UNIVERSITAT DE BARCELONA

Conservation Planning of the Endangered Pyrenean frog by integratingnatural history, landscape and population genomics under Global Changes Scenarios

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"Conservation planning of the Endangered Pyrenean frog by integrating natural history, landscape and population genomics under Global Change Scenarios."

Memoria presentada por **Marcos Peso Fernández** para optar al título de Doctor por la Universidad de Barcelona.

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“**Life always finds its way**”

Jurassic Park

This doctoral thesis was financed by a doctoral grant ((202402/2011-8) from CNPq (*Conselho Nacional de Desenvolvimento Científico e Técnologico)* under the Science without Borders Program from the Ministry of Science - Brazil.

Essa Tese de doutorado foi financiada por uma bolsa (202402/2011-8) do CNPq sob o programa Ciências sem Fronteiras do Minitério da Ciência do Brasil.

Laboratory and fieldwork were financed by a research project from the National Parks OPAN-MMARM of Spain, and fieldwork was partially financed by a research project from the Barcelona Zoo (Ayuntamiento de Barcelona).

El trabajo de campo en Pirineos fue en parte financiado por un proyecto de Investigación del Zoo de Barcelona (Ayuntamiento de Barcelona), y por un proyecto de Investigación en Parques Nacionales OPAN – MMARM de España, este último también ha financiado el trabajo de laboratorio.

Front cover: Male of *Rana pirenaica* in Barranco Comas, Añisclo Valley – PNOMP-

Credits: **David Vieites**

Back cover Arazas river in Ordesa Valley – Ordesa and Monte Perdido national park.

Credits: **Marcos Peso**

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INSTITUTIONAL ACKNOWLEDGMENTS/AGRADECIMIENTOS INTITUCIONALES

I would like to thank:

Fernando Carmena and Ignácio Gómez from SARGA and Iosu Antón from Forestal Guardness of Navarra for their inestimable collaboration in fieldwork.

Antor Casterllanau for the logistical support that was fundamental for the realization of this thesis, likewise SARGA from the Aragon Government for providing their field station in the Ordesa National Park that served as headquarters in 2011.

Aragon and Navarra´s governments for the animal capture permits during those years.

The Spanish Meteorological Agency (AEMet) for providing meteorological data from the meteo-stations in the Pyrenees.

Isaac Pozo for help with GIS and statistical analyses.

The Barcelona Zoo (Ayuntamiento de Barcelona) and National Parks Autonomous Organism (OAPN) for financing the research project that has led to this thesis.

PERSONAL ACKNOWLEDGMENTS/AGRADECIMIENTOS PERSONALES

Fueron muchas las personas que de forma muy activa directa e indirectamente han apoyado, ayudado y hecho parte de esta tesis. Muchas de estas personas han estado presentes en diferentes etapas y momentos de la tesis y de mi vida durante estos años (fueron muchas las "vidas" vividas dentro de esta tesis, muchos momentos), algunas de estas personas han estado en más de uno de estos "momentos". Seré eternamente agradecido a ellos. También me gustaría agradecer a ti, por leer esta tesis. Escribir pero sin que alguien lo lea carece de sentido. Un filósofo brasileño (Mário Sérgio Cortella) dijo una vez que el afecto y el conocimiento son dos cosas que si lo guardas, lo pierdes. Por lo tanto estas líneas a seguir son para profesar y compartir todo el afecto y el cariño que mantengo por las personas que me han ayudado en este largo proceso cuyo resultado es un poquito más de conocimiento que también lo compartiré a seguir de este apartado de agradecimiento. Me permitiréis también en este emotivo apartado cometer algún error gramatical, ortográfico y por qué no, de memoria (aunque intentaré ser lo más atento que las lágrimas que brotan de mis ojos lo permitan).

En primer lugar, y como no podía ser diferente, tengo que agradecer al **Dr David Vieites**, que al principio era "solamente" el director de mi tesis, pero durante este largo y arduo camino, se convirtió en un amigo y un referente. No tengo la menor duda, ni la más mínima vergüenza en asegurar que sin David, esta tesis nunca llegaría a su término. Así que David, queda eternizado en estas líneas mi más sincero agradecimiento a ti como investigador y como persona. Estaré eternamente agradecido por todo lo que me enseñaste, soy sumamente importante de formar parte de tus f1 científicos.

También debo agradecer al **Dr. Xavi Ferrer**, mi tutor en la Universitat de Barcelona, gracias Xavi por el cariño con el que siempre me has acogido en tu despacho y por las palabras de aliento. Aprovecho para disculparme por los inúmeros trámites administrativos que has tenido que gestionar a lo largo de esta tesis. Tampoco debería olvidarme de las dos directoras que tuvieron este programa de doctorado y que me han facilitado la vida incluso a veces más allá del extremo de la flexibilidad que institucionalmente era posible, **Dra Dolors Vinyoles y Dra Maria José López Fuster**

I also want to say thanks to **Dra Kathryn Elmer** to support me in her lab in Galsgow, for helping me to make the genomics libraries and for having shared fieldwork in Asturias. At this point I will also never forget the invaluable lab work help of **Arne Jacobs**, thanks man for the field station tour, for the lab help, and the weekend lab conversations. And many thanks to the others Elmer´s lab members, **Hans, James, Robyn**; I am grateful to you that you have converted your coffee time into our coffee time those days.

También soy verdaderamente grato a los **gobiernos de Aragón y Navarra** por conceder los permisos necesarios para realizar los trabajos de campo imprescindibles para la realización de esta tesis. También me gustaría nombrar a **Elena Villagrasa**, Técnico del **Parque Nacional de Ordesa y Monte Perdido**, muchas gracias Elena por facilitar nuestro trabajo, con una notable organización para que pudiéramos coordinar con los agentes de protección de la naturaleza (APN) actuantes dentro del parque.

Los agradecimientos a los APN no podrían faltar, su labor como protectores de la naturaleza va más allá de su puesto, es una forma de entender la vida, dedicación de quien de veras ama la naturaleza. Sin la colaboración de ellos, mi trabajo en campo hubiera sido mucho más difícil. Así que gracias a **José Antonio, Javier Campo, Javi Fanlo, Emilio Ramón, Ricardo, Carlos Tarazona, Rafa Vidaller, Manolo Grasa** y a todos los demás que por desgracia no he podido conocer o mi memoria me traiciona. Con todos vosotros he compartido gratos momentos en el campo y os soy muy agradecido. **Rafa, Manolo, Carlos y Javier**, con vosotros he pasado algo más de tiempo, y quiero recalcar la gran admiración que os profeso personal y profesionalmente. Gracias por compartir estos momentos, espero haber correspondido de alguna forma con esta tesis para la conservación de nuestras cucharetas y ranitas.

Además de los APN, hay otros apasionados de la naturaleza que han hecho lo posible para que el trabajo en el campo fuera excepcional, son los señores que, para mí, personifican la empresa Sarga (antigua Sodemasa): **Nacho Gómez y Fernando Carmena**. No tengo palabras para agradeceros, recibirme en vuestras casas, tener el teléfono siempre disponible para cualquier duda, siempre dispuestos a cuadrar agendas para unificar las idas al campo. Todavía guardo con cariño las memorias de aquel lejano julio del 2010, cuando vosotros y David me enseñasteis por primera vez una rana pirenaica. Solamente puedo deciros gracias de todo corazón!

En total fueron más de doce meses de trabajo de campo en los pirineos aragoneses, navarros y franceses distribuidos a lo largo de cuatro años para adquirir los datos necesarios para esta tesis. En este tiempo fueron muchos los acompañantes que tuve en el campo, algunos que venían a visitarme, otros a ayudarme, a hacer sus propios proyectos de tesis, máster o fin de carrera, pero al fin y al cabo acompañantes que han hecho que el campo fuera mas ameno. Para los que no tuvisteis la suerte de hacer trabajo en campo, sepan que la soledad es la mejor y también la peor compañera de trabajo. Así que quiero agradecer a **Bea** (la primera en visitarme en pirineos, no podría ser diferente), **David Vieites, Javi, Carlitos, Rigo, Guille, Ana, Miguel Peñalver, Cristina, Davi TVS, Nina, Ori, Jorge, Rubén, Daniel, Marti**. Perdonarme si me olvido de alguien, pero fuisteis muy importante en aquellos momentos, no sé si sois conscientes de cuanto, pero quiero que lo sepáis.

Después del trabajo de campo, y entre medias, hubo también el trabajo de laboratorio. Mucha gente hizo con que aprendiera mucho y disfrutara del (muchas veces frustrante) trabajo en el labo, gracias a **David, a Sandra, a Nina y a Piluchi. También muchas gracias a Michel, Ivan, Juanes, Samu, David Osca, Melinda, Violeta, Paula Carolina, Chechu, Rugé, Silvia, Tania, Miriam, Jimena y Andrés**. La más sencilla duda que me habéis solucionado en el laboratorio ha sido muy valioso para mí, serían horas para encontrar la solución (o el pocillo, la caja, la pipeta, el termociclador, las puntas, etc…) por mí mismo, también vosotros (y Rock FM, cuyo playlist ya lo sabemos todos de memoria) han sido de gran ayuda para sobrellevar las horas de laboratorio.

Hablando de horas en el laboratorio, también soy enormemente agradecido a todos los funcionarios del Museo Nacional de Ciencias Naturales de Madrid, especialmente a los guardias de seguridad de la noche en aquel tiempo (**Pedro y Santi**). Muchas gracias por la compañía y las charlas en las noches (y madrugadas) que me quedé en el laboratorio para sacar adelante este proyecto. Y por abrir el portón de la

entrada una y otra vez sin poner ninguna oposición al hecho de que estuviera en las dependencias del museo fuera del horario de funcionamiento.

Después del laboratorio (y durante) vino el momento de analizar los datos, y ahí fuisteis muchísimos los que me ayudasteis. No puedo dejar de agradecer a **Isaac**, tío ni sé cómo agradecerte por tantas veces que te he molestado con preguntas de Gis, **Joaquin Calatayud, Angel, Cristina Romero y Chechu** sin vosotros R me hubiera devorado; **Ruben** gracias por clusterizar varios de mis análisis, **Chio**, has hecho que Excel fuera mas amable. Muchos otros me ayudasteis quitándome dudas y explicándome sobre el funcionamiento de ciertas herramientas. Gracias a todos!

También durante el doctorado estuve de estancia durante 3 meses en el Cibio de Porto (Vairão) en Portugal, me gustaría agradecer al **Dr. Nuno Ferrand** por la oportunidad. Gracias también a todos que de cierta forma enriquecieron esta aventura portuguesa, **Xavi, Angelica, Eva, Walter, Alice, Zbyzsek, Raquelina.** Pero sin duda lo mejor de la estancia fue haber compartido despacho con **Il Signore Marco Sannolo**. Grazie mille amico per invitarmi a fare surf. Casi morimos con aquellas olas, pero sirvió para repensar muchos aspectos de la vida que todavía hoy sigo buscando comprender. Tu amistad y tu apoyo durante la estancia y después de ella es lo mejor que me llevo de Portugal.

Tengo que agradecer a todas estos amigos y compañeros que compartimos la investigación durante estos largos años de tesis en el Museo Nacional de Ciencias Naturales de Madrid, técnicos, estudiantes de doctorado, postdocs y allegados. Quizás lo más fácil sería resumir agradeciendo a los PESTUZOS, que quizás sea el nombre que englobe todos los subgrupos (padelfuckers, 1111, 1212, lab, jaketeros, tupperos, pestugym, pajareros, herpetólogos, futboleros, basqueteros, gentes del ventorrillo). Siempre me acordaré de la primera cena de navidad en aquella chinosidrería. Y después de eso, las cañas y tintos de verano en el asador, retinto, el gominolas, 53, etc. Los cafés de los viernes, tras comida jaketera, en la residencia. Los pacharanes, las pizzas en la 1111, la casa rural, las pachangas. Muchos recuerdos de alegrías, pero también recuerdos, de los *jornal clubs*, seminario de los viernes, aquellas charlas en la máquina de café, las reivindicaciones por un lugar digno para comer de tupper. Los momentos de desespero por el rechazo de un paper, un deadline, por la compleja relación con los supervisores, una beca perdida, las dudas existenciales. Estos también son momentos compartidos con vosotros. Por todos estos momentos y por vuestra inestimable apoyo para conseguir finalizar esta tesis, Gracias a: **Juanin** (el experto cervecero); **Jaime; Jose Manuel**; **Carlos Ponce** (gran pajarero mor); **Paloma Plus; Carol** (La padelfucker master del universo y máxima liante. “La última y nos vamos”.); **Eva y Rogga** (algún día me gustaría volver a cantar con vosotros); **Elisa y Dani** (aquellas noches de cine eran una gota de agua en el desierto); **Cantarero** (Puro nervio, gracias pestu); **Miguel Peñalver, Merel y Cristina** (siempre fuisteis de los mejores oyentes de mis penas, estoy muy agradecido de haber contado con vuestra amistad); **Sergio Alfonso; Iván; Rafa chuchu** (muchas gracias por todos tus consejos); **Miguel Matias** (Nossas conversas sempre foram uma fonte inesgotável de motivação), **Diego** (Gallú, eres un fenómeno); **Michael y Melinda** (No tengo palabras para agradecer vuestra amistad y la cantidad de veces que me habéis ayudado, espero encontraros en Ecuador, Guiana, o donde sea); **Marti; Gemmita** (mi gemmita siempre fuiste la mejor consejera, aunque yo no quisiera hacerte caso, gracias también por venirte a las Cíes); **Chechu** (el mejor amigo indio que cualquiera puede tener, el integrante frustrado del pestupiso, muchísimas gracias por todo durante la vida en el museo

y todavía más por los ánimos en estos últimos meses de la tesis en Vigo, espero que te estés sintiendo un poquito en casa en Galicia); **Octavio** (te lo dije ya en persona, fuiste de los únicos que creíste en mi cuando la mayoría, yo incluido, lo dudaba. Eso fue y es muy importante para mi, espero poder ir a visitarte a Australia); **Laura**; **Esther**; **Roberto**; **Jimena**; **Lucia, Mireia**; **Ibañez** (pintan bastos); **Idiaquez, Jorge y Gonzalo** (Hicisteis del vetorrillo un lugar a recordar); **Isaac** (cuidado con los cuchillos franceses); **Rigo** (Hemos compartido muchas cosas, ideas, papers, proyectos, piso, gracias por tu amistad). Con cada uno de vosotros he aprendido algo que lo llevaré conmigo para toda la vida y vuestro apoyo, del más pequeño detalle al más grande, ha sido de ayuda para esta tesis. Y perdonarme si me olvido de alguien, todos sabéis que escribir la tesis consume demasiadas neuronas.

A mis queridos compañeros de despacho 1212 (doce doce) que han hecho con que el ambiente de trabajo fuera el más sano y agradable. Algunos ya estaban cuando llegué y fueron terminando sus tesis, algunos llegaron después y otros simplemente estuvieron una temporada, gracias a **Melinda** (He cuidado las plantitas lo mejor que pude cuando te ibas de estancias, muestreos, cursos…), **Silvia; Ramón, Maria y Raul** (nunca olvidaré aquel lab retreat en el ventorrillo) , **Shirin** (gracias por dejarme en herencia la mejor mesa del despacho), **Paco, Guida, Marga, Jorge, Chio, Raquel, Javi, Adelmo, Ángel, Nagore, Marisa, Valentina, Geize, Fernanda** (cunha)**, Indra, Joaquin** y todos los demás que pasasteis por el mejor despacho de becarios del museo, lo siento 1111, un despacho que tiene una estantería con alcohol y una cafetera tiene que ser considerado el mejor.

Sin duda no puedo dejar de agradecer de forma especial a la familia **VieitesLab**, he compartido mucho con vosotros, no ha sido solo una tesis, han sido momentos de desesperación, de incertidumbres, de éxtasis, con muchos he compartido despacho, mesa, ordenador. Hemos compartido campo, largos y cortos recorridos en coche, en metro, viajes y papers. Compartimos ideas, discusiones, congresos, cursos, críticas y abrazos. Fundamentalmente compartimos director de tesis, y también programas informáticos, informaciones de la universidad y sus gestiones administrativas. Algunos hemos compartido piso, comida, silla e incluso lágrimas. Hasta hemos llegado a compartir pensamientos, miedos, frustraciones, alegrías y fiestas. A todos en general mis gracias por todos estos años y estas experiencias (**Raquel, Angel, Carlos, Javi, Guille, Ana, Salva, Nina, Rubén, Valentina, Thijs, David y Sandra**), pero en especial a **Raquelina**, sin ti todo hubiera sido muchísimo más difícil, la tesis, el cibio, el doctorado en general y principalmente esta recta final. Eres una gran persona, gran amiga y gran investigadora, estoy muy orgulloso de haber hecho la tesis en el mismo laboratorio que tu y mas orgulloso todavía de poder decir que te tengo como amiga. A **Angelín** por ayudarme con R, con teamviewer, a esquiar y por tu tesón en la tesis aun sin tener una beca, me ha servido siempre de gran inspiración. A mi **Carlitos**, que nada más conocerlo el primer día en pirineos hemos hablado de todos los temas que normalmente se evitan hablar con un desconocido, gracias tío, siempre estuviste ahí principalmente en los momentos amargos, siempre tendrás a alguien que vibra por tus éxitos. A **Valentina**, ni me puedo creer que solo has estado 4 meses en Madrid, me ha hecho aprender mucho de genética y me has hecho repensar muchas cosas de la vida, Grazie mille, sempre ti ricorderò. Gracias **Javi**, por todos estos años de tesis, empezamos la tesis a la vez y también a la vez la terminaremos, siempre que te busqué pidiendo ayuda nunca dijiste que no, a pesar de todas nuestras diferencias. Esto para mi ha significado mucho. **Salva**, eres un gran ejemplo de perseverancia y fortaleza,

gracias por todos estos momentos de templanza. Y finalmente gracias a **Nina**, que empezamos compartiendo conocimiento, me has enseñado muchísimo en el labo, y yo te enseñé de donde venían las muestras de tejido que analizabas, y acabamos compartiendo mucho más, jamás lo olvidaré, gracias.

Não posso me esquecer de agradecer a meus amigos do Brasil que mesmo longe, sempre foram uma grande fonte de inspiração e exemplos a seguir para mim. Desculpa por distsanciar-me em muitos momentos, sempre pude sentir o apoio de vocês através daquela brincadeira, daquele áudio ou de um simples gesto. Sinto-me afortunado por ter vocês na minha vida e poder-me espelhar nas suas atitudes. **Ganega Lili, Cabelo, Linam, Omar, Davi, João, Felipe, Pedrinho, Mcfly, Ernestão, Tiago, Aline, Fefeu, Jamile, João Cláudio, Thiago, Diego, Niquinha, Erik. Vocês são foda**, sinto muita saudade de vocês, mas fico muito feliz em sentir como a nossa amizade sofre do mesmo efeito que o vinho, só melhora com o tempo. Sem vocês essa tese seria muito mais complicada. Especialmente **Avid**, irmão você foi de grande apoio antes (para conseguir a bolsa) e durante esse processo, obrigado de coração.

Y mis amigos de vigo, también siempre fuisteis un porto seguro, e incondicional fuente de apoyo. Gracias a **Popi, Markitos, Samu, Noe, Rubas, kely, Iria, Mejo y Javi. Y en especial a Dani**, hermano tu ayuda expresada de diversas formas ha sido fundamental para conseguir concluir esta tesis, no sé si una sola vida será suficiente para agradecer y devolverte toda esa ayuda. Sería injusto si no agradeciera también a **Bea**, viviste y me apoyaste en los pasos previos y en los iniciales de esta tesis. Soy muy agradecido a ti, esta tesis también es un poco tuya.

En este párrafo mezclaré el castellano y *o Português*. Porque así me siento, una mezcla entre a *minha familia baiana e* mi familia gallega. **Esta tesis JAMÁS sería posible sin el apoyo incondicional de mi familia**, no recuerdo ningún momento de cobranzas, *de dúvidas*. *Eles sempre acreditaram, muito mais do que eu mesmo acreditei em mim.* ***Minha mãe (Mari)*** **, mi padre (Roberto)**. *Obrigado mãe*, Gracias pai. *Vocês são minha principal* inspiración.Gracias también a mis tíos (**Elisa, Mari, Jesús**) que han colaborado con su apoyo muchas veces invisible para algunos (pero yo si lo veía), soy grato también a mi prima **Paty** y mi primo (más que político) **Alejandro** por todo el apoyo que me dieron. *Obrigado a minha irmã, que no final da tese me ajudou de uma forma que nunca pensei que iria precisar, e menos que acudiria a ela por essa ajuda, Obrigado* **Marilia**. A **mi abuela**, que pese a su edad y su desconocimiento sobre qué es y para qué sirve una tesis, también me ha dado toda la ayuda que ha sabido dar. *Não deixo para o final* **meus tios e padrinhos** (**Pilar e Luis**) *Jamais terei suficientes palavras para agradecer tudo o que vocês têm feito por mim*, *sou e serei eternamente grato a vocês*.

Esta tesis es para todos vosotros y un poco vuestra. Obrigado, gracias, Thanks.

GENERAL INTRODUCTION

The Quaternary climatic oscillations, with several consecutive periods of cold glaciations and warm interglacials, had a profound impact on the distribution, spatial genetic diversity, demography and evolution of extant species (e.g. Hewitt 2004). All European mountain ranges experienced dramatic changes, both in climatology and landscapes during this period, as glaciers invaded the valleys during cold periods, and retreated during interglacials allowing species to recolonize high elevations in a cyclical process. The Alps and the Pyrenees were basically covered by ice sheets that melted during interglacials in parallel to global increase of temperatures (Crowley & North 1991, Ehlers & Gibbard 2004).

The north of the Iberian Peninsula was particularly affected by those Pleistocene climatic changes (Crowley & North 1991, Arribas 2004). The level of perpetual snow in the central part of the Cantabrian mountain range remained around 750 m during the last glaciation, while in the Pyrenees many glacial cirques remained frozen even during interglacial periods, with the level of perpetual snow around 1400-2000 m a.s.l. during the last glacial maximum, well below the current one that is around 3000 m (Uzquiano 1995, Arribas 2004, Ehlers & Gibbard 2004). In parallel to this continuous advance and retreat of the ice, the fauna and flora of these mountain systems have had opportunities to colonize high mountain areas when conditions were favorable, retreating to lower areas during the colder periods where they persisted in refugia, such as deep valleys or coastal areas with more favorable climatic conditions. In consequence, the distribution and genetic structure of many species that inhabit these areas were shaped by retreat and posterior re-colonization patterns from the different refugia (Alexandrino *et al.* 2000, Guillaume *et al.* 2000, Gómez & Lunt 2007).

The most recent data on the extent of the glaciers during the last glacial maximum (Ehlers & Gibbard 2004), suggest that in the Pyrenees the glaciated areas occupied a great extension, including the majority of valleys up to low elevations (Fig. 1). Two spatial hypotheses are available from these works, one suggesting a continuous ice sheet covering the whole mountain range in the West and more fragmented towards the East (Fig. 1a), and the other showing a larger extent of glacier tongues but many small unglaciated areas within the Western ice sheets that may have functioned as nunataks, microrefugia for relict populations of mountain species (Fig. 1b). In fact, there is recent

evidence of the existence of unique genetic lineages in some of these putative nunataks as in the European common frog, *Rana temporaria* (Vences *et al.* 2013). Both scenarios suggest that the majority of species retreated to low elevations and that these could had a high degree of interconnection through the central depression that separates the Pyrenees from the pre-Pyrenees. This connectivity would have facilitated gene flow and genetic mixing between populations from different Spanish valleys, but the ice sheet setting would have implied a significant disconnection between the French and the Spanish slopes. Genetic homogeneity for mitochondrial markers and allozymes in several species is in agreement with this model (Carranza & Arribas 2008, Carranza & Amat 2005, Montori *et al.* 2008, Veith *et al.* 2002, 2012). Moreover, there is recent fossil evidence of low elevation populations of mountain species during the Pleistocene in the pre-Pyrenees, including *Rana temporaria*, the Pyrenean newt, *Calotriton asper*, as well other cold-adapted mammal and reptile species (López-García *et al.* 2010). During cold periods at high elevations, the non-glaciated areas within ice sheets could had functioned as microrefugia for isolated populations, allowing the persistence of endemic species of restricted distribution and maintaining the phenotypic diversity observed in many species, which still needs to be further explored (e.g. Bettin *et al.* 2007, Escobar-García *et al.* 2012, Schönswetter *et al.* 2005, Stehlik *et al.* 2002, Wachter *et al.* 2012, Westergaard *et al.* 2011, Milá *et al.* 2010, Vences *et al.* 2013).

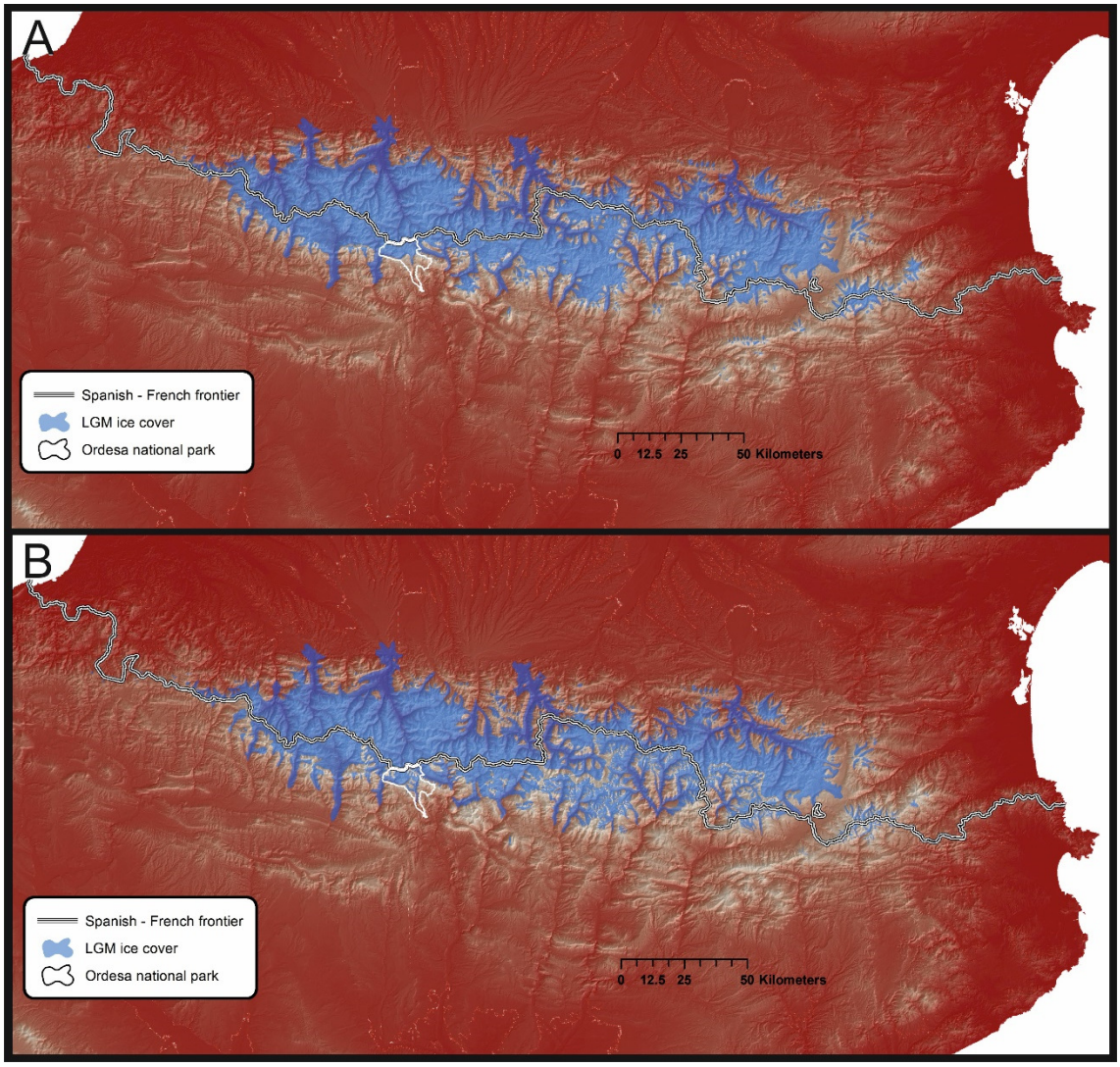

Figure 1. Maps showing different hypotheses on the extent of ice sheets during the last glacial maximum from Ehlers & Gibbard (2004). Map A suggests a rather uniform ice sheet in the western and central Pyrenees where *R. pyrenaica* occurs, and more fragmented in the East, while scenario B suggest that the ice tongues were much longer in the West, reaching lower elevations, and the existence of many unglaciated small areas within the ice sheets.the computation of Species Distribution Models (SDMs). Species occurrence data and environmental data linked with an algorithm predict models such as geographical space models or environmental space models.

Since the last glacial maximum there has been an overall increase in the temperature of the planet, which has accelerated since the 1970's, as well as an important transformation of the environment linked to human-related activities (Scheffers *et al.* 2016). This Global Change has allowed many species to (re)colonize high elevations in the Pyrenees in the last millennia where they have established stable populations. These altitudinal distribution shifts, causing range contractions and expansions, have led to considerable demographic changes in many species, sometimes population extinctions,

generating spatial genetic structure, and providing opportunities for microevolutionary adaptations in this mountain range (Dubois 1982, Carranza & Amat 2005, Charrier *et al.* 2014, Montori *et al.* 2008, Veith *et al.* 2002, 2012, Valbuena-Ureña *et al.* 2018).

Although many temperate species are also found both at low (Mediterranean/temperate climates) and high elevations (subalpine and alpine climates), their natural history and phenology are quite different between these two environments. Among them, several species of amphibians have been widely studied, like the European common frog (*Rana temporaria*) (Veith *et al.* 2002, 2012, Vieites *et al.* 2004, Vences *et al.* 2013), while for others we know less about their biology, distribution and natural history, like the Endangered Pyrenean frog (*Rana pyrenaica*) (Vieites & Vences 2003). These are sister taxa that occur in the Pyrenees, but one of the key differences between them is that while *R. temporaria* is a lentic species, *R. pyrenaica* inhabits in mountain streams. Both are ectotherms with limited dispersal capacities, distributed between mid to high elevations in the Pyrenees, as a result of a post-glacial colonization during the Holocene Global Warming. However, the Pyrenean frog does not reach high elevations like *R. temporaria* (2100 vs 2600 m a.s.l.) (Vences *et al.* 2003). *R. temporaria* shows morphological variability in the Pyrenees, which has led several authors to propose different subspecies and morphotypes creating some taxonomic uncertainty, while the Pyrenean frog was confused for a long time with *Rana iberica*, another Iberian endemism (Dubois 1982, 1983, Veith *et al.* 2002, 2003, 2012). Although both species of brown frogs share part of their distribution ranges in the central Pyrenees, there is a strong contrast between them in terms of their natural history, ecology, morphological and likely genetic diversity, which suggests that past environmental changes may have affected them differently. Their distribution, natural history and particular evolution makes them ideal model organisms to compare the imprint of the glaciations in their distribution, genetic and phenotypic structure, as well as the connectivity and gene flow between Pyrenean populations, both between valleys and across altitudinal gradients.

The Pyrenean frog is a medium-sized brown frog endemic to the Pyrenees, which was discovered and described not long ago (Serra-Cobo 1993), resolving the controversy of the presence of the Iberian frog in the Pyrenees. *R. pyrenaica* is an eminently lotic species, its typical habitat being mountain streams and torrents, with cold and oxygenated fast waters (Serra-Cobo 1993, 1997). It has also been occasionally found in fountains, springs and pools where there is continuous water renewal (Serra-Cobo 1997, 2002,

Serra-Cobo *et al.* 1998, 2000). The reproductive period in this species begins after the thaw, between February and April. The females deposit the clutches under stones, vegetation, or in areas of the riverbed where the stream current is less rapid (Serra-Cobo 1993, Serra-Cobo *et al.* 1998). The larvae are found in fast-flowing streams, usually in small pools, and metamorph during the summer. The juveniles after the metamorphosis are dispersed around the torrents (Serra-Cobo *et al.* 1998), as well as adults which have been found by us ca. 100 m far from the water. These ecological requirements contrast significantly with those of *R. temporaria*, which reproduces mainly in ponds and lakes avoiding fast water torrents.

Rana pyrenaica is distributed on the southern slopes of the central and Western Pyrenees, from the western slopes of the Mendizar mountain in Navarra to the Ordesa and Monte Perdido National Park in Aragón. There are several locations in France, in the forest of Irati, but near the border with Spain (Serra-Cobo 1993, 1997, 2002, Llamas *et al.* 1994, 1998, Ortega-Martínez & Ferrer-Justes 2000, Duguet & Melki 2003), and it has been recently found in two French sites further north (Duchateau *et al.* 2012). It is generally distributed from 1000 to 1800 meters of altitude (Serra-Cobo 1993, Vences *et al.* 1997, Serra-Cobo *et al.* 1998, 2000), although the altitudinal range spans from 440 m to 2100 m (Vieites & Vences 2003, Duchateau *et al.* 2012). By analyzing a few individuals with mitochondrial DNA, a single mutation in cytochrome b has been reported, suggesting a very low level of genetic variability in the species (Carranza & Arribas 2008). However, there are no genetic data for a sufficient number of individuals and populations, covering the species' distribution area, neither nuclear data to confirm this low genetic variability and if there is any phylogeographic structure in the species. Such data would also allow testing several hypotheses on potential barriers to gene flow, like major rivers or steep slopes.

In the late 1990s and the beginning of the 2000s, Serra-Cobo and collaborators carried out several studies for the government of Aragon in which they gathered the first distribution data for the species. Those studies were not published and are available through the Aragon Government. The same happened in Navarra. Since then, there has not been a similar study to determine the real distribution of the species, if there have been changes, or the size of the populations. Punctual observations by herpetologists suggest that the species is no longer found in some of the localities where it was present 20 years ago, since no adults or larvae have recently been found, which are easily

detected in spring-summer. The appearance and expansion of chytridiomycosis, a fungal disease that is decimating amphibian species throughout the world (Olson *et al.* 2013), may be the cause of the possible population decline of this species, but data are lacking on population sizes or prevalence of chytrid infection. The available data from the 1990s suggest a higher population density towards the central zone of distribution of the species, whereas it was low in populations located in the western limit of its distribution (Navarra), as well as in low altitude areas (Llamas *et al.* 1994, Serra-Cobo 2002). The IUCN Red List (Bosch *et al.* 2009) suggests that the species is Endangered based on a continuous decline, both in its area of occupation, the extent and quality of its habitat and in the number of localities, but it is unclear where the data to support these claims came from. In Spain is catalogued as Vulnerable (VU B1ab+2ab), in Aragón as sensitive to habitat alteration, in Navarra as a threatened species and in France as Endangered B2ab(iii,v). However, there are no published data about potential significant changes in the range of the species, as well as in its population sizes in the last decade. The factors limiting its geographical distribution are still unclear and the potential impact of changes in its typical habitats, connectivity between populations, actual population sizes or phylogeographic structure and genetic diversity have not been assessed. Considering the lack of such critical information about the species, as well as scarce observations suggesting that some of its populations may be suffering a significant decline, this project has focused on collecting data that allow us to assess the current situation of this Endangered species, and develop the bases for a conservation strategy that will help in decision-making conservation actions. The National Park of Ordesa and Monte Perdido plays a fundamental role in the conservation of the species because it is the only one that houses populations.

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STRUCTURE OF THE THESIS AND OBJECTIVES

In this thesis we have focused on clarifying the current situation, delimiting the distribution and estimating the size of the populations, evaluating the genetic variation and phylogeographic pattern, and potential threats such as chytridiomycosis in the Pyrenean frog, the only amphibian species in danger of extinction in the Pyrenees. These data suppose an important advance in the knowledge about the species and are discussed in the context of a *R. pyrenaica* conservation strategy and the central role that the Ordesa and Monte Perdido National Park should play in it.

These objectives are developed in the different chapters of the thesis

Chapter 1: we aimed to clarify and update the distribution range of *Rana pyrenaica*, by revisiting all known localities, and based on potential species distribution models determine its potential distribution and guide fieldwork into new areas where the species could potentially occur. As there is a lack of data on the population sizes, we also wanted to have an assessment of the population size for each population, based on counting clutches and larval groups that will allow us to estimate a minimum number of reproducing females.

Chapter 2: we wanted to verify the presence of the chytrid fungus, *Batrachochytrium dendrobatidis*, a fungus that is killing amphibian species and populations all over the world, in skin swabs of *R. pyrenaica* across its range. By doing this we wanted to determine if the chytrid is located in few areas or it is widespread, as well as where it does occur across the elevation gradient. By forecasting growth models of the chytrid into future climatic scenarios we wanted to assess if this fungus will move upslope an affect high elevation amphibian populations.

Chapter 3: we here describe the complete mitochondrial genome of *R. pyrenaica*, which is the base to develop specific mitochondrial primers and detect intraspecific genetic variation in the mitochondrion.

Chapter 4: we aim to reconstruct the mitochondrial phylogeography of *R. pyrenaica* based on nearly complete mitochondrial genomes. Reference genomes were amplified to determine which loci show genetic variation to further amplify those in a set

of specimens across the species range. With those data we wanted to assess the spatial genetic structure, intraspecific genetic variation and reconstruct the historical demography to test past population expansions since the last glacial maximum. We integrated genetic data with paleo-distribution modeling to test whether several refugia arose during glacial periods.

Chapter 5: in this chapter we expanded the genetic dataset to the nuclear genome by amplifying a set of single nucleotide polymorphisms (SNPs) for a representation of individuals across the distribution range of the species. With those we wanted to reconstruct the fine scale spatial genetic structure, determine the existence of genetic clusters, develop landscape genetic models and integrate those to detect potential barriers to gene flow and connectivity. With those analyses we assessed the conservation situation from a population genetic view.

General discussion: in this final chapter we integrate all the data generated to discuss the actual conservation situation of the species and propose conservation actions that can be implemented by managers and authorities.

Chapter 1:

Current distribution, population estimates and conservation status of the Endangered Pyrenean frog (*Rana pyrenaica*)

This chapter reproduce entirely the manuscript:

Peso M., Nieto-Román S., & Vieites D. R. Current distribution, population estimates and conservation status of the Endangered Pyrenean frog (*Rana pyrenaica*). Manuscript in preparation.

Current distribution, population estimates and conservation status of the Endangered Pyrenean frog (*Rana pyrenaica*)

Abstract

The Pyrenean frog, *Rana pyrenaica*, is a narrowly-distributed endemic amphibian, occurring mainly in the western and central southern Pyrenean slopes of Navarra and Aragón, with few localities in France. It has been catalogued as Endangered by the IUCN. Its distribution range and the variables that determined it are not yet clearly known despite previous efforts on this respect, as well as other key aspects of relevance for its conservation, including the actual status of its populations, degree of fragmentation or populations sizes. Here we present new data on the distribution of the species, the size of its populations and the survival of larval stages in different types of environments. The species has been found in 170 localities, increasing its range into five new 10x10 UTM squares in respect to the previous available information. Its range is fragmented, with most populations having low or very low adult population sizes, and in several historical localities the species has not been found or is nearly extinct. Fine-scale species distribution modeling suggests a distribution mainly determined by mean annual temperature and distance to streams. Monitoring of larval survival in five localities during two years suggests that droughts and storm floods can be important factors determining recruitment, mainly in localities where river pools are not deep enough. Our data support the IUCN conservation status of Endangered and suggest that urgent conservation actions to manage those small populations are needed to prevent their disappearance in the next decades.

Keywords: *Rana pyrenaica*, distribution, population estimates, distribution models, survival, conservation

INTRODUCTION

The Pyrenean frog, *Rana pyrenaica*, is a medium-sized brown frog endemic to the Pyrenees, which was discovered and described just twenty five years ago (Serra-Cobo 1993). It has been catalogued as Endangered by the IUCN (www.iucnredlist.org/), but there is a general lack of information about many aspects of its life history, real distribution, population sizes, genetic variation or real conservation threats. *R. pyrenaica* is an eminently lotic species, inhabiting mountain streams and torrents, with cold and oxygenated fast waters (Serra-Cobo 1993, 1997). It has also occasionally been found in fountains, springs and pools where there is continuous water renewal (Serra-Cobo 1997, 2002, Serra-Cobo *et al*. 1998, 2000). The reproductive period begins after snowmelt (March–April) and larval development lasts until July when metamorphs leave the streams (Vieites & Vences 2003). The females attach the clutches under stones, vegetation, or in areas of the riverbed where the stream current is less rapid, and clutches can be easily detected (Fig. 1) (Serra-Cobo 1993, Serra-Cobo *et al*. 1998, Vieites & Vences 2003). The larvae are usually found in small ponds within the brooks or streams. After the metamorphosis, the imagos stay nearby or disperse not far from the torrents (Serra-Cobo *et al*. 1998). The adults are found near the water but can disperse further away, being the maximum distance from water detected by us ca. 100m (pers. obs.), although outside the reproductive period are very hard to detect. The species' ecological requirements significantly contrast with those of the European common frog, *R. temporaria*, the conspecific sister taxon to *R. pyrenaica*, which reproduces mainly in pools and lakes avoiding brooks and torrents, and in high elevations they remain at lakes and nearby areas all year round (Vieites 2003).

