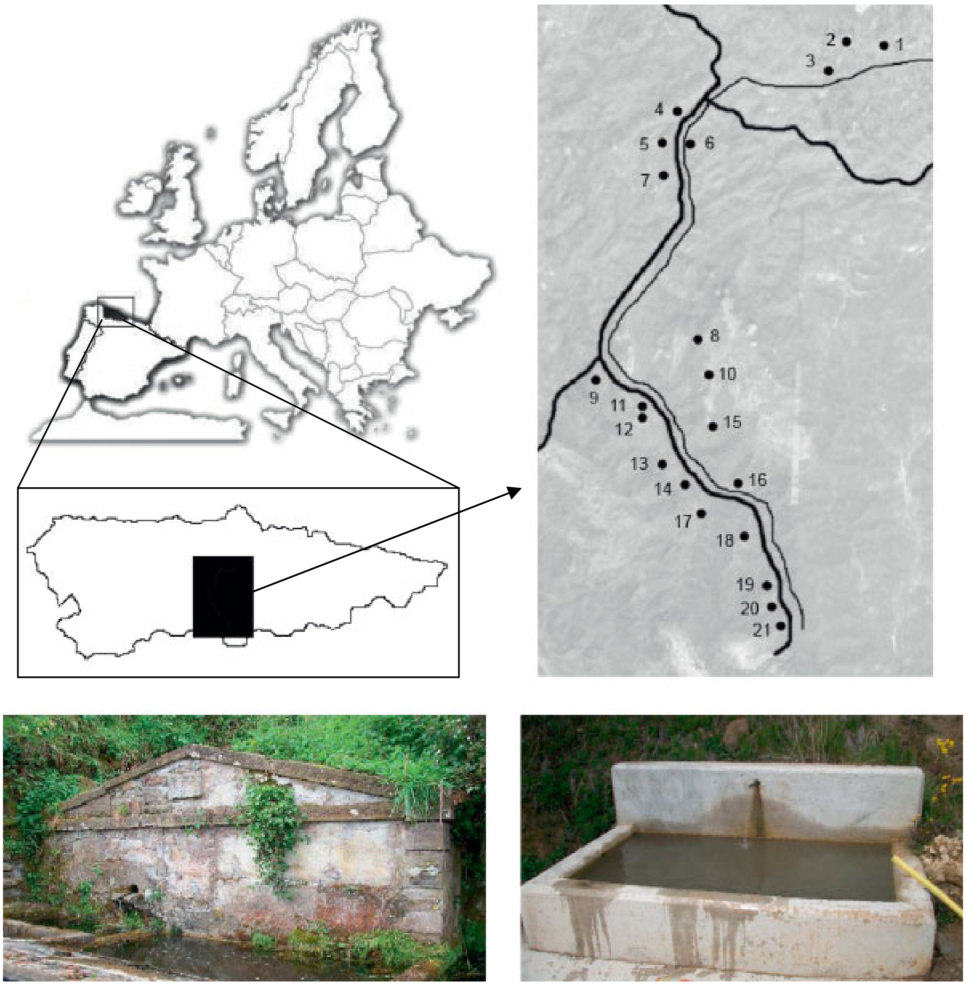

Figure 1. Map of the area studied in north-west Spain, showing the situation of the Trubia valley in the region of Asturias and the location of the 21 troughs with amphibians. Rivers are marked in black and rural roads in grey. 1, Pista W; 2, Pista E; 3, Olivares; 4, S. Andres; 5, Tuñon; 6, Sabadia; 7, Villanueva; 8, Aciera; 9, Villaorille; 10, Bermiego; 11, Toriezo Bajo; 12, Toriezo Alto; 13, Fresnedo; 14, Coañana; 15, Salcedo; 16, Rano; 17, Villamarcel; 18, Cienfuegos; 19, Cortes; 20, Lindes; 21, Carrilon. Below the maps, photographs of a trough managed in a traditional (left) and a modern (right) way in the region.

Table 1. Amphibian species recorded in the region studied (Martinez-Solano *et al.*, 2001), IUCN categories (v.2010.1) and presence in this study (Trubia valley, 6.6% surface of the Asturian region)

Species	Taxonomy	IUCN Status/Population trend	Presence
Alytes obstetricans	Anuran	Status: Least Concern Pop. trend: decreasing	Yes
Bufo bufo	Anuran	Status: Least Concern Pop. trend: unknown	Yes
Epidalea calamita	Anuran	Status: Least Concern Pop. trend: decreasing	No
Chioglossa lusitanica	Urodeles	Status: Vulnerable B2ab(ii,iii,iv) Pop. trend: decreasing	No
Discoglossus galganoi	Anuran	Status: Least Concern Pop. trend: decreasing	No
Hyla arborea	Anuran	Status: Least Concern Pop. trend: decreasing	No
Rana iberica	Anuran	Status: Near Threatened Pop. trend: decreasing	No
Rana perezi	Anuran	Status: Least Concern Pop. trend: stable	No
Rana temporaria	Anuran	Status: Least Concern Pop. trend: stable	Yes
Salamandra salamandra	Urodeles	Status: Least Concern Pop. trend: decreasing	Yes
Mesotriton alpestris	Urodeles	Status: Least Concern Pop. trend: decreasing	Yes
Lissotriton boscai	Urodeles	Status: Least Concern Pop. trend: stable	Yes
Lissotriton helveticus	Urodeles	Status: Least Concern Pop. trend: stable	Yes
Triturus marmoratus	Urodeles	Status: Least Concern Pop. trend: decreasing	Yes

Pop. trend: population trend.

In addition to climate threats, northern Iberia is in a process of accelerated social change owing to prolonged economic recession and decreasing population. Social and economic shifts threaten the traditional rural communities in this area. One of the consequences of these changes is the abandonment of traditional agricultural practices (Dopico *et al.*, 2009), with enormous impact on the biodiversity of farmlands. Natural pools and wetlands are disappearing, dried or destroyed, with subsequent loss of suitable habitats for amphibian reproduction. Although ponds used for livestock watering may show reduced amphibian reproductive success (Knutson *et al.*, 2004), troughs artificially constructed for cattle watering may represent the only permanent water points available as breeding sites of amphibians in some parts of the region.

The main objective of the present study was to determine the importance of traditional troughs for amphibian conservation. For this purpose amphibians present in these constructions in a valley in the northern Iberian Peninsula were surveyed and sampled. The Trubia valley was chosen as a case study because it is in the central region of northern Iberia and possesses a rural profile with traditional husbandry and agriculture (Dopico *et al.*, 2009). Trough characteristics that favour amphibian diversity were identified for recommending management actions aimed at amphibian conservation.

MATERIALS AND METHODS

Socioeconomic evolution of the studied region

The study site was the River Trubia valley (*ca* 700 km^2), located in the central part of the Asturian region in northern Iberia (Figure 1). Major socioeconomic changes have taken place in the region in the last three decades. The sectors traditionally considered in official employment statistics in Spain are agriculture and fisheries, industry, construction and services. For a more detailed analysis of the type of changes occurring within the agricultural sector, recent socioeconomic evolution over the last decade was studied focusing on two indicators: the official number of livestock per year as an indicator of husbandry activity, and the number of person–nights in rural hotels per year as an indicator of tourism-based economic activity. The data source was the official Regional Institute of Statistics SADEI (www.sadei.es, accessed in July 2009).

Trough inventory and features

The Trubia valley was thoroughly explored in a first visit, following the pathways and roads previously visualized in maps and photographs (SIGPAC, page http://sigpac.princast.es/visor/, last accessed July 2009) and walking private areas with the owner's permission. The objective was to identify and record all natural or artificial permanent ponds, small water points and troughs. Twenty-seven points were recorded, 21 of them with amphibians. Both ancient and modern troughs were considered. Modern troughs are constructed with the same structure and location as traditional troughs and are equally accessible to amphibians.

The following features were recorded:

- Altitude, as metres (m) above sea level at which they are located.
- Size, estimated by surface area (cm^2) and depth (cm).
- Type of substrate (rocky or sedimentary — mud on the bottom).
- Distance to the nearest trough (km).
- Vegetation (floating, emerged, submerged and total), measured as a percentage of trough covered.
- Presence (1)/absence (0) of amphibian predators (dragonfly larvae genus *Aeshna* and water beetles of the family Notonectidae; fish were not found).
- Type of management, classified by intensity as 0 (no management, that is, troughs abandoned), 1 (moderate management including vegetation control and surface cleaning once a year, generally conserving sediments — this is the traditional type of management) and 2 (intensive management with periodic cleaning, disinfection and removal of substrate sediments).
- Terrestrial connectivity for amphibian adults between adjacent troughs, determined by physical barriers to migration such as roads and rivers, as 0 (trough isolated by physical barriers), 1 (trough partially isolated, potentially connected with other troughs only in one direction), and 2 (trough bi-directionally connected with their two neighbouring troughs).
- Species richness using the program SPECRICH (Burnham and Overton, 1979), which provides a means of estimating species richness taking into account variation in species detectability.
- Number of species sampled.
- Abundance of amphibians (total number of specimens in a trough).
- Presence (1)/absence (0) of each species (always considering all stages: eggs, larvae and adults).

Sampling methodology

All the troughs were sampled in autumn 2007 (October, November and December), spring 2008 (April) and summer 2008 (July). Dipnet surveys were conducted. New clean fine pore nets were employed in each trough to prevent the spread of disease and parasites. Blind sweeps were carried out, covering the trough systematically and exhaustively. One individual of each invertebrate species was photographed for taxonomic confirmation. The amphibian specimens were photographed and re-released into the trough after sampling. For specimens that could not be identified or that were very small, a fragment of tail approximately 1 mm long was cut and collected for identification by genetic analysis. Tail fragment samples were also obtained from all *Lissotriton helveticus* found (larvae and adults). Tissue samples were stored in ethanol at room temperature until analysis.

Molecular analysis

DNA extraction was carried out following the protocol described by Estoup *et al.* (1996). For species identification and for the study of genetic diversity in *Lissotriton helveticus*, the mitochondrial genes cytochrome oxydase I (COI; Ward *et al.*, 2005) and 16S rDNA (Palumbi *et al.*, 1991) were chosen for genetic identification of small young larvae and eggs. PCR amplification of partial 16S rDNA and/or the COI gene provided accurate and unambiguous identification. Two markers were used because the 16S rDNA region could not be amplified with the universal primers by Palumbi *et al.* (1991) for *Mesotriton alpestris*, nor could the COI with primers for *Triturus marmoratus* and *Rana temporaria* samples (Ward *et al.*, 2005).

PCR amplification was performed in a GeneAmp® PCR System 2700 (Applied Biosystems, Foster City, USA) in a total volume of 40 µL containing 50 ng DNA, 5 × Go Taq® Flexi Buffer (Promega), 250 µmol L^{-1} of each dNTP, 2.5 mmol L^{-1} MgCl, 1 µmol L^{-1} of each primer (direct and reverse), 1 unit of

Go Taq® Polymerase (Promega) and bidistilled water. The PCR amplification programme was: initial denaturing at 95°C for 5 min; then 35 cycles of: 95°C for 30 s, 30 s annealing of primers at 53 or 58°C for 16S rDNA and COI gene, respectively, and 30 s at 72°C; and a final extension at 72°C for 15 min. PCR products were separated by electrophoresis in 2% agarose gels with ethidium bromide in TBE buffer ($45\,\mathrm{mmol\,L^{-1}}$ Tris, $45\,\mathrm{mmol\,L^{-1}}$ boric acid, $1\,\mathrm{mmol\,L^{-1}}$ EDTA) and viewed under UV light. The amplified bands were excised from the gel and DNA was purified using the kit Wizard® SV Gel and PCR Clean-Up system. The purified products were quantified and pre-sequencing reactions were performed using the kit Big-Dye 3.1 Terminator system. Sequencing was performed in an ABI Prism 3100 Genetic Analyzer (Applied Biosystems) at the Sequencing Unit of the University of Oviedo.

Chromatograms were viewed and sequences edited using the BioEdit Sequence Alignment Editor software (Hall, 1999). Sequences were aligned with the application ClustalW (Thompson *et al.*, 1994) and compared with the GenBank database (http://www.ncbi.nlm.nih.gov/GenBank) using the program BLASTn (Basic Local Alignment Search Tool).

Statistical analysis

Principal component analysis was carried out using the PAST (PAlaeontological STatistics, version 1.90; Hammer *et al.*, 2001) software. The software was employed for finding hypothetical variables (components) which account for variance in multidimensional datasets of trough characteristics. The data were analysed with default settings and the 'correlation matrix' option. Only those components not significantly auto-correlated ($P > 0.01$, $|r| \geq 0.8$; Bowerman and O'Connell, 1990) were considered for the PCA. For identifying correlated factors, Pearson's r-values and their significance were calculated using the Microsoft Excel software (XP version).

For a model species vulnerable to trough management (*Lissotriton helveticus*) the following parameters of genetic diversity were calculated: haplotypic (Hd) and nucleotidic (π) diversity. They were calculated within troughs and in the whole region studied, using the program DnaSP version 4.50.3 (Rozas *et al.*, 2003).

The effect of trough management on amphibian diversity in the region was estimated by removing different types of troughs from the dataset (troughs with intensive management type 2, or troughs with moderate management type 1, or troughs without management type 0) and recalculating the following parameters for the remaining sites: amphibian abundance (1, 2, 3, 4 and 5 based on the number of amphibian counts, up to 25, 26–50, 51–75, 76–100 and >100 specimens, respectively), species richness, number of species, presence of each species, genetic diversity of the model species *L. helveticus* (number of haplotypes, haplotype diversity and nucleotide diversity).

To assess the effects of management of amphibian presence, abundance and richness, a generalized linear model (GLM) was used. A model with binomial errors was employed for presence of *L. helveticus*; a normal error for amphibian abundance; and a Poisson error for species richness. The data were analysed based on GLMs with the program R version 2.11.1 (R Development Core Team, 2009; www.r-project.org).

RESULTS

Socioeconomic changes in the region

In this region, agriculture experienced a drastic reduction (Figure 2(a)); in only 26 years, the number of employees in the sector decreased from 24 900 in 1981 to only 4700 in 2007 (the last official statistics). In the Trubia valley studied here, the type of socioeconomic activity has recently shifted from agriculture and husbandry to rural tourism (Figure 2(b)). The number of livestock decreased significantly ($R^2 = 0.947$, $y = -15706\,x + 488034$) whereas the occupancy of rural hotels experienced a simultaneous and very significant increase ($R^2 = 0.9441$, $y = 76580x + 116343$).

Amphibian inventory

In the River Trubia valley, natural ponds were not found in this survey. Although of course their occurrence cannot be excluded (there may be temporal freshwater points after rainfall, or small ponds out of sight in dense bush areas), troughs are probably the most abundant permanent water points providing amphibian habitat in the area. Twenty-one of 27 (77.8%) troughs examined in the Trubia valley contained amphibians corresponding to eight species (Table 1). These eight species represent 57% of the amphibian species recorded in the region.

The six troughs that did not contain amphibians had been transformed into decorative elements without fauna and flora;

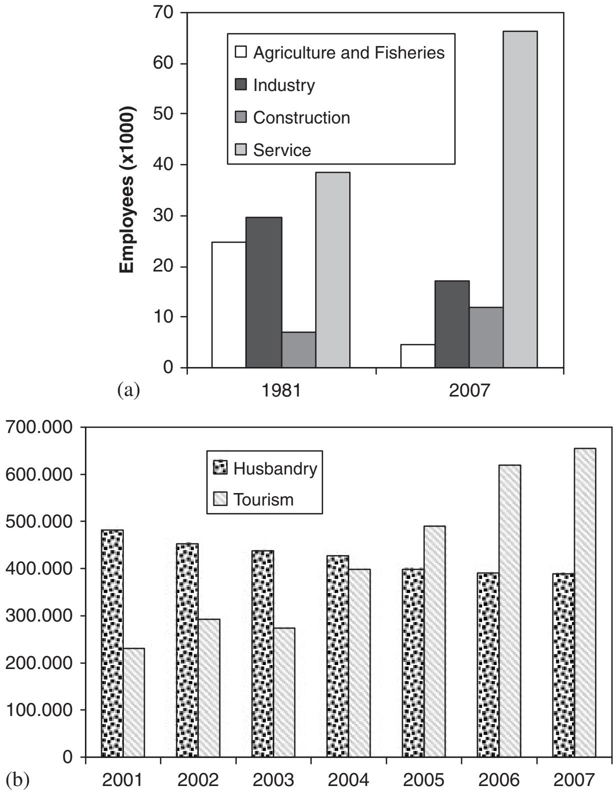

Figure 2. Socioeconomic changes in the region studied. (a) Employment (number of employees × 1000) in different economic sectors in the Asturian region in 1981 and 2007. (b) Change in rural activities in the area studied during the last decade. Husbandry indicator: number of livestock (cattle) registered per year. Tourism indicator: number of person–nights in rural hotels per year.

the 21 troughs with amphibians were still in use for cattle drinking and contained plants and animals. Of these, five troughs were not managed, silted and covered with bush (Table 2). Amphibians were absent only from the six decorative troughs; even the abandoned troughs contained at least one species. Two troughs lost their primary function during this study: Villamarcel and Lindes were cleaned and disinfected to eliminate micro- and macro-organisms, after the first and the second sampling visit, respectively, completely losing all their wildlife.

In the 21 troughs with amphibians 1136 samples were collected. Some of them (*Bufo bufo*, *Salamandra salamandra* and *Lissotriton boscai*) appeared sporadically only in one or two troughs (Table 2).

PCR amplification of partial 16S rDNA and/or COI gene provided accurate and unambiguous identification of eggs and larvae. *Alytes obstetricans* was present in the 21 troughs all year round and in all samples. *Lissotriton helveticus* was the second species both in relative abundance (in 20.3% of troughs) and also in absolute abundance. *T. marmoratus*, *M. alpestris*, *L. boscai*, *Salamandra salamandra* and *Bufo bufo* occurrence was lower (1.23, 0.7, 0.09, 0.18 and 0.09% respectively).

Trough features and relationship with amphibians

Some trough characteristics were significantly correlated with each other: number of species and species richness ($r = 0.937$, $P < 0.001$), and floating and total vegetation ($r = 0.863$, $P < 0.001$). Total vegetation and species richness were eliminated from the PCA to avoid internal autocorrelations.

Six components of the PCA were significant based on the Jolliffe cut-off value (Jolliffe, 1986), providing eigenvalues > 0.7. The first two components together explained half of the total variance (Table 3). The floating vegetation and the number of species composed the first component, which explained more than 26% variance, and the type of trough substrate and the management defined the second axis with 21% variance. Therefore these four characteristics can be considered the most important for explaining trough variation in the region. Submerged vegetation and predators accounted for less variance (13%). The PCA scatter diagram (Figure 3) showed that management intensity was opposite to vegetation, substrate condition and

Table 3. Loadings of trough characteristics on the first six axes extracted by PCA, and the proportion of variance accounted for each axis

Variable	PC axis					
	1	2	3	4	5	6
Altitude				0.4271		
Surface area						
Depth						
Substrate		0.452				
Floating vegetation	0.439				0.4429	
Emergent vegetation					0.6651	
Submerged vegetation			0.5274			
Predators			0.5791			
Management		0.4433				
Connectivity				0.5572		0.6911
Abundance				0.4867	0.424	0.4833
Number of species	0.429					
Proportion of total variance (%)	26.20	21.18	13.88	11.99	6.623	6.13

For clarity, only >|0.40| loadings are listed.

Table 2. Characteristics of the studied troughs

Troughs	Trough characteristics												Amphibian life										
	Alt	Surf	Depth	Bot	Dist	Vf	Ve	Vs	Vtot	Pred	Mng	Con	Richn	N Sp	Abu	Lh	Tm	Lb	Ma	Ss	Bb	Rt
Pista W	288	9000	5	0	1.16	0	0	0	0.0	0	0	2	2.60	2	5	1	0	0	0	0	0	0
Pista E	302	18550	30	0	1.16	0	5	5	3.3	0	2	2	1.05	1	5	0	0	0	0	0	0	0
Olivares	243	10710	26	1	2.07	0	5	15	6.7	0	0	0	2.24	4	2	1	1	0	0	0	0	1
S. Andres	135	16200	26	0	2.95	10	5	30	15.0	0	1	0	2.24	3	2	1	0	0	0	0	0	1
Tuñon	175	15000	40	1	1.18	0	5	10	5.0	0	1	1	2.24	3	4	1	0	0	0	0	0	1
Sabadia	218	13000	37	1	1.18	20	5	60	28.3	0	1	2	5.00	5	3	1	1	1	0	0	0	1
Villanueva	177	18000	40	1	1.72	5	5	90	33.3	0	0	1	3.60	3	1	1	1	0	0	0	0	0
Aciera	650	20800	46	1	1.74	0	0	5	1.7	1	1	1	2.24	3	4	1	0	0	1	0	0	0
Villaorille	361	24200	44	0	1.45	0	0	20	6.7	1	2	1	3.00	2	1	0	0	0	0	0	1	0
Bermiego	668	22000	56	1	2.00	5	25	15	15.0	1	0	1	4.00	4	3	1	0	0	1	0	0	1
Toriezo Bajo	514	15300	45	0	0.07	5	0	20	8.3	1	2	2	1.05	2	5	0	0	0	0	0	0	1
Toriezo Alto	517	45000	55	0	0.07	0	10	15	8.3	1	1	2	1.05	1	5	0	0	0	0	0	0	0
Fresnedo	638	39000	55	0	1.77	10	5	50	21.7	0	2	2	1.05	1	1	0	0	0	0	0	0	0
Coañana	606	12300	50	0	1.56	5	0	30	11.7	0	2	1	1.05	1	2	0	0	0	0	0	0	0
Salcedo	814	16800	40	0	2.44	5	0	10	11.7	0	2	1	1.05	1	3	0	0	0	0	0	0	0
Rano	706	36000	60	0	1.90	5	0	60	21.7	0	1	0	2.24	2	5	1	0	0	0	0	0	0
Villamarcel	773	18000	45	0	1.90	0	0	5	1.7	0	2	0	3.00	2	1	1	0	0	0	0	0	0
Cienfuegos	765	66000	60	0	2.70	70	10	100	60.0	0	1	1	6.00	5	5	1	1	0	1	1	0	0
Cortes	807	16250	90	1	2.27	80	5	10	31.7	1	0	1	4.00	3	3	1	0	0	0	1	0	0
Lindes	928	12675	20	1	0.22	10	0	20	10.0	0	1	1	1.05	1	1	0	0	0	0	0	0	0
Carrilon	961	36800	60	0	0.22	10	1	15	8.7	1	2	1	2.24	3	5	1	0	0	0	0	0	1

Alt, altitude in metres above sea level; Surf, surface in cm^2; Depth in cm; Bot, type of substrate (0 and 1 as rock and mud, respectively); Dist, distance to the nearest trough in km; Vf, Ve, Vs and Vtot as floating, emergent, submerged and total vegetation respectively (as percentage of trough covered). Pred, amphibian predators (0 and 1, absence and presence respectively); Mng, management type (0, 1 and 2 as absent, occasional and intensive cleaning, respectively); Con, connectivity (0, 1 and 2, terrestrial connectivity with none, one or two neighbouring troughs); Richn, species richness; N Sp, number of amphibian species present in the trough; Abu, abundance of amphibians (1, 2, 3, 4 and 5 are up to 25, 26–50, 51–75, 76–100 and >100 amphibian specimens respectively). Lh, Tm, Lb, Ma, Ss, Bb and Rt are *Lissotriton helveticus*, *Triturus marmoratus*, *Lissotriton boscai*, *Mesotriton. alpestris*, *Salamandra salamandra, Bufo bufo* and *Rana temporaria*, respectively (0, absent; 1, present) number of amphibian species which were more abundant in less managed (less cleaned) troughs.

Genetic diversity of the model species *Lissotriton helveticus*

A model species was chosen as representative of vulnerable amphibians inhabiting troughs based on its sensitivity to management, which was one of the main factors contributing to the principal components of the troughs in the region (Table 3, Figure 3). *Lissotriton helveticus* was the only species significantly correlated with management type ($r = 0.569$, $P < 0.05$).

PCR amplification of the COI gene in *L. helveticus* samples yielded a fragment of 671 nucleotides. Ten different haplotypes were found based on nine variable positions. They are available in the GenBank database (Accession Numbers GQ374500 to GQ374509). In the 11 troughs with *L. helveticus* (Table 4), the number of haplotypes ranged from one (San Andres, Villamarcel) to four (Villanueva). Correspondingly, haplotype diversity (*h*) ranged from zero in the locations with only one haplotype, to 0.90 in the most diverse 'Villanueva'. Nucleotide diversity (π) was low for each individual trough, between 0.00 and 0.00234 (Table 4).

Within-trough haplotype diversity was positively significantly correlated with trough connectivity ($r = 0.667$, $P < 0.015$), type of substrate ($r = 0.651$, $P < 0.02$), and submerged vegetation ($r = 0.531$, $P < 0.05$).

Effect of trough management on amphibian diversity

To estimate the effect of each type of management on amphibians, amphibian diversity was recalculated on partial datasets containing different types of troughs (classed according to management intensity). For most indicators (Table 5), absence of troughs managed in a traditional way (moderate cleaning, type 1) would have the greatest impact on amphibians. Abundance, richness and species number experienced the greatest decrease when type 1 troughs were removed from the dataset. Similarly, presence of most newt species was associated with type 1 management; thus, when this type of trough was removed the percentage occurrence in troughs was reduced for all species except for *Bufo bufo*, which appeared only in one type 2 trough. When the most abundant type 2 troughs (intensively managed) were removed, however, little or no effect was found on amphibian diversity because most of them contained only *Alytes obstetricans* (Table 5).

GLM (generalized linear model) analyses revealed a significant correlation between the type of management and

Table 4. Genetic diversity at the mitochondrial CO-I region considered (671 bp), for *Lissotriton helveticus* sampled from the troughs studied

Trough	*n*	Number of haplotypes	Haplotype diversity (h)	Nucleotide diversity (π)
Olivares	19	2	0.52632	0.00079
S.Andres	19	1	0.00000	0.00000
Tuñon	11	3	0.56364	0.00092
Sabadia	3	2	0.66667	0.00099
Villanueva	5	4	0.90000	0.00179
Aciera	22	2	0.52381	0.00234
Bermiego	28	2	0.47619	0.00071
Villamarcel	1	1	0.00000	0.00000
Cienfuegos	77	3	0.57553	0.00096
Cortes	27	2	0.43305	0.00065
Carrilon	5	2	0.40000	0.00060
Total	217	10	0.69289	0.00146

n, number of individuals sampled.

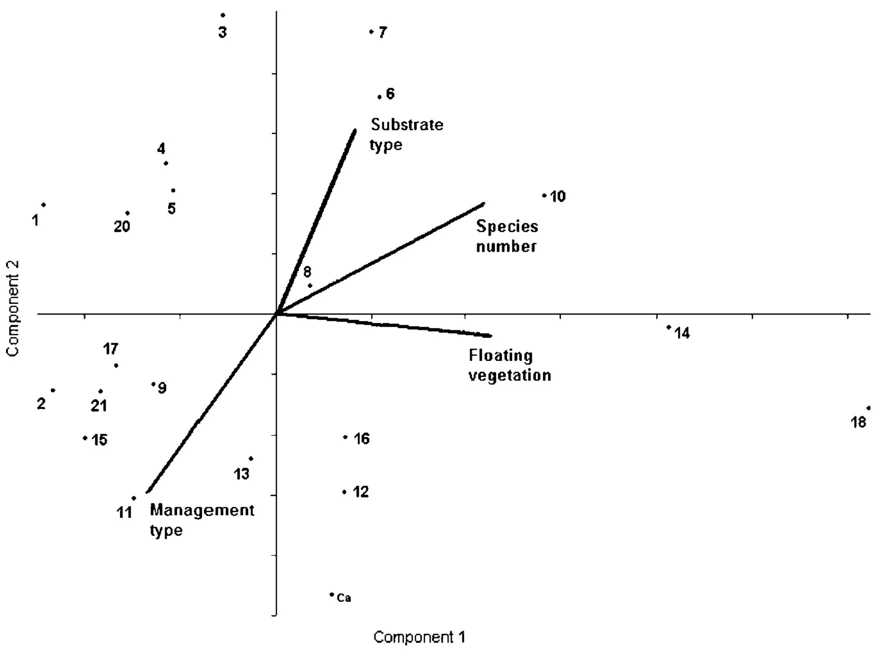

Figure 3. Graph of the dispersion of the troughs analysed with respect to the principal components: floating vegetation, type of management, type of substrate in the trough bottom and number of amphibian species. 1, Pista W; 2, Pista E; 3, Olivares; 4, S. Andres; 5, Tuñon; 6, Sabadia; 7, Villanueva; 8, Aciera; 9, Villaorille; 10, Bermiego; 11, Toriezo Bajo; 12, Toriezo Alto; 13, Fresnedo; 14, Coañana; 15, Salcedo; 16, Rano; 17, Villamarcel; 18, Cienfuegos; 19, Cortes; 20, Lindes; 21, Carrilon.

Table 5. Effect of trough management on amphibian communities

Parameters	All troughs (21)	Without 0 (16)	Without 1 (13)	Without 2 (13)
Abundance	3.143	98.2%	86.0%	100%
Richness	2.477	77.6%	77.7%	100%
Number of species	2.143	74.1%	73.5%	100%
Lh (%)	61.9%	61.6%	53.8%	84.6%
Tm (%)	19.1%	49.8%	49.8%	100%
Lb (%)	4.8%	100%	0%	100%
Ma (%)	14.3%	66.5%	33.3%	100%
Ss (%)	9.5%	50%	50%	100%
Bb (%)	4.8%	100%	100%	0%
L. helveticus diversity				
Number of haplotypes	10	70%	70%	100%
Haplotype diversity	0.6929	98.2%	99.4%	99.7%
Nucleotide diversity	0.0015	100%	86.7%	100%

Results are presented as the total regional value of each parameter, and the percentage of such regional value obtained without troughs subjected to intensive, moderate and absent management (2, 1 and 0, respectively) and the number of troughs studied in each case. Parameters are: mean amphibian abundance, richness and number of species; percentage of troughs with *Lissotriton helveticus, L. boscai, Triturus marmoratus, Mesotriton alpestris, Salamandra salamandra* and *Bufo bufo* (Lh, Lb, Tm, Ma, Ss and Bb, respectively); genetic diversity of *Lissotriton helveticus* as number of haplotypes, haplotype and nucleotide diversity.

the presence of *L. helveticus* ($P = 0.0167$) and species richness ($P = 0.0342$), but not with abundance.

The impact of removing type 1 troughs on the genetic diversity of *L. helveticus* was relatively moderate (Table 5). Three haplotypes (30%) were lost and nucleotide diversity was reduced to 87%. As for general amphibian diversity, removal of type 2 troughs had negligible effect on *L. helveticus* genetic diversity.

DISCUSSION

This study found a significant change in socioeconomic activities in northern Iberia, with traditional constructions such as troughs being converted into decorative constructions, losing their original function of livestock (cattle) watering. Traditional moderate management has been identified as the best option for preserving amphibian diversity in this region rich in amphibians. From these results, the disappearance of troughs bring about an immediate loss of species richness and also a moderate loss of genetic diversity within species.

Intensive management includes removing all vegetation, eliminating substrate sediments and disinfecting troughs for their use as fountains; this has a negative impact on newt species. For example, *Lissotriton helveticus* requires vegetation because females carefully wrap single eggs into leaves of aquatic vegetation in shallow water (Miaud, 1994). The abundance of aquatic vegetation is a recognized predictor of the presence and abundance of newts (Denoël and Lehman, 2006). Clean troughs are not suitable habitat for most amphibians; only the ubiquous *Alytes obstetricans* seems to remain in troughs subjected to intensive management.

Trough management influences not only amphibian diversity but also within-species genetic diversity. The type of substrate (preferentially with mud) and the presence of submerged vegetation are positively correlated with haplotype diversity of *L. helveticus*. Those two features clearly depend upon management type, and are replaced by stone bottoms and clean water in intensively managed troughs. The greatest loss in diversity would occur if traditionally managed troughs (moderate management) disappeared. This suggests that an action as simple as moderate management of troughs could contribute to amphibian conservation in the studied region, mitigating other habitat losses.

Another crucial characteristic is genetic diversity, which increases with connectivity ($r = 0.67$, $P < 0.02$). This result emphasizes the negative effect of habitat fragmentation on amphibians, already reported for newts and other species (Cushman, 2006). Larval stages of *L. helveticus* were found in samples taken in different seasons, because this species reproduces several times per year in this region. This continuity of aquatic forms requires the presence of permanent freshwater points even for species with short development periods, if they reproduce more than once per year.

All the newt species (*L. helveticus, T. marmoratus, M. alpestris* and *L. boscai*) and the anuran *Alytes obstetricans* found in this study are considered of special interest under Spanish environmental laws (Spanish National Catalogue of Threatened Species, R.D. 439/1990). This protection category is applied to species with a particular scientific, ecological or cultural value. In addition, sporadic presence of other species with shorter development, not detected in the present survey, cannot be excluded. Artificial agricultural ponds represent alternative breeding habitats for amphibians (Beebee, 1997; Knutson *et al*., 2004; Denoël and Ficetola, 2008). Although some authors found that pond size positively affects amphibian richness (Parris, 2006), the conservation value of much smaller artificial constructions such as troughs has been clearly shown in the present study. High levels of haplotype diversity, such as 0.9 in Villanueva or 0.6 in Sabadia, occurred in freshwater points as small as $0.8\,m^3$. In fact, trough size was not a principal variance component, and not significantly correlated with amphibian richness or abundance. In agreement with other authors (Snodgrass *et al*., 2000; Oertli *et al*., 2002; Ficetola and De Bernardi 2004; Denoël and Lehmann, 2006; Denoël and Ficetola, 2008) we concluded that small water points can significantly contribute to supporting amphibian diversity.

In the absence of other permanent water bodies, troughs should be considered amphibian shelters. Moderate cleaning to prevent silting of the trough with mud and an excess of vegetation is the traditional way in which Asturian farmers managed troughs. Beja and Alcazar (2003) found negative effects on amphibians following conversion of ponds into irrigation reservoirs or with intensification of agricultural land use. Although preventing the modernization of agriculture is not possible and would be undesirable from a socioeconomic viewpoint, keeping some of the traditional habits, such as using troughs for cattle watering, could be of environmental value. Moderate management is easy and can be adapted to water points occurring in urban areas that can also act as amphibian shelters (Parris, 2006).