Rana pyrenaica is distributed on the southern slopes of the central and western Pyrenees, from the western slopes of the Mendizar mountain in Navarra to the Ordesa and Monte Perdido National Park in Aragón. It is very rare on the northern slope of the Pyrenees. There are several localities in France, near the forest of Irati close to Spanish-French border (Serra-Cobo 1993, 1997, 2002, Llamas *et al*. 1994, 1998, Ortega-Martínez & Ferrer-Justes 2000, Duguet & Melki 2003), and it has recently been found in two isolated French sites further north (Duchateau *et al.* 2012). It is generally distributed from 1000 to 1800 meters a.s.l. (Serra-Cobo 1993, Vences *et al.* 1997, Serra-Cobo *et al.* 1998, 2000), although the altitudinal range spans from 440 m to 2100 m (Vieites & Vences 2003; Duchateau *et al.*, 2012). In the late 1990s and the beginning of the 2000s, Serra-

Cobo and collaborators carried out several studies for the government of Aragon in which they gathered the first distribution data for the species. Those studies were not published and are available through the Aragon Government. The same happened in Navarra. The historical distribution of Pyrenean frog is compiled in the Atlas of Amphibians and Reptiles of the Iberian Peninsula of the Ministry of the Environment (Pleguezuelos *et al.* 2002) as UTM mesh of 10x10 kilometers. Since these efforts, there has not been a similar initiative to determine the real distribution of the species, if there have been distribution changes, or perform any assessment about the status and populations' size. The lack of observations of adult and larvae in some historical localities in the recent years, has led to conclusions of population declines, but no real data are available to confirm these. The available data from the 1990s suggest a higher population density towards the central zone of distribution of the species, whereas it was low in populations located in the western limit of its distribution (Navarra), as well as in low altitude areas (Llamas *et al.* 1994, Serra-Cobo 2002). The IUCN Red List (Bosch *et al.* 2009) suggests that the species is Endangered based on a continuous decline, both in its area of occupation, the extent and quality of its habitat and in the number of localities, but it is unclear where the data to support these claims came from. So far, there are no published data about potential significant changes in the species' range, as well the actual population sizes or population trends.

Moreover, spatially explicit distribution models are not available for the species and the variables and historical processes that have shaped its distribution are unknown. Considering the lack of such critical information about the species, as well as the scarce observations suggesting that some of its populations may be suffering a significant decline, we here wanted to: 1) update and complete the distribution information for this Endangered species to clarify its real distribution range, 2) gather the first indirect estimates of population sizes, 3) model its distribution and determine the variables that better predict it, and finally 4) monitor the survivorship of larvae in different sites with contrasting characteristics to assess the potential recruitment capacity of the species, which directly relate to its survival. Under future Global Change scenarios, climatic instability is a factor of paramount importance (IPCC AR5 2014). In the Pyrenees, a mean decrease between 10.7% and 14.8% in precipitation, as well as an increase in mean temperatures between 2.8 and 4ºC have been proposed (López-Moreno *et al.* 2008). Moreover, the effects of those changes are expected to be more pronounced in the

southern slopes of the Pyrenees (López-Moreno *et al.* 2008), where *R. pyrenaica* occurs. Although a negative historical trend in river flood intensity and duration has been detected for the Pyrenees, mainly linked to the increase in forest cover at mid-low elevations (López-Moreno *et al.* 2006), stochastic events like big storms or heat waves are expected to be more frequent and intense (Meehl & Tebaldi 2004), that can cause higher larval mortalities by desiccation before metamorphosis or tadpole dragging to lower elevations due to mountain storms. By integrating those data we discuss the current conservation situation of the species as well as its conservation status and potential management actions.

MATERIALS AND METHODS

Fieldwork

Between the spring of 2010 and the autumn of 2014 intensive fieldwork was carried out in the Pyrenees covering a territory between Roncesvalles in Navarra to the Aran Valley in Catalonia, and in the French side from Irati forests to the eastern limit of the Parc National des Pyrenées. This territory covered the whole known distribution range of the species as well as other potential areas where it could occur. We sampled 757 localities, including all the historical localities where the species was reported from several unpublished reports of the Government of Aragon from 1997 to 2004, reports from the Government of Navarra and the scientific literature. To determine the new zones to be sampled, potential species distribution models (SDM) were done in Maxent (see details below) based on the historical distribution data. This was an iterative exercise repeated every year to improve the model with new presences and absences, which guided us to potential new areas for the species not reported before. In order to confirm the presence of the species, we visited all historical localities in 2010, and if the species was not found in 1 or 2 visits, we revisited the place again in 2011, 2012 and 2013 to confirm the actual absence of the species in the area. If in none of these visits the species was not found then we considered the population to be extinct.

The period of activity of the Pyrenean frog begins in March-April and ends in autumn. The best period to detect the species is in the early spring, when the adults are breeding, until the month of June when the tadpoles are still in the water. Therefore, we planned the visits within this period, with a first visit always during or just after the

breeding period when clutches are laid, in order to detect not only the presence of the species but its reproduction and presence of larvae. Sampling consisted on visiting localities on foot or by 4x4 vehicle whenever possible. Many localities were on steep slopes and locations far from communication routes so it was necessary to make bivouacs in that area. Transects were made in rivers, streams, small torrents and springs, locating adults, clutches and larvae. In these transects were counted the number of clutches, total number of larvae and larvae groups, juveniles and adults, recording their precise situation by GPS. The sampling was intensive and included above and underwater photography to detect spots that are usually under rocks where clutches are laid (Fig. 1). Environmental data were collected from each location and photographs were taken of the surroundings. These data included the type of bottom substrate of the stream, the characteristics of the margins, type of dominant vegetation, geology, presence of algae, trichoptera and other species of amphibians, presence of fish, human and livestock presence.

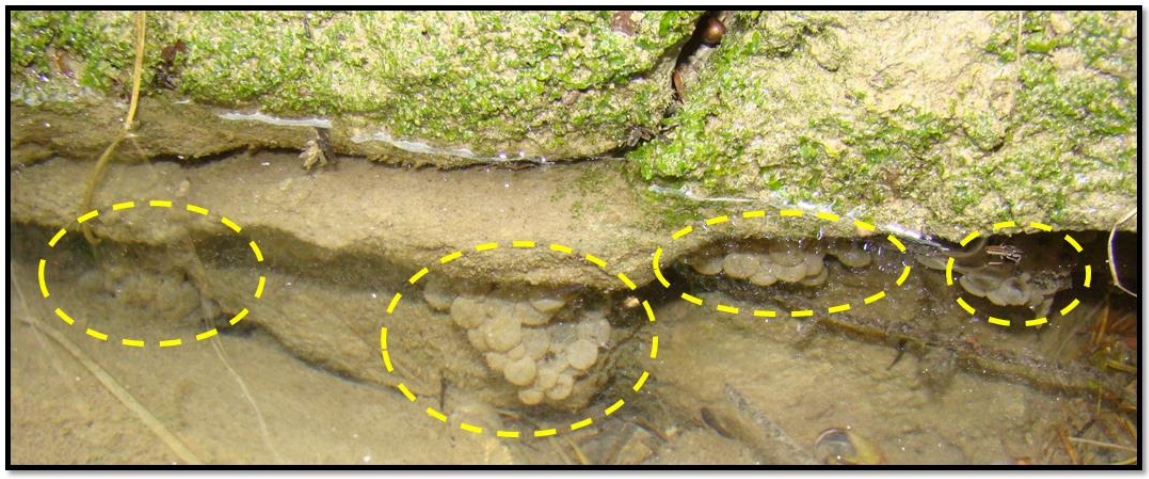

Figure 1. Photo of clutches of the Pyrenean frog, which are typically attached under rocks in stream pools where they can be easily located and counted.

Population estimates

In order to have an approximation to the population sizes by locality, relative density estimates of breeding adults were made based on observations and counting clutches, tadpole masses and adults. These estimates allow for the first time an approximate idea of the number of breeding individuals of the species per each locality. The application of mark and recapture methods, although more reliable, was not viable at the scale we aimed to work, since a tagging and recapture program is impossible for the

entire distribution area: Hence, an approximation has been used according to the number of clutches, counted larvae in each sampling and their distribution in the streams. During the reproductive period, adult frogs and clutches were located, they were counted one by one in each stream and georeferenced. Since it is likely that we do not detect them all in one visit, each locality was visited several times during early spring to locate clutches and masses of tadpoles. During development, when the first Gosner tadpole stages are reached (when they still do not swim), the small tadpoles fall into the bottom of the pond, but still form homogeneous masses that correspond to one or two clutches, depending if several females laid their clutches close to each other. In those cases we assigned each group to a clutch that corresponds to a reproductive female. Each female deposits a single clutch per year (pers. obs.), hence the number of clutches parallels the number of reproductive females. In some cases, directly estimations of the number of clutches were not possible because the larvae were scattered in the stream, or, after a flood, were concentrated at the lower sections. Therefore, in 2012 and 2013 we made the effort to revisit such localities at the beginning of the breeding season to locate the clutches and thus better estimate the number of breeding females. After these years, we have managed to have a fairly realistic estimate of the number of clutches per sections of streams sampled and therefore of breeding females. In order to have an estimate of the total number of adults, we applied a sex ratio of 1.5 males per female based on unpublished data from Serra-Cobo. By doing this we can have an approximate estimate of adult population sizes, obtaining an estimated value of adult breeding individuals per each studied locality. As we usually sampled between 100 and 200 m per stream, we then extrapolated those numbers to the total length of the streams towards headwaters, as frogs likely are also present higher up, to get a rough estimate of the potential population size in all known localities where the species occurs.

Species distribution modeling

In order to generate a spatial hypothesis for the current distribution of the species, as well as to determine the variables that could have shapes its current range, we performed several species distribution models (SDMs) at different scales and with different datasets. We first generated models using as predictive variables the bioclimatic layers from the WorldClim database (Hijmans *et al.* 2005) at 30 arcsec resolution (ca.

1km^2). Those variables are highly autocorrelated as they all are generated from the minimum, maximum and mean temperatures and precipitation. Hence, we assessed the autocorrelation of the bioclimatic layers by generating 8000 random points within the extent of the sampled area across the Pyreneesm, and performing Pearson pairwise correlations in SPSS v. 25 (IBM 2017). As most variables are autocorrelated, we removed from each pair one variable if the correlation was significant and with a Pearson coefficient value higher than 0.8. The final set of variables showing the least degree of correlation where annual mean temperature, mean temperature of wettest quarter, mean temperature of driest quarter, precipitation seasonality (coefficient of variation), precipitation of the wettest quarter, precipitation of the driest quarter, precipitation of warmest quarter. We also included slope [derived from the 90x90 m SRTM 90m elevation model (Jarvis *et al*. 2006)], distance to rivers and Corine landcover categories (EEA 2006). Those layers were available both for Spain and France, but they may be too coarse for a species like *R. pyrenaica*. Hence, we performed SDMs' using a different set of climatic layers from the "Atlas Climático Digital de la Península Ibérica" (Ninyerola *et al.* 2005). This Atlas consists on monthly and annual means of radiation, temperature and precipitation from 40 years of data for the Iberian Peninsula, based on the complete set of meteorological stations from Spain and a high number from Portugal. As it is a high resolution layer (200x200 m grid-cell size), it improves significantly other datasets like Woldclim that uses less stations and provides lower resolutions. We rescaled elevation (and the derived slope), Corine landcover and distance to rivers to the same resolution as those climatic layers. Although these Atlas climate layers do not cover France, the majority of the distribution of *R. pyrenaica* is in Spain, so the model can be helpful predicting most of the distribution of the species. We followed a similar approach than with WorldClim variables to detect autocorrelation, by performing pairwise Pearson correlations between each pair or variables. From these, we selected six variables that showed the least correlation values: distance to rivers, spring precipitation (May), annual radiation, mean annual temperature, Corine landcover and slope. All GIS processing and final maps were performed in Quantum GIS (QGIS 2018). For the modeling, we used Maxent v. 3.4.1 (Phillips & Dudik 2008). Final models were done using 170 localities where the species has beenfound and 581 real absences. We used the 75% of the data to build the model and the remaining 25% to test it. Performances of the SDMs were

evaluated using the AUC (Area Under the Curve) values obtained from the model (Elith *et al.* 2006).

Egg and larval survival models

A survival analysis from egg to metamorphosis was performed using the Kaplan-Meier method implemented in SPSS v. 25 (IBM 2017). This statistic uses population estimates or counts through time to estimate survival rates for every sampled event in time, using censored cases to calculate conditional probabilities at each time point at which an event occurs through the product limit of those probabilities. Monitoring censuses were used as input for those analyses. We visited five localities where *R. pyrenaica* is common: "Pista circular" at the Tena Valley (42.70104 N, 0.35772 W), which is a small running water stream, flat, surrounded by grass and no forest cover, shallow pools that dry out during hot and dry summers; "Iguarra" also at the Tena Valley (42.64116 N, 0.29921 W), a forest locality where the stream form deep permanent pools usually in the shadow of trees; "Buesa" at the Ara Valley (42.59807 N, 0.10291 W), a forest locality but with shallow pools that dry out in hot summers; "Barranco Comas" at the Añisclo Valley (42.66428 N, 0.12169 W), a small stream surrounded by grass and no forest cover, shallow pools that dry out during hot and dry summers and frequent presence of cows; and "Pools in Bellós river" (42.64052 N, 0.05964 E), a semi forested locality with shallow pools next to the Bellós river, where the persistence of the pools depends on river floods. We visited each locality between four and five times per season, counting the number of eggs or tadpoles in each visit in a delimited section of the stream. The year 2012 was very hot and many pools and streams dried out, while 2013 was a wetter year with major storms in early summer, allowing comparisons of mortality profiles between a hot and dry and a wet year. Hence, we monitored Pista circular, Iguarra and Buesa in 2012, and again in 2013 plus the Bellós and Comas localities.

RESULTS

Between 2010 and 2014 we visited 751 localities within the potential distribution range of the species, including all historical localities. *R. pyrenaica* was detected in 170

localities. Twenty-three new localities have been located in the valleys of Ansó, Tena, Vió; Acumuer, Bujaruelo and Hecho, as well as a new isolated population in France. This supposes an extension of the distribution area of the species in five UTM grids in Spain, compared to the available information from the Atlas of Amphibians and Reptiles of the Iberian Peninsula (Pleguezuelos *et al*. 2002) (Fig. 2).

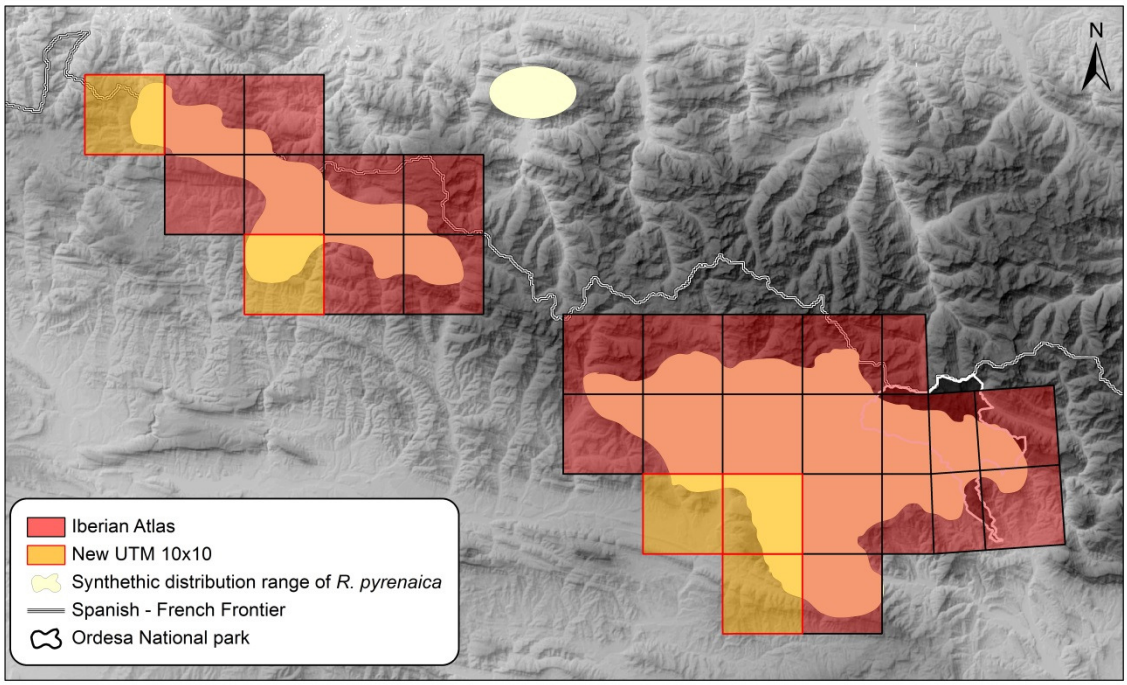

Figure 2. Synthetic distribution map of *Rana pyrenaica* showing the UTM 10x10 km grid cells from the Atlas of Amphibians and Reptiles of the Iberian Peninsula, and the five new cells were it was detected in Spain.

The new localities complete the known distribution area for the species, extending it to the west and south. In the case of grid cell 30TXN84, corresponding to Valle de Hecho, the new locality found is of special relevance, as it had never been found in that valley until now, despite the great effort of sampling in previous years. There is a discontinuity in its distribution between the eastern and western ranges, and this new locality shortens that distance between them. The gap between the eastern and western ranges is confirmed in 30TXN83 and 30TXN93 UTM 10x10 grid cells, where the species has not been found despite a very intensive sampling in the area. The origin of this Hecho valley locality is likely a result from a recent expansion from the west rather than a relict locality since the Pleistocene.

Figure 3 shows the sampling effort made during this study. The species has been searched in 751 mountain locations, many of them in stream headwaters with difficult

access. The distribution of localities clearly shows two cores spatially disconnected from each other. Between 2012 and 2014 we re-visited again most of the historical localities in order to confirm absences. It should be noted that in thirty historical localities the species has not been relocated, and the localities close to Villanúa are of special relevance. The species was described from specimens from this area, however in the whole valley we did not find any individual in 2010, one tadpole and one adult after an intense sampling in 2011 at a single place, and again in 2013. Reproduction was confirmed after finding a single tadpole in the month of June, which indicates that the species is very rare in the valley of the river Aragón.

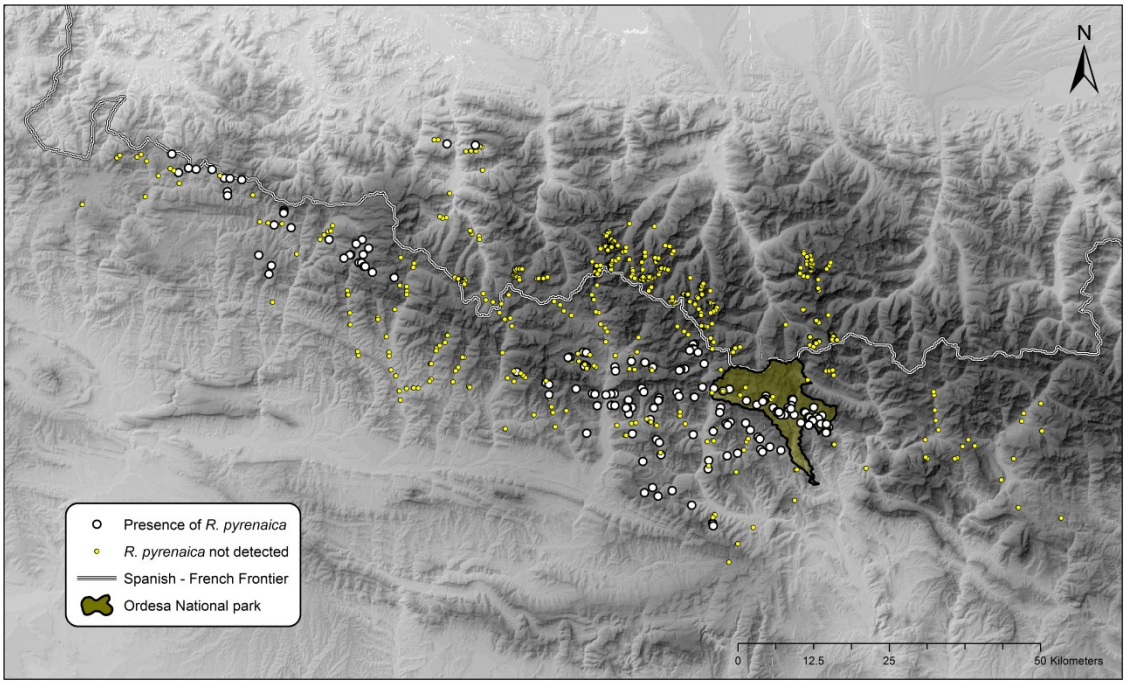

Figure 3. Sampled localities between 2010 and 2014, showing the ones where the species was detected (white) and in which it was absent (yellow).

In the Figure 4 is represented the SDM based on 1 km^2 layers. The training AUC was 0.983 and the testing AUC 0.973, suggesting that it is a good model. The variables that contributed most to the model were: annual mean temperature (43.9%), mean temperature of the wettest quarter (31.8%), mean temperature of the driest quarter (11.8%) and precipitation of the warmest quarter (7.1%), contributing all the rest of variables less than 5% to the model. The model suggests a continuous distribution from Navarra to Catalonia, including parts of the pre-Pyrenees, most of the French slopes of the Pyrenees and Catalonia where the species does not occur. Most of the known real

absence localities are predicted as presences, suggesting that the model may be overpredicting a lot within and outside the real range of the species.

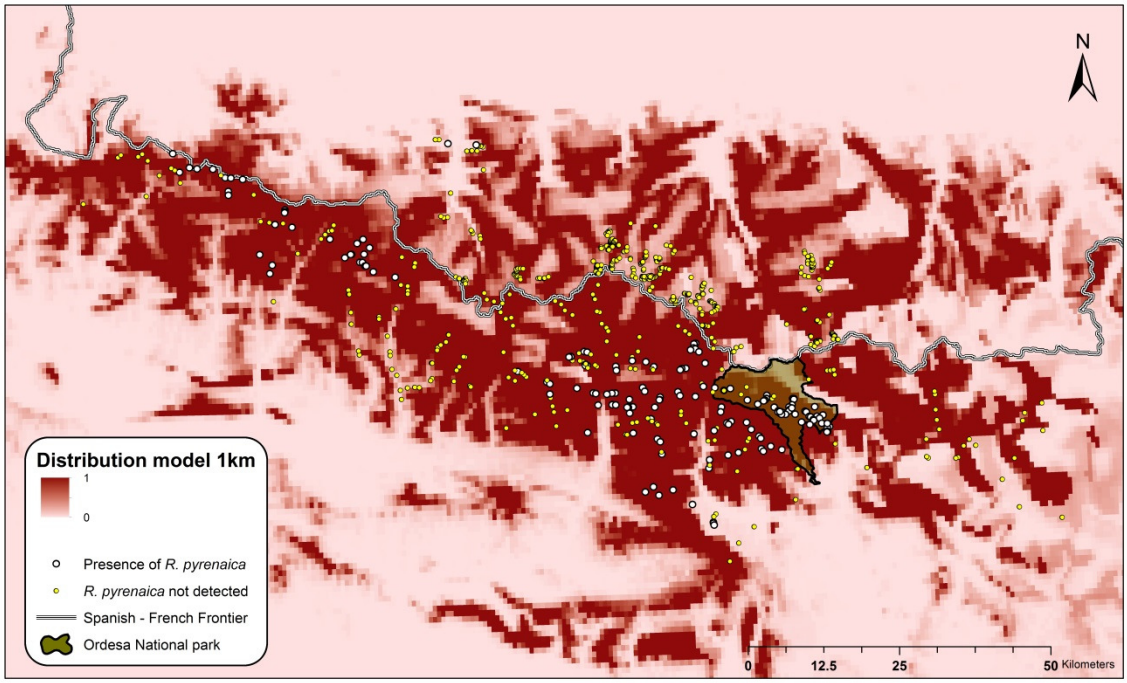

Figure 4. Species distribution map showing the probability of occurrence of *R. pyrenaica* with a spatial resolution of 1km^2 (from 0 to 1). Presences are represented by white dots and real absences by yellow dots.

The SDM based on a finer resolution, with grid cells of 200x200 m, shows a very different and more realistic pattern (Figs. 5 and 6). The training AUC was 0.975 and the testing on was 0.955, suggesting that is also a good model. The variables that contributed the most to the model were: annual mean temperature (38.3%), distance to rivers (33.8%) and spring (May) precipitation (23.6%), with the rest of variables contributing less than 5% to the model. The potential distribution area is now much smaller than the one suggested by the 1km^2 model, being restricted close to streams and mainly in mid-high elevations. There is overprediction in Catalonia, where the habitat is suitable for the species but the Cinca river acts as a barrier to dispersal from Aragón; and also in Navarra, where more streams are suitable west of its currently known distribution range. Nearly all the localities where the species occur are well predicted. Figure 6 shows the same map but reclassified to presence/absence using a cut off value when sensitivity and specificity cross. The real gap between the eastern and western core range areas is predicted as suitable for the species.

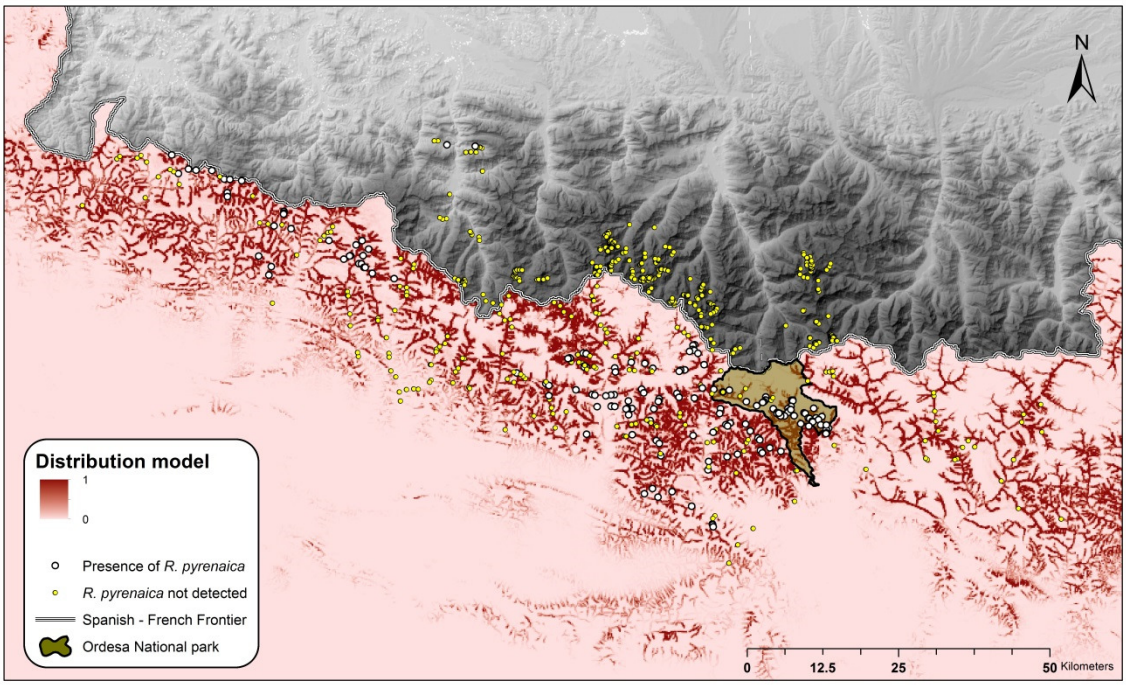

Figure 5. Species distribution map showing the probability of occurrence of *R. pyrenaica* with a spatial resolution of 200x200 m (from 0 to 1). Presences are shown by white dots and real absences by yellow dots.

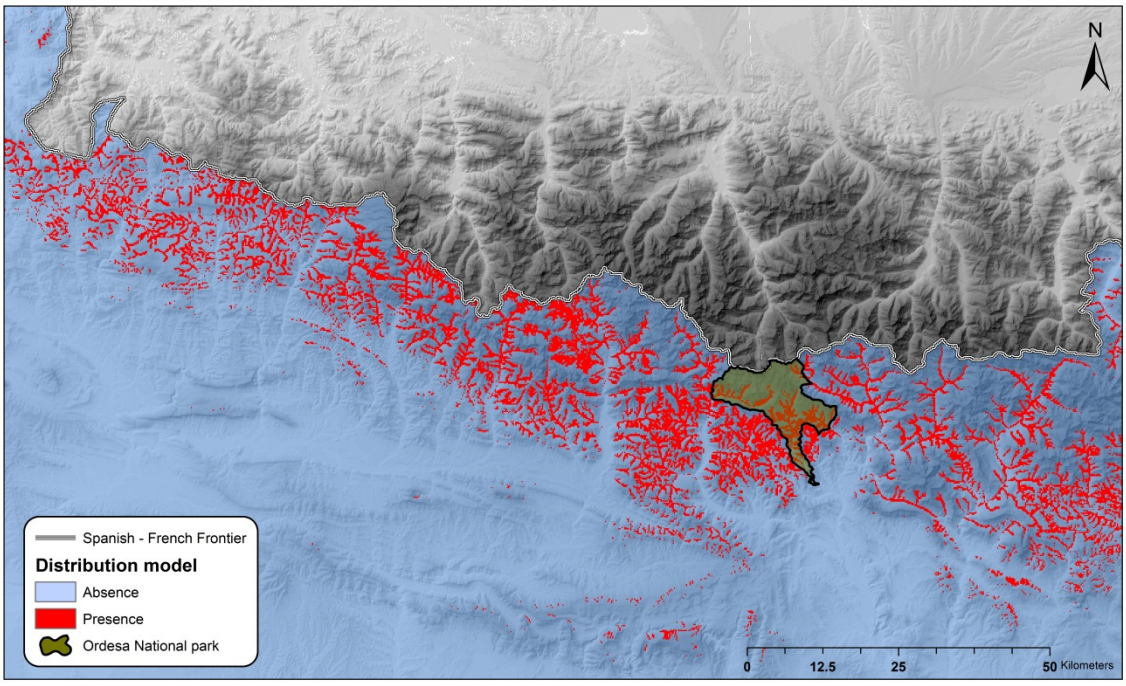

Figure 6. Same map as Figure 5 but reclassified as predicted presence and absence areas.

Our results indicate that the species is more abundant in Aragón, with a general pattern of small populations with a high degree of fragmentation. The relative density estimates of *Rana pyrenaica* suggest that in the localities where the species is present (ca. 31 km of streams out of 140 total sampled kilometers of streams)(see Appendix for more details) , the breeding adult population ranges between 1166 and 1377 individuals (Fig.

7). If we look at the distribution of the relative density per population (Fig. 7), most localities show low to very low densities (less than 30 or 15 individuals respectively). However, there are 13 localities with a higher density of individuals (>50 adult individuals), two of them within the National Park of Ordesa and Monte Perdido. If we extrapolate those values to the potential suitable distribution upstream in the same rivers (ca. 211 km), the estimated population size of *R. pyrenaica* would range between 13333 and 16438 adult frogs (Fig. 8).

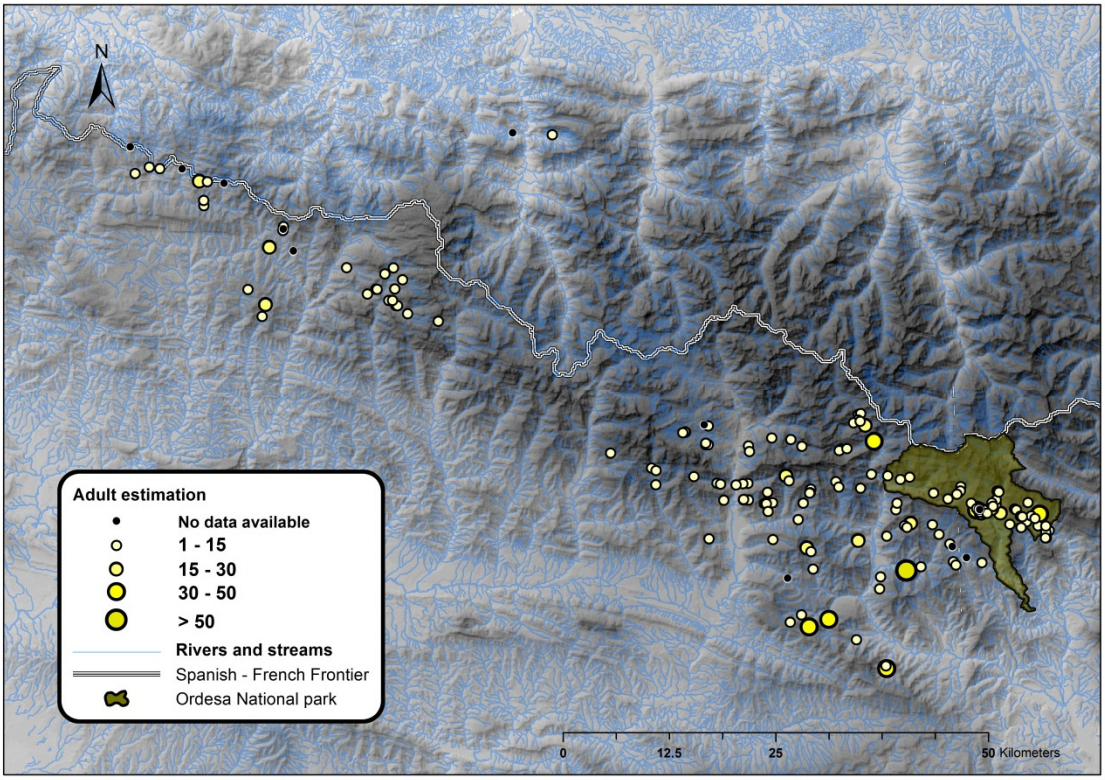

Figure 7. Relative density estimates of breeding adults per population in *R. pyrenaica* in the sampled localities. Circle sizes are proportional to the number of individuals.

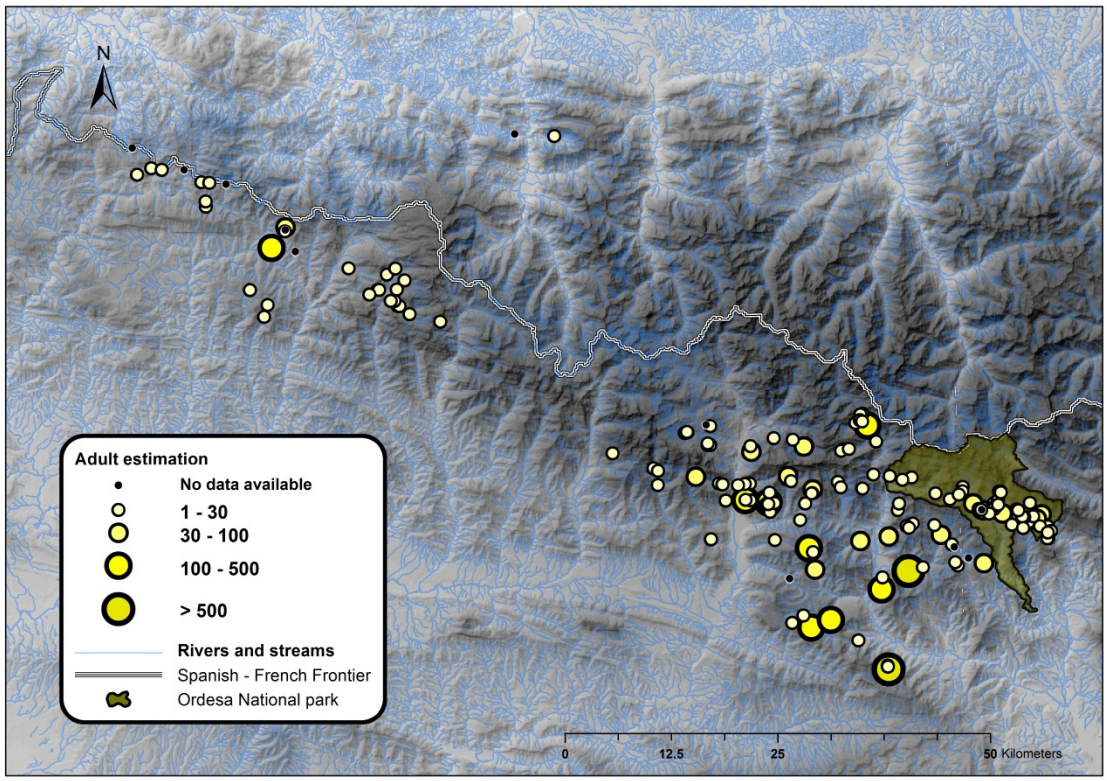

Figure 8. Relative density estimates of breeding adults per population in *R. pyrenaica* extrapolating to the sections of the same streams were *R. pyrenaica* occurs but were not sampled. Circle sizes are proportional to the number of individuals.

The survival Kaplan-Meier models for the five different populations analyzed in 2012 and 2013 are shown in Figure 9. At the Pista circular, with shallow pools and no forest cover, the mortality curve in 2012 (a very dry year that dried out pools) is very different from a wet year like 2013. While in 2013 the mortality rate is low (with a survival percentage around 90%), in the dry 2012 we can observe a much lower survival rate (around 57%), that increased in early summer. A similar pattern is found in Buesa, a locality with shallow pools that mainly dried out in 2013, giving a survival rate of ca. 63%. As a contrast, the mortality profiles from the Iguarra locality, a forest stream with deep pools, are very similar both in the dry and wet years, with a survival rate around 80%. If we look at the data from the five localities in 2013, we can see how the survival goes down in three of them, coinciding with major storms that washed tadpoles downstream, reaching survival rates of less than 15% in Bellós and Barranco Comas.

DISCUSSION

Conservation actions should be based on data that support them. Despite being one of the most threatened European amphibian species, the Endangered Pyrenean frog still is poorly known. There are many gaps of knowledge about its natural history, biology, genetic variation and phylogeographic structure or barriers to gene flow, despite that some have been addressed in previous works (Serra-Cobo 1997, 2002, Serra-Cobo *et al.* 1998, 2000, Vieites & Vences 2003). Moreover, so far, the distribution range, degree of fragmentation and current population estimates are not clearly defined or not known in the case of population sizes. Here we provide some of those data that are critical and the base of sound conservation actions.

We have dedicated many years and effort to visit all the known localities of the species, as well as sampling a large area in the Pyrenees, both in Spain and France, to try to locate new populations. We have succeeded by increasing the known distribution range of the species, as well as confirming real absences across its potential range. We have used SDMs to guide our fieldwork, by locating potentially suitable areas for the species. In several of those predicted areas we have found novel populations. The distribution range of this species is divided in two main core areas: a western one that spans from Navarra reaching the Hecho valley in Aragón, and an eastern one that goes from the Aragón River valley to Ordesa and Monte Perdido National Park. Several historical populations do not harbor this species anymore, and its situation in the Aragón River Valley in Villanúa seems not good, with very low densities to total absences per site.

The distribution models based on a resolution of 1 km^2 and Worldclim data have been widely used to predict and forecast the distribution of many species including some close relatives of *R. pyrenaica* like *R. temporaria* or *R. iberica* (Vences *et al.* 2013, Teixeira *et al.* 2018). However, it has been shown that finer layer resolutions can improve SDMs (Sardá-Palomera & Vieites 2011) as we show here for *R. pyrenaica*. While the 1 km^2 resolution model predicts a wide distribution with a high occupancy of the territory, it significantly overpredicts both within and outside the range of the species, which was confirmed by our real absence data. However, at higher resolution (200 m), the pattern is more realistic, being the distribution mainly determined by annual temperature and distance to streams, with a smaller influence of spring precipitation. Those variables make sense when understanding the distribution of the species. This is a lotic species that

occurs in and close to streams (Serra Cobo 1997, Vieites & Vences 2003), and we never have found it more than 100 m far away from streams. Its dispersal capacities are not known, but there are no observations far from streams. The mean annual temperature is a good predictor of the elevational temperature gradient in the Pyrenees. The altitudinal range where the species occurs spans from a few lowland localities to high elevations, but the species is more common at mid elevations, where the temperature is not too cold (alpine) or hot (lowlands).

If we compare the models, the one at 200 m resolution presents a more fragmented distribution linked to streams, and with most of the area of occupancy being much smaller than the predicted extent of occurrence. Overall, the species presents a very fragmented distribution, of many isolated populations that seem poorly connected in some areas. We located the species in 31 km of streams, and if we consider the total length of those streams where the species is present (including the lengths that we did not sample within the streams), the area of occupancy for the species would be less than 250 km^2. If we consider the full range, the extent of occurrence will be less than 3100 km^2. Within this area, the estimated population size would be around 13000 to 17000 adult frogs. If we compare those data with the Pyrenean populations of its sister taxon *R. temporaria*, we can see that in a single locality (Ibón de las Ranas, Circo de Piedrafita, Valle de Tena, Aragón, Spain), the estimated population size at one lake was between 500 to 1100 individuals depending on the year (Vieites 2003), hence the population sizes of *R. pyrenaica* in the same area are objectively very small. Despite that they occur in the same valleys of the Pyrenees, and were subject to the same historical and climatic processes, both species responded differently to them. While *R. temporaria* has populations with high densities in lakes across the Pyrenees, the population size of any *R. pyrenaica* locality is far below from the observed values in *R. temporaria*. A potential hypothesis of these differences is habitat specializations. Streams are not as stable as lakes and stagnant pools. They are affected by floods, droughts, and during the glaciations they were covered by ice, forcing a lowland retreat to any stream species (López-García *et* al. 2010).

We considered modeling the species potential distribution under future climate scenarios for the 2050 and 2070 following IPCC RCPs predictions (IPCC 2014). Those climatic layers are available at 1 km^2, and we have shown that at that resolution the model under current climate is not very realistic, hence, we discarded this option. However, future climate is predicted to be hotter, with an increase in temperatures at the southern

slopes of the Pyrenees, as well as a decrease in precipitation (López-Moreno *et al.* 2008). This means an increase of droughts and pool dry outs in areas where *R. pyrenaica* occurs. Survival data suggest that the mortality of larval stages and clutches is mainly determined by two factors: dry out of stream pools and stochastic storm floods that wash away and kill tadpoles. Both factors can be critical or not depending on the microhabitat structure. In streams protected by forests and with deep permanent pools, the impact of both floods and droughts seems non-existent. However, in localities where the is no forest cover, and especially where the pools are not deep enough, the mortality rates increase significantly both in hot years because of dry outs, and in wet years because of storm floods that are frequent in summer. The intensity and frequency of floods seems to be decreasing in the Pyrenees, especially in forested areas (López-Moreno *et al.* 2008), but at least half of the localities where *R. pyrenaica* occur do not have forest cover, mainly in Aragón. Under future climatic conditions, it is likely that these two factors will be a main issue for the local survivorship of the species. However, management actions to solve this issue are easy to implement, as making pools deeper is a cheap and affordable conservation action. It would be interesting to perform experiments where the mortality rates in artificially deepened pools and controls are compared to measure the utility of this action.