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Capítulo 2

Urban ponds, neglected Noah's ark for amphibians

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ABSTRACT

Urban areas can contain habitat that support amphibian populations, such as artificial ponds. We sampled a < 3m pond in the city of Oviedo (North Iberia) and found abundant populations of two anurans (*Alytes obstetricans*, *Discoglossus galganoi*) and one urodele (*Lissotriton helveticus*). The three species present at the urban site exhibited high diversity in their mitochondrial DNA, comparable to levels found for populations in nearby rural areas that contained more and larger water bodies. Our results suggest that urban ponds should be incorporated into amphibian conservation plans.

Key words: *Alytes obstetricans*; Amphibians; Conservation targets; *Discoglossus galganoi*; Diversity; Genetic diversity; *Lissotriton helveticus*; Urban ponds

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INTRODUCTION

Amphibians are perhaps the most endangered group of vertebrates inhabiting wetlands. Their populations are declining worldwide more rapidly than either birds or mammals (Stuart *et al.*, 2004). Most amphibians are exposed to aquatic and terrestrial habitats during their ontogeny. This, plus the fact that most species have highly permeable skin, makes amphibians more sensitive to environmental pollution and changes in patterns of temperature and precipitation than other terrestrial vertebrates. Because the survival of amphibians is closely linked to water availability, anthropogenic alterations of hydrology can trigger mortality events that accelerate current large scale population declines likely due to combined habitat degradation, climate change and disease outbreaks (Beebee, 1995; Berger *et al.*, 1998; Laurance, 1996; Kiesecker *et al.*, 2001). Under these circumstances, all types of freshwater sources and wetlands become valuable and in urgent need of protection.

Urban ponds have rarely been considered priority conservation targets, particularly when they are artificial and have been recently created. Although there is little work on the ecology and landscape ecology of urban ponds (Gledhill *et al.*, 2008), some outstanding studies have highlighted their value as habitats for amphibians (e.g. Gledhill *et al.*, 2008; Parris, 2006; Birx-Raybuck *et al.*, 2009). Amphibians constitute metapopulations (e.g. Cushman, 2006), taking the word "metapopulation" in the broadest sense to refer to a subdivided population that has a spatial structure (e.g. Griffiths *et al.*, 2010), and even very small ponds can contain stable populations of some species (Halley *et al.*, 1996). In areas with high levels of human activity, urban ponds are likely the last available habitat for amphibian species. Their role for preserving between-and within species diversity, however, has not been sufficiently studied to date.

In the present study we calculated between and within species diversity for amphibians in one small urban pool located in the city of Oviedo (North Iberia), and compared results with patterns of amphibian diversity from much larger natural rural areas nearby. Mitochondrial DNA variation at the COI (Cytochrome Oxidase I) gene was employed for measuring within-species genetic diversity in three representative species (*Alytes obstetricans*, *Discoglossus galganoi* and *Lissotriton helveticus*).

MATERIAL AND METHODS

Study sites

The study was conducted in North Iberia, within the region of Asturias (Figure 1). The climate is humid Atlantic temperate, with annual mean temperature of 12.9ºC (last four decades) and average annual rainfall of 973 mm. Ecosystems are typical of the Atlantic Arc region with forest dominated by caducean trees like chestnut (*Castanea sativa)*, oak (*Quercus robur*) and beech (*Fagus sylvatica*) forests, numerous short rivers in deep valleys, and small family farms dedicated to agriculture (corn *Zea mays*, beans *Phaseolus vulgaris*, apple trees *Malus domestica*) and livestock (mainly cows *Bos taurus*, sheep *Ovis orientalis aries*, and horses *Equus ferus caballus*).

The small pond "Charca Pista Finlandesa" (CPF, Figure 1), < 3 m^2 in surface area and 30 cm maximum depth, is located into a small depression within an urban park of approximately 10000 m^2 in Oviedo (GPS coordinates 43º22 N, 541 5º51 W, 723). It was constructed in 2000 and was naturally colonized by amphibians and invertebrates. The pond is surrounded by grass and scattered shrubs. The area surrounding the pond is urban and contains small houses and apartments, a primary school, a restaurant and two historic chapels.

Wetlands from two rural areas near Oviedo (Figure 1) were selected for comparison with the urban pool. These rural areas are located at similar mean altitude as Oviedo and share similar vegetation, clime and geology. Four stable water bodies were sampled within each rural area. A maximum distance of approx. 2 km separated ponds within an area (Figure 1). The distance between the urban pond and the nearest rural area was approx. 13.5 km and the distance between the two rural areas was 10.5 km. Two additional sites, one within each rural area, were sampled to increase the sample size for *D. galganoi*, making a total of six ponds sampled in each area.

Sampling methodology.

CPF and the two rural areas were sampled in autumn 2007 (October, November and December), spring 2008 and 2009 (April), and summer 2008 (July). We conducted dipnet surveys. New clean fine mesh nets were employed during each visit to prevent the spread of disease and parasites. Blind sweeps were carried out, covering systematically and exhaustively each pond. Captured amphibians were photographed and released at the sampling site. For animals that could not be identified or were very small, the tip of the tail (approximately 1 mm long) was removed and preserved for genetic analysis.

We collected tail tips from urodele larvae and adults, and anuran larvae with sterilized forceps and scissors; tail tips were stored in 100% ethanol. Sampled animals were immediately re-released into the pond. Due to rapid growth and regeneration of tail-tips in tadpoles and newts, we chose this sampling method as the least invasive (Arntzen *et al.*, 1999).

Genetic analysis

Total DNA was extracted from a small piece of tail (about 1 mm^3) using a Chelex Resin (Bio-Rad laboratories) following the protocol by Estoup *et al.* (1996) and kept at 4º C prior to analysis.

Unknown larvae were genetically identified using the 16S rDNA or the COI (Cytochrome Oxidase I) gene as barcoding tools, employing the primers and methods described by Palumbi *et al.* (1991) and Ward *et al.* (2005), respectively. Variation in the COI gene was analyzed to estimate genetic diversity within and among sampling sites for the three representative species *Alytes obstetricans*, *Discoglossus galganoi* and *Lissotriton helveticus*.

PCR reactions contains 5x Go Taq® Flexi Buffer, 2.5 mM MgCl, 250 µM of each dNTP, 1 µM of each primer, 1 unit of Go Taq® Polymerase 5U/µl (Promega) and 50 ng of DNA template in a final reaction volume of 40 µl. They were performed in a GeneAmp® PCR system 2700 (Applied Biosystems) with the following conditions: an initial denaturing step at 95 ºC for 5 min, followed by 35 cycles of denaturing at 95 ºC for 30 s, annealing (for 30 s) at 53 or 58ºC for 16S rDNA (Palumbi *et al.*, 1991) or COI gene (Ward *et al.*, 2005) respectively, and an extension at 72 ºC for 30 s, followed by a final extension at 72 ºC for 15 min.

PCR products were visualized in 2 % agarose gels stained with ethidium bromide. Bands were excised from the gel and DNA was purified with a Wizard® SV Gel and PCR Clean-Up System (Promega) before sequencing. Automated fluorescence sequencing was run on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems) with BigDye 3.1 Terminator system, in the Unit of Genetic Analysis of the University of Oviedo (Spain).

Diversity estimates

Amphibian species richness in each area sampled was calculated with the online software SPECRICH (http://www.mbr-pwrc.usgs.gov/software/specrich.html) based on methods described by Burnham & Overton (1979). Diversity was estimated employing the Shannon index (Pielou, 1975; Magurran, 1988), which is widely used to compare diversity between habitats (Clarke & Warwick, 2001). The index was calculated with the program Divers (Franja, 1993) using the formula H'=-Σ pi Ln pi, where pi= proportional abundance of the i-esim species = number of individual of the i-species/total number of individuals.

Both juveniles and adults were sampled from each site, but diversity has to be estimated based on the same life stage (generally adults). As the survival juvenile-adult may vary depending on environmental conditions, diversity estimates were made considering different published survival rates for each species (the lowest and the highest rates) for transforming the number of juveniles into number of adults. The survival rate of juveniles to metamorphose has been estimated 0.64-0.83 for *Alytes obstetricans* in other Spanish regions by other authors (Richter-Boix *et al.*, 2006), and we have applied this survival range for the two Discoglossidae found in our study. The range of juvenile-adult survival published for different newts is 0.22-0.8 (Beebee, 1996). We applied this range for the newt species found in our region.

Within-species genetic diversity indices employed considered both between and within population diversity for COI sequences. Within-population variation was estimated through haplotype diversity (Hd), equivalent to the expected heterozygosity for diploid data (the probability that two randomly chosen haplotypes are different in the sample), and nucleotide diversity (π) (the mean number of nucleotide differences per site between all pairs of haplotypes in the sample), for each population. They were calculated with the program DnaSP v.4.50.3 (Rozas *et al.*, 2003). Population pairwise F_{ST} values measure genetic distance based on genetic polymorphism, and indicate the degree of differentiation between two populations, from panmixia (one single interbreeding population) to completely independent populations. F_{ST} were calculated employing the program ARLEQUIN (Schneider *et al.*, 2000). The same program was employed for carrying out a global exact test of population differentiation, which tests the hypothesis of random distribution of different haplotypes among populations, and is a measure of the global population differentiation based on haplotypic frequencies. This test produces an unbiased p-value estimate for the null hypothesis that the genotypic distribution is the same between two populations using a Markov chain procedure (Goudet et al., 1996) with 99 999 steps, 10 000 of which were burn-in, steps before beginning to reach a random starting point, ('dememorization') steps.

Haplotype networks were chosen for inferring relationships between haplotypes and illustrating their spatial distribution. During evolution there are different rates of replacement between one nucleotide by another. Substitution models employ different parameters to describe the replacement rate expected along a nucleotide sequence. The jModelTest v.3.06 software (Posada, 2008) was employed to determine the model of sequence evolution that best fit our dataset, and to calculate the proportion of invariable sites and the value of the gamma distribution shape parameter, which are necessary for the construction of haplotype networks. Median joining (MJ) networks were constructed with the program Network 4.5.1.0 (http://www.fluxus-engineering.com).

To illustrate the relationship among populations a neighbour-joining tree (Saitou & Nei, 1987) was constructed based on genetic distances using the program MEGA 4 (Tamura *et al.*, 2007), the results obtained with the program jModelTest (cited above) were used to set the sequence evolution model for each species.

Demographic history

To investigate the demographic history of the amphibian populations considered, we employed Tajima's *D* and Fu's *Fs* tests under the assumption of neutrality for the mitochondrial COI region. Both tests measure whether the observed frequencies of segregating mutations are compatible with the frequencies expected under the standard neutral model. The Tajima's *D* is based on the expectation of a constant population size at mutation-drift equilibrium, and is widely used to detect changes in population size (Aris-Brosou & Excoffier, 1996). Very high negative values, caused by an excess of rare haplotypes, may indicate a recent population expansion (Fu, 1997). Tajima's *D* and Fu's *Fs* tests were computed by the program ARLEQUIN (Schneider *et al.*, 2000).

To detect whether the populations have undergone demographic expansion, we compared the observed frequency of pairwise sequence differences (mismatch distribution) to the expected distribution under a sudden expansion model with the SSD statistic (sum of squared differences) using 1 000 bootstrap replicates, in ARLEQUIN. This method assumes that population growth leaves a distinctive signature in the DNA sequences compared with a constant population size. Recent growth is expected to generate a unimodal distribution of pairwise differences between sequences (Roger & Harpending, 1992).

RESULTS

1. Amphibian richness and diversity

In the areas sampled (one urban and two rural) we found six amphibian species: two anurans (midwife toad, *A. obstetricans* and the painted frog, *D. galganoi*) and four urodeles (the newts *Mesotriton alpestris*, *Lissotriton boscai*, *L. helveticus* and *Triturus marmoratus*). Despite its small size, three species were present in the urban CPF: *A. obstetricans*, *D. galganoi* and *L. helveticus*. *D. galganoi* was not found in the rural areas during the first round of sampling and was only encountered after additional sampling effort. CPF had similar species richness as recorded for Rural B. The Shannon index, which characterized amphibian diversity in the three sampling areas, also yielded similar values for CPF and for Rural B (Table 1).

2. Intraspecific genetic variation

2.1. Alytes obstetricans

A 671 bp long COI sequence was obtained for *A. obstetricans* and was submitted to GenBank with the accession numbers HM032694-HM032700. Nine polymorphic sites were found (Table 2A), yielding seven synonymous haplotypes. Both haplotype and nucleotide diversity were higher for CPF than for the two rural areas (Table 3); in fact, the 40 individuals sampled from Rural B were monomorphic. The exact test for global population differentiation was highly significant (P<0.0001). Pairwise comparisons between CPF and the two rural areas showed FST values to be highly significantly different (Table 4), but no significant difference was found between the rural areas.

The sequences analyzed followed a Tamura-Nei evolution model, which gives different probabilities for six different types of nucleotide change. The haplotypes were all derived from one central lineage typical of rural areas (Figure 2A). Differentiation of the urban pond, in terms of haplotype frequencies, was complete for *A. obstetricans*, as no haplotypes were shared with rural areas. This differentiation was confirmed by the neighbour-joining tree derived from genetic distances where the two rural areas appeared within the same cluster, separated from the urban CPF (Figure 3A).

The demographic parameter Tajima'D was negative and significant for Rural A (Table 3), indicating significant variation in population size, although selection can be discarded because all nucleotide substitutions were synonymous. Significant positive SSD values for both CPF and Rural A suggest recent population expansion in the two areas.

2.2. Discoglossus galganoi

PCR amplification with the COI primers by Ward *et al.* (2005) yielded a 671 bp (base pairs) long DNA fragment for *D. galganoi* (GenBank accession numbers GU797351-GU797354). Four polymorphic sites were found within this region (Table 2B). All the substitutions were synonymous and yielded four haplotypes. The three parameters that measured within-population genetic variability yielded higher values for CPF than for the two rural areas (Table 3). As in *A. obstetricans*, the exact test for global population differentiation was highly significant (P<0.0001). Pairwise comparisons indicated that F_{ST} values (Table 4) differed significantly between CPF and the two rural areas, but not between Rural A and B. The neighbour-joining tree revealed the same association between the two rural areas which were clearly different from CPF (Figure 3B).

The COI sequence analyzed for this species followed an evolution model of Kimura, characterized by give two different probabilities for mutational transitions and for transversions. The haplotype variation found for this gene in *D. galganoi* indicated the existence of two main lineages present across the three sites, with one haplotype derived from each of these lineages but present only in CPF (Figure 2B).

The demographic parameters examined were not significant for *D. galganoi*, except SSD in Rural A (Table 3), indicating that populations were increasing only in this area, and stable in CPF and Rural B.

2.3. Lissotriton helveticus

The 671 bp COI sequence obtained (GenBank accession numbers GQ374500-GQ374506, GQ374508-GQ374509, HM032701- HM032705) was polymorphic at 12 sites and identified 14 haplotypes for the *L. helveticus* (Table 2C). As was true for the other two anurans, the urban CPF site exhibited more haplotypes than the rural areas (Table 3), nine versus five (Rural A) and three (Rural B) haplotypes, although haplotype and nucleotide diversities were higher for Rural B. The three pairwise FST values differed significantly between sample sites (Table 4), as well as the global test for population differentiation (P<0.0001), indicating spatial population differences in the region for this species. The neighbour-joining tree revealed an association between CPF and

Rural A, the nearest rural area from the urban site (Figure 3C).

The sequences analyzed followed a Kimura-two parameters evolution model, with a gamma value of 0.011. The haplotype network indicated the existence of one central, common haplotype present in the three sampling areas and other, less frequent haplotypes derived from it through one or two mutational events (Figure 2C). Significant negative Fs values were found for CPF and Rural A (Table 3), a significant negative Tajima's D parameter was obtained for Rural A, and significant, positive SSD values were found for CPF and Rural B, indicating recent demographic expansion at all three sites (Table 3).

DISCUSSION

We have found three amphibian species, two anurans and one urodele, in a very small urban pond. This assemblage represented more than 20% of the 14 amphibian species known from the Asturian North Iberian region (Figure 1), including both high mountain and coastal habitats (Álvarez López, 1927; Martínez-Solano, 2001). Three more species of newts were found in our survey of rural areas. Relatively high species diversity in a recently constructed urban wetland, only 3 m^2, demonstrates the capacity of amphibians from nearby areas to colonize new ponds rapidly as has been reported for rural landscapes (Baker & Halliday, 1999).

The three model species exhibited different levels of within and between-population genetic diversity. In general, the anurans *D. galganoi* and *A. obstetricans* were less variable than *L. helveticus*, which exhibited the twice number of haplotypes as the anurans. Difference in genetic variation could be partially explained by the small sample size of the anurans, which were less abundant than *L. helveticus* in CPF and Rural B. Between-population diversity also exhibited similar patterns of divergence for the two anurans. The two rural samples did not differ from each other. The anurans sampled from the urban pond, however, were significantly different and more variable than those found in rural areas.

Differentiation of the urban pond, in terms of haplotype frequencies, was complete for *A. obstetricans*, as no haplotypes were shared with rural areas. Large scale spatial differentiation of phylogeographic significance has been reported in Iberia for the family *Discoglossidae* (Busack, 1986; Capula & Corti, 1993; Martínez-Solano, 2004a; San Mauro, 2004; Zangari *et al.*, 2006; Gonçalves *et al.*, 2007), and our results point out that spatial differentiation also occurs at much smaller (intra-specific) and even local spatial scale, suggesting fine population structuring. Abundant literature also exists on the phylogeography of the genus *Alytes* on the Iberian Peninsula; five lineages have been described (e.g. Martínez-Solano, 2004b). However, differentiation at such a small scale, less than 20 km, has not been reported previously. It indicates strong population structuring, possibly due to anthropogenic barriers between the urban and rural sites. Barriers associated with urban habitats have promoted genetic population differentiation in other anurans (e.g. Hitchings & Beebee, 1997). Another possible explanation for the occurrence of different haplotypes in a small area, compared to the whole distribution of the species, could be a secondary contact in the area studied, as has been reported for *Alytes cisternasii* (Gonçalves *et al.*, 2009), *Alytes obstetricans* (Martínez-Solano *et al.*, 2004b) and *Discoglossus* (Zangari *et al.*, 2006). In this case, our study sites could be a contact area between two populations that have merged after a period of allopatric differentiation. Our preliminary results require more samples and larger spatial coverage for confirmation.

The spatial pattern of population variation for *Lissotriton helveticus*, with significant differences among the three samples, suggests isolation by distance or an effect of barriers to migration between the two rural zones. Although this can not be generalized based on the limited number of sampling sites of our study, strong effects of habitat fragmentation on newt population persistence (e.g. Gibbs, 1993) and structuring (e.g. Cushman, 2006) have been described elsewhere and our results point in the same direction. As noted for the two anurans, further and extended sampling is needed to confirm the initial observation for *L. helveticus*.

Understanding metapopulation dynamics is a complex objective as they can be influenced by a variety of factors. The maintenance of corridors between urban and rural ponds to promote gene flow among separated wetlands is crucial. Corridors, in appropriate number and arrangement, can offset the negative effect of population isolation (Saunders *et al.*, 1991; Mech & Hallett, 2001). This has been reported for several amphibian species. For example, for the Rocky Mountain tailed frog in Idaho (USA), *Ascaphus montanus*, gene flow among populations in managed forests followed multiple corridors (Spear & Storfer, 2010); and for *Ambystoma maculatum* strong overall connectivity among ponds may be facilitated by a network of riparian corridors (Purrenhage *et al.*, 2009). In the present study, we have found different haplotypes based on only by one change in the sequence of nucleotides in some areas for the three representative species, suggesting that they have been recently generated and may be signals of recent expansion. Changes in barriers, such road tunnels constructed by humans, could act as corridors and explain a recent population expansion (Podloucky, 1989; Jackson, 1996).

Our results, although based on modest sample sizes, illustrated the value of an urban pond as reservoirs for amphibians, as already noted by other authors (Parris, 2006; Gledhill *et al.*, 2008), and the capacity of these habitats for conserving diversity even if they are small. They could be considered potential amphibian refuges. However, without protection of adjacent terrestrial habitats, urbanization may lead to a reduction in the number of amphibian species persisting in urban ponds (Parris, 2006). We propose to expand our study of urban ponds to confirm their value as conservation targets.

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TABLES

Table 1. Amphibian diversity in an urban pond and in two rural areas in North Iberia. TWS, total water surface (m^2); SR, species richness; range of Shannon diversity indices calculated for each sampling site for different juvenile-adult survival rates.

		A. obstetricans		*D. galganoi*		*L. helveticus*		*T. marmoratus*		*L.boscai*		*M. alpestris*			
	TWS	L	A	L	A	L	A	L	A	L	A	L	A	SR	Shannon
CPF	<3	27	-	23	-	58	-	-	-	-	-	-	-	3.60	1.017-1.091
Rural A	6.2	49	-	36	-	20	18	4	3	-	1	-	-	6.00	1.278-1.285
Rural B	4.5	40	-	15	-	36	14	-	-	-	-	-	2	4.00	1.079-1.140

Table 2. Number of individuals found in the urban pond CPF and in the two rural areas A and B of each haplotype (Ha) within the COI region for the three study species.

A) *Alytes obstetricans*

Ha	1	2	3	4	5	6	7
CPF		20	3	1	3		
Rural A	44					1	4
Rural B	40						

B) *Discoglossus galganoi*

Ha	1	2	3	4
CPF	6	6	10	1
Rural A	8	7		
Rural B	19	17		

C) *Lissotriton helveticus*

Ha	1	3	4	2	5	6	7	9	10	11	12	13	14	15
CPF	41	2	8	1						2	1	1	1	1
Rural A	30	1			5			1	1					
Rural B	10					11	29							

Table 3. Genetic diversity of the three studied species in the urban pond (CPF) and two rural areas A and B. N, sample size genetically analyzed; Haplotype diversity, *p*; Nucleotide diversity, *π*; Number of haplotypes, *n*; *D*, Tajima´s D; *Fs*, Fu´s Fs; *SSD*, sum of squared differences Significance levels: *P < 0.05

	Alytes obstetricans			*Discoglossus galganoi*			*Lissotriton helveticus*		
	CPF	Rural A	Rural B	CPF	Rural A	Rural B	CPF	Rural A	Rural B
N	27	49	40	23	36	15	58	38	50
p	0.442	0.191	0	0.704	0.513	0.533	0.486	0.367	0.587
π	0.0014	0.0008	0	0.0017	0.0008	0.0008	0.0008	0.0006	0.0018
n	4	3	1	4	2	2	9	5	3
D	-0.248	-1.26*	-	0.564	1.658	1.503	-1.028	-1.29*	2.709
Fs	0.152	0.531	-	0.793	1.801	1.318	-4.34*	-2.59*	3.648
SSD	0.334*	0.039*	-	0.010	0.025*	0.030	0.326*	0.001	0.476*

Table 4. Pairwise F_{ST} values between the samples obtained in the urban pond CPF and in the two rural areas considered (below diagonal) and statistical significance as P-value (above diagonal). In each cell values are given for the three species; from top to bottom, *Alytes obstetricans*, *Discoglossus galganoi* and *Lissotriton helveticus*.

	CPF	Rural A	Rural B
CPF	-	0.000 0.001 0.002	0.000 0.013 0.000
Rural A	0.461 0.029 0.060	-	0.123 0.999 0.000
Rural B	0.602 0.162 0.276	0.045 -0.049 0.302	-

FIGURE LEGENDS

Figure 1. Map of Europe showing the Iberian Peninsula and the sampling region. Detail: map of the North Iberian sampling area. U, Urban pond sampled (Charca Pista Finlandesa); A and B, rural areas A and B.

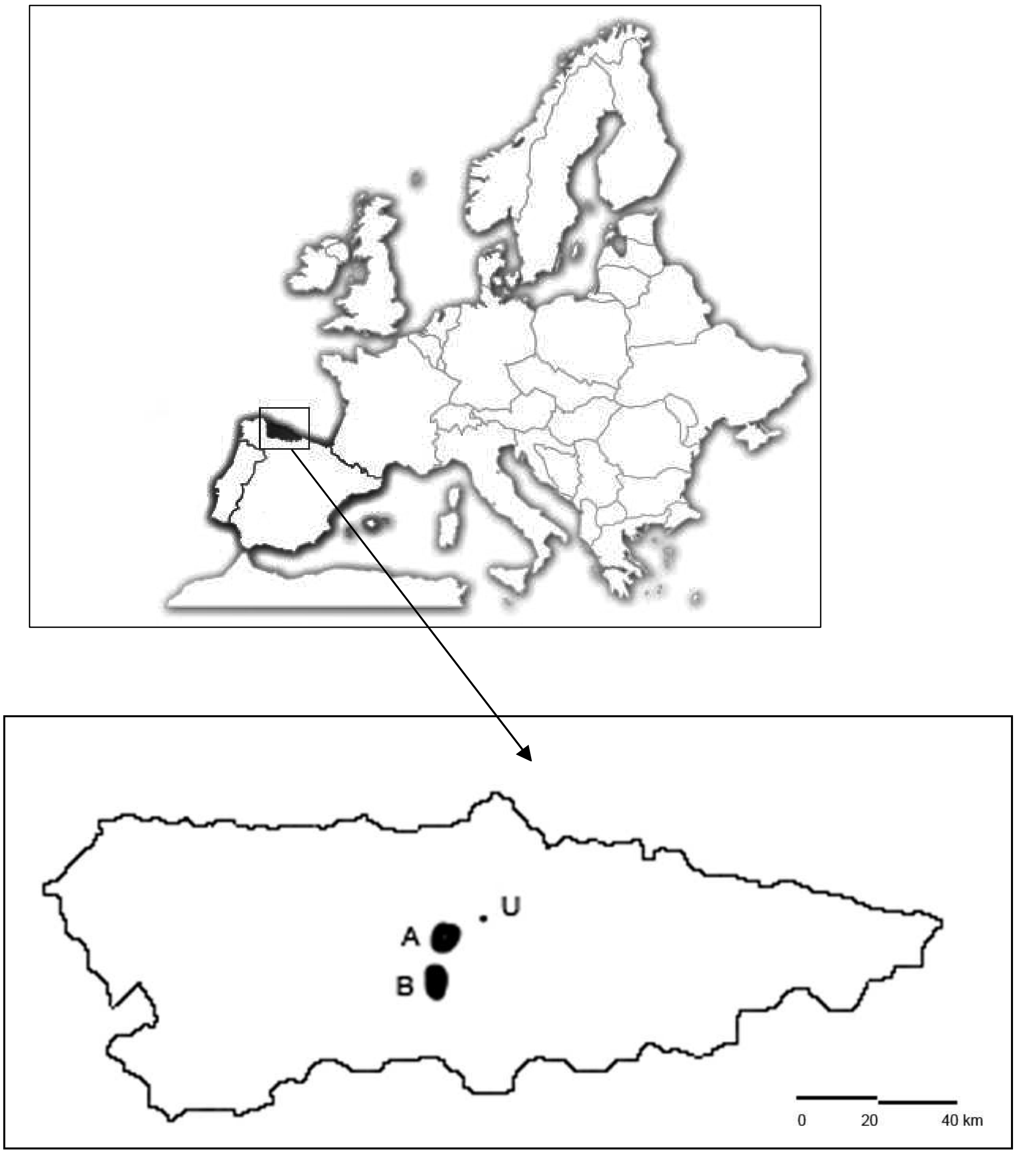
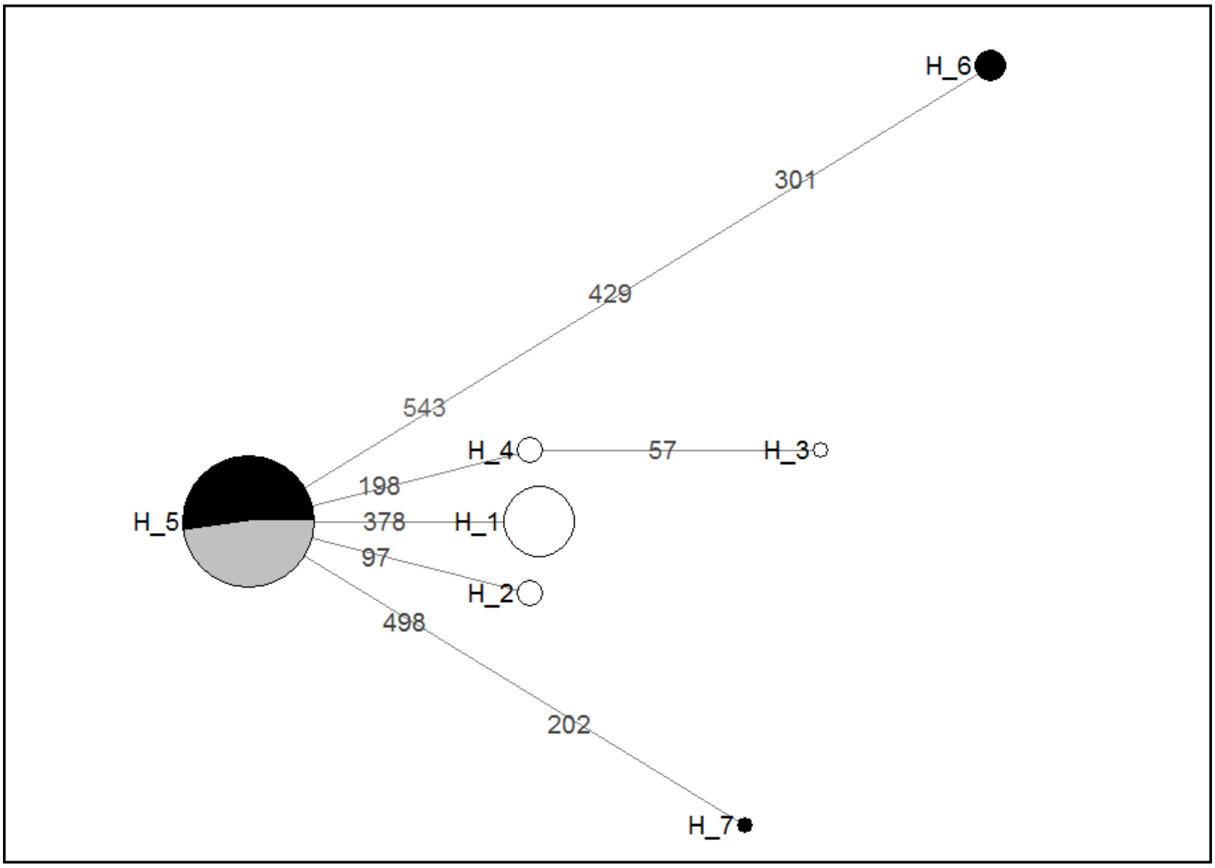
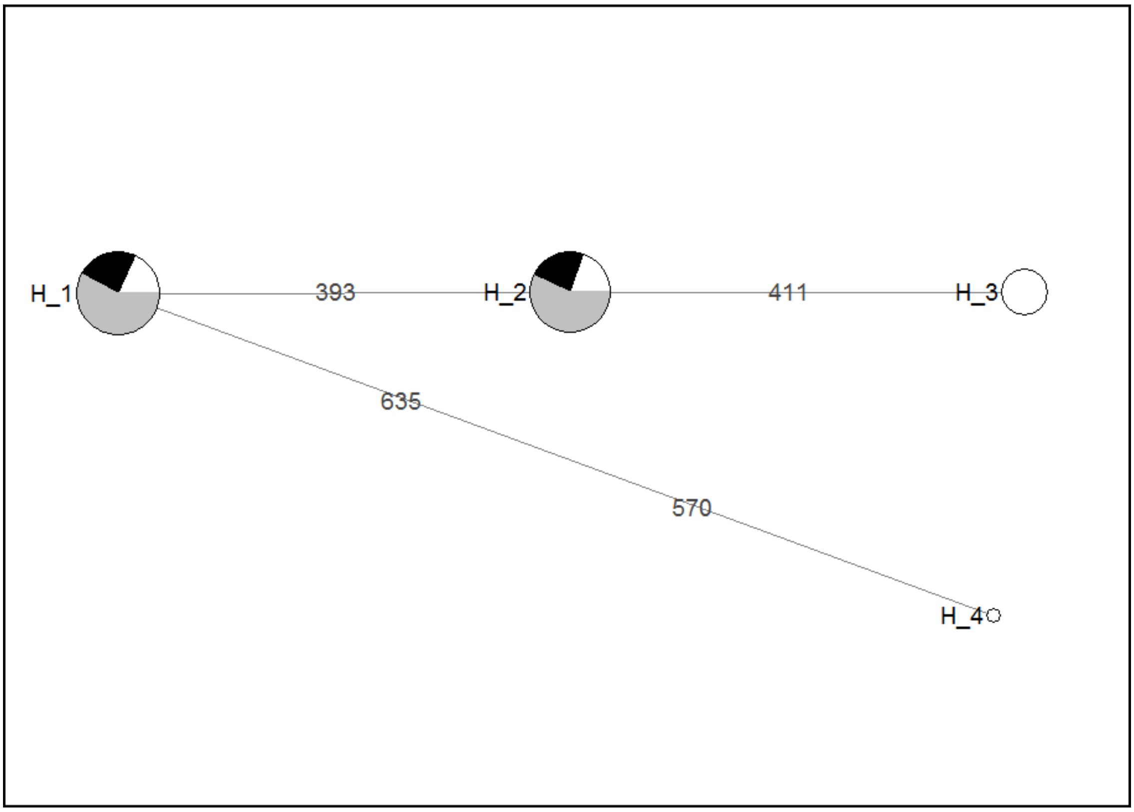


Figure 2. Median-Joining haplotype networks for COI haplotypes observed in A) *Alytes obstetricans*; B) *Discoglossus galganoi*; C) *Lissotriton helveticus*. White, CPF; black, Rural A; grey, Rural B. Each circle represents a different haplotype with size proportional to its relative frequency. Numbers on lines represent the mutation position.

A)



B)


H_1
393
H_2
411
H_3
635
570
H_4

C)

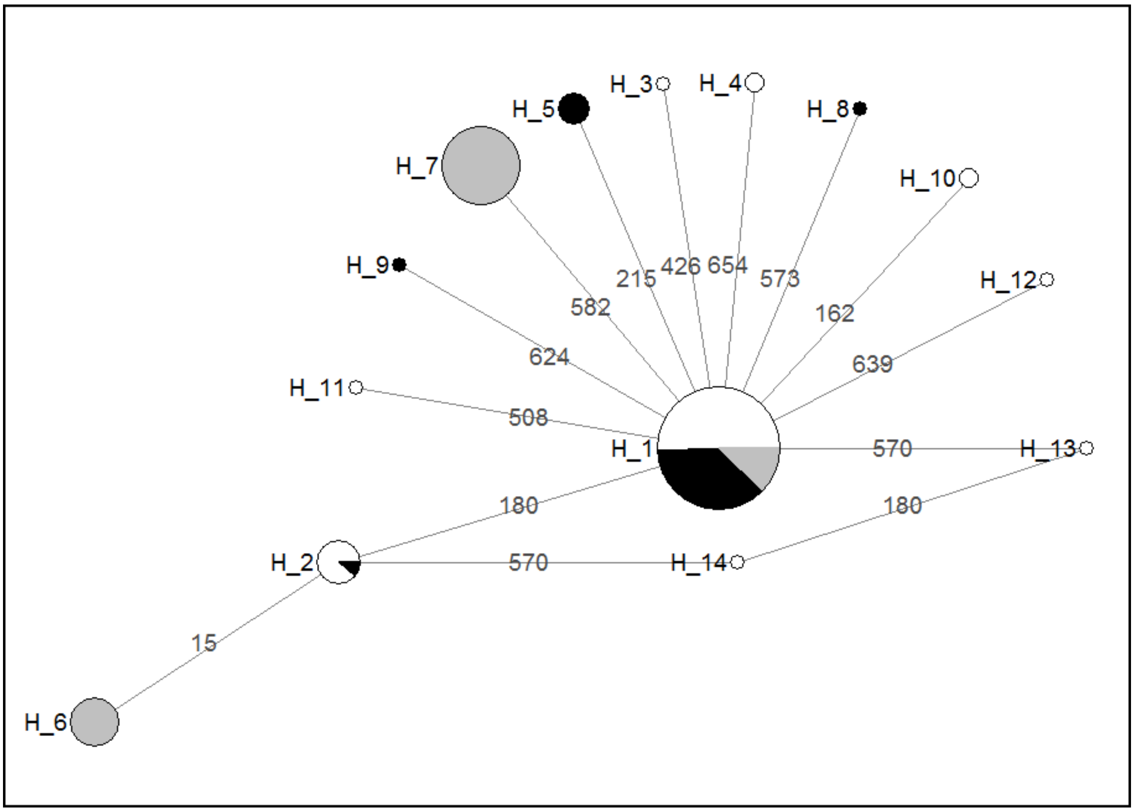
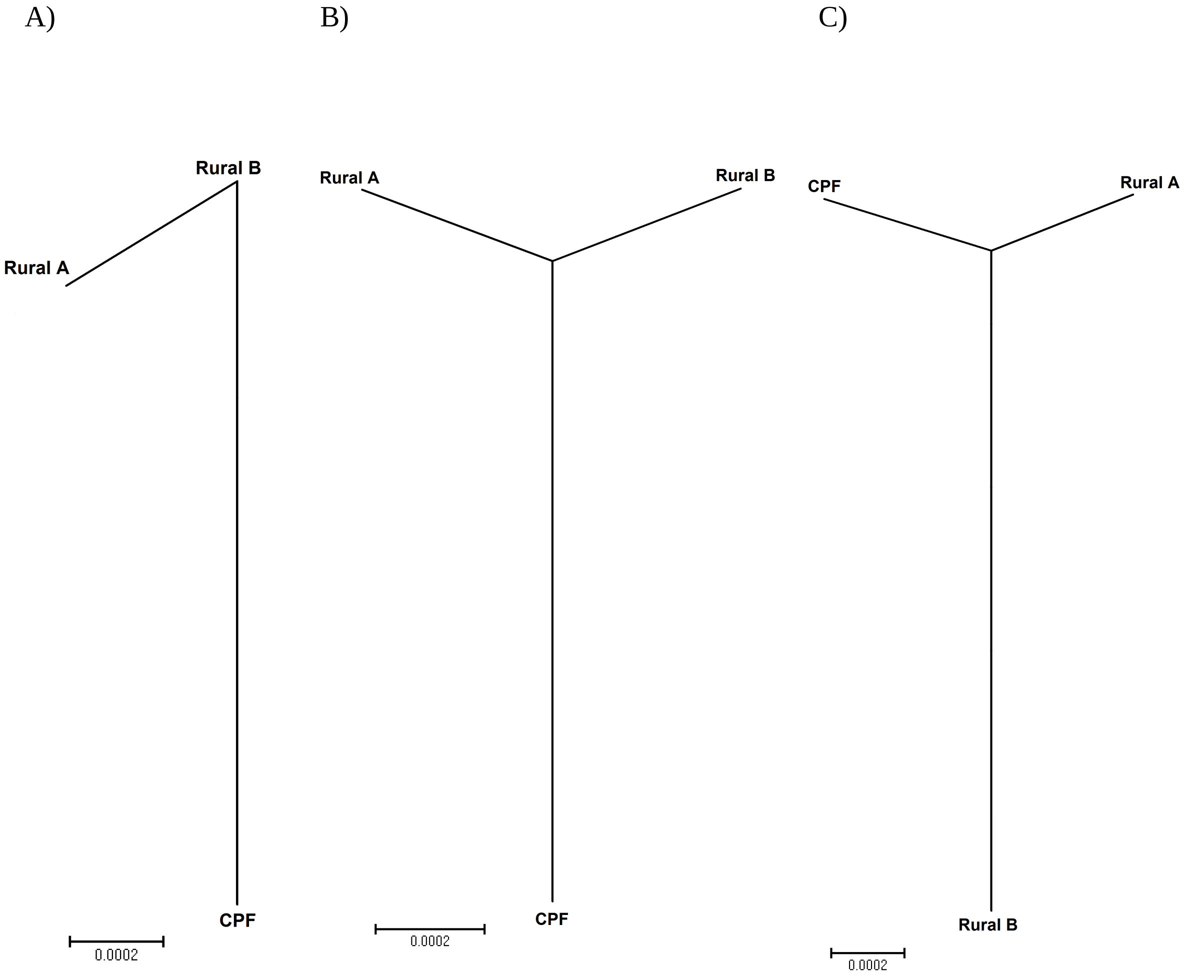
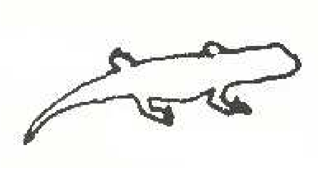

H_3
H_4
H_5
H_8
H_7
H_10
H_9
215
426
654
573
H_12
582
162
624
639
H_11
508
H_1
570
H_13
180
180
H_2
570
H_14
15
H_6

Figure 3. Neighbor-joining tree derived from genetic distances among sites inferred with the program MEGA 4 for the three studied species: A) *Alytes obstetricans*; B) *Discoglossus galganoi*; C) *Lissotriton helveticus*.





Capítulo 3

Amphibian metagenomics, a new approach for assessing the ecological value of anthropogenic habitats

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ABSTRACT

Urban spaces exhibit wildlife adapted to coexistence with humans, and some conservation target species like Amphibians inhabit water points within the cities. Understanding the characteristics of water points that enhance amphibian diversity may help to plan and prioritize management actions aimed at better supporting these vulnerable animals. In this study we have applied the novel metagenomic approach for quantifying together the intra- and inter-specific genetic diversity of all the water points found in Oviedo city (North of Spain), employing variation at the mitochondrial DNA Barcoding COI gene as genetic marker. We have identified pond size and connectivity as main factors enhancing metagenomic diversity, with species-dependent tolerance of habitat management intensity. Metagenomic indices were more sensitive at the small scale of this survey than classical diversity indices. These results confirm the value of metagenomic approaches for evaluating diversity at varied scales and indicate some priorities for amphibian conservation in urban greenspaces.

Keywords: Amphibians; diversity; metagenomics; urban water points; *Alytes obstetricans*, *Lissotriton helveticus*; *Discoglossus galganoi.*

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INTRODUCTION

Diversity is more than the number and relative abundance of species present in a habitat. It includes also intraspecific (population) diversity. Richer habitats contain more diverse populations at intra-specific level because, typically, larger stable populations conserve more genetic variants than small populations. If the genetic variants present in a habitat, as a whole, are functionally different (= code for different proteins or exhibit different regulatory properties), the resources of such habitat (space, nutrients) can be better exploited by the living community residing therein. Today, the metagenomic perspective includes both inter- and intraspecific variation and gives a holistic vision and measure of the diversity. Initially developed for prokaryotes (e.g. Riesenfeld et al. 2004; Tringe and Rubin 2005), metagenomics has commenced to be applied to eukaryotes (e.g. Creer et al. 2010; Porazinska et al. 2010) and higher eukaryotes (April et al. 2011; Ardura et al. 2011). In higher eukaryotes it has been principally measured as phylogenetic diversity (April et al. 2011; Mouillot et al. 2011), and very recently by nucleotide and haplotypic diversity and the number of putative proteins (Ardura et al. 2011) employing the cytochrome oxidase I (COI) gene as a marker.

The COI is the main marker of Barcoding projects. Such projects, aimed at collecting DNA sequences (= barcodes) for identification of all living organisms (Hebert et al. 2003), have been equally criticised (e.g. Moritz and Cicero 2004) and encouraged (e.g. Rubinoff 2006). They are accumulating an enormous amount of new sequences supported by a curator team and voucher specimens, that confer them some advantages over other databases which had not been so efficiently curated in the past and may contain specimens wrongly identified (e.g. Vilgalys 2003; Meier et al. 2006). In any case they can serve as a reference for supporting metagenomic projects of ecosystems where not all specimens can be visually identified. The COI being a coding gene, it is possible to use COI sequences for deducing the diversity of functional variants. It may serve as a shortcut for revealing the status of metagenomic diversity of communities of higher eukaryotes (Ardura et al. 2011), whose large genome size makes it difficult to carry out whole-genome metagenomics.

Amphibians are likely the most endangered higher eukaryotes and should be considered an urgent target for diversity estimates aimed at identifying priority protection measures (Collins and Halliday 2005). They are declining worldwide because they are vulnerable to a variety of factors associated with ecosystem change and degradation (Stuart et al. 2004), including climate warming (Beebee 1995), habitat pollution (critical review by Schiesari et al. 2007) and fragmentation (Hitching and Beebee 1997; Cushman 2006), deforestation (Becker et al. 2010), change of use of rural resources (Meyer-Aurich et al. 1998; Garcia-Gonzalez and Garcia-Vazquez 2011) and others. Their presence and resilience in a habitat can be considered a signal of environmental quality good enough for sheltering wildlife.

Not all the aquatic habitats containing amphibians are equally valuable for all amphibian species. Depending on their homing behaviour (e.g. Blaustein et al. 1994), dispersal capacity (e.g. Smith and Green 2005), development time (e.g. Newman 1992) and resistance to pollution (e.g. Garcia-Muñoz et al. 2010), each species has different requirements in terms of habitat connectivity, distance between ponds, seasonal permanence of water points and water quality, respectively. When water points are scarce and their maintenance is costly (not only in terms of budget but also in ecosystem management effort), prioritizing those which can shelter more species and larger and healthier populations seems to be a sensible strategy. Urban areas are a good example of limited wild aquatic spaces where giving priority to water systems that shelter most amphibian diversity could be useful for contributing to wildlife preservation. Although heavily anthropized, urban water points are shelters for many amphibian species (Parris 2006; Gledhill et al. 2008; Birx-Raybuck et al. 2009)

In this study we have applied the metagenome approach to amphibian communities inhabiting different types of urban water points as a case study. We have employed the COI gene as a marker, treated the sequences of each water point, regardless the species, as a genetic unit (= community genetics), and estimated genetic (= metagenetic) diversity for each type of water point. ANOVA-based statistical tests and Principal Component Analysis have served for estimating the significance of the observed differences among types and the main characteristics of the water points that favour amphibian metagenomic diversity.

MATERIALS AND METHODS

Urban water points analyzed

The urban area considered as case study was Oviedo city (200,000 inhabitants), which is located in the north of Spain (GPS coordinates 43º22 N, 541 / 5º51 W, 723; see Fig. 1). Eight water points were considered (Table 1) within an area of approximately 10 km^2 (Fig. 1): two spontaneous ponds originated from rainfall and local water sources (West Park and Pista Finlandesa as P1 and P2 respectively); three decorative fountains (F1, F2 and F3); three troughs (T1, T2 and T3). The eight water points were surrounded by lawn and the vegetation coverage was similar (cultivated trees and green hedges in their proximity), since they were all in the middle of public green areas (urban gardens/parks).

The characteristics considered for these water points were: depth (as average depth, measured in cm in at least four sites within a water point when its bottom was irregular), surface (approx. surface, in cm^2), vegetation corridors (adjacent vegetation connecting the water point to other areas containing Amphibians) and management. Corridors were measured as: 1/[distance in kilometres to the closest water point], considering only continuous paths with vegetation usable by Amphibians (i.e. grass paths, bushes, flower beds). In this way, higher values correspond to shorter corridors connecting near locations with Amphibians. Management is important for Amphibian diversity (Garcia-Gonzalez and Garcia-Vazquez 2011); in the present case it depends on the function of the urban water points. Spontaneous ponds are not managed in any way (absent management, intensity 0), fountains are cleaned monthly and all vegetation and bottom substrate removed (intense management, intensity 2), and troughs are employed for animal watering (horses, dogs and other domestic animals) and are cleaned sporadically without removing bottom vegetation (moderate management, intensity 1).

Sampling methodology

Dipnet surveys were conducted in the eight water points in autumn 2007, spring 2008 and summer 2008. New clean fine pore nets were employed in each point to prevent dissemination of diseases and parasites. Many blind sweeps were carried out, covering exhaustively each water point.

Tail tips were sampled from Urodeles larvae and adults and from Anuran larvae, excised with sterilized twins and scissors. Gloves were employed for specimen manipulation. The tail grows very fast in newts and tadpoles, thus this method of tissue sampling can be considered non-harmful (Arntzen et al. 1999). Tissue samples were stored in absolute ethanol until laboratory analysis. After sampling, specimens were immediately released into the water point. The species was visually identified in adults and big larvae. The species of very small larvae of ambiguous phenotype was genetically identified.

Genetic tools

Total DNA was extracted from small tail biopsies (about 1 mm^3) using a Chelex-based protocol (Estoup et al. 1996) and kept at 4º C until analysis. Briefly, tissue sample is submerged in a suspension of deionized water and 5% Chelex® 100. The alkalinity of the suspension and the boiling process disrupts the cell membranes, destroys cell proteins, and denatures the DNA. The suspension is then centrifuged, separating the resin and cellular debris from the supernatant containing the denatured DNA. The DNA can then be PCR amplified.

The mitochondrial locus analyzed was the Cytochrome oxydase I (COI) gene. A sequence fragment was PCR amplified employing the primers described by Ward et al. (2005) for the Barcoding project. The COI primers by Ward et al. (2005) did not amplify DNA from *Triturus marmoratus* samples (Garcia-Gonzalez and Garcia-Vazquez 2011); new primers were designed for this species in the neighbouring 5' and 3' regions of the COI Barcode fragment. These primers were:

COITmar-F: 5'-TCATAAAGATATTGGCACCCTCT-3'
COITmar-R: 5'-AAGAATATAGACCTCGGGGTGA-3'.

PCR reactions were carried out in a total volume of 40 µl containing 5x Go Taq® Flexi Buffer, 2.5 mM MgCl, 250 µM of each dNTP, 1 µM of each primer, 1 unit of Go Taq® Polymerase 5U/µl (Promega) and 50ng of DNA template. They were performed in a GeneAmp® PCR system 2700 (Applied Biosystems) with the following conditions: an initial denaturing step at 95 ºC for 5 min, 35 cycles of denaturing at 95 ºC for 30 s, annealing (for 30 s) at 58ºC and 59ºC for COI (Ward et al., 2005) and COI Tmar (this study) gene respectively, and an extension at 72 ºC for 30 s, followed by a final extension at 72 ºC for 15 min.

PCR products were visualized in 2 % agarose gels stained with ethidium bromide. Visible bright bands were excised from the gel and DNA was purified with a Wizard® SV Gel and PCR Clean-Up System (Promega) before sequencing. Automated fluorescence sequencing was run on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems) with BigDye 3.1 Terminator system, in the Unit of Genetic Analysis of the University of Oviedo (Spain).

Sequences were checked visually and edited employing the BioEdit Sequence Alignment Editor software (Hall 1999). They were aligned with the application ClustalW (Thompson 1994). When necessary for species identification, they were compared against the GenBank database (http://www.ncbi.nlm.nih.gov/GenBank) with the program BLASTn (Basic Local Alignment Search Tool) for retrieving the species exhibiting highest similarity with the problem sequence.

Diversity estimates

Three parameters were employed for estimating Amphibian diversity: species richness, the Shannon index (Shannon 1948; Magurran 1988) and Simpson index (1949), expressed as 1–Simpson. Species richness was calculated with the online software SPECRICH (Hines, 1996, http://www.mbr-pwrc.usgs.gov/software/specrich.html) based on the methods described by Burnham and Overton (1979). The Shannon and Simpson index were estimated for each water point employing the program DIVERS (Franja 1993), with the formula $H'=-\Sigma$ pi Ln pi for Shannon and the formula $D = \Sigma pi^2$ for Simpson index, where $p_i=$

proportional abundance of the i-esim species = number of individual of the i-species/total number of individuals.

Metagenomic diversity, that is, genetic diversity estimated across species (intra- and inter-specific together), was estimated from all the amphibian COI sequences obtained from each sample by: number of haplotypes (h); haplotype diversity as Hd= (n/(n-1)) (1-$\sum x_i^2$), where x_i is the (relative) haplotype frequency of each haplotype in the sample and n is the sample size; nucleotide diversity as: $\pi = (\sum d_{ij}) / k(k-1)/2$, where d_{ij} is the number of nucleotide differences between *i* and *j* haplotypes and k is the number of haplotypes in a sample; and metagenetic diversity as h*π, as a combined measure of intra- and inter-specific sequence variation, accounting for most h (most mutations are expected to be synonymous and would occur within-species) and π (most nucleotide substitutions would occur between sequences from different species) values respectively. Calculations of Hd and π were made employing the DnaSP software (Nei 1987; Librado and Rozas 2009).

Functional diversity was measured as the number of putative polypeptides obtained from COI sequences. DNA-protein translation was made with the program MEGA version 5 (Tamura et al. 2011).

Reconstruction of the polypeptide tree

The relationship between the functional variants present in the sampling sites was visualized constructing a Neighbor-Joining tree (Saitou and Nei 1987) with the putative polypeptides. First, the evolutionary model best-fitting the polypeptide dataset was estimated with the program PROTTEST (Abascal et al. 2005). Employing the Akaike information criterion (AIC), the JTT Matrix (Jones et al. 1992) without a gamma shape, showing the lowest AIC score was chosen. Then this model was applied in the settings for reconstructing the tree, employing the software MEGA version 5 (Tamura et al. 2011). Robustness of the NJ topology was assessed testing 1,000 bootstrap pseudo-replicates.

Identification of principal factors contributing to dataset variation

With the Principal Component Analysis (PCA) we can reduce a number of variables to a few indices (called the principal components, PCs) that are linear combinations of the original variables (Manly 1994; Sharma 1996; Yu et al. 1998; Heycox 1999). PCA provides an objective way of ‘aggregating’ indicators so that variation in the data can be accounted for as concisely as possible. To identify the most important factors accounting for Amphibian life in the considered urban water points, PCA was carried out employing the PAST (PAlaeontological STatistics, version 1.90; see Hammer et al. 2001) software. The software was employed for finding hypothetical variables (components) which account for as much of the variance in these multidimensional datasets of water point characteristics as possible. The data were analyzed with default settings, choosing the “correlation matrix” option for variables measured in different units. Only components not significantly auto-correlated ($P > 0.01$, $|r| \geq 0.8$; Berry and Feldman 1985; Bowerman and O´Connell 1990) were considered for the PCA. The same program was employed for calculating correlation values between factors and their statistical significance.

RESULTS

In total four Amphibian species were found in this survey (Table 1): two Anurans (*Alytes obstetricans, Discoglossus galganoi*) and two Urodeles (*Lissotriton helveticus, Triturus marmoratus*). The amplified COI fragments of 661 base pairs (bp) and 671 bp, for *T. marmoratus* and the rest of species respectively, allowed to unambiguously identifying all specimens, including small larvae.

The total surface was similar for the three categories of water points, between 42000 cm^2 and 50000 cm^2, but great differences in species richness and diversity were found among the eight water points studied (Table 2), from the most rich in amphibians T1 (three different species: *Alytes obstetricans*, *Lissotriton helveticus*, *Triturus marmoratus*) to F2 and P2 with only one species (*Alytes obstetricans* and *Lissotriton helveticus* respectively). The highest species richness was found at the troughs, which were globally the most diverse for amphibian species diversity from the two considered indices (H’ and 1/Simpson). The lowest species diversity and richness corresponded to the decorative fountains.

The metagenomic analysis was carried out based on COI sequences. The 27 haplotypes obtained from the four species were sent to the GenBank, where they are available with the Accession Numbers given in Table 3. In the eight urban water points a total of 27 haplotypes were found: nine from *Alytes obstetricans*; four from *Discoglossus galganoi*; 13 from *Lissotriton helveticus*; and one from *Triturus marmoratus*. They were aligned and cut down to the 659 nucleotides that overlap in the four species considered. Metagenomic diversity values obtained for each water point (Table 4) were generally higher for ponds and troughs than for fountains. The number of haplotypes was higher for ponds, as well as the haplotypic (Hd) and nucleotide (π) diversity. The metagenetic diversity h*π exacerbated the difference among water points, being maximum for P1 and minimum for F2 in an order >4000 times.

The differences among water points for metagenomic diversity were also of functional nature. Not all the haplotypes within a species were synonymous, some of them yielded different proteins (Table 3): four for *A. obstetricans*, two for *D. galganoi*, three for *L. helveticus* and one from *T. marmoratus*. The different proteins were logically clustered by species in a phylogenetic tree (Fig. 2), except the protein obtained from *T. marmoratus* which was identical to the protein yielded by most haplotypes of *L. helveticus*. As expected, the different proteins were not associated to a particular type of water point, being distributed equally in all types of urban habitats. However, and accordingly with greater DNA metagenetic diversity, the number of functional variants was greater for spontaneous ponds than for the other two types of water points (Table 4).

The Principal Component Analysis (Table 5) revealed that the variation in the dataset was explained principally by three main components, providing in total more than 85% variance (Table 5A). The factors that contributed mostly to each component (Table 5B) were: amphibian metagenetic diversity h*π and functional diversity as represented by the number of different COI proteins for the most important Component 1; depth and surface for Component 2; and connectivity and the Shannon index within Component 3. In the scatter plot (Fig. 3), metagenetic (h*π) and functional (Np) diversity were in the same direction as Surface and Connectivity, emphasizing the importance of these two habitat variables for metagenomic amphibian diversity.

Ecological and metagenomic diversity indices were significantly correlated (Table 6). As expected, h*π and the number of proteins were strongly positively correlated. Interestingly, water point surface was also strongly positively correlated with h*π, but not with the number of proteins. Other significant associations occurred between ecological and metagenomic diversity parameters (Np, Hd and Shannon index), and among ecological parameters (species richness and Shannon), but h*π did not correlate with any ecological diversity parameter. It did, however, with other characteristic of the water points: connectivity (Table 6), as also pointed out in the PCA scatter plot (Fig. 3) with h*π and connectivity in the same direction (positive X axis). The positive association between surface and depth of the water points can be considered of no biological value because troughs and fountains were constructed by humans and the mentioned association does not imply any ecological principle; actually, depth appeared in a different quadrant of that occupied by metagenomic diversity parameters in the PCA scatter plot (Fig. 3) and was not significantly associated with them (Table 6).

Finally, the four species did not exhibit similar preferences for the different types of water points. The two most abundant species were unequally distributed among water points: while *A. obstetricans* was more abundant in decorative fountains (Fig. 4), *L. helveticus* was the dominant species in spontaneous ponds. Both species were similarly abundant in troughs. The other two sporadic species appeared in ponds (*D. galganoi*) or troughs (*T. marmoratus*), but none in decorative fountains.

DISCUSSIONS AND CONCLUSIONS

The main innovation of this study is to apply a metagenomic approach for quantifying amphibian diversity, considering together intra- and inter-specific genetic variation. This has been proposed a useful method for measuring the total genetic diversity of a community at larger scales (Ardura et al. 2011), and to discover hidden diversity also at large-scale surveys (e.g. Fonseca et al. 2010; Porazinska et al. 2010). In our study we have revealed its value for distinguishing between habitats at a small-scale, as clearly revealed by significant correlations between habitat traits and the parameter h*π in amphibians from only eight spatially small water points.

The results presented in this small-scale study, although necessarily limited given the scarce number of water points found in Oviedo city, emphasize the value of urban water points as shelters for Amphibians, perhaps the most endangered vertebrates in this moment of global change. Our results indicate that even very small fountains, and ponds spontaneously formed without human intervention, constitute habitats suitable for toad and newt reproduction and are confirmed as main contributors to species richness in urban greenspaces (e.g. Gledhill et al. 2008).

Metagenomics considers together intra-and inter-specific variation. As a consequence, although high metagenetic diversity is intuitively expected to be associated with high functional diversity, it is not always true; it rather may depend on the population size and the evolutionary history of the different species that form a community. For example, in populations subjected to a recent expansion, and in large populations, most variants are synonymous (e.g. Kreitman 2000). In the metagenomic approach, the combination of a high metagenetic diversity and a low functional diversity may indicate that the community is composed by large populations of a few different species. This could be the case of the pond P2, with relatively high h*π for only one species (*L. helveticus*) with only one functional variant of the COI fragment analyzed. P2 likely shelters a large population of palmate newts. Although the species diversity was null there, P2 has an added value as a good habitat for a large population of a species which is vulnerable to the current climate warming due to reduced female fecundity at warm temperatures (Galloy and Denoel 2010). All types of water points should therefore be protected for preserving urban amphibians.

Metagenomic diversity is more informative than species diversity because it adds information on the internal variation of each population. As an example, in our study the pond size does not seem to represent a limit for the diversity of species (SR, Shannon, Simpson indices), which is not correlated with surface, supporting results previously obtained by other authors (Snodgrass et al. 2000; Oertli et al. 2002; Ficetola and Bernardi 2004; Denoël and Ficetola 2008). In contrast, we have detected a strongly significant association between water point size (surface) and metagenetic diversity, indicating that large pond size is associated with larger population size, h (haplotype number) being directly associated with effective population size (e.g. Frankham 1996) and, numerically, constituting the main part of the metagenetic diversity index. Pond size appears here as an important determinant of the amphibian population size, as pointed out by Laan and Verboom (1990), Semlitsch and Bodie (1998), Snodgrass et al. (2000).

On the other hand, as already suggested by other authors (e.g. Parris 2006; Hamer and McDonnell 2008; Stevens and Baguette 2008), connectivity between water points has been revealed in our study to be significantly associated with amphibian diversity in urban landscapes. Vegetation corridors and pond size are therefore crucial factors for the conservation of amphibians in these anthropogenic habitats. Creating new vegetation corridors among urban water points would favour Amphibians and enhance population diversity, at least for species of low dispersal capacity (Blaustein et al. 1994; Beebee 1996; Gibbs 1998).

The type of habitat management is also important for amphibians, and Garcia-Gonzalez and Garcia-Vazquez (2011) suggested that moderate management contributes positively to amphibian diversity in rural settings. In the present small-scale study focused on urban areas, however, habitat characteristics and management were not significantly associated with species diversity; only with metagenetic parameters, more sensitive than classical biodiversity estimators (Ardura et al. 2011). In fact, management contributed only in a minor extent to the total variance (Table 4B). This does not imply that all species adapt equally to all types of managed water points, as clearly seen in Fig. 4, because the palmate newt *T. helveticus* (in moderate decline; Beebee et al. 2009) and the midwife toad *A. obstetricans* exhibit different preferences depending on habitat management, intensive management being an obstacle for the development of all species except *A. obstetricans* (Garcia-Gonzalez and Garcia-Vazquez 2011).

From these results it is possible to categorize action priorities aimed at amphibian conservation, taking into account that the reaction of each species may be different. For similar water point surface, the greatest number of species, diversity and species richness were found at the functional troughs (with moderate management) followed by the spontaneous ponds (without management). Therefore a simple and null-cost action to be promoted could be reducing the intensity of management of urban water points.

Cleaning them only sporadically, respecting their floating vegetation and avoiding silting in the bottom could be enough for maintaining diverse Amphibian life (Garcia-Gonzalez and Garcia-Vazquez 2011) and, from these results on urban ponds, also for contributing to preservation of newt populations.

On the other hand, pond size and connectivity are main factors for enhancing metagenomic diversity. Increasing the number and quality of vegetation corridors and favouring large ponds would no doubt have a higher cost than moderating management, but if Amphibian conservation is important for urban citizens, such cost would be worthy. Protection of Amphibians in urban landscapes provides some services to citizens that have not been fully evaluated by now. For example, networks of urban wetlands would harbour abundant Amphibian populations (Cushman 2006) and help to better controlling mosquito populations (Chase and Shulman 2009). Another service of preserving amphibian life could be educational. Amphibians are rarely considered in this aspect, but these small animals are able to adapt to very limited and even marginal habitats and constitute natural lessons of wildlife resistance in heavily modified environments. Children are often attracted by Amphibians, which are unique examples of aquatic wildlife in most big cities. If we can help individuals and institutions to recognize the value of nature, then this should greatly increase investments in conservation, while at the same time fostering human well-being (Daily et al. 2009). Most measures can be a part of the normal activities of city organizations without excessive extra costs: employees of park services could be trained for managing water points in a way compatible with Amphibian life, and regular programs of environmental education could include valorisation of Amphibians as vulnerable and valuable animals. These are very simple low-cost measures that can be implemented and make a valuable contribution to conservation in urban landscapes.

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TABLES

Table 1. Amphibian samples found in the surveyed urban water points. Number of individuals of each species. P, F and T are spontaneous ponds, decorative fountains and functional troughs, respectively.

Sampling location	***A. obstetricans***	***L. helveticus***	***D. galganoi***	***T. marmoratus***	***Total***
P1	27	58	23	0	***108***
P2	0	19	0	0	***19***
Total Ponds	27	77	23	0	***127***
F1	43	1	0	0	***44***
F2	44	0	0	0	***44***
F3	10	1	0	0	***11***
Total Fonts	97	2	0	0	***99***
T1	14	19	0	1	***34***
T2	17	19	0	0	***36***
T3	15	11	0	0	***26***
Total Troughs	46	49	0	1	***96***
Total	170	128	23	1	***322***

Table 2. Characteristics of the analyzed urban water points and their amphibian fauna. Depth, surface, number of Amphibian species (NSp), species richness (SR), species diversity (Shannon index H'; 1/Simpson index), connectivity (Conn: 1/[distance in kilometres to the closest water point]), management (Mgmt) as 0 (unmanaged), 1 (moderate management), 2 (intensive management).

Acronym	Type	Depth (cm)	Surface (cm^2)	NSp	SR	Conn	Mgmt	Shannon	1/Simpson
P1	Pond	30	30000	3	3.6043	11.11	0	1.00982	2.56004
P2	Pond	40	20000	1	1.0500	2.78	0	0.00000	0.00000
Total Ponds			50000	3	3.6043			0.94201	2.26657
F1	Fountain	10	9000	2	3.0000	11.11	2	0.10847	1.04762
F2	Fountain	30	18550	1	1.0500	1.35	2	0.00000	0.00000
F3	Fountain	45	18000	2	3.0000	0.30	2	0.30464	1.22222
Total Fountains			45550	2	2.0000			0.09882	1.04166
T1	Trough	26	10710	3	4.0000	2.78	1	0.79427	2.14122
T2	Trough	26	16200	2	2.2414	0.34	1	0.69160	2.05212
T3	Trough	40	15000	2	2.2414	0.79	1	0.68127	2.03125
Total Troughs			41910	3	4.0000			0.74334	2.06242

Table 3. Haplotypes and putative proteins from the four Amphibian species sampled in the north Iberian urban setting, and sites where each haplotype was found. P, F and T are pond, fountain and trough respectively.

Species	Sites	Haplotype	Accession Number	Polypeptide
A. obstetricans	P, F	1-Ao	HM032698	Ao-1
A. obstetricans	F	2-Ao	JN379849	Ao-1
A. obstetricans	P, F	3-Ao	HM032695	Ao-1
A. obstetricans	P, F	4-Ao	HM032696	Ao-2
A. obstetricans	P, F	5-Ao	HM032697	Ao-1
A. obstetricans	F, T	6-Ao	HM032694	Ao-1
A. obstetricans	F	7-Ao	JN412596	Ao-3
A. obstetricans	T	8-Ao	HM032699	Ao-1
A. obstetricans	T	9-Ao	HM032700	Ao-4
D. galganoi	P	10-Dg	GU797351	Dg-5
D. galganoi	P	11-Dg	GU797352	Dg-5
D. galganoi	P	12-Dg	GU797353	Dg-5
D. galganoi	P	13-Dg	GU797354	Dg-6
L.helveticus	P, F, T	14-Lh	GQ374500	Lh-7
L.helveticus	P	15-Lh	GQ374502	Lh-7
L.helveticus	P	16-Lh	GQ374503	Lh-7
L.helveticus	P, T	17-Lh	JN379830	Lh-7
L.helveticus	P	18-Lh	HM032701	Lh-7
L.helveticus	P	19-Lh	HM032702	Lh-8
L.helveticus	P	20-Lh	HM032703	Lh-7
L.helveticus	P	21-Lh	HM032704	Lh-7
L.helveticus	P	22-Lh	HM032705	Lh-7
L.helveticus	F	23-Lh	JN412595	Lh-7
L.helveticus	P	24-Lh	JN379834	Lh-7
L.helveticus	T	25-Lh	GQ374504	Lh-9
L.helveticus	T	26-Lh	GQ374508	Lh-7
T.marmoratus	T	27-Tm	JN379821	Tm-7

Table 4. Metagenomic amphibian diversity in the urban water points analyzed.