Our data support the Endangered status for this species following IUCN criteria, as it shows a very fragmented distribution, with small populations sizes, an extent of occurrence <5000 km^2, and an area of occupancy <500 km^2. This species is not yet included in the European Habitat's directive as a priority species for conservation, which should be implemented being a priority for local and state governments.

Acknowledgements

We are grateful to many people that made this project possible. Several students and collaborators helped in fieldwork in the Pyrenees, including Carlos Zaragoza, Javier Santos, Rubén González, Miguel Vences, Nina Bernard, Guillermo Ponz and Isabel Perandones. Fernando Carmena and Ignacio Gómez from SARGA in Aragón, and Iosu Antón in Navarra were extremely helpful in the field, allowing locating many known populations. Ramon Antor Casterllanau provided logistic help to prepare and carry on fieldwork. Manuel Alcántara, David Guzmán and Jose Luis Burrel from the Aragón government showed their total collaboration and help during the whole length of the project. Forest rangers helped us during the fieldwork, and the public company Sodemasa, now SARGA, provided logistic support in Ordesa in 2011. MPF was financed with a grant by CNPq under Science Without Borders program by Brazil Ministry of Science. We thank the Governments of Aragon and Navarra for collecting permits. This work was funded by a research project of the Zoo de Barcelona (Ayuntamiento de Barcelona) as well as by a research project from the Parques Nacionales OPAN – MMARM to DRV.

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Appendix

As this is an endangered species we decided not to publish coordinates for all localities to prevent potential conservation problems. These locality information has been provided only to the pertinent authorities in Aragón and Navarra governments.

Tables

Table A.1. Sampled river length and estimated number of adults (minimum and maximum) for each locality.

Locality	Sampled river length (m)	Minimum population estimated	Maximum population estimated
Kontrasario	290	10	10
Afluente Kontrasario	100	3	3
Errekabeltza	100	No data	No data
Erlán	404	13	13
Errekaundia	363	23	23
Landatxikina	338	3	3
Ibarrondoa	100	No data	No data
Gonostierreka	323	3	3
Koitxa	262	5	5
Kumuxiloa	100	13	13
Burguiarte	201	No data	No data
Iriola	108	3	3
Arroyo	174	23	23
Olerrea	100	No data	No data
Maze (pool)	1	3	3
Petrechema (pool)	1	3	3
Maz	86	3	5
Gamueta	336	3	5
As Eras (pool)	1	5	8
La Contienda	171	3	3
La Contienda 2	88	5	5
Manantial de Estebe	100	3	3
Belabarze	350	3	3
Pinaré (pool)	1	5	8
Fuen Fria	183	5	5
Birriés	85	25	25
Baldagrás	400	10	13
Paralelo al Baldagras	269	8	10
Mazandú	771	3	3
Vidangoz	276	5	5

Locality	Sampled river length (m)	Minimum population estimated	Maximum population estimated
Tortiellas	748	3	3
Cardal	100	5	5
Anterior al Cardal (pool)	1	5	8
Embalse lado puente (pool)	1	3	3
Turbera Ordiso (pool)	1	3	3
Ordiso tras 1 caida	100	3	3
Bajo Rocín Veriza	110	No data	No data
Salto Pitx	100	25	25
Parpalo	146	3	3
Cuneta1 (pool)	1	5	5
Afluente As Vacas	195	15	15
As Vacas	568	3	3
Trabenosa	978	3	3
Afluente Ripera	131	3	3
Cuneta pista Bujaruelo (pool)	1	38	38
Pista circular f (pool)	1	3	3
Pista circular e	315	3	3
Pista circular sv	105	8	8
San Lorenzo	78	3	3
Afluente Ripera 2	276	8	8
Afluente del Ordiso 1	224	3	3
Afluente del Ordiso 2	344	10	10
Río Ordiso	353	8	8
Coronazo	73	3	3
Aljibe Pista Collarada (pool)	1	3	3
Manantiales Costera	100	3	3
Cantal	68	1	3
Comas	683	3	3
Infierno	284	30	30
Sopeliana	100	3	3
Puerto Biescas	100	3	5
Carriata	100	3	5
Canal (pool)	1	3	3
Artica	375	3	3
Suaso	530	3	3
Planas de Abozo	100	3	3
Afluente Lasieso	82	5	5
Lasieso (pool)	1	5	5
Cuneta2	100	3	3
Cecutar	100	3	3
Afluente Lasieso 2	1376	3	3

Locality	Sampled river length (m)	Minimum population estimated	Maximum population estimated
Fuen Mochera (pool)	1	5	8
Sarieso (pool)	1	3	3
San Antón	500	10	13
Puerto Yésero	155	8	8
Afluente Arazas 1	569	3	3
Afluente Arazas 2	113	3	3
Afluente Arazas3	223	10	10
Pozas Bellós (pool)	1	3	3
Fuen deros Baños	100	3	3
Cuneta3 (pool)	1	3	3
Cascata (pool)	1	3	3
Ollas	100	5	5
Afluente Arazas 4	100	5	5
Praderas Ordesa	734	3	3
Canal del Señor	100	3	3
Cuneta Estabuen (pool)	1	3	3
Estabuen	100	10	10
Capradiza	100	3	5
Iguarra	100	10	10
Aljibe Puerto Biescas (pool)	1	5	5
Bartolomé de Gavin	88	8	8
Lapayón	100	3	3
Aljibe (pool)	1	3	3
Caprariza	62	3	3
Embalse Diazas (pool)	1	5	5
Afl. Puerto Yesero (pool)	1	3	3
Afluente Arazas5	100	3	13
San Bartolomé (pool)	1	3	3
Fuen Carduso	100	3	3
Paralelo a Fuen Carduso	300	5	8
Yaba	300	20	25
Afluente Pardina	100	5	10
Paralelo Refoba	300	3	5
Afluente Berná 2	400	5	28
Afluente Fuen Berná 1	385	No data	No data
Bco Diaza	261	5	8
Fuen Berná	400	No data	No data
Refoba	300	3	3
Coma	119	35	45
Afluente Bartolomé Gavín	300	8	20
Artica Torre	200	1	3

Locality	Sampled river length (m)	Minimum population estimated	Maximum population estimated
Afluente Comas	400	8	10
San Vicenda	351	30	30
Afluente Yaga 2	150	1	3
Afluente Fuen Berná 2	156	20	25
Garganta Escuaín	132	28	38
Calzil	200	3	3
La Ralla	300	3	3
Brocal	300	3	3
Yaga	100	3	3
Charca	100	1	3
Gurrundué 1	97	8	8
Gurrundué 2	52	5	5
Piñal	490	20	20
Mallo Sasé	375	3	3
Borrué	100	3	3
Chate	371	5	8
Afluente Yaga 3	161	8	8
Furcos	291	3	3
Rosico	100	3	3
Del Lugar (pool)	1	5	8
Afluente Lugar (pool)	1	5	8
As Gloces	100	3	3
Val de Jalle	100	13	13
La Fuen	100	5	8
Afluente la Fuen	100	5	8
Manabí	100	3	5
Escuer alto	100	3	3
Dos Lucas	600	5	5
Labate bajo	254	23	23
La Grada	200	10	10
Guampe	71	No data	No data
Otal	165	13	18
Artosa	205	3	3
Aso (pool)	1	No data	No data
Fuen del Obispo	100	3	3
Espierlo	100	3	3
La Valle (pool)	1	3	3
Otal 2	118	3	5
Pista Rosada	100	78	105
Pardinas	308	5	5
Escuer	82	13	13

Locality	Sampled river length (m)	Minimum population estimated	Maximum population estimated
Anterior al Lata	100	3	3
Sasa	100	25	33
La Valle Fiscal	762	3	5
La Lata	100	25	33
Fiscal	304	5	5
San Juste (pool)	1	3	3
Camino (pool)	1	3	3
Afluente San Juste	82	38	50
Fuen de Olivan (pool)	1	3	5
AfluenteYaga 4	430	5	5
Biandisco Aso	92	10	10
Mosquera	71	8	8
Cuneta San Barotomé (pool)	1	5	5
Cabecera Yaga (pool)	1	3	3
Arpeko Oihaneko	no data	No data	No data
Francia 1	no data	No data	No data
Saillen	100	3	3
TOTAL	**31232**	**1166**	**1377**

Table A.2. Upstream river length not sampled at localities with *R. pyrenaica* presence and minimum and maximum extrapolated adult population size.

Locality	Upstream river lenght (m)	Minimum population estimated	Maximum population estimated
Kontrasario	1494	53	53
Afluente Kontrasario	2293	58	58
Errekabeltza	1115	No data	No data
Erlán	872	28	28
Errekaundia	363	25	25
Landatxikina	739	8	8
Ibarrondoa	2272	No data	No data
Gonostierreka	1593	13	13
Koitxa	1280	25	25
Kumuxiloa	1017	128	128
Burguiarte	869	No data	No data
Iriola	1465	35	35
Arroyo	2312	300	300
Olerrea	1229	0	0
Maze (pool)	0	0	0
Petrechema (pool)	0	0	0

Locality	Upstream river lenght (m)	Minimum population estimated	Maximum population estimated
Maz	419	13	25
Gamueta	1557	13	25
As Eras (pool)	0	0	0
La Contienda	1020	15	15
La Contienda 2	507	30	30
Manantial de Estebe	1265	33	33
Belabarze	350	3	3
Pinaré (pool)	0	0	0
Fuen Fria	487	15	15
Birriés	85	25	25
Baldagrás	653	18	23
Paralelo al Baldagras	334	10	13
Mazandú	871	5	5
Vidangoz	2618	48	48
Tortiellas	2895	10	10
Cardal	1004	53	53
Anterior al Cardal (pool)	0	0	0
Embalse lado puente (pool)	0	0	0
Turbera Ordiso (pool)	0	0	0
Ordiso tras 1 caida	1397	35	35
Bajo Rocín Veriza	1040	No data	No data
Salto Pitx	1559	390	390
Parpalo	1213	23	23
Cuneta1 (pool)	0	0	0
Afluente As Vacas	671	53	53
As Vacas	4490	20	20
Trabenosa	2117	8	8
Afluente Ripera	668	15	15
Cuneta pista Bujaruelo (pool)	0	0	0
Pista circular f (pool)	0	0	0
Pista circular e	599	5	5
Pista circular sv	209	15	15
San Lorenzo	1971	65	65
Afluente Ripera 2	2658	73	73
Afluente del Ordiso 1	900	13	13
Afluente del Ordiso 2	1155	35	35
Río Ordiso	1251	28	28
Coronazo	3419	118	118
Aljibe Pista Collarada (pool)	0	0	0
Manantiales Costera	2415	63	63
Cantal	1538	1	58

Locality	Upstream river lenght (m)	Minimum population estimated	Maximum population estimated
Comas	1633	8	8
Infierno	524	58	58
Sopeliana	347	10	10
Puerto Biescas	3713	95	188
Carriata	1246	33	63
Canal (pool)	0	0	0
Artica	2182	15	15
Suaso	156	3	3
Planas de Abozo	1428	38	38
Afluente Lasieso	452	30	30
Lasieso (pool)	0	0	0
Cuneta2	666	18	18
Cecutar	2353	60	60
Afluente Lasieso 2	3846	8	8
Fuen Mochera (pool)	0	0	0
Sarieso (pool)	0	0	0
San Antón	538	13	15
Puerto Yésero	2076	103	103
Afluente Arazas 1	569	3	3
Afluente Arazas 2	1765	40	40
Afluente Arazas3	223	10	10
Pozas Bellós (pool)	0	0	0
Fuen deros Baños	1031	28	28
Cuneta3 (pool)	0	0	0
Cascata (pool)	0	0	0
Ollas	649	33	33
Afluente Arazas 4	649	33	33
Praderas Ordesa	734	3	3
Canal del Señor	200	5	5
Cuneta Estabuen (pool)	0	0	0
Estabuen	2668	268	268
Capradiza	400	10	20
Iguarra	524	53	53
Aljibe Puerto Biescas (pool)	0	0	0
Bartolomé de Gavin	3500	300	300
Lapayón	2000	50	50
Aljibe (pool)	0	0	0
Caprariza	1679	70	70
Embalse Diazas (pool)	0	0	0
Afl. Puerto Yesero (pool)	0	0	0
Afluente Arazas5	532	15	68

Locality	Upstream river lenght (m)	Minimum population estimated	Maximum population estimated
San Bartolomé (pool)	0	0	0
Fuen Carduso	463	13	13
Paralelo a Fuen Carduso	483	10	13
Yaba	583	40	50
Afluente Pardina	412	23	43
Paralelo Refoba	503	5	10
Afluente Berná 2	881	13	63
Afluente Fuen Berná 1	615	No data	No data
Bco Diaza	2286	45	68
Fuen Berná	881	No data	No data
Refoba	503	5	5
Coma	0	0	0
Afluente Bartolomé Gavín	653	18	45
Artica Torre	1152	1	15
Afluente Comas	600	13	15
San Vicenda	705	63	63
Afluente Yaga 2	255	1	5
Afluente Fuen Berná 2	0	0	0
Garganta Escuaín	734	155	210
Calzil	2475	33	33
La Ralla	649	8	8
Brocal	944	10	10
Yaga	4900	123	123
Charca	2055	1	53
Gurrundué 1	1249	98	98
Gurrundué 2	487	48	48
Piñal	1049	45	45
Mallo Sasé	1154	10	10
Borrué	1024	28	28
Chate	1037	15	23
Afluente Yaga 3	193	10	10
Furcos	770	8	8
Rosico	200	5	5
Del Lugar (pool)	0	0	0
Afluente Lugar (pool)	0	0	0
As Gloces	5403	138	138
Val de Jalle	1100	138	138
La Fuen	770	40	60
Afluente la Fuen	750	38	58
Manabí	700	18	35
Escuer alto	291	8	8

Locality	Upstream river lenght (m)	Minimum population estimated	Maximum population estimated
Dos Lucas	2821	25	25
Labate bajo	1443	130	130
La Grada	1202	63	63
Guampe	765	No data	No data
Otal	2199	168	235
Artosa	1742	23	23
Aso (pool)	0	No data	No data
Fuen del Obispo	1292	33	33
Espierlo	2250	58	58
La Valle (pool)	0	0	0
Otal 2	2190	48	95
Pista Rosada	3321	2575	3488
Pardinas	1047	18	18
Escuer	2155	330	330
Anterior al Lata	730	20	20
Sasa	2219	555	723
La Valle Fiscal	762	3	5
La Lata	2482	623	808
Fiscal	2313	40	40
San Juste (pool)	0	0	0
Camino (pool)	0	0	0
Afluente San Juste	5935	2715	3620
Fuen de Olivan (pool)	0	0	0
AfluenteYaga 4	1119	15	15
Biandisco Aso	1169	128	128
Mosquera	71	8	8
Cuneta San Barotomé (pool)	0	0	0
Cabecera Yaga (pool)	0	0	0
Arpeko Oihaneko	no data	No data	No data
Francia 1	no data	No data	No data
Saillen	1000	25	25
TOTAL	**179669**	**12167**	**15061**

Table A.3. Total stream length at *R. pyrenaica* populations and total minimum and maximum population estimated.

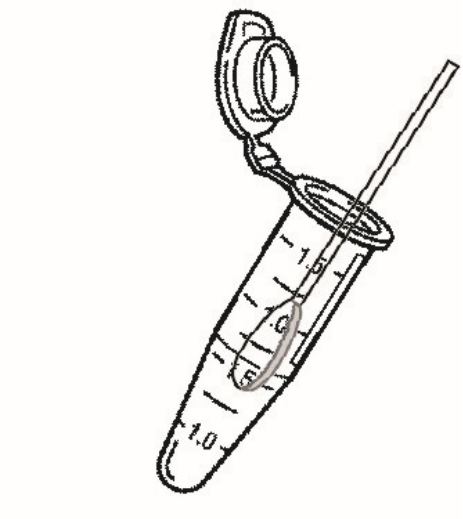
Locality	Total river lenght (sampled + potential) (m)	Minimum Total population (Sampled + Potential)	Maximum Total population (Sampled + Potential)
Kontrasario	1784	63	63
Afluente Kontrasario	2393	61	61
Errekabeltza	1215	No data	No data
Erlán	1276	41	41
Errekaundia	726	48	48
Landatxikina	1077	11	11
Ibarrondoa	2372	No data	No data
Gonostierreka	1916	16	16
Koitxa	1542	30	30
Kumuxiloa	1117	141	141
Burguiarte	1070	No data	No data
Iriola	1573	38	38
Arroyo	2486	323	323
Olerrea	1329	No data	No data
Maze (pool)	1	3	3
Petrechema (pool)	1	3	3
Maz	505	16	30
Gamueta	1893	16	30
As Eras (pool)	1	5	8
La Contienda	1191	18	18
La Contienda 2	595	35	35
Manantial de Estebe	1365	36	36
Belabarze	700	6	6
Pinaré (pool)	1	5	8
Fuen Fria	670	20	20
Birriés	170	50	50
Baldagrás	1053	28	36
Paralelo al Baldagras	603	18	23
Mazandú	1642	8	8
Vidangoz	2894	53	53
Tortiellas	3643	13	13
Cardal	1104	58	58
Anterior al Cardal (pool)	1	5	8
Embalse lado puente (pool)	1	3	3
Turbera Ordiso (pool)	1	3	3
Ordiso tras 1 caida	1497	38	38
Bajo Rocín Veriza	1150	No data	No data
Salto Pitx	1659	415	415
Parpalo	1359	26	26

Locality	Total river lenght (sampled + potential) (m)	Minimum Total population (Sampled + Potential)	Maximum Total population (Sampled + Potential)
Cuneta1 (pool)	1	5	5
Afluente As Vacas	866	68	68
As Vacas	5058	23	23
Trabenosa	3095	11	11
Afluente Ripera	799	18	18
Cuneta pista Bujaruelo (pool)	1	38	38
Pista circular f (pool)	1	3	3
Pista circular e	914	8	8
Pista circular sv	314	23	23
San Lorenzo	2049	68	68
Afluente Ripera 2	2934	81	81
Afluente del Ordiso 1	1124	16	16
Afluente del Ordiso 2	1499	45	45
Río Ordiso	1604	36	36
Coronazo	3492	121	121
Aljibe Pista Collarada (pool)	1	3	3
Manantiales Costera	2515	66	66
Cantal	1606	2	61
Comas	2316	11	11
Infierno	808	88	88
Sopeliana	447	13	13
Puerto Biescas	3813	98	193
Carriata	1346	36	68
Canal (pool)	1	3	3
Artica	2557	18	18
Suaso	686	6	6
Planas de Abozo	1528	41	41
Afluente Lasieso	534	35	35
Lasieso (pool)	1	5	5
Cuneta2	766	21	21
Cecutar	2453	63	63
Afluente Lasieso 2	5222	11	11
Fuen Mochera (pool)	1	5	8
Sarieso (pool)	1	3	3
San Antón	1038	23	28
Puerto Yésero	2231	111	111
Afluente Arazas 1	1138	6	6
Afluente Arazas 2	1878	43	43
Afluente Arazas3	446	20	20
Pozas Bellós (pool)	1	3	3
Fuen deros Baños	1131	31	31

Locality	Total river lenght (sampled + potential) (m)	Minimum Total population (Sampled + Potential)	Maximum Total population (Sampled + Potential)
Cuneta3 (pool)	1	3	3
Cascata (pool)	1	3	3
Ollas	749	38	38
Afluente Arazas 4	749	38	38
Praderas Ordesa	1468	6	6
Canal del Señor	300	8	8
Cuneta Estabuen (pool)	1	3	3
Estabuen	2768	278	278
Capradiza	500	13	25
Iguarra	624	63	63
Aljibe Puerto Biescas (pool)	1	5	5
Bartolomé de Gavin	3588	308	308
Lapayón	2100	53	53
Aljibe (pool)	1	3	3
Caprariza	1741	73	73
Embalse Diazas (pool)	1	5	5
Afl. Puerto Yesero (pool)	1	3	3
Afluente Arazas5	632	18	81
San Bartolomé (pool)	1	3	3
Fuen Carduso	563	16	16
Paralelo a Fuen Carduso	783	15	21
Yaba	883	60	75
Afluente Pardina	512	28	53
Paralelo Refoba	803	8	15
Afluente Berná 2	1281	18	91
Afluente Fuen Berná 1	1000	No data	No data
Bco Diaza	2547	50	76
Fuen Berná	1281	No data	No data
Refoba	803	8	8
Coma	119	35	45
Afluente Bartolomé Gavín	953	26	65
Artica Torre	1352	2	18
Afluente Comas	1000	21	25
San Vicenda	1056	93	93
Afluente Yaga 2	405	2	8
Afluente Fuen Berná 2	156	20	25
Garganta Escuaín	866	183	248
Calzil	2675	36	36
La Ralla	949	11	11
Brocal	1244	13	13
Yaga	5000	126	126

Locality	Total river lenght (sampled + potential) (m)	Minimum Total population (Sampled + Potential)	Maximum Total population (Sampled + Potential)
Charca	2155	2	56
Gurrundué 1	1346	106	106
Gurrundué 2	539	53	53
Piñal	1539	65	65
Mallo Sasé	1529	13	13
Borrué	1124	31	31
Chate	1408	20	31
Afluente Yaga 3	354	18	18
Furcos	1061	11	11
Rosico	300	8	8
Del Lugar (pool)	1	5	8
Afluente Lugar (pool)	1	5	8
As Gloces	5503	141	141
Val de Jalle	1200	151	151
La Fuen	870	45	68
Afluente la Fuen	850	43	66
Manabí	800	21	40
Escuer alto	391	11	11
Dos Lucas	3421	30	30
Labate bajo	1697	153	153
La Grada	1402	73	73
Guampe	836	No data	No data
Otal	2364	181	253
Artosa	1947	26	26
Aso (pool)	1	No data	No data
Fuen del Obispo	1392	36	36
Espierlo	2350	61	61
La Valle (pool)	1	3	3
Otal 2	2308	51	100
Pista Rosada	3421	2653	3593
Pardinas	1355	23	23
Escuer	2237	343	343
Anterior al Lata	830	23	23
Sasa	2319	580	756
La Valle Fiscal	1524	6	10
La Lata	2582	648	841
Fiscal	2617	45	45
San Juste (pool)	1	3	3
Camino (pool)	1	3	3
Afluente San Juste	6017	2753	3670
Fuen de Olivan (pool)	1	3	5

Locality	Total river lenght (sampled + potential) (m)	Minimum Total population (Sampled + Potential)	Maximum Total population (Sampled + Potential)
AfluenteYaga 4	1549	20	20
Biandisco Aso	1261	138	138
Mosquera	142	16	16
Cuneta San Barotomé (pool)	1	5	5
Cabecera Yaga (pool)	1	3	3
Arpeko Oihaneko	No data	No data	No data
Francia 1	No data	No data	No data
Saillen	1100	28	28
TOTAL	**210901**	**13333**	**16438**



Chapter 2:

Widespread presence of *Batrachochytrium dendrobatidis* across the full geographic range of the Endangered Pyrenean frog.

This chapter reproduce entirely the manuscript:

Peso M, Bernard N, Nieto-Roman S and Vieites DR. Widespread presence of *Batrachochytrium dendrobatidis* across the full geographic range of the Endangered Pyrenean frog. Manuscript in preparation.

Widespread presence of *Batrachochytrium dendrobatidis* across the full geographic range of the Endangered Pyrenean frog.

Abstract

Chytridiomycosis is a disease decimating amphibian populations and species worldwide. It has been recently found in the Pyrenean mountain range where a genetic study suggests a recent colonization based on the genetic homogeneity from different sites. According to this scenario, endemic Pyrenean amphibian species like the Pyrenean frog, *Rana pyrenaica*, or the Pyrenean newt, *Calotriton asper*, should have been in contact with the chytrid fungus that causes this disease only in recent times. Here, we assessed the prevalence of the fungus *Batrachochytrium dendrobatidis* (Bd) in *R. pyrenaica* across its full distribution range, finding that Bd is widespread. Low and mid elevation populations show higher percentage of infected individuals in respect to higher elevations. Growth models for Bd, based on temperature profiles, suggest that its optimum growing range centers at mid elevations, while forecasts under future climate scenarios suggest that the optimum growing conditions for Bd will reach high elevation lakes in few decades, with a potential impact on alpine populations that are less affected today. It is unknown the effect that Bd could have had on *R. pyrenaica* populations, and it could be one of the agents explaining the low populations sizes observed in the species. The high prevalence of Bd across the range of this Endangered species raises concerns about the future viability of *R. pyrenaica* populations as well as for other mountain amphibians.

Keywords: *Rana pyrenaica*, Chytridiomycosis, Conservation, *Batrachochytrium dendrobatidis*, Pyrenees.

INTRODUCTION

During the last three decades, overwhelming data have been accumulated to confirm that amphibian populations and species are declining worldwide, being one of the groups with the highest extinction risk among vertebrates (Stuart *et al.* 2004, Wake & Vredenburg 2008). In the late 20th century, population declines were reported from many species, mainly mountain amphibians, caused by increasing UV-B radiation exposure (Kiesecker *et al*. 2001, Vieites 2003), as well as other human-related causes mainly involving habitat transformation (e.g. deforestation of tropical forests, urbanization, etc.) and climate change (Blaustein & Kiesecker 2002, Stuart *et al.* 2004, Wake & Vredenburg 2008). In parallel to those impacts, emerging infectious diseases started to appear in the equation of amphibian losses, now being one of the main focus of research in amphibian declines for its global occurrence and impact on populations and species (e.g. Rödder *et al*. 2009, Olson *et al*. 2013, Lips 2016, O'hanlon *et al*. 2018). Among those, the relatively recent discovery of several species of chytrid fungi that kill frogs and salamanders, *Batrachochytrium dendrobatidis* (Bd) (Longcore *et al*. 1999) and *B. salamandrivorans* (Martel *et al*. 2013), is of paramount importance. Chytrid fungi have been at the core of the disappearance of many amphibian populations across the Planet (Rödder *et al*. 2009, Olson *et al*. 2013, O'hanlon *et al*. 2018), as well as the putative extinction of many species (Fisher *et al*. 2009, Cheng *et al*. 2011). They are expanding, being reported from areas that where chytrid free just few years ago (e.g. Vredenburg *et al*. 2012 vs. Bletz *et al*. 2015), with pet trade as an important factor for its continuous spread (Fisher & Garner 2007, Fisher *et al*. 2009, Schloegel *et al*. 2009, Martel *et al*. 2013), threating with extinction many populations and species that were never exposed to these fungi before.

Chytridiomycosis, the disease caused by these fungi, has been reported both in tropical and temperate amphibian species (Fisher *et al*. 2009), being Bd introduced in many places by humans where it persists. This is in part because *B. dendrobatidis* shows a relatively wide thermal tolerance. Its thermal physiology has been studied in the lab, showing that it has an optimum growth between 10^{o}C and 25^{o}C with a suitability temperature range between 5^{o}C and 28^{o}C (Piotrowski *et al*. 2004). Although its growth can be different in the field influenced by other factors or the different strains of Bd, this temperature range overlaps with the distribution of many amphibian temperate-latitude species. Mountains seem a particularly suitable environment for these fungi. In fact, massive die offs have been reported for many mountain frog species (Rachowicz *et al*.

2006, Catenazzi *et al*. 2013, Gillespie *et al*. 2015) across different continents and mountain ranges.

In the Pyrenees, a mountain range that separates France from Spain, there have been reports of Bd presence in several species, including *Calotriton asper*, *Salamandra salamandra*, *Triturus helveticus*, *Alytes obstetricans*, *Bufo bufo*, *Pelophylax perezi*, *Rana temporaria* and the Pyrenean frog, *Rana pyrenaica* (Global Bd-Mapping Project 2018, Walker *et al*. 2010, Clare *et al*. 2016). And recent colonization of the Pyrenees by Bd, based on the finding of identical genotypes from different sites, has been proposed (Walker *et al*. 2010). This scenario implies that whatever effects Bd has on Pyrenean amphibians should be recent and that endemic Pyrenean amphibian species were only recently exposed to the pathogen. Two species of amphibians are endemic of these mountains: *R. pyrenaica* and the Pyrenean newt *Calotriton asper*, both with fast flowing mountain streams as their typical habitat. The Pyrenean frog has one of the smallest ranges for an European amphibian and has been catalogued as Endangered according to IUCN criteria (Bosch *et al.* 2009). It is a mountain frog that inhabits mainly headwater streams, with a fragmented distribution with most populations of reduced size (Chapter 1). Together with the small population sizes, the genetic variation of the species is very small (Chapters 4, 5) with low effective population sizes. This makes this species prone to decline. Among the major threats for the species are the presence of fishes that were introduced in all streams for fishing, and stochastic events like storm floods that in the mountains can cause the sweep of all the tadpoles (Chapter 1). So far, massive die offs have not been reported for this species, but yes for *Rana temporaria*, *Alytes obstetricans* or *Lissotriton helveticus* in this area (obs pers.). However, as a stream species if there were any die offs the corpses could be washed again with the current. It is not known why the populations of this species are so small, but chytridiomicosis could have been a recent potential cause. Here we wanted to assess if the presence of Bd is common or not in this species, and what is the prevalence in the populations across the full distribution range of the species. As an Endangered species of special conservation concern, gathering the first data on the presence of Bd across its range will be very helpful to make management decisions and identify which areas may be affected by Bd to monitor them and plan applied conservation actions. We also used the thermal profile of Bd to model its presence in the Pyrenees under current climate and future climatic scenarios. These forecasts provide a spatial hypothesis of Bd's future distribution at a local scale, including

potential changes in its altitudinal range that may affect high elevation amphibian populations that are now outside the optimum of chytrid growth.

MATERIAL AND METHODS

Fieldwork

We visited all the 170 known localities where *Rana pyrenaica* occurs, covering the full distribution range of the species. Sampling was performed from 2010 to 2014, including several visits to localities to detect the species as population densities are low. The Pyrenean frog inhabits in high mountain stream headwaters, hence long hiking to remote localities was necessary in most cases. We collected swabs for chytrid analyses in 108 localities. At each locality we located adults and tadpoles, which were collected by hand or net. Each specimen was placed in an independent plastic bag before collecting swab and in some cases tissue samples. We changed gloves with every specimen to avoid potential cross-contaminations. We swabbed the skin of adults following Boyle *et al.* (2007) using MW113 2mm sterile synthetic cotton swabs (Medical Wire & Equipment). We swabbed each individual with 30 strokes: five times each hind foot on the toe webbing, each thigh was swabbed five times, and the sides of the ventral skin of the frogs five times each. Swab tips were stored into a sterile Nalgene tube filled with Ethanol 99%. For tadpoles, we swabbed both mouth and skin. Between each site, we sterilized all field equipment using 10% bleach.

PCR Bd detection

Genomic DNA was extracted from swabs preserved in ethanol, using the Qiagen DNeasy 96 Tissue Kit (Qiagen, Valencia, CA, USA), following the protocol provided by the manufacturer. We performed a nested double PCR of the ITS regions between 18S-28S fragment of the chytrid genome. A first PCR was done with specific primers Bd18SF1 (5′-TTTGTACACACCGCCCGTCGC-3′) and Bd28SR1 (5′-ATATGCTTAAGTTCAGCGGG-3′) (Goka *et al*. 2009). A second PCR was performed using as template the PCR product from the first PCR, and using the internal Bd primers Bd1a (5′-CAGTGTGCCATATGTCACG-3′) and Bd2a (5′-CATGGTTCATATCTGTCCAG-3′) (Annis *et al*. 2004). PCRs where repeated twice with positive and negative controls to confirm results.

PCR conditions for the first PCR included an initial denaturation at 95 ºC for 9 min, 35 cycles at 94 ºC for 30 sec, annealing at 50 ºC for 30 sec, extension at 72 ºC for 2 min; and a final extension of 7 min at 72 ºC. The conditions for the second PCR included an initial denaturation at 95 ºC for 10 min, 35 cycles at 94 ºC for 30 sec, annealing at 58 ºC for 30 sec, extension at 72 ºC for 30 sec; and a final extension of 7 min at 72 ºC. PCR products were loaded onto 1% agarose gels, stained with GelStar gel stain (Cambrex), and visualized in a Dark reader transilluminator (Clare Chemical). We considered that the sample was positive if in two independent PCRs there was a clear band in electrophoresis gels.

Bd distribution and forecast under IPCC scenarios

Two approaches can be done to model the distribution of Bd in the area where *R. pyrenaica* occurs: a correlative species distribution model (with Maxent or any other popular modeling software), or a mechanistic approach using the available thermal growth profiles of Bd. When modeling only the positive records of Bd known for *R. pyrenaica* using Maxent, we obtained a similar predictive map as in Chapter 1, as it is based on *R. pyrenaica* distribution records. Hence, we here followed a different approach by using one of the available Bd's thermal growth models (Piotrowski *et al*. 2004). Piotrowski's growth model suggests that the optimum Bd growth is reached between 10ºC and 25ºC and the suitability temperature is within the range of 5ºC to 28ºC. Based on those data, we calculated the number of months in which Bd has an optimum or suitable growth, and summarized them for the whole year. For doing that, we applied the growth model to the maximum and minimum monthly temperature maps available from WorldClim at ca. 1 Km^2 resolution (Hijmans *et al*. 2005) under current climate (based on the period 1950-2000). For each month, optimum growth maps were created for the minimum and maximum temperatures, and the same was done for the suitable growth. We reclassified the values of minimum or maximum temperature from all climatic layers to one (1) if the temperature was within optimum or suitable Bd's growth ranges respectively, and zero (0) if the temperature was outside those ranges. Then we summed all months to create maps in which each pixel represents how many months Bd has optimum or suitable growth conditions.

To forecast the future distribution of Bd under IPCC's RCP Coupled Model Intercomparison Project Phase 5 (CMIP5) scenarios (IPCC AR5 2014), we applied the

same approach for the years 2050 and 2070, using an ensemble of five climatic models: CCSM4, Miroc 5, CSIRO MK3-6-0, CNRM-CM5 and BCC-CSM1-1 (Karger *et al*. 2017). Those models were selected based on their differences according to their output in model comparison analyses (Knutti & Sedláček 2013, Sanderson *et al*. 2015). For each model we used a Representative Concentration Pathway (RCP) of 4.5 as an intermediate scenario, and a RCP 8.5 as a high warming scenario. We used functions from diverse libraries in R (raster, rgdal, adehabitat and SDMTools) for operations with raster layers.

In order to explore the relationship between elevation and the number of optimum or suitable months for Bd growth within the range of *R. pyrenaica*, we generated one thousand random points within this range and extracted the altitude as well as every growth map model values using ARCMap 10.1. We then explored different regression´s models that best explain the relationship between altitude and the number of months available for Bd's optimum or suitable growth, under present and future climatic conditions in SPSS v23. We finally gathered all available Bd positive (presences) and Bd negative (absences) distribution records for all Pyrenean amphibians [mainly from the Global Bd-Mapping Project (2018) and our own data], to plot the altitudinal distributions of positive and negative tested samples.

RESULTS

Presence of Bd in *Rana pyrenaica* skins

We analysed 666 samples from 106 localities throughout the whole distribution range of *R. pyrenaica* (Fig. 1). No swabs were available from the two French isolated populations. Bd was detected in 184 frog swab samples (27.6%) from 46 localities (43.4%), distributed in elevations between 857 m a.s.l. and 2079 m a.s.l. The distribution of positive localities suggests that Bd is present across the full range of the species and it is widespread in the Pyrenees. Most of the populations that yielded negative results correspond to sites from which one or few samples were available due to low population sizes and the difficulty to find the species. In localities with higher sample sizes Bd was always detected. The proportion of Bd positive samples within each locality is variable, with some areas with prevalence of Bd negative (Eastern Navarra to Hecho valley, or localities south of Ordesa – Monte Perdido National Park) and other areas with prevalence of Bd+ independently of sample size (Añisclo or Tena valleys, Fig. 1). Only in

two main valleys Bd was not detected, but those have few *R. pyrenaica* localities and low population sizes, hence few samples could be collected and analyzed from there. It is remarkable that Bd was found in the three main valleys within the Ordesa – Monte Perdido National Park.

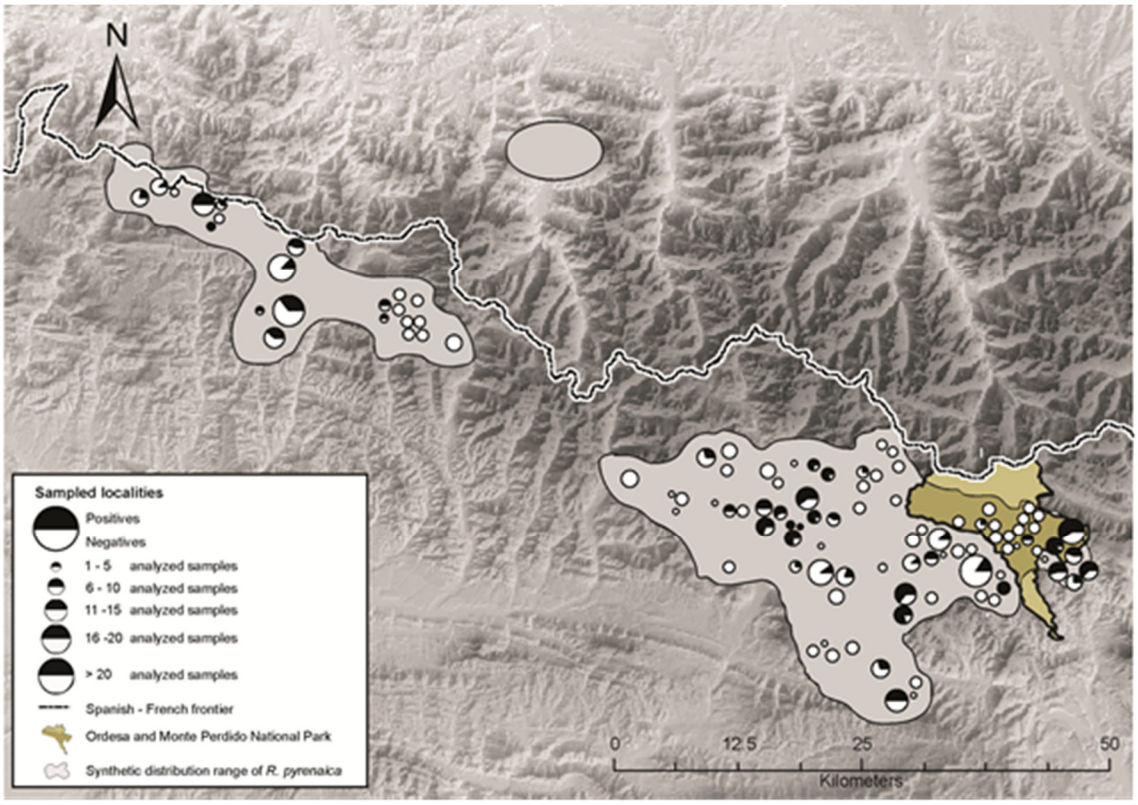


Figure 1. Map showing the distribution of the sampled localities for *Batrachochytrium dendrobatidis* in swabs from *Rana pyrenaica*, and the sample size per locality. Samples cover the whole distribution range of the Pyrenean frog. In each pie, the percentage of samples that yielded Bd positive (Bd+) is shown in black, and Bd- in white.

The growth models under current climate show that for the minimum temperature climate layer, Bd has an optimum growth during one month in most of the western Pyrenees; although if we consider the other extreme, under the maximum temperature layer, it has between 3 to 5 months of optimum growth, mainly in the valleys (Fig. 2). In both models the chytrid does not grow well in high elevations. If we consider the suitable growth range of temperatures, under the minimum temperature climate layer, the suitable Bd growth spans between 2 to 6 months, while under the maximum temperatures it spans throughout the whole year at mid and lower elevations but in the highest elevations (above 2200 m a.s.l.) Bd cannot grow well. Considering as a reference elevations of 1500 m and 2200 m a.sl., at 1500 m the chytrid cannot grow under its optimum temperature if we conssider the minimum temperature layers, but it can during seven months under the

maximum ones (hereafter: number of suitable months considering the minimum temperature – suitable months considering the maximum temperature). At 2200 m a.s.l. Bd can grow in its optimum between 2 and 4 months. If we consider the suitable climate, at 1500 m a.s.l. spans from 4-10 months and at 2200 m a.s.l between 2 and 6 months.

Under future climate scenarios for the year 2050, climatic conditions using a moderate mitigation scenario (RCP 4.5) show that both optimum growth models under minimum and maximum environmental temperatures predict an increase in the number of months suitable for the chytrid when compared to current climatic conditions (Fig. 3). Bd will be able to grow optimally in higher elevations during 2-4 months per year at 1500 m a.s.l. and between 0 to 4 months at 2200 m a.s.l. The suitable growth maps show a similar pattern, with an increase to 3-6 months of suitable grow at 1500 m a.s.l. and between 3 and 6 at 2200 m a.s.l. (Fig. 3).