Sampling site	n	h	Hd	Var. Hd	π	Var. π	h * π	Np
P1	108	17	0.806	0.0289	0.20272	0.097003	3.44624	6
P2	19	5	0.6374	0.1045	0.001858	0.001378	0.00929	1
Total Ponds	127	18	0.7913	0.0299	0.186847	0.089323	3.363246	6
F1	44	6	0.8055	0.0204	0.017636	0.009042	0.105816	3
F2	44	3	0.1723	0.074	0.000265	0.000396	0.000795	2
F3	11	3	0.5636	0.134	0.060267	0.032047	0.180801	2
Total Fountains	99	9	0.7283	0.0382	0.014771	0.007556	0.132939	4
T1	34	5	0.7736	0.0274	0.171573	0.083949	0.857865	2
T2	36	2	0.5127	0.0219	0.168314	0.082219	0.336628	2
T3	26	5	0.7385	0.0553	0.166968	0.082525	0.83484	4
Total Troughs	96	8	0.7094	0.0283	0.168153	0.08065	1.345224	4
Total	322	27	0.8383	0.0127	0.183469	0.087293	4.953663	9

Table 5. Principal Components Analysis. A, Eigenvalues and percent of variance contributed by each component. B, values contributed by each factor to each component (Axis). Jolliffe's cut-off value = 0.9; in bold, values > |0.4|.

A)

Eigenvalue	% variance
4.27386	**47.487**
2.12759	**23.64**
1.12452	**12.495**
0.784812	8.7201
0.422958	4.6995
0.244308	2.7145
0.0219597	0.244

B)

Variable	Axis 1	Axis 2	Axis 3
SR	0.3338	-0.3175	0.3987
Connectivity	0.3203	-0.2991	**-0.5356**
Depth	-0.07863	**0.5645**	0.35
Surface	0.2323	**0.5198**	-0.3447
Hd	0.3423	-0.2451	0.2693
Mgmt	-0.2714	-0.36	-0.01723
h*Pi	**0.4587**	0.1624	-0.09229
Np	**0.4202**	0.02976	-0.1877
Shannon	0.3847	0.06388	**0.443**

Table 6. Correlation coefficients between pairs of variables. Sr, species richness; Hd, haplotypic diversity; Mng, management; Np, number of proteins. In bold, significant values of biological meaning (*, P<0.05; **, P<0.01).

	SR	Connectivity	Depth	Surface	Hd	Mng	h*Pi	Np
Connectivity	0.431	-						
Depth	-0.294	-0.623	-					
Surface	-0.093	0.199	0.427	-				
Hd	**0.686***	0.557	-0.207	-0.093	-			
Mng	-0.019	-0.184	-0.246	-0.576	-0.421	-		
h*Pi	0.400	**0.640***	-0.032	**0.805****	0.338	-0.545	-	
Np	0.473	0.617	-0.125	0.505	0.468	-0.2988	**0.831****	-
Shannon	**0.690***	0.140	0.004	0.282	0.503	-0.454	0.576	**0.646***

LEGENDS FOR FIGURES

Figure 1. Map with the sampling sites within the city of Oviedo (North Spain). F1, F2 and F3, are fountains; P1 and P2 are spontaneous ponds; T1, T2 and T3 are functional troughs.

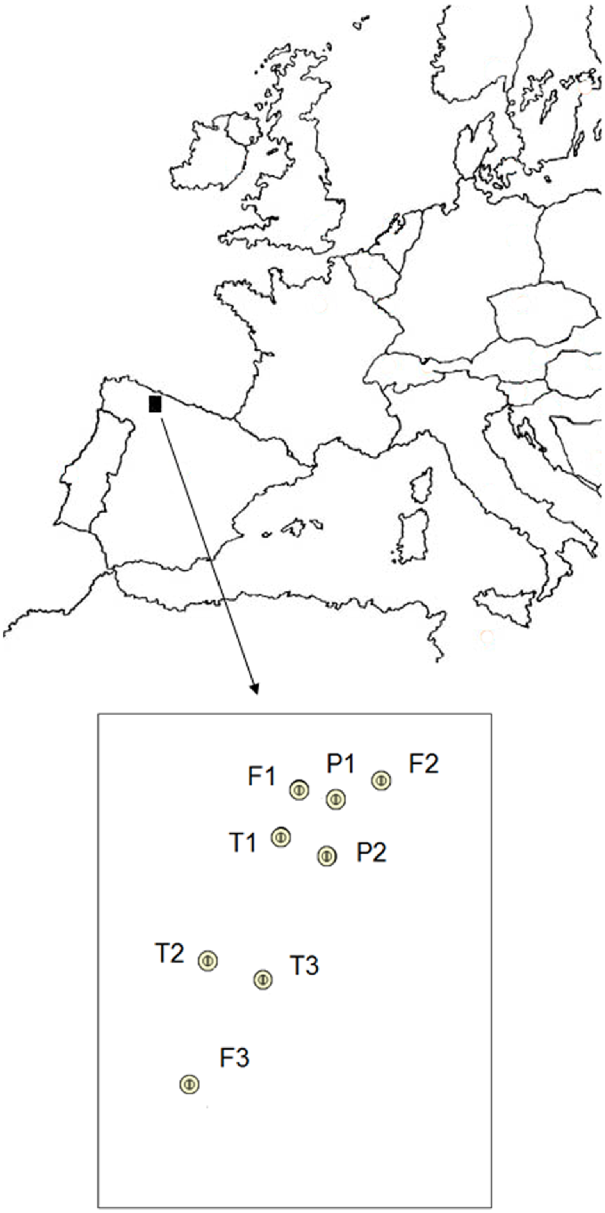


Figure 2. Neighbour-Joining tree constructed from the putative polypeptides obtained from the COI sequences of the analyzed amphibians. Acronyms correspond to: type of water point (P, F and T as pond, fountain and trough respectively); initials of the genus and species (Ao, Dg, Lb, Lh and Tm as *Alytes obstetricans*, *Discoglossus galganoi*, *Lissotriton boscai*, *Lissotriton helveticus* and *Triturus marmoratus* respectively); number of the protein variant for that water point and species.

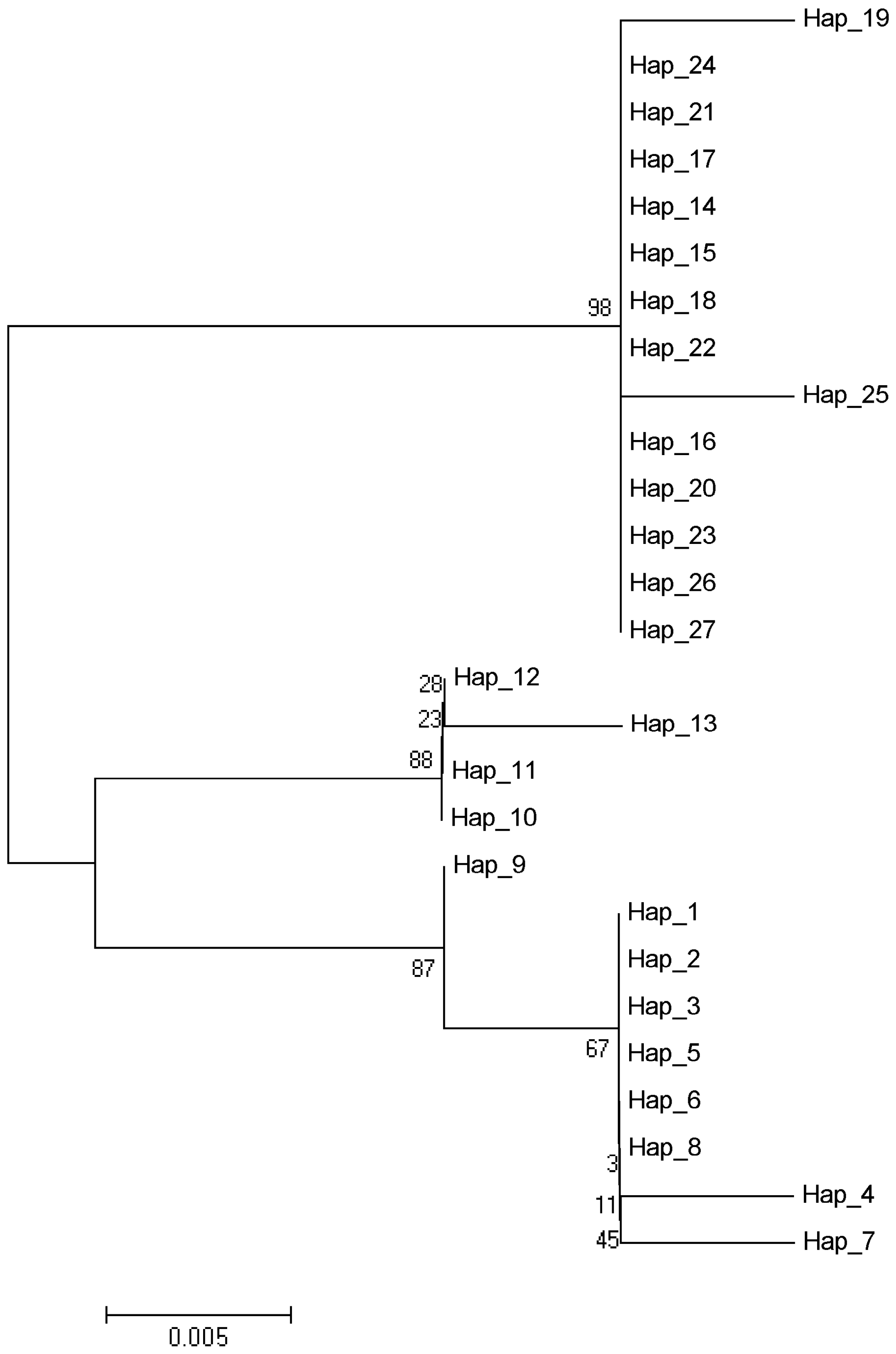


Figure 3. Scatter plot of the analyzed water points showing the six main factors identified in the PCA. h*Pi = h*π, metagenetic diversity.

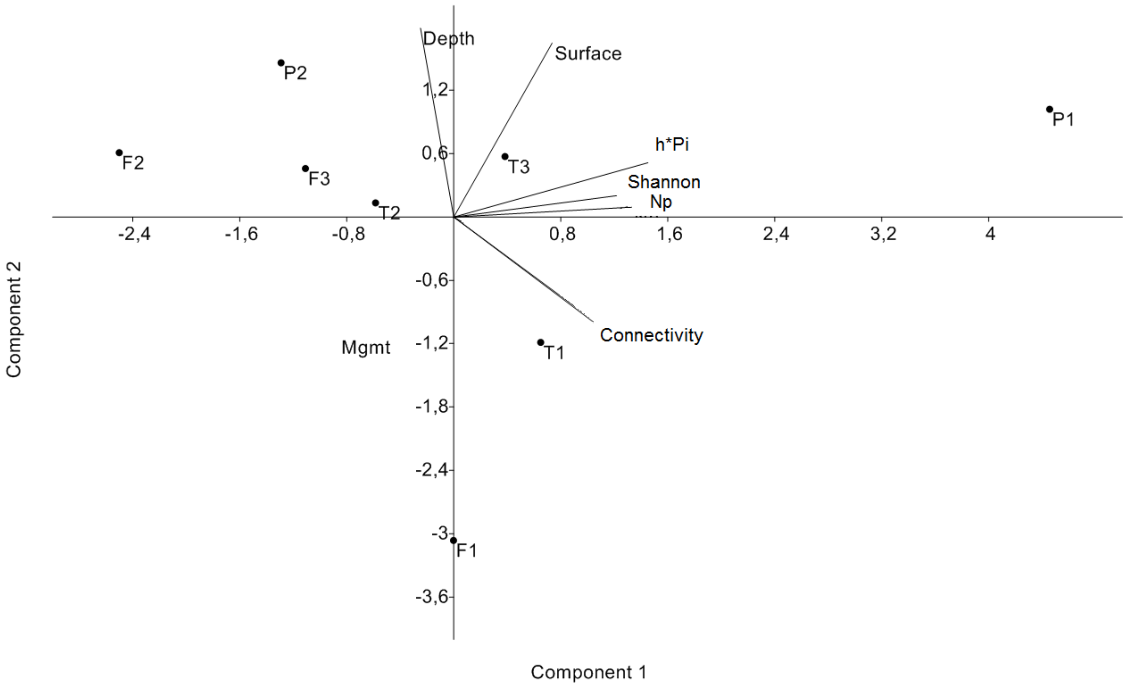
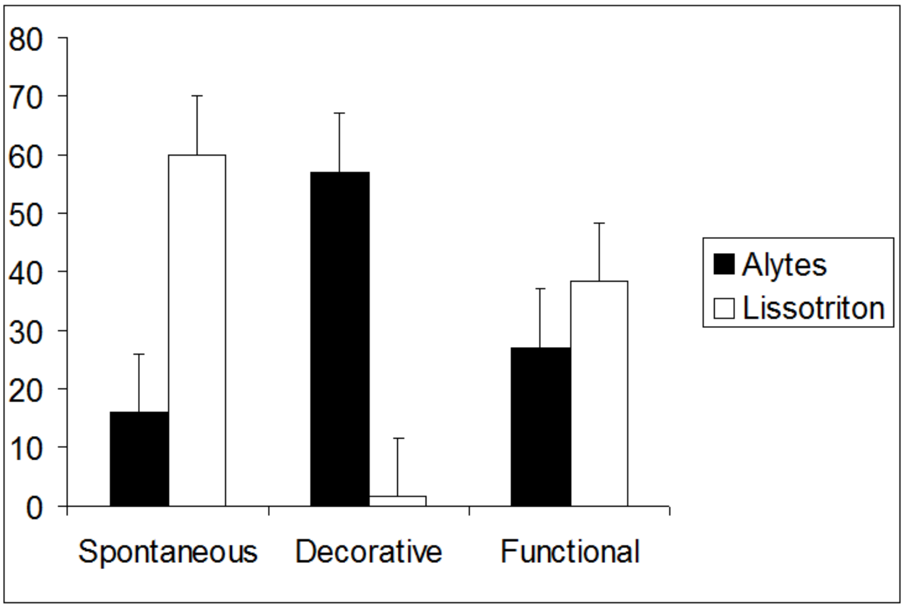


Figure 4. Distribution of the most abundant species *Alytes obstetricans* and *Lissotriton helveticus* measured as percentage (%) of each species at the three different categories of water points.



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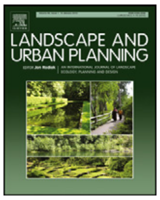

Rural road networks as barriers to gene flow for amphibians: Species-dependent mitigation by traffic calming

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ABSTRACT

Population structuring based on mitochondrial DNA variation along the rural landscape of the Trubia valley in North Iberia revealed significant association between road density and genetic distance between populations of two amphibian species, the midwife toad *Alytes obstetricans* and the palmate newt *Lissotriton helveticus*. Traffic calming (concentration of flows on minor rural roads at a few highways to decrease volumes and speeds) near urban settlements mitigates the population fragmentation of *L. helveticus* but not that of *A. obstetricans*, indicating that even small roads with low-intensity traffic act as barriers for the latter species. We suggest that the construction of passages for amphibians across rural roads would potentially mitigate population fragmentation of endangered species like the anuran *A. obstetricans*.

1. Introduction

Road networks cross landscapes and constitute a primary cause of habitat fragmentation for many species, subdividing populations with enormous demographic and genetic consequences (reviewed by Forman & Alexander, 1998). Although the negative effects of roads for ground-dwelling animals are often underestimated (Vos & Stumpel, 1996), for some species mitigating road-kill seems to be the most effective management solution, even more than controlling diseases and predation (Roger, Laffan, & Ramp, 2011). Construction of underway passages and other wildlife-crossing structures helps to mitigate wildlife mortality by enhancing road permeability and habitat connectivity, although the efficiency of such measures is difficult to evaluate (Glista, DeVault, & DeWoody, 2009). The spatial concept of the traffic-calmed rural area (TCRA), consisting of concentrating the diffused flows on minor rural roads at a few rural highways for a decrease of volumes and speeds within the region (Jaarsma, 1997; Jaarsma & Willems, 2002), has been also proposed as a solution for mitigating negative effects of road networks on rural spaces. Their effectiveness has been positively evaluated for mammals (Jaarsma, van Langevelde, & Botma, 2006) and models suggest that traffic calming can largely increase the population persistence of some species in landscapes with dense road networks (van Langevelde & Jaarsma, 2009).

Wildlife conservation in habitats modified by humans requires a careful examination of the spatial distribution of population variation of target species, for harmonizing management measures beneficial for as many of them as possible. The importance of physical barriers or habitat fragmentation is not identical for all species but it rather depends on population structuring of each one. Allowing physical connectivity between populations is crucial for conservation of sink populations in source–sink networks (Stevens & Baguette, 2008) and island subpopulations under continent–island models (Halley, Oldham, & Arntzen, 1996), but may be less important for patchy systems of discontinuous populations with little or null gene flow (Gibbs, 1993). Some groups of animals, like amphibians, are especially sensitive to habitat fragmentation, with habitat connectivity appearing to play a key role in their regional viability (Cushman, 2006). Due to their dependence on both freshwater and terrestrial conditions, amphibians have strict habitat specificity and physiological requirements (Duellman & Trueb, 1994; Kiesecker, Blaustein, & Belden, 2001; Vences & Wake, 2007). In the last decades amphibians have experienced dramatic declines worldwide and have become a protection target (Beebee & Griffiths, 2005; Stuart et al., 2004). Amphibians are generally poor dispersers (DeMaynadier & Hunter, 2000; Diego-Rasilla & Luengo, 2007; Gibbs, 1998; Joly, Miaud, Lehmann, & Grolet, 2001) and exhibit high costs of locomotion (Bennett & Licht, 1974; Gatten,

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Miller, & Full, 1992). Therefore landscape features often impact genetic structure of populations by disrupting dispersal patterns (Duellman & Trueb, 1994; Funk et al., 2005; Lynch & Duellman, 1997; Spear, Peterson, Matocq, & Storfer, 2005; Vences & Wake, 2007), although natural obstacles like mountain ridges not always represent significant barriers to gene flow. As an example, the Tsinling and Daba Mountains (China), with elevations up to 3100 m, do not significantly isolate Chinese wood frog *Rana chensinensis* populations (Zhan, Li, & Fu, 2009). Artificial barriers like roads, however, seem to be more important for amphibians (Cushman, 2006; Vos & Stumpel, 1996). Anurans are particularly sensitive to road traffic. This traffic, negatively impacts on their density (Fahrig, Pedlar, Pope, Taylor, & Wegner, 1995) through increased mortality (Hels & Buchwald, 2001), which seems to be also elevated in newts (e.g. Elzanowski, Ciesiolkiewicz, Kaczor, Radwanska, & Urban, 2009). Amphibian road mortality seems to be associated to traffic intensity, more clearly in Anurans than in salamanders (Mazerolle, 2004). The high site fidelity of amphibians (their phylopatry for the breeding pond), well documented for many species (Diego-Rasilla & Luengo, 2007; Joly & Miaud, 1989; Rowe, Beebee, & Burke, 2000), may enhance mortality because adults move from hibernation to native breeding sites (Hels & Buchwald, 2001). Although substitute breeding ponds can be progressively adopted at least by some species like *Bufo bufo* (Schlupp & Podloucky, 1994), it is clear now that the negative effect of roads is at least as great as the negative effect of deforestation for amphibians (Eigenbrod, Hecnar, & Fahrig, 2008).

Newts of the genus *Lissotriton* and *Alytes* toads are typical representative of urodeles and anuran taxa in Europe. There are several species of each group in almost all humid climates. They occupy similar habitats and larvae and metamorphs share common spaces of shallow water in lakes and ponds. In Spain they are declared vulnerable species and some of them are subjects of legal protection (Spanish National Catalogue of Threatened Species, R.D. 439/1990). *Alytes obstetricans* and *Lissotriton helveticus* are of special interest because their conservation demands the adoption of measures of protection. This is a category for the taxa which do not meet the criteria for inclusion in the threatened categories but have a particular value in scientific, ecological and/or cultural terms. In addition, *A. obstetricans* appears as near threatened (NT) in the Spanish Red Book of amphibians and reptiles. The phylogeography of the two species within the Iberian Peninsula is relatively well known. Several species of Discoglossidae, including the genus *Alytes*, are distributed in Iberian regions according to old climatic events and big mountain barriers which separated species or sub-species (Busack, 1986; Capula & Corti, 1993; Martínez-Solano, 2004; Martínez-Solano, Gonçalves, Arntzen, & García-París, 2004; Zangari, Cimmaruta, & Nascetti, 2006). The phylogeography of newts has also been studied. In Iberia there are eight species of newts, which likely colonized the Peninsula after the Last Glacial Maximum (Zagwijn, 1992). The spatial structure of their populations at small scales, however, is less known. To our knowledge there are no published studies of population structuring of Trituridae and Discoglossidae at the regional level in North Iberia. This area represents the southernmost edge for *L. helveticus* in the Northern hemisphere (Barbadillo, Lacomba, Pérez-Mellado, Sancho, & López-Jurado, 1999; Barbadillo & Sánchez-Herráiz, 1997; Gasc, 1997). Understanding the impact of road networks on their natural population structuring is necessary and urgent for regional and local management of rural spaces.

In this article we investigate spatial population structure of the midwife toad *A. obstetricans* (Laurenti, 1768), (Discoglossidae) and the palmate newt *L. helveticus* (Razoumowsky, 1789) in a rural landscape of the Atlantic North Iberia with a network of secondary and main rural roads which includes a rural highway (traffic calming) constructed near the main urban settlement of the region (Oviedo city). The genetic tool used was the variation of mitochondrial genes. The objective of this article is to determine whether significant barriers to gene flow is associated with road density and to evaluate the role of the traffic calming on mitigating population fragmentation, if any. In the light of the results obtained, we recommend some mitigation measures for protecting amphibians in the study region.

2. Materials and methods

2.1. Study area and road classification

The Trubia valley is a rural area of approximately 1200 km^2 located in Asturias, north Spain. The main economic activities in Trubia valley are agriculture and husbandry (Dopico, Linde, & Garcia-Vazquez, 2009). The city of Oviedo, capital of Asturias (200,000 inhabitants), is located in the north of the valley. The Trubia valley is crossed by a network of rural roads with a highway for traffic calming near Oviedo city (Fig. 1). Rural roads were constructed for communicating coal mines located up in the valleys with the central region in the XIX century (more than 150 years ago). The highway around Oviedo city was constructed 35 years ago.

Since traffic intensity is usually positively associated with road width (van Langevelde & Jaarsma, 2004; van Langevelde, van Dooremalen, & Jaarsma, 2009), studies of the effects of roads as barriers usually consider road width as an indicator of traffic intensity (e.g. Rico, Kindlmann, & Sedlacek, 2007). Roads across the studied region were therefore classified based on their width into three main categories:

(a) *Secondary roads*. These are rural one lane roads, paved, generally less than 5 m width and 2-way traffic; they support local traffic.
(b) *Main roads*. Larger than the secondary roads. They are two-lane roads (double direction), paved, with approximately 7–12 m width. They also support traffic in both directions.
(c) *Traffic calming highway*. It crosses the north of the valley, with a by-pass constructed around the city of Oviedo (an area about 20 km diameter).

The number of roads of each type that are located between two consecutive sampling sites were counted from maps at a scale 1:5000 and simplified at Fig. 1 to better understanding. The road crosses, measured as total number of roads between two sampling sites, was calculated by two alternative methods: unweighted (giving the same value of 1 to all roads), and weighted. We weighted road categories as follows: we assigned a value of 1 and 1.5 to secondary and main rural roads respectively, based on their relative width. As the objective of the traffic calming highways is to alleviate secondary roads from traffic intensity, traffic calming was considered to counteract these secondary roads which were allowed a 0.1 value, and only main rural roads were counted for population pairs with traffic calming sections between them.

2.2. Sampling

A total of 474 adult and larvae individuals of *L. helveticus* (275) and *A. obstetricans* (199) were collected from nine different locations of the Trubia Valley (Fig. 1 and Table 1). The samples analyzed were collected between October 2007 and July 2008 from different water points (troughs and ponds). A tiny fragment of tissue from the tail (about 1 mm) was cut off from each specimen with sterilized tweezers and scissors, and stored in absolute ethanol. The specimens were immediately released in the same sampling site. No animal was killed during sampling.

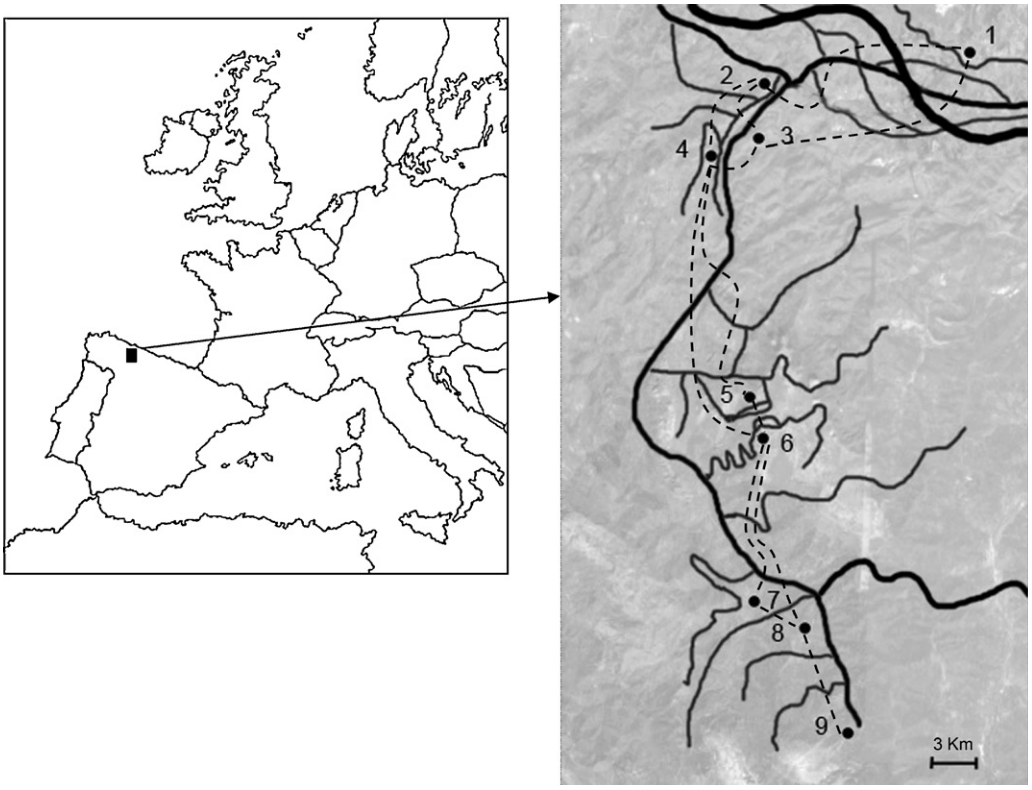

Fig. 1. Map showing the areas sampled (dots): 1, Oviedo; 2, San Andres; 3, Tuñon; 4, Santo Adriano; 5, Aciera; 6, Bermiego; 7, Villamarcel; 8, Cienfuegos; 9, Alto Quiros. Roads across the studied region are represented in decreasing order or thickness, as: traffic calming highway, main rural roads, and secondary roads. In dashed black lines, "least-cost paths" for amphibians traced taking into account the easiest way.

2.3. DNA extraction, PCR amplification and sequencing

Total DNA was extracted using a Chelex Resin (Bio-Rad laboratories) protocol following Estoup, Largiader, Perrot, and Chourrout (1996) and kept at 4 °C until analysis. Partial sequences of two mitochondrial (mt) genes were used for genetic analyses: a 671 base pairs (bp) long fragment of the subunit I of the cytochrome c oxidase (COI) and a 777 bp long fragment of the cytochrome b (Cyt b). The latter was only sequenced for *A. obstetricans*, since this species exhibited very low polymorphism in the COI fragment examined. PCR reactions were carried out in a total volume of 40 µl containing 5× Go Taq® Flexi Buffer, 2.5 and 2.0 mM $MgCl_2$ for COI and Cyt b respectively, 250 µM of each dNTP, 1 µM of each primer, 1 unit of Go Taq® Polymerase 5 U/µl (Promega) and 50 ng of DNA template. They were performed in a GeneAmp® PCR system 2700 (Applied Biosystems) with the following conditions: an initial denaturing step at 95 °C for 5 min, followed by 35 cycles of denaturing at 95 °C for 30 s, annealing (for 30 s) at 53 °C and 58 °C for Cyt b (Moritz, Schneider, & Wake, 1992; Smith & Patton, 1993) and COI (Ward, Zemlak, Innes, Last, & Hebert, 2005) respectively, and an extension at 72 °C for 30 s, followed by a final extension at 72 °C for 15 min.

PCR products were visualized in 2% agarose gels stained with ethidium bromide. Bands were excised from the gel and DNA was purified with a Wizard® SV Gel and PCR Clean-Up System (Promega) before sequencing. Automated fluorescence sequencing was run on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems) with BigDye 3.1 Terminator system, in the Unit of Genetic Analysis of the University of Oviedo (Spain).

2.4. Sequence editing

All the sequences were visually checked, edited using the BioEdit Sequence Alignment Editor software (Hall, 1999) and aligned with the ClustalW application (Thompson, Higgins, & Gibson, 1994) included in BioEdit. To ensure accuracy all the sequences were compared with those existing in the GenBank database

Table 1
Sampling sites considered within the rural area of the Trubia Valley (North Iberia). Altitude, meters above sea level, GPS coordinates. *N*, sample size of *Alytes obstetricans* and *Lissotriton helveticus* as *A.o.* and *L.h.* respectively.

Site number	Name	Altitude	GPS coordinates	*N* (*A. o.*)	*N* (*L. h.*)
1	Oviedo	225	N43.22043 W5.52097°	52	77
2	San Andres	135	N43.18973 W5.59501°	17	19
3	Tuñon	175	N43.17477 W5.58774°	15	11
4	Santo Adriano	350	N43.16451 W5.59513°	17	8
5	Aciera	650	N43.12476 W5.59641°	14	22
6	Bermiego	668	N43.11699 W5.58854°	26	28
7	Villamarcel	773	N43.08327 W5.58140°	10	1
8	Cienfuegos	765	N43.08023 W5.55939°	28	77
9	Alto Quiros	890	N43.05411 W5.54294°	20	32
Total				199	275

(http://www.ncbi.nlm.nih.gov/GenBank). Since both mitochondrial regions analyzed correspond to protein coding sequences, the presence of stop codons or indels, which could reveal pseudogene sequences, was checked with MEGA 4.1 (Tamura, Dudley, Nei, & Kumar, 2007).

2.5. Population genetic diversity

To investigate demographic history of the amphibian populations considered, we employed both Tajima's *D* and Fu's *F*s tests under the assumption of neutrality for the mt COI region. The Tajima's *D* was originally developed to test the neutral hypothesis. However, since Tajima's *D* is based on the expectation of a constant population size at mutation-drift equilibrium, it is also widely used to detect changes in population size (Aris-Brosou & Excoffier, 1996). Both tests assess whether the observed frequencies of segregating mutations are compatible with the frequencies expected under the standard neutral model. Tajima's *D* and Fu's *F*s tests were computed with the program ARLEQUIN v3.01 (Excoffier, Laval, & Schneider, 2005).

To detect whether the populations have undergone demographic and/or range expansion, we compared the observed frequency of pairwise sequence differences (mismatch distribution) to the expected distribution under a sudden expansion model with the SSD statistic (sum of squared differences) (Slatkin, 1995) using 1000 bootstrap replicates, in ARLEQUIN.

Haplotype diversity (Hd) and nucleotide diversity (π) for each population were calculated with the program DnaSP v.4.50.3 (Rozas, Sanchez-DelBarrio, Messeguer, & Rozas, 2003).

To determine the model of sequence evolution that best fits our datasets, and to calculate the proportion of invariable sites and the value of the gamma distribution shape parameter, the jModelTest v.3.06 software (Posada, 2008) was employed. Population pairwise F_{ST} values were calculated employing Tamura-Nei distances (Tamura & Nei, 1993) for *Alytes* and Kimura two-parameters model (Kimura, 1981) and a value of the gamma distribution shape parameter of 0.011 (results from the jModelTest) with the program ARLEQUIN.

Haplotype networks were chosen to illustrate the spatial distribution of the variants found and for inferring relationships between haplotypes. Median-Joining (MJ) networks were constructed with the program Network 4.5.1.0 (http://www.fluxus-engineering.com).

To perform an analysis of the molecular variance (AMOVA; Excoffier, Smouse, & Quattro, 1992) ARLEQUIN was also employed, to test its hierarchical distribution at three levels: within populations (Φ_{ST}), among populations (Φ_{SC}) and among groups of populations (Φ_{CT}). This analysis was performed to investigate how the overall genetic variation was distributed among groups of populations and evaluates the statistical significance of a genetic structure defined *a priori*. Different population groups were tested according to their geographical situation in the valley to find a significant population grouping. For both analyses (pairwise F_{ST} and AMOVA) the statistical significance was tested through 99,999 permutations.

A Mantel test was performed to test isolation by distance using the program ARLEQUIN. This test estimates the amount of correlation between two matrices, one with geographic distances (as minimum distance in km) and other with genetic distances ($F_{ST}/(1-F_{ST})$). Significance level was estimated with 10,000 permutations.

2.6. Identification of spatial genetic barriers

We employed Monmonier's maximum difference algorithm (Manni, Guerard, & Heyer, 2004) to identify and quantify spatial genetic discontinuities, using the program BARRIER v.2.2 (Manni et al., 2004). Geographical coordinates were used for each sample and connected by Delauney triangulation with the pairwise F_{ST} genetic matrix generated from the above cited program ARLEQUIN. Putative spatial genetic boundaries were identified across the studied landscape (Manni et al., 2004).

Barriers can be simple or composed by more than one segment. The program BARRIER assigns distance values for each significant segment of the barriers, corresponding to genetic distances between population pairs. Correlations of those genetic distances with the road density (weighted and unweighted number of roads separating the two populations of each pair) and their statistical significance were calculated with the statistics Spearman's *r* of ranks, employing PAST (PAlaeontological STatistics, version 1.90; Hammer, Harper, & Ryan, 2001) software. The same program was employed for testing the correlation between the significant genetic distances identified with BARRIER and the linear geographic distance between the corresponding population pairs.