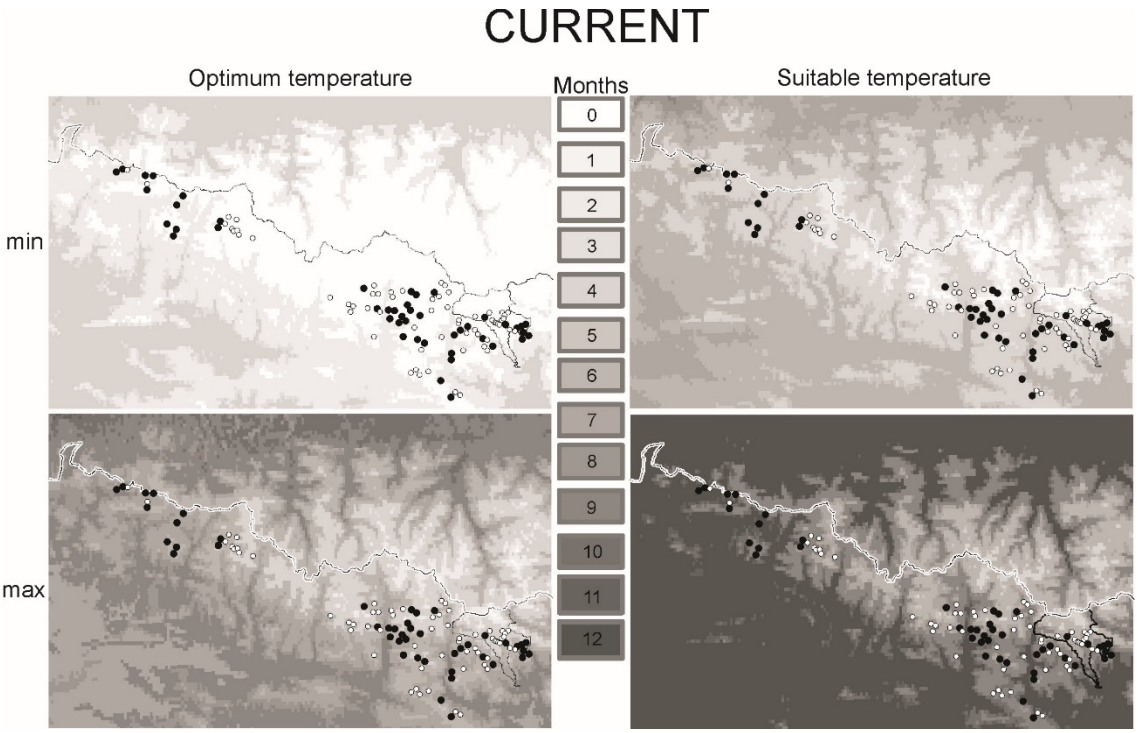


Figure 2. Map representing the total number of months of optimum growth temperature (17-25 ºC) and suitable growth temperature (4-25 ºC, sensu Piotrowski *et al*. 2004) of Bd under current climatic conditions. Maps above are built under the minimum monthly temperature layers which were aggregated to get a yearly value, while bottom maps are based on the maximum monthly temperature. Black dots represent Bd+ localities and white dots are the localities where Bd was not detected in any *R. pyrenaica* sample.

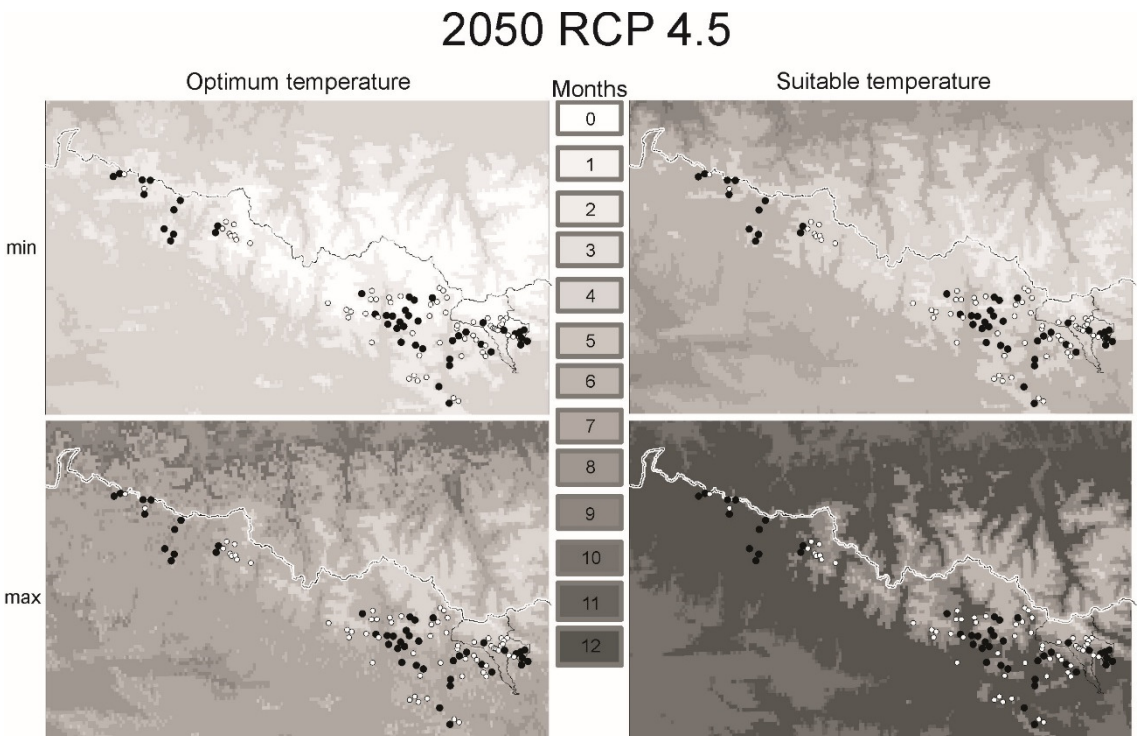


Figure 3. Map representing the total number of months of optimum growth temperature (17-25 ºC) and suitable growth temperature (4-25 ºC, sensu Piotrowski *et al*. 2004) of Bd under 2050 climatic conditions and RCP 4.5 scenario. Maps above are built under the minimum monthly temperature layers which were aggregated to get a yearly value, while bottom maps are based on the maximum monthly temperature. Black dots represent Bd+ localities and white dots are the localities where Bd was not detected in any *R. pyrenaica* sample.

The same approach under a high emission and low mitigation scenario (RCP 8.5) for the year 2050, provides a pattern that is similar to the results from the RCP 4.5 for the same period (Fig. 4). The maps for the year 2070 under a RCP 4.5 (Fig. 5) and RCP 8.5 (Fig. 6) scenarios show also an increase in the number of suitable months for chytrid growth in respect to current climate. RCP 4.5 models for 2070 are almost identical to RCP 4.5 models from 2050, however, in the RCP 8.5 models for 2070 the optimum growth is ca. one month longer at high elevations than in the same models in 2050, and two months longer than under current climate. All future scenarios suggest that high elevation areas where the chytrid has a limited growth period now will switch to a climate where the chytrid can grow optimally for 3 to 6 months at 2200 m a.sl..

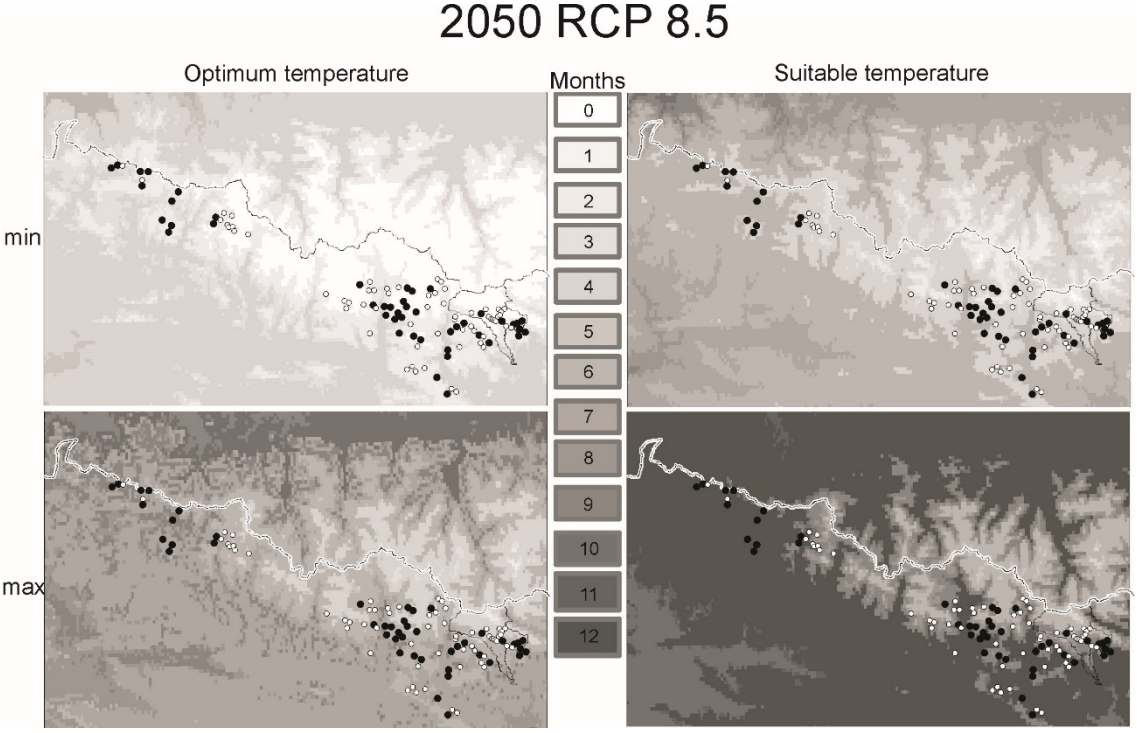


Figure 4. Map representing the total number of months of optimum growth temperature (17-25 ºC) and suitable growth temperature (4-25 ºC, sensu Piotrowski *et al*. 2004) of Bd under 2050 climatic conditions and RCP 8.5 scenario. Map details are the same as in figures 2 and 3.

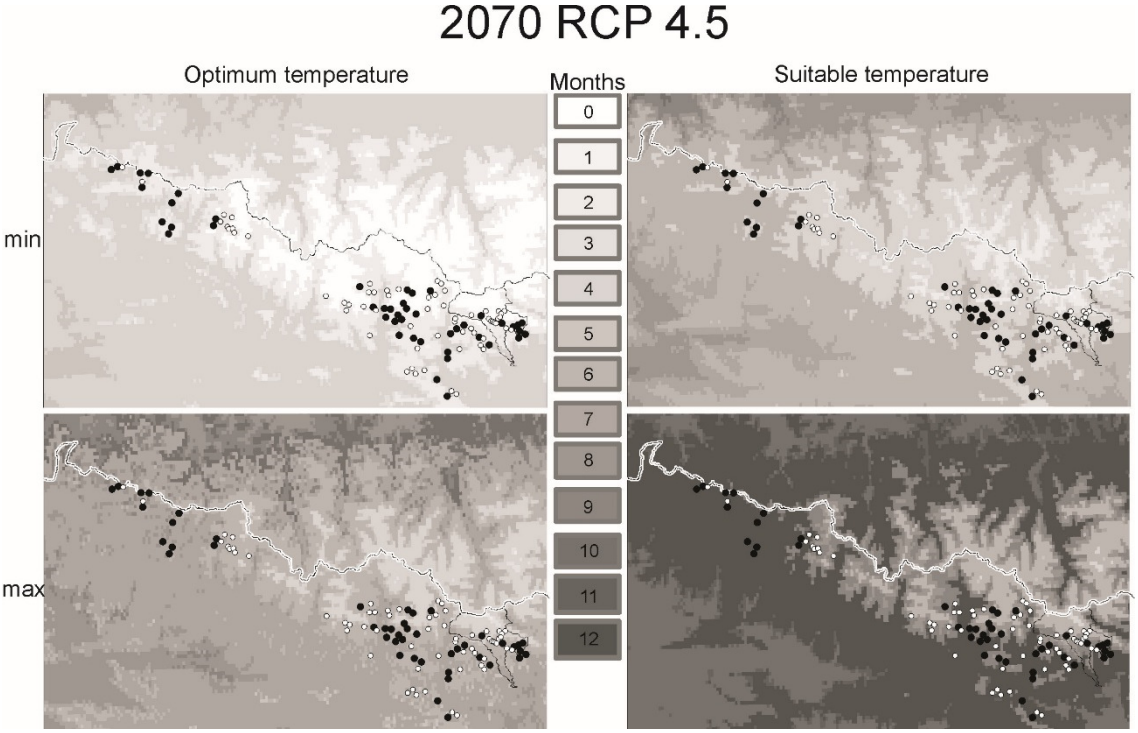


Figure 5. Map representing the total number of months of optimum growth temperature (17-25 ºC) and suitable growth temperature (4-25 ºC, sensu Piotrowski *et al*. 2004) of Bd under 2070 climatic conditions and RCP 4.5 scenario. Map details are the same as figure 2 and 3.

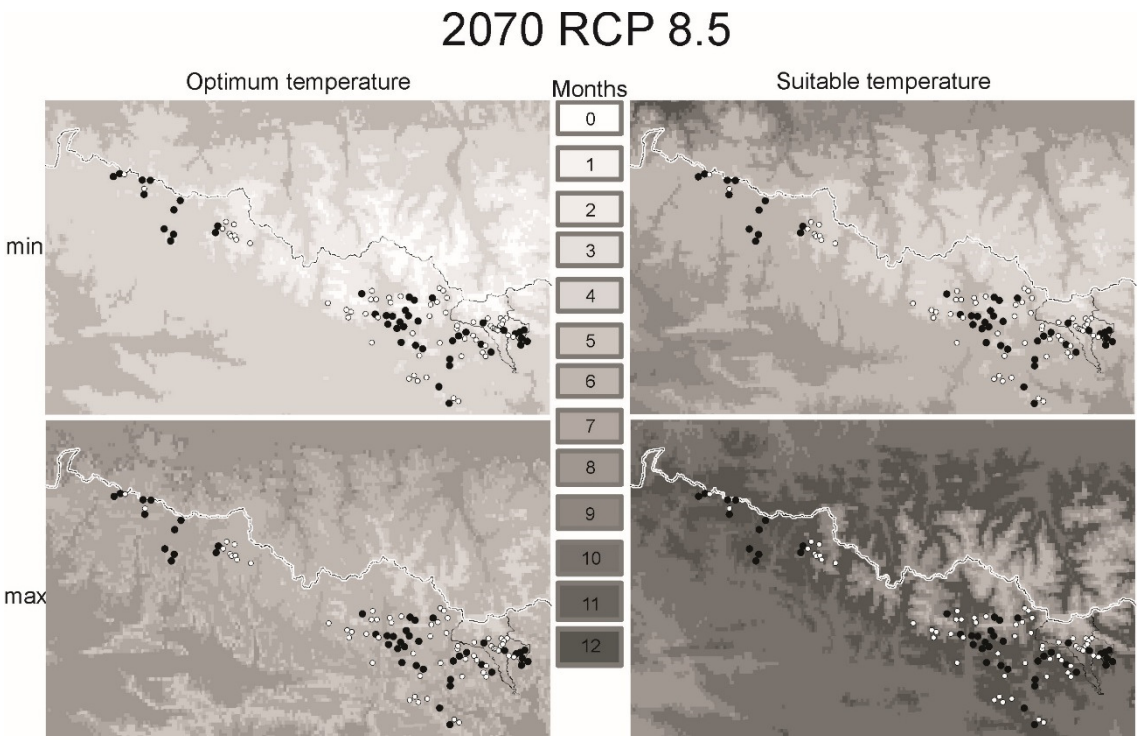


Figure 6. Map representing the total number of months of optimum growth temperature (17-25 ºC) and suitable growth temperature (4-25 ºC, sensu Piotrowski *et al*. 2004) of Bd under 2070 climatic conditions and RCP 8.5 scenario. Map details are the same as in figures 2 and 3.

The relationships between Bd's optimum and suitable growth temperatures and elevation are shown in figure 7, both under aggregated monthly minimum and maximum temperature climate. Quadratic regressions better explain this relationship. Under current minimum monthly temperature climate there is a significant negative relationship (Fig. 7a, $p<0.001$, $R^2=0.841$) between Bd optimum growth and elevation, reaching zero value at ca. 1800 m a.s.l.; while under future climate models the slopes increase reaching zero the optimum at ca. 2400 m a.s.l., save the 2070 RCP 8.5 scenario in which the optimum reaches zero ca. at 2900 m a.s.l. If we consider the monthly maximum temperatures for optimum growth, the relationship is also negative and significant (Fig. 7c, $p<0.001$, $R^2=0.773$), but under current climate the optimum crosses zero at ca. 2800 m a.s.l, basically including every water body suitable for amphibians in the Pyrenees. If we look at suitable temperatures for chytrid growth, both under current minimum (Fig. 7b, $p<0.001$, $R^2=0.889$) and maximum (Fig. 7d, $p<0.001$, $R^2=0.89$) monthly temperature climate, the relationships are negative with elevation in all cases, reaching zero at ca. 3000 m, hence including all Pyrenean water bodies. Future climate scenarios also show this relationship when increasing elevation. In the insets are shown the frequency distributions of Bd+ records (grey) and Bd- records (white) for all Pyrenean amphibians,

which are in agreement with the models. Bd+ samples concentrate at elevations lower than 2000 m a.s.l., but showing a wide overlap whith Bd- samples (see also Fig. 8). A one way ANOVA for all Pyrenean amphibian samples does not show significant differences in the means of the altitudinal distributions of Bd+ and Bd- ($p= 0.0247$). However, when considering only our *Rana pyrenaica* samples, there are significant differences between Bd+ and Bd- altitudinal distributions ($p= 0.00184$), with populations at lower and mid elevations being more prone to infection.

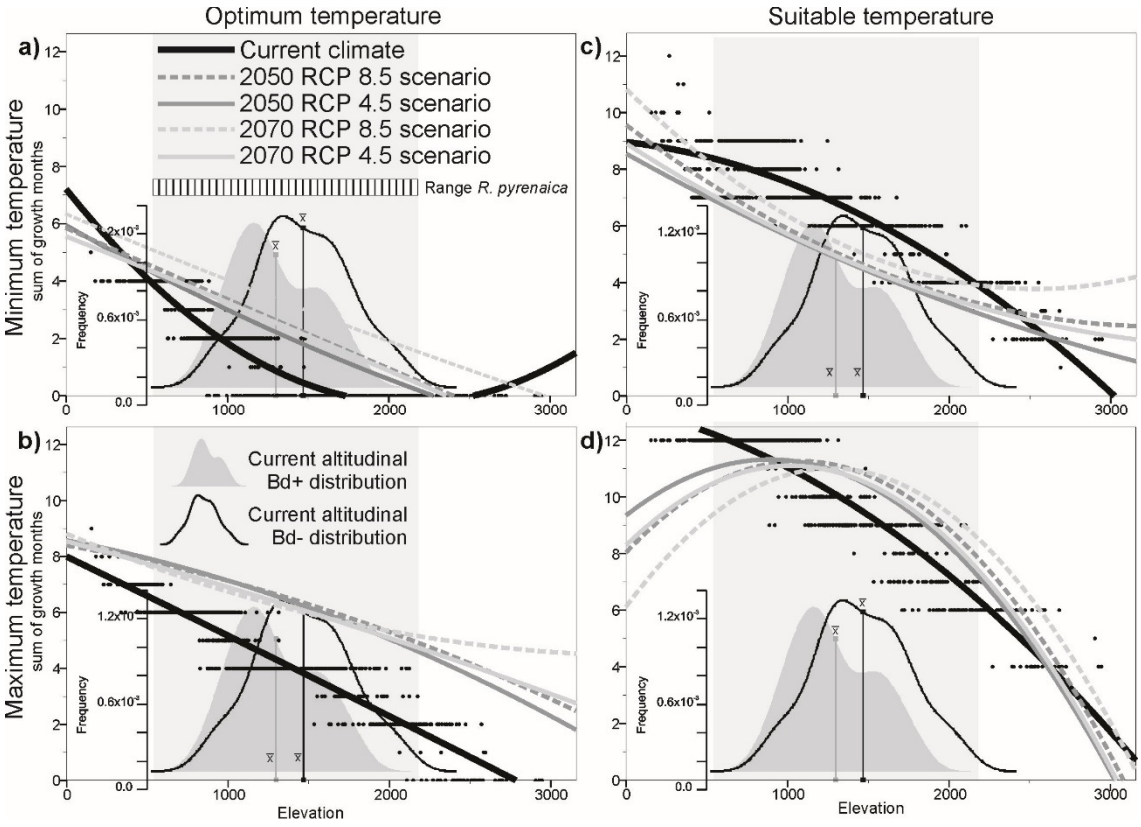


Figure 7. Plots of quadratic regressions between the number of months of Bd growth temperature under minimum (a, b) and maximum (c, d) monthly climatic data for current and future climatic scenarios. Analyses were done for the optimum growing temperature (a, c) and suitable Bd growing temperature (b, d). The inset shows the altitudinal distribution of Bd+ and Bd- analyzed samples for all Pyrenean amphibians. The altitudinal distribution range of *R. pyrenaica* is shaded in grey.

We gathered available data on amphibian samples analysed for chytrid presence from the Pyrenees, mainly from the Global Bd-Mapping Project (2018), which were assembled to our dataset. Those included eight species of amphibians and a total of 1461 samples (38% Bd positive) from 145 localities (33.8% where Bd was detected), distributed from 100 m.a.s.l. to 2586 m.a.s.l. The species included were *Alytes*

obstetricans, Rana temporaria, Triturus helveticus, Pleurodeles waltl, Pelophylax perezi, Bufo spinosus, Salamandra salamandra and *Calotriton asper*. For *Rana pyrenaica* there were only 9 samples available from 3 localities in these Atlas project. In figure 8 is represented the elevation pattern of chytrid positive and negative samples for all Pyrenean amphibians in the elevation gradient. Although there is an overlap between Bd+ and Bd- analyzed samples, a clear drop in the frequency of Bd+ samples is evident at 2000 m.a.s.l., which is also in agreement with the regression models of chytrid growth versus elevation.

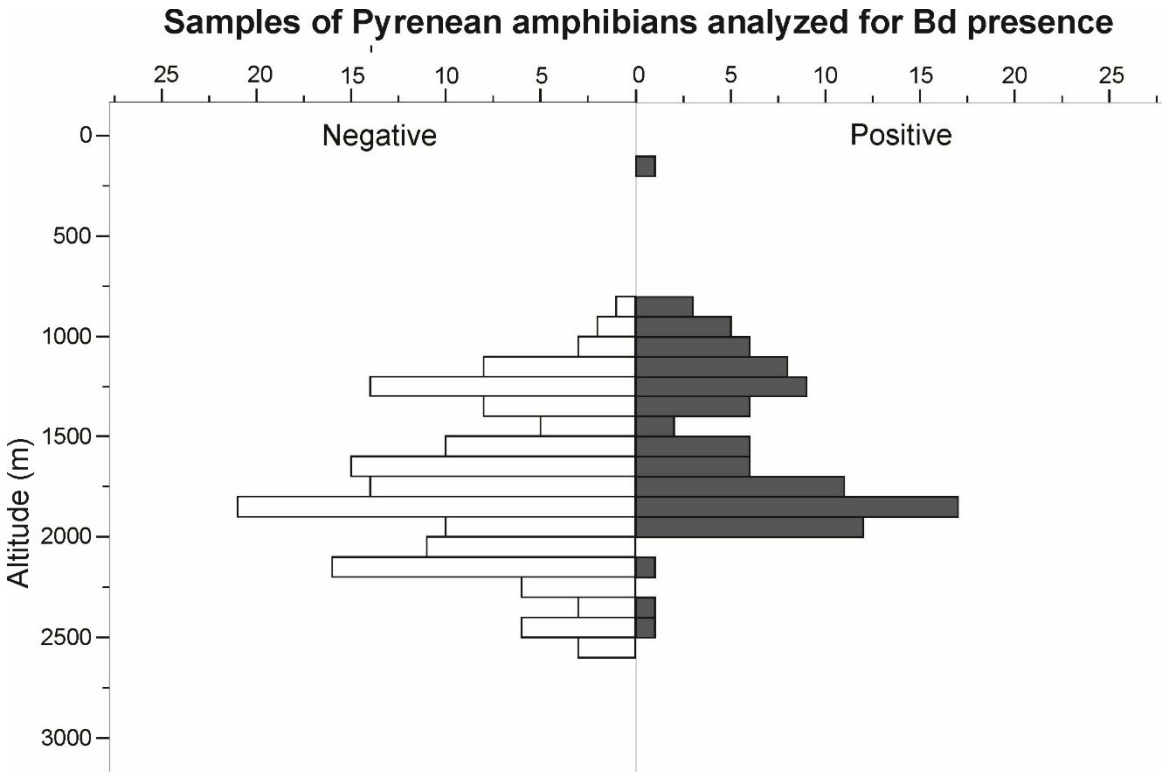


Figure 8. Bar plots of Bd positive and Bd negative analysed samples for all amphibian species in the Pyrenees. Top scale is in percentages. Bd positive samples are rare above 2000 m a.s.l.

DISCUSSION

Our results demonstrate that *Batrachochytrium dendrobatidis* is widespread across the whole distribution range of *R. pyrenaica*. The presence of Bd in the Pyrenees has been detected not long time ago, and genetic studies suggest a single recent introduction event and subsequent spread of a unique haplotype in the Pyrenees (Walker *et al*. 2010). This scenario implies that Pyrenean amphibians had been in contact with Bd for just a short period of time, hence the consequences of Bd's expansion in this area and its amphibian

fauna are not yet fully explored. Die-offs caused by Bd in the Pyrenees have been reported for *Rana temporaria* or *Alytes obstetricans* (Walker *et al*. 2010, Clare *et al*. 2016), as well as for *Lissotriton helveticus* (pers. obs.). However, there are no extensive data for the two key amphibian Pyrenean endemisms, the Pyrenean newt, *Calotriton asper*, and frog *Rana pyrenaica*.

Our data show that Bd is already present in most of the localities where the Pyrenean frog occurs, with higher presence at mid and high elevations, being detected in their skins. The elevation range of Bd presence in the Pyrenees spans across the altitudinal gradient, from lowlands to high elevations, but its presence in amphibian skins above 2000 m a.s.l. is anecdotal. This observation fits well with the available growth models (Piotrowski *et al*. 2004) that applied to the landscape suggest an upper elevation limit close to 2000 m a.s.l. under current climate. Climate models for the Pyrenees suggest a continuous warming in the next decades (López-Moreno *et al.* 2008) and a reduction of precipitation. Those models as well as our forecasted growth models, suggest an increase in the number of suitable and optimal growth months for the chytrid, and this is in part a consequence of earlier springs and later winters. This situation is not good for amphibians, as it has been shown that earlier springs increment frog mortality infected by Bd in these mountains (Clare *et al.* 2016). In high elevations, *Alytes obstetricans* spends one to two years as tadpole before leaving the water, acting as a reservoir of the chytrid (Clare *et al.* 2016), hence our growth models not only imply a Bd distribution uplift but also an increase in the probability of infection for the whole community at higher elevations. Other species, like crayfishes have been confirmed as host species for Bd (McMahon *et al.* 2013), and in the Pyrenees conservation of autochthonous crayfishes its being promoted in headwater streams.

It is possible that in the last decade, with a continuous warming and very hot years, Bd was already moving uplift towards higher elevations. Forecasted models to 2050 and 2070 suggest in all analysed scenarios that the optimal conditions for chytrid growth will move to higher elevations. We hypothesize that this is already happening and that the chytrid presence will be more frequent at alpine lakes in the next years, affecting alpine amphibian communities to an unknown extent. So far, massive die offs have not been reported for *R. pyrenaica*. However, other species with which it coexists, like *Alytes obstetricans* or eventually *R. temporaria*, are already experiencing population declines (Walker *et al*. 2010, Clare *et al*. 2016). If there is any die off in a lotic species like this, it

will be hard to detect, as dead corpses will likely be washed away by the fast spring currents. In fact, population declines did not parallel the finding of corpses in closely related stream species like *Rana iberica* (Bosch *et al*. 2018). It is pure speculation to relate the small population sizes found across the whole distribution range of *R. pyrenaica* with a potential Bd cause, however considering that the whole range of this frog is affected by Bd and that the population sizes are so small (Chapter 1), the incidence of Bd and its evolution needs to be closely monitored. Small population sizes, patchy distributions that difficult gene flow and low genetic diversity (see Chapters 4 and 5) are key ingredients for population declines and extinctions by themselves. If we add a disease that is causing massive mortalities across the planet, leading species to extinction and global warming that will make the growth conditions better for the fungus, the cocktail put this species in danger of population declines or even extinction in the next few years. It has been shown that some populations of anurans can survive the chytrid infection while others collapse, and these intraspecific differences can happen at different developmental stages, being sometimes related to skin microbiota that may protect frogs or tadpoles (Blaustein *et al.* 2005, Briggs *et al.* 2005, Woodhams *et al.* 2007). We do not have data on *R. pyrenaica* to expect any kind of resistence to Bd. We urge the authorities to not take the chance and implement a monitoring scheme to assess the extent of the problem, if there are any die offs, and in the case of a beginning of population collapses consider a captive breeding program. It is remarkable that within the Ordesa and Monte Perdido National park the presence of Bd is widespread, so having the highest protection in Europe there, conservation and management actions should focus on this national park as a first test to try to prevent amphibian declines in the next few years.

Acknowledgements

We are grateful to many people that made this project possible. Several students and collaborators helped in fieldwork in the Pyrenees, including Carlos Zaragoza, Javier Santos, Rubén González, Miguel Vences, Nina Bernard, Guillermo Ponz and Isabel Perandones. Angelica Crottini provided advice on PCR conditions. Fernando Carmena and Ignacio Gómez from SARGA in Aragón, and Iosu Antón in Navarra were extremely helpful in the field, allowing locating many known populations. Ramon Antor Casterllanau provided logistic help to prepare and carry on fieldwork. Manuel Alcántara, David Guzmán and Jose Luis Burrel from the Aragón government showed their total collaboration and help during the whole length of the project. Forest rangers helped us during the fieldwork, and the public company Sodemasa, now SARGA, provided logistic support in Ordesa in 2011. MPF was financed with a grant by CNPq under Science Without Borders program by Brazil Ministry of Science. We thank the Governments of Aragon and Navarra for collecting permits. This work was funded by a research project of the Zoo de Barcelona (Ayuntamiento de Barcelona) as well as by a research project from the Parques Nacionales OPAN – MMARM to DRV.

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Chapter 3:

The complete mitochondrial genome of the Endangered European brown frog *Rana pyrenaica* through RNAseq.

This chapter reproduce entirely the manuscript:

Peso-Fernandez. M, Ponti. R., Pons. G, Gonzalez. R Arcones, A. and Vieites D.R. (2016) The complete mitochondrial genome of the Endangered European brown frog *Rana pyrenaica* through RNAseq, Mitochondrial DNA Part B, 1:1, 394-396, DOI:10.1080/23802359.2016.1174087

The complete mitochondrial genome of the Endangered European brown frog *Rana pyrenaica* through RNAseq.

Abstract:

We sequenced the complete mitogenome of the Pyrenean frog *Rana pyrenaica*, which was determined from an Illumina Hi-seq RNAseq run. The genome is 17,213 bp in size, including 13 protein-coding genes, 21 transfer RNAs, 2 ribosomal RNAs and a control region. It shows the typical gene order of previously available frog mitogenomes, although it lacks the tRNAPhe. This is the first complete mitogenome described for a Western Palearctic brown frog species.

Keywords: Amphibia, mitogenome, RNAseq, Pyrenees, *Rana pyrenaica*.

INTRODUCTION

The Pyrenean frog (*Rana pyrenaica*) is an endangered narrowly-distributed European endemism (Sillero *et al*. 2014). Despite that its sister species *Rana temporaria* shows a considerable degree of mtDNA genetic variation across its range, including several divergent lineages in the Pyrenees (Vences *et al*. 2013), a preliminary mtDNA study across the range of *R. pyrenaica* showed a single mutation difference using three mitochondrial genes (Carranza & Arribas 2008). This potential lack of genetic variation has important conservation implications for this endangered species.

RNAseq is becoming a common approach for gathering transcriptome data using Next Generation Sequencing, being complete mitogenomes a potential output. We explored the use of RNAseq to describe the complete mitogenome of *R. pyrenaica*, which will benefit future phylogeographic, population genetic and conservation studies.

MATERIALS AND METHODS

An adult *R. pyrenaica* (Museo Nacional de Ciencias Naturales, Madrid, collection number MNCN 46671, Fieldnumber DRV7602) was collected in Uztárroz (42º35´38´´N, 0º59´27´´W), NE Spain. We extracted RNA from several tissues, which were quantified

with Qubit HS and normalized. A RNAseq library was prepared using the NEBNext Ultra RNA kit for Illumina. Quantification and size estimation were performed on a Bioanalyzer 2100 High Sensitivity DNA chip, and sequenced on half lane on a Illumina HiSeq (2x100 bp pair-end reads). After quality control and trimming with Trimmomatic (v 0.32.2) (Bolger *et al*. 2014), assembly was done with Trinity (v 2.0.6) (Haas *et al*. 2013). Trinity recovered the mitogenome except part of the control region; hence we used this assembly as input for MITObim (Hahn *et al*. 2013) to complete the reconstruction. Genome annotation was done through nucleotide sequence alignments with other ranids. The genome is deposited in GenBank (KU720300).

RESULTS

The complete mitogenome of *R. pyrenaica* is 17,213 bp in length, including 13 protein-coding genes, 2 rRNAs, 21 tRNAs and a control region. Gene order, lengths and codon compositions are shown in Table 1. The overall base composition of the heavy strand is 27.7% for A, 28.3% for T, 14.9% for G and 29.1% for C, with an A+T bias of 59.9%, similar to other ranid species (eg. Hofman *et al*. 2014, Li *et al*. 2014 a,b, Ni *et al*. 2015). The genome shows a similar gene organization as other ranids (eg. Kurabayashi *et al*. 2010, Xia *et al*. 2014), but it is the only anuran known so far lacking the $tRNA^{Phe}$.

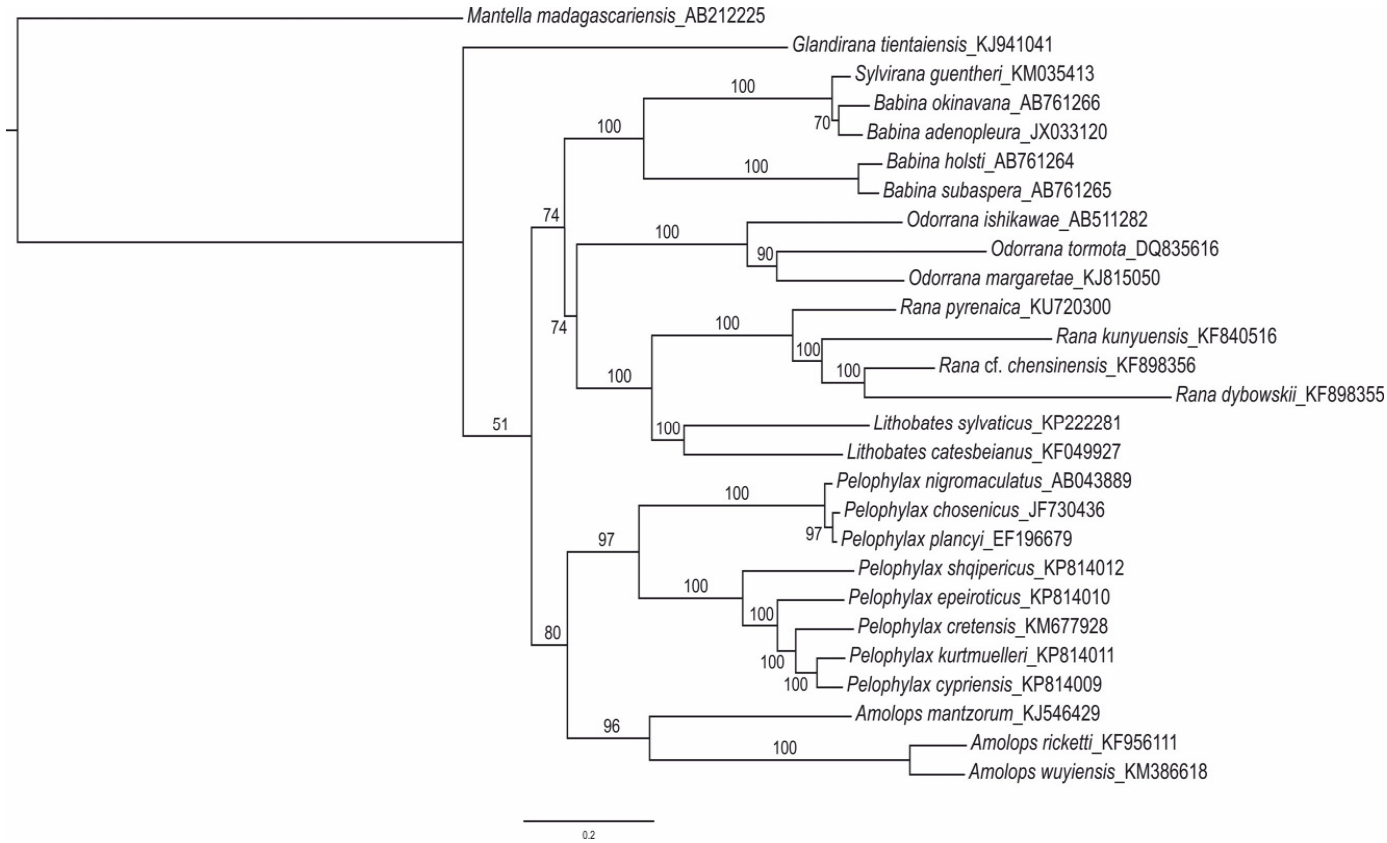

Figure 1. Phylogenetic reconstruction of the relationships between ranid frogs, based on available complete mitochondrial genomes except control regions. Maximum-likelihood analyses using a partitioned dataset by codon and gene were performed in RaxML, running for 1000 generations. ML support values are provided above branches. Genbank accession numbers are provided after the species names.

Table 1. Location of features in the mtDNA of *R. pyrenaica*

Gene/region	Start position	Stop position	Length (bp)	Spacer (+) overlap (-)	Start codon	Stop codon	Strand
$tRNA^{Leu}$	1	72	72	2			H
$tRNA^{Thr}$	75	144	70	0			H
$tRNA^{Pro}$	145	176	32	3			H
12S rRNA	180	1109	930	0			H
$tRNA^{Val}$	1109	1177	69	-1			H
16S rRNA	1178	2758	1581	1			H
$tRNA^{Leu}$ (UUR)	2760	2832	73	0			H
NAD1	2833	3793	961	0	ATT	T--	H
$tRNA^{Ile}$	3794	3863	71	0			H
$tRNA^{Gln}$	3864	3934	71	-1			H
$tRNA^{Met}$	3934	4002	69	-1			H
NAD2	4003	5035	1033	0	ATT	T--	H
$tRNA^{Trp}$	5036	5105	70	0			H
$tRNA^{Ala}$	5106	5175	70	0			L
$tRNA^{Asn}$	5176	5248	73	0			L
OL	5249	5278	30	0			L
$tRNA^{Cys}$	5276	5340	65	-3			L
$tRNA^{Tyr}$	5341	5407	67	3			L
COI	5411	6961	1551	0	ATA	AGG	H
$tRNA^{Ser}$ (UCN)	6953	7023	71	-9			L
$tRNA^{Asp}$	7025	7093	69	0			H
COII	7094	7781	688	0	ATG	T--	H
$tRNA^{Lys}$	7782	7850	69	1			H
ATP8	7852	8013	162	0	ATG	TAA	H
ATP6	8007	8688	682	-7	ATG	T--	H
COIII	8689	9472	784	0	ATG	T--	H
$tRNA^{Gly}$	9473	9540	68	0			H
ND3	9541	9880	340	0	ATG	T--	H
$tRNA^{Arg}$	9881	9949	69	0			H
ND4L	9950	10234	285	0	ATG	TAA	H
ND4	10228	11587	1360	-7	ATG	T--	H
$tRNA^{His}$	11588	11655	68	0			H
$tRNA^{Ser}$ (AGY)	11656	11722	67	30			H
ND5	11753	13544	1792	105	ATG	T--	H
ND6	13650	14150	501	0	ATG	AGA	L
$tRNA^{Glu}$	14151	14218	68	3			L
Cyt b	14222	15364	1143	0	ATG	TAA*	H
Control region	15365	17211	1846	0			H

*Stop codon completed with the addition of an A

CONCLUSION

The number of available complete mitochondrial genomes is increasing but surprisingly there are very few available for European frogs. A maximum likelihood phylogenetic analysis of ranid frogs based on available complete mitogenomes (Fig. 1) recovers the European *R. pyrenaica* as the sister taxon to the clade of Asian brown frogs.

RNAseq has proven to be a very fast and useful approach to gather complete mitogenomes. Although using common assembly tools like Trinity was not enough to gather the full genome, the combination with MITOBim has performed well to fill the assembly gaps. This approach can be routinely used to gather mitogenomes as a byproduct of transcriptome sequencing.

Acknowledgments

This work was supported by the Spanish OAPN- Ministry of Environment under Grant 206/2010; Zoo de Barcelona (Ayuntamiento de Barcelona); and the Spanish Ministry of Economy under Grant CGL2013-40924-P. MP was supported by a CNPQ fellowship. We thank the CETA/CIEMAT for the use of their infrastructures, and the Navarra authorities for collecting permits.

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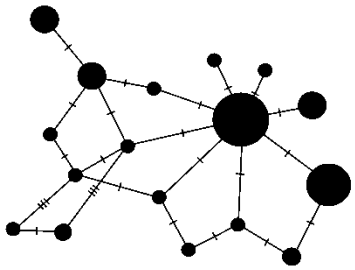

Chapter 4:

Mitogenomic phylogeography of the endangered Pyrenean frog reveals a lack of genetic variation and a recent Holocene expansion

Mitogenomic phylogeography of the endangered Pyrenean frog reveals a lack of genetic variation and a recent Holocene expansion.

Abstract:

The Pyrenean frog, *Rana pyrenaica*, is narrow range endemic species mainly distributed in northern Aragón and part of Navarra (Spain), with few isolated populations in France. It is one of the species of European amphibians with the smallest distribution range. A distribution gap divides its range in two unconnected areas, which lead to the hypothesis that this gap originated during Pleistocene glaciations, and that the two areas of occurrence correspond to postglacial expansions from two putative isolated refugia. Here, we reconstructed the phylogeography of the Pyrenean frog across its full distribution range using nearly complete mitochondrial genomes to test this hypothesis, which should be reflected in the genetic data by showing spatial genetic geographic structure. By sequencing mitochondrial genomes we were able to assess the degree of genetic variation across the full genome in the species. We amplified the most variable regions in a set of individuals covering its full distribution range finding 17 haplotypes with no clear geographic structure, suggesting recent gene flow and population connectivity. The reconstructed demography suggested small effective population sizes and a recent geographic expansion. Hindcasted species distribution models show in fact that during present times and glacial periods the gap persisted in the mountains, separating two main areas, but those were likely connected in the south across the pre-Pyrenean mountain range and the central valley. The small effective and estimated adult population sizes, together with very low genetic diversity in comparison with other species and a patchy distribution range, raise concerns about the capacity of the species to face current and future threats.

Keywords: *Rana pyrenaica*, Pleistocene, Mitochondrial genome, Phylogeography, genetic variation.

INTRODUCTION

The current distribution ranges and genetic architecture of species reflect historical processes that shaped them, mainly since the Pleistocene. In the northern hemisphere, Pleistocene glacial and interglacial periods are known to have reduced species' distribution ranges at high latitudes and altitudes by the expansion of ice sheets and glaciers, which turned into a genetic pattern of lower genetic variation in those areas after being recolonized in interglacial periods, and higher genetic diversity in the so-called refugia were species retreated during cold periods (Taberlet *et al.* 1998; Hewitt 1999, 2000, 2004, Weiss & Ferrand 2007). Being the switch between glacial and interglacial periods an intermittent process, the actual distribution ranges and phylogeographic structure should reflect the pattern originated since the last glacial maximum up to today, both across latitudinal gradients at a continental scale, and altitudinal gradients in mountain ranges (Hewitt 1999, 2000, Schmitt *et al.* 2006, Canestrelli *et al.* 2008, Schmitt 2009). However, the genetic variation and origin of lineages present in refugia can predate Pleistocene times (e.g. Recuero & García-París 2011)

Identifying refugia has received a lot of attention, with the phylogeographic structure of many species being studied in the last decades. One of the predictions of the refugium theory is that refugia showed more stable climatic and ecological conditions that allowed species to persist during cold and interglacial periods, hence in those areas persisted most of the genetic legacy of species and they should have higher genetic diversity than recolonized areas (Schneider *et al.* 1998, Carnaval *et al.* 2009). At the European scale, continental patterns in widely distributed species have shown postglacial recolonizations at higher latitudes reflecting low genetic variation (Hewitt 2000, 2004), with main refugia located in the Mediterranean Peninsulas (Iberian, Italic and Balcanic) (Hewitt 1999, Weiss & Ferrand 2007). Within the European Peninsulas there is also spatial genetic structure which has been proposed as a “refugia within refugia” model (Gómez & Lunt 2007, Abellán & Svenning 2014) linked to long-term climatic stability, which may have arose by allopatric differentiation related to climatic and/or geographic barriers to gene flow and posterior secondary contacts.

At smaller geographical scales, like in mountain ranges, and especially with cold-adapted species, this refugia theory does not necessarily fits well in space. During glaciations, many northern hemisphere species where present far south, with fossil

evidence of bison (*Bison bison*), snowy owls (*Bubo scandicus*) or black grouses (*Tetrao tetrix*) present in the Pyrenees (Arribas 2004). These species now retreated to northern latitudes with no remaining populations in the southern European refugia where the climate is not suitable anymore for them. A similar pattern likely happened with many mountain species that are now present at high elevations but absent in lowlands, while there is fossil evidence of their lowland presence during glaciations (eg. Arribas 2004, López-García *et al.* 2010). Hence, in high mountain ranges with steep elevational gradients like the Alps and the Pyrenees, there likely were not many ecologically and climatically stable areas through time, and species went up and down tracking climatic changes. Interestingly, within this overall pattern, there is increasing evidence of the existence of nunataks, small areas that remained unglaciated in the mountains (Ehlers & Gibbard 2004), that promoted genetic differentiation (Bettin *et al.* 2007, Escobar-García *et al.* 2012, Schönswetter *et al.* 2005, Stehlik *et al.* 2002, Wachter *et al.* 2012, Westergaard *et al.* 2011). This could explain the persistence of divergent and isolated lineages in some amphibian species, including a basal divergent lineage of the European common frog (*Rana temporaria*) in the Pyrenees and Cantabrian mountains (Vences *et al.* 2013).

Species with low dispersal capacities like *Rana temporaria* or *R. pyrenaica* will likely be more affected by climate changes, as their ability to track their preferred climate is lower than more mobile species. This is the case of most amphibians, and their spatial genetic structure is likely to reflect fine scale effects related to past climatic events as well as barriers. In mountain areas like the Pyrenees, the interaction of past climatic changes and the appearance and disappearance of barriers (like glaciers that covered many valleys with ice), may have lead an imprint in species' current phylogeographic structure. Recent works have recovered two main genetic clusters in several species (plants, invertebrates and vertebrates), corresponding to the western and eastern Pyrenees (considering the full mountain range from Cataluña to Navarra), and those are interpreted as the result of two main Pleistocene refugia in the Pyrenees, one in the eastern Pyrenees, and the other in the west-central area (Schmitt *et al*. 2006, Mouret *et al.* 2011, Valbuena-Ureña *et al.* 2013, 2018, Charrier *et al.* 2014, Bidegaray-Batista *et al.* 2016). From these works strong glacial bottlenecks during the Plesitocene were hypothesized, followed by demographic and population expansions during interglacials. *R. pyrenaica* is not distributed in the eastern Pyrenees, so its full range fits within the putative western-central refugium.

The Pyrenean frog, *Rana pyrenaica*, is an endangered species that is endemic to the Pyrenees, being distributed in a relatively small area with many small fragmented and unconnected populations (Chapter 1). Its current range spans 440 m to 2.100 m a.s.l. (Serra-Cobo 2002, Duchateau *et al*. 2012), not being present at higher elevations like its sister taxon *Rana temporaria* that reaches ca. 2500 m a.s.l. in these mountains (Vences *et al.* 2003). Most of its range is below the distribution range of ice sheets during the last glacial maximum (LGM) (Ehlers & Gibbard 2004), with some areas being recolonized during the Holocene warming. Its distribution range is also at the western part of the extent of Pyrenean glaciated areas, and likely was affected by potential barriers like the large glaciers that covered the Gállego or Ara rivers, as being a stream species its habitat had to retreat to lower unglaciated elevations. There is no fossil evidence of Pyrenean frogs at lowlands during glaciations, but there is of *Rana temporaria* and *Calotriton asper* at the Colomera cave (670 m a.s.l.) in the pre-Pyrenees, as well as other cold adapted mammals and reptiles species (López-García *et al.* 2010), which probes a glacial retreat of now mountain species to lower elevations. The Pyrenean frog shows an actual distribution gap that splits its range in two (Chapter 1). If this gap was a consequence of an allopatric split caused by glaciations, we can hypothesize that the species may have persisted in two microrefugia and there should be some phylogeographic structure related to this. However, the only genetic data available for few individuals and markers show a very low genetic variation in the species from both areas (Carranza & Arribas 2008). Most phylogeographic studies have focused on mitochondrial loci that have universal primers that allow a PCR amplification across amphibian species (Vences *et al.* 2005), but those loci may not always reflect the real degree of genetic variation within species. In mitochondrial genomes there are conserved and variable regions, hence, considering the technological advances in next generation sequencing, it is feasible to sequence full mitochondrial genomes in order to really assess the mitogenomic genetic variation within species (Peso *et al.* 2016). This is of outmost relevance in species with likely low genetic variation like small range endemics.

Here, we first wanted to reconstruct the phylogeographic pattern of *Rana pyrenaica*, covering its full range and all the areas that were potentially affected by barriers in the past and the present; by doing so we aim to test the two refugia hypothesis by integrating molecular analyses with hincasted species distribution modeling. And second, we wanted to assess the degree of genetic variation and diversity within the

species and across its full mitochondrial genome, as this is of extreme relevance for conservation genetic purposes in this endangered species.

MATERIALS AND METHODS

In order to assess the degree of mitochondrial genomic variation across the full range of *Rana pyrenaica*, we sequenced several complete mitochondrial genomes across its distribution range to identify regions that harbor genetic variation. Briefly, we followed this sequential procedure: first, we amplified one complete mitochondrial genome from the eastern range of the species using transcriptome RNAseq next generation sequencing (Peso *et al.* 2016), and another from the western range using the same approach (transcriptome data will be published elsewhere). Those two genomes served as a reference to develop a set of primers that were used to amplify overlapping fragments across the mitochondrial genome of brown frogs. The sequences, position in the genome and primer characteristics are shown in Table 1. We used these primers to amplify 30 more mitochondrial genomes across the species range which were used to target genomic informative regions, and then we selected primer pairs to amplify those fragments in 20 more individuals covering the full range of the species. The total number of samples for the whole phylogeographic analysis is 52, including 32 mitochondrial genomes and these 20 partial mitochondrial sequences.

Table 1. Novel primers developed and used to amplify the mitochondrial genomes of *Rana pyrenaica*. The position in the genome, primer sequence, GC content and melting temperature are shown, as well as the sense (F=forward, R=reverse).

Position	Primer	Primer name	Sequence	GC	Tm	Direction
850	CR2F	RANAMTGENOM_CR2_DRV_F	5'- GGT TAC TGA CGG ATG TGA ATC -3'	47.6	53.1	F
920	CR1R	RANAMTGENOM_CR1_DRV_R	5'- GGT AYT TAA CGC ATA GGA GGG -3'	50	53.7	R
3701	128	RANAMTGENOM_128_DRV_F	5'- GGT TTG GTC CTG GCC TTA TTA TCA -3'	45.8	56.9	F
3820	CR2R	RANAMTGENOM_CR2_DRV_R	5'- AGG GCG TTC TCA CGG GTG TGC -3'	66.7	64.7	R
3823	1	RANAMTGENOM_1_DRV_R	5'- GTG AGA CTA GGT GTTG TGG GCA -3'	57.1	59.2	R
4514	6	RANAMTGENOM_6_DRV_F	5'- CAC ACC GCC CGT CAC CCT -3'	72.2	63.3	F
4579	8	RANAMTGENOM_8_DRV_R	5'- CAC TTA CCA TGT TAC GAC TTG CCT -3'	45.8	57.1	R
4834	6B	RANAMTGENOM_6b_DRV_R	5'- GCC TGT ACT AAG ATG TTA AAA -3'	33.3	44.7	R
5207	145	RANAMTGENOM_145_DRV_F	5'- TAA AGT RGG CCT AAA AGC AGC CAC -3'	47.9	58.5	F
5527	18	RANAMTGENOM_18_DRV_R	5'- TGG TAA ACA GGC GAG GCT AAA ATT -3'	41.7	57	R
6005	23	RANAMTGENOM_23_DRV_F	5'- CAA GAG CCC ATA TCG ACA AGT AGG -3'	50	57	F
6092	24	RANAMTGENOM_24_DRV_R	5'- CAC GTA GGG TTT TAA TCG TTG AAC -3'	41.7	54.3	R
6350	23B	RANAMTGENOM_23b_DRV_R	5'- AGC TCT GCC ATA CTA ACT GCC -3'	52.4	56.7	R
6802	A	RANAMTGENOM_A_DRV_F	5'- GCC TTA ATT GGR GCC CTC CGA -3'	59.5	60.7	F
6991	154	RANAMTGENOM_154_DRV_R	5'- TGG GGC TCG RTT GGT TTC GGC -3'	64.3	63.9	R
7408	30	RANAMTGENOM_30_DRV_F	5'- GGG AGG CYA ATA GGG GTT CAA -3'	54.8	57.8	F
7554	35	RANAMTGENOM_35_DRV_R	5'- AAG GAA GGA TTT TAA CCR ACA TTG -3'	35.4	52.8	R
8352	162	RANAMTGENOM_162_DRV_F	5'- CTC ACA GGA TTT GCC CCC AAA -3'	52.4	57.9	F
8760	165	RANAMTGENOM_165_DRV_R	5'- GGA TCG AGG CCC GTC ATT CTA -3'	57.1	58.6	R
8964	168	RANAMTGENOM_168_DRV_F	5'- CAA TCC GCC GCC TGC TCG -3'	72.2	62.4	F
9044	169	RANAMTGENOM_169_DRV_R	5'- CGG CYC AGG CCC CAA AGA -3'	69.4	62.2	R
9615	49	RANAMTGENOM_49_DRV_F	5'- CTC CTC ACA GAC CGA AAT CTA -3'	47.6	53.5	F
9843	52	RANAMTGENOM_52_DRV_R	5'- GTG GTG GGC TCA AAC AAT GAA GCC -3'	54.2	61	R
10431	176	RANAMTGENOM_176_DRV_F	5'- CGT CTG TTC CCC GGG GCA -3'	72.2	62.2	F
10682	64	RANAMTGENOM_64_DRV_R	5'- CCG AGT TGG GTR GGG TGT GCC -3'	69	64	R
11149	176B	RANAMTGENOM_176b_DRV_R	5'- ACG TCT TCA GCA GTG ATA AGG -3'	47.6	54.5	R
11160	69	RANAMTGENOM_69_DRV_F	5'- CAC TCC TGA GCT GTC CCC GCC -3'	71.4	64.7	F
11266	71	RANAMTGENOM_71_DRV_R	5'- GCC CCG CAG ATT TCA GAA CAT TG -3'	52.2	59	R
11786	78	RANAMTGENOM_78_DRV_F	5'- CCA GCC CAC AAA TGA GCC TTC -3'	57.1	58.9	F
11962	193	RANAMTGENOM_193_DRV_R	5'- GGG AGA AAA TGT GCT AGR GAG TGG -3'	52.1	58.3	R
12499	198	RANAMTGENOM_198_DRV_F	5'- GTT CAA AAA GGC CTT CGT TAC GGC -3'	50	59.1	F
12920	200	RANAMTGENOM_200_DRV_R	5'- GTC GGA GGA GRC AGG CAA TAA -3'	54.8	57.9	R
13328	100	RANAMTGENOM_100_DRV_F	5'- AAT CGC AYT ACT TCT CCC AAC CCC -3'	52.1	60	F
13526	101	RANAMTGENOM_101_DRV_R	5'- GGT CAC RGG GGT ATT ATG GGT -3'	54.8	57.5	R
13688	B	RANAMTGENOM_B_DRV_F	5'- GCC ACA GCC CGC TCC CAC -3'	77.8	65	F
14204	204	RANAMTGENOM_204_DRV_R	5'- GGC GCC TCA GCG GGT AAT AAC AAT -3'	54.2	61.7	R
14536	C	RANAMTGENOM_C_DRV_F	5'- GGC ATC CTA GCC ACY GCA CTC -3'	64.3	61.5	F
14711	102	RANAMTGENOM_102_DRV_R	5'- TCA TGG TGA AGA GAT TAG GAC GGC -3'	50	58.4	R
15267	205	RANAMTGENOM_205_DRV_F	5'- GGG AAC TGC TAA TTA CCC ACG -3'	52.4	55.6	F
15736	103	RANAMTGENOM_103_DRV_R	5'- GAG CAG ACA AGA AKA ATT ATA GCG AGA -3'	38.9	55.4	R
16113	105	RANAMTGENOM_105_DRV_F	5'- AGT AGC AGG CAT YTT TCT TCT CAT -3'	39.6	55.4	F
16235	209	RANAMTGENOM_209_DRV_R	5'- TGG CAG CGR AGA ATG TGG ATA TGG -3'	52.1	60.3	R
16572	300	RANAMTGENOM_300_DRV_F	5'- AGC AGC AAG CAC ATC CTA TGT -3'	47.6	56.9	F
16812	217	RANAMTGENOM_217_DRV_F	5'- ACA TAA AAC TAA CCG CCT TAA -3'	33.3	49.6	F
16865	301	RANAMTGENOM_301_DRV_R	5'- TCG TAA CAA TTA GGG CGG TTA -3'	42.9	53.8	R
17288	113	RANAMTGENOM_113_DRV_R	5'- GTT TTT GAA GTG GTG CGA GGG C -3'	54.5	59.5	R
17543	115	RANAMTGENOM_115_DRV_F	5'- CTC CGC CAC CAG AGC AGC ACA -3'	66.7	64.6	F
17706	120	RANAMTGENOM_120_DRV_R	5'- GGA CTT TTG GCG GTG GCT TCA -3'	57.1	60.7	R
18352	230	RANAMTGENOM_230_DRV_F	5'- CTC ACC CGA TTC TTY ACA TTC CAC -3'	47.9	56.7	F
18656	125	RANAMTGENOM_125_DRV_R	5'- AGC GGA GRA TGG CGT AGG CGA -3'	64.3	64.4	R
18729	233	RANAMTGENOM_233_DRV_F	5'- TCT CTT CCT AAT ACC YCT CAC CCA -3'	47.9	57.5	F
18911	127	RANAMTGENOM_127_DRV_R	5'- AAG TGG GAA CRA GGA GGA CAA AGA -3'	47.1	59.1	R
18993	CR1F	RANAMTGENOM_CR1_DRV_F	5'- CAA CCG GTA GAA GAC CCA TTT -3'	47.6	54.8	F

Tissue samples of *Rana pyrenaica* were taken in the field between 2010 and 2014 and preserved in 99% ethanol. Table 2 and Figure 1 show the list of localities and their geographic spread across the range of the species. DNA was extracted using standard salt protocols (e.g. Vieites *et al.* 2006) and different overlapping fragments covering most of the mitochondrial genome were amplified for 30 individuals using different combinations of forward and reverse primers from table 1 following standard protocols. PCRs were performed in 25 μL reactions using ca. 50 ng genomic DNA, 10 pmol of each primer, 15 nmol of each dNTP, 50 nmol additional $MgCl_2$ and the Taq PCR buffer (10 mm Tris-

HCl, pH 8.3, 50 mm KCl, 1.1 mm $MgCl_2$ and 0.01% gelatine) and 1 U of standard Taq DNA polymerase. PCR conditions follow Vieites *et al.,* (2006): an initial denaturation step at 94°C for 90s; 35 cycles at 94°C for 30s, annealing temperature two degrees below Tm of each primer (see table 1) for 45s, extension at 72°C for 60 s; final extension of 10 min at 72°C. PCR products were purified using spin columns in a robot prior to cycle sequencing. A 10 μL sequencing reaction included 1–2 μL of template, 1 μL of sequencing buffer, 2 μL of 2 pmol primer, 1.8 μL of ABI sequence mix (BigDye Terminator version 3.1 Sequencing Standard, Applied Biosystems) and 3.2–4.2 μL of water. The sequence reaction was 33 cycles of 10 s at 96°C, 10s at 50°C and 4 min at 60°C. These were subsequently resolved on a 3100 ABI automated sequencer. For this subset of individuals we amplified 15135 basepairs (bp) covering most of the mitochondrial genome. From these sequences we identified which regions across the full genome showed some variation and we amplified them in 20 more individuals. The final dataset for phylogeographic analyses consisted in 2396 bp including most of the genetic variation found. This included 888 basepairs from the 12S to 16S rRNA, 219 bp from the 16S rRNA to the NADH dehydrogenase subunit 1, 651 bp of the Cytochrome c oxidase subunit I, 411 bp of the NADH dehydrogenase subunit 5, and 227 bp from the NADH dehydrogenase subunit 5 to the NADH dehydrogenase subunit 6. Sequences were aligned by eye. Newly determined sequences will be submitted to Genbank (accession numbers #####-##### to be added upon manuscript acceptance).

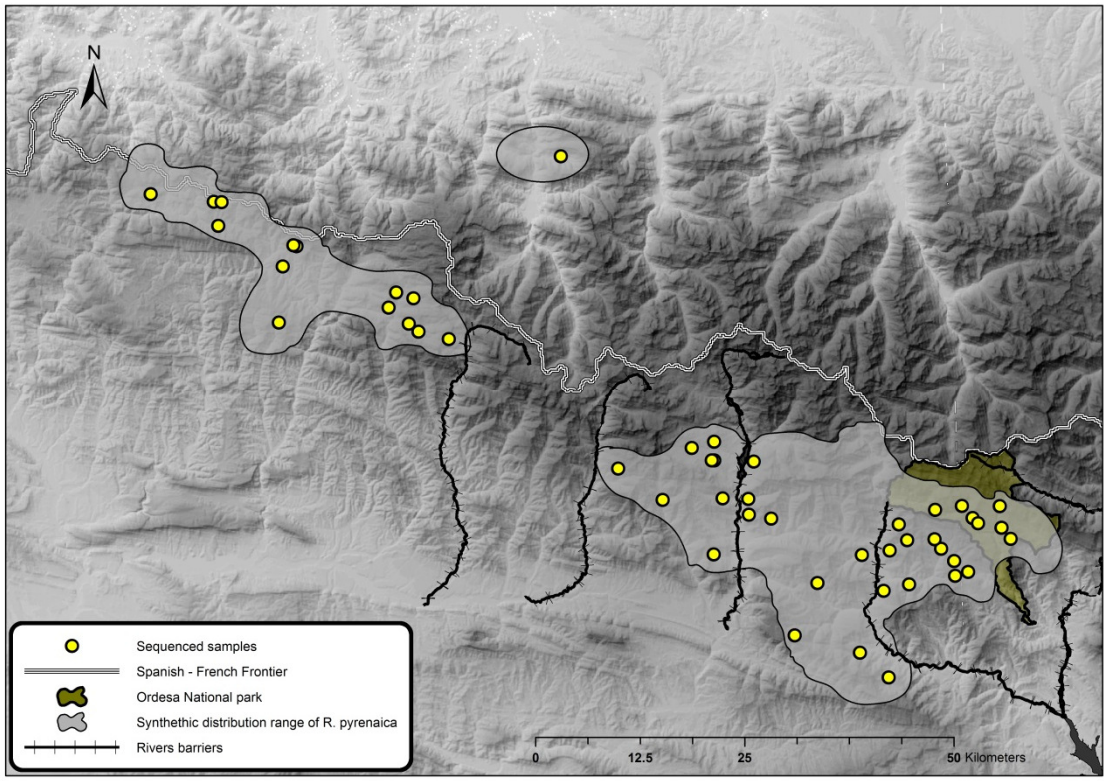

Figure 1. Distribution map of the Pyrenean frog showing the sampled localities for mitochondrial phylogeographic analyses (yellow dots).

We used PartitionFinder v. 1.0.1 (Lanfear *et al.* 2012) to select the best-fit model of nucleotide substitution considering both partition by locus, codon or concatenation of the full dataset. Because of the overall observed low genetic variation in the alignments, a concatenated dataset including all loci without partitioning was favored, and an HKY model was selected. A phylogenetic analysis was performed using Mrbayes 3.1 (Ronquist & Huelsenbeck 2003) without an outgroup. Analyses consisted of four Markov chains that were run for 20 million generations, sampled every 100 generations, with a random starting tree and default priors. The burn-in was empirically estimated by plotting –ln L against the generation number, and the trees corresponding to the first 5 million generations discarded. A consensus phylogram was computed after discarding trees reconstructed during the default burn-in period, showing no structure (a "comb", not shown).

Table 2. Fieldnumbers and locality information of sequenced tissue samples. The different areas considered are explained in the text..

Sample	Locality	Latitude	Longitude	Area
2012/458	Altos Ordesa	42.629942	0.019567	Añisclo-Escuaín
2012/474	Altos Ordesa	42.624038	0.027156	Añisclo-Escuaín
2012/934	Rio Bellós	42.64153	0.059703	Añisclo-Escuaín
2012/750	Barranco de San Vicenda	42.618325	0.061162	Añisclo-Escuaín
2012/751	Barranco de San Vicenda	42.618325	0.061162	Añisclo-Escuaín
2012/812	Barranco Mallo Sasé	42.606182	0.073771	Añisclo-Escuaín
2012/824	Ordesa	42.643361	0.004639	Ordesa
2012/849	Ordesa	42.640007	-0.03466	Ordesa
2010/481	Fuen Obispo	42.56867	-0.00891	Ara East
2011/862	Rio Aso	42.58447	-0.00951	Ara East
2011/865	Barranco Biandico Aso	42.572207	0.010119	Ara East
2010/510	As Gloces	42.59803	-0.02803	Ara East
2010/466	Borrué	42.60863	-0.03706	Ara East
2010/595	Pista Rosada	42.56117	-0.07635	Ara East
2011/744	Barranco de Chate	42.60868	-0.07687	Ara East
2010/544	Diaza	42.62574	-0.08838	Ara East
2011/738	Barranco de Arán tributario	42.59807	-0.10291	Ara East
2011/759	Bco. pista de las pardinas	42.55518	-0.11377	Ara East
2011/818	Bco. San Juste	42.46208	-0.11059	Ara West
2010/123	Labate bajo	42.59437	-0.14353	Ara West
2011/806	Barranco de San Salvador	42.48965	-0.15124	Ara West
2011/1107	Otal	42.56586	-0.20962	Ara West
2011/1119	Otal	42.56586	-0.20962	Ara West
2011/926	Barranco de san Bartolomé	42.6367	-0.2738	Tena East
2011/707	Barranco de San Lorenzo	42.69851	-0.29662	Tena East
2011/852	Barranco Estabuen	42.64165	-0.30625	Tena East
2011/946	Bco del asieso	42.65861	-0.30628	Tena East
2010/317	La valle (Fiscal)	42.51044	-0.24505	Tena East
2012/434	Afluente Barranco puerto de Biescas	42.66004	-0.343496	Tena West
2011/509	Pista circular	42.72098	-0.35339	Tena West
2010/259	Pista circular E	42.70111	-0.35407	Tena West
DRV7601	Buesa	42.701043	-0.357721	Tena West
2012/973	Barranco Escuer	42.600116	-0.359002	Tena West
2011/542	Pista circular	42.71506	-0.3863	Tena West
2010/286	Cecutar	42.66037	-0.43111	Tena West
2011/002	Aljibe Pista:del Collarada	42.69545	-0.49456	Villanúa
2010/613	Tortiellas	42.84079	-0.73636	Hecho
2010/235	Mazandú	42.84956	-0.78031	Zuriza
2010/211	Gamueta	42.8855	-0.78634	Zuriza
2010/187	Paralelo al Baldagrás	42.8584	-0.79434	Zuriza
2011/151	Río Erlán	43.00433	-1.16753	Navarra
2011/352	Regata Errekaundia	42.99479	-1.07513	Navarra
2011/372	Regato de Koitxa	42.96873	-1.06941	Navarra
2011/343	Regata de Landatxikina	42.99402	-1.0638	Navarra
2010/227	Maz	42.89225	-0.81149	Navarra
2011/637	Barranco de la Contienda	42.87625	-0.82316	Navarra
2011/574	Barranco Kumuxiloa	42.94481	-0.95567	Navarra
DRV7602	Barranco de Burgiarte	42.945946	-0.960102	Navarra
2011/470	Barranco de Tropo	42.9234	-0.97649	Navarra
2011/422	Aroyo Birriés	42.86309	-0.98405	Navarra
DRV2014/1	Ruisseau de Saillen	43.03397	-0.56489	France

To explore the relationships between mitochondrial haplotypes we calculated a Minimum spanning network (Bandelt, *et al.* 1999) using the software PopART (Leigh & Bryant 2015). We also explored other network methods implemented in PopART, including median joining and TCS networks, but they showed very similar results (not shown). To infer the recent demographic history of the species we reconstructed a Bayesian Skyline plot (Drummond & Rambaut 2007) using BEAST 2. First, we explored the dataset using a lognormal relaxed molecular clock prior that provided ucld.mean and coefficient of variation priors that were close to zero, suggesting that a strict molecular clock cannot be discarded. Hence, we ran further analyses with a strict clock with an uniform prior for the mutation rate set at 0.013 substitutions/site/My, within the variation range observed in other brown frog species (Canestrelli *et al.* 2014, Teixeira *et al.* 2018). As nucleotide substitution model we used HKY as suggested by PartitionFinder. We ran the analyses twice for 100 million generations in our local cluster, sampling every 10000 generations. Tracer v 1.7 (Rambaut *et al*. 2018) was used to check whether effective sample sizes of parameters (ESSs) were above 200 as well as for convergence of parameter estimates between both analyses. To build the final Bayesian Skyline plots we also used Tracer. To also test for potential population expansion within the species we performed Tajima's D (Tajima 1989), and Fu's Fs tests (Fu 1997) as well as a pairwise mismatch distribution analysis, as implemented in DNAsp v5 (Librado & Rojas 2009). Finally, *Rana pyrenaica* mitochondrial genetic diversity was estimated by the haplotype diversity (Hd) and nucleotide diversity indexes (π) as implemented in DNAsp v5.

In order to generate a spatial hypothesis of the last glacial maximum (LGM) distribution of the species, we modeled its current distribution using present climate and hindcasted it to the LGM using available paleoclimatic layers at 30 arcsec resolution. There are some paleoclimatic layers suitable for species distribution modeling (SDM), but the most commonly used ones (from Worldclim) are available at 2.5 arcsec, which is a grid too coarse for species with a small distribution range like *R. pyrenaica*. Hence, we used MIROC-ESM layers from the CHELSA climate project (Karger *et al.* 2017) that are available at 30 arcsec resolution (ca. 1km^2). Temperature layers are calculated in Kelvins so they were processed and transformed to Celsius degrees. For the modelling, we cut the layers around the distribution range of the species, with a wide margin, and we assessed the autocorrelation of the bioclimatic layers by generating 8000 random points within the extent of the climatic layers. Climate data was extracted in these coordinates for all layers

and pairwise Pearson correlations where performed in SPSS v 25 (IBM 2017). As most variables are autocorrelated, we removed from each pair one variable if the correlation was significant and with a Pearson coefficient value higher than 0.8. The final set of variables showing the least degree of correlation where annual mean temperature, mean temperature of wettest quarter, mean temperature of driest quarter, precipitation seasonality (coefficient of variation), precipitation of the wettest quarter, precipitation of the driest quarter, precipitation of warmest quarter. All GIS processing and final maps were performed in Quantum GIS (QGIS 2018). For the modeling, we used Maxent v 3.4.1 (Phillips & Dudik 2008), and all the locality records available for the species (see Chapter 1), which comprises 170 localities across the full species' distribution range. We used the 75% of the data to build the model and the remaining 25% to test it. Performance of the SDM was evaluated using the AUC (Area Under the Curve) values obtained from the model (Elith *et al.* 2006).

RESULTS

The complete mitochondrial genome of the species has 17213 basepairs and we amplified 15135 basepairs (bp) in 32 individuals, covering most of the genome save the control region in which primers did not work well across individuals. From these almost complete genomes, we recovered 19 unique haplotypes, and a total of 13 mutations, 9 of which were parsimony informative. Five regions were selected to further amplify in 20 individuals, as they represent most of the observed genetic variation and parsimony informative sites. The final dataset for 52 individuals consisted in 2396 bp with 17 haplotypes, 11 polymorphic (segregating) sites of which 2 were singleton variable sites and only 9 were parsimony informative across the genome.

In the Figure 2 is shown the haplotype network of the mitochondrial genomic variation across the full species range. In warm colours are represented the western populations and in cold colours the eastern ones. As it can be inferred from the network, there is no clear geographic structure as the eastern and western populations are separated by single steps. No haplotype is shared between the eastern and western populations, and no clades within the species were detected by any analysis including BEAST and MrBayes ones. We grouped the samples in areas that can be isolated by potential barriers like rivers or cliffs. The Añisclo and Escuaín canyons were isolated from Ordesa canyon

in the last ice age. Ordesa may have acted as an unit as it was a closed glaciated valley, and may be different from other populations east of the Ara river. As another potential barrier we included the Gállego River that divides the Tena valley in two, and the western Tena populations there are on a different slope from the next western valley in Villanúa (Aragón River). None of these zones are genetically distinct as some haplotypes are shared between all of them. In the west there is a similar pattern, with little differences between the Hecho valley, Zuriza and the rest of Navarra. The isolated French population presents a different haplotype very similar to other Navarra and Aragón haplotypes. The observed overall nucleotide diversity is very low (π= 0.00075), and the genetic diversity is Hd= 0.832 (h=17).

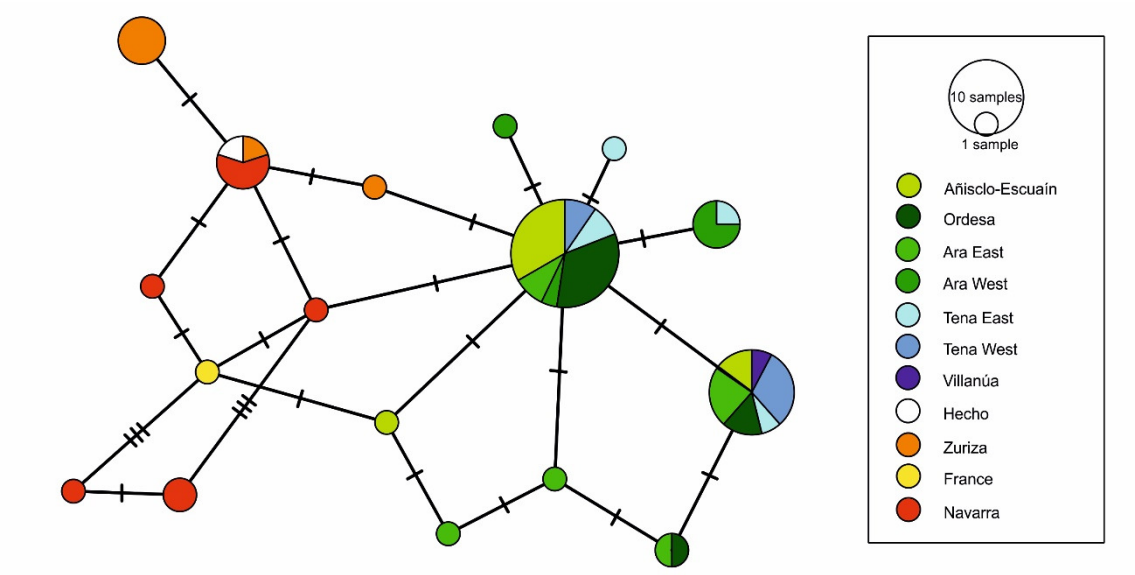

Figure 2. Haplotype network across the distribution range of the species and based on most of the genomic variation observed across the mitochondrial genome.

All the demographic analyses are in agreement and are congruent with a recent demographic change. The Bayesian skyline plot indicates that the species suffered a recent Holocene demographic expansion (Fig. 3), starting ca. 10000-12000 years ago. However, the effective population size was, and still is, very small through time. The mismatch distribution (Fig. 4) is unimodal with a nearly perfect match between observed and expected pairwise differences between alleles. Fu's Fs statistic was negative (Fs= -9.349, p=0.000) detecting a deviation from neutrality, as well as Tajima's D (D= -0.55398, p>0.10), with are in agreement with a population expansion.

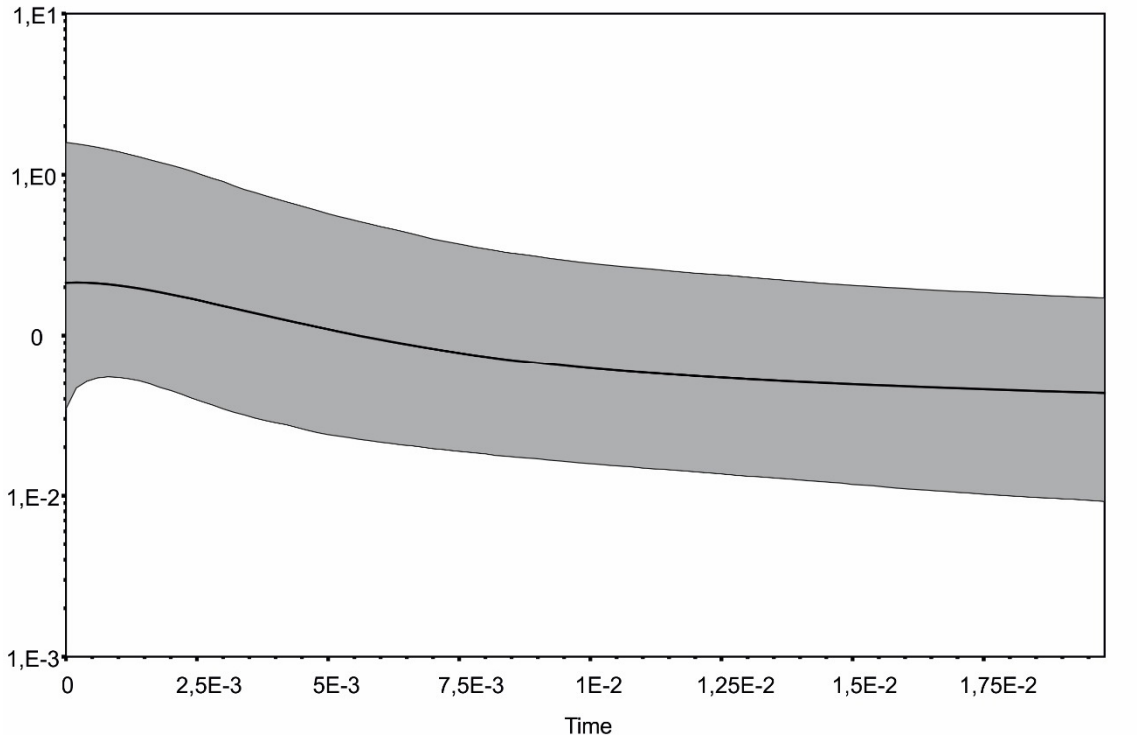

Figure 3. Bayesian Skyline plot (BSP) showing the recent demographic history of *Rana pyrenaica*. The estimated population size through time (y axis) is shown as Net units resulting from the log transformed product of the effective population size (EPS) and generation length in years. Time is shown in scientific annotation in thousands of years before present (X axis). Note the very small population size through time.

In the Figure 5 is represented the SDM for *R. pyrenaica* under the last glacial maximum climate, which was hindcasted from a current climate distribution model. This model is presented in Chapter 1, having an AUC of 0.973 for testing data. The model suggests a retreat to lower elevations and the central Pyrenean valley in the axis Sabiñanigo-Jaca-Navarra, as well as in the pre-Pyrenees. There are no pixels of 90-100% probability of occurrence and few areas are above 0.6 to 0.85 probability range. The model suggests that the eastern and western parts of the range persisted in two different and separated areas in the mountains, with a clear gap in the distribution coincident with the current gap in the distribution range of the species (from the Hecho valley to the Aragón river valley). However, the model also suggests that these eastern and western areas could have been connected across the central southern valley, as it can be seen in the probability distribution model. The French isolated populations are predicted to occur in a stable area through time, non-glaciated during the LGM, and outside the ice-sheet extent, which may have been connected to Navarra populations through a narrow territory close to the ice sheet border in the French side.

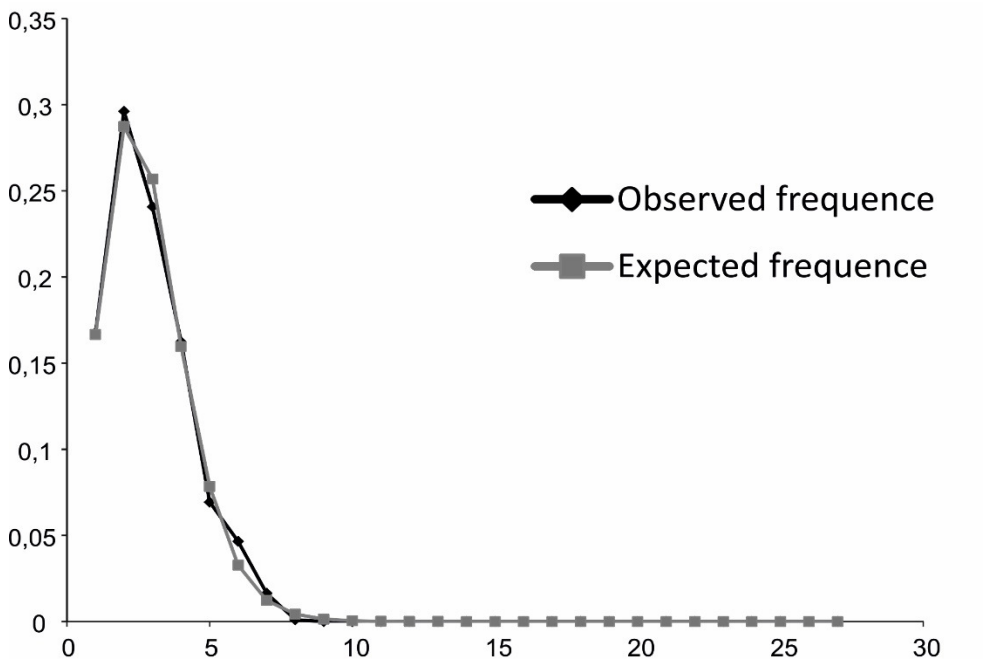

Figure 4. Mismatch distribution graph of the observed (black) and expected (grey) pairwise differences between alleles.