3. Results

After editing and aligning all the sequences, total datasets of 199 and 275 sequences were obtained for *A. obstetricans* and *L. helveticus*, respectively. For *A. obstetricans* we found 10 haplotypes and for *L. helveticus* we found 15 (Table 2a and b respectively). These sequences were deposited and are available in the GenBank database under the Accession numbers HM032694–HM032700 and HM020695–HM020700 (for *A. obstetricans*) and GQ374500–GQ374509 and HM032701–HM032705 for (*L. helveticus*). As said above, the haplotypes for *A. obstetricans* were obtained from a concatenation of COI and Cyt b sequences, and the haplotypes of *L. helveticus* were inferred only from COI sequences. In both cases, some of them were shared by more than one population, while others were private haplotypes (i.e. present in a single location). One singleton (haplotype present only in one individual) was found for *A. obstetricans* and six for *L. helveticus* (Fig. 2 and Table 2).

Haplotype diversity (H_d) values were quite different among the populations sampled (Table 3), with maximum values in Oviedo for *A. obstetricans* (Table 3a) and in Santo Adriano for *L. helveticus* (Table 3b). Some populations were monomorphic (San Andres and Aciera for *A. obstetricans*, San Andres and Villamarcel for *L. helveticus*). Significant signals of demographic expansion (negative Fu's *F*s values) were found for Oviedo and Santo Adriano palmate newts (Table 3b), but not for any midwife toad population (Table 3a). Although sample sizes were variable among localities for both species, significant correlation between sample size and haplotype diversity or number of haplotypes per site was not found for any species.

Different haplotype networks were obtained for each species (Fig. 2). The network shape obtained for *A. obstetricans* (Fig. 2a) showed three high frequency haplotypes and some less frequent variants separated by one or more mutational steps. Few haplotypes were shared by more than two populations, and their distribution did not follow a geographical pattern. Conversely, *L. helveticus* exhibited a star-like network shape (Fig. 2b), with a central and most common haplotype from which many less frequent haplotypes (or singletons) derive, generally by only one mutational step.

The models of evolution obtained with ModelTest as best fitting our datasets were the Tamura-Nei (Tamura & Nei, 1993) for *A. obstetricans* and the Kimura two-parameter model (Kimura, 1981) for *L. helveticus*, with a gamma distribution shape parameter of 0.011 for *L. helveticus*. Population pairwise F_{ST} (Table 4) were calculated for each species following the respective models and

Table 2
Number of individuals of each haplotype found in the studied populations of *A. obstetricans* (a) and *L. helveticus* (b).

a

		Haplotypes									
Locations	*N*	1	2	3	4	5	6	7	8	9	10
Oviedo	52	11	3	20	3	1	5	9			
San Andres	17								17		
Tuñon	15	3							8	4	
Santo Adriano	17	5					1		11		
Aciera	14								14		
Bermiego	26	16							10		
Villamarcel	10			3					4		3
Cienfuegos	28	4		11					12		1
Alto Quiros	20	6							14		
Total	199	45	3	34	3	1	6	9	90	4	4

b

		Haplotypes															
Locations	*N*	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Oviedo	77	50	12	8	1							2	1	1	1	1
San Andres	19	19														
Tuñon	11	7				3				1						
Santo Adriano	8	4		1		2					1					
Aciera	22						11	11								
Bermiego	28	10						18								
Villamarcel	1	1														
Cienfuegos	77	37			6				34							
Alto Quiros	32	20							12							
Total	275	148	12	9	7	5	11	29	46	1	1	2	1	1	1	1

uploaded in the software BARRIER for identifying and quantifying genetic distances between population pairs in the studied spatial landscape. Significant correlation between genetic and linear geographic distances was not found for any of the species studied, and correlation coefficients of the Mantel test were not significant ($R^2 = 0.0009$ for *Alytes* and 0.0004 for *Triturus*, $P \gg 0.05$ for both). This indicates that these species do not fit a population structure consistent with a model of isolation by distance in the region considered (there is no association between genetic and geographic distance).

Table 3
Genetic diversity in the populations sampled from the Trubia valley (Spain). *N*, number of sequences; *H*, number of haplotypes; H_d, haplotype diversity; π, nucleotide diversity; *D*, Tajimaĭs *D*; *Fs*, Fu's *Fs*; SSD, sum of squared differences.

a[a]

Sampling location	*N*	*H*	H_d	*D*	*Fs*	SSD
Oviedo	52	7	0.77602	−0.15290	−0.22003	0.00331
San Andres	17	1	0.00000	0.00000	0.00000	0.00000[**]
Tuñon	15	3	0.64762	1.05589	2.73696	0.09185
Santo Adriano	17	3	0.52206	−0.67315	0.24066	0.00507
Aciera	14	1	0.00000	0.00000	0.00000	0.00000[**]
Bermiego	26	2	0.49231	1.4372	1.52295	0.02030
Villamarcel	10	3	0.73333	1.15198	1.15966	0.00508
Cienfuegos	28	4	0.66402	0.96867	0.53353	0.02911
Alto Quiros	20	2	0.44211	1.02588	1.16902	0.01185
Total	199	10	0.71448	0.53484	0.79364	0.01851

b[b]

Sampling location	*N*	*H*	H_d	*D*	*Fs*	SSD
Oviedo	77	9	0.54887	−0.87187	−3.79075[*]	0.00074
S. Andres	19	1	0.00000	0.00000	0.00000	0.00000[**]
Tuñon	11	3	0.56364	−0.28956	−0.31406	0.03052
Santo Adriano	8	4	0.75000	0.07663	−0.90342[*]	0.00633
Aciera	22	2	0.52381	3.99872	5.7414	0.30768
Bermiego	28	2	0.47619	1.36038	1.50279	0.01718
Villamarcel	1	–	–	–	–	–
Cienfuegos	77	3	0.57553	2.93628	2.96512	0.10216
Alto Quiros	32	2	0.48387	4.42342	3.33164	0.18238
Total	275	15	0.66950	1.29267	0.94808	0.07189

Values involving Villamarcel (7) for *L. helveticus* were not calculated because of its small sample size.

[a] *Alytes obstetricans*.

[b] *Lissotriton helveticus*.

[*] Significance levels: $P < 0.05$.

[**] Significance levels: $P < 0.01$.

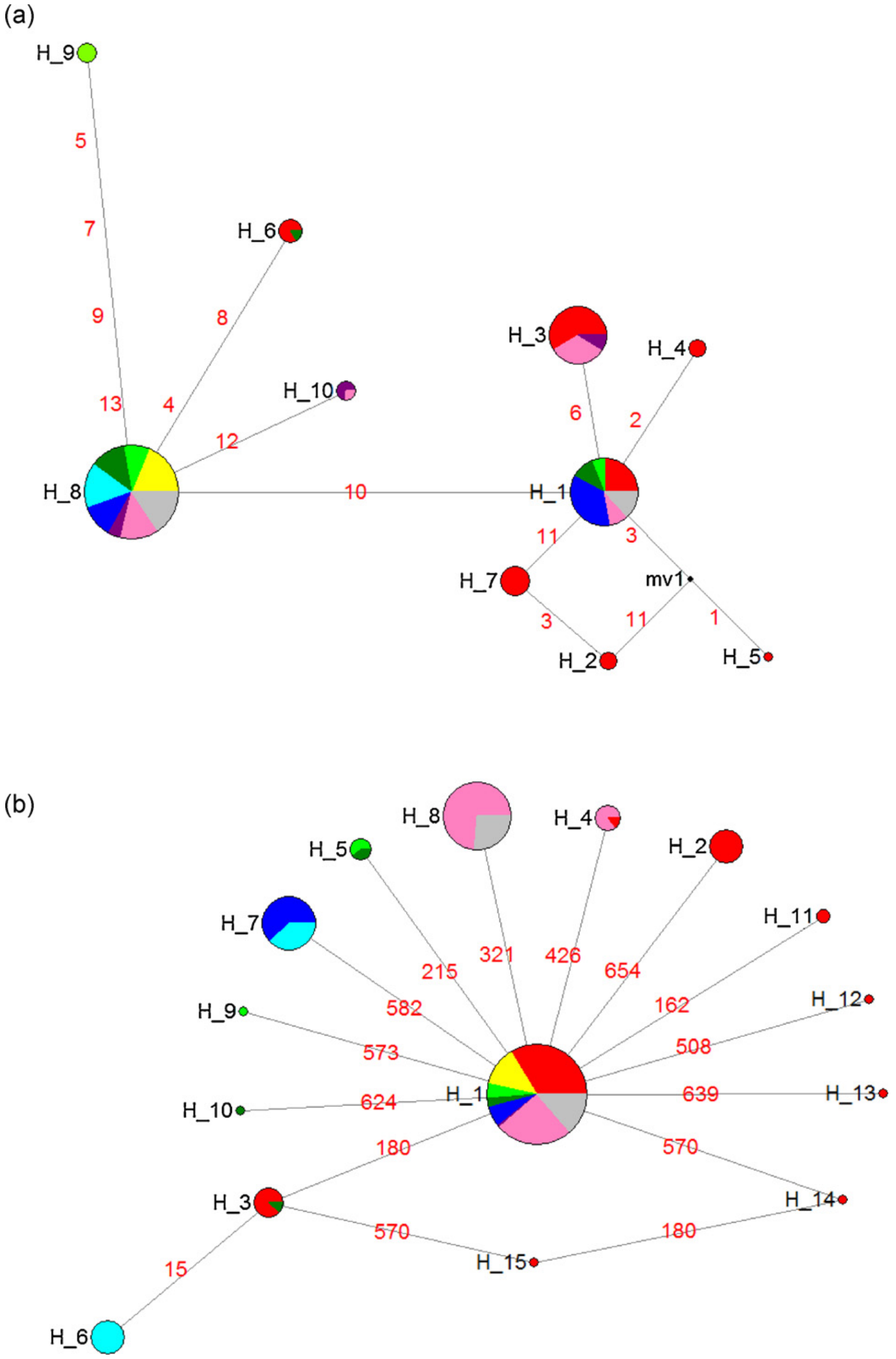

Fig. 2. Median-Joining Network constructed from the haplotypes found for (a) midwife toad and (b) palmate newt in the region studied. Each circle represents a different haplotype with size proportional to its relative frequency. Numbers on lines represent number of mutations required to explain transitions among haplotypes. Median vector is labeled as mv1. Red, Oviedo; yellow, San Andres; bright green, Tuñon; dark green, Santo Adriano; light blue, Aciera; dark blue, Bermiego; purple, Villamarcel; pink, Cienfuegos; grey, Alto Quiros. a, *Alytes obstetricans*; b, *Lissotriton helveticus*. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

The program BARRIER identified seven significant spatial barriers to gene flow in the region studied for *A. obstetricans* (Fig. 3a, barriers A to G), some of them composed by more than one segment. All populations were separated by such barriers, except two, Cienfuegos and Villamarcel (8 and 7 respectively; Table 5). This high number of significant barriers was consistent with the haplotype network shape. On the other hand, four main barriers were found for *L. helveticus* (Fig. 3b, barriers A to D). The barrier A occurred between 6 and the group 7–8–9 (Bermiego and Villamarcel–Cienfuegos–Alto Quiros). The barrier B was located between the geographically close 5 and 6 populations (Aciera and Bermiego), the barrier C between Aciera and Tuñon-Santo Adriano (5 and 3–4), and the barrier D between 2 and 3–4 (San Andres and Tuñon-Santo Adriano).

Association of genetic barriers with road density was explored with Spearman's correlation test. The intensity of the barriers between population pairs, measured as genetic distances estimated by the BARRIER software (Table 5), was significantly associated

Table 4
F_{ST} values (below diagonal) and their significance (above diagonal) among pairs of populations. For names of populations see Table 1.

a[a]

	1	2	3	4	5	6	7	8	9
1	–	0.00000	0.00000	0.00000	0.00000	0.00000	0.00079	0.00149	0.00000
2	0.43939	–	0.00178	0.04029	0.99990	0.00000	0.00050	0.00040	0.02317
3	0.34861	0.21972	–	0.03782	0.05079	0.00198	0.02782	0.00069	0.02445
4	0.26977	0.17857	0.14359	–	0.04703	0.05099	0.05108	0.01069	0.85516
5	0.42550	0.00000	0.19411	0.15626	–	0.00000	0.00099	0.00139	0.05999
6	0.17678	0.54810	0.27886	0.12783	0.52703	–	0.00782	0.00990	0.04168
7	0.21484	0.31333	0.15518	0.10724	0.27835	0.23795	–	0.16988	0.04703
8	0.10819	0.37663	0.25544	0.15965	0.35585	0.14218	0.04538	–	0.01297
9	0.29057	0.24276	0.17127	−0.04357	0.22006	0.14319	0.14163	0.17722	–

b[b]

	1	2	3	4	5	6	7	8	9
1	–	0.14438	0.00852	0.03876	0.00000	0.00000	–	0.00000	0.00000
2	0.02384	–	0.03962	0.01897	0.00044	0.00000	–	0.00018	0.00456
3	0.13545	0.20499	–	0.68685	0.00172	0.00004	–	0.00032	0.00343
4	0.09104	0.16389	−0.05466	–	0.01075	0.00004	–	0.00106	0.00481
5	0.40488	0.37009	0.34179	0.27143	–	0.00037	–	0.00000	0.00000
6	0.44062	0.58372	0.50472	0.48015	0.32984	–	–	0.00000	0.00000
7	–	–	–	–	–	–	–	–	–
8	0.27291	0.25035	0.29232	0.28665	0.51445	0.52005	–	–	0.45884
9	0.22543	0.29450	0.28199	0.26392	0.44797	0.54678	–	−0.00587	–

Values involving Villamarcel (7) for *L. helveticus* were not calculated because of small sample size.

[a] *Alytes obstetricans*.

[b] *Lissotriton helveticus*.

with the unweighted road density ($r = 0.643$, $P = 0.024$) as well as strongly correlated with the number of secondary rural roads ($r = 0.670$, $P = 0.017$) for *A. obstetricans*, therefore all types of roads may account with similar intensity as obstacles for midwife toad gene flow. Significant association was not found between weighted road density and genetic distance for this species ($r = 0.175$, $P = 0.587$, not significant), indicating that the traffic calming highway does not counteract habitat fragmentation by minor roads for

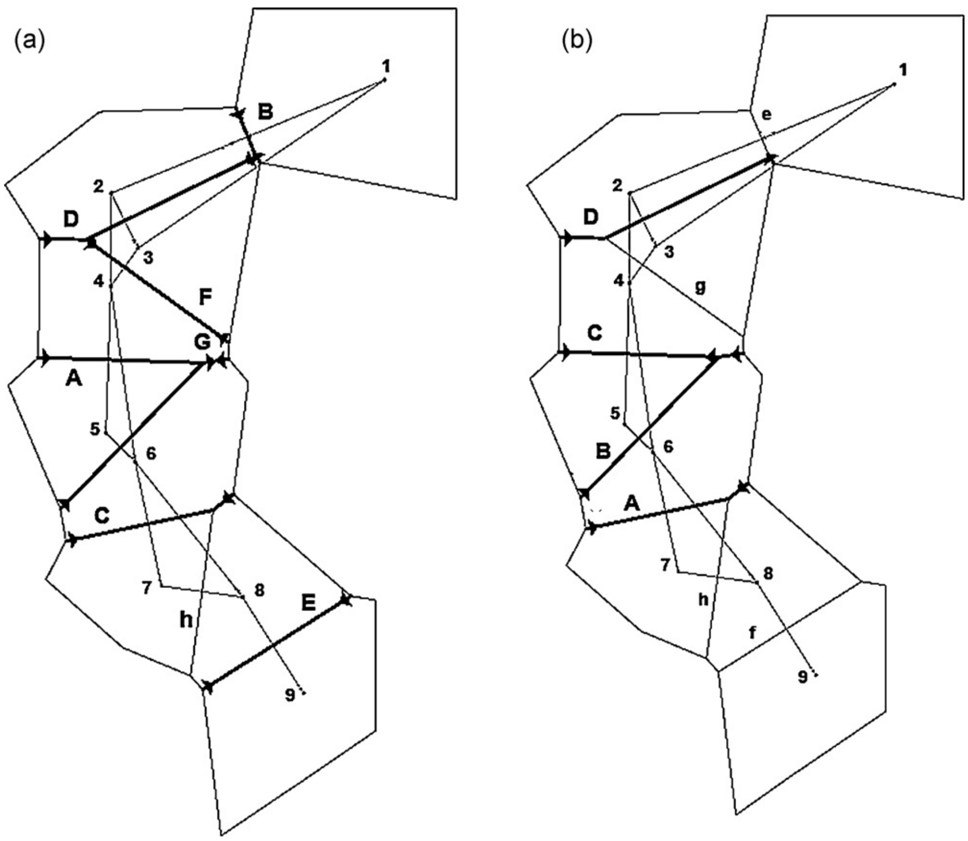

Fig. 3. Genetic barriers predicted by BARRIER (version 2.2). Barriers with distance value > 0.05 (significant) are remarked as double width and labeled with capital letters which are ordered by magnitude from most to least significant (A–h). Sampling areas: 1, Oviedo; 2, San Andres; 3, Tuñon; 4, Santo Adriano; 5, Aciera; 6, Bermiego; 7, Villamarcel; 8, Cienfuegos; 9, Alto Quiros. a, *Alytes obstetricans*; b, *Lissotriton helveticus*.

Table 5
Number and category of rural roads, road density, genetic distance estimated with BARRIER between pairs of contiguous sampling locations for each species and distance between locations measured in km (geographic distances, as linear distances or "least-cost paths" for amphibians).

Locations	Number of roads			Road density		Genetic distances		Geographic distances
	Secondary	Main	Traffic calm	Weighted	Unweighted	*A. obstetricans*	*L. helveticus*	
1–2	4	2	1	3.4	7	0.439	0.039	11.4
1–3	4	1	1	1.9	6	0.349	0.094	12.3
2–3	2	1	0	3.5	3	0.22	0.232	2.92
2–4	3	0	0	3	3	0.179	0.195	4.14
3–4	1	1	0	2.5	2	0.144	−0.044	1.99
4–5	2	1	0	3.5	3	0.156	0.273	7.92
4–6	2	1	0	3.5	3	0.128	0.432	9.44
5–6	4	0	0	4	4	0.527	0.379	1.79
6–7	1	1	0	2.5	2	0.238	–	5.89
6–8	2	1	0	3.5	3	0.142	0.448	7.86
7–8	1	0	0	1	1	0.045	–	3.26
8–9	2	0	0	2	2	0.177	−0.009	5.1

this species. For *L. helveticus*, however, strongly significant correlation was found only between genetic distance and weighted road density ($r = 0.794$, $P = 0.006$) but not with unweighted road density ($r = 0.221$, $P = 0.540$, not significant), indicating that not all roads have the same value as barriers and that traffic calming counteracts fragmentation due to small roads. No significant correlation was obtained between geographic distance and genetic distance for any of the species ($r = 0.035$, $P = 0.914$ for *A. obstetricans* and $r = 0.042$, $P = 0.907$ for *L. helveticus*, both not significant), allowing to discarding geographic distance as the main factor for population differentiation, as already suggested by the absence of IBD in the two species reported above.

Consistently with high spatial fragmentation, for *A. obstetricans* we did not find any AMOVA that yielded significant population grouping, with all populations being statistically different from each others. For *L. helveticus* the spatial population structuring revealed from the BARRIER results was statistically significant (Table 6). The five population groups defined by BARRIER reunited populations by geographical proximity: one group contained Oviedo and San Andres, another group Tuñon and Santo Adriano, other with the three populations of the upper valley, and finally Aciera and Bermiego as independent units. Variation among these five groups of populations gathered 37.6% variance and was significant ($\Phi_{CT} = 0.233$, $P = 0.002$; Table 6), whereas variation among populations within groups was negligible. On the other hand, most molecular variation was explained by within population variability (63%), which agrees with the high levels of haplotype diversity found in many samples (Table 3b).

4. Discussion

According to Cushman (2006), habitat fragmentation is one of the main anthropogenic impacts on amphibians. The results obtained in the present work support such statement. Although at this type of geographical scale microsatellites are often used as genetic markers because of their high variability, in the present case the variation at mitochondrial level has been enough to detect barriers between populations; probably the time elapsed since the construction of the rural roads, more than 150 years (generation time for these amphibians is one year), was sufficient for allowing population differentiation. At a relatively small regional scale, the genetic structure of the midwife toad populations considered in our study is highly impacted by rural roads, even by small secondary roads. Population seems to be isolated, with very little connectivity between them. Such a high level of fragmentation, significantly associated with road density, strongly contrasts with the natural structuring of toad populations in networks interconnected by gene flow, following source–sink models (Halley et al., 1996; Stevens & Baguette, 2008). The absence of association between genetic and geographic distance found in our study, together with strong significant association between genetic distance and roads, is a compelling indication of the impact of road density on midwife toad (as it is also for palmate newt). Narrow secondary rural roads are sufficient for isolating close populations, even if their traffic intensity has been alleviated by traffic calming as suggested by correlation with unweighted road density (Table 5), and apparently the impact of secondary and main roads is similar. The importance of minor roads and their impact on habitat destruction and traffic mortality have been reported yet by van Langevelde et al. (2009), suggesting that the success of mitigation on roads drastically increases when major and minor roads are integrated in the planning of traffic flows. Moreover, very low dispersal capacity of most of Anurans (Beebee, Denton, & Buckley, 1996; Blaustein, Wake, & Sousa, 1994; Gibbs, 1998) may be the cause of high road mortality (Hels & Buchwald, 2001) and particularly strong impact of roads on this group of animals (Eigenbrod et al., 2008).

In contrast, habitat fragmentation by roads did not have the same impact for the palmate newt *L. helveticus* in the region studied, in agreement with differential road mortality of anurans and urodeles found by other authors (Fahrig et al., 1995; Mazerolle, 2004). We found significant correlation between weighted road density and genetic distances between populations. Thus, the impact of high road density on *L. helveticus* population fragmentation may be alleviated by traffic calming measures, as suggested for mammals (Jaarsma et al., 2006; van Langevelde & Jaarsma, 2009).

Becker, Fonseca, Haddad, Batista, and Prado (2007) identified "habitat split" – defined as human-induced disconnection between habitats used by different life history stages of a species – which forces forest-associated amphibians with aquatic larvae to make risky breeding migrations between suitable aquatic and terrestrial

Table 6
Results of the analysis of the molecular variance (AMOVA) for *Lissotriton helveticus*.

Source of variation	Sum of squares	Variance components	% variation	*P*-value
Among groups	45.915	0.23338	37.62	0.00196
Among populations within groups	1.023	−0.00169	−0.27	0.41251
Within populations	103.382	0.38865	62.65	0.00000
Total	150.320	0.62035		

habitats. Both midwife toad and palmate newt have aquatic larvae, and road networks have high effects on their population declines, as demonstrated in the present work and reported for other anurans and urodeles (e.g. Elzanowski et al., 2009; Fahrig et al., 1995). However, and being all low dispersers, it is now clear that the effect of roads is not identical for all species (Mazerolle, 2004). The two species seem to have experienced very different recent evolution in the studied area. *L. helveticus* exhibits a star-like haplotype network, which is considered a signal of recent population expansion (Lee & Boulding, 2007; Mirol, Routtu, Hoikkala, & Butlin, 2008). This haplotype network shape, together with significant indicators of demographic expansion such as negative *F*s and SSD values, suggest that the palmate newt is undergoing positive population growth in the region studied, despite progressive urbanization in the last century (Dopico et al., 2009). On the other hand, *A. obstetricans* exhibited signs of demographic growth only in one area, the city of Oviedo, which is quite consistent with strong population fragmentation in rural zones. The two species seem thus to exhibit differential sensitivity to habitat fragmentation, a feature reported for other assemblages of amphibians (Birx-Raybuck, Price, & Dorcas, 2010; Gibbs, 1993), and consequently mitigation actions should be also varied when conservation of amphibians is a priority.

Traffic calming could be taken into account as a possible measure to alleviate fragmentation of amphibian's habitat. From our results, traffic calming seems to benefit differentially the two species considered, since a positive correlation between weighted road density and the intensity of barriers between populations was obtained for *L. helveticus* but not for *A. obstetricans*. For species highly sensitive to habitat fragmentation as *A. obstetricans*, narrow secondary roads act as a barrier to gene flow, even with traffic calming mitigation. But for species less affected by habitat fragmentation, as the palmate newts, traffic calming helps to mitigate fragmentation, likely contributing to their conservation.

The present results have implications for designing wildlife conservation measures in landscape planning. Constructing passages for amphibians under rural roads, small subterranean corridors like those constructed under highways (Glista et al., 2009; Lesbarreres, Lode, & Merila, 2004; Noël & Lapointe, 2010), would facilitate anurans crossing and connect isolated populations. In addition and in parallel, traffic calming solutions (Jaarsma, 1997; Jaarsma & Willems, 2002) would contribute to reduce population fragmentation in newts. Combining multiple restoration actions seems to be the best strategy for mitigating road impacts (e.g. Cuperus, Canters, de Haes, & Friedman, 1999) and would benefit not only amphibians but also the rest of wild fauna inhabiting rural spaces.

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Capítulo 5

Species-dependent impact of rivers on amphibian population connectivity at regional scale: implications for conservation.

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ABSTRACT

The biogeographic patterns of six pond- breeding amphibian species (three anurans and three urodeles) were investigated to determine the effect of habitat discontinuities in a patchy landscape crossed by several rivers at the Cantabric region of North Iberia. The Cytochrome Oxidase c subunit I gene was amplified from more than 400 individuals and employed as a molecular tool for identifying main spatial barriers to gene flow. Rivers were identified as geographical barriers to gene flow for all the analyzed species except for the Iberian toad (*Bufo bufo*). The degree of isolation was weaker in some species like *Discoglossus galganoi* and stronger for others like *Alytes obstetricans* and *Triturus marmoratus*. As a consequence, different population structuring was found at regional scale for the analyzed species. Spatial coincidence of different genetic lineages suggests a possible secondary contact in the region for *Lissotriton boscai* and *Triturus marmoratus.* Conservation measures for amphibians would need to consider rivers as potential barriers to population connectivity, in a different extent for each species. When setting up management guidelines, special attention should be paid to the specific requirements of each amphibian species.

Keywords: *Lissotriton helveticus, Lissotriton boscai, Triturus marmoratus, Alytes obstetricans, Discoglossus galgaoni, Bufo bufo,* habitat fragmentation, population genetic structure.

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1. INTRODUCTION

Amphibians are especially sensitive to population declines, reductions at the distribution areas and local population extinctions (Barinaga 1990), due to features like low dispersal capacity (Blaustein et al. 1994; Semlitsch 2003), homing behaviour (Sinsch 1990; Diego-Rasilla and Luengo 2002; Diego-Rasilla et al. 2008) and critical dependence on water availability and humid environments (Duellman and Trueb 1994). They are extremely sensitive to the habitat fragmentation by reason of their spatial and temporal population dynamics (Gibbs 1998). Since dispersal is likely essential to long-term population persistence (Marsh and Trenham 2001; Cushman 2006), the knowledge of barriers to gene flow is essential for planning conservation measures and understanding the regional dynamics of these vulnerable animals.

Most studies on amphibian species are focused on large-scale geographical dimension phylogenetic analyses (Martínez-Solano 2004; Martínez-Solano et al. 2006; Gonçalves et al. 2007). However, investigations on population genetics at fine-scale geographical scope are necessary for amphibian conservation. Basic information on genetic variation and patterns of gene flow are crucial for scientifically informing about management and landscape planning measures at regional and local level, in a closer approach for finding the best local solutions for local situations and problems. Amongst other geographical features, rivers have been documented to act as efficient barriers to gene flow in amphibian species (Zeisset and Beebee 2008). They seem to shape population structure in some species like túngara frogs (Lampert et al. 2003), American spotted frogs (Funk et al. 2005), montane frogs (Monsen and Blouin 2003), golden-stripped salamanders (Alexandrino et al. 2000) and others, although not all the rivers seem to be principal drivers of current amphibian population features and their role as a barrier has been discussed (e.g. Gascon et al. 2000).

To increase the understanding of the influence of patchy landscapes on amphibian dispersal, we investigated gene flow for six pond-breeding species from the northwest of the Iberian Peninsula. The region studied corresponds to the archaeological Cantabric region, known as an important refugial area during the Last Glacial Maximum (Zagwijn 1992; Martínez-Solano 2006). It is crossed by a mountain range, the Cantabric Mountains, with abundant pre-littoral valleys and hills. The region has a temperate humid Atlantic climate, it is rainy and is latitudinally crossed by many short but permanent rivers draining South-North (Figure 1). Therefore there are potential longitudinal barriers which may have shaped the population structure of species with relatively low dispersal capacity as amphibians are (e.g. Blaustein et al. 1994; Semlitsch 2003).

Three out of 8 anurans (*Alytes obstetricans*, *Bufo bufo* and *Discoglossus galganoi*) and three out of six urodeles (*Lissotriton boscai*, *Lissotriton helveticus* and *Triturus marmoratus*) occurring in the region were chosen for this study. They share the available aquatic habitats and many of them co-habit the same water point (Garcia-Gonzalez and Garcia-Vazquez 2011). All of them are considered of Least Concern by the IUCN Red List of Threatened Species (v.2011.1.), but population trends of *A. obstetricans*, *D galganoi* and *T. marmoratus* are decreasing in Iberia and all of them except *B. bufo* are considered of special interest being worthy of particular attention in terms of their scientific, ecological, cultural importance or their singularity. The Spanish Herpetological Association (AHE; http://www.herpetologica.es/) requests for *A. obstetricans* being reviewed and listed as Vulnerable, and *B. bufo* included in the List of Wildlife Special Protection Regime. Their population structure is not well known at small scale. Only a few studies are focused at the fine-scale population genetics for these species. A network of populations interconnected with gene flow has been described for *Lissotriton boscai* (Martínez-Solano et al. 2006) and population expansion was detected for northwest Iberian populations of *L. helveticus* (Recuero and García-París 2011). Unidirectional gene flow from small populations towards a central one (source-sink) has been shown in *Bufo bufo* (Martínez-Solano and González 2008) and restricted gene flow with isolation by distance was described for northwest Iberian *D. galganoi* of the northwest of the Iberian Peninsula (Martínez-Solano 2004). Finally, in a fine-scale study of *T. marmoratus* most ponds were identified as significant genetic entities, clustering by spatial proximity (Jehle et al. 2005).

The aim of this study was to evaluate population diversity and inter-population gene flow in North Iberia for the six model species with a focus on the role of river networks as barriers to gene flow, employing the mitochondrial DNA region coding for the Cytochrome Oxidase c subunit I (COI) as a genetic marker. This region is the main marker of Barcoding projects (e.g. Hebert et al. 2003). The specific population pattern of each species will be traced and the results will be focused on setting up, if possible, recommendations for management of these valuable and vulnerable wild animals.

2. MATERIALS AND METHODS

2.1. Study site and organisms

Amphibians were sampled from the central Northwestern Iberia crossed by rivers draining to the Cantabric Sea and Bay of Biscay, in the humid temperate Atlantic zone of North Spain. For this study we made a division of the region in seven areas (coded as A to G), separated by largest rivers: Navia and Esva between A and B, Narcea between B and C, Nalón and Trubia between C and D, Nora and Piloña between D and E, Sella between E and F, Cares, Nansa, Saja, Besaya, Pas, Asón and Nervión between F and G (Fig. 1). For completing the geographical range of sampling some additional sequences were downloaded from the GeneBank database: EF525955-EF525956 for *L. boscai* (from the F area) and EF525986, EF525989 for *L. helveticus* (from the G area).

2.2. Sampling methodology

We collected a total of 421 adults (urodeles) and larvae (anurans and urodeles) of six different species (Table 1). All samples were collected between October 2007 and July 2008 from at least two different water points (troughs and ponds) in each area for each species. Sampling was carried out employing dip nets. Tissue samples from individuals were obtained by non-destructive tail clipping, removing a tail segment about 1 mm long which was stored in absolute ethanol. After sampling, the animals were immediately released into the water point. New clean nets were employed in each water point to prevent from spreading possible diseases and parasites.

2.3. DNA extraction and sequences amplification

Whole genomic DNA was extracted using a Chelex Resin (Bio-Rad laboratories) protocol following Estoup et al. (1996) and preserved at -20ºC until analysis. The mitochondrial Citochrome oxidase subunit I (COI) region was amplified by polymerase chain reaction (PCR) employing the primers developed by Ward et al. (2005) for all the species except for *T. marmoratus*, which did not yield positive amplification with those primers. A new set of primers was designed for this species for the same COI region employing the program Primer 3 v.0.4. (Rozen and Skaletsky 2000). BioEdit software (Hall 1999) was employed for aligning our COI sequences of amphibians and the complete *T. marmoratus* COI sequence from GenBank EU880337.1 to select the same region of the COI for all the amphibian species. The new primers were COITmar-F (5’-TCATAAAGATATTGGCACCCTCT-3’) and COITmar-R (5’-AAGAATATAGACCTCGGGGTGA-3’).

PCR amplification was carried out in a reaction solution (total volume of 40 µl per tube) containing 5x Go Taq® Flexi Buffer, 2.5 mM $MgCl_2$, 250 µM of each dNTP, 1 µM of each primer, 1 unit of Go Taq® Polymerase 5U/µl (Promega) and 50ng of DNA template. They were performed in a GeneAmp® PCR system 2720 (Applied Biosystems) with the following conditions: an initial denaturing step at 95 ºC for 5 min, followed by 35 cycles of denaturing at 95 ºC for 30 s, annealing (for 30 s) at 58 and 59 ºC for COI (Ward et al. 2005) and COI Tmar (this study) respectively, and an extension at 72 ºC for 30 s, followed by a final extension at 72 ºC for 15 min.

PCR products were electrophoresed in 2 % agarose gels stained with ethidium bromide and DNA bands were excised. Before the sequencing reaction DNA bands were purified with a Wizard® SV Gel and PCR Clean-Up System (Promega) DNA was purified before sequencing. Automated fluorescence sequencing was run on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems) with BigDye 3.1 Terminator system, in the Unit of Genetic Analysis of the University of Oviedo (Spain).

2.4. Sequence editing

Sequences were aligned using the algorithm CLUSTAL W (Thompson et al. 1994) and checked visually with the program BioEdit (Hall 1999). To ensure correct species identification all the sequences were compared with those existing in the GenBank database (http://www.ncbi.nlm.nih.gov/GenBank) for the studied species. The presence of pseudogenes was discarded translating the sequence of nucleotides to aminoacids; stop codons or indels were not found. Translation was carried out using the program MEGA 4.1 (Tamura et al. 2007).

2.5. Population genetic diversity

Standard indices of molecular diversity, including haplotype (*Hd*) and nucleotide diversity (π), were calculated using the program DnaSP v.4.50.3 (Rozas et al. 2003).