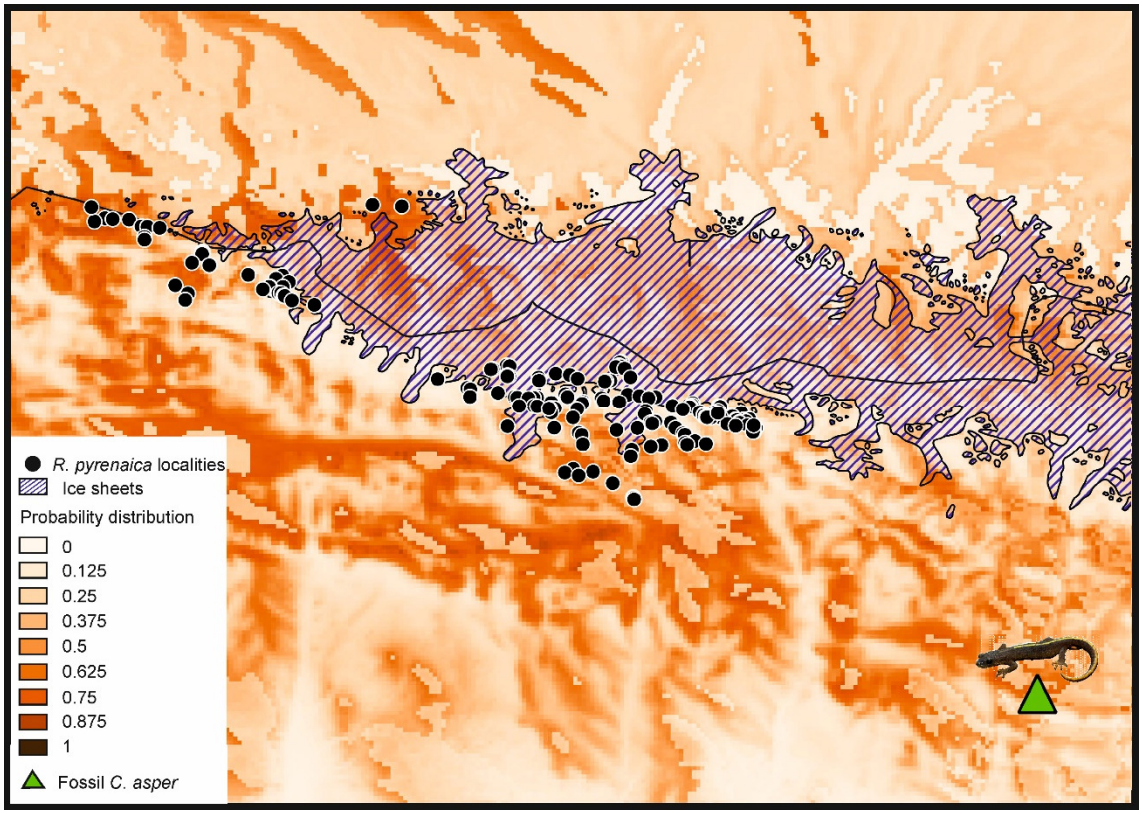

Figure 5. Spatial probability distribution of the distribution of *Rana pyrenaica* in the last glacial maximum. Black dots represent current known localities, while dashed polygon outlines the ice sheet limits during the last glacial maximum (sensu Ehlers & Gibbard, 2004). The green triangle shows the Pleistocene lowland locality (Colomera cave) where fossils of *Rana temporaria* and *Calotriton asper* have been found (López-García *et al.,* 2010).

DISCUSSION

The only previous work looking at the genetic variation of the Pyrenean frog sequenced eleven individuals from four localities for three mitochondrial DNA fragments of Cytochrome B, Cytochrome oxidase I and 12S (Carranza & Arribas 2008), and finding a single mutation in 1423 bp. This mutation was close to the priming site of the cytochrome b and we have not found it in any of our complete mitochondrial genomes, but in any case that dataset suggested that the species has a low genetic variation. In order to really assess the genetic diversity, phylogeographic structure and genetic variation of the species, we assembled a much larger dataset covering the full range of the species, sampled populations across all potential barriers to gene flow, and sequenced the whole mitochondria.

In mitochondrial phylogeographic studies, and especially in species with low genetic variation, our sequential sequencing approach has clear advantages over using available general primers for few mtDNA fragments. Now, when the price of NGS sequencing is low and long fragment sequencing is becoming more reliable, it is possible to sequence full mitochondrial genomes in a rapid and effective way. By doing so for several individuals we were able to target the regions across the genome that actually show genetic variation and develop specific primers for the loci of interest, which supposes a clear advantage to the use of general primers for short random mtDNA mitochondrial fragments. In the particular case of species with low genetic variation this is critical as otherwise we would not be able to assess the real degree of genetic diversity and where it does occur across the genome. In the next years it will be possible to massively and routinely sequence full mitochondrial genomes without the development of any primers (Liu *et al.* 2016).

Our dataset shows that *Rana pyrenaica* has a very low mitogenomic genetic diversity, being the lowest of all European brown frogs (e.g. Canestrelli *et al.* 2008, Vences *et al.* 2013, Teixeira *et al.* 2018). From a biogeographical perspective, it seems clear that *Rana pyrenaica* suffered a range contraction during the Pleistocene when a likely population bottleneck caused an important decrease in genetic variation and haplotype diversity in the species. When compared to its conspecific and sister taxon, the European common grass frog *Rana temporaria*, the differences are striking. *Rana temporaria* has a much wider distribution range, but more interestingly it shows very

divergent mitochondrial and nuclear lineages, some of which occur in the Iberian Peninsula, including one in the Pyrenees (Vences *et al.* 2013). This Pyrenean lineage likely persisted in a high altitude refugium near Benasque that was not glaciated (nunatak, pers. obs.), and it is surrounded by the most common central Pyrenean haplotype (Vences *et al.* 2013). Both species overlap in the Pyrenees but there are two main differences between them: the distribution range of the Pyrenean frog is much smaller and mostly circumscribed to the Spanish side of the Pyrenees, and *R. temporaria* is a pond breeding species while *R. pyrenaica* breeds mainly in streams (Vieites & Vences 2003). During the glaciations, the main rivers in the Pyrenees where frozen and the valleys covered by glaciers. Although some common frog populations could have persisted in ponds close to the ice that melted during summer, it is likely that the Pyrenean frog had to retreat to lower reaches of the rivers and streams that were not frozen. This likely forced a retreat of its distribution range to the pre-Pyrenean central valleys. This range contraction occurred likely in parallel to a population demographic collapse, leading to the small effective population sizes observed in the present and reconstructed in the BSP analysis. In agreement with the paleo-range reconstructed by the hindcasted SDM, our data suggest a recent Holocene range and demographic expansion to higher altitudes, although the actual distribution range overlaps with many ice-free areas during the LGM, with little high altitude colonization if we compared it with the rather common presence of *Rana temporaria* in high Pyrenean habitats (Vences *et al*., 2003). Fossil evidence support this range retreat into the pre-Pyrenean lowlands and small mountains, as fossils of the two mountain amphibians *Rana temporaria* and *Calotriton asper* have been found there, as well as many other mammal and reptile species now occurring at higher elevations (López-García *et al.* 2010).

The actual distribution range of the species shows a gap between eastern and western populations in the Hecho valley. In fact, we were able to find the first Hecho valley population in the last few years (Chapter 1), and confirmed that the distribution gap is real. This lead to the hypothesis that two main refugia could have occur during the Pleistocene, one in the drainages that go to the west (Navarra and western Aragón), and the other in the drainages that drain to the South from the Aragón and Tena rivers to the Ara river and west of the Cinca river. The paleo-distribution model shows a clear gap during the LGM in the area where today's gap exists, with a suitable climatic zone south of this gap in the central valley. This suggests that the species was never able to

recolonize higher elevations in the Holocene expansion in this area as it did east and west of it. This two refugia hypothesis should have some signature in the genetics of the species with two genetic clusters corresponding to the eastern and western range populations. Our genetic data do not support this scenario as there is not a clear genetic differentiation between eastern and western populations, despite the existing gap. The paleo-distribution model suggests and alternative scenario compatible with the existence of the mountain gap and the genetic data as well, that is a connectivity belt between eastern and western population across the central valley (Navarra-Jaca-Sabiñanigo) and the pre-Pyrenees. The model suggests a Pleistocene much southern distribution across the pre-Pyrenees where the species is not found today, that allowed gene flow between populations and could in part explain the genetic homogeneity of the species. However, as a stream species, in the much flatter areas south of the Pyrenean range the species likely had not that much available habitat, which could explain the low effective population size reconstructed for the last millennia in the BSP analysis.

Our results have important conservation genetic implications. It has been shown for long time that the combination of small distribution ranges, small effective and actual population sizes and patchy distributions can lead a species to extinction (Shaffer 1981, Soulé 1987). The Pyrenean frog can be considered a small range rare endemic species. Compared to any other European brown frog species it has the smallest range covering ca. 2400 km2 according to the IUCN (IUCN), and 30000 km2 to our own data (Chapter 1), which is very patchy, with disconnected populations most with few individuals (Chapter 1). Despite the numerous potential barriers to gene flow in the mountains, such as deep canyons, cliffs and rivers, the observed genetic differentiation is very low. The mismatch distribution is unimodal indicating that the species suffered a recent bottleneck and rapid expansion (Rogers and Harpending 1992). This population expansion is also supported by a negative departure from zero (neutrality) of Tajima's D, an index that measures the difference between the number of segregating sites and the average number of pairwise nucleotide differences (Tajima 1989). Fu's Fs test identifies the excess of rare alleles in an expanding population when compared to the number of expected alleles to be found in a stable population (Fu 1997), and the departure we observe suggests also a demographic expansion.

As this species inhabits streams, it makes sense that such habitats do not behave always as a barrier, save the presence of big fish that could predate on the frogs, as well

as other amphibians like the Pyrenean newt (Serra-Cobo *et al.* 2000). Compared to recent phylogeographic studies on other brown frogs, *Rana pyrenaica* has likely the lowest mtDNA variation. Although other Iberian Peninsula species like *R. temporaria* (Vences *et al.* 2013) or *R. iberica* (Teixeira *et al.* 2018) show high intraspecific genetic variation and distinct mitochondrial lineages, there are other widespread species like *Rana dalmatina* with a low genetic diversity across its full range (Vences *et al.* 2013), yet the amount of mtDNA data available are much lower than in our study in any species to made proper comparisons.

The reality of the species is that it has a narrow distribution range, with a very patchy distribution and restricted in many cases the small streams in headwaters where fish do not occur. Fish populations were artificially incremented and expanded by local governments to favor fishing activities, preventing the frogs to occur in most streams, habitats that likely they naturally occupied in the Pleistocene and Holocene. The genetic variation within the species is very low, and this situation likely was the same in the Pleistocene, where populations where small and the suitable habitat availability (streams) low in lowlands, despite the availability of a suitable climate as suggested by the SDM. Whether there is a fine-scale genetic structure reflecting current potential barriers to gene flow it has to be explored with different genetic markers such as SNPs (Chapter 4). The actual very low mitogenomic variation, patchy and disconnected distribution, low effective and real population sizes, as well other threats like the quitrid fungus (Chapter 2) or the massive presence of natural and introduced fish in most suitable habitats, urge the development of conservation actions that prevent the extinction of the species in this century.

Acknowledgements

We are grateful to many people that made this project possible. Several students and collaborators helped in fieldwork in the Pyrenees, including Carlos Zaragoza, Javier Santos, Rubén González, Miguel Vences, Nina Bernard, Guillermo Ponz and Isabel Perandones. Fernando Carmena and Ignacio Gómez from SARGA in Aragón, and Iosu Antón in Navarra were extremely helpful in the field, allowing locating many known populations. Ramon Antor Casterllanau provided logistic help to prepare and carry on fieldwork. Manuel Alcántara, David Guzmán and Jose Luis Burrel from the Aragón government showed their total collaboration and help during the whole length of the project. Forest rangers helped us during the fieldwork, and the public company Sodemasa, now SARGA, provided logistic support in Ordesa in 2011. MPF was financed with a grant by CNPq under Science Without Borders program by Brazil Ministry of Science. We thank the Governments of Aragon and Navarra for collecting permits. This work was funded by a research project of the Zoo de Barcelona (Ayuntamiento de Barcelona) as well as by a research project from the Parques Nacionales OPAN – MMARM to DRV.

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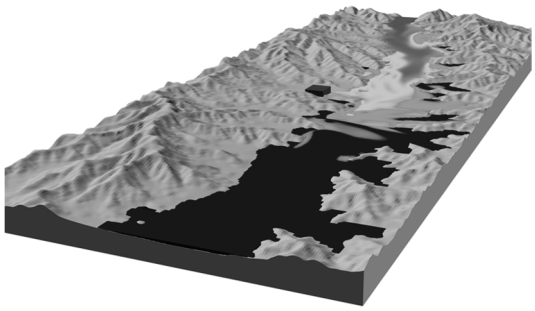


Chapter 5:

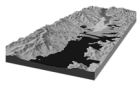
Conservation genomics, historical demography and landscape genetics of the Endangered Pyrenean frog

This chapter reproduce most of the manuscripts:

Vieites D.R., Van den Burg, M. P., Peso, M., Nieto-Román, S., Elmer, K., Jacobs, A., Genomic phylogeography and biogeographic history of a small-range endemic mountain species. Manuscript in preparation.

And

Peso, M., Van den Burg, M. P., Nieto-Román, S. & Vieites DR. Whole range landscape genomic analysis of an endemic mountain species. Manuscript in preparation.



Conservation genomics, historical demography and landscape genetics of the Endangered Pyrenean frog

Abstract

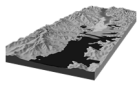
Conservation genetic studies aim to assess the diversity and geographic differentiation of one of the most essential biodiversity variables, intraspecific genetic diversity. This field is starting to incorporate novel genomic approaches to study species and populations of conservation concern; however their implementation in amphibians remains rare. Here, we utilize next-generation sequencing to study population genomics of the Endangered Pyrenean frog, *Rana pyrenaica* a narrowly distributed Pyrenean endemism. Using a distribution-wide dataset, we explored population structure and gene flow barriers using three different methods, and used approximate Bayesian computations to test different scenarios of divergence among identified genetic clusters. We assessed the genetic diversity at different scales, explored the presence of inbreeding and used a conservation and landscape genetic approach to identify conservation units. Our results show significant genetic differentiation suggesting that current population structure was shaped by the Pyrenean river drainage system (including valley glaciers), with the main river valleys as well as steep slopes acting as gene flow barriers among genetic clusters. Based on demographic-scenario probabilities, major-clade divergence occurred during the middle-Pleistocene, with subsequent divergence among eastern populations during the late-Pleistocene. Overall, *R. pyrenaica* shows very low levels of genetic diversity, with extremely low levels in isolated populations. In addition, we identified weak evidence of inbreeding. Besides inbreeding, the low genetic diversity and most populations having small effective population sizes, together with a fragmented distribution and low number of adults per population, suggest that the long-term survival of *R. pyrenaica* is severely threatened. We conclude that this species is of major conservation concern and identify six management units to aid future research and conservation-management actions.

Keywords: Pyrenean frog, *Rana pyrenaica*, genomics, conservation genetics, phylogeography, endemism.

INTRODUCTION

The field of conservation biology and the importance of conservation genetics both have their origin around the 1980s (Frankel 1974; Frankel & Soulé 1981). Advancing techniques and an increasing implementation of the genetic methodology to guide conservation biology and management have strongly embedded conservation genetics within biological research (Allendorf *et al*. 2010, 2013). However, for most species, the availability of genetic data is still minor or lacking; especially important are data on their genetic diversity, population structure, landscape genetics, genetic adaptation and demographic history. Many endangered species in need of conservation management are mostly non-model organisms for which such data are usually lacking.

Intraspecific genetic diversity has been regarded as one of the most essential biodiversity variables (May & Godfrey 1994). In conservation biology and management, the variation and structure of intraspecific genetics and local adaptation can be used to guide conservation priorities and eventually decision-making (Avise 1989, Moritz 2002); however, in practice such data are rarely used by conservation planners (Pierson *et al*. 2016). To facilitate this, geneticists identified concepts along the individual-to-species continuum, other than subspecies or populations, which capture intraspecific variation and local adaption (Moritz 1994). Of these, two conservation management units have most commonly been used to categorize genetic and adaptive variation: evolutionary significant units (ESU) and management units (MU) (Moritz 1994). Although the assignment of both type of units is based on demographic isolation, ESU's should also be reciprocally monophyletic for mitochondrial markers, evolve independently, show differences in nuclear loci and preferably have adaptive differences (Moritz 1994, for an overview of ESU definitions see Funk *et al*. 2012). On a smaller scale, MUs should be regulated by local population processes and dynamics only, thus in general MUs are smaller than ESUs and several MUs can make up one ESU (Moritz 1994). Both ESUs and MUs are conservation units that should ideally capture all adaptive variation present within a species; knowledge that is important for conservation management practices (Fraser & Bernatchez 2001, Funk *et al*. 2012), especially information on the magnitude and distribution of adaptive variation within a species. High throughput sequencing now allows conservation geneticists to acquire large genomic datasets necessary to identify genetic regions under selection (Funk *et al*. 2012).



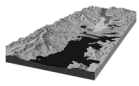
The genomic era has led to the development of multiple reduced representation genome (RRG) techniques that allow the cheap generation of genome-wide datasets for non-model organisms; restriction site-associated DNA sequencing (RADseq) (Baird *et al*. 2008), double digest RADseq (ddRAD) (Peterson *et al*. 2012) and Genotyping-By-Sequencing (GBS) (Elshire *et al*. 2011); see Andrews *et al*. (2016) for an overview. Therefore, the usage of these RRG techniques has great potential to apply to endangered species to help guiding conservation management (Funk *et al*. 2012, Twyford & Ennos 2012, Reitzel *et al*. 2013). In addition, RRG techniques are especially interesting for species with low genetic diversity identified by previous works using traditional markers given the larger number of loci in RRG datasets (but see Dimens 2016). Besides larger datasets, major advantages of RRG methods are the low cost-to-data ratio and their implementation without *a priori* genetic reference, using *de novo* assembly. However, although *de novo* assembly is a major advantage, reference-based studies are still recommended and are less prone to challenges (Alkan *et al*. 2011, Shafer *et al*. 2016). Similar to other emerging techniques, RRG methods and downstream bioinformatics have childhood limitations that still need to be overcome (Davey *et al*. 2013, Puritz *et al*. 2014b). For an overview of genotyping errors see Mastretta-Yanes *et al*. (2015).

Besides genotyping errors caused by methodological limitations or human-caused errors, the genomic architecture of study species can also hamper the successful generation of genomic data (Christensen *et al*. 2013). This is especially evident for taxa that underwent whole genome duplication events (WGD) and became polyploid (Mable *et al*. 2011). Here, the obvious problem is the increase in highly similar but paralogous sequences that, depending on the selective pressures or mutation rates affecting these genomic regions, can be hard to distinguish from homologous loci bioinformatically (Christensen *et al*. 2013), this is mainly because very small DNA fragments are usually sequenced, although new methods are being developed to better deal with paralogous loci (Willis *et al*. 2017). Next to fishes, amphibians underwent WGD events and are known for their large genome sizes (average 4.99pg for Anura, 35.30pg for Urodela; Gregory 2018), having the second largest average genome size among eukaryotes, after lungfishes (Gregory *et al*. 2007). These features could explain why RRG methods and complete genome sequencing have only sparsely been applied to amphibians (but see e.g. Hellsten *et al*. 2010, Streicher *et al*. 2014, Nunziata *et al*. 2017, Roland *et al*. 2017, Reyes-Velasco *et al*. 2018).

Patterns of intraspecific genetic variation are commonly used in phylogeography to help understand historic biogeographical processes including retreat to refugia and population expansions. A major area of research has focused on the influence of Pleistocene climatic oscillations on distribution changes and patterns of genetic diversity in European taxa (Taberlet *et al*. 1998, Hewitt 2000, 2004, Weiss & Ferrand 2007). Effects of these oscillations on species distribution and occurrence were especially evident around and due to mountain ranges given the small-scale latitudinal differences and complex topography (Hewitt 2004), particularly for terrestrial fauna. There, large temperature oscillations including associated expansions and subtractions of ice sheets (Ehlers & Gibbard 2004) lead to diverse distribution patterns driven by reoccurring range shifts, extinctions and retreat to refugia (Hewitt 1999, 2000, Schmitt *et al*. 2006, Weiss & Ferrand 2007, Canestrelli *et al*. 2008, Schmitt 2009). Across a broad range of taxa these oscillations thus had pronounced effects on patterns of distribution and genetic diversity through e.g. vicariance, subsequent secondary contact, and adaptive responses (Hewitt 2000, Davis & Shaw 2001). However main research focus has been on temperate species while these responses in cold-adapted species have been less studied and are presumed to differ from temperate species (Schönswetter *et al*. 2005, Schmitt *et al*. 2006, Schmitt 2009).

Besides (high-) Arctic systems, the Pyrenean and Alps mountain ranges in lower latitudes harbor many cold-adapted species. Since these species inhabit climatic niches along sharp altitudinal gradients, temperature oscillations likely had large impacts on their past and current distribution. Moreover, narrow climatic niches combined with geographical complexity of mountain systems suggest strong differentiation within distributions of cold-adapted species, although the time for differentiation build-up was shorter during interglacial periods compared to glacial periods. Phylogeographic studies on responses of cold-adapted Pyrenean species are especially limited (but see Mouret *et al*. 2011, Liberal *et al*. 2014, Bidegaray-Batista *et al*. 2016, Valbuena et al. 2018).

The closely-related Western Palearctic brown frogs (Amphibia; Ranidae) form a well-studied group of European frogs for most of which the phylogeographic and demographic history of some species have been studied (Veith *et al*. 2003, Canestrelli *et al*. 2008, Vences *et al*. 2013, 2017, Teixeira *et al*. 2018). Comparison of these features shows large differences in historic and current patterns of genetic variation among these European frogs (Veith *et al*. 2003, Vences *et al*. 2013). The widespread *Rana temporaria*



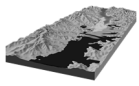
has many divergent lineages, with additional high genetic diversity at smaller scales (Vences *et al*. 2013, 2017), a pattern that is presumably the result of an initial large range with several range contractions during glacial periods with several glacial refugia and subsequent admixture between them during interglacial periods (Vences *et al*. 2013). Contrastingly, another widespread species, *R. dalmatina,* shows distribution-wide low genetic variation and evidence of few glacial refugia and rapid range expansion, which is comparable to brown frogs with smaller ranges (Vences *et al*. 2013). Among those smaller-ranged species are *R. iberica* and *R. italica*, which both show genetic signatures of multiple glacial refugia, low genetic diversity throughout their range and patterns of intraspecific post-glacial admixture (Canestrelli *et al*. 2008, Teixeira *et al*. 2018). For *R. pyrenaica*, these characteristics have not been assessed using a comprehensive range-wide dataset. However, mitochondrial DNA data suggest genetic homogeneity with connectivity between glacial refugia (Chapter 4). Interestingly, this species inhabits mid to high elevations in the Pyrenees that underwent dramatic changes during the Pleistocene cycles, with a current divided distribution range separated by a distribution gap in Aragón. These separated regions may be a consequence of the existence of at least two glacial refugia which should have a hitherto unknown genetic signature, although paleoclimatic modelling suggests connectivity across the central valley (Navarra-Jaca-Sabiñánigo) and the pre-Pyrenees.

The Pyrenean frog (*Rana pyrenaica*, Serra-Cobo 1993) is an endemic amphibian of the western and central Pyrenees where it is restricted to mountain streams with cold, fast-flowing and oxygen-rich water between 440-2100m a.s.l. (Vieites & Vences 2003, Vieites *et al*. 2016, Chapter 1). Due to the region's geography, infrastructure and water bodies with fish, the distribution of *R. pyrenaica* is extremely fragmented (Chapter 1). Given its limited extent of occurrence (<3,000 km^2), range-wide fragmentation, habitat loss and degradation, and decreasing number of locations, *R. pyrenaica* is listed as Endangered following the International Union for Conservation of Nature (IUCN) Red List guidelines (Bosch *et al*. 2009, IUCN 2017). Besides these threats, we identified that most localities consist of small populations and widespread presence of chytrid fungus (*Batrachochytrium dendrobatidis*; *Bd*) (Chapter 2), although the impact of the latter on *R. pyrenaica* remains unknown (Vieites *et al*. 2016).

Given these threats and its conservation status, *R. pyrenaica* has been a target species of genetic studies, which mainly aimed to identify and help guide conservation

efforts (Carranza & Arribas 2008, Chapter 4, Peso *et al*. 2016). First, a preliminary genetic assessment of few mitochondrial DNA (mtDNA) samples reported only a single mutation within a 1423bp region (Carranza & Arribas 2008). Hereafter, we sequenced 32 mitochondrial genomes finding very low genetic variation compared to other brown frog species (Chapter 4). This lack of genetic diversity and seeming absence of genetic clusters within *R. pyrenaica* stands in high contrast to its sister species as aforementioned. Combining these data, the restricted and endemic *R. pyrenaica* shows a reduced mitochondrial genetic variation, which, together with numerous threats to its survival, highlights the need to generate genomic data using RRG methods in order to guide conservation efforts at a fine scale.

In recent years much progress has been made in the theoretical framework of landscape ecology, especially in spatial connectivity and isolation, which has been enriched by the incorporation of new programs and genetic analyses. Since Wright (1943) proposed the idea that at greater geographic distances less connectivity between populations and therefore greater genetic differentiation (known as "isolation by distance"), this hypothesis has been tested in numerous organisms, demonstrating that it is a common phenomenon. In the field of landscape genetics, the concept of "isolation by resistance" has been recently introduced (McRae & Beier 2007), which is defined as the correlation between genetic distances and "resistance" distances. A resistance matrix is simply a classification of the landscape in a function of probability of use of the different components of the landscape. For example, one species can be able to cross the landscape without problems (0 resistance) while for others the same landscape features can behave as total barriers (100% resistance). This relationship does not have to be related to geographical distance, since many barriers are specific and are located in specific places. In the case of mountains with steep gradients this becomes more relevant, as for example cliffs can be barriers for frogs, despite that the geographic distance between both sides of the cliff is very small. Another new concept has been recently introduced that is "isolation by the environment" (IBE). It is defined as the pattern in which genetic differentiation between populations increases in relation to environmental differences, regardless of geographical distance (Wang & Summers 2010, Bradburd *et al*. 2013, Sexton *et al*. 2014, Wang & Bradburd 2014). In the recent review by Wang & Bradburd (2014), the processes that can generate IBE are identified, which include examples of clinal temperature variation, natural or sexual selection against immigrants, reduction of hybrid



fitness or biased dispersion among habitats. This opens new possibilities for testing different hypotheses on spatial connectivity based on pure distance, resistances and environment, which can be tested with genetic distance data from population genomic studies.

This project utilizes RADseq data to assess the conservation genomics and fine-scale phylogeography of *R. pyrenaica*. We also want to assess the degree of connectivity (or lack thereof) between populations and test different connectivity scenarios under a landscape genetics approach. Specifically, we aim to identify: 1) the genetic diversity at the species- and population levels; 2) assess its genetic population structure and compare that to the previously proposed population structure based on different methods; 3) identify historical barriers to gene flow and connectivity areas; 4) assess the genetic diversity and confirm signatures of inbreeding; 5) reconstruct the biogeographic and phylogeographic history of *R. pyrenaica* and the occurrence of glacial refugia. Findings provided through this project are of great importance from a conservation perspective as these can aid future conservation programs, both in- and ex-situ, aimed at securing the long-term survival for one of the most endangered European vertebrates.

MATERIAL AND METHODS

Sampling

Rana pyrenaica tissue samples (tail or finger-tip samples, or whole specimens) from 178 individuals were collected during a range-wide sampling effort in the Pyrenees between 2010 and 2014 (see previous chapters). During this multi-year effort, fieldwork was conducted from February to July. To prevent bias caused by sibling-sibling relation in our analyses, we only sampled one tadpole per clutch. In addition, we included one *Rana temporaria* tissue sample. This dataset compiles individuals from all life stages, but with a strong bias towards individuals in the larval stage (n=129, 72.5%) (Appendix), which are more detectable than adults.

DNA sample and optimization

We extracted DNA using DNAeasy kits (QIAGEN, Germany) and assessed all concentrations prior to library preparation. For all samples DNA concentrations were measured with a Qubit spectrophotometer. As tadpole tail tips yielded low DNA

concentrations (<10 ng/µl), and that for RADseq higher concentrations are required, we applied a novel technique to increase DNA concentration per sample, which uses *bacterial* DNA ligasepolymerase that performs without supplemented primers and is able to bind to any site, identify the sequence and recruit complement nucleotides to construct a primer (Sygnis TruePrime WGA Kit). This treatment led to a median increase in DNA concentration of 312%.

Library preparation and sequencing

Library preparation and Illumina ddRAD sequencing were performed using a modified protocol by Peterson *et al*. (2012) to discover and genotype SNPs. Here, optimized DNA samples were digested using *Pst*I-HF and *Msp*I enzymes where after barcodes were ligated using a cost-effective method. Samples were then pooled into two paired-end libraries including controls within and between these two libraries: each library consisted of 85 unique individuals, with 5 duplicates per library, and 10 individuals shared by both libraries. Samples were randomized both within and between libraries based on sample locality (Meirmans 2015). Thereafter, these libraries were PCR amplified and sequenced on 2 lanes using an Illumina HiSeq 2500 (Illumina Inc., San Diego, CA).

De novo assembly and SNP calling

All bioinformatic analyses were run using the dDocent v2 pipeline (Puritz *et al*. 2014a) on our local cluster at the Natural History Museum of Madrid (MNCN). First, raw sequences were demultiplexed and trimmed for each library separately. Then, as *de novo* assembly and SNP output might be influenced by low-quality data, we implemented a preliminary data exploration step to assess data quality as SNP output using default dDocent settings. This step revealed a high variation in SNP presence, both between libraries and individuals, especially within the first library (Fig. 1A). Thereafter, we optimized our dataset by excluding samples with low quality with more than 23% of the SNPs missing; Fig. 1B), removing 89 individuals (library 1: 76; library 2: 13). This step also greatly reduced subsequently necessary computational power and duration. The remaining 111 samples were combined into one dataset for subsequent analyses.

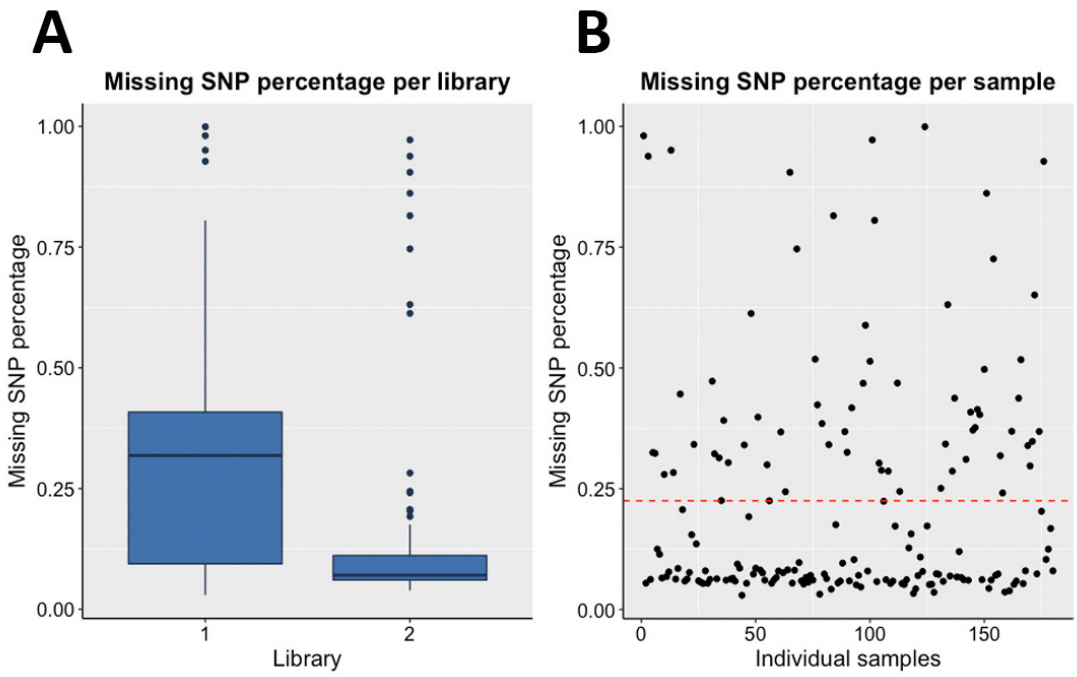


Figure 1. Variation in SNP presence both for Illumina libraries (A) and within individual samples (B), n=198. Red line represents the missing percentage cut off value.

Predictably, parameter settings during *de novo* assembly affect assembly and SNP output (Parchman *et al*. 2010, Wences & Schatz 2015). As raw RRG data are a 'black box' compared to previous data (e.o. microsatellites), data exploration provides a way of understanding data characteristics. Here, we evaluated final contig and SNP output for multiple dDocent runs with differing similarity mapping thresholds and Burrows-Wheeler Aligner (BWA) parameter combinations for both datasets (for details see Appendix): similarity threshold, gap penalty (O), mismatch penalty (B) and matching score (A). For runs with identical similarity thresholds, we used one *de novo* reference assembly. Given the large and potential repetitive genome of amphibians and observed low mtDNA genetic diversity within *R. pyrenaica* we used a 98% similarity threshold and relaxing BWA parameter values for the final dataset.

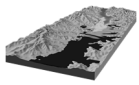
Raw SNP variants were then filtered using VCFtools v.1.15 (Danecek *et al*. 2011). First, we filtered loci with a minor allele count of <3, phred quality score of <20 and <50% call rate. Then, individuals with >50% missing data were removed. Next, loci were removed with a <0.9 call rate and minor allele frequency lower than 0.05. In addition, *vcflib* was used to select loci based on average allele balance, keeping loci with <0.01 and 0.3>X<0.7 balance values. Hereafter, we filtered loci with large discrepancies between the reference and alternate allele, with overlapping reads and paired status

discrepancy between the reference and alternate allele (Puritz *et al*. 2014a). Then, following Li & Wren (2014) and Puritz *et al*. (2014a), we removed loci for which the quality score was less than 0.75 of the depth and loci with higher than average depth and quality of <2 times the depth. Lastly, after filtering based on Hardy Weinberg equilibrium (HWE) ($p<0.001$), we implemented a new script, *rad_haplotyper*, to filter the remaining loci based on paralogs, mean depth per loci, mean quality score and a final ≥80% presence cutoff (Willis *et al*. 2017). Further details on stepwise SNP filtering are provided in the Appendix.

Although control and duplicate samples are fundamental in scientific research and crucial in experimental setup, their implementation in NGS studies seems rare or at least rarely mentioned. Here, we included several between- and within-library duplicate samples which allowed the assess quality of remaining filtered SNPs. Since only high quality samples were retained for analyzes, our final dataset included seven duplicates; three within library 2 and four between both libraries. Alignment of these duplicates identified that 33.1% of our filtered SNPs had inconsistent nucleotides between duplicates, retaining 763 consistent SNPs for further analysis.

Contamination

The presence and effect of contamination in NGS projects seem rarely considered (Alkan *et al*. 2011, Schmieder & Edwards 2011). Problems due to contamination might arise while generating sequence data and/or when it is included in the eventual working dataset. Contamination can, for example, cause contigs to be misassembled or lead to erroneous conclusions during subsequent bioinformatic analyses. However, despite these potential limitations, the identification and removal of contamination DNA in genomic datasets do not appear to be common practice as shown by Schmieder & Edwards (2011); who compiled a dataset of 202 published metagenomes and identified contaminated sequences within 72% of the analyzed genomes. Similar results were obtained by Forster (2003) who identified that 58% of 137 human mtDNA studies published between 1981-2002 contained errors. A more recent assessment of publically available sequence data by Laurence *et al*. (2014) identifies the bacteria genus *Bradyrhizobium*, a contaminant of ultrapure water systems, as a common contaminant in RRG studies.



Here, we assessed the presence of contaminated reads in 10% of raw sequence data (10 individuals) by blast searches against the National Center for Biotechnology Information (NCBI) database. Also, raw Illumina reads were mapped against the *Escherichia coli* and *Bradyrhizobium* (sp. DFCI-1) genome in Geneious using Medium/Low sensitivity and 5 iterations. In addition, both final *de novo* assemblies were mapped against the *E. coli*, *Bradyrhizobium* and *Homo sapiens* genomes using identical settings in Geneious v11.0.5. These analyses showed no contamination of *E. coli*, *Bradyrhizobium* or *H. sapiens* in either of the *de novo* assemblies. However, minor contamination was found since raw Illumina reads from 82 of the 111 high-quality samples mapped against the *E. coli* and/or *Bradyrhizobium* (sp. DFCI-1) genomes. Specifically, with an average of 3.4 million Illumina reads per sample, an average of 42 and 29 reads mapped against the *E. coli* or *Bradyrhizobium* genome respectively, and were removed.

Identification of SNPs under selection

Loci, or regions, under selection are of interest for their insights into possible selective and adaptive processes (Nosil *et al*. 2009, Moore *et al*. 2014). Such loci show higher expected F_{ST} values than under neutrality, which can be identified using genome outlier scans. Caution should be taken to assign these loci as such, however, if presence of spatial population structure is suspected (Excoffier *et al*. 2009). Using simulated data, Narum & Hess (2011) assessed robustness of three outlier scan methods and found BayeScan v2.1 (Foll & Gaggiotti 2008) to have the lowest number of type I and II errors. We followed the recommendations by Narum & Hess (2011) and use both BayeScan and FDist, only assigning and excluding outlier loci when both results are in agreement. BayeScan v2.1 was ran twice using 100K burn-in with differing priors odds of 10 and 100 (Foll & Gaggiotti 2008). For FDist (Beaumont & Nichols 1996), we used 100K simulations in a hierarchical island model as implemented in Arlequin v3.5 (Excoffier & Lischer 2010). Both programs were run using the two East and West areas as populations, corresponding to the distribution areas separated by a gap in the range of *R. pyrenaica*.

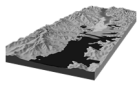
Genetic diversity

We identified observed and expected heterozygosity (H_O and H_S) and fixation index (G_{IS}) on the species and population level using GenoDive (Meirmans & Van Tienderen 2004). Hereafter we implemented a new R package *biotools* to visualize the spatial distribution of genetic diversity (as H_E) for the complete distribution range of *R. pyrenaica* (da Silva *et al*., 2017; R Core Team, 2018), using a 4 kilometer (km) radius and a minimum of two samples per pixel to calculate H_E. Furthermore, we calculated three multilocus heterozygosity (mlh) parameters, internal relatedness (IR; Amos *et al*. 2001), homozygosity by locus (HL; Aparicio *et al*. 2006) and standardized heterozygosity (SH; Coltman *et al*. 1999), using the R package *Rhh* (Alho *et al*. 2010).

Range-wide population structure

We assessed the genetic structure within the entire species range of *R. pyrenaica* using Structure v2.3.4 (Pritchard *et al*. 2010). First, we ran an admixture model with correlated frequencies and no prior location info to identify major genetic clusters. Hereafter, since Structure identifies the uppermost hierarchical structure (Evanno *et al*. 2005), we ran a similar model within the east cluster to investigate more fine-scale structure. All runs consisted of 10 replicates for K values between 1-10, using 10^6 MCMC iterations with 10^5 burn-in repeats. Hereafter the most probable value of K was inferred using the ΔK parameter in Structure-Harvester (Evanno *et al*. 2005, Earl & vonHoldt 2012, Welch *et al*. 2017). We visualized Structure results using Distruct v1.1 (Rosenberg 2004).

Given the potential bias of Structure results due to possible violations of assumptions (Pritchard *et al*. 2010), the lacking ability to detect isolation by distance (Frantz *et al*. 2009, Meirmans 2012) and uneven sample sizes (Puechmaille 2016), we compared our results to additional methods. First, we identified clusters of genetically-related individuals with a maximum between-group variation using a discriminant analysis of principal components (DAPC) using the R package *adegenet* (Jombart & Ahmed 2011). Additionally, we inferred the optimal range of genetic clusters that best explain genetic variation in our dataset based on Bayesian Information Criterion (BIC) values obtained from sequential clustering (Jombart & Ahmed 2011). Secondly, we implemented a method that uses non-negative matrix factorization algorithms to assess



population structure using the R package *LEA* (Frichot & François 2015) that we compare to Structure and *adegenet* results. Similar parameters to the Structure analyses were chosen with 10 repeats per value of K (1-10) using the *snmf* command and clustering based on cross-entropy criterion (MCE). Lastly, since spatial clustering can be explained by Isolation-By-Distance (IBD) (Meirmans 2012), we examined the effect of IBD and genetic clustering on calculated F_{ST} values (van den Burg *et al*. 2018). Specifically, we used GPS coordinates and assigned population data to assess variation in allele frequencies using redundancy analyses (RDA) in the R package *vegan* (Oksanen *et al.* 2014).

We used the obtained clusters as input to group individuals into smaller clusters: western clade (Navarra), Valle de Tena, Yésero, Bujaruelo, Ordesa and Añisclo. Then, we assessed the partitioning of genetic variation between all clusters using Analysis of Molecular Variance (AMOVA) and by calculating pairwise F_{ST} values in Arlequin v3.5 (Excoffier & Lischer 2010) and GenoDive (Meirmans & Van Tienderen 2004). We performed two AMOVAs using 99,999 permutations: 1) between the hypothesized western and eastern clades, 2) among all identified clusters.

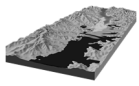
Landscape genetics

To analyze the spatial connectivity between populations, dispersal cost matrices were first elaborated from five main data sets, from which we derived several more: 1) Map of vegetation and land cover occupation with a resolution of 100x100 meters from the European program Corine Land Cover (EEA 2006); 2) Digital terrain model with a resolution of 90x90 meters from the Shuttle Radar Topography Mission of NASA (Jarvis *et al.* 2006); from which we derived an elevation map, and from this one a slope map was generated; 3) Map of water bodies and streams including fish; 4) Map of reservoirs and dams from the IGN (National Geographic Institute); and a "distance to streams" map was generated from the previous ones. 5) We used the equations relating beginning of the breeding period in function of elevation in Pyrenean *R. temporaria* from Vieites (2003), and the end of the activity period to estimate the length of the period of activity in Pyrenean brown frogs in function of elevation. Both *R. pyrenaica* and *R. temporary* are explosive breeders that have a similar phenological pattern, so we assumed that the

available pattern for *R. temporaria* can be applied to *R. pyrenaica* in absence of other data from this species. All maps were rescaled to 100x100 meters resolution in raster format.