The genetic differentiation among populations was evaluated by calculating F_{ST} values (Weir and Cockerham 1984), the significance of which was tested by comparison to 95 and 99% confidence intervals acquired from 1,000 bootstrap permutations with the program ARLEQUIN v.3.5.1.2 (Excoffier et al. 2005).

The best nucleotide substitution model for each species was determinate using jModeltest 0.1.1 (Posada 2008) and Akaike's Information Criterion (AIC) to choose between models. The best fitted model was considered for the settings of phylogenetic and demographic analyses. The phylogenetic relationships between haplotypes were deduced from neigbour-joining (NJ) networks (Bandelt et al. 1999), constructed with the software Network 4.1.0.1 (available at http://www.fluxus-technology.com/sharenet.htm) for each species.

2.6. Demographic analyses

Demographic trends were inferred from mismatch distributions and statistical tests of neutrality. The Tajima's test of selective neutrality *D* (Tajima 1989) was calculated to assess evidence for population expansion, as well as Fu's *Fs* test of neutrality (Fu 1997). Both tests are classically used to test neutrality, but they can also be used to test population growth since a population that has experienced expansion may result in a rejection of the null hypothesis of neutrality. Significant, large negative values of *D* value or *Fs* indicate an excess of recent mutations and reject the null hypothesis of stationary population. These analyses were performed with the program ARLEQUIN v.3.5.1.2 (Excoffier et al. 2005).

We used the mismatch distribution (Rogers and Harpending 1992) and Harpending raggedness index (Harpending 1994) among COI sequences to evaluate various models of population growth, stability, and spatial distribution. We compared mismatch distributions of pairwise nucleotide differences with expectations of a sudden-expansion model (Rogers 1995) using the program ARLEQUIN v.3.5.1.2 (Excoffier et al. 2005). When the distribution of the observed number of differences between pairs of haplotypes is multimodal the populations are stationary with low migration rates (Ray et al. 2003), whereas unimodal waves are expected for sudden population expansion (Rogers and Harpending 1992) or through a range expansion with high levels of migration between neighbouring demes (Excoffier 2004; Ray et al. 2003).

2.7. Spatial analyses

Isolation by distance (IBD) was examined for each species by testing the correlation between Rousset's (1997) $F_{ST}/(1-F_{ST})$ and geographical distance between population pairs using Mantel tests implemented at the software XLSTAT v.7.5.2 (Addinsoft, New York, USA) for Microsoft Excel. Geographic distance data were obtained using the path measurement tool in Google Earth (earth.google.com).

To determine the most prominent barriers to dispersal of each species, we employed the software BARRIER v.2.2 (Manni et al. 2004) for identifying geographically continuous and discontinuous assemblages of sampling sites independent from a priori knowledge of geographical population structure. Pairwise F_{ST} distances were mapped onto a matrix of geographical coordinates connected by Delaney triangulation (Brassel and Reif 1979). Barriers in the triangulation were identified using Monmonier's maximum distance algorithm, to identify regions with sharp genetic change or discontinuity on the landscape (Manni et al. 2004).

3. RESULTS

3.1. Alytes obstetricans

The COI fragment amplified for this species was 658 base pairs (bp) long. Among 101 individuals analyzed we found 11 polymorphic positions which yielded nine different haplotypes, which are available in GenBank under Accession numbers JN379848- JN379851 for this study and HM032694- HM032698 previously described at Garcia-Gonzalez & Garcia-Vazquez, in press. Eight of them were private, occurring in only one location (Table 2). Overall haplotype diversity (*Hd*) was relatively high but not the nucleotide diversity (π), with values of 0.81366 and 0.00406 respectively. At population level, the highest haplotypic and nucleotide diversity occurred in the G area (0.58667 and 0.00147 respectively) while the lowest variation corresponded to the F area where only one haplotype was detected (diversity = zero).

jModeltest identified TrN as the best model of nucleotide substitution for the sequence dataset. Pairwise F_{ST}'s were high and significant between all population pairs except for C and D areas (Supplementary table S1a). The haplotype network shows that these two areas are the only that share one haplotype (Fig. 2). All the haplotypes found in the G area were highly different from the other areas.

The Mantel test was not significant (r = 0.907, P = 0.449), indicating no association between genetic and geographic distances. The Barrier prediction analysis using Monmonier's maximum difference algorithm identified a sharp genetic discontinuity at two strong barriers (Table 3), the first between G and F, and the second between F and the western areas D and C.

None of the demographic tests (*D* and *Fs*) were significant, and results from SSD test rejected demographic expansion in all cases (Table 2) except for the area G, indicating stationary populations. In addition, the mismatch distribution showed a multimodal distribution expected for a non-growing population (Slatkin and Hudson 1991), with the exception again for the G area (Supplementary material Fig. S1). However, non-significant raggedness values suggested a possible population expansion for the areas D and G, while significant values for the area C confirmed population stationary there (Table 2).

3.2. Bufo bufo

A total of 87 specimens were sequenced for a 660 bp of the COI gene. The seven polymorphic sites yielded seven haplotypes (Table 2) with GenBank Accession numbers JN379841- JN379847. Six of the haplotypes were private. The levels of haplotype and nucleotide diversity were 0.32745 and 0.00057 respectively. The diversity was low, with global haplotype diversity of 0.32745, and global nucleotide diversity of 0.00057.

TPM1uf was identified as the best evolutionary model fit to the dataset. The haplotype network conducted by NJ method revealed one unique clade, exhibiting a star-like shape centered around one frequent haplotype shared by all the areas (Fig.2). The areas F and G contained private haplotypes. The only significant F_{ST} value was obtained between the areas F and G (Supplementary table S1b).

Isolation by distance could not be checked because the Mantel´s test can not be computed with a matrix of n = 2. Mommonier's maximum difference algorithm, implemented in BARRIER identified only one weak discontinuity between the areas G and F (Table 3), accordingly with the genetic differentiation observed from F_{ST} values.

Values for Fu's *Fs* and Tajima's *D* were significant and negative for the G area (Table 2). The SSD and Harpending's raggedness tests did not reject the demographic expansion hypothesis. Mismatch distribution were unimodal, suggesting demographic expansion (Fig. S1 for the overall). Demographic expansion could not be tested for the area E because it contained only one haplotype.

3.3. *Discoglossus galganoi*

A total number of 74 individuals were analyzed, obtaining sequences 671bp long which were polymorphic at four sites, yielding four different haplotypes from which two were private (Table 2). The haplotypes are available in GenBank under Accession numbers GU797351-GU797354 for a previous study (Garcia-Gonzalez and Garcia-Vazquez in press). Global haplotype diversity was 0.62680, and global nucleotide diversity 0.00118. The D area exhibited the highest haplotype and nucleotide diversity: 0.70356 and 0.00168 respectively.

The best nucleotide substitution model for this dataset was F81. The haplotype network revealed two principal haplotypes shared by the three areas and other two haplotypes only present at the D area (Fig. 2). The differences between these haplotypes were one or two substitutions. F_{ST} probability values showed significant differences between the D area and the other two (C and E), which were not significantly different to each other (Table S1c).

Isolation by distance could not be checked by the reason explained above (only three samples). The BARRIER analysis indicated the presence of two weak barriers to gene flow separating the area D from the neighboring C (east) and E (west) (Table 3).

All *Fs* and *D* values were not significant (Table 2). However, Harpending's raggedness index and SSD tests were not significant for each population, indicating a possible expansion in these areas. Mismatch distributions for the total data and for the individual areas exhibited a unimodal distribution which could be attributed to a sudden population expansion model (Fig. S1 for the overall).

3.4. *Lissotriton boscai*

For the Bosca's newt 24 individuals were sequenced yielding 644 bp long sequences with 12 polymorphic sites and six different haplotypes, four of them private and two shared among all the areas analyzed (Table 2). The haplotype sequences were deposited in GenBank with the accession numbers JN379835- JN379840. The global haplotype diversity was 0.72727, ranging from zero (C and F with only one haplotype per area) to 0.65934 in B. The global nucleotide diversity was 0.00313.

HKY was identified as the best model fit to the data of this species. The network analysis revealed two clearly distinct groups separated by seven mutational steps (Fig. 2). One clade contained only one haplotype and included F and C areas; the other, corresponding to A and B areas, included one common and four private haplotypes (derived from the central one by one or two mutations). Significant F_{ST} values were found between A, B and F areas (Table S1d).

The Mantel test was not significant (r = -0.25228, P = 0.890). Strong genetic discontinuities were detected using the Mommonier's algorithm (Table 3). The main barrier was detected between the area C and the western areas A and B. A less strong barrier was found between A and B, consistent with significant F_{ST} values (Table S1d).

Unimodal mismatch distributions were consistent with the sudden population expansion model (raggedness index and SSD not significant), (Table 2; Fig. S1 for the overall). In addition, *Fs* and *D* (Tajima 1989) were significant and negative for the whole samples and the B area, indicating deviations from neutrality or demographic expansion (Table 2).

3.5. *Lissotriton helveticus*

We sequenced 598 bp for 92 individuals of palmate newt, containing 16 polymorphic sites and 12 haplotypes, nine of them private (Table 2). Some of the haplotypes founded coincided with those of a previous study (GQ374500, GQ374502, GQ374503, GQ374507, GQ374508) (Garcia-Gonzalez and Garcia-Vazquez 2011). The new haplotypes have been deposited in the GenBank under accession numbers JN379829-JN379834. The haplotype diversity ranged from zero (A and G areas) to 0.71429 (area B), with a global value for all the region of 0.78071. The nucleotide diversity was highest value at the F area (0.00325), with an overall value of 0.00275.

The substitution model that better fits for this data set was TPM1uf. The haplotype network revealed two central haplotypes closely related, diverging by only one mutational step (Fig. 2). One of the central haplotypes included all the areas at the west and center of the region (B, C, D and E) with satellite haplotypes in areas C and D. The other central haplotype occurred in all areas except the ones from the center of the studied region (C and D), with private haplotypes derived from it occurring at the areas B, F and G. F_{ST} values were significant between all populations pairs (Table S1e).

The Mantel test revealed significant IBD (r = 0.884, P = 0.030). The BARRIER analysis detected five major barriers to gene flow (Table 3). The first and most important of them separated the areas G and F. The second occurred between the areas B and C. The three next barriers (D-E, E-F, C-D) were weaker.

The demographic indicators Fs and *D* yielded positive and no significant values (Table 2). However, Harpending's raggedness index was not significant for the C, D, E and F areas, not rejecting the hypothesis of demographic expansion. Similarly, the SSD test was not significant for C, D and E, also suggesting demographic expansion. The areas D and E and the overall region exhibited a unimodal distribution expected for populations in expansion (Fig. S1 for the overall).

3.6. *Triturus marmoratus*

Forty three individuals were analyzed for 661 bp long COI sequences, detecting eight different haplotypes involving 13 variable sites (Table 2). Only one haplotype was shared by all the populations and the other seven were private haplotypes, each present in only one population. Haplotypes were deposited in GenBank under accession numbers JN379821- JN379828. High differences of haplotype diversity were found among the different study areas, ranging from zero in C and D to 0.82051 in B with a global value of 0.59358. The same applies to the nucleotide diversity with overall of π = 0.00294 and the highest value at the B area with a value of π = 0.59358.

With the program jModelTest, HKY+G was identified as the best model fit to the dataset, with a gamma shape of 0.0170. The haplotype network showed a central haplotype shared by all the areas (Fig. 2). Five private haplotypes derived from the central by only one mutation step were present in A and B. Other haplotype appeared in B, separated by eight mutational steps from the central and most common haplotype. The presence of so different haplotypes in B suggests that this area is the confluence of two different lineages.

We found no evidence of IBD in the Mantel test (r = 0.724, *P* = 0.361). The BARRIER analyses identified one discontinuity between areas A and B (Table 3).

Overall non-significant values of Fs and *D* tests were obtained (Table 2). Non-significant raggedness indices and SSD tests suggested population expansion, but only the A area exhibited a unimodal mismatch distribution according a population expansion. As the area B and the overall data exhibited multimodal distributions, population expansion was not supported for this species in this region (Fig. S1 for the overall).

4. DISCUSSION

The main question raised in this study was the role of rivers (and other landscape discontinuities) for shaping the geographic distribution of genetic variation in amphibian populations, as documented by Ziesser and Beebee (2008) for other regions. The results on population structuring of the six North Iberian species analyzed provide some interesting answers. Except for the Iberian toad *B. bufo* without any detected geographical barrier, the rest of species exhibited clear discontinuities between zones that are physically separated by rivers flowing South-North across the region. The most conspicuous barriers affected the species *A. obstetricans* and *L. helveticus* (Table 3) and occurred at the east of the region, approximately in the border of the Basque country, where the River Bidasoa and close smaller rivers cut longitudinally the territory. Those were followed in magnitude by a big discontinuity at the level of the Narcea River (*L. boscai*, *L. helveticus*) and apparently less important obstacles to gene flow represented by the Sella river (accounting a barrier of 0.724 of magnitude for *A. obstetricans*. The crested newt *T. marmoratus* exhibited a discontinuity in the west, at the level of the rivers Navia and Esva. As a whole, these results indicate that the rivers can be barriers to dispersal for these five species, as they are for other anurans (e.g. Lampert et al. 2003; Monsen and Blouin 2003; Funk et al. 2005). Fine-scale studies often use microsatellites because they are potentially more powerful, but to date this kind of genetic markers has not been developed for all the species studied here. In the present case, the mitochondrial COI marker has revealed enough variability for detecting barriers to dispersal of different amphibian species, as already documented for determining the role of roads as barriers to gene flow (Garcia-Gonzalez et al. in press).

A second and important conclusion is that the impact of a river for isolating amphibian populations is strongly species-dependent. As an example, the River Narcea was an almost absolute barrier for *L. boscai* but did not seem to impede gene flow between *T. marmoratus* populations, and two smaller rivers was the main barrier for this species. The rivers *per se* not always act as geographical barriers for all the species, it may depend on the rest of the landscape and on the species. For example, in the Amazonian communities orographic features seem to exert more effect on shaping biotic diversity than the large River Juruá does (Gascon et al. 1998; Gascon et al. 2000). In our study, rivers do not seem to represent physical obstacles for *B. bufo*. This can be explained based on the ecology of these toads. For this genus, rivers are suitable habitats and contain larvae, contributing to population connectivity (e.g. Stevens et al. 2006 study on *Bufo calamita*) instead to isolation.

Another important result of our study is that we have defined population structuring and genetic diversity for the six species, in most cases for the first time at regional scale. It was different in the six species analyzed. *B. bufo* exhibited low diversity and absence of spatial differentiation across a range of 300 km. This result contrasts with a high diversification of populations of the same species inhabiting central Iberia (Martínez-Solano and González 2008), in a similar fine-scale study. Martínez Solano and González (2008) employed the mitochondrial control region and microsatellites as genetic markers, much more polymorphic markers; but even considering that COI is less variable, total absence of spatial structuring was not expected at so relatively long distance. The density of rivers in the region analyzed could explain the absence of population differentiation for this species, rivers favoring connectivity in *Bufo*. Consistently with our results, Martínez-Solano and González (2008) did not find a role of river basins for structuring genetic variability in this species, and suggested that the wide range of ecological conditions tolerated by this species and its high vagility, compared with other amphibians, encourage the gene flow. The haplotype network of *B. bufo* was star-like, which is a typical signature of a recent population expansion following a population bottleneck (Slatkin and Hudson 1991), an expansion confirmed by all the demographic indicators analyzed (Table 2) and private haplotypes (Harpending and Rogers 2000). Such signals of expansion after a bottleneck suggest a relatively recent colonization of the region, may be after the last glaciation.

The population structuring exhibited by the midwife toad *A. obstetricans* in the region was completely different, with great discontinuities in the eastern part and absence of isolation by distance suggesting a patchy population structuring may be caused by rivers acting as barriers to gene flow, as explained above. Alternatively, a smaller-scale more recent habitat fragmentation could have isolated small populations and created a more intricate spatial pattern; roads and other human constructions could be responsible, as they are for other species (Vos 1998; Cushman 2006). Such structured pattern is reflected in the haplotype network (Fig. 2). This species exhibited a combination of low nucleotide diversity and high haplotypic diversity, which is a signal of a species experiencing a fast expansion after a small effective population size or bottleneck; frequent haplotypes would have emerged during a demographic expansion and haplotype polymorphism accumulated by unique mutations, not increasing nucleotide diversity (Avise 2000). This could be again a signal of relatively recent colonization. Two different lineages were detected, one of them only in the G area, with three private haplotypes, and the other in the rest of the region. Considering that more descendant haplotypes will be associated with the oldest haplotypes (Posada and Krandall 2001), the haplotype 9, found in the west, would be the oldest one and suggests expansion from that area.

The results for the other Discoglossidae here analyzed, *D. galganoi*, were less conclusive. *D. galganoi* follows an IBD pattern in the northwest of the Iberian Peninsula at a larger scale (Martínez-Solano 2004) but in the studied area it did not exhibit any geographical structure, probably due to relatively small geographical coverage. This species exhibited here an extremely low nucleotide diversity, indicating a prolonged bottleneck in the studied region (Avise 2000) and confirmed by not significant demographic indicators, suggesting stationary populations. The number of haplotypes was small (*Hhap* = 4), but within the range obtained by Martínez-Solano (2004) across the entire range of distribution of this species employing other sequences (*nad4* and *cyt-b* genes) as genetic markers. From our study, the overall perspective for this species would be a quite homogeneous population at spatial level, rivers not being significant barriers for it.

The palmate newt *L. helveticus* followed a clear pattern of isolation by distance (significant Mantel test) in the region, exhibiting a consistent haplotype network highly structured with private haplotypes in most samples, which is typical of populations isolated by distance. The studied region corresponds with the “Asturian” clade reported by Recuero and García-París (2011), who found signals of expansion in this clade. Such possible expansion has been confirmed with our data, where high haplotypic and low nucleotide diversity together with *SSD*, raggedness index and unimodal mismatch distributions support the hypothesis of population expansion.

Two very different clades, separated by seven mutational steps, occurred in this region for *L. boscai*. The clade corresponding with the west of the sampled area is connected to other less frequent haplotypes but the clade from the east has only one haplotype. As indicates Martínez-Solano et al. (2006), this pattern of decreasing variability along a west to east axis, is common for lineages that have colonized areas along the Cantabric coast in the northern Iberian Peninsula. A possible explanation of the high differentiation between clades could be an allopatric isolation with a population bottleneck followed by an expansion at the western clade and a posterior secondary contact of previous isolated populations. Clear signals of population expansion appeared for this species in our data, with significant values for Tajima’s *D* and Fu’s *Fs,* non-significant values of Harpending’s raggedness index and *SSD* tests and unimodal mismatch distributions. All together, our results point out to a secondary contact between two different lineages coincident with relatively recent expansion in the region. Martínez-Solano et al. (2006) found patterns of genetic variability typical of refugial areas of the westernmost of the Iberian Peninsula, and the pattern of genetic variability found in the region studied here for the COI gene support their hypothesis.

Finally, the crested newt *T. marmoratus* exhibited in the western area B a combination of high haplotypic and also high nucleotide diversity, while in the rest of the region the diversity was minimal. This can be interpreted as a conjunction of two different lineages at the west of the studied region. Secondary contact of previously isolated populations after the last glacial maximum in Europe are well known for many higher eukaryotes (Hewitt 2000), and have been described for the newt *Mesotriton alpestris* in Poland (Pabijan and Babik 2006), as well as for *L. boscai* and *Alytes sp*. in the Iberian Peninsula (Martínez-Solano et al. 2004; Martínez-Solano et al. 2006; Gonçalves et al. 2009). This could also be the case of *T. marmoratus* in north Iberia. The possibility of a northwestern refugial area for the Iberian herpetofauna was discussed by Martínez-Solano et al. (2006), and our results for *T. marmoratus*, as well as those of *L. boscai*, are in agreement with that hypothesis. Lineages that have colonized the region from western refugia show signs of recent demographic expansion, like lower values of genetic diversity, starlike phylogenies and/or significant results in Tajima’s or Fu’s neutrality tests (Alexandrino et al. 2002; Paulo et al. 2002; Martínez-Solano 2004). Following these premises, all these signals are present in the populations of *T. marmoratus* and *L. boscai* studied here. Notwithstanding it, we can not exclude the possibility that this low nucleotide diversity of the species also may be due to habitat fragmentation or a recent expansion from a smaller but more stable refugial population (Mirol et al. 2008).

In conclusion, our study has revealed the occurrence of species-dependent impacts of rivers on population connectivity, acting as barriers for some species like *A. obstetricans* , *L. boscai*, *L. helveticus* and probably *T. marmoratus*. This effect is less clear for *D. galganoi* and inexistent for *B.bufo*. Demographic trends and haplotype network shapes indicate expansion in the region of genetic lineages for all species except *D. galganoi*. The whole data support the hypothesis of a glacial refugium for herpetofauna in Northwest Iberia proposed by Martínez-Solano et al. (2006).

5. MANAGEMENT RECOMMENDATIONS

Some recommendations for management can be deduced from this study. In order to establish a strategy to protect amphibians in this region we should pay attention to the specific requirements of each species due to their different capacity of connecting populations across rivers, and likely other obstacles. Priority should be given to the most endangered species, like *A. obstetricans*, *D. galganoi* and *T. marmoratus*. At least two of them, *A. obstetricans* and *T. marmoratus*, would likely benefit from incorporating passages for amphibians in extant and new bridges across rivers. The effectiveness of amphibian passages for helping individuals to overcome barriers using culverts, guidance structures, tunnels or green bridges has been well documented (Jackson 1996; Puky et al. 2007; Patrick et al. 2010). Creating paths for amphibians under existing bridges, or constructing small green bridges, could help to connect populations which otherwise would remain isolated. Further research into population genetics, natural history and ecology of these species is needed to effectively manage and conservation of amphibian diversity in this area.

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TABLES

Table 1. Number of individuals of each species sampled from in the different areas.

Species	A	B	C	D	E	F	G	Total
Alytes obstetricans			28	27		21	25	101
Bufo bufo					15	37	35	87
Discoglossus galganoi			36	23	15			74
Lissotriton boscai	7	14	1			2		24
Lissotriton helveticus	1	7	39	19	9	15	2	92
Triturus marmoratus	21	13	5	4				43
Total	29	34	109	73	39	75	62	421

Table 2. Genetic diversity in the populations sampled. *Nhap*, number of haplotypes; *p*, number of private haplotypes; *Hd*, haplotype diversity; *π*, nucleotide diversity; Tajima´s *D*; Fu´s *Fs*; *SSD*, sum of squared differences; *r*, Harpending raggedness index. Significance levels: **P*<0.05, ** *P*<0.01, *** *P*<0.001.

Species	Areas	A	B	C	D	E	F	G	TOTAL
A. obstetricans	*Nhap*	-	-	2	4	-	1	3	9
	p	-	-	1	3	-	1	3	8
	Hd	-	-	0.49471	0.44160	-	0.00000	0.58667	0.81366
	π	-	-	0.00076	0.00144	-	0.00000	0.00147	0.00406
	Tajima's *D*	-	-	14.753	-0.24858	-	N.A.	-0.25338	0.65865
	Fu's *Fs*	-	-	15.772	0.15253	-	N.A.	134.216	10.315
	SSD	-	-	0.02081*	0.33445***	-	N.A.	0.03324	0.03838
	r	-	-	0.24485*	0.52670	-	N.A.	0.20053	0.07667
B. bufo	*Nhap*	-	-	-	-	1	2	6	7
	p	-	-	-	-	0	1	5	6
	Hd	-	-	-	-	0.00000	0.24024	0.50084	0.32745
	π	-	-	-	-	0.00000	0.00036	0.00093	0.00057
	Tajima's *D*	-	-	-	-	N.A.	0.00423	-1.37331*	-1.67199***
	Fu's *Fs*	-	-	-	-	N.A.	0.47892	-2.43021**	-4.69283***
	SSD	-	-	-	-	N.A.	0.28848	0.00002	0.00091
	r	-	-	-	-	N.A.	0.32762	0.07703	0.21632
D. galganoi	*Nhap*	-	-	2	4	2	-	-	4
	p	-	-	0	2	0	-	-	2
	Hd	-	-	0.51270	0.70356	0.53333	-	-	0.62680
	π	-	-	0.00076	0.00168	0.00079	-	-	0.00118
	Tajima's *D*	-	-	165.802	0.5638	150.272	-	-	0.07141
	Fu's *Fs*	-	-	180.124	0.79317	131.784	-	-	0.74714
	SSD	-	-	0.02488	0.009678	0.03032	-	-	0.01787*
	r	-	-	0.26351*	0.08575	0.28888	-	-	0.15237***
L. boscai	*Nhap*	2	4	1	-	-	1	-	6
	p	0	2	0	-	-	0	-	2
	Hd	0.57143	0.65934	0.00000	-	-	0.00000	-	0.72727
	π	0.00089	0.00143	0.00000	-	-	0.00000	-	0.00313
	Tajima's *D*	134.164	0.87665*	N.A.	-	-	N.A.	-	-0.84680*
	Fu's *Fs*	0.85642	-0.59492*	N.A.	-	-	N.A.	-	0.60722
	SSD	0.04272	0.01225	N.A.	-	-	N.A.	-	0.03774
	r	0.34694	0.13199	N.A.	-	-	N.A.	-	0.09747
L. helveticus	*Nhap*	1	3	3	4	2	4	1	12
	p	-	2	1	2	0	3	1	9
	Hd	0.00000	0.71429	0.61808	0.5731	0.55556	0.60000	0.00000	0.78071
	π	0.00000	0.00246	0.00128	0.00123	0.00096	0.00325	0.00000	0.00275
	Tajima's *D*	N.A.	0.75467	312.095	0.83782	140.117	0.18515	N.A.	0.89997
	Fu's *Fs*	N.A.	0.66806	254.397	0.20354	101.511	123.348	N.A.	0.80917
	SSD	N.A.	0.28899*	0.06441	0.02501	0.03716	0.51283***	N.A.	0.13263
	r	N.A.	1.10204*	0.24000	0.12096	0.32099	0.27302	N.A.	0.29386
T. marmoratus	*Nhap*	3	6	1	1	-	-	-	8
	p	2	5	0	0	-	-	-	7
	Hd	0.34286	0.82051	0.00000	0.00000	-	-	-	0.59358
	π	0.00055	0.00702	0.00000	0.00000	-	-	-	0.00294
	Tajima's *D*	-0.05406	175.861	N.A.	N.A.	-	-	-	-0.80857
	Fu's *Fs*	-0.02168	107.146	N.A.	N.A.	-	-	-	-0.59535
	SSD	0.01119	0.09355	N.A.	N.A.	-	-	-	0.01874
	r	0.26603	0.15154	N.A.	N.A.	-	-	-	0.07064

Table 3. Values of genetic barriers predicted by the software BARRIER (version 2.2).

<table>
<tr><th>Species</th><th>AB</th><th>BC</th><th>CD</th><th>DE</th><th>EF</th><th>FG</th></tr>
<tr><td><i>Alytes obstetricans</i></td><td></td><td></td><td><0.1</td><td colspan="2">0.724</td><td>0.956</td></tr>
<tr><td><i>Bufo bufo</i></td><td></td><td></td><td></td><td></td><td><0.1</td><td>0.107</td></tr>
<tr><td><i>Discoglossus galganoi</i></td><td></td><td></td><td>0.179</td><td>0.223</td><td></td><td></td></tr>
<tr><td><i>Lissotriton boscai</i></td><td>0.228</td><td>0.923</td><td colspan="3"><0.1</td><td></td></tr>
<tr><td><i>Lissotriton helveticus</i></td><td><0.1</td><td>0.517</td><td>0.279</td><td>0.282</td><td>0.281</td><td>0.847</td></tr>
<tr><td><i>Triturus marmoratus</i></td><td>0.315</td><td><0.1</td><td><0.1</td><td></td><td></td><td></td></tr>
</table>

FIGURE LEGENDS

Figure 1. Map showing the sampled areas and the major rivers between them. 1, Navia; 2, Esva; 3, Narcea; 4, Pigüeña, 5, Trubia; 6, Caudal; 7, Nalón; 8, Nora; 9, Piloña; 10, Sella; 11, Dobra; 12, Cares; 13, Nansa; 14, Saja; 15, Besaya; 16, Pas; 17, Asón; 18, Nervión and 19, Ibaizabal.

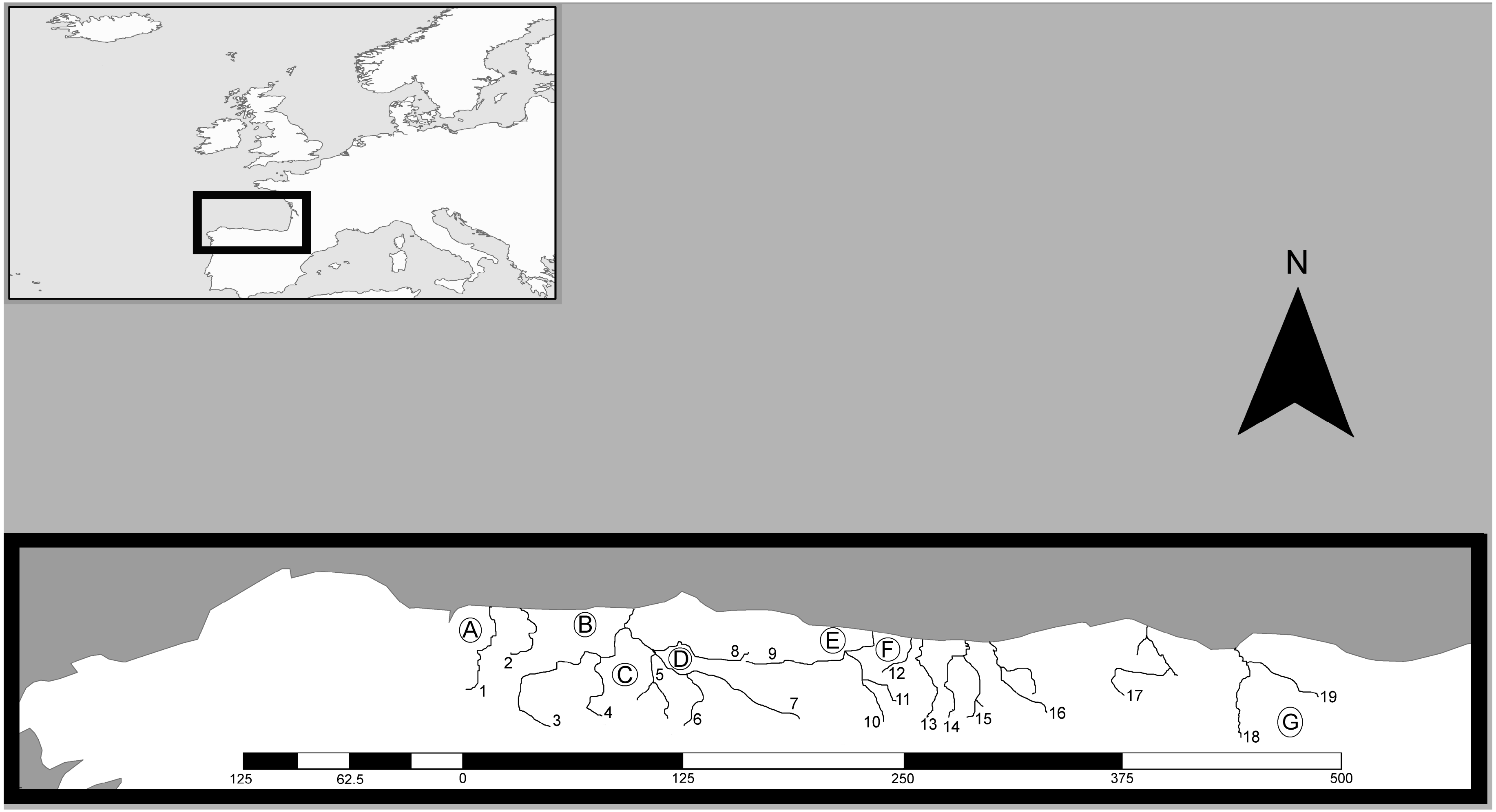
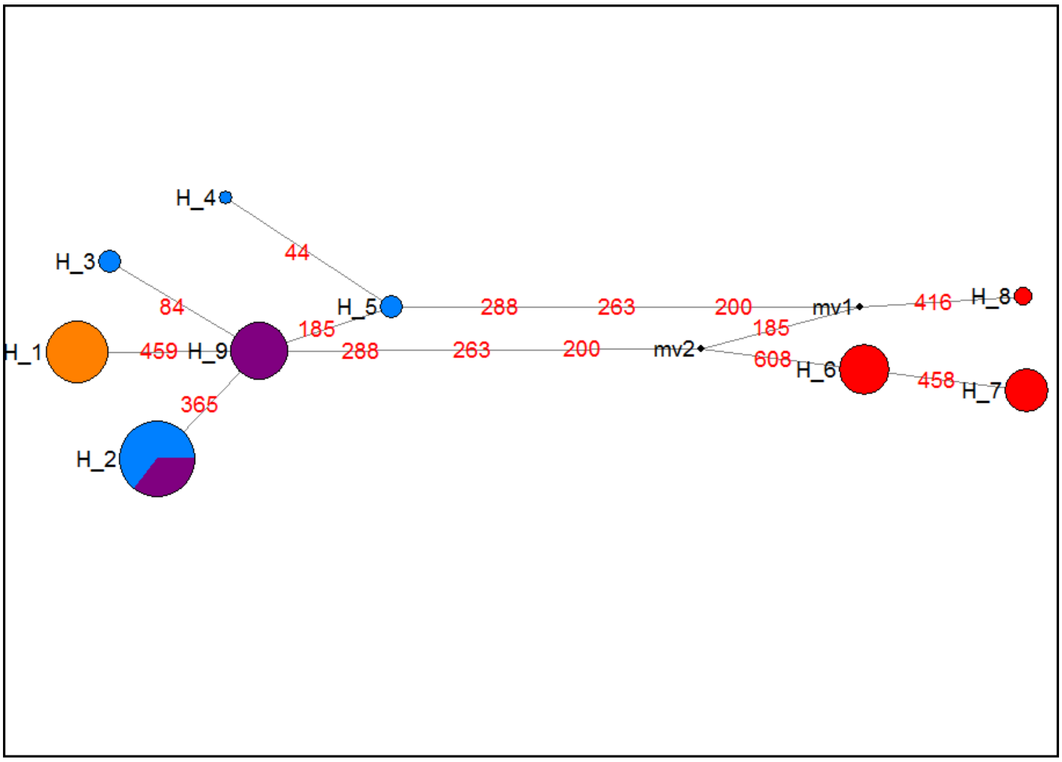
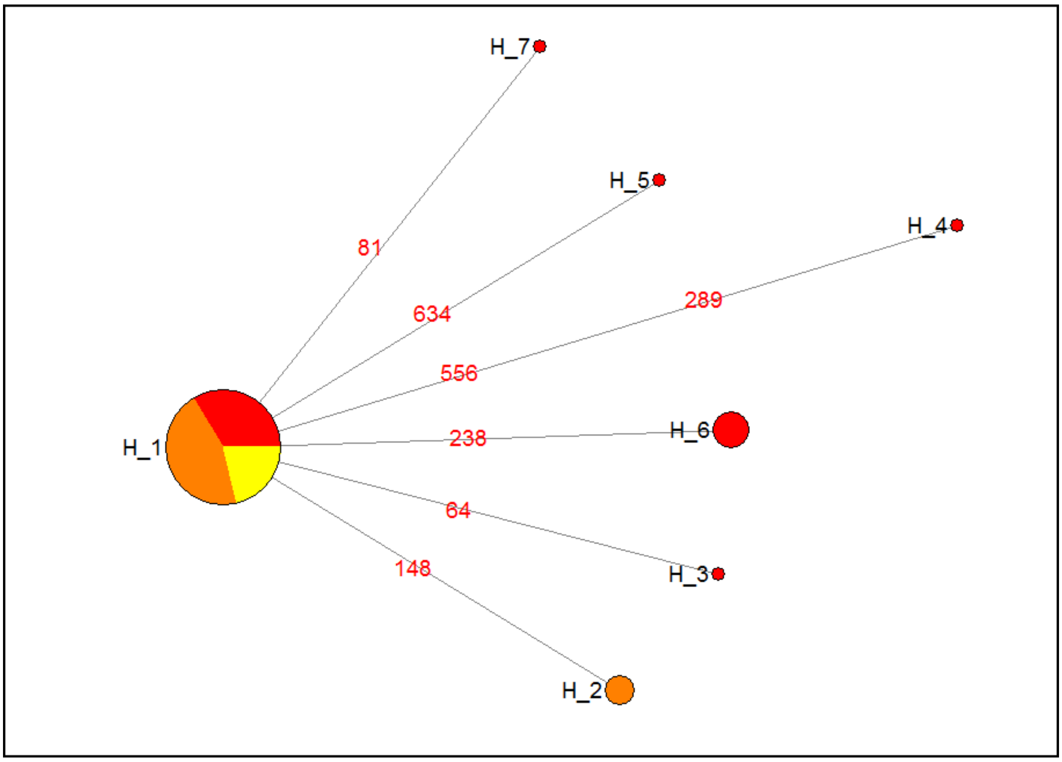


Figure 2. Median-joining network based on COI gene. The haplotypes are indicated by circles, the size of each circle being proportional to the observed frequency of each type. The number of mutations required to explain transitions among haplotypes is indicated along the lines of the network, median vectors are labeled mv1 and mv2. Dark green, A area; light green, B area; purple, C area; blue, D area; yellow, E area; orange, F area; red, G area. a) *A. obstetricans*, b) *B. bufo*, c) *D. galganoi*, d) *L. boscai*, e) *L. helveticus*, f) *T. marmoratus*.

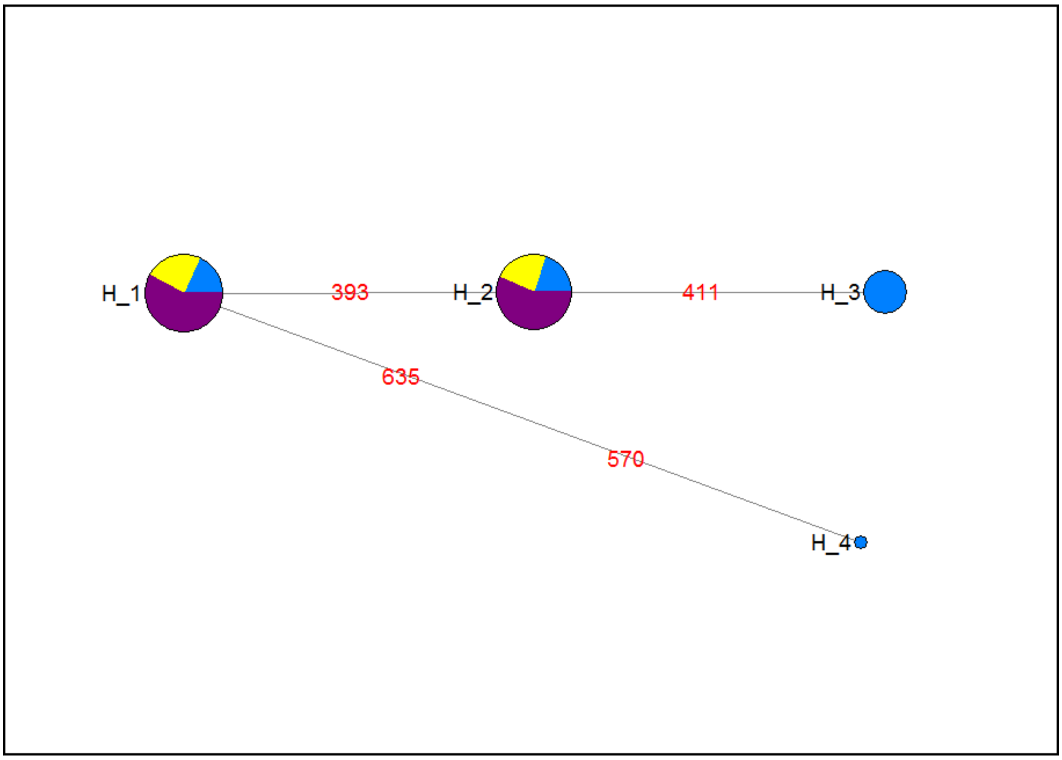
a)



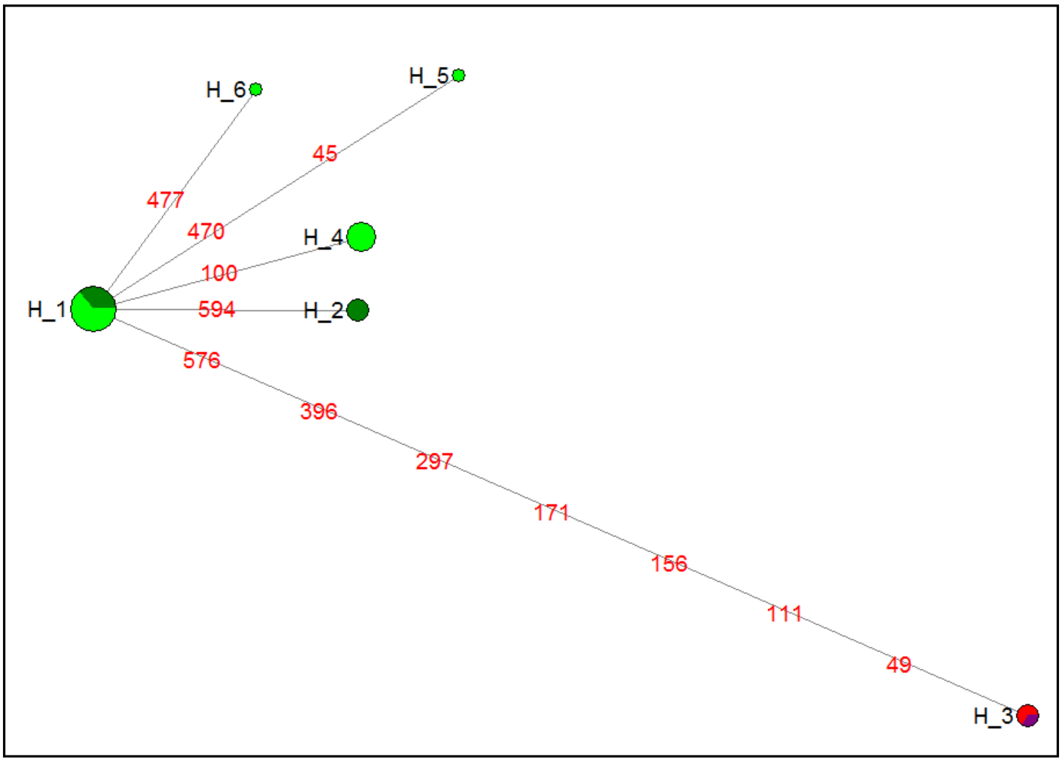
b)



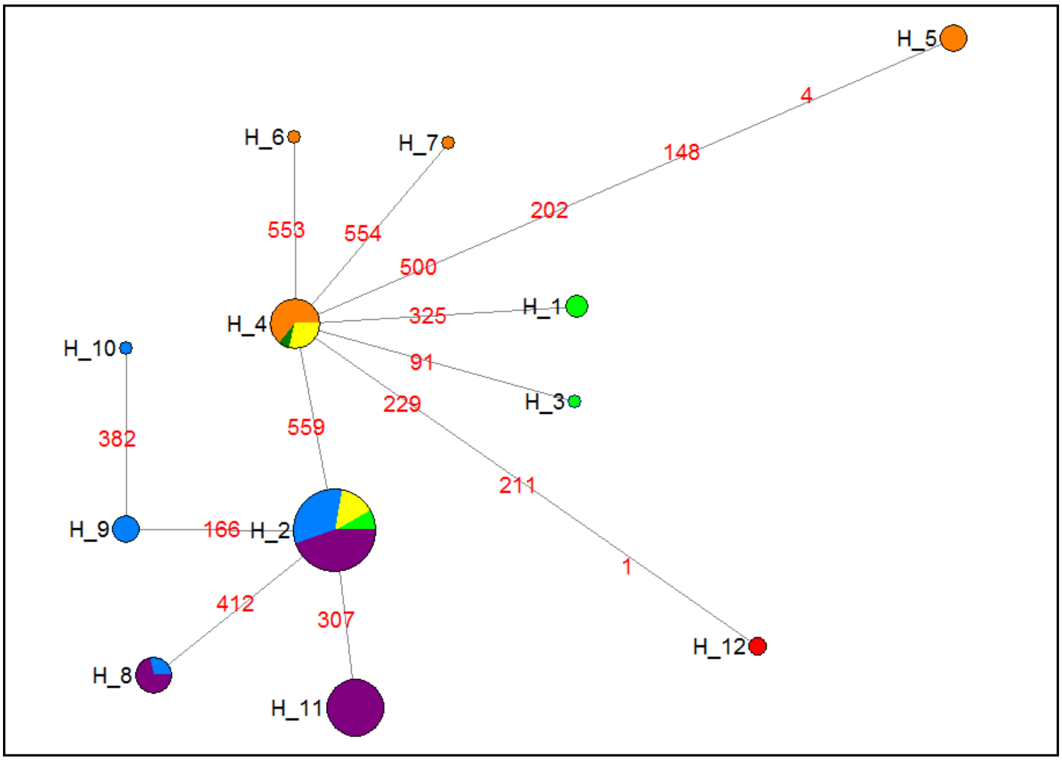
c)


H_1
393
H_2
411
H_3
635
570
H_4

d)


H_6
H_5
45
477
470
H_4
100
H_1
594
H_2
576
396
297
171
156
111
49
H_3

e)


H_5
4
148
202
H_6
H_7
553
554
500
H_4
325
H_1
H_10
91
H_3
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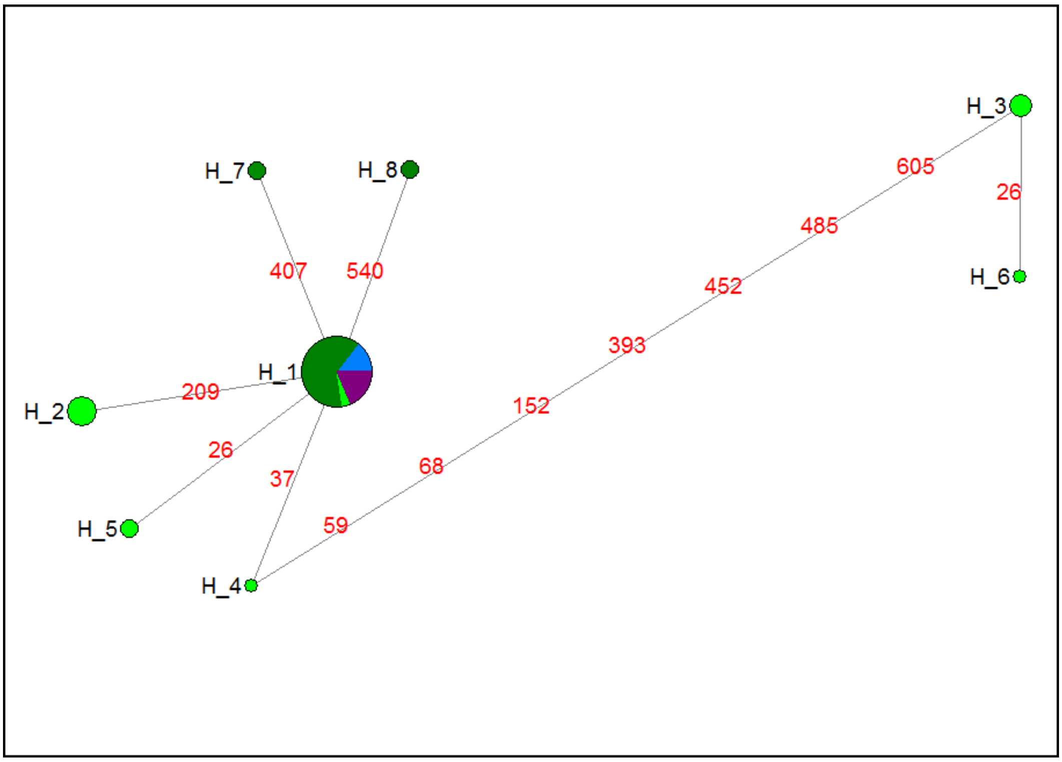
H_12
H_8
H_11

f)


H_3
605
26
485
H_7
H_8
H_6
452
407
540
393
H_1
152
209
H_2
26
68
37
59
H_5
H_4

SUPPLEMENTARY MATERIAL

Supplementary Table 1. F_{ST} values indicating genetic differentiation between population pairs. a) *A. obstetricans*, b) *B. bufo*, c) *D. galganoi*, d) *L. boscai*, e) *L. helveticus*, f) *T. marmoratus*.

a)

Sampling location	G	F	D	C
G	-			
F	0.95614***	-		
D	0.93190***	0.72409***	-	
C	0.92985***	0.84706***	-0.03172	-

b)

Sampling location	G	F	E
G	-		
F	0.10705***	-	
E	0.05062	0.05547	-

c)

Sampling location	D	E	C
D	-		
E	0.16167**	-	
C	0.20989***	-0.04950	-

d)

Sampling location	F	C	B	A
F	-			
C	0.00000	-		
B	0.88947**	0.87808	-	
A	0.93483*	0.92308	0.22778*	-

e)

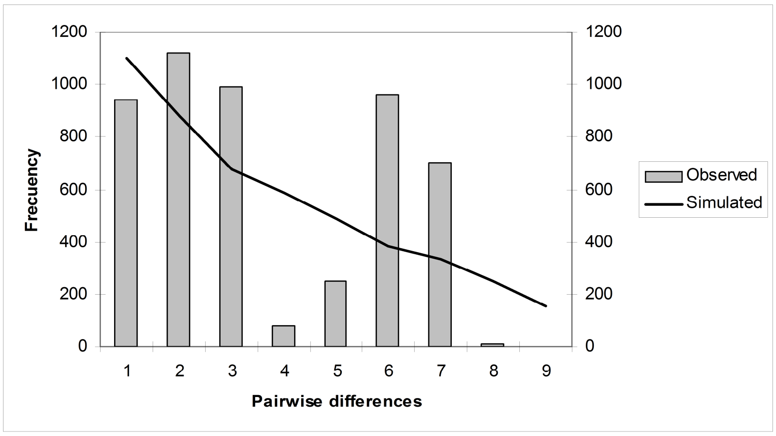
Sampling location	G	F	E	D	C	B	A
G	-						
F	0.84734***	-					
E	0.96179*	0.28086**	-				
D	0.95972**	0.57038***	0.28192**	-			
C	0.95887***	0.65449***	0.36969***	0.27853***	-		
B	0.90480*	0.27911**	0.08141	0.43656***	0.51722***	-	
A	1.00000	-0.69960	0.00000	0.52353	0.56337	-0.68665	-

f)

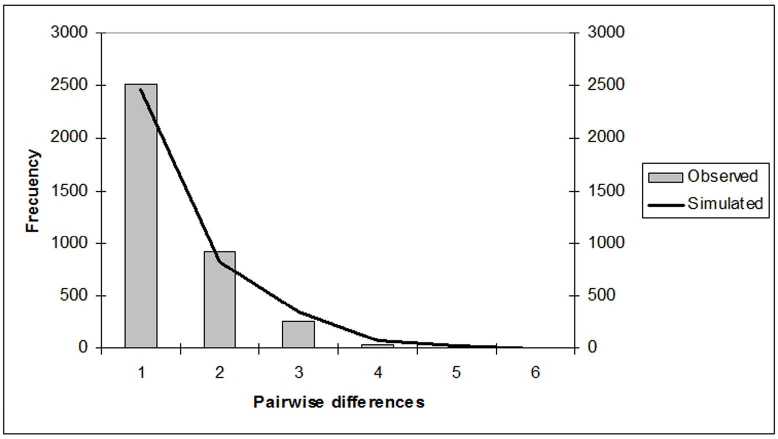
Sampling location	D	C	B	A
D	-			
C	0	-		
B	0.01763	0.05217	-	
A	-0.10401	-0.07162	0.25948*	-

Supplementary Figure 1. Mismatch distributions of the overall dataset of the COI sequences. Grey bars represent the observed frequency distribution of pairwise differences and black lines shows the distribution expected under the sudden-expansion model.

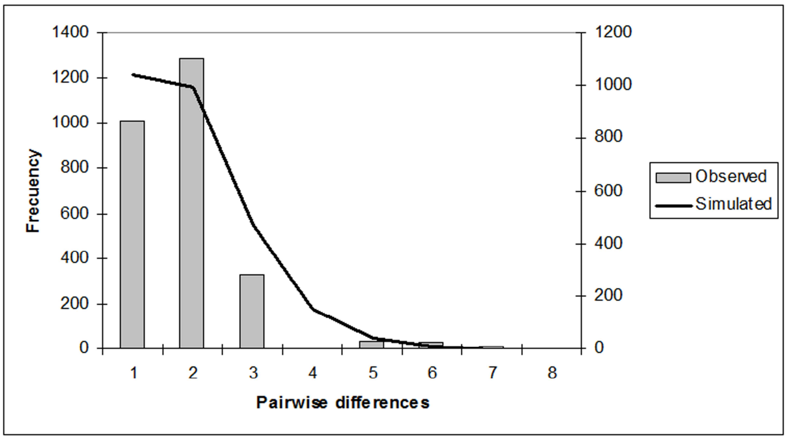
a)



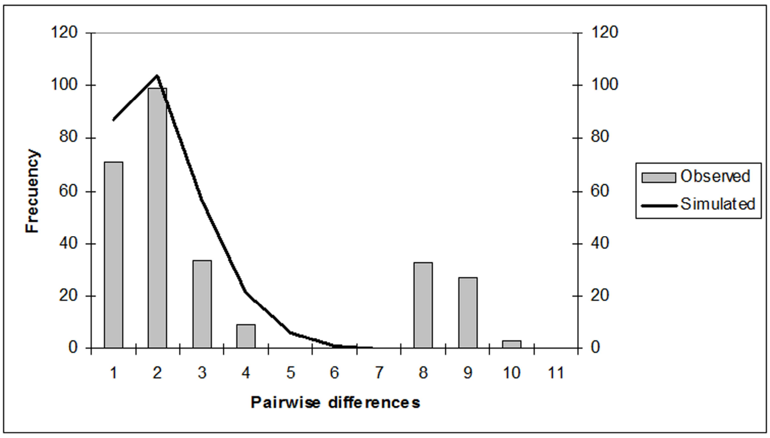
b)



c)



d)


Frecuency
Pairwise differences
Observed
Simulated
0
20
40
60
80
100
120
1
2
3
4
5
6
7
8
9
10
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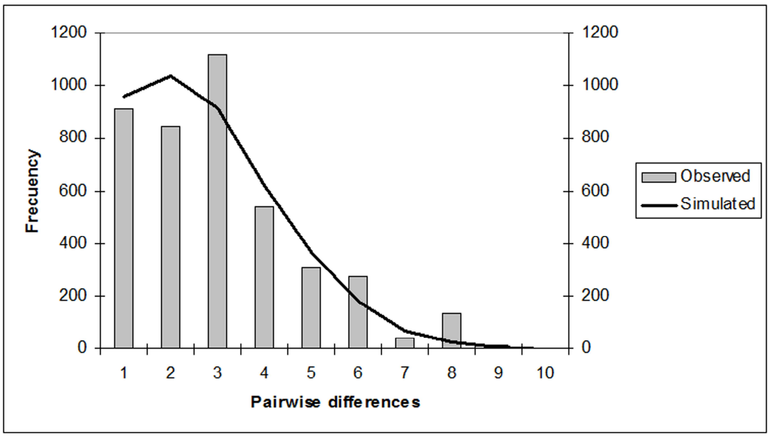
e)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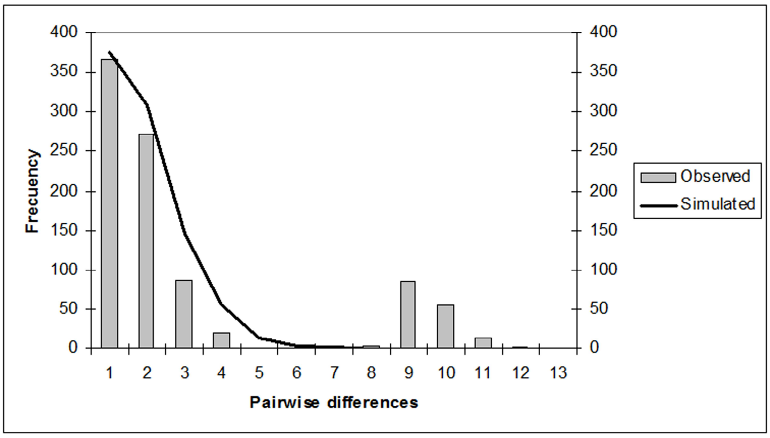
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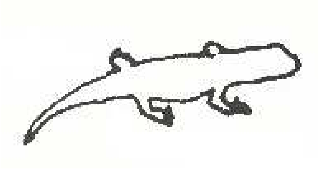

Capítulo 6

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Reasonable Economic Costs of Amphibian Conservation in Urban Environments: A Case Study in North Spain

Claudia Garcia-Gonzalez · Eva Garcia-Vazquez

Introduction

Amongst the wild animal inhabitants of urban landscapes, amphibians are likely to be the most endangered group and one whose species are declining worldwide (Beebee 1995; Stuart *et al.* 2004). They are increasingly considered protection targets (e.g., Meyer-Aurich *et al.* 1998; Collins and Halliday 2005). Some water points in urban landscapes, for example water features and retention ponds, offer good habitats for amphibian species (Parris 2006; Gledhill *et al.* 2008; Birx-Raybuck *et al.* 2010). For purposes of conservation management actions that enhance amphibian abundance in urban settings should be identified and encouraged.

Not all actions that contribute to improving ecosystems will succeed in the long term, only those whose costs are lower than benefits can expect to be of long duration (Engel *et al.* 2008). Therefore, cost-benefit analyses are convenient tools with which to prioritize conservation efforts, particularly when target species inhabit urbanized areas where human wellness is the main objective. However, in the case of herpetofauna it is not easy to determine measurable benefits, since they are not a prized resource among urbanites. A possible approach, one employed by some researchers, is to assign a value to each wild animal based on fines legally established for removing one specimen of such species from an ecosystem and multiplying that value for the number of animals saved (e.g., Engeman *et al.* 2002, 2003; Shwiff *et al.* 2007). Conservative valuations applied to individual losses can form the basis of a cost-benefit analysis aimed at identifying the thresholds at which remediation expenditures would be justified (Shwiff *et al.* 2007).

A relatively easy management action to enhance amphibian populations could be moderate cleaning of water points, which is a traditional way of managing rural troughs that seems to contribute to preserving amphibian numbers in rural settings (Garcia-Gonzalez and Garcia-Vazquez 2011). However, it does entail a cost in staff salary and time, at least if compared with the simple absence of management of spontaneous ponds. In this case study we carry out a cost-benefit analysis of urban water point management considering the value of amphibian life in a city of North Spain, Oviedo. The results may serve as a model for conservation plans for other endangered animals lacking a clear value as a resource.

Materials and Methods

Case Study

Oviedo is a northern Spanish city of 200,000 inhabitants (GPS coordinates 43º22 N, 541 5º51 W, 723). Water points were localized by systematic walking in the city in winter (February), spring (April), summer (July) and fall (October) of 2007, 2008 and 2009.

Management Classification

The water points were classed based on the management employed for their maintenance. Some of them are spontaneous ponds or water sources, generally occurring in parks or suburban areas, and are not managed in any way. They are classed as type U (Unmanaged). Other points are fountains with anaesthetic function (decorative). They are regularly cleaned once a week by removing all

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vegetation and washing them with cleaning products (containing, for example, bleach). These water points are classed as I (intensive management). Finally, some water points are generally designed to water animals (horses, dogs) and are managed by removing the excess of vegetation monthly and cleaning the bottom sediments once a year. This type of management is generally employed for rural troughs (Garcia-Gonzalez and Garcia-Vazquez 2011) and is here called moderate (type M).

Amphibian Sampling

Dipnet surveys were conducted in the water points found in Oviedo. We employed new clean nets in each water point to avoid transporting diseases. Each water point was exhaustively covered by many sweeps in order to recover all amphibians present. They were examined *de visu*, photographed and, when visual identification was not easy (for example, small tadpoles or newt larvae), tail tips (1 mm) were excised with sterilized tweezers and scissors and stored in absolute ethanol for further genetic identification. The animals were immediately released into the water point after sampling.

Genetic Identification of Visually Ambiguous Specimens

Small young larvae and eggs were genetically identified based on COI or 16S rDNA sequences. Total DNA was extracted from small tail biopsies (about 1 mm^3) using the protocol by Estoup *et al.* (1996) and kept at 4º C until analysis.

The mitochondrial locus examined first was the Cytochrome oxydase I (COI) gene, and in cases of negative amplification of the COI (*Triturus marmoratus*, *Rana temporaria*) we analyzed the 16S rDNA gene. COI and 16S rDNA were PCR amplified employing the primers described by Ward *et al.* (2005) and Palumbi *et al.* (1991) respectively. PCR reactions were carried out in a total volume of 40 µl with 5x Go Taq® Flexi Buffer, 2.5 mM MgCl, 250 µM of each dNTP, 1 µM of each primer, 1 unit of Go Taq® Polymerase 5U/µl (Promega) and 50 ng of DNA template, in a GeneAmp® PCR system 2,700 (Applied Biosystems) with the following conditions: initial denaturing step at 95°C for 5 min; 35 cycles of denaturing at 95°C for 30 s, annealing (for 30 s) at 53°C and 58°C for 16S rDNA and COI gene respectively; extension at 72°C for 30 s; final extension at 72°C for 15 min.

PCR products were visualized in 2% agarose gels with ethidium bromide. Visible bright bands were excised from the gel and DNA was purified with a Wizard® SV Gel and PCR Clean-Up System (Promega). Automated fluorescence sequencing was run on an ABI PRISM 3,100 Genetic Analyzer (Applied Biosystems) with BigDye 3.1 Terminator system, in the Unit of Genetic Analysis of the University of Oviedo (Spain).

Sequences were edited employing the BioEdit Sequence Alignment Editor software (Hall 1999). They were aligned with the application ClustalW (Thompson *et al.* 1994) and compared with reference sequences within the GenBank database (http://www.ncbi.nlm.nih.gov/GenBank) employing the program BLASTn (Basic Local Alignment Search Tool).

Benefit-cost Analysis

The benefit-cost ratio (BCR), that is, the standard ratio of benefits to costs (Loomish and Walsh 1997), is calculated as:

$$\mathrm{BCR} = [\text{Total value of animals saved}]/[\text{Management costs}]$$

Ratios higher than one could be considered economically efficient because the benefits are higher than the costs. Different management strategies (in this case U, I and M) can be compared based on their respective operational efficacy and economic efficiency, as did Engeman *et al.* (2002) when assessing the operational efficacy and economic efficiency of turtle management.

The benefit estimates are based on the number of amphibians obtained from a type of management multiplied for the individual amphibian value. The value of each amphibian was estimated based on the Spanish Law on Animal Protection (Decreto Legislativo 2/2008, 15 of April of 2008; it can be consulted online at: http://noticias.juridicas.com/base_datos/CCAA/ca-dleg2-2008.t7.html). The species *Alytes obstetricans*, *Lissotriton helveticus*, *Rana temporaria* and *Triturus marmoratus* appear within the category D. Possessing animals (including eggs and larvae or tadpoles) of this category is considered a minor infraction penalized with a fine of 100–400 €, and killing such animals is considered a major infraction punished with a 401–2,000 € fine. Species that are not in the list were not be considered for calculating benefits. Value estimates were be conservative as recommended by Shwiff *et al.* (2007), therefore we have chosen €100 as a reference. Exceptions are be made here for benefit estimates from eggs. The survival of juveniles to metamorphose has been estimated 0.64–0.83 for *Alytes obstetricans* tadpoles in other Spanish regions (Richter-Boix *et al.* 2006), and between 0-22-0.8 for different newts (Beebee 1996), but egg-adult survival rate, although variable depending on many environmental factors, is much lower for all amphibians (e.g., Beebee 1996). Therefore valuing each individual egg as an adult would exaggerate the benefit of a water point. For eggs a range of conversion factors of 0.05 – 0.01 are be applied.

As the water points were different in size (surface), we standardized the absolute number of amphibians, presenting instead the number of amphibians/surface unit for each management type, the surface unit being the average surface of a water point (= total surface/number of water points).

Management costs were estimated taking into account three main issues: salary corresponding to the hours allocated to water point management, materials (clothes, protective gloves, tools for vegetation and sediment removal) and cleaning products. We have taken as reference an average Spanish salary for a technician, including all charges (social security, taxes, insurance etc.), which is approximately 23,000 € per year (one working day=63 €). We have estimated that cleaning each water point requires one working day of one employee. The difference between M and I water points is the number of cleaning days per year: 12 and 48 (monthly and weekly) respectively, and the intensity of the cleaning. Annual salary costs for management of each type of water point will therefore be the number of days dedicated to each water point multiplied by the daily salary.

The materials are the same for M and I management. Gloves and other tools should be sterilized after cleaning each water point to prevent diseases spreading. We have estimated 300 € for materials + 100 € of sterilization costs, adding up 400 €. Cleaning products (estimated approximately 100 €/years/water point on average) are employed only for I-management.

Summarizing, costs per water point and year for M-management would be:

Staff time, 1 day/month: 63×12=756 €
No cleaning products
Equipment: 400 €
Total estimated: 1,156 €/year

For I-management would be:

Staff time, 4 days/month: 3,024 €
Equipment: 400 €
Cleaning products: 100 €
Total estimated: 3,524 €

Unmanaged water points are not without costs because they need some maintenance work like periodic cleaning for prevention of litter accumulation, bad odors and visual contamination. It is difficult to set up a standard time periodicity for cleaning such spontaneous water points because it will depend upon location within urban spaces (for example, a pond in the middle of a park may require less cleaning than a pond located close to a childcare institution or a hospital). The minimum maintenance could be reasonably assumed to occur, on average, twice a year (spring and autumn, for example). Cleaning products are not expected to be employed. Precautions about prevention of disease spreading should be taken as in the other cases, therefore the equipment should also be sterilized after cleaning each site. Costs for U-management would thus be:

Staff time, 1 day/month: 63×2=126 €
No cleaning products
Equipment: 400 €
Total estimated: 526 €/year

Results

Amphibian Inventory

Eight water points containing amphibians were found within Oviedo city (approximately ten km^2) (Table 1): two unmanaged temporary spontaneous ponds (type U), three decorative fountains (type I) and three natural fountains with type M management. The total surface of each type of water point was 50,000, 45,550 and 41,910 cm^2 for U, I and M-managed water points, respectively, with an average size of 17,182.5. Therefore U sites contain habitat equivalent to 2.91 units of average surface (50,000/17,182.5), and I sites and M sites contain 2.65 and 2.44 units respectively.

Table 1 Characteristics and amphibian abundance (measured as number of individuals of each species) found in eight water points inside Oviedo city (north of Spain). Mgmt, management as U = unmanaged, I = intense management, M = moderate management. *A. o.*, *Alytes obstetricans*; *D. g.*, *Discoglossus galganoi*; *L. h.*, *Lissotriton helveticus*; *R. t.*, *Rana temporaria*; *T. m.*, *Triturus marmoratus*. A, adults; L, larvae; E, eggs

Water point	Mgmt	Depth (cm)	Surface (cm^2)	*A. o.*	*D. g.*	*L. h.*		*R. t.*	*T. m.*
				L	L	L	A	E	A
P1	U	30	30,000	27	23	58	–	–	–
P2	U	40	20,000	–	–	–	19	–	–
Total U				27	23	58	19	–	–
F1	I	10	9,000	43	–	–	1	–	–
F2	I	30	18,550	44	–	–	–	–	–
F3	I	45	18,000	10	–	–	1	–	–
Total I				97	0	0	2	–	–
T1	M	26	10,710	14	–	–	19	102	3
T2	M	26	16,200	17	–	9	10	120	–
T3	M	40	15,000	15	–	8	3	51	–
Total M				46	0	17	32	273	3

Table 2 Benefit-cost analysis of three alternative management types (U, I and M as unmanaged, intense and moderate management respectively) for abundance of amphibians in urban water points. The value of each amphibian was estimated in 100 € from the Spanish law of Animal protection. BCR: Benefit-cost ratio, as [Estimated benefit/Estimated cost]. Low and high survival are respectively 0.01 and 0.05 egg-adult survival of *Rana temporaria* and was applied to frog eggs found in M water points

	Number of amphibians		Amphibians per surface unit		Estimated benefit		Estimated cost	BCR	
Management	Low survival	High survival	Low survival	High survival	Low survival	High survival		Low survival	High survival
U	104		35.74		3,574 €		526 €	6.79	
I	99		37.36		3,736 €		3,524 €	1.06	
M	100.73	111.65	41.28	45.76	4,128 €	4,576 €	1,156 €	3.57	3.96

In total five species of amphibians were found in our survey (Table 1): the Anurans *Alytes obstetricans* (in all water points with amphibians except one U site), *Discoglossus galganoi* (in one U site) and *Rana temporaria* (in the three M sites), and the Urodeles *Lissotriton helveticus* (in all except one I water points) and *Triturus marmoratus* (in one M site). The total number of amphibians found in each type of water point was different, as well as their life stage. Anurans were all juveniles (tadpoles or eggs in the case of *Rana temporaria*) whereas most Urodeles were adults. The total number of amphibians sampled from U, I and M water points was 127, 99 and 371 specimens respectively (adding individuals from all life stages). The number of species was three for U sites, two for I sites and four for M sites.