To reclassify the Corine layer into a cost matrix, we calculated from the distribution records of *R. pyrenaica* the observed frequencies in all sampled localities (presences and absences, see chapter 1). This reclassification was made on a percentage scale, in which 100% represent a high conductance or total permissiveness in the matrix for the movement of individuals, while 0% representing the areas of no conductance, that is to say, zero permissiveness to the movement of the individuals and an effective barrier. The altitude layer was re-classified according to the real altitudinal range of the species in the Pyrenees, with values of zero dispersion outside the altitudinal range, and 100% within it. The slope and climate layers were reclassified in the same way based on the data obtained from all the sampled localities. The fish are large aquatic predators that make almost impossible frog reproduction, as well as frog's dispersion through them, therefore the bodies of water with the presence of fish were reclassified as 0, acting as barriers. Following Dudaniec *et al*, 2013, global cost matrices were made with permutations of these parameters, to measure the potential impact of all possible combinations of variables in spatial connectivity between populations. In total we created 370 different conductance matrices combining elevation, climate, distance to rivers, fish presence, slope and landcover. During fieldwork we recorded different environmental and habitat variables from the localities where *R. pyrenaica* occurs which helped to assess these reclassifications.

These conductance (inverse of resistance) matrices, were used as input in Circuitscape v 4.0 (Shah & MacRae 2008), to perform landscape connectivity analyses. This approach builds upon electronic circuit theory to predict connectivity in heterogeneous landscapes and gene flow (McRae 2006, McRae et al. 2007, 2008). We ran the analyses with the 370 different conductance matrices and presence localities for the species. We then tested which one of these 370 spatial hypotheses correlated better with Fst distances between localities using partial Mantel tests with 1000 permutations, correlating the resistance with the genetic distances and considering the Euclidean distance. We then performed another correlation to test of "isolation by barriers" where we defined the main potential barriers based on the genetic clustering (main rivers and steep slopes) and tested it with a mantel test correlating it with genetic distance. Mantel



test results with higher correlation values are expected to be the more likely hypothesis to explain spatial connectivity considering genetic data (Shah & MacRae 2008).

Historical demography and divergence times

To investigate the potential role of ancient demographic processes in shaping the current distribution and genetic variation within *R. pyrenaica* we implemented an approximate Bayesian computation (ABC) framework using diyABC v2.1 (Cornuet *et al*. 2014). diyABC does not consider several biological processes (migration, gene flow, bottlenecks and natural selection) when testing hypotheses (Cornuet *et al*. 2014). Here, we tested scenarios to understand the timing and pattern of divergence between the western and eastern clades, and within the eastern clade using two diyABC runs (Fig. 2). Time estimates in diyABC are given as generation times; although this is unknown for *R. pyrenaica* we assumed a generation time similar to its sister species *R. temporaria* (8 years, Miaud *et al*. 1999). In the first run we assessed divergence of the west and east clade from an ancestral population at t_1 (Fig. 2A). This run consisted of five scenarios with differing timing intervals for t_1 (kya = thousand years ago): 1) present-LGM (0-21kya); 2) late Pleistocene (21-460kya); 3) middle Pleistocene (460-940kya), 4) early-middle Pleistocene (940-1,200kya); early Pleistocene (1,200-2,595kya). In the second run we used the divergence timing of the scenario with the highest probability from run one to root the time scales for all five scenarios (Fig. 2B-E). In addition to divergence of the west clade these scenarios included splits among the Valle de Tena, Yésero, Bujaruelo, Ordesa and Añisclo clusters. Scenario one was set with a rapid inter-east split and simultaneous divergence of all five clusters diverged simultaneously with the western clade at t_1 (Fig. 2B). In scenario two, the inter-east split was again rapid and simultaneous but occurred post-west divergence (Fig. 2C). Scenario three involved an early divergence of the Añisclo cluster at t_2 with rapid divergence among the other four eastern clusters thereafter at t_1 (Fig. 2D). The fourth scenario consisted of a single divergence event of the Añisclo and Bujaruelo clusters with a subsequent rapid divergence among the remaining three clusters (Fig. 2E). The fifth scenario involved separate divergence from Añisclo and Bujaruelo, with again rapid divergence between the remaining clusters (Fig. 2F). Lastly, the final scenario involved separate divergence of each clusters; Añisclo, Bujaruelo, Ordesa, Valle de Tena and Yésero (Fig. 2G). We ran all scenarios in both runs with 10^6

simulated datasets. Hereafter we compared the generated posterior probabilities of each scenario for consistency to our observed dataset using PCA plots. Then, for consistent scenarios, we performed simulations of posterior parameter distributions to test the compatibility between simulated and observed data, and to identify 95% time intervals for divergence events.

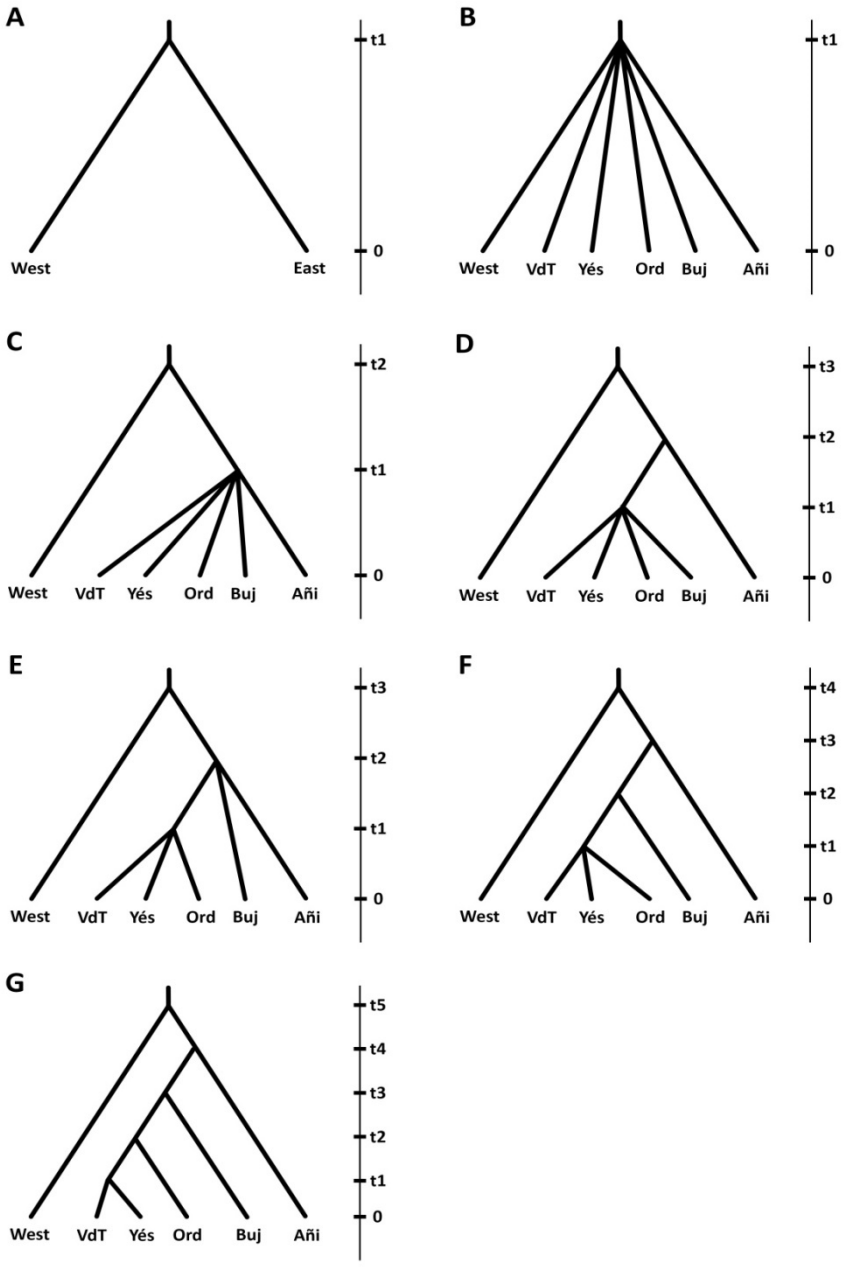
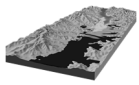


Figure 2. Historical demographic scenarios tested in diyABC, for the west and east clade and Valle de Tena (VdT), Yésero (Yés), Ordesa (Ord), Bujaruelo (Buj) and Añisclo (Añi) clusters. Time scale with ti represents time steps as generations and is not scaled. All scenarios were run without population size limitations.



RESULTS

After excluding duplicates, individuals and loci with >20% missing data, and two samples with erroneous GPS data, 93 individuals and 763 SNPs were retained. Additional assessment of HWE violations indicated that 101 SNPs deviated from HWE after Bonferroni correction. Furthermore, 2% of 75,078 pairwise comparisons were significant at $p<0.05$, although results were inconsistent between populations we removed another 23 loci with high numbers (≥10) of significant comparisons. Then, after sequential removal of 274 SNPs that showed signatures of possible paralogs ($H_O>0.8$), 365 SNPs were retained for subsequent analyses. From this dataset, FDist and BayeScan identified 55 and 0 SNPs respectively as being under selection, however as no locus was flagged by both methods none were excluded from further analyses. FDist results using Bonferonni correction identified 21 SNPs as outliers. Samples sizes (n) for each analyzed cluster were: Navarra, 19; Valle de Tena, 10; Yésero, 17; Bujaruelo, 6; Ordesa, 24; Añisclo, 17.

Genetic structure

The global F_{ST} was 0.514 ($p>0.001$) with genetic variation in *R. pyrenaica* best explained for K=2-6 as indicated by summary statistics generated by Structure, *snmf* and DAPC software (ΔK, MCE and BIC, respectively) (Appendix). First, Bayesian analysis in Structure with number of populations set to K=2 separated all western from eastern individuals, with n = 19 and 74 respectively (Fig. 3A). This was strengthened by a pairwise differentiation of F_{ST} = 0.538. Consistently both DAPC and *snmf* results were identical, assigning similar individuals to a western or eastern cluster (Fig. 3C and Appendix). Secondly, the assessment of genetic clusters within eastern populations showed a presence of 2-5 clusters (Fig. 3A). Again, all three methods (ΔK, MCE and BIC) were in agreement showing K=2-5 as the likely number of genetic clusters within the eastern range. Discriminant PCA analysis, excluding the Navarra cluster, separated individuals into five clusters: Valle de Tena, Yésero, Bujaruelo, Ordesa and Añisclo (Fig. 3D); although the genetic clustering is less clear between the Yésero, Bujaruelo and Ordesa populations. Indeed, pairwise F_{ST} between adjacent populations of Añisclo-Ordesa, Valle de Tena-Yésero, Yésero-Bujaruelo, Bujaruelo-Ordesa and Yésero-Ordesa were 0.313, 0.200, 0.194, 0.286 and 0.166 respectively (Table 1). Results of pairwise F_{ST} calculated using GenoDive (Meirmans & Van Tienderen 2004) and Arlequin (Excoffier

& Lischer 2010) were consistent (Table 1). The RDA indicated that the genetic variation in *R. pyrenaica* was better explained by the population subdivision of the five identified populations than by IBD. Although both variables explained a significant part of the total genetic variance, and while using the other variable as a co-variate, population structure and IBD explained 73.6% (p=0.001) and 4.6% (p=0.001), respectively.

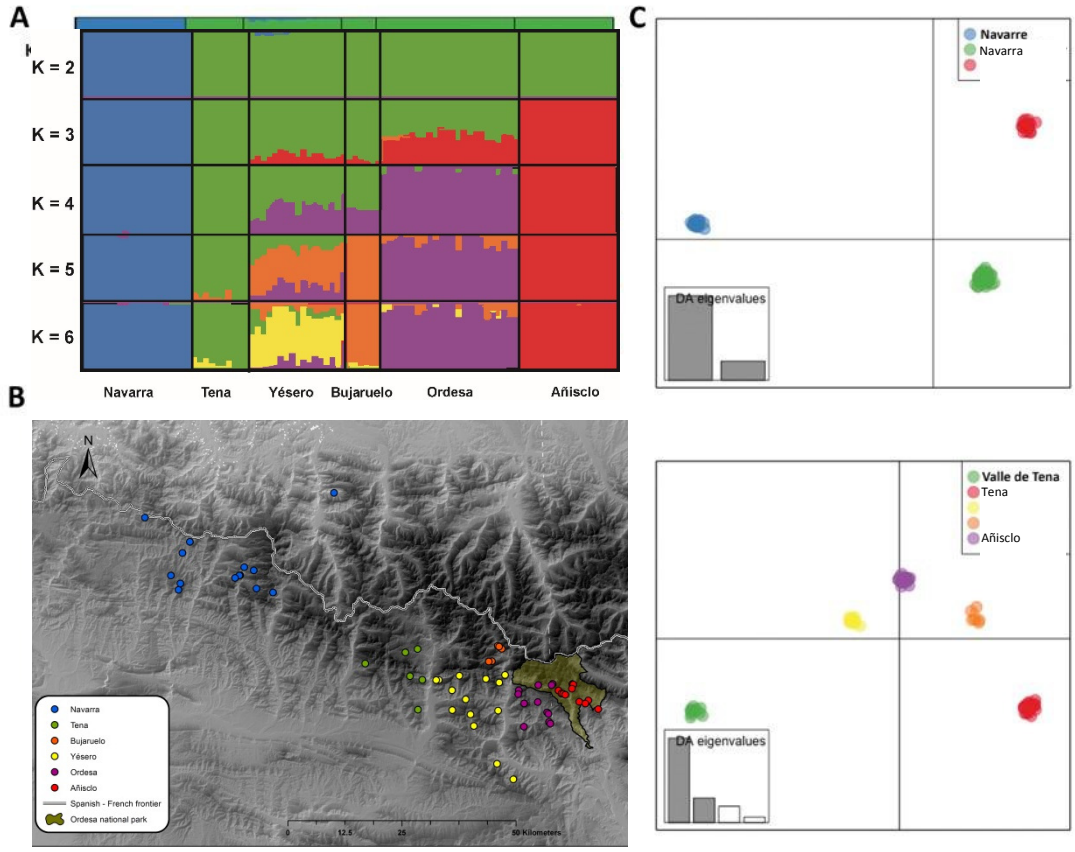


Figure 3. Genetic population structure in *Rana pyrenaica*. A) Structure barplots showing individual population assignment for five analyses with different values of K; B) Map showing sample localities included in genetic analyses with population assignment; C) Principal Component Analysis plot for K=3; D) Principal Component Analysis plot for K=4, excluding western populations (Navarra).

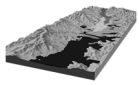


Table 2. Pairwise F_{ST} values between genetic populations of *R. pyrenaica*. Upper triangle are values generated by Genodive, lower triangle by Arlequin. Values in bold represent comparisons between adjacent populations.

	Navarra	Tena	Yésero	Bujaruelo	Ordesa	Añisclo
Navarra		**0.747**	0.609	0.768	0.659	0.825
Tena	**0.747**		**0.190**	0.366	0.349	0.601
Yésero	0.614	**0.200**		**0.184**	**0.168**	0.396
Bujaruelo	0.768	0.369	**0.194**		**0.288**	0.582
Ordesa	0.658	0.348	**0.166**	**0.286**		**0.311**
Añisclo	0.825	0.602	0.401	0.584	**0.313**	

Genetic diversity

Genetic diversity evaluated across all individuals and loci, showed average H_O and H_S values of 0.226 and 0.199, respectively (Table 2). Overall, there was a small excess of homozygosity in *R. pyrenaica* (F_{IS} = 0.068, P<0.001.) with inbreeding coefficients ranging from -0.317 to 0.01 between genetic clusters. Furthermore, a spatial assessment of genetic diversity, calculated as H_E, shows relative low and homogenous genetic diversity within the western clade (Navarra) (Fig. 4). In contrast, genetic diversity within the eastern clade is heterogeneous and overall higher (Fig. 4).

Table 2. Genetic diversity parameters for all samples, and geographically and genetically supported clusters.

	N_A	H_O	H_S	G_{IS}
Full range	2.000	0.155	0.165	0.093
Navarra	1.236	0.061	0.062	0.012
Tena	1.529	0.156	0.179	0.126
Yésero	1.753	0.221	0.263	0.159
Bujaruelo	1.499	0.200	0.186	-0.079
Ordesa	1.699	0.203	0.208	0.023
Añisclo	1.296	0.089	0.092	0.038

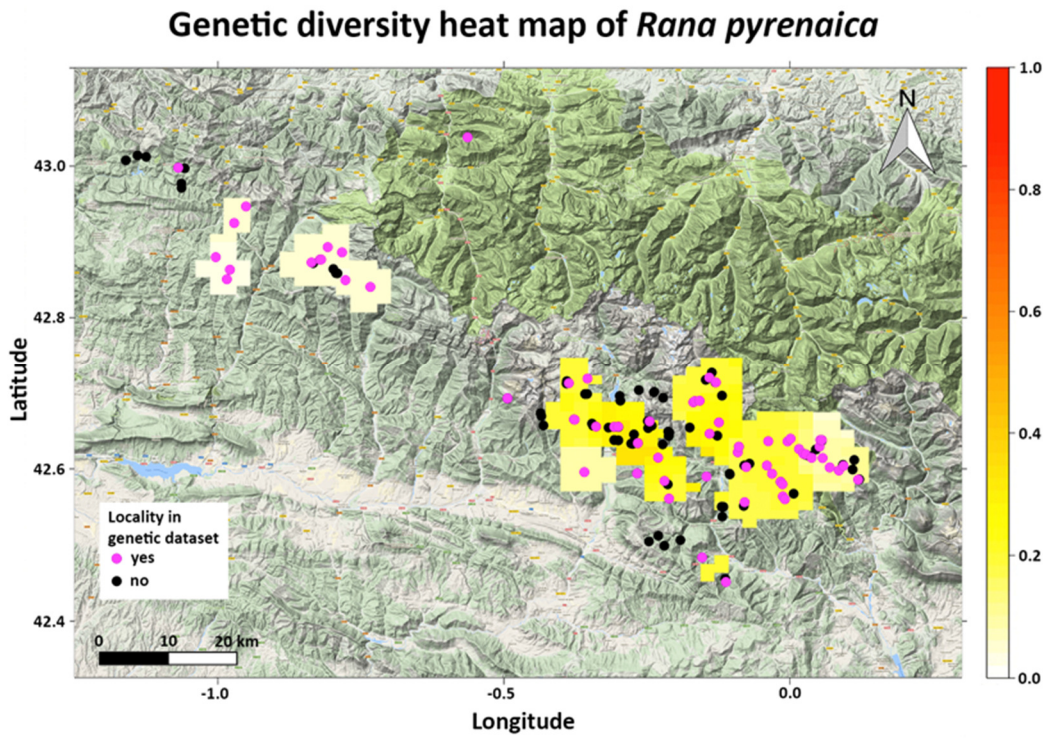


Figure 4. Spatial genetic diversity (H_E) heat map of *Rana pyrenaica*. Black dots represent known localities for this species, purple dots represent localities included in our SNP dataset.

Mean estimates of mlh parameters, including standard deviations, are summarized in Table 3. Overall, species-wide Internal Relatedness (IR) was 0.48, the eastern cluster showed an identical positive IR (0.32) and the western cluster (Navarra) a negative IR of -0.07. Populations within the eastern cluster also showed lower IR values ranging between -0.29 (Bujaruelo) and 0.11 (Yésero). A similar pattern was found for estimates of Homozygosity by Locus (HL), which was lower for the western compared to the eastern cluster. Again, within the eastern cluster, values ranged from 0.54 for Bujaruelo to 0.66 in Yésero. For Standardized Heterozygosity (SH), estimates for each clustering scale were similar.

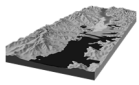


Table 3. Multilocus heterozygosity values for *R. pyrenaica* populations. Mean values in bold, and 2.5% and 97.5% quantiles between parentheses.

	Internal Relatedness	Standardized Heterozygosity	Homozygosity by locus
All data	**0.48** (0.09, 0.86)	**1** (0.31, 1.75)	**0.82** (0.67, 0.96)
Navarra	**-0.07** (-0.33, 0.38)	**1** (0.58, 1.44)	**0.61** (0.51, 0.82)
East cluster	**0.32** (-0.00, 0.69)	**1** (0.41, 1.54)	**0.76** (0.63, 0.90)
Tena	**0.01** (-0.16, 0.30)	**1** (0.66, 1.19)	**0.65** (0.57, 0.75)
Yésero	**0.11** (-0.07, 0.28)	**1** (0.83, 1.25)	**0.66** (0.57, 0.73)
Bujaruelo	**-0.29** (-0.41, -0.03)	**1** (0.74, 1.16)	**0.54** (0.47, 0.64)
Ordesa	**-0.03** (-0.32, 0.19)	**1** (0.77, 1.39)	**0.62** (0.50, 0.71)
Añisclo	**-0.03** (-0.33, -0.24)	**1** (0.70, 1.30)	**0.62** (0.49, 0.73)

Results for historical demographic analyses are shown in Fig. 5, and Appendix. The west-east divergence occurred pre-LGM, however highest probability calculated by two estimation methods was inconsistent for scenarios two (21-460kya; late Pleistocene) and three (460-940kya; middle Pleistocene) (Fig. 5A). Therefore, a 21-940kya-divergence interval was included in the second run. For this run probability estimates consistently identified scenario six (Fig. 2G) as the most probable divergence scenario (Fig. 5B). Time divergence estimates for scenario 6 (run 2), including 95% intervals, indicated that west-east divergence occurred 578 thousand years ago (kya) (200-920kya). Thereafter, within the east clade, populations diverged separately; Añisclo 310kya (102-646kya); Bujaruelo 264kya (106-501kya); Ordesa 141kya (48-282kya); Valle de Tena and Yésero 168kya (24-350kya). All supported scenarios showed a good model fit as observed data fell within simulated data distribution (Appendix).

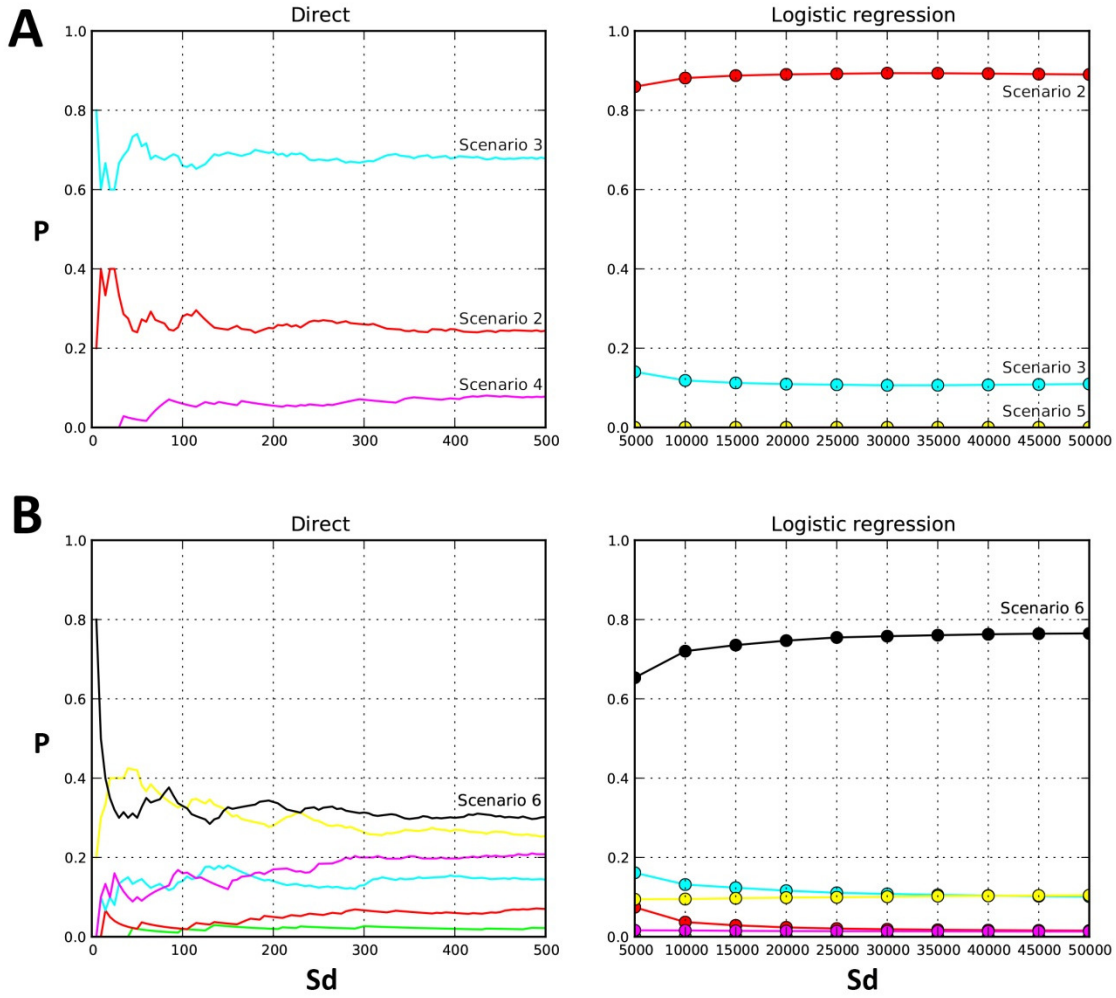


Figure 5. diyABC direct and logistic regression probability results for two approximate Bayesian computation runs. A) results for west-east divergence estimate, B) results for divergence estimate including all six populations. Figures show direct and logistic estimates of posterior probability, y-axis is probability (P), x-axis is number of simulated data sets (Sd).

Partial Mantel tests suggest that the best spatial hypotheses of landscape connectivity, considering isolation by resistance, are the ones involving the variables: distance to rivers and streams, elevation, slope and landcover (Mantel statistic r=0.4402, p=0.0001). There are resistance matrices combining some of those variables that show also significant results. The best analysis resulted in a connectivity map that is shown in Figures 6 and 7.

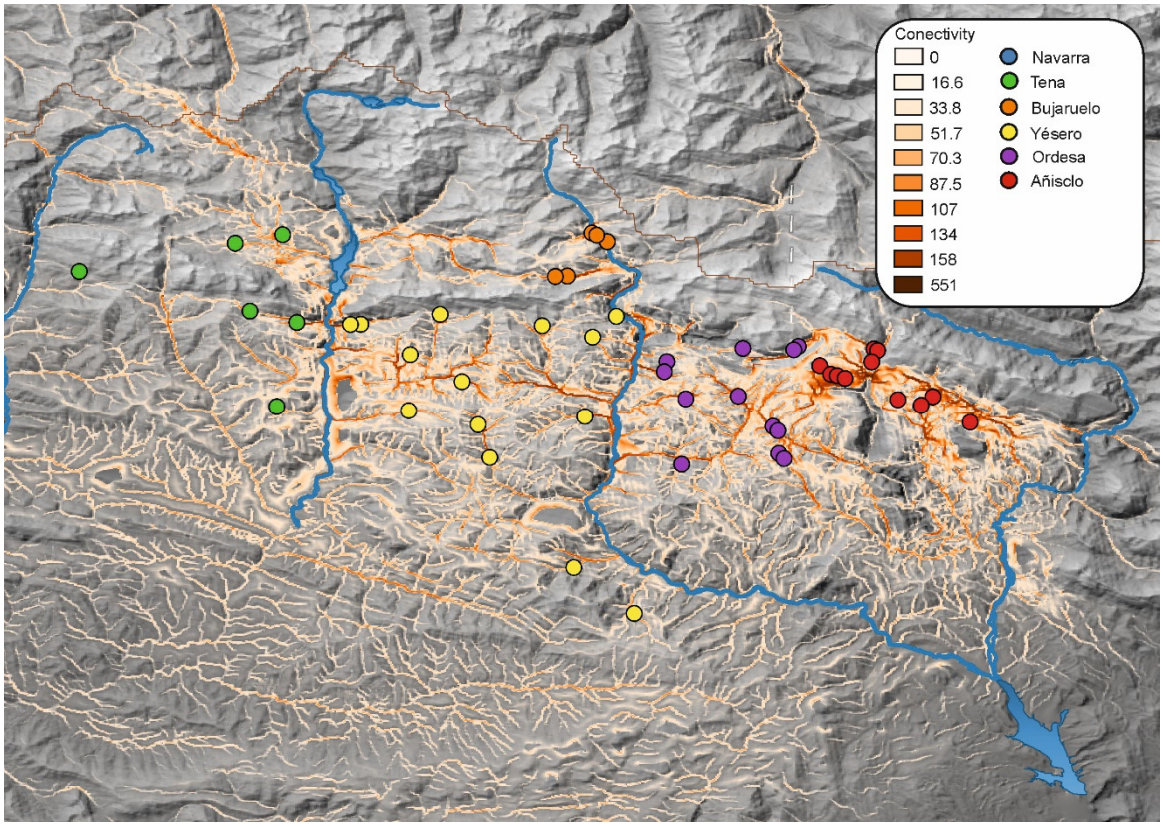


Figure 6. Connectivity map based on a conductance matrix constructed with distance to rivers and streams, elevation, slope and landcover variables. Light colors represent low connectivity areas (zero was excluded for visualization purposes) and red-brown colors represent high connectivity areas. The genetic clusters found in the population genomic analyses are color coded with the same colors as in previous figures to allow direct comparison. Main rivers are also shown. In this map we show the area corresponding to the Eastern populations (Aragón).

The results of connectivity analyses suggest that the streams function as corridors in this species, with low connectivity between most populations, especially in Navarra. However, there are some areas showing high connectivity between different genetic cluster, for example Ordesa and Añisclo, which do not correspond to the reality, as they are separated by evident barrier (big cliffs in the case of Ordesa). Other barriers are well recovered, like the Telera and Tendeñera massifs that difficult connectivity between low and high elevations in the Tena Valley, and the French side of the Pyrenes as frogs cannot cross high mountain peaks (<3000 m a.s.l.).

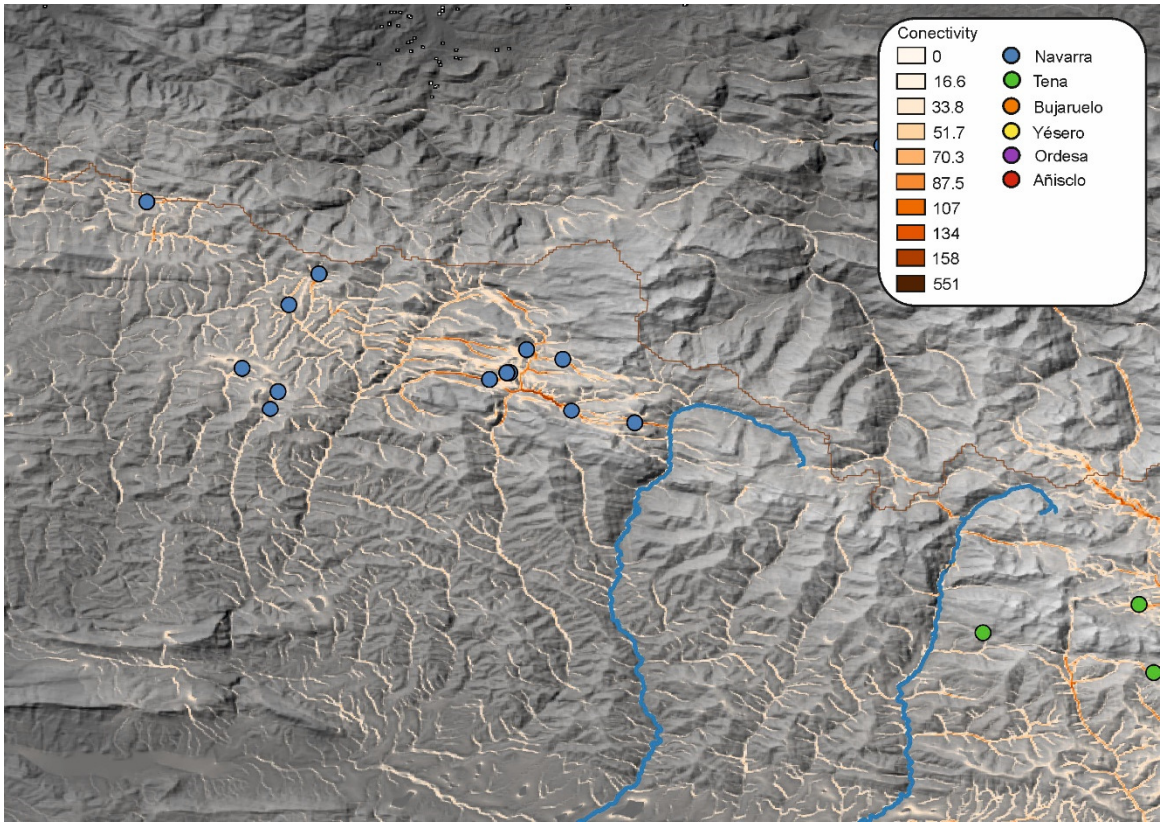
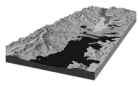


Figure 7. Connectivity map based on a conductance matrix constructed with distance to rivers and streams, elevation, slope and landcover variables. Light colors represent low connectivity areas (zero was excluded for visualization purposes) and red-brown colors represent high connectivity areas. The genetic clusters found in the population genomic analyses are color coded with the same colors as in previous figures to allow direct comparison. Main rivers are also shown. In this map we show the area corresponding to the Western populations (Navarra and part of Aragón).

We also tested the hypothesis of isolation by barrier with partial Mantel tests correlating with genetic distances. Those were done by pairwise comparisons between each pair of genetic clusters: eastern vs. western populations (r=0.7733, p=0.0001), Tena vs. Yésero (r=0.5328, p=0.0001), Ordesa-Yésero (r=0.3516, p=0.0001), Ordesa-Añisclo (r=0.5279, p=0.0001).

DISCUSSION

Here we utilized high-throughput SNP data to study the spatial genetic structure of an endemic European amphibian that is of conservation concern, being listed as Endangered by the IUCN. Analyses of genetic parameters within *Rana pyrenaica* show that there are substantial differences in genetic diversity and occurrence of moderate



inbreeding among sampled localities. In contrary to previous studies (Carranza & Arribas 2008, Chapter 4), our results show the presence of multiple genetically differentiated population clusters within *R. pyrenaica* that diverged in the middle-to-late Pleistocene. These results suggest a presence of several potential management units within this species.

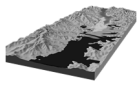
Phylogeography and population structure

Our study is the first to identify a geographic signature in genetic variation within *R. pyrenaica*. Previous studies found an absence of geographic structure for mtDNA genes and genomes, several non-coding nuclear markers and protein-coding genes (Carranza & Arribas, 2008, Chapter 4). Although the two major clades lack mtDNA monophyly (Carranza & Arribas 2008, Chapter 4), our DAPC results indicate the largest genetic variation within *R. pyrenaica* indeed separates western and eastern populations (Fig. 3A and C). Similarly, both Structure and *snmf* assignments of individuals for K=2 separate the west and east clade individuals (Fig. 3A, Appendix). Based on simulations of the demographic history, East-West divergence occurred ~578kya, during the middle Pleistocene. The middle Pleistocene, 460-940kya, is known for stronger glacial cycles and ice accumulation compared to the early Pleistocene (1,200-2,595kya), hence presumably caused longer periods of isolation between subpopulations. Interestingly, this geographical separation between both clades is identical to major haplotype groups in species of plants, spiders and other species of amphibians (Van Dijk & Bakx-Schotman 1997, Charrier *et al*. 2014, Liberal *et al*. 2014, Gonçalves *et al*. 2015, Bidegaray-Batista *et al*. 2016). Moreover, although divergence estimates were not always performed in these studies, *Alytes obstetricans* and *Harpactocrates ravastellus* divergence predates those found here for *R. pyrenaica* and occurred during the early Pleistocene (Gonçalves *et al*. 2015, Bidegaray-Batista *et al*. 2016). Alternatively, the lack of mtDNA divergence between both major clades, despite their relatively recent origin, could suggest an additional recent origin of the distribution gap after earlier gene flow occurred among different populations. Divergence among populations in the eastern clade (141-310kya) occurred within the late Pleistocene (21-460kya), during which glacial periods intensified and prolonged to ~100kya. These estimates therefore indicate that all six populations inhabited their own local LGM refugia, which especially for Bujaruelo was likely very

small given the glacial extent and distribution during the LGM (Fig. 6). In addition to the multiple-species boundary around the Valle de Tena, the Ainsa valley also constitutes a gene flow or distribution barrier for multiple species. Besides the eastern distribution boundary for *R. pyrenaica*, the Bielsa-Ainsa valley seems to be a widely occurring barrier in other species (Muster & Berendonk 2006, Valtueña *et al*. 2012, Vences *et al*. 2013, Charrier *et al*. 2014, Gonçalves *et al*. 2015, Bidegaray-Batista *et al*. 2016). Even though the phylogeographic history of *R. pyrenaica* shows similarities to that of other species, still little is known as to how Pyrenean species and ecosystems responded to Quaternary glacial cycles, which is surprising given the high level of endemism. These questions should be addressed by comparative phylogeographic studies.

Although diyABC is a regularly implemented program to reconstruct species historical demographic history based on genetic data (e.g. Portnoy *et al*. 2014), a notable limitation of this methodology is the assumption of migration absence (Cornuet *et al*. 2008). Inclusion of migration might lead to more recent divergence estimates between populations in *R. pyrenaica*. However, our results suggest a lack of migration between these clusters because river and mountain barriers have been present in their actual configuration since the LGM. It is possible that migration only occurs in this system when these barriers weaken, possible during glacial periods throughout the lowlands (see Chapter 4). Future additional analyses should perform *R. pyrenaica* divergence computations using more robust programs, preferably using complete contigs instead of SNPs.

In addition to both clades, our results indicate previously undescribed genetic diversity within the eastern clade. High genetic differentiation and, consequently, low levels of gene flow between adjacent populations suggest that the eastern clade of *R. pyrenaica* constitutes of five physically separated populations (Fig. 3A, D). Our analyses of pairwise differentiation identified both moderate (>0.15) to high (>0.2) F_{ST} values, similar to values for populations in other amphibian species (Rowe *et al*. 2000, Palo *et al*. 2003, Arens *et al*. 2007, Knopp *et al*. 2007). Geographic assessment of these proposed populations and their distribution suggest that the river drainage system acts as a strong gene flow barrier between the Valle de Tena-Yésero, and Yésero-Ordesa populations. Similar results were found in distribution ranges of other high-elevation amphibians (Li *et al*. 2009, Yan *et al*. 2012), separating divergent mtDNA lineages. However, in *R. pyrenaica*, gene flow seems not to only be restricted by river systems but also by steep



elevation changes, specifically the southern slopes of the Ordesa valley and the northeastern slope of Valle de Jalle that separates the Ordesa from Añisclo populations. Although additional localities occur closer to the southern Añisclo canyon, from which no genetic samples were sequenced, it is sound to assign the narrow and altitudinally steep Añisclo canyon as another strong gene flow barrier. Landscape genetic analyses using Circuitscape failed to recover this barrier, likely because the slope values we used as limiting value for the frog (42^0 slope) may need to be revised as the frogs could be able to climb upon higher slopes as long as there is water. Although the isolation by resistance models came significant, a pure barrier model in pairwise correlations using partial Mantel test showed that for all comparisons between genetic clusters the barriers explain the observed genetic distances, hence it is likely that big rivers and steep slopes are real barriers to gene flow. Big streams were frozen with glaciers during cold Pleistocene periods, resulting in long ice tongues covering the rivers (Ehlers & Gibbard 2004), and hence the habitat of the frogs, but more importantly those were barriers then that isolated populations at both sides. The estimated divergence times between genetic clusters suggest that their origin occurred during the Pleistocene, and it could be the result of these ice barriers that isolated populations, rather than during the actual streams and rivers where the main potential cause that prevent frogs to cross them are big fish. It is interesting that all landscape genetic analyses recover a low connectivity between Navarra populations where there are no evident geographic or climatic barriers, there were no big glaciers and they are genetically homogeneous. The observed pattern of genetic variation, high eastern and low western, could have several reasons; 1) founder effect resulting from an eastern origin range expansion (Comps *et al*. 2001), 2) western isolation with subsequent genetic drift and inbreeding, and 3) increased eastern habitat heterogeneity and geographic complexity (deep valleysm higher altitudinal gradients, more rivers and steep slopes).

Each of three clustering methods used assigned the small isolated population in France to the Navarra cluster (Fig. 3A and C; Appendix). The complete absence of *R. pyrenaica* between the French and geographically closest localities, even though suitable habitat seems available, can have two potential explanations: or it is a relict isolate of the last glacial ice expansion, as suggested by the ice-sheet maps (see chapter 4) that show that the area where it occurs today was ice-free in the LGM; or it was recently introduced there from Navarra, as we could expect some degree of genetic differentiation as

observed in the eastern populations but they are genetically the same, and landscape genetic analyses do not support connectivity between those isolated French and the Navarra populations.

It remains unclear why the Navarra population cluster is confined to its current distribution, mainly Spain and the close French frontier, and not dispersing further into France. It could (partially) be explained by several hypotheses: migration limitation due to e.g. the orientation of local river systems and low species dispersal rate combined with recent extirpation of populations within the current distribution gap; larger effect of *Bd* on the Navarra populations. Although we showed here that river systems seem to act as migration and gene flow barriers for *R. pyrenaica*, no study has so far assessed this species' annual dispersal rate. It is likely to be lower than of its sister species *R. temporaria* (>2000m; Kovar *et al*. 2009, Decout *et al*. 2012), given its fragmented and patchy distribution, and smaller body size. A low dispersal rate is also strengthened by the occurrence of only one population in a northward flowing river drainage system (Manenti & Bianchi 2011). The distribution range could also be limited through low reproductive success, which is dependent on water temperature, predatory fish, and oxygen and acidity levels (Serra-Cobo *et al*. 1998).