Benefit-cost Analysis

For calculating benefits only the species listed in the Spanish legal normative were taken into account, therefore *Discoglossus galganoi* tadpoles were excluded from this analysis. *Rana temporaria* eggs were transformed into adult units employing two different correction factors (0.05 and 0.01) for being conservative with benefits. The number of amphibians per surface unit (average surface of Oviedo water points) was higher for M managed (whatever survival estimate) than for the other types of water points (Table 2), the minimum being near 36 for unmanaged ponds. Even if *Rana temporaria* eggs were not considered, M points would yield maximum values (40 amphibians per surface unit). Accordingly the highest estimated benefit corresponded to M points.

The maximum BCR (6.8) corresponded to the management type with lowest costs, type U, while the minimum was near 1 for I water points (Table 2), i.e., no net benefits or costs. Depending on survival estimates for *Rana temporaria*, BCR will be 3.6 or near 4 for M water points. Comparatively, the operational efficacy was clearly higher for type M management while the economic efficiency was higher for U management. I management yielded an intermediate value for operational efficiency and the lowest for economic efficacy.

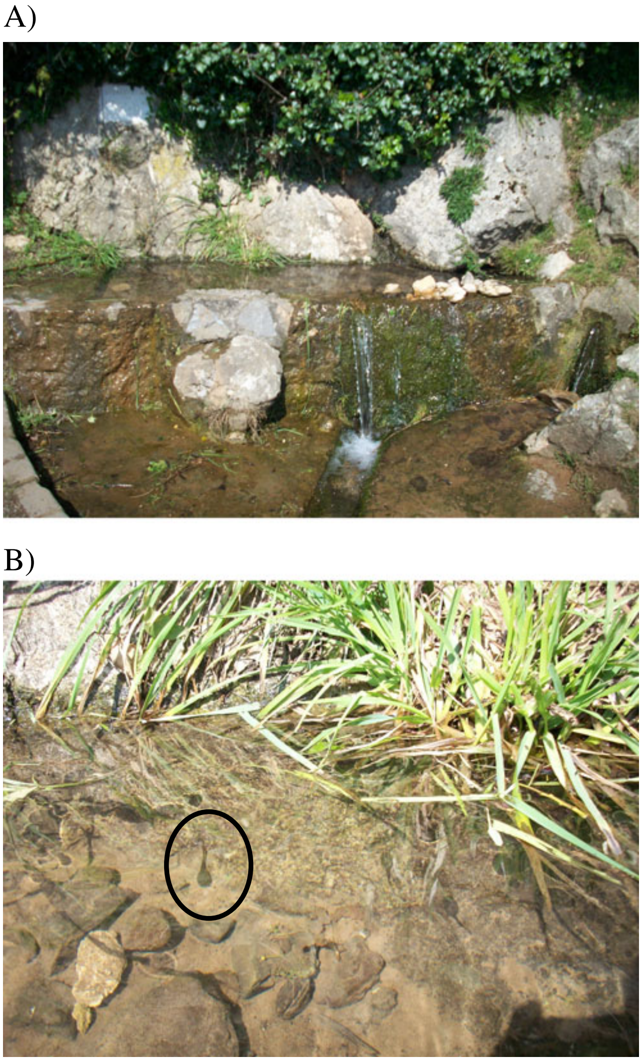

Fig. 1 Picture of a fountain with amphibians subjected to moderate management. **a** General vision. **b** Detail of the inside showing a larvae of *Alytes obstetricans*

If costs are not considered, estimated benefits place M management as the best one, followed by I and U respectively. In terms of amphibian abundance, without taking into account the number of species (diversity), I management would thus be better than U management. If diversity is considered an added value, management would be ranked from better to worse as M, U and I, with four, three and two species respectively.

Discussion

In this pilot study we have carried out a benefit-cost analysis of management protocols of urban water points considering amphibian life as a benefit. The most cost-efficient management procedure, U (the least intensely managed), did not offer the level of operational efficacy regarding the production of amphibians, as did the intermediate procedure of moderate cleaning (M). Intense cleaning (management type I) did not result in net benefits. Net benefits were obtained with moderate clearing, which could be considered the best management strategy for urban water points.

Some considerations, however, should be made for improving this type of approach if it is to be generalized for management decision-making. First, *Discoglossus galganoi* was not considered for estimating benefits because it is not listed in the current Spanish normative of animal protection. However, this species has been listed by the European Community as a species of interest whose conservation requires the designation of special areas of conservation (Council Directive 92/43/EEC; http://europa.eu.int/comm/environment/nature/habdir.htm). Its populations should be evaluated case by case in each region because marginal and sparse populations, which possibly represent distinct genetic lineages, are threatened and in some cases became extinct because of the destruction of their habitats (Gasc 1997). If *D. galganoi* is considered and the economic aspect of fines is not taken into account, the best management strategy is U. This species appears in the unmanaged pond P1 and increases the number of amphibians there, elevating P1 in the rank of favorable sites for amphibians up to the first position.

More complex than simply amphibian abundance is amphibian diversity, which is another parameter that should be taken into account when calculating ecosystem benefits, although in our case it is not easy to monetarize this because urban areas are not protected spaces unlike the interesting study by Carvalho *et al.* (2010). If this is considered, moderate management seems to be also the most beneficial strategy, as it is in rural settings (Garcia-Gonzalez and Garcia-Vazquez 2011): species richness is higher in M water points, with four different species (3.3 per water point in average) than in the other sites (2 and 1.7 for U and I points respectively).

On the other hand, economic theory implies that benefits should be measured in terms of people's willingness-to-pay for valued goods. The present analysis uses criminal or civil penalties as a surrogate for willingness-to-pay, which may not be accurate as a measure of the underlying value of the good. The study could be grounded in the use of nonmarket valuation methods, but, as for amphibian diversity, it is very difficult to give a monetary value to potential benefits of urban water points. They have obvious value beyond supporting amphibian life. If urban spaces are planned principally for human services, amphibians are not a priority. However, an ecosystem approach to urban spaces considering wildlife maintenance as a service is possible (e.g., Tzoulas *et al.* 2007), and moderate management of water points can combine services to both humans and to amphibians: water can be potable and the external appearance of the fountain can be aesthetically attractive (Fig. 1). Moderate management has been identified as a durable way for conserving wildlife in rural water points, because the long-term result of type U management is silting and finally deterioration of the water point, and I management often encompasses destruction of fragile larvae of threatened species (Garcia-Gonzalez and Garcia-Vazquez 2011). On the other hand, amphibian life can be easily integrated into urban human life if explanations about the species present in the fountain, their life cycle and their role in the ecosystem (for example controlling mosquito populations; Becker 2006) are exhibited in panels nearby. Environmental education is an intangible and unquantifiable benefit that should be valued as a long-term strategy of conservation and this simple strategy could use amphibians as a vehicle for enhancing environmental awareness in citizens.

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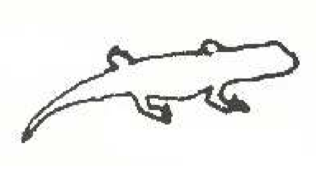


Discusión

REFUGIOS DE ANFIBIOS EN ZONAS RURALES Y URBANAS

Los resultados de esta Tesis muestran que los bebederos tradicionales constituyen puntos de agua permanentes en los cuales pueden reproducirse tanto anuros como urodelos. Especialmente para aquellas especies que necesitan agua permanente a lo largo de todo su ciclo de vida, o cuyo desarrollo larval se extiende por más de un año, como es el caso de *Alytes obstetricans*, las charcas temporales no son adecuadas. En la región estudiada, los bebederos generalmente se encuentran en el centro de los pueblos o en sus cercanías para facilitar el acceso al agua para el ganado comunal. Tradicionalmente, estos bebederos construidos en piedra eran limpiados ocasionalmente, sólo para eliminar el exceso de sedimentos y vegetación acumulados, sin rascar la vegetación adherida a las paredes ni los limos acumulados en el fondo. Pero la función tradicional de los bebederos está cambiando junto con el resto de las actividades de la zona rural asturiana (Dopico *et al.*, 2009) y con ella el tipo de manejo de los mismos. El abandono de la ganadería tradicional conlleva el abandono del uso de estos bebederos, que con el tiempo, se van colmatando de sedimentos y maleza, perdiendo su función como hábitat acuático de anfibios y otros animales. En otros casos, el aumento del turismo rural hace de estos elementos funcionales meros elementos decorativos, que son tratados mediante una limpieza exhaustiva que elimina toda vegetación y sedimentos, impidiendo el desarrollo de vida anfibia.

Se han encontrado ocho especies de anfibios en bebederos construidos para el ganado en el valle del Trubia (Principado de Asturias), lo que representa un 60 % de las 14 especies registradas en esta región (Martínez-Solano *et al.*, 2001). Estos resultados coinciden con un número creciente de estudios que identifican las charcas agrícolas artificiales como un buen hábitat acuático para la conservación de anfibios (ej. Beebee, 1997; Crochet *et al.*, 2004; Knutson *et al.*, 2004; Denoël y Ficetola, 2008; Rannap *et al.*, 2009; Hartel *et al.*, 2010, Curado *et al.*, 2011). Sin embargo, ha habido un debate acerca de su idoneidad para conservar poblaciones de anfibios a largo plazo. Uno de los principales problemas es la contaminación, causada principalmente por el aumento de microorganismos, pesticidas, fertilizantes y nitrógeno disuelto en el agua (Knutson *et al.*, 2004; Schmutzer *et al.*, 2008; revisado por Mann *et al.*, 2009). Parece probable que afecten con mayor severidad al ganado que a las poblaciones de anfibios, ya que la época de reproducción de los anfibios suele tener lugar antes de la época de mayor pérdida de calidad del agua (Canals *et al*., 2011). De cualquier forma, estos problemas de zonas agrícolas asociados a la presencia de fertilizantes o plaguicidas y al deterioro general de la calidad de las aguas no afectarían a los bebederos de ganado. Generalmente, este tipo de estructuras se abastecen de un flujo de agua continuo que proviene de la canalización de un arroyo próximo, por lo que la renovación del agua está asegurada durante todo el año y se pueden considerar puntos de agua permanentes. La presencia de compuestos químicos derivados de la agricultura no les afecta de una forma tan intensa como a las charcas, que suelen estar afectadas por la escorrentía del agua de regadío con la presencia de restos de estos compuestos nocivos. Las especies *A. obstetricans*, *M. alpestris*, *L. helveticus* y *L. vulgaris* parecen tener preferencia por los bebederos de ganado en vez de por las charcas semi-naturales o charcas para el ganado, ya que es en los bebederos tradicionales donde se encontró una mayor riqueza de especies en otras zonas (Curado *et al.*, 2011). Este tipo de estructuras suelen localizarse en zonas umbrías y esto, unido a un flujo contínuo de agua, permite el desarrollo de especies con una fase larval larga, como *A. obstetricans*, o de aquellos adultos que residan en el medio acuático todo el año. Los resultados de esta Tesis permiten identificar los bebederos tradicionales asturianos como verdaderos refugios para los anfibios.

De acuerdo con otros autores (ej. Parris, 2006; Gledhill *et al.*, 2008), estos resultados también indican que las zonas urbanas pueden contener hábitats-refugio que sustentan poblaciones de anfibios, como las charcas artificiales. Es evidente que la antropización conlleva efectos negativos para los anfibios y estos resultados apuntan hacia la importancia de establecer acciones estratégicas de gestión de estas especies en áreas urbanas. Se ha intentado desarrollar diversos modelos para intentar definir qué factores son los más determinantes para favorecer la riqueza de especies en anfibios en áreas urbanas. Varios autores concuerdan en que las características del medio que rodea las charcas en unos 1000 m aproximadamente son fundamentales, sobre todo la presencia de espacios verdes en estas áreas (Simon *et al.*, 2009; Hamer y Parris, 2011). Como medidas a tener en cuenta se destacarían mejorar el hábitat terrestre y la conectividad en torno a charcas y otros puntos de agua próximos, unido a acciones locales como mejorar la calidad del agua, eliminar las especies depredadoras y favorecer la presencia de plantas acuáticas y vegetación en los sitios de reproducción (revisión de Hamer y McDonnell, 2008; Hamer y Parris, 2011). Los resultados de esta Tesis concuerdan plenamente con las conclusiones obtenidas por estos autores; resaltan el valor de las charcas urbanas y sugieren que se debería profundizar en su conocimiento e incorporarse en los planes de conservación de anfibios. Contrariamente a lo apuntado por autores como Hitchings y Beebee (1997), Arens *et al.* (2007), Gagne y Fahrig (2007) y Nöel *et al.* (2007), que postulan los hábitats urbanos como empobrecedores de la diversidad de anfibios, en este trabajo se ha confirmado que pueden albergar una considerable riqueza en estas especies. Tomando muestras de una charca de muy pequeño tamaño (menor de 3 m^2) en la ciudad de Oviedo (Asturias), se encontraron abundantes poblaciones de dos anuros (*Alytes obstetricans*, *Discoglossus galganoi*) y un urodelo (*Lissotriton helveticus*). Comparada con el conjunto de muestras obtenidas de zonas rurales próximas, se encontró que la diversidad de especies en esta charca urbana de reciente construcción es del mismo orden, lo que indica la capacidad de los anfibios de áreas próximas de colonizar y establecerse en hábitats antropizados (Baker y Halliday, 1999) y nos permite resaltar el valor de los hábitats urbanos para estas especies.

La capacidad de dispersión de los anfibios puede verse significativamente reducida en los ambientes urbanos y suburbanos. Sin embargo, este principio general y lógico no es aplicable por igual a todas las especies, que presentan respuestas muy distintas frente a la urbanización. Los anfibios generalistas o con requisitos de dispersión relativamente bajos parecen ser más capaces de sobrevivir en ambientes urbanos y suburbanos (Hamer y McDonnell, 2008). En esta Tesis, las tres especies presentes en el pequeño hábitat urbano tomado como modelo exhiben una alta diversidad genética en su ADN mitocondrial, comparable o superior a los hábitats acuáticos rurales próximos de mayores dimensiones. Estos resultados son contrarios a otros estudios que encontraron menor riqueza de especies en charcas urbanas que en agrícolas o forestales (Gagne y Fahrig, 2007). La elevada variabilidad en la charca ovetense estudiada quizás podría ser debida a que corresponda con un área de contacto secundario, como ha sido observado para *A. cisternasii* (Gonçalves *et al.*, 2009), *A. obstetricans* (Martínez-Solano *et al.*, 2004) y *Discoglossus* (Zangari *et al.*, 2006). En cualquier caso, es evidente que en la zona en estudio las charcas urbanas son elegidas por los anfibios como medio acuático de vida.

Utilizando una perspectiva metagenómica en la misma área urbana, utilizando el gen COI como marcador, se comprobó que incluso fuentes muy pequeñas y charcas formadas espontáneamente sin intervención humana constituyen un hábitat adecuado para la reproducción del sapo partero y del tritón palmeado y permiten el establecimiento de comunidades de anfibios de gran diversidad. De esta manera, estos puntos de agua serían los mayores contribuyentes a la riqueza de especies de anfibios en hábitats urbanos, apoyando a autores como Gledhill *et al.* (2008), que destacan el gran valor de los puntos de agua urbanos como hábitats de anfibios. Esta visión metagenómica permite añadir la variación interna de las poblaciones a la variación genética interespecífica, algo que con los índices clásicos de diversidad no se recoge (Ardura *et al.*, 2011). En el caso concreto de la cuantificación de la diversidad en anfibios urbanos se comprobó que la diversidad genética intraespecífica (medida, por ejemplo, como diversidad haplotídica) no está correlacionada con la diversidad metagenómica ni con el número de variantes funcionales (proteínas). Esta estrategia de análisis permitió sacar a la luz la diversidad completa encontrada en los diferentes hábitats urbanos, tal como habían citado otros autores como ventaja del método en el estudio de comunidades de eucariotas inferiores (Fonseca *et al.*, 2010; Porazinska *et al.*, 2010).

BARRERAS PAISAJÍSTICAS PARA ANFIBIOS EN EL NORTE DE LA PENÍNSULA IBÉRICA

Para las tres especies encontradas en las charcas urbanas de Oviedo (*A. obstetricans*, *D. galganoi* y *L. helveticus*) se encontraron altos niveles de diferenciación poblacional a pequeña escala. También se encontraron diferencias significativas entre bebederos rurales próximos. Esta diferenciación podría estar causada por barreras de diferentes tipos ya que tanto para anuros (Vos *et al.*, 2001; Funk *et al.*, 2005; Johansson *et al.*, 2005; Stevens *et al.*, 2006) como para urodelos (Spear *et al.*, 2005; Marsh *et al.*, 2005), se ha observado un fuerte impacto del paisaje en la distribución de la diversidad genética, y sobre todo un efecto negativo del uso antropogénico del territorio (por ejemplo, carreteras, vías férreas, áreas urbanas). La fragmentación del hábitat es uno de los principales impactos antropogénicos en anfibios (Funk *et al.*, 2005; Cushman, 2006). Evaluando dos tipos diferentes de barreras, una antropogénica (las carreteras) y otra natural (los ríos), en este trabajo se ha podido comprobar cómo ambos tipos contribuyen a configurar la estructura de las poblaciones de anfibios, determinando el flujo genético entre demes vecinos.

La estructuración poblacional, medida a partir de la variación del ADN mitocondrial a lo largo del paisaje rural del valle del Trubia en el norte de España, reveló una asociación significativa entre la densidad de carreteras y la distancia genética para las poblaciones de dos especies de anfibios, el sapo partero común *A. obstetricans* y el tritón palmeado *L. helveticus*. La mitigación del tráfico cerca de asentamientos urbanos parece reducir la fragmentación de las poblaciones de *L. helveticus*, pero no la de las de *A. obstetricans*, lo que indica que incluso las pequeñas carreteras con baja intensidad de tráfico actúan como barreras para esta última especie. Suele suceder que el impacto de las carreteras en los anfibios presente diferencias según cuál sea la especie que tengamos en cuenta. Carr y Fahring (2001) han descrito que la mortalidad causada por el tráfico puede ocasionar mayores declives poblacionales en especies con gran capacidad de dispersión que en aquellas cuyos movimientos migratorios son menores. Las especies que se desplazan a larga distancia tienen más oportunidades de cruzar carreteras, y serían las más beneficiadas de medidas de moderación del tráfico porque se reduciría el riesgo de mortalidad durante los movimientos de los individuos de estas especies (van Langevelde y Jaarsma, 2009). Otros autores apuntan que la fragmentación causada por las carreteras afecta de forma diferente a anuros y a urodelos (Fahring *et al.*, 1995; Mazerolle, 2004). En este trabajo también se ha encontrado un efecto diferente de las carreteras en *A. obstetricans* y *L. helveticus*, con un impacto mucho mayor sobre el sapo partero que no es mitigado por circunvalaciones viarias. Este tema se retomará más adelante.

En cuanto a barreras naturales como los ríos, de acuerdo con los resultados obtenidos, no se puede describir un patrón general para el papel que desempeñan en la estructura espacial poblacional de las seis especies estudiadas en el norte de la Península Ibérica. Para cinco de las seis especies analizadas (*A. obstetricans, D. galganoi*, *L. boscai*, *L. helveticus* y *T. maroratus*) se ha visto que los ríos actúan como barreras al flujo génico, de forma similar a lo que sucede en otras especies de anuros (Lampert *et al.*, 2003; Monsen y Blouin, 2003; Funk *et al*., 2005). Sin embargo, para *B. bufo* no se apreció ningún indicio de aislamiento poblacional en el rango de los 300 km estudiados, en contraste con la alta diversificación de poblaciones documentada en el centro de España (Martínez-Solano y González, 2008). Esta especie puede utilizar los ríos como hábitat de desove, y a menudo se encuentran larvas en zonas de poca corriente de los ríos asturianos, que evidentemente no actúan de barrera al flujo génico para esta especie, dada su ecología reproductora.

El impacto de los ríos en el aislamiento de poblaciones es fuertemente dependiente de la especie de anfibio. Un mismo río puede actuar como barrera impidiendo casi totalmente el flujo genético en una especie y no suponer apenas un obstáculo para otras. En la Península Ibérica algunos ríos han sido descritos como barreras para anfibios. Por ejemplo, para *T. pygmaeus* el río Tajo actúa como barrera en el centro de Portugal en su avance hacia en Norte (Arntzen y Themudo, 2008), y el río Miño fragmenta las poblaciones de la salamandra *Chioglossa lusitanica* (Alexandrino *et al.,* 2000). Sin embargo, para otras especies como *A. obstetricans*, representan barreras abruptas específicas para el flujo genético. Su patrón de variación genética podría describirse como conjunto de pequeñas poblaciones aisladas y fuertemente diferenciadas unas de otras, una diferenciación que no puede explicarse con un modelo de aislamiento por distancia. La baja diversidad nucleotídica y alta diversidad haplotídica indicarían una rápida expansión después de un cuello de botella o un tamaño efectivo pequeño (Avise, 2000), lo que reforzaría la idea de pequeñas poblaciones muy aisladas unas de otras. En contraste, especies como *L. helveticus* presentan un modelo poblacional que se ajustaría al aislamiento por distancia, como ya fue documentado por Recuero y García-París (2011) en el clado “Asturiano” de su estudio. *D. galganoi* no presentó una estructura geográfica diferenciada, debida a la homogeneidad de sus poblaciones. La baja diversidad encontrada sugiere que la población haya pasado por un prolongado o aún reciente cuello de botella (Avise, 2000), al igual que el otro anuro Discoglossidae de este estudio, *A. obstetricans*. Tanto para *L. boscai* como para *T. marmoratus* se identificaron dos clados altamente diferenciados, con una disminución de la variabilidad genética del oeste hacia el este del área de estudio. Para *L. boscai* este patrón ya fue descrito por Martínez-Solano *et al.* (2006) en el que un aislamiento alopátrico con un cuello de botella seguido por una expansión en el clado del oeste, con un posible contacto secundario, podrían explicar su patrón de variación. Como ya sugirieron estos autores, este patrón se correspondería con la presencia de un refugio glaciar en la zona oeste de la Península Ibérica. Contactos secundarios en poblaciones previamente aisladas después del último glaciar máximo en Europa han sido ampliamente descritas en anfibios, como para *Mesotriton alpestris* en Polonia (Pabijan y Babik, 2006), o en la Península ibérica para *L. boscai* (Martínez-Solano *et al.*, 2006) y el grupo de *Alytes* (Martínez-Solano *et al.*, 2004b).

En general, en la región estudiada se han encontrado niveles de polimorfismo relativamente bajos en todas las especies estudiadas. Esto parece indicar que las poblaciones de anfibios en la región han sufrido probablemente fenómenos de cuellos de botella (Nei, 1975; Allendorf, 1986). Probablemente la estructuración poblacional de la mayoría de las especies consideradas no esté determinada por un único tipo de barrera. La conjunción de diferentes tipos de obstáculos al flujo genético, como ríos, carreteras y montañas, junto con características intrínsecas de las especies respecto a sus estrategias reproductoras y capacidad de dispersión (Zhao *et al.*, 2009), probablemente actúen de forma combinada para establecer la diferenciación genética que se observa entre las poblaciones de cada especie, que como se ha visto en este trabajo tienen una gran diversidad.

APLICACIONES PARA LA CONSERVACIÓN DE LOS ANFIBIOS EN LA REGIÓN ESTUDIADA

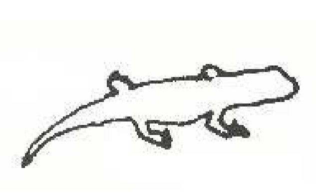
Por lo expuesto anteriormente, no parece oportuno hablar de una estrategia única para proteger los anfibios en la región estudiada, sino que hay que prestar atención a las necesidades específicas de cada especie, debido a su diferente sensibilidad a barreras físicas espaciales. Crear circunvalaciones o vías rápidas en áreas rurales pueden ayudar a favorecer la conectividad de ciertas especies como *L. helveticus*. En cambio, para la mitigación de la fragmentación de poblaciones de anfibios como *A. obstetricans*, que probablemente tiene más instinto de retorno o dificultad de dispersión, se podría proponer la construcción de pasajes a través de las carreteras rurales. Muchas especies podrían beneficiarse de la construcción de pasos para anfibios en los puentes ya existentes o de la construcción de nuevos puentes con zonas verdes como vías para atravesar los ríos. Conocer la genética de poblaciones, el rango de dispersión y los requerimientos ecológicos de cada especie sería clave para atender las necesidades particulares de cada especie de anfibio y sus limitaciones.

Los cambios en las actividades agrícolas de la región, consistentes en la disminución de la ganadería y aumento del turismo rural, son los principales motivos de la transformación del uso tradicional de estos bebederos, que pierden su función (por abandono) o se transforman en elementos decorativos. Una limpieza moderada, la presencia de sedimentos en el fondo y una abundante vegetación flotante, fueron identificados como los principales factores que contribuyen a la riqueza de especies anfibias. El manejo tradicional para prevenir la colmatación de los bebederos con un exceso de barro o residuos vegetales sería la estrategia que más favorece la presencia de anfibios.

Dada la importancia de los puntos de agua urbanos, establecer una estrategia de conservación de los mismos es fundamental. Esto no es sencillo porque las áreas urbanas no suelen ser espacios protegidos, excepto en casos concretos (Carvalho *et al.*, 2010). El análisis de coste-beneficio, teniendo en cuenta la abundancia de anfibios como beneficios y los salarios, material y productos de limpieza, como costos de mantenimiento de las fuentes y estanques urbanos, permitió identificar alguna estrategia sencilla de muy bajo coste. Una intensidad de limpieza intermedia, en la que se eliminan mensualmente los restos vegetales y anualmente el exceso de sedimentos, similar a la descrita para los bebederos de las zonas rurales, resulta ser también en zonas urbanas la más eficaz en términos de abundancia y diversidad de anfibios, y en beneficios netos. Este sistema de mantenimiento de bajo coste, favorecería el desarrollo de vida anfibia en zonas urbanas, resultando más eficaz que la ausencia de manejo de puntos de agua (abandono). La limpieza moderada de los puntos de agua en zonas urbanas podría equilibrar la conservación de los anfibios y el uso humano de los espacios públicos evitando la acumulación excesiva de larvas de insectos y el aspecto descuidado de las zonas húmedas.

El análisis de componentes principales con diferentes parámetros del hábitat permite concluir que hay dos tipos principales de acciones que favorecerían la conservación de los anfibios en los paisajes urbanos: a) el manejo de puntos de agua, que mejoraría las poblaciones de tritón, y b) la conservación o creación de corredores vegetales para la conexión entre puntos de agua, como prioridad para mejorar la diversidad de anuros. Nuevamente aparece la diferencia entre especies como una característica que afecta directamente a las potenciales estrategias y medidas de conservación, que tendrían distinta eficacia para cada especie. Para especies con una baja capacidad de dispersión, la creación de corredores vegetales que permitan conectar puntos de agua ayuda a mejorar la diversidad poblacional (Blaustein *et al.*, 1994; Beebee, 1996; Gibbs, 1998). En cambio, para otros con mayor capacidad de dispersión como los tritones, el manejo concreto de los puntos de agua es más importante para su conservación.

A modo de consideraciones finales, debido a la generalizada antropización de los hábitats, los cambios en el uso del suelo parecen un fenómeno en extensión. Los estudios llevados a cabo en esta Tesis se han centrado en el conocimiento de la pérdida y fragmentación de hábitats, realizando análisis especie-específicos, a diferentes escalas geográficas, mediante herramientas de genética molecular para conocer el flujo génico y la conectividad entre poblaciones, tal y como recomiendan autores reconocidos en conservación de anfibios como Cushman (2006) y Hamer y McDonnell (2008), entre otros. Pero también han servido para destacar el valor que tienen ciertos hábitats antropizados para la conservación de los anfibios, y para identificar acciones sencillas que sin embargo pueden contribuir a la supervivencia de estos animales en contacto con el ser humano. La transformación de los ecosistemas, la pérdida de hábitat y la degradación de la calidad del mismo son una realidad en Europa, y probablemente sean imparable en muchas regiones en desarrollo. Sin embargo, no necesariamente conducirán a la extinción de estas especies vulnerables de forma irremediable. Una manera sencilla y económica de evitarlo puede ser la preservación de estructuras antrópicas como los bebederos de ganado tradicionales y un manejo moderado de los puntos de agua. La antropización no tiene por qué ser necesariamente deletérea para anfibios. El ser humano también puede ayudar a estas y otras especies aportando nuevos hábitats y manteniéndolos en un estado apropiado de conservación. Dado que las barreras, tanto antropogénicas como naturales, pueden limitar la comunicación entre poblaciones, favorecer la conectividad mediante túneles bajo las carreteras o pasos para anfibios en los puentes, aliviaría estas limitaciones. Aunque las conclusiones de este trabajo son necesariamente limitadas por tratarse de un estudio a pequeña escala, permiten tener una primera aproximación práctica a estrategias a seguir para la conservación de anfibios en un entorno modificado por los humanos.



Conclusiones

1. Los bebederos tradicionales constituyen puntos de agua permanentes en los cuales pueden reproducirse tanto anuros como urodelos. Un manejo moderado, la presencia de sedimentos en el fondo y una abundante vegetación flotante, fueron identificados como los principales factores que contribuyen a la riqueza de especies anfibias. Cambios en el uso tradicional de los bebederos, debidos al abandono de la las actividades agrícolas y ganaderas, pueden poner en peligro estos refugios para anfibios; se recomienda un manejo de los mismos de tipo tradicional.

2. Las zonas urbanas contienen hábitats adecuados para poblaciones de anfibios, como pueden ser las pequeñas charcas artificiales. Estas charcas pueden albergar una alta diversidad genética mitocondrial, comparable a los niveles encontrados en áreas rurales cercanas, tanto en diversidad como en número de especies. Este resultado sugiere que las charcas urbanas deberían incorporarse en los planes de conservación de anfibios.

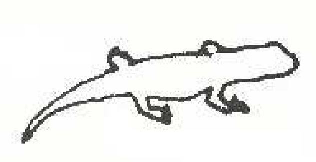
3. La perspectiva metagenómica, que combina la diversidad inter- e intraespecífica del gen COI, es más sensible a pequeña escala para evaluar la diversidad de comunidades anfibias que los índices clásicos de diversidad ecológica. Utilizando este método, se identificaron el tamaño y la conectividad como los principales factores que favorecen la diversidad de anfibios en los puntos de agua de la ciudad de Oviedo.

4. Se ha detectado en Asturias una asociación positiva entre la densidad de carreteras y la distancia genética entre poblaciones de *Alytes obstetricans* y *Lissotriton helveticus*. Las circunvalaciones o vías rápidas en áreas rurales conseguirían mitigar la fragmentación de poblaciones de *L. helveticus* pero no de *A. obstetricans*. La construcción de pasos para anfibios bajo las carreteras podría ayudar de forma significativa a la conservación de algunas especies.

5. Se han identificado los ríos cantábricos como barreras para el flujo génico para las especies *A. obstetricans*, *Discoglossus galganoi*, *L. boscai*, *L. helveticus* and *Triturus marmoratus*, pero no para *Bufo bufo*. Para establecer medidas de conservación se deberían tener en cuenta las necesidades particulares de cada especie. Se sugiere la construcción de pasos a través de puentes ya existentes para favorecer la comunicación entre poblaciones.

6. Una limpieza mensual de los puntos de agua urbanos proporciona la mayor eficiencia en coste-beneficio para favorecer la abundancia y diversidad de anfibios. Este sencillo

manejo conseguiría equilibrar la conservación de anfibios con el uso humano de los espacios públicos.



Conclusions

1. Traditional troughs are permanent water points where anurans and urodeles can reproduce. A moderate management, the presence of bottom sediments and abundant floating vegetation were identified as principal factors enhancing amphibian species richness. Changes in the traditional use of troughs due to the abandon of agricultural activities and husbandry may endanger these rural shelters for amphibians; conservation of their traditional management is recommended.

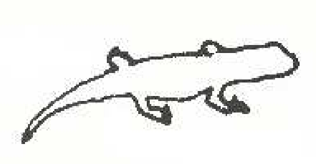
2. Urban areas contain suitable habitats for amphibian populations, amongst them small artificial ponds. Such ponds can harbour high mitochondrial genetic diversity, comparable to levels found from rural areas both in diversity and number of species. The incorporation of urban ponds in plans for amphibian conservation would be recommended.

3. A metagenomic approach, combining inter-and intra-specific diversity at the COI gene, exhibited greater small-scale sensitivity to diversity variation of amphibian communities than standard ecological diversity indices. Employing this approach, pond size and connectivity were identified as principal factors contributing to amphibian diversity in water points in the city of Oviedo.

4. Positive and significant association between road density and genetic distance between populations of *Alytes obstetricans* and *Lissotriton helveticus* has been detected in the Asturian region (North of Spain). Traffic calming in rural areas would mitigate the fragmentation of *L. helveticus* but not of *A. obstetricans* populations. Construction of passages for amphibians under the roads would significantly contribute to conservation of some amphibian species.

5. Rivers of the Cantabric region were identified as barriers to gene flow for the species *A. obstetricans*, *Discoglossus galganoi*, *L. boscai*, *L. helveticus* and *Triturus marmoratus* but not for *Bufo bufo*. Therefore, conservation measures should take into account the particular connectivity requirements of each species. Construction of amphibian passages along existing bridges would facilitate communication between populations.

6. Monthly cleaning of urban water points provided the highest cost-benefit efficiency for abundance and diversity of amphibians. This simple management action would contribute to conciliate amphibian conservation with human use of urban public spaces.



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