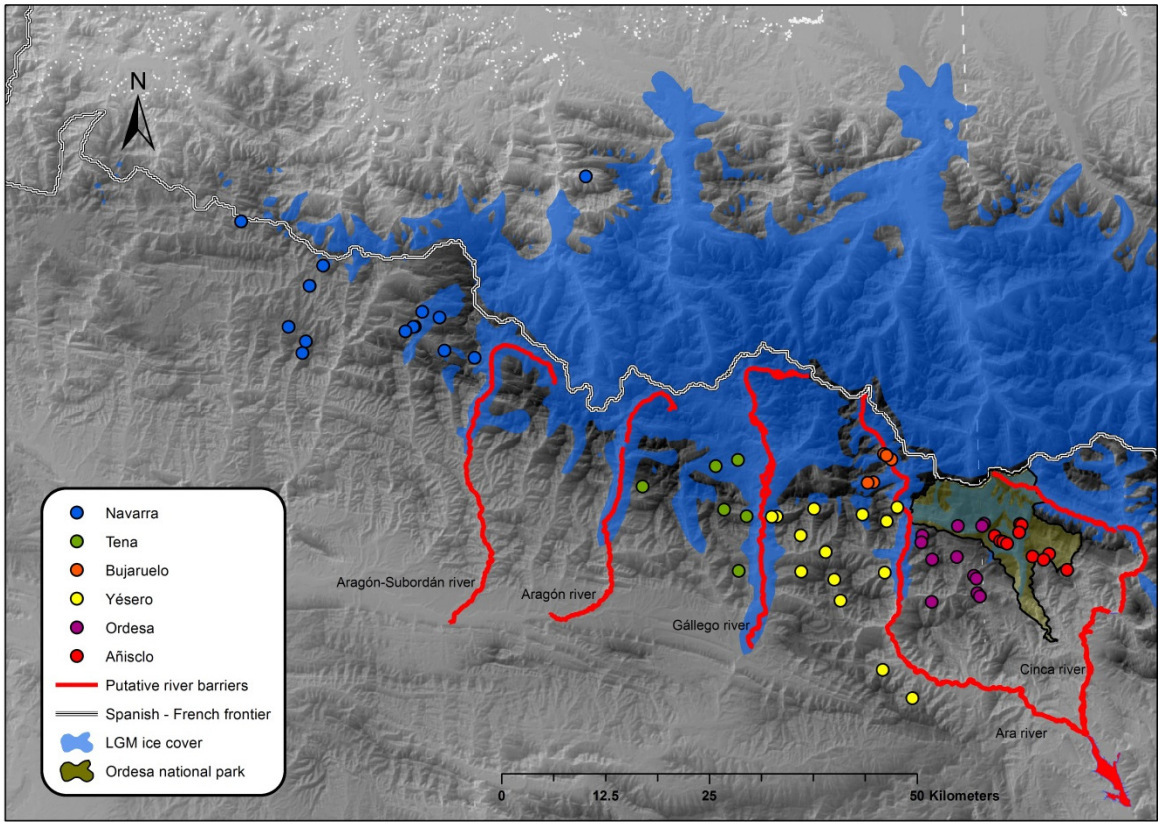


Figure 6. Current genetic population structure in *Rana pyrenaica* with ice extent during the Last Glacial Maximum. and Putative river Barriers (Cinca, Ara, Gállego, Aragón and Aragón-Subordón rivers).

Genetic diversity and conservation

Our results suggest caution must be taken when assessing population trends and identifying conservation efforts aimed to protect *R. pyrenaica*. Namely, in addition to the formerly proposed western and eastern clades, we identified multiple previously unknown genetic clusters within the eastern clade; implying that their inconsideration in management efforts could lead to local extirpation and loss of genetic diversity. Although this genetic structure is higher than previously anticipated by the results of complete mitochondrial genomes (Chapter 4), still is low, with overall very low genetic diversity (H_S=0.165), and higher genetic diversity within the eastern compared to the western clade, which shows the lowest genetic diversity (H_S=0.062). This pattern is presumably a combination of higher genetic variation within the eastern LGM refugia, a strong historical population size bottleneck within the western clade and presence of multiple isolated eastern populations prone to mechanisms that increase differentiation among them. Luckily, two of the eastern clusters occur within the Parque Nacional de Ordesa y

Monte Perdido, and hence already have the highest degree of protection possible in the European Union.

Amphibians in general are believed to be more prone to low genetic diversity and its decrease than other vertebrates (Allentoft & O'Brien 2010). This is due to several factors: 1) generally low effective population sizes in amphibians (Funk *et al*. 1999) 2) combined with clutch reproduction and potential of entire clutch mortality, and 3) low dispersal rate (Allentoft & O'Brien 2010). However similar low levels of genetic diversity are rarely reported in the amphibian literature and to our knowledge not from a species-wide dataset. For example, Seppa & Laurila (1999) found low diversity within Finnish island populations of *R*. *temporaria* (H_E<0.2) and *B*. *bufo* (H_E<0.1). Other vertebrate groups present identical low genetic diversity (H_E<0.1) in island populations of species of lizard and wallaby (Lennon *et al*. 2011, van den Burg *et al*. 2018). Furthermore, although this low genetic diversity might be caused by local population declines, which have mainly been recorded from the valley of the type locality (Serra-Cobo 1993, Vieites *et al*. 2016), another explanation could be collection for pet trade. In the digital age, locality information of endangered and protected species should be treated as sensitive, and each study should assess whether sharing specific localities in scientific publications is justified (Lindenmayer & Scheele 2017).

An additional line of evidence that points to low genetic diversity within *R*. *pyrenaica* is the SNP dataset (n=365) presented here, which is well below the final filtered number of SNPs in datasets that implemented ddRAD (Peterson *et al*. 2012, Wang *et al*. 2013, Kai *et al*. 2014, Portnoy *et al*. 2015). However, we cannot assess the potential loss of SNPs caused by sequencing issues during Illumina sequencing, which led to low quality and genetic data for 87 individuals divided over both Illumina libraries. Similarly, sequencing issues might have caused the high presence of control sample discrepancies, further decreasing our SNP dataset. As bioinformatics pipelines and SNP calling perform better with a reference genome (Alkan *et al*. 2011, Shafer *et al*. 2016), we recommend repeating these steps whenever a high quality *R*. *pyrenaica* genome becomes available.

A negative effect of inbreeding and low genetic diversity is a potential elevated expression of deleterious alleles. The occurrence of such alleles is presumably higher in populations following recent bottlenecks compared to populations that went through

historic bottlenecks experiencing lower fitness and subsequent selection against deleterious alleles (purging). Therefore, from a conservation standpoint, the pre-LGM origin of all six populations of *R. pyrenaica* suggests that currently, negative risks associated with inbreeding and low genetic diversity are expected to be low.

The high number of loci that significantly deviated from HWE equilibrium suggests non-random mating in *R. pyrenaica* or strong inbreeding, which might be caused by one or several violated HWE assumptions e.g. migration, selection, population size. The occurrence of moderate and high F_{ST} values, and the inability to detect loci under selection suggest that assumptions of migration and selection are not violated. Instead, the assumption of infinite population size is violated as shown in chapter 3, where we recover very low population sizes. Hence, we propose continuing population size monitoring and a viability assessment for implementation of methods to increase local population size.

Although genetic diversity is low across the entire range of *R. pyrenaica*, from a conservation perspective, two populations deserve major attention given the combination of geographic and genetic isolation and extremely low genetic diversity (H_S<0.1). The Añisclo and Navarra populations are therefore relatively more vulnerable to negative consequences associated with low genetic diversity and low effective population size, such as inbreeding depression and limited adaptive ability to changing environmental variables (Charlesworth & Willis 2009, Allendorf *et al*. 2013). Indeed summer temperatures and droughts are projected to increase in southern Europe (Gao *et al*. 2006). Presence of inbreeding depression within genetically low diverse populations could be assessed in future studies, similar to previous heterozygosity-fitness correlation studies on other amphibian species (see review in Allentoft & O'Brien 2010). However, given the long history of isolation and small effective population size, most genetic load must have been purged, otherwise the species would have become extinct a long time ago

Several lines of evidence suggest that the six populations function independently (Moritz 1994): physical separation, presence of strong gene flow barriers and medium to high genetic differentiation. Therefore we recommend that all identified populations are considered as separate management units (MU's), should be considered during planning and implementation of conservation actions. The isolated nature of these, mostly small, populations suggests genetic diversity could quickly deplete further due to inbreeding and genetic drift (Frankham 1996). Moreover, especially amphibians are prone to potential

large effects of genetic drift given their reproduction strategy and the potential of whole clutch mortality (Dubois 2004). In addition, preliminary data suggests that tadpole survival decreases during warm years as water in shallow pools evaporates during warm summer conditions, as well as with storm floods (see chapter 1). With projected temperature and drought increase (Gao *et al*. 2006) and stochastic events like stronger storms and floods, tadpole survival is expected to decrease further leading to even smaller population sizes and further increase in fragmentation. These features and significant but weak inbreeding in *R. pyrenaica* highlight the need of continued monitoring and conservation management of all MU's.

Following the IUCN guidelines, the conservation status of each species is ideally reassessed every five years (IUCN 2017), which is overdue for *R. pyrenaica* (Bosch *et al*. 2009). Since 2009, and in addition to this work, a large amount of data on this species has been generated and analyzed (Vieites *et al*. 2016, in prep, Peso *et al*. 2016; the current study). Therefore we propose a reassessment of the Red List status for *R. pyrenaica* that summarizes the current knowledge and guides both future research and conservation efforts.

As main conclusions, we here utilized a RRG method to infer the phylogeographic and demographic history, spatial connectivity and conservation genetic parameters to guide conservation management for an endemic endangered European brown frog. Population subdivision is formed by the river drainage system leading to high population differentiation. *Rana pyrenaica* shows weak inbreeding and very low levels of genetic diversity, which is in line with its isolated small populations. We identify six management units within the species range that deserve separate conservation management and evaluation. Because there is strong evidence for a long history of evolution in isolation for this species of poorly connected populations, conservation priority lies on reducing environmental change and stochasticity in its habitat. Before further studies have been performed on the effects of interbreeding among different populations on population viability (outbreeding depression), genetic management such as translocating individuals among populations or even clusters to increase genetic diversity is not recommended.

Acknowledgements

We are grateful to many people that made this project possible. Several students and collaborators helped in fieldwork in the Pyrenees, including Carlos Zaragoza, Javier Santos, Rubén González, Miguel Vences, Nina Bernard, Guillermo Ponz and Isabel Perandones. Fernando Carmena and Ignacio Gómez from SARGA in Aragón, and Iosu Antón in Navarra were extremely helpful in the field, allowing locating many known populations. Ramon Antor Casterllanau provided logistic help to prepare and carry on fieldwork. Manuel Alcántara, David Guzmán and Jose Luis Burrel from the Aragón government showed their total collaboration and help during the whole length of the project. Forest rangers helped us during the fieldwork, and the public company Sodemasa, now SARGA, provided logistic support in Ordesa in 2011. MPF was financed with a grant by CNPq under Science Without Borders program by Brazil Ministry of Science. We thank the Governments of Aragon and Navarra for collecting permits. This work was funded by a research project of the Zoo de Barcelona (Ayuntamiento de Barcelona) as well as by a research project from the Parques Nacionales OPAN – MMARM to DRV.

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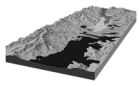

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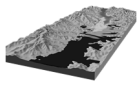

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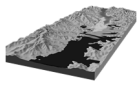

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Appendix Chapter 5

Figures

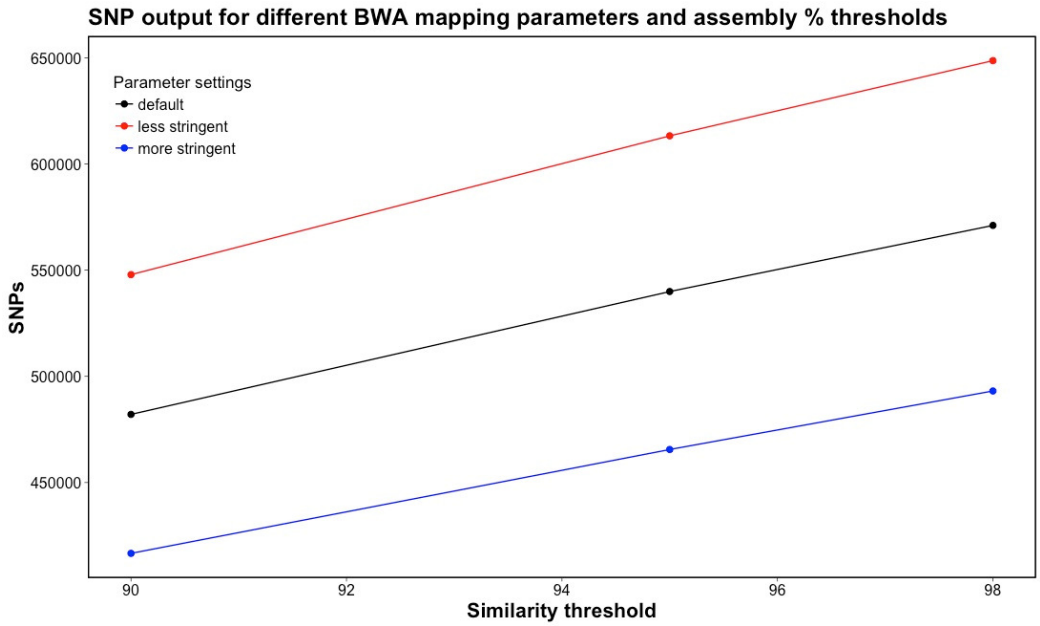


Figure A.1. Raw SNP output for nine SNP calling runs with different BWA settings for assemblies with differing similarity threshold

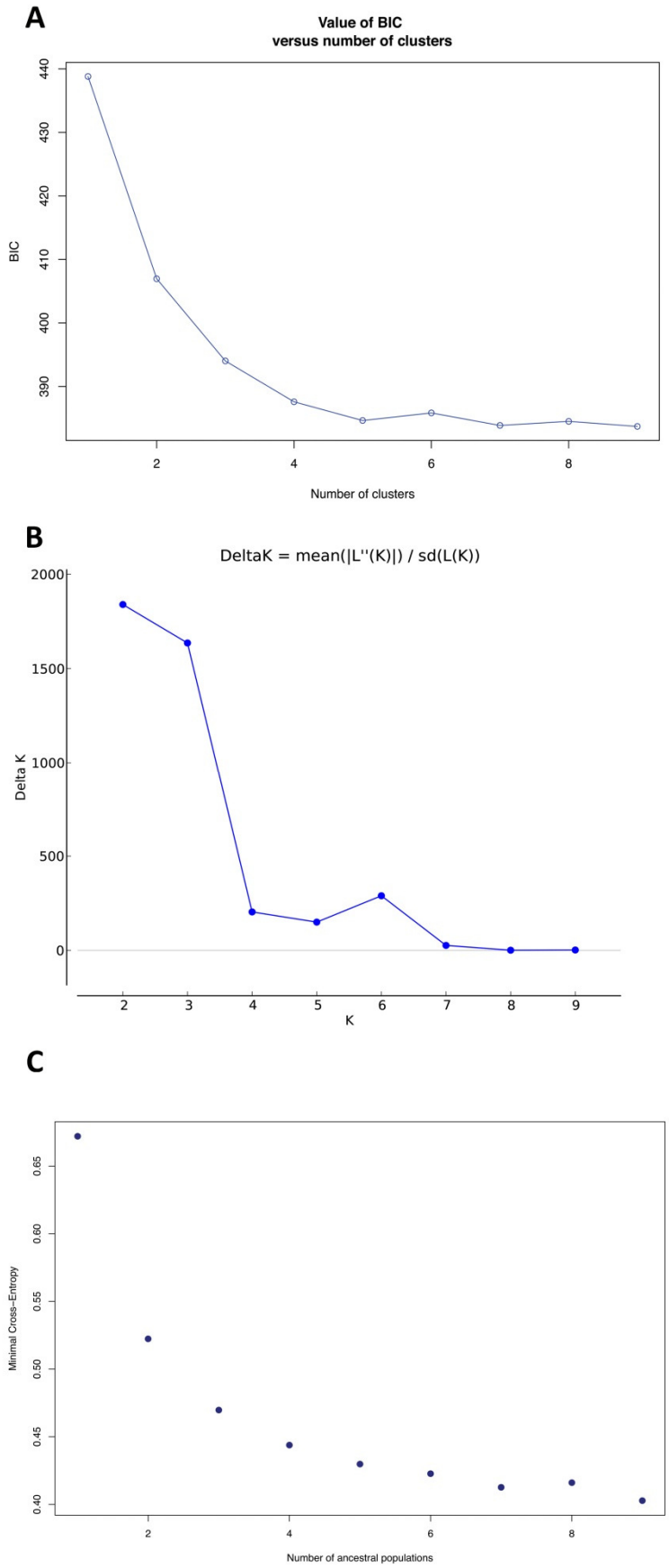


Figure A.2. Number of genetic clusters as assessed by different software programs: *adegenet* (A), Structure (B) and *snmf* (C).

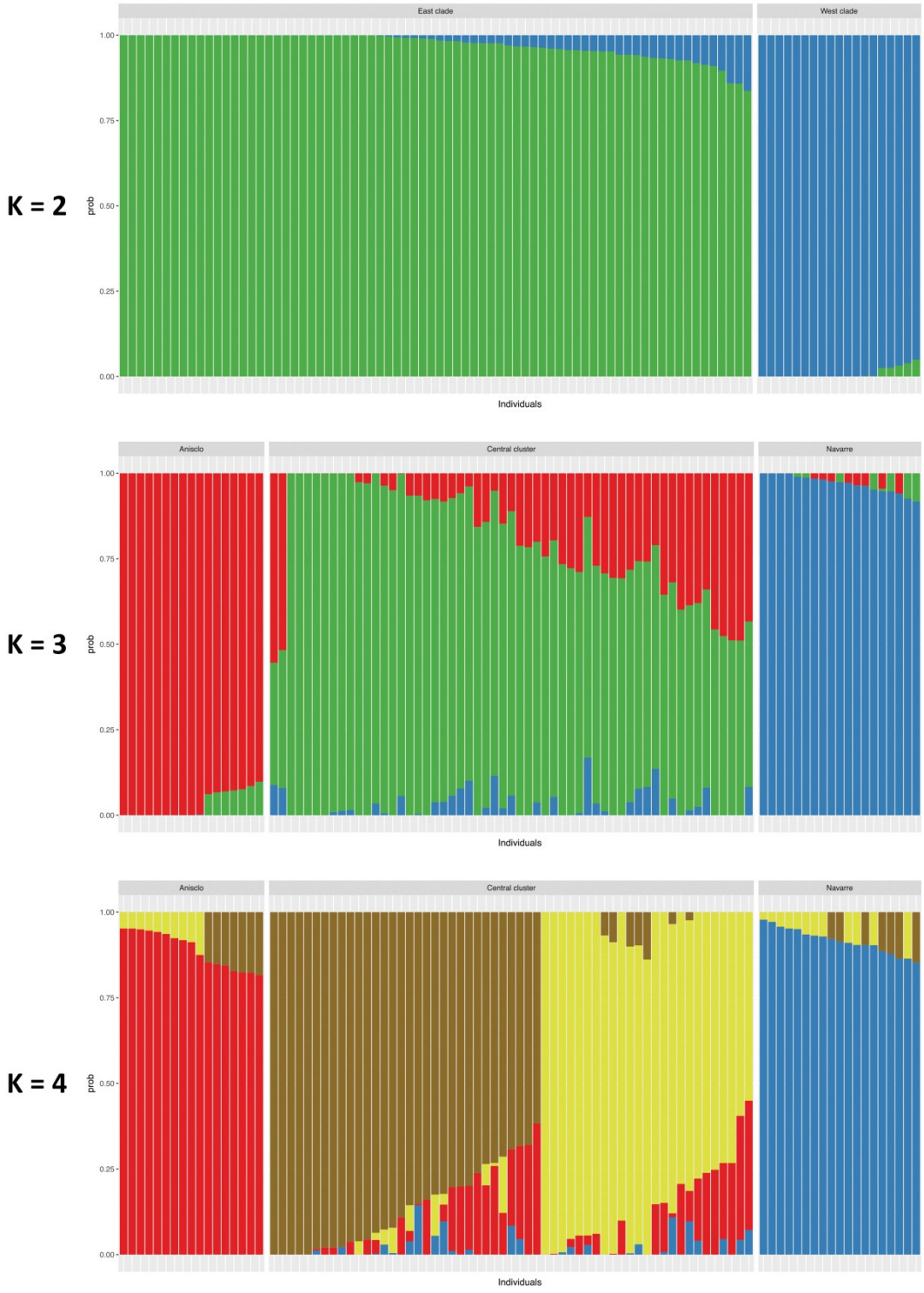


Figure A.3. *snmf* barplots showing individual probability assignments for numbers of populations (K=2-4) within *R. pyrenaica*.

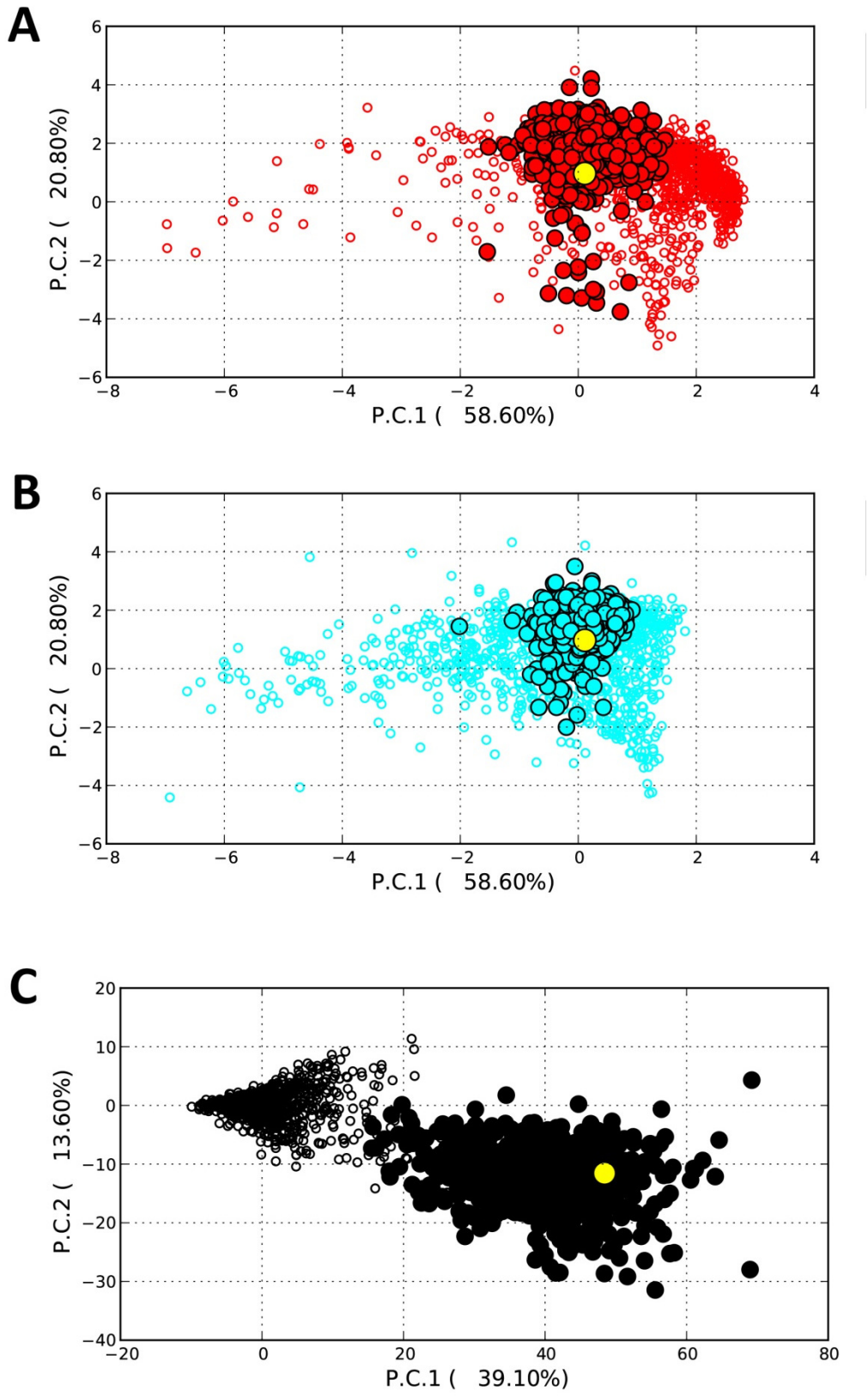
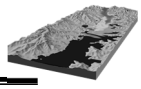


Figure A.4. Fit of prior and posterior data to scenarios with highest probability. Open and closed circles represent simulated dataset from parameters generated using prior and posterior datasets. A) Run 1, scenario 2; B) Run 1, scenario 3; C) Run 2, scenario 6. Large yellow datapoint represents *R. pyrenaica* dataset.



Tables

Table A.3. Life stage of SNPs sequenced samples of the *Rana pyrenaica* specimens.

Life Stage	Count
Larvae	129
Juvenile	13
Subadult	7
Adult	19
Imago	1
Missing	9
Total	**178**

Table A.2. Parameter settings and SNP output from nine assembly runs. BWA Mapping parameters A, B, O; Default: 1, 4, 6; Less stringent: 1, 3, 5; More stringent: 1, 5, 7.

Contig similarity	Mapping parameters	RAW SNPs	Contigs
90%	Default	482085	103954
	Less stringent	547898	
	More stringent	416645	
95%	Default	539942	114820
	Less stringent	613256	
	More stringent	465557	
98%	Default	571084	121067
	Less stringent	646996	
	More stringent	493107	

Table A.3. Number of raw SNPs and remaining SNPs after filtering steps using the dDocent pipeline.

Raw SNPs and applied filters	SNPs
TotalRawSNPs	646996
--max-missing 0.5 --mac 3 --minQ 30 --minDP 3	325694
--max-missing 0.9 --maf 0.03 --min-meanDP 20	72716
"AB > 0.3 & AB < 0.7 | AB < 0.01"	21514
"SAF / SAR > 100 & SRF / SRR > 100 | SAR / SAF > 100 & SRR / SRF > 100"	16980
MQM / MQMR > 0.93 & MQM / MQMR < 1.03	5870
"PAIRED > 0.05 & PAIREDR > 0.05 & PAIREDR / PAIRED	4961
QUAL / DP > 0.75	4875
<2 * mean depth	4682
Hardy Weinberg p>0.001	1628
rad_haplotyper filter	1140
Filtering control and duplicate sample discrepancies	**763**

DISCUSSION

This thesis is the result of a long-term project on Pyrenean amphibians, in which we wanted to better understand different subjects related to the presence, diversification, adaptation and threats of the amphibian species present in the Pyrenees. One of the least known but very interesting species is the Pyrenean frog, *Rana pyrenaica*, an Endangered endemism of the Pyrenees. We here wanted to generate data on several poorly known or directly unknown aspects of the species to help management and conservation actions. Our goal is that the results obtained in this project lay the foundations for the management and conservation strategy of *R. pyrenaica*.

Previous works laid the foundations on determining its distribution range, but its distribution limits were not clearly known (Serra-Cobo 1997, Vieites &Vences 2003). This, together with the finding of new isolated populations in France, far from the main distribution range (Duchateau *et al*. 2012), suggested that the species may have a wider distribution being present in the Northern Slope of the Central Pyrenees in France. Also, there was a lack of recent information on the current situation of the known populations, and if the species persisted in all of them and their current population sizes. For these reasons, we carried out a long-term and intense fieldwork program mainly between 2010 and 2014.

The newly generated distribution data for the species show that the area of historical occupation is maintained to a great extent, although there are localities that have disappeared. It is worth noting the practical disappearance of the species in the Aragón River valley localities including Villanúa, where the population size is less than 5 individuals. The species was described from specimens from this area, and the reasons for its local strong decline are not clear. In total, the species has disappeared from 30 historical locations, although it persists in the rest. As expected, we increased the distribution range of the species in new five Spanish UTM 10x10km squares where it was not known. In chapter 1 we present all the localities where the species is present but also the large number of visited suitable localities (according to potential distribution models) where it was not found. After intense fieldwork we have not found the species in other places in France, neither other herpetologists did, so we can assume that the isolated French localities are a relic of its past distribution or a recent introduction due their genetic similarity to the Navarra populations (see below). We anticipate that, based on our

fine-scale predictive model, the species will appear in new localities within its range, a small population can be located en few pools of remote streams.

Perhaps the most relevant factor in terms of conservation is the small population size of most localities, as well as the apparent high degree of fragmentation of the range. Although there are a number of localities with high densities for the species (although much lower when compared to *Rana temporaria*), most of them harbor a small number of breeding adults. Our relative estimate of the number of adults is the first and only attempt so far to get an estimate of the total population size of the species. Other approaches, like mark and recapture programs, can provide precise population size estimates, but they could never be applied to such a large whole-range scale. Our approach, although time consuming, provided a direct estimation of the number of reproductive females in a section of each stream, and by extrapolation to the rest of the stream a rough estimate of potential population sizes. Even if these numbers are slightly underestimating the total population of the species, the observed densities are very low in direct comparison with its sister taxon *R. temporaria*, which in a single lake can have more than a thousand reproductive individuals. In any case, the estimated population size is far below the limits to be considered an Endangered species by the IUCN.

Species distribution ranges are shaped by current and historical processes. The existence of a gap within the distribution range has led to hypothesize that it could be a consequence of a recent extinction or a legacy of Quaternary ice ages. During the Pleistocene, the extent and intensity of glaciations was important in the Pyrenees, with most of the main rivers frozen and harbouring long glaciers that extended way to the south in the Spanish slope (Ehlers & Gibbard 2004). As a lotic species, *R. pyrenaica* was likely severly affected, as well as many other cold vertebrates that are known to have retreated to lower elevations (López-García *et al*. 2010). This distribution break lead to hypothesize that, if it is a legacy from the ice ages, the actual range is the result of a postglacial colonization from two independent refugia, which should be reflected in the spatial genetics of the species. In chapter 4 we explored the mitochondrial filogeography of the species based on the genetic variation observed in nearly complete mitochondrial genomes. We integrated those data with hindcasted paleoclimatic models to reconstruct the biogeographic and spatial genetic variation history of the species. The models suggest that the gap existed already during the ice ages, but there was potential connectivity between these two core areas across the lowlands and the pre-Pyrenees. This agrees with

the observed lack of spatial genetic structure in mtDNA. The isolated French populations occur in an area that was not glaciated, hence they could be a relict of the past distribution of the species; if so they should show genetic differences from other *R. pyrenaica* populations, but this is not the case as they are rather similar to the Navarra populations. Considering this, or they were connected somehow during the Pleistocene glacial and interglacial periods to Navarra´s population, or they were recently introduced by humans there.

Demographic reconstructions and analyses suggested a recent population expansion towards higher elevations, although most of the current distribution range is within unglaciated areas, and few places within the range of the last glacial maximum ice were recolonized. This contrasts with *R. temporaria* who was able to recolonize high elevations where it is abundant. Those data also show that the species has a very low genetic intraspecific diversity, and that the effective population sizes have been low since the Pleistocene. In fact, *R. pyrenaica* has less genetic variation in the entire mitochondrial genome than in 150 pairs of *R. temporaria* mtDNA. In order to further explore this, and to try to recover spatial genetic structure related to potential barriers, we generated a nuclear genomic dataset to provide us with SNPs. This nuclear dataset allowed us to reconstruct the fine-scale spatial genetic structure of the species, which now shows spatial differences between some areas. Two main clusters were recovered, one constituted by the Navarra and western Aragón populations plus the isolated populations in France, and the other including the eastern range localities. However, within the eastern cluster, we found further genetic structure recovering five small clusters delimited by big rivers and steep slopes. We tested several divergence hypotheses with approximate Bayesian statistics, which suggested that the main east-west divergence originated ca. 600000 y. a., during the Pleistocene.

Those data also allowed to explore the potential role as a barrier of big rivers and steep slopes using a landscape genetics approximation. Our analyses favor an isolation by barrier model, where these rivers and cliffs limit the distribution of the species. According to the nuclear genomic data, those are not recent barriers but behaved like that already during the Pleistocene, where those valleys harbored large glaciers that extended far south covering the valleys (Ehlers & Gibbard 2004). The combination of landscape genetics, population genetics and species distribution modelling, lead to generate a map of potential conservation units for the species (Fig. 1).

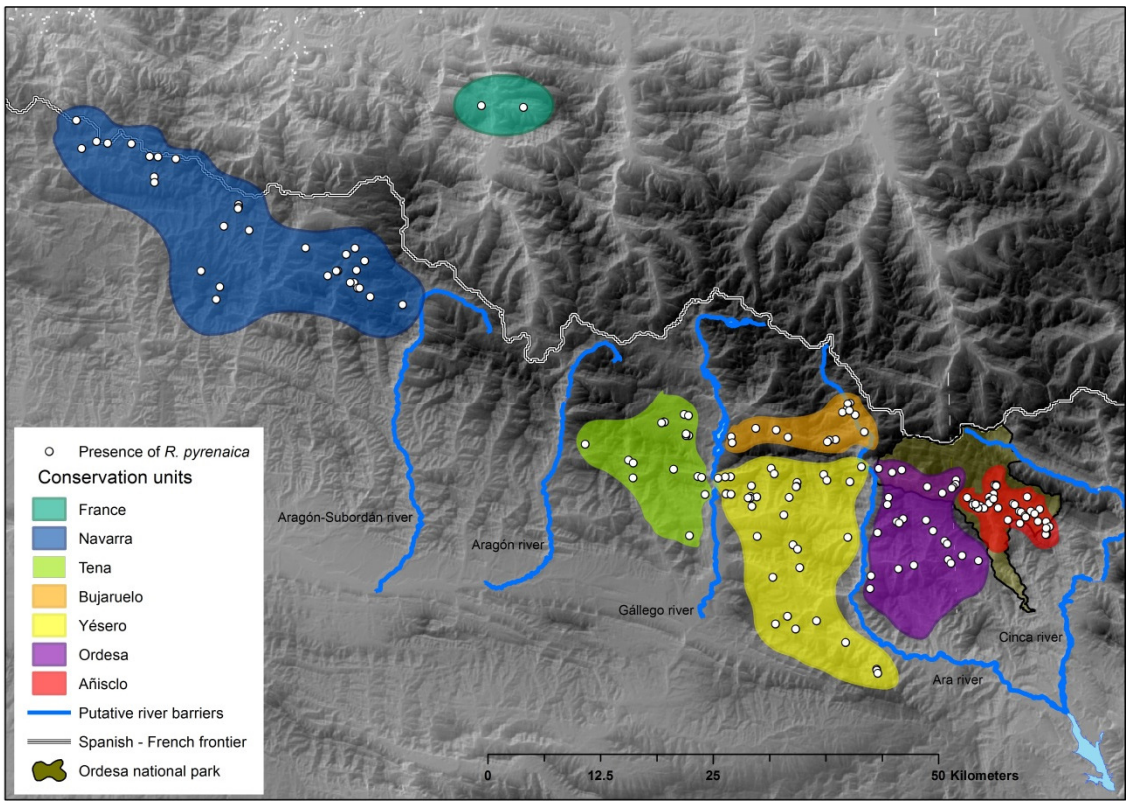


Figure 1. Proposed conservation units for *R. pyrenaica* based on the data generated in this thesis.

Those units are: the isolated French populations, the western core area including all Navarra populations to the Hecho Valley in Aragón; the populations between the aragón River and the Gállego River in the Tena Valley; the populations between the Gállego River and the Ara River (Yésero); the Bujaruelo Valley that is separated from the previous one by the Tendeñera massif; and the Ordesa and Añisclo clusters, both of which harbor populations within the Ordesa – Monte Perdido National park. It is very relevant that two of the most divergent genetic clusters occur already within a National park, which should help applying proper conservation actions to maintain their populations.

Together with the small and fragmented range, small populations sizes and lack of genetic variation, there other factors that can affect the survival of this species. The presence of large trout limits the presence of the frog, not being present in streams with certain entity with fish. Tadpole survival analyses suggested that the type of habitat greatly influences survival as well as the climate of each year. The highest mortality observed occurs in streams with shallow pools with no or little forest cover, that can dry out before metamorphosis or suffer intense floods during storms that kill all larvae. Many of these pools are clogged with sediments, so the available water volume to tadpoles is

very limited. Our data suggest that in areas where the pools are deep, and have water all the time, the survivorship is greater, serving as refugia against floods. Another related factor is the impact of the summer storm floods that wash down the tadpoles and produce rapid mass mortalities. There are places with less slope in which the flood effects are small although in others they can be very significant. Global Change models indicate that in the coming years we will experience winters with less snow and precipitation and dry summers, with seasonal storms, which is not a very hopeful scenario for the species. To remedy, in part, the problem the most effective management measure for the species right now is to decouple the pools, by removing the sediments and increasing their depth. This could ensure that there will be enough water for the tadpoles to complete the metamorphosis and reinforce recruitment for the next few years, especially taking into account predictions of prevalence of dry years in the future.

The presence of the fungus *Batrachochytrium dendrobatidis* throughout the Pyrenean frog distribution area is very worrying. The area with the greatest presence of the fungus is inside the National Park. Although the presence of the fungus has not been detected in three zones, the number of samples available from these valleys is small, and in the rest of the valleys the proportion of samples with negative BD is greater. This fungus is killing amphibian populations throughout the world, although the effects on *R. pyrenaica* are yet unknown. Our models suggest that we can expect a spread of the fungus towards high elevation water bodies in the next few years if warming continues as expected. It is necessary to monitor the affected populations to see the degree of incidence, if it affects more species, and track its potential expansion. We have not observed mass mortalities of adults or tadpoles, although chytridiomycosis perhaps is one of the causes that explain the small population size observed in many localities. The phenology of reproduction in brown frogs in the Pyrenees is directly related to snow thaw in the breeding localities. Both the historical (Ballcells 1975) and more recent (Vieites 2003) show a clear correlation between the altitude and onset of breeding period in *Rana temporaria*, which happens when snow melts. A similar pattern is expected for *R. pyrenaica*. Earlier springs, will suppose higher incidence of chytrid infections when frogs reproduce (Clare *et al*. 2016), as well as expansion of the Bd towards higher elevations.

Considering our findings, a series of management measures can be implemented to help maintain frog populations. *In situ*, it would help to improve connectivity and manage their habitat, for example through decoupling of sediments in stream pools. In

this sense, the maintenance and proper management of Ordesa's National park populations, the only area with the maximum protection for the species, is critical. Some measures can be taken to increase spatial connectivity and reinforce populations with the incorporation of new individuals, like translocating genetically similar specimens (for example larvae from desiccating pools) to places where they can develop and survive. The reinforcement of populations with low density but with suitable habitat can be effective to improve the situation of the species. For the moment we do not recommend mixing individuals from different genetic clusters to increase genetic variation.

There are also arguments for an *ex situ* captive breeding program. The low genetic diversity and low population density, as well as the presence of a lethal pathogen for amphibians such as the chytrid, are important arguments to consider the reproduction in captivity of the species in order to ensure its future survival. This strategy is already being done in the Peninsula with other species, it would be compatible with in situ conservation, and it would allow repopulating areas where the species has disappeared, reinforcing populations and improving spatial connectivity.

Our data suggest that the current threat category of the species should be maintained. *Rana pyrenaica* is cataloged by the IUCN as an Endangered species, in category B1ab (II, III, IV), in which the limited extent of the geographical area of the species is used as a classification criterion. The low density of breeding individuals, small extent of occurrence and area of occupancy justify this category. However, given that the Habitats Directive was made before the species was described, *R. pyrenaica* is not included on it. Therefore, it is necessary that the Ministry for the Energetic Transition (Former Ministry of Environment) requests the inclusion of this species in a new annex to that European Community Directive. In this way, it would be given the degree of official protection that it deserves as a priority species. In any case, both national (Spain and France) and regional governments (Aragón and Navarra) should develop conservation programs that need to be implemented as soon as possible to prevent the potential extinction of the species in this century.

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CONCLUSIONS

1.- The Pyrenean frog, *Rana pyrenaica*, is a narrow range endemic Pyrenean species, with a conservation status of Endangered according to IUCN criteria.

2.-We increased the known distribution range of the species to 170 localities, distributed within 30 10x10 UTM grid cells in Spain and 2 in France. Its estimated area of occupancy would be less than 250 km^2, and the extent of occurrence is less than 3100 km^2.

3.- The species was not relocated in thirty historical localities after several visits during four years, suggesting that it is likely extinct there.

4.- The estimated population size within the area of occupancy ranges between 13000 and 17000 adult frogs, which is low when compared to other brown frog species.

5.- The microhabitat where tadpoles develop, especially pools, is key to their survival, which increases in streams with deep ponds and forest cover. Dry years that desiccate pools or storm floods that cause tadpole downstream washing, can be alleviated by managing the habitat and increasing pool depth.

6.- The chytrid fungus, *Batrachochytrium dendrobatidis*, is widespread across the range of the species. Infection is rare above 2000 m a.s.l. but forecasts under future cliamtes of chytrid optimum and suitable growths suggest that it will affect high elevation populations as well in few decades. It is not known if Bd is causing massive die-offs in this species.

7.- The mitogenomic genetic variation of the species, as well as its genetic diversity, are very low, with 17 unique haplotypes detected. No phylogeographic structure was detected with mtDNA. The reconstructed demography suggest a small effective population sizes since the Pleistocene, and a plostglacial recolonization of higher elevations in the Holocene.

8.- The distribution gap between the eastern and western range is confirmed, and hindcasted paleoclimatic models suggest that it was already present in the Pleistocene, but the two range core areas were likely connected through the lowlands and the pre-Pyrenees.

9.- Radseq nuclear data (SNPs) support the existence of 2 to 6 genetic clusters. The eastern and western populations split ca. 600000 years ago. Within the eastern range, there are five small clusters that correspond to the areas of Añisclo, Ordea, Yésero and East Tena Valley, Bujaruelo valley and west Tena valley. We propose these areas as conservation units for management purposes.

10.- The origin of the observed genetic structure can be explained by river and steep slope barriers that prevent gene flow and connectivity.

11.- Nuclear genomic data also suggest low genetic intraspecific diversity in the species.

12.- The integration of these data support maintaining the category of Endangered for the species according to IUCN criteria.